Isolation and Characterization of Membranes from Normal and Transformed Tissue-Culture Cells

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Homogenates of baby-hamster kidney cells and rat embryo fibroblasts prepared by nitrogen cavitation contain a small population of slowly sedimenting mitochondria or mitochondrial fragments, which contaminate the microsomal fraction. This appears to limit the resolution of surface membrane and endoplasmic reticulum on magnesium-containing dextran gradients. The microsomal material and mitochondria can, however, be completely separated on a 10–60% (w/w) sucrose zonal gradient containing a 30% sucrose plateau. On magnesium-containing dextran gradients this mitochondria-free microsomal material can be resolved into at least two surface membrane fractions and at least two endoplasmic reticulum fractions. Comparison of polyoma virus-transformed and normal baby-hamster kidney cells reveals some interesting differences in their microsomal fractionation patterns and the characteristics of the $\text{Na}^+/\text{K}^+-\text{Mg}^{2+}$ adenosine triphosphatase of their surface membranes, in particular a tenfold lower K_m in the virus-transformed cells. The fractionation patterns of normal and spontaneously transformed rat embryo fibroblasts are also briefly discussed, particularly in relation to the significance of the observation that both the surface membrane and endoplasmic reticulum from these cells can be subfractionated.

Changes in composition (Buck *et al.*, 1970; Dijong *et al.*, 1971; Kryukova *et al.*, 1971; Perdue *et al.*, 1971a; Renkonen *et al.*, 1972), function (Hatanaka & Hanafusa, 1970; Peery *et al.*, 1971; Perdue *et al.*, 1971b) and structure (Inbar *et al.*, 1972a,b) of the surface membrane of tissue culture cells have been implicated in the phenomenon of 'transformation' of these cells by viruses. Central to much of this work is the preparation of a surface membrane fraction of well-established purity that is functionally intact. Clearly, every effort should be made to decrease the contamination of one membrane type by another; this is particularly important when making comparisons between the membranes from two different cell types. Variability in the characteristics of the contamination of the surface membrane preparation from the two types of cell could lead to anomalous results regarding any compositional and functional differences observed in the surface membrane from these cells.

Studies on the surface membranes from normal and virus-transformed tissue-culture cells have employed one of two general methods for the isolation of these membranes. One technique, developed by Warren *et al.* (1966), is a modification of the methods devised by Neville (1960) and Emmelot *et al.* (1964) for rat liver. The efficacy of the liver fractionation relies on the presence of desmosomes between the lateral surfaces of adjacent liver cells to maintain the membranes from these surfaces as whole sheets

during homogenization. Warren *et al.* (1966) used surface-hardening agents such as Zn^{2+} to stabilize the surface membrane of tissue-culture cells against fragmentation during homogenization. This method produces tissue-culture cell 'ghosts'. A Zn^{2+} -stabilizing technique was also used by Perdue & Sneider (1970). Cell 'ghosts' have also been obtained by hypotonic lysis of HeLa cells (Bosmann *et al.*, 1968). The disadvantages of using surface-hardening agents is the impairment of functional activity of the surface membrane, and the disadvantage of using hypotonic lysis is that rupture of mitochondria may occur.

The second technique was developed by Kamat & Wallach (1966) with Ehrlich ascites tumour cells. In this method the surface membrane and endoplasmic reticulum are converted into vesicles by nitrogen cavitation. The vesicles are ultimately fractionated on magnesium-containing ficoll or magnesium-containing dextran gradients. The latter were introduced by Avruch & Wallach (1971), who extended the method to adipocytes. Rat liver (Graham *et al.*, 1968) and baby-hamster kidney cells (Gahmberg & Simons, 1970; Makita & Seyama, 1971) have been successfully fractionated by using this method. The advantage of the Kamat & Wallach (1966) method is that surface membrane, endoplasmic reticulum and mitochondria can be recovered in functionally intact states. Some cross-contamination between surface membrane and endoplasmic reticulum has been reported by all

workers using this method: the low-density fraction from the magnesium-containing dextran (or ficoll) gradient normally contains 75–80% surface membrane, whereas the high-density fraction contains about 80–85% endoplasmic reticulum.

In the present paper we are concerned primarily with the fractionation of baby-hamster kidney cells and polyoma virus-transformed baby-hamster kidney cells by the Kamat & Wallach (1966) method. Modifications in the method have led to an improvement in the resolution of surface membrane and endoplasmic reticulum and also to subfractionation of both surface membrane and endoplasmic reticulum. This subfractionation has revealed some possible differences between the normal and polyoma virus-transformed cells. The behaviour of rat embryo fibroblasts and spontaneously transformed rat embryo fibroblasts in the modified system is also presented.

Part of this work was presented in a paper given to the Second International Zonal Symposium at the University of Surrey, Guildford, Surrey, U.K. in December 1971 (Graham, 1973).

Materials and Methods

Tissue culture

The following cell lines were used: (a) baby-hamster kidney 21-C13 cells; (b) re-cloned baby-hamster kidney 21-C13 cells transformed by polyoma virus; (c) primary cultures of rat embryo fibroblasts, before passage 25 (classified as 'normal'); (d) primary cultures of rat embryo fibroblasts, after passages 35–40 (classified as 'spontaneously transformed'); (e) a cloned line of spontaneously transformed rat embryo fibroblasts.

