Human Fibrinogen Heterogeneities

I. STRUCTURAL AND RELATED STUDIES OF PLASMA FIBRINOGENS WHICH ARE HIGH SOLUBILITY CATABOLIC INTERMEDIATES*

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SUMMARY

The heterogeneity of human plasma fibrinogen manifested as differences in solubility reflects the presence of early catabolic intermediates, which are more soluble, have a longer thrombin-clotting time, and are of a lower molecular weight than the parent material from which they are formed. In this study the S-sulfob subunits from fibrinogen fractions of low (I-4) and high (I-8 and I-9) solubility were compared. Separation of $\alpha\beta$, $\beta\gamma$, and $\gamma$ chains was achieved by gradient elution chromatography on CM-cellulose. The tryptic peptide maps were characteristic for each type of chain. The only observable differences were in the maps of $\alpha\alpha$ chains; in those of I-8 and I-9 a considerable number of spots were absent or reduced relative to I-4. NH$_2$-terminal analyses of isolated chains before and after thrombin treatment showed the characteristic residues of the $\alpha\alpha$, $\beta\beta$, and $\gamma$ chains, respectively. Thus the NH$_2$-terminal portions of all chains of I-4, I-8, and I-9 were intact.

Molecular weight estimation of subunit chains was made by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate under conditions in which differences in mobility due to the presence or absence of $\alpha$ or $\beta$ peptides were distinguishable. Electrophoresis in this medium before and after reptilase or thrombin treatment served for identification of the chains. The $\gamma$ chains moved as a single band in this system (molecular weight, 50,700) and were indistinguishable from one another; the same was true of $\beta\beta$ chains (molecular weight, 60,400). However, electrophoresis of $\alpha\alpha$ chains revealed the presence of 13 more-or-less distinct bands (numbered in order of increasing anodal mobility), of which all but two (Band 3, a $\beta\beta$ contaminant and Band 5, a $\gamma$ chain contaminant) were shown to be intact $\alpha\alpha$ chain or COOH-terminally degraded $\alpha\alpha$ remnants. The molecular weights ranged from that of intact $\alpha\alpha$ chain (70,900) to 15,400 (Band 13). The highest proportion of intact $\alpha\alpha$ chains was in I-4; the lowest was in I-9, which had virtually no detectable intact $\alpha\alpha$ chain. Conversely, I-9 contained the highest proportion of $\alpha\alpha$ remnant chains; I-4, the lowest. These data suggested that cleavage at any or all of at least 10 sites along the $\alpha\alpha$ chain resulted in the formation of early catabolic intermediates.

Comparisons with highly clottable derivative fractions I-8D and I-9D produced by plasmin treatment of I-4 in vitro showed that they were quite similar to their respective plasma counterparts (I-8 and I-9) in that the $\gamma$ and $\beta\beta$ chains appeared to be intact, but few or no intact $\alpha\alpha$ chains were present. These observations thus strengthen the notion that it is plasmin which catalyzes the formation of early intermediates in vivo. Examination of Fragment 'X,' another fibrinogen derivative produced by plasmin digestion, revealed considerable depletion of intact $\beta\beta$ as well as of $\alpha\alpha$ chains, indicating that Fragment 'X' was more extensively degraded than I-8D or I-9D.

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1 Sherman et al. (11) termed the plasmin-degraded material "I-8D,' to indicate that it was a derivative (D) and that there were five subfractionation (D) steps involved in its isolation and repurification to high clottability. In the present paper, that fraction is referred to simply as I-8D; by the same token, therefore, plasmin-degraded material having solubility equivalent to that of plasma fraction I-9 is termed I-9D.
intermediate derived from higher molecular weight species. Conclusive support for the catabolite hypothesis was provided by turnover studies of radioactively labeled rabbit fibrinogen; a classical precursor-product relationship (12) was shown between the low and higher solubility forms of clottable material (7). The fact that in both I-8 and I-9D the transformation from low to higher solubility material occurred by release of peptide material from the COOH-terminal portion of the parent species (8, 11) suggested that the formation of the \( \varepsilon \) in vivo derivatives was catalyzed by plasmin. More recent studies of the sequence of plasmin degradation of fibrinogen (13, 14) have shown that the \( \alpha \) chain is the first to be degraded; related studies of fibrin subunits formed from human fibrinogen of high and low solubility indicated that the high solubility material lacked intact \( \alpha \) chains (13), an observation which further supported the notion that formation of these metabolites in vivo is catalyzed by plasmin.

