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STUDIES ON SUBCELLULAR FRACTIONS OF HUMAN PLATELETS BY THE LACTOPEROXIDASE-IODINATION TECHNIQUE

INGER HAGEN, TERJE OLSEN and NILS OLAV SOLUM

Institute for Thrombosis Research, University of Oslo, Rikshospitalet, Oslo (Norway)

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SUMMARY

Lactoperoxidase-catalyzed ^{125}I iodination and sodium dodecyl sulphate-polyacrylamide gel electrophoresis have been performed on whole, washed platelets as well as on isolated platelet membranes and granules. Electrophoresis of the whole platelets demonstrated two major radioactive peaks, corresponding to glycopolypeptides of estimated molecular weights of 120 000 and 100 000. A small, but consistent amount of radioactivity was also associated with a 147 000 dalton glycopolypeptide.

The membranes showed the same pattern of radioactivity as the whole platelets, whereas only negligible amounts of labeled material was found in the soluble and granule fractions.

Practically all the polypeptides were labeled in membranes iodinated after their isolation.

A glycopolypeptide of 147 000 molecular weight was observed also in the soluble and the granule fractions, but no radioactivity was associated with these substances. In unreduced form, the granule glycopolypeptide penetrated only slightly into the polyacrylamide gel. Thrombin induced the release of this granule-located substance from whole platelets, as observed by gel electrophoresis of the supernatant after release reaction (secretion).

The granule glycoproteins were only partly exposed on the granule membrane since about 50 % of the acid-hydrolyzable sialic acid could be liberated by neuraminidase treatment of isolated granules.

In whole, iodinated granules the bulk of the radioactivity was associated with a polypeptide of estimated molecular weight 46 000 (possibly actin). This polypeptide was not seen in the supernatant after removal of the thrombin-degranulated platelets by centrifugation, which indicates that the granule membrane is retained with the platelets during the secretion process.

INTRODUCTION

Many stimuli can induce the rapid secretion process termed release reaction in blood platelets [1–3]. The substances extruded are located in specific, membrane-limited organelles, the dense bodies and the α-granules [4–6]. Ultrastructural observa-

tions indicate that the granule content may be transported to the extracellular medium through an open channel system [7–9]. A fusion between the granule and the cytoplasmic membranes is thought to be the first event in this process [10], but, the fate of the granule membrane is not known. There is evidence that in other secretory cells the granule membrane is retained as part of the plasma membrane [11–13].

In the present work, we examined various aspects of the composition and structure of the subcellular organelles of human platelets. One main aspect studied is whether the platelet granule membrane is extruded during the release reaction. Another purpose was to see whether glycopeptides of whole platelets represented mixtures of proteins located in different subcellular organelles within the platelet. This may be important since the surface membrane glycoproteins are thought to exert vital functions in platelet adhesion and aggregation reactions [14–17].

The lactoperoxidase-iodination technique [18] and sodium dodecyl sulphate-polyacrylamide gel electrophoresis were used to study the proteins of the platelet subcellular fractions and the extracellular medium after the release reaction had occurred. This would enable us to describe the composition and availability of the proteins of the intracellular organelles as well as the plasma membrane. It is evident from these experiments that one protein available for the iodine label in isolated, whole granules was not present in the extracellular medium after the release reaction had occurred, whereas the releaseable, granule-located substances, platelet fibrinogen and a glycopolypeptide of molecular weight 147 000, were labeled to far less an extent in whole granules.

We conclude therefore that the granule membrane is retained in the platelets during the release reaction. Further, we find that the glycopolypeptide band corresponding to a molecular weight of 147 000 as observed with reduced samples of whole platelets, actually represents a mixture of at least two glycopeptides originating from the plasma membrane and the granules, respectively.

