Biochemistry of Blood Platelets. Interaction of Activated Factor X with Platelets*

Sidney Jevons and Peter G. Barton†

ABSTRACT: Exposure of washed rabbit platelets to activated factor X resulted in platelet aggregation. This effect was characterized by a prolonged lag period prior to the onset of aggregation. These phenomena were shown to be dependent on the presence of Ca^{2+} ions. Evidence is presented that these observations were not due to contamination of the activated factor X with thrombin which is known to cause aggregation. Variations in the responses of different platelet preparations to activated factor X were shown to be principally due to differences in the amounts of prothrombin associated with the platelets and to differences in the availability of platelet factor 3, a phospholipid or lipoprotein component of the membrane.

Data obtained with selected inhibitors together with information in the literature suggested that the action of activated factor X on platelets is mediated through a surface reaction leading to generation of thrombin and subsequent release of ADP.

Blood platelets are small, nonnucleated fragments of megakaryocytes that are believed to play an important role in hemostasis. In circulating blood, platelets exist as discrete cellular entities possessing an outer membrane and containing numerous intracellular structures such as mitochondria, lysosomes, and storage granules. Also present are α granules containing large amounts of phospholipids. At the site of vascular injury, the platelets are observed in large aggregates which constitute the primary hemostatic plug. This process has been considered to be quite distinct from the process of blood coagulation and to precede the formation of a fibrin clot.

Aggregation of the platelets can be induced *in vitro* by adenosine diphosphate (Gaarder *et al.*, 1961, Born, 1962). Treatment of platelets with thrombin can lead initially to aggregation but with subsequent release of many cellular constituents followed eventually by platelet disintegration (Fonio, 1940, 1948). Aggregation induced by thrombin and other reagents is probably mediated through release of platelet ADP (Haslam, 1964, 1967).

It has been proposed that several mechanisms involved in blood coagulation converge at the activation of factor X (Papahadjopoulos *et al.*, 1964). Activated factor X is then responsible for the conversion of prothrombin into thrombin (Milstone, 1955; Barton *et al.*, 1967; Baker and Seegers, 1967; Jobin and Esnouf, 1967; Hemker *et al.*, 1967), a reaction which is accelerated greatly by the presence of factor V, calcium ions and platelets or phospholipids.

Davey and Lüscher (1965, 1967) have demonstrated a correlation between the ability of several snake venoms to activate either factor X or prothrombin and the ability of these venoms to induce platelet aggregation. We have investigated the effect of purified X_a on washed rabbit platelets *in vitro* in order to gain some insight into the sequence of events resulting from their interactions. Aggregation of platelets induced by addition of X_a has not to our knowledge been reported hitherto.

Experimental Section

Materials. Adenosine 5′-diphosphate sodium salt (Grade 1, Lot 109B-7190), adenosine (Lot A92B-251), hirudin (1000 units/mg, Grade III, Lot 69B, 0090), pyruvate kinase from rabbit skeletal muscle (Type 2, Lot 38B-4290), and phosphoenolpyruvate tricyclohexylamine salt (Lot 109B-5230) were obtained from Sigma (St. Louis, Mo.). Albumin (bovine) fraction V powder (Pentex) (Lot 72353) was obtained from Calbiochem (Los Angeles, Calif.). Diabutal (sodium pentabarbital) was from Diamond Laboratories (Des Moines, Iowa) and ethylene glycol bis(β-aminoethyl ether)-*N*,*N*-tetracetic acid[1] (EGTA) from K and K Laboratories, Plainview, N. Y. SC-87 Dri-Film siliconizing fluid was obtained from General Electric (Waterford, N. Y.) and used as 10% v/v solution in CCl_4. U. S. Standard human thrombin (21.7 units/mg, Lot B-3) was obtained from the Division of Biologics Standards, N.I.H. (Bethesda, Md.), through the courtesy of Dr. D. Aronson. Bovine thrombin from Parke-Davis (Kalamazoo, Mich.) was purified chromatographically according to Yin and Wessler (1968). Fibrinogen was prepared according to the method of Blombäck and Blombäck (1956) and was 95% clottable. For routine assays, a solution containing 2 mg/ml in 0.145 M NaCl was used. Prothrombin was prepared by a modification of the method of Cox and Hanahan (1970) in which the citrate elution from DEAE-cellulose was replaced by phosphate elution according to Seegers and Landabaru (1960). The product, specific activity 2 units/mg, appeared as a single homogeneous component when examined over a wide concentration range by sedimentation velocity in the ultracentrifuge with an $s_{20,w} = 5.1S$ when extrapolated to zero concentration. A single band was observed on disc electrophoresis at pH 8.6 and the amino acid composition was closely similar to that reported by Cox

* From the Department of Biochemistry, University of Alberta, Edmonton, Alberta, Canada. *Received August 3, 1970.* This investigation was supported by the Medical Research Council of Canada, Grant MA-2402, and the Life Insurance Medical Research Fund. S. J. was Fellow of the Canadian Heart Foundation and thanks the Wellcome Trust for a travel grant. This paper is intended as the first in a series dealing with the role of platelets in blood coagulation.

† To whom correspondence should be addressed.

