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Incubation of Exogenous Fatty Acids with Lymphocytes. Changes in Fatty Acid Composition and Effects on the Rotational Relaxation Time of 1,6-Diphenyl-1,3,5-hexatriene†

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ABSTRACT: Lymphocytes were incubated with various long chain fatty acids bound to albumin. Incubation for 20 h with unsaturated fatty acids resulted in uptake into the neutral lipids and phospholipids. The addition of concanavalin A enhanced the uptake. With the unsaturated fatty acids the rotational relaxation time of 1,6-diphenyl-1,3,5-hexatriene (DPH) was decreased, while with saturated fatty acids there was only a very small effect. The effect on the rotational relaxation times with unsaturated fatty acids was found to be due to the formation of lipid droplets in the cytoplasm. When plasma membrane free of lipid droplets was prepared, there was no effect on the rotational relaxation time despite incorporation of either linoleate or palmitate into the membrane phospholipids. Phospholipid liposomes prepared from plasma membranes obtained from cells which had been cultured with and without exogenous linoleate gave identical rotational relaxation times regardless of the history of the cells from which they were obtained. It is concluded that although the fatty acid composition of lymphocyte plasma membrane can be modified by exogenous fatty acids, there is little effect on the degree of order of the membrane phospholipid fatty acyl chains as monitored by the effects on the rotational relaxation time of DPH.

An increasing interest has been directed toward the elucidation of the functional roles of lipid in animal cell membranes. The starting point for investigations has often been to study how the membrane lipid composition of certain cells could be varied in response to the addition of exogenous fatty acids. The phospholipid fatty acids of various cell types have been successfully modified in culture after supplementation with both saturated and unsaturated fatty acids. The cell which has been most commonly used is the fibroblast (Williams et al., 1974; Glaser et al., 1974; Ferguson et al., 1975; Doi et al., 1978; Spector et al., 1979), while other cells which have been

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modified include chinese hamster ovary cells (Rintoul et al., 1978), Erlich ascites cells (King & Spector, 1978; King et al., 1977), and lymphocytes (Weyman et al., 1977; Stubbs et al., 1977; Mandel et al., 1978).

It has been suggested that changes in the physical state of the membrane lipids arising from alterations in fatty acid composition could have a modulating effect on membrane-mediated functions such as enzyme activities and transport processes (Grover et al., 1975; Solomonson et al., 1976; Kaduce et al., 1977). The physical state of membrane lipids has also been invoked as being responsible for the effects of fatty acid replacement on the agglutination of lectin receptors in fibroblasts (Horwitz et al., 1974). With unsaturated fatty acid incorporation into fibroblasts, Williams et al. (1977) have shown that at 36 °C there was no correlation between the effect of fatty acid supplementation on proliferation and any change in lipid physical state which the fatty acids might have been likely to produce. With lymphocytes the physical state of membrane lipids has been implicated as being responsible for the shift in the optimum temperature for stimulation by concanavalin A after supplementation with unsaturated fatty acids (Maccecchini & Burger, 1977). Concanavalin A transformation of lymphocytes is, however, inhibited by incubation with both saturated and unsaturated fatty acids (Weyman et al., 1977; Tsang et al., 1977), a result unlikely to correlate directly with possible effects of fatty acid saturation on the lipid physical state. Although various studies have established effects of supplementation on the physical state of membrane lipids (King & Spector, 1978; Rintoul et al., 1978; Gilmore et al., 1979a,b), the links between fatty acid supplementation and changes in the lipid physical state and between the latter and membrane functions such as enzyme activities remain to be clearly established.

One method which can be used to investigate the physical properties of membrane lipids is to see how changes in composition are reflected in the motional properties of a fluorescent hydrophobic molecule incorporated into the lipid phase. The fluorescent probe 1,6-diphenyl-1,3,5-hexatriene (DPH)[1] has been widely used in such investigations to detect the effects of changes in sterols (Shinitzky & Inbar, 1976; Veatch & Stryer, 1977; Kinosita et al., 1977; Gilmore et al., 1979a; Hildenbrand & Nicolau, 1979), phospholipid head groups (Esko et al., 1977; Schroeder, 1978; Pessin et al., 1978; Gilmore et al., 1979a,b), triglycerides (Esko et al., 1977; Pessin et al., 1978; Van Hoevan et al., 1979; Johnson & Robinson, 1979), and phospholipid fatty acids (Stubbs et al., 1977; Holloway & Holloway, 1977; Martin & Thompson, 1978; Martin & Foyt, 1978; Gilmore et al., 1979a,b).

In order to investigate further the effect of fatty acid supplementation on the physical properties of membrane lipids, we have used lymphocytes to ascertain the membrane lipid modifications that can be achieved and the effect of these modifications on the physical properties of membrane lipids as monitored by the fluorescent probe DPH.

