*Biochimica et Biophysica Acta*, 426 (1976) 688–696


BBA 77239

# ISOLATION OF MEMBRANES FROM NORMAL AND THROMBIN-TREATED GEL-FILTERED PLATELETS USING A LECTIN MARKER

SUSAN RITTENHOUSE-SIMMONS and DANIEL DEYKIN

*Boston VA Hospital and Departments of Medicine and Biochemistry, Boston University School of Medicine, Boston, Mass. (U.S.A.)*

(Received September 29th, 1975)

## SUMMARY

We report a technique for the isolation of plasma membranes from gel-filtered platelets exposed to thrombin, using $^{125}$I-labeled lentil lectin as an external marker. Labeled cells not exposed to thrombin could be lysed on a gradient of glycerol. Those cells incubated with thrombin (without external $Ca^{2+}$) were made more susceptible to breakage on a gradient of glycerol-EDTA, and homogenized with a zero-clearance homogenizer. Lysates were spun on gradients of sodium diatrizoate. The membranes obtained from such gradients have been examined by electron microscopy and by assays for enzymes and $^{125}$I label. Membranes from platelets incubated without and with thrombin were found to be enriched as follows: lectin marker, 8- and 9-fold, respectively; phosphodiesterase, 9- and 12-fold; acid phosphatase, 2.5- and 2-fold. There is thus a particularly close correlation of lectin marker with phosphodiesterase, an enzyme characteristic of normal purified membranes.

Monitoring for $^{125}$I-labeled lentil lectin appears to be a useful procedure for following platelet membranes during isolations from relatively small quantities of blood.

## INTRODUCTION

Our laboratory has been studying alterations in lipid metabolism associated with the action of agents that induce shape change and aggregation in gel-filtered human platelets. Since the isolation of membrane from such altered platelets is central to our studies, and since the classical techniques of membrane isolation from normal platelets [1, 2] have not been shown to yield pure preparations of membranes from thrombin-treated platelets, we decided to investigate plasma membrane purification using a known external marker. Ideally, the marker had to meet the following criteria: (1) ease of detection at low concentrations; (2) low potential for altering membrane lipids; (3) labeling process requiring a minimum of manipulation of cells; (4) strong affinity for the cell surface and nonpenetration to the interior; (5) lack of interference with thrombin-induced aggregation; and (6) failure to cause aggregation and/or release of granule contents.

In order to minimize possible oxidations of lipids, we avoided the widely used and otherwise successful technique of lactoperoxidase-catalyzed iodination [3], and drew instead on the findings of Feagler et al. [4], Greenberg and Jamieson [5], and Kornfeld et al. [6] that lentil phytohemagglutinin, a plant lectin binding to human platelet membranes with great affinity ($K_{diss} = 10^{-7}$ M) [4] (a) causes neither aggregation nor release of granule contents (at low concentrations) [5], (b) does not interfere with thrombin-induced aggregation and release and (c) can be labeled with $^{125}I$ without impairment of binding to platelets. Our procedure for the isolation of membranes from gel-filtered human platelets exposed to thrombin therefore involves prior labeling of the intact cells with radio-iodinated lentil lectin. We have discovered, since starting our investigation, that Chang et al. [7] have employed a similar technique with success, using labeled wheat germ agglutinin and Concanavalin A to mark fat cell, lymphocyte and liver membranes.

## MATERIALS AND METHODS

Typically, 160 ml of blood were drawn from a normal human volunteer who had not ingested aspirin for 10 days, using modified acid/citrate/dextrose [8] as anticoagulant. All manipulations were performed at room temperature. Blood was spun at $150 \times g$ for 15 min. The resulting supernatant platelet-rich plasma was mixed with 0.01 vol. of 10 mM adenosine and allowed to sit for 10 min. The platelet-rich plasma was divided in 4 ml aliquots, underlaid with a cushion of 0.5 ml dialyzed 34 % bovine serum albumin (pH 6.2), and spun at $740 \times g$ for 15 min. The interfacial platelets were then carefully removed [9], combined, and layered over a 50–60 ml column of Sepharose 2B [10] (Sigma Chemical Co.). The previously washed Sepharose had been equilibrated with a modified buffer described by Lindon [11, 12], containing 129 mM NaCl, 10.9 mM sodium citrate, 8.9 mM $NaHCO_3$, 1 mg · $ml^{-1}$ dextrose, 10 mM Trizma base, 2.8 mM KCl and 0.8 mM $KH_2PO_4$, titrated to pH 6.5 with HCl (Buffer A). Passage of the cells through the column was monitored with a Uvicord II flow cell spectrophotometer, and the main body of the platelet peak collected (here designated gel-filtered platelets I). All glassware used was siliconized (Siliclad).