[1] Abbreviations used are: X_a, activated factor X; PF3, platelet factor 3; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*-tetraacetic acid; PK, pyruvate kinase; PEP, phosphoenolpyruvate.

and Hanahan (1970). Factor X was not detectable by the standard procedures of Bachmann *et al.* (1958). No fibrinogen clotting activity was observed in 18 hr when 14 units of prothrombin was incubated with 0.2 ml of fibrinogen (2 mg/ml) at pH 7.35 and 37°. X_a was isolated according to Yin and Wessler (1968) and was further purified in our laboratory to a final specific activity of 5000 units/mg of protein (R. Radcliffe, unpublished data). Disc electrophoresis of 50 μg of protein at pH 8.6 gave one major band and one or two faintly visible bands. No fibrinogen clotting activity was observed in 18 hr when 23.2 units of X_a was incubated with 0.2 ml of fibrinogen (2 mg/ml) at pH 7.35 and 37°. No thrombin was detected when 0.2 ml of 0.025 M $CaCl_2$ was included. Dilutions of the X_a stock solution were made in plain Tyrode's buffer containing 1% bovine serum albumin. Factor V was prepared by previously published methods (Esnouf and Jobin, 1967; Barton and Hanahan, 1967) to a specific activity of 5–8 units per mg or approximately 600-fold purification from plasma. Platelet substitute (brain cephalin) was prepared according to Bell and Alton (1954). Dextrose and inorganic compounds were ACS standard reagents.

Buffer Solutions. Plain Tyrode's buffer (PT buffer), pH 7.35, contained 8.0 g of NaCl, 0.2 g of KCl, 1.0 g of $NaHCO_3$, and 0.05 g of NaH_2PO_4 per l. and was adjusted to pH 7.35 with 0.1 N HCl. This buffer was used as solvent for all reagents added to the platelet suspensions in the aggregation studies.

Calcium-free Tyrode's buffer (CFT buffer), pH 6.2, contained 8.0 g of NaCl, 0.2 g of KCl, 1.0 g of $NaHCO_3$, 0.05 g of NaH_2PO_4, 3.5 g of serum albumin, 1.0 g of dextrose, 0.2 g of $MgCl_2$, per l. and was adjusted to pH 6.2 with 0.1 N HCl.

Tyrode's albumin buffer (TA buffer), pH 7.35, contained 8.0 g of NaCl, 0.2 g of KCl, 1.0 g of $NaHCO_3$, 0.05 g of NaH_2PO_4, 3.5 g of serum albumin, 1.0 g of dextrose, 0.1 g of $MgCl_2$, and 0.222 g of $CaCl_2$ per l. and was adjusted to pH 7.35 with 0.1 N HCl.

Albino rabbits of both sexes, weighing 3.6–5.4 kg, were obtained locally.

Preparation of Rabbit Platelets. Rabbits were anesthetized with sodium pentabarbital by injection into an ear vein. Blood was collected through cannula inserted in the carotid artery into plastic syringes containing acid citrate dextrose anticoagulant (1 part to 6 parts blood) (Aster and Jandl, 1964). Platelet-rich plasma was obtained by removal of red cells by centrifugation at 120*g* for 15 min in a Sorvall RC2 centrifuge using a fixed angle rotor (r = 4.25 cm). Platelets were obtained by centrifugation of platelet-rich plasma at 1100*g* for 15 min in an International PR-6 centrifuge with a No. 269 rotor. The platelets were then washed twice with CFT buffer according to the method of Ardlie *et al.* (1970). The platelets were finally suspended in TA buffer and stored at room temperature.

Platelet counts were performed in a Spencer Bright-Line hemocytometer and the platelet concentration adjusted to a final value, normally within the range 0.5–1.0 $\times$ 10^6 platelets/mm^3. For aggregation studies aliquots of 1.0 ml were first warmed to 37°.

Platelet Aggregation. Aggregation was studied using a Payton aggregation module with a Beckman 11-in. flatbed recorder. The stirring speed was set at 10^3 rpm.

Glassware. All glassware used in the preparation and storage of platelets was previously siliconized.

Coagulation Assays. X_a was assayed by a modification (Papahadjopoulos *et al.*, 1964) of the method of Bachmann *et al.* (1958). Prothrombin was assayed by the method of Koller *et al.* (1951). Thrombin was assayed by its ability to clot purified fibrinogen. Factor V was assayed by the method of Kappeler (1955).

One unit of a plasma clotting factor is defined as the activity present in 1 ml of normal human plasma. One unit of X_a is the activity evolved from 1 ml of normal human plasma when the precursor is fully activated by Russell's viper venom.

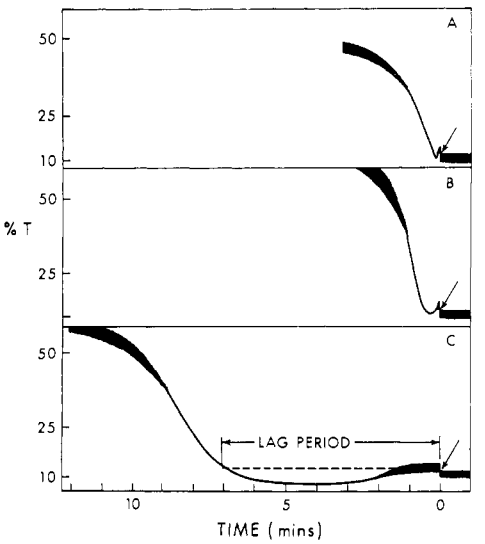

FIGURE 1: Platelet aggregation induced by 0.1 ml (A) of 10^{-4} M ADP; (B) 1.0 NIH unit/ml of thrombin; (C) 2.3 units/ml of X_a. In each case the arrow indicates the point of addition of reagent. Platelet concentration in each experiment was 0.5 $\times$ 10^6/mm^3. Time scale read from right to left.

