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FLUORESCENCE POLARIZATION MEASUREMENTS ON NORMAL AND TUMOUR CELLS AND THEIR CORRESPONDING PLASMA MEMBRANES

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Summary

Using 1,6-diphenyl-1,3,5-hexatriene as a probe, the degree of fluorescence polarization (P) at 25°C of intact and disrupted cells and isolated plasma membranes were compared for a variety of systems.

1. Human erythrocytes, mouse thymocyte and leukemia cells, rat liver and hepatoma cells, and human and mouse milk fat globules displayed P values ranging from 0.300 to 0.120.

2. P values or probe labelling rates of intact and disrupted cells were similar.

3. As compared with whole or disrupted cells, the higher to much higher P values of plasma membranes isolated from the corresponding cells showed only a limited mutual variation.

4. ΔP values, being the difference in P values between plasma membranes and whole cells were attributed to the extent to which endomembranes and non-membrane lipids contributed. Among these, triglycerides had the greatest relative effect.

5. Though a particular isolation procedure for plasma membranes may select for more rigid fragments, this effect is by far not sufficient to account for the observed ΔP values.

It is concluded that the fluorescence polarization technique with a lipophilic probe applied to whole cells represents a measure of the average fluidity of all lipids being present in a cell and thus does not exclusively monitor the cell surface membrane.

Abbreviation: DPH, 1,6-diphenyl-1,3,5-hexatriene.

Introduction

Following the finding that proteins may show lateral displacement in the plane of the cell surface, the state of fluidity of the lipid matrix has received particular attention [1,2].

One recent method for studying the fluidity of membranes is the fluorescence polarization technique [3] which uses an apolar fluorophore, e.g. DPH, as a reporter molecule for the hydrocarbon regions of the lipid bilayer. This technique has previously [4] been applied to isolated plasma membranes and exfoliated cell-surface vesicles of mouse leukemia cells and thymocytes, and found to monitor exclusively the lipid domain of these membranes. The technique has also been used for whole cells, in particular leukemia and lymphoma cells [5], on the assumption that it would measure the fluidity of the cell surface only. However, as previous results have shown [4], P values obtained for whole cells are (much) lower than those for the corresponding isolated plasma membranes. These results were interpreted to mean that either the procedure used for plasma membrane isolation selects for the more rigid fragments of the disrupted cell surface, or that the fluorescence polarization technique also monitors endomembranes and, or cytosol lipoproteins and lipid droplets, if present.

The present experiments were designed to resolve this question using a variety of cell systems with divergent characters, and plasma membranes isolated therefrom. Although tumour and their homologous normal cells feature in this study, its main objective is to determine the significance of fluorescence polarization measurements with DPH on cells in general; nevertheless some conclusions from the physical and chemical comparison of tumour and normal plasma membranes are included.

Materials and Methods

(1) Cells and tissues; plasma membrane isolation. Human erythrocytes were obtained from blood bank supply. Cells were washed free from plasma and other blood cells in isotonic Tris buffer. Preparation of red cell ghosts was performed through the use of osmotic lysis according to Hanahan and Ekholm [6].

Thymocytes were isolated from baby GR/A mouse thymuses as described earlier [7]. Spontaneously arisen leukemias of thymus origin in GR/A mice were transplanted intraperitoneally in syngeneic mice. The resulting ascites tumour cells (GRSL cells) were harvested on day 4 or 7—8 after inoculation [8]. Plasma membranes from these cells were isolated as described before [7,8].

The presence of intracytoplasmic lipid droplets was determined with the light microscope on 1-μm sections of plastic embedded cells.

Rat liver cells (RLC) and Reuber H35 hepatoma cells [9] (strain H-4-II-E-c-3) were kindly provided by Dr. R. van Wijk, University of Utrecht. These cells and GRSL cells were grown in vitro in RPMI 1640 medium [10], supplemented with 10% fetal calf serum. Cells were used 7 and 3 days after subculturing, respectively. H35 cells (about $2 \cdot 10^7$) were also inoculated intra-

peritoneally in AXC rats. Plasma membranes from the resulting tumour were isolated, following standard procedures, after about 4 weeks of growth [11].

