Tissue-Culture Cell Fractionation

ZONAL CENTRIFUGATION OF LETTRÉE CELLS HOMOGENIZED BY GLYCEROL-INDUCED LYSIS: SUBFRACTIONATION OF MEMBRANES IN SUCROSE GRADIENTS

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1. Lettrée cells were grown intraperitoneally in MF-1 mice. 2. Cells that were loaded with glycerol were swollen in 0.1 M-sucrose and disrupted by Dounce homogenization. 3. Early-passage Lettrée cells were more easily disrupted than late-passage cells by this method, and the former produced larger fragments of plasma membrane. 4. The membranes were fractionated initially in sucrose gradients (on the basis of sedimentation rate) in a BXIV zonal rotor. 5. Fractions from this gradient were further resolved in isopycnic sucrose gradients. 6. Plasma-membrane and endoplasmic-reticulum fractions were recovered in good yield and high purity.

Barber & Jamieson (1970) devised a method for homogenizing platelets that involved the uptake of glycerol by these cells from a 4.2 M solution followed by transfer of the glycerol-loaded cells to a relatively hypo-osmotic solution of 0.25 M-sucrose: the osmotic gradient generated across the plasma membrane caused the platelets to swell and burst. The present paper is concerned with the large-scale application of this method to Lettrée cells and the subsequent fractionation of the released membranes in a zonal rotor.

The separation of plasma-membrane fragments from either nuclear or mitochondrial fractions or from whole homogenates in zonal rotors has been previously reported by various workers. The large sheets of membrane from the lateral walls of liver parenchymal cells have been resolved from the nuclear fraction in low-speed A-type zonal rotors (Evans, 1970; El-Aaser *et al.*, 1966). The smaller fragments from rat hepatoma that have sedimentation rates lower than those of mitochondria were separated in a medium-speed HS rotor by Prospero & Hinton (1973). A similar approach has been made in the experiments described in the present paper for the fractionation of Lettrée-cell plasma membranes.

Materials and Methods

Cell culture and harvesting

Lettrée cells (second passage) were grown intraperitoneally in male MF-1 mice. Each passage was initiated by the injection of 0.1 ml of 5×10^6 – 10^7 cells; after 8–9 days the peritoneal fluid was harvested. The cells have a doubling time of approx. 24 h and the

Abbreviation used: ATPase, adenosine triphosphatase.

yield per mouse was 10^9 – 2×10^9 cells. Cells were kept in continuous culture for up to 13 passages. The cell suspension was diluted with 3 vol. of phosphate-buffered saline (0.12 M-NaCl/2.6 mM-KCl/8.1 mM- Na_2HPO_4 /1.5 mM- KH_2PO_4) and centrifuged at $500g_{av}$ for 5 min. Viability of the cells was checked by exclusion of Trypan Blue.

Homogenization

After two further washes in phosphate-buffered saline, the cells were suspended in 4.2 M-glycerol/5 mM-Tris buffer, pH 7.4 (3×10^9 – 4×10^9 cells/40 ml), and stirred at 4°C for 15 min. The cells were centrifuged at $1000g_{av}$ for 5 min and as much of the supernatant as possible was aspirated. Approx. 30 ml of 0.1 M-sucrose/0.5 mM-MgCl₂/0.5 mM-CaCl₂ buffered with 5 mM-Tris, pH 7.4, was added to the pellet, which was rapidly resuspended in the medium by vigorous vortex mixing. The cells were disrupted by Dounce homogenization until at least 95% of the cells had ruptured, as determined by phase-contrast microscopy.

Late-passage Lettrée cells are less easily disrupted than are early-passage cells: the latter usually require five gentle strokes of the pestle, whereas the former may require 15 vigorous strokes for complete disruption. Consequently the size of fragments produced from late-passage cells tends to be smaller than that of early-passage cells. Thus in the sedimentation-rate zonal gradient the banding position of plasma membrane will depend on the cell passage number.