The studies to be reported here were concerned with the detailed subunit structure of fibrinogen fractions of low (I-4) and high solubility (I-8 and I-9). These were compared to ascertain the exact features which account for previously shown differences in solubility and thrombin-clotting times (3). Also included were comparisons with certain plasmin-mediated in vitro derivatives, viz. Fractions I-8D and I-9D and Fragment \( \varepsilon \) (15), to determine their structural relationships to one another and to the intermediates formed in vivo.

## MATERIALS AND METHODS

### Fibrinogen Subfractions and Derivatives

Fractions I-4, I-8, and I-9 were prepared from outdated human ACD plasma as described by Mossesson and Sherry (3). The clottability of I-9, usually about 90% when prepared by the original method, was increased to more than 96% (four preparations) by the following modifications. After the usual procedure of precipitating Fraction I-8 at 2.1 g NaCl/100 ml solution (25), the precipitate was dissolved in 0.27 M NaCl-0.01 M sodium phosphate buffer, pH 6.4, to a protein concentration of 1 to 2%; resuspension by adding solid \( \beta \)-alanine to a final concentration of 2.4 M (250 g of \( \beta \)-alanine per liter of solution) was carried out at room temperature, followed by cooling to 1–2°C for 30 min or more and centrifugation at 10,000 to 12,000 \( \times \) g. The resulting Fraction I-9 was more than 96% coagulable with thrombin (range for four preparations, 96 to 99%).

Limited plasmin degradation of Fraction I-4 (original clottability >98%) was carried out as previously described (11). Clottability of the digest prior to fractionation was 88%, Fractions I-8D and I-9D isolated from this digest by ethanol and fractional precipitation >98%) was carried out as previously described (11).

### Electrolyte and Related Procedures

- **Gel Electrophoresis:** Gel electrophoresis was performed in slab gels (21) containing freshly deionized urea (8 M; buffer pH 8.6) or no urea (buffer pH 8.4). The former conditions were very similar to those described by Takagi and Iwanga (22). A vertical electrophoresis assembly (E-C Apparatus Corp., Philadelphia, Pa.) and a pulsed power supply (Ortec, Oak Ridge, Tenn.) were employed. Samples were applied in 40 μl of 20% (w/v) sucrose.

- **Polyacrylamide Gel Electrophoresis:** Polyacrylamide gel electrophoresis in acetic acid-urea solutions (23) was carried out in a disc gel apparatus with a regulated power supply (Buchler Instruments) in tubes, 5 × 75 mm, at a gel concentration of 10% (pH 2.7, 2 M urea).

### Protein Markers

- Phosphorylase A (rabbit muscle), molecular weight 94,000 (24), and aldolase (rabbit muscle), 40,000 (24), from Worthington Biochemicals Corp., albumin (bovine), 68,000 (24), from Sigma, chymotrypsinogen A (bovine pancreas), 25,700 (24), and cytochrome c (horse heart), 11,700 (25), from Mann Research Laboratories, the γ chain, 47,000 (25), and Bα chain, 56,000 (25), of human fibrinogen. The precision in estimating the molecular weight of unknown bands from these markers was ±1.5 to 3.5%.

### S-Sulfotyrosine Fibrinogen

Isolated S-sulfotyrosine fibrinogen fractions were reacted with reptilase (prepared from the venom of Bothrops atrox; a gift from Penta Pharm Ltd. Basel, Switzerland) or thrombin in the following way. Freeze-dried samples were solubilized in deionized 10 M urea to a concentration of about 10 mg per ml and then dialyzed overnight against 300 to 500 volumes of 0.2 M sodium phosphate, pH 7.0. After dialysis samples contained a uniform fine granular suspension. To 50 μl of each sample (estimated final protein was provided by Dr. Victor Marder and was about 40% clottable.

