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Effect of dietary cholesterol on microsomal membrane composition, dynamics and kinetic properties of UDPglucuronyl transferase

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The effect of cholesterol administration in vivo on the lipid composition, dynamic properties of the microsomal membrane of guinea pig livers and the kinetic properties of UDPglucuronyl transferase were studied. Cholesterol administration in the diet evoked an increase of microsomal cholesterol, but no significant changes in the fatty-acid composition of total lipids or of each phospholipid class. Instead, the phosphatidylethanolamine, phosphatidylcholine molar ratio of the membrane was markedly decreased from 0.57 to 0.38. This decline was not enough to counterbalance the overall 'ordering' effect of cholesterol and consequently, the fluorescence anisotropy of the membranes labeled with 1,6-diphenylhexatriene was increased. The lateral diffusion evaluated by measuring the pyrene excimer formation was decreased by the cholesterol incorporation. These physical changes were associated with changes in the kinetic properties of UDPglucuronyl transferase: V_{max} increased, while the K_m of the different steps of the reaction decreased in the modified microsomes. Furthermore, a shift of the non-michaelian kinetics to michaelian, equivalent to a decrease of a negative homotropic effect and apparent cooperativity of UDPglucuronic acid was observed since the Hill coefficient changed, approaching 1. A non-michaelian kinetics of this enzyme is an indication of boundary lipids in the gel phase and a shift to michaelian, a change of the surrounding lipids to a liquid-crystalline structure. In consequence, our results suggest that cholesterol incorporation in the microsomal membrane while producing a condensing effect of bulk lipids would produce an opposite effect on the UDPglucuronyl transferase boundary lipids.

Introduction

The catalytic activities of several enzymes of biological membranes have a demonstrated dependence on interactions with the phospholipid environment of the bilayer [1,2]. UDP-glucuronyl transferase belongs to this type of enzymes [3–5] and shows a complex lipid induced modulation of its kinetic properties.

Two schools of thought have tried to explain the mechanism of this modulation. One school [6–8], the so called compartmentation theory, considers that the enzyme is deeply embedded in the membrane with the active center exposed to the luminal surface of the microsomes [8]. The modulating effect will be produced in the hydrophobic

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Abbreviations: Cyt, cytochrome; PC, phosphatidylcholine; DPH, diphenylhexatriene; PE, phosphatidylethanolamine; *p*-NP, *p*-nitrophenol.

barrier of the bilayer altering the transport of the substrates to the enzyme [9] through this barrier.

The alternative theory developed by Zakim and Vessey [10] considers that the active center of the enzyme is not deeply embedded and the transport of the substrate to the enzyme is not the regulatory step. According to them, glucuronyl transferase for any substrate exists in several different conformational isomers, each possessing its own kinetic properties. The phospholipid perturbing agents would modify the lipid structure and correspondingly would evoke a redistribution of conformational isomers of the enzyme.

Besides, it has been shown that reactions requiring apparently lateral or rotational diffusion or a vertical transport across the membrane could be accelerated by an increase of membrane fluidity or decreased packing [11–15]. As regards the microsomal membrane, it has been found [16] that fluidizing agents as isoamyl and *n*-butyl alcohol decrease both the fluorescence anisotropy of membrane labeled with 1,6-diphenylhexatriene and the excimer formation of microsomes labeled with pyrene. Correspondingly, they increase the activity of the NADH-Cyt *c* reductase and glucose-6-phosphohydrolase.

Moreover, it has been shown that essential fatty-acid deficiency produces a modification of the fatty-acid composition of the phospholipids of guinea-pig liver microsomes. It is represented by a progressive decrease of the unsaturated/saturated fatty-acid ratio evoking both an increase of the fluorescence anisotropy of membrane labeled with 1,6-diphenylhexatriene and a progressive decline of the apparent negative cooperative effect of UDP-glucuronic acid on UDP-glucuronyl transferase [17].

Since cholesterol incorporation alters the physical properties of the lipid bilayers [18–20] decreasing the cross-sectional area per molecule [21] and flexibility of fatty-acid chains increasing the membrane rigidity above the gel–liquid crystalline phase transition, it was considered important to investigate the effect on the kinetic properties of UDP-glucuronyl transferase. The present results show that dietary cholesterol incorporated in liver microsomes, evoking a change in the phosphatidylcholine (PC); phosphatidylethanolamine (PE) ratio, increasing fluorescence anisotropy of membranes labeled with diphenylhexatriene and decreasing the efficiency of pyrene excimer formation as well as a modification of kinetic properties of UDP-glucuronyl transferase with an increase of the Hill coefficient for UDP-glucuronic acid.

Materials and Methods

Dietary regimens

Guinea pigs weighing 150–250 g were divided in two groups of five animals each. The control group was fed a synthetic diet according to Reid and Briggs [22] and the other group the same synthetic diet plus 3% cholesterol. Both groups were given food in restricted amounts so that the dietary intakes of test and control animals were of equal energy value. Water was given ad libitum.

Isolation of microsomes

Over a 25-day-period animals were killed by decapitation without anesthesia. Livers from each animal were rapidly excised and placed immediately in ice-cold 0.25 M sucrose (1 : 3, v/v). The microsomal fraction was separated by differential ultracentrifugation as previously described [17]. The microsomal pellet was washed once and resuspended with the same homogeneizing solution (1 : 2, v/v). Microsomes were stored at $-80°C$ under N_2 atmosphere until needed. Protein was determined by the method of Lowry et al. [23].

Steady-state fluorescence anisotropy measurements

1 μl of 2 mM 1,6-diphenylhexatriene in tetrahydrofuran was added to 4 ml of microsomal suspension (0.1 mg protein/ml) in 0.25 M sucrose/1.5 mM EDTA (pH 7.0) with rapid agitation. The final lipid concentration was 25 μM and final label concentration 0.25 μM. Another aliquot of the same suspension mixed with an equivalent volume of tetrahydrofuran was used as a reference blank in order to correct fluorescence intensities from non-specific light-scattering excitation. Samples were gently swirled for at least 1 h to allow equilibration of the probe with membrane lipids. The incorporation of 1,5-diphenylhexatriene can be easily followed by recording fluorescence enhancement with time, since this probe has nearly zero fluorescence signal in an aqueous environment.

