Comparison of Human Plasma Fibrinogen Subfractions and Early Plasmic Fibrinogen Derivatives*

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MICHAEL W. MOSESSON, DENNIS K. GALANAKIS, AND J. S. FINLAYSON

From the State University of New York, Downstate Medical Center, Brooklyn, New York 11203 and Bureau of Biologics, Food and Drug Administration, Bethesda, Maryland 20014

SUMMARY

Human plasma fibrinogen Subfractions I-8 and I-9 have previously been shown to consist of catabolic intermediates of fibrinogen characterized by the lack of COOH-terminal portions of Aα chains. The present study was undertaken to determine the structural relationships between these catabolites and core derivatives formed in vitro by the action of plasmin. First, these fractions were compared with plasma Subfraction I-4, which is representative of "native" fibrinogen, by polyacrylamide gel electrophoresis of unreduced samples in sodium dodecyl sulfate. Fraction I-4 resolved into two major bands, designated I and II (the higher the number the greater the anodal migration and the smaller the molecular size), and a trace component (Band IIIa). Fractions I-8 and I-9 exhibited four bands (IIIa, IV, V, and VI) of which Band IIIa was the major component and VI, the least abundant.

Fractions I-8 and I-9 were also compared with subfractions isolated from stage 1 plasmic digests of Fragment I-4 (treated I-9Dn, where n = clottability of the digest from which the subfraction was obtained). An early plasmic subfraction, I-9Dn, contained Bands IIIa (major component), V, and VI plus a minor amount of Band VII; a subfraction from a more advanced stage 1 digest (I-9D50) contained a faster migrating region of one BP chain; material within Bands VI and VII. Despite the fact that Fraction I-9D50 closely resembled its plasma counterpart (I-9) in clottability (96%), electrophoretic, chromatographic, and solubility behavior, several differences were apparent. Band IV was detected only in the plasma fractions; Band VII was present only in the plasma derivatives. Each of the 10 core Aα chain remnants (via, Aα/n, Aα/n, Aα/n to Aα/10) identifiable by gel electrophoresis of reduced plasma fibrinogen subfractions can apparently be generated in vitro by plasmin digestion. However, whereas Aα/n and Aα/n were the most abundant of the small Aα/n remnants (mol. wt <40,000) in Fractions I-8 and I-9, Aα/11 predominated in I-9Dn. Moreover, Bβ chain degradation was somewhat more advanced in I-9Dn than in its plasma counterpart. The failure to demonstrate complete identity between I-9 and I-9Dn underscores the present uncertainty as to whether plasmin alone catalyzes the formation of circulating catabolites in vivo.

Experiments involving gel slicing, reduction of the eluted protein, and re-examination by gel electrophoresis, plus related electrophoretic procedures and chromatographic analyses, yielded the following results. Formation of species migrating within Bands II, IIIa, IV, and V could be accounted for by the loss of COOH-terminal portions of the Aα chains; species migrating in Band IIIa or more anodally exhibited no intact Aα chains. Formation of Band IIIb (from species like those in Band IIIa) occurs by the loss of the NH2-terminal region of one Bβ chain; material within Bands VI and VII is associated not only with the removal of this region from the other Bβ chain but also with internal cleavages such as those eventually leading to the separation of Fragments D and E.

Heterogeneity of human plasma fibrinogen manifested by differences in solubility reflects the presence of early catabolic intermediates (e.g. those occurring in Fractions I-8 and I-9) that are more soluble, have a longer thrombin clotting time, and are of lower molecular weight than the parent material (occurring in Fraction I-4) from which they are formed (1-3). This molecular weight reduction occurs by proteolytic attack on Aα chains (3, 4) resulting in release of various portions of their COOH-terminal regions (2, 3). Ten cleavage sites along the Aα chain have been identified by gel electrophoresis of fibrinogen subfractions isolated from plasma; that is, 10 or less discrete remnants, smaller than intact Aα chain but retaining an intact NH2 terminus, were demonstrable (3).

The mechanism of fibrinogen catabolism is not known with certainty; it is possible that a number of circulating proteolytic enzymes play a role. Nevertheless, much of the catabolic activity resembles that of plasmin. For example, it has been shown in vitro that the initial plasmin attack on fibrinogen occurs on Aα chains (3-8) and gives rise to derivative fractions (e.g. I-9Dn) that are similar (3, 9) to the plasma fibrinogen fractions of relatively high solubility (e.g. I-9). Furthermore, increased amounts of fibrinogen catabolites are found in the plasma of patients undergoing urokinase (9) or streptokinase (10) infusions, and similar fibrinogen derivatives can be produced in vitro by the addition of urokinase (2, 11) or streptokinase (2, 10) to plasma. It has been shown (12) that plasminogen activated
in plasma can retain a portion of its enzymic activity by virtue of the formation of a complex between plasmin and α2-macroglobulin. This finding suggests at least one possible mechanism by which plasmin or plasmin-like enzymes could express their effects in blood.

