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# Effect of fibrinogen degradation products on platelet aggregation

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SYNOPSIS The digestion of fibrinogen with various concentrations of trypsin results in the formation of a variety of degradation products. Degradation products formed in this way have been purified by DEAE cellulose column chromatography and their effects on platelet aggregation investigated.

Two methods have been used to study platelet aggregation: a turbidimetric method which assesses platelet aggregation by the ability of adenosine diphosphate (ADP) to clump platelets and a method which assesses platelet adhesiveness by their ability to adhere to glass and to each other (modified Hellem technique, 1960).

Three breakdown products produced by trypsin-digested fibrinogen were studied and all showed 'antithrombin' activity: two inhibited platelet aggregation, but one accelerated aggregation in both systems. Another product prepared by digestion of fibrinogen with urokinase-activated plasminogen has been shown to possess the ability to enhance ADP-induced platelet aggregation.

In this paper is presented an account of experiments in which the effect on platelet aggregation of degradation products of fibrinogen proteolysis were studied. The work was prompted by the observation previously reported from this laboratory that trypsin can enhance platelet aggregation induced in plasma by adenosine diphosphate (ADP) (Wilson, McNicol, and Douglas, 1966). This effect of trypsin could not be explained wholly on the basis of the ability of trypsin to generate thrombin in plasma and accordingly it was thought worthwhile to investigate the effect of fibrinogen digests on platelet aggregation in this system.

Kowalski, Kopeć, and Wegrzynowicz (1963) have shown that washed and unwashed platelets treated with streptokinase-activated plasminogen lose their ability to aggregate in response to ADP. It has also been demonstrated that platelets treated with trypsin lose their ability to aggregate in fresh serum or in solutions of thrombin and calcium chloride (Schmid, Jackson, and Conley, 1962; Morse, Jackson, and Conley, 1965). It therefore appeared likely that trypsin was not enhancing aggregation by direct action on the platelets but was altering one or more plasma proteins which in turn influenced the platelet aggregation system.

Fibrinogen, an important factor in ADP-induced platelet aggregation (McLean, Maxwell, and Hertler, 1964), has also been demonstrated to be an integral platelet constituent (Grette, 1962; Schmid *et al.*, 1962; Gokcen and Yunis, 1963; Nachman, Marcus, and Zucker-Franklin, 1964; Castaldi and Caen, 1965; Morse *et al.*, 1965). In addition, fibrinogen has been shown to be closely associated with the platelet surface by its presence in the 'plasmatic atmosphere' of the platelet (Iatridis and Ferguson, 1965), and it was considered possible that the influence which the enzyme trypsin exerted on platelet aggregation might be mediated through its action on fibrinogen. The fibrinogen digests were purified by column chromatography before appraisal of their physical and chemical characteristics and of their effect on platelet aggregation.

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## REAGENTS

Human lyophilized fibrinogen grade L and human lyophilized plasminogen grade B were obtained from A.B. Kabi Stockholm. Trypsin as the 2 × crystalline salt-free preparation was obtained from L. Light and Company, and the soya bean trypsin inhibitor type 1-S was obtained from the Sigma Chemical Company St. Louis. Urokinase was obtained from Abbot Laboratories, Illinois. Adenosine diphosphate (ADP) was obtained as the sodium salt from the Sigma Chemical Company, St. Louis.

## METHODS

PREPARATION OF FIBRINOGEN DEGRADATION PRODUCTS Four degradation products were prepared, three by tryptic digestion of fibrinogen and one by digestion with urokinase-activated plasminogen. The tryptic degradation products were each prepared by incubating 0·3 ml of the various trypsin solutions with a solution containing 200 mg fibrinogen dissolved in 4 ml 0·1M phosphate buffer, *p*H 7·6. At the end of incubation 0·3 ml of the appropriate concentration of soybean trypsin inhibitor was added to the system. The urokinase degradation product was prepared by incubating 0·1 ml of the enzyme preparation with a solution composed of 200 mg fibrinogen dissolved in 4 ml 0·1M phosphate buffer *p*H 7·6 and 1 ml of plasminogen solution containing 17·5 Sgouris units/ml.