Our previous studies showed that the time required for 1,6-diphenylhexatriene penetration under these experimental conditions was less than 50 min. Final concentrations of lipids and label were chosen after the subsequent dilutions of samples demonstrated no further effect on measured anisotropy, due to depolarization of fluorescent light by scattering. Moreover, this label-to-lipid ratio was also suitable to avoid probe–probe interaction and perturbation of the lipid domain.

Measurements were made at 37°C in an Aminco–Bowman spectrofluorometer equipped with two glan polarizers. 1,6-Diphenylhexatriene was excited at 350 nm and its fluorescence was detected at 430 nm using a 2.0 M $NaNO_2$ solution as a cut-off filter for wavelengths below 300 nm. Notwithstanding the use of this device, blank corrections were still needed. The rotational motion of the probe molecule is characterized by the steady-state fluorescence anisotropy defined by

$$r_s = \frac{I_{\parallel} - GI_{\perp}}{I_{\parallel} + 2GI_{\perp}}$$

where $I_{\parallel}$ and $I_{\perp}$ are the fluorescence intensities parallel and perpendicular, respectively, to the plane of polarization of the excitation beam and G, the grating correction factor, takes account of parallel diffraction anomalies introduced by the monochromator [24].

Apparent microviscosity and order parameter S_{DPH}

The apparent microviscosity of the membrane as measured in the present work should be regarded as an operational term which can be estimated by comparing the fluorescence anisotropy in the membrane system with that observed in a reference solvent of known viscosity.

The apparent microviscosity was calculated using Perrin's equation [25] as proposed by Shinitzky and Barenholz [26] according to

$$\bar{\eta} = \frac{24\, r_s}{r_0 - r_s}$$

where r_0 is the limiting fluorescence anisotropy of the probe in a freeze solvent in absence of rotation.

Recently, the application of Perrin's equations has been submitted to criticism [27–29] because of anisotropy demonstrated by membranes. In view of this fact, fluorescence anisotropy is resolved in a fast decaying component (r_f) and an infinitely slow decaying component (r_∞) by the procedure of Van Blitterwijk et al. [27–29] using the empirical curves of these authors. The order parameter S_{DPH} referring to the mean position of the diphenylhexatriene (DPH) along the lipid acyl chains [28] was calculated by the equation

$$S^2_{DPH} = \frac{r_\infty}{r_0}$$

where r_0 is the limiting anisotropy of the probe here considered equal to 0.4 [29].

Pyrene excimer formation

The excimer formation is a diffusion-limited process and for this reason suitable to measure lateral mobility in membranes [21]. A microsomal suspension containing 0.5 mg of protein/ml was prepared with deaerated sucrose 0.25 M/EDTA 1.5 mM (pH 7.0). Different volumes of a pyrene solution in acetone were mixed with the microsomal suspension reaching final concentrations between 2 and 7 μM in pyrene. Samples without pyrene were prepared to discount the light scattering and intrinsic fluorescence contribution to the fluorescence signal. All procedures and spectra recording were carried out under N_2.

The emission spectra were obtained at 25°C in an Aminco–Bowman spectrofluorometer with an Aminco X–Y recorder. The excitation wavelength was 320 nm. The fluorescence intensity ratio of excimer to monomer (I_E/I_M) was calculated from the ratio of fluorescence intensities at 472 nm and 392 nm after blank correction.

Enzyme assays

p-Nitrophenol was used as aglycon in all studies. Glucuronidation was measured according to Zakim and Vessey [31]. The incubation mixture contained 50 mM Tris-HCl (pH 7.4), *p*-nitrophenol (0.2–0.5 mM), UDP-glucuronic acid (0.2–25 mM) and 1–1.5 mg of microsomal protein in a final volume of 0.5 ml. The reaction was started by addition of the enzyme and the disappearance of *p*-nitrophenol at 400 nm was followed [32] by removal of several serial aliquots from assay tubes during the course of each reaction. Absorbance

data were then fitted in a polynomial of the form $ax^3 + bx^2 + cx + d$, where c is the inital rate of reaction calculated at 'zero time'.

Lipid analysis

Aliquots of the suspended microsomes were extracted by the procedure of Folch et al. [33]. Fatty-acid composition was determined by gas–liquid chromatography as previously described [17]. Polar lipids were separated by TLC with a double development [34] using the solvents $Cl_3CH/CH_3OH/NH_3/H_2O$ (70 : 25 : 35 : 1.5) in the first run and Cl_3CH/CH_3OH/acetic acid/H_2O (80 : 10 : 2 : 0.75) in the second run.

Neutral lipids were separated by TLC in rods coated with silica gel using successively two solvent mixtures. In the first place, hexane/ethyl ether/acetic acid (80 : 20 : 1) and the lipids from the top to the free-acid spot were burnt and quantitated by flame ionization [35]. The lipids remaining in the low part of the rod were now developed with hexane/ethyl ether/acetic acid (50 : 50 : 1) and quantitated by flame ionization as in the first part.

Cholesterol was determined by the procedure of Huang et al. [36]. Phosphorus of lipids was measured by the method of Chen et al. [37].

Results

Effect of cholesterol on the lipid composition of guinea-pig liver microsomes

Dietary cholesterol is incorporated in guinea-pig liver microsomes. After 25 days of feeding on a diet containing 3% cholesterol guinea pigs showed an approximate duplication of the microsomal percentage (Table I). When cholesterol was incorporated in liver microsomes, the molar ratio cholesterol/phospholipids increased from 0.31 to 0.46 (Table I). This value approaches, but does not reach, the maximum value observed in most membranes, a 1 : 1 molar ratio suggested by line width of NMR measurements and X-ray difraction [38,39]. Cholesterol administration also evoked other changes in the lipid composition of the microsomal membranes. Data of Table I indicate an important variation of the phosphatidylcholine/phosphatidylethanolamine ratio evoked by the treatment. A slight relative decrease of PC and the more pronounced decrease of PE are responsible for the change of the PE/PC molar ratio from 0.57 to 0.38 (Table I).

