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SOME SYMMETRY CONSIDERATIONS FOR THE FIBRINOGEN-FIBRIN ASSEMBLY SYSTEM

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The formation of fibrin gels involves many operations which are fundamental to other assembly schemes, including limited proteolysis, spontaneous associations, and covalent stabilization. Despite a quarter century of intensive effort by a large number of laboratories, the orientation of the fundamental units in the gel is not known, nor, for that matter, is the arrangement of the subunit chains within the parent fibrinogen molecule. In this article some symmetry considerations are discussed in light of the geometry of the starting molecules and conditions necessitated by the covalent stitching which occurs after gel formation. Only a dimeric molecule in which the twofold symmetry axis coincides with a minor axis of an elongated fibrinogen molecule satisfies all the conditions.

The transformation of fibrinogen into fibrin during vertebrate blood clotting involves a number of fundamental molecular operations which occur also in many other biological assembly systems, including limited proteolysis, spontaneous association, and covalent stitching (1). In the first instance, an enzyme (thrombin) catalyzes the release of a few polar peptides (fibrinopeptides) from the fibrinogen molecule, resulting in a slightly smaller unit ("fibrin monomer") which then spontaneously polymerizes. Another enzyme (factor XIII) then catalyzes the introduction of covalent bonds between certain sidechains of adjacent molecules in the polymer, thereby stabilizing the fibrin clot.

(1) fibrinogen $\xrightarrow[H_2O]{\text{thrombin}}$ "fibrin monomers" + fibrinopeptides

(2) "fibrin monomers" $\xrightarrow{\text{(spontaneous)}}$ fibrin polymer

(3) fibrin polymer $\xrightarrow{\text{factor XIII}}$ crosslinked fibrin

Although this general outline of events has been accepted for a long time, the molecular details of the process remain shrouded in mystery, primarily because the structure of the fibrinogen molecule has been unusually resistant to analysis. Fibrinogen is a large protein (mol. wt. = 340,000) composed of three pairs of nonidentical subunits ($\alpha_2\ \beta_2\ \gamma_2$). Its physicochemical parameters have not lent themselves to simple interpretation, but it is generally accepted that the molecule is elongated along a central axis since it exhibits both flow and electric birefringence. The best estimates on the basis of hydrodynamic measurements put the axial ratio (a/b) at 5–10 for a molecule 450–500 Å long. Electron microscopy of fibrinogen has yielded a variety of proposed shapes and sizes, but at least one of these falls into the realm of these dimensions (2). Although no x-ray diffraction results have yet been reported, electron microscopy of microcrystals prepared from a partially degraded fibrinogen molecule have been analyzed by optical diffraction methods and have revealed dimensions of the order of 90 × 450 Å (3).

In the realm of chemical characterization, it is known that there are two different pairs of fibrinopeptides – A and B – and that they are the amino-terminal segments of the α-chains and β-chains, respectively (4). All six chains ($\alpha_2\ \beta_2\ \gamma_2$) are held together by disulfide bonds, and what little we know of the overall chain arrangement within the molecule has been mostly derived from considerations of which portions of which chains are disulfide bonded, as determined after either enzymatic or chemical fragmentation of the parent molecule. Similar information has been obtained about the orientation of units within the fibrin gel by determination of which chains are covalently stitched together during the factor-XIII-catalyzed stabilization process.

Unhappily, not all of the chemical results have been consistent with the picture which had emerged on the basis of earlier physicochemical characterization. In particular, the fibrinopeptides A had been thought to exist at the two extremities of the elongated molecule (one at each end) (5), but the chemical data clearly indicate that the two A peptides must be very near each other since the α-chains are disulfide linked just a few residues away from the junctions split by thrombin (6). Beyond that particular embarrassment, there has not been much serious consideration given to the problem of arranging three pairs of nonidentical chains into a fundamental scheme consistent with the principles of macromolecular symmetry, on the one hand, and the gel packing requirements on the other. In this article an attempt is made to use these principles not only to reconcile the chemical and the physical observations, but also to search among various possibilities of chain arrangement to find the one most consistent with the orientation of individual chains in the parent molecule and fibrin units in the final gel.

