UNIVERSITY OF MANITOBA

STUDIES ON GLYCOSIDASES IN CONCANAVALIN A-SENSITIVE AND RESISTANT CHINESE HAMSTER OVARY CELLS GROWN IN TISSUE CULTURE

by

OREST WILLIAM BLASCHUK

A Thesis Submitted to the Faculty of Graduate Studies in Partial Fulfilment of the Requirements for the Degree of Master of Science.

Department of Chemistry

WINNIPEG, Manitoba

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To Kathi

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ABSTRACT

Three Chinese hamster ovary cell lines (wild-type, concanavalin A-resistant variant, and a concanavalin A-sensitive revertant) were analyzed for N-acetyl-β-D-hexosaminidase, α-L-fucosidase, and α-D-mannosidase activities. Ion-exchange chromatography revealed that each cell line had three hexosaminidase forms (I, II, and III), but only hexosaminidases I and III were secreted. The specific activities of the glycosidases varied when the growth conditions of the cells were changed. The specific activities of the glycosidases in extracts of the wild-type and revertant cells were similar, but differed from the specific activities of the glycosidases in extracts of the variant cells. The specific activities of the wild-type extracellular hexosaminidase forms differed from the specific activities of the variant extracellular hexosaminidase forms.

The properties of the three wild-type hexosaminidase forms were investigated and found to be different. These hexosaminidase forms were also partially purified.

The wild-type and variant membrane glycopeptides were analyzed by affinity chromatography and gel filtration. The wild-type cells were found to contain a group of large molecular weight glycopeptides that was absent in the variant cells.

ABBREVIATIONS

Glc	Glucose
Man	Mannose
Gal	Galactose
Fuc	Fucose
Fru	Fructose
Xyl	Xylose
4 KDM	4-Keto-6-deoxy-D-mannose
GlcN	Glucosamine
GlcUA	Glucuronic acid
GlcNAc	N-Acetylglucosamine
GalNAc	N-Acetylgalactosamine
ManNAc	N-Acetylmannosamine
NANA	N-Acetylneuraminic acid
Asn	Asparagine
Ser	Serine
Thr	Threonine
UDP	Uridine 5'-diphosphate
GDP	Guanosine 5'-diphosphate
CMP	Cytidine 5'-monophosphate
cAMP	Adenosine 3:5'-cyclic monophosphate
cGMP	Guanosine 3':5'-cyclic monophosphate
P	Phosphate
Dol	Dolichol

Cer	Ceramide
CM	Carboxymethyl
DEAE	Diethylaminoethyl
Hex	Hexosaminidase
EDTA	Ethylenediamine tetraacetic acid
Tris	Trishydroxymethylaminomethane
Mn	Manganese
Mg	Magnesium
W.T. CO_2	Wild-type Chinese hamster ovary cell line
C^R-7	Concanavalin A-resistant cell line
RC^R-7	Concanavalin A-sensitive revertant cell line

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INTRODUCTION

INTRODUCTION

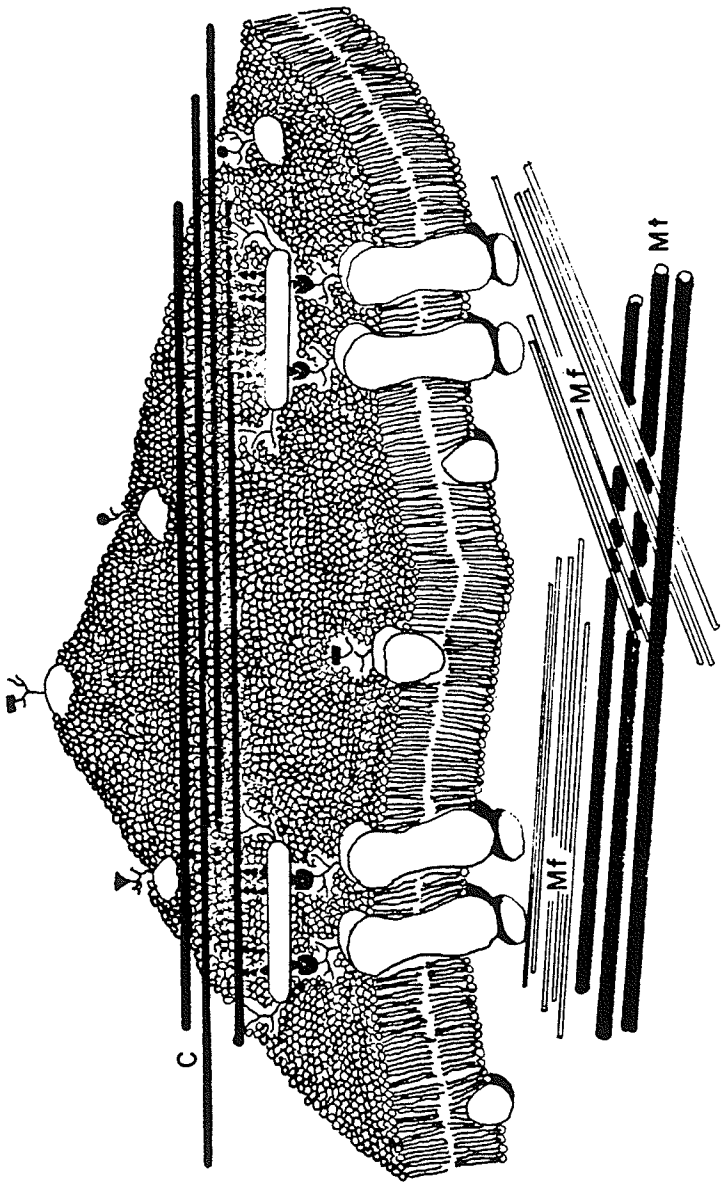
Membrane structure - an overview

The plasma membrane mediates cellular behaviour. Cell adhesion, contact, movement, and recognition are all thought to be functions of the membrane (1). The membrane can be rigid and unresponsive, or fluid and vital, depending on the circumstances. This wide variety of response is indicative of the membrane's complexity. Although the membrane is composed of only three major elements (lipid, protein, and carbohydrate), the interaction of these elements with one another and with themselves creates the complexity (2).

The fluid-mosaic model proposed by Singer and Nicolson (3) is the currently accepted concept of membrane structure (Figure 1). Lipids are shown to form an impermeable barrier between the extracellular and intracellular environments. Proteins are embedded in, or attached to, the lipid bilayer, either traversing the entire membrane, or facing the inside or outside of the cell. The proteins may act as receptors, or enzymes, or they may form channels for the passage of metabolites through the lipid (4). Movement of proteins within the lipid bilayer can be either free or restricted, thereby establishing a means of controlling membrane rigidity (4).

Membranous protein and lipid can be found associated with carbohydrate in the form of glycoproteins and glycolipids (2). These molecules usually have their carbohydrate portions orientated toward the extracellular milieu

Figure 1. A model of the cell surface[9]. The cell surface is shown to be connected to the internal cytoskeleton by protein complexes composed of collagen (c), fibronectin (capsule-shaped molecule), and integral membrane proteins. Interactions between these components are depicted to involve the oligosaccharide chains of the integral proteins. The integral proteins also link the complex to the microfilaments (Mf) and microtubules (Mt) of the internal cytoskeleton.


C
Mf
Mf
Mt

(2). Only recently has the function of the carbohydrate been elucidated. Glycoproteins and glycolipids are thought to be involved in cell-cell interactions, cell adhesion, and recognition (1, 5, 6, 7, 8).

In this thesis, the emphasis is placed on glycoprotein and glycolipid structure, biosynthesis, degradation, and function.

Structure of mammalian glycoproteins

Glycoproteins do not contain all the monosaccharides found in nature. Only the sugars listed in Table I have been shown to be involved in glycoprotein structure.

The most distinguishing characteristic of glycoproteins is the covalent linkage between the first sugar residue in the oligosaccharide chain and an amino acid in the polypeptide chain. The carbohydrate-protein linkages may be either N-glycosidic or O-glycosidic (10). Three main types of amino acid-sugar linkage have been identified in glycoproteins. The most common is the glycosylamine linkage involving the amide group of asparagine and the hydroxyl on C-1 of N-acetylglucosamine (11). Glycoproteins such as ovalbumin, fetuin, thyroglobulin, and the immunoglobulins contain this linkage (11, 12, 13, 14). Human erythrocyte membrane glycoproteins also contain the N-acetylglucosaminyl-asparagine linkage (15). The other two types of linkages found in glycoproteins involve O-glycosidic bonds. In the mucins, the glycosidic bond involves the hydroxyl groups of serine or threonine linked to N-acetylgalactosamine, while in collagen,

TABLE 1 *

Monosaccharide constituents of glycoproteins†

Hexoses	Galactose
	Mannose
	Glucose
Deoxyhexoses	L-Fucose
Hexosamines	N-Acetylglucosamine
	N-Acetylgalactosamine
Sialic acids	Acylneuraminic acids
Pentoses	Xylose
	L-Arabinose

* taken from reference 10.

† the typical sugar constituents of mucopolysaccharides are not listed.

the glycosidic bond is between the hydroxyl group of hydroxylysine and galactose (10). A detailed discussion of sugar-amino acid linkages has been written by Zinn et al. (16).

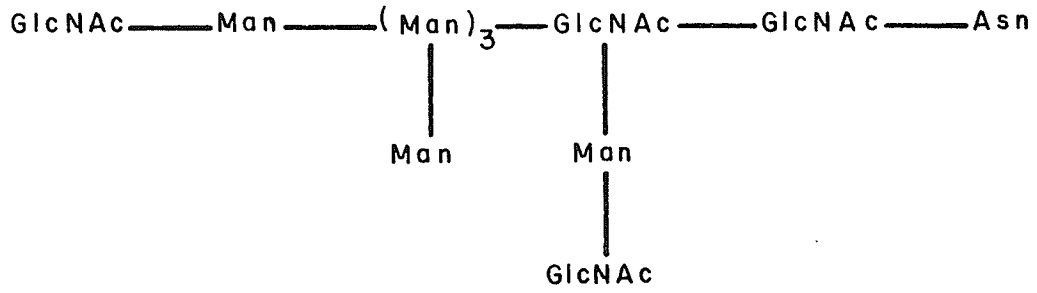
Although only a few glycoprotein oligosaccharide chains have been sequenced in their entirety, two main types of chain seem to predominate; these are referred to as simple and complex chains (12). Ovalbumin is an example of a glycoprotein with simple carbohydrate chains, containing only the sugars mannose and N-acetylglucosamine (Figure 2A). Complex chains contain the additional sugars N-acetyl-neuraminic acid, fucose, and galactose (Figure 2B). These latter chains are characteristic of the serum glycoproteins (17) as well as some membrane glycoproteins (15).

Biosynthesis of mammalian glycoproteins

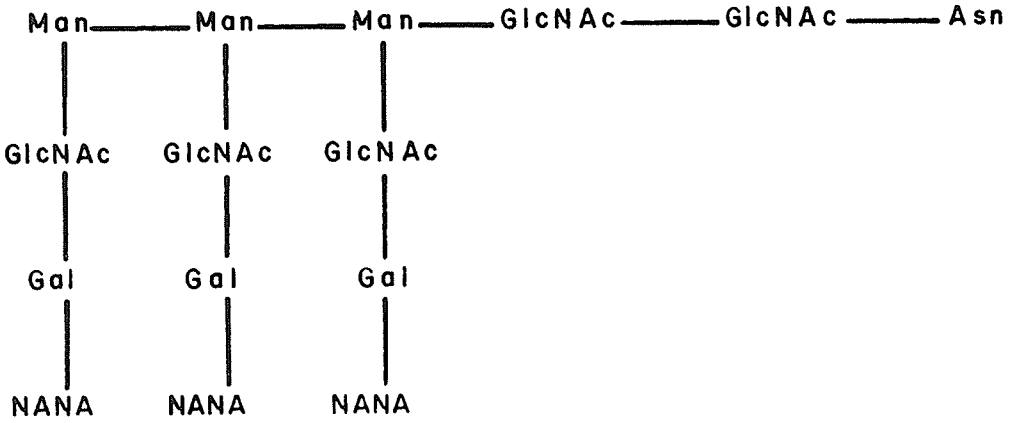
The immediate precursors of the sugars in the glycoprotein oligosaccharide chains are the nucleotide sugars. All of the nucleotide sugars can be synthesized from glucose, as is illustrated in Figure 3. Sugar nucleotide synthesis occurs in the cellular cytoplasm (18), while glycoproteins are assembled in the endoplasmic reticulum and Golgi apparatus (19). The nucleotide sugars must consequently pass through cellular membranes in order to be utilized (20).

Lipid-sugar intermediates have recently been implicated in the synthesis of glycoprotein oligosaccharide chains (2). In mammals, the lipid intermediate is a long chain polyprenol called dolichol (20)

Figure 2. Examples of simple (A) and complex (B) carbohydrate chains (12).

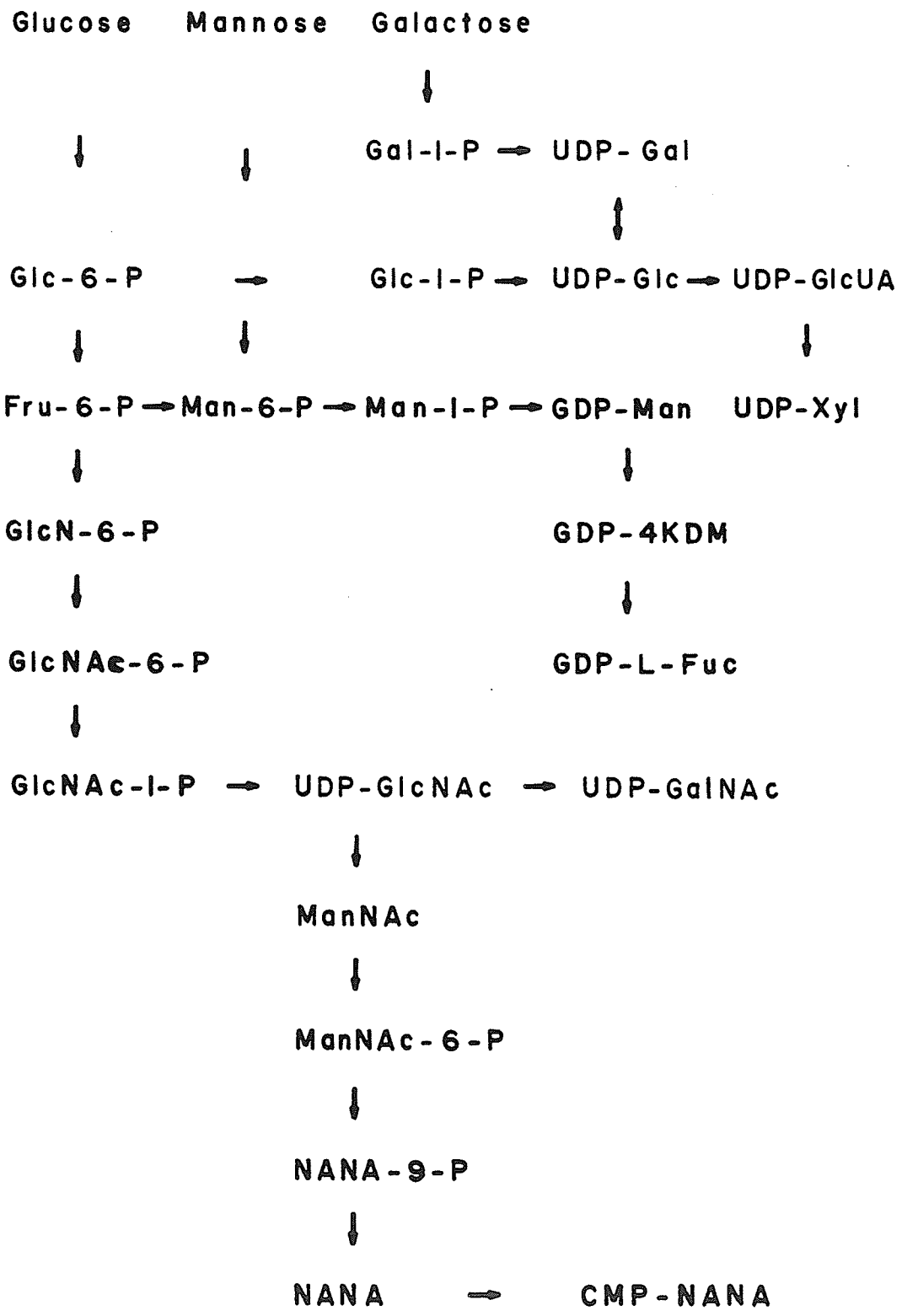

GlcNAc
Man
(Man)3
GlcNAc
GlcNAc
Asn
Man
Man
GlcNAc

A


Man
Man
Man
GlcNAc
GlcNAc
Asn
GlcNAc
GlcNAc
GlcNAc
Gal
Gal
Gal
NANA
NANA
NANA

B

Figure 3. Pathway of sugar nucleotide synthesis (10).


Glucose
Mannose
Galactose
Gal-1-P
UDP-Gal
Glc-6-P
Glc-1-P
UDP-Glc
UDP-GlcUA
Fru-6-P
Man-6-P
Man-1-P
GDP-Man
UDP-Xyl
GlcN-6-P
GDP-4KDM
GlcNAc-6-P
GDP-L-Fuc
GlcNAc-1-P
UDP-GlcNAc
UDP-GalNAc
ManNAc
ManNAc-6-P
NANA-9-P
NANA
CMP-NANA

(Figure 4A). The linkage of the monosaccharides glucose, xylose, N-acetylmannosamine, mannose, and N-acetylglucosamine to polyprenol phosphates has been demonstrated (20). The structure of α-D-manno-pyranosyl dolichol phosphate is given in Figure 4B.

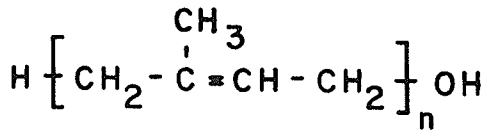
Oligosaccharide-lipid intermediates have also been shown to exist (21, 22). The sugars N-acetylglucosamine and mannose are thought to be attached to dolichol monophosphate by the pathway shown in Figure 5. The enzymes responsible for catalyzing the synthesis of the oligosaccharide chain are called glycosyltransferases (15).

The glycosyltransferases are very specific enzymes, requiring both the correct sugar nucleotide and acceptor molecule for catalysis to occur (23). The acceptor molecule may be either a glycoprotein, glycolipid, protein, lipid (ceramide), or, as mentioned previously, an oligosaccharide-lipid intermediate (Figure 6). In view of the substrate specificity of these enzymes, there are thought to be a large number of transferase systems present in the various cellular organelles, each system being specific for a particular type of acceptor molecule (23).

Glycoprotein assembly and secretion

Palade (24) has divided the process of protein secretion into six steps: synthesis, segregation, intracellular transport, concentration, intracellular storage, and discharge. Secretory proteins appear to be synthesized on polyribosomes attached to the rough endoplasmic reticulum (25). According to the "multisite" model for the biosynthesis of

Figure 4. The structures of polyprenol (A) and α-D-manno-pyranosyl dolichol phosphate (B) (15).

A 

n = 5 - 24

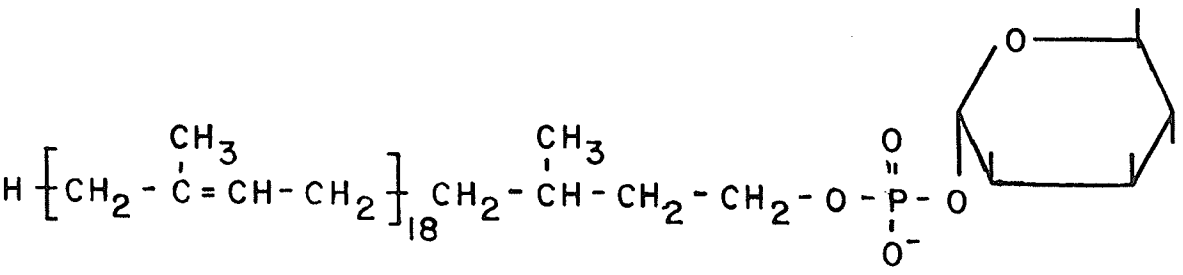
B 

Figure 5. Proposed pathway for the assembly of oligosaccharide-dolichol intermediates (20).

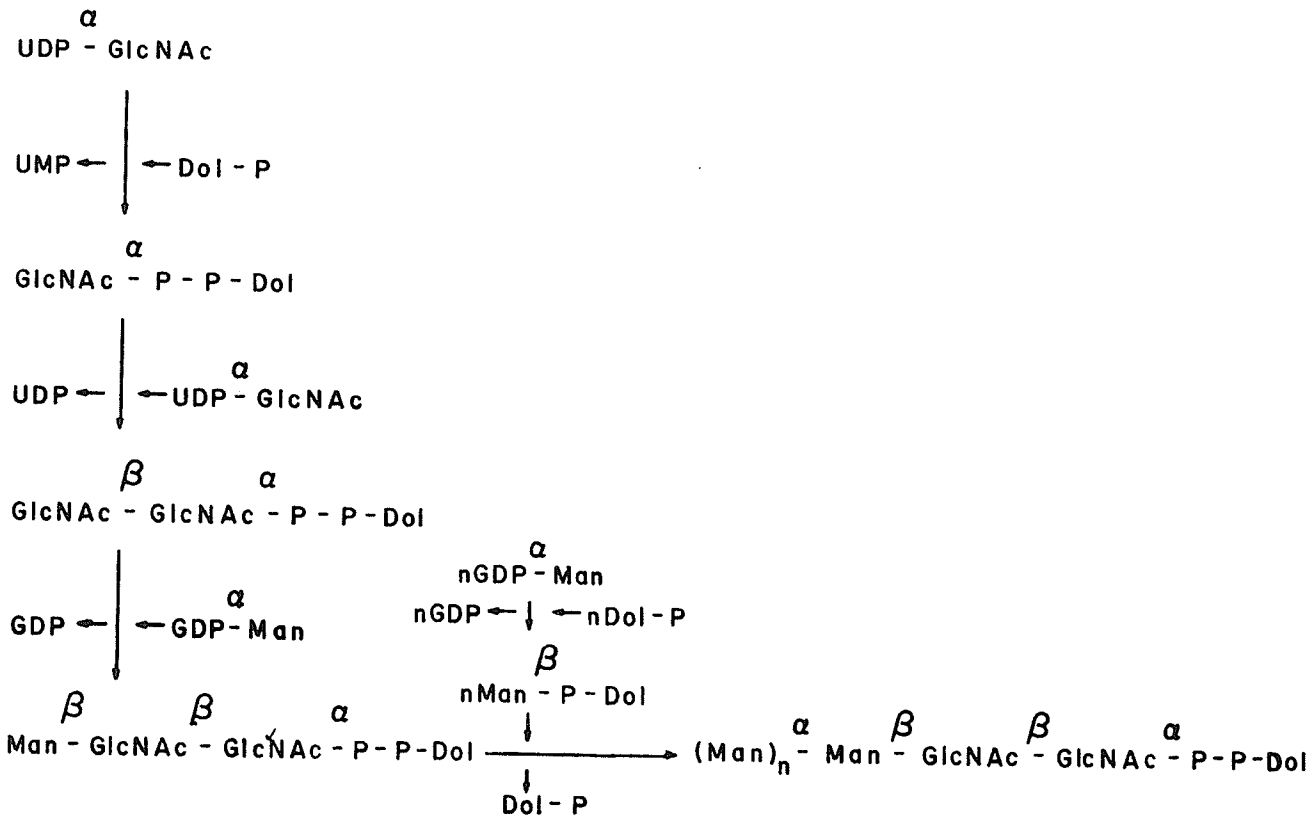
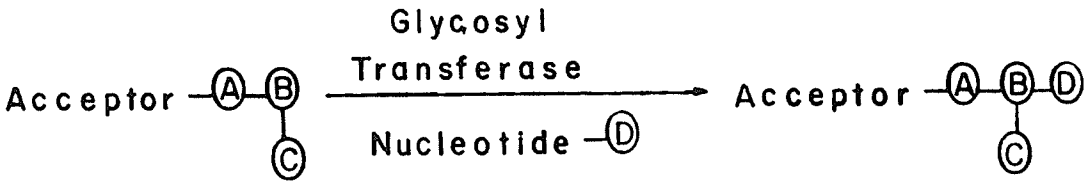

α
UDP - GlcNAc
UMP
Dol - P
α
GlcNAc - P - P - Dol
UDP
α
UDP - GlcNAc
β α
GlcNAc - GlcNAc - P - P - Dol
GDP
α
GDP - Man
α
nGDP - Man
nGDP
nDol - P
β
nMan - P - Dol
β β α
Man - GlcNAc - GlcNAc - P - P - Dol
Dol - P
α β β α
(Man)n - Man - GlcNAc - GlcNAc - P - P - Dol

Figure 6. The reaction catalyzed by glycosyltransferases (23).



SUBSTRATE PRODUCT

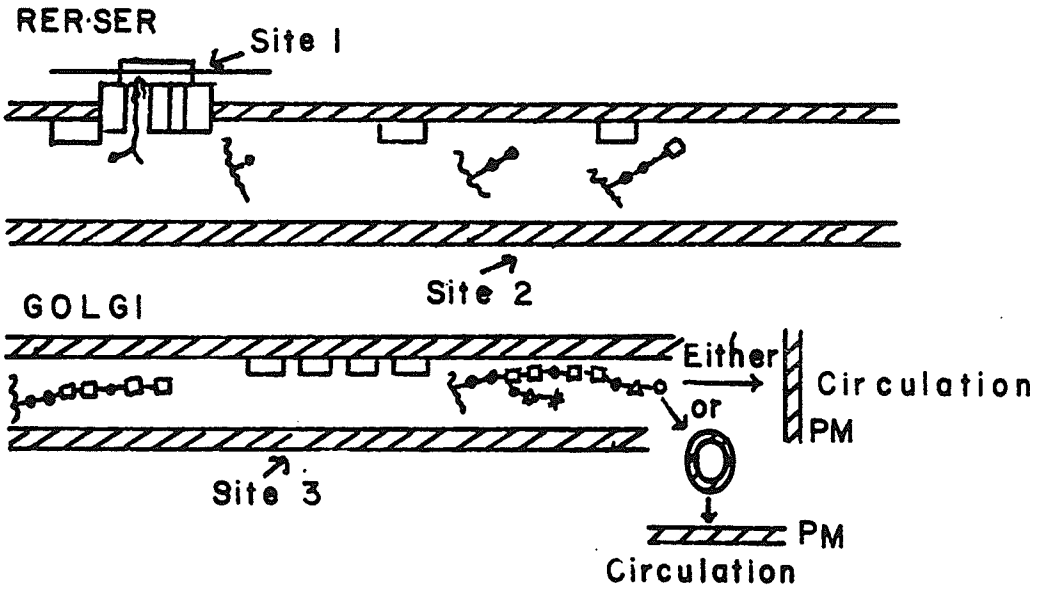
A,B,C,D are sugars

glycoproteins (26, 27), the protein is glycosylated while it is still in the process of being synthesized (Figure 7). The initial sugar, usually N-acetylglucosamine, is attached to the protein at the first asparagine residue capable of forming a linkage. Only asparagine residues which are part of the tripeptide sequence -Asn-X-($\frac{\text{Ser}}{\text{Thr}}$)- are capable of being glycosylated (28). This sequence has been recognized as a coding unit for carbohydrate chain attachment and is referred to as a sequon (Figure 8).

In 1976, Molnar (19) postulated that the synthesis of glycoprotein oligosaccharide chains occurred in a sequential manner, only one sugar being attached to the chain at a time by an appropriate glycosyltransferase (Figure 7). Although oligosaccharide-lipid intermediates were known to exist (20), their involvement in glycoprotein oligosaccharide chain synthesis had not been demonstrated. Recently, Chen and Lennarz (29) have shown that oligosaccharide chains can be transferred en bloc from lipid carriers to unglycosylated proteins (29). The concept of oligosaccharide chain synthesis has consequently been modified. Figure 9 illustrates the current concept of glycoprotein synthesis. The synthesis of oligosaccharide-lipid intermediates has been discussed previously and is shown in Figure 5. The oligosaccharide-lipid intermediates, in addition to containing mannose and N-acetylglucosamine, also contain terminal glucose residues (29, 31). Studies have shown that these glucose residues, and some mannose residues, are removed by specific glycosidases after the oligosaccharide chain has been attached

Figure 7. The "multisite" model for the biosynthesis of glycoproteins (19). The symbols RER, SER, and PM denote the rough and smooth endoplasmic reticulum, and the plasma membrane, respectively. Three sites of sugar attachment are depicted.

Figure 8. A typical oligosaccharide chain of a glycoprotein. The sites at which each sugar of the chain is attached are shown (19).


RER·SER
Site 1
Site 2
GOLGI
Either
or
Circulation
PM
Site 3
PM
Circulation

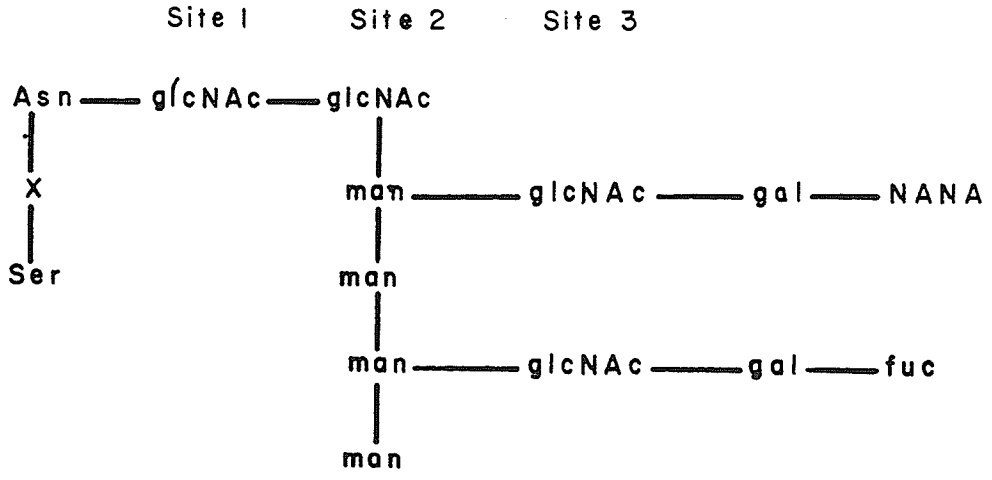
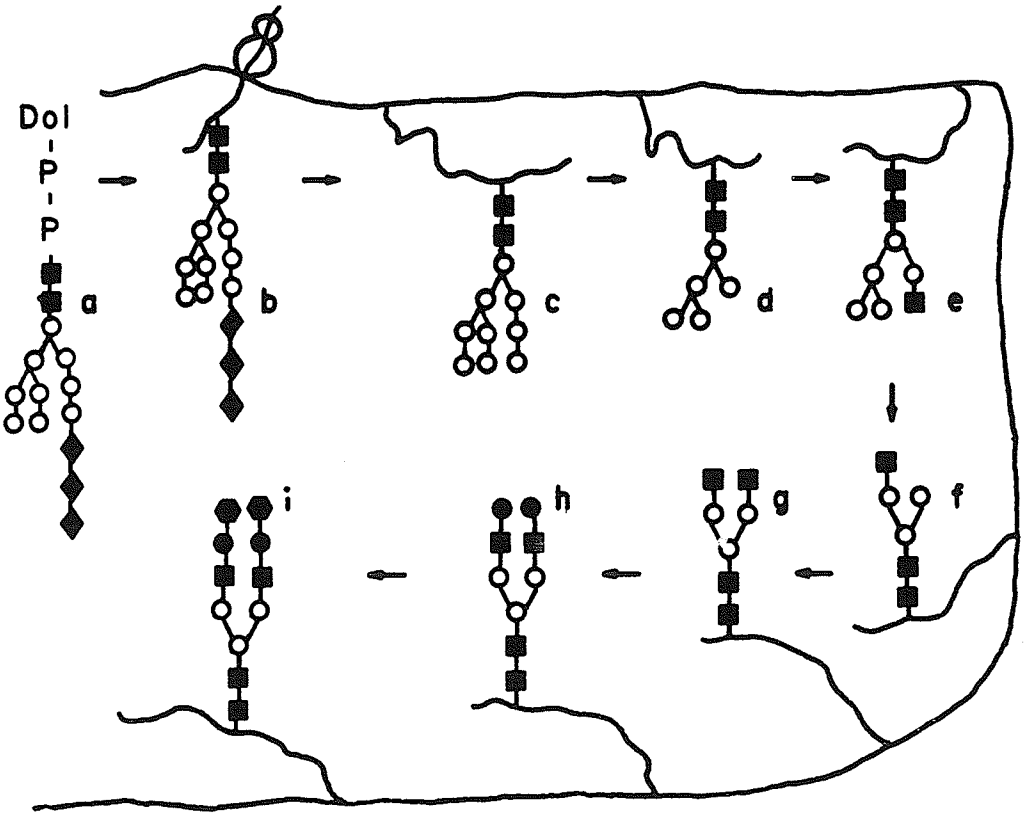

Site 1
Site 2
Site 3
Asn
glcNAc
glcNAc
X
Ser
man
glcNAc
gal
NANA
man
man
glcNAc
gal
fuc
man

Figure 9. A model illustraing the current concept of glycoprotein synthesis (30). Precursor oligosaccharide is assembled on a lipid intermediate (a), and then transferred to the nascent glycoprotein (b). Glycosidases remove glucose (◆) (c) and mannose (○) (d) residues. A glycosyltransferase then adds on an N-acetylglucosamine (■) residue (e). Additional mannose residues are then removed (f). Finally, glycosyltransferases add the sugars N-acetylglucosamine (g), galactose (●) (h), and N-acetylneuraminic acid (⬢) (i) onto the glycoprotein.


Dol
P
P
a
b
c
d
e
f
g
h
i

to the unglycosylated protein. This partial degradation of the oligosaccharide chain is referred to as processing (30). The processed glycoprotein is thought to progress from the rough endoplasmic reticulum to the smooth endoplasmic reticulum, and finally to the Golgi apparatus, where the sugars fucose, galactose, and N-acetylneuraminic acid are attached by sequential addition of the appropriate sugars (30). Subcellular fractionation studies have confirmed the predominance of fucosyl, galactosyl, and sialyltransferase activities in this organelle (32, 33), thus supporting the theory that the Golgi complex is the subcellular site for the completion of the sugar chains. In the Golgi complex, the completed glycoproteins are compartmentalized into vesicles and either discharged from the cell, stored within the cell, or incorporated into the cell's plasma membrane (24).

The involvement of glycoproteins in cell surface phenomena

Evidence is rapidly accumulating in favor of the theory that cell surface glycoproteins play a central role in cell-cell and cell-ligand interactions, intercellular adhesiveness, and the control of cell division (1, 6, 8).

Fibronectin serves as an excellent example of the involvement of glycoproteins in cellular adhesion and malignant transformation (7). This cell surface glycoprotein has been described as fibroblast surface antigen (34), cell surface protein (35), LETS (large external

transformation sensitive) protein (8), z protein (36), and fibronectin (37). All of the glycoproteins listed above are thought to be related, having a common subunit molecular weight of 200,000 - 250,000 daltons (8) and being immunologically cross-reactive (38).

Cultured fibroblasts, as well as containing fibronectin on their cell surface, secrete the glycoprotein into the medium (7). The secreted fibronectin is thought to mediate cell attachment and spreading on surfaces (39). Cells deposit fibronectin onto their tissue culture substratum, which leads to the speculation that the molecule is required for cellular adhesion (40).

Studies have shown that fibronectin is either reduced or absent on the surfaces of virally transformed (1, 8) and neoplastic cells (38). Interestingly, both transformed and neoplastic cells display a reduced adhesion to extracellular matrices (41). By adding purified fibronectin to transformed cells, workers have been able to partially restore a more fibroblastic appearance to the cells and an increased adhesiveness (42).

Membrane glycoproteins have also been implicated in cell-ligand interactions. Mammalian livers possess plasma membrane glycoproteins capable of specifically binding asialo* serum glycoproteins (43). Morell et al. (44) showed that the injection of asialo serum glycoproteins into rabbits resulted in their rapid clearance from the circulation by

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*glycoproteins which have had their terminal sialic acid residues removed.

the liver. Subsequent studies confirmed the presence of specific liver membrane glycoproteins which bound the asialo glycoproteins by interaction with galactose residues exposed by the removal of sialic acid (45, 46, 47). Conversely, neuraminidase treatment of the purified membrane glycoprotein abolished the receptor's binding activity (46). The terminal sialic acid residues of both the circulating glycoproteins and the liver glycoprotein receptors were thus shown to be crucial in the recognition and uptake processes.

Cell-ligand interactions, such as the one discussed above, are thought to be intimately involved in the process of cell-cell adhesion. Slime molds, for example, exhibit cellular interactions that are mediated by membrane glycoprotein receptors (48). The life cycle of Dictystelium discoideum can be divided into three distinct phases. Vegetative unicellular amoebae exist at the beginning of the cycle. When the food supply is exhausted, the amoebae aggregate to form a multicellular slug. The cells of the slug, in turn, differentiate into spore and stalk cells (48). Cells can be isolated at any of the various stages of the life cycle, making the study of the aggregation phenomenon possible. Antibodies have been raised against cells from the aggregating state, and their Fab* fragments have been shown to block the aggregation of amoebae (49). This finding indicated that specific membrane proteins were responsible for the aggregation phenomenon. Subsequently, a membrane glycoprotein, designated cs-A, was purified with the ability

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*antigen binding fragment

to block the action of the Fab fragments (6).

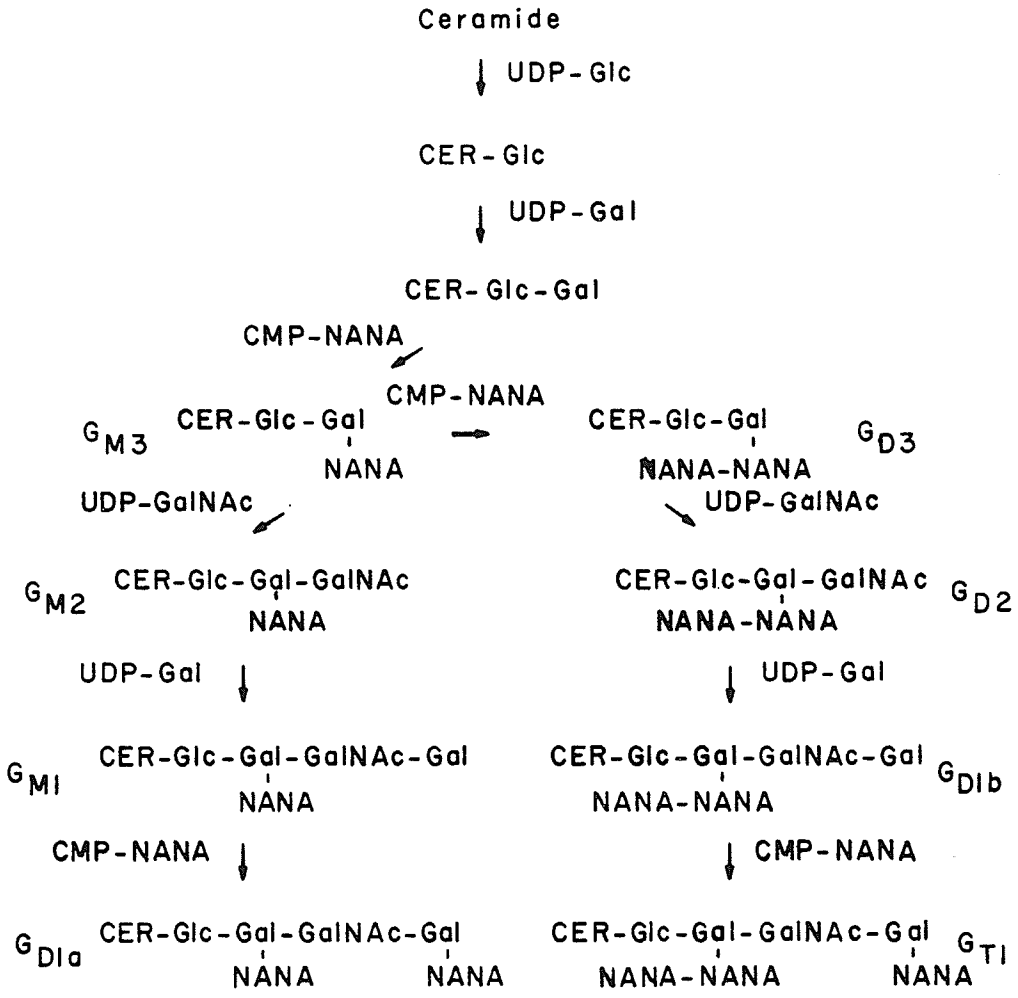
Two cell surface carbohydrate-binding proteins, discoidin I and II, have also been isolated from cells in the slime mold's aggregating state (50, 51). Lactose was found to be the best inhibitor of discoidin II, while N-acetyl-D-glucosamine was the prime inhibitor of discoidin I (51). Furthermore, the aggregation of developing cells could be blocked by N-acetyl-D-glucosamine. Rabbit antibodies directed against these two discoidins were found to bind aggregating cells, but not the vegetative cells (52). Clearly, cell surface glycoproteins and receptors play a role in cell-cell interactions.

Glycolipids

The most common mammalian glycolipids are the gangliosides. These molecules are sialoglycolipids and consequently possess a strong negative charge (5). The hydrophobic (lipid) subunit of these compounds is ceramide, a compound between an amino alcohol sphingosine and a long chain (C_{14}-C_{26}) fatty acid (53).