S-Sulfotyrosine fibrinogen was prepared according to the method of Pechère et al. (16).
concentration, 4.5 mg per ml) was added two 5-ml portions of reptilase at an interval of 30 to 60 min, or 2.5 μl of thrombin. The suspension was allowed to incubate with stirring for 3 hours at room temperature and overnight at 5°. The amount of enzyme added caused unmodified fibrinogen (Fraction I-4, 2 mg per ml, which had been carried through the same dialysis procedure) to clot 30 s after the first reptilase addition and in less than 10 s after treatment with thrombin. A portion of the enzyme-treated S-sulfop sample was mixed with an equal volume of 9 M urea to prepare the individual buffers resulted in a fall in pH; e.g. to 4.7. Urea solutions were varied from 3 to 25% of the total eluted protein depending upon the particular preparation and upon the amount of material loaded on the column. The nature of the frontal fraction is not relevant to these studies but is considered in Paper III of this series (28).

RESULTS

Chromatographic, Electrophoretic, and Immunochemical Characterization of Unmodified I-4, I-8, and I-9—These experiments were carried out to extend the results of previous studies of I-4 and I-8 (8) by comparing them with I-9, which has an even higher solubility and longer thrombin clotting time than I-8 (3).

Electrophoresis of these unmodified fractions in 5% polyacrylamide gels at pH 8.4 showed that they migrated as single bands; Fractions I-8 and I-9 were indistinguishable from one another although both had a slightly greater anodal mobility than I-4. Agarose electrophoretic studies indicated a marginally greater anodal mobility of I-8 and I-9 compared with I-4, but this difference could not be shown by the positions of the fibrinogen precipitin arcs in concomitant immunoelectrophoretic experiments. Immunodiffusion experiments comparing I-4, I-8, and I-9 resulted in fibrinogen precipitin lines of complete identity. Thus, by these several criteria, I-9 could not be distinguished from I-8.

The chromatographic elution pattern of I-8 on DEAE-cellulose was very similar to that of I-4 (Fig. 1) except for the previously observed (8) tendency for slightly earlier elution of the major peak of I-8. I-9 displayed a shoulder on the ascending limb of Peak 1 which was not present in either I-4 or I-8 (Fig. 1), a relatively minor but consistent (three preparations examined) distinguishing feature of this material.

Chromatographic Separation of Subunit Polypeptide Chains—Chromatography of S-sulfop fibrinogen preparations on CM-cellulose resulted in satisfactory resolution of the αα, ββ, and γ chains at a 1% gradient. Column chromatography of unmodified fibrinogen (Fraction I-4) except for the previously observed (8) tendency for slightly earlier elution of the major peak of I-8. I-9 displayed a shoulder on the ascending limb of Peak 1 which was not present in either I-4 or I-8 (Fig. 1), a relatively minor but consistent (three preparations examined) distinguishing feature of this material.

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chains were consistent with their elution behavior during CM-cellulose chromatography. In addition to the major band corresponding to each S-sulfo chain, small amounts of other bands were detected in I-4 and I-9 Aα chains but not in those from I-8. The presence of a γ chain contaminant, shown by other techniques to be present in all Aα preparations (see below), was suggested in a single I-8 Aα preparation by the presence of detectable terminal tyrosine in thrombin-treated or untreated samples. Because the NH2-terminal pyroglutamylation of the Bβ chain is unreactive with DTS chloride, Bβ contamination of Aα samples could not be evaluated by this analysis, but other data have indicated the consistent presence of small amounts of Bβ chains in Aα preparations (see below).

Owing to the unreactive NH2-terminal residue, Bβ chains (two preparations of each fraction) revealed only trace amounts of terminal amino acids. The most evident of these was tyrosine after thrombin treatment. Prior to thrombin treatment, small amounts of NH2-terminal glycine were detected in I-4 and I-8 Aα chains but not in those from I-9. The presence of a γ chain contaminant, shown by other techniques to be present in all Aα preparations (see below), was suggested in a single I-8 Aα preparation by the presence of detectable terminal tyrosine in thrombin-treated or untreated samples. Because the NH2-terminal pyroglutamylation of the Bβ chain is unreactive with DNS chloride, Bβ contamination of Aα chains could not be evaluated by this analysis, but other data have indicated the consistent presence of small amounts of Bβ chains in Aα preparations (see below).