SYMMETRY ASPECTS

It is generally accepted that all proteins with dimeric subunits have a twofold axis of symmetry (dyad axis) such that a rotation of 180° results in an exact superposition of identical atoms. The evolutionary basis for this phenomenon has been discussed by Monod et al. (7), and for purposes of this discussion we will presume that the rule will also be found to apply to fibrinogen. In essence, what we want to decide here is whether the dyad

axis coincides with the principal axis of an elongated fibrinogen molecule, on the one hand, or with one of its minor axes, on the other (Fig. 1). As we shall see, the two situations (DS = dyad-short axis and DL = dyad-long axis) differ significantly with regard to the kinds of assembly patterns which can ensue.

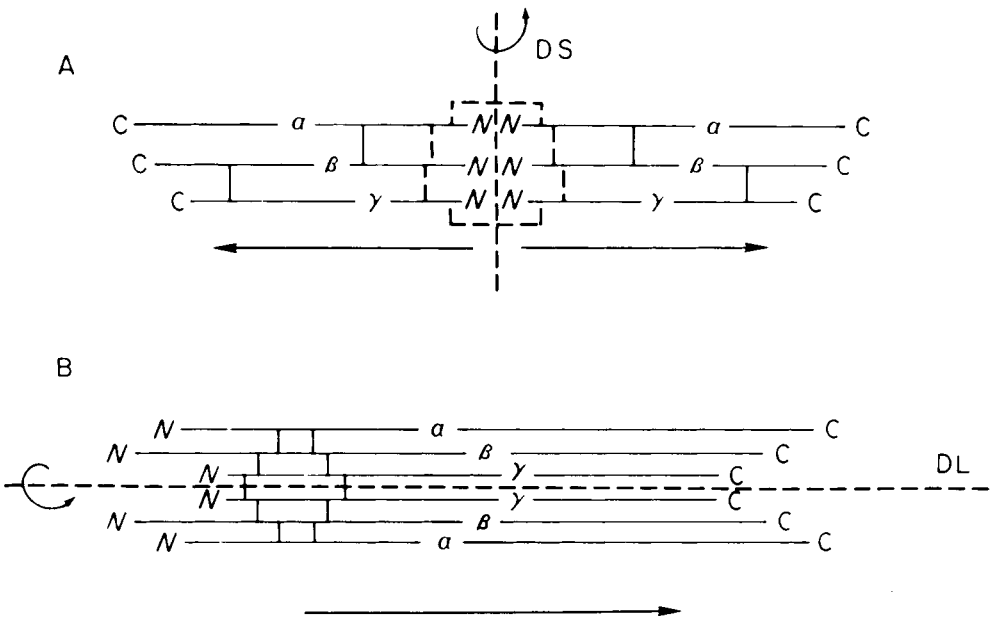

Fig. 1. Highly schematic depictions of two possible chain arrangements for the α_2 β_2 γ_2 subunit structure of vertebrate fibrinogen. In A (top) the dyad axis of the molecule is the short axis (DS = dyad-short). In B (bottom) the dyad axis of symmetry is the long axis (DL = dyad-long).

MODE OF POLYMERIZATION

Almost a quarter century ago, Ferry (8) proposed that fibrinogen molecules assembled themselves into fibrin polymers by way of a half-staggered overlap. His reasons for suggesting this were based on many data collected on the properties of intermediate fibrin polymers, which appeared to be two molecules thick and many units long. Furthermore, electron microscopy of fibrin consistently reveals a repeat unit of about 225 Å (2), and if the starting molecule is indeed 450 Å long, then a half-molecule stagger would fit perfectly (Fig. 2). In the ensuing discussion, it will be assumed that the polymerization of fibrin monomeric units does proceed by a half-molecule stagger, noting that this can be accomplished by either of our two fundamental starting units, DS or DL, using a sequence of interactions similar to those originally proposed by Ferry and his colleagues (9). The results are intrinsically different in the two cases, however, for in the case of DS molecules the equivalent chains in neighboring molecules come to lie *antiparallel* to each other,