Ganglioside oligosaccharide chains are believed to be assembled in a manner similar to glycoprotein carbohydrate chains (54). As shown in Figure 10, monosaccharide residues are added to the ceramide by glycosyltransferases. In cultured fibroblasts, the glycosyltransferases are associated with membrane fractions and are thought to be located, not only within the cell, but also on the cell surface (55, 56). Keenan et al. (57) have demonstrated the presence of these enzymes in the Golgi

Figure 10. The biosynthetic pathway of gangliosides (5).


Ceramide
UDP-Glc
CER-Glc
UDP-Gal
CER-Glc-Gal
CMP-NANA
CMP-NANA
G_{M3}
CER-Glc-Gal
NANA
CER-Glc-Gal
NANA-NANA
G_{D3}
UDP-GalNAc
UDP-GalNAc
G_{M2}
CER-Glc-Gal-GalNAc
NANA
CER-Glc-Gal-GalNAc
NANA-NANA
G_{D2}
UDP-Gal
UDP-Gal
G_{M1}
CER-Glc-Gal-GalNAc-Gal
NANA
CER-Glc-Gal-GalNAc-Gal
NANA-NANA
G_{D1b}
CMP-NANA
CMP-NANA
G_{D1a}
CER-Glc-Gal-GalNAc-Gal
NANA
NANA
CER-Glc-Gal-GalNAc-Gal
NANA-NANA
NANA
G_{T1}

apparatus of the liver and brain.

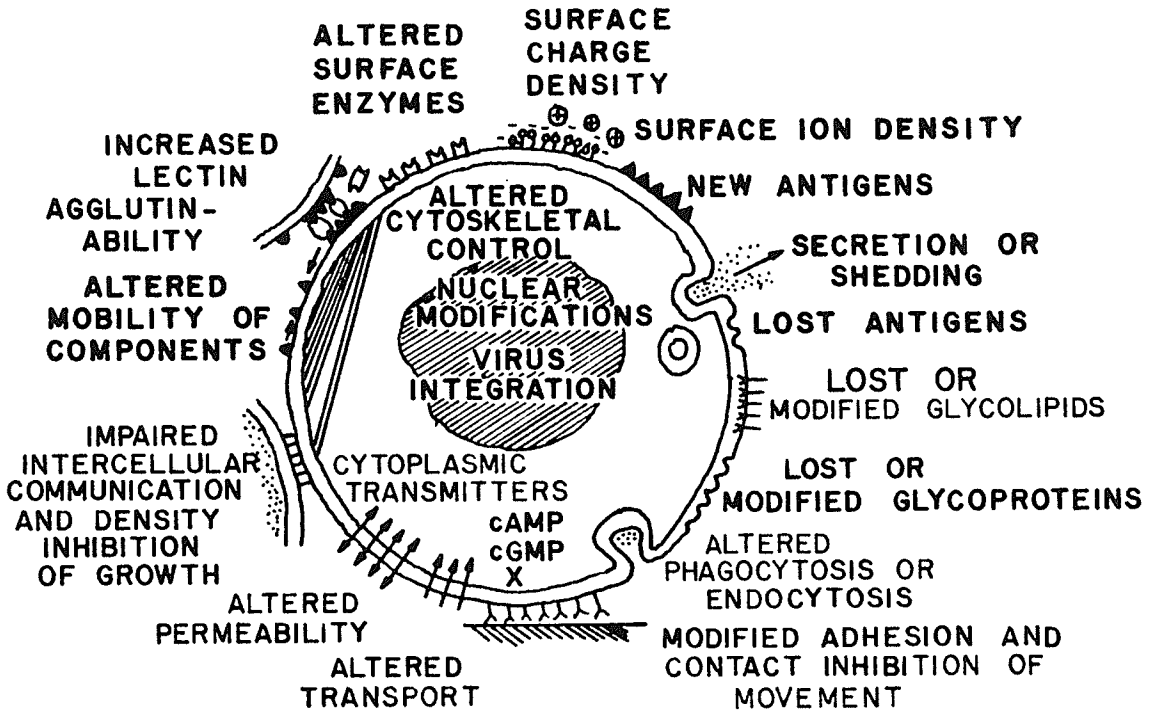
The physical nature of gangliosides makes them ideally suited as membrane receptors, the ceramide portions of these molecules being embedded in the membrane with the negatively charged oligosaccharide chains exposed to the external environment (58, 59). The prime example of a ganglioside acting as a membrane receptor is the monosialoganglioside, G_{M1} (Figure 10), which has been shown to act as a receptor for cholera toxin (60, 61). Gangliosides have also been implicated as hormone receptors. The ganglioside, G_{D1b} (Figure 10), inhibits the binding of thyroid-stimulating hormone to thyroid membranes (62). Interestingly, G_{D1a} (Figure 10), the structural isomer of G_{D1b}, has little effect on the thyroid-stimulating hormone binding. Thus, hormone interaction with membrane receptors appears to be highly specific for carbohydrate structure.

Cell surface changes accompanying transformation

In the previous sections, the role of membrane glycoproteins and glycolipids in cellular behaviour has been discussed. Clearly, the modification of these cell surface components would result in abnormal responses to environmental stimuli. Transformed and neoplastic cells display gross glycoprotein and glycolipid changes, as well as many other membrane alterations (Figure 11).

Several workers have made the observation that the glycoproteins of malignant cells contain more carbohydrate groups of a larger molecular

Figure 11. Cell surface alterations found after neoplastic transformation (1).


ALTERED SURFACE ENZYMES
SURFACE CHARGE DENSITY
SURFACE ION DENSITY
INCREASED LECTIN AGGLUTIN-ABILITY
NEW ANTIGENS
ALTERED CYTOSKELETAL CONTROL
SECRETION OR SHEDDING
ALTERED MOBILITY OF COMPONENTS
NUCLEAR MODIFICATIONS
LOST ANTIGENS
VIRUS INTEGRATION
LOST OR MODIFIED GLYCOLIPIDS
IMPAIRED INTERCELLULAR COMMUNICATION AND DENSITY INHIBITION OF GROWTH
CYTOPLASMIC TRANSMITTERS
cAMP
cGMP
X
LOST OR MODIFIED GLYCOPROTEINS
ALTERED PHAGOCYTOSIS OR ENDOCYTOSIS
ALTERED PERMEABILITY
MODIFIED ADHESION AND CONTACT INHIBITION OF MOVEMENT
ALTERED TRANSPORT

weight than the normal cells from which they are derived (63, 64, 65). Tuszynski et al. (66) have recently shown that practically all Rous sarcoma virally-transformed baby hamster kidney cell membrane glycoproteins differ from the normal hamster cell membrane glycoproteins. The conclusion reached was that viral transformation produces altered glycosylation of glycoproteins. Nest and Grimes (67), working with spontaneously transformed Balb 3T3 cells, reached the same conclusion. Apparently, transformation leads to extensive modification of membrane glycoproteins.

The ganglioside and neutral glycolipid content of neoplastically transformed cell lines also differs from normal cell lines (2). Virally transformed mouse fibroblasts exhibit a reduction of the gangliosides G_{M2}, G_{M1}, and G_{D1a} (68, 69). Other strains, when transformed, accumulate a single ganglioside. As an example, Balb 3T3 cells, when transformed with the Kirsten strain of murine sarcoma virus, accumulate G_{M2} (70). Additionally, hamster cell lines, rat kidney fibroblasts, and human fibroblasts have been examined and all display glycolipid changes when transformed by various viruses (2). These observations, together with the known functional role of glycoproteins and glycolipids, strongly imply the involvement of glycoproteins and glycolipids in the altered behaviour of neoplastically transformed cells.

The membrane glycoprotein and glycolipid changes described above are accompanied by alterations in the levels of glycosyltransferases

and glycosidases (1, 2). Clearly, if the glycoprotein and glycolipid carbohydrate chains are modified upon viral transformation, changes must be occurring in either the biosynthetic or degradative pathways of these molecules. As discussed earlier, the glycosyltransferases are involved in the biosynthesis of the oligosaccharide chains of glycoproteins and glycolipids (23). Increased or decreased levels of these enzymes would result in either larger or smaller carbohydrate chains, or perhaps, fewer carbohydrate chains. Glycoprotein and glycolipid degradation, conversely, is controlled by a system of equally specific oligosaccharide hydrolytic enzymes, called glycosidases (2). Elevated or reduced glycosidase levels would also result in glycoprotein and glycolipid modifications (71).

Bosmann and Hall (72) showed that human neoplastic tissue contained elevated levels of glycoprotein: sialyltransferase activity as well as elevated levels of certain proteases and glycosidases. These observations led to the theory that changes in the levels of enzymes involved in glycoprotein and glycolipid metabolism may lead to modifications in tumor cell surfaces resulting in the altered behaviour of these cells.

Studies have shown that glycosidase levels also increase when cells grown in tissue culture are transformed. Kijimoto and Hakomori (73) found higher β-galactosidase activities in transformed hamster-fibroblast cells. Bosmann et al. reported elevated glycosidase levels in transformed mouse (74) and chicken (75) cell lines. These observations seem to indicate a possible correlation between changes in

glycosidase and glycosyltransferase levels and transformation, as well as neoplasia.

Concanavalin A

The cell surface glycoprotein and glycolipid changes displayed by transformed cells may be detected with the aid of lectins, a group of proteins capable of agglutinating a variety of cells (76). The lectin of primary concern to this study is concanavalin A. This agglutinin was isolated from Jack Bean (Canavalia einsformis) meal (77). Concanavalin A can exist in either the monomeric, dimeric, tetrameric, or multimeric form, depending on pH and ionic conditions (78, 79). Below pH 4.6, concanavalin A dissociates into monomers of molecular weight 25,500, while from pH 5.6 to pH 7.0, the lectin is in a tetrameric form (79). The monomers are identical (80) and each contains one sugar binding site as well as a calcium and magnesium binding site (81). The two cations are required for sugar binding (81). Concanavalin A specifically binds α-D-mannopyranosyl and α-D-glucopyranosyl residues (82, 83). The C-3, C-4, and C-6 hydroxyl groups of the D-arabino configuration are essential for concanavalin A interaction (84). The lectin is also capable of binding the non-exposed α-D-mannose residues present in the core region of glycoprotein oligosaccharide chains (84).

The ability of concanavalin A to bind glycoproteins makes it an ideal membrane probe (76). As discussed previously, the membrane glycoproteins and glycolipids of transformed cells are drastically altered (1).

Consequently, concanavalin A interacts with transformed cells differently than it does with normal cells (76). Oncogenically transformed cells are preferentially agglutinated by concanavalin A at concentrations that do not affect normal (wild-type) cells (85).

Concanavalin A has many other biological properties (76). A noteworthy property is the lectin's toxicity to animal cells (86). The precise mechanisms involved in concanavalin A toxicity are not clear. Kornfeld et al. (87) have shown that the initial step involves binding of the lectin to carbohydrate receptors on the plasma membrane. The lectin is then thought to enter the cell by pinocytosis and cause cell death by an as yet unknown mechanism (2). Cytotoxic lectins such as concanavalin A, wheat germ agglutinin, ricin, and phytohemagglutinin have been used as selective agents to obtain a variety of lectin-resistant variants from established cell lines (30). A large number of these variants are incapable of binding the lectins due to alterations in their glycoprotein oligosaccharide chains (30). Lectin-resistant variants are now being used to study how these oligosaccharide chain alterations occur.

Introduction to the work presented in this thesis

The cytotoxic effects of concanavalin A were used by Ceri and Wright (88) to select several concanavalin A-resistant variants from independent wild-type, concanavalin A-sensitive, Chinese hamster ovary cell clones. The variant used in this thesis, C^{R}-7, is a subclone

which was selected from a mixed population of concanavalin A-resistant cells. This mixed population of cells was obtained after ten passages of a wild-type population (W.T. CO_2) at 34°C in growth medium containing 40 μg/ml concanavalin A (89, 90). The C^R-7 cell line was found to be temperature-sensitive, growing at 34°C, but not at 39°C (89). A concanavalin A-sensitive revertant (RC^R-7) was selected from the C^R-7 cell line by making use of the variant's temperature-sensitivity. Briefly, the C^R-7 cells were incubated at the non-permissive temperature, 39°C, and the colonies that appeared were tested for concanavalin A-sensitivity. In this manner, the cloned cell line called RC^R-7 was selected (88).

The concanavalin A-resistant variant exhibited a complex phenotype characterized by a modification in growth properties and changes in various membrane associated properties (88, 89, 91). The C^R-7 cells, as compared to the W.T. CO_2 and RC^R-7 cells, showed the following changes in phenotype: temperature-sensitive growth properties, altered cellular morphology on culture plates, enhanced sensitivity to membrane-active drugs such as phenethyl alcohol and sodium butyrate, increased resistance to the cytotoxic effects of concanavalin A, decreased cellular adhesiveness to substratum, and defective lectin-receptor mobility (88, 89, 90, 91). Additionally, surface labelling techniques have revealed the presence of an extra glycoprotein on the C^R-7 cell surface that is absent in W.T. CO_2 and RC^R-7 cells (91). Changes in glycoprotein and glycolipid metabolism could result in some or all of the alterations

described above, particularly in the appearance of the extra glycoprotein on the C^R-7 cell surface.

As mentioned previously, a correlation seems to exist between the altered membrane properties of transformed cells and changes in glycosidase and glycosyltransferase levels (1, 72, 73, 74, 75). Since the C^R-7 cells display similar membrane alterations, the need for an investigation of the glycosidase and glycosyltransferase levels in the W.T. CO_2, C^R-7 and RC^R-7 cell lines became obvious. Although changes in the glycosyltransferase levels of other lectin-resistant variants have previously been reported (30), the glycosidase levels of these variants have not been investigated. In this thesis, the emphasis was placed on the cellular glycosidases in an attempt to correlate their levels with the concanavalin A-resistant phenotype. The membrane glycoproteins of the three cell lines were also investigated to gain a better understanding of the glycoprotein changes on the variant cell surface.

MATERIALS AND METHODS

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Materials

Cell growth material

Material	Source
alpha-minimal essential medium	Flow Labs, Rockville, Md.
fetal calf serum	GIBCO Ltd., Calgary, Alta.
penicillin G	GIBCO Ltd., Calgary, Alta.
streptomycin	GIBCO Ltd., Calgary, Alta
Brockway bottles	Brockway Glass Co., Brockway, Pa.
culture plates	Lux Sci. Corp., California.
spinner bottles	GIBCO Ltd., Calgary, Alta.

Experimental material

Material	Source
p-nitrophenyl-N-acetyl-β-D-glucosaminide	Sigma Chemical Co., St. Louis, Mo.
p-nitrophenyl-α-L-fucoside	Sigma Chemical Co., St. Louis, Mo.
p-nitrophenyl-α-D-mannoside	Sigma Chemical Co., St. Louis, Mo.
Triton X-100	Sigma Chemical Co., St. Louis, Mo.
trypsin	Sigma Chemical Co., St. Louis, Mo.
Bio-Rad protein determination kit	Bio-Rad Labs, Mississauga, Ont.
Eppendorf micro test tubes, 1.5 ml.	Brinkman Instruments, Westbury, N.Y.
Amicon cell and filters	Amicon, Lexington, Mass.
DEAE-cellulose	Sigma Chemical Co., St. Louis, Mo.
CM-cellulose	Sigma Chemical Co., St. Louis, Mo.
concanavalin A-Sepharose 4B	Pharmacia, Dorval, P.Q.
Sephadex G-200	Pharmacia, Dorval, P.Q.
Sephadex G-50	Pharmacia, Dorval, P.Q.
Sephadex G-75	Pharmacia, Dorval, P.Q.
Dextran T-500	Pharmacia, Dorval, P.Q.
Dextran Blue	Pharmacia, Dorval, P.Q.
polyethylene glycol	J.T. Baker Chemical Co., Phillipsburg, N.J.

D-[6-^{3}H] glucosamine (20 Ci/mmole)	Amersham, Oakville, Ont.
Aqueous Counting Scintillant (ACS)	Amersham, Oakville, Ont.
pronase	Calbiochem, San Diego, Calif.
α-methylmannoside	Sigma Chemical Co., St. Louis, Mo.
3,3-dimethylglutaric acid	Sigma Chemical Co., St. Louis, Mo.

Cells and culture conditions

Three Chinese hamster ovary cell lines were used in these experiments: a wild-type cell line (W.T. CO_2), a concanavalin A-resistant variant cell line (C^R-7), and a concanavalin A-sensitive revertant cell line (RC^R-7). These cell lines were kindly provided by Dr. J.A. Wright. The cells were grown in alpha-minimal essential medium (92) containing 10% (v/v) fetal calf serum. Penicillin G (100 units/ml) and streptomycin sulfate (100 μg/ml) were also added to the medium. All the cell lines were grown at 34°C in a 5% CO_2 atmosphere. The cells were grown either in 16 oz. Brockway bottles, tissue culture dishes (100 mm X 15 mm), or in suspension. Cell density was measured by diluting the cells in isotonic saline (0.18 M NaCl) and counting in a particle counter (Coulter Electronics Ltd.).

Cells were grown in suspension by adding 1-2 X 10^7 cells to 200 ml of growth medium in 500 ml medium bottles. The cells were kept in suspension by continuous stirring with a Teflon coated magnetic stirring bar. The cultures were incubated at 34°C in a water bath. Cell density was maintained at 2-5 X 10^5 cells/ml by dilution with fresh medium.

Preparation of Triton X-100 cell extracts

The glycosidase activities were assayed in crude Triton X-100 extracts of cells grown either in tissue culture dishes or in suspension. The following procedure was used for cells grown in dishes. The medium in which the cells were growing was decanted and the cells were washed with phosphate buffered saline (0.2 gm KCl, 0.2 gm KH_2PO_4, 8.0 gm NaCl, 1.15 gm Na_2HPO_4 in 1 litre of water). The cells were removed from the dishes by trypsinization; 0.05% (w/v) trypsin in phosphate buffered saline was added to the dishes followed by incubation for 5 minutes at room temperature. An aliquot (0.2 ml) of the cell suspension was counted (as described previously) while the remaining cell suspension was centrifuged for 1 minute using an International Clinical centrifuge (Model CL) set at speed 7. The supernatant was decanted and the cell pellet was solubilized with 2.5% (v/v) Triton X-100 dissolved in phosphate buffered saline. The cell extract was cooled on ice for 5 minutes. Undisrupted whole cells and nuclei were then removed by centrifugation for 1 minute in an Eppendorf centrifuge (Model 3200). The supernatant was taken and assayed.

The procedure used for cells grown in suspension is given below. An aliquot (2 ml) of the cell suspension was taken to determine the cell density. The cells were then harvested by centrifugation at 6,000 rpm (6,370 g) for 10 minutes in the JA-10 head of a Beckman Model J-21 centrifuge. The supernatant was decanted and the cell pellet resuspended in phosphate buffered saline. This cell suspension was centrifuged for

1 minute in the International Clinical centrifuge (Model CL) at speed 7. The cell pellet obtained was extracted with Triton X-100 as described above.

Assay for glycosidase activities in Triton X-100 extracts

The assay mixtures for glycosidase activities contained: 5-20 µl cell extract, 0.8 mM substrate (either p-nitrophenyl-N-acetyl-β-D-glucosaminide, p-nitrophenyl-α-L-fucoside, or p-nitrophenyl-α-D-mannoside), and 0.1 M citric acid - 0.2 M Na_2HPO_4 buffer (mixed to give the required pH) in a final volume of 500 µl. The hexosaminidase activity was normally assayed at pH 4.2 and pH 6.5. The fucosidase and mannosidase activities were normally assayed at pH 5.6 and pH 6.3, respectively. The incubations were performed at 37°C for 15-60 minutes. The reactions were stopped by the addition of 1.5 ml 0.2 N NaOH-glycine buffer, pH 9.5, centrifuged for 2 minutes in an Eppendorf centrifuge (Model 3200), and the absorbance of each supernatant determined at 400 nm using a Gilford spectrophotometer (Model 2400-2). The molar extinction coefficient of p-nitrophenyl at 400 nm was 1.77×10^4 M^{-1} cm^{-1}. The units of enzyme activity were defined as the number of µmoles substrate hydrolysed per minute. Protein was assayed using the Bio-Rad protein determination kit (93).

Intracellular hexosaminidase forms

Ion-exchange chromatography was used to resolve the hexosaminidase

(N-acetyl-β-D-glucosaminidase) forms present in the W.T. CO_2 and C^R-7 cells. The procedure involved disrupting the cells by sonication instead of treatment with Triton X-100. Ten Brockway bottles of W.T. CO_2 or C^R-7 cells were harvested by trypsinization. An aliquot of the cells was counted to determine the cell density and the remaining cells were washed and collected as described previously. Ten cell pellets were obtained, one from each Brockway bottle of cells. Each of the ten pellets was suspended in 1 ml of cold 20 mM ammonium acetate, pH 6.0, and transferred to a 1.5 ml Eppendorf centrifuge tube. These cell suspensions were chilled on ice for 10 minutes. Each tube of cells was sonicated (Artek Sonic Dismembranator set at 300 watts and fitted with a P-300-1 probe) for 15 seconds, chilled on ice for 5 minutes, then sonicated again for 15 seconds. The disrupted cell suspensions were then centrifuged (Eppendorf centrifuge Model 3200) for 2 minutes. The resulting supernatants were pooled and the pH adjusted to 6.0 with 1% (v/v) acetic acid. The pooled supernatants were centrifuged (Sorvall Superspeed RC2-B fitted with a SS-34 head) for 15 minutes at 20,000 rpm (65,274 g). The supernatant obtained from this centrifugation was used for the DEAE-cellulose chromatography (see below).

Cells grown in suspension were also analyzed for hexosaminidase forms. The volume of suspended cells (either W.T. CO_2 or C^R-7) used was 1200 ml. The cells were counted and harvested as described previously. These pellets were sonicated and centrifuged in the manner described above. The resulting supernatants were pooled and diluted to 30 ml with cold 20 mM ammonium acetate, pH 6.0. The pooled, diluted

supernatants were then treated as described above.

Ion-exchange chromatography of intracellular hexosaminidase forms

The extracts obtained from the sonicated W.T. CO_2 and C^R-7 cells were chromatographed on DEAE-cellulose columns. The extract obtained from either the W.T. CO_2 or C^R-7 cells grown in Brockway bottles was applied to a 1.7 cm X 15 cm column of DEAE-cellulose equilibrated with 20 mM ammonium acetate adjusted to pH 6.0 with acetic acid. The column was washed successively with 3 solutions of 20 mM ammonium acetate, pH 6.0 containing 30 mM, 150 mM, and 300 mM NaCl, respectively. Fractions of 2.5 ml were collected and the absorbance at 280 nm was determined using a Coleman spectrophotometer (Model 101). Appropriate volumes from each fraction were assayed for hexosaminidase activity as previously described. The extracts obtained from W.T. CO_2 and C^R-7 cells grown in suspension were treated in the manner described above, with the exception that the column dimensions were 1.7 cm x 20 cm. The fractions containing hexosaminidase activity were pooled and used for kinetic studies. The hexosaminidase form eluted with 30 mM NaCl was designated Hex II, while the hexosaminidase forms eluted with 150 mM and 300 mM NaCl were designated Hex I and Hex III, respectively.

Extracellular hexosaminidase forms

The secretion of hexosaminidase forms by W.T. CO_2 and C^R-7 cells grown either in Brockway bottles or in suspension was examined. Since fetal calf serum contains low levels of hexosaminidase activity, the serum was heat inactivated at 62^oC for 2 hours prior to use in this

study (94, 95). Either W.T. CO_2 or C^R-7 cells were added to 4 Brockway bottles and the cells were cultured for 48 hours. After this time period, the medium was decanted, and 25 ml of fresh medium containing 10% (v/v) heat inactivated fetal calf serum was added to each of the 4 Brockway bottles. The cultures were then incubated for an additional 24 hours. Following this incubation, the medium was decanted from each Brockway bottle and pooled. The pooled medium was immediately centrifuged for 10 minutes at 6,000 rpm (6,370 g) in a Beckman centrifuge (Model J-21) fitted with a JA-10 head to remove any suspended cells. The cells attached to the Brockway bottles were removed by trypsinization and counted to determine the cell density (as described previously). After centrifugation of the medium, the supernatant (100 ml.) was taken and dialyzed against 4 litres of 20 mM ammonium acetate, pH 6.0 (dialyzing buffer) for 48 hours at 4^oC. During this period, the dialyzing buffer was changed every 12 hours. The dialyzed medium was collected and concentrated to 30 ml using an Amicon cell fitted with an XM-100 A filter. A 2.7 cm X 30 cm column of DEAE-cellulose equilibrated with 20 mM ammonium acetate, pH 6.0 was loaded with 25 ml of the concentrated medium. The column was eluted as described above. Fractions of 5 ml were collected and analyzed for hexosaminidase activity and protein as described previously.

The following procedure was used to examine the hexosaminidase forms secreted by W.T. CO_2 and C^R-7 cells grown in suspension. Both the W.T. CO_2 and C^R-7 cells were grown in suspension 48 hours prior to use

in the experiments. After the cells were conditioned for growth in suspension, they were harvested by centrifugation for 1 minute in an International Clinical centrifuge (Model CL) set at speed 7. The cell pellet obtained was resuspended in 100 ml medium containing 10% (v/v) heat inactivated fetal calf serum. The cells were then cultured in suspension for 24 hours. After this cultivation period, the medium was collected and treated in the same manner as described above. The DEAE-cellulose fractions containing hexosaminidase activity were pooled and used for kinetic studies.

Attempted purification of W.T. CO_2 hexosaminidase forms

The W.T. CO_2 cells were grown in suspension to a density of 5 X 10^5 cells/ml. The volume of cell suspension used was 6.4 litres. The cells were harvested and washed as described previously. The resulting cell pellets were resuspended in water (2.5 ml water per cell pellet). The cell suspensions were pooled and the cells allowed to swell for 30 minutes at room temperature. The pooled cell suspension was then chilled on ice for 30 minutes and the cells were disrupted by homogenization using 200 strokes of a Potter-Elvehjem homogenizer rotating at 1000 rev/minutes. The homogenate was centrifuged at 10,000 rpm (16,319 g) for 30 minutes using a Sorvall Superspeed RC2-B centrifuge fitted with a SS-34 head. The supernatant was kept and adjusted to pH 6.0 with 1% acetic acid. This solution was centrifuged at 20,000 rpm (65,274 g) for 30 minutes and the resulting supernatant was applied

to a 1.7 cm X 24 cm column of DEAE-cellulose and eluted as before. Fractions of 2.5 ml were collected and the hexosaminidase activity assayed as described previously. The fractions collected during elution with 30 mM NaCl were pooled and the pH adjusted to 5.0 with 10% acetic acid. This solution was loaded on a 1.7 cm X 15 cm CM-cellulose column equilibrated with 20 mM ammonium acetate adjusted to pH 5.0 with acetic acid. The column was eluted with 120 ml of a linear NaCl gradient (0.0 to 0.9 M NaCl). Fractions of 2.5 ml were collected and assayed for hexosaminidase activity as described previously. The hexosaminidase form eluted was designated Hex II. The fractions containing Hex II activity were pooled and run on a 1.5 cm X 7.5 cm column of concanavalin A-Sepharose 4B equilibrated with 0.1 M sodium acetate, pH 6.0 containing 3 mM $MnCl_2$ and 3 mM $MgCl_2$ (equilibrating buffer). The column was washed with equilibrating buffer containing 1 M NaCl (washing buffer) until no more 280 nm absorbing material was eluted and then the Hex II activity was eluted with washing buffer containing 0.1 M α-methylmannoside. Fractions of 2.5 ml were collected and assayed for hexosaminidase activity as before. The fractions containing Hex II activity were pooled. The hexosaminidase form eluted from the DEAE-cellulose column with 150 mM NaCl was designated Hex I. The fractions containing Hex I activity were pooled and purified using the same concanavalin A-Sepharose 4B chromatographic method described above. The hexosaminidase form eluted from the DEAE-cellulose column with 300 mM NaCl was designated Hex III. The fractions containing Hex III

activity were pooled and concentrated to 10 ml using an Amicon cell fitted with a UM-20 membrane. This concentrate was applied to a 2.9 cm X 33 cm column of Sephadex G-200 equilibrated with 20 mM ammonium acetate, pH 6.0. Fractions of 5 ml were collected and assayed for hexosaminidase activity as before. Several of the fractions collected formed a precipitate. This precipitate was removed by centrifugation at 20,000 rpm (65,274 g) on a Sorvall Superspeed RC2-B fitted with a SS-34 head for 15 minutes. There was no hexosamindase activity in the precipitate. The fractions containing Hex III activity were pooled and chromatographed on a 1.7 cm X 14 cm column of DEAE-cellulose equilibrated with 20 mM ammonium acetate, pH 6.0. The column was washed with 50 ml of 20 mM ammonium acetate, pH 6.0 containing 150 mM NaCl. The column was then washed with 120 ml of a linear NaCl gradient (150 - 300 mM NaCl). Fractions of 2.5 ml were collected and assayed for hexosaminidase activity as described previously. The fractions containing Hex III activity were pooled.

Preparation and analysis of W.T. CO_2 and C^R-7 membrane glycopeptides

Six dishes of either W.T. CO_2 or C^R-7 cells were grown to confluence. The cells were labelled with ^{3}H-glucosamine (10 μCi/dish) for 18 hours before harvesting. The media was decanted and the cells were washed with phosphate buffered saline. The cells were then covered with fresh phosphate buffered saline and scraped off the dishes into the saline. The cell suspensions obtained were pooled and centrifuged for 1 minute

using an International Clinical centrifuge (Model CL) set at speed 7. The supernatant was decanted and the membranes were isolated from the cell pellet by the method of Brunette and Till (96). The cell pellet was suspended in 10^{-3}M $ZnCl_2$ and the suspension was allowed to stand at room temperature for 15 minutes. The suspension was then chilled on ice for 5 minutes. After cooling, the cells were homogenized using 200-300 strokes of a Potter-Elvehjem homogenizer rotating at 1000 rev/minute. The homogenate was then centrifuged at 1,400 rpm (367 g) for 15 minutes in the SS-34 head of a Sorvall Superspeed RC2-B centrifuge. The supernatant was decanted and the pellet was suspended in 40 ml of a polyethylene glycol-dextran T500 emulsion (96). This suspension was centrifuged at 8,500 rpm (11,790 g) for 10 minutes in the SS-34 head of a Sorvall Superspeed RC2-B centrifuge. The cell membranes were removed from the interface that formed between the dextran T500 and polyethylene glycol. The membranes were suspended in 0.01 M Tris·HCl, pH 7.5 containing 0.1 M NaCl and centrifuged for 1 minute in an International Clinical centrifuge (Model CL) set at speed 7. The supernatant was decanted and the membranes suspended in 0.5 ml of 1 M Tris·HCl, pH 8.4 containing 0.1 M $CaCl_2$ and 3 mg pronase (97). This suspension was incubated for 24 hours at 37°C, then another 3 mg pronase was added and the reaction was allowed to continue an additional 24 hours. The reaction was stopped by immersing the mixture in a boiling water bath for 1 minute. The mixture was centrifuged for 2 minutes in an Eppendorf centrifuge (Model 3200). The supernatant was collected, diluted to 1 ml

with 0.1 M sodium acetate, pH 6.0 containing 3 mM $MgCl_2$ and 3 mM $MnCl_2$ (equilibrating buffer) and applied to a 0.5 cm X 50 cm column of concanavalin A-Sepharose 4B equilibrated with the same buffer. The glycopeptides that did not bind to the affinity column were removed by washing the column with equilibrating buffer containing 1 M NaCl. The glycopeptides that were retained by the affinity column were eluted with equilibrating buffer containing 0.1 M α-methylmannoside. Fractions of 2.5 ml were collected. Aliquots were withdrawn from each fraction and added to scintillation vials containing 10 ml ACS. The radioactivity was determined with a Packard Model 3003 Tri-Carb liquid scintillation spectrometer at a gain setting of 72% and a discriminator setting of 10-1000 units. Blanks and standards were counted routinely. The glycopeptides that were not retained by the affinity column were pooled and freeze-dried. The residue was dissolved in 1 ml of 0.05 M ammonium acetate, pH 6.0 and applied to either a 0.9 cm X 90 cm column of Sephadex G-50 or a 0.9 cm X 110 cm column of Sephadex G-75 equilibrated with the same buffer. Fractions of 1.5 ml and 2.0 ml were collected from the Sephadex G-50 and Sephadex G-75 column, respectively. The radioactivity of each fraction was determined as described previously. The glycopeptides that were retained by the affinity column were treated in the same manner as described above.

RESULTS

RESULTS

Glycosidase activities in Triton X-100 extracts of W.T. CO_2, C^R-7 and RC^R-7 cells

Each of the three cell lines (W.T. CO_2, C^R-7, and RC^R-7) was analyzed for hexosaminidase (N-acetyl-β-D-glucosaminidase) (EC 3.2.1.30), α-L-fucosidase (EC 3.2.1.51), and α-D-mannosidase (EC 3.2.1.24) activity. The pH optima, K_m, and V_{max} values of the glycosidases studied are given in Table II. The pH optima and K_m values of each glycosidase remained relatively constant in all three cell lines (Figures 12-19 and Table II). Maximum hexosaminidase activity occurred at pH 4.2 and pH 6.3 (Figures 12 and 13). The hexosaminidase activity was subsequently found to be due to three hexosaminidase forms (see below). At pH 4.2, two hexosaminidase forms, designated Hex I and Hex II, were shown to be active. A third hexosaminidase form, Hex III, was demonstrated to be active at pH 6.3. In all of the enzyme assays, linear relationships were established between the enzyme activities and increasing protein concentration (Figures 24-27), as well as the time of incubation (Figures 20-23).

The specific activities of the glycosidases in extracts of W.T. CO_2, C^R-7 and RC^R-7 cells grown either in dishes or in suspension were determined. The results obtained are shown in Table III. Specific activities of Hex III were several-fold (2 - 13 times) higher in all of the cell lines when the cells were grown in suspension as compared to in

TABLE II

Values of K_m and V_{max} for glycosidase activities in Triton X-100 extracts of W.T. CO_2, C^R-7, and RC^R-7 cells.

	pH Optimum	W.T. CO_2		C^R-7		RC^R-7	
		K_m [1]	V_{max} [2]	K_m	V_{max}	K_m	V_{max}
Hexosaminidases I and II	4.2	0.75	9.4	0.85	4.8	0.99	8.9
Hexosaminidase III	6.3	0.56	9.6	0.58	22.6	0.34	5.2
α-L-Fucosidase	5.6	0.16	3.3	0.15	0.87	0.18	2.4
α-D-Mannosidase	6.3	0.19	2.0	0.25	2.2	0.25	1.8

[1] mM

[2] units per mg protein

Figure 12. The pH-activity profiles of hexosaminidase I and hexosaminidase II in Triton X-100 extracts of W.T. CO_2 (⊙), C^R-7 (⊡), and RC^R-7 (△) cells.

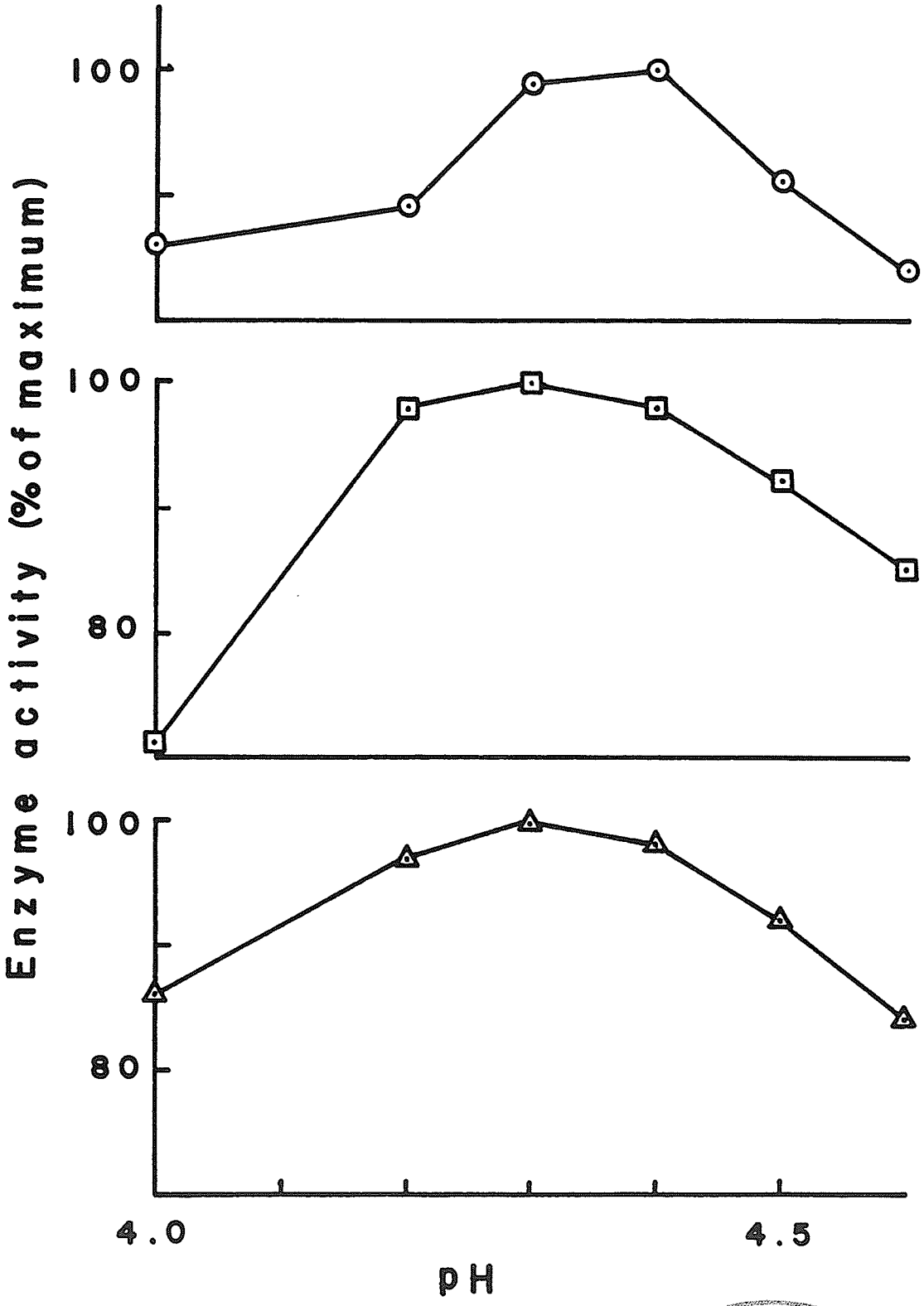


Figure 13. The pH-activity profiles of hexosaminidase III in Triton X-100 extracts of W.T. CO_2 (⊙), C^R-7 (⊡), and RC^R-7 (△) cells.

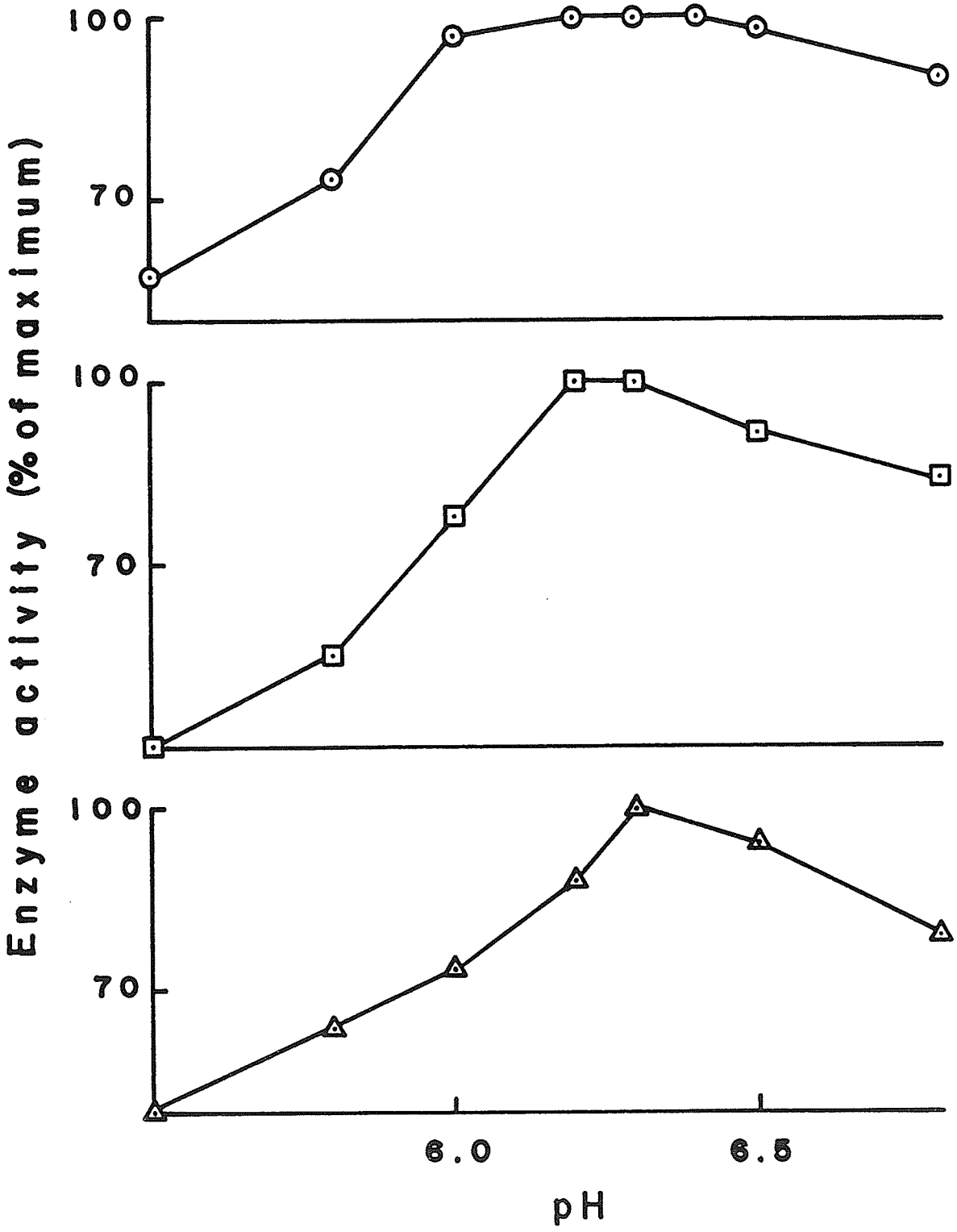

100
70
100
70
100
70
Enzyme activity (% of maximum)
6.0
6.5
pH

Figure 14. The pH-activity profiles of α-L-fucosidase in Triton X-100 extracts of W.T. CO_2 (⊙), C^R-7 (⊡), and RC^R-7 (⧋) cells.