The effect of dietary cholesterol on the fatty-acid composition of liver microsomes of guinea pigs is shown in Table II. The comparison of these animals with the controls indicates no significant differences, although some slight variation in linoleic, palmitoleic and arachidonic acids can be seen. In spite of the constancy of the fatty-acid composition of total microsomes, it was possible that individual phospholipids might show special differences. For this reason, the separation of PC, PE and phosphatidylinositol was carried out as stated in Materials and Methods and their fatty-acid composition analyzed (Table III). Once again, no

TABLE I

LIPID COMPOSITION OF GUINEA-PIG LIVER MICROSOMES (% WEIGHT)

Results are the mean of five animals analyzed separately ± S.E.

$$\frac{\text{Double bond index}}{\text{Saturated fatty acid}} = \frac{\Sigma(\text{number unsaturated mol} \times \text{number double bond})}{\Sigma \text{ number saturated mol}}$$

Lipids	Normal	Normal + cholesterol
Cholesterol	13.4 ±0.4	28.7 ±2.3
Cholesterol esters	2.8 ±0.1	4.6 ±0.8
Triacylglycerol	3.6 ±0.2	3.9 ±0.9
Phosphatidylcholine	47.8 ±1.8	41.5 ±1.9
Phosphatidylethanolamine	25.3 ±0.4	13.7 ±0.5
Phospahtidylinositol	7.1 ±0.1	7.6 ±0.6
Double bond index/saturated fatty acid	2.97 ± 0.03	3.04 ± 0.04
Cholesterol/phosphatidylcholine (mol/mol)	0.31 ± 0.10	0.46 ± 0.08
Phosphatidylethanolamine/phosphatidylcholine (mol/mol)	0.57 ± 0.04	0.36 ± 0.06

TABLE II

FATTY-ACID COMPOSITION OF LIVER MICROSOMES (% WEIGHT)

Results are the mean of five animals analyzed separately ± S.E.

Fatty acid	Normal	Normal + cholesterol
14:0	0.8 ± 0.1	0.5 ± 0.1
16:0	15.6 ± 0.4	14.3 ± 0.3
16:1	1.1 ± 0.2	1.0 ± 0.5
18:0	26.2 ± 0.1	26.0 ± 0.6
18:1	9.2 ± 0.6	8.5 ± 0.4
18:2	32.5 ± 0.7	34.7 ± 0.7
20:3	1.2 ± 0.1	1.0 ± 0.4
20:4	10.5 ± 0.2	12.0 ± 0.2
22:5	1.3 ± 0.3	1.0 ± 0.3
22:6	1.6 ± 0.1	1.0 ± 0.2

significant differences were found, except for the small changes in the fatty acids mentioned above.

Fluorescence anisotropy and pyrene excimer formation

Structure properties of both normal and cholesterol-enriched microsomes were studied by physical methods. Table IV shows fluorescence anisotropy measurements converted either in microviscosities (η) or in order parameter (S). They were remarkably increased when cholesterol was incorporated in accordance with the well-known ordering effect of the sterol molecule above the transition temperature of the lipid bilayer. To corroborate that this effect is closely related to a condensation of the bilayer structure, lateral diffusion of microsomes was estimated by measuring the pyrene excimer formation. Fig. 1 shows the effect of cholesterol on the plots of I_e/I_m vs. pyrene concentration. The efficiency of pyrene excimer formation increased linearly with the pyrene concentration for both normal and treated microsomes, but cholesterol evoked a decrease of the I_e/I_m ratio indicating a condensing effect on the membrane.

Since the cholesterol-rich diet evoked a decrease of the PE/PC ratio of the microsomal membrane in addition to the cholesterol incorporation, it was significant to investigate the contribution of this phospholipid change on the physicochemical properties of the membrane. For this reason, PC and PE of the guinea pig liver microsomes were

TABLE III

FATTY-ACID COMPOSITION OF PHOSPHOLIPIDS FROM NORMAL AND CHOLESTEROL-ENRICHED MICROSOMES

NPC, phosphatidylcholine from normal microsomes; +CPC, phosphatidylcholine from cholesterol-enriched microsomes; NPE, phosphatidylethanolamine from normal microsomes; +CPE, phosphatidylethanolamine from cholesterol-enriched microsomes; NPI, phosphatidylinositol from normal microsomes; +CPI, phosphatidylinositol from cholesterol-enriched microsomes. Results are the mean of five animals on average.

Fatty acid	NPC	+CPC	NPE	+CPE	NPI	+CPI
14:0	–	0.3	11	–	2.2	1.4
16:0	16.8	13.5	8.9	7.6	11.8	7.3
16:1	0.8	0.9	1.3	0.5	3.9	1.8
18:0	25.0	24.3	26.5	27.2	29.4	36.2
18:1	10.9	8.9	9.3	8.6	16.1	10.9
18:2	42.1	41.7	28.7	31.0	17.6	20.8
18:3	–	1.0	2.0	0.8	4.4	3.6
20:3ω9	0.5	1.0	1.1	0.5	3.2	2.8
20:4ω6	2.8	4.4	11.9	15.5	8.1	11.0
20:5ω3	–	1.2	1.0	2.0	–	1.7
22:3ω3 / 22:4ω6	0.5	1.0	2.3	1.6	1.5	1.1
22:4ω3 / 22:5ω6	0.2	0.7	2.0	0.8	1.8	–
22:5ω3	0.1	0.5	1.4	1.2	–	0.1
22:6ω3	0.3	0.6	2.5	2.7	–	1.3

TABLE IV

EFFECT OF CHOLESTEROL ON THE LIPID COMPOSITION, AND FLUORESCENCE ANISOTROPY OF LIVER MICROSOMES

3% of cholesterol in the diet during 20 days. Fluorescence anisotropy (r_s) was determined by fluorescence polarization as described in Materials and Methods. Apparent microviscosity (η) as well as S_{DPH} were calculated as previously described. Experiments were carried out at 37°C. Results are the mean of 5 animals analyzed separately ± S.E.