Novikoff ascites hepatoma cells [12] (strain N_1S_1-67, obtained through the courtesy of Dr. E.F. Walborg Jr., University of Texas, Houston) were maintained intraperitoneally in Sprague-Dawley rats and harvested on day 8. For isolation of plasma membranes, cells were washed three times in phosphate-buffered saline to remove erythrocytes and other cells in the ascites fluid. They were then swollen in hypotonic medium (10^{-3} M $NaHCO_3$, $2 \cdot 10^{-3}$ M $CaCl_2$) prior to homogenization in a Dounce-type homogenizer (20—30 strokes, 1400 rev./min until about 90% of the cells were broken, as inspected by phase contrast microscopy. Further processing of the homogenate was performed in the same way as described for other hepatomas [11].

Single-cell suspensions of Hepatoma-484A were obtained from solid intraperitoneal tumours in R/A rats by incubating the tissue (about 5 g) on the 7th day after grafting in hyaluronidase-collagenase, followed by pronase, as described by Wiepjes and Prop [13]. Single-cell suspensions of normal and regenerating rat liver hepatocytes were prepared as described earlier [14,15].

Fresh human milk (obtained from Slotervaart Hospital, Amsterdam) or milk from Balb/cxGR mice was diluted with 3 volumes medium (0.05 M Tris, 0.025 M KCl, 0.008 M $MgCl_2$, pH 7.6), and centrifuged 10 min at 4000 × *g* to separate the cream from somatic cells and skim milk. The cream was washed two more times, yielding intact milk fat globules. To isolate the membranes the globules were churned in 0.5 M sucrose in medium with a dispersing homogenizer (Ilado X10/20, Dottingen, W. Germany). After centrifugation at 4000 × *g* to remove the liberated milk fat (triglycerides) the membranes were recovered from the buttermilk after centrifugation in a discontinuous sucrose gradient during 90 min in a Beckman SW27-1 rotor at 100 000 × *g* at the interface *d* 1.16/1.21 (cf. refs. 16 and 17). The resulting preparation was washed with medium. Examination by electron microscopy showed a uniform homogeneous membrane preparation. Full details will be published elsewhere.

Rat-liver plasma membranes, enriched in sinusoidal fronts, were isolated according to Touster et al. [18]. Plasma membrane subfractions, enriched in bile canaliculi and contiguous phase, were isolated according to Wisher and Evans [19] (for nomenclature of obtained fractions, cf. ref. 20).

An 'endomembrane' preparation from rat liver was isolated from the low-speed supernatant of a standard liver plasma membrane isolation [11] in 10^{-3} M $NaHCO_3$ by precipitation at 105 000 × *g* during 1 h. Cytosol lipoprotein and part of the lipid dispersions of the original tissue will arrive in the resulting 105 000 × *g* supernatant. The bulk of the latter lipids was recovered as floating lipids after the first low-speed centrifugation step. The lipids in this fraction consisted of about 85% triglycerides and 10% cholesteryl esters.

(2) Fluorescence polarization measurements. Cells were washed with phosphate-buffered saline at least three times, to remove lipids from contaminating sources like serum, ascites fluid or growth medium. They were inspected for viability by trypan-blue exclusion. Not more than about 5% leaky cells were tolerated. Cells were disrupted by 0.5 min ultrasonic irradiation (Branson Sonifier B30 at 100 W output), nitrogen cavitation [7], standard homogenization [11] or churning. Cells (intact or disrupted), membranes or liposomes

(prepared from lipid dispersions by ultrasonics) were stained with DPH ($2 \cdot 10^{-6}$ M in phosphate-buffered saline) during 30 min at 37°C [4,21]. About 10^6 cells (or 200 μg membrane protein or 100 μg lipid) were used for measurement of the degree of fluorescence polarization P. Excitation was with polarized light (366 nm from a mercury arc). Readings were taken at 25°C in an Elscint MV-1a apparatus (Elscint Ltd., Haifa, Israel). This instrument displays $I_{\parallel}$ and $I_{\perp}$ and $P = (I_{\parallel} - I_{\perp})/(I_{\parallel} + I_{\perp})$. Total fluorescence was calculated using $F = I_{\parallel} + 2I_{\perp}$. Correction for light scatter was carried out by successive dilutions of the suspension to be measured with phosphate-buffered saline, until a plateau value of P was obtained [3].

