# *Reevaluation of the Role of the Polar Groups of Collagen in the Platelet–Collagen Interaction*

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**Chemical modification of collagen is a tool for exploring the platelet–collagen interaction. Since collagen must polymerize prior to the initiation of platelet aggregation and secretion, modification must be shown to affect platelet–collagen interaction and not collagen–collagen interaction. To address this point, the authors carried out the following chemical modifications on soluble monomeric collagen and preformed fibrillar collagen in parallel: 1) N-and O-acetylation, 2) esterification of the carboxyl groups, 3) succinylation of the free amino groups, 4) esterification of succinylated collagen. Intrinsic viscosity studies of the modified soluble collagens were consistent with normal triple helix conformation. Electron microscopy revealed all modified fibrillar collagen to maintain a fibrillar structure. Platelet aggregation and secretion of $^{14}$C-serotonin and platelet factor 4 by soluble and fibrillar collagen, respectively, were studied in human platelet-rich plasma. Neutralization of polar groups by 1) totally abolished aggregation and secretion by both collagens, while blocking acidic groups 2) resulted in enhanced aggregation and secretion by both soluble and fibrillar collagen. Blockage of amino groups by 3) abolished aggregation and secretion by both collagens. Esterified succinylated collagen 4) caused aggregation and secretion at relatively high collagen concentrations. These data support the theory that positive groups of collagen are important in platelet–collagen interaction. (Am J Pathol 1983, 112:200–206)**

IN STUDYING the platelet–collagen interaction, one must differentiate between platelet–collagen interaction and collagen–collagen interaction, because the quaternary structure of collagen has been shown to be important for platelet–collagen interaction.[1–4] Soluble tropocollagen does not appear to aggregate platelets or cause secretion. The tropocollagen molecules must associate to form microfibrils in order to effect platelet aggregation and secretion. However, the quaternary structure of native collagen is not required for platelet aggregation, as segment long-spacing collagen, fibrous long-spacing collagen, and nonstriated amorphous collagen fibers precipitated by alcohol all induce platelet aggregation.[5] The specific site(s) on the collagen molecule required for platelet–collagen interaction remains to be elucidated. It does not appear to involve the telopeptides[6] or carbohydrates.[7,8] Polar groups, specifically ε-amino groups of lysine have been reported to be critical.[9–11] However, past studies of modified collagens have not distinguished between effects on tertiary and quaternary structure. The present study examines modifications of soluble collagen in parallel with modification of preformed fibrils. Specific attention is given to monitoring the quaternary structure of the collagen by electron microscopy and assuring that the collagen has not been denatured. Platelet aggegation as well as secretion of $^{14}$C-serotonin and platelet factor 4 ($PF_4$) by collagen were measured.

## Materials and Methods

### Reagents

All reagents were reagent grade.

### Collagen Preparations and Modifications

Acid-soluble calf skin collagen was purified from commercially available acid-soluble collagen (Wor-

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thington) by the method of Rubin et al.[12] All soluble collagens were centrifuged for 1 hour at 100,000*g* prior to testing with platelets. Fibrillar forms of control and modified collagens, respectively, were formed by dialysis of the soluble collagen in 0.05% acetic acid against 0.02M $NA_2HPO_4$ as described by Bruns and Gross.[13] Esterification of glutamic and aspartic carboxyl groups of collagen with acidified methanol was carried out as described by Wilner et al.[14] Succinylated collagen was prepared by the method of Gustavson.[15] Esterification of succinylated collagen with acidified methanol was carried out according to the method of Wilner et al.[14] Acetylation of the free N- and O-group with glacial acetic acid and acetic anhydride was accomplished by the method of Wilner et al.[9] Available ε-amino groups of lysine were determined by the method of Kakade and Liener[16] as modified by Wilner et al.[11]

### Hydroxyproline Determination

Collagen concentrations were determined from the measurement of hydroxyproline.[17] The assumption is made that 14% of the weight of collagen is composed of hydroxyproline and that collagen concentration (milligram/milliliter) is calculated by multiplying the hydroxyproline values by 7.1.

