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Aqueous two-phase partition applied to the isolation of plasma membranes and Golgi apparatus from cultured mammalian cells

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Abstract

Partitioning in dextran–poly(ethylene)glycol (PEG) aqueous–aqueous phase systems represents a mature technology with many applications to separations of cells and to the preparation of membranes from mammalian cells. Most applications to membrane isolation and purification have focused on plasma membranes, plasma membrane domains and separation of right side-out and inside-out plasma membrane vesicles. The method exploits a combination of membrane properties, including charge and hydrophobicity. Purification is based upon differential distributions of the constituents in a sample between the two principal compartments of the two phases (upper and lower) and at the interface. The order of affinity of animal cell membranes for the upper phase is: endoplasmic reticulum<mitochondria<Golgi apparatus<lysosomes and endosomes< plasma membranes. Salt concentrations and temperature affect partitioning behavior and must be precisely standardized. In some cases, it is more fortuitous to combine aqueous two-phase partition with other procedures to obtain a more highly purified preparation. A procedure is described for preparation of Golgi apparatus from transformed mammalian cells that combines aqueous two-phase partition and centrifugation. Also described is a periodic NADH oxidase, a new enzyme marker for right side-out plasma membrane vesicles not requiring detergent disruptions for measurement of activity.

Keywords: Aqueous two-phase systems; Plasma membranes; Golgi apparatus

1. Introduction

Aqueous two-phase partitioning is a simple, rapid, relatively inexpensive and highly reproducible method that can be carried out without the use of sophisticated equipment. In contrast to chromatography, no solid supports or adsorptive surfaces are required which makes aqueous two-phase systems ideal for separation of cells and preparation of plasma membranes from cultured cells [1]. Partitioning in two-polymer phases consisting of dextran and poly(ethylene)glycol (PEG) represents a mature technology that is not only sensitive on the basis of surface properties [2,3], but also mild and protective of the partitioned particulate [4]. For example, in the presence of polymers, erythrocytes in hypotonic solution do not lyse immediately as they do in the absence of polymers and organelles and membranes isolated by aqueous two-phase partition retain their biological functions [4].

When separating organelles and membranes it is advantageous to use a rapid procedure in order to better preserve biological activities. Optimally, one wants to partition the material of interest either to the

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one phase or one of the phases plus the interface and the rest of the material to the opposite phase.

Preparations containing, for example, mitochondria and plasma membranes (or particles limited by the plasma membranes), are easily separated by phase partition since plasma membranes have a high affinity for the upper phase while mitochondria prefer the lower phase. Lôpez-Pérez et al. [5] found that mitochondria and synaptosomes from adult rat forebrain could easily be separated by counter-current distribution in an aqueous two-phase system composed of Dextran T500 and poly(ethylene glycol) 4000. But they also found that both particles could be separated by a batch procedure in which the same phase system was used. Electron microscopy and enzyme assays showed that a high purity of the mitochondria were obtained from the dextran-rich lower phase and that intact synaptosomes were recovered from the PEG-rich upper phase.

Another feature of the aqueous two-phase partition method is that small amounts of material can be used for purification of plasma membranes. For instance, the purification of fat cell plasma membranes by differential and density-gradient centrifugation is based on using 16–24 rats for each preparation. Partitioning in aqueous two-phase systems allows purification of plasma membranes from fat cells isolated from as few as one or two rats [6]. Sometimes it is necessary to combine aqueous two-phase partition with another procedure to obtain the desired fractions. Our laboratory used preparative free-flow electrophoresis [7,8] and aqueous two-phase partition to obtain a plasma membrane-enriched fraction from adipocytes of epididymal fat pads of rat together with a fraction enriched in small vesicles with plasma membrane characteristics. Consistent with an inside-out orientation, the electrophoretic mobility of the small vesicles was much less than that of right side-out plasma membrane.

Decisive fractionation of tissue culture cells, especially the preparation of plasma membranes free of internal membranes, is often difficult to achieve by conventional gradient methods. A major contributory factor is the extensive cytoskeletal system that tends to result in poor dispersion of membranes from the cytosolic network. We have devised a method whereby the cells are incubated in $NaHCO_3$ containing EDTA to swell the cells prior to homogenization followed by partitioning using an aqueous two-phase system adapted for tissue culture cells [9]. Progress in isolation of Golgi apparatus has been reported for immortalized (cultured) cells [10] but not from transformed cells. Due to the potential central role of the Golgi apparatus in cell transformation [11], basic studies on the origin and specificity of cell surface would be served by the development of procedures for Golgi apparatus isolation from transformed cells. Aqueous two-phase partition in combination with differential centrifugation provides a method to prepare a fraction substantially enriched in Golgi apparatus from cultured cells [12]. Also described is a new enzymatic marker for assay of right side-out plasma membrane vesicles, a periodic hydroquinone oxidase [13] capable of oxidizing NADH as a non-physiological substrate.

2. Materials and methods

Unless otherwise specified, the chemicals were from Sigma (St. Louis, MO, USA).

