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A Rapid Method for the Isolation of L-Cell Surface Membranes Using an Aqueous Two-Phase Polymer System

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Summary. A dextran-polyethylene glycol aqueous two-phase system has been used to separate cell surface membranes from other cellular organelles. The surface membranes have been identified on the basis of morphology, content of Na^+, K^+-ATPase, and presence of surface antigen as detected by a ^{51}Cr release method. Contamination of the surface membrane preparations by smooth endoplasmic reticulum, mitochondria, and nuclei has been found to be minimal. An average of 6.5% of the total protein was found in the membrane fraction. Less than two hours is required to isolate the membrane fraction after preparation of a Dounce homogenate. Fractionation by aqueous two-phase polymer systems appears to be a rapid and effective method for the isolation of surface membranes.

A number of methods for the isolation of mammalian cell surface membranes have been described (for reviews *see* Warren, Glick & Nass, 1967; Korn, 1969). Almost exclusively, these methods rely on differences in density or sedimentation characteristics to achieve separation of the surface membranes from other cell organelles. These methods do not always result in satisfactory yields or purity of the isolated material (Wallach, 1967). As the various cellular membranes are known to differ in chemical composition (Bosmann, Hagopian & Eylar, 1968), it is possible that their surface properties reflect these differences. Thus, a technique that separates cellular fractions on this basis would be of some value. The aqueous two-phase polymer systems developed by Albertsson (1960) have the characteristic of separating particles on the basis of differences in their surface properties. In this paper, we present a method for the rapid isolation of L-cell surface membranes in good purity and high yield using an aqueous two-phase dextran-polyethylene glycol system.

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Materials and Methods

Membrane Isolation

L-cells were grown in suspension culture in CMRL 1066 cell culture medium (Parker, 1961) supplemented with 10% fetal calf serum (Flow Laboratories, Rockville, Md.) and 100 μg/ml of penicillin and streptomycin. The homogenization conditions were a slight modification of the method of Warren *et al.* (1967). The cells were washed with 0.15 M NaCl, and the cell pellet (approximately 8×10^8 cells, obtained from 2 liters of suspension culture) was suspended in 40 ml of 10^{-3} M $ZnCl_2$ for 15 min at room temperature, cooled in an ice-bath for a further 5 min and then ruptured in a large Dounce homogenizer with a tight-fitting (type B) pestle (Kontes, Vineland, N.Y.). Approximately 50 to 200 strokes were required. The homogenization, as well as the subsequent steps in the procedure, were monitored by means of phase-contrast microscopy. The homogenization was stopped when greater than 90% of the cells were ruptured. The homogenate was placed in two Sorvall 50-ml polycarbonate tubes and subjected to a low-speed centrifugation for 15 min at 1,400 rpm in the 269 head of an International PR 2 centrifuge. The supernatant was designated fraction *S*, and the pellet was used for the next stage of separation in a two-phase system.

Stock solutions of the two-phase system were prepared as follows: 200 g of 20% (w/w) dextran 500 (Pharmacia) in distilled water, 103 g of 30% polyethylene glycol (PEG) (Carbowax 6000, Union Carbide), in distilled water, 99 ml of distilled water, 333 ml of 0.22 M phosphate buffer (Na salts) at pH 6.5, and 80 ml of 10^{-2} M $ZnCl_2$ were mixed in a separatory funnel and allowed to settle in the cold. The two phases were then collected. Some precipitate formed, which was collected along with the bottom phase and allowed to settle out. The pellet of cells from the low-speed centrifugation was suspended in 10 ml of top phase, and then 10 ml of the bottom phase were added. The two phases were mixed, placed in a Sorvall HB 4 (swinging-bucket) rotor and spun at 8,500 rpm for 10 min. On completion of this centrifugation, the membranes were found at the interface of the two-phase system. The pellet was suspended in water and labelled fraction *P* (Fig. 1). The supernatant (i.e., the membranes and the two-phase system) was poured into another tube, remixed and spun again at 8,500 rpm for 10 min to remove any trapped contaminants. The supernatant of the second two-phase centrifugation was similarly remixed and spun at 8,500 rpm for 10 min. The material at the interface was then collected and diluted with approximately four times its volume of distilled water. At this concentration, dextran 500 and polyethylene glycol 6000 no longer form a two-phase system. The membranes were then sedimented out of the suspension by a second low-speed centrifugation at 1,000 rpm for 15 min in the 269 head of an International PR 2 centrifuge. The pellet was suspended in water and labelled fraction *M*. The total time required for isolation of the *M* fraction after preparation of the homogenate was less than 2 hr.

