Deglycosylation of Fibrinogen Accelerates Polymerization and Increases Lateral Aggregation of Fibrin Fibers

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Fibrinogen, the major structural precursor of blood clots, was deglycosylated by peptide-N-(N-acetyl-β-glucosaminy1)asparagine amidase without denaturation of the polypeptide chains. Deglycosylated fibrinogen behaved normally in clinical coagulation assays, although it is less soluble than normal fibrinogen. However, the turbidity of clots formed from deglycosylated fibrinogen always rose faster and higher than that of clots from normal fibrinogen. Scanning and transmission electron microscopy demonstrated that fibrin made from clots of deglycosylated fibrinogen consisted of thicker, less-branched fiber bundles in a more porous network. Moreover, the degree of lateral aggregation was directly related to clot turbidity and inversely related to branching. Deglycosylation promoted turbidity development, lateral aggregation, and porosity of clots under all conditions tested. All other steps in the coagulation pathways appeared to be unaffected by the absence of carbohydrate. These results suggest that carbohydrate constitutively affects the behavior of deglycosylated fibrinogens by 1) contributing a repulsive force that promotes fibrinogen solubility and limits fibrin assembly and 2) sensitizing fibrin to conditions that influence assembly and clot structure.

Fibrinogen is transformed during blood coagulation into fibrin monomers that spontaneously polymerize into an insoluble network of fibers or clot. The structural and mechanical properties of clots are determined by experimental conditions impinging on particular sites on this complex molecule (Ferry and Morrison, 1947; Blomback and Okada, 1982; Privalov and Medved, 1982; Weisel et al., 1985; Galanakis et al., 1987). Fibrinogen is a glycoprotein composed of three pairs of nonidentical chains (AαBβγ)3 with molecular masses of 63.5, 56, and 47 kDa, respectively (McKee et al., 1966). The carbohydrate consists of Sia, Gal, Man, and GlcNAc (Blomback, 1972), and occurs as a complex, biantennary oligosaccharide (2404 Da) (Townsend et al., 1982) N-linked to Asn-364 of each Bβ (Topfer-Petersen and Henschen, 1977; Watt et al., 1979) and to Asn-52 of each γ chain (Iwanaga et al., 1968). The two glycosylation sites on the Aα chain do not contain carbohydrate (Gaffney, 1972) because they are blocked by pro residues (Henschen et al., 1983).

Results of previous studies in which the role of carbohydrate in the function of fibrinogen was investigated did not distinguish the effects of carbohydrate from those of the protein backbone. Fibrinogens were compared which may have differed in their protein backbone (Gati and Straub, 1978; Witt and Hasler, 1972; Galanakis et al., 1983) and/or in their content of carbohydrate; in no instance were both properties fully characterized. Nevertheless, these fibrinogens generally coagulated at different rates and produced clots which differed in their structural and mechanical properties (Burstein et al., 1954). In comparison to clots formed from the glycosylated (G) fibrinogen of normal adults, clots from the hyperglycosylated fibrinogens (present during fetal life and as congenital or dysfibrinogens) assembled more slowly and developed less turbidity (Tesch et al., 1979; Müller et al., 1981; Guillain and Menache, 1973; Galanakis et al., 1983; Galanakis and Mosesson, 1976). Partial (Gralnick et al., 1978; Galanakis et al., 1983; Martinez et al., 1983) or complete (Martinez et al., 1978) desialation of hyperglycosylated (Martinez et al., 1983) and normal (Martinez et al., 1977; Park et al., 1986) adult fibrinogen generally resulted in faster coagulation and in a higher level of turbidity. However, in seeming contradiction of these results, when either Sia or 40% (Nishibe and Takahashi, 1981) or 80% (Gilman et al., 1984; Barsigian et al., 1986) of total carbohydrate content was removed, then the hypoglycosylated fibrinogen coagulated at a normal rate or retained normal catabolic properties, respectively. While these last few studies accomplished the technical feat of generating hypoglycosylated fibrinogens, their ability to distinguish the effect of the absence of carbohydrate from the effect of the polypeptide backbone on the function of fibrinogen was precluded for two reasons: Hypoglycosylated fibrinogens were not generated in sufficient amounts, and most articles were transformed during blood coagulation into fibrin monomers that spontaneously polymerize into an insoluble network of fibers or clot. The structural and mechanical properties of clots are determined by experimental conditions impinging on particular sites on this complex molecule (Ferry and Morrison, 1947; Blomback and Okada, 1982; Privalov and Medved, 1982; Weisel et al., 1985; Galanakis et al., 1987). Fibrinogen is a glycoprotein composed of three pairs of nonidentical chains (AαBβγ)3 with molecular masses of 63.5, 56, and 47 kDa, respectively (McKee et al., 1966). The carbohydrate consists of Sia, Gal, Man, and GlcNAc (Blomback, 1972), and occurs as a complex, biantennary oligosaccharide (2404 Da) (Townsend et al., 1982) N-linked to Asn-364 of each Bβ (Topfer-Petersen and Henschen, 1977; Watt et al., 1979) and to Asn-52 of each γ chain (Iwanaga et al., 1968). The two glycosylation sites on the Aα chain do not contain carbohydrate (Gaffney, 1972) because they are blocked by pro residues (Henschen et al., 1983).

