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ENZYMATIC AND CHEMICAL ANALYSES OF PIG PLATELET MEMBRANE SUBFRACTIONS ISOLATED BY ZONAL CENTRIFUGATION

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SUMMARY

1. A mixed membrane fraction prepared from pig platelets was subfractionated, using the "B 14" zonal rotor, into two distinct subpopulations of membrane vesicles, each associated with a different phosphodiesterase activity.

2. The lighter subfraction (MI) was enriched 7–8 fold with bis-(*p*-nitrophenyl) phosphate phosphodiesterase activity and the denser subfraction (MII) showed a similar degree of enrichment of 5′-dTMP-*p*-nitrophenyl ester phosphodiesterase activity.

3. Assays for other enzyme activities revealed slight enrichment (approx. 2 fold) of acid phosphatase, 3′-dTMP-*p*-nitrophenyl ester phosphodiesterase and β-glucuronidase activities in MI, and β-galactosidase in MII. Cyclic AMP phosphodiesterase, lactate dehydrogenase and *N*-acetyl-β-glucosaminidase showed negligible activity in both MI and MII, and succinate dehydrogenase activity could not be detected in either subfraction.

4. Chemical analyses of the membrane subfractions demonstrated that MI contained approx. twice as much cholesterol, phospholipid, sialic acid and hexosamine per unit weight of protein than MII. These results are consistent with our previously reported observations from surface-labelling experiments, which indicated that MI was derived principally from the platelet surface-exposed membranes and that MII was probably intracellular in origin.

5. Analysis of the membrane polypeptides by sodium dodecyl sulphate-polyacrylamide gel electrophoresis revealed the presence of 12–15 components, in each subfraction, in the mol. wt. range 12 000–200 000, including a prominent band of approx. mol. wt. 46 000, which has been identified to be actin. Qualitative as well as possible quantitative differences were apparent in that MII contained three components in addition to those present in MI.

6. Analysis of the periodate-Schiff staining components by sodium dodecyl sulphate-polyacrylamide gel electrophoresis demonstrated the presence of 4 major glycoproteins in both subfractions with apparent mol. wt. ranging from approx. 95 000 to 150 000; in addition two minor components were also present. Further, a very fast-migrating band, which did not stain with Coomassie blue, was observed in both MI and MII and probably represents lipid material.

INTRODUCTION

The haemostatic activities of blood platelets have been well documented (for example, refs. 1 and 2) and their most important function is the formation of a stable white thrombus to seal injury sites in blood vessel walls. These activities depend upon the responsiveness of the surface membranes to stimuli provided by adjacent platelets, vascular endothelial cells or connective tissue exposed at the damaged site. Examples of effectors providing these stimuli are ADP, certain biogenic amines such as 5-hydroxytryptamine and the catecholamines, collagen fibres and thrombin.

At present there is little information concerning the molecular nature of the platelet surface membrane, particularly with reference to the protein or glycoprotein complexes which may act as receptors for the various haemostatic stimuli, as transducers to initiate the intracellular events leading to the release reaction, or which may be involved in the uptake of the various compounds which platelets absorb e.g. 5-hydroxytryptamine [3]. One reason for this is that studies on platelet membranes have been hampered by difficulties in preparing fractions derived solely from the platelet surface and free from the intracellular membranes, the open canalicular system and the dense tubular system, which may be derived from the megakaryocyte endoplasmic reticulum [4].

Several procedures have been described [5–8] for the subcellular fractionation of platelets by which it is possible to obtain fractions enriched with membrane vesicles and essentially free from contamination by the various granular organelles. However, it is usually recognised that such preparations are "mixed membrane" fractions, containing fragments not only of the surface membrane, but also of the intracellular membranes and probably also membranes of disrupted granules [9]. A further complication is the lack of clearly identified marker enzyme activities for the various platelet membrane structures, although bis-(*p*-nitrophenyl) phosphate phosphodiesterase activity has been reported to be surface membrane-associated in human platelets [10, 11].

In a preliminary communication [12] we described a procedure, using the "B14" zonal rotor, for the subfractionation of a platelet mixed membrane preparation into two clearly separated subpopulations of membrane vesicles. The basis for this subfractionation was the separation of two distinct phosphodiesterase activities, which were both enriched approx. 3-fold (with respect to the homogenate) in the mixed membrane fraction; the lighter subfraction showed considerable enrichment with bis-(*p*-nitrophenyl) phosphate phosphodiesterase activity and the denser subfraction was enriched similarly with phosphodiesterase activity to 5′-dTMP-*p*-nitrophenyl ester. In addition, by specific surface membrane labelling techniques, including the use of ^{125}I-labelled antiplatelet membrane antibody and a lactoperoxidase-catalysed radioiodination procedure [13], we showed that the less dense vesicles (i.e. those associated with bis-(*p*-nitrophenyl) phosphate phosphodiesterase activity) were of surface origin. Since the higher density vesicles were not labelled, we believe them to be derived from intracellular membrane structures.

In this paper we present results which substantiate the separate identities of these pig platelet membrane subfractions, based on assays for enzyme activities, chemical analyses and electrophoretic separations of the polypeptides and glycoproteins from sodium dodecyl sulphate-solubilised material.

MATERIALS AND METHODS

Preparation and homogenisation of platelets

Platelets were isolated from freshly collected pig blood, mixed in a volume ratio of 4 : 1 with an anticoagulant solution of 1.5 % (w/v) disodium EDTA in 0.85 % NaCl by the procedure described previously [8].

The platelets were weighed and suspended in 0.3 M sucrose, buffered with 5 mM Tris/HCl, pH 7.4, to a ratio of 2 ml sucrose per g wet wt. of cells. The suspension was then homogenised in an M.S.E. top-drive blender operated at top speed for five 1-min periods with 1 min between each homogenisation period for cooling. The lysate was centrifuged at $3000 \times g_{av}$ for 20 min at 4 °C, the supernatant retained and the pellet of unbroken cells and debris resuspended in 1 ml 0.3 M sucrose per g of cells and rehomogenised. After further centrifugation ($3000 \times g_{av}$, 20 min at 4 °C), the final pellet was discarded and the two supernatants combined. These pooled supernatants then constituted the starting homogenate for all subsequent subfractionations.

Preparation of platelet whole membrane fractions

A platelet whole membrane fraction was prepared by a modification of the tube density gradient procedure of Harris and Crawford [8]. Platelet homogenate (approx. 12 ml per tube) was layered on to sucrose gradients prepared in 40 ml polycarbonate tubes, by successively layering 1 ml 2.0 M sucrose, 4 ml each of 1.6, 1.4, 1.2, 1.0 and 0.8 M sucrose and finally 2 ml 0.6 M sucrose. All sucrose solutions were buffered with 5 mM Tris/HCl, pH 7.4. The tubes were centrifuged in an M.S.E. 3×43 ml swing-out rotor at $50\,000 \times g_{av}$ for 3 h at 4 °C.