Fractionation

The homogenate was initially fractionated on the

basis of sedimentation rate in a sucrose gradient contained within a BXIV zonal rotor. Stepped gradients were first introduced into the zonal rotor while it was spinning at 2000–2500 rev./min; the optimal design of the gradient varied with the cell passage number. All gradient solutions are expressed in terms of % (w/w). For passage-4 cells the following sucrose solutions were used: 50 ml of 20%, 80 ml of 30%, 100 ml of 40%, 150 ml of 50% and 150 ml of 55%; for passage-8 cells: 50 ml each of 10%, 20% and 30%, then 100 ml each of 35%, 40%, 45% and 50%; for passage-13 cells: 50 ml each of 10%, 20% and 30%, then 100 ml each of 35%, 45% and 50%. In all cases sufficient 60% sucrose was introduced to fill the rotor and all solutions contained 5 mM-Tris buffer, pH 7.4, 0.5 mM-MgCl₂ and 0.5 mM-CaCl₂. To smooth out the discontinuities in the gradient and to ensure the rotor contents were at 4°C, the rotor was centrifuged 20000 rev./min for 2 h before introduction of the sample. The rotor was decelerated to 2000–3000 rev./min; the homogenate was made 0.25 M with respect to sucrose and fed to the centre of the rotor, followed by 40 ml of 0.1 M-sucrose/0.5 mM-MgCl₂/0.5 mM-CaCl₂/5 mM-Tris buffer, pH 7.4, as overlay. The rotor was then centrifuged at 20000 rev./min for 45 min and unloaded at 2000–3000 rev./min by feeding 60% sucrose to the wall of the rotor; the 280 nm absorption of the effluent from the centre was measured by passage through a continuous-flow cell and it was

collected in approximately 15 ml fractions. Material in these fractions was harvested by centrifugation at 100000 *g*_{av} for 30 min after dilution with an equal volume of 5 mM-Tris buffer, pH 7.4.

The pelleted material was washed once in 0.25 M-sucrose/5 mM-Tris buffer, pH 7.4, and it was then either resuspended in 1–2 ml of this medium for compositional analysis or in 4 ml of 50% sucrose/5 mM-Tris buffer, pH 8.0, for subsequent refractionation in an isopycnic sucrose gradient formed from 5 ml each of 20%, 30%, 35% and 40% sucrose in 5 mM-Tris buffer, pH 8.0. After centrifugation for 16 h at 90000 *g*_{av}, the gradient was unloaded by upward displacement with 60% sucrose; the appropriate fractions were bulked together, diluted with 2 vol. of 5 mM-Tris buffer, pH 7.4, sedimented and resuspended in 1–2 ml of 0.25 M-sucrose/5 mM-Tris buffer, pH 7.4.

Compositional assays

For the methods used for determination of all enzyme activities and protein see the preceding paper (Graham & Sandall, 1979).

Chemicals

Metrizamide was purchased from Nyegaard and Co. A.S., Oslo 4, Norway. Radiochemicals and other materials were obtained as described in the preceding paper (Graham & Sandall, 1979).

Fig. 1. Fractionation of a homogenate of Lettée cells (passage 4) in a BXIV zonal rotor. For experimental details see the Materials and Methods section. —, A_{280} ; ○—○, density [% (w/w) sucrose].

Results

Sedimentation-rate zonal centrifugation

The distribution of subcellular material from passage-4, passage-8 and passage-13 Lettrée-cell

homogenates is shown in Figs. 1, 2 and 3 respectively. For convenience, the gradient (Fig. 1) was divided into six major fractions (A–F), the enzyme contents of which are shown in Table 1. In accordance with the ease of disruption of early-passage cells, most of

Fig. 2. Fractionation of a homogenate of Lettrée cells (passage 8) in a BXIV zonal rotor For experimental details see the Materials and Methods section. —, A_{280} ; ○—○, density (% sucrose).

