The Size of Collagen Fibrils that Stimulate Platelet Aggregation in Human Plasma

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Suspensions of collagen fibrils of different size were prepared from solutions of radioactive tropocollagen type I by either differential centrifugation or differential incubation at elevated temperature. The fractions were compared with respect to their ability to stimulate human blood platelet aggregation in plasma, their binding to human platelets, and their morphology, as seen in the electron microscope. Although small particles with a sedimentation coefficient as low as 4.5S bound to platelets, aggregation was not observed in the presence of collagen multimers and protofibrils without visible cross-bands in stained specimens. The onset of platelet-aggregating activity before the appearance of turbidity in collagen solutions incubated at elevated temperature is due to the formation of a few banded fibrils; this early onset and the fibrils do not appear in collagen solutions that have been ultracentrifuged before incubation.

The importance of collagen in stimulating platelets to form thrombi was first inferred from observations of these cells adhering to collagen fibres in injured blood vessel walls (Hugues, 1953). It was surmised from subsequent studies *in vitro*, however, that collagen had to be in a particulate form to stimulate platelets, the activity disappearing when the collagen was centrifuged to remove particles of approx. 100S (Hovig, 1963). Jaffe & Deykin (1974) and Muggli & Baumgartner (1973) showed that the lag period observed in aggregometer experiments with soluble collagen is due to the time required for collagen fibrils to form. These results mean that all kinetic studies *in vitro* up to the present time could only have been semiquantitative, because collagen in solution only reacts with platelets after it has formed fibrils, which are not of uniform size or perfection. It is not known how the activity of the collagen depends on the structural parameters of these fibrils. Wang *et al.* (1978) found, from controlled precipitation experiments, that the average fibril length in an active suspension could be as low as 900nm. Muggli & Baumgartner (1973) found that the development of platelet-stimulating activity in a collagen solution incubated at 37°C long precedes the onset of turbidity. On the basis of the evidence from these controlled incubations, nevertheless, it cannot be discerned whether the early onset of activity is due to a sufficiency of non-turbid small early fibrils to stimulate the reaction with platelets, as inferred by Muggli

(1978) and by Wang *et al.* (1978), or to early formation of a few large fibrils. This question of effective size relates also to the activities of collagen fibrils of different sizes found in tissue (Barnes *et al.*, 1976).

The activity of collagen fibrils and the inactivity of tropocollagen might be attributed, as suggested by some workers (Jaffe & Deykin, 1974; Wang *et al.*, 1978), to differences in binding to platelets, but the experiments cited above all involved mixtures of the two, presumably active and inactive, components. The active components themselves, which might have been a subfraction of the fibrils, were not isolated for characterization. This comment also applies to a study of the actual binding of collagen to platelets (Gordon & Dingle, 1974), in which the component to which the platelets bound appeared in the collagen solution only after it had been incubated at 37°C. Again, neither the component that bound, nor the one that did not, was isolated or characterized. These binding measurements, like the earlier aggregation experiments, were performed with mixtures of tropocollagen and fibrils of various sizes.

The above questions of the identity of the active form of collagen can be elucidated by separating the variously polymerized species. In the present study we have isolated such species from suspensions of collagen fibrils and determined their binding to platelets, their platelet-stimulating activities and the morphologies of their constituent fibrils. We find that tropocollagen is only a minor fraction of the

collagen that binds to platelets and that there is an optimum range of sizes for active fibrils.

Materials and Methods

Preparation of platelets for aggregation experiments

Platelet-rich plasma was prepared by centrifuging citrated blood (Gibson *et al.*, 1957) from healthy volunteers at room temperature for 10 min at 250g. The supernatant, containing 450 000–700 000 platelets per mm³, was removed by aspiration.

[¹⁴C]Collagen

[¹⁴C]Collagen was prepared by incubating embryonic-chick tendons (White Leghorn) at 37°C in medium containing 5 μ Ci of [¹⁴C]proline/ml (New England Nuclear Corp., Boston, MA, U.S.A.) (250 μ Ci/ μ mol), 50 μ g of β -aminopropionitrile/ml, 5 mM-CaCl₂ and 0.01 M-4-(2-hydroxyethyl)-1-piperazine-ethanesulphonate (pH 7.4). After 3 h of incubation excess unlabelled proline was added, and the incubation was continued for 30 min to allow secretion of intracellular collagen precursors and their conversion to tropocollagen (Jimenez *et al.*, 1971). Labelled tropocollagen was extracted into 10 ml of 0.45 M-NaCl/50 mM-Tris/HCl (pH 7.4) for 48 h at 4°C. The radioactive extract was centrifuged at 14 000g for 30 min, and the collagen in the supernatant was precipitated by addition of an equal volume of 4.4 M-NaCl. The precipitate was collected by centrifugation at 30 000g and redissolved in the neutral salt buffer to yield stock solution containing 1 mg of collagen/ml. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, agarose-gel filtration in sodium dodecyl sulphate, and amino acid analysis showed no evidence of components other than type-I collagen.