### Preparation of Platelets

Human blood from normal volunteers taking no medication was collected into an anticoagulant solution (1 part 3.8% sodium citrate to 9 parts whole blood) and centrifuged at 23 C for 13 minutes at 160*g*. The supernatant platelet-rich plasma (PRP) was then removed by aspiration and used for the studies. Whole blood and plasma were exposed only to plastic surfaces. The platelet count was adjusted to 200,000–300,000/μl with platelet-poor plasma, which was prepared by centrifugation of PRP at 1600*g* for 15 minutes.

### Platelet Aggregometry

Platelet aggregation was performed on a Payton Dual Channel Aggregometer (Buffalo, New York) according to the method of Born.[18]

### Platelet $^{14}$C-Serotonin Secretion

Secretion of prelabeled $^{14}$C-serotonin from platelets by collagen was determined according to the method of Jerushalmy and Zucker.[19]

### Platelet Factor 4 ($PF_4$) Secretion

$PF_4$ in the supernatant obtained after stimulation of PRP by collagen was measured by radioimmunoassay with the use of a commercial kit (Abbott Laboratories, North Chicago, Ill).

### Electron Microscopy

Aliquots of PRP that had been stirred with collagen were fixed in 2% (vol/vol) glutaraldehyde in Sorenson's phosphate buffer, pH 7.4, at room temperature for 60 minutes. The mixture was centrifuged at 3000*g* at 4 C for 15 minutes. After removal of the supernatant, the fixed pellets were stored at 4 C in Sorenson's buffer with 7.5% (wt/vol) sucrose until further processing within 2–5 days. The day prior to postfixation, the platelet button was cut into 1-cu mm pieces and placed in the same buffer. The specimens were postfixed in chilled 1% (wt/vol) buffered osmium tetroxide for 1 hour, dehydrated in graded ethyl alcohols, and infiltrated in Spurr low viscosity media (Polyscience) at 60 C for 8–16 hours. Thin sections (600 Å) were stained with uranyl acetate and lead citrate and examined in a Zeiss 109 electron microscope.

## Results

### Platelet Aggregation and Secretion by Unmodified Soluble and Fibrillar Calf Skin Collagen

Table 1 shows the results of platelet aggregation and secretion of $^{14}$C-serotonin and $PF_4$ by acid-soluble calf skin collagen and preformed fibrils of this collagen, respectively. The soluble collagen shows a long lag time before eliciting aggregation. High concentrations of this collagen are required to produce aggregation and secretion. These results are consistent with previous studies that have shown that soluble or monomeric collagen does not cause platelet aggregation or secretion but must first associate into microfibrils to produce this effect. Fibrillar collagen produces aggregation and secretion at much lower concentrations and requires a very short lag time. With both preparations there was secretion of $PF_4$ by concentrations of collagen too low to induce secretion of $^{14}$C-serotonin or aggregation.

### Effect of N- and O-Acetylation of Collagen on Aggregation and Secretion

Neutralization of the polar groups of collagen by acetylation of N and O groups by glacial acetic acid

Table 1—Unmodified Calf Skin Collagen

| Collagen | | Aggregation | | Secretion | |
|---|---|---|---|---|---|
| State | Concentration (μg/ml) | % | Lag time (min) | $^{14}$C-serotonin (%) | $PF_4$ (μg/10$^9$ platelets) |
| Soluble | 108 | 0 | >20 | 0 | 1.15 |
| | 137 | 68 | 10.4 | 16 | 6.39 |
| | 180 | 80 | 10.6 | 23 | 7.07 |
| Fibrillar | 8 | 0 | >10 | 0 | 0.64 |
| | 24 | 5 | 0.8 | 0 | 1.71 |
| | 48 | 31 | 2.2 | 2 | 3.71 |
| | 55 | 75 | 2.3 | 25 | 7.21 |
| | 78 | 71 | 2.3 | 21 | 6.77 |

The results are representative of duplicate studies. Similar results were obtained with three different collagen preparations.

and acetic anhydride resulted in total inhibition of platelet aggregation and secretion of $^{14}$C-serotonin (Table 2). Very small amounts of $PF_4$ appeared to be secreted.

