# THE USE OF THE HAMSTER TRACHEAL EPITHELIAL CELL SYSTEM FOR THE ISOLATION AND INVESTIGATION OF MUCUS GLYCOPROTEINS

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A DISSERTATION

SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS

FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

IN THE GRADUATE SCHOOL OF THE

TEXAS WOMAN'S UNIVERSITY

COLLEGE OF NATURAL AND SOCIAL SCIENCES

BY

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DECEMBER, 1986

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11/14/86
Date

To the Provost of the Graduate School:

I am submitting herewith a dissertation written by Patricia Mary Jones entitled "The Use of the Hamster Tracheal Epithelial Cell System for the Isolation and Investigation of Mucus Glycoproteins". I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Molecular Biology.

Michael J. Rudick
Michael Rudick,
Major Professor

We have read this dissertation
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ACKNOWLEDGMENTS

I would like to thank Dr. Sandra Magie for her support and invaluable assistance in the final stages of this work.

I would also like to thank my committee members for their support and patience.

The encouragement of Pat Burgoon is greatly appreciated, as is the assistance of Dr. Mary L. Higgins in photography.

## ABSTRACT

Cystic fibrosis (CF) is a disease that is the most common genetic disorder among Caucasian populations. CF appears to be epithelial in nature, involving the majority of the glandular epithelial tissues in the body, and thereby resulting in a usually systemic symptomology.

While the basic genetic defect has not yet been determined, one of the central aspects of CF symptomology is an apparent abnormality in the mucus secretions in CF individuals. Therefore, the investigation of mucus glycoproteins is necessary in order to gain a more complete understanding of cystic fibrosis.

Mucus glycoproteins, or mucins, are not single well-defined entities, but are large, highly complex molecules. They apparently vary in composition depending on their source, and even mucins from the same source appear to be extremely heterogeneous with regard to their acidity and the lengths of carbohydrate chains.

This study involved the isolation and characterization of mucins from different sources. These mucin isolates are compared and contrasted. The study also involved attempts to demonstrate that hamster tracheal epithelial (HTE) cells grown in culture synthesize and secrete mucins, and that the HTE cell system may prove useful in the investigation of mucin synthesis and secretion. Attempts at

mucin antibody production and immunoprecipitation from the HTE cells are also included.

The aim of this study is to investigate mucins from different sources, comparing and contrasting them, and also to show the possible usefulness of the HTE cell system in the study of the synthesis and secretion of mucus glycoproteins. In this way it may be possible to gain a better understanding of the nature, structure, and eventually, the function of this complex molecule.

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# INTRODUCTION

Cystic fibrosis (CF) is a disease, apparently transmitted as an autosomal recessive trait (Wright and Morton 1968; Nadler et al. 1978; Bowman and Barnett 1976; Sing et al. 1982), that is the most common genetic disorder among Caucasian populations. CF appears to be epithelial in nature, involving the majority of the glandular epithelial tissues in the body, and resulting in a frequently systemic symptomology. Clinical manifestations of the disease include dysfunction of the exocrine glands, chronic pulmonary disease, and gastrointestinal involvement (Bowman and Barnett 1976; Nadler et al. 1978). CF patients, however, show a considerable amount of variability in the expression of these symptoms, manifesting anywhere from only one to nearly all.

While the basic genetic defect has not yet been determined, one of the central aspects of CF symptomology is an apparent abnormality of mucus secretions in CF patients (Carlson 1977; Boat and Cheng 1978; Aldaheff 1978). Some investigators have suggested that mucus secretions in CF individuals appear to be abnormally viscous (Boat et al. 1977; Boat and Cheng 1978), produced in greater quantities in CF individuals (Carlson 1977; Boat and Cheng 1978), more highly sulfated and/or having different amino acid ratios than mucins from unaffected persons (Chace et al. 1985). Most of the symptoms of CF appear

to be derived from this apparent alteration in glycoprotein metabolism. For instance, the thick, excessive mucus secretions cause nutritional problems due to malabsorption in the mucus-blocked gastrointestinal tract. Chronic pulmonary disease and respiratory failure are due to accumulations in the lungs; and blockage of organ passages by mucus causes many other problems. Because of this central role that these mucin-like glycoproteins, or mucins, appear to play, the study of mucins themselves is an important facet of CF research.

Mucins are not single, well-defined entities, but are large, highly complex molecules. They vary in composition depending on their source (Allen 1983; Silverberg and Meyer 1982) and even mucins from the same source appear to be extremely heterogeneous with regard to their acidity and the length of their carbohydrate chains (Allen 1983; Van Halbreek et al. 1982; Silverberg and Meyer 1982; Kim et al. 1985). This microheterogeneity among mucins is discussed in many places in the literature (Allen 1983; Forstner 1978; Rose et al. 1984; Harding et al. 1983; Roussell et al. 1983; Lamblin et al. 1984a; Carlson 1968); and this reflects the problems associated with isolating and characterizing "mucin" molecules. Therefore, in order to achieve some sort of isolation and characterization, the focus must be on the similarities inherent in mucus glycoproteins. Mucins, or mucin-like glycoproteins, do have several characteristics in common: (a) In their native state they are uniformly large molecules, usually

with a molecular weight in excess of 1 x $10^6$ (Carlson 1977; Allen 1983; Silverberg and Meyer 1982; Kim et al. 1985; Chace et al. 1985); (b) They are comprised of 50 to 85% carbohydrate, composed of fucose, galactose, N-acetyl-galactosamine, N-acetyl-glucosamine, and sialic acid (Allen 1983; Carlson 1977; Forstner 1978; Kim et al. 1985); (c) The carbohydrate chains are frequently negatively charged due to terminal sialic acid and esterified sulphate residues (Allen 1983; Forstner 1978; Kim et al. 1985; Silverberg and Meyer 1982); (d) The carbohydrate chains are linked to the peptide backbone via O-glycosidic linkages between the hydroxyl groups of the serine and/or threonine residues and the N-acetyl-galactosamine at the sugar chain's reducing end (Allen 1983; Carlson 1977; Forstner 1978; Silverberg and Meyer 1982); (e) This O-glycosidic linkage is very alkali labile, releasing intact sugar chains upon alkaline $\beta$-elimination (Carlson 1977; Sharon 1975; Kim et al. 1985). Figure 1 gives a diagrammatic representation of a mucin molecule. Oligosaccharide side chains which are typically found in mucins are shown attached to the protein backbone via O-glycosidic linkages to serine and threonine residues (Slomiany et al. 1984; Lamblin et al. 1984a; Van Halbeek et al. 1982; Hill et al. 1977; Lamblin et al. 1984b). With these common characteristics in mind, it is possible to have, if not a "well-defined" molecule at least a working definition of "mucins" to use as a starting point in the

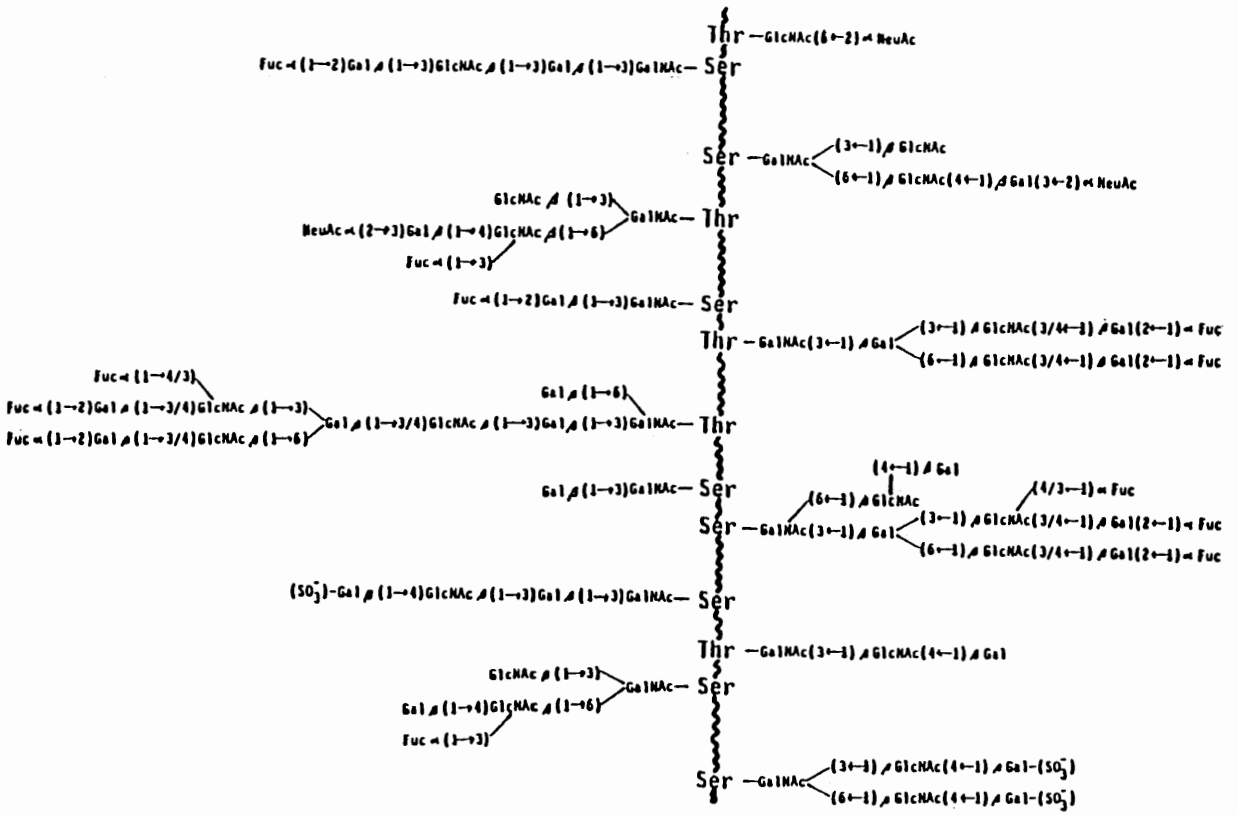



Figure 1. A representative section of a mucin molecule. The carbohydrate side chains, attached to serine and threonine residues via O-glycosidic linkages, are some that are typically found in mucins.

isolation and characterization of these complex secretions. Using this working definition, mucin-like glycoproteins from different sources can be studied and compared.

The importance of investigating mucins from human sources is obvious because of their role in such disease states as CF. Studying mucins from other sources, however, can also be important. Research has shown that hamster tracheal epithelial (HTE) cells grown in culture secrete high molecular weight, polyanionic, or mucin-like, glycoproteins (Rudick et al. 1984). The addition of serum from CF patients to the HTE cells causes stimulation of the secretion of these mucin-like molecules (Wooten et al. 1984), suggesting that a CF serum factor alters the rate of glycoprotein synthesis and/or secretion. Since comparatively little is known about the synthesis of mucin-like glycoproteins, a system, like the HTE cell system, that could lend itself to the study of these molecules, could prove to be invaluable. Investigating the addition of O-linked oligosaccharide chains, their time and site of addition during the course of glycoprotein synthesis, as well as investigating the method of action of secretion, might be feasible using this system. The biochemical regulation of mucin synthesis and secretion, and its relationship to the etiology of CF could much more easily be looked at in a cell culture system than in whole organs. However, first it is necessary to determine more about the mucin-like glycoproteins produced by the HTE cells in order to

gain insight into the regulation of their secretion.

This study involves isolation and characterization of mucus glycoproteins from both human and hamster tracheal sources. The structures of the mucin isolates from the two sources were compared and contrasted. Also included in the study were attempts at producing antibody to the isolated human mucin. This anti-human mucin antibody could be very useful in examining mucin synthesis in cells suspected to produce mucins, i.e. HTE cells, by immunoprecipitation of mucins from HTE cells. Eventually, this study could lead to the use of HTE cells to investigate the function of this complex molecule, and to an understanding of its role in CF.

## MATERIALS AND METHODS

### Materials

The following were obtained from Bio-Rad Laboratories, Richmond, CA: acrylamide, bis-acrylamide, N,N,N'N'-tetraethylmethylenadiamine (TEMED), ammonium persulfate, Bio-Rad Silver Stain Kit, DEAE-Affigel Blue, Bio-Gel A-5M, Bio-Gel $P_2$, Bio-Gel $P_{10}$, Dowex 1-x2 ($Cl^-$), Dowex 50-x2 ($H^+$) and Dowex 50-x8. Sephadex G-200 was purchased from Pharmacia, Piscataway, N.J. Freund's complete and incomplete adjuvant were from Difco Laboratories, Detroit, Mich. The following radiolabelled materials were purchased from ICN, Irvine, CA: ($^3H$)-$NaBH_4$ (100 mCi/mmol), $^{35}S$ as sulfuric acid in $H_2O$ (10 mCi); (4,5-$^3H$) L-leucine (55 Ci/mmol), (1-$^3H$) D-glucosamine hydrochloride (4-10 Ci/mmol); (U-$^{14}C$) L-threonine (180-220 mCi/mmol), and (2-$^3H$) D-mannose (25 Ci/mmol). Pansobin was obtained from Calbiochem-Behring Corporation, LaJolla, CA. ($^{35}S$) L-methionine (1200 Ci/mmol) was obtained from New England Nuclear, Boston, Mass. Neuraminidase from Clostridium perfringens and Bovine testicular hyaluronidase were purchased from Sigma Chemical Co., St. Louis, Mo. Sodium Dodecyl Sulfate (SDS) was from Pierce Chemical Co., Rockford, Ill. Beta phase scintillation cocktail was from WestChem, San Diego, Cal. Kodak X-OMAT film and Kodak x-ray developer were obtained from Eastman Kodak Co., Rochester,

N. Y. All other chemicals were reagent grade or the highest purity available.

Methods

Human Mucin Isolation.

Human mucin was isolated from human tracheal lavage samples obtained from Ft. Worth Childrens Hospital. After receiving the samples, they were dialysed against deionized water and lyophilized. The isolation was then carried out on these dialysed and lyophilized samples by means of column chromatography using Sephadex G-200 (2.5 cm x 100 cm) followed by Bio-Gel A-5M (1.5 cm x 90 cm). Both columns were run in a phosphate-saline buffer, pH 7.6, containing 0.01 M sodium phosphate, 0.15 M NaCl, 5mM $\beta$-mercaptoethanol and 0.59 mM EDTA. Fractions having a volume of 2 ml each were collected and absorbance of these fractions was read at 220 or 280 nm for protein. Void volume material was retained in each case. The samples were treated with bovine testicular hyaluronidase, as previously described (Gallagher and Kent 1975), between the two chromatographic steps in order to degrade any glycosaminoglycans present. Samples were dissolved in a phosphate-citrate buffer, pH 5.5, containing 0.05 M sodium phosphate, 0.05 M citric acid and 0.1 M NaCl. Hyaluronidase was added to the sample to a final concentration of 0.25 mg/ml, and then the samples were incubated at 37 $^{o}$C for 18 h. After A-5M fractionation, the samples were dialysed against deionized water and

lyophilyzed. Figure 2 shows the overall isolation scheme used.

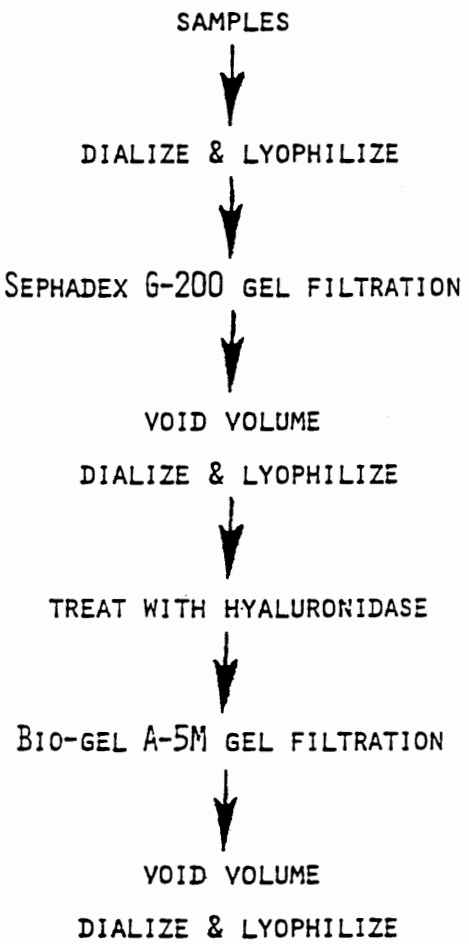
HTE Cell Culture.

Hamster tracheal epithelial cells were isolated and grown in culture as previously described (Wooten et al, 1984; Rudick et al. 1984). The cells were routinely grown in Ham's $F_{12}$ medium containing 10% FCS in a 95% air:5% $CO_2$ incubator at 37 $^{o}$C. One hundred millimeter dishes of near confluent cells were trypsinized and then transferred to roller bottles. Two 100 mm dishes were used for each roller bottle. The cells were grown in 100 ml of the medium in the roller bottles under normal cell culture conditions. When the cells were nearly confluent, they were labelled with either 20 $\mu$Ci/ml ($^{35}$S), 5 $\mu$Ci/ml ($^{3}$H)-glucosamine or 5 $\mu$Ci/ml 2-($^{3}$H)-mannose in 50 ml of medium containing only 5% FCS, and were incubated for 24 hours. The "conditioned" medium was removed from the radioactively labelled HTE cells by aspiration, centrifuged 10 min at 10,000 rpm in a Beckman J-21B centrifuge using a JA-20 rotor to remove large debris and floating cells, dialysed against deionized water and lyophilized. The HTE mucin was then isolated from these crude media samples by running them through the same chromatographic and enzymatic steps as used for the isolation of the human mucin samples.

Characterization of the Mucins.

The isolated mucins were characterized by the following means:

# MUCIN ISOLATION



a) Protein determination. Protein was measured by the method of Bradford (1976) using bovine serum albumin as a standard.

b) Hexose determination. Hexose concentration was measured by the Anthrone method (Seifter et al. 1950) using glucose as a standard.

c) Hexosamine determination. Hexosamine was measured by the method of Johnson (1971) using galactosamine as a standard.

d) Sialic Acid determination. The concentration of sialic acid was measured as previously described (Jourdian et al. 1971) using N-acetyl-neuraminic acid as a standard.

e) $\beta$-elimination. Mucin was analyzed by alkali-catalyzed $\beta$-elimination, because the presence of alkali-labile O-glycosidic linkages is a characteristic of mucus glycoproteins. The $\beta$-elimination was carried out and followed spectrophotometrically as previously described (Planter and Carlson 1975). Samples were dissolved in 0.05 N NaOH to a final concentration of 2 mg of sample/ml, and the absorbance of the sample was followed at 240 nm. $\beta$-elimination not only indicates the presence of O-glycosidic linkages, but also serves to remove the carbohydrate side chains of the mucin from their protein core. The $\beta$-elimination was done using 0.5 N NaOH in 0.5 N $NaBH_4$. After $\beta$-elimination the mixture was acidified with glacial acetic acid added dropwise, deionized using Dowex 50-x8 batchwise and dried on a rotary evaporator. The borate, as methyl borate, was then evaporated off on the rotary evaporator by 2 to 3 additions of methanol.

