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ISOLATION OF PIG PLATELET MEMBRANES AND GRANULES DISTRIBUTION AND VALIDITY OF MARKER ENZYMES

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SUMMARY

A method for the subcellular fractionation of pig platelet homogenates by sucrose density gradient centrifugation is described. The procedure is simple, highly reproducible and yields two major particulate fractions and a soluble phase. One particulate fraction consists almost entirely of membrane fragments and is relatively free from granule contamination. The other particulate zone contains the platelet granules and mitochondria. The distribution on the gradients of the enzymes lactate dehydrogenase, succinate dehydrogenase, 5′-nucleotidase, leucyl β-naphthylamidase and cholinesterase has been studied and organelle localisation further substantiated by electron microscopy. The degree of solubilisation of certain marker enzymes during homogenisation has been investigated and the parallel release of these enzymes with the soluble phase marker enzyme lactate dehydrogenase, suggests they have a true biphasic location between the soluble and particulate components of the cell. No significant difference was found in the molar ratios of cholesterol to phospholipid in the subcellular fractions but the content of each lipid was twice as high in the membrane fraction as in the granule fraction.

INTRODUCTION

Following the development of better processing techniques in electron microscopy and particularly the use of glutaraldehyde in fixation, it became clear that blood platelets, although not nucleated, contain within their cytoplasm a variety of quite well defined organelles. Of these, the mitochondria and at least two different kinds of granule-like particles predominate. In addition to these structures, a complex of intracellular membranes and a system of microtubles and microfilaments are also now known to be present in the cytoplasmic matrix: The microtubules being particularly evident when pseudopodia are formed and in certain other motile activities of the cell. During the last few years our understanding of the behaviour of whole platelets in phenomena such as adhesiveness, aggregation and the highly specific "release reaction" has been considerably enlarged, but the part played by the various intracellular structures in these activities is by no means certain. The principal difficulty

Abbreviations: DTNB, 5,5-dithiobis-2-nitrobenzoic acid; INT, 2-*p*-iodo phenyl-3-*p*-nitrophenyl-5-phenyltetrazolium chloride.

encountered here, is that in contrast to liver and other tissue, the establishment of optimum and reproducible conditions for the isolation of platelet subcellular particles for study has proved most difficult. Platelets are extremely labile cells and rapidly undergo morphological and structural changes in response to various stimuli so that rapid procedures for isolation and processing for subfractionation are necessary.

The techniques for platelet disruption which have recently been described include freezing and thawing[1,2], sonication[3–5] and both pestle and blender type homogenisation[6–10]. More recently, in a study of five different cell rupture procedures, lysis by glycerol loading has also been a recommended approach[12].

Separation of the subcellular particles from the lysate, sonicate or homogenate has usually been achieved by differential centrifugation[1], or continuous[6,10–12] or discontinuous[7,9] sucrose gradients. A continuous Urograffin gradient has been used by Da Prada and Pletscher[5] for isolating the serotonin storage granules from rabbit platelets. In an earlier study from this department[10], which was also concerned with the intracellular compartmentalisation of endogenous and absorbed serotonin, we described a fractionation procedure based upon the use of a "nil clearance" teflon homogeniser and sucrose density gradients. This procedure, while satisfactory for small volume fractionation, lacked good reproducibility when scaled up for larger homogenate volumes. In the present investigations we have used a blender homogenisation coupled with a sucrose density gradient fractionation which has proved to be highly reproducible and allows the isolation of membrane and granular fractions cleanly separated by an intermediate non-particulate zone. The low density membrane fraction is free from granular organelles and the integrity of the mitochondrial and other granular elements in the higher density sucrose zone seem relatively well preserved. The fractions have been characterised with marker enzymes and by electron microscopy. Since certain enzymes, normally almost entirely organelle associated in other tissues, are found in the soluble phase fractions of the blood platelet and have been commented upon by other workers[6,8] a study has also been made of the extent of solubilisation of these enzymes during the preparative procedures.

METHODS AND MATERIALS

Chemicals

All standard chemicals were of analytical grade where possible and obtained from either British Drug Houses Ltd, or Fisons Ltd. With the following exceptions, all other reagents (enzyme substrates and inhibitors *etc.*) were obtained from Sigma Chemical Co. Ltd.

Lactate dehydrogenase Test Combination was obtained from C. F. Boehringer und Soehne, GmbH, Mannheim, Germany. Eserine (physostygmine) was a gift from Dr C. E. Rowe. Bovine serum albumin was purchased from Armour Pharmaceuticals Ltd. ATP (disodium salt) was obtained from Kyowa Hakko Kagyo Co. Ltd, 1–4 Ohtemachi, Chiyadoku, Tokyo, Japan. This product was found to be almost entirely free from AMP and ADP by column and thin-layer chromatography and the Tris salt was prepared from it by the method of Epstein and Whittam[14]. Centrifugation was performed in an MSE Mistral 6L general purpose, refrigerated centrifuge, or in an MSE Superspeed 40 Ultracentrifuge. Homogenisation was routinely carried out with an MSE top drive blender-type homogeniser (Cat. No. 7700-A.).

Preparation of platelets

Pig blood was collected from the slaughter house. The animal was bled from the throat immediately after electrical killing and the blood allowed to flow freely into 10 l polythene vessels containing approximately one-eighth volume of 1.5% disodium EDTA in 0.15 M NaCl, as anticoagulant. Processing began within 1 h of collection. The blood was centrifuged in 1250 ml polypropylene bottles at $300 \times g$ for 40 min at 4 °C. The upper two-thirds of the supernatant platelet rich plasma was removed by siphoning through wide bore polythene tubing and again centrifuged ($300 \times g$, 20 min). The upper three quarters of the supernatant were then centrifuged, $2200 \times g$, 20 min, to deposit the platelets. The platelets were gently suspended in a solution prepared by mixing 0.154 M NaCl, 0.154 M Tris–HCl buffer (pH 7.4) and 0.077 M disodium EDTA in a volume ratio 90:8:2. This suspension were centrifuged ($300 \times g$, 30 min) to remove any residual red cells. This sequence of low speed red cell spin, high speed platelet deposition, and resuspension was repeated until the platelet suspension was microscopically free from red cells. The yield of platelets varied between 1 and 2 g wet wt/l of whole blood.

