MACROPHAGE PLASMA MEMBRANES

I. Isolation and Studies on Protein Components*

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Membranes serve many essential roles in the organization and function of cells. In addition to their obvious structural role in delimiting various intracellular compartments and in separating the cell from its environment, membranes also participate in various cellular activities, including (*a*) providing a framework for sequential catalytic processes in certain metabolic pathways; (*b*) transporting ions, nutrients, and waste products so as to regulate the cellular "milieu interieur;" (*c*) functioning in various recognition and communication processes.

The two major constituents of cell membranes are lipids and proteins. The relative amounts and nature of these materials varies, but in general, biological membranes are approximately half lipid and half protein. Currently there is much interest in the problem of how these two components are arranged in the membrane (1). Information on the variety and the properties of membrane proteins is of obvious value in relation to these structural models.

The study of membrane proteins is difficult because of technical problems such as the: (*a*) isolation of total membranes, or of a single type of membrane from a homogeneous population of cells; (*b*) assessment of the purity of the membrane preparation by morphologic and/or biochemical means; (*c*) solubilization of the membranes by methods which do not destroy their proteins; and finally, (*d*) separation of the proteins and characterization of their individual properties. Many of the studies on mammalian cell membranes have been done on red cells because these provide a ready source of a homogeneous cell population from which pure membranes can easily be prepared (2). Information is also available on certain organelle membranes in mitochondria (3) or chloroplasts (4), and on plasma membranes of platelets (5) and of hepatocytes (6). In general, these studies have indicated that cell membranes have numerous protein components (approximately 15–30) and that the number and type of proteins varies from one class of membranes to another and from one cell type to another.

In this paper we report on the isolation of plasma membrane from rabbit

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alveolar macrophages and on the partial characterization of the protein components of this membrane. The following paper presents studies on the labeling properties and metabolic turnover rate of macrophage plasma membrane proteins (7).

Materials and Methods

Calmette-Guerin Bacillus (BCG)—Induced Rabbit Alveolar Macrophages.—Macrophages were obtained from rabbit lungs by the method of Myrvik (8), as modified by Cohn and Wiener (9), 3–4 wk after the intravenous injection of lyophilized BCG. Each rabbit yielded approximately 1×10^9 cells, 90–95% of which were large macrophages. Preparations containing erythrocytes or large numbers of granulocytes were discarded.

Preparation of Macrophage Plasma Membranes.—The intact cells were washed twice with phosphate-buffered saline (PBS),[1] pH 7.4, and collected by centrifugation. 1×10^9 cells were suspended with gentle shaking in 90 ml of distilled water for 10 min at 22°C. 10 ml of 10% glutaraldehyde was added yielding a final concentration of 1% glutaraldehyde. The cells were immediately centrifuged at 400 *g* for 10 min, washed in distilled water, spun down and resuspended in 100 ml of distilled water, and cooled to 4°C. Homogenization was performed in a large 40 ml Dounce homogenizer fitted with a tight pestle (Kontes Glass Co., Vineland, N. J.) utilizing 10–15 gentle strokes. The breakage was monitored by phase microscopy. Disruption of approximately 90% of the swollen fixed cells was taken as the homogenization end point.

The isolation of the macrophage plasma membrane from the homogenate was carried out essentially as described by Warren, Glick, and Nass (10). An equal volume of 60% sucrose was added to the homogenate. 50 ml of the 30% sucrose homogenate suspension was carefully layered over 40 ml of 45% sucrose in four 100 ml centrifuge bottles. The bottles were centrifuged at 200 *g* for 40 min at 4°C. The top layer was carefully aspirated to within 5 mm of the interface and carefully relayered over 40 ml of 45% sucrose and the centrifugation procedure repeated. The top layer was again removed down to but not including the interface, diluted with distilled water to 15–20% sucrose, and centrifuged at 12,800 *g* for 40 min at 4°C. The pellets were suspended in 10 ml of 35% sucrose and homogenized in a small Dounce homogenizer with a loose-fitting pestle utilizing one stroke. 5 ml of the partially purified membrane suspension was then layered on a linear 40–65% sucrose gradient and centrifuged at 25,000 *g* for 2 hr in a Spinco ultracentrifuge using the SW 25.1 rotor. The narrow membrane band appearing at a density of 1.2028 was removed with a needle and syringe.

Preparation of Interiorized Plasma Membrane (Phagolysosomal Membrane).—These membranes were prepared from unfixed cells by the method of Wetzel and Korn (11). 1×10^9 washed alveolar macrophages were suspended in 90 ml of Eagle's minimal essential medium (MEM) (12) and 10 ml of normal rabbit serum in a plastic Ehrlenmeyer flask under 5% CO_2 and 95% O_2. The cells were shaken gently for 10 min at 37°C. Polystyrene latex beads of diameter 1.099 μ (Dow Chemical Co., Midland, Mich.) were washed in MEM, resuspended in MEM, sonicated to disperse clumps, and added directly to the culture flask (approximately 1×10^{11} beads or 100 beads/cell). The cells and beads were allowed to incubate for 30 min with constant shaking.

The cells were then harvested by centrifugation at 400 *g* for 7 min and washed two times with cold PBS to remove uningested beads. The cells were brought to a volume of 12 cc with PBS and 12 ml of 60% sucrose was added. Homogenization was performed in a 40 ml Dounce homogenizer with a tight-fitting pestle. Approximately 35 strokes were required to bring about 90% cell rupture as monitored by phase microscopy.

[1] *Abbreviations used in this paper:* MEM, Eagle's minimal essential medium; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate.

4 ml of this suspension of disrupted cells in 30% sucrose was placed at the bottom of a centrifuge tube for the No. SW 41 rotor of a Spinco Model L ultracentrifuge and then overlayed with 4 ml each of 20% and 10% sucrose and centrifuged for 90 min at 100,000 *g*. The "phagocytic vacuoles" at 10–20% sucrose interface were collected. 5 ml fractions of this phagocytic vacuole suspension were disrupted by exposure to four 15 sec sonic pulses at 0°C in the Branson Sonifier (Branson Instruments Co., Stamford, Conn.). The suspension was diluted to 5% sucrose with water and centrifuged for 20 min at 5000 *g* to remove the latex beads. The phagocytic vacuole membranes were collected as a pellet after centrifugation of the supernatant solution for 2 hr at 100,000 *g*.

