

## Incorporation of fatty acids by concanavalin A-stimulated lymphocytes and the effect on fatty acid composition and membrane fluidity

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The fatty acid compositions of the neutral lipid and phospholipid fractions of rat lymph node lymphocytes were characterized. Stimulation of rat lymphocytes with the T-cell mitogen concanavalin A resulted in significant changes in the fatty acid composition of both neutral lipids and phospholipids (a decrease in the proportions of stearic, linoleic and arachidonic acids and an increase in the proportion of oleic acid). Membrane fluidity was measured using nitroxide spin-label e.s.r., and increased during culture with concanavalin A. Culturing the lymphocytes in the absence of mitogen did not affect fatty acid composition or membrane fluidity. The uptake and fate of palmitic, oleic, linoleic and arachidonic acids were studied in detail; there was a time-dependent incorporation of each fatty acid into all lipid classes but each fatty acid had a characteristic fate. Palmitic and arachidonic acids were incorporated principally into phospholipids whereas oleic and linoleic acids were incorporated in similar proportions into phospholipids and triacylglycerols. Oleic acid was incorporated mainly into phosphatidylcholine, palmitic and linoleic acids were incorporated equally into phosphatidylcholine and phosphatidylethanolamine, and arachidonic acid

was incorporated mainly into phosphatidylethanolamine. Supplementation of the culture medium with particular fatty acids (myristic, palmitic, stearic, oleic, linoleic,  $\alpha$ -linolenic, arachidonic, eicosapentaenoic or docosahexaenoic acid) led to enrichment of that fatty acid in both neutral lipids and phospholipids. This generated lymphocytes with phospholipids differing in saturated/unsaturated fatty acid ratio, degree of polyunsaturation, index of unsaturation and  $n-6/n-3$  ratio. This method allowed the introduction into lymphocyte phospholipids of fatty acids not normally present (e.g.  $\alpha$ -linolenic) or usually present in low proportions (eicosapentaenoic and docosahexaenoic). These three  $n-3$  polyunsaturated fatty acids replaced arachidonic acid in lymphocyte phospholipids. Fatty acid incorporation led to an alteration in lymphocyte membrane fluidity: palmitic and stearic acids decreased fluidity whereas the unsaturated fatty acids increased fluidity. It is proposed that the changes in lymphocyte phospholipid fatty acid composition and membrane fluidity brought about by culture in the presence of polyunsaturated fatty acids are responsible for the inhibition of lymphocyte functions caused by these fatty acids.

### INTRODUCTION

T-lymphocytes are activated by antigens, mitogens or antibodies directed against cell-surface structures. This activation is a complex process which involves a number of plasma-membrane-associated events including activation of phospholipase C with generation of the second messengers 1,4,5-inositol trisphosphate and diacylglycerol,  $\text{Ca}^{2+}$  mobilization, protein kinase C and tyrosine kinase activation, substrate and ion transport into the cells, appearance of cell-surface receptors and secretion of cytokines [1,2]. These processes ultimately lead to proliferation of the activated cell. The activation process results in an increase in cell size, with the cell diameter almost doubling. This increase in size is accompanied by both *de novo* synthesis and turnover of membrane phospholipids [3–7]. Indeed, alterations in membrane phospholipid metabolism are among the earliest changes observed, beginning within minutes of T-lymphocyte activation [3–7]. Such metabolic activity leads to changes in the fatty acid composition of the membrane phospholipids which can be observed within 4 h of contact with the mitogen [3–7]. There is, however, very little information on changes in the fatty acid composition of lymphocytes over a longer period.

A change in the fatty acid composition of membrane phospholipids might be expected to affect membrane fluidity. There have been a number of studies of lymphocyte membrane fluidity after activation [8–16]. However, these studies have yielded conflicting

results, some finding no change in fluidity [8,9] and others reporting an increase [10–16]. Some of these studies have measured fluidity during the period immediately after activation [9–12] whereas others [8,13–16] investigated longer-term changes.

Anel et al. [17] have reported significant changes in both the fatty acid composition and membrane fluidity of phytohaemagglutinin-activated human peripheral blood lymphocytes (PBL) during long-term culture (up to 144 h). These authors concluded that the changes in fatty acid composition and membrane fluidity that they observed are involved in the maintenance and development of the activated state and in sustaining the proliferation of activated cells [17].

Unsaturated fatty acids inhibit the proliferation of rat [18,19] and human [20,21] T-lymphocytes *in vitro*. Furthermore, polyunsaturated fatty acids (PUFA) suppress production of the immunoregulatory cytokine, interleukin 2, by these cells [20,22]. Although several eicosanoids, in particular prostaglandin  $\text{E}_2$ , have similar effects to these, it was shown that the actions of fatty acids on lymphocyte functions are independent of eicosanoid production [19,21,23]. We speculated that the effects of fatty acids might be due to their incorporation into phospholipids which could result in a change in plasma-membrane fluidity [18–20,22]. In other tissues and cell types, such changes have been shown to affect receptor functioning, the activity of membrane-bound enzymes and signal-transduction mechanisms [24–27]. As T-lymphocyte activation and proliferation involve

Abbreviations used: Con A, concanavalin A; DOXYL, 4',4'-dimethyloxazolidine-N-oxyl; g.c./m.s., gas chromatography/mass spectrometry; PBL, peripheral blood lymphocytes; PUFA, polyunsaturated fatty acids;  $S_{\text{eff}}$ , effective order parameter.

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the plasma membrane in numerous ways (see above), it is tempting to suggest that fatty acids act by interfering with the optimal membrane environment and/or by preventing changes in membrane composition which may be essential components of the proliferative process itself. Therefore in this study the ability of lymphocytes to incorporate fatty acids into different lipid types and the effect on fatty acid composition and membrane fluidity have been investigated. Fatty acids used were myristic ( $C_{14:0}$ ), palmitic ( $C_{16:0}$ ), stearic ( $C_{18:0}$ ), oleic ( $C_{18:1, n-9}$ ), linoleic ( $C_{18:2, n-6}$ ),  $\alpha$ -linolenic ( $C_{18:3, n-3}$ ), arachidonic ( $C_{20:4, n-6}$ ), eicosapentaenoic ( $C_{20:5, n-3}$ ) and docosahexaenoic ( $C_{22:6, n-3}$ ) acids. This is the first study to compare directly the effects of adding such a wide range of fatty acids on both fatty acid composition and membrane fluidity of lymphocytes; in particular, this is the first time that the effects of the  $n-3$  PUFA have been investigated in this way. It is important to investigate these effects, as the mechanism of the inhibitory action of fatty acids on lymphocyte functions is not known. The  $n-3$  PUFA are the most potent inhibitors of lymphocyte functions *in vitro* [18–22] and it has been suggested that fish oils containing these fatty acids could be of use in the therapy of diseases involving T-cell activation (see the Discussion). Thus knowledge of the mechanism of action of these fatty acids is of great importance.

## MATERIALS AND METHODS

### Animals and chemicals

Male Wistar rats were purchased from Harlan-Olac, Bicester, Oxon., U.K. They were housed in the Department of Biochemistry animal house and were allowed access to food and water *ad libitum*.

The sources of chemicals, culture medium and medium supplements were as described previously [18,28]. In addition, radioactively labelled fatty acids were obtained from Amersham International, Amersham, Bucks., U.K.; lipid standards for t.l.c. were obtained from Sigma Chemical Co., Poole, Dorset, U.K.; silica-gel 60 t.l.c. plates, iodine and all solvents were obtained from BDH, Poole, Dorset, U.K. 4',4'-dimethylloxazolidine-*N*-oxyl (DOXYL)-stearic acid was obtained from Aldrich Chemical Co., Gillingham, Dorset, U.K.; and Lymphoprep (a solution of sodium metrizoate/Ficoll with a density of 1.077 g/ml) was obtained from Nycomed Pharma AS, Oslo, Norway.

### Lymphocyte preparation and culture

Cervical lymph nodes were dissected free of adipose tissue and were gently ground. The cells were washed once and lymphocytes were collected by centrifugation (1500 *g*, 20 min) on Lymphoprep. The lymphocytes were washed once more and resuspended in culture medium.

Lymphocytes were cultured at 37 °C in an air/CO<sub>2</sub> (19:1, v/v) atmosphere at a density of  $5 \times 10^6$  cells per well (final volume 2 ml) in 24-well plates in Hepes-buffered RPMI supplemented with 10 mM glucose, 2 mM glutamine, 10% fetal calf serum and antibiotics (streptomycin and penicillin). The medium also contained fatty acids (final concentration 100  $\mu$ M), added as a 1:1 complex with BSA. Fatty acid-BSA complexes were formed as described elsewhere [18,28]. Where the cells were stimulated with mitogen, the medium contained 5  $\mu$ g/ml concanavalin A (Con A).