Enzyme Assays

DPNH-diaphorase was assayed by the method of Wallach and Kamat (1966). Cytochrome *c* oxidase was measured by the method of Cooperstein and Lazarow (1951). Na^+, K^+-activated-ATPase was assayed in a reaction mixture containing 0.1 M Tris-HCl at pH 7.5, 2 mM ATP (Na salt), 2 mM $MgSO_4$, 60 mM NaCl, 5 mM KCl, and 0.1 mM ethylenediaminetetraacetate (EDTA). Control tubes contained the same reaction mixture, except that the KCl was omitted. Samples of the reaction mixture were removed at two different times, and the reaction was terminated by the addition of 30% trichloroacetic acid. The precipitated material was removed by centrifugation and the inorganic

phosphate present in the supernatant was measured by the method of Lowry and Lopez (1946). The enzyme activity was calculated from the increase in the rate of inorganic phosphate released from ATP which occurs when K^+ is added to a system already containing Na^+. This K^+-dependent response could be completely eliminated by the addition of 1 mM ouabain (Sigma).

Protein determinations were done by the method of Lowry, Rosebrough, Farr and Randall (1951), using bovine serum albumin as a standard.

Labelling of DNA

L-cells were labelled for 16 hr in medium containing 0.25 μg/ml of ^{3}H-thymidine (Amersham/Searle), specific activity 21.6 C per mmole. After cell membrane isolation, dilutions of the fractions were precipitated with an equal volume of 20% trichloroacetic acid and filtered through a Millipore (0.45 μ) filter. The filters were then dried, scintillation fluid was added (Omnifluor, New England Nuclear), and they were counted on an Ansitron liquid scintillation counter.

^{51}Cr Release Test

A modification of the ^{51}Cr release technique developed by Sanderson (1964) and Wigzell (1965) as a quantitative index of cell lysis was used to detect the presence of cell surface antigen. Antiserum to intact L-cells was prepared by injection of 10^8 washed L-cells in phosphate-buffered saline (PBS; Dulbecco & Vogt, 1954) into the ear vein of a rabbit on days 0, 7, 17 and 28. The animal was bled and sacrificed 5 days later. The collected blood was allowed to clot and to stand in the refrigerator overnight. The clot was then removed, and the antiserum was clarified by centrifugation.

The L-cells to be labelled with ^{51}Cr were first washed with PBS and resuspended in CMRL 1066. The cells at a concentration of 5×10^6 cells per ml were then labelled by incubation with 300 μC/ml of ^{51}Cr (supplied as sodium chromate in sterile isotonic saline, Frosst) at 37 °C with gentle shaking for 45 min. The cells were then washed twice with PBS and resuspended in CMRL 1066. To test ^{51}Cr release in the presence of antibody, 0.5 ml of labelled cells (2.5×10^6 cells) were incubated with 0.5 ml of antiserum (50-fold diluted in PBS) and 0.5 ml guinea pig serum for 1 hr at 37 °C. At the end of the incubation, 3 ml of ice-cold PBS was added to each tube (tube No. 2058, Falcon Plastics) to stop lysis. The tubes were then centrifuged in the 269 head of an International PR 2 centrifuge at 1,000 rpm for 15 min. The supernatant was carefully decanted and counted in a Nuclear Chicago well-type scintillation counter. Non-specific lysis was evaluated in control tubes containing PBS instead of antiserum.

The presence of surface antigen in membrane preparations was detected by testing for the ability of the preparation to block release of ^{51}Cr from labelled L-cells by the antiserum against intact cells. The material to be tested was incubated for 45 min with the antiserum at 37 °C with occasional agitation. Antiserum treated in this way was then compared with unblocked antiserum for its ability to cause cell lysis in the ^{51}Cr release test.

Results

Membrane Isolation Scheme

The membrane isolation procedure is summarized in Fig. 1. Three fractions were collected: the supernatant obtained after the first low-speed centrifugation, which contained smaller cellular particles and soluble protein

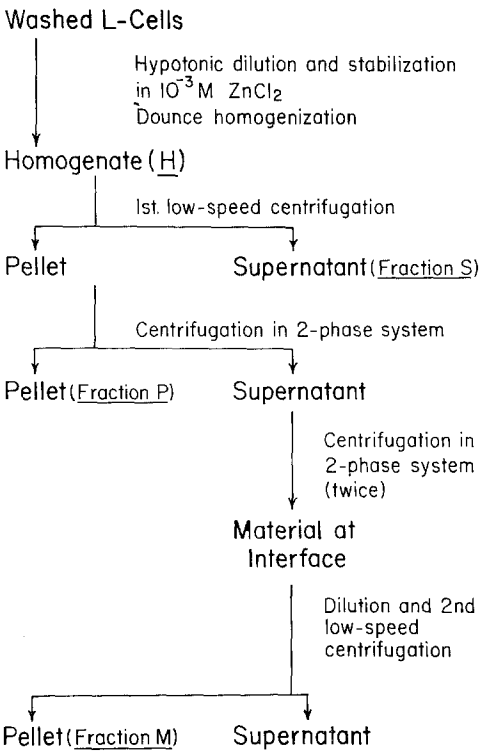

Fig. 1. Summary of the procedure used for isolation of L-cell surface membranes using an aqueous two-phase polymer system