2.1. Growth of cells

HeLa S (human cervical carcinoma) cells were grown on Dulbecco's Modified Eagles Medium (D-MEM) (Joklik modified) (Gibco-BRL, Life Technologies, Gaithersburg, MD, USA) with glutamine (244 mg/l) and phosphate (1.3 g/l Na_2HPO_4) and without $CaCl_2$ plus 5% donor horse serum (Gibco-BRL). Gentamicin sulfate (50 μg/l) and sodium bicarbonate (Sigma) (2 g/l) were added. CFK (Crandall Feline Kidney) and BAL-17 (Leukemia Mouse B) cells were grown in RPMI 1640 (Gibco-BRL) medium with glutamine (2.92 g/100 ml PBS), gentamicin sulfate, 10 m*M* pyruvate and 5% fetal calf serum (Gibco-BRL).

2.2. Isolation of plasma membranes

Cells were collected by centrifugation for 6 min at 1500 *g* and 4°C, resuspended in 0.2 m*M* EDTA in 1 m*M* $NaHCO_3$ in an approximate ratio of 1 ml per 10^8 cells and incubated on ice for 10–30 min to swell the cells. Homogenization was with a Polytron homogenizer (Brinkman Instruments, Westbury, NY,

USA) for 40 s at 10 000 rpm using a 10 ST-probe and 7-ml aliquots. To estimate cell breakage, before homogenization an aliquot of cells was placed on a microscope slide, stained with Giemsa (Sigma) and the cells were viewed by light microscopy. After homogenization, an aliquot of the homogenate placed on a microscope slide was stained with Giemsa and viewed for the presence of intact nuclei and unbroken cells. At least 90% cell breakage without breakage of nuclei was achieved routinely.

The homogenates were centrifuged (Beckman Instruments, St. Louis, MO, USA) for 10 min at 175 *g* at 4°C to remove unbroken cells and nuclei and the supernatant was centrifuged a second time at 25 000 *g* for 30 min at 4°C to prepare a plasma membrane-enriched microsome fraction. The supernatant was discarded and the pellets were resuspended in 0.2 *M* potassium phosphate buffer (pH 7.2) in a ratio of approximately 1 ml per pellet from 5×10^8 cells. The resuspended membranes then were loaded onto the two-phase system with a polymer mixture containing 6.6% Dextran T500 (Pharmacia Biotech, Alameda, CA, USA), 6.6% (w/w) poly(ethylene glycol) 3350 (Fisher Scientific, Pittsburg, PA, USA) and 0.2 *M* potassium phosphate, pH 7.2. The weight of the system was brought to 14 g with distilled water. Resuspended microsomes (2 g) were added to the two-phase system to a final weight of 16 g. The tubes were inverted vigorously for 40 times in the cold (4°C). The phases were separated by centrifugation at 1150 *g* in a Beckman JS-13.1 (Beckman Instruments) rotor for 5 min at 4°C. The upper phase containing primarily plasma membranes was diluted with 1 m*M* bicarbonate and collected by centrifugation. The purity of the plasma membranes was determined to be >85% by electron microscopy. The yield was about 20 mg plasma membrane protein from 10^{10} HeLa cells.

2.3. Right side-out versus inside-out membrane vesicles

HeLa plasma membranes prepared by aqueous two-phase partition, frozen and thawed four times to invert some of the vesicles [14], were sheared by 55 excursions through a 28.5-gauge needle fitted to a syringe and subfractionated by repeating the phase partition step. The lower phase was enriched in inside-out vesicles while the upper phase contained the remaining right side-out vesicles. The vesicles were collected by centrifugation at 95 000 *g* for 45 min at 4°C and resuspended in 50 m*M* Tris–MES, pH 7.2. The membranes were collected by centrifugation for 30 min at 95 000 *g* (Beckman SW-28 rotor) (Beckman Instruments), and 4°C.

2.4. ATPase latency assays to determine sidedness

Total ATPase activity was determined in a 500-μl reaction volume containing 25 m*M* Tris–Mes, pH 6.5, 1 m*M* NaN_3, 1 m*M* sodium molybdate, 0.1 m*M* EDTA, 3.0 m*M* $MgSO_4$, 3.0 m*M* disodium ATP, 50 m*M* KCl and 5–10 μg plasma membrane protein with or without the addition of 0.2% Triton X-100. After incubation at room temperature for 30 min the reaction was stopped by the addition of 50 μl 25% TCA and 300 μl SDS. The released phosphate was determined by addition of 2.5 ml freshly prepared reagent containing 0.8% ammonium molybdate, 0.27% ascorbic acid and 0.045% $SnCl_2$ in 1 N H_2SO_4. The absorbance was measured at 723 nm after 30 min.

2.5. Concanavalin A (Con A) binding to determine vesicle orientation

After twice washing the membrane vesicles with 1.5 ml of 5 m*M* Hepes–KOH, pH 7.2, containing 0.25 *M* sorbitol by centrifugation in a microfuge (Eppendorf) for 5 min, the pellets were resuspended in the same buffer to give a 100-μg protein/100-μl solution. An equal volume of a solution of 10 μg Con A conjugated with 20 nm immunogold (Sigma)/100 μl was added to the membrane suspension. After incubation for 40 min at room temperature, 1.5 ml of 2.5% glutaraldehyde in 0.1 *M* sodium phosphate, pH 7.2, was added followed by centrifugation in the microfuge for 5 min. The supernatant was discarded, fresh phosphate-buffered glutaraldehyde (Ladd, Burlington, VT, USA) solution was added and the material was processed for electron microscopy.

