The Fluidity of Normal and Virus-Transformed Cell Plasma Membrane

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1. The phospholipid composition and cholesterol/phospholipid ratio of plasma membrane is the same in normal as in transformed BHK (baby-hamster kidney) cells; no significant difference in length or degree of unsaturation of the contributing acyl chains is apparent. 2. The turnover of acetate-labelled phosphatidylcholine species in the plasma membrane of normal and transformed BHK cells is the same. 3. Intramembranous particles of normal and transformed 3T3-cell plasma membrane are randomly distributed, whether at 4°C or at 37°C, in sparse or in dense cultures. There is no correlation between distribution of particles and the movement of concanavalin A receptor sites. 4. It is concluded that transformation of fibroblastic cells by oncogenic viruses does not lead to major changes in the lipid fluidity of the plasma membrane. 5. Details of the phospholipid composition of nuclei, mitochondria and endoplasmic reticulum in normal and transformed BHK cells have been deposited as Supplementary Publication SUP 50061 (5 pages) at the British Library Lending Division, Boston Spa, Wetherby, West Yorkshire LS23 7BQ, U.K., from whom copies may be obtained on the terms given in *Biochem. J.* (1976) 153, 5.

The concept of membrane fluidity has been introduced to explain, among other things, the lateral movement of particles within a membrane (Singer, 1971; Singer & Nicolson, 1972); the temperature-dependence of the phenomenon may be ascribed to the fact that lipid dispersions undergo a phase change from a rigid to a more fluid structure as the temperature is increased (Luzzati, 1968; Engelman, 1970). The realization that the observed difference in agglutinability of normal and transformed cells by lectins (Aub *et al.*, 1963; Burger, 1969; Inbar & Sachs, 1969) is temperature-dependent (Comoglio & Guglielmone, 1972; Inbar *et al.*, 1973; Nicolson, 1973; Rosenblith *et al.*, 1973) and that it involves lateral movement (Nicolson, 1972; Martinez-Palomo *et al.*, 1972) of receptor sites, has led to the proposal (Barnett *et al.*, 1974) that the fluidity of the plasma membrane of normal and transformed cells is different. Since fluidity has been related to the lipid composition (cholesterol/phospholipid ratio, length and degree of unsaturation of acyl side chains) (Chapman & Wallach, 1968), it was decided to make a comparative analysis of plasma membrane from normal and transformed cells.

Previous comparisons between the lipids of normal and transformed cells have been of whole cells (Cunningham, 1972; Diringer *et al.*, 1972) and minor differences owing to variation of plasma-membrane phospholipids would not have been detected. Other

comparisons have been between plasma membrane of normal liver and of various hepatomas grown *in vivo* (Van Hoesen & Emmelot, 1972, 1973); considerable differences between rat liver and hepatoma were noted, whereas differences between mouse liver and hepatomas were less pronounced. It was therefore pertinent to compare in detail the phospholipid composition of the plasma membrane of two closely related types of cell. In the present study, normal and virally transformed BHK (baby-hamster kidney) cells were used. Since the structure of the acyl chains of phospholipids appears to be determined largely by the activities of a deacylation/reacylation cycle (Hill & Lands, 1970; Numa & Yamashita, 1974), located partly in the plasma membrane itself (Ferber, 1971), the relative turnover of acyl-labelled phospholipids was also assessed.

Because we were unable to detect any significant differences in phospholipid composition or turnover, it seemed worthwhile to examine the distribution of intramembranous particles, since this has been implicated in membrane fluidity (Barnett *et al.*, 1974). No difference between normal and transformed fibroblasts was detected, nor was a correlation apparent between clustering of concanavalin A-receptor sites and the disposition of intramembranous particles.

Methods and Materials

Growth and fractionation of cells

BHK C13 cells and a polyoma-virus-transformed variant, BHK J1 (Graham, 1972), were grown on

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plastic Petri dishes in minimal essential medium (Eagle, 1959) containing 10% newborn-calf serum (Gibco Bio-Cult Ltd., Paisley, Scotland, U.K.). Cultures were re-plated every 2–3 days, so that both types of cell were growing exponentially during the experimental period.

