Studies on Glycopeptides Released by Trypsin from Intact Human Erythrocytes*

Richard J. Winzler, Edward D. Harris, Darold J. Pekas, Clarence A. Johnson, and Peter Weber

ABSTRACT: Glycopeptides containing about one-third to one-half of the sialic acid of human erythrocytes are released from the intact cell surface by incubation with trypsin. These glycopeptides fall in a single size group with a molecular weight of about 10,000, contain galactose, acetylglucosamine, acetylgalactosamine, and *N*-acetylneuraminic acid, and are rich in serine and threonine. The glycopeptides have part of their carbohydrate as relatively small oligosaccharides linked to the peptide chain through *O*-glycosidic bonds between *N*-acetylgalactosamine and the hydroxyl groups of serine and threonine.

The glycopeptides also contain oligosaccharides linked to the peptide chain by alkali-stable bonds.

A number of investigators have demonstrated that treatment of intact erythrocytes with trypsin releases glycopeptides into the medium (Seaman and Heard, 1960; Eylar and Madoff, 1962; Cook *et al.*, 1960; Mäkelä *et al.*, 1960; Miller *et al.*, 1963; Eylar *et al.*, 1962; Ohkuma and Ikemoto, 1966). As part of a program on the study of cell surfaces, we have been interested in the structure of these glycopeptides. It seemed possible that the glycopeptides released from intact cells by trypsin might be components of the inhibitors of viral hemagglutination and of the M or N substances previously isolated from erythrocyte stroma (deBurgh *et al.*, 1948; McCrea, 1953; Baranowski *et al.*, 1959; Klenk and Uhlenbruck, 1960; Kathan *et al.*, 1961; Eylar and Madoff, 1962; Howe *et al.*, 1963; Kathan and Winzler, 1963; Romanowska and Baranowski, 1963; Morawiecki, 1964; Uhlenbruck and Weber, 1963; Cook and Eylar, 1965; Bezkorovainy *et al.*, 1966; Maddy, 1966; Springer *et al.*, 1966).

Methods

Hexose was determined by the orcinol reaction (Winzler, 1955) or by the phenol–sulfuric acid method (Dubois *et al.*, 1956). Total hexosamine was assayed by the Elson–Morgan reaction (Boas, 1953) after hydrolysis in 3 N HCl at 100° for 4 hr. Total hexosamine and the proportions of glucosamine and galactosamine were determined following hydrolysis with 3 N HCl for 4 hr using a short Dowex 50 column on an amino acid analyzer as described by Kominz (1962). Sialic acid was determined by the direct Ehrlich reaction (Werner and Odin, 1952) or by the thiobarbituric acid method of Warren (1959). Fucose was determined by the method of Dische and Shettles (1948).

Amino acids were assayed after hydrolysis for 16 hr in 5.7 N HCl at 110–115° under nitrogen using a Technicon AutoAnalyzer as well as a Beckman system. Correction was made for a 10% destruction of serine and threonine under the standard conditions of hydrolysis. This correction was determined by hydrolyzing a sample of trypsin fragment for 12, 16, 24, 36, and 72 hr and extrapolating the threonine and serine content to zero time. Tryptophan was determined by the method of Bencze and Schmid (1957). Tritium-labeled components eluted from the column were determined by passing the effluent through a liquid-flow scintillation counter (Packard Model 314 AX 500) prior to the colorimetric analysis in the Technicon AutoAnalyzer.

Sedimentation velocity and approach to equilibrium studies for molecular weight estimation were performed with a Spinco Model E analytical ultracentrifuge, equipped with a schlieren optical system. For these studies samples were dissolved at a concentration of 1% in 0.9% NaCl containing 0.01 M pH 7 phosphate buffer. Molecular weight determination by the Archibald technique followed the procedure outlined by Schachman (1957). Partial specific volumes were calculated from the amino acid and carbohydrate composition by the method of Schachman (1957); partial specific volumes used for the sugars were 0.62 for hexose and hexosamine and 0.59 for sialic acid (Bezkorovainy and Doherty, 1962). Diffusion measurements were made in a Spinco Model H electrophoresis diffusion apparatus measured over a period of 96 hr, and diffusion coefficients were computed by the fringe method (Schachman, 1957).

Tritium-labeled sodium borohydride was prepared by dissolving 2 mc (sp act. 50 mc/mmole, New England Nuclear Corp.) in 10 ml of 0.01 N NaOH containing

* From the Department of Biochemistry, State University of New York at Buffalo, Buffalo, New York, and Department of Biological Chemistry, University of Illinois, College of Medicine, Chicago, Illinois. *Received February 15, 1967.* Supported by grants from the U. S. Public Health Service (A2520 and CA-08492) and from the Armed Forces Epidemiological Board (MD 2931 and MD 2803).