Electron Microscopy.—A sample of the freshly harvested alveolar macrophage suspension containing approximately 10^7 cells was centrifuged at room temperature (200 *g* for 10 min). The cell pellet was suspended in 4 ml of 2.5% glutaraldehyde (Biological Grade, Fisher Scientific Co., Fairlawn, N. J.) in cacodylate buffer, pH 7.4, at room temperature. After 5 min the suspension was chilled in ice. Processing then followed the method described previously (13), involving fixation in a mixture of glutaraldehyde and osmium, postfixation in uranyl acetate, embedding in agar, dehydration in ethanol and propylene oxide, and embedding in Epon. Thin sections were cut with diamond knives on a Porter-Blum II microtome, stained with uranyl and lead ions (14), and examined in a Siemens Elmiskop I.

The plasma membranes from glutaraldehyde-fixed macrophages were handled in much the same way, except that the initial 5 min exposure to glutaraldehyde was omitted. The membrane band from the final sucrose gradient was diluted 1:5 into an ice-cold mixture of one part 2.5% glutaraldehyde and two parts 1% osmium tetraoxide in cacodylate buffer at pH 7.4. At each step of the washing and fixation the membranes were centrifuged for 2 min at 750 *g*.

The fixation and washing of the polystyrene phagolysosomes and of the membranes isolated from them was modified in order to avoid extraction of the polystyrene. Fixation was for 15 min in ice-cold 1% osmium tetraoxide, rather than a mixture of glutaraldehyde and osmium, and dehydration was accomplished with ethanol alone (no propylene oxide). Centrifugation at each step of the fixation and washing was for 2 min at 750 *g*.

Enzyme Assays.—These studies were performed on samples that were frozen after isolation and thawed before the assay. 5′ nucleotidase was assayed at pH 7.9 in the presence of β-glycerphosphate as described by Belfield and Goldberg (15). Acid phosphatase was assayed using alpha-napthol acid phosphate as substrate (16). Glucose-6-phosphatase was assayed as described by Hers, Beaufaye, and de Duve (17). Succinic dehydrogenase was assayed as described by Green et al. (18).

Chemical Assays.—Protein was measured by the method of Lowry et al. (19). Chloroform methanol extraction for lipid analysis was performed as described by Folch et al. (20). Cholesterol determinations were performed as described by Webster (21). Phospholipids were ashed to inorganic phosphate by the method of Chen, Toribara, and Warner (22) and measured as described by Ames and Dubin (23). Total neutral sugars were estimated by the anthrone reaction using galactose as standard (24). Sialic acids were determined by the barbituric assay method (25). Fractions of isolated membranes were extracted and assayed by a modification of the Schmidt–Tannhauser procedure as described by Shatkin (26). Purified yeast RNA was used as standard and samples were carried through the extraction procedure to check for recovery. For DNA determinations, the diphenylamine colorimetric reaction of Burton was used (27). Thymus DNA was used as standard and samples were carried through the extract in the procedure to check for recovery.

After hydrolysis for 20 hr in 6% HCl, amino acid analysis was performed on the lyophilized isolated plasma membrane using a Beckman amino acid analyzer by Dr. Martin Spencer.

For solubility studies, weighed amounts of lyophilized membrane were solubilized in 1 ml of solvent with stirring at room temperature for at least 1 hr. After high speed centrifugation (100,000 *g* for 1 hr), the insoluble pellet was dialyzed, lyophilized, and weighed.

Polyacrylamide Gel Electrophoresis.— Optimum resolution of membrane bands was obtained using the Takayama gel system (28) as modified by Baum et al. (29). Lyophilized membranes (4 mg/ml) were dissolved in a phenol–urea–acetic acid reagent: 4 g of phenol, 2.4 g of urea, 2 ml of glacid acetic acid, and 2 ml of water. 40 μl of the solubilized membrane solution was placed on each gel and electrophoresis was performed for 1 hr at 5 ma/tube. The gels were stained in 1% amido Schwartz in 7.5% acetic acid for 90 min. Destaining was performed with the use of an electrolytic destainer (Shandon Scientific Co., Inc., Sewickley, Pa.). Molecular weight estimations in sodium dodecyl sulfate acrylamide gels were performed as previously described (5).

Lipid staining of membrane bands in phenol gels was performed by prestaining with a saturated solution of Sudan black B in ethylene glycol as described by Narayan et al. (30). Carbohydrate staining of the membrane bands in phenol gels was performed using the Schiff reagent as described by Van Neerbas (31).

Immunological Studies.—These were performed as described previously (5). Guinea pigs were immunized with 2 mg portions of the isolated plasma membrane fraction mixed with complete Freund's adjuvant. The animals were injected in two intramuscular and one subcutaneous site at each injection period. Booster injections in adjuvant were given at 14–21 day intervals for a total of four injections. Blood was obtained by cardiac puncture. For absorption studies, the guinea pig serum was mixed for 2 hr at 37°C with 3 mg of lyophilized normal rabbit serum and 1 mg of pooled rabbit fraction II (Pentex Biochemical, Kankakee, Ill.). The mixture was then incubated overnight at 4°C and the serum separated by centrifugation.

RESULTS

Two different methods were used for recovery of plasma membranes from alveolar macrophages. One of these methods involved the isolation of large plasma membrane sheets liberated on gentle homogenization of swollen, glutaraldhyde-fixed cells. The other technique employed separation of membranes from phagocytic vacuoles recovered from cells disrupted soon after they had ingested large numbers of polystyrene spheres.

Many of the membrane-bounded polystyrene spheres were histochemically positive for acid phosphatase, indicating that the phagocytic vacuoles had fused with macrophage lysosomes (preexisting dense bodies or Golgi vesicles). Membranes isolated from these structures were thus possibly altered by exposure to lysosomal hydrolases and were certainly contaminated to some degree by lysosomal membrane. These phagolysosomal membranes were therefore not studied in terms of the biochemical criteria for purity but were used only for comparison in some of the gel electrophoretic studies reported below. Plasma membrane isolated by the glutaraldehyde procedure was used for qualitative and quantitative chemical studies and for the observations on labeling and turnover presented in the following report (7). In this and the subsequent report, the term plasma membrane referes to the external membrane obtained from the glutaraldehyde-fixed preparation. "Interiorized" or phagolysosomal membrane refers to the membrane coating the ingested polystyrene particles prepared from cells not exposed to glutaraldehyde.

