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# MEMBRANE ALTERATIONS IN CONNECTION WITH THE RELEASE REACTION IN HUMAN PLATELETS AS STUDIED BY THE LACTOPEROXIDASE-IODINATION TECHNIQUE AND BY AGGLUTINATION WITH BOVINE FACTOR VIII-RELATED PROTEIN *

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## Summary

Changes in the platelet membrane surface as a result of the thrombin-induced release reaction have been examined by lactoperoxidase-catalyzed iodination and agglutination of the platelets with bovine factor VIII-related protein. The distribution of labeled substances after iodination was studied after sodium dodecyl sulphate polyacrylamide gel electrophoresis of platelet proteins. The major radioactive peaks in the control samples were associated with (glyco)polypeptides of estimated molecular weights about 100 000 and 120 000, and a smaller peak was observed at 147 000 daltons.

Platelets labeled after the release reaction had been performed incorporated markedly less radioactive iodine into the membrane proteins than control platelets, i.e. 57.7% ± 9.2 (147 000), 26.6% ± 13.0 (120 000) and 20.6% ± 5.5 (100 000) of the corresponding peaks of the control. These platelets agglutinated poorly with bovine factor VIII-related protein. These effects were not seen when the platelets were preincubated with metabolic inhibitors to prevent secretion.

After sodium dodecyl sulphate polyacrylamide gel electrophoresis of the soluble fraction from released platelets, the main glycoprotein band (GPS), when examined in unreduced form, showed slower migration than the corresponding band in the control sample. It is concluded, that the surface structure of thrombin-secreted platelets is different from that of untreated cells. This change is independent of the direct action of thrombin on the membrane, but may be a result of conformational alterations during the secretion process and/or adsorption of released material to the external membrane surface.

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## Introduction

Upon various stimuli, the platelets undergo a secretion process, "release reaction", whereby granule-stored material is extruded to the extracellular milieu [1—3]. Thrombin is a potent inducer of the release reaction, and Grette [1] suggested that the first step in the thrombin-platelet interaction involved proteolysis of a platelet protein by thrombin. A few papers on the effects of thrombin on platelet membrane proteins have been published [4—8]. More of these studies have been performed with much higher concentrations of thrombin and longer periods of incubation than needed for the release reaction to occur.

The purpose of the present investigation was to study membrane alterations in connection with the release reaction, specifically. Platelets were examined before and after mild thrombin treatment, and in the presence or absence of inhibitors of the release reaction. The surface structure was examined in two different test systems, firstly by lactoperoxidase-catalyzed iodination of exposed membrane proteins [9], and secondly by agglutination of the platelets with bovine factor VIII-related protein. With the lactoperoxidase-iodination technique we wanted to observe membrane alterations as changes in the distribution of radioactive label. The agglutination system was used to study membrane alterations as manifested by changes in availability of membrane receptors.

Studies on the surface structure of released platelets seems important, since such platelets circulate in the blood stream in various pathological conditions. Also there is evidence that degranulated platelets form an apparent non-thrombogenic monolayer on the subendothelium, which might substitute for the endothelium in this respect [10].

## Materials

*Chemicals and radiochemicals*

Bovine thrombin (Topostasine, Roche, F. Hoffmann La Roche and Co., Ltd., Basel, Switzerland) was made up to 300 N.I.H. units/ml with 0.12 M NaCl, 0.03 M Tris · HCl and 0.003 M EDTA, pH 7.4 and stored at −20°C.

Antimycin A (type III), 2-deoxy-D-glucose, lactoperoxidase, β-mercaptoethanol, Coomassie brilliant blue R and ADP (Grade I) were obtained from Sigma Chemical Co., St. Louis, U.S.A. Hirudin (pure) was from V.E.B. Arzneimittelwerk, Dresden, G.D.R., Schiff's reagent from Raymond A. Lamb, London, England and acetylsalicylic acid from Norsk Medisinaldepot, Oslo, Norway.

*Biological materials*

Platelets were obtained from 500 ml of human blood collected into 40 ml of 0.077 M EDTA/HCl, pH 7.4, and processed as described elsewhere [11].