Microsomes	Double bond index	Saturated fatty acid		
		Fluorescence anisotropy r_s	Microsviscosity η	Order parameter S_{DPH}
Normal	2.97 ± 0.03	0.112 ± 0.004	1.14	0.392
+ Cholesterol	3.04 ± 0.04	0.131 ± 0.003	1.36	0.452

separated by thin-layer chromatography. They were then mixed in the same proportion found in the microsomes of normal or treated animals and sonicated with appropriate amounts of cholesterol to achieve the relative concentration of the same microsomes.

Fluorescence anisotropy of vesicles labeled with 1,6-diphenylhexatriene and pyrene excimer formation were measured in all these 'reconstituted' systems. Table V indicates that the increase of PC in the binary mixture vesicles decreases the fluorescence anisotropy, but does not greatly modify the pyrene excimer formation.

Fluorescence polarization of bilayers labeled with 1,6-diphenylhexatriene is considered to measure changes in the rotational diffusion of lipid chains, whereas pyrene excimer formation detects changes in the lateral diffusion. Moreover, a lack of relationship between fluorescence polarization and lateral diffusion has been evidenced [40]. Therefore, the increase of PC in the vesicles would modify the rotational diffusion, but would leave unaltered the lateral diffusion in the conditions of our experiment. The incorporation of cholesterol

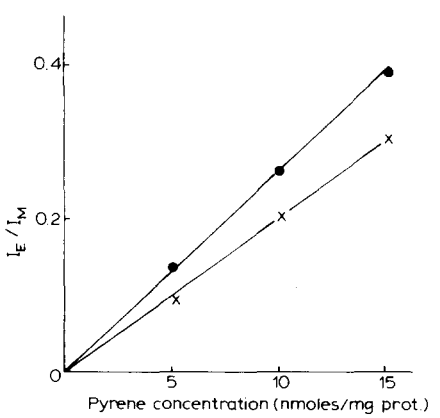

Fig. 1. Efficiency of pyrene excimer formation measured at different pyrene concentrations. The ratio I_e/I_m was evaluated as stated in Materials and Methods. Normal microsomes, ●; cholesterol-enriched microsomes, ×.

TABLE V

PREPARATION OF LIPOSOMES FROM NORMAL AND CHOLESTEROL-ENRICHED MICROSOMES

Liposomes A and B are prepared with the amounts of PC and PE shown in Table I, excluding the other phospholipids and so keeping the original ratio. Liposomes C and D prepared with the amounts of PC, PE and cholesterol shown in Table I excluding the other phospholipids. N cholesterol and + C cholesterol correspond to the cholesterol content in normal and cholesterol-enriched microsomes, respectively. The other symbols are those used in Table III. The meaning of r_s is explained in Table V and that of I_E/I_M in Materials and Methods.

Vesicles	Composition (% weight)	r_s	I_E/I_M
A	NPC: 65.4 NPE: 34.6	0.102	0.42
B	+ CPC: 75.2 + CPE: 24.8	0.089	0.41
C	NPC: 53.5 NPE: 28.3 N cholesterol: 18.2	0.108	0.38
D	+ CPC: 46.9 + CPE: 15.5 + C cholesterol: 37.6	0.120	0.31

to the vesicles (Table V) produces an increase of fluorescence anisotropy and a decrease of pyrene excimer formation. In consequence, results show on one hand that both types of motion were retarded by cholesterol and on the other that the condensing effect of cholesterol even overcomes the fluidizing effect of PC increase (compare vesicle C with D in Table V).

Effect of cholesterol on the kinetic properties of UDP-glucuronyl transferase

The mechanism of the reaction proposed by Vessey and Zakim [42] was a random-ordered sequence (Fig. 2). To study the effect of cholesterol on the kinetic parameters of the different steps of the reaction, initial rates of UDP-glucuronyl transferase activity were measured as a function of variable concentrations of UDP-glucuronic acid at several fixed concentration of *p*-nitrophenol and as a function of varying concentrations of *p*-nitrophenol at several fixed concentrations of UDP-glucuronic acid [43]. These data, when plotted on double reciprocal form (Figs. 3 and 4) indicate that the interception on the $1/S$ axis for either substrate, corresponding to the K_m, depends on the concentration of the second substrate. Secondary plots of the interception on the $1/V$ vs. $1/$(concentration of the fixed substrate) yield the value $1/V_{max}$ and a second $K_{m'}$ for each substrate. $K_{m\,UDPGA}$ and $K_{m\,p\text{-}NP}$ will be the constants of the binary enzyme substrate complex, and K'_{UDPGA} and $K'_{p\text{-}NP}$ are the dissociation constants for the ternary enzyme-substrate complex (Fig. 5).

Table VI summarizes the kinetic properties estimated for microsomes of normal and treated animals. It indicates that UDP-glucuronic acid shows lower affinity than *p*-nitrophenol for the enzyme and the incorporation of the first substrate for both alternative reactions increases the affinity for the second. The cholesterol diet evoked an

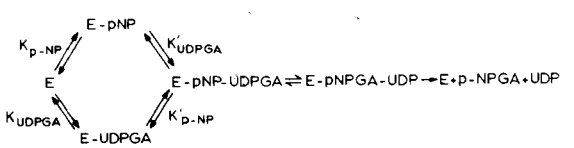

Fig. 2. Postulated reaction mechanism for UDP-glucuronyl transferase. Symbols correspond to explanations given in the text.