In a preceding study designed to elucidate the essential features of the fibrinogen molecule from the structures of the plasmic degradation products (13) attention was, of necessity, directed toward derivative species formed during the later stages of digestion (stages 2 and 3 in the terminology of Marder et al., Ref. 14). It was clear, however, that the degradation undergone by fibrinogen at these stages was far beyond that seen in even the most soluble plasma fibrinogen fractions (3, 13). In the present work, therefore, emphasis was placed on the detection and analysis of plasmic derivatives arising during digestive stage 1. Moreover, to determine the structural relationships between circulating catabolic fibrinogen intermediates and core derivatives formed in vitro by the action of plasmin, a detailed comparison was made between the plasma subfractions and those isolated from stage 1 plasmic digests.

**Terminology**

The terminology applied in this study is the same as that developed previously (1, 3, 9, 13) and conforms (insofar as guidelines are available) to the tentative recommendations (August 1972) of the Subcommittee on Nomenclature of the International Society on Thrombosis and Haemostasis. A pertinent summary follows.

The degradative phases of plasmin hydrolysis of fibrinogen have been divided into stages 1, 2, and 3 according to the terminology introduced by Marder et al. (14). Stage 1 is characterized by the presence of coagulable species.

Plasma fibrinogen subfractions are designated according to their relative solubilities (e.g. I-4, I-8, I-9); the higher the number, the greater the solubility. The subfractions (comprising core derivatives) isolated from stage 1 digests of Fraction I-4 (representing "native" fibrinogen) have been given designations similar to those of plasma Subfractions I-8 and I-9 (1), inasmuch as the latter consist primarily of coagulable fibrinogen catabolites (3). Like the fractions composed of naturally occurring fibrinogen catabolites, the plasma subfractions are also identifiable by their relatively high solubilities and by their delayed aggregation rates (9). Therefore, to differentiate a plasmic derivative subfraction prepared in vitro from the corresponding plasma subfraction, the suffix "D" is included in the name, and the clottability of the digest mixture from which it was isolated is shown as a superscript (i.e. I-9Dn, where n = clottability of the digest mixture).

Terminology for the plasmic core Fragments D and E identifiable in intermediate and advanced digests is the same as that proposed by Nussenzweig et al. (15). Core species larger than Fragment D, identifiable by their migration in sodium dodecyl sulfate-containing gels, have been designated as suggested by Mills and Karpatick (5), with Roman numerals corresponding to decreasing size (i.e. I, largest; II, next largest; and so forth). In a preceding study (13), only derivative Bands VII and VIII (corresponding to Fragments X and Y, respectively, in the terminology of Marder et al., Ref. 14) were analyzed. Under the electrophoretic conditions used, Band VII appeared as a single component; heterogeneity was evident in Band VIII, but resolution was poor. In the present study Band VII was resolved into at least two discrete bands, designated VI and VII, respectively.

The intact subunit chains of fibrinogen are designated Aα, Bβ, and γ. Cleavage at sites other than those attacked by thrombin is indicated by a solidus on the COOH- or NH2-terminal side. Thus Aα = Aα/+ /α; Bβ = Bβ/+ /β, and so forth. Chain fragments are further designated with a numerical subscript (3, 13, Table I); within a given set (e.g. Aα/core remnants), a higher subscript indicates a lower molecular weight.

**Materials and Methods**

**Preparation of Fibrinogen Subfractions and Derivatives**

Plasma Subfractions I-4 (clottability >98%), I-8, and I-9 (each >96% clottable) were isolated by a modification (1) of the Blomback procedure (10). Fraction I-4 was the starting material for preparation of plasmic derivative Subfraction I-9D from stage 1 digests. To obtain such subfractions, the contaminating plasminogen in I-4 was activated by addition of streptokinase (10 units per ml, final concentration) to a solution of fibrinogen under the conditions described by Sherman et al. (9). When the digestion had proceeded to the desired degree, diisopropyl phosphorofluoridate (Schwarz-Mann, Orangeburg, N.Y.) was added in the manner previously described (9, 13) for the inhibition of proteolytic activity. The mixture was then fractionated (1, 3, 9) to obtain Fraction I-9D. Increases in the clottability of Subfraction I-9D isolated at any phase of digestion could be achieved by subjecting the subfraction to the reprecipitation procedures recently reported.
PLASMA FRACTION STAGE I

