Biochimica et Biophysica Acta, 508 (1978) 55—64

BBA 77954

TRANSFER OF PHOSPHATIDYLCHOLINE BETWEEN DIFFERENT MEMBRANES IN *TETRAHYMENA* AS STUDIED BY SPIN LABELING

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(Received September 12th, 1977)

Summary

Transfer of phosphatidylcholine molecules between different membrane fractions of *Tetrahymena pyriformis* cells grown at 15, 27 and 39.5°C was studied by electron spin resonance (ESR). Microsomes were labeled densely with a phosphatidylcholine spin label and the spin-labeled microsomes were incubated with non-labeled cilia, pellicles or microsomes. The transfer of the phosphatidylcholine spin labels was measured by decrease in the exchange broadening of the electron spin resonance spectrum. In one experiment, the lipid transfer was measured between ^{32}P-labeled microsomes and non-labeled pellicles by use of their radioactivity. The result was in good agreement with that by ESR. The fluidity of the membrane was estimated using a fatty-acid spin label incorporated into the membranes. Transfer between lipid vesicles was also studied. The results obtained were as follows: (1) The transfer between sonicated vesicles of egg- or dipalmitoyl phosphatidylcholine occurred rapidly in the liquid crystalline phase, with an activation energy of 20 kcal/mol, whereas it hardly occurred in the solid crystalline phase. (2) The transfer rate between microsomal membranes increased with temperature, and an activation energy of the reaction was 17.8 kcal/mol. (3) The transfer from the spin-labeled microsomes to subcellular membranes of the cells grown at 15°C was larger than that to the membranes of the cells grown at 39.5°C. The membrane fluidity was larger for the cells grown at lower temperature. (4) Similar tendency was observed for the transfer between microsomal lipid vesicles prepared from the cells grown at 15°C and at 39.5°C. (5) The transfer from microsomes to various membrane fractions increased in the order, cilia < pellicles < microsomes. The order of increase in the membrane fluidity was cilia < microsomes < pellicles, although the difference between microsomes and pellicles was slight. These results indicate a crucial role of the membrane fluidity in the transfer reaction. (6) Some evidence supported the idea that the lipid trans-

fer between these organelles occurred through the lipid exchange rather than through the fusion.

Introduction

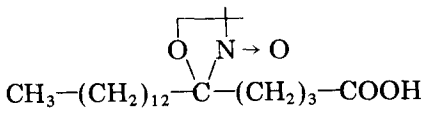
Much attention has been paid to the fluidity of biological membranes in relation to their functions and several lines of evidence have shown close correlations between physical state of phospholipids and the membrane functions [1]. In the present paper, we have studied transfer of phospholipid between various membrane fractions of *Tetrahymena pyriformis* cells in relation to the fluidity.

It is well known that the phospholipid exchange contributes to formation of biomembranes or dynamic equilibrium in membrane constituents of cell organelles. Using radioisotope technique, Nozawa and Thompson [2] have shown that exchange was involved in membrane formation in *T. pyriformis*. In spite of its popularity, this method inevitably requires much time for separation of the two membrane fractions by centrifugation or chromatography and cannot give information on the lipid transfer between the same kind of membranes. Recently, Maeda and Ohnishi [3] have developed a faster method using spin-labeled lipids which can be used to follow the rapid lipid transfer between the same kind of membranes as well as that between different membranes without separations. In the present investigation, we have densely labeled microsomes of *T. pyriformis* with a phosphatidylcholine spin label and incubated with non-labeled subcellular organelle membranes of the cells grown at various temperatues. The transfer of spin-labeled phosphatidylcholine was detected by decrease in the exchange broadening of the ESR spectrum.