Materials and Methods

Preparation of Cells. Spleens from rats (Wistar, 10–12 weeks) and mice (Balb C, 5–8 weeks) were removed and the cells teased out into Hank's BSS (Gibco, U.K.). Erythrocytes were removed by layering the cells over ficoll–triosil (Lymphoprep; Nyegaard, Oslo) according to Böyum (1968) followed by centrifugation for 8 min at 300*g*. Lymphocytes were removed from the interface and were washed and resuspended in RPMI 1640 medium (Gibco, U.K.). Alternatively, erythrocytes were lysed in distilled water at 4 °C for 12 s and, after restoring to tonicity, the remaining lymphocytes were washed twice in Hank's BSS. Cells prepared in either way gave identical results, but the latter method yielded more cells and was used for the preparation of subcellular fractions where more material was needed. Calf thymocytes were prepared by first teasing out the cells from one thymus and then washing twice in Hank's BSS to remove the dead cells. After preparation, the viability of the lymphocytes was always greater than 90% according to the trypan blue exclusion test (Boyse et al., 1964).

Incubation with Fatty Acids. The fatty acids myristate, palmitate, stearate, heptadecanoate, oleate, and linoleate were bound to bovine serum albumin (fraction V; Armour, U.K.) according to the method of Spector & Hoak (1969). Various conditions of incubation (as specified) were used: (A) round-bottom tubes (100 × 16 mm; polystyrene; Nunc, U.K.), 4×10^6 cells, 0.1 mL of fetal calf serum (heat inactivated; Sera Lab, U.K.), 0.1 mL of 4 μmol/mL albumin-bound fatty acid, and RPMI 1640 to give a final volume of 2 mL; (B) flat-bottom tubes (100 × 16 mm; polystyrene; Nunc, U.K.), 1.3×10^6 cells/mL, 0.2 mL of fetal calf serum, 0.1 mL of 4 μmol/mL albumin-bound fatty acid, and RPMI 1640 to give a final volume of 2 mL; (C) petri dishes (30 × 10 mm; modified polystyrene; Sterilin, U.K.), 4×10^7 cells, 0.25 mL of fetal calf serum, 0.125 mL of 4 μmol/mL albumin-bound fatty acid, and RPMI 1640 to give a final volume of 2.5 mL; (D) bottles (100-mL glass Gibco media bottles), 3×10^9 cells, 5 mL of fetal calf serum, 5 mL of 4 μmol/mL albumin-bound fatty acid, 10 mL of RPMI 1640, and Hank's BSS to give a final volume of 100 mL. For controls, albumin without fatty acid was added. Concanavalin A (3× recrystallized; Miles, U.K.), where added, was at the concentration giving maximum incorporation of [^{3}H]thymidine at 72 h determined as described by Weyman et al. (1977). Cells were incubated for 20 h at 37 °C. For condition D the bottles were rolled to keep the cells in suspension. After incubation the cells were washed 3 times in PBS (phosphate-buffered saline, pH 7.3; Oxoid, U.K.), after which the viability was found to be over 90%.

Electron Microscopy. Pellets of intact cells and subcellular fractions (see below) were processed for examination by electron microscopy by fixation in 1% (w/v) osmium tetraoxide in PBS for 40 min followed by dehydration according to Luft (1961) and embedding in Spurr resin (Agar Aids, U.K.). Ultrathin sections were cut on an MT2 ultramicrotome, mounted on copper grids, stained with uranyl acetate and lead citrate according to Watson (1958), and examined with a Philips EM300 electron microscope.

Preparation of Subcellular Fractions. Cells labeled with DPH (see below) were homogenized by using the cell disrupter as described by Wright et al. (1974). Cell homogenates were fractionated according to Allan & Crumpton (1970). The fractions obtained were (I) a 300*g* crude nuclear pellet, (II) a 4000*g* crude mitochondrial pellet, and (III) a 20000*g* crude plasma membrane pellet. Similar fractions have been characterized (Johnson & Robinson, 1979). The 20000*g* pellet from calf thymocytes was further fractionated by layering over a discontinuous sucrose density gradient consisting of 30, 40, and 50% w/v sucrose steps. Purified plasma membrane (fraction IV) was removed from the 30–40% interface after centrifugation for 18 h at 25000*g*. The identity of the subcellular fractions (Allan & Crumpton, 1970) was checked by 5′-nucleotidase, a plasma membrane maker, assayed according

[1] Abbreviations used: DPH, 1,6-diphenyl-1,3,5-hexatriene; PBS, phosphate-buffered saline.

to Heppel & Hilmoe (1955). For the mouse the specific activity (mean of three experiments ± SEM) was 2.96 ± 0.087 μmol of inorganic phosphate released per h per mg of protein for fraction III and 0.72 ± 0.033 μmol/(h mg of protein) for fraction II. The mitochrondrial marker, succinate dehydrogenase, assayed according to Green et al. (1955) gave a specific activity of (2.7 ± 0.52) × 10^{-2} μmol of succinate oxidized per h per mg of protein for fraction III and (12.6 ● 2.83) × 10^{-2} μmol/(h mg of protein) for fraction II. For the calf, the 5′-nucleotidase activity was 0.86 μmol/(h mg of protein) for fraction III and 2.35 μmol/(h mg of protein) for fraction IV. The latter result compared well with that of purified calf thymocyte plasma membranes from other studies (Van Blitterswijk et al., 1973; Kornfeld & Siemers, 1973; Monneron & d'Alayer, 1978).

