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EFFECT OF PHASE TRANSITION ON THE DISTRIBUTION OF MEMBRANE-ASSOCIATED PARTICLES IN MICROSOMES

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SUMMARY

(1) Rat liver microsomes were studied by freeze-fracture electron microscopy. The distribution of membrane-associated particles indicated the right-side-out orientation of microsomal vesicles. Studies at different temperatures were performed. At 30 °C membrane-associated particles are randomly distributed on membrane A-faces, while aggregations of particles are observed at 4 °C.

(2) Aggregation is dependent on the cooling rates. It can be prevented by shock-freezing.

(3) Particle aggregation is also prevented by cholesterol, added to the microsomes in equal molar ratio to the microsomal phospholipid content.

(4) These findings suggest that particle aggregation is caused by a partial freezing-out of phospholipid molecules during the phase transition from the liquid-crystalline to the gel state.

(5) The results are discussed with respect to an observed increase in activation energy of microsomal drug monooxygenation at lower temperature.

INTRODUCTION

Recent studies in one of the authors' laboratory indicated a temperature-dependent phase transition in microsomal membranes isolated from rat liver (Duppel and Ullrich [1]). As this may cause a change in the morphology of these membranes, we studied the effect of temperature by freeze-cleaving electron microscopy. During this process of freeze-cleaving, membranes are split in their hydrophobic core and large areas of the interior of the membrane are exposed. These are characterized in microsomes and other membranes by the appearance of membrane-associated particles. The distribution of membrane-associated particles, which are interpreted to be proteins (Branton and Deamer [2]), is dependent on temperature and membrane composition. These effects have been described in membranes of microorganisms by

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Verkleij et al. [3], James and Branton [4], Speth and Wunderlich [5], Rottem et al. [6], Kleemann and McConnell [7], Heast et al. [8], Shechter et al. [9] and in membranes of lymphocytes, erythrocytes and sarcoplasmic reticulum by Wunderlich et al. [10], Speth et al. [11] and Packer et al. [12], respectively. The reported changes in the distribution of membrane-associated particles have been attributed to a phase transition in the membrane bilayer.

Therefore we investigated in this study whether hepatic microsomes, too, exhibit a change in the distribution of membrane-associated particles, concomitant with the observed phase transition. In addition, we tested whether this distribution is affected by shock-freezing or by the addition of cholesterol.

METHODS

Liver microsomes have been prepared from male Sprague-Dawley rats as described previously [13]. The protein concentration was about 40 mg/ml and the phospholipid content was estimated according to Glaumann and Dallner [14]. Samples of microsomes were equilibrated for 5 min in small tubes at 4 or 30 °C and fixed as described below.

In some experiments microsomes were equilibrated at 30 °C, subsequently sprayed into a liquid N_2-cooled stainless steel vessel, transferred to a water bath at 4 °C and equilibrated again for 5 min before fixation.

Cholesterol in concentration equimolar to the microsomal phospholipids has been added as a sonicated suspension to microsomes, which were incubated for 20 min at 30 °C and then equilibrated at 4 °C.

Freeze-fracture

Samples were fixed by the addition to the microsomal suspension of an equal volume of phosphate buffer containing 2 % glutaraldehyde and after 5 min glycerol was added to give a final concentration of 30 %.

After 10 min small droplets of the samples were transferred to a golden specimen holder and rapidly frozen in Freon 22, cooled by liquid N_2.

Fracturing was performed in a Balzers BAF 300 freeze-fracture device, according to the method described by Moor and Mühletaler [15]. Pt-C shadowing (15 Å) and carbon backing (200 Å) were performed with an electron beam gun. Replicas were cleaned in a sodium hypochlorite solution and washed in distilled H_2O. They were picked up by formvar- and carbon-coated one hole grids and examined in an electron microscope (Siemens Elmiskop 101). All pictures are printed as positives (platinum depositions black). The direction of shadowing is indicated by an encircled arrow head. The nomenclature of membrane faces is according to NcNutt and Weinstein [16].

Evaluation of data

Since there is no standard procedure available to quantitatively evaluate membrane particle aggregations, we defined an aggregation index. Vesicles containing particle-free membrane areas larger than 1120 nm^2 (corresponding to 6×6 mm in micrographs with a total magnification of 180 000) with an unchanged total num-

ber of particles were defined as aggregated. An aggregation index was obtained from the ratio of counted vesicles with and without particle aggregation.