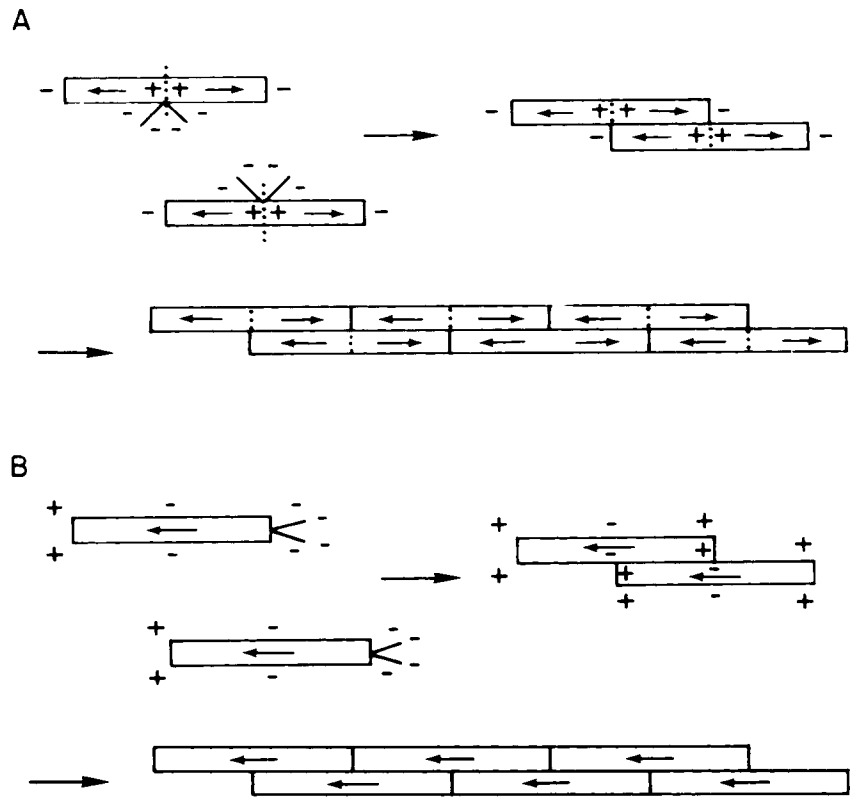

Fig. 2. Comparison of two ways of attaining a staggered overlap of fibrin units by removal of fibrinopeptide charge clusters. In A (top) the starting molecules are of the DS type, being symmetrical about their short axis. The fibrinopeptides are positioned centrally. In B (bottom) the fibrinopeptides are located at one extremity of the molecule, the symmetry being of the DL type.

whereas in the DL situation the molecules are aligned in a parallel mode* and are out of register.

ELECTRIC BIREFRINGENCE STUDIES

In theory it should be possible to distinguish between the two kinds of starting molecules (DS or DL) on the basis of their electrical asymmetry, a property which can be measured by electrical birefringence experiments. In this approach, molecules are subjected to a strong electric field and the extent of their alignment determined by the ensuing differences in the optical qualities of the solution (10). A molecule of the DS type

*The special case of where the initial dimerization is exactly end-to-end and the stagger introduced in subsequent associations is not considered here.

should have no permanent dipole moment along its principal axis because of its exact symmetry (although it might have one along a minor axis) (Fig. 3). In contrast, a DL molecule would be expected to have a permanent dipole along its principal axis, although it might be small or large, depending on the distribution of charged amino acid sidechains. Collagen, which represents an extreme case of a directionally oriented protein, has a permanent dipole moment of approximately 15,000 Debye units. The problem is complicated, however, by the fact that virtually all elongated molecules are susceptible to polarizations induced by the electric field itself, and the presence of these induced dipoles makes it much more difficult to measure permanent dipoles. Various analytical methods are available for distinguishing the two in theory, but direct demonstrations are difficult to achieve (10).

At its best, then, electric birefringence ought to be able to establish the electrical asymmetry of the starting molecule and perhaps even determine the nature of the polymerization by extending the measurements to initially ocurring dimers. Moreover, it was realized that very much could be learned by focusing on intermediate species which ought to exist when thrombin has released only one of a molecule's two fibrinopeptides A. The fibrinopeptides are usually quite electronegative, and depending on the distance of the moiety from the molecule's center of electrical symmetry, removal of a single peptide ought to change the dipole characteristics of the parent molecule (Fig. 3).