(fraction *S*); the pellet obtained after centrifugation in the two-phase system, which contained nuclei, nuclei with cytoplasmic tags, whole cells and occasional membranes (fraction *P*); and the pellet obtained after the second low-speed centrifugation (fraction *M*). The *M* fraction, when viewed under a phase-contrast microscope, or by electron microscopy (Fig. 2), consisted of large fragments several microns in size. Some whole-cell ghosts and smaller membrane pieces were also present.

Na^+, K^+-ATPase Activities

Na^+, K^+-activated ATPase activity was chosen as a marker for plasma membrane. Table 1 shows the results of six experiments in which specific activities of this enzyme were measured in the homogenate and the *S*, *P* and *M* fractions. It is apparent that the *M* fraction contained the great majority of Na^+, K^+-ATPase activity. This enzyme was either indetectable or barely detectable in the *S* and *P* fractions.

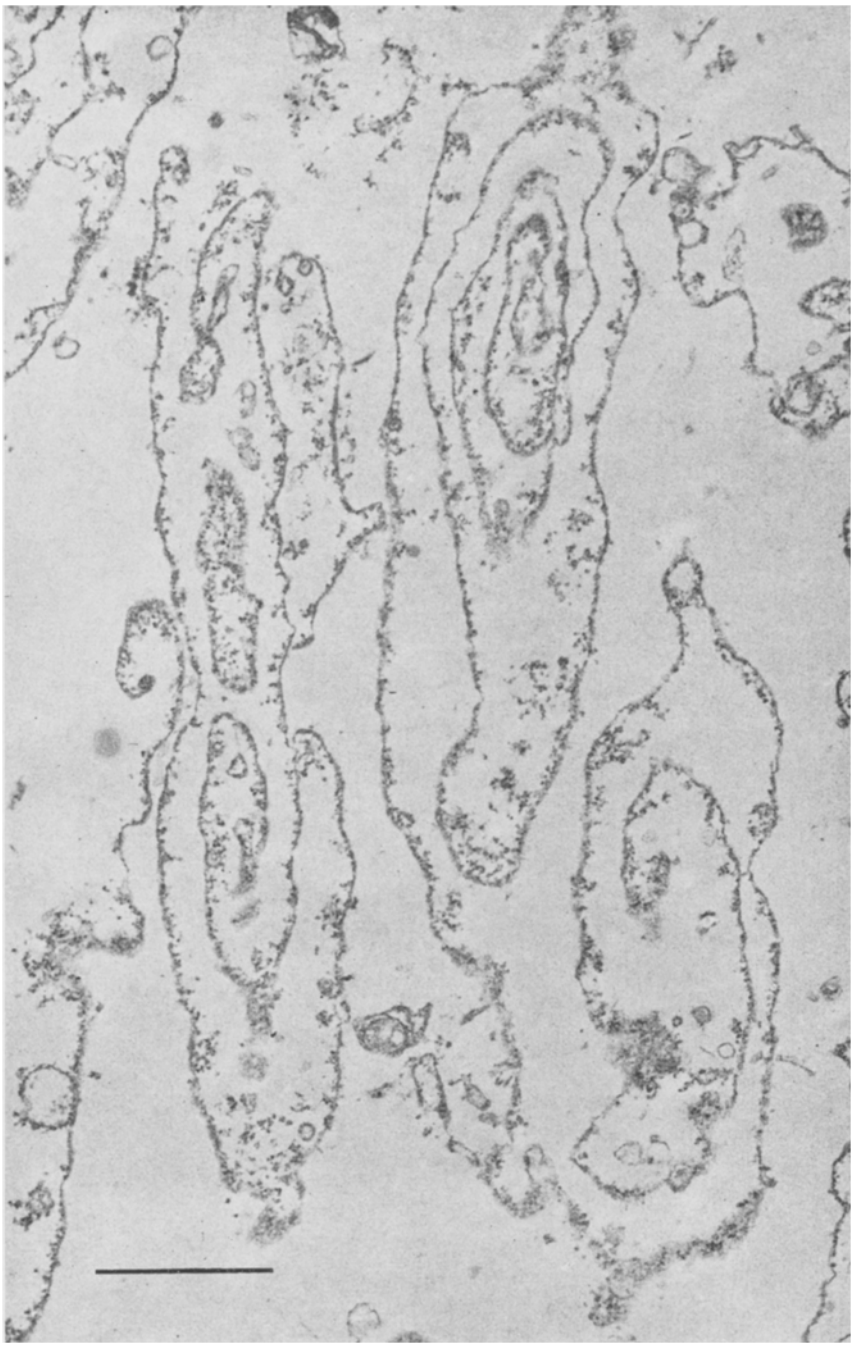

Fig. 2. Electron micrograph of the *M* fraction (courtesy of Dr. A. F. Howatson). The horizontal bar represents 1 μ

Table 1. *Specific activities of Na^+, K^+-activated ATPase in different fractions*

Exp. no.	Homogenate	Fraction		
		S	*P*	*M*
1	0.42[a]	0.23	0.61	2.9
2	0.48	0.42	0.35	5.6
3	0.38	0.62	0.22	2.0
4	0.49	nda[b]	0.28	3.8
5	0.15	0.13	nda	6.5
6	nda	0.33	nda	2.8

[a] Specific activity expressed as μmoles phosphorus released/mg protein/hr.
[b] No detectable activity.

The average specific activity of Na^+, K^+-ATPase in the *M* fraction was 3.9 μmoles phosphorus released per mg protein/hr. This activity is somewhat higher than the values of 1.4 and 0.7 μmoles *P* per mg protein/hr reported by Warren *et al.* (1967) for L-cell membranes also prepared using 10^{-3} M $ZnCl_2$ as a stabilizing agent. Differences in the method of assay could account for some of this discrepancy.

DPNH-Diaphorase Activities

DPNH-diaphorase was used as a marker for smooth endoplasmic reticulum. The results of three experiments are presented in Table 2. The majority of DPNH-diaphorase was found in the *P* fraction (the values given in Table 2 for the *S*, *P* and *M* fractions do not add to 100% because of material lost or discarded during the preparation procedure). The average activity in the *M* fraction was 3.6% of the activity present in the homogenate. This result is similar to that obtained by Wallach and Kamat (1966) using