The column chromatography of the fibrinogen degradation products was carried out by a modification of the method of Nussenzweig, Seligmann, Pelmont, and Grabar (1961). A separate column was prepared for each.

The digest obtained by incubating fibrinogen with the enzyme was layered onto a DEAE cellulose column, 37·5 cm long and 2·5 cm in diameter. Elution was with a buffer system of 0·1M sodium carbonate *p*H 8·9-0·1M sodium carbonate *p*H 8·9 + 0·2M sodium chloride with a linear concentration gradient. The flow rate for each column lay within the range 80-100 ml per hour and the eluate was collected in 10 ml amounts using an Aimer fraction collector with a drop counter attachment. The eluate samples were screened for protein content by estimating optical density at 280 m$\mu$ on a Unicam S.P. 500 spectrophotometer. The protein concentration in the samples was determined by the method of Lowry, Rosebrough, Farr, and Randall (1951). The eluate samples which contained fibrinogen degradation products were concentrated with Sephadex G-25 using Seitz filters.

REACTION KINETICS The approximate number of peptide bonds broken during the action of trypsin on fibrinogen was determined using a titrator type TT1c connected to an autoburette type ABU 1b and titrigraph type SBR 2c (Radiometer Electronic Measuring Instruments, Copenhagen).

ULTRACENTRIFUGATION Sedimentation and diffusion coefficients were determined using a Spinco, model E, ultracentrifuge, equipped with a Schlieren optical system.

PLATELET AGGREGATION The effect of pure fibrinogen and fibrinogen degradation products on platelet aggregation was investigated in the Born turbidimetric system in which platelet aggregation in response to ADP is measured (Born and Cross, 1963). Blood was collected by clean venepuncture using a plastic syringe from healthy colleagues or from hospital patients suffering from a variety of diseases none of which are known to influence platelet aggregation. The blood was citrated by adding 9 volumes of blood to 1 volume of 3·8% sodium citrate in a silicone-treated tube. Platelet-rich plasma was obtained by centrifuging the citrated whole blood at 600 *g* for five minutes. Each plasma sample was divided into two portions. Fibrinogen or fibrinogen degradation products at final concentrations of 100 $\mu$g/ml were added to one sample and an equal volume of saline to the second sample. Both samples were then incubated at 37°C for 10 minutes and then each sample was placed in an EEL titrator connected to a galvanometer (EEL type 20). Platelet aggregation was induced by the addition of ADP (0·5 $\mu$g/ml) and the platelet aggregation assessed by measuring the fall in optical density shown on the galvanometer (Wilson *et al.*, 1966).

PLATELET ADHESIVENESS The effect of fibrinogen and the various fibrinogen degradation products on platelet adhesiveness was estimated by a modification of the Hellem (1960) glass bead column method (Hirsh, McBride, and Wright, 1966). Blood was collected by clean venepuncture and citrated by the addition of 9 volumes to blood to 1 volume of 3·8% sodium citrate. The citrated blood was allowed to stand at room temperature for a minimum of 30 minutes and a maximum of 45 minutes before the experiments were carried out. The blood sample was divided into two portions, each of 2 ml amounts. To one portion was added 0·2 ml saline (control) and to the other 0·2 ml of degradation product to give a known final concentration. Both samples were incubated at 37° for 10 minutes before being passed through a glass bead column by means of a constant speed pump. The glass bead column consisted of 2·5 g glass beads (Ballotini, 0·57 mm diameter) enclosed by nylon gauze in a vinyl tube (Portland Plastics, Kent N.T.13) to give a column length of 6 cm. The contact time of the blood and glass beads was estimated and any observation which lay outside the range 30 $\pm$ 1 sec was discarded. The percentage platelet adhesiveness was calculated from the difference observed between the platelet counts performed on the blood sample before and after passage through the column.

PLATELET COUNTS These were performed by the method of Dacie, 1956.

THROMBIN CLOTTING TIMES These were estimated using the method of Fletcher, Alkjaersig, and Sherry (1959) as described by McNicol and Douglas (1964).