The γ chains (two preparations of each fraction) revealed only tyrosine detectable before or after thrombin treatment.

Electrophoretic Analysis (Figs. 4 to 9)—Urea-polyacrylamide gel electrophoresis at pH 8.6 resulted in clear separation of I-4 S-sulfo subunit chains (Fig. 4). The relative mobilities of the chains were consistent with their elution behavior during CM-cellulose chromatography. In addition to the major band corresponding to each S-sulfo chain, small amounts of other bands were usually seen.

The γ chains of I-4, I-8, and I-9 were indistinguishable from one another in this analytical system, as were the respective Bβ chains. In contrast to that of I-4, the Aα bands of I-8 and I-9 were more diffuse, somewhat more anodal than that of I-4 and tended to overlap the Bβ position. Additionally, in one of two preparations of I-8 (Fig. 4) there was a second major band which migrated more slowly than any Aα band.

FIG. 2. Elution patterns of S-sulfo chains of I-4 (39 mg), I-8 (75 mg), and I-9 (84 mg) subjected to gradient elution chromatography in 8 M urea on CM-cellulose (columns, 1.5 × 30 cm). The theoretical Na+ gradient is at the top. Each major band was separated under identical conditions. Each tube contained 4.5 ± 0.1 ml; pooling was as indicated by the shaded areas.

Reaction of any S-sulfo chain with DTT usually results in bands with slower anodal mobility (pH 8.6) because of the lower anionic charge resulting from reducive displacement of SO3− groups. After such DTT treatment, the two main bands in the I-8 Aα sample (Fig. 4) migrated as a single band with a mobility intermediate between those shown in the figure. This experiment suggested that the slower band was a disulfide-bonded polymeric form of the Aα chain separated by molecular sieving, and it effectively excluded the possibility that this might have represented α polymer (30, 31).

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The foregoing results suggested that the differences between Fractions I-4, I-8, and I-9 were related to differences in the ACY chain population. This notion was investigated, and ultimately substantiated, by the following series of experiments in which SDS-polyacrylamide gel electrophoresis was the principal technique. It had already been shown (see above) that the NH2-terminal portions of the ACY and Bβ chains were intact and could be cleaved by thrombin. It was also established at the outset, through analyses of isolated ACY, Bβ, and γ chains, that the individual chains, regardless of their molecular size, could be differentiated by comparing their gel patterns before and after reaction with reptilase or thrombin* (Figs. 5 and 7). This was possible because the electrophoretic technique was capable of distinguishing molecular weight differences in chains resulting from the loss of an A or B peptide (molecular weight 1500 to 1600); actual computed reductions were between 700 and 2800, a result consistent with the precision of the method.

The Bβ chains of I-4, I-8, and I-9 moved as single bands and were indistinguishable from one another; the same was true of γ chains (Fig. 5). Upon treatment with thrombin, the position of Bβ chains shifted toward the anode whereas that of the γ chains did not. After reaction with reptilase, most of the Bβ band underwent no change in mobility; however, two additional minor bands (arrows, Fig. 5) were detected. The band of higher molecular weight was barely appreciated as a faint band continuous with the anodal portion of the Bβ band in I-4 and I-9 or as a faint but discrete band in I-8. Its appearance was consistent with cleavage of Peptide B from a small percentage of Bβ chains. The lower band, molecular weight 56,000, differed from that of the intact Bβ chain (60,400) by 4,400, an amount suggesting that it had been derived from a Bβ chain by cleavage at a site more internal than the thrombin-susceptible site at position 14 (perhaps at position 42). Thus, in spite of the fact that reptilase does not act exclusively on the ACY chain (35), the reaction was sufficiently specific to enable ACY and Bβ chains (and by inference, γ chains as well) to be distinguished from one another.