Table 2. *Activities of DPNH-diaphorase in different fractions*

Exp. no.	Relative activity (% of activity in homogenate)		
	S fraction	*P* fraction	*M* fraction
1	22	33	3.5
2	10	37	2.9
3	20	64	4.3

Ehrlich ascites tumor cells and a Ficoll barrier technique of membrane isolation. These authors found 3.7% of the DPNH-diaphorase activity in the surface membrane-rich fraction.

Cytochrome Oxidase

Cytochrome *c* oxidase was used as a marker for mitochondria. The results of three experiments are presented in Table 3. The majority of cytochrome *c* oxidase activity was found in the *S* fraction while variable amounts were found in the *P* fraction. As no detectable amounts of this enzyme were found in the *M* fraction, the possibility of gross contamination of this fraction by mitochondria can be eliminated.

Table 3. *Cytochrome c oxidase activities in different fractions*

Exp. no.	Relative activity (% of activity in homogenate)		
	S fraction	*P* fraction	*M* fraction
1	105	10	0
2	75	21	0
3	82	6	0

DNA Content

To test for the presence of DNA in the various fractions, L-cells were labelled with ^{3}H-thymidine, the *S*, *P* and *M* fractions were isolated, and their content of ^{3}H in acid-precipitable material was determined. The results of two experiments are given in Table 4. Again, the values given for the fractions do not add to 100%; this is because additional nuclei were removed and discarded in the pellets from the second and third centrifugation in the two-phase system. In both experiments, approximately 85% of

Table 4. *Distribution of ^{3}H radioactivity in fractions obtained from cells labelled with ^{3}H-thymidine*

Exp. no.	Relative activity (% of activity in homogenate)		
	S fraction	*P* fraction	*M* fraction
1	3.7	85	0.06
2	0.9	86	0.08

the ^{3}H label was found in the *P* fraction, and only very low levels of radioactivity (0.07%) were detected in the *M* fraction. Examination of the *M* fraction by phase-contrast microscopy confirmed these results, as nuclei and whole cells were rarely found in this fraction.