### Effect of Esterification of the Carboxyl Groups of Glutamic and Aspartic Acid by Treatment With Acid-Methanol

This modification resulted in enhanced aggregation and secretion of both soluble and fibrillar collagen. Soluble collagen, which was centrifuged for 1 hour at 100,000*g* prior to testing, was more effective than an equal concentration of fibrillar collagen of the same material. The lag time of this soluble material was quite short, as can be seen in Table 3. Secretion of both serotonin and $PF_4$ occurs at concentrations of soluble and fibrillar collagen too low to induce detectable aggregation.

### Effect of Succinylation of Collagen on Platelet Aggregation and Secretion

Succinylation of the ε-amino groups of lysine by treatment with succinic acid anhydride resulted in complete inhibition of platelet aggregation and secretion of $^{14}$C-serotonin (Table 4). However, there was a small but detectable amount of $PF_4$ released into the supernatant. Determination of available ε-amino groups of lysine of the succinylated collagens revealed them to be 87–100% (range of 3 separate preparations) blocked by the succinylation method (data not shown).

### Effect of Esterification of Succinylated Collagen on Platelet Aggregation and Secretion

When succinylated soluble collagen was subjected to acid-methanol treatment, both soluble and fibrillar collagens were capable of inducing aggregation and secretion (Table 5). This modified collagen was not as effective as esterified collagen (Table 3) but was more effective than unmodified collagen (Table 1). Secretion of $PF_4$ occurs at lower concentration of collagen than secretion of serotonin or aggregation (Table 5). Determination of available ε-amino groups of lysine of these modified collagens revealed them to be 87–100% blocked (range of 3 separate preparations).

### Effect of Chemical Modifications on the Intrinsic Viscosity of Collagen

Because chemical modification of collagen might result in some denaturation of collagen, it is important to monitor a property of collagen, such as viscosity, which depends on the triple helical structure

Table 2—N- and O-Acetylation

| Collagen | | Aggregation | | Secretion | |
|---|---|---|---|---|---|
| State | Concentration (μg/ml) | (%) | Lag time (min) | $^{14}$C-serotonin (%) | $PF^4$ (μg/10$^9$ platelets) |
| Soluble* | 81 | 0 | >10 | 3.3 | 0.10 |
| | 163 | 0 | >10 | 0 | 0.30 |
| | 266 | 0 | >10 | – | 0 |
| Fibrillar | 120 | 0 | >10 | 0 | 0.24 |
| | 240 | 0 | >10 | 0 | 0.24 |

The results are representative of duplicate studies. Similar results were obtained with three different collagen preparations.

* This preparation was not really soluble. Particulate material, which contained no fibrillar structure as seen by electron microscopy, was tested.

Table 3—Esterification by Acid-Methanol

| Collagen | | Aggregation | | Secretion | |
|---|---|---|---|---|---|
| State | Concentration (μg/ml) | (%) | Lag time (min) | $^{14}$C-serotonin (%) | $PF_4$ (μg/10$^9$ platelets) |
| Soluble | 1.6 | 0 | >10 | 5 | 2.50 |
| | 2.4 | 20 | 0.6 | 2 | 2.83 |
| | 3.2 | 60 | 0.4 | 20 | 8.40 |
| | 4.0 | 75 | 0.4 | 27 | 11.00 |
| Fibrillar | 3.7 | 0 | >5 | 5 | 0.80 |
| | 7.4 | 75 | 0.4 | 32 | 8.01 |
| | 18.8 | 92 | 0.2 | 45 | 9.68 |
| | 29.6 | 88 | 0.2 | 64 | 11.15 |

The results are representative of duplicate studies. Similar results were obtained with three different collagen preparations.

of the collagen. As can be seen in Table 6, all the modified collagens shown maintained an intrinsic viscosity within 2 dl/g of unmodified tropocollagen. Therefore, it is unlikely that significant denaturation of these collagens occurred as a result of modification. Soluble collagen which was subjected to N- and O-acetylation (data not shown) was not very soluble. Visible particulate matter, which did not show fibrillar structure by electron microscopy, was sedimented by centrifugation at 1600*g* for 10 minutes. The viscosity of the supernatant was the same as that of the acetic acid.

