STUDIES OF THE CARBOHYDRATE COMPOSITION OF A PHYTOHEMAGGLUTININ FROM *PHASEOLUS VULGARIS* AND THE STRUCTURES OF ITS GLYCOPEPTIDES ISOLATED FROM PRONASE HYDROLYSATES

by

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INTRODUCTION

In 1888 Stillmark (1), according to Tobiška (2), and Sharon and Lis (3), reported the agglutination of erythrocytes from various sources by extracts of castor beans. However, not until World War II did the possibilities of finding cheap plant sources of blood-grouping reagents and of isolating reactive proteins in pure form for mechanistic studies of agglutination prompt extensive screening of plant seed extracts (4). This screening led to the discovery of agglutinating activities in extracts from a wide variety of plant seeds. Legumes were an especially rich source. While the majority of the activities reported were nonspecific, many showed specificity for known blood groups while others showed species specificity, or specificity for undescribed determinants within a species (5). Progress in this field has recently been reviewed by Boyd (5), Tobiška (2), Dechary (6), and Sharon and Lis (3).

The plant substances responsible for erythroagglutination have been called plant agglutinins, phytohemagglutinins, phytagglutinins, or lectins (7). The term "lectin", meaning to pick or choose, was proposed by Boyd (8) to

denote the antibody-like specificities of these substances while distinguishing them from iso-agglutinins, or other agglutinins formed in response to antigens.

The few purified phytohemagglutinins have been found to be glycoproteins, with the exception of concanavalin A from jack beans, which contains no carbohydrate (3,6). Recently Galbraith and Goldstein (9) proposed that phytohemagglutinins are metalloproteins, since protein bound Mn^{++} and/or Ca^{++} are required for hemagglutination by concanavalin A (10), and by the phytohemagglutinins of soybean, wax, and lima beans (9).

The most extensively characterized phytohemagglutinin is concanavalin A. The molecule is composed of identical subunits of 27,000 molecular weight (11,12), which aggregate to form dimers from pH 3 to pH 6 and tetramers at pH 7 (13,14). It is estimated to contain three interdependent binding sites per 27,000 molecular weight, one site each for the saccharide moiety, the transition metal ion, Mn^{++}, and for Ca^{++} (10,15,16). Binding of Mn^{++} at site 1 is required for Ca^{++} binding at site 2, which changes the subunit packing (17) and forms the saccharide binding site. Molecules containing nonreducing terminal α-D-mannopyranosyl

or α-D-glucopyranosyl residues are strongly bound (18).

The renewed interest in phytohemagglutinins has resulted not only in greater understanding of their properties, but also in their use as investigative tools (3). As a group of compounds with diverse binding specificities for carbohydrates, they provide a battery of structural probes. As such they are used in the description and study of blood group substances (2,5,19,20), in structural studies of glycoproteins and polysaccharides (21-24), and as adsorbents in the purification of carbohydrate-containing molecules (25-27). Phytohemagglutinins labelled with electron dense ferritin or fluorescent groups have become powerful tools in electron or fluorescent microscopic studies of carbohydrate distribution on cellular surfaces (28-31). Their availability has made them particularly suitable for mechanistic studies of carbohydrate-protein interactions and precipitin reactions.

However, one of the observations most stimulating to phytohemagglutinin research was made by Nowell (32) in 1960 when he reported that human lymphocytes cultured in vitro were induced to undergo mitosis by phytohemagglutinin commercially prepared from red kidney beans by a modifica-

tion of the method of Rigas and Osgood (33). Subsequently, this phytohemagglutinin has been extensively used as an *in vitro* mitogenic agent in lymphocyte cultures in studies of mammalian chromosomes (karyotyping), mechanisms of lymphocyte transformation and division, and as an indicator of immunological competence in various diseased states (34). *In vivo* it has been reported to restore erythrocyte production in aplastic anemia, and to protect against immunosuppressant drugs and irradiation. Naspitz and Richter (35) have recently reviewed its *in vitro* and *in vivo* effects, and Landy and Chessin (36) have reviewed its effects on immune responses.

Several other phytomitogens were subsequently found and purified. These are: the pokeweed mitogen (37); concanavalin A (38,39); and the phytohemagglutinins from *Phaseolus vulgaris* (wax bean) (39,40), *Lens culinaris* (lentil) (41,42), and *Agaricus campestris* (mushroom) (41,43). Extracts from a variety of other plants have also been reported to contain mitogenic agents (44-46).

The Phytohemagglutinin from Red Kidney Beans

According to Rigas (47), "During the late nineteen-forties, J. G. Li, while working with E. E. Osgood on the

development of methods for long term culture of human leukocytes *in vitro*, conceived the idea that beans, being plant embryos, may contain growth promoting substances similar to those believed to be present in chick embryo extracts and to be essential for the *in vitro* culture of mammalian cells." The mitogenic activity of red kidney bean extracts that were used to test this idea was missed at that time. However, the strong erythroagglutinating activity of the extracts prompted the proposal by Li and Osgood (48) that phytohemagglutinin (PHA) be used for separating leukocytes from erythrocytes. Although the erythroagglutinin from *Phaseolus vulgaris* (red kidney bean) had been partially purified much earlier, Rigas and Osgood (33) published the first purification methods yielding highly active and purified PHA in 1955. In 1959 Hungerford, Donnelly, Nowell and Beck (49) observed significant mitoses in leukocyte cultures while employing the PHA separation technique of Li and Osgood, and in 1960 Nowell (32) reported that phytohemagglutinin was an initiator of mitosis in normal but not leukemic blood lymphocytes *in vitro*.

Rigas and Osgood (33) first isolated PHA* as a muco-protein (MPHA)* containing 50% carbohydrate. At acidic pH's the electrophoretically pure MPHA dissociated into an inactive polysaccharide and a protein (PPHA)* with an agglutinating activity a 100 fold more potent than MPHA. This suggested that dissociation of the polysaccharide component had unmasked carbohydrate binding sites involved in agglutination. Employment of acidic conditions (pH 1) during the initial extraction procedures yielded predominantly PPHA (33).

Following Nowell's report (32), both MPHA and PPHA preparations were found to be mitogenic. However, the impurity of mitogenically-active commercial preparations and the presence of a minor component in PPHA preparations raised the question whether both the mitogenic and erythro-agglutinating activities were due to the same molecule or different molecules. Rigas and coworkers (47,50,51) prepared PPHA which was homogeneous by various types of electro-phoresis; immunoelectrophoresis; ion-exchange, adsorption,

*MPHA and PPHA will be used as abbreviations referring to the preparations of Rigas and coworkers, whereas PHA will be used to refer to the phytohemagglutinin of the red kidney beans.

gel permeation, and affinity chromatography; ultracentrifugation; immunodiffusion; and solubility. This PPHA contained both mitogenic and erythroagglutinating activities, and lymphoagglutinating activity, which had been reported by other investigators for PHA preparations (35). This highly purified PPHA was reported to be a glycoprotein containing 2.4% glucosamine and 8.0% neutral carbohydrate (47). It contained no uronic or sialic acid.* It was rich in aspartic acid, threonine and serine, and contained very low levels of sulfur containing amino acids (50). Alanine was the only detectible N-terminal amino acid (47,50).

The biophysical characteristics of PPHA were determined (47,50-52): the isoelectric point was 6.5; the sedimentation coefficient $s^{\circ}_{20,w,}$ 7.05$\pm$.02 S; the diffusion coefficient $D^{\circ}_{20,w,}$ (5.08$\pm$.015) x 10^{-7} cm^2 sec^{-1}; the partial specific volume $\bar{v}_{20,}$ 0.738 $\pm$ 0.001 cm^3 g^{-1}; and the molecular weight, 128,400 $\pm$ 900, based on sedimentation equilibrium and on sedimentation and diffusion coefficients.

* Rigas, D.A. Personal Communication, 1970

Urea, quanidine hydrochloride, or sodium dodecyl-sulfate (SDS) effectively dissociated PPHA into subunits at neutral or alkaline pH producing a slower sedimenting symmetrical Schlieren peak with a sedimentation coefficient, $s_{20,w}$, of approximately 1.5 S. Rigas (47) estimated PPHA to contain eight subunits from sedimentation velocity data in 8 M urea, assuming a constant partial specific volume and spherical symmetry for the undissociated and dissociated molecule. Although the question of number of subunits awaits a conclusive answer, the ultracentrifugal studies did strongly suggest that the dissociated components vary little in molecular weight. This was further supported by the observation of a single band in polyacrylamide gel electrophoresis in 0.1% SDS at pH 7.1* of SDS-dissociated PPHA.

While the subunits appear indistinguishable hydrodynamically, many observations point to their nonidentity. Polyacrylamide gel electrophoresis of urea-dissociated PPHA in 8 M urea at pH 8.6 produces eight prominent anodic bands which stain either with Amidoschwartz or periodic acid-Schiff reagent for protein or carbohydrate, respec-

*Rigas, D.A., Personal Communication, 1970

tively. Following separation and elution of the bands, the slower contained the agglutinating activity while the faster contained mitogenic activity (53). Furthermore, when homogeneous PPHA was chromatographed on IRC-50 utilizing gradients of increasing pH and sodium ion concentration, seven major peaks were observed (47). These components were interpreted to represent the formation of subunit recombinants from nonidentical subunits based on the following observations: (a) all components had lower solubilities than native PPHA in 0.1 M NaCl; (b) most of their sedimentation coefficients differed from that of PPHA; (c) starch gel electrophoresis at pH 9 revealed heterogeneity of five of the seven components examined, and starch gel electrophoresis in 8 M urea at pH 9 showed changes in the relative concentration of the bands normally observed for dissociated PPHA; (d) there were significant differences between the amino acid compositions of the components and PPHA; (e) the tryptic peptide patterns of the components differed from each other and from PPHA; (f) the components displayed a wide distribution of erythroagglutination titers ranging from one-tenth to five times the potency of PPHA; (g) the components ranged from weakly mitogenic to potencies comparable to

PPHA: and (h) the mitogenic and erythroagglutinating activities of the components appeared to vary independently.

Attempts to determine the molecular structures responsible for mitogenic and erythroagglutinating activity have been frustrated by the complexity of the molecule. However, the effects of various treatments on PPHA activity (52) point strongly to primary, secondary and possibly tertiary and quaternary polypeptide chain structural requirements. Both activities are destroyed by boiling, 6 M guanidine hydrochloride at 56° C for 25 hours, or by proteolysis with papain, pepsin, or chymotrypsin. The hemagglutinating activity is significantly reduced, with little effect on the mitogenic activity, by dissociation in 8 M urea or 6 M guanidine hydrochloride at 4° C, or by proteolysis with pronase or nagarse. Pronase digestion in the presence of 0.4% SDS destroys both activities, whereas SDS alone has little effect. The mitogenic, but not the erythroagglutinating activity, is destroyed by incubation at pH 12 for 24 hours at 6° C.

Recently, Goldberg, Rosenau and Burke (54), suggested that the mitogenic material they isolated from Phaseolus vulgaris was not a protein, since its activity persisted after 99.8% of the protein in the preparation had been

removed. This suggestion is not supported by the data on PPHA, and Goldberg himself, according to Oh and Conard, has since expressed the belief that the mitogenic activity was due to a protein (55).

PPHA contains 0.5 moles Mg^{++} and 1.5 Ca^{++} per mole.* A functional role for these metal ions has not been established but has been suggested by the report of Borjeson, Chessin and Landy (56) that treatment of PHA with EDTA destroys the hemagglutinating activity and impairs the leucoagglutinating and mitogenic activities. They state that hemagglutination is fully restored by the addition of Ca^{++} or Mg^{++}. However, no supporting data were presented. Lindahl-Kiessling (57) also found that lymphocytes, when incubated with PHA for one hour in the presence of EDTA, followed by incubation in fresh culture medium, were not stimulated, whereas stimulation was observed in the absence of EDTA.

The role of carbohydrate in the molecule is also unclear. Punnett and Punnett (58) and Breitner (59) observed that the mitogenic activity of PHA was destroyed

*Rigas, D.A. Personal Communication, 1971.

by periodate oxidation. Weber, Aro and Nordman (60) also reported the destruction of mitogenic activities, but only marginal decreases in the agglutinating activities of erythroagglutinating and leucoagglutinating components isolated from Difco PHA-P. However, the oxidation of critical amino acids was not precluded by the evidence presented in any of these reports. Cysteine, cystine, methionine, tryptophan, tyrosine and histidine are susceptible to periodate oxidation even when both the α-amino and α-carboxyl groups are blocked (61).

The erythroagglutinating activity of PHA is directed toward carbohydrate receptor sites on the erythrocyte membrane. Kornfeld and Kornfeld (62,63) have structurally characterized a glycopeptide from trypsin digested human erythrocyte membranes with potent PHA receptor site activity. The structural determinants reside in the oligosaccharide moiety, the predominant being a galactose residue. However, glycoproteins extracted from the membranes had a much more potent activity, 90% of which was destroyed by trypsin treatment, demonstrating an effect of the peptide backbone on receptor activity. The glycopeptide both binds to PHA and inhibits PHA binding to lymphocytes.

The mechanism of lymphocyte stimulation has been the subject of numerous and often conflicting reports (35). PHA appears to bind first to plasma membrane receptors (57,64-66). Binding of PHA to the cell surface is necessary but not sufficient for stimulation (64,66). Similar amounts of ^{125}I-PHA are bound to responsive mouse spleen and thymus cells as to weakly responsive bone marrow cells (64). Moreover, treatment of mouse spleen cells with neuraminidase abolished PHA migration into the cells and stimulatory activity, but not plasma membrane binding, when examined by fluorescent antibody techniques (66). Stanley, Frenster and Rigas (67) studied the localization of ^{3}H-PPHA in lymphocytes and monocytes for periods ranging from 15 minutes to 24 hours by radioautography of ultrathin sections. In 15 minutes lymphocytes and monocytes both showed significant uptake of label, which was predominantly located in the plasma membrane and cytoplasm, with lesser amounts in the nucleus. After 24 hours, lymphocytes contained localized label predominantly in the heterochromatic regions of the nucleus; whereas monocyte label was still predominantly in the cytoplasm. Rubin, Davis and Schultz (68) provided support for a direct action of PHA on the nucleus by demonstrating

that incubation of isolated human peripheral blood lymphocyte nuclei with high dosages of PHA produced marked increases in actinomycin-D sensitive incorporation of tritiated RNA precursors into acid precipitable materials, in the actinomycin-D binding by lymphocyte nuclei, and in the nuclear incorporation of ^{14}C-acetate. These data were consistent with the suggestion of Frenster (67) that PHA acts directly on heterochromatin converting it to euchromatin, which could serve as a template for actinomycin-D sensitive RNA polymerase, and bind actinomycin-D.

However, Greaves and Bauminger (69) recently reported that PHA covalently bound to Sepharose activates lymphocytes. They suggested that binding of the mitogen to the plasma membrane may trigger a second messenger. The significance of their results is not yet clear.

Glycoproteins - General Comments

A brief review of the basic features of glycoproteins provides clues on the plausible structural arrangements and functional roles of the carbohydrate in PPHA. Glycoprotein research has been extremely active in the last decade, and the following comments were taken from a number of recent reviews (70-80). In a 1960 review Winzler (81) commented, "We are, however, in a primitive

stage of information in the area - we are still in a position of cataloguing the proteins with respect to their carbohydrate content, and determining how much and what kind of sugar derivatives they contain. Questions of the nature and size of the sugar units and of their linkages to the peptide chain are almost unanswered."

The burst of activity in the area in the 1960's resulted largely from an arousal of interest with the realization that sialic acid and other carbohydrates played key roles in the structure and function of many proteins and cellular surfaces. This same period witnessed the development of sophisticated isolation procedures, microquantitation techniques for carbohydrates, such as gas-liquid chromatography, and the development of sequencing methods employing proteolytic enzymes, glycosidases, methylation, or sequential periodate oxidation. Consequently, this interest coupled with improved technology has led to the clarification of many features of glycoprotein structure and function.

Glycoproteins, or polypeptides containing covalently bound carbohydrate, are widespread in plants, animals, and microorganisms. Spiro (73) notes that in animal tissues, these compounds account for the major portion of carbo-

hydrate in the polymer form. They have diverse properties and function: examples of enzymes, hormones, blood-group active substances, mucins, connective tissue components and many plasma proteins have been shown to be glycoproteins. Their carbohydrate content may vary from less than one percent to more than 80% in various glycoproteins and generally includes from two to seven different monosaccharides. Some proteins, such as bovine, porcine and whale ribonucleases, have been isolated in glycosylated as well as nonglycosylated forms (89).

The function of the carbohydrate moiety has been of great interest but is not well understood in most instances (77). It appears directly involved in the supposed biological functions of the mucins and mucopolysaccharides, whose characteristic properties arise largely from the charges on their carbohydrate, and in blood-group active substances, in which removal of small carbohydrate units profoundly alters their activity. Indirect roles in the function of the protein have also been indicated. Removal of carbohydrate from a number of enzymes and plasma proteins does not significantly alter their enzymic or transport activities. However, removal of sialic acid from certain plasma proteins and glycoprotein hormones signals

their removal from circulation. Periodate oxidation of glycoamylase I has little direct effect on its catalytic activity but drastically reduces the stability of the enzyme, prompting the suggestion that carbohydrate stabilizes the enzyme conformation in glycoenzymes (83). Recently it was proposed that the carbohydrate moiety constitutes a means of coding for the topographical location of the glycoprotein within the organism (77). Accordingly, it would form a recognition site for a target organ or site, or could provide a hidden code whereby removal of terminal carbohydrate reveals the coded site, as is observed in the elimination of certain glycoproteins from the circulatory system.

General glycoprotein structural features are emerging, but they come primarily from studies of animal glycoproteins. The carbohydrate moiety is typically bound through a glycosidic bond from the reducing terminal monosaccharide to the side chain of an amino acid residue. Two types of carbohydrate-protein linkage have been confirmed: the N-glycosidic linkage from C-1 of N-acetyl-D-glucosamine directly to the amide nitrogen of asparagine; and the O-glycosidic linkage of any of several monosaccharides to the hydroxyl group of a hydroxyamino acid. The only example

of the former linkage type, 2-acetamido-1-N-(4-L-aspartyl)-2-deoxy-β-D-glucopyranosylamine, has been reported in glycoproteins from animals, plants, and microbes. Examples of the latter type include: O-β-D-xylopyranosyl-L-serine in chondroitin sulfate, 5-O-β-D-galactopyranosyloxy-L-lysine in collagen, O-(2-acetamido-2-deoxy-D-galactopyranosyl)-L-serine or -threonine in mucins and blood group substances, O-arabinosyl-hydroxyproline in plant cell walls, and a proposed O-mannosyl-serine or -threonine in yeast cell walls (84) and in β-fructofuranosidase (85). A third type, an S-glycosidic bond between galactose and the sulfhydryl group of cysteine, was recently reported for a glycopeptide isolated from urine, but it has not been found in any intact glycoprotein (86).

A glycoprotein may contain many covalently bound oligosaccharide units, the linkages of which may be of more than one type. Thus both O- and N-glycosidic linkages have been reported to occur in rabit γ-G-globulin, γ-A myeloma protein, lactotransferrin, ox-aorta glycoprotein and in human chorionic gonadotrophin.

The sequence of the peptide linkage region of a vast majority of glycoproteins containing the N-glycosidic

linkage is Asn-X-Thr/Ser, in which X is any amino acid, and asparagine is glycosylated. This has prompted the hypothesis that this sequence is a recognition site for glycosylation.

The carbohydrate moieties are typically highly branched structures in glycoproteins and essentially linear in mucopolysaccharides. Heterogeneity in the carbohydrate moiety is a common feature of many glycoproteins. This variability is pronounced in the nonreducing termini, as for example in chicken ovalbumin (87,88) in which the proposed structure of the oligosaccharide consists of a core of two N-acetylglucosamine and 5 mannose residues to which are bound various additional amounts of mannose and N-acetylglucosamine. This is thought to originate from varying stages of chain elongation during biosynthesis, which is considered to proceed from the reducing to the nonreducing termini by the sequential transfer of monosaccharides from the appropriate nucleotide donor to the glycoprotein acceptor.

The Glycoprotein Nature of PPHA

Earlier studies of PPHA did not conclusively prove that carbohydrate was covalently bound to the protein molecule (47). However, the presence of glucosamine in all

of the IRC-50 fractions previously mentioned, and the staining of all the electrophoretic bands of dissociated PPHA for carbohydrates (53) strongly suggested carbohydrate-protein covalent linkages. These observations also indicated that the carbohydrate in PPHA was distributed among several oligosaccharide chains.

Johnson (89,90) examined the carbohydrate constituents of PPHA. He demonstrated by two dimensional thin layer chromatography that mannose and glucosamine were the major monosaccharide constituents with much smaller amounts of fucose, xylose and arabinose.

Johnson (84,90) went on to purify glycopeptides by gel permeation and ion-exchange chromatography of tryptic hydrolysates of PPHA. Eight chromatographically different glycopeptides were detected. All were not completely resolved, and two were not obtained in sufficient quantity for composition analysis.

Five of the glycopeptide fractions each contained 9-11 different amino acids. All contained aspartic acid or asparagine, threonine, glutamic acid or glutamine, isoleucine, phenylalanine, and glucosamine. Differences in amino acid compositions suggested that all five were different, although one of the fractions was known to

contain two partially resolved glycopeptides. One major glycopeptide, GP-A_1, contained no arginine or lysine, in contrast to the others which all contained arginine. It was proposed that it had occupied a C-terminal position in the parent polypeptide. Thin layer chromatography showed this glycopeptide to contain glucosamine and mannose, with smaller amounts of fucose, arabinose, and xylose, and traces of galactose and glucose. The second major glycopeptide fraction, GP-$B_{1\&2}$, contained only mannose and glucosamine. The neutral carbohydrate compositions of the other glycopeptides were not examined.

The isolation of glycopeptides from PPHA confirmed that it is a glycoprotein, since preparative conditions were used which make the noncovalent binding of carbohydrate to the peptides unlikely. The amino acid and carbohydrate compositions of the examined glycopeptides suggested as many as five polypeptide linkage regions, to which at least two types of oligosaccharides were bound. The occurrence of about two glucosamine molecules per glycopeptide indicated that there were several oligosaccharides per mole of PPHA, but no conclusive statements about the size and number of oligosaccharides could be made due to the lack of neutral carbohydrate composition data.

In addition, the identities of the amino acid linkage sites were not apparent, since all the glycopeptides contained three amino acids known to be linkage sites in other glycoproteins, i.e., aspartic acid, or asparagine, threonine, and serine.

Glycopeptides have been isolated from two other hemagglutinins and have been characterized. Five glycopeptides were detected chromatographically in the pronase hydrolysate of wax bean phytohemagglutinin (91). The major glycopeptide fraction contained about 25% of the carbohydrate of the parent molecule and had a calculated molecular weight of 4380. It differed considerably from those studied by Johnson in that it contained tyrosine, and significant amounts of arabinose, galactose, and glucose. Lis, Sharon and Katchalski (92) isolated a glycopeptide from the pronase hydrolysate of soybean hemagglutinin. It had a molecular weight of 4600 and contained mannose, N-acetylglucosamine, and aspartic acid. In contrast to PPHA and wax bean phytohemagglutinin, all the carbohydrate of the parent molecule was recovered in this single glycopeptide. Chemical and enzymatic degradation of the glycopeptide yielded a linkage region species consisting of aspartic acid and N-acetylglucosamine, which behaved

chromatographically like synthetic 2-acetamido-1-N-(4-L-aspartyl)-2-deoxy-β-D-glucopyranosylamine (93).

Objectives

The covalent binding of carbohydrates to proteins to form glycoproteins makes the determination of glycoprotein primary structure much more complex than that of simple proteins. In addition to the determination of amino acid sequence, one must also examine: the qualitative and quantitative monosaccharide composition of the glycoprotein; the number, compositions and structures of the carbohydrate moieties; the structures of the carbohydrate-protein linkages, and their location in the primary amino acid sequence.

The primary amino acid sequence of PPHA has not been examined. Such studies have been hindered by the lack of sufficient quantities of purified subunits. However, quantities of highly purified PPHA were available, and recently developed chromatographic procedures made quantitative studies of the carbohydrate constituents possible on quantities of material comparable to those used for amino acid analyses. It was thus decided to approach the basic questions listed above pertaining to the carbohydrate in PPHA. Since the quantitative mono-

saccharide composition and its variability had not been examined, the first objective of this investigation was to obtain this information by employing recently developed gas-liquid chromatographic procedures.

No definitive work had yet been done on the structures of the carbohydrate moieties in PPHA, their mode of linkage to the protein, or on the amino acid sequences of the linkage regions. Thus, the second objective of this work was to develop procedures for preparing and purifying glycopeptides from appropriate enzymatic hydrolysates of PPHA. The third, and most important objective was the structural characterization of the isolated glycopeptides, i.e., the determination of the carbohydrate-peptide linkage constituents and the sequence of residues in the peptide and oligosaccharide moieties.

MATERIALS AND METHODS

All chemicals used were of analytical reagent grade unless otherwise noted.

Red kidney beans (green pod bush) were purchased from Chas. H. Lilly Co., Portland, Oregon. Pronase (B grade, lot no. 801261, 45,000 PUK/g), 2-deoxy-D-glucose (A grade, lot no. 72920) and D-glucosamine hydrochloride, (lot no. 63670) were obtained from Cal-Biochem., Los Angeles, California. Tetracycline hydrochloride was supplied by Chas. Pfizer Pharmaceuticals, New York, N.Y. Phenyliso-thiocyanate (b.p. 89-90° C at 10 mm Hg), sym-collidine (b.p. 169-170° C), $NaBH_4$ (98% assay), phenylacetaldehyde (98-100% purity, b.p. 78-80° C at 10mm Hg), and technical grade sodium dodecyl sulfate (95%) were Matheson, Coleman and Bell reagents, (Norwood, Ohio). Pyridine and $NaIO_4$ (99.8-100% assay) were supplied by Mallinkrodt, (St. Louis, Mo.). $NaIO_3$ was a Baker-Adamson reagent, (Allied Chemical Corp., New York, N.Y.). The anion exchange resin used in preparation of carbohydrate samples for gas-liquid chromatographic analysis, AG 1-x8 (200/400 mesh, lot no. 25522) and $(NH_4)_2SO_4$ (99.7% assay) were Baker Analyzed

Reagents (Phillipsburg, N.J.). L-isoleucine was Sigma Grade, lot no. 127B-2690 (St. Louis, Mo.). Carboxypeptidase A (20 mg/ml, 52U/mg, DFP-treated) was a Worthington enzyme (Freehold, N.J.). Ninhydrin (lot no. 07018-1) was purchased from Pierce Chemical Co., Rockford, Ill. Guanidine hydrochloride was Mann Ultra-Pure Reagent Grade (New York, N.Y.). Acetic anhydride (Merck, Rahway, N.J.), commercial 2,4,6-tri-2-pyridyl-sym-triazine (K&K Laboratories, Hollywood, Cal.), and reagent grade orcinol monohydrate (Fisher Scientific Co., Fair Lawn, N.J., lot no. 785967) were used as supplied. Orcinol monohydrate, lot no. 378-2220, obtained from Sigma, was recrystallized from benzene.

Alditol acetate standards (Regis Chemical Co., Chicago, Illinois) were supplied as 0.05 M solutions in chloroform with a specified concentration tolerance of $\pm$2.5%. The chromatography resins, Bio-Gel P-6 (lot no. 54744), Bio-Gel P-2 (lot no. 45684), AG 50W-x2 (H^+) (lot no. 10107F) and AG 1-x2 (acetate form, lot no. 653.2) were all 200/400 wet mesh and were obtained from BioRad Laboratories (Richmond, California).

Pyridine and sym-collidine were refluxed with ninhydrin and redistilled. Phenylisothiocyanate was redistilled

under vacuum, and constant boiling hydrochloric acid was prepared by distillation of 6 N hydrochloric acid.

Mettler balances, either an analytical type H16 or a top loading precision Model P120 (Mettler Instrument Corp., Hightstown, N.J.), were used for weight determinations.

Spectrophotometric measurements were made in 1.0 cm quartz cells in a Zeiss PMQ II Spectrophotometer (Carl Zeiss, Oberkochen, Wuerttemberg).

A Radiometer pH meter 26 and combination electrode GK2302B (The London Company, Cleveland, Ohio) were used for pH measurements and were standardized with commercially prepared standard buffers. Samples were at room temperature (26-28^{o} C) unless otherwise stated.

Sample drying was carried out in a Virtis Freezemobile mechanically refrigerated freeze dryer (Virtis Co., Inc., Gardiner, New York) by conventional lyophilization techniques, or in a Virtis centrifugal "Bio-Dryer" when small volumes (10 ml or less) were handled. The "Bio-Dryer" is a 1500 rpm, evacuated centrifuge, which either contains desiccant or may be attached to a conventional lyophilizer manifold. Samples in test tubes are placed in the "Bio-Dryer" without prior freezing and spun under

vacuum. The vacuum effects freezing while centrifugation prevents sample "bumping".

Where more rigorous drying was required, an Abderhalden drying pistol (H.S. Martin & Co., Evanston, Illinois) containing P_2O_5 was used at 77° C, the temperature of refluxing carbon tetrachloride. The sample chamber was continuously evacuated to a pressure of about 5 microns.

Preparation of Phytohemagglutinin

Protein phytohemagglutinin (PPHA) was isolated from red kidney beans by the procedure of Rigas and Johnson (50). The resulting salt-free, lyophilized protein was either used without further purification, or was further refined by gel permeation chromatography on Sephadex G-200 (51) using the procedure and column described by Johnson (89) for Bio-Gel P-200 chromatography of PPHA. In Figure 1 is a representative chromatogram obtained from 250 mg of protein loaded in a 2.2 x 85 cm G-200 column with a bed height of 80 cm and developed with 0.15 M NaCl. The load was quantitatively recovered and the components are labelled in the order of their elution. The resolution of C_3 is not readily apparent, but it is typically observed as a leading shoulder on the major peak (51). The major peak was conservatively cut and as such represented 86% of the

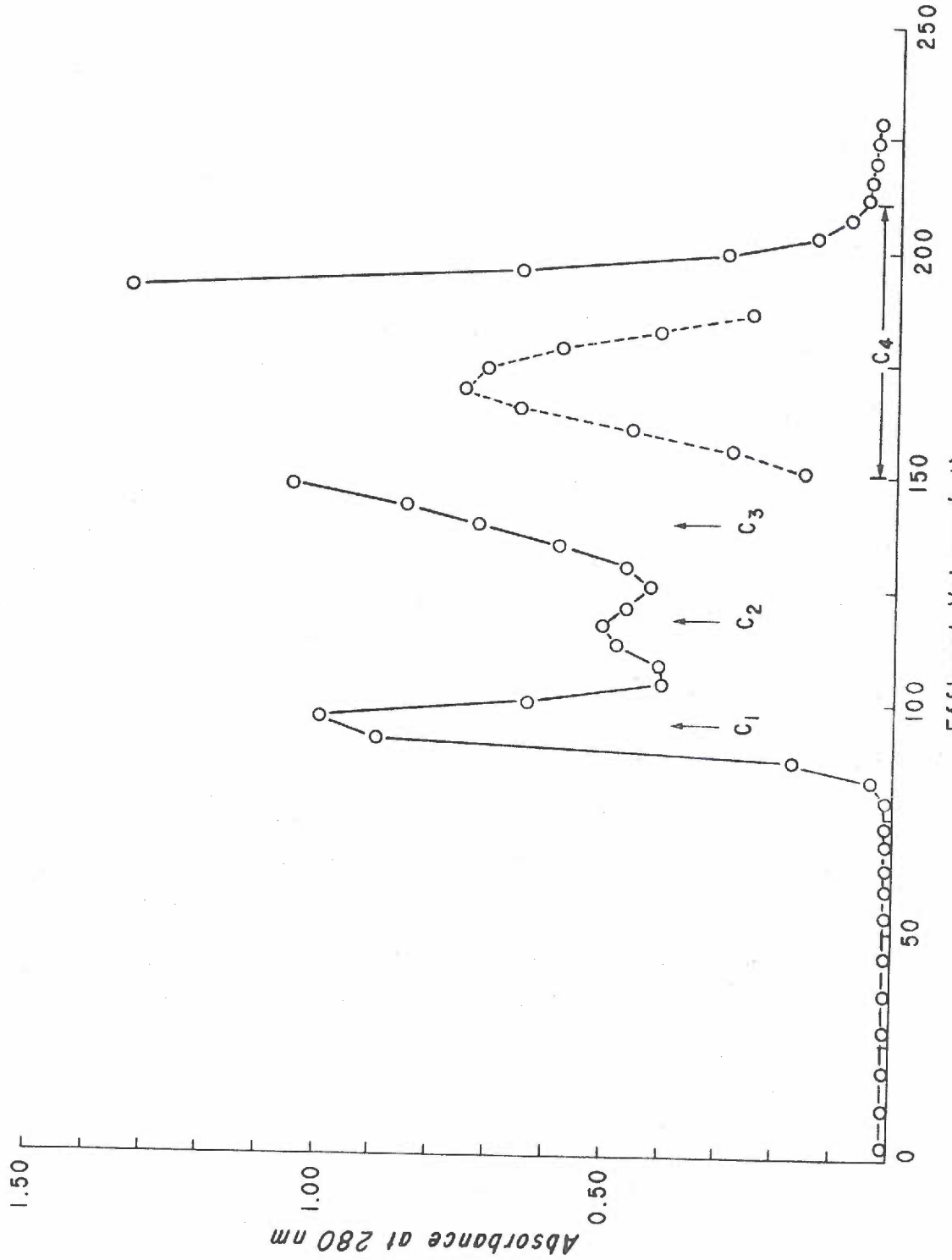


Figure 1. Sephadex G-200 chromatogram of 67-A-P_8. Broken line indicates 10-fold scale expansion.

recovered material on the basis of absorption at 280 nm. This estimate is low however, since the C_1 fractions were turbid, producing falsely high readings. The fraction pool was concentrated by ultrafiltration, dialyzed against distilled water, and lyophilized by standard procedures (89).

Different PPHA preparations will be indicated by code names in the text. Thus, 67-A-P_8 signifies the eighth precipitation product in the preparative scheme, of the first preparation, A, in 1967. And, 67-A-$P_8(C_4)_1$ signifies the fourth component (major peak) in order of elution from the first G-200 chromatography of 67-A-P_8. The preparations 67-A-P_8, 70-A-P_8 and 72-A-P_8, referred to in the text, were isolated from red kidney bean lot numbers 4-6, 7-0, and 9-3, respectively. Preparation 62-E-P_5 was obtained from an unidentified bean lot which was different from those given.

Preparation and Purification of Glycopeptides

I. Pronase Hydrolyses

Glycopeptides were prepared by proteolysis of PPHA with "Pronase", which is a mixture of endo- and exopeptidases with broad specificity from Streptomyces griseus (94).

Three hydrolytic procedures differing primarily in the methods of PPHA denaturation were employed in an attempt to maximize the susceptibility of PPHA to "Pronase" action. A solution of PPHA, either 67-A-P_8 or 67-A-$P_8(C_4)_1$, was denatured by heating alone, or by heating in the presence of dissociating agents. The denatured protein (25 to 40 mg/ml) was hydrolyzed in unbuffered media containing 0.0015 to 0.015 M Ca^{++} for 18 to 25 hours at 37° C with additions of "Pronase" totalling 2.5 to 3% w/w. To prevent bacterial contamination and growth, the hydrolysis vessel and all other accessories contacting the hydrolysis medium were cleaned and rinsed with 70% ethanol, and tetracycline was included in the hydrolysis medium at concentrations of 5 to 10 ug/ml. The pH was maintained at 7.5 by automatic or manual addition of NaOH with magnetic stirring, and the progress of the reaction was monitored by recording the quantity of NaOH added to maintain that pH. When base consumption was no longer apparent, the pH of the reaction mixture was adjusted to between 3 and 4 with glacial acetic acid. The hydrolyzed samples were then subjected to Bio-Gel P-6 gel permeation chromatography as described later.

In the first hydrolysis procedure, 301 mg of lyophilyzed 67-A-$P_8(C_4)_1$ was dissolved in 10 ml of 0.015 M $CaCl_2$. The

solution was heated to 84° C for 30 minutes in a test tube immersed in a water bath, producing a heavy precipitation. Following heating, 0.1 ml of 0.5 mg/ml tetracycline hydrochloride in 0.015 M $CaCl_2$ was added to the suspension. The suspension and a 1 ml 0.015 M $CaCl_2$ rinse of the test tube were pooled in a 15 ml, jacketted Radiometer pH stat vessel maintained at 37° C with a Haake circulating constant temperature water bath (Brinkman Instruments, Westbury, New York).

The hydrolysis was monitored with a Radiometer type TTT1c titrator and type ABU1a autoburette equipped with a 0.25 ml syringe, and base consumption was recorded with the Radiometer type SBR2c titrigraph (The London Company, Cleveland, Ohio). The titrant was 1.0 N NaOH.

The pH of the protein suspension was raised from about 5.5 to 7.5 with 1 N NaOH and 4.75 mg "Pronase" was added. After 10 hours base consumption had subsided and an additional amount of 4.1 mg "Pronase" was added. Little base was subsequently added, and after 8 more hours any reaction was stopped by adjustment of the pH to 3 with glacial acetic acid. A total of 0.50 meq of NaOH was consumed during the reaction.

The hydrolysate was lyophilized to a volume of 1.7 ml and diluted to 5.0 ml with distilled H_2O. The resulting solution contained suspended material which was removed by centrifugation and subsequently assayed for neutral carbohydrate. The supernatant was subjected to Bio-Gel P-6 chromatography.

In the second procedure, 50 mg 67-A-P_8 was dissolved in 1.5 ml of a 0.4% w/v sodium dodecyl sulfate (SDS) containing 5 µg/ml tetracycline ("Tetracyn"). The solution was heated in a test tube immersed in a water bath for 30 minutes at 91° C. In contrast to procedure 1, no precipitation occurred. The solution and a 0.5 ml (0.4% SDS) rinse of the vessel were pooled in a pH stat vessel maintained at 37° C with a Haake constant temperature water bath. The vessel was sealed with a polyethylene cap through which were inserted a Radiometer combination electrode no. GK2302B and the delivery tip of a 0.5 µl/division syringe microburet, model SB2 (Micro-Metric Instrument Co., Cleveland, Ohio). The pH was monitored with a Radiometer pH meter 26.

The pH of the protein solution was adjusted to 7.5 with 0.25 N NaOH and 0.05 ml of 10 mg/ml "Pronase" in 0.1 M NaCl was added. The pH was maintained at 7.5 by manual

addition of 0.25 N NaOH. After three hours 0.006 ml 0.5 M $CaCl_2$ was added giving an estimated concentration of 0.0015 M Ca^{++}. After 20 hours base consumption had subsided and 0.5 mg "Pronase" in 0.03 ml of 0.1 M NaCl was added. During the ensuing 5 hours base consumption was negligible, so the reaction mixture pH was adjusted to 3.5 with glacial acetic acid. Base consumption totalled 0.092 meq. A small amount of precipitate formed following storage at 4° C for two days. This was removed by centrifugation, and the supernatant was diluted to 5.1 ml with 0.1 N acetic acid and subjected to P-6 gel permeation chromatography.

The third procedure was prompted by the observation that PPHA formed a gel when heated to 56° C for 24 hours in 6 M guanidine hydrochloride*. This suggested extensive denaturation thereby giving maximal susceptibility to proteolysis. The protein, 601 mg of 67-A-$P_8(C_4)_1$, was dissolved in 6 ml 6 M guanidine hydrochloride in a 13 x 150 mm test tube. The tube was incubated in a "Temp-Blok Module Heater" (Scientific Products, Evanston, Illinois) for 3 hours at 90° C. Upon heating, the tan hue of the

*Rigas, D.A. Personal Communication, 1969

solution became much more intense, but no precipitate was formed. The solution was transferred to 8/32", Visking dialysis tubing (HMC, 52 Gloucester Pl. W.L.) followed by 2-1 ml rinses of the test tube with 2 M urea - 0.015 M $CaCl_2$. Guanidine hydrochloride was removed by dialysis against 2 liters of 2 M urea - 0.015 M $CaCl_2$ overnight with constant stirring at 4° C. Dialysis produced a slightly tan opalescent gel.

The gel was broken up and transferred to a Metrohm pH stat vessel followed by several rinses of 2 M urea - 0.015 M $CaCl_2$ totalling 7 to 8 ml. The final volume was approximately 15 ml with a protein concentration of 40 mg/ml. The vessel temperature was maintained at 37° C with a Haake constant temperature water bath and was sealed with a polyethylene cap through which were inserted a titrant delivery tube and a Sargent S-30072-15 combination electrode connected to a Metrohm E300B pH meter (Brinkman Instruments, Westbury, New York). Base addition was controlled and recorded with a Metrohm E473 "Impulsomat" and "Dosimat" equipped with a 1.0 ml syringe containing 1.0 N NaOH titrant.

