Tissue-Culture Cell Fractionation

FRACTIONATION OF MEMBRANES FROM TISSUE-CULTURE CELLS HOMOGENIZED BY GLYCEROL-INDUCED LYSIS

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1. The disruption of various types of tissue-culture cells by (a) incubation in solutions of 1.2M-glycerol and (b) transfer of the glycerol-loaded cells to relatively hypo-osmotic solutions of 0.25M-sucrose was studied. 2. Bivalent cations (2mM-Mg²⁺) were generally included to preserve the nuclei, but some cells (polyoma-virus-transformed baby-hamster kidney cells) failed to be disrupted adequately under these conditions. 3. Other cells (mouse-embryo fibroblasts) required additional gentle Dounce homogenization to effect complete cell breakage. 4. Purification of the whole homogenate was carried out by a combination of differential centrifugation and sedimentation or flotation through sucrose gradients. 5. Enzyme analysis showed that plasma-membrane, endoplasmic-reticulum and mitochondrial fractions were obtained in good yield and purity.

The production of plasma membranes from tissue-culture cells poses many problems, the chief of which is that of homogenization, in particular the resistance of most tissue-culture cells to disruption by liquid shear in iso-osmotic conditions and the tendency for them to fragment into particles of various sizes after cell breakage. Various techniques have been adopted to overcome this problem: the use of nitrogen cavitation (Kamat & Wallach, 1965; Graham, 1972, 1975) to convert all the plasma membrane into vesicles; the use of hypo-osmotic media to swell the cells before disruption by Dounce or Potter–Elvehjem homogenization (Neville, 1960; Evans, 1970; Demus, 1973; Forte *et al.*, 1973); exposure of the cells to some surface-hardening agent such as Zn²⁺ or Fluorescein mercuric acetate (Warren *et al.*, 1966) so that the plasma membrane remains completely intact during the subsequent isolation procedure.

Although nitrogen cavitation is the most reproducible of disruptive techniques and all the plasma membrane is converted into vesicles of a similar size, the separation of these vesicles from endoplasmic-reticulum vesicles is difficult, and cavitation conditions are often critical inasmuch as the pressure and/or equilibration time requirements for cell rupture are only slightly less than those that will also disrupt the nuclei. Hypo-osmotic swelling of cells to facilitate their rupture by Dounce homogenization is simple and applicable to a wide range of cell types, but the exposure of the released cell organelles such

as mitochondria and lysosomes to the hypo-osmotic medium may be detrimental, although if the medium volume/cell ratio is not too high the released soluble components partially protect the osmotically sensitive organelles. Furthermore the homogenate may be returned directly to iso-osmoticity by addition of sucrose. Hardening of the cell membrane, although beneficial to the recovery of plasma membrane from tissue-culture cells, causes the inhibition of many surface enzymes, and it has proved difficult to recover other membranes, particularly the endoplasmic reticulum, from homogenates of hardened cells.

An alternative approach to swelling the cells in hypo-osmotic media is to swell them by a method that was developed for platelets by Barber & Jamieson (1970). This involves exposure of the cells to 4.2M-glycerol: the cells take up glycerol; they are then transferred to buffered 0.25M-sucrose, which is relatively hypo-osmotic to the cell contents. Water is taken up by the glycerol-loaded platelets, which therefore swell and lyse. The application of this method to hamster-embryo fibroblasts, mouse-embryo fibroblasts and baby-hamster kidney cells, and the subsequent fractionation of the various membranes, are described in the present paper.