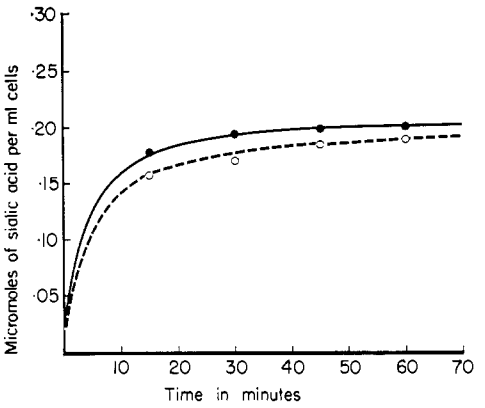

FIGURE 1: Release of sialic acid containing compounds from erythrocytes by trypsin. Washed red cells (25 ml) were incubated with the indicated amounts of trypsin in 50 ml of 0.9% NaCl containing 0.01 M phosphate buffer (pH 7.8). At the times indicated, 5-ml aliquots were withdrawn and treated with an equal volume of 10% trichloroacetic acid, and the acid-soluble sialic acid was determined by the Ehrlich method. (○) Trypsin, 0.25 mg/ml of cells. (●) Trypsin, 0.50 mg/ml of cells.

0.2 mmole of nonradioactive sodium borohydride. Aliquots (1 ml) of this solution were lyophilized in test tubes and stored until used. When ready for use, the lyophilized borohydride was dissolved in 0.2 N NaOH. The specific activity of the reagent was 2.2×10^5 cpm/μmole. Glycopeptide samples were dissolved in this reagent and incubated at room temperature, usually for 48 hr. Reaction with borohydride was stopped by acidifying the samples with acetic acid. For radioactivity determination, 0.05-ml aliquots were placed in scintillation vials, 1–2 ml of water was added, and the samples were lyophilized. The scintillation liquid (4 g of 2,5-diphenyloxazole and 0.1 g of 1,4-bis-2(phenyloxazolylbenzene) to 500 ml each of toluene and methyl Cellosolve) was added, and counting was carried out in a Packard liquid scintillation counter.

Outdated blood was pooled without regard to blood group types. Packed cells were collected by centrifugation at 700*g* for 45 min, and were washed four times with 0.9% NaCl containing 0.01 M pH 7 sodium phosphate buffer.

Results

Preparation of Trypsin Fragments. In order to determine the most suitable conditions for release of glycopeptides, packed red cells were suspended in two volumes of phosphate-buffered saline and incubated at 37° with gentle agitation with various amounts of trypsin (Nutritional Biochemicals, twice crystallized). Release of glycopeptides was determined at various times by centrifuging off the cells, precipitating the proteins with 5% trichloroacetic acid, and determining the total sialic acid in the supernatant by the direct Ehrlich reaction. The result of such an experiment is shown in Figure 1. It is evident that glycopeptides are most rapidly released in the first 15 min, and that the subsequent slow rate of release occurs at slightly different levels depending on the initial concentration of the enzyme. There was considerable variability in the total sialic acid released, with values ranging from 0.2 to 0.35 μmole of sialic acid released/ml of packed cells. The sialic acid content of red cells is 0.45–0.56 μmole/ml of packed cells (Eylar *et al.*, 1962; Mäkelä *et al.*, 1960). Thus about one-third to one-half of the total sialic acid of the red cell is released by trypsin.

In the large-scale preparative experiments, 400 ml of

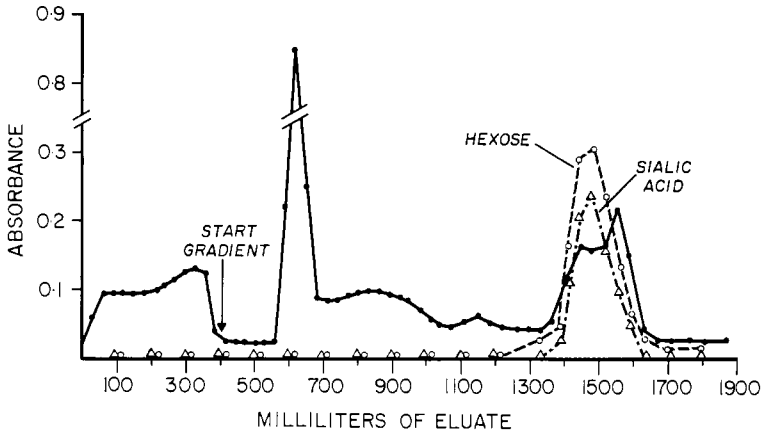

FIGURE 2: Purification of trypsin fragments by DEAE chromatography. Up to 200 mg of the trichloroacetic acid soluble, desalted fragments was passed through a 1.5 × 15 cm DEAE column equilibrated with 0.05 M sodium formate buffer (pH 6.8) and eluted as described in the text. The solid line represents absorbance at 280 mμ. Hexose is shown as absorbance at 540 mμ using the orcinol test where an absorbance of 0.1 corresponds to 0.12 mg of hexose/ml of effluent. Sialic acid is shown as absorbance at 575 mμ using the Ehrlich test where an absorbance of 0.1 corresponds to 0.05 mg/ml of effluent.

packed cells was added to 400 ml of phosphate-buffered saline containing 100 mg of trypsin. Incubation was for 1 hr at 37° with gentle agitation. The suspension was then centrifuged at 700*g* in the cold, and the red-tinted supernatant was removed by suction. To this supernatant was added one-quarter volume of 25% trichloroacetic acid to precipitate the proteins. The clear supernatant was recovered by centrifugation, neutralized with NaOH, and lyophilized. Salts were removed by dialysis after dissolving the samples in small volumes of water, and then preparations were lyophilized.

