

# Collagen-Mediated Platelet Aggregation

## EVIDENCE FOR MULTIVALENT INTERACTIONS OF INTERMEDIATE SPECIFICITY BETWEEN COLLAGEN AND PLATELETS

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**ABSTRACT** We have shown previously that periodate oxidation of collagen carbohydrate does not affect its ability to aggregate platelets. We now describe an additional characterization of periodate-modified collagen which demonstrates that collagen devoid of intact carbohydrate is fully capable of fibril formation, and we confirm its capacity to initiate platelet aggregation. Furthermore, we demonstrate that the platelet aggregating abilities of Types I, II, and III fibrillar collagen are quite similar despite differences in carbohydrate content and amino acid sequence. We also demonstrate that monomeric, pepsin-solubilized Type I human collagen is ineffective in inhibiting aggregation by preformed fibrils derived from the same molecule, thus establishing that the affinity of platelets for collagen depends upon prior polymerization of collagen. We interpret these and other findings to demonstrate that the hydroxyllysyl glycoside regions of collagen are not highly specific sites involved in platelet-collagen interactions leading to "physiological" aggregation, and that the possibility must be considered that multiple interactions involving collagen sites of comparatively low structural specificity may be the initiating events in release of platelet ADP and the ensuing aggregation.

### INTRODUCTION

Although it is well established that adhesion of blood platelets to collagen initiates platelet aggregation, the chemical basis for the platelet-collagen adhesion reaction is not understood. Aggregating activity of collagen preparations is observed when the collagen is in its native triple-helical conformation and self associated to

form fibrils (1-3). Speculation on the chemical nature of the site on collagen to which platelets bind has centered on the hydroxyllysyl glycoside region. The demonstration of a platelet membrane glucosyltransferase active toward hydroxyllysyl galactose has led Jamieson et al. (4) and Barber et al. (5), to suggest that platelet-collagen adhesion is a special case of the Roseman hypothesis (6) that cell to cell adhesion is based on interaction of cell specific glycosyltransferases with cell-specific heterosaccharides.

In an earlier study we reported (7) that periodate oxidation of collagen failed to diminish its aggregating potential. Other laboratories (8, 9), however, have reported evidence which appears to support a direct involvement of periodate modification on collagen structure and on fibril formation. We have now extended our earlier study of the periodate reaction to include an examination of its effect on collagen structure as well as on the collagen-platelet interaction. We have also examined the ability of various collagen types and of the monomeric and fibrillar forms of collagen to interact with platelets in the light of the postulated role of hydroxyllysyl glycosides in initiation of platelet aggregation.

### METHODS

**Platelet aggregation.** This determination, based on changes in the turbidity of platelet-rich human plasma, is essentially the routine clinical laboratory procedure. The experimental parameters we have employed are: (a) the quantity of collagen or related material that must be added to achieve aggregation; (b) the lag time between addition of such a sample and the beginning of aggregation; and (c) the percentage of aggregation achieved as compared with the extent of aggregation obtained in the presence of an excess of native fibrillar collagen. In a typical experiment 25  $\mu$ l of aggregating agent was added to 500  $\mu$ l platelet-rich plasma, prepared as previously described (7), in the aggregometer.

**Collagen preparations.** Human Types I, II, and III collagens prepared by pepsin digestion (10) and human Type I

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collagen prepared by acid extraction (11) were generous gifts of Dr. E. J. Miller, University of Alabama, Birmingham, Ala. Salt- and acid-soluble collagens from lathyritic rat skin were prepared by the method of Bornstein and Piez (11). Human skin insoluble collagen was obtained by a modification (12) of the method of Veis et al. (13).

**Aggregating agents.** Monomeric collagens were dissolved in 0.05% acetic acid. Fibrillar collagens were prepared by dialysis of collagen solutions in 3% acetic acid against 0.02 M  $\text{Na}_2\text{HPO}_4$  as described by Bruns and Gross (14). Insoluble collagen was homogenized in a Potter-Elvehjem tissue grinder in 0.9% NaCl.

