The influence of fatty acid unsaturation and physical properties of microsomal membrane phospholipids on UDP-glucuronyltransferase activity

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The relationship between lipid composition, the physical properties of microsomal phospholipids and the kinetics of liver UDP-glucuronyltransferase was studied in microsomes from guinea pigs supplied with a normal or a fat-free diet for 28 days. Fatty acid deficiency did not modify either the cholesterol/phospholipid molar ratio or the polar head group composition, but exclusively redistributed the unsaturated fatty acid pattern, by partially exchanging oleic for linoleic acid. This phenomenon accounts for the decrease of both rotational and translational mobilities of the fluorescent probes 1,6-diphenyl-1,3,5-hexatriene (DPH) and pyrene respectively. When the thermotropic behaviour of the different systems was assessed, no transition temperature (gel–liquid-crystalline) between 10 and 40 °C was seen as a consequence of the lower degree of unsaturation, either in the microsomal membranes or in the total lipid or total phospholipid extracts from the treated animals. In spite of this, the polarization ratio of *trans*-parinaric acid and the fluorescence intensity of merocyanine 540 revealed that a significant lateral phase separation occurred at 20–22 °C in the extracted phospholipids, which was smoother in the total lipid fractions and in the native microsomal membranes. Fatty acid deficiency caused an upward shift of the midpoint temperature of the lateral phase separation. Furthermore, the phosphatidylcholine extracted from the ‘normal’ microsomes showed a lateral phase separation centred at a lower temperature than that extracted from ‘fat-deficient’ microsomes. In contrast, the Arrhenius plot of UDP-glucuronyltransferase from ‘normal’ microsomes exhibited a change in slope at a higher temperature than that from treated microsomes. These results would suggest that fatty acid deficiency in guinea-pig liver microsomes, while rigidizing the bulk lipids, would segregate the most unsaturated phosphatidylcholine molecules towards the UDP-glucuronyltransferase microenvironment, in accordance with our previous results with cholesterol incorporation [Castuma & Brenner (1986) *Biochemistry* 25, 4733–4738].

INTRODUCTION

There are two main strategies with which to study the relationship between lipid composition, physical properties and membrane enzyme activity. The protein may be purified in a delipidated form and subsequently reconstituted with vesicles of defined composition (Bennett *et al.*, 1978; Singh *et al.*, 1981; East & Lee, 1982) or, alternatively, membrane lipids may be modified *in vivo* or *in vitro* (Brenner *et al.*, 1981) in order to analyse the impact of such treatments on the activity of intrinsic proteins. Using the latter approach, we have investigated the complex phospholipid effects on UDP-glucuronyltransferase activity (EC 2.4.1.17) (Castuma & Brenner, 1983, 1986*a,b*). In one of these previous studies (Castuma & Brenner, 1983), we showed that fatty acid deficiency in guinea-pig liver microsomes resulted in a decrease of the double bond index (DBI) together with an increase in the fluorescence anisotropy of 1,6-diphenyl-1,3,5-hexatriene (DPH) without changes in the cholesterol/phospholipid ratio. Moreover, the dietary treatment brought about a change from the non-hyperbolic kinetics of control UDP-glucuronyltransferase

(double-reciprocal plots deviating from linearity) to hyperbolic kinetics.

Recent reviews concerning the influence of double bonds on membrane physical properties and function (Brenner, 1984; Stubbs & Smith, 1984) have emphasized the lack of a simple correlation between fatty acid unsaturation and fluidity of biomembranes. In some cases, changes in the fatty acid composition failed to reveal significant differences in the electron-spin-resonance order parameter (Poon & Clark, 1982) or DPH fluorescence anisotropy (Jähnig, 1979), whereas in others, the fluidity variations could be artefacts (Stubbs *et al.*, 1980; Owen *et al.*, 1982). Nevertheless, our study (Castuma & Brenner, 1983) has been cited (Stubbs & Smith, 1984) as one of the few in which the increase in the fluorescence anisotropy derived solely from alterations in the fatty acyl group composition.

The present work examines closely the effect of fatty acid deficiency, not only on total acyl group composition of liver microsomes, but also on the distribution and fatty acid composition of individual phospholipids. To investigate the thermotropic physical properties, we used some well-characterized fluorescent probes: DPH to

Abbreviations used: DBI, double bond index (mol of unsaturated fatty acids × number of double bonds/mol of fatty acids); DPH, 1,6-diphenyl-1,3,5-hexatriene; I_e , fluorescence intensity of the pyrene excimer; I_m , fluorescence intensity of the pyrene monomer.

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sense the rotational mobility (Shinitzky & Barenholz, 1978), pyrene to measure translational mobility (Pownall & Smith, 1973), *trans*-parinaric acid to detect the formation of small amounts of solid-phase lipids (Sklar, 1980), and merocyanine 540, which is sensitive to the degree of lipid packing (Williamson *et al.*, 1983).