WHOLE DIGEST 88%

CLOTTABLE I-9D²⁰

Fig. 1. SDS-electrophoresis of unmodified samples of plasma fibrinogen fractions and stage 1 plasmic core derivatives stained with Coomassie brilliant blue. Electrophoresis was carried out at 6 mA per tube for 10 hours in 0.2% gels. Bands IV and V, Gels 2 and 3, are difficult to appreciate as distinct bands in the photograph, although they were evident upon visual inspection.

(3), but these manipulations yielded I-9D which was more than 95% clottable only when the clottability of the digest itself was at least 88%. Similar observations were reported by Sherman et al. (9).

S-sulfo derivatives of fibrinogen or of plasmic subfractions were prepared according to the method of Pechère et al. (17).

Electrophoretic and Related Procedures—SDS-electrophoresis was performed essentially as described by Weber and Osborn (18). This technique was also used for the identification of chains retaining peptide A or B by treating with reptilase or thrombin prior to electrophoresis (3, 13). Throughout the investigation samples were examined after sulfotolysis or treatment with DTT or in unmodified form, and the concentration of acrylamide in the gels was varied, according to the requirements of a given experiment. In certain studies the gels were sliced (transversely) at 1.5-mm intervals after electrophoresis of unmodified samples. Protein was eluted from the individual slices, reduced with DTT, and re-examined by SDS-electrophoresis. This procedure, which for brevity is denoted simply “gel slicing”, has previously been described in detail (13). In the present work it permitted assessment of Ao and A0/C, chains as well as of Ao/4 remnants with molecular weight <40,000, but Ao/4 remnants of intermediate size were obscured by intact B0 or γ chains.

Chromatographic Procedures—DEAE-cellulose (Whatman DE23) gradient elution chromatography of fibrinogen or plasmic subfractions was carried out as reported elsewhere (19). CM-cellulose gradient elution chromatography in 8 M urea (Whatman CM23) for separation of S-sulfo derivative chains was carried out as recently described (3).

RESULTS

Characterization of Plasma Fibrinogen Fractions by SDS-Electrophoresis—Solubility may be regarded as an index of the average molecular size of fibrinogen catabolites present in plasma fibrinogen fractions. SDS-electrophoresis of unrediced samples (Fig. 1) provided a means for determining the distribution of molecular sizes within each fraction. Unmodified Fraction I-4 exhibited two major bands (I and II); Fractions I-8 and I-9 exhibited four other bands (IIIa to VI). Bands I and II were unique to I-4, although there was some overlap of the anodal margin of Band II with material present in I-8 or I-9. Band IIb, which was a trace component of I-4, constituted the major band of Fractions I-8 and I-9. Bands IV and V were absent from Fraction I-4 but were present as minor components of both I-8 and I-9. Band VI, containing the fastest migrating material in I-8 and I-9, appeared as a minor, diffuse zone. The relative distribution of derivative bands in I-8 and I-9 was consistent with the average molecular weights of these fractions: Band IIb, the highest molecular weight component, was more abundant in I-8; Bands IV and V (lower molecular weight) were more abundant in I-9.

The degree of intactness of Aα chains has been shown (3) to account for differences among the various plasma fibrinogen fractions (cf. Fig. 2, Gels 1 and 2). To correlate these findings with the size heterogeneity of core species observed within a given plasma fraction (Fig. 1), preparative amounts of unmodified samples were subjected to SDS-electrophoresis, followed by gel slicing as described under “Materials and Methods.” After DTT reduction, all protein slices from gels of Fraction I-4 displayed αα and A0/4 chains, whereas these chains were virtually absent from the slices of Fraction I-9 (not shown). Thus, fibrinogen species migrating in Bands I and II are characterized by the presence of αα and A0/4. Successively anodal slices from I-4 exhibited a progressively decreasing ratio of αα to A0/4. This observation, however, does not imply that the difference between Bands I and II is due solely to the content of remnant A0/4; additional differences are probably attributable to smaller A0/4 remnants present in Fraction I-4 (3). In fact, no slice from I-4 contained exclusively A0/4 or Aα. The latter finding suggested that even species migrating within Band I had under-
gone some Aα chain degradation. Similar analyses of I-9 revealed major amounts of Aα/ remnants with molecular weight <40,000 in all samples (i.e. Aα/ or smaller, Table 1). Additionally, there was an association between the anodal position of a slice and the distribution of these remnants; that is, successive gel slices showed increases in the amount of Aα/α and Aα/β relative to Aα/γ or Aα/β, although the latter two were always the most abundant. Bands I11a, IV, and V were thus characterized by high levels of small Aα/ remnants, but conclusions regarding their specific contribution to individual derivative bands could not be drawn.