All tissue-culture cells were grown as a monolayer in Roux bottles (1 litre) containing 100ml of Eagle's minimal essential medium containing 10% (v/v) calf serum (inactivated by heating to 56°C for 30min), 2% (v/v) foetal calf serum, 10% (v/v) tryptose phosphate broth (Difco), 0.347% NaHCO₃, 0.01% streptomycin and 0.006% benzylpenicillin. Bottles were seeded at a cell concentration of 1.3×10^5 cells/ml and at confluence each bottle yielded approx. 4×10^7 cells.

Cell harvesting and homogenization

The cell monolayer from 30 Roux bottles was removed by scraping with a rubber 'policeman', into 0.01M-phosphate-buffered 0.15M-NaCl, pH 7.4 (about 5ml/bottle). All ensuing operations were done at 0–4°C. Cells were sedimented at 190g_{av.} for 15min. The pellet was resuspended in 40ml of 0.25M-sucrose – 0.2mM-Mg²⁺ – 5mM-tris – HCl buffer, pH 7.4, and centrifuged at 240g_{av.} for 15min. After two more washes in the sucrose–Mg²⁺–tris–HCl

medium the cells were suspended in 30ml of this medium. Homogenization was achieved by nitrogen cavitation. The stirred cell suspension was equilibrated with oxygen-free nitrogen at 5516kN/m² (800lb/in²) for 20min in a nitrogen pressure vessel (Artisan Industries Ltd., Waltham, Mass., U.S.A.), and the homogenate collected in a solution of EDTA such that the final concentration was 1mM. Material trapped within the foam caused by the abrupt pressure release was liberated by gently stirring the homogenate for 1–2min, in a large-volume beaker (approx. 500ml), the large surface area enabling rapid subsidence of the foam.

Isolation of microsomal material (microsomes) by differential centrifugation (method a)

Nuclei were removed from the homogenate by centrifugation at 2000g_{av.} for 5min. The pellet was washed once with 10ml of 0.25M-sucrose–5mM-tris–HCl–1mM-EDTA buffer, pH 7.4, and the two supernatants were combined and designated the 'post-nuclear supernatant'. Mitochondria were removed by centrifugation at either 15000g_{av.} for 15min or 22000g_{av.} for 15min (MSE 8 × 50ml angle-rotor). The mitochondrial pellet was washed once with 20ml of the sucrose–tris–EDTA medium and the two supernatants were bulked. Microsomes were sedimented from the combined supernatants at 80000g_{av.} for 90min (MSE 8 × 50ml angle-rotor).

Isolation of microsomal material (microsomes) by rate-zonal centrifugation (method b)

All zonal centrifugations were performed with an MSE BXIV titanium zonal rotor. Gradients were generated by using a Beckman model 131 gradient pump, the flow rate into the rotor never being greater than 20ml/min. The rotor was pre-cooled to 4°C, as were the sucrose solutions, which contained 5mM-tris–HCl–1mM-EDTA, pH 7.4. During loading of the gradient at 2500rev./min, however, minimal or no refrigeration of the rotor chamber was employed, to prevent condensation.

The zonal gradient was generated by loading the rotor with (a) 75ml of a linear 10–30% sucrose gradient, (b) 130ml of 30% sucrose and (c) 115ml of a linear 30–60% sucrose gradient. The remaining rotor volume was filled with 60% sucrose. Baby-hamster normal kidney cell or polyoma virus-transformed kidney cell post-nuclear supernatant (40ml) was then introduced at the core of the rotor and moved into the gravitational field by 100ml of 5% sucrose. All sucrose concentrations are given in % (w/w). After centrifugation at 80000g_{av.} for 90min, the rotor was decelerated to 2500rev./min and unloaded by pumping 60% sucrose to the wall of the rotor. The effluent from the core of the rotor was

passed through a variable-path-length flow-cell and its E_{280} monitored; 10ml fractions were collected. Those containing the microsomal band were pooled, diluted with an equal volume of 5mM-tris-HCl, pH7.4, and centrifuged at $80000g_{av}$ for 90min.

Resolution of microsomal material

The microsomal pellet obtained by either method (a) or method (b) was then treated by the method of Kamat & Wallach (1966) and Avruch & Wallach (1971). The microsomes were washed successively with 10mM-tris-HCl and 1mM-tris-HCl (both pH8.6), by using an MSE 10×10 ml angle-rotor at $100000g_{av}$ for 30min to recover the washed material. Finally, the microsomes were suspended in 2mM- Mg^{2+} -1mM-tris-HCl, pH8.6, at a concentration of 1-3mg of protein/ml and dialysed against this medium for 90min.

A sample (3ml) of the dialysed suspension was placed on top of a linear 5-25% (w/w) dextran (mol.wt. 40000) gradient (total volume 17.5ml), containing 2mM- Mg^{2+} -1mM-tris-HCl, pH8.6.

The gradients were then centrifuged at $80000g_{av}$ for 16h (MSE 3×22 ml swing-out rotor) at 2-5°C. The gradients were unloaded, by upward displacement, by introducing 30% (w/w) dextran to the bottom of the tube. To facilitate unloading the 30% dextran solution was made from dextran of mol.wt. 10000, which is less viscous than a solution of the dextran of higher mol.wt. Fractions of 0.5 or 1.0ml were collected.

