THE CARBOHYDRATE COMPONENT OF A HUMAN GAMMA GLOBULIN

by

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by

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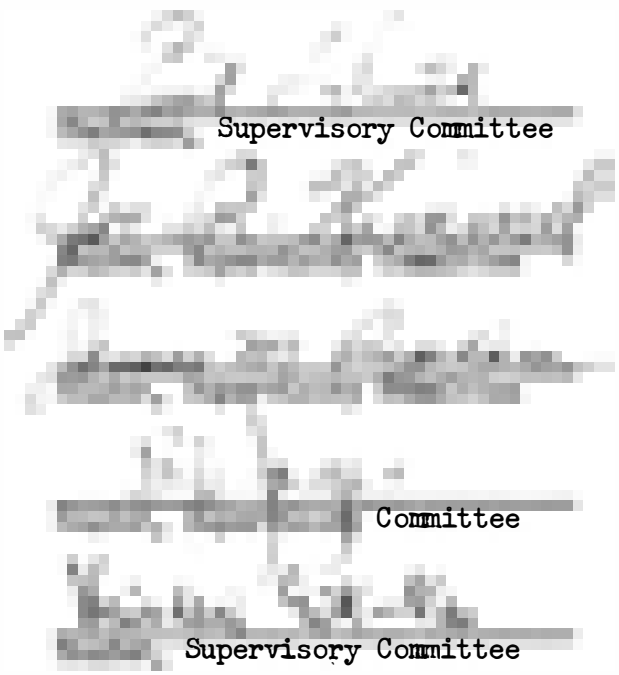

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF ILLUSTRATIONS

I. INTRODUCTION

The ability of animals to develop immunity to bacterial and virus infections, as well as to toxic agents of plants and animals, has been recognized for many years. In 1880 Pasteur discovered that parenteral injection of non-virulent preparations of bacteria and viruses produced an immunity to virulent preparations (38). Soon after this discovery von Behring demonstrated, in serum from immune animals, a principle which could produce a temporary immunity when transferred to another animal (38). This principle is now called an antibody. Many additional contributions to the concept of immunity and the role of the antibody have been made since these discoveries of Pasteur and von Behring. Antibody production was related, at first, only to the immune response; however, it was soon recognized as a general response of animals to parenteral injection of most foreign proteins and many foreign carbohydrates (71). Any substance which can produce an antibody response is now called an antigen. The chemical nature of antibodies has been disputed for many years (76). At present, antibodies are regarded as a distinct class of serum proteins with similar composition and physical properties. Most antibodies are considered to be γ-globulins (13). In fact, most γ-globulins are suspected of being antibodies (57).

Antibodies are recognized by their ability to interact with antigens and thus bring about a detectable precipitation, neutralization, alteration, or destruction of the antigens. Furthermore, antibodies show their

strongest interaction with the antigens which caused their production; whereas unrelated antigens interact very poorly or not at all. The ability of an antibody to interact specifically with only one type of antigen is referred to as antibody specificity. Antigen-antibody interaction is an excellent example of the specificity of most biological reactions and has frequently been studied as an illustration of biological specificity. Landsteiner (71) has shown that the extent of antigen-antibody interaction is influenced by minute differences in the chemical structure of the antigen. This substantiates Ehrlich's theory that antibody specificity is based on a structural correspondence or "complementariness" between the combining regions of antibody and antigen. Such a structural relation would allow many points of contact between the atoms and functional groups of the antigen and antibody (cf. Campbell (19) p. 681). Pauling (101) has explained the antigen-antibody interaction on the theoretical basis of electronic van der Waals forces, electrostatic forces, and hydrogen-bond forming forces. Individually, these forces would be insufficient to form a stable antigen-antibody bond at ordinary temperatures, but the interaction of many such forces would result in a stable antigen-antibody bond. The hydrophobic bond of Kauzmann (64) and Tanford (137) probably also contributes to antigen-antibody interaction. The strongest interaction would occur when the spatial pattern of the bond-forming elements of the antibody is completely complementary to the bond-forming elements of the antigen. Weaker interaction would occur when the bond-forming elements of the antibody and antigen are less complementary.

The antibody combining sites have been studied by observing the effects of physical and chemical treatments on antibody activity. Such studies indicate that some labile structure is necessary for activity; however, the amount is still unsettled. The effect of enzymic digestion on antibody activity is especially noteworthy. Digestion of the diphtheria antitoxin with pepsin or trypsin has been shown to yield a molecule about one-half the original size, which is fully active with respect to toxin precipitation and neutralization (90,104). Similar digestion of anti-pneumococcus antibodies, however, destroys the biological protective activity, although considerable precipitating activity remains (144). Essentially, these studies are an attempt to correlate changes in immunological properties with changes in structure and thus explain these properties in terms of antibody structure.

The correlation of antibody function and structure is one of the most important current problems in biochemistry, since it forms the basis for understanding not only a type of biological specificity, but also the limitations, abnormalities, and potentialities of antibody function in health and disease. Antibodies now appear to be involved in more than just the simple infectious diseases. They have been implicated recently in cancer, allergies, and rheumatoid diseases. However, before any features of structure and function can be definitely related, knowledge of the primary chemical structure of antibodies must be obtained. At present, little has been reported about the primary chemical structure of antibodies or γ-globulins other than the findings of Porter (108), Putnam (110), and Smith (77,79,80, 118,121,127) concerning the free amino groups, the N-terminal amino sequence,

and the apparent similarity in chemical composition of antibody preparations.

A basic question to be answered by relating structure and function is how can antibodies which are chemically and physically very similar have distinctly different specificites and functions. Different antibodies, obviously, in some manner, must have different structures; but, are these differences only physical, or are they chemical as well as physical? At present, this question cannot be answered, since significant chemical differences could very well be overlooked by current analytical methods. The significant differences might involve only five to ten of the more than 1,500 residues that make up most antibody molecules. However, differences in the structure of antibodies cannot be evaluated until the primary structure of antibodies is first established. One current controversial question about the primary structure of antibodies concerns the less than 4% carbohydrate which has frequently been recognized in antibody and γ-globulin preparations. Is this carbohydrate part of the primary structure of the antibody molecule? If so, how is it attached to the protein? Studies of the carbohydrate in an antibody-containing human γ-globulin were undertaken in this work.

The controversy over the significance of carbohydrate in antibody and γ-globulin preparations actually began when hexose and hexosamine were first discovered in serum globulin, serum albumin, and egg albumin between 1893 and 1902 by Mörner, Pavey, Krawkoff, Jacewicsch, Seeman, Hofmeister, Eichholz, and Langstein (cf. Rimington (111) and Neuberger (88)). For many years, however, the carbohydrates of this group of proteins were considered to be impurities and not truly part of the protein structure, because, first,

only small amounts of carbohydrate were found; second, the proteins studied were believed to be inadequately purified; and third, the denaturability and physical properties of these proteins were like those of non-carbohydrate containing proteins and not at all similar to the mucous-like proteins, which were known to contain large amounts of carbohydrate (cf. 88,111). Since the discovery of this group of proteins, evidence in favor of the belief that carbohydrate does form part of the primary structure has been contributed by many workers. The quantitative significance of most of this evidence, however, is questionable in view of current analytical and preparative methods.

Fränkel and Jellinek (36), in 1927, and Levene and Mori (73), in 1929, obtained polysaccharides from egg albumin following $Ba(OH)_2$ hydrolysis. The polysaccharides contained mannose and glucosamine. Levene and Mori (73) reported that the polysaccharide was nonreducing and was probably a trisaccharide composed of 1 glucosamine and 2 mannose residues. Neuberger (88), in 1938, recrystallized egg albumin to a constant 1.8% hexose content, then hydrolyzed the purified protein with trypsin and obtained a polysaccharide of 1,250 molecular weight which contained 4 mannose and 2 glucosamine residues in addition to an unidentified nitrogenous component. The polysaccharide contained 95% of the original hexose and was believed to be a single prosthetic group with one point of attachment to the molecule of natural egg albumin. However, the means of attachment was not determined.

Bierry (5), in 1929, prepared a polysaccharide from serum albumin by hydrolysis with KOH and reported that it contained galactose, mannose, and glucosamine. Rimington, in 1929, prepared polysaccharides from serum

albumin and serum globulin by hydrolysis with $Ba(OH)_2$. He reported that the polysaccharides from both proteins were trisaccharides and contained 1 glucosamine and 2 mannose residues. These polysaccharides were believed to be true constituents of the protein molecules and not just part of mucoprotein contaminants. The trisaccharides now appear to be degradation products. Sörensen and Haugaard (123), in 1933, confirmed the presence of galactose, as well as mannose, in serum proteins by the orcinol color reaction but found that the hexose content of crystalline serum albumin could vary from 0.47% to 0.02%. This led to the recognition of a carbohydrate-free and a carbohydrate-containing serum albumin (cf. Meyer (82) and Hughes (57)). The carbohydrate-containing albumin is now recognized as an α_1-globulin by its electrophoretic mobility (81).

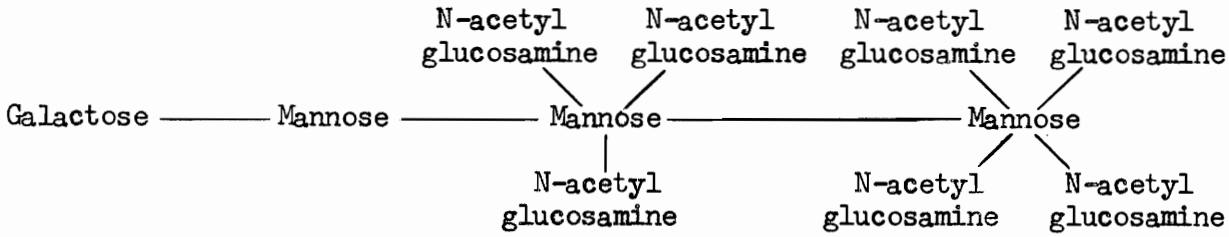
The discovery of carbohydrate-free serum albumin led to speculations concerning the existence of similar carbohydrate-free serum globulins. Hewitt (48), in 1934, found that the hexose of diphtheria antitoxin, a serum globulin, remained with the antitoxin even when it was specifically precipitated with diphtheria toxin. Peterman and Pappenheimer (97,104) have hydrolyzed diphtheria antitoxin with pepsin and isolated a fragment with half the molecular weight and twice the specific activity of the original antitoxin. This active fragment contained all the carbohydrate of the original antitoxin. Northrup (90) also found carbohydrate in the crystalline, active fragment obtained from trypsin digests of the antitoxin.

Few additional contributions appeared until more adequate methods for evaluating protein purity were developed by Tiselius (140) and Svedberg (135), and until Cohn et al. (21) developed methods for fractionating the

serum proteins into characteristic and well-defined groups. Relatively large amounts of exceptionally pure serum proteins could then be prepared. Smith et al. (118) found that the purest antibody-containing fractions from human, bovine, and equine sera contained very similar amounts of hexose and hexosamine. The carbohydrate of antibody proteins was thus recognized as a characteristic component.

Firmly bound carbohydrate is now recognized in an even larger group of proteins. It has been found in all the serum protein fractions studied except the albumin fraction (cf. Hughes (57), p. 737), in many structural proteins, such as reticulin and collagen (cf. Kendrew (65)) and in many mucous-like proteins secreted by epithelial tissues (cf. Werner (149)). Qualitatively, the carbohydrate composition of this entire group of "glycoproteins" appears to be similar (cf. Winzler (151), Stacey (132), and Meyer (82)). Not included in this group are the proteins that contain only loosely bound carbohydrates such as hyaluronic acid and chondroitin sulfate, which have not yet been found firmly bound to proteins (cf. Meyer (82) and Stacey (132)).

Several studies of the carbohydrate of the mucous-like proteins are especially noteworthy. Stacey et al. (130, 131) studied the carbohydrate of purified ovomucoid by methylation and saponification. His results indicated that the carbohydrate of ovomucoid is a single polysaccharide with the following probable structure:



No indication of the attachment to protein was found. In view of more recent work this polysaccharide does not account for all the carbohydrate of ovomucoid (cf. Warner (146), p. 467). At the time of this work, the occurrence of fucose (15) and sialic acid (148) in similar proteins was unknown. However, this work does present significant evidence for a branched, rather than linear, carbohydrate structure.

Gottschalk (42) and Blix (7) have studied the carbohydrate group of an apparently homogeneous mucous-like protein prepared by the methods of Tamm and Horsfall (136) from urine. Gottschalk concluded from semi-quantitative paper chromatographic analyses that the smallest carbohydrate unit contained 6 residues of hexosamine (glucosamine and galactosamine), 4 of galactose, 2 of mannose, and 1 of fucose, as well as an unidentified component. The studies of Blix (7) indicate that the unidentified component is probably sialic acid. Gottschalk has calculated that each molecule of this protein contains about 200 such carbohydrate units.

At present, the complete quantitative composition, the structure, and the means of attachment of a protein-bound carbohydrate group remain to be established. Furthermore, until the means of attachment is established, glycoproteins in general cannot be considered more than a very stable

addition complex of polysaccharide and protein.

The work herein described was specifically directed toward the quantitative composition and means of attachment of the bound carbohydrate group of an antibody-rich human γ-globulin. The preparation studied was fraction II-1,2 of Oncley et al. (95), Method 9. It was selected because of its known purity, its availability and its high antibody content. Jager et al. (61) verified the homogeneity of this preparation by electrophoresis, ultracentrifugation, and immunological methods. Other serum proteins could not be detected by electrophoresis, although some of the patterns did show greater spreading than would be expected for electrophoretically homogeneous particles. Ultracentrifugation demonstrated that it contains about 75% of a component with 6.5 to 7.0 $S_{20,w}$ units and 25% of a heterogeneous, heavier material which sediments over a range of 9 to 17 $S_{20,w}$ units. In regard to antibody content, this preparation is known to be very heterogeneous (cf. Oncley (95), Hughes (57), and Boyd (13)). However, it behaves as a homogeneous antigen towards rabbit antisera; thus, the particles of different mobility, sedimentation constants and antibody activity are very similar γ-globulins.

The complete amino acid analysis and free amino groups of this same preparation have been reported by Smith, et al. (77,118).

II. MATERIALS

Acetylacetone (2,4-pentanedione), Eastman 1088, Lot No. 84, B.P. 133-135° C.

Acid glycoprotein, from human plasma (120,147), given to Dr. E. L. Smith by Dr. Weimer and Dr. Winzler, Lot P.

α-Amino butyric acid, 3x recrystallized from water.

BAL (2,3-dimercaptopropanol), Bios Laboratories, Inc.

Carboxypeptidase, obtained from Worthington Biochemical Laboratories, 3x recrystallized, and dialyzed before use. A typical solution had a C_1 of 12.4.

Chymotrypsin, Armour Laboratories, crystallized, bovine origin, Lot 90492, containing not more than 50% $MgSO_4$.

p-Dimethylaminobenzaldehyde, Eastman 95, Lot No. 66. Recrystallized from ethanol-water and benzene-pet. ether.

Ethanol, absolute, U.S. Industrial Chemicals Co.

Fucose, L(-), Nutritional Biochemicals Corp.

Galactose, Merck 3655 (24846), M.P. 167-168° C.

Glucosamine hydrochloride, D(+), Pfanstiehl Chemical Co. 1713, specific rotation +72.5°.

Human γ-globulin, fraction II-1,2, obtained by Dr. E. L. Smith from E. R. Squibb and Sons, combined batches 324-329. The amino acid composition, free amino groups, and immunological, electrophoretic, and sedimenta-

tion properties of this preparation have been previously reported (61,77,118).

Hydrochloric acid, constant boiling, about 6 N. All HCl used in acid hydrolysis was prepared by distilling a 20% solution in Pyrex glass three times.

Isopropyl alcohol, Eastman 212, (In glass containers only.) Must not give opalescent solution when diluted with water.

Leucine aminopeptidase, prepared in this laboratory by Dr. R. L. Hill according to the method of Hill and Smith (49). Preparation varied in C_1 from 40 to 53.

Mannose, D(+), Eastman 2034.

Mercuripapain, prepared by the members of this laboratory according to the method of Kimmel and Smith (66). Preparations varied in C_1 from 0.6 to 1.0.

Multiple myeloma protein, Boyack (Middle No. 30), prepared by D. M. Brown in this laboratory.

Multiple myeloma protein, LeRoy (Middle No. 38), prepared by D. M. Brown in this laboratory.

Orcinol, Eastman 2112, Lot No. 46.

Pepsin, Cudahy, crystallized.

Starch, Baker Chemical Co. 4006, soluble-potato powder for iodometry, Lot No. 4064.

Trypsin, Armour Laboratories, crystallized bovine origin, Lot 9041X containing not more than 50% $MgSO_4$.

TRIS (tris[hydroxymethyl] aminomethane), Sigma Chemical Co., buffer grade,

recrystallized before use.

Versene (disodium ethylenediamine tetra-acetate), Bersworth Chemical Co.

Water, demineralized-distilled, obtained by passing distilled water through a Crystalab Deeminer, giving less than 0.2 parts per million conductivity.

III. ANALYTICAL METHODS

A. Carbohydrate Analysis

1. Paper Chromatography of Carbohydrates. The methods for carbohydrate chromatography have been reviewed by Hough (56), Kowkabany (69), Block, Durrum, and Zweig (9), and Lederer and Lederer (72).

a. Solvent systems. Isherwood and Jermyn (60) found that carbohydrates separate in the same order in all except phenolic solvents, that the R_F of aldohexoses and aldopentoses is roughly inversely proportional to their melting points, and that maximum resolution is obtained with solvents that give R_F values below 0.2 (63).

Jeanes et al. (62) have shown that, in a series of solvents of increasing R_F, the resolution of a pair of carbohydrates increases to a maximum and then decreases. For chromatography of most carbohydrates they recommend solvents containing an alcohol, a pyridine type base, and water. Solvents containing phenols or carboxylic acids should be reserved for resolutions found to be inadequate in pyridine type solvents. They found that the pyridine type solvents resolved most common monosaccharides except mixtures of mannose, arabinose, and fructose. For these mixtures phenolic solvents were more effective (99).

The following solvents were used in this work:

1) n-butanol-pyridine-water (6:4:3, v/v), developed by Jeanes et al. (62).

2) sec-butanol-acetone-acetic acid-water (3.0:3.0:1.5:2.5, v/v), developed by Zilliken et al. (152).

3) n-propanol-pyrophosphate buffer (70:30, v/v), developed by Hanes (46). The pyrophosphate buffer contained 0.05 M sodium pyrophosphate, pH 7.2-7.3 and 0.1 M NaCl.

4) Ethyl acetate-acetic acid-ethanol-water (400:114:290:180, v/v), developed in this work.

5) Ethyl acetate-acetic acid-ethanol-benzene-water (325:93:236:200:146, v/v), developed in this work.

6) sec-butanol-pyridine-formic acid (87%)-water (340:40:22:100, v/v), developed in this work.

7) n-butanol-acetic acid-water (200:30:75), developed by Thompson (139) for amino acid chromatography.

Whatman No. 1 paper was used for all except solvent 3. For this solvent Whatman No. 2 was used after being dipped in the pyrophosphate buffer and dried. The solvents were used in descending systems and allowed to drip off the serrated lower edge of the paper in order to obtain satisfactory movement of the carbohydrates. In addition, the multiple development technique (62) was sometimes used to improve the resolution. Table 1 lists typical results obtained in this work using the chromatographic solvents listed above. The values listed cannot be relied upon for identification purposes, but they are a guide to the relative positions of the sugars.