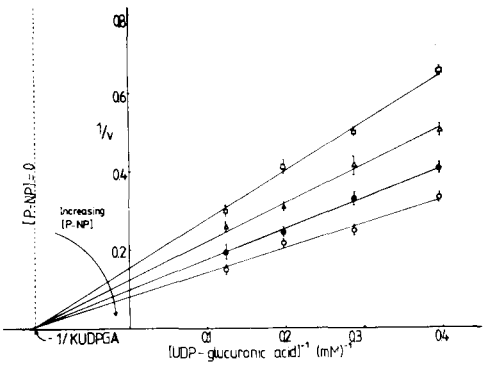

Fig. 3. Double reciprocal plots of initial rates of UDP-glucuronyl transferase as a function of varying concentrations of *p*-nitrophenol at different fixed concentrations of UDP-glucuronic acid. Each point is based on initial rate determination of UDP-glucuronyl transferase activity as described in Materials and Methods and expressed as nmoles of *p*-nitrophenol per min per mg microsomal membrane. The different concentrations of UDP-glucuronic acid were: 2.5 mM (□); 3.5 mM (△); 5.0 mM (●) and 8.0 mM (○). Vertical bars correspond to ± S.E. of the mean values of five animals.

increase of the affinity for all the reaction steps and it also increased the specific activity of the UDP-glucuronyl transferase.

Besides, the UDP-glucuronyl transferase of normal microsomes showed the typical non Michaelis–Menten kinetics already recognized by Vessey and Zakim [44] that may be described as an apparent negative cooperativity of the enzyme for

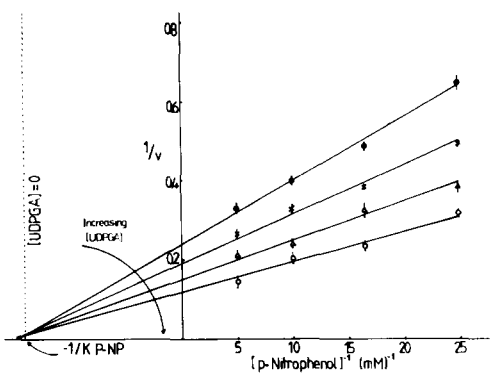

Fig. 4. Double reciprocal plots of initial rate of UDP-glucuronyl transferase activity as a function of UDP-glucuronic acid concentration at fixed levels of *p*-nitrophenol: 0.04 mM (●); 0.06 mM (×); 0.1 mM (△); 0.2 mM (○). Vertical bars correspond to ± S.E. of the mean values of five animals.

TABLE VI

KINETIC CONSTANTS FOR THE FORWARD REACTION OF UDP-GLUCURONYL TRANSFERASE

Kinetic constants were determined from bisubstrate. Kinetic analyses of initial rate data for UDP-glucuronyl transferase as explained in Materials and Methods. Results are the mean of five animals analyzed separately ± S.E.

	K_{UDPGA} (mM)	K'_{UDPGA} (mM)	$K_{p\text{-NP}}$ (mM)	$K'_{p\text{-NP}}$ (mM)	V_{max}	Hill coefficient
Normal	12.3 ± 0.4	10.6 ± 0.2	0.12 ± 0.03	0.10 ± 0.01	10.5 ± 1.1	0.40 ± 0.02
+ Cholesterol	8.1 ± 0.3	7.2 ± 0.1	0.07 ± 0.01	0.06 ± 0.01	15.9 ± 1.3	0.68 ± 0.03

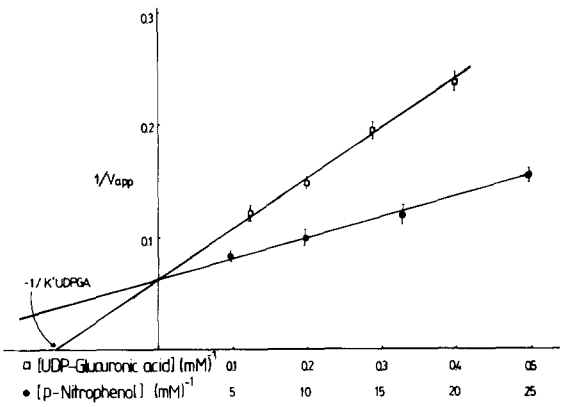

Fig. 5. The interceptions of the $1/v$ axis in Figs. 3 and 4 are replotted against $1/$[UDP-glucuronic acid] (□) and $1/$[*p*-nitrophenol] (●) in order to obtain $1/V_{max}$, K'_{UDPGA} and K' *p*-NP. Vertical bars correspond to ± S.E. of the mean values of five animals.

UDP-glucuronic acid. The degree of deviation may be expressed by the Hill coefficient [17] and Table VI shows that the cholesterol diet evoked an increase of this coefficient, that indicates a shift of the non-michaelian kinetics to michaelian.

Discussion

Effect of cholesterol on the structural and dynamic properties of microsomal membrane

Since cholesterol is an important component of many biomembranes, the understanding of its interaction with other membrane components is essential for a complete description of the hydrophobic environment in which various membrane processes occur [45,46]. At present, it is still discussed whether cholesterol forms specific complexes with a number of lipid molecules [47] or is randomly distributed throughout the lipid bilayer [48]. There is a number of well-defined physical changes which occur upon incorporation of cholesterol into model membrane systems and there are some structural requirements for the incorporation of the sterol moiety in phospholipid bilayers: a planar ring system, a side chain at C_{17} and a 3β -OH [49]. Cholesterol lipid bilayer interaction is highly hydrophobic in nature, but the effects in the polar region may be ascribed to the 3β -OH group. Above the T_m, cholesterol decreases the mean cross-sectional area per molecule within the bilayer. This effect can be understood in terms of a decrease in the flexibility of the acyl chains of the lipid due to the influence of cholesterol on the antiperiplanar-synclinal rotamery of the methylene group [49] and decrese of long-range swinging motion of these chains [50]. This so-called condensing effect is restricted to a defined part of the hydrophobic region primarily the first ten carbon atoms of the acyl chain [39]. Below the transition temperature, measurements of the acyl chain order parameter show that the presence of cholesterol inhibits the cooperative crystallization of the hydrocarbon chains. By disordering the chain packing at low temperature and reducing the chain flexibility at high temperatures, cholesterol gives origin to the so-called 'intermediate' fluid condition [51].