2.6. Measurement of NADH oxidase activity

NADH oxidase activity was determined from the disappearance of NADH measured at 340 nm with

430 nm as reference using an SLM Aminco DW-2000 spectrophotometer (SLM Instruments, Urbana, IL, USA) in the dual wavelength mode of operation. The reaction mixture contained in a final volume of 2.5 ml, 50 m*M* Tris–Mes buffer, pH 7.2, 1 m*M* KCN, 100–200 μg membrane protein, and 150 μ*M* NADH. The assay was at 37°C with constant stirring and with continuous recording over 1-min intervals over 1.5 min once a steady-state rate was reached. A millimolar extinction coefficient of 6.22 was used to determine specific activity of the NADH oxidase activity. Autocorrelation analyses were carried out [15,16]. Decomposition fits were calculated along with MAPE, MAD and MSD values.

2.7. Isolation of Golgi apparatus

The lower phase was diluted 10-fold with 1 m*M* sodium bicarbonate and centrifuged for 30 min at 25 000 *g*. The supernatant, containing the Golgi apparatus was diluted 2-fold with 1 m*M* sodium bicarbonate and centrifuged for 45 min at 31 700 *g*. The supernatant was discarded and the pellet containing the Golgi apparatus was resuspended in 50 m*M* Tris–Mes buffer, pH 7.2 [12].

2.8. Electron microscopy

A portion of the pellet containing the Golgi apparatus was fixed in 2% (v/v) glutaraldehyde (Ladd) in 0.2 *M* phosphate buffer, pH 7.2. The glutaraldehyde was removed by rinsing three times with 0.2 *M* phosphate buffer, pH 7.2, and the pellet was post-fixed overnight in cold 1% osmium tetroxide (Ladd) in the same buffer and then dehydrated in an acetone series. Samples were embedded in Epon (Ladd) [17], sectioned, stained with lead (Ladd) and examined and photographed with a Philips EM/200 electron microscope (Philips Electronic Instruments, Mahwah, NJ, USA).

2.9. Enzyme assays

2.9.1. 5′-Nucleosidase

The marker enzyme for plasma membrane was assayed in the presence of 55 m*M* Tris, pH 8.5, 5.5 m*M* magnesium chloride, 11 m*M* adenosine-5′-monophosphate and 200 μg protein in a final volume of 1 ml [18]. The mixture was incubated for 15 min at 37°C and the reaction was stopped with the addition of 1 ml cold 10% trichloroacetic acid. The amount of inorganic phosphate liberated was determined by the colorimetric procedure of Fiske and Subbrow [19].

2.9.2. Succinate-INT reductase

Mitochondrial marker enzyme was assayed in the presence of 50 m*M* monobasic potassium phosphate, pH 7.4, 0.1% 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyl tetrazolium chloride (INT), 50 m*M* sodium succinate and 200 μg protein in a final volume of 1 ml [20]. The mixture was incubated for 15 min at 37°C. One ml cold 10% trichloroacetic acid was added to stop the reaction. After 30 min, 4 ml ethyl acetate were added to each sample. After vortexing and centrifuging each sample at room temperature for approximately 3 min, the ethyl acetate layer was transferred to a glass cuvette. The absorbance was read at 490 nm in a double beam spectrophotometer (UV-160, Shimadzu Scientific Instruments, Columbia, MD, USA).

2.9.3. NADPH cytochrome c reductase

The disappearance of NADPH used to determine the activity at 340 nm of the marker enzyme for endoplasmic reticulum was assayed in the presence of 55 m*M* KPO_4 buffer, pH 7.5, containing 0.5 m*M* KCN, 0.33% cytochrome c, 22 m*M* NADPH and 200 μg protein [21].

2.9.4. Galactosyltransferase

N-Acetylglucosamine galactosyltransferase (EC 2.4.1.13), a lumenal transmembrane enzyme, was assayed according to a modified procedure of Bretz and Staubli [22] with ovomucoid as acceptor after solubilizing the membrane with Triton X-100 detergent.

Proteins were determined by the bicinchoninic acid (BCA) assay [23] using bovine serum albumin as standard.

