Preparation and Characterization of the Plasma Membrane of Pig Lymphocytes

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Lymphocyte plasma membrane was isolated from minced pig mesenteric lymph node by differential centrifugation and by centrifuging through a sucrose density gradient. The yield of membrane was approx. 0.1% (dry wt. relative to wet wt. of lymph node). The purified material had a sucrose density of 1.14 g/cm³ and consisted mainly of smooth vesicles. The membrane fraction contained, apart from protein and lipid, 59 µg of carbohydrate, 11 µg of sialic acid and 28 µg of RNA/mg of protein; no DNA was detected. The cholesterol/phospholipid molar ratio was 1.01. Specific activities (µmol of product/h per mg of protein) of 5'-nucleotidase, succinate dehydrogenase, acid phosphatase and glucose 6-phosphatase were 10.1, 0, 0.51 and 0.30 respectively. The membrane vesicles were aggregated by an antiserum against pig lymphocytes and adsorbed the agglutinins to whole lymphocytes present in the antiserum; the membrane fraction was 28 times as effective as whole cells (on a dry wt. basis) in removing the lympho-agglutinins. Antisera against the membrane fraction agglutinated whole lymphocytes. It is concluded that the preparation represents the plasma membrane of small lymphocytes. The plasma membrane of pig thymocytes was isolated by using the same procedure. Its properties were similar to those of the lymphocyte plasma membrane.

Although well-characterized plasma membranes have been isolated from a variety of mammalian cell types (e.g. erythrocytes; Dodge, Mitchell & Hanahan, 1963; HeLa; Bosmann, Hagopian & Eylar, 1968; kidney; Fitzpatrick, Davenport, Forte & Landon, 1969; liver; Evans, 1970), it appears that relatively little effort has been devoted to the preparation of the plasma membrane of lymphocytes (Warren, Glick & Nass, 1966). This is somewhat surprising in view of the important roles that have been ascribed to the surface of lymphocytes in mediating antigen recognition (Raff, Sternberg & Taylor, 1970), histocompatibility (Kahan & Reisfeld, 1969) and other immunological phenomena (Dumonde *et al.* 1969). It is evident that purified preparations of lymphocyte plasma membrane would be invaluable for the investigation of the chemical bases of these phenomena as well as being suitable for studies of the structure of cellular membranes.

Small lymphocytes should provide an eminently suitable source of plasma membrane because the ratio of surface area to cell volume is high. Also, as these cells possess a thin layer of cytoplasm only and contain little endoplasmic reticulum, contamination of the plasma membrane fraction with cytoplasmic bodies and intracellular membranes

should be minimal provided that the nuclei are not broken during the initial disruption of the cells. The primary problem was in obtaining large amounts of a homogeneous population of small lymphocytes. This was largely overcome by using mesenteric lymph nodes from young pigs that had been bred for bacon.

The aim of the present study was to establish a simple, reproducible and mild method for preparing the plasma membrane of small lymphocytes in good yield. This paper describes the separation of smooth membrane vesicles from pig mesenteric lymph nodes, and the characterization of these vesicles as lymphocyte plasma membrane on the basis of their morphological, biochemical and immunological properties.

MATERIALS AND METHODS

Glass-distilled water was used in all experiments. Chemicals were of analytical grade. Bovine serum albumin (fraction V) was purchased from Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex, U.K., and cholesterol from British Drug Houses Ltd., Poole, Dorset, U.K. Calf thymus DNA was obtained from Sigma (London) Chemical Co. Ltd., London S.W.6, U.K., and AMP and β -glycerophosphate were from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany.

Scheme 1. Homogenization and subcellular fractionation of pig mesenteric lymph node. The use of the terms nuclear, mitochondrial, ribosomal and plasma membrane is based on the subsequent characterization of the fractions.

Preparation of lymphocyte plasma membrane

The procedure is summarized in Scheme 1.

Lymph nodes. Mesenteric lymph nodes were obtained from young pigs (average age 7 months) killed in a commercial abattoir (T. Wall and Sons Ltd., London W.3, U.K.). They were removed within a few minutes of the

death of the animal and were placed immediately into a Dewar flask containing crushed ice at 0°C. About 30 min later the node was freed from surrounding tissue and fat at 0°C. The excised node appeared as an extended greyish-pink organ of varying thickness; unusually large or small nodes were discarded. Stained sections showed that each node consisted primarily of small lymphocytes

(approx. 5×10^{10} cells/30g wet wt.) together with some connective tissue; large lymphocytes, plasma cells and macrophages were rarely detected.

Tissue homogenization. All operations were carried out at 0–2°C. One node (wet wt. 30g) was chopped with scissors into small pieces and 3g portions were successively forced through a perforated plate (1.5 cm diam. containing 50 equidistant 1mm-diam. holes) in a stainless-steel hand-operated mincer with a screw-driven plunger. After each portion had been minced the layer of connective tissue that accumulated on the plate was removed; this was essential to ensure reproducible disruption of the cells. The minced tissue was stirred for 2min with 30ml of 10mM-tris-HCl–0.15M-NaCl, pH7.4 (tris buffer-saline), and then filtered through one layer of muslin cloth to remove clumps of material. The residue (wet wt. approx. 16g) consisted mainly of connective tissue together with disrupted cells, and some amorphous masses of deformed nuclei. Examination of the filtrate (approx. 40ml) by phase-contrast microscopy revealed free whole nuclei (approx. 1.3×10^{10}), mitochondria and fragments of membrane. Approx. 95% of the cells of the lymph node were broken under the above conditions of homogenization. These relatively mild conditions of cell breakage were chosen so as to minimize the rupture of nuclei and the release of viscous DNA that interfered with the subsequent fractionation procedures.