As a control the maximum particle density in an equal area (1120 nm^2) was determined. An area of 1120 nm^2 was chosen arbitrarily, because in an area of this size membrane particle aggregations as well as particle-free regions could be observed.

To show that no loss or addition of membrane-associated particles has occurred, the total number of particles has been determined in a larger area ($6.15 \cdot 10^{-3}$ $\mu m^2 = 1 \times 2$ cm on micrographs with a total magnification of 180 000) and expressed as particles per μm^2.

Only the A-faces of vesicle membranes (see Results), exposing areas larger than about $5 \cdot 10^{-3}$ μm^2, were used for counting.

RESULTS

The endoplasmic reticulum is the predominant membrane system in hepatocytes. Fig. 1 represents a typical freeze-fracture electron micrograph of rat liver endoplasmic reticulum. It is characterized by double lamellar structures separating the cytoplasm (C) and the intracisternal space (I). Branton [17] and Pinto da Silva and Branton [18] have shown that membranes are split in the hydrophobic region during the cleavage process. In the endoplasmic reticulum the inside view of the

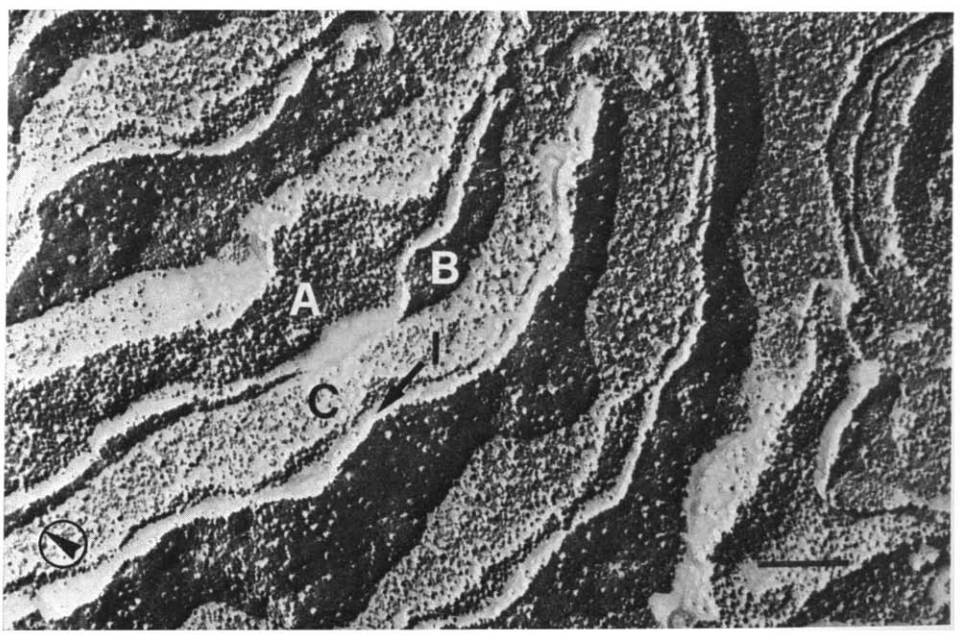

Fig. 1. Freeze-fracture electron micrograph of rat hepatocyte endoplasmic reticulum. The cytoplasm (C) is traversed by several membrane layers, which enclose the intracisternal space (I). Two different aspects of the membrane are visible; the leaflet attaching the cytoplasm is particle rich (A), the leaflet attaching the intracisternal space has a low particle density (B). Magnification 60 000 ×, bar represents 0.2 μm.

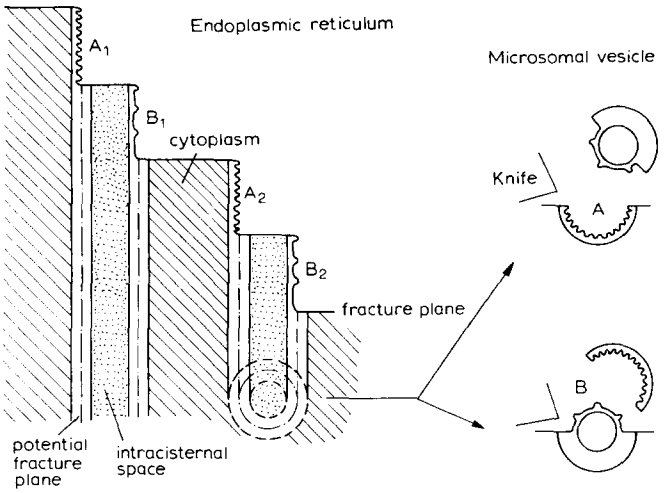

Fig. 2. Schematic representation of endoplasmic reticulum and its exposure by freeze-fracturing. On the right hand side the formation of right-side-out microsomal vesicles and their exposure by freeze fracturing is drawn. From the membranes of microsomal vesicles concave A-faces (A) and convex B-faces (B) are obtained.