For turnover studies, cells were exposed to sodium [^{14}C]acetate (1 $\mu\text{Ci/ml}$) for 4 days; at sub-culture, cells were transferred to medium containing sodium [^3H]acetate (10 $\mu\text{Ci/ml}$) and incubated for a further 8 h. Cells were harvested, washed in phosphate-buffered saline (Dulbecco & Vogt, 1954) and resuspended at approx. $2 \times 10^6/\text{ml}$ before subcellular fractionation. For labelled cells, this density was attained by addition of an excess of unlabelled cells.

Balb/c 3T3 and SV40-virus-transformed 3T3 cells (given by Mrs. M. Williams, Dunn School of Pathology, Oxford, U.K.) were grown on plastic Petri dishes (Sterilin) in minimal essential medium (Eagle, 1959) containing 10% foetal calf serum for 3T3 cells and 10% newborn-calf serum for SV40/3T3 cells.

Cells were disrupted by nitrogen cavitation and subcellular organelles isolated as previously described (Graham, 1973; Graham *et al.*, 1975).

Lipid extraction and analysis

Subcellular organelles were twice extracted with 20 vol. of chloroform/methanol (2:1, v/v); the lower layer obtained after addition of 0.2 vol. of 0.58% NaCl was washed (Folch *et al.*, 1957), evaporated to dryness, and redissolved in chloroform/methanol (2:1, v/v). Phospholipids were separated by two-dimensional chromatography on silica gel H (chloroform/methanol/7M- NH_3 , 46:18:3, by vol., followed by chloroform/methanol/water/acetic acid, 50:50:4:1, by vol.) and detected by radioautography or by exposure to iodine vapour. Selected areas were extracted and analysed by g.l.c. or, for labelled phosphatidylcholine, by argentation chromatography (see below).

Fatty acid methyl esters were prepared from individual phospholipid species by the method of Pfeiffer *et al.* (1968) and analysed in a Pye series 104 dual-column chromatograph equipped with H_2 flame detectors. Columns were packed with 15% poly(ethylene glycol succinate) on Diatomite S, and run at 186°C. Phospholipid phosphorus was determined by a modification of the method of Bartlett (1959); cholesterol was determined as described by Wycoff & Parsons (1957). Labelled phosphatidylcholine species were separated (without making apolar derivatives) on AgNO_3 -impregnated silica gel H, eluted and AgNO_3 was removed as described by Arvidson (1968). The dried residue was suspended in dioxan, containing 8 g of 2,5-diphenyloxazole and

20 g of naphthalene/litre, and radioactivity assayed in an LKB liquid-scintillation counter.

Electron microscopy

Cell cultures were fixed with 1% glutaraldehyde in phosphate-buffered saline for 30 min at either 4° or 37°C. In some experiments cells were first incubated for 10 min at 37°C with concanavalin A (100 $\mu\text{g/ml}$), rinsed in phosphate-buffered saline, incubated for 10 min with haemocyanin from *Busycon canaliculatum* (Smith & Revel, 1972) (500 $\mu\text{g/ml}$; generously given by Dr. M. J. Karnovsky, Department of Pathology, Harvard Medical School, Boston, MA, U.S.A.) and rinsed; cells were then incubated in medium for an additional 10–60 min before glutaraldehyde fixation.

For surface replicas, cells grown on glass cover-slips were fixed in OsO_4 , dehydrated in acetone and critical-point dried. After drying, a platinum/carbon replica was cast of the surface of the cells.

For freeze-fracture, cells were gradually impregnated with glycerol solutions (in phosphate-buffered saline) up to 30% (v/v) glycerol, removed from the culture dishes with a Teflon spatula and pelleted by centrifugation. Cells were mounted on gold-alloy specimen holders, frozen in freshly melted liquid Freon 22 and fractured at -110°C in a Denton freeze-fracture apparatus.