Subcellular fractionation. The above filtrate was centrifuged at 300g_{av.} for 15min in an MSE Mistral 4L centrifuge. The pellet consisted mainly of nuclei together with some intact erythrocytes. The supernatant was fractionated by differentially centrifuging at 4000, 20000 (Sorvall RC2-B centrifuge) and 105000g_{av.} (MSE 50 centrifuge). The pellets obtained by centrifuging at 4000 and 20000g_{av.} were loosely packed and care was taken not to disturb them when removing the supernatant. Each pellet was resuspended in 40ml of the tris buffer-saline and resedimented under the same conditions as before. Examination of the fractions by electron microscopy and by enzymic analysis revealed that the 20000g_{av.} pellet consisted mainly of smooth-membrane vesicles and that the 5'-nucleotidase activity, which was used as a putative marker for the plasma membrane, was associated primarily with this fraction.

The 20000g_{av.} pellet was fractionated further by centrifuging through a 40–30% (w/v) sucrose step-gradient. First, the pellet was gently resuspended in an equal volume of 80% (w/v) sucrose in 10mM-tris-HCl buffer, pH7.4, by using four to eight slow strokes with a tight-fitting glass homogenizer (internal volume 2ml; 'tissue grinder'; S. Farley and Sons Ltd., Ponders End, Middx., U.K.) until no clumps were visible in the light microscope; the formation of air pockets during homogenization was avoided. The suspension was then diluted to 45ml with 40% (w/v) sucrose in 10mM-tris-HCl buffer, pH7.4, divided equally between three Beckman SW 25.1 cellulose nitrate tubes of 32ml total capacity, and overlaid with 30% (w/v) sucrose (16ml/tube). The tubes were centrifuged for 18h at 80000g_{av.} in a modified MSE 50 centrifuge. After centrifugation, the interfacial band was carefully removed with a Pasteur pipette, diluted fourfold with 10mM-tris-HCl buffer, pH7.4, and the membrane fragments were sedimented at 20000g_{av.} for 1h (Sorvall RC2-B centrifuge). The pellet was resuspended in 40ml

of 10mM-tris-HCl buffer, pH7.4, with several strokes of the tight-fitting glass homogenizer and recentrifuged as before in a Sorvall centrifuge. This step was repeated once and the membranes were finally resuspended in 3–5ml of 10mM-tris-HCl buffer, pH7.4.

Continuous density-gradient centrifugation. Initially the 20000g_{av.} pellet obtained by differential centrifugation was further fractionated by centrifuging on continuous gradients of sucrose [30ml of 45% (w/v) against 30ml of 30%] dissolved in 10mM-tris-HCl–1mM-MgCl₂, pH7.4. The 20000g pellet was resuspended in 9ml of 10mM-tris-HCl–1mM-MgCl₂, pH7.4, by using the tight-fitting glass homogenizer and 3ml samples were layered under the gradients, which were then centrifuged (MSE 65 centrifuge; 3 × 65 rotor) at 70000g_{av.} for 18h. After centrifugation, 2ml fractions were removed from just above the small pellet by using a syringe and analysed; the sucrose concentration was determined by measurement of the refractive index.

Assay of enzymic activities

5'-Nucleotidase (EC 3.1.3.5) activity was measured as described by Michell & Hawthorne (1965). Glucose 6-phosphatase (EC 3.1.3.9) was assayed by the method of Swanson (1950), and acid phosphatase (EC 3.1.3.2) by the method of Gianetto & de Duve (1955), with β -glycerophosphate as substrate; the P_i released was determined by the method of Fiske & Subbarow (1925). Succinate dehydrogenase (EC 1.3.99.1) activity was measured as described by Earl & Korner (1965).

Analytical methods

Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951) with bovine serum albumin as standard; a 1% (w/v) solution of bovine serum albumin was assumed to have $E_{280}^{1\text{cm}}$ 6.60 (Cohn, Hughes & Weare, 1947). The RNA content of membranes was measured as described by Munro & Fleck (1966); $E_{260}^{1\text{cm}}$ 1.00 for the final solution was taken to be equivalent to 32 μ g of RNA/ml. Amounts of RNA determined by the above method were about 5% lower than those measured by the orcinol reaction (Schneider, 1945). DNA was determined by using the diphenylamine reaction with purified calf thymus DNA as standard. The precipitate obtained with HClO₄ in the RNA determination was hydrolysed with 3ml of 5% (w/v) trichloroacetic acid for 15min at 90°C. Samples (1.0ml) of the solution were added to 2.0ml of the diphenylamine reagent (Schneider, 1945) and, after heating at 100°C for 20min, the extinction at 600nm was read in cells of 1cm path-length. For the determination of total lipid, cholesterol and phospholipids, a known weight of dry plasma membrane was extracted three times with 10ml of chloroform-methanol (2:1, v/v) at room temperature over a period of 24h. The extract was washed once with 10ml of water. The organic phase was evaporated at 40°C in a current of air and the residue was dried to constant weight *in vacuo* over P₂O₅ and paraffin wax at 20°C. The weight of residue was taken to be the amount of total lipid. The cholesterol content of a sample of the residue was measured by the method of Brown, Zlatkis, Zak & Boyle (1954). The remainder