The peptide core was separated from the sugar chains via column chromatography on Bio-Gel $P_{10}$ (1.5 cm x 50 cm). The peptide was then dialyzed against water and lyophilyzed and its purity checked by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli 1970).

f) Oligosaccharide chain analysis. Human mucin: When the oligosaccharide side chains were removed from the human mucin isolate, the $\beta$-elimination was carried out in the presence of $(^3H)$-$NaBH_4$. This served to reduce and, thus, radioactively label the reducing ends of the released carbohydrate chains. These labelled chains were then analyzed by ion-exchange chromatography using Dowex 1-x2 ($Cl^-$) and Dowex 50-x2 ($H^+$) (75 mm x 15 cm). The sugar chains were also run on a Bio-Gel $P_2$ sizing column (1.5 cm x 85 cm). Ten microliter aliquots of the fractions off these columns were added to 4 ml of beta-phase scintillation fluid each and counted by liquid scintillation spectrometry.

Negatively charged oligosaccharide chains (i.e, those adsorbing to Dowex 1-x2) were assayed for the presence of sialic acid using neuraminidase (Cassidy et al, 1965). The chains were dissolved in water at a 1% concentration with the pH adjusted to 5.0 with acetic acid. Neuraminidase was added to a concentration of 0.25 mg/ml, and the mixture was incubated at 37 $^oC$ for 1 h. Once the sialic acid was thus removed from the carbohydrate chains, they were rerun through the Dowex 1-x2 ($Cl^-$) ion-exchange column to determine loss of

negative charge. Negatively charged chains were also assayed for sulfate by releasing the sulfate via methanolysis (Slomiany et al. 1981). Methonolysis was performed with 0.05 M HCl in dry methanol at room temperature for 4 h. The sulfate released by this means was assayed by the method of Terho and Hartiala (1971).

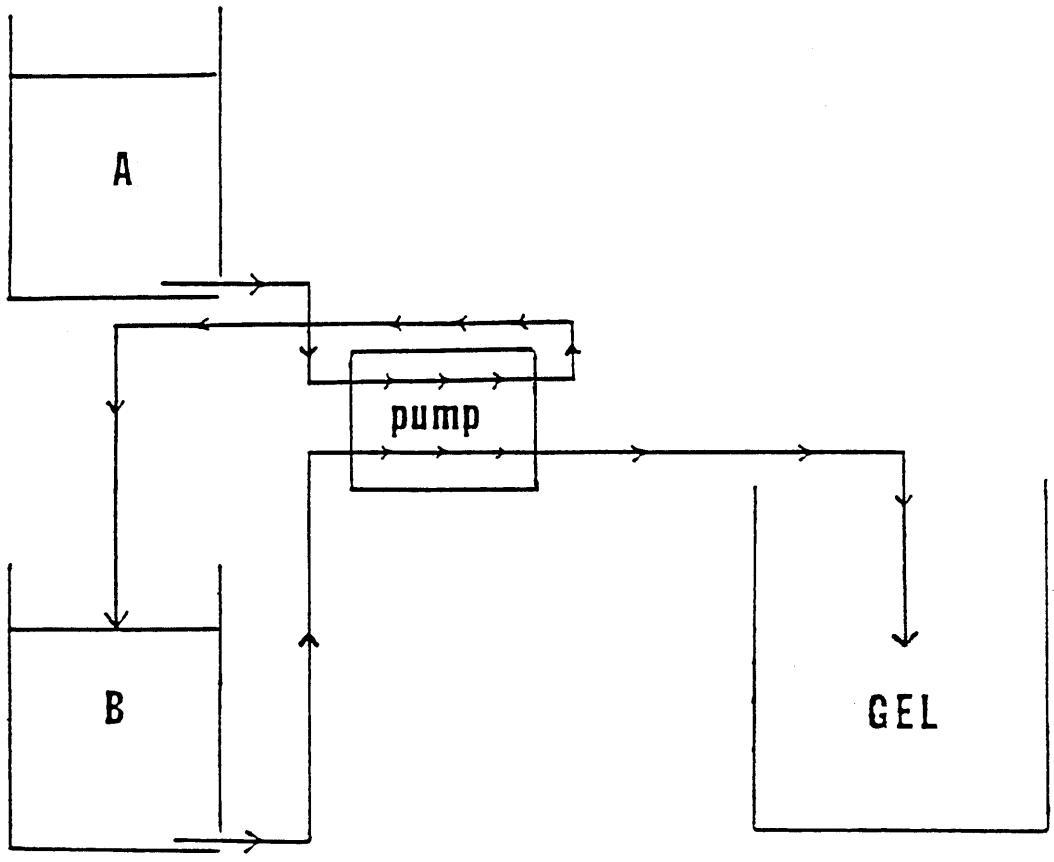
g) Oligosaccharide chain analysis; HTE mucin: When the carbohydrate side chains were removed from the HTE mucin isolate, the $\beta$-elimination was carried out both in the presence and in the absence of ($^3$H)-$NaBH_4$. Running the $\beta$-elimination in the absence of ($^3$H)-$NaBH_4$ allowed the metabolic label, originally introduced into the HTE cell culture, to be followed when analyzing the side chains. The analysis of the carbohydrate chains was essentially the same as that done on the human mucin side chains. Sulfate was not assayed for, as one of the metabolic labels was ($^{35}$S).

Polyacrylamide Gel Electrophoresis.

Mucin was analyzed on 5% and 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli 1970) and on 3.5 to 7.5% and 2.2 to 6% linear gradient gels. $\beta$-eliminated mucin was run on 10% gels and 3.0 to 7.5% gels. The gradient gels were prepared in the following manner. A gel mixture was prepared in which the acrylamide concentration was equal to that of the high end of the gradient, i.e. 7.5% for a 3.0 to 7.5% gel. This mixture was placed in a small flask with a stir bar and then placed on ice on a stir plate. A

second gel mixture was prepared having a much lower acrylamide concentration. The concentration of this second gel mixture was either 1.0% or 0% depending on whether the acrylamide concentration at the top of the gel was 3.0% or 2.2%. The gradient gel was cast by pumping gel material from the high-percentage acrylamide solution (7.5%) into the slab gel mold, and simultaneously at the same rate pumping gel material from the low-percentage solution into the high-percentage solution. Since the high-percentage gel solution was being constantly stirred, the percentage of acrylamide in it was gradually decreasing as the low-percentage solution was added. Therefore the concentration of acrylamide being cast in the gel gradually decreased. An LKB peristalic pump (LKB 2115 Multiperpex) equipped with dual channels was used to generate the linear gradient and to insure equal mixing and constant flow during the casting period. Figure 3 shows a diagram of the gradient gel casting system. TEMED and ammonium persulfate were added to both gel solutions just prior to the casting, and the solutions were kept on ice throughout, in order to slow down polymerization of the acrylamide until the casting was complete. The casting should take approximately 20 minutes.

In all the gels run, once the gel was cast, water was layered on top until polymerization of the acrylamide had occured. Then the water was poured off, and a stacking gel of 3.0% acrylamide was applied to the top. This was done with a comb in place in order to


A
pump
B
GEL

form wells to contain the samples once polymerization had occured.

Samples to be electrophoresed were prepared in a dissociating buffer containing 800 µl 0.125M Tris-HCl pH 6.8, 200 µl 10% SDS and 50 µl β-mercaptoethanol. This was done in a sample:buffer ratio of 1:2 v/v. The samples were boiled from 3 to 5 minutes, and then 10 µl glycerol and 2 to 5 µl of a 0.1% bromophenol blue tracking dye were added to each sample, followed by vortexing.

Electrophoresis was carried out on the gels after sample application at 15 mA during stacking and then at 30 mA. The electrophoresis was discontinued when the tracking dye reached the bottom of the gel. In all cases, protein detection in the gels was accomplished by silver staining, as described in the Bio-Rad Silver Stain Kit (Bio-Rad Laboratories).

Antibody preparation

Antibody to the human mucin isolate was prepared by immunizing with 0.5 mg human mucin isolate in 0.5 ml water mixed with an equal volume of Freund's complete adjuvant. This mixture was injected intramuscularly once a week for 6 weeks and then once a month thereafter. Attempts to elicit antibody to the peptide portion only of the human mucin isolate were done by two different methods. One rabbit was immunized by the same technique used for whole mucin antibody production, but only the peptide portion was applied. A separate rabbit was immunized with 0.5 mg of isolated human mucin peptide in water mixed with an

equal volume of Freund's incomplete adjuvant to a total volume of 1 ml. This mixture was then injected subcutaneously in 10 sites on the rabbit's back at 0, 2, and 4 weeks and once a month thereafter.

Antibody detection.

Blood samples were drawn with a 10 cc syringe from an ear vein once a week from each rabbit. These blood samples were assayed for the presence of antibody by means of precipitin line formation in double immunodiffusion plates as described by Ouchterlony (1968). The plates were photographed unstained or stained, as previously described (Uriel 1971) and then photographed. Ouchterlony plates to be stained were washed in 0.9% saline for 2 to 3 days to wash out unprecipitated proteins, dried, and then stained with 0.5% Amido black (w/v) for 10 to 20 min. Excess stain was removed by washing the plates in 1 M acetic acid until the background was clear. Antibody production was also assayed for by means of a dot-immunobinding assay run as previously described (Hawkes et al. 1982) but using nonfat dry milk (Blotto) as a blocking agent instead of bovine serum albumin (Johnson et al. 1984) The antigen was dotted on a nitrocellulose filter and dried thoroughly. Blotto was then added to block all non-specific binding sites on the filter. Serum from the rabbit was added and incubated overnight at room temperature. Serum was then removed, and the filters were washed in TBS (50 mM Tris-HCl, 200 mM NaCl, Ph 7.4), and then incubated for 2 h at room temperature

in peroxidase-conjugated goat anti-rabbit IgG. Filters were washed again and developed in chloronapthol and hydrogen peroxide.

Antibody Isolation.

Once antibody production was detected, 20 ml of blood was drawn from the rabbit at 4 week intervals. The rabbit was sedated first to make handling easier. A portion of the ear was shaved so that the veins were visible. A small slice was made across a lateral vein and blood was withdrawn via a vacuum pump and suction apparatus that fit over the ear.

The 30 ml of blood was then allowed to sit refrigerated for 20 min, and was then centrifuged at 10,000 rpm for 10 min in a Beckman J-21B centrifuge using a JA-20 rotor. The serum (10-15 ml) was then aspirated off the top and retained. The immunoglobulin was isolated from this rabbit serum via DEAE-Affigel Blue chromatography, as described in the Bio-Rad DEAE-Affigel Blue product information sheet (Bio-Rad Laboratories). A modification (Thaxton 1983) of the buffer used to equilibrate the column was made to get the best IgG binding possible. The buffer used was 0.02 M Tris-HCl, 0.035 M NaCl, pH 8.0. The immunoglobulin was the first unbound protein peak off the column. This isolated antibody was then dialyzed against deionized water and lyophilyzed.

Indirect Immunoprecipitation.

The antibody produced against the total human mucin molecule, anti-human mucin immunoglobin, was used with protein A-bearing strains of the bacterium Staphylococcus aureus in attempts to indirectly immunoprecipitate mucin, or mucin-like molecules from HTE cells, in order to show the production of this substance by these cells. The technique used is based upon the substitution of Protein A from S. aureus for the second anti-IgG antibody in the indirect immunoprecipitation (Kessler 1975; Kessler 1976).

Confluent HTE cells were first labelled overnight with either 2 μCi/ml ($^{14}$C)-leucine, 2 μCi/ml ($^{14}$C)-threonine or 4.2 μCi/ml ($^{35}$S)-methionine. The medium was removed from the cells, which were then scraped loose from the plates in phosphate buffered saline (PBS). The cells were than centrifuged and resuspended twice in PBS, centrifuged and finally resuspended in approximately 100 μl of 10% SDS. This mixture was then boiled for 10 min and centrifuged again. The supernatant from this final centrifugation was used for the indirect immunoprecipitation of mucin.

A modification (Hadzopoulou 1982) of the original technique of Kessler (1975) was employed that uses Pansorbin (heat killed bacteria of S. aureus with protein A present). This method allowed preincubation of the HTE cellular material with the protein A adsorbent and non-immune immunoglobulin, thus initially removing any

nonspecifically adsorbed material and allowing specific immunoprecipitation to occur in the presence of the anti-human mucin antibody.

In this procedure, 50 µl of the cellular material was diluted 10-fold with PBS-plus (PBS, pH 7.4, containing 0.1% SDS, 0.5% nonidet P-40, 1 mM EDTA, and 40 mM cold leucine, threonine or methionine, depending on which label was used). The mixture was incubated with 30 µl Pansorbin for 1 h at room temperature and then centrifuged 4 min in a microfuge to remove all non-specifically bound proteins. The supernatant was incubated for 20 min at room temperature with 20 µg non-immune immunoglobin, followed by the addition of 30 µl Pansorbin and another 1 h incubation. Material was pelleted again by centrifugation and discarded. The supernatant was then incubated with 20 µg of anti-human mucin immunoglobin at 4 °C for 24 h. Then 20 µl of the bacterial adsorbent was added for 10 min at room temperature, and the mixture was centrifuged in a microfuge to collect the immunoreactive material. The pellet was suspended in 200 µl PBS-plus, layered on top of 1 ml of 1.5 M sucrose and centrifuged for 10 min in the microfuge. Pellets were then washed in the PBS-plus until the radioactivity in the washes reached background. The pellets were then dissociated and analyzed by SDS-PAGE as described above.

Fluorographic detection of radiolabelled proteins.

Radiolabelled immunoprecipitates that were run on SDS-PAGE were then analyzed by fluorography. After staining, the gels were

impregnated with EN$^3$HANCE (New England Nuclear) for 1 h and then dried under vacuum with a Savant SGD-200 slab gel dryer. The dried gel was then placed in contact with Kodak X-OMAT AR film and exposed at -80 °C. The film was then developed using Kodak X-ray developer.

Hemagglutination.

Since it has been determined that human mucin has some of the same antigenic determinants as red blood cells (Forstner 1977), antibody produced in response to human mucin, anti-human mucin immunoglobin, should not only react with the human mucin but should also cross-react with red blood cells. This was tested by standard hemagglutination procedures (Gibbs 1977). Fresh human red blood cells were washed 3 times in physiological saline and then diluted to 2% in saline. 50 µl dilutions of the anti-human mucin antibody were set up in microtiter plates, along with 50 µl dilutions of anti-A or anti-B antiserum and saline blanks. The 2% suspension of red blood cells was added to the microtiter plates in a 1:1 (v/v) ratio with the dilutions. The plates were shaken to mix the solutions, allowed to sit for 30 min at room temperature, and then overnight at 4 °C. The plates were then observed for the presence or absence of "buttons" of red cells in the bottom of the wells.

# RESULTS

## Mucin Isolation: Human

Human mucin was isolated from tracheal lavage samples as described in Methods. Dialysed and lyophilyzed lavage samples were dissolved in buffer by overnight stirring in the cold and then run on a Sephadex G-200 column (2.5 x 100 cm). Figure 4 shows the profile of proteins eluted from the G-200. The first protein peak, the mucin fraction, was the void volume peak, as determined by Blue Dextran exclusion. The fractions contained in this peak were combined, dialysed against deionized water and lyophilyzed. This sample was then dissolved in the phosphate citrate buffer described in Methods. Bovine testicular hyaluronidase was added (0.25 mg/ml), and the mixture was incubated at 37 $^{o}$C for 16 h. The mixture was then run through a Bio-Gel A-5M column (1.5 cm x 90 cm). Figure 5 shows the protein profile eluted from the A-5M column. Again, the void volume peak was the first protein peak off the column. This material was combined, dialysed and lyophilyzed and considered to be the isolated mucin. This isolation scheme was used to isolate mucin 20 times, with comparable results each time.

## Mucin Isolation: Hamster Tracheal Epithelial

HTE cells were incubated in radiolabelled medium as described in

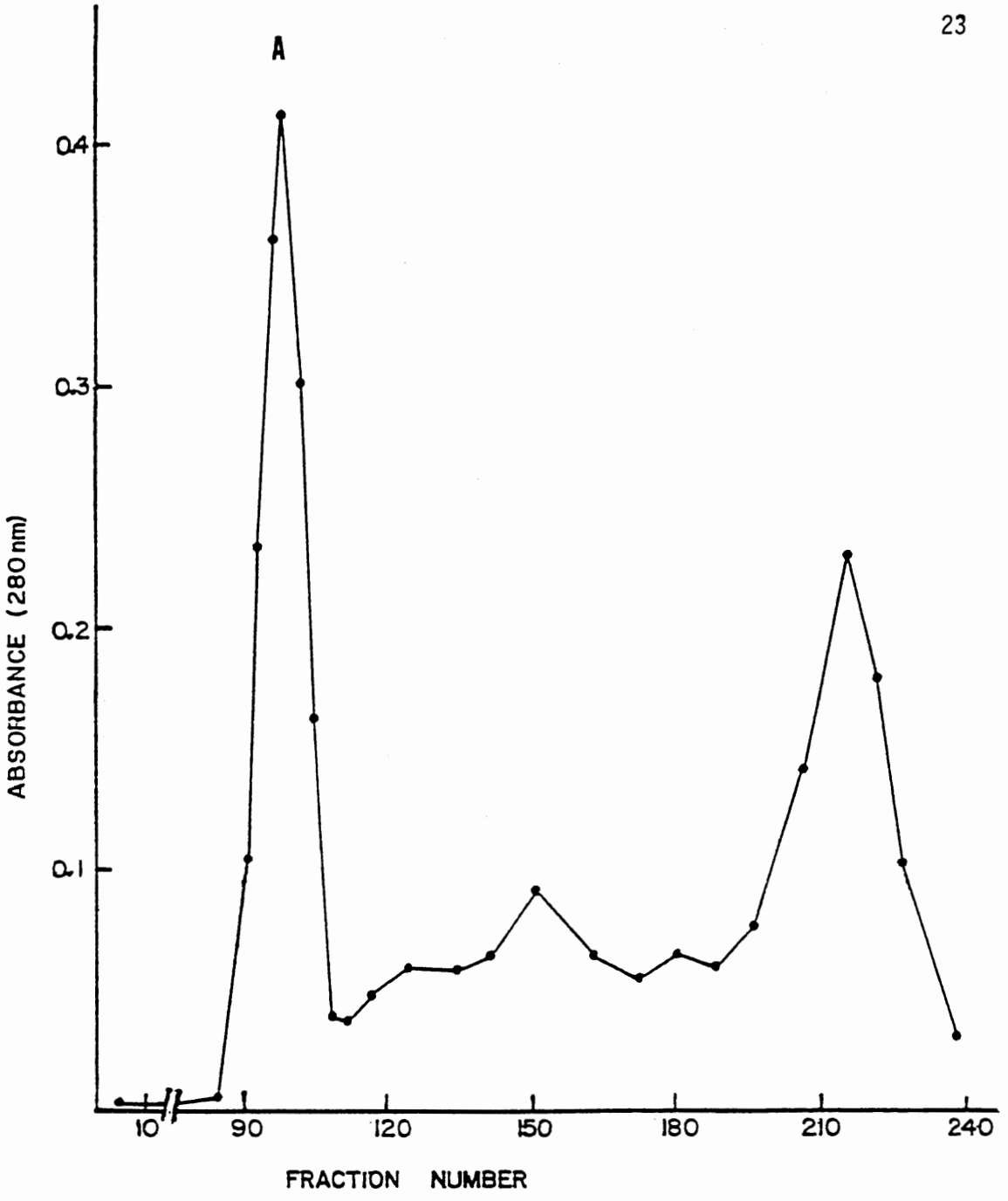



Figure 4. Sephadex G-200 chromatography of human mucin sample. Samples were dissolved in a 0.01 M sodium phosphate, 0.15 M NaCl, pH 7.6, and applied to the G-200 column equilibrated with the same buffer. Two ml fractions were collected. A: retained, void volume or "mucin" peak. Each dot represents 20 experiments.