Morphologic Evidence of Purity of the Membrane Preparations.—

(*a*) *Plasma membranes isolated from glutaraldehyde-fixed cells:* The morphologic

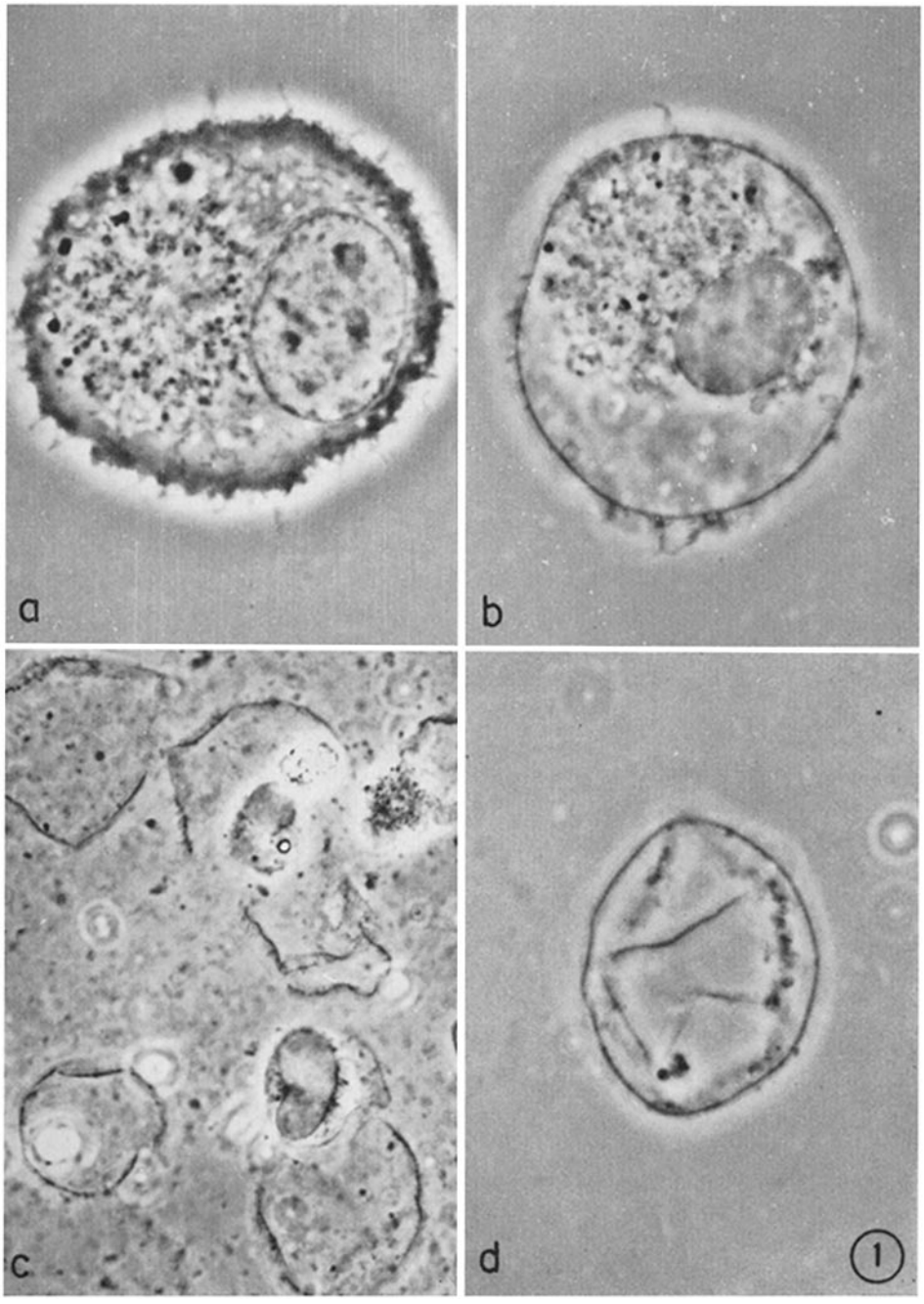
appearance of the cells at various steps of the separation procedure is shown in Fig. 1. The characteristic features of the normal alveolar macrophage as seen under phase microscopy (panel *a*) include an oval nucleus, centrosomal vesicles and small phase-dense granules, scattered accumulations of carbon, and an irregular surface contour. After hypotonic swelling in water and glutaraldehyde fixation (panel *b*) the nucleus and granular cytoplasmic elements appeared as a central mass separated by a clear space from the plasma membrane which appeared smooth and stretched. After Dounce homogenization (panel *c*) large membrane sheets were seen admixed with nuclear debris and heterogeneous small granular cell components. The final membrane band after sucrose gradient centrifugation (panel *d*) was composed almost entirely of intact membrane sheets which appeared to be relatively clean except for a few phase-dense particles.

The characteristic features of the intact cell as seen under electron microscopy (Fig. 2 *A*) included a large oval nucleus, well developed Golgi zone surrounded by numerous large granules, and many fingerlike projections of the cell surface. Electron microscopy of the isolated membrane fraction (Fig. 2 *B*) revealed large sheets of membranous structures. There was slight contamination with amorphous electron-opaque material. The typical trilaminar membrane structure was seen under high magnification (Fig. 2 *C*).

(*b*) *Phagolysosomal membranes:* Electron microscopic evaluation of the phagolysosomal band isolated at the 10–20% sucrose interface revealed polystyrene particles surrounded completely or in part by a membrane. In many instances the membrane sacs contained, in addition to the polystyrene, granular electron-opaque material or small vesicular or lamellar elements (see Fig. 3 *A*). Electron microscopy of the membrane pellet obtained after sonic disruption of the phagolysosomes and removal of the latex particles revealed numerous microvesicular structures (Fig. 3 *B*). The presence of acid phosphatase in many of the membrane-bounded polystyrene sacs, and the abundance of acid phosphatase in the membrane fraction isolated from these structures, indicated that they were to a large extent phagolysosomes formed by fusion between polystyrene phagocytic vacuoles and the lysosomal granules present in abundance in the alveolar macrophages.

Biochemical Evidence for Purity of the Plasma Membrane Preparation.—In order to evaluate further the purity of the plasma membrane fraction, enzyme profile analyses were performed. Evidence for the biochemical purification of a cellular component fraction depends on demonstrating increased specific activity of known marker enzymes while at the same time establishing maximum contamination by other cellular organelles using characteristic marker enzymes for these "contaminating" cell fractions.