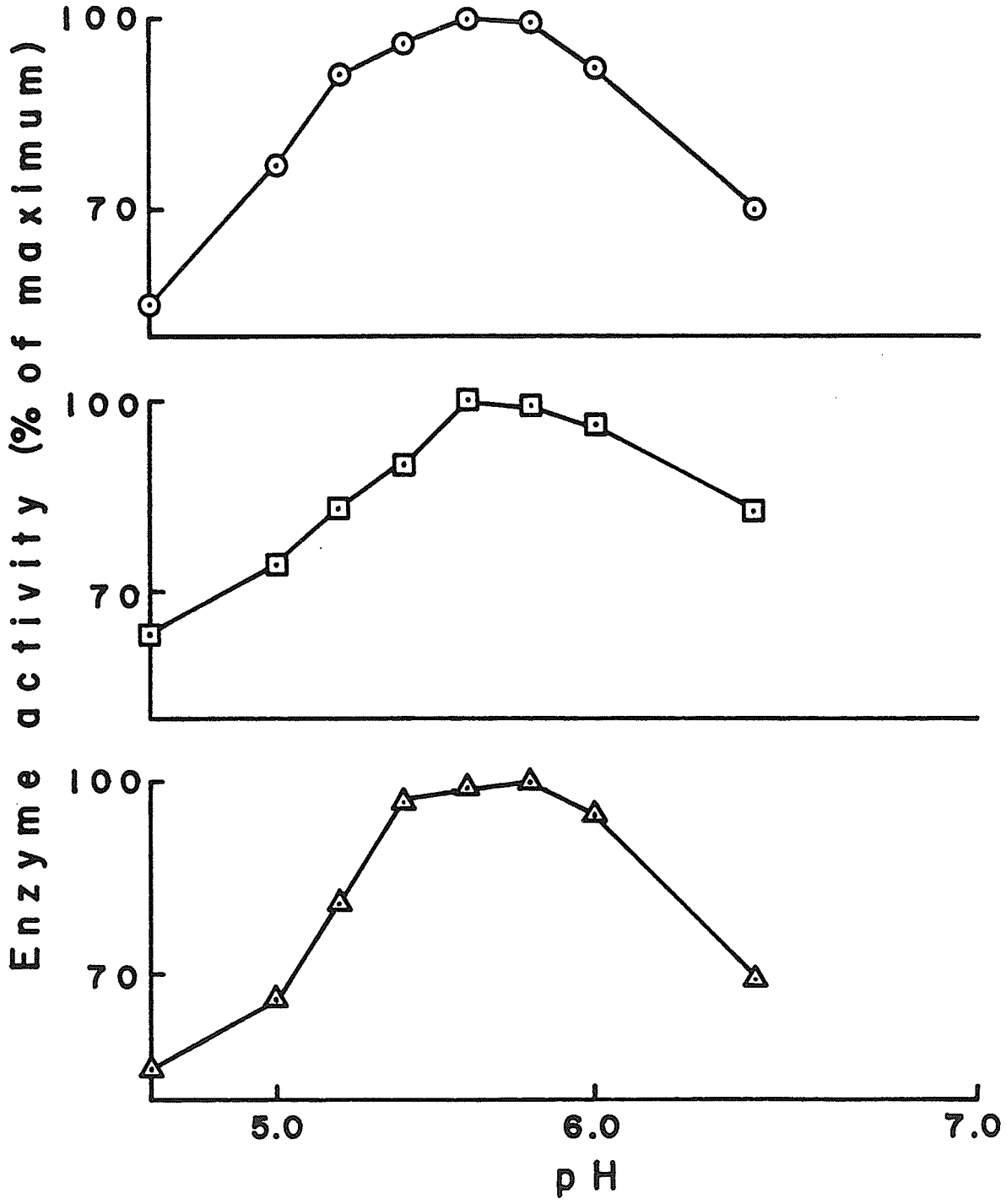

Enzyme activity (% of maximum)
100
70
100
70
100
70
5.0
6.0
7.0
pH

Figure 15. The pH-activity profiles of α-D-mannosidase in Triton X-100 extracts of W.T. CO_2 (⊙), C^R-7 (⊡), and RC^R-7 (△) cells.

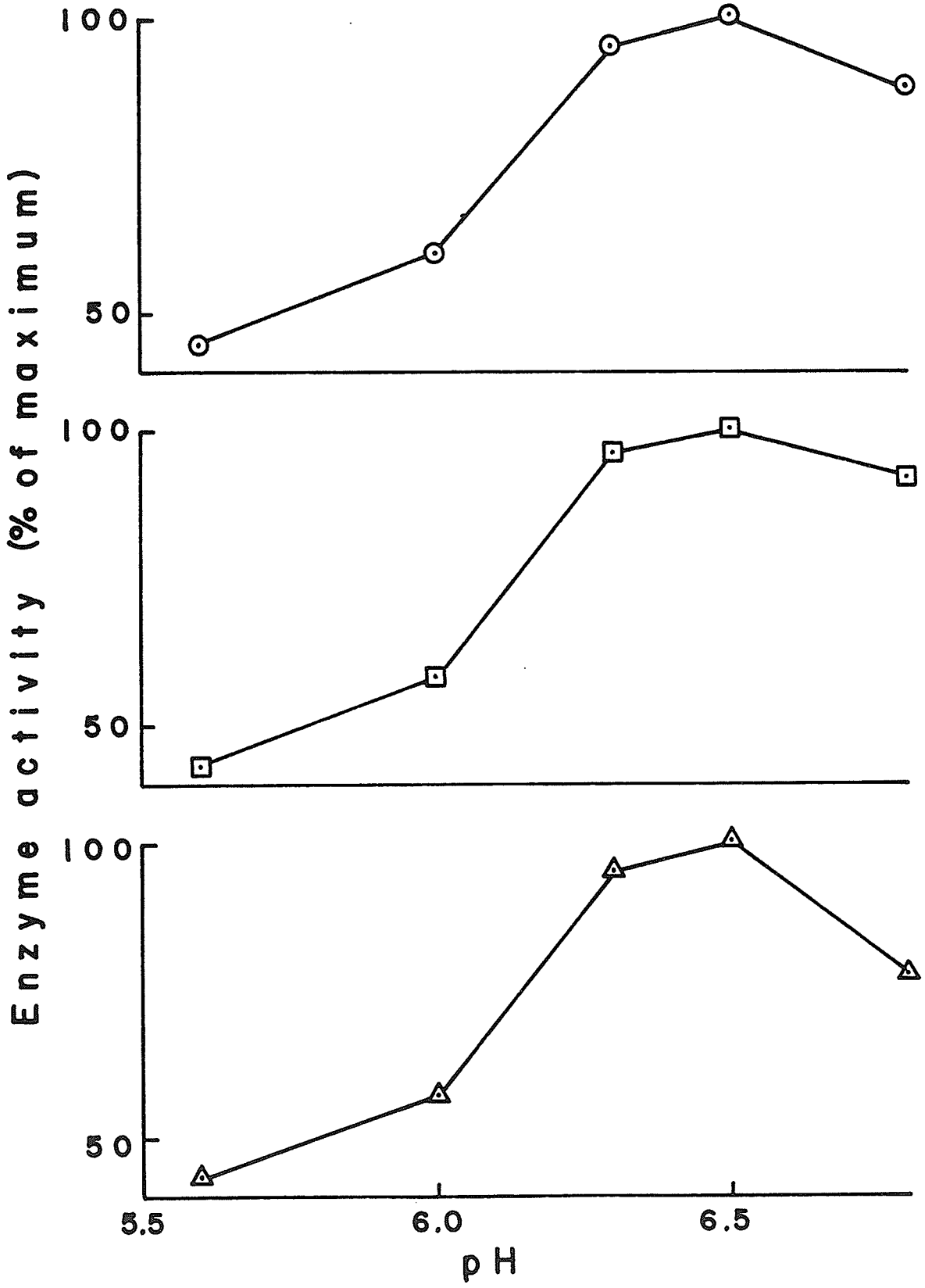

100
50
100
50
100
50
Enzyme activity (% of maximum)
5.5
6.0
6.5
pH

Figure 16. Eadie-Hofstee plots of hexosaminidase I and hexosaminidase II activities in Triton X-100 extracts of W.T. CO_2 (⊙), C^R-7 (⊡), and RC^R-7 (△) cells.

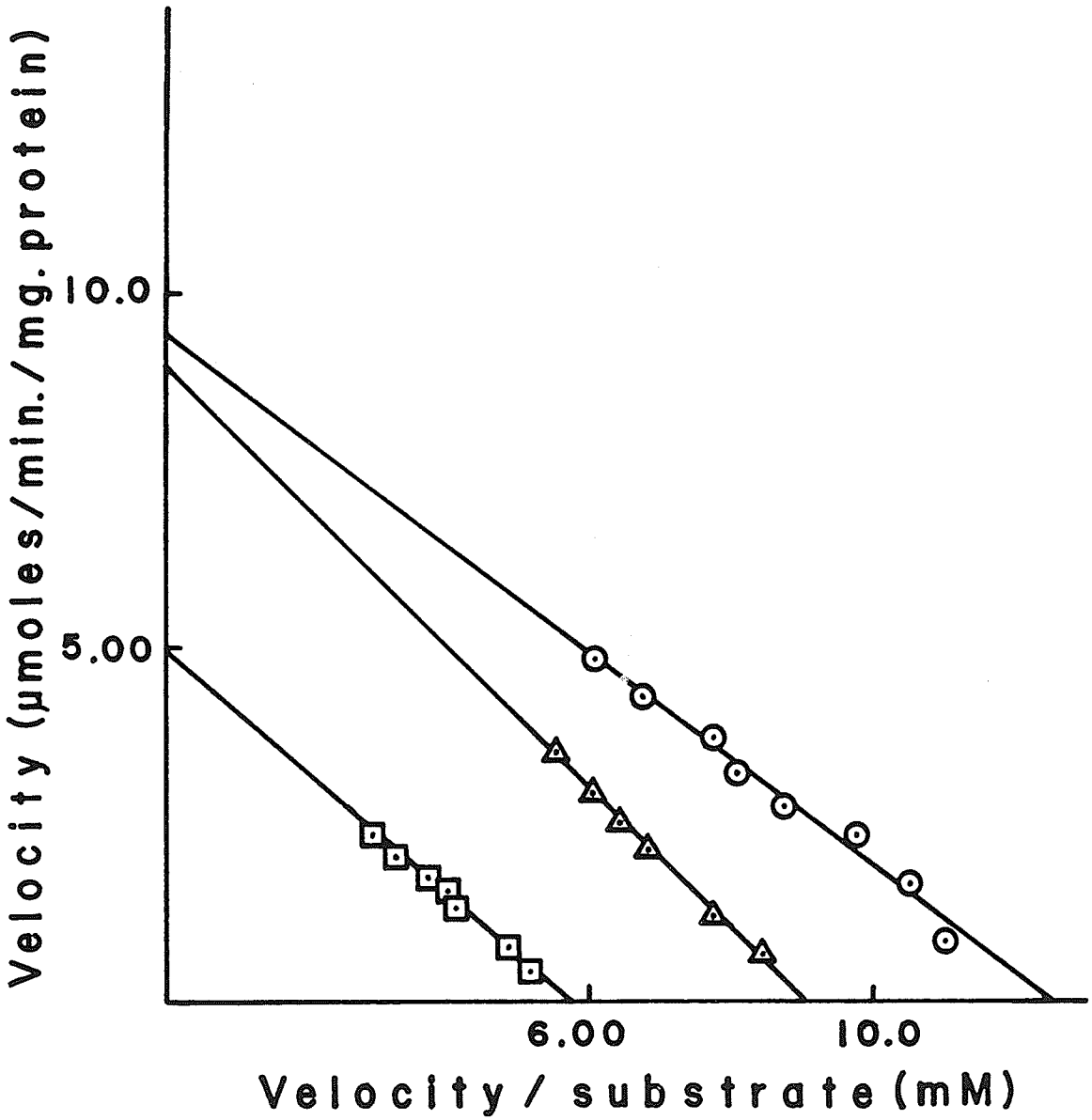

Velocity (μmoles/min./mg. protein)
10.0
5.00
6.00
10.0
Velocity / substrate (mM)

Figure 17. Eadie-Hofstee plots of hexosaminidase III activity in Triton X-100 extracts of W.T. CO_2 (⊙), C^R-7 (⊡), and RC^R-7 (△) cells.

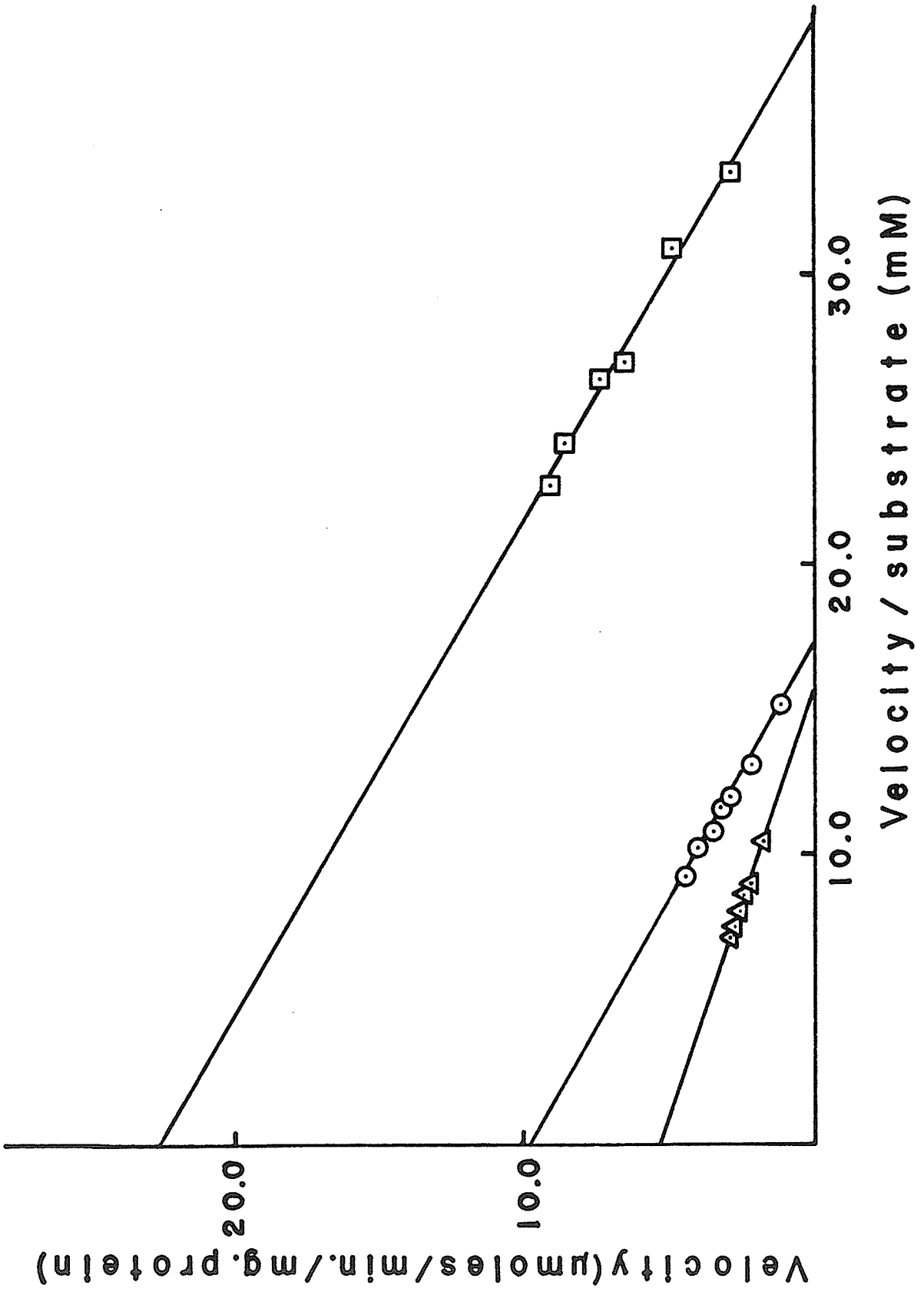

Velocity (μmoles/min./mg. protein)
20.0
10.0
10.0
20.0
30.0
Velocity / substrate (mM)

Figure 18. Eadie-Hofstee plots of α-L-fucosidase activity in Triton X-100 extracts of W.T. CO_2 (⊙), C^R-7 (⊡), and RC^R-7 (⚠) cells.

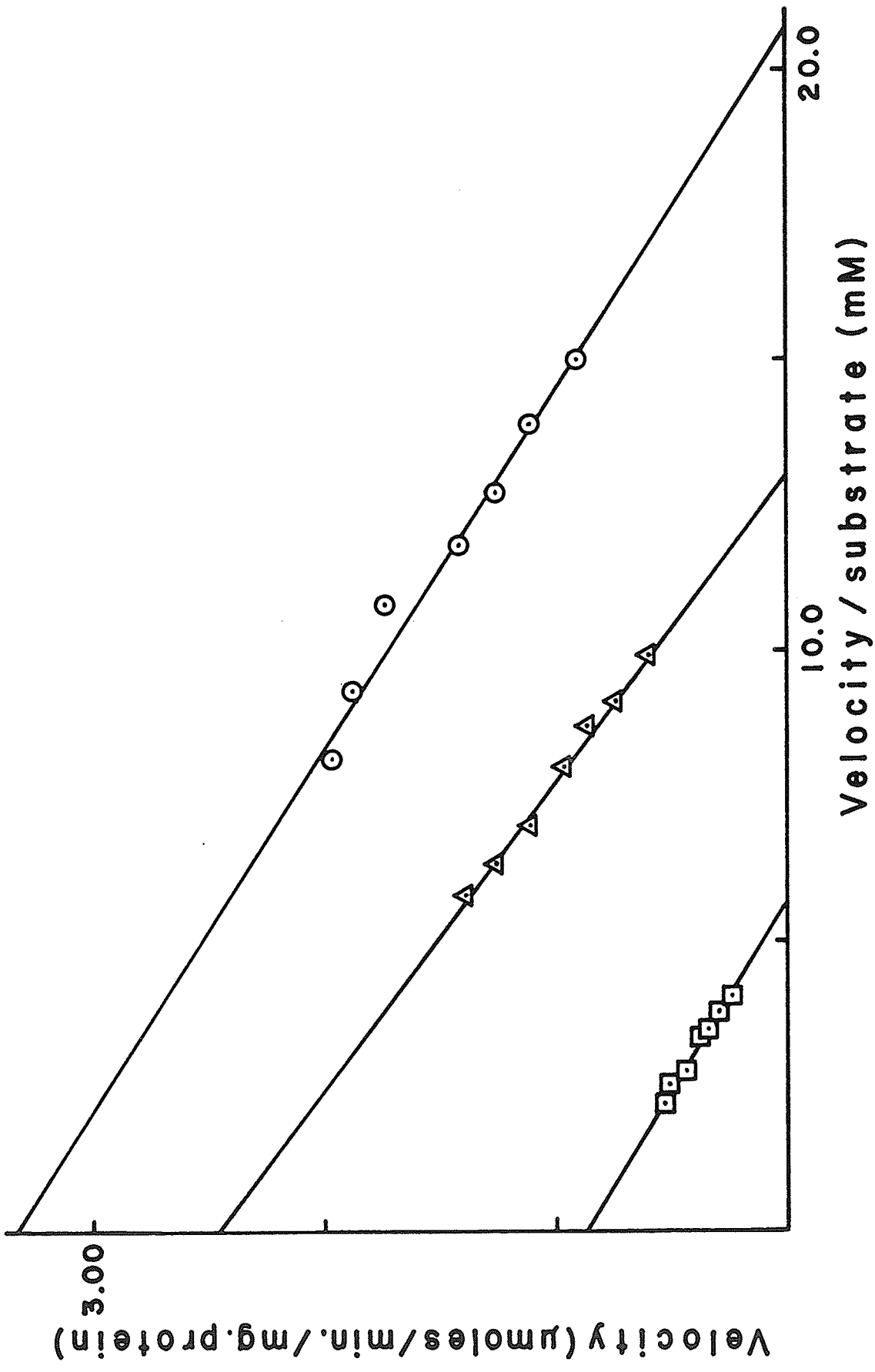

Velocity (μmoles/min./mg.protein)
3.00
10.0
20.0
Velocity / substrate (mM)

Figure 19. Eadie-Hofstee plots of α-D-mannosidase activity in Triton X-100 extracts of W.T. CO_2 (⊙), C^R-7 (⊡), and RC^R-7 (△) cells.

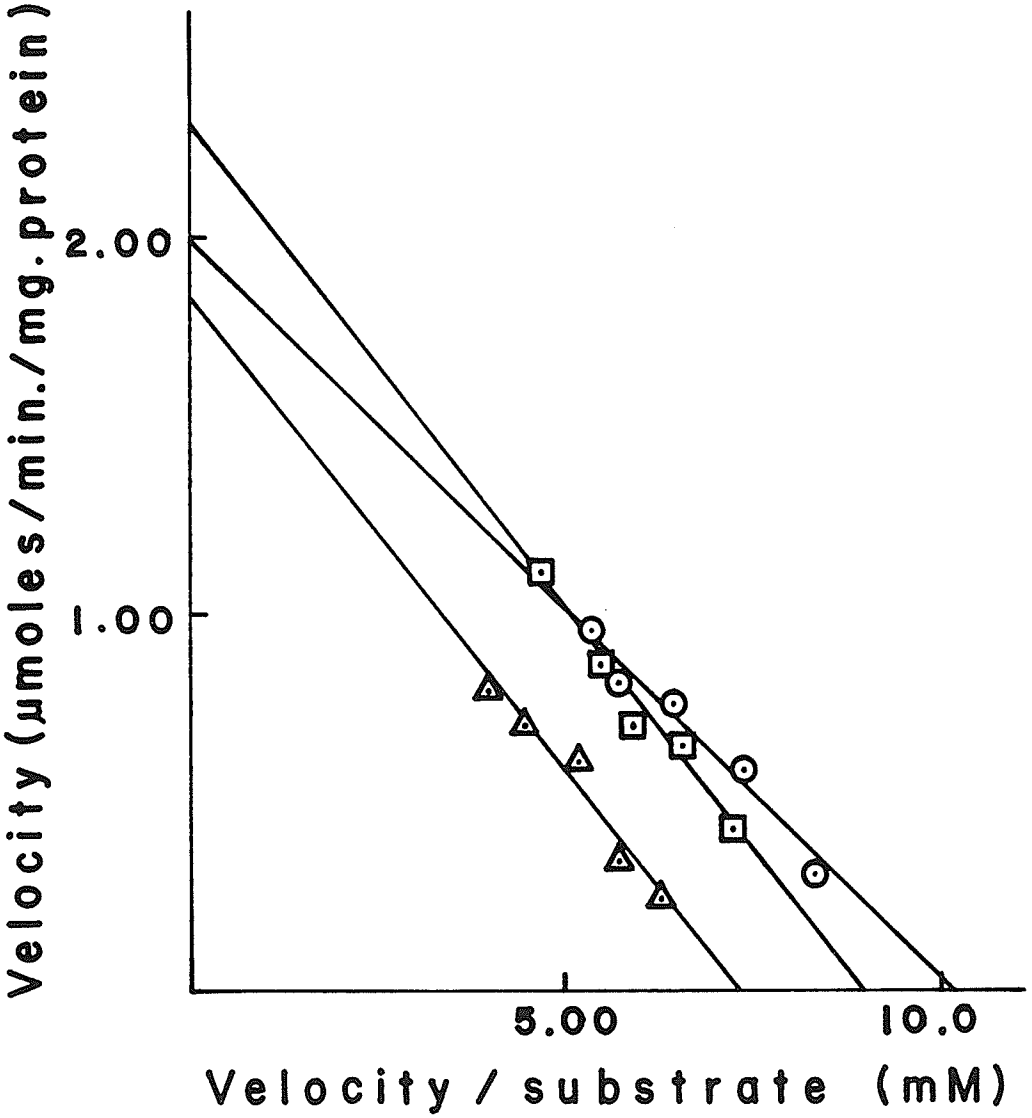

Velocity (µmoles/min./mg.protein)
2.00
1.00
5.00
10.0
Velocity / substrate (mM)

Figure 20. The effect of the time of incubation on hexosaminidase I and hexosaminidase II activities in Triton X-100 extracts of W.T. CO_2 (⊙), C^R-7 (⊡), and RC^R-7 (△) cells.

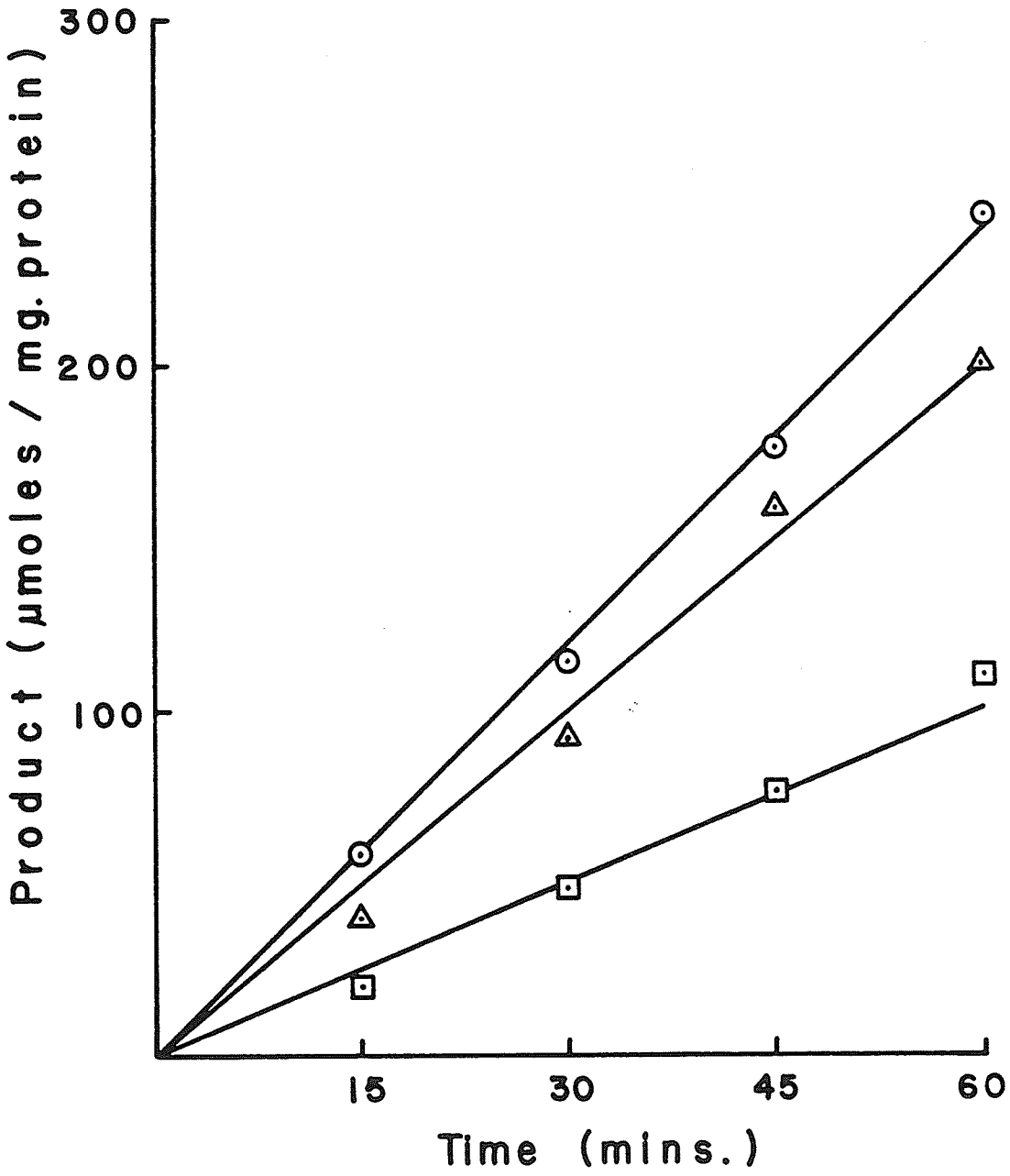

300
200
100
Product (µmoles / mg. protein)
15
30
45
60
Time (mins.)

Figure 21. The effect of the time of incubation on hexosaminidase III activity in Triton X-100 extracts of W.T. CO_2 (⊙), C^R-7 (⊡), and RC^R-7 (△) cells.

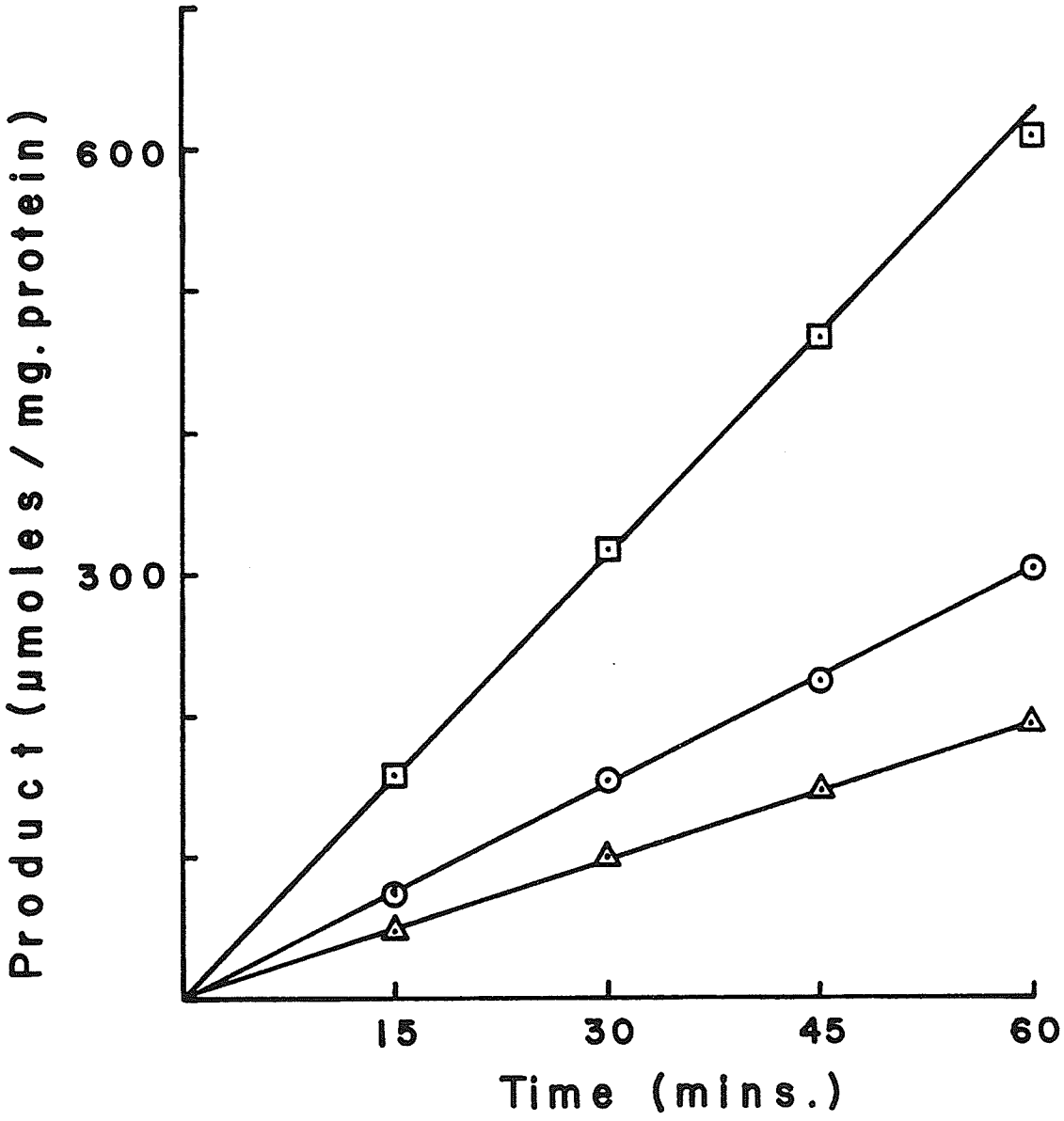

Product (µmoles / mg. protein)
600
300
15
30
45
60
Time (mins.)

Figure 22. The effect of the time of incubation on α-L-fucosidase activity in Triton X-100 extracts of W.T. CO_2 (⊙), C^R-7 (⊡), and RC^R-7 (△) cells.

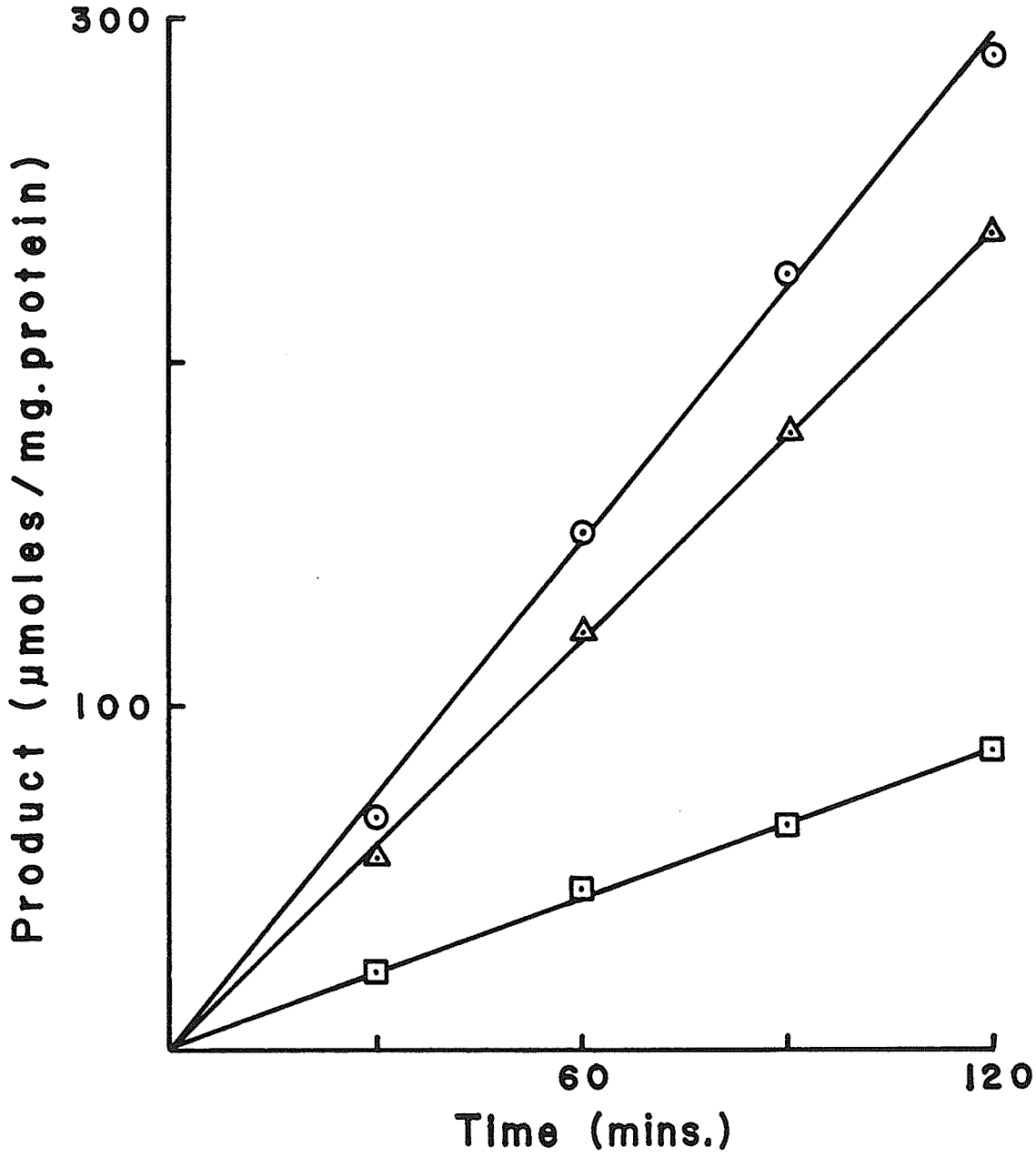

300
100
Product (µmoles / mg. protein)
60
120
Time (mins.)

Figure 23. The effect of the time of incubation on α-D-mannosidase activity in Triton X-100 extracts of W.T. CO_2 (⊙), C^R-7 (⊡), and RC^R-7 (△) cells.

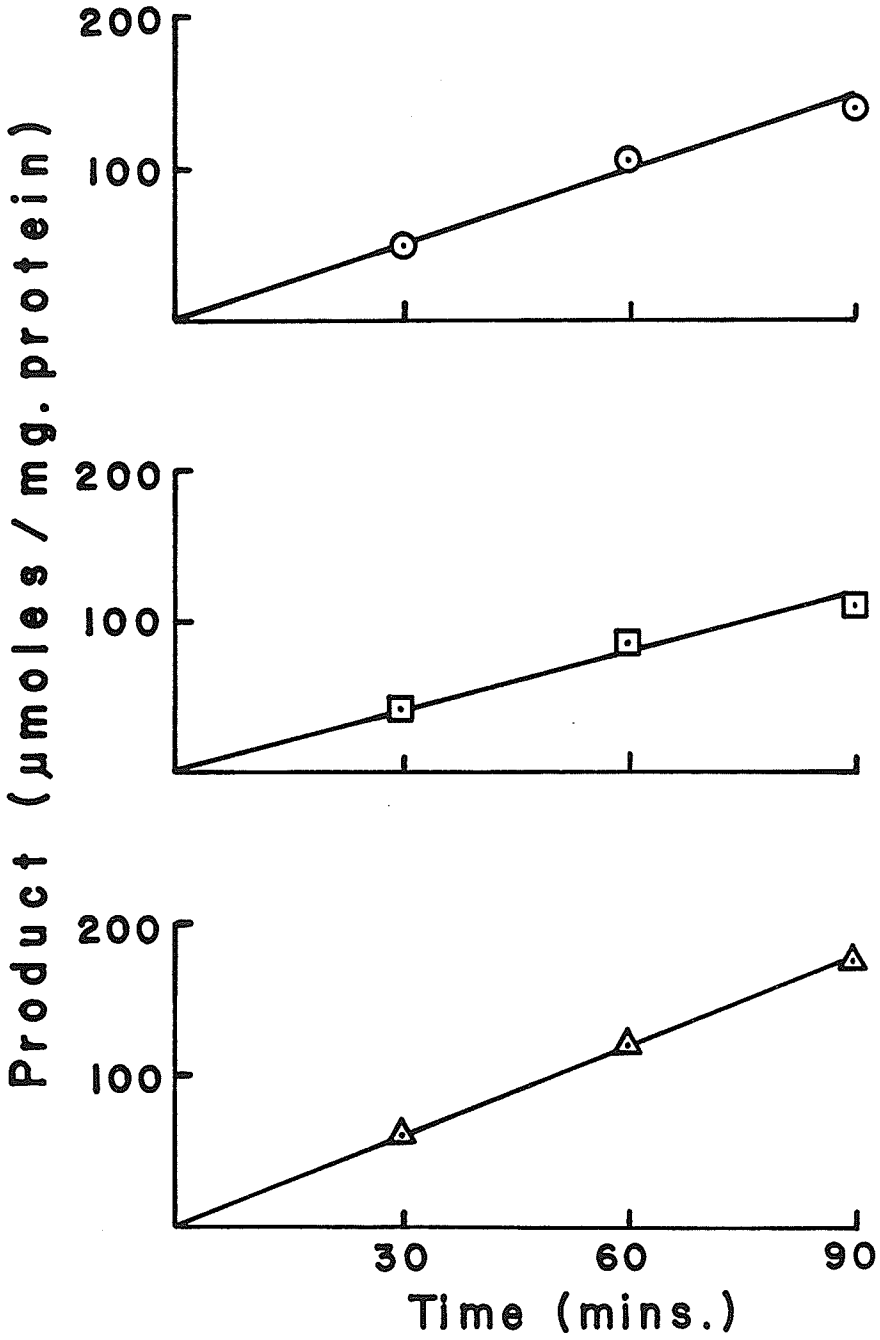

200
100
200
100
200
100
Product (μmoles / mg. protein)
30
60
90
Time (mins.)