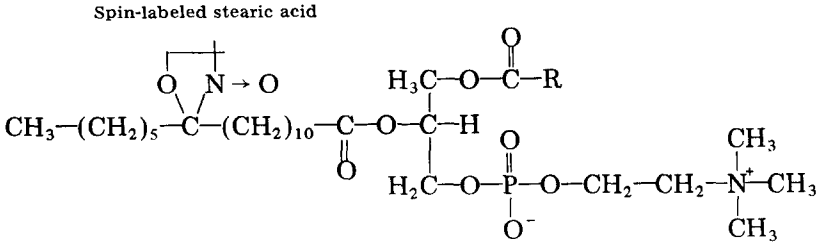
Materials and Methods

Lipids and spin-labeled derivatives

Egg yolk phosphatidylcholine was obtained by the method of Singleton et al. [5] and dipalmitoylphosphatidylcholine was prepared from anhydride of palmitic acid and glycerylphosphorylcholine as described by Robles and Van der Berg [6]. Spin probes, *N*-oxyl-4′-4′-dimethyloxazolidine derivative of 5-ketostearic acid (spin-labeled stearic acid) and spin-labeled phosphatidylcholine were synthesized according to the procedures of Waggoner et al. [7] and Hubbell and McConnell [8], respectively.



Spin-labeled stearic acid



Spin-labeled phosphatidylcholine

Preparation of membrane fractions and microsomal lipid from T. pyriformis cells

A thermotolerant strain of *T. pyriformis* NT-1 cells [9] was grown at 15, 24 and 39.5°C in an enriched proteose peptone medium as described previously [10]. Cultures (200 ml) were harvested when the cells reached a mid-logarithmic phase of growth with incubation for 24 h at 39.5°C, 36 h at 24°C and 135 h at 15°C, respectively. Subcellular fractions, cilia, pellicles and microsomes were isolated essentially according to the method of Nozawa and Thompson using a concentrated phosphate buffer (0.2 M K_2HPO_4/0.2 M KH_2PO_4/3 mM EDTA/0.1 M NaCl, pH 7.2) [10].

Lipids were extracted from individual membrane fractions by the method of Bligh and Dyer [11]. Phospholipid phosphorus content was determined by the method of Bartlett [12] with a modification of decomposition in 70% perchloric acid described by Marinetti [13].

Measurement of lipid transfer

Spin-labeled phosphatidylcholine (2 mg) was suspended in 1 ml of the concentrated phosphate buffer, sonicated for 10 min in an ice bath under a nitrogen stream with a 20 kHz tip-type sonifier (T-A-4201, Kaijo Denki, Co., Tokyo) and centrifuged at 4°C for 60 min at 100 000 × *g* to remove the small amount of undispersed lipid. The microsomes (1 mg phospholipid/ml) taken from the cells grown at 24°C were incubated with the sonicated vesicles of spin-labeled phosphatidylcholine (0.5 mg/ml) at 30°C for 40 min. After the incubation, the spin-labeled microsomes were washed once with bovine serum albumin solution (5 mg/ml) and once with phosphate buffer to remove unincorporated spin-labeled phosphatidylcholine. The incorporated spin-labeled phosphatidylcholine amounted to approx. 16.7% of the total microsomal phospholipid. 1 vol. of the spin-labeled microsomes (8 mg of phospholipid/ml) was mixed with 2 vols. of unlabeled subcellular membrane fractions (4 mg phospholipid/ml) in ice and the mixture was put into a quartz capillary for ESR measurements. The ESR spectra were measured at various temperatures using an X-band commercial spectrometer (JEOL ME-2X) equipped with a variable temperature control.

The transfer rate was estimated from the initial increase in the central peak height that was caused by decrease in the exchange broadening of the ESR spectrum due to dilution of spin-labeled phosphatidylcholine. The peak height of the spin label signal depends on the membrane fluidity and a calibration was made for the difference using non-exchange-broadened ESR spectrum of cilia, pellicles and microsomes at each temperature. In some experiments, the amount of the transferred spin-labeled phosphatidylcholine was estimated from the analysis of the changes in ESR spectra by a computer simulation. For the simulation, a series of model spectra was recorded with dispersions of egg yolk phosphatidylcholine containing various amounts of spin-labeled phosphatidylcholine.

Transfer between sonicated vesicles of total lipid extract from microsomes was measured in a mixture of the vesicles containing spin-labeled phosphatidylcholine (10% of total phosphorus content) and non-labeled vesicles (2.8 mg of phospholipid/ml each). Transfer between spin-labeled phosphatidylcholine and

egg yolk phosphatidylcholine or dipalmitoyl phosphatidylcholine vesicles was measured as described previously [3].

