Tissue-Culture Cell Fractionation

FRACTIONATION OF CELLULAR MEMBRANES FROM ^{125}I /LACTOPEROXIDASE-LABELLED LETTRÉE CELLS HOMOGENIZED BY BICARBONATE-INDUCED LYSIS: RESOLUTION OF MEMBRANES BY ZONAL CENTRIFUGATION AND IN SUCROSE AND METRIZAMIDE GRADIENTS

By John M. GRAHAM and Kathleen H. M. COFFEY
*Department of Biochemistry, St. George's Hospital Medical School,
Cranmer Terrace, London SW17 0RE, U.K.*

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1. Lettrée cells were grown intraperitoneally in MF-1 mice and labelled extrinsically by the ^{125}I /lactoperoxidase technique. 2. The cells were swollen in 1 mM- NaHCO_3 and disrupted in a Dounce homogenizer. 3. Crude fractions of endoplasmic reticulum, plasma membrane and mitochondria were separated from a post-nuclear supernatant by sedimentation-rate gradient centrifugation in a BXIV zonal rotor. 4. Further resolution of these membranes was carried out in isopycnic sucrose gradients. 5. Bands of material from the latter were subfractionated in gradients of metrizamide. Some very pure subfractions of plasma membrane and endoplasmic reticulum were obtained. In addition, one subfraction containing ^{125}I and NADPH-cytochrome *c* reductase but no Na^+ + K^+ -stimulated adenosine triphosphatase and another containing these two enzymes but no ^{125}I were resolved.

The use of enzyme markers for the plasma membrane largely arose from the work on rat liver. By inference, enzymes such as 5'-nucleotidase (Allan & Crumpton, 1972; Gahmberg & Simons, 1970; Bingham & Burke, 1972; Lelievre, 1973; Kornfeld & Siemers, 1974; Fletcher *et al.*, 1977; Laurent *et al.*, 1977) and Na^+ + K^+ -stimulated ATPase (Gahmberg & Simons, 1970; Graham, 1972, 1973; Lelievre, 1973; Graham *et al.*, 1975; Nigam & Brailovsky, 1977) have been used to identify plasma-membrane fractions from other cell types [see also the preceding papers (Graham & Sandall, 1979; Graham & Coffey, 1979)].

Although it is clear that these two enzymes do exist in the plasma membrane of various animal cells, there arise in membrane subfractionation a number of ambiguities that are difficult to resolve by use of these two marker enzymes alone. A plasma-membrane fraction containing 5'-nucleotidase but no Na^+ + K^+ -stimulated ATPase, for example (Vandenburgh, 1977), has been isolated from muscle cells; and, whereas rat and guinea-pig liver produced plasma membranes demonstrating a 16–19-fold enrichment of 5'-nucleotidase over the homogenate, no enrichment whatsoever was obtained with rabbit liver (Lauter *et al.*, 1972); and there are many examples of multiple fractions of plasma membrane displaying different enzymic functions (Graham,

1972; Lelievre & Paraf, 1973; Van Leeuwen *et al.*, 1976). We have also shown (see Graham & Coffey, 1979) that 5'-nucleotidase activities in Lettrée-cell plasma membrane fall markedly with continuous passage; assay of this enzyme activity in minor membrane fractions is thus difficult. For these reasons it is beneficial to include some 'extrinsic' marker for the plasma membrane.

One of the most widely used methods to label the plasma membrane extrinsically is the ^{125}I /lactoperoxidase technique (Phillips & Morrison, 1971; Hubbard & Cohn, 1972; Hynes & Humphries, 1974; Graham *et al.*, 1975; Shopsis & Sheinin, 1976).

Lettrée cells whose surfaces have been labelled by the ^{125}I /lactoperoxidase technique become very resistant to glycerol-induced swelling (see Graham & Coffey, 1979). The reason for this appears to be associated with the exposure of the cells for long periods to approx. 1 mM- Mg^{2+} and 1 mM- Ca^{2+} during the labelling procedure and during the previous washing steps. These ions are required to maintain the cells in an intact and viable state so that entry of the high-molecular-weight components of the iodination reaction (glucose oxidase and lactoperoxidase) is denied and thus the iodination is restricted to cell-surface structures. It was found that glycerol-induced swelling produced too variable a result for routine application to ^{125}I /lactoperoxidase-labelled Lettrée cells. For this reason it was decided to use 1 mM- NaHCO_3 in the presence of low

Abbreviation used: ATPase; adenosine triphosphatase.

concentrations (<0.5 mM) of bivalent cations to swell the cells before disruption by Dounce homogenization.