Fluorescence Techniques. The physical properties of the cell lipids were monitored by the incorporation of the hydrophobic molecule DPH (Aldrich, U.K.). The average rotational relaxation time of DPH, embedded in the cell lipids, was determined and used to give an indication of the dynamics of DPH as affected by the physical properties of the lipids [see the recent review by Shinitzky & Barenholz (1978)].

Cells were labeled with DPH by incubation for 30 min at 37 °C with a 10^{-6} M solution in PBS, prepared as described by Shinitzky & Inbar (1976). Fluorescence polarization values of whole cells were read by using an Elscint MV-1 microviscometer at a concentration of 1 × 10^7 cells/mL. The polarization values for subcellular fractions were corrected for light scattering as described by Johnson & Nicolau (1977). The fluorescence polarization (P) is related to the mean rotational relaxation time ($\bar{\rho}$) by the Perrin equation (Weber, 1953)

$$\frac{1}{P} - \frac{1}{3} = \left(\frac{1}{P_0} - \frac{1}{3}\right)\left(1 + \frac{3\tau}{\bar{\rho}}\right)$$

where τ is the lifetime of the excited state and P_0 is 0.460, the polarization in the absence of rotational motion (Shinitzky & Inbar, 1974). The fluorescence lifetimes of DPH were measured at 37.0 ± 0.5 °C by using an Applied Photophysics nanosecond spectrometer with Ortec electronics.

Lipid Analysis. Lipids were extracted by the method of Bligh & Dyer (1959), which these authors have shown to give essentially the same results as the method of Folch et al. (1956). To avoid changes in lipid composition, it is important to extract the fresh membranes in aqueous solution (Johnson, 1979). Phospholipids and neutral lipids were separated on columns of silicic acid. Saponification of the lipid fractions and the subsequent analysis of the fatty acids by gas–liquid chromatography were carried out as previously described (Weyman et al., 1977), and the composition of the fatty acids was calculated as the percentage by weight. Multilamellar liposomes of total lipid extracts and phospholipids were prepared in PBS according to the method of Bangham et al. (1965). Since the DPH was extracted along with the lipids, it was not necessary to relabel the samples.

Results

Whole Cells. In lymphocytes from both rat and mouse spleens the rotational relaxation time of DPH was lowered after supplementation with the unsaturated fatty acids linoleate and oleate (Table I). The effect on the fluorescence polarization was found to be gradual over a period of 0–20 h, after which time there was little further decrease. The leveling off was not due to exhaustion of the exogenous albumin-bound fatty acid which was in relatively abundant supply. The fact that the effects on the fluorescence polarization/rotational relaxation times were gradual argues against any immediate effect of the free fatty acids alone and in favor of an effect due to gradual changes in the cell lipids. The decrease in the rotational relaxation times with the unsaturated fatty acids was paralleled by the appearance of lipid droplets in the cytoplasm of the cells as revealed by electron microscopy (Figure 1). The lipid droplets resembled those found in fibroblasts by Mackenzie et al. (1967) and by Pessin et al. (1978).

Table I: Fluorescence Polarization, Fluorescence Lifetimes, and Rotational Relaxation Times of DPH for Lymphocytes Incubated for 20 h with Various Fatty Acids, with and without Concanavalin A[a]

	rat			mouse		
addition	P	τ (ns)	$\bar{\rho}$ (ns)	P	τ (ns)	$\bar{\rho}$ (ns)
none	0.229	7.3	18.3	0.233	6.9	18.0
albumin (control)	0.232	7.3	18.9	0.233	7.2	18.8
18:2	0.210	7.3	15.6	0.205	6.5	13.3
18:1	0.224	7.5	19.0	0.200	6.6	12.9
14:0	0.226	7.4	18.2	0.231	ND	ND
16:0	0.236	7.4	19.8	0.231	7.2	18.5
17:0	0.230	7.5	19.1	0.195[b]	ND	ND
18:0	0.236	ND	ND	0.189[b]	ND	ND
albumin + Con A (control)	0.232	7.5	19.4	0.237	6.9	18.7
18:2 + Con A	0.200	7.3	14.3	0.152	6.3	7.9
18:1 + Con A	0.219	7.2	16.7	0.170	6.4	9.5
14:0 + Con A	0.229	7.5	18.9	0.231	ND	ND
16:0 + Con A	0.240	7.5	20.8	0.228	7.0	17.5
17:0 + Con A	0.232	7.7	19.9	0.195[b]	ND	ND
18:0 + Con A	0.234	ND	ND	0.189[b]	ND	ND