TABLE 1

CARBOHYDRATE CHROMATOGRAPHY[a]

Constituent	Solvent							
	1	1	2	3	4	5	6	7
	Time (Hrs.)							
	20	3x20	25	26	22	24	25	24
Mucic acid							1.4 5.4 10.1	
Sialic acid	4.3 & 6.8		23.0 to 28.0	3.5 & 11.5				
Galacturonic acid							10.2	
Glucuronic acid	3.3 & 5.6		22.0 to 26.5	5.5		8.1	10.5	3.6 to 7.3
Galactosamine		30.6						
Glucosamine	13.0	33.3		9.8	11.8		14.3	
Gluconolactone							14.8	
Galactose	15.6	35.5	24.0	14.2	16.1	11.3	16.7	8.0
Glucose					17.8	12.5	18.3	
Sorbitol							19.8	
Sorbose							20.5	
Dulcitol							20.6	
Mannitol							21.4	
Fructose							22.1	
Arabinose							22.8	
Mannose	20.1	40.9	29.0	20.4	20.8	15.2	22.9	10.7
Xylose							26.7	
Lyxose							27.6	
Fucose	23.4	43.7	35.0	25.5			29.7	15.4
Ribose							30.2	
Galactonolactone							32.1	
Glucuronolactone	31.2		41.0	32.0	35.6	34.8	32.3	16.7
Rhamnose							34.9	

[a] Values given are approximate distances (cm.) constituents move in indicated time.

b. Detection of carbohydrates. The sugars on the paper chromatogram were detected with the reagents usually applied to sugar analysis and some new reagents developed especially for paper chromatography. A visible color or an ultraviolet fluorescence was formed in the area occupied by the sugar. The following reagents were used in this work:

1) Aniline phthalate (Partridge (100)). Aniline, 0.93 g.; phthalic acid, 1.66 g.; and water-saturated n-butanol, 100 ml. The developed chromatogram was sprayed with the reagent and heated in a 105° C. oven for 5 min. The following colors resulted: Aldopentoses, red; 6-deoxyaldohexoses, gray; aldohexoses, brown; aldohexuronic acids, pink; hexosamines, faint brown. Hough (54) has suggested that glacial acetic acid be substituted for n-butanol to improve the colors.

2) p-anisidine. p-anisidine, 1.2 g.; phthalic acid, 1.7 g.; stannous chloride (dihydrate), 10 mg.; and ethanol or water saturated n-butanol, 100 ml. The developed chromatogram was sprayed with the reagent and heated in a 105°C. oven for 5 min. The following colors resulted: Aldopentoses, red; aldohexuronic acids, red-brown; aldohexoses, green-brown; 6-deoxyaldohexoses, brown; ketoses, yellow; sialic acid, no color. This reagent was modified from the one described by Hough (54) by using a non-aqueous solvent and thus avoiding possible streaking.

3) Ammoniacal silver. Ag_2O, 2 g.; NH_4OH (28%), 25 ml.; and methanol, 75 ml. The chromatogram was sprayed with the reagent and warmed gently with a hot plate or hot air blower. The unreduced silver was removed with a rinse of sodium hyposulfite acidified with acetic acid and water. All reducing sugars formed black spots. This reagent was developed from the

silver reagents of Partridge (98,99) and Hough (17,55). It was easily applied without streaking, and gave a white background.

4) Basic silver.

a) $AgNO_3$, 2 g.; water to just dissolve $AgNO_3$; and methanol, 100 ml.

b) NaOH, 2 g.; water to just dissolve NaOH; and methanol, 100 ml. The chromatogram was sprayed with a), allowed to dry and sprayed with b). As soon as the background began to darken, the chromatogram was dipped into a solution of sodium hyposulfite acidified with acetic acid. The chromatogram was then washed and dried. Intense black spots were given by all sugars and other polyhydroxy compounds. This reagent was very sensitive, but completely non-specific and difficult to control. It was developed from the reagent of Trevelyan (141).

5) p-dimethylaminobenzaldehyde (Partridge (99)).

a) Acetylacetone, 1% in n-butanol, 100 ml.; ethanolic KOH, 5 ml.; KOH, 50% aqueous solution, 5 ml.; and ethanol, 20 ml.

b) p-dimethylaminobenzaldehyde, 1 g.; ethanol, 30 ml.; n-butanol, 180 ml.; and HCl, conc., 30 ml.

The chromatogram was sprayed with reagent a) and heated in a 105° C. oven for 5 min. It was then sprayed with reagent b) and gently warmed with a hot air blower. A cherry red color was given by hexosamines.

6) Orcinol-T.C.A. (Klevstrand and Nordal (68). Orcinol, 0.5 g.; trichloracetic acid, 15 g.; and water-saturated n-butanol, 100 ml. The chromatogram was sprayed with the reagent and heated in a 105° C. oven for 15 min. Sialic acid (diacetylneuraminic acid), acetylneuraminic acid and neuraminic acid gave

purple spots. This reagent was developed by Klevstrand and Nordal (68) as a spray reagent for detecting ketoheptoses. Blix (8) found it useful for detecting sialic acid.

c. Quantitation. The amount of unknown carbohydrate was determined either by visual comparison of the unknown with known levels of the suspected carbohydrate, or by analyzing the known and the unknown in a Spinco Analytrol as follows: The chromatogram was cut lengthwise into 3 cm. strips and analyzed using a Corning 5031 blue filter. The areas of the peaks given by the Analytrol were then used to calculate the amount of the unknown present.

2. Photometric Hexose Determination. The method used in this work was the Vasseur (145) modification of the Tillmanns-Philippi orcinol reaction essentially as applied to serum glycoprotein by Winzler (151).

a. Reagents.

1) Standard (2.00 micromoles of galactose-mannose (1:1) per ml. in 10% isopropyl alcohol). Galactose, 0.1802 g.; mannose, 0.1802 g.; isopropyl alcohol, 100 ml.; and water to 1 liter.

2) Orcinol-H_2SO_4. Orcinol, 1.6% (w/v), in 1 N H_2SO_4, 100 ml.; and 3:2 H_2SO_4-water (v/v), 900 ml.

b. Procedure. The blank, samples, and standards, 1.0 ml. each, containing 10% isopropyl alcohol, were placed in six-inch test tubes. Orcinol-H_2SO_4, 10.0 ml., was added to each test tube and then mixed with a glass stirring rod flattened on the end. The tubes were heated in an 80° C. water bath for 15 min., then placed in cold water. The developed color was read in a Coleman Jr. Spectrophotometer at a wave length of 540 mμ

against distilled water. The galactose-mannose equivalent of the samples was then determined by comparing the spectrophotometer color readings with those of the galactose-mannose standards.

The optical density was found to be directly proportional to the quantity of standard up to 2.0 micromoles of galactose-mannose. This gave a maximum optical density of about 0.8. The isopropyl alcohol was at first included in the samples only as a preservative, but it was also observed to improve the linearity of the optical density curve. The curve without isopropyl alcohol was slightly "S" shaped; with isopropyl alcohol, the curve was linear up to an optical density of about 0.8. A reagent blank on 1.0 ml. of 10% isopropyl alcohol in water was included in every assay. The optical density of the blank was usually about 0.003.

Galactose-mannose in a 1:1 molar ratio (1:1 G-M) was used as the assay standard. Sialic acid and glucosamine are reported not to interfere in this determination (8,145,151). The relative color yields of the common aldohexoses and fucose in this determination are given in Table 2.

TABLE 2

ORCINOL COLOR YIELDS COMPARED
TO 1:1 GALACTOSE-MANNOSE

Carbohydrate	Color Yields
Galactose	1.11
Mannose	0.88
Glucose	0.37
Fucose	0.62
Galactose-mannose, 3:5	0.98

The final results were corrected for the fucose content determined by the cysteine-H_2SO_4 reaction (Section III-A-5) and for the hexose ratio determined by paper chromatography (Section III-C-1), for example:

$$\mu M \text{ of } 3{:}5 \text{ G-M} = \frac{(\mu M \text{ of } 1{:}1 \text{ G-M}) - (0.619)(\mu M \text{ of fucose})}{0.98}$$

This orcinol method gives the predicted color yield only with monosaccharides. The color yield for polysaccharides is expected to be slightly lower than predicted. Vasseur (145) found it to be approximately 90% of that of equivalent monosaccharides. This low color yield, however, is sometimes more than compensated for by a brown color which is produced by the protein of the sample. Although the interference of this color cannot be completely avoided (145, 151), this method does reduce the contribution of this color to a minimum.

3. Photometric Hexosamine Determination. Elson and Morgan (33) have developed an assay for hexosamines using acetylacetone and p-dimethylaminobenzaldehyde. Many modifications of this procedure have been reported (6,10,37,58,96,134,151). The procedure used in this work, based on the studies of Blix (6) and his recommended modifications, was as follows:

a. Reagents.

1) NaCl, 2.0 N

2) Acetylacetone, 4 ml. plus 96 ml. of 1.25 N Na_2CO_3. (Made up just before each use.)

3) p-dimethylaminobenzaldehyde, 1.6 g.; ethanol, absolute, 30 ml.; and HCl, conc., 30 ml.

4) Standard glucosamine, 1.00 micromoles/ml. Glucosamine·HCl, 0.2156 g.;

isopropyl alcohol, 100 ml.; and water, to 1 liter.

5) HCl, 4.0 N.

6) NaOH, 4.0 N (standardized against 5) using methyl red indicator).

b. Acid hydrolysis. (When the hexosamine was free, hydrolysis was unnecessary.) Samples containing about 0.5 micromoles of hexosamine were placed in six-inch test tubes, diluted with water to 1.0 ml., and 1.0 ml. of 4.0 N HCl acid were added, giving 2 N HCl. The tubes were capped with glass marbles and placed in a 90° C. water bath with 4 in. of the tube extending above the water level. The tubes were removed after 12 to 14 hrs., cooled in ice-water, and 1.0 ml. of 4.0 N NaOH (standardized against the 4.0 N HCl using methyl red indicator) were added carefully.

c. Assay procedure. The blank, samples (all reagents plus water), and standards (0 to 1 micromole of hexosamine) were placed in six-inch pyrex test tubes and 1.0 ml. of 2 N NaCl were added to the blank, standards, and to the samples that were not hydrolyzed. The samples, standards, and blanks were diluted with water to give 3.0 ml., and 3.0 ml. of fresh 4% acetylacetone in 1.25 N Na_2CO_3 were added to each tube. The tubes were heated in a 90° C. water bath for 1 hr., and cooled in ice-water. Then 10.0 ml. of absolute ethanol were added and mixed, and 1.0 ml. of p-dimethylaminobenzaldehyde reagent were added slowly with stirring. The tubes were placed in a 30° C. water bath for 1 hr. The color intensities were read in a Coleman Jr. Spectrophotometer at a wave length of 530 mμ against distilled water. The hexosamine equivalent of the samples was determined by comparing the spectrophotometer readings with those of the glucosamine standards. Glucosamine and galactosamine

are reported to give the same color yield (33). The plot of the optical density versus the quantity of standard was linear up to about 0.7 micromoles or 0.6 optical density.

This modification has the advantage of increasing the sensitivity of the determination, reducing the number of sample transfers, and allowing the use of test tubes instead of volumetric flasks. The amount of ethanol used in the procedure was found to affect the quantity of colored product formed as well as its concentration. This fact is not mentioned in the literature. The 10.0 ml. of ethanol used here were found to be optimal. It was also discovered that the optical density of the blank (all reagents plus water) could be reduced to 0.05 and frequently below 0.02; first, by adding the p-dimethylaminobenzaldehyde reagent after all the ethanol had been added; second, by adding the reagent to ice-cold solutions, and third, by allowing the color to develop at 30° C. Under these conditions the color intensity reached a maximum in about 1 hr.

Since the ionic strength of the samples influenced the development of the final color, NaCl was included in the standard tubes to give them the same ionic strength as the hydrolyzed samples.

If the sample contained hexosamine in the bound form, the sample was hydrolyzed with acid before analysis. The conditions used for acid hydrolysis, 2 N HCl at 100° C. for 14 hrs., were recommended by Blix (6) for mucoproteins. The conditions for maximum yield of the hexosamine appear to vary with the type of sample. Strange and Powell (134) obtained maximum yield of hexosamine from peptides of bacterial spores using 6 N HCl at 108° C. for 20 hrs. Even then, recovery experiments indicated that 11% of the hexo-

samine was destroyed. Blix (6) has also noted an apparent destruction of hexosamine during acid hydrolysis; however, he was able to reverse the apparent destruction by increasing the heating time of the acetylacetone treatment step. The longer heating time recommended by Blix was used in this work.

4. Photometric Sialic Acid Determination. The methods for quantitative determination of sialic acid have been studied by Werner and Odin (148), and Winzler (151). Two methods were used in this work, the direct Ehrlich and the Bial-orcinol methods described by Werner and Odin (148). The standard for the assay was the acid glycoprotein of Weimer, Mehl, and Winzler (147), which has been reported to contain 10.1% sialic acid by the Ehrlich method and 9.8% by the orcinol method (148).

5. Photometric Fucose Determination. The cysteine-H_2SO_4 method of Dische (28), as applied by Winzler (151), with an external L-fucose standard and a 3 min. heating period, was used for the determination of fucose. Optical densities were measured at 396 and 430 mμ with a Beckman DU Spectrophotometer.

6. Photometric Hexuronic Acid Determination. The carbazole method of Dische (28) was used for the determination of hexuronic acids. The absorption spectra of the reaction products were determined by a Beckman DU Spectrophotometer.

B. Nitrogen Determination

The nitrogen content of the sample solutions was determined by a photometric micro-Kjeldahl method. Since only a few milligrams of the samples were available, it was necessary to develop a convenient method for assaying

samples containing about 0.1 micromole of nitrogen (about 1.4 micrograms of nitrogen). A method was developed using the ninhydrin color reaction. The sample was digested by the Kjeldahl method using a mercury catalyst as recommended by Hiller, Plazin, and van Slyke (50). After digestion, sodium acetate was added to raise the pH to about 5.5. The ninhydrin-hydrindantin reagent of Stein and Moore (87) was then used to determine the amount of ammonia present. The samples were run in duplicate. Digestion mixture, but no sample, was added to two tubes which were used as blanks. Tubes were also included for four levels of α-aminobutyric acid standard. The procedure used was as follows:

To six-inch heavy-walled pyrex test tubes, 0.10 ml. of the following digestion mixture was added: H_2SO_4, 50 ml.; HgO, 100 mg.; K_2SO_4, 7 g., and water, 50 ml. Aliquots of sample and standard, containing less than 0.3 micromoles of nitrogen in 0.1 ml. or less of solution, were then added. The tubes were placed on a 300° C. sand bath about 8 cm. deep, pushed down into the sand to a depth of about 1 cm., and heated for about 1 hr. The tubes were then capped with glass marbles, pushed down into the sand to a depth of about 5 cm., and heated for about 4 hrs. At the end of the digestion, the tubes were allowed to cool and 2.0 ml. of 2 N sodium acetate were added to each tube. Ninhydrin-hydrindantin (87), 1.0 ml., was added to each tube. The tubes were placed in a boiling water bath for 30 min., shaken for 30 seconds, and 10.0 ml. of 50% n-propanol in water were added. The optical density of each tube was read in a Coleman Jr. Spectrophotometer at 570 mμ against distilled water.

The color yield of the α-aminobutyric acid standard was approximately

0.62 micromoles per optical density unit. The optical density of the blanks was usually about 0.10. The nitrogen content of the samples was calculated from the net optical density of the samples and the α-aminobutyric acid color yield determined for each assay. Several other procedures were tried, including the use of selenium and copper as catalysts, but they all gave results which were lower than predicted from the ninhydrin color yield. The method described above does not have the precision of the methods using larger samples, but it compares well with the other photometric methods used in this work. The duplicates usually checked within 5%.

C. Amino Acid Analysis

1. Paper Chromatography. In this work, two solvents were used in standard descending systems. The bottom edge of the paper was serrated to allow the solvent to drip off. After development with solvent from 18 to 24 hrs., the paper sheets were dried at room temperature, sprayed with the ninhydrin reagent of Levy and Chung (74), and gently warmed with a hot air blower, while still wet with the color reagent, until the colored spots developed their full color. The following solvents were used:

1) n-butanol-acetic acid-water (200:30:75, v/v) (139).

2) n-propanol-pyrophosphate buffer (70:30, v/v) (46). (See Section III-A-1-a). Typical results are shown in Table 3.

The ninhydrin color reagent contained: ninhydrin, 0.1% in 95% ethanol, 50 ml.; acetic acid, 15 ml.; and collidine, tech., 2 ml.

2. Ion Exchange Resin Chromatography. The chromatographic procedure of Moore and Stein (85) was modified to permit a complete amino acid analysis

TABLE 3

AMINO ACID CHROMATOGRAPHY[a]

n-propanol-pyrophosphate		n-butanol-acetic	
	cm./22 hrs.		cm./20 hrs.
CYS	3.6	CYS	0.9
LYS	5.1	LYS	2.2
ASP, $CYSO_3H$	5.8	ARG, $ASP-NH_2$, HIS	3.1
GLU, HIS	6.9	$GLC-NH_2$, SER	4.2
ARG	8.3	ASP	5.4
$GAL-NH_2$, $ASP-NH_2$	8.7	GLY	6.5
SER, $GLC-NH_2$	10.0	GLU	8.3
$GLU-NH_2$	10.6	THR	9.5
GLY	11.3	ALA	12.2
THR, HO-PRO	16.2	PRO	13.2
ALA	18.7	TYR	15.2
PRO	24.4	VAL	22.0
TYR	31.2	PHE	25
MET	32.5	ILEU	28
VAL	35.0	LEU	30
TYR	36.8		
ILEU	40.0		
PHE	40.6		
LEU	41.9		

[a] Including the amino sugars glucosamine ($GLC-NH_2$) and galactosamine ($GAL-NH_2$). The abbreviations used for the amino acids were suggested by Brand and Edsall (14).

in two days. Pressure sufficient to give a flow rate of about 12 ml./hr., an operating temperature of 50° C., and three eluents for the two columns were used as suggested by Spackman, Stein, and Moore (124,125). The columns were 150 x 0.8 cm. and 15 x 0.8 cm. and contained Amberlite IR 120 (XE165), passed through a 200 mesh sieve when wet, and then further separated by suspending in water, allowing to settle, and decanting the upper 10% three times. The finer particles of resin obtained in this manner were used in the 15 cm. column; the coarser particles were used in the 150 cm. column. In addition to amino acids, glucosamine and galactosamine can also be separated by the column (37,126). The amino acids and hexosamines emerging from the column in the effluent fractions were quantitated with the ninhydrin-hydrindantin color reagent of Moore and Stein (87). The entire 1.5 ml. of each effluent fraction was analyzed. The optical density of the fractions was determined with a Coleman Jr. Spectrophotometer, and an elution diagram was obtained by plotting the optical densities of the effluent fractions against the fraction number on linear graph paper. The quantity of each component, represented by a peak in the elution diagram, was then determined from the integrated value of the peak. The selection of the correct base line and the integration of the peaks has been thoroughly discussed by Moore and Stein (84,85,86,133). The leucine equivalent of each component was obtained by multiplying the integrated value by the leucine color factor (0.412 micromoles/optical density unit) determined in this laboratory for the color development and photometric procedures used. The quantity of each component was then obtained by multiplying the leucine equivalent of the component by its relative color yield as established by Stein and Moore (87).

This method is reported to recover 100 ± 2% of all the amino acids except glutamic acid (85). Under these conditions of temperature, acidity, and time, about 5% of the glutamic acid was converted to pyrrolidonecarboxylic acid, as determined by Hirs, Moore, and Stein (51,85). The value of the glutamic acid was corrected for this loss. The buffers used are given in Table 4.

TABLE 4

BUFFERS FOR ION EXCHANGE CHROMATOGRAPHY
(86,124)

Buffer	Citric Acid·H_2O g.	Sodium Hydroxide (97%) g.	Hydrochloric Acid (conc.) ml.	Final Volume liters	BRIJ-35[a] ml.	Thiodiglycol[a] ml.	Water[a] ml.
pH 2.2 ± .03 0.2 N sodium citrate	105	42	80	5			
pH 3.25 ± .03 0.2 N sodium citrate	168	66	80	8	40	80	
pH 4.25 ± .03 0.2 N sodium citrate	168	66	36	8	40	80	
pH 5.15 ± .02 0.35 N sodium citrate	224	132	60	8	40		1144

[a] Added after pH adjustment and dilution to final volume.

IV. EXPERIMENTAL METHODS AND RESULTS

A. Component Carbohydrates of Human γ-Globulin

The component carbohydrates of human γ-globulin, fraction II-1,2 (95) were determined by paper chromatographic and photometric methods. The determinations were performed on either the air equilibrated powder or a stock solution prepared from the powder. The protein content of the stock solution was evaluated by the micro-Kjeldahl method described in Section III-B and expressed in terms of the nitrogen content of the anhydrous γ-globulin, 15.65% (118). The moisture content of the air equilibrated powder, as determined by McFadden (78), was 5.8%.

1. Paper Chromatography. In preparation for paper chromatography the carbohydrate components of γ-globulin were hydrolyzed with Dowex 50 in the hydrogen cycle. Ion exchange resins have been similarly used to hydrolyze many carbohydrates and proteins (26,31,39,40,41,102,112,143,150). Only about 10% of the hexose of γ-globulin was recovered by this method, but this recovery was adequate for qualitative identification.