The size of the plasma-membrane fragments produced by this method were shown, in preliminary experiments, to sediment with, or slightly more slowly than, the mitochondria, and thus a zonal gradient system similar to that described by Prospero & Hinton (1973) was used. Subfractionation of the various membranes was carried out by isopycnic centrifugation in both sucrose and metrizamide gradients. Metrizamide is a relatively new gradient compound (Rickwood & Birnie, 1975): aqueous solutions of metrizamide, in comparison with sucrose solutions of similar densities, exhibit much lower viscosities and osmolarities. It is the huge decrease in the latter that makes metrizamide an important gradient medium: membrane vesicles that are osmotically active will behave very differently in these gradients compared with sucrose gradients. Although the plasma membrane may be in the form of relatively large fragments during the zonal centrifugation, subsequent manipulations (high-speed centrifugation, resuspension of pellets and hypo-osmotic washing) will lead to comminution of these fragments towards smaller vesicular structures.

Materials and Methods

Iodination and homogenization

Lettrée cells were grown and harvested as described in the preceding paper (Graham & Coffey, 1979). Approx. 3×10^9 cells were washed once in phosphate-buffered saline (0.12 M-NaCl/2.6 mM-KCl/8.1 mM- Na_2HPO_4 /1.5 mM- KH_2PO_4) (without bivalent cations) and once in phosphate-buffered saline (containing 1 mM- CaCl_2 , 1 mM- MgCl_2 and 5 mM-glucose), and then resuspended in 30 ml of this latter medium. To 0.1 ml of the suspension (10^7 cells) was added 0.4 ml of this medium, 400 μCi of carrier-free Na^{125}I and 10 μl of a solution containing 1 mg of lactoperoxidase/ml and 5 units of glucose oxidase/ml in phosphate-buffered saline (without bivalent cations or glucose). After 10 min at room temperature with occasional agitation, 10 ml of phosphate-buffered iodide (0.13 M-NaI/2.8 mM-KCl/8.4 mM- Na_2HPO_4 /1.5 mM- KH_2PO_4) (containing 1 mM- CaCl_2 and 1 mM- MgCl_2) was added to stop the reaction; the cells were sedimented at 500 g_{av} for 5 min, washed twice in this medium and once in phosphate-buffered saline, and then combined with the unlabelled cells and re-centrifuged at 1000 g_{av} for 5 min. After the supernatant had been aspirated totally the cell pellet was resuspended in 30 ml of 1 mM- NaHCO_3 /0.2 mM- MgCl_2 . The suspension was maintained at 4°C for about 5 min and then homogenized by ten strokes of the pestle in a tight-fitting Dounce homogenizer; complete cell disruption was

checked by phase-contrast microscopy. Then 2 M-sucrose/1 mM- NaHCO_3 /0.2 mM- MgCl_2 was added to the homogenate to make it 0.25 M with respect to sucrose. In experiments in which the cells were not labelled with ^{125}I , the preparation for homogenization was similar; the cells were washed once in phosphate-buffered saline and then twice in phosphate-buffered saline supplemented with MgCl_2 and CaCl_2 .

Sedimentation-rate zonal centrifugation

Nuclei were sedimented from the homogenate at 1000 g_{av} for 5 min. The total post-nuclear supernatant was then fractionated in a sedimentation-rate zonal gradient.

The following sucrose solutions were introduced into a BXIV rotor rotating at 2500 rev./min: 50 ml each of 15%, 20%, 25% and 30%, 100 ml of 35%, 50 ml of 40%, 100 ml of 50% and 200 ml of 55% (all containing 0.2 mM- MgCl_2 and 1 mM- NaHCO_3). All sucrose gradient solutions are expressed in terms of % (w/w). When the light end of the gradient emerged from the centre of the rotor, the flow was reversed and the sample plus 30 ml of 0.1 M-sucrose/1 mM- NaHCO_3 /0.2 mM- MgCl_2 overlay were introduced at the centre of the rotor. After centrifugation at 16000 g_{av} for 1 h, the gradient was unloaded by feeding 60% sucrose into the edge of the rotor; the effluent from the centre was passed through a continuous-flow cell for monitoring of 280 nm absorption and collected in 10 ml or 15 ml fractions. Membrane material was harvested from the gradient fractions by dilution of the latter with an equal volume of 5 mM-Tris buffer, pH 7.4, and centrifugation at 100000 g_{av} for 40 min.