[a] Details of cell culturing (culture condition A), fluorescence polarization (P) and fluorescence lifetime (τ) measurements, and calculations of the rotational relaxation times ($\bar{\rho}$) were carried out as described under Materials and Methods. ND = not determined. [b] Culture condition C used; control P, 0.207.

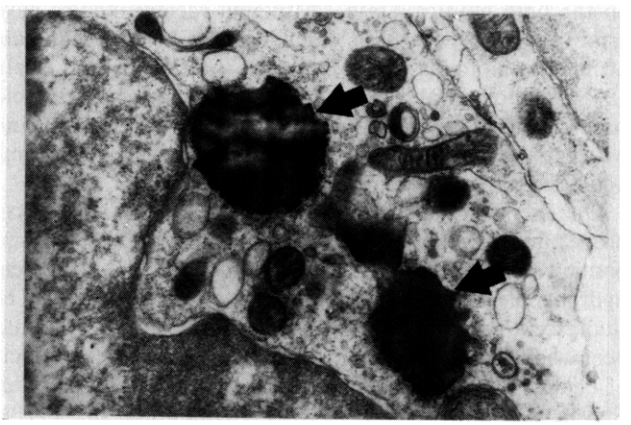

FIGURE 1: Inclusion bodies of lipid (arrowed) in a lymphocyte incubated for 20 h with linoleate (culture condition A; see Materials and Methods). Magnification ×13000.

Analysis of the neutral lipids revealed a large increase in the level of the particular fatty acid supplemented (Table II). Neutral lipids of whole cell lipid extracts were identified by thin-layer chromatography, using known standards, and there was an increase in the amount of triglycerides (results not shown). There was also an increase in the level of the supplemented fatty acid in the phospholipids.

The effect of differing cell culture densities on the fluorescence polarization of lymphocytes is shown in Figure 2. The results show that the fluorescence polarization can be lowered by a higher cell culture density. At the higher densities electron microscopy again revealed the presence of

Table II: Fatty Acid Composition (Percent) of Whole Mouse Lymphocytes after 20-h Incubation with Various Fatty Acids[a]

	additions						
	control	+18:1	+18:2	+14:0	+16:0	+17:0	+18:0
				Neutral Lipids			
14:0				29.7			
16:0	26.6	22.5	21.3	22.6	43.9	28.9	25.7
16:1	6.6	5.0	5.1	6.4	5.9	4.9	5.6
17:0						11.2	
18:0	13.6	11.0	10.1	11.9	15.1	16.6	29.0
18:1	34.7	54.1	16.0	22.2	26.0	26.5	28.4
18:2	10.1	4.3	41.3	4.5	4.6	7.5	5.1
20:4	8.3	3.0	6.2	2.7	4.5	4.4	6.2
				Phospholipids			
14:0				9.5			
16:0	28.4	24.0	27.3	25.8	33.3	22.8	24.7
16:1	2.3	6.0	3.0	2.7	3.0	1.7	2.5
17:0						12.2	
18:0	22.3	16.0	17.3	18.2	17.4	15.7	23.7
18:1	19.3	34.6	12.3	16.1	19.9	16.5	22.3
18:2	7.7	5.5	20.7	8.1	6.8	7.4	7.0
20:4	20.0	13.3	19.4	19.6	19.6	23.7	19.9
%S	50.7	40.0	44.6	53.0	50.7	50.7	48.4

[a] Details of cell culturing (culture condition C), separation of neutral lipids and phospholipids, and fatty acid analysis are described under Materials and Methods. This and subsequent tables show only the major fatty acids present; the remainder were minor components and have been omitted. The percentage of saturated fatty acids (% S) has been calculated for the phospholipids.

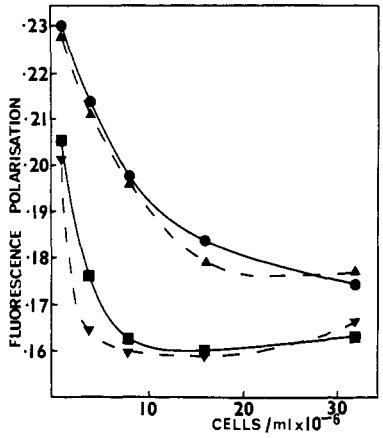

FIGURE 2: Effect of linoleate and concanavalin A on the fluorescence polarization of mouse spleen lymphocytes cultured at various densities (culture condition B; see Materials and Methods). (●) Control; (▲) concanavalin A; (■) linoleate; (▼) concanavalin A plus linoleate.

lipid droplets in the cytoplasm.