Figure 24. The effect of increasing protein on hexosaminidase I and hexosaminidase II activities in Triton X-100 extracts of W.T. CO_2 (⊙), C^R-7 (⊡), and RC^R-7 (⧋) cells.

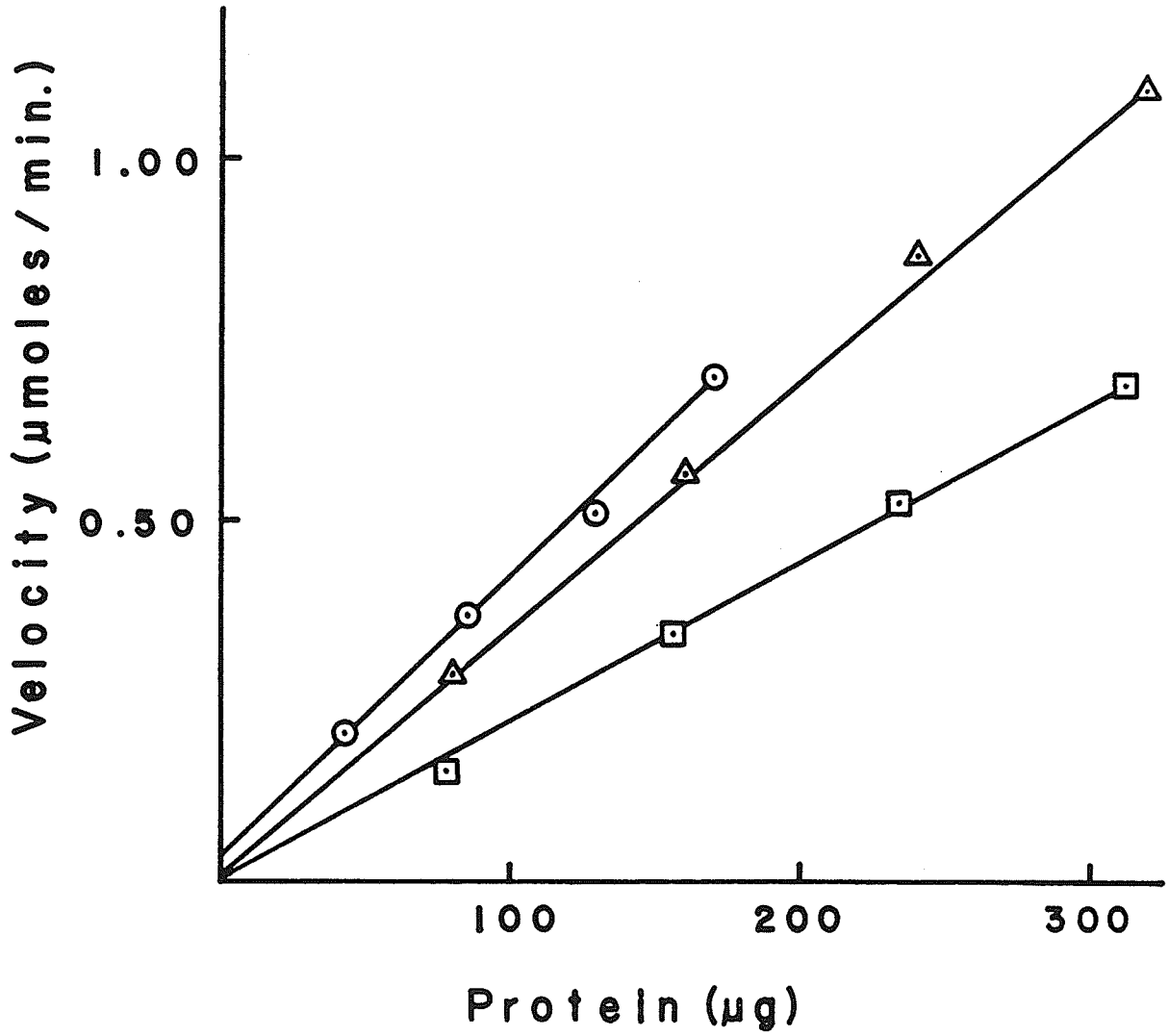

Velocity (µmoles / min.)
1.00
0.50
100
200
300
Protein (µg)

Figure 25. The effect of increasing protein on hexosaminidase III activity in Triton X-100 extracts of W.T. CO_2 (⊙), C^R-7 (⊡), and RC^R-7 (△) cells.

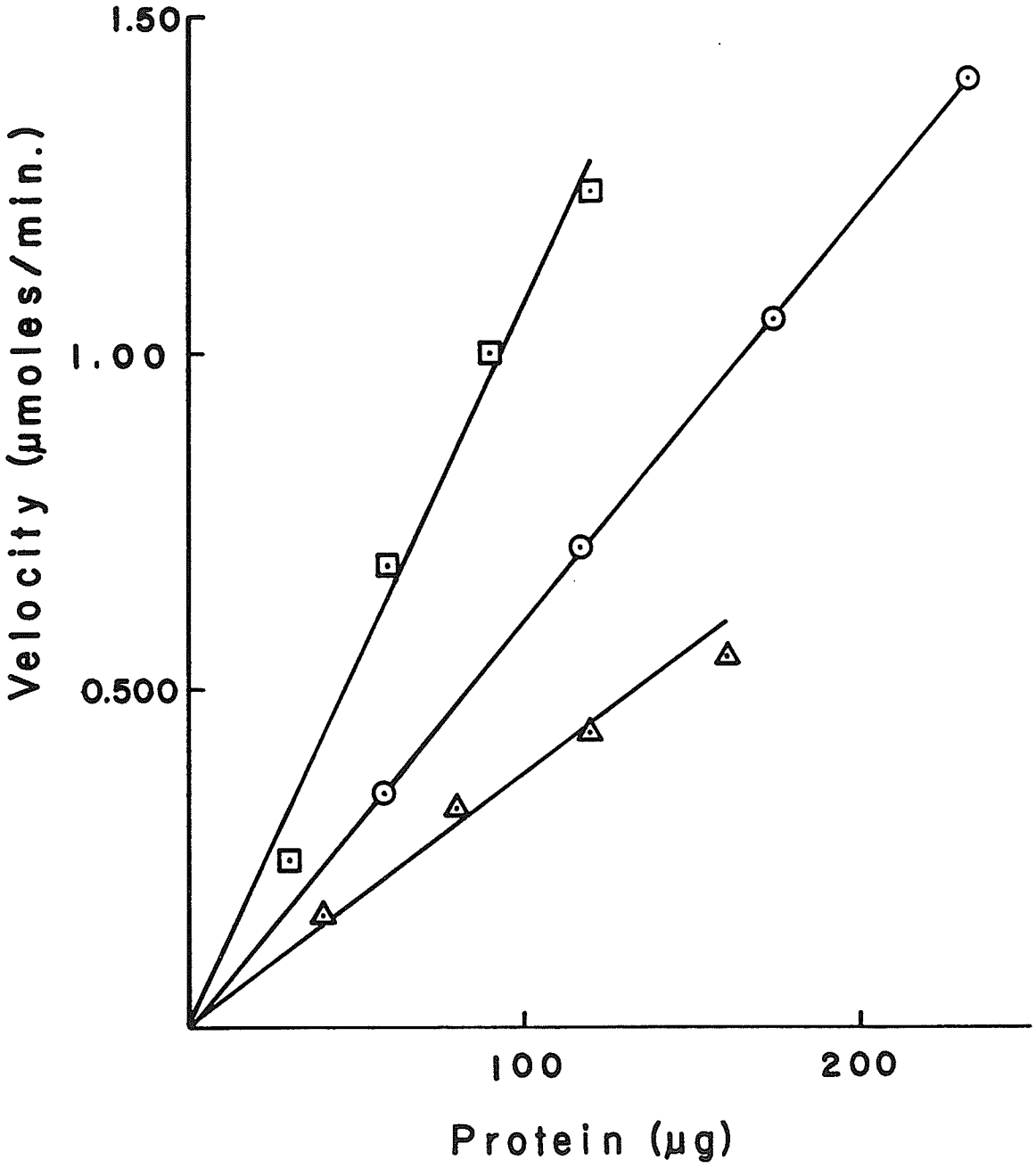

1.50
1.00
0.500
Velocity (µmoles/min.)
100
200
Protein (µg)

Figure 26. The effect of increasing protein on α-L-fucosidase activity in Triton X-100 extracts of W.T. CO_2 (⊙), C^R-7 (⊡), and RC^R-7 (△) cells.

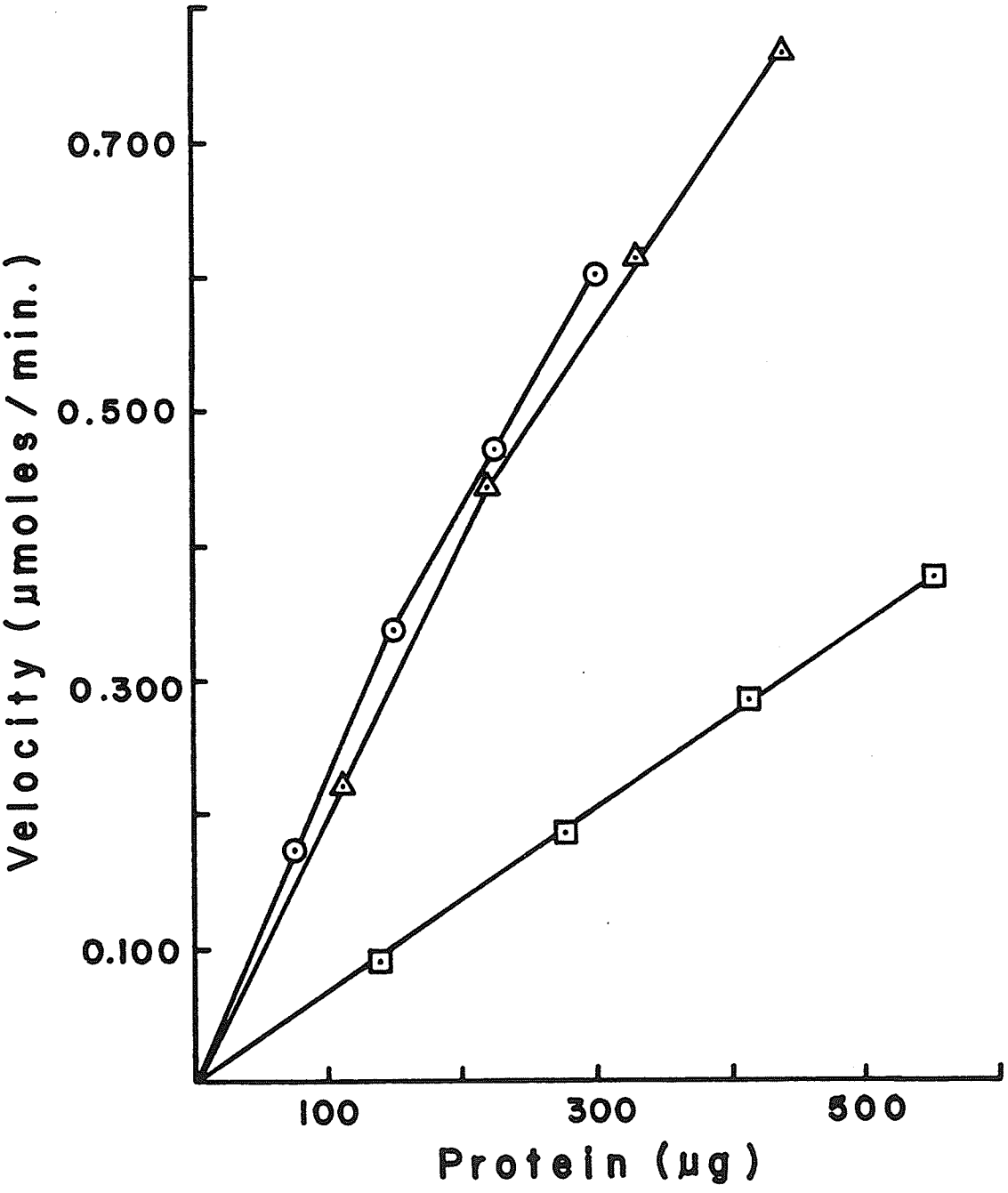

0.700
0.500
0.300
0.100
Velocity (µmoles / min.)
100
300
500
Protein (µg)

Figure 27. The effect of increasing protein on α-D-mannosidase activity in Triton X-100 extracts of W.T. CO_2 (⊙), C^R-7 (⊡), and RC^R-7 (△) cells.

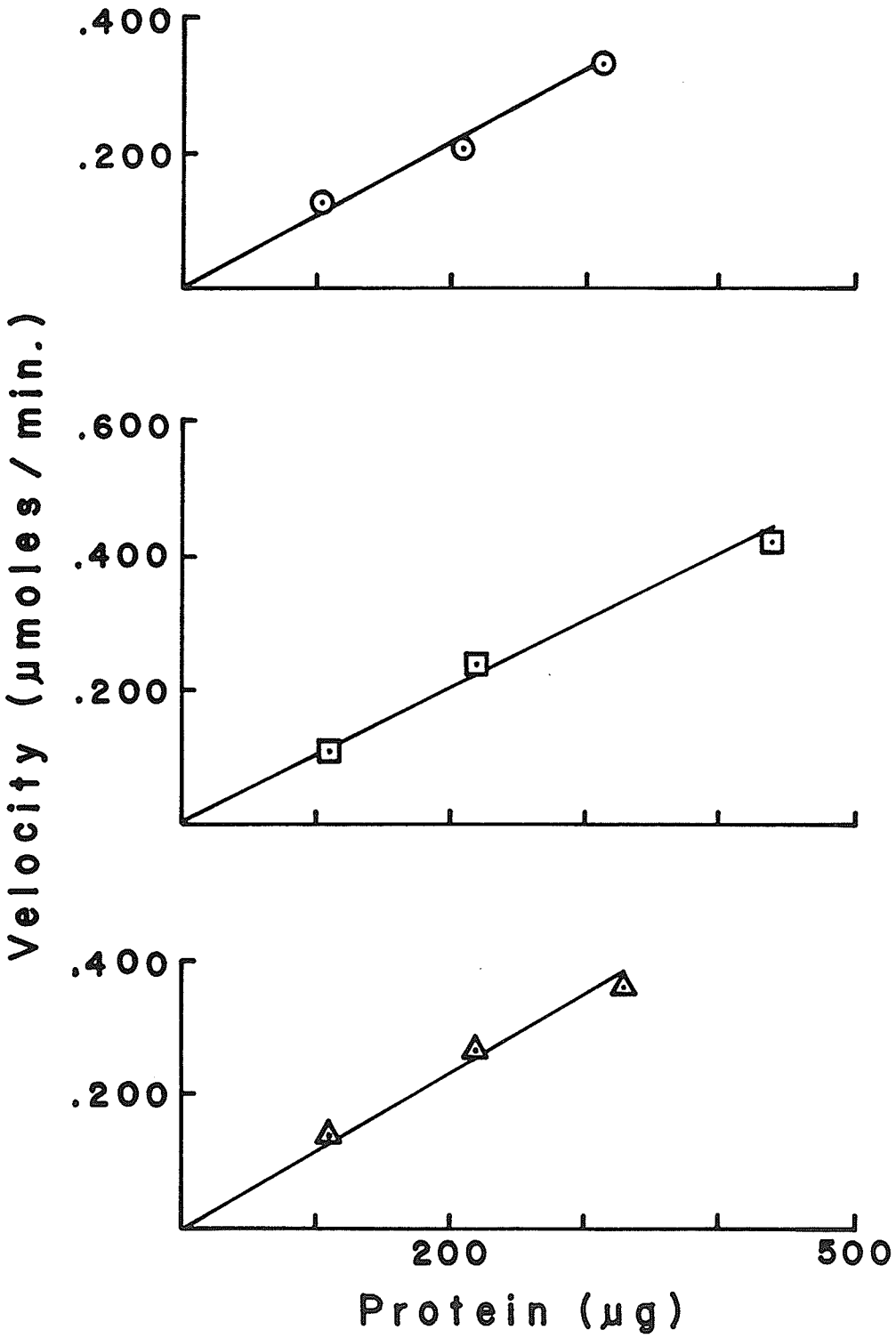

.400
.200
.600
.400
.200
.400
.200
200
500
Velocity (µmoles / min.)
Protein (µg)

TABLE III

Specific activities[1] of glycosidases in Triton X-100 extracts of W.T. CO_2, C^R-7, and RC^R-7 cells grown in dishes and in suspension.

Enzyme	Cell Line	Growth Condition		
		Subconfluence	Confluence	Suspension
Hexosaminidases I and II	W.T. CO_2	4.3±0.2	4.5±0.3	4.4±0.3
	RC^R-7	4.7±0.1	3.7±0.6	3.8±0.2
	C^R-7	6.6±0.1	2.0±0.5	2.1±0.3
Hexosaminidase III	W.T. CO_2	1.7±0.1	1.5±0.2	6.7±1.4
	RC^R-7	1.7±0.1	1.6±0.2	3.6±0.2
	C^R-7	2.4±0.2	1.2±0.3	16.0±2.2
α-L-Fucosidase	W.T. CO_2	tr[3]	2.4±0.3	n.d.[4]
	RC^R-7	tr	2.1±0.3	n.d.
	C^R-7	tr	0.5±0.1	n.d.
α-D-Mannosidase	W.T. CO_2	1.1±0.2	tr	n.d.
	RC^R-7	1.2±0.1	tr	n.d.
	C^R-7	1.4±0.2	tr	n.d.

[1]Results are expressed in units per mg protein and represent mean values of 8 to 12 separate estimations within the given range of cell densities.
[2]Subconfluence is defined as 1-6X10^6 cells per dish. Confluence is defined as 1-2X10^7 cells per dish. Suspension is defined as 2-5X10^5 cells per ml.
[3]Trace amounts of activity (less than 0.1 units per mg protein).
[4]Not determined.

dishes (Table III). The combined specific activities of Hex I and Hex II in extracts of W.T. CO_2 and RC^R-7 cells did not vary significantly with the method of culture, but Hex I and Hex II levels in C^R-7 cells increased three-fold when the cells were grown at subconfluence instead of at confluence or in suspension. The combined specific activities of Hex I and Hex II in extracts of W.T. CO_2 and RC^R-7 cells grown to confluence in dishes were about two-fold higher than the specific activities of Hex I and Hex II in extracts of comparable C^R-7 cells. When the three cell lines were grown in dishes at subconfluence, the W.T. CO_2 and RC^R-7 Hex I and Hex II activities were about two-thirds of that found in the C^R-7 cells. All three cell lines had similar specific activities for Hex III when grown in dishes either at confluence or subconfluence. In suspension, the specific activity of the C^R-7 Hex III was approximately three times greater than the specific activity of either the W.T. CO_2 or RC^R-7 Hex III.

The specific activities of α-L-fucosidase and α-D-mannosidase in extracts of cells grown in suspension were not determined. All three cell lines, when grown at subconfluence in dishes, had the same specific activities for α-D-mannosidase (Table III). At confluence, the α-D-mannosidase levels decreased drastically and only trace amounts of the enzyme were detected in all three cell lines. The specific activities of α-L-fucosidase were reduced when the cells were grown at subconfluence as compared to confluence. At confluence, the specific activities of α-L-fucosidase in extracts of W.T. CO_2 and RC^R-7 cells were four times

greater than that found in C^R-7 extracts.

Intracellular and extracellular hexosaminidase forms

Extracts of W.T. CO_2 and C^R-7 cells grown in Brockway bottles and in suspension were analyzed for hexosaminidase forms using ion-exchange chromatography. Both cell lines possessed three hexosaminidase forms (Figures 28-31). Hexosaminidase II was eluted from the DEAE-cellulose columns with 30 mM NaCl. Hexosaminidase I and hexosaminidase III were eluted with 150 mM and 300 mM NaCl, respectively. The W.T. CO_2 and C^R-7 secreted only Hex I and Hex III (Figures 32-35). No Hex II activity was found in the media. The intracellular and extracellular hexosaminidase activities of each cell line are summarized in Table IV. These activities are expressed relative to the protein applied to the DEAE-cellulose columns in Table V. The values shown in Table V are an estimate of the specific activities of the individual hexosaminidase forms in the original samples. There was no difference between the specific activities of the W.T. CO_2 and C^R-7 intracellular Hex II, but the specific activities of the Hex II in both cell lines were elevated two-fold when the cells were grown in Brockway bottles as opposed to suspension (Table V). In suspension, the specific activity of the C^R-7 intracellular Hex III was 1.7-fold greater than the specific activity of the W.T. CO_2 intracellular Hex III. In contrast, the specific activity of the W.T. CO_2 extracellular Hex III was 1.7-fold greater than the specific activity of the C^R-7 extracellular Hex III. The specific activities of the W.T. CO_2

Figure 28. Ion-exchange chromatography of the W.T. CO_2 intracellular hexosaminidase forms on DEAE-cellulose. The W.T. CO_2 cells were grown in Brockway bottles.

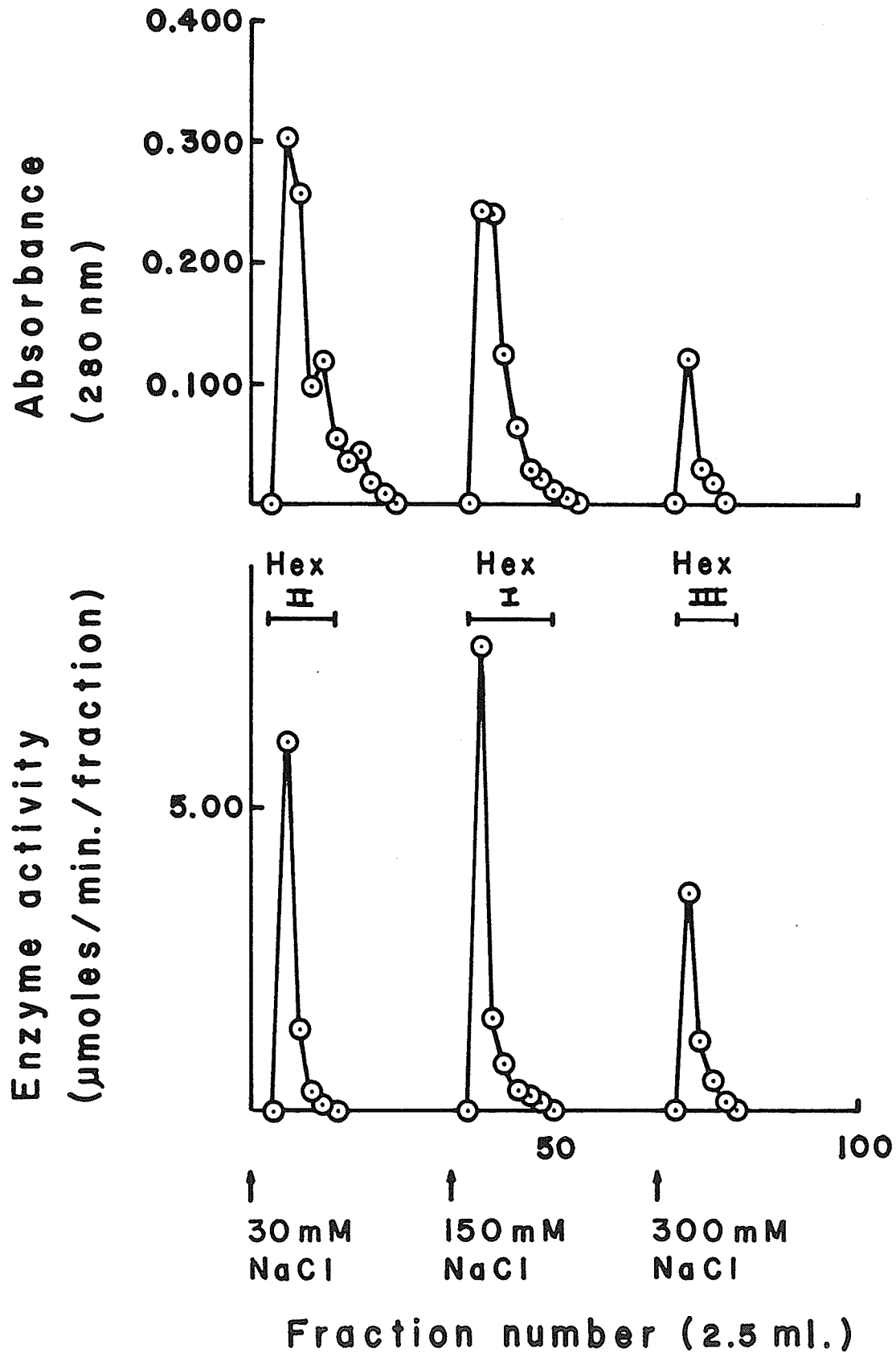

0.400
0.300
0.200
0.100
Absorbance
(280 nm)
Hex
II
Hex
I
Hex
III
5.00
Enzyme activity
(μmoles/min./fraction)
50
100
30 mM
NaCl
150 mM
NaCl
300 mM
NaCl
Fraction number (2.5 ml.)

Figure 29. Ion-exchange chromatography of the W.T. CO_2 intracellular hexosaminidase forms on DEAE-cellulose. The W.T. CO_2 cells were grown in suspension.

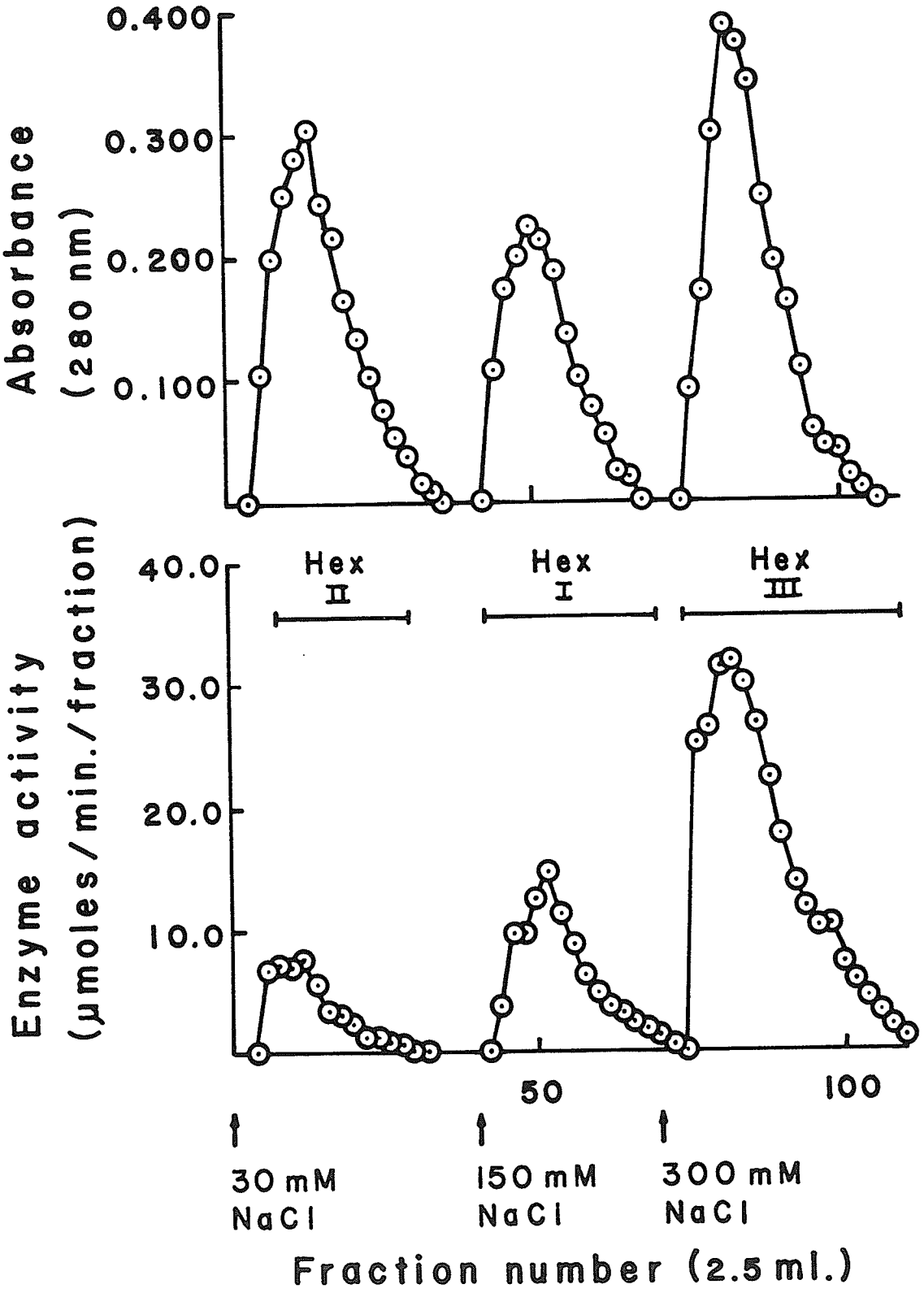

0.400
0.300
0.200
0.100
Absorbance
(280 nm)
40.0
30.0
20.0
10.0
Enzyme activity
(μmoles/min./fraction)
Hex
II
Hex
I
Hex
III
50
100
30 mM
NaCl
150 mM
NaCl
300 mM
NaCl
Fraction number (2.5 ml.)

Figure 30. Ion-exchange chromatography of the C^R-7 intracellular hexosaminidase forms on DEAE-cellulose. The C^R-7 cells were grown in Brockway bottles.

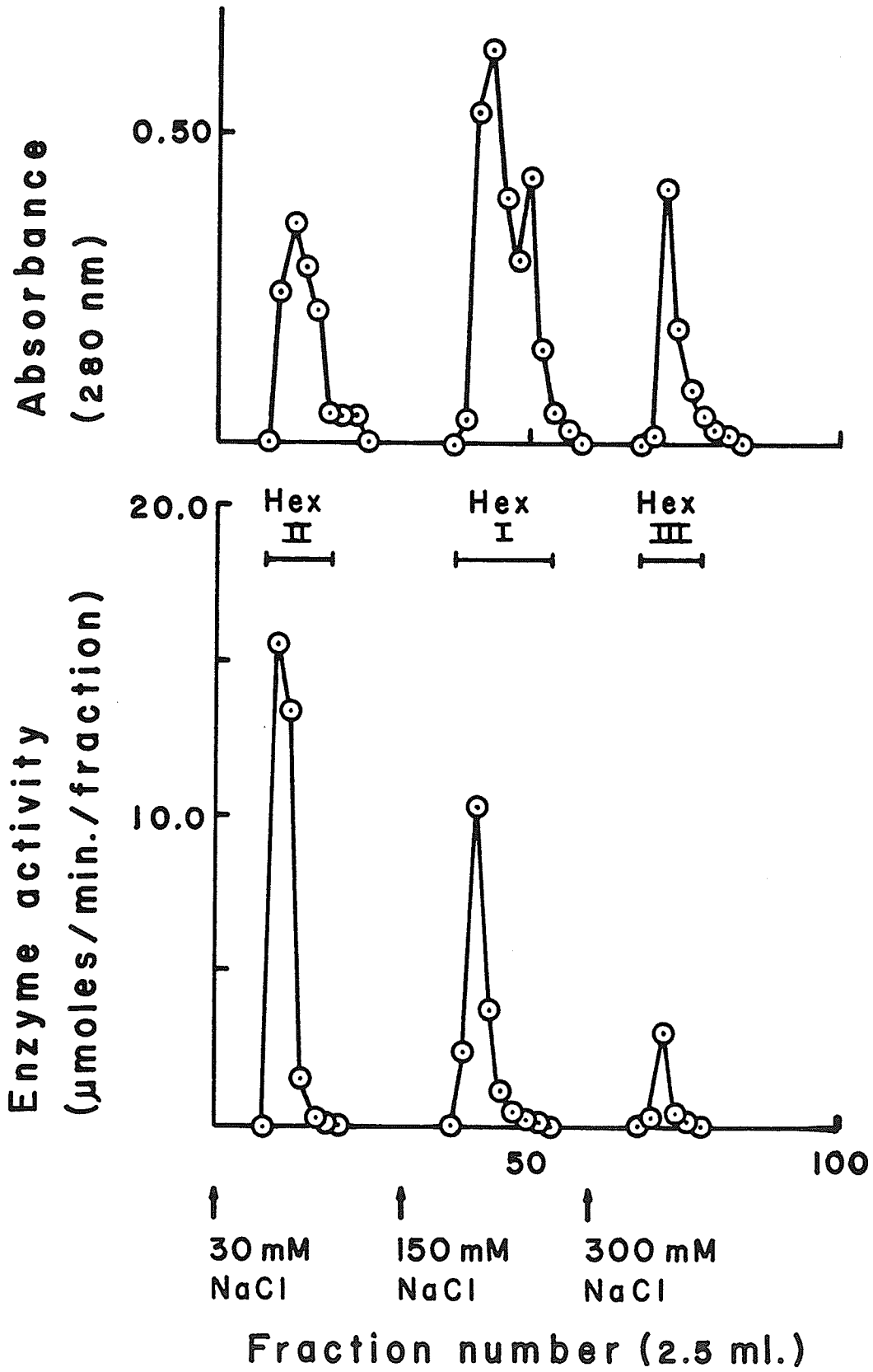

Absorbance
(280 nm)
0.50
Hex
II
Hex
I
Hex
III
20.0
Enzyme activity
(μmoles/min./fraction)
10.0
50
100
30 mM
NaCl
150 mM
NaCl
300 mM
NaCl
Fraction number (2.5 ml.)

Figure 31. Ion-exchange chromatography of the C^R-7 intracellular hexosaminidase forms on DEAE-cellulose. The C^R-7 cells were grown in suspension.

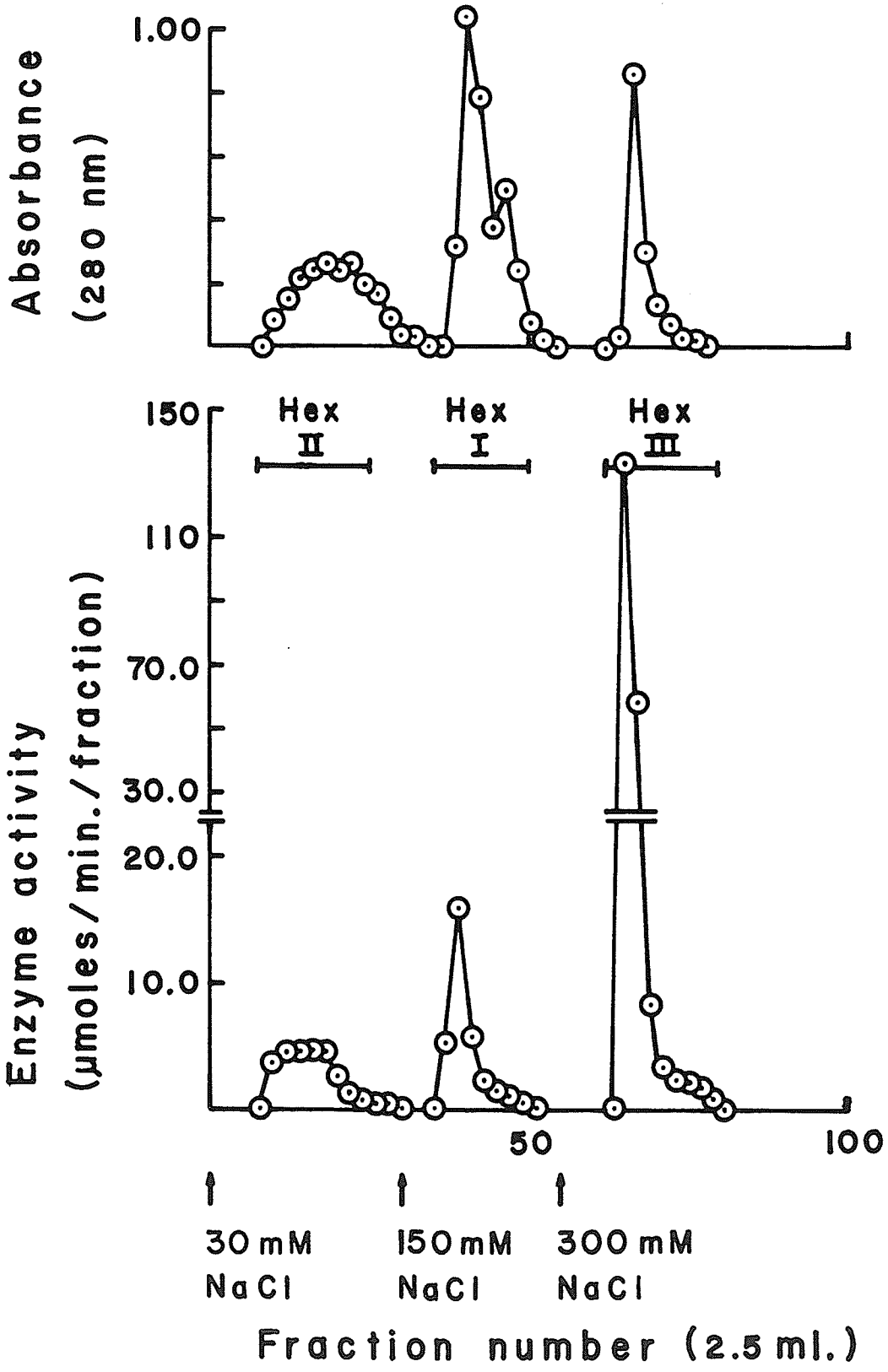

Absorbance
(280 nm)
1.00
Hex
II
Hex
I
Hex
III
150
110
70.0
30.0
20.0
10.0
Enzyme activity
(μmoles/min./fraction)
50
100
30 mM
NaCl
150 mM
NaCl
300 mM
NaCl
Fraction number (2.5 ml.)

Figure 32. Ion-exchange chromatography of the W.T. CO_2 extracellular hexosaminidase forms on DEAE-cellulose. The W.T. CO_2 cells were grown in Brockway bottles.

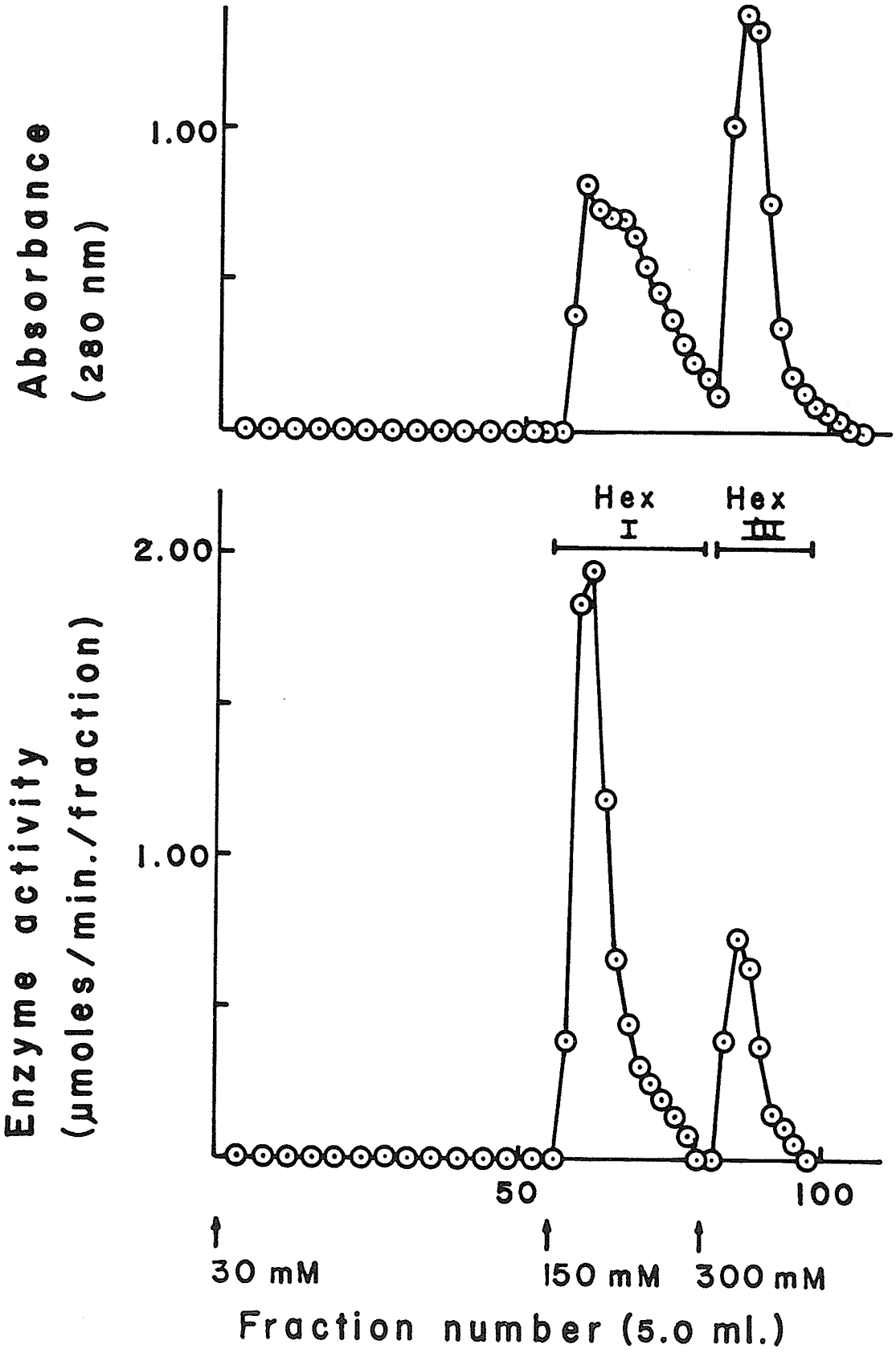

Absorbance
(280 nm)
1.00
Hex
I
Hex
III
2.00
Enzyme activity
(μmoles/min./fraction)
1.00
50
100
30 mM
150 mM
300 mM
Fraction number (5.0 ml.)