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significantly, the requisite removal of all carbohydrate (complete deglycosylation) was not achieved.

In this investigation, fibrinogen that had been completely deglycosylated by peptide-N-\(-\text{N-acetyl-}\beta\)-glucosaminyl)asparagine amidase (PNG-Asn amidase) was examined to determine the effect of the absence of carbohydrate on coagulation. When PNG-Asn amidase, which has been freed of contaminating proteases by HPLC (Langer et al., 1987), was used in stoichiometric amounts under nondenaturing conditions, the reaction proceeded to completion rather than yielding hypoglycosylated fibrinogen. Differences in coagulation between deglycosylated (DG) and G fibrinogens were easily measured due to the availability of large amounts (hundreds of mg) of DG fibrinogen and were attributable to the absence of carbohydrate on DG fibrinogen. The results of the present experiments demonstrate that deglycosylation decreased the solubility of fibrinogen and affected fibrin assembly by accelerating polymerization, increasing lateral aggregation, and decreasing branching, but did not influence other steps in the coagulation pathways. This is the first case in which the behavior of a DG coagulation factor has been characterized and demonstrates the utility of HPLC-purified PNG-Asn amidase for deglycosylating protease-sensitive glycoproteins under nondenaturing conditions.

**EXPERIMENTAL PROCEDURES**

Fibrinogen-Fibrinogen was purified (Kazal et al., 1985) from plasma obtained from normal donors 1 and 2, from patients very severely deficient in FXIII, and afibrinogenemic patients who had given their informed consent. Normal fibrinogen contained sufficient endogenous FXIII to cross-link all of the y and half of the a chains when activated by thrombin and 2.5 mM CaCl₂ (Table I). Fibrinogen purified from plasma very severely deficient in FXIII had only a trace of cross-linking activity (Table I).

Deglycosylation of Fibrinogen under Native Conditions—Preparation of protease-free PNG-Asn amidase is described elsewhere (Langer et al., 1987). Deglycosylation of native, G fibrinogen by PNG-Asn amidase was performed in Tris buffer (20 mM Tris, 0.1 M NaCl, 5 mM EDTA, 0.02% NaN₃, pH 7.1) or Hepes buffer (20 mM Hepes, 0.13 M NaCl, 1 mM EDTA, 0.02% NaN₃, pH 7.7) for 3–24 h at 37–39 °C over a range of molar ratios of PNG-Asn amidase:fibrinogen from 1:1 to 1:20. The enzyme was omitted from control, for each of these steps. Subsequently, fibrinogen was concentrated by affinity chromatography on a 4°C, separated from the enzyme over a 1.5 × 100-cm column of S-200 (Pharmacia, LKB Biochemicals Inc.) equilibrated at pH 7.3 without EDTA in modified Tris (also containing 0.14 M NaCl) or modified Hepes buffer, and stored at 4 or 70 °C until use. Because of its significantly lower solubility and predisposition to aggregation, the recovery of DG fibrinogen was generally lower than that of G fibrinogen.