MATERIALS

Chemicals and radiochemicals. Bovine thrombin, Topostasine Roche (F. Hofmann La Roche & Co., Ltd., Switzerland) was made up to 300 N.I.H. units/ml with 0.12 M NaCl containing 0.03 M Tris and 0.003 M EDTA, pH 7.4, and stored at −20 °C. Lactoperoxidase, *N*-acetylneuraminic acid, β-mercaptoethanol, antimycin A (type 3) and 2-deoxyglucose were from Sigma Chemical Co., St. Louis, U.S.A. Hirudin (pure) was supplied by VEB Arzneimittelwerk, Dresden, G.D.R., neuraminidase (from *Vibrio comma*) by Behringwerke AG, G.F.R., and Schiff's reagent by Raymond A. Lamb, London, U.K. Carrier free $^{125}I^-$ was purchased from NEN Chemicals, GmbH, G.F.R.

Biological materials. Platelets were obtained from 500 ml of human blood collected into 40 ml of 0.077 M EDTA, pH 7.4, and processed as described elsewhere [19].

METHODS

The iodination of isolated platelets was performed mainly as described by Phillips [18]. The iodinated samples were washed once, and aliquots examined on

sodium dodecyl sulphate-polyacrylamide gel electrophoresis [20]. The gels were stained for protein with Coomassie brilliant blue, and for carbohydrate with the periodic acid-Schiff's reagent [21]. Scanning was performed in a Joyce Chromoscan densitometer, and the radioactivity in 1.5-mm gel pieces was measured in a Packard Gamma Scintillation Spectrometer.

Platelets were homogenized as described by French and Holme [22] in the Aminco French Pressure Cell (2×1 min, 1361 atm), and the subcellular fractionation performed as previously published [20].

The release reaction was induced by thrombin (1 unit/ml, 1 min, 37 °C), and measured as described earlier [19]. After the incubation period, hirudin (5 ATU/ml) was added, and the platelets separated by centrifugation ($2000 \times g$, 20 min, 4 °C). In some experiments, the release reaction was inhibited by incubation of the platelets for 30 min at 37 °C with antimycin (4 μg/ml) and 2-deoxyglucose (15 μM) before the addition of thrombin [23].

Sialic acid liberated after acid hydrolysis (0.05 M H_2SO_4, 60 min, 80 °C) or neuraminidase treatment (25 units, 60 min, 37 °C) was measured as described by Warren [24]. Protein was determined by the method of Miller [25].

RESULTS

Carbohydrate staining after sodium dodecyl sulphate-polyacrylamide gel electrophoresis of reduced samples from whole platelets showed three major, pink

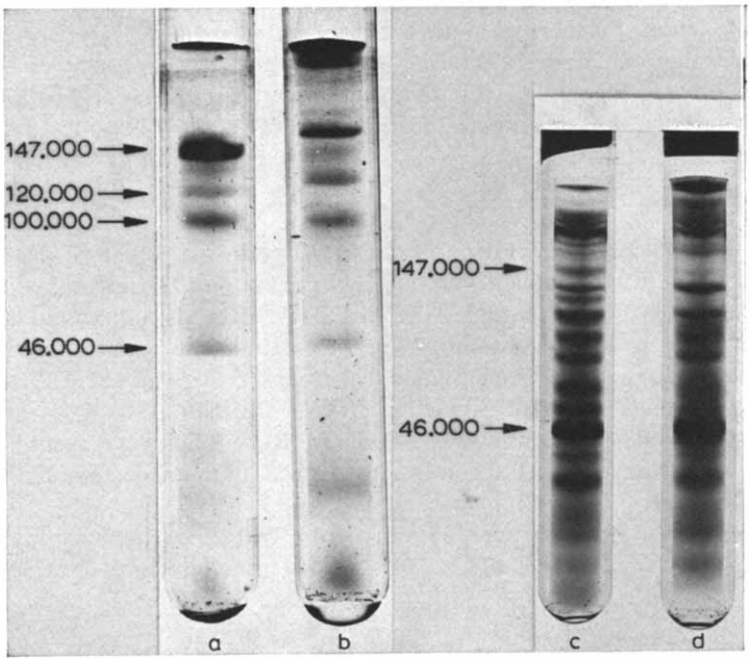

Fig. 1. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis of whole platelets, stained for carbohydrate with the periodic-acid-Schiff's reagent (PAS), and for protein with Coomassie brilliant blue. (a) Glycoprotein pattern, reduced sample; (b) glycoprotein pattern, unreduced sample; (c) protein pattern, reduced sample; (d) protein pattern, unreduced sample.