Concerning the UDP-glucuronyltransferase-lipid interactions, our kinetic findings (Castuma & Brenner, 1986a,b) were consistent with a structural rearrangement in the enzyme microenvironment when the microsomal membrane was enriched with cholesterol. We postulated that cholesterol incorporation, while rigidizing the bulk lipids, fluidized the enzyme-boundary phospholipids by segregating the unsaturated phosphatidylcholines. Hitherto, it is uncertain if the reorganization is only due to the cholesterol incorporation or whether it reflects a more general regulation mechanism. In this paper we test the validity of the model, by relating the discontinuities in the Arrhenius plots of UDP-glucuronyltransferase (Pechey *et al.*, 1978) in 'normal' and 'fat-deficient' microsomes with the phospholipid thermotropic transitions detected by fluorescent probes. The purpose of this study is to clarify, by using different methods, the influence of lipid unsaturation on membrane physical properties and on UDP-glucuronyltransferase activity.

EXPERIMENTAL

Animals and their treatment

Male guinea pigs weighing 250–300 g were kept under conditions of controlled lighting and feeding and were divided into two groups. The control group were fed on a complete semisynthetic diet according to Reid & Briggs (1953), while the fat-deficient group was given the same purified diet but with glucose substituted for the corn oil. Water was given *ad libitum*. Over the period of time studied (28 days) the two dietary treatments did not result in significant differences in the weights of either the animals or their livers.

Separation of microsomes

The animals were starved overnight and killed by decapitation without anaesthesia. All subsequent procedures were performed individually. The microsomal membranes from both normal and treated guinea pigs were prepared in 0.25 M-sucrose, as previously described (Castuma & Brenner, 1986a). Protein was determined by the method of Lowry *et al.* (1951) using bovine serum albumin (Sigma Chemical Co., St. Louis, MO, U.S.A.) as standard.

Extraction and analysis of lipids

Total microsomal lipids were extracted according to Folch's procedure (Folch *et al.*, 1957) with chloroform/methanol (2:1, v/v) and separated into neutral lipids and phospholipids on columns of silicic acid (1:20, w/w) (20 parts of silicic acid in the column for every 1 part of lipid mixture) washed with chloroform and methanol respectively. Neutral lipids were separated by one-dimensional t.l.c. on silica gel G using light petroleum/diethyl ether/acetic acid (160:38:3, by vol.). The spots were revealed by spraying the plates with a solution of $K_2Cr_2O_7$ in H_2SO_4 (7%, w/v) and further heating at 180 °C for 30 min. Quantification was performed by densitometry with authentic standards (Blank *et al.*, 1964).

Polar lipids were separated by one-dimensional t.l.c. on a mixture of silica gel H/Fluorisil (9:1, w/w) with double development solvent system (Ascott & Heaven, 1968); chloroform/methanol/ammonium/water (140:50:7:3, by vol.) in the first run and chloroform/methanol/acetic acid/water (320:40:8:3, by vol.) in the second run. Lipids were visualized by brief exposure to iodine vapour and identified by comparison with phospholipid standards. The bands were scraped off and their phosphorus contents measured by the method of Chen *et al.* (1956).

For fatty acid analysis, the total lipid Folch extract and the methanolic extract of unexposed phospholipid bands were trans-esterified in methanol/3 M-HCl (3 h at 64 °C). The resulting methyl esters were separated on a 1.8 m glass column filled with 10% 2330 on 100–200 mesh Chromosorb WAW by using a Hewlett–Packard 5840 A gas-liquid chromatograph fitted with a 5840 A GC integrator. The fatty acids were identified by comparing their relative retention times with those of reference standards. The cholesterol content was assayed by the method of Huang *et al.* (1961).

Preparation and labelling of membrane vesicles

Lipid vesicles were prepared in 0.25 M-sucrose/0.1 M-sodium-phosphate buffer, pH 7, by sonication under N_2 for 15 min in an Ultrasonic sonicator, and centrifuged at 8000 g for 20 min to recover the supernatant. Stock solutions (1 mM) of merocyanine 540, *trans*-parinaric acid and pyrene were prepared in ethanol, and DPH was dissolved in tetrahydrofuran. Appropriate amounts of the labels were added to membrane suspensions previously swirled with N_2 and the mixture was gently agitated for 30 min. For DPH (Aldrich Chemical Co., Milwaukee, WI, U.S.A.), *trans*-parinaric acid (PL Biochemicals, Uppsala, Sweden), merocyanine 540 (Molecular Probes, Eugene, OR, U.S.A.) and pyrene (Aldrich), the final lipid membrane concentrations were 50 μ M, 0.1 mM, 0.2 mM and 0.4 mM respectively. In all cases, the molar ratio of label/lipid was 1:200 or lower, to prevent energy transfer between probe molecules.

