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### Research Article

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# Evidence for a Structural Requirement for the Aggregation of Platelets by Collagen

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**ABSTRACT** This study investigates whether soluble collagen can initiate platelet aggregation or whether a higher degree of polymerization is required. Purified rat skin collagen was prepared in four states. Soluble monomeric collagen, containing 2  $\mu$ M calcium chloride, was maintained at 4°C until use. A previously uncharacterized form of collagen, soluble microfibrillar collagen, was prepared from monomeric collagen containing calcium chloride by allowing it to polymerize at 23°C. Viscometric and electron microscopic characterization of microfibrillar collagen indicated polymerization to ordered native filaments. Particulate native macrofibrillar collagen was prepared from monomeric collagen by allowing it to polymerize at 37°C in the absence of calcium. Particulate collagen, in which the fibers were randomly associated, was prepared by salt precipitation of calcium-free monomeric collagen. Microfibrillar and native macrofibrillar collagen initiated platelet aggregation, with a lag phase of approximately 60 s. Monomeric collagen initiated aggregation with a lag phase of approximately 180 s. The duration of the lag phase for platelet aggregation initiated by monomeric collagen was independent of the dose. Salt-precipitated particulate collagen did not initiate platelet aggregation. Agents which prolong the transition from monomeric collagen to fibrillar collagen (urea, arginine) retarded or prevented the aggregation of platelets by monomeric collagen. Sodium borohydride, which stabilizes the intra- and intermolecular cross-links of collagen did not affect platelet aggregation. Penicillamine, which displaces the intermolecular cross-links and binds the intramolecular cross-links of collagen, did not prevent platelet aggregation. The data suggest that an architectural requirement exists for the initiation of self-perpetuating platelet ag-

gregation; that tropocollagen units do not fulfill this requirement; that a soluble collagen preparation, microfibrillar collagen, contains the minimal structural unit; and that cross-linkages within collagen do not play a critical role in platelet aggregation.

## INTRODUCTION

The normal hemostatic sequence begins when a blood vessel is injured and culminates in the formation of a fibrin-platelet meshwork that is a structural barrier to the escape of blood. The trigger that initiates these hemostatic reactions is the separation or disruption of the endothelium, thereby allowing flowing blood to contact subendothelial connective tissue. Platelets immediately adjacent to the site of injury adhere to the connective tissue. Subsequent reactions initiated by the adherence of platelets to connective tissue lead to the formation of a definitive hemostatic plug. Initially, Hugues (1) suggested, as has been repeatedly confirmed in other laboratories (2-5), that collagen was the specific component of connective tissue to which platelets first adhere.

There have been several reports which have examined the properties of collagen necessary to promote platelet adherence. Initially, it was shown that heat-denatured or collagenase-digested preparations were inactive (2, 5). Nossel and his associates (6-8) showed that treatment of collagen with reagents that either blocked or oxidized the free  $\epsilon$ -amino groups of collagen, impaired platelet-aggregating activity. They suggested a specific role for these amino groups, rigidly spaced in the collagen molecule, in the reaction between platelets and collagen. Jamieson, Urban, and Barber (9, 10) have described UDPGlucosyltransferases bound to platelet membranes which link glucose or galactose to collagen. The suggestion that the carbohydrate side chain of collagen is essential in the platelet-collagen reaction has been supported by Chesney, Harper, and Colman (11).

Within the past few years the structure of collagen has been clarified (12, 13). Schmitt (14) has proposed

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that the reconstitution of collagen *in vitro* proceeds through three states of increasing complexity: monomeric, microfibrillar, and particulate. Monomeric collagen (tropocollagen, mol wt 300,000) is a triple-stranded coiled coil composed of  $\alpha$ - (single chain) and  $\beta$ - (double chain intramolecularly cross-linked) subunits. Microfibrillar collagen is postulated to form when monomeric collagen polymerizes between 12 and 27°C. It is thought to exist as a highly ordered structure of varying molecular weights ranging from  $10^8$  to  $10^9$ . Soluble microfibrils are capable of additional association to form macrofibrils which are particulate. This further polymerization occurs slowly at 23°C but it is accelerated at 37°C. Macrofibril formation can be inhibited by low concentrations of calcium (15).

We have prepared purified rat skin collagen in several states of association and have utilized them to examine which can initiate platelet aggregation. Our data indicate that a soluble, transitional structure more complex than monomeric collagen is required to initiate platelet aggregation. Our findings suggest that modifications of collagen which impair its platelet-aggregating ability may do so at least in part by preventing the polymerization of collagen into a critical conformation which supports aggregation.

## METHODS

Neutral salt and acetic acid-soluble rat skin collagen was prepared and purified by a minor modification of the method of Gallop and Seifter (16). Amino acid analysis of the collagen revealed residue content consistent with published values for purified rat skin collagen. The purified collagen contained 320 residues of glycine per 1,000 amino acids and had a hydroxyproline to proline ratio of 0.9, characteristic of collagen (17). Purified, lyophilized collagen was reconstituted at a final concentration of 1  $\mu\text{g}/\mu\text{l}$  (based on hydroxyproline content) (18) in 0.05 N acetic acid at 4°C. This material was treated in four ways to yield different states of collagen. Monomeric collagen was prepared immediately by adjusting the pH of the reconstituted collagen slowly to pH 7.2 with 0.1 N NaOH, and then making the solution 2  $\mu\text{M}$  in  $\text{CaCl}_2$  to prevent transition to microfibrils. The monomeric collagen was maintained at 4°C until used. Microfibrillar collagen was prepared from monomeric collagen by allowing portions to stand at 23°C for 90 min. At the concentration of collagen (1 mg/ml) and calcium (2  $\mu\text{M}$ ) employed, macrofibril formation does not occur at 37°C, and the collagen solution remains soluble indefinitely. In plasma, no fibril formation was detected over the range 0.01–0.50 mg/ml. Macrofibrillar, native particulate collagen was prepared by a modification of the previous techniques. Reconstituted collagen was prepared at 4°C and adjusted to pH 7.2 in the absence of calcium. It was then allowed to polymerize at 37°C for 20 min. After centrifugation at 15,000 *g* the supernatant material was discarded. The pellet was resuspended in distilled water, finely minced, and utilized as a particulate suspension. The preparation was maintained at 4°C until used. Material so prepared has been characterized as having ultrastructure and cross-links indistinguishable from native collagen (19, 20). Another form of particulate