In contrast to Bβ and γ chains, a complex band pattern was obtained with chromatographically isolated ACY chains. There were 11 to 13 more-or-less distinct bands, which were numbered consecutively in order of increasing anodal mobility (Figs. 6 and 7). For clarity, bands were further classified as Group I, II, or III if their relative migration was close to that of intact
FIG. 5 (upper left). SDS-polyacrylamide gel electrophoresis (9% gel) of chromatographically isolated S-sulfo Bβ and γ chains before and after treatment with reptilase (R) or thrombin (T). The Bβ and γ chains were recombined for this experiment prior to enzyme treatment. The arrows point to minor bands which became visible after reptilase treatment. The gel was stained with Coomassie blue.

FIG. 6 (upper right). SDS-polyacrylamide gel electrophoresis (9% gel) of chromatographically isolated Aα chains of I-4 and I-9 (15 μg each). The gel was stained with Coomassie blue. On the right the isolated, recombined Bβ and γ chains from I-4 are shown for reference. Eleven distinct bands were visible in this experiment and are numbered consecutively in order of increasing anodal mobility.

FIG. 7 (lower). SDS-polyacrylamide gel electrophoresis (9% gel) of chromatographically isolated S-sulfo Aα chains of I-4, I-8, and I-9 before and after treatment with reptilase (R) or thrombin (T). The gel was stained with Coomassie blue. Thirteen more-or-less distinct bands were visible and are numbered consecutively according to increasing anodal mobility. Bands 1 to 11 correspond to those in Fig. 6 and Table I in which Bβ (Band 3) and γ chain (Band 4) contaminants are indicated. The estimated molecular weights (before enzyme treatment) are indicated on the left.
Summary of the data relating to SDS-polyacrylamide gel electrophoretic estimation of the molecular weight of S-sulfo chains of I-4, I-8, and I-9. The numbered bands are further classified as Group I, II, or III if their relative migration is close to that of intact Aα, Bβ, or γ chains, respectively, and as Group IV if their migration is considerably faster than that of the intact chains. Inasmuch as Group IV bands were difficult to resolve by densitometric scanning, their relative staining intensities have been estimated visually and graded Tr (trace) to ++++. Failure to detect a given band is indicated by −.

| Group | Band No. | n* | Mean mol wt x 10^2 | Relative distribution of Group IV bands |
|-------|----------|----|-------------------|---------------------------------------|
| I     | 1        | 7  | 70.9              | 1-4 1-8 1-9  |
|       | 2        | 7  | 67.3              | 1-4 1-8 1-9  |
| II    | 3 (Bβ)*  | 8  | 60.4              | 1-4 1-8 1-9  |
|       | 4        | 7  | 57.7              | 1-4 1-8 1-9  |
| III   | 5 (γ)*   | 8  | 50.7              | 1-4 1-8 1-9  |
|       | 6        | 7  | 46.5              | 1-4 1-8 1-9  |
| IV    | 7†       | 7  | 37.6              | + + + + + + |
|       | 8        | 7  | 33.9              | + + + + + + |
|       | 9        | 7  | 31.8              | + + + + + + |
|       | 10       | 7  | 29.2              | + + + + + + |
|       | 11       | 6  | 25.0              | + + + + + + |
|       | 12       | 4  | 22.6              | + + + + + + |
|       | 13       | 3  | 15.4              | + + + + + + |

* Number of gels in which this band was measured.
† This band has the same molecular weight and susceptibility to reptilase and thrombin as the Bβ chain.
‡ This band has the same molecular weight and susceptibility to reptilase and thrombin as the γ chain.
§ This band was frequently difficult to appreciate as a discrete band when there was smearing of the stain in that region (cf. Gel 1, Fig. 6).

Aα, Bβ, or γ chains, respectively; Group IV bands were those which migrated considerably faster than any of the intact chains.

