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GROWTH DEPENDENT ALTERATIONS OF CELL SURFACE GLYCOPEPTIDES DERIVED FROM NORMAL HUMAN FIBROBLASTS

City University of New York PH.D. 1979

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1979

GROWTH DEPENDENT ALTERATIONS OF CELL SURFACE GLYCOPEPTIDES DERIVED FROM NORMAL HUMAN FIBROBLASTS

by

PATRICK R. CAMMARATA

A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

1979

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This manuscript has been read and accepted for the Executive Committee in Biology in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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To my Mother and Father

ABSTRACT

Mannose-labeled cellular glycopeptides derived from human diploid fibroblasts (KL-2) were separated into two classes by QAE Sephadex chromatography. High voltage paper electrophoresis and Sephadex G-50 chromatography were used to further characterize the glycopeptides. At least five distinct neutral species were isolated with molecular weights ranging from 1050 to 2000 daltons. A population of growing cells labeled with (^{3}H) mannose and non-growing cells labeled with (^{14}C) mannose were mixed and analyzed by the above method. Growth dependent alterations were observed. The higher molecular weight neutral glycopeptides decreased in the non-growing cells relative to the growing cellular glycopeptides. Cell surface glycopeptides (removal by mild proteolytic digestion) from both states of growth after a 20-24 hour labeling period also indicate demonstrable growth dependent alterations of the neutral species. Further, the data show that the proportion of neutral species on the cell surface is different from that associated within the cells. The predominant neutral cellular glycopeptide has an apparent molecular weight of 1050. This species is also the major neutral species on the surface of the growing cell. Conversely, on the surface of the non-growing cell, this species is present in reduced amounts with respect to both the cell material (growing and non-growing) and the growing cell surface.

A mixture of growing and non-growing cell surface neutral glyco-peptides (isolated by high voltage paper electrophoresis) was digested with endo-β-N-acetylglucosaminidase H and analyzed by paper chromato-graphy. Two products were released, glycopeptides resistant to the

endoglycosidase hydrolysis and "large core oligosaccharides," larger than $GlcNAc(Man)_6$. A second sample of the growing and non-growing mixture was digested with endo-β-N-acetylglucosaminidase H in the presence of β-N-acetylglucosaminidase and β-galactosidase, releasing an array of oligosaccharides ranging from $GlcNAc(Man)_6$ to possibly $GlcNAc(Man)_4$. "Large core oligosaccharides" were also present albeit in reduced amounts. Susceptibility of the cell surface neutral glycopeptides to the endoglycosidase H does not seem to be markedly growth dependent.

Three major species and several minor species of complex glycopeptides were fractionated by QAE Sephadex chromatography. Growth dependent alterations were apparent in the complex glycopeptides obtained from a population of growing and non-growing cells labeled with (^3H) mannose. Molecular weight determination indicated that those isolated from growing cells were always larger than those derived from non-growing cells. Treating the glycopeptides with neuraminidase decreased their apparent molecular weight by multiples equivalent to a sialic acid residue; however, glycopeptides from growing cells could still be distinguished by molecular weight estimates, from those derived from non-growing cells. The data suggest increased specific sialylation of glycopeptides derived from growing cells. Failure of neuraminidase treatment to convert the glycopeptides from both states of growth to identical size suggests a growth dependent heterogeneity of the peptide and/or oligosaccharide moiety remaining after the enzyme digestion. One peak was identical in size in both states of growth and appeared to be resistant to neuraminidase.

The complex glycopeptides were digested with endo-β-N-acetylglucosaminidase D, and analyzed by paper chromatography, high voltage

paper electrophoresis and Sephadex G-25 chromatography. All of the complex glycopeptides examined released two products: an oligosaccharide and glycopeptides resistant to the endoglycosidase hydrolysis. In all cases the oligosaccharides migrated on paper chromatography similar to a tetrasaccharide prepared from fetuin, with composition $(Man)_3GlcNAc$. Susceptibility of the complex species to the endoglycosidase D was markedly growth dependent. The amount of released oligosaccharide ranged from 13% to 61%, depending on which species was examined and its growth status. Successive digestion with α- and β-mannosidase of one of the species indicated that the oligosaccharide contained two terminal α-mannosyl residues and a β-mannose probably linked to N-acetylglucosamine, suggesting the composition $(Man\,\alpha)_2$ $Man\,\beta\,GlcNAc$.

The kinetics of assembly of mannose-containing plasma membrane glycoproteins was studied in growing and density-inhibited fibroblasts (WI38). (^3H) mannose incorporation into trichloroacetic acid soluble and insoluble material rapidly declines as cells go from an exponentially growing state to one of density inhibition. The fate of the exogenous radioactive precursor was followed by monitoring the intracellular accumulation of (^3H) mannose into the soluble pools, intracellular glycoprotein and incorporation into the plasma membrane. The transit time of newly synthesized intracellular glycoprotein into the plasma membrane of density inhibited cells was estimated to be 32 minutes. The accumulation of newly made glycoprotein into the cell surface of growing cells appears to be significantly faster.

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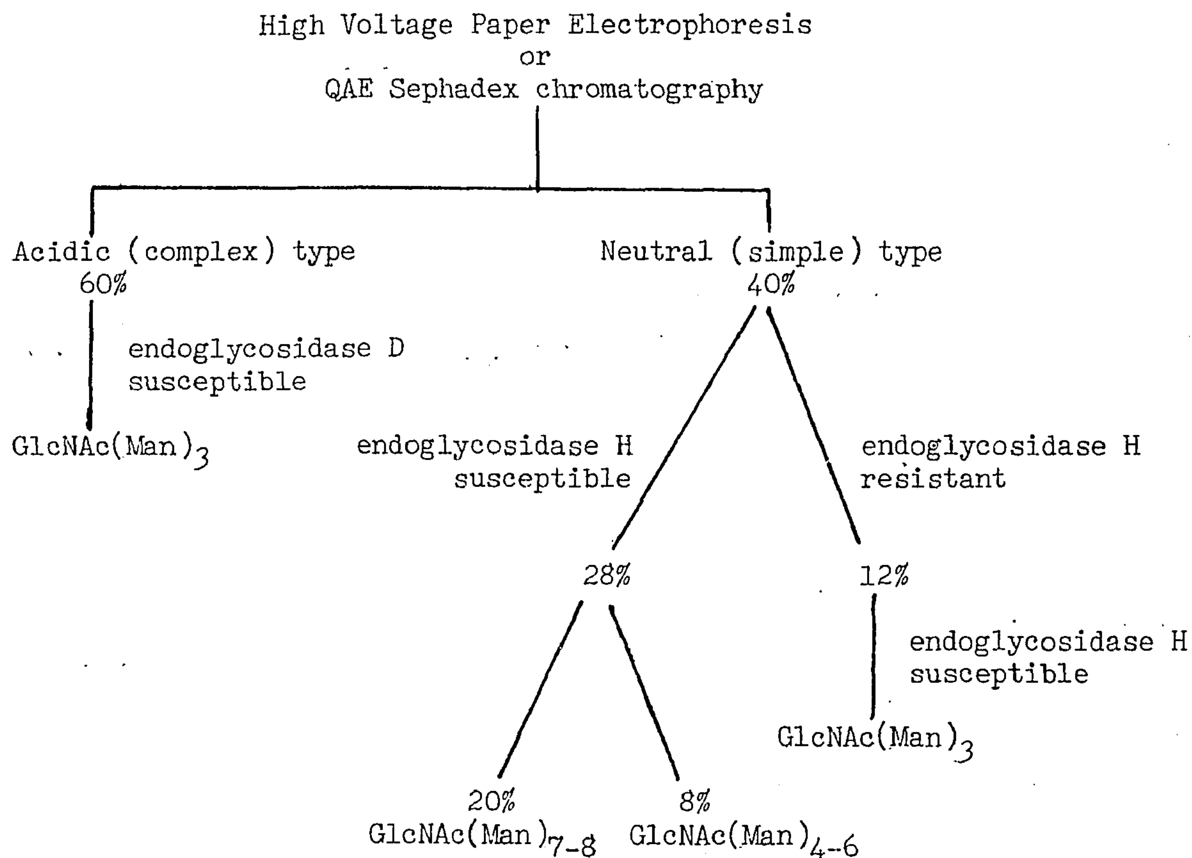
SUMMARY



Growth Dependent Alteration

1. Neutral glycopeptides may be fractionated into at least five distinct species by QAE Sephadex chromatography. The higher molecular weight cellular neutral glycopeptides in non-growing cells were markedly reduced relative to the growing cells (Fig. 2a, Table 2).

2. The distribution of growing cellular glycopeptides is closely reflected on the cell surface. In contrast, the distribution of neutral species on the non-growing cell surface does not reflect the distribution of cellular glycopeptides (Table 2).

3. The neutral species I and II exhibit an inverse relationship on the cell surface subject to the growth status of the cell (Fig. 4).

4. Susceptibility of the surface neutral glycopeptides to endoglycosidase H does not seem to be markedly growth dependent (Fig. 9a). However, in all cases examined, the QAE Sephadex-derived growing glycopeptides did release slightly more oligosaccharide material than their non-growing counterparts (Fig. 10).

5. Susceptibility of surface neutral glycopeptides I and II to endoglycosidase D (Fig. 13) and α-mannosidase (Table 5) is markedly growth dependent.

6. Complex surface glycopeptides derived from growing cells are higher in molecular weight than similar material derived from non-growing cells because of a) increased sialylation and b) increased oligosaccharide and/or peptide (Table 6).

7. Susceptibility of surface complex glycopeptides to endoglycosidase D is subject to which QAE Sephadex-derived peak is examined and its growth status (Fig. 16, Table 7).

INTRODUCTION

1. Glycoprotein biosynthesis

There is now in the literature an overwhelming amount of data that points to the participation of lipid-linked oligosaccharides in glycoprotein biosynthesis (Behrens et al., 1973; Waechter et al., 1973; Lucas et al., 1975; Chen et al., 1975; Struck and Lennarz, 1977; Lennarz, 1975; Waechter and Lennarz, 1976). This work involved a demonstration that oligosaccharide-lipids, not containing glucose, could be transferred to protein acceptors *in vitro*. Leloir and co-workers first demonstrated that dolichol-linked oligosaccharides, containing glucose, could be involved with the glycosylation of protein (Behrens et al, 1971; Parodi et al., 1972). However, this work did not demonstrate that the glucose-containing oligosaccharide being transferred contained mannose and N-acetylglucosamine as well. Recent work by Spiro, Spiro and Bhoyroo (Spiro et al., 1976a, b, c) has shown that oligosaccharide-pyrophosphoisoprenol lipids contain mannose, N-acetylglucosamine and, as an integral part of the structure, small amounts of glucose. This has been demonstrated in thyroid, oviduct, kidney, thymus and liver, but only trace amounts of glucose were present in pancreatic slices. Jeanloz and co-workers (Herscovics et al., 1977) however, demonstrated the presence of glucose in oligosaccharide-lipids of pancreatic microsomes. Robbins and co-workers demonstrated that mannosyl lipid-linked oligosaccharide synthesized by cell-free enzyme preparations can be increased by 1 or 2 glycose units in the presence of UDP-glucose (Robbins et al, 1977a), and subsequently used to transfer glucose to endogenous protein acceptors. It was shown that the glucose containing oligosaccharide-

lipid is more efficiently transferred to endogenous protein acceptors *in vitro* than its mannosyl oligosaccharide-lipid counterpart (Turco *et al.*, 1977).

A general overall pathway for intracellular glycoprotein biogenesis has been established and a model accounting for membrane asymmetry postulated (Rothman and Lenard, 1977). The antibiotic tunicamycin, which appears to inhibit the glycosylation of newly synthesized protein (Takatsuki and Tamura, 1971), has been selectively used to study the assembly and secretion of IgA and IgE by plasma cells (Hickman *et al.*, 1977). At present, it is unclear if newly synthesized proteins not glycosylated because of inhibition by tunicamycin are unable to be transported to the cell surface membrane (Leavitt *et al.*, 1977), or if the nonglycosylated proteins are transported to the surface but subject to increased turnover due to protease susceptibility (Olden *et al.*, 1978). The mechanism of action suggests that tunicamycin inhibits the formation of N-acetylglucosamine-lipid intermediates (Tkacz and Lampen, 1975) and thus prevents the attachment of the core $(GlcNAc)_2(Man)_n$ region to the newly made polypeptide.

Surface and cellular glycopeptides can be separated into two general classes: neutral glycopeptides (containing N-acetylglucosamine and mannose) and acidic glycopeptides (terminating in sialic acid) (Ceccarini *et al.*, 1975; Cammarata and Ceccarini, 1979). While it is known where the sugars are added to growing polypeptides within the cell (Whur *et al.*, 1969; Molnar, 1975), little is known about how the two classes of glycoproteins are assembled. Evidence for one mode of synthesis of the complex (acidic) glycoproteins has been found in a study of rat liver microsomes. Incorporation of L-fucose from GDP-

fucose into a glycopeptide acceptor requires at least one terminal mannose linked by a GlcNAc residue before attachment may be facilitated (Wilson et al., 1976). What has subsequently proven to be a promising approach is the use of mutants defective in glycoprotein synthesis. A Chinese hamster ovary (CHO) cell line selected for resistance to a phytohemagglutinin from Phaesolus vulgaris (Stanley et al., 1975a; Narasimhan et al., 1976) has been used to study the synthesis of the complex (acidic) glycoproteins. The wild type cell contains two GlcNAc transferase activities, I and II. The first catalyzes the addition of a terminal GlcNAc residue to mannose

```
Man
    \
     Man —— GlcNAc-R
    /
Man
```

and the second catalyzes the addition of a second GlcNAc to the acceptor

```
GlcNAc —— Man
              \
               Man —— GlcNAc-R
              /
          Man
```

The mutant cell line is lacking transferase I but has transferase II activity, i.e., the mutant is unable to synthesize the acidic glycoproteins. Similar results have been obtained from CHO cells selected for resistance to the toxin from Ricinus communis (Gottlieb et al., 1975; Stanley et al., 1975b), the agglutinin from wheat germ (Stanley et al., 1975b) and agglutinin from Lens culinaris (Stanley et al., 1975b). Summers and co-workers have described specific structural changes in the carbohydrate moiety of the G glycoprotein of vesicular stomatitis virus (VSV) prepared from three distinct lectin resistant mutants (Robertson et al., 1978). Cifone and Baker have isolated a mammalian

cell line resistant to toxic concentrations of concanavalin A (ConA). These mutants are reduced in the amount of mannosyl oligosaccharide-lipid produced compared to the wild type cells (Krag et al., in press). As such, these mutants offer an excellent tool for the study of glycoprotein synthesis.

It has previously been demonstrated in such diverse systems as a cell-free system (Robbins et al., 1977a), in vitro calf thyroid tissue slices (Spiro et al., 1976a), and Sindbis virus (Sefton, 1977) that the core glycopeptide (consisting of N-acetylglucosamine, mannose and in some cases, glucose) initially synthesized is of high molecular weight, containing 8-12 mannose residues and 0-2 or -3 glucose residues. However, the full impact of this did not become obvious until the work of Robbins and co-workers and Summers and co-workers (Robbins et al., 1977b; Hunt et al., 1978). These groups have independently used VSV and Sindbis virus as probes and demonstrated that the initial glycoprotein synthesized is of the "high mannose type." Using endo-β-N-acetylglucosaminidase H (Tarentino and Maley, 1974) and/or D (Koide and Muramatsu, 1974) they demonstrated that initially the glycoproteins synthesized were susceptible to hydrolysis by endo-β-N-acetylglucosaminidase H. However, with time, the "completed" glycopeptides were increasingly resistant to endoglycosidase H or became more susceptible to endoglycosidase D. The Robbins' group and Summers' group both postulate that the initially high molecular weight neutral species are transferred en bloc from the oligosaccharide-lipid and later trimmed via an intracellular process to an array of the so-called high mannose types (neutral species) eventually leading to the complex types (acidic glycopeptide). Kornfeld and co-workers have recently described a possible mechanism for the

processing of the G protein of VSV in CHO infected cells (Tabas *et al.*, 1978a). They postulate that the initial lipid-linked oligosaccharide contains two N-acetylglucosamine residues and approximately ten additional monosaccharide residues, at least six of which are mannose. By 30 minutes after transfer to a protein acceptor, three monosaccharide residues are excised, followed by a rapid removal of four more residues. The final product is $(Man)_3(GlcNAc)_2Asn$ and can serve as the acceptor for the outer branches leading to the complete "complex" glycoprotein whose structure has been determined by Summers and co-workers (Etchison *et al.*, 1977; Reading *et al.*, 1978). The implication is that the initial high mannose glycoprotein synthesized by *en bloc* transfer of the high molecular weight oligosaccharide-lipid must undergo considerable processing if it also serves as the intermediate to the complex glycoproteins. Further, they describe a mutant of CHO cells which accumulates $(Man)_5(GlcNAc)_2$ G viral protein. They postulate that the removal of the final two mannose residues is a mandatory prerequisite, and may be coupled to the transfer of N-acetylglucosamine to the oligosaccharide core. A possible trimming enzyme may be similar to the recent findings of Touster and co-workers (Tulsiani *et al.*, 1977), who describe the purification of a Golgi enriched form of α-mannosidase from rat liver. The Golgi α-mannosidase seems to prefer 1-2 linkages to 1-3 linkages (Opheim *et al.*, 1978).

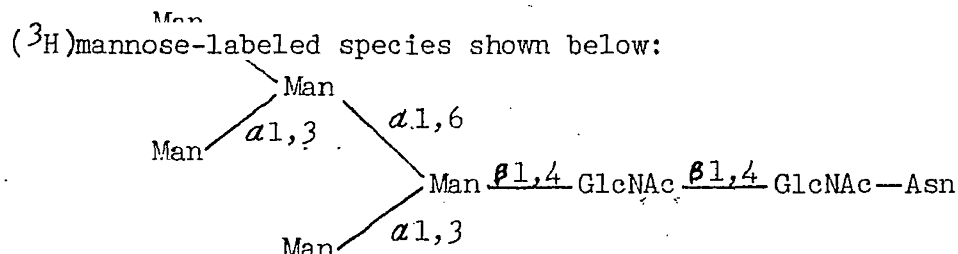
A recent series of reports by Kornfeld and colleagues have helped to further elucidate the processing scheme (Li *et al.*, 1978, Kornfeld *et al.*, 1978; Tabas and Kornfeld, 1978b). Using a combination of endo-β-N-acetylglucosaminidase C_{II} digestion, α-mannosidase digestion, acetolysis, Smith periodation degradation, methylation analysis, and periodate oxidation, the structure of the oligosaccharide moiety of the

lipid-oligosaccharide intermediate has been elucidated (Li et al., 1978). The structure contains 2 N-acetylglucosamine residues, 9 mannosyl residues and 1-3 glucosyl residues and is branched as shown below:

```
                                    Man α1,2 Man \ α1,6
                                                  Man \ α1,6
                                    Man α1,2 Man / α1,3   Man β1,3(4)GlcNAc-GlcNAc-
±Glu 1,2 ±Glu 1,3 Glu 1,3 Man α1,2 Man α1,2 Man       / α1,3
```

These workers made the further observation that the proposed structure is identical (minus the glucose residues) to Unit A of calf thyroglobulin (Ito et al., 1977). The further removal of mannosyl residues gives rise to some of the neutral glycopeptides isolated from ovalbumin (Tai et al., 1975a) and human IgM (Chapman and Kornfeld, 1978). They speculate that the neutral (or high mannose type) glycopeptides may be the result of incompletely processed intermediates destined to become complex type glycopeptides, but this remains to be demonstrated. In a second set of experiments, the processed intermediates generated after en bloc transfer of the oligosaccharide moiety from the lipid-linked precursor were isolated and characterized by brief pulse labeling with mannose, glucosamine and galactose followed by a cold chase (Kornfeld et al., 1978). Based on the proposed structures of these intermediates, a model for the processing event was proposed. Soon after en bloc transfer of the oligosaccharide intermediate from the dolichol carrier, 2 glucosyl residues are rapidly excised, so that the major species are $(Glu)_1(Man)_9(GlcNAc)_2Asn$ and $(Man)_9(GlcNAc)_2Asn$, but small amounts of $(Man)_{8-6}(GlcNAc)_2Asn$ are also present. Excision of four more mannosyl residues (all Man α1,2 linked) gives rise to $(Man)_5(GlcNAc)_2Asn$. Evidence for the conversion

of $(Man)_5(GlcNAc)_2Asn$ to $(GlcNAc)_2(Man)_3(GlcNAc)_2Asn$ was presented in the third report of this series (Tabas and Kornfeld, 1978b). Using the (^3H)mannose-labeled species shown below:



incubated in the presence of crude membrane fractions of wild type CHO cells with and without UDP-GlcNAc, it was shown that a 10-fold greater release of mannose occurred in the sample incubated in the presence of the nucleotide sugar. Further, the final processed "complex"-type intermediate was isolated and was characterized as $(GlcNAc)_2(Man)_3$ $(GlcNAc)_2Asn$, where each mannosyl arm of the inner core was substituted with N-acetylglucosamine linked β 1-2 to the α mannosyl residue. They propose that the initial transfer of an N-acetylglucosaminyl residue to the α mannosyl residue linked 1,3 to the β mannose signals a specific α-mannosidase to remove the outer branched mannosyl residues. The α1,6 linked mannosyl residue exposed is then an acceptor for a second N-acetylglucosamine residue. That species may then go on to accept fucose, galactose and sialic acid, successively.

Chen and Lennarz have demonstrated that incubation of a membrane preparation from hen oviduct with UDP-Glucose will synthesize a high molecular weight glucose-containing oligosaccharide-lipid, the oligosaccharide moiety of which may be transferred en bloc to endogenous protein and exogenous protein acceptors (Chen and Lennarz, 1978a). Further studies have verified that subsequent to the en bloc transfer of the oligosaccharide moiety, the glucosyl residues are enzymatically excised. (^{14}C)

glucose-containing glycoprotein (α-(^{14}C)glucose-lactalbumin) also released free glucose (Chen and Lennarz, 1978b). Thus it would appear that this carefully planned approach, utilizing glycoprotein biosynthesis defective mutants, specific endoglycosidases and conventional carbohydrate analysis, will rapidly lead to the complete elucidation of the processing scheme.

2. Specific endoglycosidases as tools for structural analysis of asparagine-linked oligosaccharides

In 1971 the first demonstration of an endoglycosidase capable of acting on mammalian glycoproteins appeared in the literature (Muramatsu, 1971). Incubation of a radiolabeled myeloma γ-globulin glycopeptide derived from a cell line of mouse plasma cell tumor (MPC-11) with an enzyme preparation (containing the endoglycosidase, β-galactosidase and β-N-acetylglucosaminidase) from the cultural fluid of Diplococcus pneumoniae released two products, both containing sugar residues. One product was a neutral oligosaccharide containing mannose and N-acetylglucosamine, with glucosamine at the reducing end, and the other was a charged species containing fucose, N-acetylglucosamine and amino acids. Hydrolysis of an intact radiolabeled myeloma γ-globulin under similar conditions also released a neutral oligosaccharide. It was concluded that the enzyme does not cleave peptide bonds or the carbohydrate-amino acid linkage, but rather acted as an endoglycosidase cleaving internal carbohydrate linkages, later shown to be the di-N-acetylchitobiose structure.

The purification and partial structural specificity of the enzyme was described (Koide and Muramatsu, 1974). Using the acetylated ovalbumin glycopeptide as substrate, (^{14}C)acetylAsn(GlcNAc)$_2$(Man)$_5$, endo-β-N-acetyl-

glucosaminidase D was purified 2400-fold over the cultural filtrate of <u>Diplococcus pneumoniae</u>. Using a relatively exo-glycosidase free preparation of the endoglycosidase, the di-N-acetylchitobiose linkage of the acetylated glycopeptide was cleaved releasing equimolar amounts of (^{14}C)acetylAsn(GlcNAc) and $(Man)_5(GlcNAc)$. Although essentially 100% of the (^{14}C)acetylAsn$(GlcNAc)_2(Man)_5$ could be hydrolyzed by the enzyme, only 20% of the (^{14}C) acetylated, unfractionated ovalbumin glycopeptides were susceptible. Since it was known that the di-N-acetylchitobiose structure was common to all of the ovalbumin glycopeptides (Lee and Scocca, 1972), they concluded that the structure around this linkage must also influence the hydrolytic event (Koide and Muramatsu, 1974). It was shown that a (^{14}C) acetylated bovine IgG glycopeptide failed to release the expected products if incubation was carried out with the purified endoglycosidase in the absence of β-galactosidase and β-N-acetylglucosaminidase. Unit B glycopeptides from porcine thyroglobulin (containing sialic acid, galactose, N-acetylglucosamine, mannose and fucose) were also resistant to the endoglycosidase unless treated with neuraminidase, β-galactosidase and β-N-acetylglucosaminidase, suggesting that terminal substitutions of the inner core glycopeptides affected susceptibility. Likewise, Unit A glycopeptides of calf thyroglobulin (thought to be the high mannose type) were similarly resistant, indicating that the size of the oligomannosyl core also affected the susceptibility of the asparagine-linked glycopeptides to endo-β-N-acetylglucosaminidase D hydrolysis.

The structural specificity of endoglycosidase D was further elucidated by a careful sequential degradation of bovine IgG glycopeptide with exo-glycosidases (Ito <u>et al</u>., 1975a). It was shown that one branch of

the oligomannosyl core of the IgG glycopeptide was incompletely galactosylated. Treatment of these glycopeptides with β-N-acetyl-glucosaminidase made them susceptible to endo-β-N-acetylglucosaminidase D. However, further digestion with α-mannosidase made the glycopeptide again unsusceptible, indicating that an unsubstituted α-mannosyl residue was a mandatory requirement for endoglycosidase D susceptibility. A subsequent report (Tai et al., 1975b) verified that the unsubstituted α-mannosyl residue was linked 1, 3 to the β mannosyl residue.

In 1972, Tarentino and co-workers described an endoglycosidase isolated from the culture filtrate of Streptomyces griseus which was able to hydrolyze Asn(GlcNAc)$_2$(Man)$_1$ (prepared by α-D-mannosidase digestion of the ovalbumin glycopeptide, Asn(GlcNAc)$_2$(Man)$_5$) releasing AsnGlcNAc and GlcNAc(Man). The disaccharide had the characteristics of a β-mannosidic linkage. The enzyme preparation was also able to hydrolyze the ovalbumin glycopeptide, Asn(GlcNAc)$_4$(Man)$_6$,[1] releasing the expected AsnGlcNAc and an oligosaccharide (Tarentino et al., 1972). It was later shown that the partially purified enzyme preparation from the culture filtrate of Streptomyces griseus contained two enzymes: endo-β-N-acetylglucosaminidase L, which hydrolyzed the di-N-acetylchitobiose linkage of Asn(GlcNAc)$_2$(Man)$_1$ and endo-β-N-acetylglucosaminidase H which was shown to hydrolyze species with larger oligomannosyl cores (Tarentino and Maley, 1974). Endo-β-N-acetylglucosaminidase H was used to hydrolyze the Unit A oligosacchairde from bovine thyroglobulin which was subsequently shown to possess a β-mannose linkage in the inner core glycopeptide (Tarentino et al., 1973). The purification and properties

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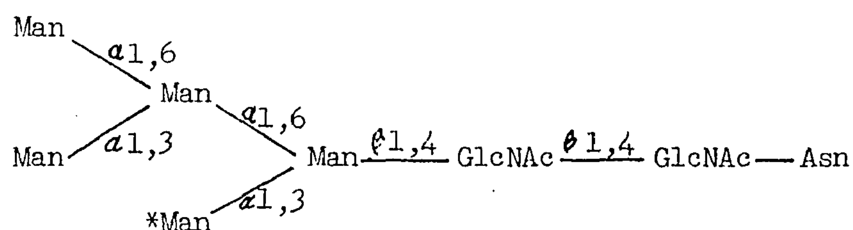
[1]This structural composition is based on the data of Huang et al (1970). A recent report indicates that this material is a mixture of three distinct glycopeptide species (Tai et al., 1977a).

of endo-β-N-acetylglucosaminidase H have been described (Tarentino and Maley, 1974). The endoglycosidase was able to cleave $Asn(GlcNAc)_2(Man)_6$ and $Asn(GlcNAc)_2(Man)_6(GlcNAc)_2$ to release the products AsnGlcNAc and $(GlcNAc)(Man)_6$ and $(GlcNAc)(Man)_6(GlcNAc)_2$, respectively. The dansyl derivative of the latter glycopeptides as well as the intact oligosaccharide were also susceptible to hydrolysis. Endo-β-N-acetylglucosaminidase H was inactive towards $Asn(GlcNAc)_2(Man)_1$, but the glycopeptide was susceptible to endo-β-N-acetylglucosaminidase L. The former enzyme could be isolated free of the latter by ion exchange chromatography followed by Sephadex G-100 chromatography. That endoglycosidase H could be used to release neutral oligosaccharides from intact glycoproteins was demonstrated in a succeeding report (Tarentino _et al._, 1974). The enzyme was able to effect the hydrolysis of neutral oligosaccharides from sulfitolyzed ovalbumin, bovine pancreatic deoxyribonuclease A and ribonuclease A, and an invertase from _Saccharomyces cerevisiae._ Those glycoproteins known to contain both neutral and acidic oligosacchairdes were also tested for susceptibility to endoglycosidase H. Thyroglobulin, IgM and Porcine ribonuclease released only their neutral chains in the presence of endo-β-N-acetylglucosaminidase H.

Comparative studies of endoglycosidase D and H have been performed by both Muramatsu and Tarentino (Arakawa and Muramatsu, 1974; Tarentino and Maley, 1975). Endo-β-N-acetylglucosaminidase H completely hydrolyzed the (^{14}C) acetylated calf thyroglobulin Unit A glycopeptides, but was unable to cleave the (^{14}C) acetylated intact Unit B glycopeptides and (^{14}C) acetylated bovine IgG glycopeptide, as well as their "side-chain-free" and "side-chain-free," defucosyl derivatives. On the other hand, endo-β-N-acetylglucosaminidase D could cleave the Unit B glycopeptides and IgG glycopeptide if the peripheral sugars were first removed by exo-

glycosidase treatment (Arakawa and Muramatsu, 1974). In contrast, Tarentino and Maley have shown that while the presence or absence of fucose does not affect the hydrolysis rate of a "side-chain-free" human IgM glycopeptide by endoglycosidase D, and the fucosylated species is resistant to endoglycosidase H, the removal of the fucosyl residue made this species (containing a 3 mannosyl inner core) susceptible to endoglycosidase H hydrolysis. They noted that longer incubation times and higher enzyme concentrations are needed to effect the hydrolysis. Further, it was observed that, in general, endoglycosidase H prefers glycopeptides with oligomannosyl cores larger than $GlcNAc(Man)_5$ and as such is ideal for neutral ("high mannose") glycopeptides. Endoglycosidase D has maximal activity for glycopeptides with oligomannosyl cores of 3-5 residues, providing that the peripheral sugars are removed first, making it an excellent choice for acidic glycopeptides (Tarentino and Maley, 1975).

An elegant demonstration of the use of endoglycosidases D and H for glycopeptide structural analysis has been reported for the characterization of two ovalbumin glycopeptide species (Tai _et al_., 1975a). These studies were also useful in further elucidating the structural specificity of both enzymes. Using a combination of endoglycosidase digestion, methylation analysis, acetolysis, NaB^3H_4 reduction and α-mannosidase digestion, the complete structure of $Asn(GlcNAc)_2(Man)_5$ and $Asn(GlcNAc)_2(Man)_6$ was determined. The proposed structure of the two glycopeptides are identical except that in the latter species the inner branched mannosyl residue, linked α1,3 to the βmannosyl residue is substituted by an α1,2 mannosyl residue. Whereas the 5 core species is unsubstituted as shown below:



While Asn(GlcNAc)$_2$(Man)$_5$ is highly susceptible to endoglycosidase D, Asn(GlcNAc)$_2$(Man)$_6$ is markedly resistant. Therefore the unsubstituted α1,3 mannosyl residue linked to the βmannosyl residue is critical for the recognition of and susceptibility to endo-β-N-acetylglucosaminidase D. A similar conclusion was previously reached using an IgG glycopeptide treated with β-N-acetylglucosaminidase to free the α1,3 linked mannosyl residue (Ito, et al., 1975a).

At about this time, two new endoglycosidases (named C_I and C_{II}) were isolated and their properties partially defined (Ito et al., 1975b). Endo-β-N-acetylglucosaminidase C_I is derived from the cultural fluid of Clostridium perfringens and appears to share an identical specificity with that of endo-β-N-acetylglucosaminidase D, isolated from Diplococcus pneumoniae. A second enzyme, endo-β-N-acetylglucosaminidase C_{II} could be purified free of the former and appeared to be similar in properties to endo-β-N-acetylglucosaminidase H from Streptomyces griseus. However, the relative activities of the C_{II} and H enzymes differed with respect to their hydrolytic rate for ovalbumin glycopeptides and Unit A glycopeptides. They suggested that the two endoglycosidases must recognize different structures around the oligomannosyl core. Further insight into the specificities of the C_{II} and H enzymes was provided in a careful analysis of ovalbumin glycopeptide III (Tai et al., 1977a). It was already known that endoglycosidase H could cleave all of the ovalbumin glycopeptides (Tai et al., 1975a). Endoglycosidase C_{II} cannot

cleave ovalbumin glycopeptides I and II, which are longer chain species. Only 75% of ovalbumin glycopeptide III was susceptible to the C_{II} enzyme, the resistant 25% being susceptible to endoglycosidase H, suggesting that glycopeptide III was a mixture. Characterizing the structure of the resistant species has helped to elucidate the structural specificity of endoglycosidase C_{II}. The composition of GP-III-C is $Asn(GlcNAc)_5(Man)_4$. The key recognition site is the α-mannosyl residue linked 1,3 to the β mannose. If substituted at the C_2 position with N-acetylglucosamine or mannose, susceptibility to endoglycosidase C_{II} is unaffected. However, further substitution of the residue at the C_4 position by another sugar makes it resistant to the C_{II} enzyme, but remains susceptible to the H enzyme. Recently these workers have isolated an ovalbumin species with composition $Asn(GlcNAc)_2(Man)_4$ (Tai et al., 1977b). The α mannosyl residue linked 1,6 to the β-mannose is substituted at the C_3 position. This species was found to be susceptible to both the C_{II} and H enzymes. $Asn(GlcNAc)_2(Man)_4$ has been prepared by limited α-mannosidase digestion of the ovalbumin glycopeptide, $Asn(GlcNAc)_2(Man)_5$. This species differs from the abovementioned glycopeptide in that the α mannosyl residue linked 1,6 to the β mannose is substituted at the C_6 position, but was also found to be highly susceptible to endoglycosidase H. This study also extended the application of endoglycosidase H, in that neutral species with as few as 2 mannosyl residues were found to be susceptible to hydrolysis (Trimble et al., 1978).

Other examples of endoglycosidases was reported from crude fig latex (Chien et al., 1977). F_I hydrolyzes the di-N-acetylchitobiose linkage from an human IgG glycopeptide much faster than from $Asn(GlcNAc)_2(Man)_5$ or $Asn(GlcNAc)_2(Man)_6$ derived from ovalbumin, and as such appears to be similar to endo-β-N-acetylglucosaminidase D. On the other hand, F_{II} could

effect the hydrolysis of both ovalbumin glycopeptides, but the IgG glycopeptide was resistant. Interestingly, both enzymes could not cleave larger ovalbumin glycopeptides, terminally substituted with N-acetylglucosamine. The possibility of whether or not larger oligomannosyl cores, such as found in Unit A thyroglobulin glycopeptides, could be susceptible to these enzymes was not examined. The presence of endoglycosidases has also been reported in mammalian tissues (Nishigaki _et al_., 1974). Extracts from rat liver, kidney and spleen, and porcine liver and spleen could hydrolyze the di-N-acetylchitobiose linkage of (^{14}C) acetylated Asn(GlcNAc)$_2$(Man)$_5$, Asn (GlcNAc)$_2$(Man)$_6$ and Asn(GlcNAc)$_4$(Man)$_6$. The endoglycosidase(s) appear to be distinct in that IgG glycopeptides and Unit A thyroglobulin glycopeptides were markedly resistant. A single endoglycosidase purified from hen oviduct extracts seems to have a broad specificity comparable to both the endoglycosidases from _Diplococcus pneumoniae_ and _Streptomyces plicatus_[1] (Tarentino and Maley, 1976). The enzyme was able to effect the hydrolysis of the di-N-acetylchitobiose linkage in compounds ranging from Asn(GlcNAc)$_2$ (Man)$_{3-6}$, as well as Asn(GlcNAc)$_2$(Man)$_3$(Fuc)$_1$. Thus it would appear that as the specificity of these endoglycosidases is elucidated, their careful use in conjunction with conventional carbohydrate chemistry should lead to rapid advances in glycoprotein structural analysis.

3. Oligosaccharide changes in asparagine-linked glycopeptides

a. growth dependent alterations

The general approach most often utilized to study growth dependent alterations in the oligosaccharide moiety of asparagine-linked glycopeptides has been to examine the size distribution of the glycopeptides by gel filtration chromatography. Cells are usually labeled metabolically with

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[1]formally _Streptomyces griseus_

either glucosamine, fucose or mannose, and the plasma membrane glycopeptides collected by 1) mild extraction with EDTA; 2) proteolytic extraction, most often with trypsin or Pronase, leaving the remaining cells intact and viable; 3) preparation of purified surface membranes; or 4) isolation of crude membrane fractions with little or no subsequent purification from subcellular membrane fractions. The radiolabeled samples collected by one of the above means are then subjected to extensive proteolytic digestion with Pronase, ultimately yielding a glycopeptide with few remaining amino acids.

It is noteworthy to stress at this point that direct comparisons of studies performed in various laboratories must be evaluated carefully, dependent upon the radioactive precursor used to obtain the results. That is to say, the radioactive precursor is used to permit the easy isolation and subsequent monitoring of glycopeptide material by gel filtration, but the choice of precursor may in fact introduce limitations as to the type of asparagine-linked glycopeptides being studied. For instance, isotopic fucose is an excellent choice in that it will label primarily glycoproteins and glycolipids, and can be recovered as fucose since it is not generally metabolized by the cell. However, fucose is generally considered to be associated with the complex (acidic) glycopeptides, thought to be similar to Unit B glycopeptides of thyroglobulin (Toyoshima _et al_., 1972, 1973), containing N-acetylglucosamine, galactose, mannose, fucose and sialic acid. On the other hand, mannose is found in all of the asparagine-linked glycopeptides, also with little conversion. Therefore studies utilizing mannose encompass both the complex glycopeptides and simple (neutral) glycopeptides, thought to be similar to Unit A glycopeptides of thyroglobulin (Arima and Spiro, 1972; Tarentino _et al_., 1973), consisting primarily of N-acetylglucosamine and clusters of mannose. Whereas, if glucosamine is used as a general probe, one might reasonably expect to label glycoproteins,

glycolipids and glycosaminoglycans, as well as finding not only N-acetylglucosamine, but conversion to N-acetylgalactosamine and sialic acids.

In 1969, the first study contrasting glycoproteins and glycopeptides from doubly-labeled fibroblast populations was published (Meezan _et al._, 1969). Although the study dealt primarily with a comparison of subcellular fractions isolated from 3T3 mouse fibroblasts and the same cell transformed by Simian virus 40 (cf. transformation dependent alterations), growth dependent alterations in nuclear fractions labeled with glucosamine were evident. Pronase-digested glycopeptides derived from growing cells were shifted toward higher molecular weight when compared to those from confluent cells, as analyzed by Sephadex G-50 chromatography. The first demonstration that cell surface glycopeptides changed in a growth dependent fashion was provided by Warren and co-workers (Buck _et al._, 1971a). Fucose-labeled glycopeptides derived from purified cell surface membranes or collected by mild trypsin digestion from normal baby hamster kidney (BHK) cells could be resolved into two broad peaks by Sephadex G-50 chromatography. Glycopeptides from rapidly growing (log) cells were enriched in the high molecular weight component relative to slowly growing (plateau) cells. The fact that either the cell membranes or trypsinates gave similar results underscores the point that the trypsinates were representative of the cell surface. In a follow-up study utilizing BHK cells arrested in metaphase by vinblastin sulfate and similarly treated cells not harvested in metaphase, the fucose-labeled trypsinates of the mitotic cells were enriched in higher molecular weight glycopeptides compared to the cells not in mitosis (Glick and Buck, 1973). The authors concluded that the glycopeptides

expressed by the mitotic cells were similar to that previously seen in exponentially growing cells (Buck *et al.*, 1971a), and as such must be related to the growth status of the cell. Growth-related alterations were also reported with the EDTA-extracts of glucosamine-labeled glycopeptides derived from 3T3 mouse fibroblasts (Sakiyama and Burge, 1972). A shift toward higher molecular weight glycopeptides was seen in rapidly dividing cells compared to nondividing cells. A similar result was also noted for the membrane fraction. In the latter case, glycopeptides derived from growing cells could no longer be distinguished from those of nondividing cells if sialic acid was first removed by mild acid hydrolysis, i.e., differences in size distribution were attributed to increased sialylation in dividing cells. Van Nest and Grimes were not able to detect any growth dependent changes in the gel filtration profiles of glucosamine-labeled glycopeptides derived from crude membrane fractions of normal BALB/c mouse cell lines (Van Nest and Grimes, 1977). Enhancement of high molecular weight fucose-labeled glycopeptides were reported for the normal human fibroblast, KL-2 (Muramatsu *et al.*, 1973). Cell surface glycopeptides harvested by mild Pronase digestion as well as cellular glycopeptides displayed an enrichment of high molecular weight glycopeptides from exponentially growing cells. Ceccarini (1975) confirmed these results in comparisons of exponentially growing and density inhibited WI38 cells. Surface glycopeptides were shifted toward higher molecular weight in growing cells relative to confluent cells, regardless of whether fucose or mannose was used as the radioactive precursor. In the former studies (Muramatsu *et al.*, 1973), possible size alterations due to the peptide-carbohydrate linkage region were also examined. Purified glycopeptides were treated with endo-β-N-

acetylglucosaminidase D, in the presence of β-N-acetylglucosaminidase, β-galactosidase and neuraminidase (cf. section 2, introduction, for enzyme spcificity) and subsequently analyzed by Sephadex G-25 chromatography. The glycopeptide fragment from non-growing cells was more heterogeneous and slightly shifted to higher molecular weight. Unlike previous studies, which implicate sialic acid content as the reason for growth-dependent size differences, this work suggested that the linkage region could also influence size distribution. Thus, the peptide-carbohydrate region, as well as the more distal portion of the oligosaccharide moiety may contribute to apparent growth dependent alterations.