Materials and Methods

Preparation of homogenate

Monolayers of hamster-embryo fibroblasts (Nil 1, Nil 2 and Nil 8), hamster-sarcoma-virus-transformed hamster-embryo fibroblasts (Nil 8-HSV), polyoma-

Abbreviation used: ATPase, adenosine triphosphatase.

virus-transformed baby-hamster kidney cells (BHK-py) and mouse-embryo fibroblasts (3T3) were grown on plastic Petri dishes or glass roller bottles in Eagle's medium (Dulbecco & Freeman, 1959) supplemented with calf serum (10%, v/v). They were harvested at confluence by scraping with a rubber 'policeman'. All cell types (5×10^8 – 2×10^9 cells) were washed three times in approx. 50 ml of phosphate-buffered saline (0.12 M-NaCl/2.6 mM-KCl/8.1 mM- Na_2HPO_4 /1.5 mM- KH_2PO_4) before resuspension in 40 ml of the glycerol-containing medium buffered with 5 mM-Tris, pH 7.4. The cell suspension was stirred gently during the glycerol-uptake phase, and then centrifuged at $1000g_{av}$ for 5 min. After the supernatant had been aspirated as completely as possible, the cell pellet was resuspended in the residual glycerol solution. This suspension was taken up into a Pasteur pipette and ejected into a stirred solution of 0.25 M-sucrose to allow swelling. The optimal conditions for glycerol uptake and sucrose solution swelling depend on the cell type and are presented in the Results section.

Fractionation of the homogenate

Various fractionation schemes have been investigated for the different cell types: for convenience these have been designated A–F. All sucrose gradient solutions are expressed in terms of % (w/w).

Differential centrifugation (A). The nuclear fraction from the homogenate was sedimented at $1000g_{av}$ for 5 min. The pellet was washed twice in 20 ml of 0.25 M-sucrose/0.2 mM- MgCl_2 /5 mM-Tris buffer, pH 7.4, and the three supernatants were combined and centrifuged at $10000g_{av}$ ($5000g_{av}$ for 3T3 cells) for 10 min to give the mitochondrial pellet. The $10000g$ supernatant was centrifuged at $100000g_{av}$ for 30 min to give the microsomal pellet. If the pellets were to be analysed directly, they were washed once in 0.25 M-sucrose/5 mM-Tris buffer, pH 7.4, and resuspended in 1–2 ml of this medium; if they were to be fractionated further, they were resuspended in the appropriate gradient medium (see E and F).

Gradient fractionation of the homogenate (B). The homogenate was made 20% with respect to sucrose (in 0.2 mM- MgCl_2 /5 mM-Tris buffer, pH 7.4). Then 4 ml was layered over a 20 ml linear sucrose gradient (30–60%) also containing 5 mM-Tris buffer, pH 7.4, and 0.2 mM- MgCl_2 and it was centrifuged at $70000g_{av}$ for 1 h.

Gradient fractionation of the homogenate (C). The homogenate was made 50% with respect to sucrose and 1 mM with respect to MgCl_2 . Then 12 ml of this was layered between 9 ml of 55% sucrose and 2 ml of 20% sucrose (both containing 5 mM-Tris buffer, pH 7.4, and 1 mM- MgCl_2), and the gradient was centrifuged in a 3×25 ml swing-out rotor at $90000g_{av}$ for 2 h.

Gradient fractionation of the homogenate (D). The homogenate was made 50% with respect to sucrose

and 1 mM with respect to MgCl_2 and adjusted to a total volume of 70 ml. Then 35 ml of this suspension was layered between 5 ml each of 55% sucrose and 20% sucrose (both containing 1 mM- MgCl_2 and 5 mM-Tris buffer, pH 7.4). Centrifugation was carried out in a 8×50 ml angle rotor at $120000g_{av}$ for 1 h.

Fractionation of membrane material (E). The membrane-containing fraction from either the differential centrifugation scheme (A) or some other gradient (B–D) was suspended in 4–5 ml of 20% sucrose/5 mM-Tris buffer, pH 7.4. This suspension was then layered on top of a linear 30–50% sucrose gradient (20 ml) in a 3×25 ml swing-out rotor and centrifuged at $90000g_{av}$ for 1 h. In this gradient membrane vesicles and membrane fragments do not reach their isopycnic density, and hence they are separated from each other and from other more rapidly sedimenting particles such as mitochondria and nuclei mainly on the basis of sedimentation rate. Although the more rapidly sedimenting particles may attain their isopycnic density, the system is described in the present paper as a 'sedimentation-rate gradient'.