The gel suspension pH was adjusted to 7.5 with 1 N NaOH and 9 mg of "Pronase" in 0.05 ml 2 M urea - 0.015 M

$CaCl_2$ was added. After several hours base consumption ceased, and at 16.5 hours an additional amount of 6 mg "Pronase" in 0.05 ml 2 M urea - 0.025 M $CaCl_2$ was added. The reaction was then followed for 7.5 hours with a 0.1 N NaOH titrant, during which time only about 10^{-3} meq of NaOH was added. Total base consumption was 0.591 meq.

The pH of the hydrolysate was adjusted to 4.0 with glacial acetic acid and was evaporated to dryness in the "Bio-Dryer". The residue was dissolved in 0.1 N acetic acid to a volume of 5.0 ml and subjected to P-6 gel permeation chromatography.

II. Column Chromatography of Glycopeptides

Glycopeptides were resolved from hydrolysates by gel permeation chromatography followed by chromatography on ion-exchange resins. Ion-exchange resins were washed thoroughly and fine particles were removed by the procedures described by Schroeder, Jones, Cormick & McCalla (95). AG 50W-x2 was stored as the H^+ form, at room temperature, and AG 1-x2 was stored at 4° C, in the acetate form.

All chromatographic columns were equipped with 1/16" teflon input and output lines (Penntube Products, Pennsylvania Fluorocarbon Co., Inc., Clifton Heights,

Pennsylvania, Stock no. M-659, AWG #22 HW Natural). The columns were operated at constant flow rates by positive displacement pumps, either a small capacity controlled volume "Mini-Pump" (Milton Roy Co., Philadelphia, Pennsylvania) or an "Accu-Flo" pump (Beckman Instruments, Inc., Spinco Division, Palo Alto, California). Column developer gradients for ion-exchange chromatography were produced with a Buchler "Varigrad." Chromatographic fractions were collected at 4° C with a Buchler refrigerated continuous fraction collector (Buchler Instruments, Fort Lee, New Jersey). Fraction volumes were determined by weighing the collected fractions in tared collection tubes.

Routinely, columns were loaded by removing the developer above the resin and percolating the sample into the resin bed by gravity flow or by air pressure. Sample vessel rinses were used to rinse the sample from the column walls into the resin after all the original sample had flowed into the resin bed. Following flow of rinses into the bed, the column was filled with developer and connected to the developer pump and fraction collector. The first fraction collection commenced following loading of the column.

Aliquots of the loaded samples were assayed for neutral carbohydrate and these data were used to determine recoveries of glycopeptides in column eluates unless otherwise noted.

The fraction tubes which were pooled are indicated on the chromatograms by double pointed arrows. The elution profiles, have been plotted such that effluent volume excludes the sample load and rinse volumes, and data collected on discreet fraction volumes have been plotted at the fraction volume midpoints. All fraction assay data were corrected for the contributions of reagent blanks unless otherwise noted.

A. Bio-Gel P-6 Gel Permeation Chromatography

Pronase hydrolysates of PPHA were routinely subjected to gel permeation chromatography on Bio-Gel P-6 to separate glycopeptides from partially digested protein, small peptides, and amino acids. Bio-Gel P-6 (25 g), with an operating range of 1000-6000 daltons, was hydrated overnight in 2 liters of distilled H_2O. Fine particles were removed from the hydrated resin by decantation following settling of the resin from suspension for 30-45 minutes. The resin was resuspended in 2 liters of distilled water and the procedure was repeated until no fine particles

were apparent in the supernatant. A 1:1 v/v resin slurry was made in 0.1 N acetic acid for pouring the column.

The chromatographic column was 0.9 x 150 cm (University of Oregon Medical School, Research Instrument Services), and was equipped with standard 18/9 socket inlet and 12/5 ball outlet fittings and with a water jacket. The base of the column was fitted with a medium porosity sintered glass disc and a capillary bore outlet.

Prior to pouring the resin, a 0.9 cm disc of Whatman No. 1 filter paper was placed on top of the sintered glass disc. The outlet was closed, and 0.1 N acetic acid was poured into the column to a height of 10 cm. The resin slurry was poured into the column and allowed to settle until about a 4 to 5 cm bed had formed. The outlet was then opened and slurry was added alternately with application of 7 psi air pressure until a bed height of approximately 146-147 cm was formed. The column was typically equilibrated with 7 or more bed volumes of 0.1 acetic acid supplied by an "Accu-Flo" pump at a flow rate of 16 ml/hr (about 13 psi) giving an equilibrated bed height of 143 to 147 cm. Following equilibration an MER #313 porous teflon disc (MER chromatographic, Mountain View, California) was inserted on top of the resin bed.

Samples were loaded in a 5 ml volume, followed by a one ml rinse of the sample vessel. The column was developed with 0.1 N acetic acid at a flow rate of 16 ml/hr. Fractions of 2.7 ml or less were collected. Typically, elution profiles of sample components were established by measuring the absorbance of fractions at 280 nm and the neutral carbohydrate content with the manual orcinol method as described below.

The column void volume was 24 ml, corrected for the sample volume, based on the chromatography of 5 mg of PPHA loaded in a 0.5 ml volume and chromatographed as described for glycopeptide samples.

B. Bio-Gel P-2 Gel Permeation Chromatography

Gel permeation chromatography on Bio-Gel P-2 was used to desalt samples, check P-6 column efficiency, and resolve reaction products in glycopeptide sequencing studies. Bio-Gel P-2, with an operating range of 100 to 1800 daltons, was hydrated in 50 or more volumes of distilled water per gram of dry resin for three or more hours. The fine particles were removed from the hydrated resin and a slurry was prepared in 0.1 acetic acid as described for Bio-Gel P-6.

A 0.9 x 65 cm column of the type used for P-6 gel chromatography was prepared and poured by the same procedure as described for preparation of Bio-Gel P-6 columns. The resin bed was equilibrated at room temperature with 10 or more bed volumes of 0.1 N acetic acid at a flow rate of 20-22 ml/hr supplied by either a "Mini-Pump" or an "Accu-Flo" pump. Operation pressure was typically 6-8 psi.

Samples were loaded in a 1 ml volume followed by one or two 0.1 N acetic acid rinses totalling 0.5 to 1.0 ml. Approximately 2 ml fractions were collected. Elution profiles were obtained by analyzing collected fractions by one or more of the manual procedures described below.

C. AG 50W-x2 Chromatography

An attempt to resolve the glycopeptide fraction obtained from gel permeation chromatography was made by employing a 0.9 x 45 cm column of AG 50W-x2 at 40° C with the pH 3.1 pyridine-acetic acid buffer (0.2 N pyridine) described by Schroeder *et al* (95) at a flow rate of 90 ml/hr. This attempt was unsuccessful, as the glycopeptide load eluted sharply in one unretarded peak. Subsequently other AG 50W-x2 procedures were tested.

The first procedure employed AG 50W-x2 at low ionic strength and pH 2.3 as adapted by McKelvy and Lee (96) from the method of Cunningham, Ford, and Rainey (97).

Approximately a 300 ml settled volume of AG 50W-x2 was washed in a Büchner funnel with 1 liter each of 2.5 N NaOH, distilled water, 6 N HCl and 1.5 liters of distilled water. Fine particles were removed by decantation following settling of the resin from a 1:1 suspension in distilled water for 30 minutes, which was repeated until no fine particles were apparent. The resin was then washed with 0.5 liter of pH 2.3, 0.002 M pyridine - 0.2 M formic acid buffer (17.05 ml concentrated formic acid + 0.316 gm pyridine per two liter volume) in a Büchner funnel and suspended in pH 2.3 buffer to a volume of 500 ml. The slurry was poured into the 0.9 x 150 cm column described for P-6 gel chromatography by the procedure described for P-6 column preparation. The column was equilibrated at room temperature with 700 ml pH 2.3 buffer supplied by an "Accu-Flo" pump at 33 ml/hr at a pressure of 11 psi. The equilibrated bed height was 148 cm.

The lyophilized glycopeptide mixture from gel permeation chromatography (64 mg) was dissolved in 0.2 N formic acid to a volume of 5.0 ml and loaded on the column

followed by two 0.5 ml rinses with pH 2.3 pyridine-formic acid buffer. The column was operated at room temperature and a flow rate of 33 ml/hr with 1440 ml pH 2.3 pyridine-formic acid buffer followed by a gradient formed with 200 ml pH 2.3 buffer in chamber 1 and 200 ml of pH 6.1 0.2 M pyridine (15.8 gm redistilled pyridine + 100 ml 0.2 N formic acid per liter) in chamber 2 of a Büchler "Varigrad." Samples were manually assayed for neutral carbohydrate with the orcinol reaction.

The second procedure resulted from Saunder's report (98) that oligosaccharides, monosaccharides, isopropylidene derivatives of sugars and methyl glycosides are separable on Dowex 50-x4(K^+) using water as the eluent.

Sufficient AG 50W-x2 to fill a 0.9 x 150 cm column was washed in a Büchner funnel with 2 N HCl until no color appeared in the washes, followed by one liter of distilled water. The resin was converted to the K^+ form by further washing with one liter of 1 N KOH, followed by two liters of distilled water. A 1:1 v/v resin slurry was prepared in distilled water and poured into the 0.9 x 150 cm column used for P-6 gel by the procedure outlined for P-6 column preparation. The column was equilibrated at room temperature

with 60 or more bed volumes of distilled water supplied by an "Accu-Flo" pump at 38 ml/hr at a pressure of 13 psi, giving a final bed height of 145-147 cm.

Samples were typically loaded in 5 ml of distilled water followed by a 1 ml water rinse. The column was developed under the conditions used for equilibration with distilled water and fractions of either 5 or 10 ml were collected. Effluents were monitored continuously with the automated orcinol assay for neutral carbohydrate described below.

A modification of this procedure employed development of the column with 50% v/v ethanol in distilled water (1580 ml 95% ethanol + 1420 ml distilled water). AG 50W-x2 (K^+) was washed with 50% ethanol and a 2:1 v/v 50% ethanol-resin slurry was poured into the 0.9 x 150 cm column as described above. The column was equilibrated at room temperature with 4 to 5 column volumes 50% ethanol supplied by an "Accu-Flo" pump at 31-32 ml/hr at 30 psi. Final bed height was 142 to 145 cm.

Samples were dissolved in 50% ethanol to a volume of 5 ml and loaded, followed by up to a 1 ml rinse of 50% ethanol. The column was developed under the conditions used for equilibration and fractions of 4 to 8 ml were

collected. Effluents were monitored continuously with the automated orcinol assay.

D. AG 1-x2 Chromatography

Chromatography of glycopeptide mixtures on AG 1-x2 with pH 5.7 (30° C), 0.1 N pyridine-acetic acid was used in conjunction with P-6 gel permeation chromatography and AG 50W-x2 pH 2.3 pyridine-formic acid chromatography, to prepare highly purified glycopeptides for structural studies. However, applicability of several volatile buffer systems was examined in the course of developing this system.

AG 1-x2 slurries were prepared by mixing 1 volume of resin with 1-2 volumes of buffer, either 1% collidine adjusted to pH 8.7 with glacial acetic acid or 0.1 N pyridine adjusted to pH 6.1 with glacial acetic acid. The slurry was deaerated under vacuum and poured into a 0.63 x 70 cm water jacketted Technicon column (Technicon Corp., Tarrytown, New York). The column was typically equilibrated at 45° C with 20 or more bed volumes of starting buffer supplied by a "Mini-Pump" at 41 ml/hr at approximately 5 psi. Equilibrated bed heights ranged from 62 to 67 cm, with those used in pH 5.7 pyridine-acetic acid runs ranging from 66-67 cm.

Glycopeptide samples were dissolved in starting buffer to a 5.0 ml volume with the pH adjusted to at least 0.5 pH units higher than that of the starting buffer. For pH 5.7 pyridine-acetic acid chromatography, 0.1 N pyridine (pH 8.5) was used. For other systems, either adjustment with NaOH, or development buffer with the same concentration of organic base, but with less acid, gave the desired pH. The sample was loaded on the column followed by two 0.5 ml rinses and was chromatographed under the conditions used for equilibration. Fractions of 5 ml were collected and column effluents were monitored continuously with the automated orcinol assay, and also with the automated ninhydrin assay in earlier runs.

The following gradient systems were tested: (1) 150 ml 1% collidine (10 ml collidine + glacial acetic acid to pH 8.7 per 1) and 150 ml 0.1 N acetic acid; (2) 200 ml pH 7.1 collidine-acetic acid (735 ml 1% collidine + 335 ml 0.1 N acetic acid) and 200 ml pH 4.1 acetic acid-collidine (200 ml 0.1 N acetic acid + 41 ml 1% collidine); (3) 150 ml pH 6.6, 0.1 M pyridine (7.9 g pyridine + 0.4 ml glacial acetic acid per 1) and 150 ml pH 5.0, 1 M pyridine (79.1 g pyridine + 56 ml glacial acetic acid per 1) and (4) 150 ml pH 6.1, 0.1 M pyridine (15.8 g pyridine + 1.7

ml glacial acetic acid per 2 l) and 150 ml pH 4.9, 0.1 N pyridine (pH 5.0 1 M pyridine-acetic acid diluted 10 fold with distilled water). The first buffer of each pair was put in chamber 1 of the Buchler "Varigrad" and the second in chamber 2. Two constant pH and ionic strength developments were also tested: (1) pH 5.7 0.1 M pyridine-acetic acid (31.64 g pyridine + 6.2 ml glacial acetic acid per 4 l); and (2) pH 6.0, 0.1 M pyridine-acetic acid (15.82 g pyridine + 2.0 ml glacial acetic acid per 2 l).

E. Chromatographic Column Effluent Monitoring

Collected fractions were analyzed with one or more of the following manual methods, or column effluents were continuously monitored by the automated system described below.

Neutral carbohydrate was determined by a modification of the orcinol method described by Winzler (99). Reagents were prepared as recommended (99), but the assay volume was scaled down and the incubation temperature was increased about 15° C. Samples (0.24 ml) were mixed with H_2SO_4-orcinol reagent (2.0 ml) in 13 x 100 mm stoppered pyrex test tubes. The tubes were immersed for twenty minutes in a 94-98° C water bath. The incubated samples were cooled for 5 minutes in a water bath at room tempera-

ture and their absorbances were measured at 420 nm in 2 ml quartz cuvettes. Reagent blanks were prepared with a sample of H_2O.

Under these assay conditions, 0.07 µmole of mannose produced a net absorbance of 1.0, and Beer's law was observed up to an absorbance of 1.5. The PPHA, however, displayed about a 5% negative deviation from Beer's law which was most apparent above an absorbance of about 0.7.

Free amino groups were determined by either spectrophotometric or spectrofluorimetric ninhydrin assays. Spectrophometric assays were conducted with the ninhydrin reagent of Yemm and Cocking (100). The samples (0.7 ml) were mixed with pH 5, 0.5 M citrate buffer (0.4 ml) and ninhydrin reagent (0.6 ml) in 13 x 100 mm stoppered pyrex test tubes. These were incubated for 15 minutes at 94-98° C in a water bath, followed by cooling for five minutes to room temperature. A volume of 2.5 ml 50% v/v aqueous ethanol was added to each sample and absorbances were read at 540 nm. Reagent blanks were prepared with a sample of H_2O. Under these conditions 0.26 µmoles of leucine produced a net absorbance of 1.0 and Beer's law was observed up to an absorbance of 1.5.

The spectrofluorimetric ninhydrin procedure was performed as described by Samejima, Dairman, Stone, and

Udenfriend (101). The samples (0.1 ml) were mixed with 2.0 ml of 0.2 M, pH 7.2 phosphate buffer, 0.2 ml of 50 mM ninhydrin, and 0.1 ml of 10 mM phenylacetaldehyde in ethanol, in 13 x 100 mm stoppered pyrex test tubes. These were incubated for 60 minutes in a water bath at 60° C, followed by cooling to 0° C in ice. Cooling the samples increased their fluorescence. Fluorescence was measured at 490 nm with an excitation wavelength of 390 nm using an Aminco-Bowman Spectrofluorimeter (American Instrument Co., Silver Spring, Maryland). With the instrument settings and apertures used throughout these assays, 50 nmoles of glycine gave a relative fluorescence of about 12 to 15.

Conductivity measurements were made with a Radiometer conductivity meter type CDM2e with a type CDCS67021 electrode. The direct meter readings represented the specific conductance x 2.3 cm. Samples were equilibrated to room temperature prior to measurement.

Some column effluents were automatically monitored during column operation with either ninhydrin and orcinol assays, or only the latter. The monitoring apparatus was assembled from the following Technicon "Autoanalyzer" modules (Technicon Corp., Tarrytown, New York): a 2-speed proportioning pump; an adjustable circulating constant

temperature bath; 2 colorimeters equipped with 15 mm flowcells, #3 apertures and constant voltage power supplies, one with 570 nm interference filters, and the other with 420 nm interference filters; and a dual channel recorder. The system was designed to provide monitoring of small sample volumes continuously without the bubble pattern normally employed in "Autoanalyzer" systems. The chromatographic columns were driven with constant volume pumps and constant time fractions were collected. A schematic of the system for simultaneous ninhydrin and orcinol assay is given in Figure 2. When only the orcinol assay was used, the sample was fed totally to the orcinol channel as described. All tubing except that in the proportioning pump was made of teflon.

Referring to Figure 2, column effluent enroute to the fraction collector passed through a specially constructed Y-junction (Inset a) where part of the stream was continuously drawn through a 0.015" i.d. teflon tube to the proportioning pump. The sizes of the sample and water manifold tubes were varied depending on the glycopeptide load, but the total flow rate was maintained between a nominal 0.23 and 0.26 ml/min. The sample was diluted with water and the stream was divided in a specially constructed

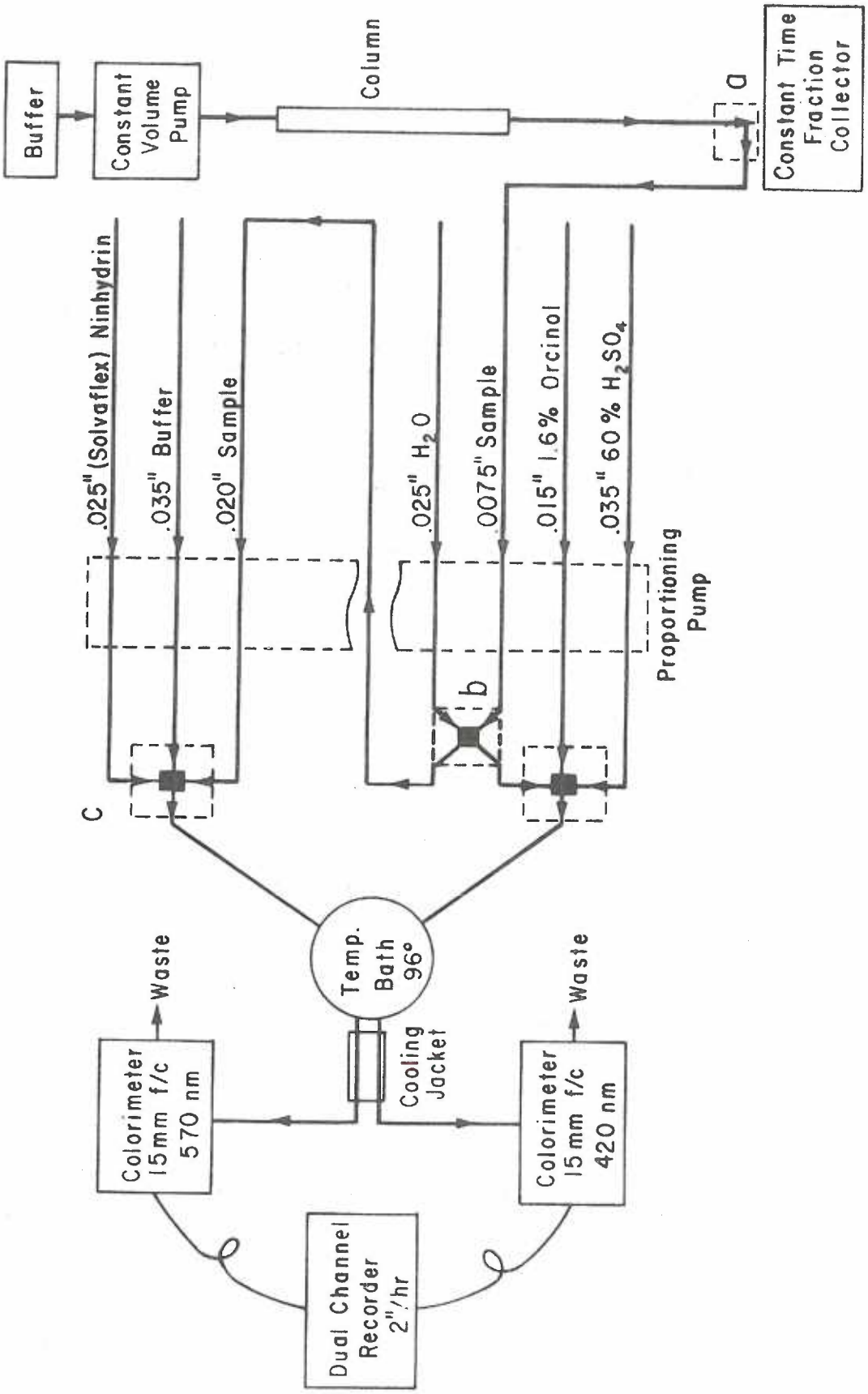


Figure 2. Schematic diagram of the automated orcinol and ninhydrin assay system for continuous monitoring of chromatographic column effluent. Components a, b, and c are shown in detail in Figure 3.

4-port manifold (Inset b). One part of the sample was passed directly into a #309 MER four-port Kel-F manifold and mixed with orcinol and sulfuric acid. The second part was directed back through the proportioning pump and into a specially constructed 4-port manifold (Inset c) where it was mixed with ninhydrin reagent and buffer. Each assay mixture then flowed through a 70 foot coil of 1/16" Penntube teflon tubing (0.138 ml/ft capacity) in the adjustable temperature bath maintained at 96° C. At the temperature bath outlet the teflon lines passed through a 4 inch chromatography column water jacket through which cold tap water was continuously circulated. Ninhydrin effluent was monitored at 570 nm and orcinol at 420 nm. Both colorimeter signals were simultaneously recorded with a dual channel recorder. The assay systems were phased such that a sampled mixture of amino acid and carbohydrate produced superimposable recorder signals.

As noted, the sampling Y, and two 4-port manifolds were specially constructed, as illustrated in Figure 3. The sampling junction (Figure 3a) was about 0.01 ml from the drop delivery point. The junction was made from 1/16" o.d. Penntube teflon tubing and 0.033" o.d. #T21195 Bel Art teflon spaghetti tubing (Joymar Scientific, Inc.,

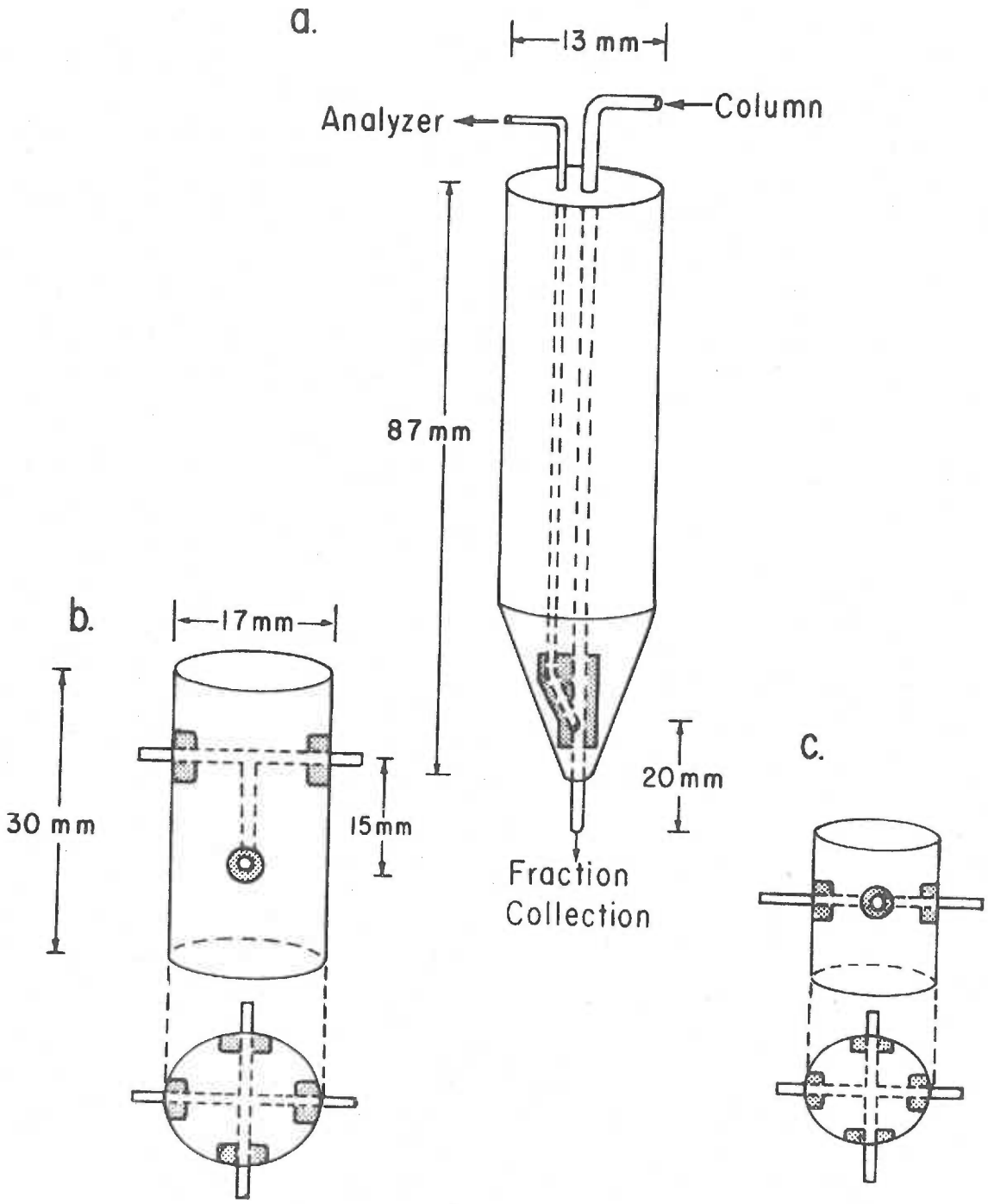


Figure 3. Specially constructed components of the automated analysis system: a. sampling junction; b. four-port manifold for sample dilution and stream splitting; and c. four-port mixing manifold for ninhydrin assay.

New York, New York). The tubing was mechanically held together while the junction was cast in Dolphon epoxy resin. Following setting, the assembly was cast in acrylic casting resin (TAP Plastics, Inc., Portland, Oregon) using a 13 i.d. x 120 mm polyethylene conical centrifuge tube for a mold.

The 4-port manifolds, for sample dilution and stream splitting (Figure 3b) and for mixing ninhydrin assay reagents (Figure 3c) were made from 20 gauge stainless steel needle sections and casting resin. The resin blocks were cast with well polished needle sections passing through the casting where holes were desired. Following setting, resin shrinkage allowed the needle sections to be withdrawn. The holes were countersunk with a 3/32" bit to a depth of about 1/8". The needle sections were then inserted to about 1 mm from the mixing chambers and held in place by epoxy resin in the countersunk holes.

The sample stream splitter was designed so that water and sample would be fed in through directly opposed inlets and would have adequate time to mix before the stream was divided at the outlets which were rotated 90^{o} from the inlets.

The adjustable temperature bath was modified for use with teflon reaction coils. The coils were supported by a

7" diameter 5" cylinder of #304 16 gauge stainless steel 3/16" mesh screen (Western Wire Works, Portland, Oregon). Technicon high temperature bath oil could not be used since bubbles appeared in the ninhydrin reaction coil effluent. Use of a 50% aqueous glycerol bath eliminated this bubble problem.

Reagents were prepared for the orcinol assay as described by Winzler (99) and for the ninhydrin assay as described by Johnson, Rigas and Jones (102).

Typically, a chromatographic column was loaded while the analyzer system was establishing a baseline. Before turning on the column pump, the analyzer manifold pump was shut off briefly and an aliquot of mannose solution, or of mannose and glycine, was injected into the column effluent line as a marker of the first fraction. The column, fraction collector and analyzer were then turned on.

The segments of the analyzer tracings were manually correlated with their corresponding collected fractions, since the recorder was not equipped with an event marker. The recorder chart speed, column flow rate, fraction collection time, and the analyzer sampling rate were each held constant. Thus each fraction corresponded to a constant recorder chart length. The appropriate length interval was marked beginning at the front of the marker pulse peak.

Analytical Methods

I. Gas-Liquid Chromatographic Analysis of Neutral Monosaccharides.

Neutral monosaccharides were qualitatively and quantitatively determined as their alditol acetates by the gas-liquid chromatographic procedure of Lehnhardt and Winzler (103). Typically, PPHA or glycopeptide samples of one mg or less were dissolved in 0.1 ml of a 20% w/v suspension of AG 50W-x2(H^+) in 0.01 N HCl. Hydrolysis was performed in silicone rubber-stoppered 6 x 50 mm culture tubes immersed in a refluxing water bath. This prevented artifacts produced by the distillation of the liquid from the suspension to the top of the tube leaving the resin dry.

Following hydrolysis, aliquots of aqueous internal standard solution containing 0.015 to 0.075 μmole of 2-deoxy-D-glucose were injected through the stoppers and mixed thoroughly with the resin suspension. The hydrolyzed neutral sugars were then separated from amino acids, amino sugars, peptides and salts by elution through small columns prepared with 0.05 ml of a 20% w/v aqueous suspension of AG 1-x8 (HCO_3^-). Eluates were evaporated to dryness in the "Biodryer". The dried eluates were reduced

in 0.10 ml aqueous 0.11 M $NaBH_4$ for one hour at room temperature. Excess borohydride was destroyed by addition of 0.02 ml glacial acetic acid followed by evaporation to dryness and removal of borate as methyl borate through 3X evaporation of the sample dissolved in 0.1 ml 1000:1 (v/v) methanolic-HCl. The samples were then stored over silica gel in an evacuated desiccator at least overnight to insure dryness.

The reduced samples were acetylated in 0.05 to 0.10 ml (v/v) acetic anhydride; pyridine for 30 minutes at 100° C in a "Temp-Blok Module Heater". The acetylated samples in acetic anhydride-pyridine were injected into the gas chromatograph.

Analyses were performed with an F & M Model 810-DR-12 gas chromatograph (Avondale, Pa.) equipped with dual columns and flame ionization detectors. The glass columns were 6 ft. x 1/4" o.d. and were packed with 0.75% HiEFF-1BP (diethylene glycol succinate), 0.25% EGSS-X (ethylene succinate-methylsilicone polymer) and 0.1% 144-B (phenyl-diethanolamine) by weight on 60/80 mesh Gas-Chrom Q (Applied Science Laboratories, Inglewood, California).

The instrument adjustments were the following:

Column temperature	160-200° C
Program rate	1°/min (nominal)
Detector temp.	325° C
Inj. port temp.	225° C
H_2 to detector	50 ml/min.
Air to detector	300 ml/min.
N_2 carrier	50 ml/min.
Chart speed	30 in/hr.

Alditol acetate standard mixtures were chromatographed under the conditions listed to determine retention times and relative peak areas. Relative retention times (RRT) were computed for each alditol acetate by dividing its retention time by that of the 2-deoxy glucose derivative. Peak areas were measured with a Keuffel & Esser #4236 compensating polar planimeter (Hoboken, N.J.). Relative molar response factors (RRF) were computed relative to the alditol acetate of deoxyglucose with the expression:

$$RRF = \frac{\text{moles deoxyglucose / area deoxyglucose}}{\text{moles monosaccharide / area monosaccharide}}$$

Chromatographic peaks of protein or glycopeptide samples were identified by comparison of their relative retention times with those of the standards. The areas of these peaks in conjunction with the appropriate response factors

were used to quantitate the monosaccharides according to the equation:

$$\frac{\text{moles of X}}{\text{mg sample hydrolysed}} = \frac{(\text{area X})\ (\text{moles deoxyglucose added})}{(RRF_X)\ (\text{area deoxyglucose})\ (\text{mg sample hydrolyzed})}$$

The linear dependence of the released neutral carbohydrate on the amount of protein hydrolyzed was tested. Samples of lyophilized 67-A-$P_8(C_4)_1$ containing 0.5 mg, 1.0 mg and 1.5 mg of protein were hydrolyzed by the standard procedure for 40 hours and analyzed.

The dependence of monosaccharide yield on hydrolysis time was examined to establish an optimal hydrolysis time for routine analyses. Ideally the time chosen would produce maximal hydrolysis with minimal decomposition of the released monosaccharides. Single samples of lyophilized 67-A-P_8(1.0 mg) were hydrolyzed for periods of 0 to 40 hours and analyzed.

The neutral monosaccharide composition of 67-A-$P_8(C_4)_1$ was determined by analysis of seven replicate samples hydrolyzed for 25 hours. The protein and deoxyglucose were dried under vacuum over P_2O_5 in a drying pistol for 51 hours at 77° C. Longer drying times at this temperature produced dried protein which was difficult to dissolve totally, indicating heat denaturation. Samples containing

no protein and no internal standard were also carried through the total procedure to check for spurious peaks not due to protein and for the absence of 2-deoxyglucose in protein hydrolysates, respectively. The relative response factors of Winzler and Lehnhardt (103) were used to compute μmoles of monosaccharide per mg PPHA, and residues monosaccharides per mole PPHA, based on a molecular weight of 128,400 (51). Computations of grams of monosaccharide per 100 grams protein were made assuming n moles of H_2O were lost in bond formation for n residues of monosaccharide. All means, and standard deviations were computed with the Wang Statistical program no. 1996A/ST1 for ungrouped data.

II. Amino Acid Analysis

Amino acid analyses were performed by the method of Spackman, Stein and Moore (104) with a Beckman 120-C amino acid analyzer equipped with long light path flow-cells for increased sensitivity (105). Glycopeptide samples were dissolved in 0.5 to 1.0 ml constant boiling HCl and were hydrolyzed in evacuated ampules at 108-110° C in a "Temp-Blok Module Heater". The hydrolysis time was 22 hours unless otherwise stated. After hydrolysis the samples were evaporated to dryness in the "Bio-Dryer" and the residue was dissolved in pH 2.2 citrate diluent buffer.

Peak areas were determined by the height x width method and compared with those obtained with aliquots of Beckman amino acid calibration mixture. In later analyses, a known amount of internal standard, L-isoleucine, was subjected to hydrolysis with the sample. Quantitation was based on the peak areas relative to added isoleucine using the equation:

moles i^{th} amino acid =

$$\frac{(\text{area}_i)\ (\text{moles Ileu added})}{(\text{area Ileu})} \times \frac{(\text{Ileu standard area})}{(\text{standard area}_i)}$$

The last term in the equation was evaluated from profiles obtained with the amino acid calibration mixture. The values used were: Asp 1.08, Thr 1.12, Ser 1.03, Glu 1.06, Gly 1.05, Ala 1.03.

III. Glucosamine Analysis

Glucosamine analyses were performed with the basic amino acid column of the Beckman 120-C amino acid analyzer. Samples were hydrolyzed in 0.05-0.20 ml of 4 N hydrochloric acid (0.6% w/v or less) in silicone-rubber-stoppered 6 x 50 mm culture tubes immersed in a refluxing water bath. Unless otherwise stated, glycopeptide samples were hydrolyzed for 4 hours and PPHA samples for eight hours.

Following hydrolysis, the samples were quantitatively transferred to test tubes with three or more rinses of pH

2.2 citrate buffer to a final volume adjusted to contain approximately 0.01 to 0.05 μmole/ml of glucosamine. Aliquots of 1.0 ml were loaded on the analyzer basic amino acid column.

Quantitation was based on the comparison of sample peak areas with those obtained with D-glucosamine standards. Following drying the glucosamine hydrochloride to constant weight, standard solutions were prepared in pH 2.2 citrate buffer containing 0.03-0.038 μmole/ml glucosamine and analyzed in the same manner as were the samples.

The reliability of the hydrolysis and analysis technique as applied to PPHA was checked by analyzing aliquots which had been hydrolyzed in the presence of known amounts of glucosamine. Sample peak areas were plotted against the quantity of glucosamine added and a straight line was fitted to the points by linear regression analysis and extrapolated to zero peak area.

Glucosamine was also estimated from the chromatograms of the acidic and neutral amino acids from 22 hour hydrolysates. The values obtained were corrected for 40% destruction based on the observed destruction in analyses of the glycopeptides.

Glycopeptide amide nitrogen was estimated from the area of the NH_3 peak concurrently with determination of glucosamine following sample hydrolysis in 4 N HCl for 4 hours at 100° C. Mixed glucosamine and NH_3 standard solutions were prepared from the same stock solutions in the same ratios as used for sample treatment. Reagent blanks containing no standard NH_3 or glucosamine were run to determine background NH_3. The NH_3 standard was $(NH_4)_2SO_4$, dried to constant weight. Sample ammonia values were corrected for reagent contributions, but not for free NH_3 in the unhydrolyzed peptide unless otherwise noted.

IV. Molecular Weight Determination

Glycopeptide molecular weights were determined by sedimentation equilibrium in a Beckman Model E analytical ultracentrifuge equipped with Rayleigh optics (Beckman Instruments, Spinco Division, Palo Alto, California). The procedure was basically that described by Chervenka (106) for low speed conventional sedimentation equilibrium. Lyophilized glycopeptide samples were dissolved in 0.10 M NaCl to concentrations of 2-3 mg/ml. The sample (0.12 ml) and FC-43 fluorocarbon oil (0.03 ml) were put in the sample sector of a 12 mm double sector synthetic boundary cell equipped with sapphire windows, and 0.10 M NaCl (0.17 ml)

was added to the solvent sector. Centrifugation was conducted in an An-D rotor with an interference counterbalance at 52,640 rpm at 20.0° C. The optical system was fitted with an interference offset condensing lens mask and a 77 A filter over the light source.

Interference photos were recorded at four hour intervals on Eastman Spectroscopic II-G plates, commencing after 16-20 hours of centrifugation. The plates were measured with a two dimensional "Gaertner" microcomparator (Chicago, Illinois). The run was assumed to be at equilibrium when photos taken four hours apart gave superimposable plots of vertical fringe shift versus distance from the center of rotation. The speed was checked by clocking the odometer reading change for a period of four or more hours.

The horizontal displacements of light fringe centers from the reference wire were measured for two different equilibrium photos from each run. The magnification factor was 2.156, based on the photographed distance from the inner to outer reference edge of the Schlieren 12 mm counterbalance divided by the same distance directly measured with the microcomparator.

Molecular weights were calculated using the Nazarian equation (107). The standard equation describing the

equilibrium distribution of a solute with molecular weight, M, in an ideal solution is:

$$C_{(q)} = C_{(o)} e^{AMq}$$

where $q = r^2$, r is the radial distance from the center of rotation, $C_{(q)}$ is the solute concentration at q, $C_{(o)}$ is the hypothetical concentration at the center of rotation, M is the solute molecular weight, and $A = \frac{(1-\bar{v}\rho)\omega^2}{2RT}$, in which $\bar{v}$ is the solute partial specific volume, ρ is the solution density, ω is the angular velocity, R is the gas constant, and T is the absolute temperature. The absolute fringe number, J, is related to concentration by the relationship:

$$J_{(q)} = \frac{an}{\lambda} C_{(q)}$$

in which a is the cell thickness, n is the specific refractive index increment, and λ is the light wavelength. Since the fringe shift, not the absolute fringe number, is directly measured on conventional sedimentation equilibrium interference photos, Nazarian (107) derived from the standard equation an equation expressing M in terms of fringe shift:

$$\log \Delta_Q J = \log \left[J_{(q+Q)} - J_{(q)}\right] = \frac{AMq}{2.303} + \log \frac{an}{\lambda} \left[C_{(q)} - C_{(o)}\right]$$

or in differentiated form solved for M,

$$M = \frac{2.303}{A} X \frac{d\log \Delta_Q J}{dq}$$

in which $\Delta_Q J$ is the fringe shift over a constant interval, Q cm^2, from q to q+Q. The undifferentiated form of the equation is mathematically exact for any size Q. Thus a series of log $\Delta_Q J$ values are determined for a given Q and a series of q values and the molecular weight can be calculated from the slope of the plot of log $\Delta_Q J$ versus q if $\bar{v}$ and ρ are known.

