THE ROLE OF N-LINKED OLIGOSACCHARIDE STRUCTURE IN REGULATION OF IMMUNOGLOBULIN SECRETION AND FUNCTION

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by

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ABBREVIATIONS

The abbreviations used in this thesis are those recommended in the Instructions To Authors of the Biochemical Journal (1986), with the following additions :-

A_{492}	Absorbance readings at 492nm.
ADCC	Antibody-dependent cellular cytotoxicity
BSA	Bovine serum albumin
CFA	Complement fixation assay
CHO	Carbohydrate
CON	Control
Con A	Concanavalin A
CSP	Castanospermine
DARIg	Donkey anti-rabbit immunoglobulin
dGlc	2-deoxyglucose
dMM	1-deoxymannojirimycin
DMSO	Dimethyl sulphoxide
dNM	1-deoxynojirimycin
Dol	Dolichol
3D-TKM	Three-detergent cocktail in TKM buffer
Elisa	Enzyme linked immuno sorbent assay
Endo H	Endo-α-N-acetylglucosaminidase H
FCS	Foetal calf serum
γ	Heavy chains of IgG
HA	Haemagglutinin
HRP	Horse radish peroxidase
HR(S)	Hours(s)

Ig	Immunoglobulin
L	Light chains
LPS	Lipopolysaccharide
μ	Heavy chains of IgM
mem	Membrane-form immunoglobulins
MHC	Major Histocompatibility Complex
M_r	Relative molecular mass
NANase	Neuraminidase
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
RArIg	Rabbit antibody to rat immunoglobulin heavy And light chains
sec	Secretory immunoglobulins
SW	Swainsonine
TCA	Trichloroacetic acid
TEMED	N,N,N',N'-Tetramethylethylene diamine
TKM	Tris-potassium-magnesium buffer
TM	Tunicamycin (-treated)
TM-1, -2 or -3	Analogues of tunicamycin (-treated)
VSV	Vesicular Stomatitis Virus

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SUMMARY

The functions of carbohydrate moieties of glycoproteins are subject to much controversy. Their role in the regulation of intracellular glycoprotein transport is obscured by the discovery of distinctive effects that result from treatment either with inhibitors of N-glycosylation or intracellular N-linked oligosaccharide processing. It is therefore the purpose of this study to ratify and to broaden the perspective in this area of ambiguity.

The experimental approach used in the investigation generally involves biosynthetic labelling of rat hybridoma cells with various radioactive precursor and an isolation of intracellular and/or extracellular immunoglobulins by immunoprecipitation technique. Through properly-timed, pulse-chase methods, it was possible to determine the effects of various inhibitors of glycosylation or oligosaccharide processing on the kinetics of immunoglobulin export. The isolated immunoglobulins were also subjected to other analyses to identify and to confirm the structures of carbohydrate molecules that were incorporated, as well as to assess the effects of inhibition of N-glycosylation or N-linked oligosaccharide processing on the biological functions of immunoglobulins.

The first part of this study involves characterization of three structural analogues of tunicamycin, TM-1, TM-2 and TM-3. Results from this investigation demonstrate that minor modifications to the structure of the antibiotic results in the loss of its biological activity (i.e., with respect to inhibition of N-glycosylation). The analogues do not inhibit N-glycosylation of immunoglobulins and have no effects on the kinetics of IgM and IgG_{2b} secretion from rat hybridomas. The data, therefore, suggest that the selective inhibition of immunoglobulin secretion that is observed when cells are treated with tunicamycin is really due to the absence of

carbohydrate moieties from the immune molecules and not a direct effect of the antibiotic.

The data also demonstrate, that unlike normal tunicamycin, the three structural analogues are not cytotoxic. Their presence has no pronounced effect on the cellular uptake of tritiated-thymidine, -uridine and -leucine. This suggests that the well-recognized cytotoxic effects of tunicamycin are also a secondary effect due to the action of the drug on other cellular functions. In addition, enzymatic analysis of isolated immunoglobulins produced and secreted in the presence of the three tunicamycin analogues demonstrates that the drugs have no effect on the N-acetylglucosaminyl transferase I and II activities located in the Golgi apparatus. Susceptibility to endo H digestion is only observed in immunoglobulins isolated from within the cells. Immunoglobulins secreted in the presence of the analogues of tunicamycin display complete resistance to the enzyme.

To provide a wider perspective on the current understanding of the role of N-linked carbohydrate processing in the regulation of glycoprotein transport, a study of the effects of four specific N-linked oligosaccharide processing inhibitors (i.e., CSP, dNM, dMM and SW) on the secretion of IgM and IgG_{2b} from rat hybridoma lines was carried out. The data clearly demonstrate that inhibition of the processing of N-linked oligosaccharide at specific stages of the pathway does not lead to any significant interference with the rate of IgM and IgG_{2b} secretion.

Resolution of the reduced immunoglobulin components secreted from cells that were treated with the individual processing inhibitors on SDS-PAGE demonstrate distinctive heavy chain structures. While no apparent difference could be seen from the μ- and γ-heavy chains that were secreted in the presence of the mannosidase Ia/b and II inhibitors (i.e., dMM and SW, respectively), treatment

with glucosidase inhibitors (i.e., CSP and dNM) results in the production of μ- and γ-heavy chains with higher M_r.

Experiments were also performed by using mixtures of the carbohydrate processing inhibitors. Treatment of I1A1.4 and 4A3 cells with selective pairs of glucosidase and mannosidase inhibitors (i.e., CSP/SW and dNM/dMM) or with all the four processing inhibitors simultaneously, also demonstrate no significant changes of the rate of immunoglobulin export. The μ- and γ-heavy chains that were isolated in all of these cases, however, demonstrate the higher-type M_r structures.

The oligosaccharide chains of IgG_{2b} from I1A1.4 cells, under normal circumstances, although bearing the complex-type structures, do not possess terminally-linked sialic acid residues. By using neuraminidase digestion analysis, it was shown that when the processing of the carbohydrate moieties was inhibited by any of the four processing inhibitors, individually, or with all of them simultaneously, γ-heavy chains become susceptible to the enzyme treatment, suggesting the presence of terminally-linked sialic acid residues. This effect may either be a result of a direct activation of intracellular sialyl transferase or due to other processing resulting in carbohydrate configurations which can act as substrates for the transferase.

N-linked carbohydrate moieties of immunoglobulins have been strongly implicated to be involved in the C1q-binding interaction. Their absence from immunoglobulins have rendered the molecules less effective in activating the complement cascade. The data from experiments performed in this study are also compatible with this interpretation. A similar result was obtained when tunicamycin-treated non-N-glycosylated IgG_{2b} from I1A1.4 cells was subjected to complement fixation assay.

In the case of immunoglobulins with high mannose structures that were secreted in the presence of oligosaccharide processing inhibitors, the data indicate otherwise. These immunoglobulins apparently possess potentiated capability in fixing complement. Complement fixation assays were also performed on normal IgG_{2b} in the presence of free mannose, galactose and N-acetylglucosamine at 5mM concentration. The monosaccharides have no effect on the complement fixation activity.

The effects of inhibition of N-glycosylation or oligosaccharide processing on Fc-binding interactions, which may involve carbohydrate moieties, have also been studied. The data indicate that neither the inhibition of N-glycosylation nor carbohydrate processing have any consequence on antibody-dependent haemagglutination by IgG_{2b} from I1A1.4 cells. In addition, inhibition of N-glycosylation or the processing of carbohydrate moieties of IgG_{2b} was also shown to have no effect on antigen binding capacity of the immunoglobulins.

CHAPTER I

INTRODUCTION

1.1. Glycoproteins.

Glycoproteins are proteins containing carbohydrate chains covalently attached to selected amino acid residues. The carbohydrate chains of glycoproteins range from simple disaccharide molecules to very complex, highly branched structures. The term glycoprotein, however, excludes proteins with extensive polysaccharide linkages. These carbohydrate-linked proteins are specially classified as proteoglycans. Glycoproteins, proteoglycans and glycolipids fall within a single class of macromolecule known as the glycoconjugates (Hughes, 1983).

Carbohydrate moieties of glycoproteins are linked either O-glycosidically, from N-acetylgalactosamine to the hydroxyl groups of serine or threonine residues of the peptide, or N-glycosidically, via the amino group of N-acetylglucosamine to the amide nitrogen of a peptidyl asparagine. O-linked oligosaccharide linkages have also been reported to exist between other sugars and amino acids, e.g., mannose, hydoxyproline and hydroxylysine (Hughes, 1983). This thesis focuses only on N-linked oligosaccharide moieties of glycoproteins, with particular reference to those associated with immunoglobulin molecules.

1.2. N-Linked Oligosaccharides of Glycoproteins.

1.2.1. Assembly and Transfer of Lipid-Linked Oligosaccharide.

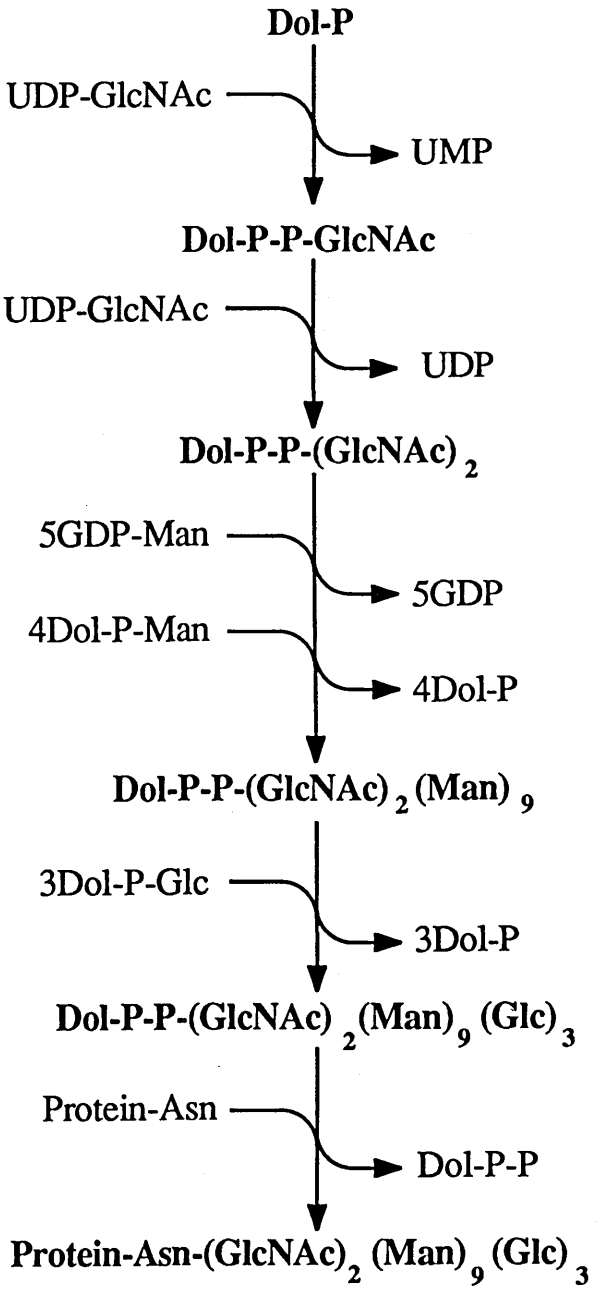
Prior to glycosylation, N-linked oligosaccharide moieties are initially built on an activated lipid carrier, dolichol phosphate. Dolichol is a family of polyisoprenols which are found in the tissues of a variety of eukaryotes (Struck and Lennarz, 1980). Figure 1 demonstrates the biosynthetic pathway of the lipid-linked oligosaccharide complex. Upon phosphorylation, dolichol sequentially acquires the various monosaccharides starting with the transfer of a single N-acetylglucosamine residue (Parodi and Leloir, 1979). This step can be specifically inhibited by tunicamycin, a drug that abrogates N-glycosylation of glycoproteins (see section

Figure 1. Assembly of lipid-linked oligosaccharides.

Prior to their transfer to nascent proteins, oligosaccharides are initially built on dolichol phosphate. Sugars are added sequentially with the first seven monosaccharides derived from nucleotide-sugar complexes whereas the next seven monosaccharides are derived from lipid intermediates (for references, see section 2.1).

KEY:

Dol-P	-	dolichol phosphate
GlcNAc	-	N-acetylglucosamine
Man	-	mannose
Glc	-	glucose


Dol-P
UDP-GlcNAc
UMP
Dol-P-P-GlcNAc
UDP-GlcNAc
UDP
Dol-P-P-(GlcNAc)2
5GDP-Man
5GDP
4Dol-P-Man
4Dol-P
Dol-P-P-(GlcNAc)2(Man)9
3Dol-P-Glc
3Dol-P
Dol-P-P-(GlcNAc)2(Man)9(Glc)3
Protein-Asn
Dol-P-P
Protein-Asn-(GlcNAc)2(Man)9(Glc)3

1.3.1.2). Addition of another N-acetylglucosamine, 9 mannose and 3 glucose units, follow sequentially (Li *et al.*, 1978; Hubbard and Robbins, 1979). While the internal sugars are transferred from nucleotide-linked precursors, peripheral monosaccharides are derived from lipid-linked intermediates (Elbein, 1979; Parodi and Leloir, 1979). The complete precursor-type oligosaccharide structure is then transferred to the proteins.

The acceptor-site on the nascent polypeptide has been demonstrated to comprise a specific tripeptide sequence consisting of asparagine-X-threonine/serine, where X may be any amino acid with the exceptions of aspartic acid and proline (Marshall, 1972 and 1974). The requirement for the specific sequence has been assessed by Bause and Legler (1981) by experimenting on a series of oligopeptides. They have come to the conclusion that a nearby hydroxylated amino acid is required for the formation of a hydrogen bond with the amide of asparagine to achieve a higher reactivity toward the glycosyl donor. Studies have also shown that not all tripeptide sequences of this nature are glycosylated (Kronquist and Lennarz, 1978). Glycosylation is apparently dependent on the tertiary conformation of the protein, and has been demonstrated to be favoured by a β-turn or loop structures (Aubert *et al.*, 1976; Beeley, 1977; Bause, 1983).

1.2.2. N-Linked Oligosaccharide Processing and Modification.

The N-linked oligosaccharide processing pathway and the subcellular locations of the various reactions involved is schematically represented in figure 2. Once transferred to the nascent polypeptide, N-linked oligosaccharides undergo extensive processing, initiated by removal of the terminal glucose residues by the action of α(1,2)-specific glucosidase I (Hettkamp *et al.*, 1984). This creates the substrate for glucosidase II which subsequently cleaves the remaining α(1,3)-linked glucose units (Burns and Touster, 1982; Brada and Dubach, 1984). Also within the

Figure 2. Processing of N-linked oligosaccharides of glycoproteins.

The N-linked oligosaccharide processing pathway and the subcellular locations of the various reactions involved is schematically illustrated (for references, see section 2.2). In the rough endoplasmic reticulum (RER) all of the glucose and one of the mannose residues are firstly being trimmed. Upon entry into the *cis* compartment of the Golgi apparatus, oligosaccharides of lysosomal glycoproteins undergo phosphorylation reaction. Secretory and membrane-associated glycoproteins, on the other hand, are subjected to the actions of the various mannosidases and transferases as they traverse the Golgi stack.

KEY:

Carbohydrate residues :-

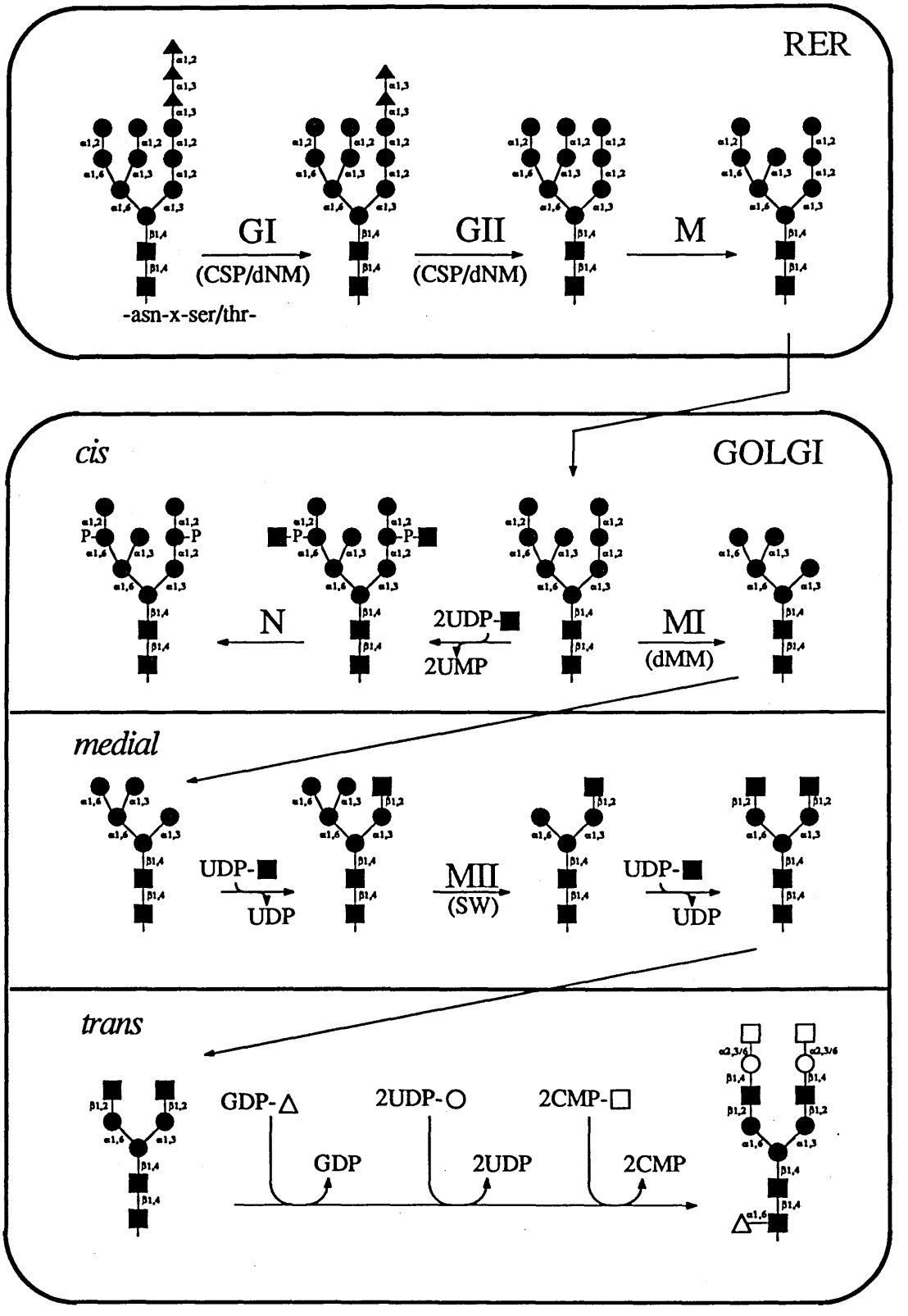
▲ - glucose
○ - galactose
△ - fucose
□ - sialic acid
● - mannose
■ - N-acetylglucosamine

Processing enzymes :-

G - glucosidase
M - mannosidase
N - N-acetylglucosaminyl-1-phosphodiester-α-N-acetylglucosaminidase

Processing inhibitors :-

CSP - castanospermine
SW - swainsonine
dNM - 1-deoxynojirimycin
dMM - 1-deoxymannojirimycin


RER
GI
(CSP/dNM)
GII
(CSP/dNM)
M
-asn-x-ser/thr-
cis
GOLGI
N
2UDP-■
2UMP
MI
(dMM)
medial
UDP-■
UDP
MII
(SW)
UDP-■
UDP
trans
GDP-△
2UDP-○
2CMP-□
GDP
2UDP
2CMP

rough endoplasmic reticulum, at least one α(1,2)-linked mannose residue is specifically trimmed by an α-mannosidase (Bischoff and Kornfeld, 1983).

The deglucosylated glycoproteins are then transported into the Golgi apparatus. This interorganelle movement occurs by means of vesicles which pinch off the endoplasmic reticulum and fuse with the Golgi membranes (Jamieson and Palade, 1967). However, since the rate of glycoprotein movement varies between protein species (Lodish *et al.*, 1983; Gabel and Kornfeld, 1984), a receptor-mediated transport mechanism is also thought to be involved. This notion is further supported by the finding that maturation of some glycoproteins into the Golgi apparatus is blocked when the endoplasmic reticulum deglucosylation process is inhibited (Gross *et al.*, 1983; Lodish and Kong, 1984; Lemansky *et al.*, 1984). Alternatively, the glucose removal may also be necessary for the glycoproteins to mature to a correct conformation (Schlesinger *et al.*, 1984).

The Golgi apparatus can be separated into at least three distinct functional compartments based on the localizations of the distinct N-linked oligosaccharide processing activities (reviewed by Farquhar, 1985). Independent studies utilizing different experimental approaches have demonstrated that the processing of N-linked oligosaccharides occurs sequentially as the glycoproteins traverse the Golgi apparatus. Upon entry into the cisternal face of the Golgi stack (i.e. the *cis* compartment), a subset of glycoproteins that are destined for the lysosome undergo specific mannose phosphorylation catalyzed by the enzyme N-acetylglucosaminyl-1-phosphotransferase (Hasilik *et al.*, 1981; Reitman and Kornfeld, 1981). This is followed by the action of another enzyme, N-acetylglucosaminyl-1-phosphodiester-α-N-acetyl-glucosaminidase (Varki and Kornfeld, 1980 and 1981), that cleaves off the terminal N-acetylglucosamine residues (see figure 2). The mannose-6-phosphate configurations acquired through these reactions act as specific ligands that interact with the intracellular mannose-6-phosphate receptors and facilitate the

glycoprotein transport to the lysosome (reviewed by von Figura and Hasilik, 1986). As these phosphorylated N-linked oligosaccharide moieties of lysosomal glycoproteins are not subject to further processing in the late Golgi compartments, the mannose phosphorylation may also confer protection against further enzymatic action.

Unlike the lysosomal hydrolases, secretory and membrane-associated glycoproteins are firstly subjected to demannosylation processes. The trimming of terminal α(1,2)-mannose units of the N-linked oligosaccharide by the enzyme mannosidase I is believed to occur in the *cis* or the *medial* compartment of the Golgi apparatus (Rothman *et al.*, 1984a and 1984b). This is followed by transfer of one N-acetylglucosamine residue in the *medial* Golgi compartment (Dunphy and Rothman, 1983), a process of which is catalyzed by N-acetylglucosaminyl-transferase I (Harpaz and Schachter, 1980a; Oppenheimer *et al.*, 1981). Also within the *medial* compartment of the Golgi apparatus, further removal of two mannose residues by the α(1,3)- and α(1,6)-specific mannosidase II (Tulsiani *et al.*, 1982; Tulsiani and Touster, 1983) and the transfer of another one or two N-acetylglucosamine residues by N-acetylglucosaminyltransferase II takes place (Harpaz and Schachter, 1980a; Mendicino *et al.*, 1981).

The action of the N-acetylglucosaminyltransferase II forms the initiation of the complex conversion reaction. Before being deposited into the plasma membrane or secreted into the extracellular environment, many oligosaccharides of glycoprotein are finally transformed into the "complex type" structure. This conversion is generally followed by the additions of one fucose unit to each of the innermost N-acetylglucosamine residues of the N-linked oligosaccharide chains (Longmore and Schachter *et al.*, 1982), and the subsequent transfer of galactose and sialic acid units, sequentially, to the terminal carbohydrate residues (Beyer *et al.*, 1981). The transferases that catalyze the two latter reactions have been

localized to the *trans* cisternae of the Golgi stack (Dunphy *et al.*, 1981; Roth and Berger, 1982; Slot and Geuze, 1983). Transformation to the "complex type" N-linked structures is a rapid process and occurs immediately before the glycoproteins exit into the extracellular environment (Peyrieras *et al.*, 1983).

1.2.3. Structural Diversity of N-Linked Oligosaccharides.

Studies locating the specific site of the various enzymes involved in the processing of N-linked oligosaccharide chains have clearly indicated that modifications of the carbohydrate moieties of glycoproteins occur as they traverse the Golgi apparatus. It is now apparent that the Golgi apparatus is really a well organized "processing factory" where a great variety of specific and systematic modification reactions occur. Characterization of the structures of N-linked oligosaccharide moieties of glycoproteins have, however, demonstrated the existence of diverse structural forms (Yamashita *et al.*, 1983). A few examples of these variations are illustrated in figure 3.

Several distinct structural forms have also been shown to exist between the N-linked oligosaccharides of the same glycoprotein. One common example is the monomeric IgM molecule, in which two of its five N-linked oligosaccharide moieties are able to escape complex-conversion and retain their high mannose structures (Shimizu *et al.*, 1971). Such partial transformation implicates the importance of the protein conformations in the determination of the glycoproteins modification.

The extent of the complex conversion reaction is another factor that contributes to the different carbohydrate configurations. Perhaps the best example to illustrate this phenomenon comes from the report by Parekh *et al.*, 1985, which demonstrates that the IgG of human serum may consist of at least 30 different

Figure 3. Structural diversity of N-linked oligosaccharides.

Structures of selective N-linked oligosaccharides of glycoproteins are schematically illustrated;

a) Precursor-type N-linked oligosaccharide.

b) High mannose-type N-linked oligosaccharide of human IgM.

c) Hybrid-type N-linked oligosaccharide of VSV G protein.

d) Monoantennary N-linked oligosaccharide of human urinary glycoprotein.

e) Biantennary complex-type N-linked oligosaccharide of human IgG.

f) Triantennary complex-type N-linked oligosaccharide of VSV G protein.

g) Tetraantennary complex-type N-linked oligosaccharide of VSV G protein.

Note that, with the exception of (d), all other N-linked oligosaccharides possess common "core" carbohydrate structure (i.e., N-acetylglucosamine$_2$Mannose$_3$). For references, see Yamashita *et al.*, 1983 and Kornfeld and Kornfeld, 1985).

KEY:

▲ - glucose

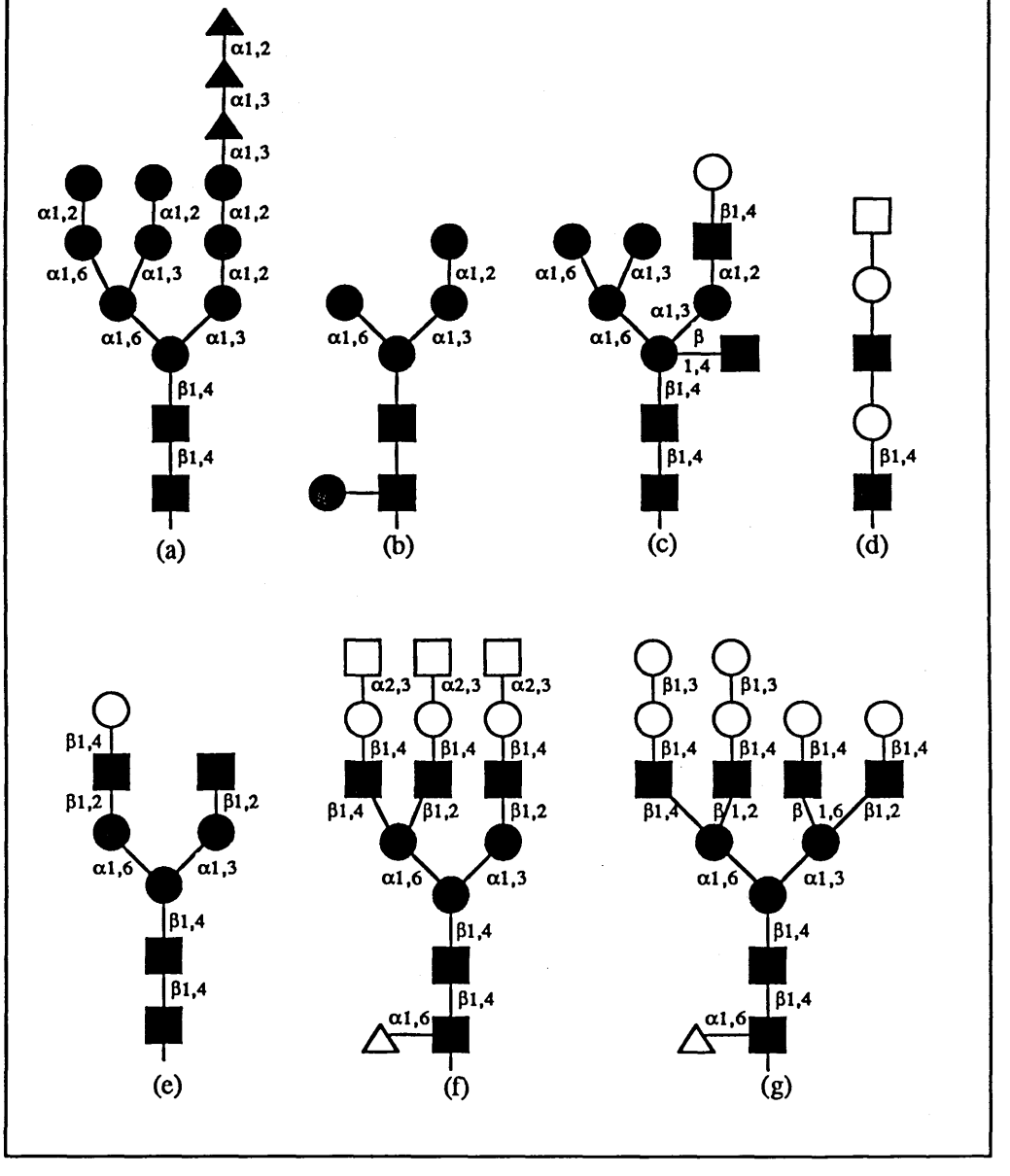
△ - fucose

● - mannose

○ - galactose

□ - sialic acid

■ - N-acetylglucosamine


α1,2
α1,3
α1,3
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α1,2
α1,6
α1,3
α1,2
α1,6
α1,3
β1,4
β1,4
(a)
α1,2
α1,6
α1,3
(b)
β1,4
α1,6
α1,3
α1,2
α1,3
β
1,4
α1,6
β1,4
β1,4
(c)
β1,4
β1,4
(d)
β1,4
β1,2
β1,2
α1,6
α1,3
β1,4
β1,4
(e)
α2,3
α2,3
α2,3
β1,4
β1,4
β1,4
β1,4
β1,2
β1,2
α1,6
α1,3
β1,4
β1,4
α1,6
(f)
β1,3
β1,3
β1,4
β1,4
β1,4
β1,4
β1,4
β1,2
β
1,6
β1,2
α1,6
α1,3
β1,4
β1,4
α1,6
(g)

complex-type biantennary oligosaccharide structures. In addition, it was also reported that approximately 5% of the total IgG population is disialylated; a further 20% exists as monosialylated forms with the vast remainder having neutral structures. Interestingly, such structural heterogeneity is not confined to the polyclonal IgG population. Relatively similar proportions have also been observed in monoclonal immunoglobulins secreted from a hybridoma cell line (Radamacher and Dwek, 1983). This observation certainly implies that the structural heterogeneity is more likely to arise from random processing and not by means of any specific regulatory mechanism in individual cells. Thus, it seems reasonable to propose that N-linked oligosaccharides are only modified if they happen to encounter the various intracellular site-specific processing enzymes in their route to exocytosis.

In certain cell types, further structural variations have been detected in which a "bisecting" N-acetylglucosamine has been attached to the core β-mannose residue. These cells have been shown to possess an enzyme, N-acetyl-glucosaminyltransferase III, that specifically transfers one N-acetylglucosamine molecule to the core β-mannose (Harpaz and Schachter, 1980b). This reaction causes inhibition of the subsequent action of the mannosidase II, which then, leads to the formation of the "hybrid-type" N-linked structure (see oligosaccharide structure c in figure 3; Harpaz and Schachter, 1980b).

In fact, a vast majority of the final oligosaccharide structures are dependent on such a regulatory mechanism, where order and specificity of the structures of substrate molecules are absolute requirements for further modification reactions. For instance, it has been shown that fucosylation and sialylation of asialotransferrin are two mutually exclusive reactions, i.e., a prior action of one enzyme will subsequently inhibit the other (Paulson *et al.*, 1978; Beyer *et al.*, 1979). Similarly, galactosylation of terminal carbohydrate chains would only occur

if a prior addition of α(1,6)-linked N-acetylglucosamine residues has taken place (Blacken *et al.*, 1982). A more detailed discussion of these regulatory mechanisms has been reviewed by Kornfeld and Kornfeld (1985).

Diversity that arises from the structures of N-linked oligosaccharides from different glycoproteins is even more pronounced. Biochemical analyses have demonstrated that N-linked oligosaccharides of glycoproteins can exist as mono-, bi-, tri- and tetraantennary structures (see figure 3). Structural conformations in these cases are more likely to be regulated by specific endogenous branching enzymes.

Structural variations may also arise from other post-translational modifications of the asparagine-linked oligosaccharides. The more common reactions include the phosphorylation of mannose residues (see section 1.2.2) and the O-sulphation of N-acetylhexosamine (Prehm *et al.*, 1979; Parsons and Pierce, 1980; Edge and Spiro, 1984) or mannose units (Freeze *et al.*, 1984). While the phosphorylation of mannose residues has been shown to be involved in the transport mechanism (von Figura and Hasilik, 1986), the function of sulphation of carbohydrate moieties remains unknown.

Although ample evidence has demonstrated the existence of microheterogeneity in the structures of N-linked oligosaccharides chains, no general physiological role has been attributed to this diversity. However, together with the other carbohydrate heterogeneity from other types of glycoproteins and glycoconjugates (Yamashita *et al.*, 1983), these variations in the structures of carbohydrate residues certainly provide a wide spectrum of epitopes for specific receptor-ligand interactions.

1.3.1. Inhibitors of N-Glycosylation.

Inhibitors of N-glycosylation are commonly used in studies assessing the functional roles of N-linked carbohydrate moieties of glycoproteins. Development of assays for measuring the biosynthesis of lipid-linked oligosaccharides and monosaccharides has provided the means to investigate the mechanisms of actions of the inhibitors and the exact step(s) which they inhibit (Behrens and Tabora, 1978). Such understanding is imperative in order to critically evaluate any experimental data obtained from studies involving use of glycosylation inhibitors. Currently, two distinct categories of inhibitors of N-glycosylation have been recognized; sugar analogues and antibiotics.

1.3.1.1. 2-Deoxy-D-Glucose.

One of the early inhibitors of N-glycosylation utilized in biochemical analyses of protein glycosylation was 2-deoxy-D-glucose (dGlc) (Farkas *et al.*, 1969). In order to exert its inhibitory effect, this sugar analogue must be metabolically converted into its nucleotide esters, GDP- and UDP-dGlc (Schmidt *et al.*, 1974 and 1976; Datema *et al.*, 1981).

The effects of this drug are numerous. Both the nucleotide esters GDP- and UDP-dGlc inhibit the formation of Glc-P-Dol by trapping of dolichol phosphate (Dol-P) as dGlc-P-Dol (Datema and Schwarz, 1978; Datema *et al.*, 1981). This product would subsequently, inhibit the glucosylation of Man_9(N-GlcNAc)$_2$-P-P-Dol by incorporating dGlc instead of glucose (Datema *et al.*, 1981). GDP-dGlc has also been demonstrated to inhibit the assembly of the lipid-linked oligosaccharide by incorporation of the sugar analogue in place of mannose (Schwarz *et al.*, 1978).

Experiments in cell-free systems have demonstrated that if dGlc was

incorporated instead of mannose into the lipid-linked oligosaccharide, transfer of the carbohydrate moiety to the nascent polypeptide could not occur (Datema and Schwarz, 1978). Paradoxically, labelled dGlc at non-inhibitory concentrations, has been shown to be incorporated into glycoproteins (Kaluza *et al.*, 1973; Steiner *et al.*, 1973).

In other studies, data obtained from utilization of this compound has proved to be doubtful. For instance, in yeast, this sugar analogue appears to inhibit the secretion of newly synthesized mannan-associated enzymes (Farkas *et al.*, 1970; Kuo and Lampen, 1972). However, subsequent analysis later revealed that the reduced rate of secretion is due to the decreased rate of synthesis of the enzyme, and not due to non-glycosylation of the macromolecules (Kratky *et al.*, 1975).

A similar situation also arises in experiments studying the immunoglobulin system. Melchers (1973) has reported that secretion of IgG from MOPC 21 cell line is blocked in the presence of this sugar analogue and suggested that it was a result of the absence of carbohydrate molecules. However, studies using tunicamycin, which is also an inhibitor of N-glycosylation (see section 1.3.1.2), have all indicated that the secretion of this isotype of immunoglobulin is not significantly reduced in the non-glycosylated state (see section 1.4.1).

1.3.1.2. Tunicamycin.

The structure of tunicamycin is illustrated in figure 7, panel a. Tunicamycin was firstly extracted from *Streptomyces lysosuperificus* as an antibiotic with antiviral activity. Subsequent analysis later revealed that the antibiotic also inhibits the replication of gram-positive bacteria, fungi and yeast, and more importantly, prevents the N-glycosylation of nascent polypeptides (Takatsuki *et al.*, 1971; Takatsuki and Tamura, 1971a and b; Takatsuki *et al.*, 1972). Unlike 2-deoxy-D-glucose, this inhibitor of N-glycosylation does not interfere with either

sugar or nucleotide metabolism (Kuo and Lampen, 1974; Tkacz and Lampen, 1975) and is not metabolizable under either *in vivo* or *in vitro* conditions (Kuo and Lampen, 1976).

The mechanism of action of tunicamycin has been extensively investigated by a number of workers. The drug has been shown to specifically inhibit the synthesis of N-acetylglucosaminylpyrophosphorylpolyisoprenol, the first product of the lipid-linked oligosaccharide formation pathway (Tkacz and Lampen, 1975; Takatsuki *et al.*, 1975), but has no effect on the subsequent steps of the chain elongation reactions or upon the final transfer of oligosaccharide to nascent polypeptides (Lehle and Tanner, 1976; Struck and Lennarz, 1977). This specific inhibition has been shown to occur by non-competitive and irreversible interaction of tunicamycin with UDP-GlcNAc:dolichyl phosphate GlcNAc-1-phosphate transferase, the enzyme that catalyzes the first step in the biosynthetic pathway of the lipid-linked oligosaccharide complex (Heifetz *et al.*, 1979).

Apart from its high-affinity interaction with the enzyme UDP-GlcNAc:dolichyl phosphate GlcNAc-1-phosphate transferase, tunicamycin has also been suggested to possess another intracellular binding site, with a much lower affinity (Heifetz *et al.*, 1979). An earlier report by Kuo and Lampen (1974) demonstrated that the binding of tunicamycin to yeast protoplasts can be inhibited by the inclusion of phospholipid with an unsaturated fatty acid moiety. Thus, it was speculated that tunicamycin may interact non-specifically with a lipophilic component of a cellular membrane, in addition to its high affinity binding to UDP-GlcNAc:dolichyl phosphate GlcNAc-1-phosphate transferase.

Tunicamycin is, however, not without cytotoxic effects. Its presence has been demonstrated to caused impairment in the synthesis of macromolecules and

induces the degradation of cellular RNA and DNA (Takatsuki and Tamura, 1971b; and Takatsuki *et al.*, 1971 and 1972). The mechanisms involved in these reactions are not defined. Although there is no direct evidence, it is believed that such cytotoxicity is due to secondary effects resulting from interference of the inhibitor with other cellular reactions, and not a direct drug-induced response (Struck and Lennarz, 1980; this thesis).

