Biochimica et Biophysica Acta, 314 (1973) 382–389

BBA 46598

CALCIUM ION CONTROL OF PLATELET THROMBOSTHENIN ATPase ACTIVITY*

JOHN P. HANSON, DORIS I. REPKE, ARNOLD M. KATZ** and LOUIS M. ALEDORT

Divisions of Hematology and Cardiology, Department of Medicine, Mount Sinai School of Medicine of the City University of New York, 100th Street and 5th Avenue, New York, N.Y. 10029 (U.S.A.)

(Received March 26th, 1973)

SUMMARY

This study demonstrates that Ca^{2+} regulates thrombosthenin ATPase activity, likening the control of platelet contraction to that of cardiac and skeletal muscle. Thrombosthenin, the platelet contractile protein, was isolated by repeated low ionic strength and isoelectric precipitation. Thrombosthenin superprecipitation and ATPase activity were measured in 10^{-4} M $CaCl_2$ (high ionized Ca^{2+}) and 0.25 mM ethylene glycol bis-(β-aminoethyl ether)-*N,N'*-tetraacetic acid (EGTA) (low ionized Ca^{2+}). In both high and low Ca^{2+}, superprecipitation, measured as an increase in turbidity, ocurred shortly after addition of ATP. ATP hydrolysis by thrombosthenin, which proceeded linearly for several hours, was greater in high Ca^{2+} (approx. 2.3 nmoles·$mg^{-1}\cdot min^{-1}$) than in low Ca^{2+} (approx. 1.8 nmoles·$mg^{-1}\cdot min^{-1}$). This difference, when analyzed by the Student's *t*-test for paired samples was highly significant ($P < 0.001$). Thrombosthenin ATPase activity was not significantly altered by azide, an inhibitor of mitochondrial ATPase, nor by ouabain, an inhibitor of $(Na^+ + K^+)$-activated ATPase. The dependence of thrombosthenin activation on ionized Ca^{2+}, measured with the use of CaEGTA buffers, was studied. The Ca^{2+}-dependent portion of thrombosthenin ATPase was half maximal at $4.5\cdot 10^{-7}$ M Ca^{2+}. This corresponds to an apparent binding constant of $2.2\cdot 10^6$ M^{-1}, a value that is comparable to that of skeletal and cardiac muscle. These data suggest that a Ca^{2+} control mechanism similar to that of the troponin–tropomyosin complex of muscle exists in the platelet.

INTRODUCTION

Thrombosthenin, a contractile system which has been implicated in clot retraction, can be extracted from human platelets by methods similar to those used to isolate actomyosin from muscle[1]. Like actomyosin, thrombosthenin exhibits Ca^{2+}- and Mg^{2+}-activated ATPase activities[2,3]. Thrombosthenin gels undergo

Abbreviation: EGTA, ethylene glycol bis-(β-aminoethyl ether)-*N,N'*-tetraacetic acid.

* A preliminary report of the work was presented at the 3rd International Society of Thrombosis and Hemastasis (Abstracts, p. 200), August, 1972.

** This author is Philip J. and Harriet L. Goodhart Professor of Medicine (Cardiology).

shrinkage and increased turbidity in the presence of ATP[1], properties that are similar to the superprecipitation of actomyosin. The electronmicroscopic appearance of thrombosthenin filaments is similar to that of actin and myosin prepared from striated muscle[4,5], and specific cross-reactions between platelet and skeletal muscle proteins have been demonstrated[6].

At the present time, the mechanisms which control the contractile activity of thrombosthenin are unknown. In both cardiac and skeletal muscle the troponin complex, along with tropomyosin, regulates contractile activity by modulating the interactions between actin and myosin. Thus, the troponin–tropomyosin complex inhibits the contractile process in the absence of Ca^{2+}, whereas this inhibition is reversed and both shortening and tension development occur when Ca^{2+} is bound to one component of the troponin complex. These control mechanisms are manifest in isolated actomyosin systems as an increase in the Mg^{2+}-activated ATPase activity and a more rapid onset of superprecipitation when ionized Ca^{2+} concentration is raised in the range between 10^{-7} and 10^{-5} M[7,8]. In view of the many similarities between the contractile proteins of platelets and muscle we examined the Ca^{2+} dependence of thrombosthenin superprecipitation and ATPase activity.

