Solubilization of Pig Lymphocyte Plasma Membrane and Fractionation of some of the Components

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The degree of solubilization of pig lymphocyte plasma membrane by sodium deoxycholate was determined at a variety of temperatures and detergent concentrations. Approx. 95% of the membrane protein was soluble in 2% deoxycholate at 23°C. Some of the biological activities of the membrane survived this treatment. The leucine β -naphthylamidase activity was more readily soluble than the 5'-nucleotidase and these enzymes could be separated by extraction with 0.5% deoxycholate at 0°C. Membrane solubilized in 2% deoxycholate at 23°C was fractionated by sucrose-density-gradient centrifugation in 1% deoxycholate. The phospholipid was separated from the protein, which formed a fairly symmetrical peak that sedimented slightly slower than ovalbumin; the leucine naphthylamidase and 5'-nucleotidase activities were resolved from each other and from the main protein peak. Similar separations were achieved by elution from Sephadex G-200 and Sepharose 6B in 1% deoxycholate. The main proteins, however, appeared to possess much higher molecular weights than those indicated by sucrose-density-gradient centrifugation. This disparity suggests that many of the membrane proteins have a rod-like shape, especially since the results of experiments with [^{14}C]deoxycholate revealed that the proteins did not bind significant amounts of deoxycholate. In contrast, 5'-nucleotidase and leucine naphthylamidase appeared to be globular. Polyacrylamide-gel electrophoresis of membrane solubilized in sodium dodecyl sulphate gave a similar distribution of protein to that achieved by sucrose-density-gradient centrifugation. Trace amounts only of polypeptides of molecular weight less than 10000 were detected.

It is generally accepted that the molecular structure of the cell surface determines many of the functions of the cell. If this view is correct then an intimate knowledge of the structure of the surface membrane is essential to define the molecular bases of the biological activities that are mediated by the cell surface. One approach to the problem of determining the surface structure is to completely solubilize the cell plasma membrane by using conditions that do not destroy the biological activities, and then to separate and characterize the biologically active molecules. Although a large variety of methods have been employed to dissolve cell membranes (a comprehensive selection of references is given by Hoogeveen, Juliano, Coleman & Rothstein, 1970), complete solubilization coupled with retention of biological activity has rarely been achieved. The most promising results have, however, in general been obtained by using detergents such as Lubrol W-X, Triton X-100 and sodium deoxycholate (e.g. Fitzpatrick, Davenport, Forte & Landon 1969; van't Riet & Planta, 1969; Dulaney & Touster, 1970; Miller, 1970).

The plasma membrane of pig small lymphocytes was chosen for investigation because it mediates important immunological phenomena, and because it is readily available in a purified form that retains many of the biological activities associated with the surface of the whole cell (Allan & Crumpton, 1970; Allan, Auger & Crumpton, 1971). Sodium deoxycholate was selected as the detergent since the results of preliminary experiments indicated that it dissolved the membrane almost completely and that at least some of the biological activities survived solubilization. The present paper describes the solubilization of the lymphocyte plasma membrane by sodium deoxycholate, and the separation of some of the membrane components by sucrose-density-gradient centrifugation and by gel filtration. A preliminary account of the work has been published (Allan & Crumpton, 1971), and a report has appeared (Allan *et al.* 1971) of the solubilization and fractionation of the lymphocyte plasma-membrane receptors for the lymphoagglutinating and lymphocyte-stimulating activities of *Phaseolus vulgaris* phytohaemagglutinin.

MATERIALS AND METHODS

Materials. Chemicals were of analytical grade and glass-distilled water was used in all experiments. Sodium deoxycholate (lot 58B-2440) was purchased from Sigma (London) Chemical Co. Ltd., London S.W.6, U.K.; the pH of a 1% (w/v) solution in water was 7.7 and $E_{1\text{cm}}^{1\%}$ at 280 nm was 0.01. [*carboxy*- ^{14}C]Deoxycholic acid (lot no. 3216; 7.4 mCi/mmol) was from Mallinckrodt Radiochemicals, St Louis, Mo., U.S.A.; the radioactive material occupied an identical position with that of added carrier sodium deoxycholate when examined by t.l.c. in a variety of solvent systems (Dr P. M. D. Hardwicke, personal communication).