After incubation with the saturated fatty acids the rotational relaxation times of DPH in whole cells were little different from those of the control. There was, however, an increase in the supplemented fatty acid in the neutral lipids, but the increase was less than that with the unsaturated fatty acids. Lipid droplets were not found in the cytoplasm when cells supplemented with the saturated fatty acids were examined by electron microscopy.

When concanavalin A was included in the incubation medium with linoleate and oleate, the relaxation times of DPH were lower than those without concanavalin A. The optimum effect of concanavalin A on the fluorescence polarization, in the presence of linoleate, coincided with the optimum concentration of concanavalin A for transformation as detected by the uptake of [^{3}H]thymidine (Figure 3). From other studies it is known that after 20 h or more of incubation with concanavalin A there is an increased turnover of neutral lipids

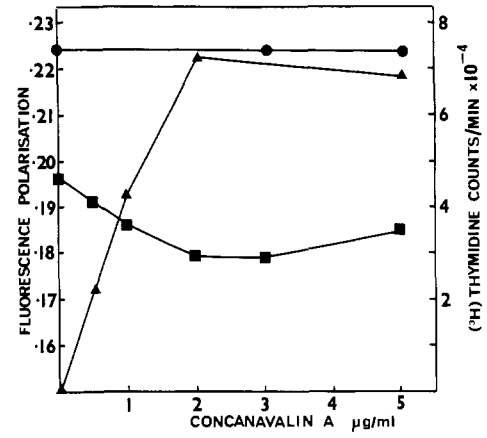

FIGURE 3: Effect on varying the concanavalin A concentration on the fluorescence polarization with and without linoleate and on [^{3}H]thymidine uptake of mouse spleen lymphocytes with linoleate by using culture condition B as described under Materials and Methods. (●) Effect of concanavalin A concentration on fluorescence polarization without linoleate; (■) effect with linoleate; (▲) effect of concanavalin A concentration on [^{3}H]thymidine uptake with linoleate.

Table III: Fatty Acid Composition (Percent) of Whole Mouse Lymphocytes after 20-h Incubation with Various Fatty Acids in the Presence of Concanavalin A[a]

	additions						
	control	+18:1	+18:2	+14:0	+16:0	+17:0	+18:0
				Neutral Lipids			
14:0				29.9			
16:0	28.4	20.1	15.9	22.7	50.0	28.5	24.1
16:1	4.1	2.6	2.4	2.9	4.0	4.7	3.8
17:0						19.4	
18:0	16.5	7.6	4.5	11.9	12.9	15.5	40.3
18:1	36.4	61.8	9.7	23.2	24.4	23.8	22.4
18:2	10.1	3.6	62.9	5.1	5.1	4.3	5.2
20:4	4.5	4.3	4.6	4.3	3.6	3.8	4.2
				Phospholipids			
14:0				17.6			
16:0	29.7	24.4	26.6	23.2	33.7	22.8	24.1
16:1	3.1	2.4	4.1	2.8	2.6	2.6	3.3
17:0						14.9	
18:0	20.6	17.4	13.9	15.9	14.2	15.7	24.0
18:1	19.2	33.3	13.2	18.4	21.1	18.0	21.2
18:2	9.1	5.4	28.3	7.0	9.2	7.8	8.0
20:4	18.3	17.1	13.9	15.1	19.2	18.2	19.3
%S	50.3	41.8	40.5	56.7	47.9	53.4	48.1

[a] Details of cell culturing (culture condition C), separation of neutral lipids and phospholipids, and fatty acid analysis are described under Materials and Methods. The percentage of saturated fatty acids (% S) has been calculated for the phospholipids.

(Resch & Ferber, 1972; W. M. Tsang, C. D. Stubbs, and A. D. Smith, unpublished experiments), and in the present study with concanavalin A there was also a greater incorporation of linoleate into the neutral lipid (Table III). The further lowering of the fluorescence polarization at 20 h, when concanavalin A is present in addition to exogenous linoleate, appears to be due therefore to an increase in the level of triglycerides, formed mainly from the incorporated linoleate. In the absence of linoleate supplementation, there was little effect of concanavalin A on the fatty acid pattern of the phospholipids. After 20 h of incubation with concanavalin A alone, there was no effect on the rotational relaxation time of DPH in whole cells (Table I), there was no formation of lipid droplets, and there was no significant effect on the fatty acid levels in the neutral lipids or phospholipids (cf. Tables II and III). With concanavalin A the saturated fatty acids still had no