The use of fucose as radioactive precursor probably monitors, for the most part, the complex glycopeptides, thereby excluding an entire class of asparagine-linked glycopeptides. Mannose is present in both the simple and complex glycopeptides, thus permitting the simultaneous examination of both types of species. Cell surface glycopeptides derived from the normal human fibroblast, KL-2, displayed growth-dependent alterations when examined by endo-β-N-acetylglucosaminidase D (Ceccarini _et al_., 1975). The simple (neutral) glycopeptides from growing cells were markedly more resistant to endoglycosidase D digestion than those from non-growing cells, suggesting the presence of large oligomannosyl cores (cf. section 2, introduction, for enzyme specificity). Glycopeptides from growing cells were also more susceptible to α-mannosidase digestion, lending further support for the presence of larger mannose clusters in exponentially growing cells relative to density inhibited cells. The cellular mannose-labeled glycopeptides were examined in a subsequent report (Muramatsu _et al_., 1976). Using endo-β-N-acetylglucosaminidases D and H, the neutral glycopeptides were converted to

arrays of oligosaccharides, ranging from GlcNAc(Man)$_3$ to GlcNAc(Man)$_{7-8}$. Growth dependent alterations were evident in that the higher molecular weight neutral glycopeptides were decreased in the non-growing cells, relative to the growing cells. Similar results for the cell surface neutral species of WI38 cells has been reported (Ceccarini and Atkinson, 1977).

b. transformation dependent alterations

Since the initial observations of Meezan (Meezan et al., 1969) and Buck (Buck et al., 1970), a great deal of information has accumulated concerning transformation dependent alterations. The purpose of this brief review is to touch upon some of the highlights of this topic, namely a) transformation related changes in asparagine-linked glycopeptides, b) carbohydrate content, c) sialyl transferase activities and d) alterations as they relate to tumorogenicity. As the literature in this field has become extensive, easily requiring an entire chapter to deal with it, no attempt has been made to cite every publication in the field. Rather, key publications dealing with the abovementioned subtopics will be discussed so as to make the reader aware of the current data. The reader is advised to refer to any of the excellent review articles presently available for a more general discussion of the topic (Nicolson, 1976; Hakomori, 1975; Hynes, 1976; Warren et al., 1978).

Interest in transformation dependent alterations was initially stimulated by the observations of Meezan (Meezan et al., 1969). Mouse fibroblasts (3T3) and the same cells transformed by Simian virus 40 (SV-3T3) were doubly-labeled with (^{3}H) glucosamine and (^{14}C) glucosamine, thus permitting the simultaneous comparison of glycoproteins and glycopeptides derived from the two cell populations. Subcellular fractions

including mitochondria, nuclei, endoplasmic reticulum and surface membranes were compared. Quantitative differences in the Sephadex G-150 profiles of the intact glycoproteins were observed. The subcellular fractions were extensively digested with Pronase and the glycopeptides analyzed by Sephadex G-50 chromatography. At least four distinct peaks were apparent and quantitative differences in each were noted. Interestingly, a broad peak, containing most of the radioactivity, was shifted to lower molecular weight in all transformed subcellular fractions, except for the surface membrane. In subsequent studies by Warren and co-workers, the surface glycopeptides from normal baby hamster kidney (BHK) cells and the same cells transformed by the RNA tumor virus, Rous sarcoma, were doubly-labeled with either L-fucose or D-glucosamine. Pronase-digested surface glycopeptides harvested either by mild trypsinization or from purified surface membranes were examined by Sephadex G-50 chromatography. Irrespective of the radioactive precursor used, the transformed cells always displayed an enrichment of high molecular weight glycopeptides relative to the normal cells (Buck *et al.*, 1970). This was true for both the surface trypsinate and purified surface membrane. The subcellular fractions of control and virus-transformed BHK cells have also been examined with both radioactive fucose and glucosamine (Buck *et al.*, 1974). The Sephadex G-50 profiles of subcellular fractions labeled with fucose were consistent in one major point. Just as previously reported with the surface trypsinates (Buck *et al.*, 1970), fractions containing nuclei, lysosome, mitochondria and endoplasmic reticulum (smooth and rough) all displayed similar enhancements of high molecular weight glycopeptides. It was noted that a low molecular weight peak (approximately 1500 daltons), thought to contain primarily N-acetyl-

glucosamine and mannose was greatly reduced if fucose was used to label the cells. In contrast, using glycosamine as the radioactive precursor (generally labelling all of the asparagine-linked glycopeptides), produced some fascinating data. While the same overall conclusions could be made about transformation dependent enrichment of high molecular weight glycopeptides, it was also noted that there was an enhancement of the lower molecular weight species in fractions containing rough endoplasmic reticulum and ER supernatant, with relatively little high molecular weight material present. On the other hand, the smooth endoplasmic reticulum gave a profile similar to the surface membrane. In addition, treatment with neuraminidase of glycopeptides derived from subcellular fractions greatly reduced, but could not completely eliminate, the size differences observed between transformed and normal BHK cells. The data strongly suggest that some differences in size may be attributed to increased sialylation, but does not exclude the possibility that other alterations in the carbohydrate moiety exist. A previous report (Warren et al., 1972a) demonstrated that neuraminidase treatment of surface trypsinates from normal and transformed BHK cells effectively eliminated all differences between the two profiles. These studies have been extended to include hamster cells transformed by DNA tumor viruses, mouse cells transformed by Rous sarcoma virus and murine sarcoma virus, and primary chick cells transformed by Rous sarcoma virus. In all cases, the same transformation depedent enrichment of high molecular weight glycopeptides was displayed (Buck et al., 1971b). Using chick embryo fibroblasts infected with a temperature-sensitive Rous sarcoma virus (T5) it was shown that the enrichment of high molecular weight material was dependent upon the phenotypic

expression of transformation (Warren et al., 1972b). T5-infected cells express a transformed phenotype at 36° but appear normal at 41°. Virus production may continue at either temperature, underscoring the point that it is not the virus production that causes the enhancement of high molecular weight glycopeptides, but rather a factor that permits the phenotypic expression of transformation. In an attempt to study relatively homogeneous glycoproteins from normal and transformed BHK cells, a systematic purification was employed involving preparative SDS electrophoresis and isoelectric focusing. Pairs of corresponding glycoproteins, doubly-labeled with glucosamine, were eluted from the focusing gel and digested with Pronase. The gel filtration of 20 of 24 pairs demonstrated marked differences in the carbohydrate moiety (Tuszynski et al., 1978). They conclude that the enrichment of high molecular weight glycopeptides seen in crude membrane fractions must be the net result of carbohydrate alterations in many glycoproteins. Similar studies have been performed on crude membrane fractions of normal mouse BALB/c cell lines compared to several virally and spontaneously transformed cell lines (Van Nest and Grimes, 1977). In general, all of the transformed cell lines displayed an enhancement of high molecular weight glycopeptides relative to the normal BALB/c cells (with the exception of C5, a tumor cell that always regresses in BALB/c mice). Corresponding glycopeptides from a normal cell and a malignant cell. (3T12T) were isolated by SDS gel electrophoresis of intact glycoproteins, followed by extensive proteolytic digestion. A transformation dependent enrichment of high molecular weight glycopeptides was observed in two out of the three SDS gel regions examined. Neuraminidase treatment shifted the profiles to smaller molecular weight, but size differences

could still be distinguished. Most interesting was the observation that the largest glycoproteins (on SDS gel electrophoresis) displayed the smallest glycopeptides (by gel filtration), while the reverse situation was found for the smallest glycoproteins. Sakiyama and Burge compared the glycoproteins and glycopeptides of normal and SV40 transformed 3T3 cells by polyacrylamide gel electrophoresis and gel filtration. They found that the glycoproteins and glycopeptides of normal and transformed cells could not be distinguished by polyacrylamide gel electrophoresis or Bio-Gel P-10 columns (Sakiyama and Burge, 1972). Ceccarini could detect no differences in the size distribution of Pronase-digested surface glycopeptides from the human fibroblast, WI38 and an SV40 transformed cell, WI18Va, when fucose was used to monitor the material. However, if the glycopeptides were labeled with mannose, the transformed glycopeptides were markedly reduced in size. He concluded that the size differences may be growth dependent, but not related to transformation (Ceccarini, 1975). The oligosaccharide moiety of mannose-labeled cell surface glycopeptides was examined in WI38 cells and SV40 transformed WI18Va (Ceccarini and Atkinson, 1977). The neutral glycopeptides from transformed cells were enriched in endoglycosidase D resistant species, relative to the rapidly growing normal cells. The data suggest the presence of larger oligomannosyl cores in the transformed state. On the whole, however, while some exceptions have been shown (Sakiyama and Burge, 1972; Ceccarini, 1975), the common rule of thumb would appear to be that transformed cells, at least *in vitro*, display an enrichment of high molecular weight glycopeptides relative to their normal counterpart.

A number of studies have attempted to correlate total cellular

carbohydrate content with the normal and transformed state, but no general consistency has emerged. Rather, it is clear from the published literature that various factors, including cell type investigated, growth conditions, cell shape, methods of extraction, transforming agent and tumorgenecity, influence the patterns of carbohydrate content seen in comparisons of normal vs. transformed cells. Certainly, the general contention that transformed cells contain decreased amounts of neutral and amino sugars, particularly sialic acid (Ohta et al., 1968; Wu et al., 1969; Grimes, 1973) has met with exceptions (Hartmann et al., 1972; Makita and Shimojo, 1973; Emmelot and Bos, 1972; Grimes and Greegor, 1976). Perhaps the single best example of the inability to make any hard and fast rules pertaining to carbohydrate content and the transformed state comes from the studies of Warren and co-workers (Hartmann et al., 1972). These workers contrasted normal baby hamster kidney cells with both DNA virus transformed and RNA virus transformed cells, as well as primary chick embryo fibroblasts and RNA virus transformed chick cells. They noted that dependent on the method used to extract and prepare cells, different interpretations were possible. Notably, they advise that cell extracts and cellular washes be included in order to determine total carbohydrate content. Nevertheless, differences were observed depending upon whether BHK or chick cells were examined, as well as the transforming agent used. In general, BHK cells transformed by DNA viruses display similar amounts of mannose and galactose compared to normal cells if both the cells and washes are accounted for. Fucose levels were consistently lower, while sialic acid content appeared similar in polyoma transformed cells, but depressed in SV40 transformed cells. On the other hand, BHK cells and their RSV

transformed counterparts showed that mannose and hexosamines were increased, while sialic acid remained about the same. Galactose and fucose were decreased (but the former by only 6%). Chick cells and their RSV transformed counterparts displayed little difference in content of mannose and hexosamines. Fucose was again depressed in transformed cells, but galactose and sialic acid content was increased. Only fucose was consistently depressed in the transformed cells, whereas clearly no general rule could be made for the other neutral sugars and amino sugars. A most fascinating trend was described for the carbohydrate content of malignant and regressing tumors (Grimes and Greegor, 1976). The chloroform:methanol soluble and insoluble fractions from crude cellular membranes of normal BALB/c mice and virally and spontaneous transformed cells were examined for carbohydrate composition. Killing tumors had total carbohydrate and sialic acid content largely similar to normal cells, whereas tumors that regress (in young immunocompetent BALB/c mice) are markedly reduced in both hexoses and hexosamines, as well as sialic acid. The data strongly suggest that the degree of tumorogenicity influences carbohydrate content. In a series of on-going studies, Perdue and co-workers have correlated carbohydrate content with oncogenic virus infection and cell shape. Studies contrasting the plasma membranes of chick embryo fibroblasts transformed either by an oncogenic avian sarcoma virus (RBA) or an avian leukosis virus (RAV-49) demonstrated that the former displayed increased neutral sugar content and decreased sialic acid content, whereas the latter had values similar to normal cells. As virus infection and maturation is taking place in either case, they conclude that the changes in the cell membrane are the result of phenotypic expression by an oncogenic virus

(Perdue *et al.*, 1971). In a subsequent report it was demonstrated that cell shape influenced neutral sugar content of plasma membranes (Perdue *et al.*, 1972). Avian sarcoma virus, morph^f Fujinami and morph^r Fujinami, both displayed decreased sialic acid content relative to normal cells and RAV-49. The former cells were fusiform in shape and displayed decreased neutral sugar content relative to the morph^r cells, which were rounded and enhanced in neutral sugars. The fact that cell shape influenced neutral sugar content, but not malignancy, indicates a second virus-directed function able to alter the cell membrane (the first being reduced sialic acid content in oncogenic viruses). That oncogenic transformation in cell culture resembles neoplastic change *in situ* with respect to carbohydrate content was demonstrated by infecting chicken breast muscle and wing web with morph^r Fujinami avian sarcoma virus. Similar neutral sugar and sialic acid content was reported as previously seen *in vitro* (Perdue *et al.*, 1973).

Numerous studies have been performed to determine glycosyl transferase activities in normal and transformed cells. Most particularly, sialyl transferase has been the subject of intensive studies in an effort to correlate its activity with altered carbohydrate content (Grimes, 1973) or enhancement of high molecular weight glycopeptides (Warren *et al.*, 1972a) in the transformed state. Using desialized bovine submaxillary mucin and fetuin as substrate acceptors, cellular particulate extracts from normal and transformed mouse and hamster cell lines were assayed for the ability to transfer sialic acid from CMP-(^{3}H) NANA to the exogenous acceptors. In all cases but one (a polyoma transformed mouse 3T3 cell), mouse cells transformed spontaneously, and by SV40 or polyoma and hamster sarcoma viruses exhibited reduced

sialyl transferase activity relative to the normal cells (Grimes, 1973). There was a strict correlation between lack of density dependent growth regulation and decreased levels of sialic acid content and decreased sialyl transferase activity. The probable structure of the acceptor substrates were reported to be polypeptide-N-acetylgalactosamine-galactose. It should be stressed that an oligosaccharide acceptor of this nature is quite different from the asparagine-linked, sialic acid containing species mentioned throughout this introduction. The reason for pointing this out is to help make clear the differences between this work and that of Warren (see below). Using the Pronase-digested trypsinates of fucose-labeled high molecular weight glycopeptides isolated from Rous sarcoma virus infected BHK cells, and subsequently desialyzed by neuraminidase, the sialyl transferase activity from cellular extracts of normal and transformed cells was determined (Warren _et al._, 1972a). Higher activities were reported for the transformed cells. Using desialyzed fetuin or bovine mucin as substrate acceptors, no differences were detected in the sialyl transferase activity of extracts from normal and virally transformed cells. It should be stressed that Warren and Grimes were probably looking at different enzymes. The Warren enzyme was detected because its specific substrate was made available (most probably of the asparagine-linked complex variety), whereas the Grimes enzyme would appear to be specific for a very different type of oligosaccharide composition. The Warren enzyme appeared to be growth dependent, as well as transformation dependent, as it was markedly reduced in non-growing cells and showed greater activity in log transformed cells compared to log normal cells. In addition, chick embryo fibroblasts, transformed by a temperature

sensitive Rous sarcoma virus, showed that sialyl transferase activity was enhanced at the permissive temperature, but reduced similar to normal levels at the nonpermissive temperature (Warren et al., 1973). It should be pointed out that the above work utilized "specific acceptors" from the surface of virally transformed cells. No comparison with "specific acceptors" from normal cell surface was reported. Hence, the possibility exists that viral extracts have preferred affinity for acceptors made from virally transformed cells and therefore show enhanced activity for viral sialyl transferase. This was made less likely by the work of Bosmann (Bosmann, 1972). He utilized a desialyzed, trypsinized "acceptor fraction" from the cell surface of both normal 3T3 and 3T3 cells transformed by Rous sarcoma virus, Murine sarcoma virus and polyoma virus. The virally transformed cells contained 1.5 to 6.1 times as much sialyl transferase activity as did the normal 3T3, irrespective of the origin of the acceptors. However, he also makes the interesting observation that the transformed acceptor fractions contain about 1.5 times as many sites for addition of NANA as the 3T3 cell acceptors.

The question as to whether enhancement of high molecular weight glycopeptides is an artifact of transformed cells in culture, or wider in scope, encompassing tumorogenic cells in vivo has been the subject of intensive investigations. A number of laboratories have found a positive correlation between tumorogenicity and the presence of high molecular weight material (Glick et al., 1974; Warren et al., 1975; Van Beek et al., 1977). Enrichment of high molecular weight glycopeptides has been demonstrated not only for transformed fibroblasts, but for malignant lymphoblasts and epithelioid cells as well. Doubly-

labeled fucose trypsinates from Novikoff rat hepatoma vs. rat liver cells, and glucosamine labeled malignant vs. nonmalignant lymphoblasts, exhibited an increased level of high molecular weight material in the malignant cells, maintained in vitro (Van Beek et al., 1973). These studies were extended to include a comparison of human leukemic cells against controls of normal resting and phytohaemagglutinin stimulated lymphocytes, as well as patients with mononucleosis. They concluded that the enrichment of high molecular weight material was characteristic of malignant cells (Van Beek et al., 1975). The possibility of these results being an artefact of cell culture was made less likely by a subsequent study by Van Beek (Van Beek et al., 1977). The cell surface glycoproteins of 3 rat hepatoma strains and late-embryonic liver cells were metabolically labeled in vivo with (^{3}H) or (^{14}C)-fucose. In all cases, the Pronase-digested trypsinates yielded enhanced high molecular weight glycopeptides for the hepatoma cells. One result was most interesting, especially as similar results will be reported in the present work (cf. Chapter III), namely that the presence of neuraminidase insensitive but mild acid hydrolysis sensitive sialic acid was demonstrated. Further, these workers concluded that the presence of sulphated sugar residues could only be affecting their findings to a limited extent. They postulate that an unknown molecular weight determinant or sialic acid residue insensitive to mild acid hydrolysis may also be affecting the results of at least one hepatoma cell. Glick found a positive correlation between the ability of cells to form tumors and the expression of phenotypic properties of transformation (Glick et al., 1974). Hamster embryo cells infected with polyoma virus could be selected for low tumorogenicity (delayed transformation) or high tumorogenicity (rapid transformation). The expression

of transformed properties increased in the delayed transformed cells (i.e., high saturation density *in vitro* and ability to form tumors in adult hamsters) as cells were passaged *in vitro*. A marked enrichment of high molecular weight glycopeptides could be detected as cells became more tumorogenic. A marked increase of sialic acid rich, fucose-containing glycopeptides was also detected in the surface and internal membranes of melanotic tumors of mice compared with normal liver and lung tissue labeled *in vitro* and *in vivo* (Warren *et al*., 1975). Thus it would seem that earlier results demonstrating an enrichment of high molecular weight glycopeptides for cells transformed *in vitro*, may be extended to include tumorogenic cells *in vivo*.

4. Fibronectin

Perhaps the single best studied cell surface glycoprotein to date is fibronectin. It has appeared in the literature under a variety of names and has been found on the surface of normal fibroblasts, but is markedly reduced or absent on the surface of many transformed cells. Fibronectin is highly protease-sensitive and can be recovered by mild extraction conditions, leaving the cell population intact. It exists as a dimer, with a monomer molecular weight between 200,000 and 250,000 daltons. A similar, if not identical molecule has been found to be present in serum and plasma. Fibronectin has been implicated in cell attachment and adhesion *in vitro*, as well as providing the matrix for cells to anchor and orient themselves *in vivo*. Fibronectin has high affinity for collagen and both macromolecules have been found to be associated in the extracellular network of fibroblast monolayers and adult human tissues.

One of the earliest demonstrations of fibronectin involved the

lactoperoxidase-catalyzed iodination of mouse-embryo fibroblasts (Hogg, 1974). A large surface protein, designated L1, was heavily labeled in untransformed cells, but was absent from transformed cells. The approximate molecular weight of the material was 250,000 daltons. Similar results were reported by Hynes using hamster fibroblasts and clonal derivatives transformed by sarcoma virus or polyoma virus. The protein (later designated large external transformation sensitive or LETS) was externally located on the cell surface and very susceptible to mild proteolytic removal. A comparison of the normal cell to its transformed counterpart showed that in the latter the protein was absent or markedly reduced (Hynes, 1973). Gahmberg et al. provided evidence that this material was a glycoprotein with molecular weight approximately 200,000 daltons (Gahmberg et al., 1974). Fibronectin was labeled externally by using galactose oxidase and tritiated sodium borohydride. The glycoprotein was present in normal cells and could not be labeled in transformed cells. It seemed to correlate with the growth status of the cell, i.e., rapidly growing, sparse or subconfluent cells showed little fibronectin labeling, whereas it was labeled when cells were grown to high saturation density. Yamada and Weston isolated a 220,000 dalton surface protein by sequential extraction in serum-free medium, and 0.2 M urea (Yamada and Weston, 1974). Cell surface protein (CSP) was found to be a glycoprotein but not affected by collagenase. The protein was trypsin sensitive and its recovery was density dependent. It has been shown that a surface antigen synthesized by cultured chick embryo fibroblasts was also present in normal chick serum (Ruoslahti et al., 1973). Fibroblast surface antigen (SF) is released in significant quantities into the

culture medium of chicken fibroblasts, and both are immunologically indistinguishable. This data provided direct evidence that this cell surface and serum antigen have extensive structural similarity. They conclude that the secretion and/or shedding process may be occurring in vivo. The circulating form of the glycoprotein may be described as "cold insoluble globulin" because of its co-precipitation with fibrinogen in the cold (Morrison et al., 1948). Human plasma SF antigen is identical to what has previously been termed "cold insoluble globulin" (Ruoslahti, 1975).

Fibronectin is a protein looking for a functional role. Transformed cells have rounded morphology when grown in monolayers and display reduced adhesiveness to the substratum. Fibronectin is markedly reduced or absent on the surface of transformed cells. Recent reconstitution experiments have provided evidence that the role of fibronectin may be to promote cell adhesiveness (Ali et al., 1977; Yamada et al., 1976). The addition of fibronectin to transformed cells not only increased adhesiveness, but restored a more fibroblast-like morphology to the cells, both in flattening and spreading. Hynes and co-workers (Ali et al., 1977) noted that actin and tubulin bundles took on a more oriented appearance in the presence of the exogenous fibronectin, suggesting that fibronectin was mediating an effect on the cytoskelton. Addition of fibronectin to aggregates of transformed cells maintained on monolayers appears to promote cell motility (Yamada et al., 1978), possibly by providing a means by which the cells may anchor and polarize themselves.

The work described below primarily involves the partial characterization of cell surface simple and complex glycopeptides derived from the normal human fibroblast, KL-2. These studies relied heavily on the introduction of the use of ion exchange chromatography to fractionate both the simple and complex glycopeptides, permitting a degree of resolution heretofore not attainable by Sephadex G-50 chromatography or high voltage paper electrophoresis alone. Thereafter, isolated glycopeptides were digested with specific exo- and endoglycosidases and the products were analyzed by column and paper chromatography as well as high voltage paper electrophoresis. Evidence will be provided for the structure of the oligomannosyl cores of the simple and complex glycopeptides. Direct comparisons of growing and density inhibited glycopeptides were made in order to correlate alterations in their oligosaccharide moieties with the growth status of the cells. Preliminary experiments, utilizing another normal human fibroblast, WI38, were performed in order to study the uptake characteristics of radioactive mannose. The kinetics of assembly of mannose-containing plasma membrane glycoproteins was investigated by following the fate of exogenous mannose as it was taken up by growing and density inhibited cells.

MATERIALS AND METHODS

Cell labeling and maintenance

Human diploid fibroblasts (KL-2) were cultured in Eagle's minimum essential medium supplemented with 5% calf serum and 5% fetal calf serum and buffered to maintain pH at optimal growth conditions (Muramatsu et al., 1973). Unless otherwise mentioned, cultures were generally plated at a density of 0.7 - 1.0 x 10^5 cells/ml, 3.0 ml per T15 closed culture flask (1.4 - 2.0 x 10^4 cells/cm^2) and fed every 24 hours with medium adjusted to pH 7.6 (one liter of Eagle's MEM is brought to pH 7.6 by the addition of 10 ml (1M) N-tris(Hydroxymethyl)methyl-2-aminoethanesulfonic Acid; 15 ml (1M) N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic Acid (Sigma, St. Louis, Mo., U.S.A.); and 5 ml (1M) N-2-Hydroxethylpiperazine-N'-2-propanesulfonic Acid (Calbiochem, LaJolla, Calif., U.S.A.). Cells were labeled either during exponential growth or in the non-growing state (cf. Fig. 20 inset) with D-(2-^{3}H)mannose (5 µc/ml, specific activity 1 Ci/mmol, Amersham-Searle Radiochemical Centre, Arlington Heights, Ill., U.S.A.) or D-(^{14}C)mannose (0.5 µc/ml, specific activity 240 mCi/mmol, Schwartz-Bioresearch, Inc., Orangeburg, N.Y., U.S.A.). Cell surface material was harvested by washing the cultures 3 times with medium lacking Ca^{+2}, Mg^{+2} and serum and treating each flask with 2 ml of 100 µg/ml Pronase (Calbiochem, LaJolla, Calif., U.S.A.) for 4 minutes at room temperature. Under these conditions cells are seen to round up but do not detach from the glass. The Pronase-released cell surface material is decanted and centrifuged at 1500 rpm for 2 minutes to remove any intact cells. The material remaining in the flasks is designated as cell remainder (cellular glycopeptides). The number of flasks used in each experiment varied, but generally, 50 to 60 T15 flasks were common. Cell surface material was extensively digested with Pronase (120 mg of enzyme in 0.14 M

Tris-HCl-0.014 M $CaCl_2$ buffer, pH 8.4, added over 3 days). Cellular glycopeptides were treated as above with 180 mg of Pronase. Variations in the number of flasks used for any experiment or the concentration of Pronase used in the digestion mixture will be reported in the Results section of each succeeding chapter.

Preparation of glycopeptides and oligosaccharides

Sephadex chromatography

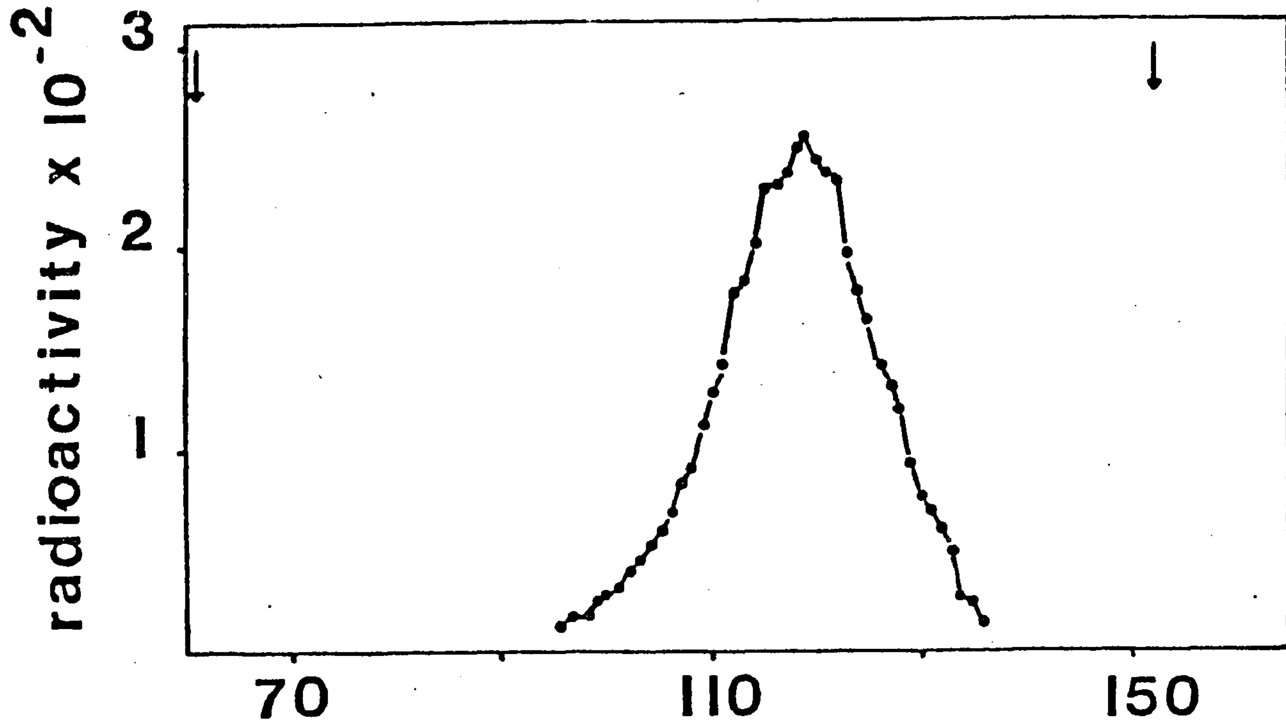
Mannose-labeled material which had been extensively digested with Pronase was immersed in boiling water, lyophilized and redissolved in a minimal amount of water. When excluded and included markers were required, 0.2 mg blue dextran and 2 mg fucose were added to the mixture. The material was applied to a column (0.9 x 110 cm) of Sephadex G-50 Fine (Pharmacia, Piscataway, N.J., U.S.A.) which had previously been equilibrated with 0.05 M ammonium acetate. Fractions of 0.6 ml were collected and an aliquot tested for radioactivity. The glycopeptide region thus detected was pooled for further analysis (Fig. 1).

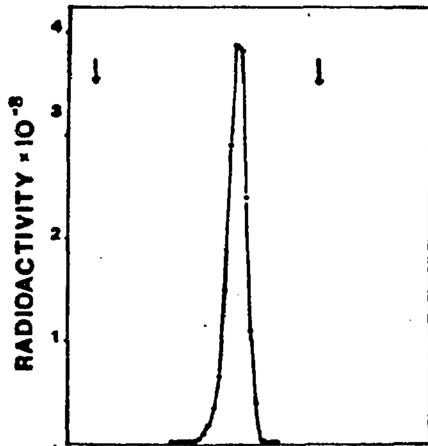
Analytical Sephadex G-50 chromatography was carried out in a similar manner except blue dextran and NaN_3 were used to calculate the excluded and included volumes, respectively, and 0.45 ml fractions were collected. The column was calibrated with markers of known molecular weight, namely (^{14}C)-acetylAsn$(GlcNAc)_2(Man)_5$ (molecular weight 1393), (^{14}C)-acetylAsn$(GlcNAc)_2(Man)_6$ (molecular weight 1555), (^{14}C)-acetylAsn$(GlcNAc)_4(Man)_6$ (molecular weight about 1961), (^{14}C)-acetylfetuin glycopeptide (molecular weight about 3000), raffinose (508) and fucose (164). A typical calibration curve is shown in Figure 1. The distribution coefficient (K_d) was calculated from the expression: K_d=Ve-Vo/Vi-Vo, Ve, Vo and Vi are the elution volumes, blue dextran and NaN_3, respectively. In one case (cf. Chapter I) calf

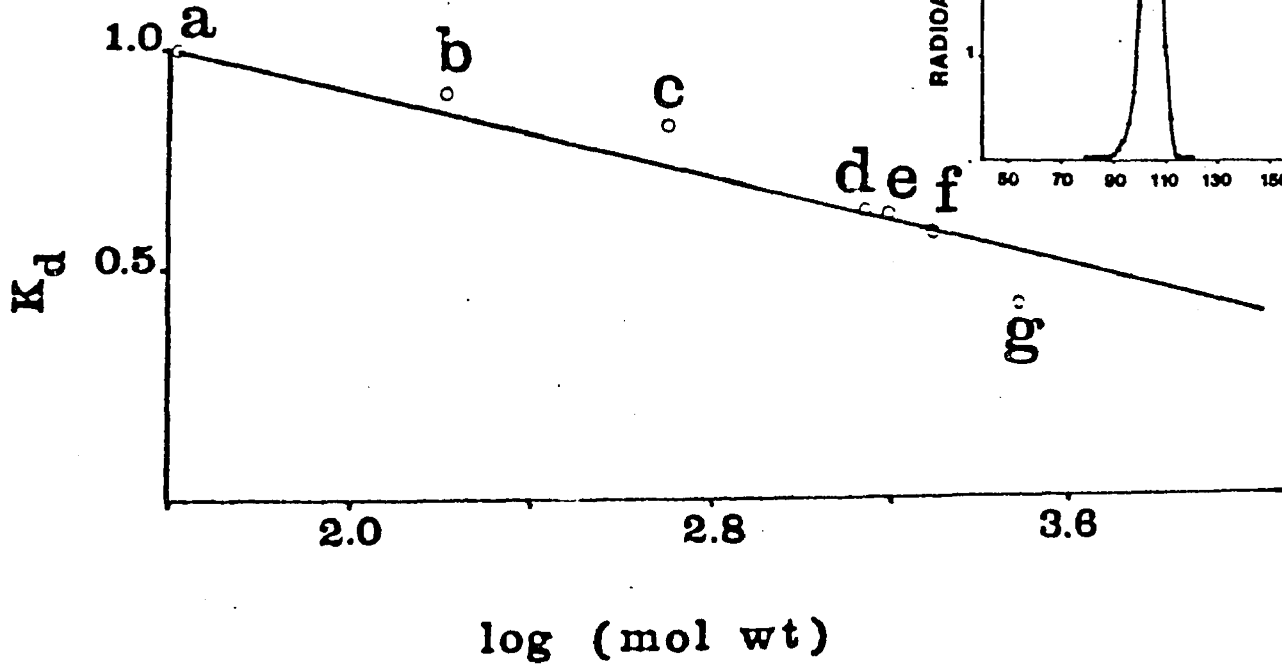
Figure 1. (Top) Typical Sephadex G-50 profile of cell surface glycopeptides. KL-2 cell surface glycopeptides from exponentially growing cells were prepared as described and partially purified by Sephadex G-50 chromatography (Materials and Methods). Blue dextran and fucose eluted at fractions 61 and 152, respectively.

(Bottom) Calibration of the Sephadex G-50 column by standard substances. K_d (distribution coefficient) is determined by Ve-Vo/Vi-Vo, where Vo, Ve and Vi represent the void volume of the column, the volume in which the standard substance elutes and the internal volume. Vo and Vi and determined by the eluted position of blue dextran and sodium azide, respectively. Conditions for the chromatography are described in Materials and Methods. Standard substances: sodium azide (43), fucose (164), (^{14}C)acetylAsn(GlcNAc)$_2$(Man)$_6$ (1555), (^{14}C)acetylAsn (GlcNAc)$_4$(Man)$_6$ (1961) and (^{14}C)acetylfetuin glycopeptide (3000).

<u>Inset</u> Sephadex G-50 profile of standard substance, (^{14}C) acetylAsn(GlcNAc)$_2$(Man)$_6$. Conditions for the chromatography are described in Materials and Methods. Blue dextran and sodium azide elute at fractions 50 and 136, respectively.


radioactivity x 10^{-2}
1
2
3
70
110
150
fraction number


RADIOACTIVITY × 10^{-8}
1
2
3
4
50
70
90
110
130
150


K_d
1.0
0.5
a
b
c
d
e
f
g
2.0
2.8
3.6
log (mol wt)

thyroglobulin Unit A (molecular weight about 2000) and calf thyroglobulin Unit B (molecular weight about 3000) were substituted for (^{14}C)-acetyl Asn(GlcNAc)$_4$(Man)$_6$ and (^{14}C)-acetylfetuin glycopeptide. Flow rates were generally established at 5.4 ml per hour. Molecular weight determinations of unknown samples were performed successively on the same calibrated Sephadex G-50 column. The eluted position of internal standards was assumed to be constant.

A column (0.9 x 145 cm) of Sephadex G-25 (Pharmacia) was similarly prepared with 0.05 M ammonium acetate. Fractions of 0.45 ml were collected at a flow rate of 5 minutes per tube. For the ovalbumin glycopeptide purification (cf. Appendix A), a column (5 x 70) of Sephadex G-25 (Pharmacia) was equilibrated and eluted with either 1 M acetic acid (purification of the first Pronase digest) or 0.1 M acetic acid (second Pronase digest). Fractions of 14 ml were collected at a flow rate of 20 minutes per tube. Hexose-containing fractions were detected by the phenol-H_2SO_4 method (Dubois et al., 1956).

Dowex AG-50W x 2 chromatography

A column (1.5 x 150 cm) of Dowex AG-50W x 2 (Bio-Rad Laboratories, 200-400 mesh, Richmond, Calif., U.S.A.) was converted to the hydroxyl form by alternate washes with 2 N HCl and 2 N NaOH, followed by successive washings with 2 N NaOH. The resin was washed with distilled water to remove excess alkali until neutral and repeatedly washed with sodium acetate buffer, pH 2.6 which was mM in Na^+ ions. This buffer is routinely prepared by the addition of 10 ml of 0.3 M sodium acetate into 2.8 liters of water containing 100 ml of glacial acetic acid. The final volume is brought to 3 liters by the addition of water or glacial acetic acid so that a final pH range between 2.60 and 2.65 is obtained. The strict adherence of this pH range is most important, and the Na^+ ion content must not exceed 3×10^{-3} M

(R. Montogomery, personal communication). The column is equilibrated with this buffer until the pH of the effluent is identical to the buffer being applied. Fractions of 13.5 ml were collected at a flow rate of 20 minutes per tube. Hexose-containing fractions were determined by the phenol-H_2SO_4 method (Dubois et al., 1956). A smaller column (0.9 x 40 cm) was prepared in an identical manner and used for cellular neutral glycopeptide fractionation (cf. Chapter II).

Amberlite MB-1 chromatography

Ion exchange chromatography for obtaining purified oligosaccharide samples (cf. oligosaccharide standards) was performed in a Pasteur pipette (0.5 x 8.5 cm) plugged at the bottom with cotton and filled with dry Amberlite MB-1 (Mallinkrodt, St. Louis, Mo., U.S.A.). The column was equilibrated with distilled water. Hexose-containing samples were passed through the column in 5 ml of water, followed by an additional 50 ml volume, successively applied in 5 ml aliquots.

QAE Sephadex chromatography

QAE Sephadex A25 (Pharmacia, Piscataway, N.J., U.S.A.), 3.0 ± 0.4 meq/gm, was charged with 1 M ammonium acetate brought to pH 8.5 (at room temperature) with ammonium hydroxide and washed repeatedly with 5 mM ammonium acetate, pH 8.5 until conductivity readings were stable. Care should be taken to avoid excessive stirring of the beads as this will affect the flow rate. A column (1.5 x 42 cm) was prepared and thereafter all operations were done at 4°. Glycopeptides partially purified by Sephadex G-50 chromatography were initially eluted with 5 mM ammonium acetate pH 8.5 until only background radioactivity was detected. Five ml samples were collected at a flow rate of 20 minutes per tube. Thereafter, a linear ammonium acetate gradient, pH 8.5, from 5 mM - 300 mM was used to elute the material remaining on the column. At completion

of the gradient, elution by 1 M ammonium acetate recovered no significant radioactivity.

Mannose-containing glycopeptides were routinely checked for the presence of free mannose before application to QAE Sephadex by descending paper chromatography.

<u>High voltage paper electrophoresis (HVPE)</u>

Mannose-containing glycopeptides (or oligosaccharides) were dissolved in a minimal amount of water and applied to 1.5 inch wide strips of Whatman 1 MM paper (Arthur H. Thomas Comp., Philadelphia, Pa., U.S.A.). Unless otherwise stated, aliquots never exceeding 160 µl were applied to the paper, 20 µl at a time, and allowed to dry between each application. Electrophoresis was generally carried out at 3600 volts for 3.5 hours in pyridine:acetic acid:water, 10:0.4:89.6, pH 6.3-6.5. Strips were dried, cut into 1 cm fractions, eluted with 0.5 ml water for 30 minutes and counted aqueously in a liquid scintillation counter (Packard 3375 or Beckman LS8000). Mannose-labeled material was also analyzed at pH 1.9, 7% formic acid, for 2 hours at 3600 volts.

<u>Glycosidases</u>

Endo-β-N-acetylglucosaminidase D, containing β-galactosidase and β-N-acetylglucosaminidase from <u>Diplococcus pneumoniae</u> was kindly provided by Dr. P. H. Atkinson (Albert Einstein College of Medicine, New York). α-Mannosidase was prepared according to the method previously described (Snaith and Levvy, 1968). Endo-β-N-acetylglucosaminidase H was prepared as described previously (Tarentino and Maley, 1974). β-Mannosidase from hen oviduct was the generous gift of Dr. A. Tarentino (New York State Department of Health, Albany). Neuraminidase

from Vibrio cholerae was purchased from Grand Island Biological Co. (Grand Island Biological Co., Grand Island, New York, U.S.A.).

Enzyme activities for β-galactosidase and β-N-acetylglucosaminidase were determined using the appropriate p-nitrophenylpyranoside in 0.075 M phosphate buffer, pH 6.5 or 0.0125 M/0.0375 M citrate/phosphate buffer, pH 5.0, containing 0.15 M NaCl (cf. Appendix B). α-Mannosidase activity was determined by the method of Li (Li, 1967) and found to contain less than 0.15% β-galactosidase and 0.12% β-N-acetylglucosaminidase. Specific activities for endo-β-N-acetylglucosaminidase D and H were determined using (^{14}C)-acetylAsn(GlcNAc)$_2$(Man)$_5$ and (^{14}C)-acetylAsn(GlcNAc)$_2$(Man)$_6$ in 0.075 M phosphate buffer, pH 6.5 and 0.0125 M/0.0375 M citrate/phosphate buffer, pH 5.0 at 37°, respectively. (Muramatsu et al., 1976; Koide and Muramatsu, 1974; cf. Appendix B).

Unless otherwise specified, digestion mixtures with endo-β-N-acetylglucosaminidase D were performed with 0.0185 units of endoglycosidase in the presence of 0.0052 units β-galactosidase, 0.037 units β-N-acetylglucosaminidase and supplemented with 10 units of neuraminidase in a final volume of 200 μl 0.075 M phosphate buffer, pH 6.5 for 15-20 hours. Endo-β-N-acetylglucosaminidase H digestion was carried out with 0.023 enzyme units in a final volume of 200 μl of 0.0125 M/0.0375 M citrate/phosphate buffer, pH 5.0, containing 0.15 M NaCl for 15-20 hours. α- and β-Mannosidase digestion (units varied and will be noted in each experiment) was carried out in 200 μl 0.05 M/0.15M citrate/phosphate buffer, pH 4.0 for 3 days. Neuraminidase digestion mixtures contained 10-15 units in 200 μl of 0.075 M phosphate buffer, pH 6.5 for 15-20 hours. All digestions were carried out under toluene at 37°. An endoglycosidase unit corresponds to 1 μmole of product released per minute from an appropriate substrate.