Homogenisation and subcellular fraction

The platelets were weighed and resuspended in 0.3 M sucrose containing 0.1 M Tris–HCl (pH 7.4) and 0.001 M EDTA to a total volume of 5 ml for every g of wet cells. The suspension was placed in a vortex flask and homogenised with an MSE blender at full speed for a total blender time of 5 min but with three short intervals for recooling. After homogenisation, the unbroken cells and large cell debris were removed by centrifugation ($5000 \times g$, 30 min at 4 °C). The cell debris pellet was again resuspended in a further volume of the Tris–sucrose–EDTA medium and rehomogenised. This second homogenate, after removal of the cell debris, was combined with the first debris-free homogenate. For the sucrose density gradient centrifugation, 2 ml of the pooled homogenate was carefully layered onto the upper surface of each sucrose gradient. The gradients also contained 0.001 M EDTA and were prepared as discontinuous layers of sucrose of decreasing molarity. Seven layers of equal volume (2 ml) of 2.0 M, 1.8 M, 1.6 M, 1.4 M, 1.2 M, 1.0 M and 0.8 M sucrose were used in MSE clear polycarbonate tubes of 20 ml nominal capacity. The gradients were allowed to diffuse at 4 °C for 16–25 h and a linearity check of these after overnight diffusion and centrifugation revealed only very slight distortion at the low and high density ends of the gradients. Centrifugation was performed in a 3×20 MSE swing out rotor for 90 min at $50000 \times g$. For the larger MSE tubes (nominal capacity 35 ml) for use with the 3×40 swing out rotor, sucrose volumes of 4 ml were used and aliquots of 5 ml homogenate applied. These too, were centrifuged at the same g_{av} for 90 min.

After gradient centrifugation, the fractions were removed successively from the upper surface by a "J" type pasteur pipette with care to avoid cross contamination of the particulate with the non-particulate zones. The appearance of the gradient and the scheme for subfractionation is presented diagramatically in Fig. 1. All fractions taken from the gradient were immediately diluted with water to a sucrose concentration between 0.25 and 0.3 M and the major particulate zones B and D centrifuged ($100000 \times g$, 4 °C, 60 min) to deposit the particles, giving subfractions, B particulate and B soluble (B_p and B_s) from the upper zone and the corresponding

subfractions D_p and D_s from the lower particulate zone. The deposited particles were resuspended in 0.3 M sucrose in 0.1 M Tris–HCl buffer (pH 7.4) containing 0.001 M EDTA.

Analytical methods

Protein was determined either by the method of Lowry *et al.*[15] as modified by Price[16] using bovine serum albumin as a standard or by the automated procedure of Schuel and Schuel[17]. Lipid extraction was performed by the method of Garbus *et al.*[18], and the extract was usually divided into two aliquots, one for the cholesterol assay and one for phospholipid phosphate determination. Cholesterol was determined by the ferric chloride/sulphuric acid method of Crawford[19] and phospholipid phosphate by the Martin and Doty[20] modification of the procedure of Berenblüm and Chain[21]. This assay was applied to sulphuric acid/peroxide digests prepared according to Parker and Peterson[22].

Assay of enzyme activities

For 5'-nucleotidase (EC 3.1.3.5) activity, the method of Michell and Hawthorne[23] was used with AMP as substrate. Experiments with other substrates for this enzyme are referred to in the experimental section of this paper. Glucose-6-phosphatase (EC 3.1.3.9) was determined by the procedure of Hubscher and West[24]. This enzyme has been used as a microsomal marker in platelet fractionation studies by a number of investigators and we have referred to it as glucose-6-phosphatase in the experimental section of this paper. However, our comments on its identity which we now believe to be a less specific enzyme appear later in the discussion section. The specific activities of both these phosphomonoesterases have been expressed in terms of μmoles P_i released per mg protein per h. Phosphodiesterase (EC 3.1.4.1) was assayed by following the liberation of *p*-nitrophenol from bis(*p*-nitrophenol) phosphate (Sigma) at pH 4.5 by the procedure of Koerner and Sinsheimer[25].

Succinate dehydrogenase (EC 1.3.99.1), measured as succinate–2-*p*-iodophenyl-3-*p*-nitrophenyl-5-phenyltetrazolium chloride (INT) oxidoreductase, was determined by the procedure of Pennington[26]. Activities were calculated as μmoles formazan liberated per mg protein per h using the molar extinction coefficient of formazan in ethyl acetate at 490 nm, $20.1 \cdot 10^3\ M^{-1} \cdot cm^{-1}$.

For lactate dehydrogenase (EC 1.1.1.27) a Boehringer Biochemica Test combination was used based upon the procedure of Wroblewski and La Due[27]. Activities have been calculated using the expression $A_{340}/\text{min} \times 5053$ and are given as μmoles NADH oxidised per min per mg protein.

Leucyl-β-naphthylamidase (EC 3.4.1.1) was determined by the method of Green *et al.*[28]. The procedure of Bratton and Marshall[29] was used for the determination of β-naphthylamine, and activities have been expressed as μmoles β-naphthylamine liberated per mg protein per h.

Cholinesterase was assayed using acetylthiocholine iodide as substrate and by the reaction of 5,5-dithiobis-2-nitrobenzoic acid (DTNB) with the liberated free thiol groups. This is essentially the method of Ellman *et al.*[30] as modified by Wheeler and his colleagues[31] for batch assays. Activities were calculated using the molar extinction coefficient of the yellow 5-thio-2-nitrobenzoic acid at 412 nm ($E = 1.36 \cdot 10^4\ M^{-1} \cdot cm^{-1}$). The difference in activity in the presence and absence of eserine at 10^{-4} M

was taken as a measure of total cholinesterase activity. In a few experiments activity differences in the presence and absence of the drug 1,5-bis-(4-trimethylammoniumphenyl)pentan-3-one diiodide (62C47 Burroughs and Wellcome) also at 10^{-4} M concentration, was used as a measure of acetylcholinesterase activity and the cholinesterase (pseudocholinesterase) activity was obtained by difference.

RESULTS

Characterisation of subcellular fractions: appearance of gradients, and electron microscopy

After density gradient centrifugation of pig homogenates, prepared exactly as described in the Methods section, two major particulate zones could be seen (Fig. 1).

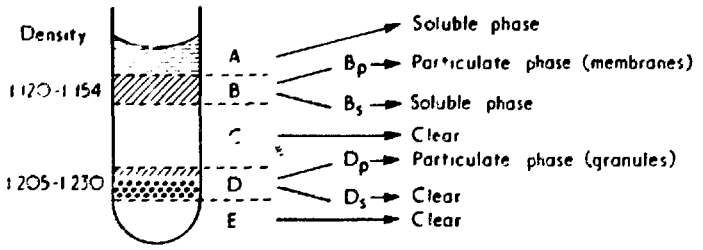


Fig. 1. Appearance of sucrose density gradients after centrifugation.

The upper, low density particulate zone B, located in the density range 1.120–1.154 and the lower, higher density zone D, in the range 1.205–1.230. Often the lower band contained two visually distinct regions, the upper part of this having a "wrinkled" sheet-like appearance and below this was a zone of fine aggregated particles. This splitting was not, however, a consistent feature of the gradients and it was rarely possible to cleanly separate these two subzones of fraction D. Fraction A, although turbid and high in protein content was found to contain no particulate material sedimentable at $100000 \times g$.