In addition to the spin-label method, the transfer of phosphatidylcholine was measured by radioisotope technique. Radioactive orthophosphate (carrier-free, 0.5 mCi, 2 ml) was added to 200 ml of cell culture an hour before the harvest, and radioisotope-labeled microsomes were prepared according to the method described above. After the ^{32}P-labeled microsomes and non-labeled pellicles were incubated for 30 min under the same conditions as those used in spin-label method, the membrane fractions were separated by centrifugation and their lipids were extracted by the method of Bligh and Dyer [11]. The individual extract was developed in a thin layer chromatograph of silica gel. The amount of phosphorus and the radio activity were determined by the method of Bartlett [12] and by a liquid scintillation counter [2], respectively. The transferred phosphatidylcholine was estimated from two experiments with the extracted lipids from microsomes and pellicles, independently. These values were almost equal.

The order parameter was measured by using spin-labeled stearic acid incorporated into various membrane fractions as described previously [4] and was used as a convenient measure for the membrane fluidity, although this parameter is directly related to flexibility of the lipid alkyl chain.

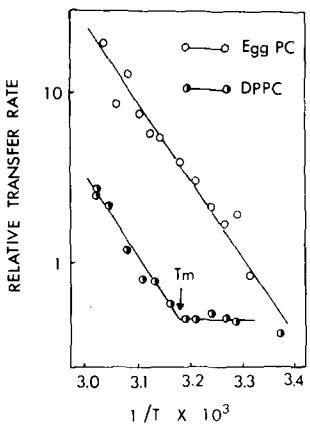
Results

Transfer between phosphatidylcholine bilayer membranes

To measure the effect of membrane fluidity on the transfer of phospholipid, we have measured the temperature dependence of the transfer rate of spin-labeled phosphatidylcholine molecules between spin-labeled phosphatidylcholine and egg yolk phosphatidylcholine or dipalmitoyl phosphatidylcholine vesicles. Immediately after mixing vesicles of spin-labeled phosphatidylcholine with non-labeled vesicles of phosphatidylcholine, a sharp three-line spectrum appeared superimposed on the exchange-broadened one and its intensity grew with time. The transfer rate was estimated from the increase in peak height of the three-line component [3]. As shown in Fig. 1, the Arrhenius plot of the rate constant gave a straight line from 25°C to 54°C for egg yolk phosphatidylcholine and above 41°C for dipalmitoyl phosphatidylcholine. The activation energy was calculated to be 19.5 kcal/mol for egg phosphatidylcholine and 22.8 kcal/mol for dipalmitoyl phosphatidylcholine. However, the transfer rate to dipalmitoyl phosphatidylcholine vesicles below the phase transition temperature (41°C) was very small and almost independent of temperature. This indicates that vesicular collision is not the rate determining step, and that the physical state of bilayer membrane plays an important role in the transfer reaction.

Transfer between microsome membranes

Microsomes were taken from *T. pyriformis* cells grown at 24°C and labeled densely with spin-labeled phosphatidylcholine molecules. The ESR spectrum showed exchange broadening because of the high concentration of spin-labeled phosphatidylcholine in the membrane (16.7%). When the densely-labeled



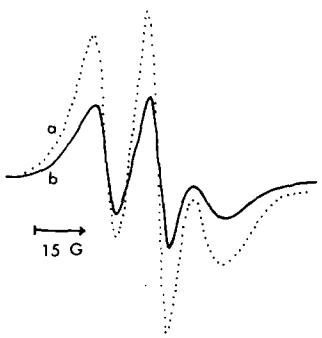


Fig. 1. Temperature dependence of lipid transfer from sonicated spin-labeled phosphatidylcholine vesicles to dipalmitoylphosphatidylcholine (○) or egg yolk phosphatidylcholine (○) vesicles. The vesicles of spin-labeled phosphatidylcholine (0.2 mM) was mixed with those of dipalmitoyl phosphatidylcholine (5 mM) or egg yolk phosphatidylcholine (2 mM) in 0.1 M KCl/50 mM Tris · HCl, pH 8.0. The initial rate of the lipid transfer between the vesicles was estimated from the increase of the sharp three lines which appeared superimposed on an exchange-broadened spectrum after the mixing. A calibration was made for the difference in membrane fluidity at various temperatures.