Because of the acidic nature of the glycopeptides, they could be separated from neutral peptides on DEAE-cellulose columns. Quantities (200 mg) of the glycopeptide fraction were dissolved in 50 ml of water and placed on a DEAE-cellulose column (1.7 × 15 cm) previously equilibrated with pH 6.8, 0.05 M sodium formate buffer. The column was then washed with 25 ml of distilled water to elute neutral components. The acidic glycopeptides were then eluted with a six-chamber gradient system containing 250 ml each of (1) water, (2) 0.05 M sodium formate, (3) 0.05 M formic acid, (4) 0.2 M formic acid, (5) 0.4 M formic acid, and (6) 0.4 M formic acid in 0.3 M sodium chloride. Fractions (7 ml) were collected and analyzed for absorbance at 280 mμ as well as for hexose and sialic acid. Figure 2 shows the results for a typical separation. It is clear that all of the bound sialic acid and hexose in the trypsin fragments were eluted together in a single major peak with a contaminating shoulder high in ultraviolet absorption and low in carbohydrate. The carbohydrate-rich peak of Figure 2 was pooled and lyophilized. The yield of ten preparations averaged 21 ± 5 mg/100 ml of packed red cells. The composition of the native trypsin fragments is shown in the third column of Table I.

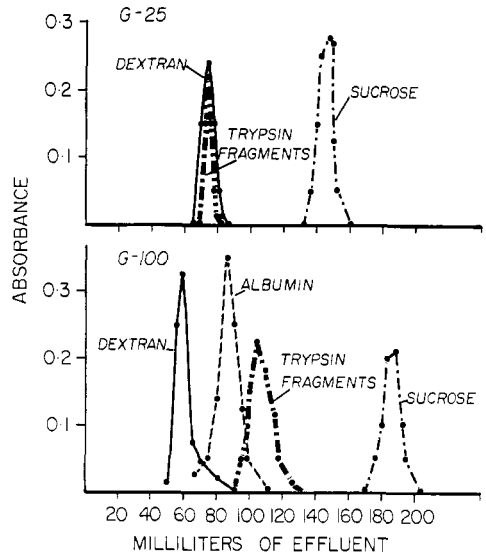

FIGURE 3: Gel filtration studies of trypsin fragments on G-25 and G-100 Sephadex columns. Both columns were of the same dimensions (2.5 × 50 cm). Blue Dextran (mol wt 2,000,000) was detected by absorbance at 500 mμ, bovine serum albumin by absorbion at 280 mμ, sucrose by the orcinol reaction, and the trypsin fragments by sialic acid analyses using the direct Ehrlich reaction.

TABLE I: Composition of Trypsin Fragments and of the High Molecular Weight Residue after Treatment with Alkaline Borohydride.

Substance	% of Moisture-Free Wt	
	Native	Peak I of Fig 6
Hexose[a]	24.4	22.7
Acetylgalactosamine[b]	13.5	0.8
Acetylglucosamine[b]	7.1	11.5
N-Acetylneuraminic acid[c]	33.8	27.2
Fucose[d]	1.8	2.4
Amino acids[e]	20.8	37.7
Ash	4.5	1.5
Total	105.9	103.8

[a] Hexose was determined by the orcinol method (Winzler, 1955). Galactose and trace amounts of mannose were detectable by paper chromatography. [b] Amino sugars were determined using an automated amino acid analyzer after hydrolysis in 3 N HCl for 4 hr at 100° (Kominz, 1962). Results are expressed in terms of the acetylated sugars since present evidence indicated that all amino sugars are acetylated (Kathan and Adamany, 1967). [c] Sialic acid was determined by the method of Warren (1959). [d] Fucose was determined by the method of Dische and Schettles (1948). [e] Determined with automated amino acid analyzer. Tryptophan was determined by the method of Bencze and Schmid (1957).

The trypsin fragments emerged with the void volume during gel filtration on G-25 Sephadex columns. They emerged in a single somewhat retarded peak during gel filtration on Sephadex G-100 columns (Figure 3). The elution volume from a Sephadex G-100 column calibrated with albumin according to the method of Andrews (1965) corresponded to an apparent molecular weight of 34,000. The fragments showed a single symmetrical peak with a sedimentation constant of 1.5 S when examined in the analytical ultracentrifuge (Figure 4). The trypsin fragments are not homogeneous, however, since all preparations have shown a broad negatively charged, ninhydrin-positive, sialic acid containing zone when subjected to high-voltage paper electrophoresis in pyridine–acetic acid buffers at pH 3.7. Also, the amino acid composition indicates heterogeneity, as will be discussed later.