Platelet membrane vesicles located in the upper of the two particulate zones (corresponding to zone B of Harris and Crawford [8]) and this membrane fraction was removed by pasteur pipette with care to avoid contamination by soluble phase. Usually fractions from 6 tubes were pooled and diluted 2 : 1 (v/v) with 5 mM Tris/HCl, pH 7.4, before loading on to the zonal rotor.

Preparation of platelet membrane subfractions by zonal centrifugation

The platelet membrane suspension was subfractionated using a sucrose linear density gradient in an M.S.E. "B14" titanium zonal rotor, operated in the M.S.E. Super Speed "75" ultracentrifuge. All sucrose solutions were prepared in 5 mM Tris/HCl, pH 7.4.

A 60 ml sample of the whole membrane suspension was applied to approx. 500 ml of a linear gradient prepared from 18 % and 40 % (w/w) sucrose, followed by 25 ml 40 % (w/w), with a cushion of approx. 25 ml 60 % (w/w) sucrose. The rotor was then operated at 47 000 rev./min for 18 h at 4 °C, after which 20-ml fractions were collected, by displacing the gradient with 60 % (w/w) sucrose.

The membrane subfractions were then obtained by pooling, respectively, the zonal fractions containing 24–28 % (w/w) sucrose (density range 1.10–1.12) for subfraction MI, and 30–34 % (w/w) sucrose (density range 1.129–1.150) for subfraction MII. The pooled fractions were diluted 1 : 1 with 5 mM Tris/HCl pH 7.4 and centrifuged at $100\,000 \times g_{av}$ for 60 min at 4 °C. The membrane pellets were finally resuspended in 0.3 M sucrose for enzyme analysis or in 5 mM Tris/HCl, pH 7.4 for chemical analysis.

Enzyme assays

The assays were designed so that samples of zonal fractions, containing high concentrations of sucrose, were diluted sufficiently to avoid any interference by sucrose [14] and incubation temperatures were 37 °C unless otherwise stated. The assays were performed at pH optima previously determined using platelet homogenates.

Three phosphodiesterase (EC 3.1.4.1) activities were assayed as follows, using *p*-nitrophenyl derivatives: up to 0.2 ml of diluted fraction was incubated in a final volume of 1.0 ml containing (a) 1.0 μmol bis-(*p*-nitrophenyl) phosphate (Sigma Ltd.) and 0.2 mmol sodium acetate buffer, pH 5.5; (b) 1.0 μmol 5′-dTMP-*p*-nitrophenyl ester (Boehringer Corporation) and 0.8 ml of a buffer system consisting of 3 mmol sodium acetate, 120 mmol sodium succinate, 120 mmol sodium maleate and 120 mmol Tris per l, adjusted with HCl to pH 7.9; (c) 0.5 μmol 3′-dTMP-*p*-nitrophenyl ester (Sigma Ltd.) and 0.8 ml of the acetate/succinate/maleate/Tris/HCl buffer, pH 7.0. In all assays the released *p*-nitrophenol was determined spectrophotometrically at 410 nm.

Cyclic AMP phosphodiesterase (EC 3.1.4.c) was assayed as follows: 0.1 ml of sample in a final volume of 0.5 ml containing 0.3 ml of acetate/succinate/maleate/Tris/HCl buffer, pH 8.5, 5 μmol $MgCl_2$ and 1.25 μmol cyclic AMP. The reaction was stopped by immersion in boiling water for 3 min and after cooling and preincubation at 37 °C for 5 min, 0.05 ml 5′-nucleotidase (Sigma Ltd., grade II, 2 mg/ml in pH 8.5 buffer) was added. After further 20 min incubation, 0.95 ml 15 % (w/v) trichloroacetic acid was added, and after centrifuging, 1 ml samples of the supernatants were assayed for inorganic phosphate [15].

Two acid phosphatase (EC 3.1.3.2) activities were measured: (a) by the fluorimetric procedure of Robinson and Willcox [16] in a final volume of 1.0 ml containing 0.2 mmol sodium acetate buffer, pH 5.5 and 0.2 μmol 4-methylumbelliferyl phosphate (Koch-Light Laboratories Ltd.); (b) in a final volume of 1.0 ml containing 0.2 mmol sodium acetate buffer, pH 6.0 and 10 μmol *p*-nitrophenyl phosphate, with the released *p*-nitrophenol determined at 410 nm.

N-Acetyl-β-glucosaminidase (EC 3.2.1.30), β galactosidase (EC 3.1.1.23) and β-glucuronidase (EC 3.2.1.31) were assayed by modifications of the procedures of Robinson et al. [17] using 4-methylumbelliferyl derivatives (Koch-Light Laboratories Ltd.) as substrates: in a final volume of 1.0 ml containing 0.9 ml of the acetate/succinate/maleate/Tris/HCl buffer described above and either 0.25 μmol *N*-acetyl-β-glucosaminide, 0.5 μmol β-galactoside or 0.1 μmol β-glucuronide at pH 4.1, 4.1 and 5.1 respectively.

Succinate dehydrogenase (EC 1.3.99.1) was measured by the method of Pennington [18] and lactate dehydrogenase (EC 1.1.1.27) was assayed at 25 °C by a procedure based on that of Wroblewski and La Due [19].

Polyacrylamide gel electrophoresis

Electrophoresis was carried out on 7.5 % (w/v) polyacrylamide stick gels (9.0×0.5 cm) prepared in a buffer system of 0.4 M boric acid adjusted with Tris (approx. 0.1 M) to pH 7.0 and containing 0.1 % sodium dodecyl sulphate. The same buffer was used in the electrode compartments and the gels were run at 3 mA/gel until the marker dye (bromophenol blue) located approx. 0.5–1.0 cm from the anodic end. Membrane samples and protein standards were solubilised in Tris/

borate buffer, containing 4 % (w/v) sodium dodecyl sulphate, 8 M urea and 0.1 M 2-mercaptoethanol, by heating in a boiling water bath for 10 min.

To indicate the separated polypeptides, gels were stained with 0.1 % Coomassie brilliant blue R in methanol/acetic acid/water (45 : 10 : 45, by vol.) and destained in methanol/acetic acid/water (1 : 1 : 8, by vol.). Glycoproteins were stained by a periodic acid-Schiff procedure [20].

The following protein preparations were used as standards to calibrate the gels for molecular weight determination and were purchased from Sigma Ltd., except rabbit skeletal muscle myosin (mol. wt. 200 000) which was prepared by the method of Trayer and Perry [21]: phosphorylase *a* (mol. wt. 94 000), catalase (bovine liver, mol. wt. 60 000), ovalbumin (grade V, mol. wt. 43 000), trypsin (type III, mol. wt. 23 500) and ribonuclease A (type I-A, mol. wt. 12 700). Relative mobility was calculated as the ratio of the distance travelled by the protein, compared with that travelled by the marker dye, and a linear relationship was established between relative mobility and log molecular weight.

