Preparation of Inside-Out Vesicles of Pig Lymphocyte Plasma Membrane†

Frank S. Walsh,‡ Brian H. Barber,§ and Michael J. Crumpton*

ABSTRACT: Between 30 and 50% of pig lymphocyte plasma membrane vesicles were not bound by concanavalin A (Con A)–Sepharose. Various results suggest that the Con A-unretarded fraction represents "inside-out" membrane vesicles. First, an alternative cell surface ligand, anti-lymphocytic serum, gave a similar fractionation to Con A. Second, lack of binding by Con A was not due to lack of carbohydrate or to masking of carbohydrate by extraneous protein, because the unfractionated membrane and the unretarded fraction had similar carbohydrate and polypeptide compositions. Third, although the carbohydrate of the unretarded membrane vesicles was accessible to ^{125}I-labeled Con A and to release by soluble trypsin, it was not accessible to ferritin–Con A or trypsin–Sepharose. Fourth, antisera against the external surface of the Con A-unretarded vesicles strongly agglutinated the unretarded membrane, but caused negligible agglutination of whole lymphocytes. When attached to Sepharose these antisera bound all of the Con A-unretarded fraction, but failed to bind the membrane that adhered to Con A–Sepharose.

It is generally accepted that many aspects of cell behavior are regulated by the interaction of extracellular ligands with specific receptors exposed on the cell surface. A key event in the regulatory process is the mechanism by which biochemical signals generated at the cell surface are transmitted across the plasma membrane. One particularly attractive mode of informational transfer is via membrane proteins that are exposed on both the outer and inner faces of the lipid bilayer. Such "transmembrane" proteins are especially well documented in erythrocyte plasma membranes (Bretscher, 1971; Boxer et al., 1974; Mueller and Morrison, 1974; Steck, 1974a; Whiteley and Berg, 1974; Reichstein and Blostein, 1975), but most probably occur in all other cell types (e.g., mouse L cells: Hunt and Brown, 1975). We are interested in identifying these proteins in resting and activated T and B lymphocytes.

The identification of transmembrane proteins is usually achieved by comparative labeling of the outside and inside (cytoplasmic) membrane surfaces (e.g., see Reichstein and Blostein, 1975). The application of this approach is, however, dependent upon inverted ("inside-out"; IO)[1] plasma membrane vesicles being available. Such vesicles can be prepared by using various procedures (Bennett and Cuatrecasas, 1973; Steck, 1974b; Hunt and Brown, 1975). One especially promising method depends first upon plasma membrane preparations containing inverted membrane vesicles (Bennett and Cuatrecasas, 1973) and secondly, upon the availability of ligands that are specific for either the IO or the RO membrane surface. The latter requirement would be satisfied by carbohydrate-binding proteins with a broad specificity such as Con A if, as seems likely, plasma-membrane carbohydrate is located exclusively on the outer surface (Hirano et al., 1972; Keenan et al., 1974; Nicolson and Singer, 1974). Indeed, Con A has been claimed to bind RO vesicles of mouse plasmocytoma plasma membrane and thereby separate them from IO vesicles (Zachowski and Paraf, 1974; Zachowski et al., 1975). The present paper describes the application of both Con A and ALS covalently attached to Sepharose-4B to the separation of a fraction of pig lymphocyte plasma membrane that according to various criteria behaves as IO vesicles.

Materials and Methods

Chemicals. Con A covalently bound to Sepharose-4B (10 mg of Con A/ml of gel sediment; batch no. 5067) and CNBr-activated Sepharose-4B (batch no. 5100) were obtained from Pharmacia Fine Chemicals. Ferritin–Con A was a gift from Durward Lawson (Dept. of Zoology, University College, London). It was prepared by conjugating ferritin (twice crystallized; Koch-Light Laboratories) to Con A using glutaraldehyde (Taab, Reading, Berks.) as described by Stobo et al. (1972) and was separated from unconjugated material according to de Petris and Raff (1972). Con A (Pharmacia Fine Chemicals) was radioiodinated with carrier-free Na^{125}I (The Radiochemical Centre, Amersham) by using chloramine T (Greenwood et al., 1963). The product had a specific activity of 7.5 nCi/μg and 97% of the radioactivity was precipitated by 10% (w/v) trichloroacetic acid. Trypsin pretreated with diphenylcarbamoyl choride, methyl α-D-mannopyranoside, and methyl α-D-glucopyranoside were purchased from Sigma Chemical Co. Trypsin was coupled to CNBr-activated Sepharose-4B (3 mg of trypsin/ml of gel sediment) at 24 °C for 2 h using the procedure recommended by Pharmacia Fine Chemicals.

Antisera. ALS was prepared according to Levey and Medawar (1966) by injecting rabbits intravenously with 10^9 pig mesenteric lymph node lymphocytes in 1 ml of 0.15 M NaCl on two occasions separated by 14 days. Antiserum was collected 7 days after the second injection. Some properties of the ALS employed in this study have been described by Allan and Crumpton (1970). The immunoglobulin fraction of ALS, prepared by precipitating with 18% (w/v) and then 14% Na_2SO_4 (Kekwick, 1940), was coupled to CNBr-activated Sepharose-4B (20 mg of protein/ml of gel sediment) at 24 °C for 2 h.

