STUDIES ON THE ISOLATION, PURIFICATION AND CHARACTERIZATION OF THE TRYPTIC GLYCOPEPTIDES OF THE PHYTOHEMAGGLUTININ FROM RED KIDNEY BEANS (*PHASEOLUS VULGARIS*)

by

Edward Andrew Johnson, B.S.

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I. INTRODUCTION

The presence of erythrocyte agglutinins in extracts of castor beans, according to Cushny (1), was first described in 1888 by Stillmark (2) during his studies on the highly toxic substance, ricin, also present in these beans. Early efforts to ascertain the chemical nature of ricin, stimulated a debate as to whether it was a protein or some other complex molecule. In an attempt to resolve this question, Osborne, Mendel and Harris (3) fractionated and partially purified the protein components of the castor bean (Ricinus communis). In their "physiological experiments" section there is a description of the effects of their ricin preparations upon the erythrocytes of the laboratory animals used in their experiments. They state, "The characteristic influence of ricin preparations on extravascular blood or suspensions of corpuscles is exhibited in the agglutination of the erythrocytes followed by the sedimentation of flocky masses upon the bottom of the retaining vessel."

This observation aroused the interest of the immunologists of the time and in 1891 Ehrlich (4) reported his studies on antibody formation against ricin in the mouse. Later, working as students of Ehrlich, Landsteiner and Raubitschek (5) reported the presence of erythrocyte agglutinins in the seeds of a number of other legumes. Landsteiner (6) also demonstrated that the degree of agglutination of a given extract was not the same for different animal species.

Boyd and Reguera (7) extended these observations with the purpose of finding specific or semispecific action on the erythrocytes of different individuals within a given species. They reasoned that if such

specificity could be demonstrated, a cheap source of blood grouping reagents would be made available and studies on the mechanism of agglutination could be made by chemical modifications of the purified agglutinating component. In 1949, they reported the results of studies on 262 varieties of plants. The only specific agglutinin found was in the lima bean, Phaseolus limensis, which agglutinated human erythrocytes of the A blood group.

Since that time many investigators have expanded this work and, as reviewed by Boyd (8), over the past fifteen years there have been found plant extracts which demonstrate different specificities toward a number of human blood groups with the notable exception of the Rh antigens. In addition to specific leguminous agglutinins, there are found specific anti-A agglutinins in the mushroom, Clitocybe nebularis, anti-B agglutinins in the tree fungus, Polyporus fomentarius, and anti-H agglutinins in the mushroom, Xylaria polymorpha. A non-specific hemagglutinin present in potatoes has been described by Marinkovich (9).

Boyd (8) has proposed that the term "lectin" be used for these plant agglutinins and has divided them into three general categories: direct "lectins" which will agglutinate cells suspended in saline; indirect "lectins" which require plasma-suspended cells or cells treated with certain proteolytic enzymes; and nonagglutinating "lectins"[1] which

[1] Although Boyd (8) proposed the term "lectin" for the plant agglutinins, the term "phytohemagglutinin" seems to be more commonly used and for consistency will be used throughout the remainder of this paper or abbreviated PHA.

combined with cells but do not agglutinate them in either system. The majority of the known phytohemagglutinins are of the first category but as Boyd points out, this may be because most workers have only tested for this type of phytohemagglutinin.

A source list of phytohemagglutinins has been compiled by Boyd, Waszczenko-Zacharczenko and Goldwasser (10) which demonstrates the wide distribution of these substances in the plant kingdom.

Although plant extracts have been extensively studied in terms of erythrocyte agglutinins, their activity is not limited to the red blood cells alone. Kashwabara, Tanaka and Matsumoto (11) have observed a tail-to-tail agglomeration of bull spermatozoa when soybean extracts were added to their suspensions. Nordman, de la Chapelle and Granbeck (12) have presented suggestive evidence for the existence of two types of agglutinins in bean (Phaseolus vulgaris) extracts, one primarily a leukoagglutinin and the other primarily an erythroagglutinin, but neither being devoid of the opposite activity. Using red cell stroma adsorbed extracts of the red kidney bean, Tunis (13) has demonstrated the presence of a non-erythroagglutinin capable of agglutinating Sarcoma 180 cells of the ascites form and other nonerythroid nucleated cells.

The presence of phytohemagglutinins in bean extracts (Phaseolus vulgaris) was rediscovered in the late 1940's by Li and Osgood (14) while developing in vitro methods for the long term culture of human leukemic leukocytes. According to Rigas et al. (15), Li speculated that the bean seed, the plant embryo, might contain growth promoting factors analogous to those found in chick embryo extracts used for in vitro

mammalian cell cultures. Although they did not demonstrate the presence of a growth promoting factor (mitogen) in the bean extracts, in 1949 Li and Osgood (14) reported a simple method employing the phytohemagglutinin for the rapid and efficient separation of leukocytes from whole blood.

Ten years later Li's speculation found support when Nowell (16) observed an increased number of mitotic figures in his leukocyte preparations obtained by the phytohemagglutinin separation method. Through controlled studies, Nowell demonstrated the phytohemagglutinin to be responsible for this mitogenic effect and suggested that the mononuclear leucocytes, lymphocytes and monocytes, appeared to be the only leucocytes induced into mitosis. Later studies by Marshall and Roberts (17), Cooper, Barkhan and Hale (18) and others have provided good evidence that the small lymphocytes convert in vitro into the dividing cell. In addition to the peripheral lymphocytes, Nowell (16) reported leukemic blasts and bone marrow cells to be stimulated and undergo mitosis upon the addition of phytohemagglutinin to their cultures. Recently Sarkany and Caron (19) have reported that epithelial cells in organ culture of human skin are induced to undergo mitosis by phytohemagglutinin.

The first benefits of Nowell's observation were obtained by the geneticists, as culture techniques like those described by Mellman (20) utilizing the mitogenic property of phytohemagglutinin yield large numbers of metaphase chromosomes suitable for chromosome studies. Recent investigations by Berman and Stulberg (21), Pearmain and Lycette (22) and others concerning the role that phytohemagglutinin plays in initiating

mitosis have led them to postulate that mitosis is an immune response elicited by the cells against the phytohemagglutinin. If this proves to be the case, phytohemagglutinin will also be of value in future immunologic studies. As the phytohemagglutinin has the ability to transform and stimulate lymphocytes normally incapable of division to rapidly proliferate future studies on the mechanisms of cellular division and differentiation will be aided by the use of phytohemagglutinin (15).

Most studies on the mitogenic activity of phytohemagglutinin have been made using an impure commercial preparation. Punnett and Punnett (23) reported that in some lots of commercial preparations they were unable to demonstrate mitogenic activity by their methods, although they could demonstrate hemagglutinating activity. They concluded the mitogen is actually a contaminant in the commercial preparations. Thus the identity of the erythroagglutinin with the mitogen has been challenged. The efforts to resolve this question can best be presented in light of the studies made on the purification and characterization of phytohemagglutinin.

Early work by Osborne et al. (3) on ricin implicated the association of the toxicity of their preparations with the erythroagglutinating activity. The criterion of purity used by these investigators was comparative lethal dose analyses. Since the modern physicochemical methods used to demonstrate protein homogenity were not available to these workers, little can be said about the purity of their preparations. However, Kabat, Heidelberger and Bezer (24) have since demonstrated their

ricin preparations to be electrophoretically, ultracentrifugally and immunochemically homogeneous. Their preparations possessed both toxicity and erythroagglutinating activity. The purified ricin was found to be a protein with a molecular weight of about 80,000 and to have an isoelectric point of 5.3.

Sumner and Howell (25) observed a similar association with their preparations of the mild toxin, Concanavalin A, isolated from jack beans (Canavalia ensiformis).

An electrophoretically homogeneous preparation of soybean erythro-agglutinin was also shown to be toxic (26). Later studies on this agglutinin revealed it to be a protein with a molecular weight of 96,000 (27).

In contrast, the kidney bean (Phaseolus vulgaris) and navy bean (Phaseolus communis) agglutinins have been found to be non-toxic (28, 29). It is from these beans that the commercial preparations have been prepared by modifications of the methods of Rigas and Osgood (30).

Using a combined ethanol and ammonium sulfate fractionation procedure, Rigas and Osgood (30) isolated an active erythroagglutinating mucoprotein (MPHA) from red kidney beans which contained 50% reducing substances. This preparation appeared to be homogeneous when subjected to moving boundary electrophoresis between pH 5.8 and 8.6. However, below pH 5.8 the electrophoretic patterns showed progressive asymmetry and at pH 2 complete dissociation was observed with one component migrating as a cation while the other remained stationary. Samples of the two components were taken directly from the electrophoretic cell and analyzed chemically

and for agglutinating activity. The cationic component was found to be a protein retaining the agglutinating activity, whereas the stationary component was an inactive polysaccharide. From this observation, a fractionation procedure employing ammonium sulfate at pH 1 was developed and the active protein (PPHA) isolated. This preparation appeared homogeneous by electrophoretic analysis between pH 2 and 8.0. When subjected to analytical ultracentrifugation, a minor component was observed sedimenting slightly faster than the larger homogeneous component. The protein was shown to have an isoelectric pH of 6.5, a sedimentation coefficient of 7.2 S (M.W. = 128,000), a nitrogen content of 14.6% and a 3.4% content of reducing substances. The preparation gave a positive agglutination test at concentrations of 0.1 microgram per milliliter. Since these studies were prior to Nowell's discovery of the mitogenic activity of phytohemagglutinin preparations, no mitogenic assays were reported.

Shortly after Punnett et al. (23) presented their hypothesis on non-identity of the mitogen with the erythroagglutinin, Barkhan and Ballas (31) reported that they found the mitogenic activity to remain in their phytohemagglutinin preparations from which the erythroagglutinin had been removed by red cell adsorption. In neither case was a mitogenic substance isolated and characterized that was devoid of erythroagglutinating activity. Michalowski, Jasinska and Madalinski (32) repeated the work of Barkhan et al. and found that losses in the erythroagglutinating activity resulted in parallel losses of the mitogenic activity.

Further studies by Rigas et al. (15, 33) were made on a PPHA

preparation that was shown by them to be homogeneous by 11 different criteria of homogeneity. The same preparation has recently been shown to be homogeneous by immunoelectrophoresis and immunodiffusion techniques (34). This preparation possessed both activities.

However, when subjected to column chromatography on erythrocyte stroma and on the weak cation exchange resin IRC-50, multiple components were observed. From the erythrocyte stroma a minor component (about 5% of the total) was eluted from the column at 7°C and appeared to have no affinity for the stroma. The major portion of the column load was removed from the stroma when the temperature was raised to 56°C. Analyses of the two components for the two activities indicated the minor component possessed a greater mitogenic activity than the major component in relation to the agglutinating activity. Successive rechromatography of the major component on the stroma column failed to elucidate the possible relationship of these two components as inactivation of both activities occurred.

From the IRC-50 chromatography eight or more components were obtained which possessed different degrees of the two activities, the proportions of which varied independently from each other. The tryptic peptide patterns, sedimentation coefficients, solubilities and electrophoretic patterns of these components demonstrated them to be different from each other and from the parent preparation.

When the pure PPHA was subjected to 8 M urea and then analyzed by ultracentrifugation, two components were found to be present. The slower sedimenting component was estimated to have one-eighth the

molecular weight of the untreated PPHA (about 128,000). The sedimentation coefficient of the faster sedimenting component was 7.2 S, i.e., the same as the untreated PPHA. Upon longer exposure to the urea the relative concentrations of the two components changed with the slower becoming the greater of the two.

When the 8 M urea-treated PPHA was subjected to starch gel electrophoresis employing 8 M urea, pH 9 borate buffers, nine protein bands were obtained. The concentration of the fastest migrating band diminished with longer periods of treatment of the PPHA with urea before electrophoresis.

When the IRC-50 components were treated with urea and electrophoresed, several of the 8 bands were missing in two of the components, while the other components differed in their relative concentrations as indicated by the color density of the bands.

In vitro dose response studies of human leucocytes with the pure PPHA were made. Utilization of protein, RNA and DNA radioactive precursors by the leucocytes was used as a measure of the mitotic activity. From these studies it was demonstrated that there is an optimum dose, above and below which the leucocytes will not respond and undergo mitosis.

On the basis of these results it was concluded by these workers that the PPHA is composed of 8 electrophoretically different polypeptide chains (subunits), each having a molecular weight one-eighth that of the undissociated molecule. The IRC-50 resin causes the molecule to dissociate and when reassociation occurs, combinations other than that

of the original molecule result. Since the agglutinating and mitogenic activities of the IRC-50 components varied independently from each other, it was postulated that one type of subunit is responsible for the mitogenic activity and another type is responsible for the agglutinating activity of the PPHA molecule. A neutral type of subunit was also postulated to explain the finding that one of the IRC-50 components exhibited activities, both of which were greater than that of the original PPHA preparation. The fact that there is an optimum dose for leucocyte response was considered as suggestive evidence that one of the subunits is toxic at high concentrations. Not precluding the possibility of another substance in the bean having only mitogenic activity, their findings conclusively demonstrate that the PPHA molecule possesses both biological activities.

Few of the phytohemagglutinins from the many sources described have been chemically characterized. Of those that have there appears to be basic similarities between them. They are large proteins with molecular weights between 80,000 and 130,000 and isoelectric points between 5.2 and 6.5. They contain large amounts of aspartic acid and their content of histidine, tryptophan and the sulfur containing amino acids cysteine and methionine is low. The phytohemagglutinins are glycoproteins for about 10% of their weight is composed of glucosamine and neutral sugars (9,15,24,27,33,35-39). The red kidney bean phytohemagglutinin contains 19 glucosamine residues and 63 neutral monosaccharide residues per molecule of the protein (15).

The purpose of the work presented in this thesis was to study the

carbohydrate portion of the molecule in terms of the number, site of attachment and monosaccharide composition of the oligosaccharide moieties present in the protein. Since the possibility existed that the oligosaccharide units might be the same but have different sites of attachment, it was felt that the greatest information would be gained by isolating the tryptic glycopeptides and analyzing them for differences in amino acid and carbohydrate composition. Thus this work has involved establishing methods by which the tryptic glycopeptides may be separated, isolated and studied in terms of their structure.

II. METHODS AND MATERIALS

A. Physical Measurements

Optical densities of protein solutions were determined at 280 mμ with a PMQ II Zeiss spectrophotometer (Carl Zeiss, Germany). The quartz cuvettes with a 10 mm light path manufactured by Pyrocell Mfg. Co. were used. In all determinations the solvent of the protein solution was used as the blank.

Measurements of pH were made with a Model G Beckman pH meter, standardized with the appropriate Beckman standard buffer. The temperature of the standard buffer was always adjusted to that of the sample before the meter was standardized.

Conductivity measurements were made with a Klett Conductivity Bridge (Klett Mfg. Co., New York). The conductivity cell was of Jones and Bollinger design and obtained also from Klett Mfg. Co. The cell constant was 14.4. The volume of solution between the two electrodes was 1 ml. The cell was connected in parallel with a standard resistance of 10^4 ohms having a ~0.01% deviation (Type 500-J, General Radio Co.) After rinsing the cell 3 times each with 5 ml of the solution to be measured, the cell was filled and the bubbles between the electrodes and solution were removed. Following equilibration of the cell in a 20°C water bath for 30 minutes, the total resistance of the cell and standard resistance was determined.

The resistance of the cell was calculated using the equation:

$$\frac{1}{R_c} = \frac{1}{R_t} - \frac{1}{R_s} \qquad (1)$$

where R_t is the total resistance as determined with the conductivity bridge, R_s the resistance of the standard resistor and R_c the resistance of the cell. Since conductivity is defined by the equation

$$C = \frac{1}{R} \qquad (2)$$

then

$$C_c = \frac{1}{R_c} = \frac{1}{R_t} - \frac{1}{R_s} \qquad (3)$$

To find the specific conductance (κ) of the solution the equation

$$\kappa = K\frac{1}{R_c} = KC_c \qquad (4)$$

is used, where K is the cell constant.

Weight measurements were made with models H16 and P-120 Mettler balances. All materials stored in the cold were brought to room temperature before weighing.

B. Physical Methods

Protein solutions and suspensions to be dried were lyophilized with a VirTis model 10-145MR-BA mechanically refrigerated Freeze-Mobile (The VirTis Company, Inc., Gardiner, N.Y.). Before lyophilization the protein solutions were placed in flasks adapted for the Freeze-Mobile and frozen solid in a dry ice-methyl cellosolve bath.

Preparatory centrifugation procedures were performed using a RC-2 Super-Speed Servall refrigerated centrifuge (Ivan Sorvall, Inc., Norwalk, Conn.).

Concentration of protein solutions was carried out according to the ultrafiltration method described by Rigas (40). The special flask was

fitted with a length of presoaked and washed 8/32" Visking tubing (HMC, 52 Gloucester Place W.1) and filled with the protein solution. Ultrafiltration was effected by a vacuum (6.25 ± 0.75 cm Hg) controlled with a U-tube manometer and 3-contact electronic relay (Emil Greiner Co., New York) especially modified for this purpose (40).

Series F Haake thermoregulated circulating water baths (Brinkmann Instruments, Inc., New York) and Precision Temptrol Circulating water baths (Precision Scientific Co., Chicago, Ill.) were used in experiments requiring constant temperature control.

Fractions of chromatographic column eluates were collected with Buchler fraction collectors (Buchler Instruments, Fort Lee, N.J.).

Concentration or drying of peptide solutions and their acid hydrolysates was performed using a model PTFE-10 Buchler Portable Flash evaporator (Buchler Instruments, Fort Lee, N.J.). The temperature of the evaporator flask was maintained at 40°C.

All chemicals used were of reagent grade quality.

C. Purification of the Protein Phytohemagglutinin

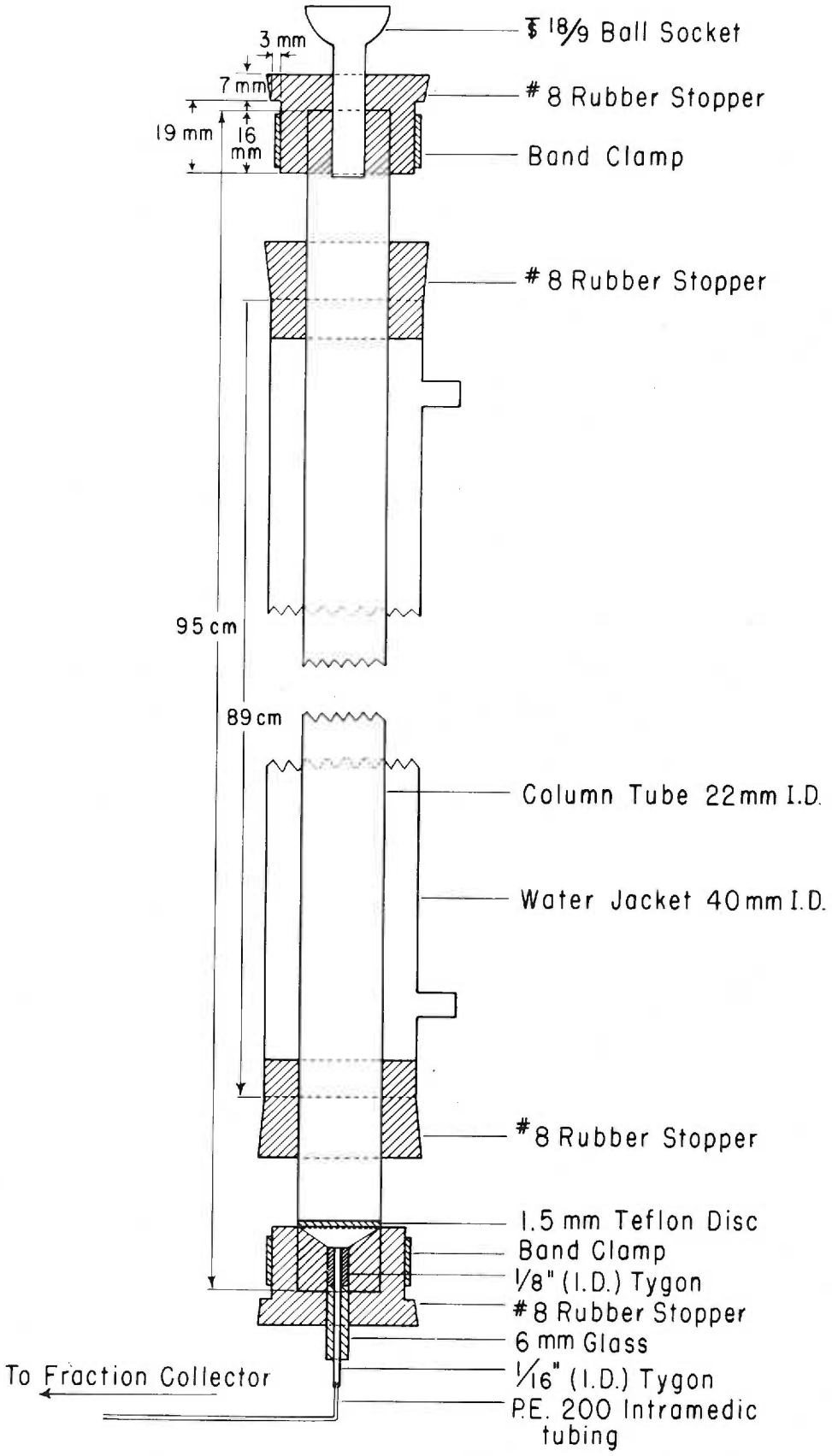
Twenty-five pounds of red kidney beans (Lilly Brand, germination standard Lot 4-C) were sorted and only healthy beans retained. Ten kilograms of the sorted beans were rinsed 3 times with tap water and allowed to air dry at room temperature for 24 hours. Using a F-4 corn mill (Quaker City Mill, Philadelphia), the beans were ground to a fine flour consistency. With the exception of removal of the insoluble material from the initial 0.1 M HCl extract by centrifugation, the bean flour was processed according to the procedure of Rigas et al. (15, 33).