collagen was prepared from monomeric collagen by the addition at 4°C of 1 part of a solution of cold NaCl (20 g/100 ml) to 4 parts of monomeric collagen. The fluffy white precipitate which formed on slow stirring for an hour was centrifuged at 2,500 *g* for 20 min. The precipitate was washed three times with cold water and was finally resuspended in deionized water at a final concentration of 1  $\mu\text{g}/\mu\text{l}$ . This material was used as a fine particulate suspension, maintained at 4°C until used.

The physical states of the various preparations of collagen were assessed by electron microscopy and by viscometry. The collagen preparations were prepared for electron microscopy by a modification of the method of Rautenberg and Kühn (21). Wet preparations were stained directly on a Formvar-coated grid with phosphotungstic acid (0.4 g/100 ml, pH 3.5) for 12 min. The grid was then washed with water and was counterstained with uranyl acetate (1 g/100 ml) for 10 min. Micrographs were made with an RCA EMU-3G electron microscope. Monomeric preparations gave barely detectable staining patterns at the resolution employed. Macrofibrillar, native particulate collagen appeared as broad sheets of typical collagen fibers, arranged in a dense meshwork. Particulate, salt-precipitated collagen appear as an amorphous material, in which no native, cross-banded fibrils could be seen. This appearance was consistent with previous descriptions of similarly prepared material (22). The electron micrographic appearance of microfibrillar collagen is shown in Fig. 1. The fibrils were approximately 600 Å wide, with lengths ranging from 3 to 30  $\mu\text{m}$  (mean of 50 measurements, 11.5  $\mu\text{m} \pm 2.7$  SD).

The viscometric properties of the two soluble collagen preparations, monomeric and microfibrillar, were carried out in a size 100 Ostwald-Cannon-Fenske capillary viscometer. Temperature was controlled to within 0.05°C in a constant temperature, circulating water bath. Before being tested, all collagen solutions were centrifuged at 30,000 *g* for 30 min. The solutions were then pre-equilibrated at the temperature to be tested. Each preparation was tested, in triplicate, at five concentrations. The reduced intrinsic specific viscosity was determined by extrapolation (Fig. 2). When reconstituted, monomeric collagen was assessed at 4°C, pH 3.8, an intrinsic specific viscosity of 15.2 dl/g was observed, in accordance with published values for purified native tropocollagen (23). Adjusting the pH of monomeric collagen to 7.1 at 4°C increased the specific intrinsic viscosity to 18.2 dl/g. When the preparation was allowed to equilibrate for 30 min at 23°C the intrinsic viscosity rose to 27.4 dl/g. There was no further increase in viscosity on standing for as long as 90 min at 23°C. The increase in viscosity was reversible. Reducing the temperature of the collagen (after 60 min of standing at 23°C) to 4°C lowered the intrinsic viscosity to its original value of 18.2 dl/g. After maintaining the temperature at 4°C for a further 60 min, the temperature was then raised again to 23°C, and the reduced intrinsic viscosity rose to 27.0 dl/g as before.

The combined electron microscopic and viscometric data thus established the four forms of collagen as distinct from each other.

Platelet-rich plasma (PRP)<sup>1</sup> was prepared from normal subjects by a two-syringe technique with plastic disposable syringes and thin-walled 19-gauge needles. Whole blood was transferred to plastic centrifuge tubes containing 0.5

<sup>1</sup>Abbreviation used in this paper: PRP, platelet-rich plasma.