Fig. 2. Change in the ESR spectrum of the spin-labeled microsomes on mixing with non spin-labeled microsomes. One volume of the spin-labeled microsomes (8 mg of phospholipid/ml) was mixed with 2 vols. of unlabeled microsomes (4 mg of phospholipid/ml) in a concentrated phosphate buffer (0.2 M K_2HPO_4/0.2 M KH_2PO_4/3 mM EDTA/0.1 M NaCl, pH 7.2) and incubated for (a) 30 min at 37°C and (b) 0 min in a quartz capillary. Spectral analysis by computer simulation showed that the amount of the transferred spin-labeled phosphatidylcholine in spectrum (b) to (a) corresponded to 6% of the total phospholipids.

microsome sample was incubated with non-labeled one, the ESR spectrum became narrowed and the peak height increased (see Fig. 2). The spectral change is attributable to dilution of spin-labeled phosphatidylcholine molecules due probably to transfer and intermixing of spin-labeled phosphatidylcholine between microsomes. Almost no spectral changes were observed when the spin-labeled microsomes were incubated in the absence of other membranes. Fig. 3 plots the central peak height as a function of incubation time. An analysis of changes in the ESR spectra indicated that 6% of total phospholipids was transferred by incubation at 37°C for 30 min between microsomes from cells grown at 24°C. It is clearly shown that the transfer rate was dependent on temperature. The Arrhenius plot of the relative rate obtained from the initial slope gave a straight line and the activation energy obtained was 17.8 kcal/mol. This value was quite similar to that for the phosphatidylcholine vesicles.

Effect of fluidity of acceptor membrane on transfer

Various membrane fractions were taken from cells grown at different temperatures (15 and 39.5°C), and the transfer of spin-labeled phosphatidylcholine from spin-labeled microsomes to these membranes was measured. The fluidity of these membranes was dependent on the cell-growth temperature [4]. Fig. 4 shows the order parameter of spin-labeled stearic acid incorporated into these

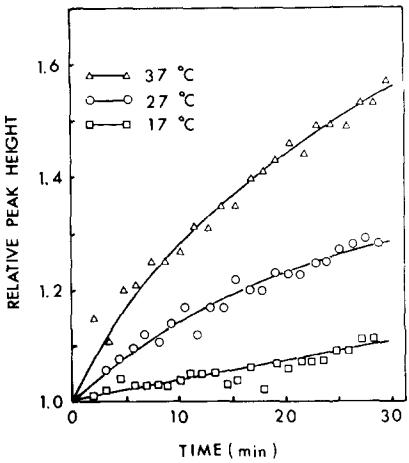


Fig. 3. Lipid transfer between microsome membranes at various temperatures. The spin-labeled microsome, which was prepared from cells grown at 24°C, was incubated with non-labeled one at 17°C (□), 27°C (○) and 37°C (△). The increase in the central peak height of the ESR spectrum was plotted on the ordinate. Other experimental conditions were the same as those in Fig. 2.

membrane fractions at various temperatures. The membranes prepared from cells grown at 15°C had distinctly greater fluidity than those grown at 39.5°C when compared at the same temperature. The transfer to these membranes is shown in Fig. 5. In all cases the transfer of spin-labeled phosphatidylcholine to the