Analytical Methods—Protein concentrations were determined spectrophotometrically with the following values of E₁⁰₀₀ of fibrinogen, 15.1; FXIII, 13.4; PNG-Asn amidase, 10.0. Concentrations and pH values are indicated at their final levels in the figures and tables. Sample buffer for SDS-PAGE (Laemmli, 1970) was supplemented with 9 M urea and/or reducing agents as indicated. Electrophoretic slabs of SDS gels (Towbin et al., 1979) were overlaid with 125I-concanavalin A (125I-ConA), washed, and subjected to autoradiography for 1–24 h. Carbohydrate was released from fibrinogen and decoratetylated by acid hydrolysis and its glucN content measured by the method of Elson and Morgan (1953). A standard curve of increasing GlCN content corresponding to increasing amounts of G fibrinogen was generated, assuming 16 mol of GlCN/mol of G fibrinogen. Absorbance readings for increasing amounts of DG fibrinogen were plotted assuming the same extinction coefficients. Release of fibrinopeptides from fibrinogen by thrombin was determined by HPLC (Ebert and Bell, 1985). DG and G fibrinogen (2 mg/ml) were partially digested for 24 h with plasmin (0.17 casein units/ml; Kab) in 50 mM Tris, 0.15 M NaCl, pH 7.2, supplemented with either 10 mM EGTA or 5 mM CaCl₂. The reaction was stopped by boiling in nonreducing sample buffer. Plasmin was omitted from controls. All clinical assays were carried out in plasma reconstituted with purified DG and G fibrinogens and modified Tris buffer essentially as described previously (Ebert and Bell, 1983). These assays included: monitoring of the extrinsic and intrinsic coagulation pathways by the PT (one-stage prothrombin time) and PTT (partial thromboplastin time), respectively; individual determinations of factors V, VIII:C, IX, and X; and Clauss (Clauss) and immuno-electrophoretic (anti-F) determinations of fibrinogen.

**Turbidity Measurements**—Fibrinogen samples (0.625 mg/ml in modified Tris or Hepes buffer with 2.5 mM CaCl₂ or 7.5 mM NaCl, ±0.6 μg/ml exogenous FXIII, and ±0.1 M A huh were equilibrated for 10 min at 33 °C in quartz cuvettes in a Varian DMS 90 spectrophotometer. The A₅₀₀ of the samples was adjusted to 0 and fresh thrombin was rapidly added at time 0 to a final concentration of 1 or 1.25 units/ml. The A₅₀₀ was recorded at 30-s intervals until it stabilized, which was often within 120 min. After reaching A₅₀₀, clots were synergized by blotting on Whatman No. 1 filter paper. Each key, compacted fibrin clot was boiled for 5 min in sample buffer containing 5 M urea and reducing agents and run on 10% SDS-PAGE. The amount of γ dimer and α multimer was estimated visually on stained gels according to a predetermined scale (Table I). TEM and SEM.—In so far as possible, clots were prepared for electron microscopy in the same way and with the same protein preparations and reagents that were used for turbidity measurements and other biochemical experiments. Methods used to prepare clots for TEM were described previously (Weisel, 1987). Dispersal of clots just after the gelation point usually yielded the best images of fibrin fibers, which were aligned parallel to the direction of flow for measurement of the maximum fiber diameter. Fibrin fibers on 300 or 400 mesh carbon-coated Formvar grids were negatively contrasted with 1% uranyl acetate and examined in a Phillips 400 electron microscope outfitted with a low dose kit. The microscope magnifications were calibrated with negatively contrasted tropomyosin, which has a repeat of 39.5 nm.

Specimens for SEM were prepared in plexiglass microdissection cells performed for solvent perfusion. Clots were formed under the conditions indicated and allowed to stand ~20 times the gelation time to assure that fiber assembly was complete. Specimens were washed three times with phosphate buffer, air-dried for 10 min, fixed for 30 min in 2% glutaraldehyde, and rinsed three times. Samples were post-stained for 15 min with 1% osmium tetroxide, rinsed, and dehydrated in a graded series of ethanol concentrations through 100% over a period of 1.5 h. The clots were critical point-dried with CO₂ for ~45 min in a Denton apparatus, mounted, and finally coated with ~12.5 nm of gold-palladium prior to examination in an AMRAY 1400 scanning electron microscope.