Results

Aggregation of Platelets by ADP, Thrombin, and Activated Factor X. The platelet aggregometer measured the transmission of light through a suspension of platelets in a standard sized cuvet. The data were recorded as a plot of the per cent transmission (%T) *vs.* time. In an untreated platelet suspension an oscillating tracing was observed as a result of variable transmission through the disc-shaped platelets. When ADP or thrombin was added there was an initial sharp increase in transmission due to dilution, then as the platelets increased in volume, became spherical and more randomly distributed both the %T and amplitude of oscillation decreased. During platelet aggregation a much larger increase in %T occurred and a large amplitude tracing was obtained. In cases where spontaneous deaggregation followed, the light transmission again decreased. These observations were in accordance with previous reports (O'Brien, 1962; Born, 1962; Haslam, 1964). Such curves are shown in Figure 1 in order to facilitate comparison with our own results obtained with X_a.

Addition of 0.1 ml of X_a solution (2.32 units per ml) to a suspension of 1 ml of washed platelets (0.5 $\times$ 10^6/mm^3) caused an initial increase in light transmission followed by a period which showed no further change either in the per cent transmission of the platelet suspension or in the amplitude of the oscillations on the tracing. There followed a gradual decrease in the light transmission and amplitude of the oscillations after which the transmission of the suspension increased, indicating aggregation. The presence of large aggregates of platelets also gave rise to a large amplitude tracing. On occasion, this sequence of events was followed by a characteristic deaggregation curve.

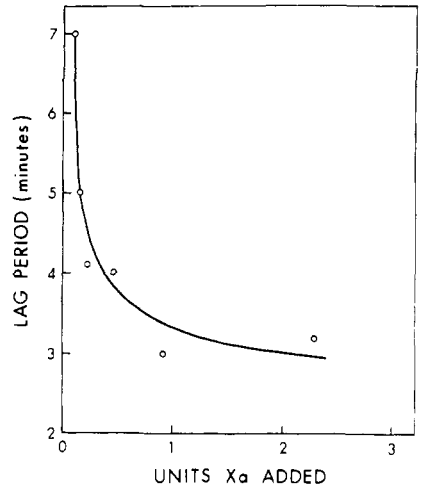

FIGURE 2: Effect of different amounts of X_a on the duration of the lag period preceeding platelet aggregation. Platelet concentration $0.5 \times 10^6/mm^3$. X_a added in 0.1-ml volume in each case.

The interval between the addition of X_a and the onset of platelet aggregation is characteristic of X_a-induced aggregation and henceforth will be designated as the lag period. The duration of this effect has been found to vary considerably from one platelet preparation to another. This observation prompted us to investigate the conditions which might affect the duration of the lag period.

Effect of Concentration of Activated Factor X on Platelet Aggregation. The relationship between the amount of added X_a and the length of the lag period preceding aggregation is shown in Figure 2. The data shown in this figure were obtained using aliquots from a single platelet preparation. A similar pattern was observed with three other platelet preparations. It can be seen that the length of the lag period was dependent on the amount of X_a added to 1.0-ml suspension up to a limit of one unit of X_a. Higher concentrations of X_a failed to shorten the lag period significantly.

Effect of Platelet Concentration on the Aggregation Induced by X_a. The characteristic aggregation induced by activated factor X was found to be dependent also on the concentration of platelets in the suspension (Figure 3). Increasing the concentration of platelets in the suspension decreased the duration of the lag period and increased the extent of aggregation observed. Similar results were obtained in four separate experiments.

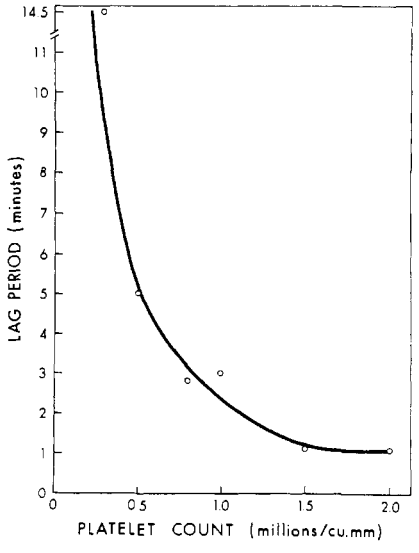

FIGURE 3: Effect of platelet count on the duration of the lag period preceeding aggregation. Amount of X_a added was 2.3 units.

TABLE I: Effect of Washing Platelets with CFTA on the Duration of the Lag Period and on the Amounts of Associated Prothrombin and Factor V.

	No Wash	1 Wash	2 Washes	4 Washes
Lag (min)[a]	0.5	1.7	3.0	4.9
Prothrombin (units/ml)	0.0160	0.0030	0.0027	0.0025
Factor V (units/ml)	2.35	0.68	0.68	0.68

[a] Platelets treated with 0.23 unit of X_a. [b] Volume of each wash was 10 ml.