Bovine factor VIII-related protein was purified as previously published [12] by modifications of the methods described for the corresponding human protein [13,14].

Human fibrinogen was purified as described by Blombäck and Blombäck [15].

## Methods

The release reaction was induced by incubation of the washed platelets with thrombin (1 N.I.H. unit/ml) for 1 min at 37°C. Aggregation was minimized by the presence of 0.003 M EDTA in the suspending medium. Hirudin (5 A.T.U./ml) was added at the end of the incubation period. When metabolic inhibitors were used to prevent the release reaction, the platelet suspension was preincubated for 30 min at 37°C with antimycin A (4 μg/ml) and 2-deoxy-D-glucose (15 mM) [16]. The platelets were always sedimented and resuspended in fresh medium before the further examinations. Subcellular fractions were prepared as described previously [17,18]. The iodination process was performed as described by Phillips [9]. Platelets were labeled either before or after thrombin treatment, washed once, and then examined by sodium dodecyl sulphate polyacrylamide gel electrophoresis [18,19].

The radioactivity present in 1.5 mm gel pieces was measured in a Packard Gamma Scintillation Spectrometer. The radioactivity values for all pieces within each peak of radioactivity were summed up, and the sum expressed in percentage of the corresponding peak in the control sample.

Agglutination was induced by addition of 50 μl of purified bovine factor VIII-related protein to 500 μl platelet suspension (300 000 platelets/μl). The suspending fluid contained 0.003 M EDTA to avoid ADP-mediated aggregation. The aggregometer (a modified EEL titrator with a Varian G 15-1 recorder) was calibrated in such a way that the difference in light transmission between suspensions of 150 000 and 300 000 platelets/μl corresponded to 50 chart divisions (0.5 mV or half of full scale). The agglutination was expressed as Δ chart divisions per 25 s [20].

## Results

The carbohydrate bands seen after gel electrophoresis of whole platelets correspond to glycopolypeptides of molecular weight of 147 000, 120 000 and 100 000, respectively. The band at 147 000 has previously been shown to represent three different glycopolypeptides [21], one of which is located in the platelet granules. This granule glycoprotein is released during the specific platelet release reaction, and migrates only slightly into the gel in its unreduced state (Fig. 1, top). In the soluble fraction isolated from control platelets, one main carbohydrate band is observed which migrates similarly both in its reduced and unreduced state (Fig. 1a, below). The corresponding fraction isolated from thrombin-released platelets revealed a similar carbohydrate pattern when reduced samples were electrophoresed, whereas the main band showed a slower migration in unreduced samples (Fig. 1b, below). This difference between reduced and unreduced samples was not observed in thrombin-treated platelets which had been pretreated with antimycin A and 2-deoxy-D-glucose to inhibit secretion (Fig. 1c, below).

No effect of thrombin could be observed on the membrane glycoprotein pattern. Also, the pattern of radioactivity was unchanged after thrombin treatment.