In experiments in which the incorporation and fate of fatty acids were studied, the medium contained [ $1-^{14}$ C]palmitic, [ $1-^{14}$ C]oleic, [ $1-^{14}$ C]linoleic or [ $1-^{14}$ C]arachidonic acid.

After various times of culture (see the Results section) the cells were collected, washed three times with PBS and resuspended.

### Lipid extraction and analysis

Lymphocytes were resuspended in a small volume of PBS and sonicated ( $3 \times 15$  s at an amplitude of 12  $\mu$ m) in an MSE sonicator. Lipid was extracted using chloroform/methanol (2:1, v/v).

In experiments where the incorporation and fate of radioactively labelled fatty acids were investigated, lipid classes were separated by t.l.c. on silica-gel 60 plates using hexane/diethyl ether/acetic acid (35:15:1, by vol.) to separate neutral lipids and chloroform/methanol/ammonia (65:30:3, by vol.) to separate phospholipids. After t.l.c., lipids were detected by staining with iodine. The position of each lipid class was determined by comparison with a commercially available standard lipid mixture which was separated on the same plate as the samples. Each identified spot was scraped from the t.l.c. plate and lipid was extracted with chloroform/methanol (2:1, v/v). The lipid extract was transferred to scintillation counting vials, dried and incorporation of radioactivity determined by liquid-scintillation counting.

In experiments where the fatty acid composition of lymphocyte lipids was investigated, neutral lipids were separated from phospholipids by chromatography through a column of activated silicic acid prewashed with chloroform. The neutral and phospholipids were eluted from the column with chloroform and chloroform/methanol (1:1, v/v) respectively. Fatty acids were prepared by saponification in methanolic 0.5 M KOH for 60 min at 70 °C and were extracted into ethyl acetate. After evaporation to dryness, fatty acids were prepared by reaction with an excess of diazomethane in ether. Fatty acid methyl esters (dissolved in methyl acetate) were separated by gas chromatography as described in detail elsewhere [28]. Fatty acid methyl esters were identified by comparison with standards run previously or by gas chromatography/mass spectrometry (g.c./m.s.) of fatty acid trimethylsilyl esters. Trimethylsilyl esters of fatty acids were prepared by heating with *NO*-bis(trimethylsilyl)trifluoroacetamide for 10 min at 60 °C. G.c./m.s. data were obtained as described previously [28].

### Plasma-membrane fluidity measurements

Lymphocytes were suspended in a small volume of PBS and incubated briefly with a dry film of 5-DOXYL-stearic acid, a spin label with the nitroxide group situated at carbon 5 of the hydrocarbon chain. The dry film of spin label was obtained by transferring 10  $\mu$ l of a 1 mg/ml solution of 5-DOXYL-stearic acid in chloroform to a small glass tube and evaporating to dryness. The spin-labelled lymphocyte suspension was transferred to a 100  $\mu$ l glass capillary tube with a sealed end. The cells were concentrated by centrifugation (1500 *g*, 5 min). The upper aqueous phase was removed before e.s.r. measurements were made. The time between spin labelling the lymphocytes and beginning e.s.r. measurements was less than 30 min.

The effective order parameter ( $S_{\text{eff}}$ ), determined by e.s.r. spectroscopy, is a measure of the amplitude and rate of motion of acyl chains [29] or membrane 'fluidity' [29].

For e.s.r. measurements the capillary tube containing spin-labelled lymphocytes was inserted into a standard 4 mm e.s.r. tube. E.s.r. spectra were obtained using a Bruker ESP 300 spectrometer equipped with a nitrogen gas-flow variable temperature unit. Spectra were digitized using the Bruker ESP 1600 data system. Spectra were recorded at various temperatures (see

the Results section). The maximum and minimum hyperfine splittings ( $A_{\parallel}$  and  $A'_{\perp}$  respectively) were measured from the spectra and were used to calculate  $S_{\text{eff}}$ , as a measure of membrane fluidity [29]:

$$S_{\text{eff}} = \frac{A_{\parallel} - A'_{\perp}}{A_{zz} - \frac{1}{2}(A_{xx} + A_{yy})} \times \frac{a'_0}{a_0}$$

where  $A_{xx}$ ,  $A_{yy}$  and  $A_{zz}$  are the single-crystal principal hyperfine splittings for the DOXYL group [29].  $A_{\parallel}$  and  $A'_{\perp}$  are the experimentally determined hyperfine splittings;  $A'_{\perp}$  was adjusted to give  $A_{\perp}$  according to [29] in order to accommodate spectral overlap in the powder spectra:

$$A = A'_{\perp} + 1.4[1 - (A_{\parallel} - A'_{\perp})]/[A_{zz} - \frac{1}{2}(A_{xx} + A_{yy})]$$

The term  $a'_0/a_0$  was used to normalize order parameter determinations for the sensitivity of nitroxide hyperfine splittings to environmental polarity, a relative measure of which is given by the isotropic splitting factor  $a_0$ , where  $a_0 = \frac{1}{3}(A_{\parallel} + 2A_{\perp})$  and  $a'_0 = \frac{1}{3}(A_{zz} + A_{xx} + A_{yy})$  (see [29]).

E.s.r. measurements were always begun within 30 min of contact between spin label and cells; a set of measurements on a single sample was complete within 1 h. 'Flip-flop' motion of fatty acids takes several hours. Therefore, during the e.s.r. measurements made in this study, the spin-labelled stearic acid would have remained in the lymphocyte plasma membrane rather than becoming redistributed to intracellular membranes. Thus the  $S_{\text{eff}}$  values presented in this study give a measure of the plasma-membrane fluidity.

### Other procedures

Protein concentrations were measured by the method of Bradford [30]. Statistical comparisons were made using Student's unpaired *t* test.

## RESULTS

### Lymphocyte fatty acid composition

The fatty acid composition of the neutral lipid fraction of lymphocytes from the rat is shown in Table 1. The neutral lipid fraction includes acylglycerols, cholesterol, cholesterol esters and non-esterified fatty acids; triacylglycerols are the major fatty acid-containing component of this fraction. These lipids largely represent an intracellular store of fatty acids. The neutral lipids of freshly prepared cells contained high proportions of the saturated fatty acids (particularly palmitic and stearic acids) and lower proportions of PUFA, which accounted for less than 20% of the fatty acids (Table 1). Culture of the cells in the absence of mitogen (i.e. in the quiescent state) for 24 or 48 h did not cause any alteration in the neutral lipid fatty acid composition (Table 1). In contrast, mitogenic stimulation caused significant changes (Table 1). After 24 h stimulation with Con A, the proportion of stearic acid was decreased compared with cells cultured in the absence of Con A. Greater changes were observed after 48 h of culture in the presence of Con A: the proportions of stearic, linoleic and arachidonic acids were decreased compared with those in fresh cells, whereas that of oleic acid was increased. Compared with cells cultured quiescently for 48 h, the proportions of stearic and linoleic acids were decreased (Table 1). These changes in fatty acid composition resulted in a small, but not statistically significant, decrease in the saturated/unsaturated fatty acid ratio.

The fatty acid composition of the phospholipid fraction of lymphocytes is shown in Table 2. The phospholipid fraction of