Electron micrographs of the particulate material (sedimented at $100000 \times g$ 60 min) from the fractions B and D are presented in Fig. 2 and a full description of the various intracellular elements identifiable in these fractions is presented in the legends to the figures. Also included (Fig. 2) is an electron micrograph of the homogenate before application to the sucrose density gradient. The upper zone of the gradients (B) contained predominantly membrane fragments and vesicles and the lower zone (D) consisted mainly of the granular organelles (α granules, 5HT storage organelles and mitochondria).

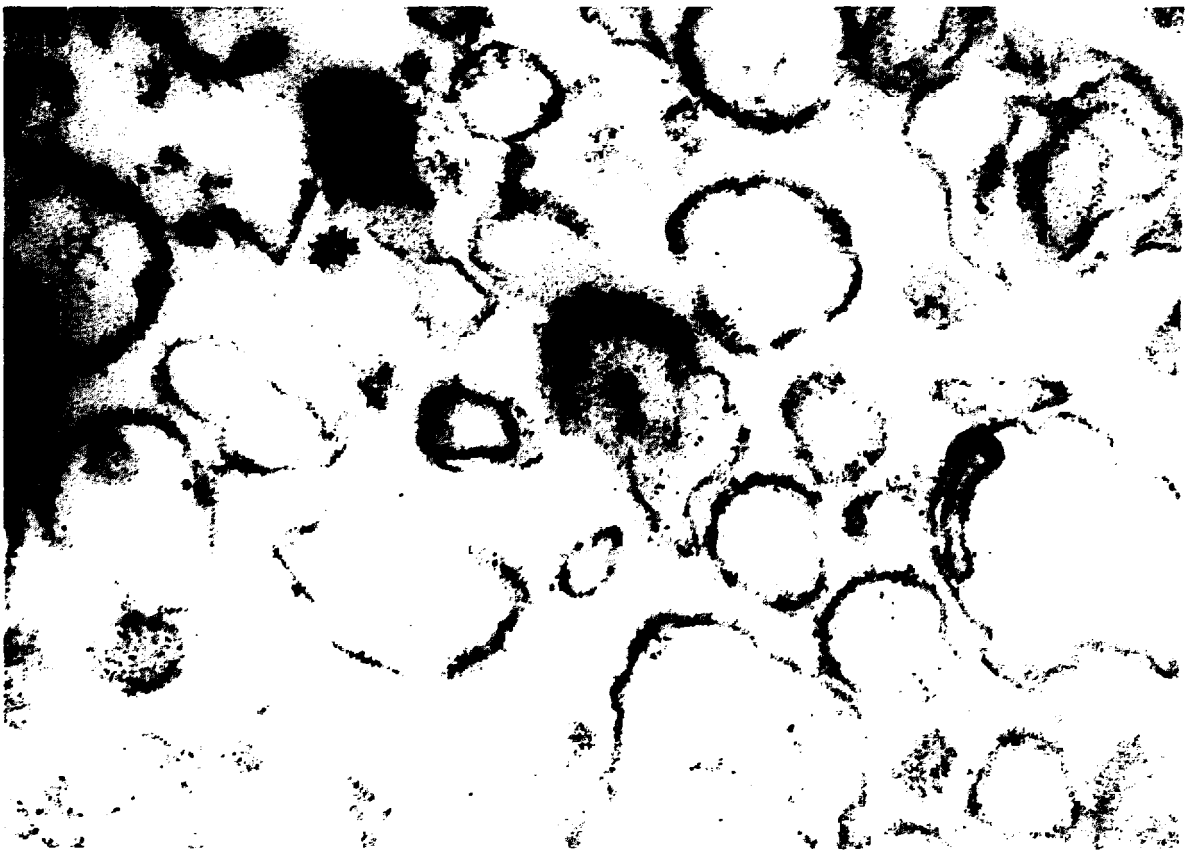
Protein distribution and marker enzyme localisation

Table I shows the distribution of protein (expressed as percentage of total recovered protein) in the fractions from thirteen sucrose density gradients prepared from different platelet homogenates. Recoveries with respect to the homogenate pay load to the gradients varied in the range 91.0–109.7% (mean $100.0 \pm 6.4\%$). Table I also indicates that with this procedure $81.4 \pm 4.8\%$ of the total homogenate protein is associated with the soluble phase of the cell as calculated from the sum of the protein in fraction A, which is a non-particulate fraction, and the protein in the soluble

Fig. 2. (a) Electron micrograph of homogenate (magnification × 27200) showing mitochondria (Mit), ɤ granules (Gra), small osmiophilic dense bodies (db) and membranes (Mem). (b) Electron micrograph of membrane fraction (B_p) (magnification × 52000). (c) Electron micrograph of granule fraction (D_p) showing whole and distorted ɤ granules and small dense bodies. Mitochondria are also found in this fraction. (Magnification × 25500).

component of fraction B (B_s). Only small quantities of protein were found in the intermediate zone C, the soluble component of fraction D (D_s) and in the fraction taken from the bottom of the gradient, zone E.

The distribution of the various subcellular elements in the gradients have been followed by using 5'-nucleotidase as a surface membrane marker, succinate dehydrogenase for mitochondria, glucose-6-phosphatase for the microsomal fraction