**Chromatographic and Related Analyses of Unmodified and S-Sulfo Derivatives of I-9D**

In previous studies, comparisons of plasma fibrinogen fractions had included DEAE-cellulose chromatography of unmodified Fractions I-4, I-8, and I-9 (2, 3) and CM-cellulose chromatography of their S-sulfo derivatives (3). These analytical procedures were therefore employed to compare plasma fractions with an early plasmic derivative subfraction, I-9D (clottability 96%), which had previously been shown by SDS-electrophoresis of whole reduced samples (3) to be very similar to Fraction I-9 (cf. Fig. 2, Gels 2 and 3). Chromatography on DEAE-cellulose had shown (3) that the elution profile of Fraction I-9, unlike I-4 or I-8, contained a shoulder on the ascending limb of the first chromatographic peak (i.e. Peak 1). The elution pattern of I-9D (Fig. 2) obtained under the same conditions (Fig. 3) exhibited this shoulder as well as all of the major characteristics of the profile of I-9. (Consistent with the fact that separation on DEAE-cellulose occurs primarily on the basis of charge rather than molecular size, SDS-electrophoresis of reduced samples from various regions of the I-9 chromatogram provided no explanation for the observed chromatographic distribution.)

Chromatography (Fig. 4) of the S-sulfo derivative of I-9D on CM-cellulose yielded an elution profile virtually indistinguishable from that previously obtained for I-9 (cf. Ref. 3). Furthermore, SDS-electrophoresis of pooled column fractions showed that each chromatographic peak of S-sulfo I-9D contained the type of chains found in the corresponding peak of S-sulfo I-9. That is, the first major peak ("γ") contained mostly γ chains; the second ("Bβ"), mostly Bβ chains; and so forth (Fig. 4). Material eluted in the "Aα" peak is considered in a later section; that eluted in the "γ" and the "Bβ" regions is described in the following paragraph.

**SDS-electrophoresis of I-9D**

SDS-electrophoresis of the "γ" peak of S-sulfo I-9D demonstrated, in addition to intact γ chains, a band known (13) to be occupied by plasmic derivatives of Bβ chains (Bβ/α and Bβ/β, Table I) or γ chains (Bβ/β, Table I) or both. Small amounts of such a band had also been evident in electrophoretic patterns of the γ chain peaks from plasma fractions (cf. Ref. 3). Intact Bβ chains were the predominant species in the "Bβ" peak, but derivative chains of Bβ origin (Bβ/β and Bβ/α, Fig. 4, and Table 1) were present as well. The Bβ/α remnant has been detected in plasma fibrinogen fractions (i.e. remnant band 5, Ref. 3); however, the Bβ chain appears to be unique to plasmic derivative fractions.

**Identification of Plasmic Cleavage Sites on Aα Chain. Comparison of Aα Remnants Found in Plasma Fibrinogen Fractions and in I-9D**

Ten cleavage sites along the Aα chain of circulating fibrinogen have been identified; hydrolysis at these sites produces Aα/α remnants ranging in molecular weight from 15,400 (Aα/α3) to 67,300 (Aα/α) (Table 1 and Ref. 3). The similarities between Aα/α chains in plasmic derivatives and those in plasma subfractions were explored by comparing I-9D with Fractions I-4 and I-9. SDS-electrophoresis of reduced I-9D (Fig. 2, Gel S) showed, as in the case of I-9 (Gel 2), virtually no intact Aα chains or Aα/α remnants. Instead, several Aα/α remnants (identified by their increase in migration rate after treatment with reptilase or thrombin) had previously been identified in circulating fibrinogen catabolites (3). Remnant Aα/α appeared as a minor band continuous with the anodal margin of the γ chain (Fig. 2, Gel S); its presence in either I-9 or I-9D was demonstrated by treating the sample with reptilase or thrombin (not shown). Since Aα/α is detectable in I-4 only as a minor component of isolated S-sulfo Aα chain preparations (3), its identification in