In the case of erythrocytes the staining solution was of double concentration and the amplifier set at high sensitivity in order to supersede the quenching of the fluorescent light by hemoglobin.

Fluorescence polarization techniques can be practiced for the determination of fluidity parameters of lipid dispersions. Unlike fluorescence polarization decay measurements in which the complex motions of fluorophores in biomembranes can be resolved [34], steady-state measurements only yield weight averaged fluidity parameters. The degree of fluorescence polarization (P), estimated in this manner, relates to the 'apparent microviscosity' of the lipid domain through the Perrin equation [3,22]. In this equation the excited state lifetime and a parameter relating to the molecular shape of the fluorophore appear. However, for comparative purposes, simple presentation of P values can serve as a scale of fluidity. Theoretically, P values range from −0.333 to +0.500 [22]. In practice, using DPH for lipid bilayer probing, the range is from about 0.100 (highly fluid) to the limiting value 0.460 (highly viscous).

Results and Discussion

Fluorescence polarization values of intact and disrupted cells and isolated plasma membranes

The various cell systems studied are listed in Table I grouped according to their homology and roughly in order of decreasing P values obtained for intact cells. These values cover a wide range from $P = 0.301$ for human erythrocytes to $P = 0.120$ for milk fat globules.

The plasma membranes isolated from the corresponding cells showed much less mutual variation in their P values, which in all cases examined were higher or much higher than those of the intact cells. According to the difference in P values between plasma membranes and whole cells (ΔP in Table I) the cell systems studied could be divided into three groups consisting of erythrocytes, thymocytes and GRSL leukemia cells ($\Delta P = 0.030$—0.061); liver and hepatoma ($\Delta P = 0.099$—0.139); and milk fat globules ($\Delta P = 0.187$).

For representative examples of these three groups (see Table I) the P values of intact cells were compared with those measured after the cells had been disrupted prior to incubation with the DPH probe. The latter values were similar to those of intact cells. The same P values were also obtained, as demonstrated on GRSL cells, if prelabelled cells were disrupted (by nitrogen cavitation or sonic irradiation) and were either incubated with extra probe or not, prior to measurement. These results strongly favour the view that DPH labels

TABLE I

DEGREE OF FLUORESCENCE POLARIZATION (P) OF CELLS AND PLASMA MEMBRANES MEASURED AT 25°C AFTER LABELLING WITH 1,6-DIPHENYL-1,3,5-HEXATRIEN (DPH)

Data are expressed as mean ± S.D. with number of preparations in parentheses. ΔP is defined as the difference between P values of plasma membranes and intact cells.

Cell type	Intact cells	Disrupted cells	Plasma membranes	ΔP
Human erythrocytes	0.301 ± 0.002 (3)	0.298 ± 0.001 (2) [a]	0.332 ± 0.000 (2)	0.031
Mouse thymocytes, GR/A	0.269 ± 0.010 (5)	0.278 ± 0.001 (2) [b]	0.306 ± 0.006 (3)	0.037
GRSL cells, in vitro	0.232 ± 0.011 (5)	n.d.	0.287 ± 0.008 (5)	0.055
GRSL cells on GR/A (4 days old)	0.237 ± 0.012 (4)	n.d.	0.275 ± 0.009 (4)	0.038
GRSL cells on GR/A (7—8 days old)	0.208 ± 0.014 (9)	0.215 ± 0.003 (3) [b,e]	0.269 ± 0.015 (7)	0.061
RLC, in vitro line of rat liver cells	0.268 ± 0.000 (2)	0.278 ± 0.001 (2) [a]	n.d.	—
Reuber H35 hepatoma, in vitro line	0.263 ± 0.001 (2)	0.264 [a]	n.d.	—
Reuber H35 hepatoma on AXC (solid, 28 days old)	—	—	0.310	—
Novikoff ascites hepatoma on Sprague-Dawley (8 days old)	0.212	n.d.	0.351 ± 0.004 (4)	0.139
Novikoff hepatoma on Sprague-Dawley 70 passages later	0.250 ± 0.003 (3)	0.264 [a]	0.349	0.099
Novikoff hepatoma, on Sprague-Dawley again 20 passages later	0.190 ± 0.002 (2)	0.216 ± 0.013 (2) [a]	n.d.	(0.160)
Rat hepatoma 484A on R/A (solid, 8 days)	0.203 ± 0.017 (4)	n.d.	0.337 ± 0.013 (4)	0.134
Rat liver, R/A	—	0.183 [c]	0.322 ± 0.011 (11)	—
Rat hepatocytes, single cells, RxU	0.190 ± 0.004 (2)	n.d.	0.311 ± 0.007 (2)	0.121
Rat hepatocytes, single cells, from 24 h regenerating liver RxU	0.165 ± 0.007 (2)	n.d.	0.288 ± 0.010 (2)	0.123
Human milk fat globules	0.123 ± 0.003 (4)	0.132 ± 0.005 (3) [d]	0.310	0.187
Mouse milk fat globules, Balb/cxGR	0.120	0.115 ± 0.004 (3) [a,d]	n.d.	—