Further purification of the phytohemagglutinin was effected by gel filtration on a 2 X 88 cm column of polyacrylamide gel P-200 (Bio-Rad Laboratories), 50-150 wet mesh (Control No. 3335). The dry gel was added slowly with stirring to distilled, deionized water (25 gm gel per 2 liters). The gel suspension was then boiled gently for 5 minutes, cooled and allowed to settle to 3/5 the suspended volume. The supernatent and fines were aspirated off and the gel washed three times with 0.2 M NaCl. Each wash was performed by addition of 0.2 M NaCl to bring the suspension to its original total volume, stirring for 30 minutes, settling to 3/5 the suspended volume and removal of the supernatent by aspiration. After the third wash, the gel was allowed to settle to 1/2 the suspended volume, the supernatent removed by aspiration and a 1:1 suspension of the gel made in 0.2 M NaCl.

Since the large column desired for this purification step was not available, a satisfactory column was assembled from common laboratory supplies. A diagrammatic representation with dimensions of the column is given in Fig. 1. The column tube and water jacket were made of pyrex glass tubing. The ground glass ball socket fitting on the top of the column was purchased from Owens-Illinois Glass Company (Kimax #33604). The filter at the bottom was made by first cutting the circular disc from a 5 mm piece of porous teflon (Fluoro-Plastics, Inc., Philadelphia, Pa.) and then slicing the disc with a razor blade to make it 2.5 mm thick. The bottom glass fitting was fashioned from a 6 mm piece of pyrex tubing by melting one end shut and blowing out a bubble. The distal half of the bubble was removed and the remaining edge was

Figure 1.

Schematic cross section of preparative chromatograph column assembled out of common laboratory supplies.


3 mm
7 mm
19 mm
16 mm
95 cm
89 cm
To Fraction Collector
$ 18/9 Ball Socket
# 8 Rubber Stopper
Band Clamp
8 Rubber Stopper
Column Tube 22mm I.D.
Water Jacket 40mm I.D.
8 Rubber Stopper
1.5 mm Teflon Disc
Band Clamp
1/8" (I.D.) Tygon
8 Rubber Stopper
6 mm Glass
1/16" (I.D.) Tygon
P.E. 200 Intramedic tubing

carefully melted down to fit the well in the #8 rubber stopper. The well in the rubber stopper was cut so that a 2 mm ridge of rubber would remain between the glass fitting and the wall of the column tube. In so doing, a tight seal was made between the column tube, rubber stopper and glass fitting. The glass fitting was designed to make the mixing volume as small as possible (approximately 0.5 ml) and with the purpose of keeping the protein solution from coming in contact with the rubber. Both the top and bottom rubber stoppers were tightly secured to the column with screw-type band clamps (Breeze M-16, Union, N.J.) to prevent leakage.

Before pouring the column the temperature was lowered and maintained at 7°C by circulating water through the column jacket. A small volume of 0.2 M NaCl was added to the column and the air in the teflon filter and bottom of the column was forced out under 5 psi air pressure and displaced by the saline leaving a 5 cm height of saline above the filter. The tip of the tubing leading from the bottom of the column (transfer tip) was raised and the column filled to its top with the well-mixed 1:1 gel suspension. After about one hour of settling the transfer tip was lowered several centimeters from the top and a 4 ml/hr flow rate maintained. When the gel had settled 12-15 cm from the top of the column, the supernatent above the gel was removed to within 3 cm of the surface of the gel, the gel 4 cm below the surface resuspended and the column filled to its top with well mixed 1:1 suspension. This procedure was continued until the settled gel bed was about 90 cm in height. A Marriott flask connected to the column via polyethylene tubing

(PE 250 Intramedic) was used as a constant hydrostatic pressure head. By raising or lowering the Marriott flask, the flow rate was adjusted to and maintained at 4 ml/hr. The hydrostatic head, measured in cm, was found by measuring the distance from the inside end of the air tube of the flask and the open end of the transfer tube. This distance should never exceed 20 cm because at distances greater than this the gel bed will pack and the flow rate will approach zero.

After stabilizing the bed by equilibrating the column with 0.2 M NaCl for 72-96 hours at a 4 ml/hr flow rate, the column was loaded with the phytohemagglutinin obtained by the ammonium sulfate fractionation procedure above. One or two hundred milligrams of the lyophilized protein were dissolved in 3 or 5 ml of 0.2 M NaCl respectively. With the transfer tip raised to the top of the column the protein solution was layered with a syringe and needle below the saline and above the gel. The protein was eluted with 0.2 M NaCl at a 4 ml/hr flow rate maintained with the constant pressure head described. The column temperature was at 7°C throughout the elution of the protein. One hour fractions were collected in a Buchler refrigerated fraction collector.

Protein determinations on the fractions were made by optical density measurements at 280 mμ using 0.2 M NaCl as the blank. Carbohydrate and ninhydrin analyses were performed using the Technicon Autoanalyzer method (vide infra).

D. Ultracentrifugation

Ultracentrifugal analyses were performed with a Spinco Model E ultracentrifuge. All analyses were maintained at 20°C with the RIST

temperature control unit. Protein solutions were prepared in 0.1 M NaCl which was adjusted to pH 7 by the addition of 0.1 M NaOH. Analyses were carried out using the Analytical D rotor and the standard 15 mm cell. A 1° wedge window was used for the double cell comparative analyses.

Ultracentrifugal separations were made with the 30 mm fixed partition cell in the Analytical E rotor. The fixed partition plate was covered with Whatman #5 filter paper. To fill the cell the upper portion of the cell was filled with the protein solution and forced into the lower portion by centrifugation at 3000 rpm. The upper portion was then replenished with the protein solution and the separation was made at 50,740 rpm. After the separation was complete the cell was immediately removed and the solutions above and below the partition were individually removed and kept separate.

The schlieren optics system with a green filter was used to follow the progress of the ultracentrifugation and the results were recorded on Kodak metalographic film plates.

E. Starch Gel Electrophoresis

Starch gel electrophoreses of the phytohemagglutinin preparations were carried out as described by Rigas et al. (33). The two buffer systems of pH 8.1 and 2.0 were employed. The pH 8.1 buffer was the discontinuous tris-borate system and the pH 2.0 buffer was the 0.005 M glycine-HCl system. The gels were prepared with hydrolyzed potato starch (Lot 199-1, Connaught Medical Research Lab., Toronto, Canada) in the appropriate buffers.

To 2 mg of the preparation to be electrophoresed, 0.04 ml of the appropriate gel buffer was added. The solutions were applied to the gel by soaking a 0.6 X 10 mm piece of Whatman 3 mm filter paper with the solution and placing them 5 mm apart in a cross-sectional slit cut in the gel. A model EC451 electrophoresis assembly and EC453 power supply was used in these experiments (E-C Apparatus Co., Swarthmore, Pa.). The gel was connected to the electrode-containing buffer vessels with four buffer-soaked sheets of Whatman 3mm filter paper. Electrophoresis was effected by a potential of 50 V/15 cm for the pH 2.0 system and 150 V/15 cm for the pH 8.1 system. Each system was electrophoresed for 4 hours at cold tap water temperatures (approx. 10°C).

After electrophoresis the protein bands were visualized by staining with amido-schwartz stain ("Buffalo Black NBR", Allied Chemical Co., New York). The results were recorded by photographing the gel with transmitted white light.

F. Titration of Agglutinating Activity

The agglutinating activity of the phytohemagglutinin preparations was determined by the titration method of Salk (41) as modified by Rigas et al. (15).

G. Tryptic Hydrolyses

The tryptic hydrolyses were performed by a modification of the method of Ingram (42). A Radiometer pH Stat assembly including a type TTT 1c Titrator, type ABU 1a Auto-buret and SBR2c Titrigraph was used to control the hydrolysate pH and record the progress of hydrolysis

(Radiometer, Copenhagen, Denmark). The Auto-buret was fitted with the 2.5 ml buret (B 150). The glass electrode (G 202B) and calomel electrode (K 401) were used to monitor the hydrolysate pH. The instrument was standardized with Beckman pH 7 and 10 buffers at 40°C before use. Prior to use all glassware used in the procedure was autoclaved. The electrodes and plastic parts of the pH stat were sterilized with 70% ethanol. All solutions were prepared with pre-boiled, distilled, deionized water.

A weighed amount of salt-free, lyophilized phytohemagglutinin was placed in a 15 X 70 mm tube having a female ℨ 19/38 ground glass joint. The protein was dissolved in 0.05 M acetate buffer pH 6.5 (100 mg/5 ml). The tube was then fitted onto a Pyrex West type condenser having a male ℨ 19/38 ground glass joint. The protein was denatured by immersing the tube in a boiling water bath while cold tap water was passing through the water jacket of the condenser. After 30 minutes of boiling the tube was cooled to room temperature and the coagulated protein was transferred to the large, jacketed pH stat vessel (type V512) with four 1 ml water washes. At this point 0.5 ml of a tetracycline in water solution was added, making a concentration of 0.05 mg tetracycline per ml before hydrolysis. The protein suspension was brought to 40°C and the pH was adjusted to 8.0 with carbonate-free 0.1 M NaOH. Immediately after the pH adjustment 0.75 ml of a 10 mg/ml solution of trypsin in 0.005 M HCl was added to the vessel and the titrator and titrigraph turned on simultaneously. The chart speed of the titrigraph was 7 cm/hr. The hydrolysis was carried out at 40°C and the pH maintained at 8.0

with carbonate-free 0.1 M NaOH. For all hydrolyses twice recrystallized, salt-free, lyophilized trypsin was used (Worthington Biochemical Co. Lot 6119B). Before adding the trypsin solution to the protein suspension, it was passed through a 0.45 micron millipore filter (Millipore Filter Co., Bedford, Mass.).

The tetracycline used was in the hydrochloride form lot 4Y535 ("Tetracyn", Chas. Pfizer and Co., Inc., N.Y.).

H. Bacterial Assays

The tryptic hydrolysate was checked for bacterial contamination by placing 0.05 ml of the hydrolysate into a tube of sterile trypticase-soy broth and 0.05 ml hydrolysate spread on sterile blood agar and Mueller-Hinton agar plates. The inoculated media were incubated for 24 hours at 37°C and checked for growth.

Antibiotic sensitivity tests were carried out by suspending some of the bacteria growing on a blood agar plate in a sterile broth extract tube. A volume of 0.05 ml of this suspension was spread onto a blood agar plate and sensitivity discs were applied. The plate was incubated for 24 hours at 37°C and checked for inhibition of growth around the discs. Table I lists the antibiotics tested.

Identification of the contaminating bacteria was made by the results obtained from the Gram stain, a motility test by light microscopy of a wet mount of the bacterial suspension and triple sugar-iron slants containing lactose, glucose, sucrose and iron in the form of $FeSO_4$. Fermentation of each of the sugars, lactose, sucrose and glucose was determined by observing for an acid reaction to pH indicators and gas

production by the bacteria in separate tubes containing sterile broth extract and one of the three sugars. These tubes were incubated for 24 hours at 37°C and checked for reaction.

All procedures and materials used for these studies are described in the bacteriology manual (43).

I. Gel Filtration of the Tryptic Hydrolysates

The tryptic hydrolysates were first subjected to gel filtration on columns of P-2 and P-30 polyacrylamide gels (Bio-Rad Laboratories, Richmond, Calif.).

The P-2 gel (lot 2993) used had an operating range of 200-2000 molecular weight and a wet mesh size of 100-200. The dry gel was added slowly with continuous stirring to distilled deionized water (50 gm gel/l). The gel was then allowed to stand at room temperature for 24 hours to swell. The supernatent and fines above the settled gel were removed by decantation. Following resuspension of the gel in the original volume of fresh water, it was boiled gently for 5 minutes with occasional stirring. After allowing the gel to settle the clear supernatent was removed by decantation. The gel was further washed 3 times with water and 3 times with the desired buffer to be used for chromatography. Each washing included suspension of the gel in the original volume of water or buffer, stirring 30 minutes, settling for 1 hour and removing the supernatent by decantation. Following the third buffer wash, a suspension of 1 part gel to 2 parts buffer was made for pouring the column.

A 2 x 60 cm jacketed column (Pharmacia Fine Chemicals, Inc., New Market, N.J.) fitted with a teflon filter disc was used for the P-2 gel chromatography. Before pouring the column the transfer tip was raised and 10 ml of the buffer was poured into the column. A 1.9 x 38 cm column extension (Scientific Glass Co., Bloomfield, N.J.) was connected to the top of the column. The column temperature was adjusted to 25°C by circulating water from a constant temperature water bath through the jacket of the column. The column and extension were filled with well-mixed 1:2 gel suspension and the gel was allowed to settle by gravity. When the settled gel surface was 4-5 cm from the bottom of the column the transfer tip was lowered and the packing proceeded under buffer flow. After the packed gel height was approximately 15 cm, 6 psi air pressure was used to pack the remainder of the column to a height of 55 cm.

Following packing, the gel column was equilibrated with 500 ml of buffer at a flow rate of 2 ml/min before use. To load the column the buffer above the gel was removed and not more than 10 ml of the material to be chromatographed was layered above the gel. The load material was allowed to drain into the column by gravity flow and the column sides were rinsed 2 times with 2 ml of buffer. After refilling the column to its top with buffer the material was eluted from the column with the buffer at a 2 ml/min flow rate. The flow rate was maintained with a Spinco Accu-flo chromatographic pump (Beckman Instruments, Inc., Palo Alto, Calif.) connected to the column via polyethylene tubing (PE-190 Intramedic) and a Spinco PVC ball joint

filter assembly (Part No. 313-347). The temperature of the column was maintained at 25°C during the development. Fractions of 3 ml each were collected and analyzed for peptide and carbohydrate. Several buffers were used for this chromatography, the descriptions of which are presented in the results section.

The P-30 gel (lot 2953) used had an operating range of 20,000-50,000 molecular weight and a wet mesh size of 50-150. The gel was treated in a manner similar to the P-2 gel, with the exception that 20 gm gel per liter of water were used and the boiling step was eliminated. After the water washes the gel was washed with 0.2 M NaCl.

Two different columns were used for the P-30 gel filtration. For the preparative work the large column used for the P-200 gel filtration earlier described in this section was used. The pouring procedure described for the P-2 gel was only modified by using 4 psi air pressure to pack the column to a height of 85 cm. The column was equilibrated with one liter of 0.2 M NaCl at a 10 ml/hr flow rate. The flow rate was maintained with a Model H Milton Roy Mini Pump (Milton Roy Co., Philadelphia, Pa.) connected to the column as described for the P-2 column. The column temperature was 20°C during the development. The column was loaded as before with 15 ml of the whole hydrolysate and 3 ml fractions were collected and analyzed with the autoanalyzer method. The void volume of the column was determined by passing a 9 ml load (20 mg/ml) of the protein phytohemagglutinin through the column at a 30 ml/hr flow rate. The 3 ml fractions collected were analyzed by the autoanalyzer method.

For smaller amounts of material the 2 x 60 cm column used for the P-2 gel was packed with the P-30 gel at 4 psi air pressure to a height of 50 cm and equilibrated with 250 ml of 0.2 M NaCl at a 10 ml/hr flow rate. The column temperature was at 20°C during the development. The whole hydrolysate was loaded as described and eluted with 0.2 M NaCl at a 10 ml/hr flow rate. The 3 ml fractions collected were analyzed for peptide and carbohydrate.

J. Ion Exchange Chromatography of Glycopeptides

The separation and isolation of the glycopeptides was performed by cation and anion exchange column chromatography.

The sulfonated polystyrene resin AG 50W-X2 used for the cation exchange chromatography was obtained in the hydrogen form from Bio-Rad Laboratories (lot 4399-29, B 1219). The given mesh size was 270-325 and the exchange capacity was 0.7 meq/ml resin bed. The resin and buffers were prepared according to the methods of Schroeder, Jones, Cormick and McCalla (44). The final wash was substituted for by a 0.1 M pH 2.78 pyridine buffer[2].

A 0.9 x 70 cm jacketed column (Scientific Glass Co., Bloomfield, N.J.) with a medium porosity filter was used. The column temperature was raised to 50°C and 5 ml of the pH 2.78 buffer was poured into the column. A well-mixed suspension of 1 part resin and 2 parts pH 2.78 buffer was then poured into the column and allowed to settle. When the settled resin height was 3 cm, 20 psi air pressure was applied and the

[2] This buffer was prepared by using half the quantity of pyridine required for the pH 3.1 buffer.

packing continued. To avoid layering effects additions of the mixed resin suspension were made when the top of the unsettled resin was about 5 cm from the surface of the packed resin. The final packed resin height was 60 cm.

Before loading the column it was equilibrated with 250 ml of the pH 2.78 buffer at a 30 ml/hr flow rate maintained with a Model H Milton Roy Mini Pump[3]. The peptide solutions to be loaded were concentrated to 3-4 ml by flash evaporation and adjusted to pH 2.0 with glacial acetic acid.

Separation of the peptides was effected by developing the column with a linear gradient produced by a 2-chambered Buchler Varigrad assembly. Two different buffer systems were used. System A: The mixing vessel which was connected to the pump contained 250 ml of the pH 2.78 buffer. The second vessel contained 250 ml of pH 5, 2 N pyridine buffer. System B: 250 ml of the pH 2.78 buffer was placed in the mixing vessel. The second vessel contained 250 ml of a 0.3 N pyridine buffer pH 3.6. The 0.3 N buffer was prepared by using 96.6 ml pyridine per 4 liters of buffer instead of the 645 ml required for the pH 5 buffer. The acetic acid and water proportions remained the same.

The columns were eluted with a 30 ml/hr flow rate. The column temperature was 50°C during development. The 3 ml fractions collected were analyzed for peptide and carbohydrate.

[3] Kindly loaned for use by Dr. J. T. Van Bruggen.

The strongly basic quarternary ammonium resin AG 1-X2 used was obtained in the chloride form from Bio-Rad Laboratories (lot 5572-26, B 1278). The given wet mesh range was 80-100 and the exchange capacity was 0.8 meq/ml resin bed. The resin was prepared according to the methods of Schroeder et al. (44). The resin was converted to the borate form by substituting the final wash with a 0.1 M potassium tetraborate solution. Three further washings were made with 2 liter portions of a 0.01 M, pH 9.0 potassium borate buffer. The latter buffer was prepared by mixing 0.01 M potassium tetraborate and 0.01 M boric acid solutions together to obtain the desired pH 9.0.

A 0.9 x 50 cm jacketed column (Scientific Glass Co.) with a medium porosity filter was used. The column temperature was adjusted to 25°C. The transfer tip of the column was raised and 4 ml of the pH 9.0 buffer was poured into the column. A 0.9 x 40 cm column extension (Scientific Glass Co.) was connected to the column and both were filled with a well-mixed suspension of 1 part resin and 2 parts pH 9.0 buffer. When the settled resin height reached 5 cm, the transfer tip was lowered and the resin packed with 10 psi air pressure. The final packed resin height was 42 cm. The column was then equilibrated with 250 ml of the pH 9.0 buffer at a 15 ml/hr flow rate maintained with a Milton Roy Mini Pump.

Immediately before loading the column, the top 2 cm of yellowish resin was removed and the peptide solutions applied. The peptide solutions were obtained from P-2 gel filtration chromatography columns which had been eluted with the 0.01 M, pH 9.0 borate buffer.

The peptide separation was effected by developing the column with

a 3-chamber gradient system produced by a Buchler Varigrad assembly. The mixing vessel contained 0.01 M pH 9.0 potassium borate buffer. The second vessel contained 0.01 M, pH 9.0 potassium borate buffer which was made 0.5 molar in NaCl. The addition of the NaCl lowered the pH to 8.78. The third vessel contained 0.8 M boric acid (pH 3.68). As the densities of these buffers are quite different, 100 ml of the described buffer was placed in the second vessel. Both the mixing and third vessels were balanced against the second.

This gradient system was started immediately after the column was loaded. The column was eluted at a 15 ml/hr flow rate. The column temperature was 25°C during development. The 3 ml fractions collected were analyzed for peptide and carbohydrate.

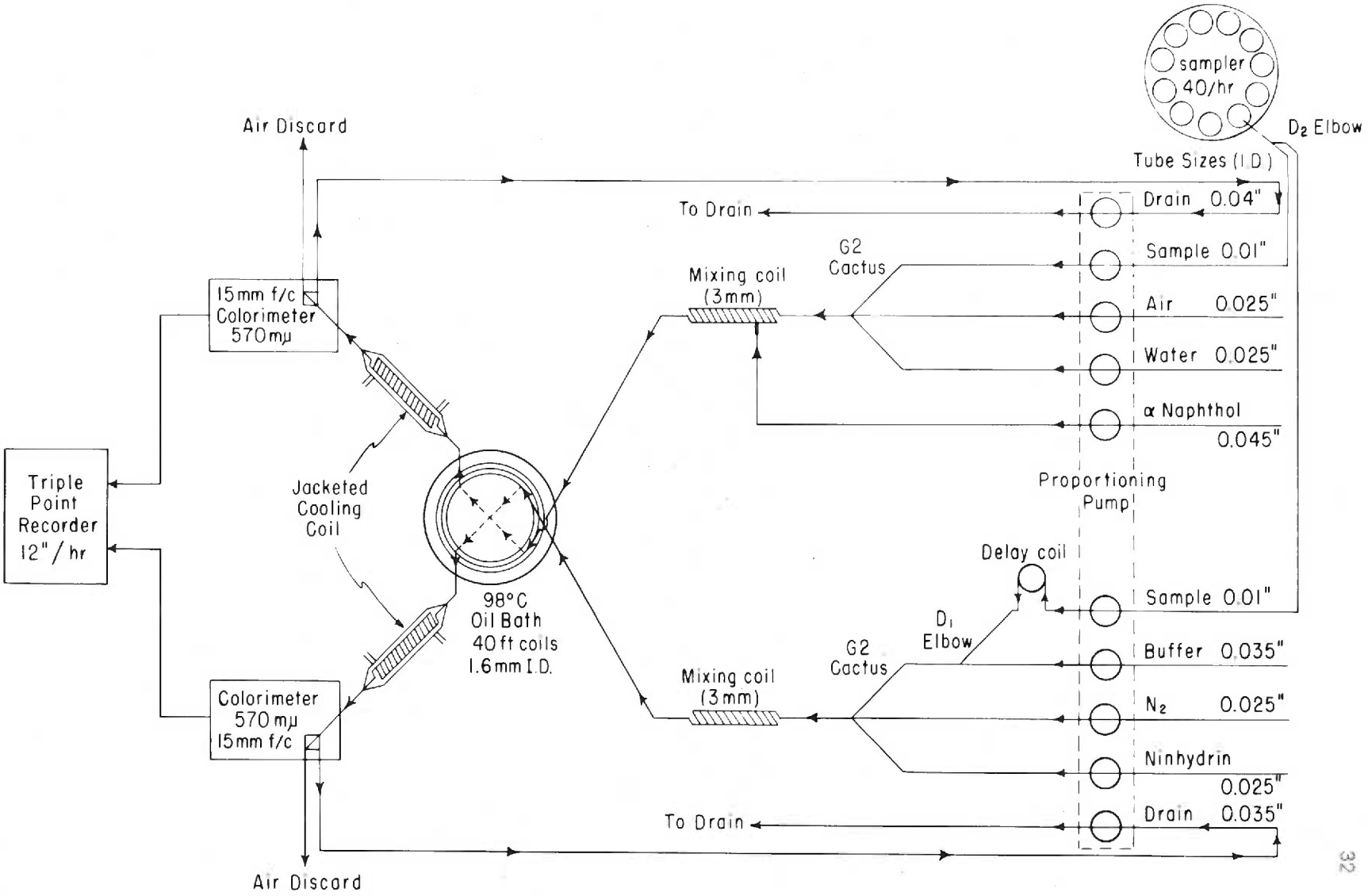
K. Automated Analyses of Column Eluate Fractions

To locate the glycopeptides in the chromatographic fractions an automated procedure employing a Technicon AutoAnalyzer (Technicon Chromatography Corp., Chauncey, N.Y.) was developed. Fig. 2 is a schematic diagram of the system used. The upper manifold (upper 5 tubes on the proportioning pump) and its train were used for carbohydrate analyses on the fractions. The carbohydrate reagent used was a 0.2% (w/v) α-naphthol solution in concentrated sulfuric acid (45). The α-naphthol was dissolved in the sulfuric acid by continual stirring and the solution was allowed to stand at room temperature in the dark for 8 hours before use.