Fractionation of membrane material (F). For isopycnic separation the membrane-containing fraction either from the differential centrifugation scheme (A) or some other gradient (B–E) was suspended in 6 ml of 50% sucrose/5 mM-Tris buffer, pH 7.4. This suspension was then incorporated into a stepped gradient of 6 ml each of 20%, 30% and 40% sucrose in 5 mM-Tris buffer, pH 7.4, and centrifuged in a 3×25 ml swing-out rotor for 16 h at $90000g_{av}$. If the fraction contained nuclei then the above volumes were decreased to 5 ml, and 4 ml of 60% sucrose/5 mM-Tris buffer, pH 7.4, was included at the bottom of the gradient.

Collection of tube gradients

The bands were removed by using a flat-tipped metal cannula attached to a syringe or collected by upward displacement with dense sucrose. Fractions from the gradient were diluted with at least 2 vol. of 5 mM-Tris buffer, pH 7.4, and sedimented at $100000g_{av}$ for 30 min (membranes) or $20000g_{av}$ for 30 min (mitochondria and nuclei). The pellets were washed twice in 0.25 M-sucrose/5 mM-Tris buffer, pH 7.4, and resuspended either in this medium (1–2 ml) for analysis or in the appropriate gradient medium if they were to be refractionated.

Compositional analysis

Assays for the following enzymes were carried out: Na^+ + K^+ -stimulated Mg^{2+} -dependent ATPase (EC 3.6.1.3) and 5'-nucleotidase (EC 3.2.2.4) by the methods of Avruch & Wallach (1971); succinate-cytochrome c reductase (EC 1.3.99.1) by the method of Mackler *et al.* (1962); NADPH-cytochrome c reductase (EC 1.6.2.4) by the method of Williams &

Kamin (1962). Protein was measured by the method of Lowry *et al.* (1951), with bovine serum albumin as standard. RNA and DNA were measured colorimetrically by the methods described by Munro & Fleck (1966) and Burton (1956) respectively.

Chemicals

[γ - ^{32}P]ATP (sodium salt) and [U- ^{14}C]AMP (ammonium salt) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Cytochrome *c* (horse heart) was purchased from the Boehringer Corporation, Lewes, Sussex, U.K. All other chemicals were AnalaR grade from BDH Chemicals, Poole, Dorset, U.K.

Results

Glycerol-induced lysis

The conditions required to produce more than 95% disruption while at the same time causing minimal nuclear damage vary with the cell type. Optimal glycerol-loading conditions for each cell type are given in Table 1. The inclusion of 0.2mM-Mg $^{2+}$ in the glycerol-loading medium for all types of Nil cells and for 3T3 cells maintained the nuclei in a more condensed state but decreased the subsequent cell breakage from approx. 99% to approx. 95%. Addition of Mg $^{2+}$ to the glycerol-loading medium in the case of BHK-py cells decreased the subsequent cell breakage from approx. 95% to below 50%.

When glycerol-loaded Nil 1, Nil 2, Nil 8, Nil 8-HSV and BHK-py cells were taken into 0.25M-sucrose/0.2mM-MgCl $_2$ /5mM-Tris buffer, pH 7.4, they swelled and lysed; lysis was aided by gentle pipetting or stirring. As an alternative, the osmoticity of the glycerol solution was simply lowered by addition of 2 vol. of 0.2mM-MgCl $_2$ /5mM-Tris buffer, pH 7.4, to the stirred cell suspension. Although Nil 1, Nil 2 and Nil 8 cells were disrupted satisfactorily by this method, and indeed it was the preferred method for Nil 8-HSV cells, BHK-py cells remained more or less intact when treated in this manner.