Chemical analyses

Protein was determined by the method of Lowry et al. [22] using bovine serum albumin for the standards. Zonal fractions were diluted 1 : 10 to overcome interference by sucrose, and compared with standards prepared in 3 % (w/w) sucrose.

All subsequent chemical analyses on membrane fractions were conducted on material that had been extensively dialysed against 1 mM Tris/HCl, pH 7.4, to remove sucrose. Lipids were extracted with chloroform/methanol (2 : 1, by vol.) [23]; the cholesterol contents were determined by the method of Crawford [24] and phospholipid phosphorus measured by the procedure of Chalvardjian and Rudnicki [25] after perchloric acid digestion.

Individual phospholipids were separated by thin-layer chromatography on Kieselgel H (E. Merck) in a solvent system containing chloroform/methanol/acetic acid/water (50 : 25 : 8 : 4, by vol.) at 4 °C. The component phospholipids were visualised after treatment with iodine and identified by comparison with standards (Lipid Products Ltd.) chromatographed simultaneously. After allowing the iodine to evaporate, the areas of absorbant containing the lipid classes were scraped directly into digestion tubes and, after digestion with perchloric acid, the inorganic phosphorus contents were determined [26]. Results are expressed as percentages of total phospholipid recovered and are the means of duplicate analyses.

The sialic acid contents were assayed by the method of Aminoff [27] using the correction factor of Warren [28] after samples had been hydrolysed with 50 mM H_2SO_4 at 80 °C for 1 h; *N*-acetylneuraminic acid was used as a standard. The total hexosamine contents were determined [29] after hydrolysis of the membrane fractions with 6 M HCl at 100 °C for 4 h in sealed tubes; an equimolar mixture of glucosamine/HCl and galactosamine/HCl was used as a standard. Neutral sugars were estimated after hydrolysis of samples in 2 M trifluoroacetic acid at 100 °C for 6 h in sealed tubes, followed by separation as their borate complexes, on a Jeol JLC-6AH sugar analyser by a procedure modified from Hough et al. [30]. Amino acid analysis was carried out by the procedure described by Wilkinson et al. [31], using a Locarte analyser.

The density of sucrose in the zonal fractions was determined from the refractive index measured by means of an Abbe refractometer.

Electron microscopy

Membrane samples were fixed in 6.25 % (v/v) glutaraldehyde in 0.2 M cacodylate buffer, pH 7.4, for 30 min, washed in cacodylate buffer for 1 h and post fixed in 1 % OsO_4 in 0.285 M barbitone sodium/sodium acetate/HCl buffer, pH 7.4, for 2 h. The fixed pellets were washed in barbitone/acetate buffer, dehydrated through graded alcohols and propylene oxide and embedded in Araldite. Ultra-thin sections were stained with uranyl acetate for 15 min and viewed with a Philips model 301 electron microscope.

RESULTS

Distribution of enzyme activities in zonal fractions

The distribution profiles for protein and several enzyme activities across the zonal gradient after centrifugation of platelet membrane material are shown in Fig. 1. It can be seen that the crude membrane fraction had been resolved into three major protein peaks; peak 1 at the lower density end of the gradient probably contained residual soluble phase protein and located between 15 and 23 % (w/w) sucrose (density range 1.061–1.095); peak 2 occurred between 24 and 35 % (w/w) sucrose (density 1.100–1.154) and peak 3 at the end of the gradient, close to the cushion layer, between 40 and 50 % (w/w) sucrose (density 1.178–1.232).

The distribution of bis (*p*-nitrophenyl) phosphate phosphodiesterase activity showed a major peak at the lower density margin of protein peak 2, with maximum activity in the fraction containing 25–26 % (w/w) sucrose (density 1.105–1.110). The phosphodiesterase activity towards the 5′-thymidine substrate, however, had a distribution which was clearly distinct from the bis-(*p*-nitrophenyl) phosphate phosphodiesterase activity, with maximum activity occurring at 31–32 % (w/w) sucrose (density 1.133–1.139).

Of the other enzyme activities assayed, acid *p*-nitrophenyl phosphatase and 3′-dTMP-*p*-nitrophenyl ester phospodiesterase showed profiles with the highest activities in fractions containing the bis-(*p*-nitrophenyl) phosphate phosphodiesterase peak, with considerable activity also associated with protein peak 3; the 3′-dTMP phosphodiesterase also exhibited significant activity in the region of the 5′-dTMP-*p*-nitrophenyl ester activity peak. 4-Methylumbelliferyl phosphatase activity was distributed quite evenly between the three protein peaks. These results suggest that the protein at the denser end of the gradient (peak 3) was probably derived from granular material. Similarly, the distributions of lactate dehydrogenase and cyclic AMP phosphodiesterase activities, which were predominantly associated with protein peak 1, indicate that the protein at the light end of the gradient was derived from residual soluble phase. *N*-acetyl-β-glucosaminidase and β-galactosidase activities were also associated with protein peak 1, with the latter enzyme showing considerable activity in the region of the 5′-dTMP derivative phosphodiesterase peak. β-glucuronidase activity was principally associated with the bis-(*p*-nitrophenyl) phosphate-phosphodiesterase peak.