Q was set at 2.0 cm^2 to minimize the relative magnitude of measurement errors. Values of log $\Delta_Q J$ were computed for successive q values at 0.2 cm^2 intervals from about the meniscus to the fluid column bottom by linear interpolation (108). The corresponding log $\Delta_Q J$ values from two series of measurements on different photos were averaged. The slope of the plot, log $\Delta_Q J$ versus q and the coefficient of correlation were computed with a Wang 700 advanced programming calculator using the Wang linear regression program No. 1987 A/ST3. The standard deviation, s_b, of the slope was calculated as described by Snedecor and Cochran (109). A confidence interval for the molecular weight estimate resulting from a difference of one standard

deviation unit in the slope was provided by multiplying the ratio of s_b to the slope by $\frac{4.606RT}{(1-\bar{v}\rho)\omega^2}$.

Peptide partial specific volumes were estimated from the composition data and the calculated partial specific volumes of the constituent residues with the equation (110):

$$\bar{v} = \frac{\sum \bar{v}_i w_i}{\sum w_i}$$

in which $\bar{v}$ is the peptide partial specific volume, $\bar{v}_i$ is the partial specific volume of the i^{th} residue, and w_i is the weight percent of the i^{th} residue. The values of $\bar{v}_i$ used were the following:

Residue	$\bar{v}_i$ (cc/g)	Reference
Asp	0.59	(110)
Asn	0.60	"
Glu	0.66	"
Thr	0.70	"
GlcNAc	0.666	(111)
Man	0.613	"
Fuc	0.678	"
Xyl	0.628	Calculated

The value for xylose was calculated from that of fucose through multiplication by 146.1, the residue weight of

fucose, subtraction of the volume of one CH_2 group, estimated at 16.1 cc/mole (111), and division by 132.1, the residue weight of xylose.

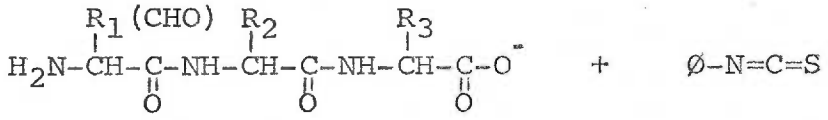
The density of the solution, ρ, was estimated as 1.0023 gm/cc, the measured density of 0.10 M NaCl at 20.0° C (112).

Theoretical molecular weights were calculated from composition data using the minimum number of residues giving approximately integral residue ratios. The molecular weights of n hydrolyzed residues were summed and corrected for n-1 moles of H_2O lost in bond formation.

Structural Analysis

I. Edman Degradation

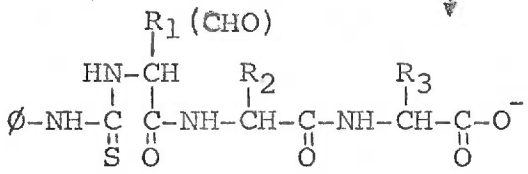
The glycopeptide amino acid sequence and site of carbohydrate attachment were studied with the Edman degradation (113,114). The following reaction sequence illustrates the rationale of the Edman degradation procedure for a hypothetical tripeptide to which an oligosaccharide (CHO) is bound through the side chain of the N-terminal amino acid:



Tripeptide

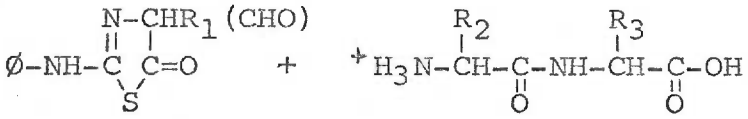
Phenylisothiocyanate (PITC)

(Coupling) OH^-



Phenylthiocarbamyl (PTC) derivative

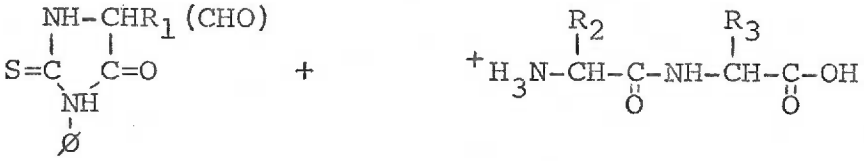
(Cleavage) H^+



Anilino-thiazolinone (ATZ) Derivative

Dipeptide

(Conversion) H_3O^+



Phenylthiohydantoin (PTH) Derivative

Dipeptide

For the coupling reaction, approximately 0.1 μmole of glycopeptide was dissolved in 2 ml of pH 9.0 pyridine-triethylamine buffer (50 ml redistilled pyridine + 2 ml triethylamine + 0.75 ml glacial acetic acid brought to 100 ml with distilled water) in a 13 x 100 mm dichromate-H_2SO_4-washed "Kimax" screwcap culture tube. A volume of 0.05 ml phenylisothiocyanate (PITC) was then added. The tube was flushed with nitrogen, capped, and incubated for 3 hours at 40° C in a "Temp-Blok Module Heater". The incubated samples were evaporated to near dryness in the "Bio-Dryer" followed by extraction three times with 2 ml analytical reagent grade benzene to remove unreacted PITC and phenythiourea. Extracted samples were stored overnight in a vacuum desiccator to insure dryness.

Cyclization and cleavage were effected by incubation in 1.0 ml of anhydrous trifluoroacetic acid for 1 hour at room temperature. Trifluoroacetic acid was removed in a vacuum desiccator containing NaOH pellets. To convert the anilinothiazolinones to phenylthiohydantoins, 1.0 ml of 0.2 N acetic acid was added to the residue and the solution was incubated for 15 minutes at 40° C in the "Temp-Blok". The samples were then extracted three times with 1.0 ml analytical reagent grade ethyl acetate to remove any amino

acid phenylthiohydantoin (PTH) derivatives. PTH derivatives containing an oligosaccharide, as illustrated above, were anticipated not to be extractable due to the polarity of the carbohydrate moiety. This expectation was supported by observations which will be presented under "Results".

To determine which amino acid had been removed from the N-terminus, an aliquot of the degraded peptide in 0.2 N acetic acid was hydrolysed in constant boiling HCl for 16 hours and analyzed for amino acid composition as described earlier. This hydrolysis time was chosen since: 1) in timed hydrolysis experiments on the glycopeptides the levels of free amino acids reached a maximum by this time; and 2) hydrolysis for 16 hours was expected to give less hydrolysis of PTH-Asx or PTH-Glx to the free amino acids than the standard 22 hours hydrolysis. In connection with the last point, the PTH-derivatives of aspartic and glutamic acid had been hydrolyzed for 24 hours in 6 N HCl at 120^{o} C to give 48 and 39%, respectively, of the theoretical yields of the free amino acids. Under these same conditions, no threonine or serine were recoverable from their PTH-derivatives (115).

The difference between the amino acid compositions of the peptide before and after a degradation step often provides the identity of the amino acid. However, this technique does not distinguish the acid and amide forms of aspartic or glutamic acid, since the amides are destroyed during hydrolysis, nor does it distinguish whether the carbohydrate was bound to the PTH-amino acid or to the residual peptide. To answer these questions, the ethyl acetate extract was subjected to thin layer chromatography as described below. This chromatographic system clearly resolved the acid and amide forms of PTH-Asx or of PTH-Glx. Thus, the acid or amide forms of aspartic and glutamic acid were identified by the difference analysis coupled with the chromatographic behavior of the extractable PTH-derivative.

However, no extractable PTH-derivative was expected when the derivative contained carbohydrate as illustrated above. Whenever no PTH-derivative was extracted, the solution of degradation products in 0.2 N acetic acid, which was anticipated to contain a glycosylated PTH-derivative and a residual peptide, was chromatographed on Bio-Gel P-2.

Chromatographic fractions were analyzed for neutral carbohydrate with the orcinol assay (99) and for absorbance

at 264 nm, the measured adsorption maximum for PTH-Asn dissolved in 0.1 N acetic acid. Since the presence of an oligosaccharide in the PTH-derivative significantly increases its molecular weight, the resulting chromatogram would be expected to have: a) an orcinol positive peak with 264 nm absorbance at or near the column void volume, and b) a ninhydrin positive peak at a larger elution volume. In practice, the low levels of peptide employed in these degradations precluded standard ninhydrin analysis so that the fractions in the early orcinol positive zone and later fractions, where smaller peptides should elute, were separately pooled and analyzed for amino acid composition.

Thin layer chromatography was performed with 20 x 20 cm Eastman #6060 silica gel thin layer sheets with fluorescent indicator. Samples and phenylthiohydantoin standards dissolved in acetone were spotted along a line 2 cm from the bottom edge. A standard mixture containing 3-4 μg of each PTH-derivative was loaded. The plates were developed with 90:10 (v/v) chloroform:methanol to a height of 18-19 cm and were visualized under ultraviolet light (116). A standard load of 2 μg was easily visualized.

Standard derivatives were prepared from aspartic acid, asparagine, glutamic acid, glutamine and threonine.

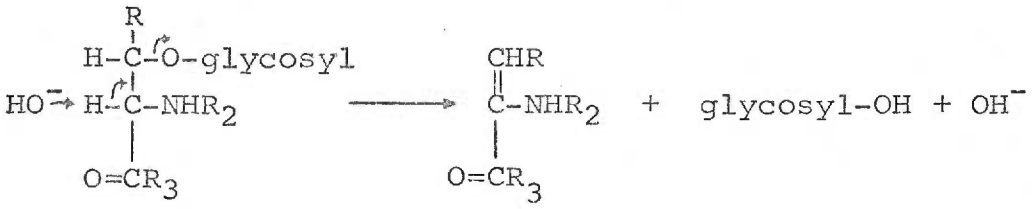
Approximately 20 mg of each was treated in the same manner as were peptides, except that sodium hydroxide was required to adjust the pH's of the acidic amino acid solutions to 9 for the reaction with PITC. The ethyl acetate extracts were evaporated to dryness and the products were recrystallized from aqueous ethanol. Uncorrected melting points of the dried products, with the literature melting points in brackets, were: PTH-Asp, 226° C [229° C] (117); PTH-Asn, 231° C [234° C] (117); PTH-Glu, 165-168° C [166-167° C] (117); PTH-Gln, 204° C [201-211° C, decomp.] (118); and PTH-Thr, 190-195° C [193° C] (119).

Each standard produced a single spot when chromatographed in the system described, and the amides were well resolved. However, the corresponding forms of aspartic acid and glutamic acid, i.e., the acid or the amide PTH-derivatives were only partially resolved.

II. β-Elimination Reaction

When threonine was a plausible carbohydrate linkage site following Edman degradation, the glycopeptide was subjected to conditions which can produce a β-elimination of the carbohydrate moiety. This elimination of carbohydrate bound via O-glycoside linkages to the side chains of threonine or serine proceeds rapidly in 0.1 to 0.5 N NaOH at temperatures from 0° to 25° C if the α-carboxyl and α-amino

groups of the threonyl or seryl residues are blocked. Thus when a threonyl or seryl residue is known to occupy a nonterminal position in the glycopeptide and is covalently bound to carbohydrate, alkali should give the reaction (120).



R=H for serine or CH_3 for threonine

where elimination destroys the threonyl or seryl residue and produces a free reducing oligosaccharide. Inclusion of a reducing agent such as $NaBH_4$ in the reaction medium provides reduction of the reducing terminal monosaccharide of the glycosyl moiety and partial reduction of the less stable unsaturated amino acid residue. Thus, with $NaBH_4$, appearance of an α-amino butyryl residue accompanies threonine destruction and an alanyl residue results from serine.

The procedure was carried out two ways. In the first, approximately 0.02 µmole peptide was dissolved in 0.5 ml H_2O and 0.1 ml 2.5 N NaOH was added giving a base concentration of 0.41 N. The sample stood at 30° C for 24 hours and then was acidified with acetic acid and evaporated

to dryness. The resulting residue was hydrolyzed and subjected to amino acid analysis as previously described.

In the second procedure, 0.025 μmole of lyophilized peptide was treated with 0.1 ml of 0.4 M $NaBH_4$-0.1 N NaOH (121) for 48 hours at 25° C in a 6 x 50 mm culture tube. The sample was then acidified with HCl to destroy $NaBH_4$ and evaporated to dryness. Borate was removed as methyl-borate by 4X evaporation to dryness of the sample dissolved in 0.2 ml of 1/1000 (v/v) concentrated HCl in absolute methanol. The residue was then hydrolyzed and subjected to amino acid analysis as described.

III. Carboxypeptidase Digestion

Carboxypeptidase-A digestion was used to determine whether carbohydrate was bound to glycopeptide C-terminal threonine.

Approximately 0.3 μmole peptide was dissolved in 0.5 ml 0.1 M NaCl, and 0.01 ml of 0.5 M aqueous tris(hydroxymethyl)aminomethane was added to maintain the pH at about 9. Hydrolysis was begun with the addition of 0.005 ml of Worthington carboxypeptidase-A, and the sample was incubated for four hours at 37° C in a constant temperature water bath. Carboxypeptidase was precipitated from the hydrolysate by the addition of 0.5 ml 10% trichloroacetic acid followed by centrifugation. The supernatant was drawn

off with a pipet, and the precipitate was washed with 0.5 ml 10% trichloroacetic acid and recentrifuged. Both supernatants were pooled and extracted three times with 2 ml aliquots of ethyl ether to remove trichloroacetic acid. The aqueous solution was evaporated to dryness in the "Bio-Dryer" and subjected to P-2 gel chromatography as described earlier to separate the fragment containing carbohydrate from salt and amino acids. The fractions were examined with the orcinol (99) and fluorescent ninhydrin (101) assays and conductivity measurements.

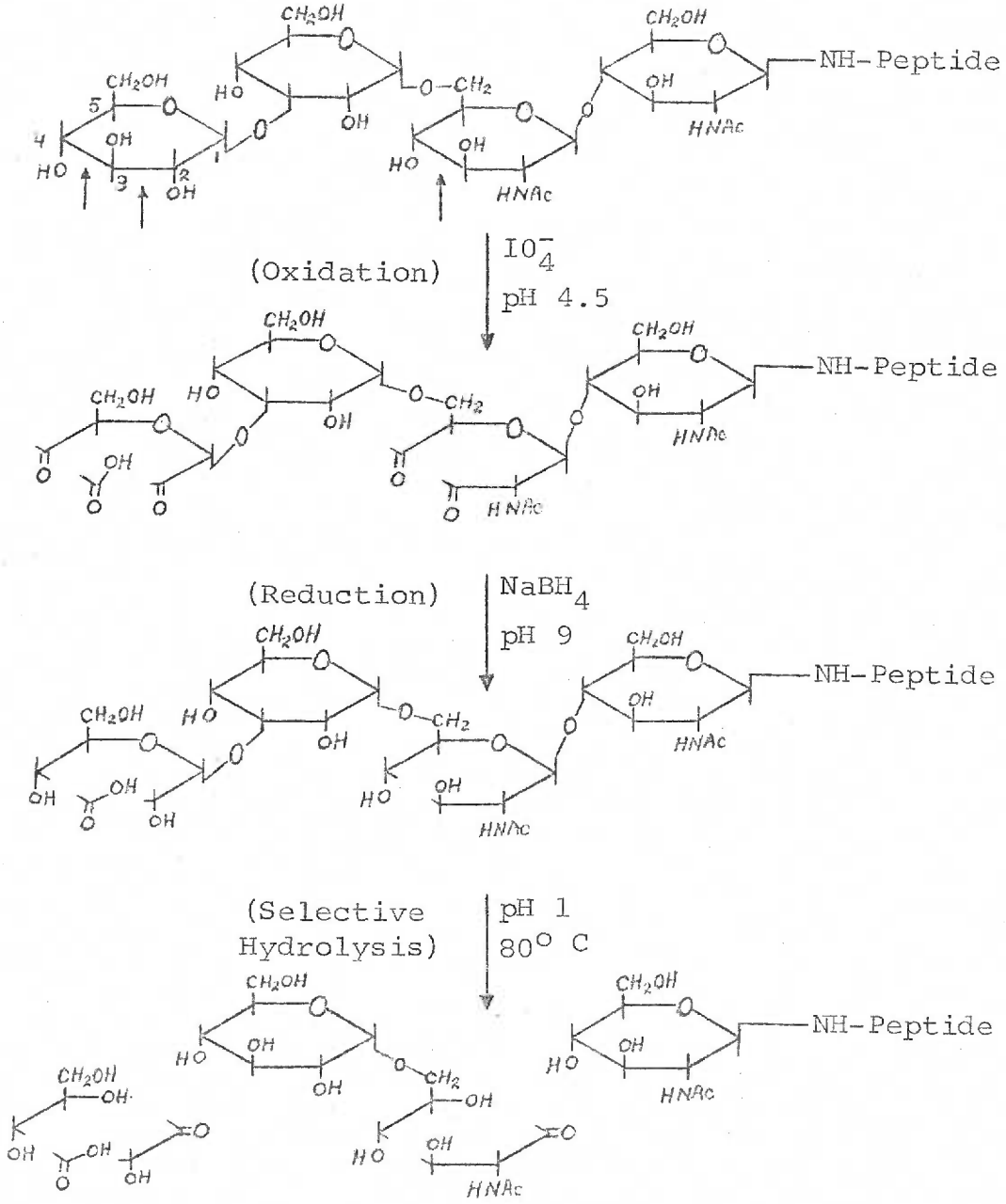
Carbohydrate positive fractions were pooled and analyzed for amino acid composition as previously described.

IV. Periodate Oxidation

The identity of the carbohydrate residue directly bound to the peptide chain and the general sequence of carbohydrate residues in the glycopeptide were examined by sequential periodate oxidation or "Smith degradation" (122).

The procedure employed periodate oxidation under recommended conditions (123,124), sodium borohydride reduction of the oxidized products to form acid labile "polyalcohols" (125), and mild acid hydrolysis of these acyclic acetals (126).

One cycle of a sequential periodate oxidation for a hypothetical glycopeptide containing a tetrasaccharide composed of two mannosyl and two N-acetylglucosaminyl residues would be expected to proceed as follows:



In the first step, periodate oxidizes all the 1,2 diol groups at the points indicated by arrows and cleaves the carbon-carbon bonds of these groups. Theoretically one mole of periodate is consumed per 1,2 diol group. Thus, the terminal mannosyl and the nonterminal N-acetyl glucosaminyl residues are destroyed by the consumption of 3 moles of periodate per mole of glycopeptide. The oxidized glycopeptide is illustrated as containing free carbonyl groups, although such reactive groups have been indicated to reversibly condense to form other structures (122,124). The oxidized compound is reduced with $NaBH_4$ to convert reactive carbonyl groups to less active alcohol groups. This step is critical for two reasons: 1) no meaningful quantitation of the remaining unoxidized monosaccharides is otherwise possible since acid hydrolysis would result in substantial monosaccharide decomposition (125); and 2) reduction makes it possible to hydrolyze the fragments of the oxidized monosaccharide residues from the unaltered part of the saccharide under conditions which give little hydrolysis of glycosidic bonds between unaltered monosaccharides (122). In the third step the oxidized-reduced glycopeptide is hydrolyzed under mild acid conditions. The products are monosaccharide fragments, one free mannose which was separated from the peptide core

by a periodate-susceptible monosaccharide, and a core peptide containing N-acetylglucosamine. The latter is now susceptible to periodate oxidation and may be carried through another cycle.

Several features of the sequence are worth noting. Under well controlled conditions where nonspecific oxidation, or overoxidation, is minimized, the moles of periodate consumed represents the moles of 1,2 diol groups oxidized. The positions of the glycosidic bonds determines the types of fragments resulting from oxidation. Thus qualitative and quantitative analysis of the unaltered monosaccharide residues and of the fragments after each oxidation-reduction cycle provides information which significantly limits the number of plausible sequences and glycosidically bound positions in the parent glycopeptide.

Referring to the example, one can see that pyranosides such as mannose will consume a maximum of 2 moles of periodate, whereas N-acetylglucosamine will consume a maximum of one. Furthermore, resistance to periodate oxidation is only conferred by blockage of sites C-2, C-3, or C-4 on a mannosyl residue such that no 1,2 diol groups remain. Thus, blockage of C-3 only, or of any two of these three sites will make the residue resistant. In N-acetyl-glucosamine, blockage of either of the two sites, C-3 or

C-4, will make the residue resistant.

As a precautionary note, the core structure resulting from a given degradation cycle should be separated from the other products of the reaction before composition analysis. Although the sequence of monosaccharide destruction in glycopeptides generally proceeds from the nonreducing termini to the peptide bound terminus without producing free monosaccharides, as illustrated above, such a behavior should not be assumed a priori. Thus, without isolation of the core structure, one might conclude from the composition data on the above product mixture that the two oxidized residues were located near the nonreducing terminus while the two unoxidized residues were both still bound to the peptide.

Various conditions were used for the periodate oxidation of the glycopeptides, and these will be presented in the "Results" section. In general, the glycopeptide was dissolved in pH 4.7, 0.025 M sodium acetate buffer and transferred to a quartz cuvette. Sodium metaperiodate in the same acetate buffer was added to give a 3-4 fold excess of periodate. In a second cuvette, the same volume of sodium metaperiodate solution was added to an aliquot of acetate buffer, containing no glycopeptide, to give the same final concentration as used in the sample oxidation. A

third cuvette was filled with acetate buffer only. The cuvettes were capped with "Parafilm" and placed in the sample compartment of a Zeiss PMQ-II spectrophotometer maintained at 25° C with an Ultrathermostat type K-2 circulating constant temperature water bath (Messgerate Werk Lauda, W. Germany). The progress of the reaction was then followed spectrophotometrically (127), except at 275 nm, with a slit width of 0.70 mm. The maximum absorption wavelength for metaperiodate is 222.5 nm, where at 25° C and pH 4-6 the molar extinction coefficient is approximately 1.04×10^4 l/mole-cm (128). Monitoring at 275 nm facilitated direct measurement of much higher concentrations of periodate.

Adherence to Beer's law at 275 nm was observed up to an absorbance of 1.8 with concentrations of sodium metaperiodate in pH 4.7, 0.025 M acetate buffer ranging from 0.5-5 mM. The extinction coefficient at 275 nm, 25° C, and at a slit width of 0.7 mm was 3.56×10^2 l/mole-cm. The absorbance of 5 mM $NaIO_3$ in acetate buffer under the same conditions was 0.056.

Absorbances of the three cuvettes were manually recorded periodically, and periodate consumption was calculated with the equation:

$$\frac{\text{moles } IO_4^- \text{ consumed}}{\text{mole substrate}} =$$

$$\frac{(\text{Abs } IO_4^- - \text{blank}) - (\text{Abs Sample} - \text{blank})}{(\text{Abs } IO_4^- - \text{blank})} \times$$

$$\frac{[IO_4^-]}{[\text{Substrate}]} \times \frac{(\text{Abs } IO_4^-)}{(\text{Abs } IO_4^- - \text{Abs } IO_3^-)}$$

where $[IO_4^-]$ and [substrate] are the molar concentrations used in the experiment. The last term is a correction for the absorbance of IO_3^- which is produced by the reduction of IO_4^-. The sample blank was measured before IO_4^- addition and divided by the appropriate dilution factor, or was measured after IO_4^- destruction and was corrected for the contribution of IO_3^-. Between readings the cuvettes were positioned out of the light path.

The oxidation was allowed to proceed until rapid oxidation had ceased and a slow but constant oxidation had proceeded for at least several hours. Periodate consumption was estimated by extrapolating the slow phase of the oxidation curve to zero time. Overall oxidation time ranged from 48 to 96 hours. Periodate was then destroyed by addition of an excess of ethylene glycol followed by spectrophotometric monitoring of the periodate disappearance.

The pH of the sample solution was adjusted to about pH 9 with sodium hydroxide, and $NaBH_4$ was added to a final concentration of 0.4 M. After standing for 5 hours at

room temperature, the sample was mixed with a small aliquot of glacial acetic acid to destroy excess borohydride.

Samples were subsequently hydrolyzed at pH 1 and subjected to Bio-Gel P-2 chromatography, as described earlier, to resolve the remainder of the glycopeptide from oxidation fragments and reagents. The oxidized-reduced glycopeptide was dissolved in water and aliquots were analyzed for amino acid, hexosamine, and neutral carbohydrate composition as previously described.

To provide information on the susceptibility of its carbohydrate moieties to periodate oxidation, PPHA (70-A-P_8) was oxidized once with IO_4^-. The procedure differed from that used above for glycopeptides. An amount of 30 mg of lyophilized salt-free 70-A-P_8 was dissolved in 1.0 ml pH 4.6, 0.05 M sodium acetate buffer. A volume of 2.0 ml 15 mM $NaIO_4$ in acetate buffer was added, followed by incubation at 4° C in the dark. A reagent blank containing no protein was similarly prepared. Due to the absorption of PPHA in the spectral region used previously for IO_4^- determination, the colorimetric periodate assay of Avigad (129) was used to monitor the reaction. This assay is based upon the rapid oxidation by periodate of the violet ferrous-2,4,6-tri-2-pyridyl-S-triazine complex to a colorless compound. Aliquots (0.02 ml) of the oxida-

tion sample were drawn and mixed with 10 ml of the Avigad reagent (diluted 4.5 parts + 0.5 parts H_2O), and the absorbance was determined at 593 nm, with a slit width of 0.03 mm. The periodate consumption was calculated from the disappearance of color in the assay due to periodate using a standard curve prepared by analyzing a series of standards containing 0 - 160 nmoles of sodium metaperiodate in a final volume of 5 ml.

After 5.5 hours IO_4^- consumption had ceased and excess periodate was destroyed with 0.03 ml 50% v/v aqueous ethylene glycol. The pH was adjusted to 9 with sodium hydroxide. Sodium borohydride (12 mg) was added to give a concentration of 0.2 M followed by incubation for 2 hours at room temperature. The sample was then transferred to 8/32" "Visking" cellulose tubing and dialyzed at 4^{o} C against two 1 liter volumes of 0.1 N NaCl overnight, followed by three 1 liter volumes of distilled water for 48 hours, which precipitated the protein. The retentate was lyophilized yielding 23.2 mg of an expected 28 mg of oxidized-reduced protein. This material was very insoluble. Therefore, a fine suspension was prepared in 2 M acetic acid, and aliquots were drawn and analyzed for neutral carbohydrates and glucosamine as described.

RESULTS

Carbohydrate Composition of PPHA

I. Neutral Carbohydrate Composition of PPHA

The average relative retention times (RRT) and relative molar response factors (RRF) computed from gas-liquid chromatographic analyses of alditol acetate standard mixtures are listed in Table I. The RRF values of Lehnhardt and Winzler (103) are included but have been recalculated based on an RRF = 1.00 for the alditol of 2-deoxyglucose. The experimental RRF values agreed well with those of Lehnhardt and Winzler. It was therefore concluded that the previously reported values were applicable to the data obtained from the F & M gas chromatograph, and they were subsequently used for all analyses.

A typical gas-liquid chromatogram tracing is illustrated in Figure 4a for a 40 hour hydrolysate of 67-A-P_8 (1.0 mg) to which 2-deoxyglucose (0.075 μmole) had been added. Omission of 2-deoxyglucose resulted in the absence of any 2-deoxyglucose peak, whereas analysis of only 2-deoxyglucose gave a single peak corresponding to 2-deoxyglucose. Peaks appear in positions corresponding to the alditols of fucose, arabinose, xylose, mannose, galactose, and glucose, with an unknown just prior to xylose. The

TABLE I. Gas-liquid chromatographic relative retention times (RRT) and relative molar response factors (RRF) for alditol acetate standards

Alditol	RRT[a]	(n)[b]	RRF[a]	(n)[b]	Literature RRF[c]
Fuc	0.524±.005	(13)	1.00±.05	(19)	0.989
Ara	0.720±.004	(13)	0.906±.032	(19)	0.908
Xyl	0.880±.002	(6)	0.847±.032	(19)	0.876
Man	1.31±.01	(13)	1.06±.03	(13)	1.07
Gal	1.42±.01	(13)	1.10±.04	(18)	1.10
Glc	1.46±.01	(5)	1.10±.06	(18)	1.09

a. Means ± probable error

b. Number of determinations

c. Computed from values relative to arabitol given by Lehnhardt and Winzler (103)

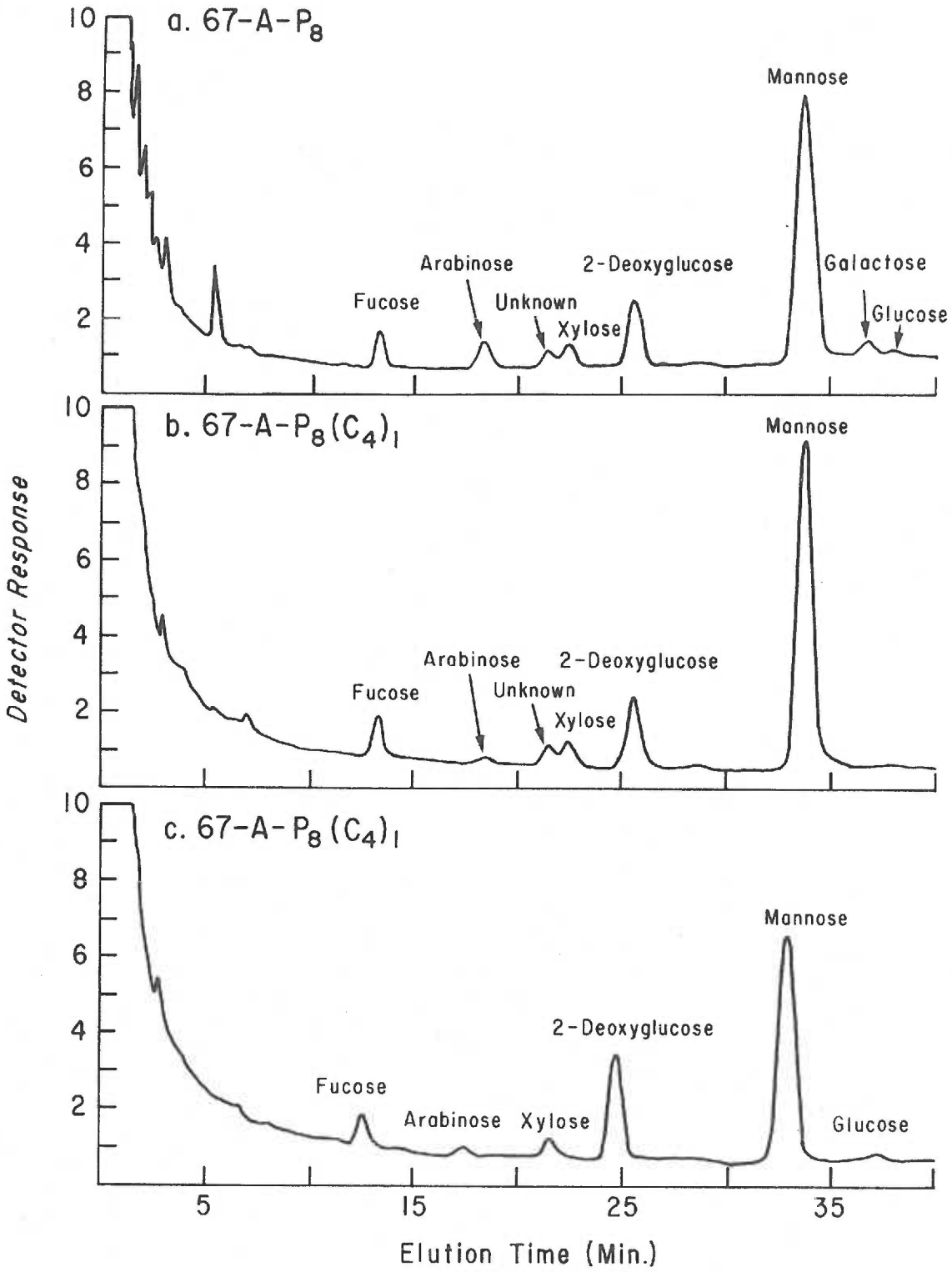


Figure 4. Tracings of gas-liquid chromatograms of PPHA hydrolysates with added 2-deoxyglucose (0.075 μmole): a. 1.0 mg 67-A-P_8, 40 hour hydrolysate; b. 1.0 mg 67-A-$P_8(C_4)_1$, 25 hour hydrolysate; and c. 0.5 mg 67-A-$P_8(C_4)_1$, 40 hour hydrolysate.

large peak at one to two minutes was the sample solvent peak. Small peaks of unknown origin were occasionally observed being eluted at less than 10 minutes. One at 6-8 minutes was observed to increase in size as the intensity of the brown color of the pyridine-acetic anhydride sample solution increased. None of these early peaks appeared to be correlated with the levels of monosaccharides observed. Figure 4b shows a tracing for a 25 hour hydrolysate of 67-A-$P_8(C_4)_1$ (1.0 mg) plus 0.075 μmoles deoxyglucose. Inspection of the two indicates that lesser amounts of arabinose and galactose are present following purification of PPHA by Sephadex G-200 chromatography. Consequently, 67-A-P_8 was used to determine an optimal routine hydrolysis time which would give maximal yields of the true monosaccharide constituents as well as the contaminants of the PPHA preparation.

Figure 5a shows the monosaccharide yield per mole of PPHA as a function of hydrolysis time. Single samples were prepared and analyzed for each time. Following 20 hours of hydrolysis, fucose, arabinose, xylose, and the unknown component reached maximal values, while galactose and mannose attained 90% or more of their observed maxima. Glucose was difficult to quantitate due to its low level in the samples and due to its incomplete resolution from

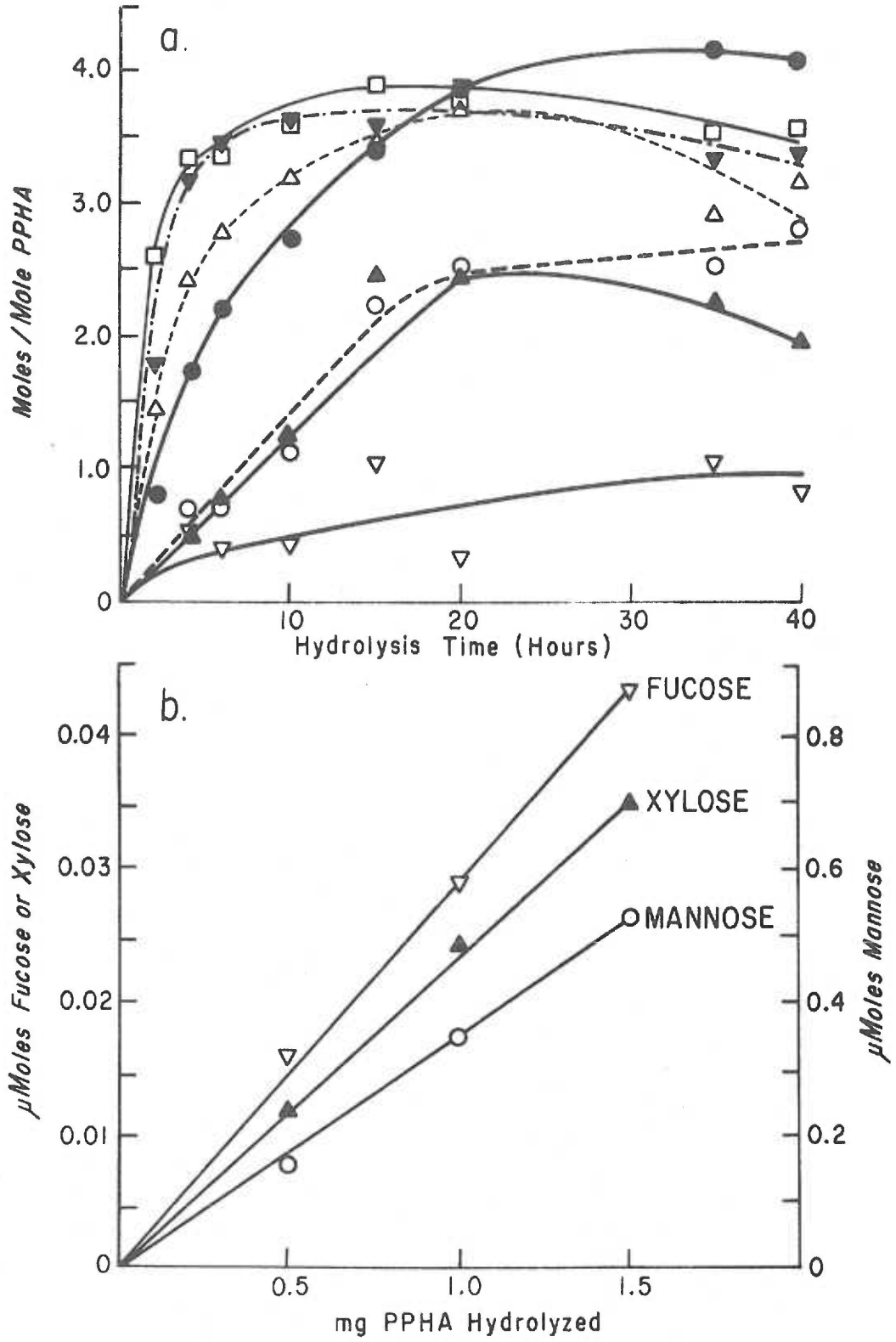


Figure 5. Dependence of neutral monosaccharide yield on: a. hydrolysis time for 67-A-P_8, ●—● Man, ▽ ··· ▽ Fuc, □—□Ara, △--△ Xyl, o--o Gal, ▲—▲ unknown, and ▽—▽ Glc, where Man values are plotted at 1/10 the observed values; and b. amount of 67-A-$P_8(C_4)_1$ hydrolyzed.

galactose. The timed hydrolysis experiment indicated that the monosaccharide yields should reach plateau values by about 25 hours. Therefore, twenty-five hour hydrolysis was chosen for routine analyses of PPHA.

The results from single analyses of 7 replicate samples of dried 67-A-$P_8(C_4)_1$ hydrolyzed for 25 hours are tabulated in Table II.

The average RRT's observed agreed well with their corresponding standard values listed in Table I except for the unknown component. Its RRT did not correspond to any of approximately twelve alditol derivatives studied by this method (103). The mean values of μmoles monosaccharide/mg dry protein are listed with the probable errors, i.e.,±0.6746 standard deviation units.

The nature of the unknown component was not determined. It is probably a neutral molecule at pH 2 since charged molecules are removed from the hydrolysate by the ion-exchange resins. It was not observed unless protein was present in the hydrolysis medium. However, its yield was correlated with hydrolysis time as shown in Fig. 5a. Therefore, it was decided to test the assumed linear dependence of the monosaccharide and the unknown yields on the amount of protein hydrolyzed. Single samples of lyophilized 67-A-$P_8(C_4)_1$ containing 0.5 mg, 1.0 mg and

TABLE II. Gas-liquid chromatographic analyses of the neutral carbohydrates of dried 67-A-$P_8(C_4)_1$

Residue	RRT	μmoles / mg	g Residue / 100 g	moles / mole PPHA
Fuc	0.528	0.0319±.0009	0.466	4.10±.12
Ara	0.722	0.0059± 0008	0.078	0.76±.10
Unknown	0.844	0.0183±.0015[a]	---	2.35±.19
Xyl	0.880	0.0301±.0014	0.398	3.87±.18
Man	1.308	0.3444±.0106	5.586	44.22±1.36
Gal	1.419	Trace[b]		
Glc	1.463	Trace[b]		
TOTAL			6.53	52.95[c]

a. Calculated assuming an RRF = 1.00

b. Trace quantities corresponding to about 0.5 residues per mole PPHA or less were obscured by the mannitol peak and were not quantitated

c. Does not include unknown

1.5 mg were hydrolyzed for 40 hours and single aliquots were analyzed. A forty hour hydrolysis was applied to further check for destruction of fucose, xylose and mannose. Surprisingly, the unknown was not observed in any of the samples as illustrated by the tracing in Figure 4c for the 0.5 mg hydrolysate. Linear dependencies were observed, however, for fucose, xylose and mannose as illustrated in Figure 5b. These plots gave values of 3.71 fucose, 2.99 xylose, and 44.7 mannose residues per mole PPHA. The values for fucose and xylose were both lower by 10% and 23% respectively, than those previously determined for this PPHA preparation hydrolyzed for 25 hours, whereas the mannose value was the same. These decreases in fucose and xylose could not be explained by the moisture content of the lyophilized protein which was about 1-2%. Thus the observed decreases appeared consistent with the destruction of these residues indicated in Figure 5a. Furthermore, if the unknown component had been derived from either of these residues, an increase, not a decrease, in their levels would have been expected in this experiment since the unknown appeared to represent about two residues of monosaccharide, or 50% of the original level of either fucose or xylose. In neither Figure 5a nor 5b does the level of destruction of fucose or xylose appear to be large enough

to mask such an increase. However, correlation of the levels of the unknown with those of mannose was not possible, since the unknown was estimated to occur at only 5% of the concentration of mannose. The 50% confidence interval for the mannose level was 3% of the mean value, so that a 5% increase in the mannose level would not be statistically significant. It was concluded that fucose and xylose were partially destroyed by 40 hour hydrolysis and that the unknown could not have been derived from them. On the other hand, the level of mannose appeared to be independent of the time of hydrolysis, but the possibility that the unknown was derived from mannose could not be excluded. However, the unknown was observed in both pistol dried and lyophilized samples of 67-A-$P_8(C_4)_1$, and in lyophilized 67-A-P_8, but not in any of the other pistol dried PPHA preparations, nor in reagent blanks, samples of only 2-deoxyglucose, or any of the glycopeptides prepared from 67-A-P_8 or 67-A-$P_8(C_4)_1$. Consequently, its occurrence could only be correlated with the presence of protein in the hydrolysis medium, but not with the drying operation, G-200 chromatography, or with any of the reagents used.