Blue Dextran, Sephadex G-200 and Sepharose 6B were purchased from Pharmacia (G.B.) Ltd., London W.13, U.K. Proteins of known molecular weight, used for calibration of sucrose density gradients, columns of Sephadex G-200 and Sepharose 6B, and polyacrylamide disc-gels were obtained as follows: glucagon (cryst.; mol.wt. 3483), bovine insulin (cryst.; mol.wt. 5734), horse heart cytochrome *c* (type VI; mol.wt. 12384), ovalbumin (grade V; mol.wt. 43000) and bovine serum albumin (cryst.; mol.wt. 69000) were from Sigma (London) Chemical Co. Ltd.; soya-bean trypsin inhibitor (mol.wt. 21500) was from Worthington Biochemical Corp., Freehold, N.J., U.S.A.; ^3H -labelled mouse immunoglobulin M and light chains (mol.wt. assumed to be 900000 and 25000 respectively; Parkhouse & Askonas, 1969) were donated by Dr R. M. E. Parkhouse; sperm-whale metmyoglobin (mol.wt. 17816) was prepared as described by Crumpton & Wilkinson (1965); the *N*-terminal 53-amino acid peptide of myoglobin (mol.wt. 6416; Edmundson, 1963) was obtained from apomyoglobin by cleavage with cyanogen bromide and was purified by elution from Sephadex G-50 with 6M-urea-m-propionic acid; pig immunoglobulin G [mol.wt. assumed to be 151000 by analogy with horse immunoglobulin G (Pain, 1963)] was prepared from serum by precipitation with Na_2SO_4 (Kekwick, 1940) and subsequent elution from DEAE-Sephadex A-50 equilibrated with 10 mM-sodium phosphate buffer, pH 7.0 (Fráněk & Lankaš, 1963).

Acrylamide, *N*:*N*-methylene-bisacrylamide and sodium dodecyl sulphate (specially pure) were purchased from BDH (Chemicals) Ltd., Poole, Dorset, U.K., and *NNN'*-tetramethylethylenediamine was from Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K. Naphthalene Black 10B was obtained from G. T. Gurr Ltd., London N.W.9, U.K., and Coomassie Brilliant Blue R was from E. Gurr Ltd., London S.W.14, U.K.

L-Leucine β -naphthylamide hydrochloride was purchased from Koch-Light Laboratories Ltd., *N*-1-naphthylethylenediamine dihydrochloride was from BDH (Chemicals) Ltd., and the disodium salt of ATP was from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany.

Mesenteric lymph nodes and serum were obtained from young pigs (average age 7 months), which had been bred for bacon, and were given by T. Wall and Sons Ltd., London W.3, U.K. The lymphocyte plasma membrane was prepared as described by Allan & Crumpton (1970). The protein and RNA contents, and 5'-nucleotidase activities of the preparations were determined; the values agreed closely with those reported previously.

Freshly prepared samples (within 48 h of the death of the animal) only were used.

Solubilization of membrane. This was usually achieved at room temperature by addition of a suitable volume of a 10% (w/v) solution of sodium deoxycholate in water (pH 7.7) to a suspension of plasma membrane (about 5 mg of membrane protein/ml of water). After 15 min the mixture was centrifuged (Spinco model L) at 100000 g_{av} for 1 h; the material present in the supernatant was assumed to be soluble. Under these conditions, 2% sodium deoxycholate dissolved 95% of the membrane protein.

Fractionation of the solubilized membrane. This was carried out either by sucrose-density-gradient centrifugation or by elution from columns of Sephadex G-200 and Sepharose 6B.

Samples of the above supernatant (1 ml containing 4–5 mg of protein in 2% sodium deoxycholate) were layered over continuous gradients of sucrose [9.5 ml of 30% (w/v) against 9.5 ml of 10%] dissolved in 1% sodium deoxycholate and were centrifuged (MSE 65 centrifuge; 8 $\times$ 25 Ti angle rotor) at 45000 rev./min for 17 h at 0°C. After centrifugation, 1 ml fractions were collected from the bottom of the tube by siphoning the contents with an Everett automatic syringe. A plot of $s_{20,w}^0$ ($\times 10^{-13}$ s) for pig immunoglobulin (7.2), bovine serum albumin (4.41), ovalbumin (3.55) and myoglobin (2.08) versus their peak positions after centrifuging under the above conditions gave a straight line.