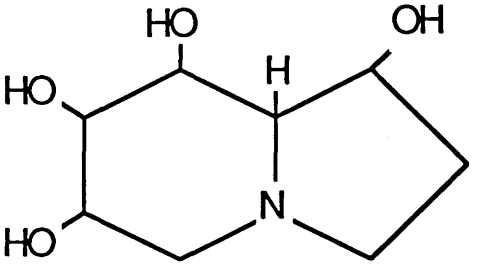
One of the main drawbacks in studies employing inhibitors of N-glycosylation is the difficulty in deducing the precise effect resulting from non-N-glycosylation against a background of sequalae that are indirectly induced by the drug. Thus, in this respect, tunicamycin offers certain advantages over 2-deoxy-D-glucose or other sugar analogues due to its non-involvement in general cellular metabolism. However, tunicamycin itself is not without side-effects. One example is its inhibition of cellular RNA, DNA and protein synthesis. Thus, experimental data that arise from utilization of this antibiotic may, under certain circumstances, prove to be equally erroneous especially when a long-term exposure to the drug is required.

1.3.2. Inhibitors of N-Linked Oligosaccharide Processing.

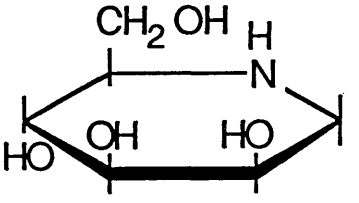
Discoveries of inhibitors of N-linked oligosaccharide processing events are a recent advance (reviewed by Fuhrmann *et al.*, 1985 and Elbein, 1987). Their use in experimental analysis would certainly provide additional information on the biological role of oligosaccharide processing (see section 1.4). Two classes of processing inhibitors are currently known; sugar analogues, that are derived from microorganisms, and plant alkaloids. Both sets of inhibitors function by mimicking the substrate molecules of the specific trimming enzymes. The biochemistry of the four selective processing inhibitors employed in this study (the structures of which are illustrated in figure 4) are discussed briefly below.

Figure 4. Structures of N-linked oligosaccharide processing inhibitors.

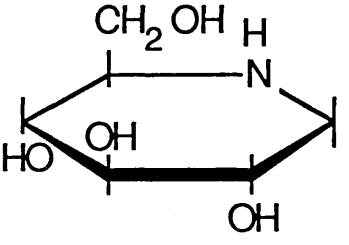
Structures of N-linked oligosaccharide processing inhibitors that are used in this study are illustrated. The specific actions of the inhibitors in the N-linked oligosaccharide processing pathway are schematically illustrated in figure 2. CSP and dNM inhibit glucosidase I and II; dMM, mannosidase Ia/b and SW, mannosidase II. For references, see sections 1.3.2.1 - 1.3.2.4.



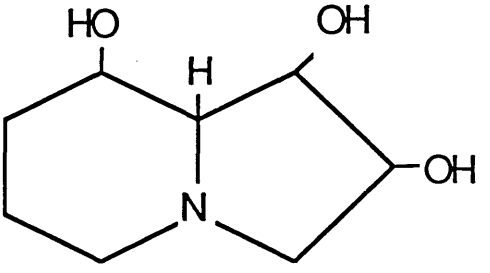
Castanospermine
(CSP)



1-deoxynojirimycin
(dNM)



1-deoxymannojirimycin
(dMM)



Swainsonine
(SW)

1.3.2.1. Castanospermine (CSP).

Castanospermine (1,6,7,8,-tetrahydroxyoctahydroindolizidine) was first isolated from the plant, *Castanospermum australe* (Hohenschutz *et al.*, 1981). It has been shown to inhibit the action of both α- and β-glucosidase (Saul *et al.*, 1983), and the processing of influenza viral haemagglutinin (Pan *et al.*, 1983), soybean cell glycoproteins (Hori *et al.*, 1984) and human hepatoma cell glycoproteins (Sasak *et al.*, 1985). Castanospermine inhibits both the actions of glucosidase I and II of the N-linked oligosaccharide processing pathway (Pan *et al.*, 1983; Sasak *et al.*, 1985; Szumilo *et al.*, 1986).

Bio-gel P4 chromatographic resolution of the Endo H-cleaved carbohydrate, synthesized in the presence of castanospermine, has revealed that the majority of the oligosaccharide possess $hexose_{10}$-GlcNAc structures and enzymic methods have identified the oligosaccharides as being mostly of the $Glc_3Man_7GlcNAc$ configuration (Pan *et al.*, 1983). This strongly suggests that the carbohydrate chains are still subjected to further trimming processes despite having intact glucose residues on one of their branched terminals.

1.3.2.2. 1-Deoxynojirimycin (dNM).

1-Deoxynojirimycin (dNM) also inhibits both the glucose trimming enzymes in the N-linked oligosaccharide processing pathway (see figure 2). However, its specificity and magnitude of inhibition appear to differ according to species. In yeast, it has been shown that the I_{50} (i.e., the concentration of inhibitor achieving a 50% inhibition of enzyme activity) for glucosidase I and II, are 20μM and 2μM, respectively (Saunier *et al.*, 1982). The converse occurs in calf liver cells, in which the I_{50} for glucosidase II (20μM) is much higher than that for glucosidase I (3μM) (Hettkamp *et al.*, 1982).

At 10mM concentration, dNM has been demonstrated to inhibit the N-

glycosylation process (Gross *et al.*, 1983). This is apparent from the higher M_r band of the inhibitor-treated samples analyzed by SDS-polyacrylamide gel electrophoresis. Like most other sugar analogues, dNM may be involved in other cellular metabolic pathways, although no report on this aspect has yet been documented.

Because of its inhibition of both glucosidase I and II activities of the oligosaccharide processing pathway, the carbohydrate structures that are formed in the presence of this drug may be expected to consist of a mixture of oligosaccharides with different number of glucose residues. This has indeed been confirmed by resolution of the Endo H-cleaved oligosaccharides by Bio-gel P4 chromatography (Gross *et al.*, 1983). Furthermore, the presence of oligosaccharides that have been subjected to further mannose trimmings was also detected. Other evidence has indicated that at least part of the oligosaccharide may have been subjected to complex conversion (Peyrieras *et al.*, 1983).

1.3.2.3. 1-Deoxymannojirimycin (dMM).

The mannose analogue of dNM, 1-deoxymannojirimycin (dMM) or 1,5-dideoxy-1,5,-imino-D-mannitol, has been synthesized and shown to be an inhibitor of the mannosidase Ia/b of the N-linked oligosaccharide processing pathway (Fuhrmann *et al.*, 1984). Structural analysis of the carbohydrate chains of IgM and IgD synthesized in the presence of this amino-sugar has demonstrated about 70% of the high-mannose oligosaccharides are of the $Man_9GlcNAc_2$ structure. Unlike in the case of dNM, these oligosaccharides have been demonstrated not to undergo further processing as indicated by N-acetylneuraminidase digestion analysis.

1.3.2.4. Swainsonine (SW).

The final N-linked oligosaccharide trimming enzyme of the Golgi

apparatus has been shown to be inhibited by an alkaloid, swainsonine (8α-indolizidine-1,2,8-triol), firstly isolated from a leguminous plant, *Swainsona canescens* (Colegate *et al.*, 1979). Swainsonine, which also inhibits the mannosidases of the lysosomes (Dorling *et al.*, 1980), was shown to act only on the late mannosidase II enzyme, without affecting the mannosidase Ia/b (Tulsiani *et al.*, 1982). N-linked oligosaccharides produced in the presence of this inhibitor appear to be of the hybrid-type (Tulsiani *et al.*, 1982). Since inhibition of mannosidase II only affects one half of the carbohydrate moiety (see figure 2), the other is therefore normally processed to the complex form.

1.4. Roles of Carbohydrate Moieties of Glycoproteins.

N-linked oligosaccharide moieties of glycoproteins have been implicated in many diverse functions. Participation of the carbohydrate moieties could be seen either by their contribution to the optimal structural or physicochemical properties of the glycoproteins, or by regulating their transport via receptor-mediated mechanisms. These diverse roles of N-linked oligosaccharide moieties of glycoproteins are elaborated in the following discussion.

1.4.1. Facilitating Secretion.

An hypothesis of carbohydrate moieties as controlling elements in the secretion of glycoproteins has been advanced. In an attempt to generalize the functional role of the carbohydrate structures of glycoproteins, Eylar (1965) has forwarded an hypothesis which regards carbohydrate moieties as chemical markers that discriminate secretory and intracellular proteins. In addition, it was also envisaged that there exist receptors for these carbohydrate units in carriers or cellular membranes that, upon interaction with the glycoproteins, will facilitate their export.

This hypothesis is still open to criticism. To date, no such membrane or vesicular receptors have been discovered, and more importantly, inhibition of N-glycosylation only affects the transport of certain glycoproteins (see tables 1 and 2). The inhibitory effect observed on the transport of the non-N-glycosylated glycoproteins provides some support for Eylar's hypothesis and implicates the role played by the carbohydrate moieties in the export of the macromolecules. However, the fact that other non-N-glycosylated glycoproteins are able to escape inhibition of secretion suggests a more complex situation. The selective inhibition of specific non-N-glycosylated glycoproteins has also been shown to occur in a hybridoma cell line that simultaneously secretes both IgG and IgM; while the secretion of the non-N-glycosylated IgG is efficiently maintained, the export of the non-N-glycosylated IgM is profoundly inhibited (Blatt and Haimovich, 1981). Hence, the possibility that the selective inhibition is due to different secretory mechanisms adopted by individual cell types is apparently ruled out. This observation would also negate the notion that carbohydrate moieties are the sole controlling element in the secretion of glycoproteins. Other hypotheses that could account for this intriguing selective inhibitory transport phenomenon are therefore proposed;

1) there is an alternative secretion controlling mechanism that non-N-glycosylated glycoproteins could employ to secure export (e.g., sulphation on tyrosine residues, Baeuerle and Huttner, 1984) and/or,

2) other parameters are involved in the control of glycoprotein secretion, e.g., solubility, size, the extent of glycosylation and the presence of other types of carbohydrate moieties.

The first postulate is somewhat weak since it arises from a single experimental observation. When the glycosylation of murine IgG_{2a} is inhibited, the molecules have been shown to be sulphated at tyrosine

Table 1. Effects of tunicamycin on secretory glycoproteins.

Glycoproteins	Result	References
Invertase and acid phosphatase of yeast	secretion inhibited	Kuo and Lampen, 1976
IgA of MOPC 315	secretion inhibited; blocked within RER	Hickman *et al.*, 1977
IgE of IR 162	secretion inhibited	Hickman *et al.*, 1977
IgA of 5 mouse plasmacytoma lines and IgM of MOPC 104E	secretion inhibited	Hickman and Kornfeld, 1978
IgG of 6 mouse plasmacytoma lines	secretion not affected	Hickman and Kornfeld, 1978
Fibronectin of chick embryo fibroblast	secretion not affected; enhanced proteolysis	Olden *et al.*, 1978
Rat liver transferrin and chick liver apoprotein B	secretion not affected	Struck *et al.*, 1978
Ovalbumin of oviduct	secretion not affected	Keller and Swank, 1978
Carboxypeptidase Y of yeast	secretion not affected	Hasilik and Tanner, 1978
Mouse L cell interferon	secretion not affected	Fujisawa *et al.*, 1978
Human fibroblast interferon	secretion not affected	Mizrahi *et al.*, 1978
Procollagen of chick embryo fibroblast	secretion not affected	Duksin and Bornstein, 1979
IgM of X63/38C-13	secretion inhibited	Blatt and Haimovich, 1981
IgG of X63/38C-13	secretion not affected	Blatt and Haimovich, 1981
IgA of DAKIKI Arosros-1 and IgM of BJAB	secretion inhibited	Cushley *et al.*, 1982
IgG of EB-4, Bec 11 and MAJA	secretion not affected	Cushley *et al.*, 1982
IgD of B1.8δ	secretion not affected	Vasilov and Ploegh, 1982

Table 2. Effects of tunicamycin on plasma membrane glycoproteins.

Glycoproteins	Result	References
Sindbis viral glycoproteins	membrane insertion inhibited; enhanced proteolysis	Schwarz *et al.*, 1976
Semliki viral glycoproteins	membrane insertion inhibited	Leavitt *et al.*, 1977
Acetylcholine receptor in muscle cells	transport not affected; enhanced proteolysis	Trowbridge *et al.*, 1978
Vesicular stomatitis viral G protein	membrane insertion not affected	Gibson *et al.*, 1978
IgA of MOPC 315	membrane insertion not affected	Hickman and Wong-Yip, 1979
Erythrocyte glycophorin	membrane insertion not affected	Gahmberg *et al.*, 1980
HLA-A and -B antigens of JY	membrane insertion not affected	Ploegh *et al.*, 1981
IgM of W279	membrane insertion not affected	Sibley and Wagner, 1981
Transferrin receptor of CCRF-CEM	membrane insertion not affected	Omary and Trowbridge, 1981
LDL receptor of human fibroblasts	membrane insertion inhibited	Chatterjee *et al.*, 1981
IgA of DAKIKI-Aros-ros-1 and IgM of BJAB and DAUDI	membrane insertion inhibited	Cushley *et al.*, 1981
Murine leukemia viral glycoproteins	membrane insertion inhibited	Polonoff *et al.*, 1982
IgM of DAUDI	membrane insertion inhibited	Kubo and Pelanne, 1983
Asialoglycoprotein receptor of HepG-2	membrane insertion not affected	Breitfeld *et al.*, 1984
Mouse hepatitis viral glycoprotein E-2	membrane insertion inhibited	Repp *et al.*, 1985

residues (Baeuerle and Huttner, 1984). Although sulphation is a widespread occurrence, its function is still unknown (Huttner, 1982). However, with the exception of a single case (Liu and Baenziger, 1985), all other reported proteins that possess tyrosine sulphated residues are destined for secretion. In addition, comparative analysis on the plasma proteins and the proteins from various tissues have also demonstrated that the former has a much higher level of tyrosine sulphate content (Hille *et al.*, 1984). These observations strongly implicate the involvement of tyrosine sulphation as a secretory mechanism. Thus, in the case of the non-N-glycosylated IgGs, sulphation has been suggested as an alternative mechanism in ensuring their export (Baeuerle and Huttner, 1984).

The second scenario is best illustrated in the immunoglobulin system. The data of Hickman and Kornfeld (1978) have clearly demonstrated that the extent of inhibition of immunoglobulin secretion correlates with the carbohydrate content and the overall molecular size of the various immunoglobulin isotypes. Accordingly, the secretion of the IgG, which is a monomer with (in most cases) a single N-linked oligosaccharide moiety on each of its gamma chains, is not significantly reduced (Hickman and Kornfeld, 1978). In addition, the absence of carbohydrate moieties have also been demonstrated to cause an alteration on the physicochemical property of the proteins as well as decreasing their stability (Tarentino *et al.*, 1974; Chu *et al.*, 1978; Hickman and Wong-Yip, 1979). Such changes in conformation and solubility would be most likely to have a greater effect on the highly glycosylated molecules like IgA, IgM and IgE, although other factors like the primary structure of the protein and the location of the oligosaccharide structures may be equally important. The presence of other types of carbohydrate moieties, e.g., O-linked oligosaccharide chains of IgA or IgD (Dawson and Clamp, 1968; Jefferis *et al.*, 1975), not affected by tunicamycin could also contribute to the extent of blockade of the glycoprotein secretion.

1.4.2. Facilitating Intracellular Routing.

While secretion involves the ultimate export of macromolecules, carbohydrate moieties could also play a role in the sorting and the regulation of intracellular trafficking of glycoproteins. N-linked oligosaccharides of glycoproteins are extensively processed as they traverse the Golgi apparatus (see section 1.2.2). The oligosaccharide processing may play a role in acquiring a specific configuration involved in trafficking mechanism. Indeed, transport of lysosomal glycoproteins have been shown to be regulated by such a mechanism; via the mannose-6-phosphate residues of their N-linked oligosaccharide chains (see section 1.2.2). The involvement of carbohydrate moieties of secretory or membrane-associated proteins, on the other hand, is less definite.

By utilizing specific processing inhibitors, numerous studies have shown that while the trimming of terminal glucose residues in some glycoproteins is essential for their efficient maturation from the rough endoplasmic reticulum to the Golgi apparatus, such processing is less crucial in other glycoproteins (see tables 3 and 4). Inhibition of mannosidases of the Golgi apparatus have, however, been consistently reported to have no inhibitory effect on the kinetics of the export of glycoproteins (see tables 3 and 4).

The above selective inhibitory phenomena would again indicate different trafficking mechanisms for individual glycoproteins unless the regulation of transport is dependent upon some other controlling parameters. Currently, at least two separate exocytic transport pathways are recognized (reviewed by Farquhar, 1985). Secretory proteins could either be transported via a regulatory (i.e., dependent on an appropriate signal) or a constitutive mechanism (i.e., continually maintained) (Moore and Kelly, 1985). These distinct transport mechanisms provide excellent rationalization of the above selective inhibitory phenomena. While the

Table 3. Effects of N-linked oligosaccharide processing inhibitors on secretory glycoproteins.

Glycoproteins	Processing Inhibitor	Result	References
IgD of B1.8δ	dNM	secretion inhibited	Peyrieras *et al.*, 1983
IgM of B1.8μ	dNM	secretion not affected	Peyrieras *et al.*, 1983
IgM of B1.8μ and IgD of B1.8δ	SW	secretion not affected	Peyrieras *et al.*, 1983
α_1-antichymotrypsin and α_1-antitrypsin of HepG-2	dNM	secretion inhibited	Lodish and Kong, 1984
Transferrin, albumin and glycoprotein C3 of HepG-2	dNM	secretion not affected	Lodish and Kong, 1984
IgM of B1.8μ and IgD of B1.8δ	dMM	secretion not affected	Fuhrmann *et al.*, 1984
Caeruloplasmin and α_1-antitrypsin of HepG-2	CSP	secretion inhibited	Sasak *et al.*,1985
α-acid glycoprotein and α-proteinase inhibitor of rat hepatocytes	dMM	transport not affected	Gross *et al.*,1985
Secretory glycoproteins of HepG-2	SW	transport and secretion accelerated	Yeo *et al.*,1985
α-acid glycoprotein and α-proteinase inhibitor of rat hepatocytes	Methyl-dNM dNM and CSP	transport not affected	Gross *et al.*,1986
Thyroglobulin of porcine thyroid cells	dNM and SW	secretion not affected	Franc *et al.*,1986
IgE of a mouse hybridoma cells	CSP, methyl-dNM and SW	secretion not affected	Granato and Neeser, 1987
IgM of 4A3 and IgG of I1A1.4	CSP, dNM, dMM and SW	secretion not affected	Hashim and Cushley, 1987b,c

Table 4. Effects of N-linked oligosaccharide processing inhibitors on plasma membrane glycoproteins.

Glycoproteins	Processing Inhibitor	Result	References
Vesicular stomatitis viral glycoproteins	SW	membrane insertion not affected	Kang and Elbein, 1982
HLA-A, -B and -C antigens of BAU	dNM and dMM	membrane insertion not affected	Burke *et al.*, 1984
Asialoglycoprotein receptor of HepG-2	SW	membrane insertion not affected	Breitfeld *et al.*, 1984
Influenza viral glycoproteins	dMM	membrane insertion not affected	Elbein *et al.*, 1984
Rous sarcoma viral glycoproteins	dMM and SW	membrane insertion not affected	Bosch *et al.*, 1985
Mouse hepatitis viral glycoprotein E-2	methyl-dNM and CSP	membrane insertion inhibited	Repp *et al.*, 1985
Mouse hepatitis viral glycoprotein E-2	dMM and SW	membrane insertion not affected	Repp *et al.*, 1985
Feline sarcoma viral oncogene products	methyl-dNM and CSP	membrane insertion inhibited	Nichols *et al.*, 1985
Feline sarcoma viral oncogene products	SW	membrane insertion not affected	Nichols *et al.*, 1985
Insulin and Insulin-like growth factor I receptors of IM-9	SW	membrane insertion not affected	Duronio *et al.*, 1986

Key; dNM: 1-deoxynojirimycin dMM: 1-deoxymannojirimycin
CSP: Castanospermine SW: Swainsonine

transport of the inhibited glycoproteins reflects that of the regulated-type mechanism, that of the non-inhibited glycoproteins would presumably be of the constitutive-type.

In the case of immunoglobulins, further controversy surrounds the different effects reported on the transport and ultimate insertion of the membrane-form non-N-glycosylated IgA and IgM (see table 2). While certain data exhibit no interference in the membrane localization of the surface molecules (Hickman and Wong-Yip, 1979; Sibley and Wagner, 1981), others demonstrate inhibition (Cushley, 1981; Yuan, 1982; Kubo and Pelanne, 1983; Sitia *et al.*, 1984). Much of these discrepancies appear to arise from the different experimental approaches that were adopted, and several arguments have already been proposed to counter the apparent detection of the non-N-glycosylated immunoglobulins at the plasma membrane (Cushley, 1981; Yuan, 1982; Kubo and Pelanne, 1983; Sitia *et al.*, 1984), i.e., there is no evidence to indicate that the immunoglobulins detected on the cell surface are indeed the membrane-bound form and are of the non-N-glycosylated type.

However, the distinct effects that are observed would suggest involvement of different transport mechanisms or the existence of separate transport pathways followed by the secreted and membrane-bound immunoglobulins. Indeed, the possibility of a single cell type having two separate exocytic pathways has been demonstrated to occur in a mouse pituitary tumour cell line, i.e., by possessing both the constitutive and regulatory mechanisms (Gumbiner and Kelly, 1982). Although the secretory mechanism in plasma cells has been implicated to be of the constitutive type (Tartakoff and Vassalli, 1977 and 1978), such a mechanism could perhaps account only for the major synthetic products of the cells, i.e., the immunoglobulins, or alternatively, multiple constitutive intracellular transport channels could be in existence. Thus, it is still highly possible that membrane-

bound immunoglobulins could be transported and deposited via a different pathway of similar or dissimilar mechanisms.

In further support of this hypothesis, the work of Strous and Lodish (1980) have demonstrated that plasma membrane glycoproteins may be synthesized at twice the rate of a secreted glycoprotein, implying that the two processes have to differ at some stage. In an extended study, they have also reported that the maturation of secreted proteins from the rough endoplasmic reticulum to the Golgi apparatus in human hepatoma HepG-2 cells occur at different and characteristic rates (Lodish *et al.*, 1983).

Currently, the accepted view on the structures of the distinct monomeric secretory and membrane forms of a single immunoglobulin isotype is that the latter slightly differ by possessing an extra hydrophobic peptide sequence at the carboxy termini of its heavy chains, that functions as an anchorage in the plasma membrane (Williams *et al.*, 1978; Singer *et al.*, 1980; Cushley, 1981; Cushley *et al.*, 1982). If the controversial immunoglobulins detected on the cell surface are indeed the non-N-glycosylated membrane-bound form, then the obvious implication would be that the hydrophobic sequence of the cell surface immunoglobulins is somehow involved in the prevention of blockade at the membrane of the rough endoplasmic reticulum.

Alternatively, the selective inhibition of secretory IgM and IgA is perhaps due to the absence of carbohydrate moieties, which may hinder their proper assembly, or the requirement for intact carbohydrate moieties of J chains of the polymeric molecules. The J chains of the polymeric immunoglobulins have been demonstrated to be indirectly involved in facilitating secretion (reviewed by Koshland, 1985). However, a recent report by Cattaneo and Neuberger (1987) has

demonstrated that in absence of J chains, assembly and secretion of IgM can occur as efficiently.

1.4.3. Protection Against Degradation.

In certain glycoproteins, e.g., fibronectin and the acetylcholine receptor, the failure to detect secretion upon inhibition of glycosylation is due not to the inability of the non-glycosylated proteins to be exported, but rather is because they are being degraded intracellularly. Interference with N-glycosylation of these proteins has been shown to enhance their intracellular degradation (reviewed by Olden *et al.*, 1982). Non-glycosylated IgM has similarly been shown to be more rapidly catabolized within the cell (Dulis *et al.*, 1982). Therefore, the carbohydrate, in these cases, serves to protect glycoproteins against specific proteases. The exact mechanism involved in such protective function is still unresolved. Enhanced proteolysis could be due to the exposure of sensitive sites of the proteins in the non-glycosylated state or alternatively it could be a result of the co-packaging of lysosomal hydrolases with surface or secretory products during export.

While the total carbohydrate structures provide protection for sensitive sites on the proteins from the actions of proteases, the transfer of terminal sugars to oligosaccharide moieties have also been demonstrated to serve a similar purpose but in a slightly different context. Most glycoproteins are known to have terminal sialic acid residues in the oligosaccharide chains, and are thus termed the "complex type" (see section 1.2.2). One of the functions of this process is to prevent the inner sugar residues (normally galactose) from being recognized by specific receptors mediating pinocytosis and catabolism of glycoproteins. Asialoglycoproteins have been demonstrated to be efficiently internalized by hepatocytes, fibroblasts and macrophages through this mechanism (reviewed by Ashwell and Harford, 1982). Similarly, it has been shown that the enzymic removal of sialic acids from whole

erythrocytes leads to their rapid clearance from the blood stream (Schauer, 1982). These erythrocytes were then detected to be bound to macrophages and were later phagocytosed (Schauer, 1982; Schauer *et al.*, 1984).

Another example of the protective roles played by carbohydrate moieties is the glucosylation of the lipid-linked oligosaccharide occuring in the rough endoplasmic reticulum. In rat splenic lymphocytes, it has been discovered that non-glucosylated lipid-linked oligosaccharides were selectively degraded by a phosphodiesterase, clearly suggesting the protective role of the glucose residues (Hoflack *et al.*, 1981). In addition, Parodi and his co-workers (1983 and 1984) have also shown that protein-bound non-glucosylated high mannose units may be transiently reglucosylated by an endoplasmic reticulum glucosyltransferase. This reaction has been demonstrated in mammalian, avian, protozoan and plant cells but is not detectable in yeast (Parodi *et al.*, 1983 and 1984). While no function for this reglucosylation has been demonstrated, it was speculated that the glucosylation served to protect protein-linked oligosaccharides from extensive degradation by the mannosidase of the endoplasmic reticulum.

1.4.4. Involvement in Ligand-Receptor Interactions.

As described in section 1.2.3, although all N-linked oligosaccharide chains of glycoproteins arise from a common biosynthetic pathway, subsequent modification processes have resulted in a diverse structural array of final oligosaccharide conformations. Carbohydrate molecules are well known for their high antigenicity (Feizi, 1984). Thus, the fine-structural differences could serve as specific ligands or receptors to meet the various specialized functions. The roles of these receptors could be further classified by functional criteria.

1.4.4.1. Interactions in Transport Mechanisms.

Cell surface lectin receptors, which are themselves glycoproteins, have been clearly demonstrated to be involved in mediating pinocytosis (reviewed by Ashwell and Harford, 1982). In liver cells, where hydrolases are being actively taken up and waste products rapidly catabolized, receptors for specific carbohydrate configurations have been detected. By means of *in vivo* serum clearance studies and actions of glycosidases to obtain specific structure of the carbohydrate moieties, receptors for terminal galactose (Morell *et al.*, 1968; Hickman *et al.*, 1970), mannose/N-acetylglucosamine (Stahl *et al.*, 1976; Achord *et al.*, 1977), fucose (Prieels *et al.*, 1978) and mannose-6-phosphate (Fischer *et al.*, 1980) residues have been detected on the plasma membrane of hepatocytes. Analysis of these receptors have clearly indicated that they are actively involved in recognizing and transporting the appropriate glycoproteins into the cells. Whether they are also involved in directing the glycoproteins to be degraded in the lysosome remains to be resolved. Studies assessing the importance of the carbohydrate units of these receptors have led to different conclusions. While the N-linked carbohydrate moieties of the mannose-6-phosphate receptors are crucial for their efficient ligand-binding, that of the asialoglycoprotein receptors are not quite as important (Breitfeld *et al.*, 1984).

Macromolecular internalization of low density lipoprotein (LDL), transferrin, epidermal growth factor (EGF) and insulin has also been reported to occur via the receptor-mediated endocytic mechanism (reviewed by Brown *et al.*, 1983, Pastan and Willingham., 1983 and Helenius *et al.*, 1983). One common feature shared by the receptors for the macromolecules is the possession of N-linked carbohydrate chains. However, this does not distinguish the receptors from other cell-surface proteins, such as histocompatibility antigens, that do not participate in endocytosis. In the case of the insulin receptor, although its

carbohydrate units have been shown to be crucial for ligand binding and signal transmission processes (Reed *et al.*, 1981; Ronnet and Lane, 1981; Cherqui *et al.*, 1982), their conversion to the complex-type structure is not as important (Duronio *et al.*, 1986). The role of the carbohydrate moieties in regulating the functions of the other receptors have yet to be assessed.

The involvement of lectin receptors in mediating exocytosis or secretion of glycoproteins has only begun to emerge. Following the elucidation of the oligosaccharide processing pathway of N-linked carbohydrate chains of glycoproteins (see section 1.2.2), specific lectins have been suggested to facilitate the transport of the macromolecules and their destination-targeting processes (reviewed by Olden *et al.*, 1982). Many membrane receptors that are engaged in pinocytosis, described in the above paragraph, have apparently been discovered to be more concentrated in intracellular compartments like the rough endoplasmic reticulum and the Golgi apparatus (Bleil and Bretscher, 1982). However, with the exception of mannose-6-phosphate receptors, their exact roles are still undetermined. The only established receptor-mediated intracellular transport mechanism is that of the lysosomal glycoproteins. These proteins have been shown to be phosphorylated in the *cis*-cisternae of the Golgi stack (reviewed by Sly and Fischer, 1982), and their transport to the lysosomes is mediated by receptors for mannose-6-phosphate, shown to reside in the rough endoplasmic reticulum and the Golgi apparatus (reviewed by von Figura and Hasilik, 1986).

The question of whether a similar receptor-mediated exocytic mechanism also applies to secretory and membrane-associated glycoproteins is intriguing. Preliminary studies appear to indicate the existence of such a mechanism, at least for some glycoproteins, and at certain stages of the oligosaccharide processing. The various inhibitors of the asparagine-linked oligosaccharide processing (see section

1.3.2) that are available certainly assure rapid progress in this area. Already, studies utilizing glucosidase inhibitors demonstrate selective inhibitory effect on glycoproteins (see table 2). However, interference with Golgi mannosidases appears not to exert any inhibitory effect on the kinetics of the glycoprotein transport (see table 2). The selective inhibition in these cases suggests the existence of rate-limiting steps and the possible involvement of a receptor-mediated trafficking mechanism, especially at the level of inter-compartmental transport between endoplasmic reticulum and Golgi apparatus. This is most appropriate in cases where the physiological concentrations of the glycoproteins need to be carefully monitored and strictly controlled. Such a mechanism may therefore be less relevant for cells at a differentiation stage fully committed in the secretion of glycoproteins (e.g., immunoglobulin secretion from plasma cells).

1.4.4.2. Interactions Mediating Biological Functions.

Carbohydrate molecules have been implicated in numerous interactions mediating biological functions. One of the most intriguing discoveries in assessing the functional role of carbohydrate constituents of glycoproteins is the identification of the various blood group antigens. To date, at least twenty different blood group systems have been recognized in man (Race and Sanger, 1975). Distinctions between the groups vary from a single sugar substitution at the erythrocyte surface carbohydrate molecules, as in the case of A and B blood group antigens, to a few sugar residues. Antibodies to foreign erythrocyte surface antigens are naturally produced.

Carbohydrate units have also acted as antigenic epitopes in numerous other systems. Studies utilizing monoclonal antibodies have revealed that most cell surface differentiation and tumour associated antigens from diverse cell types are indeed saccharide structures of glycoproteins and glycolipids (Thorpe and Feizi, 1984; Feizi *et al.*, 1984). These antigenic markers belong to a family of

carbohydrate structures which also include the major blood group antigens.

The Major Histocompatibility Complex (MHC) encodes other surface antigens that mediate immunological specificity. These glycoproteins which possess N-linked glycans are essential for reactions of specific immune recognition. Foreign cells are recognized by their distinct MHC molecules and are thus acted upon by the cytotoxic T cells (reviewed by Burakoff *et al.*, 1984). Recognition and lysis of syngeneic virally-infected cells is regulated by the MHC as well as the viral antigens (Zinkernagel and Doherty, 1974; Shearer, 1974). Oligosaccharide moieties of the MHC molecules and of the G proteins of the vesicular stomatitis virus-infected cells, however, have so far been found not to confer any specific immunological function as assessed by their non-glycosylated form reactivity with alloantisera, MHC-directed monoclonal antibody as well as cytolysis by alloimmune cytotoxic cells (Parham *et al.*, 1977; Ploegh *et al.*, 1981; Black *et al.*, 1981).

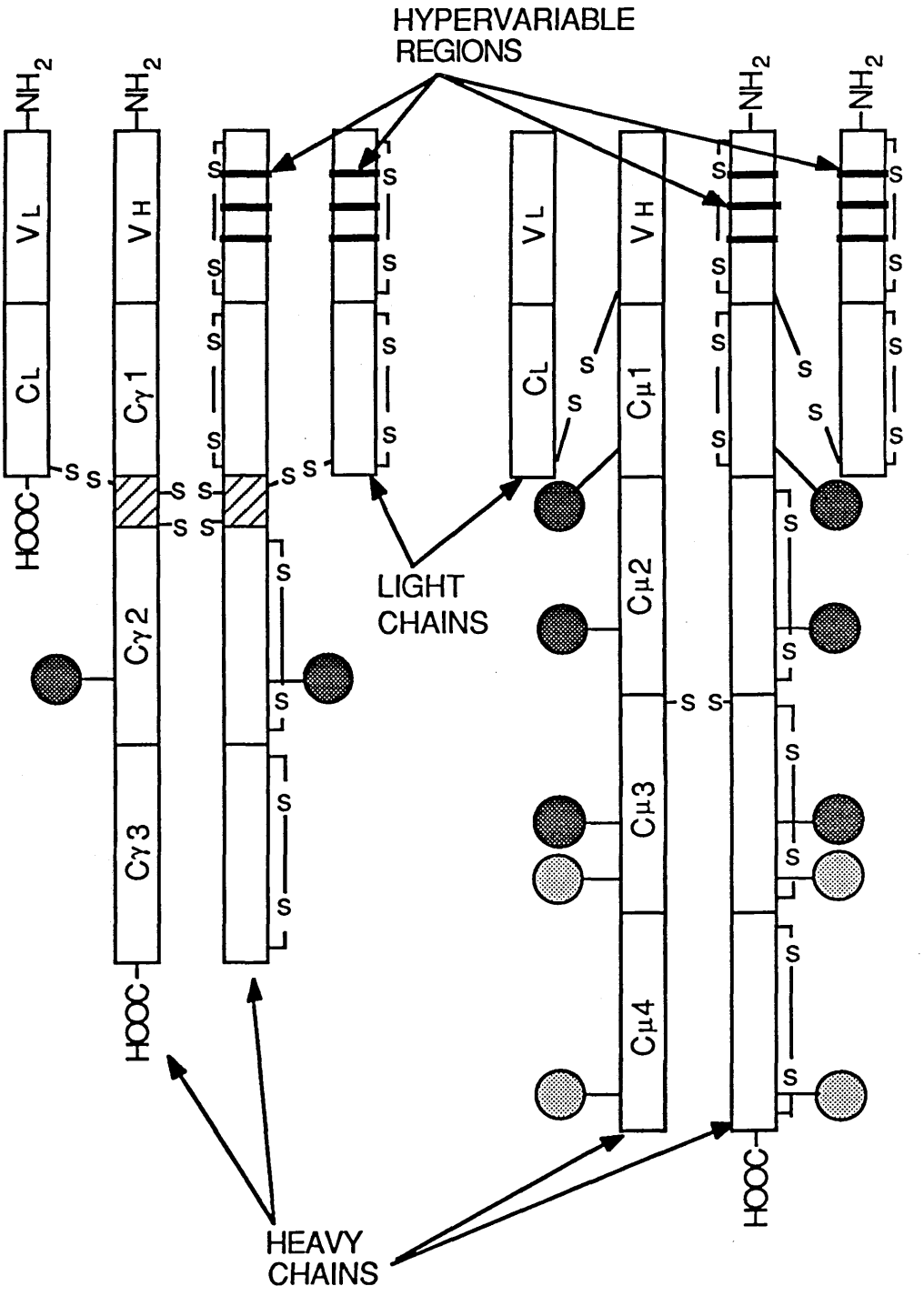
Another of the body's defence mechanisms involved in the destruction of foreign elements is the activation of the enzyme cascades of the complement system. Generally, the complement's three major functions are; the activation of the immune system (e.g., macrophages), opsonization, where complement facilitates the phagocytosis of antigens, and cytolysis of target cells (reviewed by Perlmutter and Cotton, 1986 and Muller-Eberhard, 1986). One of the routes involved in the fixation of complement is through the binding of C1q subcomponent of the cascade by bound antibodies, i.e., the classical complement pathway. In IgG isotypes, C1q binding has been shown to occur at the Cγ2 domains of the antibody molecule (Ellerson *et al.*, 1972; Yasmeen *et al.*, 1976), which are also known to contain N-linked oligosaccharide chains (see figure 5). It has further been shown that binding of C1q subcomponent to non-glycosylated IgG is markedly impaired compared with that of fully-glycosylated molecules, implicating the involvement of the

Figure 5. The basic structures of IgG and monomeric IgM molecules.

Structures of IgG and monomeric IgM molecules are schematically illustrated. The amino terminal end is characterized by sequence variability (V) in the heavy (H) and light (L) chains. The rest of the molecules have relatively constant structures. The constant portion of the light chain is termed the C_L region. The constant portion of the heavy chain is further divided into three discrete domains for IgG (i.e., Cγ1, Cγ2 and Cγ3) and four domains for IgM (i.e., Cμ1, Cμ2, Cμ3 and Cμ4). Each of the γ-heavy chains possess one complex-type N-linked carbohydrate moiety (Asn-297). The μ-heavy chain contains five N-linked oligosaccharide chains, of which three are complex-typed (Asn-170, -332 and -395) and the other two having high mannose structures (Asn-402 and 563) (reviewed by Winkelhake, 1978).

<u>KEY:</u>

- hinge region
- complex-type N-linked oligosaccharide
- high mannose-type N-linked oligosaccharide


HYPERVARIABLE
REGIONS
NH2
VL
CL
VH
Cγ1
Cγ2
Cγ3
HOOC
LIGHT
CHAINS
Cμ1
Cμ2
Cμ3
Cμ4
HEAVY
CHAINS

carbohydrate units (Koide *et al.*, 1977; Winkelhake *et al.*, 1980; Nose and Wigzell, 1983). This phenomenon has been demonstrated to arise from the increase in the K_d values of the binding interactions of C1q with non-N-glycosylated IgG, although the binding capacity is not affected (Leatherbarrow *et al.*, 1985). Interestingly, the presence of free sugar molecules has also been observed to have a slightly inhibitory effect (Koide *et al.*, 1977).