Compositional assays

5'-Nucleotidase (EC 3.1.3.5), Na^+/K^+ -stimulated Mg^{2+} adenosine triphosphatase (EC 3.6.1.3) and NADH diaphorase (EC 1.6.99.3) were assayed as described by Avruch & Wallach (1971); glucose 6-phosphatase (EC 3.1.3.9) was assayed by the method of Hübscher & West (1965) and succinate-cytochrome *c* reductase (EC 1.3.99.1) was assayed by the method of Mackler *et al.* (1962). Protein was assayed by the method of Lowry *et al.* (1951) with dry bovine serum albumin as standard. If no more than 50μ l of the membrane suspension is sampled for protein assay, then neither sucrose nor dextran interferes significantly with the colour development.

Results

Fractionation of microsomes obtained by differential centrifugation on magnesium-dextran gradients

By using the differential rate centrifugation scheme to isolate microsomes from any of the four cell types (baby-hamster normal kidney cells or polyoma virus-transformed kidney cells, rat embryo normal fibroblasts or spontaneously transformed fibroblasts) we

obtained essentially similar results in the resolution of surface membrane and endoplasmic reticulum on magnesium-containing dextran gradients as those of Gahmberg & Simons (1970) and Makita & Seyama (1971). The protein concentration profile describing the distribution of baby-hamster kidney cell microsomal material within the gradient is shown in Fig. 1. Fig. 2(a) demonstrates that the highest specific activity of glucose 6-phosphatase and NADH diaphorase (endoplasmic reticulum markers) occur at the high-density end of the gradient (around $\rho = 1.090$). This high-density band contains essentially all of the endoplasmic reticulum within the gradient (Fig. 2b). The surface membrane enzyme markers, on the other hand, achieve their highest concentration in the low-density end of the gradient (Fig. 2a). There is moreover some subfractionation of the surface membrane: the highest specific activity of the Na^+/K^+ - Mg^{2+} adenosine triphosphatase occurs at $\rho = 1.033$, whereas that of the 5'-nucleotidase occurs at $\rho = 1.023$. The positions of these specific activity peaks do not, however, coincide with those of the low-density protein peaks (Fig. 1); much of the least-dense protein peak contains little or no enzyme activity. The total activity profiles of these two enzymes (Fig. 2b) demonstrate that most of the surface membrane material bands at higher densities,

Fig. 1. Protein concentration profile of baby-hamster kidney cell microsomal material fractionated on a 5-25% dextran gradient containing 2mM- $MgSO_4$.

The microsomes were isolated by method (a) and fractionated as described in the Materials and Methods section.

Fig. 2. Enzyme activity profiles of baby-hamster kidney cell microsomal material fractionated on a 5–25% dextran gradient containing 2mM-MgSO₄.

The microsomes were isolated by method (a) and fractionated as described in the Materials and Methods section. (a) Specific activity expressed in arbitrary units/h per mg of protein; (b) total activity expressed in arbitrary units. ■, 5'-Nucleotidase; □, Na⁺/K⁺-Mg²⁺ adenosine triphosphatase; ○, glucose 6-phosphatase; ●, NADH diaphorase.

Table 1. Enzyme composition of two baby-hamster kidney cell microsomal subfractions recovered from a 5–25% dextran gradient containing 2mM-MgSO₄ and of baby-hamster kidney cell mitochondria

The microsomes and mitochondria were prepared by differential centrifugation by method (a) and the microsomes were fractionated as described in the Materials and Methods section.

Membrane fraction	Na ⁺ /K ⁺ -Mg ²⁺ adenosine triphosphatase (μmol of ATP hydrolysed/h per mg of protein)	NADH diaphorase (μmol of NADH oxidized/h per mg of protein)	Succinate–cytochrome <i>c</i> reductase (μmol of cytochrome <i>c</i> reduced/h per mg of protein)
Low-density gradient band	2.6	63	0.005
High-density gradient band	0.3	197	0.010
Mitochondria	Not tested	Not tested	0.350

coincident with the endoplasmic reticulum. The preponderance of the latter, however, diminishes the degree of contamination of the endoplasmic reticulum fraction by surface membrane material to tolerable levels. If a low-density fraction ($\rho = 1.018$ – 1.035)

and a high-density fraction ($\rho = 1.080$ – 1.095) are compared, then the specific activities of Na⁺/K⁺-Mg²⁺ adenosine triphosphatase and NADH diaphorase in these two baby-hamster kidney cell microsomal subfractions is as shown in Table 1. The

degree of cross-contamination in each fraction is similar to that previously reported (Gahmberg & Simons, 1970; Makita & Seyama, 1971) and essentially identical results were obtained with polyoma virus-transformed kidney cells, rat embryo normal fibroblasts and spontaneously-transformed fibroblasts.

In all preparations, even those in which the mitochondria were removed at $22000g_{av}$ for 15 min, it was observed that the surface membrane and endoplasmic reticulum were contaminated with a very small but significant amount of succinate-cytochrome *c* reductase (Table 1). This appears to originate from a minor contamination of the total microsomes with mitochondria.

Isolation of microsomes by zonal centrifugation

Attempts to resolve the mitochondria and microsomes on linear sucrose gradients (10–60%, w/w) with either an MSE 3×22 ml swing-out rotor or an MSE 8×50 ml angle rotor were unsuccessful.