Fluorescence measurements

All the fluorescence studies were performed as previously described (Castuma & Brenner, 1986b) in an Aminco Bowman spectrofluorimeter equipped with two glan prisms for polarization measurements. Temperature scans (10–40 °C) were performed by cooling the cuvette holder at an average rate of 20 °C/h and monitoring with a thermistor probe inserted into the cuvette, just above the excitation beam.

To measure the efficiency of pyrene excimer formation, the steady-state emission spectra were recorded at 25 °C in an Aminco x-y recorder. The samples were excited at 320 nm and flushed with N_2 to avoid oxygen collisional quenching. The fluorescence intensity of the pyrene excimer divided by fluorescence intensity of the pyrene monomer (I_e/I_m) was calculated from the fluorescence intensities at 472 nm and 392 nm respectively. For merocyanine-labelled membranes, fluorescence was excited at 540 nm and the peak height at 590 nm was registered. Samples without labels served to count light scattering and stray light contributions to the fluorescence signal.

For polarization measurements, the sample was excited with vertically polarized light, while vertical (I_v) and horizontal (I_h) emission intensities were recorded. The

instrumental anisotropy (G) was determined by measuring vertical and horizontal emission values using horizontally-polarized light as described by Azumi & McGlynn (1962). Polarizer alignment was adjusted using a dilute suspension of glycogen in water (1%, w/v) (Lakowicz, 1983), and light scattering was avoided by measuring the anisotropy as a function of vesicle concentration (Lentz *et al.*, 1979). Sample absorbances were < 0.05 and no reabsorption of the emitted light occurred. Results were expressed either as fluorescence anisotropy (A) for DPH, or polarization ratio (P), for *trans*-parinaric acid after blank and instrumental anisotropy corrections according to the following equations:

$$A = \frac{[I_{\parallel}/I_{\perp} \times G] - 1}{[I_{\parallel}/(I_{\perp} \times G)] + 2} \quad P = \frac{I_{\parallel}}{I_{\perp} \times G}$$

For DPH, light was excited at 357 nm and the fluorescence recorded at 440 nm, while for *trans*-parinaric acid these values were 320 nm and 430 nm respectively.

Enzymic measurements

UDP-glucuronyltransferase activities of normal and fat-deficient animals were determined as previously described (Castuma & Brenner, 1986a) by using *p*-nitrophenol as aglycone. The incubation mixture contained 50 mM-Tris/HCl (pH 7.0), 0.4 mM-*p*-nitrophenol and 25 mM-UDP-glucuronic acid (Sigma) in a final volume of 0.6 ml. The reaction was started by the addition of the enzyme, and the disappearance of *p*-nitrophenol at 400 nm was monitored by removal of several serial aliquots from assay tubes during the course of each reaction. Velocities (nmol/min per mg of microsomal protein) were calculated from initial rates.

For Arrhenius plots, activities were determined at various temperatures between 5 and 40 °C with varying amounts of microsomal protein in the incubation mixtures (from 0.5 to 7 mg). The pH of the buffer used changed from 7.2 at 0 °C to 6.4 at 50 °C. Lines and break points were fitted by linear-regression least-squares analysis. The data were fitted to either a single straight line or two lines intersecting at a break temperature, which was varied over the temperature range measured. The slopes so calculated presented significant differences at a 95% level of confidence and their correlation coefficients were greater than those for a single line.

To assess the integrity of the microsomal permeability barrier, the latency of mannose 6-phosphatase (Arion, 1981) and UDP-glucuronyltransferase (Pechey *et al.*, 1978) were measured according to the equation:

$$\% \text{ latency} = \frac{A_T - A_U}{A_T} \times 100$$

where A_T is the activity of Triton X-100-treated microsomes and A_U is the activity of untreated microsomes.

RESULTS AND DISCUSSION

Lipid analysis

In previous work (Castuma & Brenner, 1983), we showed that fat deprivation in guinea pigs for 28 days brought about a decrease in the double bond index (DBI) together with an increase in the measured DPH fluorescence anisotropy, without any change in the

Table 1. Lipid distribution of normal and fat-deficient guinea-pig liver microsomes

Microsomes were separated and the lipids extracted and separated as detailed in the Experimental section. The results are expressed as means $\pm$ S.E.M. for four independent experiments run in duplicate. The statistical significance between test and control groups was evaluated by using the Student's *t* test.