Oligosaccharide standards

The asparagine-linked glycopeptides of fetuin were isolated according to the method of Spiro (Spiro, 1960). 25.8 mg (by weight) of this material was treated with endo-β-N-acetylglucosaminidase D (0.11 units) in the presence of β-galactosidase (0.031 units), β-N-acetylglucosaminidase (0.22 units) and 15 units of neuraminidase in 1 ml of 0.075 M phosphate buffer, pH 6.5 for 48 hours at 37° under toluene. At completion of the reaction, the digest was desalted by Sephadex G-50 chromatography and the high molecular weight region (oligosaccharide) was collected. This material was reduced in volume and applied to a column of Amberlite MB-1 as described. The recovered oligosaccharide was 6.5 mg (by weight). An aliquot of this material was subjected to descending paper chromatography on Whatman 1 MM paper in ethyl acetate:pyridine:water, 12:5:4 (Solvent I) for 3 days. The oligosaccharide spot was located by staining with alkaline silver nitrate (Trevelyon et al., 1950; cf. descending paper chromatography). The composition of the oligomannosyl core has been shown to be GlcNAc(Man)$_3$ (Spiro, 1973). All subsequent KL-2 glycopeptides subjected to endoglycosidase digestion were analyzed by the same chromatographic conditions. The strips were cut into 1 cm fractions and counted aqueously.

The asparagine-linked glycopeptides of ovalbumin were isolated according to the method previously described (Huang et al., 1970; cf. Appendix A). 10.4 mg of species D (Asn(GlcNAc)$_2$(Man)$_6$) and 11.5 mg of species E (Asn(GlcNAc)$_2$(Man)$_5$) were digested with 0.0458 units of endo-β-N-acetylglucosaminidase H and 0.037 units of endo-β-N-acetylglucosaminidase D, in the presence of 0.0104 units β-galactosidase, 0.074 units β-N-acetylglucosaminidase and 5 units of neuraminidase, respectively.

The final volume of each digestion mixture was 1 ml in the appropriate buffer as described above (cf. glycosidases). Digestion mixtures were incubated at 37° for 42 hours under toluene. As control, 5 µl of (^{14}C)-acetylAsn$(GlcNAc)_2(Man)_6$ (about 36,300 cpm) and (^{14}C)-acetylAsn$(GlcNAc)_2$ $(Man)_5$ (about 40,000 cpm) was added to the appropriate digestion mixture. Prior to ion exchange chromatography with Amberlite MB-1, an aliquot of each was tested by HVPE at pH 6.5 along with the intact acetylated standards. In both cases, the control substrate was hydrolyzed completely, releasing the radiolabeled product, (^{14}C)-acetylAsnGlcNAc and an oligosaccharide. Therefore, under these conditions it may be assumed that 100% of the unlabeled glycopeptides were hydrolyzed to release the oligosaccharide product. The digests were applied directly to Amberlite MB-1 as described above. The recovery of species D oligosaccharide was 4 mg and species E was 8 mg. An aliquot of each was analyzed by descending paper chromatography as described. The purported disaccharide Man$\overset{\beta}{\text{—}}$GlcNAc was prepared from Asn$(GlcNAc)_2(Man)_6$ and provided on various occasions by Drs. Atkinson or Ceccarini. Fetuin and all monosaccharide standards were purchased from Sigma (Sigma, St. Louis, Mo., U.S.A.).

Characterization of sugars in labeled glycopeptides

Hydrolysis of any glycopeptide sample under conditions to hydrolyze hexoses (1 N HCl for 4 hours at 100° in sealed vials) released radioactivity comigrating with authentic mannose. Less than 5% of the radioactivity was found to comigrate with authentic glucosamine. Under conditions to hydrolyze fucose (0.1 N HCl at 80° for 45 minutes in sealed vials) less than 5% of the radioactivity was found to migrate with authentic fucose. Descending paper chromatography using Whatman 3 MM paper was carried out with butanol:pyridine:water, 6:4:3 (Solvent II), and developed for 15 hours.

Selective removal of terminal sialic acid residues was carried out with 0.1 N H_2SO_4 at 80° for 60 minutes and no radioactivity was detected in the sialic acid region.

Descending paper chromatography

Samples are prepared for paper chromatography by successive application of 5 µl aliquots allowed to dry between each application. Generally, sample volumes applied never exceeded 100 µl. For radioactivity detection, the paper was allowed to dry overnight and cut into 1 cm fractions, eluted with 0.5 ml water for 30 minutes and counted aqueously. Oligosaccharide markers (Solvent I) and monosaccharides (Solvent II) were detected by staining with alkaline silver nitrate (Trevelyon et al., 1950). A solution of saturated silver nitrate (1 ml) is added to acetone (200 ml) and the paper dipped, patted dry, and allowed to air-dry. A solution of ethanolic NaOH (ethanol:10 N NaOH:water, 16:1:3) is sprayed onto the chromatogram. Sugars are detected by the appearance of deep-brown to black spots. The reaction may be terminated by rinsing the paper in a solution of Kodak rapid fixer (Eastman Kodak Comp., Rochester, New York, U.S.A.) or 6 M NH_4OH followed by rinsing in distilled water.

Incorporation experiments

Cell maintenance

Human diploid fibroblasts, WI38, were cultured in Eagle's minimum essential medium, supplemented with 5% calf serum and 5% fetal calf serum. Cells were plated at a density of 0.7-1.0 x 10^5 cells/ml, 3.0 ml per closed T15 culture flask. At the end of the initial 24 hour period, cells were fed daily with medium adjusted to pH 7.6 to optimize growth conditions (Muramatsu et al., 1976). Under these

conditions, WI38 will grow exponentially for at least 5 days and are density inhibited by 12-14 days. In a second set of experiments (mannose incorporation into neutral and acidic glycopeptides) cells were cultured in 150 cm^2 closed flasks, maintained on the same feeding schedule as above. Cells were plated at a density of $1.4\text{-}1.8 \times 10^6$ cells in 50 ml of medium.

Radioactive labelling and assay

Growth studies: Cells were labeled with D-(2-^{3}H)mannose (2 µc/ml, specific activity 1 Ci/mmol, Amersham-Searle Radiochemical Centre, Arlington Heights, Ill., U.S.A.) or L-(6-^{3}H)fucose (2 µc/ml, specific activity 13.4 Ci/mmol, New England Nuclear, Boston, Mass., U.S.A.) for 3 hours on selected days throughout a 14 day growth period. Incorporation was stopped by removal of the radioactive medium and rapidly washing the cells three times by filling the T15 culture flask with ice-cold Earle's solution (Earle, 1943). The cultures were drained by aspiration and extracted with 8% trichloroacetic acid (TCA) at 4° for 15-30 minutes. The extract was decanted and saved (TCA soluble) and the culture flasks washed again, three times with ice-cold Eagle's solution and drained overnight at 4°. The cells were dissolved in 5 ml of 0.1 M NaOH, 0.2 M Na_2CO_3 (TCA insoluble) (Ceccarini and Eagle, 1971). Aliquots of the TCA soluble and insoluble material were counted aqueously after neutralization, in a liquid scintillation counter (Packard 3375) and corrected for quenching. An aliquot of the insoluble material was used to determine protein concentration (Oyama and Eagle, 1956).

Accumulation studies

Cells were labeled on day 5 (exponentially growing) and day 14

(density inhibited) with D-(2-^{3}H)mannose (5 µc/ml, specific activity 1.0-1.5 Ci/mmol). Cultures were labeled continuously and replicate T15 flasks were removed at preselected time points. A total of 4 T15 flasks were needed for each time point. Two of the flasks were used to determine the soluble pool (cell soluble) and the total insoluble material (cell total insoluble). Incorporation was stopped by removal of the labeled medium and rapid washing of the cells with Earle's solution as described above. Cultures were twice-extracted with 2.5 ml 60% ethanol on a heating plate for 10 minutes at 60°. The extract was decanted, brought to a final volume of 5.0 ml (from loss due to heating), centrifuged at 2500 rpm to remove intact cells and saved. The insoluble material was washed three times with ice-cold Earle's solution and drained overnight at 4°. Cells were dissolved in 5 ml of 0.1 M NaOH, 0.2 M Na_2CO_3 as above, and an aliquot used to determine protein concentration. The remaining two flasks were used to determine the accumulation of labeled material on the cell surface (surface glycoprotein) and the residual material (cell remainder). Incorporation was stopped as above and the flasks drained by aspiration. Cell surface material was harvested by the addition of 2 ml of 100 µg/ml Pronase for 7-8 minutes at room temperature. The Pronase-released cell surface material is decanted and centrifuged at 2500 rpm to remove loose cells. This material was heated at 80° for 2 minutes to inactivate the Pronase and extensively dialyzed against water to remove any free mannose. The cells remaining on the glass were shaken off in 2.0 ml of Earle's solution, to which 2.0 ml of 20% TCA was added and extracted 15-30 minutes at 4°. The cells were centrifuged at 2500 rpm and the supernatant discarded. To the pellet was added two successive portions of 2.5 ml 60% ethanol and treated as

above (cell soluble). The cells were centrifuged and the ethanol discarded. Any residual ethanol was removed by nitrogen-flushing. The pellet was dissolved in 5 ml of 0.1 M NaOH, 0.2 M Na_2CO_3 as before and an aliquot used to determine protein concentration. Aliquots of the cell soluble material, cell total insoluble, cell surface material and cell remainder were counted aqueously in a liquid scintillation counter (Packard 3375). The samples were corrected for quenching by the use of appropriate standards. Generally, this involved the use of a (^{3}H)-toluene standard counted aqueously under the identical conditions as each of the various fractions collected. Counting efficiencies were determined and the radioactivity was converted to disintegrations per minute.

CHAPTER I - FRACTIONATION OF MANNOSE-LABELED SIMPLE AND COMPLEX GLYCOPEPTIDES BY QAE SEPHADEX CHROMATOGRAPHY

Cellular glycoproteins constitute a very heterogeneous population of molecules (Nicolson, 1976; Beeley, 1971). Extensive proteolytic digestion has been used to reduce this material to glycopeptides with relatively few amino acids (Spiro, 1965). Among the techniques used to isolate and characterize glycopeptides have been gel filtration (Buck *et al.*, 1970; Meezan *et al.*, 1969), high voltage paper electrophoresis (Ceccarini *et al.*, 1975; Atkinson *et al.*, 1976), affinity chromatography (Ogata *et al.*, 1975), and ion exchange chromatography. Anionic exchangers have been used to separate net negatively charged molecules containing sialic acid (Kornfeld and Kornfeld, 1970; Brown, 1972; Onodera and Sheinin, 1970) or, possibly, charged amino acids. Cation exchangers have been used to separate neutral mannose-rich glycopeptides (Huang *et al.*, 1970; Javaid *et al.*, 1975). The work to be described concerns an alternative method of fractionating both acidic and neutral species of glycopeptides with one column whereas, otherwise, a combination of the abovementioned techniques would be required. The technique permits the large-scale separation of glycopeptides with essentially 100 percent recovery.

RESULTS

Human diploid fibroblasts (KL-2) were cultured in Eagle's minimum essential media supplemented with 5% calf serum and 5% fetal calf serum, and buffered to maintain pH at optimal growth conditions (Muramatsu *et al.*, 1973; cf. Materials and Methods). Cells were labeled for 20-24 hours either during exponential growth or in the non-growing state with

D-(2-^{3}H)mannose(5 μc/ml, specific activity 1 Ci/mmol, Amersham-Searle Radiochemical Centre, Arlington Heights, Ill., U.S.A.) or D-(^{14}C)mannose(0.5 μc/ml, specific activity 240 mCi/mmol, Schwartz-Bioresearch, Inc., Orangeburg, N.Y., U.S.A.). Cell surface material was removed by mild Pronase digestion, and the remaining material was designated as cellular glycopeptides. The cell material was extensively digested with Pronase and partially purified by Sephadex G-50 chromatography as described (Materials and Methods).

Mannose-containing cellular glycopeptides partially purified by Sephadex G-50 chromatography contain a mixture of acidic species (whose carbohydrate portion terminates with sialic acid) and neutral species (terminating with neutral sugars) (Ceccarini _et al._, 1975). As shown below, when a mixture of these glycopeptides is placed on QAE Sephadex, neutral species have a weak affinity, since they rapidly elute at low salt concentration, while acidic species (carrying a net negative charge) are retained but elute with a linear gradient of higher salt concentrations.

Glycopeptides collected by Sephadex G-50 chromatography were lyophilized, redissolved in a minimal amount of water, and applied to a column of QAE Sephadex. The mannose-labeled glycopeptides were initially eluted from the QAE Sephadex with 5 mM ammonium acetate, pH 8.5. As shown in Figure 2a, they separated into at least five distinct peaks. The molecular weight of each peak was estimated by Sepadex G-50 chromatography based on their mobilities in relation to known standards. The standards used were two (^{14}C) acetylated ovalbumin glycopeptides of known structure (Tai _et al._, 1975a), namely (^{14}C)-acetylAsn(GlcNAc)$_2$(Man)$_5$ (molecular weight 1393) and (^{14}C)-acetylAsn(GlcNAc)$_2$(Man)$_6$ (molecular weight 1555); and calf thyroglobulin unit A (molecular weight about 2000).

Each sample eluted within the glycopeptide region and no radioactivity was recovered in the region of free mannose. The apparent molecular weight for the components ranged from approximately 1050 to 2000 daltons; they were neutral (see below). In general, neutral glycopeptides contain $(Man)_n(GlcNAc)_2$, or terminate in other neutral sugars, linked to asparagine (Tarentino et al., 1970; Arima and Spiro, 1972). The difference in molecular weight between each peak reported here (cf. Fig. 2a) can be accounted for by one or two monosaccharide residues. The results are consistent with the "oligomannosyl cores" ranging from 3-7 or 8 mannose residues, as has been previously proposed (Muramatsu et al., 1976) to exist in neutral glycopeptides obtained from diploid fibroblasts. However, studies on peak I (Fig. 2a) suggest that it may contain both glycopeptide and oligosaccharide material (cf. Chapter II).

When a linear gradient of 5-300 mM ammonium acetate was applied to the QAE column, a heterogeneous mixture of acidic mannose-labeled material was obtained between 30 mM and 150 mM salt (Fig. 2b). With the present material it was not possible to detect distinct species in the eluted material. However, a similar analysis of cell surface material from exponentially growing and non-growing cells, gave three distinct major peaks and several minor ones (cf. Chapter III).

Glycopeptides partially purified by Sephadex G-50 chromatography can also be separated into acidic and neutral populations by high voltage paper electrophoresis (HVPE) (Fig. 3a). Peaks I-V, Figure 2a, were also analyzed by HVPE. All five migrated as neutral glycopeptides (Fig. 3b-f). Of particular interest is the fact that the five species, while differing significantly in molecular weight, migrated only 3-4 fractions apart, and thus would be virtually indistinguishable from each other by HVPE. It was noted that the cellular glycopeptide

Figure 2. QAE Sephadex fractionation of cell material. Exponentially growing cells were labeled with D-(2-^{3}H)mannose (5 µc/ml, 1 Ci/mmol) and non-growing cells with D-(^{14}C)mannose (0.5 µc/ml, 240 mCi/mmol) as described in text. The mannose-containing glycopeptides eluted and pooled from Sephadex G-50 columns were lyophilized and further chromatographed by QAE Sephadex. a) The sample (1.1 x 10^{6} ^{3}H cpm and 3.0 x 10^{5} ^{14}C cpm) was applied to a column (1.5 x 42 cm) and eluted with 5mM ammonium acetate, pH 8.5. Five ml fractions were collected at a flow rate of 20 minutes per tube. Elution was continued until only background radioactivity was detected. b) Glycopeptides eluted with a linear gradient, 5 mM to 300 mM ammonium acetate, pH 8.5. 1500 ml of 5 mM NH_4Ac in the mixing chamber and 1500 ml of 300 mM NH_4Ac in the reservoir. No significant radioactivity was recovered by subsequent elution with 1 M NH_4Ac. For simplicity, only the growing material is plotted in 2b, but is similar for the non-growing.

a

I II III IV V

CPM ^{3}H •—•

1200 600 200

CPM ^{14}C o—o

200 120 40

60 120 180

b

500 200

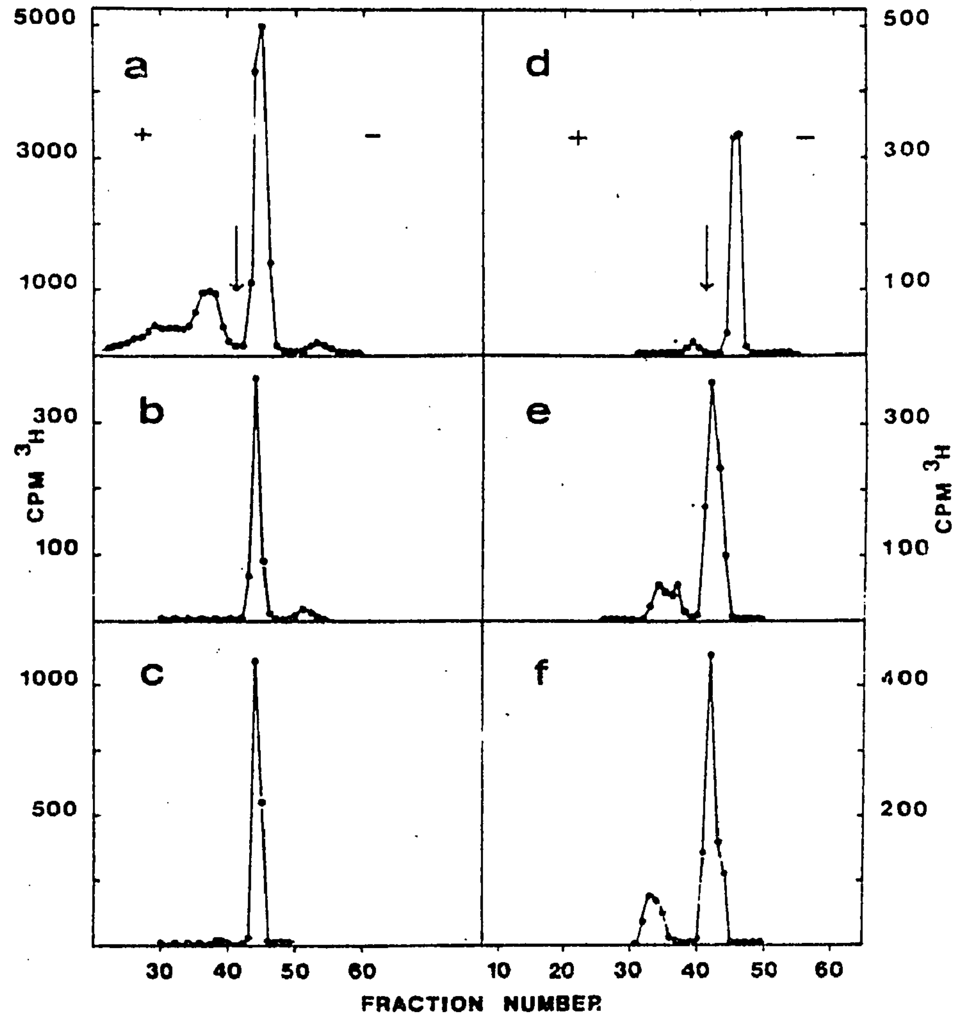
.150 .120 .075 .030

MOLARITY (NH_4Ac)

0 80 160 240 320

FRACTION NUMBER

Figure 3. Electrophoretic analysis of neutral glycopeptides. Mannose-labeled glycopeptides were prepared as outlined in the legend of figure 2 and further characterized by high voltage paper electrophoresis at pH 6.5. a) Cell material was partially purified by Sephadex G-50 chromatography and analyzed by HVPE, at 4000 volts for 3.5 hours. Acidic glycopeptides migrate to the positive pole (fractions 20-40) and neutral glycopeptides move slightly to the negative pole (fractions 41-46). Neutrality is defined by the characteristic migration of neutral sugars under identical conditions. The arrow indicates the origin (fraction 41). b) Species I eluted with 5 mM ammonium acetate; c) Species II; d) Species III; e) Species IV and f) Species V. Apparent acidic contamination for each peak was as follows (measured as percent acidic cpm of total radioactivity recovered from paper): Species I - 0%; Species II - 0%; Species III - 5%; Species IV - 22%; and Species V - 33%. For simplicity, only the (^{3}H)-labeled glycopeptides from growing cellular material are shown. The (^{14}C)-labeled non-growing cellular material gave similar results. The paper strip was cut into 1 cm fractions and counted for radioactivity. Fraction 41 represents $+\frac{1}{2}$ cm and $-\frac{1}{2}$cm.


a
b
c
d
e
f
+
−
CPM ^{3}H
FRACTION NUMBER
5000
3000
1000
300
100
500
400
200
10
20
30
40
50
60

profile (Fig. 3a) contains an apparent basic mannose-labeled species, comprising about 3-4% of the total material. Only peak I (Fig. 3b) was found to contain this component. With respect to the order of elution from the column, the neutral peaks appear to become progressively more contaminated with acidic material, ranging from no contamination in peak I to 33% contamination in peak V.

The heterogeneous peak of mannose-labeled glycopeptides eluted from the column between 30 mM and 90 mM salt concentration was collected (fractions 80-116, 120-140, 150-160 and 170-190) and an aliquot of each fraction was analyzed by HVPE; 83-98% of the radioactivity migrated as acidic glycopeptides. In general it was noted that each of the four fractions contained decreasing amounts of neutral material ranging from 17% in fractions 80-116 to 2% in fractions 170-190. The apparent cross contamination of neutral and acidic glycopeptides observed is not likely to be due to overlapping peaks on the QAE column since elution of the neutral glycopeptides is routinely continued to fraction 240 (only background radioactivity is recovered beyond fraction 180) before the linear gradient is applied.

A comparison of the neutral glycopeptides derived from non-growing and growing cellular material suggested growth dependent alterations (Fig. 2a). The neutral glycopeptides derived from non-growing cellular material were markedly reduced in the high molecular weight species (IV and V) relative to those derived from growing cellular material. A similar result has previously been reported (Muramatsu et al., 1976). They used two specific endo-β-N-acetylglucosaminidases (Tarentino and Maley, 1974; Koide and Muramatsu, 1974) to convert the mixture of cellular neutral glycopeptides isolated by HVPE into an array of oligosaccharides, and paper chromatography to identify different neutral

species of glycopeptides. HVPE separates cellular glycopeptides into two heterogeneous classes based on charge differences (Ceccarini *et al.*, 1975; cf. Fig. 3a). It should be stressed that the present technique, however, permits the separation of cellular glycopeptides into acidic material and at least five distinct neutral peaks whose presence could not be readily predicted without prior glycosidase digestion. Thus the technique has the advantage of making direct comparisons of intact glycopeptides derived from two different cell populations. Thereafter, fractions of interest may be further studied using specific enzymatic probes and the digested products analyzed by paper chromatography.

CHAPTER II - STUDIES ON THE SIMPLE GLYCOPEPTIDES

Previous studies on the cellular and surface glycopeptides of the normal human fibroblast cells (KL-2) have demonstrated growth dependent alterations of the neutral mannose-containing glycopeptides. Specific endoglycosidases were used to show that cellular neutral glycopeptides from growing cells were enriched in higher molecular weight oligomannosyl cores relative to the non-growing cells (Muramatsu _et al_., 1976). A similar result was found with the surface neutral glycopeptides in as much as the growing surface material was more resistant to endo-β-N-acetylglucosaminidase D but more susceptible to α-mannosidase (Ceccarini _et al_., 1975). The endoglycosidase D resistant material was later shown to be higher molecular weight oligomannosyl cores, susceptible to hydrolysis by endo-β-N-acetylglucosaminidase H (Muramatsu _et al_., unpublished observation). The present studies deal with growth dependent alterations of surface neutral glycopeptides revealed through the use of ion exchange chromatography. The data to be presented supports and further extends the previous finding that the cell surface neutral glycopeptides are rich in endo-β-N-acetylglucosaminidase H susceptible species.

RESULTS

Normal human fibroblast cells (KL-2) were labeled with (^{3}H)mannose either during early log phase (day 3) or in a confluent, non-growing state (day 16). The cell surface glycopeptides were harvested by mild proteolytic digestion, extensively digested with Pronase and partially purified by Sephadex G-50 chromatography (Materials and Methods). This material was lyophilized, redissolved in a minimal amount of water, and applied to a column of QAE Sephadex.

<u>QAE Sephadex chromatography</u>

(^{3}H)mannose labeled glycopeptides derived from the surface of growing and non-growing cells were applied to columns of QAE Sephadex, previously equilibrated and eluted with 0.005 M ammonium acetate, pH 8.5. Under these conditions, approximately 40% of the radioactivity can be recovered as neutral material, in either state of growth (Table I). The remaining 60% of the label is eluted when a linear gradient of 5 mM - 300 mM ammonium acetate is applied to the column. This material has been shown to contain complex glycopeptides (cf. Chapter III). The QAE Sephadex profiles of the neutral glycopeptides from both states of growth are shown in Figure 4. Panel A represents the growing cell surface and appears to be similar to the cellular neutral glycopeptides (cf. Chapter I, Fig. 2a) i.e., peak I is the predominant surface neutral species, comprising 51% of the neutral material (Fig. 4a, Table 2). The distribution of the remaining surface neutral species was similar to that found with the cellular neutral glycopeptides (Table 2). However, growth dependent alterations were evident on the surface of the non-growing cells (Fig. 4b). Peak I is significantly reduced, while peak II has become greatly enriched. This contrasts sharply to the distribution of neutral species found within the non-growing cell (Fig. 2a, Table 2). In other words, the relative proportion of neutral species found within the non-growing cell is not reflected on the non-growing surface.

In order to elucidate the structure of the QAE Sephadex fractionated neutral species, each isolated peak was further examined. The approach taken was to use a combination of five distinct methods to detect differences in the glycopeptides. They are 1) further purification of the neutral species by Dowex AG-50W x 2 chromatography, 2) susceptibility to

Table 1

Percentage of simple and complex glycopeptides isolated by QAE Sephadex chromatography[a]

	Cellular glycopeptides	Surface glycopeptides
growing neutral	62.7	42.9
non-growing neutral	66.6	39.8
growing acidic	37.3	57.1
non-growing acidic	33.4	60.2

[a]Cellular and surface glycopeptides were prepared as described in Materials and Methods. The glycopeptide mixtures were fractionated by QAE Sephadex chromatography as described in the legends of figures 2, 4 and 16. Percentages represent the total radioactivity recovered as simple or complex glycopeptides.

Figure 4. QAE Sephadex fractionation of cell surface neutral glycopeptides. Exponentially growing and non-growing cells were labeled with D-(2-^{3}H)mannose (5 µc/ml, 1 Ci/mmol) and the cell surface glycopeptides harvested as described in Materials and Methods. The mannose-containing glycopeptides partially purified by Sephadex G-50 chromatography were applied to columns (1.5 x 42 cm) of QAE Sephadex. Simple glycopeptides elute with 5 mM ammonium acetate, pH 8.5. Five ml fractions were collected at a flow rate of 15 ml per hour. Approximately 5.7×10^5 cpm growing material and 7.5×10^5 cpm non-growing material were applied. a) Growing surface glycopeptides; b) Non-growing surface glycopeptides.

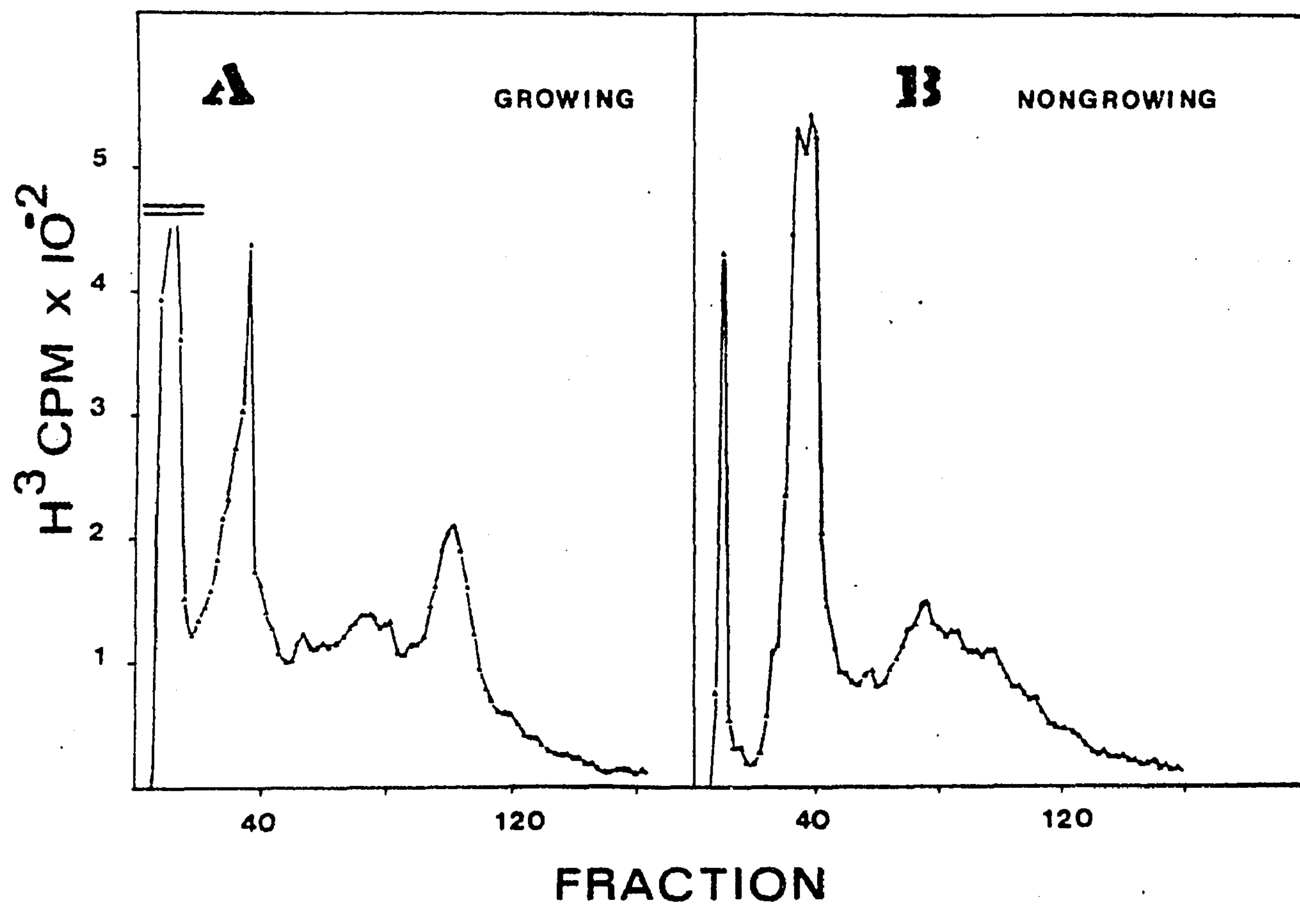

A
GROWING
B
NONGROWING
H^3 CPM x 10^{-2}
5
4
3
2
1
40
120
40
120
FRACTION

Table 2

Percentage of cellular and surface simple glycopeptide species isolated by QAE Sephadex chromatography[a]

	Cellular glycopeptides		Surface glycopeptides	
	species	percentage	species	percentage
growing	I	39.45	I	50.94
	II	16.05	II	19.30
	III	11.14	III	5.28
	IV	21.71	IV	9.35
	V	11.65	V	15.13
non-growing	I	65.62	I	8.48
	II	8.39	II	46.64
	III	11.00	III	6.64
	IV	9.65	IV	18.45
	V	5.34	V	19.85

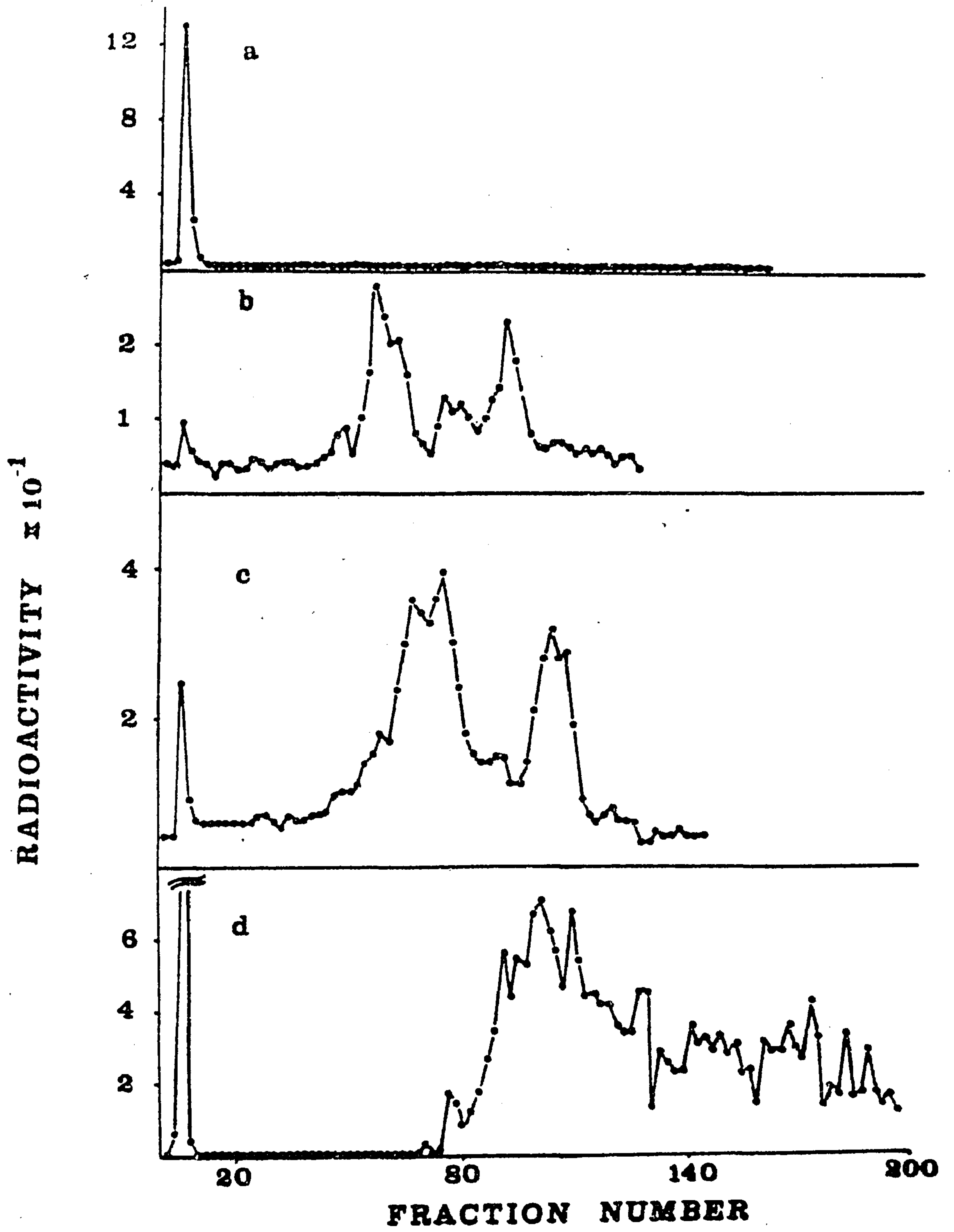
[a]Cellular and surface glycopeptides were prepared as described in Materials and Methods. The glycopeptide mixtures were fractionated by QAE Sephadex chromatography as described in the legends of figures 2 and 4, respectively. Percentages represent that radioactivity recovered as each simple species.

endo-β-N-acetylglucosaminidase D and H, which leads to information about the size of the "oligomannosyl core," 3) Sephadex G-25 chromatography, which aids in identification of released oligosaccharides and endoglycosidase resistant glycopeptides, 4) paper chromatography, which leads to information about the composition of the released "oligomannosyl core," and 5) susceptibility to α-mannosidase, which helps to quantitate the relative amount of exposed, terminal α-mannosyl residues on the glycopeptides.

<u>Studies on the growing cellular neutral species purified by QAE Sephadex chromatography: Dowex AG-50W x 2 fractionation</u>

Cells were previously labeled with (^{3}H)-mannose (rapidly growing) or (^{14}C)-mannose (non-growing) and the cellular glycopeptides extensively digested with Pronase, mixed and partially purified by Sephadex G-50 chromatography. The mixture of growing and non-growing glycopeptides was separated into neutral and acidic species by QAE Sephadex chromatography (Materials and Methods, Chapter I). A column (0.9 x 40 cm) of Dowex AG-50W x 2 was prepared as described in Materials and Methods and equilibrated and eluted with sodium acetate buffer, pH 2.6. Fractions of 3 ml were collected at a flow rate of 9 ml per hour. Aliquots of each of the fractions were counted for radioactivity. Generally, the (^{14}C)-mannose cpm were too low to be judged significant. Therefore the data in this section will be presented solely for the growing glycopeptides labeled with (^{3}H) mannose. As shown in figure 5, species I-IV, derived from QAE Sephadex (Fig. 2a), could be further fractionated by Dowex AG-50W x 2 chromatography. (The recovered radioactivity for species V was very low and so is not presented.) Of particular interest was the correlation between the order of elution of the neutral species from QAE Sephadex (Fig. 2a) and a similar order of elution from Dowex AG-50W x 2 (Fig. 5). That is, the earlier a neutral species eluted from the QAE column, the

Figure 5. Dowex AG-50W x 2 fractionated cellular neutral glycopeptides. The preparation and isolation of these neutral glycopeptides were done as described in the legend of figure 2. The QAE Sephadex-derived neutral glycopeptides were applied to columns (0.9 x 40 cm) of Dowex AG-50W x 2 and eluted with sodium acetate buffer, pH 2.6 (Materials and Methods). Fractions of 3.0 ml were collected at a flow rate of 20 minutes per tube. a) Species I; b) Species II; c) Species III; d) Species IV. Only the ^3H-labeled growing cellular glycopeptides are shown.



a
b
c
d
12
8
4
2
1
4
2
6
4
2
RADIOACTIVITY x 10^-1
20
80
140
200
FRACTION NUMBER

earlier it also eluted from the Dowex AG-50W x 2 column.

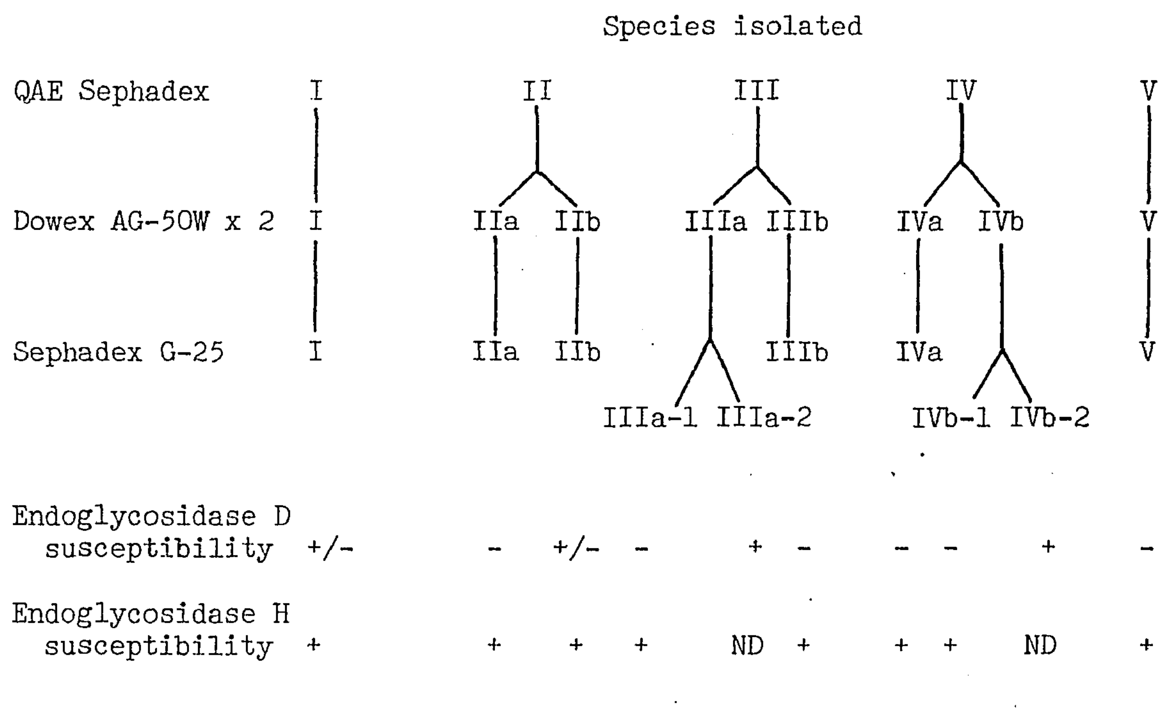
On the Dowex resin, peak I eluted as a single, sharp component between fractions 4-10 (Fig. 5a). Peaks II and III could be further separated into at least two distinct components: peak IIa (fractions 50-68), peak IIb (fractions 82-110), peak IIIa (fractions 50-90) and peak IIIb (fractions 96-110) (Fig. 5b,c). Peak IV was found to be extremely heterogeneous and was pooled as peak IVa (fractions 76-130) and peak IVb (fractions 131-200) (Fig. 5d). At the completion of each fractionation, a further elution with 0.05 M sodium acetate, pH 6.0, could recover no significant radioactivity. Peak V was pooled from fraction 110-220. A flow chart of the components isolated is presented in Table 3. Peaks II-IV appeared to be contaminated (to varying extents) with a peak I-type component. This material was not further examined. The finding that each of the QAE Sephadex-derived species elutes at a very distinct position on the Dowex AG-50W x 2 column strongly argues that each peak is different in structure. Moreover, the data is consistent with the possibility that the QAE Sephadex-derived peaks are heterogeneous and contain more than one glycopeptide component.