Lentil lectin was prepared from dried lentils according to Howard et al. [13] as modified by Kornfeld et al. [6]. It was iodinated with carrier-free $Na^{125}I$ (New England Nuclear) as described by Majerus and Brodie [14]. All plastic and glassware for use with lectin-labeled material had been soaked overnight in cold 0.5 % albumin and rinsed three times in distilled water. 4–6 ml of gel-filtered platelets I were incubated for 20 min at room temperature with less than 25 $\mu g \cdot ml^{-1}$ $^{125}I$-labeled lectin. The incubation mixture was then passed through a 50–60 ml Sepharose 2B column equilibrated with the above-described buffer, but at pH 6.8 and lacking dextrose (Buffer B), since dextrose has competing affinity for lentil lectin. The distribution in size of interfacial platelets, gel-filtered platelets I and gel-filtered platelets II was monitored with a Packard Multichannel Analyzer.

The morphology of normal gel-filtered platelets II and gel-filtered platelets II treated with 0.5 units $ml^{-1}$ of thrombin in the absence of $Ca^{2+}$ was observed in electron micrographs of 3 % glutaraldehyde-fixed preparations post-fixed with $OsO_4$ and stained with uranyl acetate and lead citrate. Spurr-embedded thin sections were observed and photographed using a Phillips 300 electron microscope.

The following procedure was found to be effective with thrombin-treated,

$Ca^{2+}$-free cells: a cushion of 0.3 ml of 80 % glycerol/2 mM EDTA in buffer B was overlaid with a continuous linear gradient of 0–40 % glycerol and 0–1.5 mM EDTA in buffer B. The cell suspension was laid over this gradient after incubation and spun first for 30 min at $1100 \times g$ (Sorvall RC-5B HS 4 swinging bucket rotor), and then for 10 min at $5800 \times g$. The resulting loose pellet was suspended in 1.5–2.0 ml of 10-fold diluted buffer B containing 1 mM EDTA, and homogenized in two portions, as described by Marcus [2], in a zero-clearance homogenizer. Solutions of 20 % sodium diatrizoate [15, 16], density 1.11 $g \cdot ml^{-1}$ or linear 20–30 % gradients, maximum density = 1.19 $g \cdot ml^{-1}$, were employed to separate a membrane fraction, as an interface from unbroken cells, granules and mitochondria. Pooled homogenates were layered over 12 ml of 20 % sodium diatrizoate/1 mM EDTA in buffer B, or a 20–30 % linear gradient. Gradients were spun in an SW27 rotor at $63\,500 \times g$ for 150 min at 4 °C, using a Beckman L2-65 centrifuge. Normal (unexposed to thrombin) lectin-labeled cells could be lysed either by centrifugation on graded glycerol and vortex swirling of the pellet in buffer [1] or by the method described above.

The material at the buffer-20 % sodium diatrizoate interface was diluted with buffer B and spun in an SW 50.1 rotor at $114\,000 \times g$ for 45 min. Supernatants were removed and the pellets fixed for electron microscopy, as described previously, or assayed for enzymatic activity, or counted in a Packard Auto-Gamma Scintillation Spectrometer 5230. Protein determinations were made using the Lowry technique [17] with appropriate blanks to correct for the diatrizoate, where necessary. Successive 1 ml fractions were taken above and below the interface for assessment of $^{125}$I-labeled lectin and protein content, and the pellet from the 150 min spin was assayed for enzymes, protein and lectin. A control was run in which whole cells were incubated in various media for 150 min at 4 °C and then spun in an SW 50.1 rotor at $100\,000 \times g$ for 45 min.

In order to obtain membrane preparations for enzymatic assay, we processed about 450 ml of blood as described in the foregoing procedure.