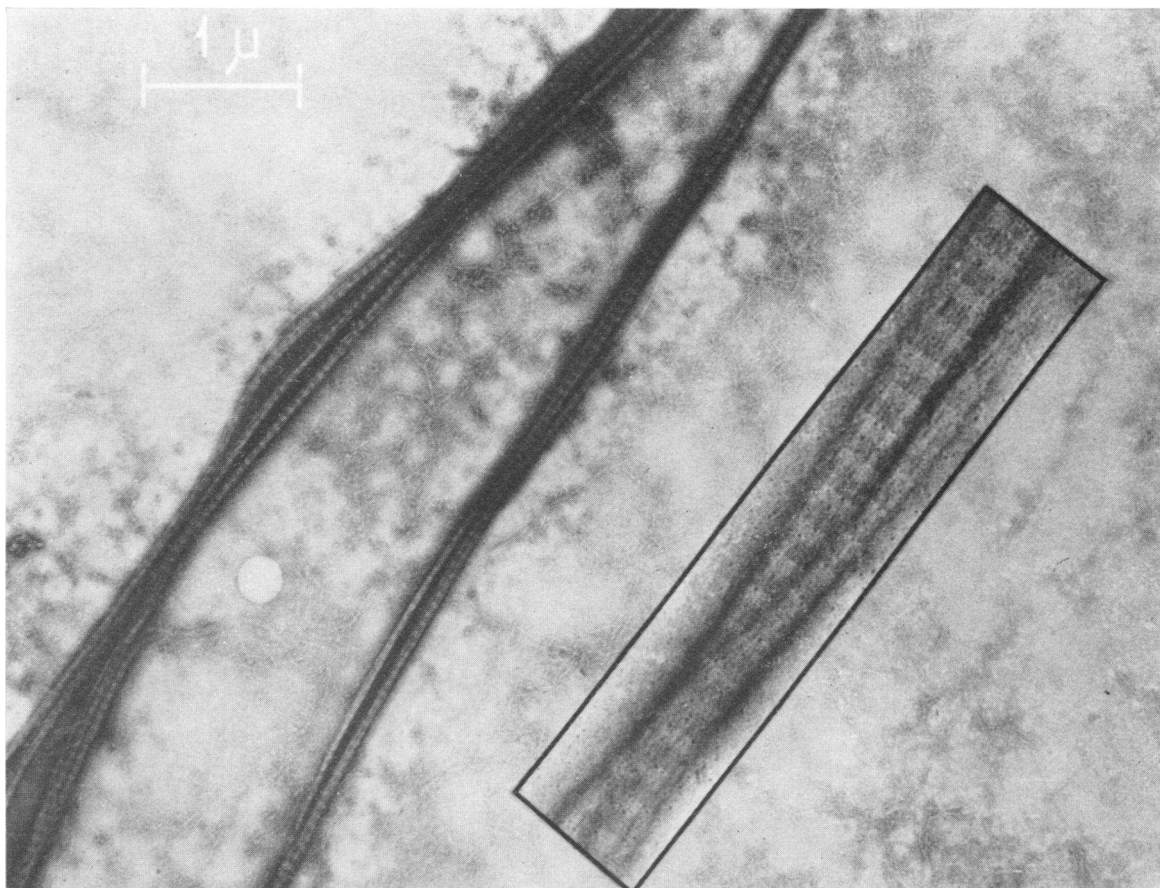


FIGURE 1 Microfibrillar collagen. Acid- and salt-soluble collagen ( $1 \mu\text{g}/\mu\text{l}$ ) containing  $2 \mu\text{M}$   $\text{CaCl}_2$  was adjusted to pH 7.2 at  $4^\circ\text{C}$  and allowed to polymerize at  $23^\circ\text{C}$ . Wet preparations stained directly on grid. Insert: magnification,  $\times 10$ . Original magnification,  $\times 20,300$ .

ml of 3.8% trisodium citrate. Sufficient blood was added to reach a volume of 5 ml, and the anticoagulated blood was centrifuged at room temperature at  $180 g$  for 10 min. The PRP was then removed with silicone-coated Pasteur pipettes. The platelet count of the PRP ranged from 150,000 to 400,000/ $\mu\text{l}$ . Platelet-poor plasma was prepared in a similar fashion except that the whole blood was centrifuged at  $2,400 g$  for 20 min.

The interaction of collagen with platelets was assayed by the technique of platelet aggregation (24, 25). The change in optical density of stirred PRP induced by platelet aggregation was measured at  $37^\circ\text{C}$  in a platelet aggregometer (Chrono-Log Corp., Broomall, Pa.) with a 609-nm red filter. 0.5 ml of PRP was added to a 0.312-inch diameter cuvette containing a  $1 \times 4$ -mm stirring bar (cut segment of a paper clip). All collagen preparations were in 0.05 M Na acetate, pH 7.1. When used, calcium concentration was  $2 \mu\text{M}$ . A measured volume of the collagen preparation to be tested was added, and changes in optical density were continuously recorded.

The final change in optical density is a complex function, one of platelet adhesion to collagen and then of a series of self-perpetuating platelet-aggregation reactions initiated by the adherence of platelets to collagen. This technique, therefore, is only an indirect assay of platelet

adherence to collagen, the necessary first step in the sequence.

In some experiments urea was added to the PRP. Urea solutions were freshly prepared and filtered through a mixed bed resin (Dowex  $50 \times 8$ , Dowex  $1 \times 8$ ) to remove cyanate.

Sodium borohydride reduction (26) ( $0.18 \text{ mg}/\text{mg}$  of collagen) of both monomeric and microfibrillar collagen was performed under carefully controlled conditions to prevent fibril formation during the exothermic reaction. Reduction was performed at pH 7.0,  $23^\circ\text{C}$ . Excess borohydride was removed by dialysis. To prevent fibril formation during dialysis, the pH of the dialysis solution (0.05 M Na acetate) was raised gradually from 7.0 to 7.2 by sequential dialysis against solutions of pH 7.0, then 7.1, and finally pH 7.2, each with sufficient  $\text{CaCl}_2$  to attain  $2 \mu\text{M}$ . Reduction of monomeric collagen was carried out as described above except that all reactions were carried out at  $4^\circ\text{C}$ . Dialysis was then conducted as with the microfibrillar collagen. The reduced monomeric collagen was maintained at  $4^\circ\text{C}$  until use.

To assay the degree of reduction, standardized tritiated sodium borohydride (26) was added to both forms of soluble collagen. The nonspecific radioactivity was removed by dialysis, and the bound radioactivity was measured after

acid and alkaline hydrolysis of the collagen, separation of the amino acids on an amino acid analyzer equipped with a stream-splitter, and detection of the radioactivity in the alcohols and secondary amines formed during the reduction of the native cross-links by liquid scintillation spectrometry.

## RESULTS

Fig. 3 depicts representative tracings produced by the addition of 30  $\mu\text{g}$  of the native particulate, microfibrillar, or monomeric collagen. Both particulate macrofibrillar and soluble microfibrillar collagen initiate aggregation in 60 s or less. Monomeric collagen also initiated aggregation, but only after a delay in excess of 3 min. In contrast, particulate, salt-precipitated collagen (not shown) did not aggregate platelets at any concentration employed (30–300  $\mu\text{g}$ ).