Analogous to the fixation of complement, immunoglobulin carbohydrate moieties have also been implicated to be involved in other Fc-mediated phenomena, i.e., direct binding with monocytes or macrophages or by rosette formation with sheep erythrocytes, and the induction of antibody dependent cellular cytotoxicity (ADCC) (Koide *et al.*, 1977; Nose and Wigzell, 1983). Although most early reports on the binding interactions in these biological activities have indicated involvement of the Cγ3 domains of IgGs (Okafor *et al.*, 1974; Ciccimarra *et al.*, 1975; Barnett-Foster *et al.*, 1980), studies by Burton and co-workers (Woof *et al.*, 1984 and 1986; Partridge *et al.*, 1986) have conversely suggested that the monocyte binding domain on human IgG is found on the Cγ2 domain. Thus, a direct involvement of carbohydrate moieties in the Fc binding interactions is still highly possible.

1.4.4.3. Interactions in Disease-Associated Phenomena.

Autoimmune disorders are diseases in which the body abnormally generates antibodies against self-antigens. This could either be a consequence of an external induction leading to the generation of antibodies to the self-antigens or a result of abnormal changes of the body's own self-antigens. In many of the reported autoimmune disorders, autoantibodies are demonstrated to be directed against carbohydrate epitopes (Parekh *et al.*, 1985; Feizi and Childs, 1985).

The sera of patients with the autoimmune hemolytic disorder known as cold agglutinin disease (which is associated either with chronic lymphoproliferative disorders or may transiently follow infections with *Mycoplasma pneumoniae* or Epstein Barr virus) have been reported to consist of monoclonal antibodies directed against the developmentally regulated carbohydrate I and i antigens of human erythrocytes (Feizi and Childs, 1985). The i antigen (consisting of repeating N-acetyllactosamine units) is a marker of human foetal erythrocytes and is largely substituted by the I antigen (consists of a "branched-type" repeating N-acetyllactosamine units) at approximately one year of age.

Rheumatoid arthritis is a well known disease in which the immune complexes that are present consist exclusively of immunoglobulins. The sera of patients suffering from this disease have been reported to consist largely of antibodies with transformed N-linked oligosaccharides. These antibodies could both act as the autoantigen (most commonly IgG) as well as the rheumatoid factor. Characterization of the carbohydrate moieties of IgGs from these patients demonstrates that they largely consist of the agalactoside-type oligosaccharide chains (Parekh *et al.*, 1985). These novel carbohydrate moieties of IgGs have also been shown to exist in the sera of primary osteoarthritic patients (Parekh *et al.*, 1985).

1.5. Models of Intracellular Routing of Glycoproteins.

1.5.1. Mechanisms of Intracellular Routing.

Accumulated evidence has indicated no unifying mechanism of glycoprotein transport. Carbohydrate moieties of glycoproteins are involved in the mechanism of transport of some glycoproteins but appear to have no function in others (see section 1.4.2).

At least three classes of glycoproteins, i.e., plasma membrane, secretory

and lysosomal glycoproteins use a common site, the rough endoplasmic reticulum, for their membrane translocation. Current evidence suggests that only in case of the latter, do carbohydrate moieties play a role in the regulation of transport of glycoproteins (see section 1.4.4.1). Studies from different systems appear to indicate that oligosaccharides are not crucial for the transport of secretory and membrane glycoproteins (see section 1.4.4.1; Greene *et al.*, 1981). In specific cases, where inhibition of N-glycosylation leads to the termination of protein export, disruption is more likely to be due to the severe changes in the physicochemical properties of the proteins (Hickman and Kornfeld, 1978).

The intracellular sorting of proteins is an unequivocally clear phenomena, as demonstrated in the epithelial cells, MDCK. Membrane proteins of these cells are observed to be segregated into two distinct plasma membrane domains, i.e., apical and basolateral, which are biochemically, physiologically and morphologically distinct (Misfeldt *et al.*, 1976; Cereijido *et al.*, 1978 and 1980; Louvard, 1980). Carbohydrate moieties have been shown not to be involved in the trafficking of these proteins (Green *et al.*, 1981). However, the existence of the sorting phenomena certainly demonstrates multiple mechanisms of intracellular protein segregation and also implicates the involvement of a receptor-mediated transport process.

The common existence of two distinct transport mechanisms within a single cell has also been unravelled in the endocrine cell line AtT-20 (Gumbiner and Kelly, 1982). While most of plasma membrane glycoproteins of these cells follow the constitutive mechanism, export of secretory glycoproteins requires appropriate signals. These proteins are firstly retained in storage vesicles and only released when the appropriate signals are acquired. Regulation of exocytosis often involves the activation of G protein, although the mechanisms that are associated vary

between distinct cell types (reviewed by Burgoyne, 1987). Transport via the constitutive mechanism may occur either by a distinct receptor-mediated process or simply by non-carrier-mediated bulk flow (Moore and Kelly, 1985).

While the transport of selective proteins clearly demonstrates involvement of specific signals, existing evidence appears to indicate that most of the other proteins that are destined for export or deposition into the plasma membrane are simply transported by bulk flow. Studies on the export of simple glycopeptides that follow normal intracellular routing via endoplasmic reticulum and Golgi apparatus to the cell surface have demonstrated an equal or a slightly faster rate of transport compared to those of the plasma membrane and secretory proteins (Wieland *et al.*, 1987). With the existence of such a rapid and efficient intracellular bulk flow, the export of proteins certainly does not require any form of receptor-mediated mechanism. Proteins that are free to diffuse through the membrane of the rough endoplasmic reticulum may be swiftly swept to the cell surface by default. Thus, the view that there may exist receptors that may promote the transport of proteins (Fitting and Kabat, 1982) is becoming more unpopular.

Another line of evidence that strongly support this notion is the identification of signals that act to retain specific proteins in particular intracellular compartments. Several reports have already described that deletion of part of glycoproteins that normally reside in the endoplasmic reticulum resulted in their transportation to the cell surface or secretion into the extracellular environment, suggesting that the truncated fragment may contain the specific signal for retention (Poruchynsky *et al.*, 1985; Paabo *et al.*, 1987). More direct evidence comes from the recent work of Munro and Pelham (1987). They have observed that a number of proteins found in the endoplasmic reticulum possess common tetrapeptide sequence, Lys-Asp-Glu-Leu. Deletion of this sequence from one of the proteins

was subsequently demonstrated to result in its secretion. Moreover, when the sequence was transplanted to the C-terminus of lysozyme, which is a protein that is normally secreted, the lysozyme derivative was found to be retained in the endoplasmic reticulum.

Compartmental retention of proteins may also be accomplished by a slightly different mechanism. It is well known that the subunit assembly of immunoglobulins and histocompatibility antigens is a prerequisite for their secretion. Non-assembled heavy chains of immunoglobulins have been found to be associated with a cellular protein located in the lumen of the endoplasmic reticulum, known as BiP, i.e., immunoglobulin heavy chain binding protein (Haas and Wabi, 1983; Gething *et al.*, 1986). Immunoglobulin heavy chains are only released from these proteins when their proper assembly with the light chains has taken place. Bole *et al.* (1986) have accordingly suggested that BiP functions as a safeguard measure in the prevention of export of improperly assembled molecules. Another protein that may be regulated by the same mechanism is the influenza viral haemagglutinin (HA). Non-assembled HA molecules have also been found to be associated with BiP, and release of these molecules may only occur after completion of their trimerization process.

1.5.2. Rationale of Distinctive Mechanisms of Intracellular Routing.

The different types of intracellular trafficking mechanisms that have been proposed appear to be constructed to meet the functional requirements of individual types of glycoproteins and cells. The transport mechanism involving mannose-6-phosphate ligand-receptor interactions is exclusively designed for the lysosomal enzymes to be routed to the lysosomes (reviewed by von Figura and Hasilik, 1986). In the absence of carbohydrate moieties, transportation of these enzymes is chaotically disrupted. Involvement of such mechanisms are also observed in the transport of other intracellular-site-specific proteins such as those residing in the

mitochondria (reviewed by Colman and Robinson, 1986; Hurt and van Loon, 1986), although signal sequences may not be carbohydrate residues.

In addition, signal sequences may also act to retain proteins in specific compartments (reviewed by Rothman, 1987). Most secretory and plasma membrane-associated proteins are strongly believed to be transported to the cell surface, en routing the rough endoplasmic reticulum and Golgi apparatus, by bulk flow (Wieland *et al.*, 1987). In order to abstain from being swept by such a mechanism, resident proteins of the endoplasmic reticulum and Golgi apparatus have acquired specific retention signal sequences that embed themselves to their respective compartments. A slightly different retention mechanism may also exist in the cell to prevent the secretion of improperly assembled macromolecules. The cell has developed certain proteins that function to retain defective molecules from further transportation (Bole *et al.*, 1986).

In the case of endocrine cells, a similar constitutive mechanism is also in existence to handle the trafficking of integral proteins. However, in order to maintain the physiological concentration of various hormones, another channel, that may be efficiently controlled, is also required. This is ingeniously achieved by the alternative regulatory mechanism of the same cell.

The physiological concentration of immunoglobulins of the body is also rigorously regulated. However, in this case, and conforming to the clonal selection hypothesis, regulation is postulated to be at the level of differentiation of plasma cells. Enhancement of antibody production is speculated to be associated with plasma cell blastogenesis. This notion is supported by the work of Tartakoff and Vassalli (1977 and 1978) who have indirectly shown that the export of immunoglobulins from actively secreting plasma cells occurs constitutively, without

involvement of any regulatory mechanism.

1.5.3. Immunoglobulins as a Model System for Studying Carbohydrate Moieties of Glycoproteins.

Immunoglobulins offer an excellent model system to study the role of N-linked oligosaccharides of glycoproteins. Advantages of using immunoglobulins are;

a) the availability of well established cell lines and hybridomas which secrete homogeneous immunoglobulins of specific isotypes and at specific differentiation stages;

b) the availability of specific anti-immunoglobulin antisera of high avidity, thus permitting easy isolation and detection of radiolabeled immunoglobulins;

c) the variations in types and numbers of oligosaccharide components within particular immunoglobulin isotypes provide a wide range of distinctive examples;

d) the potential role of oligosaccharides in many effector functions of immunoglobulins that warrants further resolution; and

e) the existence of numerous well established assays of immunoglobulins for low-level quantitation purposes.

1.6. Objectives of study.

The functions of carbohydrate moieties of glycoproteins are surrounded by much controversy. Their role in the regulation of glycoprotein transport is obscured by the finding that only certain glycoproteins have their secretion impaired under non-N-glycosylated condition (see section 1.4.1).

Many of the studies which have addressed the function of oligosaccharide side chains in the transport of glycoproteins have employed tunicamycin as the inhibitor of N-glycosylation (see section 1.3.1.2). One particular criticism of the data obtained from experiments using this inhibitor is that the drug itself may act to disrupt cellular transport processes in addition to its inhibition of protein glycosylation. To address this particular point, a study of the biological activities of three structurally-modified tunicamycin analogues with respect to macromolecular biosynthesis, glycosylation and immunoglobulin export from rat hybridoma cells was undertaken (this thesis, and Hashim and Cushley, 1987a).

The recent advent of N-linked oligosaccharide processing inhibitors provides a means to investigate the role of carbohydrate processing in the regulation of glycoprotein transport. Already, studies in many systems have indicated a similar selective inhibition of transport of glycoproteins (see section 1.4.2). To provide a wider perspective to this area of endeavour, a study was carried out to determine the effects of N-linked oligosaccharide processing inhibition on the transport of IgM and IgG_{2b} from rat hybridoma cells (this thesis, and Hashim and Cushley, 1987b and c).

N-linked oligosaccharide moieties of immunoglobulins have been strongly implicated to be involved in various effector functions of immunoglobulins. Non-N-glycosylated IgGs have been reported to be less effective in activating the

complement cascade, in direct binding with monocytes or macrophages or by rosette formation with sheep erythrocytes, and the induction of antibody dependent cellular cytotoxicity (see section 1.4.4.2). To extend these observation(s), a study examining the effects of IgG_{2b} synthesized in the presence of processing inhibitors on the expression of effector functions of immunoglobulins was undertaken.

CHAPTER II

MATERIALS AND METHODS

2.1. General Materials.

The materials used during the course of this study and their respective suppliers are described;

2.1.1. Cell Culture Materials.

RPMI-1640 medium, foetal calf serum (FCS) and penicillin/streptomycin solution were obtained either from Gibco Biocult Ltd., Paisley, Scotland or Northumbria Biologicals Ltd., Northumbria, England. Tissue culture flasks were purchased from Costar Northumbria Biologicals Ltd., Northumbria, England and accessory sterile plastics were supplied by Sterilin Ltd., Feltham, England.

2.1.2. Fine Chemicals.

All chemicals used were of the highest grade available commercially. With exceptions of the following, all other chemicals were supplied by BDH Chemicals, Poole, Dorset, U.K.

Tris (hydroxymethyl) aminomethane (i.e., trizma base) was obtained from Boehringer Corporation, Lewes, U.K.

Hydrogen peroxide and sodium hydrogen carbonate were purchased from Fisons Scientific Apparatus Ltd., Loughborough, Leics., U.K.

Sodium chloride and sucrose were supplied by Formachem Ltd., Strathaven, Scotland, U.K.

Calcium chloride, calcium nitrate tetrahydrate and sodium hydroxide were obtained from Hopkins and Williams, Chadwell Heath, Essex, U.K.

Dimethylsulphoxide and trichloroacetic acid were purchased from Koch Light Laboratories, England.

Amino acids, D-galactose, D-mannose, methyl-α-D-glucopyranoside, methyl-α-D-mannopyranoside, N-acetylglucosamine, O-phenylene diamine, salicylic acid, sodium deoxycholate, sodium pyruvate, and vitamins were supplied by Sigma Chemical Co. Ltd., Poole, Dorset, England.

2.1.3. Tunicamycin and Analogues.

Tunicamycin was purchased either from Sigma Chemical Co. Ltd., Poole, Dorset, England, Boehringer Corporation, London, England or Miles Laboratories Ltd., Slough, U.K. Analogues of tunicamycin were kindly provided by Drs. R.T. Hotstons and A.R. Williamson of Glaxo Group Research Ltd.

2.1.4. Inhibitors of Oligosaccharide Processing.

Castanospermine (CSP), 1-deoxynojirimycin (dNM), 1-deoxymanno-jirimycin (dMM) and Swainsonine (SW) were obtained from Boehringer Corporation, Lewes, U.K.

2.1.5. Radiochemicals.

All radiochemicals were obtained from the Amersham International plc., Amersham, England. The specific activities of the radiochemicals used are described in table 5.

2.1.6. Enzymes.

All enzymes were purchased from Boehringer Corporation, Lewes, U.K. The specific activities of the enzymes are described in table 6.

Table 5. Specific activities of radiochemicals.

Radiochemicals	*Specific Activities*
L-[^{35}S]-methionine	>1200Ci/mmol
L-[4,5-^{3}H]-leucine	53Ci/mmol
[methyl-^{3}H]-thymidine	52Ci/mmol
[5-^{3}H]-uridine	28.5Ci/mmol
D-[2-^{3}H]-mannose	19Ci/mmol

Table 6. Specific activities of enzymes.

Enzymes	*Specific Activities*
Endoglycosidase H (*S. plicatus*)	45I.U./mg
Glycopeptidase F (*F. meningosepticum*)	25,000U/mg
Neuraminidase (*C. perfringens*)	3U/mg

2.1.7. Serological Reagents.

Rabbit anti-rat IgG (RArIg)	Miles Laboratories Ltd., Slough, U.K.
Donkey anti-rabbit IgG (DARIg)	Scottish Antibody Production Unit.
Guinea pig complement	Sera-Lab, Sussex, U.K.
Haemolysin	Flow Laboratories Ltd., Irvine, Scotland.
RArIg-Horseradish-peroxidase	Miles Laboratories Ltd., Slough, U.K.

2.1.8. Electrophoretic Materials.

Acrylamide, 2-mercaptoethanol, N,N'-methylene bis acrylamide, N,N,N'N'-tetramethylenediamine (TEMED) and ammonium persulphate were obtained from BDH Chemicals, Poole, Dorset, U.K.

2.1.9. Photographic Materials.

X-Omat-H X-ray film	Kodak (U.K.) Ltd., London.
SX-80 developer	Kodak (U.K.) Ltd., London.
FX-40 X-ray liquid fixer	Kodak (U.K.) Ltd., London.

2.1.10. Miscellaneous Reagents.

Vibrio cholerae serotype Inaba (3S A3) was obtained from The David Bruce Laboratories, Wiltshire, U.K. Lipopolysaccharide (*E. Coli* 012.B8) and Concanavalin A-sepharose 4b were purchased from Sigma Chemical Ltd., Poole, Dorset, England. Fresh sheep erythrocytes were supplied weekly by Gibco Biocult. Ltd., Paisley, Scotland.

2.2. Standard Solutions.

Unless otherwise stated, all buffers were stored at 4°C.

2.2.1. Phosphate-Buffered Saline (PBS-A).

170mM NaCl

3.4mM KCl

10mM Na_2HPO_4

1.8mM KH_2PO_4 pH 7.2

2.2.2. TKM Buffer.

5mM MgCl2

0.1M KCl

0.1M Tris-HCl pH 8.2

2.2.3. Three-Detergent Lysis Buffer (3D-TKM).

The following detergents were added to TKM buffer;

1% (W/V) Triton X-100

1% (W/V) Deoxycholic acid

0.5% (W/V) SDS

2.2.4. Immunoprecipitate Wash Buffer.

1% (W/V) SDS

0.01% (W/V) Phenol red

50mM Tris-HCl pH 8.8

(Stored at room temperature)

2.2.5. SDS-PAGE Reducing Sample Buffer.

2% (W/V) SDS

10% (W/V) Glycerol

0.001% (W/V) Bromophenol blue

5% (V/V) 2-mercaptoethanol

62.5mM Tris-HCl pH 6.8

(Stored at -20°C)

2.2.6. Con A buffer.

1mM $CaCl_2$

1mM $MgCl_2$

1mM $MnCl_2$

0.3M NaCl

0.02% SDS

10mM Tris-HCl pH 7.5

2.2.7. Glycopeptidase F Buffer.

1% (v/v) 2-mercaptoethanol

0.2% (W/V) SDS

1% (W/V) Triton-X 100

10mM EDTA

50mM Potassium phosphate buffer pH7.2

2.2.8. McIlvaines Buffer.

Stock solutions;

A) 0.1M Citric acid

B) 0.2M $Na_2HPO_4 12H_2O$.

Working diluent;

17.9ml of A and 32.1ml of B were mixed and the volume made up to 100ml to give a solution of pH 6.0.

2.2.9. Complement Fixation Assay (CFA) Buffer.

Stock solutions (10 x);

81.6g NaCl (1.4M)

12.1g Tris (0.1M)

33ml of 0.15M $MgSO_4$

15ml of 0.1M $CaCl_2$

The pH was adjusted with HCl to 7.4 and the final volume was made up to 1 litre.

Working diluent;

1g of BSA was dissolved in 100ml of stock solution and the final volume was made up to 1 litre.

2.3. Tissue Culture.

2.3.1. Rat Hybridoma Cell Lines.

Rat hybridoma 4A3 and I1A1.4 cell lines synthesize and secrete IgM and IgG_{2b}, respectively (Damato *et al.*, 1986; Ghosh and Campbell, 1986). Both cell lines were generated in this department and were kindly provided by Dr. A.M. Campbell.

2.3.2. Tissue Culture Medium.

The composition of RPMI-1640 medium is described in table 7. For "complete" tissue culture medium, the RPMI-1640 was supplemented with 10% (V/V) foetal calf serum, 2mM fresh L-glutamine, penicillin (10^5 I.U./litre) and streptomycin (100mg/litre). Foetal calf serum was heat inactivated at 56°C for 30 minutes and stored at -20°C, prior to use. L-glutamine and penicillin /streptomycin solutions were kept at -20°C as 100 x stock.

2.3.3. Routine Culture.

Rat hybridoma 4A3 and I1A1.4 cells were maintained in complete

Table 7. Composition of RPMI-1640 medium.

Amino acids	*mg/litre*	*Amino acids*	*mg/litre*
L-Arginine	200.0	L-Lysine HCl	40.0
L-Asparagine	65.0	L-Methionine	15.0
L-Aspartic acid	20.0	L-Phenylalanine	15.0
L-Cystine (2HCl)	65.0	L-Proline (hydroxy-L-	
L-Glutamic acid	20.0	proline free	20.0
Glycine	10.0	L-Serine	30.0
L-Histidine (free base)	15.0	L-Threonine (allo free)	20.0
L-hydroxyproline	20.0	L-Tryptophan	5.0
L-Isoleucine (allo free)	50.0	L-Tyrosine	15.0
L-leucine (methionine free)	50.0	L-Valine	20.0
Vitamins		*Inorganic salts*	
Biotin	0.2	$Ca(NO_3)_2.4H_2O$	100.0
D-calcium pantothenate	0.25	KCl	400.0
Choline chloride	3.0	$MgSO_4$	48.84
Folic acid	1.0	NaCl	6000.0
i-Inositol	35.0	Na_2HPO_4	800.0
Nicotinamide	1.0	$NaHCO_3$	2000.0
para-Aminobenzoic acid	1.0	*Other components*	
Pyrodoxine hydrochloride	1.0	Glucose	2000.0
Riboflavin	0.2	Phenol red	5.0
Thiamine hydrochloride	1.0	Reduced Glutathione	1.0
Vitamin B12	0.005		

medium in Costar flasks at 37°C in a 95% O_2:5% CO_2 humid incubator, and subcultured when confluent. Routine cell culture was performed under sterile conditions in a Laminar-flow hood.

2.3.4. Storage and Recovery of Cells.

Log-phase cells were harvested, washed and resuspended at 2 x 10^6 cells per ml of storage medium (i.e., RPMI-1640 supplemented with 20% (V/V) foetal calf serum and 10% (W/V) DMSO). 1ml of cell suspension was aliquoted into cryostat ampoules (1.5ml capacity). Ampoules were kept at -70°C overnight before being transferred into a gas-phase liquid nitrogen freezer. For recovery, cells were quickly thawed at 37°C, washed twice with incomplete medium, resuspended in complete RPMI medium (20% foetal calf serum) and incubated at 37°C in a humidified incubator. The percentage of foetal calf serum was subsequently reduced to 10% over several subcultures.

2.3.5. Determination of Cell Viability.

Trypan blue solution was prepared by mixing 1 part of 1% (W/V) trypan blue with 4 parts of 1% (W/V) NaCl. Cell viability was determined by mixing equal volumes of cell suspension and the trypan blue solution. Numbers of live and dead cells were estimated by counting in a Neubauer haemocytometer.

2.4. Radiolabelling Procedures.

2.4.1. Analysis of Macromolecular Synthesis.

Cells were washed twice with serum-free RPMI-1640 medium and resuspended at 2.5 x 10^6 cells/ml. 100µl aliquots of the cell suspension were dispensed into individual wells of microtitre trays containing appropriate concentrations of tunicamycin or analogues. After 3 hours at 37°C, each well was pulsed with 1.0µCi/well of radioactive macromolecular precursor and further

incubated for 1 hour at 37°C. Samples were then automatically harvested using a Skatron cell harvester. Radioactivity was determined by liquid scintillation spectrometry (section 2.5.7).

2.4.2. Pre-Treatment of Cells.

Prior to labelling, 2.5 x 10^6 viable cells were harvested from log-phase cultures and exposed to the appropriate drugs. In case of tunicamycin and its analogues, cells were subjected to three hours exposure at 2μg/ml in order to exhaust the pre-existing glycosylated products within the cell. Treatment with the processing inhibitors, however, involved a one hour pre-exposure. The final concentrations of the processing inhibitors used were as follows;

Castanospermine	75ug/ml
1-deoxynojirimycin	5mM
1-deoxymannojirimycin	1mM
Swainsonine	10ug/ml

These processing inhibitors were dispensed from 100 x stock solutions that were stored at 4°C. Control samples were treated similarly but in absence of any drugs.

2.4.3. Biosynthetic Labelling.

After treatment with the various inhibitors (section 2.4.2), cells were washed twice with serum-free RPMI-1640 lacking methionine (i.e., labelling medium). The cells were resuspended in 200μl of the labelling medium supplemented with 100μCi ^{35}S-methionine and incubated at 37°C for 60 minutes. For mannose labelling, cells were washed with and resuspended in RPMI-1640 medium containing sodium pyruvate as energy source instead of glucose. Tritiated mannose was normally obtained in 50% (V/V) ethanol solution and therefore had to be lyophilized and resuspended in labelling medium, prior to use. 50-75μCi of tritiated mannose were normally used for each labelling and incubation was carried

out for 90 minutes. In case of drug-treated cells, the washing and labelling medium also contained the appropriate concentrations of the drugs.

2.4.4. Pulse-Chase Experiments.

Cells were biosynthetically radiolabeled as described in section 2.4.3. After the labelling period, cells were pelleted and resuspended in 500µl of complete RPMI-1640. Aliquots of 125µl were immediately collected upon resuspension; the cells were pelleted and subjected to lysate preparation (section 2.5.1). The remainder of cell suspensions were reincubated at 37°C for further 4-6 hours. At the appropriate chase intervals (see individual figures), 125µl aliquots of cell suspensions were collected. Cells were pelleted and the culture supernatants were collected and prepared for immunoprecipitation as described in section 2.5.2.

2.5. Analysis of Cellular Proteins.

2.5.1. Preparation of Lysates.

Cell lysates were prepared in the three-detergent buffer system described by Mosmann *et al.* (1978 and 1979). Radiolabeled cells were washed twice with ice-cold TKM buffer (section 2.2.2), resuspended in 8 volumes of the same buffer and lysed by addition of 1 volume of 10% (W/V) triton X-100 in TKM buffer. The suspension was allowed to stand on ice for 20 minutes. Nuclei were removed by centrifugation (3,000 x g for 30 minutes) and the resulting supernatant was added to 1 volume of 10% (W/V) deoxycholate/5% (W/V) SDS in TKM buffer. Insoluble debris was removed by high speed centrifugation (30,000 x g for 30 minutes) and the supernatant was either used immediately for serological analysis or stored at -70 °C. If stored, the high speed centrifugation step was repeated on the thawed lysate.

2.5.2. Preparation of Culture Supernatants.

Prior to immunoprecipitation, culture supernatants, following the appropriate chase periods, were obtained by pelleting the cells (500 x g) and

subjecting the cell supernatant to high speed centrifugation (30,000 x g for 30 minutes).

2.5.3. Total Protein Precipitation.

Determination of total cellular proteins was done by acid-precipitating radiolabeled lysates on glass fibre discs. Aliquots of cell lysates were dispensed onto glass fibre discs, air-dried and immersed into two successive baths of 10% (W/V) ice-cold trichloroacetic acid for 60 minutes. Discs were rinsed with absolute alcohol, air-dried and counted by liquid scintillation spectrometry (section 2.5.7).

2.5.4. Serological Titration of RArIg and DARIg.

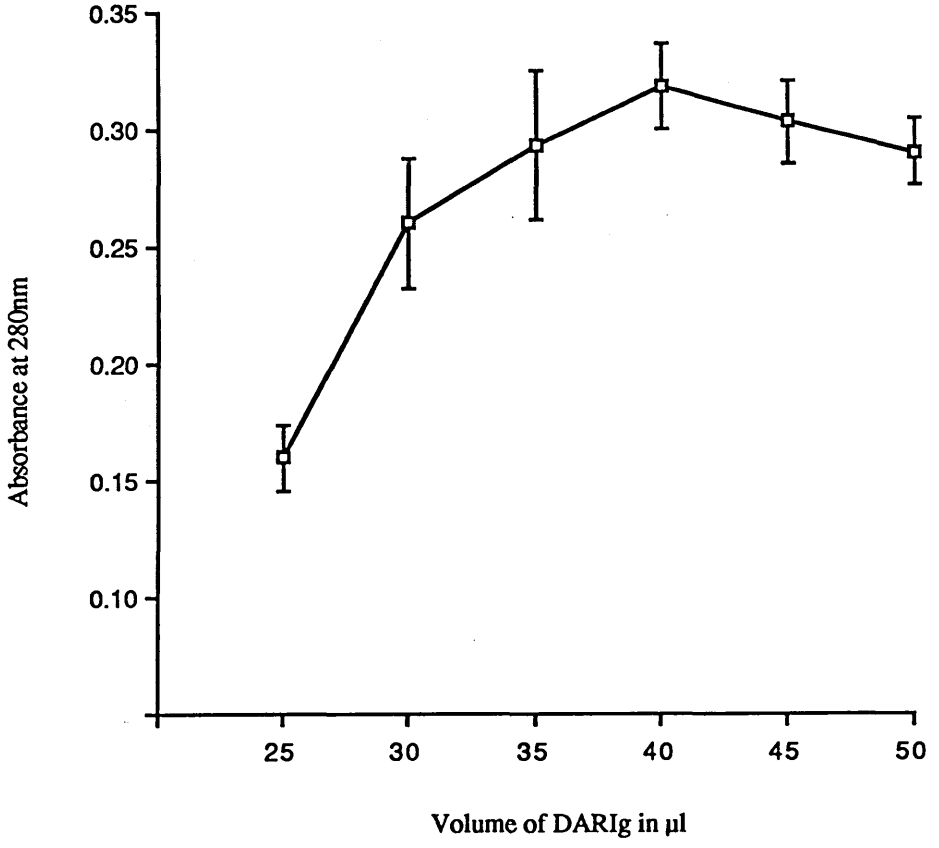
To determine the optimal volumes of RArIg and DARIg, prior to usage in indirect immunoprecipitation procedures (section 2.5.5), a titration of the two antibodies was performed. Different volumes of DARIg were added to 10µl of RArIg (1:10 dilution) and the solution was made up to 250µl with PBS. Mixtures were incubated at room temperature for 1 hour and overnight at 4°C. Precipitates were collected by centrifugation, washed four times with PBS and resuspended in 1ml of 0.1M sodium hydroxide. Absorbance was measured spectrophotometrically at 280nm. Figure 7 demonstrates the titration profile of the two antibodies. The optimal serological titration of the two antibodies was represented by the mixtures exhibiting the maximum absorbance reading, i.e., 40µl DARIg to 1µl RArIg.

2.5.5. Indirect Immunoprecipitation.

10µl of 1:10 dilution of RArIg was added to an aliquot of cell lysate or culture supernatant and incubated at room temperature for 30 minutes. A serological equivalence of DARIg, as determined in section 2.5.4, was added and the mixture was incubated overnight at 4°C. Immunoprecipitates were harvested by centrifugation.

Figure 6. Serological titration of DARIg and RArIg.

Determination of the optimal serological titre of DARIg and RArIg is described in section 2.5.4. Mixtures of 10µl (1:10 dilution) of RArIg and different concentrations of DARIg were incubated at room temperature for one hour and overnight at 4°C. Absorbance of the precipitates was measured spectrophotometrically at 280nm. Each point represents mean absorbance values of duplicate samples. Bars indicate standard deviation. The optimal serological titration of the two antibodies are represented by the mixtures exhibiting the maximum absorbance reading, as indicated by arrow (i.e., 40µl DARIg to 1µl RArIg).


0.35
0.30
0.25
0.20
0.15
0.10
Absorbance at 280nm
25
30
35
40
45
50
Volume of DARIg in µl

2.5.6. Purification of Serological Precipitates.

Harvested immunoprecipitates from section 2.5.5 were resuspended in 100µl 3D-TKM buffer and layered onto the top of two-step discontinuous sucrose gradient. The gradient consisted of 300µl of 1M sucrose in 3D-TKM and 300µl of 0.5M sucrose in 3D-TKM. After addition of sample, the gradients were centrifuged at 3,000 x g for 30 minutes and rinsed with immunoprecipitate wash buffer (section 2.2.4) without disturbing the precipitates. The washed immunoprecipitate was either dissolved in SDS-PAGE reducing sample buffer or stored in 3D-TKM buffer at 4°C.

2.5.7. Liquid Scintillation Counting.

Samples were added to scintillation vials containing 3ml of ecoscint and counted in a Beckman LS Liquid Scintillation Spectrometer.

2.6. Enzyme Analysis.

2.6.1. Endo H Digestion.

Washed immunoprecipitates were precipitated three times with 5 volumes of acetone and resuspended in 50mM citrate buffer (pH 5.5) containing 0.01% SDS. After the final wash, the precipitates were suspended in 50µl of the citrate buffer, boiled and 5mU of endo H were added (the enzyme was stored in aliquots of 5mU/20µl citrate buffer at -70°C). Digestion was allowed to proceed at 37°C for 18 hours. Mock-treated samples were treated under the same buffer conditions in absence of enzyme. Reactions were terminated by addition of concentrated reducing sample buffer and boiling for 5 minutes (Tarentino *et al.*, 1978).

2.6.2. Glycopeptidase F Digestion.

Digestion of immunoprecipitates with Glycopeptidase F was performed as in section 2.6.1 using Glycopeptidase F buffer pH 7.2. Samples were treated with

1U of the enzyme (Plummer *et al.*, 1984).

2.6.3. Neuraminidase Digestion.

Prior to enzyme digestion, immunoprecipitates were acetone precipitated as in section 2.6.1 and resuspended in 50mM sodium acetate buffer (pH 5). 300mU of neuraminidase was then added to the samples. Digestion was allowed to proceed at 37°C for 18 hours. Control samples were treated under the same buffer conditions in absence of enzyme. Reactions were terminated as in section 2.6.1.

2.6.4. Pronase Digestion.

Digestion of immunoprecipitates with Pronase was performed as in section 2.6.1 using 25mM Ammonium bicarbonate solution. Samples were treated with a final concentration of 0.1mg/ml of the enzyme.

2.6.5. Concanavalin A-Sepharose Chromatography.

Tritiated-mannose labelled IgG_{2b} were immunoprecipitated as described in section 2.5.5. Immunoprecipitates were treated with various enzymes (see individual figure legend). Reaction was stopped by boiling. Samples were diluted with Con A buffer (section 2.2.6) and loaded on to a Con A column (200µl bed volume) equilibrated with the same buffer. This is followed by elution with 1.5ml of Con A buffer, 10mM methyl-α-D-glucopyranoside and 100mM methyl-α-D-mannopyranoside, respectively. Radioactivity of the elutions as well as the Con A-sepharose beads was determined. Results are expressed in percentage of radioactivity.

2.7. Polyacrylamide Gel Electrophoresis.

SDS-polyacrylamide gel electrophoresis was performed according to the discontinuous buffering system of Laemmli (1970).

2.7.1. Stock Solutions.

Unless otherwise stated all solutions were stored at 4°C.

Solution A.

45% (W/V) Acrylamide

1.2% (W/V) N,N'-methylene bis acrylamide

Solution was deionized with amberlite MB-1,filtered and stored in a dark bottle.

Solution B.

1.5M tris-HCl (pH 8.8)

0.13% (V/V) TEMED

Solution C.

12% (W/V) SDS.

solution was kept at room temperature.

Solution D.

10% (W/V) Ammonium persulphate.

Solution E.

0.65M Tris-HCl (pH 6.8)

Reservoir Buffer (5 X).

125mM Trizma base

660mM Glycine

1% (W/V) SDS

Solution was kept at room temperature.

2.7.2. Separating Gel Preparation.

Separating gels (10% acrylamide) were prepared from stock solutions as follows;

Solution A (ml)	13.3
Solution B (ml)	15.0

Solution C (ml)	0.5
Solution D (ml)	0.5
Deionized water (ml)	30.7

The solution was immediately poured into casting apparatus and allowed to polymerize.

2.7.3. Stacking Gel Preparation.

Stacking gels were prepared from stock solutions as follows;

Solution A	2.64ml
Solution C	0.20ml
Solution D	0.20ml
Solution E	2.40ml
Deionized water	18.32ml
TEMED	24µl

The solution was poured onto the top of the separating gel and allowed to polymerize around a 20 well teflon template.

2.7.4. Sample Preparation and Electrophoresis.

Immunoprecipitates were dissolved in 20-25µl of reducing sample buffer (section 2.2.5) and heated to 100°C for 2 minutes. Samples were immediately loaded into individual wells in the stacking gel. Electrophoresis was performed either for 4-5 hours at a constant current of 45mA or 18-20 hours at 10mA.

2.7.5. Fluorography.

Fluorography was performed according to Chamberlain (1979). Gels were immersed into two successive baths of 1M sodium salicylate for one hour. The gels were then dried onto a Whatman 3mm chromatography paper under vacuum at 80°C and exposed to X-ray films at -70°C.

2.7.6. Development of Films.

After exposure, fluorographic images were visualized by immersing the film in DX-80 developer for 5 minutes, rinsing with water and then immersing in FX-40 fixer for 2 minutes. The film was rinsed with water and allowed to air-dry prior to inspection. All operations were done in the dark room using filtered lighting facilities.

2.8. Immunoglobulin Assays.

2.8.1. Treatment with Inhibitors.

2×10^6 I1A1.4 cells were pre-treated with the various inhibitors as described in section 2.4.2. Cells were then washed twice and resuspended in 2ml of complete RPMI-1640 medium containing the appropriate concentrations of inhibitors. Suspensions were then incubated at 37°C for 4 hours in a humidified incubator. Cells were then pelleted and the supernatant collected and screened by ELISA (section 2.8.2). Samples were then subjected to the respective functional assays (sections 2.8.3 - 2.8.6).

2.8.2. Enzyme-Linked Immuno Sorbent Assay (ELISA).

ELISA was performed according to Ghosh and Campbell (1986). 96-well microtitre plates (Dynatech MicroElisa Systems) were washed with distilled water and coated with 10^6 Inaba vibrios/100µl/well in PBS. Plates were centrifuged at 800 x g for 5 minutes at room temperature. Supernatant was removed by flicking the plates which were then washed twice with PBS. Plates were then blocked with 0.5% (W/V) gelatin in PBS for 30 minutes to saturate the unreacted sites in the wells. Serial dilutions of samples containing antibodies were added to the plates, which were then incubated at room temperature for 2 hours. Plates were washed 7 times with PBS before addition of 100µl of RArIg covalently linked to horseradish peroxidase (1:1000 dilution of conjugated antibody in PBS containing 0.9% (W/V) NaCl and 5% (W/V) BSA) to each well. Plates were left at room temperature for

another hour and subsequently washed 3 times with PBS. 100µl of substrate solution (0.4mg/ml O-phenylene diamine with 0.01% (V/V) fresh hydrogen peroxide in McIlvaines buffer) was added to each well and the plates were incubated in the dark for 15-20 minutes. Reaction was terminated by addition of 50µl of 4N H_2SO_4 to each well and the colour was measured at 492nm in a Titretek Multiskan Spectrophotometer.

2.8.3. Sensitization of Sheep Erythrocytes.

500µl of fresh sheep erythrocytes were washed with CFA working diluent until a clear solution was obtained. The packed sheep erythrocytes were then resuspended in 10ml of CFA working diluent, to which 10µl of haemolysin was also added. Suspension was incubated at 37°C for 30 minutes. The freshly sensitized sheep erythrocytes were then used for assay of complement fixation (section 2.8.4).