Antisera against the Con A-unretarded membrane vesicles

† From the National Institute for Medical Research, London NW7 1AA, England. *Received February 23, 1976.*

‡ Recipient of a MRC Research Studentship.

§ Recipient of a Canadian MRC Centennial Fellowship.

[1] Abbreviations used are: IO, inside-out; RO, rightside-out; Con A, concanavalin A; ALS, anti-lymphocytic serum; TBS, 0.01 M Tris-HCl–0.15 M sodium chloride buffer (pH 7.4).

(see below) were prepared using vesicles that had been fixed with glutaraldehyde in order to preserve vesicle orientation during immunization. The conditions used for fixation were those recommended by Anderson and Dresser (1971) as having a negligible effect upon the antigenicity of mouse thymocytes. A suspension of unretarded vesicles (1 mg of protein/ml) was incubated at 24 °C for 5 min with an equal volume of 0.2% glutaraldehyde, diluted tenfold with TBS and pelleted at 75 000*g* for 45 min. The pellet was resuspended in water (3 mg of protein/ml), and emulsified with an equal volume of Freund's complete adjuvant, and 1 ml was injected into rabbits at multiple sites. Antisera were collected after 21 days. The antisera possessed negligible agglutination titres against whole lymphocytes (*2). These agglutinins and any other antibodies that may have been formed against the lymphocyte outer surface were removed by absorbing 1 ml of antiserum twice with 10^8 live lymphocytes (>98% viable as judged by exclusion of eosin) at 24 °C for 1 h and centrifuging at 1000*g* for 10 min.

Pig lymphocyte plasma membrane was prepared from mesenteric lymph node as described by Allan and Crumpton (1970) except that the lymph node was disrupted by using a larger model of the tissue press (Snary et al., 1976b) and the plasma membrane fraction recovered from the discontinuous sucrose gradient was washed twice in TBS by resuspending with a Pasteur pipet and centrifuging at 75 000*g* for 30 min. The purified membrane consisted predominantly of vesicles within the range 100–800 nm diameter, but the presence of some membrane sheets and spirals cannot be excluded. According to various morphological, biochemical, and immunological criteria, the plasma membrane fraction was not contaminated by significant amounts of endoplasmic reticulum or golgi membrane (Allan and Crumpton, 1970). Various arguments suggest that the purified plasma membrane fraction was derived primarily from small lymphocytes (Allan and Crumpton, 1970; Chavin et al., 1975). This interpretation is endorsed by the observation that the polypeptide composition of the plasma membrane preparations, as revealed by polyacrylamide gel electrophoresis in sodium dodecyl sulfate, did not differ significantly from that of plasma membrane prepared from suspensions of pig mesenteric lymph node lymphocytes (B. H. Barber, M. J. Crumpton, and F. S. Walsh, unpublished observations).

Fractionation of Plasma Membrane. The purified plasma membrane (4 mg of protein/ml of TBS) was fractionated at 24 °C on a column (5 × 1 cm) of either Con A–Sepharose or ALS–Sepharose supported in a syringe barrel on a plug of polypropylene. The column was eluted with TBS at a flow rate of 10 ml/h. Fractions were assayed for protein and the total protein eluted was taken to represent the amount of unretarded membrane. The protein-containing fractions were pooled, stored at 0 °C and normally used within 24 h. All columns were used once only. The recovery of the adsorbed membrane has not been extensively investigated, but 0.1 M methyl α-D-mannopyranoside failed to elute significant amounts of protein. The lack of elution is most probably due to the high affinity of binding arising through multivalent interactions. Elution of bound cells from various insoluble ligands, including Con A–Sepharose, by competitive inhibitors has also proven difficult to achieve (e.g., Edelman et al., 1971).

Agglutination. The capacities of antisera to agglutinate pig lymphocytes were measured as described by Allan and Crumpton (1970), except that the suspensions of mesenteric lymph node lymphocytes used had been separated from dead cells and erythrocytes on Isopaque–Ficoll according to Parish et al. (1974). Agglutination of membrane vesicles was determined as follows: 50 μl of membrane suspension (1.6 mg of protein/ml of TBS) was incubated at 37 °C for 1 h with an equal volume of antiserum and agglutination was read by phase-contrast microscopy after the tubes had been vigorously shaken. Antisera were diluted serially 1:2 and the greatest dilution at which agglutination was detected was taken as the end point. The reciprocal of this serum dilution is referred to as the agglutination titre. Normal serum collected before immunization failed to agglutinate whole lymphocytes at a dilution of 1:2.

Con A Binding. The capacity of membrane vesicles to bind Con A was determined by incubating a fixed amount of membrane (230 μg of protein) with varying amounts of ^{125}I-labeled Con A in a total volume of 0.1 ml. The samples were rotated end-over-end for 45 min at 25 °C, diluted to 1.5 ml with TBS and the membrane sedimented for 4 min in a microfuge (model 320; James A. Jobling & Co. Ltd., Stone, Staffs.). The pellets were washed twice by resuspending in TBS prior to measuring their radioactivity. Nonspecific binding was assessed by including identical samples except for the presence of 0.1 M methyl α-D-glucopyranoside.

Release of Carbohydrate by Tryptic Digestion. Membrane preparations (600 μg of protein in 0.4 ml of TBS) were incubated at 37 °C for 1 h with either 300 μg of soluble trypsin or 300 μl of trypsin–Sepharose containing about 900 μg of bound trypsin. The membrane was sedimented at 75 000*g* for 30 min and the carbohydrate released into the supernatant was estimated by gas-liquid chromatographic analysis.