Hydrolysis was undertaken with 100 mg. of the γ-globulin, 10 ml. of Dowex 50-X2 (200-400 mesh, hydrogen cycle), and 25 ml. of demineralized-distilled water in a sealed, evacuated 50 ml. hydrolysis tube at 105° C. The hydrolysis was continued for 66 hrs. to allow sufficient time for clumps of undissolved globulin to disintegrate. The hydrolysate was filtered, evaporated to dryness in an evacuated desiccator containing NaOH and con-

centrated H_2SO_4, and dissolved in water. Appropriate aliquots were chromatographed with solvent 5 (Section III-A-1-a) and developed with the aniline phthalate color reagent. Clearly resolved spots of galactose, mannose, and fucose were detected. The chromatogram was analyzed with a Spinco Analytrol using cam C and a Corning 5031 blue filter. The optical density-area products for galactose, mannose, and fucose were 61, 91, and 13, respectively. This indicates a galactose-mannose ratio of about 3 to 5. The amount of fucose present, however, was too low for significant evaluation.

Pentoses, uronic acids, and other hexoses could easily have been detected if present. Glegg et al. (39,41) were able to detect ribose and glucuronic acid, as well as galactose, mannose, and fucose in reticulin by similar hydrolysis and chromatographic procedures. Sialic acid was not expected to survive the conditions of hydrolysis and hexosamines would have remained bound to the Dowex 50. An attempt was made to detect hexonic acids and other non-reducing polyhydroxy compounds with the basic silver spray reagent (Section III-A-1-b); however, only a continuous smear appeared. Apparently the unresolved degradation products also reacted with the reagent, obscuring even the galactose, mannose, and fucose spots.

2. Hexose. The hexose content of γ-globulin was determined by the orcinol-H_2SO_4 method described in Section III-A-2. This determination, performed on two 10.0 mg. samples of the air equilibrated γ-globulin powder, gave a value of 62.6 ± 1.6 μM (1:1 G-M)/g. When corrected for moisture (5.8% (74)), fucose (16.1 μM/g., Section IV-A-4), and the 3:5 galactose-mannose ratio found by paper chromatography (Section IV-A-1), this value became 57.7 μM/g, 9.23 residues per mole of γ-globulin (160,000 g.), or

0.94%. The hexose value of this preparation was previously determined (118) with a glucose standard and a different orcinol-H_2SO_4 method. If the color yields given in Table 2 apply to the orcinol-H_2SO_4 method previously used, the 3:5 galactose-mannose equivalent of the previously determined value, 2.3% (118), would be 0.87%. This value agrees with the value determined in this work, 0.94%, within the expected limits. These values, however, are probably higher than the actual hexose values because of the interference of the brown color formed by the relatively large amount of protein in γ-globulin (95%). Thus, the present analytical value, 9.23 residues per mole, probably represents 8 to 9 actual hexose residues.

3. Hexosamine. The hexosamine content of γ-globulin was determined by the Elson and Morgan method described in Section III-A-3. The standard for the assay was glucosamine·HCl. The assay was performed on 1.0 ml. aliquots of a stock solution of the γ-globulin in 0.15 M NaCl containing 16.6 mg./ml., based on micro-Kjeldahl nitrogen content. The samples were hydrolyzed with 2N HCl for 14 hrs. prior to assay. The final color readings were corrected for the nonspecific color of samples treated like the other samples except for the omission of the p-dimethylaminobenzaldehyde from the color reagent. The average of two determinations was 63.0 ± 2.9 μM/g. This corresponds to 10.08 moles per mole of γ-globulin (160,000 g.) or 1.28% acetyl hexosamine, which is very close to the 1.27% previously reported for this preparation (118). Both assay values, however, are probably higher than the true hexosamine values since, according to Boas (10), similar colors are formed by other substances such as pyrroles and indoles either present in the sample or formed from hexoses and amino acids during the assay. Sialic acid may

also form a similar color (148). Thus the analytical value, 10.08 residues per mole, probably represents 8 to 10 actual N-acetylglucosamine residues.

The amino groups of the hexosamine are presumably acetylated since DNP-hexosamines were not detected after fluorodinitrobenzene treatment of either this preparation (77) or the glycopeptides prepared from this preparation (Section IV-C-7-a) and since the free amino groups of naturally occurring hexosamines are almost always acetylated (132). The hexosamine is probably glucosamine since only glucosamine was detected in the glycopeptides prepared from γ-globulin (Section IV-C-5-d).

4. Fucose. The fucose content of γ-globulin was determined by the cysteine-H_2SO_4 method described in Section III-A-5. The standard for the assay was L-fucose. Although rhamnose gives the same color yield, the assay was considered a measure of fucose since no other 6-deoxyaldohexose has been found in animal tissue (15,29,151). The assay was performed in duplicate on 1.0 ml. aliquots of a stock solution of the γ-globulin in 0.15 M NaCl containing 16.6 mg./ml., based on micro-Kjeldahl nitrogen content. The assay indicated a fucose content of 16.1 ± 0.4 μM/g. This is equivalent to 2.6 moles per mole of γ-globulin (160,000 g.). This value, however, is probably higher than the true fucose value since protein usually increases the fucose color yield (29). Thus, the actual fucose content of γ-globulin is probably 2 residues per mole.

5. Sialic acid. The sialic acid content of γ-globulin and two multiple myeloma proteins was determined by the direct Ehrlich method as described by Werner and Odin (148). The acid glycoprotein of Weimer, Mehl and Winzler (147) was used as the standard. It had been reported to contain

10.1% sialic acid (148) by this method. The samples were weighed directly into matched 0.8 x 7.5 cm. assay tubes and dissolved with 1.5 ml. of 0.2 M NaCl. Then 0.3 ml. of the p-dimethylaminobenzaldehyde reagent were added. The tubes were placed in boiling water for 30 min., cooled, centrifuged, and read in a Coleman Jr. Spectrophotometer at 565 mμ. The acid glycoprotein equivalent of each sample was read from a standard curve prepared from 0.1 to 1.0 mg. of the acid glycoprotein per tube. The results obtained are given in Table 5.

TABLE 5

SIALIC ACID CONTENT OF TWO MULTIPLE MYELOMA PROTEINS AND HUMAN γ-GLOBULIN

Sample	Equivalent Amt. of Acid Glycoprotein	g. Sialic Acid per 100 g. of Sample	Moles of Sialic Acid per 160,000 g.
Multiple myeloma protein[a], Boyack (Middle No. 30) 10 mg.	0.77 mg.	0.78	3.75
Multiple myeloma protein, LeRoy (Middle No. 38) 10 mg.	0.21	0.212	1.02
Human γ-globulin II-1,2 75 mg.	1.37	0.185	0.89

[a] The physical, chemical, and immunological properties of Boyack multiple myeloma protein were reported by Smith et al. (122), referred to as Myeloma D.

The method used here is claimed by Werner and Odin (148) to be the most specific one for sialic acid, but not the most sensitive. Tryptophan does not give a color in this determination provided peroxides or other oxidants are absent. The analytical values given in Table 5 probably represent, respectively, 4, 1, and 1 moles of sialic acid per mole of protein.

B. Preparation of Glycopeptides

To allow a more detailed study of the carbohydrate of γ-globulin, a procedure was developed for separating the carbohydrate from the protein. The globulin was first hydrolyzed with a proteolytic enzyme of broad specificity. The carbohydrate-containing fragments, referred to as glycopeptides, were then isolated by appropriate methods.

The procedures were followed by the orcinol hexose determination given in Section III-A-2 and the Moore and Stein photometric ninhydrin amino nitrogen determinations (87).

Step 1 - Digestion with Papain. A salt-free, 10 to 20% suspension of human γ-globulin was digested with 1 to 2% (w/w) recrystallized mercuripapain prepared by the procedure of Kimmel and Smith (66). The papain had a C_1 of 0.6 or higher. Ethylenediaminetetra-acetate (Versene) and cysteine-HCl or 2,3-dimercaptopropanol-1 (BAL) were used to activate the papain. Papain and activators were added at intervals during the digestion. The pH of the digest was maintained at 6.5 $\pm$ 0.5 by the intermittent addition of 1.0 N base. The digestion was conducted at 60° C. to reduce the hazard of bacterial growth and allow a rapid digestion. Papain was selected for the digestion because of its ability to digest proteins extensively (18,53,116)

and its ready availability in pure, carbohydrate-free form. The preparation, specificity, and properties of papain have been reviewed by Kimmel and Smith (67).

The digestion was allowed to continue until no further increase in ninhydrin assay value was observed. This usually took from 24 to 48 hrs. The final ninhydrin assay value was about 5 millimoles per gram of globulin. This corresponds to about 50% hydrolysis. At the end of the digestion a yellow-gray precipitate remained. Since it contained only about 1.5% of the original hexose, it was discarded. The clear supernatant, which contained about 94.5% of the original hexose, was used for the preparation of the glycopeptides. The digestions are summarized in Table 6.

Step 2 - Dowex 50 Treatment. The clear supernatant from the papain digest was treated with 300 ml. of well-washed Dowex 50x8, 20 to 50 mesh, in the hydrogen cycle, at 5° C. The eluate from the resin, which had a pH of about 2, was neutralized with lithium hydroxide. The eluate from a typical treatment contained 72% of the hexose but only 1.6% of the free amino groups applied to the resin. The major portion of the glycopeptides was not bound by the resin, probably because the glycopeptides were too large to penetrate the mesh of the resin. An abstract of the principles involved has been published by the Dow Chemical Company (32). Several other resins were used in preliminary experiments: Dowex 50x2, 200-400 mesh, hydrogen cycle, retained 72% of the hexose. Dowex 1x2, 200-400 mesh, bicarbonate cycle, and Dowex 50x2, 200-400 mesh, silver cycle, retained all the hexose. These resins were not considered useful for the preparation of the glycopeptides.

TABLE 6

PAPAIN DIGESTION OF γ-GLOBULIN

Preparation No.	Amount of Globulin	Volume of Digest	Amount of Papain	Activators Used	Extent of Digestion	Base Used to Maintain pH	Time Allowed for Digestion
I	25 g.	250 ml.	0.3 g.	Cysteine 7.5 mM. Versene 5.0 mM.	4.2 mM./g.	10 ml. 1.0 N NaOH	25 hrs.
II	50 g.	318 ml.	0.95 g.	BAL 10 mM. Versene 10 mM.	5.4 mM./g.	8 ml. 1.0 N NaOH	28 hrs.
III	50 g.	335 ml.	1.0 g.	BAL 12.5 mM. Versene 12.5 mM.	-	20 ml. 1.0 N LiOH	43 hrs.
IV	50 g.	335 ml.	1.0 g.	BAL 13.3 mM. Versene 12.5 mM.	5.5 mM./g.	21 ml. 1.0 N LiOH	98 hrs.

Step 3 - Ethanol Precipitation. The results of a preliminary experiment indicated that precipitation of the glycopeptides with about 90% ethanol gave significant purification. As illustrated in Figure 1, 90% ethanol precipitated about 90% of the hexose, but only about 40% of the free amino groups. Consequently 90 to 95% ethanol was used in preparing the glycopeptides. Precipitation was carried out by adding the glycopeptide solution to the ethanol to avoid formation of a gum. Absolute ethanol was used to keep the total volume small. The precipitates were recovered by centrifugation, dissolved in water, filtered, and reprecipitated 2 to 3 times. Lithium chloride in ethanol was used when necessary to flocculate the precipitate. The precipitates were washed with cold absolute ethanol and cold anhydrous, peroxide-free diethyl ether and then dried in a cautiously evacuated desiccator containing P_2O_5 and NaOH. A typical 3-fold precipitation yielded 84% of the hexose, 18% of the free amino groups, and 20% of the solids initially present. The carbohydrate-containing fragments prepared in this manner will be referred to as the Mixed Glycopeptide. Preparation of the Mixed Glycopeptide is summarized in Figure 2.

Step 4 - Zone Electrophoresis. Paper electrophoresis was used to study the purity of the Mixed Glycopeptide and to determine suitable conditions for purifying the glycopeptides by starch column electrophoresis. Two paper electrophoresis systems were used. One system consisted of a covered glass aquarium which used paper sheets or strips 57.5 cm. long; the other system was the Spinco Model R paper electrophoresis unit. The best resolution was obtained with 0.05 N pH 8.5 sodium diethylbarbiturate (Veronal) buffer. Similar electrophoresis patterns were obtained, however

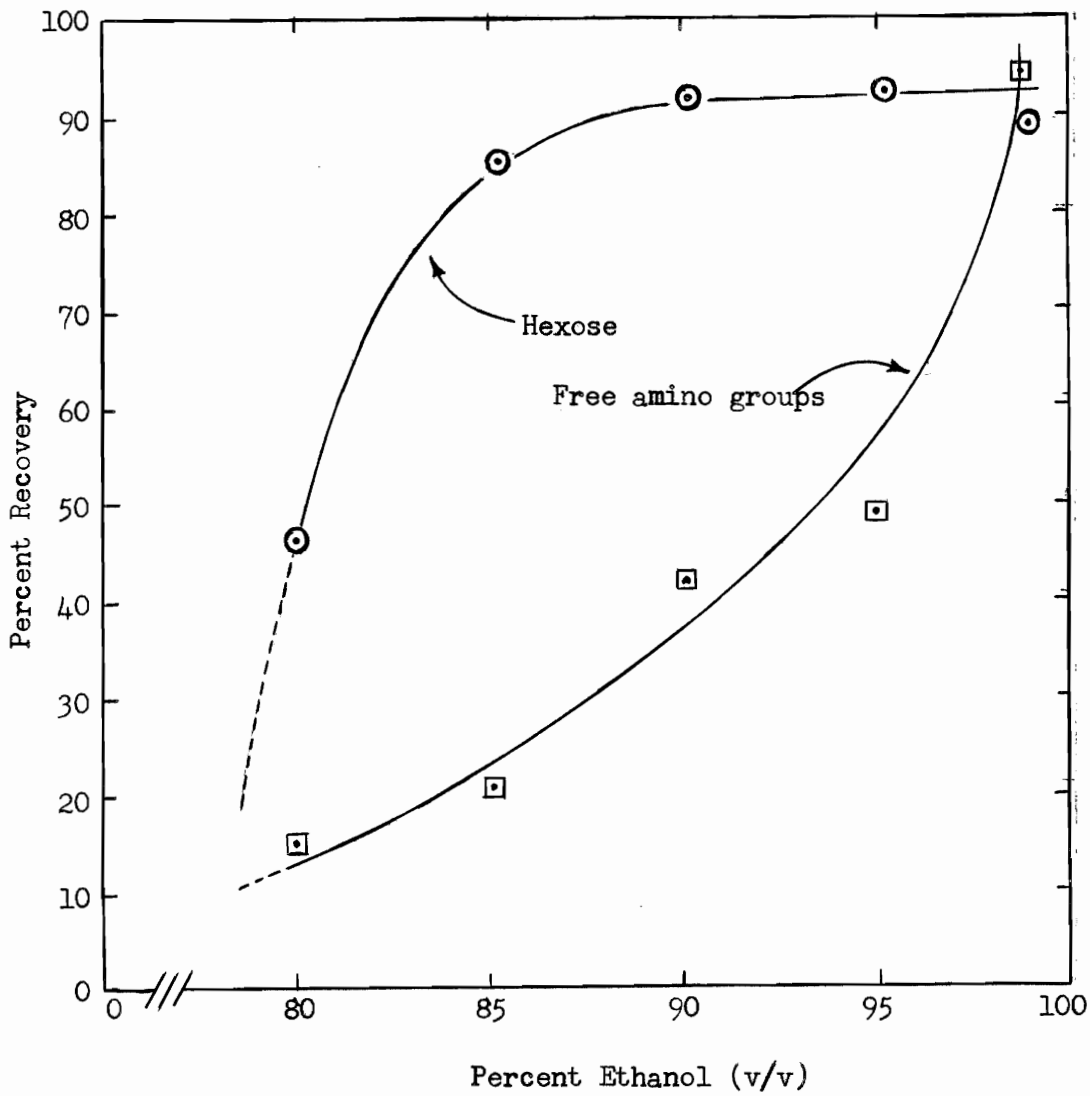


Figure 1. Comparison of the Effect of Ethanol in Precipitating the Hexose and Free Amino Groups of Papain-Digested γ-Globulin. The preparation studied was a lyophilized residue of the resin treated papain digest of Step 2. The hexose was determined by the orcinol-H_2SO_4 method of Section III-A-2. The free amino groups were determined by the ninhydrin method of Moore and Stein (87).

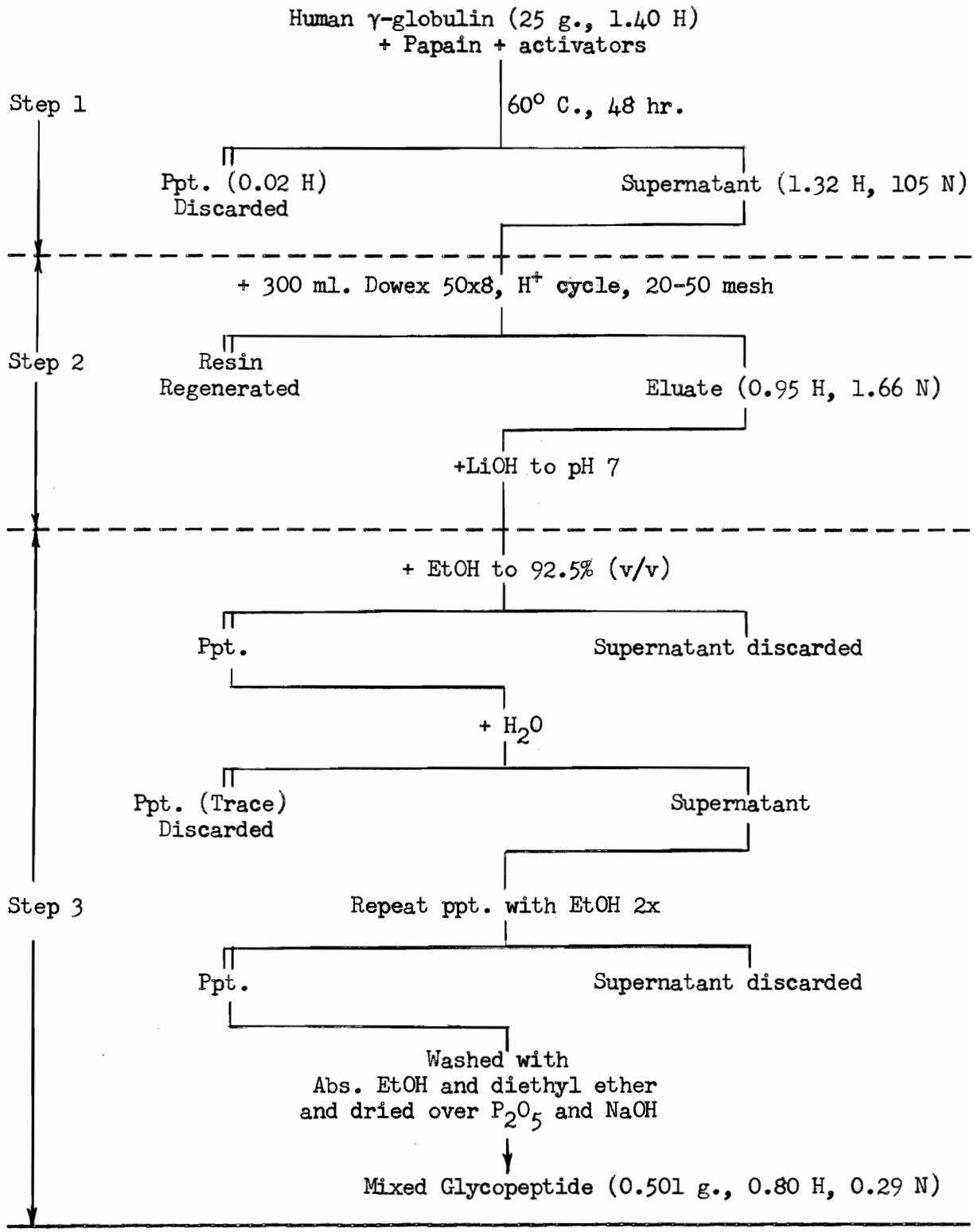


Figure 2. Summary of Mixed Glycopeptide Preparation. The H and N values represent millimoles of hexose and free amino groups derived from 25 g. of γ-globulin.

in 0.1 N pH 8.5 Veronal buffer, in 0.05 N pH 9.2 borate buffer, and in 0.1 N pH 6.8 sodium phosphate buffer. Electrophoresis was performed on 1 mg. samples of the Mixed Glycopeptide dissolved in 0.010 ml. of water and applied in narrow bands across the 3 cm. width of the paper strips with the Spinco sample applicator. Good resolution was obtained with 1.5 ma./strip and 450 volts at 5° C. for 24 hrs. in the aquarium system and with 0.6 ma./strip and 130 volts at 5° C. for 24 hrs. in the Spinco system. The components were detected with either ninhydrin or α-napthol reagents. The most useful ninhydrin reagent contained 0.025% (w/v) ninhydrin in a solution containing 80 ml. of isopropyl alcohol, 10 ml. of acetic acid, and 10 ml. of pyridine. The dried electrophoresis strips were dipped in the reagent and heated in a 105° C. oven for 5 min. Although the glycopeptides could not be detected with the usual carbohydrate reagents (9) when cellulose paper was used, they were easily detected with α-napthol and sulfuric acid (16) on glass fiber filter paper.[a] The most useful α-napthol reagent contained 0.5 g. of α-napthol, 20 ml. of conc. H_2SO_4, and 480 ml. of absolute ethanol. The dried strips were dipped in the reagent, heated in a 105° C. oven for 5 min. on a glass plate, and placed between two strips of 2 in. Scotch brand tape. The electrophoresis strips were analyzed with a Spinco Analytrol using cam 3 and Wratten Filter No. 58 (peak transmission at 530 mμ). Both color reagents detected three major peaks in the same relative positions. The close correlation of the two tracings strongly suggests that the Mixed Glycopeptide consists of at least three components which contain both carbohydrate and

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[a] Mfd. by Hulbert Paper Co., Reeve Angel and Co., 934AH.

free amino groups. Typical analytrol tracings of separate ninhydrin and α-napthol treated strips, superimposed for comparison, are illustrated in Figure 3.