Solubilized membrane was also fractionated at 2°C by using Whatman chromatography columns (W. & R. Balston Ltd., Maidstone, Kent, U.K.) containing Sephadex G-200 or Sepharose 6B (88.9 cm $\times$ 1.5 cm and 94.1 cm $\times$ 1.5 cm respectively). Samples (1 ml) were eluted with 1% sodium deoxycholate by upward-flow at a rate of 5.1 ml/h (Perpex pump; LKB Instruments Ltd., South Croydon, Surrey, U.K.). The effluent was monitored by a Uvicord II spectrophotometer at 280 nm and timed fractions (4/h) were collected. To prevent the formation of bubbles, columns were eluted continuously; the reservoir was replenished with fresh solvent at weekly intervals and was provided with a soda lime guard tube. Column void volumes were measured by using Blue Dextran (68.7 ml and 57.7 ml for Sephadex G-200 and Sepharose 6B respectively), and the total volumes were determined from the column dimensions (157 ml and 166 ml respectively). The elution volumes of standard proteins were determined by extrapolating to the peak position the leading and trailing edges of the elution zone; values determined for the same protein on different occasions differed by no more than $\pm 2\%$. Plots of K_{av} (Laurent & Killander, 1964) versus log mol.wt. for cytochrome *c*, soya-bean trypsin inhibitor, bovine serum albumin and immunoglobulin G on Sephadex G-200, and for these proteins and immunoglobulin M on Sepharose 6B, gave straight-line relationships.

Polyacrylamide-disc-gel electrophoresis. This was carried out by a modification of the method of Summers, Maizel & Darnell (1965) in columns (10 cm $\times$ 0.8 cm) of 7.5% (w/v) polyacrylamide gel in 0.1M-sodium phosphate buffer, pH 7.2, containing 0.1% sodium dodecyl sulphate; the same buffer was used in the electrode vessels. Materials for electrophoresis were dissolved in 5% (w/v) sodium dodecyl sulphate or in 5% sodium dodecyl sulphate–2.5% 2-mercaptoethanol; the solutions were immediately

heated in a boiling-water bath for 5 min to dissociate macromolecular complexes (Maizel, 1969) and to minimize proteolysis (Pringle, 1970). Samples [100 μ l of membrane protein (5 mg/ml) or 20 μ l of standard protein solution (5 mg/ml)] were mixed with 0.1 vol. of 0.02% Bromophenol Blue and 0.2 vol. of 80% (w/v) sucrose, and were layered under the electrolyte on to the top of the gel. Electrophoresis was carried out at room temperature for 17 h at a constant current of 4.5 mA/tube; the voltage gradient was about 1 V/cm. Under these conditions the Bromophenol Blue moved 7–8 cm through the gel. Gels were stained for 45 min with a 5% saturated solution of Naphthalene Black 10B in methanol–water–acetic acid (3:6:1, by vol.) and were destained in the same solvent. Alternatively, gels were thoroughly washed with 20% (w/v) sulphosalicylic acid for 24 h, stained with 0.02% Coomassie Brilliant Blue R in 20% sulphosalicylic acid for 30–40 min and destained in 7% (v/v) acetic acid. Glucagon and insulin were soluble in methanol–water–acetic acid but were satisfactorily revealed by sulphosalicylic acid–Coomassie Blue.

Other methods. Leucine β -naphthylamidase activity was measured as described by Goldburg & Rutenburg (1958). Adenosine triphosphatase was assayed in a total volume of 2 ml of 50 mM-tris-HCl buffer, pH 7.4; 2.5 mM-ATP; 10 mM-MgCl₂; 0.1 M-KCl; 0.1 M-NaCl; 10 mM-sodium potassium tartrate. After 15 min incubation at 37°C the reaction was stopped by the addition of 1 ml of 25% (w/v) trichloroacetic acid. P_i was determined at room temperature by a modification of the method of Fiske & Subbarow (1925); 2 ml of the supernatant and 0.1 ml of 'Fiske & Subbarow reducer' (Sigma Chemical Co., St Louis, Mo., U.S.A.) were used.

The radioactivities of ³H-labelled proteins and [¹⁴C]deoxycholate (0.2 and 0.1 ml samples respectively in 10 ml of Zimm scintillation fluid; Kinard, 1957) were counted for 1 and/or 10 min in a Tri-Carb Liquid Scintillation Spectrometer; results were not corrected for efficiency nor quenching. Other methods were performed as described by Allan & Crumpton (1970).

RESULTS

Solubilization of plasma membrane

The effect of increasing concentration of sodium deoxycholate on the 5'-nucleotidase, leucine naphthylamidase and Na⁺-stimulated adenosine triphosphatase activities of the lymphocyte plasma membrane is shown in Fig. 1. The 5'-nucleotidase activity increased to about 130% of its initial value in 1% sodium deoxycholate and then decreased gradually to its original value in 5% sodium deoxycholate; the apparent activation of enzyme activities by detergent was noted previously and has been ascribed to a transition of the enzyme from a 'bound' to a 'soluble' form or to the dissociation of an inhibitor (e.g. Owen & Freer, 1970; Sirakov & Kochakian, 1970). In contrast, adenosine triphosphatase and leucine naphthylamidase were apparently inactivated by sodium deoxycholate. Under the conditions described in Fig. 1 the former activity was almost completely destroyed by 0.2%

