6. E. Reczko and A. Mayr, *Arch. Ges. Virusforsch.* **13**, 591 (1963).
7. J. H. Gillespie, K. McEntee, J. W. Kendrick, W. C. Wagner, *Cornell Vet.* **49**, 288 (1959).
8. W. W. Dimock, P. R. Edwards, D. W. Bruner, *Kentucky Agr. Exp. Sta. Bull.* 426 (1942).
9. L. E. Carmichael, J. Fabricant, R. A. Squire, *Proc. Soc. Exp. Biol. Med.* **117**, 826 (1964).
10. M. F. Barile, R. Yaguchi, W. C. Eveland, *Am. J. Clin. Pathol.* **30**, 171 (1958).
11. The antiserums were provided by E. R. Doll, Univ. of Kentucky (equine rhinopneumonitis); J. H. Gillespie, Cornell Univ. (IBR); T. Tokumaro, Children's Hospital, Philadelphia (pseudorabies); Fromm Laboratories, Grafton, Wis. (canine hepatitis and canine distemper).

22 April 1965

Separation and Partial Purification of Plasma-Membrane Fragments from Ehrlich Ascites Carcinoma Microsomes

Abstract. *Membrane vesicles arising from the surface of Ehrlich ascites carcinoma cells were separated from the bulk of other microsomal components in Ficoll density gradients. Initial separation depends on the differential action of magnesium ion on various microsomal components. Immunologic and enzymic markers were used to monitor separation and purification steps.*

The membrane components of Ehrlich ascites carcinoma microsomes are made up primarily of broken endoplasmic reticulum, and also of the fragmented plasma membrane of these cells (*1–4*). We have previously examined the distribution of mixtures of microsomal membranes in density gradients of Ficoll, a sucrose polymer of low osmotic activity, and demonstrated that the component membranes exist as fluid-filled vesicles whose volumes are determined by electrostatic mechanisms and by osmotic effects (*4*). We followed the plasma-membrane fragments by means of a specific immunologic tag and by use of an enzyme marker, and showed vesicles derived from the surface membrane of these cells to be relatively insensitive to changes in ionic environment, in contrast with vesicles arising from the cell interior, which shrink with decreasing *p*H and with increasing ionic strength. We now report a purification of plasma-membrane vesicles which relies on their stability in the presence of divalent cations, which shrink and aggregate most other membrane structures. In that it relies on specific markers and depends on differences between the electrostatic properties of various membrane types, this approach differs from other membrane separation methods (*5*).

Purification of Ficoll, preparation and sampling of density gradients, density measurements, centrifugations, and determinations of protein and of rates of adenosine triphosphate hydrolysis have been described (*3, 4*). Oxidation of reduced diphosphopyridine nucleotide (DPNH-diaphorase activity) was measured in a medium containing $10^{-4}M$ DPNH, $6.6 \times 10^{-4}M$ potassium ferricyanide, $0.01M$ tris-hydroxymethyl aminomethane (Tris) (*p*H 7.4), and 4 to 20 μg of membrane protein in a volume of 1.1 ml. Oxidation of DPNH was followed by the change of absorbance at 340 mμ. The extinction coefficient used was 6.22×10^3 liter mole^{-1} cm^{-1}.

Distribution of cell-surface antigens (*2, 4*) was estimated by the ability of various fractions to absorb antibody capable of agglutinating intact tumor cells. Antiserum was prepared by immunization of a horse with whole microsomal membranes. The γ-globulin (antibody fraction) was prepared from the antiserum as described (*4*).