## RESULTS

INITIAL EXPERIMENTS To study the behaviour of pure fibrinogen, plasminogen, trypsin, soya bean trypsin inhibitor, and urokinase experiments in column chromatography were carried out.

Fibrinogen was eluted from a column as a single peak at a NaCl molarity of 0·089M; plasminogen was eluted as two peaks, one at 0·080M NaCl and the second at a NaCl concentration >0·1M NaCl; trypsin and soya bean trypsin inhibitor were eluted at a molarity of >0·1M NaCl; and the urokinase preparation was eluted as three peaks, the first at a molarity of 0·080M NaCl and the other two at molarities >0·1M NaCl.

PREPARATION AND CHARACTERIZATION OF DEGRADATION PRODUCTS The first fibrinogen degradation product was prepared by incubating 200 mg fibrinogen with 0·67 mg trypsin at 37°C for 10 minutes. Immediately after incubation 0·67 mg soya bean trypsin inhibitor was added and the mixture passed through a DEAE cellulose column. A single well defined peak was eluted at a NaCl molarity of 0·035M. This protein will be referred to as product *a*.

The second fibrinogen degradation product was prepared by incubating 200 mg fibrinogen with 6·67 mg trypsin at 37°C for 10 minutes followed at once by the addition of 6·67 mg soya bean trypsin inhibitor. From this mixture a single well defined peak was eluted at a molarity of 0·045M NaCl. This protein will be referred to as product *b*.

The third fibrinogen degradation product was prepared by incubating 200 mg fibrinogen with 6·67 mg trypsin at 37°C for 16 hours. Then 6·67 mg soya bean trypsin inhibitor was added and the mixture passed through a column. A very diffuse elution pattern was obtained but a discrete peak at an approximate concentration of 0·060M NaCl. The initial discrete peak was concentrated with Sephadex G-25 and will be referred to as product *c*.

The fourth fibrinogen degradation product was prepared by digesting at 37°C for 10 min 200 mg fibrinogen with $4 \times 10^3$ CTA units urokinase and 17·5 Sgouris units plasminogen. From this mixture a single peak was eluted at a molarity of 0·035M NaCl. This protein will be referred to as product *d*.

The physiochemical characteristics of the products *a* and *b* were analysed with a Spinco model E ultracentrifuge. It was only possible to perform one determination of the sedimentation (S) and diffusion (D) values for each of the products. For product *a* the S value was estimated as 6·72 and the D value at $6{\cdot}62 \times 10^{-7}$ which gave a molecular weight of 82,427 calculated from the Svedberg equation. For product *b*, the S value was estimated as 4·37, the D value as $1{\cdot}44 \times 10^{-6}$, and a molecular weight of 24,642 was calculated from the Svedberg equation.

The degree of digestion of fibrinogen which corresponded to the products *a* and *b* was estimated using a *p*H stat. The reactions were carried out at *p*H 8·5 with 0·0335 N NaOH as titrant at 37°C under a stream of nitrogen. In the investigation of the reaction leading to the formation of product *a*, fibrinogen (20 mg in 1 ml 0·9% NaCl), was treated with 0·067 mg trypsin and the reaction followed for 10 minutes. It was found that $1{\cdot}005 \times 10^{-6}$ moles of alkali were utilized during the reaction. This figure is equivalent to 17·085 moles of alkali used per 340,000 g of fibrinogen. Thus in the formation of product *a* 17 peptide bonds of fibrinogen were broken. For the formation of product *b*, 20 mg of fibrinogen was incubated with 0·67 mg trypsin for 10 minutes. During this reaction $>2{\cdot}680 \times 10^{-6}$ moles of alkali were required to maintain a constant *p*H. This is equivalent to 45·56 moles per mole of fibrinogen, indicating that 45 peptide bonds were broken during the reaction.