Isopycnic sucrose gradients

For tube gradients in a 3×25 ml swing-out rotor the appropriate fractions were suspended in 4 ml of 30% sucrose/5 mM-Tris buffer, pH 8.0, and made part of a stepped gradient of 4 ml each of 25%, 35%, 40%, 45% and 50% sucrose, all in 5 mM-Tris buffer, pH 8.0. The gradients were centrifuged at 90000 g_{av} for 16 h and collected by upward displacement with 60% sucrose. Membrane material was harvested from gradient fractions as described above under 'Sedimentation-rate zonal centrifugation'.

Metrizamide gradients

Fractions were suspended in 0.7 ml of 10% (w/v) metrizamide/5 mM-Tris buffer, pH 8.0, and layered over 0.7 ml each of 20%, 25%, 30%, 35% and 40% (w/v) metrizamide, all in 5 mM-Tris buffer, pH 8.0. These gradients were centrifuged in a 6×4.2 ml swing-out rotor at 300000 g_{av} for 2 h. Well-separated bands were collected by using a flat-tipped metal cannula attached to a syringe. Fractions were diluted with

2 vol. of 5 mM-Tris buffer, pH 7.4, and sedimented at 100000g_{av}. for 30 min.

Compositional analysis

All sedimented membranous material was suspended in 1–2 ml of 0.25 M-sucrose/5 mM-Tris buffer, pH 7.4, before analysis. Enzyme assays etc. were carried out as described by Graham & Sandall (1979).

Chemicals

Carrier-free Na¹²⁵I was purchased from The Radiochemical Centre, Amersham, Bucks., U.K. All other radiochemicals and chemicals were obtained as described in the preceding papers (Graham & Sandall, 1979; Graham & Coffey, 1979).

Results

Sedimentation-rate zonal gradients

Fig. 1 shows the 280 nm absorption and enzyme profile of a post-nuclear fraction from unlabelled early-passage (3–8) Lettrée cells in a sedimentation-rate zonal gradient. The NADPH–cytochrome *c* reductase activity (endoplasmic reticulum) coincided with band B of the absorption trace; the Na⁺+K⁺-stimulated ATPase activity was largely located in the shoulder (C) centripetal to the band of mitochondria

(D). It was observed, however, that a significant amount of Na⁺+K⁺-stimulated ATPase activity sedimented in band B; some of this activity co-migrated with the NADPH–cytochrome *c* reductase activity, but some activity was definitely associated with a more slowly sedimenting fraction. Band A contained soluble protein and some light components whose identity was not investigated.

With late-passage (8–13) Lettrée cells the size of plasma-membrane fragments (as shown by the profile of Na⁺+K⁺-stimulated ATPase activity in Fig. 2) was considerably more heterogeneous than with early-passage cells. The leading edge of the band containing Na⁺+K⁺-stimulated ATPase activity tended to overlap the light mitochondria more significantly. This is not necessarily detrimental to the overall separation because the prime object of this gradient is to resolve the plasma membrane from the endoplasmic reticulum. Subsequent isopycnic gradient centrifugation can separate plasma membrane and mitochondria.

Sedimentation-rate zonal and isopycnic sucrose and metrizamide gradients

¹²⁵I/lactoperoxidase-labelled Lettrée cells were fractionated in the sedimentation-rate zonal gradient (see Fig. 1) and zones B, C and D were collected.

Fig. 1. Fractionation of an early-passage Lettrée-cell homogenate in a sedimentation-rate zonal gradient. For experimental details see the Materials and Methods section. —, A_{280} ; ●-●, density (% sucrose); □-□, NADPH–cytochrome *c* reductase activity; ▲-▲, Na⁺+K⁺-stimulated ATPase activity; ○-○, succinate–cytochrome *c* reductase activity.

Fig. 2. Fractionation of a late-passage Lettrée-cell homogenate in a sedimentation-rate zonal gradient. For experimental details see the Methods and Materials section. —, A_{280} ; ●—●, density [% (w/w) sucrose]; □—□, NADPH-cytochrome *c* reductase activity; ▲—▲, $\text{Na}^+ + \text{K}^+$ -stimulated ATPase activity; ○—○, succinate-cytochrome *c* reductase activity.