### Effect of Modification on the Fibrillar Structure

All fibrillar collagens maintained a fibrillar structure after modification, as can be seen in Figure 1 (a, b, c, e, and f). No fibrillar collagen was detected in the preparations of soluble collagen (not shown). In the soluble acid-methanol collagen preparations, rare nonstriated, slender strands could be identified by electron microscopy (Figure 1d) despite the fact that the preparation was centrifuged for 1 hour at 100,000*g* prior to testing with platelets. The electron micrograph was made after the collagen was added to PRP and stirred in the aggregometer at 37 C.

## Discussion

Despite the important role of collagen in hemostasis, the mechanism of the platelet-collagen interaction remains to be elucidated. Several investigators have demonstrated a requirement for microfibrillar formation in inducing platelet aggregation. Our studies confirm this observation. Earlier studies by Wilner et al[10-12] suggested that polar groups, specifically the ε-amino groups of lysine, were critical. However, these observations were reported prior to the knowledge of the importance of microfibrillar structure. Therefore, the studies reported by Wilner were not designed to address this point. No study was carried out at that time to show whether the various chemical modifications had denatured the collagen or affected its microfibrillar characteristics. No studies of secretion were undertaken. The collagen used in the studies by Wilner et al. was Nishihara insoluble collagen[20] which was dialyzed against phosphate buffer. Though this collagen would be expected to be microfibrillar, it is possible that chemical treatment could have modified the structure.

Therefore, we designed our experiment to study soluble collagen and preformed fibrillar collagen in parallel, with careful attention to the helical structure of soluble collagen with each modification and electron-microscopic assessment of both soluble and fibrillar forms of control and modified collagens, respectively. We, like Wilner et al,[9] found that acetylation of the N and O groups of collagen by glacial acetic acid and acetic anhydride completely abolished the ability of soluble or fibrillar collagen to cause platelet aggregation, although fibrillar collagen maintained its structure, as seen by electron microscopy.

Table 4—Succinylation

| Collagen | | Aggregation | | Secretion | |
|---|---|---|---|---|---|
| State | Concentration (μg/ml) | (%) | Lag time (min) | $^{14}$C-serotonin (%) | $PF_4$ (μg/10$^9$ platelets) |
| Soluble | 100 | 0 | >10 | 0 | 0.01 |
| | 200 | 0 | >10 | 0 | 0.47 |
| Fibrillar | 100 | 0 | >10 | 0 | 0.53 |

The results are representative of duplicate studies. Similar results were obtained with three different collagen preparations.

Table 5—Succinylation Followed by Esterification With Acid-Methanol

| Collagen | | Aggregation | | Secretion | |
|---|---|---|---|---|---|
| State | Concentration (μg/ml) | (%) | Lag time (min) | $^{14}$C-serotonin (%) | $PF_4$ (μg/10$^9$ platelets) |
| Soluble | 15.0 | 0 | >10 | 0 | 0.47 |
| | 30.0 | 34 | 1.3 | 0 | 1.54 |
| | 60.0 | 94 | 2.0 | 16 | 4.88 |
| Fibrillar | 4.2 | 0 | >10 | 6 | 0.59 |
| | 10.5 | 30 | 1.3 | 1 | 1.71 |
| | 21.0 | 86 | 0.8 | 25 | 6.24 |
| | 42.0 | 91 | 0.5 | 22 | 4.27 |

The results are representative of duplicate studies. Similar results were obtained with three different collagen preparations.

These findings would, then, support the view that fibrillar structure alone is not sufficient to cause platelet aggregation but that polar groups are required.