EFFECT OF FIBRINOGEN AND FIBRINOGEN BREAKDOWN PRODUCTS ON PLATELET AGGREGATION AND ADHESIVENESS A variety of fibrinogen degradation products can be formed from fibrinogen by tryptic digestion according to the relative concentrations of fibrinogen and trypsin in the incubation mixture. The effect on platelet aggregation of three such products and of a product formed by digestion of fibrinogen with urokinase-activated plasminogen was investigated in a turbidimetric system. For comparative purposes the effect of the initial pure fibrinogen was also investigated.

Platelet-rich plasma samples were incubated with fibrinogen or one of the degradation products for 10 minutes at 37°C before platelet aggregation was induced by the addition of 0·5 $\mu$g/ml adenosine diphosphate.

Seven plasma samples were incubated with product *a* at a concentration of 100 mg/ml. At this concentration product *a* appeared to exert no influence on platelet aggregation; no significant difference could be distinguished between the changes in optical density occurring in the test samples and the saline controls (Fig. 1). Seven plasma samples were incubated with product *b* at a concentration of 100 $\mu$g/ml. This product can enhance platelet aggregation; two minutes after the onset of aggregation the mean optical density of the test samples was 0·341 and of the control samples 0·378 ($t = 3{\cdot}252$, $P < 0{\cdot}02$) (Fig. 2). Product *d* was tested in a similar manner and also to some extent enhanced platelet aggregation; one and a half minutes after the onset of aggregation the mean optical density reading of the 10 test samples was 0·216 and of the control samples 0·305 ($t = 2{\cdot}340$, $P < 0{\cdot}05$) (Fig. 3). Product *c* appears to cause some inhibition of platelet aggregation (Fig. 1). Seven plasma samples were incubated with this product at a concentration of 100 $\mu$g/ml. Two minutes after the onset of aggregation the mean optical density reading of the test samples was 0·473 and of the control samples 0·436 ($t = 3{\cdot}190$, $P < 0{\cdot}02$). Fibrinogen at a concentration of 100 $\mu$g/ml was tested in seven plasma samples. Fibrinogen was found to enhance platelet aggregation in this system, confirming the findings of Cross (1964).

The effect of products *a* and *b* on platelet adhesiveness in whole citrated blood was investigated

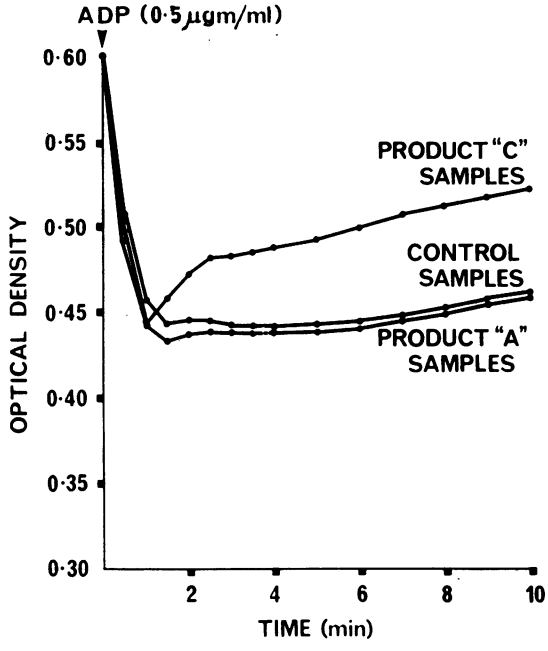


FIG. 1. *Effect of product* a (*100 μg/ml*) *and product* c (*100 μg/ml*) *on ADP-induced platelet aggregation.*