These were sedimented and washed twice in 0.25M-sucrose/5mM-Tris buffer, pH 8.0, and fractionated in an isopycnic sucrose gradient (pH 8.0). Zone B produced four major bands (B_1 – B_4) and zones C and D both produced five bands (C_1 – C_5 and D_1 – D_5), the topmost of which was a very minor fraction and not sampled (Tables 1 and 2). The trichloroacetic acid-precipitable ^{125}I radioactivity of these bands indicated that most of the plasma membrane occurs in bands C_2 and C_3 , and that plasma membrane in fraction D occurs in the equivalent bands D_2 and D_3 ; the ^{125}I radioactivity in these four bands together accounts for 68% of the total. Of the remainder 13% is in band C_4 and 10% in band B_3 . Band C_5 contained only 5% of the protein in gradient C but the ^{125}I radioactivity was higher than that in band C_4 on a specific-radioactivity basis and it contained the highest $\text{Na}^+ + \text{K}^+$ -stimulated ATPase specific activity of any fraction from the sucrose gradients (Tables 1 and 2). In the absence of glucose oxidase approx. 0.6% of the radioactivity observed with the complete system was incorporated into protein; in the absence of lactoperoxidase this value was 3.8%.

Sucrose gradients failed to achieve a reasonable

separation of the ^{125}I radioactivity and $\text{Na}^+ + \text{K}^+$ -stimulated ATPase activity from the NADPH-cytochrome *c* reductase activity from the microsomal fraction (B): although bands B_1 and B_2 were relatively enriched in $\text{Na}^+ + \text{K}^+$ -stimulated ATPase activity and ^{125}I radioactivity and band B_3 was relatively enriched in NADPH-cytochrome *c* reductase activity, the cross-contamination was significant (Tables 1 and 2).

Gradients of metrizamide (Table 3) achieved an improved resolution. Band C_4 contained ^{125}I radioactivity, $\text{Na}^+ + \text{K}^+$ -stimulated ATPase activity and succinate-cytochrome *c* reductase activity; the metrizamide gradient separated the last-mentioned enzyme activity, which occurred predominantly in band C_{4-1} (but also in bands C_{4-3} and C_{4-4}), from the $\text{Na}^+ + \text{K}^+$ -stimulated ATPase activity and ^{125}I radioactivity, which were restricted to band C_{4-5} . The identities of bands C_{4-2} , C_{4-3} and C_{4-4} , which together contained less than 10% of total gradient protein, have not been further studied. The fractionation of band C_3 is considerably more complex, inasmuch as the distributions of ^{125}I radioactivity and $\text{Na}^+ + \text{K}^+$ -stimulated ATPase activity are dissimilar. Although band C_{3-3} contains the highest

Table 1. Resolution of zonal-gradient fractions (B, C and D) in isopycnic sucrose gradients: total ^{125}I radioactivity and total enzyme activities

For experimental details see the text.

Fraction	^{125}I radioactivity (c.p.m./ml)	$\text{Na}^+ + \text{K}^+$ -stimulated ATPase activity (μmol of ATP hydrolysed/h per ml)	NADPH-cytochrome <i>c</i> reductase activity (μmol of cytochrome <i>c</i> reduced/h per ml)	Succinate-cytochrome <i>c</i> reductase activity (μmol of cytochrome <i>c</i> reduced/h per ml)
B ₁	37	<0.005	5.76	<0.05
B ₂	355	0.027	3.92	0.13
B ₃	344	0.018	4.22	0.13
B ₄	138	0.010	0.96	0.04
C ₂	1458	0.049	<0.05	0.90
C ₃	2664	0.028	<0.05	2.76
C ₄	821	0.024	<0.05	1.84
C ₅	181	0.031	<0.05	0.22
D ₂	405	0.021	<0.05	4.22
D ₃	515	0.010	<0.05	9.24
D ₄	149	<0.005	<0.05	5.02
D ₅	26	<0.005	<0.05	0.58

Table 2. Resolution of zonal-gradient fractions (B, C and D) in isopycnic sucrose gradients: ^{125}I specific radioactivity and enzyme specific activities

For experimental details see the text.