The molecular weight of the trypsin fragments has been estimated by sedimentation and diffusion procedures and by the Archibald approach to equilibrium procedure. The results shown in Table II are in agreement

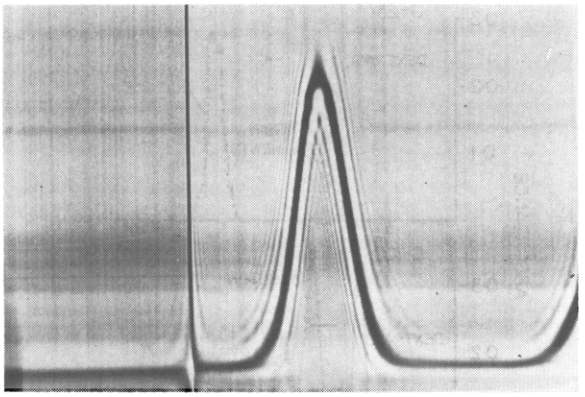

FIGURE 4: Ultracentrifugation of the trypsin fragments. Trypsin fragments were dissolved to a concentration of 1% in 0.01 M phosphate buffer at pH 7.0 and 0.15 M NaCl. The frame was taken with a bar-angle setting of 75° 16 min after the instrument had reached a speed of 59,780 and using a standard, 4° sector, 12-mm cell. Sedimentation from left to right.

TABLE II: Molecular Weight of Trypsin Fragment.

$s_{20,w}$ (S)	1.5
$D_{20,w}$ (Fick units)	10.65
$\bar{v}$ (ml/g)	0.632
Molecular weight	
Sedimentation and diffusion method	9,550
Archibald method	
At meniscus	11,070
At bottom	10,370

that the molecular weight of the trypsin fragments is in the neighborhood of 10,000. The difference from the apparent molecular weight obtained by the gel filtration method no doubt reflects the effects of the branched and extended glycopeptide chains on the exclusion volume of the glycopeptides.

Several investigators have studied the effect of alkali or of alkaline borohydride on glycoproteins and acid mucopolysaccharides, and have shown that serine and threonine are destroyed if these amino acids are linked to carbohydrate by an *O*-glycosidic linkage involving the anomeric group of a sugar and the hydroxyl group of the amino acid (Anderson *et al.*, 1965; Tanaka *et al.*, 1964; Harbon *et al.*, 1964; Carubelli *et al.*, 1965; Schiffman *et al.*, 1964; Lindahl and Roden, 1966; Adams, 1965). When alkaline treatment is carried out in the presence of sodium borohydride, the peptide-bound products from the serine and threonine formed during the β-elimination reaction are in part reduced to alanine and α-aminobutyric acid, respectively. The sugar involved in the *O*-glycosidic bond is reduced to the corresponding alcohol (Anderson *et al.*, 1965; Tanaka *et al.*, 1964; Adams, 1965; Lloyd and Kabat, 1964; Schiffman *et al.*, 1964).

This approach has been extended to trypsin fragments using tritium-labeled sodium borohydride in the presence of NaOH. Samples (5 mg) of native and trypsin fragments desialized by treatment with 0.1 N HCl for 1 hr at 80° (Warren, 1959) were incubated with 1.5 ml of a reagent containing 0.1 M sodium borohydride (2.2×10^5 cpm/μmole) and 0.2 M NaOH. After various times the reaction was terminated by transferring 0.05-ml aliquots to scintillation vials, acidifying with acetic acid, and lyophilizing. Radioactivity was determined after addition of the liquid scintillation solution.

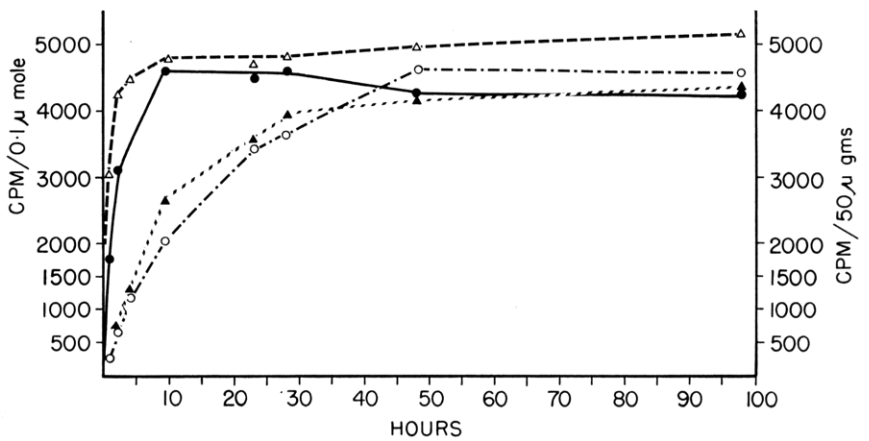

FIGURE 5: Incorporation of tritium from tritium-labeled borohydride in alkaline solution. Samples (5 mg) of native trypsin fragments and of acid-desialized trypsin fragments as well as 5 mg of galactose and of glucosamine were treated with 1.5 ml of 0.1 M sodium borohydride (sp act. 2.2×10^5 cpm/μmole) in 0.2 N NaOH. Activity was determined on 0.05-ml aliquots taken at the times indicated. For further explanation see text. (○) Native trypsin fragments, (▲) desialized trypsin fragments, (△) galactose, and (●) glucosamine.