Fig. 1. Effect of increasing concentration of sodium deoxycholate on the 5'-nucleotidase (○), leucine naphthylamidase (△) and Na⁺-(Mg²⁺+K⁺)-stimulated adenosine triphosphatase (□) activities of the lymphocyte plasma membrane. Samples of the membrane suspension (800 μ g of protein) were incubated at 0°C for 15 min with increasing amounts of a 10% (w/v) solution of sodium deoxycholate in a final volume of 1.0 ml of water. After 15 min, samples were assayed for 5'-nucleotidase (200 μ l), leucine naphthylamidase (400 μ l) and adenosine triphosphatase (200 μ l) as described in the text. A control comprising membrane in the absence of deoxycholate was included; the activities (μ mol of product liberated/h per mg of protein at 37°C) of 5'-nucleotidase, leucine naphthylamidase and adenosine triphosphatase were 8.7, 1.5 and 4.2 respectively.

sodium deoxycholate, whereas about 80% of the leucine naphthylamidase activity was destroyed by 1% sodium deoxycholate. Treatment of a much higher concentration of membrane [6 mg of protein/ml (Table 1) versus 800 μ g/ml (Fig. 1)] with 0.5% sodium deoxycholate caused, however, no detectable decrease in the leucine naphthylamidase activity, and a decrease of about 65% only in the adenosine triphosphatase activity (Table 1). These results suggest that the degrees of inactivation of leucine naphthylamidase and adenosine triphosphatase are dependent on the ratio of the amount of membrane protein to the amount of deoxycholate. However, since a smaller volume of the more concentrated membrane was used to measure the leucine naphthylamidase activity [20 μ l (Table 1) compared with 400 μ l (Fig. 1)], it is possible that, in this case, the degree of apparent inactivation was determined by the amount of sodium deoxycholate in the assay mixture. Inactivation may also be related to interference by deoxycholate with the

metal ion requirements of leucine naphthylamidase (Mahadevan & Tappel, 1967) and adenosine triphosphatase.

Fig. 2 shows the degree of solubilization of the membrane protein and the 5'-nucleotidase activity with increasing concentration of sodium deoxycholate at 0°C and 23°C. The results revealed that

Fig. 2. Degree of solubilization at 0°C and 23°C of membrane protein (○ and ● respectively) and 5'-nucleotidase activity (△ and ▲ respectively) by increasing concentrations of sodium deoxycholate. Samples of a suspension of lymphocyte plasma membrane (700 μg of protein in 0.1 ml of water; 5'-nucleotidase activity, 8.7 μmol of P_i liberated/h per mg of protein) were incubated for 15 min with increasing amounts of deoxycholate dissolved in 0.9 ml of water. The degree of solubilization was measured after centrifuging at 100 000g for 1 h; the results are expressed as % of a control sample that was not treated with deoxycholate or centrifuged.

the degree of solubilization depended on the deoxycholate concentration and the temperature. Essentially all the membrane protein and 5'-nucleotidase activity were soluble in 2% sodium deoxycholate at 23°C, and these conditions were subsequently used routinely to dissolve the membrane. In contrast, at 0°C 5% sodium deoxycholate failed to dissolve the membrane completely. The results shown in Fig. 2 also revealed that at 0°C the membrane protein was preferentially dissolved relative to the 5'-nucleotidase by low concentrations of deoxycholate. This view was confirmed by a comparison of the extents of solubilization of protein and 5'-nucleotidase by a constant amount of sodium deoxycholate at a variety of temperatures between 0° and 35°C. Maximal solubilization of the 5'-nucleotidase activity was achieved at 23°C whereas, in contrast, the leucine naphthylamidase activity was more soluble at 0°C. The relative amounts of protein, phospholipid, 5'-nucleotidase, leucine naphthylamidase and adenosine triphosphatase extracted by 0.5% sodium deoxycholate at 0°C are shown in Table 1. Subsequent solubilization of the pellet in 2% sodium deoxycholate at 23°C gave a threefold increase in the specific activity of 5'-nucleotidase relative to the plasma membrane. Further, the above procedure allowed an almost complete separation of the 5'-nucleotidase and leucine naphthylamidase activities.