An enzyme profile analysis of the purified membrane fraction is seen in Table I. The specific activities of four separate enzyme markers in the glutaraldehyde-


a
b
c
d
1

treated homogenate as well as the purified membrane fraction are shown. Although enzymatic activity was in each instance partially destroyed (50–80%) by glutaraldehyde fixation, the residual enzymatic activities were nevertheless significant and served as useful markers for the individual organelles. The values shown represent the averages of three separate determinations performed on different preparations. 5′ nucleotidase, an enzyme considered to be a relatively specific plasma membrane marker (32, 33), was enriched 40-fold in the membrane preparation when compared to the cell homogenate. In contrast, the enzyme markers for endoplasmic reticulum (glucose-6-phosphatase), mitochondria (succinic dehydrogenase), and lysosomes (acid phosphatase) showed no significant enrichment in the membrane fraction. The relative contamination was estimated by comparing the total amount of the various enzyme markers present in the final membrane fraction with the amount present in the glutaraldehyde-treated unfractionated homogenate and correcting for the incomplete membrane yield. The calculated contamination of the plasma membrane fraction with other cell organelles was reasonably small (Table II). Making the assumption that these marker enzymes are distributed in macrophages as they are in hepatocytes (34), we calculated that the plasma membrane in these preparations was approximately 92% pure with a yield of 18–30% of that originally available in the homogenate.

Overall Chemical Composition of the Plasma Membrane.—Chemical analysis of the isolated plasma membrane fraction revealed 46% protein, 41% lipid, and 8% carbohydrate (Table III). Of some interest was the relatively high content of RNA, averaging 3% of the membrane by dry weight on four separate membrane preparations. This membrane-associated RNA represented 8–10% of the total cell homogenate RNA.

Amino acid analysis of the plasma membrane fraction (Table IV) revealed a pattern similar to that described for membrane protein analyses of other cells

FIG. 1. Phase-microscopic pictures illustrating various steps in the isolation of rabbit alveolar macrophage plasma membranes. (*a*) A living alveolar macrophage. Note the large oval nucleus and the dark granules gathered about the centrosomal region in the upper part of this cell. A few clear vesicles and threadlike mitochondria are seen in the perinuclear region. The cell is round in overall shape, but the surface membrane appears to have a highly irregular detailed contour with numerous convolutions and fingerlike projections. × 2000. (*b*) An alveolar macrophage after swelling in distilled water and brief fixation with glutaraldehyde. The oval nucleus appears homogeneous. Granules and other organelles are clustered forming a perinuclear mass. The surface membrane is well defined and appears stretched. A large clear zone is seen between the nucleocytoplasmic aggregation at right and the plasma membrane over the left half of the cell. × 2000. (*c*) Large membrane sheets, nuclei, and dispersed granules are liberated after the swollen, aldehyde-fixed cells are disrupted in a Dounce homogenizer. × 800. (*d*) An example of a cell ghost present in the membrane band obtained from the final sucrose density gradient centrifugation. The band contained large membrane fragments, some of which appeared to be slightly contaminated with granular material. ×2000.

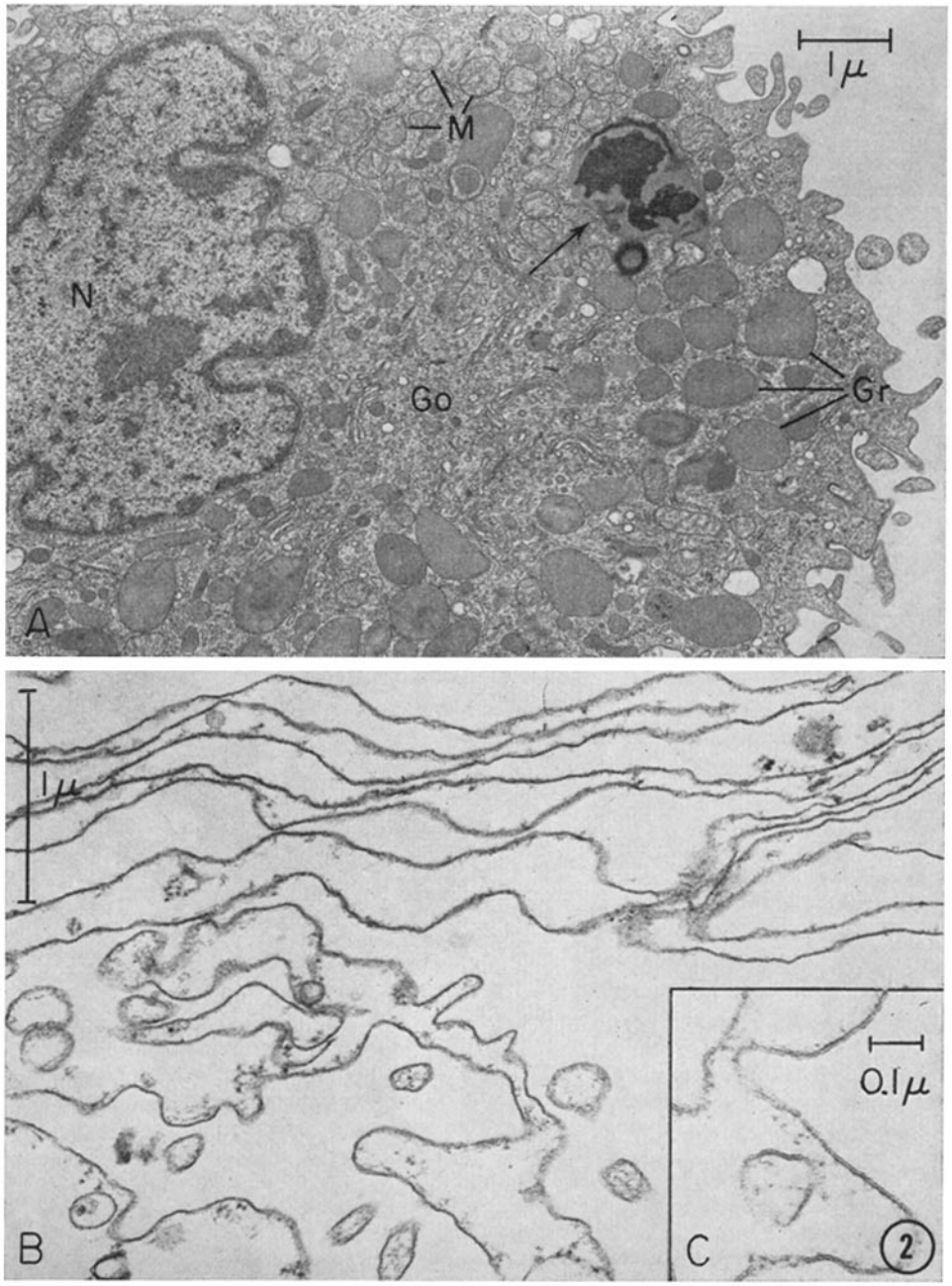


FIG. 2. Ultrastructural appearances of a rabbit alveolar macrophage (above) and of the plasma membrane fraction (below) isolated from these cells. The alveolar macrophage in panel *A* illustrates typical features of these cells: large oval nucleus (*N*), perinuclear Golgi region (*Go*) with vesicles and flattened saccules, and large numbers of dense granules (*Gr*) and mitochondria (*M*) in the peripheral cytoplasm. A residual body containing carbon is present (arrow). Note the numerous fingerlike projections of the cell surface at right. × 12,000. Panel *B* is an electron micrograph of the membrane fraction isolated from alveolar macrophages. The fraction consists of long membrane sheets in a loosely packed parallel array. A few small granular elements appear to be attached to some of the membranes. × 28,000. The insert (*C*) shows the membrane fraction at higher magnification. Typical 9–10 nm trilaminar unit structure is visible in many regions of the membrane. × 70,000.