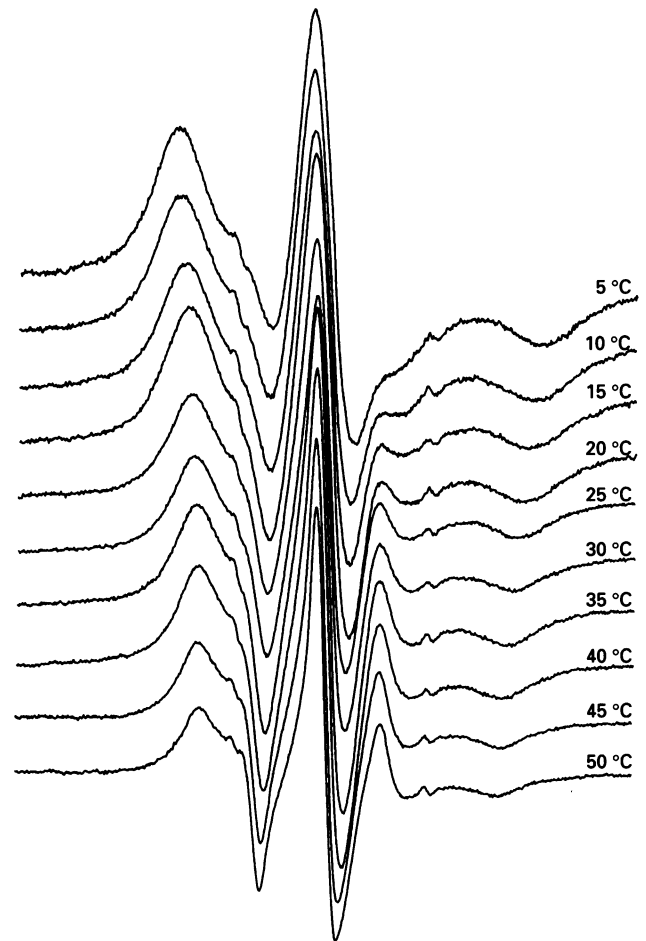
**Table 1 Fatty acid composition of the neutral lipid fraction of lymphocytes**  
Lymphocytes were prepared as described in the Materials and methods section and were cultured in the absence (quiescent) or presence (stimulated) of 5 µg/ml Con A. After 24 or 48 h the cells were collected, washed and briefly sonicated, and the lipid was extracted using chloroform/methanol. Neutral lipids and phospholipids were separated by silicic acid column chromatography. Fatty acids were prepared by saponification and methylated. The methyl esters were separated by g.c. Data are means ± S.E.M. of three separate cell preparations. Only the major fatty acids are shown. Statistical significance (Student's *t* test) is indicated as follows: versus fresh cells <sup>a</sup>*P* < 0.05, <sup>b</sup>*P* < 0.02, <sup>c</sup>*P* < 0.01; versus quiescent cells <sup>d</sup>*P* < 0.05, <sup>e</sup>*P* < 0.02; versus 24 h *P* < 0.05.

Fatty acid (mol%)	Saturated/unsaturated									
	C <sub>12:0</sub>	C <sub>14:0</sub>	C <sub>16:0</sub>	C <sub>16:1,n-7</sub>	C <sub>18:0</sub>	C <sub>18:1,n-7</sub>	C <sub>18:2,n-6</sub>	C <sub>20:4,n-6</sub>	C <sub>20:5,n-3</sub>	C <sub>22:6,n-3</sub>
Fresh	2.1 ± 0.2	4.7 ± 0.8	32.6 ± 0.5	1.8 ± 0.6	20.4 ± 1.4	16.1 ± 0.7	7.8 ± 0.5	5.9 ± 0.1	0.3 ± 0.1	0.5 ± 0.1
24 h Quiescent	1.8 ± 0.2	3.8 ± 0.7	34.9 ± 0.9	1.3 ± 0.5	23.3 ± 0.8	14.2 ± 2.1	6.7 ± 0.4	6.0 ± 0.4	0.2 ± 0.1	0.7 ± 0.1
24 h Stimulated	1.9 ± 0.3	4.4 ± 0.5	33.8 ± 2.1	1.5 ± 0.7	18.9 ± 0.7 <sup>a</sup>	18.5 ± 1.3	5.4 ± 1.3	5.5 ± 0.8	0.3 ± 0.1	0.6 ± 0.1
48 h Quiescent	1.7 ± 0.2	4.5 ± 0.4	34.9 ± 1.8	1.1 ± 0.5	22.7 ± 2.3	17.3 ± 2.2	6.7 ± 0.4	5.5 ± 0.3	0.4 ± 0.1	0.8 ± 0.2
48 h Stimulated	2.2 ± 0.3	3.9 ± 0.7	31.9 ± 1.1	1.4 ± 0.3	15.4 ± 0.8 <sup>abd</sup>	22.9 ± 1.5 <sup>b</sup>	5.1 ± 0.3 <sup>cd</sup>	4.7 ± 0.2 <sup>c</sup>	0.3 ± 0.1	0.8 ± 0.2

**Table 2 Fatty acid composition of the phospholipid fraction of lymphocytes**

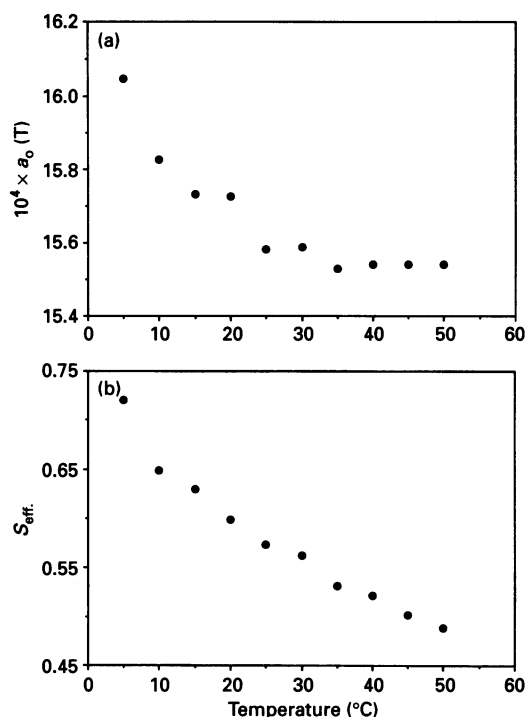
Lymphocytes were prepared as described in the Materials and methods section and were cultured in the absence (quiescent) or presence (stimulated) of 5 µg/ml Con A. After 24 or 48 h the cells were collected, washed and briefly sonicated, and the lipid was extracted using chloroform/methanol. Neutral lipids and phospholipids were separated by silicic acid column chromatography. Fatty acids were prepared by saponification and methylated. The methyl esters were separated by g.c. Data are means ± S.E.M. for three separate cell preparations. Only the major fatty acids are shown. Statistical significance (Student's *t* test) is indicated as follows: versus fresh cells <sup>a</sup>*P* < 0.05, <sup>b</sup>*P* < 0.01; versus quiescent cells <sup>d</sup>*P* < 0.05, <sup>e</sup>*P* < 0.02, <sup>f</sup>*P* < 0.01; versus 24 h <sup>g</sup>*P* < 0.05, <sup>h</sup>*P* < 0.001.

	Fatty acid (mol%)											Saturated/ unsaturated
	C <sub>12:0</sub>	C <sub>14:0</sub>	C <sub>16:0</sub>	C <sub>16:1, n-7</sub>	C <sub>18:0</sub>	C <sub>18:1, n-9</sub>	C <sub>18:2, n-6</sub>	C <sub>20:4, n-6</sub>	C <sub>20:5, n-3</sub>	C <sub>22:6, n-3</sub>		
Fresh	1.8 ± 0.2	3.8 ± 0.5	17.1 ± 0.8	2.4 ± 0.3	29.8 ± 0.7	13.4 ± 0.4	6.4 ± 0.1	22.1 ± 1.0	0.3 ± 0.1	0.9 ± 0.1	1.1 ± 0.1	
24 h Quiescent	1.6 ± 0.3	3.7 ± 0.5	16.5 ± 0.9	2.2 ± 0.2	30.5 ± 1.8	15.6 ± 2.2	6.5 ± 0.8	21.0 ± 1.9	0.3 ± 0.1	0.8 ± 0.2	1.1 ± 0.1	
24 h Stimulated	1.4 ± 0.2	2.8 ± 0.4	18.1 ± 0.8	2.6 ± 0.3	24.6 ± 1.4 <sup>a</sup>	19.0 ± 1.3 <sup>a</sup>	4.4 ± 0.7 <sup>a</sup>	18.3 ± 1.5	0.4 ± 0.1	1.1 ± 0.1	1.0 ± 0.1	
48 h Quiescent	1.5 ± 0.1	4.1 ± 0.7	15.5 ± 0.6	2.1 ± 0.8	27.2 ± 1.2	17.8 ± 2.5	5.6 ± 0.3	19.8 ± 0.8	0.4 ± 0.1	0.8 ± 0.2	1.0 ± 0.2	
48 h Stimulated	2.1 ± 0.2	3.2 ± 0.3	10.3 ± 0.4 <sup>ab</sup>	2.0 ± 0.2	19.9 ± 1.3 <sup>ab</sup>	28.9 ± 2.7 <sup>cdg</sup>	4.1 ± 0.3 <sup>cd</sup>	16.8 ± 0.6 <sup>bd</sup>	0.5 ± 0.1	1.3 ± 0.2	0.7 ± 0.1 <sup>a</sup>	

**Figure 1 Temperature-dependence of the e.s.r. spectrum of 5-DOXYL-stearic acid-labelled lymphocytes**

Lymphocytes were isolated and prepared for e.s.r. spectroscopy as described in the Materials and methods section. 5-DOXYL-stearic acid was used as the spin label. E.s.r. measurements were made at temperatures between 5 and 50 °C as described in the Materials and methods section. Spectra are from a representative cell preparation.