To test the reproducibility of these observations, we examined the time required for the initiation of aggregation (lag phase) after the addition of various forms of collagen to PRP from 30 different individuals. The lag phases observed were: macrofibrillar collagen,  $52 \pm 20$  s (mean  $\pm 2$  SD); microfibrillar collagen,  $53 \pm 14$  s; and monomeric collagen,  $179 \pm 14$  s. There was no significant difference between macrofibrillar and microfibrillar collagen. The lag phase observed with monomeric collagen was highly significantly different ( $P < 0.001$ , Student's *t*-test) from those of particulate and microfibrillar collagen.

A representative dose-response curve for increasing amounts of monomeric collagen is shown in Fig. 4. 1  $\mu\text{g}$  of monomer did not initiate aggregation, but higher

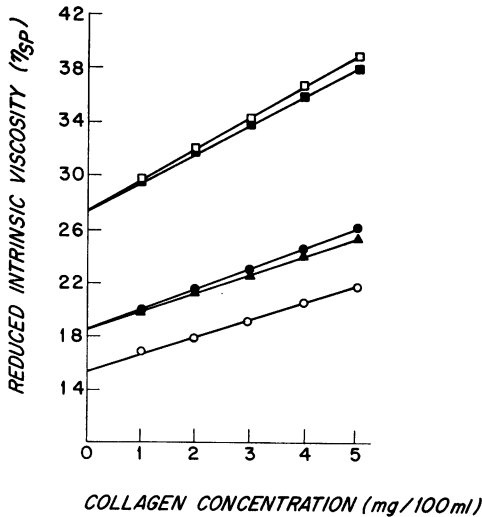


FIGURE 2 Reduced intrinsic specific viscosity of soluble collagen preparations. All determinations performed in triplicate. ○, Acid-soluble collagen (2  $\mu\text{M}$   $\text{CaCl}_2$ ), 4°C, pH 3.8; ●, same preparation, pH 7.1, 4°C; □, same preparation, pH 7.1, 23°C; ▲, same preparation, temperature lowered to 4°C after 60 min at 23°C; ■, same preparation, temperature restored to 23°C after 60 min at 4°C.

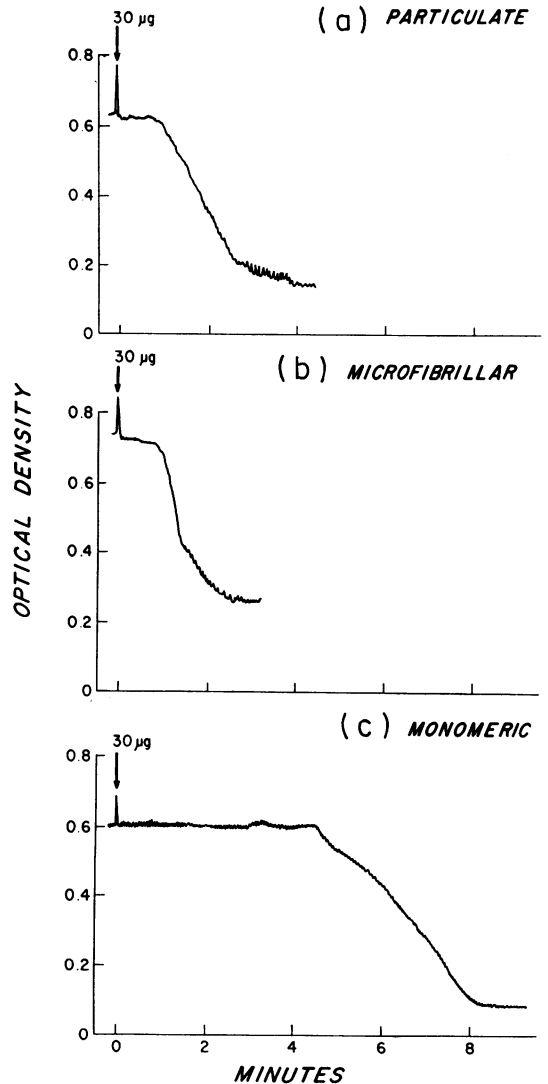


FIGURE 3 Representative tracings of platelet aggregation induced by 30  $\mu\text{g}$  of collagen added to 0.5 ml of PRP continuously stirred at 37°C. (a) Particulate native preparation; (b) soluble microfibrillar collagen; (c) soluble monomeric collagen.

doses produced increasing degrees of platelet aggregation. At all doses, however, the lag phase remained constant.

Studies were undertaken to assess the effect of urea on the transition from monomeric to microfibrillar collagen, and in parallel experiments on the initiation of platelet aggregation. The effect of urea on the polymerization of monomeric collagen (containing 2  $\mu\text{M}$   $\text{CaCl}_2$ ) was studied by viscometry (Fig. 5). The pH of a solution of monomeric collagen was raised from 3.8 to 7.1 at 4°C, and the specific intrinsic viscosity rose from an extrapolated value of 15.6 to 16.0 dl/g, consistent with our prior observations (Fig. 2). The

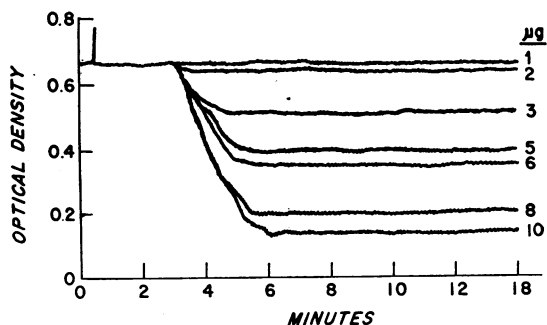


FIGURE 4 Dose-response curve for monomeric collagen. Collagen was added with a chilled syringe.