Sephadex G-25 chromatography

An aliquot of each of the neutral components isolated by Dowex AG-50W x 2 chromatography was digested with endo-β-N-acetylglucosaminidase D (0.037 units) in the presence of β-galactosidase (0.0104 units), β-N-acetylglucosaminidase (0.074 units) and 5 units of neuraminidase in a final volume of 1.7 ml of 0.075 M phosphate buffer, pH 6.5 for 24 hours at 37° under toluene. The reaction was terminated by freezing and stored in this manner until ready to be used. A column of Sephadex G-25 (0.9 x 145 cm) was prepared as described in Materials and Methods. As shown in Figure 6 the enzyme-treated material could be separated into components

Table 3

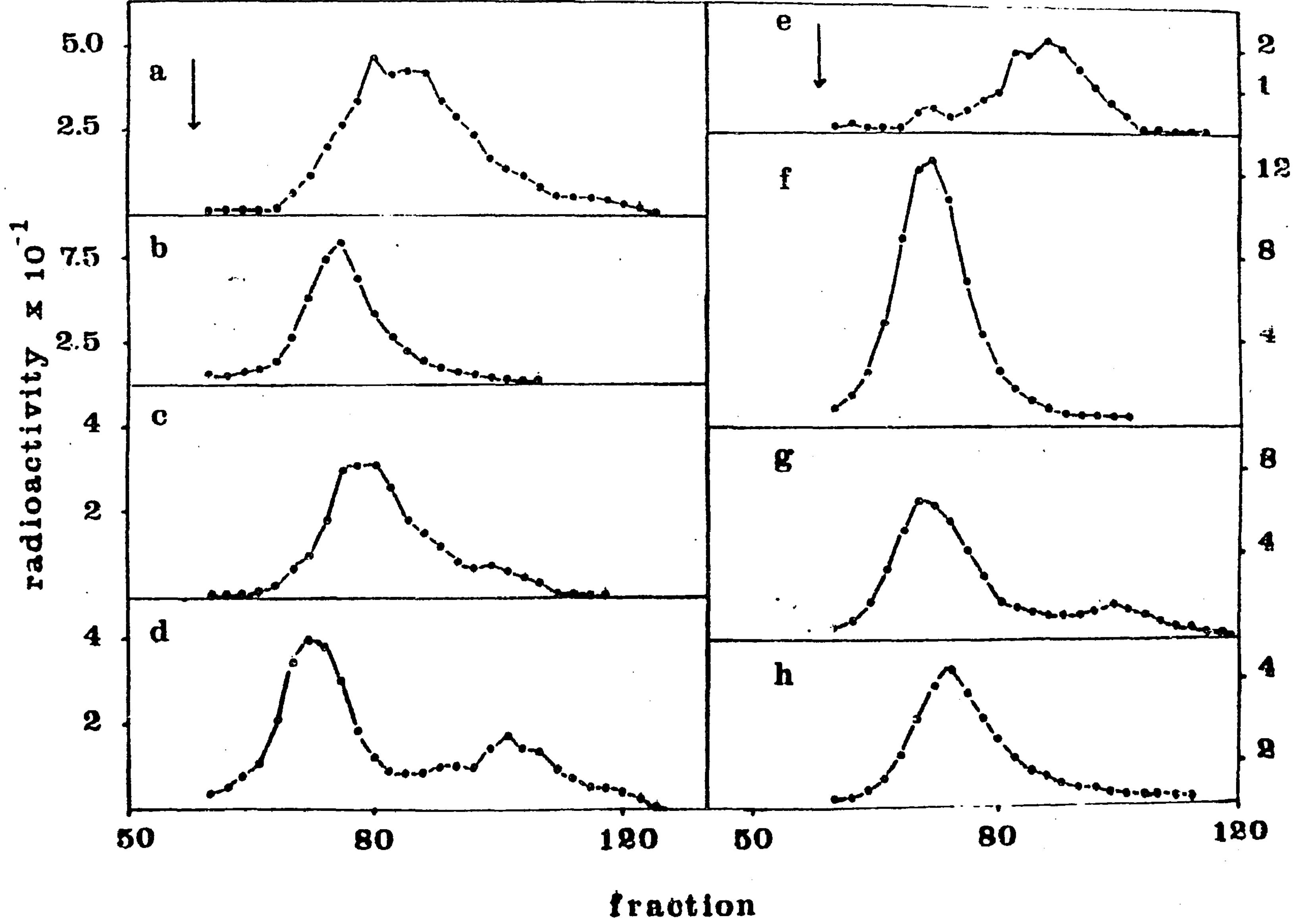
Flow chart of purification scheme for cellular neutral glycopeptides



+ susceptible, - resistant, +/- partially susceptible

ND not done

Figure 6. Endo-β-N-acetylglucosaminidase D-digested cellular neutral glycopeptides. Neutral mannose-containing glycopeptides were prepared as described in the legend of figure 5. They were digested with endo-β-N-acetylglucosaminidase D (0.037 units) in the presence of β-galactosidase (0.0104 units), β-N-acetylglucosaminidase (0.074 units) and neuraminidase (5 units) and analyzed by Sephadex G-25 chromatography (Materials and Methods). Fractions of 0.6 ml were collected and aliquots counted for radioactivity. a) Species I; b) Species IIa; c) Species IIb; d) Species IIIa; e) Species IIIb; f) Species IVa; g) Species IVb; h) Species V. Blue dextran eluted at fraction 58. Only the ^{3}H-labeled growing cellular glycopeptides are shown.

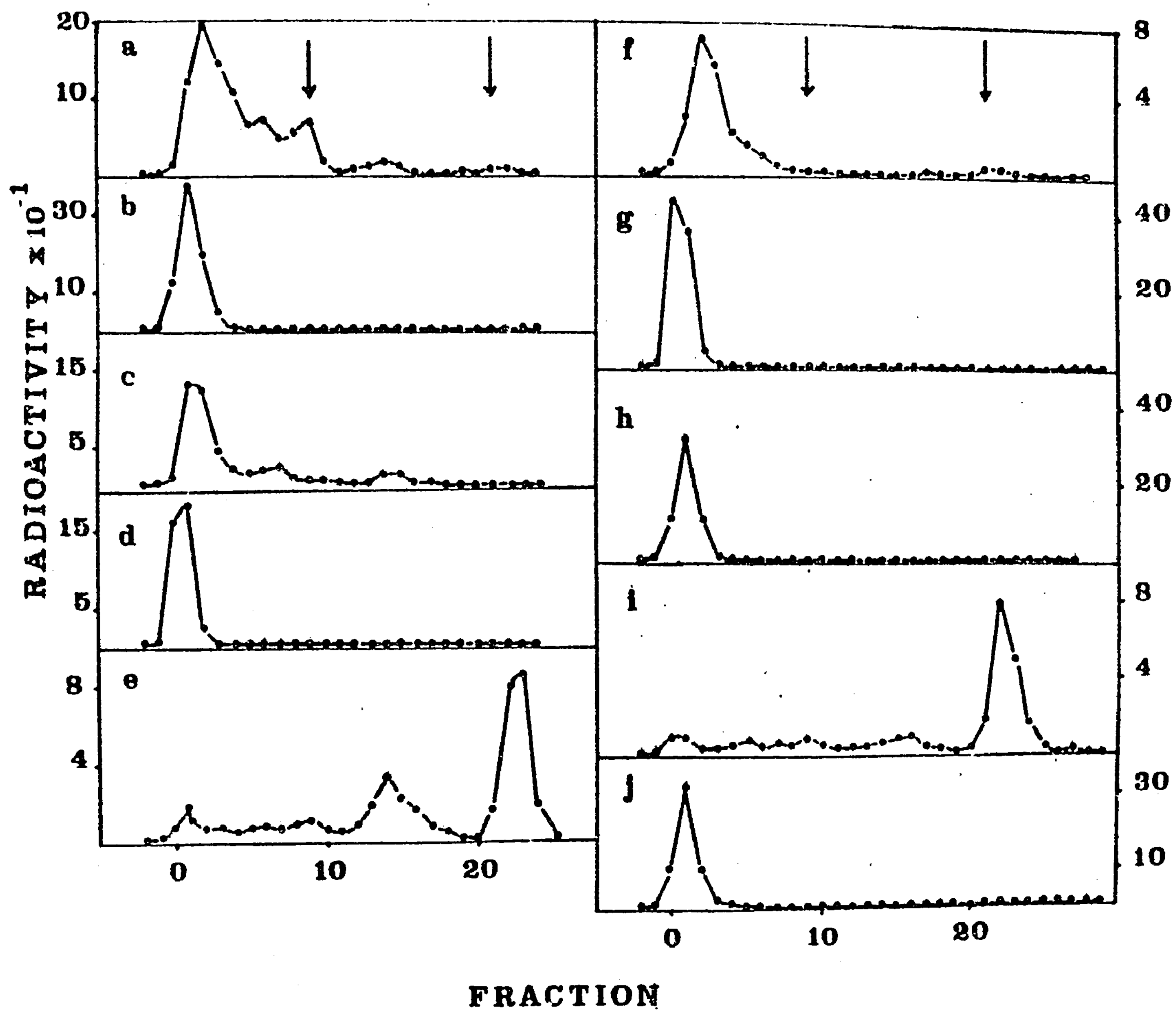

radioactivity x 10^-1
fraction
a
b
c
d
e
f
g
h
5.0
2.5
7.5
2.5
4
2
4
2
2
1
12
8
4
8
4
4
2
50
80
120
50
80
120

of high molecular weight and/or lower molecular weight. The high molecular weight component may be regarded as resistant glycopeptide(s). Peaks IIa, IVa and V migrated solely as high molecular weight components and may tentatively be regarded as glycopeptides resistant to endo-β-N-acetylglucosaminidase D hydrolysis (Fig. 6b, f, h). Peaks IIb and IVb appear to be primarily of high molecular weight, but a small amount of low molecular weight product was also released (Fig. 6c, g). In the case of the latter, the two peaks were pooled separately as IVb-1 (fractions 62-86) and IVb-2 (fractions 90-104) (Fig. 6g). Peak IIIa could be separated into a high molecular weight product (IIIa-1, fractions 62-82) and a product with apparent low molecular weight (IIIa-2, fractions 88-110) (Fig. 6d). Peak IIIb appears to elute midway between the two components of IIIa and will require further characterization by paper chromatography (Fig. 6e). Peak I elutes with a broad profile and may tentatively be regarded as a heterogeneous mixture of glycopeptides and released oligosaccharides (Fig. 6a). A summary of the products collected from the Sephadex G-25 column is shown in Table 3.

Endo-β-N-acetylglucosaminidase D treated neutral species

Aliquots of the endoglycosidase D treated material pooled from the Sephadex G-25 chromatography (Fig. 6) were analyzed by paper chromatography (Fig. 7). Samples were applied in 5 μl aliquots to Whatman 1 MM paper and run for 3 days in Solvent I, along with authentic GlcNAc(Man)$_5$ and GlcNAc(Man)$_3$ (Materials and Methods). Under these chromatographic conditions, glycopeptides resistant to endoglycosidase D hydrolysis remain at the origin (Muramatsu et al., 1976). The position of the standards GlcNAc(Man)$_3$ (fraction 20) and GlcNAc(Man)$_5$ (fraction 8) are shown with arrows (Fig. 7). Most of the species examined remained near the origin and I conclude that they are neutral glycopeptides resistant to hydrolysis by

Figure 7. Paper chromatography of endo-β-N-acetylglucosaminidase D-digested cellular neutral glycopeptides. Neutral glycopeptides were digested with endoglycosidase D in the presence of the three exo-glycosidases and prepared as described in the legend of figure 6. Aliquots of each of the pooled peaks were subjected to paper chromatography for 3 days. The arrows indicate the position of authentic GlcNAc(Man)$_5$ (fraction 8) and GlcNAc(Man)$_3$ (fraction 20). a) Peak I; b) Peak IIa; c) Peak IIb; d) Peak IIIa-1; e) Peak IIIa-2; f) Peak IIIb; g) Peak IVa; h) Peak IVb-1; i) IVb-2; j) Peak V. Only the ^{3}H-labeled growing cellular glycopeptides are shown. The origin represents +½ cm and -½ cm. Strips were cut 1 cm wide and into 1 cm fractions, eluted with water and counted for radioactivity.



RADIOACTIVITY x 10-1
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40
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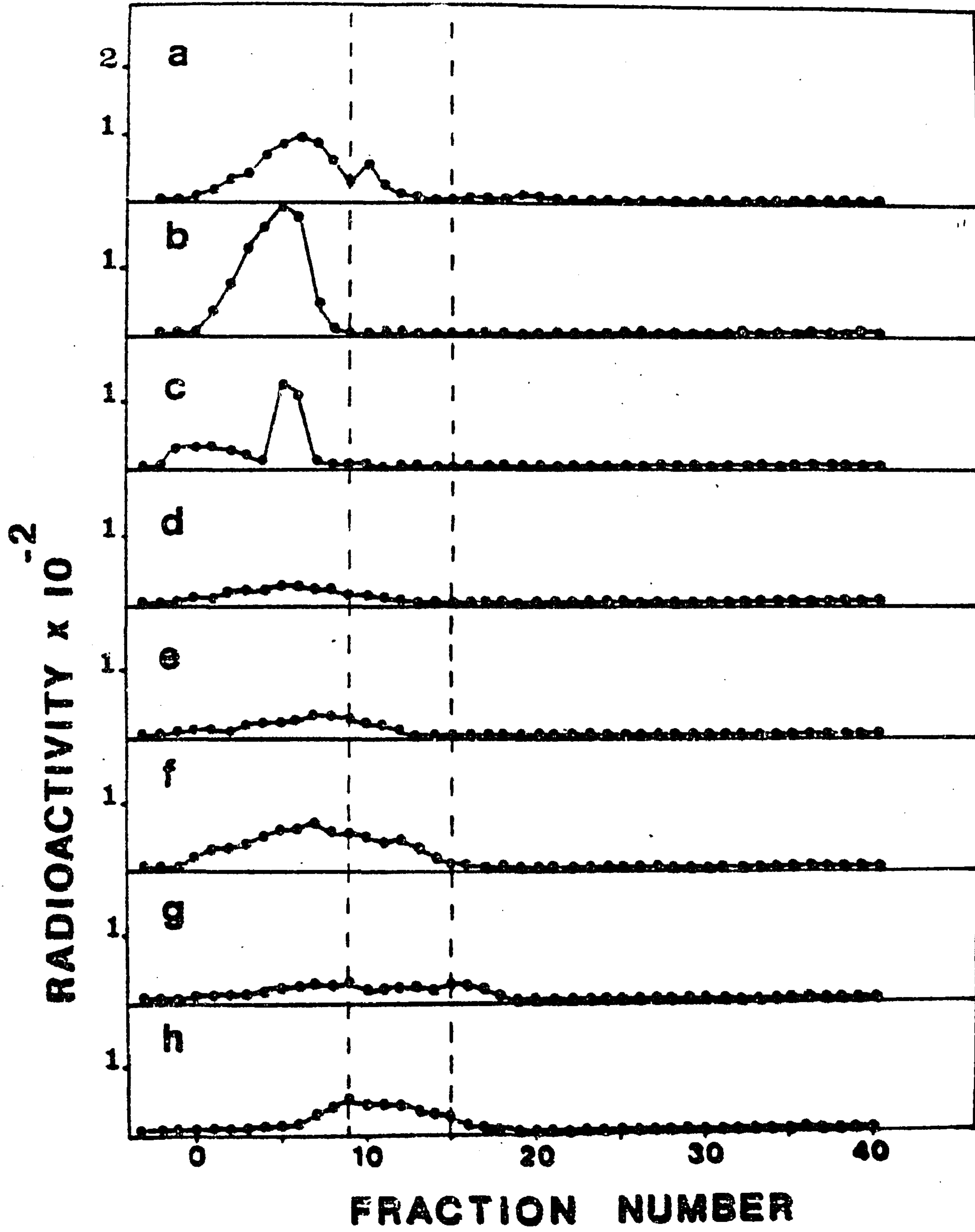
20

endo-β-N-acetylglucosaminidase D (Fig. 7b,c,d,f,g,h,j). Peaks IIIa-2 and IVb-2 migrated primarily with authentic GlcNAc(Man)$_3$ (Fig. 7e,i). Material migrating intermediate to authentic GlcNAc(Man)$_5$ and GlcNAc(Man)$_3$, possibly GlcNAc(Man)$_4$, was also detected for peak IIIa-2 (Fig. 7e). Peak I appears to be a heterogeneous mixture of resistant glycopeptides and released oligosaccharide (Fig. 7a). The data were consistent with the results obtained from the Sephadex G-25 chromatography. That is, all of the species which were of apparent high molecular weight were in fact glycopeptides resistant to endoglycosidase D (Fig. 6, 7). The lower molecular weight component consisted of released oligosaccharides with structures GlcNAc(Man)$_5$, GlcNAc(Man)$_3$, and possibly GlcNAc(Man)$_4$ (Fig. 6, 7). Approximately 79.6% of the growing cellular neutral glycopeptides were resistant to hydrolysis by endo-β-N-acetylglucosaminidase D. This was in excellent agreement with the previous report in which a mixture of cellular neutral glycopeptides purified by high voltage paper electrophoresis were found to be 76.1% resistant to endo-β-N-acetylglucosaminidase D hydrolysis (Muramatsu et al., 1976).

Endo-β-N-acetylglucosaminidase H treated neutral species

Aliquots of the mannose-labeled neutral glycopeptides isolated by Dowex AG-50W x 2 (Fig. 5) were next analyzed by endo-β-N-acetylglucosaminidase H (0.023 units), releasing an heterogeneous array of oligosaccharides by paper chromatography (Fig. 8). Most significantly, little radioactivity remained near the origin after 9 days. Knowing the specificity of endo-β-N-acetylglucosaminidase H, it has been suggested that the released products are oligosaccharides of the high mannose type (Muramatsu et al., 1976), similar in structure to Unit A of thyroglobulin (Arima and Spiro, 1972). In fact, this appeared to be the case, as the majority of the species analyzed were found to release oligosaccharides that appeared

Figure 8. Paper chromatography of endo-β-N-acetylglucosaminidase H-digested cellular neutral glycopeptides. Neutral mannose-containing glycopeptides were prepared as described in the legend of figure 5. They were digested with endo-β-N-acetylglucosaminidase H (0.023 units) and analyzed by paper chromatography for 9 days. The broken lines indicate the position of GlcNAc(Man)$_6$ (fraction 9) and GlcNAc(Man)$_5$ (fraction 15). a) Species I; b) Species IIa; c) Species IIb; d) Species IIIa; e) Species IIIb; f) Species IVa; g) Species IVb; h) Species V. Only the ^{3}H-labeled growing cellular glycopeptides are shown.



a
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g
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1
RADIOACTIVITY x 10^-2
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10
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30
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FRACTION NUMBER

to be larger than GlcNAc(Man)$_6$. It has previously been suggested that these may be neutral oligosaccharides with 7 or 8 mannosyl residues (Muramatsu et al., 1976). The data do not exclude the possibility that terminal substitutions other than mannosyl residues are also present. With the present material it was not possible to assign structures to any of the species analyzed by endoglycosidase H. However, it was clear that cellular neutral glycopeptides purified by QAE Sephadex chromatography behaved in a manner similar to neutral glycopeptides purified by high voltage paper electrophoresis in so far as susceptibility to the specific endoglycosidases. That is, some of the neutral cellular glycopeptides could be converted to smaller oligomannosyl cores with endo-β-N-acetylglucosaminidase D, while the majority may be converted to what appear to be large oligosaccharides by hydrolysis with endo-β-N-acetylglucosaminidase H.

Studies on the surface neutral glycopeptides

In the previous section it was demonstrated that cellular neutral glycopeptides purified by QAE Sephadex chromatography behaved in a manner similar to cellular neutral glycopeptides isolated by high voltage paper electrophoresis (Muramatsu et al., 1976). That is, essentially all of the neutral material could be converted into oligomannosyl cores of varying sizes by digestion with either endo-β-N-acetylglucosaminidase D or H. In this section, evidence will be provided that the cell surface neutral glycopeptides from growing and non-growing cells are rich in species susceptible to endo-β-N-acetylglucosaminidase H. The approach taken was to analyze neutral material at different stages of purification with either endo-β-N-acetylglucosaminidases D or H. That is, 1) neutral glycopeptides isolated by high voltage paper electrophoresis and 2) neutral glycopeptides fractionated by QAE Sephadex chromatography. Since demonstra-

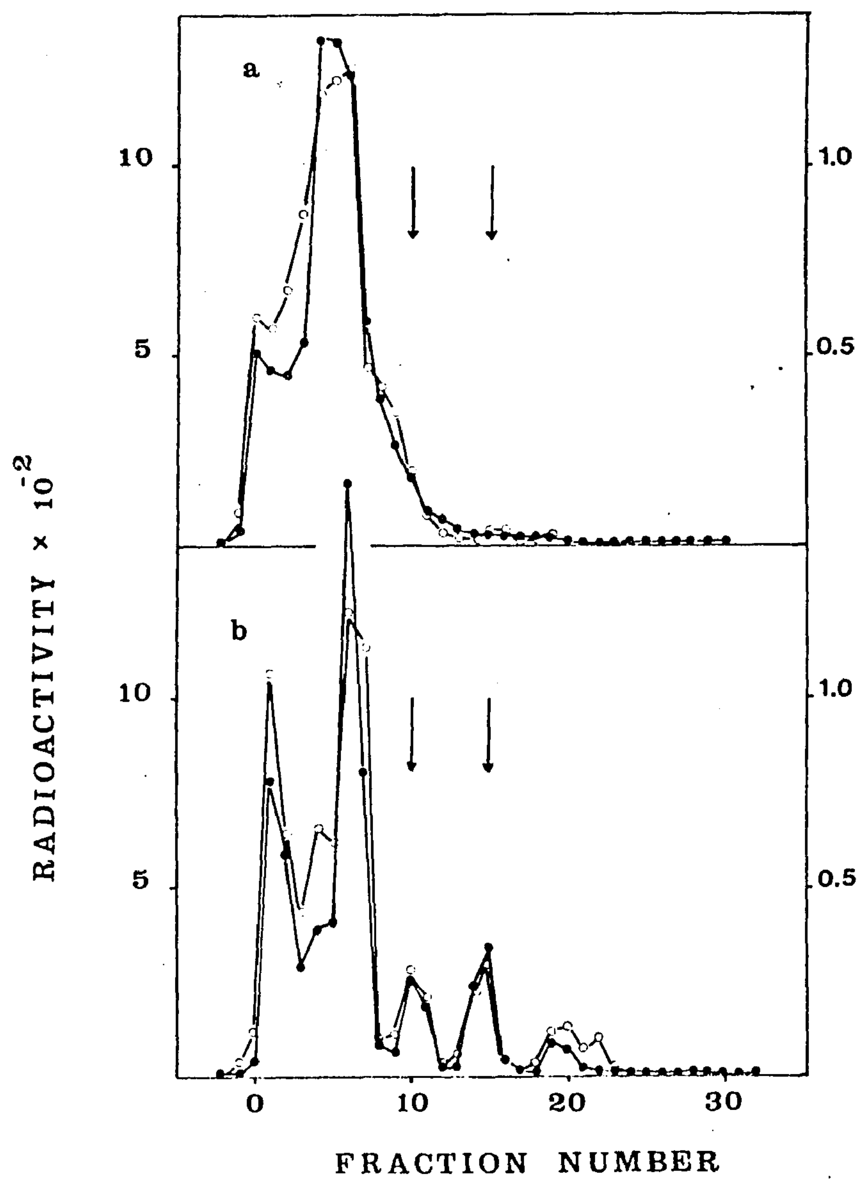
ble growth dependent alterations were evident for peaks I and II (derived from the QAE Sephadex fractionation), particular emphasis was placed on characterizing this material (Fig. 4a, b).

Isolated by high voltage paper electrophoresis

KL-2 cells were labeled with (^{3}H) mannose (non-growing) and (^{14}C) mannose (rapidly growing), the cell surface glycopeptides collected, and the mixture partially purified by Sephadex G-50 chromatography as described (Materials and Methods). A mixture of approximately 59,400 (^{3}H) cpm and 7,800 (^{14}C) cpm in 240 µl of water was separated into neutral and acidic material by high voltage paper electrophoresis, pH 6.5. Care was taken to apply a minimal amount of the mixture to the paper, as overloading the strip resulted in an incorrect distribution of neutral to acidic material. It was found that 10 µl and 20 µl aliquots gave similar results, whereas 50 µl aliquots gave distorted separations. For this reason, the 240 µl sample was applied in 10 µl aliquots to 24 separate strips. A typical separation yielded approximately 47% neutral material and 53% acidic material. The neutral glycopeptides (fractions 50-42) were collected by elution with water and reduced in volume for further analysis.

An aliquot of the growing and non-growing glycopeptide mixture was digested with endo-β-N-acetylglucosaminidase H (0.0458 units), desalted by Sephadex G-50 chromatography on a column (0.9 x 110 cm) previously equilibrated and eluted with water and subjected to paper chromatography for 14 days. The arrows indicate the position of authentic GlcNAc(Man)$_6$ (fraction 10) and GlcNAc(Man)$_5$ (fraction 15). Approximately 15-20% of the radioactivity was found near the origin after 14 days and it may be concluded that the cell surface neutral glycopeptides,

Figure 9. Paper chromatography of endo-β-N-acetylglucosaminidase H-digested surface neutral glycopeptides. Exponentially growing cells were labeled with D-(^{14}C)mannose (0.5 μc/ml, 240 mCi/mmol) and non-growing cells with D-(2-^{3}H)mannose (5 μc/ml, 1 Ci/mmol) and the cell surface glycopeptides prepared as described in Materials and Methods. The mixture of mannose-containing glycopeptides were separated into neutral and acidic material by high voltage paper electrophoresis, pH 6.5 (Materials and Methods). Neutral glycopeptides (19,800 cpm ^{3}H-labeled non-growing material ●—● and 2600 cpm ^{14}C-labeled growing material o—o) was digested with endo-β-N-acetylglucosaminidase H (0.0458 units) either alone or in the presence of β-galactosidase (0.75 units) and β-N-acetylglucosaminidase (0.8 units), desalted by G-50 Sephadex chromatography, and the products separated by paper chromatography for 14 days. The arrows indicate the position of authentic GlcNAc(Man)$_6$ (fraction 10) and GlcNAc(Man)$_5$ (fraction 15). a) endo-β-N-acetylglucosaminidase H digest; b) endo-β-N-acetylglucosaminidase H and exo-glycosidases.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in either state of growth, are enriched in species susceptible to endo-β-N-acetylglucosaminidase H hydrolysis (Fig. 9a). Under these conditions, intact glycopeptides would remain near the origin (fraction 0-3), and may be regarded as glycopeptides resistant to the endo-β-N-acetylglycosaminidase H hydrolysis. Virtually all of the released material migrate as though it was larger than GlcNAc(Man)$_6$.

A second aliquot of the growing and non-growing mixture was re-examined with endo-β-N-acetylglucosaminidase H (0.0458 units), but in the presence of β-N-acetylglucosaminidase (0.8 units) and β-galactosidase (0.75 units). This second digest was co-run with the sample previously digested solely with endoglycosidase H, under identical conditions. Figure 9b shows that under these conditions an array of mannosyl oligosaccharides are released. Oligomannosyl cores migrating with authentic GlcNAc(Man)$_6$ and GlcNAc(Man)$_5$ are now detectable, with a concurrent decrease in the amount of "large core oligosaccharides." No smaller products were detected if the paper chromatogram was developed for 3 days. Whereas, digestion with endo-β-N-acetylglucosaminidase H alone released material significantly larger than GlcNAc(Man)$_6$ and no "medium size cores" were detected (Fig. 9a). The data strongly suggest that a portion of the "large core oligosaccharides" must terminate with N-acetylglucosamine and/or galactose. However, the fact that a significant amount of material continues to migrate as "large core oligosaccharides" after treatment with endoglycosidase H in the presence of β-N-acetylglucosaminidase and β-galactosidase is consistent with the previous contention that the oligomannosyl cores of the neutral glycopeptides are mostly unsubstituted and that a portion may contain clusters with as many as 7-8 mannosyl residues (Muramatsu _et al._, 1976; cf. Table 4).

Table 4

Analysis of oligomannosyl cores of surface neutral glycopeptides digested by endo-β-N-acetylglucosaminidase H with and without exo-glycosidases[a]

	endoglycosidase H					endoglycosidase H + exo-glycosidases[b]				
	large[c] core	6[d] core	5[e] core	4[f] core	resistant material	large core	6 core	5 core	4 core	resistant material
non-growing	81.2	-	-	-	18.8	51.1	8.4	10.3	3.5	26.7
growing	85.2	-	-	-	14.8	48.8	8.2	8.3	4.7	30.0

[a]Preparation of the surface neutral glycopeptides and the conditions for the enzyme digestions are described in the legend of figure 9.

[b]N-acetylglucosaminidase and β-galactosidase

[c]assumed to be GlcNAc(Man)$_{7-8}$, but may also contain smaller oligomannosyl cores with terminal substitution by sugars other than mannose

[d]GlcNAc(Man)$_6$

[e]GlcNAc(Man)$_5$

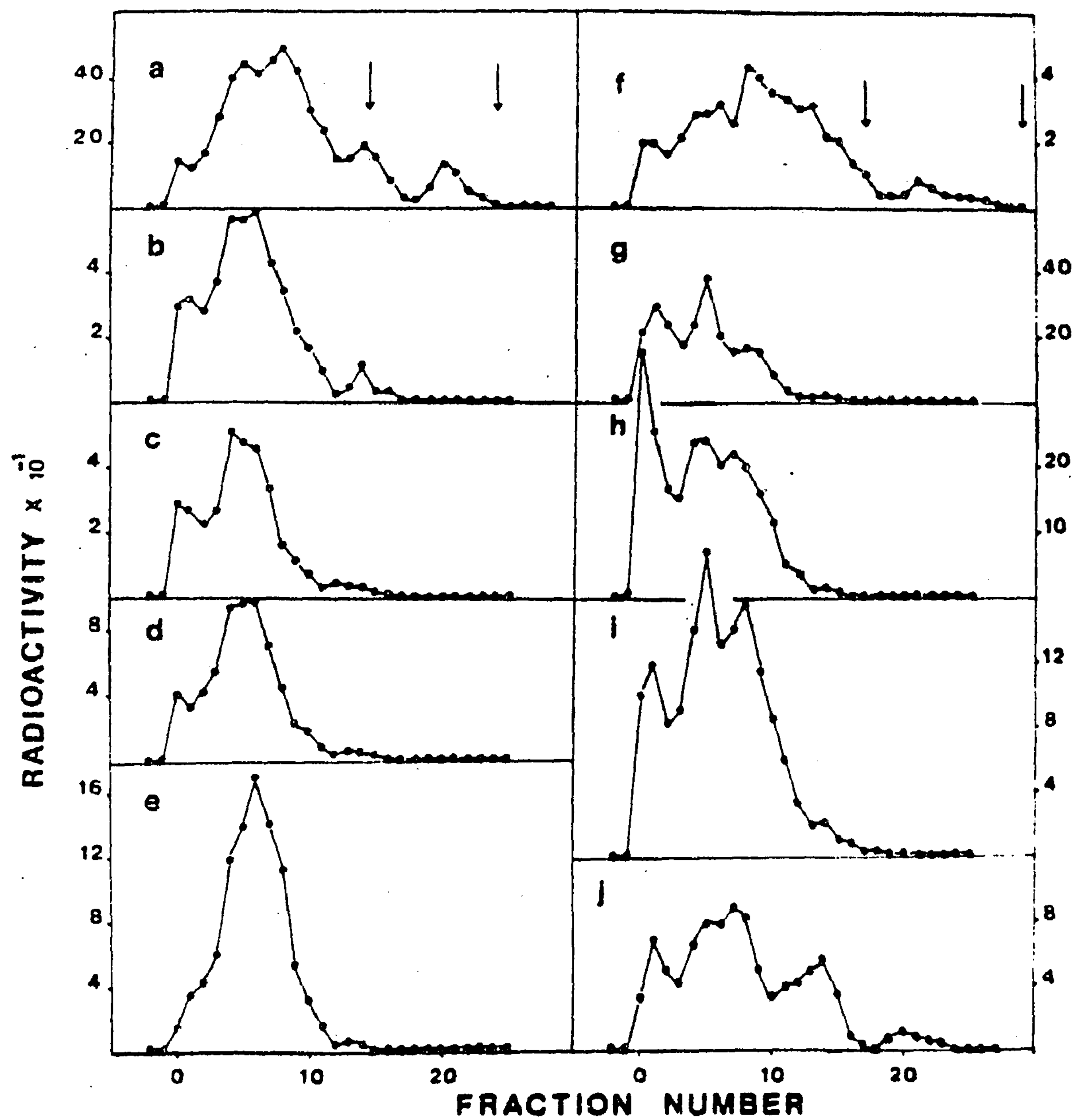
[f]assumed to be GlcNAc(Man)$_4$

Isolated by QAE Sephadex chromatography

Cell surface glycopeptides from KL-2 human fibroblasts were labeled with (^{3}H) mannose in the exponentially growing and density-inhibited state, partially purified by Sephadex G-50 chromatography and separated into neutral and acidic glycopeptides by QAE Sephadex chromatography (Materials and Methods). The neutral glycopeptides were separated into at least five distinct species (Fig. 4a, b) and each was subsequently hydrolyzed with endo-β-N-acetylglucosaminidase H. As shown in figure 10, in every case two products could be detected, released oligosaccharides, the majority of which migrated as if larger than GlcNAc(Man)$_6$ and resistant intact glycopeptides which remained at the origin. No other radioactivity could be recovered from the paper. The amount of material migratign as released oligosaccharides relative to the total amount of radioactivity recovered from the paper varied from species to species. That is, susceptibility to endoglycosidase H appears to vary depending on which QAE Sephadex-derived peak is examined and its growth status (Fig. 10a-j). More oligosaccharide material was released from the growing glycopeptides than their non-growing counterparts. It may also be significant that the oligosaccharide material released from the growing glycopeptides appears to be larger than similar material released from the non-growing glycopeptides (Fig. 10a-j). Of particular interest is that all of the peaks examined display a high susceptibility to endoglycosidase H hydrolysis, suggesting an enrichment of "large core oligocaccharides" in each of the species derived from the QAE Sephadex column.

As controls, intact surface glycopeptides from both states of growth were treated under similar conditions, but without prior incubation with endoglycosidase H. Figure 11 demonstrates that the intact glyco-

Figure 10. Paper chromatography of endo-β-N-acetylglucosaminidase H-digested surface neutral glycopeptides derived from QAE Sephadex chromatography. The preparation and isolation of these neutral glycopeptides were done as described in the legend of figure 4. They were digested by endo-β-N-acetylglucosaminidase H (0.023 units) and analyzed by paper chromatography for 15 days. The arrows indicate the position of authentic $GlcNAc(Man)_6$ and $GlcNAc(Man)_5$, respectively. a) growing species I; b) growing species II; c) growing species III; d) growing species IV; e) growing species V; f) non-growing species I; g) non-growing species II; h) non-growing species III; i) non-growing species IV; j) non-growing species V.



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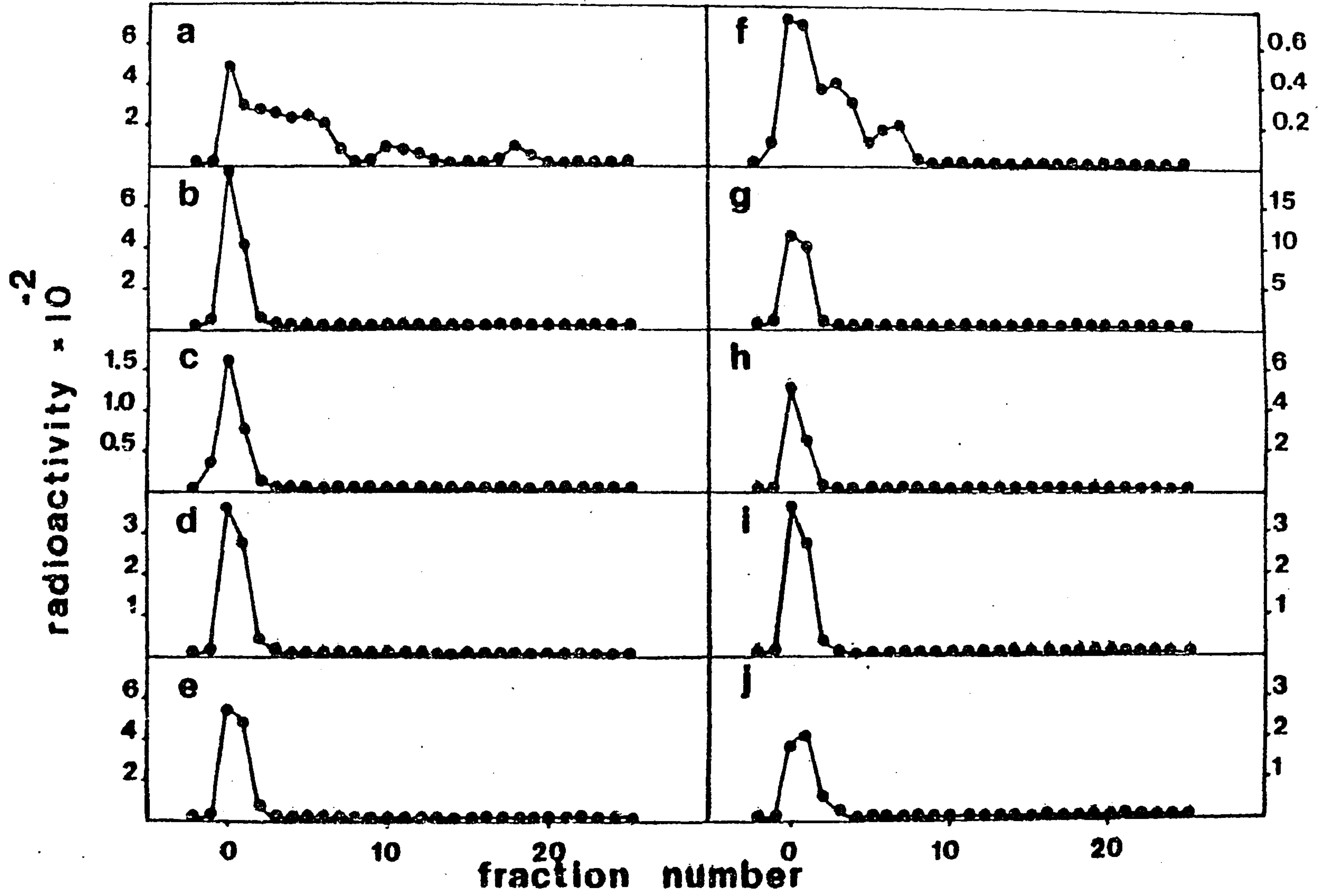
20
RADIOACTIVITY x 10⁻¹
FRACTION NUMBER

Figure 11. Paper chromatography of intact surface neutral glycopeptides derived from QAE Sephadex chromatography. The material and conditions for chromatography were the same as described in the legend of figure 10, except that no prior treatment with endo-β-N-acetylglucosaminidase H was performed. a) growing species I; b) growing species II; c) growing species III; d) growing species IV; e) growing species V; f) non-growing species I; g) non-growing species II; h) non-growing species III; i) non-growing species IV; j) non-growing species V.



radioactivity × 10^-2
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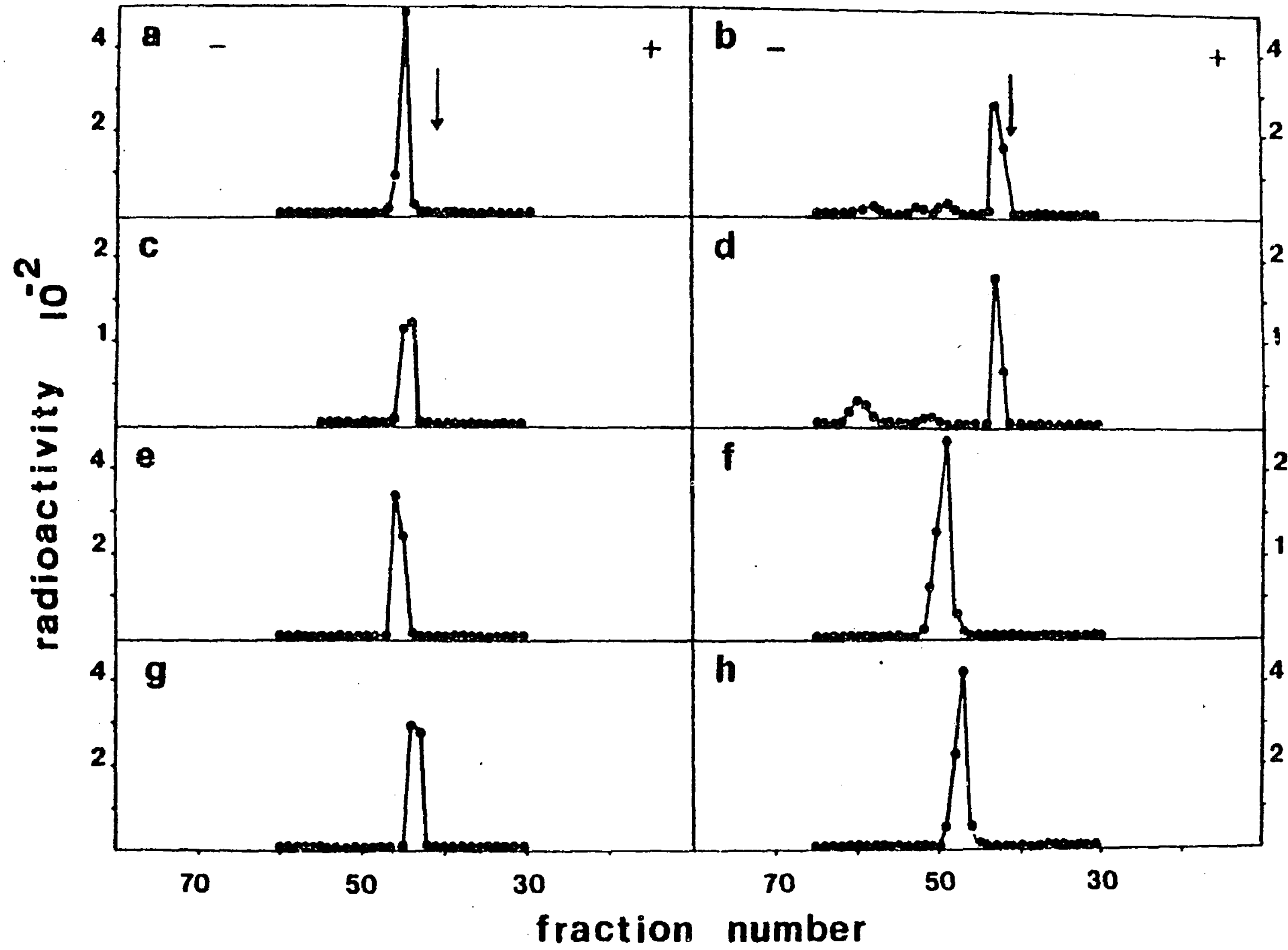
peptides remain near the origin (fractions 0-2), underscoring the point that "large core oligosaccharides" are released from the neutral glycopeptides in the presence of endoglycosidase H (Fig. 10). However, it was noted that peak I, derived from growing and non-growing cells, appears to contain oligosaccharides without prior treatment by endoglycosidase H (Fig. 11a, f). This was subsequently confirmed for growing peak I by other methods (see below).