Figure 33. Ion-exchange chromatography of the W.T. CO_2 extra cellular hexosaminidase forms on DEAE-cellulose. The W.T. CO_2 cells were grown in suspension.

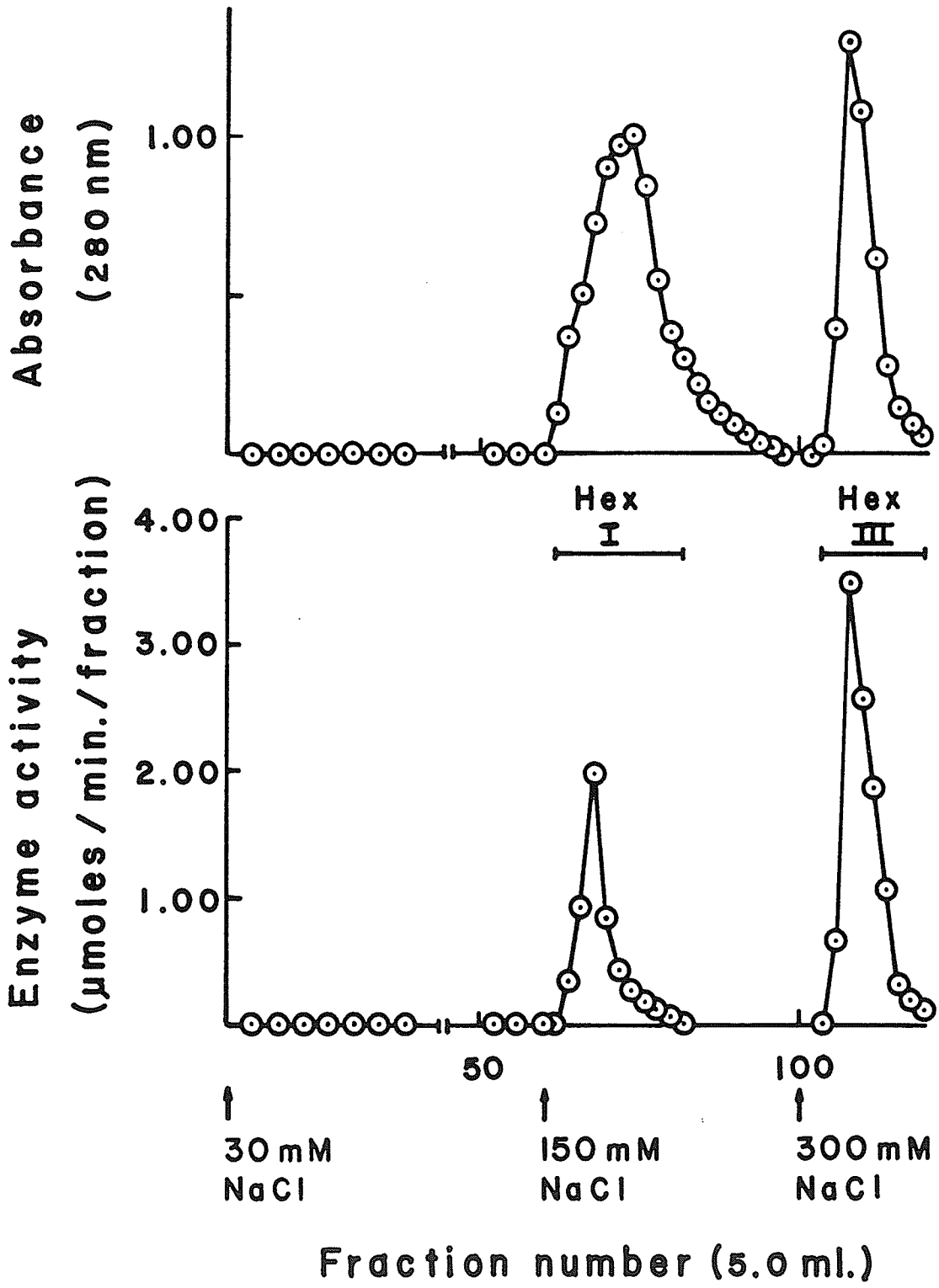

Absorbance
(280 nm)
1.00
Hex
I
Hex
III
Enzyme activity
(μmoles / min. / fraction)
4.00
3.00
2.00
1.00
50
100
30 mM
NaCl
150 mM
NaCl
300 mM
NaCl
Fraction number (5.0 ml.)

Figure 34. Ion-exchange chromatography of the C^R-7 extracellular hexosaminidase forms on DEAE-cellulose. The C^R-7 cells were grown in Brockway bottles.

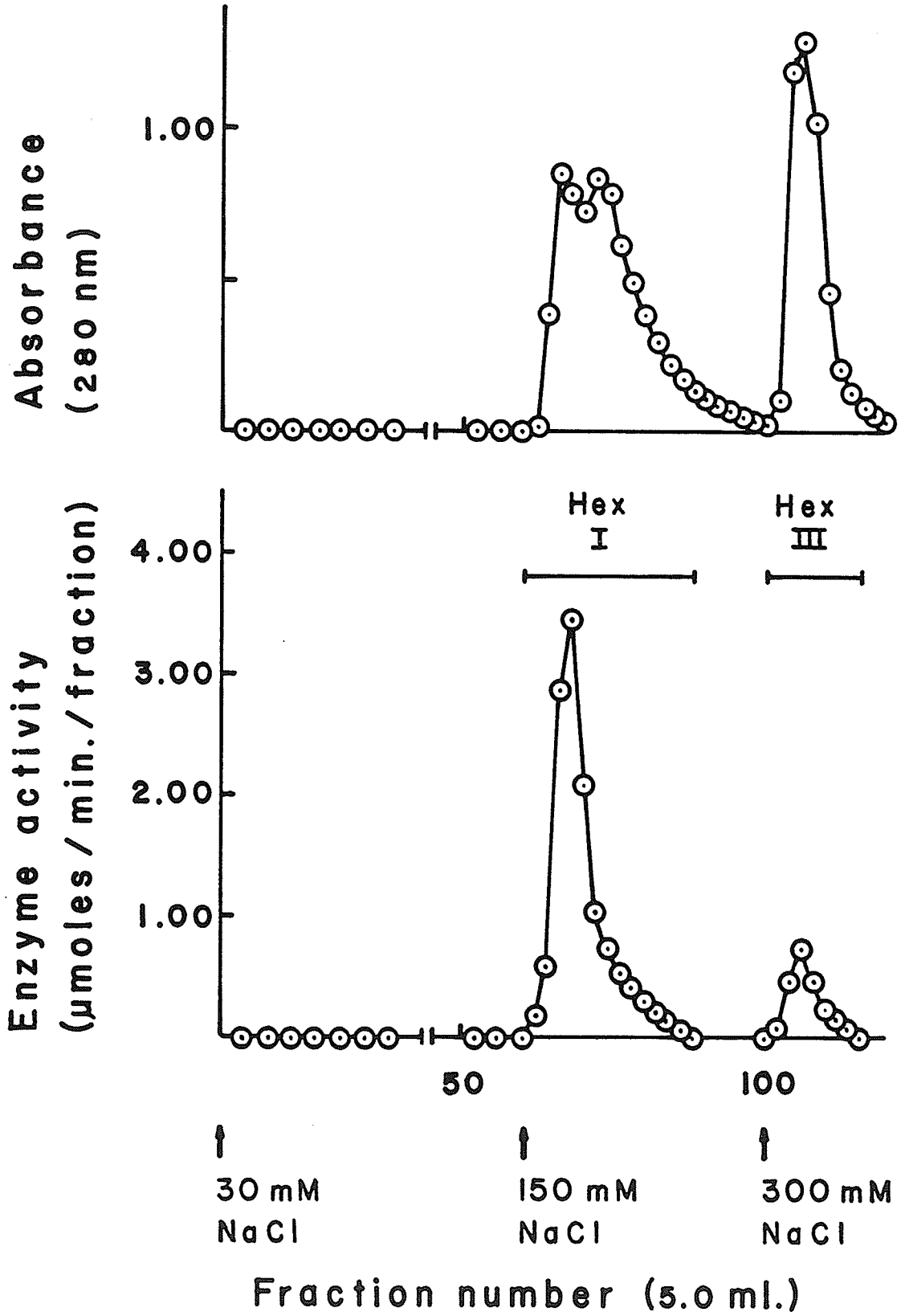

Absorbance
(280 nm)
1.00
Enzyme activity
(μmoles / min. / fraction)
4.00
3.00
2.00
1.00
Hex
I
Hex
III
50
100
30 mM
NaCl
150 mM
NaCl
300 mM
NaCl
Fraction number (5.0 ml.)

Figure 35. Ion-exchange chromatography of the C^R-7 extracellular hexosaminidase forms on DEAE-cellulose. The C^R-7 cells were grown in suspension.

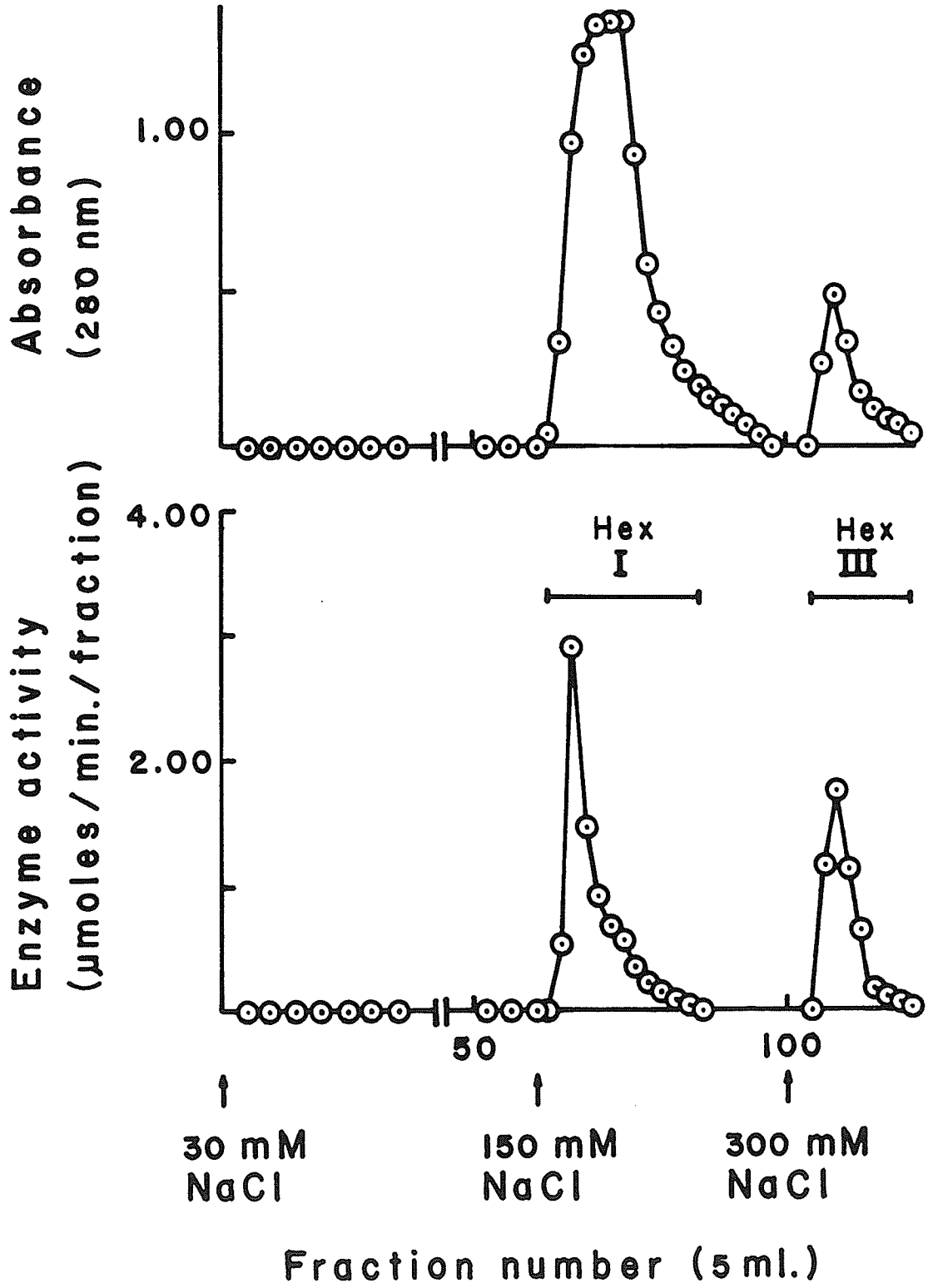

Absorbance (280 nm)
1.00
Enzyme activity (μmoles/min./fraction)
4.00
2.00
Hex I
Hex III
50
100
30 mM NaCl
150 mM NaCl
300 mM NaCl
Fraction number (5 ml.)

TABLE IV

Activities of W.T. CO_2 and C^R-7 intracellular and extracellular hexosaminidase forms.

Growth Condition	Cell Line	From Cells					From Medium			
		Cell Density[1]	Total Protein[2] Loaded (mg)	Total Intracellular Activity (units) Hex 1	Hex II	Hex III	Cell Density[1]	Total Protein[2] Loaded (mg)	Total Extracellular Activity (units) Hex I	Hex III
Brockway bottle	W.T. CO_2	1.05×10^7	5.5	15.9	14.8	2.73	2.17×10^7	440	12.6	3.99
	C^R-7	2.56×10^7	27	35.0	58.9	7.30	2.05×10^7	485	20.9	3.58
Suspension	W.T. CO_2	5×10^5	76	189	90.6	362	3.2×10^5	300	8.60	19.2
	C^R-7	3×10^5	50	55.3	54.6	396	2.3×10^5	258	14.1	9.56

[1]Expressed as either cells per Brockway bottle or cells per ml.

[2]Total protein chromatographed on DEAE-cellulose.

TABLE V

Total activities of W.T. CO_2 and C^R-7 intracellular and extracellular hexosaminidase forms relative to the total protein.

Growth Condition	Cell Line	Total Activity Relative to the Total Protein (units/mg protein loaded)				
		Intracellular			Extracellular	
		Hex I	Hex II	Hex III	Hex I	Hex III
Brockway bottle	W.T. CO_2	2.89	2.69	0.50	0.029	0.0090
	C^R-7	1.32	2.21	0.27	0.043	0.0073
Suspension	W.T. CO_2	2.48	1.19	4.76	0.029	0.064
	C^R-7	1.11	1.09	7.92	0.055	0.037

intracellular Hex I was two-fold higher than the specific activity of the C^R-7 intracellular Hex I, regardless of whether the cells were grown in bottles or in suspension. The specific activity of the C^R-7 extracellular Hex I was twice that of the W.T. CO_2 extracellular Hex I.

Properties of the intracellular W.T. CO_2 hexosaminidase forms

The properties of the three hexosaminidase forms derived from W.T. CO_2 cells grown in suspension were studied. Two buffers were used in these experiments: 0.1 M citric acid - 0.2 M Na_2HPO_4 and 0.1 M 3,3-dimethylglutaric acid - 0.2 M NaOH. The pH curves of the three hexosaminidase forms are shown in Figures 36 and 37. The hexosaminidase activities were linear with increasing time (Figures 38 and 39) and protein (Figures 40 and 41). The pH optima, K_m, and V_{max} values are shown in Table VI. The K_m values (obtained from Figures 42, 43 and 44) and pH optima for the hexosaminidase forms did not vary significantly with the different buffers. The V_{max} values of Hex I and Hex II also remained constant, but the V_{max} value of Hex III was 1.6-fold greater when the enzyme was assayed in the citric acid-Na_2HPO_4 buffer as compared to the 3,3-dimethylglutaric acid buffer.

The effects of metal ions (Mn^{++} and Mg^{++}) and EDTA on Hex III activity are shown in Figure 45. Maximum stimulation of Hex III activity occurred at an EDTA concentration of 3 mM. In contrast, Mn^{++} and Mg^{++} inhibited the Hex III activity 30% at this concentration. At higher concentrations of these metal ions (10-30 mM), Hex III activity was

Figure 36. The pH-activity profiles of hexosaminidases I (⊙), II (△), and III (⊡). The buffer used was 0.1 M citric acid - 0.2 M Na_2HPO_4.

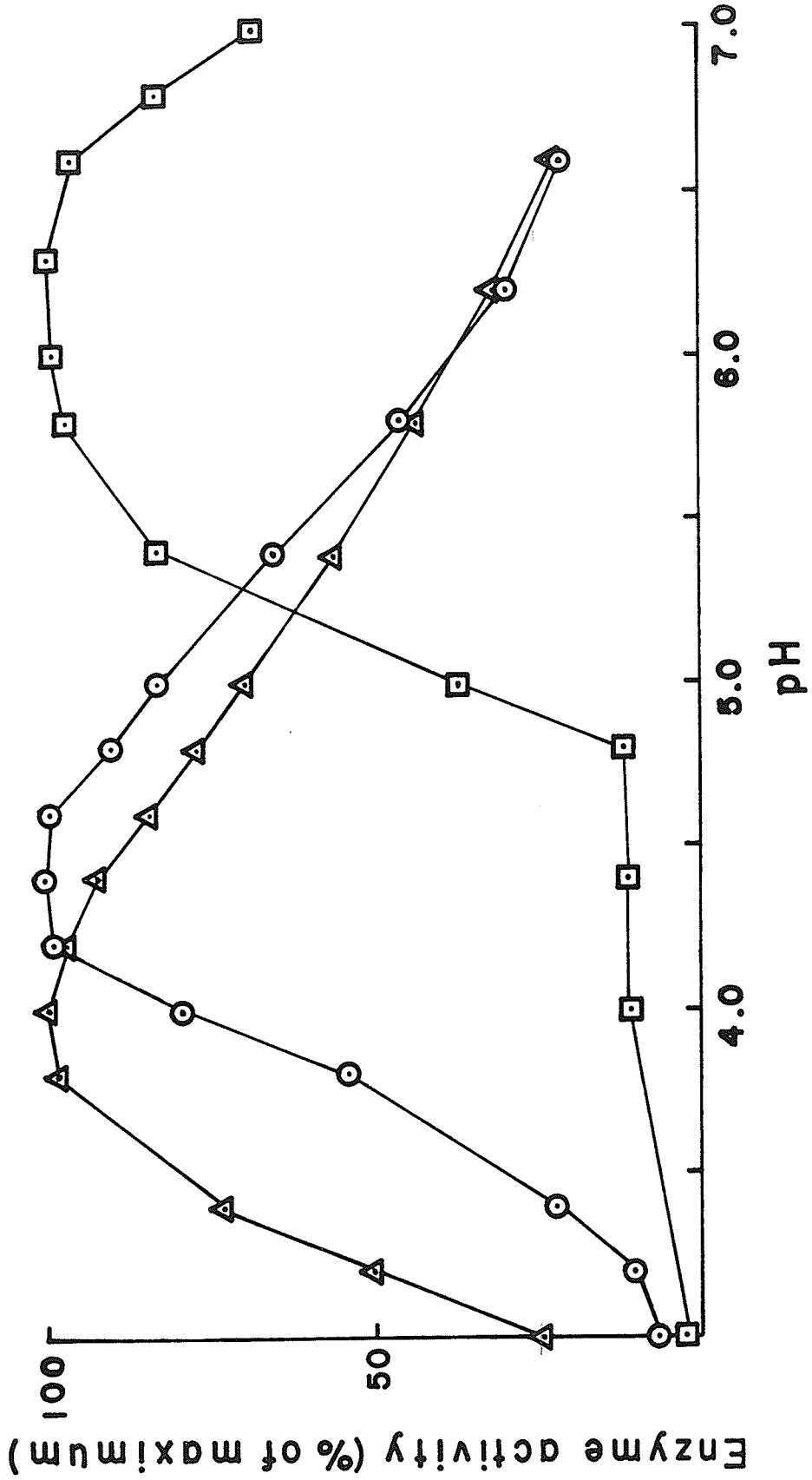

Enzyme activity (% of maximum)
100
50
4.0
5.0
6.0
7.0
pH

Figure 37. The pH-activity profiles of hexosaminidase I (⊙), II (△), and III (⊡). The buffer used was 0.1 M 3,3-dimethylglutaric acid - 0.2 M NaOH.

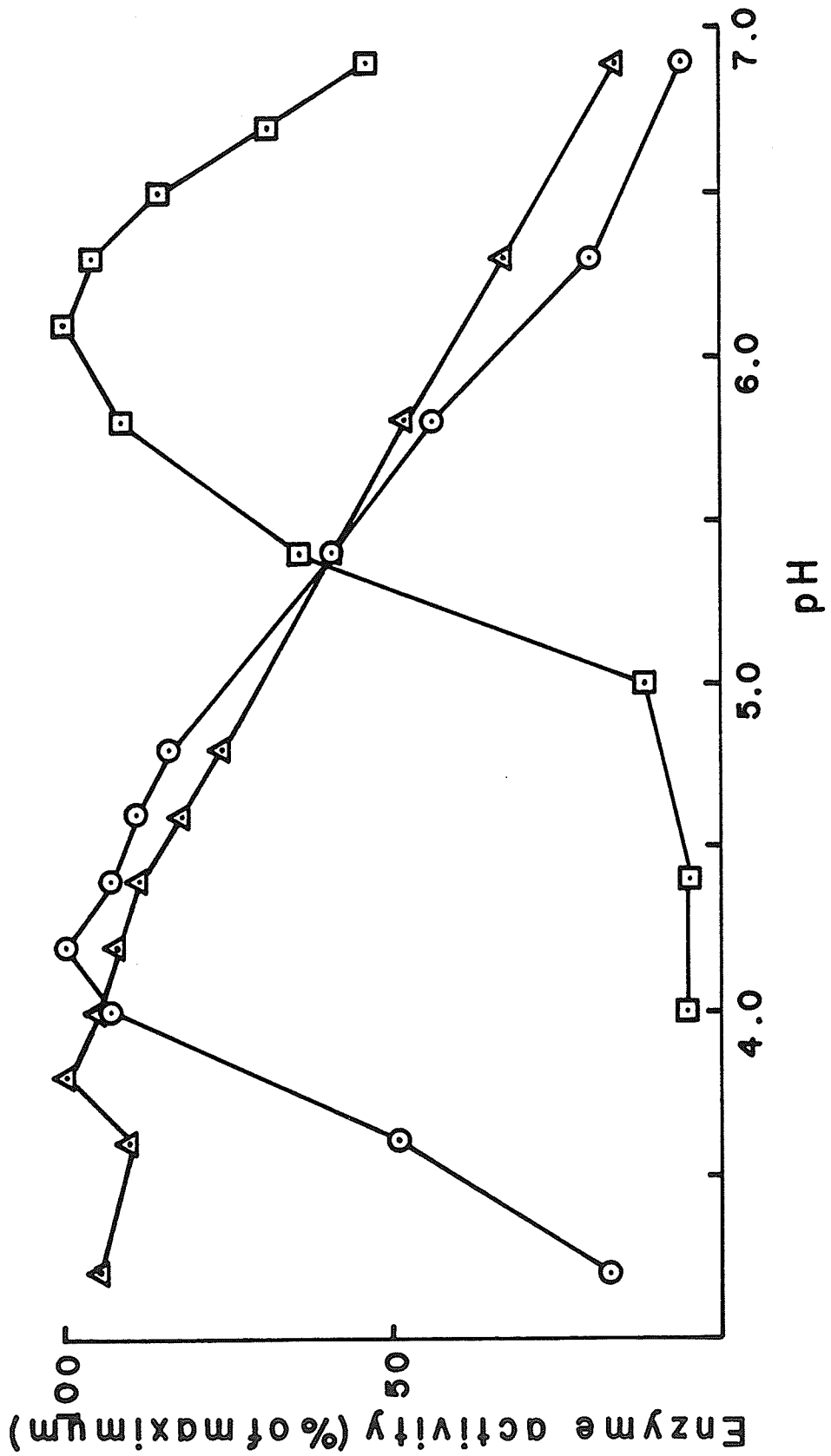

Enzyme activity (% of maximum)
100
50
4.0
5.0
6.0
7.0
pH

TABLE VI

Kinetic properties of W.T. CO_2 hexosaminidase forms.

Kinetic Property	Buffer					
	0.1 M Citric acid-0.2 M Na_2HPO_4			0.1 M 3,3-Dimethylglutaric acid-0.2 M NaOH		
	Hex I	Hex II	Hex III	Hex I	Hex II	Hex III
pH Optimum	4.4	4.0	6.3	4.2	3.8	6.1
K_m (mM)	0.89	0.63	0.37	0.91	0.63	0.48
V_{max} (units/mg protein)	3.5	2.1	6.8	3.6	2.1	4.1

Figure 38. The effect of the time of incubation on the activity of hexosaminidases I (⊙), II (△), and III (⊡). The buffer used was 0.1 M citric acid - 0.2 M Na_2HPO_4.

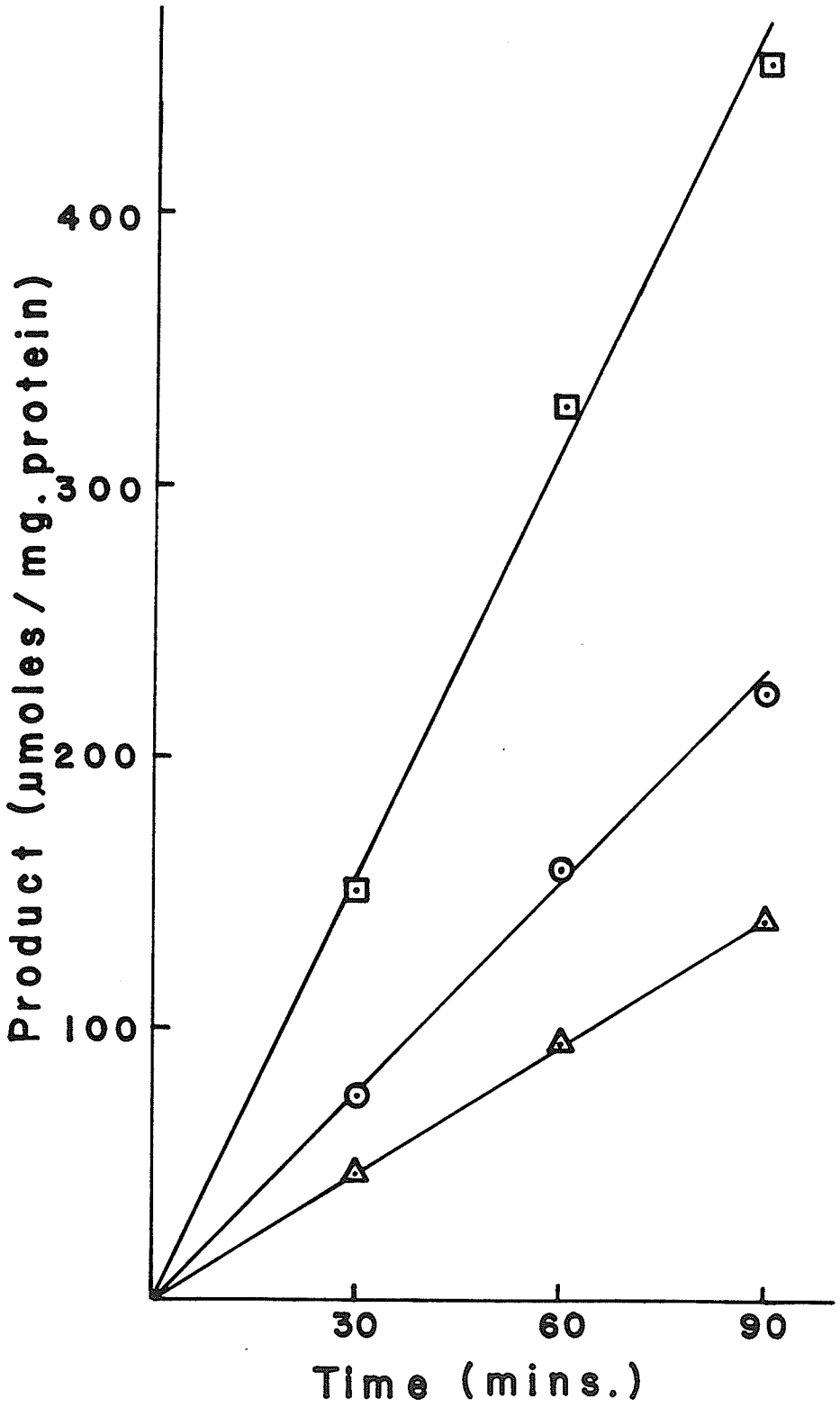

Product (µmoles / mg. protein)
400
300
200
100
30
60
90
Time (mins.)

Figure 39. The effect of the time of incubation on the activity of hexosaminidases I (⊙), II (△), and III (⊡). The buffer used was 0.1 M 3,3-dimethylglutaric acid-0.2 M NaOH.

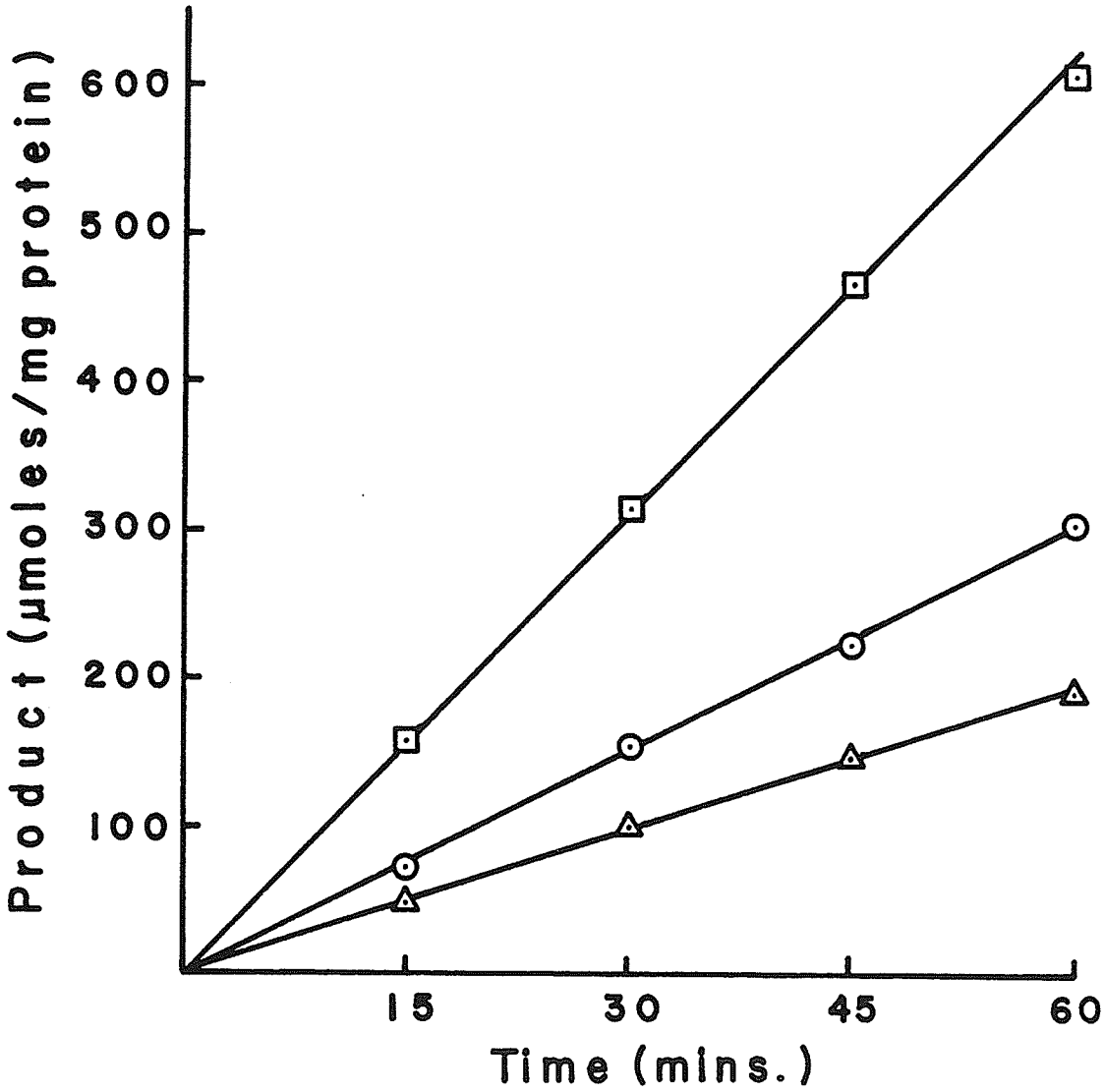

Product (µmoles/mg protein)
600
500
400
300
200
100
15
30
45
60
Time (mins.)

Figure 40. The effect of increasing protein on the activity of hexosaminidases I (⊙), II (△), and III (⊡). The buffer used was 0.1 M citric acid - 0.2 M Na_2HPO_4.

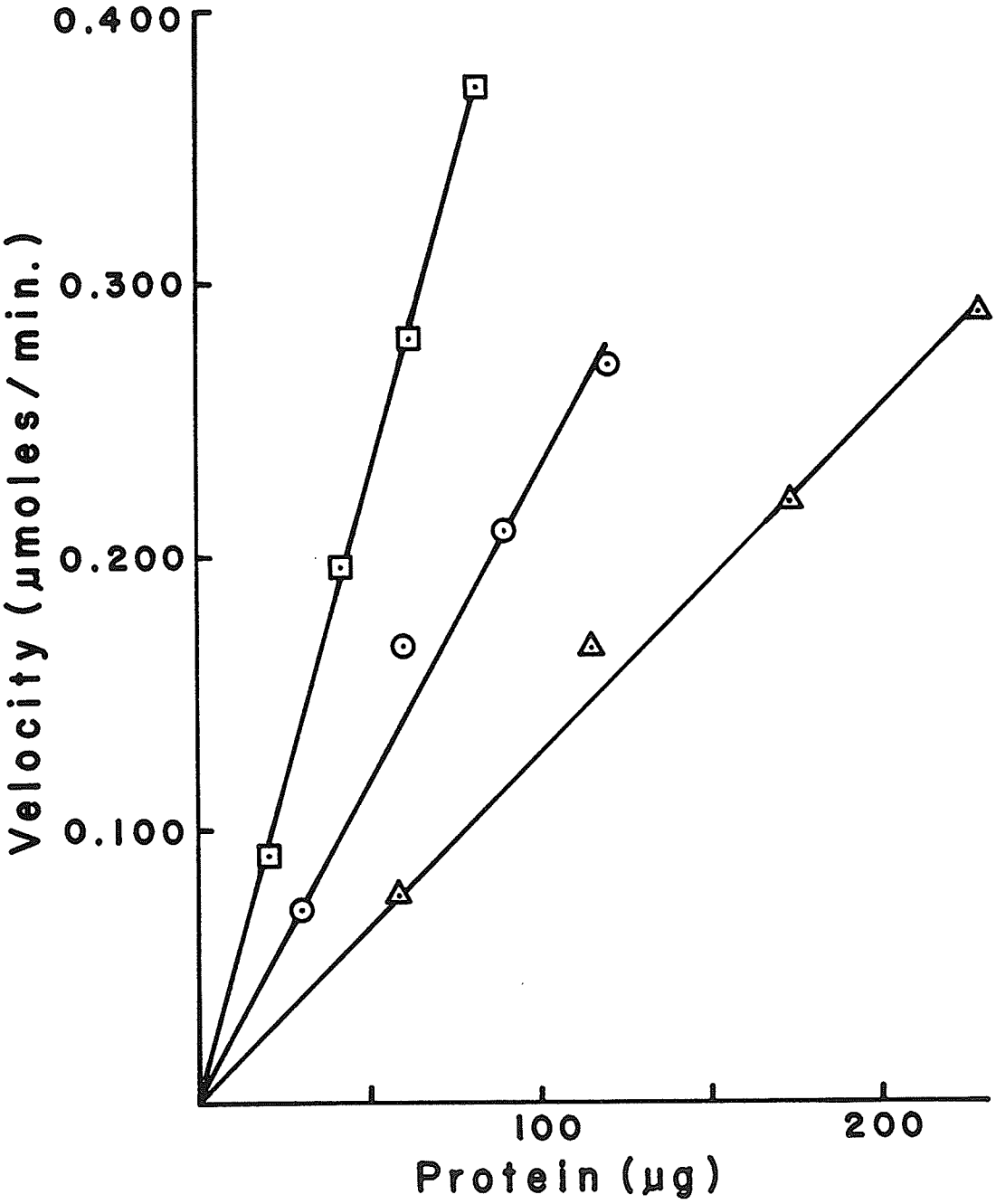

0.400
0.300
0.200
0.100
Velocity (µmoles/min.)
100
200
Protein (µg)

Figure 41. The effect of increasing protein on the activity of hexosaminidases I (⊙), II (△), and III (⊡). The buffer used was 0.1 M 3,3-dimethylglutaric acid-0.2 M NaOH.

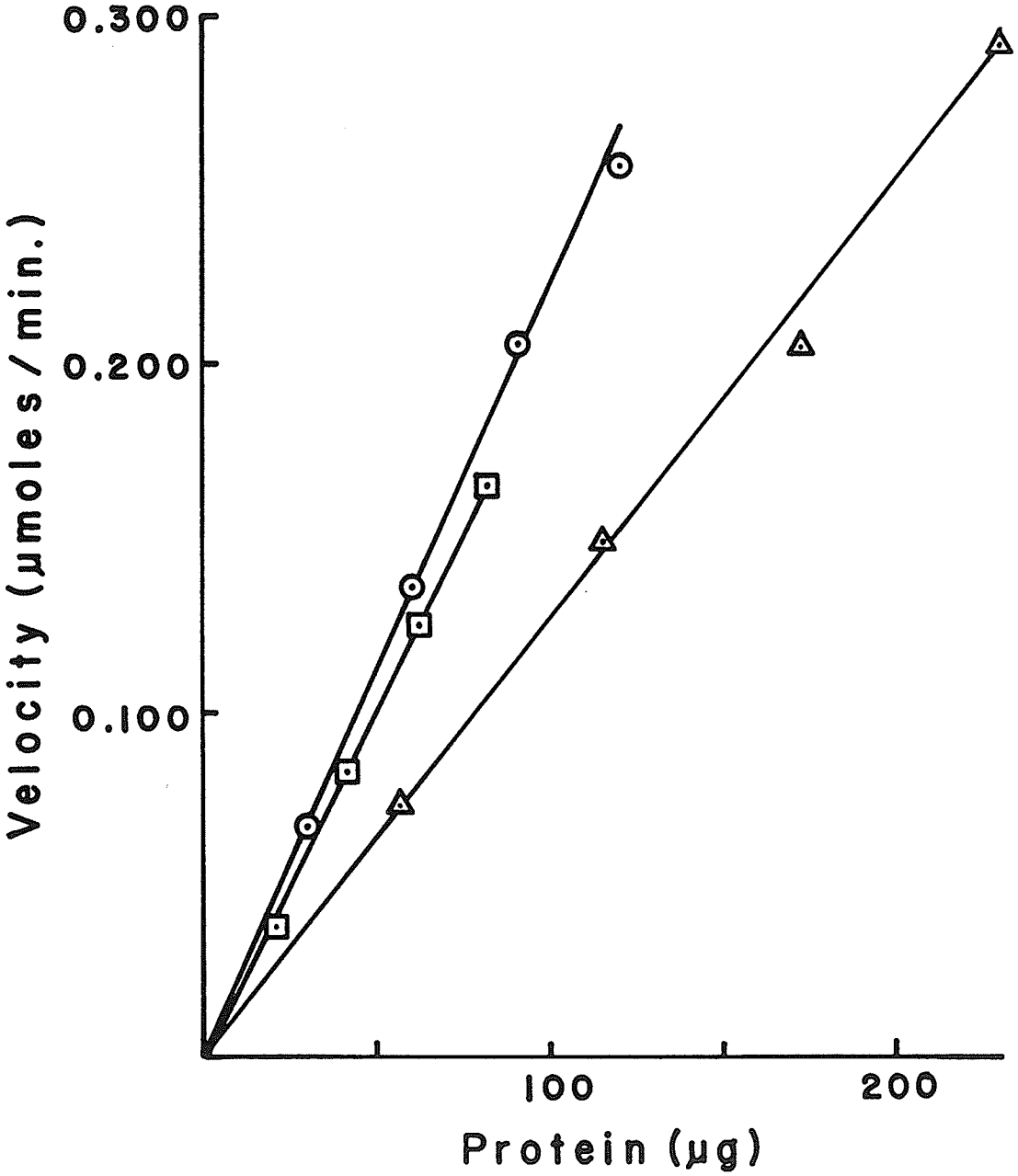

0.300
0.200
0.100
Velocity (µmoles / min.)
100
200
Protein (µg)

Figure 42. Eadie-Hofstee plots of hexosaminidase I activity in 0.1 M citric acid - 0.2 M Na_2HPO_4 (⊙), and 0.1 M 3,3-dimethylglutaric acid - 0.2 M NaOH (⊡) buffers.