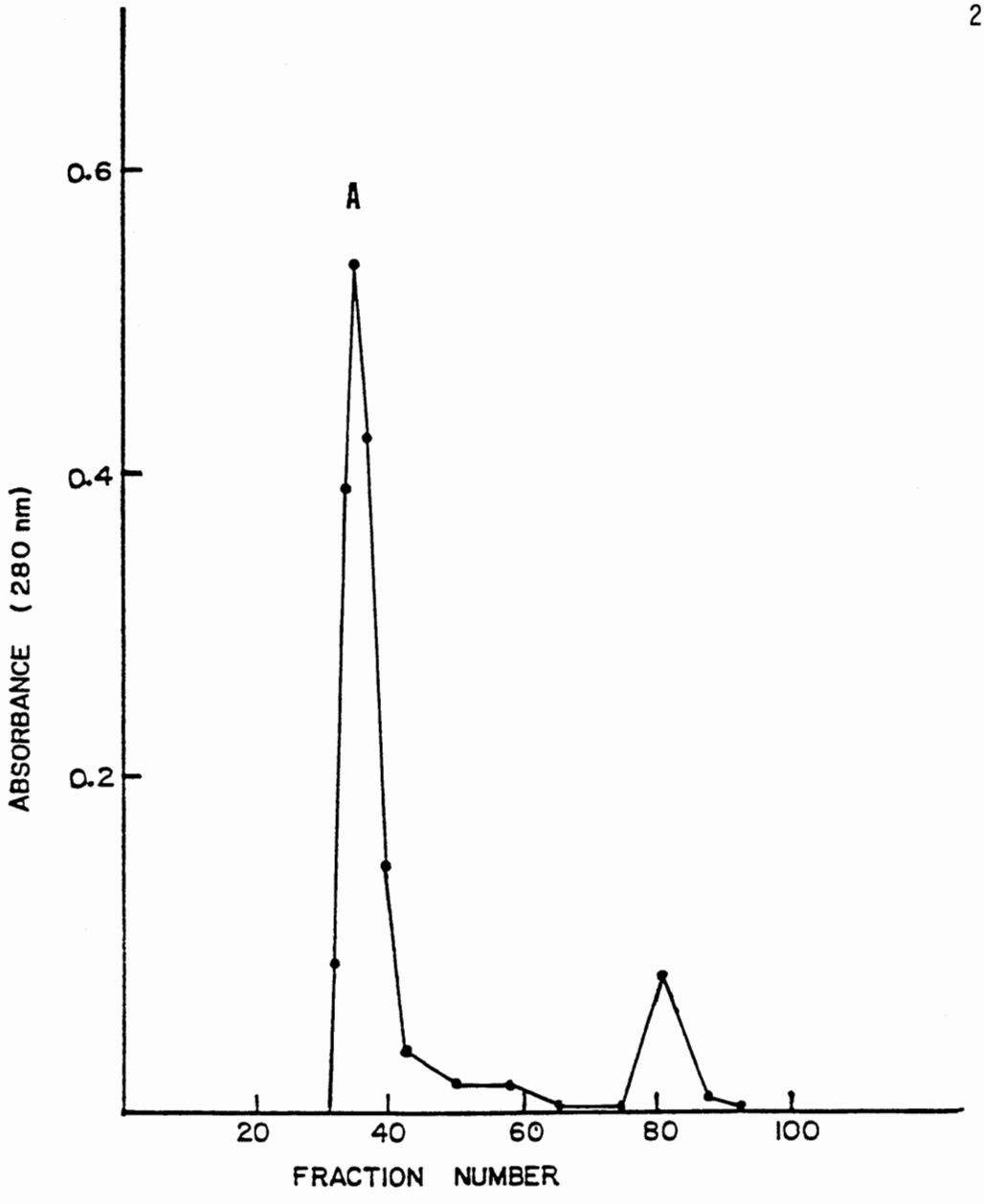



Figure 5. Bio-gel A-5M chromatography of human mucin sample. Lyophilyzed samples are treated with 0.25 mg/ml hyaluronidase for 18 h and then applied to the A-5M column equilibrated with a 0.01 M sodium phosphate, 0.15 M NaCl buffer, pH 7.6. Two ml fractions are collected. A: retained, void volume or "mucin" peak. Each dot represents 20 experiments.

the Methods section. After 24 hrs of labelling, the "conditioned" medium off these cells was dialysed against deionized water and lyophilyzed. The media samples were then run through the same isolation scheme as that for human mucin. Figure 6 shows the protein profile eluted from the G-200 column. This protein profile was the same for all samples, whether they were labelled with ($^{35}S$), ($^{3}H$)-glucosamine or 2-($^{3}H$)-mannose. The void volume peak came off first, followed by a large peak of included proteins. When these same fractions were counted for radioactivity, the results are shown in Figures 7, 8, and 9, with Figure 7 showing the profile from the ($^{35}S$) labelled cells, Figure 8 from the ($^{3}H$)-glucosamine labelled cells and Figure 9 from the (2-$^{3}H$)-mannose labelled cells. In the case of the ($^{35}S$) labelled cells, the majority of the label, approximately 90%, occured in the void volume peak, the mucin fraction. With the ($^{3}H$)-glucosamine and (2-$^{3}H$)-mannose labels, the label was split between the mucin fraction and the included proteins that elute from the colume after it. In the case of ($^{3}H$)-glucosamine, approximately 45% of the label was found in the mucin fraction. With (2-$^{3}H$)-mannose, about 50% of the label occured in the mucin fraction.

Figure 10 shows the protein profile off the A-5M column. Again, this profile was basically the same for all three radiolabeled samples. However, the radioactive counts off the A-5M are shown in Figure 11, 12, and 13, with Figure 11 representing the ($^{35}S$) labelled sample, Figure 12 representing the ($^{3}H$)-glucosamine labelled sample and

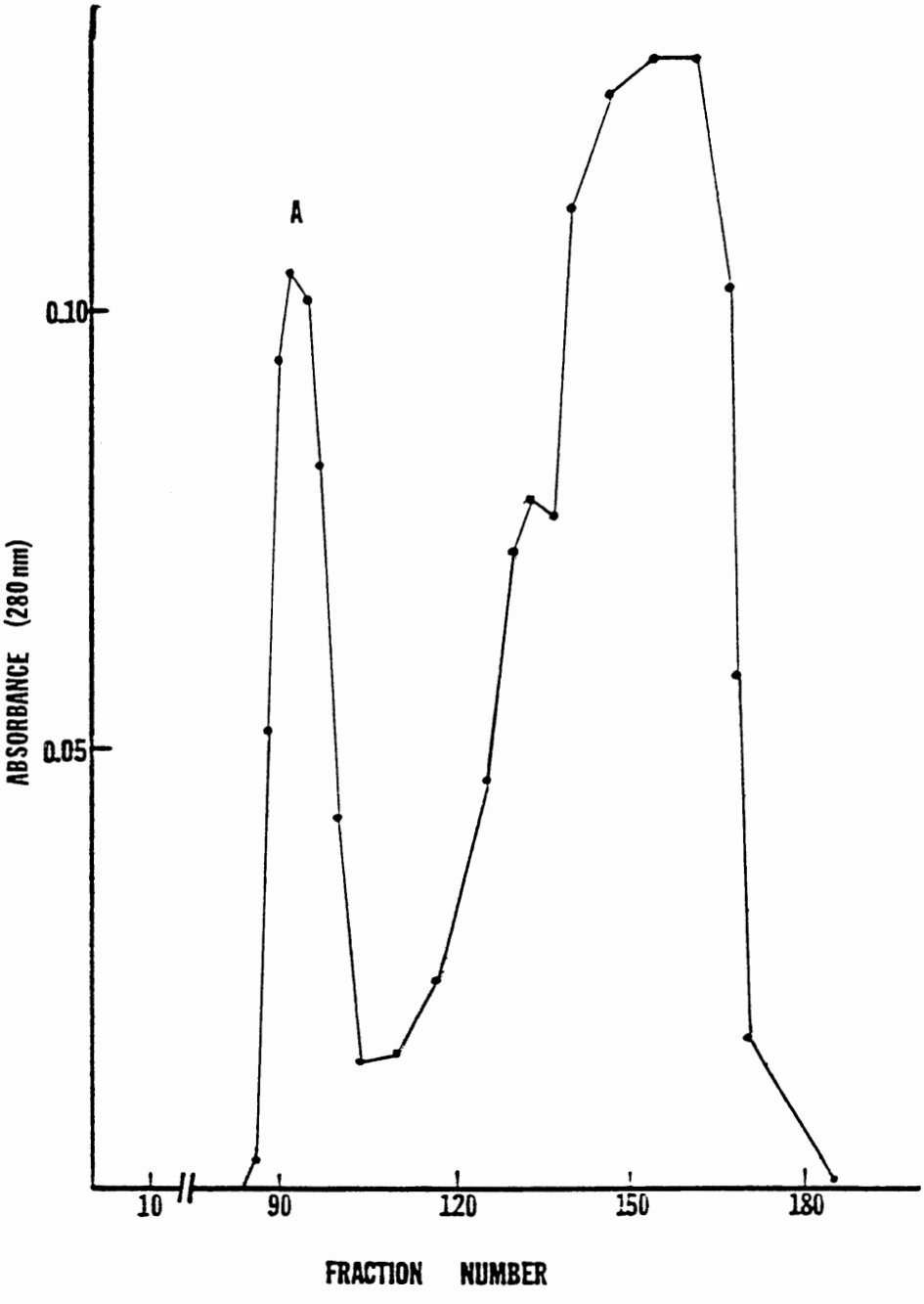



Figure 6. Sephadex G-200 chromatography of HTE cell mucin sample. Samples were dissolved in a 0.01 M sodium phosphate, 0.15 M NaCl buffer, pH 7.6, and applied to the G-200 column equilibrated with the same buffer. Two ml fractions were collected. A: retained, void volume or "mucin" peak. Each dot represents 6 experiments.

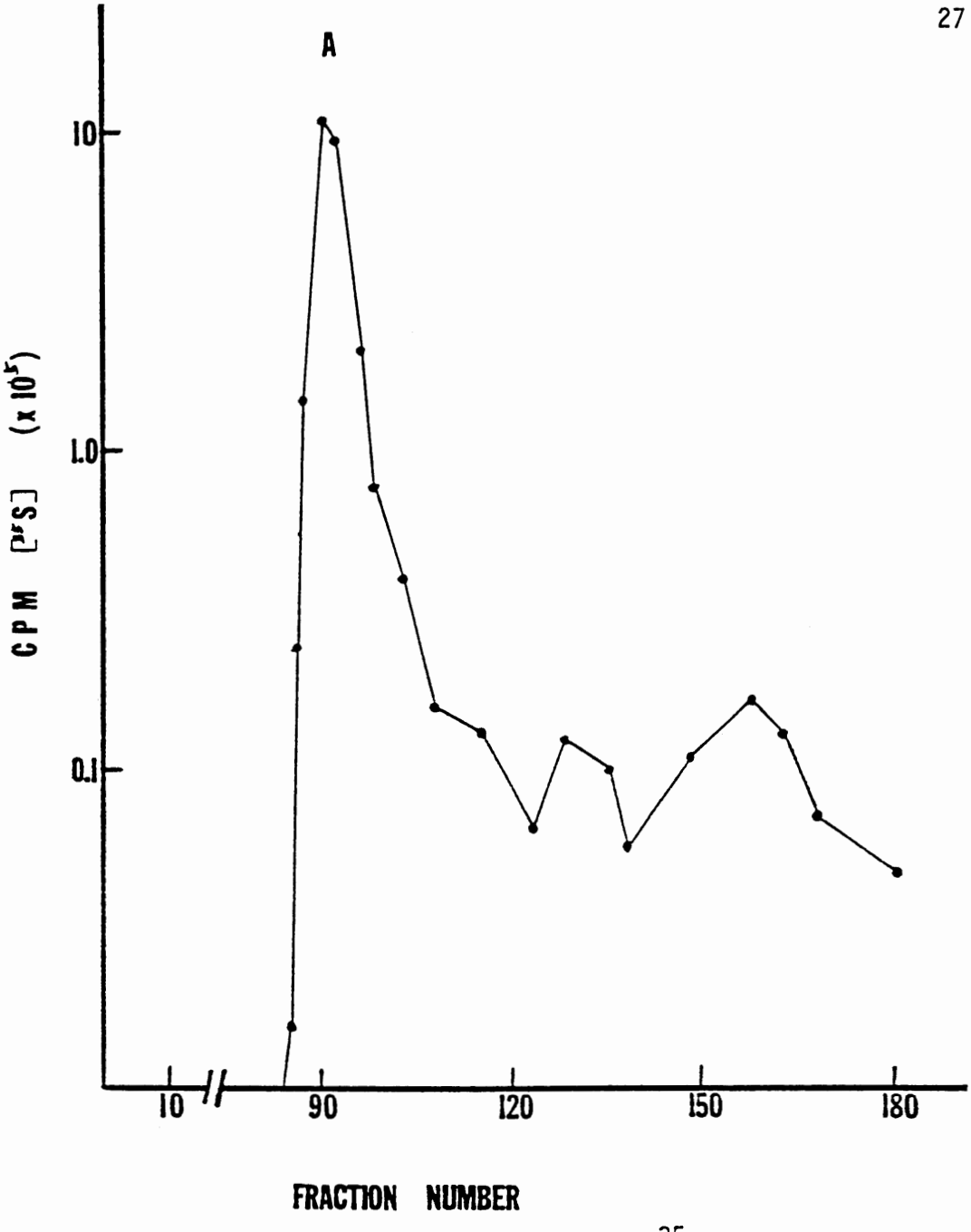



Figure 7. Sephadex G-200 chromatography of ($^{35}$S)-labelled HTE cell mucin sample. HTE cells are labelled 24 h with ($^{35}$S). Media off these cells is dialyzed, lyophilized, and then dissolved in 0.01 M sodium phosphate, 0.15 M NaCl buffer, pH 7.6. The sample is then applied to the G-200 column equilibrated in the same buffer. Two ml fractions are collected. A: void volume or "mucin" peak. Each dot represents 3 experiments.

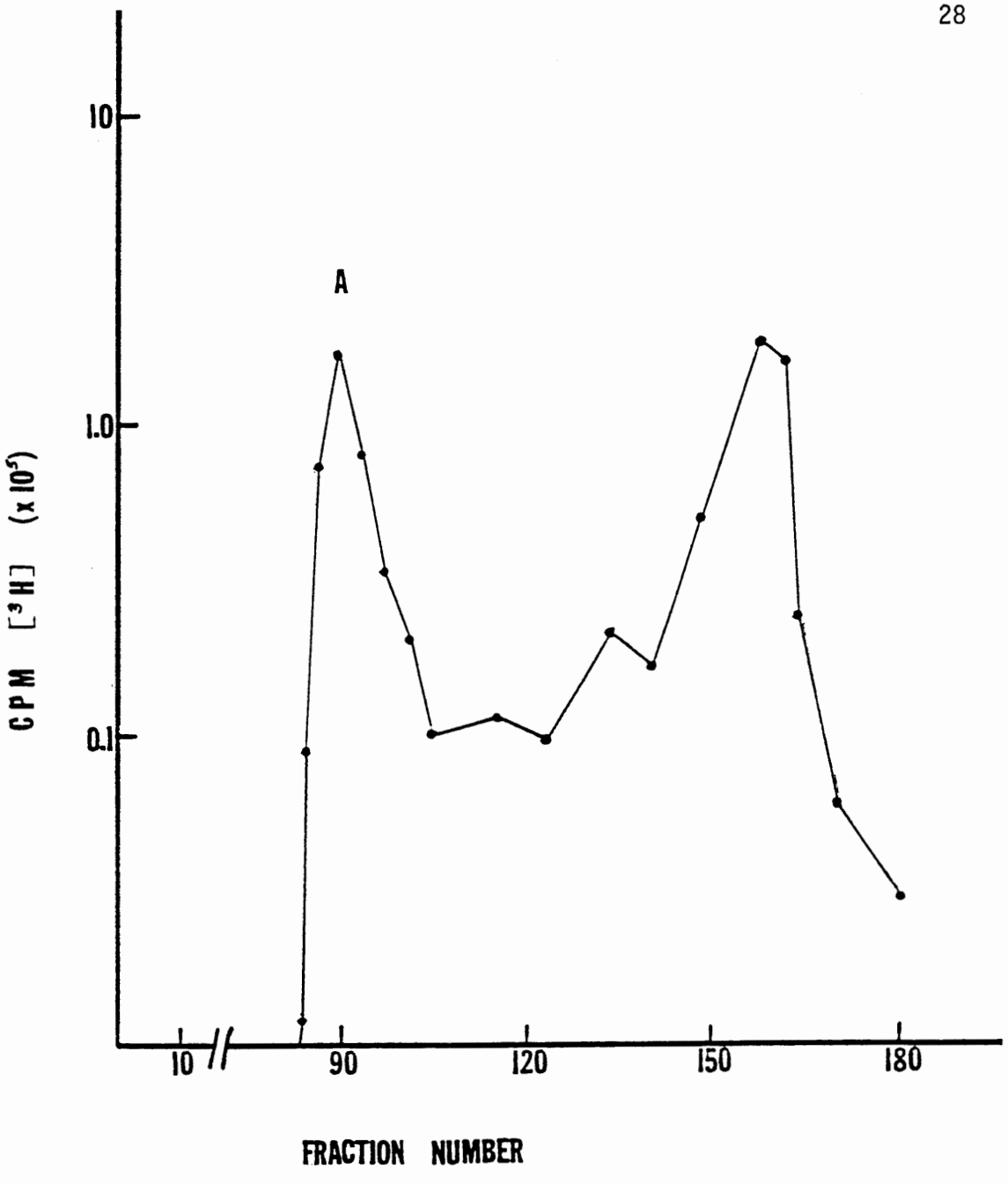



Figure 8. Sephadex G-200 chromatography of ($^{3}$H)-glucosamine labelled HTE cell mucin sample. HTE cells are labelled 24 h with ($^{3}$H)-glucosamine. Media off these cells is dialyzed, lyophilized, and then dissolved in 0.01 M sodium phosphate, 0.15 M NaCl buffer, pH 7.6. The sample is then applied to the G-200 column equilibrated in the same buffer. Two ml fractions are collected. A: void volume or "mucin" peak. Each dot represents 2 experiments.