The conditions of paper electrophoresis were then duplicated as closely as possible in starch column electrophoresis. The design and operation of a similar starch column have been described by Porath (34,106). Figure 4 shows diagramatically the essential parts of the apparatus. The column of starch was 48 cm. long and 3 cm. in diameter. A freshly poured column had a flow rate of about 12 ml./hr., but after the column was allowed to stand for several days, the flow rate decreased to about 3 ml./hr. No hexose could be detected when 1 ml. of the column eluate was assayed by the orcinol method described in Section III-A-2. The buffer, 0.05 N pH 8.5 Veronal, contained 82.5 g. of sodium diethylbarbiturate, 15 to 16 g. of diethylbarbituric acid, 800 ml. of isopropyl alcohol, and 7200 ml. of demineralized-distilled water. The maximum amount of sample adequately resolved was 200 mg. The sample was dissolved in 0.4 ml. of buffer, applied to the starch column, and washed in with two 0.2 ml. portions of buffer. A properly applied sample formed a visible, uniform brown band about 2 mm. thick and about 2 mm. down from the top of the starch. The electrophoresis was performed in a cold room at 5° C. with a constant potential of 450 volts. The current was 14 ma. initially, but it dropped to 11 ma. as soon as temperature equilibrium was attained. Adequate resolution was usually obtained in 48 hrs. In 68 hrs. the resolution was slightly improved. The positive power lead was connected to the bottom electrode so that the glycopeptides, which are negatively charged, would migrate down into the

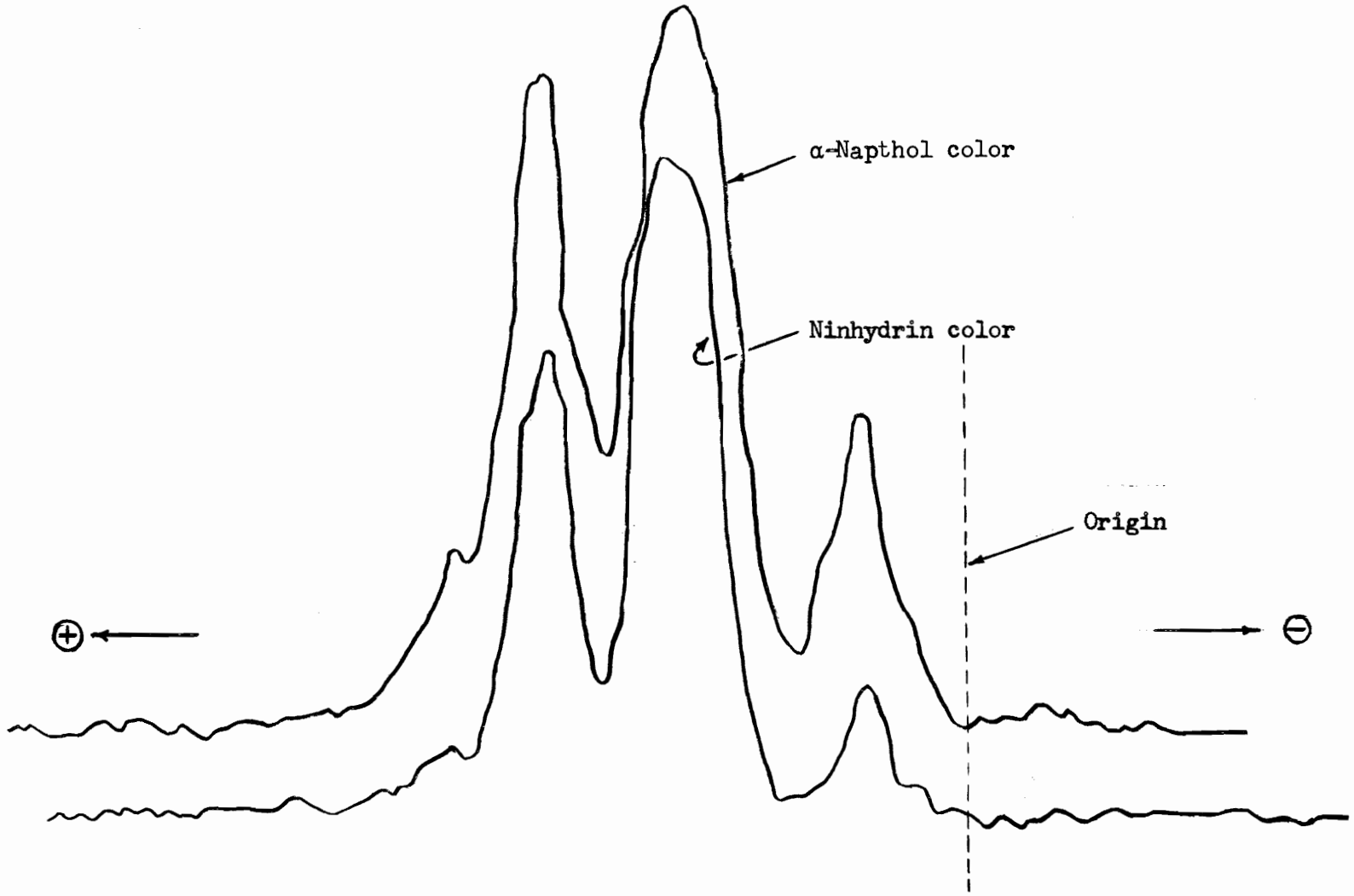

Figure 3. Paper Electrophoresis of the Mixed Glycopeptide. Analytrol tracing of separate α-napthol- and ninhydrin-treated electrophoresis strips are superimposed to illustrate the close correspondence of the peaks given by the two color reagents. The origin was corrected for endomosis (9).

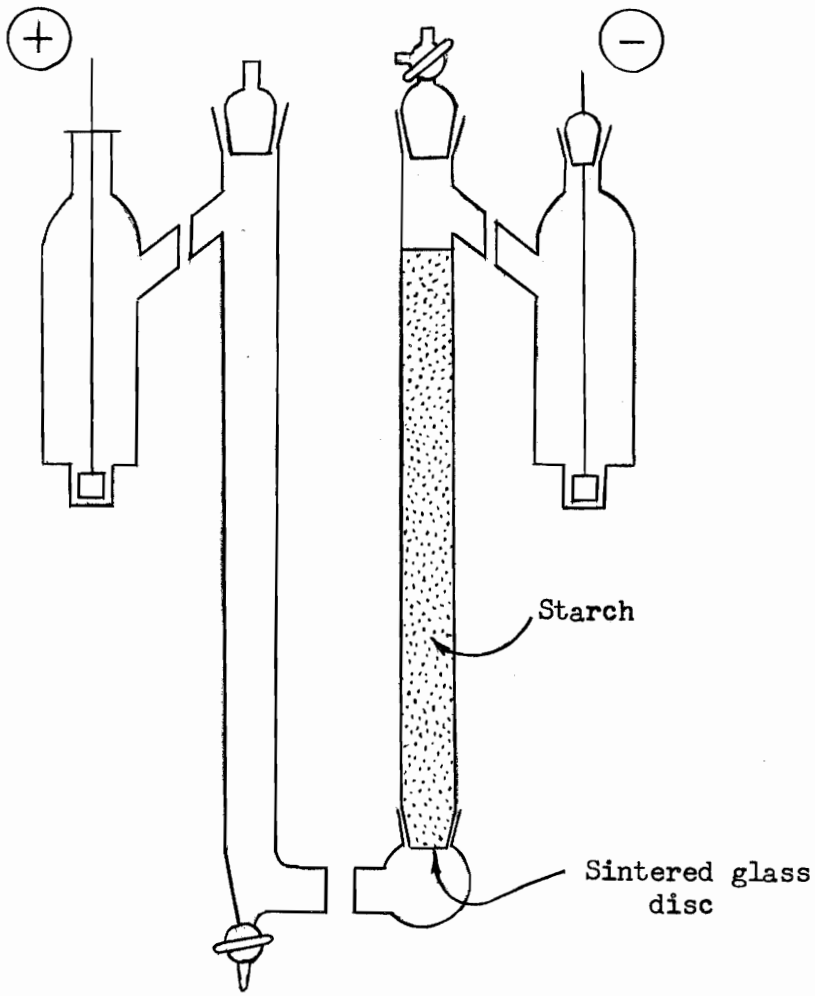

Figure 4. Starch Column Electrophoresis Apparatus.

starch. After electrophoresis, the starch column was placed on an automatic time-flow fraction collector, and a constant-height buffer reservoir was placed on the column. About 300 0.5-ml. effluent fractions were collected. Aliquots (0.10 ml.) of every fifth effluent fraction were assayed for hexose with the orcinol method of Section III-A-2 and for free amino groups with ninhydrin (87). An elution diagram such as the one shown in Fig. 5 was obtained when the optical densities of the assayed fractions were plotted against the fraction number. Here, as in Fig. 3, the close parallel of the carbohydrate and free amino group colors strongly suggests that the Mixed Glycopeptide consists of at least three components which contain both carbohydrate and free amino groups. These three components, referred to as Glycopeptides 1, 2, and 3 (G-1, G-2, and G-3), were recovered from the pooled center effluent fractions of each peak, as indicated in Fig. 5, by an alcohol precipitation similar to Step 3. The effluent fractions of doubtful purity were discarded. The precipitates were washed with cold absolute ethanol and cold anhydrous peroxide-free diethyl ether and then dried in a cautiously evacuated desiccator containing P_2O_5 and NaOH.

A typical electrophoresis run yielded 18, 42, and 12 mg., respectively, of Glycopeptide 1, 2, and 3 from 200 mg. of the Mixed Glycopeptide. This represents a 50% recovery of hexose and hexosamine but only a 36% recovery by weight. The total hexose eluted from the column, however, typically amounted to 100 $\pm$ 5%. As indicated in Fig. 5, 20.5% of the eluted hexose was contained in G-1, 42.1% in G-2, and 27.7% in G-3. Thus, G-1, G-2, and G-3 represent 90% of the hexose of the Mixed Glycopeptide, which, in turn, represents about 60% of the hexose of γ-globulin. Typical hexose yields

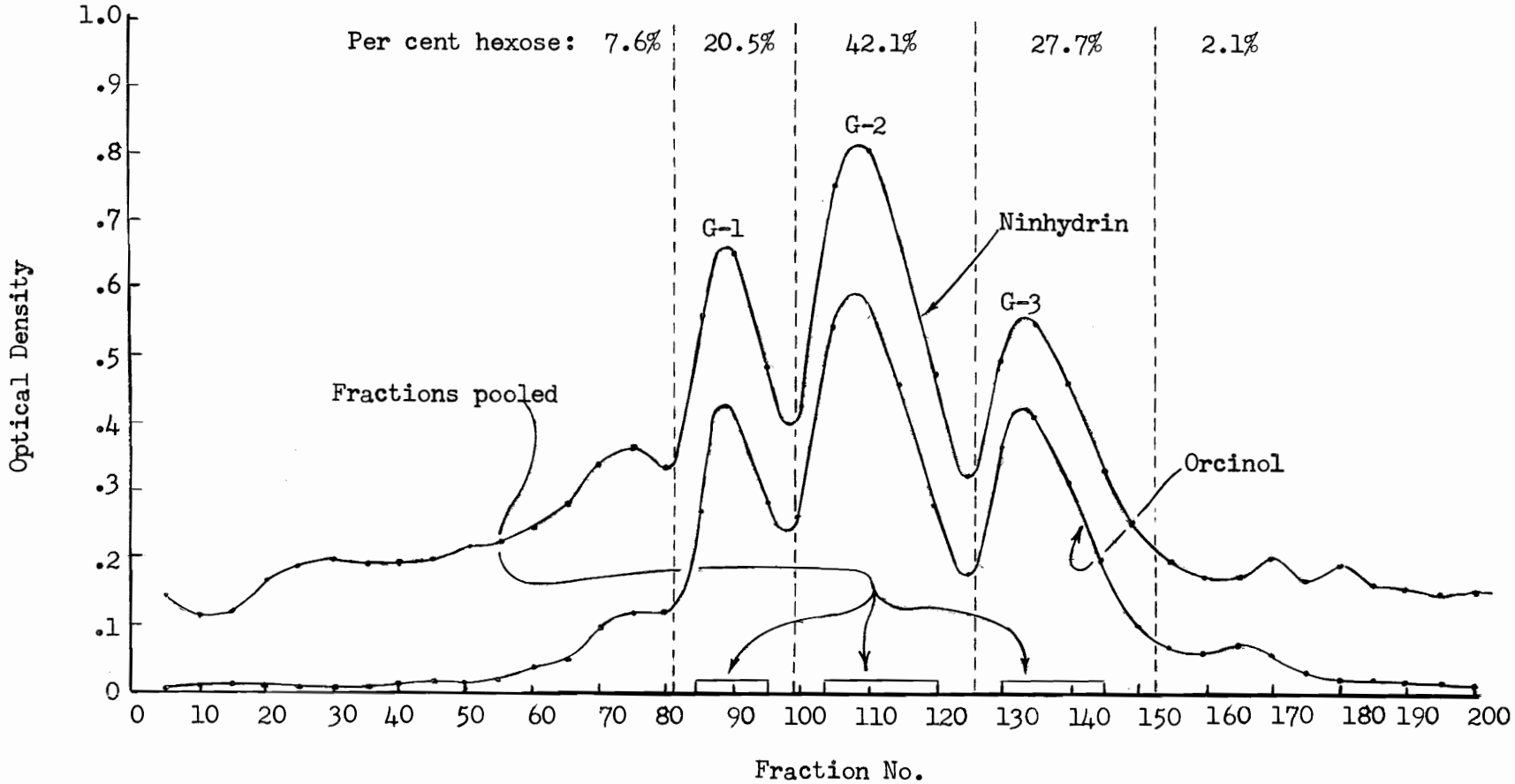

Figure 5. Starch Column Electrophoresis Elution Diagram of the Mixed Glycopeptide. Electrophoresis was performed in 0.05 N pH 8.5 Veronal buffer at 5° C. with 450 volts and 11 ma. for 48 hrs. The effluent fractions were about 0.5 ml. each. The orcinol color (minus 0.008) is equivalent to 2.58 μM of 1:1 galactose-mannose per O.D. unit. The ninhydrin color (minus 0.077) is equivalent to 0.415 μM of leucine per O.D. unit.

of the four steps of preparation are given in Table 7. Altogether, 11 experimental electrophoresis runs were performed. Four of these runs, which were used for preparing the purified glycopeptides, yielded a total of 55, 102, and 32 mg. of Glycopeptide 1, 2, and 3 respectively.

TABLE 7

GLYCOPEPTIDE YIELDS

Procedure	Hexose Yield
Papain digestion (Step 1)	95%
Resin treatment (Step 2)	72
Ethanol precipitation (Step 3)	84
Steps 1, 2, and 3	58
Column electrophoresis (Step 4)	50
Steps 1, 2, 3, and 4	29

C. Characterization of Glycopeptides

The characterization studies were performed on the Mixed Glycopeptide (Step 3), as well as on the three purified glycopeptides. To allow as many determinations as possible, a stock solution of each purified glycopeptide was prepared, from 50, 50, and 25 mg. of Glycopeptides 1, 2, and 3, respectively. The concentration of each stock solution was evaluated by micro-Kjeldahl analysis (Section III-B) and expressed in terms of the Dumas nitrogen values determined by Dr. A. Elek[a] on samples dried at 80-85° C. *in vacuo*. The Dumas nitrogen values of Glycopeptide 1, 2, and 3 were 6.29%, 6.88%, and 7.94%,

[a] 4763 W. Adams Boulevard, Los Angeles 16, California.

respectively. On the basis of these values, the stock solutions contained respectively, 10.5 (± .4),[a] 9.53 (± 0.03) and 6.96 (± 0.1) mg. of dry glycopeptide/ml. A 1 to 5 dilution of each stock solution was also prepared.

1. Electrophoresis. Glycopeptide 2 was studied for purity and relative mobility by electrophoresis in 0.1 N pH 8.5 Veronal and 0.1 N pH 5.0 acetate buffers. The Spinco Model R system was used. Human serum albumin and human serum globulin were included for mobility comparisons. The samples were applied in 0.010 ml. of 10% solutions. The electrophoresis strips were developed with ninhydrin and analyzed with the Spinco Analytrol as in Step 4 of Section IV-B. Glycopeptide 2 was found to migrate as a symmetrical peak at pH 8.5 and pH 5.0. The approximate mobility was -3.4 at pH 8.5 and -1.2 at pH 5.0. Glycopeptides 1 and 3 were not studied by paper electrophoresis since they were not as plentiful as Glycopeptide 2.

2. Sedimentation. The sedimentation rate of the Mixed Glicopeptide was studied in a Spinco Model D Ultracentrifuge (119). However, no sedimentation boundary could be detected in 90 min. at 60,000 r.p.m. with a 1% solution of the Mixed Glycopeptide in normal saline. Thus, apparently, no component of greater than 10,000 molecular weight was present. To determine the presence of components of less than 10,000 molecular weight, Dr. H. K. Schachman, Dept. of Biochemistry, University of California, was asked to study the Mixed Glycopeptide in the Synthetic Boundary Cell Ultracentrifuge (47,105,117) which he was instrumental in developing. He obtained a symmetrical boundary with a sedimentation constant of 0.61. This is equivalent to a molecular weight of 1,350 for a spherical unhydrated molecule

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[a] One-half the difference of duplicate micro-Kjeldahl determinations.

with a 0.64[a] partial specific volume (75). However, the glycopeptides are probably hydrated and somewhat elliptical in shape and thus would have a molecular weight greater than 1,350. Since the diffusion constant was not determined, the molecular weight could not be directly calculated, but it was certainly less than 6,000 since a molecule with a molecular weight greater than 6,000 and with the observed 0.61 sedimentation constant would have an unreasonable molar frictional and axial ratio (75). Furthermore, the molecular weights estimated by fractional dialysis (Section IV-C-9) and FDNB end group analysis (Section IV-C-7-a) were also less than 6,000. The total residue weight of the significant constituents found in Glycopeptide 1, the largest component of the Mixed Glycopeptide, is about 3,920 (Section IV-C-6-b). A molecule of this size with the observed 0.61 sedimentation constant would have a 2.04 molar frictional ratio and a 13 to 1 axial ratio if 50% (w/w) hydrated (75). These ratios are consistent with an essentially linear arrangement of the 17 to 23 residues found in the glycopeptides. Thus, the molecular weight of the glycopeptides was assumed to be about 4,000.

3. Optical Rotation. The optical rotation of the three purified glycopeptides was measured with a Rudolph Polarimeter, Model 118, using a one-decimeter tube and a sodium lamp. The results are given in Table 8.

4. Absorption Spectra. The absorption spectra of the Purified Glycopeptides were determined at pH 7.5 using the 1 to 5 diluted stock solutions and a Beckman Model DU Spectrophotometer. The spectra were identical in shape and are characteristic of those reported for tyrosine (4). The extinctions at 275 mμ are given in Table 9.