The diluent tube on the manifold was connected to a supply of distilled deionized water. Acidflex tubing provided by Technicon was

Figure 2.

Diagram of system used for the ninhydrin and α-naphthol analyses of chromatographic fractions with the Technicon AutoAnalyzer.


sampler
40/hr
D2 Elbow
Tube Sizes (I.D.)
Air Discard
To Drain
Drain 0.04"
Sample 0.01"
Air 0.025"
Water 0.025"
α Naphthol
0.045"
G2
Cactus
Mixing coil
(3mm)
15mm f/c
Colorimeter
570mμ
Triple
Point
Recorder
12" / hr
Jacketed
Cooling
Coil
98°C
Oil Bath
40 ft coils
1.6mm I.D.
Proportioning
Pump
Delay coil
D1
Elbow
Sample 0.01"
Buffer 0.035"
N2 0.025"
Ninhydrin
0.025"
Drain 0.035"
Colorimeter
570 mμ
15mm f/c
Air Discard

used for the manifold reagent and drain tubes and for all transfer tubing. Solvaflex tubing was used for all other tubes of the system.

The lower portion of the manifold (the 5 lower tubes on the proportioning pump) and its train were used for ninhydrin analyses on the fractions. The ninhydrin and pH 5.2 citrate diluent buffer were the same as that used for the amino acid analyzer (46).

Both colorimeters were equipped with a 570 mμ filter and a #3 aperture between the light source and the flow cell. A 570 mμ filter and #2 aperture was used between the light source and the zero control unit.

The results were recorded with a triple point recorder supplied by Technicon. Two channels were used for the carbohydrate analyses and one channel was used for the ninhydrin analyses.

The varible power control on the proportioning pump was set at 100, delivering the maximum flow through the described system.

This automated method was a rapid and time-saving procedure. It was a conservative method for only 0.15 ml of each fraction was required for both analyses. The linearity of response, although not perfect, was good enough that per cent recoveries of the column load were calculable. To decrease the effect of the non-linear portion of the response curve, four standards were prepared by diluting a sample of the load material in the buffer system used for the column. Using the following equation the total amount recovered from the column was calculated.

$$P = \frac{100 \times OD_t \times V \times Cs \times D}{OD_s \times A} \qquad (5)$$

In this equation P is the per cent recovered of the load, A, in a fraction containing a volume, V. The net optical density of a given fraction is OD_t. If this fraction was diluted before the assay, then the dilution factor, D, must be used. OD_s is the optical density of the standard having the concentration C_s. The standard used is that one which matches closest the fraction being considered.

L. Amino Acid Analyses

Amino acid and glucosamine analyses of the peptides were performed according to the methods of Moore, Spackman and Stein (47) as modified by Benson and Patterson (48), using spherical bead resins for shorter development times. A Spinco Model 120 amino acid analyzer was used for the analyses. It was equipped with the long-path flow cell described by Jones and Weiss (49) for increased sensitivity. A Model CRS-12AB digital read-out system (Infotronics Corp., Houston, Texas) connected to the photocell output of the analyzer facilitated the quantitative computations of the amino acids.

The volatile buffer solutions of the isolated peptides were flash evaporated to dryness. The dried peptides were transferred from the evaporator flasks to Kontes K-56125 round bottom drying ampules (Kontes Glass Co., N.J.) with three 1 ml washes of a 9% w/v phenol solution in 6 N HCl. The ampules were evacuated with a vacuum pump and sealed. The peptides were hydrolyzed for 22 hours at 110°C in a Temp-Blok Module Heater (Lab-Line Instruments Inc., Chicago, Ill.). Following hydrolysis the hydrolysates were transferred with three 1 ml water washes to evaporator flasks and flash evaporated to dryness. The dried amino acids

were dissolved in the pH 2.2 sample dilutor buffer and 1.0 ml of their solutions were applied to the columns of the analyzer. The volume of the sample buffer used was dependent upon the relative amount of the amino acid solutions required to give optimum color production with the analyzer system.

M. Thin Layer Chromatography

For qualitative analyses of the monosaccharide components of the phytohemagglutinin, two-dimensional cellulose thin layer chromatography was employed according to the methods of Vomhof and Tucker (50). The slurries were prepared with MN-cellulose powder 300 (Macherey, Nagel and Co., Duren, Germany) and 0.37 mm layers were spread on 20 x 20 cm glass plates. Desaga/Brinkmann (Brinkmann Instruments, Westbury, N.Y.) equipment was used for the thin layer procedures.

To liberate the sugars from the protein, 30 mgs of the salt-free, lyophilized phytohemagglutinin were placed in a Kontes K-56125 round bottom drying ampule and 4.0 ml of 1.0 N sulfuric acid were added. The ampule was evacuated with a vacuum pump and sealed with a flame. The protein was hydrolyzed for 6 hours at 100°C in a Temp-Blok Module Heater. Following hydrolysis the hydrolysate was transferred to a 15 ml conical centrifuge tube with three 1 ml water washes and neutralized with solid barium carbonate. The insoluble material was removed by centrifugation at 6000 rpm for 30 minutes. The supernatent was flash evaporated to dryness and redissolved in 1 ml of a 10% (v/v) iso-propanol in water solution. As a control 3 mgs each of D (+) galactose (lot 114B-5370), D (+) xylose (lot 114B-1880), D (+) mannose (lot 55B-1930), D (+)

glucosamine-HCl (lot 74B-2040), αD (+) glucose (lot 35B-1590), D (+) fucose (lot 124B-1600) and D (-) arabinose[4] (lot 123B-1450) Sigma grade (Sigma Chemical Co., St. Louis, Mo.) were placed in an ampule and treated in a manner identical to that described for the protein. An unhydrolyzed mixed standard was prepared by dissolving 1.0 mg of each sugar in 10 ml of a 10% (v/v) iso-propanol in water solution. A separate D galactosamine-HCl (Mann Research Laboratories, Inc., New York) standard was prepared by dissolving 1 mg in the 10% v/v iso-propanol.

After the samples were applied the plates were developed two times in the first dimension with a developer described by Fink, Cline and Fink (51) which had a composition of formic acid-methyl ethyl ketone-tert.-butyl alcohol-water (15:30:40:15, v/v). The second dimension was developed a single time with a developer described by Gordon, Thornburg and Werum (52) which had a composition of 2-propanol-pyridine-acetic acid-water (8:8:1:4, v/v). Two hundred milliliters of the developer was placed in the chromatographic tank 45 minutes before the plates were added. The temperature of development was 32°C.

The method of detection used was that of Trevelyan, Procter and Harrison (53). Since the background rapidly becomes dark by the reduced silver, the sugar spots were immediately circled with a sharp pencil when they appeared. Treatment of the plates with a wash in 5% thiosulfate solution followed by a water wash was omitted because the

[4] Kindly provided by Dr. P. J. Bentley.

layer would peal from the plate. However, when this technique was tried, a permanent light brown background would result and the sugar spots could be seen.

For a permanent record the plates with their circled spots were traced onto a piece of onionskin paper.

III. RESULTS

A. Phytohemagglutinin Preparation

The general precautions of maintaining a low pH during the ammonium sulfate extraction, keeping all solutions at low temperature, preventing the production of foam, and making clean separations of precipitates from supernatents were taken. The most important step in this preparation procedure is the adjustment of the three parameters, pH, protein concentration, and conductivity, of the dialysate obtained following a 50% volume increase by the tap water dialysis of the dissolved 75% ammonium sulfate precipitate. The reported values for these parameters are a pH of 3.90, a protein concentration of 55 mg/ml and a specific conductivity of 8×10^{-4} mho cm^{-1} (15).

For the preparation used in these studies these parameters were matched satisfactorily. Following dialysis a 49.6% volume increase was observed. The dialysate was adjusted to the correct volume by the addition of distilled water. A one to one hundred dilution of the dialysate gave an optical density reading of 0.548. This is equivalent to 54.8 mg of protein per milliliter of solution. The total resistance as measured according to the methods described was 5917.4 ohms at 20°C. Using equation (1) the resistance of the cell was calculated as 1.667×10^{4} ohms. Using equation (4) the specific conductivity of the solution was calculated as 8.64×10^{-4} mho cm^{-1}. From the ten kilograms of

beans processed, 20.4 grams salt-free, lyophilized protein were obtained. This preparation will be referred to by the code F-6. The yield obtained was the same as that reported for this purification procedure (30).

To determine the purity of this preparation 10 mg of the protein was dissolved in 1 ml of a 0.1 M NaCl solution and centrifuged at 59,780 rpm in the ultracentrifuge. The ultracentrifugal pattern is given in Fig. 3a. From this pattern it can be seen that a very minor component, sedimenting faster than the larger component, is present.

The results of the starch-gel electrophoresis of this preparation at pH's 8.1 and 2.0 are given in Fig. 4 a and b. The amount of protein loaded on these gels was approximately 1.75 mg. The F-6 preparation was compared to a preparation that was shown homogeneous by 12 criteria of homogeneity (labeled E-5). A-5 is an impure fraction taken during the purification procedure. Comparison of the patterns of the F-6 with the E-5 demonstrates that the F-6 is identical to the E-5 in terms of electrophoretic homogeneity.

Further evidence for the purity of the F-6 was obtained by comparing its agglutinating activity to that of the E-5 and A-5. The results of this titration are given in Table II. Ten mg of each preparation was dissolved in 1.0 ml of a buffered pH 7, 0.14 M NaCl solution. These protein solutions were then serially

Figure 3.

Ultracentrifuge Patterns:

a) Phytohemagglutinin preparation F-6. Concentration 10 mg/ml. First frame taken 7 min. after 59,780 rpm was reached. Following frames 16 min. apart.

b) Comparison of F-6 fractions removed from fixed partition cell. Top pattern: 11.1 mg/ml solution of bottom fraction. Bottom pattern: 14.7 mg/ml solution of top fraction. Times from right to left after reaching 59,780 rpm: 5, 8, 10, 12, 14 and 16 minutes.

c) Component III from P-200 gel filtration column - concentration 10 mg/ml. First frame taken 7 min. after 59,780 rpm was reached. Following frames 16 min. apart.

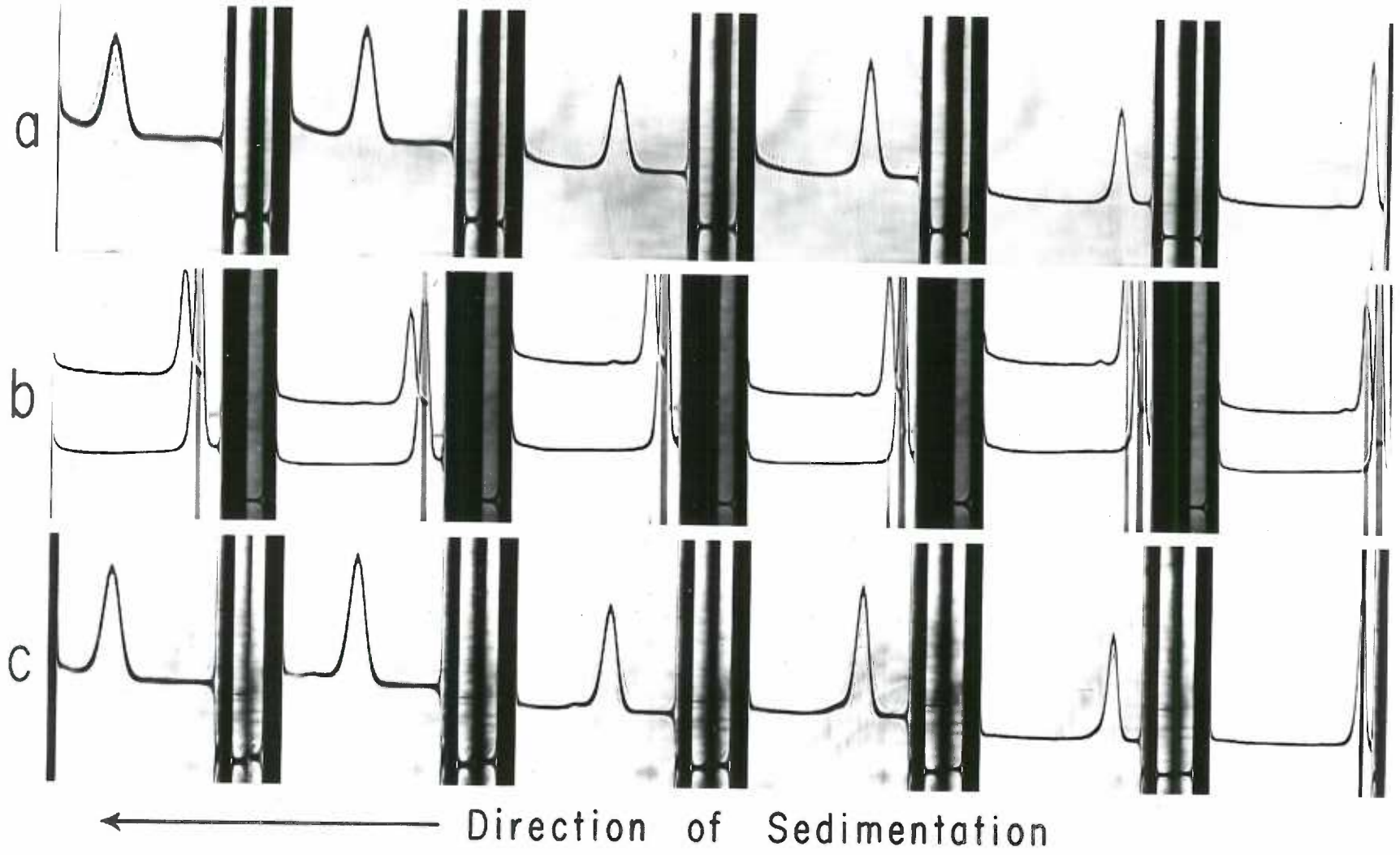
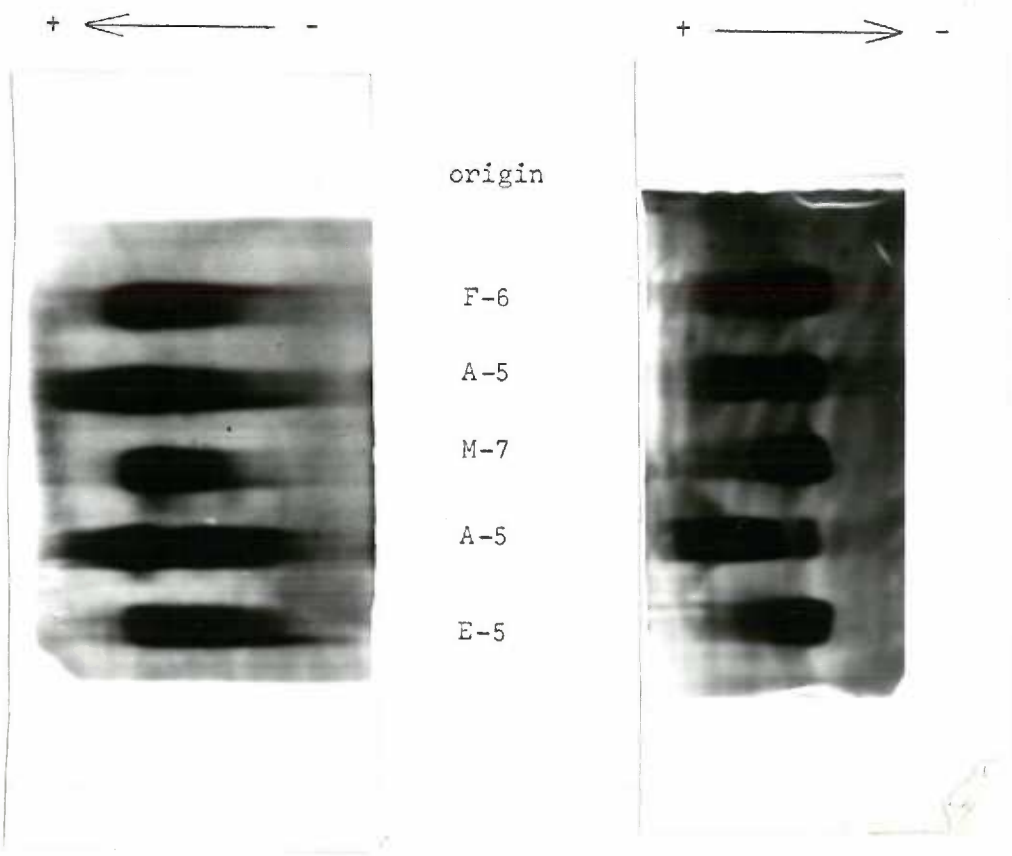

a
b
c
Direction of Sedimentation

Figure 4.

Photographs of starch gel electrophoreses of phytohemagglutinin preparations F-6, M-7 pure fractions; A-5 impure fraction; E-5 homogeneous control.

a) Discontinuous tris-borate buffer system, pH 8.1

b) Glycine-HCl buffer system, pH 2.0



a b

TABLE II

AGGLUTINATING ACTIVITY TITRATION
OF PHYTOHEMAGGLUTININ FRACTIONS

Serial Dilutions (µgm/ml)	Final Dilutions (µgm/ml)	E-5	F-6	A-5
500	250	+	+	+
200	100	+	+	+
100	50	+	+	+
50	25	+	+	+
20	10	+	+	+
10	5	+	+	+
5	2.5	+	+	±
2	1.0	+	+	-
1	0.5	+	+	-
0.5	0.25	±	±	-
0.2	0.1	-	-	-
0.1	0.05	-	-	-
0.05	0.025	-	-	-

Control (-)

diluted with buffered saline to give the concentrations in the first column. To a 0.5 ml aliquot of each dilution was added 0.5 ml of a 0.25% buffered saline suspension of fresh erythrocytes, making the final concentrations given in the second column. The tubes were incubated over night at 20°C and given positive (+) or negative (-) scores according to the described method. As a negative control 0.5 ml buffered saline was added to 0.5 ml of the 0.25% erythrocyte suspension. As can be judged by these titres on the basis of the lowest concentration which causes agglutination (i.e., 0.25 µgm) both F-6 and E-5 appear to be identical. Thus the preparation appeared pure enough to begin studies on the glycopeptides. The small component observed in the ultracentrifugal analyses was regarded as a negligible contaminant.

When the tryptic hydrolysate of F-6 was chromatographed on the AG 50W-X2 cation exchange column, employing system A as a developer, a component was eluted with the buffer front from the column which contained a considerable amount of carbohydrate but little or no ninhydrin color. In Fig. 5 this component is labeled "PSC". A similar component devoid of ninhydrin color but containing carbohydrate was eluted from the 2.2 x 85 cm P-30 preparative column (Fig. 6). This component appeared after 80 ml of the 0.2 M NaCl developer had been passed through the column, whereas the remainder of the carbohydrate did not begin to appear in the column effluent until 165 ml of the developer was collected. From this evidence it

Figure 5.

Separation of peptides in a whole tryptic hydrolysate of F-6 on AG 50W-X2 using developer system A.