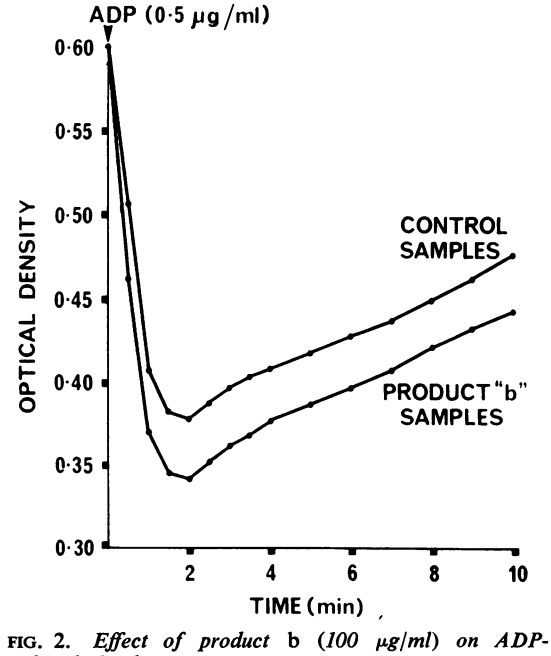


FIG. 2. *Effect of product* b (*100 μg/ml*) *on ADP-induced platelet aggregation.*

with the modified Hellem technique, as also was that of a sample of the initial pure fibrinogen solution.

In the first series of experiments 2 ml of the citrated whole blood was incubated at 37°C for 10 minutes with one of the degradation products or with fibrinogen at a concentration of 100 μg/ml before the sample was passed through the glass

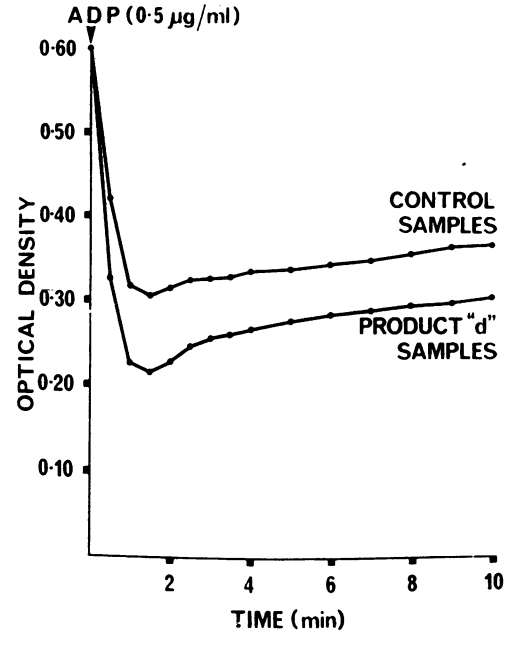


FIG. 3. *Effect of product* d (*100 μg/ml*) *on ADP-induced platelet aggregation.*

bead column. Under these conditions the results with all three protein solutions were inconclusive; no clear pattern emerged for the effect of either the degradation products or fibrinogen on platelet adhesiveness. The experiment was repeated with the degradation products *a* and *b* at a concentration of 200 μg/ml. Eleven plasma samples were investigated with each product but no significant differences were found between the platelet adhesiveness of the control and test samples.

In the turbidimetric system described above, the protein solutions added to the plasma samples are thoroughly mixed with the plasma by the stirring action which occurs during the assay procedure. The experiments with glass bead columns were therefore modified in order to mix thoroughly the protein solutions and the whole citrated blood before passage through the column. The blood sample and the substance under test were rotated on the turn-

table of a blood cell suspension mixer (Matburn Ltd. London) for 10 minutes at 37°C. A second sample with normal saline was treated similarly to act as a control. Using this modification seven blood specimens were examined with product *a*. This protein appeared to have no significant effect on platelet adhesiveness; the mean percentage adhesiveness of the control samples was 72·5 ± 12·3% and of the test samples 70·4 ± 15·6% (Table I). The same series of seven blood specimens were used for the investigation of the product *b* on platelet adhesiveness. At a concentration of 100 μg/ml this protein caused a significant increase in platelet adhesiveness. The mean platelet adhesiveness of the control samples was 72·5±12·3% and of the test samples 77·5 ± 11·6% ($t = 3{\cdot}33$, $P < 0{\cdot}02$) (Table I).

