R_F in this system.) Some yellow ultraviolet-absorbing material was detected near the front.

HEXAPEPTIDE AMIDE VI showed two strong spots with ninhydrin, R_F 0.73 and 0.81. Two slower developing (ninhydrin) spots appeared, R_F 0.58 (trace) and 0.68. Some ultraviolet-absorbing material was detected near the front.

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Isolation and Amino Acid Sequences of Glycopeptides Obtained from Bovine Fibrinogen*

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ABSTRACT: After exhaustive pronase digestion of bovine fibrinogen, the resultant glycopeptides were purified and fractionated by gel filtration and ion-exchange chromatography. Three purified fractions were obtained which contained up to 80% of the total carbohydrate of fibrinogen. The amino acid sequences of the three fractions are Asp-Lys, Gly-Glu-Asp-Arg, and Glu-Asp-Arg.

Fibrinogen is a glycoprotein composed of six polypeptide subunits with a total molecular weight of about 340,000. The carbohydrate components comprise about 3% of the total weight of the molecule; the monosaccharide residues include *N*-acetylglucosamine, galactose, mannose, and *N*-acetylneuraminic acid (Davie and Ratnoff, 1965). Our interest in the carbohydrate components of fibrinogen was stimulated initially by suggestions that these constituents might be involved in the fibrinogen–fibrin conversion. For example, several laboratories have reported that about 20% of the carbohydrate content of fibrinogen is released during its conversion to urea-insoluble fibrin (Bagdy and Szara, 1955; Blombäck, 1958; Chandrasekhar *et al.*, 1962; Chandrasekhar and Laki, 1964; Laki, 1951; Szara and Bagdy, 1953), although this has been disputed (Hörmann and Gollwitzer, 1964; Raisys *et al.*, 1966; Rosenberg and Carman, 1964). In addition, it has been reported that oxidation of the carbohydrate components of fibrinogen by periodate results in a loss of clottability by thrombin (Laki and Mester, 1952).

One approach which has been employed by several laboratories in the study of the carbohydrate components of fibrinogen or fibrin has entailed proteolytic digestion followed by purification of the resultant glycopeptide fractions (Cynkin and Haschemeyer, 1964; Haschemeyer and Cynkin, 1964; Lipinski, 1964; Mester *et al.*, 1963a,b, 1965; Mester and Moczar, 1965; Mészáros, 1964). In most of these studies, the preparations were heterogeneous with respect to amino acid composition. In the present study, pronase digestion followed by ion-exchange column chromatography has led to the isolation of three major glycopeptide fractions whose amino acid sequences have been determined.

* From the Department of Biochemistry, Tufts University School of Medicine, Boston, Massachusetts. *Received July 15, 1966.* This research was supported by the National Science Foundation (GB-2794) and by the U. S. Public Health Service (GM-08518). A preliminary report of this work was presented at the 150th National Meeting of the American Chemical Society, Atlantic City, N. J., Sept 1965.

† Research Career Development Awardee, U. S. Public Health Service.

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Experimental Section

Materials and Methods. Bovine fibrinogen was obtained from Pentex, Inc., and contained about 60% protein by weight which was determined in our laboratory to be 93–94% clottable. Pronase, a proteolytic enzyme from *Streptomyces griseus*, was obtained from Calbiochem, Inc. Carboxypeptidase B was obtained from Gallard-Schlesinger.

Quantitative Analyses. All measurements of optical density were performed on a Beckman-Gilford spectrophotometer. Amino acids and other ninhydrin-reactive materials were quantitated by the method of Rosen (1957). Carbohydrates were estimated as neutral hexose by the phenol–sulfuric acid procedure of Montgomery (1961), using a standard containing galactose and mannose (1:2). Amino sugars were quantitated by the method of Rondle and Morgan (1954). *N*-Acetylneuraminic acid was estimated by the procedure of Warren (1959). Dinitrophenyl (DNP) derivatives were quantitated by measuring their absorbances in alkaline solution at 362 mμ using an extinction coefficient of 18,000. Amino acid analyses were performed on a Beckman 120 automatic amino acid analyzer. These analyses were provided through the courtesy of Dr. Maurice Liss.