Acid phosphatase (EC 3.1.3.2) was assayed according to Michell et al. [18] using *p*-nitrophenylphosphate, in keeping with the findings of Kaulen and Gross [19].

*β*-Glucuronidase (EC 3.2.1.31) was assayed with *p*-nitrophenyl-*β*-D-glucuronide (Sigma) as described by Michell et al. [18].

Phosphodiesterase (EC 3.1.4.18) was assayed in a system based on that of Koerner and Sinsheimer [20].

Succinate dehydrogenase (EC 1.3.99.1) was measured by a modification of the method of Muller et al. [22] by which 7 mM Tris · Cl (pH 7.4), 2 mM sodium cyanide, 0.5 mM phenazine methosulfate (Sigma), 0.099 mM dichlorophenolindophenol (Sigma), 0.1 % (w/v) Triton X-100, 1 mM sodium succinate (pH 7.4) and 0.1–0.7 mg of enzyme protein were mixed in a total of 1.0 ml. The decrease in the absorbance of dichlorophenolindophenol at 600 nm was determined continuously for 5 min vs. a blank.

Activities were expressed as units · $mg^{-1}$ protein, where units = nmol of product released/h.

## RESULTS

The resistance of thrombin-treated cells to breakage presented a major barrier to the resolution of a membrane fraction from such cells. A significant improvement

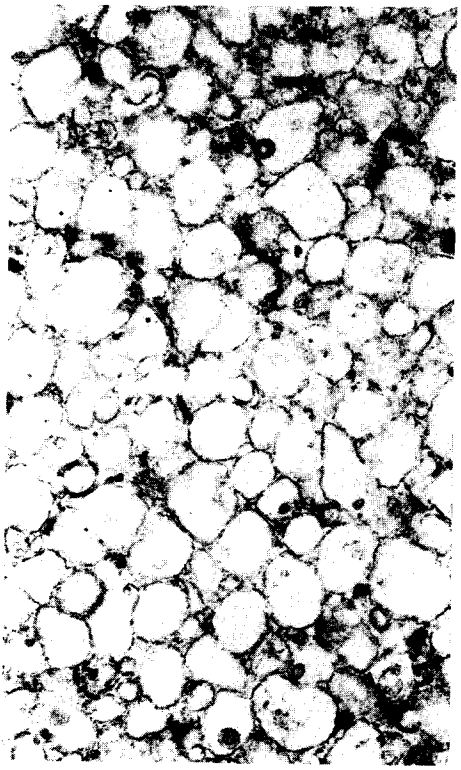

Fig. 1. Electron micrograph of normal membrane isolated from the buffer-20 % sodium diatrizoate interface. Normal cells were first broken by the standard glycerol lysis technique. Magnification: 2.7 × 4500.

in cell breakage occurred when thrombin-treated cells were subjected to glycerol-EDTA centrifugation prior to homogenization.

A preparation of interfacial membrane from normal cells lysed by glycerol loading and agitating is seen in Fig. 1, and appears to be free of unbroken cells and granules. The interfacial fraction from cells exposed to 0.5 units · $ml^{-1}$ of thrombin and lysed with glycerol and homogenization as described in the text is presented in Fig. 2. A slightly greater contamination by granules and more heterogeneity in vesicular size characterize such material. An enrichment in granules and mitochondria is seen in the pellet depicted in Fig. 3, obtained from a lysate of normal cells spun on 20 % sodium diatrizoate. This pellet was comparable to a pellet preparation derived from thrombin-treated and -lysed cells.

Gel-filtered platelets II were found to have $^{125}$I-labeled lectin which was 95 % sedimentable at 2000 × $g$ for 10 min and was not removable, when tested, by three successive washes of cold buffer B. Usually 5–6 $\mu$g of lectin were bound per $10^9$ cells, below the saturation level of 28 $\mu$g per $10^9$ cells. However, 98 % of the label was removable by exposure of the platelets to 0.1 M dextrose. Sucrose also removed lectin, and for this reason sodium diatrizoate was chosen as the density gradient medium. No loss of sedimentable counts beyond control values (approx. 10 %) occurred