membrane leaflet attaching the cytoplasm (A-face) is characterized by a high density of membrane-associated particles, while the aspect of the leaflet attaching the intracisternal space has only a low particle density (B-face).

Homogenization of the endoplasmic reticulum membranes leads to the formation of microsomal vesicles. This procedure retains the polarity of the membranes as in the intact endoplasmic reticulum: the membrane leaflet attaching the intracisternal space is now turned towards the vesicular content (right-side-out), as shown by the orientation of ribosomes in thin section electron microscopy (Leskes et al. [19, 20]). Fig. 2 shows schematically the possible aspects of membrane faces in endoplasmic reticulum membranes and microsomal vesicles derived from this membrane system.

Freeze-fractured microsomal vesicles (30 °C) are presented in Fig. 3. The aspect is characterized by concave particle-rich vesicles (A-face) and convex ones with lower particle density (B-face). This is according to the membrane aspects expected from right-side-out vesicles (see Fig. 2). The apparent diameter of the vesicle fragments is 50–200 nm, but in some instances convex vesicles with high particle density can be seen. They are often larger in diameter and may represent inside-out vesicles or, more probably, plasmalemmal contaminations. The membrane-associated particles on the A-faces of the microsomal vesicles (only those are considered in the following experiments) are randomly distributed and the number of particles per μm^2 is 3370 ± 882.

A characteristic change in the arrangement of membrane-associated particles is seen when the microsomes are equilibrated at 4 °C: particle-free areas are exposed, adjacent to areas where membrane-associated particles are aggregated (Fig. 4). Despite this alteration in particle distribution the number of particles per μm^2 is practically unchanged (3934 ± 708). A quantitative evaluation of the particle aggregation is given in Table I. Two methods were used to determine aggregations:

first we looked for areas void of particles, second we counted the maximum number of particles in a defined area (for details see Methods). In both cases there is a marked difference between vesicles equilibrated at 30 °C or 4 °C.