1 The β derivative was initially characterized as a γ chain in Ref. 3 because of its electrophoretic migration and its failure to react with thrombin. Its subsequent identification as a Bβ chain remnant has been developed in Ref. 13.
FIG. 4. Elution pattern of S-sulfo chains of I-9D88 (39 mg) subjected to gradient elution chromatography in 8 M urea on CM-cellulose (3) (column, 0.9 X 30 cm). The point at which the gradient was begun is indicated by the vertical arrow. Each tube contained 4.5 ± 0.1 ml; pooling was as indicated by the shaded areas. Results of SDS-electrophoresis carried out under standard conditions (see legend Fig. 2) are shown in the upper portion of the figure (9% gels, stained with Coomassie brilliant blue). The samples represent pooled eluate fractions from the chromatogram of S-sulfo I-9D88 (below) or the S-sulfo Aα chain fraction from a chromatogram of S-sulfo I-4. Certain samples were treated with reptilase (R) or thrombin (T) before electrophoresis.

whole samples of I-9D88 (as well as in somewhat more advanced derivative fractions) indicated that it had been formed as a result of plasmic hydrolysis.

Electrophoretic patterns of S-sulfo Aα chain preparations (Fig. 4) suggested that remnant Aα/4 was somewhat more abundant in Fraction I-9D88 than in the parent material, I-4. This notion was confirmed by densitometry, which showed that Aα/4 amounted to 9.4% (range for three analyses, 8.2 to 10.6%) of the I-4 Aα chain population and 13.3% (range for four analyses, 13.0 to 13.6%) of that of I-9D88. These values implied that plasmin had catalyzed the formation of Aα/4 in vitro. Despite the fact that these Aα/ remnants can be produced by plasmic hydrolysis in vitro, early plasmic derivatives (viz. I-9D88) differ from Fraction I-9 in the distribution of the various Aα/ remnant chains. In I-9, the major Aα/ remnants of molecular weight <40,000 are Aα/6 and Aα/11; little or no Aα/11 is evident (Fig. 2 and Ref. 3). In sharp contrast, Aα/11 is the most abundant Aα/ remnant in I-9D88 (Figs. 2 and 4). Its distribution in the core species comprising I-9D88 was further investigated by analysis of gel slices obtained by preparative electrophoresis.
of unmodified I-9D³⁸. Although the \( \text{Aa}_{/11} \) remnant was present in all protein-containing slices, the \( [\text{Aa}_{/11} + \text{Aa}_{/11}]:[\text{Aa}_{/11} + \text{Aa}_{/11}] \) ratio, as assessed either visually or by densitometry (Table II), increased with the anodal position of the gel slice. No gel slice of I-9D³⁸ exhibited an \( [\text{Aa}_{/11} + \text{Aa}_{/11}]:[\text{Aa}_{/11} + \text{Aa}_{/11}] \) ratio as low as that of Fraction I-9 (which had almost no detectable \( \text{Aa}_{/11} \) band under these load conditions).

**Further Characterization of Stage 1 Core Derivatives by SDS-Electrophoresis**—Comparison of the starting material for plasmogen digestion, viz. Fraction I-4 (Fig. 1, Gel 1), with the unfractionated 88% clottable digest (Gel 4) showed the persistence of Band 1 in the latter. Moreover, the digest contained increased amounts of material overlapping the positions of Bands II and III, indicating that material migrating as Bands II and III has been formed from Band I during this early digestive phase. Electrophoresis of unmodified I-9D³⁸ (Fig. 1, Gel 5) yielded a band pattern similar in several respects to that of plasma Fractions I-8 and I-9. The main band corresponded to Band IIIa (Fig. 1, Gels 2 and 3), although it did overlap somewhat the position of Band IIIb (Gels 6 and 7). In addition, Fraction I-9D³⁸ exhibited three faster bands, the first two of which migrated approximately in the positions of Bands V and VI. (There was no evidence of a derivative migrating as Band IV.) The fastest band (VII) had no identifiable counterpart in the plasma fractions and appeared in minor amounts at this phase.

Experiments described above showed that the feature distinguishing derivative Band IIIa of Fraction I-9 from Bands I and II of Fraction I-4 was the degree of Aa chain fragmentation. This distinction was valid for Band IIIa of Fraction I-9D³⁸ as well since, like I-9, it had undergone little attack on chains other than Aa (Fig. 2, cf. Refs. 3 and 13). However, plasmogen derivative fractions showing appreciable degradation of other chains required additional investigation to establish the structural features of their derivative bands. For example, Fraction I-9D³⁸ (Fig. 2, Gel 4) exhibited a considerable amount of the /β₂ remnant as well as a band remnant as well as a band known from previous studies (13) to be occupied by remnants of Bβ and γ origin (viz. \( [\gamma_{/11}, \beta_{/11}, \beta_{/11}] \)). The /γ₁ remnant (migrating in the cathodal region of the γ band) was present in relatively large amounts. An additional remnant (mol wt ~ 9000) of uncertain origin (Fig. 2), unreactive with reptilase or thrombin, was also evident in I-9D³⁸. In fractions like this, the composition of derivative bands separated by SDS-electrophoresis of unmodified sample (Fig. 1) was investigated by electrophoresis of reduced protein obtained from gel slicing experiments.