Lipid	Proportion of lipid (g/100 g total)	
	Normal	Fat-deficient
Cholesterol	9.5 $\pm$ 0.4	9.2 $\pm$ 0.3
Cholesterol esters	1.1 $\pm$ 0.1	1.1 $\pm$ 0.1
Triacylglycerols	2.6 $\pm$ 0.2	2.5 $\pm$ 0.2
Phosphatidylcholine	46.5 $\pm$ 2.7	46.9 $\pm$ 2.7
Phosphatidylethanolamine	24.9 $\pm$ 1.6	25.3 $\pm$ 1.4
Phosphatidylinositol	7.8 $\pm$ 0.3	7.4 $\pm$ 0.3
Phosphatidylserine	3.3 $\pm$ 0.2	3.5 $\pm$ 0.1
Sphingomyelin	4.3 $\pm$ 0.2	4.1 $\pm$ 0.2
Cholesterol/phospholipid (mol/mol)	0.31 $\pm$ 0.02	0.30 $\pm$ 0.01

cholesterol/phospholipid ratio of liver microsomes. However, to exclude the possibility that these changes in the physical properties were due to modified levels of other membrane components, we have now also examined the lipid distribution and the fatty acid composition of individual phospholipids. Table 1 shows that the distribution patterns of neutral and polar lipids from 'normal' and 'fat-deficient' microsomes are the same, with the clear predominance of phospholipids (85%) over neutral lipids typical of the endoplasmic-reticulum vesicles (Brenner *et al.*, 1981). Phosphatidylcholine is the major fraction (about 55% of the total phospholipid content) followed by phosphatidylethanolamine, with other minor contributions from phosphatidylinositol, phosphatidylserine and sphingomyelin.

Table 2 gives the fatty acid composition of total lipids and individual phospholipids. The total acyl group distribution from 'normal' and 'fat-deficient' microsomes is in agreement with our previous findings (Castuma & Brenner, 1983). The fat deprivation resulted in a highly significant decrease in the linoleic acid content and a less marked decline of arachidonic and docosahexenoic acids, together with a great increase in the monounsaturated fatty acids, especially oleic acid. No compensatory rise in the proportion of eicosatrienoic acid, as reported in fat-deficient rats (Brenner *et al.*, 1981), is found in guinea-pig liver microsomes.

In this paper, the detailed phospholipid analysis reveals that phosphatidylcholine contains the largest amount of linoleic acid, but the smallest proportion of arachidonic acid. Phosphatidylethanolamine, in contrast, shows a higher content of arachidonic acid, while phosphatidylinositol exhibits the largest amount of stearic acid (approx. 40%) among all the phospholipids. In accordance with other membrane types (Carmel *et al.*, 1985), the composition of sphingomyelin shown in Table 3 is characterized by a high proportion of saturated fatty acids and an absolute lack of arachidonic acid. The fat-

Table 2. Fatty acid distribution of total lipids and individual phospholipids from normal and fat-deficient microsomes

The methyl esters of the fatty acids were separated by g.l.c. as explained in the Experimental section. The results are expressed as means $\pm$ S.E.M. for five independent experiments run in duplicate. The statistical significance between test and control groups was evaluated by using the Student's *t* test (**P* < 0.001; ***P* < 0.005; ****P* < 0.01).

Lipid	Source	Proportion of fatty acid (g/100 g total)												DBI
		C _{14:0}	C _{16:0}	C _{16:1}	C _{18:0}	C _{18:1}	C _{18:2}	C _{20:3-6}	C _{20:3-9}	C _{20:4-6}	C _{22:5-3}	C _{22:6-3}		
Total lipid	Normal	0.9±0.2	14.1±0.9	1.2±0.2	26.1±1.3	9.8±0.6	34.5±1.2	1.0±0.1	0.6±0.1	9.4±0.4	0.8±0.1	1.6±0.2	1.30±0.05	
	Fat-deficient	1.4±0.3	18.4±1.1	2.6±0.3***	25.3±1.3	21.7±0.9*	19.5±0.8*	1.2±0.2	0.7±0.1	7.5±0.3*	1.0±0.1	0.7±0.1***	1.07±0.02**	
Phosphatidylcholine	Normal	0.3±0.1	13.9±0.5	0.3±0.1	28.3±1.4	9.1±0.3	42.7±1.4	0.3±0.1	0.5±0.1	3.8±0.2	0.2±0.1	0.6±0.1	1.14±0.02	
	Fat-deficient	0.5±0.1	15.1±0.7	1.1±0.2***	31.6±1.8	10.9±0.6	36.2±1.3***	0.5±0.1	0.4±0.1	2.9±0.1**	0.3±0.1	0.5±0.1	1.00±0.01	
Phosphatidylethanolamine	Normal	0.6±0.1	9.4±0.8	1.2±0.1	28.9±1.8	8.4±0.4	32.6±1.7	0.5±0.1	0.8±0.2	13.2±0.5	1.6±0.7	2.8±0.2	1.51±0.06	
	Fat-deficient	1.2±0.1**	12.3±0.6	2.4±0.3***	33.3±1.9	12.6±0.6*	23.9±1.1**	1.2±0.1**	0.5±0.1	9.6±0.3*	1.3±0.1	1.7±0.1**	1.26±0.03	
Phosphatidylinositol	Normal	0.8±0.1	6.7±0.3	1.3±0.1	39.6±1.7	10.3±0.5	19.4±0.8	2.0±0.1	2.9±0.2	16.2±0.7	0.5±0.1	0.3±0.1	1.30±0.05	
	Fat-deficient	1.3±0.3	7.5±0.5	2.2±0.2***	43.3±1.9	13.6±0.8***	10.9±0.5*	3.7±0.2*	3.0±0.1	14.0±0.6	0.3±0.1	0.2±0.1	1.03±0.02*	
Phosphatidylserine	Normal	1.5±0.1	7.2±0.3	1.3±0.1	37.8±1.8	9.2±0.6	27.7±1.3	1.1±0.1	1.5±0.2	10.2±0.6	1.3±0.1	1.2±0.1	1.22±0.02	
	Fat-deficient	2.1±0.4	8.3±0.4	1.6±0.1	43.2±2.1	13.0±0.7**	19.3±0.9*	1.5±0.1	2.1±0.2	6.7±0.3*	1.2±0.1	1.0±0.1	1.00±0.04**	