TABLE III: Effect of Alkaline Borohydride on Amino Acid Composition of Trypsin Fragments.

	Native[a,b]		$OH^--BH_4^-$[c] Treated (moles/ 10,000 g)	Significant Changes (moles/ 10,000 g)
	g/100 g	moles/10,000 g		
Lys	1.05	0.72	0.70	–
His	1.29	0.83	0.82	–
Arg	1.13	0.65	0.65	–
Asp	1.69	1.27	1.26	–
Thr	4.78	3.98	0.93	−3.05
Ser	4.19	3.98	1.30	−2.68
Glu	1.13	0.77	0.77	–
Pro	0.71	0.67	0.66	–
Gly	0.50	0.58	0.58	–
Ala	0.92	1.03	1.57	+0.54
Cys	–	–	–	–
Val	0.96	0.82	0.82	–
Met	–	–	–	–
Ile	0.62	0.47	0.48	–
Leu	0.35	0.27	0.28	–
Tyr	0.45	0.25	0.24	–
Phe	–	–	–	–
Trp	0.96	0.47	*d*	–
α-Aminobutyrate	–	–	0.25	+0.25
Acetylgalactosamine	13.4	6.05	0.39	−5.66
Acetylglucosamine	7.1	3.20	3.05	–
Galactose	24.3	13.52	13.40	–
Sialic acid	33.8	10.95	10.65	–

[a] Average of ten determinations on four preparations. [b] All values expressed on a moisture-free basis. [c] Average of six determinations on four preparations. [d] Not done.

Correction was made for counts fixed by a distilled water control. The results are shown in Figure 5, along with comparative results using 5 mg of galactose and of glucosamine similarly treated. It is evident that galactose took up the label most rapidly. Glucosamine was reduced somewhat more slowly and the trypsin fragments were most slowly labeled under the conditions employed. Tritium uptake was complete in about 36 hr. Removal of sialic acid had little effect on uptake of tritium by trypsin fragments under the very alkaline conditions of these experiments.

Labeled trypsin fragments were hydrolyzed in 5.7 N HCl for 16 hr at 105° and were then subjected to separation on Dowex 50 columns monitored for radioactivity during amino acid analysis. Radioactive alanine and α-aminobutyric acid were found, as well as radioactive components emerging as several peaks in the hexosamine area. The nature of these radioactive peaks has not been investigated.

The results shown in Figure 5 suggested that reaction of the native trypsin fragments with alkaline sodium borohydride was complete by 48 hr. Therefore, native trypsin fragments were incubated for 48 hr at room temperature with a reagent containing 0.1 M sodium borohydride in 0.2 M NaOH. The reaction was stopped by acidification to 5.7 N HCl for determination of amino acids, by acidification to 3 N HCl for determination of hexosamines, or by acidification to pH 4 with acetic acid for determination of sialic acid and hexose. Amino acids, hexosamines, hexose, and sialic acid of the treated and untreated samples were determined by the methods already indicated. The results of these analyses, along with results obtained with untreated trypsin fragments, are given in Table III. The native fragments average about 17 amino acid residues/mole (mol wt 10,000), 8 of which are serine and threonine present in equal amounts. With the exception of these two amino acids and aspartate and alanine, the other amino acids are present to the extent of less than one residue per mole. This is additional evidence for the heterogeneity of these trypsin fragments. The results also show that treatment with alkaline borohydride resulted in a loss of serine, threonine, and hexosamine, in an increase in the amount of alanine, and in the appearance of some α-aminobutyric acid. The latter two were radioactive in experiments in which tritium-labeled borohydride was employed.

It is particularly interesting that the alkaline borohydride treatment selectively destroys galactosamine but not galactose, glucosamine, or sialic acid. This observation has also been made by Kathan and Adamany

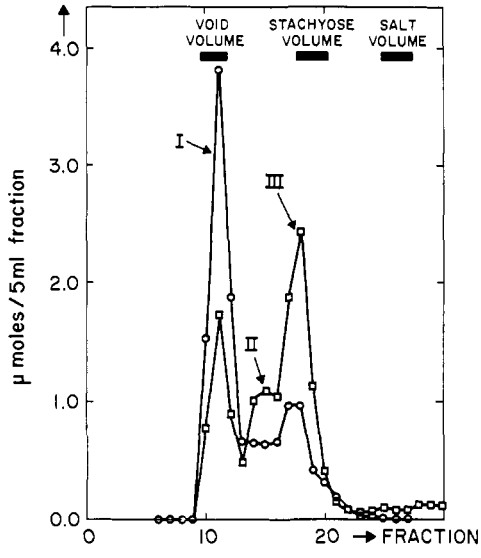

FIGURE 6: Gel filtration of trypsin fragment after treatment with alkaline borohydride. Trypsin fragments (14.88 mg) treated at room temperature with 0.1 M $NaBH_4$ in 0.1 N NaOH for 50 hr were passed through a Sephadex G-25 column (1.8 × 41.5 cm gel bed). Elution was with 0.1 N acetic acid and 5-ml fractions were collected. Ordinate: μmoles/5-ml fraction. Abscissa: fraction number. (⊡-⊡-⊡) Sialic acid, determined with Ehrlich's reagent. (⊙-⊙-⊙) Hexose as galactose equivalents, determined with the phenol–sulfuric acid method.