METHODS

Blood was obtained from 18–50-year-old apparently health volunteers who were instructed to take no drugs for one week prior to the donation. Approximately 400 ml whole blood were drawn into chilled Nalgene bottles containing 100 ml acid–citrate–dextrose solution *plus* 1 mM dithiothreitol. All subsequent procedures were carried out at 4 °C.

Platelet-rich plasma was obtained by centrifugation at $225\times g$ for 40 min. The platelets were collected from the supernatant by centrifugation at $2250\times g$ for 20 min and washed once with 50 ml of 0.15 M NaCl, 0.1% EDTA, 1 mM dithiothreitol. The wash solution had been adjusted with Tris–acetate to pH 6.8. To extract thrombosthenin, the platelets were suspended in 2 vol. of Weber–Edsall solution (0.6 M KCl–0.01 M Na_2CO_3–0.04 M $NaHCO_3$) containing 1 mM dithiothreitol and homogenized for 12 min at 15000 rev./min in a Virtis Model 45 Homogenizer equipped with two stainless steel blades. ATP (Sigma, disodium salt) was then added to a final concentration of 2 mM and homogenization was repeated for 1 min as described above. After thrombosthenin was extracted overnight, the solution was centrifuged at $20000\times g$ for 60 min and the pellet discarded. Thombosthenin was precipitated from the supernatant by addition of 12 vol. of distilled water containing 1 mM dithiothreitol. The pH was adjusted to 6.3 with 0.125 M sodium acetate buffer at pH 4.9. The glass electrodes were calibrated at room temperature with the temperature compensator set to 0 °C. The suspension was stirred slowly for 90 min after which the thrombosthenin was collected by centrifugation at $1500\times g$ for 15 min. The precipitate was dissolved in 0.6 M KCl, 1 mM dithiothreitol and 20 mM Tris–acetate at pH 6.8. The low ionic strength and isoelectric precipitations were repeated, after which the final precipitate was dissolved in 1–2 ml of 0.6 M KCl, 1 mM dithiothreitol and 20 mM Tris–acetate at pH 6.8 and dialyzed overnight against the same solution.

Protein concentration was determined by the method of Lowry *et al.*[9]. Hydrolysis of ATP was measured at 25 °C by determinations of P_i liberation[10]. Super-

precipitation was measured in a Gilford Recording Spectrophotometer[11]. CaEGTA (ethylene glycol bis-(β-aminoethyl ether)-*N,N'*-tetraacetate, calcium salt) buffers were prepared by methods previously described[12] with EGTA obtained from Sigma Chemical Co. All chemicals used were reagent grade. Distilled water was passed through an ion-exchange resin and redistilled in glass prior to use.

RESULTS

Specificity of thrombosthenin ATPase activity

In order to exclude the possibility that contaminating membrane ATPases contribute to that of thrombosthenin, the effects of ouabain, an inhibitor of the plasma membrane (Na^+–K^+)-activated ATPase, and sodium azide, an inhibitor of mitochondrial ATPase, were examined. The effects of azide were examined in solutions containing 0.1 mM $CaCl_2$ and ouabain sensitivity was examined in EGTA as described previously[12]. Neither azide nor ouabain significantly inhibited the ATPase activity of these thrombosthenin preparations (Table I). Measurements of succinate cytochrome *c* reductase[13] further demonstrated the virtual absence of mitochondrial contamination. The rate of cytochrome *c* reduction by thrombosthenin was approx. 2 μmoles·mg^{-1}·min^{-1}, a value that is approx. 1% of purified mitochondria[12].

TABLE I

EFFECTS OF INHIBITORS OF MEMBRANE ATPases ON THROMBOSTHENIN ATPase ACTIVITY

Reaction mixtures are described in the legend to Fig. 1. Experiment I was carried out with 10^{-4} M $CaCl_2$. Experiment II was carried out with 0.25 mM EGTA.

	ATPase activity (*nmoles·mg^{-1}·min^{-1}*)
Experiment I	
Control	2.0
5 mM sodium azide	2.2
Experiment II	
Control	1.23
10^{-6} M ouabain	1.15

Time course of ATP hydrolysis and superprecipitation

The initial ATPase activity of thrombosthenin gels was extremely low. The average value in the presence of 10^{-4} M $CaCl_2$, 2 mM $MgCl_2$ and 1 mM ATP was approx. 2 nmoles·mg^{-1}·min^{-1}, approx. 1% that of skeletal muscle actomyosin[14].