In contrast to that of I-9 or I-9D³⁸, most of the Band III material from I-9D³⁸ or I-9D³⁸ was distinctly more anodal (i.e. Band IIIb, Fig. 1). Band V was no longer evident in either of the latter preparations, but there were increased amounts of Bands VI and VII. The subunit chain structure of these derivative bands, assessed as described above, indicated that the Bβ chain was enriched in gel slices taken from the cathodal region (i.e. from Band IIIb) but was virtually absent from the anodal slices (Fig. 5). Conversely, the \( [\gamma_{/11}, \beta_{/11}, \beta_{/11}] \) region was relatively abundant in anodal slices and depleted in cathodal slices. The finding that cathodal samples contained the /β₂ remnant as well as the Bβ chain suggested that core species migrating as Band IIIb, in addition to having undergone extensive degradation of both Aα chains (a feature also characteristic of Band IIIa), had released the NH₂-terminal region of one Bβ chain as a Bβ remnant (cf. Fig. 6). The absence of Bβ chains in anodal gel slices permitted the additional conclusion that species migrating as Bands VI and VII had released the NH₂-terminal regions of both Bβ chains. However, the migration of Bands VI and VII (Fig. 1) was so rapid relative to Band IIIb that the mere release of the NH₂-terminal region of the second Bβ chain appeared insufficient to account for the difference. The cathodal to anodal distribution of the /γ₁, /β₄, Bβ/4 band suggested that internal plasmin attack related to the ultimate separation of Fragments D and E might account for the formation of Bands VI and VII. This possibility is considered again below and depicted schematically in Fig. 6.

**DISCUSSION**

**Consideration of Heterogeneity Shown in Previous Polyacrylamide Gel Electrophoretic Studies**—Heterogeneity of circulating fibrinogen as a function of size was first suggested by studies of its solubility (1) and proved by molecular weight determination on plasma Subfractions I-4 and I-8 (2). Disc electrophoresis (20) carried out at that time showed that material from I-8 migrated farther than did that from I-4 (2). Since I-4 and I-8 did not differ significantly with respect to charge, as assessed by chromatography on DEAE-cellulose (2) or by electrophoresis.
Fig. 6. Schematic diagram of core species migrating as Bands I to VII. The general chain structure of core species migrating within the various bands and of chain remnants released from the core during their formation are depicted by the drawings. The interchain disulfide bridges (—) which join the \( \alpha \) (I), \( \beta \) (II), and \( \gamma \) (V) chains are shown forming two hexagons linking all six chains in two regions (13). No attempt has been made to indicate the exact location of intrachain disulfide bridges (13) or the precise spatial orientation of the chains. Cleavage sites are indicated by numbers corresponding to terminology summarized in the text and in Table I. **Heavy lines** show conversion pathways. **Thin solid lines** show remnants released during these conversions. **Thin broken lines** show regions from which remnants originate.

Band VI and VII become the major core derivatives during the late phases of stage I. Subsequently, these derivatives can undergo cleavage resulting in the separation of Fragments D and E or, prior to separation of Fragments D and E, they may release a large remnant containing the COOH-terminal region of one \( \beta \) chain (i.e. \( \beta_\alpha \)) to form derivatives migrating as Band VIII (13). Electrophoresis of reduced samples prepared from gel slices (Fig. 5 and Ref. 13) has indicated that virtually all available \( \alpha \) and \( \beta_\alpha \) remnants are released during the formation of Bands VI and VII. However, the electrophoretic positions of these bands relative to other core derivatives strongly indicate by appropriately placed question marks.

on cellulose acetate strips (1), the differences in migration in polyacrylamide gels appeared to be due primarily to the molecular sieving properties of this medium. Electrophoretic heterogeneity was also found within each plasma subfraction (2) as well as within early plasmic derivatives (9), although the significance of these observations was not fully appreciated. Mills and Karpatkin (21) identified six core components by SDS-electrophoresis of solubilized clots prepared from plasma fractions differing in solubility; these components appear to correspond to Bands I to VI described above. Their results and the present work strongly imply that the electrophoretic heterogeneity observed within each subfraction in earlier studies (2, 9) can indeed be attributed to size differences.