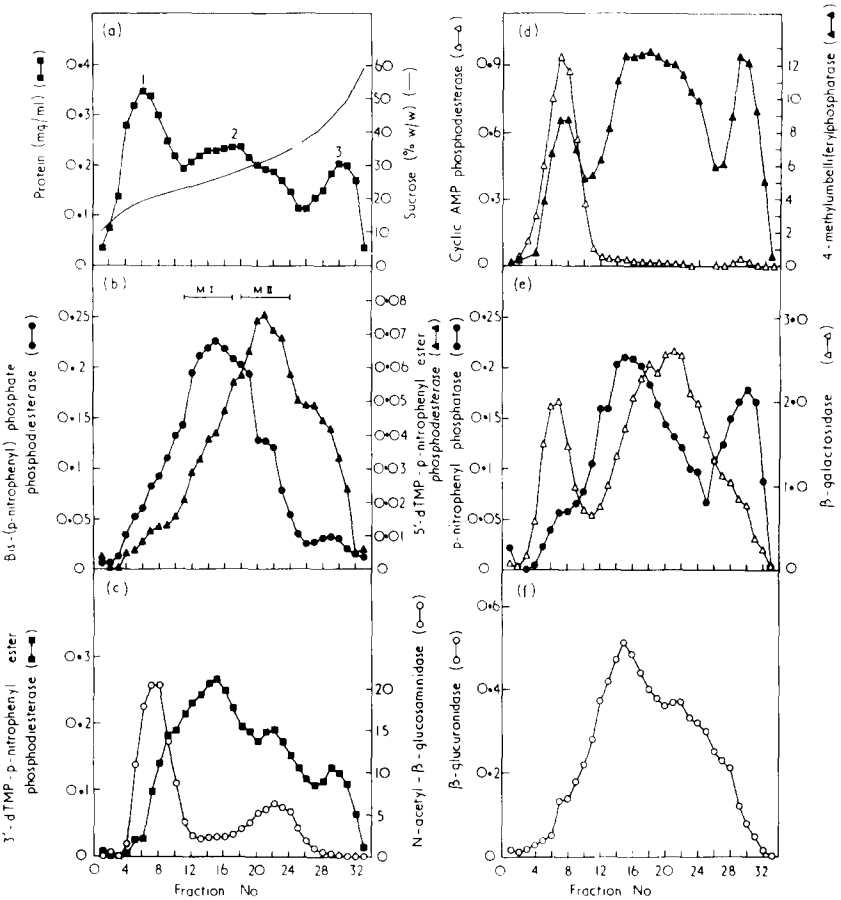

Fig. 1. Distribution of protein and enzyme activities in fractions obtained by zonal subfractionation of platelet membranes. Details of the preparative procedures are given in the Methods section. The protein distribution is plotted as mg/ml fraction and enzyme profiles are expressed as μmol product released/h/ml fraction (nmol/h/ml for 4-methyl umbelliferyl phosphate, *N*-acetyl-β-glucosaminidase, β-galactosidase and β-glucuronidase). The fractions pooled to prepare membrane subfractions MI and MII are indicated in (b) by the solid lines.

Enzyme activities in the two membrane subfractions

The membrane subfractions MI and MII were obtained by pooling, respectively, the zonal fractions around the bis-(*p*-nitrophenyl) phosphate phosphodiesterase activity peak (24–28 % (w/w) sucrose) and those around the 5'-dTMP derivative activity peak (30–34 % (w/w) sucrose), as described in the methods section. Tables I and II list the specific activities and percentage total activities for several enzymes in the whole membrane preparation (M) from the preliminary fractionation and the two membrane subfractions MI and MII obtained from the zonal gradient.

In subfraction MI, the bis-(*p*-nitrophenyl) phosphate phosphodiesterase activity was enriched 7–8 fold relative to the homogenate, and contained approx. 20 % of the homogenate activity. Similarly, subfraction MII showed a 7–8 fold

TABLE I

SPECIFIC ENZYME ACTIVITIES IN PLATELET MEMBRANE FRACTIONS

The preparation of the original membrane fraction and the two membrane subfractions is described in the text. The values are specific activity expressed as μmol of product released/h/mg protein (nmol/h/mg protein for *N*-acetyl-β-glucosaminidase, β-galactosidase and β-glucuronidase) ±S.D. in the homogenate (H), the original membrane fraction (M) and the two membrane subfractions (MI and MII). The values in parentheses refer to the enrichment of enzyme activity relative to the homogenate; *n* refers to the number of preparations assayed.

	n	Specific activity			
		H	M	MI	MII
Bis-(*p*-nitrophenyl) phosphate phosphodiesterase	3	0.109 ± 0.003	0.363 ± 0.113 (3.33)	0.791 ± 0.139 (7.26)	0.351 ± 0.107 (3.22)
5′-dTMP-*p*-nitrophenyl ester phosphodiesterase	3	0.058 ± 0.009	0.174 ± 0.037 (3.00)	0.125 ± 0.039 (2.16)	0.431 ± 0.027 (7.43)
3′-dTMP-*p*-nitrophenyl ester phosphodiesterase	3	0.655 ± 0.069	0.679 ± 0.046 (1.04)	1.216 ± 0.032 (1.86)	0.807 ± 0.10 (1.23)
Cyclic AMP phosphodiesterase	1	1.586	0.344 (0.22)	0.146 (0.09)	0.136 (0.09)
4-Methylumbelliferyl phosphatase	2	0.034 ± 0.004	0.032 ± 0.002 (0.94)	0.051 ± 0.006 (1.50)	0.035 ± 0.012 (1.03)
p-Nitrophenyl phosphatase	3	0.720 ± 0.294	0.842 ± 0.371 (1.17)	1.095 ± 0.310 (1.52)	0.738 ± 0.266 (1.03)
N-Acetyl-β-glucosaminidase	3	47.0 ± 9.0	27.0 ± 2.0 (0.57)	7.0 ± 2.0 (0.15)	19.0 ± 5.0 (0.40)
β-Galactosidase	3	6.4 ± 1.4	5.3 ± 1.1 (0.82)	5.6 ± 1.2 (0.88)	11.4 ± 2.0 (1.78)
β-Glucuronidase	3	1.01 ± 0.11	1.26 ± 0.10 (1.25)	2.22 ± 0.31 (2.20)	1.30 ± 0.06 (1.29)
Succinate dehydrogenase	3	0.055 ± 0.013	0.001 ± 0.001 (0.02)	0	0
Lactate dehydrogenase	1	12.21	3.33 (0.27)	1.71 (0.14)	1.23 (0.10)

TABLE II

PERCENTAGE DISTRIBUTION OF PROTEIN AND ENZYME ACTIVITIES IN THE PLATELET MEMBRANE FRACTIONS

The details of the preparation of the original membrane fraction and the two subfractions are described in the text. M, MI and MII refer to the original membrane fraction, and the two subfractions respectively. The values presented are percentages of homogenate total activity and protein ±S.D. recovered in the membrane fractions; *n* refers to the number of preparations assayed. Total recoveries for enzyme activities and protein in all the fractions at all stages in the preparative procedure were in the range 90–110 % of the homogenate activity and protein.

	n	Percentage distribution		
		M	MI	MII
Protein	3	22.9 ± 5.7	4.1 ± 1.3	4.1 ± 1.0
Bis-(*p*-nitrophenyl)phosphate phosphodiesterase	3	46.3 ± 3.2	18.5 ± 0.6	10.3 ± 1.1
5′-dTMP-*p*-nitrophenyl ester phosphodiesterase	3	50.7 ± 0.8	8.6 ± 1.7	19.6 ± 4.6
3′-dTMP-*p*-nitrophenyl ester phosphodiesterase	3	23.0 ± 7.9	7.6 ± 2.5	4.7 ± 1.6
Cyclic AMP phosphodiesterase	1	4.3	0.25	0.20
4-Methylumbelliferyl phosphatase	2	15.9 ± 3.1	4.7 ± 0.8	4.2 ± 0.7
p-Nitrophenyl phosphatase	3	19.2 ± 1.5	5.6 ± 1.5	4.2 ± 0.4
N-Acetyl-β-glucosaminidase	3	12.9 ± 1.9	1.4 ± 0.3	2.7 ± 0.8
β-Galactosidase	3	15.5 ± 0.4	2.6 ± 0.4	5.5 ± 0.7
β-Glucuronidase	3	17.1 ± 2.3	4.6 ± 0.6	3.5 ± 1.2
Succinate dehydrogenase	3	0.5 ± 0.6	0	0
Lactate dehydrogenase	1	4.1	0.5	0.3

enrichment for the enzyme acting on 5′-dTMP-*p*-nitrophenyl phosphodiester, with approx. 20 % of the homogenate activity.