Presence of Surface Antigens

The presence of cell-surface antigens was detectable by the ability of sub-cellular fractions to inhibit complement-dependent cell lysis induced by antiserum prepared against intact L-cells. The degree of inhibition was calculated as the ratio of ^{51}Cr activity released using antiserum incubated together with cellular material, relative to the ^{51}Cr activity released using unblocked antiserum. Non-specific lysis, represented by the ^{51}Cr activity released when antisera were replaced by saline, was subtracted in each case. The results of three experiments in which *M* fractions from three different preparations were used to block the antiserum are shown in Fig. 3. It was found that the percent inhibition of ^{51}Cr release increased linearly with the amount of *M* fraction protein used to block the antiserum, up to a maximum of 100% inhibition for amounts of protein in excess of 100 μg. Shown in the same figure is the inhibition obtained when the homogenate (*H*) was used as the blocking material. The percentage inhibition again increased linearly with the amount of protein added but much larger quantities of protein were required to reach a given percentage inhibition by the homogenate in comparison with the *M* fraction. Data obtained for larger quantities of homogenate protein than those shown in Fig. 3 demonstrated that 50% inhibition of cell lysis was attained by using 640 μg of *H* protein. As only 50 μg of *M* protein was required to reach 50% inhibition (Fig. 3), the

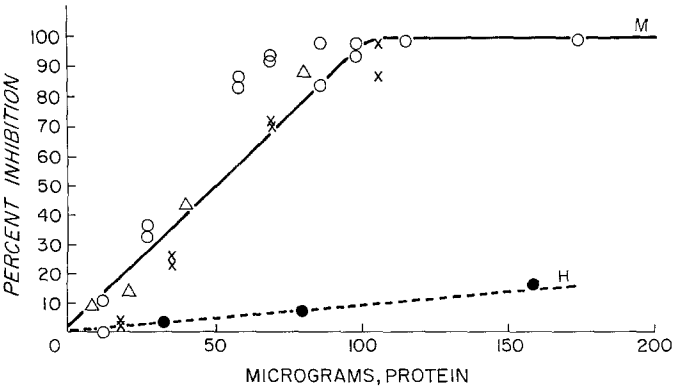

Fig. 3. Percentage inhibition of ^{51}Cr release as a function of quantity of material used to block antiserum prepared against intact L-cells. Solid line: *M* fraction. Dashed line: Homogenate. Different symbols represent results of separate experiments

serum blocking activity was enriched approximately 13-fold in the *M* fraction.

As a test of the specificity of the inhibition assay, the ability of the *P* fraction to inhibit release of ^{51}Cr was also measured. A maximum inhibition of ^{51}Cr release of 18% was obtained using 155 μg of protein from the *P* fraction. Most of this low level of inhibition probably resulted from unruptured cells present in the *P* fraction. As a second test of the inhibition assay, the ability of fetal calf serum (which was present in the medium used to grow the L-cells) to inhibit ^{51}Cr release was also measured. No detectable inhibition of cell lysis occurred in the presence of up to 125 μg protein of fetal calf serum.

These results demonstrate that the *M* fraction contains surface antigen. They also provide evidence that the ^{51}Cr release technique can be adapted for use as a quantitative assay of the amount of surface material present in various subcellular fractions.

Yield

In a total of eight experiments, an average of 6.5% (SE 1.3%) of the total protein was found in the *M* fraction. This is in the same range as that reported by Warren and Glick for L-cell membranes. They found from 3–5% (Warren & Glick, 1968) to 10–12% (Glick & Warren, 1969) of the total L-cell protein in the membrane fractions.

Discussion

The isolation of plasma membranes using aqueous two-phase polymer systems has several advantages. The method is rapid, requiring less than 2 hr for the isolation of the *M* fraction from the homogenate. The yield of total protein in the membrane fraction and the purity of the preparations compare favorably with results obtained using other methods. The aqueous two-phase polymer systems utilize exceptionally mild conditions; as Albertsson (1970) has noted, these polymers often exert a protective effect on proteins and cell particles. Previous work (Brunette, McCulloch & Till, 1968) has indicated that these systems may even be used for the fractionation of populations of intact cells and that hemopoietic stem cells retain their viability during prolonged exposures to dextran-polyethylene glycol systems. The technique is flexible, in that the behavior of particles in two-phase systems may be made to vary as a result of alterations in the pH, ionic environment, and polymer composition of the two-phase system (Albertsson, 1970). R. Sheinin and K. Onodera (*personal communication*, 1970) have shown that the technique described here may be extended to other mamma-

lian cell lines; they have prepared membranes from mouse 3*T*3 cells using the two-phase system described above, but with lower-speed centrifugation. We conclude that fractionation by an aqueous two-phase dextran-polyethylene glycol system is a rapid, mild, flexible and effective method for the isolation of cell-surface membranes.

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