Considerations of Core Species Identified by SDS-Electrophoresis of Unreduced Samples (Fig. 6)—The present study has shown that plasma fibrinogen includes a number of (multichain) derivative species indentifiable by their migration rates in SDS-electrophoresis. These derivatives, which were found in the electrophoretic bands designated II, IIIa, IV, and V, were shown (by experiments involving separation of the subunit chains) to arise via the release of certain portions of the COOH terminal region of \( \alpha \) chains. Species migrating in Bands II, IIIa, and V are also formed during early phases of the plasmatic hydrolysis of "native" fibrinogen (Fraction I-4) in vitro (Fig. 1). Since subfractions comprised of these plasmatic derivatives (e.g. I-9D0\textsuperscript{m}) contain almost no intact \( \alpha \) chains but manifest only minor attack on chains other than \( \alpha \), it may be concluded that they too are formed by the release of COOH-terminal peptides from both \( \alpha \) chains. Band IIIb, occurring in appreciable amounts at a somewhat later phase of plasmatic degradation (i.e. I-9D\textsuperscript{m} and I-9D\textsuperscript{p}, Fig. 1), is formed from species like those in IIIa by the release of material from the NH\textsubscript{2}-terminal region of one \( \beta \) chain.

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suggest that the release of $\beta/3$ or B$\beta/3$ remnants is insufficient to account for the formation of species migrating as Bands VI and VII. This apparently requires additional attack on internal sites, such as those eventually cleaved to separate Fragments D and E. The following considerations of this attack are based upon information obtained by carrying plastic digestion beyond stage 1 (13) and on the amino acid sequence of the NH$_2$-terminal portion of fibrinogen (22).

Since remnants $\beta_1$ and $\beta_2$ both contain the COOH-terminal portion of the B$\beta$ chain (Ref. 13 and Table 1) and each is produced by cleavage at a different site, cleavage at both of these sites should give rise to a derivative chain ($\gamma/3$) of molecular weight $\approx 6,000$. However, such a remnant has not yet been identified. Remnant $\alpha/12$ (mol wt 6,700, Ref. 13), comprises part of Fragment D and appears to account for a portion of the $\alpha$ chain between $\alpha/12$ (Table 1), which is formed during stages 1 and 2, and $\alpha/14$ (Table 1), which has been identified in early forms of Fragment E (13). The fate of the remainder of this $\alpha$ chain segment is uncertain. It is not known whether such a $\alpha/3$ chain or the $\beta/3$ remnant alluded to above is released or whether it remains covalently linked to the core. The NH$_2$-terminal sequence of the $\gamma$ chain (22) indicates that scission at appropriate combinations of plasmic cleavage sites (i.e. positions 35, 53, 58, and 62) should release part of the $\gamma$ chain as one or more $\gamma/3$ remnants (Fig. 6).

In their studies of the plastic digestion of fibrinogen, Mills and Karpatkin (5) identified two early core components by SDS-electrophoresis. These products, which they designated "I" and "II", correspond to our plasmic derivative Bands I1b and [VI + VII], respectively. They attributed the formation of product "I" to the release of the COOH-terminal region from one $\alpha$ chain and that of product "II" to the release of the corresponding portion from the other. In addition, these investigators indicated that other degradative events, such as the loss of the NH$_2$-terminal fragment from the B$\beta$ chain (i.e. B$\beta/3$), were associated with the formation of derivative species within product "II" (5). Our present analyses strongly indicate that products "I" and "II" could not have been formed in the manner proposed. Neither $\alpha$ chain of product "I" (i.e. derivative Band I1b) is intact, and appreciable degradation of the B$\beta$ chain has occurred as well. Even the release of all available $\alpha/1$ and B$\beta/3$ remnants does not result in the formation of product "II" (i.e. Bands VI + VII). Pizzo et al. (8) have also examined unreduced plasmic digests by SDS-electrophoresis. They concluded that there are several major species of "Fragment X" formed although they did not provide data bearing on the specific structural features of the various derivatives. Discrepancies between certain of their conclusions (e.g. the site of initial B$\beta$ chain attack) and ours relating to the formation of early core derivatives are attributable to several of their underlying assumptions, which are not consistent with presently available information (3, 13).