Effect of Washing Procedures on the Aggregation of Platelets. It was observed that platelets prepared under standard conditions, when treated with standard samples of X_a, responded in a somewhat variable manner. Occasionally, preparations failed to aggregate at all. At the same time the different platelet preparations responded in a more or less constant way to treatment with adenosine diphosphate or thrombin. It was suspected that the variability observed with X_a might be due to slight differences in the effects of the washing procedures on the platelets. Therefore, platelets from different stages of the washing procedure were suspended in TA buffer at a final concentration of $0.5 \times 10^6/mm^3$. These suspensions were then treated with X_a and the aggregation curve was determined. It can be seen from Table I, line 1, that the length of the lag period increased after each wash. Secondly, the amounts of accessory clotting factors associated with platelets were determined. In each of the coagulation assays (prothrombin, factor V), 0.1 ml of platelet suspension was substituted for the source of the clotting factor in the assay. These results are also shown in Table I. These data confirm the known association of clotting factors with platelets and show that these are partially removed by successive washing procedures (Bounameaux, 1957). Nonetheless, substantial amounts of factor V and much smaller amounts of prothrombin remain associated with the platelets after several washings. These results were not affected by the time allowed for each washing between 2 and 15 min.

Effect of Supplements of Prothrombin and Factor V. The addition of 0.1 ml of X_a solution (1.30 units/ml) to a particular platelet suspension containing 0.5×10^6 platelets/mm^3 caused aggregation after 12.5 min (Figure 4A). When 0.1 ml of prothrombin (0.007 unit/ml) was added to an identical aliquot of platelets and X_a was again added the aggregation became evident after 4 min (Figure 4B). Similar treatment of another aliquot of platelets with 0.1 ml of prothrombin (0.007 unit/ml) followed by 0.1 ml of factor V (15.3 units/ml) resulted in a further reduction in the lag period to 2.9 min from the addition of X_a (Figure 4C). The addition of 0.1 ml of factor V solution followed by X_a did not result in aggregation in 12 min. Factor V and prothrombin, either separately or together, had no effect on the platelets unless X_a was also added.

TABLE II: Effect of Inclusion of Prothrombin in the Washing Media on the Duration of the Lag Period and on the Amount of Prothrombin Associated with the Platelets.

	N[a]	P[b]	N[a]	P[b]	N[a]	P[b]
Preparation	I		II		III	
Lag (min)[c]	No aggregation in 20 min	1.2	No aggregation in 20 min	1.3	4.6	0.9
Prothrombin (units/ml)	<0.001	0.012	<0.001	0.012	0.003	0.050

[a] N = normal washing procedure. [b] P = prothrombin included in washing media. [c] Platelets treated with 0.23 unit of X_a.

Effect of Inclusion of Prothrombin in the Washing Solutions. The platelet button was prepared from 60 ml of platelet-rich plasma and resuspended in 10 ml of CFTA buffer. A portion of this suspension (2.9 ml) was then added to a solution of 0.1 ml of prothrombin (17 units/ml). After 15 min the two portions were centrifuged, and the platelet buttons resuspended in the second CFTA buffer. Platelets washed without prothrombin were resuspended in 10.0 ml of CFTA buffer while those washed with prothrombin were transferred to 2.9 ml of CFTA buffer plus 0.1 ml of prothrombin solution. Finally, after 15-min incubation the platelets were centrifuged and resuspended in TA buffer and the suspensions adjusted to 0.5×10^6 platelets/mm^3. The extent of aggregation induced by either ADP or thrombin did not vary in the two preparations by more than 1% of the maximum aggregation observed. However, treatment of the two portions with 0.1 ml of X_a (2.3 units/ml) revealed a striking difference. Prothrombin-treated platelets aggregated after a much shorter lag period (Table II, line 1) and to a slightly greater extent than the untreated platelets. This effect was reproducible with four separate platelet preparations. Furthermore, the variability in the lag period between different preparations was essentially abolished by the prothrombin treatment (Table II). The lag period of the platelets washed normally was shortened to 1 min by the addition of prothrombin (0.17 unit) prior to addition of the X_a. Assay of the platelets for prothrombin showed a greater concentration of the latter in the prothrombin washed preparation than in the platelets washed normally (Table II).

Effect of Aging of Platelets. A standard platelet suspension (0.5×10^6/mm^3) was prepared and allowed to remain at 37° for up to 3 hr. Aliquots of the suspension were removed at intervals and each treated with 0.1 ml of X_a (2.3 units/ml). It was found that as the platelets aged the lag period became shorter (Table III) and that the extent of aggregation increased.

Samples of the platelet suspensions were taken at intervals and assayed for prothrombin as described previously. No difference in prothrombin concentration could be found throughout the period of the experiment. However, aging of the platelets was found to increase their effectiveness as a substitute for "cephalin" (Bell and Alton, 1956) in the modified factor X assay (Table III). Furthermore, addition of 0.1 ml of Bell and Alton cephalin (50 μg of phospholipid/ml) to fresh platelets prior to addition of X_a (0.1 ml, 2.3 units/ml) resulted in aggregation with a lag period of 1.2 min.