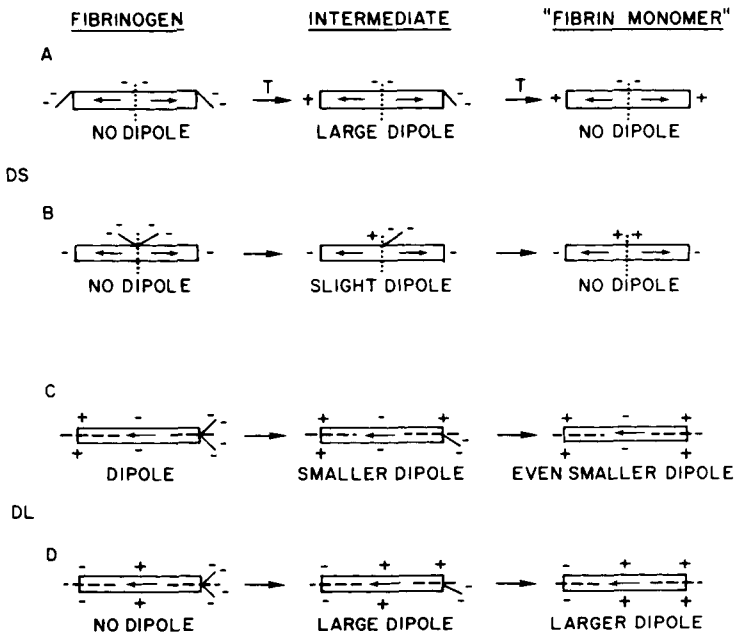

Fig. 3. Hypothetical changes in dipole moment for dimeric fibrinogen molecules losing first one fibrinopeptide A and then the other. In A, a molecule of the DS type has its fibrinopeptides located at opposite ends. In B, a DS type molecule has its fibrinopeptides in a central position. C and D are both models of the DL type, the difference being in the intrinsic distribution of charge in the parent molecules. In theory, electric birefringence should be able to distinguish among the four models. Only longitudinal dipoles are considered.

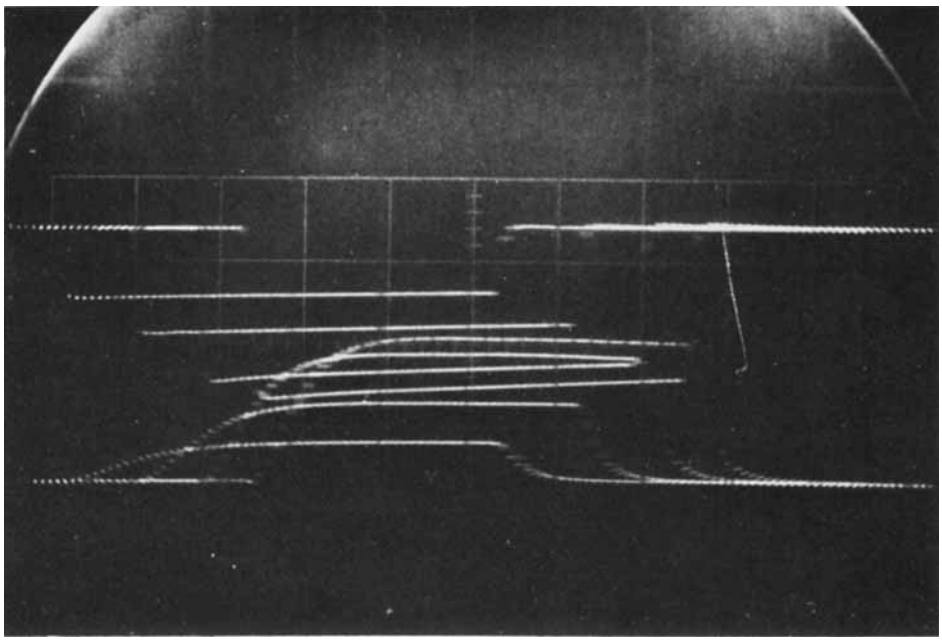

Fig. 4. Birefringence exhibited by bovine fibrinogen in an electric field at pH 7.2 (11).

In 1963 Haschemeyer (5) reported the results of such an experiment in which she found a large transient dipole correlating with the release of one molecule of fibrinopeptide A. Subsequent removal of the remaining fibrinopeptide A restored charge symmetry, and straightforward calculation indicated that the fibrinopeptides were 220 Å from the electric center of the molecule, consistent with their being at the ends of a molecule 450 Å long. As indicated above, however, a wealth of chemical evidence has accumulated in recent years which makes it clear that the two fibrinopeptides A (which are the amino-terminal segments of α-chains) must be located very near each other. The complete disagreement of the two approaches (chemical and electrical) has led us to attempt to repeat the electric birefringence experiments (Fig. 4), and although we are not yet in a position to furnish a detailed report, it seems clear that our data are most consistent with model B in Fig. 3 (11). Seen in retrospect, it seems that the complications associated with electric birefringence measurements and certain peculiarities of the fibrinogen molecule itself conspired to defeat those early attempts, elegant as the experimental concept itself was.