**RESULTS**

Fibrinogen Is Carbohydrate-free after PNG-Asn Amidase Treatment—Deglycosylation of normal, native fibrinogen by PNG-Asn amidase was assessed in three independent assays. First, as shown in Fig. 1A, the relative molecular mass of the Bβ and γ chains decreased approximately 2400 each, which in turn corresponds to the relative molecular mass of the released oligosaccharides; the masses of the Aα chain and its normal fragments (Galanakis and Mosesson, 1983) were unchanged. These results indicate, moreover, that no proteolysis of fibrinogen occurred as a consequence of treatment with PNG-Asn amidase. Second, as shown in Fig. 1B, reactivity of the two G chains to 125I-ConA decreased after treatment with increasing concentrations of PNG-Asn amidase. Complete elimination of reactivity occurred at approximately a 10-fold lower enzyme concentration for the Bβ than for the γ chain. With or without enzyme treatment, the Aα chain was unreactive with the lectin. Third, GlCN content was undetectable in enzyme-treated protein (Fig. 1C) but was approximated by proportional to protein concentration for G fibrinogen. PNG-Asn amidase is often contaminated with another glycosidase, endo-β-N-acetylglucosaminidase F, which leaves stubs of GlcNAc remaining on the protein backbone. However, PNG-Asn amidase purified by HPLC is free of other glycosides as well as protease. Thus, fibrinogen treated with this PNG-Asn amidase did not retain...
any stubs of Man or chitobiose cores from its oligosaccharides, and was therefore regarded as DG. DG fibrinogen was less soluble than G fibrinogen; i.e., the minimum ionic strength and/or temperature necessary to maintain solubility and counteract self-association (aggregation) was greater for DG than for G fibrinogen. Conversely, for any given set of conditions, a lower concentration of DG than G fibrinogen would remain soluble.

**Fibrinogen Activation, Fibrin Stabilization, and Lysis by Plasmin Are Unaffected by Removal of Carbohydrate**—To determine whether these reactions are influenced by carbohydrate, DG and G fibrinogens were compared as substrates for the enzymes involved in each step. The rate and extent of release of fibrinopeptides A, AP, B, and Bα-14 catalyzed by thrombin were identical for DG and G fibrinogens (Fig. 2A). In the presence of either endogenous, exogenous, endogenous and exogenous, or no FXIII, fully coagulated DG and G fibrinogens contained essentially the same amounts of γ dimer or αA tetramer (Table I). Cross-linking did not occur in the absence of either FXIII, Ca²⁺, or thrombin. Partial digestion by plasmin generated the same fragments from DG and G fibrinogens, except that the DG migrated slightly more rapidly than the G fragments due to the absence of their carbohydrate (Fig. 2B). Calcium ions protected both fibrinogens identically from proteolysis. Thus these three coagulation steps are not affected by the removal of carbohydrate from fibrinogen.

**Coagulation Pathways Preceding Fibrinogen Activation Are Unaffected by Removal of Carbohydrate**—While neither fibrinogen nor its carbohydrate are known to influence the steps in the coagulation pathways preceding release of the fibrinopeptides, these possibilities were examined as follows. Plasma from a patient with a severe deficiency of fibrinogen was reconstituted with DG or G fibrinogen and then assayed in routine clinical tests for the extrinsic and intrinsic coagulation pathways. Both fibrinogens completed a clotting cascade which functioned within normal limits free of any detectable deficiency, inhibition, or other abnormal coagulation (Table II). In the absence of added fibrinogen, the afibrinogenemic plasma was essentially unclottable even though it contained normal amounts of functional factors VII, IX, and VII:C. Thus, by these methods, removal of carbohydrate does not appear to affect any step in the clotting cascade, and the aglycan may be considered undenatured.

**Removal of Carbohydrate from Fibrinogen Increases Turbidity Development of Fibrin Clots**—In striking contrast to the functional similarities between DG and G fibrinogens described so far, two differences in turbidity development occurred between them (Fig. 3 and Tables III and IV). The rate (the inverse of t₀₅₀, the time to reach half-maximal turbidity) was always greater (1.5- to 60-fold, depending on conditions) (Table III and Fig. 3), and the maximum extent (Aₙ₅₀) was always greater (1.27- to 65-fold, depending on conditions) (Table IV and Fig. 3) for DG than G fibrinogen. Thus, the effects of the absence of carbohydrate on turbidity development were qualitatively the same under all conditions tested: The rate and extent were always greater than in the presence of carbohydrate. SDS-PAGE revealed that all three polypeptide chains were intact in clots formed from DG and G fibrinogens (gels not shown).