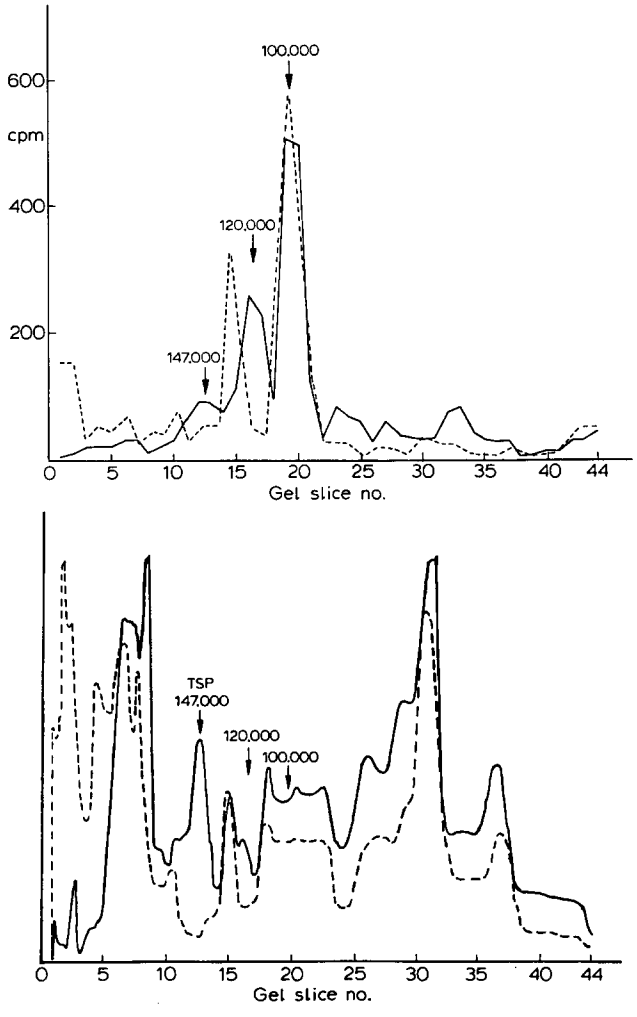

Fig. 2. Distribution of protein (lower curves) and radioactivity (upper curves) after sodium dodecyl sulphate-polyacrylamide gel electrophoresis of whole, iodinated platelets. 150 μg of protein was applied to the gels. Reduced samples, ;——— unreduced samples, -----. TSP, thrombin-sensitive protein.

bands (147 000, 120 000 and 100 000 daltons, respectively) previously [18] termed GP I, GP II and GP III (Fig. 1a). The band seen at 46 000 daltons represented an unstained, opaque zone. The intensity of the band at 147 000 daltons was lower in the unreduced samples, and an additional band appeared only a slight distance from the application surface (Fig. 1b and d).

The radioactivity in the reduced samples was mainly recovered in areas corresponding to polypeptides of 100 000 and 120 000 molecular weight (Fig. 2). A smaller peak was observed at 147 000 daltons in addition to a variable number of minor peaks corresponding to polypeptides of lower molecular weight.

When H_2O_2 or lactoperoxidase was omitted during the iodination procedure, the iodine retained with the platelets was 19 % and 3 % of the control, respectively.

TABLE I

^{125}I RETAINED IN THE ABSENCE OF EITHER H_2O_2 OR LACTOPEROXIDASE AND THE EFFECT OF PLATELET WASHING AFTER IODINATION

Sample	Activity (cpm)	Protein (mg)	cpm/mg protein	% of control
Control*	8180	10.0	818	100
$-H_2O_2$	1457	9.2	159	19.4
−lactoperoxidase	247	9.8	25	3.1
10 × washing after iodination	4255	5.5	780	95.5

* Standard system, see Methods.