Fractionation of solubilized membrane

Sucrose-density-gradient centrifugation. Lymphocyte plasma membrane was extracted with 2% sodium deoxycholate at 23°C and the soluble material (95% of the membrane protein) was fractionated by centrifugation for 17 h on a continuous sucrose density gradient. The profile across the sucrose density gradient of a typical run is

Table 1. *Relative amounts of protein, phospholipid, 5'-nucleotidase, leucine naphthylamidase and adenosine triphosphatase extracted from lymphocyte plasma membrane by 0.5% sodium deoxycholate at 0°C*

Plasma membrane (6 mg of protein) suspended in 1 ml of 0.5% sodium deoxycholate was incubated at 0°C for 15 min and then centrifuged at 100 000g for 1 h. The supernatant was removed and the pellet was resuspended in 1 ml of 0.5% sodium deoxycholate. The volume of samples used for assay of enzymic activities were as follows: 5'-nucleotidase, 100 μl; leucine naphthylamidase, 20 μl; adenosine triphosphatase, 200 μl. The enzymic activities of the original membrane are expressed as μmol of product liberated/h per mg of protein.

	Before extraction	After extraction (% of original)	
		Supernatant	Pellet
Protein (mg)	6	60	40
Phospholipid (μg/mg of protein)	440	45	55
5'-Nucleotidase	10.3	5	95
Leucine naphthylamidase	1.5	95	5
Na ⁺ -(Mg ²⁺ + K ⁺)-stimulated adenosine triphosphatase	3.6	21	14

Fig. 3. Fractionation of solubilized lymphocyte plasma membrane by sucrose-density-gradient centrifugation. Plasma membrane (4.8 mg of protein) was incubated for 15 min at 23°C in 1.1 ml of 2% sodium deoxycholate and then centrifuged at 100000g for 1 h. The supernatant (1 ml) was layered on to the gradient [19 ml of 10–30% (w/v) sucrose dissolved in 1% sodium deoxycholate], and after centrifuging at 45000 rev./min for 17 h in a MSE 8×25 Ti angle rotor, 1 ml fractions were removed from the bottom of the tube by using a syringe. Samples were analysed for protein (○) by the method of Lowry, Rosebrough, Farr & Randall (1951), phospholipid (●), 5'-nucleotidase (Δ) and leucine naphthylamidase (□). Enzymic activities are expressed as μmol of product liberated/h and the molecular weight of phospholipid was assumed to be 775. The arrows indicate the positions of standard proteins centrifuged in separate tubes under the same conditions: IgG, pig immunoglobulin G; BSA, bovine serum albumin; Ov, ovalbumin; Mb, sperm-whale myoglobin. The top of the gradient is at the right of the figure.

Fig. 4. Sucrose-density-gradient centrifugation of lymphocyte plasma membrane solubilized in the presence of [¹⁴C]deoxycholate. The experimental conditions were identical with those described for Fig. 3 except that 5 μl of 0.21 M [¹⁴C]deoxycholic acid (7.4 mCi/mmol) was added to 1 ml of soluble membrane (3.6 mg of protein in 2% sodium deoxycholate) before addition to the gradient. Fractions were analysed for protein (○) by measurement of E_{280}^{1em} , and for radioactivity (●; 0.1 ml samples).

shown in Fig. 3. The phospholipid remained near the top of the gradient and was resolved from the majority of the membrane proteins which formed a fairly symmetrical peak that sedimented slightly slower than ovalbumin. A small pellet containing no more than 5% of the protein added to the gradient was usually present. Measurement of protein as E_{280} (see Fig. 4) and E_{220} gave similar profiles to that shown in Fig. 3 except for an increase in extinction at the top of the gradient due to light-scattering. The 5'-nucleotidase and leucine naphthylamidase activities were partly resolved from each other and from the majority of the protein. 5'-Nucleotidase sedimented at the same rate as pig immunoglobulin G, whereas the results of experiments in which the gradients were centrifuged at a slower speed than that employed in Fig. 3 suggested that leucine naphthylamidase had a sedimentation coefficient of about 12S.

Sucrose-density-gradient centrifugation of lymphocyte plasma membrane which had been dissolved in the presence of sodium [¹⁴C]deoxycholate revealed that the radioactivity remained at the top of the gradient and that the majority of the proteins did not bind significant amounts of radioactivity (Fig. 4).

Fig. 5. Elution of solubilized lymphocyte plasma membrane from Sephadex G-200. Plasma membrane (4.5 mg of protein in 1 ml of 2% sodium deoxycholate) was added to a column (88.9 cm $\times$ 1.5 cm) of Sephadex G-200 and eluted with 1% sodium deoxycholate. Fractions (1.28 ml) were collected and were analysed for protein (○) by the method of Lowry *et al.* (1951), phospholipid (●), 5'-nucleotidase (△) and leucine naphthylamidase (□). Enzymic activities are expressed as μ mol of product liberated/h, and the molecular weight of phospholipid was assumed to be 775. The arrows indicate the positions of standard proteins eluted under the same conditions: IgG, pig immunoglobulin G; BSA, bovine serum albumin; STI, soya-bean trypsin inhibitor; Cyc, cytochrome *c*.