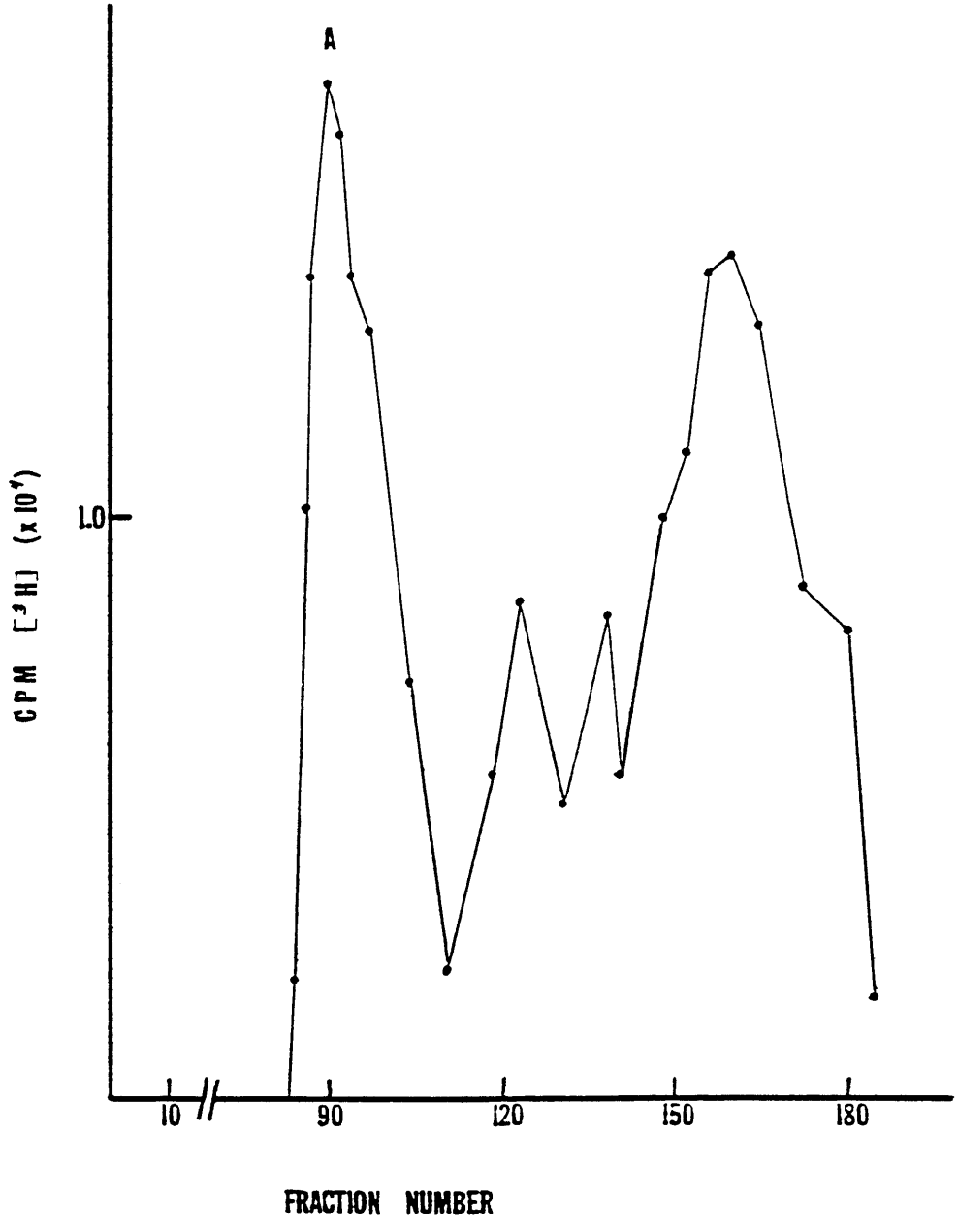



Figure 9. Sephadex G-200 chromatography of (2-$^{3}$H)-mannose labelled HTE cell mucin sample. HTE cells were labelled 24 h with (2-$^{3}$H)-mannose. Media off these cells was dialyzed, lyophilized and then dissolved in 0.01 M sodium phosphate, 0.15 M NaCl buffer, pH 7.6. The sample was then applied to the G-200 column equilibrated in the same buffer. Two ml fractions were collected. A: void volume or "mucin" peak. Each dot represents 2 experiments.

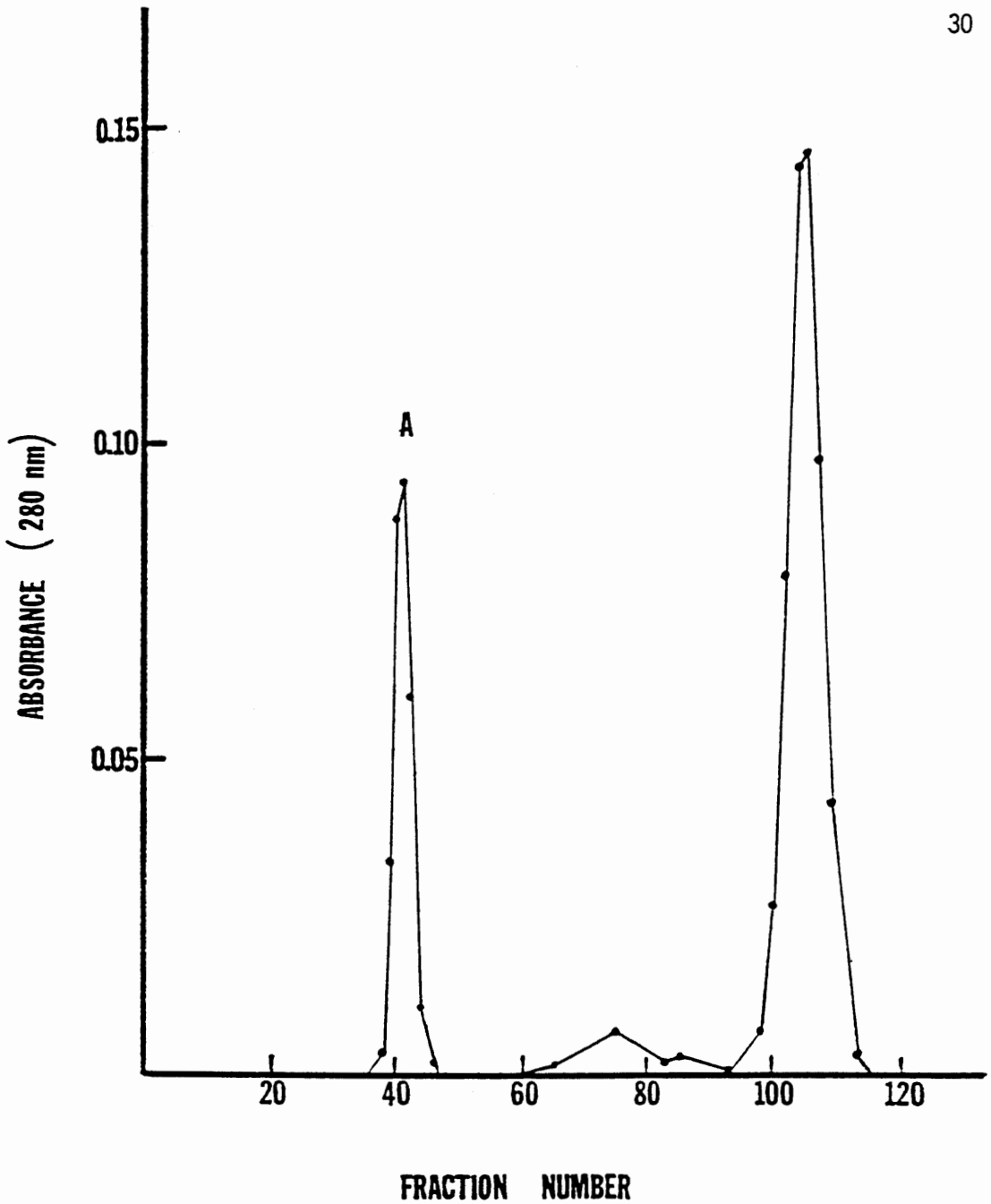



Figure 10. Bio-gel A-5M chromatography of HTE cell mucin sample. Lyophilized samples were treated with 0.25 mg/ml hyaluronidase for 18 h and then applied to the A-5M column equilibrated with 0.01 M sodium phosphate, 0.15 M NaCl, pH 7.6. Two ml fractions were collected. A: void volume or "mucin" peak. Each dot represents 6 experiments.

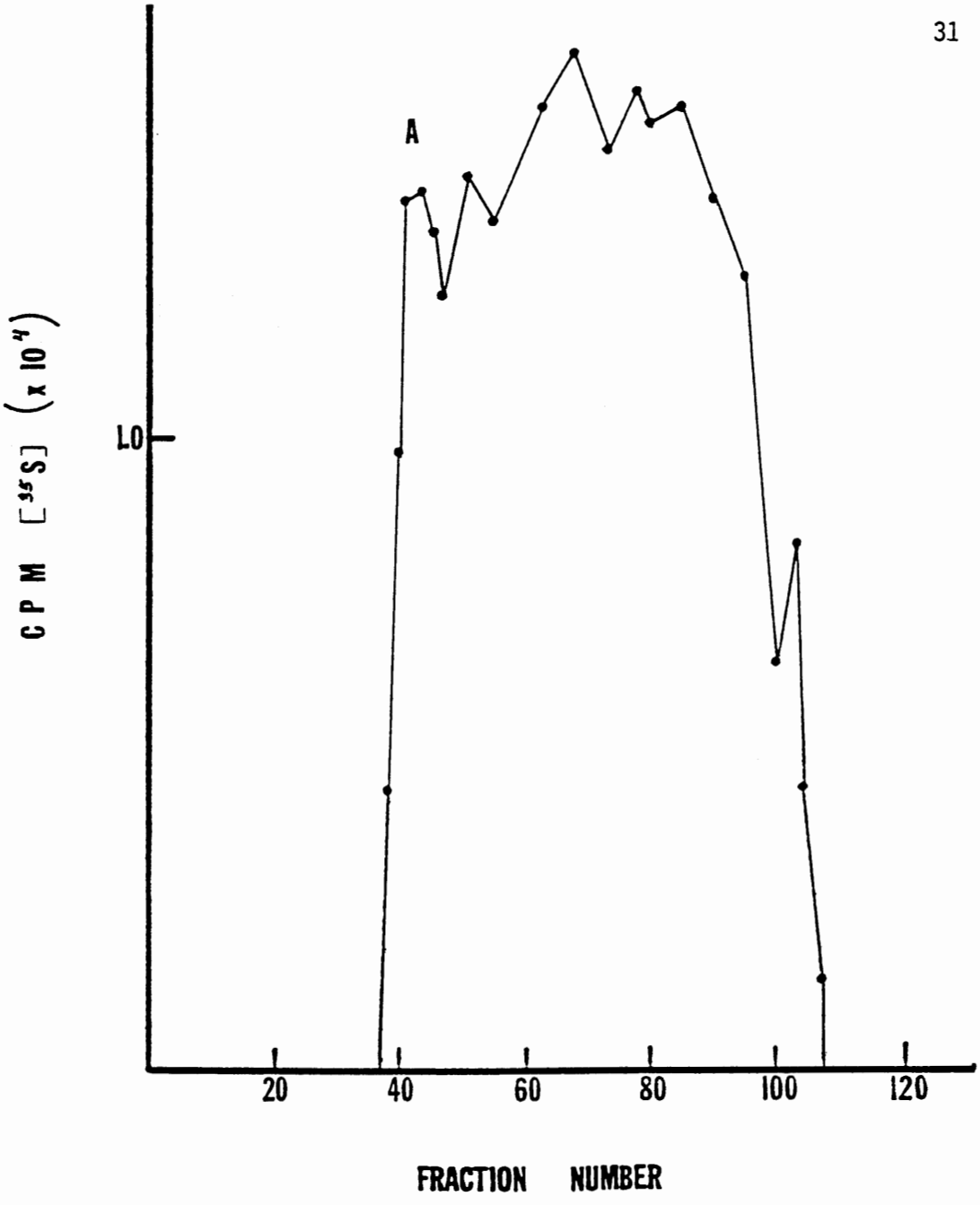



Figure 11. Bio-gel A-5M chromatography of ($^{35}$S)-labelled HTE cell mucin sample. HTE cells were labelled 24 h with ($^{35}$S). Media off these cells was run through a Sephadex G-200, treated with hyaluronidase for 18 h and then applied to the A-5M column equilibrated in a 0.01 M sodium phosphate, 0.15 M NaCl buffer, pH 7.6. Two ml fractions were collected. A: void volume or "mucin" peak. Each dot represents 3 experiments.

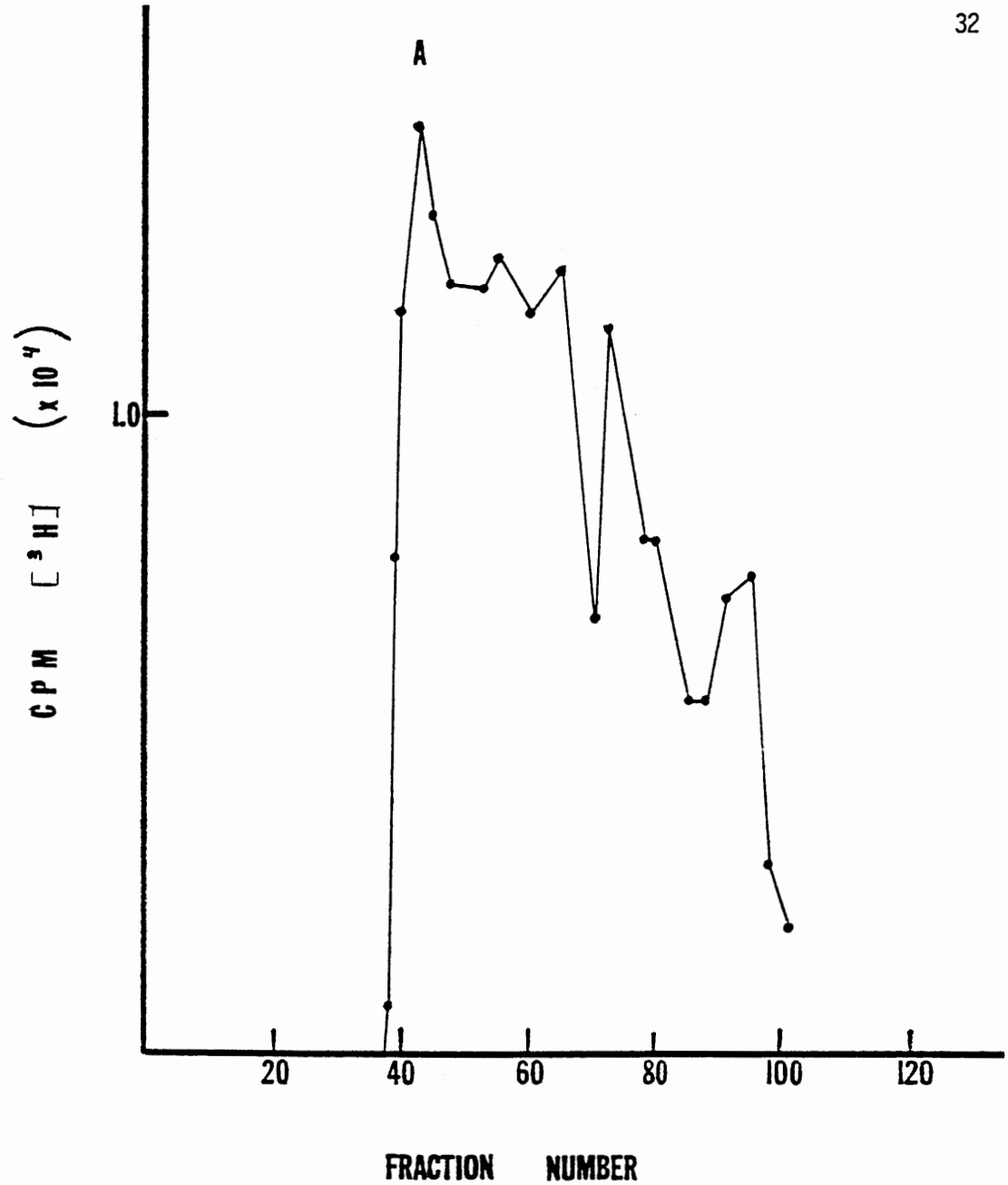



Figure 12. Bio-gel A-5M chromatography of ($^{3}$H)-glucosamine labelled HTE cell mucin sample. HTE cells were labelled 24 h with ($^{3}$H)-glucosamine. Media off these cells was run through a Sephadex G-200, treated with hyaluronidase, and then applied to the A-5M column equilibrated in a 0.01 M sodium phosphate, 0.15 M NaCl buffer, pH 7.6. Two ml fractions were collected. A: void volume or "mucin" peak. Each dot represents 2 experiments.