For 3T3 cells, transfer of the glycerol-loaded cells

to the relatively hypo-osmotic sucrose medium, though causing cell swelling, failed to cause disruption; for these cells additional shearing forces were required. 3T3 cells, loaded with glycerol as indicated in Table 1, were stirred in 30ml of 0.25M-sucrose/0.2mM-MgCl $_2$ /5mM-Tris buffer, pH 7.4, at 4°C for 5–10min and then disrupted by five strokes of the pestle in a loose-fitting Dounce homogenizer.

Fractionation of homogenates

Methods A+F. Differential centrifugation of a BHK-py cell homogenate showed that more than 98% of the total DNA remained with the nuclei, i.e. that little or no nuclear breakage occurred (Table 2). Most of the plasma membrane sedimented in the mitochondrial (10000g) fraction, which contained 59% of the total Na $^{+}$ +K $^{+}$ -stimulated ATPase activity; of the remainder 22% sedimented with the microsomal fraction (100000g) and 10% with the nuclei (1000g). The RNA and NADPH-cytochrome *c* reductase in the microsomal fraction accounted for 84% and 77% respectively of the total, i.e. this fraction contained most of the endoplasmic reticulum. From the mitochondrial pellet three major bands were resolved by isopycnic centrifugation (method F). Table 3 shows that band 2 was considerably enriched in Na $^{+}$ +K $^{+}$ -stimulated ATPase and represents a purification of 13-fold over the homogenate; band 3 contained most of the mitochondria (succinate-cytochrome *c* reductase activity).

When Nil 8 cells were fractionated in a similar manner, most of the Na $^{+}$ +K $^{+}$ -stimulated ATPase activity sedimented with the mitochondria, and in the subsequent isopycnic gradient this enzyme was heavily concentrated in band 1 (see Table 4). Although the application of methods A+F successfully resolved the mitochondria and nuclei from the plasma membrane of Nil 8 cells, the endoplasmic-reticulum contamination of the plasma-membrane fraction was high. In contrast with the results obtained with BHK-py cells the RNA and NADPH-cytochrome *c* reductase were not heavily concentrated in the microsomal pellet (Table 4).

The plasma-membrane fragments from 3T3 cells appeared to be larger than those from BHK-py or Nil 8 cells; 60% of the Na $^{+}$ +K $^{+}$ -stimulated ATPase sedimented in a heavy-mitochondrial fraction (5000g $_{av}$ for 10min), whereas most of the RNA and NADPH-cytochrome *c* reductase remained in the supernatant (see Table 5) of this fraction.

Method B. Four major bands and a pellet were separated from BHK-py-, Nil 8- and 3T3-cell homogenates (Table 6). In all cases the DNA was contained entirely within the pellet. Microsomal fragments were largely restricted to bands 1 and 2; mitochondria were mainly distributed between bands 3 and 4 in the case of BHK-py and Nil 8 cells, and between bands 2 and 3 in the 3T3 cells. The presence of most of the

Table 1. Glycerol loading conditions for various cell types

Cell type	Conditions
Nil 1, Nil 2, Nil 8 and 3T3 cells	Stirred at 4°C for 10min in 1.2M-glycerol/5mM-Tris buffer, pH 7.4 ($\pm$ 0.2mM-MgCl $_2$)
Nil 8-HSV cells	Stirred at 4°C for 20min in 1.2M-glycerol/5mM-Tris buffer, pH 7.4 ($\pm$ 0.2mM-MgCl $_2$)
BHK-py cells	Stirred at 4°C for 20min in 1.2M-glycerol/5mM-Tris buffer, pH 7.4

Table 2. *Differential centrifugation of BHK-py-cell homogenate*
For experimental details see the text.