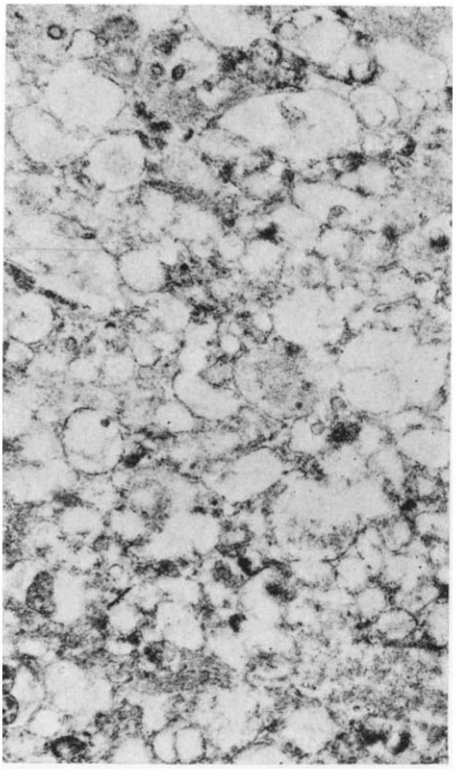

Fig. 2. Electron micrograph of thrombin-treated membrane isolated from the buffer-20 % sodium diatrizoate interface. Thrombin-exposed cells were lysed by glycerol plus homogenization as described in the text. Magnification: 2.5 × 4500.

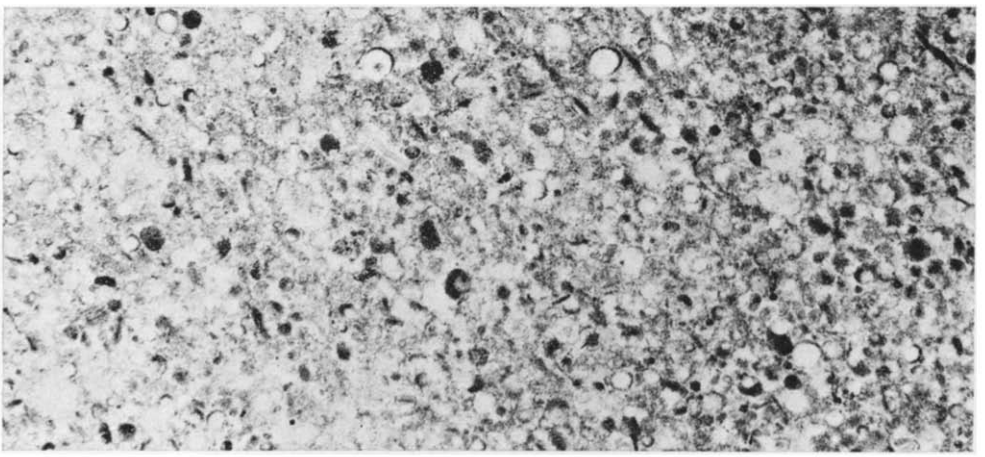

Fig. 3. Electron micrograph of a pellet obtained after centrifugation of a normal platelet lysate on 20 % sodium diatrizoate. A predominance of granules, mitochondria and small vesicles is evident. Magnification: 3.2 × 4500.

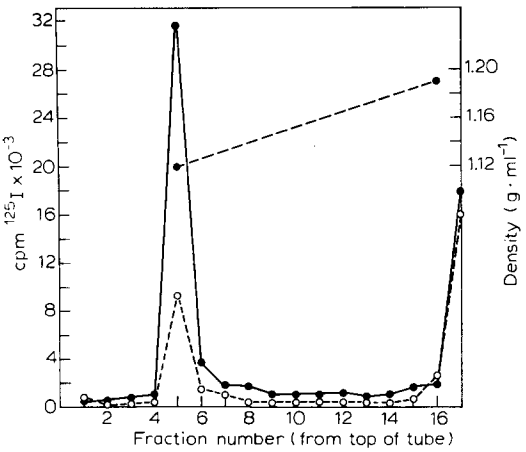


Fig. 4. Distribution of $^{125}$I-labeled lectin over linear gradients of 20–30 % diatrizoate. Glycerol lysates of normal cells (○) and glycerol homogenates of thrombin-treated cells (●) were layered over density gradients in separate experiments.