of the residue was digested with 1 ml of 70% (w/v) HClO_4 at 200°C for 1 h and the phosphorus determined by the method of Fiske & Subbarow (1925); phospholipid was calculated by assuming 25 μg of phospholipid/ μg of phosphorus (Kopaczynk, Perdue & Green, 1966). The carbohydrate content of plasma membrane that had been washed once with water by centrifuging was determined by using the anthrone (Scott & Melvin, 1953) and indole (Ashwell, 1957) reactions with glucose as standard. Sialic acid was determined by the method of Warren (1963) after hydrolysis in 50 mM- H_2SO_4 at 80°C for 60 min. The values were expressed as *N*-acetylneuraminic acid, although it seemed possible by analogy with pig erythrocyte-stroma mucoprotein (Klenk & Uhlenbruck, 1958) that the sialic acid of pig lymphocyte plasma membrane was *N*-glycolylneuraminic acid. Amino acid analyses were performed with an automatic analyser (Benson & Patterson, 1965); the length of the short column was adjusted to 20 cm so that glucosamine and galactosamine were eluted as separate peaks. Samples of dry defatted membrane (1–2 mg) were hydrolysed *in vacuo* with constant-boiling HCl for 24 and 72 h as described by Crumpton & Wilkinson (1963).

Immunological methods

Lymphocyte suspensions. Single-cell suspensions of lymphocytes for immunization and agglutination were prepared by gently squeezing small fragments of pig mesenteric lymph node with a loose-fitting glass plunger in a plastic tube containing 0.15 M-NaCl. The dispersed tissue was filtered through one layer of muslin to remove clumps and centrifuged at 600 g_{av} for 5 min. The pellet was gently resuspended in 0.15 M-NaCl by using a Pasteur pipette and resedimented at 600 g_{av} ; this process was repeated three times. Under these conditions nuclei and broken cells clumped, and were subsequently removed by two passages through a column of absorbent cotton wool. The filtrate contained 5% erythrocytes, 2% large lymphocytes and 93% small lymphocytes; more than 90% of the small lymphocytes were viable as judged by the exclusion of Trypan Blue.

The dry weight of pig lymphocytes was determined by centrifuging (600 g_{av} for 5 min) a suitable volume of the lymphocyte suspension (5×10^9 cells), and by drying the pellet (approx. 1 ml) to constant weight at 110°C.

Antisera. A sample of normal serum was obtained from each animal before immunization. Antisera to small lymphocytes were prepared in New Zealand White rabbits by two injections intravenously, separated by 14 days, of 10^9 cells in 1 ml of 0.15 M-NaCl. The animals were bled 7 days after the second injection. An identical procedure was used to prepare antisera to the purified plasma-membrane fraction; an amount of membrane equivalent to 2 mg of protein was injected on each occasion. Before use all antisera were incubated at 56°C for 30 min to inactivate complement.

Lymphoagglutination test. Serial $\frac{1}{2}$ dilutions of anti-serum were prepared in 0.1 ml volumes with 0.15 M-NaCl as diluent, and an equal volume of a lymphocyte suspension (2×10^7 cells/ml) was added to each dilution. The tubes were shaken and incubated at 37°C for 1 h. Agglutination was read microscopically after the tubes had been vigorously shaken. The greatest dilution at which

agglutination could be detected was taken as the end point; the reciprocal of this serum dilution is referred to below as the agglutinin titre. Serial $\frac{1}{2}$ dilutions of normal serum with lymphocytes were included as a control in each test. Anti-lymphocyte sera possessed agglutinin titres of between 600 and 1200, whereas the titre of normal serum was usually not more than 2 and never greater than 8.

Adsorption of lymphoagglutinin. The capacities of whole lymphocytes and of the various subcellular fractions to adsorb the lymphoagglutinins of anti-lymphocyte sera were determined in the following way. Increasing amounts of lymphocytes (2×10^7 – 18^8 cells) or subcellular fractions (10–1000 μg of protein) in 1 ml of 0.15 M-NaCl were incubated for 1 h at 37°C with a constant volume (0.1 ml) of anti-lymphocyte serum. The tubes were centrifuged at 20000 g_{av} for 5 min (Sorvall RC2-B centrifuge) and the lymphoagglutinin titres of the supernatants were determined as described above.

Microscopy

All fractions were examined by phase-contrast microscopy during the various stages of the fractionation procedure. For electron microscopy, pellets of the various subcellular fractions were fixed for 1 h at 0°C in a 2:1 (v/v) mixture of 1% (w/v) osmium tetroxide and 2.5% (v/v) glutaraldehyde in 0.1 M-sodium cacodylate buffer, pH 7.4 (Hirsch & Fedorko, 1968). The fractions were then dislodged carefully from the tubes, rinsed twice with 0.15 M-NaCl at 0°C and 'post-fixed' for 15 min in 0.25% uranyl acetate in 0.1 M-veronal acetate buffer, pH 6.2. Specimens were dehydrated with ethanol and embedded in Epikote 812 epoxy resin. Thin sections were stained with uranyl acetate and lead citrate and examined in a Philips EM 300 microscope.