ATP hydrolysis by thrombosthenin increased in a linear fashion during the first 2–3 h of the reaction (Fig. 1). After a brief initial clearing phase, lasting approx. 10 min, superprecipitation took place, as evidenced by an increased turbidity at 620 nm (Fig. 1). Unlike skeletal muscle actomyosin[15], however, an initial slow rate of ATP hydrolysis could not be detected during the brief clearing phase. A striking

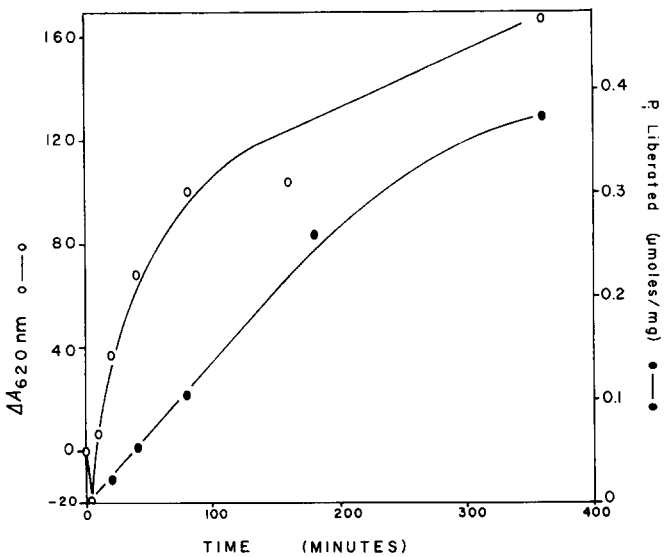

Fig. 1. Time course ATP hydrolysis (●–●) and superprecipitation (○–○) by thrombosthenin. Reactions were carried out simultaneously with 0.8 mg/ml thrombosthenin at 25 °C in 10^{-4} M $CaCl_2$, 2 mM $MgCl_2$, 1 mM ATP, 0.1 M KCl, 15 mM Tris–acetate at pH 6.8.

difference in the behavior of thrombosthenin as compared to that of skeletal actomyosin was the early onset superprecipitation when a small fraction of the ATP was hydrolyzed (less than 2% of the total added ATP (Fig. 1)). This behavior, which was seen consistently in both the presence and absence of Ca^{2+}, made it impossible to establish a direct correlation between ATPase activity and superprecipitation such as is seen in skeletal muscle actomyosin[11]

Effects of Ca^{2+} on ATP hydrolysis

To evaluate the Ca^{2+} sensitivity of thrombosthenin, the ATPase activity of each of nine thrombosthenin preparations was examined in the presence (10^{-4} mM $CaCl_2$) and virtual absence (0.25 mM EGTA) of Ca^{2+}. All preparations showed activation by Ca^{2+} although the extent of Ca^{2+} sensitivity varied. The average ATPase activities of seven preparations are presented in Table II. Activation by Ca^{2+},

TABLE II

Ca^{2+} SENSITIVITY OF THROMBOSTHENIN

Samples were taken after 90 min from reactions, carried ou as described in Fig. 1. Data were analyzed by the Student's *t*-test for paired samples.

	ATPase activity ($nmoles \cdot mg^{-1} \cdot min^{-1}$)
$CaCl_2$ (10^{-4} M)	2.33 ± 0.76*
EGTA (0.25 mM)	1.84 ± 0.68*

* Mean ± S.E., $t = 4.877$, $P < 0.001$, $n = 7$.

analyzed by the Student's *t*-test for paired samples, was highly significant ($P<0.001$), being found invariably in all seven preparations. The extent of activation by Ca^{2+}, when expressed as the ratio of ATPase activities in the presence and virtual absence of Ca^{2+}, was greatest with shorter reactions times although Ca^{2+} activation presisted throughout the reaction (Fig. 2).

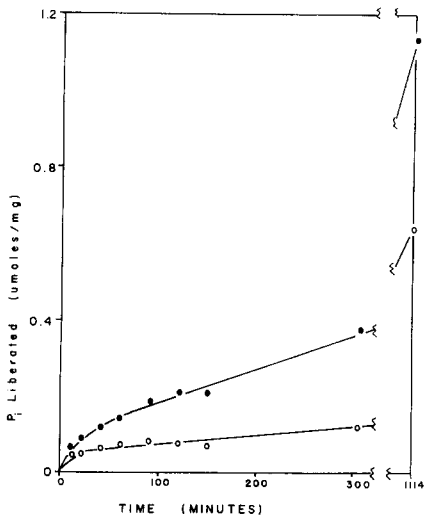

Fig. 2. Ca^{2+} dependence of ATP hydrolysis by thrombosthenin. Reactions were carried out in 10^{-4} M $CaCl_2$ (●–●), and 0.25 mM EGTA (○–○) with the remaining conditions as given in Fig. 1.