Preparation of unlabelled collagen

Unlabelled type-I collagen was obtained from acetic acid extracts or from neutral 0.15 M-NaCl extracts of lathyrus chick-embryo tendons by neutral-salt precipitation as described previously (Jimenez *et al.*, 1977).

Formation of active collagen species during incubation

The incubations were performed under two sets of conditions. The first set reproduced the conditions of Muggli & Baumgartner (1973). Labelled collagen dissolved in 0.15 M-NaCl/50 mM-Tris/HCl (pH 7.4) was incubated at 37°C with intermittent stirring in the sample compartment of a Cary 15 spectrophotometer (Varian Instruments, Palo Alto, CA, U.S.A.). Samples were taken periodically during the incubation before the absorbance had begun to rise, during the rise, and after the turbidity had reached its maximum value. In one experiment the collagen

solution was incubated at a concentration of 407 μ g/ml in the spectrophotometer, and in the other at 102 μ g/ml. Fig. 3(a) shows the course of the turbidity change and the times at which samples were taken during the second of these incubations.

A portion (50 μ l) of each sample taken from the collagen solutions was added to 0.5 ml samples of platelet-rich plasma in an expanded-scale photometer that provides 700 rev./min. stirring at 37°C (ChronoLog Aggregometer, Broomall, PA, U.S.A.). Each sample found from this test to be active was used to titrate the platelet-aggregation reaction (Born, 1962), and the platelet aggregates were analysed for bound collagen, by following an aggregation-binding procedure previously described (Kronick & Jimenez, 1979). Also, a drop of each sample was applied to a carbon-coated electron-microscope grid, stained with phosphotungstic acid (pH 4) and rinsed with 0.1 M-acetic acid.

The second set of conditions for the incubations were those used by Williams *et al.* (1978) and Gelman *et al.* (1979), with slight modification. The incubation mixture was first centrifuged at 100 000g (Beckman rotor type 50, r_{av} 7 cm) for 1 h to remove particulate fibrillogenesis nuclei. This solution contained unlabelled collagen at 6.2 mg/ml in 0.15 M-NaCl/30 mM-sodium phosphate/10 mM-Tris/HCl at pH 7.4. The solution was incubated in the sample compartment of a Cary 14 spectrophotometer (Varian Instruments) at 29°C for 55 min with intermittent stirring and withdrawal of samples, and continuous monitoring of turbidity with 600 nm light. A 50 μ l portion of each sample withdrawn was added at a concentration of 0.62 mg/ml to platelet-rich plasma at 37°C in the aggregometer; another portion was deposited on carbon-coated electron-microscope grids for examination of the fibrils. No more than 30 s was required to handle each sample.

For this second set of conditions, we examined several parameters of the aggregometer curves (Born, 1962) to characterize the platelet response to collagen. Maximal aggregation was not suitable, since the platelets eventually aggregated completely to all of the collagen samples, although only after lag periods of various lengths for the preturbid ones. These lag periods decreased progressively with collagen incubation time, falling below 30 s after the collagen had been incubated for 18 min. The most reproducible index of platelet response was the rate of increase of light transmission determined 30 s after the addition of collagen. For the samples of high and intermediate activity, this point lay on the linear part of the aggregometer curve. When the response was delayed, for the preturbid samples, the slope at this point was very small, indicating lack of aggregating activity, but the platelets always began to respond immediately to samples of low turbidity, reaching a steady rate in less than 30 s.