The 3′-dTMP phosphodiesterase, β-glucuronidase and the two phosphatase activities all showed slight enrichment in subfraction MI, but represented only small percentages of the homogenate activities of these enzymes. β-galactosidase showed up to a 2-fold enrichment in subfraction MII, but again this represented only a very small amount of the homogenate activity. There was negligible activity of cyclic AMP phosphodiesterase and lactate dehydrogenase in both of the subfractions and succinate dehydrogenase activity could not be detected in either MI or MII.

Morphology of the membrane subfractions

Electron microscopy of the two membrane subfractions revealed that both were composed of small membrane vesicles, up to 0.5 μm in diameter. There were no obvious morphological differences between them, either in size or character of vesicle and both fractions resembled the original whole membrane fraction.

Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis, in the presence of sodium dodecyl sulphate, of the two membrane subfractions, treated with sodium dodecyl sulphate, urea and 2-mercaptoethanol, revealed 12–15 polypeptide bands in both subfractions (Fig. 2). Most of these bands were clearly resolved and had comparable mobilities in the two subfractions, corresponding to polypeptides with approx. mol. wt. of 12 000, 15 000, 28 000, 46 000, 54 000, 64 000, 75 000, 80 000, 95 000, 110 000,

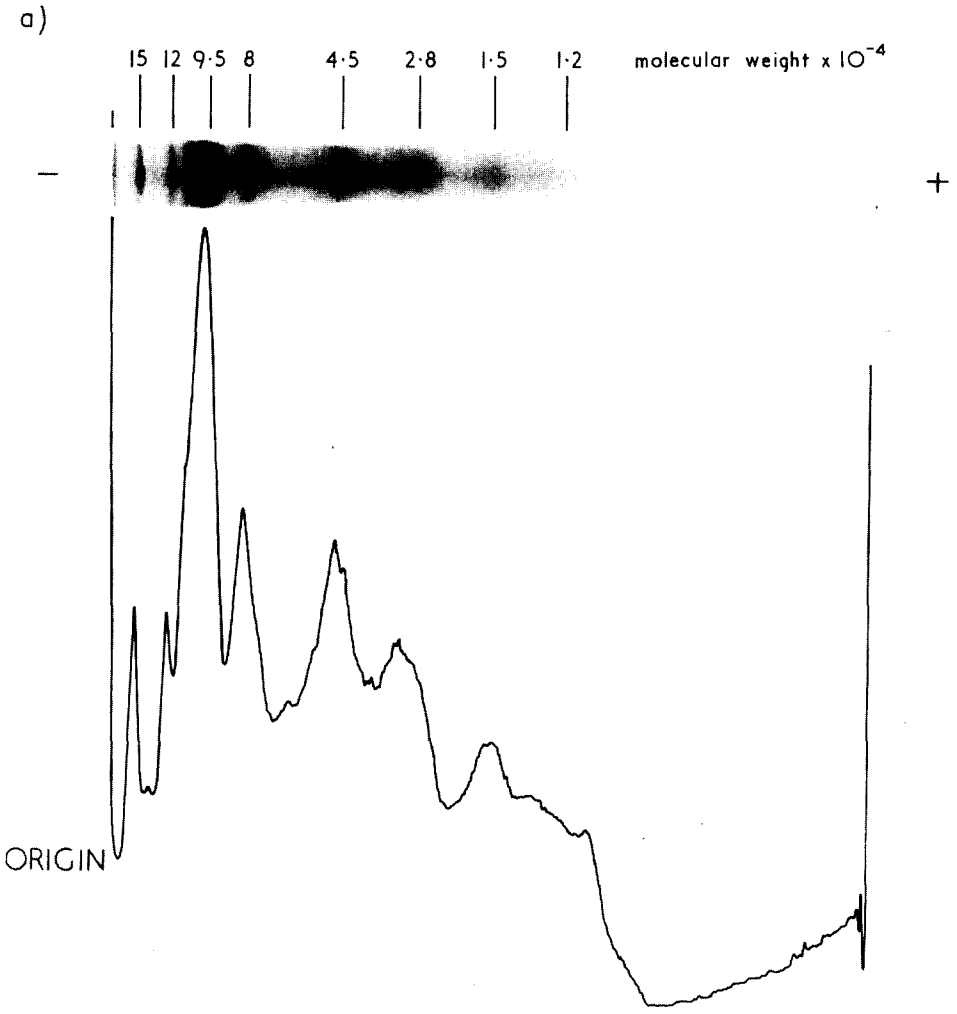

Fig. 2a. For legend see opposite page.

120 000 and two closely adjacent bands with mol. wt. between 140 000 and 150 000. In addition, a polypeptide of at least 200 000 daltons was present in both MI and MII, but had not migrated into the gel matrix. There were, however, qualitative as well as possible quantitative variations between the polypeptide profiles of the two subfractions, in that MII also contained polypeptides of apparent mol. wt. of 39 000, 130 000 and 135 000 which were not represented in MI.

Of particular interest is the presence of the approx. 46 000 dalton component in the membrane subfraction. This co-electrophoresed with purified rabbit skeletal muscle actin and pig platelet actin and, as discussed later, there is now considerable evidence to suggest that actin is firmly associated with platelet membranes.

Fig. 3 shows the results obtained from polyacrylamide gels stained with the periodic acid-Schiff reagent. Four major glycoprotein bands migrating with mo-