Table 3. Fatty acid distribution of sphingomyelin from normal and fat-deficient microsomes

The methyl esters of the fatty acids were separated by g.l.c. as explained in the Experimental section. The results are expressed as means $\pm$ S.E.M. for five independent experiments run in duplicate. The statistical significance between test and control groups was evaluated by using the Student's *t* test (**P* < 0.001).

Lipid	Source	Proportion of fatty acid (g/100 g total)												DBI	
		C _{14:0}	C _{16:0}	C _{16:1}	C _{18:0}	C _{18:1}	C _{18:2}	C _{20:0}	C _{20:1}	C _{20:2}	C _{22:0}	C _{22:2}	C _{24:0}		C _{24:1}
Sphingo- myelin	Normal	1.4±0.1	10.8±0.5	1.4±0.1	30.7±1.4	9.1±0.4	17.3±0.8	1.2±0.1	0.7±0.1	2.5±0.2	19.3±0.9	1.8±0.1	2.2±0.2	1.6±0.1	0.67±0.02
	Fat- deficient	1.6±0.1	11.3±0.6	2.0±0.2	33.8±1.5	9.3±0.5	8.9±0.4*	1.6±0.2	0.9±0.1	3.0±0.1	21.2±1.2	2.0±0.1	2.6±0.1	1.8±0.1	0.51±0.01

free diet results in a decrease in linoleic acid, mainly in phosphatidylcholine and phosphatidylethanolamine and less markedly in phosphatidylinositol, together with an increase in the oleic acid content, especially noticeable in the phosphatidylethanolamine and phosphatidylserine fractions. Hence, the fat deprivation decreases the DBI of all the phospholipid classes, primarily phosphatidylinositol and less significantly phosphatidylethanolamine (Table 2).

While the DBI may be thought of as an average measure of the double bond distribution, a more accurate picture can be obtained by subdividing the contributions of the individual molecules. In this experiment, by adding the molecular content of the major fatty acids in all the phospholipids, it appears that normal phospholipids contain roughly (per 100 mol) 54 mol of saturated fatty acids, 8 mol of oleic acid, 30 mol of linoleic acid and 8 mol of arachidonic acid. The fat-free diet results in an increase of 2 mol of saturated fatty acids and 8 mol of oleic acid, together with a decrease of 9 mol of linoleic acid and 1 mol of arachidonic acid. In consequence, our results now demonstrate that fat deprivation in the guinea-pig microsomal membrane is a good experimental system for studying the influence of unsaturation on physical properties, since the dietary treatment does not modify either the cholesterol/phospholipid molar ratio or the polar head group composition, but exclusively redistributes the unsaturated fatty acid pattern by partially exchanging oleic for linoleic acid.

Relationship between lipid composition and physical properties

The relationship between the lipid composition and the dynamic properties of the 'normal' and 'fat-deficient' membranes was examined from the points of view of the rotational and translational mobility of DPH and pyrene respectively.

Fig. 1 shows the thermotropic analysis of total lipids and total phospholipid dispersions labelled with DPH. In all cases the straight lines provide no evidence of phase transition within the temperature range scanned. Since higher anisotropy values indicate a more restricted motional freedom of the probe molecule, and the anisotropy of the lipid fraction was always lower than that of the corresponding membranes for the two preparations, it can be concluded that microsomal proteins play a role in controlling the physical state of the membrane lipids (Pugh *et al.*, 1980). The higher anisotropy values for total lipids compared with total phospholipids confirm here that the rigidizing effect of the cholesterol cannot be offset by the presence of small amounts of triacylglycerols (Stubbs *et al.*, 1980). These results are in accordance with those obtained in our laboratory by Garda & Brenner (1985) using rat liver microsomes. Moreover, in all types of membranes analysed, the fluorescence anisotropy of the labelled vesicles from 'fat-deficient' animals is higher than that of the normal ones, clearly showing a decreased rotational mobility of the probe in the less unsaturated environment.