Correlation of binding of collagen fibrils with their activity

A solution of radioactive collagen (0.15 mg/ml, 5.2×10^6 c.p.m./ml) was incubated at 37°C for 10 min, when it could cause platelets to aggregate at a concentration of 15 µg/ml in an aggregometer with a lag time less than 30 s. A 0.25 ml portion of this collagen solution was layered on a NaCl gradient from 0.15 to 0.20 M, buffered with 50 mM-Tris/HCl, pH 7.4, in a plastic 5 ml (1.3 cm × 5 cm) centrifuge tube. After centrifuging at 1000g for 45 min in an IEC 26 cm swing-out rotor at room temperature, we punctured the tube and collected drops alternately in 0.5 ml samples of platelet-rich plasma for platelet-aggregation assay and in scintillation tubes for radioactivity assay: one drop into the platelets, four into the scintillation vial. Some 70% of the applied counts was recovered, which is consistent with the expected loss at the wall in the particular combination of cylindrical centrifuge tube and rotor used here.

Each platelet tube was immediately placed into the aggregometer at 37°C with stirring so that the platelet reaction could be monitored. In order to collect the platelets for determination of the amount of collagen bound to them, 10 µM-ADP was added to each tube to cause complete aggregation after the reaction with collagen was complete. After the platelet aggregates had settled by gravity, the plasma supernatants were drawn off into scintillation vials. The platelets were washed three times with buffered saline (0.15 M-NaCl/50 mM-Tris/HCl, pH 7.4) and transferred to scintillation vials. All radioactive samples (plasmas and pellets) were digested with NCS organic base (Amersham-Searle Corp.) and counted for radioactivity in scintillant made from 0.67 g of 1,4-bis-(5-phenyloxazol-2-yl)benzene, 6.7 g of 2,5-diphenyloxazole, 1.3 litre of toluene, and 300 ml of Triton X-100 (Amersham-Searle Corp.). We have previously shown (Kronick & Jimenez, 1979) that this method of collecting the platelets and washing them reliably removes free collagen, and that a quantitative assay of the bound collagen results from this treatment. Aggregation of the platelets with ADP is necessary because single platelets cannot be separated conveniently from large collagen aggregates by centrifugation or filtration.

Binding of tropocollagen and collagen multimers to platelets

Freshly prepared labelled collagen (1 mg/ml, 4×10^5 c.p.m./ml) in 0.45 M-NaCl/50 mM-Tris/HCl (pH 7.4) was incubated for 40 min at 26°C. Then 0.5 ml was layered on top of a Ficoll (polymerized sucrose, Pharmacia Chemical Corp.) density gradient, running from 7.5 to 30% (w/v) Ficoll in 0.15 M-NaCl/10 mM-Tris/HCl, pH 7.4. After sedimentation at 100000g

and 4°C for 20 h, the column was fractionated, from the top, into scintillant, every third fraction being added instead to 2 ml of platelet-rich plasma containing 10^{10} platelets. These human platelets had been prepared from citrate/phosphate/dextrose (Gibson *et al.*, 1957) platelet-rich plasma from 450 ml of blood by centrifuging this plasma with 5 mM-EDTA at room temperature and resuspending in one-third of the original volume of buffered saline (0.15 M-NaCl/10 mM-Tris/HCl, pH 7.4). After addition of labelled collagen from the gradient, each portion of platelets was centrifuged through 8% Ficoll/0.15 M-NaCl/10 mM-Tris/HCl (pH 7.4) at 12000g for 10 min, and the radioactivity of each platelet pellet was determined as described above.

As a control, we did a similar experiment, substituting erythrocyte 'ghosts' at a concentration of 2 mg of protein/ml, prepared by the method of Dodge *et al.* (1963), for the platelets. The gradient was calibrated for sedimentation coefficients with tropo-collagen ($s = 2.8S$) and haemoglobin ($s = 4.5S$). These two reference points are sufficient to describe the binding of collagen aggregates to platelets.

Results

Size fractionation and activity

The binding of collagen fibrils to platelets is correlated with their stimulating activity in Fig. 1. Fig. 1(c) shows an even distribution of radioactivity along the salt gradient. Most of the protein is in the form of heavy particles. Fig. 1(b) shows the location on the gradient of the collagen that binds to platelets: 92% of the binding fraction is found between fractions 3 and 7. These fractions are the only ones that cause platelets to aggregate (Fig. 1a). They comprise only a minority of the total collagen, even though it is nearly all in particulate form.