Preparation of Samples for Amino Acid Analyses. Samples for amino acid analysis were dissolved in 6 N HCl, sealed in fusion tubes under vacuum, and hydrolyzed for 22 hr at 110°. The solutions were evaporated to dryness repeatedly to remove HCl. The dried samples were redissolved in buffer and suitable aliquots were taken for analysis.

Column Chromatography. The Sephadex G-25 used for gel filtration was washed exhaustively and equilibrated with 0.1 N acetic acid. Elution was carried out with the same solvent in a 4.6 × 44 cm column. Dowex 50-X2, 100–200 mesh, in a 1 × 41 cm column, was washed successively with 2 N NaOH, water, 2 N HCl, and again with water. The column was then equilibrated with 0.005 N pyridine–acetate buffer, pH 3.1. Dowex 1-X2, 100–200 mesh, was converted to the formate form. The resin was washed on a Büchner funnel with 3 N sodium formate until free of chloride. The resin was then poured into a 0.8 × 50 cm column, treated first with ten bed volumes of 6 N formic acid–1 N sodium formate (1:1), and then with several bed volumes of 90% formic acid. Finally, the column was irrigated with distilled water until a pH of 5 was reached.

Terminal Analyses. The DNP derivatives of the glycopeptides were formed by reaction with FDNB[1] in the presence of trimethylamine as described by Fraenkel-Conrat *et al.* (1955). After preliminary ether extraction, the DNP-glycopeptides were chromatographed using silica gel tlc with chloroform–benzyl alcohol–acetic acid (70:30:3) as solvent. Unreacted FDNB and dinitrophenol moved at the solvent front and were thus separated from the glycopeptides (which remained at the origin). The area of the chromatogram containing the glycopeptide was moistened slightly to facilitate its removal with a spatula, and the DNP-glycopeptide was extracted into 1.5 M NH_4OH for several minutes on a Vortex mixer. The concentration of glycopeptide was calculated from the absorbancy at 342 mμ assuming a molar extinction coefficient of 18,000 (or 36,000 for the lysine-containing glycopeptide). The reaction with FDNB was complete as judged by the absence of ninhydrin-reacting material, and the material remaining at the origin during chromatography was all of high molecular weight (*i.e.*, 1500 or greater) as judged by molecular-exclusion tlc on Sephadex G-25 or Bio-Gel P-2.

The DNP-glycopeptides were hydrolyzed for 16 hr at 105° in constant-boiling HCl. The hydrolysate was diluted 1:9 with water and extracted with ether. The DNP derivative of peak IV-B was also hydrolyzed for 2 hr at 105° in 90% formic acid–glacial acetic acid–60% perchloric acid (10:5.5:1.5) in order to minimize the destruction of DNP-glycine; the DNP derivative was extracted into *t*-amyl alcohol in benzene (Hanes *et al.*, 1952).

DNP-amino acids were identified by tlc on silica gel using chloroform–benzyl alcohol–acetic acid (70:30:3) and *n*-propyl alcohol–30% NH_3 (7:3) for separation of the ether- and water-soluble derivatives, respectively. In a few cases, particularly after extraction with *t*-amyl alcohol in benzene, the R_F values of the DNP-amino acids were sufficiently altered by salt effects to require either two-dimensional chromatography (using the same solvent in both directions) or by cochromatographing standards with the extracted unknown.

Reaction with Carboxypeptidase B. The presence of C-terminal basic amino acids was determined by comparing the electrophoretic patterns of the glycopeptides obtained before and after incubation with COP-B. The reaction with COP-B was carried out at 37° for 8 hr using about 0.5 μmole of the glycopeptide in 100 μl of phosphate buffer (pH 7) and 2 μl of the commercial COP-B solution. The blank reactions devoid of glycopeptide or COP-B were carried out simultaneously.