RESULTS

Preparation of plasma membrane

Various methods of preparing lymphocyte plasma membrane from pig mesenteric lymph node were investigated. The procedure described in the Materials and Methods section was that which gave the highest yield of plasma membrane with the lowest degree of contamination by other cellular components. The presence of 0.25 M-sucrose during the disruption of the tissue and the initial stages of the fractionation (cf. Boone, Ford, Bond, Stuart & Lorenz, 1969) was found to be undesirable since it caused extensive breakage and aggregation of the nuclei. Also, the presence of salt (0.15 M-sodium chloride and/or 1 mM-magnesium chloride) promoted the aggregation of membranes during the fractionation of the 20000 g pellet on the 40–30% sucrose step-gradient; under these conditions the plasma-membrane fraction was contaminated with larger amounts of RNA (60–100 $\mu\text{g}/\text{mg}$ of protein). As a result, in the method finally adopted sucrose was not added during the initial stages of the fractionation and the concentration of salt was kept

Fig. 1. Separation of lymphocyte plasma membrane fraction by sucrose-density-gradient centrifugation. The pellet obtained by differential centrifugation at $20000g_{av}$ was homogenized in a tight-fitting homogenizer with 9 ml of 10 mM-tris-HCl-1 mM-MgCl₂, pH 7.4, and 3 ml samples were layered under the gradients. After centrifuging at $70000g_{av}$ for 18 h in a MSE 3×65 rotor, 2 ml fractions were removed from just above the small pellet by using a syringe. Samples (0.1 ml) were analysed for protein (E_{660}) and 5'-nucleotidase activity (E_{660}); cholesterol (E_{560}) was determined in 1.5 ml samples. ○, Protein; □, 5'-nucleotidase; △, cholesterol; ●, sucrose concentration.

to a minimum during density-gradient centrifugation.

Initially, plasma membrane present in the $20000g$ pellet was further purified by centrifuging for 18 h to equilibrium on a continuous sucrose density gradient. The profile across the sucrose gradient of a typical run is shown in Fig. 1. One peak that was enriched in cholesterol and 5'-nucleotidase activity was found at a sucrose density of 1.14 g/cm^3 . Observation of this peak by electron microscopy showed that it consisted primarily of smooth-membrane vesicles. In later experiments it was found more convenient to use a step-gradient of 40–30% (w/v) sucrose; under these conditions the smooth-membrane vesicles formed a sharp band at the interface.

The yield of plasma membrane obtained from 30 g of wet lymph node by using the final procedure was 15–25 mg of protein or 26–43 mg dry wt.

Characterization of subcellular fractions

The various subcellular fractions were characterized chemically, enzymically and morphologic-

ally. The results described were obtained in one particular experiment performed on a lymph node of 30 g wet wt.; similar results were obtained on 12 separate occasions.

Chemical analysis. The chemical compositions of the various fractions are summarized in Table 1. The results revealed that 96% of the DNA present in the initial filtrate (Scheme 1) sedimented at $300g$. It was concluded that very few nuclei were fragmented during the disruption of the cells. Although RNA was fairly evenly distributed between the subcellular fractions obtained by differential centrifugation, the $105000g$ pellet contained the largest total amount of RNA and the highest RNA/protein ratio. These results were in agreement with the morphological examination of whole lymphocytes and indicated that most of the ribosomes were not membrane-bound. The plasma-membrane fraction contained no detectable DNA and a small amount of RNA only ($21\text{ }\mu\text{g/mg}$ of protein). A comparison of the cholesterol/protein ratios for the subcellular fractions obtained by differential centrifugation revealed that the $20000g$ pellet possessed the highest value ($95\text{ }\mu\text{g/mg}$ of

Table 1. *Chemical composition of fractions obtained during the purification of plasma membrane from pig lymphocytes*

The fractions are described in Scheme 1. Total weight refers to the total amount of component present in the respective fraction obtained from a lymph node of 30g wet wt. N.D., Not determined.

	Protein (mg total wt.)	DNA		RNA		Cholesterol	
		(mg total wt.)	(μ g/mg of protein)	(mg total wt.)	(μ g/mg of protein)	(mg total wt.)	(μ g/mg of protein)
Mince residue	N.D.	250	N.D.	77	N.D.	80	N.D.
Filtrate	1330	104	78	77	58	45	34
Nuclear pellet (300g _{av.})	257	100	390	10.2	40	4.1	16
Mitochondrial pellet (4000g _{av.})	120	1.2	10	9.3	78	6.7	56
20000g _{av.} pellet	86	0	0	7.4	87	8.2	95
Ribosomal pellet (105000g _{av.})	70	0	0	19.3	277	4.7	67
Supernatant	520	0.37	0.7	15.2	29	10.9	21
Plasma membrane	17.5	0	0	0.37	21	4.06	231

Table 2. *Enzymic activities of subcellular fractions*

Total enzymic activities present in the fractions isolated from a lymph node of 30g wet wt. are expressed as μ mol of product liberated/h. Specific activities are given as μ mol of product liberated/h per mg of protein at 37°C.