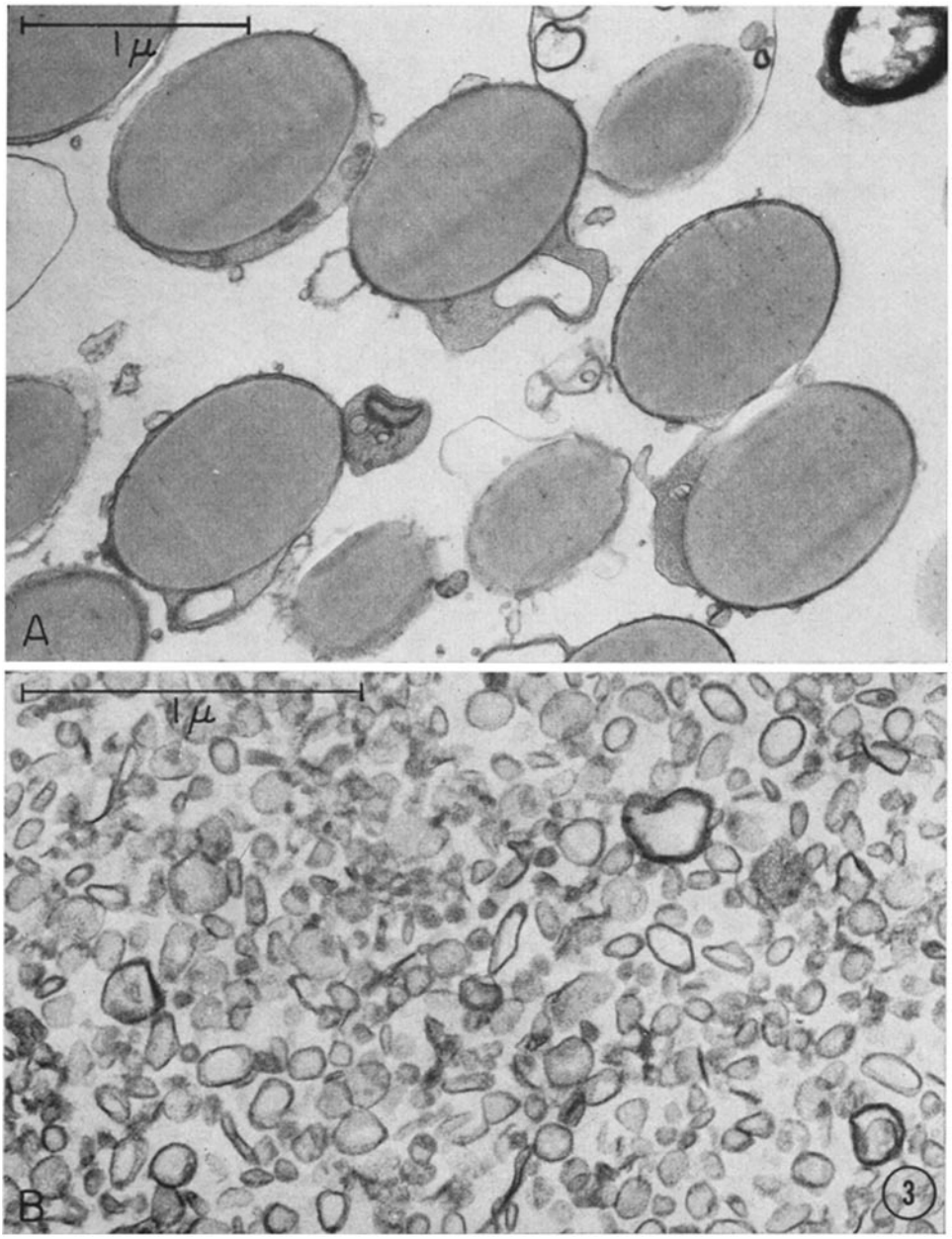


FIG. 3. Phagolysosomes isolated from alveolar macrophages which had ingested polystyrene spheres (above), and a membrane fraction recovered from disrupted phagolysosomes (below). As is seen in panel *A*, the polystyrene spheres appear as moderate electron-opaque structureless oval bodies in this material processed so as to avoid extraction of the plastic. The spheres are surrounded completely or in part by membranes. In many instances the membrane sacs contain, in addition to polystyrene, finely granular electron-opaque material or small vesicular or lamellar elements. × 30,000. Panel B illustrates the ultrastructure of the membrane fraction recovered from phagolysosomes disrupted by sonication. The material consists entirely of very small (50–200 mμ) vesicles with generally electron-transparent content. × 45,000.

(2). Of note was the characteristically high aspartic and glutamic acid content.

Studies of the Number of Protein Components and their Size by Gel Electrophoresis.—

(*a*) *Solubility:* In order to study adequately the individual membrane components, it was necessary to solubilize the membrane protein. The glutaraldehyde-fixed plasma membranes proved to be relatively insoluble in the usual buffer systems (Table V). They were essentially insoluble in a saline buffer, and only slightly soluble in 2% sodium dodecyl sulfate (SDS). More drastic procedures such as 90% formic acid followed by dialysis into a mercaptoethanol–urea solution followed by dialysis into 2% SDS yielded somewhat better final solubility. Maximum solubility (60–70%) of the plasma membrane proteins

TABLE I

Enzyme activity of macrophage plasma membrane fraction

Enzyme marker	Enzyme specific activity*		Specific activity ratio Membrane / Homogenate
	Homogenate	Membrane fraction	
5′ Nucleotidase (Plasma membrane)	0.31 ± 0.07	12.5 ± 1.6	40.3
Glucose-6-phosphatase (Endoplasmic reticulum)	0.83 ± 0.17	0.63 ± 0.07	0.75
Succinic dehydrogenase (Mitochondria)	0.22 ± 0.05	0.35 ± 0.14	1.6
Acid phosphatase (Lysosomes)	6.6 ± 1.1	6.6 ± 1.4	1.0

* Units: nucleotidase, μM/mg per min; glucose-6-Phosphatase, μM inorganic P/mg per 30 min; dehydrogenase, μM indophenol/mg per min; acid phosphatase, μg/napthol per mg/30 min.