2.8.4. Complement Fixation Assay (CFA).

CFA was performed according to Wasserman and Levine (1961). 100µl attenuated Inaba vibrios (10^7 vibrios/100µl) and 600µl guinea pig complement (1:400 dilution in CFA working diluent) were added to tubes with serial dilutions of antibody (in duplicate). A panel of control tubes (in absence of antibody, antigen and complement, respectively) and tubes solely consisting of complement solution (i.e., "complete lysis tubes") were also prepared. All preparations were assembled on an ice bath. Mixtures were incubated at 4°C overnight whereupon 200µl of haemolysin-sensitized sheep erythrocytes (section 2.8.3) were added to the individual tubes. Tubes were immediately and simultaneously incubated at 37°C with gradual shaking. Incubation was performed until complete lysis of sheep erythrocytes was observed from the "complete lysis tubes". Tubes were simultaneously centrifuged (3,000 x g) at 4°C for 5 minutes and supernatants were

then measured for absorbance readings at 492nm using the Beckman Spectrophotometer. Percentage of complement fixation are expressed as follows;

$$\frac{A_{492} \text{ of "complete lysis"} - A_{492} \text{ of samples}}{A_{492} \text{ of "complete lysis"}} \times 100\%$$

2.8.5. Coating of LPS on Sheep Erythrocytes.

1ml of fresh sheep erythrocytes was washed with PBS until a clear solution was obtained. 500µl of the packed sheep erythrocytes were then added to 1ml of LPS solution (1mg/ml) and incubated at 37°C for 60 minutes. The cell suspension was then washed twice with PBS, resuspended at 2×10^6 cells/ml and used as soon as possible.

2.8.6. Haemagglutination.

50µl of antibody samples at various dilutions were plated (in duplicate) into the wells of Dynatech microtitre plates. This was followed by the addition of 50µl of LPS-coated sheep erythrocytes (section 2.8.5) into each well. Plates were left at room temperature overnight prior to inspection for agglutination.

CHAPTER III

RESULTS AND DISCUSSION

SECTION 3.1.

CHARACTERIZATION OF ANALOGUES OF TUNICAMYCIN.

3.1.1. Model Systems.

In the course of this study, two rat hybridoma lines secreting IgM and IgG_{2b} were used as model systems. These rat lines were generated in this department and were kindly provided by Dr. A.M. Campbell.

Rat hybridoma 4A3 cell line was generated by fusion of spleen cells of DA rats immunized with human uveal melanoma cells, with the Y3 Ag 1.2.3 rat myeloma line (Damato *et al.*, 1986). Isotypic determination of the immunoglobulins synthesized and secreted by 4A3 cells demonstrates that they are of the IgM class.

Rat hybridoma I1A1.4 cell line was generated by fusion of spleen cells of DA rats immunized with 35A3 (Inaba) strain of *Vibrio cholerae*, with the Y3 Ag 1.2.3 rat myeloma line (Ghosh and Campbell, 1986). I1A1.4 cells synthesize and secrete anti-cholerae antigens of the IgG_{2b} isotype. Characterization studies of this cell line by several different immunological assays has demonstrated that the IgG_{2b} molecules that are secreted, although exhibit preference to the Inaba antigen, could also react with the Ogawa antigen of the *Vibrio cholerae* (Ghosh and Campbell, 1986).

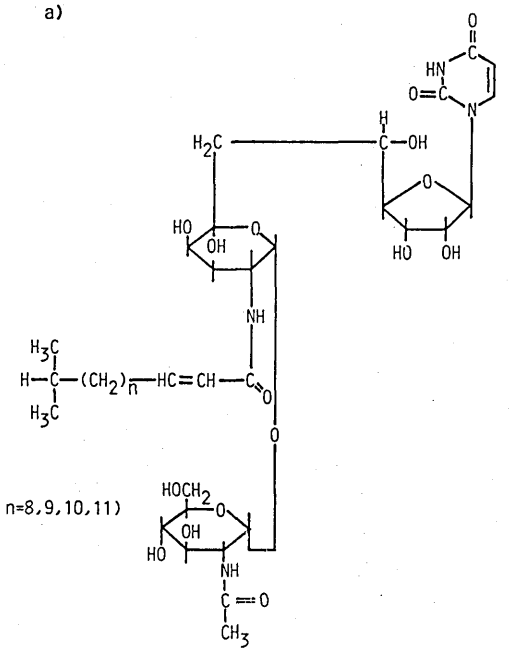
3.1.2. Analogues of Tunicamycin.

The structures of the three analogues of tunicamycin characterized in this study are illustrated in figure 7. The analogues are referred to as tunicamycin-1, -2 and -3. Tunicamycin-1 has an open ring structure at the site of the uracil base and a saturated aliphatic side chain. The fatty acid side chain of tunicamycin-2 is similarly modified, but the integrity of the uracil ring is preserved. In this case, however, the furanose sugar group to which the pyrimidine is bound is substituted by a pyranose ring. Analogue tunicamycin-3 is modified by methylation of the nitrogen residue in

Figure 7. Structures of tunicamycin and its analogues.

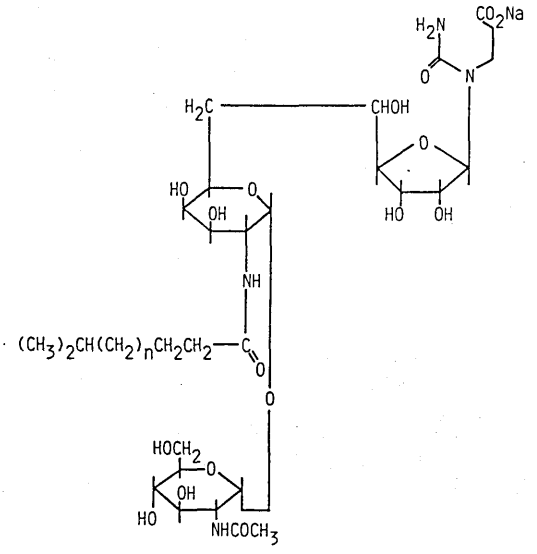
The structure of normal tunicamycin is shown in *panel a*. The analogues are; *panel b*: tunicamycin-1; *panel c*: tunicamycin-2; *panel d*: tunicamycin-3.

a)

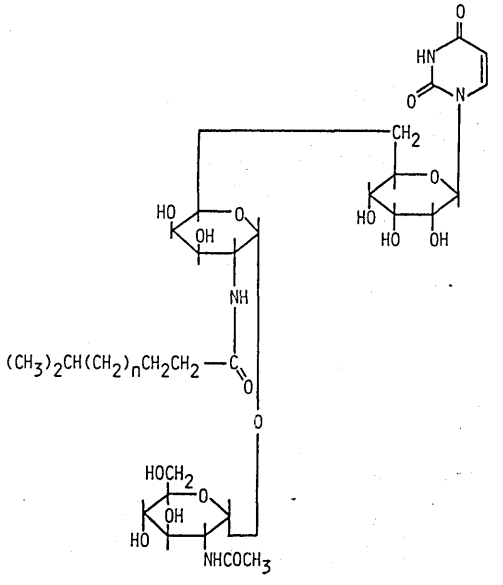


n=8,9,10,11)

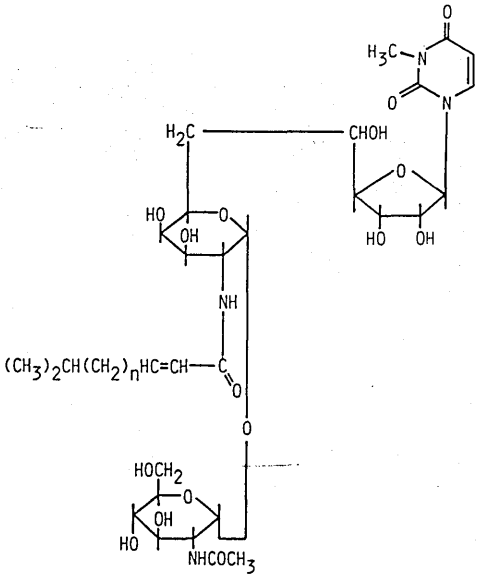
b)



c)



d)



the uracil moiety.

3.1.3. Macromolecular Biosynthesis.

For analysis of macromolecular synthesis, log-phase 4A3 cells were incubated with the indicated concentrations of the drugs for three hours prior to a one hour pulse with the appropriate radioactive precursor. Cells were then automatically harvested onto glass fibre discs, and incorporation of radioactivity was determined.

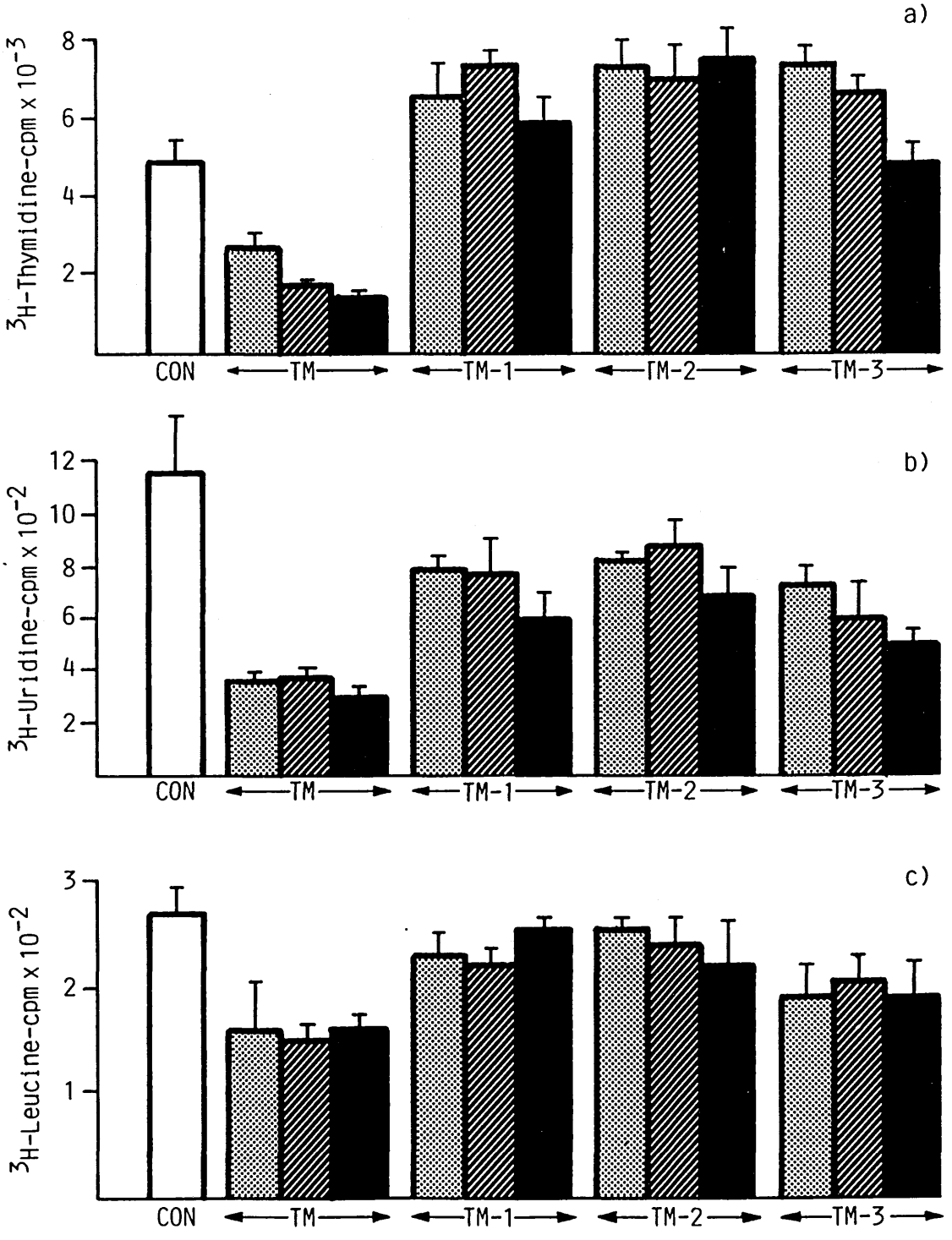
Figure 8 illustrates the effects of tunicamycin and analogues upon cellular macromolecular biosynthesis. Only normal, unmodified tunicamycin had any inhibitory effect upon DNA synthesis in 4A3 cells, and the extent of the inhibition was apparently dose-dependent (figure 8, panel a). None of the tunicamycin analogues had any inhibitory effect at the concentrations tested, but all appeared to have a slightly mitogenic effect upon the cells.

By contrast, RNA synthesis, as assessed by tritiated uridine incorporation, was inhibited by all forms of the antibiotic, particularly at the highest concentration tested (figure 8, panel b). This is expected since uridine components are present in the structures of all of the drugs and are likely to provide active competition for the radiolabeled precursor. As observed in the analysis of DNA synthesis, normal tunicamycin caused the greatest inhibition of RNA synthesis.

Protein synthesis in treated 4A3 cells was inhibited to the greatest extent by normal tunicamycin, and to a small but significant degree by tunicamycin-3 (figure 8, panel c). Neither of the tunicamycin-1 or tunicamycin-2 analogues had any pronounced inhibitory effect upon cellular protein synthesis. Cellular cytotoxicity caused in the presence of tunicamycin has been frequently observed in

Figure 8. Effects of tunicamycin and analogues upon cellular macromolecular biosynthesis.

4A3 cells were incubated at 37°C for 3 hours in the presence of tunicamycin or analogues prior to a 1 hour pulse with appropriate tritiated macromolecular precursor. *Panel a* illustrates the data for thymidine incorporation, *panel b* uridine incorporation, and *panel c* refers to tritiated leucine incorporation. The open bar in each case represents control, untreated cells. The concentrations of tunicamycin or analogues were: stippled bar, 1μg/ml; hatched bar, 2μg/ml; closed bar, 4μg/ml.


a)
^{3}H-Thymidine-cpm x 10^{-3}
8
6
4
2
CON
TM
TM-1
TM-2
TM-3
b)
^{3}H-Uridine-cpm x 10^{-2}
12
10
8
6
4
2
CON
TM
TM-1
TM-2
TM-3
c)
^{3}H-Leucine-cpm x 10^{-2}
3
2
1
CON
TM
TM-1
TM-2
TM-3

several systems (see section 1.3.1.2). The question that is often raised is whether this inhibitory effect is a direct drug-induced response or a secondary effect due to interference of the drug on other cellular reactions.

The above data confirm the hypothesis that tunicamycin does inhibit general cellular macromolecular synthesis, but that none of the analogues tested exhibit pronounced toxic effects. Since the analogues, although possessing close structural homologies to normal tunicamycin, have not mimicked the antibiotic with respect to inhibitory effects upon general macromolecular synthesis, it is therefore reasonable to deduce that the cellular toxicity demonstrated in the presence of tunicamycin is a secondary effect due to the action of the drug on other cellular functions.

3.1.4. Quantitative Glycosylation.

4A3 cells were labelled with tritiated mannose and the level of incorporation into acid-precipitable glycoprotein and into immunoprecipitable IgM was assessed. Figure 9, panel a, illustrates that none of the tunicamycin analogues inhibited the incorporation of tritiated sugar into acid-precipitable glycoprotein; normal tunicamycin virtually abolished mannose incorporation. These data strongly suggest that the tunicamycin analogues are unable to inhibit the activity of the UDP-GlcNAc:dolichyl phosphate GlcNAc-1-phosphate transferase and, hence, protein glycosylation.

The data for incorporation of mannose into IgM are entirely consistent with those obtained for total acid-precipitable material. That is, that the tunicamycin analogues have no inhibitory effect upon tritiated mannose incorporation into IgM molecules, and that normal tunicamycin completely inhibits this process. This, therefore, leads to the conclusion that only native tunicamycin is capable of inhibiting the N-glycosylation of proteins. Further support of this notion is

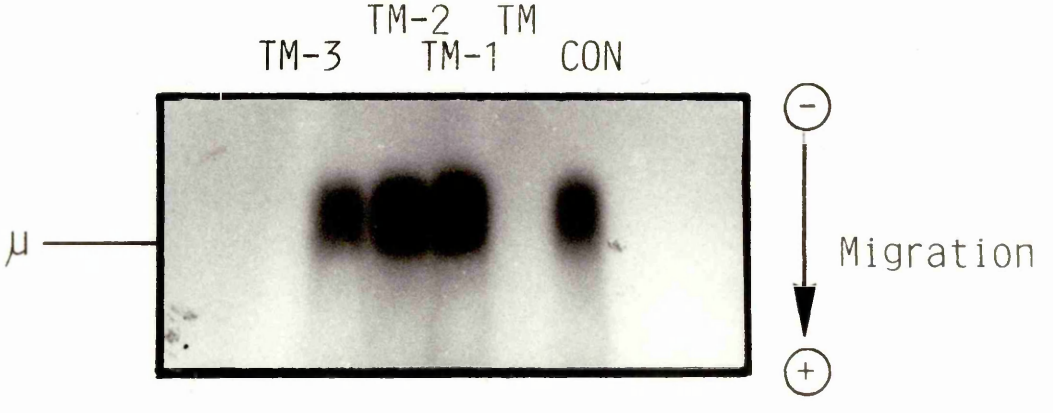
Figure 9. Effects of tunicamycin and analogues upon tritiated mannose incorporation.

Panel a; 4A3 cells were pre-incubated with 2μg/ml tunicamycin or analogues for 3 hours prior to a 90 minutes pulse with 25μCi of tritiated mannose. Lysates were prepared in a final volume of 125μl, and immunoprecipitates were prepared from 100μl aliquots of lysate. Washed immunoprecipitates were resuspended in 100μl lysis buffer. 5μl aliquots of total lysate or washed immunoprecipitate were used for assay of radioactivity. All determinations were performed in triplicate and are expressed as counts per minute.

Panel b; aliquots of the immunoprecipitated IgM secreted from tritiated mannose labelled 4A3 cells were reduced and subjected to SDS-PAGE (10% (W/V) acrylamide) and fluorography. CON refers to control; TM: tunicamycin; TM-1, -2 and -3 are analogues tunicamycin-1, -2 and -3, respectively.

	Total Acid-insoluble (CPM)	Immunoprecipitable IgM (CPM)
Control	223 ± 33	163 ± 9
TM	52 ± 17	17 ± 9
TM-1	295 ± 21	263 ± 12
TM-2	210 ± 17	189 ± 14
TM-3	395 ± 20	143 ± 8

^{3}H-mannose labelling of μ chains



observed from the fluorograph of immunoprecipitates of secretory IgM analysed by SDS-polyacrylamide gel electrophoresis (figure 9, panel b). Bands of similar migration are detected in all of the tracks except that loaded in immunoprecipitate of samples synthesized in the presence of normal unmodified tunicamycin.

3.1.5. Biosynthesis and Transport of IgM.

4A3 cells were pre-incubated in the absence and presence of tunicamycin or its analogues at 37°C for three hours, pulsed with ^{35}S-methionine for one hour, and chased with complete RPMI for the indicated time intervals.

Figure 10 illustrates a kinetic analysis of the secretion of IgM from 4A3 cells. Although the tunicamycin-3 analogue can apparently decrease the rate at which IgM is secreted from the cells, normal tunicamycin-treated 4A3 cells show very little, if any, export of IgM over a 6 hour chase. Beyond this time point, any secretion may be attributable more to cell death and lysis rather than to an active export process.

Figure 11 shows the SDS-PAGE profile of isolated IgM from cell lysates and supernatants of 4A3 cells. In the lysates, only normal tunicamycin (lane B) gives rise to a lower-M_r species of μ-heavy chain. The analogues (lanes C,D and E) have no effect upon the electrophoretic mobility of the isolated μ-chains. With respect to secreted IgM, very little is specifically immunoprecipitable immediately following the pulse (0 hour), but significant amounts of IgM are precipitable from 6 hour chase supernatants in control (lane A) and analogue-treated (lanes C,D and E) culture supernatants. Only in the case of normal tunicamycin-treated cells is there an absence of IgM in the supernatant of 4A3 cells. The extra light chain bands that are visualized in the SDS-PAGE profile of immunoprecipitated IgM are due to synthesis encoded by the parent myeloma used in the generation of this hybridoma.

Figure 10. Kinetics of IgM secretion from treated 4A3 cells.

4A3 cells were pre-incubated with tunicamycin or analogues and pulsed with 150μCi of [^{35}S]-methionine and chased for the indicated periods. Aliquots of immunoprecipitates prepared from culture supernatants at the indicated times were spotted onto glass fibre discs and precipitated with ice-cold 10% (W/V) trichloroacetic acid. Dried discs were analysed by liquid scintillation spectrometry. All determinations were made in triplicate.

KEY:

- ○ - control
- □ - normal tunicamycin-treated
- △ - tunicamycin-1-treated
- ▲ - tunicamycin-2-treated
- ● - tunicamycin-3-treated

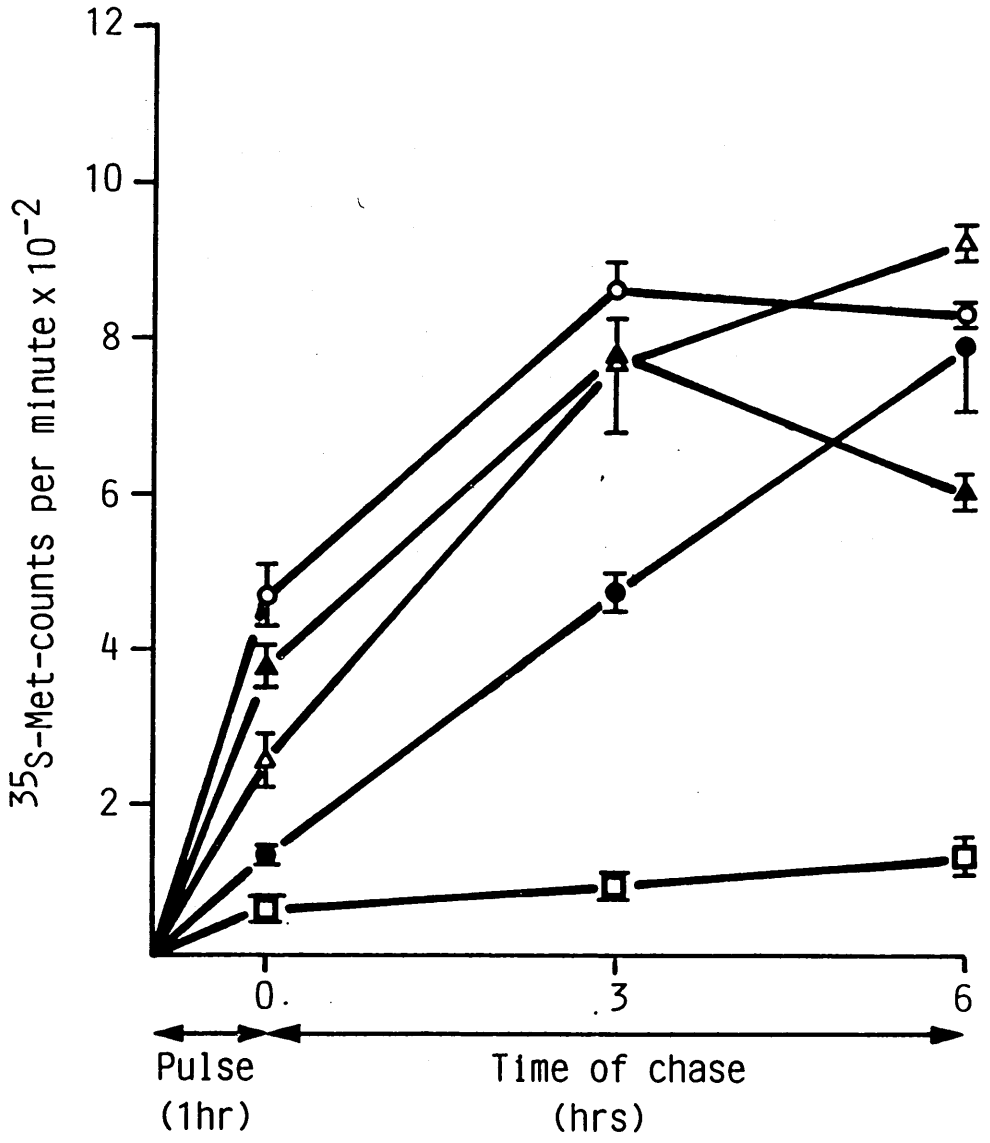

12
10
8
6
4
2
^{35}S-Met-counts per minute x 10^{-2}
0
3
6
Pulse
(1hr)
Time of chase
(hrs)

Figure 11. SDS-PAGE profiles of IgM isolated from 4A3 cell lysates and supernatants.

4A3 cells were pre-treated with tunicamycin or analogues and pulsed with 150μCi of [^{35}S]-methionine and chased for the indicated periods. Aliquots of the washed immunoprecipitates were run on a 10% (W/V) acrylamide gel following reduction.

KEY:

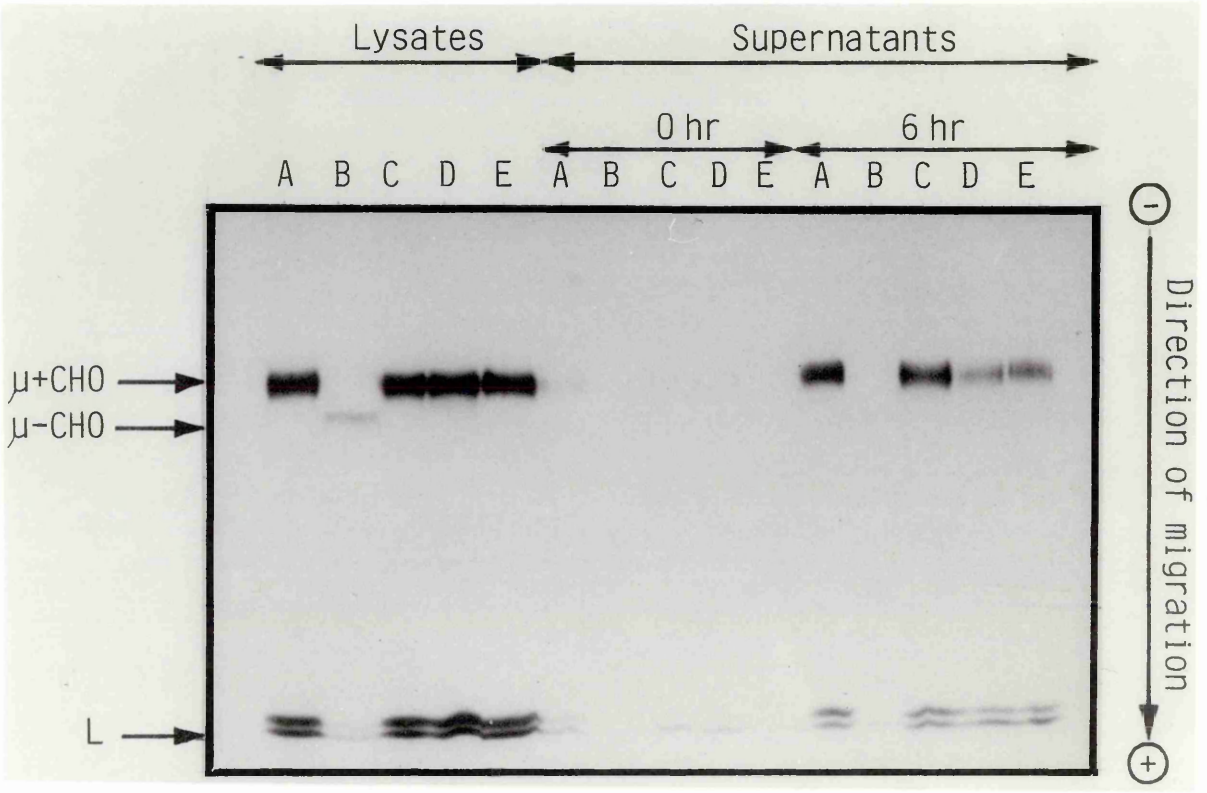
A - control

B - normal tunicamycin-treated

C - tunicamycin-1-treated

D - tunicamycin-2-treated

E - tunicamycin-3-treated


Lysates
Supernatants
0 hr
6 hr
A B C D E A B C D E A B C D E
μ+CHO
μ-CHO
L
Direction of migration
-
+

3.1.6. Biosynthesis and Transport of IgG_{2b}.

To analyse the effects of the analogues of tunicamycin on biosynthesis and transport of rat hybridoma IgG_{2b}, a pulse-chase experiment was carried out using I1A1.4 cells. Only native tunicamycin has any effect upon the M_r of the γ-chain immunoprecipitated from both lysates and culture supernatants (figure 13). In this case, however, and consistent with previous observations in a number of laboratories (Hickman and Kornfeld, 1978; Blatt and Haimovich, 1981), IgG secretion is not inhibited by tunicamycin or, in these experiments, by any of the three analogues (figures 12 and 13). The results are a close parallel to those of figures 10 and 11, and support the hypothesis that the structure of tunicamycin cannot be modified without loss of inhibitory activity.

Heifetz *et al.* (1979) have suggested that there are two classes of tunicamycin binding sites on the membranous elements of the cell. The high-affinity sites are represented by UDP-GlcNAc:dolichyl phosphate GlcNAc-1-phosphate transferase, while low affinity sites are characterized by non-specific binding of tunicamycin to lipid. The data may be interpreted in terms of the structure of the analogues (figure 7) and their abilities to interact with two classes of tunicamycin receptors.

A minor modification in the uracil moiety as in tunicamycin-3 appears to be sufficient to abolish the activity of the antibiotic. This may be explained in terms of ability of the modified base to effectively enter the catalytic site of the UDP-GlcNAc transferase. Thus, it is binding to high affinity tunicamycin receptors which would be predicted to be impaired by this structural modification to tunicamycin. Tunicamycin-3 might be expected to behave as a competitive inhibitor of the transferase, but it may be bound in the transferase active site with only very low affinity, and be readily displaced by UDP-GlcNAc molecules themselves, with

Figure 12. Kinetics of IgG secretion from I1A1.4 cells.

Treatment and analysis of immunoprecipitates were performed exactly as described in the legend to figure 3.1.4. All determinations were made in triplicate.

KEY:

- ○ - control
- □ - normal tunicamycin-treated
- △ - tunicamycin-1-treated
- ▲ - tunicamycin-2-treated
- ● - tunicamycin-3-treated

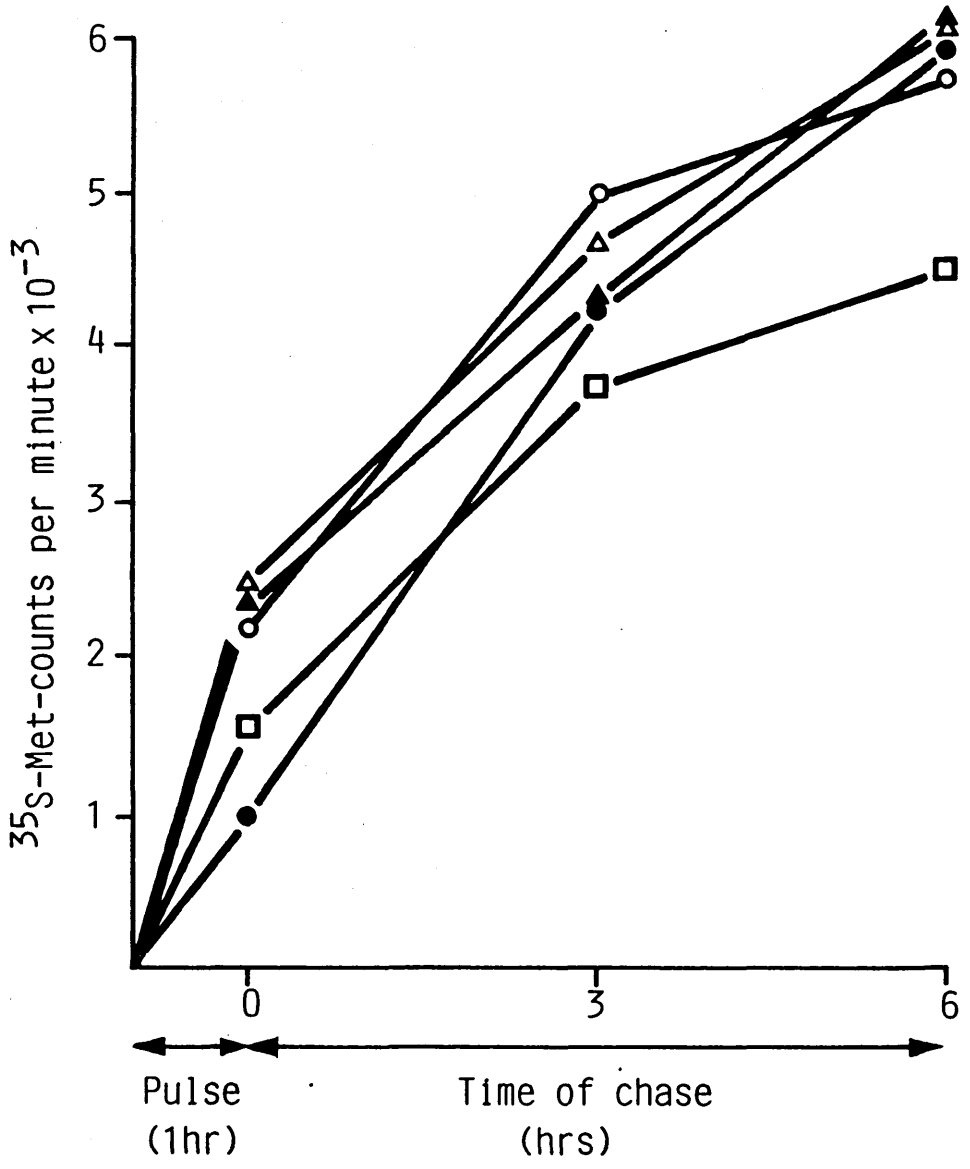

6
5
4
3
2
1
^{35}S-Met-counts per minute x 10^{-3}
0
3
6
Pulse
(1hr)
Time of chase
(hrs)

Figure 13. SDS-PAGE profiles of IgG isolated from I1A1.4 cell lysates and supernatants.

I1A1.4 cells were pre-treated with tunicamycin or analogues and pulsed with 150μCi of [^{35}S]-methionine and chased for the indicated periods. Aliquots of washed immunoprecipitates were run on a 10% (W/V) acrylamide gel following reduction.

KEY:

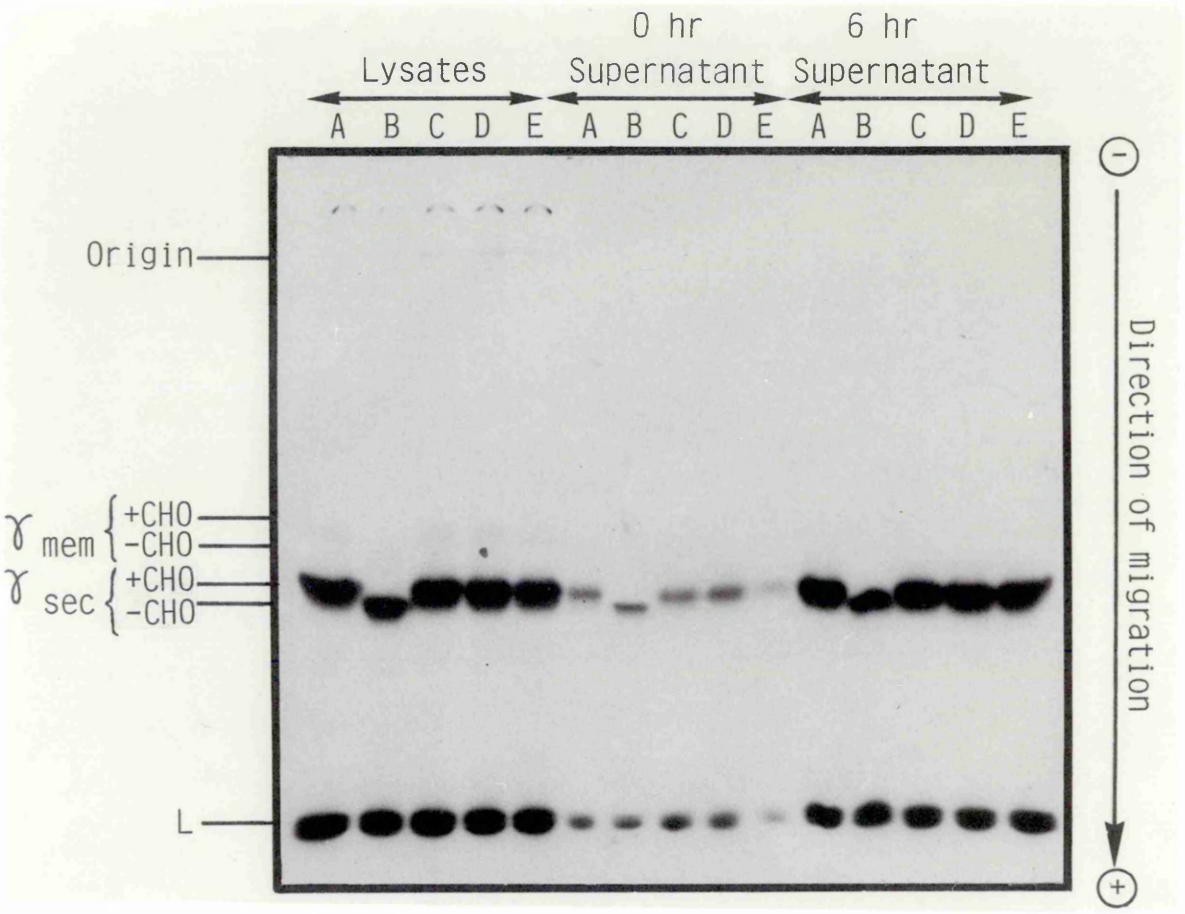
A - control

B - normal tunicamycin-treated

C - tunicamycin-1-treated

D - tunicamycin-2-treated

E - tunicamycin-3-treated


0 hr
6 hr
Lysates
Supernatant
Supernatant
A B C D E A B C D E A B C D E
Origin
γ mem
+CHO
-CHO
γ sec
+CHO
-CHO
L
Direction of migration

the net result being no detectable inhibition of the activity of the transferase. Reversible competitive inhibition of the transferase by tunicamycin-3 might be visualized by the isolation of partially glycosylated molecules which are observed in IgM-producing cells which are treated with low concentrations (less than 0.5μg/ml) of tunicamycin (Cushley *et al.*, 1983). In order for partially glycosylated μ-chains to be observed with tunicamycin-3, it is predicted that high concentrations of this analogue would have to be employed.

Previous studies (Kuo and Lampen, 1974) have demonstrated that the binding of tunicamycin to yeast protoplasts can be inhibited by the inclusion of phospholipid in the incubation medium. Furthermore, the inclusion of phospholipid could inhibit tunicamycin binding only if an unsaturated fatty acid structure was present. The above data suggest that the lack of an unsaturated aliphatic tail in intact tunicamycin leads to an impairment of antibiotic activity. Neither tunicamycin-1 nor tunicamycin-2 analogues have unsaturated structures in their respective aliphatic tails, and this particular modification may result in an inability of the analogues to interact with non-specific or low affinity tunicamycin binding sites. If the tunicamycin analogues are unable to bind to the membranous elements of the cell, then it is unlikely that they will be in the correct cellular compartment to interact with the high-affinity tunicamycin binding sites, and consequently will be unable to inhibit glycosylation.

Both tunicamycin-1 and tunicamycin-2 analogues possess modifications other than that to the aliphatic tail (see figure 7). The open ring structure at the site of the uracil base of tunicamycin-1 may result in the failure of this analogue to bind to the transferase active site (i.e., the high-affinity tunicamycin binding sites) and an inability to block protein N-glycosylation. The pyranose sugar of tunicamycin-2 may also act in a similar manner. The data suggest that minor modifications to the structure of the uracil moiety alone can eliminate the ability of tunicamycin to inhibit

glycosylation. However, in the case of analogues tunicamycin-1 and tunicamycin-2, it is not possible to distinguish the contribution of lipid modification and ribose/base modification in the loss of antibiotic activity. Further studies with other tunicamycin analogues may help to resolve this question.