TABLE I

EFFECT OF FIBRINOGEN DEGRADATION PRODUCTS a AND b ON PLATELET ADHESIVENESS

| | *Percentage Platelet Adhesiveness* | | |
|---|---|---|---|
| *Subject No.* | *Control* | *Product a (100 μg/ml)* | *Product b (100 μg/ml)* |
| 1 | 80·1 | 73·4 | 88·6 |
| 2 | 63·8 | 67·4 | 74·9 |
| 3 | 76·2 | 74·5 | 80·2 |
| 4 | 82·7 | 89·3 | 83·0 |
| 5 | 55·3 | 48·6 | 62·6 |
| 6 | 61·3 | 54·3 | 62·4 |
| 7 | 87·9 | 87·9 | 91·8 |
| Mean | 72·5 | 70·4 | 77·6 |
| ±S.D. | ±12·3 | ±15·6 | ±11·7 |

The effect of fibrinogen (100 μg/ml blood) on platelet adhesiveness was investigated with a series of seven samples using the modified glass bead column method. Fibrinogen was found to cause a significant increase in platelet adhesiveness (Table II); the mean percentage adhesiveness of the control samples was 61·7 ± 11·0 and of the fibrinogen-treated samples 78·6 ± 9·0 ($t = 5{\cdot}166$, $0{\cdot}001 < P < 0{\cdot}005$).

THROMBIN CLOTTING TIME The effects of products *a* and *b* were examined in a thrombin clotting time system over the range of concentration 100 μg/ml to 2 mg/ml plasma. The results are shown in Table III. It would appear that both these fibrinogen degradation products can cause prolongation of the thrombin clotting time, presumably mediated through defective fibrin polymerization.

## DISCUSSION

The digestion of fibrinogen by trypsin can result in the formation of a variety of degradation products.

TABLE II

EFFECT OF FIBRINOGEN (100 μg/ml) ON PLATELET ADHESIVENESS

| | *Percentage Platelet Adhesiveness* | |
|---|---|---|
| *Subject No.* | *Control* | *Fibrinogen* |
| 1 | 71·3 | 71·3 |
| 2 | 58·8 | 83·6 |
| 3 | 47·5 | 70·1 |
| 4 | 64·3 | 82·7 |
| 5 | 79·8 | 94·0 |
| 6 | 55·2 | 69·4 |
| 7 | 55·2 | 79·0 |
| Mean | 61·7 | 78·6 |
| ±S.D. | ±11·0 | ± 9·0 |

TABLE III

EFFECT OF FIBRINOGEN DEGRADATION PRODUCTS a AND b IN A THROMBIN CLOTTING TIME ASSAY

| *Sample* | | *Thrombin Clotting Time (sec.)* |
|---|---|---|
| Saline control | | 16·5, 16·0 |
| Product a (mg/ml) | 2 | 30·2, 30·0 |
| | 1 | 25·2, 25·4 |
| | 0·5 | 25·5, 25·5 |
| | 0·2 | 25·0, 25·5 |
| | 0·1 | 25·5, 25·2 |
| Product b (mg/ml) | 2 | 28·5, 28·8 |
| | 1 | 26·2, 26·2 |
| | 0·5 | 24·5, 25·0 |
| | 0·2 | 23·2, 23·8 |
| | 0·1 | 23·4, 23·8 |

The effects of two such products on platelet aggregation in a turbidimetric system, on platelet adhesiveness in a glass bead column assay system, and on thrombin clotting times have been investigated. Some of the physiochemical properties of these products have also been examined.

Conflicting reports have appeared in the literature concerning the effect of fibrinogen degradation products on ADP-induced platelet aggregation. Degradation products prepared by digestion of fibrinogen with streptokinase-activated plasminogen have been shown to inhibit both ADP-induced aggregation (Jerushalmy and Zucker, 1966; Kowalsk *et al.*, 1963) and fibrinogen-induced aggregation (Kopeć, Budzynski, Stachurska, Wegrzynowicz, and Kowalski, 1966) *in vitro*. However, degradation products prepared by plasmin digestion of fibrinogen have also been shown to exert no influence on ADP-induced platelet aggregation (Hirsh, Fletcher, and Sherry, 1965) except when ADP is present in trace amounts (Larrieu, Marder, and Inceman, 1966). In our experiments, product *a* produced an effect on platelet aggregation in the ADP system in agreement with the results of Hirsh *et al.* (1965) and product *c* produced results in agreement with those of Kowalski *et al.* (1963). Barnhart, Cress, Henry, and Riddle (1967) have

published a report on the identification of a fibrinogen degradation product which possesses the ability to decrease the number of circulating platelets after infusion into dogs and can enhance platelet aggregation *in vitro*; this product may be similar to our products *b* and *d*.