Succinylation of soluble and fibrillar collagen, respectively, resulted in 87–100% blockage of the ε-amino groups of lysine. These collagens were incapable of causing platelet aggregation or secretion of serotonin. The succinylated microfibrillar collagen maintained fibrillar structure, though the diameter of these modified fibers appeared to be slightly less than those of unmodified fibrillar collagen. These results support the importance of the ε-amino groups of lysine in inducing platelet aggregation and serotonin secretion. It is of interest that our results differ significantly from those of Wilner et al, who reported variable results. In their hands, succinylation of ε-amino groups did not inhibit platelet aggregation in one study[11] and partially inhibited it in another.[10] However, when these same authors blocked ε-amino groups of collagen by other techniques such as N-acetylation,[9] treatment with 2,4,6-trinitrofluorobenzene sulfonic acid[11] and diazotization with nitrous acid,[9] respectively, platelet aggregation was abolished.

Esterification of the glutamic and aspartic carboxyl groups of collagen by methanol resulted in enhanced platelet aggregation and secretion by both soluble and fibrillar collagen. By viscosity determination the soluble esterified collagen was similar to unmodified collagen. By electron microscopy the soluble material did not show any fibrillar structure. However, an occasional nonstriated slender strand form was identified. The fibrillar modified collagen maintained its fibrillar structure. Decreasing the negative charges by esterification appeared to allow a more effective interaction of the collagen with platelets. Wilner et al[11] reported that esterification of collagen had no effect on the ability of the collagen to aggregate platelets. They do not present data on soluble collagen. It is intriguing to postulate that the rigid microfibrillar structure of the unmodified collagen is required to give enough positively charged groups at strategic points. The presence of negatively charged groups as well along the rigid matrix may negate some effect of the positive charges. It is possible that removal of some of the negative charges by esterification of carboxyl groups of acidic amino acids such as aspartic and glutamic acids may render more effective the positive groups of lysine and hydroxylsine. The platelet aggregation and secretion seen with the soluble esterified collagen may in fact be due to the presence of nonstriated fibrils. Muggli has shown that such amorphous fibers are capable of causing platelet aggregation.[5] Alternatively, soluble collagen in which 90–100% of carboxyl groups of glutamic and aspartic acids have been esterified may possess enough positive charges strategically placed to bind to platelets and elicit aggregation and secretion. Other cationic substances such as lysozyme, trasylol, and protamine sulfate,[21] which lack microfibrillar structure, are able to aggregate platelets.

Interpretation of the data obtained from collagen in which succinylation was followed by esterification with acid methanol is somewhat difficult to interpret. The process of esterification restored the ability of succinylated collagen to cause aggregation and secretion. Our results differ from those of Wilner et al, who found that their modified collagen did not cause platelet aggregation. The cause of this discrepancy is

Table 6—Viscosity of Soluble Collagens

| Type of collagen modification | Viscosity (dl/g) |
|---|---|
| Unmodified | 18.5 |
| Acid-methanol | 19.8 |
| Succinylation | 16.0 |
| Succinylated and esterification | 20.5 |
| Denatured unmodified | 4.2 |

Viscosity was measured in an Ostwald viscometer in a 20 C water bath. Determinations were performed in triplicate.