A model accounting for the structural requirements necessary to explain the above-stated results considers that the ring system is contiguous to the proximal segment of the phospholipid acyl chains, while the side chain extends deeply into the bilayer interior. Differential Van der Waal's interactions, resulting in a fluidity gradient along the sterol-phospholipid contact regions are in consequence, to be expected [52].

Our results obtained by the incorporation of cholesterol in guinea-pig liver microsomes agree with the demonstrated effects of cholesterol: on one side, cholesterol produces an increase in the measured anisotropy and the order parameter (Table IV) above the T_m of microsomes and, on the other decreases the efficiency of pyrene excimer formation which is dependent on the rate of translational diffusion of pyrene in the plane of the membrane that determines the frequency of collisions (Fig. 1).

However, Table I shows that dietary cholesterol evokes not only an increase of microsomal cholesterol, but also a decrease of the PE/PC ratio of the membrane. This decrease is consistent with the finding of Lim et al. [54] that an increse of PC synthesis is induced in the livers of young rats by feeding a diet enriched with cholesterol and cholate. The increase of PC biosynthesis was correlated with an increase of the activity of CTP: phosphocholine cytidylyl-transferase and its translocation to the microsomes. The afore-mentioned translocation has been considered to regulate the activity of this enzyme [55] that determined the rate of PC biosynthesis [56]. To what extent cholesterol incorporation in the microsomes alters the affinity of the enzyme for the membranes by a mechanism similar to the one proposed for fatty acid effect [57] or takes part in another proposed mechanism, is subject of speculation and research.

Anyhow, the practical effect of the changes of the PE/PC ratio on the microsomal membrane properties is important, since the variation of this ratio modifies the physicochemical properties of the membrane. It is known [53] that saturated or trans-unsaturated phosphatidylethanolamines undergo a lipid phase transition some 20–30°C higher than the corresponding phosphatidylcholine.

Phosphatidylethanolamines compared to PC have a small polar head group which is thought to be oriented tangential to the plane of the bilayer. This small head group allows a very close packing of the phosphatidylethanolamine molecules, and there is also less water penetration into the polar head region [53].

Therefore, the decrease of PE/PC ratio would decrese the gel-liquid crystalline phase transition temperature of the membrane without modifying the enthalpy of the transition, but favouring the bilayer structure and decreasing the packing [59]. Results in Table V show in this respect that the decrease of PE/PC ratio in PC-PE vesicles decrease the fluorescence anisotropy of the labeled bilayer indicating an increased rotational diffusion and decreased packing. In consequence, the high cholesterol diet promoting such increase (Table I) evokes by this mechanism an unpacking of the bilayer which is not enough to compensate totally the condensing effect of the cholesterol binding (Tables IV and V). Therefore, the overall effect of cholesterol was an increased 'ordering' of the whole microsomal membrane, but less than that expected without PE-PC ratio decrease.

Effect of cholesterol on the kinetic properties of UDP-glucuronyl transferase

The high cholesterol diet evoked a change in the lipid composition, structrure and physicochemical properties of liver microsomes and at the same time modified the kinetic parameters of the membrane bound UDP-glucuronyl transferase (Table VI). All the K_m values of the forward steps of the reaction (Fig. 2) were decreased, the V_{max} was incresed and the Hill coefficient that was less than unity was enhanced, approaching this value. Cholesterol evoked these effect, but, up to this moment, we cannot ascribe the variations of the kinetic constants to the cholesterol molecule only. These effects may be well attributed to a direct effect of cholesterol or to the corresponding modification of the PE/PC ratio or to both causes.

However, the present results indicating a shift of the non-michaelian kinetics of the enzyme to michaelian in correspondence to an increase of the bilayer packing by effect of cholesterol, are consistent with a similar effect found in microsomes in which the increase of the order parameter arose from an essential fatty acid deficiency [17].

Moreover, Hochman et al. [60] found that the delipidated form of pig liver UDP-glucuronyl transferase produces a non-Michaelis–Menten kinetics of the type shown in the present work when the enzyme was reconstituted into unilamellar vesicles of phosphatidylcholine in gel phase. The basis of this kinetic pattern inferred by binding studies is the presence of one high-affinity and one low-affinity binding site for UDP-glucuronic acid. The two classes of binding sites seemed to be

generated by two subunits that bind the substrate within a single molecule of the enzyme. Besides, they found that the melting of the phospholipids from the gel phase to the liquid crystalline phase was associated with a switch from non-Michaelis–Menten to Michaelis–Menten kinetics and the disappearance of the functionability of one of the binding sites.

However, the bulk lipids of microsomal membranes are at the physiological temperature of the animal, in the liquid crystalline state [61,62] and the UDP-glucuronyl transferase bound on the intact membrane shows non-Michaelis–Menten kinetics [14,44]. Hence, these data would be in an apparent discrepancy with the data of reconstituted artificial enzyme bilayer systems [60]. To explain this apparent discrepancy, Hochman et al. [60] suggested that the lipid milieu surrounding the UDP-glucuronyl transferase in the membrane would be in a gel state at about 37°C and so in different packing conditions than bulk lipids. In a later paper, Hochman and Zakim [63] presented evidence that this is the case for the UDP-glucuronyl transferase of pig liver microsomes.