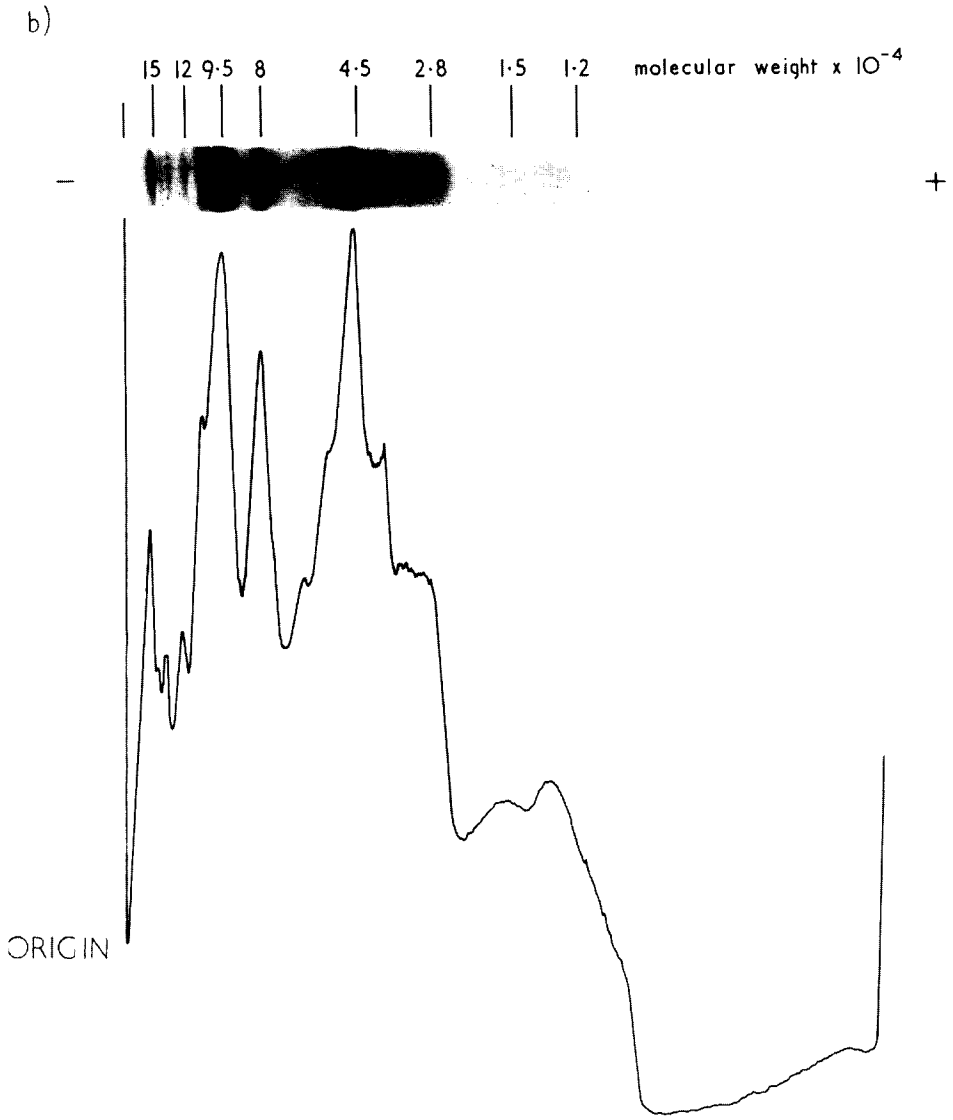

Fig. 2. Analysis of the polypeptide components of platelet membrane subfractions by electrophoresis on sodium dodecyl sulphate-polyacrylamide gels. Membrane subfractions were prepared and solubilised as described in the text; samples applied contained approx. 100 μg of protein. Details of the electrophoretic procedure are given in the methods section. Polypeptide bands were stained with Coomassie brilliant blue R and densitometry traces were obtained at 570 nm using a Gilford model 2000 spectrophotometer with gel-scanning attachment. (a) Membrane subfraction MI; (b) membrane subfraction MII.

bilities corresponding to apparent mol. wt. of approx. 95 000, 110 000, 120 000 and 140–150 000 were present in both subfractions. In addition, periodate-Schiff positive bands with apparent mol. wt. of 80 000 and 65 000 were present as minor components in both MI and MII. Further, a fast-migrating, intensely staining periodate-Schiff

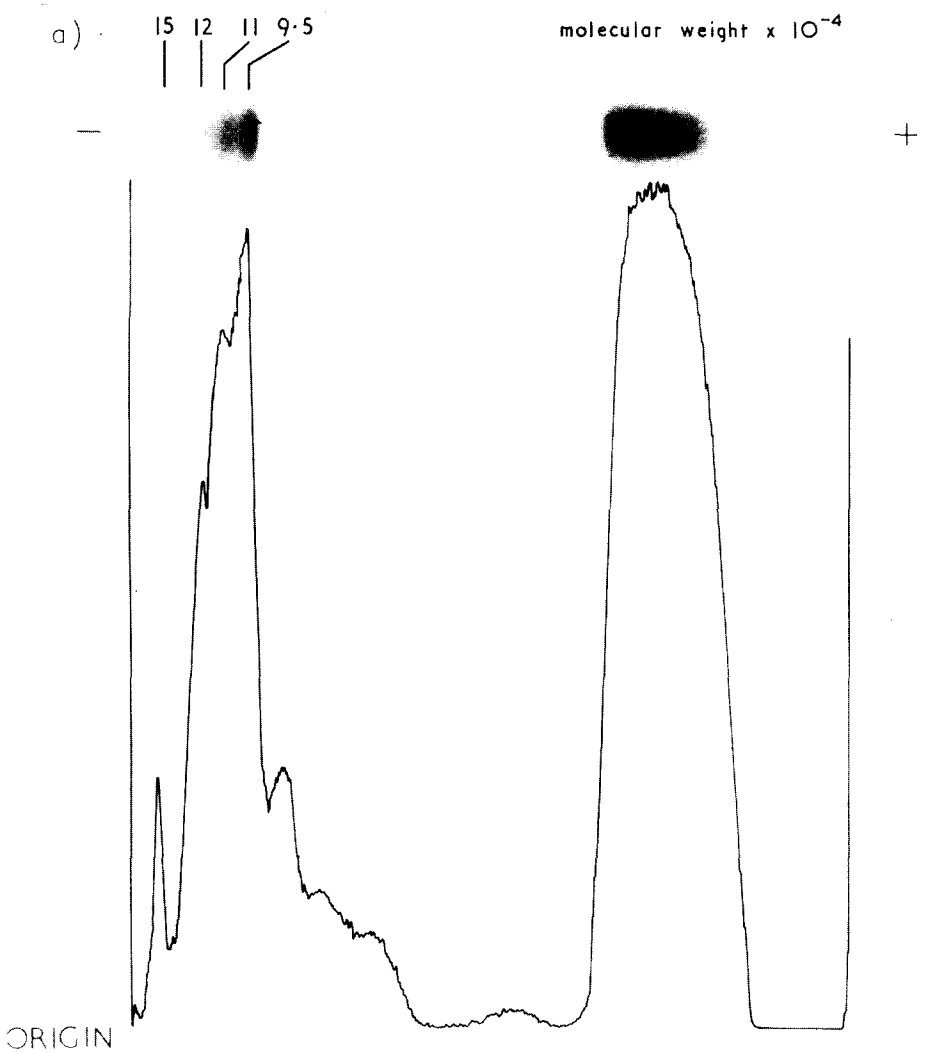

Fig. 3a. For legend see opposite page.

positive band, running in front of the most anodic Coomassie blue-staining component, was observed in both subfractions. However, this fast-moving band was not seen in membrane samples from which lipid had been removed by chloroform/methanol extraction [23].