TABLE II

SUBCELLULAR FRACTIONATION OF WASHED, IODINATED PLATELETS. DISTRIBUTION OF RADIOACTIVITY AND PROTEIN

	Radioactivity (% of total)		Protein (mg)	cpm/μg Protein
Fraction A (soluble)	22.1		3.000	46.5
Fraction B (membranes)	64.7		3.440	130.0
Fraction C	8.1		0.190	88.9
Fraction D (granules, mitochondria)	4.5		0.396	54.5
Fraction E	0.5		0.017	74.7
		Total	7.043	
Homogenate			7.1	79.5

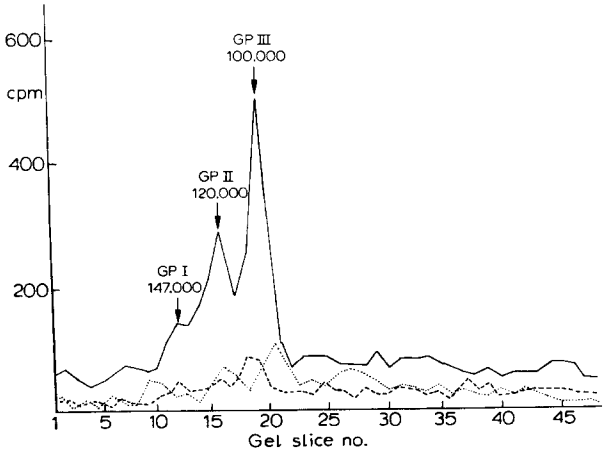

Fig. 3. Distribution of radioactivity after sodium dodecyl sulphate-polyacrylamide gel electrophoresis of the soluble fraction, ·····; membrane fraction, ———; and granule fraction, – – – – isolated from whole, iodinated platelets. Reduced samples. The same amount of protein was applied to each gel (100 μg).

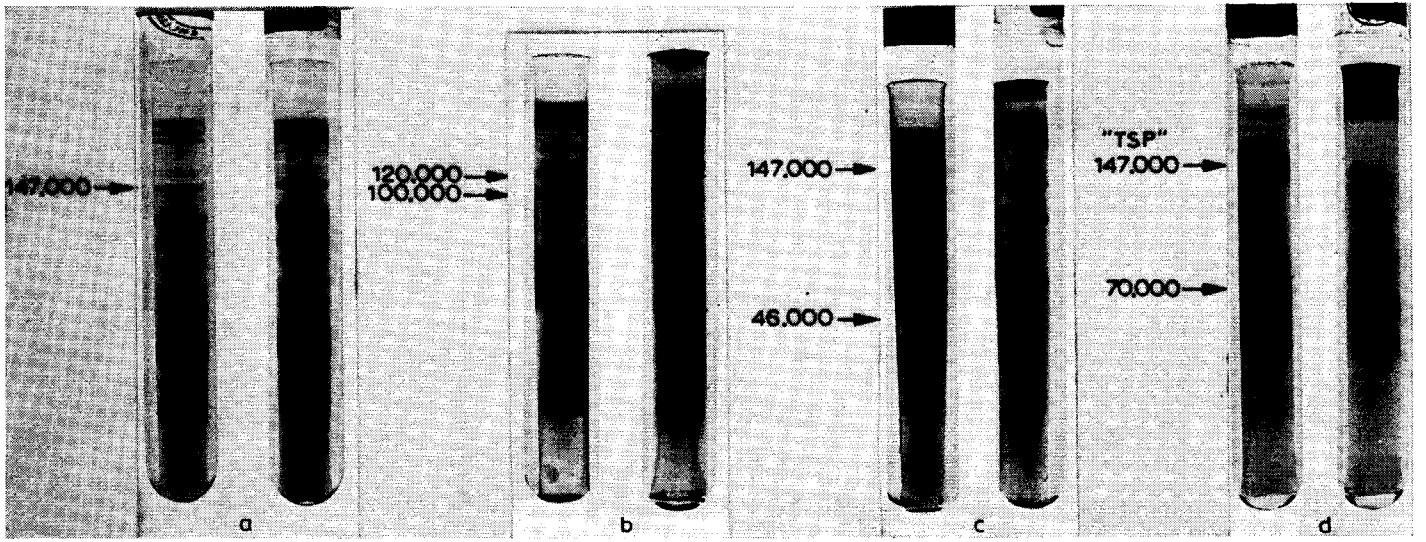

Fig. 4. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis of the soluble (a), membrane (b), granule fraction (c) and the extracellular medium (supernatant) after thrombin-induced release reaction (d) stained for protein with Coomassie brilliant blue. Reduced samples to the left, unreduced to the right. TSP, thrombin-sensitive protein.