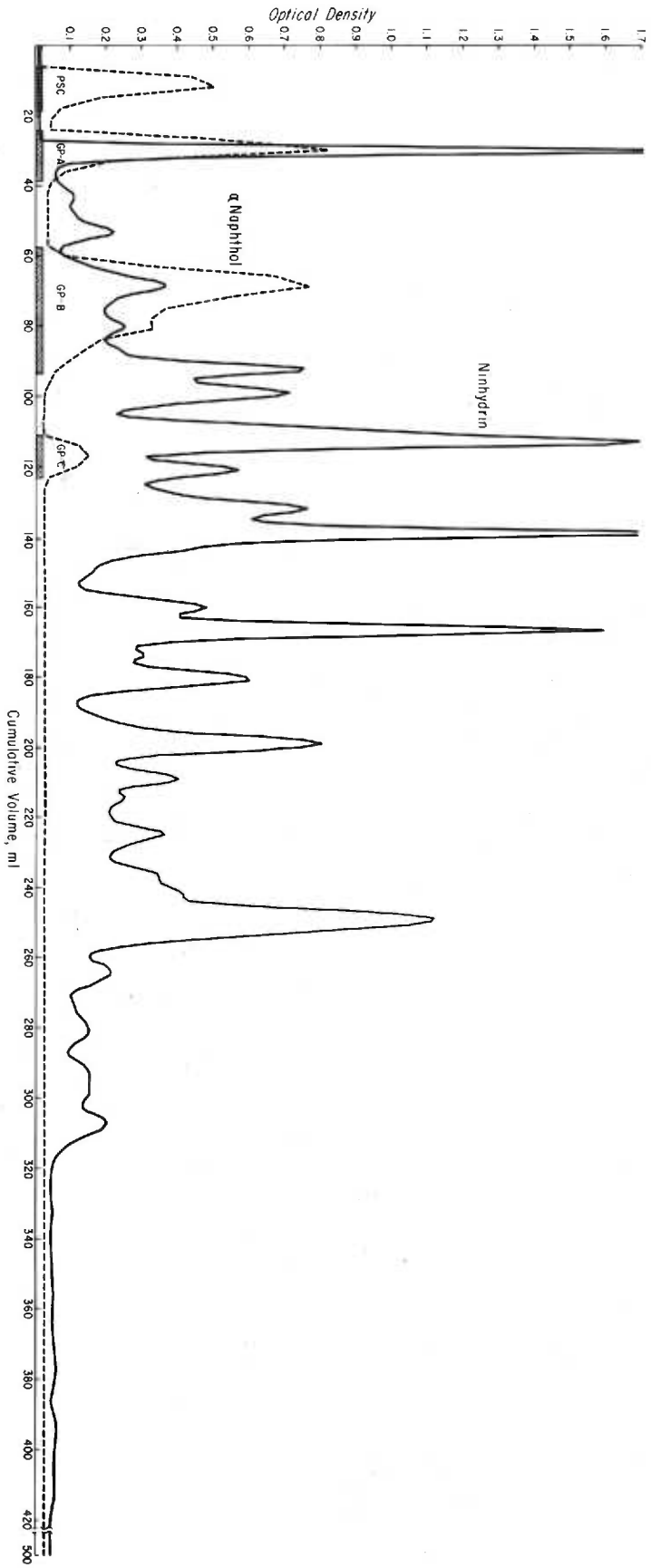
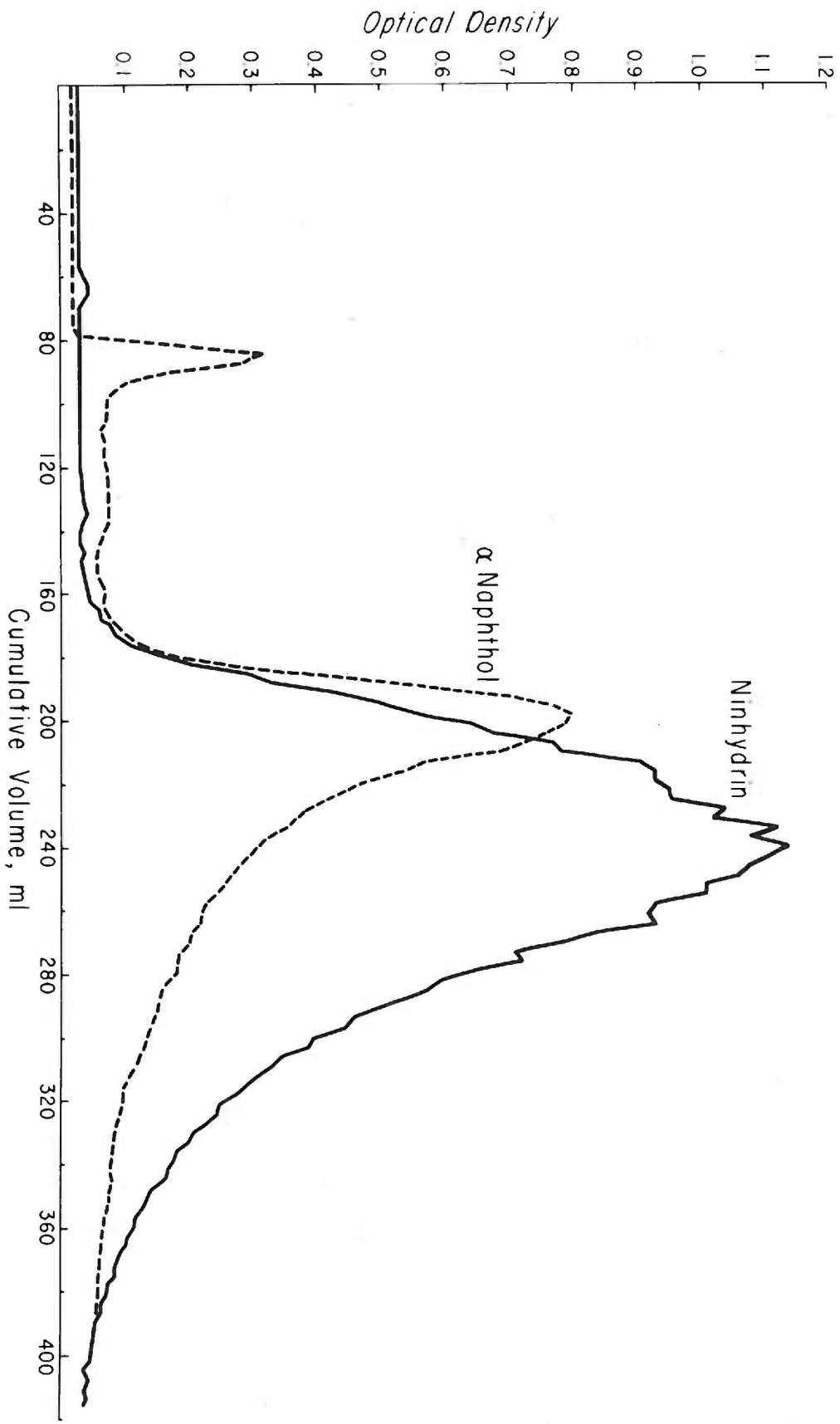

Optical Density
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
1.1
1.2
1.3
1.4
1.5
1.6
1.7
PSC
GP A
GP B
GP C
α Naphthol
Ninhydrin
Cumulative Volume, ml
20
40
60
80
100
120
140
160
180
200
220
240
260
280
300
320
340
360
380
400
420
500

Figure 6.

Chromatogram from P-30 polyacrylamide gel filtration of a whole tryptic hydrolysate of T-6. Column 2.2 x 85 cm. Developer 0.2 M NaCl


Optical Density
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
1.1
1.2
40
80
120
160
200
240
280
320
360
400
Cumulative Volume, ml
α Naphthol
Ninhydrin

was postulated that the PSC was a large molecular weight polysaccharide contaminant in the F-6 preparation. This contaminant represented 5% of the total carbohydrate present in the preparation. To conclusively demonstrate that this component was in fact a contaminant, the fast sedimenting component observed by ultracentrifugal analyses was separated from the larger, slower component by ultracentrifugation employing the fixed partition cell. A 40 mg/ml solution of the F-6 was prepared in 0.1 M NaCl. Two milliliters were placed in the 30 mm fixed partition cell and centrifuged at 50,740 rpm. When the major component had sedimented to a position half way between the miniscus and the partition, the ultracentrifuge was stopped and the solutions above and below the partition were removed and kept separate. Three runs of this type were performed.

The top and bottom fractions were then analyzed in separate cells. A 1° wedge window was used so that the solutions could be compared under identical conditions. In Fig. 3b the upper pattern is that of the bottom fraction taken from the partition cell. The lower pattern of the plate is that of the top fraction. The concentrations of the top and bottom fractions as measured by the optical density of their solutions were 14.7 and 11.1 mg/ml, respectively. As can be observed, the fast minor component is no longer present in the top fraction, whereas its concentration in the bottom fraction appears to be greater than that of the original F-6 shown in Fig. 3a.

The two fractions were hydrolyzed with trypsin and their hydrolysates were chromatographed on the 2 x 50 cm column of P-30 polyacrylamide gel. In both cases 45.3 mg of protein, as measured by the optical densities of their solutions, were hydrolyzed. The whole hydrolysate of each fraction was placed on the P-30 column and eluted with 0.2 M NaCl at a flow rate of 10 ml/hr. Only the carbohydrate analyses were performed on the fractions. The chromatograms of the two fractions are given in Fig. 7. Chromatogram (a) is that of the top fraction and (b) is that of the bottom fraction. Although a small amount of the contaminant was still present in the top fraction, considerably more was found in the bottom fraction. This evidence indicates that the first carbohydrate component of the tryptic hydrolysate of F-6 eluted from the P-30 polyacrilamide gel columns originates from the small contaminant of F-6 observed by ultracentrifugation.

To study the carbohydrate portion of the F-6 it is evident that this contaminant must be removed from the preparation. This separation was performed with the large 2.2 x 85 cm preparative column packed with P-200 polyacrylamide gel. The operating range of this gel was given as 80,000 to 130,000 molecular weight. Both 100 and 200 mg loads were applied to the column and in both cases the results were the same. Fig. 8 is a representative chromatogram of a run in which a 100 mg load was used. It can be seen that a contaminant, I, very rich in carbohydrate is readily

Figure 7.

Chromatograms of whole tryptic hydrolysates of 43 mg of the F-6 fractions removed from the fixed partition ultracentrifuge cell. a) Top fraction b) Bottom fraction Column 2 x 5 cm. Developer 0.2 M NaCl.

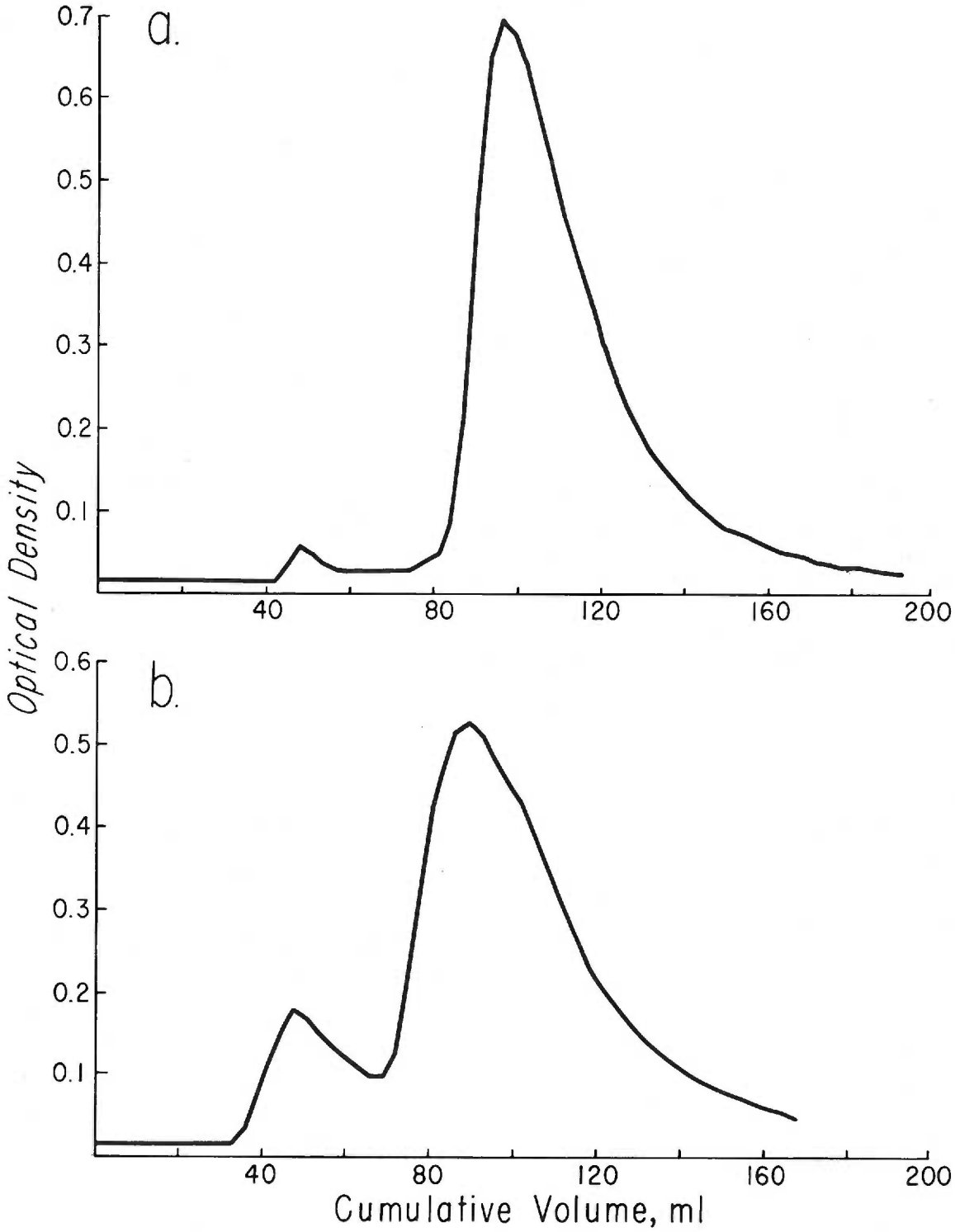
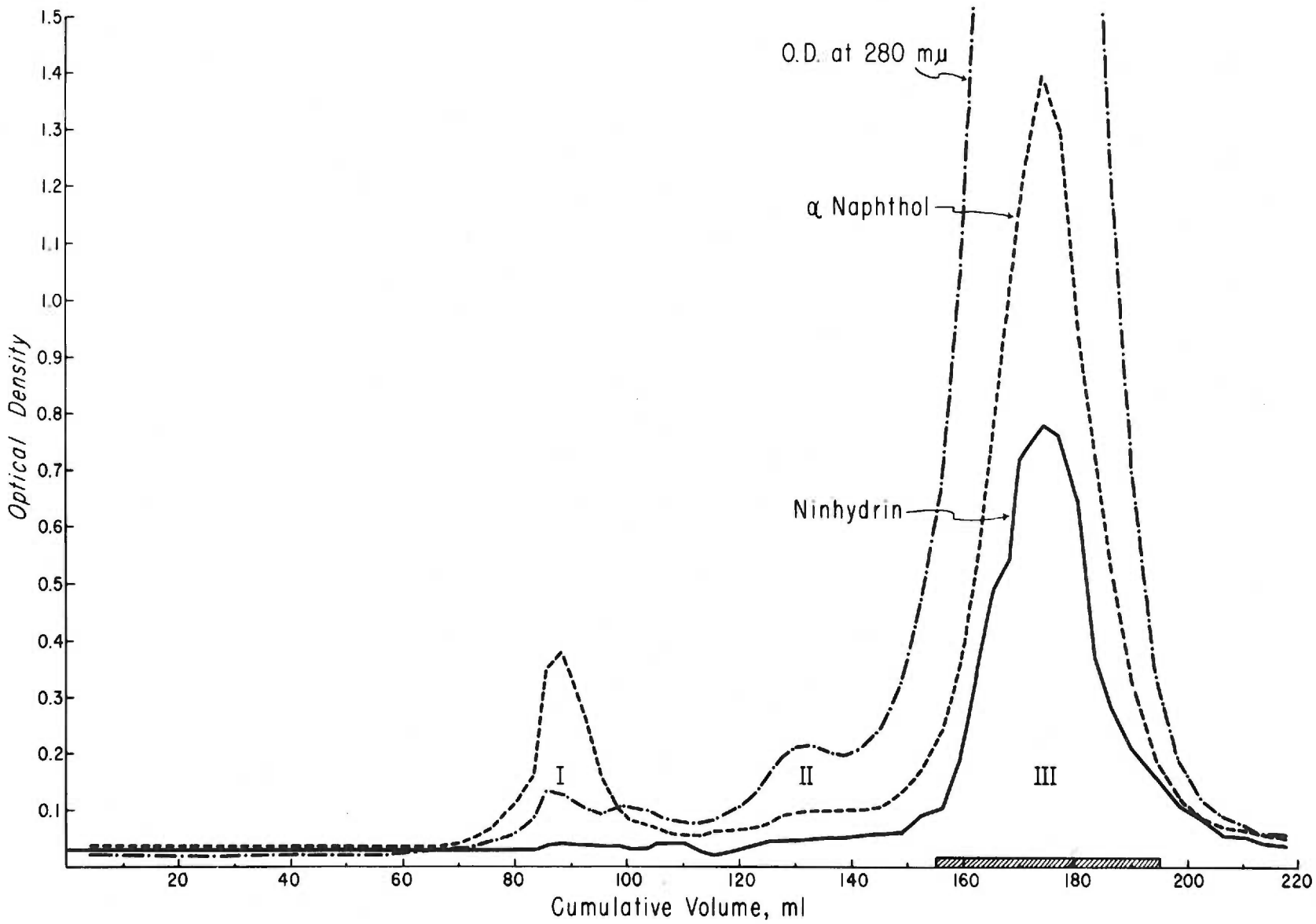

a.
b.
0.7
0.6
0.5
0.4
0.3
0.2
0.1
40
80
120
160
200
Optical Density
Cumulative Volume, ml

Figure 8.

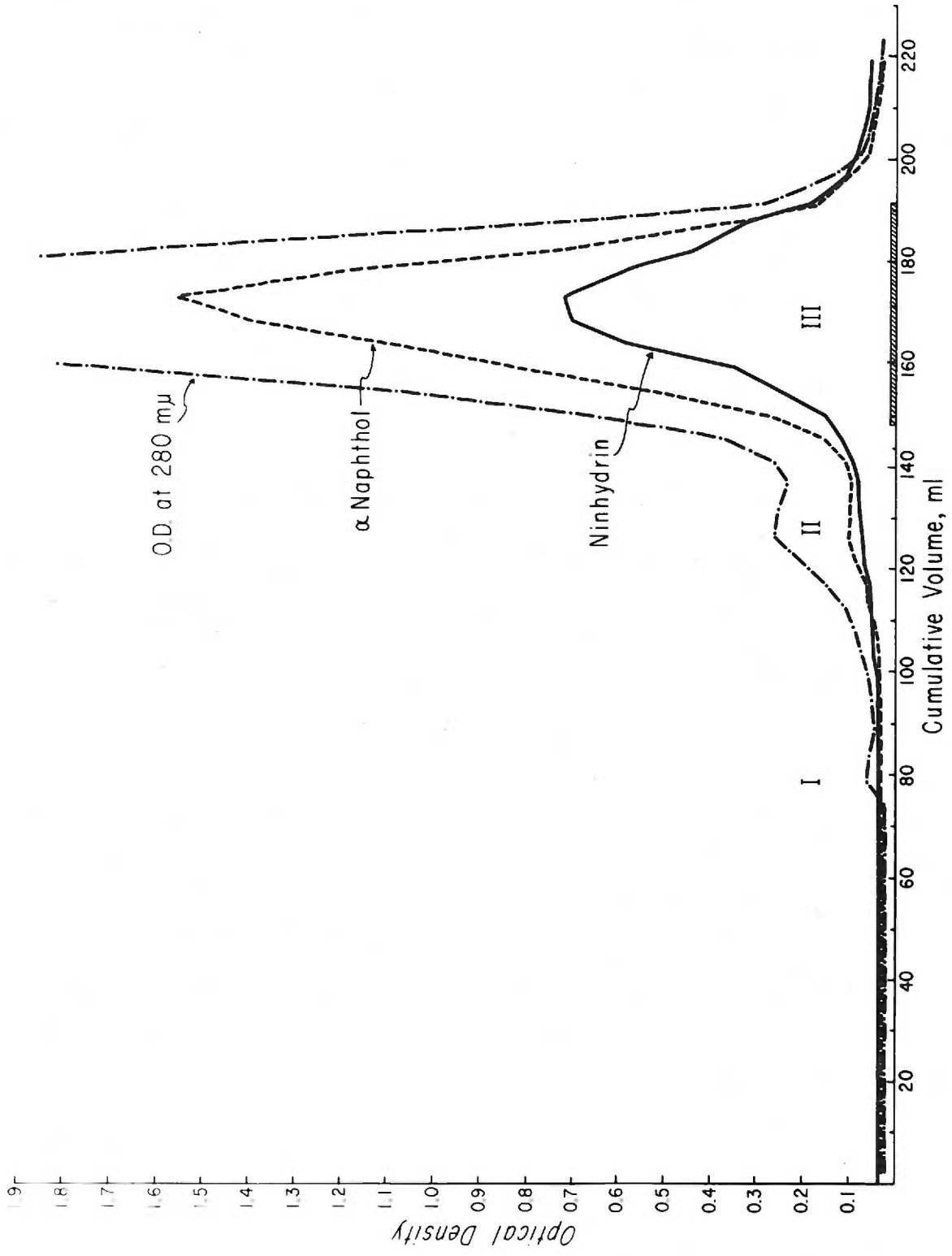
Chromatogram of the P-200 polyacrylamide gel filtration of F-6. Load: 100 mg in 3 ml. Column 2.2 x 85 cm. Developer: 0.2 M NaCl. Hatched rectangle marks fractions pooled.


O.D. at 280 mμ
α Naphthol
Ninhydrin
Optical Density
Cumulative Volume, ml
I
II
III
1.5
1.4
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
20
40
60
80
100
120
140
160
180
200
220

separated from the protein, III. This contaminant is presumably identical to the fast sedimenting component in the ultracentrifuge, which is responsible for the appearance of the PSC component in the tryptic hydrolysates of F-6. Another component, II, with a molecular weight slightly larger than that of component III, but of very similar carbohydrate content, was also observed. One of the fractions collected from the maximum of each component was tested for agglutinating activity by placing a drop of the fraction on a glass slide and adding to it a drop of a 1:1 dilution of whole blood with buffered saline. No agglutination was observed in component I. The activity of component III was much greater than that of component II. Thus component III was taken to be the phytohemagglutinin. The fractions of III included under the hashed rectangle (Fig. 8) were pooled, concentrated by ultrafiltration, dialyzed against three changes of distilled, deionized water and lyophilized. One hundred milligrams of the lyophilized material was dissolved in 3 ml of 0.3 M NaCl and rechromatographed on the P-200 column. Fig. 9 is the chromatogram of this run. Although contaminant I was no longer present, a small peak of ninhydrin color and 280 absorbing material was present in its position. Components II and III were both present in what appeared to be the same relative concentrations as before. Ten milligrams of the lyophilized component III was dissolved in 1 ml of 0.1 M NaCl and analyzed in the ultracentrifuge. The ultracentrifugal pattern

Figure 9.