	5'-Nucleotidase		Acid phosphatase		Glucose 6-phosphatase		Succinate dehydrogenase	
	Total activity	Specific activity	Total activity	Specific activity	Total activity	Specific activity	Total activity	Specific activity
Filtrate	1126	0.85	898	0.68	477	0.36	110	0.083
Nuclear pellet (300g _{av.})	50	0.19	113	0.44	53	0.21	9.6	0.037
Mitochondrial pellet (4000g _{av.})	194	1.62	286	2.38	23	0.19	74	0.616
20000g _{av.} pellet	380	4.45	121	1.41	51	0.59	9.9	0.115
Ribosomal pellet (105000g _{av.})	153	2.19	19	0.26	32	0.46	5.6	0.080
Supernatant	135	0.26	211	0.41	206	0.40	0	0
Plasma membrane	173	9.9	8	0.46	6	0.34	0	0

protein). This ratio was increased markedly in the plasma-membrane fraction (231 μ g/mg of protein).

Enzymic activities. The separation of plasma membrane was followed by using 5'-nucleotidase as a marker, and the presence of mitochondria, lysosomes and endoplasmic reticulum was detected by assaying for succinate dehydrogenase, acid phosphatase and glucose 6-phosphatase respectively. The distributions of these enzymes among the subcellular fractions are shown in Table 2. It can be seen that 76% of the total succinate dehydrogenase activity of the initial filtrate (Scheme 1) was recovered in the 300g and 4000g pellets, and

that none of the succinate dehydrogenase activity of the 20000g pellet was detected in the purified plasma-membrane fraction. Although the acid phosphatase activity of the filtrate was mainly concentrated in the 4000g pellet, approx. 24% of the total acid phosphatase activity was associated with the 105000g supernatant. The latter activity may have been due to the presence of soluble cytoplasmic non-specific phosphatases or may indicate that some breakage of lysosomes had occurred during the disruption of the cells. The 105000g supernatant also contained the major portion (43%) of the total glucose 6-phosphatase activity, although

each fraction possessed a similar specific activity. 5'-Nucleotidase activity was distributed among all the subcellular fractions but was mainly associated with the 20000g pellet (34% of the total activity). Further, approx. half of the 5'-nucleotidase present in the 20000g pellet was concentrated in the purified plasma membrane.

Electron microscopy. Sections of mesenteric lymph node showed that at least 90% of the nucleated cells had a diameter of 8μm or less. Single cells obtained by gently disrupting the node had the typical appearance of small lymphocytes with a large nucleus surrounded by a thin rim of cytoplasm containing little rough endoplasmic reticulum, many free ribosomes and relatively few mitochondria (Plate 1; the field shown was selected so as to illustrate the cellular components that may contaminate the plasma-membrane fraction). The cells also contained internal smooth-membrane vesicles and some cells possessed small dark granules bounded by membrane; these granules may represent lysosomes (Brittinger, Hirschhorn, Douglas & Weissmann, 1968).

The appearance of the pellets obtained by centrifuging the filtrate of disrupted cells at 300, 4000 and 20000g is shown in Plates 2(a), 2(b) and 3 respectively. The 300g pellet contained mainly whole nuclei associated with various amounts of cytoplasmic constituents. Mitochondria represented the major constituent of the 4000g pellet together with dense lysosome-like particles and rough and smooth membranes, some of which enclosed ribosomes and/or cytoplasm. Examination of the 20000g pellet revealed numerous smooth-membrane vesicles of varying size and some vesicles of rough membrane; many of the vesicles appeared to contain cytoplasm and in some cases free ribosomes. In contrast, the purified plasma-membrane fraction consisted almost entirely of smooth-membrane vesicles (see below). The 105000g pellet was composed of ribosomes together with a few very small vesicles and amorphous fragments.

Characterization of plasma membrane

Biochemical properties. The chemical composition and enzymic activities of the plasma-membrane fraction are summarized in Table 3. It can be seen that different preparations possessed similar properties. The dominant features of the chemical composition were the absence of DNA, the relatively small amount of RNA and the high value for the cholesterol/phospholipid molar ratio. The protein and total lipid contents were similar to those reported for the plasma membrane of HeLa cells (Bosmann *et al.* 1968), whereas the amounts of

Table 3. *Composition of plasma-membrane fraction*

Values are given $\pm$ s.d. with the numbers of determinations in parentheses. The protein content of a known weight of dried membrane was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as standard. The weight of phospholipid was calculated by multiplying the weight of phosphorus by 25. The molecular weight of phospholipid was assumed to be 775. Enzymic activities are expressed as μ mol of product liberated/h per mg of protein.