Dependence of ATPase activity on Ca^{2+} concentration

When the dependence of thrombosthenin ATPase activity on Ca^{2+} concentration was measured, activation was found to be maximal at approximately 10^{-5} M Ca^{2+} (Fig. 3). That portion of the total ATPase activity which was Ca^{2+} sensitive was determined in another experiment by substracting the ATPase activity measured in 0.25 mM EGTA from that measured at various Ca^{2+} concentrations. When a Lineweaver–Burk plot was constructed from these data, the straight line drawn through all five points with the aid of a PDP-8e Computer crossed the ordinate at 1.2 $(\text{nmoles}\cdot\text{mg}^{-1}\cdot\text{min}^{-1})^{-1}$, corresponding to a V for the Ca^{2+}-activated ATPase of 0.83 $\text{nmole}\cdot\text{mg}^{-1}\cdot\text{min}^{-1}$ (Fig. 4). More significantly, the intercept along the abcissa, where $1/V=0$, was $2.2\cdot10^{6}$ M^{-1}. This corresponds to an apparent K_m of $4.5\cdot10^{-7}$ M Ca^{2+}. Similar estimates were obtained for two other thrombosthenin preparations.

DISCUSSION

The present findings demonstrate that the ATPase activity of thrombosthenin responds to Ca^{2+} in a manner very similar to that of both cardiac and skeletal muscle actomyosin. Thus ATP hydrolysis by thrombosthenin was significantly and invariably higher in 10^{-4} M $CaCl_2$ than in 0.25 mM EGTA (Fig. 2, Table II). In order to exclude significant contamination by membrane fragments, ATPase activity was measured in the presence and absence of azide and ouabain. Neither azide, an inhibitor of mitochondrial ATPase[16],nor ouabain, which inhibits platelet (Na^+–K^+)-activated

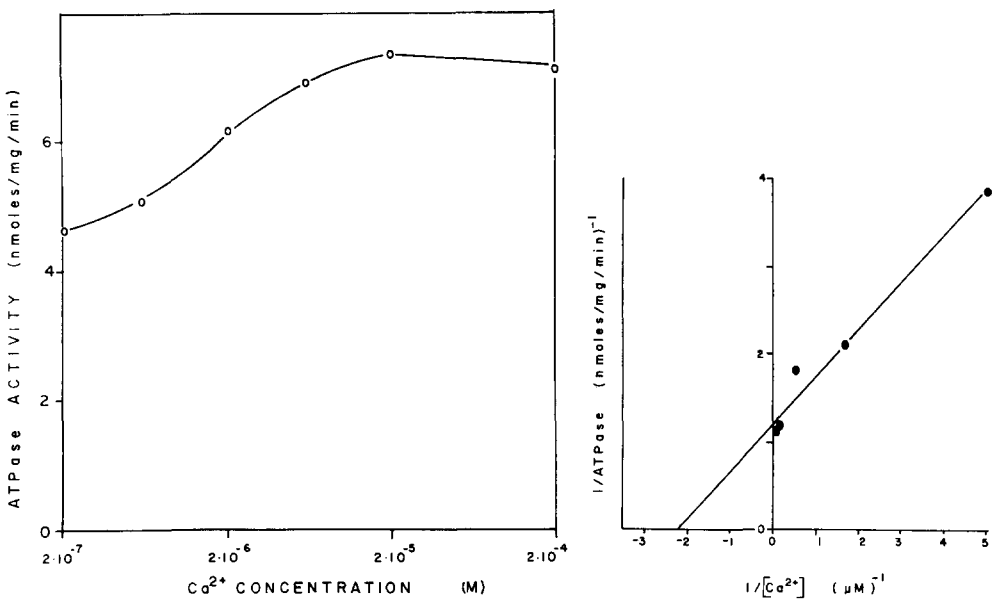

Fig. 3. Ca^{2+} dependence of thrombosthenin ATPase activity. Reactions were carried out in Ca-EGTA buffers under conditions described for Fig. 1.