Gel filtration. Fig. 5 shows the fractionation of solubilized lymphocyte plasma membrane by elution with 1% sodium deoxycholate from a column of Sephadex G-200 calibrated with standard proteins. Analysis of protein as E_{280} and E_{220} gave similar results except for a small peak in the phospholipid position. No evidence was obtained for the presence of significant amounts of protein with a molecular weight less than that of cytochrome *c*. As observed previously by sucrose-density-gradient centrifugation, the phospholipid was resolved from the protein, and the 5'-nucleotidase and leucine naphthylamidase activities were partly separated from each other. Also, the 5'-nucleotidase occupied a position identical with that of immunoglobulin G, and the elution position of leucine naphthylamidase corresponded to a molecular weight of about 300 000. However, the majority of the protein behaved as if it possessed a much higher molecular weight than that indicated by sucrose-density-gradient centrifugation. This discrepancy was confirmed by elution from Sephadex G-200 of the protein peak separated by sucrose-density-gradient centrifugation; fraction nos. 11–16 inclusive (Fig. 3) were concentrated by pressure dialysis against 1% sodium deoxycholate before addition to

the column of Sephadex G-200. Under these conditions the protein was resolved into two peaks of approximately equal area and whose positions corresponded to molecular weights of about 100 000 and 200 000.

Elution of solubilized membrane from a column of Sepharose 6B in 1% sodium deoxycholate (Fig. 6) gave similar results to those obtained by using Sephadex G-200. The majority of the membrane protein formed a fairly symmetrical peak, the position of which corresponded to a molecular weight of about 100 000 and which was separated from material eluted in the void volume of the column. The area of the excluded peak, measured in terms of E_{220} or E_{280} , represented about 10% of the total area of eluted material. The E_{220} peak in the position of cytochrome *c* was due to light-scattering and did not represent protein.

Fractionation of membrane that had been solubilized in 2% sodium [14 C]deoxycholate on columns of Sephadex G-50 eluted with 1% sodium deoxycholate gave an almost complete separation of protein from radioactivity.

Polyacrylamide-gel electrophoresis. Plate 1(a) shows the protein-band pattern of lymphocyte plasma membrane solubilized in sodium dodecyl

EXPLANATION OF PLATE I

Polyacrylamide-gel electrophoresis in 0.1% sodium dodecyl sulphate of plasma membrane of pig lymphocytes. (a) unfractionated plasma membrane; (b) and (c), fraction nos. 5-10 and 11-16 respectively of membrane solubilized in sodium deoxycholate and centrifuged on a sucrose density gradient (Fig. 3); (d) unfractionated plasma membrane; (e) *N*-terminal 53-amino acid peptide of sperm-whale myoglobin. (a), (b) and (c), Samples were dissolved in 5% sodium dodecyl sulphate before electrophoresis and were stained with Naphthalene Black. (d) and (e), Samples were dissolved in 5% sodium dodecyl sulphate-2.5% 2-mercaptoethanol and stained with Coomassie Blue. The arrows indicate the positions of the centres of the bands given by standard proteins dissolved in 5% sodium dodecyl sulphate. IgG, pig immunoglobulin G; BSA, bovine serum albumin; Ov, ovalbumin.

Fig. 6. Elution of solubilized lymphocyte plasma membrane from Sepharose 6B. Plasma membrane (4.0 mg of protein in 1 ml of 2% sodium deoxycholate) was added to a column of Sepharose 6B (94.1 cm $\times$ 1.5 cm) and eluted with 1% sodium deoxycholate. Fractions (1.28 ml) were collected and were analysed for protein by measurement of $E_{220}^{1\text{cm}}$ (○) and $E_{280}^{1\text{cm}}$ (●). The arrows indicate the positions of standard proteins eluted under the same conditions: IgM, mouse immunoglobulin M; IgG, pig immunoglobulin G; BSA, bovine serum albumin; Cyc, cytochrome *c*.

sulphate, electrophoresed in polyacrylamide gel and stained with Naphthalene Black; an identical pattern was obtained by fixing the protein bands with sulphosalicylic acid and staining with Coomassie Blue. A small amount of material was excluded from the gel; this probably corresponds to protein eluted in the first peak from Sepharose 6B. Material entering the gel was resolved into about 20 bands of differing intensities and a turbid unstained band, due to lipid, in the position of the Bromophenol Blue marker. No protein was detected in front of the lipid band. A comparison of the positions of the protein bands with those of standard proteins run under the same conditions indicated that the more intensely-staining bands had molecular weights within the range 40 000–100 000. This interpretation was confirmed by examination of the faster-sedimenting material and the main protein peak that had been separated by sucrose-density-gradient centrifugation (Plates 1b and 1c respectively). It was concluded that the separation of membrane proteins achieved by polyacrylamide-gel electrophoresis in sodium dodecyl sulphate resembled more closely the fractionation obtained by sucrose-density-gradient centrifugation in 1%

sodium deoxycholate than that given by elution from Sepharose 6B.