The above data support the previous findings of Tkacz and Wong (1978) who demonstrated that generation of tunicamycin fragments by acid hydrolysis or acetolysis led to loss of ability to inhibit N-glycosylation. Their data have suggested that the intact tunicamycin was required for expression of antibiotic biological activity, and the data herein extend those observations by demonstrating that small modifications to the structure of intact tunicamycin can also result in loss of the ability of the drug to inhibit protein N-glycosylation. The data are therefore consistent with the interpretation that unmodified tunicamycin is required for expression of antibiotic inhibitory activity, and because the analogues of tunicamycin do not mimic its effects upon secretion of immunoglobulin molecules, it is also reasonable to conclude that such inhibition of transport is due not to side-effects of the antibiotic but to absence of carbohydrate side chains.

3.1.7. Qualitative Glycosylation.

Tunicamycin irreversibly inhibits the activity of UDP-GlcNAc:dolichyl phosphate GlcNAc-1-phosphate transferase, possibly by acting as a substrate-product transition state analogue (Heifetz *et al.*, 1979). The discussed data suggest that a small modification to any of the base, aliphatic or nucleotide components of tunicamycin will abrogate its ability to inhibit the activity of this enzyme. However, a further series of UDP-N-acetylglucosamine transfer reactions occur during oligosaccharide processing in the Golgi apparatus (see section 1.2.2), and these N-acetylglucosaminyl transferase I and II activities might be subject to inhibition by the tunicamycin analogues.

To address this question, immunoprecipitates of IgG molecules derived from lysates and supernatants of I1A1.4 cells were subjected to endo H digestion. In this situation, if the tunicamycin analogues inhibit the activity of the UDP-N-acetylglucosaminyl transferase then a "complex", processed oligosaccharide cannot be generated, and a "high mannose" form of carbohydrate side chain will remain. Only the latter form of oligosaccharide structure is susceptible to endo H digestion (Tarentino *et al.*, 1978).

Figure 14 illustrates the SDS-PAGE profile of digested and mock-digested immunoprecipitates derived from cell lysates. In all cases, except tunicamycin-treated cells, the γ-chains showed susceptibility to endo H digestion. However, when secreted γ-chains are analysed following endo H treatment, all forms of the heavy chain polypeptide are resistant to the action of endo H, which implies that the oligosaccharides present on the secreted molecules are fully processed and are of the complex form (figure 15). This observation suggests that not only do tunicamycin analogues fail to inhibit the first stage in assembly of the oligosaccharide-dolichol molecule, but also that they have no effect on the N-acetylglucosaminyl transferase I and II activities located in the Golgi apparatus.

The above study has demonstrated that modification to the structure of tunicamycin results in the loss of its ability to inhibit N-glycosylation. The analogues have also been shown to have no pronounced inhibitory effect on the synthesis of macromolecules. From these observations, it is therefore reasonable to deduce that the selective inhibition of immunoglobulin secretion and the cytotoxic effect that is observed when cells are treated with tunicamycin are secondary responses of the cell. In addition, analogous to tunicamycin, the analogues also have no effect on the N-acetylglucosaminyl transferases of the Golgi apparatus.

Figure 14. Endoglycosidase H susceptibility of intracellular IgG derived from I1A1.4 cells.

Purified immunoprecipitates of I1A1.4 cell lysates were treated with endoglycosidase H (+) or with buffer only (-). CON refers to control; TM: tunicamycin; TM-1, -2 and -3 are analogues tunicamycin-1, -2 and -3, respectively.

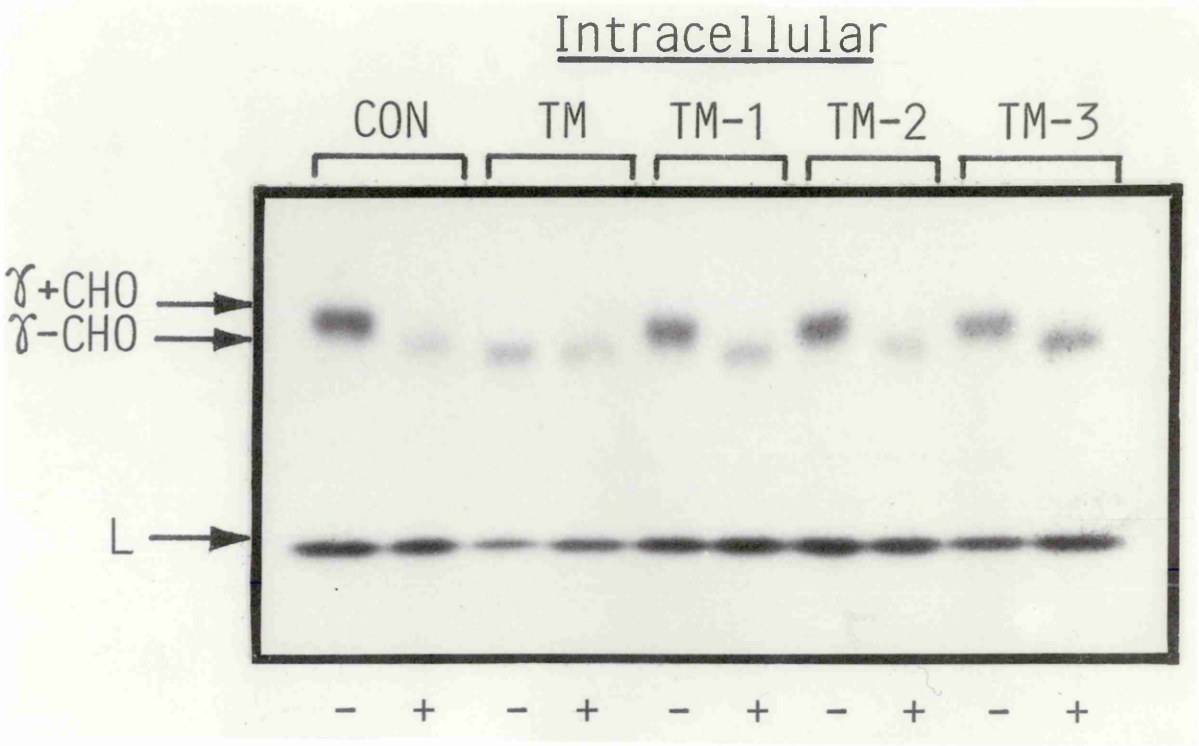
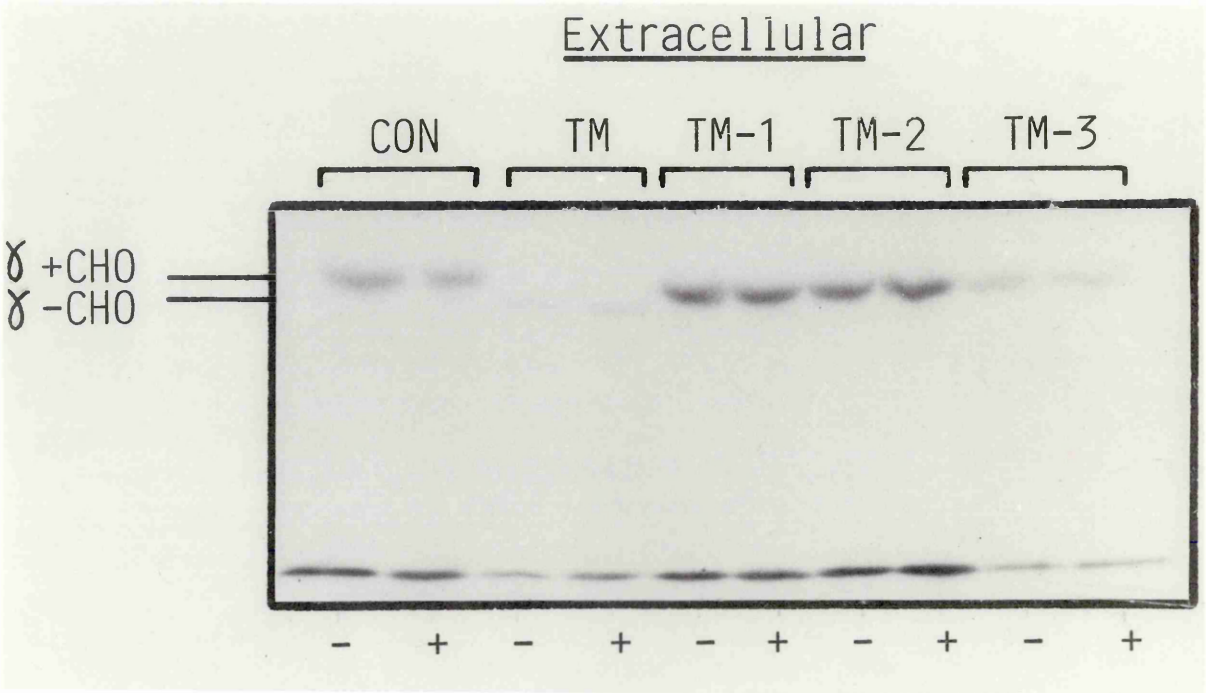

Intracellular
CON
TM
TM-1
TM-2
TM-3
γ+CHO
γ-CHO
L
- + - + - + - + - +

Figure 15. Endoglycosidase H susceptibility of secretory IgG derived from I1A1.4 cells.

Purified immunoprecipitates of I1A1.4 culture supernatants were treated with endoglycosidase H (+) or with buffer only (-). CON refers to control; TM: tunicamycin; TM-1, -2 and -3 are analogues tunicamycin-1, -2 and -3, respectively.


Extracellular
CON
TM
TM-1
TM-2
TM-3
γ +CHO
γ -CHO
- + - + - + - + - +

SECTION 3.2.

N-LINKED OLIGOSACCHARIDE PROCESSING INHIBITION - EFFECTS ON SECRETION OF RAT HYBRIDOMA IMMUNOGLOBULINS.

3.2.1. Processing Inhibition of N-Linked Oligosaccharides of IgM.

4A3 cells were treated with the indicated concentrations of processing inhibitors for one hour prior to pulse chase and immunoprecipitate preparation. Incorporation of radioactivity was determined by counting 5µl aliquots of immunoprecipitates. Figure 16 demonstrates the kinetics of IgM secretion from various N-linked oligosaccharide processing inhibitor-treated 4A3 cells. With the exception of SW-treated cells, which exhibit a slightly reduced rate of secretion, the rates of export of IgM from 4A3 cells treated with the other processing inhibitors are comparable to that observed for control cells. No appreciable secretion of IgM was detected from TM-treated 4A3 cells.

3.2.1.1. IgM Secretion; I. Castanospermine.

The kinetic data are supported by the individual SDS-PAGE profiles of radioactive bands of immunoprecipitated material. Figure 17, panel a, shows the analysis of IgM secreted from CSP-treated 4A3 cells. The fluorographic intensity of µ-chain bands can be seen to increase with time of chase for both control and CSP-treated samples. This is in agreement with kinetic data which suggest that treatment of 4A3 cells with CSP does not result in any changes to the rate of export of IgM. However, the electrophoretic mobility of the µ-chains immunoprecipitated from cultures of CSP-treated cells is slower than that of the µ-chains derived from control cultures. A similar observation has been earlier reported in experiments using CSP (Peyrieras *et al.*, 1983; Pan *et al.*, 1983; Hori *et al.*, 1984). The higher M_r µ-chains secreted in the presence of CSP has been suggested to be due to the presence of precursor-type oligosaccharide structures.

Effect of treatment with CSP is further demonstrated by endo H analysis of the immunoprecipitated IgM (figure 17, panel b). µ-chains of samples from untreated culture supernatant are observed to be partially resistant to the enzyme

Figure 16. Kinetics of IgM secretion from N-linked oligosaccharide processing inhibitor-treated 4A3 cells.

Aliquots of immunoprecipitate derived from culture supernatants at the indicated times were spotted onto filter discs, and counted by liquid scintillation spectrometry. All determinations were performed in triplicate. *Panel A*: CSP-treated, *B*: dNM-treated, *C*: dMM-treated and *D*: SW-treated. In each panel (o - o) represents control, (Δ - Δ): processing inhibitor-treated and (□ - □): TM-treated.

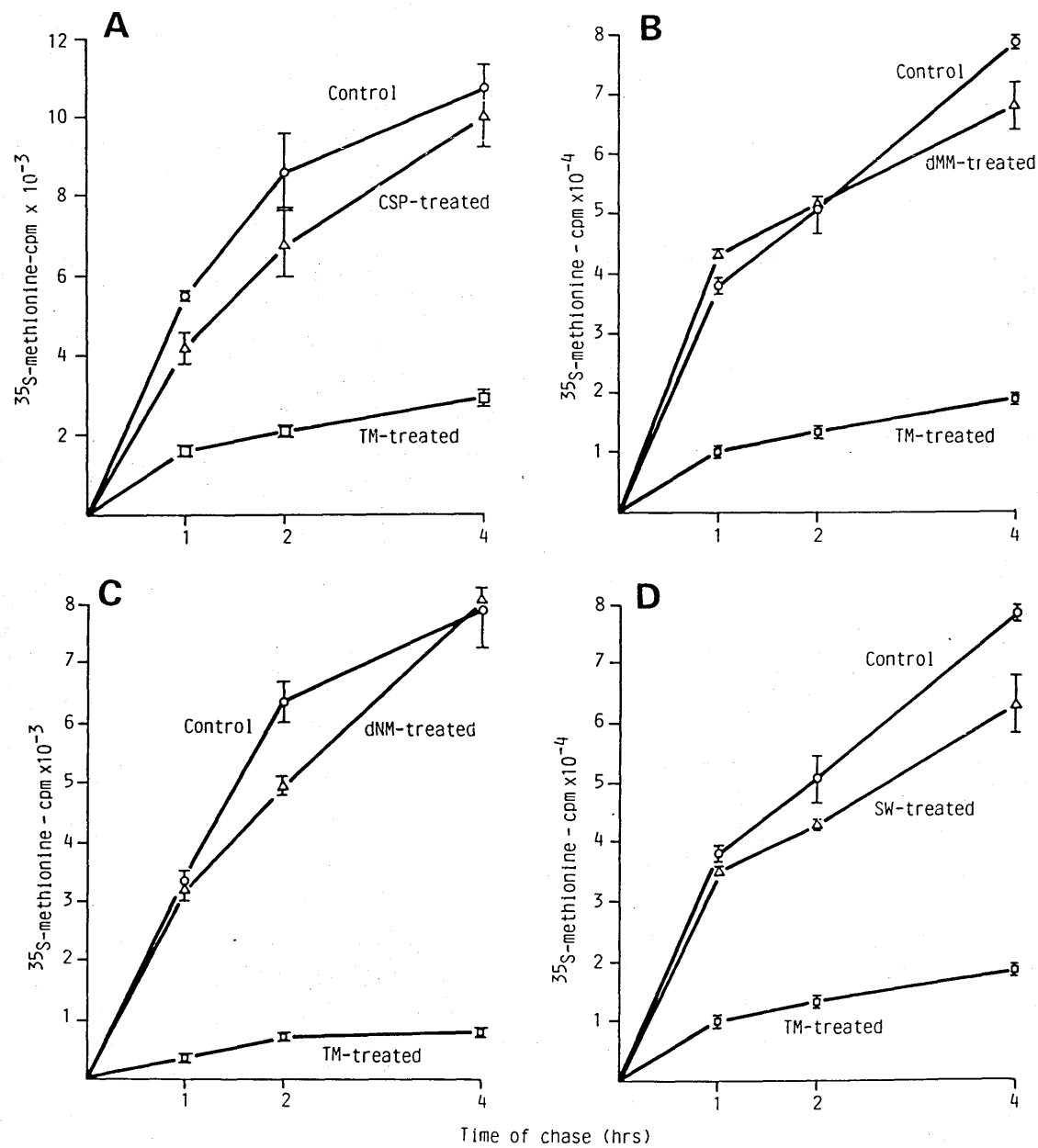
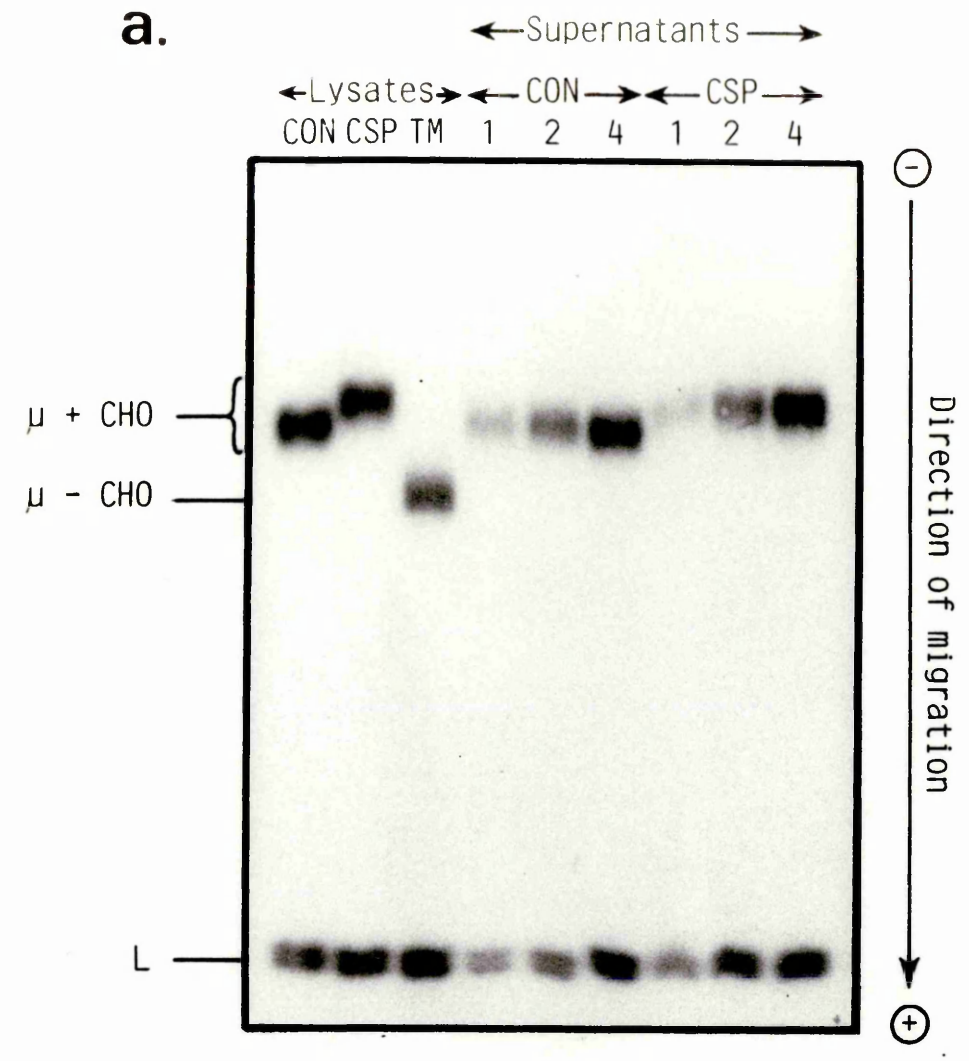
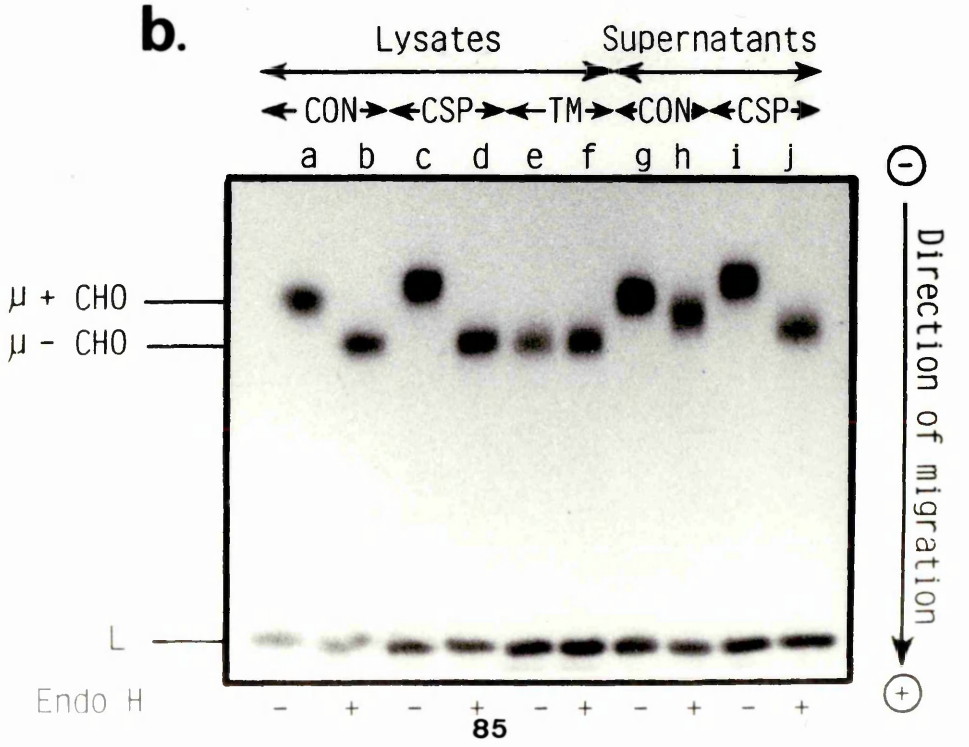

A
Control
CSP-treated
TM-treated
35S-methionine-cpm x 10-3
B
Control
dMM-treated
TM-treated
35S-methionine - cpm x10-4
C
Control
dNM-treated
TM-treated
35S-methionine - cpm x10-3
D
Control
SW-treated
TM-treated
35S-methionine - cpm x10-4
Time of chase (hrs)

Figure 17. Analysis of IgM secreted from Castanospermine-treated 4A3 cells.

Panel a; Aliquots of immunoprecipitate prepared from either cell lysates or supernatants from 4A3 cultures treated with CSP were boiled in sample buffer containing 2-mercaptoethanol and electrophoresed on 10% (W/V) acrylamide slab gels. Numbers for supernatant samples refer to duration of chase in hours.

Panel b; Small portions of immunoprecipitate prepared from 4 hour chase supernatants was precipitated with 5 volumes of acetone and resuspended in a small volume of 50mM citrate buffer, pH 5.5, containing 0.1% (W/V) SDS. 5 milliunits of endo H were added and the sample incubated at 37°C for 18 hours; a non-digested control was also prepared. Samples were analysed on 10% (W/V) acrylamide slab gels. Lysate (lanes a-f) and supernatant (lanes g-j) samples, both endo H-treated (+) (lanes b, d, f, h and j) and untreated controls (-) (lanes a, c, e, g and i) were analysed for each of control, TM- and CSP-treated cultures.


a.
Supernatants
Lysates
CON
CSP
CON CSP TM 1 2 4 1 2 4
μ + CHO
μ - CHO
L
Direction of migration


b.
Lysates
Supernatants
CON
CSP
TM
CON
CSP
a b c d e f g h i j
μ + CHO
μ - CHO
L
Endo H
- + - + - + - + - +
Direction of migration

treatment (lane h). This is because a single μ-chain generally contains five N-linked oligosaccharide moieties which exist as a mixture of complex and high mannose forms (see figure 5). Thus, partial endo H susceptibility is a result of the presence of these high mannose oligosaccharide structures in IgM. The μ-chains secreted in the presence of CSP are, however, fully susceptible to the enzyme treatment (lane j) indicating modified non-complex-type N-linked oligosaccharide structures.

3.2.1.2. IgM Secretion; II. 1-Deoxynojirimycin.

The electrophoretic mobility of μ-chains resolved by SDS-PAGE of reduced immunoprecipitates isolated from dNM-treated cells are illustrated in figure 18, panel a. In this case, however, there appear not to be any differences in the electrophoretic mobilities of μ-chains derived from dNM-treated cells to that of control cells. This is most likely due to the insufficient resolution of the bands since immunoprecipitate samples were subjected to a much shorter gel electrophoresis. In other cases, immunoglobulin heavy chains that are isolated from cells exposed to dNM demonstrate slower Mr electrophoretic migration as compared to control samples (e.g., see figure 23). The data, however, demonstrate that inactivation of cellular glucosidase I and II activities does not impair the secretion of IgM from 4A3 cells.

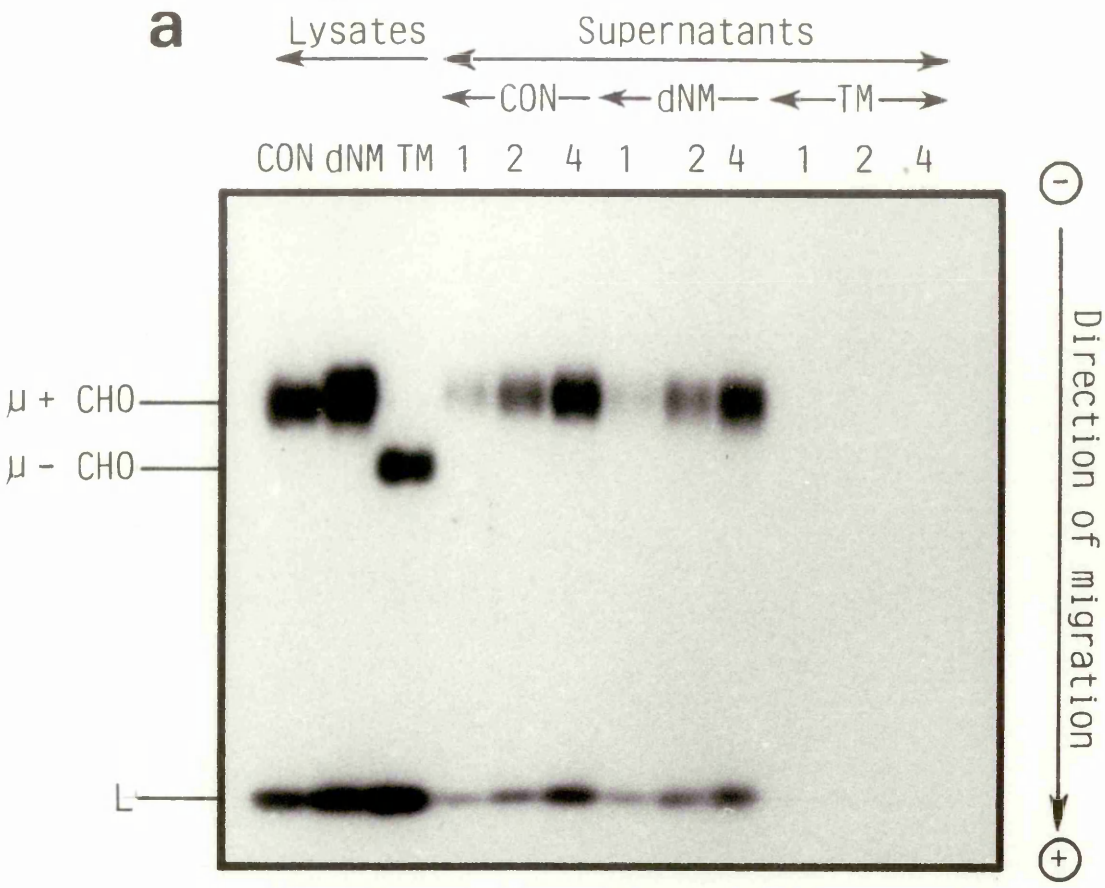
Endo H digestion analysis of the untreated and dNM-treated IgM immunoprecipitates confirms the effects of the sugar analogue on N-linked oligosaccharide processing; while the μ-chains isolated from the dNM-treated culture supernatants are susceptible to the enzyme (lane j), control μ-chains are resistant (lane h, figure 18, panel b). Intracellular immunoglobulins are, however, susceptible to the enzyme treatment in both cases (lanes b and d).

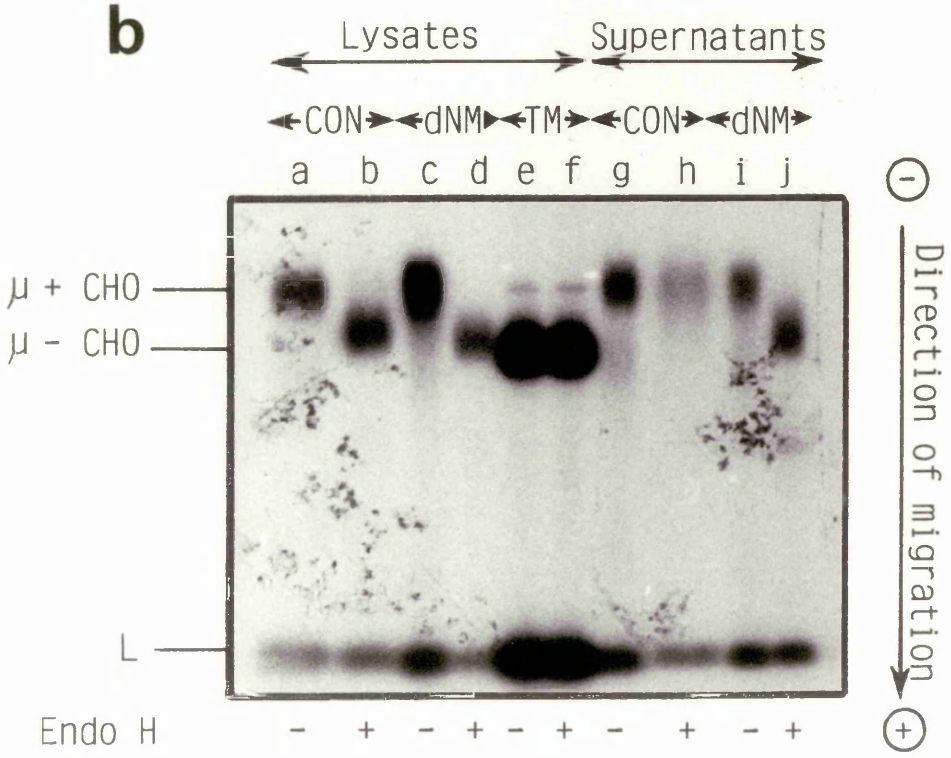
Figure 18. Analysis of IgM secreted from 1-deoxynojirimycin-treated 4A3 cells.

Panel a; SDS-PAGE analysis of immunoprecipitated IgM from dNM-treated 4A3 cells was performed on 10% (W/V) acrylamide gels as described in the legend to figure 17(a).

Panel b; Endo H digestion of immunoprecipitates prepared from aliquots of cell lysates or from 4 hours chase supernatants and analysis was performed as described in the legend to figure 17(b).

Endo H treatment of control secreted samples in this case demonstrates that carbohydrate moieties of the IgM are resistant to enzymatic cleavage. This is quite unexpected and differs from other data that show that carbohydrate moieties of IgM are partially susceptible to the enzyme due to the presence of high mannose N-linked oligosaccharide moieties. This may be due to the fact that the IgM may have not been completely denatured (a requirement for the enzyme to act) or that the enzyme that was used may have lost its activity.


a
Lysates
Supernatants
CON
dNM
TM
CON dNM TM 1 2 4 1 2 4 1 2 4
μ + CHO
μ - CHO
L
Direction of migration


b
Lysates
Supernatants
CON
dNM
TM
CON
dNM
a b c d e f g h i j
μ + CHO
μ - CHO
L
Endo H
- + - + - + - + - +
Direction of migration

3.2.1.3. IgM Secretion; III. 1-Deoxymannojirimycin.

Figure 19, panel a, demonstrates the fluorograph of a similar pulse-chase experiment performed in the presence of the mannosidase Ia/b inhibitor, dMM. Intensity of the μ-chain bands increases with the time of chase. However, there is no difference in the electrophoretic mobility of the μ-chains immunoprecipitated from cultures of dMM-treated cells with that of the μ-chains derived from control cultures. This is compatible with earlier findings from studies using this drug (see section 1.3.2.3). A similar result is also obtained from the endo H digestion analysis (figure 19, panel b). While control μ-chains exhibit partial susceptibility to the enzyme (lane h), total susceptibility is seen in the case of μ-chains isolated from the mannosidase Ia/b inhibitor-treated culture supernatants (lane j).

3.2.1.4. IgM Secretion; IV. Swainsonine.

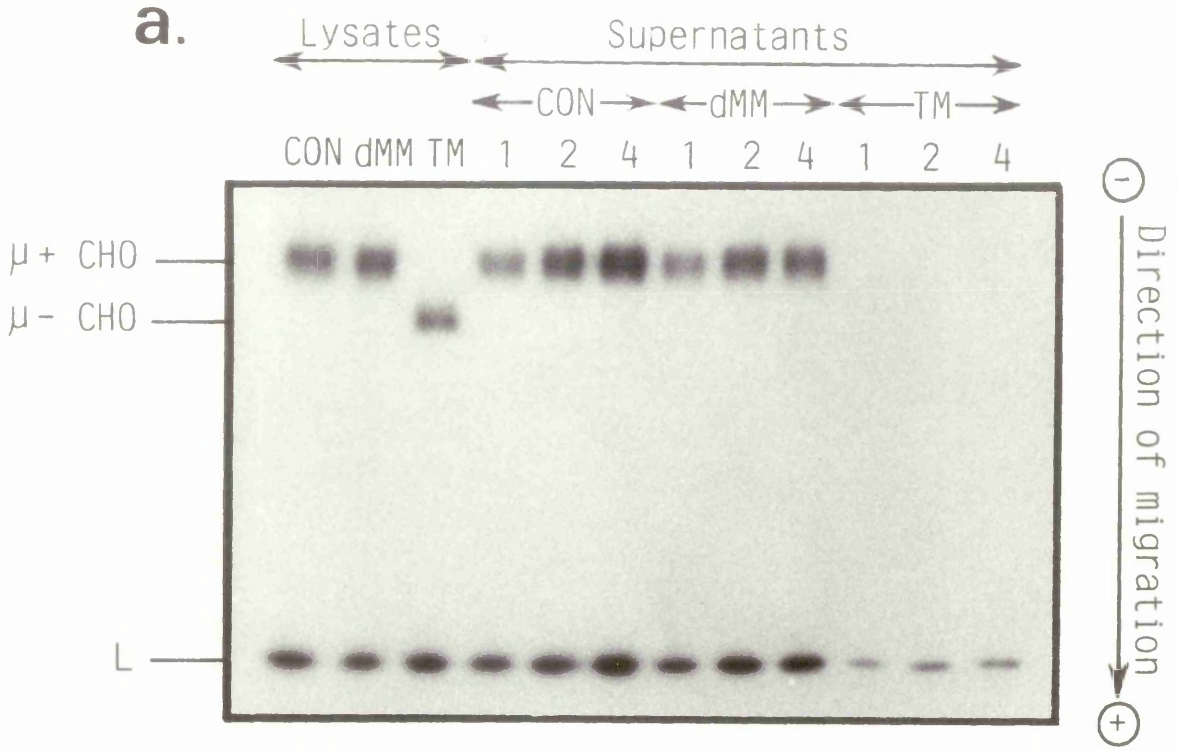
In the case of pulse-chase experiment with SW-treated 4A3 cells, identical electrophoretic resolution to that achieved in the presence of dMM was obtained (figure 20, panel a). The μ-chain bands are observed to increase in intensity accordingly with duration of chase. The effect of the mannosidase II inhibitor is further confirmed by the endo H analysis since, unlike the partially digested μ-chains secreted from control cells (lane h), μ-chains secreted in the presence of the drug are fully susceptible to the enzyme (lane j, figure 20, panel b).

The data from pulse-chase experiments carried out on 4A3 cells clearly demonstrate that inhibition of the processing of N-linked oligosaccharide at various stages of the pathway (glucosidase and mannosidase activities) does not lead to any significant interference with the rate of IgM secretion. The data fully support that previously reported by Peyrieras *et al.* (1983) and Fuhrmann *et al.* (1984), i.e., inhibition of oligosaccharide processing in B1.8μ cells by dNM, dMM and SW does not affect the secretion of IgM molecules. However, The B1.8μ cell line used in their study is unique and does not provide a suitable model for transport analysis.

Figure 19. Analysis of IgM secreted from 1-deoxymannojirimycin-treated 4A3 cells.

Panel a; SDS-PAGE analysis of immunoprecipitated IgM from dMM-treated 4A3 cells was performed on 10% (W/V) acrylamide gels as described in the legend to figure 17(a).