when cells were incubated with thrombin, or with 20 % sodium diatrizoate in Buffer B containing 1 mM EDTA. Normal gel-filtered platelets II applied to the above-described glycerol gradients lost 50–72 % of their protein, while retaining 92 % of their $^{125}$I label. In addition to demonstrating the avidity of lectin binding to gel-filtered platelets, this information reveals that platelets were breaking to some extent during centrifugation through glycerol. Comparable breakage was not observed for thrombin-exposed cells. However, centrifugation through glycerol and EDTA prior to homogenization seemed to render these cells more fragile, since homogenization of cells with EDTA directly after incubation with thrombin was ineffective in yielding a resolvable lysate. The distribution of activities for normal cells subjected to lysis by glycerol, and for thrombin-treated cells spun through glycerol and homogenized, is given in Fig. 4. These results were obtained in separate experiments with $^{125}$I-labeled lectin preparations of different specific activity. Consequently, the difference in the magnitudes of activity in the two fractions 5 is not significant. The most salient feature of this figure is that much activity was present at the interface (fraction 5) in both preparations. Material present in this fraction (typically containing 30–50 % of the total activity applied to the gradient) displayed enrichment of specific activities, relative to whole platelets, of 7.3- and 13-fold for thrombin-free and exposed preparations, respectively. Pellets (fraction 17) derived from these centrifugations were not significantly enriched in lectin. Their ratios of specific activities were 1.3 and 1.9 for thrombin-free and exposed cells, relative to the whole cell lysate from which each was derived.

The contents of lectin and enzymatic activities of the two major fractions (membranes and pellet) from the centrifugation on 20 % sodium diatrizoate are given in Table I. Interfaces (density = 1.11 g · ml$^{-1}$) were notably enriched in lectin and phosphodiesterase relative to whole gel-filtered platelets II, and depleted in the $\alpha$-granule and mitochondrial enzymes, $\beta$-glucuronidase and succinate dehydrogenase. This was the opposite of the case for pellets.

TABLE I

RELATIVE ACTIVITIES OF ENZYMES AND LECTIN IN LYSATE FRACTIONS

Activities are presented as the means of three experiments. The ranges are given in parentheses. Interfaces were obtained from centrifugation of lysates on 20 % sodium diatrizoate. Pellets were at the bottom of this density medium.

| Marker | Cell lysate (units/mg protein or μg/mg protein) | Ratio of specific activity in fraction as compared with cell lysate | | | |
|---|---|---|---|---|---|
| | | Normal cells | | Thrombin-exposed cells | |
| | | Interface | Pellet | Interface | Pellet |
| Phosphodiesterase | 1.8(0.8–3.1) | 9.0(7.1–11) | 3.4(3.2–3.7) | 12(11–14) | 3.3(1.4–5.3) |
| Acid phosphatase | 6400(4800–7800) | 2.5(1.9–3.8) | 0.43(0.13–0.73) | 2.1(0.9–3.5) | 0.51(0.24–0.73) |
| β-Glucuronidase | 36(31–44) | 0.41(0.23–0.60) | 1.5(1.1–1.8) | 0.73(0.42–0.88) | 2.5(1.7–2.9) |
| Succinate dehydrogenase | 150(128–160) | 0.090(0.041–0.13) | 2.3(1.7–2.9) | 0.15(0.11–0.22) | 2.7(2.1–3.4) |
| Lentil lectin | 2.9(2.8–3.1) | 8.2(7.3–9.0) | 3.9(1.3–6.6) | 9.2(5.4–13) | 1.9(1.4–2.0) |

The yield of membrane protein with respect to whole cell (gel-filtered platelet II) protein was 4 %.

## DISCUSSION

In keeping with the findings of Barber and Jamieson [1] and Kaulen and Gross [21], we have shown that membranes from gel-filtered platelets display substantial phosphodiesterase activity. In addition, we note similar observations for membranes from thrombin-treated cells.

The distribution of $^{125}$I-labeled lectin and its enrichment in the interfacial fraction (8- to 9-fold) parallel those of phosphodiesterase, which shows a 9- to 12-fold enhanced activity in the interfacial fraction. The labeled lectin, as a marker, allowed preliminary assessment of techniques feasible for the isolation of membrane from cells exposed to thrombin, using relatively small amounts of blood.