Electrophoresis of membrane that had been solubilized in sodium dodecyl sulphate and mercaptoethanol gave a somewhat different band pattern from that of membrane dissolved in sodium dodecyl sulphate alone; Plate 1(d) shows the pattern revealed by staining the reduced proteins with Coomassie Blue. Reduction was associated with the almost complete disappearance of material excluded from the gel, the loss of some protein bands and the appearance of some new bands. Negligible amounts of stain only were detected in front of the lipid band whereas the *N*-terminal 53-amino acid peptide of myoglobin, which was used as a marker of low molecular weight, stained intensely (Plate 1e). It was concluded that the lymphocyte plasma membrane probably does not contain significant amounts of polypeptides of molecular weight less than 10 000 (cf. 'miniproteins'; Laico, Ruoslahti, Papermaster & Dreyer, 1970).

DISCUSSION

A wide variety of approaches to the solubilization of membrane proteins have been explored previously. Extraction of the membrane with either detergent or organic solvent has been widely employed, and the results have varied from the complete solubilization of the membrane to the selective removal of one or more components. Solubilization of the membrane has, however, been frequently associated with a loss of the biological activities, and the subsequent fractionation of the soluble proteins has, apart from some notable exceptions, proved relatively unrewarding. The present work has shown that sodium deoxycholate solubilizes almost completely the plasma membrane of pig lymphocytes while preserving at least some of the biological activities. Further, the potential fractionation of the soluble extract by affinity chromatography appears promising in view of the observation that specific molecular interaction may be retained in the presence of the concentration of sodium deoxycholate used for solubilization (Crompton, 1971).

The degree of solubilization of the lymphocyte plasma membrane by sodium deoxycholate depends on the concentration of membrane, the absence or presence of salt, the concentration of detergent and the temperature. In the absence of salt, maximal solubilization of 5 mg of membrane protein/ml was achieved in 2% sodium deoxycholate at 23°C. Under these conditions 95% of the protein was not sedimented by centrifugation at 100 000*g* for 1 h and, further, 90% of the supernatant protein was not excluded by Sepharose 6B in 1% sodium deoxycholate (Fig. 6). It was concluded that at

least 85% of the membrane protein was truly soluble in sodium deoxycholate. The 5'-nucleotidase and leucine naphthylamidase activities of the membrane survived solubilization whereas, in agreement with previous results (Chignell & Titus, 1969; Towle & Copenhaver, 1970), the Na⁺-stimulated adenosine triphosphatase activity was destroyed by low concentrations (0.2%) of the detergent (Fig. 1). The leucine naphthylamidase activity was more readily soluble than the 5'-nucleotidase activity, especially at 0°C. Extraction of the membrane with 0.5% sodium deoxycholate at 0°C dissolved about 70% of the protein and 95% of the leucine naphthylamidase but only 5% of the 5'-nucleotidase. On increasing the sodium deoxycholate concentration to 2% and the temperature to 23°C a further 25% of the protein and over 90% of the 5'-nucleotidase activity dissolved. This differential solubilization procedure allowed an almost complete separation of these enzyme activities and gave a threefold increase in the specific activity of 5'-nucleotidase; the enzymes were also partly resolved from each other by elution of the solubilized membrane from Sephadex G-200 (Fig. 5) or by centrifuging through a sucrose density gradient (Fig. 3). The different solubilities of membrane proteins probably reflect different degrees of binding to the membrane. In this case, 5'-nucleotidase is more firmly bound or occupies a less superficial position in the membrane layer and, as a result, may be more characteristic of membrane proteins than leucine naphthylamidase.

Lipid-protein interactions have been suggested to play an important role in determining membrane structure (Wallach, 1969). Solubilization of the membrane in 2% sodium deoxycholate was associated with the almost complete destruction of these interactions, since the majority of the phospholipid was resolved from the protein by fractionating the solubilized membrane on sucrose density gradients or Sephadex G-200 (Figs. 3 and 5); separation of lipid from protein on treatment of membranes with detergents has also been reported by other workers (Engelman, Terry & Morowitz, 1967; Salton & Schmitt, 1967; Jones & Kennedy, 1969). However, the possibility that a fraction of the lipid in the membrane is more firmly bound to protein than the remainder, and the presence in the soluble extract of some lipoprotein complexes, cannot be ruled out at present. Indeed it seems likely, in view of the results of Widnell & Unkeless (1968), that the soluble 5'-nucleotidase is still associated with lipid.