Panel b; Endo H digestion of immunoprecipitates prepared from aliquots of cell lysates or from 4 hours chase supernatants and analysis was performed as described in the legend to figure 17(b).


a.
Lysates
Supernatants
CON
dMM
TM
CON dMM TM 1 2 4 1 2 4 1 2 4
μ + CHO
μ - CHO
L
Direction of migration

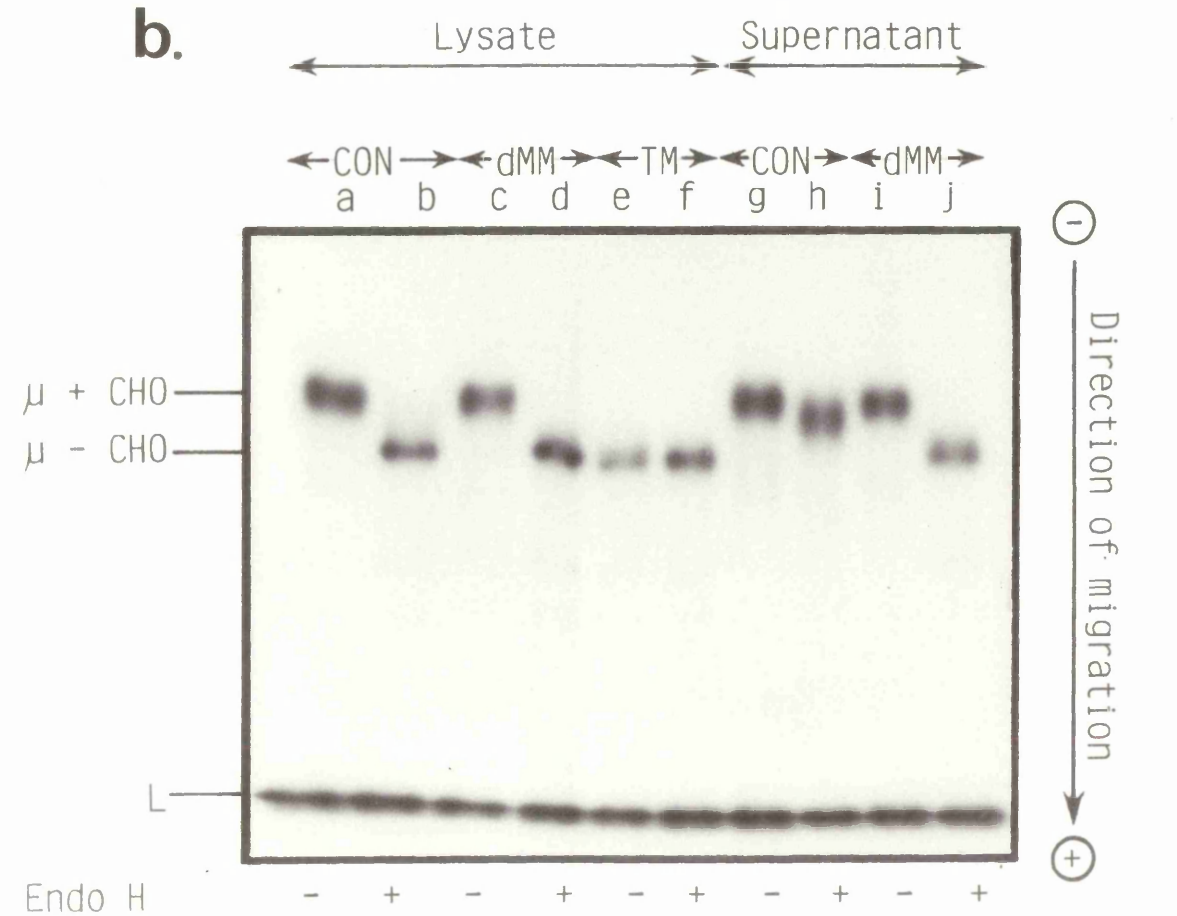
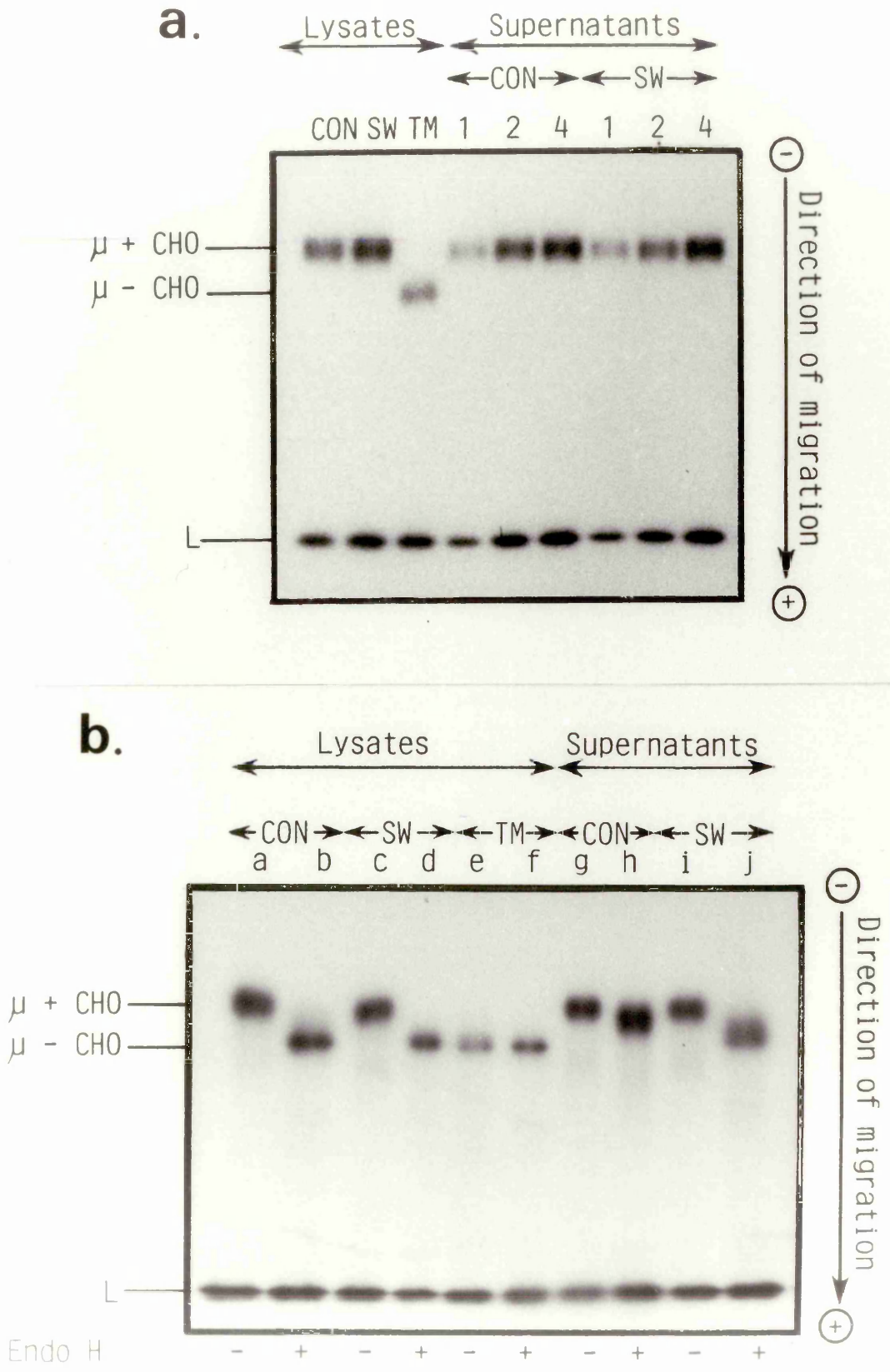

b.
Lysate
Supernatant
CON
dMM
TM
CON
dMM
a b c d e f g h i j
μ + CHO
μ - CHO
L
Direction of migration
Endo H
- + - + - + - + - +

Figure 20. Analysis of IgM secreted from Swainsonine-treated 4A3 cells.

Panel a; SDS-PAGE analysis of immunoprecipitated IgM from SW-treated 4A3 cells was performed on 10% (W/V) acrylamide gels as described in the legend to figure 17(a).

Panel b; Endo H digestion of immunoprecipitates prepared from aliquots of cell lysates or from 4 hours chase supernatants and analysis was performed as described in the legend to figure 17(b).


a.
Lysates
Supernatants
CON
SW
CON SW TM 1 2 4 1 2 4
μ + CHO
μ - CHO
L
Direction of migration
b.
Lysates
Supernatants
CON
SW
TM
CON
SW
a b c d e f g h i j
μ + CHO
μ - CHO
L
Endo H
- + - + - + - + - +
Direction of migration

The carbohydrate moieties of the IgM synthesized from the cell line have been reported not to be crucial for maintaining immunoglobulin secretion (Vasilov and Ploegh, 1982). Since IgM of B1.8μ can apparently be secreted in the non-N-glycosylated state, it is unlikely that inhibition of oligosaccharide processing would block secretion.

The study that was carried out using 4A3 cells is more definitive in that the cell line is compatible with most other IgM producing cells with respect to the importance of its carbohydrate moieties in controlling transport. Inhibition of N-glycosylation of IgM from 4A3 cells leads to blockade in secretion (see section 3.1.5). Thus, results that were obtained from the oligosaccharide processing inhibition experiments are more conclusive than those that have been previously reported. In addition, the above data also extends the existing observation by demonstrating that interference with CSP also fails to block intracellular transport of IgM.

3.2.2. Processing Inhibition of N-Linked Oligosaccharides of IgG.

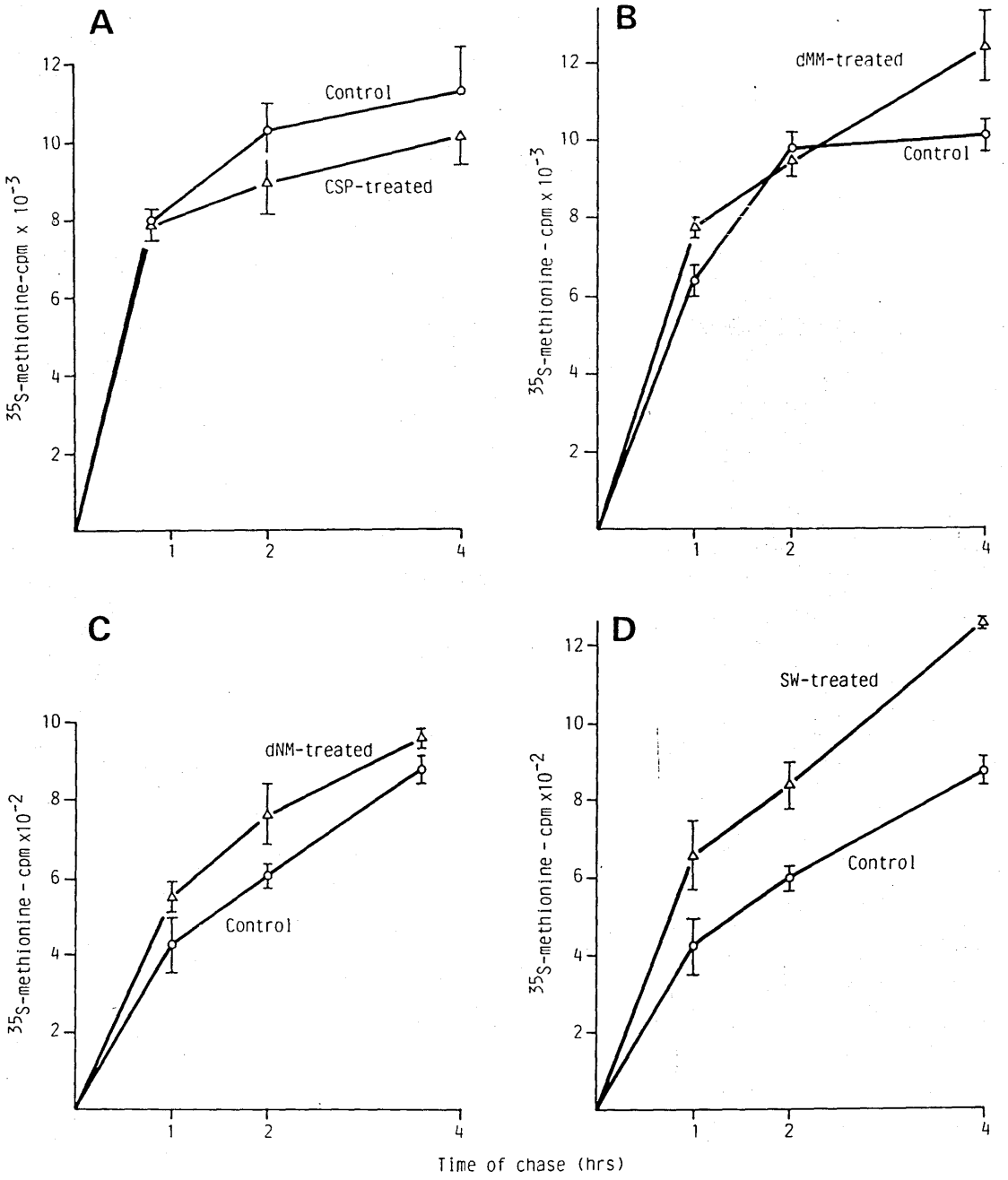
I1A1.4 cells were treated with the indicated concentrations of processing inhibitors. After pulse-chase, IgG was isolated from aliquots of lysate and supernatant of the cells by immunoprecipitation. Incorporation of radioactivity was determined by counting 5μl aliquots of immunoprecipitates. Figure 21 shows a kinetic analysis of the secretion of IgG from control and processing inhibitor-treated I1A1.4 cells. Treatment of the cells with SW appears to result in a slightly enhanced secretion. The rates of export of IgG from I1A1.4 cells treated with the other processing inhibitors are identical to those observed for control cells.

3.2.2.1 IgG Secretion; I. Castanospermine.

The SDS-PAGE profiles of IgG component polypeptides isolated from

Figure 21. Kinetics of IgG secretion from N-linked oligosaccharide processing inhibitor-treated I1A1.4 cells.

Aliquots of immunoprecipitate derived from culture supernatants at the indicated times were spotted onto filter discs, and counted by liquid scintillation spectrometry. All determinations were performed in triplicate. *Panel A*: CSP-treated, *B*: dNM-treated, *C*: dMM-treated and *D*: SW-treated. In each panel (O - O) represents control and (Δ - Δ): processing inhibitor-treated.


A
B
C
D
Control
CSP-treated
dMM-treated
Control
dNM-treated
Control
SW-treated
Control
35S-methionine-cpm x 10-3
35S-methionine - cpm x 10-3
35S-methionine - cpm x10-2
35S-methionine - cpm x10-2
Time of chase (hrs)

control, CSP- and TM-treated cultures are illustrated in figure 22, panel a. The data demonstrate that CSP-treatment does not result in any decrease in the rate of export of IgG from cells exposed to the alkaloid, and the M_r of the γ-chain isolated from the culture supernatants is slightly greater than that of control γ-chains.

The oligosaccharide structure present on the IgG_{2b} molecules secreted by I1A1.4 cells is complex in form and is resistant to endo H digestion (see section 3.1.7). Figure 22, panel b, illustrates that following treatment with CSP, the intracellular form of the γ-chain in the treated cells shows susceptibility to endo H, and also that those isolated from the culture supernatants are also digested by the enzyme. The data suggest that CSP has inhibited the glucosidase activity of I1A1.4 cells, since the treated cells have not converted the N-linked oligosaccharides to a complex, endo H-resistant state in a manner analogous to control, untreated cells. The data are consistent with the notion that CSP can inhibit processing of the N-linked oligosaccharides of the IgG molecule, but that such interference does not impair secretion of IgG from treated cells.

3.2.2.2. IgG Secretion; II. 1-Deoxynojirimycin.

Figure 23, panel a, shows the SDS-PAGE profiles of IgG isolated from control, dNM- and TM-treated I1A1.4 cultures, respectively. The data show a similar trend to that obtained using CSP, i.e., dNM-treatment does not result in any decrease in the rate of export of IgG from cells and may indeed cause a slight increase in the kinetics of IgG secretion. The γ-chains isolated from lysates or supernatants of dNM-treated cells also exhibit a slightly slower electrophoretic mobility relative to control γ-chains.

Figure 23, panel b, demonstrates that dNM treatment alters susceptibility

Figure 22. Analysis of IgG secreted from Castanospermine-treated I1A1.4 cells.

Panel a; Aliquots of immunoprecipitate prepared from either cell lysates or supernatants from I1A1.4 cultures treated with CSP were boiled in sample buffer containing 2-mercaptoethanol and electrophoresed on 10% (W/V) acrylamide slab gels. Numbers for supernatant samples refer to duration of chase in hours.

Panel b; Small portions of immunoprecipitate prepared from 4 hour chase supernatants was precipitated with 5 volumes of acetone and resuspended in a small volume of 50mM citrate buffer, pH 5.5, containing 0.1% (W/V) SDS. 5 milliunits of endo H were added and the sample incubated at 37°C for 18 hours; a non-digested control was also prepared. Samples were analysed on 10% (W/V) acrylamide slab gels. Lysate (lanes a-f) and supernatant (lanes g-l) samples, both endo H-treated (+) (lanes b, d, f, h, j and l) and untreated controls (-) (lanes a, c, e, g, i and k) were analysed for each of control, TM- and CSP-treated cultures.

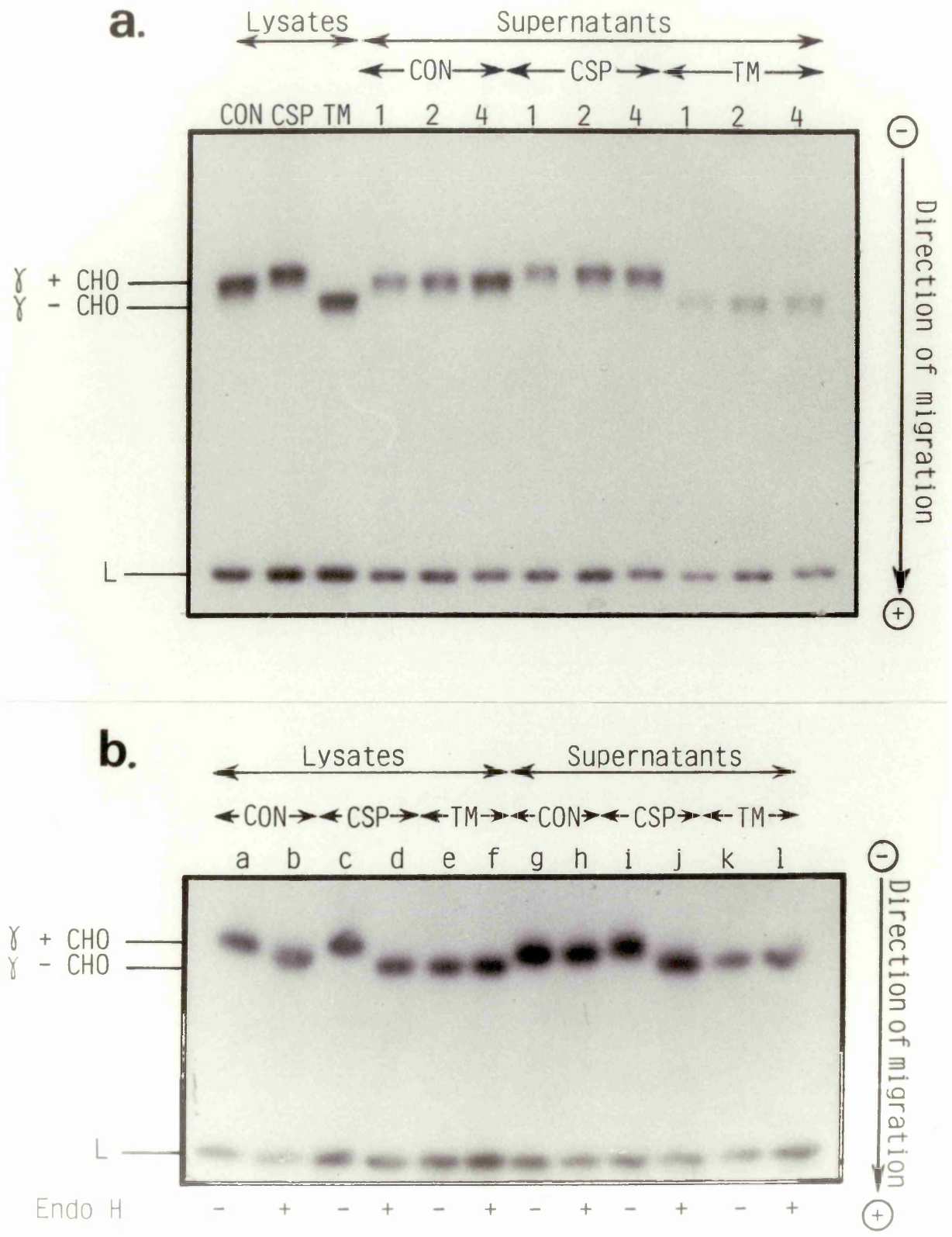
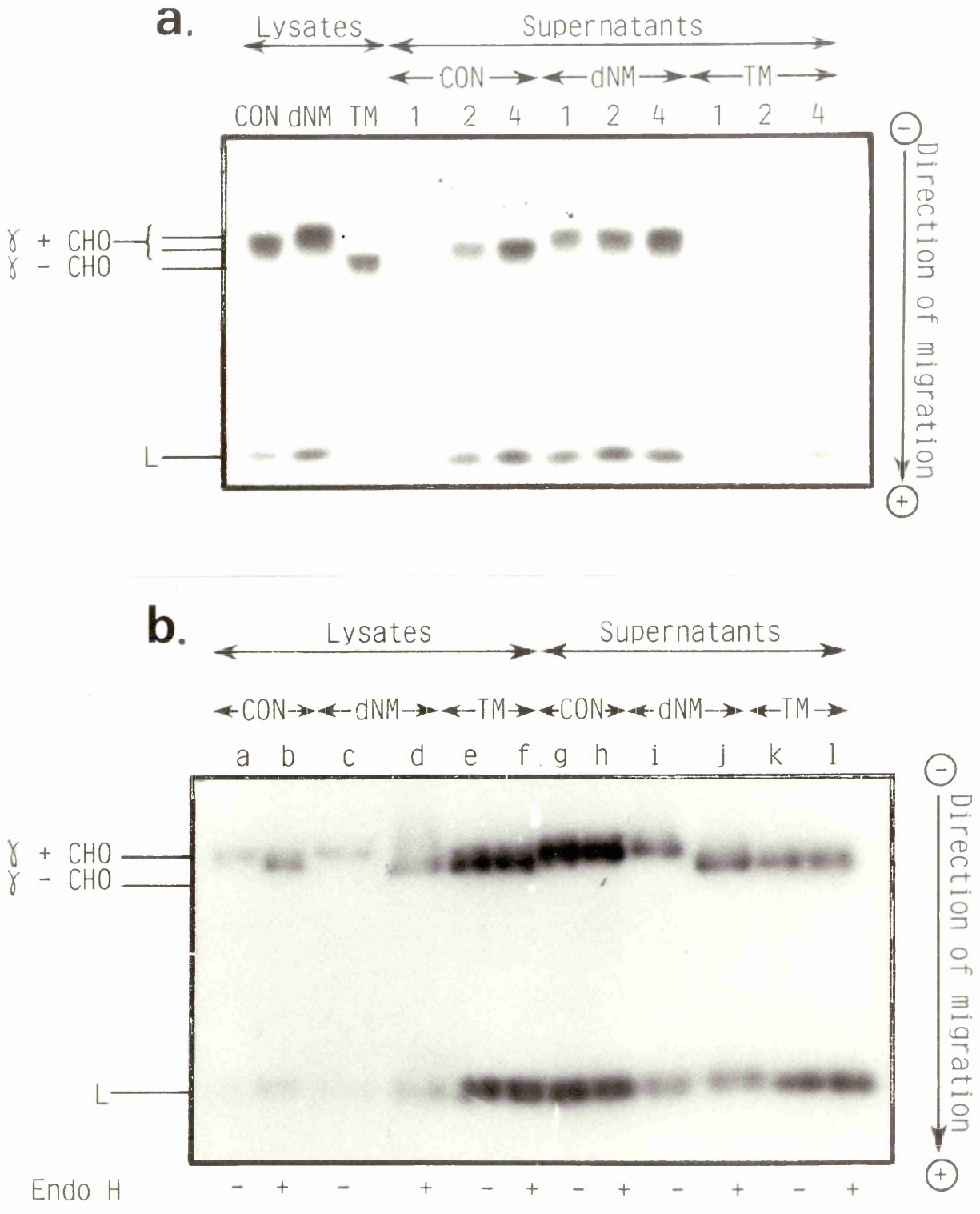

a.
Lysates
Supernatants
CON
CSP
TM
CON CSP TM 1 2 4 1 2 4 1 2 4
Y + CHO
Y - CHO
L
Direction of migration
b.
Lysates
Supernatants
CON CSP TM CON CSP TM
a b c d e f g h i j k l
Y + CHO
Y - CHO
L
Endo H - + - + - + - + - + - +
Direction of migration

Figure 23. Analysis of IgG secreted from 1-deoxynojirimycin-treated I1A1.4 cells.

Panel a; SDS-PAGE analysis of immunoprecipitated IgG from dNM-treated I1A1.4 cells was performed on 10% (W/V) acrylamide gels as described in the legend to figure 22(a).

Panel b; Endo H digestion of immunoprecipitates prepared from aliquots of cell lysates or from 4 hours chase supernatants and analysis was performed as described in the legend to figure 22(b).


a.
Lysates
Supernatants
CON
dNM
TM
CON dNM TM 1 2 4 1 2 4 1 2 4
γ + CHO
γ - CHO
L
Direction of migration
b.
Lysates
Supernatants
CON
dNM
TM
CON
dNM
TM
a b c d e f g h i j k l
γ + CHO
γ - CHO
L
Direction of migration
Endo H
- + - + - + - + - + - +

of the γ-chain to endo H digestion. While control secreted γ-chains are resistant to the effects of the enzyme, IgG secreted from dNM-treated cells contains γ-chains which are susceptible to digestion by endo H. The data therefore parallel those obtained from studies with CSP in illustrating that while dNM can prevent oligosaccharide processing and thereby alter the qualitative structure of the N-linked glycans of the IgG molecule, it has no inhibitory effect upon secretion of IgG from the cell.

3.2.2.3. IgG Secretion; III. 1-Deoxymannojirimycin.

Figure 24, panel a, demonstrates the fluorograph of pulse-chase experiment carried out in the presence of dMM. There is no difference in the electrophoretic mobility of the γ-chains immunoprecipitated from cultures of the sugar analogue-treated cells with that of the γ-chains derived from control cultures. Intensity of the γ-chain bands also corresponds with the increasing time of chase. Result from the endo H analysis on the immunoprecipitated IgG produced from control, dMM- and TM-treated I1A1.4 cells is illustrated in figure 24, panel b. The carbohydrate chains of IgG secreted in the presence of the mannosidase Ia/b inhibitor has been structurally modified as demonstrated by the γ-chains susceptibility to endo H digestion (lane j).

3.2.2.4. IgG Secretion; IV. Swainsonine.

Pulse-chase experiments with I1A1.4 cells treated with SW yield similar results. Identical electrophoretic resolution to that done in the presence of dMM was obtained (figure 25, panel a). Modification of the N-linked oligosaccharides of IgG synthesized in the presence of this processing inhibitor is further confirmed by the endo H analysis where only the drug-treated sample is fully susceptible to the enzyme (lane j, figure 25, panel b).

The data obtained from experiments using I1A1.4 cells demonstrate that

Figure 24. Analysis of IgG secreted from 1-deoxymannojirimycin-treated I1A1.4 cells.

Panel a; SDS-PAGE analysis of immunoprecipitated IgG from dMM-treated I1A1.4 cells was performed on 10% (W/V) acrylamide gels as described in the legend to figure 22(a).

Panel b; Endo H digestion of immunoprecipitates prepared from aliquots of cell lysates or from 4 hours chase supernatants and analysis was performed as described in the legend to figure 22(b).

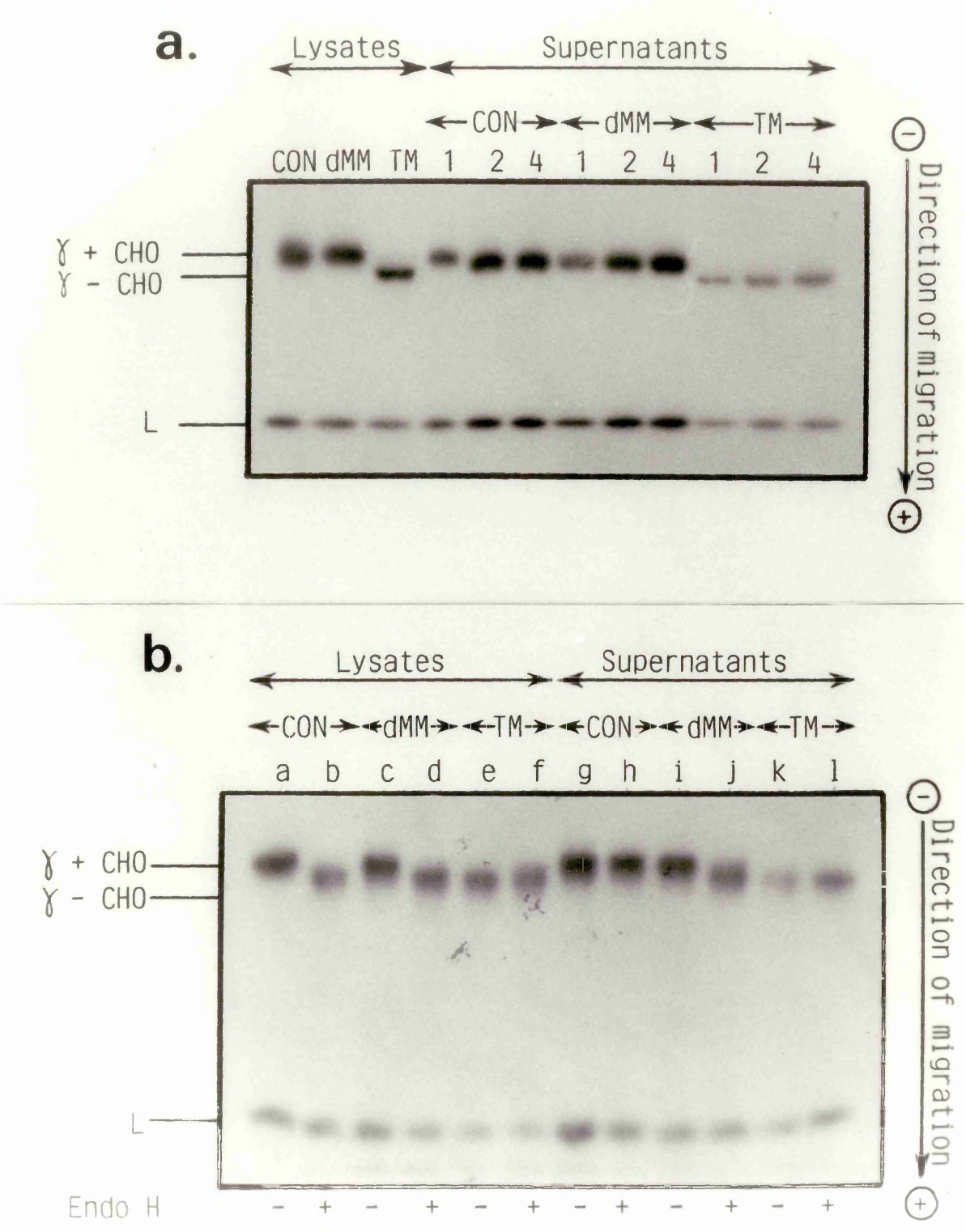
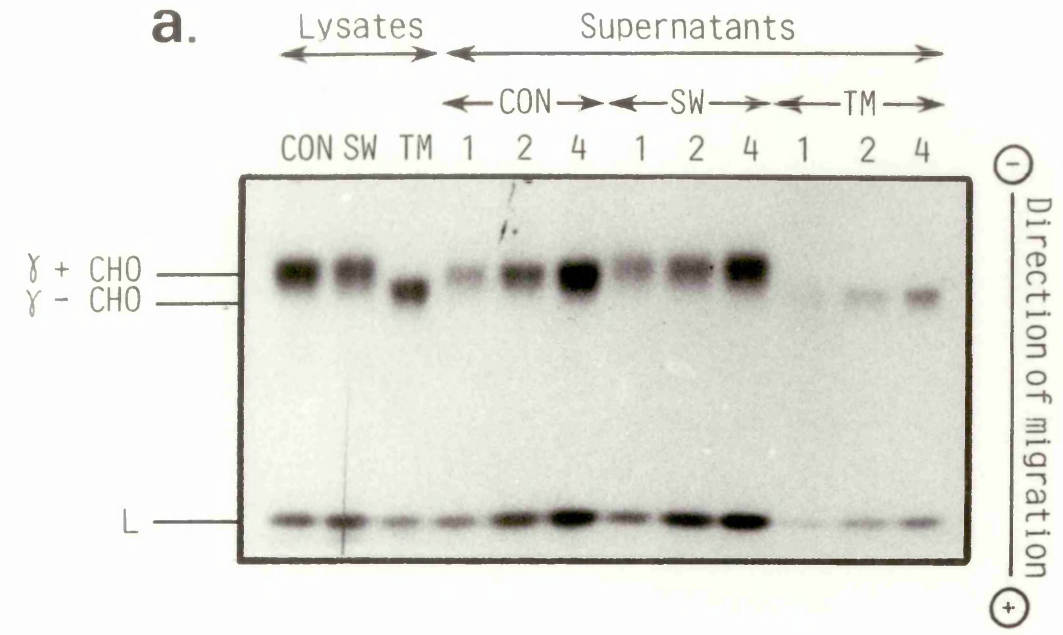
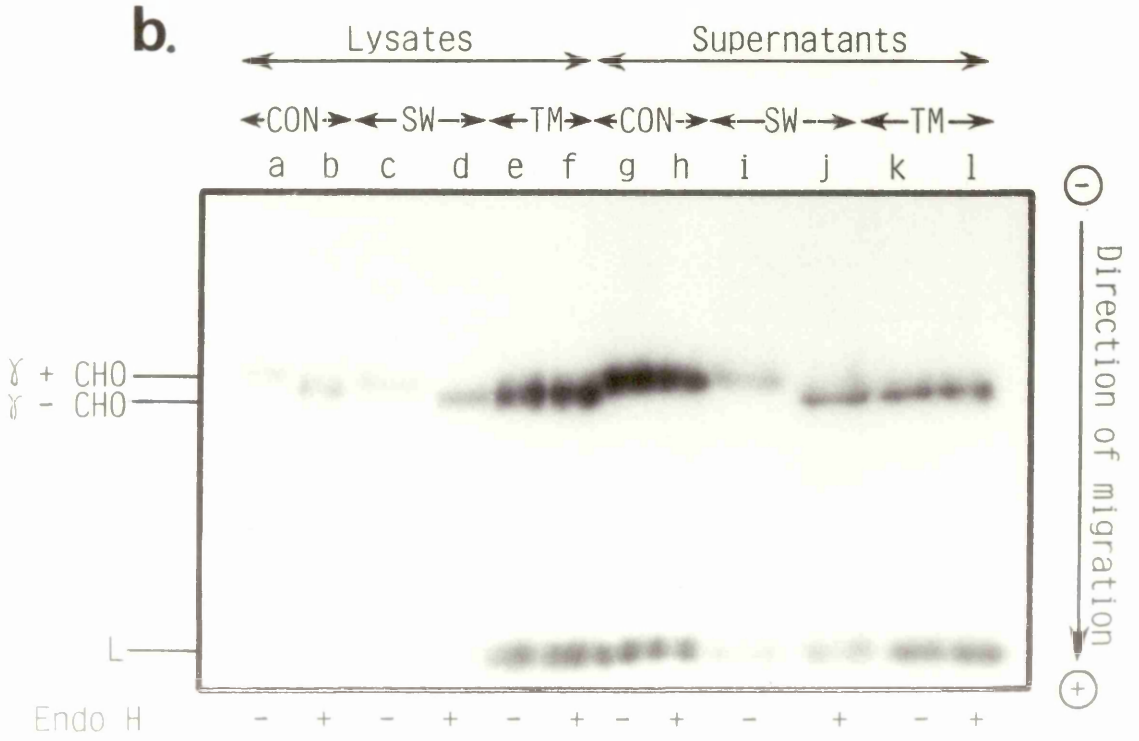

a.
Lysates
Supernatants
CON
dMM
TM
CON dMM TM 1 2 4 1 2 4 1 2 4
γ + CHO
γ - CHO
L
Direction of migration
b.
Lysates
Supernatants
CON
dMM
TM
CON
dMM
TM
a b c d e f g h i j k l
γ + CHO
γ - CHO
L
Endo H
- + - + - + - + - + - +
Direction of migration

Figure 25. Analysis of IgG secreted from Swainsonine-treated I1A1.4 cells.

Panel a; SDS-PAGE analysis of immunoprecipitated IgG from SW-treated I1A1.4 cells was performed on 10% (W/V) acrylamide gels as described in the legend to figure 22(a).

Panel b; Endo H digestion of immunoprecipitates prepared from aliquots of cell lysates or from 4 hours chase supernatants and analysis was performed as described in the legend to figure 22(b).


a.
Lysates
Supernatants
CON
SW
TM
CON SW TM 1 2 4 1 2 4 1 2 4
γ + CHO
γ - CHO
L
Direction of migration


b.
Lysates
Supernatants
CON
SW
TM
CON
SW
TM
a b c d e f g h i j k l
γ + CHO
γ - CHO
L
Endo H - + - + - + - + - + - +
Direction of migration

the rate of secretion of IgG is not diminished by inhibition of either glucosidase or mannosidase activities. Since IgG can be efficiently secreted in the non-N-glycosylated state (see table 1), it is unlikely that inhibition of oligosaccharide processing would block secretion. Such a possibility is, however, not totally excluded, as has been demonstrated in case of the IgD of the B1.8δ cells. While transport of the non-N-glycosylated IgD is efficiently maintained (Vasilov and Ploegh, 1982), inhibition of glucosidase I and II activities by dNM lead to markedly impaired immunoglobulin export (Peyrieras *et al.*, 1983). The effects of carbohydrate processing inhibition by CSP on the expression of membrane-form immunoglobulins are also similarly observed. Membrane-form IgD cell surface expression is markedly retarded in the presence of the glucosidase inhibitor but is not affected in the presence of mannosidase inhibitors (Cushley *et al.*, personal communication).

The capacity of dNM to inhibit secretion is apparently random with the secretion of α1-antichymotrypsin and α_1-antitrypsin, but not transferrin, albumin or C3, from the human HepG-2 cell line being inhibited (Lodish and Kong, 1984). Similarly, in rat hepatocytes, dNM treatment inhibited the secretion of α_1-acid glycoprotein and α_1-proteinase inhibitor by approximately 50%. CSP treatment, however, caused no appreciable inhibition of secretion of any of these macromolecules from the hepatocytes (Gross *et al.*, 1986). CSP also has apparently selective effects upon secretion of proteins from hepatocytes. For example, the secretion of α_1-antitrypsin, caeruloplasmin and, to a much lesser extent, antithrombin III can be inhibited by CSP treatment. In the same cells, however, the rates of secretion of the protein albumin and of the exclusively O-glycosylated glycoprotein apolipoprotein E were not affected.

Exposure to mannosidase inhibitors have, however, resulted in consistent

observations, i.e., their interference does not lead to any retardation of glycoprotein export (Peyrieras *et al.*, 1983; Fuhrmann *et al.*, 1984; Gross *et al.*, 1985; Hashim and Cushley, 1987b). Indeed, several groups have observed that the export of glycoproteins can apparently be accelerated in the presence of SW (Yeo *et al.*, 1985; Fuhrmann *et al.*, 1985). In hepatocytes, the rates of secretion of caeruloplasmin and α2-macroglobulin were increased, but not those of transferrin, albumin or α1-antitrypsin (Yeo *et al.*, 1985).

The data obtained from this study may be interpreted in terms of the overall sensitivity of a given immunoglobulin isotype to interference with glycosylation as assessed by ultimate secretory fate. For example, treatment of B cell lines with tunicamycin results in different effects upon secretion depending on the isotype of the immunoglobulin synthesized by the cell line. Many laboratories have observed that IgM secretion is inhibited by tunicamycin treatment, but IgG secretion proceeds quite efficiently (see table 1). This may be reflected in the experiments with SW, where the secretion of IgM, but not IgG, is slightly retarded by the presence of the mannosidase II inhibitor. The two most obvious explanations which could be advanced to account for the data would be the number of oligosaccharide groups per heavy chain, and the overall size and solubility of the assembled immunoglobulin (19S IgG as opposed to 7S IgG). Neither of these views can be readily accommodated with data obtained from hepatocytes systems. Thus, both α2-macroglobulin and caeruloplasmin are high molecular weight, multiply N-glycosylated molecules, and the secretion of both is potentiated rather than retarded by SW.

3.2.3. Simultaneous Inhibition of N-linked Oligosaccharide Processing of IgM and IgG.

The data from the above SDS-PAGE analysis, however, do not reveal the

precise structures that evolved from manipulations involving use of individual N-linked oligosaccharide processing inhibitors. Several reports have already described that inhibition of carbohydrate processing by using CSP, dNM or SW does not totally prevent further modification reactions (Peyrieras *et al.*, 1983; Tulsiani *et al.*, 1983; Gross *et al.*, 1986). Terminal carbohydrate branches that are not affected by the actions of the processing inhibitor are normally further processed and may indeed be transformed to the complex type structures. Thus, if N-linked oligosaccharide moieties may be partly processed and undergo complex conversion reactions, there is still a possibility of configurations that are generated becoming involved in the putative ligand-receptor-mediated transport processes. Accordingly, a study assessing the effects of simultaneous inhibition of glucosidase and mannosidase activities, which theoretically assures complete prevention of complex conversion reactions, on the export of rat hybridoma IgM and IgG was carried out.

Figure 26 demonstrates the kinetics of IgM (panels a, b and c) and IgG (panels d, e and f) secretion in the presence of mixtures of processing inhibitors. Cells were exposed to the mixtures of processing inhibitors prior to the routine pulse-chase procedures. The data demonstrate that, in all cases, simultaneous blockade of the glucosidase and mannosidase activities does not interfere with the rate of immunoglobulin secretion from rat hybridoma cells.