**Periodate oxidation.** The conditions were slightly modified from those previously reported (7). A concentrated solution of aqueous-sodium periodate was added to rat skin salt-soluble collagen dissolved in 0.05 M sodium acetate pH 4.5 at 2 mg/ml to a final concentration of 30 mM. The reaction was continued with gentle stirring in a foil-wrapped flask for 30 h at 5°C. The reaction was terminated by the addition of a twofold molar excess of ethylene glycol followed by dialysis for 48 h against 3% acetic acid in the cold.

**Borohydride reduction.** Periodate-oxidized collagen prepared as described above was dialyzed in the cold, without prior drying, against 0.05 M sodium phosphate buffer, pH 7.4, containing 0.2 M NaCl. Then 10 mg of sodium borohydride was added to 10 ml of solution containing 1.8 mg/ml periodate-oxidized collagen. The mixture was stirred in an ice bath for 1 h. After acidification with glacial acetic acid the preparation was dialyzed against 3% acetic acid in the cold.

**Thermal denaturation studies.** Circular dichroic spectra were obtained at temperatures ranging from 15° to 40°C using a Cary 60 spectropolarimeter (Varian Associates, Palo Alto, Calif.) with a model 6002 CD attachment and a thermostatted cell holder. Collagen samples were dissolved in 3% acetic acid at a concentration of 0.45 mg/ml. The path length was 0.5 mm. Samples were allowed to equilibrate for 15 min at each temperature. The spectrum was then recorded twice in succession to insure that equilibrium was obtained. Only values at 225 nm were plotted in Fig. 1 to indicate changes related to denaturation.

**Electron microscopy of collagen fibrils.** Native type fibrils were prepared by dialysis against 0.02 M  $\text{Na}_2\text{HPO}_4$  as described by Bruns and Gross (14). A drop of the fibril suspension was applied to 200 mesh carbon-coated nickel grids (Ladd Research Industries, Burlington, Vt.). The preparations were stained with phosphotungstic acid and uranyl acetate as described by Stark et al. (15).

**Gel electrophoresis.** Electrophoresis was conducted in the presence of sodium dodecyl sulfate on gels containing 5% acrylamide according to the procedure of Weber et al. (16). The gels were stained for protein with Coomassie Blue by the method of Fairbanks et al. (17).

**Amino acid and carbohydrate analysis.** Amino acid analyses were performed on a Beckman 120 Amino Acid Analyzer (Beckman Instruments, Inc., Fullerton, Calif.) after hydrolysis in 6 N HCl for 24 h at 110°C (18). The collagen hydroxylysyl glycosides were determined on the analyzer after hydrolysis in 2 N NaOH for 24 h at 110°C in Teflon tubes (19). Glucose and galactose contents of collagen were assayed with glucostat and galactostat reagent kits (Worthington Biochemical Corp., Freehold, N.J.). The samples were hydrolyzed in 2 N HCl for 4 h at 110°C (20), neutralized with Dowex AGI-X8 ( $\text{HCO}_3^-$  form; Bio-Rad Laboratories, Richmond, Calif.), filtered, and lyophilized. The lyophilized materials were then assayed for glucose and galactose.

**Protein determinations.** Protein concentrations of fibril preparations were determined by the biuret reaction (21), using rat skin acid-soluble collagen as a standard.

## RESULTS

**Characterization of periodate-oxidized rat skin salt-soluble collagen.** Oxidation of rat skin salt-soluble collagen with periodate at pH 4.5 leads to destruction of almost all of the galactose and glucose in collagen. The small residual quantity of glucose,  $\approx 8\%$ , indicated in Table I, appears to reflect a background absorbance problem in the gluco-stat assay. The hydroxylysine residues of collagen which are unsubstituted with carbohydrate are also modified by periodate through oxidative deamination and introduction of an aldehyde in the five position.