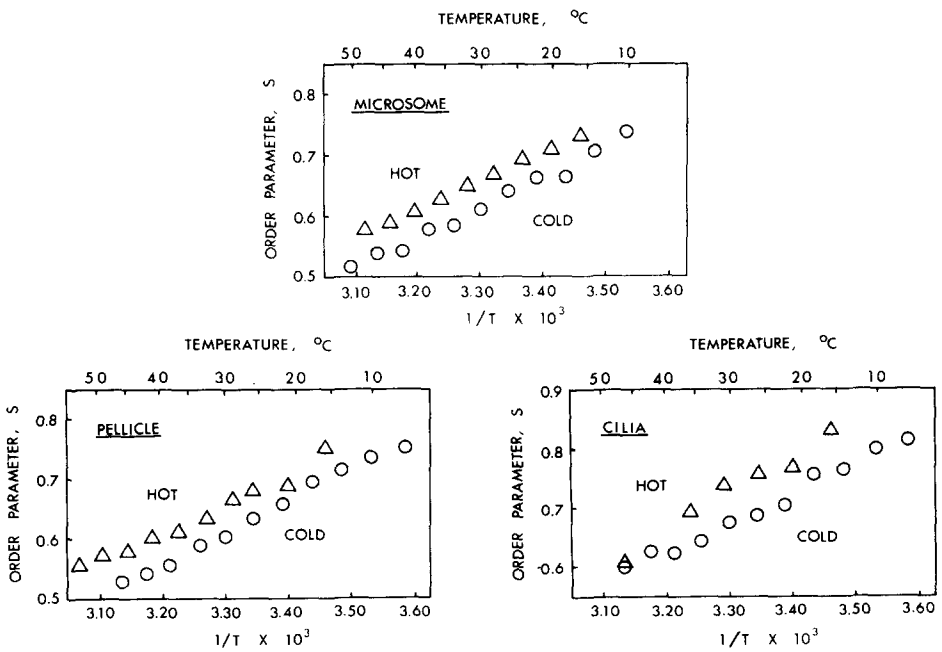


Fig. 4. Dependence of the membrane fluidity on the growth temperature of host cells. Microsomes (a), pellicles (b) and cilia (c) were taken from cells grown at 15°C (○) or 39.5°C (△) and spin-labeled with spin-labeled stearic acid. The order parameter obtained from the ESR spectrum was plotted against $1/T$ ($^{\circ}K^{-1}$).