When platelet degranulation was prevented with the use of metabolic inhibi-

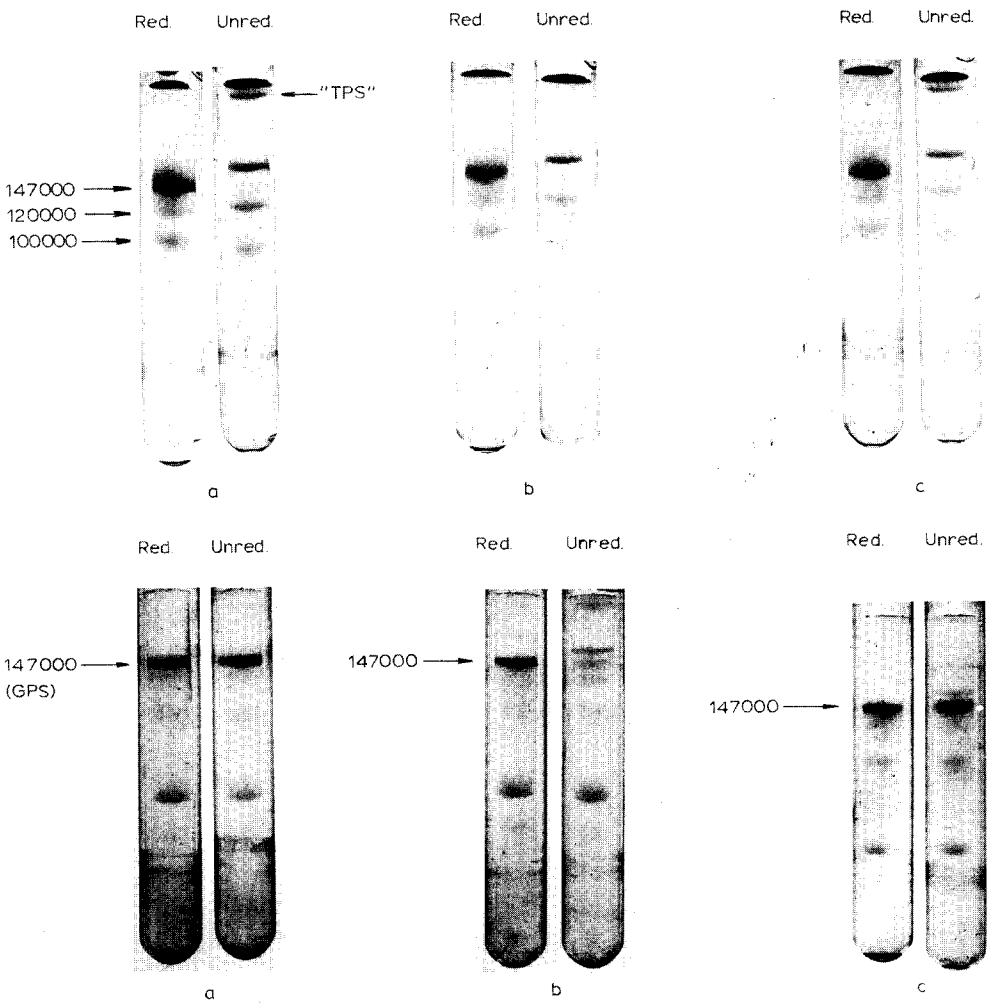


Fig. 1. Gel electrophoresis of whole platelets (top) and the soluble fraction (bottom) stained for carbohydrate with the periodic acid-Schiff's reagent. Top: (a) Control platelets. (b) Thrombin-released platelets. (c) Platelets treated with thrombin, release reaction prevented with metabolic inhibitors. Bottom: (a) Soluble fraction from control platelets; (b) Soluble fraction from thrombin-released platelets; (c) Soluble fraction from thrombin-treated platelets, release reaction prevented with metabolic inhibitors. "TPS", granule glycoprotein.

tors, the radioactive iodine was incorporated to the same degree both into control and thrombin-treated platelets. However, when labeled subsequently to the release reaction, markedly less radioactive iodine was incorporated into the membrane proteins of the treated platelets than into the corresponding proteins of the control platelets (Fig. 2), even if the secreted material had been removed and the treated platelets had been suspended in fresh buffer before iodination. The incorporated radioactivity represented 57.7% ± 9.2 (147 000), 26.6% ± 13.0 (120 000) and 20.6% ± 5.5 (100 000) (mean of 7 experiments ± S.D.) of the corresponding peaks in the control samples. Thrombin-released platelets which had been washed 5 times after degranulation incorporated on