Electron Microscopy. Binding of ferritin–Con A by membrane vesicles was carried out using an identical procedure with that described above for ^{125}I-labeled Con A, except that membrane vesicles (500 μg of protein in 0.5 ml) were incubated with 50 μl of ferritin–Con A in the presence or absence of 0.1 M methyl α-D-glucopyranoside. The washed pellets were fixed as described by Hirsch and Fedorko (1968) by adding a mixture (2:1 v/v) of 1% osmium tetroxide and 2.5% (v/v) glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for 1 h at 0 °C. The residues were 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 sodium barbiturate adjusted with acetic acid to 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 an AEI EM6B microscope.

Other Methods. Protein was estimated according to Lowry et al. (1951). The neutral sugar, amino sugar, and sialic acid contents of membrane preparations were determined by gas-liquid chromatography after methanolysis and trimethylsilylation as described by Snary et al. (1976a). Polyacrylamide gel electrophoresis in sodium dodecyl sulfate was performed on 7.5% (w/v) slab gels using the Tris–glycine buffer system of Laemmli (1970). Membrane samples were dissolved by heating at 100 °C for 2 min in 2% (w/v) sodium dodecyl sulfate, 0.1 M dithiothreitol, 10% (w/v) glycerol, and 0.02% bromophenol blue. Gels were stained with Coomassie blue in methanol, water, acetic acid (41:52:7, v/v).

Results

Fractionation of Plasma Membrane. The purified preparations of pig lymphocyte plasma membrane were eluted from a column of Con A–Sepharose with TBS. Between 30 and 50% of the membrane protein passed straight through the column, whereas when an equivalent amount of Sepharose-4B was used in place of the Con A–Sepharose 90 ± 10% (average of six

determinations) of the membrane protein was eluted. The above results were independent of the membrane preparation (12 separate preparations examined) and the amount of membrane protein (within the range 2–5 mg) added to the column. If the membrane that was not bound by Con A–Sepharose (the "Con A-unretarded fraction") was immediately reapplied to a second column of Con A–Sepharose, then >80% of the added protein was eluted. The length of time elapsing between the two column runs could be extended to at least 24 h at 0 °C with no significant change in the amount of the unretarded fraction. The results of preliminary experiments suggest that the proportion of the initial plasma membrane preparation that is not bound by Con A–Sepharose is determined by the prior treatment of the membrane. Thus, the unretarded portion was increased from 30–50% to 70–80% by homogenizing the membrane recovered from the discontinuous sucrose gradient twice in a tight-fitting, all-glass homogenizer (Uniform; 0.08–0.12 mm all-round clearance; Jencons (Scientific) Ltd., Hemel Hempstead, Herts.) in hypotonic (10 mM) Tris-HCl buffer, pH 7.3 (see Figure 3 of Snary et al., 1976b); the membrane was recovered after each wash by centrifuging at 100 000*g* for 30 min. It was concluded that the preparations of pig lymphocyte plasma membrane contain a reproducible, stable fraction that is not bound by Con A–Sepharose.

A similar fractionation of the plasma membrane preparations was achieved by elution from a column of ALS–Sepharose.[2] Thus, about 50% of the membrane added to ALS–Sepharose passed freely through the column. The close resemblance of this fractionation to that achieved by using Con A is emphasized by the observation that >80% of the Con A-unretarded membrane was not bound by ALS–Sepharose. In contrast, antibodies produced by immunization with the Con A-unretarded membrane bound >80% of the Con A-unretarded membrane. When this antiserum, attached to Sepharose, was used to fractionate the lymphocyte plasma membrane, about 50% of the membrane passed straight through the column and >80% of this fraction was bound by Con A–Sepharose. It was concluded that the antibodies to the Con A-unretarded membrane bound the complementary membrane fraction to that bound by Con A. This conclusion is supported by the observation that Con A and the antibodies to the Con A-unretarded membrane collectively adsorbed >96% of the plasma membrane preparation.

Composition of Con A-Unretarded Membrane Fraction. The polypeptide chain compositions of the initial plasma membrane preparation and the fractions that were not bound by Con A, ALS, and the antibodies to the Con A-unretarded membrane, were assessed by polyacrylamide gel electrophoresis in sodium dodecyl sulfate. As shown in Figure 1, a comparison of the results revealed no significant differences between any of the membrane fractions apart from an extra band in the Con A-unretarded membrane. This band had a molecular weight of about 27 000 and was coincident with the major polypeptide chain of Con A (Wang et al., 1971). It most probably represented Con A derived from the column. The amount of contaminating Con A would apparently cover no more than 1% of the potential Con A binding sites of the membrane. This estimate was based on the assumptions that Con A represents 1% of the unretarded membrane protein