Results are means ± 1 SD.

TABLE II

Maximum calculated contamination of plasma membrane fraction

Cell component	Contamination
	%
Endoplasmic reticulum (Glucose-6-phosphatase)	1.8
Mitochondria (Succinic dehydrogenase)	3.2
Lysosomes (Acid phosphatase)	2.5
Nuclei (DNA)	<1.0

was achieved in a phenol–urea–acetic acid mixture. The phagolysosomal membrane was approximately 85% soluble in the phenol–urea reagent and 60–75% soluble in the SDS reagent. Solubility of this membrane in aqueous buffers was not determined.

TABLE III

Chemical composition of macrophage plasma membrane

Component	%
Protein	46
Lipid	41
Carbohydrate*	8
RNA	3
DNA	0.02

* Hexosamine not determined.

TABLE IV

Amino acid composition of macrophage plasma membrane

Amino acid	Residues/100 residues
Lysine	3.86
Histidine	2.15
Arginine	5.76
Aspartic acid	10.70
Threonine	6.53
Serine	8.16
Glutamic acid	11.74
Proline	6.14
Glycine	8.62
Alanine	8.24
Half-cystine	1.88
Valine	6.53
Methionine	2.11
Isoleucine	4.03
Leucine	8.41
Tyrosine	2.90
Phenylalanine	4.05

(*b*) *SDS gel studies:* Acrylamide gel electrophoresis of plasma membrane proteins partially solubilized in SDS revealed several polypeptide bands (Fig. 4). For this preparation, membrane protein was first dissolved in 90% formic acid, dialyzed into mercaptoethanol–urea, and then dialyzed into 2% SDS. Approximately 25% of the membrane protein was solubilized. It has been previously shown that marker polypeptides migrate in these gels primarily as a function of molecular size (35). The molecular weight range of the major plasma

membrane components varied between 70,000 and 140,000. It is of interest that seven to eight bands were observed in this sytem. Similar studies performed on phagolysosomal membrane proteins solubilized in SDS also revealed seven to nine bands with the same general molecular weight range as that noted for the plasma membrane proteins. The patterns observed with the phagolysosomal membrane protein in the SDS gels were not sharply defined, thus it was not

TABLE V

Solubility of Macrophage Plasma Membrane

Solvent	Protein solubilized
	%
Phenol–urea–acetic acid	60–72
Formic acid–urea–SDS	25
Sodium dodecyl sulfate–2%	15
Phosphate saline (pH 7.4)	0–1

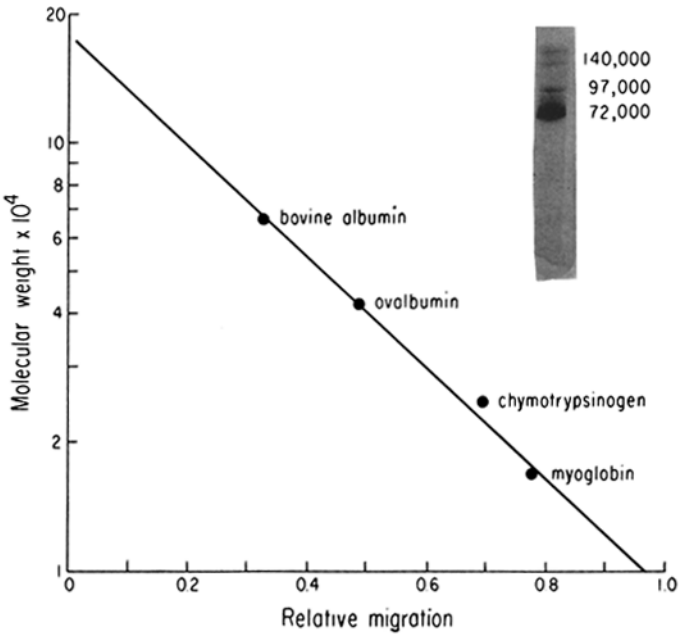


FIG. 4. Molecular weight estimations of partially solubilized macrophage plasma membrane proteins in an SDS gel. At left is shown a plot of relative migration rates in the gel of markers of known molecular weight. The insert at upper right is a stained gel of the separated membrane proteins; approximately seven bands are visible, with R_f values indicating a range of molecular weights from 72,000 to 140,000.

possible to determine accurately the molecular weights of the individual proteins.

(*c*) *Phenol–urea gel studies:* For these studies, which allowed maximum solubility, the plasma membrane proteins as well as the phagolysosomal membrane proteins were dissolved in phenol–urea–acetic acid and subjected to electrophoresis in a 7.5% gel containing 5 M urea and 35% acetic acid. The acrylamide gel patterns of both membrane preparations are compared in Fig. 5. The protein patterns were similar in number of bands and in overall size distribution. Several of the components also appeared to have the same migration rates, but

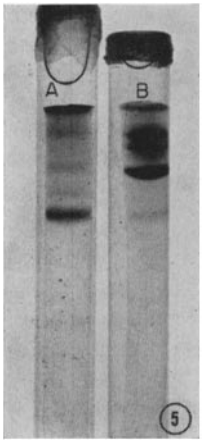


FIG. 5. Shows comparison of gel patterns of the glutaraldehyde-fixed macrophage plasma membranes (*A*) and the unfixed phagolysosomal membranes (*B*). The membranes were solubilized in phenol–urea–acetic acid and subjected to electrophoresis in 7.5% acrylamide gel containing 5 M urea and 35% acetic acid. Overall number, size distribution, and migration rates of individual protein bands were quite similar, but the relative concentrations of the individual components appeared to be different in the two membrane preparations.

they differed markedly in concentration as reflected by differences in intensity of staining.

Studies on the Nature of the Membrane Protein Components.—Chemical heterogeneity of the plasma membrane components was demonstrated by differential staining of the acrylamide gel electrophoretic bands (Fig. 6). Two anodally moving bands stained heavily for lipid. One band close to the origin stained faintly for carbohydrate. Similar differential chemical staining studies of the phagolysosomal membrane protein patterns also revealed two anodally moving lipid bands. PAS staining of the phagolysosomal membrane proteins was unsatisfactory on several different preparations.