3. Results and discussion

Cultured (HeLa, CFK and BAL-17) cells incubated in 0.2 m*M* EDTA in 1 m*M* $NaHCO_3$ used to

Table 1
Marker enzyme activities of plasma membranes purified from HeLa and CFK cells by aqueous two-phase partition

Marker	HeLa cells		CFK cells	
	Total homogenate	Plasma membrane	Total homogenate	Plasma membrane
5′-Nucleotidase (plasma membrane) (μmol/h per mg protein)	13	56	0.7	5.5
Succinate-INT reductase (mitochondria) (μmol/h per mg protein)	1.4±2.1	0.7±0.5	0.9±0.7	0.4±0.2
NADPH-cytochrome c reductase (ER) (nmol/min per mg protein)	10.9±10.5	6.0±3.7	3.1±0.6	7.4±0.4
Galactosyltransferase (Golgi apparatus) (nmol/h per mg protein)	3.6±1.9	0.3±0.1	13.4±3.4	9

swell the cells prior to homogenization followed by partitioning in an aqueous two-phase system adapted for tissue culture cells resulted in plasma membrane fractions of acceptable purity (>85%) and yield (23%) as determined by marker enzymes (Tables 1 and 2) and electron microscopy (Figs. 1–3, Table 3). The major contaminants included mitochondria (3%), endoplasmic reticulum (7%) and other membranes (<4%).

A relatively uniform population of closed vesicles following aqueous two-phase partition was revealed using electron microscopy of the isolated vesicles (Figs. 1 and 2). More than 85% of the vesicles had morphological characteristics of plasma membranes (Table 3). Large smooth (lacking ribosomes or electron transport particles) vesicles in thickness of 9–10 nm were observed. When contrasted with lead, the plasma membranes exhibited the dark–light–dark pattern characteristic of plasma membrane. There was a minimal amount of plasma membrane contaminants (endoplasmic reticulum, mitochondria, Golgi apparatus or nuclear envelope). That the vesicles were predominantly right side-out was confirmed by binding of Concanavalin A–gold (Fig. 3).

To prepare inside-out vesicles, HeLa plasma membranes obtained by aqueous two-phase partition were subjected to a series of four freeze–thaw treatments followed by hypotonic lysis facilitated by drawing the suspended vesicles approximately 55 times through a fine hypodermic needle. The procedure resulted in some of the vesicles being everted into a cytoplasmic side-out configuration. The mixture of two populations was subjected to a second aqueous two-phase partition. The majority of the right side-out vesicles partitioned to the upper phase while the inside-out vesicles predominantly partitioned to the lower phase.

Latency of ATPase activity was used to determine enzymatic activity localized to the inside surface of the plasma membrane (Table 4). Unless detergent was added to disrupt the vesicles, both the starting vesicles and the more electrophoretically right side-out vesicles exhibited low ATPase activity. The inside-out vesicles, in contrast, exhibited nearly the same high ATPase activity both before and after detergent disruption of the membranes. Concanavalin A binding visualized by electron microscopy using concanavalin A conjugated with 20 nm immunogold

Table 2
Specific activities of the plasma membrane marker enzyme, 5′-nucleotidase, for the plasma membrane-enriched upper phase and Golgi apparatus fractions from BAL-17 cells isolated by aqueous two-phase partition

Fraction	Specific activity (μmol/h per mg protein)[a]
Plasma membrane-enriched upper phase	240
Endoplasmic reticulum	100
Golgi apparatus enriched	<80

[a] μmol P_i released from AMP per h per mg protein.