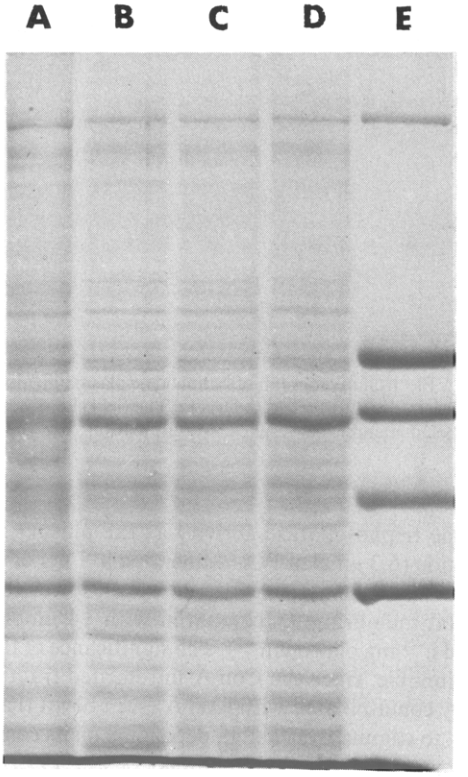

FIGURE 1: Polyacrylamide gel electrophoresis in sodium dodecyl sulfate of pig lymphocyte plasma membrane stained with Coomassie blue. (A) Initial membrane preparation, (B) Con A-unretarded fraction, (C) ALS-unretarded fraction, (D) fraction unretarded by antibodies to the Con A-unretarded membrane, (E) standards in order of decreasing molecular weight: myosin (200 000), transferrin (78 000), bovine serum albumin (69 000), tubulin (55 000), actin (43 000), and L chain (25 000). The L chain is coincident with the bromophenol blue band at the gel front.

TABLE I: Carbohydrate Composition of Initial Lymphocyte Plasma Membrane Preparation and of the Fraction Unretarded by Con A–Sepharose.

	(nmol of carbohydrate/mg of protein)	
Sugar	Initial Membrane Preparation	Con A-Unretarded Fraction
Mannose	88	79
Galactose	200	190
Glucose	114	102
Inositol	13	20
Glucosamine	99	107
Galactosamine	49	42
Sialic acid	140	120

(Figure 1), that 1 mg of membrane protein is equivalent to 10^9 lymphocytes (Allan and Crumpton, 1970), and that whole lymphocytes possess 10^7 Con A binding sites/cell (Stobo et al., 1972).

Table I shows the carbohydrate compositions of the initial membrane preparation and the Con A unretarded fraction. Although some minor differences were detected, the close similarity in carbohydrate composition indicates that a deficiency in carbohydrate was not responsible for the lack of binding of the unretarded membrane vesicles by Con A–Sepharose.

Enzymatic assays showed that the Con A-unretarded fraction possessed the same Na^+-(Mg^{2+} + K^+)-stimulated

[2] ALS is raised by immunization with whole lymphocytes and contains primarily antibodies against the lymphocyte surface structure (Levey and Medawar, 1966). Consequently, it resembles Con A in being a cell-surface ligand.

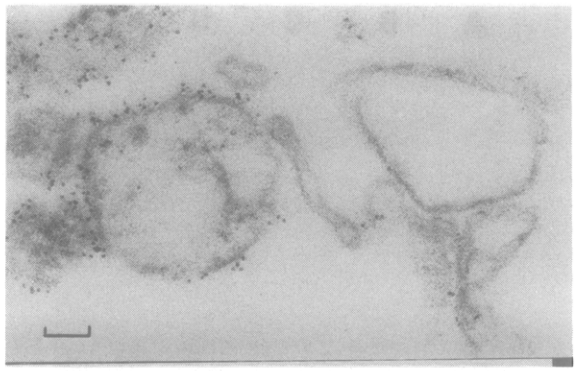

FIGURE 2: Electron micrograph of lymphocyte plasma membrane stained with Con A covalently attached to ferritin. The field shows heavily labeled and unlabeled membrane vesicles. Magnification: bar represents 200 nm.

TABLE II: Capacities of Initial Plasma Membrane and Unretarded Membrane Fraction to Bind ^{125}I-labeled Con A.

^{125}I-labeled Con A Added (μg)	Methyl α-D-Glucopyranoside[a]	% Radioactivity Bound: Initial Membrane Preparation	% Radioactivity Bound: Con A-Unretarded Fraction
18	−	70	–
22	−	–	86
100	−	81	–
113	−	–	86
125	−	85	–
177	−	–	77
185	+	2	4

[a] Added to give final concentration of 0.1 M.

adenosine triphosphatase activity as the plasma membrane preparation (6.3 μmol of PO_4^{3-} liberated h^{-1} mg of protein^{-1}). Its 5′-nucleotidase activity was, however, lower than that of the initial membrane (4 compared with 14 μmol of PO_4^{3-} liberated h^{-1} mg of protein^{-1}). The significance of these results is questionable, since the Con A-unretarded fraction is most probably contaminated with Con A (see above) that has been reported to stimulate or inhibit, depending on its concentration, both enzyme activities (Zachowski et al., 1975). The low glucose-6-phosphatase activity (0.96 μmol of PO_4^{3-} liberated h^{-1} mg of protein^{-1}) of the initial plasma membrane was not increased in the Con A-unretarded fraction.

Accessibility of Carbohydrate of Con A-Unretarded Membrane. The capacities of the plasma membrane preparation and of the Con A-unretarded fraction to bind Con A were assessed by using ^{125}I-labeled Con A; binding of radioactivity was shown to be specific by inhibition with methyl α-D-glucopyranoside. The results in Table II indicate that their Con A-binding capacities were not significantly different. It was concluded that the carbohydrate moieties of the plasma membrane preparation and the Con A-unretarded fraction were equally accessible to Con A. Although this conclusion is contrary to that deduced from the fractionation of the membrane using Con A–Sepharose, this dichotomy would be resolved if the carbohydrate of the Con A-unretarded membrane was not accessible to Con A–Sepharose due to the increased size of the ligand. This interpretation is supported by the results of two experiments.