COVALENT CROSSLINKING OF FIBRIN

Another approach to understanding the arrangement of units within the polymer,

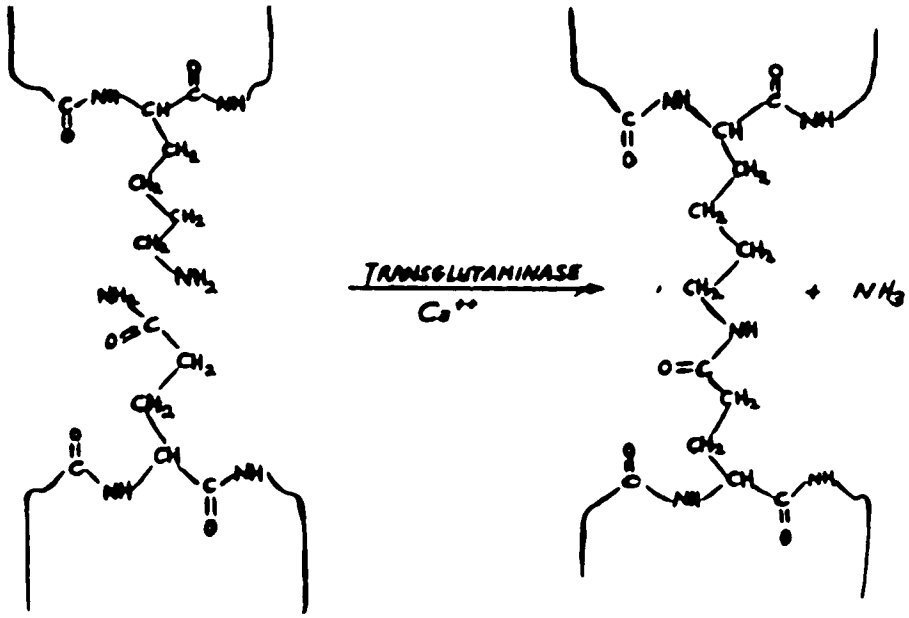

Fig. 5. Formation of ε-(γ-glutamyl)'lysine crosslinks by condensation of glutamine and lysine side-chains catalyzed by factor XIII.

as well as the individual chains within the parent molecules, stems from the covalent crosslinking of fibrin gels by the plasma transglutaminase known as factor XIII. This enzyme catalyzes the formation of ε-(γ-glutamyl) lysine crossbridges between specific glutamine and lysine sidechains in neighboring fibrin units (Fig. 5). The most interesting of these bonds are formed between the γ-chains of adjacent molecules, giving rise to γ-γ dimers (12, 13, 14). Given enough time and enzyme, these same kinds of bond are also formed between α-chains (15), the product in this case being extensive arrays of multimerically bound α-chains (Fig. 6).

In our laboratory we were able to locate and completely characterize the cross-linking unit which occurs between γ-chains (16, 17). We found that the γ-chains of neighboring molecules are oriented in an antiparallel fashion such that a pair of reciprocal crosslinks can be formed between them (Fig. 7) and that the site is very near to the carboxy-terminus of the γ-chain (it ought to be recalled that the triggering event for gelation is the removal of the fibrinopeptides from the amino-termini of the α- and β-chains). We were able to rule out the possibility that this bonding arrangement was occurring between γ-chains of the same molecule by preparing a hybrid fibrin made from bovine and human fibrinogen. The crosslinking sites of these two fibrinogens differ electrophoretically by virtue of a histidine/glutamine interchange, and we were thus able to demonstrate the existence of a hybrid crosslinking unit (as well as the two pure types) (18). The amino-acid sequences of the γ-chain crosslinking segments are such that if two α-