Partial Acid Hydrolysis of Peak IV-C. Partial acid hydrolysis of the DNP derivative of peak IV-C was done for 3 hr at 105° in 0.1 N HCl. The hydrolysate was adjusted to 1 N HCl and extracted with ether. The ether extract was chromatographed in chloroform–benzyl alcohol–acetic acid. DNP derivatives were extracted with 1 N NH_4OH, evaporated to dryness, and subjected to total hydrolysis in 6 N HCl as described above for the N-terminal analyses. The non-N-terminal amino acid composition was then determined by high-voltage electrophoresis.

Results

Preparation of Fibrinogen Glycopeptides. FIRST PRONASE DIGESTION. In a typical experiment, 7 g of

[1] Abbreviations used in this work: FDNB, fluorodinitrobenzene; tlc, thin layer chromatography; COP-B, carboxypeptidase-B.

bovine fibrinogen was suspended in 300 ml of 0.2 M Tris–HCl buffer, pH 8.0. To this suspension were added 2.8 ml of 0.4 M $CaCl_2$, 12 ml of 95% ethanol, 120 mg of Pronase, and a few drops of chloroform to prevent bacterial growth. The mixture was incubated for 1 week at 37°; 30 mg of additional Pronase was added every other day. Early experiments had established that the increase in ninhydrin reactivity, under these conditions, ceased after the seventh day of treatment. The yellow, opalescent solution was heated at 100° for 10 min, concentrated to a small volume (about 20 ml) under vacuum, and filtered, and the filtrate was made 0.1 N with respect to acetic acid.

FIRST GEL FILTRATION. The digest was chromatographed on Sephadex G-25, using a flow rate of about 60 ml/hr. Fractions (20 ml) were collected. Appropriate aliquots were assayed for neutral sugars and for ninhydrin-reactive material. The glycopeptides were eluted near the exclusion volume of the column in fractions 14–26, which were pooled and evaporated to dryness under vacuum. Quantitative recovery of the carbohydrate present in fibrinogen was normally achieved.

SECOND PRONASE DIGESTION. The crude glycopeptide fraction thus obtained was dissolved in 40 ml of 0.1 N HCl and incubated at 80° for 1.5 hr. This treatment was sufficient to remove sialic acid quantitatively, but released less than 5% of the neutral sugars of the glycopeptide fraction in the form of galactose and mannose. (This estimate is based on the measurement of carbohydrate found in the region of low molecular weight components in the second gel filtration procedure.)

In our earlier experiments, reproducibility was difficult to achieve in both the preparation of the crude glycopeptide fraction by Pronase digestion and in the subsequent isolation of purified glycopeptides by either preparative paper electrophoresis or by ion-exchange column chromatography. These difficulties were largely overcome by "stripping" sialic acid from the glycopeptides after the first Pronase digestion.

After hydrolysis, the solution was neutralized to pH 6–7 and evaporated to dryness. The dried material was dissolved in 25 ml of 0.2 M Tris–HCl buffer, to which were added 0.2 ml of 0.4 M $CaCl_2$, 1.0 ml of ethanol, and 20 mg of Pronase. Proteolytic digestion was again continued for 7 days, with 5 mg of Pronase added every other day. The solution was then evaporated to dryness.

SECOND GEL FILTRATION. The dried sample was dissolved in 10 ml of 0.1 N acetic acid and passed through Sephadex G-25 as described above. The glycopeptide fraction contained 140 mg of neutral sugar as estimated by the phenol–sulfuric acid procedure.

Column Chromatography on Dowex-50 (Pyridinium Form). About 100 mg of neutral sugar equivalents of the glycopeptide fraction were evaporated repeatedly to remove acetic acid. The material was then dissolved in 2 ml of 0.005 N pyridine–acetate buffer, pH 3.1, and applied to a column. Elution was carried out with the same buffer until 42 6-ml fractions were collected. At this point elution was continued using a linear gradient at constant pH (3.1) of 0.005–0.2 N pyridine–acetate buffer. The resulting elution pattern is shown in Figure 1. Total carbohydrate recovery from the Dowex 50 column was usually close to 100%. Of the carbohydrate applied to the column 70–80% was contained in peak IV which was further fractionated as described below.