The platinum/carbon replicas were cleaned in bleach, washed in water and examined in an AEI 801 electron microscope. In freeze-fracture micrographs the encircled arrows indicate the direction of shadow. Fractures through the plasma membrane reveal A and B fracture faces: the A face belongs to the cytoplasmic membrane leaflet and faces the cell exterior; the complementary B face belongs to the external membrane leaflet and faces the cytoplasm.

The replicas shown in Plates 1 and 2 are typical of 50 or more cells examined.

Results and Discussion

Lipid composition

In the present paper results for plasma membrane are given. The corresponding data for nuclei, mitochondria and endoplasmic reticulum have been deposited with the British Library Lending Division as Supplementary Publication SUP 50061. No indication of 'randomization' of phospholipid classes between subcellular organelles of the kind reported by Bergelson *et al.* (1970) is apparent. There is no major difference in phospholipid distribution or acyl-chain content between normal and transformed BHK cells.

The distribution of phospholipid classes within the plasma membrane of normal cells is likewise similar to that of transformed cells; in each case phosphatidylcholine and phosphatidylethanolamine account for more than 60% of all phospholipids

Table 1. Composition of plasma-membrane phospholipids

Exponentially growing cells were harvested and fractionated and phospholipids extracted, purified and analysed as described in the Methods and Materials section. Values from two separate experiments are quoted. n.d., Not detectable. The product moment correlation coefficient (r) for % distribution of total phospholipids (column 1) between normal and transformed cells is 0.94. For acyl-chain content (columns 2-12) of normal and transformed cells, $r = 0.96$ (phosphatidylcholine), 0.99 (phosphatidylethanolamine), 0.99 (phosphatidylinositol) and 0.93 (sphingomyelin).

Phospholipids (% of total)	Phospholipid class	Acyl-chain content (% of total)											
		C _{14:0}	C _{16:0}	C _{16:1}	C _{18:0}	C _{18:1}	C _{18:2}	C _{20:0}	C _{20:4}	C _{22:0}	C _{22:1}	C _{22:4}	Other*
Normal cells													
54, 44	Phosphatidylcholine	2.4, 2.9	24.8, 28.0	3.4, 3.9	10.2, 14.1	41.2, 38.2	7.3, 5.5 n.d.,	0.5	1.8, 2.6	1.3, 1.0	2.0, n.d.	3.9, 2.3	1.7, 1.0
22, 24	Phosphatidylethanolamine	1.4, 5.7	9.4, 24.3	1.5, 4.3	23.8, 21.4	25.8, 30.6	6.9, 3.9 n.d.,	0.8	1.8, 1.2 n.d.,	1.5	1.9, 1.0	13.3, 3.3 n.d.,	1.5
13, 8	Phosphatidylinositol	1.3, 3.7	4.5, 14.9	1.4, 2.3	39.6, 37.1	28.7, 26.8	5.6, 4.7	1.7, n.d.	1.2, 1.4 n.d.,	1.1	2.1, n.d.	9.1, 3.1	4.9, 2.3
11, 23	Sphingomyelin	4.2, 8.7	61.7, 27.1	1.1, 5.2	9.3, 21.6	13.0, 21.1 n.d.,	4.4 n.d., n.d.		2.4, 1.9	1.8, 2.4 n.d.,	3.2	3.6, 0.6	0.9, 3.8
Transformed cells													
41, 41	Phosphatidylcholine	2.5, 2.3	29.5, 34.4	2.5, 4.1	13.3, 11.1	31.8, 33.1	9.9, 8.9 n.d., n.d.		2.2, 2.6	2.2, 0.7	0.8, 0.3	2.3, 1.7	3.0, 0.9
23, 22	Phosphatidylethanolamine	2.8, 2.7	16.0, 13.9	1.9, 1.9	25.6, 22.5	29.2, 39.2	6.4, 6.6 n.d.,	0.6	3.9, 3.6 n.d.,	0.4	1.1, 1.1	7.9, 5.5	5.1, 2.0
20, 21	Phosphatidylinositol	1.7, 0.9	11.7, 5.0	1.9, 1.1	33.8, 38.7	25.8, 39.0	3.6, 2.6 0.6, n.d.		2.5, 1.3	2.3, n.d.	0.8, 1.7	4.6, 6.5	10.6, 3.2
15, 16	Sphingomyelin	3.8, 3.4	40.5, 35.7	2.1, 2.7	12.7, 15.3	12.5, 23.1	3.3, 2.6 n.d., n.d.		6.5, 5.2	1.6, 3.2	2.6, 5.9	7.2, 1.3	7.3, 1.7

* Unidentified long-chain polyunsaturated fatty acids.