Absorptions were performed as follows: about 100 μg of protein from each fraction was incubated with 480 μg of the γ-globulin for 15 minutes at 37°C in a mixture of 0.5 ml of $0.15M$ NaCl, $0.01M$ Tris (*p*H 7.4), and 0.2 percent gelatin. The volume was increased to 2 ml with the same medium, and the mixture was centrifuged at 40,000 rev/min for 15 minutes. The supernatants were tested for agglutinating antibody, with serial twofold dilutions (*6*). A 2-plus reaction, defined as the formation of clumps about 0.2 mm in diameter, was chosen as end point. The unabsorbed γ-globulin was used as reference in each assay. With our γ-globulin preparation and 2×10^5 cells per milliliter of assay mixture, a 2-plus reaction was obtained with 3.8 μg of γ-globulin per milliliter. Results of antibody absorptions are expressed as changes in the concentration of γ-globulin required to produce agglutination. When absorption reduced the agglutinating titer by more than four doubling dilutions, the absorption was repeated with less protein. In those fractions giving no detectable absorption in the initial test, absorptions were repeated at higher concentrations of protein.

To isolate plasma membranes, tumor cells were separated as before (*7*) and then ruptured in a mixture of $0.25M$ sucrose, $0.005M$ Tris (*p*H 7.4), and $0.0002M$ $MgSO_4$ by intracellular cavitation of nitrogen (*8*). The homogenate was then made to $0.001M$ with respect to EDTA, and nuclei, mitochondria, and lysosomes were sedimented by centrifugation for 15 minutes at 12,500 rev/min and 4°C (rotor 9RA, Lourdes Betafuge). Microsomes were sedimented by centrifugation for 45 minutes at 40,000 rev/min and 4°C (rotor No. 40, Spinco L-2 preparative ultracentrifuge). The microsomal pellet was resuspended to a concentration of 1.0 to 1.5 mg of protein per milliliter in $0.01M$ Tris (pH 8.6) and resedimented by centrifuging at 40,000 rev/min for 45 minutes. The washing was repeated with $0.001M$ Tris, *p*H 8.6. The initial washing removes trapped soluble proteins; intravesicular soluble proteins are released in the second washing step because of the transient leakiness of the vesicle membranes caused by osmotic stress at very low ionic strength (*9*). The microsomes were then homogenized in $0.001M$ Tris

Table 1. Distribution of microsomal protein and of various membrane markers after centrifugation of Ficoll-Mg barrier at 25,000 rev/min for 15 hours at 4°C in SW-25 rotor; 51.5 mg of protein per tube; percentages are of total recovery.

Location	Protein (%)	DPNH-diaphorase		Na^+-, K^+-ATPase*		Surface antigen	
		mmole mg^{-1}min^{-1}	%	Pi (μmole-mg^{-1} hr^{-1})	%	Δ(γ-globulin) mg^{-1}	%
At barrier	20.2	0.068	3.7	7.95	67.1	1.14	88.3
In barrier	11.5	.213	6.3	2.80	13.4	0.12	4.5
Pellet	62.9	.553	90.0	0.74	19.4	.03	7.3

* Adenosine triphosphatase; P_i, inorganic phosphorus.

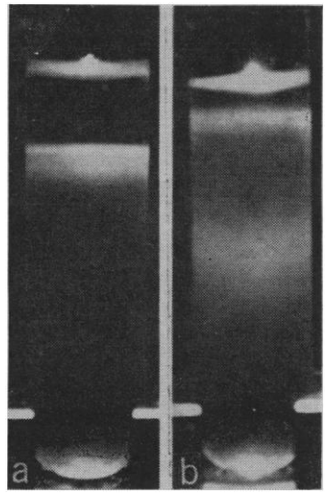

Fig. 1. *a*, Initial separation: top zone, containing the plasma-membrane fragments, consists of two closely spaced bands (SW-39 rotor, 39,000 rev/min, 5 hours, 4°C; 9.7 mg protein per tube). *b*, Separation of the top zone into its major components (SW-39 rotor, 35,000 rev/min, 16 hours, 4°C; 2.3 mg protein per tube).

(*p*H 8.6) containing 0.001*M* $MgSO_4$ to give a suspension of protein at 3 to 7 mg/ml, which was dialyzed against 200 volumes of the same medium for 2 hours at 4°C. After dialysis, the suspension (1.5 ml for rotor SW-39 and 10 ml for rotor SW-25) was layered on two volumes of Ficoll (density, 1.096 at 4°C) containing 0.001*M* Tris and 0.001*M* $MgSO_4$ and centrifuged at 39,000 rev/min for 5 hours (rotor SW-39) or at 25,000 rev/min for 15 hours (rotor SW-25).