Effect of Calcium Ions on Aggregation. The platelet button was prepared from platelet-rich plasma and washed twice in calcium-free media as in the standard procedure. An aliquot of the button from the second wash was then resuspended in modified Tyrode's albumin buffer (pH 7.35) in which the $CaCl_2$ had been replaced by an equimolar amount of NaCl.

The effect of ADP, thrombin, and X_a on these initially calcium-free suspensions and the effect of subsequent addition of calcium ions in each case is shown in Table IV.

It can be seen that elimination of Ca^{2+} ions prevented aggregation but not the shape change with ADP and thrombin

TABLE III: Effect of Aging of Platelets on the Duration of Lag Period and on the Amount of Prothrombin and Available Platelet Factor 3 Associated with the Platelets.

Aging time (min)	0	37	78	126
Lag[a] (min)	4.5	2.3	1.5	1.6
Prothrombin (units/ml)	<0.001	<0.001	<0.001	<0.001
Platelet factor 3[b] (sec)	90	47	39	38

[a] Platelets treated with 0.23 unit of X_a. [b] The platelet factor 3 activity was assayed by a modification of the factor X assay in which a standard solution of X_a was used and platelet suspension (0.1 ml) was substituted for cephalin. The results were expressed in terms of clotting times.

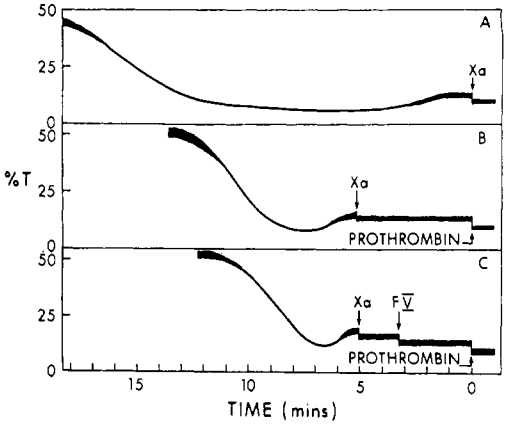

FIGURE 4: Effect of added clotting factors on the aggregation of platelets induced by X_a: (A) tracing obtained after addition of 0.13 unit of X_a to 0.5×10^6 platelets/mm^3; (B) tracing obtained with platelets suspended in 0.0007 unit of prothrombin/ml; (C) tracing obtained with platelets suspended in 0.0007 unit of prothrombin/ml and 1.53 units of factor V/ml.

TABLE IV: Influence of ADP, Thrombin, and X_a on Platelets in the Presence and Absence of Calcium Ions.

Addition	Shape Change[a]	Aggregation[a]
10^{-5} M ADP[b]	+	−
10^{-5} M ADP[b] + 2×10^{-3} M Ca^{2+}	+	+
0.1 unit/ml of thrombin[b]	+	−
0.1 unit/ml of thrombin[b] + 2×10^{-3} M Ca^{2+}	+	+
2.3 units/ml of X_a[b]	−	−
2.3 units/ml X_a[b] + 2×10^{-3} M Ca^{2+}	+	+

[a] + indicates a positive reaction; − indicates a negative reaction. [b] ADP, thrombin, and X_a added to the platelet suspension to produce the final concentration specified. Calcium chloride, where indicated, was added after a further 4 min.

but prevented both the shape change and aggregation with X_a.

When $CaCl_2$ (0.1 ml, 2.2×10^{-2} M) was added prior to addition of X_a a normal aggregation curve with a lag period of 8.0 min resulted. Addition of $CaCl_2$ alone to this Ca^{2+}-free platelet suspension had no effect in 23 min. At the same time a normal platelet suspension containing Ca^{2+} ions was prepared from the same washed platelet button and was found to aggregate on addition of X_a (0.1 ml, 23.0 units/ml) with a lag period of 4.8 min.

The addition of ADP (0.1 ml, 10^{-4} M) or thrombin (0.1 ml, 0.5 unit/ml) to this preparation resulted in aggregation curves similar to those obtained with the preparation which had been supplemented with Ca^{2+} ions.

Effect of Hirudin on Aggregation Induced by Activated Factor X. Hirudin is known to inhibit the action of thrombin in converting fibrinogen into fibrin (Markwardt, 1961). Neutralization of 1 NIH unit of thrombin by 1 unit of hirudin was found to require less than 1-min incubation at 37°. This amount of hirudin did not affect the clotting activity of 2.3 units of X_a after incubation for 13 min at 37°.

Hirudin (0.1 ml, 10 unit/ml) was added to an aliquot of a standard platelet suspension and after 1 min thrombin (0.1 ml, 0.5 NIH unit/ml) was added. No change in light transmission was observed. A second aliquot of the platelet suspension was incubated for 1 min with hirudin (0.1 ml, 10 units/ml) and X_a (0.1 ml, 23.0 units/ml) was then added. There was no change in $\%T$ of the suspension.

Effect of Adenosine on Aggregation. The aggregation induced by X_a (0.1 ml, 9.3 units/ml) was inhibited after 3–4-min preincubation of the platelets with 0.1 ml of 2×10^{-2} M adenosine. Similarly 0.1 ml of 2×10^{-2} M adenosine inhibited the aggregation induced by ADP (0.1 ml, 10^{-4} M) and thrombin (0.1 ml, 0.5 NIH unit/ml). Although adenosine prevented aggregation by X_a, ADP, and thrombin in no case was the initial shape change inhibited.