The rates of lateral diffusion in the endoplasmic-reticulum membranes were evaluated by measuring the efficiency of pyrene excimer formation. The effect of the degree of unsaturation upon the rate of lateral mobility of the probe is shown in Fig. 2, where the I_e/I_m ratio is plotted versus pyrene concentration. The efficiency of

Fig. 1. Thermotropic properties of membranes, total lipids and total phospholipid dispersions labelled with DPH

Samples were prepared as described in the Experimental section. Each point represents the mean $\pm$ S.E.M. for five independent experiments run in duplicate. Symbols: $\circ$, normal microsomes; $\square$, total lipids from normal microsomes; $\triangle$, total phospholipids from normal microsomes; $\bullet$, fat-deficient microsomes; $\blacksquare$, total lipids from fat-deficient microsomes; $\blacktriangle$, total phospholipids from fat-deficient microsomes. A, Fluorescence anisotropy.

pyrene excimer formation increases linearly with the probe concentration for the 'normal' and 'fat-deficient' membranes. For the latter, the lower I_e/I_m ratio suggests a decreased translational mobility caused by decreased levels of membrane unsaturation.

The relationship between the fatty acid unsaturation and the fluidity of biological membranes is still a subject of controversy. In some studies the physical parameter under investigation remained unaffected by changes in the degree of unsaturation of fatty acids (Jähnig, 1979; Poon & Clark, 1982), and in others (York *et al.*, 1982; Castuma & Brenner, 1983) variations in the physical properties that derived solely from alterations in the hydrocarbon chains have been reported.

In spite of this, it is well documented (Brenner, 1984; Stubbs & Smith, 1984) that the introduction of a *cis* double bond disrupts the cooperativity of the tightly packed *trans* bonds of saturated fatty acids, increasing the cross-sectional area per fatty acyl chain and, in this way, the so-called fluidity. However, recent reviews (Brenner, 1984; Stubbs & Smith, 1984) have emphasized the lack of a linear relationship between the number of double bonds and the membrane fluidity. Particularly, ideas about the role of the second double bond, i.e. of polyunsaturated fatty acids, in biological membranes

Fig. 2. Efficiency of pyrene excimer formation at 25 °C

Samples were prepared as described in the Experimental section. Each point represents the mean $\pm$ S.E.M. for five independent experiments run in duplicate. Symbols correspond to those of Fig. 1.

has been subjected to criticism (Lee *et al.*, 1986), principally based on the work of Stubbs *et al.* (1981). These authors, by performing dynamic anisotropy measurements in artificial membranes, have shown that although the first double bond increases both the range and rate of the motions, the second double bond produces a considerably lesser effect on the range of the movement and the third and fourth bring about no further increase, but rather a slight decrease.

Our present experiments help to clarify the effect of polyunsaturated fatty acids on the fluidity of biological membranes. The fatty acid deficiency partially substitutes oleic for linoleic acid, a phenomenon to which, in the absence of compensatory changes in other lipid components, the observed increase in the stationary fluorescence anisotropy has to be ascribed. The latter result can be rationalized on the basis of a decline in the rate of the motions of the hydrocarbon chains since, on close inspection, the work of Stubbs *et al.* (1981) shows a definite increase in the rate of motions with the number of double bonds per molecule.

We have also investigated the occurrence of a lateral phase separation above the gel-liquid-crystalline transition temperature. Owing to the preference of *trans*-parinaric acid for solid-phase lipids, the polarization ratio of this probe keeps fairly constant with temperature for a given percentage of solid phase (Sklar *et al.*, 1979), and increases rapidly with the appearance of a small percentage of gel phase in a fluid system, i.e. at the onset of a lateral phase separation. The temperature dependence of polarization ratio values for *trans*-parinaric acid in microsomes, total lipid and total phospholipid extracts is shown in Fig. 3. For total

Fig. 3. Thermotropic properties of membranes, total lipids and total phospholipid dispersions with *trans*-parinaric acid

Samples were prepared as described in the Experimental section. Each point represents the means $\pm$ S.E.M. for five independent experiments run in duplicate. Symbols correspond to those of Fig. 1. *P*, polarization ratio.