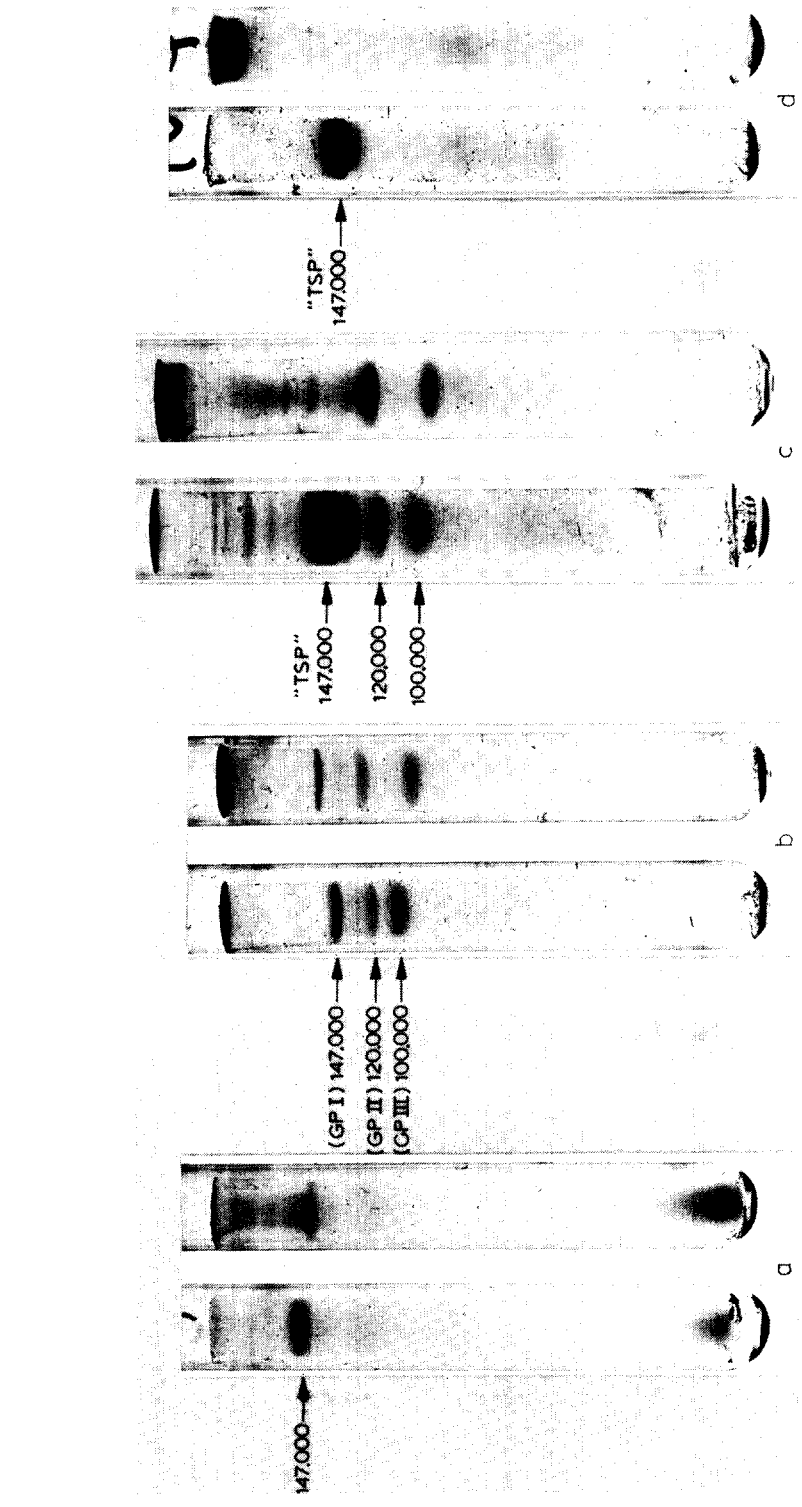

Fig. 5. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis of the soluble fraction (a), the membrane fraction (b), the granule fraction (c) and the extracellular medium (supernatant after thrombin-induced release reaction) (d) stained for carbohydrate with the periodic-acid-Schiff's reagent (PAS). TSP, thrombin-sensitive protein. Reduced samples to the left, unreduced to the right.