Effect of Pyruvate Kinase (PK) and Phosphoenolpyruvate (PEP). To a standard platelet preparation was added 0.1 ml of PK (0.25 mg of protein/ml) and 0.1 ml of PEP (20 mg/ml). After incubation for 3 min at 37°, 0.1 ml of X_a (8.3 units/ml) was added. No aggregation was observed. In control experiments in which the enzyme solutions were replaced by the solvent buffers a normal aggregation pattern was seen. Aggregation of the platelets by 0.1 ml of 10^{-4} M ADP was also abolished by preincubation with the two enzymes.

Discussion

In this paper we have directly demonstrated for the first time that the addition of X_a to washed platelets results in aggregation. This is important because X_a is an enzyme which is an obligatory intermediate in almost all known mechanisms of blood coagulation. Our findings therefore establish a new relationship between blood clotting and platelet function.

The aggregation induced by X_a exhibited two characteristic features. First, we noted a long lag period between addition of X_a and the subsequent aggregation, probably indicative of an intermediate sequence of reactions. Secondly, X_a did not induce an initial shape change in the platelets in the absence of Ca^{2+} ions. These results are qualitatively different from those obtained with thrombin and ADP (O'Brien and Heywood, 1966; Haslam, 1964). These considerations, together with the demonstration that the highly purified X_a preparations were free of fibrinogen-clotting activity, allowed us to discount the possibility that traces of thrombin were responsible for the observed aggregation.

Washed platelets can substitute for various clotting factors in a number of specific coagulation assays. Factor V (Hjört *et al.*, 1955), factor VIII (Bounameaux, 1957), and a low level of prothrombin activity (Bounameaux, 1957) are associated with the surface membrane of the platelet after washing. When a solution of pure prothrombin was included in both washings the amount of prothrombin remaining attached to the platelets in the final suspension was considerably increased over the normal amount while at the same time the duration of the lag period after addition of X_a was drastically reduced. A similar reduction of the lag period was seen when prothrombin was added to washed platelets concomitantly with X_a. When highly purified bovine factor V was also added the lag period was even further reduced.

In the experiment recorded in Figure 2, increasing concentration of X_a was shown to reduce the duration of the lag period to a limiting value of 3 min. At the higher concentrations of X_a the lag period could be further shortened by increasing the number of platelets present (Figure 3), by adding a trace of phospholipid suspension (Bell and Alton cephalin) or by aging the platelets. The increasing sensitivity of platelets to X_a during aging is in contrast to their decreasing sensitivity to ADP. Surgenor and Wallach (1961) and Marcus and Zucker (1965) have suggested that platelet factor 3 is present on the surface membrane of platelets only after a protein coat has been removed and this takes place during aging.

It may be concluded that when X_a is present in excess, factors provided by the platelets themselves, namely prothrombin, factor V, and platelet factor 3, may be rate limiting and that they act synergistically in the aggregation process induced by X_a. In platelet preparations where these factors were constant the reproducibility in the length of the lag period was $\pm 2\%$.

O'Brien and Heywood (1966) have shown that ADP induces a shape change in platelets in the absence of Ca^{2+} ions. This was also true for the shape change induced by thrombin. In contrast, X_a has no detectable effect on platelets in the absence of Ca^{2+} ions. It has been shown that X_a adsorbs to phospholipids only in the presence of Ca^{2+} ions (Papahad-

jopoulos and Hanahan, 1964). Inasmuch, as phospholipids provide a model for platelet function in blood coagulation it seems reasonable to infer that Ca^{2+} is required for binding of X_a to the platelet surface and that this interaction is the initial event following addition of X_a. The shape change which follows is probably due to slow conversion of platelet prothrombin into thrombin by X_a. Hirudin inhibits thrombin but not X_a when tested in coagulation assays (Markwardt *et al.*, 1964). However, it prevented aggregation of platelets by X_a as well as by thrombin. This result is consistent with the idea that thrombin is an intermediate in the aggregation induced by X_a. Thrombin-induced aggregation is known to be mediated through release of ADP which may then act as a high-energy phosphate acceptor from an unidentified phosphorylated intermediate in the platelet membrane (Packham *et al.*, 1970). High concentrations of adenosine, which inhibit the aggregation induced by ADP, also inhibited aggregation induced by X_a and thrombin. Likewise, conversion of liberated ADP into inactive ATP by pyruvate kinase in the presence of phosphoenolpyruvate, neutralized the effect of X_a on platelets. The sequence of events, deduced from all of these observations, is shown in Figure 5.

Finally, we may consider the possible significance of induced aggregation in intravascular coagulation. We have previously pointed out the potency of X_a–phospholipid complexes in developing stasis thrombi in a standard experimental animal model (Barton *et al.*, 1970). A further interesting finding is that X_a adsorbed on a phospholipid surface is protected to some extent against neutralization by a naturally occurring plasma inhibitor (E. T. Yin, private communication). We have now shown that X_a can cause aggregation of platelets *in vitro*, an effect which might be expected to contribute to thrombus formation in areas of the circulation subjected to local stasis. An additional component, resulting from thrombin generation, would be the release of α granules providing further platelet factor 3 activity as coagulation develops. The validity of extrapolating *in vitro* data to the *in vivo* situation remains to be established however.