Fig. 4. Effect of cholesterol addition on the thermotropic properties of total phospholipids labelled with *trans*-parinaric acid

Samples were prepared as described in the Experimental section. Each point represents the means $\pm$ S.E.M. for five independent experiments run in duplicate. Symbols: $\square$, total lipids from normal microsomes; $\triangle$, total phospholipids from normal microsomes; $\bullet$, total phospholipids from normal microsomes + cholesterol in the same amount found in the original membrane; $\blacksquare$, total lipids from fat-deficient microsomes; $\blacktriangle$, total phospholipids from fat-deficient microsomes; $\circ$, total phospholipids from fat-deficient microsomes + cholesterol in the same amount found in the original membrane. The curves corresponding to the symbols $\square$, $\triangle$, $\blacksquare$, $\blacktriangle$ are those extracted from Fig. 3. *P*, polarization ratio.

lipids, and to a lesser degree for microsomes, the change in polarization ratio is relatively smooth, although the comparison of slopes above and below 20–22 °C suggests some lateral phase separation. However, the abrupt increase of the polarization ratio in the phospholipid fraction clearly points out the appearance of a solid phase within the bilayer. The midpoint of the lateral phase separation obtained from differential plots (results not shown) occurs at 19 °C and 23 °C for phospholipids of normal and fat-deficient animals respectively.

To demonstrate that the partial obliteration of the total lipid phase separation is attributable to the presence of the sterol rather than to another component of the neutral lipid fraction, cholesterol was added to the membrane phospholipid so as to reach the amount present in the total lipid fraction. Fig. 4 compares the polarization ratio values of *trans*-parinaric acid for this mixture with those of total lipids and phospholipids from Fig. 3. The data reveal that the addition of the sterol results in a marked decrease in the amount of lipid involved in the phase separation and a curve profile similar to the total lipid fraction in both normal and fat-deficient membranes.

Merocyanine 540 is a dye sensitive to the degree of lipid packing of bilayers, and its fluorescence intensity at 590 nm increase markedly when it is inserted in membranes whose lipids are more widely spaced (Williamson *et al.*, 1983). Fig. 5 shows that both in microsomes and in the total phospholipid extracts, the

fat-deficient diet brings about a more efficient packing of the fatty acyl chains. Moreover, the dye is able to detect a phase separation at about 20–22 °C for the phospholipid fraction in a similar way to *trans*-parinaric acid (Fig. 3). When the results shown in Figs. 3, 4 and 5 using two different fluorescent probes are compared, it emerges that guinea-pig liver microsomal phospholipids undergo a lateral phase separation at about 20–22 °C, which agrees with the estimate made by Pechey *et al.* (1978). This transition is partially masked in the total lipid fraction by the effect of cholesterol and in the microsomal membranes by the smoothing effect of proteins (Gally *et al.*, 1980). To get further insight into the molecular mechanism of the lateral phase separation and its relationship with the degree of unsaturation, the phosphatidylcholines from 'normal' and 'fat-deficient' microsomes were labelled with both *trans*-parinaric acid and merocyanine 540. Since the midpoint of a phase separation can be more accurately determined from differential plots, the corresponding $\partial I/\partial t$ and $\partial P/\partial t$ for both types of phosphatidylcholines were plotted against the temperatures (Fig. 6). The fluorophores sense a thermotropic structural reorganization in accordance with that found by Rintoul *et al.* (1979). As could be expected from the decrease in the number of double bonds (Brenner, 1984), the midpoint of the lipid phase separation is shifted from 19 °C to 26 °C in the phosphatidylcholines of 'fat-deficient' compared with 'normal' microsomes.

Fig. 5. Thermotropic properties of membranes and total phospholipids labelled with merocyanine 540

Samples were prepared as described in the Experimental section. Each point represents the mean $\pm$ S.E.M. for five independent experiments run in duplicate. Symbols correspond to those of Fig. 1.

Effect of phospholipids on microsomal UDP-glucuronyltransferase

There is evidence that certain enzymes can segregate specific lipids out of the bulk lipid pool to constitute their lipid 'annulus' (Houslay & Palmer, 1978). In doing so, these enzymes create a microenvironment whose physical properties need not reflect those of the bulk lipid pool. In previous papers (Castuma & Brenner, 1986a,b) we explained our results on the effect of cholesterol on lipid-protein interactions on the basis that cholesterol incorporation in the microsomal membrane, while increasing the packing of the bulk lipids, fluidized the enzyme microenvironment by segregating the most unsaturated phosphatidylcholines. This hypothesis is also supported by our kinetic findings (Castuma & Brenner, 1986a,b), those of Hochman & Zakim (1983) and the specific requirement of the delipidated UDP-glucuronyltransferase for phosphatidylcholine demonstrated by Singh *et al.* (1981). Furthermore, it is well known that the cholesterol molecule interacts preferentially with the most saturated phosphatidylcholines (Gruyer & Bloch, 1984). However, we could not ascertain whether the model reflected a general regulation mechanism or if it was restricted to the cholesterol incorporation.