Our present experiments with cholesterol-treated guinea pigs (Table IV) and previous works with essential fatty-acid-deficient animals [17] show that the non-Michaelis–Menten kinetics of UDP-glucoronyl transferase is gradually lost in correspondence with an increase of order parameter of the whole microsomal membrane. Therefore, if these results are analyzed in the light of Hochman et al. postulate [60] would mean that while the bulk lipids of the guinea pig microsomes are in a liquid-crystalline phase (Castuma, C.E. and Brenner, R.R. unpublished data), the lipid milieu surrounding the enzyme would be in the gel phase and the essential fatty-acid deficiency [17] or cholesterol incorporation (Table IV) (Fig. 1), while increasing the packing of the bulk lipids of the microsomal membrane would produce an opposite effect in the lipid milieu of the UDP-glucuronyl transferase. That the physical state of the lipid milieu surrounding a membrane bound protein may be different to that of the bulk lipids is quite demonstrated in the light of present theories of lipid–lipid and protein–lipid interactions in the bilayers [64–69]. Moreover, the fluidizing effect produced on protein bound lipids by antifluidizing agents of the bulk lipids of the membrane as cholesterol, is also understandable in the light of present results, suggesting that molecular interaction of cholesterol and phospholipids in membranes induces 'patching' [70] and lateral phase separation [71]. A favourable condition, suitable for explaining this effect would be the existence of a specific lipid requirement of the protein and this is the case of the UDP-glururonyl transferase which has a special requirement for choline containing phospholipids [3,72]. It would fit with the effect of dietary cholesterol that not only increased the cholesterol content of the membrane, but also enhanced the PC/PE ratio (Table I). Phosphatidylcholine was shown to have lower transition temperature than PE and as it was discussed before, elicits an antagonic effect to cholesterol on the fluidity. Another way to increase the fluidity of localized domains of the membrane could also be produced if cholesterol interacts preferentially with saturated phosphatidylcholines excluding the unsaturated molecules. In this respect, experiments of Guyer and Bloch [73] show that cholesterol rigidifies phosphatidylcholine vesicles of different fatty-acid composition in the following order: bisaturated > monounsaturated > biunsaturated.

The less interaction of cholesterol with biunsaturated lecithins would give ground to our hypothesis suggesting a possible segregation of the more unsaturated and fluid lecithins excluded from cholesterol-saturated phosphatidylcholine domains. These excluded phosphatidylcholines would fluidize the boundary lipids of UDP-glucuronyl transferase evoking a decrease of Hill coefficient. Besides, independently from changes of phospholipid composition and distribution, cholesterol evokes a condensing effect in a liquid crystalline bilayer, but elicits a fluidizing effect on a bilayer in the gel phase that would be the physical state of the lipid milieu of the enzyme. In addition, it has been also suggested based on a number of studies [39,74,75] that the ordering effect of cholesterol in a liquid crystalline bilayer varies from the surface to the center of the bilayer and even may increase the mobility of the tail segments of fatty acids [39,74].

In consequence, we propose that the shift of the non-michaelian kinetics of UDP-glucuronyl transferase to michaelian by effect of dietary cholesterol

would be produced by packing changes in the lipid of the bilayer in contact with the enzyme and different from the changes produced in the bulk lipids. Further experiments to confirm this postulate are under development.