Table IV: Fluorescence Polarization, Fluorescence Lifetimes, and Rotational Relaxation Times for DPH in Whole Cells, Subcellular Fractions, and Liposomes from Mouse Lymphocytes after 20-h Incubation with Linoleic Acid in the Presence of Concanavalin A[a]

		control	+18:2
whole cells	P	0.207 ± 0.003 (13)	0.146 ± 0.006 (9)
	τ (ns)	6.90	6.30
	$\bar{\rho}$ (ns)	14.3	7.4
fraction II	P	0.227 ± 0.005 (9)	0.189 ± 0.009 (8)
(4000g pellet)	τ (ns)	7.55	6.54
	$\bar{\rho}$ (ns)	18.7	11.6
fraction III	P	0.256 ± 0.005 (9)	0.234 ± 0.006 (9)
(20000g pellet)	τ (ns)	8.67	7.97
intact tissue	ρ (ns)	27.7	20.0
total lipid	P	0.234	0.233
liposomes	τ (ns)	7.09	6.56
	$\bar{\rho}$ (ns)	18.7	16.3
phospholipid	P	0.146	0.143
liposomes	τ (ns)	6.76	6.17
	$\bar{\rho}$ (ns)	8.0	7.1

[a] Details of cell culturing (culture condition C) and subcellular fractionation, determination of fluorescence polarization (P) and fluorescence lifetimes (τ), and calculation of the rotational relaxation times ($\bar{\rho}$) are as described under Materials and Methods. Where stated, the results are the mean ± SEM (number of results is in parentheses); otherwise, the results are the mean of at least two separate experiments).

Table V: Fatty Acid Composition (Percent) of Fraction II (4000g Pellet) and Fraction III (20000g Pellet) of Mouse Lymphocytes after 20-h Incubation with Linoleic Acid in the Presence of Concanavalin A[a]

	fraction II		fraction III	
	control	+18:2	control	+18:2
	Neutral Lipids			
16:0	37.1 ± 0.6	21.1 ± 1.7	38.2 ± 1.2	28.0 ± 2.4
16:1	5.2 ± 0.6	2.8 ± 0.6	6.8 ± 0.3	4.0 ± 0.5
18:0	21.9 ± 1.7	9.5 ± 1.8	21.1 ± 3.3	15.8 ± 2.1
18:1	21.3 ± 1.1	10.0 ± 0.7	20.8 ± 1.3	17.9 ± 4.6
18:2	5.8 ± 0.7	51.3 ± 4.7	7.2 ± 1.0	28.0 ± 8.2
20:4	8.7 ± 1.7	5.3 ± 0.1	5.9 ± 0.5	6.3 ± 1.7
	Phospholipids			
16:0	28.0 ± 0.5	26.2 ± 1.0	34.1 ± 1.0	26.3 ± 1.5
16:1	3.8 ± 0.5	2.9 ± 0.4	3.4 ± 0.3	3.4 ± 0.9
18:0	17.2 ± 0.4	15.4 ± 2.0	21.0 ± 0.9	20.1 ± 1.7
18:1	20.0 ± 1.8	11.4 ± 0.5	17.2 ± 0.5	14.3 ± 2.8
18:2	10.1 ± 0.3	27.9 ± 2.2	7.4 ± 0.2	21.0 ± 2.5
20:4	20.9 ± 2.4	16.2 ± 1.5	16.9 ± 2.0	14.9 ± 1.3
% S	48.0	41.6	55.1	46.4

[a] Details of cell culturing (culture condition C), cell fractionation, separation of neutral lipids and phospholipids, and fatty acid analysis are as described under Materials and Methods. The percentage of saturated fatty acids (% S) has been calculated for phospholipids. The results are expressed as the mean ± SEM from three experiments except for the phospholipids of fraction III which were from four experiments.

Table VI: Fluorescence Polarization, Fluorescence Lifetimes, and Rotational Relaxation Times for DPH in Whole Cells and Subcellular Fractions from Calf Thymus Lymphocytes after 20-h Incubation with Linoleic Acid and Palmitic Acid[a]

fraction		control	+18:2	+16:0
whole cells	P	0.233	0.188	0.205
	τ (ns)	6.11	5.65	5.09
	$\bar{\rho}$ (ns)	16.0	9.9	16.4
fraction II (4000g pellet)	P	0.235	0.197	0.223
	τ (ns)	7.20	7.09	7.95
	$\bar{\rho}$ (ns)	18.9	13.5	19.2
fraction III (20000g pellet)	P	0.260	0.254	0.256
	τ (ns)	7.90	8.11	ND
	$\bar{\rho}$ (ns)	26.3	25.3	ND
fraction IV (30–40% w/v sucrose density interface)	P	0.285	0.285	0.287
	τ (ns)	8.09	8.27	7.43
	$\bar{\rho}$ (ns)	33.5	34.2	31.4

[a] Details of cell culturing (culture condition D) and subcellular fractionation, determination of fluorescence polarization (P) and fluorescence lifetimes (τ), and calculation of the rotational relaxation times ($\bar{\rho}$) are as described under Materials and Methods. ND = not determined. Except for palmitic acid, the results are the mean of two separate experiments.

effect on the rotational relaxation time (Table I), although there was an increased level of incorporation of fatty acid into the neutral lipids (Table III).