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[a] Calculated from the partial specific volumes of the components as obtained from the data of Cohn and Edsall (20) and Cox et al. (22) according to Oncley (94).

TABLE 8

OPTICAL ROTATION OF GLYCOPEPTIDES

Glycopeptide	Degrees Measured Rotation	g. Glycopeptide per 100 ml. Stock Solution	Specific Rotation (degrees) $(g./ml.)^{-1}(dm.)^{-1}$
1	-0.209 ± 0.008[a]	1.05 ± 0.04[b]	-19.9 ± 0.8[c]
2	-0.200 ± 0.007	0.953 ± 0.003	-21.0 ± 0.2
3	-0.167 ± 0.006	0.696 ± 0.01	-24.0 ± 0.5

[a] Ninety five per cent confidence limits.

[b] One-half the difference of duplicate micro-Kjeldahl determinations.

[c] Estimated probable variation.

TABLE 9

EXTINCTION OF GLYCOPEPTIDES AT 275 mμ

Glycopeptide	Measured Optical Density	g. of Glycopeptide per 100 ml. of Stock Solution	Specific Extinction Coefficient $(g./ml.)^{-1}(cm.)^{-1}$
1	0.812	0.210	392
2	0.970	0.191	456
3	1.117	0.139	841

The absorption spectrum of Glycopeptide 1 was also determined at pH 13 and pH 2. This was accomplished by dissolving 4.76 mg. of the glycopeptide in 3.00 ml. of 0.1 N NaOH, determining the spectrum, then adding 0.030 ml. of 12 N HCl, and again determining the spectrum. A shift in absorption spectrum characteristic of tyrosine with free phenolic hydroxyl groups (4) was noted, as illustrated in Figure 6. The 275 and 293 mμ peaks were 0.602 and 0.915 optical density units, respectively. Since the phenolic hydroxyl groups of the tyrosine were free, the carbohydrate must be attached to some other functional group of the peptide chain.

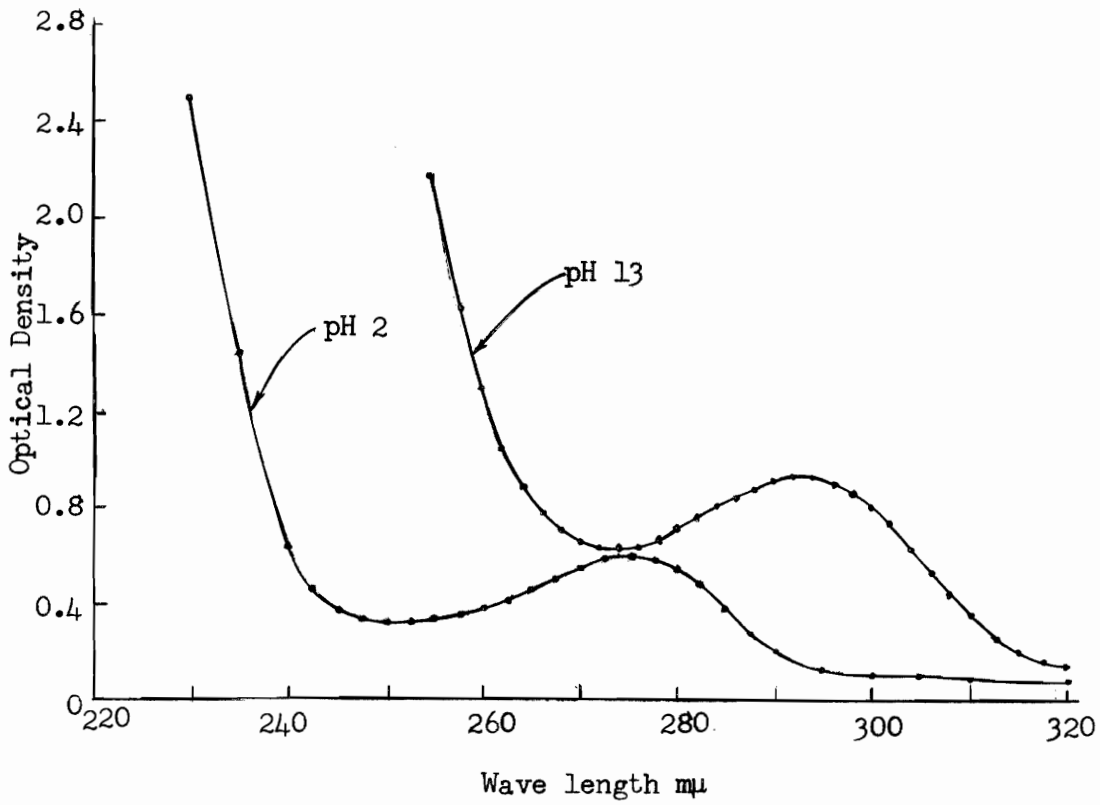

Figure 6. Absorption Spectra of Glycopeptide 1 (1.59 mg./ml.)at pH 2 and pH 13.

5. Carbohydrate Composition.

a. Paper chromatography. The hexoses of the Mixed Glycopeptide and of Glycopeptide No. 1 were determined by paper chromatography after hydrolysis with 0.25 N H_2SO_4. The procedure involved a minimum amount of handling and gave about 50% recovery, which was considered adequate for qualitative identification. Gottschalk and Ada (43) were able to recover only 80 $\pm$ 10% of the hexose using an involved hydrolysis and separation procedure. The hydrolyses were conducted on 10 mg. of the Mixed Glycopeptide and 2 mg. of Glycopeptide 1 (1.0 ml. of the 1 to 5 stock solution) in 10 ml. of 0.25 N H_2SO_4 in sealed, evacuated 50 ml. hydrolysis tubes for 22 hrs. at 105° C. After hydrolysis, the H_2SO_4 was removed by filtration through a medium sintered glass funnel. The filtrate was concentrated to dryness on a rotary evaporator. The residue was dissolved in 0.1 to 1.0 ml. of water and analyzed by paper chromatography using the butanol-pyridine-water solvent, the multiple development technique, the p-anisidine phthalate color reagent, and the Spinco Analytrol as described in Section III-A-1. Galactose, mannose, fucose, and a hexosamine were detected. Good resolution was obtained, but the spots were too diffuse for precise quantitation. The presence of degradation products probably caused the spots to spread. The ratios found were about 3 to 5 to 2 for galactose, mannose, and fucose, respectively. Since destruction factors were not considered, these ratios are only an approximation. The same results were obtained with both the Mixed Glycopeptide and Glycopeptide 1. Therefore, Glycopeptides 2 and 3 were also assumed to have the same hexoses. Traces of hexosamine were detected by this method, but other methods were required for estimation and

and identification (Section IV-C-5-d).

No other sugars were detected. Pentoses, ketokexoses, hexuronic acids, and other aldohexoses would have been detected if present. The basic silver reagent was not able to detect any other polyhydroxy components, thus ruling out the presence of hexonic acids and other nonreducing sugars.

b. Hexose. The hexose content of the purified glycopeptides was determined by the orcinol-H_2SO_4 method described in Section III-A-2. The determinations were performed on duplicate 0.050 ml. aliquots of the stock solutions. The uncorrected 1:1 galactose-mannose equivalent hexose values were 1.726, 2.085, and 3.181 millimoles per gram of Glycopeptide 1, 2, and 3, respectively. Correcting these values for fucose (Section IV-C-5-c) and for the 3:5 galactose-mannose ratio found by paper chromatography (Section IV-C-5-a) gave the hexose values of Tables 11, 12, and 13 (Section IV-C-6). However, these values could be as much as 10% low, since, according to Vasseur (145), polysaccharides give only about 90% of the color of equivalent monosaccharides.

c. Fucose. Fucose was detected in Glycopeptide 1 and in the Mixed Glycopeptide by paper chromatography of 0.25 N H_2SO_4 hydrolysates as discussed in Section IV-C-5-a. In addition, the fucose content of Glycopeptides 1, 2, and 3 was determined by duplicate analysis of the cysteine-H_2SO_4 reaction of Dische (29) described in Section III-A-5. The results are included in Tables 11, 12, and 13 (Section IV-C-6). The reported values were corrected for hexose interference as recommended by Winzler (151).

d. Hexosamine. The hexosamine content of Glycopeptides 1, 2, and 3 was determined in the course of amino acid analysis by the column chroma-

tography method of Moore and Stein as described in Section III-C-2. Aliquots of the stock solutions (1.0 ml.) containing about 10 mg. of the glycopeptides were hydrolyzed with 5 ml. of 6 N HCl in an atmosphere of SO_2 in sealed tubes at 105° C. for 20 hrs. as described in Section IV-C-6-b. The hydrolysate residues were dissolved in pH 2.2 citrate buffer (86) for application to the column. Analysis of the effluent fractions with ninhydrin (87) indicated the presence of only one hexosamine component, which emerged from the 150 cm. column shortly after phenylalanine. This was where glucosamine was expected to emerge; galactosamine was expected to emerge in later effluent fractions (37,126). The presence of hexosamine was verified by analysis of every fourth effluent fraction of the last 200 fractions from the 150 cm. column analysis of Glycopeptide 2 with the p-dimethylaminobenzaldehyde method described in Section III-A-3. The effect of the sodium citrate buffer of the effluent fractions on the color yield was taken into consideration by using the blank effluent fractions plus added glucosamine for the assay standard. The amount of hexosamine determined by the ninhydrin and by the p-dimethylaminobenzaldehyde methods agreed within the expected limits. Only ninhydrin was used for analysis of Glycopeptides 1 and 3. The results given in Tables 11, 12, and 13 (Section IV-C-6) were not corrected for acid hydrolysis loss, which, according to Boas (10), could be as much as 20%. If the results were corrected for 20% loss, the glucosamine values of Glycopeptide 1, 2, and 3 would become 7.6, 7.8, and 3.9, respectively, or 8, 8, and 4 residues per mole. In the glycopeptides, glucosamine probably occurs as N-acetylglucosamine, since DNP-glucosamine could not be detected after fluorodinitrobenzene treatment (Section IV-C-7-a) and since naturally occurring hexosamines are

almost always acetylated (132).

The hexosamine present in Glycopeptide 2 was further identified by paper chromatography[a] with butanol-pyridine-water and detection with p-dimethylaminobenzaldehyde as described in Section III-A-1. Good resolution was obtained with three 22-hr. developments. Only glucosamine was found. Two distinct spots were obtained when the unknown was mixed with galactosamine, but only one was obtained when mixed with glucosamine.

e. Sialic acid. The sialic acid content of Glycopeptides 1, 2, and 3 was determined by analysis of duplicate 0.500 ml. aliquots of the 1 to 5 stock solutions with the photometric orcinol-HCl method described by Werner and Odin (148). The acid glycoprotein of Weimer, Mehl, and Winzler (147) was used as the standard. It has been reported to contain 9.8% sialic acid (148) by the orcinol-HCl method. The results are included in Tables 11, 12, and 13 (Section IV-C-6). The absorption spectra of the colored products formed by the acid glycoprotein and the glycopeptides, determined with a Beckman DU Spectrophotometer, were similar to those reported for crystalline sialic acid by Werner and Odin (148).

The sialic acid of the Mixed Glycopeptide was identified by paper chromatography after treatment with HCl, pH 2, 100° C., for 1 hr. to hydrolyze the sialic acid-glycopeptide bond. The sialic acid was then identified by chromatography with butanol-pyridine-water and detection with orcinol-T.C.A. as described in Section III-A-1. The sialic acid from the glycopeptide chromatographed with the N-acetylneuraminic acid component (8) of bovine sialic acid.[b] Free sialic acid could not be detected before

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[a] Of a similar acid hydrolysate dissolved in water.

[b] Sialic acid was obtained from Dr. Kristen Eik-Nes.

the HCl treatment.

f. Hexuronic acid. Hexuronic acids could not be detected in the 0.25 N H_2SO_4 hydrolysates of the Mixed Glycopeptide and Glycopeptide 1 described in Section IV-C-5-a. However, hexuronic acids, if present, might have been destroyed by the acid. To consider, further, the presence of hexuronic acids, the carbazole reaction of Dische (28) was used. Glycopeptide 1, 0.5 mg., was assayed along with 0.1 mg. of sodium glucuronate. The absorption spectra of the colored products were determined in a Beckman DU Spectrophotometer. In the presence of hexuronic acids the optical density at 530 mμ would be greater than that at 440 mμ (28). The results are given in Table 10. If Glycopeptide 1 contained 1% hexuronic acid, the optical density at 530 mμ would have been 0.15 higher than that at 440 mμ. Instead, it was found to be 0.05 lower, thus indicating that the hexuronic acid content was considerably less than 1%. Even 1% hexuronic acid would not be significant in view of a molecular weight less than 6,000 (Section IV-C-2).

TABLE 10

RESULTS OF HEXURONIC ACID ASSAY

Sample	Optical Density	
	440 mμ	530 mμ
Glycopeptide 1, 0.5 mg.	0.135	0.087
Sodium glucuronate, 0.1 mg.	0.309	1.513

6. Amino Acid Composition.

a. Tryptophan. The Mixed Glycopeptide was assayed for tryptophan by the p-dimethylaminobenzaldehyde-H_2SO_4 method of Spies and Chambers (127, 128,129), procedure N (128). The color produced was equivalent to 0.11% or one residue of tryptophan per 180,000 g. The molecular weight of the Mixed Glycopeptide estimated by sedimentation (Section IV-C-2) was less than 6,000. Obviously, then, tryptophan is not a significant part of the glycopeptides.

b. Acid stable amino acids. The amino acid composition (except tryptophan) of Glycopeptides 1, 2, and 3 was determined by the ion exchange resin chromatography method of Moore and Stein (see Section III-C-2). Aliquots of the stock solutions, containing about 10 mg. of the glycopeptides, were hydrolyzed with 5 ml. of 3x distilled 6 N HCl in an SO_2 atmosphere in sealed tubes at 105° C. for 20 hrs. To obtain the SO_2 atmosphere, the sample and the HCl were placed in a 30 ml. hydrolysis tube and frozen in dry ice-acetone. While this mixture was still frozen, sodium bisulfite was added and the tube was evacuated to 10 mm. of Hg. and sealed. The amount of sodium bisulfite added, 0.1 g., was calculated to yield about 30 ml. of SO_2 at 100° C. and 1 atmosphere pressure. The SO_2 was used to reduce the destruction of amino acids, as recommended by Pederson and Baker (103). One effect of the SO_2 is probably the retarding of the "browning reaction" (52). The hydrolysates were taken to dryness on a rotary evaporator, dissolved in 2.5 to 5.0 ml. of pH 2.2 citrate buffer (86), and analyzed by the column chromatography method described in Section III-C-2. The equivalent glycopeptide concentration of each hydrolysate was evaluated by micro-Kjeldahl

analysis (Section III-B) and expressed in terms of the Dumas nitrogen values of each glycopeptide. Typical elution diagrams are shown in Figures 7 and 8. The amino acids of Glycopeptide 2 were also identified by paper chromatography of a similar acid hydrolysate (dissolved in water) with propanol-pyrophosphate. The same amino acids were found in each case.

The results, included in Tables 11, 12, and 13, were not corrected for acid hydrolysis losses or for extent of hydrolysis since many unknown factors were involved and since only enough sample was available for hydrolysis under one set of conditions. The acid hydrolysis losses, however, were expected to be greater than those usually observed because of the large amount of carbohydrate present in the glycopeptides and the resulting "browning reaction" (52). The aspartic acid, glutamic acid, and tyrosine values per mole, given in Tables 11, 12, and 13, are within $\pm$ 10% of whole numbers; thus, the acid hydrolysis losses could not have been much greater than expected. The tyrosine values, if corrected for 5% destruction (51), would become 0.96, 1.00, and 1.15 residues per mole, respectively.

The estimated residues per mole, given in the last column of Tables 11, 12, and 13 differ from the values of the previous column by less than $\pm$ 10% experimental error of the analytical methods. The residue weights of the components listed in the last column add up to about 3,920; 3,790; and 2,720 for Glycopeptide 1, 2, and 3 respectively. The molecular weights, obtained by averaging the weights that contain a whole number of residues, were 4,850; 4,200; and 2,800 for Glycopeptide 1, 2, and 3, respectively. The weights greater than 6,000, however, were not considered since the molecular weights estimated by sedimentation (Section IV-C-2), fractional

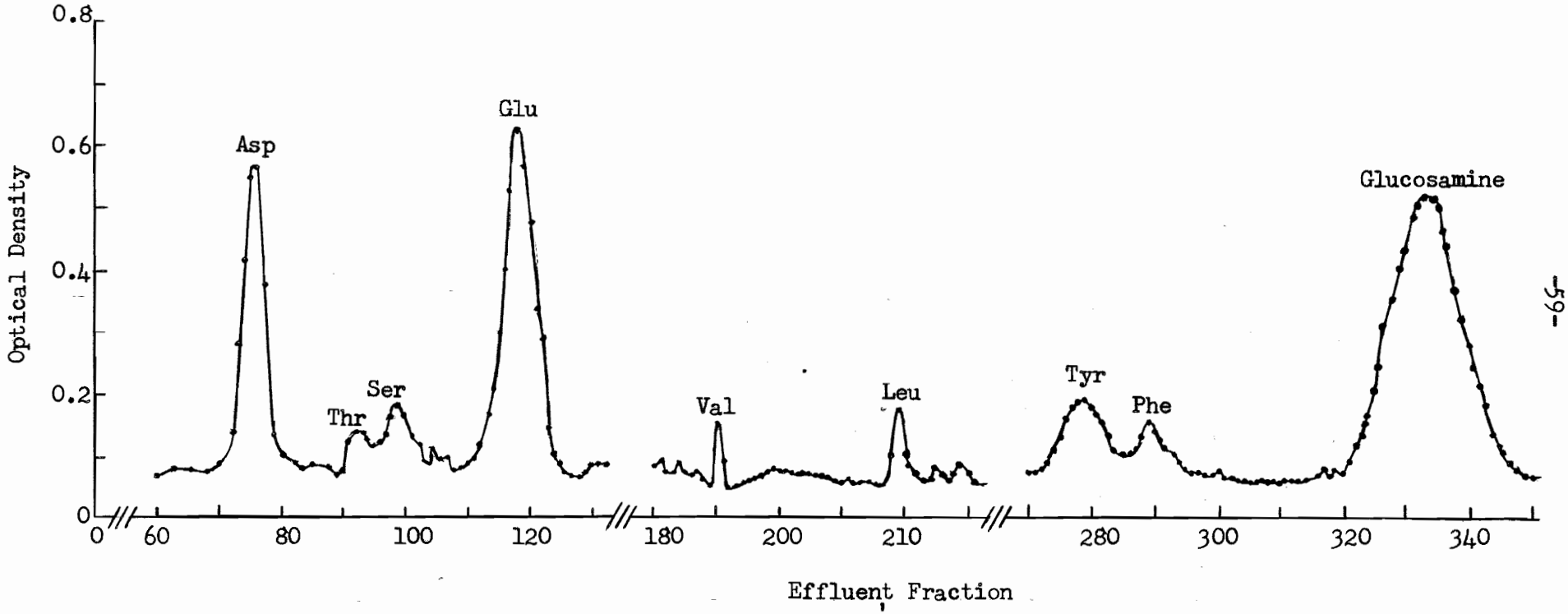

Figure 7. Column Chromatography Elution Diagram of Acid Hydrolyzed Glycopeptide 1. An aliquot containing 2.08 mg., was analyzed with a 150 cm. ion exchange column as described in Section III-C-2.