freshly prepared cells contained a higher proportion of PUFA (approx. 30%) than did the neutral lipid fraction; arachidonic acid in particular comprised over 20% of the phospholipid fatty acids (Table 2), three times its proportion in the neutral lipid fraction (Table 1). The proportion of saturated fatty acids was lower in the phospholipid fraction than in the neutral lipid fraction, although the former contained a greater proportion of stearic acid (Table 2). These differences resulted in a significantly (*P* < 0.05) lower saturated/unsaturated fatty acid ratio in the phospholipids than in the neutral lipids. Culture of lymphocytes in the quiescent state did not significantly alter the fatty acid composition of the phospholipid fraction (Table 2). In contrast, and as observed for the neutral lipid fraction (Table 1), culture in the presence of mitogen caused significant alterations in the fatty acid composition of the phospholipid fraction (Table 2). After 24 h in the presence of Con A the proportions of stearic and linoleic acids were decreased, and the proportion of oleic acid was increased, compared with the fresh cells (Table 2). More marked changes occurred after 48 h of culture in the presence of Con A: the proportions of palmitic, stearic, linoleic and arachidonic acids were reduced, and the proportion of oleic acid was increased, compared with both fresh cells and cells cultured in



**Figure 2** Temperature-dependence of the isotropic hyperfine splitting constant,  $a_0$ , and the effective order parameter,  $S_{\text{eff}}$ .

Lymphocytes were isolated and prepared for e.s.r. spectroscopy as described in the Materials and methods section. 5-DOXYL-stearic acid was used as the spin label. E.s.r. measurements were made at temperatures between 5 and 50 °C as described in the Materials and methods section and (a)  $a_0$  and (b)  $S_{\text{eff}}$  calculated. Data are from a representative cell preparation.

the absence of mitogen for 48 h (Table 2). Furthermore, the proportions of palmitic and oleic acids were different from those in the phospholipids of cells cultured for 24 h in the presence of mitogen (Table 2). These changes in the fatty acid composition resulted in a decrease in the proportion of PUFA in the phospholipids and in a marked decrease (by 40%) in the saturated/unsaturated fatty acid ratio.

The majority of PUFA present in the phospholipid fraction of rat lymphocytes were of the  $n-6$  family (Table 2). These accounted for approx. 95% of PUFA in the phospholipids of freshly prepared lymphocytes, giving a  $n-6/n-3$  PUFA ratio of approx. 20. Despite the decrease in the proportions of linoleic and arachidonic acids in the phospholipids of the cells cultured for 48 h in the presence of Con A (Table 2),  $n-6$  PUFA still comprised about 90% of the PUFA present. Although the increase in the proportion of  $n-3$  PUFA in the phospholipids of these cells was small (Table 2), the  $n-6/n-3$  ratio decreased after 48 h of culture to approx. 10.

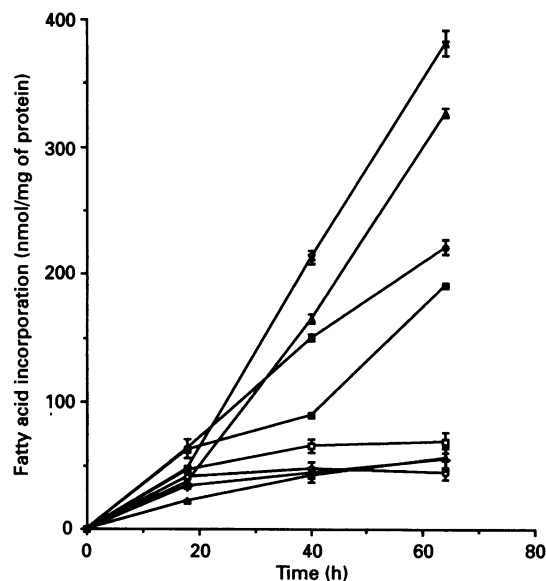
### Lymphocyte plasma-membrane fluidity

The temperature-dependence of the e.s.r. spectrum of 5-DOXYL-stearic acid-labelled lymphocytes is shown in Figure 1. The temperature-dependence of the isotropic hyperfine splitting constant,  $a_0$ , and the effective order parameter,  $S_{\text{eff}}$ , is shown in Figure 2. The values of both  $a_0$  and  $S_{\text{eff}}$  are similar to values for these parameters in other natural membrane systems [29]. There was a decrease in the value of  $S_{\text{eff}}$  with increasing temperature (Figure 2b), indicating that plasma-membrane fluidity (both rate and amplitude of acyl chain motion) increases with increasing

**Table 3** Effect of mitogenic stimulation on the effective order parameters,  $S_{\text{eff}}$ .

Lymphocytes were isolated as described in the Materials and methods section and were cultured in the absence (quiescent) or present (stimulated) of Con A (5  $\mu\text{g}/\text{ml}$ ). After the indicated time the cells were collected, washed and prepared for e.s.r. spectroscopy as described in the Materials and methods section. 5-DOXYL-stearic acid was used as the e.s.r. probe. E.s.r. spectra were recorded at 25 and 37 °C and  $S_{\text{eff}}$  was calculated, as described in the Materials and methods section. Data are means  $\pm$  S.E.M. for three separate cell preparations. Statistical significance (Student's  $t$  test) is indicated as follows: versus fresh cells  $^aP < 0.02$ ,  $^bP < 0.01$ ,  $^cP < 0.001$ ; versus quiescent cells  $^dP < 0.05$ ,  $^eP < 0.01$ .

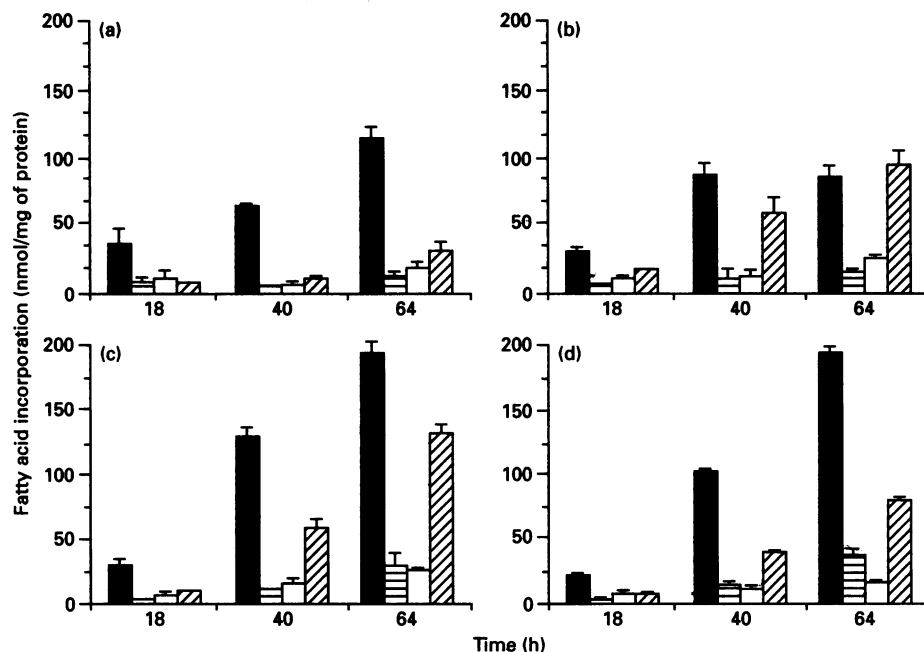
Time in culture	$S_{\text{eff}}$	
	25 °C	37 °C
Fresh	0.572 $\pm$ 0.004	0.535 $\pm$ 0.001
12 h Quiescent	0.568 $\pm$ 0.010	0.540 $\pm$ 0.009
12 h Stimulated	0.539 $\pm$ 0.006 <sup>a</sup>	0.480 $\pm$ 0.003 <sup>de</sup>
24 h Quiescent	0.564 $\pm$ 0.013	0.514 $\pm$ 0.015
24 h Stimulated	0.535 $\pm$ 0.004 <sup>b</sup>	0.478 $\pm$ 0.011 <sup>b</sup>
36 h Quiescent	0.569 $\pm$ 0.008	0.531 $\pm$ 0.004
36 h Stimulated	0.537 $\pm$ 0.006 <sup>de</sup>	0.469 $\pm$ 0.010 <sup>de</sup>
48 h Quiescent	0.564 $\pm$ 0.004	0.536 $\pm$ 0.007
48 h Stimulated	0.520 $\pm$ 0.006 <sup>de</sup>	0.461 $\pm$ 0.010 <sup>de</sup>