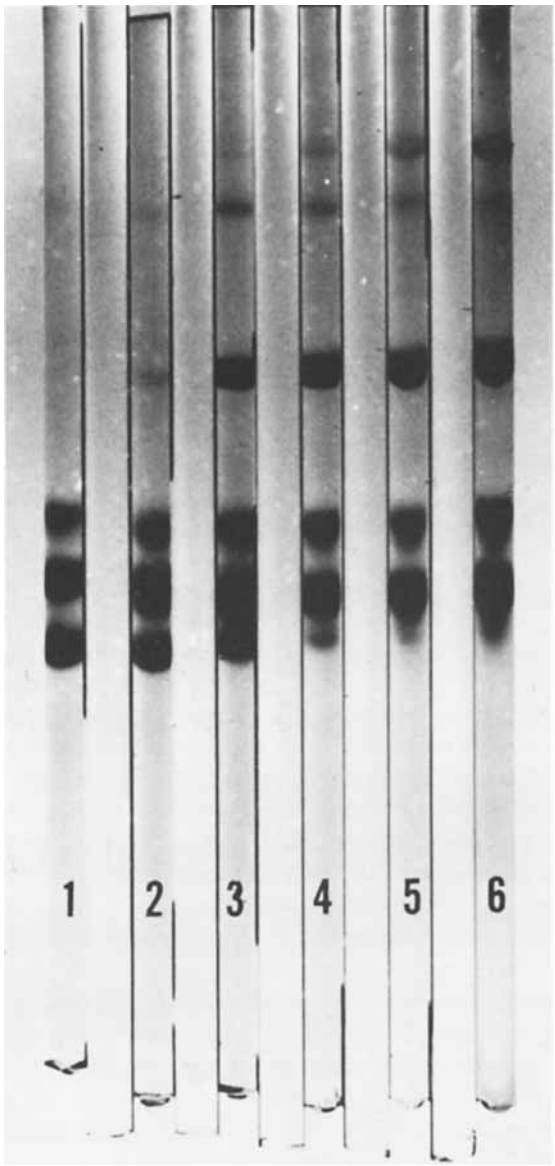

Fig. 6. SDS-gel electrophoresis of polypeptide chains produced by reduction of bovine fibrin during the course of its formation under crosslinking conditions. Samples were poisoned at various intervals by the addition of a urea-mercaptoethanol-SDS mixture. (1) zero time; (2) 1.5 min; (3) 5 min; (4) 10 min; (5) 25 min; (6) 40 min. Note disappearance of γ-chains concomitant with appearance of γ-γ dimers. α-chain dimers are strongest in (3); α-chain trimers are strong in (6).

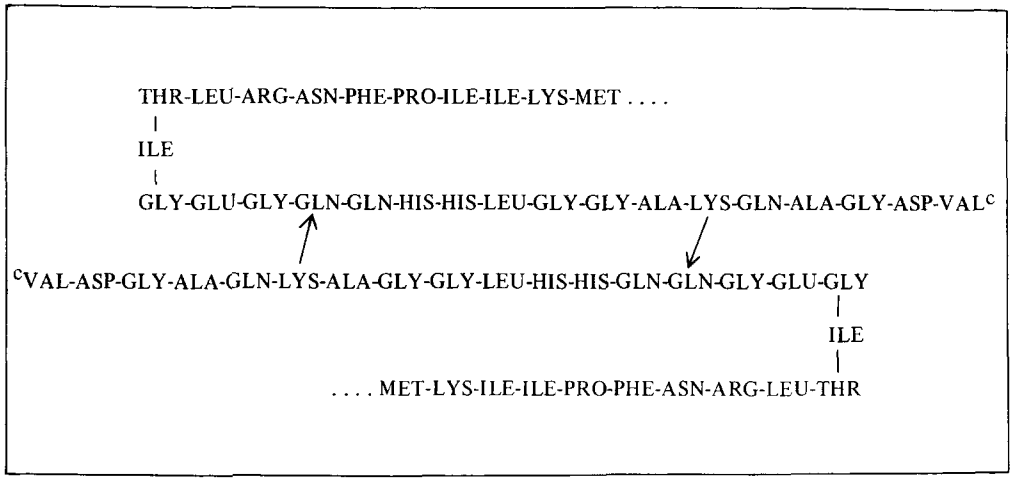

Fig. 7. Amino acid sequence of carboxy-terminal segments of γ-chain from human fibrin(ogen) showing reciprocal crosslinks (arrows) between antiparallel neighboring chains.

helical configurations are aligned in an antiparallel manner the two segments are perfectly juxtaposed for interaction (Fig. 8). It is significant that *all* γ-chains can become involved in this exact same crosslinking situation, indicating that all the units in the fibrin gel have the same orientation in this regard (Fig. 9). We have argued elsewhere (20) that these segments are contact sites in the initial stages of fibrin formation (Fig. 10).