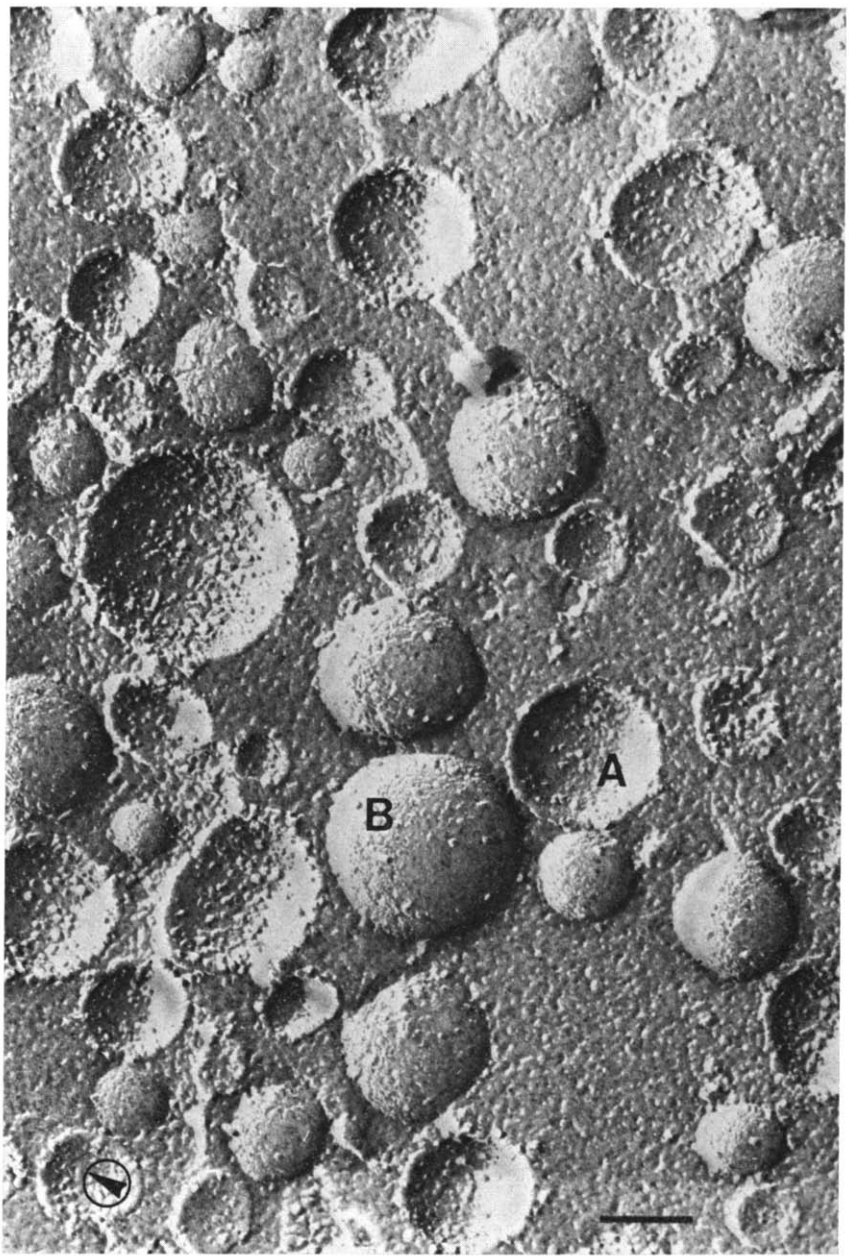

Fig. 3. Fracture faces of microsomal vesicles incubated at 30 °C. The numerous particles in the A-faces (A) are randomly distributed. Convex B-faces (B) exhibit low particle density. Magnification 120 000×, bar represents 0.1 μm.

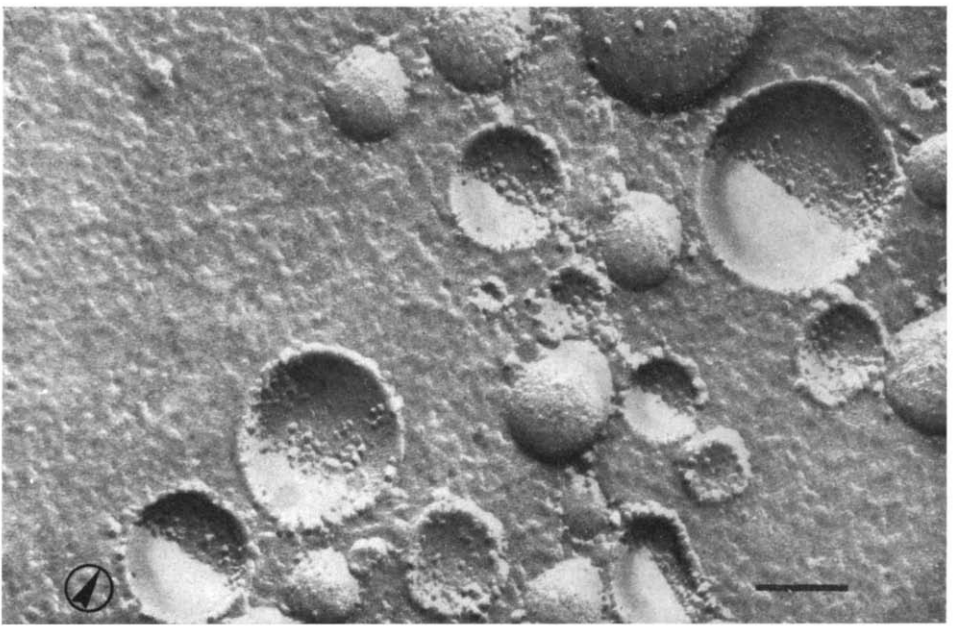

Fig. 4. Fracture face of microsomal vesicles incubated at 4 °C. The particles in the A-faces are aggregated. Magnification 120 000 ×, bar represents 0.1 μm.