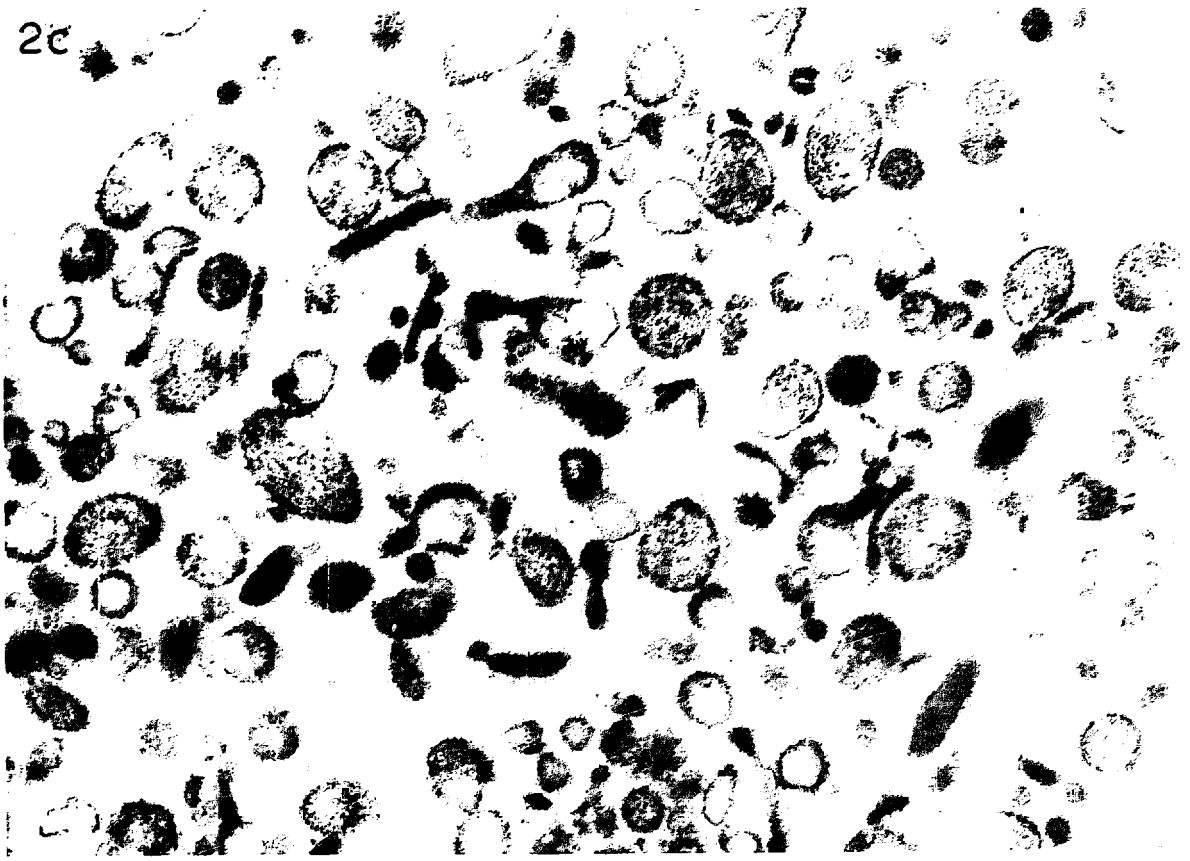

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TABLE I

DISTRIBUTION OF PROTEIN IN SUBCELLULAR FRACTIONS

For details of gradient centrifugation and fractionation see Fig. 1 and text. Results for each fraction expressed as % of total recovered protein for each gradient.

Prepn No.	*Subcellular fractions*							*Recovery (%) of homogenate*
	A	B_p	B_s	*C*	D_p	D_s	*E*	
1	50.7	7.0	27.4	3.7	5.2	4.2	1.8	107.5
2	50.5	7.9	24.6	3.9	10.1	1.8	1.2	107.5
3	53.2	8.1	28.1	2.8	3.2	2.5	1.5	98.4
4	55.9	6.0	28.2	1.9	5.3	2.6	0.0	109.7
5	52.0	5.6	29.0	2.0	5.3	4.1	2.0	102.2
6	44.2	5.6	28.3	1.7	16.4	3.4	0.4	96.4
7	42.5	3.5	49.1	1.4	2.6	1.0	0.0	93.6
8	42.1	5.1	38.1	1.3	11.4	1.9	0.2	95.8
9	46.7	3.3	38.0	2.8	5.2	3.5	0.5	98.0
10	43.2	7.3	36.8	2.3	8.3	2.2	0.0	85.7
11	45.3	5.5	37.9	1.8	9.5	0.0	0.0	91.0
12	49.0	4.9	30.6	3.6	6.7	2.3	2.9	108.7
13	57.4	3.9	28.5	2.3	4.2	0.8	2.8	95.3
Mean	48.7	5.7	32.7	2.3	7.3	2.3	1.0	100.0
±S.D.	±5.1	±1.6	±6.8	±0.9	±3.8	±1.3	±1.0	±6.4

and lactate dehydrogenase for the soluble phase of the cell. It must be noted however, that the validity of these enzymes as markers for platelet subcellular elements is less well established than with other cell systems. With the exception of 5′-nucleotidase activity the recovery of all marker enzymes, calculated with respect to homogenate gradient loads, was generally close to 100% (range 87–108%). Recovery of 5′-nucleotidase activity was always less satisfactory with occasional values as low as 40–50%. The reasons for these losses are not known but there is some evidence for an increase in lability of this enzyme after gradient fractionation. (Unpublished observations from this laboratory). The results for the succinate dehydrogenase distribution are presented in Table II. Between 83 and 93% of the recovered activity was found in the particulate component of the granule fraction (D_p). The enzyme was concentrated in this fraction to levels between 2 and 22 times that of the corresponding homogenate. The specific activities for succinate dehydrogenase in the membrane fractions (B_p) were low in comparison with those found in the corresponding granule fraction of each gradient and represents some slight contamination of the membrane fraction by mitochondrial fragments. The particulate phase succinate dehydrogenase activities measured as succinate–INT oxidoreductase were totally inhibited by 0.25 M malonate and the small apparent activities of the soluble phase fractions were unaffected by this competitive inhibitor for succinate. In a number of experiments the absence of soluble phase mitochondrial enzyme activity was confirmed by cytochrome *c* oxidase assay and the activity of this enzyme was almost exclusively confined to the particulate fraction (D_p). Fraction C from two gradients (preparations 4 and 7) showed a small amount of succinate dehydrogenase activity but since the corresponding granule

TABLE II

DISTRIBUTION OF SUCCINATE DEHYDROGENASE ACTIVITY IN SUBCELLULAR FRACTIONS

For details of gradient centrifugation and fractionation see Fig. 1 and text. Results for each fraction expressed as % of total recovered activity for gradient. Figures in parentheses are the relative specific activities, *i.e.* the ratio of the specific activity of each fraction to that of the corresponding homogenate (homogenate= 1.0). No succinate dehydrogenase activity could be detected in fractions A, B_s, D_s and E. Specific activity, μmoles formazan released per h per mg protein (Pennington[26]).

Prepn No.	*Subcellular fractions*		
	B_p	*C*	D_p
2	10.5 (0.32)	0	89.5 (2.13)
3	16.8 (0.34)	0	83.2 (3.69)
4	10.8 (1.45)	5.8 (2.48)	83.4 (12.28)
5	10.5 (0.86)	0	89.5 (7.78)
6	14.4 (2.10)	0	85.6 (4.33)
7	8.0 (1.54)	6.4 (3.72)	84.1 (22.12)
10	8.2 (0.97)	0	91.7 (9.39)
11	6.9 (0.61)	0	93.1 (4.71)
Mean	10.8 (1.02)	8.8 (3.02)	85.0 (8.30)

fraction from the same gradients showed the highest specific activities in the study (12.28 and 22.12), this can be attributed to a slight cross contamination during removal of the fractions from the gradient.

Succinate dehydrogenase activity could not be detected in the C fraction from the remaining six gradient preparations.