[a] Disrupted by ultrasonic irradiation, prior to DPH incubation.
[b] Disrupted by nitrogen cavitation, prior to DPH incubation.
[c] Disrupted by tissue homogenization, prior to DPH incubation.
[d] Disrupted by churning, prior to DPH incubation.
[e] Similar results were obtained if DPH-prelabelled cells were disrupted by either nitrogen cavitation or ultrasonic irradiation, and the disrupted cells received another incubation with DPH or not.
n.d., not determined.

all the lipid components of cells and that the fluorescence signal emitted from intracellular sites may equally well reach the detection device whether the cells are intact or broken. This conclusion is substantiated by the results of Fig. 1 in which the rate of DPH labelling of intact and disrupted GRSL cells at 25°C, as measured by total fluorescence, is compared.

Fig. 1 also illustrates the P values of intact and disrupted GRSL cells as a function of time of incubation with DPH at 25°C. The P value of disrupted cells remained constant from the earliest measurement on. However, intact cells consistently displayed a somewhat higher P value immediately after addition of

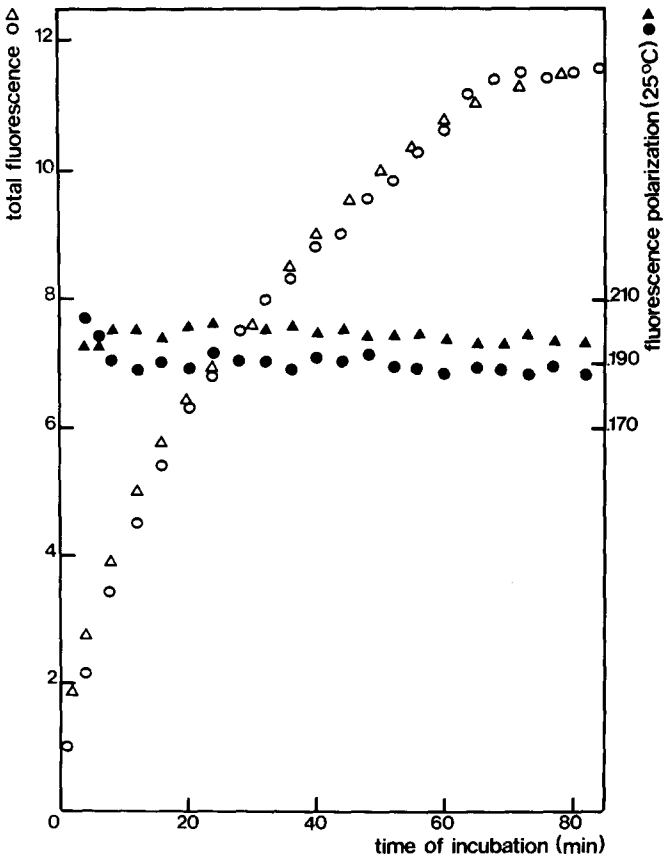

Fig. 1. Rate of DPH labelling of intact GRSL cells (○) and disrupted GRSL cells (△) as illustrated by the increase of total fluorescence as a function of incubation period at 25°C. Time course of degree of fluorescence polarization of intact GRSL cells (●) and disrupted GRSL cells (▲) upon DPH labelling ($2 \cdot 10^{-6}$ M) at 25°C. The final values for intact and disrupted cells are not equal because no correction for (constant) light scatter was employed.

the probe, followed by a rapid decline to the plateau value exhibited by the disrupted cells. A similar effect was obtained with Novikoff ascites hepatoma cells and interpreted to mean that it takes only a short time for DPH to enter into cells and label intracellular lipids so that the relative contribution of the cell surface to the recorded *P* value quickly declines to the mean value of all lipid pools present.