	Plasma membrane
Protein (% dry wt.)	57 (1)
Total lipid (% dry wt.)	42 $\pm$ 1 (3)
Phospholipid (μ g/mg of protein)	438 $\pm$ 10 (4)
Cholesterol (μ g/mg of protein)	217 $\pm$ 10 (11)
Cholesterol/phospholipid molar ratio	1.01 $\pm$ 0.03 (4)
Carbohydrate (μ g of glucose/mg of protein)	59 $\pm$ 8 (3)*
Sialic acid (μ g of N-acetylneuraminic acid/mg of protein)	11 $\pm$ 1 (3)
RNA (μ g/mg of protein)	28 $\pm$ 5 (5)
DNA (μ g/mg of protein)	0 (5)
5'-Nucleotidase	10.1 $\pm$ 1.5 (7)
Acid phosphatase	0.51 $\pm$ 0.07 (3)
Glucose 6-phosphatase	0.30 $\pm$ 0.04 (2)
Succinate dehydrogenase	0 (2)

* Determined by using the anthrone method (Scott & Melvin, 1953); the indole method (Ashwell, 1957) gave 83 $\pm$ 10 (3) μ g of glucose/mg of protein.

Table 4. *Amino acid composition of the protein moiety of the plasma-membrane fraction*

Dry plasma membrane was exhaustively extracted with chloroform-methanol (2:1, v/v) and samples (1–2mg) of the residue were hydrolysed for 24 and 72h *in vacuo* at 110°C with 1ml of constant-boiling HCl. Amounts of threonine and serine were corrected for decomposition during hydrolysis and the values of valine and isoleucine were those determined after 72h hydrolysis. The half-cystine content was measured after hydrolysis for 24h. Tryptophan was not determined.

Amino acid	Amino acid composition (mol/100mol)
Lysine	6.7
Histidine	1.9
Arginine	5.0
Aspartic acid	9.5
Threonine	5.8
Serine	7.2
Glutamic acid	11.7
Proline	4.8
Glycine	7.8
Alanine	7.6
Cystine (half)	0.6
Valine	6.9
Methionine	2.0
Isoleucine	5.1
Leucine	9.7
Tyrosine	3.3
Phenylalanine	4.3

carbohydrate (determined by the anthrone method) and sialic acid were about half of those determined, by using similar techniques, for the erythrocyte plasma membrane (Bakerman & Wasemiller, 1967). Amino acid analysis of the defatted plasma-membrane fraction (Table 4) showed the presence of approx. 14%, 21% and 31% of basic, acidic and non-polar (proline, valine, isoleucine, leucine and phenylalanine; Van Holde, 1966) amino acids respectively; these values were in close agreement with the amino acid compositions of some other plasma membranes [e.g. erythrocytes (Bakerman & Wasemiller, 1967); liver (Evans, 1970)]. The elution pattern obtained with the short column of the analyser revealed peaks in the positions of glucosamine and galactosamine. The ratio of the areas of the glucosamine to galactosamine peaks was approx. 4:1, and the total amount of hexosamine (uncorrected for decomposition during hydrolysis) was 0.8 mol/100 mol of amino acids.

The most notable feature of the enzymic activities of the plasma membrane fraction (Table 3) was the high specific activity of 5'-nucleotidase relative to those of acid phosphatase, glucose 6-phosphatase and succinate dehydrogenase. The specific activities of the latter enzymes were very low and were similar to those reported for plasma membranes of other cell types (e.g. Evans, 1970).

Morphological properties. Electron microscopy of sections of the plasma-membrane fraction (Plate 4) showed a population of smooth vesicular structures. No gross contamination with cytoplasmic elements was detected, but some vesicles enclosed amorphous material and/or a few ribosomes. The significance of the small more-electron-dense bodies is difficult to assess, but in some cases it appears that they may represent spirals of membrane associated with trapped cytoplasm or tangential sections through the membrane layer. It is, however, possible that some of these bodies are related to the small dark granules seen in whole cells (Plate 1). If this view is correct and if the granules are lysosomes (see above), then the plasma-membrane fraction is contaminated by a few lysosomes. This interpretation is consistent with the low acid phosphatase activity of the plasma membrane (Table 3). Also, if lymphocyte lysosomes have a sucrose density of 1.15 g/cm³ (Bowers & de Duve, 1967), then they would not have been separated from the plasma membrane (density 1.14 g/cm³) by centrifuging through a sucrose density gradient.

Immunological properties. Incubation of the plasma-membrane fraction with an antiserum against pig lymphocytes caused the membrane vesicles to aggregate. The aggregates were sedimented by centrifuging at 2000 *g*_{av.} for 15 min, whereas most of the membrane vesicles that had been preincubated with a sample of normal serum

were not sedimented under these conditions. Further, the plasma-membrane fraction adsorbed the agglutinins to whole lymphocytes present in anti-lymphocyte sera. The capacities of the various subcellular fractions to remove the lymphoagglutinins are compared in Table 5. The 20000 *g* pellet was more effective than the other subcellular fractions. Further, the plasma-membrane fraction was about three times as effective as the 20000 *g* pellet and about fourteen times as effective as the initial filtrate. The minimum number of whole lymphocytes required to lower the lymphoagglutinin titre of antiserum 27 by the same amount as 0.07 mg of plasma-membrane protein, under the same conditions, was $8 \times 10^7 \pm 1 \times 10^7$ cells (average of three determinations); i.e. 8×10^7 lymphocytes were equivalent to 0.07 mg of membrane protein or 0.12 mg dry wt. of membrane. As the dry weight of 5×10^9 pig lymphocytes was 208 ± 4 mg (average of 2 determinations), 0.12 mg of the plasma-membrane fraction and 3.33 mg dry wt. of whole lymphocytes possessed the same number of receptor sites for the lymphoagglutinins. If the receptor site is located exclusively on the surface of lymphocytes, then the degree of purification of the plasma membrane relative to whole cells was approx. 28-fold.