Fig. 3(a) shows the distribution of material from a baby-hamster kidney cell post-nuclear supernatant in the zonal gradient system described in method (b) of the Materials and Methods section. The mitochondria (band C) move completely through the 30% sucrose plateau whereas the microsomes (band B) do not. Succinate-cytochrome *c* reductase cannot be detected in the microsomal fraction and neither Na^+/K^+-Mg^{2+} adenosine triphosphatase nor glucose 6-phosphatase can be detected in the mitochondrial fraction. Band A contains soluble protein. If the 30% sucrose plateau is omitted from the gradient, overlap of the microsomes and mitochondria occurs. The single-plateau system is effective for the complete separation of mitochondria and microsomes from all four tissue-culture cell types. The only obvious difference consistently observed between the zonal fractionation patterns from transformed and untransformed cells was a decrease in the amount of mitochondrial material relative to the amount of microsomal material in the transformed cells. Fig. 3(b) demonstrates the distribution of subcellular components from a polyoma virus-transformed baby-hamster kidney cell post-nuclear supernatant. This relative impoverishment of the mitochondria from the transformed cell is even more noticeable when the fractionation patterns from rat embryo normal fibroblasts and spontaneously-transformed fibroblasts are compared (Figs. 4a and b).

Fractionation of microsomes from baby-hamster normal kidney cells and polyoma virus-transformed kidney cells obtained by zonal centrifugation

The succinate-cytochrome *c* reductase-free microsomes (band B, Fig. 3) from baby-hamster normal

kidney cells and polyoma virus-transformed kidney cells fractionate into four major bands and one minor band in dextran gradients containing $2mM-Mg^{2+}$. The densities of these four bands are given in Table 2. There is a clear overall similarity in the band patterns, and in different experiments a remarkable constancy exists in the actual positions of the bands from the microsomes of one particular cell type. Bands 1, 2, 3 and 5 possess identical densities in the two types of cell. The density of band 4 from polyoma virus-transformed kidney cells may be slightly higher than that from normal kidney cells, although the variability of the latter's position makes it difficult to be certain on this point. The amount of material in band 1 never accounted for more than 3% of the total. A similar band was observed by Kamat & Wallach (1966) and it has been suggested that it is derived from the Golgi membranes. Enzymic

Fig. 3. Fractionation of post-nuclear supernatants from baby-hamster normal kidney cells (a) and polyoma virus-transformed kidney cells (b) on a sucrose zonal gradient

The experimental details were as described in the Materials and Methods section. ■, E_{280} profile; ○, density profile in terms of concentration of sucrose (% w/w).

Fig. 4. Fractionation of post-nuclear supernatants from rat embryo normal fibroblasts (a) and spontaneously transformed fibroblasts (b) on a sucrose zonal gradient

The experimental details are described in the Materials and Methods section. ■, E_{280} ; ○, density profile in terms of concentration of sucrose (% w/w).

analysis, however, indicates that, at least in baby-hamster normal kidney cells and polyoma virus-transformed kidney cells under the operating conditions described, this band is probably derived from the surface membrane.

Enzyme and protein content of the dextran gradient bands from baby-hamster normal kidney cells and polyoma virus-transformed kidney cells

Tables 3 and 4 display the distribution of $\text{Na}^+/\text{K}^+-\text{Mg}^{2+}$ adenosine triphosphatase and NADH diaphorase in dextran gradient fractions from baby-hamster normal kidney cells and polyoma virus-transformed kidney cell microsomes respectively. Both the actual specific activities of the $\text{Na}^+/\text{K}^+-\text{Mg}^{2+}$ adenosine triphosphatase in individual bands and the distribution pattern of the enzyme within the gradient show good reproducibility between experiments with one particular cell type. However,

Table 2. Densities of baby-hamster normal kidney cell and polyoma virus-transformed kidney cell microsome fractions recovered from 5–25% dextran gradients containing 2mM- MgSO_4

The microsomes were isolated by method (b) and fractionated as described in the Materials and Methods section. The values are the means of six experiments with normal kidney cells and four experiments with polyoma virus-transformed kidney cells.

Band no.	Band densities (g/ml)	
	Normal cells	Polyoma virus-transformed cells
1	1.015 (± 0.001)	1.015 (± 0.001)
2	1.024 (± 0.001)	1.023 (± 0.001)
3	1.043 (± 0.002)	1.042 (± 0.002)
4	1.062 (± 0.003)	1.067 (± 0.002)
5	1.081 (± 0.001)	1.082 (± 0.002)

although the NADH diaphorase activity is confined mainly to bands 4 and 5, both the actual specific activities and the relative distribution of the enzyme in these bands show great variation between experiments. For this reason, a mean figure for the NADH diaphorase in each band would be rather meaningless: instead, the values from two experiments with baby-hamster normal kidney cells (A and B) and two experiments with polyoma virus-transformed kidney cells (C and D) are given. The discrepancies are perhaps most noticeable in band 5, and the variable state of aggregation of this band may be partially responsible.