Fig. 3. Fractionation of a homogenate of Lettrée cells (passage 13) in a BXIV zonal rotor For experimental details see the Materials and Methods section. —, A_{280} ; ○—○, density (% sucrose).

the plasma membrane was clearly in the form of large fragments, since the $\text{Na}^+ + \text{K}^+$ -stimulated ATPase was concentrated in fractions D and E. Essentially all of the endoplasmic-reticulum vesicles (NADPH-cytochrome *c* reductase activity) were contained in fraction A, and the mitochondria were primarily in fraction C.

If the 5'-nucleotidase is truly localized in the surface membrane of these cells then it appears to be associated particularly with a slowly sedimenting fraction quite distinct from that containing the $\text{Na}^+ + \text{K}^+$ -stimulated ATPase, in which the 5'-nucleotidase activity is relatively low. Although most of the 5'-nucleotidase activity occurred in fraction A, the high concentration of this enzyme in fraction B

suggests that at least part of the 5'-nucleotidase activity is associated with a slowly sedimenting fraction distinct from the endoplasmic reticulum.

In passage-8 cells (Fig. 2 and Table 2) the $\text{Na}^+ + \text{K}^+$ -stimulated ATPase is distributed broadly through the gradient, although there are two regions (B and E+F) where it is concentrated. As with passage-4 cells, there was considerable 5'-nucleotidase activity in the endoplasmic-reticulum fraction (band A); however, in addition its specific activity was relatively high in band E and also in a median fraction (C) that was relatively impoverished in $\text{Na}^+ + \text{K}^+$ -stimulated ATPase and NADPH-cytochrome *c* reductase activities and contained few mitochondria (predominantly in band D).

Table 1. Enzyme content of zonal fractions (see Fig. 1) from passage-4 Lettrée-cell homogenate
For experimental details see the text.

	Fraction ...	A	B	C	D	E	F	Homogenate
$\text{Na}^+ + \text{K}^+$-ATPase								
Recovery (%)		—	—	4.2	52.1	20.4	3.7	
Specific activity (μmol of ATP hydrolysed/h per mg of protein)		0.05	0.05	0.34	2.29	2.10	1.06	0.20
5'-Nucleotidase								
Recovery (%)		60.5	16.5	5.4	4.2	0.3	2.2	
Specific activity (μmol of AMP hydrolysed/h per mg of protein)		2.20	2.18	1.02	0.41	0.16	0.15	0.20
NADPH-cytochrome <i>c</i> reductase								
Recovery (%)		75.0	1.9	0.3	0.9	0.8	3.6	
Specific activity (μmol of cytochrome <i>c</i> reduced/h per mg of protein)		6.34	0.5	0.19	0.04	0.03	0.17	1.15
Succinate-cytochrome <i>c</i> reductase								
Recovery (%)		—	6.4	61.0	11.6	5.8	—	
Specific activity (μmol of cytochrome <i>c</i> reduced/h per mg of protein)		—	1.6	8.9	0.54	0.43	—	1.34

Table 2. Enzyme content of zonal fractions (see Fig. 2) from passage-8 Lettrée-cell homogenate
For experimental details see the text.

	Fraction ...	A	B	C	D	E	F	Homogenate
$\text{Na}^+ + \text{K}^+$-stimulated ATPase								
Recovery (%)		1.8	17.4	9.9	9.4	25.4	20.8	
Specific activity (μmol of ATP hydrolysed/h per mg of protein)		0.14	2.06	1.17	1.14	3.00	2.45	0.16
5'-Nucleotidase								
Recovery (%)		41.6	10.8	19.2	11.8	10.4	6.1	
Specific activity (μmol of AMP hydrolysed/h per mg of protein)		0.58	0.33	0.44	0.31	0.40	0.24	0.05
NADPH-cytochrome <i>c</i> reductase								
Recovery (%)		69.4	9.8	13.2	4.8	2.0	1.7	
Specific activity (μmol of cytochrome <i>c</i> reduced/h per mg of protein)		6.58	0.93	0.95	0.38	0.23	0.13	1.06
Succinate-cytochrome <i>c</i> reductase								
Recovery (%)		—	—	1.1	77.0	21.5	—	
Specific activity (μmol of cytochrome <i>c</i> reduced/h per mg of protein)		—	—	0.63	6.4	2.7	—	0.91

It should be noted that, although the fractions from each gradient are designated A–F, this does not imply an identity of density between homonymous fractions from different gradients.