Chromatogram of the rechromatography of component III on the P-200 polyacrylamide gel column. Load: 100 mg in 3 ml. Column: 2.2 x 85 cm. Developer: 0.2 M NaCl.


O.D. at 280 mμ
α Naphthol
Ninhydrin
I
II
III
Optical Density
Cumulative Volume, ml

of this analysis is shown in Fig. 3c. Two components were again present. By comparing the patterns and the time interval between each frame it can be seen that the minor sedimenting component in Fig. 3c did not move away from the major component as rapidly as the minor contaminant did in Fig. 3 a and b. Thus the ultracentrifugal analyses were consistent with the results obtained by the P-200 gel filtration.

A comparison of the AG 50W-X2 cation column chromatograms of the tryptic hydrolysates of F-6 and of component III revealed that the only difference between them was that the PSC was missing from the III preparation. Therefore, the III was used for all further studies.

B. Tryptic Hydrolyses

The procedure for the tryptic hydrolysis was adapted for the phytohemagglutinin after the hemoglobin method of Ingram (42). Numerous workers have observed the phytohemagglutinin to be relatively stable to heat (15,54). For this reason a 30 minute denaturation period in a boiling water bath was used instead of 10 minutes. The condenser arrangement which was described under methods was used to prevent evaporation of the buffer. The protein was dissolved in the 0.05 M pH 6.6 acetate buffer. In this buffer the protein was partially dissolved and partially suspended as a fine precipitate. When this solution was placed in the boiling water bath the fine precipitate was dissolved and the solution became very

clear when a temperature of about 85°C was reached. The protein coagulated and precipitated out of solution as the temperature increased.

Optimum hydrolysis by acid activated trypsin requires a pH near 8. For each peptide bond that is cleaved a proton is liberated into the solution. To maintain the pH 8 optimum, constant addition of base is required. Thus the progress of the hydrolysis was observed by the rate at which base was added to the hydrolysate. The hydrolysis was considered complete when no further additions of base occurred over a three-hour period.

The tryptic hydrolyses were carried out using 100 or 200 mg portions of the phytohemagglutinin. Approximately 8 hours after the beginning, the hydrolysate was a clear, yellow-brown solution. At this point base was still being added at a frequent rate and, therefore, the hydrolysis was allowed to continue.

During the early part of this work the end point of the hydrolysis was difficult to judge. Hydrolyses allowed to continue for as long as 72 hours were still being titrated with base and a 3-hour period in which no further base was added was never achieved. In some cases the hydrolysates would become cloudy after having been clear solutions for about 12 hours.

Two possibilities were considered that might cause this difficulty. The first was that the hydrolysate was taking up carbon dioxide from the atmosphere and the carbonic acid produced was

being titrated by the base. However, the rate of base addition was greater than one would expect from carbon dioxide dissolving in a solution at 40°C and when the pH stat vessel was sealed off from the atmosphere the problem was uncorrected. The second possibility was that bacterial contamination might produce acidic metabolic products which would be titrated by the base. A sample of a 24 hour hydrolysate was removed from the pH stat vessel and spread on the media described (vide supra). After 9 hours of incubation at 37°C all plates were covered completely by bacteria. The bacteria were found to be motile, Gram negative bacilli. The fermentation tests were negative to both gas and acid production. After 24 hours of incubation the TSI slants were characterized by an orange-red butt. From the results of these tests the bacteria were tentatively identified as the enteric bacteriam, Alcaligenes fecalis with the possibility of the pseudomonads not ruled out.

The results of the antibiotic sensitivity tests are given in Table I. After 24 hours of incubation at 37°C the greatest area of inhibition was around the tetracycline sensitivity disc.

The amount of tetracycline to be added to the hydrolysate was determined by calculating the ratio of the amount of tetracycline on the sensitivity disc to the volume encompassed by the circle of inhibition. The area inhibited was 2 cm in diameter and the gel was 0.5 cm thick. The volume of gel inhibited was calculated as 1.57 ml and the amount of tetracycline in this volume was 5 µgm. Therefore,

TABLE I

ANTIBIOTICS TESTED FOR BACTERIAL SENSITIVITY

Antibiotic	Amount Present	Incubation* Time 9	24	Source
Coly-Mycin	2 µgm	-	-	Balt. Biol. Lab. (Baltimore, Md.)
Kanamycin	30 µgm	+	+	" " "
Chloromycetin	5 µgm	+	+	Difco Lab. (Detroit, Mich.)
Novobiocin	5 µgm	m	m	" "
Ristocetin	5 µgm	-	-	" "
Tetracycline	5 µgm	+	++	" "
Penicillin	2 units	-	-	" "
Vanomycin	5 µgm	-	-	" "
Neomycin	5 µgm	-	-	" "

* + = inhibition of growth

\- no inhibition of growth

m = minimal growth

a concentration of 5 µgm/1.57 ml or 3.2 µgm/ml of hydrolysate was used. With that concentration of tetracycline in the hydrolysates no further problems with bacteria were encountered. A decisive end point occurred after 24 hours of hydrolysis.

Referring again to Ingram's procedure, immediately after the hydrolysis the hydrolysate was adjusted to pH 6.5 and boiled for four minutes to inactivate the trypsin. Following this procedure, a large quantity of a gelatinous precipitate was obtained after boiling the hydrolyzed phytohemagglutinin. This precipitate was insoluble in water and 50% acetic acid. The precipitate and supernatent were separated by centrifugation at 37,000 x g for 30 minutes. Both of these fractions contained carbohydrate. It was felt undesirable to make a fractionation of the hydrolysate at this stage and therefore, the pH adjustment and boiling steps were eliminated from the procedure. For the removal of the trypsin the whole hydrolysate was subjected to gel filtration.

C. Gel Filtration of Tryptic Hydrolysates

In addition to the removal of the trypsin from the hydrolysate, the use of the polyacrylamide gel filtration made it possible to change the buffer system of the hydrolysate as well. The possibility also existed that some separation of the glycopeptides from each other or the separation of the non-glycopeptides from the glycopeptide fraction could be achieved by this method. When the whole hydrolysate was placed on the 2.2 x 85 cm P-30 preparative column, a

chromatogram like that in Fig. 6 was obtained. Disregarding the PSC peak, the peptides were eluted as a single broad component with the glycopeptides predominantly in the front of the peak. That a buffer change did occur with the glycopeptide fraction may be assumed because the smaller non-glycopeptides which must be larger than the buffer ions were being eluted after the glycopeptide fraction.

The PSC component was found to be eluted after 83 ml of developer had passed through the column, which corresponds with the 80 ml found when the phytohemagglutinin or normal human hemoglobin was used to determine the void volume. From this it can be seen that the PSC component can be used as an internal control for the void volume and that its molecular weight must be greater than 20,000. It also can be seen that the glycopeptides were retarded by the P-30 column for their elution volume was 184 ml. For this type of chromatography the R_f value is defined by the quotient, void volume/elution volume (55). Using the above elution figures the calculated R_f for the glycopeptides that first appear from the column was 80/184 or 0.45.

Similar chromatograms were obtained when the whole hydrolysate was placed on the 2 x 50 cm column of P-30 gel (Fig. 7). The position of the PSC peak in Fig. 7b indicates that the void volume was 42 ml. The elution volume of the first glycopeptides to appear was 78 ml. The R_f for the glycopeptides on this column was 42/78

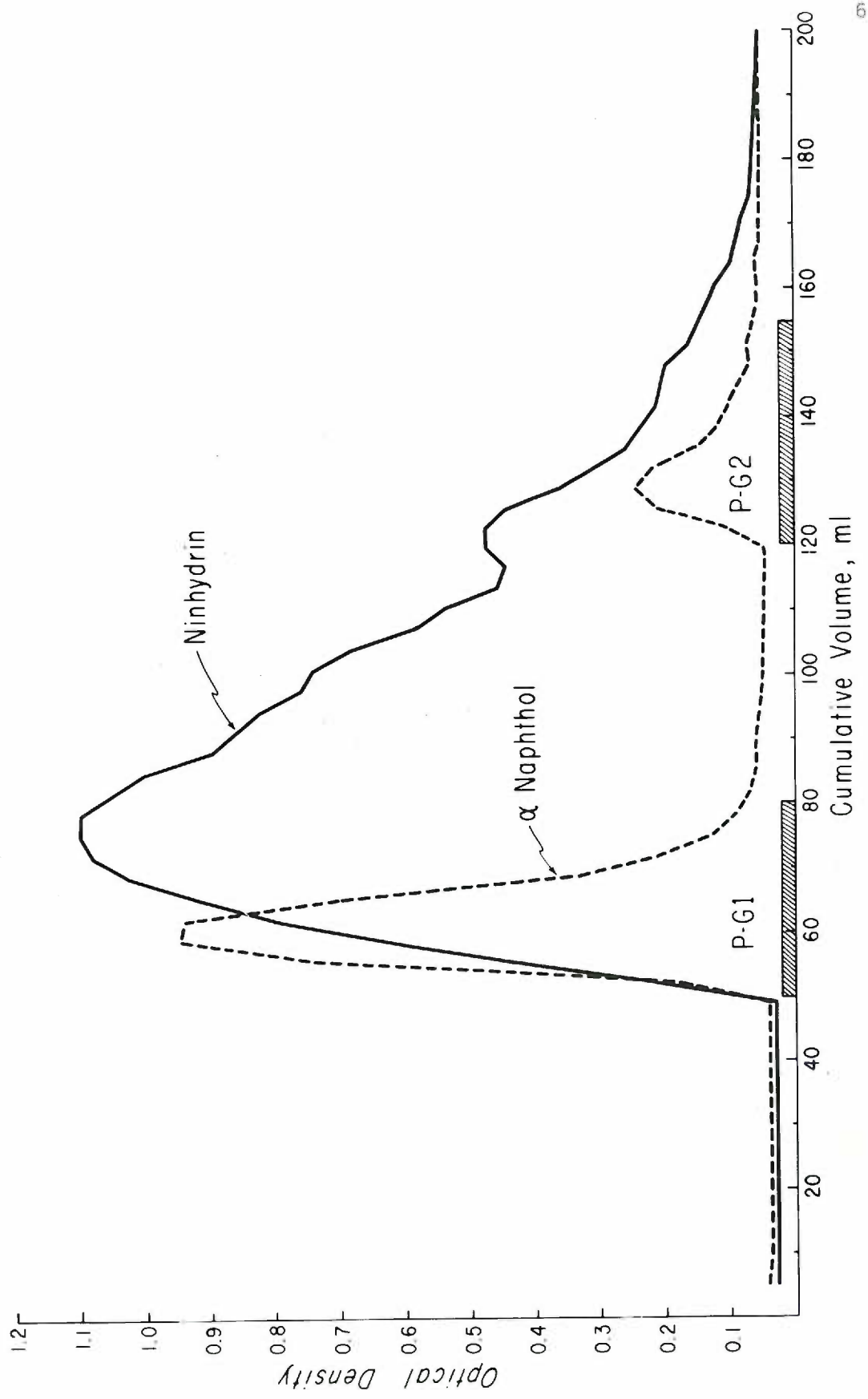
or 0.54. A plot of the log molecular weight versus R_f shows that substances with R_f values of 0.6 and lower have corresponding molecular weights of 10,000 and lower (55). The molecular weight of trypsin has been given as 23,800 (56). From the plot, trypsin should have had a R_f of 0.84. Therefore, the trypsin must have been removed from the glycopeptides as it should have been eluted somewhere between the PSC peak and the first glycopeptides. However, it was not possible to detect the trypsin because of its low concentration in the hydrolysate and to the sensitivity of the ninhydrin method.

The fact that no distinct separation of the peptides occurred is reasonable if one considers that the hydrolysate is a mixture of peptides which have a broad range of molecular weights.

A disadvantage of the P-30 gel system was the time required to elute the peptides from the column. With the only advantage of this system being its ability to change the buffer system of the peptide mixture, the P-30 was substituted by the 2 x 50 cm column of P-2 gel. The time of elution of the peptides from the P-2 column was 1.5 hours as compared to the 26 hours required for the 2 x 50 cm column of P-30. The first buffer system used for the P-2 gel column was the beginning pH 9 borate buffer employed in the AG 1-X2 anion exchange chromatography. In this buffer the peptides were soluble. A 5 ml portion of the whole hydrolysate produced a chromatogram similar to that in Fig. 10 with the exception that the

Figure 10.

Chromatogram of whole tryptic hydrolysate of component III on a P-2 polyacrylamide gel column. Developer: 0.5 M acetic acid. Column: 2 x 50 cm.


Optical Density
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
20
40
60
80
100
120
140
160
180
200
Cumulative Volume, ml
Ninhydrin
α Naphthol
P-G1
P-G2

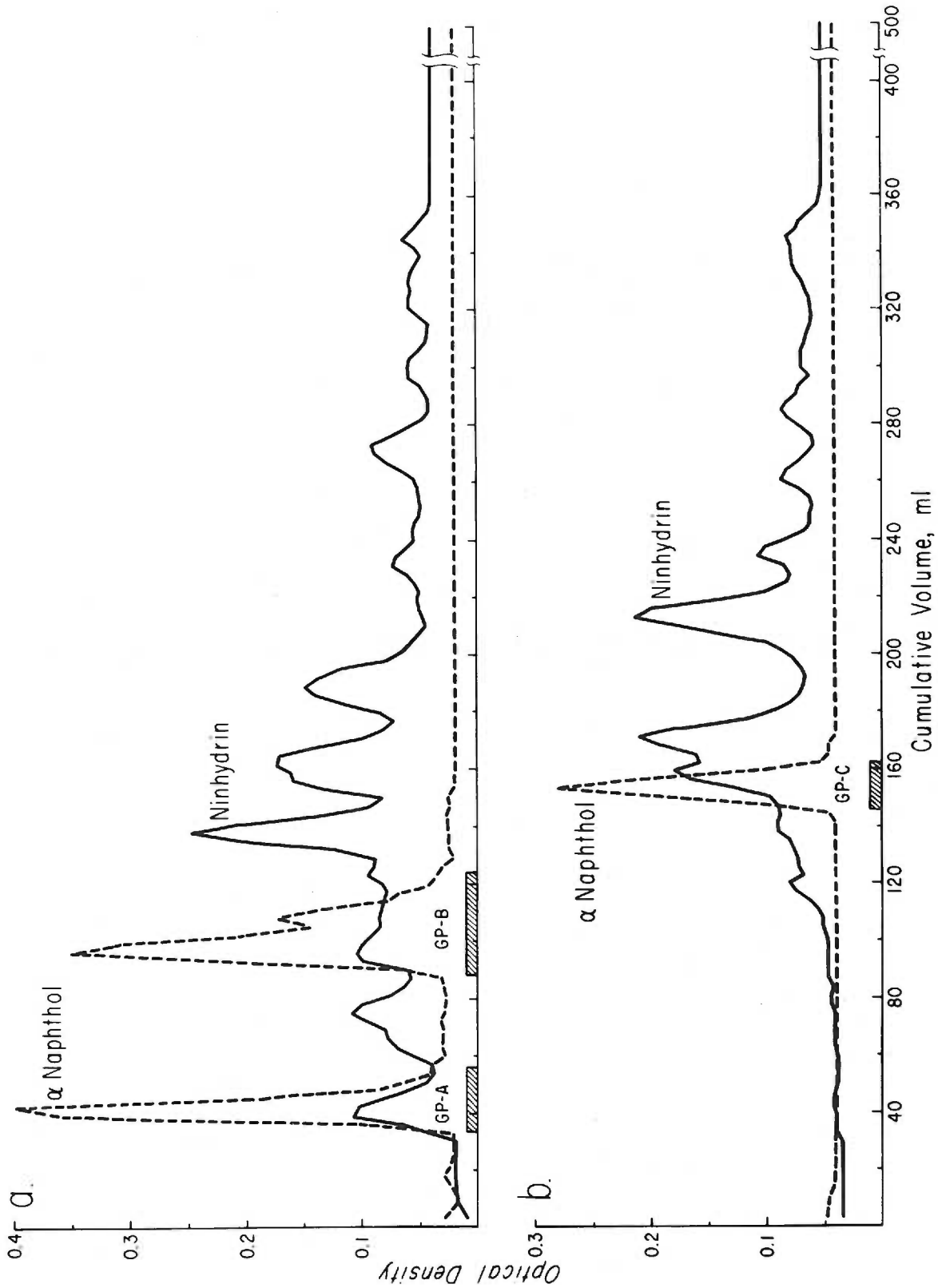
second carbohydrate peak was missing. In those runs where the PSC was present, there was no separation of it from the larger glycopeptides. Assuming that no interaction between the gel and the peptides occurred, the void volume of this column was 45 ml. By pooling just the tubes containing carbohydrate a large amount of the smaller non-glycopeptides were removed. Thus the 45-78 ml fraction was used for the AG 1-X2 chromatography.

For the AG 50W-X2 cation exchange chromatography it was desirable to have the glycopeptides in a pH 2 system before loading the column. Both 50% and 0.5 M acetic acid solutions were used. The P-2 gel column was equilibrated with 1 liter of the acetic acid solution and then loaded with a 5 ml portion of the whole hydrolysate. Fig. 10 is a chromatogram of the elution of the peptides from the P-2 column with 0.5 M acetic acid. The same results were obtained with the 50% acetic acid. With this system the two carbohydrate-containing components P-G1 and P-G2 were obtained. The fractions included under the hatched rectangles were pooled separately and chromatographed on the AG 50W-X2 cation exchange column using system A as the developer. By comparing Fig. 11 a and b with the chromatogram of the whole hydrolysate in Fig. 5 it was apparent that the P-G1 from the P-2 gel column contained the GP-A and GP-B peptides whereas the P-G2 from the P-2 gel contained the GP-C.

Making the same assumption as before, the void volume of the P-2 column was about 45 ml. The P-G2 first appeared in the effluent

Figure 11.

Chromatograms from the AG 50W-X2 chromatography of the glycopeptide fractions P-G1 and P-G2 obtained from P-2 gel column. Developer system A. a) P-G1. b) P-G2.


a.
α Naphthol
Ninhydrin
GP-A
GP-B
b.
α Naphthol
Ninhydrin
GP-C
Optical Density
Cumulative Volume, ml
0.4
0.3
0.2
0.1
0.3
0.2
0.1
40
80
120
160
200
240
280
320
360
400
500

at about 120 ml. Using these figures, the R_f of the P-G2 was calculated as 45/120 or 0.38. Referring again to the log M.W. vs R_f plot, substances with R_f values of 0.52 and lower have corresponding molecular weights of 150 and lower. At later stages of purification the P-G2 was found to produce both ninhydrin and carbohydrate color. Assuming the P-G2 contained only one monosaccharide residue and one amino acid residue, it would have a molecular weight greater than 200. It was concluded that more than filtration was taking place and that an adsorption phenomenon was occurring on the P-2 gel. The P-G1 fraction being eluted from the column in the void volume had a R_f of 1.0. From the plot substances with a R_f 1.0 have a corresponding molecular weight of 3000 or more. As earlier noted the glycopeptides had a R_f of 0.54 on the P-30 gel which corresponds to having molecular weights below 10,000. Thus it appears that the tryptic glycopeptides of the phytohemagglutinin have molecular weights in the range of 3000 to about 9000.

D. AG 50W-X2 Cation Exchange Chromatography

During the early part of this work the supernatents from the boiled tryptic hydrolysates were flash evaporated down to about 4 ml and the concentrate was adjusted to pH 2 with 1 N HCl. The pH adjustment caused a white precipitate to form which was removed by centrifugation before the solution was loaded on the cation exchange column. The peptides were eluted from the column with

developer A. The glycopeptides shown in Fig. 5 were all present in the solution and a 100% recovery of the carbohydrate load was obtained. The gelatinous precipitate recovered after the hydrolysate was boiled was resuspended in the beginning buffer of developer A, centrifuged and the supernatent was chromatographed on the same system. Repeating this procedure three times resulted in an identical glycopeptide pattern, with the relative amount of carbohydrate becoming smaller with successive washes. The washed gelatinous precipitate yielded a positive carbohydrate test.

A similar gelatinous residue was produced when the whole hydrolysate was placed on the P-2 polyacrylamide gel columns developed with the acetic acid solutions. However, the carbohydrate loaded on these columns was fully recovered and when chromatographed on the cation exchange column only those glycopeptides shown in Fig. 5 were obtained.

The GP-A from a number of runs was pooled and concentrated by flash evaporation. During the concentration a white precipitate formed that was insoluble in the beginning buffer of developer A. A sample of the suspension of the concentrate was loaded on the cation exchange column and eluted with the beginning buffer only. A small peak was eluted that appeared directly after the GP-A. In some chromatograms of the whole hydrolysate a similar peak was observed as a pronounced shoulder to the GP-A. When the precipitate was removed from the GP-A concentrate and the supernatent chromatographed, the small peak was missing from the chromatogram. The

precipitate was resuspended in the beginning developer A buffer, centrifuged and the supernatent chromatographed. From the chromatogram it was observed that a very small amount of GP-A was present in the supernatent. The washed precipitate did not appear to have decreased in its packed volume. Thus it appears probable that more than one glycopeptide was present in GP-A.

From the chromatogram in Fig. 5 it was apparent that GP-B was also heterogeneous. This glycopeptide fraction was concentrated by flash evaporation and rechromatographed on the cation exchange column using developer B. From the chromatogram in Fig. 12 it was observed that the GP-B fraction contained at least five glycopeptides. Thus, if two tryptic glycopeptides are present in GP-A, five in GP-B and one in GP-C, then there are eight tryptic glycopeptides present in the phytohemagglutinin.

E. AG 1-X2 Anion Exchange Chromatography

The glycopeptide fraction obtained from the P-2 gel column developed with the 0.01 M, pH 9.0 borate buffer was concentrated to a volume of 4 ml by flash evaporation. The concentrate was loaded on the column and eluted with the buffer gradient system described (vide supra). The results of this chromatography are shown in Fig. 13. The same figure shows the gradient curves for the borate ion and NaCl concentrations. The concentrations for these curves were calculated using a theoretical equation (57).