The following conditions favored the rapid development of high turbidity of G fibrin, but had little effect on the turbidity development of DG fibrin, which was rapid and extensive regardless of conditions (Tables III and IV): the absence of Ahx, the absence of exogenous FXIII (endogenous
by absorbance at 210 nm. Fibrinopeptides were not released in and plasmin. Fibrinopeptides were separated by reverse-phase HPLC and quantitated control reactions run in the presence of acid. The reactions were stopped with H3PO4; the released fibrinogen was reacted with thrombin for various times up to 60 min. The positive effects of these conditions on turbidity of fibrin assembly became Ca2+. The locations of fibrinogen, fragments X (M, 250,000), D (M, 100,000, 82,000, and 78,000), and E (M, 50,000) and M, standards (myosin, β-galactosidase, phosphorylase b, bovine serum albumin, and ovalbumin) are indicated.

Fibrinogen was reacted with plasmin supplemented with either 10 mM EGTA or 5 mM CaCl2 and then analyzed by 7.5% SDS-PAGE under nonreducing conditions. Lane 1, G fibrinogen control; lane 2, G fibrinogen + plasmin + EGTA; lane 3, G fibrinogen + plasmin + Ca2+; lane 4, DG fibrinogen control; lane 5, DG fibrinogen + plasmin + EGTA; and lane 6, DG fibrinogen + plasmin + Ca2+. The locations of fibrinogen, fragments X (M, 250,000), D (M, 100,000, 82,000, and 78,000), and E (M, 50,000) and M, standards (myosin, β-galactosidase, phosphorylase b, bovine serum albumin, and ovalbumin) are indicated.

 Qualitatively insensitive to these conditions after deglycosylation, the degree of insensitivity varied considerably with fibrinogens purified from different donors and with different buffers: all other conditions being the same, DG fibrinogen from donor 1 in Tris buffer (Fig. 3) was more insensitive than DG fibrinogen from donor 2 in Hepes buffer (Tables III and IV).2

As first observed by Ferry and Morrison (1947), the ease with which clots at Amax underwent syneresis was directly related to the level of Amax; the higher the level, the easier the compaction into a minute, rubbery ball and resulting expulsion of liquid. Clots with lower levels of Amax were more gelatinous and required prolonged blotting for complete syneresis, while those with the lowest levels were so friable that they were barely recoverable after blotting.

Removal of Carbohydrate from Fibrinogen Increases Lateral Aggregation and Decreases Branching of Fibrin Fibers—To determine the structural basis which underlies the difference in Amax between clots from G and DG fibrinogens, clots comparable to those in Fig. 3 were examined by SEM and TEM. While the hydrated, three-dimensional arrangement of fibers is preserved by preparation for SEM, TEM is better suited for determining the detailed features of individual fibers and their aggregation to form fiber bundles.

It was determined from SEM that clots formed from DG fibrinogen (Fig. 4A) were more porous, being composed of thicker, less branched fiber bundles with larger open spaces between them than clots of G fibrinogen (Fig. 4B). Negatively contrasted specimens were examined by TEM. The band pattern and periodicity (22.5 nm) of fibrin were identical for DG (Fig. 4C) and G (Fig. 4D) fibers. Both were highly ordered along the fiber axis. However, the DG fibers were more highly ordered in the lateral direction (Fig. 4C, inset). They aggregated with each other laterally to a much greater extent, forming thick and usually straight bundles, somewhat similar to those observed with G fibrin at subphysiological salt concentration and pH (Weisel, 1986).

2 This difference in the degree of the effect of these conditions on the growth of fibrin fibers was confirmed by comparing the diameter of fibers measured directly by electron microscopy with values calculated from turbidity analysis. Measured and calculated values were in close agreement under each condition (Langer and Weisel, unpublished observation).
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Coagulation studies of plasmas reconstituted with DG and G fibrinogens

| Fi, fibrinogen; aFi, afibrinogenemic. |
|------------------|------------------|------------------|
| s                | s/ %             | mg %            |
| Normal plasma    | 17/65/100        | 170             |
| Normal plasma + DG Fi | 18/58/74        | 285             |
| Normal plasma + G Fi | 19/61/100       | 240             |
| aFi plasma       | 167/30/60        | <10             |
| aFi plasma + G Fi | 18/90/66         | 120             |
| aFi plasma + G Fi | 18/59/63         | 105             |
| Normal range     | 15-23/60-100     | >60             |