**Figure 3** Incorporation of fatty acids into lymphocyte lipids

Lymphocytes were isolated as described in the Materials and methods section and were cultured in the absence ( $\square$ ,  $\circ$ ,  $+$ ,  $\triangle$ ) or presence ( $\blacksquare$ ,  $\bullet$ ,  $\times$ ,  $\blacktriangle$ ) of 5  $\mu\text{g}/\text{ml}$  Con A and radioactively labelled palmitic ( $\square$ ,  $\blacksquare$ ), oleic ( $\circ$ ,  $\bullet$ ), linoleic ( $+$ ,  $\times$ ) or arachidonic ( $\triangle$ ,  $\blacktriangle$ ) acid (final concentration 100  $\mu\text{M}$ ). After 18, 40 or 64 h the cells were collected, washed and briefly sonicated and the lipid extracted using chloroform/methanol. The lipid extract was dried and the incorporation of fatty acid determined by liquid-scintillation counting. Data are means  $\pm$  S.E.M. for three separate cell preparations.

temperature. In addition,  $a_0$  decreased in value from low temperature (5 °C) to 20 °C, above which a constant value of  $a_0$  was measured (Figure 2a). This shows that fast motion (on the spin-label e.s.r. timescale [29]) only occurs in these membranes at temperatures greater than 20 °C, and so  $S_{\text{eff}}$  is a more appropriate measure of the acyl chain motional contribution to membrane fluidity.  $S_{\text{eff}}$  reflects both the rate and amplitude of acyl chain motion when slow-intermediate spin-label motion occurs within the membrane.



**Figure 4** Fate of incorporated fatty acids in lymphocyte lipids

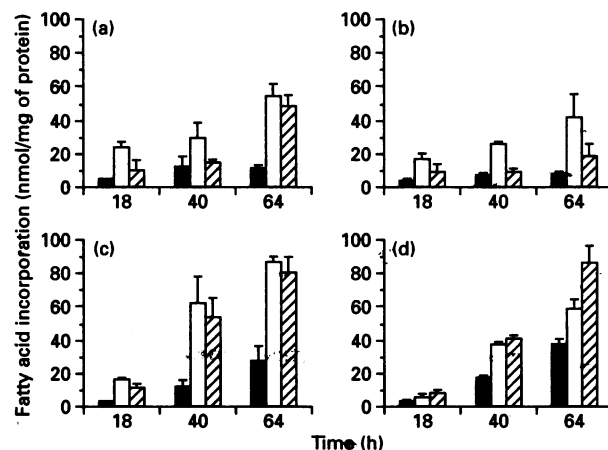
Lymphocytes were isolated as described in the Materials and methods section and were cultured in the presence of 5  $\mu\text{g}/\text{ml}$  Con A and radioactively labelled palmitic (a), oleic (b), linoleic (c) or arachidonic (d) acid (final concentration 100  $\mu\text{M}$ ). After 18, 40 or 64 h the cells were collected, washed and briefly sonicated, and the lipid was extracted using chloroform/methanol. Lipid classes were separated by t.l.c. (see the Materials and methods section) and the incorporation of fatty acid into phospholipid (■), mono- and di-acylglycerol (▨), non-esterified fatty acid (□) and triacylglycerol (▧) was determined. Data are means  $\pm$  S.E.M. for three separate cell preparations.

The effect of mitogenic stimulation on lymphocyte plasma-membrane fluidity was investigated by measuring  $S_{\text{eff}}$  at two selected temperatures (25 and 37  $^{\circ}\text{C}$ ) at various times after exposure of the cells to mitogen (Table 3). Measurements at both temperatures indicate that  $S_{\text{eff}}$  decreases during culture after mitogenic stimulation;  $S_{\text{eff}}$  does not change if the cells are cultured in the quiescent state (Table 3). These results indicate that lymphocyte membrane fluidity increases after mitogenic stimulation.

#### Incorporation of fatty acids by cultured lymphocytes

The incorporation of fatty acids into lipids of lymphocytes is shown in Figure 3. In the absence of mitogen, all fatty acids were incorporated at a similar low rate (initially approx. 25–40 nmol/20 h per mg of protein). The incorporation of all fatty acids was significantly increased during culture in the presence of Con A (Figure 3). The rate of incorporation of the four fatty acids investigated was different and varied from approx. 180 nmol/64 h per mg of protein for palmitic acid to approx. 370 nmol/64 h per mg of protein for linoleic acid (Figure 3).

The intracellular fate of the incorporated fatty acids is shown in Figure 4. There were marked differences in the pattern of incorporation of different fatty acids into various lipid classes. The incorporation of each fatty acid into each lipid class (phospholipid, mono- and di-acylglycerol, non-esterified fatty acid and triacylglycerol) increased with increasing time in culture (Figure 4). However, the proportions of the fatty acid incorporated into each lipid class varied between the fatty acids: palmitic and arachidonic acids were incorporated predominantly (more than 60%) into phospholipid, whereas oleic and linoleic acids were incorporated more equally into phospholipid and triacylglycerol (Figure 4).



**Figure 5** Fate of incorporated fatty acids in lymphocyte phospholipids

Lymphocytes were isolated as described in the Materials and methods section and were cultured in the presence of 5  $\mu\text{g}/\text{ml}$  Con A and radioactively labelled palmitic (a), oleic (b), linoleic (c) or arachidonic (d) acid (final concentration 100  $\mu\text{M}$ ). After 18, 40 or 64 h the cells were collected, washed and briefly sonicated, and the lipid was extracted using chloroform/methanol. Phospholipid classes were separated by t.l.c. (see the Materials and methods section) and the incorporation of fatty acid into phosphatidylserine (■), phosphatidylcholine (□) and phosphatidylethanolamine (▧) was determined. Data are means  $\pm$  S.E.M. for three separate cell preparations.

The fate of the fatty acids among different classes of phospholipid was also investigated (Figure 5). The incorporation of each fatty acid into each class of phospholipid increased with increasing time in culture (Figure 5) but, again, different fatty acids

appeared to have different fates: oleic acid was incorporated largely (more than 60%) into phosphatidylcholine, whereas palmitic and linoleic acids were incorporated equally into phosphatidylcholine and phosphatidylethanolamine, and arachidonic acid was incorporated more into phosphatidylethanolamine, particularly after 64 h of culture (Figure 5). Of the phospholipids, incorporation into phosphatidylserine was the lowest for all fatty acids tested (Figure 5).

#### Effect of fatty acid incorporation on lymphocyte fatty acid composition

The fatty acid compositions of the neutral and phospholipid fractions of mitogen-stimulated lymphocytes were altered after 48 h culture in the presence of a variety of fatty acids (Tables 4 and 5). Generally, both lipid fractions became enriched solely in the fatty acid added to the medium (Tables 4 and 5). For example,  $\alpha$ -linolenic acid is not normally found in either the neutral lipids or phospholipids of lymphocytes (Tables 1 and 2) but incubation with this fatty acid resulted in  $\alpha$ -linoleic acid proportions of 12% and 21% respectively in these lipid fractions (Tables 4 and 5). Similarly, the  $n-3$  PUFA eicosapentaenoic, docosapentaenoic ( $C_{22:5, n-3}$ ) and docosahexaenoic acids, which in the fresh cells comprise less than 3% of the total fatty acids, constitute more than 10% of the fatty acids of the neutral lipids and approx. 30% of those in the phospholipids after incubation of the cells with either eicosapentaenoic or docosahexaenoic acids (Tables 4 and 5). This incorporation of  $n-3$  PUFA resulted in a significant decrease in the proportion of arachidonic acid (Tables 4 and 5), so that, after incubation of the cells with  $n-3$  PUFA, the proportion of these fatty acids was greater than that of  $n-6$  PUFA (Tables 4 and 5).

Fatty acid composition data may be summarized in a variety of ways, such as the saturated/unsaturated fatty acid ratio, the percentage of PUFA or the index of unsaturation, which takes into account the number of double bonds in a fatty acid as well as its proportion. The fatty acid composition of the phospholipid fraction of lymphocytes cultured in the presence of various fatty acids is summarized in these ways in Table 6; in addition the  $n-6/n-3$  ratio is shown. These data indicate the significant effect the presence of fatty acids in the culture medium has on the phospholipid composition of lymphocytes. Culture in the presence of saturated fatty acids doubled the saturated/unsaturated fatty acid ratio and significantly decreased the percentage of PUFA and index of unsaturation (Table 6). Despite the change in fatty acid composition caused by incubation with oleic acid, none of the parameters shown in Table 6 was significantly altered. Culture in the presence of PUFA did not affect the saturated/unsaturated fatty acid ratio (Table 6), largely because the increase in the proportion of PUFA was accompanied by a significant decrease in the proportion of oleic acid (Table 5). In contrast, culture with PUFA greatly increased the proportion of PUFA (from almost 30% to over 40%; Table 6) and the index of unsaturation (Table 6). Of importance, the  $n-6/n-3$  ratio, which was approx. 10 in the control cells, was significantly increased by culture in the presence of linoleic or arachidonic acids and significantly decreased by culture in the presence of  $n-3$  PUFA (Table 6).