The importance of the reciprocal bonding cannot be overstated, since it is this feature which clearly specifies the requirement for an antiparallel chain arrangement. Thus, if we return to the problem of DS and DL starting units, it is a simple matter to envision antiparallel chain arrangement and reciprocal bonding for the DS unit in a staggered overlap polymer, but it is difficult, if not impossible, to accommodate a DL-half-stagger assembly to this kind of constraint. We feel that these findings clearly favor fibrinogen models of the DS variety.

There is not yet enough evidence to make decisive judgments about chain arrangement in the case of α-chain crosslinking, although multimer formation (the bonding together of many chains) demands that the donor and acceptor sites be spatially distant so that different parts of a given chain can be attached to at least two other chains and/or that there be two or more acceptor sites per chain. In fact, there is evidence for at least two acceptor sites (19, 20, 21). In either situation, accommodation can be made for DS *or* DL molecules aligned in a half-staggered overlap (Fig. 11). The crosslinking of α-chains occurs much more sluggishly than γ-chain crosslinking, and it is possible that it occurs only after the aggregation of families of intermediate polymers (20).

In this brief article, I have tried to delineate two fundamental problems associated with the fibrinogen/fibrin transformation: (a) the directional nature of the starting molecule and (b) the orientation of these units after their assembly into fibrin. I have emphasized the constraints imposed on the system by the existence of reciprocally crosslinked

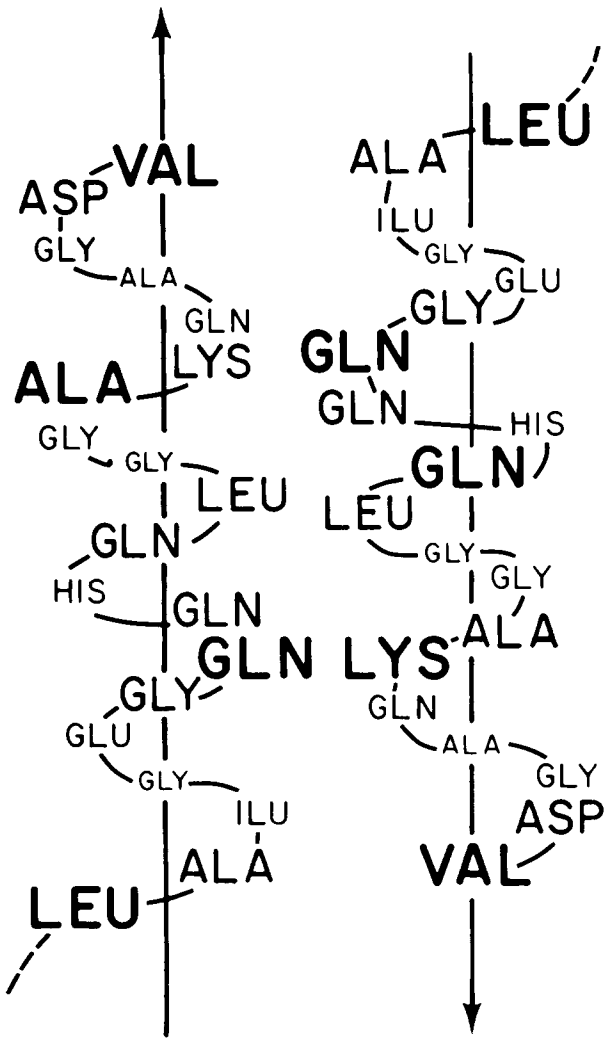

Fig. 8. Schematic depiction of two antiparallel γ-chain segments in α-helical conformations. Note how the glutamine and lysine sidechains are positioned conveniently for reciprocal bridging. The sequences shown are for the bovine molecule.

chains which have to be oriented in an antiparallel fashion, and taking this fact together with many other observations, I have argued for a starting molecule in which the twofold axis coincides with the short dimension of an elongated particle. I have also suggested that electric birefringence experiments may yet establish the existence of such a molecule.