**Comparison of Circulating Catabolites and Plasmic Derivatives—** Previous studies (9), in which Fraction I-8 and a plastic derivative Fraction I-SD$_5$ (obtained from a digest of 85% clottability) were compared, demonstrated that the latter closely resembled its plasma "solubility counterpart" in chromatographic behavior on DEAE-cellulose, electrophoretic behavior (in agarose, polyacrylamide gels, or cellulose acetate strip), NH$_2$-terminal groups, fibrinopeptide content, molecular size, clottability, and rate of aggregation of fibrin monomers prepared from these fractions. More recent observations (3) included a preliminary comparison of the subunit structures of I-8, I-9, I-8D, and I-9D (the latter two fractions from a digest of 88% clottability) by SDS-electrophoresis; these results and our present studies permit a fairly detailed summary of the similarities and differences among these fractions.

The close similarity between I-8 and I-9D* parallels that previously shown for Fractions I-8 and I-8D (3, 9). Fraction I-9 and its plasmic counterpart are virtually indistinguishable by chromatography on DEAE-cellulose (Fig. 3) or by chromatography of their S-sulfo derivatives on CM-cellulose (Fig. 4, present work and Fig. 2, Ref. 3). Analyses of unchromatographed preparations (Fig. 2) or of material obtained from the $\alpha$ chain peak of CM-cellulose chromatograms (Fig. 4) indicate that all $\alpha/1$-remnant chains (i.e. $\alpha/12$ to $\alpha/12$) identifiable in circulating fibrinogen can also be formed in vitro by plasmin action. This conclusion differs from that implied by Mills and Karpatkin; they concluded that $\alpha/1$, and $\alpha/12$ occurred only in plasmic digests (5). By contrast, our results suggest that $\alpha/1$ and $\alpha/12$ are formed in plasmic digests and in vivo, although they have a much lower frequency in the latter instance.

SDS electrophoresis of samples from plasmic digestion showed that core derivatives corresponding in size to those found in I-8 (i.e. Bands II), I-8, and I-9 (i.e. IIIa and V) were formed as the consequence of $\alpha$ chain attack. These results differ sharply and inexplicably from those of Smith and Frank (23), who reported that they were unable to produce plasmic derivatives with molecular weights similar to those of plasma catabolites.

Differences between I-9 and I-9D* include the absence of derivative Band IV from the latter and the paucity of remnants $\alpha/1$, and $\alpha/12$ in the former. In addition, $\beta/3$ chain degradation is somewhat more advanced in plasmic Fraction I-9D* than in its plasma counterpart (I-9). Remnants $\beta/2$ and $\beta/3$ can be demonstrated in the chromatographic fractions of S-sulfo I-9D* (Fig. 4) as well as in gels of cross-linked fibrin from this preparation (24). The $\beta/2$ chain is also identifiable in I-9 (3, 24), but $\beta/3$ has not been detected. A band corresponding to the $\beta/3$ chain (Fig. 2, Ref. 3). Analyses of unchromatographed preparations (Fig. 2) of material obtained from the B$\beta/3$ remnant chains (i.e. Ac$\alpha/1$ to Ac$\alpha/12$) identifiable in circulating fibrinogen fractions (3), although there was no indication of the presence of the B$\beta/3$ remnant. Thus, even as early as a plasmic subfraction as I-9D* manifests somewhat more degradation than the clottable fibrinogen catabolites isolated from plasma. Inasmuch as Fragment X (14) results from considerably more advanced digestion, the normal concentration of this fragment in circulating plasma must be very low.\(^\text{5}\)

The failure to demonstrate complete identity between I-9 and I-9D* underscores the uncertainty as to whether plasmin alone catalyzes the formation of circulating catabolites in vivo. Nevertheless, available evidence indicates that plasmin or a plasmin-like enzyme(s) catalyze(s) the formation of circulating catabolites and permits the speculation that some or all of the

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1 In agreement with the observation of Mills and Karpatkin (4) we have found an increase in the amount of $\alpha/1$ during early phases of plastic hydrolysis. By using relatively heavy loads for SDS-electrophoresis we have demonstrated small amounts of $\alpha/1$ in the "$\alpha/1$" peak of S-sulfo I-9D* (cf. Fig. 4), whereas under the same conditions this remnant was not seen in the "$\alpha/1$" peak of S-sulfo I-4.

2 Band VI was previously shown to correspond to Fragment X (13). This band was not demonstrable in plasma subfractions, hence Fragment X, if present in plasma at all, must be represented by Band VI of Fraction I-9 (Fig. 1).
observed features of these circulating derivatives may be related to the modifying effects of the plasma environment (12).

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