#### Effect of fatty acid incorporation on lymphocyte membrane fluidity

The effect of culturing Con A-stimulated lymphocytes with fatty acids on membrane fluidity was investigated by measuring  $S_{eff}$  at 25 and 37 °C (Table 7). Culture with myristic acid did not affect

**Table 4** Fatty acid composition of the neutral lipid fraction of lymphocytes cultured in the presence of fatty acids

Lymphocytes were prepared as described in the Materials and Methods section and were cultured in the presence of 5  $\mu$ g/ml Con A and 100  $\mu$ M fatty acid. After 48 h the cells were collected, washed and briefly sonicated, and the lipid was extracted using chloroform/methanol. Neutral lipids and phospholipids were separated by silicic acid column chromatography. Fatty acids were prepared by saponification and methylated. The methyl esters were separated by g.c./m.s. Data are means  $\pm$  S.E.M. for three separate cell preparations. Only the major fatty acids are shown. Statistical significance (Student's *t* test) versus control cells with no fatty acid added is indicated as follows: <sup>a</sup>*P* < 0.05, <sup>b</sup>*P* < 0.02, <sup>c</sup>*P* < 0.01, <sup>d</sup>*P* < 0.001.

Fatty acid added	Fatty acid (mol%)									
	C <sub>14:0</sub>	C <sub>16:0</sub>	C <sub>16:1, n-7</sub>	C <sub>18:0</sub>	C <sub>18:1, n-9</sub>	C <sub>18:2, n-6</sub>	C <sub>18:3, n-3</sub>	C <sub>20:4, n-6</sub>	C <sub>20:5, n-3</sub>	C <sub>22:6, n-3</sub>
None	3.9 $\pm$ 0.7	31.9 $\pm$ 1.1	1.4 $\pm$ 0.3	15.4 $\pm$ 0.8	22.9 $\pm$ 1.5	5.1 $\pm$ 0.3	n.d.	4.7 $\pm$ 0.2	0.3 $\pm$ 0.1	0.8 $\pm$ 0.2
C <sub>14:0</sub>	17.3 $\pm$ 2.1 <sup>c</sup>	34.3 $\pm$ 2.4	1.1 $\pm$ 0.2	16.1 $\pm$ 1.1	18.6 $\pm$ 0.8	4.8 $\pm$ 0.5	n.d.	3.2 $\pm$ 0.3 <sup>b</sup>	0.2 $\pm$ 0.1	0.4 $\pm$ 0.2
C <sub>16:0</sub>	4.3 $\pm$ 0.6	55.3 $\pm$ 3.3 <sup>c</sup>	0.8 $\pm$ 0.2	14.1 $\pm$ 1.2	14.3 $\pm$ 1.3 <sup>b</sup>	3.8 $\pm$ 0.2 <sup>a</sup>	n.d.	2.8 $\pm$ 0.2 <sup>c</sup>	0.2 $\pm$ 0.1	0.3 $\pm$ 0.2
C <sub>18:0</sub>	4.6 $\pm$ 0.8	24.6 $\pm$ 2.2 <sup>a</sup>	0.9 $\pm$ 0.3	36.2 $\pm$ 2.1 <sup>d</sup>	19.7 $\pm$ 1.5	3.7 $\pm$ 0.3 <sup>a</sup>	n.d.	2.5 $\pm$ 0.3 <sup>c</sup>	0.2 $\pm$ 0.1	0.4 $\pm$ 0.1
C <sub>18:1, n-9</sub>	4.2 $\pm$ 0.4	28.7 $\pm$ 1.4	1.0 $\pm$ 0.2	11.6 $\pm$ 0.8 <sup>a</sup>	39.6 $\pm$ 2.4 <sup>c</sup>	4.1 $\pm$ 0.4	n.d.	2.9 $\pm$ 0.2 <sup>c</sup>	0.2 $\pm$ 0.1	0.4 $\pm$ 0.2
C <sub>18:2, n-6</sub>	3.3 $\pm$ 0.4	27.6 $\pm$ 1.7	1.1 $\pm$ 0.3	12.2 $\pm$ 1.1	16.3 $\pm$ 1.2 <sup>a</sup>	18.7 $\pm$ 2.4 <sup>c</sup>	n.d.	6.2 $\pm$ 0.4 <sup>a</sup>	0.2 $\pm$ 0.1	0.4 $\pm$ 0.1
C <sub>18:3, n-3</sub>	3.8 $\pm$ 0.7	29.5 $\pm$ 2.1	1.6 $\pm$ 0.4	13.1 $\pm$ 2.1	17.7 $\pm$ 0.6 <sup>a</sup>	4.3 $\pm$ 0.7	12.6 $\pm$ 2.1 <sup>d</sup>	2.6 $\pm$ 0.2 <sup>c</sup>	1.4 $\pm$ 0.3 <sup>a</sup>	1.3 $\pm$ 0.3
C <sub>20:4, n-6</sub>	4.4 $\pm$ 0.4	28.1 $\pm$ 1.4	1.3 $\pm$ 0.2	11.7 $\pm$ 0.9 <sup>a</sup>	15.3 $\pm$ 0.9 <sup>b</sup>	9.2 $\pm$ 1.6	n.d.	11.6 $\pm$ 1.4 <sup>c</sup>	0.2 $\pm$ 0.1	0.4 $\pm$ 0.1
C <sub>20:5, n-3</sub>	2.9 $\pm$ 0.3	33.2 $\pm$ 2.1	2.1 $\pm$ 0.3	13.2 $\pm$ 1.1	21.1 $\pm$ 1.1	4.6 $\pm$ 0.3	n.d.	3.4 $\pm$ 0.4 <sup>a</sup>	7.9 $\pm$ 1.6 <sup>c</sup>	1.7 $\pm$ 0.4
C <sub>22:6, n-3</sub>	3.6 $\pm$ 0.5	31.6 $\pm$ 1.3	1.5 $\pm$ 0.2	16.2 $\pm$ 1.4	19.7 $\pm$ 0.7	3.8 $\pm$ 0.4	n.d.	4.1 $\pm$ 0.3	1.8 $\pm$ 0.5 <sup>a</sup>	8.2 $\pm$ 2.1 <sup>a</sup>

**Table 5 Fatty acid composition of the phospholipid fraction of lymphocytes cultured in the presence of fatty acids**

Lymphocytes were prepared as described in the Materials and methods section and were cultured in the presence of 5 µg/ml Con A and 100 µM fatty acid. After 48 h the cells were collected, washed and briefly sonicated, and the lipid was extracted using chloroform/methanol. Neutral lipids and phospholipids were separated by silicic acid column chromatography. Fatty acids were separated by saponification and methylated. The methyl esters were separated by g.c. Data are means ± S.E.M. for three separate cell preparations. Only the major fatty acids are shown. Statistical significance (Student's *t* test) versus control cells with no fatty acid added is indicated as follows: \**P* < 0.05, <sup>b</sup>*P* < 0.02, <sup>c</sup>*P* < 0.01, <sup>d</sup>*P* < 0.001. n.d. indicates not detected.