solution was then made 0.1 M in urea, and the temperature was raised to 23°C. In contrast to the prompt rise to 27.0 dl/g observed in the absence of urea (Fig. 2), the intrinsic viscosity had not changed at 30 min and rose slowly thereafter reaching 21.0 dl/g at 90 min. Urea had no effect on preformed microfibrillar collagen (Fig. 6). The specific intrinsic viscosity of a monomeric solution of collagen containing 2  $\mu$ M CaCl<sub>2</sub>, measured at pH 3.8, 4°C, was 15.6 dl/g. The solution was adjusted to pH 7.1 and allowed to equilibrate at 23°C for 30 min. The intrinsic viscosity was now 27.8 dl/g. The solution was rendered 0.1 M in urea, and the viscosity was measured after 30, 60, and 90 min standing at 23°C. There was no change in the intrinsic specific viscosity.

To assess the effect of urea on collagen-initiated platelet aggregation, sufficient urea was added to PRP to reach a final concentration of 0.1 M (no attempt was made to assess the degree of protein binding of urea). Urea did not impair the aggregation of platelets initiated

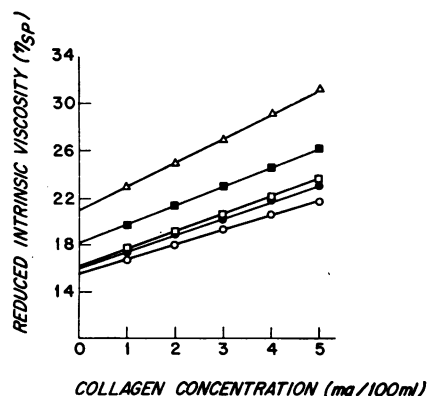


FIGURE 5 Effect of urea (0.1 M) on reduced intrinsic specific viscosity of soluble collagen. Mean of triplicate determinations. ○, acid-soluble collagen (2  $\mu$ M CaCl<sub>2</sub>), 4°C, pH 3.8; ●, same preparation rendered 0.1 M in urea, pH 7.1, 4°C; □, same preparation after 30 min at 23°C; ■, same preparation after 60 min at 23°C; △, same preparation after 90 min at 23°C.

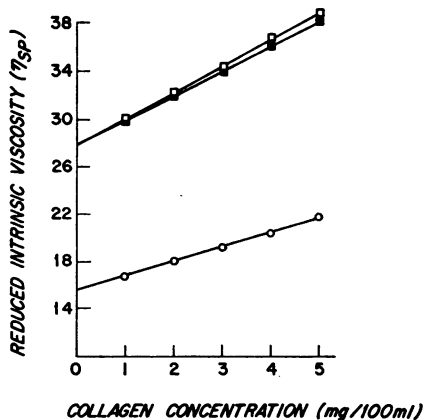


FIGURE 6 Effect of urea on preformed microfibrillar collagen: Mean of triplicate determinations. ○, acid-soluble collagen (2  $\mu$ M CaCl<sub>2</sub>, pH 3.8, 4°C; □, same preparation, pH 7.1, 23°C for 30 min; ■, same preparation rendered 0.1 M in urea, 23°C for 90 min.

by the addition of 10  $\mu$ g of microfibrillar collagen (Fig. 7a). By contrast, 0.1 M urea completely prevented the aggregation initiated by the addition of an equal amount

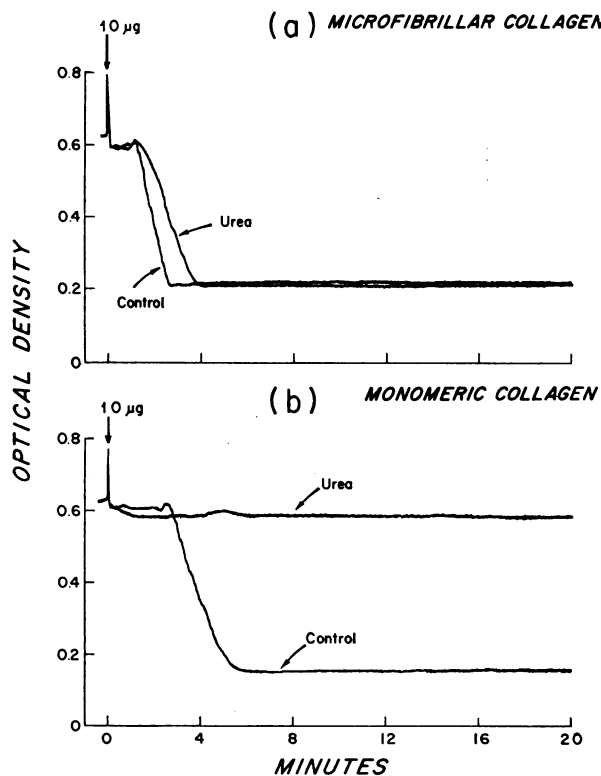


FIGURE 7 Effect of urea (0.1 M final concentration) on platelet aggregation. (a) 10  $\mu$ g microfibrillar collagen added at arrow. (b) 10  $\mu$ g monomeric collagen added at arrow.

TABLE I  
Lag Period for  $\text{NaBH}_4$ -Reduced Monomeric and  
Microfibrillar Reagent Collagens

Collagen preparation	Lag period*	
	Intact	Reduced†
Monomeric	210	195
Microfibrillar	75	60

\* Interval between addition of collagen and first detectable decrease in optical density.