FIG. 3. Development of turbidity during coagulation of DG and G fibrinogens. DG and G fibrinogens at 0.625 mg/ml were clotted with 1 unit/ml thrombin in 20 mM Tris, 0.14 mM NaCl, 2.5 mM CaCl₂, 0.02% NaN₃, pH 7.3, and the absorbance change with time monitored at 350 nm. For simplicity, A₃60 is expressed rather than the turbidity, which equals \( \frac{A_{360}}{1 \text{ cm}} \times 2.303 \). Coagulation of new evidence (Table II and see below). These changes are unlikely to account for the effects of deglycosylation, since similar effects result from desialation alone (Martinez et al., 1983; Martinez et al., 1977; Park et al., 1986). There is no specific reversion of these residues into Asn to assess their possible influence on assembly of DG fibrin (not technically feasible). Aside from this single type of modification, the polypeptide backbone of fibrinogen was apparently unaffected by deglycosylation, and the polypeptide chains of fibrin appeared to remain intact in all clots at the completion of coagulation. This conclusion is consistent with the hypothesis that, in situ, carbohydrate constructively increases solubility of G and hyperglycosylated fibrinogens and limits the assembly and porosity of clots of these fibrins. This hypothesis gains its credence from the mounting, indirect evidence with which it is consistent, and as previous findings which had seemed to be contradictory (Nishibe and Takahashi, 1981; Gilman et al., 1984; Barsigian et al., 1986) are reinterpreted in light of new evidence (Table II and see below).

DISCUSSION

The behavior of DG fibrinogen was characterized. In the absence of carbohydrate, the solubility of fibrinogen decreased, clots synerized more readily, the rate and extent of development of fibrin turbidity increased, but other coagulation steps were unaffected. The effect of the absence of carbohydrate on the rate of assembly was easily seen in kinetic profiles of turbidity development. The effect on the extent of turbidity development was reflected in the maximum turbidity of the kinetic profiles and in the direct correspondence of this parameter with the thickness of fiber bundles in clots as determined by SEM and TEM. A similar correspondence was also observed for clots formed under the conditions in Table IV. Moreover, clots of DG fibrinogen were more porous, containing fiber bundles that were less branched than G fibrin. The differences in solubility, syneresis, turbidity development, lateral aggregation, and clot porosity between DG and G fibrinogens can be attributed to the absence of carbohydrate on DG fibrinogen. Although Asn in converted to Asp at former sites of glycosylation (Trimbel et al., 1986), these changes are unlikely to account for the effects of deglycosylation, since similar effects result from desialation alone (Martinez et al., 1983; Martinez et al., 1977; Park et al., 1986). In any case, specific reversion of these residues into Asn to assess their possible influence on assembly of DG fibrin (not technically feasible). Aside from this single type of modification, the polypeptide backbone of fibrinogen was apparently unaffected by deglycosylation, and the polypeptide chains of fibrin appeared to remain intact in all clots at the completion of coagulation. This conclusion is consistent with the hypothesis that, in situ, carbohydrate constructively increases solubility of G and hyperglycosylated fibrinogens and limits the assembly and porosity of clots of these fibrins. This hypothesis gains its credence from the mounting, indirect evidence with which it is consistent, and as previous findings which had seemed to be contradictory (Nishibe and Takahashi, 1981; Gilman et al., 1984; Barsigian et al., 1986) are reinterpreted in light of new evidence (Table II and see below).

A possible relationship between carbohydrate content and clot development has been suggested previously (Martinez et al., 1977; Müller et al., 1981; Martinez et al., 1983), but other results, which failed to demonstrate an effect of carbohydrate, cast doubt on its general significance (Gilman et al., 1984; Nishibe and Takahashi, 1981; Barsigian et al., 1986). These apparently contradictory findings have been resolved and accounted for here. In agreement with previous work, carbohydrate influences neither activation, ligation, and lysis of fibrinogen (Fig. 2 and Table I) (Paisack and Martinez, 1977; Galanakis et al., 1983; Martinez et al., 1977; Park et al., 1986; Barsigian et al., 1986; Gilman et al., 1984) nor other nonassembly steps in the coagulation pathways (Table II). Thus, the influence of the partial (Nishibe and Takahashi, 1981) or complete removal (Table II) of carbohydrate on fibrin assembly is not necessarily detectable by the clinical assays, but it is obvious in the gross appearance,
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TABLE IV
Comparison of the maximum turbidity from clots of DG and G fibrinogens