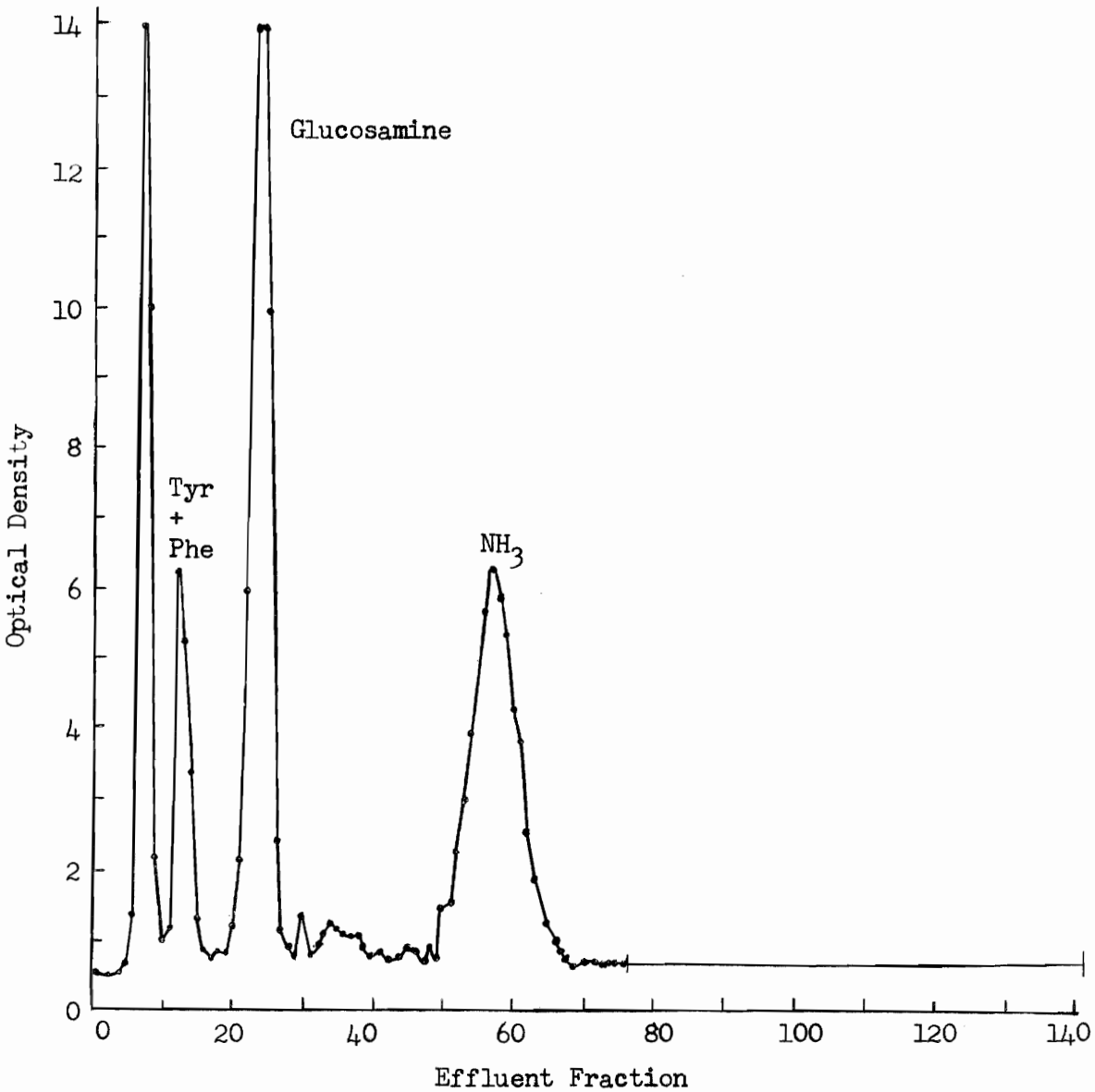

Figure 8. Column Chromatography of Acid Hydrolyzed Glycopeptide 1. An aliquot, containing 2.08 mg., was analyzed with a 15 cm. ion exchange column as described in Section III-C-2.

TABLE 11

COMPOSITION OF GLYCOPEPTIDE 1

Residue	Millimoles of Residue per Gram of Sample[a]	Grams of Residue per 100 Grams of Sample	Grams of Nitrogen per 100 Grams of Sample	Nitrogen as % of Total[b]	Grams Containing Whole No. of Residues	Residues per 4850 g.[c] of Sample	Estimated Residues per Mole
Hexose	1.502	24.3	-	-	5330/8	7.29	8
Glucosamine[d]	1.235	25.1	1.73	27.5	4860/6	6.00	6
Fucose	1.413	6.0	-	-	4840/2	2.00	2
Sialic acid	0.210	7.0	0.29	4.6	4790/1	1.02	1
Aspartic acid	0.439	5.1	0.62	9.9	4560/2	2.13	2
Glutamic acid	0.685	8.9	0.96	15.3	4380/3	3.32	3
Tyrosine	0.188	3.1	0.26	4.1	5320/1	0.91	1
Phenylalanine	0.097	1.4	0.14	2.2	10300/1	0.47	-
Threonine	0.056	0.6	0.08	1.3	17800/1	0.27	-
Serine	0.093	0.8	0.13	2.1	10700/1	0.45	-
Valine	0.050	0.5	0.07	1.1	20000/1	0.24	-
Alanine	0.029	0.2	0.04	0.6	34100/1	0.14	-
Ammonia	0.923	-	1.29	20.5	-	4.48	-
Total		83.0	5.61	89.2			23

[a] Based on Dumas nitrogen of dry glycopeptide and micro-Kjeldahl nitrogen of assayed solution.

[b] Total nitrogen, determined by the Dumas method, was 6.29% of dry weight (Section IV-C).

[c] This molecular weight was obtained by averaging the weights which contain a whole number of residues. The values greater than 6,000, however, were not included, since the molecular weights estimated by sedimentation (Section IV-C-2), fractional dialysis (Section IV-C-9-), and amino end group analysis (Section IV-C-7-a) were all less than 6,000.

[d] Calculated as N-acetyl glucosamine, assuming 100% recovery after acid hydrolysis.

TABLE 12

COMPOSITION OF GLYCOPEPTIDE 2

Residue	Millimoles of Residue per Gram of Sample[a]	Grams of Residue per 100 Grams of Sample	Grams of Nitrogen per 100 Grams of Sample	Nitrogen as % of Total[b]	Grams Containing Whole No. of Residues	Residues per 4200 g.[c] of Sample	Estimated Residues per Mole
Hexose	1.811	29.3	-	-	4420/8	7.61	8
Glucosamine[d]	1.432	29.1	2.01	29.2	4190/6	6.01	6
Fucose	0.504	7.4	-	-	3970/2	2.12	2
Sialic acid	0.136	4.5	0.19	2.8	7360/1	0.57	0-1
Aspartic acid	0.434	5.0	0.61	8.9	4650/2	1.82	2
Glutamic acid	0.562	7.3	0.79	11.5	3570/2	2.36	2
Tyrosine	0.227	3.7	0.32	4.7	4410/1	0.95	1
Phenylalanine	0.090	1.3	0.13	1.9	11100/1	0.38	-
Threonine	0.018	0.2	0.03	0.4	55900/1	0.08	-
Serine	0.051	0.4	0.07	1.0	19500/1	0.22	-
Alanine	0.022	0.1	0.03	0.4	46000/1	0.09	-
Valine	0.072	0.7	0.10	1.5	14000/1	0.30	-
Ammonia	0.804	-	1.13	16.4		3.36	-
Total		89.0	5.41	78.7			21-22

[a] See footnote a of Table 11.

[b] Total nitrogen, determined by the Dumas method, was 6.88% of the dry weight (Section IV-C).

[c] See footnote c of Table 11.

[d] See footnote d of Table 11.

TABLE 13

COMPOSITION OF GLYCOPEPTIDE 3

Residue	Millimoles of Residue per Gram of Sample[a]	Grams of Residue per 100 Grams of Sample	Grams of Nitrogen per 100 Grams of Sample	Nitrogen as % of Total[b]	Grams Containing Whole No. of Residues	Residues per 2800 g.[c] of Sample	Estimated Residues per Mole
Hexose	2.799	45.3	-	-	2858/8	7.84	8
Glucosamine[d]	1.107	22.5	1.55	19.5	2710/3	3.10	3
Fucose	0.713	10.4	-	-	2800/2	2.00	2
Sialic acid	0.071	2.4	0.10	1.3	14200/1	0.20	0-1
Aspartic acid	0.650	7.5	0.91	11.5	3080/2	1.82	2
Glutamic acid	0.321	4.1	0.45	5.7	3120/1	0.90	1
Tyrosine	0.390	6.4	0.55	6.9	2560/1	1.09	1
Phenylalanine	0.116	1.7	0.16	2.0	8640/1	0.32	-
Threonine	0.037	0.4	0.05	0.6	26800/1	0.10	-
Serine	0.067	0.6	0.09	1.1	14800/1	0.19	-
Lysine	0.138	1.8	0.39	4.9	7240/1	0.39	-
Ammonia	1.748	-	2.45	30.9	-	4.9	-
Total		103.1	6.70	84.4			17-18

[a] See footnote a of Table 11.

[b] Total nitrogen, determined by the Dumas method, was 7.94% of the dry weight (Section IV-C).

[c] See footnote c of Table 11.

[d] See footnote d of Table 11.

dialysis (Section IV-C-9), and FDNB end group analysis (Section IV-C-7-a) were all less than 6,000. The amino acids listed below tyrosine are probably due to contaminating peptides since less than 1 residue per mole was present. A preliminary column analysis of another preparation of Glycopeptide 2 gave similar values for aspartic acid, glutamic acid, and tyrosine but different values for the amino acids listed below tyrosine. This also suggests that the amino acids below tyrosine are due to contaminating peptides. These amino acids amount to 3.5%, 2.5%, and 4.5% (w/w) of Glycopeptide 1, 2, and 3, respectively. The deviations of the aspartic acid, glutamic acid, and tyrosine values from whole numbers probably represent experimental error, which could be ± 10%. The complete analytical composition given in Tables 11, 12, and 13 represents 83.0%, 89.0% and 103.1% of the weight and 89.2%, 78.7% and 84.4% of the nitrogen of the three glycopeptides respectively. These recoveries are consistent with the ± 10% experimental error of each determination and indicate that essentially all the constituents have been identified. If the glucosamine values were corrected for a possible 20% (10) acid hydrolysis loss, the analytical composition would represent 89.3%, 96.3%, and 108.7% of the weight and 96.1%, 86.0%, and 89.3% of the nitrogen of the three glycopeptides.

7. Peptide Structure.

a. Fluorodinitrobenzene end group analysis. The amino end groups of Glycopeptides 1, 2, and 3 were determined by the fluorodinitrobenzene method of Sanger (114,115). Modifications of the method have been reviewed by Porter (84) and by Fraenkel-Conrat, Harris, and Levy (35). Fluorodinitrobenzene (1-fluoro-2,4-dinitrobenzene), referred to as FDNB, can react with

free amino, imino, phenolic hydroxyl, thiol, and imidazole groups to form the corresponding dinitrophenyl derivatives, referred to as DNP derivatives. The procedures used were essentially those described by McFadden and Smith (79) and Thompson (138) and are summarized in Table 14. The results obtained are given in Table 15. An uncorrected yield of 96 μM of DNP glutamic acid was obtained per gram of Glycopeptide 1. When corrected for the usual acid hydrolysis recovery (56% (107)), this value becomes 172 μM/g. This is equivalent to 1 residue per 5,830 g. or 0.84 residues per 4,850 g., the molecular weight of Glycopeptide 1 estimated from amino acid analysis (Section IV-C-6-b). The molecular weight estimated from sedimentation (Section IV-C-2) was about 4,000 and from fractional dialysis (Section IV-C-9) was between 3,000 and 6,000. Thus, Glycopeptide 1 appears to have one free amino end group per mole.

The dinitrophenylation of Glycopeptides 2 and 3 was followed, at constant pH, with an automatic titrator.[a] The uptake of 0.02 N trimethylamine was recorded and studied by the method of Fraenkel-Conrat, Harris, and Levy (35). Glycopeptides 2 and 3 yielded, respectively, 263 and 493 μM of H^+ per gram at pH 7. These values correspond to about 37% and 46%, respectively, of theoretical dinitrophenylation, assuming 1 tyrosine and 1 free amino group per 4,200 g. and 2,800 g. of Glycopeptide 2 and 3, respectively (Section IV-C-6-b). Although the yield of DNP amino acids from Glycopeptides 2 and 3 was only about 0.1 residue/mole, uncorrected, it was adequate for qualitative identification. DNP-hexosamines were not found in any of the three glycopeptides. Thus, the hexosamines of the glycopeptides have no free amino groups.

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[a] Radiometer, Copenhagen, Type TTT 1a.

TABLE 14

DINITROPHENYLATION PROCEDURES

Sample	Conditions of Dinitro-phenylation	Removal of Excess FDNB	Hydrolysis	Separation into Aqueous and Ether Phases	Uncorrected Total Yield of DNP-amino Acids
Glycopeptide 1 1.0 mg. (0.5 ml. of 1-5 stock solution	H_2O 0.4 ml. sample 0.5 " 10% TMA 0.1 " EtOH 2.0 " FDNB 0.1 " 5 hrs. in hydrolysis tube	Extraction with 4 x 10 ml. of ether in hydrolysis tube	6N HCl 24 hrs. in sealed, evacuated tube, 105°C.	Extraction of dry residue with 4 x 1 ml. of CE-50	96 μM/g.
Glycopeptide 2 1.9 mg. (0.2 ml. of stock solution)	sample 0.2 ml. H_2O 2.0 " FDNB 0.1 " pH 7.0 with TMA and autotitrator giving 263 μMH+/g. (35) in 4 hrs.	Continuous extraction with a. ether b. EtOAc + 0.5 ml. HOAc	6N HCl 20 hrs. in sealed, evacuated tube, 105°C.	Continuous extraction with ether	(qualitative analysis only)
Glycopeptide 3 1.4 mg. (1.0 ml. of 1-5 stock solution)	sample 1.0 ml. H_2O 0.8 " FDNB 0.05" pH 7.1 with TMA and autotitrator giving 493μMH+/g. (35) in 5 hrs.	Continuous extraction with a. ether b. EtOAc + 0.5 ml. HOAc	6N HCl 20 hrs. in sealed, evacuated tube, 105°C.	Continuous extraction with ether	23 μM/g.

TABLE 15

RESULTS OF FDNB ANALYSIS[a]

Sample	Ether Phase		Aqueous Phase	
	Method	Result	Method	Result
Glycopeptide 1	Buffered celite column pH 4 CE-50 (79,138)	DNP GLU 96 μM/g. (other components not detectable)	Propanol-pyrophosphate paper chromatography (Section III-C-1)	O-DNP TYR ++ ASP ++ GLU ++ hexosamine ++++
Glycopeptide 2	1 M citrate paper chromatography (113)	DNP GLU ++++ (only traces of other components)	"	O-DNP TYR ++ ASP ++ GLU ++ hexosamine ++++
Glycopeptide 3	Buffered celite column pH 4 CE-50 pH 6.5 $CHCl_3$ (79, 138)	DNP ASP 23 μM/g. (other components not detectable)	"	O-DNP TYR ++ ASP ++ GLU ++ hexosamine ++++

[a] Uncorrected for acid hydrolysis loss.

b. Further enzymic digestion.

Papain. The Mixed Glycopeptide was retreated with papain to determine whether further digestion was possible since, during the first digestion, the high concentration of substrate and hydrolysis products present might have prevented complete digestion. A 2 mg./ml. solution of the Mixed Glycopeptide was incubated at 60° C. with 0.2 mg./ml. of recrystallized mercuripapain, C_1 = 1.0, in 0.01 M sodium phosphate buffer, pH 7.2, containing 0.01 M BAL. The digestion was followed by the ninhydrin method (87). An insignificant increase in ninhydrin color from 0.88 μM to 1.0 μM/ml. was noted in 20 min. At 90 min., the ninhydrin color was still 1.0 μM/ml. The activity of the papain was found to be undiminished at the end of the digestion. The treated glycopeptides were analyzed by paper electrophoresis (Section IV-B, Step 4) and found to be unchanged. Thus, the glycopeptides appear to be completely degraded in the initial papain digestion (Section IV-B, Step 1).

Pepsin, trypsin, and chymotrypsin. The Mixed Glycopeptide was treated with pepsin, trypsin, and chymotrypsin to determine whether digestion was possible with these enzymes. A 2 mg./ml. solution of the Mixed Glycopeptide was incubated with each enzyme, 0.1 mg./ml., at 38.3° C. for 20 hrs. Thymol was added to retard bacterial growth. The buffers used were 0.1 M pH 3.5 citrate, for pepsin treatment, and 0.1 M pH 7.1 phosphate, for trypsin and chymotrypsin. The digestions were followed by a photometric ninhydrin method (87). During the 20-hr. incubation, the assay values increased from 1.39 μM/ml., initially, to 1.40 μM/ml. for

pepsin, 1.50 to 1.55 μM/ml. for trypsin and 1.48 to 1.50 μM/ml. for chymotrypsin. These increases were not significant and indicated that the glycopeptide could not be degraded by these enzymes.

Leucine aminopeptidase and carboxypeptidase. The structure of the peptide portion of the glycopeptides was studied by digestion with leucine aminopeptidase (49) and carboxypeptidase (89). Some of the studies were followed by a photometric ninhydrin method (87). The samples taken for ninhydrin analysis were added either to 0.2 M pH 5.1 acetate buffer or directly to the pH 5 buffered ninhydrin reagent. The leucine aminopeptidase, prepared by the method of Hill and Smith (49), varied in activity from a C_1 of 40 to 53. The carboxypeptidase was dialyzed before use to remove any free amino acids or small peptides present. One typical dialyzed preparation of carboxypeptidase was found to have a C_1 of 12.4 when assayed by the Grassmann and Heyde method (25,45). Thymol was added to each incubation mixture to retard bacterial growth.

Experiment 1. The Mixed Glycopeptide was treated with leucine aminopeptidase (LAP) and carboxypeptidase (CBXP) to obtain preliminary information concerning the ability of these enzymes to degrade the glycopeptides. Separate 2 mg./ml. solutions of the Mixed Glycopeptide (5 mg. each) were incubated at 40° C. with 1.52 mg./ml. of LAP (C_1 = 50) in 0.02 M pH 8.5 TRIS (tris[hydroxymethyl]aminomethane) buffer containing 0.002 M $MgCl_2$ and with 0.1 mg./ml. of CBXP in 0.02 M pH 8.0 TRIS buffer. During the 24-hr. incubation period the ninhydrin color value of the LAP treated sample increased from 1.20 μM/ml. to 3.10 μM/ml., and the CBXP treated sample increased from 1.20 μM/ml. to 1.58 μM/ml.,

thus indicating significant degradation by both enzymes. The amino acids released were removed from the digests with Dowex 50x4 (30-50 mesh), H^+ cycle eluted with 4 N ammonium hydroxide, and identified by paper chromatography with propanol-pyrophosphate as described in Section III-C-1. Varying amounts of many amino acids were detected.

Experiment 2. Glycopeptide 1, in a 4 mg./ml. solution (1.0 ml. of the stock solution in 2.5 ml.), was treated with 1.8 mg./ml. of leucine aminopeptidase C_1 = 45, in 0.06 M pH 8.5 TRIS buffer containing 0.002 M $MgCl_2$. The incubation was continued for 24 hrs. at 41.6° C. The treated solution was dialyzed by the method of Section IV-C-9-a, first through 20/32 cellulose casing (1 hr. x 4). The dialysate (expected to contain most of the glycopeptide and free amino acids) was concentrated and then dialyzed through 23/32 cellulose casing (5 min. x 6). This dialysate was expected to contain 98% of the amino acids but only about 10% of the glycopeptide. The dialysate was analyzed by column chromatography as described in Section III-C-2 and was found to contain a ratio of 1.00 asparagine to 1.97 glutamic acid to 0.67 tyrosine. The solution from inside the 23/32 cellulose casing (expected to contain 90% of the glycopeptide) was hydrolyzed with 6 N HCl at 105° C. for 24 hrs. and analyzed for amino acids; column chromatography showed that it contained a ratio of 1.00 glutamic acid to 0.40 tyrosine, to 1.93 glucosamine. The effluent fractions containing aspartic acid were lost; however, paper chromatography with propanol-pyrophosphate (Section III-C-1) showed that the hydrolysate contained about equal amounts of aspartic and glutamic acids. Thus, the 2 aspartic acids, 3 glutamic acids, and 1 tyrosine of the glycopeptide were accounted for, leaving only 4 out

of 6 glucosamines unaccounted for. The 4 glucosamines were probably split off the glycopeptide by the conditions of handling in the course of the digestion, concentration, and dialysis.

Experiment 3. A 4.0 mg./ml. solution of Glycopeptide 1 (1.0 ml. of the stock solution in 2.5 ml.) was treated 24 hrs. at 40° C. with 0.08 mg./ml. of carboxypeptidase. The solution was adjusted to pH 8.0 with ammonium hydroxide and acetic acid. An aliquot of the incubation mixture containing 0.4 mg. of glycopeptide was chromatographed on paper with the propanol-pyrophosphate system (Section III-C-1), but only faint traces of many amino acids (CYS, ASP, GLU, SER, GLY, THR, ALA, TYR, VAL, PHE, and LEU) were detected. Thus, carboxypeptidase was unable to significantly degrade Glycopeptide 1. The traces of amino acids detected probably came from peptide contaminants of the glycopeptide and the carboxypeptidase.