Periodate-oxidized collagen produced under the conditions described here exhibits normal melting behavior as measured by circular dichroism (Fig. 1) and readily forms fibrils on dialysis against 0.02 M  $\text{Na}_2\text{HPO}_4$ . More than 90% of the protein in such periodate oxidized- and control-fibril preparations was sedimented by centrifugation at 12,000 g for 15 min. Some difficulty was experienced with aggregation of the periodate-oxidized collagen at neutral pH after fibril formation which is believed to reflect additional intermolecular cross-linking involving the additional aldehyde groups generated by the periodate oxidation. Polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulfate confirmed the presence of increased amounts of beta and gamma chains in the periodate-treated collagen. The periodate-oxidized collagen was therefore reduced with sodium borohydride and the resulting product was much easier to store and did not aggregate at neutral pH. Both the periodate-oxidized and the periodate-oxidized, borohydride-reduced collagen formed native type fibrils upon dialysis against 0.02 M  $\text{Na}_2\text{HPO}_4$  (Fig. 2).

**Platelet aggregating potential of modified rat skin salt-soluble collagen.** Fibrillar forms of normal, periodate-oxidized, and periodate-oxidized, borohydride-reduced rat skin salt-soluble collagens were produced by dialysis against phosphate buffer, and each preparation tested at a series of concentrations for its ability to induce platelet aggregation. As seen in Fig. 3, all forms of collagen were fully effective in inducing aggregation.

**Comparison of the platelet aggregating potential of Types I, II, and III human collagens.** Several dis-

TABLE I  
Carbohydrate and Hydroxylysine Analyses of Rat Skin Salt-Soluble Collagen before and after Periodate Oxidation

Collagen	Glucose	Galactose	Hydroxylysine
	% (wt/wt)	% (wt/wt)	residues/1,000 residues
Control	0.120	0.190	5.0
Periodate oxidized	0.010	0.002	0.8

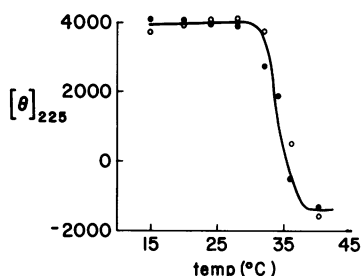


FIGURE 1 Thermal denaturation of untreated (●) and periodate oxidized (○) rat skin salt-soluble collagen. Circular dichroic spectra of collagen solutions in 3% acetic acid were recorded at the indicated temperatures after a 15-min equilibration period. The values obtained at 225 nm were used to construct the denaturation curve.

tinct forms of collagen are now known to exist and three have been isolated and characterized in some detail (10). All three have been reported to be effective in initiating platelet aggregation (7, 22, 23). A quantitative comparison of their effectiveness is shown in Table II and Fig. 4. The table also includes carbohydrate analyses of these collagens. When these collagen are all converted to the fibrillar form before testing, they are very similar in their minimal effective aggregating concentration, with Type III appearing to be the most active. Examination of the fibrils of Types I, II, and III collagen revealed that Type I fibrils were of slightly larger diameter than those of Types II and III.

The collagen types were also compared by adding solutions of the monomeric forms to platelet-rich plasma in the aggregometer. Under these conditions, as Jaffe and Deykin (2) and Muggli and Baumgartner (1) have shown for Type I collagen, a lag period is observed during which the monomeric collagen polymerizes to collagen fibrils which at some critical size and concentration can initiate platelet aggregation. Under these conditions both the lag time and the extent of aggregation depend upon the quantity and type of collagen added. It may be seen in Fig. 4 that Type III collagen exhibits the shortest lag times, Type I (prepared by the usual acid extraction) is intermediate, and Type II collagen has the longest lag time. Types II and III collagens are typically produced from the tissue sources by pepsin digestion, which removes the small nontriple-helical regions which participate in intermolecular cross-link formation, thus solubilizing the collagen. Type I collagen may be prepared in this way as well, though most studies of this most common collagen type are made

on acid- or salt-extracted material in which the nonhelical regions remain intact. Type I collagen prepared by pepsin digestion failed to initiate platelet aggregation even at the highest levels which could be conveniently tested when added in monomeric form because it does not readily form fibrils in platelet-rich plasma during the 10–15 min period maximally observed. Fibrils are, however, formed by this material upon dialysis against phosphate buffer and such fibrils are fully active in initiating platelet aggregation (24) (Table II).