With passage-13 cells (Table 3) no $\text{Na}^+ + \text{K}^+$ -stimulated ATPase activity was detectable beyond the mitochondrial band (D); instead it was concentrated entirely between the peaks of NADPH-cytochrome *c* reductase and succinate-cytochrome *c* reductase activities. The 5'-nucleotidase activity was also concentrated in this region of the gradient, but its maximum activity was still centripetal to that of the $\text{Na}^+ + \text{K}^+$ -stimulated ATPase.

Subfractionation

Fractions from the zonal gradient were further purified in isopycnic sucrose gradients. An illustration of the additional purification achieved by this procedure, the subfractionation of bands A, C and D from zonal-gradient fractions of passage-8 Lettrée cells, is presented here. The four subfractions (A_1 – A_4) resolved from band A (Table 4) demonstrate that it is possible to resolve the bulk of the NADPH-cytochrome *c* reductase activity from the 5'-nucleotidase activity. Band A_2 is particularly enriched in the latter enzyme, and band A_4 is particularly enriched in NADPH-cytochrome *c* reductase activity. It appears therefore that the 5'-nucleotidase activity associated with the zonal band A is at least partly due to a contaminating plasma-membrane fraction. The residual activity of this enzyme in band A_3 was further resolved from the NADPH-cytochrome *c* reductase activity by re-fractionation.

Band A_3 was harvested and resuspended in 0.5 ml of 25% sucrose/0.5 mM- MgCl_2 /5 mM-Tris buffer, pH 8.0, and placed atop a stepped gradient of 30%, 35%, 40%, 45% and 50% sucrose (0.7 ml of each, and

contained in 0.5 mM- MgCl_2 /5 mM-Tris buffer, pH 8.0). Centrifugation for 2 h at 200000 $g_{av.}$ produced two major fractions at the positions of the original 35%/40% and 40%/45% sucrose interfaces (A_3^1 and A_3^2 respectively) and a minor band above the 35% sucrose that was not analysed (Table 4). Bands A_3^1 and A_3^2 contained most of the 5'-nucleotidase and NADPH-cytochrome *c* reductase activities respectively.

In isopycnic sucrose gradients fractions C and D produced bands C_1 – C_3 and D_1 – D_3 (see Table 4). Band C_3 contained high NADPH-cytochrome *c* reductase activity and low 5'-nucleotidase activity; the density of this fraction suggests that it is rough endoplasmic reticulum.

Bands C_1 and C_2 are the major plasma-membrane-containing fractions; they both contain high 5'-nucleotidase activity, but band C_2 is heavily enriched in $\text{Na}^+ + \text{K}^+$ -stimulated ATPase activity. This is a further example of the dichotomy of these two enzymes in Lettrée-cell membranes.

The only minor contaminant in band C_2 was mitochondria; this fraction contained succinate-cytochrome *c* reductase activity only 7% of that of the major mitochondrial fraction D_3 . Fraction D also produced a band in the isopycnic sucrose gradient (D_1) that was chiefly plasma membrane. Band D_2 appeared to contain a mixture of mitochondria and plasma membrane (Table 4).

Discussion

The resistance of Lettrée cells to swelling and homogenization compared with the fibroblastic cells described in the preceding paper (Graham & Sandall, 1979) means that (a) a higher concentration of glycerol in the external medium is required for

Table 3. Enzyme content of zonal fractions (see Fig. 3) from passage-13 Lettrée-cell homogenate
For experimental details see the text.