(1967) and by Springer *et al.* (1966) using stromal components of higher molecular weight.

The nature of the products produced by treatment of the trypsin fragments with alkaline borohydride was investigated by subjecting the treated samples to gel filtration on Sephadex G-25 and measuring the hexose and sialic acid in the effluents. The results are shown in Figure 6. It is clear that three significant peaks are obtained. Emerging with the void volume is a component (peak I) containing large amounts of hexose and sialic acid in a molar ratio of 2:1. The composition of peak I is shown in the last column of Table I. Its amino acid composition is the same as the treated trypsin fragments shown in Table III. It is virtually free of galactosamine, and contains sialic acid, galactose, and glucosamine. A small intermediate fraction (peak II) containing sialic acid and hexose emerges with an elution volume slightly less than that of stachyose. A third major peak (peak III) emerges with the elution volume of stachyose. This peak contains no free sialic acid by the Warren (1959) procedure, but contains considerable bound sialic acid. Peaks II and III contain negligible amounts of peptides or amino acids. The nature of their components is under investigation.

Discussion

Since about one-third of the protein-bound sialic acid can be cleaved by trypsin from the surfaces of intact erythrocytes, it is evident that glycoproteins are exposed in the aqueous environment of the cell surface. It is estimated from the amount of sialic acid released and the molecular weight of the trypsin fragment that about 5×10^6 glycopeptide molecules are released from each red cell during incubation with trypsin. It is somewhat surprising to find that a molecular weight of about 10,000 is common to all the glycopeptides released by trypsin. High-voltage electrophoresis and amino acid analyses suggest that the trypsin fragments isolated from pooled cells are not completely homogeneous, although obviously they are closely related. Studies with trypsin fragments from single donors may reveal greater homogeneity than has been observed with the pooled samples employed in the present work.

A striking aspect of the amino acid composition of the trypsin fragments is their lack of cystine, methionine, and phenylalanine, and their very high complement of serine and threonine. One mole of the fragments contains eight residues of serine plus threonine and eight to nine residues of all the other amino acids combined. This raises the possibility that the trypsin fragments are made up of a sequence which, statistically at least, is serine-X-threonine-X-, etc., where X may be any other amino acid. Certain other glycoproteins, including the submaxillary mucins and the blood group substances with A, B, and H activity, are also characterized by very high content of the hydroxy amino acids (Hashimoto *et al.*, 1964).

That some, but not all, of the serine and threonine is involved in *O*-glycosidic linkages with carbohydrate is clear from the results of the alkaline borohydride experiments (Table III). About 75% of the serine and threonine was destroyed by treatment with alkaline borohydride. At the same time tritium-labeled alanine and α-aminobutyric acid appeared, though not in stoichiometric amounts. There was loss of an equivalent amount of galactosamine, strongly supporting the view that the galactosamine is linked glycosidically to serine and threonine. As is shown in Table I, glucosamine remains with the high molecular weight peptide following treatment with alkaline borohydride. This indicates that glucosamine, in contrast to galactosamine, is a part of an alkaline stable oligosaccharide in the glycopeptide. This alkaline stability makes it likely that acetylglucosamine or galactose is linked to asparagine in a β-asparaginylglycosylamine bond as described by Marshall and Neuberger (1964) and others. This possibility is being investigated using pronase digestion of the alkali-treated trypsin fragments in an attempt to isolate aspartic acid containing glycopeptides.

The nature of the low molecular weight sialic acid containing components shown in peaks II and III of Figure 6 has not yet been extensively investigated. In the Warren (1959) test, peak III before hydrolysis gives an orange color with an absorption maximum at 540 mμ prior to acid hydrolysis. After mild acid hydrolysis, the strong absorption peak obtained in the Warren procedure is at 550 mμ, corresponding to that obtained with authentic sialic acid.

In previous work (Kathan and Winzler, 1963) it was observed that treatment with borohydride of desialized preparations of an inhibitor of viral hemagglutination isolated from erythrocytes caused a conversion of hexosamine to hexosaminitol. This led to the suggestion that sialic acid is linked 2–1 to acetylhexosamine in this inhibitor. Kathan and Adamany (1967) showed that the borohydride treatment which resulted in the selective destruction of hexosamine in the previous studies was due to an increase in the alkaline lability of the *O*-glycosidic bond between hexosamine and serine or threonine, after removal of sialic acid. Therefore, the previous proposal of a sialyl 2–1 hexosamine bond now appears very improbable.