† Sodium borohydride reduction (0.18 mg  $\text{NaBH}_4$ /mg collagen) was carried out as described in Methods.

0.5 ml PRP was prewarmed at 37°C for 1 min. 3  $\mu\text{g}$  of each collagen preparation was added to the PRP. For monomeric collagen, the syringe was precooled.

of monomeric collagen (Fig. 7b). The defect was not absolute. When 50  $\mu\text{g}$  of monomeric collagen was used, aggregation was delayed and did not begin until 9 min after the addition of collagen. Arginine (0.12 M) produced similar effects to urea at both concentrations of collagen.

In a further experiment monomeric collagen (10  $\mu\text{g}$ ) was incubated for 20 min in 0.5 ml of PRP containing 0.1 M urea (final concentration), after which 5  $\mu\text{g}$  of microfibrillar collagen was added. As a control, 5  $\mu\text{g}$  of microfibrillar collagen was added to a similarly incubated sample of PRP (0.1 M urea) to which no monomeric collagen had been added. In both instances, brisk aggregation occurred with the expected lag period of approximately 1 min.

D-Penicillamine, between 0.02 and 0.2 mM, did not affect aggregation initiated by microfibrils, but increasing amount of penicillamine progressively prolonged the lag period after the addition of monomeric collagen. The first detectable prolongation of aggregation was observed at a final D-penicillamine concentration of 0.02 mM. Maximum prolongation (11 min) was observed at 0.1 mM.

In further experiments, the cross-links of both monomeric and microfibrillar collagen were reduced with sodium borohydride (Table I). There was no difference in the onset of aggregation nor in the degree of aggregation between the reduced and nonreduced forms of collagen.

## DISCUSSION

The structure of collagen and the sequence of the formation of collagen fibers from soluble monomeric precursors have been summarized recently (12, 13). Monomeric skin and tendon collagen consists of three polypeptide chains (two  $\alpha_1$  and one  $\alpha_2$ ) coiled in a helix. Each chain has an approximate molecular weight of

95,000. Throughout most of the length of the chain glycine is present as every third amino acid. Hydroxylysine residues are subsequently glycosylated by glucosyl- and galactosyltransferases to form O-galactosyl- $\beta$ -glycosyl side chains. Carbonyl groups are introduced by an enzyme(s) (27) which forms aldehydes from lysine and hydroxylysine. These aldehydes react with amino acid side chains on other collagen molecules to form intermolecular cross-links and react with each other to form aldols which serve as intramolecular cross-links. Collagen molecules in solution under physiologic conditions self-orient to facilitate the formation of aggregates.

We have utilized the previous observation of Bensusan and Hoyt (15) that low concentrations of calcium retard the formation of fibrils to prepare a form of collagen that has undergone partial but reversible polymerization. The electron microscopic and viscometric data presented in Figs. 1 and 2 clearly identify microfibrillar collagen as a more highly ordered structure than monomeric collagen. Because of the marked axial asymmetry (observed on the electron photomicrographs) of the microfibrillar preparation, the measurements of viscosity may actually underestimate the degree of polymerization by as much as 50% (28, 29), but the qualitative differences between monomeric and microfibrillar collagen are clear-cut. The reversibility of the microfibrillar preparation, its solubility, and its electron microscopic appearance differentiate it as well from native particulate collagen formed in the absence of calcium.

Our data demonstrate that our preparation of microfibrillar collagen is a potent initiator of platelet aggregation, as effective as native particulate collagen. This observation suggests that the platelets adhere as well to microfibrillar as to particulate collagen. Our observation that particulate salt-precipitated collagen does not initiate platelet aggregation indicates that randomly oriented native collagen aggregates do not afford an appropriate surface for platelet aggregation. The difference in effectiveness between monomeric and microfibrillar collagen, therefore, is not simply a function of the size of the collagen polymers.

The difference in lag phase between monomeric and microfibrillar collagen (Fig. 3) could reflect either of two possibilities. The monomeric preparation may be itself a less effective initiator of aggregation in that fewer platelets adhere to the collagen compared with the same microfibrillar preparation. Alternatively, the monomeric preparation may have no effect but must first polymerize to form microfibrils which then initiate aggregation. To examine these alternatives we determined the dose-response curve of platelet aggregation initiated by increasing amounts of monomeric collagen. It was our hypothesis that if monomers were inefficient aggregators, then increasing the dose should progres-

sively shorten the delay before aggregation becomes apparent. Conversely, since in the doses of collagen we employed the rate of polymerization of monomers to microfibrils is independent of the concentration of collagen, then if polymerization is a requirement for the initiation of aggregation by monomers, increasing the dose should not affect the lag phase. The data in Fig. 4 suggest that monomers themselves do not initiate self-perpetuating platelet aggregation but must first polymerize to microfibrils.

To test further our hypothesis that a molecular structure more complex than that present in monomeric collagen is required to initiate aggregation, experiments were carried out with urea. The viscometric data presented in Figs. 5 and 6 indicate that 0.1 M urea retards the formation of microfibrillar collagen but does not dissociate preformed microfibrillar collagen. These observations extend prior findings that urea retards fibrillar collagen formation (30). Similarly, arginine has been shown to impair the polymerization of purified collagen in a manner identical to urea (30). Our observation, that urea (Fig. 7) and arginine prevent aggregation of platelets by monomeric collagen but do not influence the effect of microfibrillar collagen, strengthens our concept that monomeric tropocollagen must first polymerize to microfibrillar collagen to initiate aggregation.