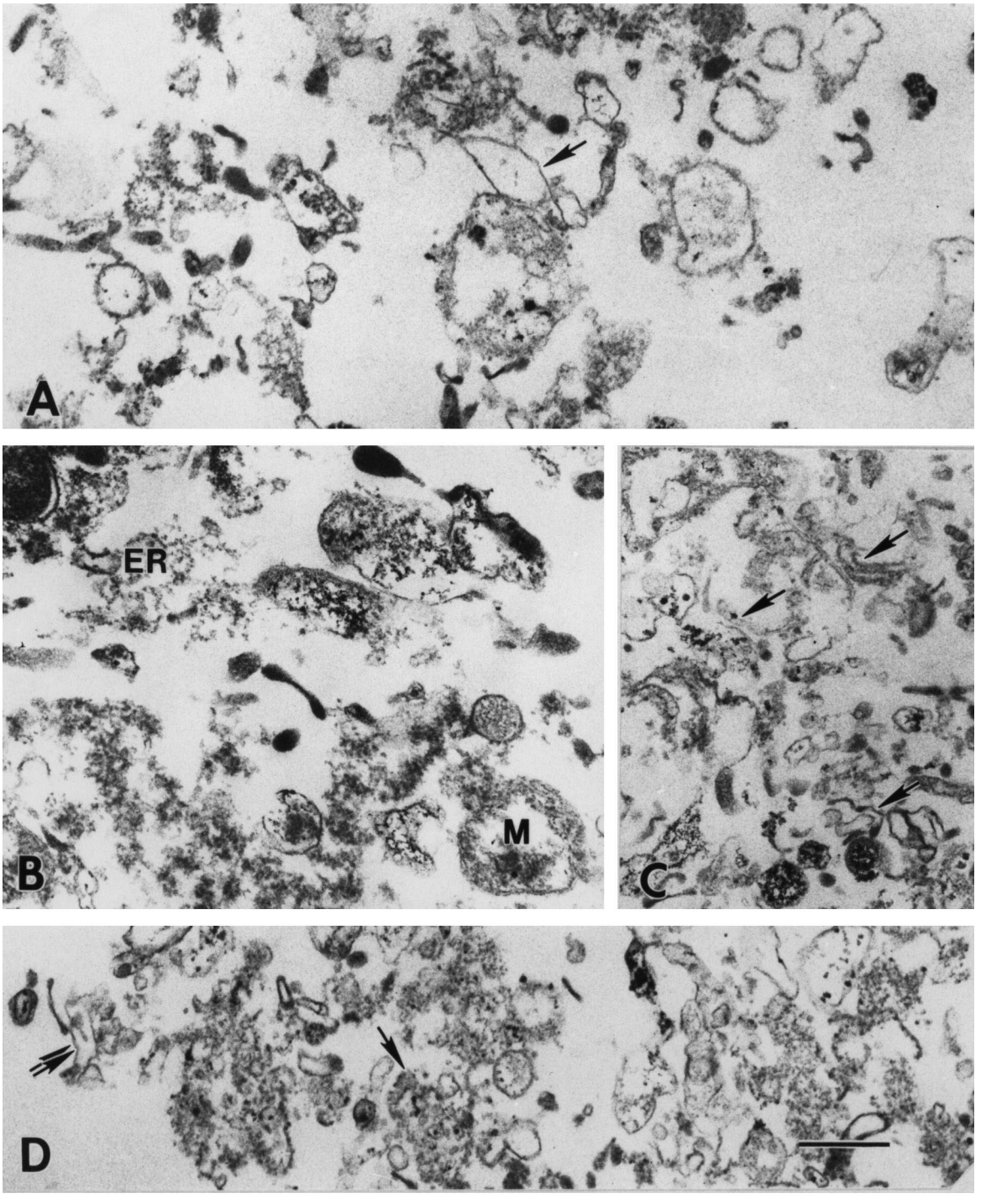

Fig. 1. Electron micrographs of HeLa cell fractions isolated by aqueous two-phase partition and centrifugation as described previously for K-562 cells [12]. (A) Plasma membrane-enriched upper phase (arrows). (B) Lower phase depleted of plasma membranes. (C) Golgi apparatus (arrows) isolated by centrifugation followed by aqueous two-phase partition. (D) Endoplasmic reticulum (arrows) fraction from plasma membrane-depleted lower phase. Bar=0.5 μm.