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References

1 Strobel, H.W., Lu, A.H., Heidema, J. and Coon, M.J. (1970) J. Biol. Chem. 245, 4851–4854
2 Zakim, D. and Vessey, D.A. (1976) in The Enzymes of Biological Membranes (Martonosi, A., ed.), Vol. 2, pp. 433–442, Plenum Press, New York
3 Erickson, R.H., Zakim, D. and Vessey, D.A. (1978) Biochemistry 17, 3706–3711
4 Singh, O.M.P., Graham, A.B. and Wood, G.C. (1981) Eur. J. Biochem. 116, 341–345
5 Singh, O.M.P., Graham, A.B. and Wood, G.C. (1982) Biochem. Biophys. Res. Commun. 107, 345–349
6 Berry, C. and Hallinan, T. (1976) Biochem. Soc. Trans. 4, 652–657
7 Pechey, D.T., Graham, A.B. and Wood, G.C. (1978) Biochem. J. 175, 115–124
8 Cummings, J., Graham, A.B. and Wood, G. (1980) Biochem. J. 185, 521–526
9 Berry, C. and Hallinan, T. (1975) Biochem. Biophys. Acta 403, 335–344
10 Zakim, D. and Vessey, D.A. (1982) in Membranes and Transport (Martonosi, A., ed.), Vol. 1, pp. 269–273, Plenum Press, New York
11 Garda, H.A. and Brenner, R.R. (1982) Acta Physiol. Latinoam. 32, 35–52
12 Strittmatter, P., Rogers, M.J. and Spatz, L. (1972) J. Biol. Chem. 247, 7188–7194
13 Rogers, M.J. and Strittmatter, P. (1974) J. Biol. Chem. 249, 895–900.
14 Strittmatter, P. and Rogers, J.M. (1975) Proc. Natl. Acad. Sci. USA 72, 2658–2661
15 Thilo, L., Träuble, H. and Overath, P. (1977) Biochemistry 16, 1283–1290
16 Garda, H.A. and Brenner, R.R. (1984) Biochim. Biophys. Acta 769, 160–170
17 Castuma, C.E. and Brenner, R.R. (1983) Biochim. Biophys. Acta 729, 9–16
18 Demel, R.A., Van Deenen, L.L.M. and Pethica, B. (1967) Biochim. Biophys. Acta 135, 11–17
19 Lapper, R.D., Paterson, S.J. and Smith, I.C.P. (1972) Can. J. Biochem. 50, 969–981
20 Gallay, J. and De Kruijff, B. (1982) FEBS Lett. 143, 133–136
21 Lecuyer, H., Dervichian, D.G. (1969) J. Mol. Biol., 45, 39–57
22 Reid, M.E. and Briggs, G.M. (1953) J. Nutr. 51, 341–354
23 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275
24 Burton, I., Litman, J. and Barenholz, Y. (1982) Methods Enzymol. 81, 678–685
25 Perrin, F. (1936) J. Phys. Radium 7, 1–11
26 Shinitzky, M. and Barenholz, Y. (1978) Biochim. Biophys. Acta 515, 367–394
27 Heyn, M.P. (1979) FEBS Lett. 108, 359–364
28 Jahnig, F. (1979) Proc. Natl. Acad. Sci. USA 76, 6361–6365
29 Van Blitterswijk, N.J., Van Hoeven, R.P. and Van der Meer, B.W. (1981) Biochim. Biophys. Acta 644, 323–332
30 Pownall, H.J. and Smith, L.C. (1973) J. Am. Chem. Soc. 95, 3136–3140
31 Zakim, D.A. and Vessey, D. (1973) J. Biol. Chem. 253, 1632–1638
32 Vessey, D.A., Goldenberg, J. and Zakim, D. (1973) Biochim. Biophys. Acta 309, 58–66
33 Folch, J., Lees, M. and Sloane-Stanley, G.A. (1957) J. Biol. Chem. 226, 497–509
34 Ascott, J. and Heaven, R.S. (1968) J. Chromatog. 35, 297–300
35 Kaitaranta, J.K. and Nicolaides, N. (1981) J. Chromatogr. 205, 339–347
36 Huang, T.C., Chen, C.P., Wefler, V. and Raftery, J. (1961) Anal. Chem. 33, 1405–1406
37 Chen, P.S., Toribara, T.Y. and Warner, H. (1956) Anal. Chem. 28, 1756–1758
38 Yeagle, R.L. (1981) Biochim. Biophys. Acta 640, 263–273
39 Darke, A., Finer, E.G., Flook, A.G. and Phillips, M.C. (1972) J. Mol. Biol. 63, 265–279
40 Kleinfeld, A.M., Dragsten, P., Klausner, R.D., Pjura, W.J. and Matayoshi, E.D. (1981) Biochim. Biophys. Acta 649, 471–480
41 Phillips, M.C. and Finer, E.G. (1974) Biochim. Biophys. Acta 356, 199–208
42 Vessey, D. and Zakim, D. (1971) J. Biol. Chem. 246, 4649–4656
43 Segel, I.H. (1975) Enzyme Kinetics, John Wiley & Sons, New York
44 Vessey, D.A. and Zakim, D. (1973) Biochim. Biophys. Acta 315, 43–48
45 Demel, R.A. and De Kruijff, B. (1976) Biochim. Biophys. Acta 457, 109–132
46 Quinn, P.J. and Chapman, D. (1980) CRC Crit. Rev. Biochem. 8, 1–117
47 Martin, R.B. and Yeagle, P.L. (1978) Lipids 13, 594–597
48 Jacobs, R. and Oldfield, E. (1979) Biochemistry 18, 3280–3285
49 De Kruijff, B., Demel, R.A. and Van Deenen, L.L.M. (1973) Biochim. Biophys. Acta 341, 1–19
50 Godici, P.E. and Landsberger, F.R. (1975) Biochemistry 14, 3927–3933
51 Chapman, D. (1968) Biological Membranes, Academic Press, New York

52 Franks, N.P. (1976) J. Mol. Biol. 100, 345–356
53 Verkley, A.J. and Van Deenen, L.L.M. (1974) Biochim. Biophys. Acta 373, 495–501
54 Lim, P.M., Pritchard, P.H., Paddon, H.B. and Vance, D.E. (1983) Biochim. Biophys. Acta 753, 74–82
55 Vance, D.E. and Pelech, S.L. (1984) Trends. Biochem. Sci. 9, 17–20
56 Vance, D.E., Choy, P.C. (1979) Trends Biochem. Sci. 4, 145–148
57 Pelech, S.L., Pritchard, P.H., Brindley, D.N. and Vance, D.E. (1983) Biochem. J. 216, 129–136
58 Bloch, K. (1983) CRC Crit. Rev. Biochem., 14, 47–92
59 Cullis, P.R. and De Kruijff, B. (1979) Biochim. Biophys. Acta 559, 399–420
60 Hochman, Y., Kelley, M. and Zakim, D. (1983) J. Biol. Chem. 258, 6509–6516
61 Mabrey, S., Powis, G., Shenkman, J.B. and Tritton, T.R. (1977) J. Biol. Chem. 252, 2929–2933
62 Brenner, R.R., Garda, H.A., De Gómez Dumm, I.N.T. and Pezzano, H. (1981) Progress in Lipid Res. 20, 315–321
63 Hochman, Y. and Zakim, D. (1983) J. Biol. Chem. 258, 11758–11768
64 Owicki, J.C., Springgate, M.W. and McConnell, H.M. (1978) Proc. Natl. Acad. Sci. USA 75, 1616–1619
65 Owicki, J.C. and McConnell, H.M. (1979) Proc. Natl. Acad. Sci. 76, 4750–4754
66 Jähnig, F. (1981) Biophys. J. 36, 329–345
67 Jähnig, F. (1981) Biophys. J. 36, 347–357
68 Recktenwald, D.J. and McDonnell, H.M. (1981) Biochemistry 20, 4505–4510
69 Stier, A., Kuhnle, W. and Rosen, R. (1977) in Microsomes and Drug Oxidations (Ullrich, V. et al., eds.), pp. 1–8, Pergamon Press, Oxford
70 Jain, M.K. (1983) in Membrane Fluidity in Biology, Vol. I (Aloia, R., ed.), pp. 1–37, Academic Press, New York
71 Melchor, D.L., Scautto, F.J. and Steim, J.M. (1980) Biochemistry 19, 4828–4834
72 Gorki, J.P. and Kasper, C.B. (1978) Biochemistry 17, 4600–4605
73 Guyer, W. and Bloch, K. (1983) Chem. Phys. Lipids 33, 313–322
74 Kutchai, H., Chandler, L.M. and Zavoico, G.B. (1983) Biochim. Biophys. Acta 736, 137–149
75 Rothman, J.E. and Engelman, D.M. (1972) Nature New Biol. 237, 42–44