Subcellular Fractions. In order to find out whether there was any effect of fatty acid supplementation on the physical properties of the membrane lipids, we studied the dynamic properties of DPH in subcellular fractions. The effect on the rotational relaxation time of incubation with linoleate for 20 h, in fractions II and III, is shown in Table IV. A decrease of relaxation time was found in both fractions, but it was greater in fraction II. The greater effect of linoleate on fraction II was matched by a high level of linoleate in the neutral lipids, whereas the level of linoleate in the fraction III neutral lipids was correspondingly lower (Table V). It therefore appeared that the effect on the relaxation times of incubation with linoleate, in fractions II and III, was merely a reflection of the amount of trapped triglycerides, as found in the similar study of Esko et al. (1977). Examination by electron microscopy confirmed the presence of lipid droplets trapped in both fractions. The influence of protein on the lipid in the intact membranes of fraction III was demonstrated by the lower relaxation times in the corresponding total lipid liposomes (Table IV). In fraction III there was an increase in the level of linoleate in the phospholipids and a decrease in the percentage of saturated fatty acids, but the relaxation times of the phospholipid liposomes were not in any way affected by changes in the level of linoleate. In order to check that the effect on the relaxation times in the intact membranes of fraction III was due entirely to triglyceride droplets, it was necessary to prepare more highly purified plasma membrane (fraction IV). The experiment therefore was repeated with calf thymocytes, which provided the larger amount of material needed, but which otherwise showed similar results to the mouse spleen lymphocytes. The purified plasma membrane from these cells (fraction IV, Table VI) showed no alteration in the relaxation time of DPH compared to that of the control. The value for the rotational relaxation time for fraction IV was higher than that for fraction III, due to the higher proportion of cholesterol in the purified plasma membranes. The lack of triglyceride droplets in fraction IV was indicated by the negligible increase of linoleate in the neutral lipids (Table VIII), compared to the much larger increase found in fraction III for the mouse (Table V). There was, however, considerable incorporation of linoleate or palmitate into the phospholipids of fraction IV of the calf thymocytes, with a small change in the percentage of saturated fatty acids.

Discussion

When the fluorescent molecule DPH is incorporated into membranes, it distributes evenly throughout the hydrophobic regions of the lipids (Lentz et al., 1976). One molecule of DPH occupies about the same space as a fatty acid chain, and it is thought to rotate within the restrictive confines of the

Table VII: Fatty Acid Composition (Percent) of Fraction II (4000*g* Pellet) and Fraction IV (30–40% Sucrose Interface) of Calf Thymus Lymphocytes after 20-h Incubation with Linoleic Acid and Palmitic Acid[a]

	fraction II			fraction IV		
	control	+18:2	+16:0	control	+18:2	+16:0
			Neutral Lipids			
16:0	34.3	16.7	55.2	38.4	38.6	44.6
16:1	5.1	2.0	2.7	11.0	10.8	7.6
18:0	15.6	6.1	10.9	16.2	15.3	14.3
18:1	30.4	14.9	18.2	27.2	21.5	23.4
18:2	8.4	56.6	8.7	6.1	9.7	6.8
20:4	6.2	3.7	4.3	1.1	4.1	3.3
			Phospholipids			
16:0	21.9	20.3	27.9	30.9	27.1	37.1
16:1	3.5	3.0	3.4	4.2	2.5	4.1
18:0	15.0	12.4	9.5	16.8	16.2	10.9
18:1	29.7	25.7	27.2	28.1	26.2	26.9
18:2	17.0	30.2	17.2	8.9	19.0	8.3
20:4	12.9	8.4	14.8	11.1	9.0	12.9
% *S*	36.9	32.7	37.4	47.7	43.3	48.0

[a] Details of cell culturing (culture condition D), cell fractionation, and separation of neutral lipids and phospholipids are as described under Materials and Methods. The percentage of saturated fatty acids (% *S*) has been calculated for phospholipids. Except for palmitic acid, the results are the mean of two separate experiments.

surrounding hydrocarbon chains (Kawato et al., 1977; Chen et al., 1977; Dale et al., 1977). We have not calculated the average microviscosity (Shinitzky et al., 1971), since it is now accepted that the calculation is based on assumptions which may be invalid and that the parameters of the motion of DPH are more accurately described by time-resolved anisotropy studies, which yield more information on both the degree of orientational constraint and the rate of rotation (Kawato et al., 1977; Chen et al., 1977; Dale et al., 1977; Hare & Lussan, 1977; Lakowicz & Prendergast, 1978; Lakowicz et al., 1979). The measurement of steady-state fluorescence polarization and rotational relaxation times can nevertheless yield important information regarding the changes of physical properties after alterations in the composition of membrane lipids (Lakowicz et al., 1979; Hildenbrand & Nicolau, 1979; Jahnig, 1979).