Tables III and IV summarize the analyses of various pistol-dried PPHA preparations hydrolyzed for 25 hours. Three sample aliquots were drawn from each preparation stock solution with the same micropipet, eliminating analysis differences between preparations due to micropipet variation. Three aliquots of each sample were chromatographed. The data for 67-A-$P_8(C_4)_1$ are the same as listed in Table II.

The most variable monosaccharides were arabinose, galactose, and glucose. Prior to G-200 chromatography all preparations contained significant but variable quantities of arabinose and galactose. Following G-200 chromatography the preparations contained less than one residue of arabinose and traces of galactose, while the glucose value actually increased in the case of 70-A-P_8.

Preliminary analyses of the C_1, C_2 and C_3 components (Figure 1) obtained from G-200 chromatography of 67-A-P_8 showed that at least 35% by weight of C_1 was arabinose and galactose in a ratio of about 1:1. Thus, less than a 10% contamination of PPHA with C_1 would account for the observed levels of galactose and arabinose.

In contrast to arabinose, galactose, and glucose, the amount of fucose, xylose and mannose varied little as a

TABLE III. Summary of carbohydrate compositions of dried PPHA preparations in grams of residue per 100 grams[a].

Res.	62-E-P_5	70-A-P_8	70-A-$P_8(C_4)_1$	72-A-$P_8(C_4)_1$	67-A-$P_8(C_4)_1$	Ave.
Fuc	0.406	0.425	0.405	0.411	0.466	0.423
Ara	0.894	0.264	0.064	0.069	0.078	0.070[b]
Xyl	0.380	0.384	0.389	0.380	0.398	0.386
Man	6.08	6.12	6.38	6.09	5.59	6.05
Gal	0.698	0.244	----	----	----	----
Glc	----	----	0.10	0.12	----	----
TOTAL	8.46	7.44	7.34	7.07	6.53	6.93[c]
GlcNAc	2.45	2.42	2.44	2.34	2.42	2.41

a. Values are means $\pm$ 0.6745 s

b. Averages of $(C_4)_1$ components only

c. Total of $(C_4)_1$ averages

TABLE IV. Summary of carbohydrate compositions of dried PPHA preparations in residues per mole[a]

Res.	62-E-P_5	70-A-P_8	70-A-$P_8(C_4)_1$	72-A-$P_8(C_4)_1$	67-A-$P_8(C_4)_1$	Ave.
Fuc	3.57±0.23	3.73±0.18	3.56±0.21	3.61±0.28	4.10±0.12	3.71
Ara	8.69±0.57	2.57±0.16	0.62±0.06	0.67±0.07	0.76±0.10	0.68[b]
Xyl	3.69±0.14	3.74±0.31	3.78±0.19	3.70±0.26	3.87±0.18	3.76
Man	48.1±1.4	48.5±2.2	50.5±2.13	48.2±1.3	44.2±1.4	47.9
Gal	5.53±0.58	1.93±0.22	---	---	---	----[b]
Glc	---	---	0.83±0.05	0.99±0.12	---	----
TOTAL	69.6	60.4	59.3	57.2	53.0	56.0[c]
GlcNAc	15.5	15.3	15.4	14.8	15.3	15.3

a. Values are means ± 0.6745 s

b. Averages for $(C_4)_1$ components only

c. Total of $(C_4)_1$ averages

consequence of G-200 chromatography, or with the bean lot. Fucose and xylose vary by less than 0.5 residue per mole. Mannose does not vary significantly in three of the four preparations from different bean lots, but there does appear to be about 10% less mannose in 67-A-$P_8(C_4)$. This variation is of questionable significance, however, because of the appearence in the analyses of 67-A-$P_8(C_4)_2$ of the unknown component, the origin of which has not been established.

II. Glucosamine Composition

Replicate samples of lyophilized 67-A-$P_8(C_4)_1$ were hydrolyzed for different times and one aliquot of each was analyzed as described. Four, eight, and twelve hour hydrolysates gave values of 13.3, 13.5 and 13.1 glucosamine residues per 128,400 g protein, respectively, demonstrating that the glucosamine yield was virtually constant following hydrolysis from 4 to 12 hours. However, eight hour hydrolysis produced less complex chromatograms facilitating determination of peak areas and was subsequently employed in analyses of PPHA.

In order to check for glucosamine destruction and to investigate the validity of the procedure, aliquots from the protein stock solution used above were hydrolyzed for

8 hours at the same protein concentration as above, but in the presence of known amounts of added glucosamine. One sample containing no added glucosamine was also hydrolyzed. A single aliquot of each sample was chromatographed. Figure 6 shows the plot of peak area versus added glucosamine. The straight line, fitted to the data by linear regression analysis, had a coefficient of correlation of 0.999998. Numerical extrapolation of this curve to the x-intercept gave 13.1 residues per 128,400 g PPHA. The agreement between this value and those above indicated that the hydrolysis method gave negligible destruction of glucosamine.

The results obtained from single analyses of duplicate hydrolsates of the same dried PPHA preparations as were analyzed for neutral carbohydrate are included with the neutral carbohydrate data in Tables III and IV. All samplings, except for 67-A-$P_8(C_4)_1$, were made from the same stock solutions with the same pipets and at the same time as those for the reported neutral carbohydrate analyses. The ranges of duplicate sample glucosamine values averaged 0.9% with an extreme of 1.8%.

The higher glucosamine contents for these PPHA preparations compared with the previously obtained value of 13.1

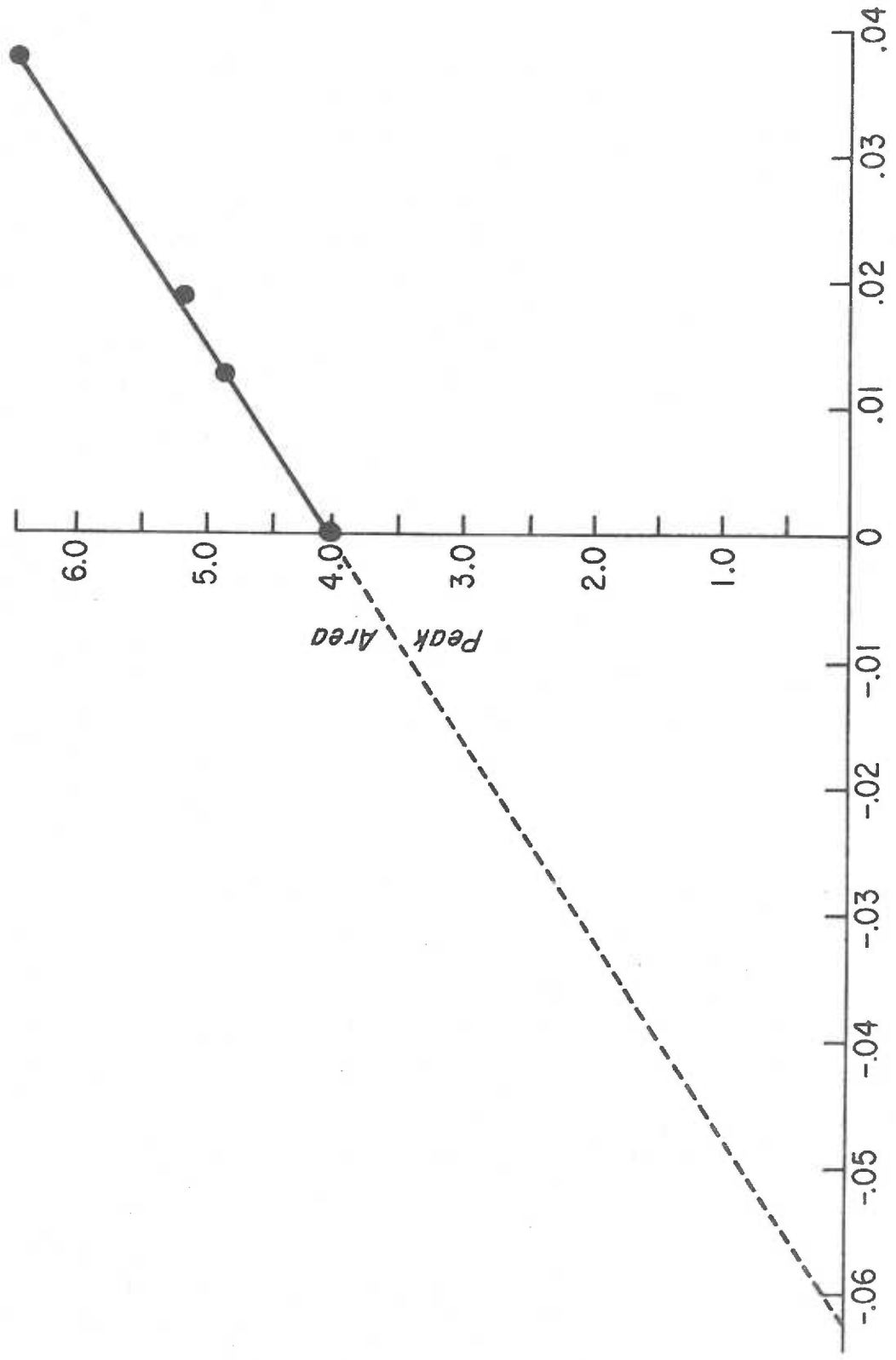


Figure 6. Assay of glucosamine content of 67-A-$P_8(C_4)_1$ by the internal standard method.

residues per mole for 67-A-$P_8(C_4)_1$ prompted a reexamination of the latter. A 5 mg sample of pistol-dried 67-A-$P_8(C_4)_1$ was hydrolyzed in 2 ml of 4 N HCl and subsequently diluted to 25 ml in a volumetric flask. Two aliquots were chromatographed giving an average of 15.3 residues per mole. The latter value was considered more valid since thoroughly dried protein was used, and volumetric errors were minimized.

The glucosamine content was almost identical for all the preparations, except 72-A-$P_8(C_4)_1$, which had about 3%, or 0.5 residue per mole, less glucosamine than the average.

Thus, on the average, PPHA subjected to G-200 chromatography contained 56 residues of neutral carbohydrate (4 fucose, 4 xylose, and 48 mannose) and 15 residues of glucosamine. The total 71 residues constitute 9.3% of the dry weight of PPHA, or a residue weight of about 12,000 out of a total molecular weight of 128,400.

III. Periodate Oxidation of PPHA

The consumption of periodate by 70-A-P_8 is shown in Figure 7. Approximately 40 moles periodate per mole PPHA were rapidly consumed, followed by a slower consumption which reached a plateau of approximately 54 moles per mole by 5.5 hours. Oxidation rendered the protein insoluble at pH 4.7,

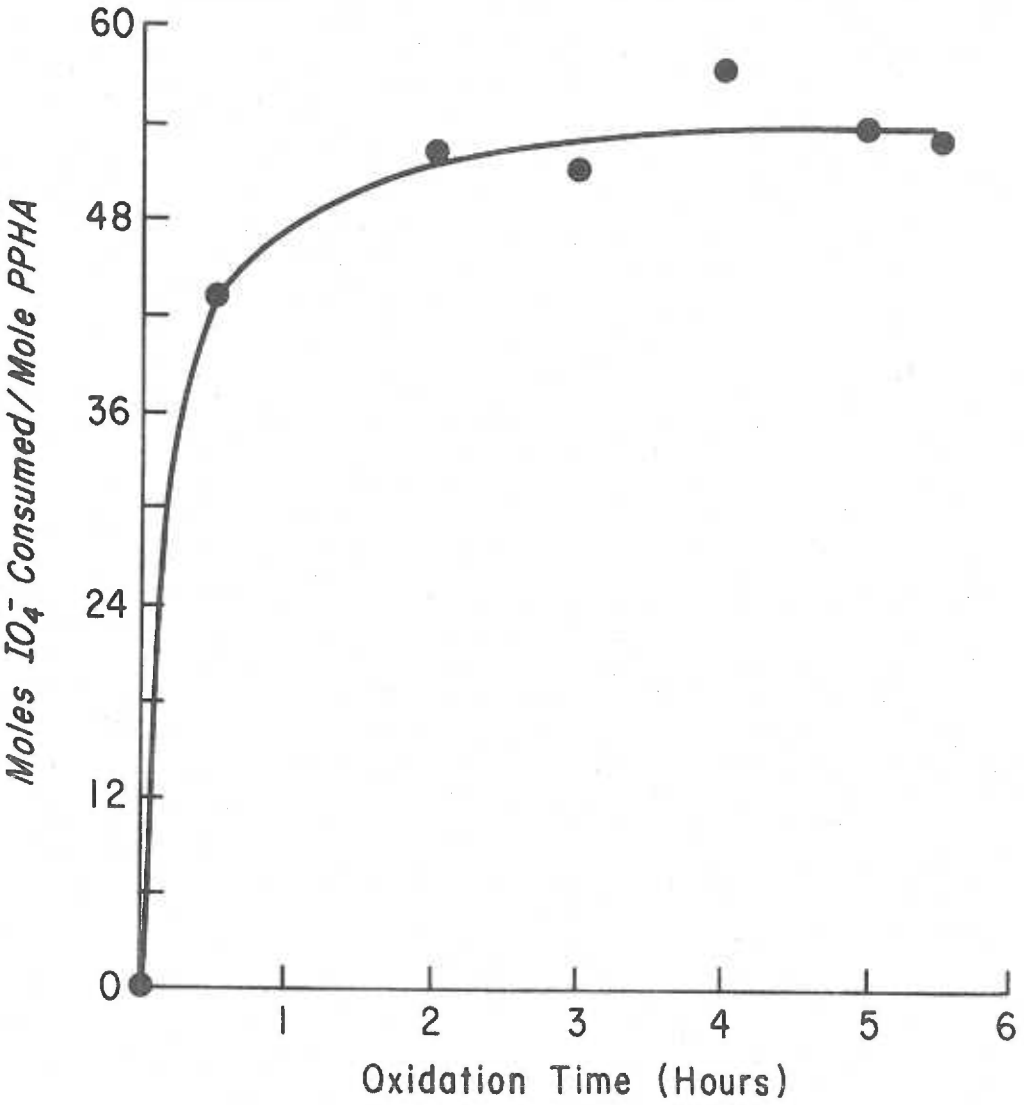


Figure 7. Periodate consumption of 70-A-P_8 at 4° C.

but it redissolved following adjustment of the pH to 9 with NaOH. Following reduction with $NaBH_4$ and dialysis to remove salts, the protein was lyophilized. The lyophilized, oxidized-reduced protein was much less soluble than the native. It would not dissolve in 2 M acetic acid (4 mg/ml), in 0.1 M NaCl at pH 7 (1 mg/ml), or in 4 N HCl (6 mg/ml). Thus lyophilization should be avoided when solutions are needed for biological assay, etc.

The results of the neutral carbohydrate and hexosamine analyses of the native and oxidized-reduced PPHA are listed in Table V. None of the glucosamine was oxidized, while 75% of the total fucose, xylose, and mannose or 42 residues per mole were oxidized. All of the fucose and 86% of the xylose were destroyed. Of the monosaccharides indicated to be contaminants of the preparation, arabinose and galactose, galactose was unaffected, while 30% of the arabinose was oxidized.

TABLE V. Comparison of the carbohydrate composition of native and periodate oxidized 70-A-P_8

	Residues/Mole					
	GlcN	Fuc	Ara	Xyl	Man	Gal
Native	15.3	3.73	2.57	3.74	48.5	1.93
Oxidized	15.3	0	1.79	0.52	13.5	2.15
Difference	0	3.73	0.78	3.22	35.0	0
% oxidized	0	100	30	86	72	0

Glycopeptide Studies

I. Development of Preparation and Isolation Procedures

The effectiveness of a variety of procedures for preparing glycopeptides was examined. The following results provide the basis for a set of procedures capable of producing highly purified glycopeptides in high yield which will be discussed later.

The first objective in glycopeptide preparation was the proteolysis of PPHA under conditions which would eliminate incomplete hydrolysis due to the native conformation of the protein. Bio-Gel P-6 chromatography was used to determine whether very large peptides persisted following proteolysis as a result of insufficient denaturation. Figure 8a shows the P-6 chromatogram of a pronase hydrolysate of PPHA obtained by procedure 1 which employed heat denaturation. Insoluble material, which was removed from the hydrolysate prior to chromatography, accounted for 1% of the orcinol positive material in the whole hydrolysate. Total recovery of orcinol positive material from the column was 83%. However, later recoveries ranged from 95-106% following allowance for negative deviations from Beer's law.

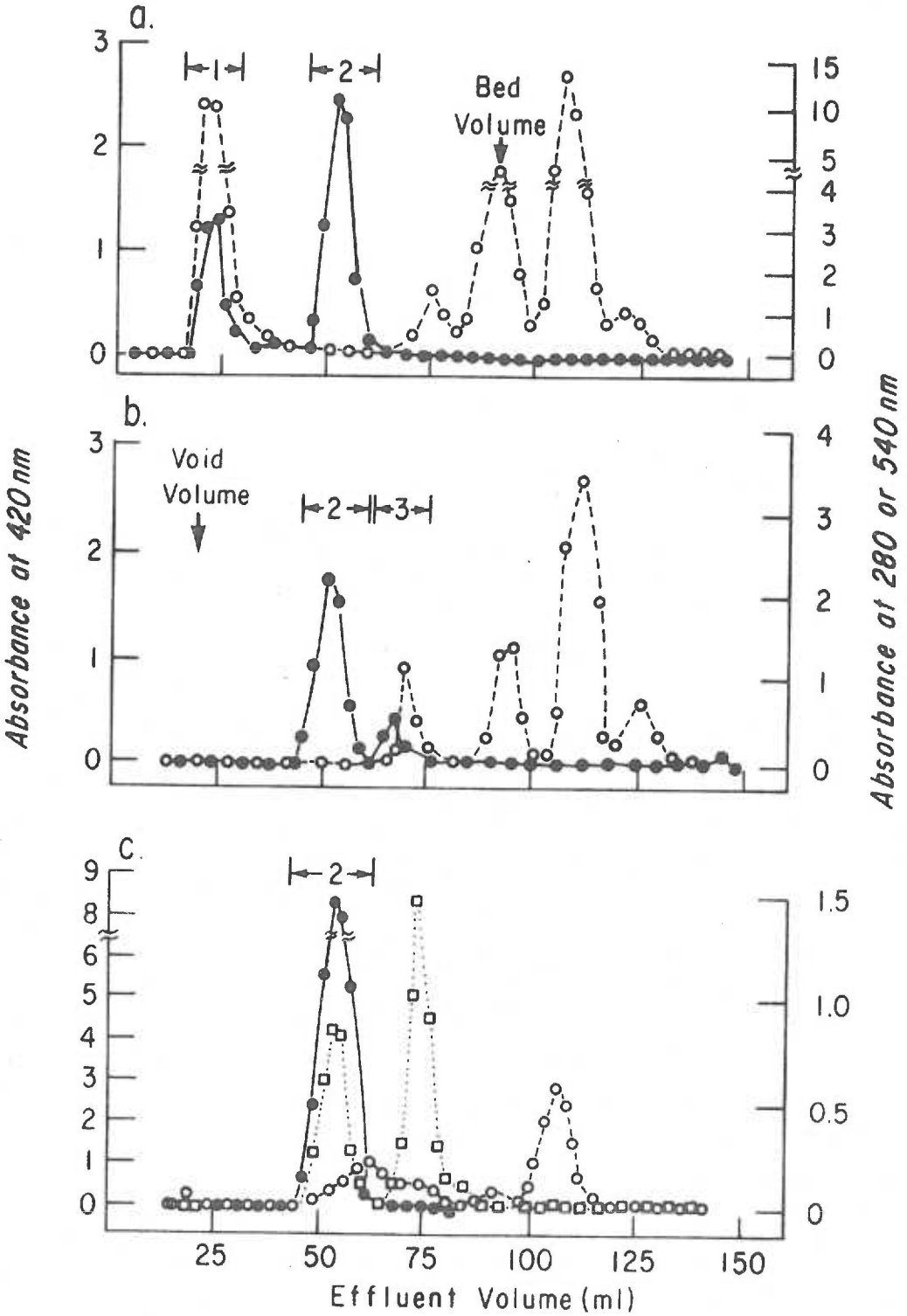


Figure 8. Bio-Gel P-6 chromatograms of pronase hydrolysates of PPHA, ●—● manual orcinol assay, o--o fraction absorbance at 280 nm, and □···□ manual ninhydrin assay: a. heat denatured 67-A-$P_8(C_4)_1$ hydrolyzed by procedure 1; b. 67-A-P_8 denatured and hydrolyzed in the presence of SDS by procedure 2; and c. re-chromatography of rehydrolyzed peak 2 from 67-A-$P_8(C_4)_1$ hydrolyzed by procedure 3.

Two orcinol positive peaks were observed. Peak 1, eluting near the column void volume, contained appreciable material absorbing at 280 nm and approximately one third of the recovered orcinol positive material. Peak 2, which was retarded by the column, contained little material absorbing at 280 nm and approximately two thirds of the recovered orcinol positive material. Pooling and lyophilization of the indicated peak 1 fractions yielded 65 mg of dry material, which represented 22% by weight of the protein hydrolyzed.

This material was rehydrolyzed with pronase and rechromatographed on P-6. Orcinol peaks were observed in positions corresponding to both peaks 1 and 2. Again, peak 1 contained material absorbing at 280 nm while peak 2 did not. Thus, peak 1 represented incompletely hydrolyzed material, since by repeated hydrolyses its orcinol positive, but not 280 nm absorbing, constituents were shifted to the peak 2 position.

Denaturation and pronase hydrolysis under similar conditions but in the presence of 0.4% SDS (procedure 2) eliminated the occurrence of peak 1. As illustrated in Figure 8b, no material was eluted in the position corresponding to peak 1. About 80% of the loaded orcinol positive

material was eluted in peak 2, while an additional 11% was eluted in a later peak designated as peak 3. Upon rechromatography, the peak 2 material was quantitatively recovered in one orcinol positive peak at the same elution volume as originally observed. In contrast, rechromatography of peak 3 failed to reproduce the original elution profile. Orcinol positive material was eluted diffusely beginning at the original elution volume of peak 3. Also, the 280 nm absorbing material that was included in the peak 3 fraction pool was eluted in a different position and independently from the orcinol positive components, as compared with its original behavior. Therefore, peak 3 appeared to be an artifact arising from the presence of SDS, since rechromatography produced a significantly different profile. Moreover, the appearance of peak 3 appeared correlated with the use of SDS, since no such peak was observed in any of the chromatograms of hydrolysates not containing SDS.

Procedure 3, employing heat and guanidine hydrochloride denaturation of PPHA followed by pronase hydrolysis in 2M urea, produced glycopeptides eluting only in the peak 2 position.

Peak 2 from Figure 8a was chromatographed on Bio-Gel P-2 to determine whether further resolution of glycopeptides, or of peptides from glycopeptides, was possible using gel permeation chromatography. Bio-Gel P-2 has greater resolving power than P-6 in the 100-1800 molecular weight range, and the elution volume of peak 2 suggested glycopeptide molecular weights of roughly 1000-2000. As illustrated in Figure 9, all the glycopeptide components eluted together producing superimposable orcinol and ninhydrin profiles. Thus, it appeared that the glycopeptides were distributed through a relatively narrow molecular weight range, since they were not resolved by chromatography on gels of these two porosities; and that P-6 chromatography, under the conditions described, provided the maximum resolution of peptides from glycopeptides achievable with gel permeation chromatography.

Since glycopeptides were not resolved by gel permeation chromatography, chromatographic systems employing ion-exchange resins were examined. The indicated fractions in Figure 9 were pooled, lyophilized, and subjected to AG 1-x2 chromatography with the volative buffer systems previously described (vide supra). The effluents were continuously monitored by the automated procedures previously described.

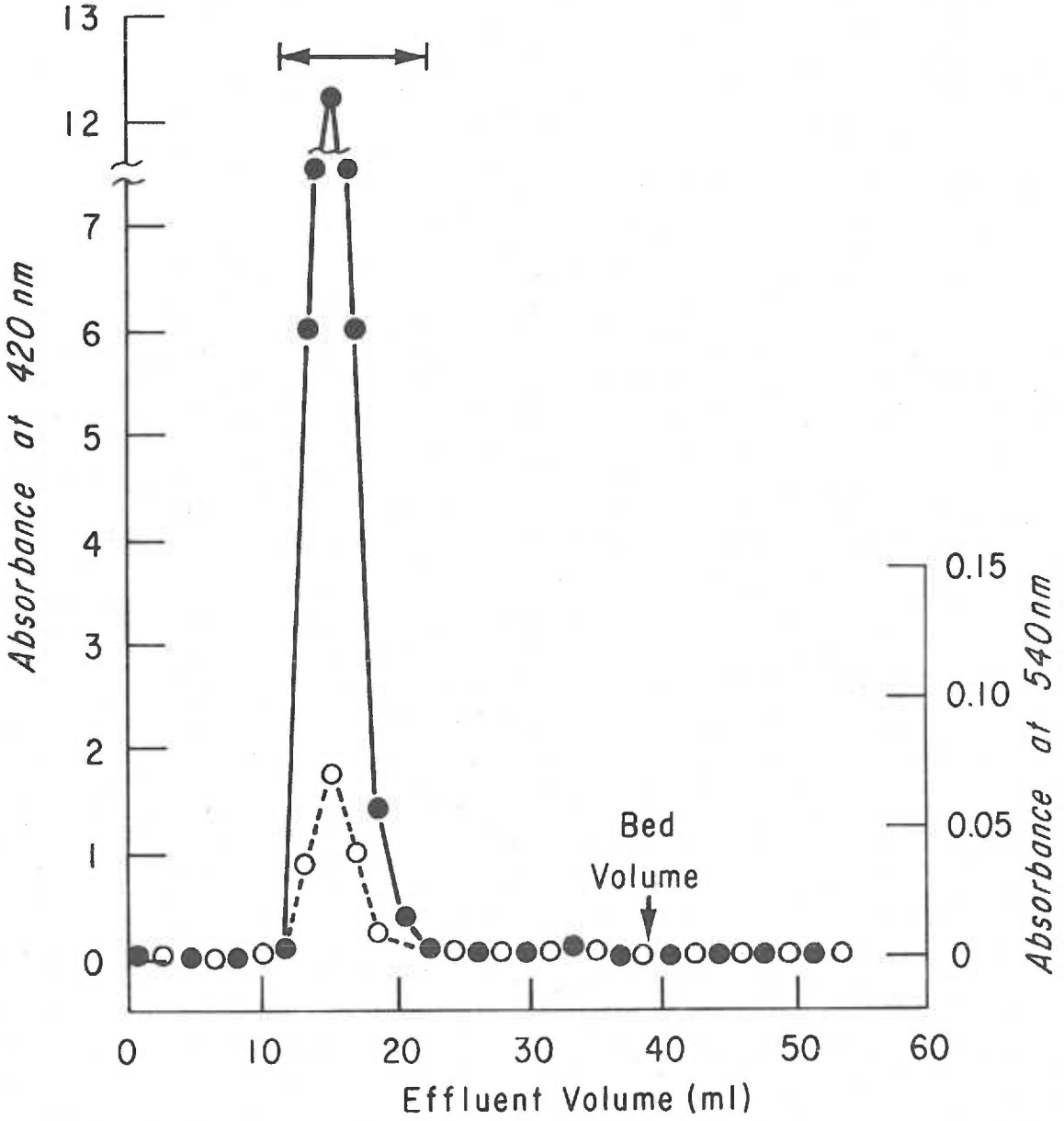


Figure 9. Bio-Gel p-2 chromatogram of the pooled peak 2 material from Figure 8a: ●—● manual orcinol assay, and o--o manual ninhydrin assay.

The results are illustrated in Figures 10 and 11, in which data from the automated orcinol and ninhydrin assay profiles have been plotted.

As illustrated in Figure 10a, the glycopeptide load was eluted as an assymmetric peak in the region of rapid pH change provided by gradient system 1. Recovery estimates were not obtained in this run. However, continuation of the run with an additional gradient produced by 75 ml 0.1 N acetic acid and 75 ml 1.0 N acetic acid failed to elute more components from the column. The indicated fractions were pooled and lyophilized.

The material was rechromatographed with gradient system 2 which provided a more gradual pH change and lower ionic strength. As shown in Figure 10b, the mixture of glycopeptides was partially resolved into at least four components. Recovery of the orcinol positive material was estimated at 105% with 40% in the first major peak and 65% in the subsequent peak complex. The first peak gave a much higher ninhydrin response, indicating a higher ratio of free amino groups to carbohydrate residues than in peak 2. While the resolution of peak 1 from peak 2 was satisfactory, the components in peak 2 were poorly resolved. Therefore, the indicated fractions were pooled, providing one pool

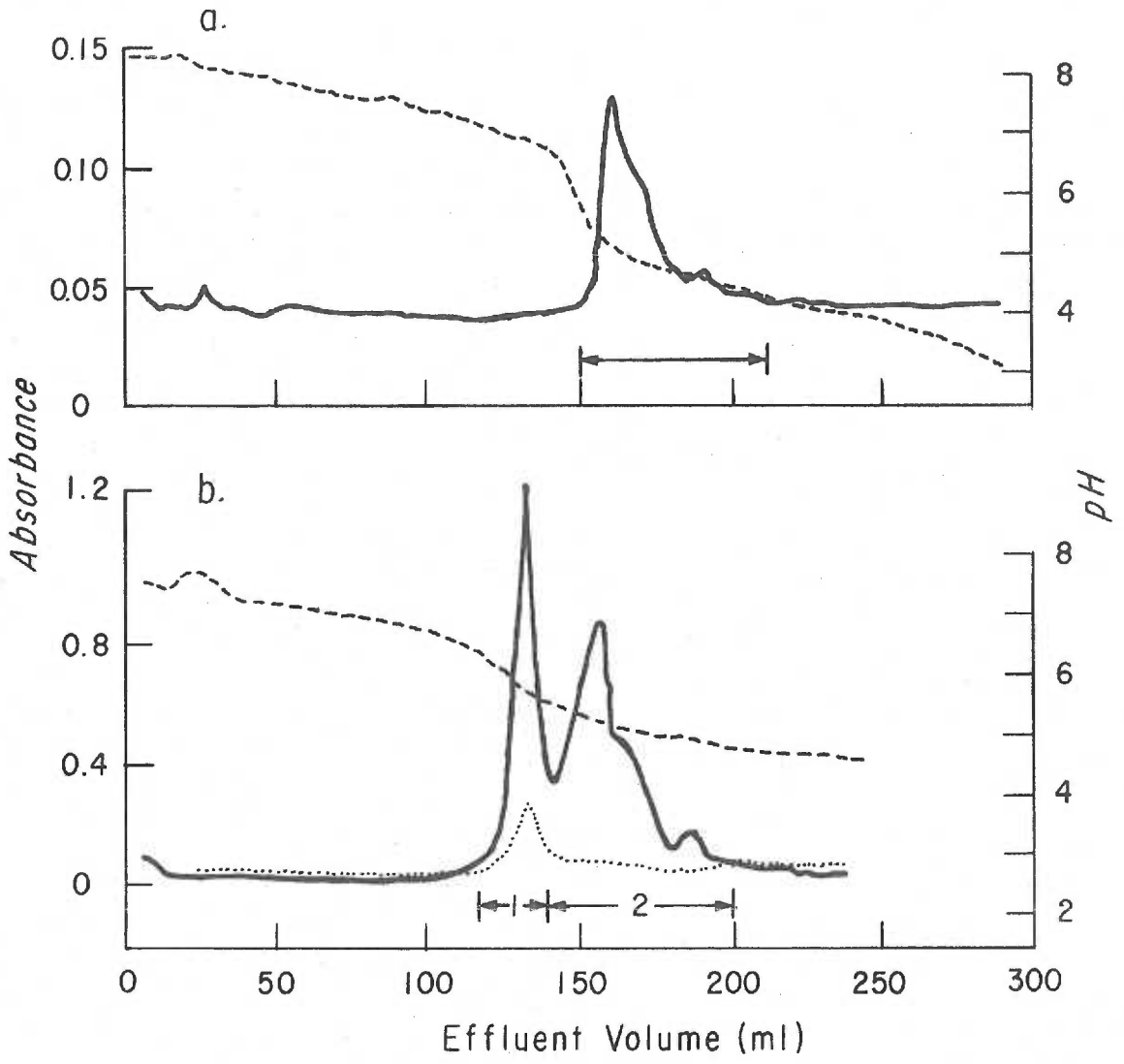


Figure 10. AG 1-x2 chromatograms of the glycopeptide mixture from Figure 9, — automated orcinol at 420 nm, ··· automated ninhydrin assay at 570 nm, and --- fraction pH: a. gradient system 1 at 40° C; and b. pooled fractions from Figure 10a chromatographed with gradient system 2 at 45° C.

containing predominantly peak 1, and a second containing peak 2 components.

The lyophilized peak 2 material was rechromatographed with gradient system 3, which provided a 1.5 pH unit gradient and a change from 0.1 M pyridine to 1.0 M pyridine. All of the material eluted in one peak at approximately 50 ml. The orcinol positive fractions were pooled and lyophilized.

Rechromatography of this material with gradient system 4 produced the profile in Figure 11a. The gradual pH change with a constant pyridine concentration further resolved at least five component peaks with a recovery of 98% of the load. This resolution was considered marginal, and the orcinol positive fractions were pooled and lyophilized.

The material was then chromatographed with a pH 5.7 pyridine-acetic acid buffer (0.1 M pyridine) producing the profile in Figure 11b. The peak at 100 ml corresponded to peak 1 in Figure 10b, since rechromatography of peak 1 with the pH 5.7 buffer gave a large peak at about 100 ml with a pronounced leading shoulder.

The pooled, lyophilized orcinol positive fractions from Figure 11b were rechromatographed with pH 6.0

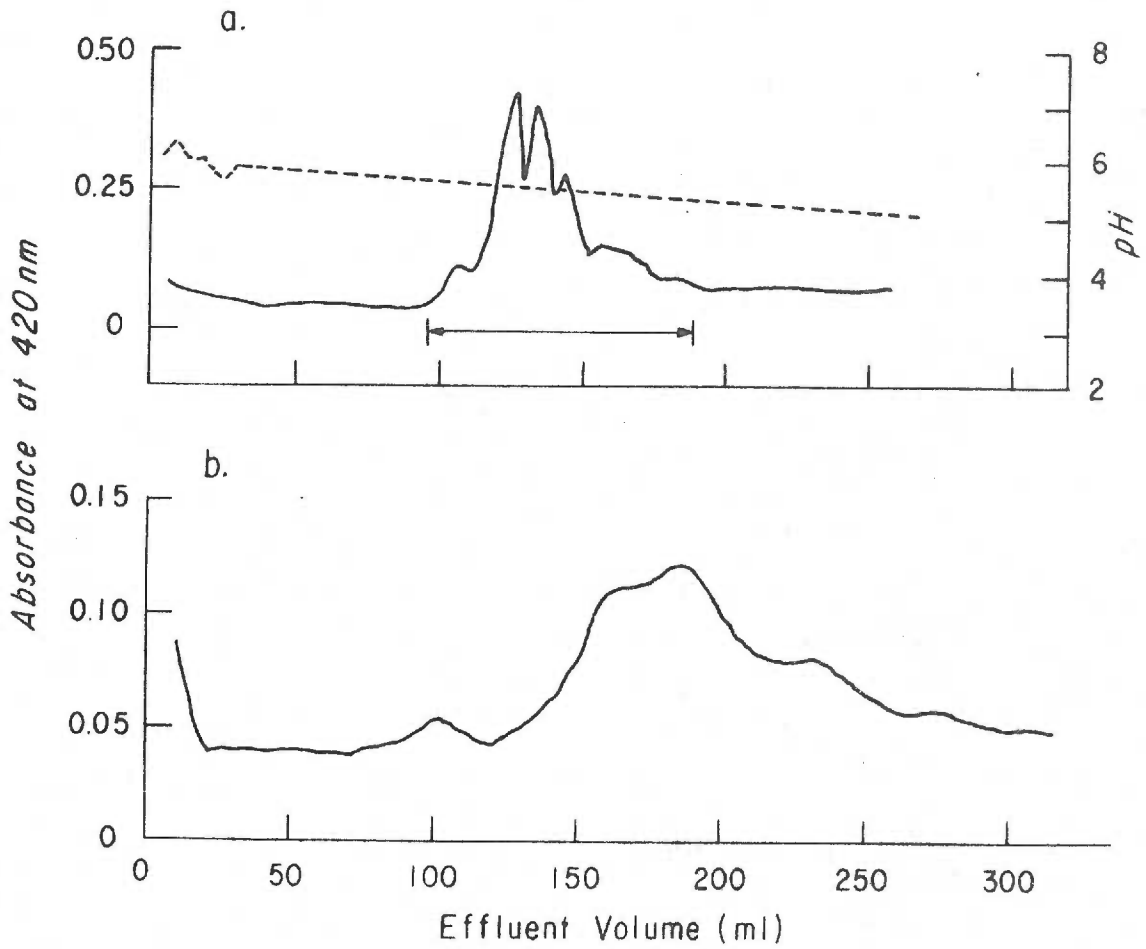


Figure 11. AG 1-x2 chromatograms of glycopeptide mixtures, — automated orcinol assay at 420 nm, and --- fraction pH: a. fraction pool 2 from Figure 10b with gradient system 4 at 45° C; and b. pooled fractions from Figure 11a with pH 5.7, 0.1 M pyridine-acetic acid at 45° C.

pyridine-acetic acid (0.1 M pyridine). The resulting chromatogram was similar to that obtained with the pH 5.7 system, except that the component peaks were broader and eluted at approximately twice the previous volumes.

Although the glycopeptide components were only partially resolved in the pH 5.7 system, this system was used for rechromatography of components which were partially purified by other techniques.

An additional series of experiments employed AG 50W-x2 (K^+) as described under "Methods" with continuous automated orcinol assay of the column effluents. The glycopeptide mixture used was prepared by P-6 chromatography of a pronase hydrolysate of 67-A-P_8 obtained by procedure 2. The P-6 profile appeared as illustrated in Figure 8b, and the peak 2 fractions were pooled and lyophilized.

The residue was dissolved in 5 ml distilled water and chromatographed with water on AG 50W-x2(K^+), giving the profile in Figure 12a. The first peak complex contained at least three components and accounted for 35% of the loaded orcinol positive material. A second well-resolved peak complex contained at least two components and accounted for 53% of the load. The two indicated fraction pools were lyophilized and rechromatographed.