Effect of intracellular membranes and non-membrane lipids on the P value of cells

Lower or much lower *P* values are exhibited by intracellular membranes and non-membrane lipids, especially triglycerides, than by plasma membranes (Table II, lower part).

Erythrocytes, thymocytes and GRSL cells. Erythrocytes lack endomembranes, whereas thymocytes and GRSL cells show a high nucleus-cytoplasm ratio leaving only a small volume of cytoplasm. This (relative) paucity of intra-

TABLE II

DEGREE OF FLUORESCENCE POLARIZATION (P) OF PLASMA MEMBRANES AND FRACTIONS, AS MEASURED WITH 1,6-DIPHENYL-1,3,5-HEXATRIENE

Data are expressed as mean ± S.D. with number of preparations in parentheses.

Preparation	P (25°C)
GRSL	
Plasma membranes	
from mitochondrial pellet	0.278 ± 0.011 (4)
from microsomal pellet	0.261 ± 0.014 (3)
Rat liver	
Plasma membranes	
(1) standard preparation	0.322 ± 0.011 (11)
(2) sinusoidal fronts	0.258 ± 0.013 (2)
(3) bile canaliculi	0.304
(4) contiguous phase plus junctions	0.329
(5) single preparation, washed with 10^{-3} M $NaHCO_3$ and spun at low speed	0.318
(6) as 5 but spun at high speed	0.322
(7) same preparation, washed with 0.9% NaCl (1 h) and spun at low speed	0.317
(8) as 7 but at high speed	0.315
Endomembranes [a]	0.208 ± 0.010 (4)
Floating lipids [b]	0.133 ± 0.013 (2)
Cytosol "lipoprotein" [c]	0.173 ± 0.000 (2)
Synthetic triolein	0.117
Milk fat globule triglyceride	0.120

a Consisting of all cellular membranes (except for nuclei and the bulk of plasma membranes) precipitable between 1500 × g and 105 000 × g in 10^{-3} M $NaHCO_3$.

b Lipids floating on top of liver homogenate after 15 min 1500 × g; twice washed. Composition approx. 85% triglycerides and approx. 10% cholesteryl esters.

c 105 000 × g supernatant minus top and bottom layers.

cellular membranes is compatible with the relatively high P and small ΔP values exhibited by these systems. The unexpected ΔP value for erythrocytes has been suggested to be caused by the tensile force present in intact cells but lacking in the isolated membranes [23].

Of interest with respect to the contribution of intracellular non-membrane lipid to the P value of intact cells is the comparative experiment carried out with GRSL cells harvested at day 4 and 7—8 after transplantation (Table I). The 7—8-day-old cells had mainly reached the resting phase and in 45% of these cells lipid droplets (triglycerides) were observed in the sections, whereas the 4-day-old cells were in the growth phase and in less than 5% of the cells lipid droplets were detected. Isolated plasma membranes in the two cases showed similar P values. However, the 7—8-day-old cells showed a smaller P (0.208) and a higher ΔP (0.061) value than did the 4-day-old cells (P = 0.237, ΔP = 0.038). These differences are attributed to the accumulation of the lipid droplets in the former cells (cf. ref. 24).

Milk fat globules. The very opposite behaviour to that of the afore-mentioned cell systems is displayed by the milk fat globules (Table I, ΔP = 0.187) which consists for 2% by weight of membranes and for about 97% of triglycerides [25]. Their low P value is apparently due to these triglycerides

because effective removal leads to membranes with a P value characteristic of plasma membranes in general.