In this study we have shown that lymphocytes incorporate exogenous fatty acids into neutral lipids (triglycerides) and phospholipids. Only when the exogenous fatty acid is unsaturated do triglycerides accumulate in the cytoplasm as droplets, and as a result the whole cell rotational relaxation time is lowered. The main effect of concanavalin A is to increase the incorporation of the supplemented fatty acid into the triglycerides, with a more modest increase in the incorporation into phospholipids. Concanavalin A alone had little effect on the fatty acid patterns or the rotational relaxation times under these conditions. An important point raised in this study, as with ethanolamine supplementation (Esko et al., 1977; Pessin et al., 1978), is that conclusions as to the effects of modifications on the physical properties of cell membranes cannot be made solely on the basis of whole cell measurements if triglyceride droplets are formed. It is therefore necessary to prepare subcellular fractions to assess the contribution of triglyceride droplets to the changes found in whole cell measurements.

The fatty acid pattern of the neutral lipids extracted from the purified plasma membrane showed very little incorporation of the exogenous fatty acid. The level of the exogenous fatty acid was very high in fractions containing lipid droplets, which shows that the triglycerides of the lipid droplets and those of the plasma membrane are distinct species. Analysis of the fatty acid pattern of the phospholipids, in contrast to the neutral lipids, revealed considerable incorporation of the exogenous fatty acid; however, there was little effect on the rotational relaxation time of DPH. Recently, Seelig & Seelig (1977) have raised the important point that the physical state of fatty acyl chains in phospholipid bilayers must be considered from the point of view of both the *ordering* of the hydrocarbon chains and the *rates* of the different types of motion of the hydrocarbon chains. On the introduction of a single cis double bond in the phospholipids of a bilayer, the degree of order was shown to decrease and the rate of motion to increase. The theory developed to describe the motion of DPH in bilayers considers the motion to be within the confines of a "cone" (Chen et al., 1977; Kawato et al., 1977; Dale et al., 1977; Kinosita et al., 1977; Weber, 1977). The "cone angle", which can be determined by time-resolved studies, can be considered as a static property of DPH which relates exactly to the degree of order mentioned above, whereas the rotational diffusion constant of DPH is governed by the rate of motion of the fatty acyl chains. The steady-state polarization and rotational relaxation times used in this study relate to both the acyl chain order and the kinetic mobility of the fatty acyl chains reflected by the rotational diffusion of DPH (Kawato et al., 1977; Chen et al., 1977; Lakowicz & Prendergast, 1978; Lakowicz et al., 1979; Hildenbrand & Nicolau, 1979; Jahnig, 1979). Hildenbrand & Nicolau (1979) and Jahnig (1979) give a method by which the selective contributions of the two terms can be calculated from steady-state measurements. Jahnig (1979) has shown that this method is very successful as applied to dipalmitoylphosphatidylcholine, but it remains to be shown whether his assumptions can be extended to the complex systems described in this paper. The results reported here, however, show that the rotational relaxation time is largely unaffected by changes in the membrane fatty acids and at the physiological temperature (i.e., above the gel to liquid-crystalline phase transition temperature). This result is in keeping with other studies, such as that of King et al. (1977), who found only small changes in the spin-label order parameter on fatty acid supplementation, while Axelrod et al. (1978), using the fluroescence photobleaching recovery technique, found that substantial oleate supplementation had little effect on the rate of diffusion, which relates to the rate of motion of the fatty acyl chains.

We have now determined the rotational relaxation times for liposomes of purified lecithins with varying degrees of unsaturation. The results (S. M. Johnson, A. D. Smith, and C. D. Stubbs, unpublished experiments) show that, over the range of fatty acid saturation normally encountered in the cell, before and after fatty acid supplementation (35–55%), changes in the rotational relaxation time are small. Large changes in the rotational relaxation time begin to occur only if the mixed phospholipids are within about 10 °C of their liquid-crystalline to solid phase transition, a situation unlikely to occur under physiological conditions, as encountered by lymphocytes and other mammalian cells.

It is common practice to infer that changes in saturation of the phospholipid fatty acyl chains, achieved by various means, must necessarily affect "membrane fluidity". This term is properly reserved for the motional state of the fatty acyl chains, which may not be related to the degree of order of fatty acyl chains (Seelig & Seelig, 1977; Schreier et al., 1978). This emphasizes the importance of relating inferred changes in "fluidity" to specific physical parameters, since the effects will differ according to the physical technique employed.

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