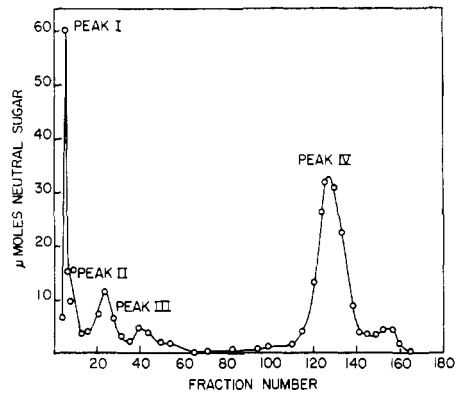

FIGURE 1: Elution pattern of glycopeptides on Dowex 50 (pyridinium form). Elution and other procedures are described in the text.

Fractionation of Peak IV on Dowex 1 (Formate). After lyophilization of the combined fractions from peak IV, a portion (about 16 mg of carbohydrate) was dissolved in 2 ml of distilled water and applied to a column of Dowex 1 (formate). Elution was carried out with distilled water until 42 fractions of 5.5 ml each had been collected. Peak IV was separated into three components during the water elution, termed peaks IV-A–C, in order of their emergence from the column. A fourth peak, IV-D, was eluted when the solvent was changed to 0.05 N formic acid. The water elution pattern is shown in Figure 2. Recoveries in various fractions are summarized in Table I.

Amino Acid Composition of Glycopeptide Fractions. The amino acids contained in the fibrinogen glyco-

TABLE I: Carbohydrate Recovery during Purification.

Fraction	% Total Recov[a]
Gel filtration I	100
Gel filtration II	100
Peak IV	70–80
Peak IV-A	38–45
Peak IV-B	17–20
Peak IV-C	11–15

[a] Recoveries are based on the carbohydrate content of fibrinogen.

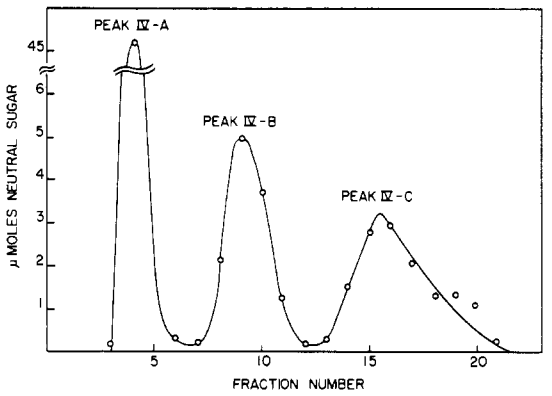

FIGURE 2: Fractionation of peak IV on Dowex 1 (formate). The figure shows one of several elution patterns obtained. In other runs, performed with higher initial loadings of glycopeptides for use in structural analyses, poorer separations were achieved as indicated by higher base lines between peaks. Recovery data was based on analyses of the pooled fractions.

peptides IV-A–C are shown in Table II.[2] Although peaks I–III and IV-D appear to be heterogeneous, as judged by their amino acid composition, they contain only 20–30% of the carbohydrate of fibrinogen,

TABLE II: Amino Acid Composition of Fibrinogen Glycopeptides.[a]

	Peak		
	IV-A	IV-B	IV-C
Lysine	1.02	0.06	0.05
Arginine	0.03	0.97	0.98
Aspartic acid	1.00	1.00	1.00
Glutamic acid	0	0.97	0.99
Glycine	0	0.74	0
Glucosamine	2.17	2.43	2.48

[a] The amino acid residues are expressed as molar ratios relative to aspartic acid.

and were not studied further. The lower value for glycine shown in Table II reflects a contamination of peak IV-B with peak IV-C, resulting in an enrichment of arginine, aspartic acid, and glutamic acid in this fraction.