TABLE III

ACTIVITY AND DISTRIBUTION OF LACTATE DEHYDROGENASE IN SUBCELLULAR FRACTIONS

Results from three separate gradients expressed as specific activity and % total recovered activity. For details of gradient centrifugation and fractionation see Fig. 1 and text. Specific activity, μmoles NADH oxidised per min per mg protein. No lactate dehydrogenase activity could be detected in fractions C, D_s and E.

	Subcellular fractions				*Homogenate*
	A	B_p	B_s	D_p	
Specific activity	3.74	0.16	2.62	0.07	1.07
	2.42	0.18	1.32	0.16	1.62
	2.76	0.10	2.22	0.16	1.14
% total recovered activity	70.3	0.7	28.7	0.3	—
	67.2	0.8	31.1	0.9	—
	72.7	0.3	26.2	0.8	—

The suitability of lactate dehydrogenase as a soluble phase marker enzyme was confirmed in earlier experiments in which platelet homogenates were separated directly into soluble and particulate phases by differential centrifugation (100000 × *g*, 90 min). No preparation of particulate material contained more than 2% of the total homogenate activity, and frequently no activity could be detected at all in the particulate fractions. The distribution of this enzyme in the major subcellular fractions from three typical sucrose density gradient preparations are shown in Table III. It will been seen that between 98.3 and 99.0% of the lactate dehydrogenase activity is located with the soluble phase of the cell (*i.e.* sum of fractions A and B_s). No lactate dehydrogenase activity could be detected in the fractions C, D_s and E and less than 1% of the total homogenate activity was associated with the granular elements, fraction D_p.

Table IV shows the distribution of glucose-6-phosphatase activity in the subcellular fractions. Here approx. 65% of the total activity towards glucose 6-phosphate is located with the soluble phase of the cell ($A + B_s$) and the remaining activity was fairly evenly distributed between the membrane (B_p) and granular (D_p) fractions. The relative specific activities for this enzyme in the two particulate fractions indicate some concentration there, with values between 1.05 and 4.85 (mean 2.55) for the membrane fraction B_p and between 1.20 and 3.16 (mean 1.89) for the granule fraction D_p. The presence of a glucose-6-phosphatase in blood platelets has been reported earlier by Fleschi[32] using histochemical procedures, and activities towards this substrate have also been measured in both microsomal and granule containing fractions prepared from human platelet lysates by Moake and his colleagues[13].

Our own studies of the pH profile of this enzyme with glucose 6-phosphate and β-glycerophosphate as substrates have revealed that both the soluble and particulate fractions of homogenates show peak activity towards glucose 6-phosphate at around

TABLE IV

DISTRIBUTION AND ACTIVITY OF GLUCOSE-6-PHOSPHATASE IN SUBCELLULAR FRACTIONS

For details of gradient fractionation see Fig. 1 and text. Results expressed as % of total recovered activity for each gradient. Figures in parentheses relative specific activities (homogenate= 1.0), see legend of Table II. No activity could be detected in fractions C, D_s and E.

Prepn No.	*Subcellular fractions*			
	A	*Bp*	*Bs*	*Dp*
2	5.1 (0.10)	14.6 (1.75)	67.3 (2.56)	13.0 (1.20)
3	15.5 (0.12)	20.7 (1.05)	45.3 (1.67)	18.2 (1.98)
4	24.5 (0.46)	16.9 (2.96)	47.8 (1.77	10.9 (2.14)
5	34.1 (0.48)	17.5 (2.27)	36.9 (0.93)	11.5 (1.58)
6	15.0 (0.53)	14.7 (4.06)	37.2 (2.05)	33.3 (3.16)
7	15.8 (0.34)	18.6 (4.85)	59.5 (1.11)	6.1 (2.18)
10	15.1 (0.16)	20.1 (1.30)	41.9 (0.54)	23.0 (1.30)
11	9.6 (0.16)	16.3 (2.19)	52.8 (1.02)	21.3 (1.64)
Mean	16.8 (0.29)	17.4 (2.55)	48.6 (1.33)	17.1 (1.89)

pH 6.0–6.5 with little or no activity measureable below pH 5.0 and above pH 8.0 (Figs 3a and 3b). With β-glycerophosphate as substrate, peak activity also occurs at pH 6.0–6.5 with both these cell fractions but in the case of the soluble phase it showed both a small alkaline and a considerable acid phosphatase component too (Fig. 3a). Two of the characteristics of the specific glucose-6-phosphatase of mammalian liver are its lability at pH 5.0 and inhibition by higher concentrations

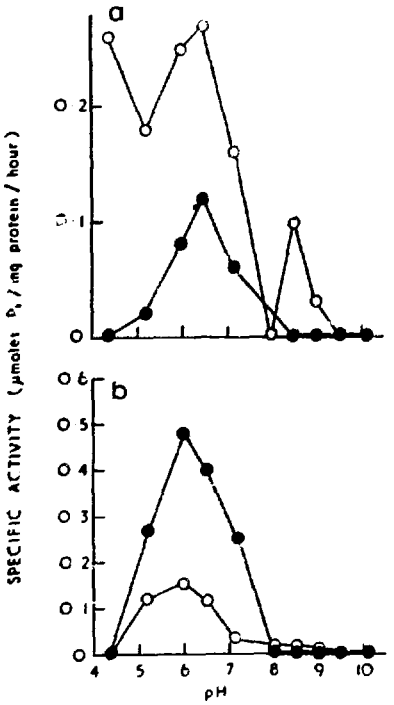

Fig. 3. pH profiles of phosphatase activity in particulate (●—●) and soluble (○—○) phases towards the substrates β-glycerophosphate (a) and glucose 6-phosphate (b).

of glucose[46,47]. However, activities measured at pH 6.0 towards glucose 6-phosphate and β-glycerophosphate were both inhibited (approx. 50%) by pretreatment at pH 5.0 before assays at pH 6.0, and unaffected by the addition of 50 and 100 mM glucose. Although throughout this paper this enzyme has been referred to as glucose-6-phosphatase it should perhaps be considered for the present to be a non-specific pH 6.0–6.5 phosphatase until the complete spectrum of phosphomonoesterases in the platelet has been fully characterised and the stability of the glycogen stores more closely examined.

The 5′-nucleotidase activity of the platelet homogenate and of all the fractions was generally very low when AMP was used as substrate. When detectable, the soluble fraction (A) accounted for between 80 and 100% of the total cell 5′-nucleotidase activity. A number of other nucleotides were found in fact, to be better substrates for the platelet enzyme than AMP and for example, assays with CMP and UMP showed between 7 and 9 times the activities recorded for AMP with the soluble phase, fraction A (Table V).