In spite of the variability in the NADH diaphorase activities, clearly bands 1–3 are predominantly surface membrane and bands 4 and 5 predominantly endoplasmic reticulum. Only $\text{Na}^+/\text{K}^+-\text{Mg}^{2+}$ adenosine triphosphatase is detectable in bands 1 and 2 (Tables 3 and 4). In band 1 the amount of material is very small (about 5–10% of that in band 2) and it is difficult to give an accurate value for the specific activity of the adenosine triphosphatase in this band. Therefore in Tables 3 and 4 a range of activities is given for band 1 rather than a mean figure. With both normal kidney cells and polyoma virus-transformed kidney cells band 2 represents a particularly pure surface membrane fraction, NADH diaphorase activity being undetectable. Baby-hamster normal kidney cells yield a surface membrane fraction with a higher maximum specific activity of $\text{Na}^+/\text{K}^+-\text{Mg}^{2+}$ adenosine triphosphatase than do polyoma virus-transformed kidney cells. Also there is a shift in the distribution of the $\text{Na}^+/\text{K}^+-\text{Mg}^{2+}$ adenosine triphosphatase activity from polyoma virus-transformed kidney cells to higher densities compared with that

Table 3. *Enzyme composition of bands recovered from 5–25% dextran gradients containing 2mM-MgSO₄ with mitochondria-free microsomes from baby-hamster kidney cells*

The microsomes were isolated by method (b) and fractionated as described in the Materials and Methods section. The Na⁺/K⁺-Mg²⁺ adenosine triphosphatase values are the means of six experiments; the NADH diaphorase values for two experiments are shown for the reasons given in the text.

Band no.	Na ⁺ /K ⁺ -Mg ²⁺ adenosine triphosphatase (μmol of ATP hydrolysed/h per mg of protein)	NADH diaphorase (μmol of NADH oxidized/h per mg of protein)	
		Expt. A	Expt. B
1	1.0–3.0	<0.5	<0.5
2	9.2 (±1.8)	<0.5	<0.5
3	1.2 (±0.4)	5.0	15.0
4	0.2 (±0.1)	24.0	45.0
5	<0.05	15.0	80.0

Table 4. *Enzyme composition of bands recovered from 5–25% dextran gradients containing 2mM-MgSO₄ with mitochondria-free microsomes from baby-hamster polyoma virus-transformed kidney cells*

The microsomes were isolated by method (b) and fractionated as described in the Materials and Methods section. The Na⁺/K⁺-Mg²⁺ adenosine triphosphatase values are the means of four experiments; the NADH diaphorase values for two experiments are shown for reasons given in the text.

Band no.	Na ⁺ /K ⁺ -Mg ²⁺ adenosine triphosphatase (μmol of ATP hydrolysed/h per mg of protein)	NADH diaphorase (μmol of NADH oxidized/h per mg of protein)	
		Expt. C	Expt. D
1	0.1–0.3	<0.5	<0.5
2	1.9 (±0.5)	<0.5	5.0
3	3.1 (±0.7)	<0.5	5.0
4	<0.05	45.0	30.0
5	<0.05	56.0	18.0

from normal cells. The maximum specific activity of the Na⁺/K⁺-Mg²⁺ adenosine triphosphatase from baby-hamster normal kidney cells occurs in band 2, whereas that of polyoma virus-transformed kidney cells occurs in band 3. This trend is emphasized by the relative lack of this enzyme in band 1 from polyoma virus-transformed kidney cells.

Table 5 gives the protein content of each gradient band for both types of cell. On a protein basis polyoma virus-transformed kidney cells contain rather more endoplasmic reticulum and rather less surface membrane than do untransformed cells. Taking the sum of the protein contents of bands 2 and 3 as total surface membrane and the sum of the protein contents of bands 4 and 5 as total endoplasmic reticulum, then the surface membrane/endoplasmic reticulum protein weight ratio is 0.54 for baby-hamster normal kidney cells and 0.25 for polyoma virus-transformed kidney cells. The shift of

a major part of the endoplasmic reticulum material into band 5 with the polyoma virus-transformed cells compared with normal cells is also significantly different; the ratio of protein in band 4 to that in band 5 is 0.18 for polyoma virus-transformed cells and 0.78 for normal cells.

By using the values in Table 5, it is possible to derive the overall specific activity of the Na⁺/K⁺-Mg²⁺ adenosine triphosphatase in the total surface membrane from both types of cell. With baby-hamster normal kidney cells the average Na⁺/K⁺-Mg²⁺ adenosine triphosphatase specific activity is 4.5 μmol of ATP hydrolysed/h per mg of protein; for the polyoma virus-transformed kidney cells the value is 2.6. The total enzyme activity within the surface membrane fractions is 1500 μmol of ATP hydrolysed/h for baby-hamster normal kidney cells and 520 μmol of ATP hydrolysed/h for polyoma virus-transformed kidney cells.

Table 5. Protein content (μg) of major bands recovered from 5–25% dextran gradients containing 2 mM- MgSO_4 with mitochondria-free microsomes from baby-hamster normal kidney cells and polyoma virus-transformed kidney cells

The microsomes were isolated by method (b) and fractionated as described in the Materials and Methods section. The total protein within the gradient has been normalized to 1 mg for each experiment.