It is of interest that the ability of the three collagen types to induce platelet aggregation does not correlate well with total carbohydrate or with either the di- or monosaccharide-hydroxylysine content.

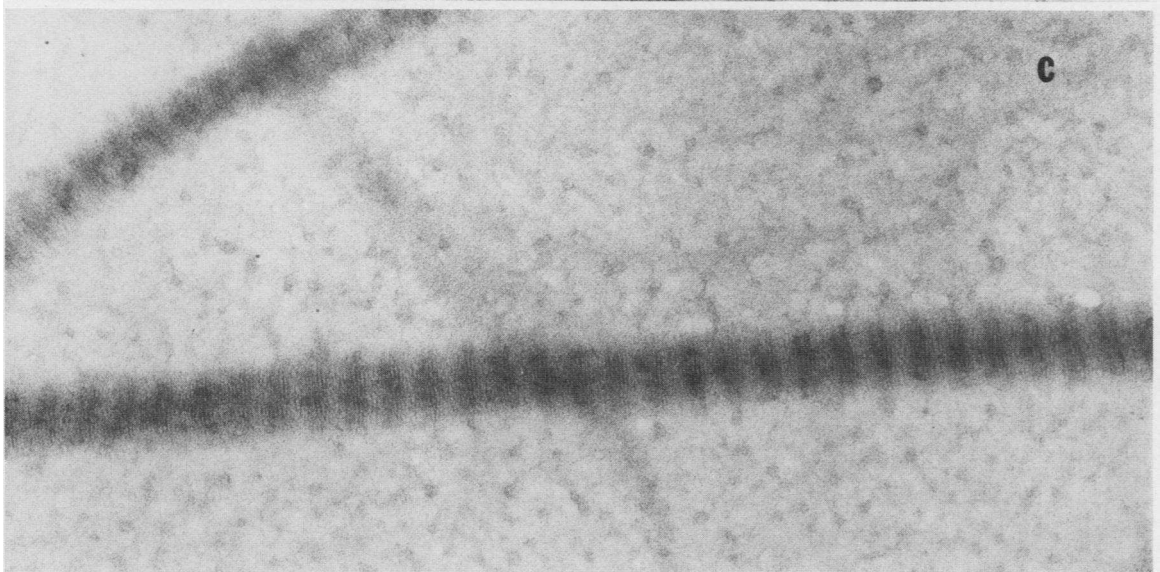
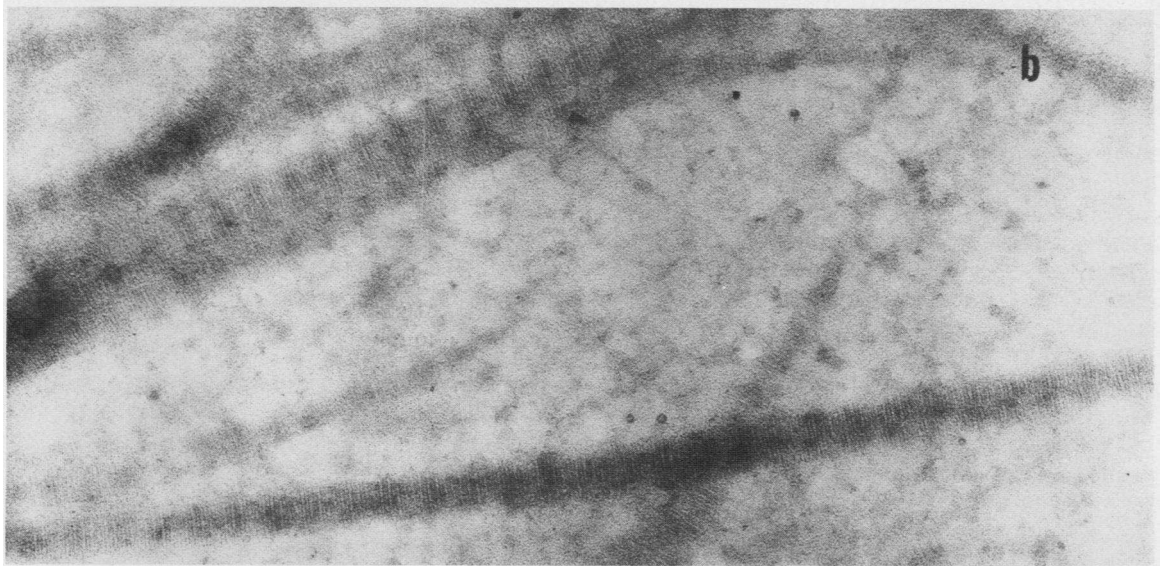
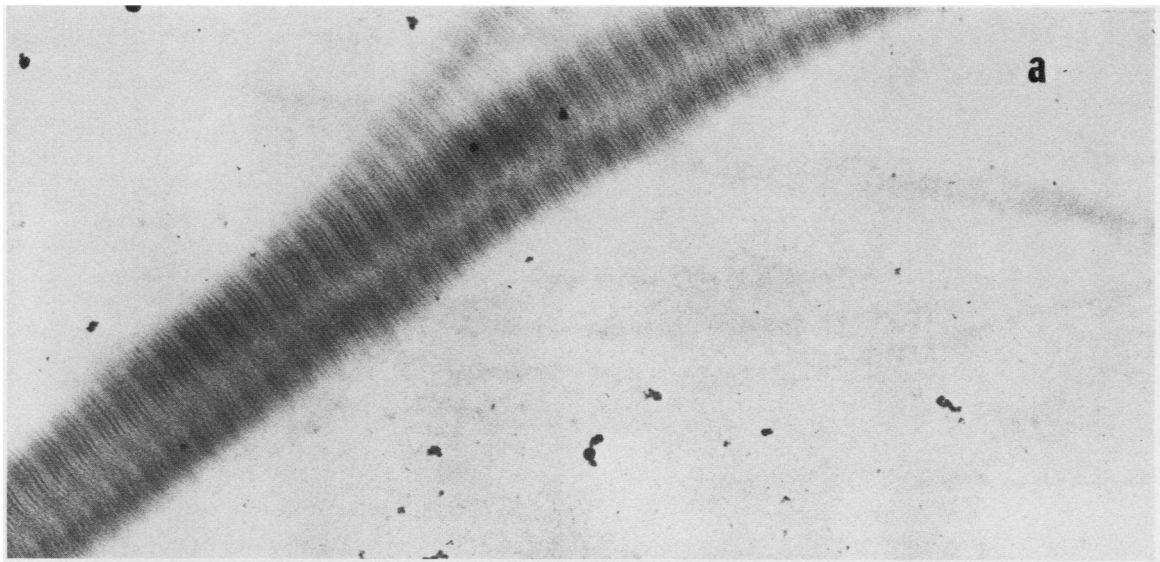
*Competition of monomeric and fibrillar forms of Type I pepsin solubilized collagen in initiation of platelet aggregation.* Several investigators have devised procedures which have demonstrated that the fibrillar form of collagen is essential for aggregation (1–3). Experiments aimed at direct demonstration of decreased binding of collagen monomer to platelets are fewer and more equivocal (25). The negligible rate of fibril formation of Type I pepsin solubilized collagen in platelet-rich plasma makes possible a rather straightforward comparison of the relative binding capacities of the monomeric and fibrillar forms of this collagen. This fibrillar form was prepared by dialysis against phosphate buffer. Addition of up to 100  $\mu\text{g}/\text{ml}$  of the monomeric form of this collagen to platelets had no effect whatever upon the aggregating activity of 12  $\mu\text{g}/\text{ml}$  of the subsequently added fibrillar form.