Washing (10 times) after iodination did not influence the amount of label per mg of protein (Table I).

After extensive dialysis, 64.7 % of the radioactivity was recovered in the membrane fraction, whereas 22.1 % and 4.5 % were recovered in the soluble and granule fractions, respectively (Table II). Some of this radioactivity may have represented free iodine as gel electrophoresis followed by gel slicing and counting revealed that only minimal amounts of radioactive iodine were bound to soluble or granule proteins, whereas membrane samples showed essentially the same pattern of radioactivity as whole platelets (Fig. 3).

Gels containing reduced and unreduced samples of the subcellular fractions, and the extracellular medium (supernatant) after thrombin-induced release reaction were stained for protein (Fig. 4a–d) and carbohydrate (Fig. 5a–d). In the soluble fraction, a glycopolypeptide with a molecular weight of 147 000 was observed. Its electrophoretic mobility was unchanged when the reducing agent was omitted (Figs. 4a and 5a).

The membranes revealed three glycopolypeptides of molecular weight 147 000 (GP I), 120 000 (GP II) and 100 000 (GP III) (Figs. 4b and 5b) as also observed by others [14, 18]. The mobilities of the two largest glycopolypeptides were slightly lower in their unreduced forms, whereas the smallest moved slightly faster, probably because of intramolecular disulfide bonds.

The granules contained three major glycopolypeptides with the same mobilities as the membrane glycopeptides GP I, GP II and GP III (Figs. 4c and 5c). However, the first band (147 000 daltons) certainly represented two different glycoproteins, which was clearly seen in the unreduced gels. In this case, the stain intensity after electrophoresis was markedly less, and a new band was seen just below the application surface.

Similarly, the extracellular medium from suspensions of platelets which had undergone the thrombin-induced release reaction contained a glycopolypeptide which migrated like the granule glycopolypeptide both in reduced and unreduced state (Figs. 4d and 5d). Consistent with this, the glycoprotein was not observed in the extracellular medium when the platelets were incubated with thrombin in the presence of antimycin and 2-deoxyglucose, which are known to inhibit the release reaction [23].

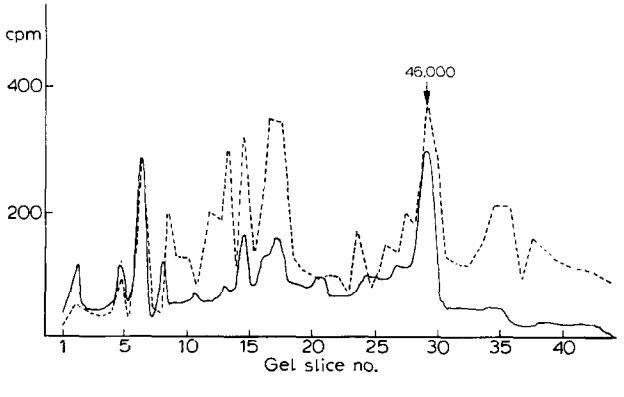

Fig. 6. Distribution of protein, ———; and radioactivity, – – – – – after sodium dodecyl sulphate-polyacrylamide gel electrophoresis of platelet membranes iodinated after their isolation.

Practically all the polypeptides were labeled after iodination of the isolated platelet membranes (Fig. 6). This has been shown previously with membranes isolated by other methods [26, 27].