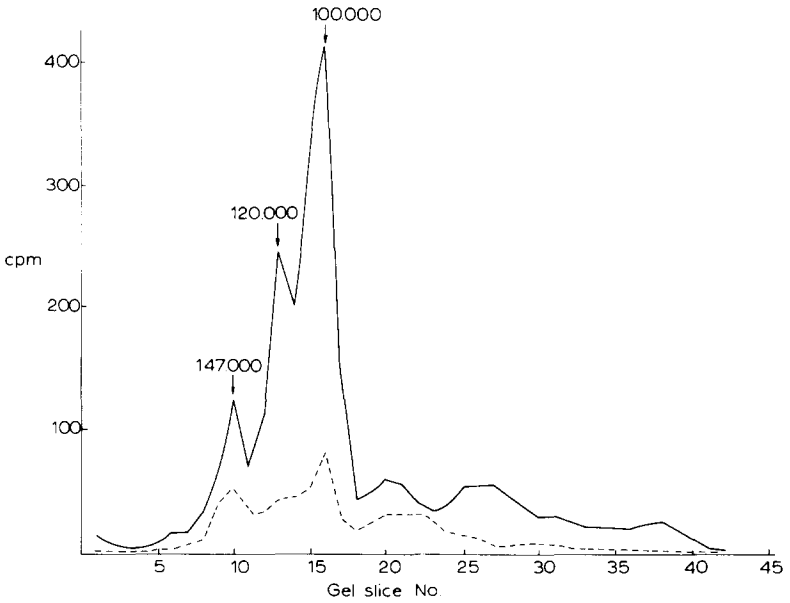


Fig. 2. Distribution of radioactive label after gel electrophoresis of whole, iodinated platelets (reduced samples). Control platelets (———). Platelets iodinated after thrombin-induced release reaction, and removal of secreted substances (- - - - -).

the other hand larger amounts of radioactive iodine than the correspondingly washed platelets which had not been exposed to thrombin (Fig. 3a). Two additional peaks corresponding to polypeptides of molecular weights between 50 000 and 70 000 were seen.

When unreduced samples were electrophoresed, these peaks disappeared concomitantly with the appearance of a peak with a similar mobility as fibrinogen (fig. 3b). In order to examine whether these alterations were caused by the released material, or structural changes in the membrane, untreated, washed platelets were suspended in the extracellular medium obtained by centrifugation of thrombin-released platelets, and incubated for 1 min at 37°C. Hirudin had been added in excess to neutralize the thrombin present. Subsequently to the incubation, the platelets were centrifuged, resuspended in fresh buffer (0.12 M NaCl, 0.03 M Tris · HCl, 0.003 M EDTA, pH 7.4) and iodinated. These platelets were markedly less able to incorporate radioactive iodine than the control platelets (Fig. 4a). However, when these platelets had been washed 5 times before iodination, their radioactivity pattern did not differ from the control any more (Fig. 4b).

Platelets were then incubated with ADP (40 $\mu$M, 1 min, 37°C) alone or ADP plus human fibrinogen (50 or 300 $\mu$g/ml) in the presence of 0.003 M EDTA. Iodination after centrifugation and resuspension of the platelets in fresh buffer showed no difference in distribution of radioactive label from the control platelets. Neither was any difference observed when platelets were iodinated in the presence of ADP (40 $\mu$M) or acetylsalicyclic acid (1.2 mM).

Thrombin-released platelets agglutinated poorly with bovine factor VIII-related protein, and the reactivity was only partially restored by washing the