Antisera prepared by immunization of six rabbits with the plasma-membrane fraction agglutinated suspensions of whole lymphocytes. The lymphoagglutinin titres of the antisera varied from 50 to 200 and were somewhat less than those for antisera against whole cells (600–1200).

Table 5. *Capacities of the subcellular fractions to adsorb the lymphoagglutinins present in an antiserum against pig lymphocytes*

The results are expressed in terms of the minimum amount of protein present in each fraction that was required to lower the lymphoagglutinin titre of antiserum 27 from 800 to 50 or less than 50; this decrease in titre is equivalent to the removal of at least 94% of the lymphoagglutinins. The values for the plasma membrane are given $\pm$ s.d. with the number of determinations in parentheses. Experimental details are given in the text.

Subcellular fraction	Protein (mg)
Filtrate	1.0
Nuclear pellet (300 <i>g</i> _{av.})	1.0
Mitochondrial pellet (4000 <i>g</i> _{av.})	0.4
20000 <i>g</i> _{av.} pellet	0.2
Ribosomal pellet (105 000 <i>g</i> _{av.})	0.4
Plasma membrane	0.07 ± 0.01 (3)
Supernatant (105 000 <i>g</i> _{av.})	$>10^*$

* This amount of supernatant caused no detectable decrease in the lymphoagglutinin titre.

EXPLANATION OF PLATE I

Electron micrograph of small lymphocytes from pig mesenteric lymph node. Magnification: $\times 21000$. The field shown was selected so as to illustrate the cellular components that could contaminate the plasma-membrane fraction.

EXPLANATION OF PLATE 2

Nuclear pellet (a) and mitochondrial pellet (b) obtained by differential centrifugation at $300g_{av}$ and $4000g_{av}$, respectively of the filtrate from the minced mesenteric lymph node. Magnifications: $\times 10250$ and $\times 41000$ respectively.

EXPLANATION OF PLATE 3

Pellet obtained by centrifuging the filtrate of minced lymph node between 4000 and 20000 g_{av} . Magnification: $\times 61\,000$.

EXPLANATION OF PLATE 4

Plasma-membrane fraction from pig small lymphocytes. Magnification: $\times 61\,000$.

Table 6. *Properties of pig thymus plasma membrane*

The values refer to two separate preparations each of which was isolated from 30g wet wt. of thymus. Enzymic activities are expressed as μmol of product liberated/h per mg of protein.

	Plasma membrane
Total protein (mg)	9.8; 13.6
RNA ($\mu\text{g}/\text{mg}$ of protein)	27; 31
Cholesterol ($\mu\text{g}/\text{mg}$ of protein)	205
5'-Nucleotidase	2.8; 4.7
Acid phosphatase	0.26
Glucose 6-phosphatase	0.05
Succinate dehydrogenase	0

Pig thymus plasma membrane

Plasma membrane was isolated from pig thymus by using a procedure identical with that described for the lymphocyte plasma membrane. Although the results were in general similar to those obtained with lymph node there were a number of differences. In particular a much smaller amount of residue was obtained on filtering the cell homogenate from 30g wet wt. of pig thymus, and the filtrate contained a much larger number of whole nuclei (5×10^{10} versus 1.3×10^{10} for lymph node). Also, the total succinate dehydrogenase activity of the filtrate was approx. 33% of that obtained with lymph node and the activity was evenly distributed by differential centrifugation between the nuclear, mitochondrial and 20000g pellets (cf. Table 2). The properties of the plasma-membrane fraction from pig thymus are given in Table 6. The most notable feature is the lower 5'-nucleotidase activity relative to that of the lymphocyte plasma membrane (cf. Table 3). The reason for the large difference between the 5'-nucleotidase activities of the two preparations of thymus plasma membrane is not known. This variation in activity was in marked contrast with that of the lymphocyte plasma membranes, which was less than 20% (Table 3).

DISCUSSION

Examination of sections of mesenteric lymph nodes from 7-month-old pigs and of single-cell suspensions prepared by gently disrupting the node indicated that at least 90% of the cells were mononuclear, had a diameter of $8\mu\text{m}$ or less and possessed a thin rim of cytoplasm only. It was concluded that most of the cells of the nodes were small lymphocytes.

A typical lymph node of 30g wet wt. contained approx. 5×10^{10} small lymphocytes together with vascular and connective tissue. During the disruption of the node the connective tissue formed a pad on the perforated plate of the mincer, and as

the cells were squeezed through the pad they were, apparently, stressed sufficiently for their surface membrane to be broken; approx. 95% of the cells were broken under these conditions. Provided that the node was disrupted in the absence of sucrose, nuclei could be removed by filtering the homogenate through muslin and by centrifuging the filtrate at 300g; 96% of the DNA of the filtrate sedimented with the nuclei. Since very little soluble DNA was released during homogenization, it appeared that most of the nuclei survived this treatment. Further, most of the nuclei recovered by centrifuging at 300g retained the integrity of their inner and outer membranes (Plate 2a). In view of these results it was considered unlikely that the cell homogenate contained significant amounts of nuclear membrane.