During centrifugation the membrane material separates into two major fractions (Fig. 1*a*, a zone at the top of the barrier, devoid of aggregates and consisting of two closely associated bands, and a heavily clumped pellet. There is a small amount of material in the barrier itself. The top zone and the barrier were withdrawn separately, and the pellet was homogenized on 0.001*M* Tris (*p*H 8.6) and 0.01*M* EDTA. The various fractions were then dialyzed first against this medium and then against 0.001*M* Tris, *p*H 8.6; this step reduces aggregation of the pellet material. Samples were taken for the various analyses, and the remainder was stored at −28°C.

The procedure was initially tested with an SW-39 rotor containing three identical Ficoll barrier tubes. In these experiments 17 to 21 percent of the applied microsomal protein was recovered at the top of the barrier. This top zone also contained 85 to 91 percent of the total surface antigen and 71 to 72 percent of the Na^+- and K^+-activated adenosine triphosphatase. The SW-25 rotor gave similar results (Table 1).

The surface membrane fragments in Ehrlich ascites carcinoma microsomes can be readily separated in high yield from the bulk of the microsomal components. With the surface antigen as criterion, the initial yield in the top zone approaches 90 percent. Those surface membrane vesicles which do go into the pellet are trapped nonspecifically and appear at the top of the Ficoll-magnesium barrier when the pellet material is recycled through a procedure identical with the original separation step.

Only about two-thirds of the Na^+- and K^+-activated adenosine triphosphatase is recovered in the top zone; a consistent proportion (20 to 26 percent) persists in the pellet even after recycling. Two explanations for the presence of this enzyme-component must be considered: (i) The enzyme system, although concentrated in the plasma membrane, is also present in some intracellular membrane structures, possibly precursors of plasma membrane; and (ii) the enzyme represents fragments of plasma membrane which closed "inside-out" during cell rupture, so that surface antigens are not accessible to antibody in our absorption procedure.

The distribution of DPNH-diaphorase suggests that this is an intracellular enzyme system, and we tentatively assume that its presence in the top zone represents contamination by membranes arising from the cell interior. If all particles bearing DPNH-diaphorases have the same specific activity, 12.5 to 32 percent of the protein in the top zone must be suspected of arising from the cell interior.

The above separation depends on the following phenomena: at *p*H 8.6, divalent cations cause shrinkage of most of the vesicles arising from the endoplasmic reticulum and related structures, but not of vesicles arising from the cell surface. In Ficoll gradients a volume decrease leads to an increase in density (*4*). The action of divalent cations is presumed to involve reduction of the surface potential of susceptible structures; that this is the case has been shown by the reduction in the electrophoretic mobility of microsomal membranes in the presence of Mg^{++} (*10*). Decreased surface potential promotes aggregation, and this in turn speeds sedimentation of the aggregated particles in the initial zone. Clumping occurs when the small aggregates are decelerated, and thus concentrated, at the Ficoll barrier; the newly formed large clumps move rapidly into the pellet. The separation process is further accelerated by coaggregation between ribosomes and vesicles arising from the cell interior (*11*). The sensitivity of