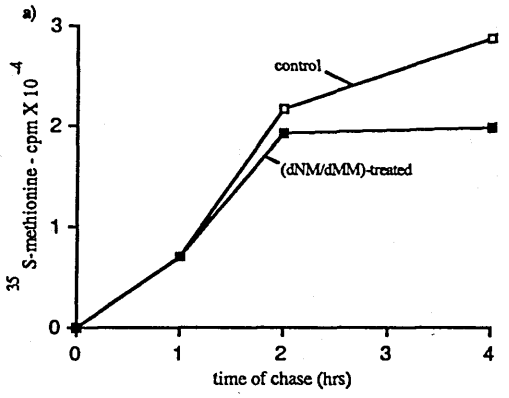
3.2.3.1. SDS-PAGE Analysis of Mixtures of Inhibitors-Treated 4A3 and I1A1.4 Cells.

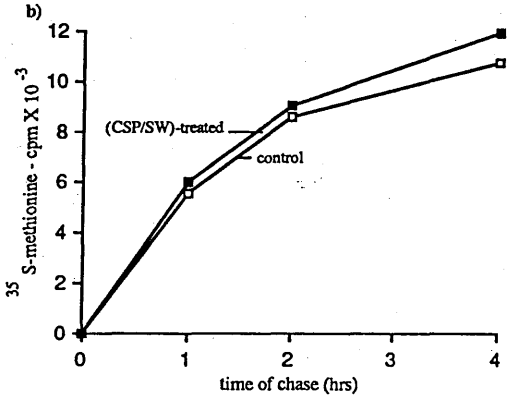
The SDS-PAGE profiles of IgM component polypeptides isolated from control and mixtures of processing inhibitors-treated cultures are illustrated in figure 27; panels a: dNM/dMM, b: CSP/SW and e: CSP/dNM/dMM/SW (4I in figure). The data demonstrate that simultaneous blockade of multiple steps involved in the processing of N-linked oligosaccharides of the immunoglobulin μ-chains does not impair the rate of IgM secretion from 4A3 cells. Intensity of μ-chain bands is

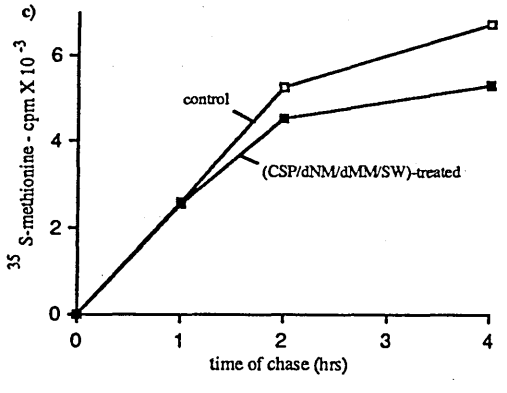
Figure 26. Kinetics of IgM and IgG secretion from cells treated with mixed oligosaccharide processing inhibitors.

Aliquots of immunoprecipitate derived from culture supernatants at the indicated times were spotted onto filter discs, and counted by liquid scintillation spectrometry. All determinations were performed in triplicate. *Panels a, b* and *c* were counts obtained from immunoprecipitated IgM molecules. *Panels d, e* and *f* were counts obtained from immunoprecipitated IgG molecules.

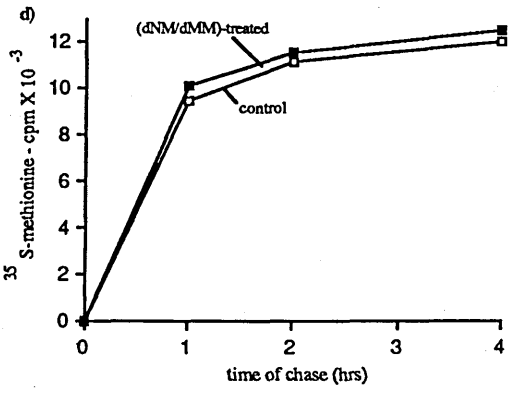
4A3

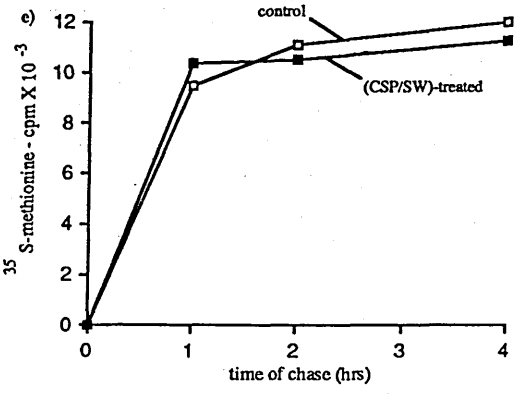

a)
control
(dNM/dMM)-treated
35 S-methionine - cpm X 10 -4
time of chase (hrs)


b)
(CSP/SW)-treated
control
35 S-methionine - cpm X 10 -3
time of chase (hrs)


c)
control
(CSP/dNM/dMM/SW)-treated
35 S-methionine - cpm X 10 -3
time of chase (hrs)

11A1.4


d)
(dNM/dMM)-treated
control
35 S-methionine - cpm X 10 -3
time of chase (hrs)


e)
control
(CSP/SW)-treated
35 S-methionine - cpm X 10 -3
time of chase (hrs)

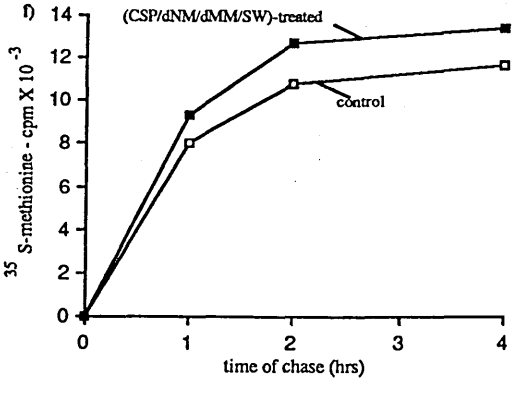
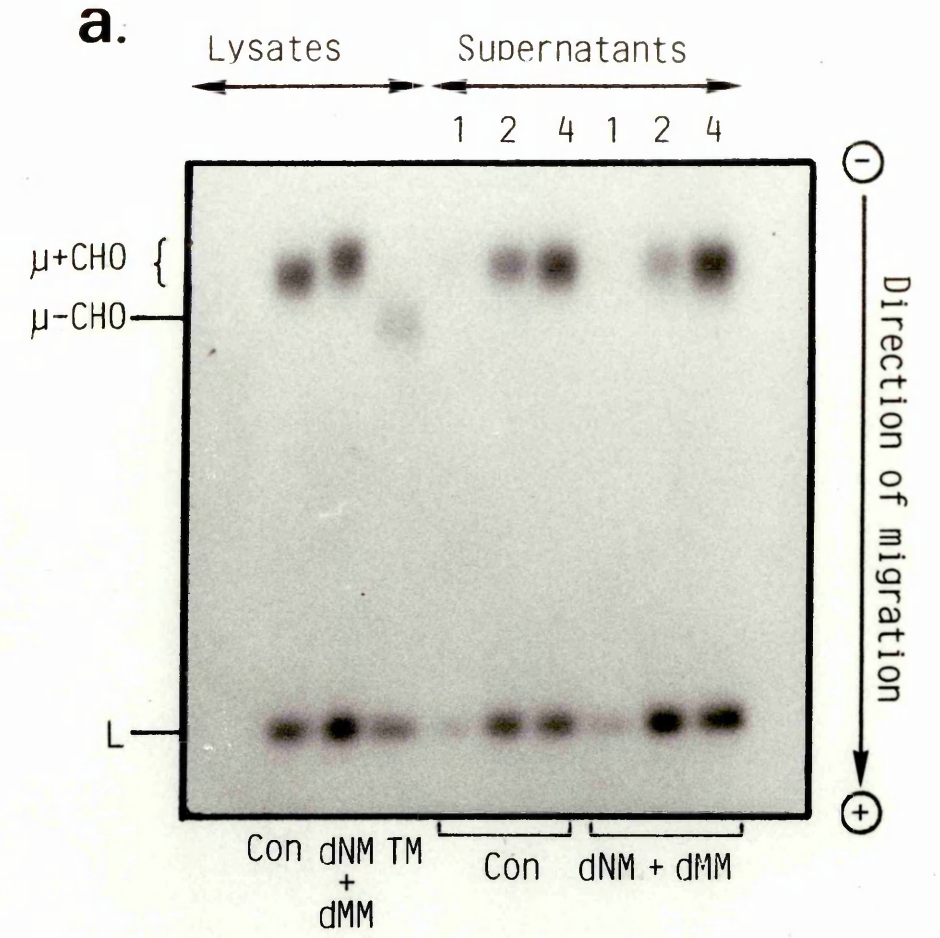
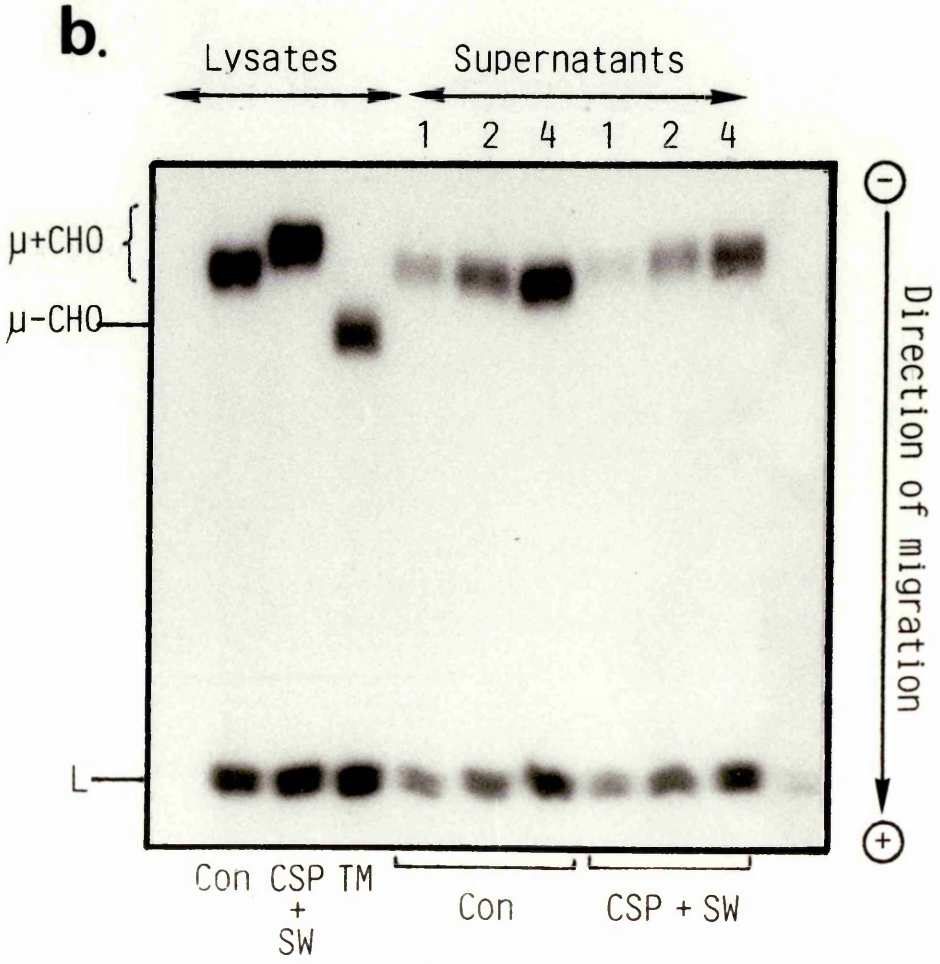

f)
(CSP/dNM/dMM/SW)-treated
control
35 S-methionine - cpm X 10 -3
time of chase (hrs)

Figure 27. SDS-PAGE profiles of immunoglobulins isolated from cultures treated with mixtures of oligosaccharide processing inhibitors.

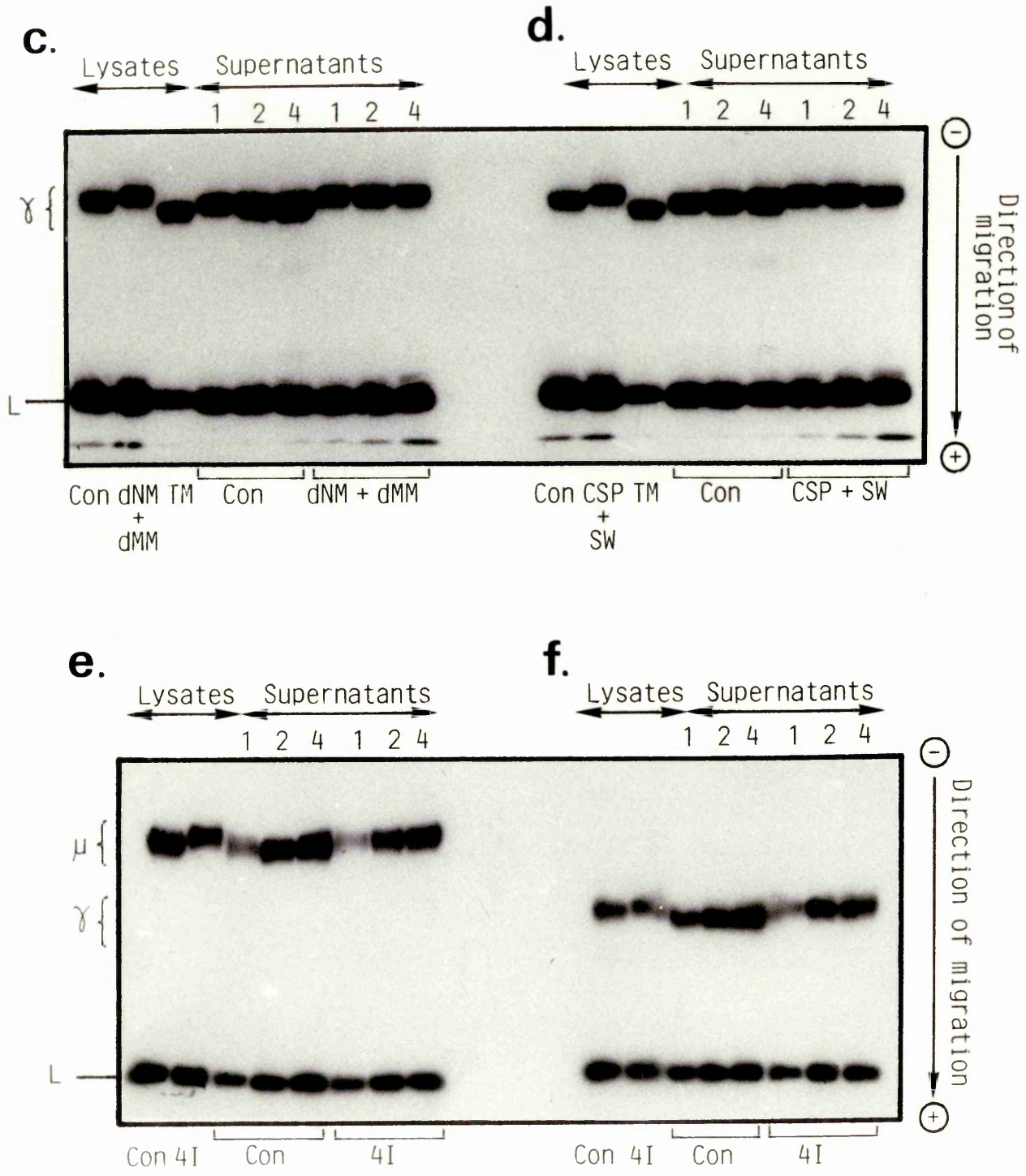
Aliquots of immunoprecipitate prepared from either cell lysates or supernatants from 4A3 and I1A1.4 cultures exposed to mixtures of processing inhibitors were boiled in sample buffer containing 2-mercaptoethanol and electrophoresed on 10% (W/V) acrylamide slab gels. Numbers for supernatant samples refer to duration of chase in hours. *Panels a* and *b* demonstrate the heavy and light chains of immunoglobulins secreted from 4A3 cells treated with dNM/dMM and CSP/SW, respectively. *Continue on next page...*


a.
Lysates
Supernatants
1 2 4 1 2 4
μ+CHO
μ-CHO
L
Direction of migration
Con
dNM + dMM
TM
Con
dNM + dMM


b.
Lysates
Supernatants
1 2 4 1 2 4
μ+CHO
μ-CHO
L
Direction of migration
Con
CSP + SW
TM
Con
CSP + SW

...continuation of figure 27.

Panels c and *d* demonstrate the heavy and light chains of immunoglobulins secreted from I1A1.4 cells treated with dNM/dMM and CSP/SW, respectively. *Panels e* (4A3) and *f* (I1A1.4) show the SDS-PAGE resolution of immunoglobulins isolated from cells treated with all of the processing inhibitors (i.e., CSP/dNM/dMM/SW or 4I).


c.
Lysates
Supernatants
1 2 4 1 2 4
γ
L
Con dNM TM + dMM
Con
dNM + dMM
d.
Lysates
Supernatants
1 2 4 1 2 4
Con CSP TM + SW
Con
CSP + SW
Direction of migration
e.
Lysates
Supernatants
1 2 4 1 2 4
μ
γ
L
Con 4I
Con
4I
f.
Lysates
Supernatants
1 2 4 1 2 4
Con 4I
Con
4I
Direction of migration

observed to increase with the time of chase. In all cases, however, μ-chains that were isolated from culture supernatant of treated cells migrated more slowly than those of untreated cells.

Panels c, d and f of the same figure demonstrate the electrophoretic mobilities of γ-chains isolated from mixtures of inhibitors-treated I1A1.4 cells resolved by SDS-PAGE (panels c: dNM/dMM, d: CSP/SW and f: CSP/dNM/dMM/SW, i.e., 4I in figure). As in IgM from 4A3 cells, the export of IgG from I1A1.4 cells is not affected when exposed to the mixtures of the processing inhibitors. Also observed in all cases is the retarded electrophoretic migration of γ-chains isolated from mixtures of drugs-treated cells.

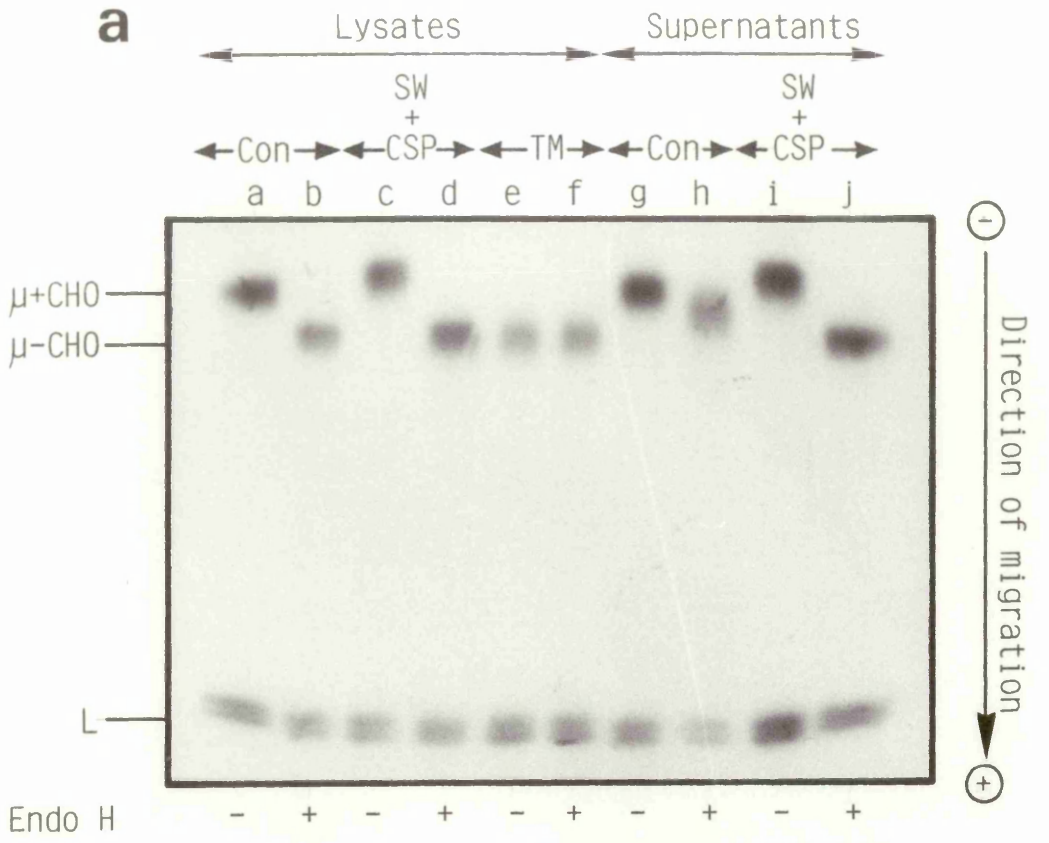
3.2.3.2. Endo H Analysis of Mixtures of Inhibitors-Treated 4A3 and I1A1.4 Cells.

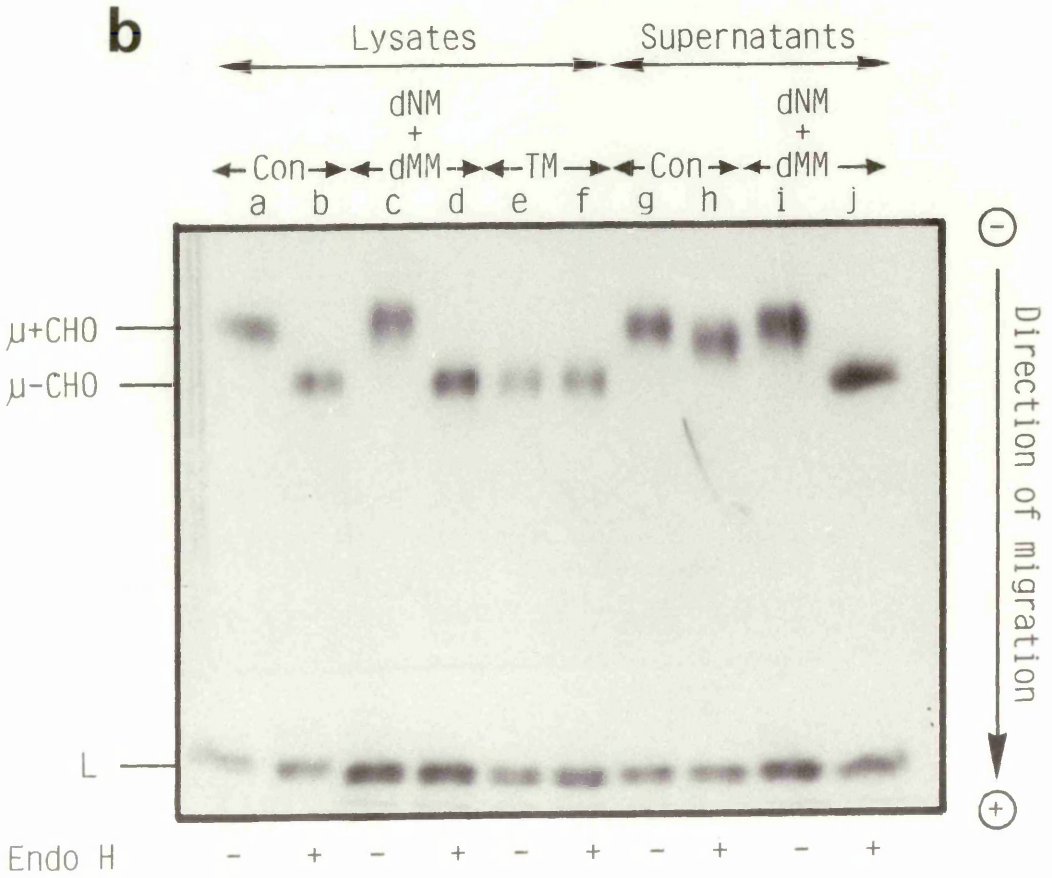
Aliquots of immunoprecipitates of IgM and IgG that were isolated from cultures of cells that were exposed to the mixtures of processing inhibitors and their respective controls were subjected to endo H analysis as described in section 2.6.1. The individual enzyme- and mock-treated samples were then reduced and resolved by SDS-PAGE. Figure 28 demonstrates that when exposed to the series of the processing inhibitors, μ- and γ-chains that were secreted are susceptible to the enzyme digestion.

The principal finding from the analysis that was carried out by simultaneously inhibiting the multiple steps involved in the processing of N-linked oligosaccharides of immunoglobulins is that such interference has no inhibitory effect upon the secretion of immunoglobulins from rat hybridoma cells. In all cases studied, however, the qualitative structure of the oligosaccharides present on the immunoglobulins was different from controls as determined by susceptibility to endo H digestion and by the M_r of the isolated immunoglobulin heavy chains on

Figure 28. Endo H analysis of mixtures of oligosaccharide processing inhibitors-treated 4A3 and I1A1.4 cells.

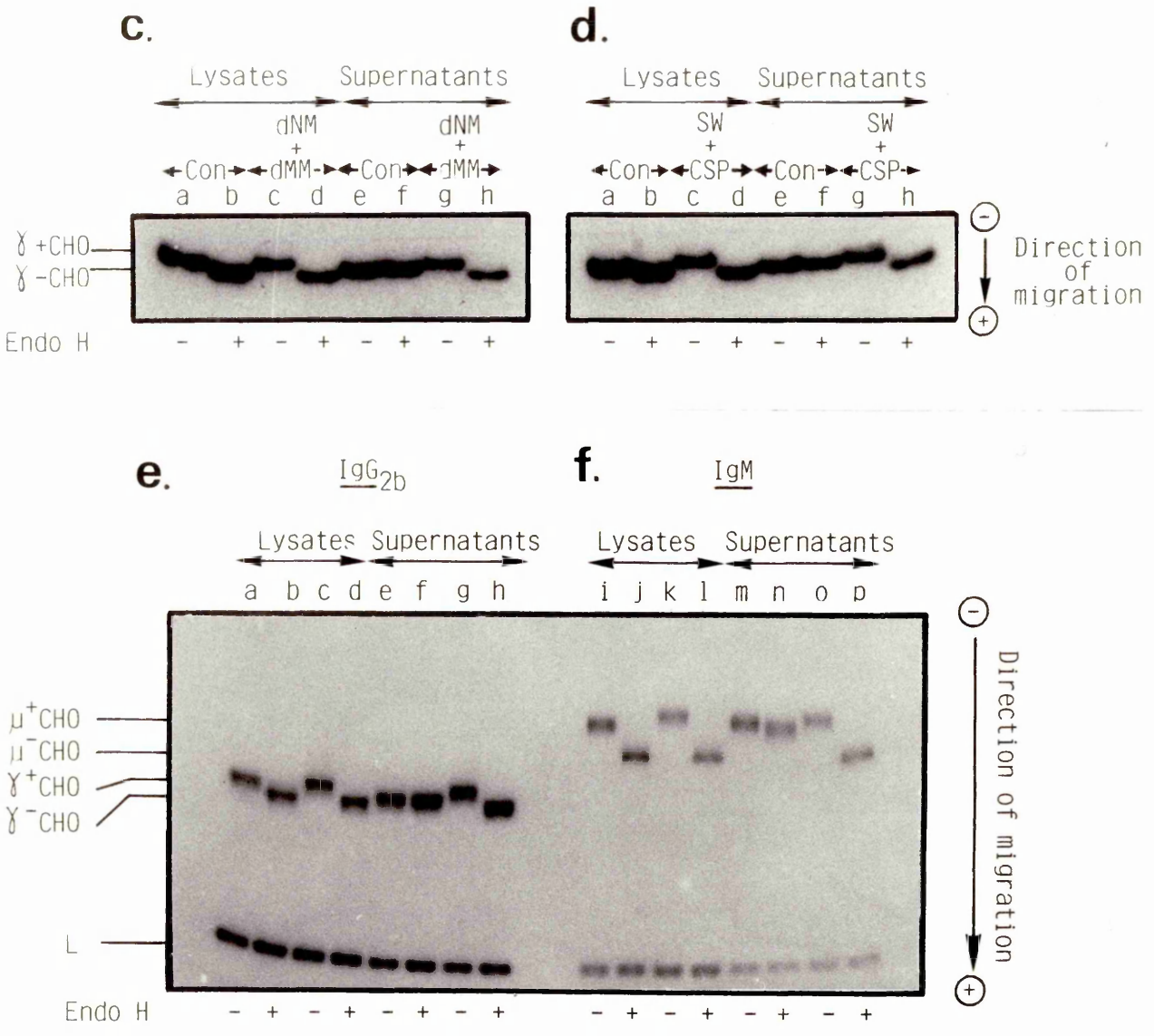
Endo H digestion of immunoprecipitates prepared from aliquots of cell lysates or from 4 hours chase supernatants and analysis was performed as described in the legend to figure 22(b). *Panels a* and *b* demonstrate the endo H analysis of immunoglobulins secreted from 4A3 cells treated with mixtures of alkaloids (CSP/SW) or sugar analogues (dNM/dMM). *Continue on next page...*


a
Lysates
Supernatants
SW + CSP
SW + CSP
Con
CSP
TM
Con
CSP
a b c d e f g h i j
μ+CHO
μ-CHO
L
Direction of migration
Endo H
- + - + - + - + - +


b
Lysates
Supernatants
dNM + dMM
dNM + dMM
Con
dMM
TM
Con
dMM
a b c d e f g h i j
μ+CHO
μ-CHO
L
Direction of migration
Endo H
- + - + - + - + - +

...continuation of figure 28.

Panels c and *d* demonstrate the endo H analysis of immunoglobulins secreted from I1A1.4 cells treated with mixtures of sugar analogues (dNM/dMM) and alkaloids (CSP/SW). *Panels e* (I1A1.4 cells) and *f* (4A3 cells) demonstrate analysis of immunoglobulins from cells exposed to all of the processing inhibitors, i.e., lanes c, d, g, h, k, l, o and p (CSP/dNM/dMM/SW). Lanes a, b, e, f, i, j, m and n are their respective controls.


c.
Lysates
Supernatants
dNM
+
dMM
Con
a b c d e f g h
γ +CHO
γ -CHO
Endo H
- + - + - + - +
d.
Lysates
Supernatants
SW
+
CSP
Con
a b c d e f g h
Direction of migration
- + - + - + - +
e.
IgG2b
Lysates
Supernatants
a b c d e f g h
μ+CHO
μ-CHO
γ+CHO
γ-CHO
L
Endo H
- + - + - + - +
f.
IgM
Lysates
Supernatants
i j k l m n o p
Direction of migration
- + - + - + - +

SDS-PAGE.

Resolution of the μ- and γ-chains that were derived from treated cells by SDS-PAGE reveals that all possess a higher M_r relative to their counterparts immunoprecipitated from control cultures. This is especially pronounced in the case of μ-chains derived from 4A3 cells (figure 17). This higher M_r form of μ- and γ-chains has been previously observed in cells treated with either of the glucosidase inhibitors, i.e., CSP or dNM (figures 17, 22 and 23; Peyrieras *et al.*, 1983). Given that higher M_r forms of μ- and γ-heavy chains can be detected when cells are exposed simultaneously to glucosidase and mannosidase inhibitors suggests that treatment with glucosidase inhibitors may be rate limiting with respect to generation of substrate configurations for later steps in the processing pathway.

The data have significant implications for the involvement of oligosaccharide processing enzymes in overall regulation of glycoprotein secretion. Kelly and co-workers (Gumbiner and Kelly, 1982; Moore and Kelly, 1985) have suggested that glycoproteins can be secreted by two distinct cellular pathways, a constitutive pathway (which operates continuously), or by regulatory pathway in which the export of particular proteins is subject to strict control. By such a classification, immunoglobulins when secreted by hybridomas, would be a member of that category of secretory glycoproteins which are exported via the constitutive pathway. This is supported by the evidence that the processing of N-linked oligosaccharides that occurs as immunoglobulin traverses the rough endoplasmic reticulum and Golgi apparatus does not have any regulatory function in controlling secretion (Peyrieras *et al.*, 1983; Fuhrmann *et al.*, 1985; this thesis). Further support of this notion comes from the work of Tartakoff and Vassalli (1977 and 1978) who have demonstrated that unlike cells that follow the regulatory pathway, export of immunoglobulins from actively-secreting plasma cells are not affected by

depletion of extracellular calcium, or by addition of hormones, cyclic nucleotide derivatives, or agents acting on microtubules or microfilaments.

The lack of involvement of oligosaccharide processing in control of secretion has been observed for many glycoproteins other than immunoglobulins (Franc *et al.*, 1986; Gross *et al.*, 1986), although there are some glycoproteins, for example, α_1-antitrypsin, α_1-antichymotrypsin and caeruloplasmin from HepG-2 cells (Lodish and Kong, 1984; Sasak *et al.*, 1985), whose secretion can be retarded by glucosidase inhibitors. The apparent division of glycoproteins into categories whose secretion are respectively sensitive or insensitive to inhibition of oligosaccharide processing supports the notion of two secretory pathways. However, an interesting question arises as to whether proteins which belong to a constitutive pathway in one state of differentiation of their biosynthesis cell, are members of that same pathway in all developmental forms of that cell. The data from this thesis and others with regard to immunoglobulin secretion (Peyrieras *et al.*, 1983; Fuhrmann *et al.*, 1984), have been made in hybridomas which are fully committed to secretion, and may not be valid for other differentiation states of B cells (e.g., lymphoma cells), where immunoglobulin secretion is a lower metabolic priority for the cell.

Support for this proposal is provided by the finding that mannosidase inhibitors can block the development of human B cells (Tulp *et al.*, 1986). Using human peripheral blood mononuclear cells stimulated by mitogens, it was noted that the presence of SW or dMM at the start of the culture or their addition up to 24 hours after initiation of culture of the lymphocytes with the mitogens inhibited IgM and IgG production assessed at day 5. The effect was apparently a specific block in the development of B cells to antibody-secreting cells and not a toxic effect of the inhibitors. Furthermore, the effect was only observed if the inhibitors were added early in the culture since immunoglobulin secretion from cells treated with SW or

dMM on day 5 of culture was not impaired, suggesting a differentiation state-dependent effect.

Several conclusions may be derived from the preceding studies involving use of oligosaccharide processing inhibitors. The data clearly demonstrate that inhibition of the processing of N-linked oligosaccharide at specific stages of the pathway, or by using all of the oligosaccharide processing inhibitors simultaneously, does not lead to any significant interference with the rate of IgM and IgG_{2b} secretion. Different structures of oligosaccharide chains are, however, synthesized from cells exposed to the oligosaccharide processing inhibitors. This is demonstrated from their susceptibility to endo H enzyme. In case of immunoglobulins synthesized in the presence CSP, dNM or mixtures of inhibitors containing either of the glucosidase I and II inhibitors, the structural difference is further demonstrated by the relatively heavier M_r heavy chains shown by their SDS-PAGE resolution.

SECTION 3.3.

N-LINKED OLIGOSACCHARIDE PROCESSING INHIBITION - STRUCTURAL ANALYSIS OF CARBOHYDRATE MOIETIES AND EFFECTS ON SECONDARY FUNCTIONS OF RAT HYBRIDOMA IgG_{2b}.

3.3.1. Neuraminidase Digestion Analysis.

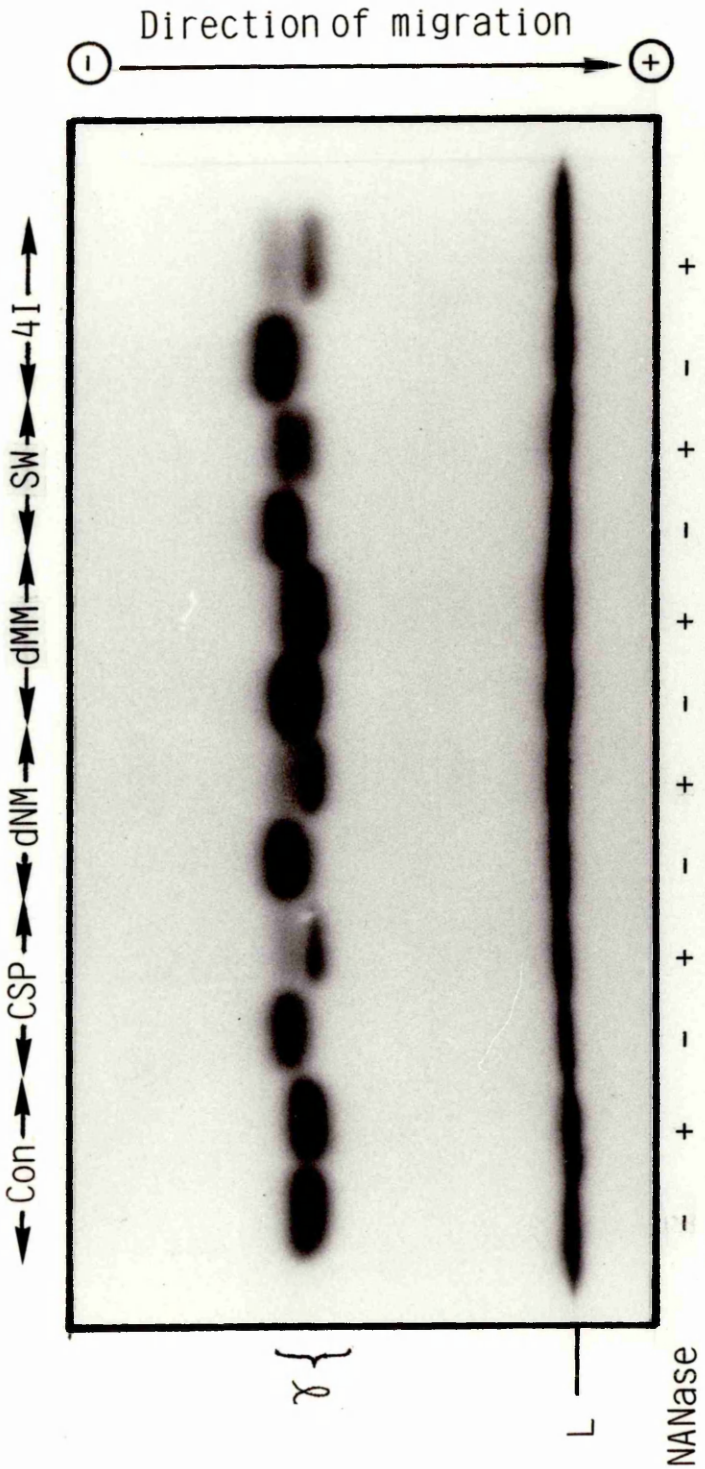
The specific site(s) of action of the various oligosaccharide processing inhibitors that are used in this study are well documented (see sections 1.3.2.1 - 1.3.2.4). However, the precise structures of carbohydrate moieties that are generated when cells are exposed to these inhibitors are not clearly defined. The only common criteria that oligosaccharide moieties have when produced in the presence of the processing inhibitor is their susceptibility to the endo H enzyme, which often lead to their being generally classified as having "high mannose" structures. Although endo H is known not to act on the complex-type oligosaccharide structures, its precise mechanisms of action on the other wide variations of oligosaccharide structures are not well understood (Fuhrmann *et al.*, 1985).

An attempt was made to identify the N-linked oligosaccharide structures of immunoglobulins that are secreted in the presence of the oligosaccharide processing inhibitors. Figure 29 demonstrates the neuraminidase analysis of immunoprecipitated IgG_{2b} secreted from I1A1.4 cells that were exposed to the oligosaccharide processing inhibitors. In control (i.e., untreated) samples, the isolated IgG_{2b} molecules from I1A1.4 culture supernatant demonstrate resistance to the enzyme. This suggests that under normal conditions, immunoglobulins that are secreted from I1A1.4 cells have no terminally-linked sialic acid residues. However, when the cells are treated with the processing inhibitors, a fraction of the immunoprecipitated immunoglobulins demonstrates susceptibility toward neuraminidase digestion, suggesting the presence of sialic acid units on some of the immunoglobulins.