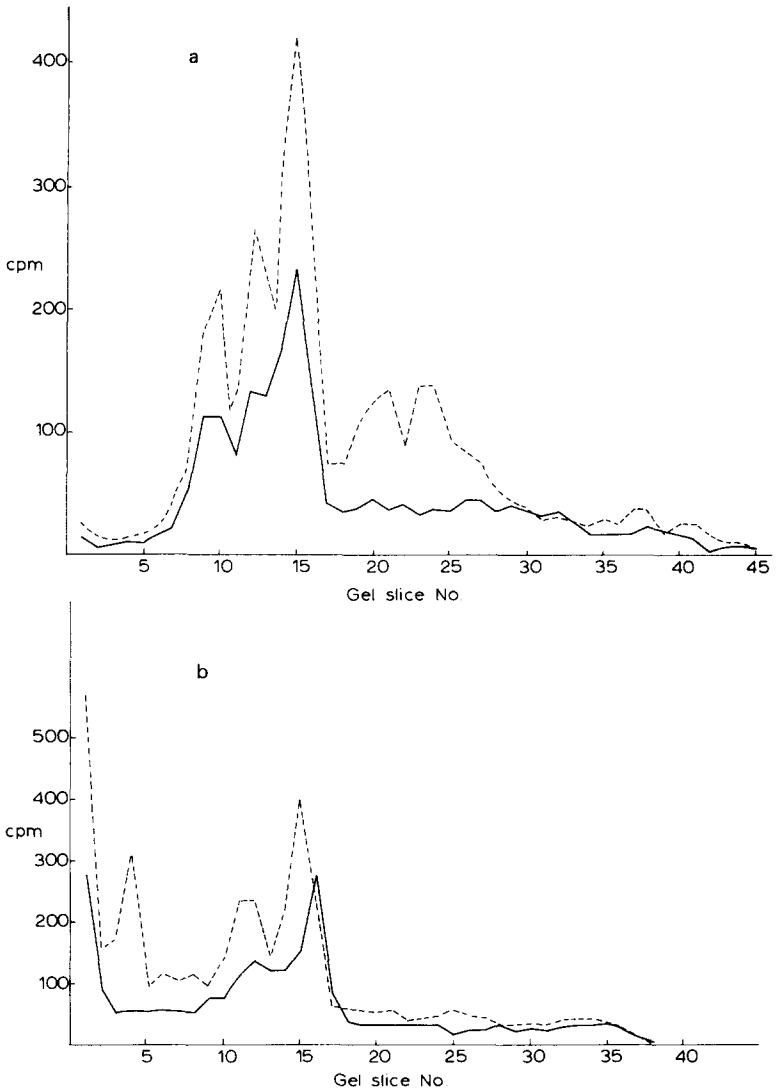


Fig. 3. (a) Distribution of radioactive label after gel electrophoresis of whole, iodinated platelets. Reduced samples. Control platelets, washed 5 times before iodination (———). Thrombin-released platelets washed 5 times before iodination (- - - - ). (b) As in (a), unreduced samples.

TABLE I

AGGLUTINATION OF PLATELETS WITH BOVINE FACTOR VIII-RELATED PROTEIN

The numbers in brackets represent the number of experiments.

| | Δ Chart division sample / Δ Chart division control |
|---|---|
| Thrombin-released platelets | 0.43 ± 0.13 (9) |
| Thrombin-released platelets, washed 5 times before tested | 0.71 ± 0.07 (6) |
| Control platelets suspended in the extracellular medium from thrombin-released platelets | 0.50 ± 0.23 (4) |
| Platelets plus ADP (40 μM) | 0.56 ± 0.18 (9) |