On the basis of the available evidence, the glycopeptides here described appear to be comprised of a family of closely related peptides in which half of the amino acid residues are serine and threonine, most of which are linked through their hydroxyl groups to the anomeric carbon of acetylgalactosamine in a low molecular weight oligosaccharide. This would account for the lability of 75% of the serine and threonine and virtually all of the galactosamine to alkaline borohydride. The remaining carbohydrate must be bound to the peptide chain in one or more alkaline-stable oligosaccharides containing glucosamine, galactose, and sialic acid, accounting for *ca.* half of the total carbohydrate in the glycopeptide.

Kathan *et al.* (1961) and Kathan and Winzler (1963) isolated about 80% of the sialic acid of erythrocyte stromal as a viral hemagglutination inhibitor with a molecular weight of 31,000. One-third to one-half of the sialic acid of the red cell is isolated in the glycopeptides described here. Therefore, the trypsin fragment must be derived in part, and probably entirely, from the inhibitor of viral hemagglutination previously described (Kathan *et al.*, 1961; Kathan and Winzler, 1963). It appears very likely that the glycopeptides represent the terminal portion of the hemagglutination inhibitor previously described, and that the inhibitor is in turn a subunit of larger membrane structures studied by Bezkorovainy *et al.* (1966), Springer *et al.* (1966), and Maddy (1966). This logic would suggest that the glycopeptides released from red cells by trypsin are the free terminal portions of cell surface glycoproteins split at trypsin-accessible lysine or arginine residues. The other end of the glycoprotein is presumably associated with the cell membrane.

From experiments based on dissolution of red cell membranes with detergents, Morawiecki (1964) has proposed that glycoprotein subunits in erythrocyte membranes have a carbohydrate-rich portion which is exposed to the aqueous environment and a lipophilic portion which is associated with the red cell membrane. The data here reported are consonant with this proposal.

References

Adams, J. B. (1965), *Biochem. J. 97*, 345.

Anderson, B., Hoffman, P., and Meyer, K. (1965), *J. Biol. Chem. 240*, 156.

Andrews, P. (1965), *Biochem. J. 96*, 595.

Baranowski, T., Lisowska, E., Morawiecki, A., Romanowska, E., and Strozecka, K. (1959), *Arch. Immunol. Terapii Doswiadczalnej 7*, 15.

Bencze, W. L., and Schmid, K. (1957), *Anal. Chem. 29*, 1193.

Bezkorovainy, A., and Doherty, D. G. (1962), *Arch. Biochem. Biophys. 96*, 491.

Bezkorovainy, A., Springer, G. F., and Hotta, K. (1966), *Biochim. Biophys. Acta 115*, 501.

Boàs, N. F. (1953), *J. Biol. Chem. 204*, 553.

Carubelli, R., Bhavanandan, V. P., and Gottschalk, A. (1965), *Biochim. Biophys. Acta 101*, 67.

Cook, G. M. W., and Eylar, E. E. (1965), *Biochim. Biophys. Acta 101*, 57.

Cook, G. M. W., Heard, D. H., and Seaman, G. V. F. (1960), *Nature 188*, 1011.

deBurgh, P. M., Yu, P.-C., Howe, C., and Bovarnick, M. (1948), *J. Exptl. Med. 87*, 1.

Dische, Z., and Shettles, L. B. (1948), *J. Biol. Chem. 175*, 595.

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., and Smith, F. (1956), *Anal. Chem. 28*, 350.

Eylar, E. H., and Madoff, M. A. (1962), *Federation Proc. 21*, 402.

Eylar, E. H., Madoff, M. A., Brody, O. V. and Oncley, J. D. (1962), *J. Biol. Chem. 237*, 1992.

Harbon, S., Herman, G., Rossignol, B., Jollès, P., and Clauser, H. (1964), *Biochem. Biophys. Res. Commun. 17*, 57.

Hashimoto, Y., Hashimoto, S., and Pigman, W. (1964), *Arch. Biochem. Biophys. 104*, 282.

Howe, C., Avrameas, S., de Vaux St. Cyr, C., Grabar, P., and Lee, L. T. (1963), *J. Immunol. 91*, 683.

Kathan, R. H., and Adamany, A. (1967), *J. Biol. Chem. 242*, 1716.

Kathan, R. H., and Winzler, R. J. (1963), *J. Biol. Chem. 238*, 21.

Kathan, R. H., Winzler, R. J., and Johnson, C. A. (1961), *J. Exptl. Med. 113*, 37.

Klenk, E., and Uhlenbruck, G. (1960), *Z. Physiol. Chem. 319*, 151.

Kominz, D. R. (1962), *J. Chromatog. 9*, 253.

Lindahl, U., and Roden, L. (1966), *J. Biol. Chem. 241*, 2113.

Lloyd, K. O., and Kabat, E. A. (1964), *Biochem. Biophys. Res. Commun. 16*, 385.

Maddy, A. H. (1966), *Biochim. Biophys. Acta 117*, 193.

Mäkelä, O., Miettinen, T., and Pesola, R. (1960), *Vox Sanguinis 5*, 492.

Marshall, R. D., and Neuberger, A. (1964), *Biochemistry 3*, 1596.

McCrea, J. F. (1953), *Yale J. Biol. Med. 26*, 191.