Fraction ...	Homogenate	1000g sediment	10000g sediment	100000g sediment
DNA (mg)	1.30	1.28	0.01	0.01
RNA (mg)	0.25	0.01	0.01	0.21
Na ⁺ +K ⁺ -stimulated ATPase				
Total activity (μ mol of ATP hydrolysed/h)	25.4	2.7	15.0	5.7
Specific activity (μ mol of ATP hydrolysed/h per mg of protein)	0.7	0.3	4.3	1.5
NADPH-cytochrome <i>c</i> reductase				
Total activity (μ mol of cytochrome <i>c</i> reduced/h)	8.83	0.49	1.41	6.85
Specific activity (μ mol of cytochrome <i>c</i> reduced/h per mg of protein)	0.24	0.06	0.43	1.81
Succinate-cytochrome <i>c</i> reductase				
Total activity (μ mol of cytochrome <i>c</i> hydrolysed/h)	15.85	0.54	10.88	2.55
Specific activity (μ mol of cytochrome <i>c</i> hydrolysed/h per mg of protein)	0.43	0.06	3.18	0.67

Table 3. *Resolution of mitochondrial fraction from BHK-py cells in an isopycnic sucrose gradient*
For experimental details see the text.

	Band no. ...	1	2	3
Density (g/ml)		1.12–1.13	1.14–1.16	1.19–1.21
Na ⁺ +K ⁺ -stimulated ATPase specific activity (μ mol of ATP hydrolysed/h per mg of protein)		2.5	9.3	3.0
Succinate-cytochrome <i>c</i> reductase specific activity (μ mol of cytochrome <i>c</i> reduced/h per mg of protein)		—	1.4	7.8

Table 4. *Fractionation of Nil 8-cell homogenate by methods A+F*
For experimental details see the text. N.D., Not determined.

Fraction ...	Method A			Method F		
	1000g sediment	10000g sediment	100000g sediment	Band 1	Band 2	Band 3
DNA (% of total recovered)	99	1	—	N.D.	N.D.	N.D.
Na ⁺ +K ⁺ -stimulated ATPase	6.6	70.6	22.7			
Activity (% of total recovered)						
Specific activity (μ mol of ATP hydrolysed/h per mg of protein)	0.05	1.26	0.80	4.45	0.21	<0.1
RNA (% of total recovered)	6.5	39.1	54.3	10.8	15.2	13.0
NADPH-cytochrome <i>c</i> reductase						
Activity (% of total recovered)	—	33.1	66.8			
Specific activity (μ mol of cytochrome <i>c</i> reduced/h per mg of protein)	<0.1	0.44	1.80	0.48	0.31	0.11
Succinate-cytochrome <i>c</i> reductase						
Activity (% of total recovered)	—	84.4	15.5			
Specific activity (μ mol of cytochrome <i>c</i> reduced/h per mg of protein)	<0.2	4.8	1.2	0.80	4.4	6.3

Table 5. *Crude fractionation of 3T3-cell homogenate by method A*
For experimental details see the text. N.D., Not determined.

	Fraction ...	1000g sediment	5000g sediment	10000g sediment	100000g sediment
Na ⁺ +K ⁺ -stimulated ATPase activity (% of total recovered)		20.7	62.8	6.9	9.4
Succinate-cytochrome <i>c</i> reductase activity (% of total recovered)		2.3	65.3	29.1	3.1
NADPH-cytochrome <i>c</i> reductase activity (% of total recovered)		—	—	13.4	85.5
RNA (mg)		N.D.	0.12	0.25	0.96

Table 6. *Crude fractionation of cell homogenates by density-gradient centrifugation (method B)*
For experimental details see the text.

	Cell type	Na ⁺ +K ⁺ -stimulated ATPase activity (% of total)	NADPH-cytochrome <i>c</i> reductase activity (% of total)	Succinate-cytochrome <i>c</i> reductase activity (% of total)	DNA (% of total)
Band 1	BHK-py cells	9.4	36.4	—	—
	Nil-8 cells	11.1	10.0	—	—
	3T3 cells	18.0	11.6	—	—
Band 2	BHK-py cells	21.8	63.6	10.4	—
	Nil-8 cells	42.4	90.0	—	—
	3T3 cells	—	71.9	40.4	—
Band 3	BHK-py cells	59.5	—	48.9	—
	Nil-8 cells	37.7	—	78.9	—
	3T3 cells	33.1	16.0	59.5	—
Band 4	BHK-py cells	9.3	—	29.0	5
	Nil-8 cells	4.4	—	19.2	3
	3T3 cells	48.9	—	—	3
Pellet	BHK-py cells	—	—	—	95
	Nil-8 cells	4.4	—	—	97
	3T3 cells	—	—	—	97