Chemical composition

Details of the chemical analyses of the membrane subfractions are given in Tables III and IV. Protein, phospholipid and cholesterol were the major constituents of both subfractions, with some carbohydrate moieties also present. There are significant differences between the compositions of MI and MII; MII had a greater content of protein (approx. 65 % by weight) than MI (approx. 50 %), so that the

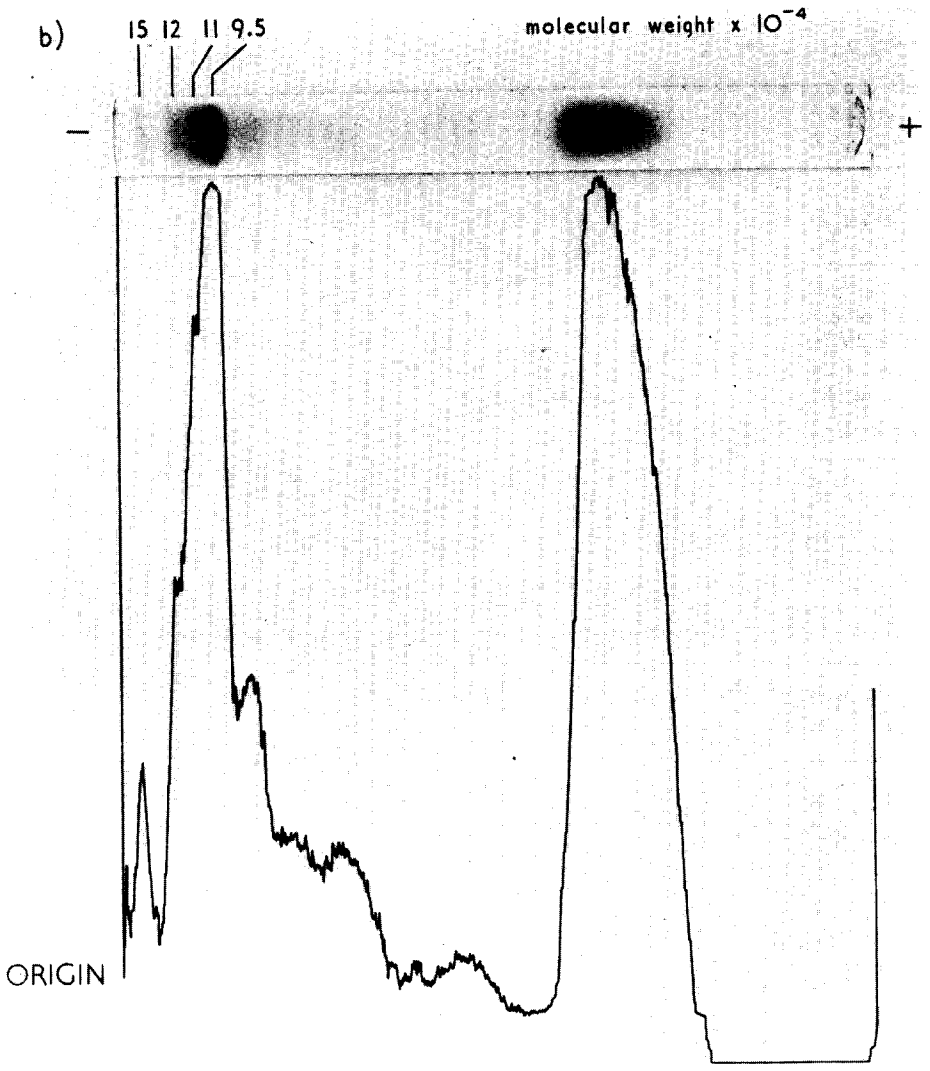

Fig. 3. Analysis of the periodate-Schiff reagent staining components of platelet membrane subfractions by electrophoresis on sodium dodecyl sulphate-polyacrylamide gels. The preparation and solubilisation of the membrane subfractions and the electrophoretic procedure are described in the methods section; samples contained approx. 200 μg of protein. Gels were stained with periodate-Schiff reagent and densitometry traces obtained at 550 nm. (a) Membrane subfraction MI; (b) membrane subfraction MII.

phospholipid : protein and cholesterol : protein ratios of MI were approx. twice those of MII. This observation is consistent with subfraction MI having a lower buoyant density than MII. Subfraction MI also had a slightly higher cholesterol : phospholipid molar ratio (0.82) than MII (0.71).

Analyses of the phospholipid classes present in the membranes revealed closely similar patterns for both subfractions, the major difference being that MII

TABLE III

CHEMICAL ANALYSIS OF THE PLATELET MEMBRANE SUBFRACTIONS

Results are expressed as μg/mg of membrane protein ± S.D. MI and MII refer to the two subfractions and n refers to the number of preparations analysed. The molecular weight of phospholipid was taken as 780.

	n	Content (μg/mg of protein)	
		MI	MII
Total phospholipid	5	517 ± 57	264 ± 95
Total cholesterol	5	209 ± 17	95 ± 43
Sialic acid	3	11.7 ± 4.5	6.3 ± 1.0
Total hexosamine	2	80.8 ± 26.6	46.6 ± 9.5
Total neutral sugars	3	54.4 ± 20.5	53.3 ± 22.9
Cholesterol/phospholipid molar ratio	5	0.82 ± 0.08	0.71 ± 0.12

TABLE IV

AMINO ACID ANALYSIS OF PLATELET MEMBRANE SUBFRACTIONS

The results of two separate analyses are presented (a and b), and are expressed as residues/100 residues of amino acid. The values represent the means of 24- and 72-h hydrolyses, with the values for serine and threonine corrected for losses during hydrolysis. n.d. = not determined.

	MI		MII	
	a	b	a	b
Asp	10.7	10.9	10.1	10.6
Thr	5.7	6.2	5.8	6.5
Ser	9.0	9.0	8.1	8.1
Glu	10.7	11.1	11.2	11.4
Pro	5.1	6.1	5.5	6.7
Gly	8.0	7.6	7.9	7.3
Ala	7.7	7.2	8.3	7.5
Half-Cys	n.d.	n.d.	n.d.	n.d.
Val	6.8	6.9	6.7	7.0
Met	1.3	1.0	1.7	1.4
Ile	4.8	4.3	4.9	4.5
Leu	10.0	9.2	9.8	9.1
Tyr	3.7	3.5	3.3	2.6
Phe	4.1	3.5	4.0	3.4
His	2.0	2.5	2.0	2.6
Lys	5.8	6.1	6.1	6.6
Arg	4.6	4.9	4.6	4.7
Trp	n.d.	n.d.	n.d.	n.d.

showed a slightly higher phosphatidyl ethanolamine content (29.4 % of the total recovered) than MI (25.9 %), with a correspondingly lower content of phosphatidyl choline (MII, 30.2 %; MI, 33.1 %). Sphingomyelin represented 25 % of the recovered phospholipid and phosphatidylserine and phosphatidylinositol together accounted for the remaining 15–16 % of both MI and MII. Cardiolipin was not detected in either subfraction, and the analysis also demonstrated that cerebrosides were absent.

Both membrane subfractions contained 7–8 % carbohydrate, with MI having twice the content of sialic acid and total hexosamines expressed per unit weight of protein than MII. The total neutral sugar contents of the two subfractions were very similar, with galactose, glucose and mannose being the major components and smaller amounts of fucose; ribose was detected in only trace amounts in both MI and MII.

The results of the amino acid analyses are presented in Table IV, from which it can be seen that both subfractions had essentially similar amino acid compositions. A separate analysis also demonstrated the presence of 3-methyl histidine in both subfractions.