Two other enzyme distributions were investigated; leucyl-β-naphthylamidase, and thiocholinesterase. None of these enzymes gave distribution profiles which would suggest they had any "marker" value in the simple subcellular fractionations

TABLE V

SUBSTRATE SPECIFICITY OF 5'-NUCLEOTIDASE IN THE SOLUBLE FRACTION A OF TWO SUBCELLULAR FRACTIONATION EXPERIMENTS

For details of assay see Material and Methods section. Substrates all at 5 mM concentration with 10 mM Mg^{2+}. Specific activity, μmoles P_i per h per mg protein.

Substrate	*Relative specific activity (relative to activity with AMP as substrate)*	
	Prepn 10	*Prepn 11*
AMP	1.0	1.0
CMP	9.1	7.4
CMP	2.5	2.2
IMP	2.8	2.8
UMP	7.4	8.5

TABLE VI

ACTIVITIES AND DISTRIBUTION OF LEUCYL-β-NAPHTHYLAMIDASE AND CHOLINESTERASE IN SUBCELLULAR FRACTIONS

Each part of the table includes the results from two separate gradients. For details of gradient preparations and subfractionation see Fig. 1 and text. For details of assay and units of specific activity see Material and Methods section. Leucine aminopeptidase measured as L-leucyl-β-naphthylamidase. Cholinesterase measured as thiocholinesterase activity inhibited by 10^{-4} M eserine. No activity of either enzyme could be detected in fractions C, D_s and E.

	Prepn No.	*Homogenate*	*Subcellular fractions*			
			A	*B_p*	*B_s*	*D_p*
Leucyl-β-naphthylamidase						
Specific activity	1	0.054	0.091	0.061	0.112	0.040
	2	0.076	0.129	0.083	0.124	0.054
Relative specific activity	1	1.00	1.69	1.13	2.07	0.74
	2	1.00	1.70	1.09	1.63	0.71
% total recovered activity	1	100	59.3	2.7	36.1	1.9
	2	100	58.2	3.8	34.7	3.3
Total cholinesterase						
Specific activity	1	0.128	0.194	0.065	0.194	0.064
	2	0.081	0.113	0.049	0.081	0.032
Relative specific activity	1	1.00	1.08	0.37	1.08	0.37
	2	1.00	1.40	0.60	1.00	0.40
% total recovered activity	1	100	50.0	2.5	44.0	3.5
	2	100	59.0	2.9	35.0	3.0

carried out here (Table VI). Between 93 and 95% of the peptidase was found to be in the soluble phase (sum of fractions A and B_s). Total eserine-inhibited cholinesterase activity was also found to be predominantly soluble with about 94% of the activity associated with the soluble phase.

Assays in the presence and absence of the acetylcholinesterase inhibitor 62C47 gave inconsistent results and recoveries suggested that the usefulness of the drug in differentiating between the two enzymes varied with the nature of the cell fraction. It was demonstrated however that the small activity in the particulate fractions was predominantly due to an acetylcholinesterase, similar in properties to the acetylcholine hydrolysing enzyme of erythrocytes and nervous tissues of most animal species. Fig. 4 shows the results of a typical gradient distribution of the six enzymes, lactate dehydrogenase, 5′-nucleotidase, glucose-6-phosphatase (pH 6.0–6.5 phosphatase), succinate dehydrogenase, leucyl-β-naphthylamidase and cholinesterase. The results have been expressed as relative activities and plotted against percentage recovered protein in the form of a histogram.

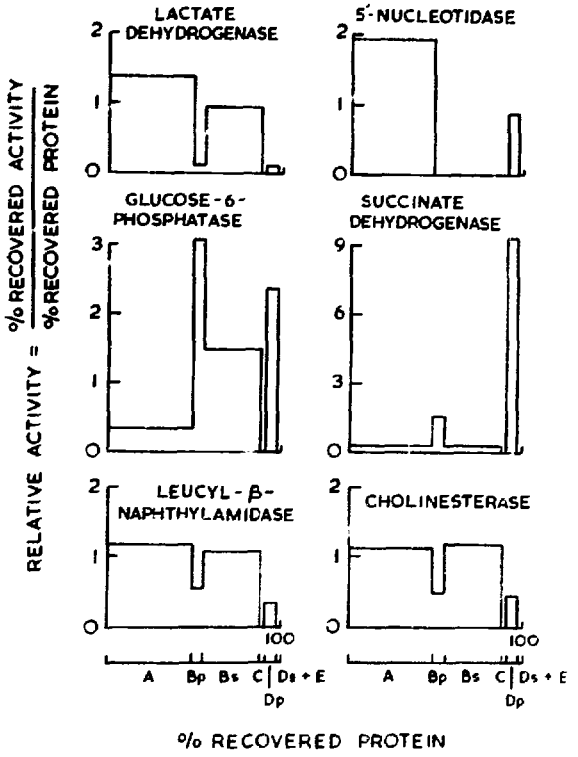

Fig. 4. Typical gradient distribution of the enzymes lactate dehydrogenase, 5′-nucleotidase, glucose-6-phosphatase, succinate dehydrogenase, leucyl-β-naphthylamidase and cholinesterase. Results presented as relative enzyme activity against percentage recovered protein in the gradient.

Extent of solubilisation during homogenisation

During the course of these studies it became clear that some of the intracellular enzymes showed a biphasic distribution between the particle and soluble phase fractions. Moreover, 5′-nucleotidase normally considered to be a plasma membrane marker in liver tissue and other cell systems was predominantly located in the soluble phase. Varying degrees of solubilisation of organelle-associated enzymes with different homogenisation procedures has been commented upon earlier by other workers[8]

in the platelet field. The following experiments were designed to determine the extent of solubilisation of certain enzymes during the preparative procedures used in this study. Using equal aliquots from the same platelet pool, the times of homogenisation were varied between a few seconds and 5 min. The homogenates were centrifuged ($5000 \times g$, 30 min) to remove unbroken cells and large debris, and the supernatant then centrifuged at high speed ($100000 \times g$, 60 min) to separate the soluble and particulate phases. The particles were washed twice in Tris–sucrose–EDTA and the washes discarded since protein assays showed negligible amounts in the first washes.

The total protein of each homogenate and of each soluble and particulate phase, and the activities of lactate dehydrogenase, 5′-nucleotidase and glucose-6-phosphatase (pH 6.0–6.5 phosphatase) in each soluble phase were determined. Lactate dehydrogenase activity was considered to be the most reliable soluble phase marker for the platelet on the basis of earlier findings of our own and other workers, and therefore the comparative liberation of this and the other two enzymes with

TABLE VII

EFFECT OF VARYING HOMOGENISATION TIMES ON PLATELET ENZYME DISTRIBUTION

For details of the homogenisation procedure see text (Method and Materials section). For expression of results (specific activities *etc.*), see legends to Tables III and V.