Na⁺+K⁺-stimulated ATPase activity in band 4 from 3T3 cells reflects the greater size of plasma-membrane fragments from these cells compared with those from BHK-py and Nil 8 cells, which distributed between bands 2 and 3. The Na⁺+K⁺-stimulated ATPase in band 1 from 3T3 cells probably reflects the presence of small fragments or vesicles of plasma membrane produced during the Dounce homogenization. If Mg²⁺ was omitted from the gradient the DNA was not confined to the pellet.

Methods C+E. A homogenate of 3T3 cells was treated as described for method C. In addition to a pellet that contained all of the DNA (Table 7), two bands, namely band 1 at the 20%/50% sucrose interface and band 2 at the 50%/55% sucrose interface, were obtained. Most of the Na⁺+K⁺-stimulated ATPase was contained in band 1, 4% was contained in band 2 and the remainder was trapped in the pellet.

Band 1 also contained almost all of the endoplasmic reticulum and about one-third of the total mitochondria. The results reported here were from one experiment: the distributions of enzymes in the bands of material were identical in three similar experiments, but, however, the amount of Na⁺+K⁺-stimulated ATPase activity in the pellet remained unpredictable and varied from 0% in one experiment to 17% in the experiment reported here.

Re-fractionation of band 1 in a sedimentation-rate gradient (type E) produced three major fractions (Table 7): band 1a contained all the NADPH-cytochrome *c* reductase activity, band 1c contained all the Na⁺+K⁺-ATPase activity and the succinate-cytochrome *c* reductase was distributed between band 1c (28% of the total applied to the gradient) and a diffuse zone (band 1b) that contained the remainder of the activity. With Nil 8 cells a similar

Table 7. *Fractionation of 3T3-cell homogenate by methods C+E*
For experimental details see the text.

Fraction ...	Gradient C			Gradient E		
	1	2	Pellet	1a	1b	1c
Na ⁺ +K ⁺ -stimulated ATPase						
Activity (% of total)	78	4	17			
Specific activity (μ mol of ATP hydrolysed/h per mg of protein)	1.26	0.18	0.29	<0.2	<0.2	3.39
NADPH-cytochrome <i>c</i> reductase activity						
Activity (% of total)	89	11	—			
Specific activity (μ mol of cytochrome <i>c</i> reduced/h per mg of protein)	0.57	0.19	<0.05	1.9	<0.05	<0.05
Succinate-cytochrome <i>c</i> reductase activity						
Activity (% of total)	35	65	—			
Specific activity (μ mol of cytochrome <i>c</i> reduced/h per mg of protein)	1.4	3.7	<0.1	<0.1	5.8	4.7
DNA (% of total)	—	—	100			

Table 8. *Fractionation of 3T3-cell homogenate by gradient systems D+E+F*
For experimental details see the text.

	Na ⁺ +K ⁺ -stimulated ATPase		NADPH-cytochrome <i>c</i> reductase		Succinate-cytochrome <i>c</i> reductase	
	Activity (% of total)	Specific activity (μ mol of ATP hydrolysed/h per mg of protein)	Activity (% of total)	Specific activity (μ mol of cytochrome <i>c</i> reduced/h per mg of protein)	Activity (% of total)	Specific activity (μ mol of cytochrome <i>c</i> reduced/h per mg of protein)
Gradient E						
Band 1	—	<0.1	63.6	1.89	7.3	0.61
Band 2	—	0.2	36.3	2.22	4.4	0.83
Band 3	20–58	1.5–3.7	—	<0.2	17.6	2.81
Band 4	80–42	2.2–1.4	—	<0.2	70.5	14.85
Gradient F						
Band 3–1		7.24		<0.1		0.56
Band 3–2		0.62		<0.1		3.44

distribution of material was obtained with methods C and E except that the Na⁺+K⁺-ATPase and succinate-cytochrome *c* reductase activities in gradient E were reversed.