## DISCUSSION

Collagen modified in its carbohydrate-side chains by periodate oxidation does not exhibit decreased thermal stability. Furthermore, upon dialysis against 0.02 M  $\text{Na}_2\text{HPO}_4$  the periodate oxidized as well as the periodate oxidized-borohydride reduced material readily forms native type fibrils as revealed by electron microscopy. Both the periodate-oxidized and the oxidized reduced material are fully effective in inducing platelet aggregation.

These studies demonstrate that collagen carbohydrate is not required to induce platelet aggregation and do not, therefore, support the glucosyltransferase theory. This conclusion is consistent with the recent observation (26) that the platelet-collagen glucosyltransferase requires as substrate the denatured rather than the native form of collagen, since the latter is required to initiate platelet aggregation.

FIGURE 2 Native type fibrils formed by untreated (a), periodate oxidized (b), and periodate oxidized-borohydride reduced (c) rat skin salt-soluble collagen. The fibril preparations were stained with phosphotungstic acid and uranyl acetate.  $\times 75,000$ .



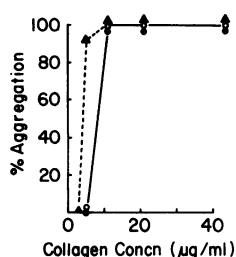


FIGURE 3 Effect of carbohydrate modification by periodate on the ability of collagen to induce platelet aggregation. Fibrils were prepared from untreated (●), periodate oxidized (○), and periodate-oxidized, borohydride-reduced (▲) rat skin salt-soluble collagen dissolved in 3% acetic acid by dialysis against 0.02 M  $\text{Na}_2\text{HPO}_4$ . The percentage of maximal aggregation induced by the indicated concentration of collagen fibrils was determined.

The observations of Kang et al. (27) and Chiang et al. (28, 29) on the binding to platelets of chick collagen peptides which contain hydroxysyl glycosides and on their capacity to initiate aggregation are difficult to relate to the well-established requirement for triple-helical structure for platelet aggregation. Furthermore, only chick and not rat (7) or bovine (27) peptides are reportedly effective in initiating aggregation. Collagen glucosyltransferase on the platelet membrane appears to be the likely source of the 650,000 hydroxylsyl glycosidepeptide binding sites reported by us (7) and by Chiang et al. (28). Although binding may reflect a specific interaction such as, for example, the hydroxylsyl glycoside-glucosyltransferase complex, this affords only a confirmation of the existence of the enzyme on the platelet membrane and need have no relationship whatever to platelet aggregation.

The natural occurrence of collagens of distinct

TABLE II  
Carbohydrate Content and the Effectiveness of Human Collagens in Initiation of Platelet Aggregation when Added to Platelet-Rich Plasma in Monomeric or Fibrillar Form

Collagen type	Carbohydrate content		Minimal effective aggregating concentration	
	gal-hyl	glc-gal-hyl	Mono- meric*	Fibrillar†
	residues/1,000 residues		µg/ml	
I-Acid solubilized	0.5	1.2	30	10
I-Pepsin solubilized	0.4	1.3	>300	9
II-Pepsin solubilized	4.3	4.2	44	10
III-Pepsin solubilized	0.4	1.0	7	4

\* Dissolved in 0.5% acetic acid.

† Formed by dialysis against 0.2 M  $\text{Na}_2\text{HPO}_4$ .

amino acid sequence (10) offers a possible probe into the essential features of the collagen molecule required for the initiation of platelet aggregation. When introduced in fibrillar form, all collagens are highly effective (Table II). The rate of fibril formation appears to account for the differences observed among the monomeric collagens (Table II, Fig. 4).

These studies indicate that the properties of fibrillar collagen required to initiate platelet aggregation are a common characteristic of the three collagen types examined. The existence of regions of variation in amino acid sequences of the various collagens, suggest the possibility that completely identical "platelet-binding sites" may not occur on all three collagens. The recent report by Balleisen et al. (30) that fibers prepared from "synthetic"  $\alpha 1(\text{I})_3$  are fully effective in initiating aggregation provides strong support for this view.