Further studies on neutral peaks I and II

Growth dependent alterations were evident for the surface neutral glycopeptides when examined by QAE Sephadex chromatography (Fig. 4a, b). The data indicated a disproportionate ratio of peak I to peak II depending on the state of growth (Table 2). As such, emphasis was placed on trying to infer the structural types present in this material. Aliquots of peaks I and II were analyzed by high voltage paper electrophoresis. All of the material migrated to the neutral zone (fractions 43-47) at pH 6.5 (Fig. 12). At pH 1.9 amphoteric behavior in peak I was noted in that a portion of the label migrated further to the negative pole (Fig. 12b, d). It may be significant that peak II migrated further to the negative pole at pH 1.9 than the majority of peak I, suggesting a difference in net positive charge under these conditions (Fig. 12f, h). This characteristic migration on HVPE as well as paper chromatography is indirect evidence that peaks I and II contain amino acids and thus are glycopeptides. Although, it must be pointed out that peak I did not appear to contain some contaminating oligosacchairdes (Fig. 11a, f). From the data in this section it may be concluded that peaks I and II are neutral, and primarily glycopeptide.

Aliquots of peaks I and II were digested with endo-β-N-acetylglu-

Figure 12. Electrophoretic analysis of neutral peaks I and II. Mannose-labeled neutral peaks I and II were prepared as described in the legend of figure 4. Aliquots of intact material were loaded onto Whatman 1 MM paper and run in pyridine:acetic acid:water, 10.0:0.4:89.6, pH 6.5 or 7% formic acid, pH 1.9 as described in the text. a) growing peak I, pH 6.5; b) growing peak I, pH 1.9; c) non-growing peak I, pH 6.5; d) non-growing peak I, pH 1.9; e) growing peak II, pH 6.5; f) growing peak II, pH 1.9; g) non-growing peak II, pH 6.5; h) non-growing peak II, pH 1.9.



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1
radioactivity 10^{-2}
70
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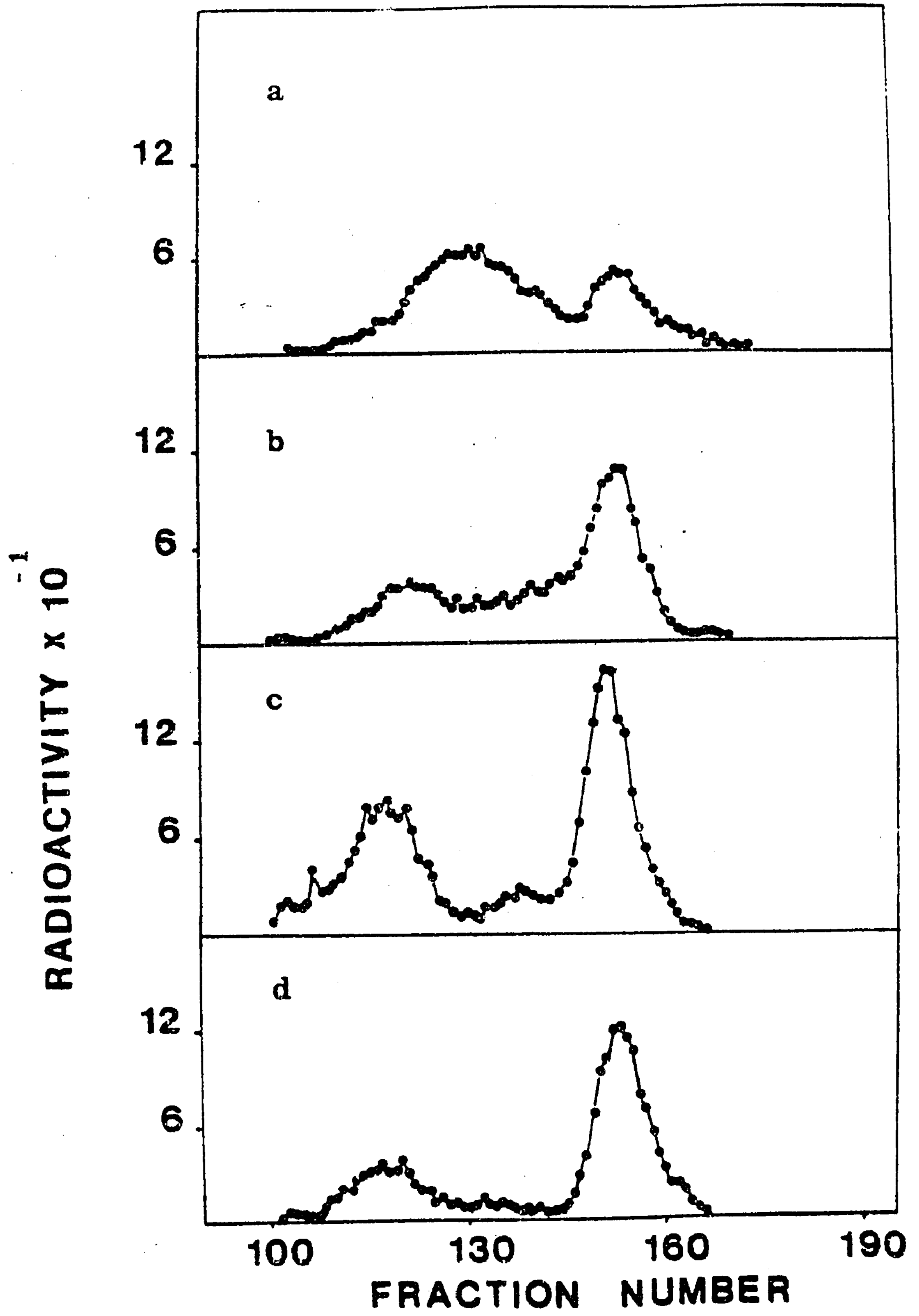
fraction number

Figure 13. Endo-β-N-acetylglucosaminidase D-digested neutral peaks I and II. Mannose-containing surface neutral glycopeptides were prepared as described in the legend of figure 4. Peaks I and II were digested with endo-β-N-acetylglucosaminidase D (0.0185 units) in the presence of β-galactosidase (0.0052 units), β-N-acetylglucosaminidase (0.037 units) and neuraminidase (10 units) and applied to columns (0.9 x 145 cm) of Sephadex G-25 (Materials and Methods). Fractions of 0.45 ml were collected and aliquots counted for radioactivity. a) Growing peak I; b) Non-growing peak I; c) Growing peak II; d) Non-growing peak II. Blue dextran eluted at fraction 95.


a
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12
6
RADIOACTIVITY x 10^-1
100
130
160
190
FRACTION NUMBER

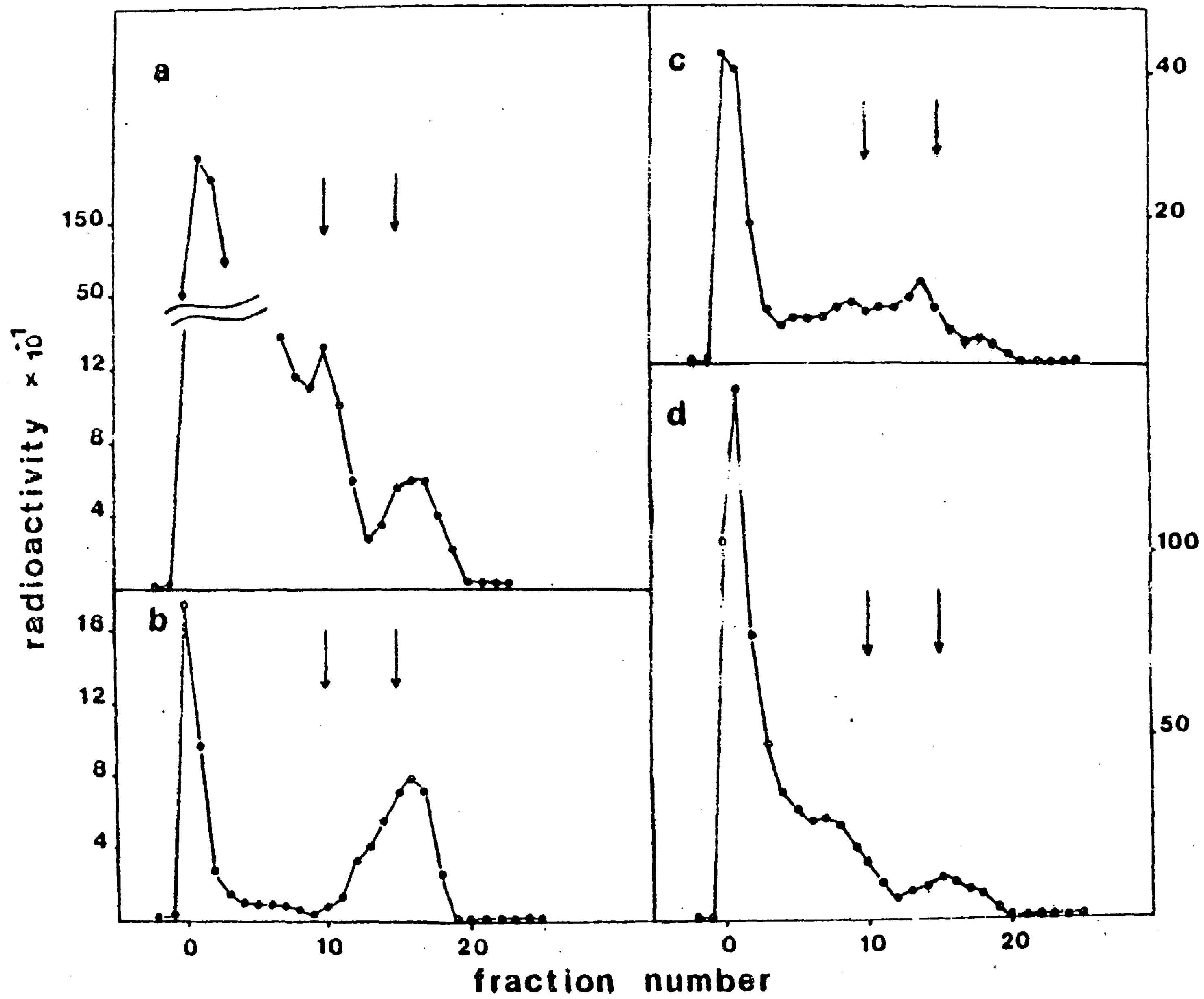
cosaminidase D, in the presence of β-galactosidase, β-N-acetylglucosaminidase and neuraminidase and subsequently analyzed by Sephadex G-25 chromatography. As shown in Figure 13, generally two products could be detected, a high molecular weight peak which may be regarded as resistant glycopeptide(s) and a low molecular weight component, consisting of released oligosaccharides. Blue dextran eluted at fraction 95. Growth dependent alterations were evident. The non-growing material from both peaks I and II was more susceptible to endoglycosidase D hydrolysis. The amount of mannose-label released as the low molecular weight component was 52% vs. 30% and 66% vs. 52% for non-growing peak I vs. growing peak I and non-growing peak II vs. growing peak II, respectively.

The endoglycosidase D treated material was pooled from the Sephadex G-25 columns, reduced in volume and analyzed by paper chromatography. Surprisingly, growing peaks I and II released oligosaccharides comigrating with authentic $GlcNAc(Man)_5$ and $GlcNAc(Man)_3$ (Fig. 14e, g) whereas, non-growing peaks I and II released only $GlcNAc(Man)_3$ (Fig. 14f, h). The data suggest that non-growing peaks I and II are enriched in carbohydrate moieties whose inner core structure must contain 3 mannosyl residues. It was of considerable interest to note that both peaks I and II, in either state of growth, contain glycopeptides whose inner core structure was $GlcNAc(Man)_3$. That is, those glycopeptides that contain common oligomannosyl cores and yet elute either as peak I or peak II species must differ in net charge due to some other factor, possibly terminal substitution with other sugars or different amino acids in their peptide moiety.

Analysis of peaks I and II by -mannosidase

When peaks I and II were digested with α-mannosidase (approximate-

Figure 14. Paper chromatography of endo-β-N-acetylglucosaminidase D-digested neutral peaks I and II. Mannose-labeled surface neutral glycopeptides were digested with endoglycosidase D in the presence of the three exo-glycosidases and prepared as described in the legend of figure 13. Aliquots of the pooled material (fractions 101-196) were subjected to paper chromatography for 3 days. The arrows indicate the position of authentic $GlcNAc(Man)_5$ (fraction 10) and $GlcNAc(Man)_3$ (fraction 15). a) Growing peak I; b) Non-growing peak I; c) Growing peak II; d) Non-growing peak II.



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10
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fraction number

Table 5

Analysis of neutral peaks I and II digested by α-mannosidase[a]

	Species	Percentage of released mannose[b]
growing	I	65
	II	61
non-growing	I	23
	II	37

[a]Surface neutral peaks I and II were prepared as described in the legend of figure 4 and subsequently digested with α-mannosidase (Materials and Methods, cf. Chapter II).

[b]The α-mannosidase-treated neutral glycopeptides were applied to columns (0.9 x 145 cm) of Sephadex G-25. Fractions of 0.95 ml were collected at a flow rate of 0.95 ml per 7 minutes. Blue dextran and (^{14}C)mannose were co-run as markers.

ly 7 units in 2 days) and analyzed by Sephadex G-25 chromatography, more mannose was released from the growing glycopeptides (Table 5). The α-mannosidase acts on terminal α-mannosyl residues (Li, 1967). The data suggest that growing peaks I and II may contain more terminal mannosyl residues. The possibility that the growing glycopeptides contain larger clusters of mannose cannot be excluded (see discussion). It should be mentioned that the Sephadex G-25 profile of growing peak I indicated that 7% of the mannose-label eluted as the disaccharide (Man)GlcNAc. The purported disaccharide is consistent with previous data suggesting that growing peak I was contaminated with oligosaccharides. Only growing peak I appeared to contain this material.

CHAPTER III - STUDIES ON THE COMPLEX GLYCOPEPTIDES

During the past ten years, several laboratories have demonstrated growth dependent alterations in surface glycopeptides. An enrichment in high molecular weight surface glycopeptides has been reported for virus transformed and exponentially growing cells versus normal and confluent cells (Meezan *et al.*, 1969; Buck *et al.*, 1970; Buck *et al.*, 1971a; Warren *et al.*, 1972b). Warren *et al.* attributed this difference to an enhancement of a class of sialylated glycoproteins of transformed cells in comparison to normal cells (Warren *et al.*, 1972a). Using radioactive fucose and endo-β-N-acetylglucosaminidase D, Muramatsu *et al.* observed that the linkage region from surface glycopeptides of non-growing cells was more heterogeneous when compared to that of growing cells (Muramatsu *et al.*, 1973). Further, growth attributed differences for neutral glycopeptides, similar in structure to Unit A of thyroglobulin (Arima and Spiro, 1972; Tarentino *et al.*, 1973), have been shown to involve an enrichment of high molecular weight glycopeptides in virally transformed and rapidly growing cells compared to non-growing cells (Ceccarini *et al.*, 1975; Muramatsu *et al.*, 1976; Ceccarini and Atkinson, 1977). The complex glycopeptides (acidic species) are thought to contain smaller oligomannosyl cores, GlcNAc$(Man)_3$, with side chains covered with N-acetylglucosamine, galactose and sialic acid (Muramatsu *et al.*, 1976). These structures are probably similar to Unit B of thyroglobulin (Toyoshima *et al.*, 1972; Toyoshima *et al.*, 1973). This chapter deals with growth dependent alterations of acidic glycopeptides, derived from human fibroblasts, labeled with mannose. The approach used was to isolate relatively homogeneous subclasses of acidic glycopeptides by ion

exchange chromatography. The isolated acidic glycopeptides were digested with specific exo- and endoglycosidases and the products were analyzed by column and paper chromatography as well as high voltage paper electrophoresis. The data are consistent with growth dependent alterations in both the oligosaccharide and peptide moiety of specific glycopeptides.

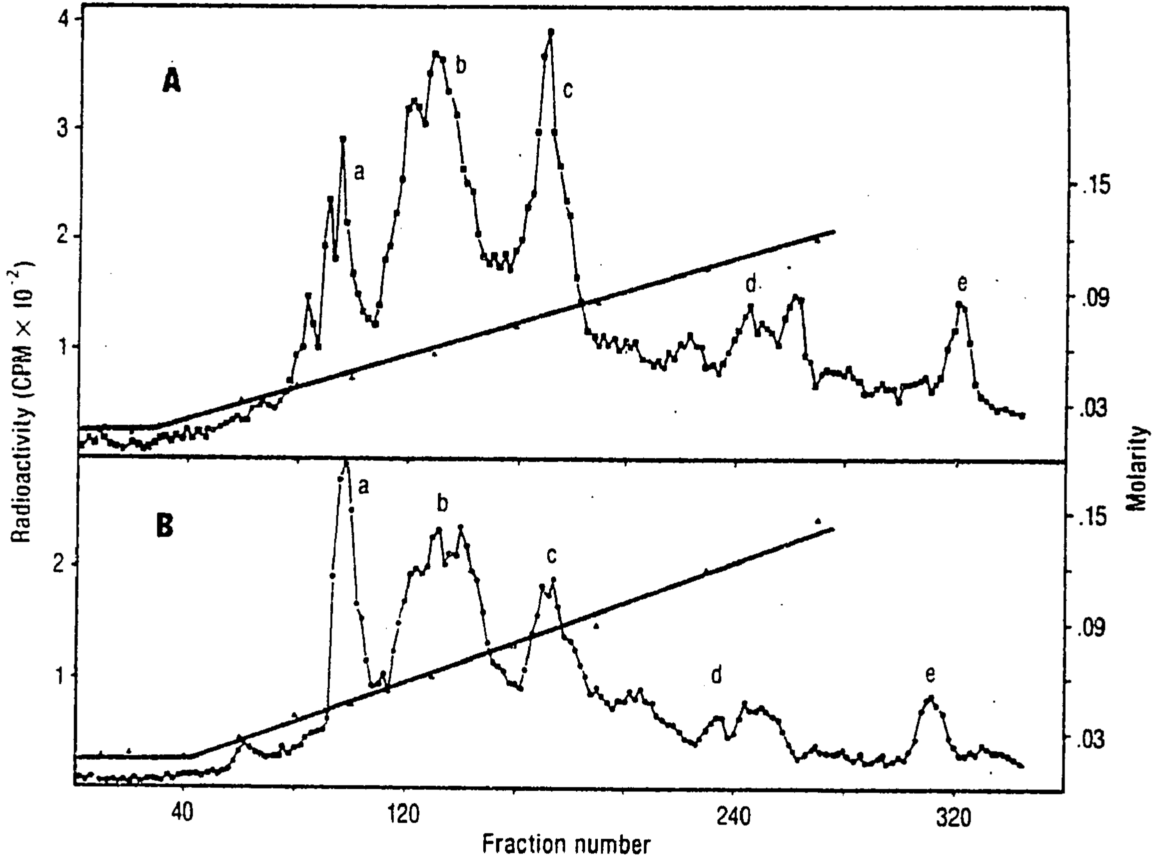
RESULTS

Purification and isolation of glycopeptides

Tritium-labeled glycopeptides from KL-2 partially purified by Sephadex G-50 columns were applied to columns of QAE Sephadex, which had been equilibrated with 5 mM ammonium acetate, pH 8.5. Approximately 40% of the radioactivity applied fractionated at this salt concentration. This material mainly contains neutral glycopeptides (possibly some oligosaccharides) (cf. Chapter II). When a linear gradient of 5 mM - 300 mM ammonium acetate was applied to the column, three major peaks from both the growing and non-growing material, eluting between 40-45 mM, 60-65 mM and 80-85 mM salt could be obtained (Fig. 15). These fractions have been designated A, B, and C respectively. Minor peaks in either state of growth eluted between 100-105 mM and 140-145 mM salt and will be referred to as D and E respectively. Approximately 60% of the mannose-labeled material was recovered as acidic surface glycopeptides. Virtually 100% of the starting material was recovered, as no appreciable radioactivity could be eluted with a 1 M ammonium acetate wash after completion of the gradient.

With respect to growth dependent alterations, no major differences between the profiles of growing and non-growing acidic surface species, as far as the appearance or disappearance of peaks in either state of

Figure 15. Chromatography on QAE Sephadex of cell surface complex glycopeptides. The surface component from a population of KL-2 cells maintained either as exponentially growing or in the non-growing state, labeled with D-(2-^{3}H)mannose (5 µc/ml, 1 Ci/mmol) was collected as described in Materials and Methods. The material was partially purified by Sephadex G-50 chromatography and applied to QAE Sephadex columns (1.5 x 42 cm). Simple glycopeptides elute at 5 mM ammonium acetate, pH 8.5 (cf. Fig. 4). The complex glycopeptides elute with an ammonium acetate gradient of 5 mM to 300 mM (▵——▵). 1500 ml of 5 mM NH_4Ac in the mixing chamber and 1500 ml of 300 mM NH_4Ac in the reservoir. Five ml fractions were collected at a flow rate of 15 ml per hour. Approximately 5.7 x 10^5 cpm growing material and 7.5 x 10^5 cpm non-growing material were applied. No detectable radioactivity was recovered with elution by 1 M ammonium acetate. a) Growing surface glycopeptides; b) Non-growing surface glycopeptides.


A
B
a
b
c
d
e
Radioactivity (CPM × 10⁻²)
Molarity
Fraction number
40
120
240
320
1
2
3
4
.03
.09
.15

growth were apparent. However, peak A from growing cells always appeared more heterogeneous than the corresponding material from non-growing cells, and the relative distribution of radioactivity differed between growing and non-growing cell surfaces, but this was not examined in further detail.

Molecular weight studies before and after neuraminidase treatment on QAE Sephadex purified species

Peaks A, B, C and E from both states of growth purified by QAE Sephadex were pooled for further analyses. The apparent molecular weight of each peak was determined on a calibrated Sephadex G-50 column. The acidic glycopeptides analyzed ranged in apparent molecular weight from a minimum of 2900 to a maximum of 4100 daltons (Table 6) and appear to elute by increasing order of molecular weight, at least for peaks A, B and C. On closer inspection growth dependent alterations for peaks A, B and C were noted in that material isolated from growing cell surfaces was always higher in molecular weight than that eluted at the same salt concentration from non-growing cells. The difference between growing and non-growing material ranged from about 400 daltons (peak A) to 850 daltons (peak C). Peak E, which eluted at the highest salt concentration (140-145 mM), had an apparent molecular weight of about 3700 daltons and is identical in both states of growth.

Peaks A, B, C and E were digested with 10 units of neuraminidase as described (Materials and Methods) and their apparent molecular weight redetermined on the same Sephadex G-50 column. As shown in Table 6, growth dependent alterations persisted after neuraminidase digestion, in that peaks isolated from the growing cell surface were still higher in molecular weight than their non-growing counterparts. Since neura-

Table 6

Molecular weight estimates of surface complex species

Complex Species	Intact Glycopeptides[a]	Neuraminidase Treated[b]	Δ Daltons	Sialic Acid Residue Equivalents[c]
growing A	3300	2450	850	2.8
non-growing A	2900	2300	600	2.0
growing B	3700	3300	400	1.3
non-growing B	3150	3000	150	0.5
growing C	4100	3500	600	2.0
non-growing C	3250	2950	300	1.0
growing D	3700	3700	0	0
non-growing D	3700	3700	0	0

[a]Complex glycopeptides were fractionated by QAE Sephadex chromatography as described in figure 15. Estimation of molecular weights were determined by Sephadex G-50 chromatography using standards as described in Materials and Methods.

[b]Complex glycopeptides were digested with neuraminidase as described in Materials and Methods.

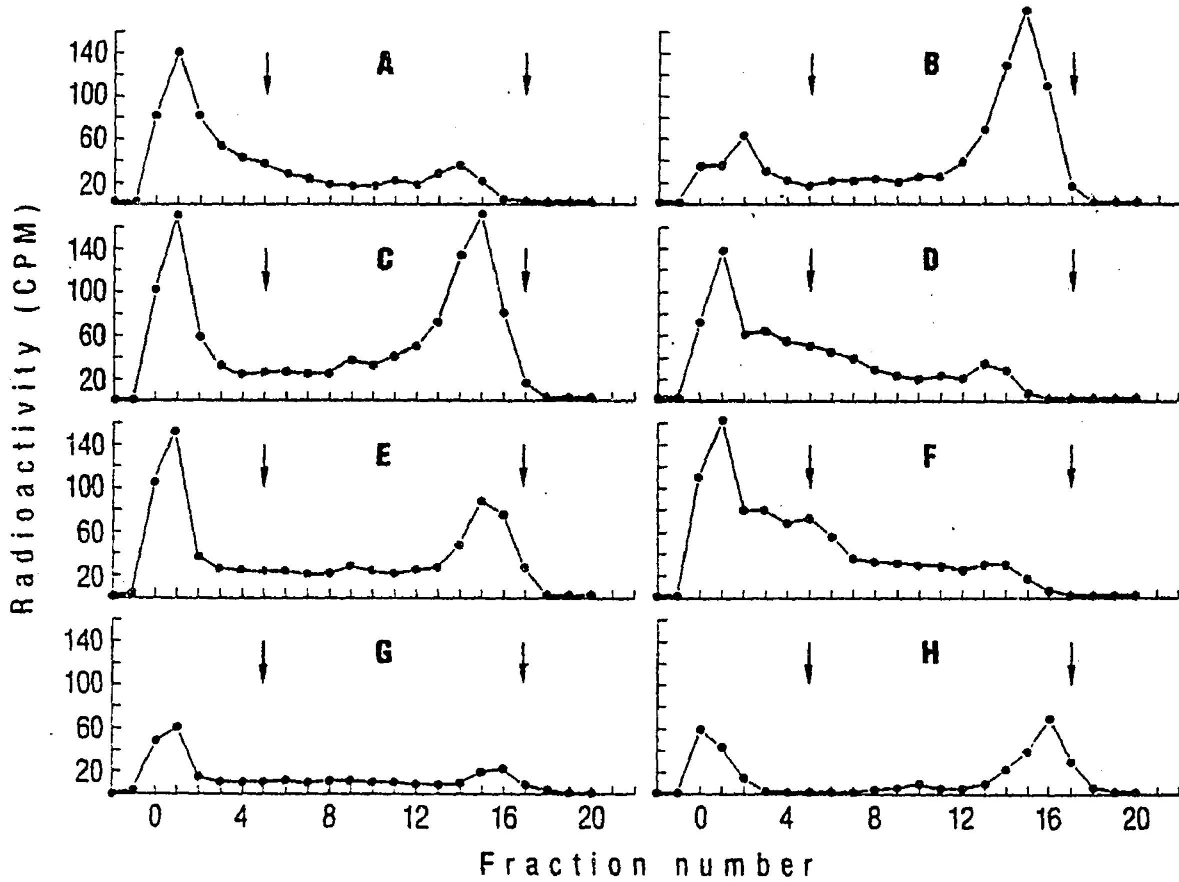
[c]Calculated difference in molecular weight between an intact species and its neuraminidase treated counterpart divided by the molecular weight of one sialic acid residue, 309.

minidase will specifically remove terminal sialic acid residues, the observed differences in molecular weight between a growing and non-growing peak, eluted at the same salt concentration, cannot be due solely to variations in sialic acid content. At present, this size difference cannot be attributed to the peptide or remaining oligosaccharide moiety. Of particular interest is peak E, which retained the same apparent molecular weight before and after neuraminidase digestion in both states of growth. This resistance to neuraminidase was further examined (see below).

Endo-β-N-acetylglucosaminidase D susceptibility of mannose-labeled acidic glycopeptides

Aliquots of the mannose-labeled glycopeptides designated as A, B, C and E (Fig. 15) were desalted by Sephadex G-50 chromatography. Glycopeptides from both states of growth were digested with endo-β-N-acetylglucosaminidase D, in the presence of β-galactosidase, β-N-acetylglucosaminidase and neuraminidase and the products analyzed by paper chromatography. As shown in Figure 16, in every case the fast moving product migrated to a similar position (13-16 cm) and was within 2-3 cm of authentic GlcNAc(Man)$_3$ (16-18 cm). Under these chromatographic conditions, glycopeptides remain near the origin (Muramatsu et al., 1976; cf. Fig. 11) and thus the material found in this region can be regarded as resistant glycopeptide species. No other radioactivity was recovered from the paper. The percentage of released material (fractions 13-16) relative to the total radioactivity recovered from the paper varied from species to species, ranging from 13 to 61%. The percentage of released oligosaccharide from each peak examined appears to depend on the state of growth. That is, susceptibility of surface acidic

Figure 16. Paper chromatography of surface complex glycopeptides digested by endo-β-N-acetylglucosaminidase D. Complex glycopeptides (1400-7400 cpm) were digested with endo-β-N-acetylglucosaminidase D (0.0185 units) in the presence of β-galactosidase (0.00523 units), β-N-acetylglucosaminidase (0.037 units) and neuraminidase (10 units) as described in Materials and Methods. Paper chromatography was carried out on Whatman 1 MM paper in ethyl acetate:pyridine:water, 12:5:4 for 3 days. The arrows indicate the position of the standards $GlcNAc(Man)_5$ (fraction 5) and $GlcNAc(Man)_3$ (fraction 17). a) Growing surface peak A; b) Non-growing surface peak A; c) Growing surface peak B; d) Non-growing surface peak B; e) Growing surface peak C; f) Non-growing surface peak C; g) Growing surface peak E; h) Non-growing surface peak E.



A
B
C
D
E
F
G
H
140
100
60
20
0
4
8
12
16
20
Radioactivity (CPM)
Fraction number

glycopeptides to endo-β-N-acetylglucosaminidase D appears to be a growth related phenomenon (Table 7). Of particular note was that all the species analyzed contained some degree of resistant material.

Finally, intact acidic glycopeptides were analyzed for susceptibility to endo-β-N-acetylglucosaminidase H. Approximately 700-3800 cpm of mannose-labeled material was digested with 0.023 units of endo-β-N-acetylglucosaminidase H. All radioactivity remained near the origin (fractions 0-3 cm) and no other radioactivity could be recovered from the paper. From this it may be concluded that the intact acidic glycopeptides examined are resistant to hydrolysis by endo-β-N-acetylglucosaminidase H.

<u>Endo-β-N-acetylglucosaminidase D susceptibility studies on glycopeptide E</u>

The nature of resistance of the acidic glycopeptides to endo-β-N-acetylglucosaminidase D was further examined. Since the enzyme functions best in the presence of β-galactosidase, β-N-acetylglucosaminidase and neuraminidase, it was felt that incomplete removal of side chains might account for the observed resistance. For this reason glycopeptide E was examined. Its apparent molecular weight had remained unchanged in both states of growth before and after neuraminidase treatment (Table 6). As such, it was felt that peak E was likely to contain sialic acid residues resistant to neuraminidase.

Intact non-growing peak E migrated to the positive pole on high voltage paper electrophoresis (HVPE) at pH 6.5 (Fig. 17A). From this it might be inferred that its net negative charge may be due to terminating sialic acid residues. However, after neuraminidase digestion (10 units for 24 hours at 37°) better than 90% of the material continued to migrate as though it retained net negative charge (Fig. 17B), i.e.,

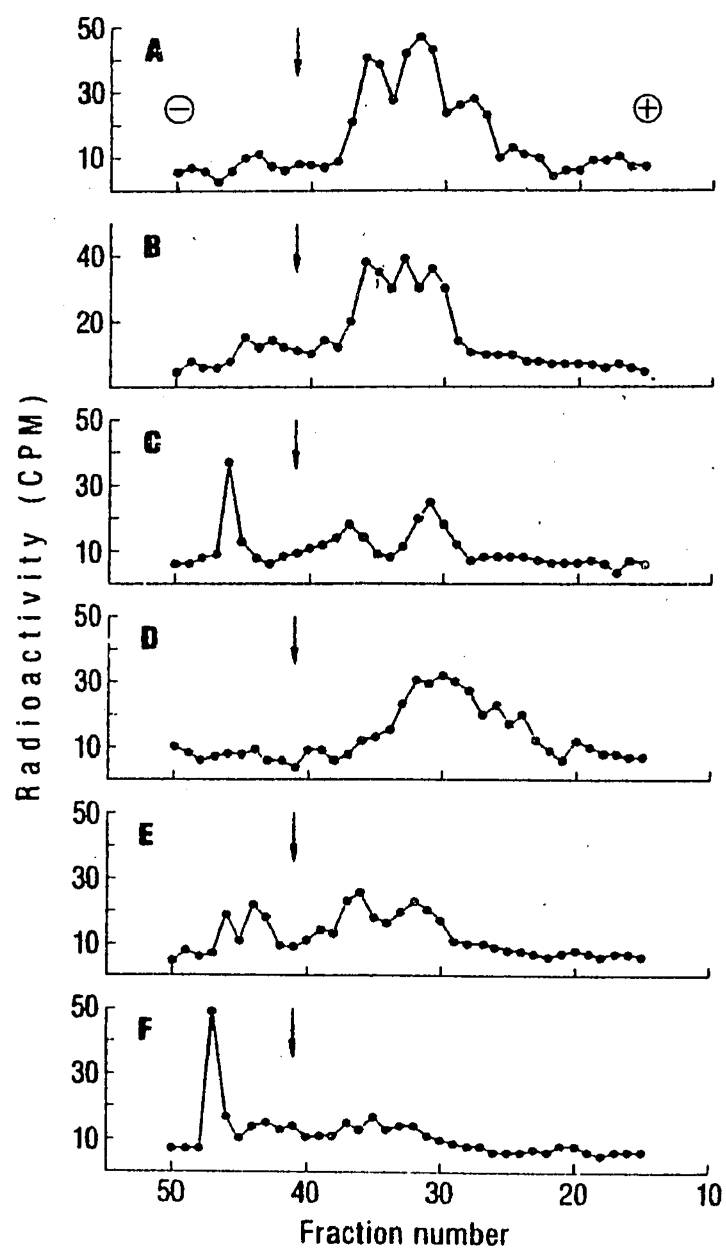
Table 7

Analysis of oligomannosyl cores released from complex glycopeptides digested with endo-β-N-acetylglucosaminidase D

Glycopeptide Sample	Percentage of oligomannosyl core released with endo-β-N-acetylglucosaminidase D[a]
growing A	16
non-growing A	61
growing B	47
non-growing B	14
growing C	36
non-growing C	13
growing E	27
non-growing E	53

[a]Complex glycopeptides were digested with endo-β-N-acetylglucosaminidase D in the presence of β-galactosidase, β-N-acetylglucosaminidase and neuraminidase as described in Materials and Methods and the products analyzed by paper chromatography as described in Figure 17. Results are expressed as the percentage of radioactivity released as oligosaccharide (fractions 12-19) relative to the total counts per minute recovered on the chromatogram.

Figure 17. Electrophoretic analysis of glycopeptide E. Intact material from non-growing and growing peak E was applied to Whatman 1 MM paper and run in pyridine: acetic acid:water, 10.0:0.4:89.6, pH 6.5. Non-growing (1720 cpm) and growing glycopeptides (1160 cpm) were digested with 10 units of neuraminidase. Non-growing E (1550 cpm) and growing E (1740 cpm) were also treated by mild acid hydrolysis. Aliquots from both experiments were analyzed by HVPE as described in the text. a) Intact, non-growing; b) Neuraminidase digested, non-growing; c) Acid treated, non-growing; d) Intact, growing; e) Neuraminidase digested, growing; f) Acid treated, growing. The arrow indicates the origin (fraction 41).


A
B
C
D
E
F
Radioactivity (CPM)
Fraction number
50
40
30
20
10

less than 10% appeared to be neuraminidase susceptible as judged by the criteria of migrating to the neutral region on HVPE. This agreed well with the failure of neuraminidase to decrease the apparent molecular weight of peak E (Table 6). However, mild acid hydrolysis with H_2SO_4 (0.1 N H_2SO_4 at 80° for one hour in sealed vials) shifted 32% of the material to the neutral region (fractions 46-42, Fig. 17C) on HVPE at pH 6.5. The remaining 68% continued to migrate to the positive pole. The data suggest that the peptide moiety appears to impart net negative charge to non-growing peak E. The possibility that the sugar moiety after mild acid hydrolysis may also contribute negative charge cannot at present be excluded.[1] Further, the profile of intact non-growing peak E (Fig. 17A) on HVPE suggests that it is heterogeneous and contains sialic acid residues resistant to neuraminidase but removable by mild acid hydrolysis (Fig. 17B, C). Those species resistant to neuraminidase would also be resistant to endo-β-N-acetylglucosaminidase D (Muramatsu, 1978).

Intact growing peak E migrated to the positive pole on HVPE at pH 6.5 (Fig. 17D). After treatment with neuraminidase 75.0% of the radioactivity still migrated to the positive pole and 25% to the neutral region (Fig. 17E).[2] Therefore, in general, intact material from growing peak E

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[1]Phosphate and sulfate groups can be excluded, the former would have been removed by mild acid hydrolysis. Furthermore, labeling cells with $H_2(^{35}S)O_4$ did not label the glycopeptide region as observed in the intial Sephadex G-50 column.

[2]It should be mentioned that although 25% of peak E from growing cells shifted to the neutral region on HVPE (Fig. 17E), a decrease in apparent molecular weight was not detected by Sephadex G-50 chromatography (Table 6). This may be due to the insensitivity of the column chromatography to detect small increments of change, which in this case was not sufficient to appreciably change the average molecular weight of the glycopeptides.

appears to contain species which are more susceptible to the action of neuraminidase than non-growing peak E. Mild acid hydrolysis to remove terminal sialic acid residues, shifted 60% of the mannose-label to the neutral region (Fig. 17F). However, a greater percentage of the sialic acid in the growing cells is resistant to neuraminidase (35% versus 22%) (Table 8).

In summary, both growing and non-growing peak E are heterogeneous containing at least 3 subclasses of glycopeptide: 1) those that are neuraminidase susceptible; 2) those that are neuraminidase resistant; and 3) those that migrate as acidic species but probably not containing sialic acid. With respect to growth dependent alterations it was noted that a) non-growing peak E contains a greater proportion of "apparent" acidic species after mild acid hydrolysis than growing peak E (Table 8, data in parentheses); b) growing peak E contains a greater proportion of species susceptible to neuraminidase than non-growing peak E (Table 8); c) growing peak E also contains a slightly larger proportion of sialic acid species which are resistant to neuraminidase but whose sialic acid residues could be removed by mild acid hydrolysis (Table 8) and d) non-growing peak E is more susceptible to endoglycosidase D, releasing almost twice as much oligosaccharide as growing peak E (Fig. 16). This last result would appear paradoxical since glycopeptide(s) E is still mainly acidic after treatment with neuraminidase (Fig. 17B, E). These results might indicate the presence of neuraminidase resistant species, and thus should not be susceptible to endoglycosidase D (Muramatsu, 1978). The fact that the intact glycopeptide(s) E releases more oligosaccharide than expected indicates that some portion of this heterogeneous class probably contains amino acids which impart a net negative charge to the

Table 8

Analysis of glycopeptide E on high voltage paper electrophoresis[a]

	Glycopeptide Sample	
	Growing E	Non-growing E
Neuraminidase	25 (75)	10 (90)
0.1 N H_2SO_4	60 (40)	32 (68)
Neuraminidase resistant[b]	35	22

[a]Intact, neuraminidase treated and mild acid hydrolyzed glycopeptide E were analyzed by high voltage paper electrophoresis at pH 6.5 as described in Figure 18. Results are expressed as the percentage of radioactivity migrating to the neutral zone (fractions 46-42) relative to the total counts per minute recovered. Numbers in parentheses represent radioactivity migrating to the positive pole.

[b]That material which failed to migrate to the neutral zone after neuraminidase digestion but behaved as neutral after mild acid hydrolysis.

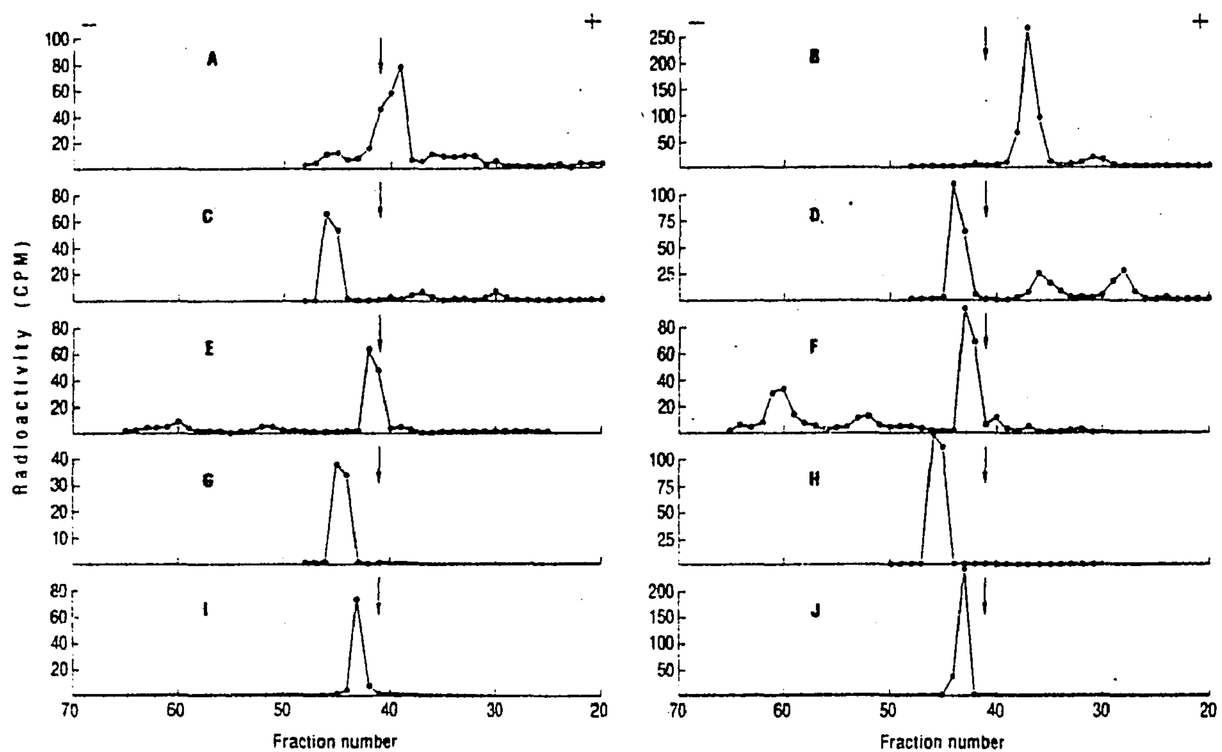
glycopeptide and could therefore be susceptible to endoglycosidase D digestion. However, the data also suggest that some resistance to endo-β-N-acetylglucosaminidase D should be due to undigested or incompletely digested glycopeptides after neuraminidase treatment (cf. Table 8).