The experiment in which monomeric collagen was preincubated with PRP before the addition of microfibrillar collagen further suggests that platelets do not adhere to monomeric collagen or that if they do, they do not undergo the release reaction, since the subsequent addition of microfibrillar collagen resulted in brisk aggregation. Since the exposure of both forms of collagen to platelets occurred in the presence of urea, the lack of aggregation with monomeric collagen cannot be ascribed solely to the urea.

Penicillamine displaces the Schiff-base intermolecular cross-links in fibrillar collagen (31). Since penicillamine did not diminish the ability of microfibrillar collagen to initiate platelet aggregation, these links are not critical to the platelet-collagen interaction. The results of penicillamine treatment of monomeric collagen are more complex, since there was a prolongation of the lag phase. The fact that aggregation did occur indicates that the binding of the aldehydic groups in monomeric collagen interfered with the rate of fibril formation, but did not prevent polymerization.

Additional evidence that neither intra- nor intermolecular cross-links are essential for collagen-initiated platelet aggregation was obtained from the sodium borohydride experiments (Table I). Stabilization of both classes of bonds results from the introduction of hydrogens by sodium borohydride, converting the aldehydes

to alcohols and forming covalent secondary amines from the Schiff bases. Therefore, neither displacing bonds with penicillamine nor fixing them by reduction interfered with platelet aggregation. This evidence, taken together, strongly suggests that the lysine and hydroxylysine-derived cross-links do not have a specific function for platelet aggregation.

Nossel and his associates (6-8) treated acid-dispersible collagen with reagents (nitrous acid, glacial acetic, and acetic anhydride, 2,4-dinitrofluorobenzene, and 2,4,6-trinitrobenzenesulfonic acid) which blocked the free amino groups of lysine. They found that the treated collagen lost its ability to aggregate platelets. They concluded that free amino groups in general and specifically the  $\epsilon$ -amino groups of lysine were critical for the platelet-aggregating activity of collagen. As shown by Rauterberg and Kühn (21), however, such forms of treatment may lead to random orientation of the collagen chains in the particulate collagen that results. Our data show that particulate collagen treated in this way is ineffective in initiating aggregation. Therefore, other factors in addition to the blocking of free amino groups of lysine may have contributed to the loss of platelet-aggregating activity.

Jamieson and his co-workers (9, 10) have suggested that a platelet membrane-bound glucosyltransferase enzymatically links the platelet membrane to a glycosidic receptor on collagen. They postulate that the link forms the basis for the platelet-aggregating ability of collagen. Chesney, Harper, and Colman, (11) extended these findings by demonstrating that treatment of acid-soluble collagen with galactose oxidase impaired the subsequent ability of that material to aggregate platelets. They concluded that galactose was essential in platelet aggregation by serving as a receptor for platelet glucosyltransferase. However, Muggli and Baumgartner (32) have presented data which agree with our original report (33) and show further that galactose oxidase treatment of soluble collagen monomers impairs their ability to polymerize.

Our observations lead us to propose an alternative hypothesis to explain much of the previously reported data. We suggest that the chemical modifications of collagen which impair its ability to initiate platelet aggregation may do so by impairing the polymerization of collagen into an oriented meshwork.

In a recent report, Katzman, Kang, and Beachy (34) stated that isolated  $\alpha_1$ -monomers and the  $\alpha_1$ -CB5 fragment of  $\alpha_1$ -monomers purified from denatured collagen aggregate platelets. It is difficult to assess their data in the absence of ultrastructural or physical evidence that reassociation had not taken place in these preparations. Since a large excess of denatured protein was added in



their experiments, small amounts of renatured collagen may have been included. Their work is, as they acknowledged, contrary to that of Wilner, Nossel, and LeRoy (6), Chesney et al. (11), as well as to our own. Katzman and his associates (34) also claim that the inhibitions of platelet aggregation that they observed with glucosamine and glucosyl-galactosyl-hydroxylysine reflected binding of a specific platelet receptor site by these agents. However, it has been previously shown that glucosamine retards collagen fibril formation (35) which may explain the inhibition of aggregation by the preparation of collagen they used. Glucosyl-galactosyl-hydroxylysine also contains a sugar-protonated group and may behave as does glucosamine.

Our demonstration that a material less complex than macrofibrillar collagen can initiate platelet aggregation *in vitro* may be pertinent to hemostasis. Baumgartner and Haudenschild (36) have shown that platelets can adhere to subendothelial amorphous material. When the subendothelial amorphous material is treated with collagenase, platelets no longer adhere. They point out that the amorphous material differs from fibrillar collagen in being sensitive to trypsin, in that it is less reactive with platelets than fibrillar collagen, and it is morphologically distinct from collagen fibers. They recognize that their arguments do not exclude the presence of collagen-like proteins in the amorphous material. To the extent that microfibrillar collagen exists as a component in the amorphous subendothelial material, it may serve as an important thrombogenic surface.