Band no.	Normal cells		Polyoma virus-transformed cells	
	Expt. A	Expt. B	Expt. C	Expt. D
2	125	161	83	76
3	204	214	120	123
4	286	296	115	135
5	400	330	680	665

Na⁺/K⁺-Mg²⁺ adenosine triphosphatase Lineweaver-Burk plots

The activity of the $\text{Na}^+/\text{K}^+-\text{Mg}^{2+}$ adenosine triphosphatase in the total surface membrane fraction from the two cell types was determined for substrate concentrations between 0.002 and 0.1 mM. Lineweaver-Burk plots for the enzyme from surface membrane fractions of baby-hamster normal kidney cells and polyoma virus-transformed kidney cells are shown in Figs. 5 and 6 respectively, and the K_m is 0.25 mM and 0.028 mM respectively. Thus, although the $\text{Na}^+/\text{K}^+-\text{Mg}^{2+}$ adenosine triphosphatase is present at a lower concentration in surface membrane fractions of polyoma virus-transformed cells, its affinity for ATP is markedly greater than is the normal kidney cell surface membrane enzyme.

Fractionation of microsomal material from rat embryo normal fibroblasts and spontaneously transformed fibroblasts

On magnesium-containing dextran gradients rat embryo normal fibroblast and spontaneously transformed fibroblast microsomes isolated from the sucrose zonal gradient system are resolved into four major bands and one minor band (Table 6 gives the densities of these bands). The overall similarity in the band pattern between the normal cells and spontaneously transformed fibroblasts and also between these two cell types and the baby-hamster kidney cell types (compare Table 2) is quite clear. Again the reproducibility in band positions for one cell type is remarkable. Bands 1 and 5 from the two rat embryo fibroblast types and the two baby-hamster kidney cell types are identical in positions: however, bands 2, 3 and 4 occur at a slightly higher density in the

Fig. 5. Lineweaver-Burk plot for $\text{Na}^+/\text{K}^+-\text{Mg}^{2+}$ adenosine triphosphatase of surface membrane from baby-hamster kidney cells

The incubation mixture contained 0.002–0.1 mM- $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, 1 mM- Mg^{2+} , 150 mM- NaCl , 30 mM- KCl , 20 mM-tris-HCl, pH 7.4, and membrane (20–30 μg of protein), in a total volume of 1 ml.

former. The only significant difference between the rat embryo normal fibroblast and the spontaneously transformed fibroblast is the position of band 3.

Analysis of the $\text{Na}^+/\text{K}^+-\text{Mg}^{2+}$ adenosine triphosphatase and NADH diaphorase within the gradients demonstrates that the fractionation patterns are essentially identical with those obtained with baby-hamster normal kidney cells and polyoma virus-transformed kidney cells. The enzyme content of the dextran gradient bands from microsomes of rat embryo spontaneously transformed fibroblasts are compared in Table 7. Bands 1–3 are derived mainly from the surface membrane and bands 4 and 5 from the endoplasmic reticulum. As with the baby-hamster kidney cells the amount of NADH diaphorase activity in bands 4 and 5 was very variable between experiments, although the relative concentration of this enzyme in band 4 was reproducible. The results for adenosine triphosphatase were, however, again subject to only slight variation between experiments. Therefore the NADH diaphorase activities shown in Table 7 have been taken from a single experiment: the $\text{Na}^+/\text{K}^+-\text{Mg}^{2+}$ adenosine triphosphatase results represent mean values from three experiments.

Table 8 describes the distribution of protein in the four major bands of rat embryo normal fibroblast

and spontaneously transformed fibroblast microsomes. As with the two baby-hamster kidney cell types, the transformed cell has a smaller surface membrane/endoplasmic reticulum protein weight ratio than the normal cell; the values are 0.43 and 0.83 respectively (taking bands 2+3 as total surface membrane and 4+5 as total endoplasmic reticulum). Another identical trend observed in the rat embryo fibroblast types is the shift of endoplasmic reticulum material from band 4 to band 5 in the transformed state. Indeed, the distribution of endoplasmic reticulum material between bands 4 and 5 is com-

pletely reversed in rat embryo spontaneously transformed fibroblasts compared with the normal fibroblasts. The protein weight ratio (band 4/band 5) in the normal cells is 3.7, whereas in spontaneously transformed fibroblasts it is 0.48. To accentuate this shift of the endoplasmic reticulum material to a higher density in spontaneously transformed cells, in three out of four experiments with these cells a sixth band containing NADH diaphorase activity was observed at $\rho = 1.098$.

The post-nuclear supernatant from the cloned spontaneously transformed cells behaved identically with the uncloned spontaneously transformed cells in the single-plateau sucrose zonal gradient. Further, the fractionation of the mitochondria-free microsomes from cloned rat embryo spontaneously transformed fibroblasts, in a magnesium-containing dextran gradient, produced surface membrane and endo-

Fig. 6. *Lineweaver-Burk plot for Na⁺/K⁺-Mg²⁺ adenosine triphosphatase of surface membrane from polyoma virus-transformed baby-hamster kidney cells*
The incubation mixture is described in Fig. 5.

Table 6. *Densities of rat embryo normal fibroblast and spontaneously transformed fibroblast microsome fractions recovered from 5-25% dextran gradients containing 2mM-MgSO₄*

The microsomes were isolated by method (b) and fractionated as described in the Materials and Methods section.