Fraction	^{125}I sp. radio- activity (c.p.m./ μg of protein)	$\text{Na}^+ + \text{K}^+$ -stimulated ATPase sp. activity (μmol of ATP hydrolysed/h per mg of protein)	NADPH-cytochrome <i>c</i> reductase sp. activity (μmol of cytochrome <i>c</i> reduced/h per mg of protein)	Succinate-cytochrome <i>c</i> reductase sp. activity (μmol of cytochrome <i>c</i> reduced/h per mg of protein)
B ₁	0.8	—	6.26	—
B ₂	2.1	0.15	1.14	0.04
B ₃	2.6	0.13	1.59	0.05
B ₄	3.2	0.25	1.14	0.05
C ₂	11.7	0.39	—	0.36
C ₃	12.3	0.13	—	0.68
C ₄	5.7	0.18	—	0.65
C ₅	6.1	1.00	—	0.08
D ₂	4.8	0.25	—	1.69
D ₃	2.9	0.06	—	3.04
D ₄	0.9	—	—	1.73
D ₅	0.4	—	—	0.04

concentration of ^{125}I radioactivity, no $\text{Na}^+ + \text{K}^+$ -stimulated ATPase was detected in this band. If the presence of ^{125}I radioactivity in the four bands C₃₋₁–C₃₋₄ identifies these fractions as plasma membrane, then some heterogeneity must exist in the plane of the membrane, since only bands C₃₋₁ and C₃₋₂ contain $\text{Na}^+ + \text{K}^+$ -stimulated ATPase activity. Mitochondria were not satisfactorily resolved by this gradient.

Subfractionation of bands B₁, B₂ and B₃ has also produced some interesting results. All bands contained NADPH-cytochrome *c* reductase activity (Table 3). Bands B₁₋₁ and B₁₋₃ were clearly very pure endoplasmic reticulum, since they did not contain

either of the plasma-membrane markers; moreover band B₁₋₃ exhibited the highest NADPH-cytochrome *c* reductase specific activity of any fraction. The identity of the other fractions is more problematical. Like band C₃₋₃, band B₂₋₂ contains a very high ^{125}I specific radioactivity, but, unlike band C₃₋₃, it also contains $\text{Na}^+ + \text{K}^+$ -stimulated ATPase and NADPH-cytochrome *c* reductase activities, both at significant values. The magnitude of the ^{125}I radioactivity indicates a surface origin for this fraction. Interestingly these fractions with high ^{125}I radioactivity, i.e. bands B₂₋₂, C₃₋₃ and C₄₋₅, are of a relatively high density compared with other fractions in the respective gradients, possibly indicating a high

Table 3. Resolution of selected isopycnic-sucrose-gradient fractions (see Tables 1 and 2) in metrizamide gradients: ^{125}I specific radioactivity and enzyme specific activities

For experimental details see the text. N.D., Not determined.

Fraction	^{125}I sp. radio- activity (c.p.m./ μg of protein)	$\text{Na}^+ + \text{K}^+$ -stimulated ATPase sp. activity (μmol of ATP hydrolysed/h per mg of protein)	NADPH-cytochrome <i>c</i> reductase sp. activity (μmol of cytochrome <i>c</i> reduced/h per mg of protein)	Succinate-cytochrome <i>c</i> reductase sp. activity (μmol of cytochrome <i>c</i> reduced/h per mg of protein)
B ₁₋₁	<0.02	<0.03	1.22	<0.01
B ₁₋₂	2.07	<0.03	2.25	<0.01
B ₁₋₃	<0.02	<0.03	9.33	<0.01
B ₂₋₁	<0.02	<0.03	1.44	<0.01
B ₂₋₂	16.5	0.12	2.11	<0.01
B ₃₋₁	<0.02	0.41	1.99	<0.01
B ₃₋₂	8.8	0.13	1.25	0.07
C ₃₋₁	6.2	0.41	N.D.	0.28
C ₃₋₂	5.4	0.32	N.D.	0.81
C ₃₋₃	16.8	<0.03	N.D.	0.16
C ₃₋₄	4.6	<0.03	N.D.	0.02
C ₄₋₁	<0.02	<0.03	N.D.	0.85
C ₄₋₂	<0.02	<0.03	N.D.	<0.01
C ₄₋₃	<0.02	<0.03	N.D.	0.08
C ₄₋₄	<0.02	<0.03	N.D.	0.06
C ₄₋₅	16.2	1.10	N.D.	<0.01