Guinea pig antiserum to the isolated plasma membrane fraction precipitated

on immunoelectrophoretic analysis a single serum protein constituent, rabbit IgG (Fig. 7). This reactivity was completely removed by prior absorption of the antiserum with normal rabbit serum or with rabbit IgG. On immunodiffusion,

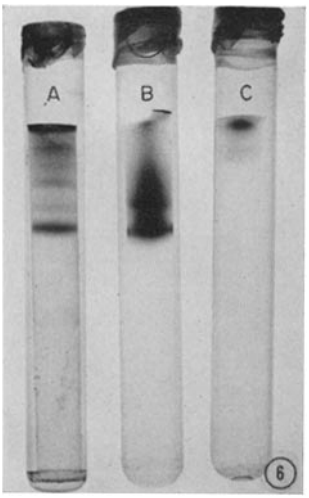


FIG. 6. Shows acrylamide gel patterns run in parallel on macrophage plasma membranes dissolved in phenol–urea–acetic acid. The gel at left (*A*) was stained for protein with amido Schwartz. Gel *B* was stained for lipids with Sudan black B. Gel *C* was stained for carbohydrate using the Schiff reagent. The two most rapidly migrating protein bands stained positively for lipid in Gel *B*. The carbohydrate stain revealed a faint band near the origin (Gel *C*).

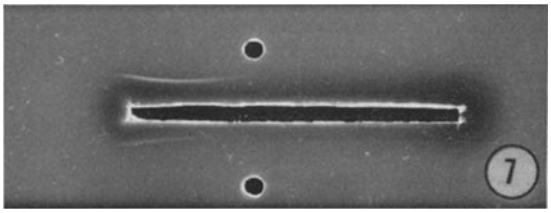


FIG. 7. The demonstration by immunoelectrophoresis of the presence of an antibody to rabbit gamma globulin in the serum of a guinea pig immunized with plasma membranes isolated from rabbit alveolar macrophages. The upper well contained purified rabbit IgG, the lower well contained whole rabbit serum. Anode at the right. The central trough was filled with guinea pig antiserum to the rabbit macrophage plasma membranes. A single precipitin arc is seen in the position characteristic for gamma globulins.

the plasma membrane proteins partially solubulized in SDS formed two precipitin bands with antiserum which had been absorbed with rabbit IgG and whole rabbit serum (Fig. 8). Thus at least two antigenic specificities were detectable in the final isolated plasma membrane preparation: IgG demonstrated

on immunoelectrophoresis, and one or two antigens other than IgG demonstrated by immunodiffusion. Immunologic studies were not performed with the phagolysosomal membrane proteins.

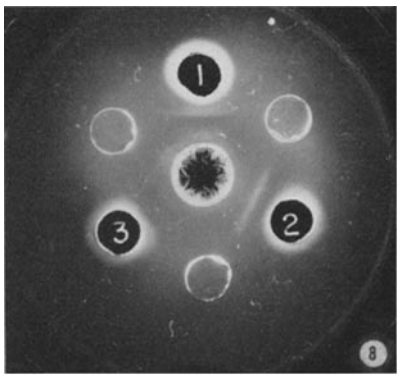


FIG. 8. Gel diffusion studies on components in the antiserum to macrophage plasma membrane. The central well contained plasma membranes solubilized in sodium dodecyl sulfate. Wells 1 and 2 contained two different guinea pig antisera, each of which had been absorbed with rabbit serum and rabbit gamma globulin. One or two precipitin bands are seen. Well 3 contained normal (preimmunization) guinea pig serum.

DISCUSSION

The Use of Aleolar Macrophages for Plasma Membrane Isolation.—Rabbit alveolar macrophages are derived from progenitors in the bone marrow which enter the blood stream as monocytes and then emigrate into pulmonary tissue. The long-term history of these macrophages is not entirely certain; they may enter the alveoli and be transported up the bronchotracheal escalator to be discharged, or they may remain at local lung sites for long periods of time. These cells play an important role in host defense against respiratory infection (36). Alveolar macrophages display activities typical of macrophages in general; they adhere to glass, they demonstrate ruffled plasma membranes, and they engage in pinocytosis and phagocytosis.

Many of these macrophage activities involve participation of the plasma membrane, making this a particularly interesting cell to study in a systematic analysis of plasma membrane proteins. Alveolar macrophages are available in large quantities as an essentially pure population on tracheobronchial lavage of rabbits which have been suitably stimulated by BCG (8).

The Isolation of Plasma Membrane from Alveolar Macrophages.—Technical difficulties were encountered in the course of the initial attempts to isolate plasma membrane from alveolar macrophages. Success was finally achieved with a modification of the procedure used by Warren, Glick, and Nass for

isolating cultured fibroblast membranes (10). The basic steps of this procedure involve hypotonic swelling of the cell suspensions, followed by brief exposure to some agent which preserves the membrane in a relatively stretched state while fixing the major intracellular components as a shrunken central cytoplasmic mass. Gentle homogenization then yields large sheets of plasma membrane which can be isolated by centrifugation. We were unable to isolate macrophage membranes with zinc salts, fluorescein mercuric acetate, or tris (hydroxymethyl) aminomethane as recommended by Warren and colleagues (10). The use of glutaraldehyde as the fixative led to the recovery of good yields of plasma membrane sheets in an excellent state of purity. The glutaraldehyde step made the isolation procedure reliable and relatively rapid, and it had the further advantage of preserving both the ultrastructure and the enzymatic activity of the membrane, so that assessment of purity could be based on morphologic and biochemical criteria. Obviously purity of the plasma membrane preparation must be established before meaningful studies can be done on its protein components.

The major disadvantage of the use of glutaraldehyde relates to its cross-linking activities on proteins. We thus had to consider the possibility that exposure to glutaraldehyde significantly modified the membrane proteins so as to alter their solubility, number, and individual properties. This consideration stimulated us to seek, for purposes of comparison, a source of unfixed plasma membrane from alveolar macrophages. Membranes recovered from polystyrene phagosomes, separated by the method of Wetzel and Korn (11), proved useful to this end. Although these phagosomal membranes are perhaps somewhat altered from the native plasma membranes by virtue of their interaction with the polystryrene spheres or their fusion with lysosomes, they did nevertheless provide us with reassurance on the validity of the results obtained on membranes isolated after exposure to glutaraldehyde, since generally similar gel electrophoretic patterns were given by solutions of these two membrane preparations. Particularly important was the fact that the unfixed phagolysosomal membranes and the glutaraldehyde-treated plasma membranes exhibited approximately the same number of major protein components (See Fig. 5). There is thus no evidence that glutaraldehyde used under these conditions produced significant cross-linking between different species of proteins in the membrane. The number of protein bands was not significantly reduced (or increased) by the glutaraldehyde. Furthermore, the degree of denaturation by glutaraldehyde within a single protein molecule is not great since enzymatic and γG-receptor activities are at least partially preserved. It is thus reasonable to assume that the results obtained by our modification of the Warren procedures are valid in terms of numbers and general types of proteins present in the macrophage membrane.