An experimental analogon of the disrupted milk fat globule was constructed by loading liposomes, consisting of phosphatidylcholine and cholesterol in a 1 : 1 molar ratio, with increasing amounts of triolein (0—3.3 molar to the former lipids). As shown in Fig. 2 incorporation of triolein led to a decrease of the P values of the liposomes, with smaller amounts of triglyceride having a relatively greater effect on the decrease in P value than had larger amounts. Plotting the molar ratio of cholesterol to total fatty acyl groups present in the

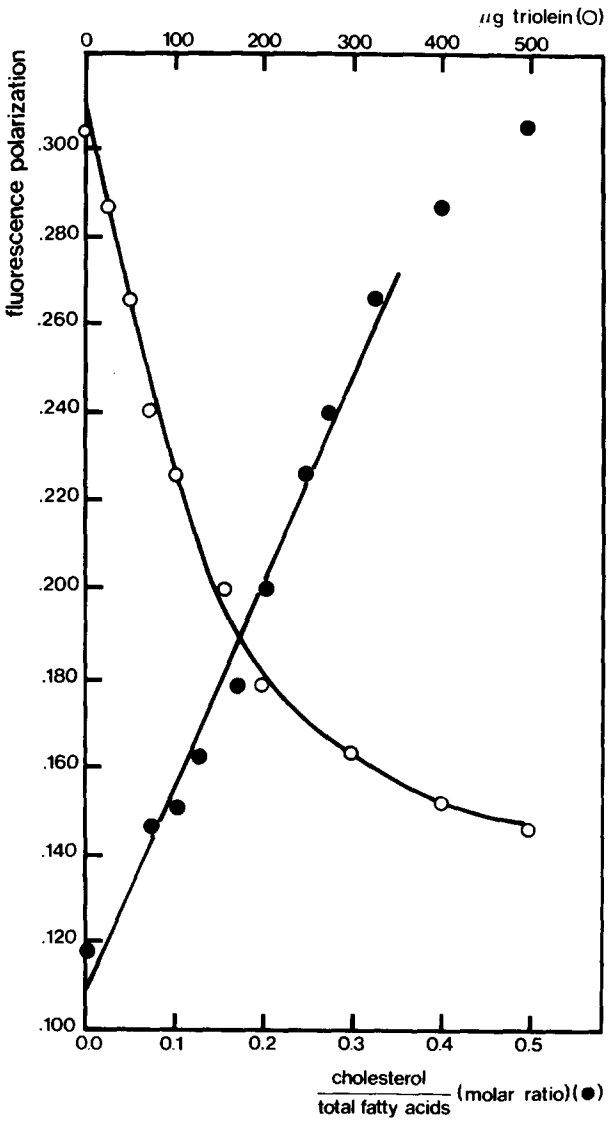

Fig. 2. A series of liposomes, consisting of a constant 1 : 1 molar ratio of egg lecithin (133 µg) and cholesterol (66 µg) with increasing amounts of triolein (0—3.3 molar to cholesterol; i.e. 0—500 µg) was labelled with DPH ($2 \cdot 10^{-6}$ M) and the degree of fluorescence polarization measured at 25° C. P values are plotted with respect to triolein content (○) and to cholesterol/total fatty acid molar ratio (●).

system, on the assumption that cholesterol is available to all these fatty acyls for binding, against the corresponding *P* values yielded a straight line for a considerable part of the curve. Extrapolation of that straight line to zero phospholipid content yielded a *P* value (0.110) like that of triglycerides of disrupted milk fat globules and triolein (0.120, 0.117).

Liver and hepatomas. Rat liver and hepatoma cells contain many cytoplasmic structures of which the *P* values are intermediary to *P* values of plasma membranes and triglycerides (Table II). This is in line with the finding that ΔP values for these cells are in between ΔP values of erythrocytes and milk fat globules, respectively (Table I). A further illustration of the contribution of lipid droplets to the *P* value of intact cells is furnished by the regenerating hepatocytes which contain lipid droplets 24 h after two-thirds hepatectomy. Here, however, a difference in plasma membrane *P* values was also apparent (as compared with intact liver); this may have been due to the observed tendency of the plasma membranes of regenerating liver to bind lipids in the course of their isolation [26]. Comparison (Table I) of different samples of Novikoff ascites hepatoma cells shows that the *P* values of whole cells may differ, while the corresponding plasma membranes exhibit a constant *P* value.