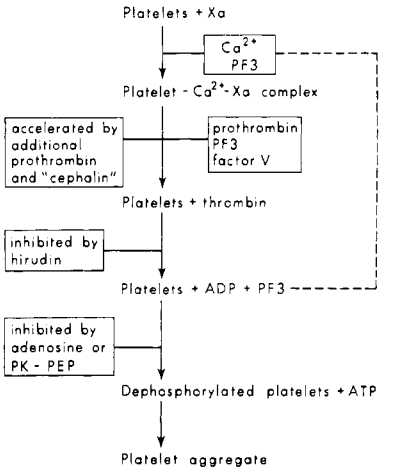

FIGURE 5: The probable sequence of events following addition of X_a to washed platelets suspended in a Ca^{2+}-containing medium. Normal cofactors are shown at the right whereas additional acceleratory or inhibitory agents are shown at left. Dashed line indicates a positive feedback effect.

Acknowledgments

The initial observation of platelet aggregation after addition of activated factor X was made while S. Jevons was receiving guidance in respect to platelet preparation in the laboratory of Dr. J. F. Mustard, Department of Pathology, McMasters University, Hamilton. We thank Drs. J. F. Mustard and E. E. Nishizawa for invaluable assistance and Dr. W. C. Day, Mr. R. K. Bull, and Mr. R. Radcliffe for preparation of some of the highly purified clotting factors used in this study. Technical assistance was provided by Mr. R. K. Kulshreshtha.

References

Ardlie, N. G., Packham, M. A., and Mustard, J. F. (1970), *Brit. J. Haematol. 19*, 7.

Aster, R. H., and Jandl, J. H. (1964), *J. Clin. Invest. 43*, 843.

Bachmann, F., Duckert, F., Koller, F. (1958), *Thromb. Diath. Haemorrh. 2*, 24.

Baker, W. J., and Seegers, W. H. (1967), *Thromb. Diath. Haemorrh. 17*, 205.

Barton, P. G., and Hanahan, D. J. (1967), *Biochim. Biophys. Acta 133*, 506.

Barton, P. G., Jackson, C. M., and Hanahan, D. J. (1967), *Nature (London) 214*, 923.

Barton, P. G., Yin, E. T., and Wessler, S. (1970), *J. Lipid Res. 11*, 87.

Bell, W. N., and Alton, H. G. (1954), *Nature (London) 174*, 88.

Blombäck, B., and Blombäck, M. (1956), *Ark. Kem. 10*, 415.

Born, G. V. R. (1962), *Nature (London) 194*, 927.

Bounameaux, Y. (1957), *Rev. Fr. Etud. Clin. Biol. 2*, 52.

Cox, A. C., and Hanahan, D. J. (1970), *Biochim. Biophys. Acta 207*, 49.

Davey, M. G., and Lüscher, E. F. (1965), *Nature (London) 207*, 730.

Davey, M. G., and Lüscher, E. F. (1967), *Proc. Congr. Eur. Soc. Haematol., 10th 1966*, 118.

Esnouf, M. P., and Jobin, F. (1967), *Biochem. J. 102*, 660.

Fonio, A. (1940), *Schweiz Med. Wochenschr. 70*, 510.

Fonio, A. (1948), *Bull. Schweiz. Akad. Med. Wiss. 4*, 470.

Gaarder, A., Jonsen, J., Laland, S., Hellem, A. J., and Owren, P. A. (1961), *Nature (London) 192*, 531.

Haslam, R. J. (1964), *Nature (London) 202*, 765.

Haslam, R. J. (1967), *in* Physiology of Hemostasis and Thrombosis, Johnson, S. A., and Seegers, W. H., Ed., Springfield, Ill., Thomas, p 88.

Hemker, H. C., Esnouf, M. P., Hemker, P. W., Swart, A. C. W., and MacFarlane, R. G. (1967), *Nature (London) 215*, 248.

Hjört, P. F., Rapaport, S. I., and Owren, P. A. (1955), *Blood 10*, 1139.

Jobin, F., and Esnouf, M. P. (1967), *Biochem. J. 102*, 666.

Kappeler, Z. (1955), *Klin. Med. (Vienna) 153*, 103.

Koller, F., Loeliger, A., and Duckert, F. (1951), *Acta Haematol. 6*, 1.

Marcus, A. J., and Zucker, M. B. (1965), The Physiology of Blood Platelets, New York, N. Y., and London, Grune and Stratton, Inc., p 21.

Markwardt, F. (1961), *Thromb. Diath. Haemorrh. 5*, 576.

Markwardt, F., Hoffmann, A., and Landmann, H. (1964), *Thromb. Diath. Haemorrh. 11*, 230.

Milstone, J. H. (1955), *J. Gen. Physiol. 38*, 757.

O'Brien, J. R. (1962), *J. Clin. Pathol. 15*, 452.

O'Brien, J. R., and Heywood, J. B. (1966), *J. Clin. Pathol. 19*, 148.