Figure 12.

Chromatogram of the rechromatography of GP-B on AG 50W-X2 using developer system B. Since GP-B_1 and GP-B_2 were not well separated, they were pooled together and given the coded GP-$B_{1\&2}$.

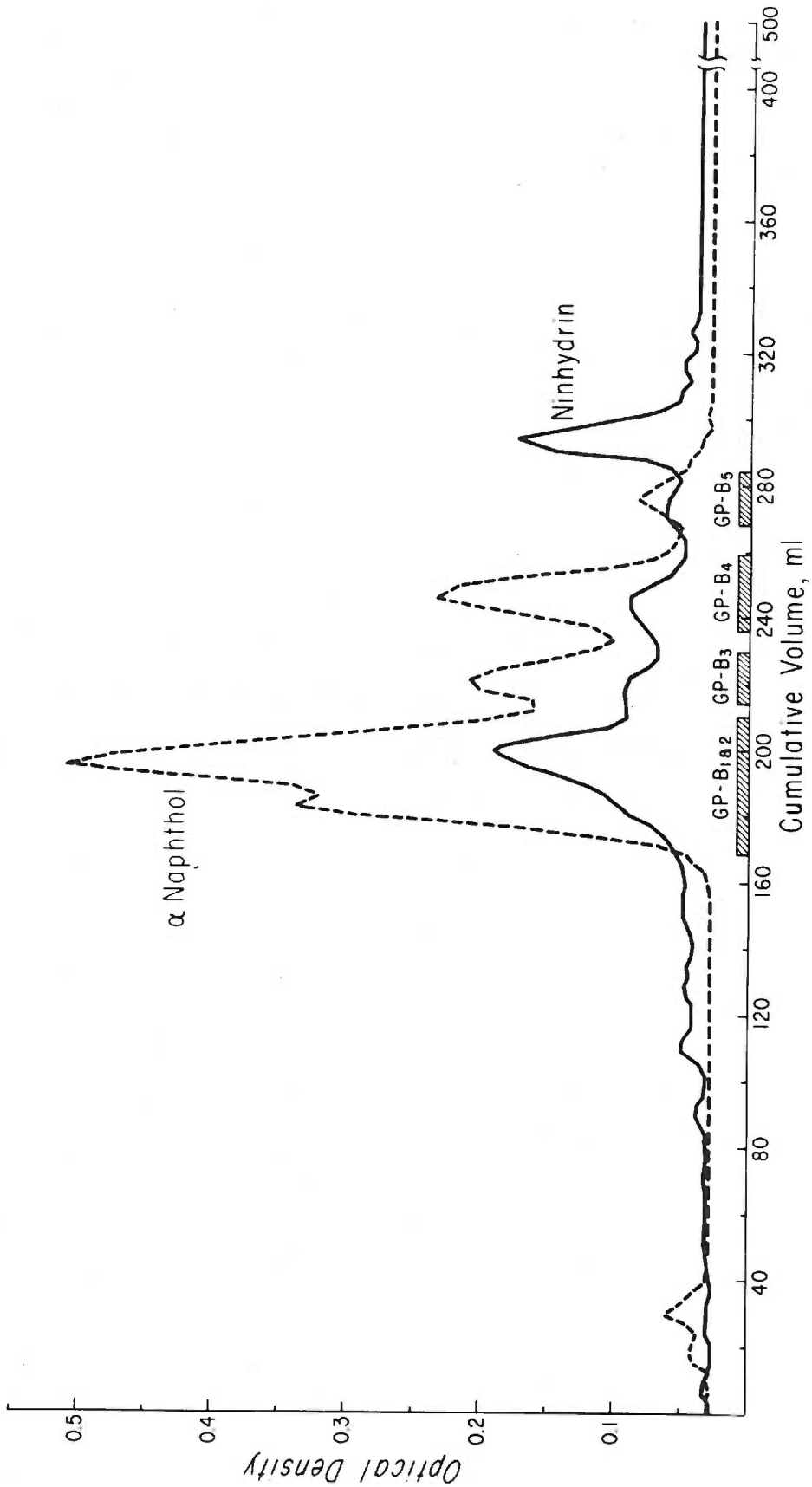
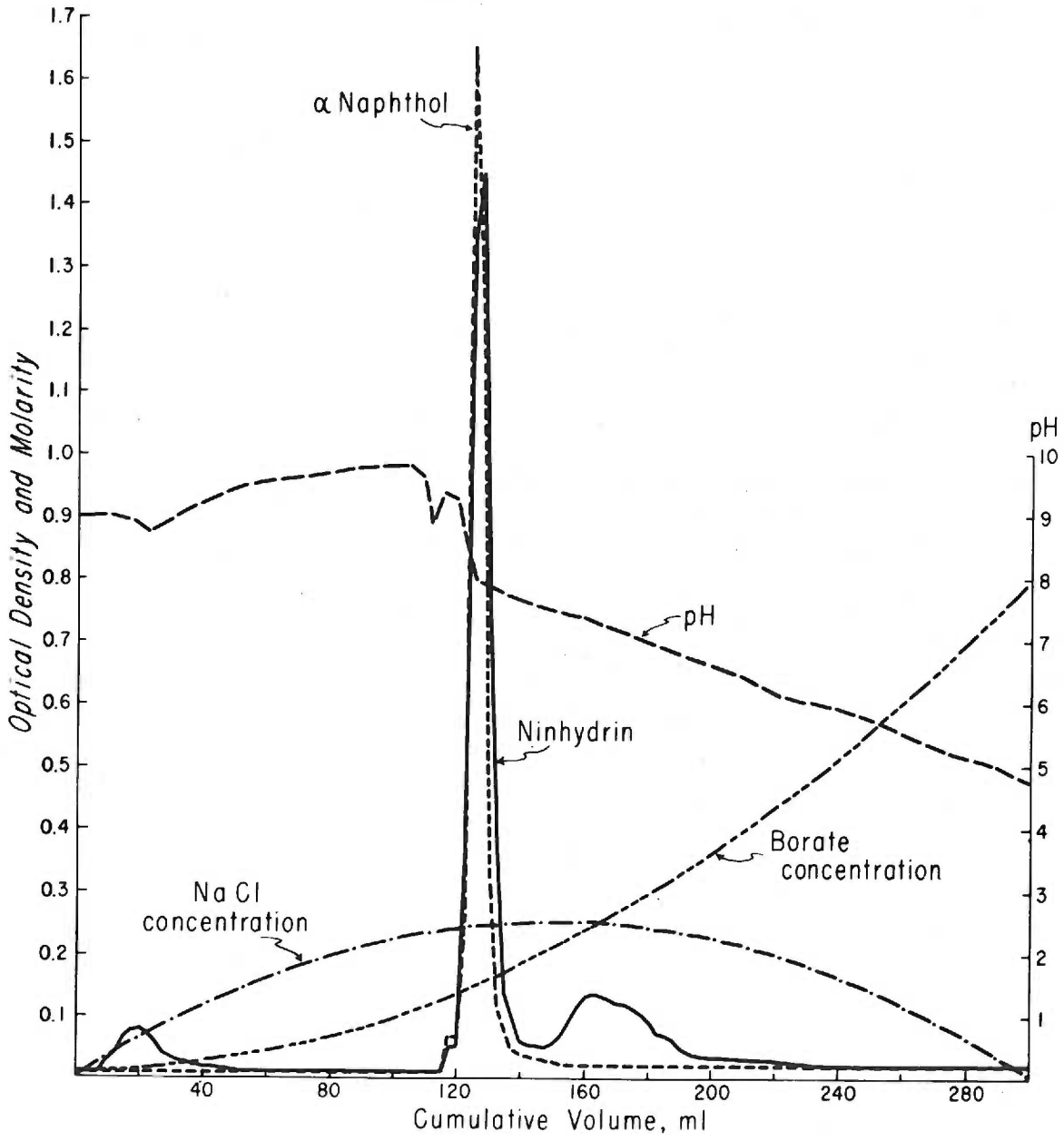

Optical Density
0.1
0.2
0.3
0.4
0.5
α Naphthol
Ninhydrin
GP-B1&2
GP-B3
GP-B4
GP-B5
40
80
120
160
200
240
280
320
360
400
500
Cumulative Volume, ml

Figure 13.

Chromatogram of the AG 1-X2 chromatography of the glycopeptide fraction obtained from a P-2 gel column. The P-2 gel column was developed with the beginning pH 9.0 borate buffer used for the AG 1-X2 system. The gradient system shown in the theoretical gradient calculated with equation (6) and used for the AG 1-X2 chromatography. The pH curve was determined empirically. Column size 0.9 x 40 cm.


α Naphthol
pH
Ninhydrin
Borate concentration
Na Cl concentration
Optical Density and Molarity
pH
Cumulative Volume, ml
1.7
1.6
1.5
1.4
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
10
9
8
7
6
5
4
3
2
1
40
80
120
160
200
240
280

$$\frac{C}{L} = \frac{(N-1)!}{(N-n)!(n-1)!}\left(1 - \frac{v}{V}\right)^{N-n}\left(\frac{v}{V}\right)^{n-1} \qquad (6)$$

In this equation C is the concentration emerging from the mixer when the volume, v, has been withdrawn from the total volume, V, of the N chambered gradient system. The equation is solved for each chamber, n, with L concentration of the substance being considered. The C's for each of the chambers are added together to find the total concentration of the substance at volume v. The chamber from which the solution is withdrawn is n = 1 and the chamber furthest removed from the exit point is n = N. It is the total concentrations that have been plotted in Fig. 13.

The pH curve was found by determining the pH of the fractions collected from the column. This curve was found to be reproducible in each of three runs using this buffer system.

Although the glycopeptides were eluted as a single peak, a certain degree of purification was achieved as some of the non-glycopeptides were eliminated. A disadvantage of this system was that only about 70% of the carbohydrate load was recovered.

The glycopeptide fraction from three runs on this system were pooled together and concentrated to a volume of 1 ml. To the concentrate were added 2 ml of the beginning buffer of developer A for the cation exchange chromatography. A clear light-brown solution was obtained. To remove the borate and sodium chloride salts, the concentrate was passed through a 0.9 x 40 cm P-2 gel

column that had been equilibrated with the beginning buffer of developer A. This column was operated at room temperature with a flow rate of 30 ml/hr. The 3.3 ml fractions collected were analyzed for carbohydrate only. The glycopeptides were eluted in the 17-30 ml fraction and the carbohydrate load was fully recovered. This fraction was pooled together and was adjusted to pH 2 by the addition of glacial acetic acid. This fraction was then chromatographed on the cation exchange column using developer system A. From the chromatogram shown in Fig. 14 it was observed that GP-A was missing and the relative amount of GP-C was greater than the GP-B. This observed difference in amounts of GP-B and GP-C was in contrast to that found when the whole hydrolysate was chromatographed on the cation exchange column (Fig. 5). It was concluded that GP-A and a portion of GP-B was firmly bound to the anion exchange resin and was not eluted from the column with the borate buffer system employed. Assuming that the GP-C was completely eluted from the anion exchange resin, it is conceivable that after its isolation from the cation exchange column the GP-C could be further purified by the borate buffer system used with the AG 1-X2 resin.

F. Amino Acid Analyses of Glycopeptides

The amino acid analyses of the 22-hour acid hydrolysates of the glycopeptides isolated are given in Tables III-VII. The GP-A was free of the minor component described earlier. The GP-B fractions were taken directly from the AG 50 W cation exchange column after

Figure 14.

Chromatogram of the AG 50W-X2 chromatography of the glycopeptide fraction obtained from the AG 1-X2 column. Developer: system A.

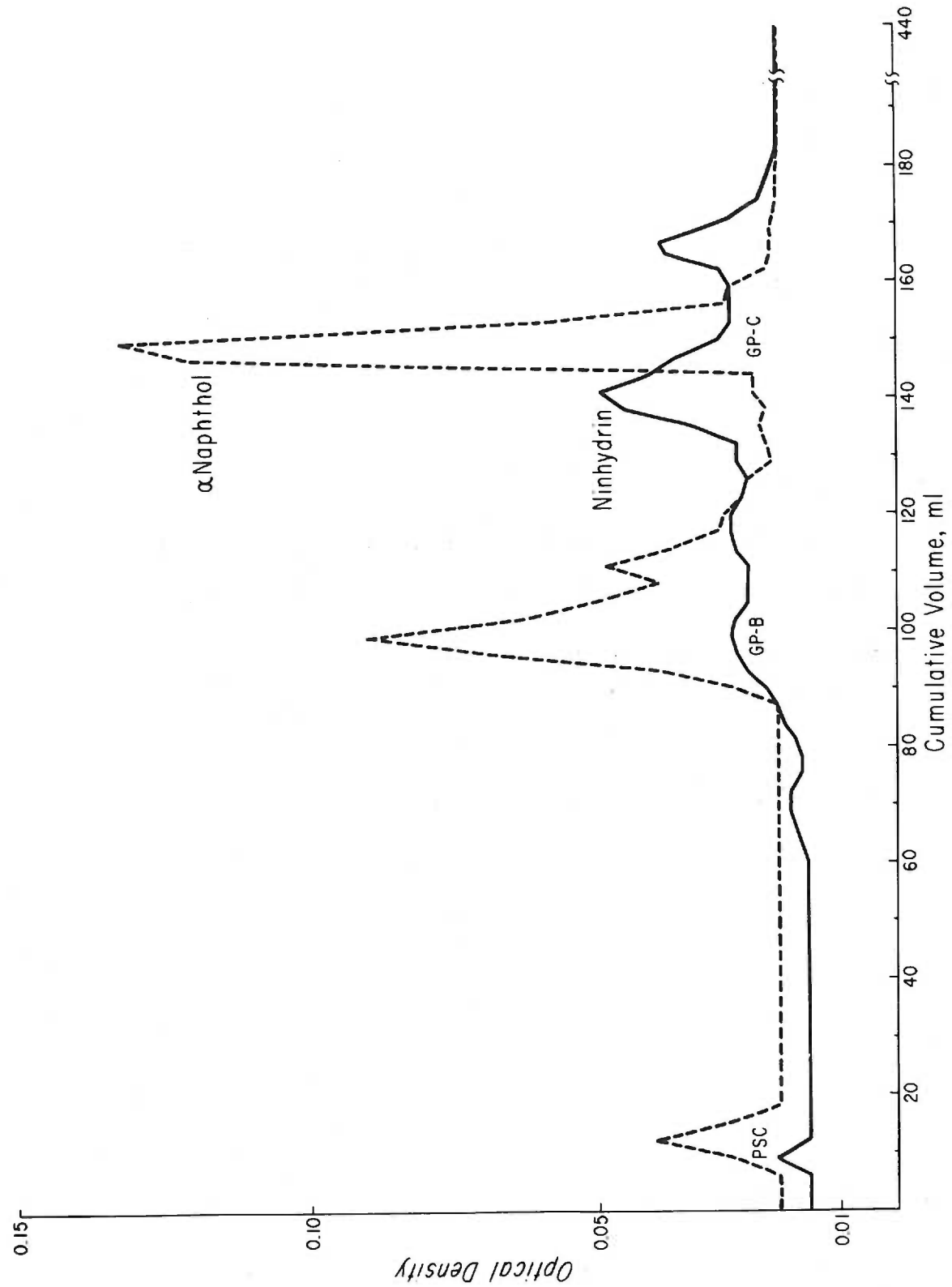

Optical Density
0.15
0.10
0.05
0.01
20
40
60
80
100
120
140
160
180
440
Cumulative Volume, ml
PSC
GP-B
GP-C
Ninhydrin
αNaphthol

TABLE III

AMINO ACID ANALYSES OF GP-$B_{1\&2}$

Amino Acid	Total µm	Cal. Ratio	Est. Ratio	Contam. µm (A_c)	Cal. Contam. Ratio	Est. Contam. Ratio
Lysine	0.0184	0.086	0	0.0184	1.00	1
Histidine						
Arginine	0.2142	1.00	1	0	0	0
Aspartic Acid	0.5435	2.54	2	0.1151	6.3	6
Threonine	0.3810	1.78	2	0	0	0
Serine	0.2955	1.38	1	0.0813	4.4	4
Glutamic Acid	0.6232	2.91	3	0	0	0
Proline	0.0365	0.17	0	0.0365	1.98	2
Glycine	0.0718	0.34	0	0.0718	3.9	4
Alanine	0.1691	0.79	1	0	0	0
Cystine	0	0	0	0	0	0
Valine	0.0542	0.25	0	0.0542	2.9	3
Methionine	0.0061	0.03	0	0.0061	.33	0
Isoleucine	0.2485	1.16	1	0.0343	1.9	2
Leucine	0.5300	2.43	2	0.1016	5.5	6
Tyrosine	0.0116	0.05	0	0.116	0.63	1
Phenylalanine	0.3658	1.71	2	0	0	0
Glucosamine	0.5769	2.69	3	0	0	0

TABLE IV.

AMINO ACID ANALYSES OF GP-B_3

Amino Acid	Total µm	Cal. Ratio	Est. Ratio	Contam. µm (A_c)	Cal. Contam. Ratio	Est. Contam. Ratio
Lysine	0.0234	0.17	0	0.0234	1.00	1
Arginine	0.1381	1.00	1	0	0	0
Aspartic Acid	0.2403	1.74	2	0	0	0
Threonine	0.1151	0.83	1	0	0	0
Serine	0.0890	0.64	1	0	0	0
Glutamic Acid	0.2140	1.55	1	0.0759	3.2	3
Proline	0.0128	0.09	0	0.0925	3.9	4
Glycine	0.0358	0.26	0	0.0358	1.53	2
Alanine	0.0873	0.63	0	0.0873	3.7	4
Cystine	0	0	0	0	0	0
Valine	0.0638	0.46	0	0.0638	2.7	3
Methionine	0	0	0	0	0	0
Isoleucine	0.1074	0.78	1	0	0	0
Leucine	0.2826	2.05	2	0	0	0
Tyrosine	0.0045	0.03	0	0.0045	0.19	0
Phenylalanine	0.1617	1.17	1	0.0236	1.00	1
Glucosamine	0.1976	1.43	1 or 2	0	0	0

TABLE V

AMINO ACID ANALYSES OF GP-B_4

Amino Acid	Total µm	Cal. Ratio	Est. Ratio	Contam. µm (A_c)	Cal. Contam. Ratio	Est. Contam. Ratio
Lysine	0.0182	0.10	0	0.0182	1.00	1
Arginine	0.1850	1.00	1	0	0	0
Aspartic Acid	0.3220	1.74	2	0	0	0
Threonine	0.1461	0.79	1	0	0	0
Serine	0.1002	0.54	0 or 1	0.1002	5.5	6
Glutamic Acid	0.2661	1.44	1	0.0811	4.45	4
Proline	0.0174	0.09	0	0.0174	0.95	1
Glycine	0.0572	0.31	0	0.0572	3.14	3
Alanine	0.0714	0.38	0	0.0714	3.92	4
Cystine	0.0010	0.00	0	0.0010	0	0
Valine	0.0849	0.46	0	0.0850	4.6	5
Methionine	0	0	0	0	0	0
Isoleucine	0.1272	0.69	0 or 1	0.1272	6.98	7
Leucine	0.3500	1.89	2	0	0	0
Tyrosine	0.0089	0.05	0	0.0089	0.5	0
Phenylalanine	0.2183	1.18	1	0.0333	1.8	2
Glucosamine	0.3392	1.83	2	0	0	0

TABLE VI

AMINO ACID ANALYSES OF GP-B5

Amino Acid	Total µm	Cal. Ratio	Est. Ratio	Contam. µm (A_c)	Cal. Contam. Ratio	Est. Contam. Ratio
Lysine	0.0212	0.37	0	0.0212	1.00	1
Arginine	0.0570	1.00	1	0	0	0
Aspartic Acid	0.1119	1.96	2	0	0	0
Threonine	0.0702	1.23	1	0.0132	0.6	0 or 1
Serine	0.0646	0.99	1	0	0	0
Glutamic Acid	0.1453	2.55	2	0.0312	1.5	1
Proline	0.0231	0.40	0	0.0231	1.1	1
Glycine	0.0836	1.47	1	0.0266	1.25	1
Alanine	0.0085	0.15	0	0.0085	0.4	0
Cystine	0.0226	0.40	0	0.0226	1.06	1
Valine	0.0371	0.65	0	0.0365	1.72	2
Methionine	0	0	0	0	0	0
Isoleucine	0.0580	1.02	1	0	0	0
Leucine	0.1292	2.26	2	0.0151	0.7	1
Tyrosine	0	0	0	0	0	0
Phenylalanine	0.0598	1.05	1	0	0	0
Glucosamine	0.0821	1.44	1 or 2	0	0	0

TABLE VII

AMINO ACID ANALYSES OF GP-A

Amino Acid	Total µm	Cal. Ratio	Est. Ratio
Lysine	0	0	0
Arginine	0	0	0
Aspartic Acid	0.2202	2.00	2
Threonine	0.1790	1.63	2
Serine	0.1121	1.02	1
Glutamic Acid	0.1310	1.19	1
Proline	0.1025	0.93	1
Glycine	0.1132	1.03	1
Alanine	0.1646	1.50	1 or 2
Cystine	0	0	0
Valine	0.1132	1.03	1
Methionine	0	0	0
Isoleucine	0.1223	1.11	1
Leucine	0.0483	0.44	0 or 1
Tyrosine	0	0	0
Phenylalanine	0.1008	0.91	1
Glucosamine	0.2158	1.96	2

GP-B was rechromatographed with the developer system B. The given micromolar amounts were found by comparison with a standard that was 0.05 micromolar in all of the amino acids, glucosamine and galactosamine.