There were usually two Group I bands (occasionally this group was resolved into a triplet) both of which were enzymically identifiable as Aα chains. Of these bands, Number 1 was assumed to correspond to intact Aα chain whereas Number 2 was regarded as an Aα remnant of slightly reduced molecular size (Table I). Of the Group II bands one proved to be a Bβ chain (Band 3) because it reacted only with thrombin, while the other (Band 4) met the enzymic criteria for an Aα chain. Band 5 (Group III), on the basis of its characteristic molecular weight and its nonreactivity with reptilase or thrombin, is most likely a γ chain contaminant. Band 6 is an Aα remnant which can also be observed in unchromatographed fibrinogen chain preparations as a discrete band or as a minor band continuous with the γ chain at its anodal border (Fig. 9); it becomes clearly evident after treatment with reptilase or thrombin. Of the Group IV chains, Bands 12 and 13 were the faintest which could be enzymically identified as Aα remnants, whereas Bands 8 and 9 were the predominant species (e.g., Fig. 7). The relative proportion of the total Aα chain population represented by Group IV varied directly (Table I; Fig. 8) with the solubility of the preparation (i.e., 1-9 > 1-8 > 1-4).

For the foregoing data it was evident that the differences between I-4, I-8, and I-9 were in the degree of intactness of their Aα chains. Densitometric scans (Fig. 8) indicated that 66% of the I-9 and I-4 gels were those shown in Fig. 6; the I-8 gel scanned was obtained by running a 45-µg sample in the same experiment as the 112-µg sample of 1-8 shown in Fig. 7.
of all I-4 Aa chains were in Group I but only 19% of I-8 and 11% of I-9 chains were of this size order. Furthermore, it could be estimated visually that Fraction I-9 had virtually no intact (i.e. Band 1) Aa chains whereas about 50% of the Group I bands in I-8 and most of those in I-4 were of this variety. Understandably, I-9 had the highest proportion of the smaller Aa remnants.

Comparisons with I-8D, I-9D, and Fragment 'X'—Comparisons of whole I-8 and I-9 with the plasmin-mediated Fractions I-8D and I-9D and Fragment 'X' were carried out by SDS-gel electrophoresis to assess and compare the type and degree of degradation of their component chains (Fig. 9). I-8D and I-9D were quite similar to their respective counterparts, I-8 and I-9, in that the Bβ and γ chains appeared to be intact and few Group I Aa chains were detectable. The I-8D and I-9D bands which were visible and identifiable as Aα remnants by enzymic analysis corresponded in size to the Aα remnants 2, 8, 9, 11, and 12 which had been identified in whole preparations of I-8 or I-9 or their isolated chains. In contrast to I-8 and I-9 there was little indication of the presence of Bands 6 and 10 in I-8D or I-9D. Furthermore, in I-8D and I-9D there were considerable quantities of the Aα remnant corresponding in size to Band 11, whose concentration was too low to be detected in equivalent amounts of whole I-8 and I-9 (Fig. 9). Fragment 'X' (two preparations) displayed considerable depletion of intact Bβ chains as well as of Aα chains. In some gels (not shown), the γ zone was resolved into two bands, probably representing degraded Bβ chains as well as γ chains. It seems reasonable to conclude from these patterns, albeit tentatively, that I-8D and I-9D resemble their plasma counterparts in lacking intact Aα chains and that Fragment 'X' differs in that substantial amounts of intact Bβ chains are lacking as well.

**DISCUSSION**

In the method previously described (3) for the isolation and purification of high solubility plasma fibrinogen fractions, Fraction I-7 was treated with 2.1 M glycine to precipitate Fraction I-8; the subsequent precipitate from the resulting supernatant solution was designated I-9. The proportion of fibrinogen precipitating in the I-8 fraction was largely dependent upon the protein concentration prior to glycine addition. Thus, Fractions I-8 and I-9 were both regarded as representative of fibrinogen of the highest solubility (3). The present studies permit the further conclusions that the measurable differences between I-8 and I-9 are of a relatively minor quantitative nature.