First, the binding of Con A by the initial plasma membrane preparation was assessed morphologically using Con A covalently attached to ferritin. Figure 2 shows that the plasma membrane preparation is composed of two types of vesicles, one of which was heavily labeled with ferritin particles, whereas the other was unlabeled. Labeling was shown to be Con A specific by being completely inhibited in the presence of 0.1 M methyl α-D-glucopyranoside. So far as could be determined, the ferritin particles were restricted to the outer surface of the labeled vesicles. A similar examination of the Con A-unretarded membrane showed that this corresponded to the unlabeled vesicles of the initial membrane preparation.

Second, the accessibility of the membrane glycoproteins was examined by measuring the amounts of carbohydrate released by trypsin and trypsin attached to Sepharose. Table III shows that soluble trypsin released similar amounts of mannose, amino sugars, and sialic acid from the plasma membrane preparation and the Con A-unretarded fraction. In contrast, trypsin–Sepharose was only 14% as effective at releasing carbohydrate from the Con A-unretarded membrane as from the total membrane.

It was concluded from the above experiments that the carbohydrate of the Con A-unretarded fraction is accessible to bind free Con A and to release by soluble trypsin, but is inaccessible to these molecules when they are attached to ferritin and/or Sepharose beads.

Immunological Properties of Con A-Unretarded Membrane. The capacities of ALS and the antisera produced by immunization with the glutaraldehyde-fixed, Con A-unretarded membrane fraction to agglutinate whole lymphocytes, and the Con A-unretarded membrane vesicles are compared in Table IV and Figure 3. These results are notable for a number of reasons. First, antisera to the Con A-unretarded membrane agglutinated strongly the unretarded membrane vesicles but caused negligible agglutination of whole lymphocytes (titre, ≤ 2). The marginal agglutination titre against whole cells was readily removed by absorption with a small number of lymphocytes (two lots of 10^8 cells/ml of serum) without causing a marked reduction in titre against the unretarded membrane. Second, ALS gave the opposite pattern of results. Thus, it agglutinated whole lymphocytes strongly, but failed to cause any detectable agglutination of the Con A-unretarded membrane.

Similar results were obtained when the antisera were attached to Sepharose. In this case, as described above, antisera to the Con A-unretarded fraction bound all of the Con A-unretarded membrane, but failed to bind the membrane that was adsorbed by Con A–Sepharose.

These results indicate that the surface antigenic determinants of whole lymphocytes are not accessible on the Con A-unretarded membrane vesicles to either free antibody, as judged by agglutination, or antibody attached to Sepharose. Conversely, the surface of the Con A-unretarded vesicles possesses a different set of antigenic determinants that are not exposed on the surface of either whole lymphocytes or the membrane vesicles that are bound by Con A–Sepharose.

Discussion

There are a number of possible explanations for the apparent fractionation of the preparations of pig lymphocyte plasma membrane vesicles by Con A and ALS attached to Sepharose. The most appealing is based upon the supposition that the membrane preparations contain two types of vesicles with different orientations, namely either RO or IO. In this case, since the plasma membrane carbohydrate is most probably

TABLE III: Comparison of Release of Carbohydrate from Initial Plasma Membrane and Con A-unretarded Fraction by Soluble Trypsin and Trypsin-Sepharose-4B.[a]

Expt No.	Membrane Fraction	Treatment	(nmol of Carbohydrate/mg of Protein) Mannose	Glucosamine	Galactosamine	Sialic Acid
1	Initial	None	ND[b]	ND	ND	ND
	Initial	Trypsin	40	78	33	65
	Unretarded	Trypsin	32	73	25	48
2	Initial	Trypsin–Sepharose	96	82	50	32
	Unretarded	Trypsin–Sepharose	22	14	8	0

[a] Equal amounts of the membrane fractions (600 μg of protein) were incubated with either soluble trypsin (300 μg) or trypsin (900 μg) covalently attached to Sepharose-4B. The membrane was sedimented by centrifugation and the carbohydrate present in the supernatant was estimated by gas-liquid chromatography. [b] ND, not detected.

TABLE IV: Agglutination Titres of Antisera Prepared by Immunization with Whole Lymphocytes and Con A-unretarded Membrane.

Antiserum	Agglutination titre: Lympho-cytes	Con A-Unretarded Membrane
Anti-whole lymphocytes (ALS)	128	0
Anti-Con A-unretarded membrane[a]	≤2	32
Anti-Con A-unretarded membrane[a,b]	0	16
Normal rabbit serum	0	0

[a] The results shown were given by one antiserum. Similar results were obtained using antisera produced in four animals. [b] After absorbing twice with whole lymphocytes (10^8 cells/ml of antiserum.)

located exclusively on the outside surface (Hirano et al., 1972; Keenan et al., 1974; Nicolson and Singer, 1974), the unretarded membrane vesicles can be equated with the IO orientation.