Homogenisation time (s)	*Protein (mg)*		*Soluble phase enzymes*					
	Soluble phase	*Particles*	*Lactate dehydrogenase*		*5′-Nucleotidase*		*Glucose-6-phosphatase (pH 6.0–6.5 phosphatase)*	
			Total act.	*Spec. act.*	*Total act.*	*Spec. act.*	*Total act.*	*Spec. act.*
45	55.3	15.7	108.2	1.97	12.6	0.227	3.25	0.059
90	77.6	20.8	144.1	1.86	17.4	0.225	4.40	0.057
180	89.8	22.0	191.7	2.13	18.3	0.204	4.43	0.049
300	118.0	22.2	255.2	2.16	25.3	0.215	6.67	0.056

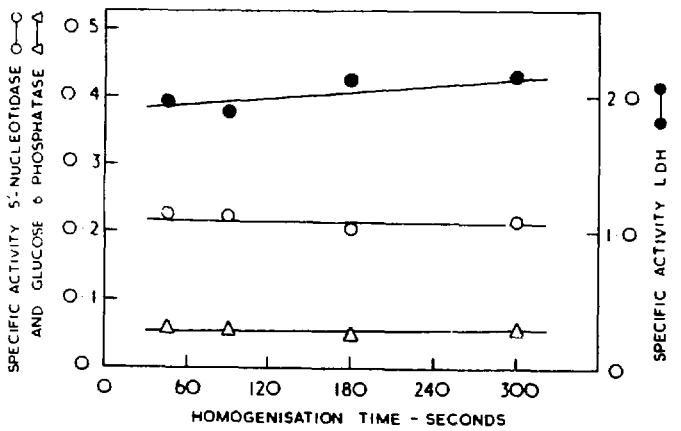

Fig. 5. Specific activities of the enzymes 5′-nucleotidase, lactate dehydrogenase (LDH) and glucose-6-phosphatase in the soluble phase after varying times of homogenisation.

increasing time of homogenisation was followed. The total homogenate yield of protein and the soluble and particulate phase protein distribution for homogenisation times of 45, 90, 180 and 300 s are shown in Table VII. Included also in Table VII are the total and specific activity values for the three enzymes lactate dehydrogenase, 5'-nucleotidase and glucose-6-phosphatase present in the soluble phase. It will be seen that the yield of total protein in the particulate and soluble phase fractions, increased with increasing homogenisation time to values which at 300 s were about twice those of the 45 s homogenate. A similar 2-fold increase in the total soluble phase activity of all three enzymes was found but the specific activity values for each enzyme did not vary significantly with the increasing times of homogenisation (Fig. 5).

Phospholipid and cholesterol distribution

Fig. 6 shows the cholesterol and phospholipid concentrations in homogenates and in the particulate fractions B_p and D_p. These composite values have been derived from the analyses of the fractions prepared from nine different pig platelet homogenates. The mean cholesterol content of the membrane fraction (0.45 ± 0.15 μmole/mg protein) was about twice as high as that of the granule fraction (0.22 ± 0.07 μmole/mg protein). This difference was found to be significant ($P<0.01$). Similarly the mean phospholipid phosphate concentration of the membrane fraction B_p was about twice as high as that of the fraction D_p containing the granular organelles. This difference too was significant ($P<0.01$). There was no statistically significant difference between the molar ratios, cholesterol/phospholipid of the homogenate and subcellular fractions.

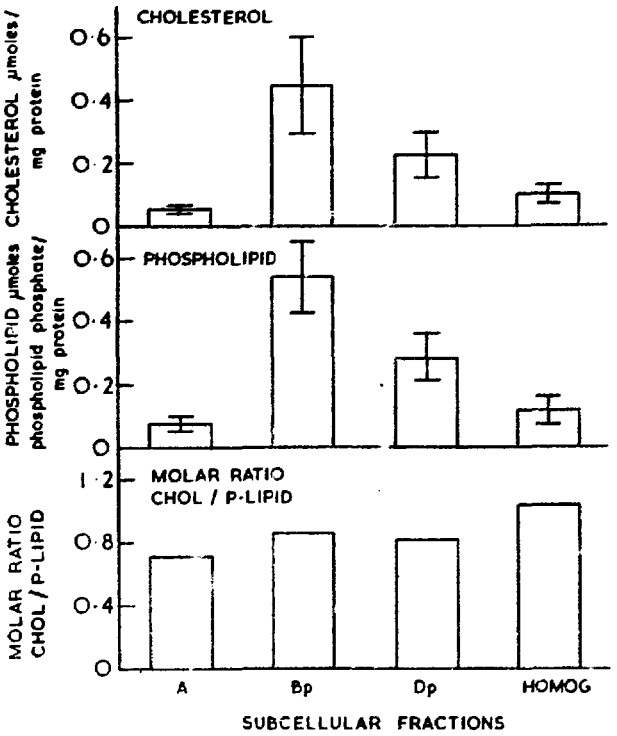

Fig. 6. Composite histogram from nine gradient preparations showing concentrations of cholesterol and phospholipid in homogenate, soluble phase and the two particulate fractions B_p and D_p. (Mean values 9 experiments ± 1 S.D.) Also shown are the molar ratios cholesterol/phospholipid for each fraction.

DISCUSSION

With the density gradient fractionation procedures described here, applied to blender homogenates of platelet suspensions, a clear separation of a membrane fraction from the various granular elements of the cell can be achieved. A broad, clear, non-particulate and virtually protein-free zone, separates the two major organelle fractions on the sucrose gradient. This has allowed clean removal of the fractions and the protein distribution profiles of nine preparations has indicated that the position of the zones in the gradient is very constant and the technique of fractionation is extremely reproducible. However, a complete separation of the many different granular organelles seen in the electron micrographs of the homogenate has not yet unfortunately been achieved and, whilst the membrane fraction is essentially uncontaminated by whole granular organelles, the granular fraction prepared in these studies is a more heterogeneous fraction containing intact mitochondria, large α-granules and small dense bodies, previously identified in the pig platelet as the 5-hydroxytryptamine storage organelles (Minter and Crawford[10]). In addition there are numerous less well-defined granular elements. Marcus *et al.*[6] obtained a very similar sucrose gradient separation of human platelet organelles with a localisation of the two major particulate zones approximating to the present study. They used in their work a "nil clearance" Teflon pestle homogeniser which in our experience (Minter and Crawford[10]) has proved to be inferior in reproducibility and give less well defined granular organelles than blender homogenisation. One particular disadvantage with "mil clearance" Teflon homogenisers is that standardisation of the homogenisation conditions is dependent upon the uniform side-to-wall clearance produced by low temperature contraction of the Teflon pestle. Our own careful measurements of both commercially available and workshop-made pestles revealed that uniform contraction rarely occurs and most pestles assume a slight conical shape with a consequent variability in shear forces. The larger the capacity of the homogeniser the more pronounced is this effect and particularly if they are constructed such that the drive rod only partially penetrates the Teflon block. Siegel and Lüscher[7] also working with human platelets, developed a blender type homogenisation procedure. Their technique separated at least five different particulate zones and although the granular structures appeared better separated than with the procedures described here, a well defined granule-free membrane fraction was not always present. Day *et al.*[8] studied three methods of homogenisation and fractionation with varying combinations of homogenisation media, methods of homogenisation (pestle and blender), pretreatment of homogenate, and different gradient compositions. They obtained two or three major gradient zones but there was not a well defined organelle subfractionation. They noted, however, (a finding confirmed during the earlier investigations in the present study), that prior separation of particulate material from the soluble phase before gradient centrifugation was a less satisfactory procedure for optimal organelle preservation than the application of the whole homogenate onto the sucrose gradient. It seems that with blood platelets the introduction of an additional preparative stage and the early removal of the very sensitive organelles from their protective intracellular colloidal environment is perhaps best avoided.