Endo-β-N-acetylglucosaminidase D susceptibility of glycopeptide B

Tritium-labeled glycopeptides isolated as peak B (Fig. 15A, B) were digested with endo-β-N-acetylglucosaminidase D (5580-11,140 cpm, 0.0185 enzyme units with 0.0052 units β-galactosidase, 0.037 units β-N-acetylglucosaminidase and 10 units neuraminidase) and the products separated into oligosaccharide and resistant material by paper chromatography in Solvent I, as described in Materials and Methods. (All of the radioactivity initially applied to the paper was recovered either as oligosaccharide or resistant material.)

Aliquots of the oligosaccharide, resistant material and intact glycopeptide were subjected to HVPE at pH 6.5 and 1.9. Intact glycopeptides migrate to the positive pole at pH 6.5 (Fig. 18A, B). The non-growing species migrates 2-3 cm faster than the growing and appears to be less heterogeneous. Resistant glycopeptides from growing and non-growing cells (fractions 0-6 on paper chromatography) migrated 78% and 60%, to the neutral region on HVPE at pH 6.5, respectively (Fig. 18C, D). From this data it may be concluded that a large proportion of this material, in either state of growth, is neuraminidase susceptible. The remaining 22% and 40%, respectively, may be due to incomplete removal of sialic acid residues by neuraminidase or to the peptide and/or remaining oligosaccharide moiety imparting net negative charge. When the growing or non-growing resistant material was analyzed on HVPE at pH 1.9 it migrated primarily to the neutral region. However, a marked amphote-

Figure 18. Electrophoretic analysis of glycopeptide B. Growing peak B (5580 cpm) and non-growing peak B (11,140 cpm) were digested with endo-β-N-acetylglucosaminidase D, and separated into oligosaccharide and resistant material as shown in figure 17. Aliquots of intact material, resistant material and oligosaccharide were loaded onto Whatman 1 MM paper and run in pyridine:acetic acid:water, 10.0:0.4:89.6, pH 6.5 or 7% formic acid, pH 1.9 as described in the text. a) Intact, growing peak B, pH 6.5; b) Intact, non-growing peak B, pH 6.5; c) Growing resistant material, pH 6.5; d) Non-growing resistant material, pH 6.5; e) Growing resistant material, pH 1.9; f) Non-growing resistant material, pH 1.9; g) Growing oligosaccharide, pH 6.5; h) Non-growing oligosaccharide, pH 6.5; i) Growing oligosaccharide, pH 1.9; j) Non-growing oligosaccharide, pH 1.9.



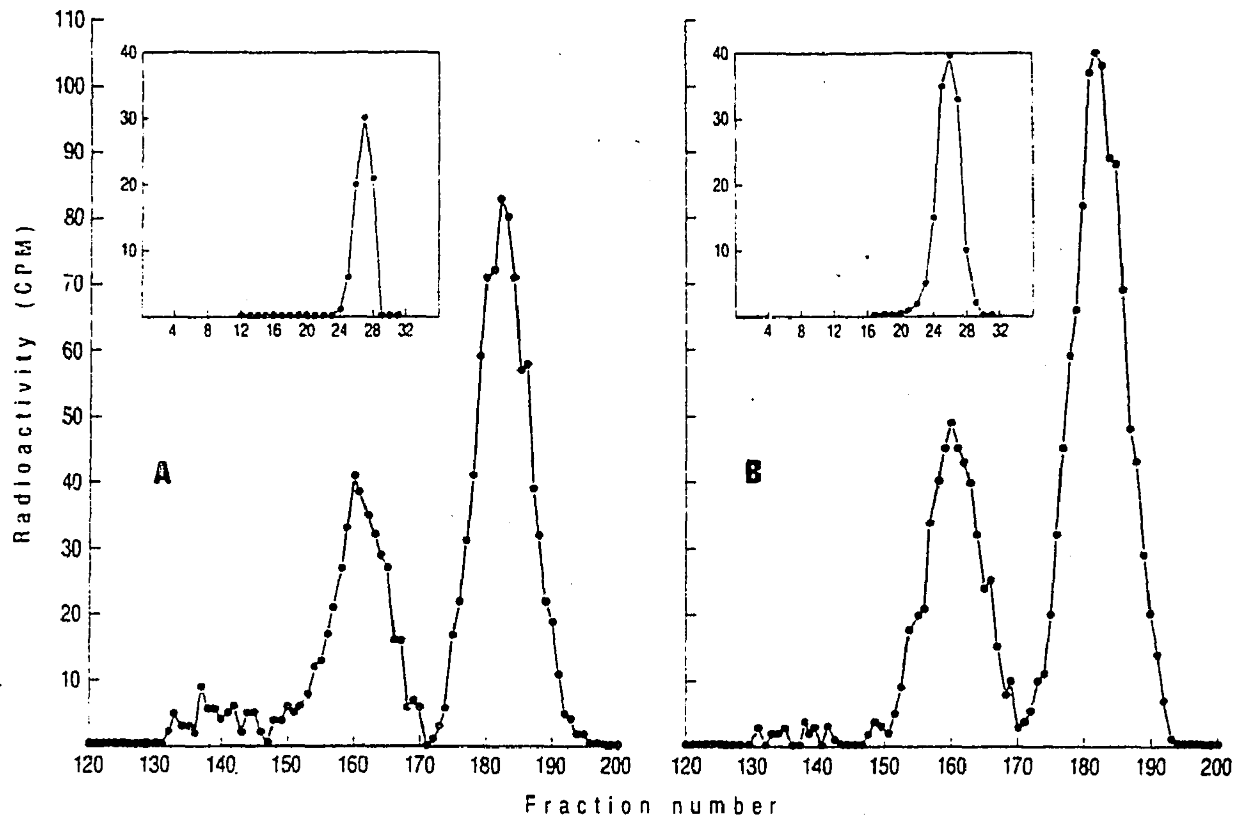
A
B
C
D
E
F
G
H
I
J
Radioactivity (CPM)
Fraction number
Fraction number

ric behavior was observed in that 32-46% of the label migrated further toward the negative pole (Fig. 18E, F). The characteristic migration on paper chromatography as well as on HVPE are indirect evidence that the resistant material contains amino acids and thus is still a glycopeptide. The fast moving mannose-labeled product isolated by paper chromatography migrated solely to the neutral region both at pH 6.5 and 1.9 (Fig. 18G, H, I, J). These results indirectly imply that this material is oligosaccharide.

Analysis of the released oligosaccharide by mannosidases

A second preparation of intact glycopeptide B was digested with endo-β-N-acetylglucosaminidase D (8920 cpm growing material and 11,140 cpm non-growing material) and separated into oligosaccharide and resistant material. Aliquots of the oligosaccharide(s) recovered from the paper chromatogram were digested with jack bean α-mannosidase (1593-2650 cpm, 7.0 units α-mannosidase) and subsequently analyzed by Sephadex G-25 chromatography (Fig. 19A, B). The low molecular weight product co-eluted with authentic mannose. The ratio of radioactivity migrating with free mannose to the larger species was 2:1 and 2.1:1 for growing and non-growing oligosaccharides, respectively. The data suggest that the fast moving product released by endoglycosidase D digestion from intact peak B in either state of growth is an oligosaccharide having the following composition: $GlcNAc(Man)_3$. The radioactivity eluted from the Sephadex G-25 column was also analyzed by paper chromatography (Solvent II) and all migrated with either authentic mannose or authentic Man—β—GlcNAc. The mannose to disaccharide ratio was 2:1. When this material was digested with β-mannosidase (98 munits, 2 days at 37° under toluene) and chromatographed on paper after desalting with Amberlite

Figure 19. Sephadex G-25 analyses with α-mannosidase of oligosaccharide region released by endo-β-N-acetylglucosaminidase D. Released oligosaccharide from growing peak B (1593 cpm) and non-growing peak B (2650 cpm) was digested with α-mannosidase (7.0 units) as described in Materials and Methods, and fractionated by a column of Sephadex G-25, 0.9 x 145 cm. Fractions of 0.45 ml were collected at a flow rate of 5.4 ml per hour. An equal aliquot of each fraction was counted for radioactivity. Blue dextran and NaN_3 eluted at fractions 88 and 215, respectively. a) Growing oligosaccharide material; b) Non-growing oligosaccharide material. Inset: Equal volumes of fractions 147-196 were pooled, lyophilized and digested with β-mannosidase (98 m units). The digested material was desalted on Amberlite MB1 (0.5 x 3.0 cm), applied to Whatman 3 MM paper and run in butanol:pyridine:water, 6:4:3 for 18 hours.


Radioactivity (CPM)
Fraction number
A
B

MBl, only free mannose could be detected (Fig. 19 insets), since the radioactivity migrated similarly to authentic (^{14}C) mannose. No radioactivity could be detected in the region of authentic Man$\xrightarrow{\beta}$GlcNAc.

Aliquots of the glycopeptides pooled as resistant to endo-β-N-acetylglucosaminidase D were also digested with α-mannosidase (3000-4500 cpm, 7.0 units) and subsequently analyzed by Sephadex G-25 chromatography. It was found that 24% and 40% of the label, in growing and non-growing material respectively, was released as free mannose. The data imply that the resistant glycopeptides do contain some exposed α-mannosyl residues. However, the failure to release more mannose indicates that resistance to α-mannosidase may be due to incomplete removal of side chain sugars, thus preventing access to the mannosyl residues. This resistance may in part be due to a failure to remove sialic acid residues with neuraminidase, but may also involve galactosyl and/or N-acetylglucosaminyl residues (see discussion). It must be stressed that failure to remove side chain sugars by exo-glycosidase digestion may also be the reason for the failure of endo-β-N-acetylglucosaminidase D to act on these species. At present, the data cannot exclude steric hindrance by the peptide moiety or a lack of α-mannosyl residues as the reason for the resistance to α-mannosidase.

CHAPTER IV - STUDIES ON MANNOSE INCORPORATION IN GROWING AND DENSITY INHIBITED HUMAN FIBROBLASTS: PRELIMINARY DATA

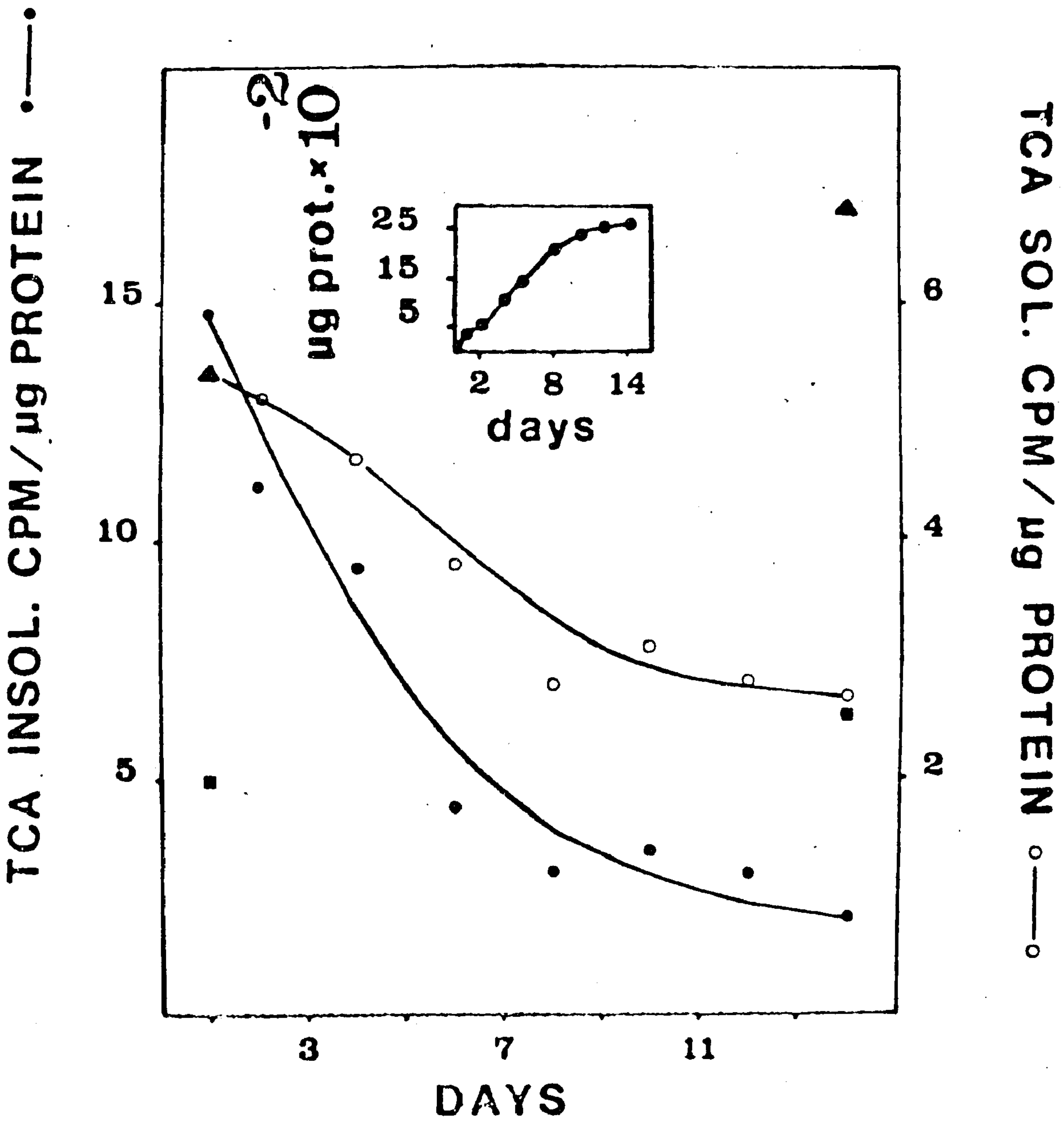
This chapter deals with the kinetics of assembly of mannose-containing glycoproteins into the plasma membrane of the human lung fibroblast, WI38. This cell was used instead of KL-2 because at the time of the experiments, KL-2 was not available in our laboratory. Other laboratories have shown that the time of entry of newly synthesized glycoproteins into plasma membranes occurs in minutes (15-30) rather than hours (Atkinson 1973; 1975; Hunt and Summers, 1976; Knipe *et al.*, 1977; Lamb and Choppin, 1977). In the work to be described here, the fate of the exogenous radioactive precursor was followed from the time it first appeared in the cytoplasm to its incorporation into the cell surface membrane. The transit time of the mannosyl glycoproteins from their initial site of synthesis to the plasma membrane has been estimated as a function of the growth status of the cell (Fig. 20 inset). The relationship of cell growth to apparent glycoprotein biosynthesis was also examined.

RESULTS

Glycoprotein synthesis and cell growth

In WI38 cells the accumulation of radioactive mannose into TCA soluble and insoluble material rapidly decreased during exponential growth while fucose accumulation increased (Fig. 20). A similar result for fucose accumulation has previously been observed (Ceccarini and Atkinson, unpublished observation). Thus it would seem that depending on which sugar is used as a probe, diametrically opposite conclusions about glycoprotein synthesis as a function of cell growth will be

Figure 20. Incorporation of radioactivity in growing and density inhibited cultures. WI38 cells incubated in T15 culture flasks were pulsed for 3 hours with D-(2-^{3}H)mannose (2 μc/ml, 1 Ci/mmol) or L-(6-^{3}H)fucose (2μc/ml, 13.4 Ci/mmol) on various days throughout a 14 day growth period. The protein, TCA soluble and TCA insoluble material were analyzed (Materials and Methods). TCA insoluble mannose cpm/μg protein ●——● ; TCA soluble mannose cpm/μg protein ○——○ ; TCA insoluble fucose cpm/μg protein ■ ; TCA soluble fucose cpm/μg ▲ . Inset: Growth curve; replicate T15 culture flasks were collected on days 1, 2, 4, 6, 8, 10, 12, 14 and protein concentration determined (Materials and Methods).


TCA INSOL. CPM/µg PROTEIN
TCA SOL. CPM/µg PROTEIN
15
10
5
6
4
2
3
7
11
DAYS
µg prot. x 10-2
25
15
5
2
8
14
days

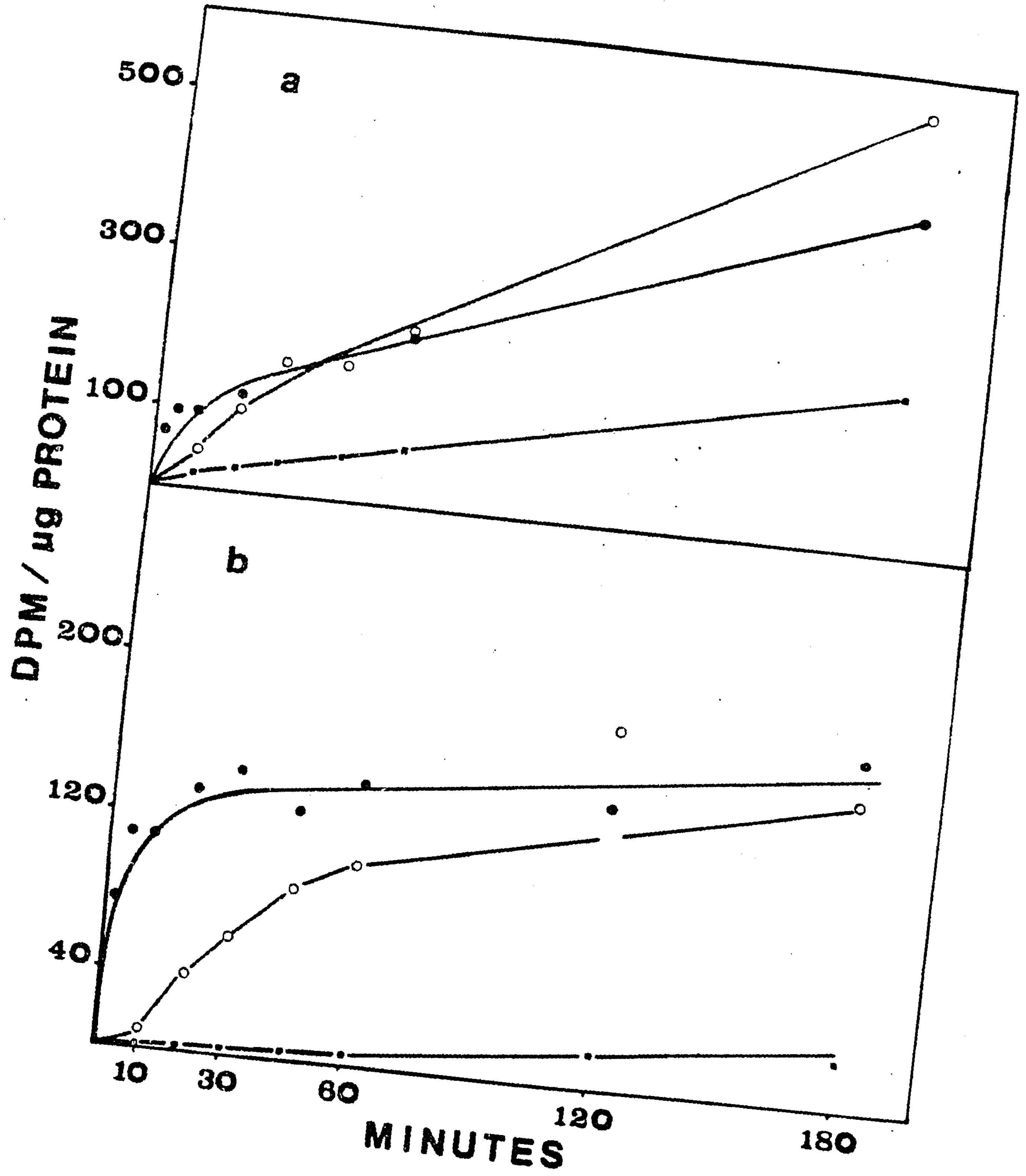
obtained. This effect would appear to be paradoxical but may be explained by alterations in the endogenous pools of the two sugars as cell confluency is approached. That is, the specific radioactivity of the intracellular sugar pools are subject to the contributions of both the exogenous radioactive precursor as well as the endogenous synthesis and storage of the sugar (see discussion). Whatever the reason, it is significant to note that mannose and fucose appear to exhibit an inverse relationship for glycoprotein synthesis as a function of cell growth.

<u>Accumulation of newly synthesized glycoprotein</u>

Cell cultures were labeled with (^3H) mannose and the distribution of radioactivity incorporated into newly made glycoproteins on the cell surface, cell remainder and ethanol soluble (cell soluble) fractions examined as a function of time. Cell surface glycoproteins are defined as that material which is removed by mild proteolytic digestion with Pronase. The ethanol insoluble radioactivity remaining after the Pronase treatment is defined as cell remainder and the ethanol soluble fraction represents free mannose precursors. The accumulation of newly synthesized glycoprotein on the cell surface and cell remainder was expressed as the radioactivity per µg of cell remainder protein. The cell soluble fraction was expressed as the radioactivity per µg of total cell protein.

Accumulation of mannose into the cell soluble fraction occurred with little discernible lag in both the growing and non-growing cultures (Fig. 21a, b), and probably appears within the cytoplasm in less than 1 minute. On the other hand, the accumulation of newly made cell surface glycoprotein in the non-growing cultures appeared with a clear lag (Fig. 21b). Extrapolation to zero from the more linear part of

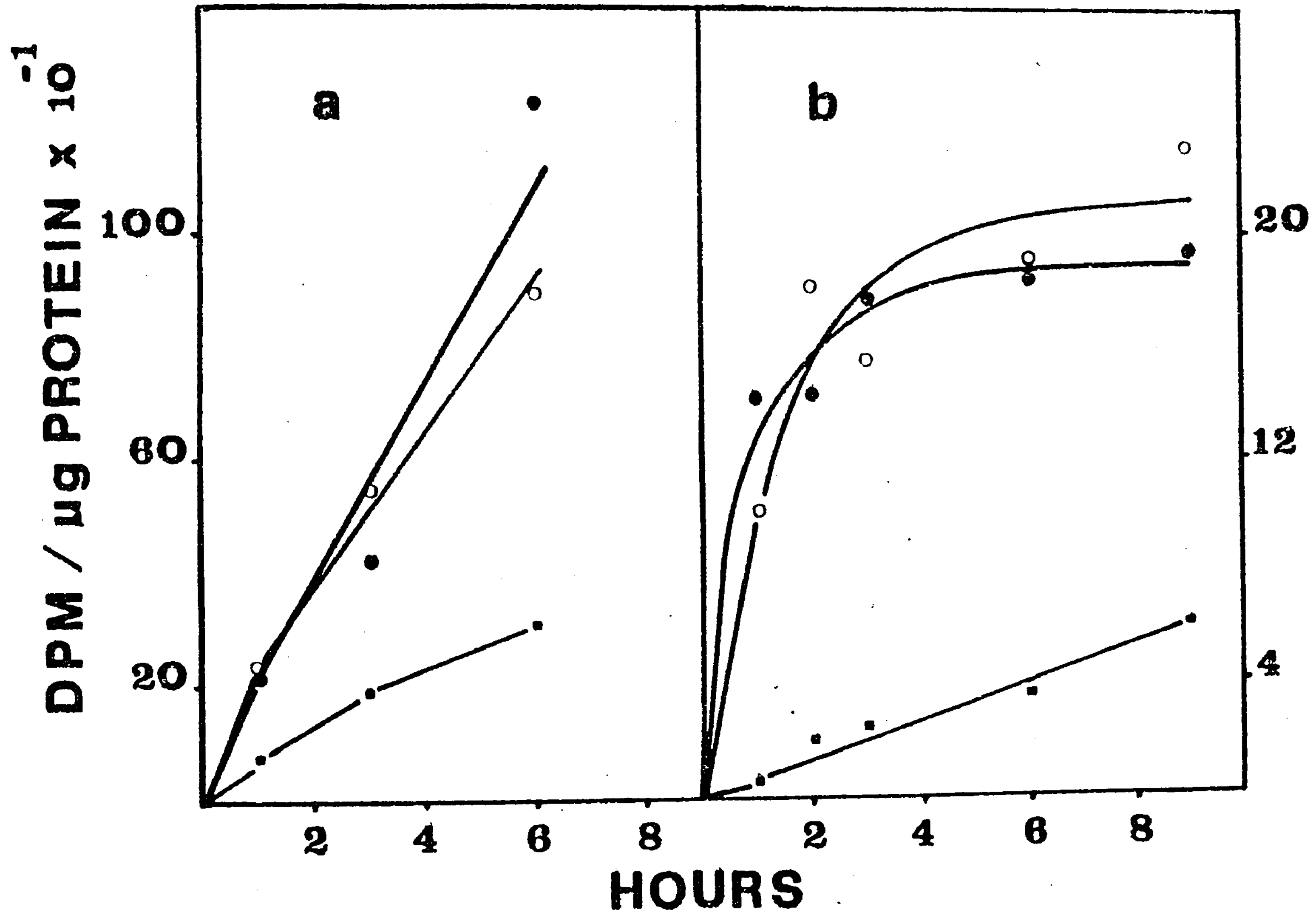
Figure 21. Accumulation of soluble precursor and newly synthesized glycoprotein in cells and plasma membrane. Cells were labeled with D-(2-^{3}H)mannose (5 µc/ml, 1.0-1.5 Ci/mmol) on day 5 and day 14 after plating. At selected labeling times replicate flasks were analyzed for cell soluble and insoluble content, and two additional flasks processed for cell surface material and cell remainder (Materials and Methods). A Growing cells, ●——● accumulation of cell soluble dpm/µg protein, ○——○ cell insoluble dpm/µg protein, ■——■ surface dpm/µg protein; B Non-growing cells, ●——● accumulation of cell soluble dpm/µg protein, ○——○ cell insoluble dpm/µg protein, ■——■ surface dpm/µg protein. The cellular insoluble (remainder) material plotted represents that material remaining after mild proteolytic digestion to remove surface glycopeptides (Materials and Methods).


a
b
500
300
100
200
120
40
DPM / µg PROTEIN
10
30
60
120
180
MINUTES

the curve indicates that the lag period is approximately 40 minutes (Atkinson, 1975). The accumulation of intracellular glycoprotein (cell remainder) proceeds with a lag of about 8 minutes (6-10). Therefore it takes about 32 minutes for newly synthesized mannose-containing glycoprotein to be transported to the surface. In contrast to this, the accumulation of newly synthesized glycoprotein on the surface of growing cells seems to proceed at a more rapid rate (Fig. 21a). It was not easy to determine a lag time but it certainly appears to be less than 30 minutes. Likewise, the accumulation of intracellular glycoprotein appears to begin within 3 minutes (2-5). Thus it would seem that, in general, the time it takes for glycoproteins to be synthesized, mixed with the pre-existing glycoprotein pool, and transported to the cell surface is faster in a growing cell population than a non-growing culture.

The same experiment was repeated over a period of hours rather than minutes to determine the uptake characteristics of mannose in both states of growth for longer time points. The accumulation of mannose into the cell soluble fraction, cell remainder and cell surface glycoprotein was linear over a 6 hour period in the growing cells (Fig. 22a). In comparison, the cell soluble and cell remainder pools appear to approach constant specific radioactivity during a 9 hour period in the non-growing cultures (Fig. 22b). However, the cell surface glycoprotein continues to accumulate in a linear fashion (Fig. 22b). At no time during the course of the experiment did the specific radioactivity of the cell surface glycoproteins exceed the cell remainder in either state of growth. The data suggest that the intracellular pool of cell soluble material and cellular glycoprotein (cell remainder) may

Figure 22. Long term accumulation of soluble precursor and newly synthesized glycoprotein in cells and plasma membranes; same as described in the legend of figure 22, except for time points of longer incubation with D-(2-^{3}H) mannose. A Growing cells, ●——● accumulation of cell soluble dpm/µg protein, ○——○ cell insoluble dpm/µg protein, ■——■ surface dpm/µg protein; B Non-growing cells, ●——● accumulation of cell soluble dpm/µg protein, ○——○ cell insoluble dpm/µg protein, ■——■ surface dpm/µg protein.


a
b
DPM / µg PROTEIN x 10^{-1}
100
60
20
20
12
4
2
4
6
8
2
4
6
8
HOURS

be smaller in the non-growing cells relative to the growing. On the other hand, the data cannot exclude the possibility that the internal pools in the growing cells are smaller than those of the non-growing cells but are subject to expansion in the presence of exogenous mannose (see discussion).

CHAPTER V - DISCUSSION

QAE Sephadex chromatography

The mixture of cellular glycopeptides derived from cultures of growing and non-growing cells were analyzed by QAE Sephadex chromatography. Using this technique it was possible to fractionate both the simple and complex glycopeptides with a single passage through the column. Growth dependent alterations were reproducibly evident at this stage of the purification. The higher molecular weight neutral glycopeptides in non-growing cells were markedly reduced relative to the growing cells (Fig. 2a, Table 2). A similar result was previously reported using neutral cellular glycopeptides isolated by high voltage paper electrophoresis and specific endo-β-N-acetylglucosaminidases (Muramatsu *et al.*, 1976). Thus it appears that the use of ion exchange chromatography will prove to be useful for detection of subtle biological differences utilizing large quantities of intact glycopeptides.

In a separate set of experiments, the cell surface glycopeptides from a population of exponentially growing and density inhibited cells were also examined by QAE Sephadex chromatography. A second growth dependent alteration was evident upon close examination of the cell surface neutral glycopeptides (Fig. 4a, b). Table 2 summarizes the distribution of mannose-labeled material present in each of the neutral species. As with the growing cellular glycopeptides, the predominant neutral species on the growing surface was peak I. Further, the distribution of cellular glycopeptides was closely reflected on the cell surface. In contrast to this, the predominant neutral species on the non-growing surface was peak II, with a concurrent decrease in peak I. It may be significant that the

distribution of neutral species associated on the non-growing cell surface does not, however, reflect the distribution of cellular glycopeptides (Table 2).

Therefore, it would appear that depending on the state of growth, cells are able to mediate the proportion of neutral species associated with the cell surface, irrespective of the distribution of neutral species present as cellular glycopeptides. The cellular glycopeptides are here defined as the intracellular pool of glycoproteins destined for transport to the cell surface, but must also include the structural glycoproteins of intracellular membranes and organelles, glycoproteins secreted to the medium and plasma membrane glycoproteins resistant to the mild proteolytic treatment. The complexity of the system makes interpretation difficult. Nevertheless, growth dependent alterations were apparent and a number of possibilities to explain the disproportionate accumulation of non-growing surface neutral glycopeptides are listed: 1) preferential selection of specific intracellular glycoproteins for transport to the cell surface, i.e., specific glycosylated proteins may be retained intracellularly (for structural purposes or reserve pool) or selectively secreted to the medium while others are destined for transport to the cell surface; 2) differential rates of degradation of neutral species on the cell surface, i.e., one class of neutral species may be protected from protease attack due to interactions with the substratum, while others are more exposed; and 3) enhancement of specific neutral species may be a surface mediated event, i.e., activation of cell surface glycosyl transferases at cell confluency. With respect to the second point, it should be stressed that the data cannot exclude the possibility that the purported growth dependent alteration is subject to the

conditions of the mild proteolytic digestion used to harvest the surface material. That is, specific neutral glycoproteins may be cryptic in the non-growing state and not so readily available to the Pronase digestion. Whichever the reason, it is of considerable interest to note that neutral species I and II exhibit an inverse relationship on the cell surface subject to the growth status of the cell. The biological significance of this is yet to be demonstrated.

Table 1 summarizes the percentage of mannose-labeled material recovered as simple (neutral) glycopeptides and complex (acidic) glycopeptides associated with growing and non-growing cells and surfaces. Approximately 60% of the mannose-labeled cellular glycopeptides, from both states of growth, were present as neutral glycopeptides. The reverse situation is found on the cell surface, where about 40% of the mannose-label is associated with neutral glycopeptides and 60% as acidic species. Thus it may be concluded that, in general, cellular glycopeptides are enriched in neutral species, whereas the surface glycopeptides are enriched in acidic species. It was also noted that the ratio of neutral to acidic glycopeptides on the growing surface was slightly higher than that of the non-growing surface. Similar conclusions have previously been suggested by independent methods (Ceccarini _et al_., 1975; Muramatsu _et al_., 1976). The following pattern begins to emerge, cells are able to 1) mediate an accumulation of acidic species on the cell surface, 2) restrict the relative amount of neutral species, and 3) exercise an even finer tuning in that the distribution of neutral species associated with the cell surface may be subject to parameters regulated by the growth status of the cells.

During the past several years a great deal of information has been accumulated on the neutral glycopeptides of KL-2 human fibroblasts (Muramatsu et al., 1973; Ceccarini et al., 1975; Muramatsu et al., 1976). The novelty of the approach used by these investigators has been the use of specific endo-β-N-acetylglucosaminidases to convert the mixture of neutral glycopeptides isolated by high voltage paper electrophoresis into an array of mannosyl oligosaccharides, and paper chromatography to infer the structure of the glycopeptides. The disadvantage of using high voltage paper electrophoresis as an initial purification step for obtaining neutral and acidic glycopeptides is that it suffers from a limited ability to accomodate large quantities of material, and neutral glycopeptides so isolated appear to be homogeneous (Fig. 3). However, when this neutral region was previously studied using specific endo-β-N-acetylglucosaminidases it clearly contained several glycopeptide species (Muramatsu et al., 1976). For this reason, studies were initiated using ion exchange chromatography as a potential means of fractionating both neutral and acidic glycopeptides. In order to test the validity of the method, my initial studies utilized the cellular neutral glycopeptides of KL-2 human fibroblasts, thus providing a means of confirming the technique. The method appears to be sensitive enough to detect growth dependent alterations (Fig. 2a). At least five distinct neutral species could be obtained.

Cellular simple glycopeptides

Other laboratories, principally that of Dr. Rex Montgomery, have used Dowex AG-50W x 2 to fractionate a mixture of Pronase-digested ovalbumin glycopeptides into relatively homogeneous sub-classes (cf. Fig. 24). Each of the ovalbumin glycopeptides was found to contain no amino acids

other than L-asparagine. Glycopeptides containing several amino acids could be recovered by elution with a sodium acetate buffer of higher molarity and pH (Huang *et al.*, 1970). A similar method was used in the present studies. The QAE Sephadex-derived neutral glycopeptides could be further purified by Dowex AG-50W x 2, under conditions similar to that described for the fractionation of ovalbumin glycopeptides (cf. Materials and Methods, Fig. 5).[1] No further radioactivity could be recovered by elution with 0.05 M sodium acetate buffer, pH 6.0. By comparison, the data indirectly provides evidence that the cellular neutral glycopeptides must contain very few amino acids, possibly only L-asparagine. The cellular neutral glycopeptides could be separated into five fractions by QAE Sephadex chromatography and appeared to be homogeneous (Fig. 2a). Nevertheless, heterogeneity persisted; peaks II-IV each contain more than one glycopeptide component (Fig. 5b-d). The data strongly suggest that the apparent heterogeneity must be due to the carbohydrate moieties of the glycopeptides. Of particular interest is peak I, which eluted first from both the QAE Sephadex and Dowex resin as a single, sharp component (Figs. 2a, 5a). The mixture of ovalbumin glycopeptides contain a fraction which behaves in a similar fashion on Dowex AG-50W x 2 (Huang *et al.*, 1970; Conchie and Strachan, 1978). That material (designated as peak F or fraction 1) has a composition of 8 mannosyl residues, 5 hexosamines, 6-13% galactose and 1 asparagine residue (Conchie and Strachan, 1978). While the molecular weight of such a species far exceeds the apparent molecular weight determined for

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[1] In general, the structure of the ovalbumin glycopeptides are probably similar to the neutral glycopeptides (Muramatsu *et al.*, 1976; Tai *et al.*, 1975a).

peak I, the possibility that terminal galactosyl residues influence the migration of peak I on both QAE Sephadex and Dowex AG-50W x 2 should not be overlooked.

Overall, approximately 80% of the cellular neutral glycopeptides isolated by QAE Sephadex chromatography and subsequently purified by Dowex AG-50W x 2 chromatography were resistant to endoglycosidase D, but susceptible to endoglycosidase H (Figs. 7, 8). The data suggest that the growing cellular neutral glycopeptides are enriched in species with oligomannosyl cores greater than $GlcNAc(Man)_5$. Although, the data does not exclude the possible presence of Asn $(GlcNAc)_2(Man)_3(X)$, where X is another mannosyl residue linked to the α1,3 mannosyl residue and thus would predictably be resistant to endoglycosidase D hydrolysis. A similar result was previously reported for the growing cellular neutral glycopeptides isolated by high voltage paper electrophoresis (Muramatsu _et al._, 1976).

Surface simple glycopeptides

Previous studies on the cell surface glycopeptides derived from the human fibroblast, KL-2, utilized endo-β-N-acetylglucosaminidase D (Ceccarini _et al._, 1975). It was found that neutral glycopeptides from growing cells were more resistant to the endoglycosidase but more susceptible to α-mannosidase than material derived from non-growing cells. It was suggested that the endoglycosidase D resistant material consisted of large oligomannosyl core glycopeptides similar to Unit A of thyroglobulin (Arima and Spiro, 1972). However, direct confirmation of this was lacking since the material was only analyzed with α-mannosidase. The present studies utilized endo-β-N-acetylglucosaminidase H, an enzyme with known specificity for the high mannose type glycopeptides (Tarentino

and Maley, 1974; Arakawa and Muramatsu, 1974).

The data establishes that endo-β-N-acetylglucosaminidase H releases oligosaccharides from the cell surface neutral glycopeptides which are similar to those previously reported for cellular glycopeptides (Muramatsu et al., 1976). Both the growing and non-growing surface neutral glycopeptides are highly susceptible to endoglycosidase H hydrolysis, converting better than 80% of the label into "large core oligosaccharides" and leaving relatively little radioactivity at the origin (Fig. 9a). However, it was surprising to find that if the endoglycosidase H digestion was performed in the presence of β-N-acetylglucosaminidase and β-galactosidase a portion of the "large core oligosaccharides" could be converted to "medium core oligosaccharides," ranging from $GlcNAc(Man)_6$ to possibly $GlcNAc(Man)_4$ (Fig. 9b). The data strongly suggest that the cell surface of KL-2 fibroblasts in either state of growth is enriched with neutral glycopeptides whose carbohydrate moieties are susceptible to endoglycosidase H hydrolysis. Of these, some portion must be substituted with N-acetylglucosamine and/or galactose. The data also confirm the existence of large oligomannosyl cores, greater than $GlcNAc(Man)_6$. These species probably contain 7-8 mannosyl residues and are probably unsubstituted, as previously suggested (cf. Table 4; Muramatsu et al., 1976). Susceptibility of the cell surface neutral glycopeptides to endoglycosidase H does not seem to be markedly growth dependent (Table 4).

All of the surface neutral glycopeptides are highly susceptible to endo-β-N-acetylglucosaminidase H hydrolysis, the amount of "large core oligosaccharides" released dependent upon which peak is examined and its growth status (Fig. 10). However, when peaks I and II were digested with endoglycosidase D, in the presence of β-N-acetylglucosa-

minidase, β-galactosidase and neuraminidase, a significant portion of the neutral material could be converted into low molecular weight oligosaccharides (Fig. 13), the majority of which were shown to be GlcNAc$(Man)_3$ (Fig. 14). Susceptibility to endoglycosidase D appears to be growth dependent. Non-growing peaks I and II release more oligosaccharide material than do their growing counterparts (Fig. 13). A similar result has previously been reported using other techniques (Ceccarini _et al._, 1975). The data indicate that a significant portion of the neutral glycopeptides which comprise surface peaks I and II must be susceptible to both endoglycosidase H (releasing "large core oligosaccharides") and endoglycosidase D, in the presence of β-N-acetylglucosaminidase, β-galactosidase and neuraminidase (releasing "small oligomannosyl cores") (Figs. 10, 13). Such a species could have a composition of Asn$(GlcNAc)_2(Man)_{3-5}(GlcNAc)_a(Gal)_b$, where a and/or b can only be indirectly demonstrated with the present techniques.

The substrate specificity of endo-β-N-acetylglucosaminidase H has been examined with substrates of known structure. Endoglycosidase H could act on calf thyroglobulin Unit A, but could not hydrolyze intact or "side-chain-free" porcine thyroglobulin Unit B or bovine IgG glycopeptides (Arakawa and Muramatsu, 1974). Treatment of these glycopeptides with α-L-fucosidase could not convert them to susceptible species. These workers routinely used 25 munits of H enzyme in their studies. Other workers have demonstrated that while intact and "side-chain-free" IgM glycopeptides are resistant to endoglycosidase H, a fucose-depleted, "side-chain-free" IgM glycopeptide was susceptible, releasing GlcNAc $(Man)_3$ (Tarentino and Maley, 1975). It was observed that a relatively large amount of enzyme was required to effect the hydrolysis. Complete

hydrolysis could be achieved in 24 hours with 100 munits of enzyme. The reason for the difference between these two results has not been fully resolved. Furthermore, it has been shown that all of the ovalbumin glycopeptides are completely hydrolyzed by the H enzyme (Tai _et al._, 1975a). While the complete structure of all the species remains to be determined, compositional data suggest that some of the species terminate with N-acetylglucosamine and contain 5-6 mannosyl residues. No fucose has ever been detected in the ovalbumin glycopeptides. The studies reported here utilized approximately 50 munits of endoglycosidase H and would appear to indicate that some neutral species with a possible core structure of $GlcNAc(Man)_3$ were susceptible to the H enzyme when substituted with N-acetylglucosamine and/or galactose (cf. Table 4, resistant material).

When the surface neutral glycopeptides were treated with endo-β-N-acetylglucosaminidase H in the presence of β-N-acetylglucosaminidase and β-galactosidase, approximately 70% of the mannose-label in either state of growth could be converted into oligomannosyl cores of composition $GlcNAc(Man)_n$, where n equals 4-7 or 8 mannosyl residues (Table 4). No smaller products could be detected when the paper chromatogram was developed for a shorter period of time, i.e., 3 days vs. 14 days. Since the neutral species represent 40% of the total surface glycopeptides, about 28% of the surface glycopeptides must have oligomannosyl cores larger than $GlcNAc(Man)_3$ (Table 1). Of this 28%, approximately 20% include the high mannose type, and are probably unsubstituted, containing 7-8 mannosyl residues. The remaining 8% consists of oligomannosyl cores ranging from $GlcNAc(Man)_6$ to possibly $GlcNAc(Man)_4$, and appears to be substituted with N-acetylglucosamine and/or galactose (Fig. 9).