protein/lipid ratio. The existence of variations in the ^{125}I specific radioactivity of fractions that are clearly derived from the plasma membrane, e.g. bands B₃₋₂, C₃₋₁, C₃₋₂ and C₄₋₅ on the basis of two markers (^{125}I radioactivity and $\text{Na}^+ + \text{K}^+$ -stimulated ATPase activity), makes it difficult to designate fractions with lower ^{125}I specific radioactivity such as band B₁₋₂; it may be a plasma-membrane fraction containing NADPH-cytochrome *c* reductase activity or a mixture of endoplasmic reticulum and a plasma-membrane fraction lacking $\text{Na}^+ + \text{K}^+$ -stimulated ATPase activity. Only in fractions where there is a complete lack of ^{125}I radioactivity can this fraction be designated an internal origin with any certainty, e.g. bands B₁₋₁, B₁₋₃, B₂₋₁ and B₃₋₁. The last-mentioned fraction is particularly noteworthy since it represents an internal membrane bearing activities of both NADPH-cytochrome *c* reductase and $\text{Na}^+ + \text{K}^+$ -stimulated ATPase, the latter being an enzyme normally associated with the plasma membrane.

Discussion

The cell/homogenate volume ratio of $3 \times 10^9/30\text{ml}$ is optimal for disruption of Lettrée cells: increasing the cell number lowers the efficiency of disruption, and decreasing the cell number leads to damage to internal organelles. Under the optimal conditions most of the $\text{Na}^+ + \text{K}^+$ -stimulated ATPase activity sediments just behind the mitochondria in the rate-zonal step. A similar situation has been observed for rat hepatoma membranes (Prospero & Hinton, 1973). With late-passage cells (8–13) the size of the plasma-

membrane fragments is far more heterogeneous than with early-passage cells (1–8); this effect, in a more severe form, was also seen with cells homogenized by glycerol-induced lysis (see Graham & Coffey, 1979). It is indeed noticeable that the major endoplasmic-reticulum band from late-passage cells is also significantly broadened compared with that of early-passage cells, whereas the mitochondria tend to separate more clearly into a light and a heavy population. What is more noteworthy, however, is that three regions containing $\text{Na}^+ + \text{K}^+$ -stimulated ATPase activity, which can be discerned in the zonal-gradient fractionation of early-passage cells: the small peak at 100 ml, the shoulder at about 270 ml and the major peak at 330–350 ml (Fig. 1), are also present in the zonal-gradient fractionation of late-passage cells, although the relative amounts of material in the three regions are different. Thus the change at the cell surface that occurs during continuous passage of these cells does not merely produce an increased fragmentation of the plasma membrane, but appears to favour the production of specific fractions of plasma-membrane fragments. This is in contrast with the method of glycerol-induced lysis, in which the zonal-gradient distribution of $\text{Na}^+ + \text{K}^+$ -stimulated ATPase activity from early-passage cells does not resemble that of late-passage cells. It is also pertinent to point out that, although there is also a change in the distribution of the endoplasmic reticulum (NADPH-cytochrome *c* reductase activity) in the gradient between early-passage and late-passage cells, the tripartite nature of the band of NADPH-cytochrome *c* reductase activity is retained.