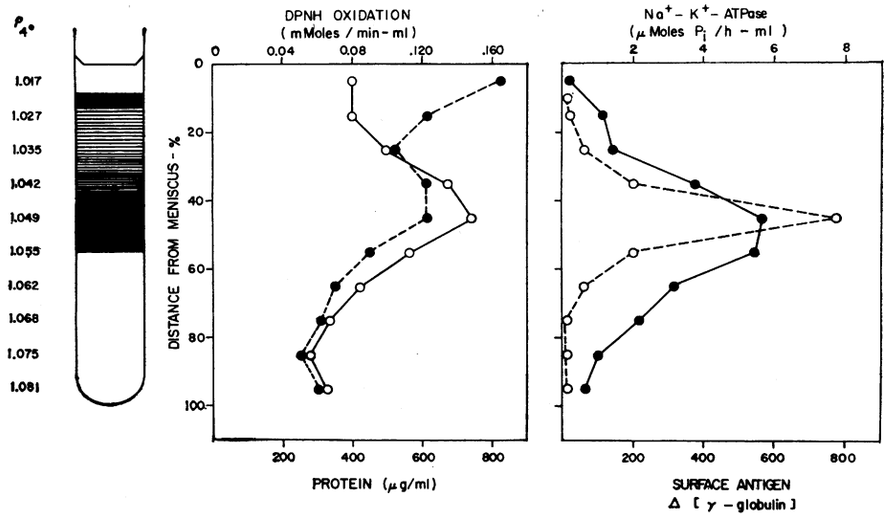

Fig. 2. Distribution of protein, DPNH-diaphorase, Na^+- and K^+-activated adenosine triphosphatase (*ATPase*), and surface antigen after equilibration of top-zone material in Ficoll density gradient (SW-39 rotor, 35,000 rev/min, 16 hours, 4°C; 2.3 mg protein per tube); o———o, protein; ●-----●, DPNH oxidation; ●———●, Na^+-, K^+-activated adenosine triphosphatase; o-----o, surface antigen; P_i, inorganic phosphorous; ρ, density.

these membranes to divalent cations is quite striking: whereas they do not coagulate grossly when concentrated isopycnically in gradients of NaBr (salt concentration $> 1M$), they form large clumps under conditions which are identical except for the presence of $0.001M$ $MgSO_4$ (*11*). The contrasting stability of the plasma-membrane fragments demonstrates an important difference between the electrical properties of membranes arising from the external surface of Ehrlich ascites carcinoma cells and those arising from the cell interior.

The plasma-membrane material in the top zone can be further purified by centrifugation in Ficoll density gradients. To do this, the material of the top zone was adjusted to appropriate density and mixed directly into Ficoll gradients containing $0.001M$ Tris, *p*H 8.6. The gradients were centrifuged in an SW-39 rotor at 35,000 rev/min for 16 hours at 4°C (Figs. 1*b* and 2). There are two principal components: a small one with a density of 1.024 at 4°C and a large one with a density of 1.050. The region between these two bands, not homogenous, consists of a series of closely spaced layers which extend into the peak at 1.050 density. The low-density component, which scatters light strongly, is rich in DPNH-diaphorase, but lacks surface antigen and Na^+- and K^+-activated adenosine triphosphatase.

The distribution of surface antigen and Na^+- and K^+-activated adenosine triphosphatase shows in three experiments that the plasma-membrane vesicles are located in the larger band with modal density at 4°C of 1.050 ± 0.001, a density considerably less than that found from distributions obtained with whole microsomal membranes (*4*). This suggests that the previous values were spuriously high because of nonspecific interactions between vesicles of different origins. The DPNH-diaphorase in the plasma-membrane zone may be due to (i) continued contamination with membranes arising only from the cell interior; (ii) presence of vesicles composed of "mixed" membranes, that is, arising from sites where the cell surface and endoplasmic reticulum were continuous at the moment of cell rupture; or (iii) engulfing of fragments of endoplasmic reticulum within large plasma-membrane vesicles at the time of cell rupture.

The striking layer formation throughout the plasma-membrane zone must be taken to indicate discontinuity in the volume or matrix density of plasma membrane-vesicles or both. Volume discontinuity appears more likely, since vesicle volume is a major determinant of density in Ficoll gradients (*4*) and since layers are not observed in gradients of sodium bromide.