Band no.	Band densities (g/ml)	
	Normal cell microsomes	Spontaneously transformed cell microsomes
1	1.015 (±0.001)	1.015 (±0.001)
2	1.027 (±0.001)	1.027 (±0.001)
3	1.055 (±0.001)	1.048 (±0.002)
4	1.068 (±0.001)	1.067 (±0.001)
5	1.083 (±0.003)	1.081 (±0.002)

Table 7. *Enzyme composition of bands recovered from 5-25% dextran gradients containing 2mM-MgSO₄ with mitochondria-free microsomes from spontaneously transformed rat embryo fibroblasts*

The microsomes were isolated by method (b) and fractionated as described in the Materials and Methods section. The Na⁺/K⁺-Mg²⁺ adenosine triphosphatase values are the mean of three experiments; the NADH diaphorase values are taken from a single experiment for the reasons given in the text.

Band no.	Na ⁺ /K ⁺ -Mg ²⁺ adenosine triphosphatase (μmol of ATP hydrolysed/h per mg of protein)	NADH diaphorase (μmol of NADH oxidized/h per mg of protein)
1	1.0-2.0	<0.5
2	4.2 (±0.7)	<0.5
3	9.6 (±2.0)	9.0
4	0.8 (±0.2)	80.0
5	<0.05	10.0

Table 8. *Protein content (μg) of major bands recovered from 5–25% dextran gradients containing 2mM-MgSO₄ with mitochondria-free microsomes from rat embryo normal fibroblasts and spontaneously transformed fibroblasts*

The microsomes were isolated by method (b) and fractionated as described in the Materials and Methods section. The total protein within the gradient has been normalized to 1 mg for each experiment.

Band no.	Normal cells			Spontaneously transformed cells		
	Expt. A	Expt. B	Expt. C	Expt. D	Expt. E	Expt. F
2	174	101	112	93	93	109
3	331	310	333	205	188	214
4	384	430	476	216	239	222
5	109	158	77	486	479	454

plasmic reticulum bands of density and protein and enzymic content almost identical with those from uncloned spontaneously transformed fibroblasts.

Discussion

All four tissue-culture cell types (baby-hamster normal kidney cells and polyoma virus-transformed kidney cells, rat embryo normal fibroblasts and spontaneously transformed fibroblasts) used in these studies, when fractionated by the methods described in this paper, yield a small amount of succinate-cytochrome *c* reductase material that does not sediment at speeds which pellet most of the mitochondria. Only under centrifugal conditions that sediment some of the heavier microsomes (25000g_{av.} for 15 min) can this contaminating material be eliminated in a differential centrifugation scheme. The presence of this slowly sedimenting mitochondrial material is not surprising, for it is quite well established that the total mitochondrial fraction from any source is extremely heterogeneous. Using a sucrose zonal gradient, Swick *et al.* (1970) found that the mean particle diameter of mitochondria from mouse liver varied from 0.80 to 1.65 μm . Loeb & Kimberg (1970) found a broad range of sedimentation rates for rat liver mitochondria in linear sucrose gradients: most of the mitochondria were in a band around 1M-sucrose, but a small fraction remained near the top of the gradient (0.5M-sucrose) and others reached the bottom of the gradient (1.5M-sucrose). Work with ox heart mitochondria is frequently performed with a particular 'heavy fraction' (Harris *et al.*, 1969). There exists therefore much evidence for the presence, in any mitochondrial fraction, of organelles with a wide spectrum of sizes and sedimentation rates.

If the microsomal pellet obtained from a tissue-culture cell homogenate is contaminated by mitochondria, then the latter will probably rupture when the microsomes are exposed to the hypo-osmotic

tris buffers as part of the Kamat & Wallach (1966) technique. Henceforth, the microsomal fraction is going to be contaminated with mitochondrial membranes in some form (either as large sheets or vesicles or both). Interestingly, Gahmberg & Simons (1970) also reported that their endoplasmic reticulum fraction contained succinate dehydrogenase activity. These workers used 13 600g_{av.} for 15 min to sediment the mitochondria from an homogenate of baby-hamster kidney cells produced in a manner identical in all respects with that reported in this paper. Since, however, they gave no activity for this enzyme in the mitochondria, a quantitative assessment of the contamination of their endoplasmic reticulum fraction by mitochondria cannot be made.

In addition the soluble mitochondrial protein, which would be released from the mitochondria during lysis in the hypo-osmotic tris buffers, may become adsorbed on to the surface membrane and/or endoplasmic reticulum membranes. Since the separation of the surface membrane and endoplasmic reticulum vesicles on magnesium-containing dextran gradients relies on the differential titration of the fixed negative charges on the internal surfaces of the surface membrane and endoplasmic reticulum membranes by Mg²⁺ (Wallach & Kamat, 1964; Avruch & Wallach, 1971), any neutralization of the negative charge by ionic interactions with basic mitochondrial soluble protein(s) will modify the effect of the Mg²⁺, and may affect the separation of surface membrane and endoplasmic reticulum on these gradients. It is well established, for instance, that cytochrome *c* is able to interact ionically with negatively charged phospholipids (Das *et al.*, 1965).

Clearly, it is impossible satisfactorily to resolve the mitochondria and microsomes from these tissue-culture cell homogenates by differential centrifugation. Avruch & Wallach (1971), using adipocytes, employed a 27.6–54.1% (w/v) linear sucrose gradient to fractionate the homogenate into microsomes, mitochondria and nuclei. A small amount of succin-

ate-cytochrome *c* reductase activity was, however, still demonstrable in their microsomal fraction. The tissue-culture cells used in this study similarly demonstrated an incomplete resolution of microsomes and mitochondria on a linear sucrose gradient, with either a swing-out rotor or a zonal rotor. The large capacity of the latter indicates that the problem of complete separation was not due to overloading of the gradient. The single plateau system which we developed achieved as complete a separation of microsomes and mitochondria as possible. The system is effective for a number of tissue-culture cell types in addition to those described in this paper, e.g. NIL-cells, L-cells and 3T3-cells.