Methods D+E+F. In system D, a homogenate of 3T3 cells produced a pellet (nuclei) and an interfacial band that was washed once in 0.25M-sucrose/5mM-Tris buffer, pH7.4, before resuspension in 20% sucrose/5mM-Tris buffer, pH7.4. This suspension was then fractionated in a type E gradient: four major bands and a pellet were obtained (Table 8). The endoplasmic reticulum was restricted to bands 1 and 2, whereas the mitochondria were distributed between bands 3 and 4. The plasma membrane (Na⁺+K⁺-stimulated ATPase activity) was also distributed predominantly between bands 3 and 4; in three experiments most of this enzyme activity

occurred in band 3 (a range of activities and recoveries is given in Table 8). This suggests that the size of the plasma-membrane fragments in the gradient E input varies; this may be associated with the shearing forces required in the two resuspension steps in the preparation of the material recovered from gradient D for the sedimentation-rate gradient. If these shearing forces are made more severe, then plasma-membrane fragments also appear in band 2. Further purification of the plasma-membrane-containing bands was carried out in isopycnic sucrose gradients (type F). Because of the variability in the distribution of the Na⁺+K⁺-stimulated ATPase both bands 3 and 4 were subfractionated routinely. Table 8 shows the results of an experiment in which band 3 contained the highest Na⁺+K⁺-stimulated ATPase activity; the plasma-membrane band (3–1) had negligible con-

tamination from mitochondria and endoplasmic reticulum and represents an 18-fold purification over the homogenate.

Discussion

It is clear from the results described above that 'glycerol-induced lysis' is an effective method for the homogenization of a variety of tissue-culture cells. For hamster-embryo fibroblasts (Nil 1, Nil 2 and Nil 8), hamster-sarcoma-virus-transformed hamster-embryo fibroblasts (Nil 8-HSV) and polyoma-virus-transformed baby-hamster kidney cells (BHK-py) the method is particularly suited because the lysis occurs when the glycerol-loaded cells are transferred to iso-osmotic sucrose; in these cases cell disruption does not require the liquid shear forces of Dounce or Potter-Elvehjem homogenization.

Although 3T3 cells do require liquid shear (Dounce homogenization) to rupture the swollen cells, the release of the cellular organelles into an iso-osmotic sucrose medium makes this method preferable over the standard hypo-osmotic-shock procedure, although the latter is required under certain conditions (see Graham & Coffey, 1979).

Most of the plasma-membrane fragments produced by this method are more rapidly sedimented than are the vesicles of the endoplasmic reticulum and they can be separated on the basis of sedimentation rate either by differential centrifugation or in a gradient. Variability in (a) the susceptibility of different cells to osmotic lysis and liquid shear and (b) the propensity of their surface membranes to fragmentation is well illustrated in the comparison of 3T3 cells with any of the Nil cell lines. Although the former require additional liquid shear for cell rupture, they produce larger plasma-membrane fragments than do the latter: the bulk of the plasma membranes from 3T3 cells sediment at 50000g-min (compared with 100000g-min for Nil cells), and in sucrose gradients (method B) the 3T3 plasma membranes sediment more rapidly than those of either BHK-py or Nil cells.

Differences in sedimentation characteristics are not confined to the plasma membrane; the endoplasmic reticulum from Nil 8 cells was significantly more rapidly sedimented than that from BHK-py cells. Consequently a simple differential centrifugation followed by an isopycnic sucrose gradient was quite satisfactory for obtaining a plasma-membrane fraction from BHK-py cells, but not from Nil 8 cells.