(Table 1). The increased concentration of phosphatidylinositol in the transformed cells may be compared with some of the density-dependent changes in total cellular phosphatidylinositol of normal and transformed NIL cells that have been reported on the basis of [¹⁴C]palmitate-incorporation data (Sakiyama *et al.*, 1972). The significance of such changes remains to be assessed. The acyl-chain distribution of individual phospholipid classes shows no significant difference between normal and transformed cells (Table 1). The distribution is similar to that of other intracellular organelles, and reflects to a large extent the distribution of fatty acids in the total extractable lipids of the calf serum in which the cells were grown (see Supplement SUP 50061), as noted previously (Bailey *et al.*, 1972).

In Table 2 possible cumulative differences in degree of unsaturation and chain length of constituent fatty acids are presented. No major difference between the plasma-membrane phospholipids of BHK and BHK J1 cells is apparent, except that the percentage of long-chain fatty acids in sphingomyelin could be higher in the transformed cells ($P = 0.15$). At best, however, this would result in a change of only 2% (on average) of the total phospholipids, and is therefore unlikely to affect the physical properties of the membrane greatly. In summary, transformation does not result in any major modification of length or degree of unsaturation of the acyl chains of any of the main phospholipids present in the plasma membrane. Plasmalogens, which are present in relatively minor amounts in plasma membrane, were not measured. Those values for normal and tumour-cell membranes that have been reported do not appear to correlate in any way with the growth rate or with tumorigenicity (Van Hoesen & Emmelot, 1973).

The cholesterol/phospholipid ratio in the plasma membrane is approximately the same in normal and transformed cells (0.80 mol/mol in normal cells as against 0.86 mol/mol in transformed cells in one experiment, and 1.2 as against 1.3 mol/mol in another; both sets of values are within the range quoted in the literature for normal cells). Van Hoesen & Emmelot (1972, 1973) have observed somewhat higher cholesterol/phospholipid ratios in the plasma membrane of rat and mouse hepatomas compared with normal liver. The conclusion of Inbar & Shinitzky (1974) and of Vlodansky & Sachs (1974), that an observed decrease in the cholesterol/phospholipid ratio of leukaemic lymphocytes, compared with normal lymphocytes, reflects a decreased ratio in the plasma membrane, is therefore not confirmed; of course, the intracellular distribution in fibroblasts and lymphocytes may be different. What is clear is that the altered behaviour of BHK cells on transformation (Stoker, 1972) cannot be ascribed to a decrease in the cholesterol/phospholipid ratio of the plasma membrane.

Table 2. *Length and degree of unsaturation of plasma-membrane phospholipids*

Mean values calculated from the data of Table 1 are re-expressed as indicated.

Phospholipid class	Mean chain length (no. of C atoms)	Long-chain acids (> C ₁₈) (% of total)	Unsaturated acids (% of total)	Polyunsaturated acids (> 2 double bonds) (% of total)
Normal cells				
Phosphatidylcholine	17.3	9	56	7
Phosphatidylethanolamine	16.5	13	49	11
Phosphatidylinositol	17.1	14	48	11
Sphingomyelin	16.6	10	31	9
Transformed cells				
Phosphatidylcholine	17.1	8	52	6
Phosphatidylethanolamine	17.1	15	58	13
Phosphatidylinositol	17.2	17	52	14
Sphingomyelin	16.9	21	42	15