Fraction ...	A	B	C	D	E	F	Homogenate
$\text{Na}^+ + \text{K}^+$ -stimulated ATPase							
Recovery (%)	9.4	63.7	20.0	—	—	—	
Specific activity (μmol of ATP hydrolysed/h per mg of protein)	1.32	7.97	2.10	—	—	—	0.30
5'-Nucleotidase							
Recovery (%)	38.2	30.4	21.7	—	—	—	
Specific activity (μmol of AMP hydrolysed/h per mg of protein)	0.66	0.50	0.28	—	—	—	0.06
NADPH-cytochrome <i>c</i> reductase							
Recovery (%)	75.1	10.2	5.2	4.0	—	—	
Specific activity (μmol of cytochrome <i>c</i> reduced/h per mg of protein)	7.94	2.1	0.9	1.6	—	—	0.84
Succinate-cytochrome <i>c</i> reductase							
Recovery (%)	—	—	28	57	10	—	
Specific activity (μmol of cytochrome <i>c</i> reduced/h per mg of protein)	—	—	1.9	7.9	2.3	—	1.62

Table 4. Subfractionation of zonal-gradient fractions from passage-8 Lettrée cells in isopycnic sucrose gradients
For experimental details see the text. N.D., Not determined.

Fraction	Density (approx. % sucrose)	5'-Nucleotidase (μmol of AMP hydrolysed/h per mg of protein)	Na ⁺ +K ⁺ -stimulated ATPase (μmol of ATP hydrolysed/h per mg of protein)	NADPH-cytochrome c reductase (μmol of cytochrome c reduced/h per mg of protein)	Succinate-cytochrome c reductase (μmol of cytochrome c reduced/h per mg of protein)
Sucrose gradients					
A ₁	31	0.84	N.D.	2.0	N.D.
A ₂	38	1.14	N.D.	3.0	N.D.
A ₃	42	0.63	N.D.	5.2	N.D.
A ₄	48	0.15	N.D.	13.3	N.D.
C ₁	32	0.78	0.58	—	—
C ₂	38	0.86	5.25	—	0.55
C ₃	43	0.34	—	2.32	—
D ₁	35	1.08	5.20	0.21	—
D ₂	40	0.34	—	—	6.2
D ₃	44	—	—	—	7.8
Sucrose/MgCl ₂ gradients					
A ₃ ¹	40	1.89	N.D.	0.4	N.D.
A ₃ ²	45	0.43	N.D.	11.8	N.D.

adequate glycerol uptake, (b) a lower concentration of sucrose is required to permit adequate swelling of the glycerol-loaded cells and (c) more severe Dounce disruption is required to rupture the cells. Interestingly, they become progressively more resistant to liquid shear forces with continuous passage. The reason for this alteration is not known, but it does appear to be paralleled by a significant decrease in 5'-nucleotidase activity (about threefold) that occurs at about passage 8. These changes appear to be correlated with a raised growth rate of the Lettrée cells that occurs between passages 6 and 10: the doubling time is shortened from 24h to 18–20h. Interestingly, changes in this enzyme have been observed during liver regeneration after partial hepatectomy: Arima *et al.* (1972) reported a marked decrease in activity, and Masuda *et al.* (1976) reported a transient increase that occurred when the rate of cell division had fallen to a minimum.

The bulk of the Na⁺+K⁺-stimulated ATPase activity is associated with rapidly sedimenting plasma-membrane fragments from passage-4 cells. Increasing liquid shear forces required to disrupt the later-passage cells results in the production of progressively smaller membrane fragments containing Na⁺+K⁺-stimulated ATPase. Thus this enzyme is distributed broadly through the gradient with passage-8 cells, indicating that the plasma membrane from such cells is broken down to fragments of a variety of sizes, whereas passage-13 cells produce exclusively small fragments (or vesicles) containing Na⁺+K⁺-stimulated ATPase. Although the distribution of this enzyme in the zonal gradient of passage-4 cells clearly points to the existence of large fragments of plasma membrane, the 5'-nucleotidase activity across the gradient indicates that this enzyme is associated predominantly with small membrane vesicles. This divergence in activities is most obvious with passage-4 cells, but also occurs in later-passage cells, although the situation, particularly with passage-8 cells, is considerably more complex. The disparity of the distribution of these two enzymes suggests that either the 5'-nucleotidase is not concentrated in the plasma membrane of these cells or it is associated primarily with regions of the plasma membrane that are predisposed to the formation of small fragments or vesicles during disruption.