The data strongly imply that the processing inhibitors have, in some way, rendered some of the immunoglobulin molecules susceptible to further modification

Figure 29. Neuraminidase analysis of carbohydrate structures of immunoglobulins secreted in the presence of oligosaccharide processing inhibitor.

[^{35}S]-methionine-labelled IgG_{2b} isolated from I1A1.4 cells that were untreated or those that were exposed to the various processing inhibitors were subjected to neuraminidase digestion for 18 hours at 37°C (+). Buffer-treated control samples were also prepared (-). Reaction was terminated by addition of reduced sample buffer and boiling for two minutes, before finally being analysed by SDS-PAGE.


Direction of migration
⊖
⊕
Con
CSP
dNM
dMM
SW
4I
+ - + - + - + - + -
γ
L
NANase

by allowing the attachment of sialic acid residues to its carbohydrate chains. Whether this is a result of a *de novo* activation of the sialic acid transferase, or due to the structural modification of the N-linked oligosaccharide moiety that gives rise to substrate configuration for the enzyme's action, is a subject for further study.

3.3.2. Concanavalin A Affinity Chromatography.

Concanavalin A (Con A) is a lectin that binds preferentially to the core carbohydrate structure (see figure 3) of N-linked oligosaccharides. Many studies have shown that the affinity of the lectin binding interaction is dependent upon the structures of carbohydrate molecules. For example, Con A binds strongly to the biantennary complex-type glycopeptides possessing two peripheral NeuAc-Gal-GlcNAc units but has no affinity to the triantennary complex-type with similar sugar constituents (Krusius *et al.*, 1976). The presence of a "bisecting" GlcNAc group linked 1-4 to the β-mannose residue of the trimannosyl core has also been shown to markedly weaken the N-linked carbohydrate moiety interaction with Con A (Narasimhan *et al.*, 1986).

In this study, two distinctive approaches were adopted in investigating the affinity interaction of the structures of N-linked oligosaccharides, synthesized in the presence of processing inhibitors, with Con A. Binding studies were performed on either, cleaved carbohydrate moieties from immunoprecipitated IgG_{2b} molecules derived from treatment with endo H (or Glycopeptidase F in case of control samples) or, glycopeptides that are obtained from pronase treated samples. The method for the Con A affinity chromatography is described in section 2.6.5, and results of the experiments are illustrated in figure 30. For convenience, oligosaccharides or glycopeptides that are eluted by Con A buffer or 10mM methyl-α-D-glucopyranoside may be interpreted as having no or low affinity with Con A, and those eluted by 100mM methyl-α-mannopyranoside or remained bound to Con

Figure 30. Concanavalin A-sepharose chromatography of oligosaccharides/ glycopeptides of IgG_{2b} secreted in the presence of processing inhibitors.

Panel a, Oligosaccharides derived from Endo H treatment (or Glycopeptidase F, in case of control) of IgG_{2b} immunoprecipitates were analysed by Con A affinity chromatography. The Con A-sepharose beads column (200µl bed volume) was eluted with Con A buffer, 10mM methyl-α-D-glucopyranoside and 100mM methyl-α-D-mannopyranoside, respectively. Eluents as well as the Con A-sepharose beads were then counted and the results are expressed in percentage of total radioactivity. *Panel b*, A similar Con A affinity chromatography was performed on Pronase-treated IgG_{2b} immunoprecipitates.

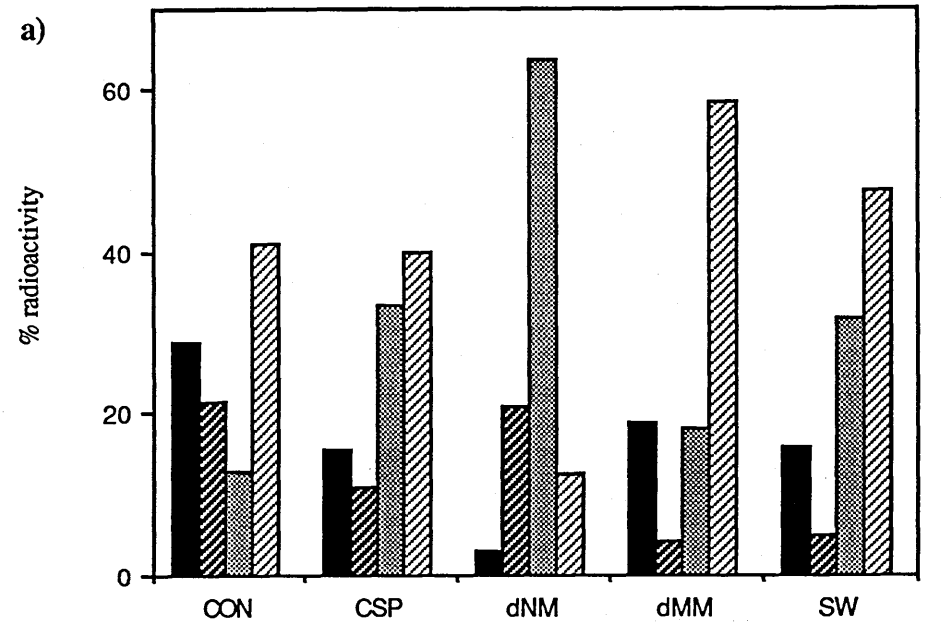
KEY:

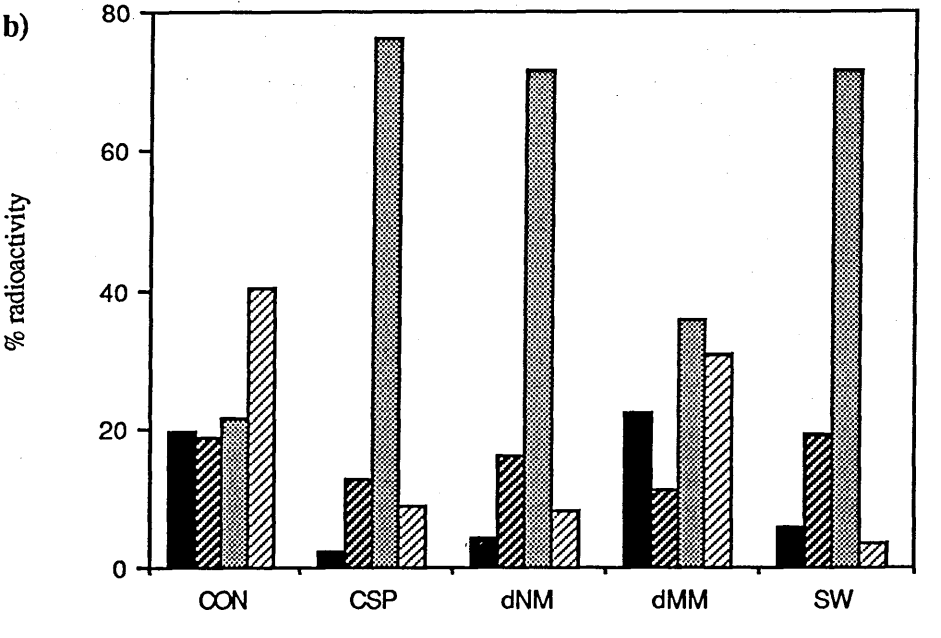
■ - Con A buffer eluent.

▨ -10mM methyl-α-D-glucopyranoside eluent

▩ -100mM methyl-α-D-mannopyranoside eluent

▨ - Con A-sepharose beads


a)
% radioactivity
60
40
20
0
CON
CSP
dNM
dMM
SW


b)
% radioactivity
80
60
40
20
0
CON
CSP
dNM
dMM
SW

A-sepharose beads may be considered as having high affinity to the lectins (Reitman *et al.*, 1982).

The elution profile of control and processing inhibitor-treated samples from both oligosaccharides and glycopeptides are significantly distinct. Elution of control oligosaccharides obtained from digestion of immunoprecipitates with glycopeptidase F demonstrate approximately equal ratio of carbohydrate moieties with low and high affinity for Con A. In the presence of processing inhibitors, however, a greater fraction of oligosaccharides with high affinity for Con A is formed (approximate ratio of 4:1). This elution pattern is also observed in case of glycopeptides derived from pronase-treated immunoprecipitates. Immunoglobulins that are synthesized in the presence of oligosaccharide processing inhibitors, therefore, generally possess carbohydrate moieties with higher affinity to Con A. In addition, the different percentages of radioactivity that were detected from the various elutions also suggest the presence of structural heterogeneity of the carbohydrate moieties of immunoglobulins synthesized in the presence of oligosaccharide processing inhibitors, although this is not clearly observed from SDS-polyacrylamide gel resolution.

3.3.3. Elisa Titration of Rat hybridoma IgG_{2b}.

The protocol for treatment of I1A1.4 cells with tunicamycin and the processing inhibitors is described in section 2.8.1. Prior to assays for secondary functions, control and drug-treated samples were screened by Elisa. Figure 31 demonstrates a typical example of the Elisa titration of IgG_{2b} from I1A1.4 cells. The assay demonstrates that total inhibition of N-glycosylation or interference with the processing of the N-linked oligosaccharide chains of immunoglobulins have no effect on the antigen binding property of the immune molecules; an observation which parallels the previously reported data of Nose and Wigzell (1983). In absence of an accurate quantitative analysis for concentrations of IgG_{2b} secreted by

Figure 31. Elisa titration of rat hybridoma IgG_{2b}.

Before subjecting to functional assays of immunoglobulins, culture supernatants from control and drug-treated samples were screened by Elisa. Microtitre plates were coated with 10^6 Inaba vibrios per well and blocked with 0.5% (W/V) gelatin before adding the I1A1.4 cell culture supernatants. Plates were incubated at room temperature for two hours and washed with PBS. 100µl of HRP-RArIg solution was subsequently added to each well and plates were left for further incubation for another hour. Plates were washed again and then added with substrate solution until formation of colour was observed. Reaction was terminated by addition of H_2SO_4 to each well and colour was measured at 492nm in a Titretek Multiskan Spectrophotometer. Samples were analysed in duplicate.

KEY:

□ - control

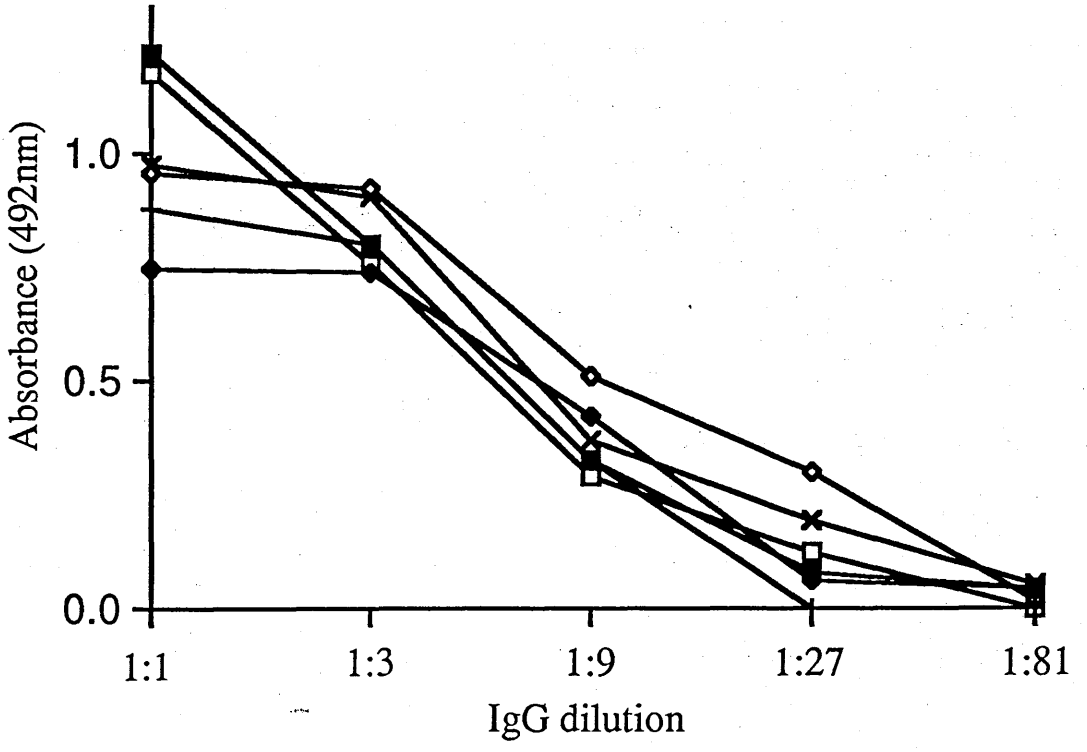
■ - TM-treated

◇ - CSP-treated

◆ - dNM-treated

+ - dMM-treated

X - SW-treated


Absorbance (492nm)
1.0
0.5
0.0
1:1
1:3
1:9
1:27
1:81
IgG dilution

cells that are untreated and those that are exposed to tunicamycin or any of the processing inhibitors, and basing on the above observation that the oligosaccharide moieties of immunoglobulins have no roles in antigen binding, this procedure was also adopted to provide a general guide to the difference in concentrations of the secreted immunoglobulins. Throughout the course of this study, relatively similar results were achieved from culture supernatants that were generated and screened by the described methods.

The data are also supported by results that were obtained from the pulse-chase procedures as discussed in sections 3.1.6 and 3.2.2. By using immunoprecipitation, it was demonstrated that the rate of synthesis and secretion of untreated IgG_{2b} is similar to the kinetics of export from those cells that were exposed to the respective processing inhibitors (figure 21). Treatment of I1A1.4 cells with tunicamycin, however, results in a slightly decreased secretion of immunoglobulins (figure 12). This effect is most likely due to the minor cytotoxicity of the glycosylation inhibitor on the cells rather than a direct inhibition on the rate of immunoglobulin export.

3.3.4. Effects of N-linked Oligosaccharide Processing Inhibition of IgG_{2b} on Ability to Fix Complement.

In light of the finding that non-N-glycosylated IgGs are less effective in triggering the complement cascade (Koide *et al.*, 1977; Winkelhake *et al.*, 1980; Nose and Wigzell, 1983), it would be interesting to determine the effects of inhibition of N-linked oligosaccharide processing of immunoglobulins upon fixation of complement. After screening by Elisa (section 2.8.2), culture supernatants that are obtained from I1A1.4 cells that are untreated and those that have been exposed to tunicamycin or processing inhibitors (section 2.8.1), are thus, subjected to assay of complement fixation (for methods, see section 2.8.4).

Figure 32 demonstrates a representative result that was obtained from the complement fixation assay of immunoglobulins from control, tunicamycin- and processing inhibitor-treated I1A1.4 cell culture supernatants. Assays were performed in duplicate. Results are expressed in terms of percentage complement fixation relative to control, i.e., complete lysis solution (section 2.8.4). The data demonstrate that non-N-glycosylated IgG_{2b} is less effective in fixing complement than control antibody. This is explained by studies which have shown that in the absence of carbohydrate moieties, immunoglobulins bind less effectively with the C1q subcomponent of the complement cascade (Leatherbarrow *et al.*, 1985). However, since the kinetic data suggest that in the presence of tunicamycin, IgG_{2b} is secreted at a slightly reduced rate (figure 12), it is not possible, in this case, to conclude precisely whether the difference in complement fixation exhibited by the control and tunicamycin-treated culture supernatants is a consequence of structural modification of the antibody or simply an effect of a less concentrated antibody solution.

On the other hand, immunoglobulins possessing high mannose N-linked oligosaccharide structures that are secreted in the presence of the four distinct processing inhibitors, i.e., CSP, dNM, dMM and SW, demonstrate apparently potentiated capability in activating the complement cascade as compared to the untreated control molecules. Since the kinetic data from pulse-chase experiments (figure 21) and the Elisa titration curves of the samples that are used in this assay (figure 31) indicate no significant difference in concentrations between the processing inhibitor-treated and control culture supernatants, it is therefore reasonable to conclude that the potentiated effect in triggering the complement cascade demonstrated by the processing inhibitor-treated samples is really a result of the modified carbohydrate structures of IgG_{2b}.

Figure 32. Effects of N-linked oligosaccharide processing inhibition of IgG_{2b} on the fixation of complement.

Complement fixation assay was performed according to the method described in section 2.8.4. Tubes consisting of different concentrations of I1A1.4 cell culture supernatants, 1×10^7 attenuated Inaba vibrios and guinea pig complement solution were incubated at 4°C overnight. A panel of control tubes (in absence of antibody, antigen and complement, respectively) and tubes solely consisting of complement solution (i.e., "complete lysis tube") were also prepared. On the next day, haemolysin-sensitized sheep erythrocytes were added to each tube. Tubes were immediately incubated at 37°C until a complete lysis of the sheep erythrocytes was obtained from the "complete lysis tube". Tubes were simultaneously centrifuged at 4°C to pellet debris and unbroken cells. Supernatants were then measured for absorbance readings at 492nm. Samples were analysed in duplicate. Results are expressed in percentage complement fixation relative to "complete lysis" control readings (section 2.8.4).

<u>KEY:</u>

□ - control

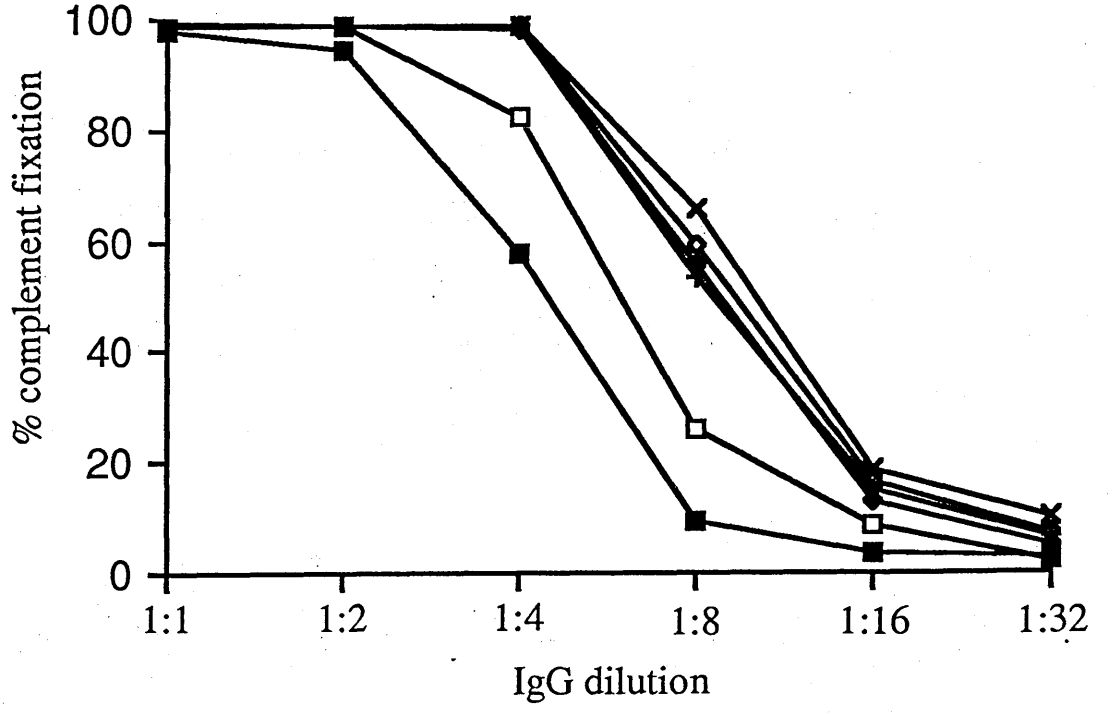
■ - TM-treated

◇ - CSP-treated

◆ - dNM-treated

+ - dMM-treated

x - SW-treated


100
80
60
40
20
0
% complement fixation
1:1
1:2
1:4
1:8
1:16
1:32
IgG dilution

This finding provides clues to the optimal structures involved in the binding interaction between antibodies and C1q protein. Currently, it is known that the IgM is generally more efficient in fixing complement than IgG (Roitt *et al.*, 1985). The C1q binding region on the immunoglobulin M has also been localized to the Cμ4 domains (Hurst *et al.*, 1971 and 1975; Bubb and Conradie, 1976), which, under normal circumstances, individually possess one N-linked oligosaccharide moiety (see figure 5). Structural analysis of the carbohydrate moieties of the N-linked oligosaccharide chains at the Cμ4 domains demonstrates that both possess high mannose configuration (Shimizu et al., 1971; Hickman et al., 1972). Taken together, these data and the data of figure 32 strongly suggest that the high mannose N-linked oligosaccharide structures of immunoglobulins (as reflected by their susceptibility to endo H digestion) are one of the critical factors that contribute to an effective interaction with the C1q protein.

The earlier data from figure 29 (neuraminidase analysis of carbohydrate structures), however, suggest that the structures of carbohydrate residues that are generated in the presence of each oligosaccharide processing inhibitor, although demonstrating susceptibility to endo H digestion (panel b of figures 22-25), are still partly processed to the complex form. This is evident from their susceptibility to neuraminidase digestion. The complex-type N-linked oligosaccharide chains from normal IgG_{2b} from I1A1.4 cells do not possess sialic acid residues as demonstrated by their resistance to the enzyme treatment. Thus, the potentiated effect in complement fixation that are observed in cases of processing inhibitor-treated immunoglobulins (figure 32) could have also arisen as a result of the differences in terminal carbohydrate structures. This is, however, refuted by reports (Koide *et al.*, 1977), which demonstrate that in case of N-linked oligosaccharides of IgGs containing terminal galactose and sialic acid residues, enzymatic removal of the sugar moieties by neuraminidase and β-galactosidase does not result in any changes to the complement fixation activity of the immunoglobulin.

The I1A1.4 cell line that is used in this study is, unfortunately, not the optimum model system with which to perform complement fixation assay. Antibodies produced by this rat hybridoma cells are directed against the LPS-coating of the Inaba strain of *Vibrio cholerae* (Ghosh and Campbell, 1986). The C1q subcomponent of the complement cascade is well known for its high non-immunological reactivity with a variety of antigens, e.g., acidic mucopolysaccharide, heparin, protamine, DNA and envelope glycoprotein of certain oncoviruses (McConnel *et al.*, 1981). In this case, the C1q molecule has also been discovered to react with lipopolysaccharide (data not shown). Consequently, it is not possible to perform direct binding assay involving radiolabeled C1q proteins.

3.3.5. Effects of N-Linked Oligosaccharide Processing Inhibitors on Assay of Complement Fixation.

One of the arguments that may be advanced against the above complement fixation data concerns the effects the processing inhibitors themselves may have on the assay. Processing inhibitor-treated culture supernatants that were used in the complement fixation assay, all contain the drugs to which the cells have been exposed, and which may, therefore, have contributed to the observed potentiation effects. To test this notion, a complement fixation assay was performed on control culture supernatants that have been supplemented with the various processing inhibitors at their characteristic concentrations.

Figure 33 shows the complement fixation curves from assays of control and processing inhibitor-supplemented I1A1.4 culture supernatants. As clearly illustrated, there is no significant difference that could be detected between the control and samples that have been added with tunicamycin or the processing

Figure 33. Effects of tunicamycin and N-linked oligosaccharide processing inhibitors on complement fixation.

To determine the effects of tunicamycin and N-linked oligosaccharide processing inhibitors on complement fixation, assay was performed on control samples supplemented with the various inhibitors at their respective concentrations. Samples were analysed in duplicate.

KEY:

□ - control

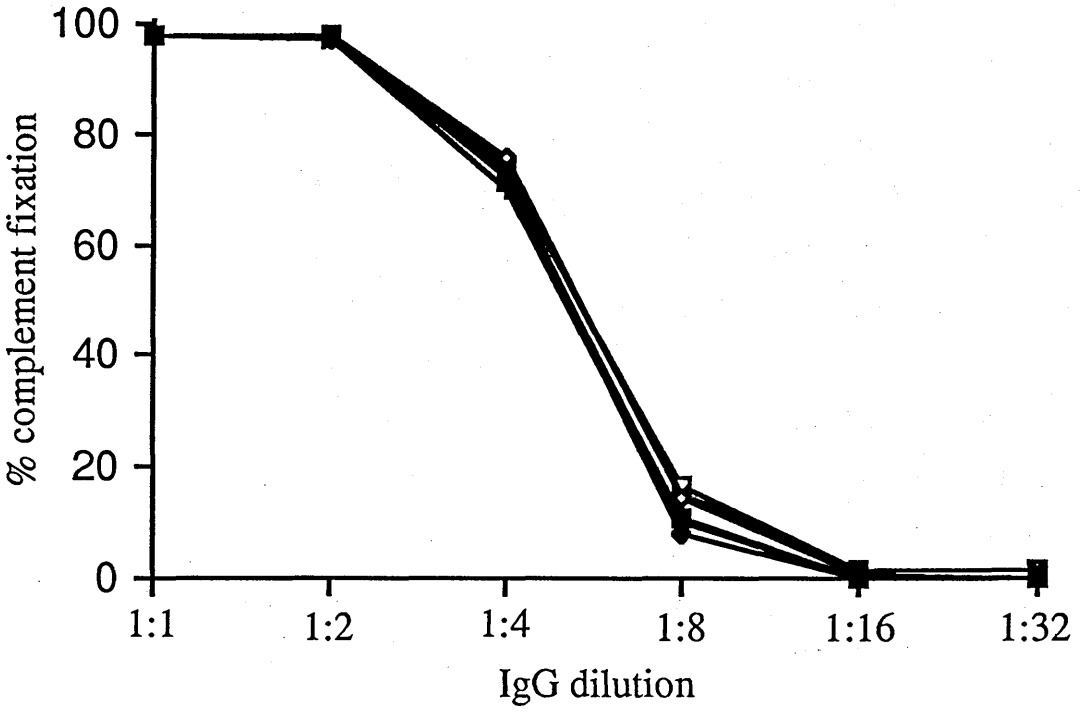
■ - plus TM (2μg/ml)

◇ - plus CSP (75μg/ml)

◆ - plus dNM (5mM)

+ - plus dMM (1mM)

x - plus SW (10μg/ml)


100
80
60
40
20
0
% complement fixation
1:1
1:2
1:4
1:8
1:16
1:32
IgG dilution

inhibitors. The data unequivocally illustrate that the processing inhibitors have no direct effect on the complement fixation activity and therefore substantiate the hypothesis that high mannose structures of the IgG_{2b} molecules are more effective in triggering the complement cascade than the naturally processed complex-type configurations.

3.3.6. Effects of Monosaccharides on Assay of Complement Fixation.

In the report by Koide *et al.* (1977), apart from demonstrating that non-N-glycosylated IgG are less effective in activating the complement cascade, it was also shown that the presence of free sugars, i.e., N-acetylglucosamine, N-acetylgalactosamine, mannose and galactose, at 100mM concentrations, results in a substantial degree of inhibition of the complement fixation activity. The data provide considerable support to the hypothesis that high mannose oligosaccharide structure provides certain advantages in fixing complement than the complex-type glycan. Free sugar residues may have acted as competitors in the binding of the first component of the complement cascade.

To verify this observation, complement fixation assays were performed on control untreated samples in the absence and presence of 5mM mannose, N-acetylglucosamine and galactose. If the free monosaccharides do act as competitive inhibitors to the C1q binding interaction, the 5mM concentration of sugars that was used should be sufficient to cause a shift in the complement fixation profile, relative to the control. Additions of higher concentrations of the sugars may indirectly interfere with the method of complement fixation assay instead.

Figure 34 demonstrates the complement fixation curves achieved from assays of I1A1.4 cell culture supernatants in the absence and presence of 5mM mannose, N-acetylglucosamine and galactose. The data clearly demonstrate that

Figure 34. Effects of monosaccharides on complement fixation.

To determine the effects of sugars on complement fixation, assay was performed on control samples supplemented with the various monosaccharides at 5mM concentration. Samples were analysed in duplicate.

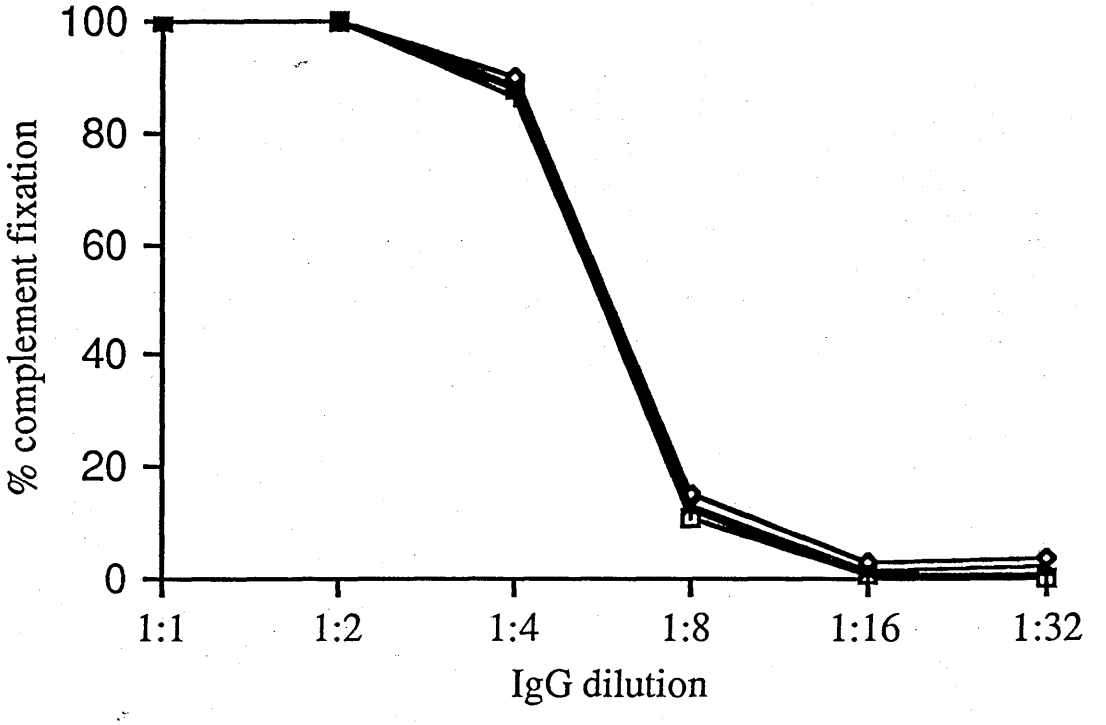
KEY:

□ - control

◇ - plus D(+)-mannose

+ - plus D(+)-N-acetylglucosamine

X - plus D(+)-galactose


100
80
60
40
20
0
% complement fixation
1:1
1:2
1:4
1:8
1:16
1:32
IgG dilution

none of the sugars that were added have any effect on the complement fixation activity by rat hybridoma IgG_{2b}. This finding contradicts the earlier published report (Koide *et al.*, 1977) which suggested that free sugar residues may act as competitive inhibitors in the C1q binding activity, although the concentration of monosaccharides that was used in the earlier study is twenty times higher than those that are described in this thesis.

3.3.7. Effects of N-Linked Oligosaccharide Processing Inhibition on Agglutinating Activity of IgG_{2b}.

While complete carbohydrate digestion of the IgG molecules causes considerable impairment on the antibody's function to fix complement, no effects was observed on its haemagglutination activity (Koide *et al.*, 1977). By subjecting the untreated I1A1.4 cell culture supernatants and those derived from cells that have been exposed to tunicamycin and the processing inhibitors to haemagglutination assay (for methods, see section 2.8.6), a similar result was also obtained.

Haemagglutination assay was performed by mixing the various culture supernatants at different dilutions with lipopolysaccharide-coated sheep erythrocytes (section 2.8.6). Figure 35 demonstrates that the end-point agglutinating titre is the same in all cases. Agglutination are observed in wells containing culture supernatants at 1:1 and 1:2 dilutions but not at the 1:4 antibody dilution.

The above structural analysis of carbohydrate moieties of IgG_{2b} isolated from cells treated with N-linked oligosaccharide processing of inhibitors has led to an intriguing observation. The carbohydrate moieties of normal IgG_{2b} molecules do not possess any terminally linked sialic acid residues as shown by their resistance to neuraminidase digestion. However, when the cells were exposed to the oligosaccharide processing inhibitors (individually or simultaneously), a fraction of

Figure 35. Effects of N-linked oligosaccharide processing inhibition on agglutinating activity of IgG_{2b}.

Haemagglutination assay was performed as in the method described in section 2.8.6, i.e., by mixing the various culture supernatants with lipopolysaccharide-coated sheep erythrocytes. Samples were analysed in duplicate.

KEY:

cRPMI - complete RPMI-1640 medium

CON - culture supernatant of untreated I1A1.4 cells.

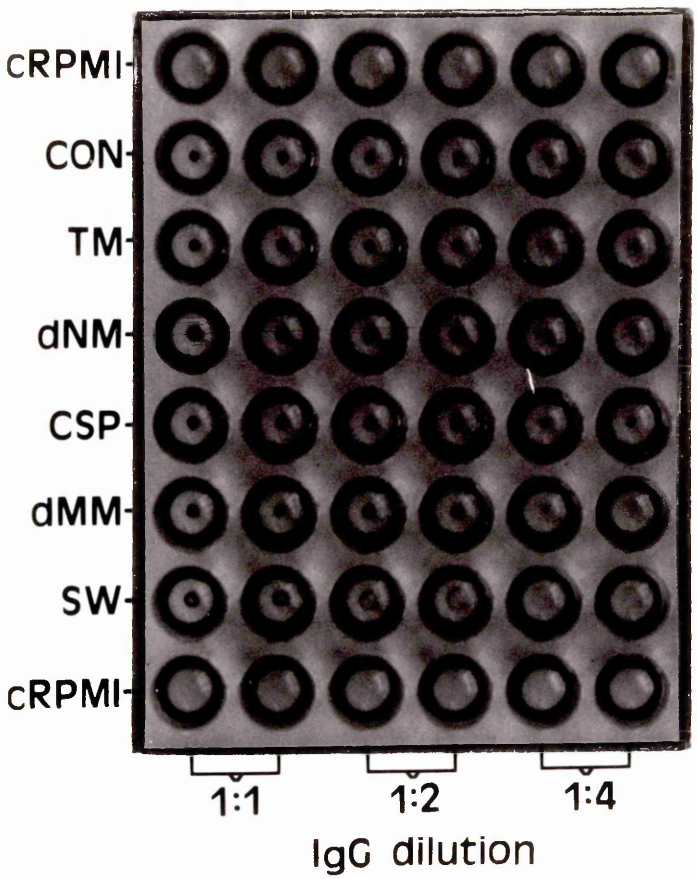
TM - culture supernatant of tunicamycin-treated I1A1.4 cells

dNM - culture supernatant of 1-deoxynojirimycin-treated I1A1.4 cells

CSP - culture supernatant of castanospermine-treated I1A1.4 cells

dMM - culture supernatant of 1-deoxymannojirimycin-treated I1A1.4 cells

SW - culture supernatant of swainsonine-treated I1A1.4 cells


cRPMI
CON
TM
dNM
CSP
dMM
SW
cRPMI
1:1
1:2
1:4
IgG dilution

the secreted immunoglobulins demonstrate susceptibility to the enzyme. In addition, the study has also demonstrated the presence of structural heterogeneity of the N-linked oligosaccharide moieties synthesized in the presence of the processing inhibitors when the oligosaccharide were analysed on their binding interaction with Con A.

Investigation of the effects of N-linked oligosaccharide processing inhibition on the functional activities of immunoglobulins have provided a few interesting observations. Non-N-glycosylated IgG_{2b} was firstly demonstrated to be less effective in fixing complement. Immunoglobulins with high mannose structures that were synthesized in the presence of processing inhibitors, on the other hand, demonstrate potentiated capabilities in activating the complement cascade. The structural modification of the carbohydrate residues of IgG_{2b} by the processing inhibitors have, however, no effect on the antigen binding and haemagglutination activities of the immunoglobulins.

SECTION 3.4.

GENERAL CONCLUSIONS.

The question that often arises from studies employing tunicamycin as the biological tool to assess the role of N-linked oligosaccharide moieties in controlling transport of glycoproteins is whether the drug may directly interfere with the biological activities of the cell. This is of prime importance since direct interference of the antibiotic may totally dissolve the usefulness of this tool and discredits the data obtained from its experiments. This thesis has described a study characterizing three different structural analogues of tunicamycin. It has shown that the integrity of every structural component of tunicamycin is highly essential for its ability to inhibit N-glycosylation as well as the intracellular transport of IgM molecules. It was further demonstrated that the modified tunicamycins have no pronounced effects on the synthesis of cellular macromolecules. From these observations, it was deduced that the tunicamycin-associated cytotoxicity and the selective inhibition of glycoprotein export are secondary responses of the cells resulting from the inhibitor's primary actions. The structural analogues of tunicamycin were then, also shown not to interfere with the N-acetylglucosaminyl transferase I and II residing in the golgi apparatus.

The role of N-linked oligosaccharide processing in the control of transport of rat hybridoma immunoglobulins were also investigated. Inhibition of the processing of N-linked oligosaccharides of rat hybridoma IgG_{2b} and IgM has been shown to have no effect on the kinetics of immunoglobulin export. Taken together, the data provide substantial support for the view that carbohydrate moieties have no general role in regulating the transport of glycoproteins. This is compatible with the recent emerging hypothesis that the intracellular transport of glycoproteins does not require any form of promoting mechanism. Glycoproteins may be channeled quite efficiently via the rapidly-occuring constitutive bulk flow process. Involvement of the regulatory ligand-receptor mechanism only applies for proteins to be retained in specific intracellular compartments. In cases where inhibition of N-glycosylation or

carbohydrate processing leads to markedly retarded protein export, other factors may have been the cause. Absence of carbohydrate moieties or interference with their intracellular processing may severely alter the physicochemical property of proteins, and thus, prevent their transport. Alternatively, the structural modifications may also result in exposure of specific sites on the molecules which may then interact with intracellular retention proteins.

The structures of carbohydrate moieties of immunoglobulins derived from cells exposed to the N-linked oligosaccharide processing inhibitors were also studied. Inhibition of N-linked oligosaccharide processing appears to be a more complex situation than initially expected. Immunoglobulins synthesized in the presence of CSP, dNM or mixtures containing either of these glucosidase inhibitors demonstrate higher M_r as resolved by SDS-PAGE. There was considerable structural variation amongst the carbohydrate structures of immunoglobulins synthesized in the presence of all of the oligosaccharide processing inhibitors. In case of the IgG_{2b} from I1A1.4 cells, inhibition of N-linked oligosaccharide processing is further associated with the transfer of sialic acid residues to the carbohydrate chains (N-linked carbohydrate moieties of normal IgG_{2b} from I1A1.4 cells do not possess any sialic acid residues).

Finally, the effects of inhibition of N-glycosylation or N-linked oligosaccharide processing on the functional activities of IgG_{2b} of rat hybridoma was investigated. The data demonstrate that non-N-glycosylated immunoglobulins are less effective in activating the complement cascade. On the other hand, immunoglobulins synthesized in the presence of the oligosaccharide processing inhibitors demonstrate potentiated capability in fixing complement. Neither the inhibition of N-glycosylation nor the carbohydrate processing has any effect on the antigen binding and haemagglutination activities of the immunoglobulins.

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