DISCUSSION

In addition to the surface membrane, platelets contain two well-defined intracellular membrane complexes, the open canalicular system and the dense tubular system [4]. Several authors [5–8] have described procedures, using tube density gradient centrifugation, for the subcellular fractionation of platelets, by which two major particulate fractions can be obtained, one of which consists predominantly of membrane vesicles, free from contamination by granular organelles. This fraction corresponds essentially to the "microsomal" fractions of other tissues and is almost certainly derived from both the surface and intracellular membrane systems of the platelet.

In our studies of the fractionation of pig platelet homogenates using the "B14" zonal rotor [32], we established that fractions containing platelet membrane vesicles showed considerable enrichment for two phosphodiesterase activities (towards bis-(*p*-nitrophenyl) phosphate and 5′-dTMP-*p*-nitrophenyl ester) and that these two activities consistently displayed slightly different distributions in the zonal gradients. Using the conditions described in this paper the platelet mixed membrane fraction, prepared by tube gradient centrifugation, can be resolved further into two distinct subfractions by zonal centrifugation. The basis of this subfractionation lies in the separation of the two phosphodiesterase activities, each of which showed 7–8 fold enrichment, with respect to the homogenate, in the respective subfractions, i.e. bis-(*p*-nitrophenyl) phosphate phosphodiesterase activity in MI, and activity towards the 5′-dTMP derivative in MII.

Other assays indicated further enzymic differences between the two subfractions. Although both MI and MII were free from mitochondrial enzyme activities, they contained small amounts of various hydrolase activities (Tables I and II); i.e. acid phosphatase, 3′-dTMP-*p*-nitrophenyl ester phosphodiesterase and β-glucuronidase locating with sub-fraction MI and β-galactosidase activity with MII. These activities, however, represent only 3–6 % of the total homogenate activity of these hydrolases. It is not clear if this indicates a genuine association of these hydrolase activities with certain platelet membrane structures or there is some contamination by hydrolase-containing granules or whether hydrolases, liberated from granules as a consequence of homogenisation, have adsorbed onto membrane fragments. The finding that the various activities are distributed differently between the two subfractions tends to support the former view, and the dual location of certain hydrolases has been reported for other tissues, for example, β-glucuronidase occurs in kidney

lysosomes and endoplasmic reticulum [33]. Several authors have also commented upon the heterogeneous nature of platelet acid hydrolase-containing elements [34, 35]. The *N*-acetyl-*β*-glucosaminidase activity present in the original mixed membrane fraction appears to be derived from residual soluble phase material, since its distribution profile is essentially the same as that of lactate dehydrogenase and cyclic AMP phosphodiesterase (Fig. 1).

The two platelet membrane subfractions, although showing these enzymic differences, are morphologically indistinguishable in electron micrographs. They can, however, be further distinguished between by cell surface labelling experiments and we have previously reported [12] the results of labelling pig platelets with an ^{125}I-labelled anti-platelet whole membrane antibody and by a lactoperoxidase-catalysed iodination technique. Using these procedures, we showed that membrane subfraction MI (i.e. the fraction with high phosphodiesterase activity to bis-(*p*-nitrophenyl) phosphate) was enriched with surface-orientated components. These results have recently been confirmed (in collaboration with Dr. G. Bullock, Ciba Research Division, Horsham, unpublished work) by morphometric analysis of electron microscopic autoradiographs of whole platelets labelled with anti-membrane antibody, and by light microscopic autoradiographic analysis of the two membrane subfractions isolated from them. The origin of the membrane material of subfraction MII has yet to be established, although these recent results infer an intracellular location.

Barber and Jamieson [10] have reported the isolation of two membrane subfractions from human platelets after lysis by a glycerol loading procedure. In their studies, however, both of the subfractions showed approx. 8-fold enrichment of bis-(*p*-nitrophenyl) phosphate phosphodiesterase activity and since both were labelled by lactoperoxidase iodination [11], it would appear that each contained surface-derived structures. The differences between their subfractionation results and those reported here may represent a species variation or be a consequence of the different homogenisation and fractionation procedures used.

Electrophoresis on sodium dodecyl sulphate-polyacrylamide gels of the membrane subfractions solubilised in sodium dodecyl sulphate/urea/2-mercapto-ethanol revealed further differences between MI and MII. Although the overall distribution patterns of the component polypeptides were similar, there were qualitative differences, as well as possible quantitative variations, in that MII contained polypeptides of apparent mol. wt. 39 000, 130 000 and 135 000 in addition to those present in MI.

Of particular interest is the presence of a polypeptide of apparent mol. wt. 43–46 000 in both subfractions. Recently, we have shown [36] that this component coelectrophoreses with purified skeletal muscle and platelet actins, and that the addition of platelet membrane subfractions to purified rabbit muscle myosin causes an elevation of the Mg^{2+}-dependent adenosine triphosphatase activity, a characteristic property of actins in polymerised form. More recent results [37], involving binding studies with ^{125}I-labelled platelet actin and solubilisation with Triton X-100, identify the 43–46 000 mol. wt. component as actin and indicate a firm association with the platelet membrane structures.

Four major glycoprotein bands were found in both MI and MII, with apparent mol. wt. ranging between 95 000 and 150 000. Previously human platelet

membranes have been reported [38, 39] to contain only three glycoprotein-staining components within this molecular weight range, although a recent paper [40] reported the presence of a fourth glycoprotein. In our study at least two other minor glycoprotein components were also observed. In addition, a fast migrating, periodate-Schiff positive band, which was not stained with Coomassie blue, was present in both subfractions. A similar component has been observed in human platelet membranes [11, 38], although there is some confusion as to whether this band represents glycopeptides or lipid residues. In our studies, this material was completely separated from the other periodate-Schiff positive components into the chloroform layer after chloroform/methanol extraction of the membrane subfractions, indicating it to be lipid in nature.

The chemical analyses of the two membrane subfractions revealed further significant differences; MI contained approx. twice as much cholesterol and phospholipid per unit weight of protein than MII, and the sialic acid and total hexosamine contents, similarly expressed, were greater in MI than MII. The chemical composition of MI compares well with those reported for surface membrane preparations from other tissues e.g. liver [41, 42] and kidney [43]. The amino acid analyses of MI and MII are essentially similar and the profiles resemble those reported for various surface membrane and intracellular membrane preparations from a variety of tissues [44], including the analyses of Barber and Jamieson [10] for human platelet membranes, except that these workers could not detect the presence of methionine. The demonstration that 3-methyl histidine is present in pig platelet membrane subfractions is consistent with our observation that actin is associated with platelet membrane structures; purified pig platelet actin contains approx. 0.6 residues of 3-methyl histidine per mol of actin [45].

Investigations to characterise further the membrane subfractions MI and MII are presently being carried out and attempts are being made to differentially solubilise component polypeptides and glycoproteins of the membranes. A possible involvement of the membrane-associated actin in the platelet's secretory and haemostatic functions is being explored and we are investigating possible natural substrates for the two major phosphodiesterase activities associated with the membrane subfractions.

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