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#### REFERENCES

- Hugues, J. 1960. Accolement des plaquettes au collagen. *C. R. Soc. Biol.* **154**: 866.
- Zucker, M. B., and J. Borelli. 1962. Platelet clumping produced by tissue suspensions and collagen. *Proc. Soc. Exp. Biol. Med.* **109**: 779.
- Hovig, T. 1963. Aggregation of rabbit blood platelets produced *in vitro* by saline "extract" of tendons. *Thromb. Diath. Haemorrh.* **9**: 248.
- Spaet, T. H., J. Cintron, and M. Spivack. 1962. Some properties of the platelet-connective tissue mixed agglutination reaction. *Proc. Soc. Exp. Biol. Med.* **111**: 292.
- Spaet, T. H., and M. B. Zucker. 1964. Mechanism of platelet plug formation and role of adenosine diphosphate. *Am. J. Physiol.* **206**: 1267.
- Wilner, G. C., H. L. Nossel, and E. C. LeRoy. 1968. Aggregation of platelets by collagen. *J. Clin. Invest.* **47**: 2616.
- Nossel, H. L., G. C. Wilner, and E. C. LeRoy. 1969. Importance of polar groups for initiating blood coagulation and aggregating platelets. *Nature (Lond.)* **221**: 75.
- Wilner, G. C., H. L. Nossel, and T. L. Procupez. 1971. Aggregation of platelets by collagen: polar active sites of insoluble human collagen. *Am. J. Physiol.* **220**: 1074.
- Jamieson, G. A., C. L. Urban, and A. J. Barber. 1971. Enzymatic basis for platelet: collagen adhesion as the primary step in haemostasis. *Nat. New Biol.* **234**: 5.
- Barber, A. J., and G. A. Jamieson. 1971. Platelet collagen adhesion characterization of collagen glucosyltransferase of plasma membranes of human blood platelets. *Biochim. Biophys. Acta.* **252**: 533.
- Chesney, C. M., E. Harper, and R. W. Colman. 1972. Critical role of the carbohydrate side chains of collagen in platelet aggregation. *J. Clin. Invest.* **51**: 2693.
- Grant, M. E., and D. J. Prockop. 1972. The Biosynthesis of collagen. *N. Engl. J. Med.* **286**: 194; 242; 291.
- Tanzer, M. L. 1973. Cross-linking of collagen. Endogenous aldehydes in collagen react in several ways to form a variety of unique covalent cross-links. *Science (Wash. D. C.)* **180**: 561.
- Schmitt, F. O., J. Gross, and J. H. Highberger. 1955. The crystallization of collagen in fiber formation. *Symp. Soc. Exp. Biol.* **9**: 148.
- Bensusan, H. A., and B. L. Hoyt. 1958. Effect of various parameters on the rate of formation of fibers in collagen solutions. *J. Am. Chem. Soc.* **80**: 719.
- Gallop, P. M., and S. Seifter. 1963. Preparation and properties of soluble collagen. *Methods Enzymol.* **6**: 635.
- Ramachandran, G. K. 1967. Treatise on Collagen. Academic Press, Inc., New York, 284.
- Prockop, D. J., and S. Udenfriend. 1960. A specific method for the analysis of hydroxyproline in tissues and urine. *Anal. Biochem.* **1**: 228.
- Gross, J. 1964. Organization and disorganization of collagen. *Biophys. J.* **4**: 63.
- Franzblau, C., A. H. Kang, and B. Faris. 1970. *In vitro* formation of intermolecular crosslinks in chick skin collagen. *Biochem. Biophys. Res. Commun.* **40**: 437.
- Rauterberg, J., and K. Kühn. 1968. The renaturation behaviour of modified collagen molecules. *Hoppe-Seyler's-Z. Physiol. Chem.* **349**: 611.
- Pease, D. C., and M. Bouteille. 1971. The tridimensional ultrastructure of collagenous fibrils cytochemical evidence for a carbohydrate matrix. *J. Ultrastruct. Res.* **35**: 339.
- Kahn, L. D., and L. P. Witnauer. 1966. The viscometric behaviour of solubilized calf skin collagen at low shear rates. *J. Biol. Chem.* **241**: 1784.
- Born, G. V. R., and M. J. Cross. 1963. The aggregation of blood platelets. *J. Physiol. (Lond.)* **168**: 178.
- O'Brien, J. R. 1962. Platelet aggregation. Part I. Some effects of the adenosine phosphates thrombin and cocaine upon platelet adhesiveness. II. Some results of a new method of study. *J. Clin. Pathol.* **15**: 446, 452.
- Gallop, P. M., and O. O. Blumfeld. 1966. Amino aldehydes in tropocollagen: the nature of a probable cross-link. *Proc. Natl. Acad. Sci. U. S. A.* **56**: 1260.
- Narayanan, A. S., R. C. Siegel, and G. R. Martin. 1972. On the Inhibition of lysyl oxidase by B-amino propionitrile. *Biochem. Biophys. Res. Commun.* **46**: 745.
- Scheraga, H. A. 1955. Observations on the viscometric properties of soluble collagen. *J. Chem. Phys.* **23**: 1526.

29. Simha, R. 1940. Physical studies of purified collagen. *J. Phys. Chem.* **44**: 25.
30. Gross, J., and D. Kirk. 1958. The heat precipitation of collagen from neutral salt solutions: some rate-regulating factors. *J. Biol. Chem.* **233**: 355.
31. Nimni, M. E. 1965. Effect of penicillamine on collagen. *Biochim. Biophys. Acta.* **111**: 516.
32. Muggli, R., and H. R. Baumgartner. 1973. Collagen induced platelet aggregation requirement for tropocollagen aggregates. Proceedings of the 4th International Congress on Thrombosis and Haemostasis, Vienna. 86.
33. Jaffe, R., and D. Deykin. 1972. Platelet aggregation initiated by microfibrillar collagen. *Fed. Proc.* **31**: 241.
34. Katzman, R. L., A. H. Kang, and E. H. Beachy. 1973. Collagen-induced platelet aggregation involvement of an active glycopeptide fragment ( $\alpha$ 1-CB5). *Science (Wash. D. C.)*. **181**: 670.
35. Le Gande, Y., J. P. Caen, and L. Robert. 1968. Effect of glucosamine on platelet-collagen reaction. *Proc. Soc. Exp. Biol. Med.* **127**: 941.
36. Baumgartner, H. R., and C. Haudenschild. 1972. Adhesion of platelets to subendothelium. *Ann. N. Y. Acad. Sci.* **201**: 22.