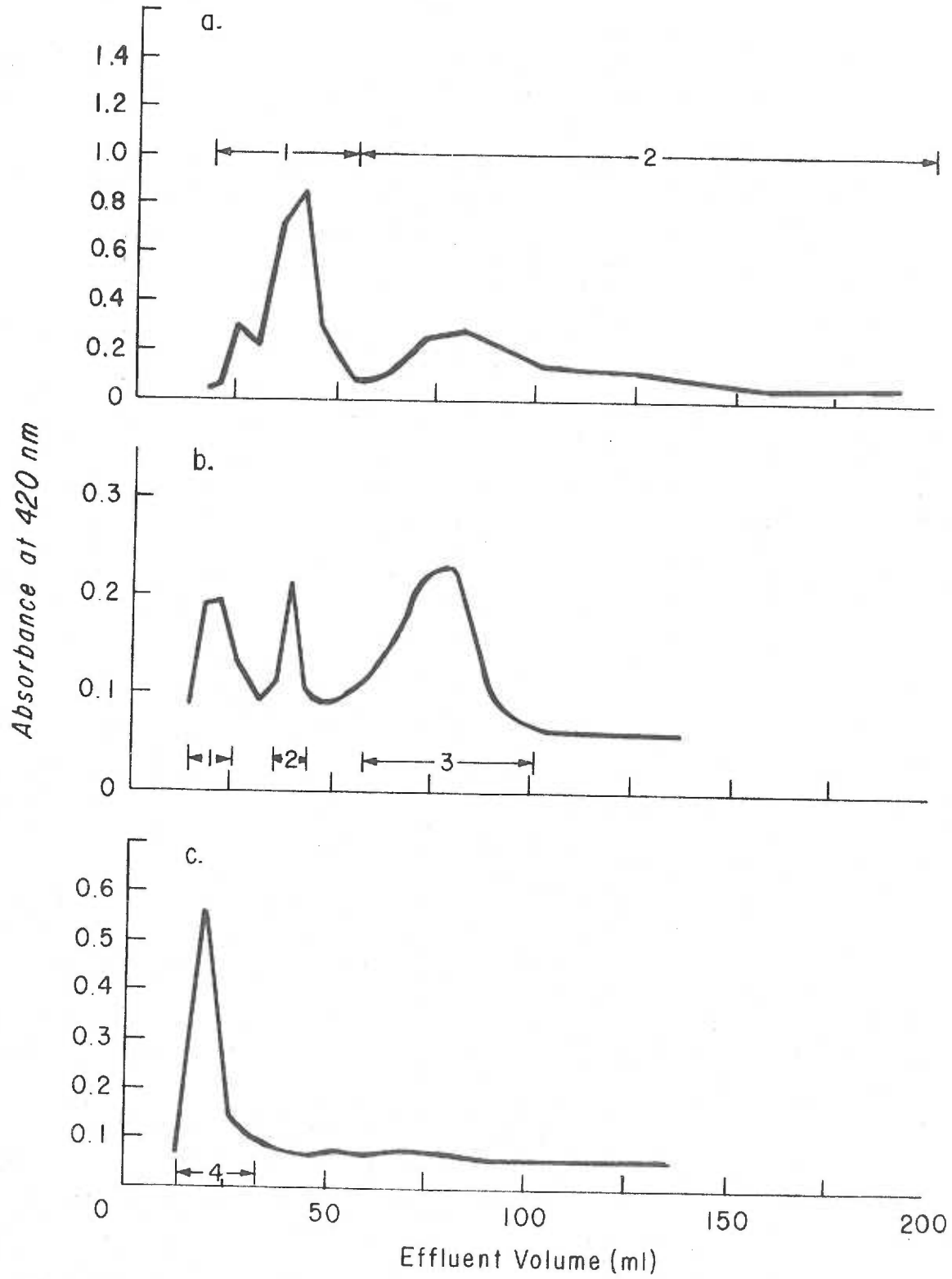


Figure 12. AG 50W-x2(K^+) chromatograms of glycopeptides prepared from an SDS hydrolysate of 67-A-P_8 by P-6 chromatography, – automated orcinol assay: a. H_2O elution (sample load in 5 ml); b. 50% ethanol elution of fraction pool 1 from Figure 12a (sample load in 10 ml); and c. 50% ethanol elution of fraction pool 2 from Figure 12a (sample load in 5 ml).

Instead of development of the column with water, 50% aqueous ethanol was used in an attempt to increase the affinity of the glycopeptides for the resin. Rechromatography of fraction pools 1 and 2 produced the profiles in Figures 12b and 12c, respectively.

In 12b, three components were resolved. Peak 1 accounted for 10%, peak 2 for 8%, and peak 3 for 67% of the load. The intermediate fractions were not assayed. In 12c, only one peak with a tail was observed. Recovery was estimated at only 21% and no other material was recovered from the column.

The fractions indicated in 12b and 12c were pooled, lyophilized and redissolved in distilled water. Aliquots were assayed for amino acid composition following 22 hour hydrolyses, and for neutral carbohydrate composition following 40 hours hydrolyses, giving the results in Table VI. No separate glucosamine determinations were made and the listed figures represent estimates from the amino acid analyzer long column chromatogram of the 22 hour hydrolysates, assuming 40% destruction.

The basis for separation of the components is not readily apparent from the composition data. However, it is

TABLE VI. Residue ratios of AG 50W-x2(K^+) fraction pools from Figure 12 b and c

Residue	Fraction Pool 1	2	3	4
Asx	(1.0)	(1.0)	(1.0)	(1.0)
Thr	0.97	0.29	0.95	0.20
Ser	0.28	0.29	0.08	0.14
Glx	0.27	0.13	0.02	0.96
Gly	0.33	0.03	0.12	0.32
Ala	0.20	0.10	0.02	0.15
Val	0.17	----	----	----
Ileu	0.20	----	----	0.23
Leu	0.15	----	----	0.36
GlcN*	1.3	0.50	0.65	Lost
Fuc	0.35	0.19	0.40	----
Xyl	0.29	0.16	0.29	----
Man	1.2	1.1	1.3	6.1

* Estimated from 22 hour hydrolysates assuming 40% destruction.

clear that two classes of glycopeptides differing in their fucose and xylose content have been resolved.

II. Preparation and Composition

Glycopeptides were prepared from 67-A-$P_8(C_4)_1$ for structural studies by hydrolysis procedure 3. The hydrolysate was subjected to Bio-Gel P-6 chromatography. Following elution of the orcinol peak corresponding to peak 2 in Figure 8a, the column pressure increased precipitously preventing collection of later fractions. This behavior appeared to be due to the high urea concentration (about 6 M) in the loaded sample, since a small shrinkage of the resin bed was observed during loading, and the pressure increased at an elution volume near that expected for urea. The estimated recovery was suspiciously high at 125%.

The orcinol positive fractions were pooled and lyophilized. The residue was dissolved in 2.5 ml of 0.015 M $CaCl_2$ containing 10 μg per ml of tetracycline and was rehydrolyzed with 0.65 mg pronase at 37° and pH 7.5 by the pH-stat procedure described. Very little base was added, and after 24 hours the sample pH was adjusted to 4.0 with glacial acetic acid. The hydrolysate was diluted to 5.0 ml with distilled water and rechromatographed on Bio-Gel P-6.

As illustrated in Figure 8c the orcinol positive material eluted as a single peak which contained 99% of the orcinol positive load and 34% of the ninhydrin positive load. An additional 54% of the ninhydrin positive load was recovered in orcinol negative peaks eluting later. The indicated orcinol positive fractions were pooled and lyophilized, yielding 64 mg of glycopeptides or 11% by weight of the protein hydrolyzed. Assuming an average carbohydrate composition of 75% from the composition data in Table VII, this yield, uncorrected for sampling and other losses, represented about 85% of the theoretical yield.

The glycopeptide mixture was chromatographed on AG 50W-x2 with pH 2.3, formic acid-pyridine buffer producing the profile in Figure 13. Recovery of orcinol positive material was 96%. Aliquots of the loaded sample and of the peak fractions indicated by vertical arrows were analyzed for amino acid composition following hydrolysis for 22 hours and for neutral carbohydrate composition following hydrolysis for 40 hours. No amino acid internal standard was used. Glucosamine was estimated from 22 hour hydrolysates assuming 40% destruction. The composition data from single analyses are listed in Table VII as ratios relative to aspartic acid.

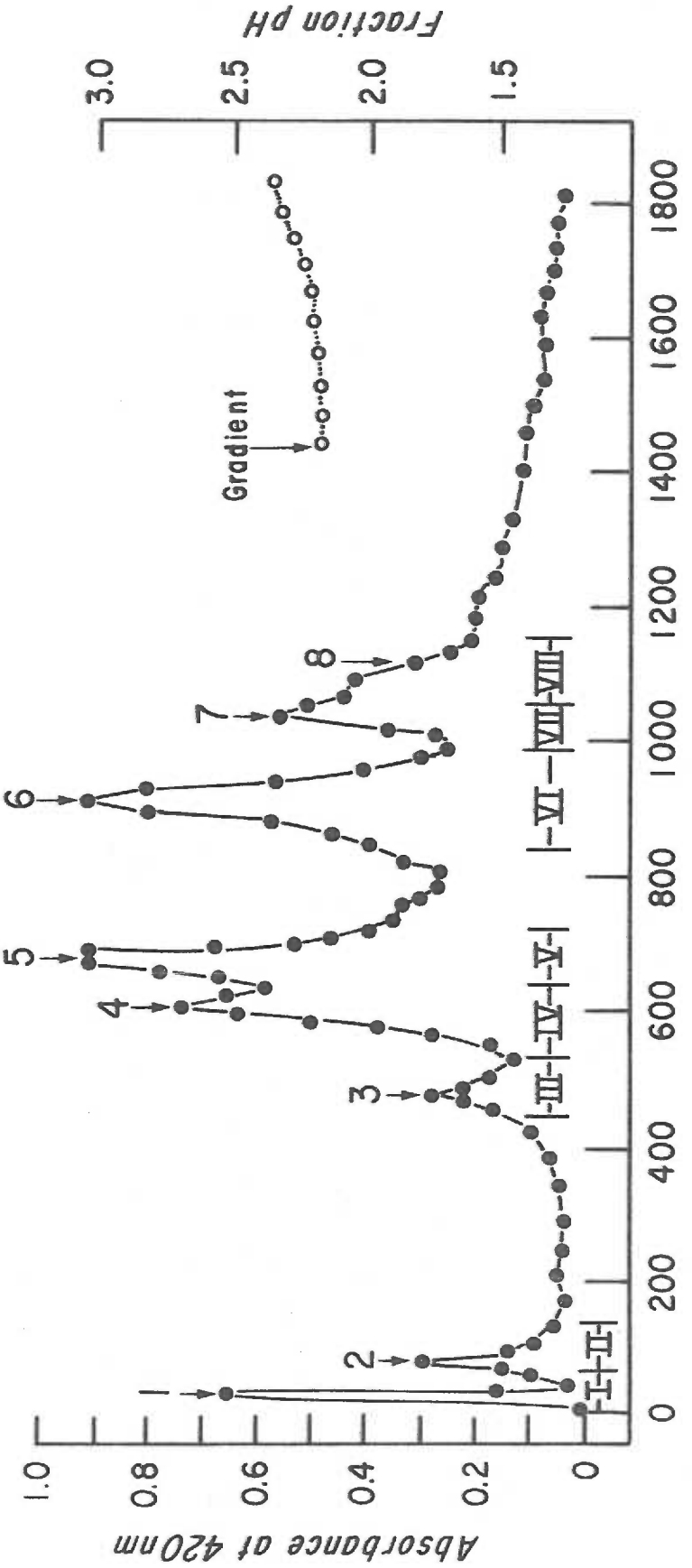


Figure 13. AG 50W-x2 chromatogram of the glycopeptide mixture from Figure 8c obtained with pH 2.3, 0.2 M formic acid-0.002 M pyridine, followed by a pH gradient from 1440 to 1800 ml: ●—● manual orcinol assay, and o·····o fraction pH.

TABLE VII. Residue ratios of AG 50W-x2 peak fractions listed in Figure 13.

Res. \ Sample	Load	1	2	3	4	5	6	7	8
Asx	N.D.	(1.0)	(1.0)	(1.0)	(1.0)	(1.0)	(1.0)	(1.0)	(1.0)
Thr	N.D.	0.85	0.61	0.88	0.61	0.67	0.92	0.62	0.72
Ser	N.D.	0.06	0.26	T	0.08	0.44	T	0.13	T
Glx	N.D.	0.37	0.56	T	0.56	0.65	0.05	0.20	0.35
Gly	N.D.	0.06	0.26	0.08	T	0.26	0.05	0.10	0.06
Ala	N.D.	0.05	0.17	T	T	0.15	T	0.06	T
GlcN*	14	1.3	0.83	0.92	1.2	1.1	0.83	0.83	1.00
Fuc	(4.0)	0.40	0.29	0.50	0.09	T	0.42	0.24	0.09
Xyl	2.7	0.28	T	0.20	T	---	0.31	0.22	0.14
Man	44.	3.9	3.4	1.6	4.8	4.6	1.7	1.9	2.3

Fract. Pool	I	II	III	IV	V	VI	VII	VIII
% of Load in Fraction Pool	1.8	2.4	3.7	12.0	14.2	18.3	7.6	6.6

* Estimates from 22 hour hydrolysates assuming 40% destruction.

T = Indicates traces of less than .05.

None of the fractions contained more than traces of basic amino acids. All yielded substantial quantities of NH_3 which was not quantitated due to lack of appropriate controls. The fractions indicated in Figure 13 were pooled and lyophilized. The percentage of the orcinol positive column load recovered in each fraction pool is also listed at the bottom of Table VII. The fraction pools will subsequently be designated by the Roman numerals listed for each in Table VII.

The material in peak VI was chromatographed on AG 50W-x2(K^+) using 50% ethanol as developer. One peak representing 98% of the load eluted between 22-32 ml (compare with Figure 12). A small peak tail was observed. The main peak fractions representing 95% of the load were pooled, lyophilized, and rechromatographed on AG 50W-x2(K^+) with water as developer. The load was quantitatively recovered in one peak eluting from 24 to 34 ml. The pooled fractions were lyophilized producing a yellow-tinted residue.

Since the preparation was colorless prior to chromatography on AG 50W-x2, the colored material appeared to represent contamination by resin castings. This material

was removed by chromatography on Bio-Gel P-2. The orcinol positive material was recovered in a single peak representing 96% of the load. The peak fractions were pooled and lyophilized yielding a colorless residue.

The amino acid, glucosamine and neutral carbohydrate compositions of the residue were determined. The results are listed in Table VIII. Aliquots for amino acid analysis, containing added isoleucine as an internal standard, were hydrolyzed for 12, 17 and 22 hours. No variation in the yields of specific amino acids was observed as a function of hydrolysis time beyond that observed for duplicate analyses of a single hydrolysate. Consequently, the data for all samples were pooled and averaged.

Glucosamine assays of samples hydrolyzed for 3, 4, 5 and 6 hours indicated no dependence of glucosamine yield on hydrolysis time in this time range. Thus, all values were averaged.

Neutral carbohydrate assays were conducted by gas-liquid chromatography following hydrolyses for 25 and 40 hours. The results reported here are for 25 hour hydrolysates. The 40 hour results were lower for all three monosaccharides; namely, 5% for mannose; 10% for fucose and 15%

TABLE VIII. Composition of AG 50W-x2 glycopeptide peak VI

Residue	g* / 100 g	μmoles** / mg	Calculated Ratio	Integral Ratio
Asp	11.28 (5)	0.972±.049	(2.00)	2
Thr	9.17 (5)	0.907±.032	1.87	2
Ser	0.30 (5)	0.035±.032	0.072	---
Glu	1.02 (5)	0.079±.006	0.164	---
Gly	0.32 (4)	0.056±.012	0.114	---
Ala	0.17 (2)	0.024±.007	0.050	---
NH_3	---- (4)	0.732±.056	1.51	1-2
GlcN	20.94 (4)	1.041±.026	2.14	2
Fuc	5.58 (4)	0.382±.028	0.786	1
Xyl	5.39 (4)	0.408±.028	0.840	1
Man	29.99 (4)	1.85 ±.104	3.80	4
TOTAL	84.16			

* Calculated with the residue weights. Numbers in parentheses are the number of determinations.

** Mean ± standard deviation.

for xylose. All subsequent neutral carbohydrate analyses of glycopeptides were made on 25 hour hydrolysates.

The AG 50W-x2 fraction pools IV, V and VI were chromatographed on AG 1-x2 with pH 5.7 pyridine-acetic acid development. Chromatographic profiles were obtained with the automated orcinol assay system.

Glycopeptide fraction VI produced the profile in Figure 14a. The elevation of the baseline at the beginning of the profile was produced by a mannose pulse which marked the commencement of fraction collection. Total recovery of orcinol positive material was 90%. Most of the carbohydrate was eluted as a large peak, VI-1, centered at 95 ml with a pronounced shoulder, VI-2, at approximately 115 ml. Both together accounted for 73% of the load. Low levels of components were observed at 60 ml (2.5%), 200 ml (6%) and 250 ml (6.0%). The indicated fraction pools were lyophilized and rechromatographed.

The profile obtained for peak VI-1 is illustrated in Figure 14b. The material was recovered in a single peak centered at 98 ml representing 91% of the load. No significant minor peaks were observed. The peak fractions were conservatively pooled as indicated to minimize contamination by traces of component VI-2.

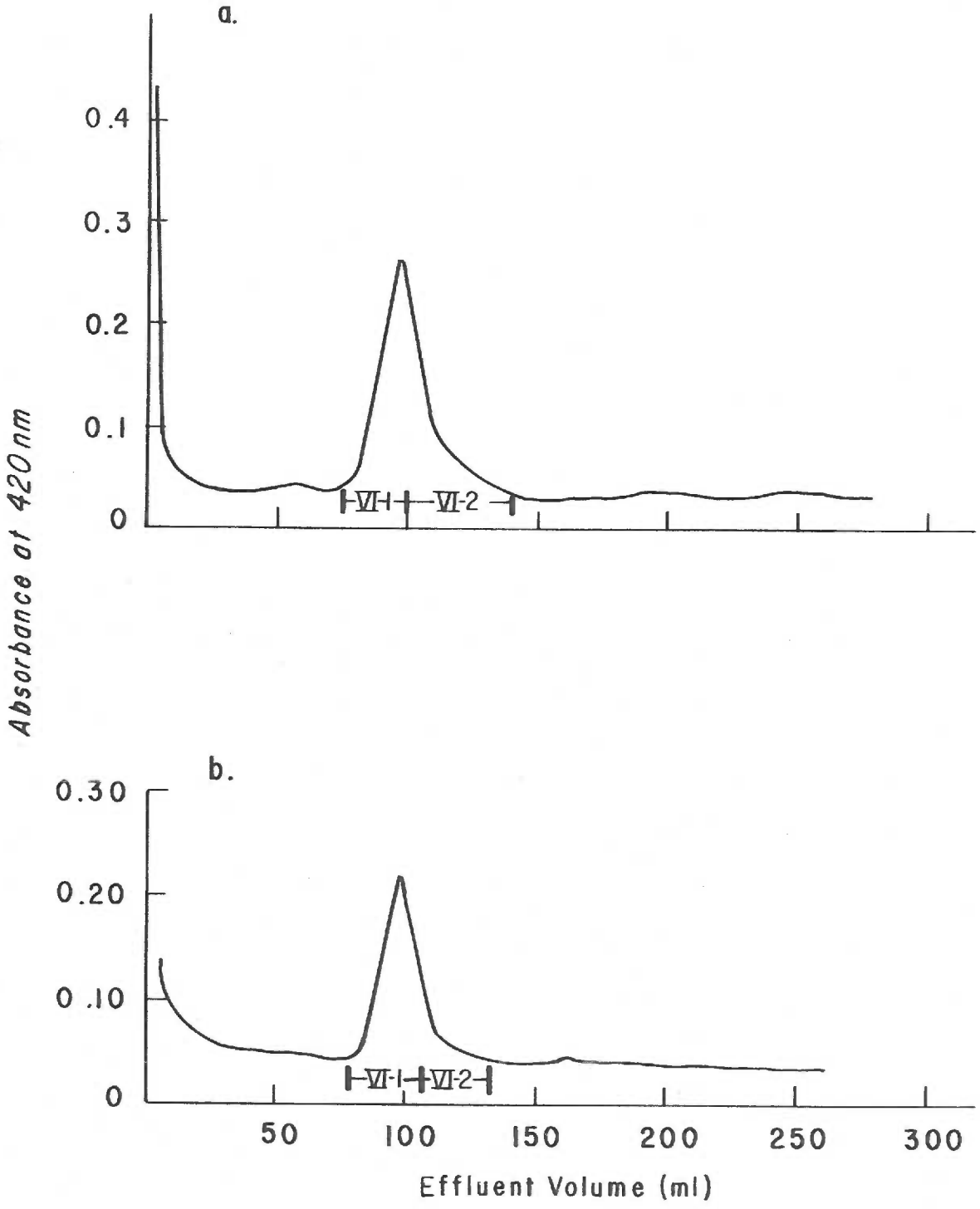


Figure 14. AG 1-x2 chromatograms of AG 50W-x2 peak VI (automated orcinol assay): a. first chromatography; and b. rechromatography of fraction pool VI-1 from a.

The VI-2 fraction pools from these runs were pooled and rechromatographed. Total recovery was 73%. Two major peaks were observed, one centered at 95-100 ml corresponding to VI-1, and a second at 115-120 ml corresponding to VI-2. Fractions were conservatively pooled from 120 to 146 ml and lyophilized.

The composition data for glycopeptides VI-1 and VI-2 are summarized in Table IX. Both peptides had very similar amino acid and carbohydrate compositions except that VI-2 contained no fucose. Both glycopeptides contained slightly less mannose than the parent material reported in Table VIII. The glutamic acid content of VI-1 was also much less than that of the parent material.

Chromatography of peak V on AG 1-x2 at pH 5.7 produced the profile in Figure 15a. Total recovery was 82%: 3% in peak V-1 near the column void volume; 39% in V-2 at about 160 ml; and 37% in V-3 at about 210 ml. A low level of orcinol positive material (3%) eluted diffusely between 20 and 120 ml. Also, the tail of peak V-3 slowly approached baseline, and not all of this low level orcinol positive material was recovered and included in the recovery assay.

TABLE IX. Compositions of AG 1-x2 peaks VI-1 and VI-2

Residue	VI-1 Calculated Ratio	VI-1 Integral Ratio	VI-2 Calculated Ratio	VI-2 Integral Ratio
Asp	2.00	2	2.00	2
Thr	1.92	2	1.88	2
Ser	0.24	0	0.20	0
Glu	----	0	0.12	0
Gly	0.02	0	0.30	0
Ala	0.04	0	0.10	0
Val	----	0	0.04	0
Leu	----	0	----	0
NH_3	1.49	1-2	1.69	1-2
GlcN	2.02	2	1.77	2
Fuc	0.88	1	----	0
Xyl	0.88	1	0.73	1
Man	3.10	3	2.84	3

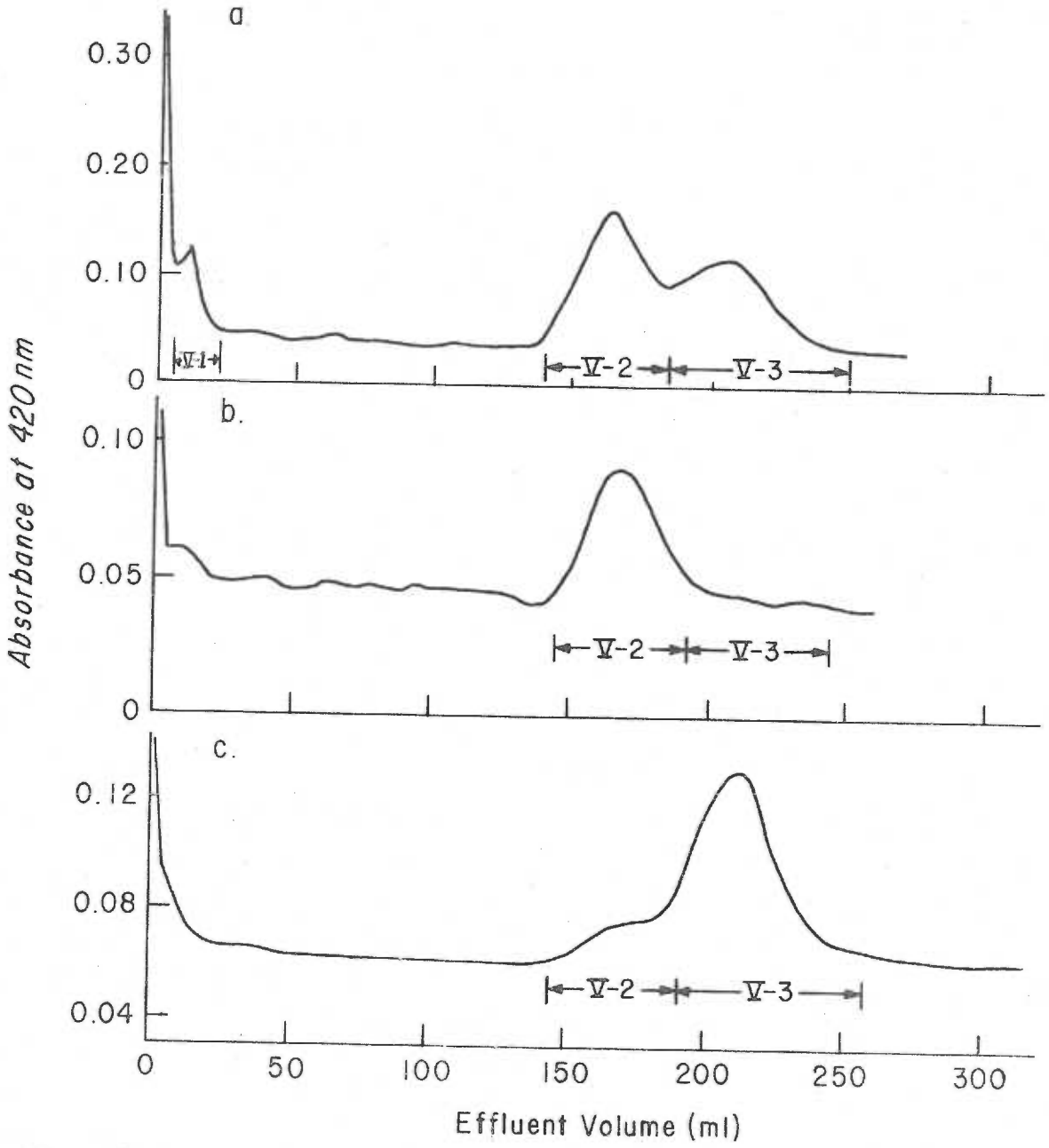


Figure 15. AG 1-x2 chromatograms of AG 50W-x2 peak V (automated orcinol assay): a. first chromatography; b. rechromatography of fraction pool V-2 from a; and c. rechromatography of fraction pool V-3 from a.

The indicated fraction pools were lyophilized. Pools V-2 and V-3 were rechromatographed giving the profiles in Figures 15b and 15c, respectively. In 15b, the total recovery was 93%, with 82% eluting in peak V-2 at 165-170 ml, and 11% in peak V-3. In 15c, the total recovery was 89%, with 77% in peak V-3 at 210 ml and 12% in peak V-2. The glycopeptide fractions V-1 (from Figure 15a), V-2 (Figure 15b) and V-3 (Figure 15c) were analyzed for amino acid, glucosamine, and neutral carbohydrate composition giving the results in Table X.

The residue ratios of V-1 suggested that it was a complex mixture, while the nearly integral ratios for V-2 and V-3 indicated high degrees of purity. The carbohydrate compositions of V-2 and V-3 were almost identical. However, V-2 contained one more residue of aspartic acid and NH_3 than V-3. In contrast to VI-1 and VI-2 (Table IX), both V-2 and V-3 contained glutamic acid and carbohydrate moieties composed only of glucosamine and mannose.

Chromatography of AG 50W-x2 peak IV on AG 1-x2 at pH 5.7 produced the profile in Figure 16. Total recovery was 85%; the double peak near the void volume accounted for 4%; peak IV-1 at 60 ml, 5%; peak IV-2 at 145-150 ml, 37%; and peak IV-3 at 170-180 ml, 38%. Aliquots were drawn for

Table X. Compositions of AG 1-x2 peaks V-1, V-2 and V-3

Residue	V-1 Calculated Ratio	V-2 Calculated Ratio	V-2 Integral Ratio	V-3 Calculated Ratio	V-3 Integral Ratio
Asp	1.00	2.00	2	1.00	1
Thr	0.44	1.03	1	0.98	1
Ser	0.87	0.13	0	0.11	0
Glu	0.34	1.08	1	1.02	1
Gly	0.98	0.10	0	0.08	0
Ala	0.41	0.05	0	0.045	0
Val	0.22	----	0	----	0
Leu	0.29	----	0	----	0
NH_3	4.43	2.48	2-3	1.50	1-2
GlcN	1.81	1.89	2	1.84	2
Fuc	4.99	----	0	----	0
Xyl	----	----	0	----	0
Man	7.19	8.20	8	7.72	8

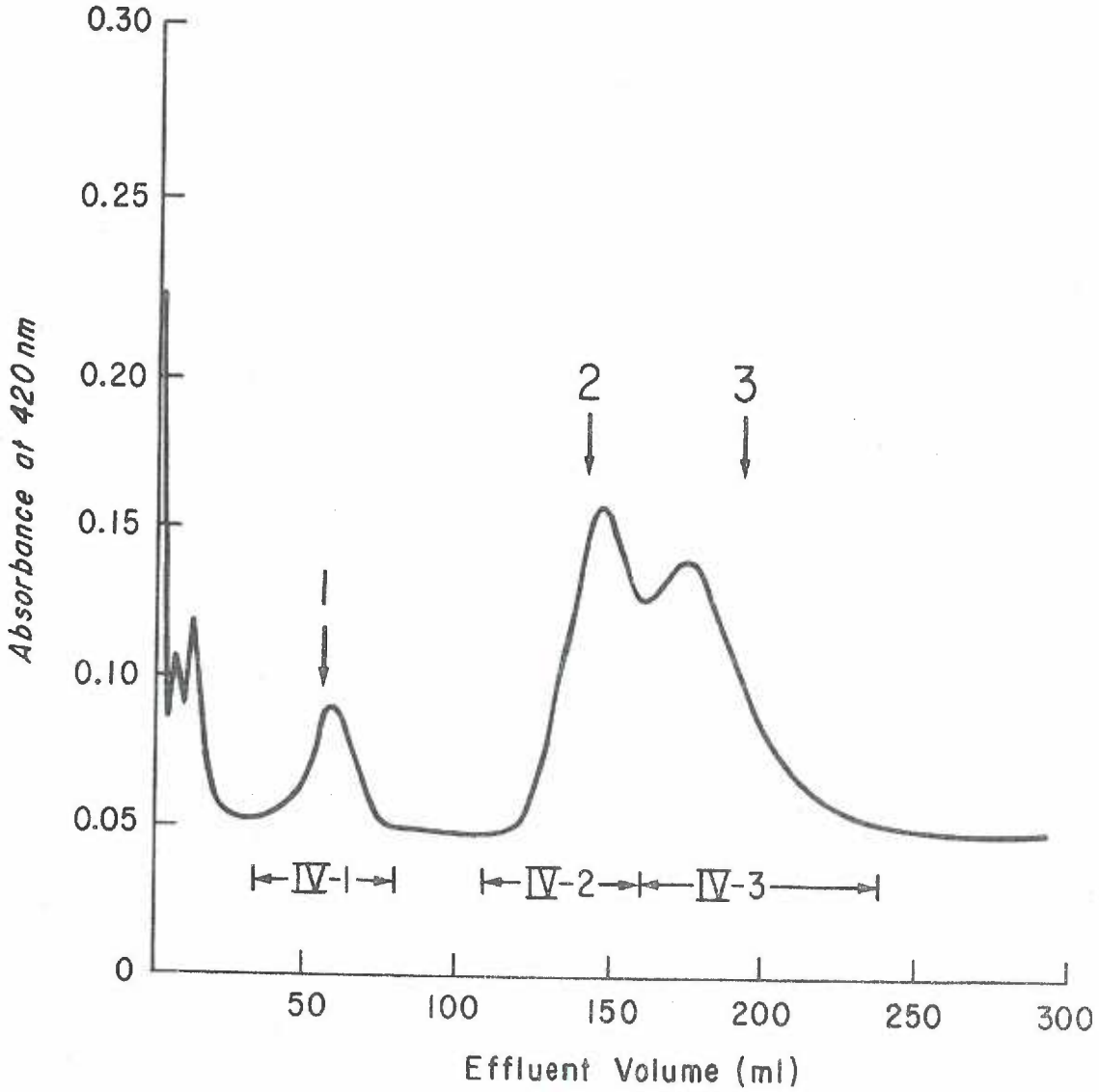


Figure 16. AG 1-x2 chromatogram of AG 50W-x2 peak IV (automated orcinol assay).

composition analyses from the peak fractions indicated by vertical arrows. The results of single analyses are listed in Table XI.

Peak IV-1 differed significantly from IV-2 and IV-3 in both amino acid and carbohydrate composition. It contained no glutamic acid and its carbohydrate composition was more similar to those of VI-1 and VI-2 (Table IX). Peaks IV-2 and IV-3 differed from each other most significantly in their aspartic acid content. Their compositions were strikingly similar to those of V-2 and V-3 (Table X). The difference between their mannose contents is questionable, since instrumental difficulties gave substantial ranges of values, i.e., 7.8 to 8.4 mannose residues for IV-2 and 6.8 to 8.1 for IV-3.

III. Molecular Weight Determination

The molecular weights of glycopeptides VI-1, V-2 and V-3 were determined by sedimentation equilibrium as described under "Methods".

The plots of log $\Delta_Q J$ versus q were straight lines for all three glycopeptides as illustrated in Figure 17, thus indicating homogeneity. Table XII summarizes the molecular weight data. Listed are the slope, dlog $\Delta_Q J/dq$ = b, the coefficient of correlation, and the ratio of the standard

Table XI. Compositions of AG 1-x2 tubes 1, 2 and 3 from peaks IV-1, IV-2 and IV-3, respectively

Residue	1 Calculated Ratio	1 Integral Ratio	2 Calculated Ratio	2 Integral Ratio	3 Calculated Ratio	3 Integral Ratio
Asp	(2.00)	2	2.00	2	1.15	1
Thr	1.22	1	1.01	1	1.00	1
Ser	T	0	T	0	T	0
Glu	0	0	1.03	1	0.97	1
Gly	T	0	T	0	T	0
Ala	T	0	T	0	T	0
NH_3	1.50	1-2	2.94	3	2.46	2-3
GlcN	1.81	2	1.72	2	1.68	2
Fuc	0.32	0	----	0	----	0
Xyl	0.71	1	----	0	----	0
Man	3.00	3	8.12	8	7.36	7

T = Trace amounts less than 0.05

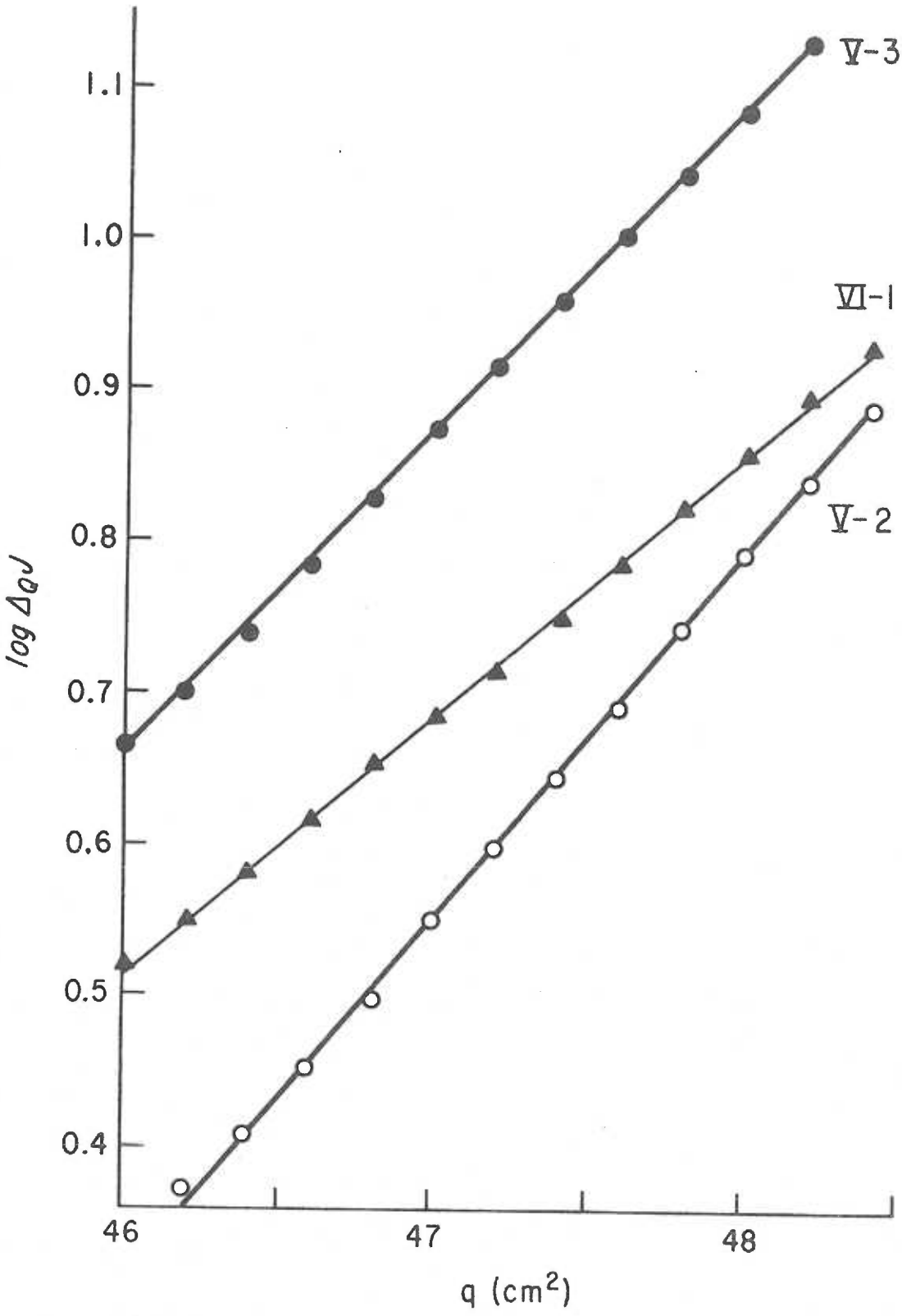


Figure 17. Sedimentation equilibrium plots of log $\Delta_Q J$ versus q for glycopeptides, VI-1, V-2, and V-3.

TABLE XII. Molecular weight data for glycopeptides VI-1, V-2 and V-3

	VI-1	V-2	V-3
$d\log \Delta_Q J/dq = b$	0.17110	0.23902	0.21382
correlation coefficient	0.99964	0.99970	0.99988
S_b/b	0.0081	0.0078	0.0050
$M(1-\bar{v}\rho)$	632.13	883.07	789.98
$\bar{v}$ (cc/gm)	0.642	0.628	0.630
$M_{app} \pm \frac{2RT\ 2.303\ S_b}{(1-\bar{v}\rho)\omega^2\ b}$	1773 ± 84	2383 ± 78	2144 ± 50
M_{comp}	1620	2180	2066
$\frac{M_{app}-M_{comp}}{M_{comp}} \times 10^2$	+9.4%	+9.3%	+3.8%

deviation of the slope, S_b, to the slope, b. The computed values of $M(1-\bar{v}\rho)$ are given with the estimates of $\bar{v}$ used to calculate the molecular weights. Since only one concentration of each peptide was examined, molecular weights are presented as apparent molecular weights, M_{app}, with the weight range resulting from a deviation, S_b, in the slope. M_{comp} is the minimum molecular weight calculated from the composition data and is used to determine the percent deviation of the measured molecular weight from this minimum value.

The apparent molecular weights establish the correctness of the assumed glycopeptide constituent ratios and demonstrate the similarity of glycopeptide molecular weights as suggested by gel permeation chromatography on Bio-Gel P-2 and P-6. All the molecular weight estimates were higher by 4-9% than the minimum values based on their compositions.

IV. Edman Degradation

Glycopeptides V-2, V-3 and VI-1 were subjected to Edman degradation. The first one to be sequenced was glycopeptide V-3, shown by composition and molecular

weight data to be (Asx, Thr, Glx, $GlcNAc_2$, Man_8)*. The amino acid difference analyses for two degradation steps are summarized in Table XIII.

TABLE XIII. Subtractive Edman degradation of glycopeptide V-3a

Step	Residue		
	Asx	Glx	Thr
Undegraded	1.02	1.04	1.00
1st degradation	<u>0.24</u>	1.04	1.00
P-2 fraction II	<u>0.08</u>	1.08	1.00
2nd degradation	--	<u>--</u>	0.74[b]

a. Underlined figures represent the residue eliminated.

b Yield of free threonine in 2nd degradation.

The first degradation removed 0.78 residues of either aspartic acid or asparagine. Thin layer chromatography of the ethyl acetate extract of the degradation products (Figure 18) showed no component corresponding to either PTH-Asp or PTH-Asn. A large diffuse component was observed

* Glucosamine is assumed to be acetylated as explained in the "Discussion".