DG and G fibrinogens at 0.625 mg/ml with or without endogenous fXIII were clotted with 1.25 units/ml thrombin at 33 °C in 20 mM Hepes, pH 7.4, 0.13 M NaCl, 0.02% NaN₃ ± exogenous fXIII at 60 μg/ml, and ± εAhx at 0.1 M. Each reaction mixture was supplemented with either 2.5 mM CaCl₂ or 7.5 mM NaCl. A₃₅₀ was recorded at 20.5-min intervals and the maximum level of each kinetic profile tabulated from the average of duplicate measurements. For simplicity, the maximum A₃₅₀ is expressed rather than the maximum turbidity, which = A₃₅₀/1 cm × 2.303. For each set of conditions, the effect of the absence of carbohydrate on the final turbidity is expressed as the ratio of the A₃₅₀ of DG and G fibrinogens.

<table>
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<tr>
<th></th>
<th>Ca²⁺</th>
<th>Na⁺</th>
<th>Ca²⁺ DG/G</th>
<th>Na⁺ DG/G</th>
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<tr>
<td>Endogenous fXIII</td>
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<td>1.32</td>
<td>1.34</td>
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<td>Endogenous fXIII + exogenous fXIII</td>
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<td>0.70 G</td>
<td>1.80</td>
<td>0.76</td>
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<tr>
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<td>0.78 G</td>
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<td>0.02</td>
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<td>0.84 G</td>
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<td>0.64</td>
</tr>
<tr>
<td>No endogenous fXIII + exogenous fXIII + εAhx</td>
<td>1.55 G</td>
<td>0.09* G</td>
<td>1.29</td>
<td>0.23*</td>
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*a* Readings made after coagulation for ~16 h.

Fig. 4. Electron microscopy of fully coagulated DG and G fibrinogens. Clots were formed under the same conditions as in Fig. 3 and then processed for SEM and TEM. 

- Visualized by SEM of clots of DG (A) and G (B) fibrins. 
- Visualized by TEM of clots of DG (C) and G (D) fibrins. 
- Magnification of A-D, bar (shown in A), 1 μm. 
- C, Inset, high magnification of a single DG fibrin fiber showing the details of the band pattern and the extent of lateral order (bar, 0.1 μm).

syneresis properties, turbidity development, and ultrastructure of clots of DG fibrinogen (Tables III and IV and Figs. 3 and 4). In light of the insensitivity of these clinical assays, the unremarkable results from them do not necessarily exclude an effect of carbohydrate on fibrin assembly. Furthermore, previous results which correlated functional effects with the carbohydrate content of fibrinogen (Tesch et al., 1979; Müller et al., 1981; Guillin and Menache, 1973; Galanakis et al., 1983; Martinez et al., 1983; Martinez et al., 1977; Park et al., 1986; Gralnick et al., 1978; Kaudewitz and Henschen, 1986; Burstein et al., 1954) are consistent with the effects of deglycosylation observed here. In fact, all of these findings are consistent with an inverse relationship between carbohydrate content and turbidity development, as well as the hypothesis that carbohydrate constitutively slows polymerization, limits lateral aggregation, and reduces porosity of clots.

The effects of deglycosylation were observed here for the first time. Detection of these effects was not possible with previous experimental designs, which could not generate or use deglycosylated fibrinogen. Instead, hypoglycosylated fibrinogen, which retained 20-60% of normal carbohydrate content, was used in some of the earlier experiments, although it was apparently unavailable in quantities necessary for conducting most coagulation assays (Nishibe and Taka-Hashi, 1981; Gilman et al., 1984; Barsigian et al., 1986). Similarly, ~82% of total carbohydrate remained on fibrinogen after desialation (Martinez et al., 1983; Gralnick et al.,
Assuming this constraint of symmetry, many other configurations of carbohydrate content of fibrinogen as it circulates or assembles but was unaffected by endogenous FXIII. The influence of concentration, as well as the absence of exogenous FXIII, on the function of many other glycoproteins of physiological significance. The remaining discussion will relate the observed effects of the absence of carbohydrate on DG fibrinogen to fibrin assembly and to possible mechanisms which might account for these effects.