Since electrophoresis was carried out on whole fractions, rather than isolated chains certain of the less intensely staining Aα remnants (e.g. Bands 7 and 13) could not be identified under the load conditions in this experiment; the possible presence of Band 4 could not be assessed because of the relatively high amount of intact Bβ chain in the unchromatographed material. In this experiment the Band 6 Aα remnant was visualized as a minor band continuous with the anodal border of the γ-band; its presence was clearly apparent in the corresponding reptilase- or thrombin-clotted fibrin preparation (not shown). Inasmuch as Bands 8 and 9 were not well resolved they were considered together.

The remnant chains of Fragment 'X' have not as yet been identified by enzymic analysis. Although certain bands visible in the Group IV region correspond in size to Aα remnants (e.g. Bands 11 and 12), unequivocal identification has yet to be made, involving the degree of intactness of Aα chains. Although both are representative of the same over-all process, in view of the facts that I-9 may now be prepared in highly clottable form, is more completely catalyzed than I-8 (in the sense of virtually complete lack of intact Aα chain), has a higher solubility and exhibits a longer thrombin-clotting time (3), it would appear that I-9 is a more suitable fraction than I-8 for future comparative studies (e.g. with Fraction I-4 or Fragment 'X'). Based upon the molecular weights of the component Aα chains (Table I) and their distribution in each preparation (Fig. 8), the mean molecular weight of I-9 can be calculated to be about 10,000 less than that of I-8. Thus, based upon a molecular weight for Fraction I-8 of 271,000, obtained by averaging the previously reported values 269,000 and 273,000 (8, 11), Fraction I-9 may tentatively be assigned a mean molecular weight of 261,000.

There were no differences in the tryptic peptide maps of Bβ chains isolated from I-4, I-8, and I-9 or in those of the γ chains isolated from these fractions. These findings have supported the NH-terminal, electrophoretic, and chromatographic data in indicating that the Bβ and γ chains are intact. In contrast, the peptide map of Aα chains from I-4 contained several spots which were clearly missing or faint in the Aα chains isolated from I-8 and I-9. The fact that there were no new peptide spots in I-8 or I-9 suggests that the primary sequence of the smaller Aα chains is present in (and thus derived from) larger Aα chains. These observations, combined with NH-terminal and electrophoretic data, add to an already convincing body of evidence (3, 7, 8, 11, 13) that lower molecular weight plasma fibrinogens are clottable catalytic intermediates.

There is as yet no conclusive evidence that plasmin mediates the catabolism of fibrinogen in vivo, although the similarities between early products of plasmin digestion in vitro (namely I-8D, I-9D) and their plasma counterparts make such a surmise most attractive (11, 39). The present studies have shown that the structural features of the chains of I-8 and I-9 are essentially the same as those of I-8D and I-9D. Differences between the naturally occurring and plasmin-mediated derivatives (e.g. the presence of larger amounts of Band 11 Aα remnant in the latter) appear to be quantitative rather than qualitative, and could reflect the modifying influences of the plasma environment on plasmin action. In a recent brief report, Mills and Karpatkin (40) concluded that plasmin was not the enzyme responsible for early degradation of fibrinogen in vivo and that thrombin or a contaminant nonplasmin fibrinolytic activity present in their thrombin preparation might be responsible. The idea that thrombin itself could mediate such a reaction in vivo is entirely inconsistent with the widely accepted specificity of thrombin and the available data on the early plasma intermediates (3, 8, this study). The conclusion that the fibrinolytic activity discernible in their experiments might not be plasmin will require more rigorous documentation.

Aα chain heterogeneity manifested as a doublet (namely Bands 1 and 2) in the Group I Aα position in SDS-electrophoretic experiments of Fraction I-4 or its equivalent has been reported from other laboratories (13, 30). Heterogeneity has also been shown by gel electrophoresis of S-carboxymethylated (31, 41) and S-sulfo chains (23) under acid conditions. Since an ob-
from these data. It is therefore convenient to regard cleavage
degradation sequence (or sequences) cannot be deduced
2 and 9, for example, occurs with relatively high frequency, the
recognized by the demonstration of at least ten Aα remnants

II peptide indicating that

is hydrolysis of the Bβ chain (13, 14) which is not associated
I-8D and I-9D (11) occurs in this manner. Following this there
occurs by COOH-terminal attack without loss of clottability

early catabolic intermediates (Fig. 10).