Fatty acid added	C <sub>14:0</sub>	C <sub>16:0</sub>	C <sub>16:1, n-7</sub>	C <sub>18:0</sub>	C <sub>18:1, n-9</sub>	C <sub>18:2, n-6</sub>	C <sub>18:3, n-3</sub>	C <sub>20:4, n-6</sub>	C <sub>20:5, n-3</sub>	C <sub>22:5, n-3</sub>	C <sub>22:6, n-3</sub>
None	3.2 ± 0.3	10.3 ± 0.4	2.0 ± 0.2	19.9 ± 1.3	28.9 ± 2.7	4.1 ± 0.3	n.d.	16.8 ± 0.6	0.5 ± 0.1	0.3 ± 0.1	1.3 ± 0.2
C <sub>14:0</sub>	18.3 ± 1.4 <sup>d</sup>	12.4 ± 1.1	2.1 ± 0.1	22.1 ± 1.2	21.4 ± 2.2	2.9 ± 0.2 <sup>a</sup>	n.d.	12.2 ± 0.5 <sup>c</sup>	0.4 ± 0.1	0.4 ± 0.2	0.9 ± 0.2
C <sub>16:0</sub>	4.7 ± 0.4 <sup>a</sup>	28.8 ± 1.3 <sup>b</sup>	1.5 ± 0.2	24.1 ± 1.1	19.7 ± 1.8 <sup>a</sup>	3.0 ± 0.2 <sup>a</sup>	n.d.	13.1 ± 0.6 <sup>b</sup>	0.4 ± 0.1	0.2 ± 0.1	0.7 ± 0.2
C <sub>18:0</sub>	4.2 ± 0.3	16.1 ± 0.7 <sup>c</sup>	1.8 ± 0.2	29.8 ± 1.3 <sup>c</sup>	21.6 ± 1.6	3.2 ± 0.3	n.d.	12.4 ± 0.7 <sup>c</sup>	0.4 ± 0.1	0.2 ± 0.1	0.8 ± 0.2
C <sub>18:1, n-9</sub>	3.8 ± 0.2	8.6 ± 0.8	1.4 ± 0.1	16.4 ± 0.8	39.2 ± 2.1 <sup>a</sup>	3.1 ± 0.1 <sup>a</sup>	n.d.	13.1 ± 0.4 <sup>c</sup>	0.3 ± 0.1	0.2 ± 0.1	0.6 ± 0.1 <sup>d</sup>
C <sub>18:2, n-6</sub>	3.1 ± 0.4	9.1 ± 0.7	1.8 ± 0.1	14.1 ± 0.9 <sup>a</sup>	20.4 ± 0.8 <sup>a</sup>	29.3 ± 1.4 <sup>d</sup>	n.d.	18.2 ± 0.8	0.4 ± 0.1	0.2 ± 0.1	0.5 ± 0.1 <sup>d</sup>
C <sub>18:3, n-3</sub>	2.2 ± 0.2	8.6 ± 0.4	1.7 ± 0.2	15.2 ± 1.0 <sup>a</sup>	21.6 ± 0.8	3.2 ± 0.2	n.d.	11.3 ± 0.5 <sup>c</sup>	3.1 ± 0.3 <sup>b</sup>	1.4 ± 0.2 <sup>c</sup>	1.2 ± 0.2
C <sub>20:4, n-6</sub>	3.4 ± 0.3	12.1 ± 0.9	2.4 ± 0.1	14.3 ± 0.7 <sup>b</sup>	19.7 ± 1.1 <sup>a</sup>	8.6 ± 0.4 <sup>d</sup>	n.d.	27.5 ± 1.1 <sup>c</sup>	0.7 ± 0.1	0.2 ± 0.1	0.8 ± 0.1
C <sub>20:5, n-3</sub>	2.8 ± 0.4	10.2 ± 1.1	1.4 ± 0.2	18.6 ± 1.1	16.7 ± 1.4 <sup>b</sup>	4.0 ± 0.1	n.d.	6.2 ± 0.4 <sup>d</sup>	20.7 ± 2.1 <sup>d</sup>	4.6 ± 0.3 <sup>d</sup>	3.7 ± 0.4 <sup>c</sup>
C <sub>22:6, n-3</sub>	2.7 ± 0.2	11.2 ± 0.6	1.8 ± 0.1	20.7 ± 0.9	17.4 ± 0.6 <sup>b</sup>	4.2 ± 0.2	0.8 ± 0.2 <sup>d</sup>	5.3 ± 0.3 <sup>d</sup>	2.1 ± 0.2 <sup>c</sup>	3.5 ± 0.2 <sup>d</sup>	23.1 ± 1.6 <sup>d</sup>

**Table 6 Summary of the fatty acid composition of the phospholipid fraction of lymphocytes cultured in the presence of fatty acids**

Data (means ± S.E.M., *n* = 3) are summarized from Table 5. Statistical significance (Student's *t* test) versus control cells with no fatty acid added is indicated as follows: \**P* < 0.05, <sup>b</sup>*P* < 0.02, <sup>c</sup>*P* < 0.01, <sup>d</sup>*P* < 0.001.

Fatty acid added	Saturated/unsaturated	PUFA (%)	Index of unsaturation	<i>n</i> - 6/ <i>n</i> - 3
None	0.7 ± 0.1	27.5 ± 2.1	135 ± 5	10.9 ± 1.2
C <sub>14:0</sub>	1.4 ± 0.2 <sup>a</sup>	18.8 ± 1.3 <sup>a</sup>	97 ± 4 <sup>c</sup>	10.1 ± 0.9
C <sub>16:0</sub>	1.5 ± 0.2 <sup>a</sup>	18.4 ± 1.2 <sup>b</sup>	91 ± 5 <sup>c</sup>	13.2 ± 1.3
C <sub>18:0</sub>	1.3 ± 0.2	20.0 ± 2.1	100 ± 3 <sup>c</sup>	8.5 ± 0.7
C <sub>18:1, n-9</sub>	0.6 ± 0.1	21.3 ± 3.1	125 ± 5	11.5 ± 0.6
C <sub>18:2, n-6</sub>	0.4 ± 0.1	48.6 ± 1.6 <sup>c</sup>	160 ± 6 <sup>a</sup>	43.2 ± 0.5 <sup>d</sup>
C <sub>18:3, n-3</sub>	0.5 ± 0.2	43.6 ± 1.2 <sup>c</sup>	178 ± 4 <sup>c</sup>	0.6 ± 0.1 <sup>d</sup>
C <sub>20:4, n-6</sub>	0.6 ± 0.1	40.8 ± 2.2 <sup>b</sup>	168 ± 4 <sup>c</sup>	16.9 ± 1.2 <sup>a</sup>
C <sub>20:5, n-3</sub>	0.7 ± 0.2	42.4 ± 1.6 <sup>c</sup>	206 ± 8 <sup>c</sup>	0.4 ± 0.1 <sup>d</sup>
C <sub>22:6, n-3</sub>	0.7 ± 0.1	40.0 ± 2.0 <sup>b</sup>	223 ± 6 <sup>d</sup>	0.4 ± 0.1 <sup>d</sup>

**Table 7 Effect of culture of lymphocytes with various fatty acids on the effective order parameter, S<sub>eff</sub>**

Lymphocytes were isolated as described in the Materials and methods section and were cultured in the presence of 5 µg/ml Con A and 100 µM fatty acid. After 48 h the cells were collected, washed and prepared for e.s.r. spectroscopy as described in the Materials and methods section. 5-DOXYL-stearic acid was used as the spin label. E.s.r. spectra were recorded at 25 and 37 °C and S<sub>eff</sub> calculated as described in the Materials and methods section. Data are from a single representative cell preparation.

Fatty acid added	S <sub>eff</sub>	
	25 °C	37 °C
None	0.547	0.463
Myristic	0.544	0.466
Palmitic	0.572	0.507
Stearic	0.613	0.469
Oleic	0.540	0.398
Linoleic	0.513	0.446
α-Linolenic	0.514	0.412
Arachidonic	0.505	0.426
Eicosapentaenoic	0.523	0.412
Docosahexaenoic	0.492	0.418

S<sub>eff</sub>, compared with culture with Con A alone. Culture with the other saturated fatty acids increased S<sub>eff</sub> at 25 °C (both palmitic and stearic acids) and 37 °C (palmitic acid only), which is indicative that these saturated fatty acids decrease membrane fluidity. The value of S<sub>eff</sub> was lower after culture with oleic acid (37 °C only). Culturing lymphocytes with PUFA (linoleic, α-linolenic, arachidonic, eicosapentaenoic and docosahexaenoic acids) resulted in a decrease in S<sub>eff</sub> at both temperatures (Table 7); each of these fatty acids had a similar effect on S<sub>eff</sub>. Thus culture of lymphocytes in the presence of PUFA increases plasma-membrane fluidity.

## DISCUSSION

This study investigated the changes in fatty acid composition and membrane fluidity which occur during lymphocyte culture and the effect of supplementation of the culture medium with a number of different fatty acids on these properties. This is the first study to have investigated the effect of such a wide variety of fatty acids, including fish-oil-derived *n* - 3 PUFA, in this way.



The fatty acids were used at a physiological concentration (100  $\mu$ M) and they were added to the culture medium as complexes with albumin in order to mimic the physiological means of presentation of fatty acids to cells.

Anel et al. [17] reported the fatty acid composition of the total lipid fraction of freshly prepared human peripheral blood lymphocytes, and the fatty acid composition of phospholipids and phospholipid classes has been reported for human PBL [17,31] and animal lymphocytes from many sources [e.g. 6,8]. The fatty acid composition of lymphocyte neutral lipids (Table 1) has not been reported previously. The fatty acid composition of the phospholipid fraction of freshly prepared rat lymph node lymphocytes (Table 2) resembled those reported previously for lymphocyte phospholipids (e.g. [6,8,17,31]). The neutral and phospholipid fatty acid compositions reported here also resemble those reported elsewhere for the mouse peritoneal macrophage [28].