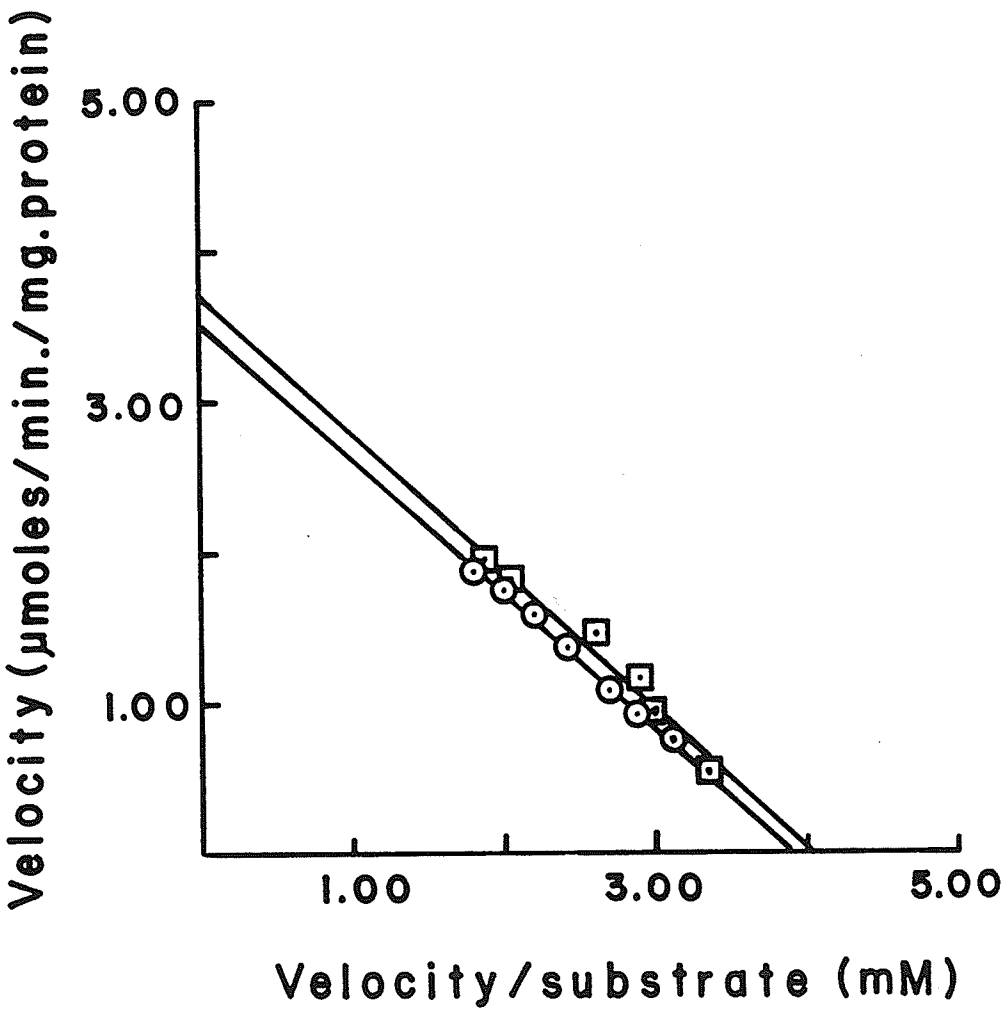

Velocity (µmoles/min./mg.protein)
5.00
3.00
1.00
1.00
3.00
5.00
Velocity/substrate (mM)

Figure 43. Eadie-Hofstee plot of hexosaminidase II activity in 0.1 M citric acid - 0.2 M Na_2HPO_4 (⊙), and 0.1 M 3,3-dimethylglutaric acid - 0.2 M NaOH (⊡) buffers.

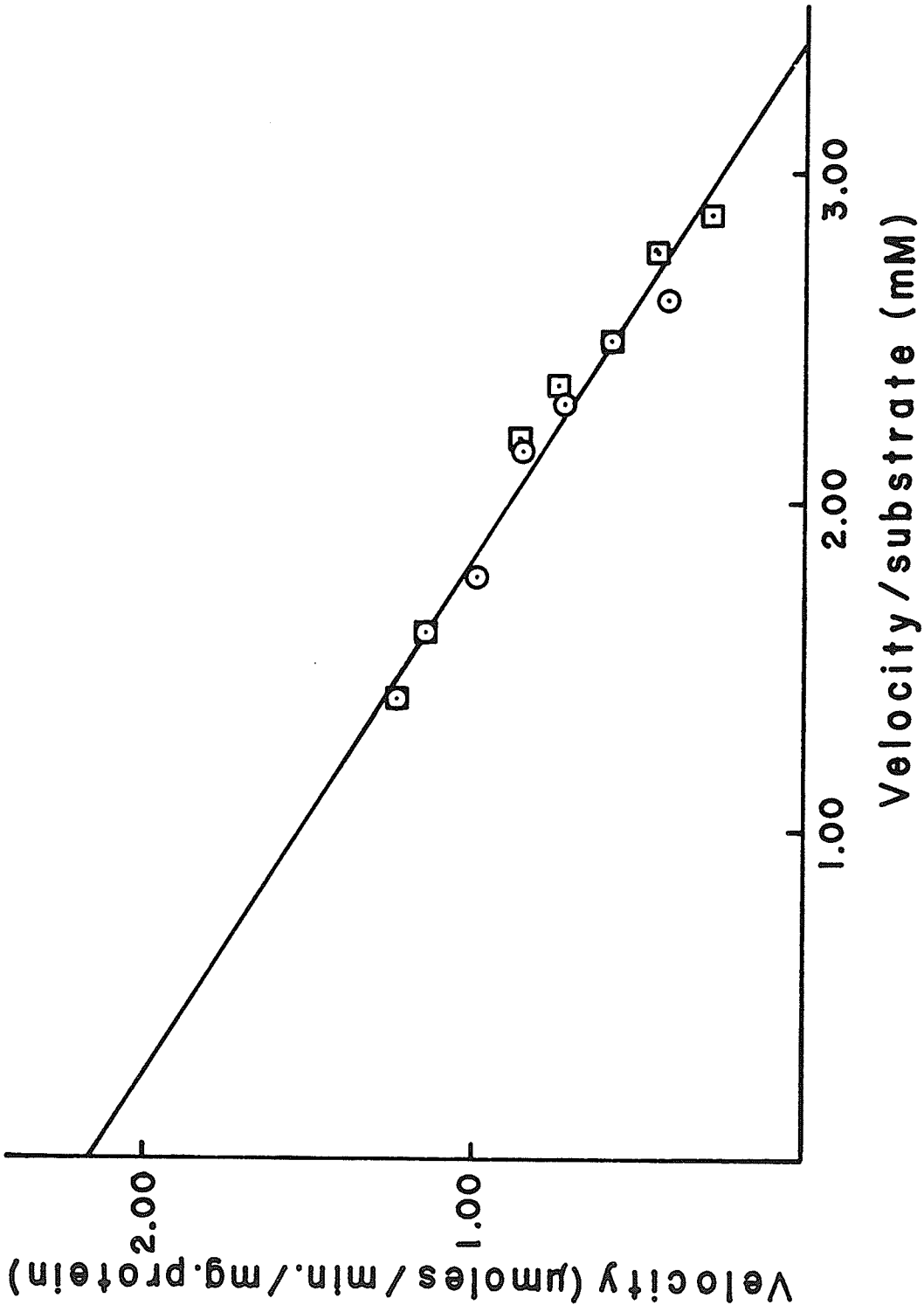

Velocity (μmoles/min./mg.protein)
2.00
1.00
1.00
2.00
3.00
Velocity/substrate (mM)

Figure 44. Eadie-Hofstee plots of hexosaminidase III activity in 0.1 M citric acid - 0.2 M Na_2HPO_4 (⊙), and 0.1 M 3,3-dimethylglutaric acid - 0.2 M NaOH (⊡).

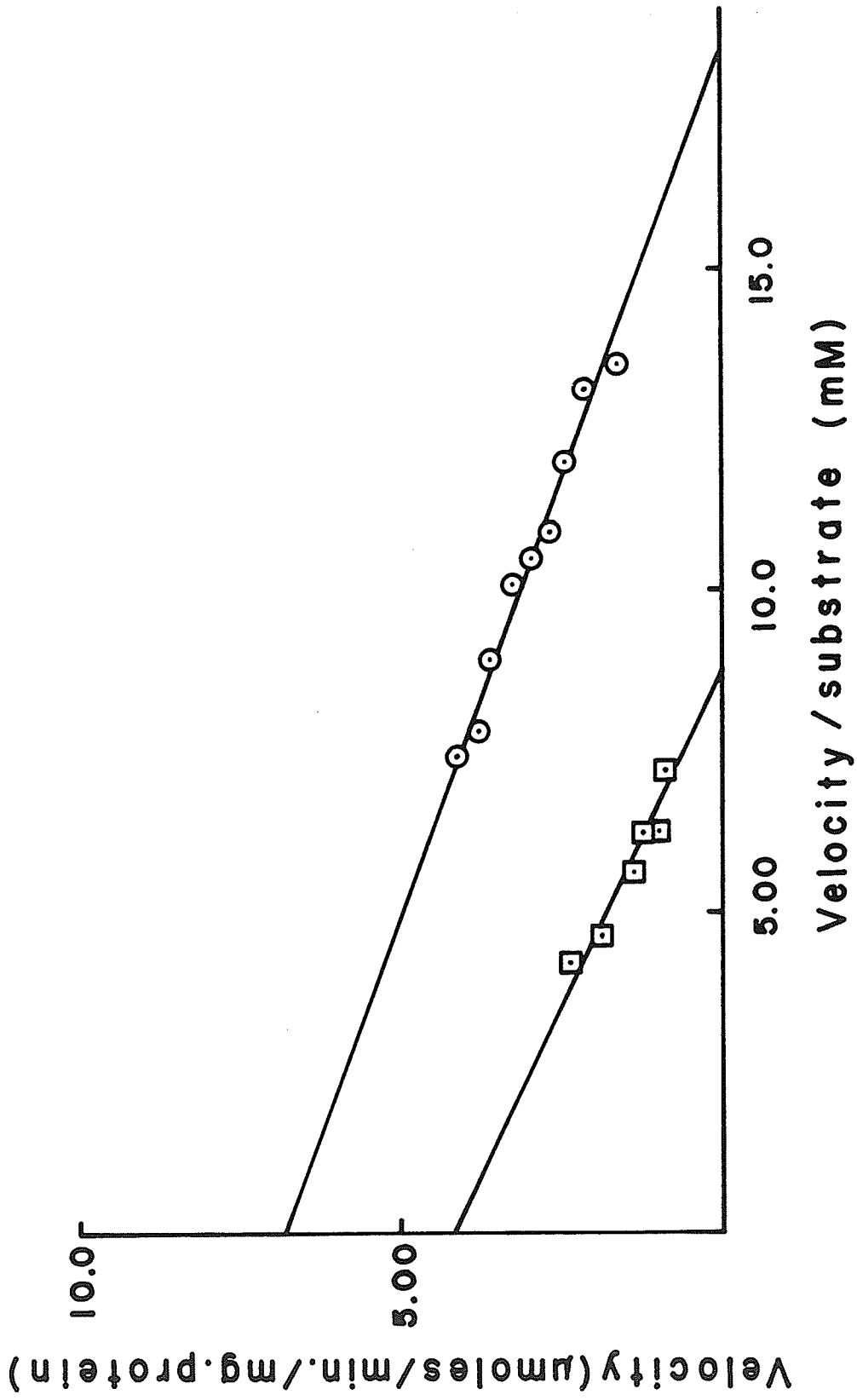
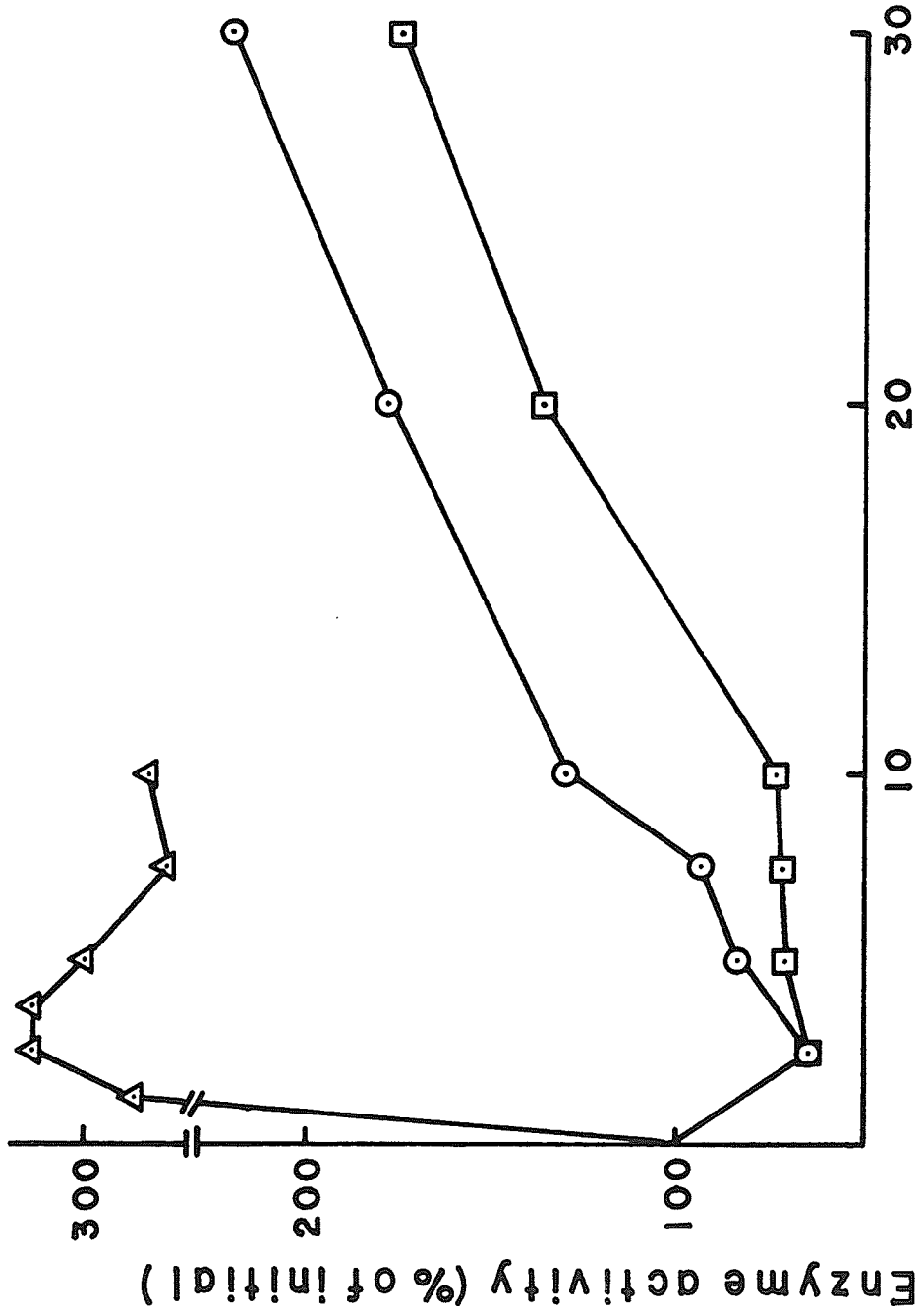

Velocity (μmoles/min./mg.protein)
10.0
5.00
5.00
10.0
15.0
Velocity / substrate (mM)

Figure 45. The effect of $MnCl_2$ (⊙), $MgCl_2$ (⊡), and EDTA (△) on hexosaminidase III activity. The buffer used was 0.1 M 3,3-dimethylglutaric acid - 0.2 M NaOH.


Enzyme activity (% of initial)
300
200
100
10
20
30
MnCl2, MgCl2, EDTA (mM)

stimulated.

The stability of the three hexosaminidase forms at 50°C was determined. Hexosaminidase II was the most stable form, retaining 53% activity after incubation at 50°C for 50 minutes (Figure 46). Hexosaminidase I retained 15% activity, while Hex III retained only 2.5% activity.

Partial purification of W.T. CO_2 hexosaminidases I, II and III

Hexosaminidases I, II, and III were partially purified from W.T. CO_2 cells grown in suspension. The three hexosaminidases were separated from one another by DEAE-cellulose chromatography (Figure 47). The fractions (1-25) collected while the DEAE-cellulose column was being washed with 30 mM NaCl were not assayed for Hex II activity, but were pooled and applied directly to a CM-cellulose column. This column was washed with a linear NaCl gradient, the Hex II activity being eluted at an NaCl concentration of 100 mM (Figure 48). The fractions containing Hex II activity were pooled and applied to a concanavalin A-Sepharose 4B column. The enzyme specifically bound to the column and was not eluted when the column was washed with buffer containing 1 M NaCl (washing buffer) (Figure 49). The Hex II activity was eluted only when the column was washed with buffer containing 0.1 M α-methylmannoside (eluting buffer). Hexosaminidase I also bound specifically to the concanavalin A-Sepharose 4B column and was eluted only when the column was washed with eluting buffer (Figure 49). The Hex III obtained by

Figure 46. The effect of temperature (50° C) on the activity of hexosaminidases I (⊙), II (△), and III (⊡).

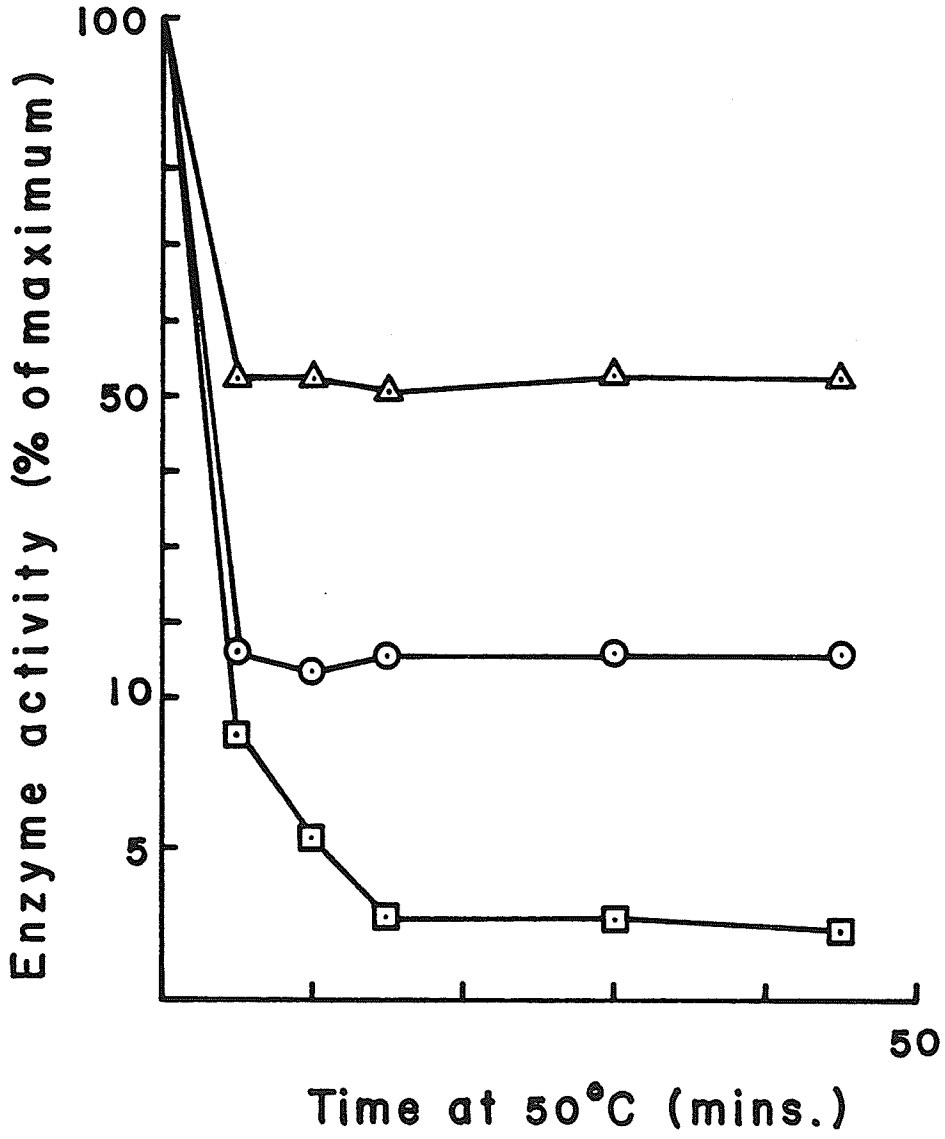

100
50
10
5
Enzyme activity (% of maximum)
50
Time at 50°C (mins.)

Figure 47. Ion-exchange chromatography of W.T. CO_2 intracellular hexosaminidase forms on DEAE-cellulose.

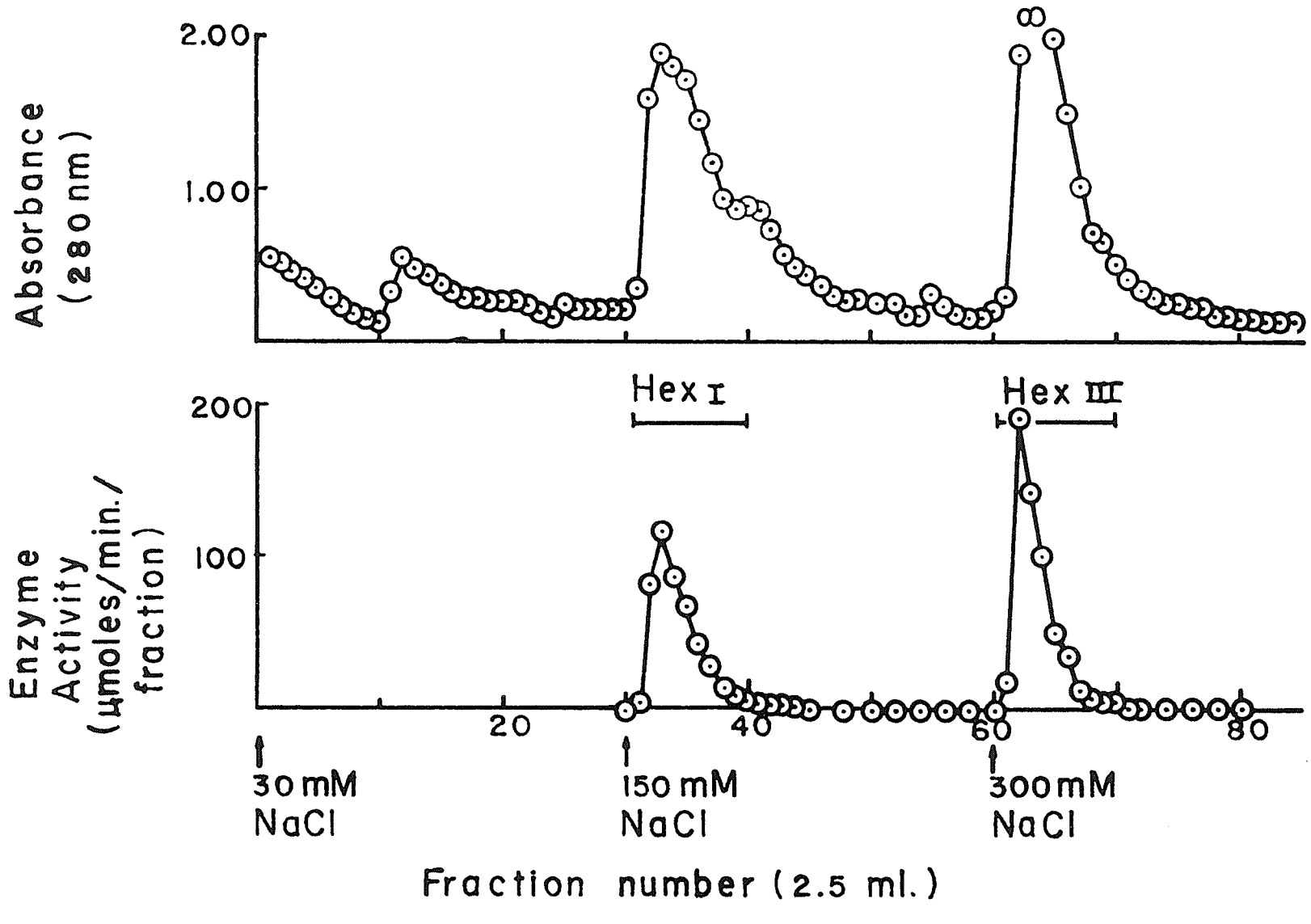

Absorbance (280 nm)
2.00
1.00
Enzyme Activity (μmoles/min./ fraction)
200
100
Hex I
Hex III
20
40
60
80
30 mM NaCl
150 mM NaCl
300 mM NaCl
Fraction number (2.5 ml.)

Figure 48. Ion-exchange chromatography of hexosaminidase II on CM-cellulose.

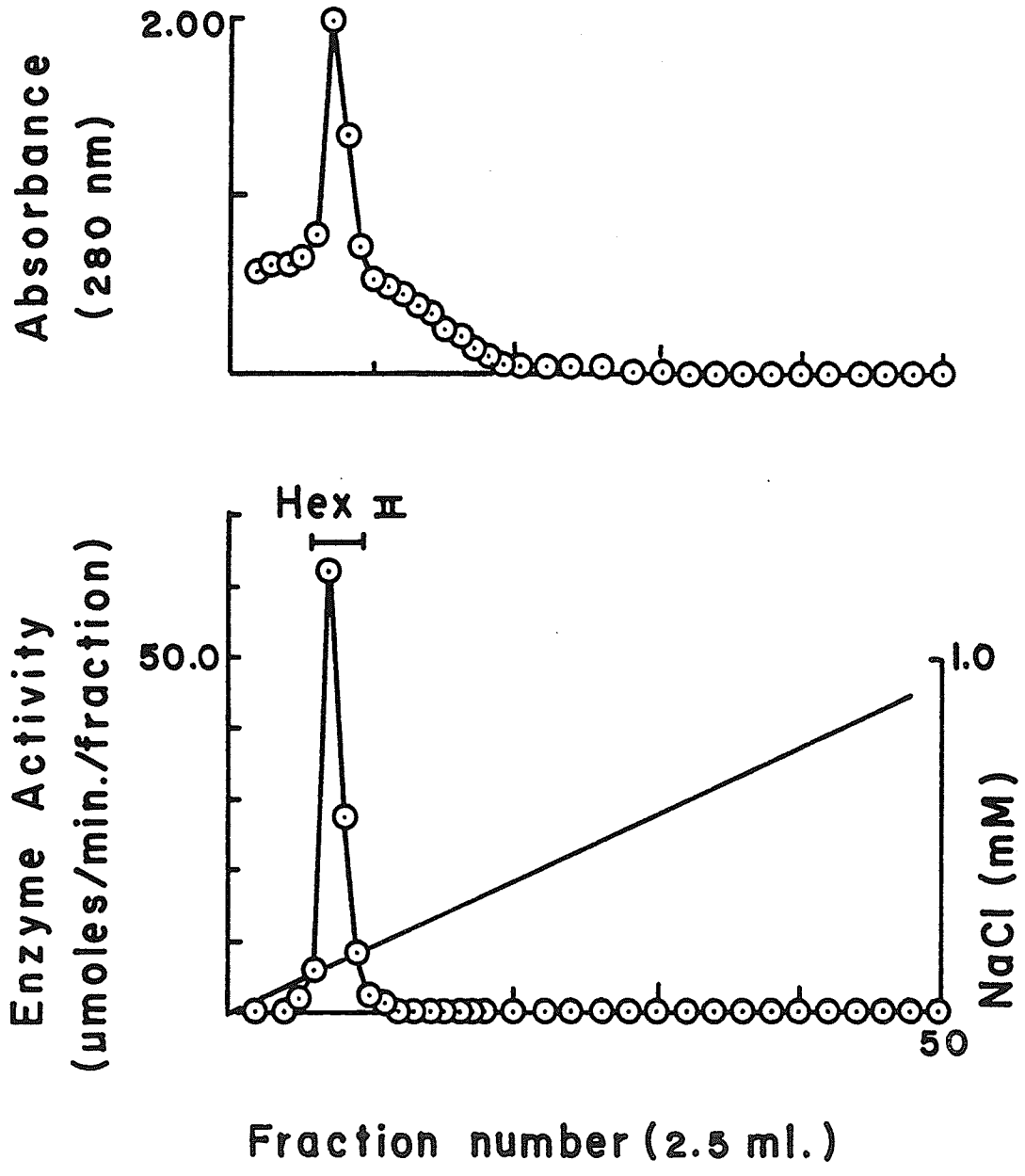
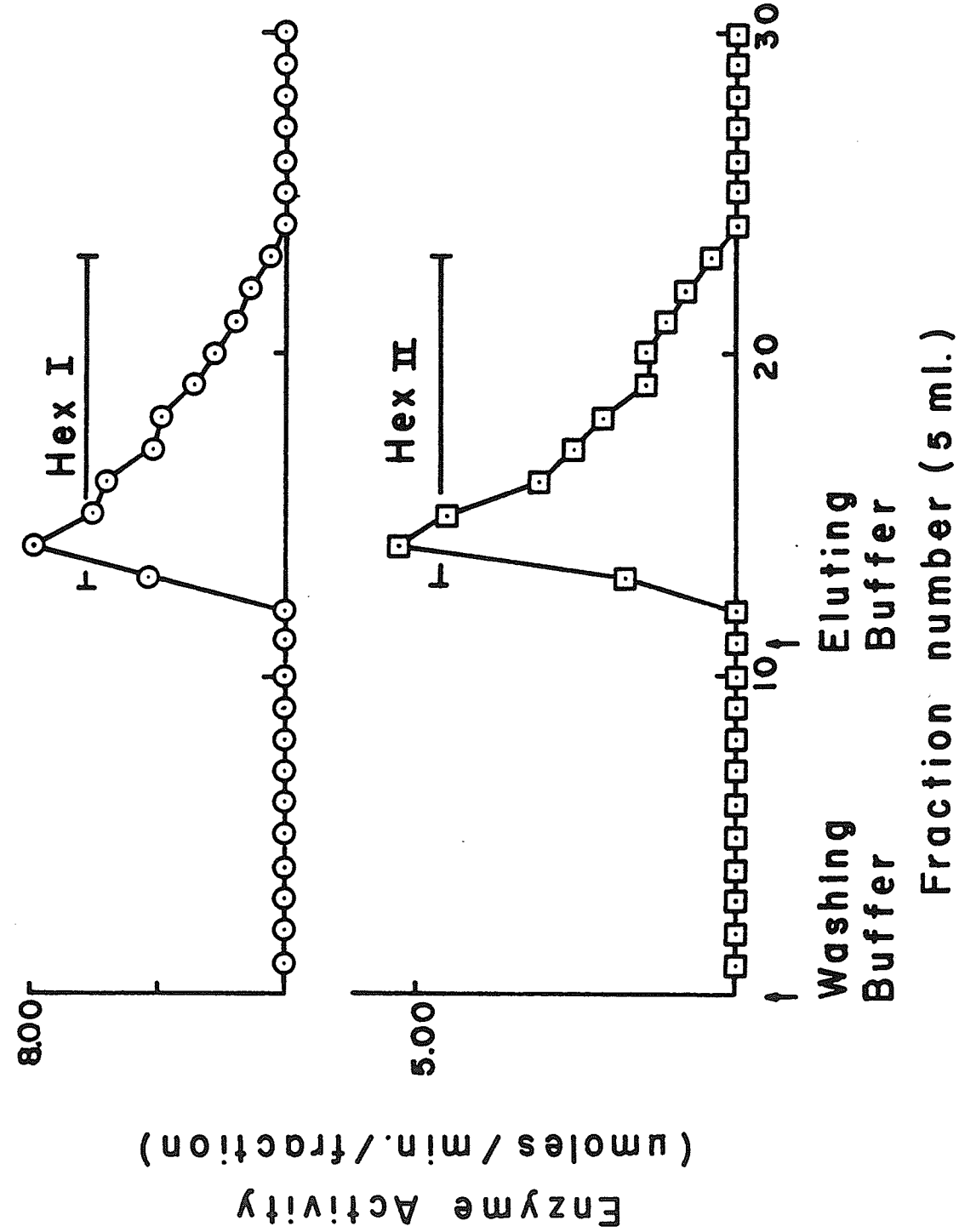

2.00
Absorbance
(280 nm)
Hex II
50.0
1.0
Enzyme Activity
(umoles/min./fraction)
NaCl (mM)
50
Fraction number (2.5 ml.)

Figure 49. Affinity chromatography of hexosaminidases I (⊙), and II (⊡) on concanavalin A-Sepharose 4B.


Enzyme Activity
(umoles / min. / fraction)
8.00
5.00
Hex I
Hex II
10
20
30
Washing Buffer
Eluting Buffer
Fraction number (5 ml.)

DEAE-cellulose chromatography was applied to a Sephadex G-200 column. This purification step was only slightly successful, as the enzyme activity was not clearly resolved from the majority of the protein (Figure 50). The fractions containing Hex III activity were pooled and rechromatographed on DEAE-cellulose. The Hex III was eluted from the column at an NaCl concentration of 150-220 mM (Figure 51). The elution pattern contained six peaks of activity. Hexosaminidase III did not bind concanavalin A-Sepharose 4B. The purification procedures are summarized in Table VII. Hexosaminidases I, II, and III were purified 30-fold, 33-fold, and 19-fold, respectively. Although the homogeneity of the final enzyme preparations could not be determined due to the low yields; 99.8%, 99.9%, and 99.6% of the starting protein was removed during the purification of Hex I, Hex II, and Hex III, respectively.

Analysis of the W.T. CO_2 and C^R-7 membrane glycopeptides

In the introduction, a hypothesis concerning the relationship between membrane glycoprotein changes and alterations in the levels of glycosidases was proposed. Differences between the specific activities of the W.T. CO_2 and C^R-7 glycosidases have been documented in this section. According to the hypothesis, the W.T. CO_2 and C^R-7 membrane glycoproteins should also differ. This corollary was investigated by analyzing the W.T. CO_2 and C^R-7 membrane glycopeptides. The W.T. CO_2 and C^R-7 ^3H-glucosamine labelled membrane glycopeptides were chromatographed on concanavalin A-Sepharose 4B (Figures 52, 54, 56, and 58). This procedure separated the glycopeptides into two classes: concanavalin A binding glycopeptides

TABLE VII

Purification of W.T. CO_2 hexosaminidase forms.

Purification Step		Total Enzyme Activity (units)	Total Protein (mg)	Specific Activity (units/mg protein)	Purification (- fold)	Yield (%)
Supernatant	Hex I & II	845	300	2.82	-	100
	Hex III	810		2.71		
Hex I						
DEAE-cellulose		419	43	9.79	3.5	50
Concanavalin A		39	0.45	86.6	31	4.6
Hex II						
CM-cellulose		113	12	9.42	3.3	13
Concanavalin A		15	0.16	92.5	33	1.8
Hex III						
DEAE-cellulose		461	72	6.40	2.4	57
Sephadex G-200		293	22	13.3	4.9	36
DEAE-cellulose		60	1.14	52.5	19	7.4

Figure 50. Elution profile of hexosaminidase III chromatographed on Sephadex G-200.

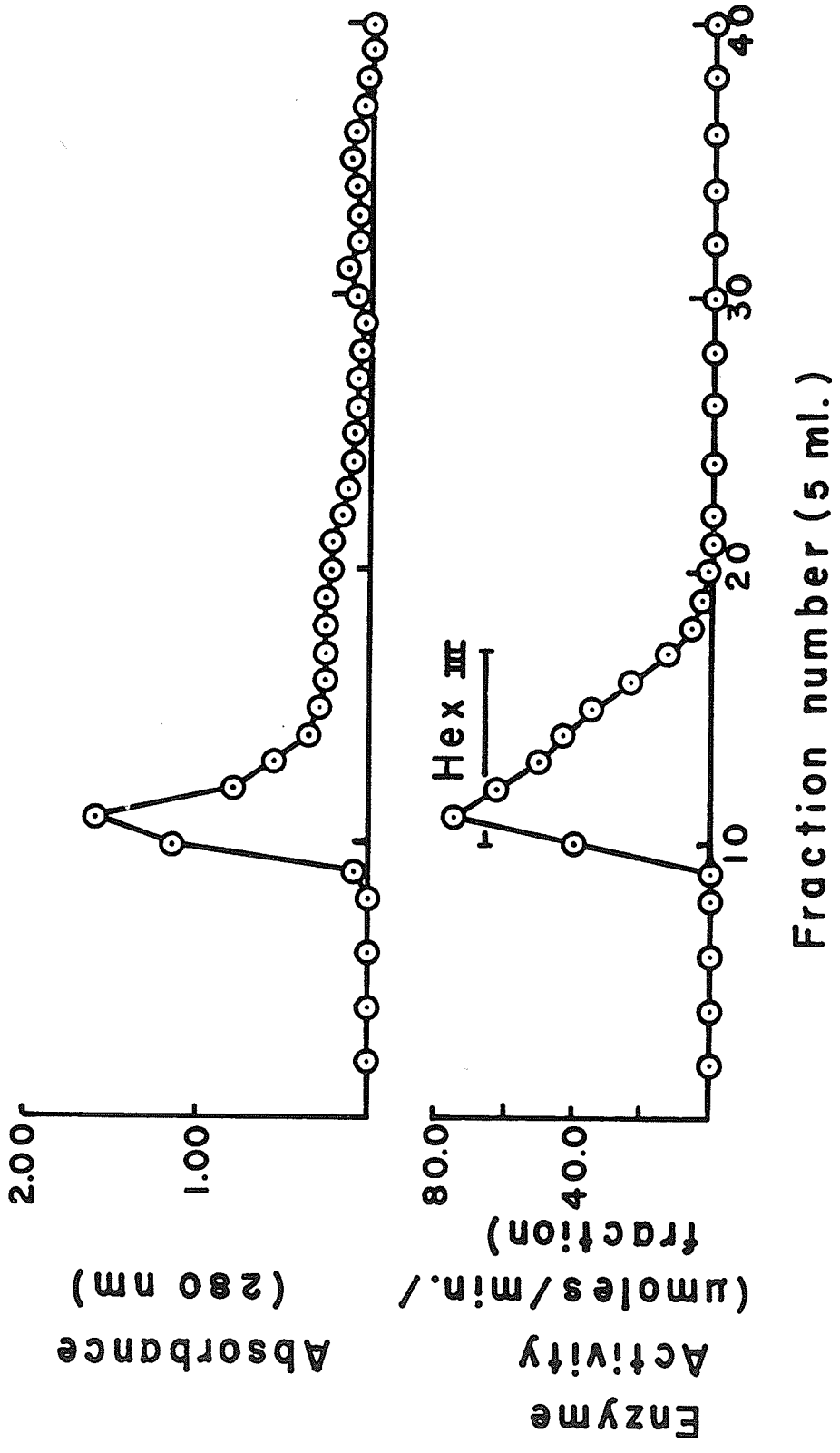
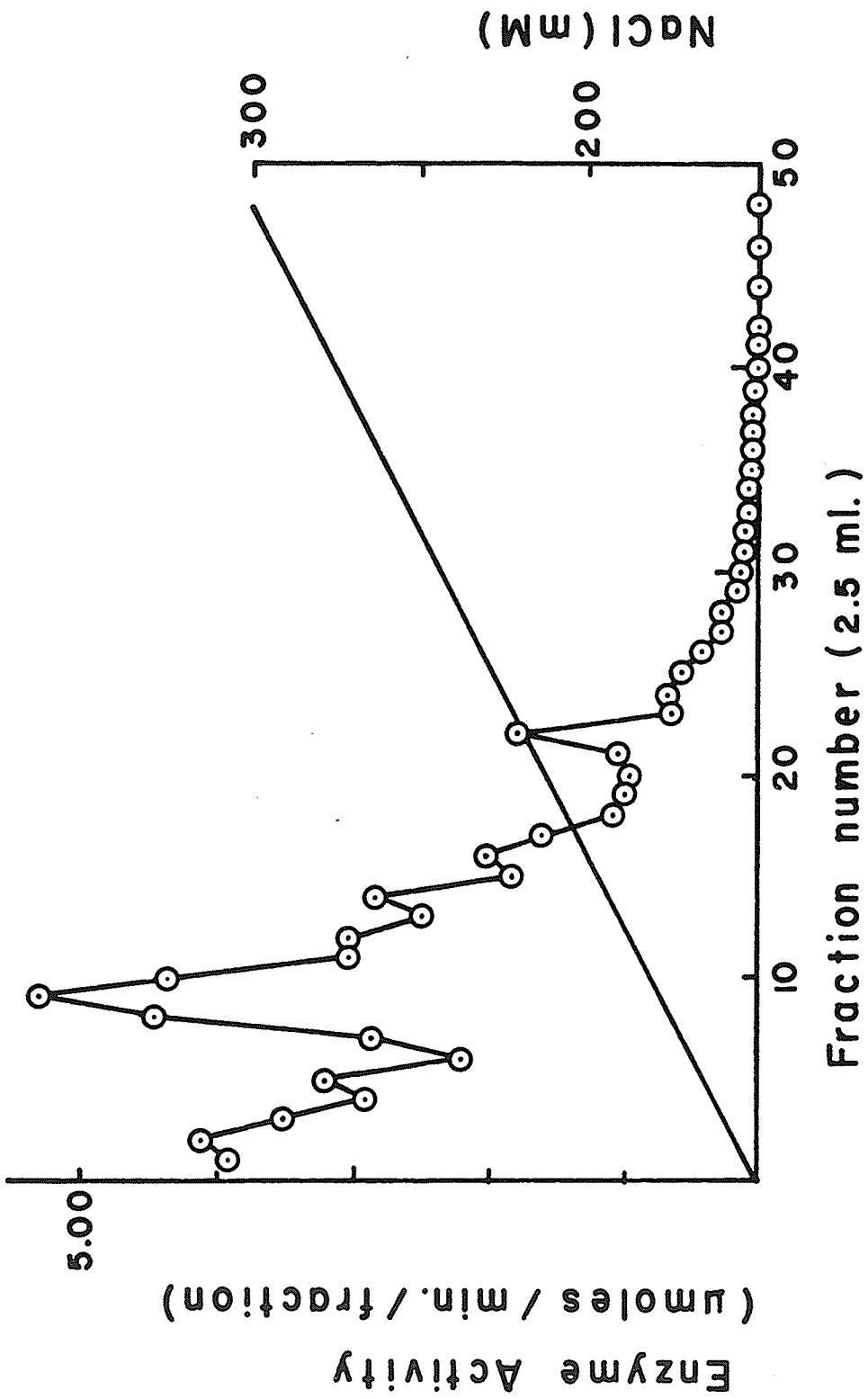

Absorbance
(280 nm)
2.00
1.00
Enzyme
Activity
(μmoles/min./
fraction)
80.0
40.0
Hex III
10
20
30
40
Fraction number (5 ml.)