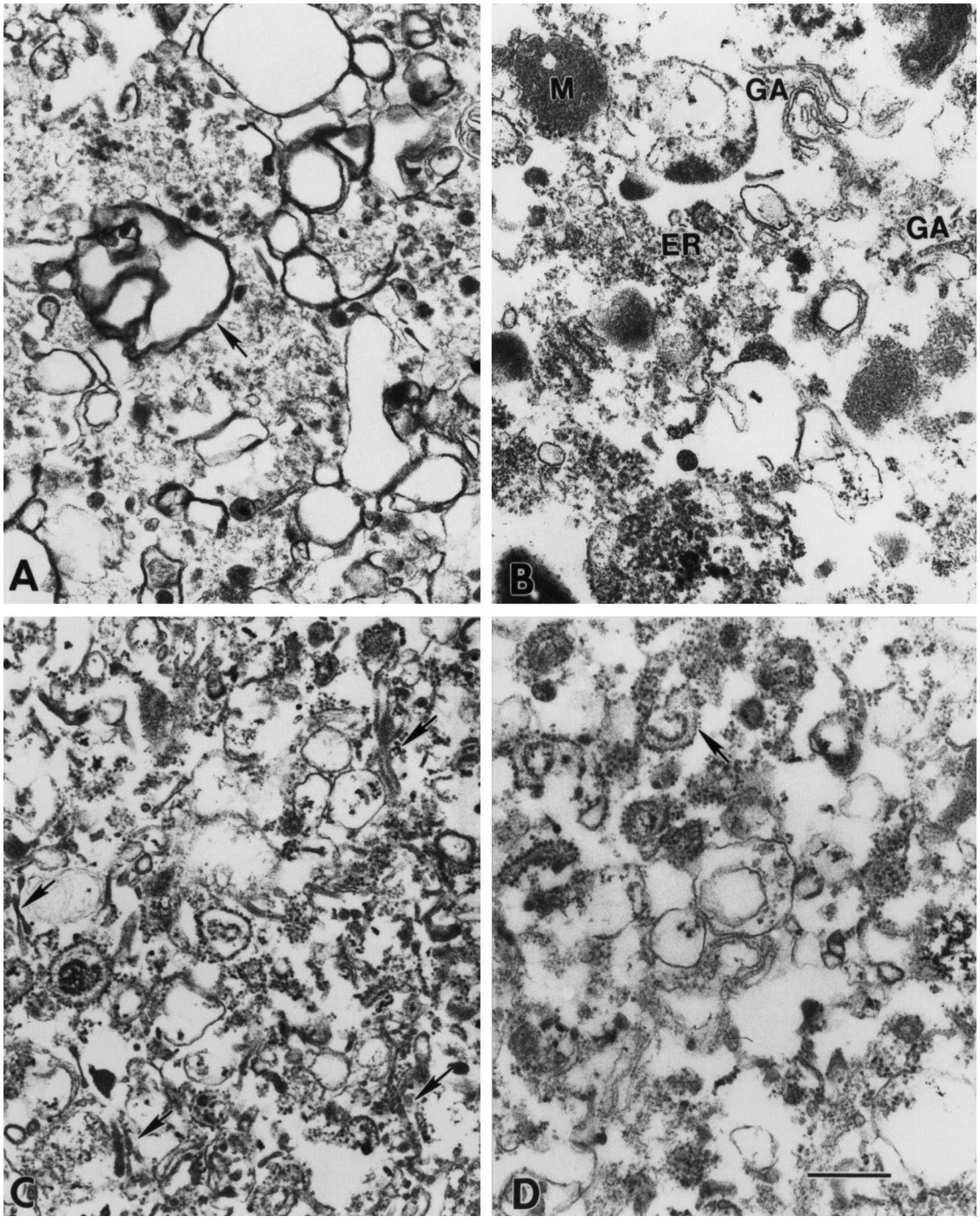

Fig. 2. Electron micrographs of BAL-17 cells isolated by aqueous two-phase partition and centrifugation as described previously for K-562 cells [12]. (A) Plasma membrane-enriched upper phase (arrows). (B) Lower phase depleted of plasma membranes. (C) Golgi apparatus (arrows) isolated by centrifugation followed by aqueous two-phase partition. (D) Endoplasmic reticulum (arrows) fraction from plasma membrane-depleted lower phase. Bar=0.5 μm.