As might be expected, the phospholipid fraction of rat lymphocytes contains a higher proportion of unsaturated fatty acids, in particular arachidonic acid, than does the neutral lipid fraction (Tables 1 and 2). Stimulating the cells with a mitogen caused significant changes in the fatty acid composition of both neutral and phospholipids: in both fractions there was a decrease in the proportions of stearic, linoleic and arachidonic acids and an increase in that of oleic acid (Tables 1 and 2). Culturing the cells in the absence of mitogen did not affect the fatty acid composition. These results can be compared with those of Anel et al. [17] who used human PBL. These authors found that after 24 h of culture the proportion of oleic acid was greater in stimulated than in quiescent cells. With longer culture the proportion of oleic acid increased whereas the proportions of stearic, linoleic and arachidonic acids decreased [17]. The changes reported in the present study using rat lymphocytes are in agreement with a previous study using human lymphocytes.

The incorporation and fate of four fatty acids (palmitic, oleic, linoleic and arachidonic) were investigated (Figures 3–5), as was the fatty acid composition of the neutral and phospholipid fractions after culture with nine different fatty acids. These fatty acids represent those whose effects on lymphocyte functions have been investigated [18–23]. In agreement with earlier studies which used radioactively labelled oleic acid [3–6], fatty acid incorporation was greatly increased into mitogen-stimulated lymphocytes. The rates of fatty acid uptake observed are difficult to compare with earlier studies as these often had not taken into account the increases in cell number and cellular protein that occur during the course of culture. However, the rates of fatty acid incorporation observed (Figure 3) are similar to those reported for mouse peritoneal macrophages [28].

Each of the fatty acids investigated had a characteristic fate; palmitic and arachidonic acids were incorporated mainly into phospholipids whereas oleic and linoleic acids were more evenly distributed between neutral and phospholipid fractions, particularly later in the culture period (Figure 4). There were also characteristic patterns of incorporation among the phospholipid classes: oleic acid was incorporated largely into phosphatidylcholine whereas the other three fatty acids were distributed more evenly between phosphatidylcholine and phosphatidylethanolamine (Figure 5).

The fatty acid compositions of both the neutral and phospholipid fractions of lymphocytes were greatly altered by culture in the presence of fatty acids (Tables 4 and 5); generally each fraction became enriched in the particular fatty acid added to the culture medium. This technique resulted in significant incorporation of fatty acids not normally found in lymphocyte lipids, such as  $\alpha$ -linolenic acid, or of fatty acids usually found in low

proportions, such as eicosapentaenoic and docosahexaenoic acids (Tables 4 and 5). The fact that these three fatty acids replace arachidonic acid in phospholipids (Table 5) may be of some importance, as eicosanoids formed from arachidonic acid have many key immunoregulatory roles [32,33]. The modification of lymphocyte phospholipids by addition of fatty acids to culture medium has been previously reported [8,31,34] but the range of fatty acids used in a single investigation has been limited. In addition, previous studies have not investigated the effects of addition of the long-chain  $n-3$  PUFA on lymphocyte fatty acid composition. It is important that the effects of  $n-3$  PUFA on cells of the immune system, such as lymphocytes, be investigated in the light of the putative immunosuppressive effects of fish oils which are rich in these fatty acids (see below).

Whether there is a change in plasma-membrane fluidity after lymphocyte activation is a controversial point, as previous studies have shown that fluidity increases [10–16] or does not change [8,9]. In contrast with the present investigation, which used e.s.r. to measure membrane fluidity, most previous studies [8,9,12–17] have used the fluorescent probe diphenylhexatriene. Experimental conditions have varied widely in the previous studies which differed according to the source of lymphocytes used (human PBL [10–12,15–17], mouse splenocytes [8,9,11,13], mouse thymocytes [9,10] and rat lymph node lymphocytes [14]), time of lymphocyte culture (1–96 h) and temperature used to make fluorescence or e.s.r. measurements. One constant feature has been the means of lymphocyte activation, which has been exclusively by a T-cell mitogen. The differences in experimental protocol cannot, however, explain the differences in the findings of previous studies. For example, studies that measured fluidity only 1 h after lymphocyte stimulation have reported no change [9] or an increase [1,12], whereas those employing longer periods of culture [8,13–17] have yielded similarly contradictory findings. The studies that most resemble the present investigation are those that used a long period of culture [13–17]; these studies have all shown an increase in membrane fluidity during the course of the culture. However, in some of these studies, unstimulated cells also underwent an increase in fluidity [15,16], such that there was no difference in fluidity between stimulated and unstimulated cells. The present investigation, in agreement with some previous studies [13,14,17], found that the fluidity of unstimulated lymphocytes did not change greatly during culture (Table 3). However, plasma-membrane fluidity was significantly increased in lymphocytes stimulated with the T-cell mitogen Con A (Table 3). This study suggests that the lymphocyte proliferative process is accompanied by a change in plasma-membrane fluidity brought about by a change in membrane phospholipid fatty acid composition.

Culture of mitogen-stimulated lymphocytes in the presence of fatty acids resulted in membrane-fluidity changes that were different from those caused by mitogen stimulation alone: the saturated fatty acids palmitic and stearic decreased fluidity whereas unsaturated fatty acids increased fluidity (Table 7). That the fatty acid composition of phospholipids is a major determinant of the membrane fluidity of rat lymphocytes is demonstrated by the correlations between the order parameter,  $S_{\text{eff}}$ , and the saturated/unsaturated fatty acid ratio ( $r = 0.753$  for  $S_{\text{eff}}$  at 25 °C and  $r = 0.713$  for  $S_{\text{eff}}$  at 37 °C; both  $P < 0.05$ ), the percentage of PUFA ( $r = -0.76$  for  $S_{\text{eff}}$  at 25 °C;  $P < 0.05$ ) and the index of unsaturation ( $r = -0.793$  for  $S_{\text{eff}}$  at 25 °C;  $P < 0.01$  and  $r = -0.695$  for  $S_{\text{eff}}$  at 37 °C;  $P < 0.05$ ) of the cells cultured in the presence of fatty acids (data taken from Tables 6 and 7).

If an increase in plasma-membrane fluidity is an inherent part of the lymphocyte activation and proliferation process, as is

suggested by this study, it can be speculated that the fluidity increase required for an optimal proliferative response must fall within particular boundaries. Thus substances that result in either decreased or increased fluidity (compared with the effect of mitogenic stimulation alone) would be expected to affect the ability of the cells to proliferate. This study provides support for this suggestion, as although saturated fatty acids decrease and unsaturated fatty acids increase lymphocyte membrane fluidity (compared with the effect of Con A alone; Table 7), both saturated and unsaturated fatty acids inhibit lymphocyte proliferation [18–22]. Further support comes from the observations that myristic acid does not affect either lymphocyte membrane fluidity (Table 7) or proliferation [18–22].

As events that occur within the plasma membrane are essential to the response of the lymphocytes, we now suggest that the changes in membrane structure (phospholipid fatty acid composition and fluidity) induced by changes in the fatty acids available to the cells prevent this response from being optimal. This may be the way by which fatty acids inhibit lymphocyte proliferation [18–21] and cytokine production [20,22]. Indeed, other agents that perturb membrane structure and/or fluidity, including ethanol, sterols and carcinogens, also inhibit lymphocyte proliferation and interleukin 2 production (see [20] for further references). There is evidence from tissues such as liver and adipose that changes in membrane properties can affect the activity of receptors, membrane-associated enzymes and signal-transduction pathways [24–27]. It is likely that such changes in lymphocyte membrane structure would have similar functional implications.

These findings have clinical implications, as suppression of lymphocyte activation and function could be of benefit in diseases in which T-lymphocytes are implicated. It is possible that dietary supplementation with PUFA-containing oils could provide a therapy for such disorders. Indeed, fish oil supplementation has been shown to be of some benefit in rheumatoid arthritis [35], multiple sclerosis [36] and psoriasis [37]. As a change in the fatty acid composition of phospholipids and membrane fluidity may be required to bring about the inhibition of lymphocyte function, it seems important to investigate these parameters in subjects undertaking dietary therapy. Furthermore, it will be important to investigate further the effects of fatty acids at the membrane level.

P.Y. is supported by an SERC Studentship. P.C.C. held a Nuffield Medical Fellowship when some of this work was carried out. We thank Peter Fisher for his assistance with carrying out e.s.r. measurements.

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