Miller, A., Sullivan, J. F., and Katz, J. H. (1963), *Cancer Res. 23*, 485.

Morawiecki, A. (1964), *Biochim. Biophys. Acta 83*, 339.

Ohkuma, S., and Ikemoto, S. (1965), *Nature 212*, 198.

Romanowska, E., and Baranowski, T. (1963), *Arch. Immunol. Therap. Exptl. 11*, 625.

Schachman, A. K. (1957), *Methods Enzymol. 4*, 32.

Schiffman, G., Kabat, E. A., and Thompson, W. (1964), *Biochemistry 3*, 113.

Seaman, G. V. F., and Heard, D. H. (1960), *J. Gen. Physiol. 44*, 251.
Springer, G. F., Nagai, Y., and Tegtmeyer, H. (1966), *Biochemistry 5*, 3254.
Tanaka, K., Bertolini, M., and Pigman, W. (1964), *Biochem. Biophys. Res. Commun. 16*, 404.
Uhlenbruck, G., and Weber, P. (1963), *Z. Immunit. Allergieforsch. 126*, 325.
Warren, L., (1959), *J. Biol. Chem. 234*, 1971.
Werner, I., and Odin, L. (1952), *Acta Soc. Med. Upsalien. 57*, 230.
Winzler, R. J. (1955), *Methods Biochem. Anal. 2*, 279.

The Isolation of the Mouse Nerve Growth Factor Protein in a High Molecular Weight Form*

Silvio Varon, Junichi Nomura,† and E. M. Shooter

ABSTRACT: The nerve growth factor is a protein which specifically stimulates the growth of sympathetic and embryonic sensory ganglia. This communication describes the isolation of the nerve growth factor protein from the adult male mouse salivary gland in a new high molecular weight form. The new nerve growth factor form has been purified 40-fold from homogenates of the gland by a procedure involving only gel filtration on Sephadex G-100, DEAE-cellulose chromatography, and a second gel filtration on Sephadex G-150. In the final product the nerve growth factor activity is associated, as judged by a number of physical criteria, with a single protein component comprising over 95% of the total protein of the fraction. It represents 2% of the soluble protein of the gland and 80% of the expressed activity of the gland homogenate. The molecular weight of this new nerve growth factor species is approximately 140,000. Chromatography on CM-cellulose at acid pH separates an active subunit(s) whose molecular weight is approximately 30,000, grossly in the range of the nerve growth factor products isolated by the older procedure.

For the past 10 years, Levi-Montalcini (1966) and her group have been investigating a protein factor which selectively enhances the growth of sympathetic and embryonic sensory ganglia. The factor is present in the sympathetic ganglia themselves (Levi-Montalcini and Angeletti, 1961; Winick and Greenberg, 1965) and appears to be essential to their preservation (Levi-Montalcini and Angeletti, 1963). Biochemical studies (Cohen, 1959; Angeletti *et al.*, 1964; Liuzzi *et al.*, 1965; Angeletti *et al.*, 1965) have shown that this nerve growth factor enhances, in the responsive ganglia, oxidation of glucose, the incorporation of acetate into lipids, and the synthesis of both protein and RNA and that the stimulation of RNA synthesis occurs independently from, and before, that of protein synthesis (Angeletti *et al.*, 1965).

Little work on the nerve growth factor protein has been reported since the earlier studies (Cohen, 1957, 1959, 1960; Levi-Montalcini *et al.*, 1965). The factor has been purified from snake venom (Cohen, 1959) and adult male mouse submaxillary gland (Cohen, 1960). The two preparations are very similar in their biological properties, but differ in their behavior on ion-exchange chromatography, in specific activity, and in sedimentation properties with sedimentation coefficients of 2.2 and 4.33 S and estimated molecular weights of 20,000 and 44,000, respectively (Cohen, 1959, 1960). It is possible that these are two different protein factors present in snake venom and the mouse submaxillary gland, respectively, but having the same biological activity. On the other hand, as Cohen (1960) pointed out, the difference in molecular weight might simply reflect differences in the isolation procedures, for example, the use of a step involving urea for the material of lower molecular weight. It has been subsequently observed (S. Varon, unpublished data) that gel filtration of crude salivary extracts on Sephadex G-100 satisfactorily separated the nerve growth factor from other biologically active materials such as the epithelial (Cohen, 1964) and the mesenchymal growth factors (Gandini-Attardi *et al.*, 1965). Two other observations were made at the same time. The nerve growth factor emerged from the Sephadex column in a position indicative of a higher molecular

* From the Department of Genetics and Lt. Joseph P. Kennedy, Jr. Laboratories for Molecular Medicine, Stanford University School of Medicine, Palo Alto, California. *Received March 10, 1967.* This research was supported by funds from the Lt. Joseph P. Kennedy, Jr. Foundation, by a U. S. Public Health Service research grant from the National Institute of Neurological Diseases and Blindness (NB-04270) and Training Grant GM-295, and by a United Cerebral Palsy Foundation research grant (R-178-64).

† On leave from the Department of Neuropsychiatry, Kyoto University Faculty of Medicine, Kyoto, Japan.