Complete removal of carbohydrate significantly affected both the rate and extent of turbidity development. These parameters correspond to two steps of fibrin assembly, protofibril formation and lateral aggregation (Hantgan and Hermans, 1979; Carr and Hermans, 1978; Medved et al., 1985). Branching, another assembly step, is not detected by turbidity development, but was directly visualized by electron microscopy. The intermolecular contacts which drive and support the first two steps may be promoted by the absence of carbohydrate, whereas deglycosylation seems to limit the contacts necessary for branching and thus favor clot porosity. Isosobrin fibrin assembly determines the mechanical properties of the clot, these are in turn determined by the absence of carbohydrate. For example, a moderate degree of lateral aggregation and porosity, which are apparently optimal for hemostasis in adults, occurs in clots formed from G fibrinogen. In contrast, extremely high or low degrees of lateral aggregation and porosity appear to contribute to the ready collapse or weakness of clots of DG or hyperglycosylated fibrinogen, respectively. Formation of normal clots also required physiological osmolarity and Ca\textsuperscript{2+} concentration, as well as the absence of exogenous FXIII, but was unaffected by endogenous FXIII. The influence which carbohydrate may have on assembly is probably established in hepatocytes upon deglycosylation of newly translated fibrinogen; there is currently no evidence for the routine regulation of coagulation by modulation of the carbohydrate content of fibrinogen as it circulates or assembles into fibrin fibers.

Carbohydrate N-linked to glycoproteins makes variable contributions to the conformation and function of the polypeptide backbone, but general rules for the mechanism of these interactions have yet to be established (Olden et al., 1985). The carbohydrate on fibrin may contribute to carbohydrate-carbohydrate and carbohydrate-protein interactions, which both occur on IgG (Parekh et al., 1985). The actual interactions would depend on the orientation of each oligosaccharide in three dimensions, which is known only in part. Oligosaccharides are situated along the molecule on the B\beta chain at the proximal end domain and on the \gamma chain in the coiled-coil at the periphery of the central domain. As a result of the half-staggered arrangement of monomers in fibers, \beta and \gamma oligosaccharides located on opposing monomers line up with each other in axial register (Weisel, 1987). However, the rotational orientation of the oligosaccharides around the molecular axis is unknown. For example, the orientation of \beta oligosaccharides is rotated by 180° in Fig. 5A relative to that in Fig. 5B. Both orientations are consistent with the 2-fold molecular symmetry axis of fibrinogen. Assuming this constraint of symmetry, many other configurations of \beta and \gamma oligosaccharides are also possible. The configurations in Fig. 5A and B have no special theoretical advantage; rather, they serve to illustrate carbohydrate-carbohydrate and carbohydrate-protein interactions, respectively. With the former interactions, \beta and \gamma oligosaccharides on opposing monomers would be contiguous with each other, whereas with the latter interactions, each oligosaccharide would be individually contiguous with the protein backbone of the opposite monomer. The former and latter configurations might enable the oligosaccharides to affect fibrin assembly jointly or separately, respectively. All of the possible configurations described here are consistent with the latest structural models of fibrinogen (Weisel et al., 1985) and carbohydrate (Carver and Brisson, 1984) and take into account the molecular dimensions of the oligosaccharides (length, 3.04-5.5 nm; height 2.4 nm; thickness 0.5 nm (Mentreuil, 1982)) and the presumed free rotation of the Asn side chains through which they are anchored.

The interactions of carbohydrate may cause molecules of fibrin or fibrinogen to be repelled, limiting their assembly and enhancing their solubility. Repulsive forces that arise from carbohydrate could be electrostatic, hydrophobic, and/or steric. For example, many investigators have suggested that interaction of negatively charged Sia with each other or with Asp or Glu could repel opposing monomers. Either interaction could account for the observation that blockage or removal of Sia promotes self-association of fibrinogen or fibrin (Diaz-Mauricio et al., 1984). However, differences in Sia content do not account for all functional discrepancies between hyperglycosylated (fetal) and G fibrinogen. The unexplained discrepancy may result from differences in either the content of neutral hexoses or in the

\[ \text{J. Carver, personal communication.} \]
protein backbone (Galanakis et al., 1983). While differences in the protein backbone have been ruled out for the fibrinogen used in the present report, it remains to be determined whether the effects of deglycosylation can be accounted for solely by the absence of Sia, or whether the absence of neutral and amino sugars also contributes to these effects.

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