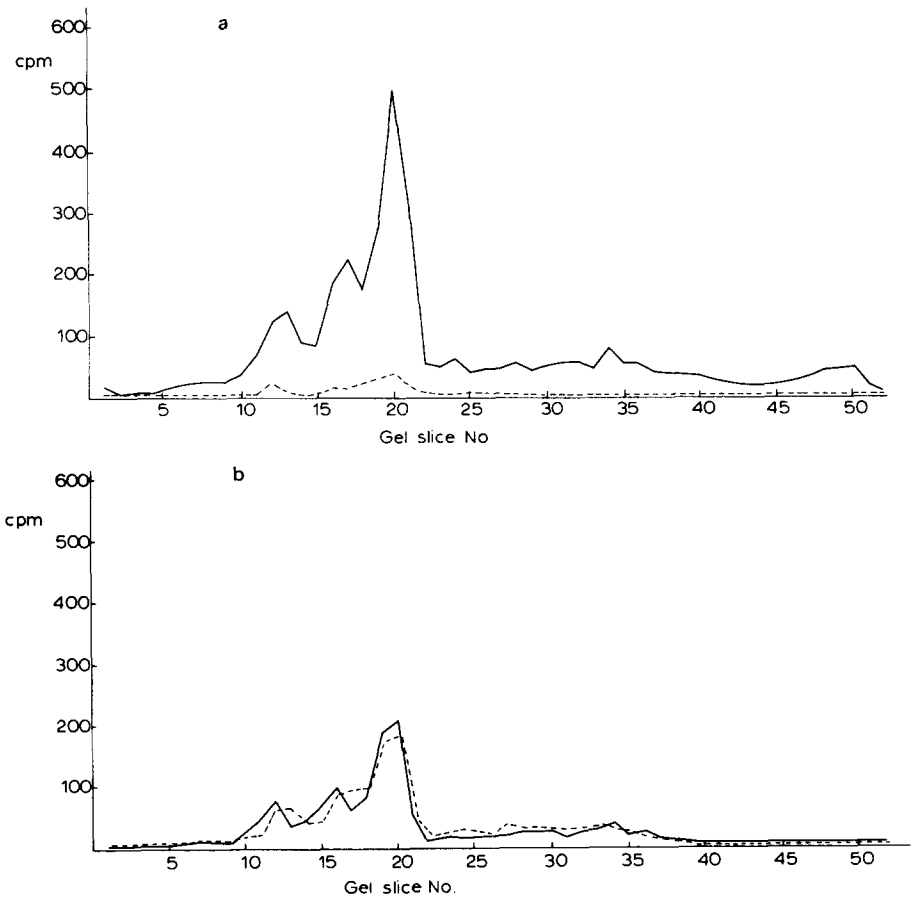


**Fig. 4 (a) Distribution of radioactive label after gel electrophoresis of whole, iodinated platelets. Control platelets (———) incubated in the extracellular medium from thrombin-released platelets (- - - - -). The platelets were washed once before iodination. (b) As in (a), the platelets having been washed 5 times before iodination.**

platelets 5 times (Table I). No difference was observed between control- and thrombin-treated platelets which had been preincubated with metabolic inhibitors to prevent secretion. The agglutination response became reduced when untreated, washed platelets were suspended in the extracellular medium from released platelets (1 min, 37°C), sedimented and resuspended in fresh buffer (Table I). This was also observed with such platelets which had been washed 5 times before the agglutination. Note that the thrombin present in the extracellular medium had been neutralized by the addition of excess hirudin, and the leakage of [$^{14}$C]serotonin was only 13.3% from the sample platelets compared to 3.3% from the control platelets, which is insignificant.

The presence of ADP (40 $\mu$M), or ADP plus human fibrinogen (40 $\mu$M and 50 $\mu$g/ml, respectively) showed an inhibitory effect on the platelet agglutination with bovine factor VIII-related protein (Table I). When these substances were removed by washing, the agglutination was as strong as in the control.

Grant et al. [22] have previously shown that ADP inhibits ristocetin-induced agglutination of platelets in PRP.

The presence of fibrinogen alone (50 μg/ml) of acetylsalicylic acid (100 μM, 1.2 mM) had no effect on the agglutination of platelets with bovine factor VIII-related protein.

## Discussion

The present investigation was performed to study membrane alterations after thrombin-induced release reaction in human platelets. Conditions were chosen so as to observe the initial effect(s) of thrombin on the platelet surface. In some experiments aimed at eliminating the effects of released material on the platelets, or changes due to the secretion step per se, thrombin treatment was performed when secretion was prevented by metabolic inhibitors. No change in radioactivity distribution or glycoprotein pattern could be observed after thrombin treatment of prelabeled platelets. Similar results were obtained by Nachman et al. [6], whereas Phillips and Agin [7] and Steiner [23] found reduced label associated with the 118 000 glycopolypeptide after more extensive thrombin treatment than needed for the release reaction. The explanation may be that splitting of only a few peptide bonds is sufficient for the release to occur [24], and that this can be observed as a proteolysis only if the experimental conditions are chosen so as to "amplify" the thrombin effect [7]. On the other hand, one cannot exclude the possibility that thrombin acts through a non-proteolytic mechanism.