Figure 51. Ion-exchange chromatography of hexosaminidase III on DEAE-cellulose.


Enzyme Activity
(µmoles / min. / fraction)
5.00
Fraction number (2.5 ml.)
10
20
30
40
50
NaCl (mM)
200
300

and concanavalin A non-binding glycopeptides. The W.T. CO_2 and C^R-7 concanavalin A binding and non-binding glycopeptides were chromatographed separately on either Sephadex G-50 or Sephadex G-75 to determine their molecular weight classes (Figures 53, 55, 57, and 59). The W.T. CO_2 and C^R-7 concanavalin A binding glycopeptides had similar elution patterns when chromatographed on either Sephadex G-50 or Sephadex G-75 indicating that both cell lines possess concanavalin A binding glycopeptides with similar molecular weights. The W.T. CO_2 and concanavalin A non-binding glycopeptides, when chromatographed on either Sephadex G-50 or G-75, exhibited elution patterns characterized by two peaks, A and B (Figures 53 and 57), while the C^R-7 concanavalin A non-binding glycopeptide elution patterns had only a single pronounced peak, B (Figures 55 and 59). Apparently, the W.T. CO_2 concanavalin A non-binding glycopeptides were composed of a class of high molecular weight glycopeptides that was either reduced, or not present, in the C^R-7 concanavalin A non-binding glycopeptide mixture.

Figure 52. Elution profile of W.T. CO_2 glycopeptides chromatographed on concanavalin A-Sepharose 4B. The concanavalin A binding and non-binding glycopeptides were analyzed separately by gel filtration on Sephadex G-50.

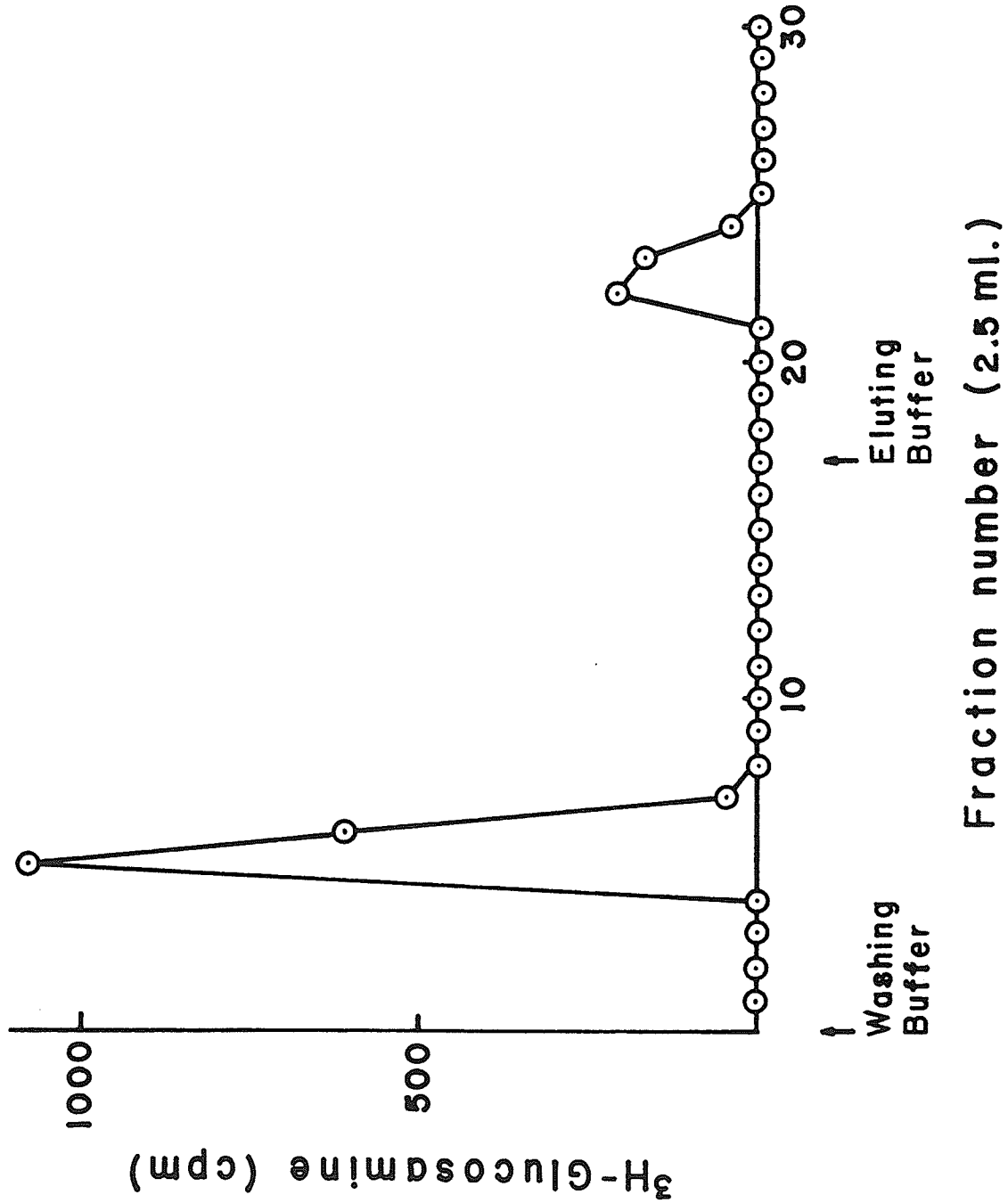

1000
500
^{3}H-Glucosamine (cpm)
10
20
30
Fraction number (2.5 ml.)
Washing Buffer
Eluting Buffer

Figure 53. Elution profiles of W.T. CO_2 concanavalin A binding (⊡), and non-binding (⊙) glycopeptides chromatographed on Sephadex G-50. Dextran blue eluted in the fraction indicated by the letter D.

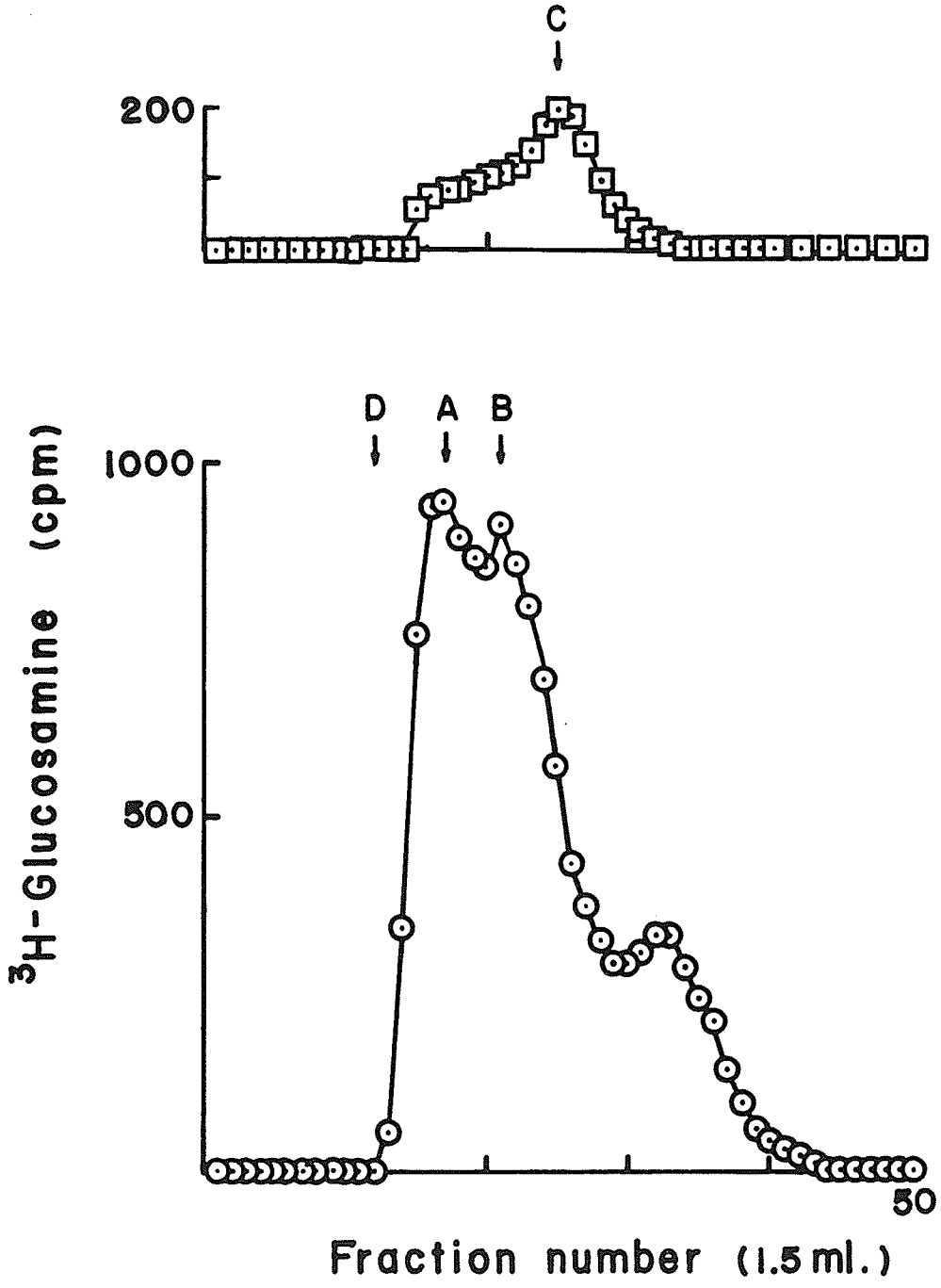

C
200
D
A
B
1000
500
^{3}H-Glucosamine (cpm)
50
Fraction number (1.5 ml.)

Figure 54. Elution profile of C^R-7 glycopeptides chromatographed on concanavalin A-Sepharose 4B. The concanavalin A binding and non-binding glycopeptides were analyzed separately by gel filtration on Sephadex G-50.

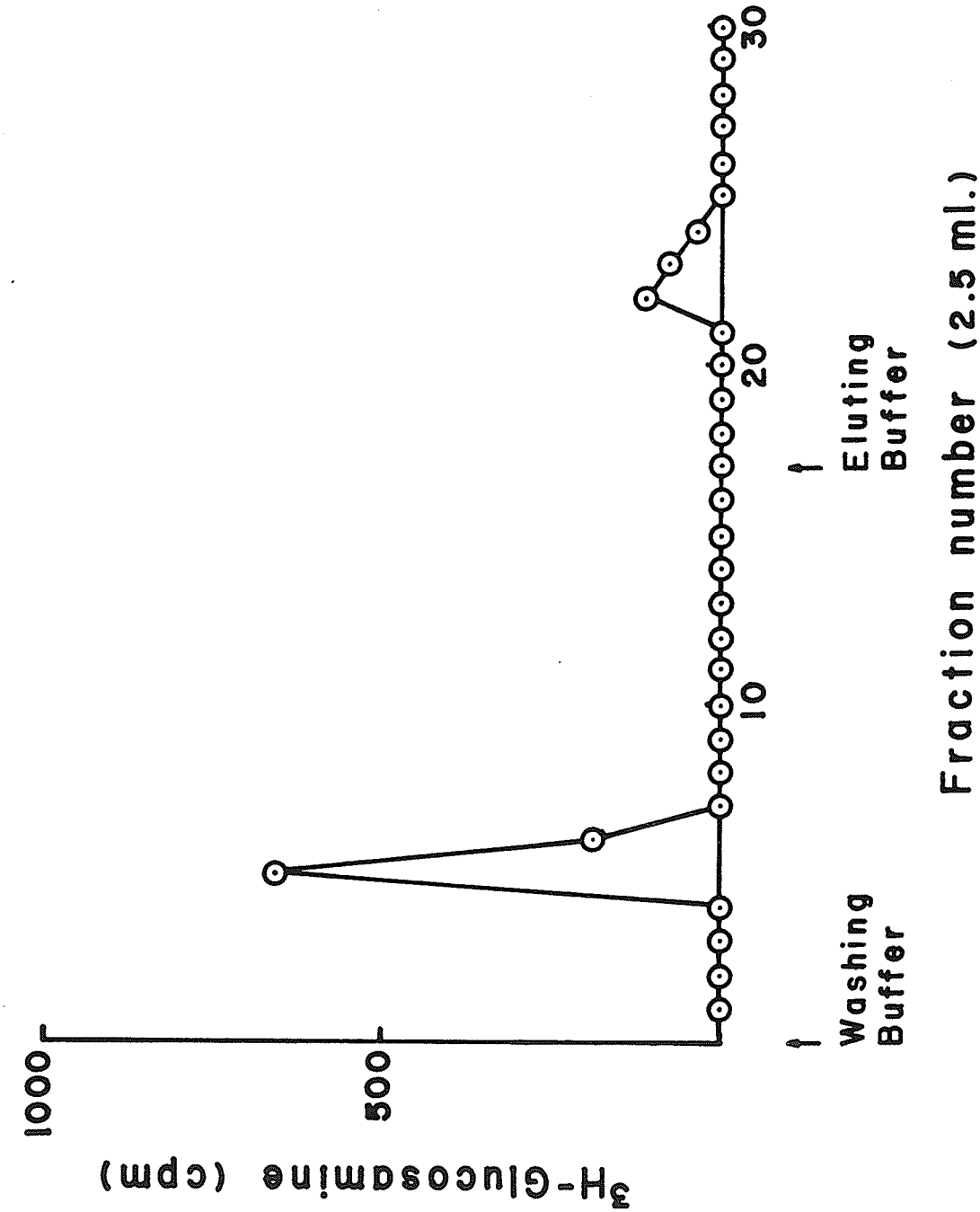

1000
500
^{3}H-Glucosamine (cpm)
10
20
30
Fraction number (2.5 ml.)
Washing Buffer
Eluting Buffer

Figure 55. Elution profiles of C^{R}-7 concanavalin A binding (⊡) and non-binding (⊙) glycopeptides chromatographed on Sephadex G-50. Dextran blue eluted in the fraction indicated by the letter D.

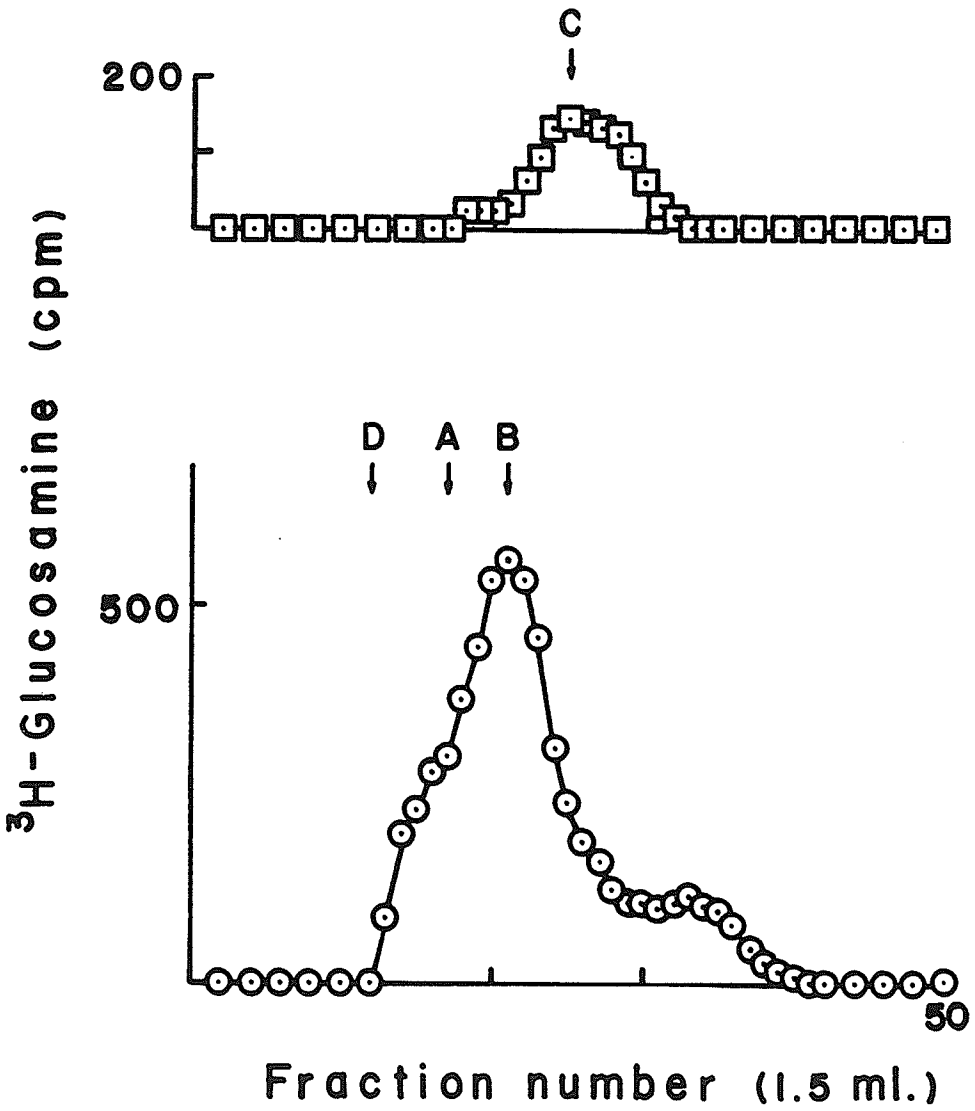

C
200
D
A
B
500
^{3}H-Glucosamine (cpm)
50
Fraction number (1.5 ml.)

Figure 56. Elution profile of W.T. CO_2 glycopeptides chromatographed on concanavalin A-Sepharose 4B. The concanavalin A binding and non-binding glycopeptides were analyzed separately by gel filtration on Sephadex G-75.

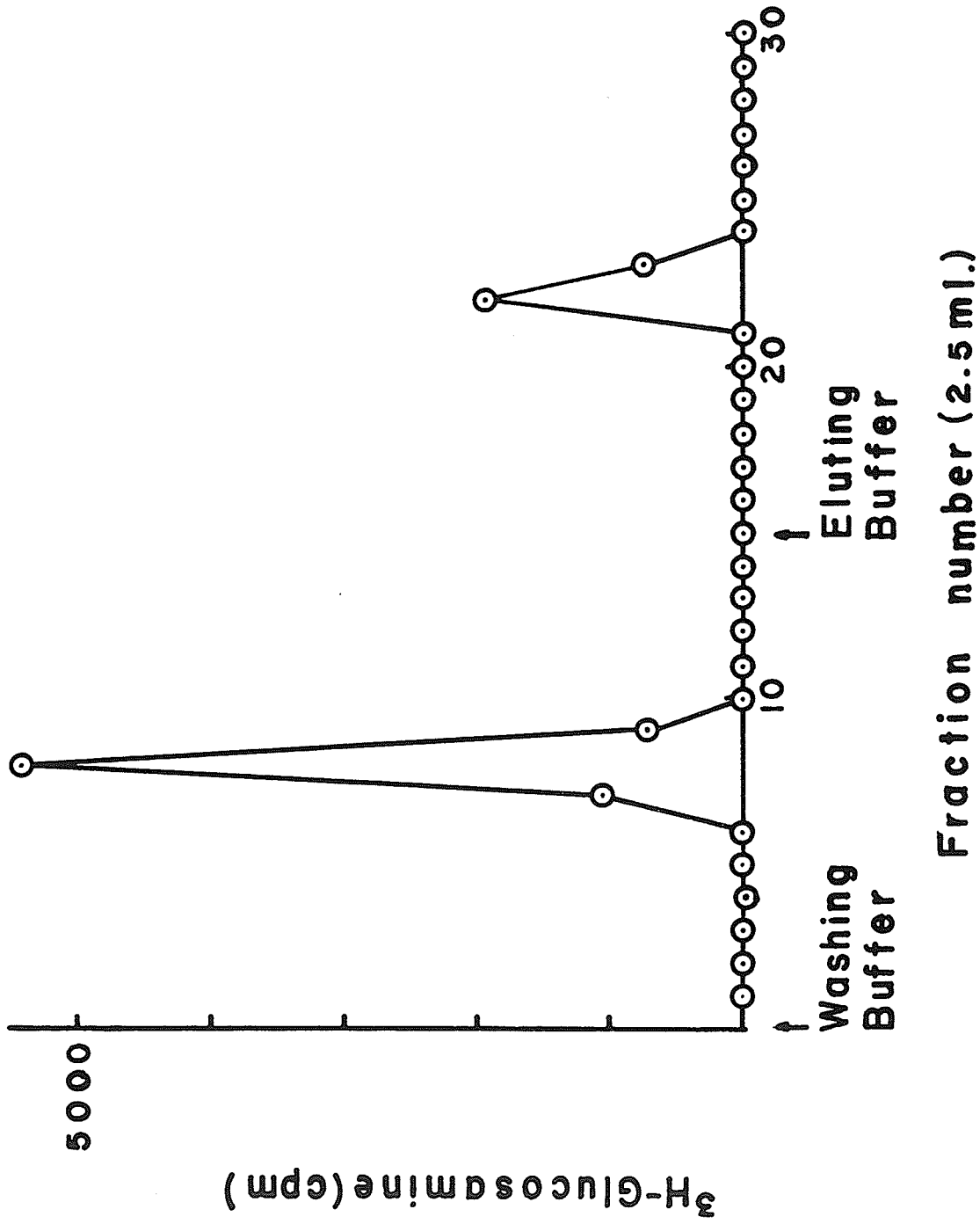

5000
^{3}H-Glucosamine (cpm)
Washing Buffer
Eluting Buffer
10
20
30
Fraction number (2.5 ml.)

Figure 57. Elution profiles of W.T. CO_2 concanavalin A binding (⊡) and non-binding (⊙) glycopeptides chromatographed on Sephadex G-75. Dextran blue eluted in the fraction indicated by the letter D.

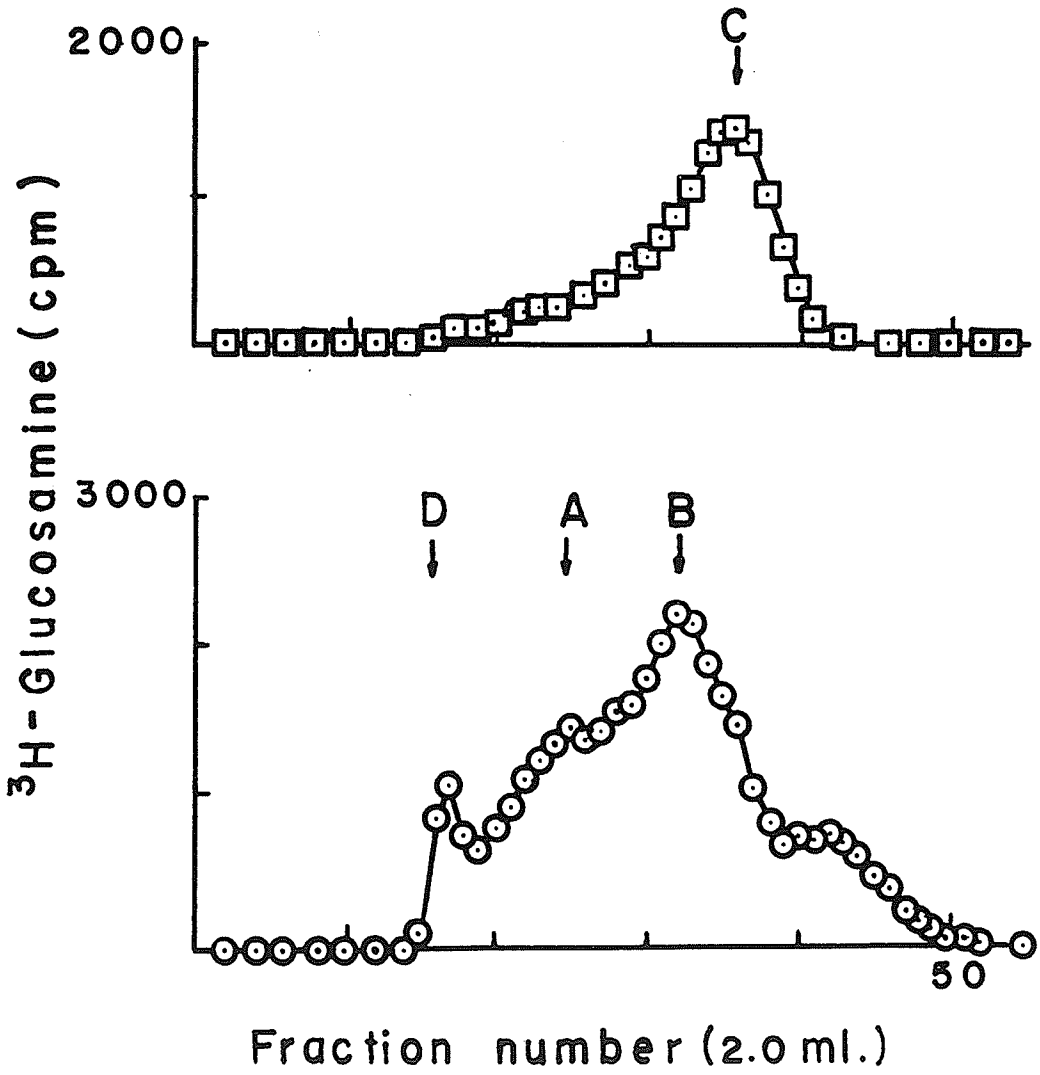

2000
C
3000
D
A
B
50
^{3}H-Glucosamine (cpm)
Fraction number (2.0 ml.)

Figure 58. Elution profile of C^R-7 glycopeptides chromatographed on concanavalin A-Sepharose 4B. The concanavalin A binding and non-binding glycopeptides were analyzed separately by gel filtration on Sephadex G-75.

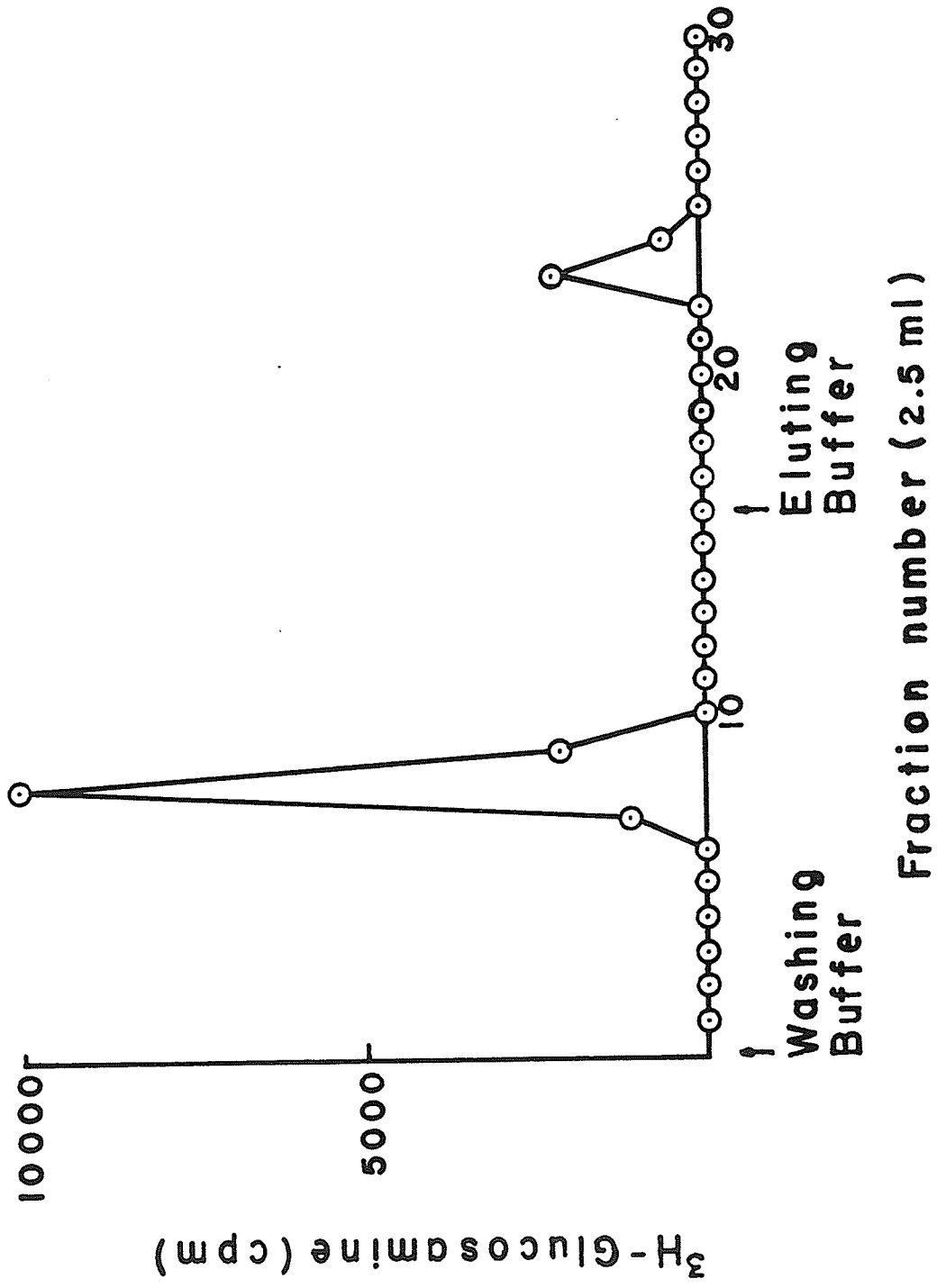
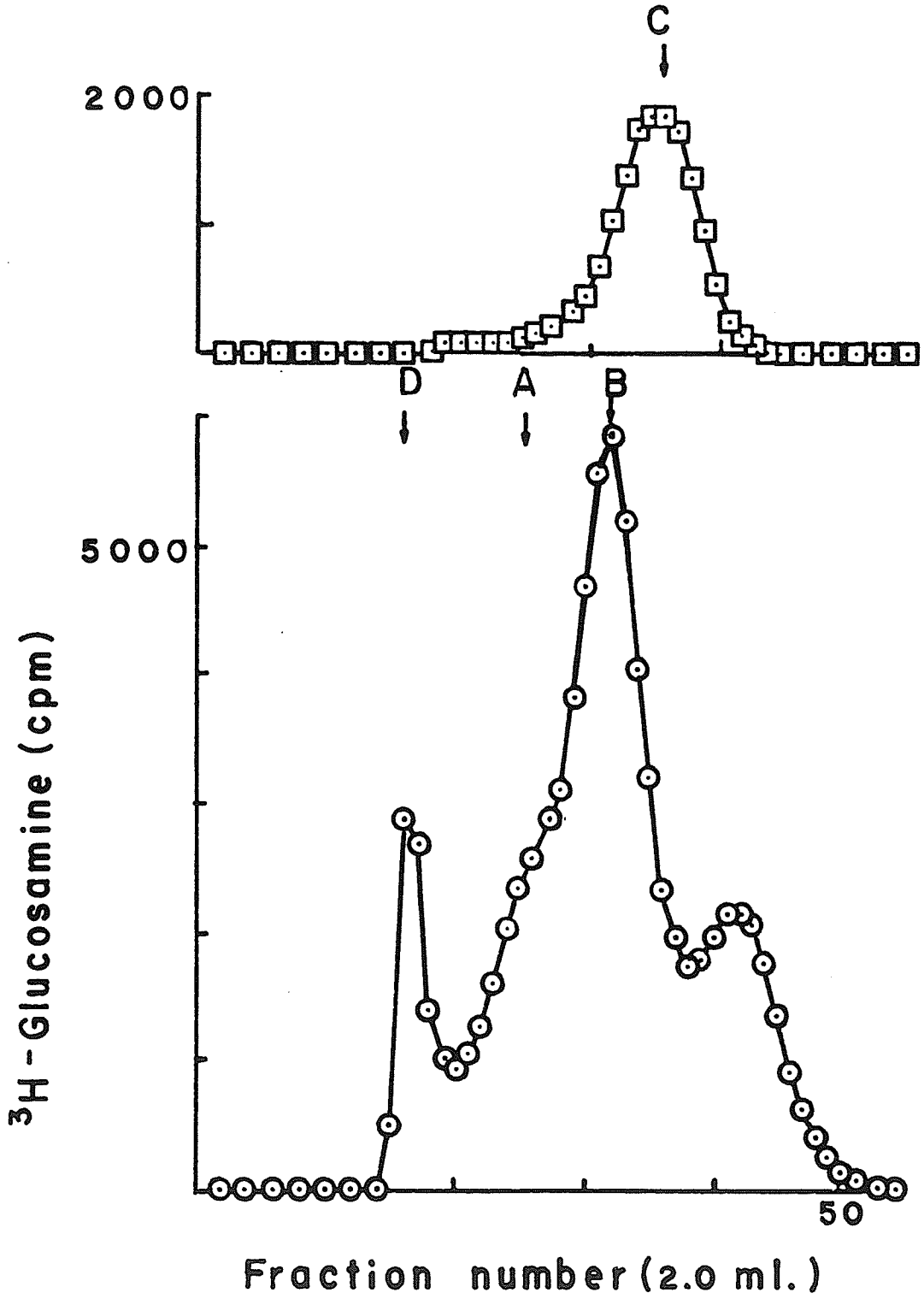

10000
5000
^{3}H-Glucosamine (cpm)
Washing Buffer
Eluting Buffer
10
20
30
Fraction number (2.5 ml)

Figure 59. Elution profiles of C^R-7 concanavalin A binding (⊡) and non-binding (⊙) glycopeptides chromatographed on Sephadex G-75. Dextran blue eluted in the fraction indicated by the letter D.


C
2000
D
A
B
5000
^{3}H-Glucosamine (cpm)
50
Fraction number (2.0 ml.)

DISCUSSION

DISCUSSION

The glycoproteins and glycolipids of normal cells change drastically upon viral or neoplastic transformation (1, 2, 63-71). These glycoprotein and glycolipid alterations are accompanied by increased glycosidase levels (72-75, 98, 99). The increased glycosidase levels in virally transformed cells are thought to cause the modification of the oligosaccharides located in peripheral regions of the cellular membrane (76, 104). The modification of cell surface components produces the heightened sensitivity of virally transformed cells to agglutination by the lectin, concanavalin A (1, 71, 76, 85). In this study, the glycosidase levels of two concanavalin A-sensitive cell lines, W.T. CO_2 and RC^R-7, were compared with the glycosidase levels of a concanavalin A-resistant cell line, C^R-7. Previous studies have shown that the C^R-7 cells were approximately 2.5 times more resistant to the cytotoxic effects of concanavalin A than the W.T. CO_2 and RC^R-7 cells (88). The C^R-7 cells, in comparison with the W.T. CO_2 and RC^R-7 cells, also exhibited altered cell surface glycoproteins (91).

The major glycosidases present in the cells examined were the hexosaminidases; their specific activities in Triton X-100 extracts being greater than the specific activities of α-L-fucosidase and α-D-mannosidase (Table III). The specific activities of hexosaminidases I and II in Triton X-100 extracts of W.T. CO_2 and RC^R-7 cells grown to

confluence, or in suspension, were approximately the same. In comparison with the specific activities of the hexosaminidases in extracts of C^R-7 cells, the W.T. CO_2 and RC^R-7 hexosaminidase levels were twice as great. Bosmann has made similar observations working with virally transformed 3T3-Va cells, an established cell line of embryonic mouse fibroblasts (Table VIII). The major glycosidase in these cells was also a hexosaminidase. The 3T3-Va cells were transformed by either DNA tumor viruses (polyoma virus, simian virus) or RNA tumor viruses (murine sarcoma virus, Rous sarcoma virus). The specific activities of the hexosaminidase in extracts of transformed cells were found to be at least two-fold greater than the specific activities of the enzyme in extracts of the untransformed 3T3-Va cells (74, 98).

The specific activities of α-L-fucosidase in extracts of confluent W.T. CO_2 and RC^R-7 cells were the same (Table III). These specific activities were elevated four times above the specific activity of α-L-fucosidase in extracts of confluent C^R-7 cells. Bosmann reported that there was no α-D-fucosidase activity in 3T3-Va cells, but found the specific activity of β-D-fucosidase in extracts of RNA tumor virus transformed 3T3-Va cells to be three times greater in comparison to the specific activity of β-D-fucosidase in extracts of normal 3T3-Va cells (98).

The α-D-mannosidase activity in extracts of confluent W.T. CO_2 C^R-7, and RC^R-7 cells was minimal. Only trace amounts of activity

TABLE VIII

Specific activities[1] of glycosidases in extracts of normal and transformed 3T3-Va cells.

	Cell line		
Enzyme[2]	3T3-Va	SV-3T3	PY-3T3
N-acetyl-β-D-glucosaminidase	520±36	1350±50	1270±21
α-D-mannosidase	90±2	156±4	138±8

	Cell line		
Enzyme[3]	3T3-Va	MSV-3T3	RSV-3T3
N-acetyl-β-D-glucosaminidase	424±21	943±81	2392±108
β-D-fucosidase	22±1	63±2	68±4
α-D-mannosidase	95±3	208±18	108±11

[1] nmoles/hr/mg protein
[2] taken from reference 98
[3] taken from reference 74.

were detected (Table III). Bosmann reported a slight increase in α-D-mannosidase activity in the transformed 3T3-Va cells; the most significant increase in α-D-mannosidase activity occurring when the cells were transformed by murine sarcoma virus (Table VIII).

A general trend can be observed by comparing the W.T. CO_2, RC^R-7 and C^R-7 cell lines with the untransformed and virally transformed 3T3-Va cell lines. The C^R-7 and 3T3-Va cells possess depressed glycosidase levels when compared with the W.T. CO_2, RC^R-7, and virally transformed 3T3-Va cells. The C^R-7 cells seem to reflect the untransformed cellular state while the W.T. CO_2 and RC^R-7 cells approximate the transformed cellular state. This analogy can be strengthened by examining the agglutination of the cells by concanavalin A. The C^R-7 and 3T3 cell lines are not readily agglutinated by concanavalin A, while the W.T. CO_2, RC^R-7, and SV 3T3 cells are comparatively sensitive to agglutination by the lectin (Table IX). Since concanavalin A specifically binds α-D-mannopyranosyl residues (82, 83), the increased sensitivity of the W.T. CO_2, RC^R-7 and SV3T3 cells to the lectin may indicate that all three cell lines have undergone the same fundamental change in their membrane receptors. The increased glycosidase levels in these cells might be responsible for the modification of the cell surface receptors, allowing them to interact with concanavalin A. The process of cell surface modification by the glycosidases has been referred to as sublethal autolysis (74, 75, 101).

The glycosidase levels in W.T. CO_2, RC^R-7, and C^R-7 cells grown

TABLE IX

Cell agglutination in the presence of concanavalin A.

Cell Line	Concanavalin A Concentration (μg/ml)					
	0	50	250	500	750	1000
W.T. CO_2[1]	-	-	++	++++	++++	++++
C^R-7	-	-	-	++	+++	++++
RC^R-7	-	-	++	++++	++++	++++
	0	62.5	125	250	500	1000
3T3[2]	-	-	-	-	-	-
SV3T3[3]	-	++	+++	++++	++++	++++

1 Taken from reference 88
2 Taken from reference 100
3 Simian virus transformed 3T3 cells

at low cell densities (subconfluence) were approximately the same, although the specific activities of hexosaminidase I and II in extracts of the C^R-7 cells were elevated 1.5-fold when compared to the hexosaminidase specific activities in extracts of W.T. CO_2 and RC^R-7 cells. The differences in the glycosidase levels of the three cell lines were readily discernable in confluent cells, or in cells grown in suspension. The specific activities of the glycosidases in the three cell lines were responsive to the growth conditions, the notable exception being the W.T. CO_2 and RC^R-7 hexosaminidase I and II levels, which remained constant regardless of growth conditions (Table III). Three significant changes in the glycosidase levels occurred as the cell density increased: the specific activities of hexosaminidases I and II in extracts of C^R-7 cells decreased three-fold, the α-L-fucosidase levels increased drastically in the W.T. CO_2 and RC^R-7 cells, but only slightly in the C^R-7 cells, and the α-D-mannosidase levels were reduced to a minimum in all three cell lines. In general, the specific activities of the glycosidases in extracts of confluent C^R-7 cells were low in comparison to the specific activities of the glycosidases in extracts of subconfluent C^R-7 cells and confluent W.T. CO_2 and RC^R-7 cells. A possible explanation for this reduction of the glycosidase levels in confluent C^R-7 cells is that the confluent cells were susceptible to contact inhibition and were growing at a slower rate in comparison to the subconfluent C^R-7 cells. Confluent W.T. CO_2

and RC^R-7 cells, being similar to virally transformed cells, would grow at a faster rate than confluent C^R-7 cells, as transformed cells are less sensitive to contact inhibition (102). A difference in the growth rates of the W.T. CO_2, RC^R-7 and C^R-7 confluent cells would explain the lower specific activities of the glycosidases in confluent C^R-7 cells.