[2] No distinction is made in this paper between aspartic acid and asparagine, or between glutamic acid and glutamine.

Identification of N-Terminal Amino Acids. The results of the N-terminal analyses of the three major glycopeptide fractions are shown in Table III. Quantitation of results necessitated chromatography, elution, and absorbance measurements of the DNP-glycopeptides as well as of the DNP-amino acids of the hydrolyzed derivatives. Since the absorbances were often 0.05 or less, experimental errors may be expected in addition to losses incurred by hydrolyses; in particular, "recoveries" in some cases were greater than 100%, reflecting a difficulty in the quantitative elution of the DNP-glycopeptide. However, no DNP-amino acids other than those reported in the table were observed, and the results clearly identify the N-terminal amino acids, namely, aspartic acid, glycine, and glutamic acid for peaks IV-A–IV-C, respectively.

In earlier experiments, DNP derivatives of peak IV-B were hydrolyzed in 6 N HCl for 16 hr at 105°, and both DNP-glycine and DNP-glutamic acid were obtained in nearly equal amounts, with poor total recovery. It was inferred that DNP-glycine was the predominant N-terminal amino acid, a conclusion which was readily confirmed by the 2-hr, 105° formic acid–glacial acetic acid–perchloric acid hydrolysis to minimize destruction of DNP-glycine. The presence of DNP-glutamic acid (~15–20%) was reproducibly demonstrated and was due to an incomplete separation of peaks IV-B and C, as was also suggested by the amino acid analyses. The DNP analysis of peak IV-A demonstrated the essential equivalence of DNP-aspartic acid and ε-DNP-lysine, establishing that the linkage of the carbohydrate must be to the β-carboxyl group of aspartic acid (or its amide).

Identification of C-Terminal Amino Acids. The glycopeptides were subjected to high-voltage paper electrophoresis (9500 v, 1.5 hr, 45°, 7% formic acid as buffer) with and without incubation with COP-B. The presence of arginine as the C-terminal amino acid of peaks IV-B and C was shown by the appearance of free arginine and the marked decrease in the relative mobilities of the glycopeptides after incubation with COP-B. The conversions appeared to be essentially quantitative.

Only slight release of lysine from peak IV-A was observed, although lysine is known to be C-terminal here. This nonrelease may be due to the interference of the carbohydrate chain or of the adjacent aspartic acid, preventing the action of COP-B. The results of the N-terminal analyses and the COP-B reaction established the sequence of peak IV-C to be Glu-Asp-Arg and peak IV-B to be Gly-(Glu, Asp)-Arg.

The Sequence of Peak IV-B. The internal sequence of peak IV-B was established by determining the composition of a DNP peptide obtained from partial acid hydrolysis of the DNP derivative of peak IV-B. After ether extraction the partial hydrolysate was resolved into three yellow "spots" on tlc (in addition to the dinitrophenol at the solvent front) using the chloroform–benzyl alcohol–acetic acid solvent. Spot 1 remained at the origin and was shown to contain all of the peak IV-B amino acids plus glucosamine by total

TABLE III: DNP Derivatives of Peaks IV-A–C.

Peak	mμmoles DNP-Peptide	DNP-Glu	DNP-Asp	DNP-Gly	ε-DNP-Lys	% Recov
IV-A	7.3		4		4	55, 55[a]
	2.7		8		5.3	...
	4.8		3.6		0.8	75, 17[a]
IV-B	11			6.2		56
	7.2			2.8		39
	11	1.8		8.7		79, 16[b]
IV-C	8.1	6.0				74
	7.6	4.2				55
	2.0	3.3				

[a] Recovery of ε-DNP-lysine. [b] Recovery of DNP-glutamic acid.

hydrolysis and high-voltage electrophoresis. Spot 2 moved just ahead of a DNP-aspartic acid standard upon total hydrolysis and yielded glutamic acid as the only amino acid. The third spot was presumed to be DNP-glycine since it cochromatographed with standard DNP-glycine and yielded no significant amount of amino acids upon hydrolysis. In all of the electrophoresis experiments traces of leucine, valine, serine, and glycine could be detected. Electrophoresis of blank elutions showed them to be contaminants from the Kodak silica gel tlc sheets.