The main glycoprotein band seen after gel electrophoresis of the soluble fraction (GPS) [20] is probably identical to glycocalicin recently described by Okumura et al. [25—27]. There is increasing evidence that this glycoprotein is surface-located in intact platelets, and that it is involved in the receptor mechanism for factor VIII-related protein (von Willebrand's factor) at the platelet membrane [20,25]. Okumura and Jamieson [25] suggested that this glycoprotein also acted as a surface receptor for thrombin. As shown in the present investigation, a change in the electrophoretic behaviour of this glycoprotein occurred as a result of the thrombin-induced release reaction. This was not seen after thrombin-treatment of platelets which had been preincubated with metabolic inhibitors. It therefore seems fair to suggest that GPS is involved in some way in the thrombin-induced release reaction, but not necessarily as the initial receptor (substrate) for thrombin on the surface membrane. Even if we favour the view that GPS represents a molecular entity of its own, there exists some evidence that GPS is derived in some way from the intrinsic membrane glycopolypeptide, GP I [26,27]. In this connection, it is interesting to note that GPS from thrombin-released platelets showed an identical electrophoretic behaviour to this membrane glycoprotein (GP I) both in the reduced and unreduced form (Hagen, unpublished observation).

Based on the prolonged bleeding time and impaired platelet adhesion in patients suffering from the classical von Willebrand's disease [28], and on certain in vitro experiments [29—31], the human factor VIII-related protein is thought to be important for the adhesion of platelets to subendothelial tissue. Defective adhesion and prolonged bleeding time, as well as inability of the

platelets to agglutinate with factor VIII-related protein was also observed in patients with Bernard-Soulier syndrome [32—35]. Platelets from such patients show a qualitative membrane defect, manifested as a reduced content of sialic acid [36] and strongly reduced intensity of a glycopolypeptide band ($M_r$, 150 000) compared to normal platelets [37,38], and it has been suggested that an interaction between the factor VIII-related protein and a platelet receptor lacking in this disease is necessary for the normal adhesion [35,39—41]. Our experiments may be explained on the basis that such a receptor is less available in normal platelets after the release reaction has taken place. This may be related to the reduced adherenece to damaged aorta of thrombin-degranulated platelets recently reported by Reimers and coworkers [42]. Also, it is interesting to note that GPS is affected by the thrombin-induced release reaction, which may explain the impaired interaction with bovine factor VIII-related protein.

In conclusion, the platelet surface is altered by the thrombin-induced secretion process. This is demonstrated as a reduced availability of the membrane proteins to undergo lactoperoxidase-catalyzed iodination, as well as an impaired platelet agglutination with bovine factor VIII-related protein. These alterations are not due to a direct effect of thrombin, but secondary to the release reaction. From the present investigation, it is not possible to conclude whether the observed alterations were caused by secreted substances, or were a result of structural rearrangements of platelet membrane proteins during secretion. However, from the experimental evidence, it seems reasonable to suggest that both phenomena may play a role in this respect.

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## References

1 Grette, K. (1962) Acta Physiol. Scand. 56 suppl. 195
2 Davey, M.G. and Luscher, E.F. (1968) Biochim. Biophys. Acta 165, 490—506
3 Holmsen, H., Day, H.J. and Stormorken, H. (1969) Scand. J. Haematol. suppl. 8
4 Ganguly, P. (1971) J. Biol. Chem. 246, 4286—4290
5 Baenziger, N.L., Brodie, G.N. and Majerus, P.W. (1971) Proc. Natl. Acad. Sci. U.S. 68, 240—243
6 Nachman, R.L., Hubbard, A. and Ferris, B. (1973) J. Biol. Chem. 248, 2928—2936
7 Phillips, D.R. and Agin, P.P. (1974) Biochim. Biophys. Acta 352, 218—227
8 Feagler, J.R., Tillack, T.W., Chaplin, D.D. and Majerus, P.W. (1974) J. Cell. Biol. 60, 541—553
9 Phillips, D.R. (1972) Biochemistry 11, 4582—4588
10 Baumgartner, H.R. (1974) in Thrombosis: Pathogenesis and Clinical Trials (Deutsch, E., Brinkhous, K.M., Hinnom, S. and Lechner, K., eds.) pp. 91—106, Schattauer Verlag, Stuttgart
11 Hagen, I. (1972) Biochim. Biophys. Acta 273, 141—148
12 Solum, N.O. and Peterka, M. (1977) in: Platelets and Thrombosis, Serono Symposium, Milan 1976 (Mills, D.C.B. and Pareti, F.I., eds.) New York, in the press
13 Newman, J., Johnson, A.J., Karpatkin, M.H. and Puszkin, S. (1971) Br. J. Haematol. 21, 1—20
14 Van Mourik, J.A. and Mochtar, I.A. (1970) Biochim. Biophys. Acta 221, 667—679
15 Blomback, B. and Blomback, M. (1956) Ark. Kemi. 10, 415—443
16 Murer, E.H. and Holme, R. (1970) Biochim. Biophys. Acta 222, 197—205