Further evidence in support of the idea that the glycosidase levels were responsive to growth conditions was the resulting increase in the specific activities of hexosaminidase III in W.T. CO_2, RC^R-7, and C^R-7 cells shifted from growth in dishes to growth in suspension (Table III). The mechanism, or mechanisms, controlling this increase in hexosaminidase III levels is unknown and remains to be investigated.

The preliminary survey of the hexosaminidases in Triton X-100 extracts of the hamster cells resulted in several interesting observations: the hamster cells contained at least two hexosaminidase forms; confluent C^R-7 cells, in comparison to confluent W.T. CO_2 cells, were deficient in hexosaminidases I and II, and C^R-7 cells grown in suspension exhibited exaggerated levels of hexosaminidase III activity. The causes and consequences of these variations in hexosaminidase activity were unknown, and formed the basis of investigation for the remainder of this section of the study.

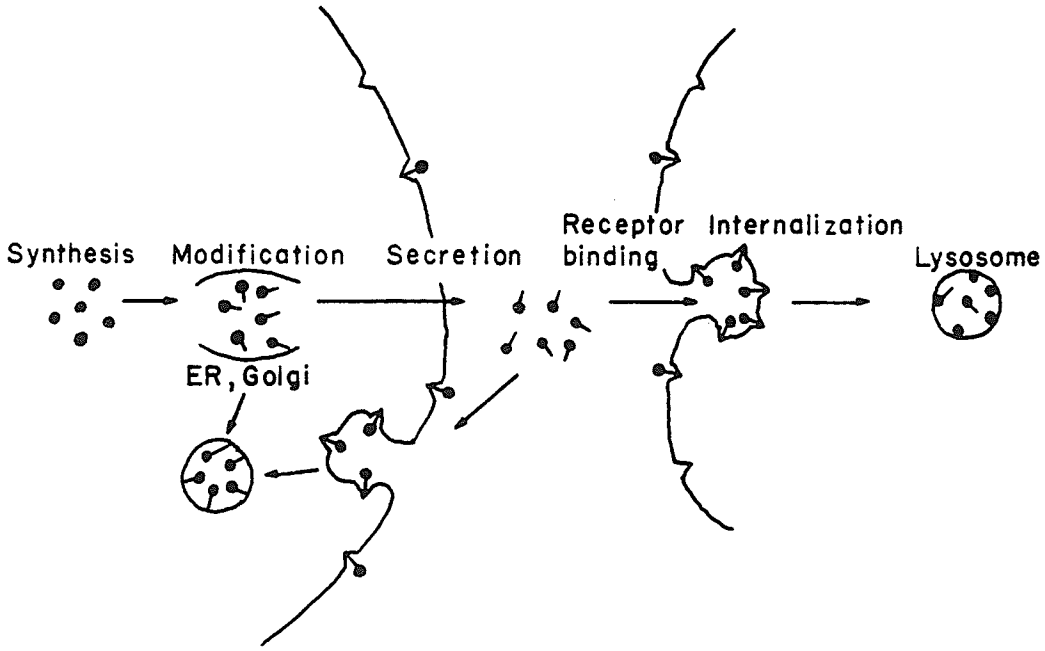
The hexosaminidase forms in human organs have been extensively investigated, as their deficiency results in many lysosomal diseases (103). The standard procedure for isolation of hexosaminidase involves

separating the hexosaminidase forms by ion-exchange chromatography (104). This procedure was applied to sonicated extracts of W.T. CO_2 and C^R-7 cells. The cells were shown to contain three hexosaminidase forms (Figures 28-31). The hexosaminidase forms in the media were also analyzed. The cells secreted only two hexosaminidase forms (I and III); no hexosaminidase II activity was found in the media (Figures 32-35). There was no difference between the specific activities of the W.T. CO_2 and C^R-7 intracellular hexosaminidase II forms, but the specific activities were two-fold greater in cells grown in bottles when compared with cells grown in suspension (Table V). The main difference between the two cell lines was in their intracellular hexosaminidase I levels. The specific activity of the intracellular hexosaminidase I in W.T. CO_2 cells was twice that of the C^R-7 intracellular hexosaminidase I. This situation was reversed in the media, the C^R-7 cells secreting 1.5 times as much hexosaminidase I as the W.T. CO_2 cells (Table V). The hexosaminidase III levels of W.T. CO_2 and C^R-7 cells grown in suspension demonstrated the same balance between intracellular and extracellular enzyme levels. The specific activity of the C^R-7 intracellular hexosaminidase III was 1.7-fold higher than that of the W.T. CO_2 intracellular hexosaminidase III, while the specific activity of the W.T. CO_2 extracellular hexosaminidase III was 1.7-fold higher than the specific activity of the C^R-7 extracellular hexosaminidase III (Table V). These results can be explained by taking into account the postulated life cycle

of lysosomal enzymes.

The life cycle of lysosomal enzymes is shown in Figure 60 and was originally proposed by Hickman and Neufeld in 1972 to explain I-cell disease or mucolipidoses II (106). Fibroblasts (I-cells) cultured from the skin of patients with mucolipidoses II were found to lack the lysosomal enzymes β-galactosidase, α-L-fucosidase, and arylsulfatase A, and had reduced levels of α-mannosidase, α-L-iduronidase, N-acetyl-β-glucosaminidase, and β-glucuronidase (106). These enzymatic deficiencies were not due to a lack of synthesis, since elevated levels of several of the enzymes were found in the culture medium of the fibroblasts (103). The I-cells appeared to have a membrane defect which caused the lysosomal enzymes to leak out (107). Hickman and Newfeld tested this hypothesis by adding bovine β-glucuronidase to the culture medium of I-cells, normal human fibroblasts, and β-glucuronidase deficient fibroblasts, and measuring the retention of this enzyme by the three cell lines. The results obtained showed the I-cells to be considerably more retentive of the enzyme than the two other cell lines (106). The I-cells did not have a membrane defect. Hickman and Neufeld then hypothesized that the intracellular enzyme deficiency of the I-cells was due to a defect in the enzymes. To test this hypothesis, they measured the uptake of N-acetyl-β-D-glucsaminidase derived from normal cells and I-cells, by fibroblasts derived from a patient with the "o" variant of Tay-Sachs disease. These Tay-Sachs fibroblasts lacked both A and B

Figure 60. Life cycle of lysosomal enzymes (105). The glycosidases are represented by the symbol ? . Details of the life cycle are given in the text.


Synthesis
Modification
ER, Golgi
Secretion
Receptor binding
Internalization
Lysosome

isoenzymes of hexosaminidase (106). The experiment showed that the enzyme derived from normal cells was readily absorbed by the Tay-Sachs fibroblasts while the uptake of the enzyme derived from the I-cells was minimal. Hickman and Neufeld proposed that the lysosomal enzymes were secreted by cells and then taken up by adjacent cells and incorporated into the lysosomes. The uptake was thought to involve recognition sites on the enzymes and receptor sites on the cell surface (106). In I-cell disease, the recognition sites on the enzymes appeared to be altered. The disease was not due to an enzyme deficiency, but rather to the inability of the cells to take up the enzymes and incorporate them into the lysosomes (103).

Evidence that lysosomal glycosidases have specific recognition sites which mediate their uptake has emerged only recently. These recognition sites are now believed to contain phosphorylated carbohydrate moieties (108, 109). This postulate is entirely feasible since glycosidases are glycoproteins (110, 111). Kaplan et al. studied the uptake of β-glucuronidase by human fibroblasts (108). They found that the uptake could be inhibited by yeast mannans, phosphorylated sugars, and sugars. The phosphorylated sugar, D-mannose-6-phosphate, was a more potent inhibitor than either D-mannose-1-phosphate or D-mannose. Treatment of β-glucuronidase with alkaline phosphatase was found to abolish uptake of the enzyme, but not its catalytic activity. The conclusion reached was that the enzyme's recognition site involved a phosphorylated D-mannose-type carbohydrate. Ullrich et al. strengthened this conclusion by

demonstrating the inhibition of ∝ and β-N-acetylglucosaminidase and α-mannosidase uptake by mannose-6-phosphate (109). Treatment of these enzymes with alkaline phosphatase also abolished their uptake by the human fibroblasts.

The observations discussed above can readily be applied to the results obtained in this thesis. According to Figure 60, there are two pathways the glycosidases can follow once they have been synthesized: the first pathway involves secretion by the cell into the medium and the second involves direct compartmentalization into the lysosomes. If the glycosidases are secreted, to re-enter the cell, they must bind a cell surface receptor via their recognition sites. No hexosaminidase II activity was found in the cellular medium (Tables IV and V). This observation may indicate that the enzyme is not secreted by the hamster cells, but transferred directly to the lysosomes, presumably from the Golgi complex. Hexosaminidases I and III were secreted into the cellular medium (Tables IV and V). The uptake of these enzymes by the cells, according to the hypothesis, is contingent upon two requirements: the enzymes must have the proper recognition sites, and the cells must have the proper surface receptors. If either of these are altered, the enzymes will not be internalized. The C^R-7 cells, whether they were grown in suspension or in bottles, had reduced specific activities of the intracellular hexosaminidase I in comparison to the W.T. CO_2 cells, but in the medium the specific activities of the C^R-7 hexosaminidase I were elevated in

comparison to those of the W.T. CO_2 hexosaminidase I. These observations suggest that the C^R-7 hexosaminidase I was incapable of being internalized by the C^R-7 cells due to a defect in the enzyme's recognition site or a defect in the cells' surface receptors. The specific activity of the intracellular hexosaminidase III in W.T. CO_2 cells grown in suspension was 1.7-fold lower than the specific activity of the intracellular hexosaminidase III in C^R-7 cells. The specific activity of the W.T. CO_2 extracellular hexosaminidase III was 1.7-fold higher than the specific activity of the C^R-7 extracellular hexosaminidase III. These observations also lead to speculation concerning the W.T. CO_2 cells' ability to internalize the extracellular hexosaminidase III.

The data presented in Table V areindicative of the differences between not only the two cell lines, but also the three hexosaminidase forms. The specific activities of hexosaminidase I remained constant in both cell lines, regardless of the growth conditions. In comparison, the specific activities of hexosaminidases II and III changed when the cells were shifted from growth in bottles to growth in suspension. The hexosaminidase II levels decreased by about a half, while the specific activities of hexosaminidase III increased 10-30-fold. Hexosaminidase III was apparently regulated by a different mechanism than hexosaminidases I and II. Further differences between the three hexosaminidase forms are shown in Table X. The data establishes the identity of each hexosaminidase form, and shows that

TABLE X

Properties of hamster cell and human hexosaminidases

	Hexosaminidase					
	W.T CO_2			Human[1]		
	I	II	III	A	B	C
K_m (mM)[2]	0.890	0.637	0.370	0.590-0.830	0.690-0.700	?[3]
pH Optimum[2]	4.4	4.0	6.3	4.0-4.5	4.0-4.5	6.0-7.0
Thermostability	labile	stable	labile	labile	stable	labile
Sensitivity to Mn^{++}, Mg^{++}	-	-	+	-	-	?
Molecular Weight	?	?	?	$1.0-1.4X10^5$	$1.0-1.4X10^5$	$2.0X10^5$
Concanavalin A Affinity	+	+	-	+	+	?
DEAE-cellulose Affinity	++[4]	+	+++	++	+	++++
Secreted	yes	no	yes	yes	no	no

[1] Data obtained from references 53, 112-119.
[2] Substrate used was p-nitrophenyl-N-acetyl-β-D-glucosaminide.
[3] Not determined.
[4] + Eluted with 30 mM NaCl
++ Eluted with 150 mM NaCl
+++ Eluted with 300 mM NaCl
++++ Bound irreversibly to the ion-exchanger or denatured.

each hexosaminidase is a unique and clearly distinguishable enzyme.

The hamster cell hexosaminidase forms are readily comparable to the hexosaminidase forms found in human tissues and fluids (Table X). The two major isozymes present in human tissues have been designated hexosaminidase A and hexosaminidase B (120). The two isozymes have been shown to differ in amino acid and carbohydrate composition (116). Hexosaminidase B was reported to contain no sialic acid, while hexosaminidase A contained 1.65 residues/mol of the enzyme (116). The presence or absence of sialic acid is thought to determine the behaviour of the hexosaminidases on DEAE-cellulose chromatographic columns (121). Since the discovery of hexosaminidases A and B, several other hexosaminidase forms have been identified. The hexosaminidase forms A, B, P, I, I_2, S_1, S_2 have all been shown to have pH optima in the range 4.0 - 5.0, while an eighth form, hexosaminidase C, has the unique pH optimum range of 6.0 - 7.0 (53, 113, 120, 122, 123). The isozymes listed above are thought to be interrelated, since they all have the ability to utilize p-nitrophenyl-N-acetyl-β-D-glucosaminide as a substrate. Structural and immunological studies of the two major isozymes have demonstrated that hexosaminidase A is a heteropolymer comprised of α and β chains $(\alpha\beta)_n$, while hexosaminidase B is a homopolymer consisting of β chains $(\beta)_n$ (116, 124, 125). Recently, Srivastava et al. have proposed that the subunit compositions of the hexosaminidase isozymes B, I_1, I_2, A, S_1, S_2 were β_6, $\beta_5\alpha_1$, $\beta_4\alpha_2$, $\beta_3\alpha_3$, $\beta_2\alpha_4$ and $\beta_1\alpha_5$, respectively (126).

Geiger and Arnon's finding, that the α subunits contained the sialic acid residues, correlates with this model, as hexosaminidase B, the homopolymer containing no sialic acid, has the lowest affinity for DEAE-cellulose chromatographic columns, while hexosaminidase S_2, being composed predominantly of sialic acid containing α subunits, has the highest affinity (116, 126).

The nature of hexosaminidase C remains a mystery. This enzyme has been shown to be present in extracts from embryonic tissues (liver, brain, and lung), normal adult brain, and neonatal spleen (118, 119). The unique nature of hexosaminidase C was suggested when the enzyme failed to interact with antisera raised against hexosaminidases A and B (118). Several other observations have been made that suggest hexosaminidase C is unrelated to hexosaminidases A and B. Patients suffering from Sandhoff's disease were found to have a complete deficiency of hexosaminidases A and B, but hexosaminidase C was present in their tissues (118). Hexosaminidase C was also present in a foetal Tay-Sachs brain, which was completely deficient in hexosaminidase A (118). Perhaps the most puzzling observation reported was the loss of hexosaminidase C activity on DEAE-cellulose chromatographic columns. The enzyme appeared to be either denatured or irreversibly bound to the ion-exchanger (118, 127). In view of the data presented, Braidman et al. (112) proposed that hexosaminidase C was under a separate genetic control from the other hexosaminidase forms. The data presented in Table V suggested that hexosaminidase III, which had similar properties to hexosaminidase C, was regulated by a

different mechanism than hexosaminidases I and II. The specific activities of hexosaminidase III changed drastically when the cells were grown in suspension as opposed to bottles. In contrast, the specific activities of hexosaminidase I did not change, indicating the lack of an interrrelationship between the two hexosaminidase forms.

The involvement of hexosaminidases A and B in the catabolism of gangliosides has been well documented (53). In view of the similarities between these isozymes and the hamster cell hexosaminidase forms (see Table X), the hypothesis that both sets of hexosaminidases catalyze the same reactions seems plausible. Studies have shown that hexosaminidases A and B are lysosomal hydrolases capable of catalyzing the hydrolysis of terminal N-acetylglucosaminyl or N-acetylgalactosaminyl moieties from glycolipids (GM_2-ganglioside, GA2-globoside) and polysaccharides (53, 128, 129). The hexosaminidases display a remarkable specificity towards natural substrates. Only hexosaminidase A is capable of hydrolyzing the GM_2-ganglioside. Hexosaminidase B is presumed to act on the GA2-globoside (128). The absence of one or both of these hexosaminidases causes Tay-Sachs disease (53). There are three variants of this disease. Sandhoff's disease (also known as variant "o" of Tay-Sachs disease) is characterized by the absence of both hexosaminidase A and B in the brain of the patient (53). As a consequence of this deficiency, GM_2-ganglioside, asialo-GM_2-ganglioside, and GA2 globoside accumulate in the brain. In variant B of this

disease, only hexosaminidase A was found to be absent, and GA2 globoside did not accumulate (53). The third variant of Tay-Sachs disease, AB, is perhaps the most interesting. The activities of hexosaminidases A and B were shown to be substantially elevated when the isozymes were assayed using synthetic substrates, but when natural substrates were utilized, the isozymes were found to be only marginally active (53). Perhaps these enzymes have a defect in their amino acid composition which alters their catalytic activity (53). The glycolipids GM_2-ganglioside and asialo-GM_2-ganglioside accumulated in this instance (53).

The observations discussed above indicate the intimate relationship between the glycolipid content of tissues and the hexosaminidase levels. The differences between the specific activities of the C^R-7 hexosaminidases I and III and the W.T. CO_2 hexosaminidase forms could result in differences between the glycolipids of the two cell lines. The validity of this hypothesis is currently under investigation.

Hexosaminidases A and B have been purified from various human tissues (placenta, kidney cortex, brain)(104, 110, 116, 130, 131, 132). The purification procedure commonly used involves concanavalin A affinity chromatography, ion-exchange chromatography, and gel filtration (104, 116). Hexosaminidase C has not been successfully purified (112, 127, 133). The specific activities of hexosaminidases A and B in homogenates of various human tissues were substantially lower (42-128-fold) than the specific activities of hexosaminidases I and II in

extracts of the hamster cells (110, 130, 132). Consequently, the purification of hexosaminidases A and B from human tissues required a complex procedure in comparison to the simplified method used to purify hexosaminidases I, II and III (see Tables VII and XI). Hexosaminidases I, II, and III were purified 31-, 33- and 19-fold, respectively (Table VII). These values were low due to the high specific activities of the hexosaminidase forms in the starting material and were not indicative of the purification achieved (Table VII). Lee and Yoshida purified hexosaminidases A and B 4,300- and 6,000-fold respectively, but the combined specific activity of these two enzymes in the starting fraction was reduced 80-fold in comparison to the initial combined specific activity of hexosaminidases I and II (Tables VII and XI). The amount of contaminating protein removed from hexosaminidases I, II, and III during their purification was 99.8%, 99.9% and 99.6%, respectively (Table VII). These values give a better indication of the purification achieved. The homogeneity of the final hexosaminidase preparations was not determined due to the low yields, but undoubtedly, the enzymes were still impure. The purification procedure remains to be modified in order to increase the yield and the purity of the hexosaminidase forms.

At the beginning of this discussion, the C^R-7 and W.T. CO_2 cells were compared to untransformed and transformed cells. The W.T. CO_2 cells were shown to be similar to the transformed cells. Both of these cell types, in comparison to the C^R-7 and untransformed cells,

TABLE XI[1]

Purification of placental hexosaminidases A and B.

Fraction	Total enzyme activity (units)	Total protein (mg)	Specific activity (units/mg)	Purification (-fold)	Yield (%)
Homogenate	13,000	$373X10^3$	0.035		100
Supernatant	10,000	$280X10^3$	0.036	1	77
Concanavalin A	5,700	2650	2.2	63	44
Hexosaminidase A DE-22	4,000	660	6.1	174	31
QAE-Sephadex	2,400	230	10	290	19
Sephadex G-200	~ 2,000	57	35	1000	15
QAE-Sephadex	~ 2,000	46	44	1250	15
Preparative disc-gel electrophoresis	1,300	11	120	3400	10
QAE-Sephadex	750	5	150	4300	5.8
Hexosaminidase B DE-22	1,800	290	6.2	180	14
Calcium phosphate	1,100	85	13	370	5.2
Sephadex G-200	880	33	27	770	6.8
QAE-Sephadex	900	33	27	770	6.8
CM-52	300	1.45	207	6000	2.3

[1] Taken from reference 110.

exhibited increased glycosidase levels and an increased sensitivity to agglutination by the lectin, concanavalin A(Tables III, VIII, IX). The membrane glycolipids and glycoproteins of normal cells were found to be altered upon viral transformation (66-70). These changes were believed to be responsible for the increased sensitivity of transformed cells to agglutination by concanavalin A (1).

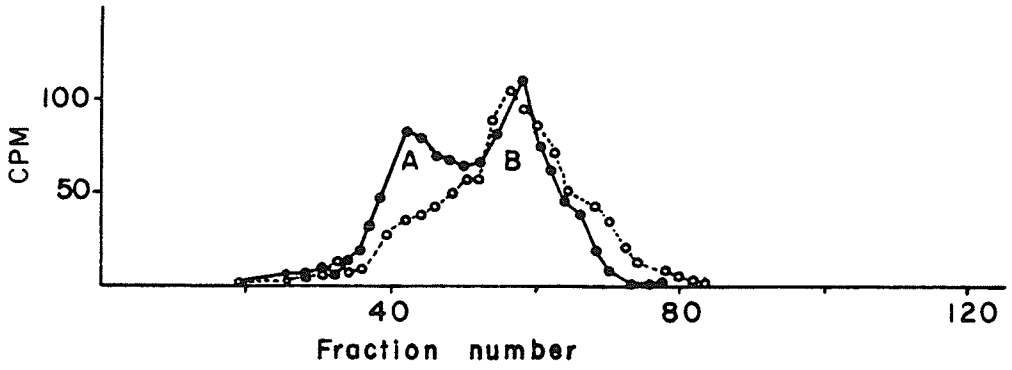
One of the procedures routinely used to examine the membrane glycoproteins of cells involves labelling the glycoproteins in vivo with radioactive sugars (97). The glycoproteins are then extracted from the cells and digested with pronase to yield a mixture of glycopeptides (97). These glycopeptides are essentially composed of oligosaccharide chains and a few amino acids (134). The glycopeptides are analyzed by gel filtration (Sephadex G-50) (66, 97, 134). Warren et al. used this procedure to analyze the glycoproteins of normal and virally transformed cells (134). The elution profiles of the glycopeptides derived from the normal and transformed cells were found to be different (Figure 61). The glycopeptides derived from the transformed cells were composed of a group of large molecular weight glycopeptides (peak A in Figure 61), that was not present in the glycopeptide mixture derived from the normal cells (134). Subsequent studies have shown that this group of large molecular weight glycopeptides (designated group A) was characteristically elevated in a wide variety of malignant cells (136). The group A glycopeptides were not retained by concanavalin A-Sepharose affinity columns (135).

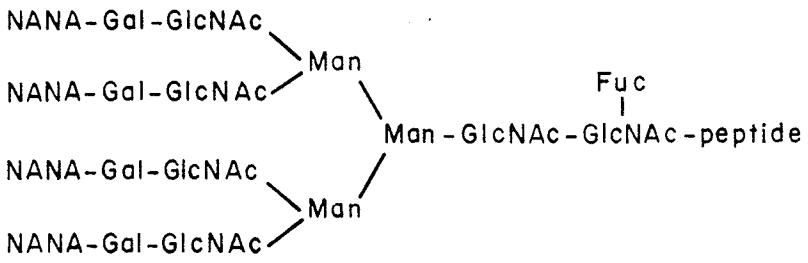
Figure 61. Elution patterns from a column of Sephadex G-50 (fine) of large-scale pronase digests from trypsin treated baby hamster kidney 21-C_{13} cells and C_{13}-B_4 cells (134).

--○-- 21-C_{13} (normal cells)

—●— C_{13}-B_4 (transformed cells)

Figure 62. A structural model of the majority of the large glycopeptides from transformed cells (135).


CPM
100
50
A
B
40
80
120
Fraction number


NANA-Gal-GlcNAc
NANA-Gal-GlcNAc
Man
NANA-Gal-GlcNAc
NANA-Gal-GlcNAc
Man
Fuc
Man-GlcNAc-GlcNAc-peptide

A structural model of these glycopeptides is shown in Figure 62.

The W.T. CO_2 and C^R-7 membrane glycoproteins were analyzed in the manner described above. The glycopeptides derived from each cell line were seperated into two groups on the basis of their affinity to a concanavalin A-Sepharose 4B column (Figures 52, 54, 56, and 58). The W.T. CO_2 and C^R-7 glycopeptides retained by the affinity column were determined to be of similar molecular weights by gel filtration on Sephadex G-50 and G-75 (Figures 53, 55, 57, 59, peak c). In comparison, the W.T. CO_2 and C^R-7 glycopeptides that were not retained by the affinity column exhibited different elution patterns when chromatographed on Sephadex G-50 and G-75 (Figures 53, 55, 57, and 59). The W.T. CO_2 glycopeptides were composed of a group of large molecular weight glycopeptides that was reduced in the C^R-7 glycopeptide mixture (peak A in Figures 53, 55, 57, and 59). Apparently, the W.T. CO_2 glycopeptides have the characteristic group A glycopeptides common to virally transformed cells. These results are another indication of the similarity between the W.T. CO_2 cells and transformed cells.

CONCLUSION

The glycosidase activity levels in confluent W.T. CO_2 and RC^R-7 cells, in comparison to the C^R-7 cells, have been shown to be elevated (Table III). Neoplastic and transformed cells also display elevated glycosidase activity levels (72, 74, 98, Table VIII). These elevated

levels may be responsible for the modification of cell surface glycoproteins and glycolipids. Certainly, an intimate relationship exists between the glycolipid composition of tissues and the hexosaminidase activity levels (53). The W.T. CO_2 and transformed cells seem to have similar membrane glycopeptide alterations (Figures 52, 56, 61). Both of these cell types possess a group of large molecular weight glycopeptides that is absent from the C^R-7 and untransformed cells (Figures 52, 54, 56, 58, 61). Perhaps the altered glycosidase activity levels are responsible for this difference. Evidence indicating the involvement of cell surface glycoproteins and glycolipids in cell-cell and cell-ligand interactions, intercellular adhesiveness and the control of cell division is rapidly accumulating (1, 6, 8). Alterations in these cell surface components would consequently effect cellular behaviour. The role of glycosidases in the control of cellular behaviour still remains unclear, but if these enzymes were capable of modifying cell surface components, they would indirectly exert some influence on cellular behaviour.

REFERENCES

REFERENCES

1. Nicolson, G.L. (1976) Biochim. Biophys. Acta 458, 1-72.
2. Wallach, D.F.H. (1975) Membrane Molecular Biology of Neoplastic Cells, pp. 9-72, Elsevier Scientific Publishing Co., New York.
3. Singer, S.J. and Nicolson, G.L. (1972) Science 175, 720-731.
4. Quinn, P.J. (1976) The Molecular Biology of Cell Membranes, pp. 80-112, University Park Press, Baltimore.
5. Fishman, P.H. and Brady, R.O. (1976) Science 194, 906-915.
6. Muller, K. and Gerisch, G. (1978) Nature 274, 445-449.
7. Yamada, K.M. and Olden, K. (1978) Nature 275, 179-184.
8. Hynes, R.O. (1976) Biochim. Biophys. Acta 458, 73-107.
9. Bornstein, P., Duksin, D., Balian, G., Davidson, J.M. and Crouch, E. (1978) Ann. N.Y. Acad. Sci. 312, 93-105.
10. Sharon, N. (1975) Complex Carbohydrates, Wesley Publishing Co., Don Mills, Ontario.
11. Fletcher, A.P., Marks, G.S., Marshall, R.D. and Neuberger, A. (1963) Biochem. J. 87, 265-273.
12. Spiro, R.G. (1973) Adv. Prot. Chem. 27: 349-467.
13. Baenziger, J. and Kornfeld, S. (1974) J. Biol. Chem. 249, 7270-7281.
14. Arima, T., Spiro, M.J. and Spiro, R.G. (1972) J. Biol. Chem. 247, 1825-1835.
15. Hughes, R.C. (1976) Membrane Glycoproteins, Butterworth and Co., Toronto.

16. Zinn, A., Plantner, J.J., and Carlson, D.M. (1975). in Mammalian Glycoproteins and Glycolipids (Pigman, W. and Horowitz, M.I., eds.), pp. 69-83, Academic Press, New York.

17. Schmid, K. (1975) in The Plasma Proteins (Putnam, F.W., ed.), Vol. 1, 2nd. edn., pp. 183-228, Academic Press, New York.

18. Warren, L. (1972) in Glycoproteins: Their composition and Function (Gottschalk, A., ed.), Part B, pp. 1097-1136, Elsevier Scientific Publishing Co., New York.

19. Molnar, J. (1976) in The Enzymes of Biological Membranes (Martonosis, A., ed.), Vol. 2, pp. 385-419, Plenum Press, New York.

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

21. Spiro, M.J., Spiro, R.G. and Bhoyroo, V.D. (1976) J. Biol. Chem. 251, 6409-6418.

22. Robbins, P.W., Kragg, S.S. and Liu, T. (1977) J. Biol. Chem. 252, 1780-1785.

23. Talmadge, K.W. and Burger, M.M. (1975) in Biochemistry Series One (Whelan, W.J., ed.), Vol. 5, pp. 43-93, University Park Press, Baltimore.

24. Palade, G. (1975) Science 189, 347-358.

25. Blobel, G. and Dobberstein, B. (1975) J. Cell Biol. 67, 835-851.

26. Molnar, J., Robinson, G.B. and Winzler, R.J. (1964) J. Biol. Chem. 240, 1882-1888.

27. Lawford, G.R. and Schachter, H. (1966) J. Biol. Chem. 241, 5408-5418.

28. Struck, D.K., Lennarz, W.J. and Brew, K. (1978) J. Biol. Chem. 253, 5786-5794.

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

30. Briles, E.B. and Kornfeld, S. (1978) T.I.B.S. 3, 223-227.

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

32. Schachter, H., Jabbal, I., Hudgin, R.L., Pinteric, L., McGuire, E.J. and Roseman, S. (1970) J. Biol. Chem. 245, 1090-1100.

33. LeeLavathi, D.E., Estes, L.W., Feingold, D.S. and Lombardi, B. (1970) Biochim. Biophys. Acta 211, 124-138.

34. Ruoslahti, E., Vaheri, A., Kuusela, P. and Linder, E. (1973) Biochim. Biophys. Acta 322, 352-358.

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

36. Blumberg, P.M. and Robbins, P.W. (1975) Cell 6, 137-147.

37. Keski-Oja, J., Mosher, D.F. and Vaheri, A. (1976) Cell 9, 29-33.

38. Chen, L.B., Gallimore, P.H. and McDougal, J.K. (1976) Proc. Natl. Acad. Sci. U.S.A. 73, 3570-3574.

39. Pearlstein, E. (1976) Nature 262, 497-500.

40. Yamada, K.M. and Pastan, I. (1976) T.I.B.S. 1, 222-224.

41. Shields, R. and Pollock, K. (1974) Cell 3, 31-38.

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

43. Ashwell, G. and Morell, A.G. (1977) T.I.B.S. 2, 76-78.

44. Morell, A.G., Irvine, R.A., Sternlieb, I., Scheinberg, I.H. and Ashwell, G. (1968) J. Biol. Chem. 243, 155-159.

45. Pricer, W.E., Jr. and Ashwell, G. (1971) J. Biol. Chem. 246, 4825-4833.

46. Hudgin, R.L., Pricer, W.E., Jr., Ashwell, G., Stockert, R.J. and Morell, A.G. (1974) J. Biol. Chem. 249, 5536-5543.

47. Kawasaki, T. and Ashwell, G. (1976) J. Biol. Chem. 251, 1296-1302.

48. Frazier, W.A. (1976) T.I.B.S. 1, 130-133.

49. Beug, H., Gerisch, G., Kempff, S., Riedel, V. and Cremer, G. (1970) Exp. Cell Res. 63, 147-158.

50. Simpson, D.L., Rosen, S.D. and Barondes, S.H. (1974) Biochemistry 13, 3487-3493.

51. Frazier, W.A., Rosen, S.D., Reitherman, R.W. and Barondes, S.H. (1975) J. Biol. Chem. 250, 7714-7721.

52. Chang, C-M., Reitherman, R.W., Rosen, S.D. and Barondes, S.H. (1975) Exp. Cell Res. 95, 136-142.

53. Schneck, L. (1975) in The Gangliosidoses (Volk, B.W. and Schneck, L., eds.), pp. 31-124, Plenum Press, New York.

54. Roseman, S. (1970) Chem. Phys. Lipids 5, 270-297.

55. Patt, L.M. and Grimes, W.J. (1974) J. Biol. Chem. 249, 4157-4165.

56. Yogeeswaran, G., Lane, R.A. and Hakomori, S. (1974) Biochem. Biophys. Res. Commun. 59, 591-599.

57. Keenan, T.W., Moore, D.J. and Basu, S. (1974) J. Biol. Chem. 249, 310-315.

58. Winzler, R.J. (1970) Int. Rev. Cyt. 29, 77-125.

59. Nicolson, G.L. and Singer, S.J. (1974) J. Cell Biol. 60, 236-248.

60. Cuatrecasas, P. (1973) Biochemistry 12, 3547-3558.

61. Heyninger, W.E., van (1974) Science 183, 656-657.

62. Mullin, B.R., Fishman, P.H., Lee, G., Aloj, S.M., Ledley, F.D., Winand, R.J., Kohn, L.D. and Brady, R.O. (1976) Proc. Natl. Acad. Sci. U.S.A. 73, 842-846.

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

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

65. Glick, M.C., Rabinowitz, Z. and Sachs, L. (1973) Biochemistry 12, 4864-4869.

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

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

68. Mora, P.T., Brady, R.O., Bradley, R.M. and McFarland, V.N. (1969) Proc. Natl. Acad. Sci. U.S.A. 63, 1290-1296.

69. Brady, R.O. and Mora, P.T. (1970) Biochim. Biophys. Acta 218, 308-319.

70. Fishman, P.H., Brady, R.O., Bradley, R.M., Aaronson, S.A. and Todaro, G.J. (1974) Proc. Natl. Acad. Sci. U.S.A. 71, 298-301.

71. Poste, G. (1972) Exp. Cell Res. 73, 319-328.

72. Bosmann, H.B. and Hall, T.C. (1974) Proc. Natl. Acad. Sci. U.S.A. 71, 1833-1837.

73. Kijimoto, S. and Hakomori, S.-I. (1971) Biochem. Biophys. Res. Commun. 44, 557-563.

74. Bosmann, H.B. (1972) Biochim. Biophys. Acta 264, 339-343.

75. Bosmann, H.B., Lockwood, T. and Morgan, H.R. (1974) Exp. Cell Res. 83, 25-30.

76. Sharon, N. and Lis, H. (1972) Science 177, 949-959.

77. Sumner, J.B. and Howell, S.F. (1935) J. Immunol. 29, 133-134.

78. McKenzie, G.H., Sawyer, W.H. and Nichol, L.W. (1972) Biochim. Biophys. Acta 263, 283-293.

79. McKenzie, G.H. and Sawyer, W.H. (1973) J. Biol. Chem. 248, 549-556.

80. Edelman, G.M., Cunningham, B.A., Recke, G.N., Becker, J.W., Waxdal, M.J. and Wang, J.L. (1972) Proc. Natl. Acad. Sci. U.S.A. 69, 2580-2584.

81. Yariv, J., Kalb, A.J. and Levitzki, A. (1968) Biochim. Biophys. Acta 165, 303-305.

82. So, L.L. and Goldstein, I.J. (1967) J. Immunol. 99, 158-163.

83. So, L.L. and Goldstein, I.J. (1967) J. Biol. Chem. 242, 1617-1622.

84. Goldstein, I.J. (1975) in Concanavalin A (Chowdhury, T.K. and Weiss, A.K., eds.), pp. 35-53, Plenum Press, New York.

85. Benjamin, T.L. and Burger, M.M. (1970) Proc. Natl. Acad. Sci. U.S.A. 67, 729-734.

86. Shohan, J., Inbar, M. and Sacks, L. (1970) Nature 227, 1244-1246.

87. Kornfeld, S., Eider, W. and Gregory, W. (1974) in Control and Proliferation in Animal Cells (Clarkson, B. and Baserga, R., eds.), pp. 435-445, Cold Spring Harbor Laboratory, New York.

88. Ceri, H. and Wright, J.A. (1978) Exp. Cell Res. 114, 217-227.

89. Ceri. H. and Wright, J.A. (1977) Exp. Cell Res. 104, 389-398.

90. Wright, J.A. (1975) Can. J. Microbiol. 21, 1650-1654.

91. Ceri, H. and Wright, J.A. (1978) Exp. Cell Res. 115, 15-23.

92. Stanners, C.P., Eliceiri, G.L. and Green, H. (1971) Nature N.B. 230, 52-54.

93. Bio-Rad Technical Bulletin 1051.

94. Elligsen, J.D., Thompson, J.E. and Frey, H.E. (1975) Exp. Cell Res. 92, 87-94.

95. Bloch, R., Betschart, B. and Burger, M.M. (1977) Exp. Cell Res. 104, 143-152.

96. Brunette, D.M. and Till, J.E. (1971) J. Membrane Biol. 5, 215-224.

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

98. Bosmann, H.B. (1969) Exp. Cell Res. 54, 217-221.

99. Bosmann, H.B. and Pike, C.Z. (1970) Life Sci. 9, 1433-1440.

100. Ozanne, B. and Sambrook, J. (1971) Nature, N.B. 232, 156-160.

101. Bosmann, H.B. (1977) Int. Rev. Cyt. 50, 1-23.

102. Thompson, J.E., Gruber, M.Y. and Kruuv, J. (1978) Exp. Cell Res. 111, 47-53.

103. Neufeld, E.F., Lim, T.W. and Shapiro, L.J. (1975) Ann. Rev. Biochem. 44, 357-376.

104. Wiktorowicz, J.E., Awasthi, Y.C., Kurosky, A. and Srivastava, S.K. (1977) Biochem. J. 165, 49-53.

105. Hughes, R.C. and Sharon, N. (1978) T.I.B.S. 3, N277.

106. Hickman, S. and Neufeld, E.F. (1972) Biochem. Biophys. Res. Commun. 49, 992-999.

107. Wiesmann, U., Lightbody, J. and Herschkowitz, N.N. (1971) N. Engl. J. Med. 284, 109-110.

108. Kaplan, A., Achord, D.T. and Sly, W.S. (1977) Proc. Natl. Acad. Sci. U.S.A. 74, 2026-2030.

109. Ullrich, K., Mersmann, G., Weber, E. and Figura, K., von (1978) Biochem. J. 170, 643-650.

110. Lee, J.S. and Yoshida, A. (1976) Biochem. J. 159, 535-539.

111. Lusis, A.J. and Paigen, K. (1978) J. Biol. Chem. 253, 7336-7345.

112. Braidman, I., Carroll, M., Dance, N. and Robinson, D. (1974) Biochem. J. 143, 295-301.

113. Beutler, E., Kuhl, W. and Comings, D. (1975) Am. J. Hum. Genet. 27, 628-638.

114. Kanfer, J.N. and Spielvogel, C. (1973) Biochim. Biophys. Acta 193, 203-207.

115. Bearpark, T. and Stirling, J.L. (1977) Biochem. J. 168, 435-439.

116. Geiger, B. and Arnon, R. (1976) Biochem. 15, 3484-3493.

117. Srivastava, S.K., Yoshida, A., Awasthi, Y.C. and Beutler, E. (1974) J. Biol. Chem. 249, 2049-2053.

118. Poenaru, L., Weber, A., Dreyfus, J.C., Overdijk, B. and Hooghwinkel, G.J.M. (1974) F.E.B.S. Letts. 41; 181-184.

119. Poenaru, L. and Dreyfus, J.C. (1973) Clin. Chim. Acta 43, 439-442.

120. Robinson, D. and Stirling, J.L. (1968) Biochem. J. 107, 321-327.

121. Willcox, P. and Renwick, A.G.C. (1977) Eur. J. Biochem. 73, 579-590.

122. Ikonne, J.U., Rattazzi, M.C. and Desnick, R.J. (1975) Am. J. Hum. Genet. 27, 639-650.

123. Stirling, J.L. (1972) Biochim. Biophys. Acta 271, 154-162.

124. Beutler, E. and Kuhl, W. (1975) Nature 258, 262-264.

125. Srivastava, S.K. and Beutler, E. (1974) J. Biol. Chem. 249, 2054-2057.

126. Srivastava, S.K., Wiktorowicz, J.E. and Awasthi, Y.C. (1976) Proc. Natl. Acad. Sci. U.S.A. 73, 2833-2837.

127. Overdijk, B., Kroef, W.M.J., van der, Veltkamp, W.A. and Hooghwinkel, G.J.M. (1975) Biochem. J. 151, 257-261.

128. Li, Y.-T., Mazzotta, M.Y., Wan, C.-C., Orth, R. and Li, S.-C. (1973) J. Biol. Chem. 248, 7512-7515.

129. Robinson, D. (1974) Enzyme 18, 114-135.

130. Srivastava, S.K., Awasthi, Y.C., Yoshida, A. and Beutler, E. (1974) J. Biol. Chem. 249, 2043-2048.

131. Aruna, R.M. and Basu, D. (1975) J. Neurochem. 25, 614-617.

132. Tallman, J.F., Brady, R.O., Quirk, J.M., Villalba, M. and Gal, E. (1974) J. Biol. Chem. 249, 3489-3499.

133. Lisman, J.J.W. and Overdijk, B. (1978) Hoppe-Seyler's Z. Physiol. Chem. 359: 1019-1022.

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

135. Ogata, S.-I., Muramatsu, J. and Kobata, A. (1976) Nature 259, 580-582.

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