There is evidence that supports both of these ideas: in rat liver this enzyme is not confined to the plasma membrane (Widnell & Unkeless, 1968; Widnell, 1972; Pletsch & Coffey, 1972), and it occurs at very low activities in the plasma membrane from rabbit liver (Lauter *et al.*, 1972) and rat adipose tissue (McKeel & Jarett, 1970). Values for relative specific activities often show that the enrichment of 5'-nucleotidase in a plasma membrane (over the homogenate) is 3–30-fold lower than that of the Na⁺+K⁺-stimulated ATPase (Bingham & Burke, 1972;

Gahmberg & Simons, 1970; Van Leeuwen *et al.*, 1976; Suzuki *et al.*, 1977), although the reverse situation was observed for human skin fibroblasts (Kartner *et al.*, 1977). Furthermore 'light' subfractions of plasma membrane tend to exhibit a preponderance of 5'-nucleotidase activity and 'heavy' subfractions tend to exhibit a preponderance of $\text{Na}^+ + \text{K}^+$ -stimulated ATPase activity (Evans, 1970; Graham, 1972; Suzuki *et al.*, 1977).

The wide separation of the $\text{Na}^+ + \text{K}^+$ -stimulated ATPase and 5'-nucleotidase activities from passage-4 Lettrée cells in the sedimentation-rate zonal gradient is clearly quite different from the relatively small differences in isopycnic banding densities reported by Evans (1970), Graham (1972) and Suzuki *et al.* (1977). Similar to the latter density discriminations, however, is the separation of 5'-nucleotidase and $\text{Na}^+ + \text{K}^+$ -stimulated ATPase activities in fractions A, B and C from the zonal gradient of passage-8 and passage-13 cells and in the subsequent isopycnic-sucrose-gradient subfractions of these zonal fractions. The resolution of zonal fraction A from passage-8 cells in an isopycnic sucrose gradient shows that the bulk of the NADPH-cytochrome *c* reductase activity can be resolved from the 5'-nucleotidase activity (Table 4).

A comparison of 5'-nucleotidase and $\text{Na}^+ + \text{K}^+$ -stimulated ATPase activities in zonal fractions A and B from both passage-8 and passage-13 cells shows the former enzyme to be associated predominantly with the lighter particles; however, a heavier fraction C contains both enzymes in almost equal activities. In the subsequent isopycnic sucrose-gradient centrifugation of fraction C the tendency for the 5'-nucleotidase/ $\text{Na}^+ + \text{K}^+$ -stimulated ATPase ratio to be high in the lighter fraction (C_1) and low in the heavier fraction (C_2) is maintained (Table 4). Although a third more-dense fraction (C_3) also contains 5'-nucleotidase activity, the high NADPH-cytochrome *c* reductase activity suggests that this fraction is predominantly endoplasmic reticulum. Whether this 5'-nucleotidase activity is indeed expressed by the endoplasmic reticulum or by co-sedimenting 5'-nucleotidase-rich plasma-membrane particles is not

clear. However, if band A_3^1 represents a particularly pure subfraction of 5'-nucleotidase-containing plasma membrane (the very low NADPH-cytochrome *c* reductase activity attests to this) then the 5'-nucleotidase activity in band A_3^2 , which contains a high NADPH-cytochrome *c* reductase activity (26-fold that in band A_3^1), suggests that this enzyme is a real component of this endoplasmic-reticulum subfraction.

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