P values and lipid composition of isolated plasma membranes

As illustrated above, the *P* values of the various plasma membranes showed only limited mutual variation. The lowest *P* values were recorded for GRSL cells. The reason for this is the relatively small contents of cholesterol and sphingomyelin in the latter plasma membranes, being (much) lower than those of thymocyte plasma membranes (quantitative data in refs. 4 and 27). This corresponds with the lower *P* value of GRSL relative to thymocyte plasma membranes. Conversely the rat hepatoma plasma membranes contain more cholesterol and exhibit an increased saturation of the fatty acyls of their (phospho) lipids as compared with rat liver plasma membranes (for the solid hepatoma 484A, see ref. 28; our results on the Novikoff ascites hepatoma cells are similar to the former). These chemical changes correspond, in turn, with a higher *P* value of the hepatoma as compared with the liver plasma membranes. Thus two further conclusions emerge. First, there is no uniform direction of change in *P* value (and lipid composition, cf. ref. 28) between tumour plasma membranes and their homologous normal plasma membranes. Secondly, the changes which are observed are not due to the type of growth of the cells (ascites vs. solid: Novikoff hepatoma and GRSL cells vs. hepatoma 484A) but are apparently cell specific.

Does plasma membrane isolation select for particular fragments of the disrupted cell surface?

The possible fractionation of plasma membrane fragments during isolation of these membranes from GRSL cells and liver was studied using *P* values as criterion.

GRSL cells. Plasma membranes were isolated as described previously [8] from a crude microsomal fraction prepared from a 13 500 $\times g$ supernatant of cells disrupted by nitrogen cavitation. In addition plasma membranes were isolated from the 13 500 $\times g$ pellet, viz. the crude mitochondrial pellet which theoretically contains larger plasma membrane fragments than does the micro-

somal fraction [27]. As shown in Table II, the two plasma membrane preparations did not significantly differ in *P* values.

Rat liver. The plasma membrane preparation obtained by our standard method [11] which makes use of the crude nuclear pellet, was compared with three plasma membrane subfractions enriched in, respectively, sinusoidal (blood front) membranes, bile canalicular fronts, and contiguous membranes containing junctional complexes, prepared as indicated under Materials and Methods. On account of the *P* values obtained, it appears that our standard liver plasma membrane preparation which electron microscopically demonstrates abundant bile canaliculi and contiguous front membranes with junctions, is relatively poor in blood front membranes. However, this is by far not sufficient to explain the difference in *P* values between whole cells and plasma membranes in terms of a fractionation of the latter. If the three subfractions would, in the order given above, represent 40, 15 and 45% of the total plasma membrane skeleton [29], the mean *P* value of the liver cell surface may be estimated [30] to be 0.300, whereas intact hepatocytes display a value of 0.190 (Table I).

Comment and conclusion

The *P* values of intact cells are lower or much lower than those of the corresponding plasma membranes; the magnitude of this difference depends on the cell type.

It has previously been shown that the *P* values of plasma membranes (thymocytes, GRSL cells [4]; liver, hepatomas (van Hoeven, R.P., Oomen-Meulemans, E.P.M. and Emmelot, P., unpublished)) are similar to those obtained for liposomes prepared from the total lipid extracted from these membranes. It has now been shown that the *P* value of intact cells is equal to that of disrupted cells. These *P* values are similar to, or at the most 15% higher than those of the liposomes prepared from the total lipid extracted from the cells (unpublished).

Accordingly, the DPH probe is capable of penetrating into intact cells (cf. refs. 31 and 32) and to label all (accessible) lipids, next to those of the plasma membrane. Furthermore the light emitted from the cell interior appears to reach the detection device. Since *P* values decrease from about 0.350 to 0.120 in the order plasma membranes > endomembranes > non-membrane lipids, the mean *P* value of cells depends on the amounts of the various membrane and non-membrane lipids being present and their chemical composition.

In a comparable system of cells (thymocytes, GRSL cells; liver, hepatoma) a difference in the *P* value of the cells may be accompanied by a difference in the *P* value of the plasma membranes. However, the *P* value of a given cell (GRSL or Novikoff hepatoma ascites cells) may differ according to certain physiological conditions, whereas that of the corresponding plasma membranes remains constant.

Our main conclusion is that the degree of fluorescence polarization as measured by lipophilic fluorescent probes on intact cells cannot be considered as a reliable parameter of cell surface lipid fluidity as has been claimed [33]. Unless appropriate corrections for the contribution of endolipids can be made, interpretation of data on intact cells should be met cautiously.

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