The identity of the amino acid adjacent to the N-terminal end was inferred to be glutamic acid from the results of the digestion of spot 2. Control experiments showed that under identical conditions of hydrolysis, neither DNP-glycine nor DNP-glutamic acid gave rise to the free amino acid in quantities sufficient to detect on the electropherogram with ninhydrin. Hence, spot 2 of the ether-extracted partial hydrolysates must have been DNP-Gly-Glu. These data when combined with the N- and C-terminal analyses given above establish that the amino acid sequence of peak IV-B is Gly-Glu-Asp-Arg.

TABLE IV: Amino Acid Sequences of Fibrinogen Glycopeptides.

Peak	Amino Acid Sequence
IV-A	H_2N-Asp-Lys-COOH | CHO
IV-B[a]	H_2N-Gly-Glu-Asp-Arg-COOH | (CHO)
IV-C[a]	H_2N-Glu-Asp-Arg-COOH | (CHO)

[a] The position of carbohydrate attachment has not been established.

Discussion

The glycopeptides obtained by Pronase digestion of fibrinogen were shown to contain the amino acid sequences summarized in Table IV. Peaks IV-A and C were homogeneous by all criteria applied (*i.e.*, amino acid analyses, DNP analyses, electrophoresis, COP-B digestion, and ion-exchange chromatography). However, peak IV-B contained about a 20% contamination with peak IV-C (see legend to Figure 2). This conclusion seems the most reasonable one based on both the N-terminal analyses (*i.e.*, some DNP-glutamic acid in addition to the major component, DNP-glycine) and the amino acid composition (*i.e.*, the results represent a mixture of two glycopeptides, 75% of which is one containing arginine, aspartic acid, glutamic acid, and glycine, and 25% an identical composition, except for glycine, which is absent).

Previous attempts to isolate "pure" Pronase glycopeptides from fibrinogen, in other laboratories as well as our own, were not successful (in terms of obtaining unit molar ratios of amino acids). We feel that the sialic acid "stripping" following the first Pronase digestion may be responsible for the homogeneity of the limit Pronase digest. We cannot say whether fractions IV-C and B (differing only in N-terminal glycine) arise from different areas of the fibrinogen molecule, or represent heterogeneous end products of an originally homogeneous population of sites. It is also possible that the minor fractions, peaks I–III and IV-D, arose as an artifact of Pronase digestion. The release by mild acid hydrolysis of 1 mole of NH_3/mole of glycopeptide IV-A (unpublished results) shows that the attachment of the carbohydrate in this fraction is through amide linkage to asparagine.

Although we have not quantitated the carbohydrate composition of our glycopeptides, Mester *et al.* (1965) have suggested that two types of carbohydrate chains are present in fibrin, differing only in that they contain one or two sialic acid residues. Since the sialic acid was removed in order to obtain our product, no comparison here is possible. Comparison of our amino acid analyses with those reported by Mester *et al.* (1963b) lead to ambiguities not readily interpretable until more of the sequences around the glycopeptides are established.

Problems also arise in attempting to correlate the present study with prior estimates on the number of glycopeptide chains in fibrinogen. As has been pointed out above, peaks IV-B and -C may arise from the same site and we have not yet ascertained the origin of 20% of the glycopeptides obtained (*i.e.*, the minor carbohydrate components may have a common origin with peaks IV-A–IV-C or represent a different site(s)); also, peak IV-A might be derived from more than one site on the fibrinogen molecule. Additional complications might result from molecular heterogeneity of the original fibrinogen from pooled plasma.

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