Unfortunately, little information about the size of the collagen particle that binds to platelets and stimulates them is forthcoming from the fast-sedimentation pattern of Fig. 1. To examine the properties of small collagen species (tropocollagen and multimers), a sample was incubated at the relatively low temperature of 26°C in 0.45 M-NaCl as described in the Methods and Materials section. This solution was not turbid, and 95% of the radioactivity applied to the Ficoll gradient appeared as tropo-collagen. Fig. 2 shows how the distribution of sedimentation velocities of small collagen aggregates compares with the platelet-binding distribution. The peak in the sedimentation-velocity curve is marked by tropocollagen ($s = 2.8S$) and the inflection in the binding curve, by the band of sedimenting haemoglobin ($s = 4.5S$), which had been added to the mixture. The collagen solution evidently contained a small number of fibrillar aggregates, although the

Fig. 1. Correlation of sedimentation speed of collagen fractions with their interactions with platelets

A solution of [^{14}C]collagen, containing 0.15 mg/ml and 5.2×10^6 c.p.m./ml, was incubated at 37°C for 10 min. Then 0.25 ml was centrifuged at 1000g for 45 min in an IEC 26 cm swing-out rotor at room temperature on a gradient from 0.15 M-NaCl to 0.20 M-NaCl. Fractions were collected directly into 0.5 ml aggregometer samples of platelet-rich plasma and alternately into scintillation vials for radioactivity assay, in the relation 1 drop to 4 drops. (a) Platelet response (measured by the change in light transmission of a platelet suspension monitored in the aggregometer) due to each collagen sample relative to that induced by a saturating amount of collagen. (b) The amount of collagen bound to platelets in each sample was determined as described in the text, and is expressed here for each fraction as the percentage of the total amount bound. (c) The total radioactivity in each platelet sample, the sum of that bound to the platelets and that free in the plasma (see the text), was averaged with that collected into the corresponding scintillation vial to give the distribution of the mass of radioactive collagen over the gradient. The diameters of the circles represent the average deviations.

Fig. 2. Binding to platelets of collagen fractions separated according to sedimentation coefficient

[^{14}C]Collagen solution (1 mg/ml, 0.5 ml) that had been incubated at 26°C was layered on a density gradient of 7.5–30% (v/v) Ficoll in buffered saline. After sedimentation at 100000g for 20 h, two out of every three fractions from the column were dropped directly into scintillant solution, every third fraction (0.2 ml) being added to 2 ml of a suspension containing 10^{10} platelets. The platelets themselves were then centrifuged through columns of 15% Ficoll and buffered saline to separate them from unbound collagen, and their radioactivities were determined. The above incubation, fractionations and experiments with platelets were done in triplicate, corresponding to the circles, squares and upright triangles. A single experiment was done with erythrocyte membranes, corresponding to the inverted triangles. Closed symbols, amount of collagen in each fraction applied to platelets. Open symbols, fraction of collagen in each fraction that bound to platelets. The arrows mark the sedimentation of tropocollagen (2.8S) and haemoglobin (4.5S). —, Total collagen; ----, percentage of collagen bound.

appearance of radioactivity toward the bottom of the gradient tube.

The direct interpretation of these two sets of data is that only a minor fraction of the collagen sedimenting at 2.8S, which includes all the tropocollagen, binds to platelets, and aggregates of $s > 4.5\text{S}$ bind appreciably. The most active fraction, however, comprises particles of intermediate size, sedimenting from 4×10^4 to $10 \times 10^4\text{S}$.

Fibrillogenesis and activity

When the collagen that had not been ultracentri-

low protein concentration, the low temperature and the salt prevented actual gelation (Wood & Keech, 1960). These aggregates are responsible for the

Fig. 3. Correlation of fibrillogenesis and appearance of platelet-stimulating activity in incubation mixtures containing $0.012 \mu\text{g}$ of collagen/ml at 37°C that had not been ultracentrifuged

(a) Progress of turbidity during the incubation, showing the times at which samples A-G were removed. (b) Aggregation response of platelets titrated with collagen samples B, C, D and F, defined in (a). The platelet response is expressed as the maximum light-transmission change in the aggregometer, relative to the difference between the transmissions of the original platelet suspension and platelet-free plasma.