During the rupture of the cells the surface membrane formed vesicles, most of which sedimented between 4000 and 20000g. Examination of the 20000g pellet (Plate 3) revealed that some of the smooth-membrane vesicles contained varying amounts of cytoplasm including ribosomes. It seemed likely that portions of the cytoplasm had been enclosed during the vesiculation of the surface membrane. The 20000g pellet also contained a small proportion of rough-membrane vesicles. These were probably derived from rough endoplasmic reticulum, although it is possible that they arose from the outer nuclear membrane, which appeared to be associated with electron-dense ribosome-like particles (Plate 2a). The fractionation of this collection of vesicles presented a problem, since the vesicles were not only heterogeneous in size but also with respect to density owing to various amounts of trapped ribosomes and/or cytoplasm, or bound ribosomes. The same problem was encountered previously during the purification of the plasma membrane of HeLa cells (Boone *et al.* 1969), and was solved by sonicating the membranes to liberate the enclosed material. In the present work this technique gave unacceptably low yields of purified membrane. Alternatively, it was decided to isolate those vesicles with the lowest density by centrifuging them to equilibrium through a step-gradient of 40–30% sucrose. Under these conditions the rough membranes and the smooth-membrane vesicles with trapped ribosomes remained at the bottom of the centrifuge tube. In contrast, the interfacial band comprised mainly smooth-membrane vesicles containing little cytoplasm and very few ribosomes (Plate 4).

The purified smooth-membrane fraction contained no detectable DNA and small amounts only of RNA (21–33 $\mu\text{g}/\text{mg}$ of protein). Although the RNA may represent an integral part of the membrane (Glick & Warren, 1969; Davidova & Shapov, 1970), it appears more likely that it is primarily due

to contamination by ribosomes (cf. liver plasma membrane; Evans, 1970). The values for the DNA and RNA contents reflect a low degree of contamination of the smooth-membrane vesicles by nuclear elements, rough endoplasmic reticulum and/or ribosomes. The low activities of succinate dehydrogenase, glucose 6-phosphatase and acid phosphatase also indicated little contamination by mitochondria, endoplasmic reticulum and lysosomes respectively. These interpretations of the results are supported by the morphological appearance of the plasma-membrane fraction (Plate 4). The above results do not, however, rule out the possibility that some or all of the purified smooth-membrane fraction was derived from the internal smooth-membrane vesicles seen in whole lymphocytes (Plate 1). This possibility is considered to be unlikely for a number of reasons. First, the smooth-membrane fraction possessed a high value for the cholesterol/phospholipid molar ratio (1.01). Similar values have been obtained for the plasma membranes of a number of cell types and have been assumed to indicate a high degree of purity of the plasma membrane (Coleman & Finean, 1966), whereas a much lower value has been reported for the internal smooth membrane of HeLa cells (Bosmann *et al.* 1968). Secondly, the specific activity of 5'-nucleotidase, which is a recognized marker of plasma membrane, was increased 12-fold in the purified smooth-membrane fraction relative to the filtrate of the minced lymph node. Similar increases in the specific activity of 5'-nucleotidase have been obtained during the purification of plasma membranes from a variety of cell types (Coleman & Finean, 1967). Thirdly, the smooth-membrane fraction possessed a high capacity to adsorb the lymphoagglutinins from antisera prepared by immunization with whole lymphocytes. This capacity was increased in the membrane fraction relative to the initial filtrate by a similar extent (14-fold) to that observed for 5'-nucleotidase (12-fold). On the other hand, the increase in this capacity was 28-fold when expressed as dry weight of membrane relative to dry weight of whole lymphocytes. The reason for the above discrepancy is not known, but it seems likely that it is related to the use of different parameters (protein versus dry weight) and that the initial filtrate is enriched in the lymphocyte surface membrane. If it is assumed that the receptor site for lymphoagglutinin is located exclusively on the surface of lymphocytes, then the degree of purification of the surface membrane was 28-fold by dry weight. In contrast, a 49-fold purification was determined for the plasma membrane of HeLa cells by using a related technique (Boone *et al.* 1969). However, since the diameter of a HeLa cell is two to three times that of a small lymphocyte, the surface area/mass ratio

for a small lymphocyte is three to five times that of a HeLa cell. The maximum possible degree of purification of the surface membrane relative to the cell mass is therefore three to five times greater for HeLa cells than for small lymphocytes. As a result the degree of purification of the lymphocyte surface membrane was probably greater than that achieved by Boone *et al.* (1969) for the plasma membrane of HeLa cells. It was concluded from the above results that the purified smooth-membrane vesicles represent the plasma membrane of small lymphocytes.

The method described for preparing lymphocyte plasma membrane from pig mesenteric lymph node was relatively mild, comparatively simple and gave reproducible results. The yield of plasma membrane (0.1% dry wt. of membrane relative to wet wt. of lymph node) was comparable with that which has been achieved with other tissues. Further, morphological examination of the side fractions suggested that purified preparations of whole nuclei, mitochondria and ribosomes could be easily obtained by using essentially the same method. Also, it appeared that the method is generally applicable to other organs (e.g. thymus) provided that they contain a suitable amount of connective tissue.

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