To reveal proteins present on the granule membrane surface, gel electrophoresis was performed on iodinated granules. The highest peak of radioactivity was associated with a polypeptide with a molecular weight of 46 000 (Fig. 7). This polypep-

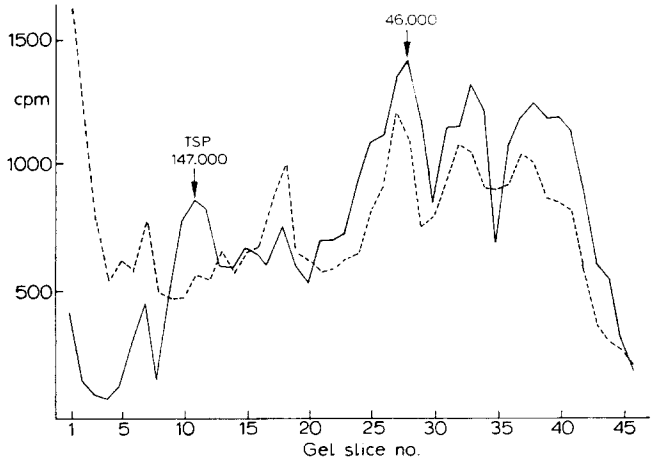

Fig. 7. Distribution of radioactive label after sodium dodecyl sulphate-polyacrylamide gel electrophoresis of platelet granules iodinated after their isolation. Reduced sample, ———; unreduced sample, – – – –.

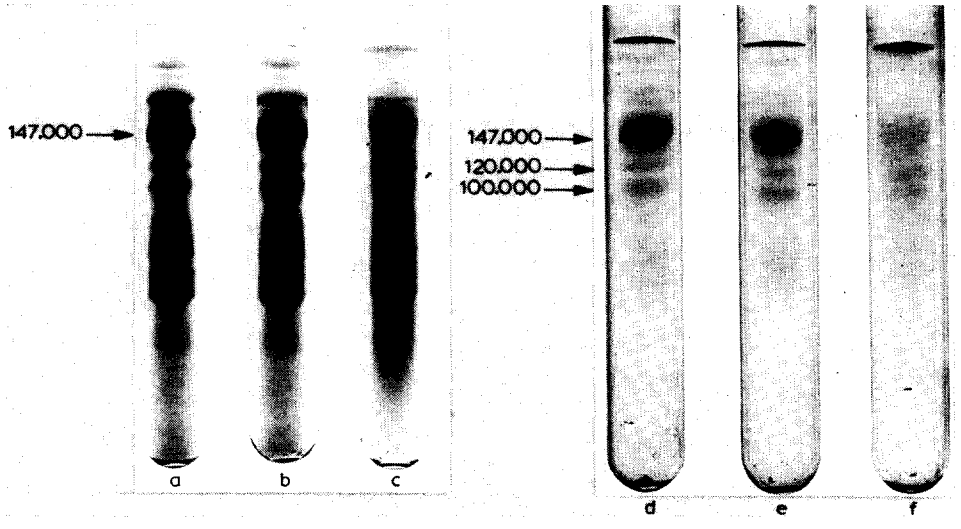

Fig. 8. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis of isolated granules, stained for protein (a–c) and carbohydrate (d–f). Control granules (a and d), neuraminidase treated granules (b and e), acid hydrolyzed granules (c and f).

tide was not seen extracellularly after the platelets had undergone the release reaction. A variable amount of radioactivity was bound to the releaseable glycoprotein as well as polypeptides of lower molecular weight.

48 % of the total acid-hydrolyzable sialic acid was released after neuraminidase treatment of whole granules. Granules treated with neuraminidase showed a protein and glycoprotein pattern identical to the control granules, whereas for the acid-hydrolyzed granules the releaseable glycoprotein and a protein of higher molecular weight showed markedly reduced staining (Fig. 8).

DISCUSSION

The distribution of radioactivity in whole platelets and isolated membranes in this study is in accordance with the early reports of Phillips et al. [18, 28] and Nachman and coworkers [27]. However, the splitting of GP II into two radioactive peaks (IIa and IIb) as described later [16] were not seen in our experiments. This may be due to the different sizes of the gel slices, and/or the resolution of the electrophoresis system. It is also important to note that different experimental conditions may alter the glycoprotein migration on sodium dodecyl sulphate-polyacrylamide gels. This may have resulted in a confusing nomenclature on platelet glycoproteins.