Fraction C from the zonal gradient produces four major fractions in the isopycnic sucrose gradient, all of which contain both protein-bound ^{125}I radioactivity and $\text{Na}^+ + \text{K}^+$ -stimulated ATPase activity. It is clear, however, that these two markers are not randomly distributed in the plasma membrane; some heterogeneity must exist to explain the existence of ^{125}I radioactivity/ $\text{Na}^+ + \text{K}^+$ -stimulated ATPase activity ratios that vary from 6.1 (band C_5) to 94.6 (band C_3). It is feasible that the low values for the $\text{Na}^+ + \text{K}^+$ -stimulated ATPase activity in bands C_3 and C_4 are due to the higher mitochondrial contamination of these two fractions compared with that of bands C_2 and C_5 : the higher Mg^{2+} -dependent ATPase activity may interfere with the assay of the $\text{Na}^+ + \text{K}^+$ -stimulated ATPase activity. However, previous experiments in which mitochondria were added to a plasma-membrane preparation showed that, so long as no more than 15% of the total ATP in the assay mixture was hydrolysed, accurate measurements could be obtained for the $\text{Na}^+ + \text{K}^+$ -stimulated ATPase activity both in the presence and in the absence of a basal Mg^{2+} -dependent ATPase enzyme activity. Furthermore, the subfractionation of band C_3 in a metrizamide gradient produces more substantial evidence for the existence of 'membrane microheterogeneity'. A comparison of the enzyme activities and ^{125}I radioactivities of bands C_{3-1} and C_{3-3} , for example, indicates that both fractions are relatively little contaminated by mitochondria (the succinate-cytochrome *c* reductase activities being only 9.2 and 5.2% of that of band D_3); both are derived predominantly from the surface membrane, as shown by their high ^{125}I radioactivities compared with those of other fractions such as bands B_{1-3} and C_{4-1} ; yet only band C_{3-1} contains a detectable amount of $\text{Na}^+ + \text{K}^+$ -stimulated ATPase activity. In this case the lack of this enzyme in band C_{3-3} cannot be accounted for by any interference from mitochondrial Mg^{2+} -dependent ATPase activity, since the mitochondrial contamination of this fraction is in fact lower than that of band C_{3-1} .

Identification of those fractions, e.g. zone B_{1-2} , containing both low ^{125}I radioactivity and low activity of an intracellular enzyme is difficult. It may be a real surface component or it may derive from the small number of cells that do not exclude the iodinating system. It is unlikely that internal membranes become labelled as a result of the internalization of the iodinating system by pinocytosis; although this process occurs significantly at 37°C , at room temperature it occurs at a negligible rate (P. Knox, personal communication).

The results emphasize the need for caution when enzyme markers are used to monitor a fractionation procedure, particularly if only one marker is used for one type of membrane. Without the ^{125}I -labelling, band C_{3-3} for instance might have been considered

as derived from the cytoplasmic membranes on the basis of a lack of $\text{Na}^+ + \text{K}^+$ -stimulated ATPase activity, yet the high ^{125}I specific radioactivity shows that this fraction certainly contains plasma membrane in high concentration. Partial or total segregation of some plasma-membrane markers has been widely reported (Graham, 1972; Van Leeuwen *et al.*, 1976; Kartner *et al.*, 1977; Monneron & d'Alayer, 1978; Vandenberg, 1977; Saccomani *et al.*, 1977).

These results support the idea that plasma membrane is indeed a functional mosaic. This concept has been proposed for the surface membranes of cells from a number of organized tissues, e.g. rat liver (Evans, 1976), kidney proximal tubule (Kenny & Booth, 1976) and intestinal epithelium (Michell *et al.*, 1976); it is becoming clear, however, that this surface functional specialization is not confined to cells that also have an obvious morphological specialization, e.g. a brush border. Although at least some protein molecules can move laterally through the lipid bilayer (Frey & Edidin, 1970), and indeed this movement may be necessary for protein molecules newly incorporated into the plasma membrane to take up a preferred location in the membrane, some restrictions must be imposed on certain membrane components to account for the subfractionation data. There is indeed abundant evidence that asymmetry exists in the distribution of both lipid and protein molecules both in the plane of the membrane and through the thickness of the membrane (for a recent review see Trams, 1977).

The 'functional mosaic' model is, furthermore, not restricted to the plasma membrane; the endoplasmic reticulum also demonstrates specialization (Beaufay *et al.*, 1974; Higgins, 1976; Nilsson & Dallner, 1977). The isolation of functionally distinct populations of vesicles from the endoplasmic reticulum may reflect the biosynthetic capabilities of these membranes. Proteins may be transferred to the surface membrane as single molecules and/or as part of a preformed membrane vesicle that fuses with the plasma membrane (Palade, 1975). If proteins and lipids are synthesized synchronously (Wilson & Fox, 1971), then cytoplasmic membranes bearing plasma-membrane determinants may be detectable during subfractionation of the endoplasmic reticulum. The light fraction described by Beaufay *et al.* (1974) may, for example, fall into this category. Subfraction B_{3-1} isolated in the present study may also represent a plasma-membrane precursor; the lack of any ^{125}I radioactivity identifies it as an internal membrane, but in addition to NADPH-cytochrome *c* reductase activity it also contains significant $\text{Na}^+ + \text{K}^+$ -stimulated ATPase activity.

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