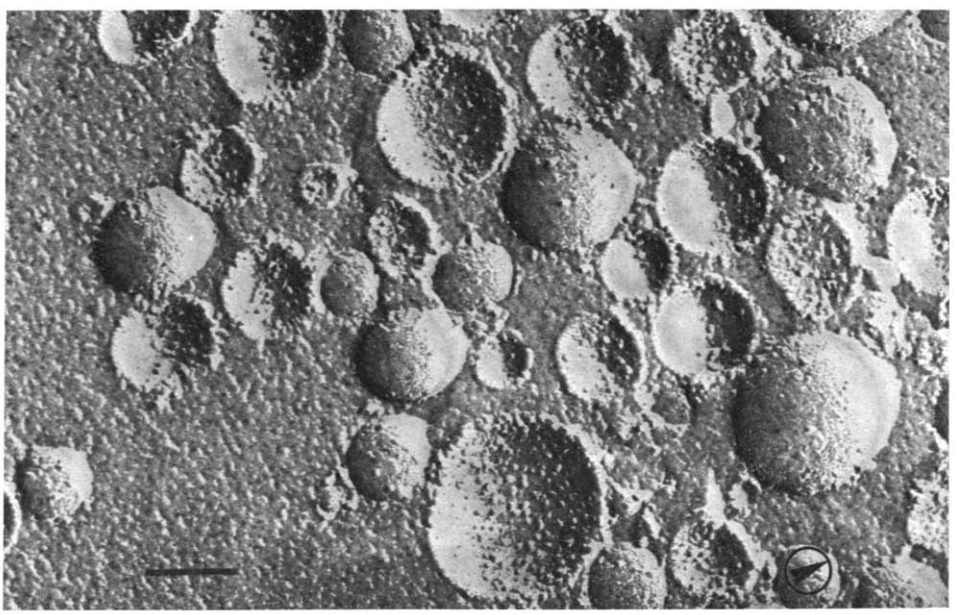

Fig. 5. Random distribution of membrane associated particles in microsomal vesicles enriched with cholesterol and equilibrated at 4 °C. Magnification 120 000 ×, bar represents 0.1 μm.

TABLE I

AGGREGATION OF MEMBRANE-ASSOCIATED PARTICLES IN RAT LIVER MICROSOMES AND ITS PREVENTION UNDER VARIOUS CONDITIONS EVALUATED FROM FREEZE-FRACTURE MICROGRAPHS

For details see Methods.

Microsomes equilibrated at	Aggregation index*			Maximum number of particles within a defined area (1120 nm^2)	Number of particles per μm^2**
	a	b	c		
4 °C	24	42	0.57	10.7 ± 1.8 ($n = 48$)	3934 ± 708
30 °C	8	53	0.151	5.8 ± 1.2 ($n = 40$)	3370 ± 882
4 °C	54	109	0.495	11.2 ± 2.0 ($n = 42$)	3252 ± 726
4 °C + cholesterol	46	170	0.27	6.8 ± 1.3 ($n = 65$)	3328 ± 616
4 °C	23	42	0.548	11.6 ± 1.9 ($n = 31$)	3492 ± 569
4 °C shock-frozen from 30 °C	18	87	0.207	6.8 ± 1.2 ($n = 52$)	3590 ± 569

* Number of vesicles with (a) and without (b) particle-free areas of 1120 nm^2. Ratio a : b = c

** A total area of 0.5–0.6 μm^2 was counted of each preparation.

To study further this aggregation phenomenon we have used cholesterol, which is known to increase the fluidity of artificial and biological membranes below the phase transition (Oldfield and Chapman [21]). When cholesterol is incorporated into the microsomal membrane in equimolar amounts to the phospholipid content the aggregation of membrane-associated particles is considerably reduced at 4 °C, almost resembling preparations from 30 °C (see Table I, Fig. 5).

To test if the aggregation of membrane-associated particles is due to an extrusion of the particles from certain areas by phospholipid molecules, comparable to a freezing-out, we performed shock-freezing experiments. In these experiments microsomes equilibrated at 30 °C were quenched to the temperature of liquid N_2. When the temperature is raised to 4 °C the particle distribution is random, as in microsomal membranes prepared at 30 °C, in contrast to the aggregation of particles when microsomes are slowly cooled from 30 to 4 °C (Table I).

DISCUSSION

Membranes are split by freeze-fracturing in the hydrophobic core as shown by Branton [17] and Pinto da Silva and Branton [18], exposing two different inner aspects of the membrane. The visible membrane face of the leaflet attaching the cytoplasm of plasma membranes (A-face, according to McNutt and Weinstein [16]) as well as of intracellular membranes is characterized by a high particle density, while the aspect of the opposite leaflet (B-face) exposes considerably less particles (Branton and Deamer [2], Meyer and Winkelmann [22]).