fused was incubated at 37°C (Fig. 3a), platelet-stimulating activity developed before turbidity, as observed by others (Muggli & Baumgartner, 1973; Jaffe & Deykin, 1974). During the incubation of the more dilute collagen solution there was a progressive increase of aggregating activity, as measured by the dose required to drive the platelet-aggregation reaction to one-half its light transmission change. The development of activity can be followed in the progressive micrographs of the collagen taken from the incubation mixtures (Plate 1). Samples taken before incubation showed no fibrillar structures at all, only a uniformly amorphous texture. Sample B, taken from the dilute solution after 3 min, before aggregating activity was observed, showed no

banded structures, but only fine protofibrils. Sample C, however, at 6 min, although still a clear liquid, already showed a few well-developed fibrils, as well as very fine ones (Plate 1a). It was slightly active, the half-stimulating dose being greater than $10 \mu\text{g/ml}$ (Fig. 3b). Similar fibrils were found at 9 min, still before the turbidity was observed to increase. Sample E, taken at 12 min, had numerous fine banded fibrils, as well as thick banded fibrils. One was estimated to be $100 \mu\text{m}$ long $\times$ $0.4 \mu\text{m}$ thick (Plate 1b). Samples E, F, and G were indistinguishable from each other in the electron microscope and seemed to have more thick fibrils than the samples taken earlier, but all samples (B-G) had the fine fibrillar background shown in Plate 1(c). These turbid samples (E, F and G) were equivalent in platelet-aggregating activity.

In the more concentrated solution of uncentrifuged collagen incubated at 37°C , thin banded fibrils were again found in the clear solutions, accompanying the onset of platelet-aggregating activity. The most active sample, taken after the solution had become turbid, was full of well-developed long banded fibrils.

The second set of experiments in which the proper-

Fig. 4. Correlation of fibrillogenesis and appearance of platelet-stimulating activity in collagen incubation mixtures at 26°C that had been ultracentrifuged

The initial portion of the curve for the turbidity of the collagen incubation mixture is expanded 10-fold. Each triangle on the response curve corresponds to a sample that was removed for both assay of the activity and for electron-microscopic examination. A portion ($100 \mu\text{l}$) of each sample was removed from the spectrophotometer with a capillary syringe; $50 \mu\text{l}$ of this was added to the platelet-rich plasma being stirred at 37°C in the aggregometer, and the rest in the syringe was placed on a carbon-coated grid, for the electron micrographs in Plates 2 and 3. The platelet response is expressed as the slope of the relative light-transmission change against time determined 30 s after the collagen sample was added to the platelet-rich plasma. —, Turbidity ($10\times$); ----, turbidity ($1\times$); Δ , platelet response.

ties of collagen were monitored while it was being incubated at elevated temperature were carried out on a solution that had been centrifuged to remove particles of about 15S (100000g for 1h). We deliberately chose a lower incubation temperature for this solution and included 30mm-phosphate to optimize the formation of native banded fibrils, following the observation of Williams *et al.* (1978), who also precentrifuged their collagen. Although one might attribute the different behaviour of this collagen to any of the three alterations in the incubation conditions (temperature, phosphate or centrifugation), it seems unlikely that those that optimize the formation of native structures would inhibit the formation of early native banded fibrils. This consideration leads us to accept the centrifugation step as the critical one in this experimental procedure. This incubation mixture behaved differently from those that had not been centrifuged, in that the appearance of platelet-aggregating activity coincided with the earliest turbidity that we could measure with our instrument (Fig. 4). The result, however, was similar to that from the set of experiments with collagen that had not been centrifuged, in that banded fibrils were found only in the samples that aggregated platelets, and all the active samples contained them. In the sample taken just at the end of the preturbid period (Plate 2a, 11.5min), only very thin (40nm) fibrils could be found, with great difficulty. At this time the platelet-aggregating activity did not exceed 5% of maximum, which is the limit of the experimental precision. At 18.5min, platelet-aggregating activity was 28% of the maximum according to our index, which was the rate of light-transmission change determined 30s after the addition of 0.62mg of collagen/ml. In this 18.5min sample, well-formed banded fibrils were easily found (Plate 2b, 18.5min). It appears from the micrograph of the 23.0min sample that banded fibrils were growing during the incubation as the platelet-aggregating activity increased and the solution was becoming more turbid (Plate 2c, 23min). Although no qualitative differences are observed, it is clear that the increase in activity is correlated with increase in turbidity from this point on (Fig. 4 and Plate 3, 26.5–40.2min). When the maximum activity was attained at 40min, the platelet response was no longer limited by the collagen, since the turbidity was continuing to increase slowly, even at 55min.