The major oligomannosyl core on the surface of growing and non-growing cells is GlcNAc$(Man)_3$ and appears to be primarily associated with the acidic species (cf. Chapter III) but was also present in the neutral species (Fig. 14). The cell surface probably represents the final site of accumulation for these two classes of glycoproteins. The biological significance for the presence of these two types of carbohydrate moieties on the cell surface remains to be demonstrated.

Surface complex glycopeptides

Acidic glycopeptides from growing and non-growing cell surfaces were separated into three major peaks and several minor ones (Fig. 15A, B). The material designated as peaks A, B, C and E were pooled for further analysis. Peak D was too poorly resolved to warrant further analysis at this time.

With the exception of peak E, it was observed that intact glycopeptides from growing cell surfaces were always higher in molecular weight than non-growing material eluted at the same salt concentration (Table 6). This pattern could in part be explained by increased sialylation in the growing glycopeptides relative to the non-growing. The data indicate that the difference in sialic acid content between a growing and non-growing peak, eluted at the same salt concentration, is one residue (column 3, 4; Table 6). Peak E was of special interest in that it appeared to be identical in size in both growing and non-growing samples. It was also resistant to neuraminidase; however, its molecular weight was reduced from 3700 daltons to 3250 daltons in both states of growth after mild acid hydrolysis.

If the glycopeptides from growing and non-growing surfaces differed only in their extent of sialylation, it would have been expected

that identical molecular weights after neuraminidase digestion would have been observed. However, it was found that growth dependent alterations persisted, i.e., the growing glycopeptides were still larger than those derived from non-growing cells, the conclusion being that the differences in the peptide or the carbohydrate moiety remaining after the neuraminidase treatment probably account for these findings.[2]

The acidic glycopeptides digested with endo-β-N-acetylglucosaminidase D in the presence of β-galactosidase, β-N-acetylglucosaminidase and neuraminidase released a fast moving product which migrates similar to authentic $GlcNAc(Man)_3$ prepared from fetuin (Fig. 16). The amount released from each species appears to depend on the growth status of the cells (Table 7). The fast moving product from glycopeptide B was also examined by high voltage paper electrophoresis at pH 6.5 and 1.9 (Fig. 18G, H, I, J). Its nonamphoteric behavior was consistent with the conclusion that this material was an oligosaccharide. Subsequent digestion with α-mannosidase released free mannose and a purported disaccharide in a ratio of 2:1 (Fig. 19). Digestion with β-mannosidase released only free mannose (Fig. 19 inset). Thus the oligosaccharide contained two α-mannosyl residues and one β-mannosyl residue linked to N-acetylglucosamine. The data suggest that it has the following composition: $(Man\,\alpha)_2Man\,\beta\,GlcNAc$. The data are consistent with the suggestion that all of the acidic glycopeptides in KL-2 human fibroblasts that are susceptible to endoglycosidase D hydrolysis contain a common oligomannosyl

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[2]Since it has previously been demonstrated that in at least one case sialic acid residues could only be removed by mild acid hydrolysis, the data cannot exclude the possibility that some of these molecular weight differences can be explained by neuraminidase resistant species.

core. It should be stressed, however, that it is not presently possible to make any conclusions about the size of the oligomannosyl core of the resistant glycopeptides.

Not all of the mannose-label of the acidic glycopeptides released an oligosaccharide product when digested with endoglycosidase D. A varying amount of radioactivity remained at the origin (Fig. 16). The resistant material from glycopeptide B (Fig. 16) has been shown to have amphoteric properties on high voltage paper electrophoresis (Fig. 18C, D, E, F). However, when this material was extensively digested with α-mannosidase it released 24.0% and 40.0% free mannose in growing and non-growing samples, respectively. Since the majority of these resistant glycopeptides in either state of growth migrate as though they were neutral, it is assumed that they were neuraminidase susceptible (Fig. 18C, D). Therefore, the data suggest that incomplete removal of other sugar residues by β-galactosidase and/or β-N-acetylglucosaminidase prevents exposure of non-reducing terminal α-mannosyl residues. Other laboratories have shown that a suitable substance for endoglycosidase D must contain no sialic acid and have a mandatory unsubstituted α-mannosyl residue linked to the 3 carbon of the β-mannosyl residue (Ito et al., 1975a; Tai et al., 1975a).

Using peak E it was demonstrated that the majority of the material in either state of growth continues to migrate to the positive pole after neuraminidase treatment, i.e., less than 10% and 25% of the mannose-containing glycopeptides, for non-growing and growing peak E, respectively, appears to be neuraminidase susceptible (Fig. 17B, E). A larger shift of the label into the neutral region could be achieved by mild acid hydrolysis of the intact glycopeptides (Fig. 17C, F).

Presumably, hydrolysis in 0.1 N H_2SO_4 removes terminal sialic acid residues, the remaining glycopeptide should have no negative charge, and so migrate as a neutral species. However, it was found that 68.0% and 40% of the label from non-growing and growing glycopeptides, respectively, continued to migrate as though it retained net negative charge (Fig. 17C, F). The data suggest that peak E is comprised of at least 3 sub-classes of glycopeptides. One class was susceptible to neuraminidase; another was neuraminidase resistant but neutral after acid hydrolysis; and the last class appeared to retain an "apparent" net negative charge even after mild acid hydrolysis. It is suggested that the negative behavior of this last class of glycopeptides might involve amino acids or an as yet unidentified sugar residue. It was noted that all three sub-classes differ in their relative proportions in growing versus non-growing peak E. In particular, if the proteins glycosylated in either state of growth were identical, it would have been expected that subsequent to mild acid hydrolysis the proportion of "apparent" acidic material would be identical in non-growing and growing cells. This does not seem to be the case (Fig. 17C, F). It is postulated that this is indirect evidence for heterogeneity in the glycoproteins, i.e., depending on the state of growth different proteins may be glycosylated. Since these studies looked at glycopeptides and not intact glycoproteins, the possibility also exists that identical proteins are glycosylated in either state of growth, but different sites on the protein backbone are recognized (giving rise to glycopeptide fragments with different net charge). Other interpretations are possible, some of which might involve the sugar moiety or differential turnover.

Enzyme purity and reactivity

A matter of concern to any investigator planning to use specific glycosidases for structural analysis of glycopeptides is the degree of purity of the enzyme preparation and incomplete removal of sugar residues due to a difference in susceptibility to enzyme hydrolysis. The former can result in the generation of "false" products, while the latter will increase the complexity of the system by creating additional heterogeneity. It was clear from these studies as well as those of other workers that exo-glycosidase treatment often results in the partial release of sugar residues (Shepherd and Montgomery, 1978; Narasimhan et al., 1977; Kornfeld and Ferris, 1975; Baenziger and Kornfeld, 1974; Toyoshima et al., 1972). The incomplete removal of sialic acid from complex glycopeptide E best exemplifies this point. A significant portion of both growing and non-growing peak E retained net negative charge after treatment with neuraminidase, but could be made to act as a neutral glycopeptide in high voltage paper electrophoresis after mild acid hydrolysis (cf. Chapter III). The problem was again evident upon close examinination of endoglycosidase D-resistant glycopeptide B. When this material was digested with α-mannosidase, a partial release of α-mannosyl residues was obtained. This was attributed to the failure of the exo-glycosidases present in the endoglycosidase D mixture to release all terminal N-acetylglucosamine and/or galactosyl residues. An alternative explanation might evoke terminal α-mannosyl residues with heretofore unknown oligosaccharide structure, not susceptible to the jack bean α-mannosidase. Both cases could explain the apparent failure of endoglycosidase D to completely hydrolyze the complex glycopeptides examined during the course of these studies.

For any experiment utilizing Pronase and/or specific exo- and endoglycosidases as tools for glycopeptide analysis, the possibility of contaminating glycosyl asparaginase (Conchie and Strachan, 1978), endoglycosidases (Kawasaki and Ashwell, 1976), and exo-glycosidases (see below) must always be considered. That enzyme purity and reactivity may have influenced the apparent growth dependent release of α-mannosyl residues from surface neutral peaks I and II (cf. Chapter II) illustrates this point. More mannose could be released from the growing glycopeptides than from non-growing glycopeptides (Table 5), suggesting the possible presence of larger mannosyl clusters in the growing material. However, digestion of the total surface neutral glycopeptides with endoglycosidase H in the presence of β-N-acetylglucosaminidase and β-galactosidase indicated that the growing and non-growing material contained approximately equal amounts of "large core oligosaccharides," presumably $GlcNAc(Man)_{7-8}$ (Fig. 9b, Table 4). On the basis of migration on paper alone, it is not possible to determine if these "large oligomannosyl cores" terminate with N-acetylglucosamine or galactose, but the assumption is that they are mainly unsubstituted (Muramatsu _et al._, 1976). The other released oligomannosyl cores must terminate with N-acetylglucosamine and/or galactose in situ (Fig. 9a, b). The α-mannosidase used in this study was purified from jack bean meal (Snaith and Levvy, 1968). During the course of its purification it was noted that a single pyridine treatment did not eliminate all of the β-galactosidase and β-N-acetylglucosaminidase activity, whereas a second pyridine treatment produced α-mannosidase which was free of contaminating exo-glycosidases, but its specificity to the ovalbumin standards $Asn(GlcNAc)_2(Man)_5$ and $Asn(GlcNAc)_2(Man)_6$ was modified. For

this reason, a single pyridine treated preparation was used during the course of these studies, containing on the average less than 0.15% β-galactosidase and 0.12% β-N-acetylglucosaminidase. Therefore, the possibility exists that the apparent growth dependent release of α-mannosyl residues from surface neutral peaks I and II was the result of the α-mannosidase being contaminated by the two exo-glycosidases. Moreover, the preferential release of exposed α-mannosyl residues may in part be due to the difference in reactivity of the three exo-glycosidases to specific linkages and oligosaccharide structure. The α-mannosidase purified from jack bean meal is known to cleave Manα1-2Man and Manα1-6Man linkages faster than Manα1-3Man (Tai et al., 1975a).

The possible presence of glycosyl asparagine and endoglysosidase activity in the commercial Pronase used in these studies is suggested mainly by the data in figure 11a and f. However, the data also show the other neutral species do not contain any oligosaccharide material (Fig. 11). It has previously been reported that a glycosyl asparagine activity can be found in Pronase (Arima and Spiro, 1972). Endo-β-N-acetylglucosaminidase H is isolated from Streptomyces griseus (now plicatus), also the commercial source of Pronase (Tarentino and Maley, 1974). As Ashwell and co-workers have already observed, the presence of contaminating exo- and endoglycosidases should always be assumed with any specific glycosidase preparation until proof of purity is established (Kawasaki and Ashwell, 1976).

Mannose incorporation in growing and density inhibited cells

The biosynthesis of sugar-containing macromolecules may be studied by following the fate of radioactive precursors (for a review see Yurchenco et al., 1978). The preliminary experiments reported here

were initiated in order to investigate the kinetics of assembly of the asparagine-linked glycoproteins from their initial site of synthesis to their site of accumulation in the cell surface of growing versus non-growing fibroblasts. The data permits the following interpretation but other interpretations are possible. As the experiment in figure 20 demonstrates, WI38 cells maintained within a closed system rapidly decline in capability to incorporate mannose as cells approach confluency and become density inhibited. In fact, the trichloroacetic acid-extracted soluble material and the trichloroacetic acid insoluble material per microgram of protein decreased 2 fold and 7 fold, respectively, as cells advanced from an exponentially growing state to one of density inhibition. The reverse situation seems to be true for fucose incorporation. It has previously been demonstrated that radioactive precursors (uridine, thymidine and amino acids) are rapidly incorporated during exponential growth but declines sharply as cells become confluent (Levine et al., 1965). Other workers have shown that the capability of non-growing cells to incorporate radioactive precursor rapidly declines as the cells age (Warren and Glick, 1968). The data in the present experiment indicate that exponentially growing WI38 cells continually lose the ability to incorporate mannose. The cells were initally plated at low density, and exponential growth (days 1-5) appeared to be normal, leading eventually to density inhibition (days 12-14) (Fig. 20, inset). The reason for the decrease in glycoprotein biosynthesis is not clear. The following possibilities for the decline in glycoprotein biosynthesis as cells approach confluency include a) a decrease in glycosylation, b) decreased synthesis of protein precursors, c) decreased transport of completed glycoproteins to the cell surface

or medium (thereby diluting the label) and d) an alteration in the relative contribution of the exogenous radioactive precursor to the endogenous source of mannose. With respect to the last point, the decrease in glycoprotein biosynthesis may only be apparent, subject either to the dilution of the exogenous precursor from mannose leakage into the medium or an increased contribution from the endogenous synthesis and storage of mannose.

When long-term mannose incorporation was examined (hours rather than minutes) it was demonstrated that for non-growing cells, the ethanol-extracted soluble pool equilibrated within 1 hour, and the intracellular glycoprotein pool probably within 3 hours (Fig. 22b). However, the specific radioactivity (expressed as radioactivity per microgram of protein) of these pools accumulated in a linear fashion from 1 to 6 hours in exponentially growing cells (Fig. 22a). The data is consistent with but does not prove that the intracellular pool sizes might be smaller in density inhibited cells than for rapidly growing cells. The data cannot exclude the possibility that subsequent to addition of the exogenous precursor, the growing intracellular pools continually expand (at least for the duration of the experiment), whereas the non-growing pools have a finite size and cannot expand. In such a situation, the growing pools might not appear to equilibrate (irrespective of the initial size of the pool), while the non-growing pools would appear to do so. Bearing this in mind, it might be possible for the non-growing intracellular pools to be larger than those of the growing, but the inability of the former to expand upon addition of exogenous precursor may give the appearance of more rapid equilibration times.

A lag of approximately 15 minutes for fucose-containing glycopeptides to incorporate into HeLa cell plasma membranes has been attributed to the transit time of this material from an intracellular pool to the cell surface (Atkinson, 1973; 1975). Similar short-term incorporation studies were carried out with WI38 cells in order to approximate the transit time of mannose-containing glycoproteins from their site of synthesis to the cell surface. The data establish that, for non-growing cells, it takes about 32 minutes from the time that newly synthesized glycoprotein first enters the cytoplasm to be incorporated into the plasma membrane (Fig. 21b). This transit time is probably somewhat overestimated, reflecting both the equilibration time of the glycoprotein precursors to the internal glycoprotein pool and the processing of the glycoprotein intermediates by trimming and/or addition of monosaccharide residues. In comparison, the transit time for intracellular glycoprotein to incorporate into the surface of growing cells would appear to be faster (Fig. 21a). Although difficult to measure, the appearance of cytoplasmic glycoprotein also seems to be faster in growing cells. Whether this is due to an increased rate of synthesis, flow time through the internal pools, transit time of glycoproteins to the plasma membrane or combination thereof is not evident from these preliminary experiments.

APPENDIX A

Purification of ovalbumin

Ovalbumin was prepared from fresh eggs by the method previously described (Kekwick and Canaan, 1936). Egg whites from 144 eggs were separated and the volume measured. To this was added an equal volume of a saturated sodium sulfate solution (36.7 grams per 100 ml of water). Care must be taken to keep the temperature above 30° as the salt has a tendency to crystallize. The mixture was slowly stirred overnight at 37° and the precipitate removed by centrifugation. The pH of the supernatant was adjusted so that it was between 4.6 and 4.8 by the slow addtion of 0.2 N sulfuric acid with continuous stirring. At this time, anhydrous sodium sulfate was added until a permanent opalescence was detected. The mixture was allowed to stand at room temperature for 1 or 2 days until the crystallization of the protein was complete. The precipitate was collected by centrifugation, redissolved in a minimal amount of water and recrystallized by the addition of solid sodium sulfate. This procedure was continued for an additional two recrystallizations. The material can easily be stored as a dry powder. Starting with 12 dozen eggs, the final yield of purified ovalbumin was approximately 200 grams.

Analytical methods

Hexoses were determined by the phenol-H_2SO_4 method as previously described (Dubois *et al.*, 1956). Unless otherwise stated, an aliquot of the hexose-containing material in 0.5 ml of water was mixed with 0.5 ml of 5% phenol. To this 2.0 ml of H_2SO_4 was rapidly pipetted and the sample immediately shaken, allowed to cool and read on a Beckman

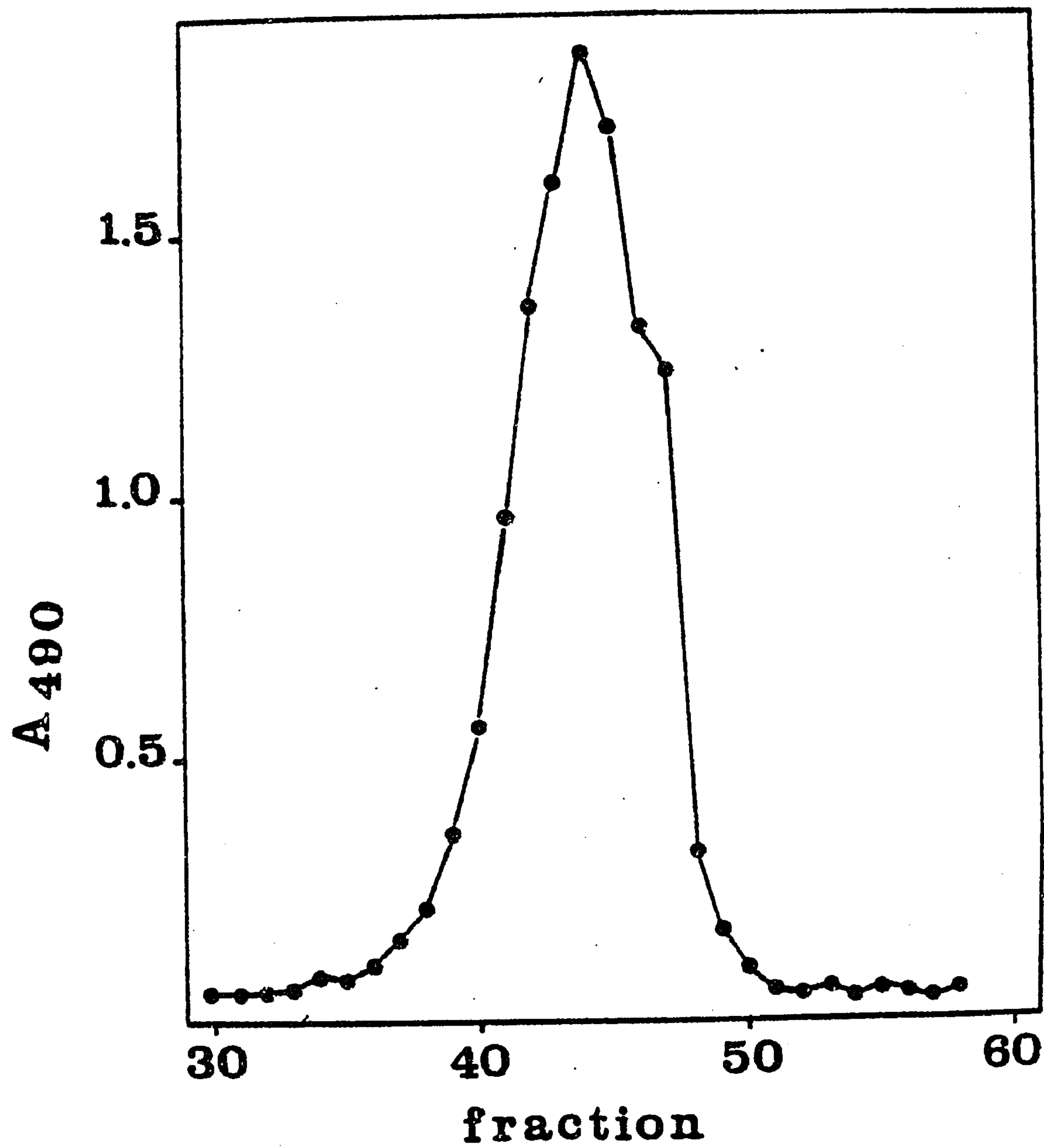
Spectrophometer (Beckman DB) at an absorbance of 490 nm. A standard curve, using mannose, ranging from 5 ug to 30 ug was used to determine hexose concentration. All dilutions were made in the appropriate volume of water:5% phenol:H_2SO_4, 1:1:4.

Purification of ovalbumin glycopeptides

The isolation of the ovalbumin glycopeptides was carried out as described (Huang et al., 1970). Approximately 43 grams of purified ovalbumin was dissolved in 1 liter of water and extensively dialyzed until free of sodium sulfate. The material was adjusted to pH 7.8 by the addition of 1 M NaOH and heated 15 to 30 minutes at 80°. The denatured protein was allowed to cool at room temperature and subsequently 330 ml of 0.2 M Tris buffer, containing 0.04 M $CaCl_2$, was added to it. The pH of the solution was adjusted to 7.4. The material was extensively digested with 1 gram of Pronase, added over a 2 day period, while being maintained at 37°, under a layer of toluene. The hydrolyzate was centrifuged and the clear supernatant decanted and lyophilized. The dry residue was dissolved in 250 ml of 0.1 M acetic acid and applied to a column (5 x 70 cm) of Sephadex G-25 in 50 ml aliquots and collected as described (Materials and Methods) with 1 M acetic acid. The initial hexose content was 510 mg. The final pooled material contained 428 mg of hexose, representing a recovery of 84%.

The pooled material was lyophilized and redissolved in 100 ml of 0.05 M Tris-buffer, containing 0.01 M $CaCl_2$. The glycopeptides were redigested with 300 mg of Pronase, added over 2 days as above. The hydrolyzate was centrifuged and the supernatant collected and desalted by Sephadex G-25 chromatography with 0.1 M acetic acid (Fig. 23). Hexose-containing fractions were pooled and lyophilized. The final

Figure 23. Purification of ovalbumin glycopeptides. Ovalbumin was isolated and the glycopeptides prepared as described in Appendix A. The Pronase-digested material was desalted by application to columns (5 x 70 cm) of Sephadex G-25. Fractions of 14 ml were collected at a flow rate of 20 minutes per tube and hexose-containing fractions detected by the phenol-H_2SO_4 method. Overall recovery was 74.5%.



1.5
1.0
0.5
A 490
30
40
50
60
fraction

content of hexose was 380 mg, representing an overall recovery of 74.5%.

Approximately 243 mg of the hexose-containing mixture was applied to a column of Dowex-AG 50 x 2 (1.5 x 150 cm) previously equilibrated and eluted with sodium acetate buffer, pH 2.6, mM in Na^+ ions. As shown in figure 24, five peaks designated A-E eluted between fractions 150-400. Each peak was pooled separately. The final hexose content of each peak was not chemically determined, but an approximation from the area under each curve suggests the following percentages: peak A - 3.71%, B - 8.55%, C - 32.90%, D - 41.29% and E - 13.55%. This was generally in good agreement with the previous findings of Huang *et al.* (Huang *et al.*, 1970). Two peaks eluted early from the column (fractions 15 to 27, not shown) and have previously been observed (Huang *et al.*, 1970). Subsequent elution with 0.05 M sodium acetate, pH 6.0, to obtain glycopeptides with additional amino acid residues was not performed.

(^{14}C) acetylation of ovalbumin glycopeptides C, D and E

The acetylation of $Asn(GlcNAc)_2(Man)_6$ (species D), $Asn(GlcNAc)_2(Man)_5$ (species E) and $Asn(GlcNAc)_4(Man)_6$ (species C)[1] was carried out similar to the method previously described (Arakawa and Muramatsu, 1974). The method will be described in detail for the acetylation of $Asn(GlcNAc)_2(Man)_6$ but was identical for the acetylation of all the above-mentioned glycopeptides. The procedure makes the assumption that the reaction will only acetylate free amino groups in the peptide moiety, and not hydroxyl groups of sugar residues (Arakawa and Muramatsu, 1974). Approximately 38.7 mg (by weight) of $Asn(GlcNAc)_2(Man)_6$ (25.6 µmoles)

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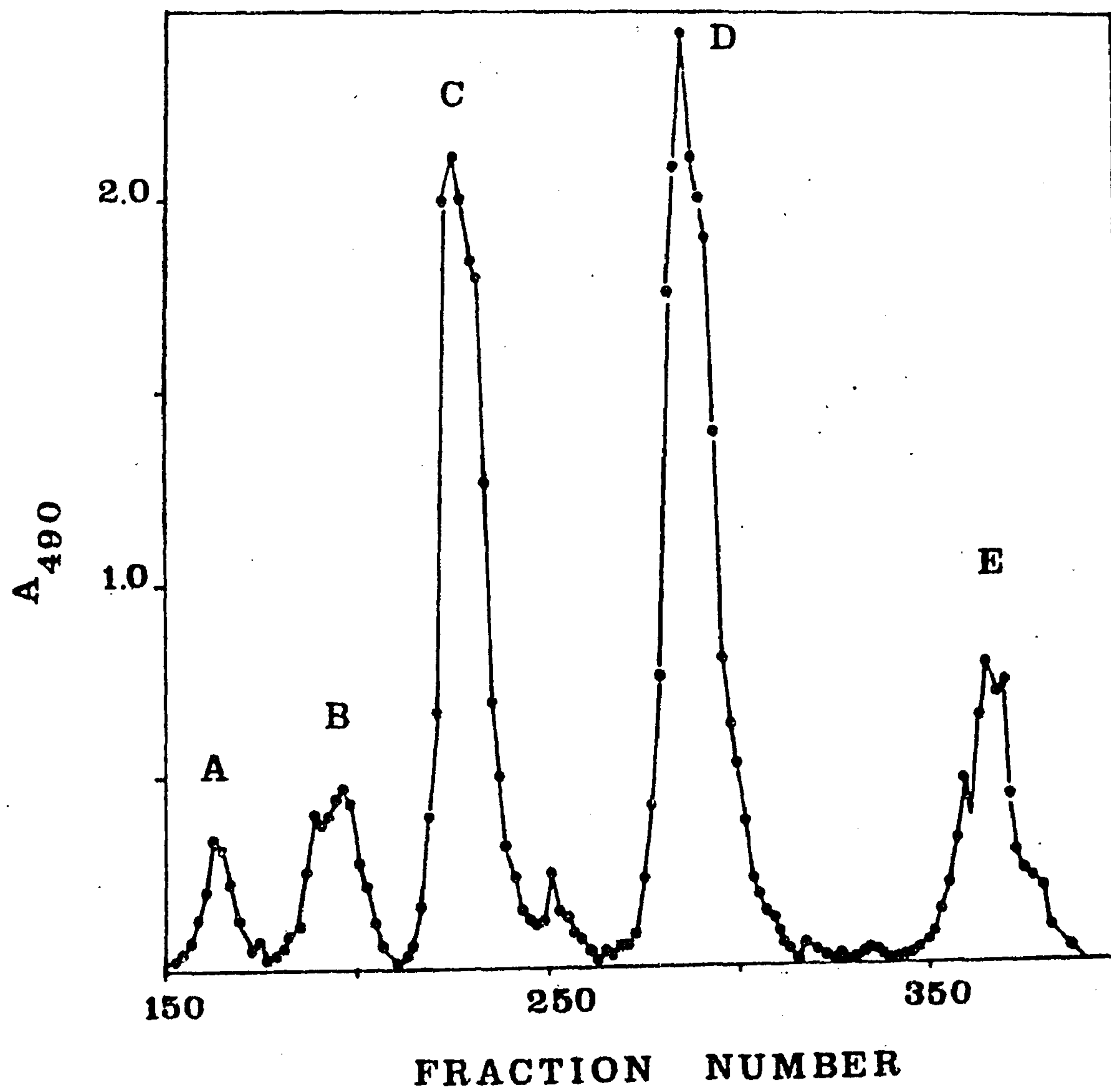
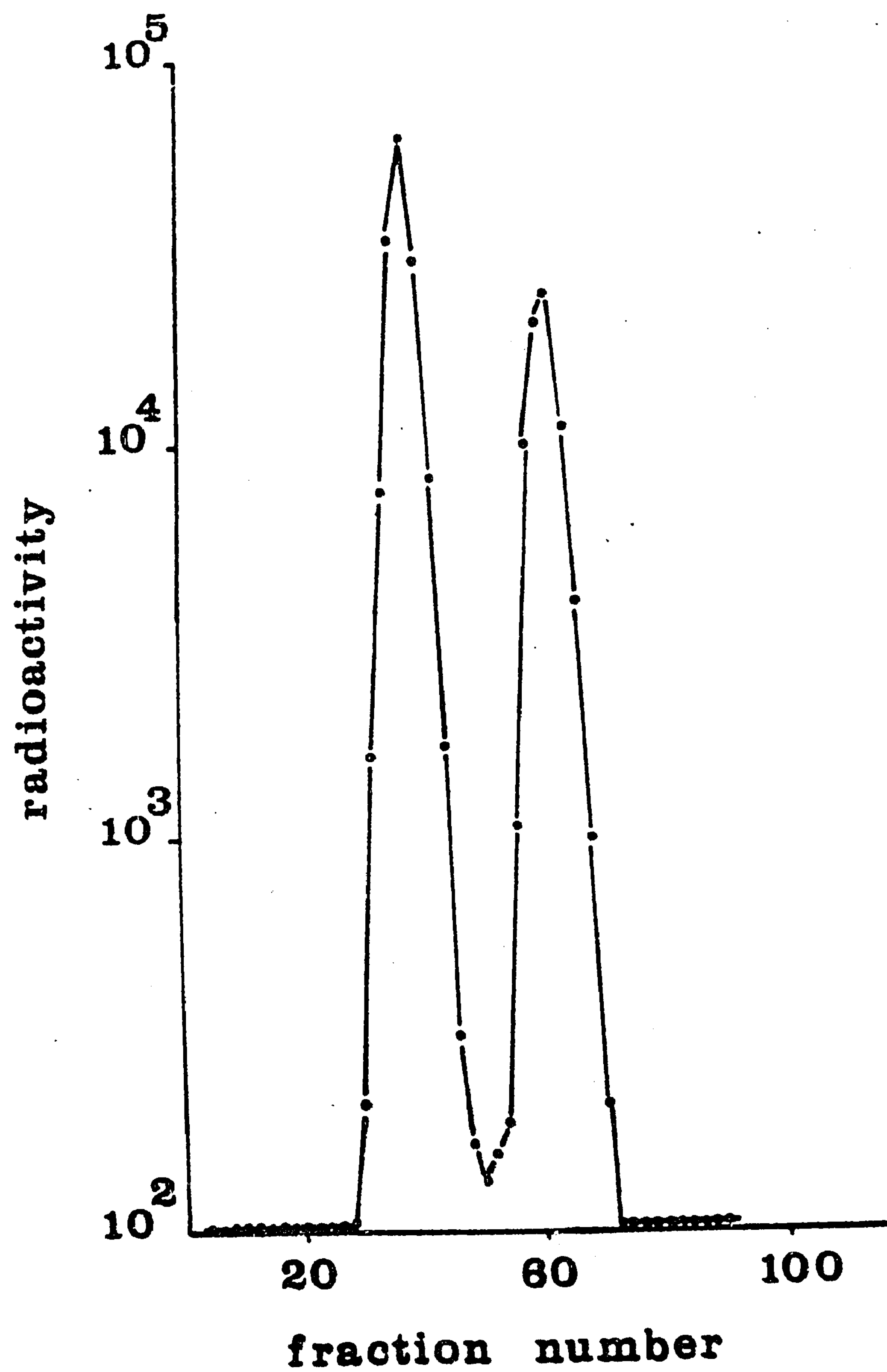
[1]This Dowex-purified peak has been shown to consist of at least three distinct glycopeptide species (Tai *et al.*, 1977a).

Figure 24. Fractionation of ovalbumin glycopeptides. The mixture of ovalbumin glycopeptides was prepared as described in the legend of figure 24 and applied to a column (1.5 x 150 cm) of Dowex AG-50W x 2, eluted with sodium acetate buffer, pH 2.6, mM in Na^+ ions. Fractions of 13.5 ml were collected at a flow rate of 20 minutes per tube. Hexose containing fractions were detected by phenol-H_2SO_4 (Materials and Methods). Peaks eluting early from the column (fractions 15-27) are not shown.


A
B
C
D
E
2.0
1.0
A_{490}
150
250
350
FRACTION NUMBER

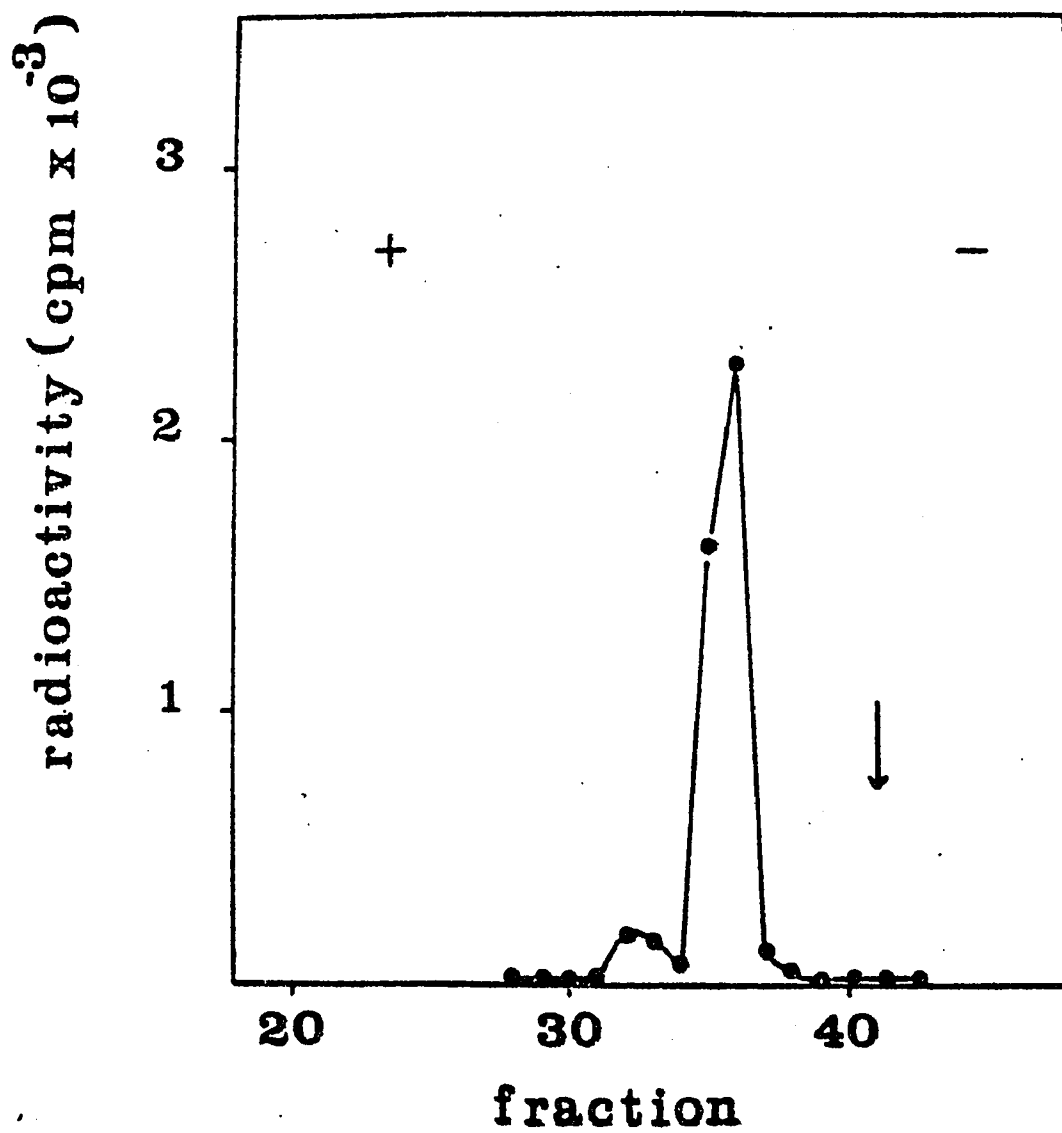
was acetylated with 8.62 umole of (^{14}C) acetic anhydride (29 mCi/mmole, Amersham-Searle Radiochemical Centre, Arlington Heights, Ill., U.S.A.) in 0.4 ml of 0.5% $NaHCO_3$ at room temperature for 2 hours. The (^{14}C) acetylated glycopeptide was precipitated by the addition of 8 ml of 95% ethanol and allowed to settle overnight at -20°. The material was centrifuged at 2000 rpm for 5 minutes and the supernatant discarded. Most of the ethanol could be decanted, and the remaining volume was blown off with nitrogen-flushing. The pellet was dissolved in 2.0 ml of 0.05 M ammonium acetate and applied to a column of Sephadex G-25 (0.9 x 145 cm), which had previously been equilibrated and eluted with 0.05 M ammonium acetate. Fractions of 1 ml were collected at a flow rate of 10 minutes per tube. Fractions 30-46 were pooled and lyophilized (Fig. 25). Approximately 16 mg (by weight) of (^{14}C) acetylAsn$(GlcNAc)_2$ $(Man)_6$ was recovered, with a final specific activity of 72,600 cpm/ 0.0205 µmole. An aliquot (about 18,000 cpm) of this material was analyzed by high voltage paper electrophoresis, pH 6.5. Figure 26 shows that the majority of the material migrates to the positive pole as a single peak. Less than 10% of the material appears to be a contaminant (possibly (^{14}C)acetylAsn$(GlcNAc)_2(Man)_5$). The specific activity of (^{14}C)acetylAsn$(GlcNAc)_2(Man)_5$ and (^{14}C)acetylAsn$(GlcNAc)_4(Man)_6$ was found to be 80,650 cpm/0.0135 µmole and 31,700 cpm/0.0105 µmole, respectively.

Figure 25. Preparation of (^{14}C)acetylAsn(GlcNAc)$_2$(Man)$_6$. 38.7 mg of Asn(GlcNAc)$_2$(Man)$_6$ (25.6 µmoles) was acetylated with 8.62 µmoles of (^{14}C)acetic anhydride as described in Appendix A. The mixture was applied to a column (0.9 x 145 cm) of Sephadex G-25, eluted with 0.05 M ammonium acetate. Fractions of 1.2 ml were collected at a flow rate of 6 ml per hour. Fractions 30 to 46 were pooled and lyophilized. The specific activity of the acetylated species was 72,600 cpm/0.0205 µmoles.



10^5
10^4
10^3
10^2
radioactivity
20
60
100
fraction number

Figure 26. Electrophoretic analysis of (^{14}C)acetylAsn(GlcNAc)$_2$(Man)$_6$. The acetylated glycopeptide was prepared and isolated as described in the legend of figure 26. An aliquot (18,000 cpm) was analyzed by high voltage paper electrophoresis, pH 6.5 in pyridine:acetic acid:water, 10.0:0.4:89.6 for 2 hours at 3,500 volts. The origin is at fraction 41. The paper strip was cut into 1 cm fractions, eluted with water and counted for radioactivity.


radioactivity (cpm x 10^-3)
3
2
1
+
−
20
30
40
fraction

APPENDIX B

Purification of endo-β-N-acetylglucosaminidase H

Enzyme assay

The digestion mixture containing 5 µl of (^{14}C)acetylAsn(GlcNAc)$_2$ (Man)$_6$ and 50 µl of enzyme solution was carried out in a final volume of 200 µl 0.0125 M/0.0375 M citrate/phosphate buffer, pH 5.0, containing 0.15 M NaCl. The reaction was carried out under toluene at 37° for 30 minutes and was terminated by rapid immersion in ice-cold water. An aliquot of the digestion mixture was applied to 1.5 inch wide strips of Whatman 1 MM paper and analyzed by high voltage paper electrophoresis, pH 6.5 for 2 hours at 3,500 volts (Fig. 27). Strips were dried, cut into 1 cm fractions, eluted with water for 30 minutes and counted in a liquid scintillation counter. Radioactivity in the substrate ((^{14}C)acetylAsn(GlcNAc)$_2$(Man)$_6$) and the hydrolyzed product ((^{14}C)acetyl Asn(GlcNAc)) was determined and the results expressed as either the percent hydrolysis of the substrate or specific activity of the enzyme. One unit is defined as the amount of enzyme needed to hydrolyze 1 µmole of substrate per minute. Protein was determined by the method previously described (Oyama and Eagle, 1956).

Sample Calculation

Reaction mixture

50 µl enzyme

50 µl 0.6 M NaCl

5 µl (^{14}C)acetylAsn(GlcNAc)$_2$(Man)$_6$ (32,521 cpm)

45 µl H_2O

50 µl 0.05 M/0.15 M citrate/phosphate, pH 5.0

cultural filtrate, no dilution

observed digested material = 5,689 cpm

total cpm recovered = 17,613 cpm

5 µl substrate = 32,521 cpm

quenching factor 5,689 x 32,521/17,613 = 10,504 cpm

0.0205 µmole = 72,600 cpm

i.e., 0.00297 µmoles digested/30 minutes

enzyme protein = 0.0362 mg/50µl

sp. act. = 0.00273 µmole/mg/min

OR Let 1 unit = 1 µmole/min

unit/ml = 0.00297 µmoles/30 minutes

= 0.000099 µmole/min

x 20 = 0.00198 units/ml

Total enzyme volume - 8500 ml

Total units = 16.83 µmole/min

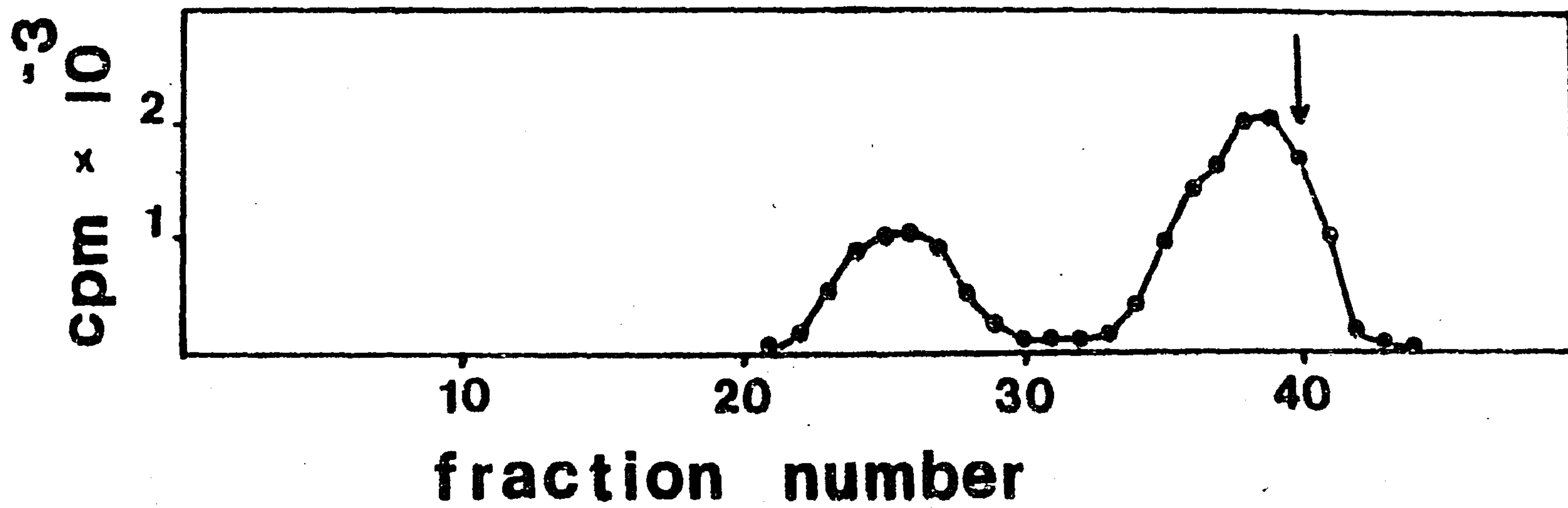
Total mg protein = 6156

sp. act. = 0.00273 µmole/mg/min

Glycosidase contamination

Contamination by exo-glycosidases was determined using the appropriate p-nitrophenyl glycoside. The digestion mixture containing 250 µl of the appropriate p-nitrophenyl glycoside (250 µg) and 50 µl of enzyme solution was carried out in a final volume of 1.0 ml 0.0125 M/ 0.0375 M citrate/phosphate buffer, pH 5.0, containing 0.15 M NaCl. The reaction was incubated at 37° and varied from 15 minutes to 21 hours. The reaction was terminated by the addition of 2.0 ml of 1 M sodium carbonate and read on a Beckman Spectrophotometer at 400 nm. A unit

Figure 27. Analysis of (^{14}C)acetylAsn(GlcNAc)$_2$(Man)$_6$ digested by endo-β-N-acetylglucosaminidase H. The acetylated substrate was hydrolyzed by endoglycosidase H as described in Appendix B and analyzed by high voltage paper electrophoresis, pH 6.5 in pyridine:acetic acid:water, 10.0:0.4:89.6 for 2 hours at 3,500 volts. Paper strips were cut into 1 cm fractions and counted for radioactivity. The percentage of intact substrate ((^{14}C) acetylAsn(GlcNAc)$_2$(Man)$_6$, fractions 33-43) and released product ((^{14}C)acetylAsnGlcNAc, fractions 21-31) were used to calculate the specific activity of the enzyme as µmole substrate hydrolyzed per minute per mg protein.


cpm x 10-3
2
1
10
20
30
40
fraction number

is defined as the amount of enzyme needed to hydrolyze 1 µmole of the appropriate p-nitrophenyl glycoside per minute.