This polarity enables us to determine whether microsomal vesicles retain their orientation (right-side-out) compared to membranes of the endoplasmic reticulum. In agreement with data obtained from thin section electron microscopy (Leskes et

al. [19, 20]) and from investigations of enzyme distribution in situ and in microsomes (Kreibich et al. [23], Kuriyama [24]) we find right-side-out microsomal vesicles. This is concluded from the observation that the particle-rich faces are concave shaped, corresponding to the leaflet neighbouring the external medium of the microsomal vesicles (compare Fig. 2). Only in very few cases are convex particle-rich vesicles found, independent of the experimental procedure, and as they are mostly irregular in shape and larger in size, they are likely to be of plasmalemmal origin.

The membrane-associated particles in microsomal vesicles are randomly distributed at 30 °C, but are aggregated at 4 °C. This can be evaluated only on A-faces, since the number of particles on B-faces is too small. The particle aggregation is expressed in terms of an aggregation index and of the maximum particle density per unit area. We are aware of the fact that the values obtained are not absolute numbers, because of the continuously changing angle of the fracture plane compared to the angle of shadowing. But as this is a statistical event, it is averaged in our experiments, and the procedure can be used to compare the different preparations. With respect to this we find a relative number of about 3500 particles per μm^2, which is identical for aggregated and non-aggregated preparations, indicating that the total number of particles in the A-faces has not changed. A recent characterization of various membranes from rat hepatocytes according to their particle densities showed similar numbers of membrane associated particles for microsomal membranes (Losa et al. [25]).

Aggregation of membrane-associated particles in different membranes (described by various authors) has been attributed to different molecular mechanisms. Pinto da Silva [26] claims an electrostatic phenomenon responsible for ionic strength- and pH-dependent particle aggregation in erythrocyte membranes. Since these two parameters have been kept constant in our experiments, such a mechanism seems unlikely to be of importance in microsomal vesicles. McIntyre et al. [27] report particle aggregations in mouse lymphocytes induced by glycerol treatment, which can be prevented by prefixation with glutaraldehyde. As we routinely fixed our preparations before the addition of glycerol for cryoprotection, glycerol cannot be the inducing factor for particle aggregation in our membrane preparations. In addition, for glycerination no further temperature adjustment was performed. Thus the glycerination step occurred at almost the same temperature for experiments originally performed at 30 °C as well as at 4 °C, but nevertheless a marked difference in particle aggregation was observed.

Temperature-dependent particle aggregations have been observed in various membrane preparations. Such aggregations have been described in several microorganisms [3–9] and in lymphocytes [10].

All these investigators claimed a phase transition of the phospholipid bilayer to be responsible for these phenomena. In experiments in one of the authors' laboratory, studying the temperature dependence of the activation energy of drug monooxygenation, indications were found for a phospholipid phase transition in rat liver microsomes (Duppel and Ullrich [1], preceding paper). To test the hypothesis that phase transitions cause particle aggregation in microsomes, we performed shock-freezing experiments. By this procedure phospholipid molecules were transferred extremely rapidly from the liquid-crystalline state to the temperature of liquid N_2, thus preventing a particle redistribution caused by a partial freezing-out of

phospholipid molecules. Indeed a particle aggregation could be prevented in these experiments.

Particle distribution could be influenced by some investigators by altering the composition of various membranes (microorganisms [3, 4, 6, 8, 9], erythrocytes [11] and sarcoplasmic reticulum [12]). Verkleij et al. [3] and Rottem et al. [6] studied the effect of cholesterol on particle distribution in *Acholeplasma* and *Mycoplasma* membranes, respectively. In accordance with their findings particle aggregation was prevented at 4 °C in the presence of cholesterol in microsomal vesicles (Fig. 4). As cholesterol is known to increase the fluidity of phospholipid membranes below the phase transition as shown by NMR [28], ESR [29], fluorescence techniques [30] and differential scanning calorimetry [31], this is a further indication that particle aggregation in microsomal membranes is caused by a partial freezing-out of phospholipids.

Considering the fact that the activation energy of drug monooxygenation reactions in hepatic microsomes is increased at lower temperatures (preceding paper [1]) the present findings offer a possibility of interpretation. If the membrane proteins involved in drug monooxygenation behave like membrane-associated particles, or if they are even integral constituents of these, they may be aggregated at lower temperature when the phospholipid molecules undergo a phase transition. The restricted mobility impedes an effective electron transfer, thus increasing the activation energy.

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