We were interested in whether the very thin protofibrils (<10nm thick), described by Williams *et al.* (1978) and Gelman *et al.* (1979), were active toward platelets. These form before the onset of turbidity and are believed to be the units of thick banded fibrils. In neither set of incubations did platelet-aggregating activity appear along with these structures in the absence of banded fibrils. They are most clearly seen in Plate 3 at 26.5min.

Discussion

The preferential binding of collagen aggregates to platelets over that of tropocollagen is shown in Fig. 2. The rise in binding affinity is abrupt, with a half-height for a particle of 4.5S. Binding to erythrocyte membranes (at a concentration greater than that of the platelet membranes) is not observed for tropocollagen or aggregates. The aggregates examined in this experiment are believed to be small because of their low values of *s* and may typify the multimers that Wang *et al.* (1978) suggested to be the smallest possible platelet-stimulating species. We see here that multimers do bind strongly to platelets, the dissociation constant being less than 1 nM.

We have, however, found the maximum platelet-stimulating activity in much larger aggregates. To isolate the active fractions, sedimentation at low force was necessary, as shown in Fig. 1. The activity appeared in fractions 3–7 of Fig. 1, which correspond to sedimentation coefficients from 4×10^4 to 10×10^4 S. The inactivity of fractions larger than this could be merely a geometrical effect. Fig. 1 shows that the collagen mass is evenly distributed along the sedimentation column; heavier species at the bottom of the column must then present less total available surface to interact with platelets, there necessarily being a larger number of thin fibrils at the top than thick ones at the bottom. The activity seems to be more dependent on the number of fibrils (or total surface area) than on their mass. Although the slowly sedimenting fractions were inactive, this might be due to their lower collagen contents.

The platelet-stimulating activity of some type of multimer was proposed by Muggli & Baumgartner (1973) to explain the appearance of activity before turbidity in collagen solutions being incubated at 37°C. Our results show that banded fibrils were actually present in these solutions (Plate 1). The fibrils are evidently too fine and few in number to be discernible as increased turbidity. When all the collagen is converted to fibrils, in sample D, the maximum activity is attained (Fig. 3).

Evidence has appeared that, in incubations such as these, collagen first forms very thin microfibrils, from about 2 to 50nm thick, which then pack laterally to make thick banded fibrils (Williams *et al.*, 1978; Gelman *et al.*, 1979). These very thin microfibrils are present during the lag period observed in the progress of turbidity during the incubation of the collagen, and therefore do not contribute to the turbidity during the early phase of fibrillogenesis. It might have been supposed that these microfibrils were the species that were active toward platelets, which also appeared during this lag period in earlier experiments (Muggli & Baumgartner, 1973). We have shown here, however, that the preturbid solutions are not active toward platelets if the

EXPLANATION OF PLATE I

Electron micrographs of collagen in samples C(a), E(b) and F(c), defined in Fig. 3(a)

When each sample was removed from the spectrophotometer, a drop was placed on a carbon-coated grid for 1 min; excess liquid was then drawn off, and the grid was stained with phosphotungstic acid (pH 4) for 2 min. Each grid was then rinsed with 0.1 M-acetic acid. The rest of the sample was used for the titration in Fig. 3(b). Samples are identified on the photographs. Sample C (a) shows the presence of a few well-developed banded fibrils, which appear with the incipient activity in the preturbid collagen solutions. Sample E(b) shows the appearance of many banded fibrils as the solution grows turbid. A background of microfibrils, best shown in the micrograph taken of sample F(c), appeared during the preturbid period and in all the samples taken afterwards.

EXPLANATION OF PLATE 2

Electron micrographs of the earlier samples of collagen incubation mixtures removed at the indicated times, corresponding to the points in the platelet-response curve of Fig. 4

Each sample was prepared and stained as described in the legend to Plate 1 and in the text. At 11.5 min (a), only a few very thin fibrils could be found. At 18.5 min (b) a small number of thicker banded fibrils were present. At 23 min (c), banded fibrils appeared to be increasing in number and size.