The use of a differential centrifugation scheme to recover the maximum amount of $\text{Na}^+ + \text{K}^+$ -stimulated ATPase in the 10000g (or 5000g for 3T3 cells) pellet depends on efficient washing of the nuclear sediment. Employment of a gradient to remove the nuclei from the homogenate overcomes any problem of nuclear breakage due to repeated washing and also

minimizes the progressive comminution of plasma-membrane fragments.

Maximization of the yield of plasma membrane is important in view of the apparent heterogeneity of the protein components in the plane of the membrane. For example, the intestinal epithelium brush border is enriched in maltase and alkaline phosphatase activities whereas the non-luminal membrane is enriched in $\text{Na}^+ + \text{K}^+$ -stimulated ATPase and adenylate cyclase activities (Fujita *et al.*, 1972), and rat liver plasma-membrane sheets can be subfractionated into separate components rich in 5'-nucleotidase and $\text{Na}^+ + \text{K}^+$ -stimulated ATPase respectively (Evans, 1970). Partial segregation of these enzymic activities in plasma-membrane subfractions has also been observed in mouse plasmacytoma cells (Lelievre, 1973), thyroid plasma membrane (Suzuki *et al.*, 1977), baby-hamster kidney cells (Graham, 1972) and chick-embryo fibroblasts (Perdue & Sneider, 1970).

In the best cases the yield of plasma membrane from sources other than liver, as measured in terms of percentage recovery of a specific enzyme marker from the homogenate, may reach 38% for skin fibroblasts (Kartner *et al.*, 1977), but normally recoveries are somewhat lower, e.g. 12–18% for HeLa cells (Cohen *et al.*, 1977), 13% for bovine thyroid-gland cells (Suzuki *et al.*, 1977) and 10–15% for human thymocytes (Allan & Crumpton, 1972). When the size of the membrane fragments produced is variable the processing of one fraction obtained by differential centrifugation of the homogenate leads to low recoveries. Forte *et al.* (1973) processed only a 7200g-min fraction and recovered only 0.1% of the protein in an Ehrlich ascites-cell homogenate as plasma membrane. Demus (1973) on the other hand harvested both the rapidly and the slowly sedimenting fragments from a human lymphocyte homogenate to obtain 1% of the total protein as plasma membrane.

To minimize the problem of selection it is therefore imperative to maximize the recovery of plasma membrane from the homogenate. In the experiments described in the present paper most of the plasma membrane from the tissue-culture cells studied sediments with the mitochondria; however, failure to process the nuclear pellet led to a variable loss of plasma membrane. This was overcome either by washing the pellet or, preferably, by gradient centrifugation of the pellet.

Since resuspension of any pelleted material requires the imposition of liquid shearing forces, it is advantageous to reduce these resuspensions to a minimum before any sedimentation or flotation rate separations, whose efficacy depends at least partly on particle size. For example, a simple three-layer gradient (method C) permitted the recovery of 78–90% of the total $\text{Na}^+ + \text{K}^+$ -stimulated ATPase activity from a 12ml 3T3-cell homogenate, whereas at least 27% of this enzyme activity was lost to

either the nuclear or microsomal pellets in a differential centrifugation scheme. Although most of the endoplasmic reticulum was also recovered with the plasma membrane, it was subsequently resolved in a sedimentation-rate gradient.

For large amounts of homogenate these simple systems are impracticable, and the combined methods (D+E+F) form a useful fractionation scheme. The requirement of two sedimentation steps (and consequently two resuspension steps) between methods D and E may lead to a further fragmentation of the plasma membrane, but it was observed that this approach improved the resolution of the Na^+ - K^+ -stimulated ATPase activity from the NADPH-cytochrome *c* reductase activity in gradient E. Thus, although washing may lead to the unpredictable distribution of plasma membrane between bands 3 and 4 of gradient E, the improved removal of endoplasmic reticulum outweighs this problem. Furthermore, if both bands are subsequently processed in an isopycnic gradient (F), the loss of Na^+ - K^+ -ATPase activity is minimized.

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