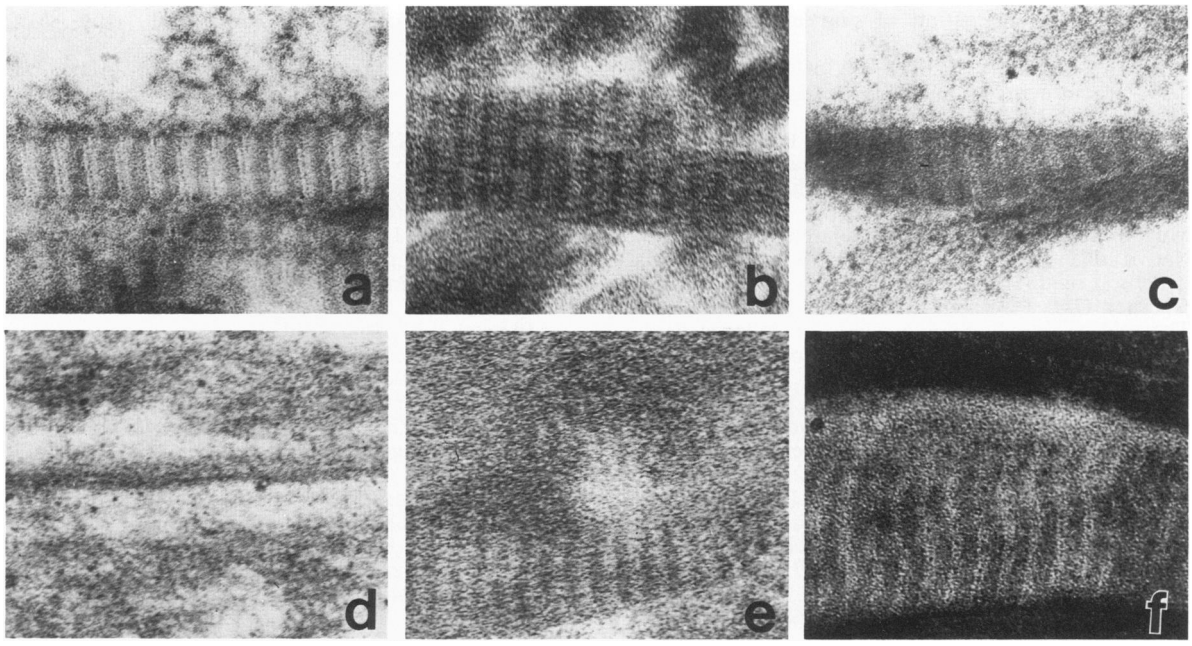


**Figure 1**—Electronmicroscopy of control and modified collagen. **A**—Unmodified native fibril. **B**—N- and O-acetylated native fibril. **C**—Succinylated native fibril. **D**—Nonstriated strand formed from soluble collagen treated with acid-methanol. **E**—Acid-methanol treated native fibril. **F**—Acid-methanol-treated succinylated native fibril. Electronmicroscopy was prepared from specimens obtained after the collagen was stirred at 37 C in the platelet aggregometer, thus reflecting the collagen structure under the conditions of platelet-collagen interaction. The soluble acid-methanol collagen preparation was centrifuged for 1 hour at 100,000*g* prior to stirring with PRP in the aggregometer. The preparations were stained with uranyl acetate and lead citrate. (×84,730)

not apparent. In our studies the ability to interact with platelets could not be attributed to ε-amino groups of lysine, which were shown by lysine determination to be blocked. One possible explanation is that the net charge distribution, rather than specific positive charges, is important. A second possibility is that the ε-amino groups of lysine are critical for the interaction of unmodified microfibrillar collagen with platelets, but collagen modified by succinylation followed by esterification elicits a response via a different mechanism requiring binding sites different from those of unmodified collagen.

It is of some interest to note that secretion of serotonin only occurs with collagen capable of inducing aggregation. However, significant amounts of $PF_4$ are secreted by collagens that do not cause aggregation. The fact that this occurs in a dose-dependent manner suggests more than a nonspecific effect. This dissociation of α-granule secretion from dense granule secretion and aggregation induced by collagen suggests that α-secretion can occur independent of dense granule secretion and aggregation and can be induced by agents that are not capable of inducing platelet aggregation and dense granule secretion. Zucker et al[22] have shown that fibrillar collagen stimulates secretion of the α-granule components, $PF_4$, β-thromboglobulin, and Factor VIII antigen at lower concentrations of collagen than that required for dense granule secretion of serotonin. Two interpretations of our data are possible. Very low concentrations of unmodified collagen are present and elicit the response as described by Zucker et al,[22] or modified collagens are capable of interacting with platelets to give an incomplete response. This latter possibility would be consistent with the theory of Santoro and Cunningham,[23] who propose that the collagen fibril facilitates the development of multiple, simultaneous, and linked interactions between sites on the platelet surface and several sites on the collagen fibril, resulting in platelet aggregation and secretion. The presence of some of the sites on the fibril might be capable of eliciting a partial response, whereas a complete response requires the availability of additional sites on the fibril. Our data support the view that polar groups, specifically the ε-amino groups of lysine, appear to be of great importance in eliciting the full response of aggregation and secretion.

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