Further information is given by the Arrhenius curves of UDP-glucuronyltransferase in 'normal' and 'fat-deficient' microsomes (Fig. 7). The plots show two linear regions intersecting with a sharp transition. The UDP-glucuronyltransferase from 'normal' membranes exhibited a change in slope at 23.5 °C and the Arrhenius activation energy (E_a) increased from 28 kJ/mol below the critical temperature (T_c) to 71 kJ/mol above this temperature, in agreement with the results of Pechey *et al.* (1978). The fat-deficient diet resulted in a small

Fig. 6. Thermotropic properties of phosphatidylcholines labelled with merocyanine 540 and *trans*-parinaric acid

Samples were prepared as described in the Experimental section. Each point represents the mean for five independent experiments run in duplicate. Symbols: $\triangle$, phosphatidylcholine from normal microsomes; $\blacktriangle$, phosphatidylcholine from fat-deficient microsomes. (a) Differential plot of phosphatidylcholines labelled with merocyanine 540. The $\partial I/\partial t$ is extracted from plots of fluorescence intensity versus temperature. (b) Differential plot of phosphatidylcholines labelled with *trans*-parinaric acid. The $\partial P/\partial t$ is extracted from plots of P versus temperature.

Fig. 7. Arrhenius plots of UDP-glucuronyltransferase

The incubation mixture and other conditions were as described in the Experimental section. Each point represents the mean $\pm$ S.E.M. for five independent experiments run in duplicate. Symbols: $\circ$, normal microsomes; $\bullet$, fat-deficient microsomes. v is expressed as nmol of *p*-nitrophenol transformed/min per mg of protein. The critical temperatures, T_c , were determined by a statistical analysis (Hoare, 1972). The values underlined are the Arrhenius activation energy (E_a , kJ/mol).

lowering of the T_c to 18.3 °C and a shift of the E_a below this temperature to 32 kJ/mol.

To confirm the validity of these results, we must consider that intact membrane phospholipid structure exerts two types of effect on UDP-glucuronyltransferase. On one hand, the enzyme depends for full activity on its association with phospholipids (Singh *et al.*, 1981; Hochman & Zakim, 1983). On the other hand, in native microsomes, the enzyme is latent and intact membrane structure restricts activity. Mild degrees of perturbation activate the enzyme (by removing latency), while more severe treatments inactivate the transferase molecule by disrupting essential interactions with phospholipids (Cummings *et al.*, 1984). For this reason, the integrity of the phospholipid permeability barrier in both types of microsomal membranes was assessed by measuring the percentage of latency of both mannose-6-phosphatase and UDP-glucuronyltransferase. For the phosphatase, these values were 90 % and 92 % for 'normal' and 'fat-deficient' microsomes and for the transferase 86 % and 87 % respectively. These results rule out the possibility of a bilayer perturbation caused by the dietary treatment. In agreement with this, Pechey *et al.* (1978) reported that biphasic Arrhenius plots were generated only by the latent microsomal enzyme.

In general, for membrane-bound enzymes, T_c values in the Arrhenius plots can be correlated with the transition temperature of the bulk lipids (Danks & Tribe, 1979), or with a phase separation of the bulk lipids (Houslay & Palmer, 1978). However, a number of biophysical and biochemical techniques indicate that the nonlinearity of the activity profile of many membrane-bound enzymes is linked to a phase separation or a phase transition of the

lipids surrounding the enzyme molecule (Brasitus, 1983).

In our experiments, the change in slope in the Arrhenius plots of the UDP-glucuronyltransferase from 'normal' microsomes occurs at some degrees higher (23.5 °C) than that for the enzyme from 'fat-deficient' microsomes (18.3 °C) (Fig. 7). By comparing Figs. 6 and 7 we can state that the discontinuity in the slope of the Arrhenius plots from 'fat-deficient' microsomes is shifted towards lower temperatures like the discontinuity temperature of the highly unsaturated phosphatidylcholines from 'normal' microsomes and vice-versa.

Taking into account (a) the preference of purified guinea-pig UDP-glucuronyltransferase for fluid phosphatidylcholines (Singh *et al.*, 1981); (b) our present results showing that the Arrhenius plot of the enzyme from 'fat-deficient' microsomes exhibits a break shifted to lower temperatures like that for highly unsaturated phosphatidylcholines; and (c) the widespread preference of the enzymes for the more fluid phases (Kleeman & McConnell, 1976), we can add new evidence to generalize our previous model. Therefore, we can suggest that the fatty-acid deficiency in guinea-pig liver microsomes, while rigidizing the bulk microsomal lipids, would segregate the highly unsaturated phosphatidylcholine molecules towards the UDP-glucuronyltransferase microenvironment.

Although Lee *et al.* (1986) have demonstrated the lack of a direct correlation between the degree of unsaturation and enzyme activities, the present work provides physical evidence indicating that changes in the levels of polyunsaturated fatty acids involve changes in the overall lipid organization which, sensed at the protein-lipid interface, may influence enzyme kinetics.

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