The value of the "marker" enzymes used in the present study is difficult to assess. Lactate dehydrogenase as in almost all other cells appears to be completely

soluble in the blood platelet and the present findings of 99% soluble lactate dehydrogenase confirms the earlier results of Marcus *et al.*[6], Moake *et al.*[13] and Day *et al.*[8] who reported this enzyme 98% and over 90% soluble, respectively. Succinate dehydrogenase (the malonate inhibited succinate–INT reductase) locates, and is concentrated to some extent, in the granular fraction (D_p) the fraction in which whole mitochondria are seen in electron micrographs. Between 83 and 93% of the total recovered activity appears to be tightly associated with the particulate components of this fraction. The mitochondria however, in the platelet are sparse, and White and Krivit[33] estimated from their electron micrographs only between two and seven identifiable mitochondria per human platelet.

Since the metabolic and behavioural activities of the platelet involve nucleotides containing the adenosine moiety, AMP was used routinely in these studies as the substrate for 5′-nucleotidase activity. Although this enzyme is now thought to be specificaliy associated with the surface membranes of liver, kidney and intestinal mucosal cells[34,36,37] and this has been confirmed histochemically for the liver plasma membrane[38,39], its activity in the blood platelet is extremely low and was almost entirely located in the soluble phase in the present studies. Day and his colleagues[8] using IMP as substrate found 5′-nucleotidase activity in only two out of ten preparations. The soluble phase of their gradients accounted for 80% of the nucleotidase activity of the cell and the specific activity values (approx. 0.007 μmole/h per mg protein) were even lower than those recorded here. The soluble 5′-nucleotidase encountered in the present investigation was found to be considerably more active towards CMP and UMP than AMP or IMP. In this context it is of interest that Ipita[40,41] has found that the sheep brain 5′-nucleotidase is strongly inhibited by nucleotide triphosphates, particularly ATP and UTP, and similar findings have been reported for the enzyme of Ehrlich's ascites tumour cells[42]. In these studies the inhibition was greatest when AMP was the enzyme substrate. The blood platelet contains very high levels of ATP and the possibility of a regulatory role for ATP with this enzyme should perhaps be further investigated.

Glucose-6-phosphatase (the pH 6.0–6.5 phosphatase) is another enzyme which has been found in the present work to be located to some extent in the soluble phase of the blood platelet. There was however some evidence of concentration of this enzyme in the particulate elements of the cell with relative specific activity values of between 1.3 and 4.0 for the membrane fraction and 1.3 and 3.2 for the granular fraction. The identity of this pH 5.0-labile enzyme, active at around pH 6.0 towards both glucose 6-phosphate and β-glycerophosphate has been commented upon in this paper. A similar enzyme activity, referred to as glucose-6-phosphatase, has been studied by Moake and his colleagues[13] in microsomal and granule-containing fractions prepared from human platelet lysates. However, although these investigators reported specific activity values between 2 and 5 times higher than the highest measured in the present study with pig platelet fractions, the substrate specificity of the enzymes was not investigated.

The experiments in which the liberation of various cell enzymes into the soluble phase was measured after different times of homogenisation indicate that there is a parallel liberation of soluble phase protein, of glucose-6-phosphatase (pH 6.0–6.5 phosphatase), 5′-nucleotidase and lactate dehydrogenase with increasing homogenisation time. The protein content of the soluble phase from the

"300-s homogenate" was more than twice that of the "45-s homogenate". Similar increases in the total activity of the three enzymes in the soluble phase were recorded but the specific activities did not significantly vary with the different experimental conditions. If one can make the assumption that for the platelet, as in other cells, lactate dehydrogenase is a valid soluble phase marker enzyme these findings strongly suggest that the soluble phase may also be the true intracellular location of a considerable proportion of the two enzymes 5′-nucleotidase and the glucose-6-phosphatase (pH 6.0–6.5 phosphatase).

Leucyl-β-naphthylamidase and the thiocholinesterase were found to be more than 90% soluble in these studies. Figures for the activity of these enzymes in platelet fractions have not been previously published but the results for the peptidase location are in general agreement with early work on this enzyme in rat tissues[43], although more recent findings[44,35] have now linked this enzyme specifically with plasma membrane structures in certain cells.

The membrane fraction was found to contain a higher concentration of both phospholipid and cholesterol than the granule fraction. Both values were higher than the corresponding homogenate values since the total lipid content of the soluble phase was very low. Similar findings were obtained for phospholipid protein ratios of platelet subfractions by Norday *et al.*[45]. These workers reported a w/w ratio of 0.65 for a predominantly membrane fraction, 0.29–0.49 for their granule fraction and a ratio of 0.1 for homogenate. Equivalent mean values found in this study calculated on a w/w basis were 0.42, 0.22 and 0.08, respectively. Marcus and his colleagues[6] reported values for total lipid/protein ratios in their membranes and granules to be very similar. For their fractions, the lipids were distributed as follows: membrane, 78% phospholipid and 20% cholesterol; granules, 75% phospholipid and 23% cholesterol. These figures reveal that the phospholipid/protein ratios for the two fractions were essentially the same, as were their cholesterol/protein ratios. Their results therefore differ in this respect from the present study but they do support the findings here with regard to the similarity of the molar ratios of cholesterol to phospholipid in the two particulate fractions of each preparation.

Although the homogenisation and fractionation procedures used in this study are still far from ideal and do not yet allow clear subdivision of the many different granule components of the cell it was felt that the reproducibility of the distribution and particularly of the localisation of discrete membrane and granule zones in the gradients warrants the use of this procedure for further studies on the membrane fraction and soluble phase of the platelet. Further processing of the particulate fractions from this type of gradient or from zonal rotor separations using a similar gradient profile may allow the identification of plasma membrane components and also a better resolution of the granular organelles.

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