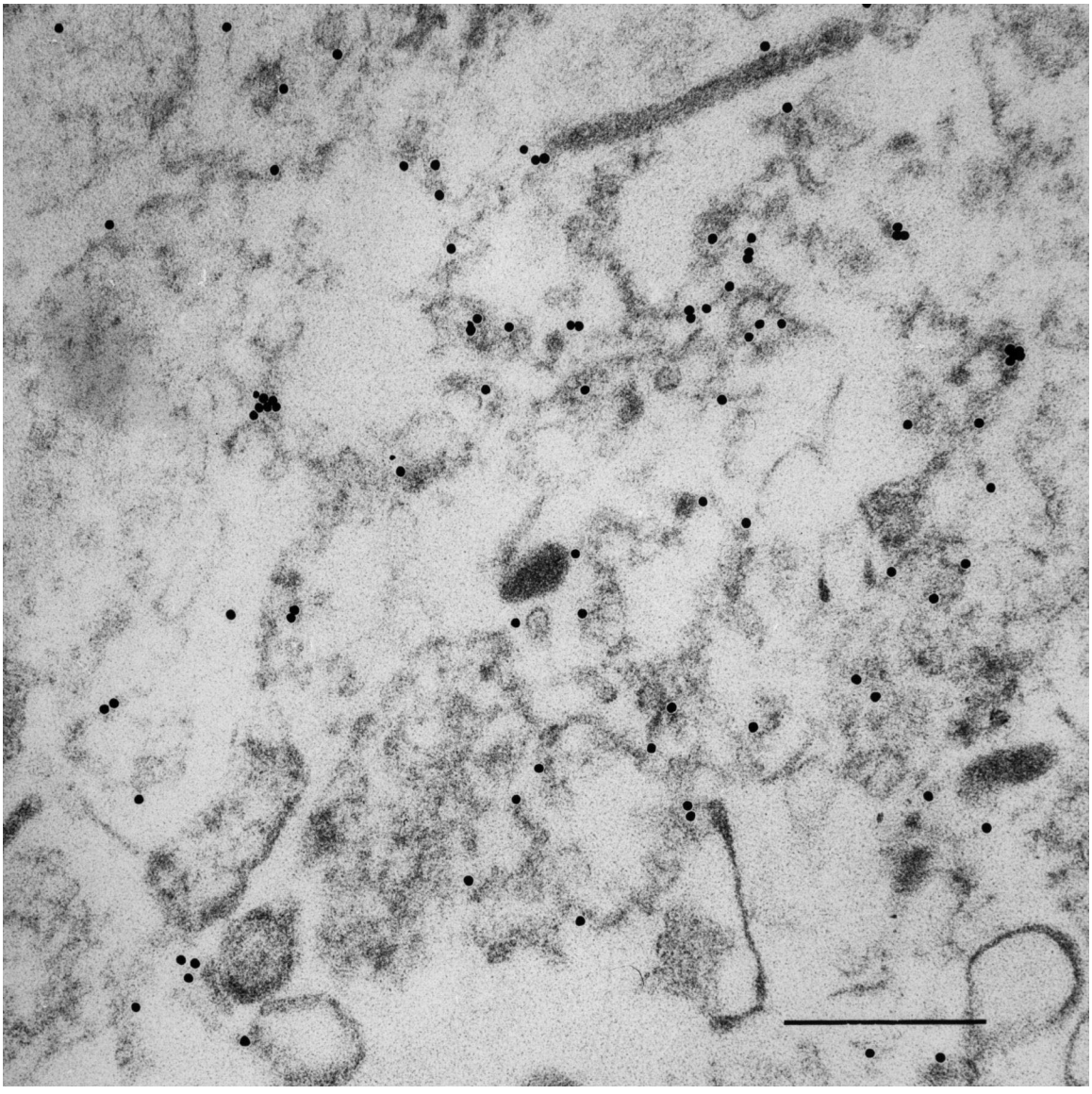

Fig. 3. Electron micrograph of the plasma membrane-enriched upper phase prepared from a plasma membrane fraction of homogenized CFK cells reacted with concanavalin A conjugated to colloidal gold in solution prior to centrifugation and washing to remove unbound conjugate. The concanavalin A binds to glycoproteins at the external surface of the cell and the bound colloidal gold particles confirm a plasma membrane origin of a majority of the vesicles and that they are, for the most part, oriented right side-out. Bar=0.5 μm.

(Fig. 3) was used to confirm orientation of the vesicles in the two fractions.

There was approximately a 3.5-fold relative enrichment of Golgi apparatus fragments in the lower phase after centrifugation and two-phase partition (Table 5). The Golgi apparatus stacks were subsequently concentrated by centrifugation (Fig. 1C) as described [12].

Golgi apparatus fractions from the BAL-17 cells (Fig. 2C) contained a capsaicin-inhibited NADH oxidase activity similar to that common to the plasma membrane of transformed cell lines [12]. The

Table 3
Composition of membrane fractions prepared from HeLa cells by aqueous two-phase partition as estimated by electron microscope morphometry

Cell component	Intercepts per 100 total intercepts	
	Upper phase	Lower phase
Plasma membrane	86±3	n.d.
Nuclear envelope	n.d.	4±4
Endoplasmic reticulum +nuclear envelope fragments	7±3	66±14
Mitochondria	3±1	19±4
Golgi apparatus	1±1	6±6
Lysosomes+endosomes	1±1	1±0
Other	2±0	4±0

Table 4
ATPase latency of HeLa plasma membrane (μmol P_i/h per mg protein) in the presence and absence of detergent (from Morré [30])

Fraction	0.2% Triton X-100	
	−	+
Fraction A (right side-out)	<0.5	6.4
Fraction B (inside-out)	5.5	7.1

initial rate of capsaicin-inhibited oxidation by NADH by isolated Golgi apparatus fractions from the BAL-17 cells appeared not to be due largely to contaminating plasma membranes vesicles which was reduced substantially by aqueous two-phase partition. The specific activity of the Golgi apparatus fractions for the plasma membrane marker enzyme 5′-nucleotidase is less than 33% that of the plasma membrane-enriched upper phase fraction (Table 2).

Aqueous two-phase partition is useful also to remove plasma membrane contamination from Golgi apparatus (Figs. 1C and 2C) or endoplasmic reticulum (Figs. 1D and 2D) fractions from cultured cells as described [12].

Table 5
Specific activity of the Golgi apparatus marker enzyme, galactosyltransferase in HeLa cell fractions isolated by two-phase partition and centrifugation

Fraction	Specific activity (nmol/h per mg protein)	Relative enrichment
Total homogenate	1.75	–
Lower phase	6.17	3.5
Upper phase	2.95	1.7

A unique external surface marker enzyme activity that can be used for the direct assay of right side-out plasma membranes is the periodic plasma membrane-associated oxidation of NADH [24,25]. The activity is periodic and can be directly measured. HeLa cell plasma membrane exhibited a rate of NADH oxidation of about 1 nmol/min per mg protein and was proportional to time and protein concentration. The K_m for NADH oxidation was about 25 μ*M* [24]. The oxidation of externally supplied NADH by plasma membrane vesicles has the potential to serve as a marker activity to guide aqueous two-phase membrane separation. The NOX protein is located at the external cell surface affording the opportunity to use this activity to identify and quantitate right side-out plasma membrane vesicles (Fig. 4). Many plasma membrane markers are associated with the cytosolic membrane surface and require the use of detergents for measurement of their activities (latency) [26].

The distinguishing feature of the NOX family of

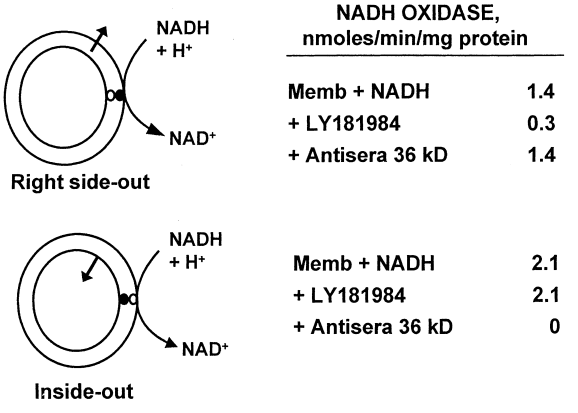

Fig. 4. Model illustrating the concept of two oxidase sites on the plasma membrane: one external and one internal. NADH, being impermeant, can reach the outside site with right side-out vesicles and is accessible to the inside site only with inside-out vesicles. The antisera to a peptide synthesized using sequence information from the purified 36-kDa NADH oxidase from the inside surface of liver plasma membrane inhibited only with inside-out vesicles. The drug-responsive NOX isoform of HeLa cells is restricted to the surface of right side-out vesicles. The tNOX-specific antitumor sulfonylurea, LY181984, *N*-(4-methylphenylsulfonyl-*N*′-(4-chlorophenyl)urea inhibits only the oxidase exposed to NADH with right side-out vesicles, i.e., the external NADH site.