as occurring in three relatively distinct stages (15), in the first
which (Stage I) clottable derivatives are present; the second
major steps in plasmin-mediated deg-
radiation in vitro involves hydrolysis of the Bβ chain, probably
in the NH2-terminal region (Step 2). The disulfide knot region (42)
the Aα chain is shown schematically. No attempt has been made to indicate the precise spatial arrangement of the

observable transition from Band 1 to Band 2 has been reported
to occur during plasmin hydrolysis (13) and since the ratio of
Band 2 to Band 1 is greater in I-8 than in I-4 and almost infinite
in I-9 (Fig. 7), only Band 1 can be regarded as an intact chain.
All other identifiable Aα-bands are intermediate catabolites
which are COOH-terminally degraded. In recent EDE-gel elec-
trophoretic experiments13 with Blomback Fractions I-1 and I-3
(4) from fresh-frozen plasma, a doublet has been noted in the
Aα position. Thus, all plasma fibrinogen fractions appear to
contain at least some degraded Aα chains. As a result of the
present studies at least ten cleavage sites on the Aα chain (pre-
sumably lysyl-X or arginyl-X bonds; see Fig. 10) have been recog-
ized by the demonstration of at least ten Aα remnants of discrete molecular weight. Although conversion to Bands
2 and 9, for example, occurs with relatively high frequency, the
exact degradation sequence (or sequences) cannot be deduced
from these data. It is therefore convenient to regard cleavage
of any or all of these bands as “Step I” in the formation of the
early catabolite intermediates (Fig. 10).

Plasmin degradation of fibrinogen in vitro is currently viewed
as occurring in three relatively distinct stages (15), in the first
of which (Stage I) clottable derivatives are present; the second
and third stages are characterized in part by the absence of clot-
table derivatives. Presently available information permits a
more complete picture of the composition of Stage I components
to be drawn and a comparison of the well characterized early
clottable derivatives (11, 15, 43) to be made. The present data,
as well as those of others (13, 14) clearly indicate that it is the
Aα chain which is first degraded (Step 1, Fig. 10) and that this
occurs by COOH-terminal attack without loss of clottability
(3, 11). The formation of the plasmin-mediated Fractions
I-SD and I-9D (11) occurs in this manner. Following this there
is hydrolysis of the Bβ chain (13, 14) which is not associated
(at least initially) with the loss of clottability (13, 44). Shainoff
and co-workers (44, 45) have reported that prior to appreciable
loss of clottability the Bβ chain loses a fragment containing the
B peptide indicating that the early fragmentation of the Bβ
which are recognizable in the acid gel system will prove to be the
same Aα remnants differentiated in the SDS system, although not
necessarily in the same relative positions.

13 Unpublished results.

14 The known sequence of the NH2-terminal portion of the Bβ
chain (40, 47) indicates that among the plasmin-susceptible sites
are three (Arg-Ala, position 42, and Lys-Ala, positions 47 and 58)
whose disruption would result in release of a fragment containing
Peptide B and the formation of new NH2-terminal alanine in the
core. In recent studies we have observed that new NH2-terminal
alanine is formed in the core molecule of the early plasmin-cat-
alyzed derivatives in which varying degrees of Bβ chain frag-
mentation have occurred. The presence of new alanine might not
have been suspected unless specific steps (such as NH2-terminal
analysis before and after thrombin treatment) were taken to
differentiate it from the alanine at the NH2 terminus of the Aα
chains. Thus, the fact that the NH2-terminal residues of Frag-
ment X’ were reported to be the same as those of fibrinogen (48),
not contradict the proposed degradative sequence.
Footnote 1), they concluded that the initial Bβ attack occurred in the COOH-terminal region of the molecule. Our demonstration of the intactness of the Bβ chains in I-SO and I-9D, as well as our subsequent studies in which the Bβ remnant corresponding to their β’ chain has been shown to be unreactive with thrombin, strongly indicates that initial cleavage of the Bβ chain takes place in the NH2-terminal region, as originally reported by Shainoff and co-workers (44, 45).

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