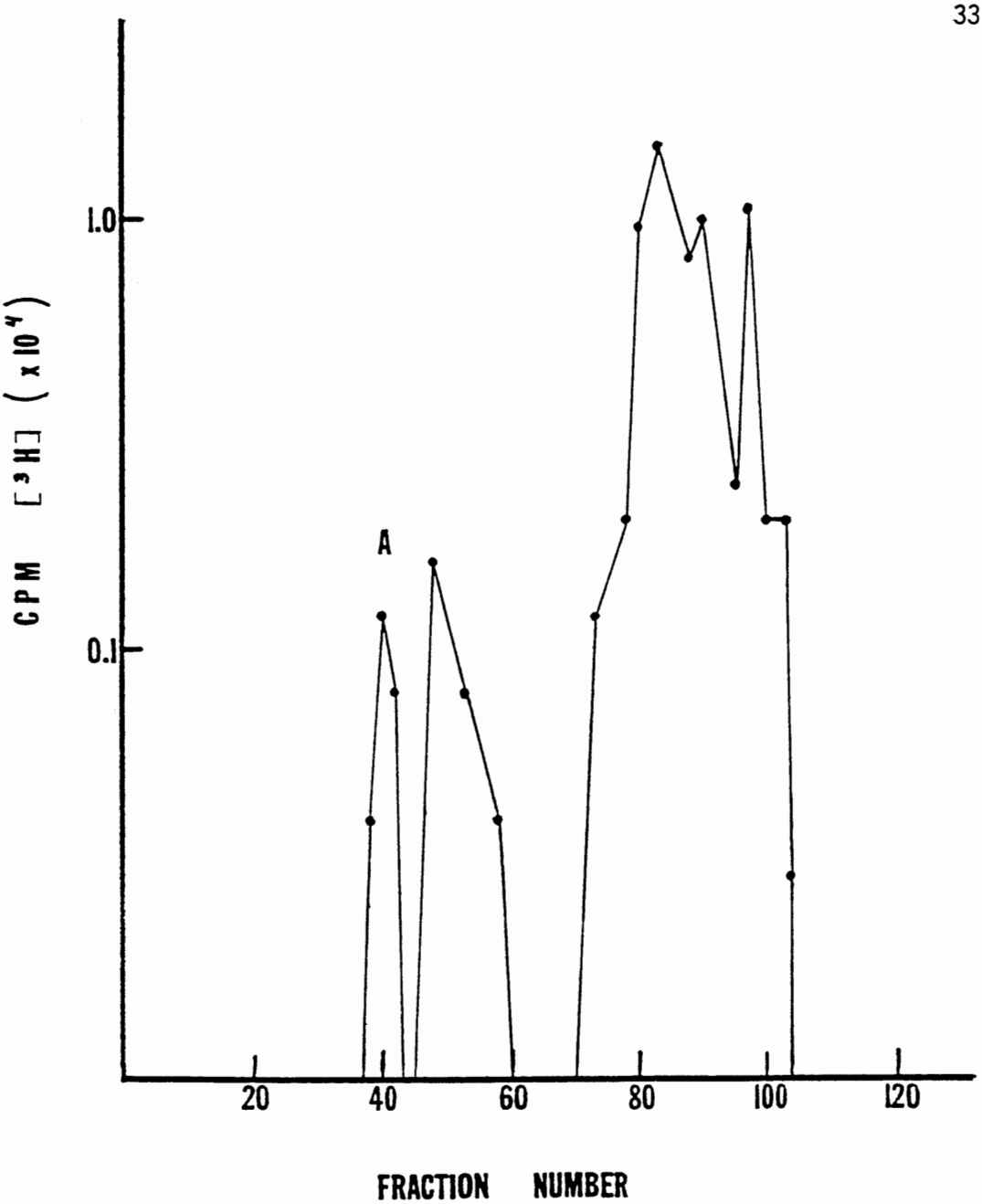



Figure 13. Bio-gel A-5M chromatography of (2-$^{3}$H)-mannose labelled HTE cell mucin sample. HTE cells were labelled 24 h with (2-$^{3}$H)-mannose, Media off these cells was run through a Sephadex G-200, treated with hyaluronidase, and then applied to the A-5M column equilibrated with 0.01 M sodium phosphate, 0.15 M NaCl buffer, pH 7.6. Two ml fractions were collected. A: void volume or "mucin" peak. Each dot represents 2 experiments.

Figure 13 representing the (2-$^{3}$H)-mannose labelled sample. In the ($^{35}$S) and ($^{3}$H)-glucosamine labelled samples, the radioactive counts were spread throughout the column, with only about 19% of the ($^{35}$S) and 32% of the ($^{3}$H)-glucosamine label found in the mucin fraction. In the case of the (2-$^{3}$H)-mannose label, most of the counts are found in the material that comes off the column after the void volume fraction, with only 1% found in the void volume. In all three cases, only the void volume fraction was retained, so much of the radioactive label was lost at this step. The retained void volume peaks off the A-5M column were combined, dialysed against deionized water and lyophilyzed. This fraction was considered to be the isolated HTE mucin.

Mucin Characterization

The isolated mucins were tested for protein, hexose, hexosamine, sialic acid and sulfate contents, as described in Methods. The results of these assays are giving in Table 1. Hexose and hexosamine levels were very similar between the human and HTE mucin samples. Sialic acid levels were lower in the human mucin than the HTE. Levels of all the compounds tested were found to be within ranges of values reported in the literature. Human mucin was found to be 13.25% protein by weight, while HTE mucin was 17.0% protein by weight.

Both HTE and human mucin isolates were $\beta$-eliminated with NaOH,

# TABLE I

## MUCIN COMPOSITION

| | HUMAN [a] | HTE [a] | REPORTED+ RANGES |
|---|---|---|---|
| HEXOSE | 0.90 | 1.04 | 0.61-1.92 |
| HEXOSAMINE | 1.90 | 1.80 | 0.82-2.27 |
| SIALIC ACID | 0.28 | 0.45 | 0.20-0.99 |
| SULFATE | 0.07 | NA | 0.03-0.61 |

[a] VALUES GIVEN ARE MG/MG PROTEIN, WHICH IS 13.25% BY WEIGHT OF TOTAL HUMAN MUCIN MOLECULE, AND 17.0% BY WEIGHT OF TOTAL HTE MUCIN MOLECULE.

+ THESE RANGES WERE TAKEN FROM VALUES REPORTED IN THE FOLLOWING SOURCES: JABBAL, ET.AL.,1976; CHACE, ET.AL.,1985; SACHDEV, ET.AL,1980; HERZBERG, ET.AL.,1979. ALL VALUES ARE MG/MG PROTEIN.

Alkaline β-elimination causes the type of reaction shown in Figure 14, where the addition of NaOH causes the release of the carbohydrate chain and the formation of an unsaturated derivative of serine or threonine. These derivatives have a characteristic absorption in the ultraviolet spectrum, so their formation can readily be detected at 240 nm as is shown in Figure 15. When this reaction was carried out in the presence of $NaBH_4$, the released sugar was converted to the corresponding sugar alcohol and the serine and threonine derivatives were converted to alanine and 2-aminobutyric acid respectively. In the presence of $(^3H)$-$NaBH_4$, both the oligosaccharide chain and the peptide chain would be radioactively labelled this way. As can be seen in Figure 16, β-elimination carried out under reducing conditions (in the presence of $NaBH_4$) gives an immediate rise in absorbance caused by the formation of the double bonds, followed by a decrease as the double bonds are saturated.

Oligosaccharide chain analysis

After β-elimination the oligosaccharide chains were separated from the peptide core of the mucin molecule by Bio-Gel $P_{10}$ column chromatography. With the human mucin samples, β-elimination was carried out in the presence of $(^3H)$-$NaBH_4$ in order to radioactively label the oligosaccharide chains. Figure 17 shows the protein profile and the pattern of radioactivity off the $P_{10}$ column. All the human mucin samples show this same basic profile, with the peptide core being

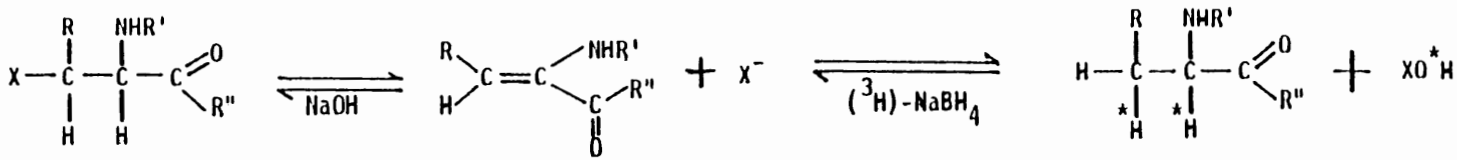



Figure 14. The $\beta$-elimination reaction. X : the oligosaccharide side chain. R : H for serine, $CH_3$ for threonine. R' and R" : other amino acids of the peptide chain. *H : tritiated hydrogen molecules from the $(^3H)$-$NaBH_4$.

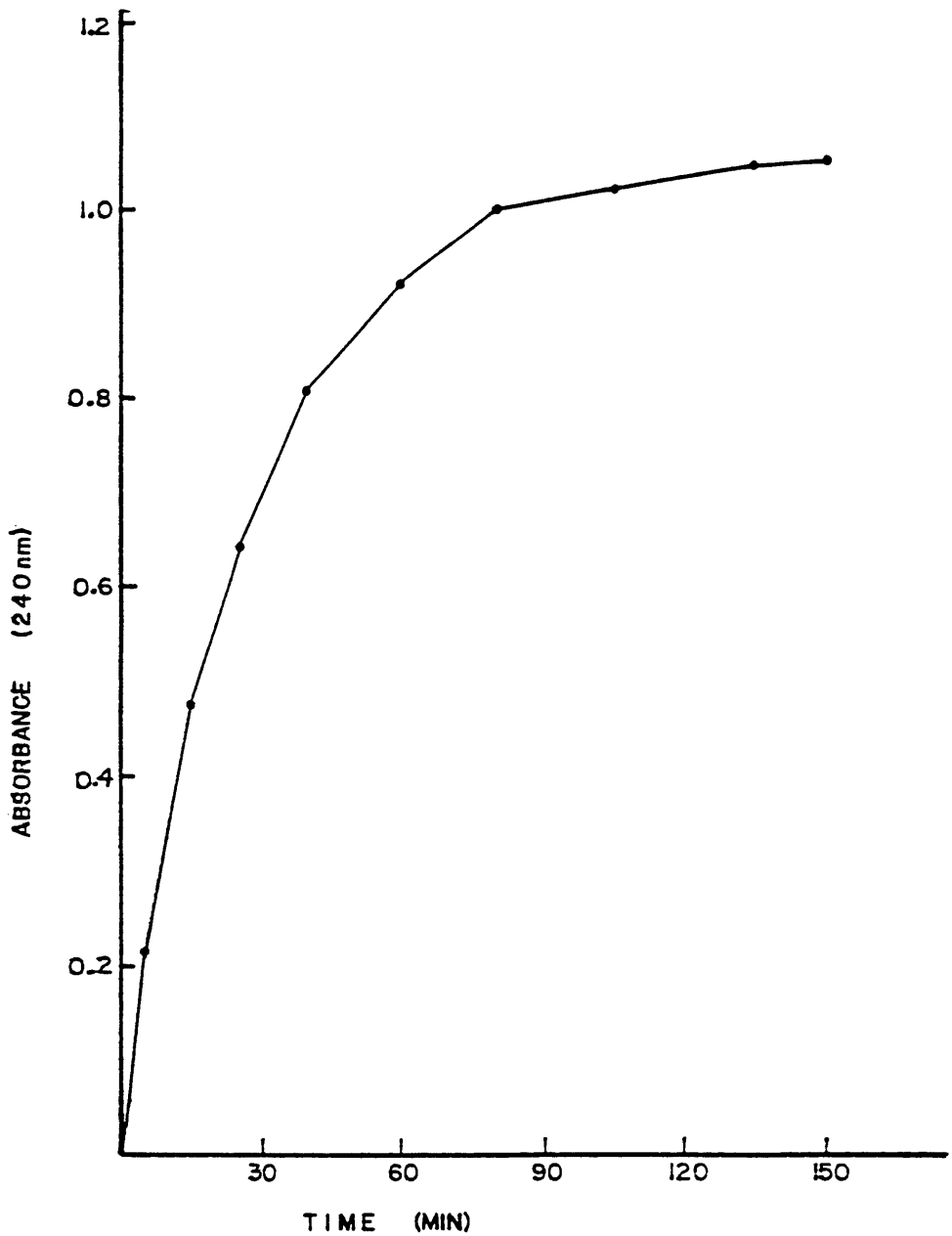



Figure 15. Alkali-catalyzed $\beta$-elimination of mucin sample. Mucin sample was dissolved in 0.5 N NaOH at a 2 mg/ml concentration and incubated at 50 °C. Absorbance at 240 nm was measured at various time points. Each dot represents 15 experiments.

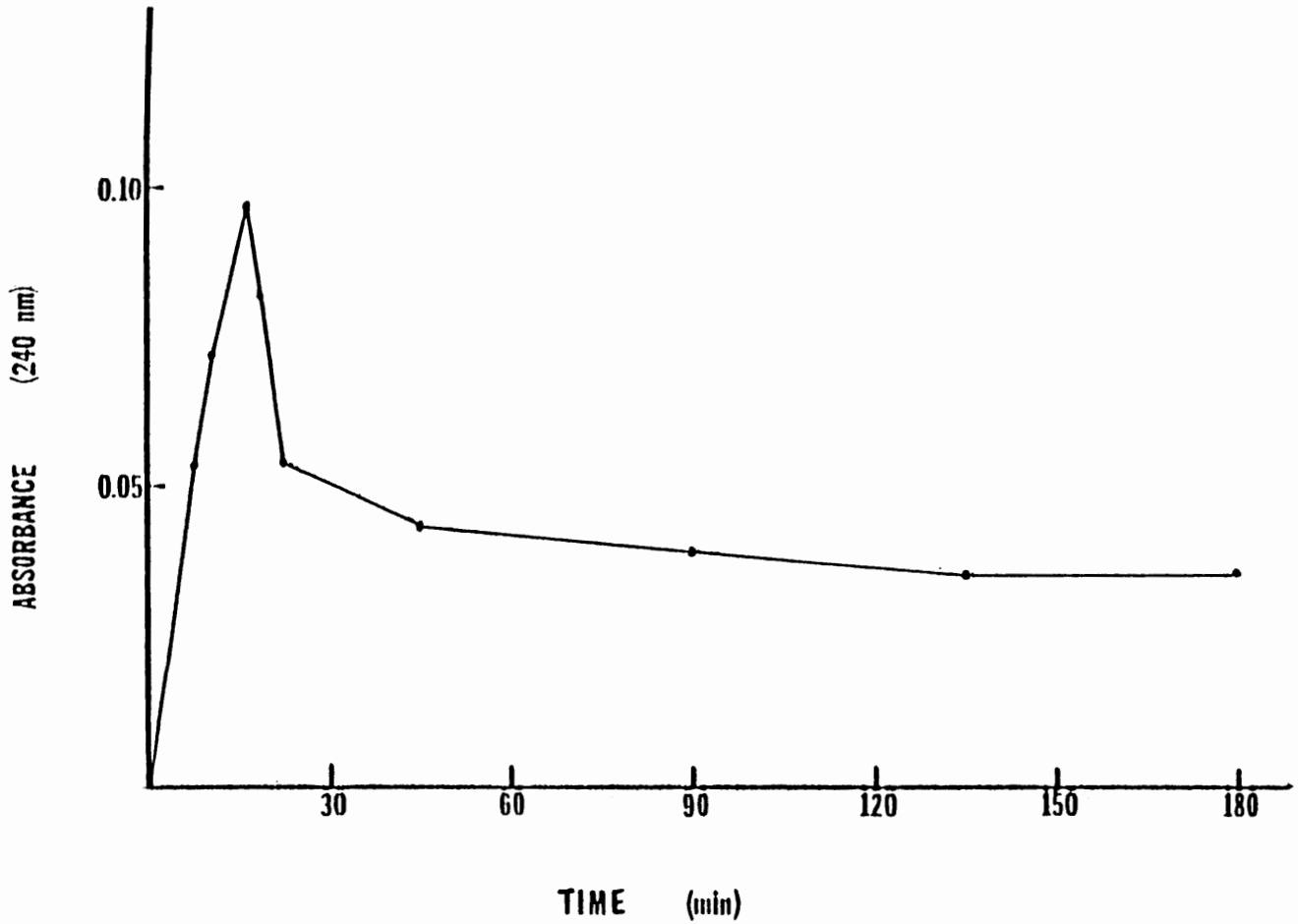



Figure 16. Alkali-catalyzed $\beta$-elimination of mucin sample under reducing conditions. Mucin sample was dissolved in 0.5 N NaOH, 0.5 N $NaBH_4$ at a 2 mg/ml concentration and incubated at 50 °C. Absorbance at 240 nm was measured at various time points. Each dot represents 8 experiments.

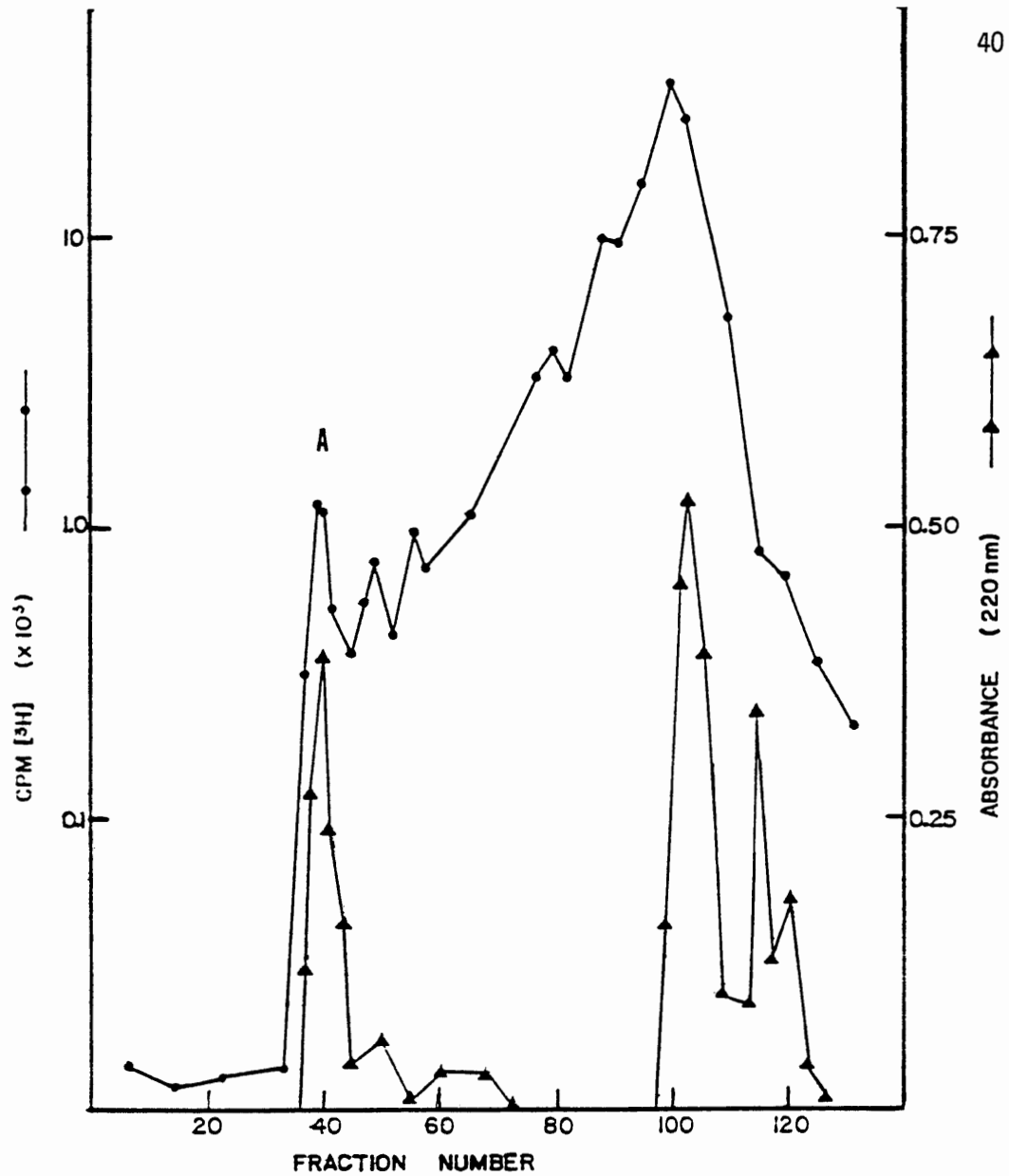



Figure 17. Bio-gel P10 chromatography of mucin sample $\beta$-eliminated in the presence of $(^3H)$-$NaBH_4$. Mucin sample was $\beta$-eliminated in 0.5 N NaOH, 0.5 N $NaBH_4$ with $(^3H)$-$NaBH_4$ present. The final product was dried on a rotary evaporator, dissolved in deionized water and applied to the P10 column equilibrated in 0.005 M sodium phosphate, 0.075 M NaCl buffer, pH 7.6. Half ml fractions were collected. ●——●——● : CPM profile off column, ▲——▲——▲ : protein profile off column, A: peptide peak. Each dot represents 10 experiments.

in the first protein peak, the void volume peak, and the oligosaccharides coming out later. While there was some radioactivity associated with the peptide core, as is expected, most of the radioactivity present is associated with the oligosaccharide chains.