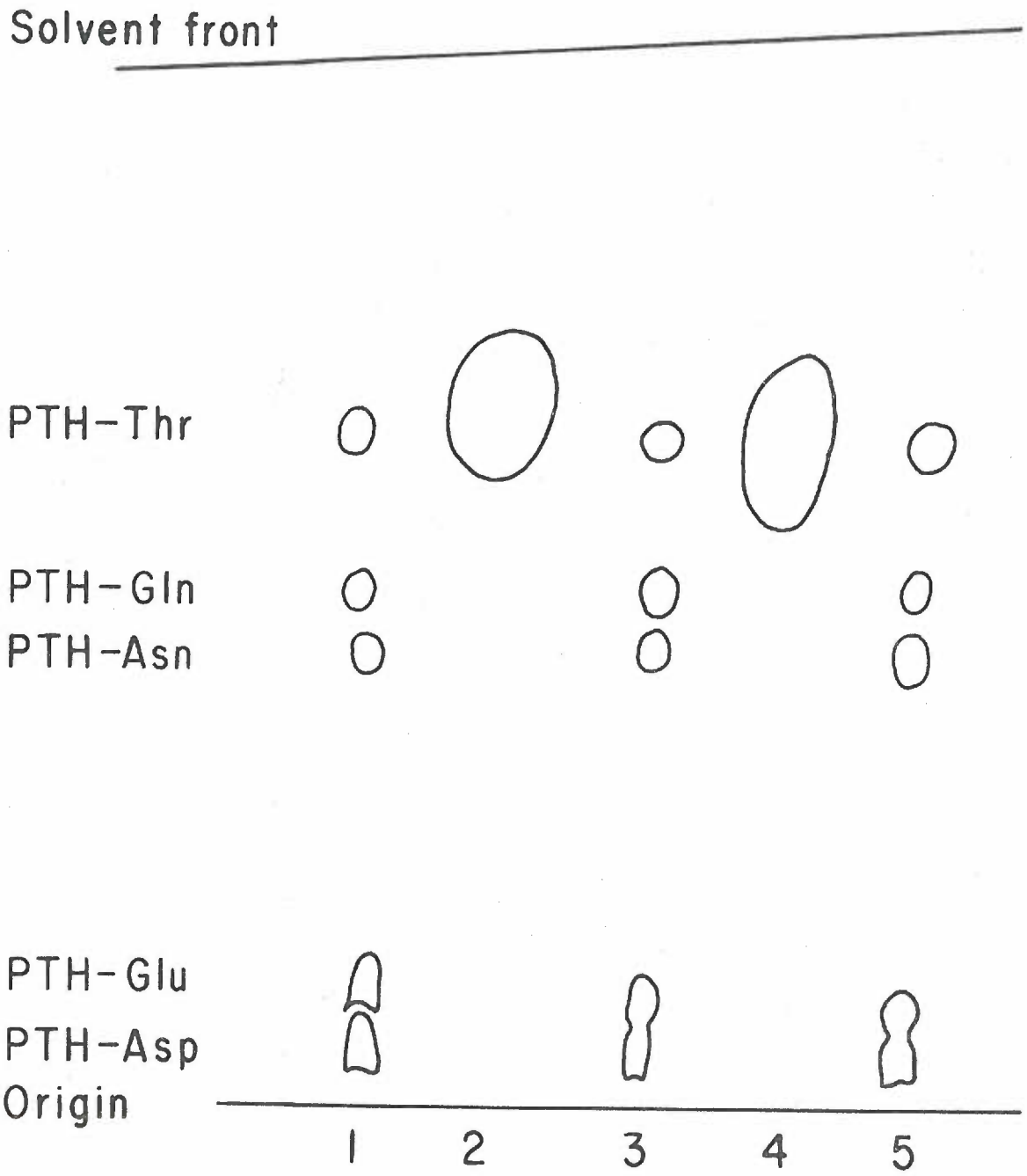


Figure 18. Thin layer chromatogram of the first Edman degradation extract from glycopeptide V-3: 2 μl of a mixture of standard PTH-drivatives at about 2 mg/ml each was loaded at 1, 3, and 5; and 2 μl out of a total of 20 μl of the extract was loaded at 2 and 4.

in the region of PTH-Thr. This component was observed in all extracts examined by thin layer chromatography.

The absence of PTH-Asn suggested that the carbohydrate had been bound to the N-terminal residue and had prevented the extraction of the phenylthiohydantoin derivative. This hypothesis was supported by Bio-Gel P-2 chromatographic separation of the degradation products. As shown in Figure 19, an orcinol positive component with strong 264 nm absorption eluted at a volume which was similar to that of the undegraded glycopeptide. A small orcinol peak eluted later in a position corresponding to that of monosaccharides. The indicated fraction pools were analyzed for amino acid composition. Fraction I contained approximately 90% of the aspartic acid of the loaded degradation mixture, while fraction II contained about 90% of the threonine and glutamic acid in a ratio of 1:1.

The remaining fraction II material was degraded again. The difference analysis value in Table XIII represents the yield of free threonine. This value was obtained by analysis of an unhydrolyzed sample of the degradation products. The corresponding acid hydrolyzed sample was lost. Thin layer chromatography of the ethyl acetate extract (Figure 20) showed the presence of a component

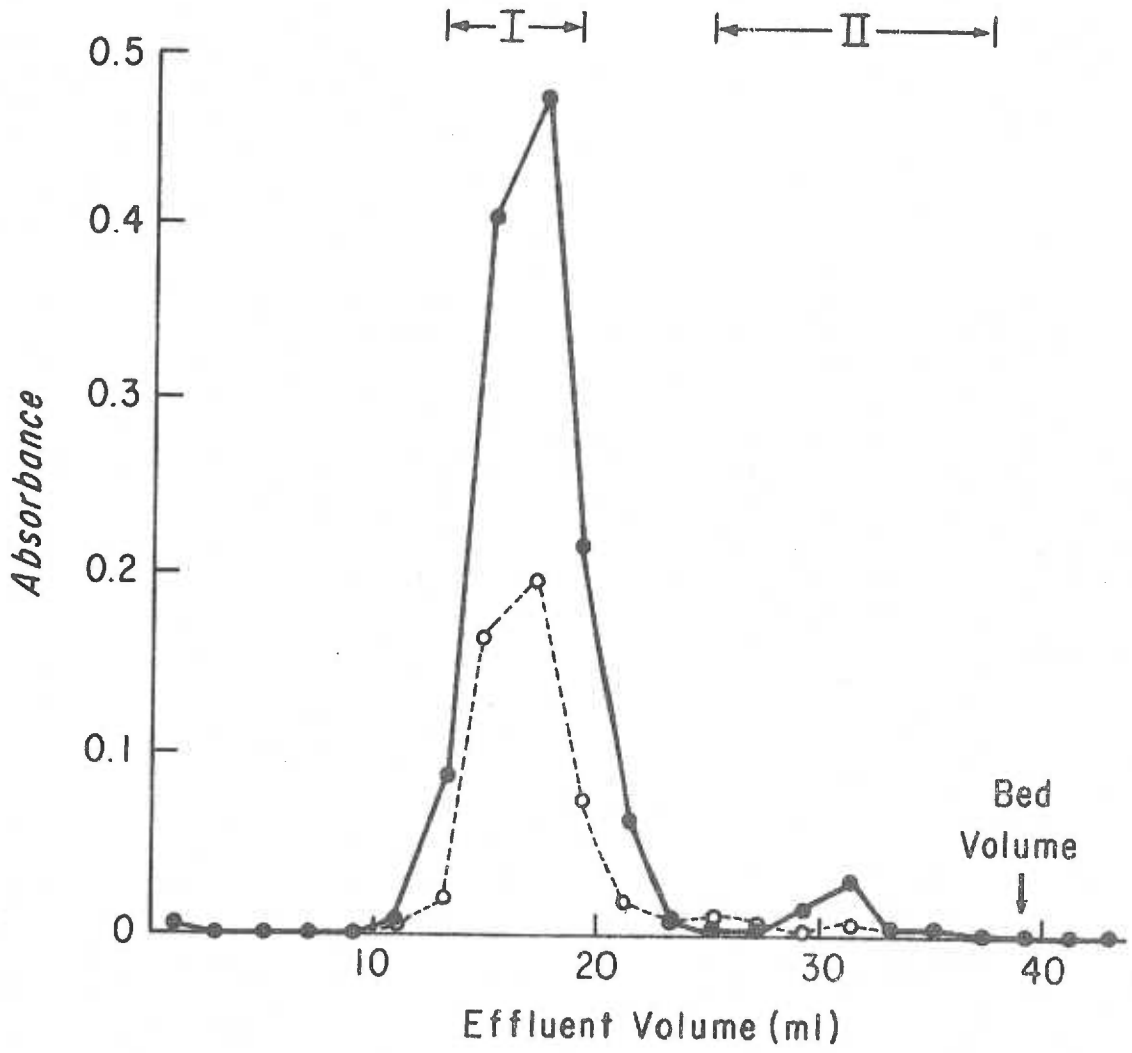


Figure 19. Bio-Gel P-2 chromatogram of the products of the first Edman degradation of glycopeptide V-3: ●—● orcinol assay at 420 nm, and o--o absorbance at 264 nm.

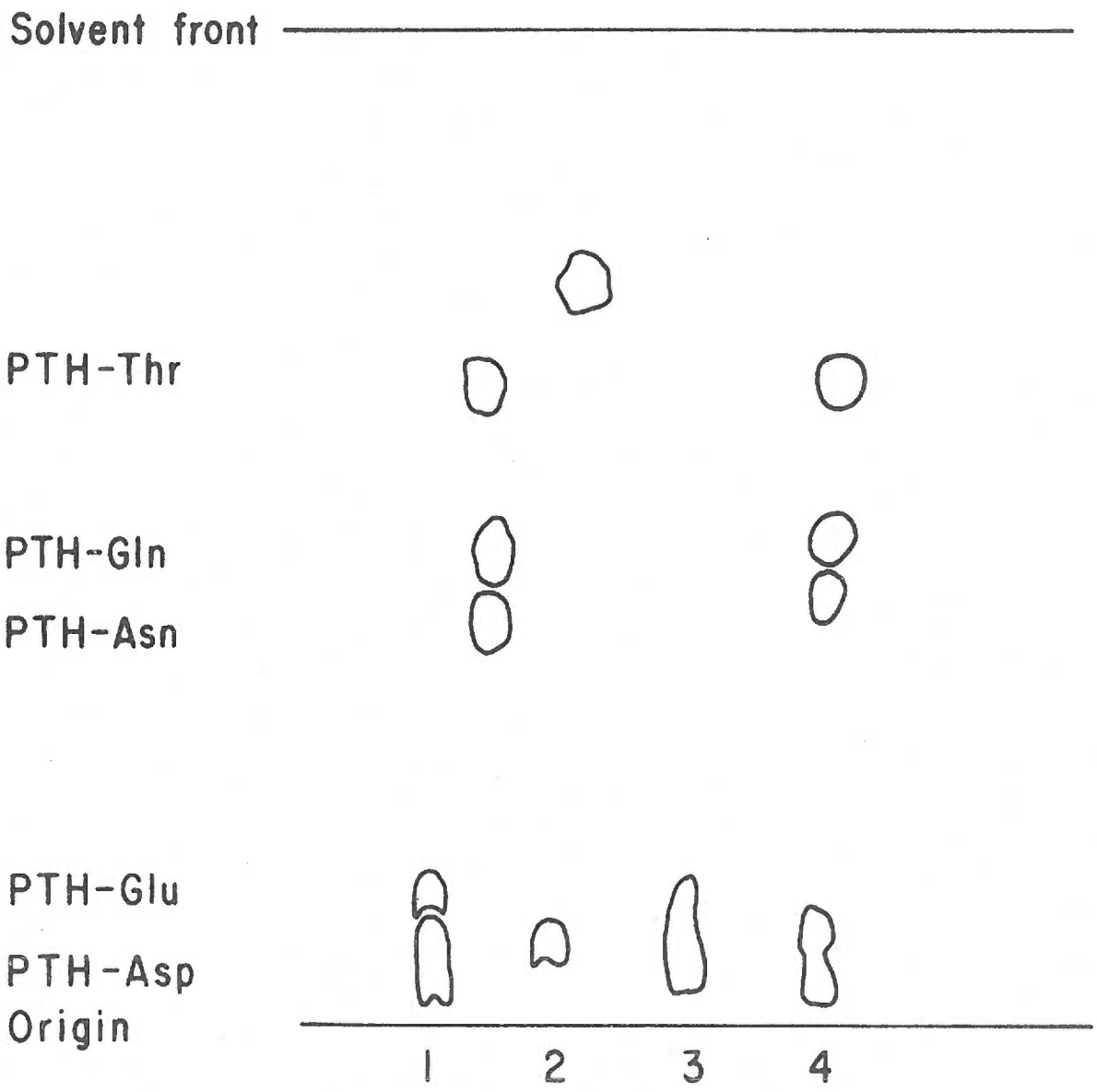


Figure 20. Thin layer chromatogram of the second Edman degradation extract from glycopeptide V-3: 3 and 2 μl of the mixed PTH-standards at 1 and 4, respectively, all of glycopeptide V-3 extract at 2, and 4 μl of 2 mg/ml PTH-Glu at 3.

corresponding to either PTH-Asp or PTH-Glu and a second component of unknown identity migrating slightly further than PTH-Thr. The former component was assumed to represent PTH-Glu since the difference analysis indicated the loss of either Glu or Gln.

Based on these data the amino acid sequence, Asx-Glu-Thr, was assigned to V-3 in which the carbohydrate was bound through the side chain of the N-terminal Asx residue.

Glycopeptide V-2, (Asx_2,Thr,Glx,$GlcNAc_2$,Man_8), was subjected to three Edman degradations. Table XIV summarizes the amino acid difference analyses.

TABLE XIV. Subtractive Edman degradation of Glycopeptide V-2*.

Step	Residue Asx	Glx	Thr
Undegraded	1.94	1.04	1.00
1st degradation	<u>1.20</u>	1.05	1.00
P-2 fraction II	<u>1.04</u>	1.28	1.00
2nd degradation	1.00	<u>0.28</u>	1.00
3rd degradation	1.00	0.27	<u>0.33</u>

* Underlined figure represents the residue eliminated

The first degradation destroyed 0.74 residue of either aspartic acid or asparagine. Thin layer chromatography of the ethyl acetate extract showed neither PTH-Asp nor PTH-Asn (Figure 21). This behavior resembled that of glycopeptide V-3. Consequently, the degradation products were chromatographed on Bio-Gel P-2 giving a profile identical to that obtained for glycopeptide V-3 in Figure 19. Fraction II from this chromatography contained the hypothetical tripeptide (Asx, Thr, Glx) in a yield of 80%. However, the recovery of glutamic acid was 99% as opposed to 79% for both aspartic acid and threonine. Moreover, significant levels of serine and glycine contamination arose during P-2 chromatography and the accompanying procedures. Thus, it appeared that the high glutamic acid ratio following P-2 chromatography represented contamination.

The residue of this fraction II was degraded twice. The first of these removed 1.0 residue of either glutamic acid or glutamine. Thin layer chromatography of the

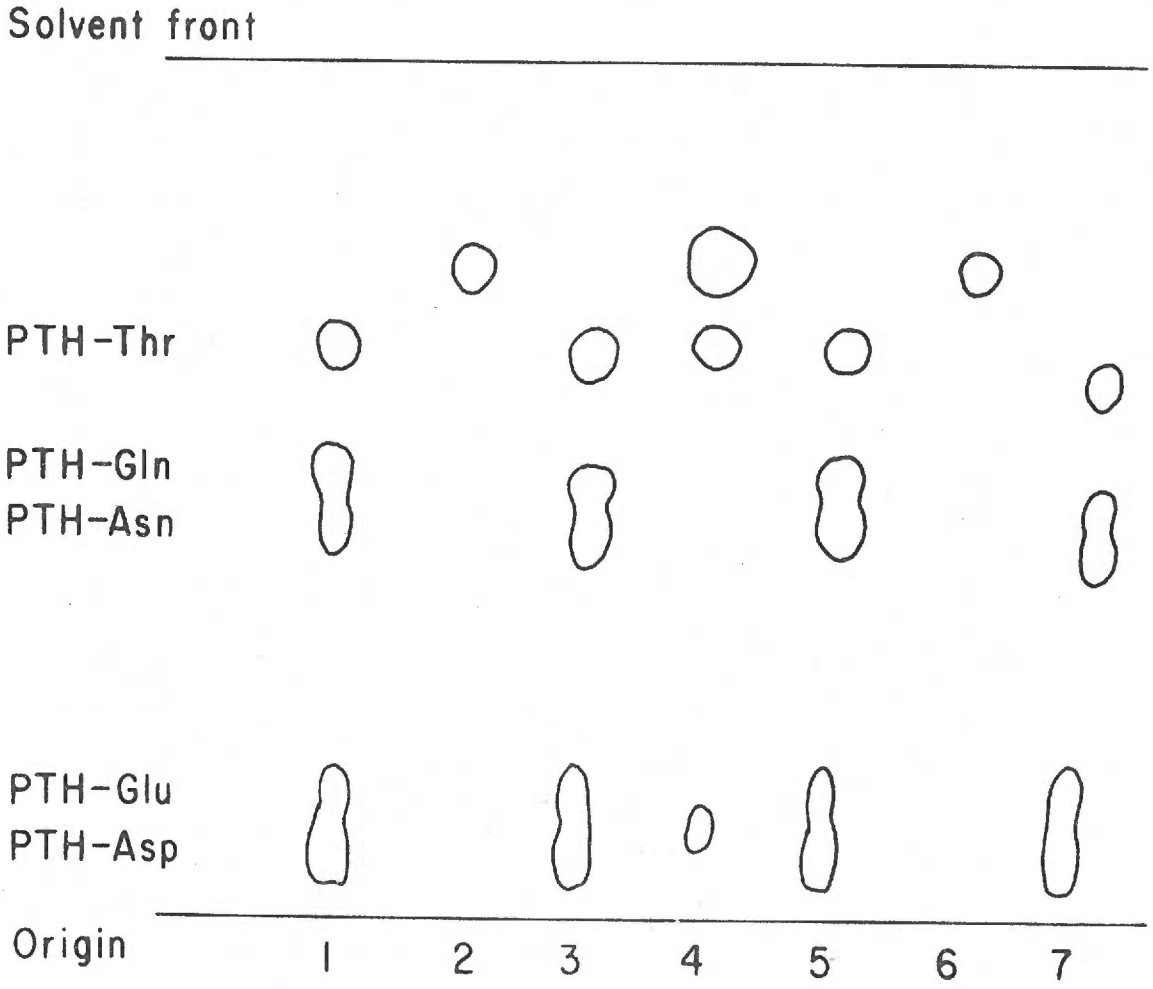


Figure 21. Thin layer chromatogram of the Edman degradation extracts from glycopeptide V-2. All of the extract from each step was loaded in a single spot: mixed PTH-standards at 1,3,5, and 7; and the extracts from the first, second, and third degradations at 2, 4, and 6, respectively.

extract (Figure 21) showed a component in the PTH-Asp to PTH-Glu region, but none in the region of the corresponding amides, indicating the presence of Glu but not of Gln in the parent peptide. Three additional components were also observed, a faint one slightly displaced from the origin (not shown in Figure 21), one corresponding to PTH-Thr and one beyond PTH-Thr. The significance of the apparent PTH-Thr component is not clear. Amino acid recovery data indicated loss of glutamic acid but not of threonine. Of the other contaminating amino acids noted above, the glycine level was slightly lower.

The second degradation of the residue of this fraction II removed 0.67 residues of threonine. However, thin layer chromatography (Figure 21) showed no component corresponding to PTH-Thr.

On the basis of these data the assigned amino acid sequence of the glycopeptide V-2 was Asx-Glu-Thr-Asx, in which the carbohydrate was bound through the side chain of the N-terminal residue.

Glycopeptide VI-1, (Asx_2, Thr_2, $GlcNAc_2$, Man_3, Fuc, Xyl), was subjected to three Edman degradations. Table XV summarizes the amino acid difference analyses.

TABLE XV. Subtractive Edman degradation of glycopeptide VI-1.*

Step	Residue Asx	Thr
Undegraded	2.08	2.0
1st degradation	<u>1.16</u>	2.0
2nd degradation	<u>0.51</u>	2.0
P-2 fraction I	<u>0.53</u>	2.0
3rd degradation	0.75	2.0

* Underlined figures represent residue eliminated.

The first degradation destroyed 0.92 residue of either aspartic acid or asparagine. Thin layer chromatography (Figure 22) showed a component in the region of PTH-Asp but none in the region of PTH-Asn.

The second degradation destroyed 0.65 residue of aspartic acid or asparagine. Recoveries of aspartic acid and threonine following the second degradation were 46%

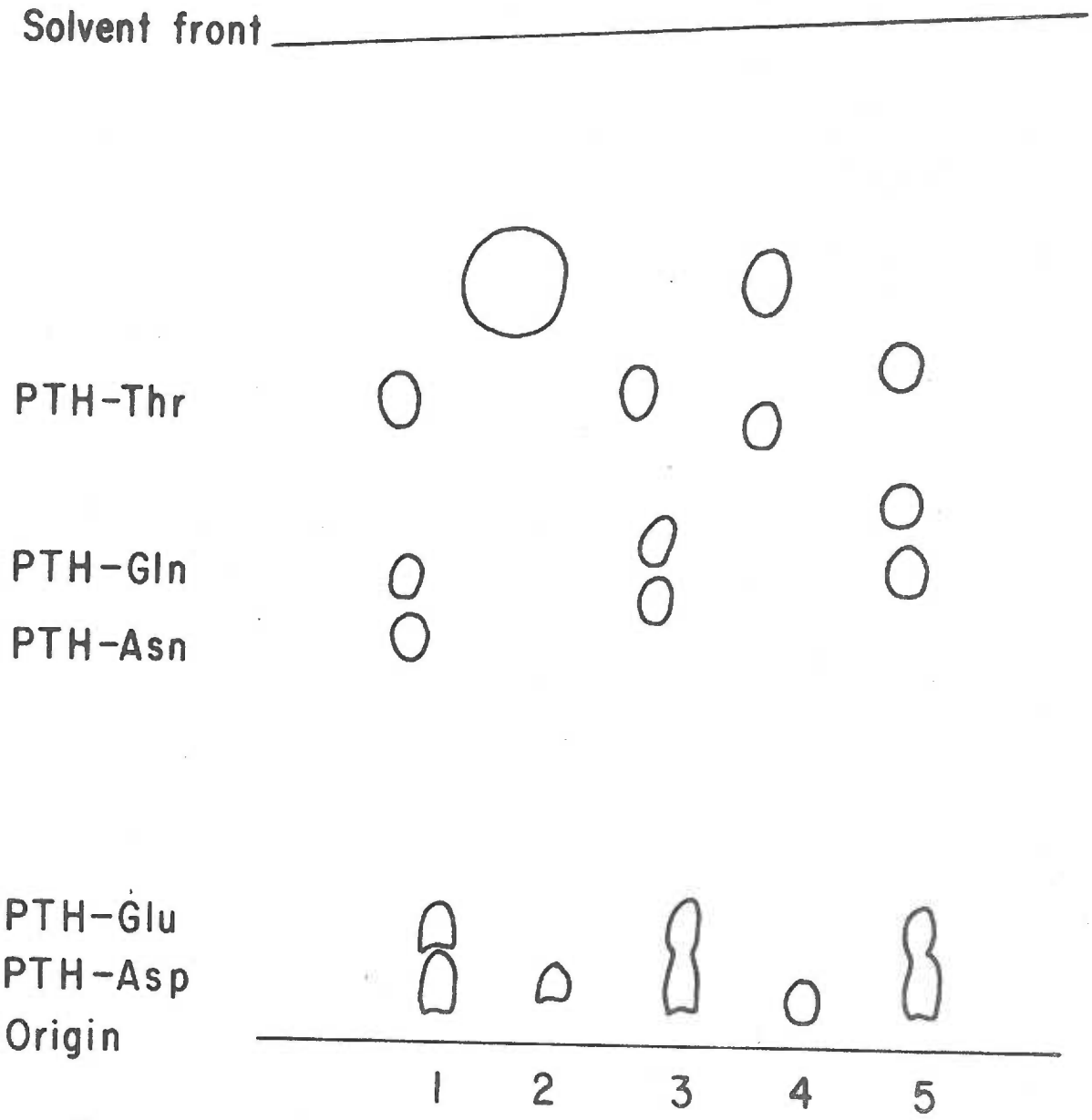


Figure 22. Thin layer chromatogram of the first and second Edman degradation extracts from glycopeptide VI-1: mixed PTH-standards at 1, 3, and 5; and 1 μl of a total of 20 μl of the first extract at 2, and 6 μl of a total of 20 μl of the second extract at 6.

and 106%, respectively. Thin layer chromatography of the ethyl acetate extract (Figure 22) showed only a faint component corresponding to PTH-Asp. A second faint component migrated between PTH-Gln and PTH-Thr, and a third component corresponded to the unknown observed in every degradation extract.

The observed low level of PTH-Asp suggested that most of the aspartic acid destroyed by the second degradation had not been extracted. Consequently, the degradation mixture was chromatographed on Bio-Gel P-2 giving the profile in Figure 23. Ultraviolet absorbance at 264 nm was associated with the major but not the minor orcinol peak. The major orcinol peak's elution volume approximated that of the undegraded glycopeptide, while the minor peak's resembled that expected for monosaccharides. The indicated fraction pools were analyzed for amino acid composition. Approximately 90% of the aspartic acid and threonine in the loaded degradation mixture was recovered in fraction I. No significant change in the ratios was observed. Fraction II contained free fucose and traces of a number of different amino acids.

Degradation of the fraction I material produced an increase in the aspartic acid: threonine ratio. Recoveries

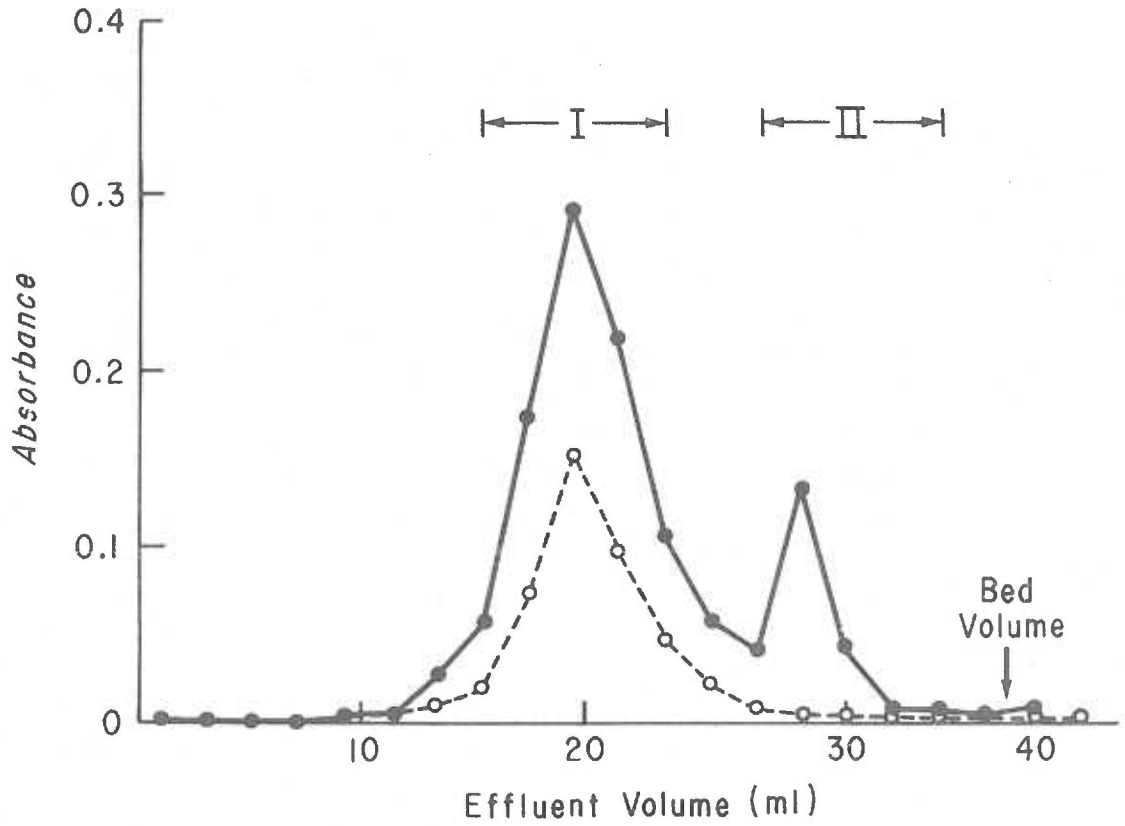


Figure 23. Bio-Gel P-2 chromatogram of the second Edman degradation products of glycopeptide VI-1: ●—● orcinol assay at 420 nm, and o--o fraction absorbance at 264 nm.

of aspartic acid and threonine were 85% and 61%, respectively. This would be the expected result if approximately 0.6 residue threonine were destroyed while aspartic acid remained unchanged. Thin layer chromatography of the ethyl acetate extract (not shown) revealed one component extending from the origin to the mid-point of the PTH-Asp region and a second component slightly ahead of PTH-Gln.

On the basis of these data the amino acid sequence, Asp-Asx-Thr-Thr, was assigned to glycopeptide VI-1. The carbohydrate linkage site was not clearly demonstrated by these studies.

V. β-Elimination Reaction of Glycopeptide VI-1

The Edman degradations of glycopeptide VI-1 failed to demonstrate clearly which amino acid was the carbohydrate binding site. The indicated sequence of the peptide was Asp-Asx-Thr-Thr. The N-terminal aspartic acid did not appear bound to the carbohydrate, leaving the penultimate Asx and the threonine residues as possible binding sites.

The peptide was treated with alkali by the two methods described under "Methods", and subsequent amino acid analyses gave the ratios shown in Table XVI. After treatment 1 no change in threonine was apparent. However, the sample appeared contaminated with serine and glycine.

Treatment 2 produced a decrease of 0.28 residue threonine. This appeared to be a marginal decrease. Therefore, glycopeptide V-2, in which the carbohydrate is bound to the N-terminal Asx residue, was also subjected to treatment 2 as a control, since it contained a non-terminal Thr residue. Unexpectedly, the treatment destroyed 0.2 threonine residues, suggesting that this procedure caused an unspecific loss of threonine.

TABLE XVI. Amino acid composition of glycopeptide VI-1 following base treatment

Residue	Untreated	(1) 0.4 N NaOH 24 hrs. 30° C	(2) 0.1 N NaOH-0.4 M $NaBH_4$ 48 hrs. 25° C
Asx	2.00	2.00	2.00
Thr	1.92	1.93	1.65
Ser	0.24	0.64	0.16
Glx	0.002	0.06	0.09
Gly	0.015	0.54	0.24
Ala	0.04	0.24	0.08

VI. Carboxypeptidase Digestion of Glycopeptide VI-1

Glycopeptide VI-1 was treated with carboxypeptidase A. Chromatography of the deproteinized hydrolysate on Bio-Gel P-2 gave the profile in Figure 24. Amino acid analysis of the orcinol and fluorescent positive peak showed the degraded glycopeptide to contain ($Asx_{2.0}$, $Thr_{1.05}$, $Ser_{0.13}$, $Gly_{0.15}$), i.e., 0.90 residue less threonine than the parent molecule. The retarded fluorescent positive peak eluted at a volume expected for amino acids and was not further examined.

VII. Sequential Periodate Oxidation of Glycopeptide V-3

Glycopeptide V-3 (0.25 μmole) was oxidized at pH 4.7 in the dark at 25° C with periodate at a concentration of 5 mM. The time course of its periodate consumption is shown in Figure 25. Six moles of periodate per mole glycopeptide were rapidly consumed, followed by a much slower consumption of approximately 3 moles per mole. During the last twenty hours the rate of periodate consumption had decreased to 1 mole /mole. Extrapolation of this latter region of the curve to zero time gave a consumption of 9.0 mole/mole of glycopeptide.

Following destruction of excess periodate and reduction of the oxidation products with $NaBH_4$, excess

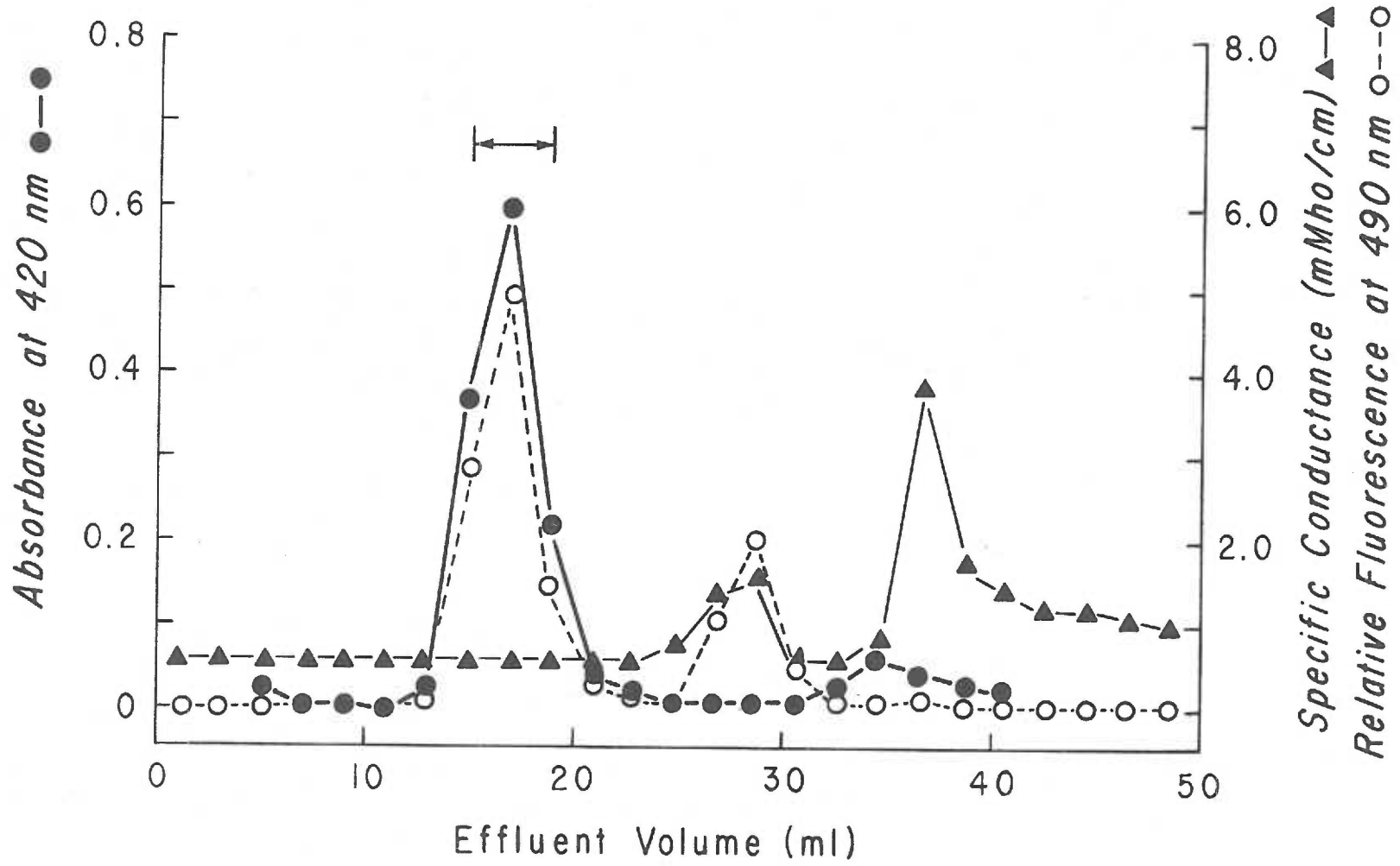


Figure 24. Bio-Gel P-2 chromatogram of the carboxypeptidase digest of glycopeptide VI-1: ●—● orcinol assay, o--o fluorescent ninhydrin assay, and ▲—▲ fraction specific conductance.

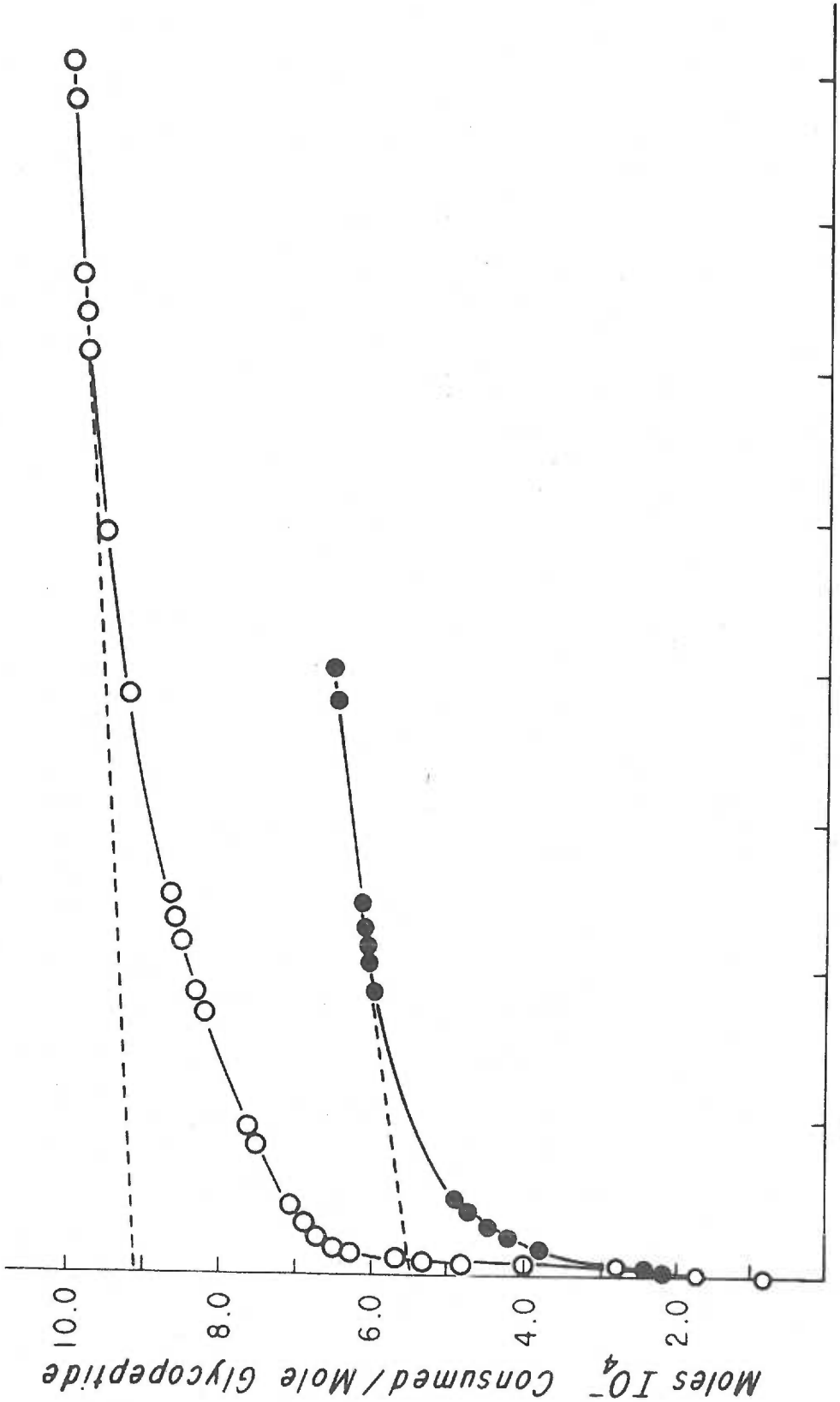


Figure 25. Periodate consumption in the first (o—o) and second (●—●) oxidations of glycopeptide V-3.

borohydride was destroyed with glacial acetic acid. The solution was evaporated to dryness and borate was removed by dissolution of the residue in methanolic-HCl and evaporation to dryness. The residue was dissolved in 1 ml of 0.1 N HCl and incubated for 18 hours at 25° C to hydrolyze the oxidized fragments from the glycopeptide. Chromatography of the hydrolysate on Bio-Gel P-2 produced the profile in Figure 26.