We have undertaken some experiments which indicate that the above-described membranes, isolated from sodium diatrizoate buffer interfaces, can be resolved further on discontinuous gradients of sodium diatrizoate into two fractions of density 1.09 and 1.12 $g \cdot ml^{-1}$. The fractions are of equal specific activity, comparable to that of the original interface. These observations are in agreement with those of Barber and Jamieson [1]. Further resolution involves centrifugation of the interfacial material for 18 h, too long a period for us to reasonably expect preservation of the normal state of unsaturation of the membrane lipids which interest us. However, we feel that our interfacial membranes are sufficiently free of contaminating granules, mitochondria and unbroken cells to justify the future analysis of changes in their lipid following exposure to thrombin as indicative of alterations in plasma membrane lipids.

## ACKNOWLEDGEMENTS

Support for this research was provided by the Veterans Administration Hospital and the Departments of Biochemistry and Medicine, Boston University, Boston, Mass. We wish to express thanks to Mr. George Nolley of the Boston VA Hospital for performing electron microscopy presented here, to Mr. Walter Gregory of Dr. Stuart Kornfeld's Laboratory at the Washington University School of Medicine for guidance in the purification of lentil lectin and, finally, to Dr. Manfred L. Karnovsky of Harvard University, for helpful comments for the preparation of this manuscript.

## REFERENCES

1 Barber, A. J. and Jamieson, G. A. (1970) J. Biol. Chem. 245, 6357–6365
2 Marcus, A. J., Zucker-Franklin, D., Safier, L. B. and Ullman, H. D. (1966) J. Clin. Invest. 45, 14–28
3 Nachman, R. L., Hubbard, A. and Ferris, B. (1973) J. Biol. Chem. 248, 2928–2936
4 Feagler, J. R., Tillack, T. W., Chaplin, D. D. and Majerus, P. W. (1974) J. Cell Biol. 60, 541–553
5 Greenberg, J. H. and Jamieson, G. A. (1974) Biochim. Biophys. Acta 345, 231–242
6 Kornfeld, S., Rogers, J. and Gregory, W. (1971) J. Biol. Chem. 246, 6581–6586
7 Chang, K.-J., Bennett, V. and Cuatrecasas, P. (1975) J. Biol. Chem. 250, 488–500

8 Aster, R. H. and Jandl, J. H. (1964) J. Clin. Invest. 43, 843–856
9 Hutton, R. A., Howard, M. A., Deykin, D. and Hardisty, R. M. (1974) Thromb. Diath. Haemmorrh. 31, 119–132
10 Tangen, O., Berman, H. J. and Marfey, P. (1971) Thromb. Diath. Haemorrh. 25, 268–278
11 Lindon, J. N. (1974) Human Platelet Manipulation by Gel Filtration: An Evaluation. Ph. D. Thesis, Mass. Inst. Technol.
12 Lindon, J. N. and Waugh, D. F. (1974) Fed. Proc. 33, 270
13 Howard, I. K., Sage, H. S., Stein, M. D., Young, N. M., Leon, M. A. and Dyckes, D. F. (1971) J. Biol. Chem. 246, 1590–1595
14 Majerus, P. W. and Brodie, G. N. (1972) J. Biol. Chem. 247, 4253–4257
15 Ghettie, V., Nilsson, K. and Sjoquist, J. (1974) Proc. Natl. Acad. Sci. U.S. 71, 4831–4835
16 Tamir, H. and Gilvarg, C. (1966) J. Biol. Chem. 241, 1085–1090
17 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
18 Michell, R. H., Karnovsky, M. J. and Karnovsky, M. L. (1970) Biochem. J. 116, 207–216
19 Kaulen, H. D. and Gross, R. (1971) Thromb. Diath. Haemorrh. 27, 353–361
20 Koerner, J. F. and Sinsheimer, R. L. (1957) J. Biol. Chem. 228, 1039–1047
21 Kaulen, H. D. and Gross, R. (1973) Thromb. Diath. Haemorrh. 30, 199–214
22 Muller, M., Hogg, J. F. and DeDuve, C. (1968) J. Biol. Chem. 243, 5385–5395