Experiment 4. A 1.4 mg./ml. solution of Glycopeptide 3 (1.0 ml. of the 1 to 5 stock solution) was treated 16 hrs. at 40° C. with 0.036 mg./ml. of carboxypeptidase. The solution was adjusted to pH 8.0 with ammonium hydroxide. An enzyme control was similarly treated. Equivalent aliquots of the treated sample (containing 0.3 mg.) and the enzyme control were chromatographed on paper with the propanol-pyrophosphate system (Section III-C-1), but only questionable traces of several amino acids were detected in both the sample and the control. Thus, carboxypeptidase was also unable to significantly degrade Glycopeptide 3.

Experiment 5. A 0.5 ml. aliquot of the 1 to 5 stock solution of Glycopeptide 3 (containing 0.7 mg.) was treated with 0.78 mg. of leucine aminopeptidase, C_1 = 53, for 48 hrs. at 40° C. in 0.65 ml. of solution containing 0.02 M $MgCl_2$. The solution was initially adjusted to pH 8.5 with ammonium hydroxide. An enzyme control was similarly treated. An aliquot of the treated glycopeptide (containing 0.22 mg.) and an equivalent aliquot of the control were chromatographed with the propanol-pyrophosphate system (Section III-C-1). Aspartic acid, glutamic acid, and tyrosine were found in a ratio of about 1 to 1 to 1. This accounted for all the amino acids of Glycopeptide 3 except 1 aspartic acid. Traces of other amino acids were found in both the sample and the enzyme control. The unaccounted for aspartic acid must be attached to the carbohydrate, otherwise it, too, would have been detected by paper chromatography. Thus, apparently, aspartic acid is linked directly to the carbohydrate of the glycopeptide and forms the link between the carbohydrate and protein of γ-globulin. The aminopeptidase was able to degrade Glycopeptide 3 more extensively than Glycopeptide 1 (Exp. 2) probably because a higher enzyme to substrate ratio was used and because the carbohydrate group of Glycopeptide 3 was smaller, thus offering less steric hindrance.

Experiment 6. The Mixed Glycopeptide was treated with both leucine aminopeptidase and carboxypeptidase to allow study of the degraded components by fractional dialysis (Section IV-C-9) and electrophoresis-chromatography (Section IV-C-8). A 40 mg./ml. solution of the Mixed Glycopeptide (100 mg. total) was treated with 8.76 mg./ml. of leucine aminopeptidase, C_1 = 40, in 0.02 M TRIS buffer, pH 8.5, containing 0.005 M $MgCl_2$, at

40° C. During 45 hrs. of incubation the ninhydrin assay value increased from 59.2 μM/ml. to 128 μM/ml. The mixture was adjusted to pH 7.5 with acetic acid and treated with 13.9 mg./ml. of carboxypeptidase. The ninhydrin color value increased from 151 μM/ml. at time zero to 160 μM/ml. at 45 hrs.

The treated Mixed Glycopeptide was then studied by electrophoresis chromatography as described in Section IV-C-8. Two glycopeptides were found that contained only carbohydrate and aspartic acid in significant amounts. This verifies the conclusion of Exp. 5 that aspartic acid is linked directly to the carbohydrate of the glycopeptide and probably forms the link between the carbohydrate and protein of γ-globulin.

The treated Mixed Glycopeptide was also studied by fractional dialysis as described in Section IV-C-9. The major component contained 70% of the hexose and dialyzed at a rate comparable with dialysis rates of known compounds of molecular weights between 3,000 and 6,000.

8. Electrophoresis-chromatography.

a. Method. The electrophoresis-chromatography method of Ingram (59) was used to study the Mixed Glycopeptide after treatment with leucine aminopeptidase and carboxypeptidase (Section IV-C-7-b, Exp. 6). The method consisted of electrophoresis followed by chromatography at right angles to the direction of electrophoresis. The electrophoresis was performed in pH 6.4 pyridine-acetate buffer (100 ml. of pyridine and 3.5 ml. of acetic acid diluted to 1 liter) at about 10 volts/cm. for 3 hrs. The samples were placed in the center near one 22-in. end of a 22 x 18 in. sheet of Whatman 3 MM paper. Electrophoresis was then performed across the 22-in. end. The

paper was dried and then placed in a descending butanol-acetic acid chromatography system (Section III-C-1) to allow chromatography down the 18 in. dimension. The chromatograms were sprayed with 0.025% ninhydrin in ethanol and developed at room temperature to allow detection of the components but leave most of the free amino groups intact.

b. Study of the Mixed Glycopeptide. A 0.025 ml. aliquot of the leucine aminopeptidase- and carboxypeptidase-treated Mixed Glycopeptide (Section IV-C-7-b, Exp. 6) was analyzed in the above manner. A diagram of the developed chromatogram is shown in Fig. 9. The chromatogram was expected to contain many amino acid spots as well as the glycopeptide spots, since the Mixed Glycopeptide was known to contain peptide impurities which could be degraded by LAP or CBXP (Section IV-C-7-b, LAP and CBXP, Exp. 1). As soon as the colored spots appeared they were marked and tentatively identified from the predicted behavior of known amino acids. Four of the spots, 1, 2, 3, and 4, which could not be identified, were cut out (as shown in Fig. 9) and washed in acetone to remove the excess ninhydrin. The contents of the spots were eluted with water, hydrolyzed with 6 N HCl in capillary tubes (79), and then chromatographed with propanol-pyrophosphate (Section III-C-1). The results obtained are given in Table 16. Spots 2 and 3 contained significant amounts of only aspartic acid and glucosamine, thus verifying the conclusion of Section IV-C-7-b, Exp. 5, that the carbohydrate of the glycopeptide is linked to aspartic acid. Traces of other amino acids, probably contaminants, were also detected.

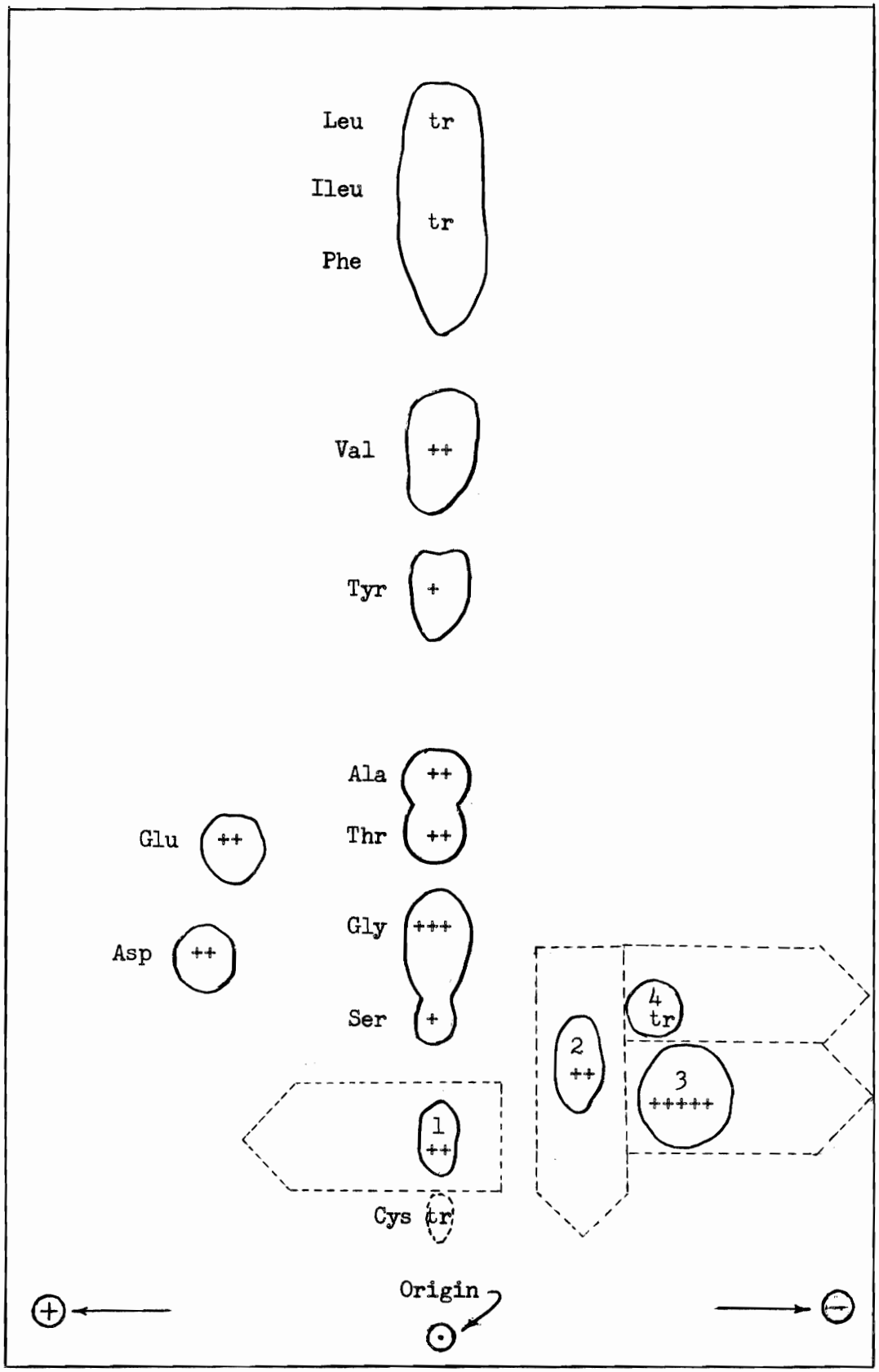

Figure 9. Electrophoresis Chromatography of LAP- and CBXP-treated Mixed Glycopeptide.

TABLE 16

ANALYSIS OF COMPONENTS OF LAP- AND CBXP-TREATED MIXED GLYCOPEPTIDE

Component	Spot 1	Spot 2	Spot 3	Spot 4
Aspartic acid	+++	++	+++	tr
Glutamic acid	+++	tr	tr	tr
Glucosamine	+++++	+++	++	tr

c. Study of the dialyzed Mixed Glycopeptide. Component 3 of the dialyzed leucine aminopeptide- and carboxypeptidase-treated Mixed Glycopeptide (Section IV-C-9-b) was also studied by electrophoresis chromatography.[a] An aliquot of dialysate No. 18 containing 0.63 μM of hexose and 0.18 μM of free amino groups was analyzed as described above (Section IV-C-8-a). The chromatogram, sprayed with ninhydrin and developed 24 hrs. at 25° C., showed three major spots (5, 6, and 7 of Fig. 10), which were not detected in the undialyzed glycopeptide, and two minor spots, which correspond to Spots 3 and 4 of the undialyzed glycopeptide (Fig. 9). The three major spots of the dialyzed glycopeptide were probably present in the chromatogram of the undialyzed glycopeptide on the positive side of Spot 1, but were not detected because of their slow color development and

[a] The undialyzed leucine aminopeptidase- and carboxypeptidase-treated Mixed Glycopeptide was studied by electrophoresis chromatography in Section IV-C-8-b.

low color yield. Component 3, which represents 70% of the Mixed Glycopeptide (Section IV-C-9-b), apparently contains several subcomponents. Thus, even after exhaustive digestion with leucine aminopeptidase and carboxypeptidase, the Mixed Glycopeptide still contains several different charged species.

9. Fractional Dialysis.

a. Method. Craig (23,24) has developed a fractional dialysis method which can be used for the estimation of molecular weights and for the determination of molecular homogeneity. A large area of dialysis membrane was used for a relatively small volume of the solution being dialyzed; for example, 45 sq. cm. of dialysis membrane for 0.6 ml. of solution. Uniform distribution of solution over the membrane surface was obtained by placing one end of a sealed glass cylinder of slightly smaller diameter inside the dialysis tubing, thus displacing the air and spreading the solution to be dialyzed into a thin film. The solution outside the tubing was 10 to 20 times the volume of the solution inside the tubing and was replaced with fresh solution at appropriate intervals to maintain a high concentration gradient and prevent the rate of back dialysis from becoming significant. Under these conditions, the escape rates were found to be very reproducible and were a first order function of the concentration of the solute present inside the dialysis tubing. Some unpublished first order 50% escape times, determined by Craig (24), are listed in Table 17.

The sealed cylinder placed in the dialysis tubing was designed to facilitate the introduction and removal of the sample from the dialysis tubing and to allow mixing of the sample each time the solution outside the tubing was changed. It had a 6 mm. tube down the center of the cylinder with a

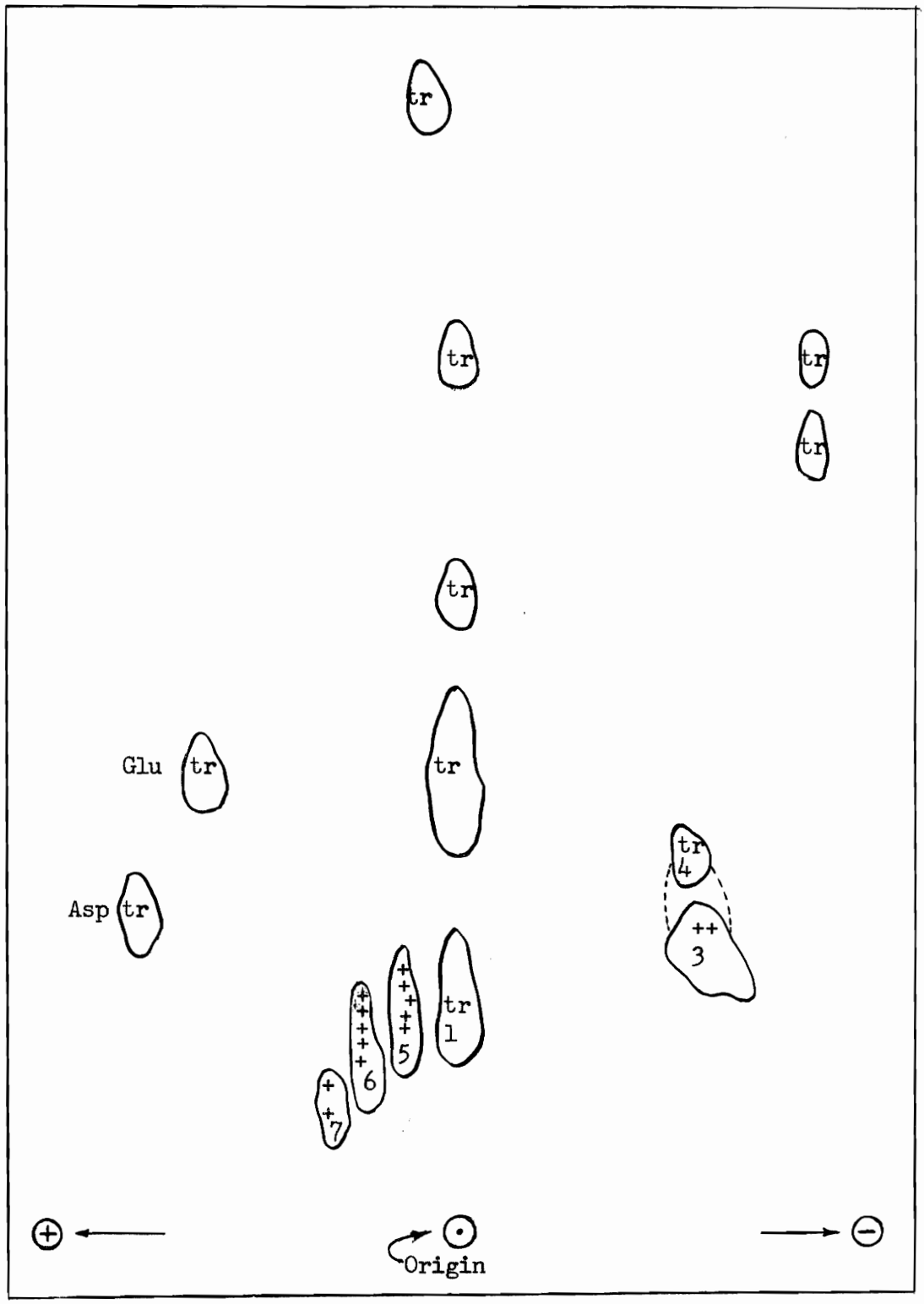

Figure 10. Electrophoresis Chromatography of Component 3 of LAP- and CBXP-treated Mixed Glycopeptide. Component 3 was obtained by fractional dialysis as described in Section IV-C-9-b.

TABLE 17

FIRST ORDER 50% ESCAPE TIMES

Solute	Molecular Weight	50% Escape Time	
		20/32 Membrane[a]	18/32 Membrane[a]
Tryptophane	204	4 min.	6 min.
Bacitracin	1,432	15 min.	21 min.
Subtilin	3,300	54 min.	138 min.
Insulin	5,733	90 min.	Will not pass.

[a] Manufactured by Visking Corporation.

ground glass B-D syringe adapter on top and an annular constriction near the top of the cylinder to trap any excess sample and allow air to escape when the sample was placed in the dialysis tubing. The sample was introduced and removed through a length of small-diameter plastic tubing which was connected to a syringe and led down the 6 mm. tube to the bottom of the dialysis tubing.

Craig has determined the 50% escape times from the slope of the usual first order rate plot of the logarithm of the per cent of the original solute remaining in the tubing against time. This method applies only to dialysis of a single solute of known concentration or to mixtures that can be treated as single solutes. To extend the applicability of this method to mixtures of solutes of unknown concentration, the first order

rate equation was changed to a form involving only time and the rate of appearance of solute outside the dialysis tubing. The equation was derived as follows from the first order rate equation:

The first order rate equation,

$$- dx/dt = kx \text{ or } \ln(x_o/x) = kt,$$

where x is the quantity of solute inside the dialysis membrane, x_o is the quantity at time zero, t is the time elapsed from time zero, and k is the rate constant, becomes

$$x/x_o = e^{-kt}$$

when changed to the exponential form. Substituting (x_o-y) for x, where y = quantity of solute dialyzed through the membrane, gives

$$(x_o-y)/x_o = e^{-kt}.$$

This can be rearranged to give $y = x_o - x_o e^{-kt}$. Differentiating gives $dy = x_o k e^{-kt} dt$ or $dy/dt = x_o k e^{-kt}$. This can be changed to

$$\ln(dy/dt) = \ln(x_o k) - kt.$$

Thus, the plot of $\ln(dy/dt)$ against t should give a straight line of slope equal to -k and intercept, I, equal to $\ln(x_o k)$. The 50% escape time equals $(\ln 2)/k$ as in the usual first order rate equation. The quantity of each solute present initially can be determined from the intercept, I, of each linear portion of the plot, x_o = (anti ln I)/k. In practice, $\Delta y/\Delta t$ can be used in place of dy/dt, provided Δt is small compared to the 50% escape time. An application of the method will be illustrated in the following section.

b. Study of degraded components of the Mixed Glycopeptide.