Direct evidence in support of the assumption that membrane preparations contain two types of vesicles, only one of which is represented in the unretarded fractions, was obtained by using Con A covalently attached to ferritin as a stain for surface carbohydrate. Thus, electron microscopy of stained membrane preparations revealed labeled and unlabeled vesicles (Figure 2), whereas no labeling of the unretarded fractions was detected. Although these results are consistent with the unretarded membrane vesicles having an IO orientation, other explanations that do not invoke any difference in orientation are possible. For example, the unretarded membrane may be deficient in carbohydrate residues or its surface structure including the carbohydrate may be masked by extraneous material. These alternative explanations are ruled out by various arguments. Thus, the marked similarity in the carbohydrate compositions of the initial membrane and the Con A-unretarded fraction (Table I), and in their capacities to bind ^{125}I-labeled Con A (Table II), indicate that the unretarded membrane is not deficient in carbohydrate, whereas the similarity in their polypeptide compositions (Figure 1) argues against the presence of extraneous masking protein(s) in the unretarded fractions. Apart from these arguments, carbohydrate alone is unlikely to mediate the fractionation of the plasma membrane preparations, since ALS is most probably not exclusively a ligand for carbohydrate.

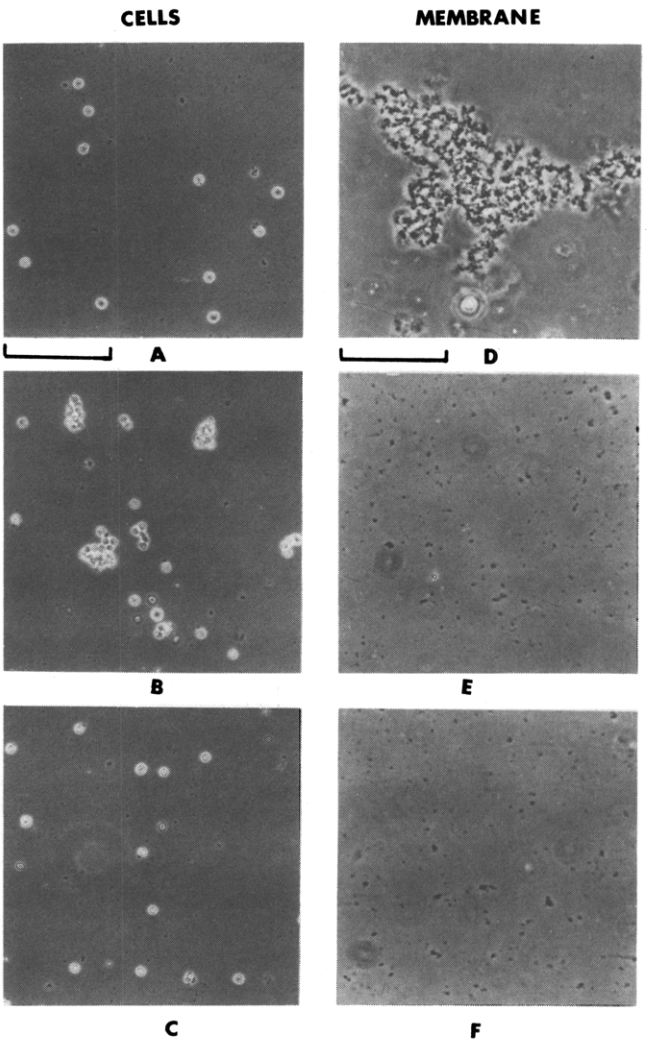

FIGURE 3: Agglutination of whole lymphocytes (A–C) and of Con A-unretarded membrane (D–F) by antiserum to Con A-unretarded membrane (A, D), ALS (B, E), and normal rabbit serum (C, F). Membrane agglutination (D–F) was assessed by phase-contrast microscopy. Magnification: (A–C), bar represents 50 μm; (D–F), bar represents 12.5 μm.

More direct evidence for the unretarded membrane vesicles having an IO orientation was obtained by comparing the reactivities of the antisera produced against whole lymphocytes (ALS) and the Con A-unretarded fraction. These antisera

possessed strikingly different properties. Thus, antisera to the Con A-unretarded fraction, when attached to Sepharose, bound the complementary plasma membrane fraction to that bound by Con A- and ALS-Sepharose. Similarly, the antisera to the Con A-unretarded fraction caused negligible agglutination of whole lymphocytes, but agglutinated strongly the unretarded membrane vesicles even after absorption with whole lymphocytes (Figure 3, Table IV). In contrast, ALS failed to bind and agglutinate the unretarded membrane. These results indicate that the antigenic determinants exposed on the surface of the unretarded membrane vesicles are completely different from the surface antigens of whole lymphocytes. The most logical explanation for this difference is that the unretarded membrane vesicles have an IO orientation.

If the above interpretation is correct, then the carbohydrate of the unretarded membrane fractions should not be accessible to bind free Con A or to release by tryptic digestion. It is, however, evident from the results shown in Tables II and III that these predictions were not satisfied. Although these results argue against the unretarded membrane being IO, an alternative explanation, namely that the vesicles are permeable to Con A and trypsin, is more probably correct. This view is supported by the reports (Steck, 1974b) that plasma membrane vesicles as normally isolated are generally permeable and require to be "sealed" before their permeability characteristics resemble those of the whole cell. More direct support for this view is provided by the observations that when the sizes of Con A and trypsin were increased by attachment to ferritin and Sepharose, then the carbohydrate of the unretarded membrane was no longer accessible to release by trypsin (Table III) and to bind Con A (Figure 2). On the other hand, it is perhaps somewhat surprising to find that the lymphocyte membrane vesicles were permeable to such large molecules as Con A (diameter of the tetramer, about 60 Å; Edelman et al., 1972).