proteins (plant, animal, cancer, etc.) is that the two activities, the oxidation of hydroquinone or NADH and the interchange of protein thiol–disulfide bonds alternate within a total period of 24 min [27]. This periodicity of function, which makes possible the distinction of the NOX protein from all other cellular oxidases and protein disulfide–thiol interchange activities, is normally highly synchronized [28]. Synchrony (entrainment) in plants is achieved by light [29].

The periodicity is given by purified proteins [27,29,30] and by the cloned protein from HeLa expressed in *E. coli* [27]. The periodic nature of the protein serves to distinguish the NOX proteins from all other cellular oxidative and protein disulfide isomerase-like activities.

All of the periodicity data were significantly autocorrelated and agreed closely with the experimental data (Fig. 5). The decomposition fit analysis, illustrated in Fig. 5, was used to validate the periodic pattern. The three measured values used to help evaluate the accuracy of the fitted values were mean average percentage error (MAPE, 75.95), a measure of the periodic oscillation, mean average deviation (MAD, 0.176), a measure of the absolute average deviations from the fitted values, and mean standard deviation (MSD, 0.067), the measure of standard deviation from the fitted values.

Decomposition was carried out by fitting a trend line to the data, then detrending the data by subtracting out the trend component. The detrended data was then smoothed by using a centered moving average of length equal to the length of the cycle. Once the moving average was obtained, it was subtracted from the detrended data. Within each period, the median value of the raw data was found. These medians made up the cycle indices. The cycle indices were in turn used to adjust the data. Classical decomposition

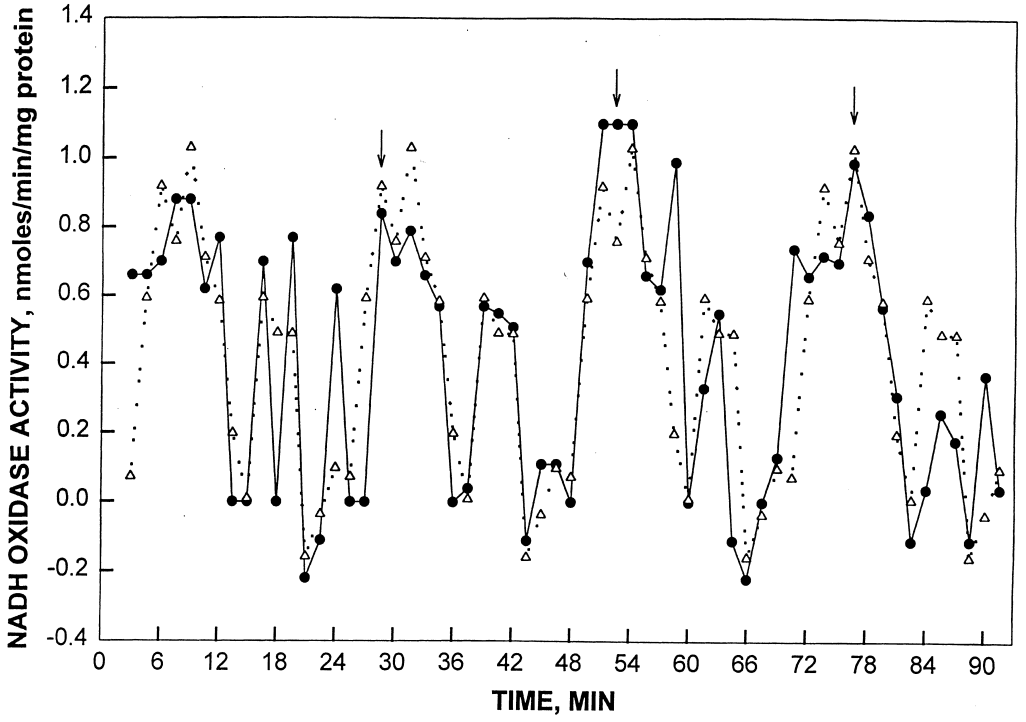

Fig. 5. Rate of NADH oxidation by a single preparation of plasma membrane vesicles of HeLa cells with time over 90 min (solid symbols and lines). The activity oscillated with a 23-min period with maxima at 23, 52 and 74 min (arrows). Rates of NADH oxidation were measured over 1 min at intervals of 1.5 min. A decomposition fit (open triangles, dotted lines) show the reproducibility of the pattern of the periodicity. The amplitudes at cycle maxima were significantly different ($P<0.001$) from the amplitudes at cycle minima and were highly autocorrelated (see text).

decomposed a time series into trend, cyclic and error components. In our analysis, the trend component was insignificant.

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