A 0.50 ml. aliquot of the leucine aminopeptidase- and carboxypeptidase-treated Mixed Glycopeptide from Section IV-C-7-b, Exp. 6, was dialyzed by the method described in the previous section using a 30 cm. sac of Visking Corporation 23/32 cellulose casing. The sample was dialyzed against 10.0 ml. portions of demineralized-distilled water containing 10% isopropyl alcohol to retard bacterial growth. The dialysates were first changed at 5 min. intervals 6 times, then 15 min. x 6, 1 hr. x 3, 3 hrs. x 2, and 12 hrs. x 6, giving a total of 23 dialysates. Each dialysate was analyzed by both the orcinol (Section III-A-2) and ninhydrin (51) methods to determine the hexose and free amino group content. The micromoles of hexose and free amino groups present in each dialysate were divided by the length of the dialysis interval (in minutes) to obtain the dialysis rates ($\Delta y/\Delta t$). The natural logarithm of these values was plotted against the mid-time of the dialysis interval of each dialysate to obtain the dialysis rate curves for the hexose and free amino groups, as shown in Fig. 11. The curve of the free amino groups showed three linear regions, whereas the curve of the hexose showed two linear regions. The calculated 50% escape times and zero time quantities of three components are given in Table 18. Orcinol and ninhydrin assay of an equivalent undialyzed sample indicated that 30 μM of total hexose and 80 μM of total free amino groups were present initially. Craig has not reported 50% escape times for the 23/32 membrane but has reported them for the similar 18/32 membrane. As estimated from the values for the 18/32 membrane in Table 17, Component 3, with a 50% escape time of 330 min., has a molecular weight between 3,000 and 6,000, and Component 2, with a 50%

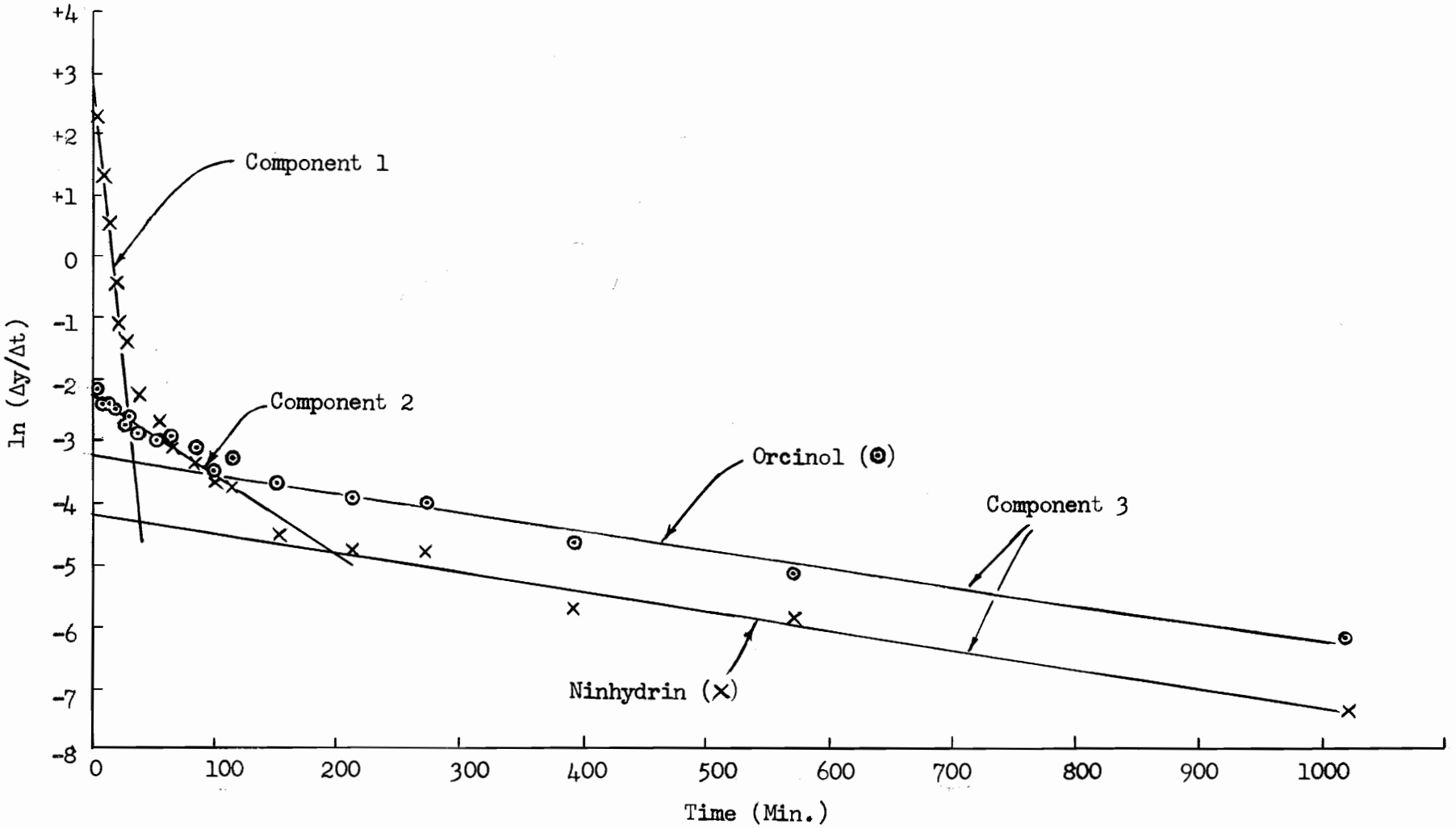

Figure 11. Fractional Dialysis of LAP- and CBXP-treated Mixed Glycopeptide. The dialysis rate, $\Delta y/\Delta t$, is expressed in micromoles per minute.

escape time of 50 min., has a molecular weight between 1,500 and 3,000. Component 1 was probably free amino acids. The molecular weight estimated for Component 3 is in the same range as the molecular weight of the glycopeptides determined by sedimentation, FDNB analysis, and amino acid and carbohydrate analysis (Section IV-C-6-b). The hexose of Component 3 (Table 18) contains about 70% of the total hexose of the Mixed Glycopeptide.

TABLE 18

FRACTIONAL DIALYSIS OF COMPONENTS OF THE MIXED GLYCOPEPTIDE

Component	50% Escape Time (Min.)	Quantity (μM)	
		Hexose	Free Amino
1	3.8	-	66.0
2	50	7.7	7.7
3	330	19.4	7.2
Total		27.1	80.9

Aspartic acid, glutamic acid, tyrosine, and glucosamine were found in Component 3 (dialysate No. 16) after acid hydrolysis and paper chromatography with propanol-pyrophosphate (Section III-C-1). Component 3 was also studied by electrophoresis chromatography as described in Section IV-C-8-c and found to contain at least three subcomponents.

10. Paper Chromatography of the Mixed Glycopeptide. Several unsuccessful attempts were made to chromatograph the Mixed Glycopeptide in

pyridine-acetic acid-water (29:21:50, v/v) and sec-butanol-formic acid (88%)-water (300:50:150, v/v). The chromatograms were developed with borax (0.05 M in H_2O) 5 ml., phenol red (0.2% in ethanol) 10 ml., and methanol, 100 ml., or the ninhydrin reagent of Section III-C-1. Only elongated smears were detected. Discrete spots were obtained, however, with butanol-acetic acid chromatography after electrophoresis as described in Section IV-C-9.

D. Summary and Conclusions

1. Glycopeptide Composition. The composition of the three purified glycopeptides is summarized in Table 19. The values given are within ± 10% of the analytical values and are based on molecular weights estimated from analytical composition, sedimentation, fractional dialysis, and amino end group analysis. Two acetylglucosamine values for each glycopeptide are included in the table. The higher values have been corrected for a possible 20% acid hydrolysis loss (10). The sialic acid content of Glycopeptides 2 and 3, as indicated, is less than 1 residue per mole. Some sialic acid was probably lost during the preparation of these glycopeptides. The 8 hexose residues appear to be composed of 3 galactose and 5 mannose.

2. Purity. The nonstoichiometric amino acids amount to about 3.5%, 2.7%, and 4.5% by weight of Glycopeptide 1, 2, and 3 respectively. Since these amino acids were released by carboxypeptidase, they are probably part of the peptide contaminants. However, amino end group analysis detected only traces of peptides other than those with aspartic and glutamic acid end groups, thus the peptide contaminants represent only a small fraction of the glycopeptides.

TABLE 19

COMPOSITION OF GLYCOPEPTIDES[a]

Component	Glycopeptide 1	Glycopeptide 2	Glycopeptide 3
Hexose	8	8	8
N-acetyl glucosamine	6 to 8	6 to 8	3 to 4
Fucose	2	2	2
Sialic acid	1	0.6	0.2
Aspartic acid	2	2	2
Glutamic acid	3	2	1
Tyrosine	1	1	1

[a] Values given are in residues per mole and are within ± 10% of the analytical values.

The essential purity of the glycopeptides was also indicated by the symmetrical sedimentation and electrophoresis peaks, and the stoichiometry of the analytical values (Table 19). The purity of the glycopeptides is thus better than 90%.

3. Comparison of Glycopeptides. As shown in Table 19, the composition of the three glycopeptides is very similar. Variations occur only in the glutamic acid, sialic acid, and glucosamine values. The similarity in composition suggests that the glycopeptides are derived from the same structure, probably represented by Glycopeptide 1. The variation in the number of glutamyl residues apparently represents differences in the peptide

chain length. Some variations in chain length can be expected with an enzyme such as papain, since enzymic hydrolysis at one site would inhibit hydrolysis at adjacent sites.

The variation in sialic acid content was probably produced by the digestion procedure and the purification methods. The sialic acid link is known to be very unstable (8,92). Glycopeptide 2 probably contains two components, one (60%) with 2 glutamic acids and 1 sialic acid and the other (40%) with 3 glutamic acids and no sialic acid. These components would thus have the same net negative charge and would account for the low sialic acid and high glutamic acid values. A similar situation would account for the low sialic acid content of Glycopeptide 3. The most unusual feature of Glycopeptide 3, however, is that it has lost one-half of its glucosamine but none of its hexose or fucose. Therefore, the missing glucosamine residues could not have been interspersed among the other sugars but must have been terminal units.

4. Peptide Structure. As indicated in Table 19, Glycopeptide 3 (G-3) contains 1 residue of glutamic acid, 1 of tyrosine, and 2 of aspartic acid. According to fluorodinitrobenzene (FDNB) analysis, the N-terminal residue of G-3 is aspartic acid. When G-3 was treated with leucine aminopeptidase (LAP), only 1 residue of aspartic acid, tyrosine, and glutamic acid was released. Thus, 1 aspartic acid residue must have remained bound to the carbohydrate. This evidence suggests that G-3 has the structure:

Asp(Tyr,Glu)Asp(CHO)

where the residues in parenthesis are of undetermined sequence and (CHO) represents the carbohydrate group.

The similar amino acid and carbohydrate composition of the three glycopeptides suggests that they are derived from the same structure. The amino acid composition is the same except that Glycopeptide 2 (G-2) has one more glutamic acid residue than G-3, and Glycopeptide 1 (G-1) has two more than G-3. According to FDNB analysis, both G-2 and G-1 have N-terminal glutamic acid residues. Thus, the additional glutamic acid of G-1 and G-2 are N-terminal to the structure shown above for G-3. This evidence suggests that G-1 has the structure:

Glu.Glu.Asp.(Tyr,Glu)Asp(CHO)

When G-1 was treated with LAP, 2 residues of glutamic acid, 1 of asparagine, and 0.6 of tyrosine were released, leaving 0.4 residues of tyrosine, 1 of glutamic acid, and 1 of aspartic acid bound to the carbohydrate. This evidence is in accord with the above structure of G-1 and suggests that tyrosine precedes the third glutamic acid residue of G-1 and follows asparagine rather than aspartic acid. Presumably, the asparagine residue of G-3 was converted to aspartic acid by the preparation methods. Thus, G-1 has the structure:

Glu.Glu.Asp(NH_2).Tyr.Glu.Asp(CHO)

Attachment of the carbohydrate group to aspartic acid was verified by additional evidence. Two glycopeptides that contained only aspartic acid and carbohydrate were obtained by electrophoresis chromatography of the LAP- and carboxypeptidase- (CBXP) treated Mixed Glycopeptide. Attachment of the carbohydrate to the phenolic group of tyrosine can definitely be excluded since O-DNP tyrosine was obtained from all three glycopeptides following treatment with FDNB, tyrosine was released from the glycopeptides

by LAP, and the shift in absorption spectrum characteristic of tyrosine with free hydroxyl groups was observed with change in pH. Attachment of the carbohydrate to the C-terminal residue of the peptide chain was verified by treatment of G-1 and G-3 with CBXP whereby only traces of amino acids were released. The carbohydrate is probably attached by an amide or ester type bond to the β-carboxyl group of aspartic acid. Attachment to the α-carboxyl group can be excluded since papain would probably hydrolyze an α-ester bond and both papain and LAP would hydrolyze an α-amide bond.

The peptide chain of the three glycopeptides most likely represents a segment of the primary peptide structure of γ-globulin rather than a separate peptide chain. Thus, the attachment to aspartic acid is believed to form the primary linkage between the carbohydrate and protein of γ-globulin.

5. Carbohydrate Group of γ-Globulin. One mole of Glycopeptide 1, as illustrated in Table 20, contains all the carbohydrate of γ-globulin within the limits of experimental error. Since γ-globulin (II-1,2) is essentially homogeneous, every molecule presumably contains 1 mole of Glycopeptide 1. The other glycopeptides, 2 and 3, are probably slight variations of Glycopeptide 1 produced by the digestion procedure and purification methods and, thus, do not necessarily represent true variations in the carbohydrate group of γ-globulin.

The three glycopeptides represent 90% of the Mixed Glycopeptide carbohydrate, which, in turn, represents 60% of the γ-globulin carbohydrate. Thus, it is very unlikely that γ-globulin contains a different type of carbohydrate group. Furthermore, several types of evidence indicate that the glycopeptides do not represent more than 1 type of carbohydrate group.

TABLE 20

CARBOHYDRATE COMPOSITION OF γ-GLOBULIN AND GLYCOPEPTIDE I

Carbohydrate	γ-Globulin[a] (Residues/Mole)	Glycopeptide 1[b] (Residues/Mole)
Hexose	8 to 9	8
Glucosamine	8 to 10	6 to 8
Fucose	2	2
Sialic acid	1	1

[a] Discussed in Section IV-A.

[b] Discussed in Section IV-C.

First, only 1 peptide chain was attached to the carbohydrate group. Second, the carbohydrate group was attached to the peptide chain only by an aspartic acid residue. And, third, the calculated molecular weights of the glycopeptides are in good agreement with the experimental molecular weights evaluated by sedimentation, fractional dialysis, and amino end group analysis. On the basis of the evidence presented, the carbohydrate of human γ-globulin, II-1,2, is a single, definite group chemically bound to the protein by a single aspartic acid residue.

V. DISCUSSION

As summarized in Section IV-D, human γ-globulin, fraction II-1,2, was found to contain a single carbohydrate group attached to a single aspartic acid residue of the protein and composed of 1 sialic acid, 2 fucose, 6 to 8 glucosamine, 3 galactose, and 5 mannose residues. Although the presence of carbohydrate in γ-globulin has been previously reported, this is the first report of the entire carbohydrate composition. Rimington (111) identified glucosamine and mannose in a mixture of equine serum globulins. Hewitt (48) concluded that galactose, mannose, and glucosamine were a true part of diphtheria antitoxin, a γ_1-globulin. Fucose was first detected in serum proteins by Dische et al. (30) and later found in γ-globulin by Micheel et al. (83). Sialic acid was identified in γ-globulin by Böhn et al. (11,12).

Similar carbohydrates have also been reported in other purified glycoproteins. Neuberger (88) found D-mannose and D-glucosamine in the carbohydrate group of ovalbumin. Morgan et al. (1,2,3) found D-galactose, L-fucose, D-glucosamine, and D-galactosamine in three different blood-group substances.[a] Gottschalk (42) found galactose, mannose, fucose, glucosamine, and galactosamine in the urinary mucoprotein characterized by Tamm and Horsfall (136). In addition, Blix (7) found sialic acid in this preparation. Odin and Werner (91) found galactose, mannose, glucosamine, galactosamine,

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[a] The presence of protein in the blood-group substances, and thus their glycoprotein nature, has been disputed. The purest preparations, however, contain 20-25% protein, and none have been obtained free of a protein or peptide group (cf. Werner (149), p. 49).

and sialic acid in the acid glycoprotein characterized by Winzler et al. (120,147). These similarities in carbohydrate composition suggest that all glycoproteins may contain the same general type of carbohydrate group.

Until this present work, however, glycoproteins could not be considered more than a very stable addition complex of polysaccharide and protein, since no evidence had been obtained for a definite linkage between the carbohydrate and protein. A covalent linkage was assumed since a linkage of this type would most likely explain the constant ratio of carbohydrate to protein in the purest preparations. Other linkages, however, such as those associated with antigen-antibody interaction (see Section I), might also explain the constant carbohydrate to protein ratio. Now, with respect to human γ-globulin at least, carbohydrate has been shown to be primarily linked to an aspartic acid residue of a protein; thus the carbohydrate and protein form part of one primary structure. The carbohydrate of other glycoproteins is probably linked to protein in a similar manner and thus also forms part of the primary structure. Human γ-globulin and possibly other carbohydrate-containing proteins, then, appear to represent a distinct class of biological compounds and not just a simple addition complex of polysaccharide and protein. Recognition of a class of true glycoproteins is now justified.

Human γ-globulin, fraction II,1-2, contains many antibodies (107). In fact, most of this γ-globulin preparation is believed to have antibody activity (cf. Boyd (13) and Hughes (57)). Even though this preparation contains many different antibodies, it is homogeneous both as an antigen and by many chemical and physical criteria (see Section I). The yield of

glycopeptides and the carbohydrate analyses summarized in Section IV-D also suggest that this preparation is essentially homogeneous. Thus, most of the antibodies of this preparation presumably contain a similar carbohydrate group. Slight variations in the carbohydrate group, however, could have been over looked by the methods used in this work. Although this present work does not directly relate differences in antibody structure, it does provide a method whereby possible differences in structure of a particular region can be studied and, in this manner, it may eventually contribute to the correlation of antibody function and structure.

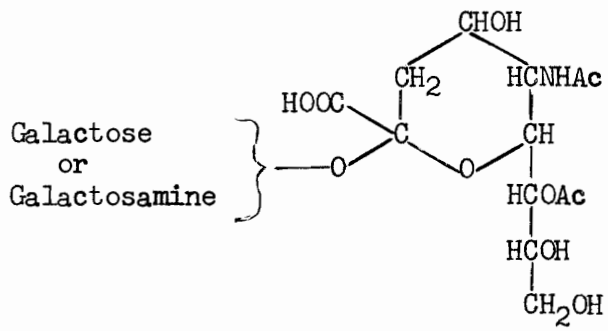
Now that antibodies have been shown to contain a definite carbohydrate group and not just a tightly adsorbed polysaccharide, the findings of Hewitt (48), Petermann and Pappenheimer (104), and Northrop (90) concerning the constant association of carbohydrate with active diphtheria antitoxin, even after enzymic digestion, becomes especially significant. These findings suggest that carbohydrate is closely associated with the combining sites of the antitoxin. The association of a tightly adsorbed polysaccharide with the combining sites would suggest only that some of the combining sites had already combined with a polysaccharide. Association of a definite carbohydrate group which is part of the primary antitoxin structure, however, suggests that, in some way, the carbohydrate group may contribute to the structure of the combining sites. Most antibodies are now assumed to have 2 antigen combining sites (cf. Campbell et al. (19)) and perhaps 2 or more sites for combining with complement, but present evidence indicates that there is only one carbohydrate group per antibody molecule.

As summarized in Section IV-D, the carbohydrate of γ-globulin was found attached to the C-terminal aspartic acid residue, probably to the β-carboxy group, of the following peptide chain:

-Glu.Glu.Asp(NH_2).Tyr.Glu.Asp(CHO)-.

This peptide most likely represents a segment of the peptide structure of γ-globulin and was probably hydrolyzed from the peptide structure, during papain digestion, at both the N-terminal glutamic acid and C-terminal aspartic acid residues. Thus, at the pH of blood, about pH 7.2, this 6-residue portion of γ-globulin would have 3 negatively charged groups, which seems to be an unusually high concentration.

In this present work, no attempt was made to study the carbohydrate structure; several structural features, however, were suggested by the evidence presented. The carbohydrate was found attached to a C-terminal aspartic acid residue, thus the linkage between the carbohydrate and protein is probably an ester or amide type bond. The sialic acid linkage appears similar to that of other glycoproteins, since the linkage was completely hydrolyzed when the glycopeptides were treated 1 hr. at pH 2 and 100° C., the usual conditions for hydrolyzing the sialic acid linkage (8). Gottschalk (44) has suggested that the sialic acid (diacetyl neuraminic acid) of bovine serum mucoid and of sialic-lactose (neuramin-lactose (142) and O-acetyl lactaminic acid-lactose (170)) is linked to a hydroxy group of galactose or galactosamine in the following manner:



No suggestion of the hexose structure was obtained. Stacey et al. (130, 131) have determined that the carbohydrate group of ovomucoid has a branched structure composed of a hexose chain surrounded by acetyl hexosamine residues (see Section I). The carbohydrate group of γ-globulin could have a similar structure.

VI. SUMMARY

1. The carbohydrate components of human γ-globulin, fraction II-1,2, were identified and quantitatively estimated.

2. Three glycopeptides were isolated from a papain digest of human γ-globulin and characterized by chemical and physical methods.

3. The amino acid sequence of the three glycopeptides was determined.

4. The three glycopeptides were found to represent a single carbohydrate group which was attached to the protein by a single aspartic acid residue. The carbohydrate group contained 3 galactose, 5 mannose, 2 fucose, 6 to 8 glucosamine, and 1 sialic acid residue.

5. The significance of these findings was discussed in relation to glycoproteins and antibodies.

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