Because of the variety of effects produced by fibrinogen breakdown products it was thought important to characterize products *a* and *b* in some detail by the investigation of the reaction kinetics involved in their formation and the study of their ultracentrifugal properties. Product *a* has been demonstrated to be formed from fibrinogen by the rupture of 17 peptide bands per molecule of fibrinogen. The resulting protein was found to have an $S_{20}$, $\omega$ of 6·72 and a molecular weight of about 82,000. Forty-seven peptide bonds per fibrinogen molecule appeared to have been split in the formation of product *b* and the $S_{20}$, $\omega$ for this protein was determined as 4·37 with a molecular weight of about 25,000. The currently accepted values for the $S_{20}$, $\omega$ of fibrinogen is 7·9 (Scheraga and Laskowski, 1957) and the molecular weight of the Kabi human fibrinogen used in the preparation of the degradation of the fibrinogen molecule may produce a molecule with a very much reduced weight, although a proportionate number of peptide bonds do not appear to have been broken. This observation may be explained by the currently accepted concept of the structure of fibrinogen which holds that the molecule is made up of three peptide chains (Blombäck and Yamashina, 1958; Blombäck, Blombäck, Edman, and Hessel, 1966; Laki and Gladner, 1964); cleavage of the bonds between the peptide chains would result in a marked fall in the molecular weight of protein.

In the present study it has been demonstrated that fibrinogen added to platelet-rich plasma can enhance ADP-induced platelet aggregation, a finding previously reported by Cross (1964), by Solum and Stormorken (1965), and by Kopeć *et al.* (1966). Gaarder and Laland (1964) have recently advanced the hypothesis that during the process of platelet aggregation a physicochemical phenomenon occurs between platelet membranes, 'bridges' being formed between the platelets, consisting of complexes of ADP, fibrinogen, and calcium ions; a protein which has a similar structure and charge characteristics to fibrinogen might perhaps substitute for fibrinogen in the formation of such complexes. While it has been demonstrated that the degradation products *a* and *b* differ sufficiently from the initial fibrinogen as to interfere with the thrombin-fibrinogen reaction, they retain partial immunological identity to human fibrinogen (unpublished observations), and it may be that product *b* possesses the necessary properties to substitute for fibrinogen in the formation of inter-platelet bridges.

Recently Kopeć *et al.* (1966) reported that unpurified degradation products prepared by the action of streptokinase-activated plasminogen inhibit the normal release of adenine nucleotides from platelets as they aggregate. Kopeć *et al.* (1966) also report that fibrinogen does not appear to affect adenosine nucleotide release, and clearly in view of its action in augmenting platelet aggregation it is improbable that our purified product *b* shares the effect on adenine nucleotide release found by Kopeć *et al.*, with a heterogeneous mixture of breakdown products.

Platelet aggregation has been shown to have a critical role in the initial stages of haemostasis (Hjort and Hasselback, 1961; Poole and French, 1961; Grette, 1962; Käser-Glanzmann and Lüscher, 1962; Marr, Barboriak, and Johnson, 1965; Hellem and Owren, 1964; Russell, 1961). The attractive theoretical concept of a haemostatic balance mechanism suggests that fibrinolysis and coagulation may be continuously operating in parallel throughout the vascular bed (Astrup, 1958; Sherry, Fletcher, and Alkjaersig, 1959) and if such is the case, products of fibrinogen proteolysis may have an important homeostatic role in displacing the equilibrium of the system towards coagulation.

The possibility of a physiological role for the fibrinogen degradation product *b* is strengthened by the circumstance that it can be produced not only with trypsin but with the intravascular fibrinolytic enzyme plasmin, produced by activation of human plasminogen by human urokinase.

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