A number of investigations (1, 2) have demonstrated that the fibrillar form of collagen is required or, at least, is several orders of magnitude more effective in the initiation of aggregation. It is not clear, however, whether this is related to a lower binding constant for monomeric collagen by sites on the platelet surface responsible for initiating aggregation or to an inherent ineffectiveness of single-bound collagen molecules. Gordon and Dingle (25) concluded that, on the basis of the radioactivity bound to the platelet surface when exposed to monomeric or fibrillar  $^{125}\text{I}$ -labeled collagen, only fibrillar collagen binds to platelets. Such an observation could, however, arise simply because many more counts are bound per collagen site when a fibril is bound than when a monomer is bound. Collagen of very high specific activity would be required

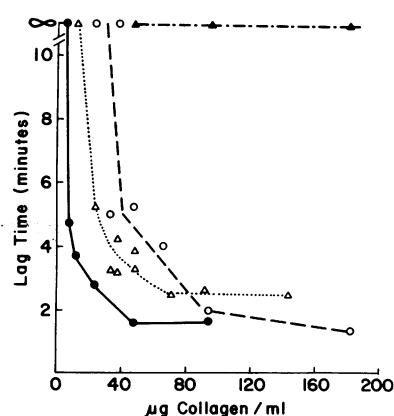


FIGURE 4 Effect of concentration on observed lag time for aggregation of platelets by Type I acid solubilized (Δ), Type I pepsin solubilized (▲), Type II pepsin solubilized (○), and Type III pepsin solubilized (●) human collagens. Collagens dissolved in 0.05% acetic acid were added to 0.5 ml platelet-rich plasma to give the indicated final concentration and the lag time before aggregation was determined.

to rule out the binding of monomeric collagen. Pepsin solubilized Type I collagen permits another approach to this question. If monomeric, pepsin solubilized Type I collagen does bind with significant affinity to sites on the platelet responsible for initiating aggregation, it should inhibit aggregation produced by subsequent addition of the fibrillar form of this collagen. The ability of fibrillar pepsin solubilized Type I collagen to induce platelet aggregation was thus titrated in the presence or absence of up to a 10-fold excess of monomeric collagen. Virtually identical curves were obtained indicating that if monomeric collagen binds to the appropriate platelet sites, the affinity constant is at least two orders of magnitude less than that of fibrillar collagen. These results suggest either that creation of a single platelet-binding site on collagen requires a multimolecular assembly of collagen molecules or that multiple, simultaneous, and "linked" collagen-platelet interactions are required to initiate aggregation. Although conceivable, it seems highly unlikely that all three collagen types could generate an identical specific supramolecular site for interaction with platelets, so that multiple, linked interactions seem the more likely basis for aggregation. The presence on the platelet surface of multiple binding sites exhibiting only modest specificity and affinity for some feature of the collagen molecule, also multiply present in the fibrillar form, could lead to high affinity adhesion (31, 32). This concept is consistent with the observed inability of monomeric collagen to inhibit aggregation induced by a chemically identical fibrillar collagen.

One possibility consistent with these observations is that the collagen fibrils serve as a rigid matrix which, when exposed by endothelial injury, interacts with multiple sites on the platelet surface to initiate aggregation. The specificity of collagen may reside solely in its character as a relatively unique biomaterial for forming such a matrix, and the nature of the binding sites on collagen may be of a considerably lower order of specificity than might be anticipated in conventional receptor-ligand interactions of biological importance. Although ordered fibrils of pure collagen afford the required matrix in *in vitro* systems, it is possible that more complex matrices, such as basement membrane, which lacks obvious fibrillar order, can also initiate platelet aggregation (33).

The cross-linking of platelet-receptor sites by the matrix may be required for initiation of aggregation, perhaps in a manner similar to that by which lectins induce the capping reaction in lymphocytes (34, 35). Transmitted through the plasma membrane by integral proteins, and, by means of the cytoskeletal network of microfilaments and microtubules, this signal could initiate the release of ADP containing granules and subsequent aggregation.

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