Referring to Tables III-VI it is seen that the micromolar amounts of arginine recovered for the GP-B glycopeptides were, in all cases, higher than the lysine. Using the micromolar quantity of arginine, the ratio of micromoles of amino acid per micromole of arginine was calculated and is given in the second column. The third column is the nearest whole number ratio. In some cases a considerable amount of difference was observed between the calculated ratio and the estimated ratio. This difference was attributed to contaminating amino acids from a lysine-containing peptide contaminant. The micromolar amount, A_c, of a given contaminating amino acid was found by the following equation:

$$A_c = A_t - (N \times A_{rg}) \qquad (7)$$

where A_t is the total number of micromoles of the amino acid recovered; N is the estimated whole number ratio of the amino acid in the glycopeptide and A_{rg} is the micromoles of arginine recovered. Thus, for aspartic acid in GP-B_1&$_2$ (Table III), A_t = 0.5435, N = 2 and A_{rg} = 0.2142. Using equation (7), A_c is calculated as 0.1151.

When the A_c's are divided by the micromolar amount of lysine, a set of ratios for the contaminating peptide's amino acids are

obtained. In Tables III-VI the A_c's are in the fourth column. The fifth column is the calculated ratios for the contaminating peptide.

Table VII is the analyses of GP-A. With lysine and arginine being absent, one half the micromolar recovery of aspartic acid was used to find the calculated ratios given.

Table VIII is a resume of the Tables III-VII with only the estimated ratios of the glycopeptides given. From these analyses it was apparent that the GP-A differs from the GP-B glycopeptides in that both lysine and arginine were absent. Other observed differences were that the GP-A contains proline and valine residues while the GP-B peptides did not. When comparing the GP-B analyses with each other, it must be taken into consideration that GP-$B_1\&_2$ was probably heterogeneous, as evidenced by the peptide chromatogram in Fig. 12. The GP-B glycopeptides appeared to be very similar, with the exception of GP-B_5 as judged by its different ratios of glycine and glutamic acid. Assuming the judgement used in the calculations to be correct, at least two or possibly more of the glycopeptides were different in their amino acid compositions.

G. Carbohydrate Analyses

The cellulose thin layer chromatogram (TLC) trace for the hydrolyzed sugar standard is shown in Fig. 15. A 5 µl load of the standard was spotted on the plate. The sugars corresponding to the spots were earlier determined by chromatographing the individual sugars under identical conditions using each developer system

TABLE VIII

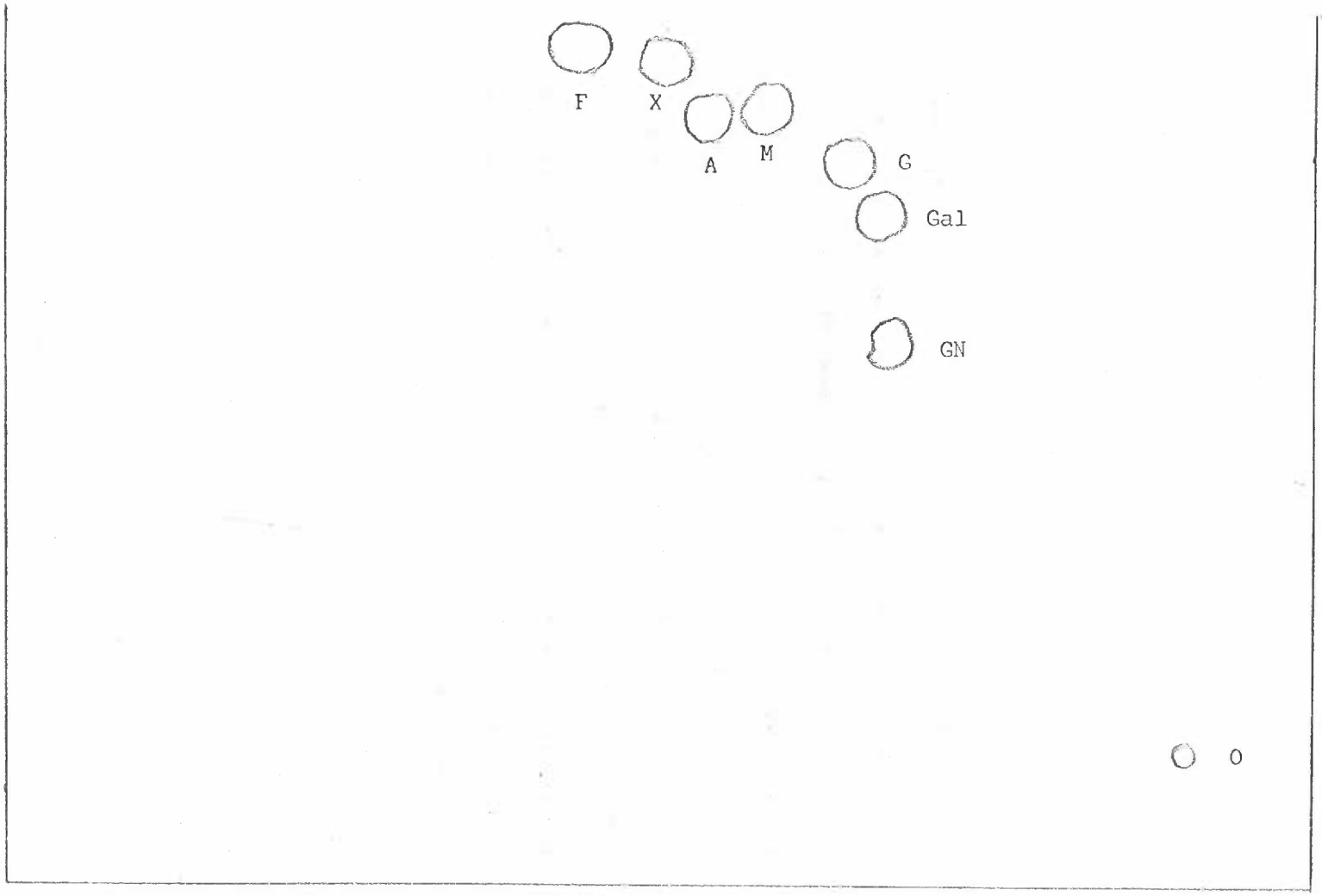
RESUME OF AMINO ACID ANALYSES OF GLYCOPEPTIDES

Amino Acid	GP-A	GP-$B_{1\&2}$	GP-B_3	GP-B_4	GP-B_5
Lysine	0	0	0	0	0
Histidine	0	0	0	0	0
Arginine	0	1	1	1	1
Aspartic Acid	2	2	2	2	2
Threonine	2	2	1	1	1
Serine	1	1	1	0 or 1	1
Glutamic Acid	1	3	1	1	2
Proline	1	0	0	0	0
Glycine	1	0	0	0	1
Alanine	1 or 2	1	0	0	0
Cystine	0	0	0	0	0
Valine	1	0	0	0	0
Methionine	0	0	0	0	0
Isoleucine	1	1	1	0 or 1	1
Leucine	0 or 1	2	2	2	2
Tyrosine	0	0	0	0	0
Phenylalanine	1	2	1	1	1
Glucosamine	2	3	1 or 2	2	1 or 2

Figure 15.

A trace of the thin-layer chromatogram of the hydrolyzed sugar standard. Load: 5 µl. Code: A = arabinose; F = fucose; G = glucose; Gal = galactose; GN = glucosamine; M = mannose; X = xylose; O = origin. This code will also be used in Figs. 16-21.

In Figs. 15-20: Double development of 15 cm from right to left with formic acid-methyl ethyl ketone-tert. butyl alcohol-water (15:30:40:15, v/v). Single development of 15 cm from bottom to top with 2-propanol-pyridine-acetic acid-water (8:8:1:4, v/v).



Bottom edge

separately in one dimension. Fig. 16 is a trace of the chromatogram where 5 µl of the sugars solution obtained from the PPHA preparation III was chromatographed. Glucosamine and mannose were found to be present. However, when 10 µl of the protein sugars were loaded and chromatographed, fucose and xylose were also found to be present (Fig. 17). About 0.025 mg of fucose, xylose and mannose were added to the protein sugar solution and 15 µl of the solution were chromatographed. From this chromatogram, Fig. 18, it was observed that in addition to the mannose, fucose and xylose, a less dense brown spot appeared in a position corresponding to arabinose.

The glycopeptides GP-A and GP-$B_{1\&2}$ were hydrolyzed and treated in a manner identical to the protein as described (vide supra). The GP-A was free of the minor glycopeptide described. A 10 µl load of their sugar solutions were chromatographed. The chromatograms for each are given in Figures 19 and 20, respectively. From Fig. 19 it was observed that the GP-A contained fucose, xylose, mannose and arabinose. Very faint spots were observed in the glucose and galactose positions. The glucosamine spot was slightly displaced from the position found in earlier chromatograms. From Fig. 20 it was observed that only mannose and glucosamine were present in the GP-$B_{1\&2}$ hydrolysate. To confirm these findings and to make more certain that glucosamine was the questioned spot in Fig. 19, a cellulose plate was spotted in the manner shown in Fig. 21. The loads of the two glycopeptide fractions were increased to 15 µl. The

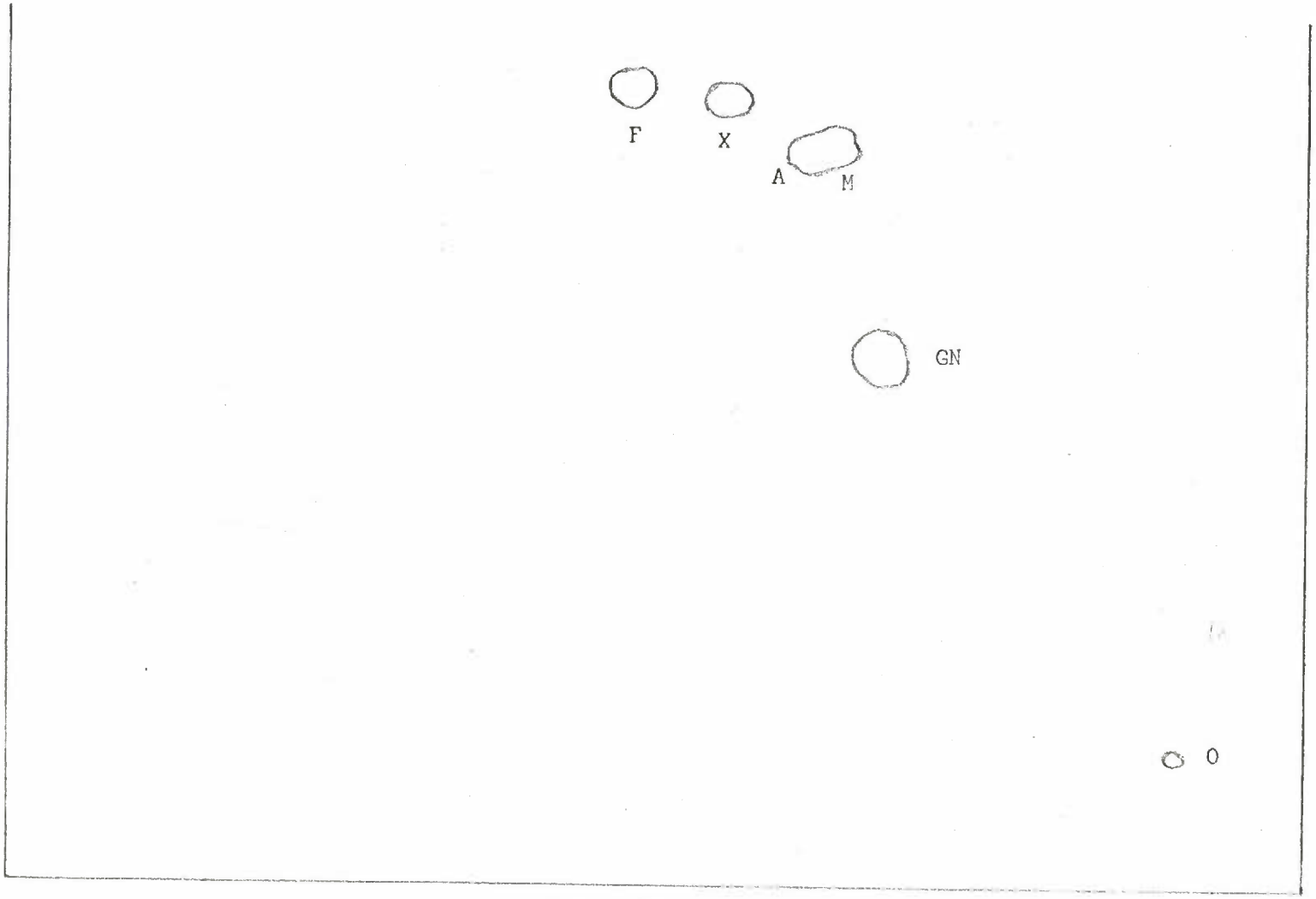
Figure 16.

Trace of the thin-layer chromatogram of the hydrolyzed component III. Load: 5 µl.

Bottom edge

Figure 17.

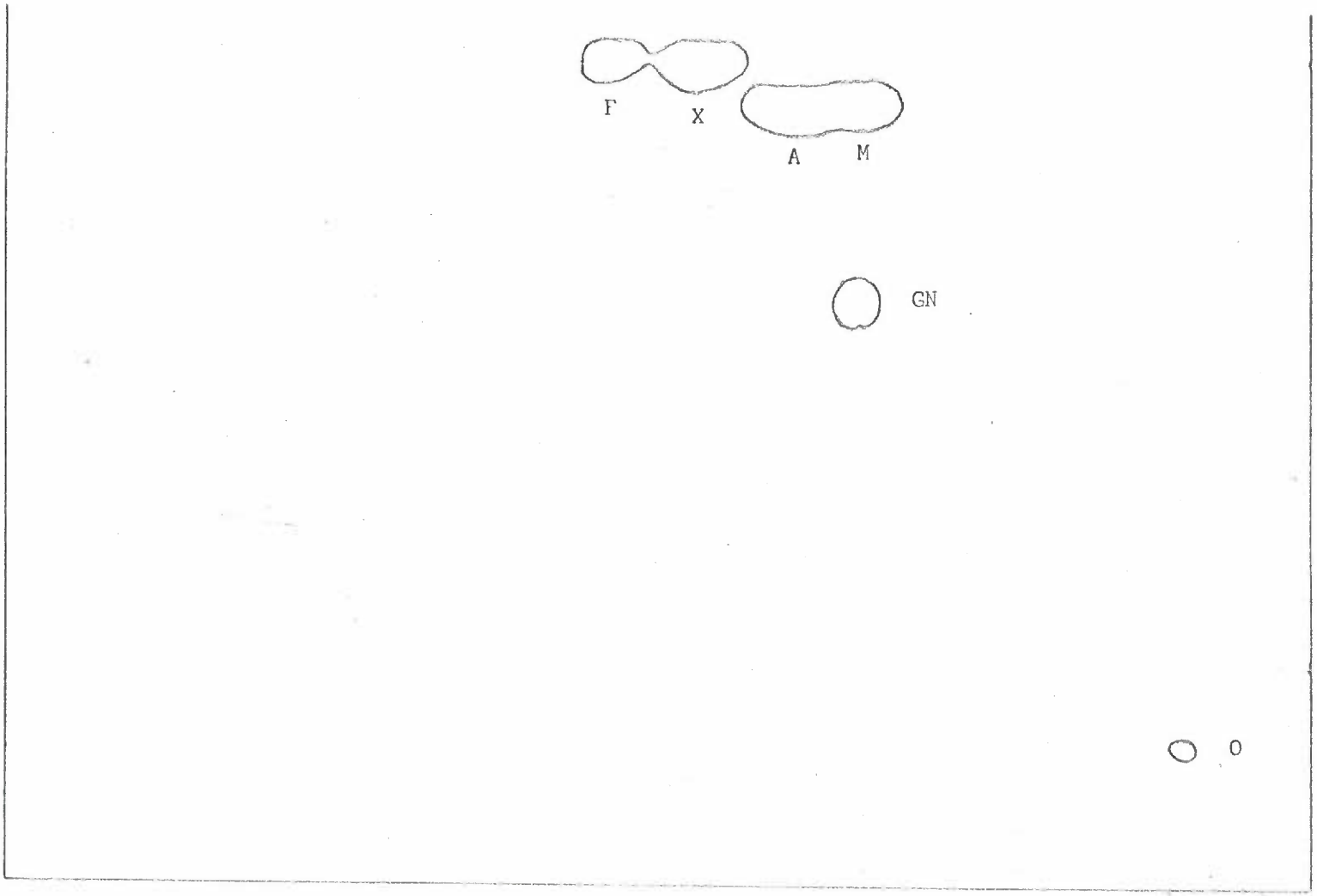
Trace of the thin-layer chromatogram of the hydrolyzed component III. Load: 10 µl.



Bottom Edge

Figure 18.

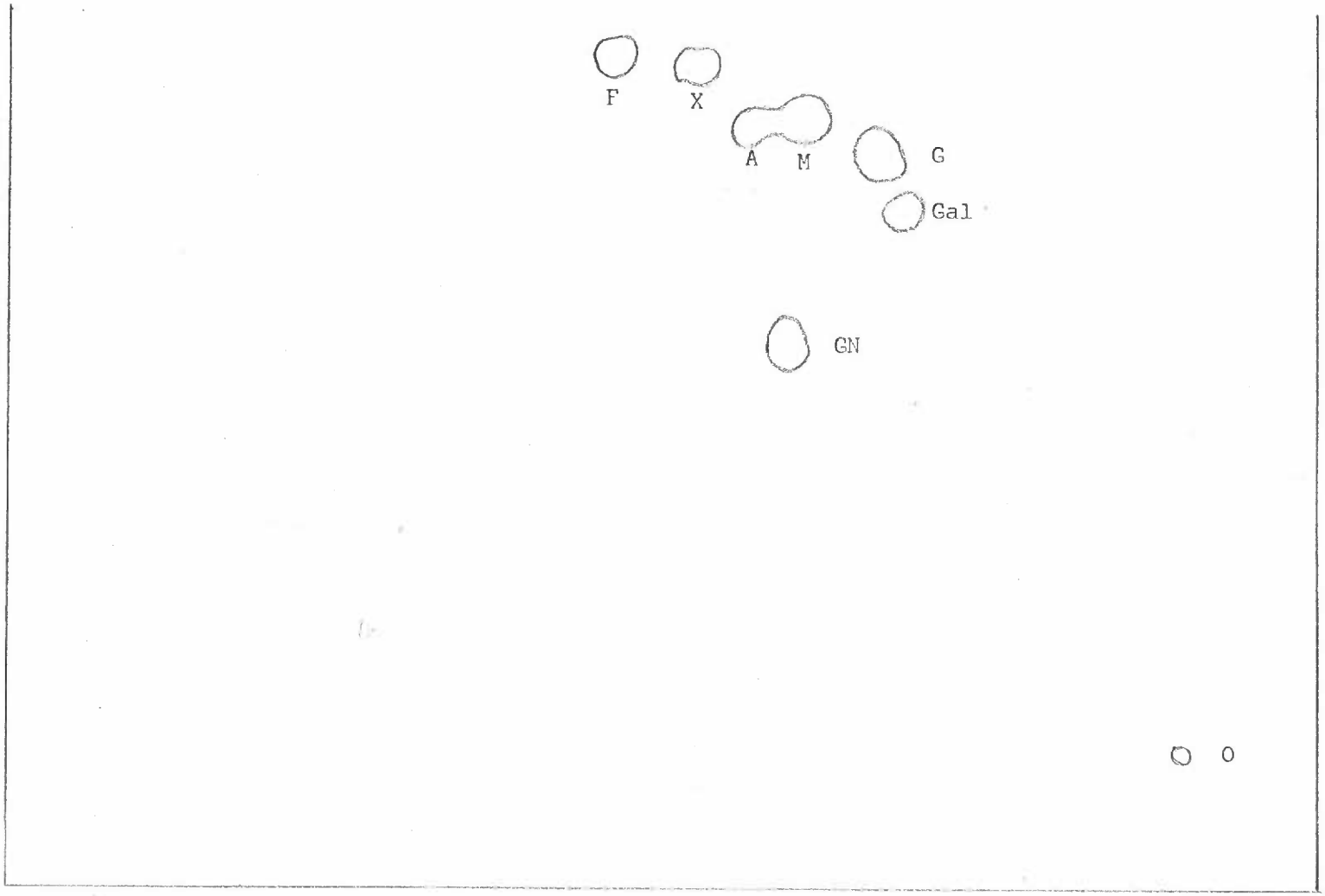
Trace of the thin-layer chromatogram of the hydrolyzed component III to which 0.025 mg of fucose, xylose and mannose was added per ml of solution. Load: 15 µl.



Bottom edge

Figure 19.