The glycopeptide fraction pool (shown by the double arrow in Figure 26) contained mannose and glucosamine in a ratio of 2.3:2.0. However, the amount of mannose determined by gas-liquid chromatography accounted for only 40% of the orcinol positive material. The excessive orcinol value was suspected to have resulted from incomplete hydrolysis of monosaccharide fragments from the glycopeptide. The lyophilized P-2 glycopeptide fraction was rehydrolyzed in 1 ml of 0.1 N HCl for 2 hours at 80° C. Chromatography of the hydrolysate on Bio-Gel P-2 gave the profile in Figure 27. Fraction I contained approximately 40% of the recovered orcinol material while fraction II contained 60%. Fraction I contained 92% of the recovered mannose and glucosamine while fraction II contained 8%. Therefore, hydrolysis for 2 hours at pH 1 and 80° C

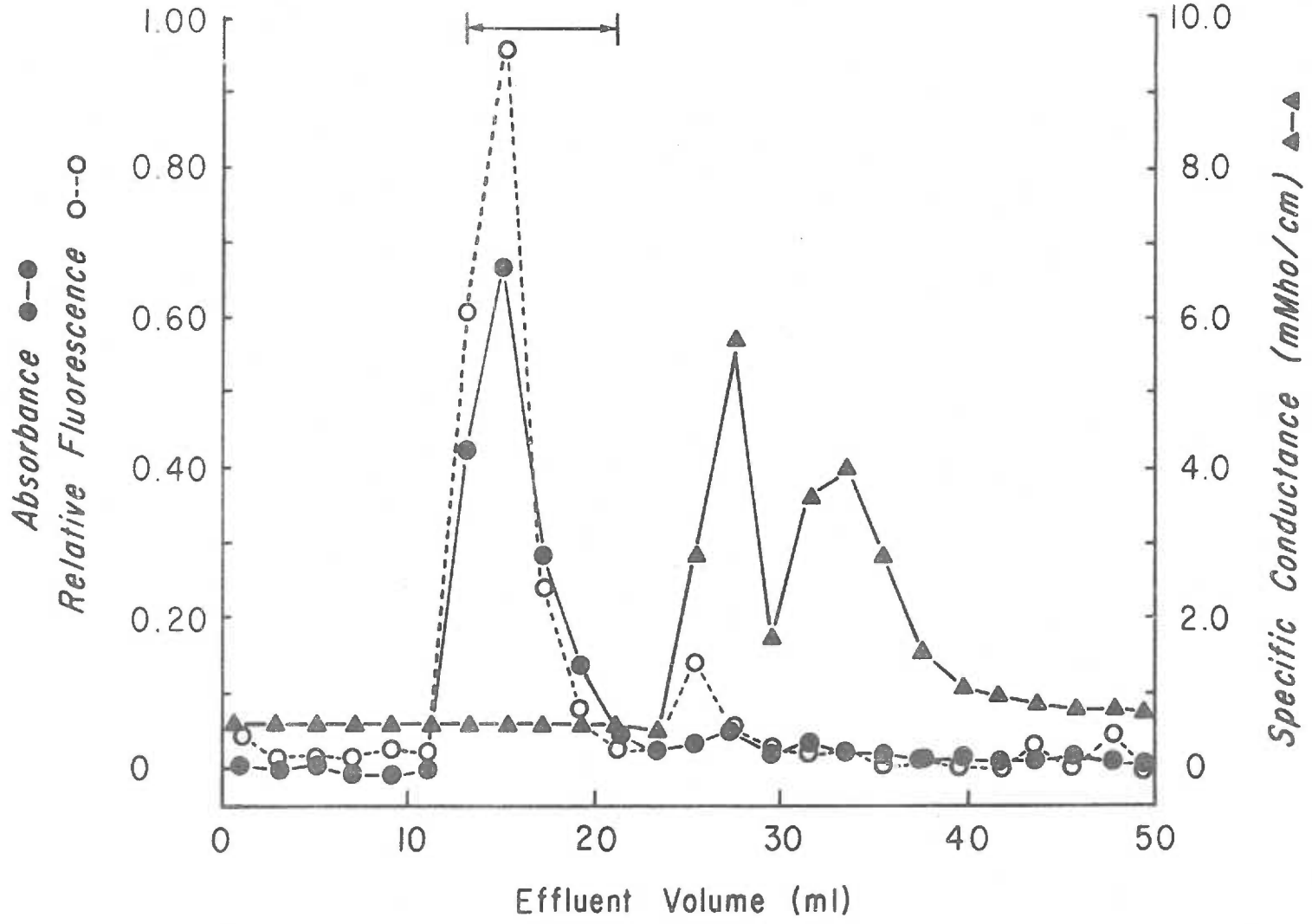


Figure 26. Bio-Gel P-2 chromatogram of the first periodate oxidation-borohydride reduction products of glycopeptide V-3 following mild acid hydrolysis (0.1 N HC1 for 18 hours at 25° C). Orcinol values are plotted at 10x the observed values. ●—● orcinol assay at 420 nm, o--o fluorescent ninhydrin assay at 490 nm, and ▲—▲ fraction specific conductance.

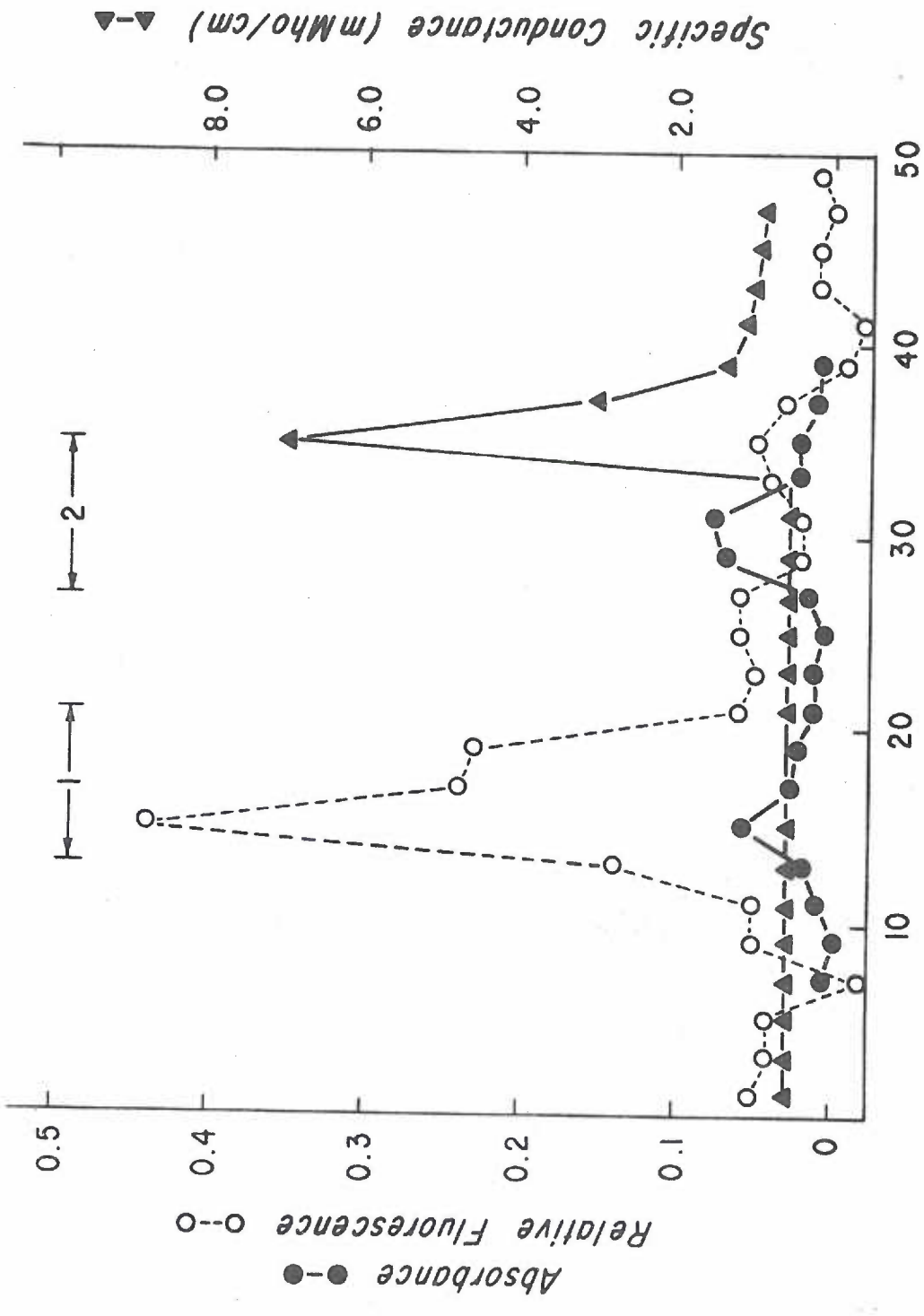


Figure 27. Bio-Gel P-2 chromatogram of the glycopeptide pool from Figure 26 following 0.1 N HC1 hydrolysis for 2 hours at 80° C: ●—● orcinol assay at 420 nm, o--o fluorescent ninhydrin assay at 490 nm, and ▲—▲ fraction specific conductance.

removed a substantial portion of the orcinol positive material which did not represent mannose, while only removing about 0.15 to 0.2 residues of each intact monosaccharide.

Fraction I contained 2.2 residues glucosamine and 2.1 residues mannose per mole glycopeptide. Thus, 5.6 residues of mannose were destroyed by the first oxidation.

Fraction I, containing 0.086 μmole of peptide, was oxidized a second time under the same conditions as those used in the first oxidation, except with a periodate concentration of 1.25 mM. The periodate consumption shown in Figure 25 rapidly reached 4 moles per mole of glycopeptide followed by a slower consumption of 2 moles per mole, until a slower but relatively constant consumption ensued at 24 hours. Extrapolation of this slow oxidation phase of the curve to zero time gave a consumption of 5.5 moles per mole. Following $NaBH_4$ reduction of the peptide and destruction of excess borohydride, the sample was desalted by Bio-Gel P-2 chromatography. The glycopeptide peak was evaporated to dryness and the residue was hydrolyzed in 1 ml of 0.1 N HCl for 2 hours at 80^{o} C.

Chromatography of the hydrolysate on P-2 gel gave the profile in Figure 28. Analysis of fraction 1 (Table XVII)

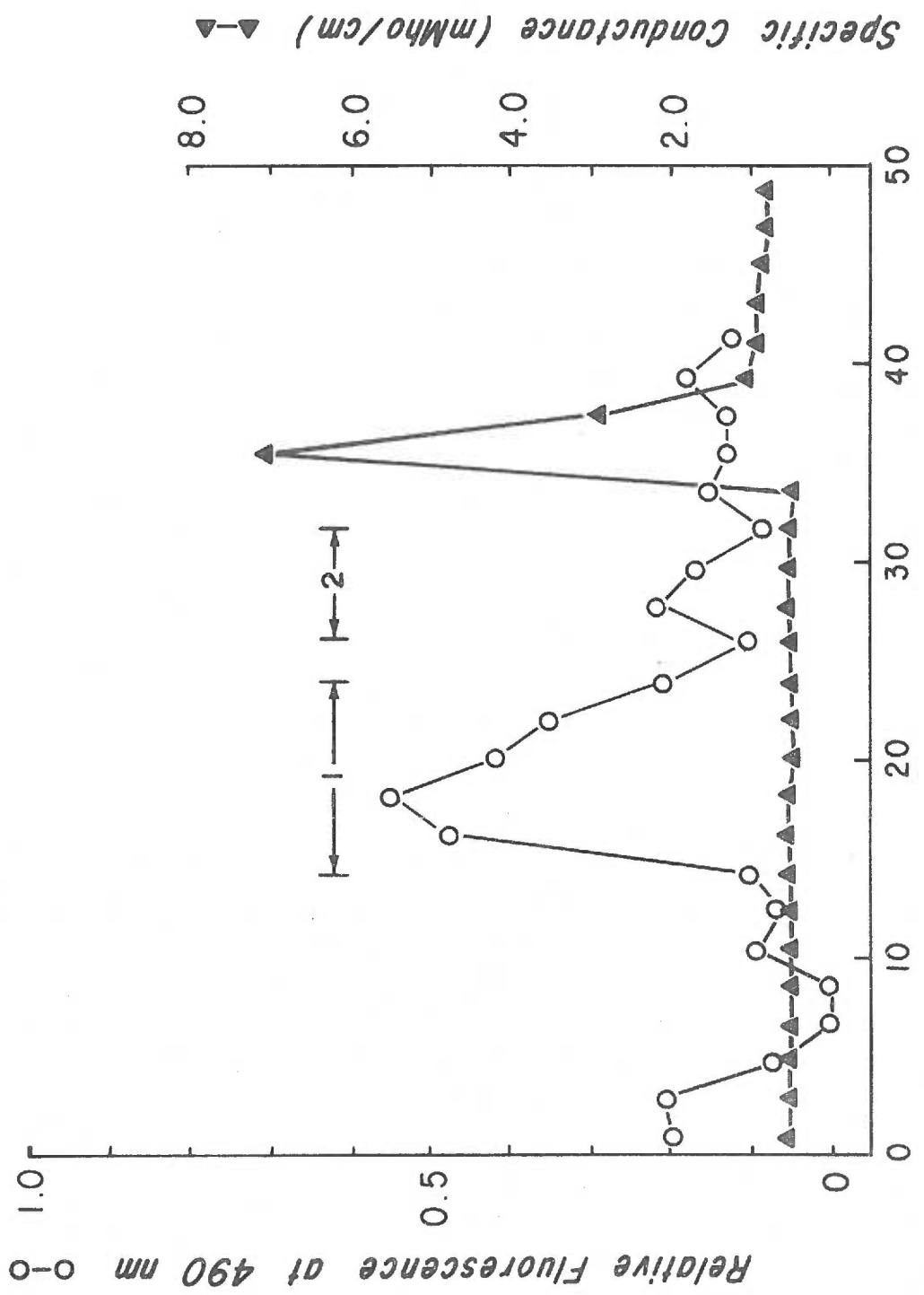


Figure 28. Bio-Gel P-2 chromatogram of the second periodate oxidation-borohydride reduction products of glycopeptide V-3 following 0.1 N HC1 hydrolysis for 2 hours at 80° C: o—o fluorescent ninhydrin assay at 490 nm and ▲—▲ fraction specific conductance.

showed a decrease of 1.6 mannose residue but no change in the glucosamine. Fraction 2 contained small quantities of amino acids and about 6% of the recovered glucosamine.

The results of the two periodate oxidations are summarized in Table XVII.

TABLE XVII. Summary of sequential periodate oxidation of glycopeptide V-3.

	Moles per Mole of Glycopeptide[a]		
	GlcN	Man	Periodate Consumed
Unoxidized	1.84 (2)	7.7 (8)	
1st oxidation[b]	2.0 (2)	2.3 (2)	
1st oxidation[c]	2.2 (2)	2.1 (2)	9
2nd oxidation	1.9 (2)	0.5 (0-1)	5.5

a. Figures in parentheses represent integral values.

b. Values for 0.1 N HCl hydrolysate (18 hours at 25° C) where glucosamine value assumed to be 2.0.

c. Values for 0.1 N HCl hydrolysate (2 hours at 80° C).

A third oxidation of an estimated 0.03 μmole of the product of the second degradation was attempted. The sample was evaporated to dryness and dissolved in 0.1 ml 2.5 mM $NaIO_4$ (4 fold excess) in pH 4.7, 0.025 M acetate buffer and incubated in the dark at 25° C for about 70 hours. Following periodate destruction, borohydride reduction, borohydride destruction and borate removal, the sample was analyzed for carbohydrate and amino acid contents as previously described. The data were poor due to instrument problems. The mannose: glucosamine ratio was 0.4:2 suggesting that little, if any, oxidation had occurred.

VIII. Periodate Oxidation of Glycopeptide VI-1

Carboxypeptidase-treated glycopeptide VI-1 (0.2 μmole) was oxidized with 5 mM periodate under the conditions previously described for the first oxidation of V-3. The course of periodate consumption is shown in Figure 29. Following the rapid consumption of 4 moles of periodate per mole peptide, 3-4 additional moles were slowly consumed. By forty eight hours, the consumption rate had decreased to 1 mole of periodate per mole per 80 hours. Extrapolation of the latter phase of the curve to zero time gave a periodate consumption of 7.1 moles per mole glycopeptide.

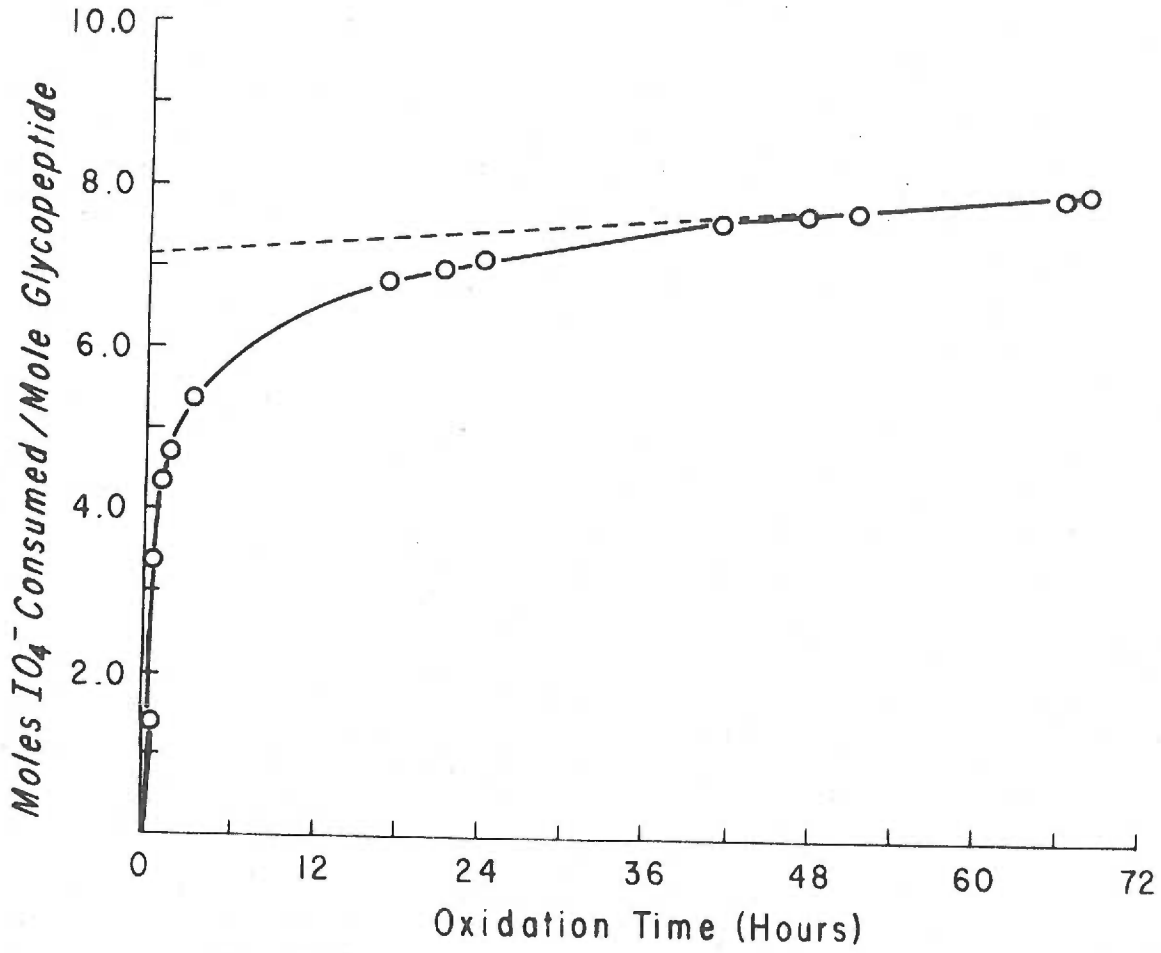


Figure 29. Periodate consumption in the first oxidation of glycopeptide VI-1.

After $NaBH_4$ reduction of the oxidation products and destruction of excess borohydride, the glycopeptide solution was desalted by P-2 gel chromatography. Orcinol assay indicated much more neutral carbohydrate than found by gas-liquid chromatography as was also observed in the oxidation of glycopeptide V-3. The glycopeptide composition was analyzed without further hydrolysis to remove fragments produced by oxidation. Table XVIII summarizes the carbohydrate composition before and after the first periodate oxidation. All of the fucose and xylose were oxidized. Two of the three residues of mannose were destroyed, while glucosamine was unchanged.

TABLE XVIII. Carbohydrate composition of glycopeptide VI-1 before and after periodate oxidation

	Residues/Mole		
Residue	Unoxidized	Oxidized	Integral Change
GlcN	2.02	1.92	0
Fuc	0.88	0	1
Xyl	0.88	0	1
Man	3.10	0.91	2

DISCUSSION

Carbohydrate Composition of PPHA

The isolation of PPHA from a mucoprotein complex (33) and the requirement for carbohydrate in cellular receptor sites for PHA, (62,63) suggest that carbohydrate contaminants may persist in purified PPHA preparations as a consequence of protein-carbohydrate interactions. Previous studies of the carbohydrate composition of PPHA were restricted to determination of the total neutral monosaccharide and hexosamine compositions of one preparation not subjected to gel chromatography (47) and to the identification of the monosaccharide constituents of a second preparation subjected to Bio-Gel P-200 chromatography (89,90). In this thesis the individual neutral carbohydrate and hexosamine compositions of various PPHA preparations are reported. These PPHA preparations differed either in their stage of purification, or in their source, i.e. bean lot. The data reported herein have certain structural implications and provide a basis for the comparison of PPHA with other PHA preparations reported in the literature.

The neutral monosaccharides in the examined PPHA preparations were identified in their reduced states as the acetylated derivatives of the reduced monosaccharides and were proposed to be the products of the parent compounds: mannose, fucose, arabinose, galactose, glucose and of an unknown compound. However, unequivocal identification of the parent monosaccharides is not possible by the exclusive examination of their reduction products, since reduction destroys one of the defining features of the parent compound. More than one parent aldose or ketose may yield the same "alditol" through reduction. For example, arabitol may result from the aldoses, arabinose and xylose, or from the ketoses, ribulose and xylulose. Similarly, a parent ketose may produce two "alditols", as in the reduction of fructose to mannitol and glucitol. Thus, confirmatory evidence is required to avoid erroneous identification and the possible resultant errors in quantitation. Such evidence was provided by the thin layer chromatographic demonstration of components with the mobilities of mannose, fucose, arabinose and xylose in acid hydrolysates of PPHA purified by P-200 chromatography (89,90). No galactose nor glucose were reported, possibly due to the low levels of these components in PPHA following gel chromatography.

The origin of the unknown component is not clear. It was indicated to be uncharged at pH 2. Its occurrence in some analyses of the whole protein and never in analyses of the glycopeptides suggested that it was an artifact. When observed, it represented about 4% or less of the total neutral carbohydrate, or roughly 2 residues per mole of PPHA. No relationship between the level of the unknown and the levels of the monosaccharides was observed. The disappearance of the unknown did not appear correlated with an appearance of additional fucose, xylose, or mannose (Figure 5b). However, the employed forty hour hydrolysis introduced an element of uncertainty, since both fucose and xylose were partially destroyed while mannose appeared unaffected by the longer hydrolysis time. Nevertheless, if the unknown was derived from fucose or xylose, its disappearance should have been correlated with a significant increase in fucose or xylose, which was not observed. If, however, it was derived from mannose, the expected change in the mannose level would have been statistically insignificant (vide supra).

To be considered a constituent of the PPHA molecule, a monosaccharide should comprise a relatively constant

proportion of the preparation composition as the preparation approaches purity, and it should be demonstrable as a major constituent of one or more glycopeptides. These criteria are met by mannose, glucosamine, fucose and xylose, but not by arabinose, galactose or glucose. The levels of arabinose and galactose were variable prior to Sephadex G-200 chromatography and were markedly reduced by chromatography to less than one residue per mole (Tables III and IV). Moreover, neither occurred at greater than trace quantities in any glycopeptides examined. Glucose was observed at variable levels of one residue or less per mole in most PPHA preparations and occasionally at trace levels in the glycopeptides. It was particularly suspect as a contaminant due to its widespread occurrence. Thus, the evidence supports mannose, glucosamine, fucose and xylose as being constituents of the PPHA molecule, while the bulk of arabinose, galactose, and glucose must be attributed to contaminants. The origin of glucose is not known, but the bulk of arabinose and galactose are accounted for by the carbohydrate-rich component, C-1, which elutes earlier than PPHA from Sephadex G-200. This component is probably the one referred to by Johnson as the polysaccharide contaminant which was removable by Bio-Gel P-200 chromatography,

and could represent the inactive polysaccharide associated with PPHA to form the mucoprotein originally isolated by Rigas (33).

The stereochemical configurations of the constituent monosaccharides and the state of glucosamine in PPHA were not determined in these studies. However, indirect evidence suggests that they occur as D-mannose, N-acetyl-D-glucosamine, L-fucose and D-xylose. These are the forms commonly found in glycoproteins. Puztai (130) characterized D-glucosamine and D-mannose as the major carbohydrate constituents in acid hydrolysates of materials extracted as proteins or glycoproteins from kidney beans. Roberts, Connor and Cetorelli (131) have demonstrated incorporation of ^{14}C-glucosamine into ethanol-insoluble materials in seedlings of corn, broad bean, and barley, and other plant tissues. Hydrolysis of these materials produced ^{14}C-glucosamine. Pronase hydrolysis of the materials from corn and sycamore produced "glycopeptides" rich in amino acids and glucosamine. Nitrous acid oxidation does not deaminate glucosamine in PPHA*, as would be expected if the amino group was not blocked. Mannose is suggested to occur as

* Streb, F. Personal Communication, 1971

α-D-mannopyranoside in non-reducing terminal positions by the binding of PPHA to concanavalin A (51). Soybean hemagglutinin, and ribonuclease B bind to concanavalin A to form precipitates (24) and the mannose residues of each may be removed from the glycopeptides with α-mannosidase (93,132). Yasuda, Takahashi and Murachi (133) released fucose from the pineapple stem bromelain glycopeptide with α-L-fucosidase; and Liao and Barber (134) demonstrated the conversion of GDP-D-mannose to GDP-L-fucose by an enzyme preparation from the leaves of green beans (Phaseolus vulgaris). Finally, xylose in the pineapple stem bromelain glycopeptide was released by β-D-xylosidase (133).

As noted in the "Introduction" heterogeneity in the oligosaccharide moieties of glycoproteins appears common. Thus, one of the basic objectives in examining PPHA prepared from different bean lots was to determine the variability of the carbohydrate composition of PPHA. The previously published values (47) of 8.02% neutral carbohydrate and 2.39% glucosamine were obtained with preparation 62-E-P_5 and agree well with the values, 8.46% and 2.45%, respectively, obtained in this study by different analytical techniques on the same preparation (Tables III and IV). However, the previously reported value of 19 glucosamine

residues per mole PPHA (47) was discovered to result from a calculation error, the elimination of which yielded a value almost identical to the value of 15.5 residues per mole reported here. This preparation had a higher neutral carbohydrate content than the later preparations which were further purified by Sephadex G-200 chromatography. However, this increase was wholly attributable to the contributions of arabinose and galactose. Thus, it is probable that 62-E-P_5 was contaminated with the polysaccharide component previously discussed, which would significantly alter its carbohydrate, but not necessarily its amino acid composition.

The mannose, glucosamine, fucose and xylose compositions of 62-E-P_5, 67-A-$P_8(C_4)_1$, 70-A-$P_8(C_4)_1$, and 72-A-$P_8(C_4)_1$, were relatively constant, even though these preparations were isolated from different bean lots (Tables III and IV). Glucosamine, fucose and xylose varied by less than one residue per mole. The mannose content did not vary significantly in three of the preparations, but was about 10% lower in 67-A-P_8. While this deviation is statistically significant, its real significance is questionable due to the anomalous appearance and questionable origin of the unknown component in the analyses of 67-A-$P_8(C_4)_1$.

Thus, until a significant difference in the mannose composition of 67-A-$P_8(C_4)_1$ is unequivocally demonstrated, the data support the view that PPHA prepared from red kidney beans by the procedure of Rigas and coworkers (33, 47,50), which includes gel-permeation chromatography on Sephadex G-200, has a characteristic qualitative and quantitative carbohydrate composition. Thus, the carbohydrate composition can be used as an additional criterion of purity and as a basis for comparison of PPHA with other preparations.

The carbohydrate composition of PPHA differs significantly from the compositions of various PHA's isolated from Difco PHA-P. Difco PHA-P is an impure commercially available PHA prepared by applying only the first steps of the method of Rigas and Osgood (33). Yachnin and Svenson (135) demonstrated at least 17 different bands by polyacrylamide disc gel electrophoresis at pH 4.5 of Difco PHA-P. From this preparation, mitogenic components with varying physical, chemical and biological properties have been isolated (55,60,135-137). Yachnin and co-workers (135,136) prepared high and low-erythroagglutination titer mitogenic components, H-PHAP and L-PHAP, respectively, by CM-Sephadex chromatography of Difco PHA-P. Both prepara-

tions had similar amino acid and qualitative carbohydrate compositions, but differed in quantitative carbohydrate composition: H-PHAP contained 2.3% by weight glucosamine, 4.76% mannose, 0.48% xylose and 0.38% fucose, or arabinose, for a total of 7.9%; and L-PHAP contained 2.10% glucosamine, 1.69% mannose, 0.17% xylose and 0.14% fucose or arabinose for a total of 4.1% (136). Employing similar techniques Weber and co-workers (60,137) prepared weakly and strongly erythroagglutinating mitogenic components similar to those of Yachnin and co-workers. No detailed carbohydrate composition data was given, except that the weakly-erythroagglutinating component contained 6.8% neutral carbohydrate, primarily as mannose, and 3.1% N-acetylglucosamine (137), in contrast to the values of Yachnin _et al_. Oh and Conard (55) isolated two mitogenic components, M-A and M-B, having low erythroagglutination potencies from Difco PHA-P by preparative disc-gel electrophoresis in 4 M urea at pH 9.0. Component M-A contained 1.15% hexosamine and 2.9% hexose, while M-B contained 2.0% hexosamine and 4.3% hexose. In contrast to PPHA, M-A had a histidine N-terminus and M-B had an asparagine.

The biological and physicochemical properties of PPHA more closely resemble those of H-PHAP and the erythroagglutinating mitogen of Weber. The hexosamine and fucose compositions of PPHA and H-PHAP are almost identical, but the mannose level of PPHA is significantly higher than that of H-PHAP, while the xylose level is lower. The neutral carbohydrate levels in L-PHAP are all lower than in PPHA, while the hexosamine is about the same. Interestingly, the carbohydrate composition of Weber's weakly-erythroagglutinating preparation does not resemble L-PHAP as much as it does H-PHAP and PPHA.

Weber (60) and Yachnin and Svenson (135) have proposed that H-PHAP is composed of two types of subunits while L-PHAP contains only one type, each molecule containing a total of four subunits. If H-PHAP is comparable to PPHA, then one would expect that there would be an average of two oligosaccharide chains per subunit (vide infra). These two oligosaccharides could be of the same or different types, i.e., a subunit could contain only mannose and glucosamine, or all of the above monosaccharides. If both oligosaccharide chain types, as described for glycopeptides V-3 and VI-1, occurred on the same subunit, then H-PHAP and L-PHAP should not differ in their carbohydrate

composition. Since this is not the case, as the results of Yachnin and co-workers indicate, one may suggest that the oligosaccharide containing mannose and glucosamine is located on one type of subunit, and the second one containing fucose, xylose, mannose and glucosamine is located on another type of subunit. However, the similarity of the weight ratios of the neutral sugars and the identical hexosamine compositions of H-PHAP and L-PHAP, suggest an alternative plausible explanation, namely, that the observed differences in their neutral carbohydrate compositions result from lack of completion of the carbohydrate chains, or from oligosaccharide degradation during the preparation of PHA-P. In this case identical polypeptide chains may carry either complete or incomplete oligosaccharide chains bound to identical points.

The various biological and chemical properties of these components in Difco PHA-P and their variable carbohydrate compositions suggest that the beans contain a number of mitogenic components which may display marked heterogeneity in their carbohydrate compositions. It would appear that the isolation procedures of Rigas and co-workers (33,47,50) select a highly homogeneous component which varies little in its carbohydrate composition. This may reflect the sensitivity of their isolation procedures to

differences in protein solubility which was indicated to be profoundly affected by the carbohydrate in the molecule. Periodate oxidation of PPHA significantly reduced its solubility, but whether the oxidation affected only the carbohydrate, and not amino acid residues as well, was not established.

Any conclusions based on comparisons between PPHA and these components must, however, be regarded with reservations. The preparative techniques and the precise source of Difco PHA-P are not known. A knowledge of these techniques is especially critical for the comparison of carbohydrate compositions since beans are a common source for glycosidases, especially mannosidase and N-acetyl-glucosaminidases (138,139). Contamination of a PHA preparation with these glycosidases would be anticipated to give substantial hydrolysis of the oligosaccharides when the solutions were at about pH 4 to 5, the typical range for exoglycosidase action. It is not possible to establish whether such degradations occur during PPHA preparation. However, if they did occur, it is to a very reproducible extent.

The location of the monosaccharides within the oligosaccharide chains of PPHA was partially revealed by

their rate of release by acid hydrolysis and their susceptibility to periodate oxidation. Nonreducing terminal monosaccharides are released by the cleavage of one glycosidic bond in contrast to nonterminal monosaccharides which require the cleavage of two or more. Thus, when the glycosidic bonds of the oligosaccharide are hydrolyzed at comparable rates, or when the individual bonds of the terminal residues are more rapidly hydrolyzed than those of the internal monosaccharides, the terminal sugars are the first to appear in the free form (123). As illustrated in Figure 5a, fucose and xylose are hydrolyzed very rapidly, in contrast to mannose. Glucosamine was not examined under comparable conditions. These data are consistent with those of the periodate oxidation of PPHA in Table V. Almost all of the fucose and xylose, 72% of the mannose, and 0% of the glucosamine were destroyed by a single oxidation cycle. Thus, fucose, xylose, and part of the mannose are indicated to be in the terminal regions of the oligosaccharides, while the remainder of the mannose and all the glucosamine may be in periodate resistant core regions adjacent to the polypeptide chain. Such resistance is typically expected when mannose and glucosamine are in such locations.

Glycopeptide Preparation and Purification

One of the more difficult facets of glycopeptide studies is the isolation of pure components. Often the methods and conditions used to purify peptides are not sufficiently sensitive to variations in carbohydrate structure to yield pure glycopeptides. Thus, the initial objectives of the glycopeptide studies were to modify or devise techniques for the preparation and isolation of pure glycopeptides in high yield from PPHA.

Pronase was selected as the proteolytic agent for its broad specificity in preference to a specific enzyme, such as trypsin. It offered the advantage of hydrolysis to small peptides and amino acids. Carbohydrate bound to a small peptide would significantly increase its molecular weight so that the glycopeptide could be easily separated from peptides and amino acids by gel permeation chromatography. Since the distribution of oligosaccharides in PPHA was unknown, glycopeptides containing large peptide chains could contain more than one oligosaccharide. Hydrolysis to small peptides minimizes this possibility. Finally, the amino acid constituent of the carbohydrate-protein linkage is more easily identified in a small peptide. The major disadvantages arise from: the possi-

bility of forming glycopeptides which have peptides of different lengths but which were products of the same region of the parent polypeptide; the problem of incorporating such short segments into the sequence of the whole protein; and the possible variation in enzyme composition of different lots of pronase.

Johnson (89,90) denatured PPHA for tryptic hydrolyses by boiling for thirty minutes. In the current studies, heating at about 85° C for thirty minutes produced protein suspensions which were difficult to handle and were still partially resistant to enzyme hydrolysis. Use of dissociating agents during protein denaturation gave protein preparations which were more easily handled and were very susceptible to enzyme hydrolysis. Thus, addition of SDS to the protein solution followed by heating at 90° C for thirty minutes rendered a solution in which the denatured protein was totally hydrolyzed by pronase to small peptides and glycopeptides. Guanidine hydrochloride (6 M) combined with heating for three hours at 90° C also produced a solution of denatured protein which, following elimination of guanidine by dialysis against 2 M urea, was also extensively hydrolyzed. It is probable that less rigorous conditions employing guanidine hydrochloride or other

dissociating agents, such as urea, would be equally effective for PPHA denaturation and would be of value in the preparation of tryptic glycopeptides.

Glycopeptides were obtained in high yield and purity from the pronase hydrolysates by chromatography on Bio-Gel P-6, on AG 50W-x2 at low pH and ionic strength, and on AG 1-x2 at pH 5.7. Chromatography on AG 50W-x2 at low pH has proven to be one of the most powerful tools in the separation of glycopeptides which vary primarily in their carbohydrate moieties (79,88). Glycopeptides from ovalbumin containing only asparagine and variable quantities of mannose and glucosamine were eluted in order of decreasing molecular size with pH 2.6 acetate buffer, 1 mM in Na^+ (87,88,97). Using pH 2.5, 0.2 M formic acid-0.002 M pyridine, McKelvy and Li (96) resolved an α-amylase glycopeptide mixture shown to be homogeneous by high voltage paper electrophoresis at pH 1.8 and 6.4. They observed similar elution profiles for glycopeptides containing aspartic acid and serine and for the same glycopeptide preparation from which serine had been removed, implying that the heterogeneity detected by the column resided primarily in the carbohydrate moieties. As in the case of ovalbumin, these glycopeptides contained only glucosamine and neutral carbohydrates.

Chromatography of glycopeptides from PPHA on AG 50W-x2 with 0.2 M formic acid-0.002 M pyridine resolved the mixture into several components. However, heterogeneity was still evident in peaks I, II, IV, and V, VII and VIII, based on non-integral ratios of their amino acid constituents. (Table VII). Chromatography on AG 1-x2 at pH 5.7 appeared more sensitive to variations in the peptide moiety since it resolved peaks IV and V into components with nearly integral ratios of amino acid residues (Tables X and XI).

Chromatography of crude glycopeptide mixtures from gel permeation chromatography on AG 1-x2 alone did not give satisfactory resolution of the glycopeptides, and recoveries were often of the order of 80% to 90%. However, there was no evidence indicating preferential loss of specific glycopeptide components on the column. The losses were probably unspecific and arose from the constant pH and ionic strength conditions, required for good resolution on the 0.63 x 70 cm column, which broadened the elution profiles to the point where minor components and the tails of all components approached the limits of the detection system. In the future, both resolution and recovery of the glycopeptides might be improved by using a longer column,

such as 0.9 x 150 cm, with an elution gradient such as illustrated in Figure 11b or 12a.

Chromatography on Dowex 50W-x4(K^+) with water development has been used by Saunders (98) to separate oligosaccharides, monosaccharides, and various monosaccharide derivatives. Such sensitivity to carbohydrate structural variation suggested that this system is potentially useful in the separation of glycopeptides. Preliminary experiments (Figure 12 and Table VI) supported this speculation in that chromatography with water resolved PPHA glycopeptides into 4 major peaks. Rechromatography with 50% ethanol further resolved the first three peaks, but resulted in the early elution of only one peak in low yield from the last peak. The compositions of the peaks (Table VI) suggested that this technique is applicable to the separation of glycopeptides containing fucose and xylose from those devoid of these two sugars and could be of limited usefulness in the preparation of glycopeptides. It was not routinely employed, however, since it did not appear to improve upon the purification of glycopeptides achieved with AG 50W-x2 chromatography at low pH and ionic strength.

The chromatographic procedures employed to prepare the glycopeptides used in the structural studies were sensitive to variations in molecular size, as well as carbohydrate and amino acid structure. They were straightforward technically and amenable to automated analysis of the column effluents. The isolated peptides which were characterized, V-2, V-3, and VI-1, appeared highly purified as judged by their compositions, behavior during Edman degradation, and their monodispersity in the ultracentrifuge.

Glycopeptide Characterization

Earlier studies of PPHA (33,53) suggested that the carbohydrate was distributed among several oligosaccharide moieties. As noted in the "Introduction", polyacrylamide-gel-electrophoresis of PPHA in 8 M urea at pH 8.6 produced at least eight prominent bands which were stained by both protein and carbohydrate stains, which suggested that all of the subunits of PPHA contain carbohydrate. Johnson's isolation of different tryptic glycopeptides, each containing about two glucosamine residues, further supported such a distribution (89,90). The absence of fucose and xylose in one of the tryptic glycopeptides, $GP\text{-}B_{1\&2}$, further suggested that more than one type of oligosaccharide occurred in PPHA. Unfortunately, further interpretation

of his results was hindered by the lack of quantitative carbohydrate composition data for whole PPHA and the tryptic glycopeptides.

As discussed earlier, PPHA contains an average of 71 monosaccharide residues per mole, totalling a molecular weight of about 12,000. The observed low levels of fucose and xylose (4 residues of each per mole of PPHA) would be limiting if the monosaccharides were distributed among more than four oligosaccharides. Thus, in such a case these residues would be found in some glycopeptides, but not others, as observed by Johnson (89,90).

The properties of the pronase glycopeptides indicated that PPHA contains 7 to 10 oligosaccharide chains ranging from about 1200 to 1700 in molecular weight, each consisting of 7 to 10 monosaccharide residues. All of the glycopeptides were eluted as a single peak during gel permeation chromatography through either Bio-Gel P-6 (Figure 8) or Bio-Gel P-2 (Figure 9), thus suggesting a relatively narrow molecular weight range for the whole glycopeptides. The AG 50W-x2 fraction compositions (Table VII) showed that the glycopeptides all contained mannose and glucosamine. Their total carbohydrate contents were approximately equal by weight (70 to 80%), establishing that the weights of the

carbohydrate moieties as well as those of the whole glycopeptides covered a relatively narrow range. These inferences were supported by the measurements of the molecular weights of the glycopeptides VI-1, V-2, and V-3, which appeared to represent the molecular weight extremes based on the compositions of their AG 5W-x2 parent peaks (Table VII). Glycopeptide VI-1 contained carbohydrate totalling about 1200 molecular weight and consisting of seven monosaccharide residues, while glycopeptides V-2 and V-3 each contained an oligosaccharide of about 1700 molecular weight, containing 10 monosaccharide residues.