The presence of multiple surface membrane fractions, which possess different, well-defined densities, and different $\text{Na}^+/\text{K}^+-\text{Mg}^{2+}$ adenosine triphosphatase specific activities, raises some interesting possibilities.

(a) Both the rat embryo normal fibroblast and spontaneously transformed cell lines used in this study are primary cultures. It is feasible therefore that these tissue-culture lines, being derived from whole-rat embryos, contain heterogeneous 'cell types'. This cellular diversity could be responsible for the observed multiplicity of surface membrane and endoplasmic reticulum bands in the dextran gradients. The cloned and uncloned lines of spontaneously transformed rat embryo fibroblasts, however, show almost identical surface membrane/endoplasmic reticulum fractionation patterns. Since the cloned line should be homogeneous with respect to 'cell type', it seems unlikely that cellular heterogeneity is responsible for the presence of multiple surface membrane and endoplasmic reticulum fractions. Moreover, at least two surface membrane and two endoplasmic reticulum bands are also recovered in the dextran gradients from microsomes of baby-hamster normal kidney cells and polyoma virus-transformed kidney cells, both lines of which have been cloned.

(b) Another possibility is that the recovery of two (or three) surface membrane fractions possessing different $\text{Na}^+/\text{K}^+-\text{Mg}^{2+}$ adenosine triphosphatase activities reflects some changes which occur in the surface membrane during the cell cycle. Already there is some evidence that the rate of phospholipid incorporation into cellular membranes varies during the cell cycle of P815Y mouse mast cells (Bergeron *et al.*, 1970).

(c) Finally, it is feasible that the multiple surface membrane fractions reflect the presence of specialized regions of the surface membrane, which are functionally and structurally distinct.

Our observations on the recovery of multiple surface membrane fractions with different densities and functional activities would support, in the broadest sense, some macromolecular specialization. They suggest that there exist within the membrane some

oligomeric units or series of oligomeric units that are devoted predominantly to one particular enzymic function.

There is, however, an increasing body of evidence which suggests that the membrane is a rather fluid structure, rather than a strictly ordered one. Frye & Edidin (1970) found that mouse cell antigens were able to move rather freely through the plane of the membrane. Other workers (Glaser *et al.*, 1970; Steim *et al.*, 1969) have emphasized the relative independence of the majority of the lipid and protein in the membrane. Steim *et al.* (1969), using differential calorimetry, also stressed the importance of a fluid lipid bilayer in maintaining the viability of cells. Green (1971) proposed that the majority of membrane proteins were 'floating', either as single molecules, or as oligomers within the lipid. The supposition that much of the protein exists in oligomeric units has also been proposed by Wallach & Gordon (1968). If the predominant lipid-protein interaction is a hydrophobic one (Wallach & Gordon, 1968) then the presence of protein-protein and protein-lipid interactions overcomes the problem of reconciling this with the notion that most of the lipid is not bound to the protein (Steim *et al.*, 1969). An oligomeric structure would also be a necessary prerequisite for the maintenance of enzyme systems such as the electron-transport system.

The changes in the specific activity and K_m of the $\text{Na}^+/\text{K}^+-\text{Mg}^{2+}$ adenosine triphosphatase in the surface membrane from baby-hamster normal kidney cells and polyoma virus-transformed kidney cells demonstrate the need for caution in drawing conclusions regarding the effect of transformation on a particular enzyme from specific activity results alone. On their own, the specific activity results on the $\text{Na}^+/\text{K}^+-\text{Mg}^{2+}$ adenosine triphosphatase activity may indicate a depletion of this particular enzyme in the surface membrane of the transformed cell. The K_m results, however, suggest a more subtle alteration in the enzyme structure. This may be achieved by a molecular variation in the enzyme protein itself or by a change in the lipid associated with the enzyme. The activity of $\text{Na}^+/\text{K}^+-\text{Mg}^{2+}$ adenosine triphosphatase is influenced by the type of phospholipid associated with it (Emmelot & Bos, 1968; Tanaka & Sakamoto, 1969; Wheeler & Whittam, 1970) and it has been frequently proposed that the membranes of transformed cells contain a different pattern of phospholipids (Bergelson *et al.*, 1970) and glycolipids (Hakomori & Murakami, 1968; Mora *et al.*, 1969; Renkonen *et al.*, 1972) compared with those of untransformed cells. It would be particularly interesting to be able to reproduce, in the surface membrane of baby-hamster normal kidney cells, the glycolipid pattern demonstrated in the surface membrane of the polyoma virus-transformed kidney cells (Renkonen *et al.*, 1972) and to determine whether

this affected the characteristics of the $\text{Na}^+/\text{K}^+-\text{Mg}^{2+}$ adenosine triphosphatase activity.

Clearly, it will be necessary to examine several clones of normal and transformed cells before any firm conclusions can be reached concerning the differences in the enzymic activity in the surface membrane from these two cell types.

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