17 French, P.C. and Holme, R. (1974) Thromb. Diath. Haemorrh. 32, 432—440
18 Hagen, I. (1975) Biochim. Biophys. Acta 329, 242—254
19 Fairbanks, G., Steck, T.L. and Wallach, D.F.H. (1971) Biocgemistry 10, 2606—2616
20 Solum, N.O., Hagen, I. and Peterka, M. (1976) Thrombosis Res. 10, 71—83
21 Hagen, I., Olsen, T. and Solum, N.O. (1976) Biochim. Biophys. Acta 455, 214—225
22 Grant, R.A., Zucker, M.B. and McPherson, J. (1976) Am. J. Physiol. 230, 1406—1410
23 Steiner, M. (1973) Biochim. Biophys. Acta 323, 653—658
24 Phillips, D.R. (1974) Thromb. Diath. Haemorrh. 32, 207—215
25 Okumura, T. and Jamieson, G.A. (1976) Thrombosis Res. 8, 701—706
26 Okumura, T., Lombart, C. and Jamieson, G.A. (1976) J. Biol. Chem. 251, 5950—5955
27 Okumura, T. and Jamieson, G.A. (1976) J. Biol. Chem. 251, 5944—5949
28 Salzman, E.W. (1963) J. Lab. Clin. Med. 62, 724—735
29 Weiss, H.J. (1968) Blood 32, 668—679
30 Bouma, B.N., Wiegerinck, Y., Sixma, J.J., van Mourik, J.A. and Mochtar, I.A. (1972) Nature New Biol. 236, 104—106
31 Tschopp, T.B., Weiss, H.J. and Baumgartner, H.R. (1974) J. Lab. Clin. Med. 83, 296—300
32 Bernard, J. and Soulier, J.P. (1948) Sem. Hop., Paris 24, 217—221
33 Bithell, T.C., Parekh, S.J. and Strong, R.R. (1972) Ann. N.Y. Acad. Sci. 201, 145—160
34 Howard, M.A., Hutton, R.A. and Hardisty, R.M. (1973) Brit. Med. J. 2, 586—588
35 Caen, J.P. and Levy-Toledano, S. (1973) Nature, New Biol. 244, 159—160
36 Grottum, K.A. and Solum, N.O. (1969) Brit. J. Haematol. 16, 277—290
37 Nurden, A.T. and Caen, J.P. (1975) Nature 255, 720—722
38 Jenkins, C.S.P., Phillips, D.R., Clemetson, K.J., Meyer, D., Larrieu, M.-J. and Luscher, E.F. (1976) J. clin. Invest. 57, 112—124
39 Weiss, H.J., Tschopp, T.B., Baumgartner, H.R., Sussman, I.I., Johnson, M.M. and Egan, J.J. (1974) Am. J. Med. 57, 920—925
40 Caen, J.P., Nurden, A.T., Janneau, C., Michel, H., Toblem, G., Levy-Toledano, S., Sultan, Y., Valensi, F. and Bernard, J. (1976) J. Lab. Clin. Med. 87, 586—596
41 Evensen, S.A., Solum, N.O., Grottum, K.A. and Hovig, T. (1974) Scand. J. Haematol. 13, 203—214
42 Reimers, H.J., Cazenave, J.-P., Senyi, A.F., Hirsch, J., Kinlough-Ratbone, R.L., Packham, M.A. and Mustard, J.F. (1975) Vth Congr. Int. Soc. Thromb. Haem., Paris, Abstr. Vol. pp. 470