Phospholipid turnover

Phospholipids turn over at distinct rates, dependent on the nature of the polar head group and of the acyl side chains. Turnover assessed by labelling of the polar head group (with choline, inositol etc.) generally measures complete degradation and resynthesis (Pasternak, 1973); labelling of the acyl chain measures reacylation, owing to operation of the Lands cycle (Hill & Lands, 1970; Numa & Yamashita, 1974), as well. Exposure to a long pulse of a radioisotopically labelled precursor, followed by a short pulse of the precursor labelled with a different radioisotope, allows an estimate of the relative rapidity of turnover of particular phospholipid species to be made (e.g. Pasternak & Bergeron, 1970). In acetate-labelled cells, plasma membrane has a slightly higher ratio of 'fast' to 'slow' species ($P < 0.05$), with the endoplasmic reticulum possibly also having a ratio greater than 1; this pattern is the same in normal and transformed cells (Table 3). The presence of phospholipid-exchange proteins (Dawson, 1973; Wirtz, 1974) in cultured cells (Pasternak, 1976) presumably limits any larger accumulation of recently synthesized phospholipids in plasma membrane or endoplasmic reticulum (Quinn & Pasternak, 1976).

Argentation chromatography (Arvidson, 1968) of intact phospholipids separates phosphatidylcholines into two broad categories (Quinn & Pasternak, 1976): a major one containing largely C_{16:0}, C_{18:0}, C_{18:1} and C_{18:2} species, and a minor band enriched with the highly unsaturated species (largely C_{20:4}, C_{22:4} and C_{22:6}). The major, more saturated species turn over relatively faster than the minor, highly unsaturated ones (Table 4; $P < 0.02$), in keeping with results of specific-activity measurements made by Arvidson (1968), by Sundler *et al.* (1972), and by Trewhella & Collins (1973*a,b*). It is noteworthy that normal and transformed cells show identical patterns. Since the operation of the Lands cycle (Hill & Lands,

Table 3. *Turnover of phosphatidylcholine in subcellular organelles*

Exponentially growing cells were exposed to a 4-day pulse of [¹⁴C]acetate, followed by an 8 h pulse of [³H]acetate. The cells were harvested, fractionated and phosphatidylcholine extracted as described in the Methods and Materials section. Of total ³H or ¹⁴C recovered from each lipid extract, 10–30% was in phosphatidylcholine, 10–30% in other phospholipids, and the remainder in neutral lipid. Ratios, which are expressed relative to a value of 1.00 assigned to unfractionated homogenate, are quoted for two or more separate experiments. The 95% confidence limit for possible differences between normal and transformed cells is 40% (plasma membrane), 30% (endoplasmic reticulum), 30% (mitochondria) and 30% (nucleus).

Organelle	³ H/ ¹⁴ C ratio	
	Normal cells	Transformed cells
Homogenate	1.00	1.00
Plasma membrane	1.43; 2.00; 1.58	1.52; 1.28
Endoplasmic reticulum	1.03; 1.41	1.33; 1.54
Mitochondria	0.91; 0.97	0.80; 0.92
Nucleus	0.99; 1.02; 1.00	0.95; 1.15

1970) involves mainly essential, i.e. serum-derived, fatty acids such as arachidonic acid (Numa & Yamashita, 1974), use of acetate as a labelling precursor gives only a limited measure of Lands-cycle activity. Nevertheless, it is clear from Tables 3 and 4 that whatever reacylation reactions of acetate-derived fatty acids take place in cultured fibroblasts, they are not significantly affected by viral transformation.

Disposition of intramembranous particles

Normal and transformed 3T3 cells, in sparse and in dense (confluent) cultures, were examined after

EXPLANATION OF PLATE I

Freeze-fracture micrographs of normal and transformed 3T3 cells

Replicas show fractures through the plasma membrane (A face) of 3T3 cells (*a, c*) and SV40/3T3 cells (*b, d*) at 37°C. In each case, intramembranous particles (approx. 8.5 nm diam.) are randomly distributed (*a, b*), with the exception of gap junctions (*c, d*) in which they are aggregated in close-packed clusters.

EXPLANATION OF PLATE 2

Micrographs of normal and transformed 3T3 cells after exposure to concanavalin A

Shadow-cast surface replicas (*a, b*) and freeze-fracture replicas (*c, d*) of 3T3 cells (*a, c*) and SV40/3T3 cells (*b, d*) after concanavalin A/haemocyanin treatment and incubation for 1 h at 37°C. In each case, concanavalin A-receptor sites are clustered (*a, b*; arrows), whereas intramembranous particles of the A fracture face remain randomly distributed under the same conditions (*c, d*).