Fig. 4. Lineweaver–Burk plot of Ca^{2+} dependence of that portion of the thrombosthenin ATPase activity that is activated by Ca^{2+}. Discussed in text.

ATPase[17], altered the ATPase activity of these thrombosthenin preparations significantly (Table I). Levels of succinate cytochrome *c* reductase, a marker for mitochondrial inner membranes, was also extremely low. Furthermore, the low ATPase of these thrombosthenin preparations, 1–3 nmoles·mg^{-1}·min^{-1} is similar to that reported by others[2,17].

The present finding that the ATPase activity of thrombosthenin, like that of actomyosin, is controlled by Ca^{2+} in the micromolar range of concentration is in accord with the many other similarities already observed between these two contractile systems. Thrombosthenin contains fibrils with a diameter of 80–100 Å and a periodicity which is comparable to that of muscle actin[5] and the arrowhead configuration seen when muscle actin complexes with heavy meromyosin is also seen when platelet actin is reacted with heavy meromyosin[4,6]. Thrombosthenin also undergoes superprecipitation in solutions containing ATP[3], findings that are confirmed in the present study. Finally, a platelet protein similar to that of skeletal muscle tropomyosin has been described[18].

Activation of thrombosthenin ATPase by Ca^{2+} was half maximal at approx. $4.5 \cdot 10^{-7}$ M Ca^{2+}*. The apparent Ca^{2+} binding constant for the Ca^{2+}-sensitive regulatory site of thrombosthenin is therefore $2.2 \cdot 10^{6}$ M^{-1}, a value in the range

* There remains some uncertainty as to the exact value for the CaETGA binding constant[21], a 4-fold difference having been found for this binding constant in phosphate and amine buffers. Because of these discrepancies, a value halfway between these published values has been chosen in these studies[8].

reported previously for the Ca^{2+} stimulation of both cardiac and skeletal muscle actomyosins ATPases[7,8], and the high affinity Ca^{2+} binding sites of skeletal[19] and cardiac[20] troponins. This similarity suggests that the platelet, like muscle, has a physiological regulatory mechanism controlled by the troponin–tropomyosin complex although further studies will be needed to isolate and characterize such a system.

Both ATPase activity and Ca^{2+} sentivity were found to vary when different thrombosthenin preparations were compared. Furthermore, by the second day after completion of the purification as much as two-thirds of the Ca^{2+} sensitivity had disappeared at the same time ATPase activity increased. In muscle actomyosins, oxidation of troponin thiol groups has been found to abolish Ca^{2+} sensitivity[8] while oxidation of myosin thiol groups increases ATPase activity at neutral pH[22]. The present observations suggest that thiol oxidation or a similar mechanism may have contributed to the variability noted in this study. The relatively slight activation of thrombosthenin ATPase activity by Ca^{2+} may be related to the lower extent of activation by actin that is seen in myosins that have intrinsically low ATPase activity[14].

Addition of ATP to thrombosthenin, as has been reported previously[3], caused optical changes similar to those described to accompany the superprecipitation of cardiac and skeletal muscle actomyosin. Immediately after addition of ATP, the turbidity of the reaction mixture decreased briefly (clearing), then increased (superprecipitation) (Fig. 1). The turbidity of thrombostenin, unlike that of muscle actomyosin, increased at a time when only a small fraction of the ATP had been hydrolyzed (Fig. 1). This observation indicates that the observed turbidity changes might be due, in part at least, to a product of ATP hydrolysis such as ADP and not, as in the case of skeletal muscle actomyosin, to the decreased ATP concentration that results from the enzymatic action of the partially dissociated actomyosin[11,15]. The observation that ADP concentrations in the range of 0.02 mM caused a marked increase in turbidity without detectable enhancement of ATPase activity might account for this phenomenon.

While this work was in progress, Ca^{2+} sensitivity of thrombosthenin ATPase was found independently by Cohen and Cohen[23]. These findings may be of significance in the understanding of a growing number of congenital and acquired qualitative platelet defects that produce hemorrhagic diatheses[24–27]. In almost all of these defects the platelets do not respond appropriately to aggregating agents[25,26]. Abnormalities also include alterations in clot retraction which are most likely due to malfunctioning of thrombosthenin. For example, in Glanzmann's Thrombasthenia platelets neither aggregate nor retract[28]. Although both aggregation and contraction are Ca^{2+} dependent, their interrelationship remains undefined[29]. In some qualitative platelet disorders there appears to be no abnormality in either intracellular Ca^{2+} content[30] or thrombosthenin ATPase activity[30,31]. It is possible therefore that some of the platelet defects represent abnormalities in the Ca^{2+} control mechanism that is defined in the present investigation.