The above results indicate that the unretarded membrane fraction most probably possesses an IO orientation. However, before it can be concluded that this fraction represents IO plasma membrane, the possibility must be ruled out that it represents endoplasmic reticulum and/or golgi whose natural orientations with respect to carbohydrate are the reverse of plasma membrane (Hirano et al., 1972). This possibility was discounted by various results. First, as judged by morphological and biochemical criteria the plasma membrane preparations were not contaminated by significant amounts of other cell membranes (Allan and Crumpton, 1970). Second, the low glucose-6-phosphatase activity (a marker for endoplasmic reticulum) of the plasma membrane preparation was not increased in the unretarded fraction. Third, various results suggest that endoplasmic reticulum, golgi, and plasma membrane have different polypeptide compositions (Neville and Glossmann, 1971; Bergeron et al., 1973). As a result, the identical compositions of the plasma membrane and unretarded fraction (Figure 1) argue strongly against the unretarded membrane not being plasma membrane. Fourth, the proportion of the unretarded fraction present in the plasma membrane preparation could be increased to 70–80% by merely homogenizing under hypotonic conditions.

It is concluded that the Con A- and ALS-unretarded membrane fractions most probably represent IO plasma membrane vesicles. The suitability of these fractions as candidates for the identification of lymphocyte transmembrane proteins is currently being assessed using comparative labeling techniques. The antiserum to the Con A-unretarded membrane should prove invaluable not only in this connection but also in the identification of the proteins that are located on the cytoplasmic surface of the lymphocyte plasma membrane. Although this study has been concerned with the application of Con A- and ALS-Sepharose to the preparation of lymphocyte IO membrane vesicles, it seems likely that this approach will also be applicable to the preparation of IO plasma membrane of other cell types.

Acknowledgments

We thank Durward Lawson for the gift of ferritin-Con A and for his assistance and advice with the electron microscopy. The sample of tubulin was kindly donated by Dr. M. Jacobs.

References

Allan, D., and Crumpton, M. J. (1970), *Biochem. J. 120,* 133.

Anderson, H. R., and Dresser, D. W. (1971), *Eur. J. Immunol. 1,* 31.

Bennett, V., and Cuatrecasas, P. (1973), *Biochim. Biophys. Acta 311,* 362.

Bergeron, J. J. M., Evans, W. H., and Geschwind, I. I. (1973), *J. Cell Biol. 59,* 771.

Boxer, D. H., Jenkins, R. E., and Tanner, M. J. A. (1974), *Biochem. J. 137,* 531.

Bretscher, M. S. (1971), *J. Mol. Biol. 59,* 351.

Chavin, S. I., Johnson, S. M., and Holliman, A. (1975), *Biochem. J. 148,* 417.

de Petris, S., and Raff, M. C. (1972), *Eur. J. Immunol. 2,* 523.

Edelman, G. M., Cunningham, B. A., Reeke, G. N., Becker, J. W., Waxdal, M. J., and Wang, J. L. (1972), *Proc. Natl. Acad. Sci. U.S.A. 69,* 2580.

Edelman, G. M., Rutishauser, U., and Millette, C. F. (1971), *Proc. Natl. Acad. Sci. U.S.A. 68,* 2153.

Greenwood, F. C., Hunter, W. M., and Glover, J. S. (1963), *Biochem. J. 89,* 114.

Hirano, H., Parkhouse, B., Nicolson, G. L., Lennox, E. S., and Singer, S. J. (1972), *Proc. Natl. Acad. Sci. U.S.A. 69,* 2945.

Hirsch, J. G., and Fedorko, M. E. (1968), *J. Cell Biol. 38,* 615.

Hunt, R. C., and Brown, J. C. (1975), *J. Mol. Biol. 97,* 413.

Keenan, T. W., Franke, W. W., and Kartenbeck, J. (1974), *FEBS Lett. 44,* 274.

Kekwick, R. A. (1940), *Biochem. J. 34,* 1248.

Laemmli, U. K. (1970), *Nature (London) 227,* 680.

Levey, R. H., and Medawar, P. B. (1966), *Proc. Natl. Acad. Sci. U.S.A. 56,* 1130.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193,* 265.

Mueller, T. J., and Morrison, M. (1974), *J. Biol. Chem. 249,* 7568.

Neville, D. M., and Glossmann, H. (1971), *J. Biol. Chem. 246,* 6335.

Nicolson, G. L., and Singer, S. J. (1974), *J. Cell Biol. 60,* 236.

Parish, C. R., Kirov, S. M., Bowern, N., and Blanden, R. V. (1974), *Eur. J. Immunol. 4,* 808.

Reichstein, E., and Blostein, R. (1975), *J. Biol. Chem. 250,* 6256.

Snary, D., Allen, A. K., Faulkes, R. A., and Crumpton, M. J. (1976a), *Biochem. J. 153,* 75.