Recently, George et al. [29] have developed a label, diazotized [^{125}I]diiodosulfanilic acid, which binds covalently to exposed proteins on the platelet surface. Using this compound, GP II was labeled to the same extent as GP III. In unreduced samples, a glycoprotein band termed GP IIa was seen between GP I and GP II. A prominent peak of radioactivity was also found in this area. With both reduced and unreduced samples, we observed only weak and inconsistent indications of four glycoprotein bands in this area. Compared to the reduced state, the relative migration of the three bands were slightly different in the unreduced system. Based on the distribution of radioactive label, it seems reasonable to suggest that the second and third band in the unreduced system correspond to GP II and GP III in the reduced system, respectively, and that the GP IIa as observed by George et al. [29] corresponds to our second band (GP II). The presence of four glycoprotein bands in reduced gels as observed by others [16] may mean that GP IIa and GP IIb represent two different glycoproteins, or that the splitting of the GP II band results from incomplete reduction.

In whole platelets, the band at 147 000 daltons showed the strongest stain intensity. However, in isolated membranes we found about equal staining intensity of the three glycoproteins. In these studies, it is important to note that there exists a glycoprotein both in the soluble and the granule fraction which migrates like GP I in the reduced state. Recent observations by Lombart et al. [30] and Okumura and Jamieson [31] indicate that the glycoprotein occurring in the soluble fraction has a surface location. However, we were unable to detect any radioactivity in this glycoprotein after iodination of whole platelets, and whether it represents a cytoplasmic constituent or is solubilized from the plasma membrane seems to be an unresolved question.

The main granule glycoprotein can easily be detected separately from GP I and the glycoprotein present in the soluble fraction due to the fact that it migrates only slightly into the gel in its unreduced state. A similar glycoprotein was observed extra-

cellularly after thrombin-induced release of platelets. Knowing from previous work that granular material disappears from the cells during the release reaction, it seems fair to suggest that the protein in question is an intragranular glycoprotein which can be secreted during the general platelet release reaction, and can thus be used as a specific release marker. We have previously suggested [20] that this glycoprotein is identical to the "thrombin-sensitive protein" (TSP) first described by Baenziger et al. [32, 33]. This has recently been confirmed by Käser-Glanzman and coworkers [34]. That the thrombin-sensitive protein has an intracellular location has also been shown by Phillips and Agin [28].

The sialic acid present in the extracellular medium after thrombin treatment of the platelets [19] is most probable bound to released thrombin-sensitive protein [33] and platelet fibrinogen [35]. About 50 % of the total acid-hydrolyzable granule sialic acid could not be liberated from whole granules by neuraminidase. This may be bound to glycoproteins from within the granules (possibly thrombin-sensitive protein and platelet fibrinogen) and/or to glycolipids.

Compared to the major radioactive peak, a small amount of ^{125}I was associated with the releaseable glycoprotein in granules. A certain degree of organelle disruption during their preparation may account for this. However, its presence both in the membrane and as a constituent of the intragranular material cannot be excluded. In this connection it is interesting to note that dopaminebetahydroxylase in the adrenal medulla is located partly in the granule membrane and intragranularly [36].

The main difference between the protein pattern of membranes and granules are the presence of thrombin-sensitive protein and platelet fibrinogen in the latter. Glycopolypeptides of molecular weights 120000 and 100000 seem to be present in both fractions and could be constituents both of the cytoplasmic and the granule membrane. Plasma membrane contamination in the granule fraction is not probable because of the minor amounts of radioactivity recovered in this fraction. However, as some granules are disrupted during homogenization, the presence of organelle membranes in the plasma membrane fraction is quite probable.

The secretory process in adrenal medulla, polymorphonuclear leukocytes and mast cells show many similar features to those of the platelets [37], and experimental data have indicated that the granule membrane is left behind after secretion in these cells [11–13, 38]. Our results show that the granule membrane protein most heavily labeled after iodination of isolated granules was not detectable in the supernatant after the release reaction had occurred. These findings strongly indicate that at least part of the platelet granule membrane is left behind after the secretion process. Recently, Taylor et al. [39] have demonstrated the presence of actin on the outer membrane of pig platelet granules. The similarity between the electrophoretic mobilities of the iodinated granule protein and actin suggests that the two proteins may be the same. However, since actin also is present in the soluble fraction [40], it cannot be excluded that this protein adheres to the granule surface.

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