Solubilization of the membrane was probably also associated with the destruction of most of the protein-protein interactions. Sucrose-density-gradient centrifugation showed (Fig. 3) that the majority of the soluble protein sedimented at about the same rate as ovalbumin, although some protein

sedimented at a faster rate. These results suggested that a major fraction of the membrane proteins had a molecular weight of about 45 000. The results of polyacrylamide-gel electrophoresis of membrane dissolved in sodium dodecyl sulphate were not inconsistent with this suggestion since under these conditions the major protein species appeared to have molecular weights within the range 40 000–100 000. Similar distributions of molecular weights for membrane proteins have been described previously. For example, Rosenberg & Guidotti (1968) reported that many of the proteins of human erythrocyte membranes have molecular weights close to 50 000, whereas Demus & Mehl (1970) concluded that 92% of erythrocyte membrane proteins have molecular weights within the range 26 000–65 000 by polyacrylamide-gel gradient electrophoresis in phenol-formic acid-water. Also, Kiehn & Holland (1970) reported that most of the proteins of animal cell membranes possess molecular weights of 45 000–85 000 after reduction and polyacrylamide-gel electrophoresis in sodium dodecyl sulphate.

In contrast with sucrose-density-gradient centrifugation, most of the soluble lymphocyte membrane protein appeared to have a much higher molecular weight on Sephadex G-200 and Sepharose 6B. The reason for this discrepancy is not known. However, since the elution positions of proteins are related to their molecular shapes (Stokes radius) rather than their molecular weights (Ackers, 1964) it seems probable that many of the membrane proteins have a rod-like shape. It is at present uncertain whether these proteins have intrinsically high axial ratios in the membrane or whether unfolding of the polypeptide chains occurred during solubilization. The validity of the latter suggestion is, however, questionable as 1% sodium deoxycholate caused no gross conformational change in sperm-whale metmyoglobin or pig immunoglobulin G (Crumpton, 1971, and unpublished observations). As a result, the presence in the membrane of extended polypeptide chains appears likely, although the possibility that the conformations of the membrane proteins are unusually sensitive to alteration by deoxycholate cannot be ruled out. In contrast with the majority of the membrane proteins, 5'-nucleotidase and leucine naphthylamidase possessed the same molecular weights (150 000 and approx. 300 000 respectively) by sucrose-density-gradient centrifugation and by elution from Sepharose 6B, and were consequently judged to be globular. It was concluded that the present results are consistent with the schematic model of membrane structure proposed by Lenard & Singer (1966) and Glaser, Simpkins, Singer, Sheetz & Chan (1970).

The results of sucrose-density-gradient centrifugation indicated that, contrary to erythrocyte membranes (Gwynne & Tanford, 1970), polypeptide

chains of very high molecular weight (about 200 000) do not represent a major constituent of the lymphocyte membrane, especially since some of the protein in fraction nos. 5–10 of the gradient (Fig. 3) is immunoglobulin (D. Allan & M. J. Crumpton, unpublished work). On the other hand, elution of solubilized membrane from Sephadex G-200 or Sepharose 6B, and polyacrylamide-gel electrophoresis in sodium dodecyl sulphate failed to reveal the presence of significant amounts of polypeptides of molecular weight less than 10 000 ('miniproteins'; Laico *et al.* 1970). It was considered unlikely that the failure to detect polypeptides of low molecular weight was due to technical reasons (cf. Laico *et al.* 1970) since fresh membrane was used (within 36 h of the death of the animal) and the low-molecular-weight polypeptides (insulin, glucagon and the N-terminal 53-amino acid peptide of myoglobin) employed as markers were readily revealed.

Fractionation of lymphocyte membrane solubilized in the presence of radioactive sodium deoxycholate (Fig. 4) showed that significant amounts of deoxycholate were not bound by the proteins. This conclusion is in agreement with a report by Rudman & Kendall (1957) that deoxycholate is not bound by human immunoglobulin and to a small extent only by serum albumin (2.3 mol/mol of albumin in 0.15 M-sodium chloride–10 mM-sodium phosphate buffer, pH 7.6), and with the observation that sodium deoxycholate has little effect on the precipitation of protein antigens by their homologous antibodies (Crumpton, 1971).

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