When the HTE mucin samples were $\beta$-eliminated, this same basic profile was observed, with the metabolic label being associated with the oligosaccharide chains (Figure 18). HTE mucin samples labelled with (2-$^3$H)-mannose were not $\beta$-eliminated since there was not enough label left after isolation to allow it to be followed further.

The fractions off the $P_{10}$ column comprising the oligosaccharide chains were combined, and dried down on a rotary evaporator. Further testing was done on this sugar pool. The sugar chains were dissolved in water and run on a $P_2$ sizing column. Figure 19 shows the sugar chain profile off the $P_2$ column. Carbohydrate chains off the $P_2$ column range in size from 1 sugar residue to 9 to 10 sugar residues long. The length can be determined by graphing the elution volume of the chains vs. molecular weight using the elution volumes of standard sugar chains of known molecular weights. Figure 20 shows the plot used for this purpose. Sugar chains from the original sugar pool were also run on ion-exchange columns, Dowex 1-x2 ($Cl^-$) and Dowex 50-x2 ($H^+$) to detect the presence of charges on these molecules. Samples were dissolved in water and applied to the columns. Adsorbed molecules were eluted off the Dowex 1-x2 ($Cl^-$) using 0.5 M NaCl and from the Dowex 50-x2 ($H^+$) using 1.5 N ammonium hydroxide. No sugar chains

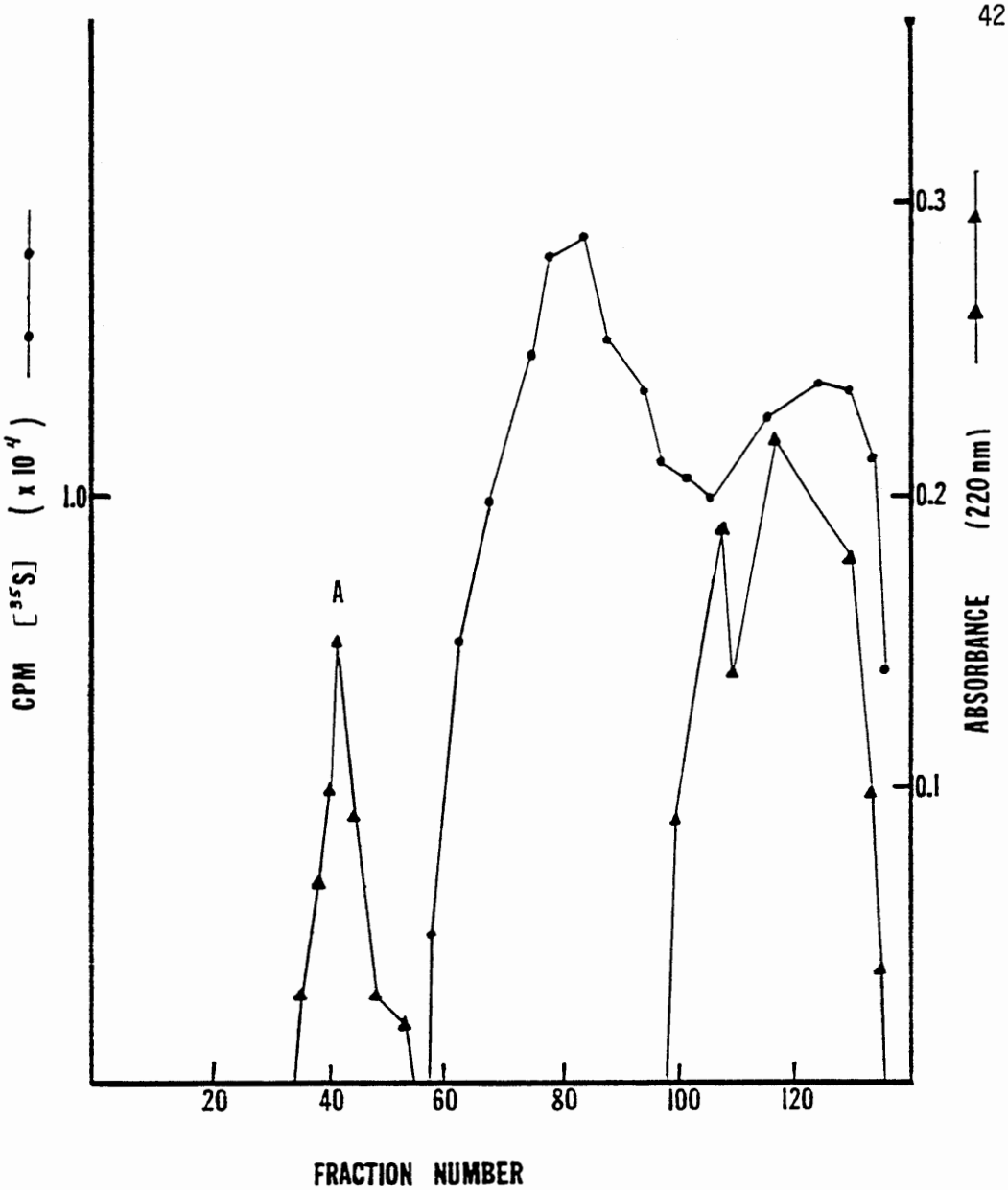



Figure 18. Bio-gel P10 chromatography of $\beta$-eliminated HTE mucin sample previously labelled with ($^{35}S$). Mucin sample was $\beta$-eliminated in 0.5 N NaOH, 0.5 N $NaBH_4$. The final product was dried on a rotary evaporator, dissolved in deionized water and applied to the P10 column equilibrated in 0.005 M sodium phosphate, 0.075 M NaCl, pH 7.6. Half ml fractions were collected. —●—●—: CPM profile off column. —▲—▲—: protein profile off column. A: peptide peak. Each dot represents 4 experiments.

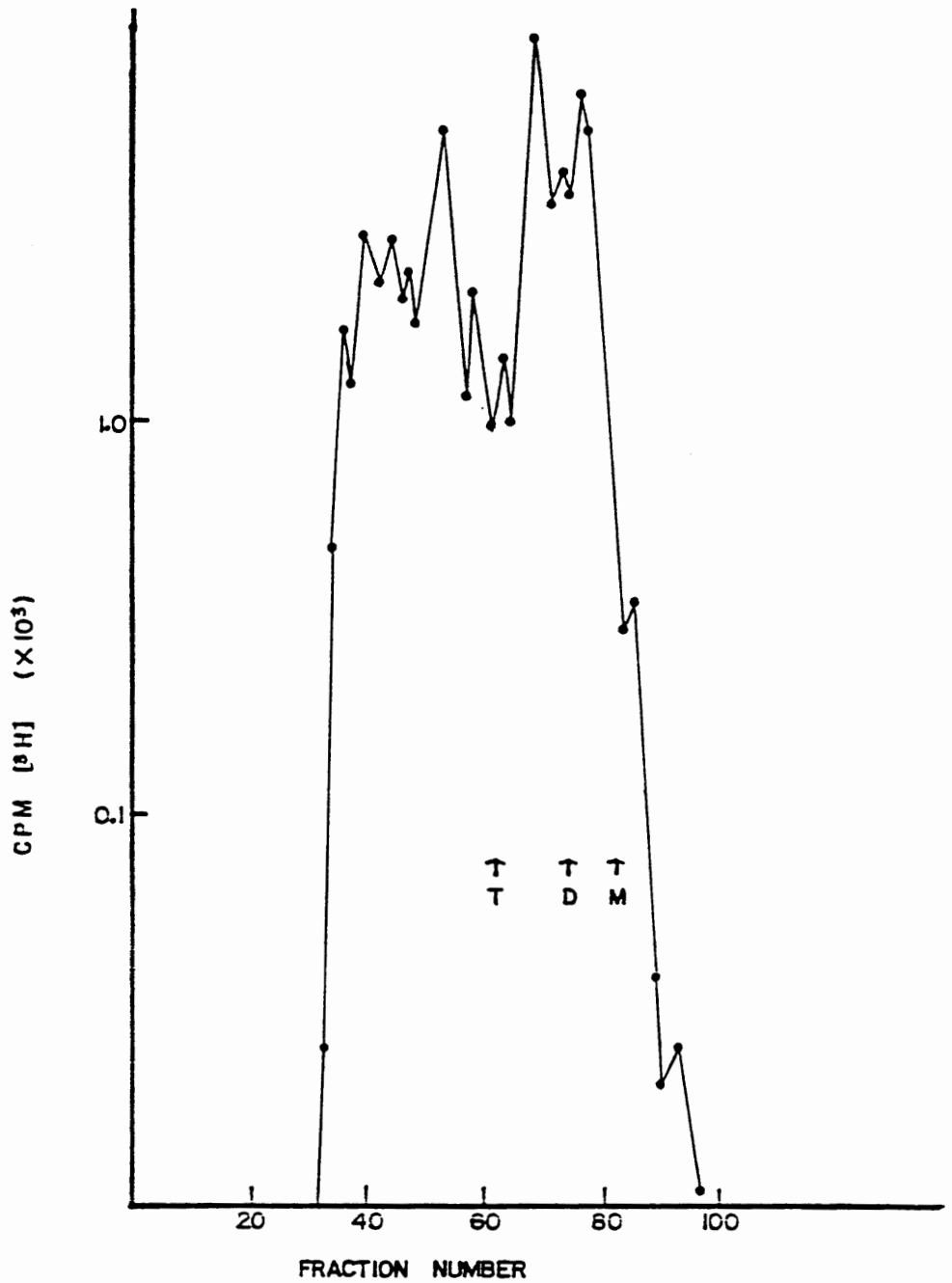



Figure 19. Bio-gel P2 chromatography of $\beta$-elimination-released oligosaccharide side chains from mucin sample. After the carbohydrate side chains were separated from the peptide core of the mucin on Bio-gel P10, they were dried down on a rotary evaporator, dissolved in deionized water and applied to the P2 column. Half ml fractions were collected. M: point where monosaccharide standard comes off the column. D: disaccharide standard. T: tetrasaccharide standard. Each dot represents 15 experiments.

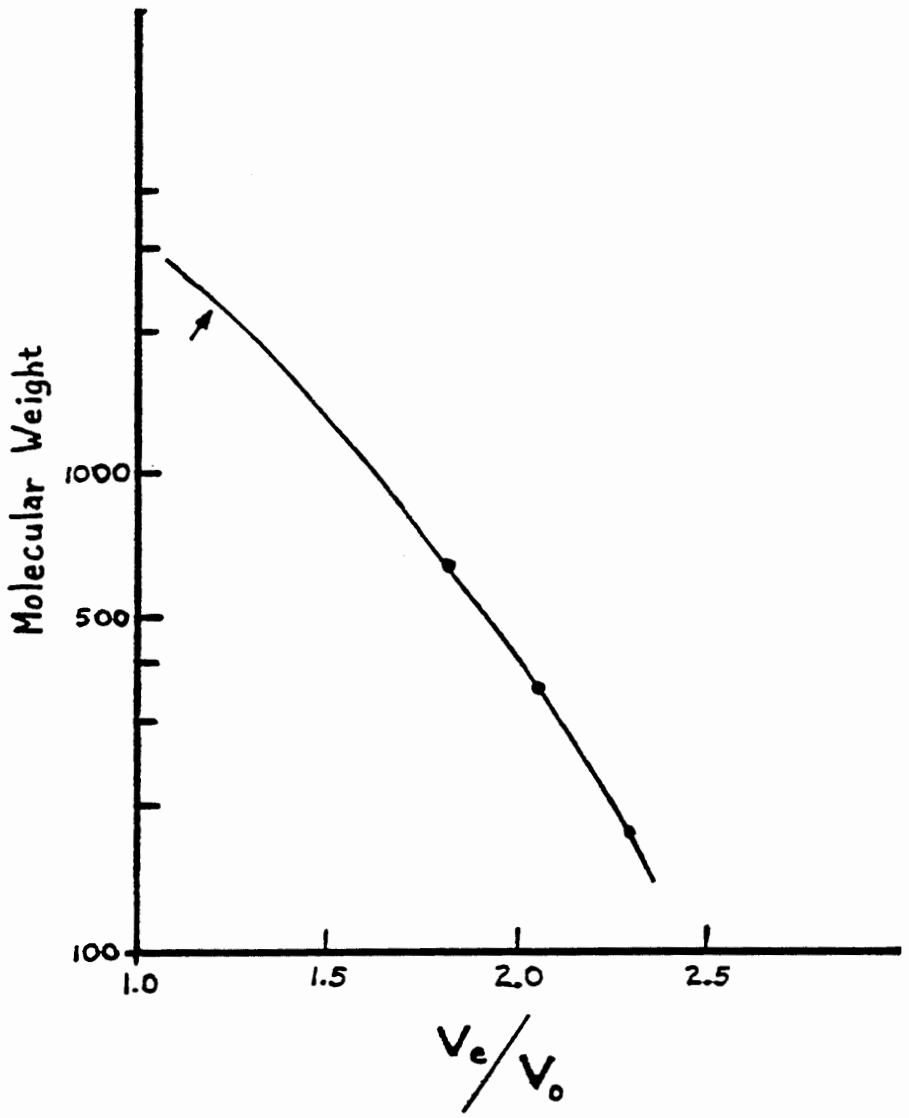



Figure 20. Molecular weight estimation of the oligosaccharide chains obtained by $\beta$-elimination. The Bio-gel P2 column was calibrated with mono-, di-, and tetrasaccharide standards (indicated by the dots). $\beta$-elimination-released sugar chains eluted from the P2 column all along this range. The arrow represents chains approximately 15 sugar residues long.

were retained on the Dowex 50-X2($H^+$) column suggesting that none of the molecules were positively charged. The pattern of radioactivity off the Dowex 1-X2($Cl^-$) column is shown in Figure 21. The two groups of carbohydrate side chains, negatively charged and neutral, were then run back on the $P_2$ sizing column separately. Figure 22 shows their profiles off the column. All the long carbohydrate chains, those approximately 7 residues long and longer, are negatively charged. The neutral sugar chains make up the medium and short chains. Oligosaccharide side chains released off the HTE mucin isolates demonstrated this same basic pattern

Negatively charged sugar chains were further tested to determine possible explanations for the negative charge. In order to test for the presence of N-acetyl neuraminic acid, the negatively charged chains were treated with neuraminidase, as described in Methods, to remove the sialic acid and then rerun through a Dowex 1-X2($Cl^-$) to determine loss of negative charge. The samples off the Dowex column were then put back through the $P_2$ column. The chromatography results are shown in Figure 23. Whenever the long, negatively charged carbohydrate chains were treated with neuraminidase and the sialic acid removed, the same results occured. There remained a group of long chains that were still negatively charged. There were also five distinct classes of chains that were shorter and no longer negatively charged.

Those carbohydrate chains still negatively charged after neuraminidase treatment were tested for the presence of esterified

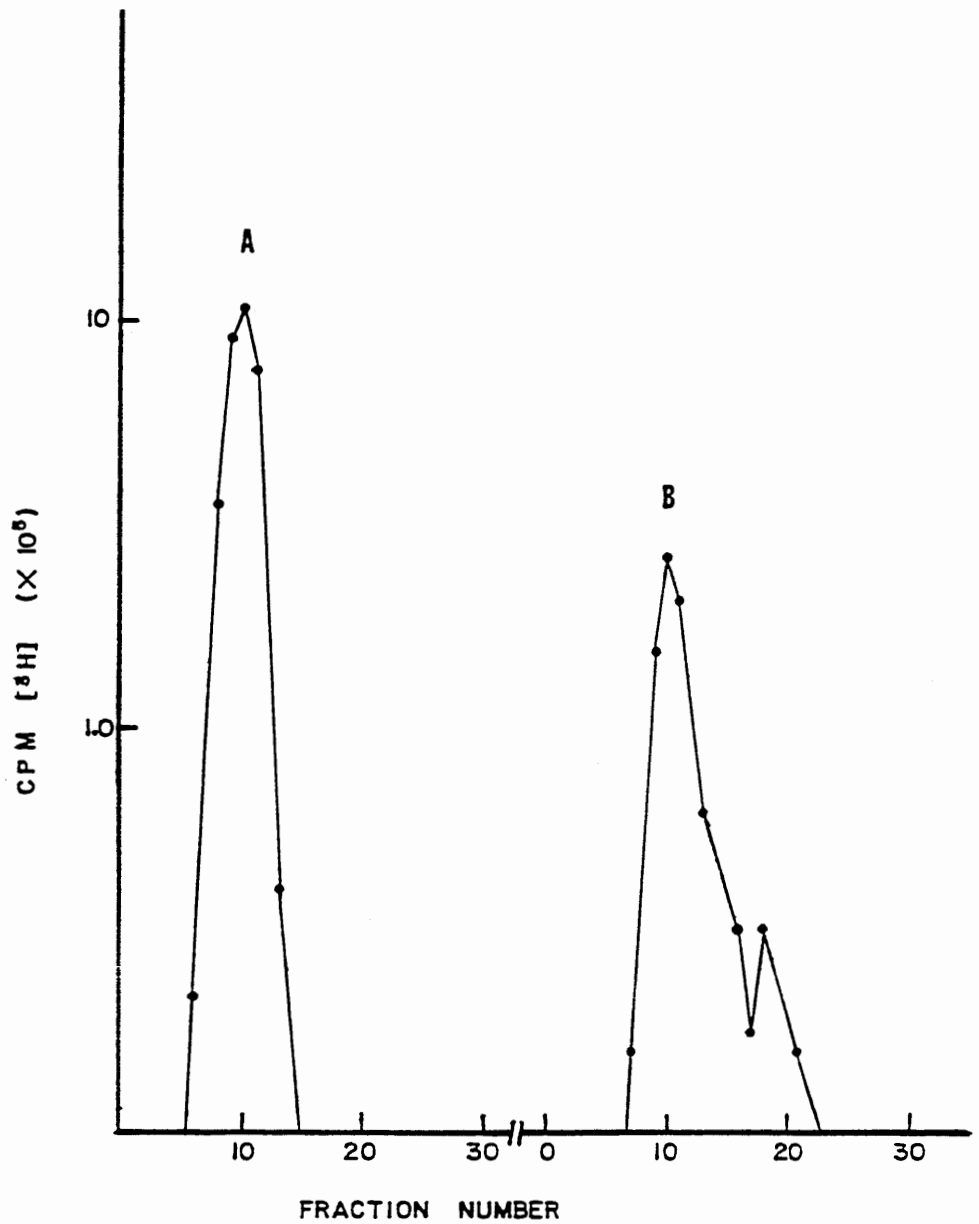



Figure 21. Dowex 1-x2 ($Cl^-$) ion-exchange chromatography of oligosaccharide side chains released from mucin sample. Oligosaccharide side chains were dissolved in deionized water and applied to the Dowex 1-x2 column. Negatively charged chains that stuck to the column were eluted using 0.5 N NaCl. One ml fractions were collected. A: neutral chains that did not stick to the column. B: negatively charged chains eluted with NaCl. Each dot represents 16 experiments.