VIRENDRA B. KAMAT
DONALD F. HOELZL WALLACH
Department of Biological Chemistry, Harvard Medical School, Boston, Massachusetts 02115

References and Note

1. D. F. H. Wallach and E. H. Eylar, *Biochim. Biophys. Acta* **52**, 594 (1961).
2. D. F. H. Wallach and E. B. Hager, *Nature* **96**, 1004 (1962).
3. D. F. H. Wallach and D. Ullrey, *Biochim. Biophys. Acta* **88**, 620 (1964).
4. D. F. H. Wallach and V. B. Kamat, *Proc. Nat. Acad. Sci. U.S.* **52**, 721 (1964).
5. D. M. Neville, *J. Biophys. Biochem. Cytol.* **8**, 414 (1960); P. Emmelot, C. J. Bos, E. L. Benedetti, P. H. Rumke, *Biochim. Biophys. Acta* **90**, 126 (1964).
6. T. L. Steck and D. F. H. Wallach, *Biochim. Biophys. Acta* **97**, 510 (1965).
7. D. F. H. Wallach and D. Ullrey, *ibid.* **64**, 526 (1962).
8. M. J. Hunter and S. L. Commerford, *ibid.* **47**, 580 (1961).
9. D. F. H. Wallach and F. L. Murphy, Biophys. Soc. abstracts (1965).
10. D. F. H. Wallach and M. Perez-Esandi, in preparation.
11. D. F. H. Wallach and V. B. Kamat, in preparation.
12. We thank M. Perez-Esandi and D. Ullrey for technical assistance. Work supported by the National Cancer Institute (grant CA 0738-2), the U.S. Army (grant DA-49-193-MD-2162), and (D.F.H.W.) by the Leukemia Society.

8 February 1965

Passive Transfer of the Action of Freund's Adjuvant by Serum of Rabbits Injected with the Adjuvant

Abstract. *Serum collected at intervals from rabbits treated with Freund's complete adjuvant was injected together with fluid diphtheria toxoid into recipient rabbits. Control rabbits received toxoid alone or toxoid mixed with serum of untreated donors. There was no antibody response in the controls or in recipients of serum obtained from donors 3 days after adjuvant treatment. Recipients of serum obtained from donors 1 to 9 weeks after adjuvant treatment responded with antibody formation. The magnitude of the response of recipients was correlated with the increase in γ-globulin concentration in the serums of the corresponding donor rabbits. Passive transfer of adjuvant action indicates that a serum factor, possibly natural antibody, is partially responsible for the immunity-enhancing activity of Freund's adjuvant.*

The theory of antibody formation proposed by Jerne (*1*) maintains that natural antibody, formed in the absence of antigenic stimulation, is required for antibody response. The natural antibody functions as the immunologic recognition system. This view is supported by the work of Segre and co-workers (*2*) on the enhancement by specific antibody of the antibody response in pigs deprived of colostrum. Terres and Wolins (*3*) also reported that specific antibody enhanced the antibody response in mice. One of the consequences of the theory would be that if the concentration of natural antibody is increased, within limits, the magnitude of the antibody response should increase, since more of the injected antigen would combine with antibody and form complexes capable of stimulating the antibody-producing cells. The secondary response may be explained in this manner (*1, 4*).

Since the antibody response to the administration of an antigen mixed with an adjuvant assumes some of the characteristics of a secondary response, the hypothesis may be formulated that adjuvants increase the concentration of natural antibody. Such action may be expected if the adjuvant caused either or both of the following: (i) leakage of natural antibody from lymphoid cells into the circulation, through destruction of the cells or alteration of their permeability; (ii) rapid proliferation of lymphoid cells which produce natural antibody.

Certain findings appear to agree with this hypothesis. Humphrey (*5*) reported that in rabbits injected with Freund's complete adjuvant alone there was an increase in the concentration of circulating γ-globulin, in that about 10 mg of γ-globulin per milliliter of serum could not be accounted for by antibodies directed against *Mycobacterium* and a number of other antigens. Paraf and Moraillon (*6*) injected rabbits repeatedly with Freund's complete adjuvant. When such rabbits were given human serum albumin within 10 days after the last injection of adjuvant, their antibody response was greater than that of rabbits not receiving prior treatment