The numbers of oligosaccharide chains per mole PPHA represented by the major AG 50W-x2 fraction pools IV, V, and VI, were estimated from the neutral carbohydrate recoveries listed in Table VII. Although each fraction pool was subsequently resolved into more than one component on AG 1-x2, the compositions of these AG 1-x2 components established that each of the pools contained predominantly one type of carbohydrate unit. The data of Table VII shows that pool IV contains the recovered equivalent of 7 residues of neutral monosaccharides per mole PPHA; pool V, 8 residues; and pool VI, 10 residues. Since the pools IV and V both contain 7 to 8 residues of mannose per mole of

glycopeptide, collectively they account for two oligosaccharide moieties composed of 2 glucosamine and about 8 mannose residues. The predominant oligosaccharide in pool VI contained 5 neutral monosaccharide residues (1 fucose, 1 xylose, 3 mannose, and 2 glucosamine). Therefore, two such chains are represented in pool VI. Thus, these four oligosaccharides account for about 50%, or 26 neutral monosaccharide and 8 glucosamine residues, of the total of 56 neutral carbohydrate and 15 glucosamine residues in PPHA. These values should be considered as estimates, but it is interesting to note that four each of the two oligosaccharide types would total 4 fucose, 4 xylose, 44 mannose, and 16 glucosamine residues per mole of PPHA. The parent PPHA preparation, 67-A-$P_8(C_4)_1$, contained 4 fucose, 4 xylose, 44 mannose, and 15 glucosamine residues per mole.

The occurrence of several oligosaccharide chains in PPHA raises the question of whether their modes of linkage to the polypeptide chain and their peptide linkage region sequences are the same or different. Johnson found different amino acid compositions for all five of the tryptic glycopeptides he examined, suggesting that at least five of the eight tryptic glycopeptides had different amino acid sequences (89,90). However, as will be discussed later,

evidence suggests that the amino acid sequence very near the carbohydrate linkage site may act as a recognition site for the enzymes which add the carbohydrate to the peptide chain. Thus, while the tryptic glycopeptides may differ in their amino acid sequences, the further removal of amino acids by pronase might be expected to give fewer unique amino acid sequences for the "recognition" regions immediately adjacent to the carbohydrate-protein linkage sites.

This expectation was apparently supported by the compositions of the AG 50W-x2 components (Table VII). Where Johnson's tryptic glycopeptides each contained significant quantities of 9 to 11 types of amino acid, the partially purified pronase glycopeptide fractions each contained predominantly two or three of a total of six types of amino acids observed. Aspartic acid, threonine, and glutamic acid predominated in the acid hydrolysates. The observed levels of serine, glycine and alanine were low and of questionable significance. All of the fractions contained aspartic acid, threonine, mannose and glucosamine, but there were significant differences in the distributions of fucose, xylose, and glutamic acid. Glutamic acid content appeared negatively correlated with fucose and

xylose content. It thus appeared that oligosaccharide chains containing fucose and xylose may be bound to linkage regions devoid of glutamic acid.

In pursuit of this possibility, the two major AG 50W-x2 peaks having the greatest compositional differences, V and VI, were further purified, and their major components sequenced. The two glycopeptides, V-2 and V-3, isolated in approximately equal proportions from V, had the amino acid sequences, Asx-Glu-Thr-Asx and Asx-Glu-Thr, respectively. The oligosaccharide in each was bound to the N-terminal Asx residue, probably via an N-glycoside linkage (vide infra). This linkage would account for one mole of NH_3 per mole of peptide. The acid hydrolysates of V-2 contained approximately one more residue each of aspartic acid and NH_3 than did V-3 (Table X), and no PTH-Gln was obtained in the Edman degradation (Tables XIII and XIV and Figures 20 and 21). Therefore, it was concluded that the C-terminal residue in V-2 was Asn. Glycopeptide VI-1, which represented the bulk of peak VI, had the amino acid sequence Asp-Asx-Thr-Thr, as explained under results. Thus, three glycopeptide amino acid sequences were established which were derived from at least two unique parent linkage region sequences.

The tetrapeptide, V-2, and the tripeptide, V-3, could have resulted from different degrees of hydrolysis of the singular parent amino acid sequence, -Asx-Glu-Thr-Asx-, or from two different parent sequences. Peak IV was also resolved into two major components, IV-2 and IV-3, in the same proportions as V-2 and V-3. The amino acid and carbohydrate compositions of IV-2 and IV-3 were remarkably similar to those of V-2 and V-3, respectively. It is, therefore, possible that they could have been derived from the same parent amino acid sequences. The compositions of AG 50W-x2 peak fraction 3, of glycopeptide IV-1 and of glycopeptide VI-2, are compatible with their representing all or part of the amino acid sequence of VI-1. However, the AG 50W-x2 peak fractions 7 and 8 contained intermediate quantities of threonine and glutamic acid. These represent glycopeptide mixtures, as judged by their compositions. Nevertheless, their compositions are compatible with the hypothesis that they may be mixtures of glycopeptides derived from the two unique sequences listed above. Although such speculations suggest that only two parent linkage region amino acid sequences may account for the observed glycopeptide sequences and compositions, additional

sequence data will be needed for all the glycopeptides before any definite conclusions may be reached in this regard.

The major amino acid constituents, aspartic acid, threonine, and glutamic acid, of these glycopeptides all contain side chain functional groups through which an oligosaccharide chain could be bound. As described in the "Introduction", aspartic acid, in the form of asparagine, and threonine, but not glutamic acid, are already established linkage sites in other glycoproteins. Thus, all the glycopeptides contain two amino acid residues commonly found as linkage sites.

The linkage sites in glycopeptides V-2 and V-3 were demonstrated to be the N-terminal aspartic acid residues, which probably occur in the amide forms as will be discussed below. In both cases, the first Edman degradation destroyed about 0.75 residues of aspartic acid, forming no ethyl acetate extractable PTH-derivatives as would be expected if the N-terminal Asx were not bound to carbohydrate. Moreover, in both instances a high molecular weight carbohydrate containing compound, with UV absorption properties characteristic of PTH-amino acids, was separated from the expected peptide fragment produced by the cleavage

reaction. Thus, the data showed that the carbohydrate occurred as a single oligosaccharide linked to the N-terminal Asx residue through its sidechain, since the amino and carboxyl groups must be blocked by the Edman degradation, in order for peptide cleavage to occur and produce the observed products.

The linkage site in VI-1 was indicated to be the penultimate Asx residue, but the evidence is not as compelling as for glycopeptides V-2 and V-3. The first Edman degradation removed the N-terminal Asx residue and formed an extractable PTH-derivative with the mobility of PTH-Asp. In the second step, the reaction appeared to remove an additional 0.6 residue of Asx, but only traces of PTH-Asp were detected by thin layer chromatography. It was thought that the second Edman degradation may have attacked the linkage site. To investigate this possibility, the products of the reaction were chromatographed on Bio-Gel P-2. The chromatogram (Figure 23) showed two orcinol positive peaks, the major one in the region expected for the parent glycopeptide, and the minor one in the monosaccharide region. The major orcinol peak was superimposable on a UV absorbance peak characteristic of PTH-derivatives. It appeared, therefore, that this peak repre-

sented the carbohydrate linked to an Asx residue which had been cleaved from the peptide by the second Edman degradation, leaving a chromatographically separable dipeptide, Thr-Thr. Surprisingly, however, the composition analysis showed that no such separation had occurred, as about 90% of the loaded aspartic acid and threonine were recovered in the major peak with their ratios unchanged. Thus there was no separable peptide cleavage product at this step, as was observed for glycopeptides V-2 and V-3, suggesting that either the expected cleavage reaction did not proceed or that the products were still chemically bound together.

In order to check for a carbohydrate bond to the penultimate threonine residue, a sample of the whole glycopeptide was treated with alkali (Table XVI). Incubation of the glycopeptide in 0.4 N NaOH at 30° C for 24 hours produced no change in the threonine content, whereas treatment with 0.1 N NaOH - 0.4 M $NaBH_4$ for 48 hours at 25° C destroyed about 0.27 residues of threonine. This loss was of a questionable nature, however, since these conditions also produced a loss of 0.2 threonine residues in glycopeptide V-2, which also contains a nonterminal Thr residue, but in which the oligosaccharide was demonstrated to be bound to the terminal Asx residue.

To determine if the linkage site was the C-terminal threonine, the whole glycopeptide was digested with carboxypeptidase A. Following gel filtration on Bio-Gel P-2 (Figure 24), the early eluting orcinol positive peak contained 2.0 Asx and 1.05 Thr, i.e., 0.9 residues of threonine had been removed leaving an apparent tripeptide to which the carbohydrate was bound. Thus, the removal of the N- and C-terminal amino acid residues, and the failure of alkali to destroy the internal threonine residue pointed to the penultimate Asx as the linkage site.

The reason for the failure of the Edman degradation in the case of glycopeptide VI-1, in contrast to V-2 and V-3, is obscure. There are, however, a number of differences between VI-1 and the other two peptides. In addition to the differences in amino acid sequence, glycopeptide VI-1 contained a different type of oligosaccharide chain which was much more labile than those of V-2 and V-3 (Figure 23). The usual problems encountered in the removal of N-terminal Asx residues do not explain the observed behavior. Cyclization of Asx to form an imide involving the β-carboxyl group was observed by Bornstein (140) in peptides from collagen. Under alkaline conditions such structures are hydrolyzed preferentially to give a peptide

bond to the β-carboxyl group (114). The structures of the rearranged peptide or the parent imide would allow the coupling reaction, but would prevent the cleavage reaction. However, since the carbohydrate appeared to be bound to the β-carboxyl carbon, such a rearrangement would be expected to free the oligosaccharide. The resulting PTH-peptide, being aromatic and much smaller than the carbohydrate moiety, would have been eluted from Bio-Gel P-2 much later than the carbohydrate, in contrast to the observed behavior. Furthermore, the indicated loss of 0.6 threonine residues in the third degradation step agreed well with the value of 0.6 Asx residues removed in the second step, and suggested that a PTH-derivative was formed in the second step, but for some unknown reason, the products remained chemically linked.

The carbohydrate linkage to the sidechain of an Asx residue most likely represents an N-glycosidic linkage in which the amide group of Asn is bound directly to C-1 of the terminal reducing monosaccharide of the oligosaccharide. The following observations support this view. Firstly, all the well characterized carbohydrate-peptide linkages are glycosides, i.e., they involve C-1 of the reducing terminal monosaccharide (70,79). Thus, if the oligo-

saccharides in PPHA were O-glycosidically bound to the β-carboxyl of aspartic acid, the linkage would be of the glycosidic ester type, which is anticipated to be quite labile. In this regard, Inch and Fletcher (141) observed that the glycosidic esters, 2-acetamido-1-O-acetyl-3,4,6-tri-O-benzyl-2-deoxy-β-D-glucopyranose and the corresponding 1-O-benzoyl-derivative, were 50% and 100% hydrolyzed, respectively, after 70 hours in aqueous dioxane at 50° C, while the corresponding α-anomers were 5-10% hydrolyzed. This lability under such mild conditions makes it doubtful that such linkages would survive the isolation procedures, i.e., the low pH of about 1, that is employed in the isolation of PPHA, the heat denaturation of the protein at about pH 5, and especially the prolonged exposure of the glycopeptides to pH 2 conditions on the AG 50W-x2 column. The effectiveness of AG 50W-x2 as a catalytic surface for hydrolytic reactions is shown by its ability to extensively hydrolyze proteins in 0.01 N HCl at 100° C under the conditions described for the gas-liquid chromatography of monosaccharides.* Furthermore, the acid hydrolysates of the

* Lehnhardt, W.F. Personal Communication, 1969.

glycopeptides VI-1, V-2, V-3, IV-1, IV-2, and IV-3, all contained significant levels of NH_3, the probable sources of which were either free amides, or amides involved in an N-glycosidic linkage. The only possible source of amide-N in glycopeptides VI-1, V-2, and V-3 was asparagine, since glutamic acid was either absent, or it was shown to be in the acid form as in V-2 and V-3. Finally, whenever carbohydrate has been shown to be bound to the aspartic acid side chain, further chemical characterization has always shown the bond to be an N-glycoside (70,79).

Only one monosaccharide, N-acetyl-glucosamine, has been found N-glycosidically bound to aspartic acid in glycoproteins (70,79). It was thus anticipated that N-acetyl-glucosamine would be the carbohydrate constituent of the linkages in glycopeptides V-2, V-3, and VI-1. The nature of the carbohydrate constituent of the linkage and the general features of the oligosaccharide sequence of glycopeptide V-3 were examined by sequential periodate oxidation. Schematically, the two steps of the sequential oxidation proceeded as follows:

$Man_8GlcNAc_2$-Asn-Glu-Thr

↓ 9 IO_4^-

$Man_2GlcNAc_2$-Asn-Glu-Thr

↓ 5.5 IO_4^-

$Man_{0.5}GlcNAc_2$-Asn-Glu-Thr

In the first degradation step the consumption of 9 moles IO_4^- per mole of glycopeptide destroyed six mannose residiues.* Since the maximum consumption per mole of mannoside residue is 2 moles, three mannose residues consumed 2 moles each of periodate, while the other three each consumed one. Thus, as outlined under "Methods", three mannose residues had unblocked C-2, C-3, and C-4 positions, while the remaining three were obligatorily blocked at either C-2 or C-4.

* This result was duplicated at 5° C in the dark using the Avigad periodate assay.

In the second degradation step the consumption of 5.5 moles of periodate per mole of glycopeptide destroyed 1.5 mannose residues. This consumption exceeds the theoretical maximum for the mannose destroyed by 2.5 moles/mole. The reason for such a high consumption is not apparent. It may have arisen from sample contamination. For example, 35 μg of cellulose fiber could have given the excess consumption. However, an earlier experiment gave an extrapolated consumption of 4.2 moles.*

The structural interpretation of these results is further complicated by the residual 0.5 residue of mannose following the second degradation. This mannose could have represented theoretically oxidizable mannose which was incompletely oxidized, or resistant residues, possibly arising either from linkage isomerization, or from a mixture of glycopeptides containing different numbers of mannosyl residues. For example, if the latter case was true, the two mannose residues remaining after the first degradation step could have been the average of one residue on one oligosaccharide and three on another.

* The conditions were similar to those listed above, except (IO_4^-) = 1.0 mM. GlcNAc was unaffected, but a shortage of material prevented the assay of mannose.

Incomplete oxidation is plausible at the low periodate concentrations used (1.25 mM), especially if a contaminant competed with the peptide for the periodate which was at an estimated four fold excess relative to mannose. Yasuda, Takahashi and Murachi (133) noted that oxidation of the pineapple stem bromelain glycopeptide in 5 mM periodate at pH 4.5 for 20 hours at 5° C in the dark did not completely oxidize the mannose, fucose, and xylose residues. When the concentration of periodate was increase to 25 mM and its ratio to the peptide doubled, these residues were completely oxidized. However, in these studies, arabitol in 5 mM periodate (5 fold excess) at pH 4.7 and 25° C in the dark consumed 96% of the theoretical amount of periodate in 45 minutes; and glucitol consumed 95% of the theoretical amount under the same conditions in 5 hours. Unfortunately, no standards were oxidized in 1.25 mM periodate.

Linkage isomerism of the mannose residues in oligosaccharides with the same composition could produce the observed results, if for example, half of the oligosaccharides contained the structure Man-(1→2,4,or 6)-Man-$GlcNAc_2$-peptide, while the remainder were Man-(1→3)-Man-$GlcNAc_2$-peptide. Both mannose residues in the former would be susceptible to oxidation, while only the terminal residue

would be oxidized in the latter. This possibility remains open, especially since it is questionable whether the AG 50W-x2 chromatography would be sensitive enough to this variation to accomplish the separation of such closely related glycopeptides.

The third possibility was suggested by the apparent heterogeneity of the glycopeptide on Bio-Gel P-2 (Figures 26, 27 and 28) following the first periodate oxidation. The early eluting glycopeptide peak in each case had a trailing shoulder. The occurrence of this shoulder in all three chromatograms and in both the fluorescent ninhydrin and orcinol traces in Figure 26 indicated that it was not the result of assay errors.

Materials eluting in such a shoulder would be expected to either have lower molecular weights or a greater tendency to adsorb to the gel than the major components eluting slightly earlier. Heterogeneity in molecular weight could result from variations in the amino acid or the carbohydrate moieties. If parts of the peptide had been hydrolyzed from the oligosaccharide during the 80^{o} C hydrolyses, one would expect variations in the ratios of amino acid constituents or increases in the glucosamine and mannose to peptide ratios. These variations were less than 10% and

were of questionable statistical significance because of the small amount of material analyzed. Since the elution volumes of the components were less than the observed 26 ml for raffinose (mol. wt. 500), the apparent molecular weight of the shoulder material in Figure 28 would be significantly greater than that of the peptide moiety (about 450). Therefore, the major and minor components would both be expected to contain carbohydrate. Since at this step, there remains only 2.5 residues of Man and GlcN per mole of peptide, it is questionable whether it is possible to have sufficient heterogeneity in their levels to give the observed chromatographic behavior.

Regardless of the nature of the residual mannose following the second periodate oxidation, one point is notable. There is not enough residual mannose in the twice degraded peptide to preclude the conclusion that GlcNAc is one of the carbohydrate constituents, if not the sole constituent, of the carbohydrate-peptide linkage. Moreover, a core structure of $GlcNAc_2$ is consistent with the data. Thus, the sequential periodate degradation of glycopeptide V-3 confirms that GlcNAc is a constituent of the carbohydrate-peptide linkage. Consequently, it is proposed

that the linkage is 2-acetamido-N-(4-L-aspartyl)-2-deoxy-β-D-glucopyranosylamine as described for ovalbumin and other glycoproteins (70,79).

Assuming, however, that the residual mannose was susceptible, but was incompletely oxidized, the following oligosaccharide structural features would be supported. Destruction of both mannose residues in the second oxidation requires that neither had blocked C-3 sites. Since these two residues were resistant to oxidation in the first step, each must have been protected in that step by the six oxidizable mannose residues. This protection could result from a single substitution on each at C-3, or from two substitutions at C-2 and C-3, C-3 and C-4, or C-2 and C-4, where the C-3 site is unblocked by the first oxidation. However, the maximum number of oligosaccharide substituents is limited to three by the results of the first oxidation.

The resistance of both GlcNAc residues to the second oxidation and their elution with the peptide from Bio-Gel P-2 (Figure 28) following 0.1 N HCl hydrolysis supports a $GlcNAc_2$-peptide core structure. GlcNAc is made resistant to periodate oxidation by a single block at C-3 or C-4. Taking into account the resistance of both GlcNAc residues and the susceptibility of both mannose residues in the

second degradation, one may write three structural formulae for the oligosaccharide core which differ in the monosaccharide to which one mannose residue is bound:

a) Man-(1→3, or 4)-GlcNAc-GlcNAc-NH-Asp-Glu-Thr
                                |
                               Man

b) Man-(1→3, or 4)-GlcNAc-(1→3, or 4)-GlcNAc-NH-Asp-Glu-Thr
                          |
                         Man

c) Man-(1→3, or 4)-GlcNAc-(1→3, or 4)-GlcNAc-NH-Asp-Glu-Thr
 |
(1→2, 4, or 6)
 |
Man

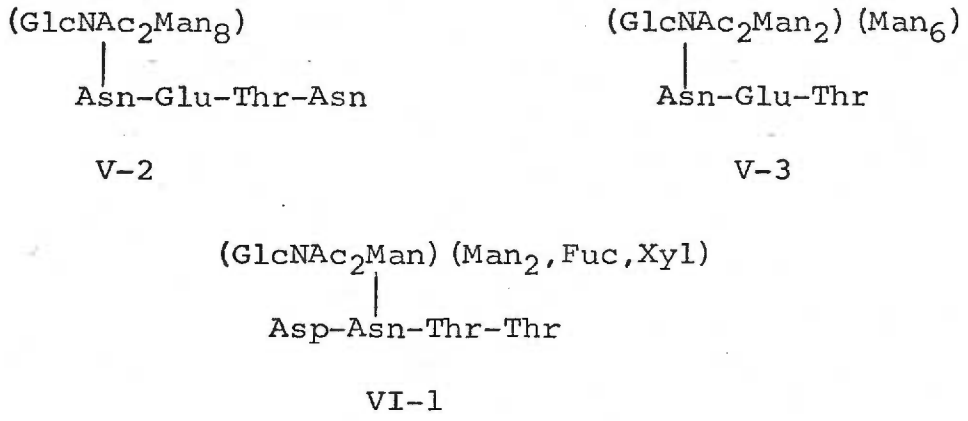
One mannose must be bound to C-3 or C-4 of the GlcNAc penultimate to the peptide to confer the observed resistance to oxidation. The second mannose could be bound to any of the other three monosaccharides, the only excluded site being C-3 of the GlcNAc-bound mannose as shown in c), which would make the penultimate mannose resistant to oxidation. Structure c) is the core structure reported for the glycopeptides of pineapple stem bromelain (133), taka-amylase (142), bovine ribonuclease B (132), and hen egg albumin (87),88,97).

Glycopeptide VI-1 was only oxidized once with periodate and reduced with borohydride. It was not subsequently hydrolyzed with 0.1 N HCl. Fucose, xylose, and 2 out of 3

of the mannose residues were oxidized with the consumption of 7 moles of periodate per mole peptide. Thus, of the four residues destroyed, three of them were not blocked at C-2, C-3, or C-4, while the other was blocked at either C-2 or C-4. Therefore, as in the case of glycopeptide V-3, VI-1 has also a maximum of three nonreducing terminal monosaccharide residues. The rapid release of fucose during acid hydrolysis of PPHA as discussed earlier, and the observation that fucose has always been situated in nonreducing terminal positions in other glycoproteins (80), suggest that fucose is one of the terminal residues.

Since the oxidized peptide was not hydrolyzed, it is not possible to establish which, if any, of the remaining monosaccharides is bound directly to the peptide. The data are, however, consistent with the expected behavior of an oligosaccharide core region consisting of one mannose and two GlcNAc residues, and consequently with the hypothesis that GlcNAc is bound directly to the peptide chain.

On the basis of the Edman degradations, periodate oxidations, and other discussed evidence, the following structural formulae are proposed for glycopeptides, V-2, V-3 and VI-1:



V-2

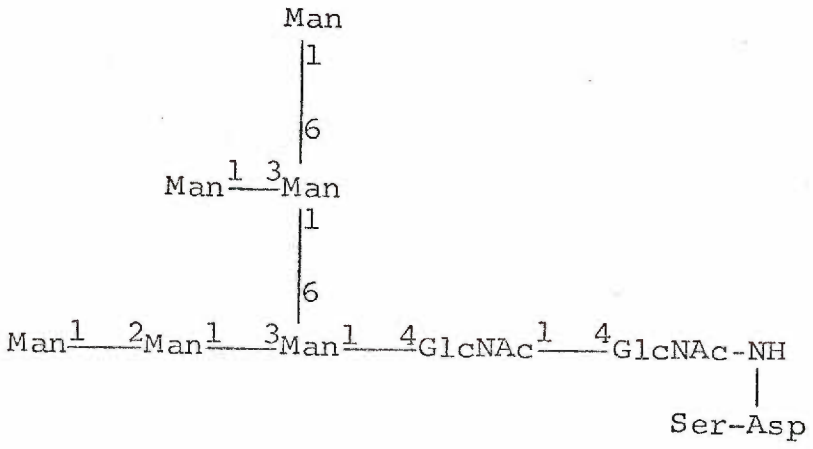
V-3

VI-1

As mentioned earlier, the monosaccharide composition of PPHA agrees well with that expected if it contained four of each of the two oligosaccharide chain types found in glycopeptides V-3 and VI-1. If such were the case, it would be predicted that periodate oxidation of PPHA would consume 64 moles of periodate per mole of protein and destroy all but 12 mannose and 16 GlcNAc residues. This prediction is in fair agreement with the observed 54 mole consumption in 5.5 hours which left 13.5 mannose and 15.3 GlcNAc residues intact.

Full elucidation of an oligosaccharide structure becomes quite complex when it contains several monosaccharides of the same type. The proposed structures on page 204 for the carbohydrate core of glycopeptide V-3 could be relatively easily distinguished given an

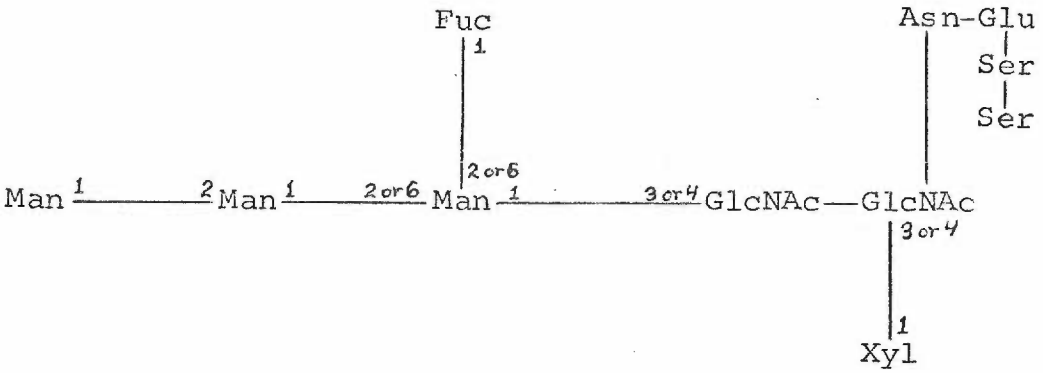
adequate quantity of material. Methylation of all the free hydroxyl groups followed by hydrolysis and identification of the methylated monosaccharides would reveal the blocked positions on each monosaccharide. Complete structural characterization of the glycopeptide would be possible employing monosaccharide-specific exoglycosidases, which cleave glycosidic bonds of a specified configuration; methylation studies as noted above; and sequential periodate oxidation, in which the oxidation fragments are identified and quantitated. The combination of these techniques was illustrated in the sequence studies of the single oligosaccharide chain in taka-amylase (142). The complete structure of this glycopeptide oligosaccharide, which is very similar to the indicated structure of glycopeptide V-3, with the exception of two less mannose residues, was proposed to be:



in which the mannoside linkages are α-anomeric and the GlcNAc linkages are β-anomeric. This structure is degraded by periodate in a manner similar to glycopeptide V-3 (if the residual 0.5 residue of mannose was susceptible but not oxidized). The first degradation step destroys four mannose residues leaving a structure identical to c) which was proposed for glycopeptide V-3; and the second degradation removes the remaining two mannose residues.

Monosaccharide specific exoglycosidases are a more powerful tool when a number of different monosaccharide types make up the oligosaccharide as is the case with glycopeptide VI-1. Yasuda et al, (133) used sequential periodate oxidation followed by characterization of the oxidation products, various combinations and sequences of digestions with exoglycosidases, and partial acid hydrolyses, to study the sequence of the single oligosaccharide in pineapple stem bromelain. The number of possible sequences of this oligosaccharide were limited to a much greater extent with just these techniques than would have been possible if the oligosaccharide were only composed of two types of carbohydrate, as in the case of the taka-amylase oligosaccharide. This glycopeptide, which has the same carbohydrate composition as glycopeptide VI-1, was arbitrarily assigned the following structure out of a

number of possible structures which were consistent with the data:



The first periodate oxidation of this oligosaccharide destroys all the fucose, xylose, and mannose, leaving the $GlcNAc_2$ core intact, in contrast to glycopeptide VI-1, which retained one mannose residue in addition to two GlcNAc residues.

The relationship of the pronase glycopeptides to the tryptic glycopeptides examined by Johnson (89,90) can only be speculated upon until well characterized tryptic glycopeptides are digested with pronase. However, consideration of the preponderance of tryptic glycopeptide fractions $GP\text{-}A_1$ and $GP\text{-}B_{1\&2}$, and their qualitative carbohydrate compositions suggest that pronase glycopeptide VI-1 is derived from $GP\text{-}A_2$, while glycopeptides V-2, V-3, and possibly IV-2 and IV-3, may be derived from $GP\text{-}B_{1\&2}$.

Both GP-A_1 and GP-$B_{1\&2}$ contain aspartic acid and threonine in a ratio of 2:2, as does VI-1, while GP-B_3, GP-B_4, and GP-B_5 have ratios of 2:1. However, GP-$B_{1\&2}$ contains only mannose and glucosamine, while both GP-A_1 and VI-1 contain fucose, xylose, mannose, and glucosamine, and are major glycopeptide components. Since GP-A_1, based on its lack of lysine or arginine, apparently represents a C-terminal polypeptide containing about 15 amino acid residues, VI-1 would be located within the terminal 15 amino acid residues of the C-terminus. The predominant pronase glycopeptides containing only mannose and glucosamine, i.e., V-2, V-3, IV-2, and IV-3, could be derived from any of the tryptic glycopeptides GP-$B_{1\&2}$, GP-B_3, GP-B_4, or GP-B_5, considering only their amino acid compositions. Unfortunately, the carbohydrate compositions of the three latter glycopeptides were not determined. The preponderance of GP-$B_{1\&2}$, coupled with its carbohydrate content of only mannose and glucosamine, strongly suggests that one or more of the peptides V-2, V-3, IV-2, or IV-3, are derived from it. Tryptic glycopeptides GP-B_3, and GP-B_4, occurred at high enough levels that each, if it contained only mannose and glucosamine, could also represent the parent peptide of one of

these pronase glycopeptides. Thus, it is plausible that tryptic glycopeptide GP-A_1 contains the linkage region sequence, Asp-$\overset{\overset{CHO}{|}}{Asn}$-Thr-Thr, while glycopeptide fraction GP-$B_{1\&2}$ contains the sequence $\overset{\overset{CHO}{|}}{Asn}$-Glu-Thr.

As noted in the "Introduction", the tripeptide sequence, Asn-X-Ser/Thr, has been found in almost all the glycopeptides of primarily animal origin which contain the N-glycosidic linkage. This observation prompted the suggestion that this amino acid sequence is a signal for asparagine glycosylation. Jackson and Hirs (143) further pointed out that the polarity of residue X may be a signal for the type of oligosaccharide to be synthesized at the adjacent asparagine. In this connection, they grouped glycopeptides according to their oligosaccharide content of only glucosamine and mannose (class II) or their content of a greater number of monosaccharides, including fucose, galactose, galactosamine, glucosamine, mannose, and sialic acids (complex polysaccharides, class I). Class I glycopeptides were observed to contain amino acid residues at X with polar sidechain groups, e.g., Ser, Glu, Arg, Lys, or Thr, while Class II glycopeptides contained residues of low polarity at X, such as Met, Leu, or Ala. The stem bromelain glycopeptide was included in class I, and contains Glu at X.

The regularity of these linkage region peptide sequences for primarily animal glycoproteins suggests that such a recognition site is plausible for glycoproteins from organisms lower on the phylogenetic scale. Moreover, an examination of the homologies of the codon sequences for the linkage site amino acids commonly found in animal glycoproteins, led Jett and Jamieson (144) to speculate that the glycosylamine linkage involving the amide group of asparagine was the most primitive of all the linkages reported, since the codons for Ser, Thr, and Lys, could each result from a single base mutation of the codon for asparagine.

Glycopeptides V-2, V-3, and VI-1, all contain the Asn-X-Thr sequence. In fact, the compositions of all the pronase glycopeptides which contain both Asx and Thr are consistent with such a sequence. Glycopeptide VI-1, according to Jackson and Hirs, would fall into class I, and in agreement with this classification it contains a polar amino acid at site X. Glycopeptides, V-2 and V-3, would both be in class II, but instead of a nonpolar amino acid at X, they contain Glu. It may be speculated that if the suggestion of Jackson and Hirs is upheld for most animal glycoproteins, then the nonconformity to this rule

of the plant glycoproteins may indicate that a divergence in the X-residue signal may have arisen during the evolution of the more complex animal systems.

Significance and Future Work

Since few plant glycoproteins have been extensively characterized, the information presented on the glycopeptide structures and the distribution of carbohydrate in PPHA constitute a significant contribution to the body of knowledge on plant glycoproteins, providing a basis for structural comparisons of glycoproteins from plants, animals, and microbes. These studies also constitute a step toward the elucidation of the primary structure of PPHA, which is critical to understanding its mechanism of action.

The established carbohydrate compositions provide an additional basis for comparing PPHA with other PHA preparations. The procedures developed for the isolation of highly purified glycopeptides from PPHA should expedite subsequent glycopeptide studies of PPHA and possibly of other glycoproteins.

A number of questions associated with the objectives of this work remain unanswered and require additional studies. The carbohydrate compositions, amino acid

sequences, and the carbohydrate linkage sites, of the remaining major glycopeptide fractions, IV, VII, and VIII, must be examined to establish the number of unique linkage region sequences immediately adjacent to the carbohydrate linkage sites and the number of unique oligosaccharides in PPHA. None of the oligosaccharides has yet been completely sequenced. Tryptic glycopeptides should be prepared, characterized, and hydrolyzed with pronase to establish the relationship between the tryptic and pronase glycopeptides, and the number of unique tryptic glycopeptide sequences in PPHA. Finally, the carbohydrate compositions of the isolated PPHA subunits and the structures of the pronase glycopeptides derived from them should be examined to show how the different types of oligosaccharides are distributed among the mitogenic and the erythroagglutinating subunits.

SUMMARY AND CONCLUSIONS

Gas-liquid chromatographic analyses of the neutral monosaccharide constituents of various PPHA preparations demonstrated the presence of mannose, fucose, xylose, arabinose, galactose and glucose. The levels of arabinose, galactose and glucose were variable in PPHA preparations not subjected to the final purification step (Sephadex G-200 chromatography). However, they were practically eliminated (< 1 residue per mole) following this final purification step, and were thus considered to represent contaminants. In contrast, fucose, xylose, mannose and glucosamine content varied little during the latter stages of PPHA purification. Since each was also a constituent of one or more of the glycopeptides, it was concluded that they are true constituents of the PPHA molecule. The quantities of these constituent sugars varied little in fully purified PPHA prepared from different bean lots. The average composition of four different preparations was found to be: 48 mannose, 15 glucosamine, 4 fucose and 4 xylose residues per mole of dry, salt-free PPHA. This total of 71 residues constitutes 9.3% of the dry weight of PPHA and accounts for

about 12,000 daltons of its molecular weight. The neutral carbohydrate composition of PPHA preparations should be a useful criterion of purity.

The carbohydrate constituents were suggested to occur as D-mannose, N-acetyl-D-glucosamine, D-xylose, and L-fucose.

Comparison of PPHA with other phytohemagglutinin preparations, all derived from Difco PHA-P, showed similar qualitative but different quantitative carbohydrate compositions.

Glycopeptides could be prepared in high yield and purity by pronase digestion of PPHA denatured in the presence of dissociating agents, followed by chromatography on Bio-Gel P-6, on AG 50W-x2 at low pH, and on AG 1-x2 at pH 5.7. All the glycopeptides eluted in one peak from either Bio-Gel P-2 or P-6. Chromatography of the glycopeptide mixture on AG 50W-x2 produced four major peaks and three minor peaks. The peak components all contained Asx, Thr, Man, and GlcNAc. Some contained Glx, while others contained Fuc and Xyl. There appeared to be a negative correlation between Glx content and the content of Fuc and Xyl. Chromatography of the three major peaks, IV, V, and VI, on AG 1-x2 resolved them into their major components which were: IV-2 having the composition

(Asx_2,Thr,Glx) ($GlcNAc_2$,Man_8); IV-3, (Asx,Thr,Glx) ($GlcNAc_2$,Man_7); V-2, (Asx_2,Thr,Glx)($GlcNAc_2$,Man_8); V-3, (Asx,Thr,Glx)($GlcNAc_2$,Man_8); and VI-1, (Asx_2,Thr_2) ($GlcNAc_2$,Man_3,Fuc,Xyl). Estimates from the recovery data indicated that the glycopeptides from peaks IV and V accounted for two oligosaccharides of the type, ($GlcNAc_2$,-Man_8), per mole of PPHA,while VI-1 represented about two of the type, ($GlcNAc_2$,Man_3,Fuc,Xyl).

The amino acid sequences of V-2 and V-3 were shown by Edman degradation to be:

$$(GlcNAc_2,Man_8)\text{-Asx-Glu-Thr-Asx} \quad \text{and} \quad (GlcNAc_2,Man_2)(Man_6)\text{-Asx-Glu-Thr}$$

respectively, in which the carbohydrate is bound through the sidechains of the N-terminal Asx residues. The C-terminal residue of V-2 was indicated to be Asn. The compositions of glycopeptides IV-2 and IV-3 are compatible with these same sequences.

Sequential periodate oxidation of V-3 established that GlcNAc is bound to the Asx side chain and that both GlcNAc residues are situated in the core of the oligosaccharide near the carbohydrate-peptide linkage. The oligosaccharide

was concluded to be a branched structure, having either two or three nonreducing termini. The similar elution behavior of V-2 from AG 50W-x2, suggests that its oligosaccharide has a similar structure.

It was proposed that the oligosaccharide-peptide linkage in V-2 and V-3 are of the N-glycosidic type consisting of 2-acetamido-N-(4-L-aspartyl)-2-deoxy-β-D-glucopyranosylamine.

The most probable amino acid sequence of VI-1 based on Edman degradation, carboxypeptidase A digestion, and alkali treatment was:

```
(GlcNAc2,Man)(Man2,Fuc,Xyl)
      |
Asp-Asx-Thr-Thr
```

in which the oligosaccharide is bound to the penultimate Asx residue. However, the data were not as conclusive as for V-2 and V-3.

The single periodate oxidation of VI-1 destroyed all but one Man and two GlcNAc residues. The oligosaccharide, similar to V-3, was indicated by its periodate consumption to be a branched structure with a maximum of three nonreducing termini. The data were consistent with a core

structure similar to that of V-3 in which both GlcNAc residues are situated in the immediate vicinity of the carbohydrate peptide linkage. It was hypothesized that the carbohydrate-peptide linkage in VI-1 is of the N-glycoside type, as in V-2 and V-3.

The amino acid sequences of these linkage regions conform to the general scheme Asn-X-Thr/Ser which occurs in almost all the glycoproteins containing the N-glycosidic linkage between C-1 of GlcNAc and the amide nitrogen of asparagine.

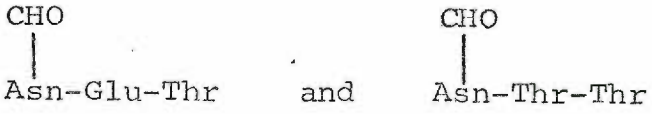
It was estimated that PPHA contains 7-10 oligosaccharide moieties ranging in molecular weight from 1200 to 1700, each consisting of 7 to 10 monosaccharides. Each oligosaccharide contains Man and GlcNAc, but not all contain Fuc and Xyl. Two oligosaccharide chain types have been established: one composed of 2 GlcNAc and 7 to 8 Man, as found in glycopeptides IV-1, IV-3, V-2, and V-3; and a second containing 2 GlcNAc, 3 Man, 1 Fuc, and one Xyl, as found in glycopeptide VI-1.

Terminal Man residues were suggested to occur as α-D-mannopyranosides based on the binding of PPHA to concanavalin A. The oligosaccharide type appeared correlated with the amino acid composition of the glycopeptides. The highly

purified glycopeptides containing only Man and GlcNAc contained glutamic acid, while those containing all the monosaccharides were devoid of glutamic acid, but rich in threonine.

Periodate oxidation of PPHA destroyed 75% of the neutral monosaccharides and none of the GlcNAc, leaving 13 to 14 Man and 15 GlcNAc residues intact. These data suggest that all the oligosaccharides may have core structures composed of GlcNAc and Man. Furthermore, since all the glycopeptides contain Asx, it is plausible that the carbohydrate-protein linkages are all of the N-glycoside type.

While further characterization work will be required to establish the maximum number of amino sequences in the immediate region of the carbohydrate protein linkages, the observed compositions of the glycopeptide fractions that were not sequenced are compatible with the hypothesis that they contain one or both of the following sequences:



It is noted that if PPHA contained four of each of the two types of oligosaccharides mentioned above, it would be expected to contain 4 Fuc, 4 Xyl, 44 Man, and 16 GlcNAc

residues per mole, which agreed remarkably well with the observed composition. If such was the case, one step of the periodate oxidation would be predicted to destroy all but 12 Man and 16 GlcNAc residues, as judged by the behavior of the glycopeptides, V-3 and VI-1. This prediction too agreed fairly well with the observed values of 13.5 Man and 15.3 GlcNAc residues.

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