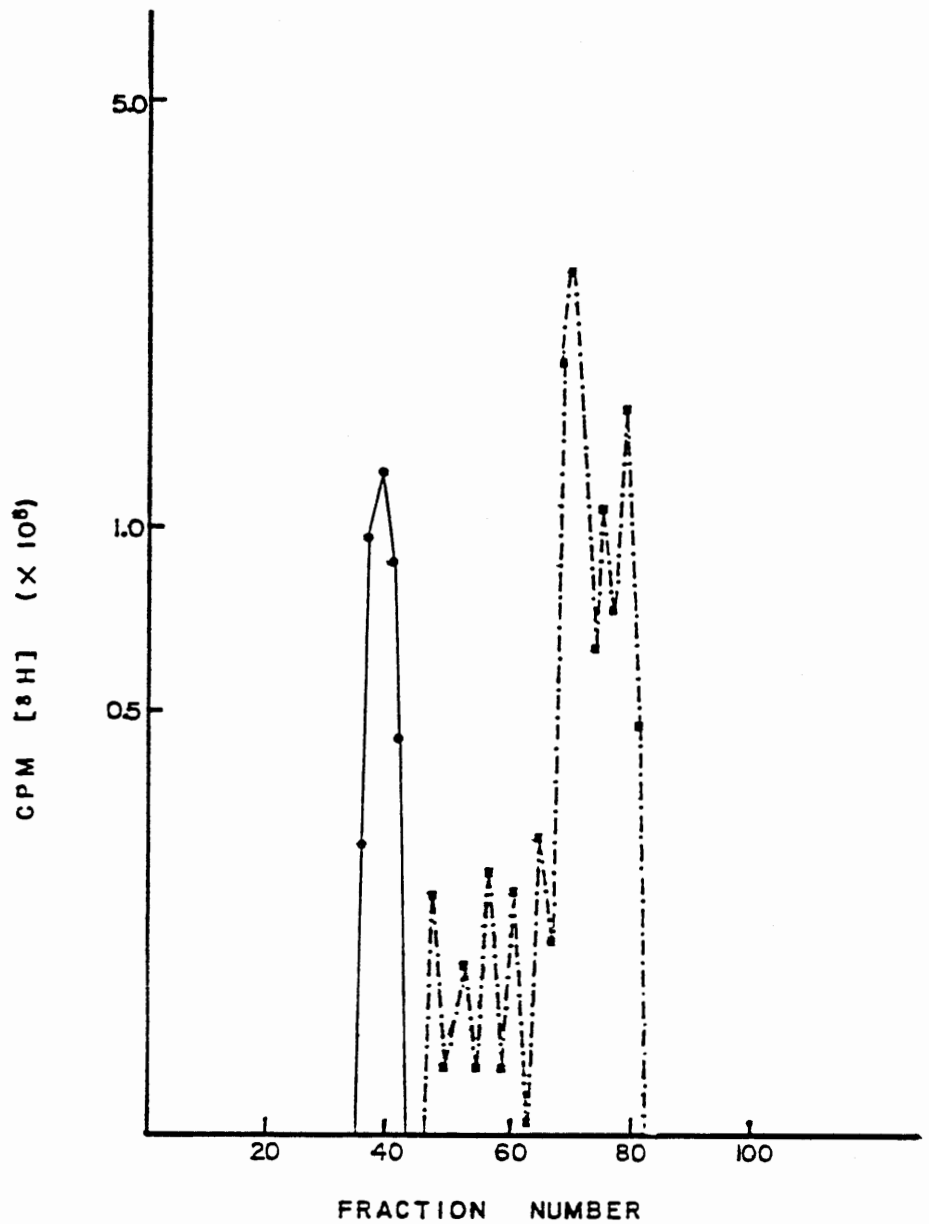



Figure 22. Bio-gel $P_2$ chromatography of oligosaccharide side chains off human mucin sample. After being separated by ion-exchange chromatography, the negatively charged and neutral chains were separately dried down, dissolved in deionized water and applied to the $P_2$ column. One and a half ml fractions were collected. —●—●—: negatively charged carbohydrate chains. —■··—·■··—: neutral carbohydrate chains. Each dot represents 8 experiments.

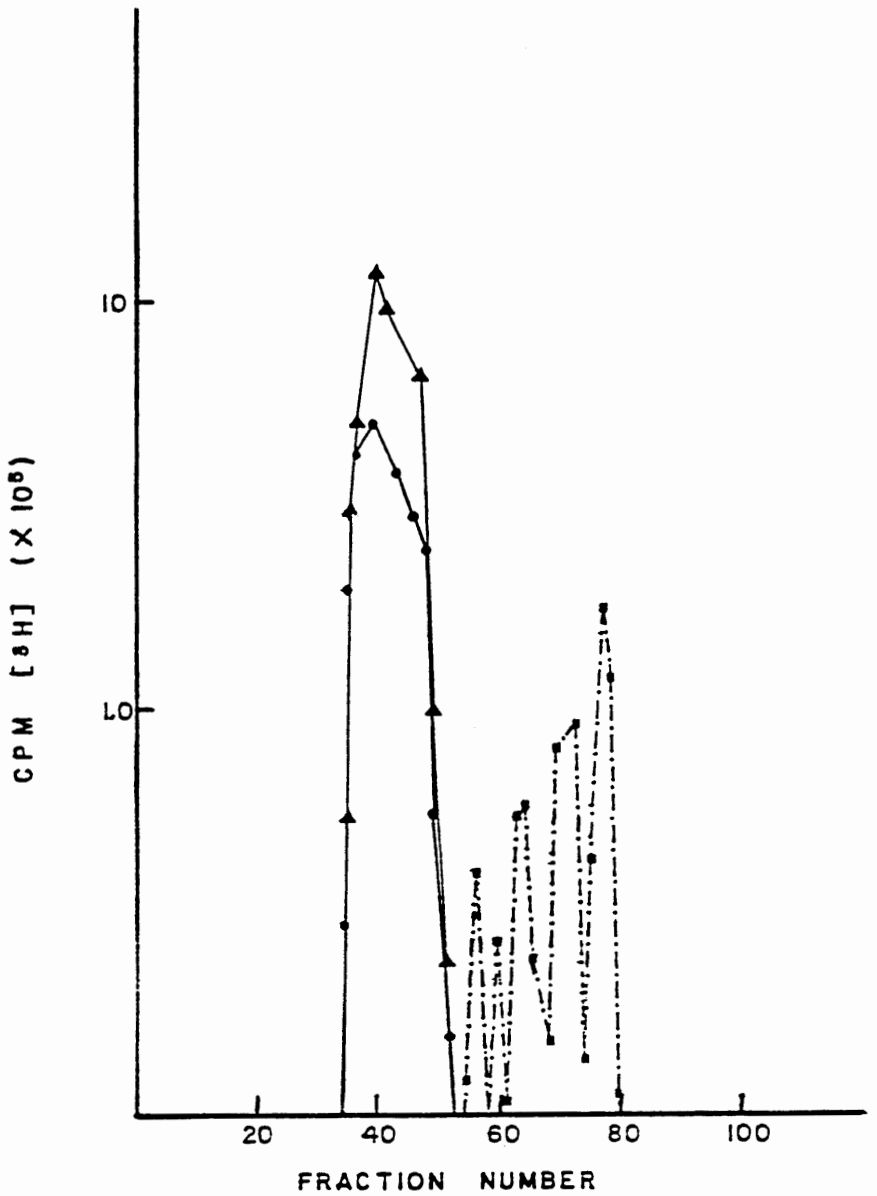



Figure 23. Bio-gel $P_2$ chromatography of neuraminidase treated oligosaccharide side chains. Negatively charged carbohydrate side chains were treated with neuraminidase, separated via ion-exchange chromatography, and then the different groups were applied to the $P_2$ column separately. One and a half ml fractions were collected.
—▲—▲—: negatively charged oligosaccharide chains;
—●—●—: negatively charged chains after neuraminidase;
-■··■-: neutral chains after neuraminidase. Each dot represents 5 experiments.

sulfate residues. The residues were removed from the sugar chains via methanolysis and then assayed for. The sugar chains were then retested for loss of negative charge on the ion-exchange column. Figure 24 shows the $P_2$ column profile of these chains after methanolysis and Dowex 1-X2($Cl^-$) chromatography. Release of the sulfate residues resulted in a range of sizes of neutral sugar chains, and there was still a group of long, negatively charged carbohydrate chains.

Polyacrylamide Gel Electrophoresis

Mucin was analysed via SDS-PAGE as described in the Methods section. Figure 25 shows total human mucin isolate run on a 2.2 to 6.0% linear gradient gel. The mucin appeared as a smear high in the gel. Figure 26 shows the peptide core from $\beta$-eliminated mucin samples run on a 10% gel. Many bands were found on the gels of the $\beta$-eliminated samples from both the human and HTE mucin samples.

Mucin Antibody Production

Antibody to the total human mucin molecule was produced in a rabbit as described in Methods. Figure 27 shows the results of double immunodiffusion experiments. Single precipitin lines were obtained between the antigen in the center well, the total human mucin molecule, and the serum obtained from the immunized rabbit's blood. The precipitin lines occured using straight rabbit's serum and also at a 1:2 dilution.

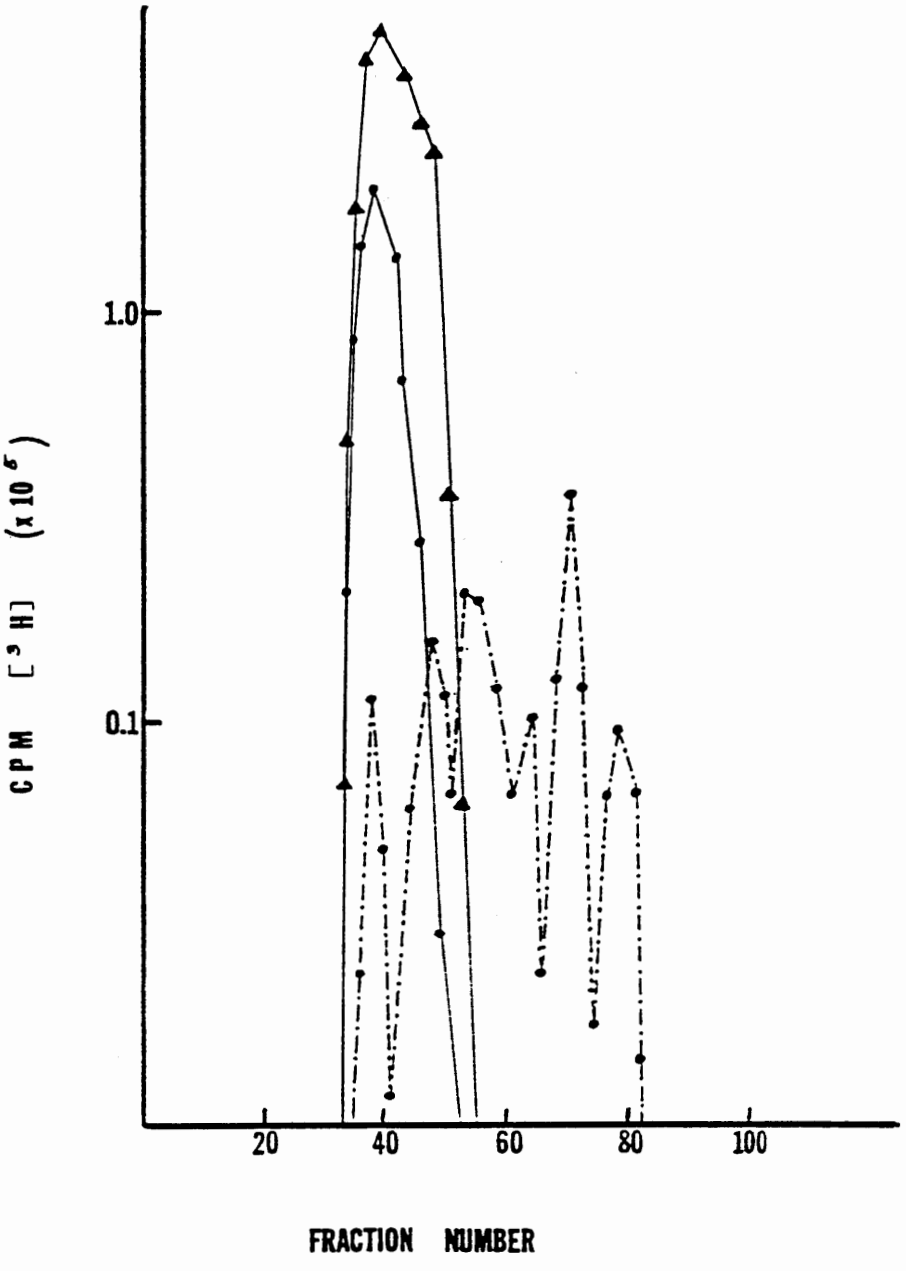



Figure 24. Bio-gel $P_2$ chromatography of oligosaccharide side chains with the sulfate removed. The sulfate residues were removed from the negatively charged chains via methanolysis. The chains were then separated via ion-exchange chromatography and the different groups were applied separately to the $P_2$ column. One and a half ml fractions were collected.

—▲—▲—: negatively charged oligosaccharide side chains
—●—●—: negatively charged chains after methanolysis
-■-·■-·-: neutral chains after methanolysis
Each dot represents 3 experiments.

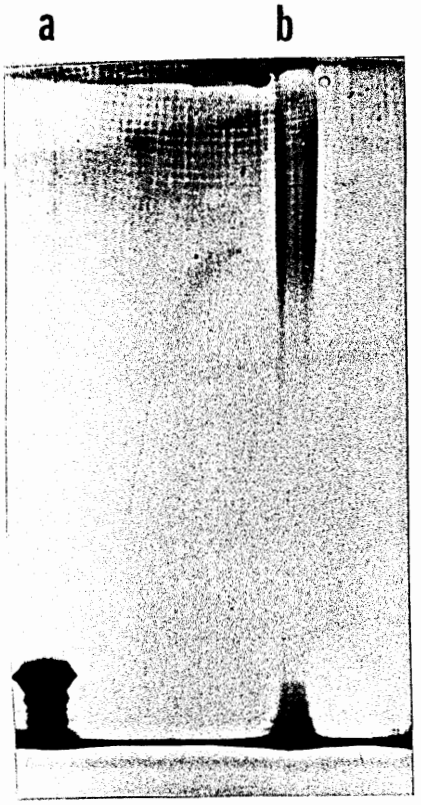



Figure 25, Human mucin isolate on a 2.2 to 6.0% SDS-polyacrylamide linear gradient slab gel. The mucin isolate was dissolved in 800 µl Tris, pH 6.8, 200 µl 10% SDS, 50 µl β-mercaptoethanol, boiled 3 min, and then vortexed with 10 µl glycerol and 3 µl tracking dye. This mixture was then applied to the gel. The gel was stained with silver stain for protein. Lane a: phosphorylase A, molecular weight marker, (93 k). Lane b: human mucin isolate.

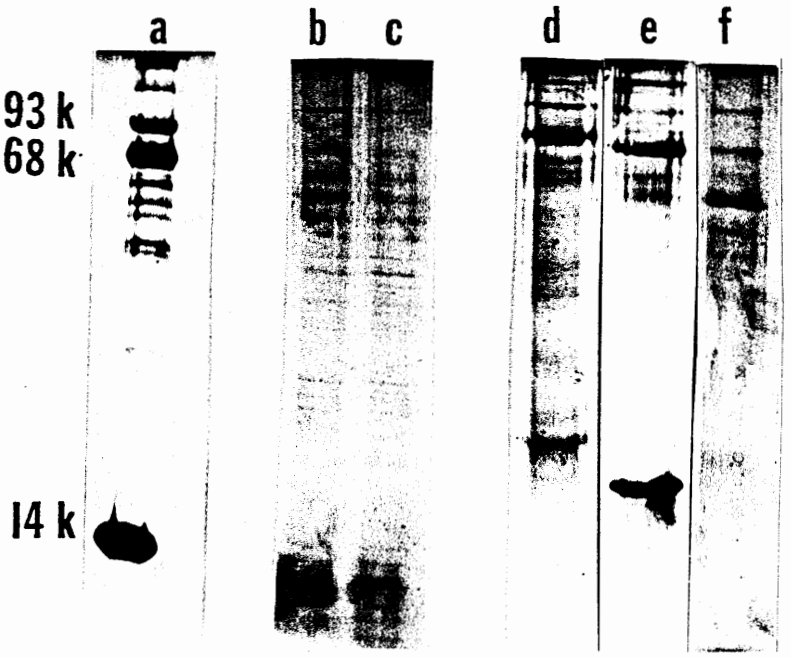



Figure 26. $\beta$-eliminated mucin sample on a 10% SDS-polyacrylamide gel. Mucin samples were $\beta$-eliminated in 0.5N NaOH, and then the oligosaccharide chains were separated from the peptide portion via Bio-gel P10 chromatography. The peptide part was dried down on a rotary evaporator, dissolved in electrophoresis buffer, boiled, vortexed with glycerol and tracking dye, and run on the 10% gel. Gels were silver-stained for protein. Lane a: molecular weight markers, phosphorylase A (93 k), bovine serum albumin (68 k), lysozyme (14 k). Lanes b and c: $\beta$-eliminated HTE mucin samples. Lanes d - f: $\beta$-eliminated human mucin samples.

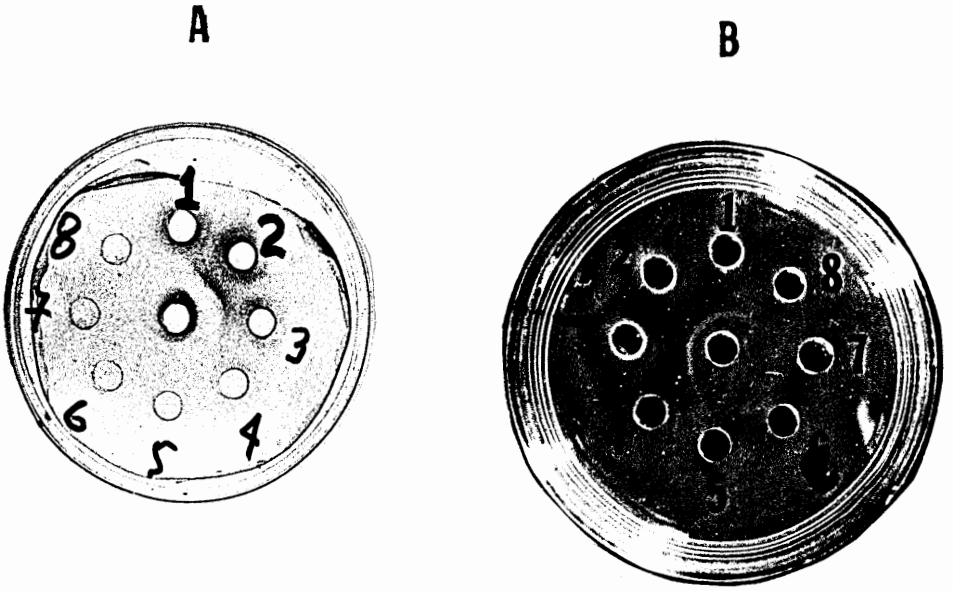



Figure 27. Double-diffusion immunoprecipitation of anti-human mucin antibody. Serum taken from the immunized rabbit's blood was tested for the presence of anti-human mucin antibody. The center well of both plates contains human mucin (20 μg). The other wells of both plates contain: 1 : non-immune serum
2 : undiluted immune serum
3-8 : serial dilutions of immune serum, with well 3 being 1:2, well 4 being 1:4, etc. Plate A is stained and plate B is unstained.