Packham, M. A., Rathbone, R. L., Guccione, M. A., Lloyd, J. V., Nishizawa, E. E., and Mustard, J. F. (1970), *Proc. Can. Fed. Biol. Soc. 13*, 107.
Papahadjopoulos, D., and Hanahan, D. J. (1964), *Biochim. Biophys. Acta 90*, 436.
Papahadjopoulos, D., Yin, E. T., and Hanahan, D. J. (1964), *Biochemistry 3*, 1931.
Seegers, W. H., and Landabaru, R. H. (1960), *Can. J. Biochem. Physiol. 38*, 1405.
Surgenor, D. M., and Wallach, D. F. H. (1961), *in* Blood Platelets, Johnson, S. A., Monto, R. W., Rebuck, J. W., Horn, R. C., Jr., Ed., Boston, Mass., Little Brown & Co., p 289.
Yin, E. T., and Wessler, S. (1968), *J. Biol. Chem. 243*, 112.

Kinetic Characterization of Oxidative Phosphorylation in *Alcaligenes faecalis**

Robert Adolfsen and E. N. Moudrianakis†

ABSTRACT: Monovalent cations were found to be capable of replacing divalent cations in the preparation of phosphorylating particles by centrifugation from cell-free crude extracts of *Alcaligenes faecalis*. Optimal phosphorylating activity was observed when the membrane fragments were sedimented in the presence of 0.1 M KCl. A comparison of particles prepared in $MgCl_2$ to particles prepared in KCl showed that the specific activity of phosphorylation was about the same, that ADP and DPNH requirements were nearly absolute, that inhibition by 2,4-dinitrophenol and KCN were almost total, and that the only product of the reaction was terminally labeled ATP. Respiratory control of DPNH oxidase activity by P_i and ADP, in contrast to previous studies on this system, was totally absent in both types of particles. Three roles for metal ions were found in this system. (1) They were required in the preparation of the particles, probably for the binding of coupling factors to membrane fragments. (2) They were required, along with 0.25 M sucrose, to stabilize the membranes to storage at −20°. (3) Divalent cations activated the phosphorylating reaction in a biphasic manner. The first phase of the activation was attributed to the formation of the Mg–ADP complex and was insensitive to K^+. The second phase was attributed to activation by free Mg^{2+}, and antagonism by K^+ was observed.

Oxidative phosphorylation has been studied in *Alcaligenes faecalis* for a number of years by Pinchot. The emphasis in that work was on the characterization of coupling factors, primarily the heat-stable factor (Pinchot, 1957; Shibko and Pinchot, 1961b), and then on a proposed high-energy intermediate (Pinchot, 1960; Pinchot and Hormanski, 1962). The purpose of this paper is to characterize the basic kinetic properties of electron transport and oxidative phosphorylation in this system. These studies are meant to provide a firm foundation for future studies on this system, which will include coupling factor purification and characterization and also probes into the mechanism of oxidative phosphorylation.

During the course of the kinetic characterization, several roles for metal ions were found. These related especially to the preparation of the phosphorylating particles, to stabilizing the activity of the system, and to activating the phosphorylating reaction. A striking independence of the phosphorylating system from the electron transport system was also observed. Variables that affected electron transport, such as DPNH concentration, affected phosphorylation similarly—as would be expected. However, variables that affected the rate of the phosphorylating reaction, such as ADP, P_i, $MgCl_2$, and KCl, had no effect at all on the rate of electron transport.

Materials and Methods

Preparation of Phosphorylating Particles. The culture medium for the growth of *A. faecalis* contained 0.2 g/l. of NaCl, 0.1 g/l. of $MgSO_4 \cdot 7H_2O$, 0.1 g/l. of $CaCl_2 \cdot 2H_2O$, 0.0038 g/l. of $Na_2MoO_4 \cdot 2H_2O$, 0.0225 g/l. of $FeSO_4 \cdot 7H_2O$, 0.15 g/l. of citric acid, 0.5 g/l. of yeast extract, and 30 g/l. of CasAmino acids. Medium (12 l. at a time) was prepared and NaOH was added to bring the pH to 8.0. The medium was autoclaved in 1-l. volumes in 6-l. flasks for 20 min at 20 psi. Slants of *A. faecalis* grown on medium containing 2% nutrient agar in addition to the usual components were stored at 4° until needed. The cells were suspended in about 15 ml of sterile medium and 1 ml of suspension was used to inoculate each flask. The cultures were grown for 21 hr at 37° on a shaker to an OD_{660} of approximately 3 (late log phase). The cells were then harvested in a refrigerated Sharples centrifuge and washed once in about 2 l. of 0.1 M KCl–0.25 M sucrose (KCl–sucrose). The wet weight yield of cells per liter of culture medium was approximately 11 g. After suspending the washed cells as a 20% (w/v) slurry in fresh KCl–sucrose, they were ruptured in an Aminco French pressure cell at a pressure of about 6 tons. Debris was removed by centrifugation at 20,000*g* for 10 min (15K in the Spinco No. 30 rotor), yielding the cell-free crude extract. This was

* From the Department of Biology, The Johns Hopkins University Baltimore, Maryland 21218. *Received August 17, 1970.* This work was supported by Grant AI-02443 from the National Institutes of Health and Grant GB-12128 from the National Science Foundation to Dr. G. B. Pinchot and by National Institutes of Health Grant GM-13518 to one of us (E. N. M.).

† To whom correspondence should be addressed.