EXPLANATION OF PLATE 3

Electron micrographs of later samples of collagen incubation mixture removed at the indicated times, corresponding to the points in the platelet-response curve of Fig. 4

At 26.5 min (*a* and *b*) thick banded fibrils, attached to a reticulate network of microfibrils, were found; the banded structure of the thick fibrils and how they are attached to the microfibrils are shown in greater detail at higher magnification (*b*). The final turbid suspension, taken at 40.2 min (*c*), comprised a network of thick and thin fibrils and microfibrils, differing only in density from the earlier turbid suspensions. Each sample was prepared and stained as described in the legend to Plate 1 and in the text.

solutions have been centrifuged before the incubation (Fig. 4), although we have seen the microfibrils form whether the collagen has been ultracentrifuged (Plate 2) or not (Plate 1c). As mentioned in the Results section, the microfibrils in Plate 1(c) were characteristic of the inactive preturbid sample B of Fig. 3. These unbanded protofibrils that formed during the lag period therefore cannot be active toward platelets. We find that only solutions that have banded fibrils are active. That the active species are actually these thick fibrils is confirmed in our experiments with fractionated collagen, in which the active fraction is found to be a fast-sedimenting one (Fig. 1). The apparent loss of activity from the heaviest fractions is likely to be a mass effect, and is consistent with occasional observation of a decrease in activity in collagen solutions that have been incubated for long times (Barnes *et al.*, 1976).

Implicit in this reasoning are assumptions about the amounts of the small microfibrils present during the period before banded fibrils are observed. The experiments of Williams *et al.* (1978) and of Gelman *et al.* (1979) did not show how the preturbid collagen was distributed between microfibrils and tropocollagen. It appears from their and our micrographs, however, that appreciable amounts were in the form of microfibrils. This is confirmed by markedly shortened lag times in the platelet responses when platelets were stimulated with collagen samples taken during this early period of the incubation. If the lack of stimulation from the early samples had merely been a matter of small amounts of microfibrils, incubating these solutions would not have shortened the platelet-response lag time, which is due to collagen fibrillogenesis in the platelet suspension.

Wang *et al.* (1978) have determined the platelet-aggregating activities of collagen incubation mixtures that had been characterized by a flow-birefringence parameter, which they used to calculate the molecular length. They found that suspensions with a calculated fibril length corresponding to the trimer were active. As these authors themselves point out, however, their collagen solutions were polydisperse with respect to size of the fibrils. That is, their suspensions, which had not been fractionated, would have contained a few long fibrils. [They did not state whether they precentrifuged their tropocollagen solutions before fibrillogenesis; as we and others (Williams *et al.*, 1978; Gelman *et al.*, 1979) have shown, a small number of fibrils form rapidly unless this is done.] We therefore see no discrepancy between our observations and theirs, although our conclusions disagree.

Collagen is found in tissue in a variety of aggregated forms. The thickest and most perfect crystalline fibres are found in tendons, composed of type-I collagen, defined by its chemical composition. Types I and III in vascular tissue are present as

fibrils that are finer than in tendons, but are identifiable as cross-banded structures with high molecular order. A poorly ordered form is type-IV collagen in vascular subendothelial basement membrane, which is not identifiable in the tissue itself as fibrils, but can be precipitated from citrate solution as very fine fibres with the dimensions of single molecules (Olsen *et al.*, 1973). It is doubtful that platelets interact with tendon collagen in any physiologically significant situations. They do adhere to subendothelium, and aggregate *in vitro* in response to type-IV collagen which has been extracted from glomeruli with pepsin (Freytag *et al.*, 1978). This activity of type-IV collagen may be due to its subfibrillar form after it is reconstituted. Secretion of a growth factor from the adherent platelets may be pathological, promoting the proliferation of atherogenic smooth-muscle cells on arterial walls. Adhesion to vessel-wall collagen is believed to be physiologically important, also, in establishing haemostasis when the vascular wall is perforated. It appears from the present study that the large number of fine collagen fibres in the vessel wall tissue presents the collagen in its optimum form for binding and aggregating platelets.

Conclusions

(1) Platelet-stimulating activity is not found in collagen solutions that are undergoing fibrillogenesis until well-developed fibrils appear. (2) Well-developed banded fibrils appear before the onset of turbidity in collagen incubation mixtures that have not been previously ultracentrifuged; when, however, nuclei are removed by centrifugation at high speed, the fibrils appear with the onset of turbidity. (3) There is an intermediate state of aggregation of collagen in which the specific platelet-stimulating activity is maximal, which probably balances the size of the fibrils with their surface area. Collagen in this state has a sedimentation coefficient of about 10^5 S. (4) Small collagen aggregates, or multimers, bind to platelets with very high affinity ($K_d < 10$ nM). (5) It is confirmed (Kronick, 1974; Gordon & Dingle, 1974) that tropocollagen does not bind measurably to platelets.

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