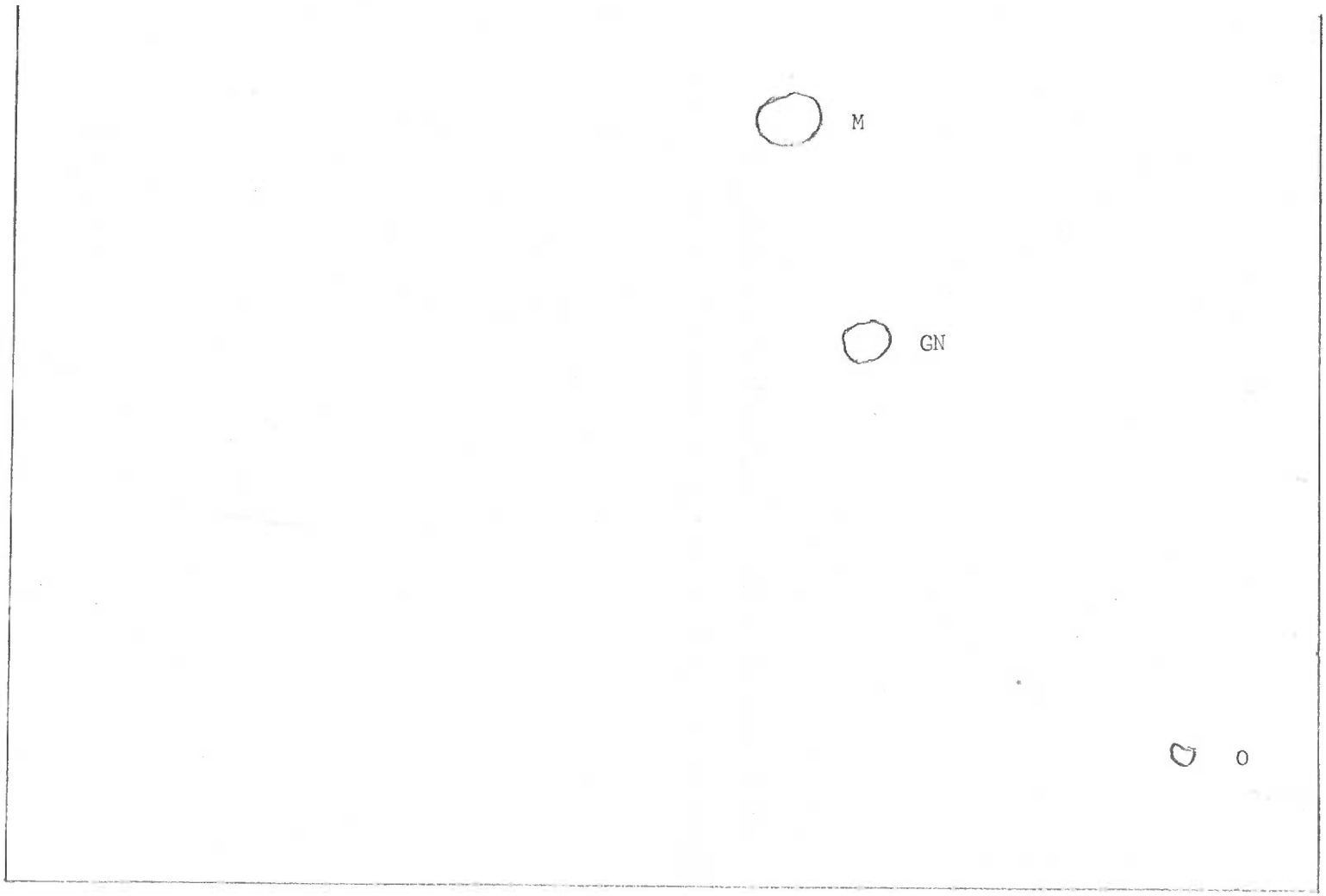
Trace of the thin-layer chromatogram of the hydrolyzed glycopeptide GP-A. Load: 10 µl.



Bottom edge

Figure 20.

Trace of the thin-layer chromatogram of the hydrolyzed glycopeptide fraction GP-$B_{1\&2}$. Load: 10 µl.



Bottom edge

Figure 21.

Trace of the thin-layer chromatogram of the hydrolyzed GP-A, GP-$B_{1\&2}$ and unhydrolyzed sugar standards.

1 & 7. Glucosamine 10 µl of a 0.1 mg/ml solution.

2 & 6. Galactosamine 10 µl of a 0.1 mg/ml solution.

3. Hydrolyzed GP-A (15 µl).

4. Unhydrolyzed mixed standard 10 µl of a solution 0.1 mg/ml in each sugar except galactosamine.

5. Hydrolyzed GP-$B_{1\&2}$ (15 µl).

Single development of 15 cm from bottom to top with 2-propanol-pyridine-acetic acid-water (8:8:1:4, v/v).

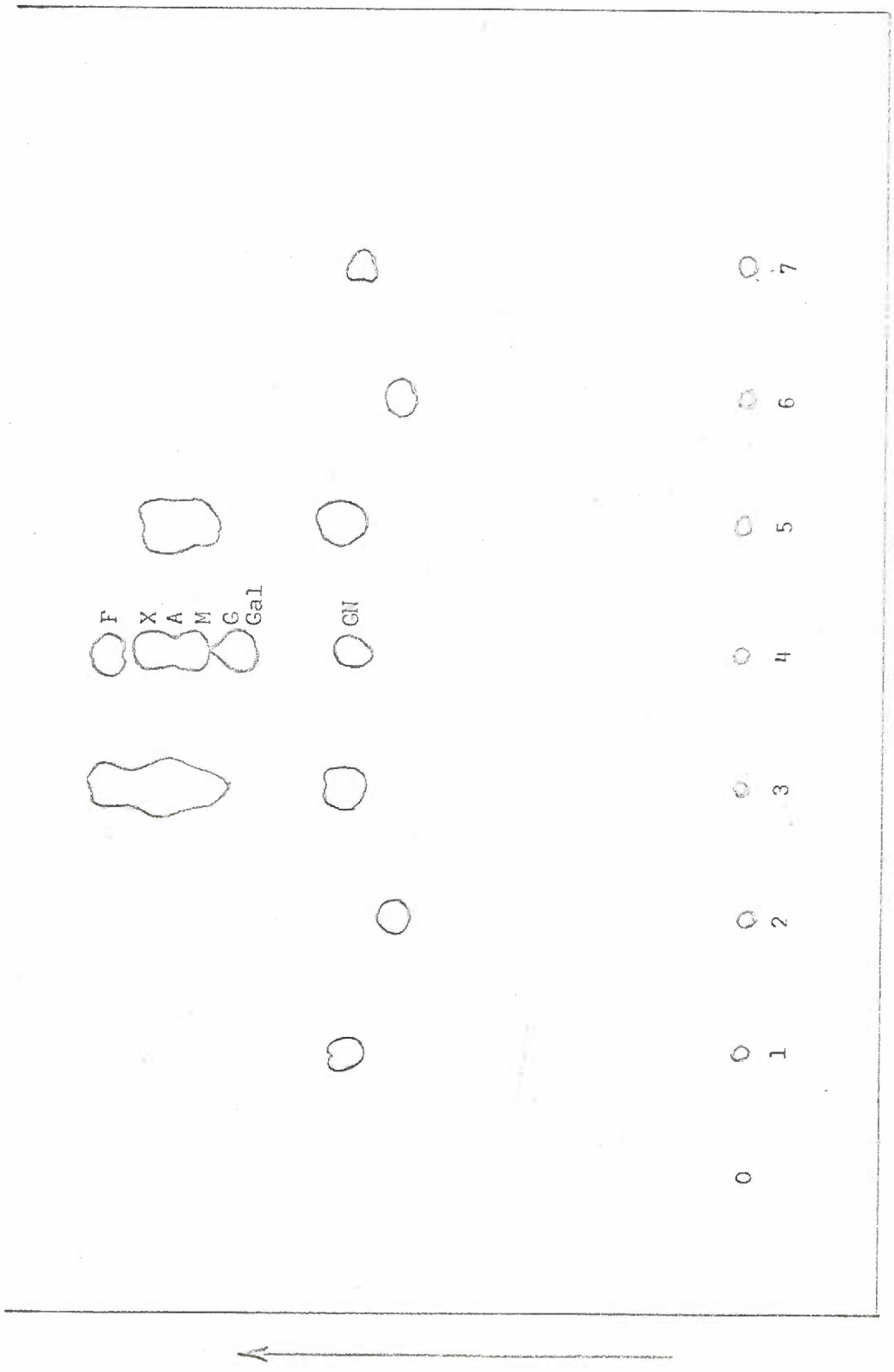

F
X
A
M
G
Gal
Gl
0
1
2
3
4
5
6
7
Bottom edge

plate was developed once in the direction shown with the 2-propanol-pyridine-acetic acid-water (8:8:1:4,v/v) developer.

By comparing these plates it appeared that the GP-A did contain glucosamine. However, the galactose and glucose spot was not observed this time with the greater load of material and, therefore, was considered a plate artifact or amino acid spot. The GP-$B_{1\&2}$ did show upon increasing the load that arabinose and possibly xylose were present. Thus it appears that the carbohydrate portion of the phytohemagglutinin contains relatively large amounts of mannose and glucosamine and comparatively smaller amounts of fucose, arabinose, and xylose. Glucose and galactose do not appear to be present in the protein. Comparison of the two glycopeptides, GP-A and GP-$B_{1\&2}$, in terms of monosaccharide content indicates that the two differ in their oligosaccharide moieties as well as their amino acid content.

IV. DISCUSSION

The origin and nature of the polysaccharide contaminant of the phytohemagglutinin preparation can only be speculated upon. Throughout the ammonium sulfate fractionation procedure some fractions were observed to have a pronounced pink color. Some of the color from the red bean hulls is presumably dissolved from them by the 0.1 M HCl extraction and then adsorbed onto other components of the bean. During the water dialysis step of the purified PPHA prior to lyophilization, the precipitate was observed to have a faint pink color. After lyophilization the dried protein was light tan in color. When the polysaccharide contaminant was removed from that preparation by the P-200 gel filtration chromatography and component III was pooled, concentrated, dialyzed and lyophilized, the dried material was no longer tan but was a very white preparation. From this it might be speculated that a chromogenic substance, either the polysaccharide or a complex molecule adsorbed to the polysaccharide, follows the PPHA during the purification procedure.

The component II which was eluted from the P-200 column is interesting in that it is not present in the preparation before the gel filtration, as evidenced by the ultracentrifugal pattern in Fig. 3a. A column artifact was ruled out by repouring the column and changing the teflon filter in the bottom of the column to a nylon net filter. In both cases the same chromatogram was obtained. When the component III was rechromatographed on the same system, in contrast

to what one would expect, component II in Fig. 9 was approximately the same height as the original peak in Fig. 8. In addition, upon ultracentrifugation of component III a minor component sedimenting faster than the major component was observed (Fig. 3c). Referring to Fig. 8, if it is assumed that peak II is symmetrical, only a small portion, if any, of the tail of component II would have been pooled with the component III. Similar remarks may be made concerning the small component observed in the component I position of Fig. 9.

These findings suggest that the phytohemagglutinin interacts with the gel and brings about a polymerization of the molecule. This polymerization may involve either association of whole molecules, or possibly dissociation into subunits, followed by reassociation. When reassociation occurs not all subunits combined with their original counterparts, thus producing higher molecular weight aggregates. If this is so, one may expect to see a component eluted after the component III which would be lower in molecular weight due to the loss of subunits. Theoretically, if a substance is not adsorbed to the gel, it should appear in the effluent by the time a volume of developer equal to the bed volume has been passed through the column (58). The bed volume of the P-200 column was approximately 300 ml. In those runs where 300 ml were passed through the column, no component smaller than III was observed. Its absence could be explained by postulating a symmetrical dissociation of the molecule into two or

four parts and upon reassociation they combined with undissociated PPHA molecules, thus producing only molecules with molecular weights larger than the original PPHA molecule. Many possibilities of this nature could be postulated. As earlier mentioned, Rigas et al. (15) have demonstrated the dissociation of the PPHA molecule by the weak cation exchanger IRC-50. After reassociation the products differed from the original molecule. Therefore, it is not an unlikely possibility that the effects observed on the P-200 gel represent some sort of molecular rearrangement.

As a glycoprotein the purified phytohemagglutinin contains 10% carbohydrate (15). The distribution of this carbohydrate in the protein molecule might occur in several ways. It could be present as one large oligosaccharide or as several smaller oligosaccharide moieties bound to the protein at different places. The amino acids to which the oligosaccharides are linked might be the same or different. The monosaccharide composition of the oligosaccharide moieties might also be the same or different. The studies presented have demonstrated that there are at least two different tryptic glycopeptides in the PPHA molecule. The absence of lysine and arginine in the GP-A glycopeptide is given as the most direct evidence for this conclusion. As judged by the results of the amino acid analyses on the GP-A it was quite free from contaminating peptides. That is, except for leucine and alanine all amino acids present were in ratio close to whole numbers. In regard to this discrepancy for these two

amino acids it is interesting to note that if the micromolar amount of leucine is added to the alanine, a calculated ratio for alanine of 1.9 results. Similarily, if the alanine ratio is taken as one and its excess micromolar amount is added to the leucine, a calculated ratio for leucine of 0.93 results. This might indicate that in some peptides an alanine is substituted for by a leucine. Since both of these are aliphatic amino acids, their separation by the cation exchanger would not have been too likely. Such a substitution may represent a genetic variation due to a difference in the gene loci which code for this polypeptide chain.

It could be argued that some other enzyme contaminating the trypsin such as chymotrypsin might have cleaved a portion, including arginine, to produce GP-A from one of the other tryptic peptides. However, the presence of proline in the GP-A does not support this argument for one would then expect to find proline in the other glycopeptides. This was not seen to be the case.

Definite conclusions about the differences in the GP-B glycopeptides are difficult because of their apparent contamination with small amounts of other peptide, as suggested by their lysine content. However, the amino acid ratios indicate their peptide portions to be very similar.

Supporting evidence for the existence of at least two glycopeptides is given by the carbohydrate analyses. The two pooled fractions, GP-A and GP-$B_{1\&2}$, were taken from the same chromatographic

run. Each fraction contained approximately the same amount of carbohydrate when comparisons were made of their color produced during the autoanalyzer method. When their sugar hydrolysates were chromatographed on the thin layer system, GP-A definitely contained fucose, whereas the GP-$B_{1\&2}$ did not, even when the load was increased by 50%.

That there may be eight different glycopeptides in the phytohemagglutinin is suggested by the chromatography on the cation exchange column. Whether some of these represent partially hydrolyzed tryptic peptides was not ruled out. However, presumptive evidence against this possibility may be derived from the results of the gel filtration of the peptides. Assuming that no adsorption of glycopeptides occurred on the gel, their molecular weight range appeared to be between 3000 and 9000. If all of the carbohydrate was present as one oligosaccharide unit containing 63 molecules of mannose and 19 molecules of glucosamine, its molecular weight would be 13,278. If mannose is substituted for by arabinose, the molecular weight of the oligosaccharide would be 11,386. From the thin layer chromatography of the sugars of the protein hydrolysate a large amount of mannose was found to be present relative to the other sugars. Thus the molecular weight of the oligosaccharide unit would be closer to 13,000. This oligosaccharide would be linked to a tryptic peptide. Using separately the amino acid ratios given for the different glycopeptides in Table VIII as the absolute

number of those amino acids in the peptide, the total weight of any of the glycopeptides would be about 14,000. If in fact, they were partial hydrolytic products, this estimate would probably be low. In any case a molecular weight this large is not consistent with the results obtained by the gel filtration. If there are eight different glycopeptides in the phytohemagglutinin, it may be possible that they are distributed equally among the eight different subunits observed by Rigas et al. (15).

Hydrolysis of a protein with trypsin involves the cleavage of the peptide bond on the carboxyl side of lysine and arginine residues. Assuming that the protein chain was completely hydrolyzed by the trypsin, two different types of peptide chains would be possible with respect to their lysine and arginine content. The C-terminal peptides of the protein chain would not contain a lysine or arginine residue except for those proteins having lysine or arginine as their C-terminal amino acids. All other peptides would have lysine or arginine as their C-terminal amino acids. The absence of both lysine and arginine in GP-A indicates that it is a C-terminal glycopeptide. Thus, one of the oligosaccharide units must be linked near the C-terminal end of a subunit.

Reports on the nature of the glycopeptides in the plant agglutinins have appeared only recently. In a preliminary communication Liener et al. (59) reported the presence of several glycopeptides in their wax bean hemagglutinin (Phaseolus vulgaris), following hydrolysis with pronase. No other information was given as

to how these glycopeptides were different. Nevertheless, their findings support the conclusions drawn from the results presented here.

Lis et al. (36) isolated a pronase glycopeptide from soybean hemagglutinin. It was found to contain mannose and glucosamine. This carbohydrate was found to be linked to an aspartic acid residue. On the basis of molecular weight and carbohydrate analyses they concluded that only one oligosaccharide unit linked to an aspartic acid residue present in that agglutinin. Thus it appears that the phytohemagglutinins from different genera will be quite different with respect to the nature of the carbohydrate in their molecules. This hypothesis is supported by the findings of Huprikar and Sohonie (38). They have found the white pea (Pisum sp.) hemagglutinin to contain xylose, glucose, rhamnose, fructose and galactose. They were unable to detect glucosamine; however, this may have been caused by their failure to elute it from Dowex 50 with 2 N HCl. Marinkovich (9) reported that the potato hemagglutinin contains only arabinose. However, comparison of the potato hemagglutinin, which has a molecular weight of 20,000 and isoelectric point of 9, to the legumenous phytohemagglutinins is anticipated to reveal pronounced differences.

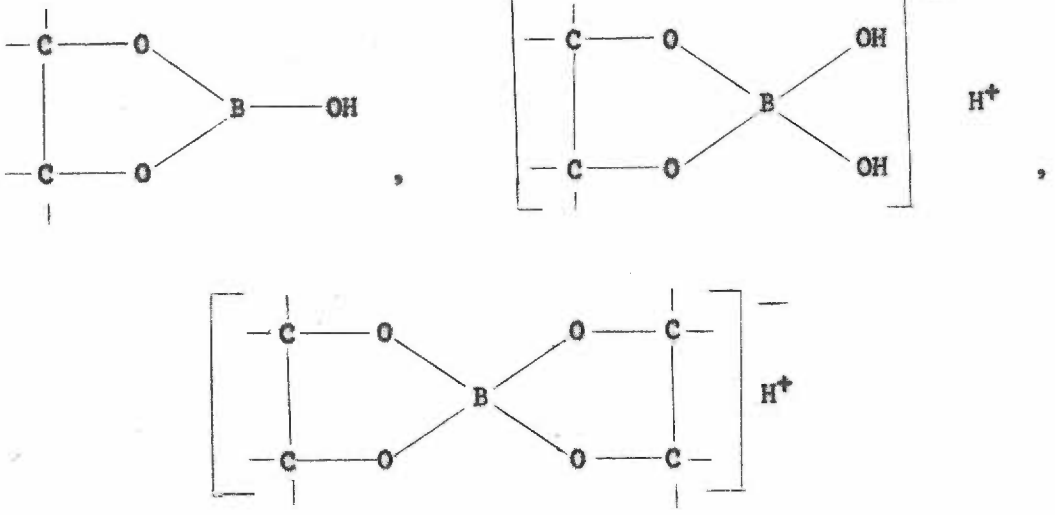
That the carbohydrate portion of the PPHA is composed of glucosamine, large amounts of mannose and smaller amounts of fucose, arabinose and xylose is in partial agreement with that found by other investigations on the phytohemagglutinins isolated from Phaseolus

vulgaris subspecies. Liener et al. (59) have recently reported that the wax bean phytohemagglutinin contains 7.3% carbohydrate consisting chiefly of mannose with smaller amounts of arabinose, xylose, glucose and galactose. Jaffe and Hannig (35) found their semi-purified preparations of black kidney bean hemagglutinin contained glucosamine, large amounts of mannose and smaller amounts of fucose, xylose, glucose and galactose.

The discrepancies in these findings with those presented here may be due to the sensitivity of the methods used, could represent impurities or could be real differences in the proteins. If the latter is shown to be correct, then the methods of purification should be examined closely. This suggestion is made in reference to the findings of Rigas and Osgood (30) that the phytohemagglutinin exists as a mucoprotein. It is possible that the different methods used for purifying the protein phytohemagglutinin might result in differences in the carbohydrate moiety remaining on the protein.

If pronase or another enzyme less specific than trypsin had been used for these studies, it is probable that the eight glycopeptides would not have been observed. Particularly so if the differences were in the oligosaccharide units alone. It was for this consideration that trypsin with its lysine and arginine specificity was used. By using trypsin more of the amino acids of either side of the oligosaccharide units were obtained. In this way the chance of observing differences in the glycopeptides was increased. However, as

evidenced by the AG 50 W cation exchange chromatography, the problem became one of separation. For this reason work of an exploratory nature was carried out to find a method which could be used for glycopeptide separations. It was conceived that if the glycopeptides could be altered in such a way that their chemical structure remained unchanged, it would be possible to effect separations that might have otherwise been impossible. For this the effect of borate on sugars was chosen. As reviewed by Boeseken (60), it has been demonstrated that by the addition of sugars to borate solutions the conductivity is increased. It was postulated that complexes were formed between the cis-hydroxyl groups of the sugar and the borate. The ability for different sugars to form these complexes differs and is very dependent upon their structures. The structures suggested for these complexes are given below.



These complexes are in equilibrium with each other, the state of which is dependent upon the pH of the solution, the ratio of borate to sugar

and the absolute concentration of the sugar in the solution. The charged complexes are favored at high pH's and low ratios of sugar to borate.

Khym and Zill (61) were the first to demonstrate that sugars could be separated utilizing this property of sugars. Using Dowex 1 anion exchange resins and pH 8 borate buffers they were able to separate mono-, di- and trisaccharides from each other.

With the notion of extending these observations to glycopeptides the AG 1-X2 anion exchange resins and borate buffers were used. Several different buffers were tried, all of which proved unacceptable. Although with some buffer systems separations were achieved, at no time was the carbohydrate load fully recovered. In Fig. 14 it was demonstrated that the GP-A was missing from the glycopeptide fraction taken from the AG 1-X2 system described. Other studies using the same resins and picoline-pyridine buffers at pH 8 indicate that the GP-A is firmly bound to the resin, even in the absence of borate. Further studies are required to determine whether borate systems used with anion exchangers may be employed to effect separations of glycopeptides which differ only in their carbohydrate moieties.

V. SUMMARY

The tryptic glycopeptides of the phytohemagglutinin from the red kidney bean (Phaseolus vulgaris) were investigated. The phytohemagglutinin was isolated and purified by ammonium sulfate fractionation and polyacrylamide gel filtration procedures.

Following tryptic hydrolysis of the purified phytohemagglutinin the peptides were subjected to polyacrylamide gel filtration. A glycopeptide fraction with a molecular weight range of 3000 to 9000 was obtained. This fraction yielded eight glycopeptides when chromatographed on cation exchange columns.

Analyses of their amino acid and carbohydrate compositions has demonstrated that two or more different tryptic glycopeptides are present in the phytohemagglutinin. The absence of lysine and arginine in one of the glycopeptides indicated that it must be a C-terminal glycopeptide and that one of the oligosaccharide units is linked near the C-terminal end of a polypeptide chain of the protein.

Carbohydrate analyses of the phytohemagglutinin have shown that it contains glucosamine, relatively large amounts of mannose and smaller amounts of fucose, xylose and arabinose. Carbohydrate analyses on three glycopeptides revealed that the C-terminal glycopeptide contains all five monosaccharides, whereas fucose is absent in the other two.

From these investigations it has been suggested that eight different tryptic glycopeptides are present in the phytohemagglutinin.

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