Snary, D., Woods, F. R., and Crumpton, M. J. (1976b), *Anal. Biochem.* (in press).
Steck, T. L. (1974a), *J. Cell Biol. 62,* 1.
Steck, T. L. (1974b), *Methods Membr. Biol. 2,* 245.
Stobo, J. D., Rosenthal, A. S., and Paul, W. E. (1972), *J. Immunol. 108,* 1.
Wang, J. L., Cunningham, B. A., and Edelman, G. M. (1971), *Proc. Natl. Acad. Sci. U.S.A. 68,* 1130.
Whiteley, N. M., and Berg, H. C. (1974), *J. Mol. Biol. 87,* 541
Zachowski, A., Migliore-Samour, D., Paraf, A., and Jolles, P. (1975), *FEBS Lett. 52,* 57.
Zachowski, A., and Paraf, A. (1974), *Biochem. Biophys. Res. Commun. 57,* 787.

Enveloped Viruses as Model Membrane Systems: Microviscosity of Vesicular Stomatitis Virus and Host Cell Membranes†

Yechezkel Barenholz, Norman F. Moore,* and Robert R. Wagner

ABSTRACT: The fluorescence probe 1,6-diphenyl-1,3,5-hexatriene was used to study and compare the dynamic properties of the hydrophobic region of vesicular stomatitis virus grown on L-929 cells, plasma membrane of L-929 cells prepared by two different methods, liposomes prepared from virus lipids and plasma membrane lipids, and intact L-929 cells. The rate of penetration of the probe into the hydrophobic region of the lipid bilayer was found to be much faster in the lipid vesicle bilayer as compared with the intact membrane, but in all cases the fluorescence anisotropy was constant with time. The L-cell plasma membranes, the vesicles prepared from the lipids derived from the plasma membranes, and intact cells are found to have much lower microviscosity values than the virus or virus lipid vesicles throughout a wide range of temperatures. The microviscosity of plasma membrane and plasma membrane lipid vesicles was found to depend on the procedure for plasma membrane preparation as the membranes prepared by different methods had different microviscosities. The intact virus and liposomes prepared from the virus lipids were found to have very similar microviscosity values. Plasma membrane and liposomes prepared from plasma membrane lipids also had similar microviscosity values. Factors affecting microviscosity in natural membranes and artificially mixed lipid membranes are discussed.

Enveloped viruses potentially provide simple, controllable model systems for studying membrane composition and function for the following reasons: (i) enveloped viruses bud from surfaces of infected cells and can be readily purified free of cell membranes; (ii) membranes of enveloped viruses appear to be homogeneous; (iii) viral membrane lipids are selected from preformed lipids of host cell membranes, although the lipid proportions differ for the virus and the cell; (iv) viral membranes contain very few protein species, all of which are coded for by the viral genome and not by the cell genome; and (v) the virus genomes do not code for lipid synthesis but evidence is available that the viral membrane proteins select cell lipids for viral membrane assembly by as yet undetermined mechanisms (Lenard and Compans, 1974; Blough and Tiffany, 1973; Choppin et al., 1971).

The purpose of the study described here was threefold: (1) to study some membrane properties of an enveloped virus system, and to investigate the relationship between the host cell plasma membrane and the virus membrane; (2) to describe the advantages and difficulties of using the technique of fluorescence polarization to measure microviscosities of biological membranes; and (3) to compare the results obtained by the fluorescence polarization technique with those obtained with other physical methods such as electron spin resonance (ESR)[1] (Landsberger et al., 1971–1973) and nuclear magnetic resonance (NMR) (Stoffel and Bister, 1975).

1,6-diphenyl-1,3,5-hexatriene was the fluorescence probe used in this study to measure the microviscosities of the hydrophobic regions of viral and host cell membranes. The same probe was used by Shinitzky and Inbar (1974) and Fuchs et al. (1975) to measure the microviscosity of the membranes of lymphocytes and 3T3 cells, respectively. Vesicular stomatitis (VS) virus grown on L cells was selected for this study because the lipid composition of both the virus membrane and the host cell plasma membrane have been described in detail (McSharry and Wagner, 1971). Furthermore the virus has only two proteins in its lipid membrane and these have been well characterized (Wagner et al., 1972b; Schloemer and Wagner, 1975; Moore et al., 1974; Bishop et al., 1975), making this system one of the simplest naturally occurring membranes.

Materials and Methods

Cells. BHK-21 cells were grown as monolayers at 37 °C in 20 ml of BHK-21 medium supplemented with 10% tryptose phosphate broth and 10% fetal calf serum and containing 1%

† From the Departments of Microbiology (N.F.M. and R.R.W.) and Biochemistry (Y.B.), University of Virginia School of Medicine, Charlottesville, Virginia 22901. *Received October 16, 1975.* N.F.M. and R.R.W. were supported by Grant BMS-72-02223 from the National Science Foundation, by Grant VC-88 from the American Cancer Society, and by Public Health Service Grant AI-11112 from the National Institute of Allergy and Infectious Diseases. Y.B. was supported by Public Health Service Grant No. HL17576 from the National Institute of Heart and Lung.

[1] Abbreviations used are: VS, vesicular stomatitis; BHK, baby hamster kidney; BME, Basal Medium Eagle; EBSS, Earle's balanced salt solution; PBS, phosphate-buffered saline; RSB, reticulocyte standard buffer; SPM, sphingomyelin; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; DPH, 1,6-diphenyl-1,3,5-hexatriene; FMA, fluorescein mercuric acetate; B, bullet; ESR, electron spin resonance; NMR, nuclear magnetic resonance; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.