It should be pointed out that the mixtures of agents used to solubilize mem-

branes, native or fixed, are themselves denaturing to a greater or lesser degree, so that the protein patterns in gels cannot be taken as an absolute reflection of the state of native proteins. These patterns are nevertheless meaningful and of significance in revealing the degree of heterogeneity (number of bands) and some general properties of the isolated materials (size, presence of carbohydrate and lipid, etc.).

The question should be raised as to the necessity for using any "fixative" agent for the isolation of macrophage plasma membranes. Hepatocyte membranes have been recovered by centrifugal separation after simple homogenization of liver tissue (6). Hepatocytes, or cells in other organs, are attached to one another or fixed in a connective tissue matrix so that the cells are readily broken by gentle homogenization. In the case of free cells in suspension, such as the macrophages used here, the cells resist disruption, and when homogenization is applied with vigor sufficient to break most of the cells, the plasma membranes and intracellular membranes are shredded and form a mixed population of small vesicles which cannot be adequately separated.

Characteristics of Macrophage Membrane Protein.—Numerous studies have been reported in recent years dealing with the fundamental characteristics of the protein in various cell membrane preparations. Green et al. (37), approximately 10 yr ago, reported the isolation of a small molecular weight homogeneous "structural" protein from heart mitochondria which was enzymatically inactive and constituted approximately 60% of the mitochondrial membrane protein. Similar proteins have been isolated from liver microsomes, chloroplasts (38), neurospora mitochondria (39), and human red cell membranes (40). From these studies arose an early simplified model of membrane protein. This held that the cell membranes of many different species were essentially similar in that the membrane backbone was composed of a small molecular weight enzymatically inactive structural protein which in effect served as a membrane building block. This concept, however, has radically changed in the last few years. The present data strongly suggest that the structural protein homogeneity of earlier studies was probably related to artifacts of denaturation and poor resolution (41, 42).

In fact, there is much evidence now from several studies that membranes are comprised of numerous proteins with no single species predominating. It has been shown in several laboratories that erythrocyte membranes contain up to 15–20 separate protein species (43, 44). Plasma membranes from several strains of mycoplasma also contain approximately 20 proteins (45). Studies with liver cell membranes show similar degrees of heterogeneity (6). We have previously demonstrated approximately 15 separate proteins in human platelet membranes (5). The most recent evidence therefore strongly suggests that cell membranes contain a complex array of heterogeneous proteins.

In comparison with these previous observations, the macrophage membrane

is remarkable in terms of the relatively small number of its protein components. At the outset of this study we held the opposite expectation; macrophage membrane would seem to be functionally as complex or more complex than erythrocyte or mycoplasma membranes, and the small number of protein elements present is thus surprising and merits further study. The number of proteins in the macrophage membrane is sufficiently small that studies on structural and functional roles of individual components seem possible.

Our studies clearly demonstrate the molecular weight as well as chemical heterogenetiy of the alveolar macrophage plasma membrane proteins. The molecular weight of the proteins solubilized in the SDS experiments ranged from 70,000 to 140,000. Of the seven to nine proteins demonstrated electrophoretically after phenol solubilization, two stained for lipid and at least one stained for carbohydrate. In view of the incomplete solubility of the preparations used in these studies (25% in SDS, 60–70% in the phenol mixture), it is possible that the number of proteins observed represents only a fraction of that originally present in the native plasma membrane. Our observations on the unfixed phagosomal membranes, which appeared to be somewhat more soluble in SDS, gave the same small number of membrane bands, tending to support the validity of the conclusion that the number of components is reasonably small.

The chemical composition and amino acid analysis of our final plasma membrane preparation are generally similar to those previously found in studies of other mammalian cell membranes (2). The relatively large amount of RNA (3% by dry weight) associated with macrophage plasma membrane is practically identical to the amount found by Glick and Warren (46) to be associated with mouse fibroblast plasma membranes. These investigators reported that the L-cell surface membranes exhibited protein synthesis in vitro. We made similar studies on the macrophage membrane preparation and found it to be totally inert in an in vitro protein-synthesizing system, showing no evidence of functionally intact ribosomes or functionally intact messenger RNA.[2] Our findings in this regard are of questionable significance in view of the likelihood that glutaraldehyde fixation may significantly compromise the synthetic system.

Immunochemical analysis of the isolated plasma membrane clearly indicated rabbit IgG in the preparation. No other known rabbit serum proteins were detectable. It has been previously shown that macrophages have specific membrane-binding sites for the F_c portion of the IgG molecules (47). Thus it would appear likely that the immunoglobulin detected in the macrophage membrane preparation represents tightly bound IgG originally present in the alveolar exudate. The demonstration of immunoglobulin in these highly purified membrane preparations illustrates the fact that some "membrane proteins" may be extracellular proteins adsorbed firmly to membrane receptor sites rather than

[2] Nachman, R., and J. Lucas Lenard. Unpublished observations.

integral structural or functional components of the membrane proper. Macrophages have also been shown to contain specific complement-receptor sites (48), but these proteins were not identified in the isolated macrophage membrane preparation.

SUMMARY

Plasma membranes have been isolated from pure populations of rabbit alveolar macrophages which were swollen in water, fixed briefly with glutaraldehyde, disrupted by Dounce homogenization, and separated by sucrose gradient centrifugation. The recovered membranes exhibited good structural preservation and enzymatic activity; both morphologic and biochemical evidence indicated a high degree of purity (>90%) of the membrane preparation.

Interiorized plasma membranes were also prepared without exposure to glutaraldehyde from phagocytic vacuoles recovered from alveolar macrophages which had ingested large numbers of polystyrene spheres. These membranes were contaminated with lysosomal constituents, but they were nevertheless of value for comparison to the "pure" membranes isolated by the glutaraldehyde procedure.

Acrylamide gel electrophoresis of the solubilized plasma membranes and phagolysosomal membranes revealed similar protein patterns, with seven to nine individual components ranging in molecular weight from 70,000 to 140,000. The two most rapidly migrating components gave positive reactions for lipid as well as protein. A band containing carbohydrate was detected near the origin of the plasma membrane gels.

Antisera were made by injecting guinea pigs with the purified rabbit alveolar macrophage plasma membranes. Gel diffusion and immunoelectrophoretic study of these antisera established the presence of rabbit immunoglobulin G and of one or two other antigenic constituents in the membrane preparation.

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