ACKNOWLEDGEMENT

This work was supported by U.S.P.H.S. Grants HL-10905 and HL-13191; the New York Heart Association; and the Anna and Ruth Lowenburg, Albert A. List, Helen B. Kuyler, and the Jack Martin Funds.

J.P.H. is an American Cancer Society Fellow.

REFERENCES

1 Bettex-Galland, M. and Lüscher, E. (1959) *Nature* 184, 276–277
2 Bettex-Galland, M. and Lüscher, E. (1961) *Biochim. Biophys. Acta* 49, 536–547
3 Bettex-Galland, M. and Lüscher, E. (1965) *Adv. Protein Chem.* 20, 1-34
4 Zucker-Franklin, D. and Grusky, G. (1972) *J. Clin. Invest.* 51, 419–430
5 Zucker-Franklin, D., Nachman, R. L. and Marcus A. J. (1967) *Science* 157, 945–946
6 Adelstein, R., Pollard, T. and Kuehl, W. (1971) *Proc. Natl. Acad. Sci. U.S.A.* 68,2703–2707
7 Ebashi, S. and Endo, M. (1968) *Prog. Biophys. Mol. Biol.* 18, 123–183
8 Katz, A. M. (1970) *Physiol. Rev.* 50, 63–158
9 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* 193, 265–275
10 Taussky, H. and Shorr, E. (1953) *J. Biol. Chem.* 202, 675–685
11 Katz, A. M. (1964) *J. Biol. Chem.* 239, 3304–3311
12 Katz, A. M., Repke, D. I., Upshaw, J. E. and Polascik, M. A. (1970) *Biochim. Biophys. Acta* 205, 473–490
13 Rabinowitz, M. and DeBernard, B. (1957) *Biochim. Biophys. Acta* 26, 22–29
14 Bárány, M., Gaetjens, K., Bárány, K. and Karp, E. (1964) *Arch. Biochem. Biophys.* 106, 280–293
15 Maruyama. K. and Gergely, J. (1962) *J. Biol. Chem.* 237, 1100–1106
16 Bielawski, J. and Lehninger, A. (1966) *J. Biol. Chem.* 24, 4316–4322
17 Aledort, L. M., Troup, S. B. and Weed, R. I. (1968) *Blood J. Hematol.* 31, 471–479
18 Cohen, I. and Cohen, C. (1971) *Fed. Proc.* 30, 1309 Abstract
19 Hartshorn, D. J. and Pyuen, H. Y. (1971) *Biochim. Biophys. Acta* 229, 698–711
20 Reddy, Y. S. and Honig, C. R. (1972) *Biochim. Biophys. Acta* 275, 453–463
21 Ogawa, Y. (1968) *J. Biochem. Tokyo* 64, 255–257
22 Kielley, W. W. and Bradley, L. B. (1956) *J. Biol. Chem.* 218, 653–659
23 Cohen, I. and Cohen, C. (1972) *J. Mol. Biol.* 68, 383–387
24 Berger, S. (1970) *Can. Med. Assoc. J.* 102, 1271–1274
25 Caen, J. P., Vainer, H., Sultan, Y. and Lukasiewicz, H. (1970) *Ser. Haematol.* 3, 83–99
26 Goldman, B. and Aledort, L. M. (1972) *Ann. Int. Med.* 76, 269–273
27 Mustard, J. F. and Packman, M. A. (1970) *Pharmacol. Rev.* 22, 97–187
28 Glanzmann, E. (1918) *Jahrb. Kinderheilkd.* 88, 1; lxxxviii, 1;3 pl; 113–141
29 Aledort, L. M. (1971) in *The Circulating Platelet* (Johnson, S., ed.), pp. 259–283, Academic Press
30 Caen, J. P., Castaldi, P. A., Leclerc, J. C., Inceman, S., Larrieu, M. J., Probst, M. and Bernard, J. (1966) *Am. J. Med.* 41, 4–26
31 Weiss, H. J. and Aledort, L. M. (1967) *Lancet* ii, 495-497