Experimental

Endo-β-N-acetylglucosaminidase H was purified essentially as previously described (Tarentino and Maley, 1974).

Step 1 Cultural filtrate - A stock strain of Streptomyces plicatus was transferred to plates of Hickey-Tresner sporulating agar (Hickey and Tresner, 1952) and grown at 37° for 3 days. The spores were removed from the plate by scraping and added to 600 ml of Jeuniaux's medium (Jeuniaux, 1966). The cells were incubated at 26° for 3 days on a New Brunswick rotary shaker at 220 rpm. At this time, 50 ml aliquots were transferred to 10 2-liter flasks each containing 1 liter of Jeuniaux's medium. The cells were grown an additional 3 to 6 days at 37° with continuous stirring at 220 rpm. Generally, at the end of the incubation period, 1 liter of cells was used to innoculate another 10 liters of medium, and the remaining 9 liters harvested by filtration through filter paper. In this fashion, 4 batch preparations were prepared. A sample batch chosen at random (batch 2) contained a final cultural filtrate volume of 8.5 liters with 0.72 mg/ml protein. The endo-β-N-acetylglucosaminidase H activity was determined to be 0.00198 units/ml.

Step 2 Zinc precipitation - Unless otherwise stated, the following data is taken from the batch 2 preparation. The cultural filtrate (8.2 liters) was raised to 0.075 M Zn^+ ions by the dropwise addition, with continuous stirring, at room temperature of 636 ml of 1 M zinc acetate. The pH was maintained at 7.0 by the periodic addition of solid Trizma base. The resulting precipitation was allowed to settle

at room temperature for 2 hours and subsequently, overnight at 4°. The supernatant was removed and the precipitate contrifuged at 4000 rpm for 10 minutes. The pellet was dissolved in 0.3 M sodium citrate, pH 5.5. The final volume was 1090 ml containing 2.02 mg/ml protein with an endoglycosidase activity of 0.0075 units /ml.

Step 3 Ammonium sulfate precipitation - The extract from the previous step (1090 ml) was raised to 40% saturation by the addition of 22.5 grams of solid ammonium sulfate per 100 ml of solution. The mixture was kept on ice and stirred continuously for 45 minutes and centrifuged at 8,500 rpm for 20 minutes. The supernatant (1200 ml) was adjusted to 90% saturation by the addition of 33.5 grams of solid ammonium sulfate per 100 ml, maintained as above. The resulting precipitate was dissolved in 135 ml of 0.01 M potassium phosphate dibasic, pH 8.45 and dialyzed against 10 liters of the same buffer with repeated changes. The extract before dialysis contained 7.7 mg/ml protein and the enzyme activity was 0.0384 units/ml.

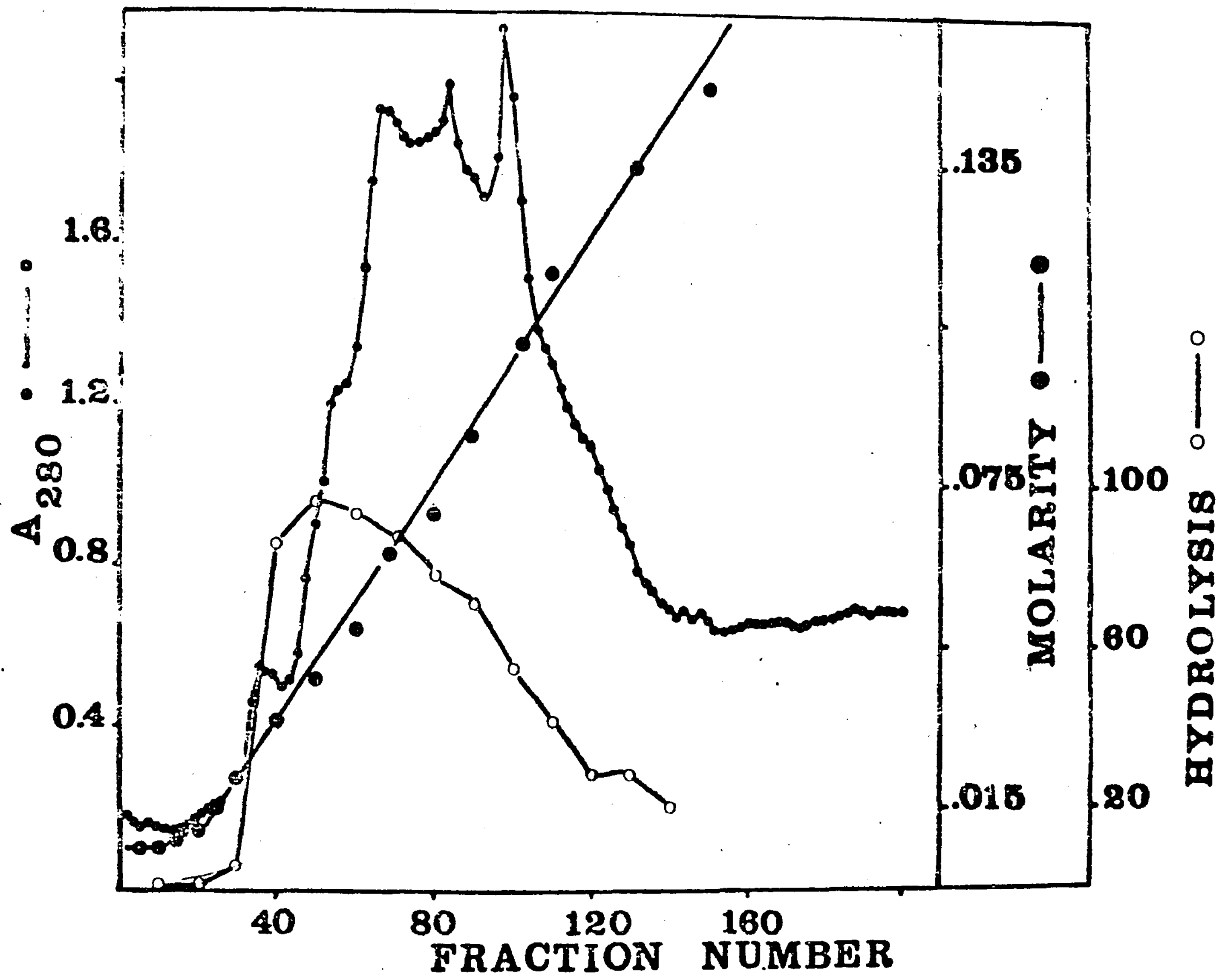
Step 4 DEAE cellulose chromatography - The dialyzed extracts from 4 batch preparations were combined (911 ml containing 6.38 grams of protein) and applied to a column (3.5 x 72 cm) of DEAE-cellulose (DE 23, Whatman, Maidstone, Kent England) at a flow rate of 120 ml per hour. The column was previously equilibrated and eluted with 0.01 M potassium phosphate dibasic pH 8.45 at a flow rate of 33 ml per hour until all unabsorbed protein was washed off. A linear gradient of potassium phosphate dibasic, pH 8.45 was applied with 2.0 liters of 0.01 M potassium phosphate dibasic in the mixing chamber and 2.0 liters of 0.32 M potassium phosphate dibasic, pH 8.45 in the reservoir. Fractions of

12.4 ml were collected at a flow rate of 50 ml per hour. Every tenth tube was assayed for endo-β-N-acetylglucosaminidase H activity and was located between fraction 20-140 (Fig. 28). Fractions 30 to 80 were pooled and reduced in volume by reverse dialysis with ethylene glycol, and subsequently dialyzed against 10 liters of 0.01 M sodium acetate, pH 4.85 for 2 days, with repeated changes. Fractions 81-150 were not further purified. The final volume after dialysis was 260 ml with a protein concentration of 2.514 mg/ml and an enzyme activity of 0.185 units/ml.

Step 5 Sulfopropanyl Sephadex chromatography - The dialyzed extract from step 4 (2 0 ml containing about 528.0 mg of protein) was applied to a column (2.5 x 25 cm) of SP-Sephadex C-25 (Pharmacia, Piscataway, N.J., U.S.A.) at a flow rate of 36 ml per hour. (A 50 ml portion of the step 4 extract containing about 126.0 mg of protein was saved.) The column was previously equilibrated and eluted with 0.01 M sodium acetate, pH 4.85 until all unabsorbed protein was washed off. Thereafter, a linear gradient containing 800 ml of 0.01 M sodium acetate, pH 4.85 in the mixing chamber and 800 ml of 0.10 M sodium acetate, pH 4.85 in the reservoir was applied to the column. Fractions of 9 ml at a flow rate of 36 ml per hour were collected. The enzyme-containing fractions were located in the effluent (see below) and pooled. The final volume was 185 ml containing 1.83 mg/ml of protein with an enzyme activity of 0.156 units/ml.

Step 6 Sephadex G-100 chromatography - The extract from step 5 (85 ml containing about 156.0 mg of protein) was lyophilized, redissolved in 10.0 ml of 0.01 M **potassium phosphate dibasic,** pH 8.45, and applied to a

Figure 28. DEAE-cellulose chromatography of endo-β-N-acetylglucosaminidase H. The enzyme was prepared as described in Appendix B and the dialyzed extract applied to a column (2.5 x 72 cm) of DEAE-cellulose, equilibrated and eluted with 0.01 M potassium phosphate dibasic, pH 8.45 until all unabsorbed protein washed off, at a flow rate of 33 ml per hour. Thereafter, a linear gradient of potassium phosphate dibasic, pH 8.45 was applied, 2.0 liters of 0.01 M potassium phosphate dibasic in the mixing chamber and 2.0 liters of 0.32 M potassium phosphate dibasic in the reservoir. Fractions of 12.4 ml were collected at a flow rate of 50 ml per hour. Aliquots of every tenth tube were analyzed for endoglycosidase H activity as described in the legend of figure 27. Fractions 30 to 80 were pooled for later use. o——o per cent hydrolysis of acetylated substrate by endoglycosidase H, •——• absorbance at 280 nm, ●——● molarity of the salt gradient.



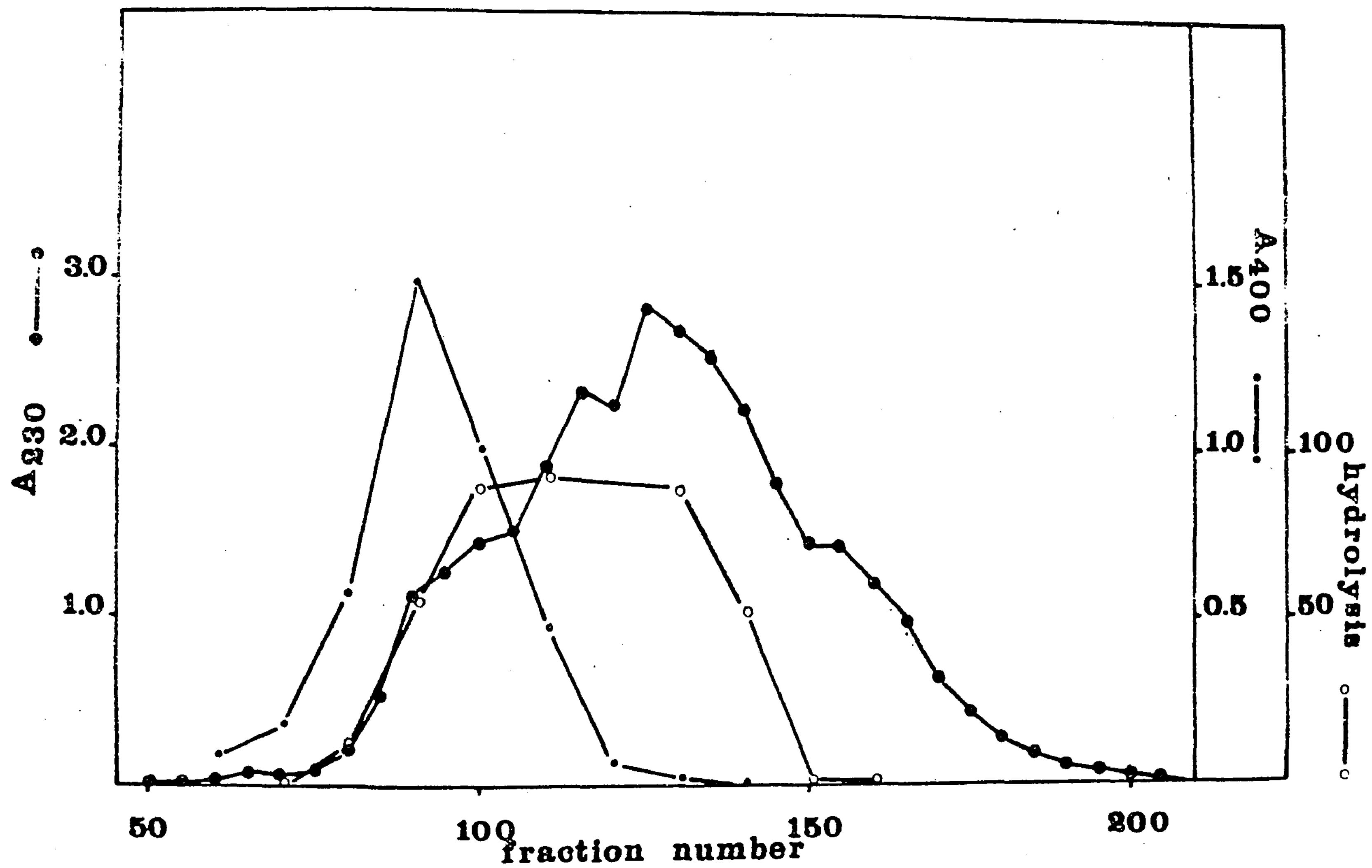
A_{280}
1.6
1.2
0.8
0.4
40
80
120
160
FRACTION NUMBER
.135
.075
.015
MOLARITY
100
60
20
HYDROLYSIS

column (2.5 x 140 cm) of Sephadex G-100 (Pharmacia, Piscataway, N.J., U.S.A.). The column was previously equilibrated with potassium phosphate dibasic, pH 8.45 and eluted with the same buffer. (100 ml of extract from step 5 containing about 183 mg of protein was saved.) Fractions of 3.0 ml were collected at a flow rate of 11 ml per hour. Endo-β-N-acetylglucosaminidase H activity was present between fractions 70 to 150 (Fig. 29). Fractions 120-150 were found to be essentially free of β-N-acetylglucosaminidase activity and pooled for later use. The final volume was 50 ml containing 0.578 mg/ml of protein and an endo-β-N-acetylglucosaminidase H activity of 0.458 units/ml.

Using (^{14}C)acetylAsn(GlcNAc)$_2$(Man)$_6$ as a substrate, endo-β-N-acetylglucosaminidase H was purified 291 fold over the cultural filtrate (Table 9). The final specific activity of the enzyme was determined to be 0.795 μmoles/mg/min. The enzyme preparation was found to be free of the following exo-glycosidases when assayed with the appropriate p-nitrophenyl glycoside; α-mannosidase, α-fucosidase and β-galactosidase. As shown in figure 29, considerable β-N-acetylglucosaminidase activity overlapped the endo-β-N-acetylglucosaminidase H activity when purified by Sephadex G-100 chromatography. For this reason, fractions 120-150 were pooled for later use. Less than 0.025% β-N-acetylglucosaminidase was found to be present, and this was judged to be low enough to allow the endo-β-N-acetylglucosaminidase H preparation to be useful in structural analysis of susceptible glycopeptides. No further purification was attempted.

With respect to the purification scheme, it must be pointed out that in

Figure 29. Sephadex G-100 chromatography of endo-β-N-acetylglucosaminidase H. The extract from step 5 (Appendix B) was applied to a column (2.5 x 140 cm) of Sephadex G-100, eluted with 0.01 M dipotassium hydrogen phosphate, pH 8.45. Fractions of 3.0 ml were collected at a flow rate of 11 ml per hour. Aliquots of every tenth tube were analyzed for endoglycosidase H activity as described in the legend of figure 28 as well as for β-N-acetylglucosaminidase activity (Appendix B). o——o endo-β-N-acetylglucosaminidase H activity, •——• β-N-acetylglucosaminidase activity, ●——● absorbance at 230 nm.



A230
3.0
2.0
1.0
A400
1.5
1.0
0.5
% hydrolysis
100
50
50
100
150
200
fraction number

Ta' le 9

Purification of endo-β-N-acetylglucosaminidase H

Stage of purification	volume (ml)	activity[a]	protein (mg)	specific activity[b]
+cultural filtrate	8500	16.83	6156	0.00273
zinc precipitation	1090	8.18	2207	0.00370
ammonium sulfate precipitation	135	5.18	1040	0.00498
++DEAE-cellulose	260	48.1	653.7	0.0735
SP Sephadex	185	28.86	339	0.085
Sephadex G-100	50	22.95	28.88	0.795

[a] μmoles/minute = 1 unit

[b] μmoles/minute/mg protein

\+ represents purification from 1 batch preparation

++ represents purification from 4 batch preparations combined

my hands, I was not able to achieve a significant purification of the enzyme using SP-Sephadex chromatography. While a significant amount of protein remained absorbed to the column after the 0.01 M sodium acetate, pH 4.85 elution, the column worked in reverse of that expected in that all of the enzyme activity desorbed with the low molarity wash. The reason for this is not clear, but may have been due to insufficient dialysis time.

DIFFERENTIAL SPECIFICITIES OF ENDO-β-N-ACETYLGLUCOSAMINIDASES*

N[14C]-Acetylated Substrates	Gp	Clostridium Perfringens C_I	Clostridium Perfringens C_{II}	Diplococcus Pneumoniae Endo D	Streptomyces Griseus Endo H
I Ovalbumin Glycopeptides					
a. $(Man)_5-(GlcNAc)_4-Asn$	III - A	R	S	R	S
b. $(Man)_7-(GlcNAc)_2-Asn$	III - B	R	S	R	S
c. $(Man)_4-(GlcNAc)_5-Asn$	III - C	R	R	R	S
d. $(Man)_6-(GlcNAc)_2-Asn$	IV	R	S	R	S
e. $(Man)_5-(GlcNAc)_2-Asn$	V	S	S	S	S
f. $(Man)_4-(GlcNAc)_2-Asn$	VI	–	S	–	S
g. Man $-(GlcNAc)_2-Asn$	•	R	R	R	R**
II Thyroglobulin Glycopeptides					
a. Unit A		R	S	R	S
b. Unit B		R	R	R	R
c. Unit B side chain free		S	R	S	R
d. Unit B defucosyl		–	–	S	R***
III IgG-Glycopeptides					
a. Intact		R	R	R	R
b. Intact (side chain free)		S	R	S	R
c. Defucosylated		–	–	R	R
d. Degalactosylated		–	–	R	R
e. Defucosylated - side chain free		–	–	S	R

R = resistant to enzyme hydrolysis

S = susceptible to enzyme hydrolysis

• Glycopeptide V digested with α-mannosidase

** Sensitive to endoglycosidase L

*** In nanogram quantities

Ovalbumin

GP-III A

Manα1→6 Manα1→6 Manβ1→C
Manα1→3 Manα1
GlcNAcβ1→2 Manα1→3 Manβ1
GlcNAcβ1→4 Manβ1

GP-III B

Manα1→2 Manα1→6 Manα1→6 Manβ1→C
Manα1→3 Manα1
Manα1→2 Manα1→3 Manβ1

GP-III C

GlcNAcβ1→4 Manβ1→C
Manα1→3 Manα1→6 Manβ1
GlcNAcβ1→2 Manα1→3 Manβ1
GlcNAcβ1→4* Manα1

GP-IV

Manα1→6 Manα1→6 Manβ1→C
Manα1→3 Manα1
Manα1→2 Manα1→3 Manβ1

GP-V

Manα1→6 Manα1→6 Manβ1→C
Manα1→3 Manα1
** Manα1→3 Manβ1

GP-VI

Manα1→3 Manα1→6 Manβ1→C
Manα1→3 Manβ1

C = 4GlcNAcβ1→4GlcNAc→Asn

* Possible recognition point of C_{II} enzyme GlcNAcβ1→4 substitution on the mannose results in a resistant substrate for the C_{II} enzyme

Thyroglobulin

Unit A

$(Man\alpha)_N$→Manα→Manα
$(Man\alpha)_N$→Manα→Manα
$(Man\alpha)_N$→Manα
→Manβ1→4GlcNAcβ1→4GlcNAc→Asn

Unit B

Sialic Acid→Galβ→GlcNAcβ→Manα
Sialic Acid→Galβ→GlcNAcβ→Manα
Galβ→GlcNAcβ→Manα
→Manβ→GlcNAcβ1→4GlcNAc→Asn
Fucα→GlcNAc

Bovine IgG

Side Chain Free

Manα1→6 Manβ1→4GlcNAcβ1→4 GlcNAc→Asn
Manα1→3 Manβ1
Fucα1→3 GlcNAc

Intact

Galβ1→4GlcNAcβ1→2Manα1→6 Manβ1→4GlcNAcβ1→4 GlcNAc→Asn
(Galβ1→4)GlcNAcβ1→2Manα1→3 Manβ1
Fucα1→3 GlcNAc

** This mannose must be terminal for the Endo D enzyme activity

(Muramatsu, 1971; Koide and Muramatsu, 1974; Arakawa and Muramatsu, 1974; Ito et al., 1975; Tai et al., 1977a and b; Nishhigaki et al., 1974; Tarentino and Maley, 1974, 1975; Tarentino et al., 1972, 1973)

*taken from Atkinson and Hakimi (1979)

BIBLIOGRAPHY

1. Ali, I. V., Mautner, V., Lanza, R., and Hynes, R. O. Cell 11, 115-120 (1977).

2. Arakawa, M. and Muramatsu, T. J. Biochem. 76, 307-317 (1974).

3. Arima, T. and Spiro, R. G. J. Biol. Chem. 247, 1836-1848 (1972).

4. Atkinson, P. H. Meth. Cell Biol. 7, 157-188 (1973).

5. Atkinson, P. H. J. Biol. Chem. 250, 2123-2134 (1975).

6. Atkinson, P. H., Moyer, S. A., and Summers, D. F. J. Mol. Biol. 102, 613-631 (1976).

7. Atkinson, P. H. and Hakimi, J. (1979) in "The Biochemistry of Glycoproteins and Proteoglycans" (ed. Lennarz, W. J.) Plenum, New York.

8. Baenziger, J. and Kornfeld, S. J. Biol. Chem 249, 7260-7269 (1974).

9. Beeley, J. G. Biochem. J. 123, 399-405 (1971).

10. Behrens, N. H., Parodi, A. J., and Leloir, L. F. Proc. Natl. Acad. Sci. U.S.A. 68, 2857-2860 (1971).

11. Behrens, N. H., Carminatti, H., Staneloni, R. J., Leloir, L. F. and Cantarella, A. I. Proc. Natl. Acad. Sci. U.S.A. 70, 3390-3394 (1973).

12. Bosmann, B. H. Biochem. Biophys. Res. Commun. 49, 1256-1262 (1972).

13. Brown, J. C. J. Supramol. Struct. 1, 1-7 (1972).

14. Buck, C. A., Glick, M. C., and Warren, L. Biochem 9, 4567-4576 (1970).

15. Buck, C. A., Glick, M. C., and Warren, L. Biochem 10, 2176-2180 (1971a).

16. Buck, C. A., Glick, M. C., and Warren, L. Science 172, 169-171 (1971b).

17. Buck, C. A., Fuhrer, J. P., Soslau, G., Warren, L. J. Biol. Chem. 249, 1541-1550 (1974).

18. Cammarata, P. and Ceccarini, C. J. Cell Bio. 75, 226a (1977).

19. Cammarata, P. and Ceccarini, C. J. Gen. Phys. 72, 2a (1978).

20. Cammarata, P. and Ceccarini, C. J. Chrom. 168, 395-400 (1979).

21. Ceccarini, C. and Eagle, H. Proc. Natl. Acad. Sci. U.S.A. 68, 229-233 (1971).

22. Ceccarini, C. Proc. Natl. Acad. Sci. U.S.A. 72, 2687-2690 (1975).

23. Ceccarini, C., Muramatsu, T., Tsang, J., and Atkinson, P. H. Proc. Natl. Acad. Sci. U.S.A. 72, 3139-3143 (1975).

24. Ceccarini, C. and Atkinson, P. Biochim. Biophys. Acta 500, 197-207 (1977).

25. Ceccarini, C. and Atkinson, P. H. unpublished observation.

26. Chapman, A. and Kornfeld, R. Fed. Proc. 37, 1299 (1978).

27. Chen, W. W., Lennarz, W. J., Tarentino, A. L., and Maley, F. J. Biol. Chem. 250, 7006-7013 (1975).

28. Chen, W. W. and Lennarz, W. J. J. Biol. Chem. 253, 5774-5779 (1978a).

29. Chen, W. W. and Lennarz, W. J. J. Biol. Chem. 253, 5780-5785 (1978b).

30. Chien, S. F., Weinburg, R., Li, S. C., and Li, Y. T. Biochem. Biophys. Res. Commun. 76, 317-323 (1977).

31. Cifone, M. A. and Baker, R. M. J. Cell Biol. 70, 77a (1976).

32. Conchie, J. and Strachan, I. Carbohyd. Res. 63, 193-213 (1978).

33. Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., and Smith, F. Anal. Chem. 28, 350-356 (1956).

34. Emmelot, P. and Bos, C. J. J. Mem. Biol. 9, 83-104 (1972).

35. Etchison, J. R., Robertson, J. S., and Summers, D. F. Virology 78, 375-392 (1977).

36. Gahmberg, C. G., Kiehn, D., and Hakomori, S. Nature 248, 413-415 (1974).

37. Glick, M. C. and Buck, C. A. Biochem. 12, 85-90 (1973).

38. Glick, M. C., Rabinowitz, Z., and Sachs, L. J. Virology 13, 967-974 (1974).

39. Gottlieb, C., Baenziger, J., and Kornfeld, S. J. Biol. Chem. 250, 3303-3309 (1975).

40. Grimes, W. J. Biochem 12, 990-996 (1973).

41. Grimes, W. J. and Greegor, S. Cancer Res. 36, 3905-3910 (1976).

42. Hakomori, S. Biochim. Biophys. Acta. 417, 55-89 (1975).

43. Hartmann, J. F., Buck, C. A., Defendi, V., Glick, M. C., and Warren, L. J. Cell Phys. 80, 159-166 (1972).

44. Herscovics, A., Bugge, B., and Jeanloz, R. W. J. Biol. Chem. 252, 2271-2277 (1977).

45. Hickman, S., Kulczycki, A., Lynch, R. G., and Kornfeld, S. J. Biol. Chem. 252, 4402-4408 (1977).

46. Hogg, N. M. Proc. Natl. Acad. Sci. U.S.A. 71, 489-492 (1974).

47. Hickey, R. J. and Tresner, H. D. J. Bacteriol. 64, 891-892 (1952).

48. Huang, C. C., Mayer, H. E., Jr., and Montgomery, R. Carbohyd. Res. 13, 127-137 (1970).

49. Hunt, L. A. and Summers, D. F. J. Virol. 20, 637-645 (1976).

50. Hunt, L. A., Etchison, J. R., and Summers, D. F. Proc. Natl. Acad. Sci. U.S.A. 75, 754-758 (1978).

51. Hynes, R. O. Proc. Natl. Acad. Sci. U.S.A. 70, 3170-3174 (1973).

52. Hynes, R. O. Biochem, Biophys. Acta 458, 73-107 (1976).

53. Ito, S., Muramatsu, T., and Kobata, A. Biochem. Biophys. Res. Commun. 63, 938-944 (1975a).

54. Ito, S., Muramatsu, T., and Kobata, A. Arch. Biochem. Biophys. 171, 78-86 (1975b).

55. Ito, S., Yamashita, K., Spiro, R. and Kobata, A. J. Biochem. 81, 1621-1631 (1977).

56. Javaid, J. I., Hof, H., and Brunngraber, E. G. Biochim. Biophys. Acta 404, 64-82 (1975).

57. Jeuniaux, C. Methods in Enzymol. 8, 644-650 (1966).

58. Kawasaki, T. and Ashwell, G. J. Biol. Chem. 251, 5292-5299 (1976).

59. Kekwick, R. A. and Cannan, R. A. Biochem J. 30, 227-234 (1936).

60. Knipe, D. M., Baltimore, D., and Lodish, H. F. J. Virol. 21, 1128-1139 (1977).

61. Koide, N. and Muramatsu, T. J. Biol. Chem. 249, 4897-4904 (1974).

62. Kornfeld, R. and Kornfeld, S. J. Biol. Chem. 245, 2536-2545 (1970).

63. Kornfeld, R. and Ferris, C. J. Biol. Chem. 250, 2614-2619 (1975).

64. Kornfeld, S., Li, E., and Tabas, I. J. Biol. Chem. 253, 7771-7778 (1978).

65. Krag, S. S., Cifone, M., Robbins, P. W., and Baker, R. M. J. Biol. Chem. in press.

66. Lamb, R. A. and Choppin, P W. Virol. 81, 371-381 (1977).

67. Leavitt, R., Schlesinger, S., and Kornfeld, S. J. Biol. Chem. 252, 9018-9023 (1977).

68. Lee, Y. C. and Scocca, J. R. J. Biol. Chem. 247, 5753-5758 (1972).

69. Lennarz, W. J. Science 188, 986-991 (1975).

70. Levine, E. M., Becker, Y., Boone, C. N., and Eagle, H. Proc. Natl. Acad. Sci. U.S.A. 53, 350-356 (1965).

71. Li, E., Tabas, I., and Kornfeld, S. J. Biol. Chem. 253, 7762-7770 (1978).

72. Li, T. T. J. Biol. Chem. 242, 5474-5480 (1967).

73. Lucas, J. J., Waechter, C. J., and Lennarz, W. J. J. Biol. Chem. 250, 1992-2002 (1975).

74. Makita, A. and Shimoto, H. Biochim. Biophys. Acta 304, 571-574 (1973).

75. Meezan, E., Wu, H. C., Black, P. H., and Robbins, P. W. Biochem 8, 2518-2524 (1969).

76. Molnar, J. Mol. Cell Biochem. 6, 3-14 (1975).

77. Morrison, P. R., Edsall, J. T., and Miller, S. G. J. Am. Chem. Soc. 70, 3101-3108 (1948).

78. Muramatsu, T. J. Biol. Chem 246, 5535-5537 (1971).

79. Muramatsu, T., Atkinson, P. H., Nathenson, S. G. and Ceccarini, C. J. Mol. Bio. 80, 781-799 (1973).

80. Muramatsu, T., Koide, N., Ceccarini, C., and Atkinson, P. H. J. Biol. Chem. 251, 4673-4678 (1976).

81. Muramatsu, T. Methods in Enzymol. Vol. 50, Part C, 555-559 (1978).

82. Muramatsu, T. Koide, N., Ceccarini, C., and Atkinson, P. H. unpublished observation.

83. Narasimhan, S., Stanley, P., Williams, D., and Schachter, H. Fed. Proc. 35, 1441 (1976).

84. Narasimhan, S., Stanley, P., and Schachter, H. J. Biol. Chem. 252, 3926-3933 (1977).

85. Nicolson, G. L. Biochim. Biophys. Acta 458, 1-72 (1976).

86. Nishigaki, M., Muramatsu, T., and Kobata, A. Biochem. Biophys. Res. Commun. 59, 638-645 (1974).

87. Ogata, S., Muramatsu, T., and Kobata, A. J. Biochem. 78, 687-696 (1975).

88. Ohta, N. Pardee, A., McAuslan, B. R., and Burger, M. Biochim. Biophys. Acta 158, 98-102 (1968).

89. Olden, K., Pratt, R. M., and Yamada, K. M. Cell 13, 461-473 (1978).

90. Onodera, K. and Sheinin, R. J. Cell Science 7, 337-355 (1970).

91. Opheim, D. J. and Touster, O. J. Biol. Chem. 253, 1017-1023 (1978).

92. Oyama, V. I. and Eagle, H. Proc. Soc. Exp. Biol. Med. 91, 305-307 (1956).

93. Parodi, A. J., Behrens, N. H., Leloir, L. F., and Carminatti, H. Proc. Natl. Acd. Sci. U.S.A. 69, 3268-3272 (1972).

94. Perdue, J. F., Kletzien, R., and Miller, K. Biochim. Biophys. Acta 249, 418-434 (1971).

95. Perdue, J. F., Kletzien, R., and Wray, V. L. Biochim. Biophys. Acta 266, 505-510 (1972).

96. Perdue, J. F., Warner, D., and Miller, K. Biochim. Biophys. Acta 298, 817-826 (1973).

97. Reading, C. L., Penhoet, E. E., and Ballou, C. E. J. Biol. Chem. 253, 5600-5612 (1978).

98. Robbins, P. W., Krag, S. S., and Liu, T. J. Biol. Chem. 252, 1780-1785 (1977a).

99. Robbins, P. W., Hubbard, S. C., Rurco, S. J., and Wirth, D. F. Cell 12, 893-900 (1977b).

100. Robertson, M. A., Etchison, J. R., Robertson, J. S., Summers, D. F., and Stanley, P. Cell 13, 515-526 (1978).

101. Rothman, J. E. and Lenard, J. Science 195, 743-753 (1977).

102. Ruoslahti, E., Vaheri, A., Kuusela, P., and Linder, E. Biochim. Bicphys. Acta 322, 353-358 (1973).

103. Ruoslahti, E. and Vaheri, A. J. Exp. Med. 141, 497-501 (1975).

104. Sakiyama, H. and Burge, B. W. Biochem 11, 1366-1377 (1972).

105. Sefton, B. M. Cell 10, 659-668 (1977).

106. Shepherd, V. and Montgome:y, R. Carbohyd. Res. 61, 147-157 (1978).

107. Snaith, S. M. and Levvy, G. A. Biochem. J. 110, 663-670 (1968).

108. Spiro, R. G. J. Biol. Chem. 235, 2860-2869 (1960).

109. Spiro, R. G. J. Biol. Chem. 240, 1603-1610 (1965).

110. Spiro, R. G. Adv. Prot. Chem. 27, 349-494 (1973).

111. Spiro, M. J., Spiro, R. G., and Bhoyroo, V. O. J. Biol. Chem. 251, 6400-6408 (1976a).

112. Spiro, R. G., Spiro, M. J., and Bhoyroo, V. O. J. Biol. Chem. 251, 6409-6419 (1976b).

113. Spiro, M. J., Spiro, R. G., and Bhoyroo, V. O. J. Biol. Chem. 251, 6420-6425 (1976c).

114. Stanley, P., Narasimhan, S., Siminovitch, L., and Schachter, H. Proc. Natl. Acad. Sci. U. S. A. 72, 3323-3327 (1975a).

115. Stanley, P., Caillibot, V., and Siminovitch, L. Cell 6, 121-128 (1975b).

116. Struck, D. K. and Lennarz, W. J. J. Biol. Chem. 252, 1007-1013 (1977).

117. Tabas, I., Schlesinger, S., and Kornfeld, S. J. Biol. Chem. 253, 716-722 (1978a).

118. Tabas, I. and Kornfeld, S. J. Biol. Chem. 253, 778-7786 (1978b).

119. Tai, T., Yamashita, K., Ogata-Arakawa, M., Koide, N., Muramatsu, T., Iwashita, S., Inoue, Y., and Kobata, A. J. Biol. Chem. 250, 8569-8575 (1975a).

120. Tai, T., Ito, S., Yamashita, K., Muramatsu, T., and Kobata, A. Biochem. Biophys. Res. Commun. 65, 968-974 (1975b).

121. Tai, T., Yamashita, K., Ito, S., and Kobata, A. J. Biol. Chem. 252, 6687-6694 (1977a).

122. Tai, T., Yamashita, K., and Kobata, A. Biochem. Biophys. Res. Commun. 78, 434-441 (1977b).

123. Takatsuki, A. and Tamura, G. J. Antibio. (Tokyo) Ser. A. 24, 785-794 (1971).

124. Tarentino, A., Plummer, T. H., Jr., and Maley, F. J. Biol. Chem. 245, 4150-4157 (1970).

125. Tarentino, A. L., Plummer, T. H., and Maley, F. J. Biol. Chem. 247, 2629-2631 (1972).

126. Tarentino, A. L., Plummer. T. H., and Maley, F. J. Biol. Chem. 248, 5547-5548 (1973).

127. Tarentino, A. L. and Maley, F. J. Biol. Chem. 249, 811-816 (1974).

128. Tarentino, A. L., Plummer, T. H., and Maley, F. J. Biol. Chem. 249, 818-824 (1974).

129. Tarentino, A. L. and Maley, F. Biochem. Biophys. Res. Commun. 67, 455-462 (1975).

130. Tarentino, A. L. and Maley, F. J. Biol. Chem. 251, 6537-6543 (1976).

131. Tkacz, J. S. and Lampen, J. O. Biochem. Biophys. Res. Commun. 65, 248-257 (1975).

132. Toyoshima, S., Fukuda, M., and Osawa, T. Biochem. 11, 4000-4005 (1972).

133. Toyoshima, S., Fukada, M., and Osawa, T. Biochem. Biophys. Res. Commun. 51, 945-950 (1973).

134. Trevelyon, W. E., Proctor, D. P., and Harrison, J. S. Nature 166, 444-445 (1950).

135. Trimble, R. B., Tarentino, A. L., Plummer, T. H., Jr., and Maley, F. J. Biol. Chem. 253, 4508-4511 (1978).

136. Tulsiani, D. P., Opheim, D. J., and Touster, O. J. Biol. Chem. 252, 3227-3233 (1977).

137. Turco, S. J., Stetson, B., and Robbins, P. W. Proc. Natl. Acad. Sci. U.S.A. 74, 4411-4414 (1977).

138. Tuszynski, G. P., Baker, S. R., Fuhrer, J. P., Buck, C. A., and Warren, L. J. Biol. Chem. 253, 6092-6099 (1978).

139. Van Beek, W. P., Smets, L. A., and Emmelot, P. Cancer Res. 33, 2913-2922 (1973).

140. Van Beek, W. P., Smets, L. A., and Emmelot, P. Nature 253, 457-460 (1975).

141. Van Beek, W. P., Emmelot, P., and Homburg, C. Brit. J. Cancer 36, 157-165 (1977).

142. Van Nest, G. A. and Grimes, W. J. Biochem 16, 2902-2908 (1977).

143. Waechter, C. J., Lucas, J. J., and Lennarz, W. J. J. Biol. Chem. 248, 7570-7579 (1973).

144. Waechter, C. J. and Lennarz, W. J. Ann. Rev. Biochem. 45, 95-112 (1976).

145. Warren, L. and Glick, M. (J. Cell Bio. 37, 729-746 (1968).

146. Warren, L., Fuhrer, J. P., and Buck, C. A. Proc. Natl. Acad. Sci. U.S.A. 69, 1838-1842 (1972a).

147. Warren, L., Critchley, D., and Macpherson, I. Nature 235, 275-278 (1972b).

148. Warren, L., Fuhrer, J. P., and Buck, C. A. Fed. Proc. 32, 80-85 (1973).

149. Warren, L., Zeidman, I. and Buck, C. A. Cancer Res. 35, 2186-2190 (1975).

150. Warren, L., Buck, C. A., and Tuszynski, G. Biochim. Biophys. Acta 516, 97-127 (1978).

151. Whur, P., Herscovics, A., and Leblond, C. P. J. Cell Biol. 43, 289-311 (1969).

152. Wilson, J. R., Williams, D., and Schachter, H. Biochem. Biophys. Res. Commun. 72, 909-916 (1976).

153. Wu, H. C., Meezan, E., Black, P. H., and Robbins, P. H. Biochem. 8, 2509-2517 (1969).

154 Yamada, K. M., Yamada, S. S., and Pastan, I. Proc. Natl. Acad. Sci. U.S.A. 73, 1217-1221 (1976).

155. Yamada, K. and Weston, J. A. Proc. Natl. Acad. Sci. U.S.A. 71, 3492-3496 (1974).

156. Yamada, K. M., Olden, K., and Pastan, I. Ann. N.Y. Acad. Sci. 312, 256-277 (1978).

157. Yurchenco, P. D., Ceccarini, C., and Atkinson, P. H. Methods in Enzymol. Vol. 50, Part C, 175-204 (1978).