Table 4. Turnover of sub-species of phosphatidylcholine

The intact phosphatidylcholines of Table 3 were separated by argentation chromatography into two bands as described in the Methods and Materials section. The major band contains mainly C_{16:0}, C_{18:0}, C_{18:1} and C_{18:2} species; the minor band contains in addition the long-chain polyunsaturated species. Ratios, which are expressed relative to a value of 1.00 assigned to phosphatidylcholine before argentation chromatography, are quoted for two separate experiments. The correlation coefficient (*r*) for the major and minor bands of plasma membrane and endoplasmic reticulum, between normal and transformed cells, is 0.98.

Organelle	³ H/ ¹⁴ C ratio	
	Major band	minor band
Normal cells		
Plasma membrane	1.18; 1.17	0.76; 0.75
Endoplasmic reticulum	1.08; 1.22	0.96; 0.76
Transformed cells		
Plasma membrane	1.20; 1.21	0.75; 0.82
Endoplasmic reticulum	1.16; 1.29	0.83; 0.82

fixation at 4° and 37°C. In every case, fractures through the plasma membrane reveal a random distribution of intramembranous particles, approximately 8.5 nm in diameter, on the A (Plates 1a and 1b) and B faces. The same is true of cells at different stages of the cell cycle (S. Knutton, unpublished work), in contrast with an earlier observation (Scott *et al.*, 1971). The few small aggregates of intramembranous particles that are seen under all conditions (except in very sparse cultures) in replicas of both normal and transformed cells can be identified as gap junctions by their location at the sites of cell contact and by their distinct morphology (Plates 1c and 1d). Such aggregates, which vary considerably in size, are more commonly seen in replicas of transformed cells than in those of normal cells; this probably reflects the increased area of cell/cell contact in cultures of transformed cells.

Incubation of cells with concanavalin A, followed by haemocyanin and preparation of surface replicas, allows one to see concanavalin A-receptor sites (Smith & Revel, 1972). With transformed cells, patches of different sizes are formed; with normal cells, few patches appear (Plates 2a and 2b), as previously noted (Rosenblith *et al.*, 1973). In neither case is any clustering of intramembranous particles apparent (Plates 2c and 2d).

Conclusions

It is clear that increased mobility of lectin-receptor sites on transformed cells (Inbar *et al.*, 1973; Nicolson, 1973; Rosenblith *et al.*, 1973) cannot be ascribed, at least in fibroblasts, to an alteration in the composition

or turnover of the plasma-membrane lipids (Tables 1–4). A similar conclusion has been reached on the basis of studies with spin-labelled fatty acids (Gaffney *et al.*, 1974; Gaffney, 1975). Such conclusions conflict with results on the fluidity of normal and transformed fibroblasts, obtained either with spin-labelled fatty acid (Barnett *et al.*, 1974) or with ¹H and ¹³C nuclear-magnetic-resonance spectra (Nicolau *et al.*, 1975), as well as with the observations of Shinitzky & Inbar (1974) on the microviscosity of normal and malignant lymphocytes assessed with a fluorescent hydrocarbon. The distribution of intramembranous particles detected by freeze-fracture electron microscopy is as random in normal- as in transformed-cell plasma membranes (Plates 1 and 2). The fact that this distribution is unaffected by clustering of concanavalin A receptor sites (Plate 2) is good reason for supposing that lateral mobility of receptor sites is controlled by factors other than membrane fluidity. The relation of such factors to actin-like filaments (Nicolson & Painter, 1973; Elgsaeter & Branton, 1974) and their involvement in the altered surface membrane of transformed cells is being investigated (see, e.g., Wickus *et al.*, 1975).

During preparation of this manuscript, a report has appeared (Pinto da Silva & Martinez-Palomo, 1975), that is in agreement with our observations on the distribution of intramembranous particles in normal and transformed cells.

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