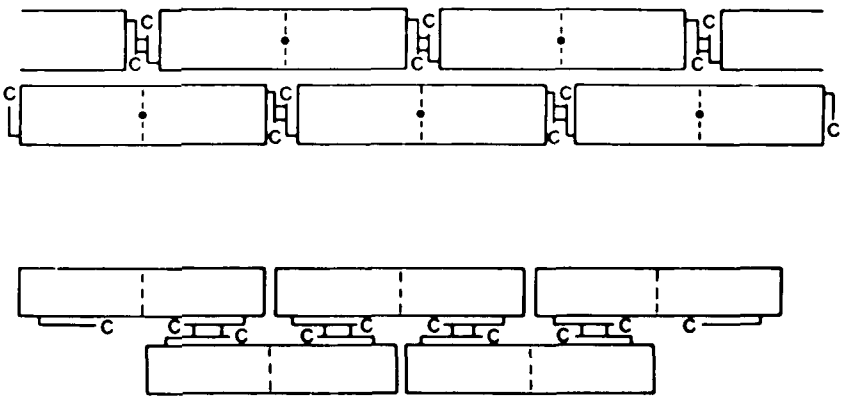

Fig. 9. Two different ways reciprocal γ-γ dimers could be involved in the formation of intermediate polymers involving staggered overlaps. In both cases the parent molecules have symmetries of the DS type. It is not immediately obvious how reciprocal linkages of this sort could be formed during the propagation of a (two-molecule thick) staggered overlap polymer involving molecules of the DL type.

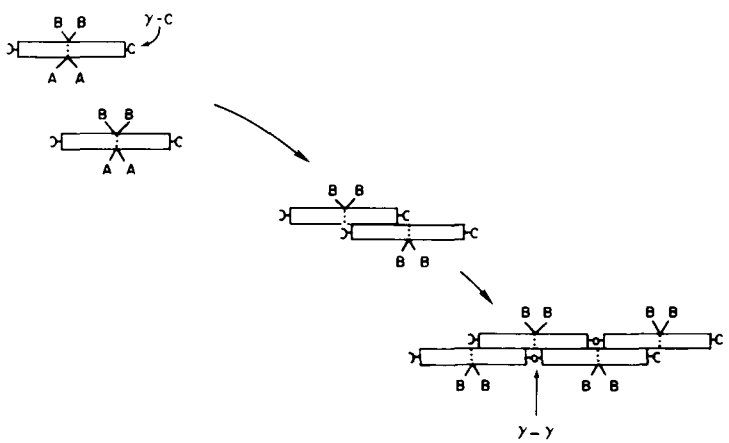

Fig. 10. Depiction of how formation of a two-molecule thick intermediate polymer formed by staggered overlaps involves two different sets of contacts. One of these comes into play at the original dimerization step and also at all subsequent lateral associations. The second set involves end-to-end abutment and takes place as a consequence of the lateral associations rather than as a primary event in its own right. In this sketch the juxtaposition of γ-chain crosslinking sites is depicted as taking place during the end-to-end abutment step.

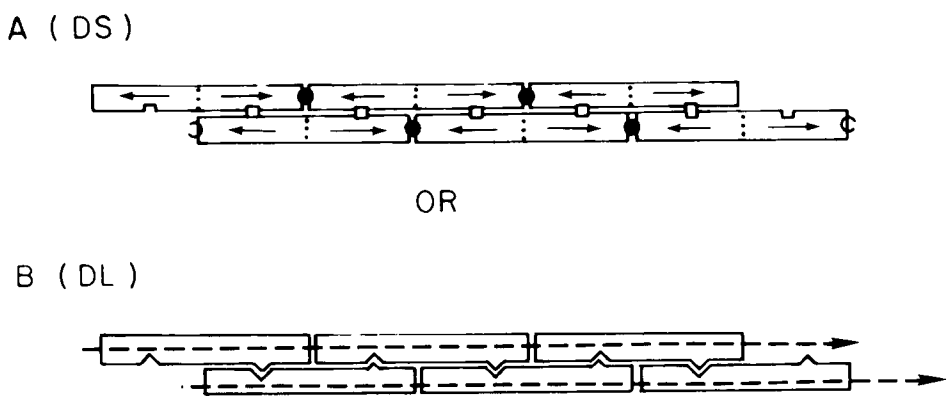

Fig. 11. Schematic depiction showing how molecules of either the DS type (A, top) or the DL type (B, bottom) can be involved in α-chain multimer formation. In the case of the DS type, however, at least two different donor and acceptor sites must exist on each half of a molecule, whereas the DL system could be managed with one of each (although more could exist, of course). In either case the multimeric array could also be formed by exclusively lateral operations after the association of intermediate polymers (20).

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