Indirect Immunoprecipitation

Using the isolated anti-human mucin antibody, attempts were made to indirectly immunoprecipitate mucins from radiolabelled HTE cells, as described in Methods. Figure 28 shows autoradiograms of the immunoprecipitates run on 5% and 10% SDS-polyacrylamide gels. Many proteins bands were immunoprecipitated by the antibody.

Hemagglutination

Isolated human mucin was tested for its antigenic similarity to blood group antigens. This was done by testing the antibody produced in response to the human mucin, the anti-human mucin antibody, for its ability to agglutinate red blood cells. Figure 29 shows the results of the hemagglutination set up as described in Methods. The absence of "buttons" of red cells in dilutions of the antibody indicated that the anti-human mucin antibody does cross-react with, and cause agglutination of red blood cells.

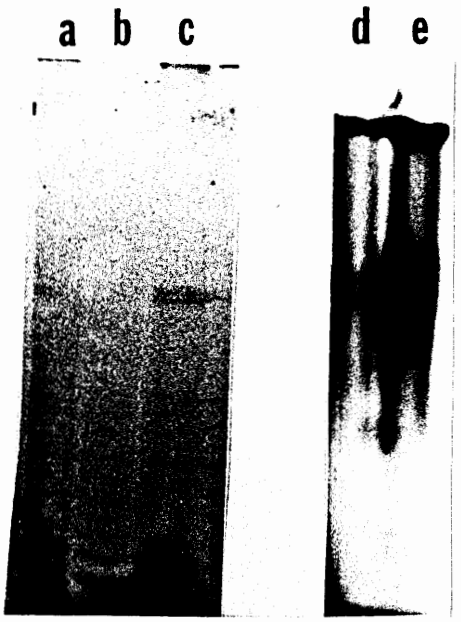



Figure 28. Autoradiographs of immunoprecipitates from HTE cells. HTE cells were labelled with ($^{14}$C)-threonine overnight, collected, washed with PBS and boiled 10 min in 10% SDS. The cells were then centrifuged and the supernatant was immunoprecipitated with anti-human-mucin antibody as described in Methods. The immunoprecipitates were run on 5% and 10% SDS-polyacrylamide gels which were then dried and autoradiographed at -80°C. Lanes a-c : 5% gel. Lanes d and e ; 10% gel.

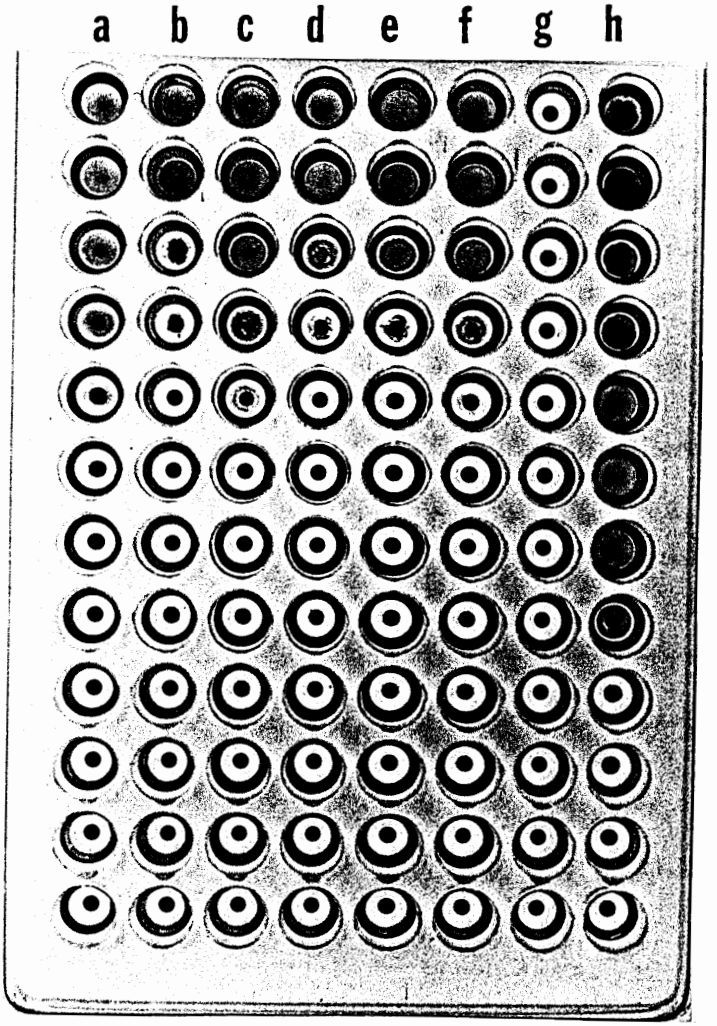



Figure 29. Direct hemagglutination of human red blood cells using anti-human-mucin antibody. Anti-human-mucin antibody was isolated from rabbit serum via DEAE-Affigel Blue chromatography. The isolated antibody was dissolved in 0.9% NaCl to a concentration of 10 mg/ml. This was then used to set up the hemagglutination in microtiter plates. Columns a - f : different isolations of anti-human-mucin antibody. The top row of wells contained 50 ul of 10 mg/ml, followed by serial 1:2 dilutions down the plate, i.e. the second row down contained 50 ul of 5 mg/ml, third row contained 50 ul of 2.5 mg/ml, etc. Column g : 0.9% NaCl with no antibody. Column h : serial 1:2 dilutions of anti-A antiserum. The top row of wells was undiluted.

# DISCUSSION

The study of mucus-type glycoproteins, including their isolation and characterization, has been an area of increasing interest over the last decade. As the malfunctioning of mucins or mucin production has been implicated in the etiology of more and more diseases, from infertility to peptic ulcers to cystic fibrosis, research in this field has grown. A comparison of the results of this research has proven to be confusing. The confusion stems from several reasons: 1. Mucins have been isolated from a myriad variety of sources; pig, monkey, toad, sheep and even human (Chace et al. 1985; Kim et al. 1985; Qureshi et al. 1979; Hill et al. 1977; Herzberg et al. 1979). 2. Mucins have been isolated and purified in a multitude of different ways, from column chromatography to enzymatic degradation and proteolysis to thiol reduction, many of which many have resulted in an isolated molecule very different from native mucin (Chace et al. 1985; Kim et al. 1985; Jabbal et al. 1976; Boat et al. 1976). 3. Mucin-like glycoproteins are extremely heterogeneous molecules, apparently having as many differences as similarities between any two mucin moledules (Allen 1983; Harding 1884). While there are certain characteristics used to classify mucins as mucins, a microheterogeneity exists in the carbohydrate side chains of these molecules that includes both differences in carbohydrates present, and differences

in charge due to varying amounts of sialic acid and esterified sulfate residues. Even mucins isolated with the same technique from different parts of the same source, e.g. human gastric vs. human bronchial vs. human cervical, apparently manifest a degree of structural dissimilarity (Qureshi et al. 1979). Mucins are excessively polydisperse.

This study dealt basically with a comparison and contrast of mucins isolated using the same technique from different sources, human tracheal and hamster tracheal epithelial (HTE) cells. The study also involved attempts to further study mucins produced by epithelial cells via immunological means using antibody produced against isolated mucins.

The substances isolated from both the HTE cells and the human tracheal lavage samples appeared to be mucin-like glycoproteins on the basis of the characteristics used to classify mucins as such. Both isolates were large molecules, being excluded in the void volume peaks from both Sephadex G-200 and Bio-Gel A-5M columns (Figures 4, 6, 5 and 10 respectively). Both appeared to be mainly carbohydrate, with the human mucin being only 13.25% protein and the HTE mucin being only 17% protein. Hexose, hexosamine, sialic acid and sulfate were present in amounts comparable to those reported in the literature (Table 1). Some of the carbohydrate side chains were negatively charged. The oligosaccharide side chains were linked to the peptide backbone via alkali-labile O-glycosidic linkages. In the presence of NaOH, these linkages were broken, releasing intact carbohydrate chains. All of

these characteristics suggest that the isolates from both sources were mucins.

When isolating mucins from the HTE cells, the cells were labelled with one of three different radioisotopes, ($^{35}S$), ($^{3}H$)-glucosamine and (2-$^{3}H$)-mannose. ($^{35}S$) was used in order to detect the presence of sulfate in the isolated mucin. ($^{3}H$)-glucosamine was used because glucosamine is a common sugar found in mucin carbohydrate side chains. (2-$^{3}H$)-mannose was used because mannose is a sugar not normally found in mucins. For this reason, (2-$^{3}H$)-mannose was used specifically instead of 1-($^{3}H$)-mannose so that if the mannose was converted to another sugar via phosphomannose isomerase the label would be lost. Only if it were incorporated as mannose would the mucin be radioactively labelled.

In looking at the results of this labelling, it can be seen that after G-200 fractionation the major of the ($^{35}S$) label was found in the void volume, mucin peak, while the ($^{3}H$)-glucosamine and (2-$^{3}H$)-mannose labels were split fairly evenly between the void volume and the included serum protein peaks (Figures 7, 8 and 9). This suggests that while most of the ($^{35}S$) was incorporated solely into the mucin fraction, the HTE cells incorporate glucosamine and mannose into both the mucus glycoproteins and other secreted proteins. This suggestion is supported by a study in the literature that reported that the sugar label and an amino acid label are incorporated at approximately the

same rate into HTE cell secreted proteins (Rudick et al. 1984).

After further chromatographic fractionation of the G-200 void volume peak on the Bio-gel A-5M column, the results were slightly different. The ($^{35}S$) and ($^{3}H$)-glucosamine labels were found not only in the void volumn, mucin peak, but throughout the column (Figures 11 and 12) suggesting that these substances were found in more than just the mucus glycoproteins secreted from the cells. In the case of (2-$^{3}H$)-mannose however, almost none of the label was found in the mucin fraction (Figure 13). This suggests that mannose was not incorporated by the HTE cells into the mucus glycoproteins which they secrete. This accords with reports in the literature that mucins contain no mannose (Allen 1983; Kim et al. 1985).

$\beta$-elimination of mucin isolates from both human and HTE cell sources resulted in the release of oligosaccharide side chains that can be separated from the peptide portion of the molecules via Bio-gel $P_{10}$ column chromatography. Figure 17 shows that a small void volume, peptide portion, came off the column first, followed by a large range of oligosaccharide chains which contain the majority of the radioactive label. These carbohydrate chains from either source range in size from one to ten or more sugar residues long (Figure 19).

The oligosaccharide chains isolated from the human and HTE mucin samples were analyzed via ion-exchange chromatography and were found to be composed of two separate groups, a group of negatively charged carbohydrate chains and a group of neutral chains. On further analysis,

by running each group separately back through the $P_2$ sizing column, it was found that the negatively charged oligosaccharide chains were specifically the long chains, those having approximately seven or more sugar residues. The neutral carbohydrate side chains were all medium length and short (Figure 22). This finding apparently holds true for the carbohydrate chains from both human and HTE mucins.

Negatively charged chains were assayed to determine if the negative charge was due to sialic acid presence by removing the sialic acid with neuraminidase and then testing for loss of negative charge. Results show that when sialic acid was removed from the long, negatively charged chains, five distinct classes of shorter, neutral carbohydrate chains result. Also, a group of long, negatively charged chains persists even after sialic acid removal (Figure 23). This group was then tested to determine if the remaining negative charge was due to esterified sulfate residues. Upon removal of this sulfate via methanolysis, a range of sizes of shorter neutral chains resulted, as well as a group of long chains that were still negatively charged (Figure 24). Therefore, although both sialic acid and esterified sulfate account for the majority of the negative charges on the oligosaccharide side chains, they apparently do not account for all of it.

Mucin isolates were also analysed via SDS-PAGE. The total human mucin, because of its size, does not easily enter polyacrylamide gels. Figure 25 shows the human mucin molecule run on a 2.2 to 6.0% linear

gradient gel. The mucin appeared as a smear near the top of the gel when stained with silver stain. Similar results have been reported in 3.3% and 4.0% acrylamide gels stained with periodic acid-Schiff (PAS) reagents for carbohydrates (Boat et al. 1976; Herzberg et al. 1979; Jabbal et al. 1976), although with 4.0% acrylamide the mucin barely enters the gel. The peptide portion of β-eliminated mucin was also analysed by SDS-PAGE. Figure 26 shows 10% gels of various β-eliminated mucin samples. The pattern of proteins appeared to be basically the same for all human samples with about 4 main bands and many smaller bands. The pattern was different for β-eliminated HTE mucin, although there were still many bands present. This multitude of peptide bands was perhaps not too surprising, since mucus glycoproteins appear to interact strongly, although nonspecifically, with other proteins (Allen 1983). Also, mucins have been reported to exist as subunits that are joined by disulphide bonds and/or a "link" peptide (Mantle et al. 1983; Allen 1983). Boat et al.(1976) have reported that β-elimination produces small glycopeptides. The peptides found on the 10% gel range in size from greater than 93K to around 14K.

Once the mucins had been isolated and characterized, attempts were made to further study them by trying to look at mucin synthesis in HTE cells via immunological means. In order to do this, the isolated human mucin was used to elicit antibody production in rabbit as described in Methods. Antibody to the whole human mucin molecule

(a-hm antibody) was produced by the rabbit, though the titer was very low. As can be seen in Figure 27, a single precipitin line occured between the mucin antigen in the center well and rabbit serum containing the elicited a-hm antibody. This precipitin reaction also occured at a 1:2 dilution, but was weaker there. Attempts to increase the titer of the antibody were not successful.

After the presence of the a-hm antibody was detected via the double immunodiffusion, the immunoglobulins were isolated from the rabbit's serum as described in the Methods section. This isolated antibody was then used to try to indirectly immunoprecipitate mucins from HTE cells. Figure 28 shows the results of autoradiographs done on the 10% and 5% gels of the immunoprecipitates from these indirect immunoprecipitation experiments. Even when preincubated to prevent non-specific binding, the a-hm antibody appears to immunoprecipitate a multitude of substances. The antibody does not appear to be very specific.

Since it has been reported in the literature that mucins have similar, if not the same, antigenic determinants as are found on the surface of red blood cells (Lloyd and Kabat 1968) the a-hm antibody was tested for its ability to agglutinate red blood cells. Figure 29 shows the result of the hemagglutination assay. Antibody produced in response to human mucin agglutinates red blood cells, suggesting that the a-hm antibody recognized the carbohydrate portion of the mucin molecule. This result is in contrast to the findings of others in

the literature. The laboratory of G. Forstner has elicited antibody production in rabbits to human intestinal goblet cell mucin (Jabbal et al. 1976; Qureshi et al. 1979) in a manner that was very similar to that used in this study. They report that their resulting antibody does not agglutinate erythrocytes, and apparently recognizes a "naked" peptide portion of the mucin molecule. They also find that a specific three dimensional configuration of the molecule is necessary for full antigenicity (Mantle et al. 1984). Other investigators suggest that both peptide and carbohydrate residues are necessary for antigenicity (L'hermitte et al. 1976).

While the antigenic determinants for the mucin molecules are still debatable, it seems to be the general consensus that antibody elicited to one type of mucin molecule exhibits variable cross-reactivity with other mucins. Antibody produced in response to human intestinal mucin shows cross-reactivity with dog, monkey and rabbit, but not with rat, pig, or toad (Qureshi 1979). Therefore, although preliminary double immunodiffusion results suggest some cross-reactivity between hamster tracheal tissue and the a-hm antibody, the inability of the antibody to immunoprecipitate mucins from HTE cells could be a function of its inability to recognize the HTE mucin. Another possibility is that the titer of the antibody was too low to immunoprecipitate appreciable amounts of mucin, or that the mucin itself is not produced in any great amount, probably less than 1% of the proteins secreted normally by the HTE cells. Attempts to

elicit antibody production to the peptide "core" of the human mucin, with the carbohydrate side chain removed, were unsuccessful.

The study of mucins is still a fertile area of investigation, and undoubtedly, the HTE cell system will prove to be invaluable in many respects. Since these cells produce and secrete mucin-like glycoproteins, mucins themselves could be studied from this system. HTE cells could also feasibly be used to study the biochemical regulation of mucin production, from the biosynthetic pathways of O-glycosylation to the final addition of sialic acid and sulfate residues. The study of mucin biosynthesis in organ culture has been hampered by the complexity of the tissue, which is not a factor in HTE cell culture. Therefore, this system may prove to be ideal for mucin studies.

The investigation of mucus glycoproteins can basically be considered a study in comparisons. Although all mucins appear to have in common the characteristics outlined in the Introduction to this work, the more reports that come out in the literature, the more differences are detected between mucins. These reported differences frequently appear to be contradictory, but this is apparently a function of the number of different sources and techniques used for mucin isolation and characterization. As each specific mucin is more definitively characterized, it can be seen that mucus glycoproteins, as a class of compounds, remain as complex and heterogeneous as ever.

This study has shown that mucins isolated from two different sources, human trachea and hamster tracheal epithelial cells, have the

inherent similarities expected of mucins, as well as predictable differences. Both contain all the characteristics of mucin-like glycoproteins, although to different degrees. As can be seen in Table 1, the composition of both mucins falls within the range of values reported in the literature for other mucin isolates. The study also has shown that although production of antibody to mucin can be elicited in rabbit, the produced antibody may not be able to be used in any system other than that to which it is produced. It appears that the antigenic determinants of different mucins could differ considerably.

While many inroads have been made in the study of mucin-like glycoproteins, a large area has yet to be covered. More research in this area will probably have to go in the direction of definitively characterizing each separate mucin molecule, using such methods as HPLC and mass spectroscopy; however, with the amount of microheterogeneity being reported for this molecule, it still may not be possible to accomplish this to the extent of defining normal versus abnormal mucins. Probably the most promising approach to take at this point is to use the HTE cell system to study the biochemistry of mucin sysnthesis and secretion, since this study has shown that HTE cells produce mucins. Immunological studies of mucins in HTE cells may still be possible, but the antibody used will probably have to be elicited against HTE mucin isolates, not mucins from other sources. Using these

methods, it may be possible to gain a clearer understanding not only of individual mucin structure, but also of the biosynthesis and secretion of these unique, complex glycoproteins.

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