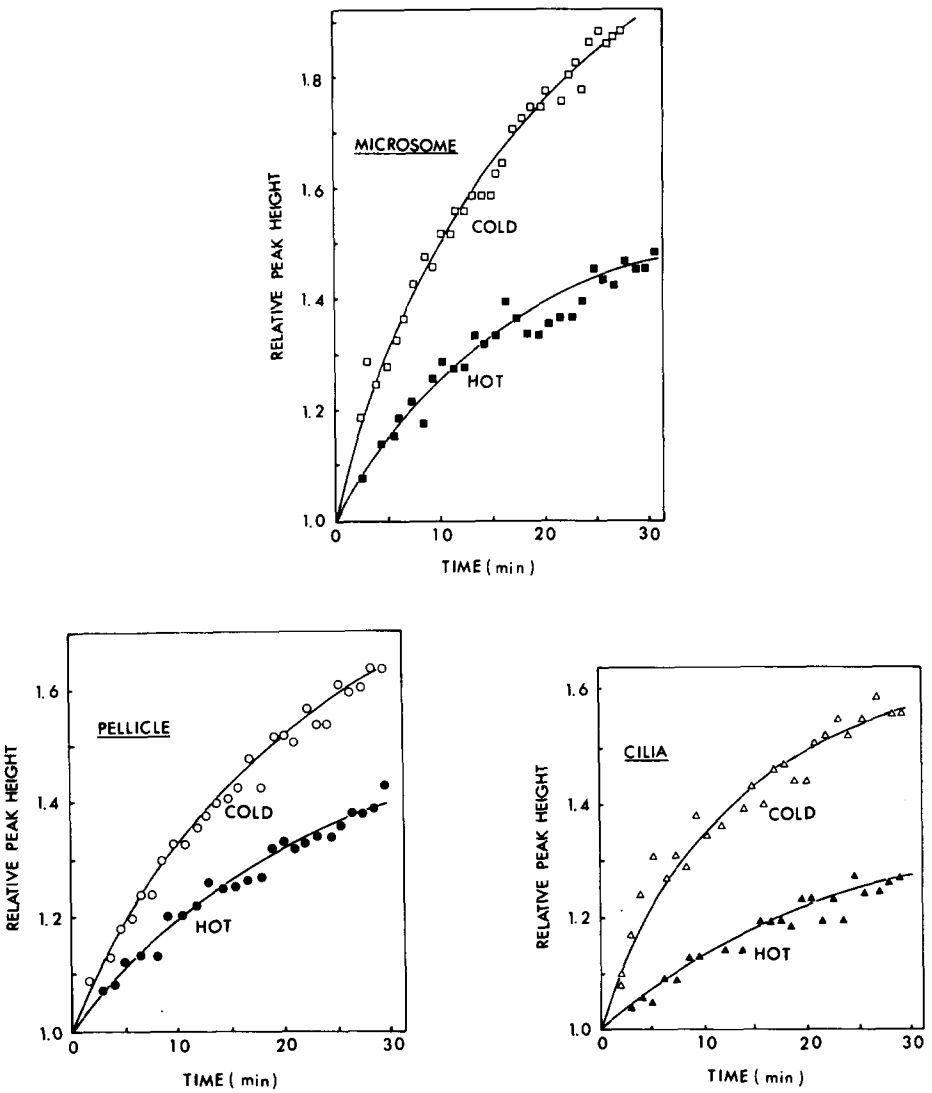


Fig. 5. Effect of the acceptor membrane fluidity on the lipid transfer. The microsome membranes were prepared from cells grown at 24°C, spin-labeled and used as the donor membranes. The acceptor membranes, microsome (a), pellicles (b) and cilia (c) were prepared from cells grown at 15°C (□,○,△) or 39.5°C (■,●,▲). The donor membrane was incubated with the acceptor membrane at 37°C and the central peak height of the ESR spectrum was plotted on the ordinate.

membrane fractions of the cells grown at the lower temperature was about 1.5—2 times faster than to those of the cells grown at the higher temperature. These results clearly indicate that the membrane fluidity plays a crucial role in the transfer of phospholipid between biological membranes. A similar tendency was observed in lipids extracted from microsomes of cells grown at 15 and 39.5°C. The transfer of spin-labeled phosphatidylcholine molecules between microsomal lipid vesicles prepared from cells at 15°C was about twice as fast as that between the microsomal lipid vesicles taken from cells grown at 39.5°C.

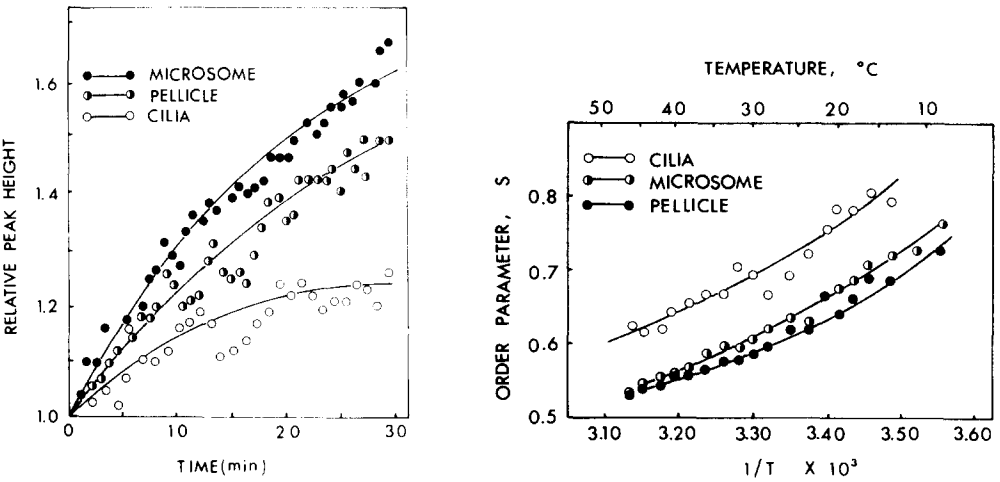


Fig. 6. Lipid transfer from spin-labeled microsomes to microsomes (●), pellicles (◑) and cilia (○). All the membrane fractions were prepared from the cells grown at 24°C. Increase in the central peak height measured at 37°C was plotted. A calibration was made for the difference of fluidity in each membrane fraction.

Fig. 7. Comparison of fluidity of various membrane fractions taken from the cells grown at 24°C. Microsomes (◑), pellicles (●) and cilia (○) were spin-labeled with spin-labeled stearic acid and the order parameter was plotted against $1/T$ ($°K^{-1}$).

Transfer between different membrane fractions

The transfer of spin-labeled phosphatidylcholine from the spin-labeled microsomes to microsomes, pellicles and cilia was measured and compared in Fig. 6. The order of transfer rate was cilia < pellicles < microsomes. The order parameter of spin-labeled stearic acid incorporated into these membranes was shown in Fig. 7 which indicates that ciliary membranes were the most rigid and pellicles were the most fluid of the membrane fractions. Microsomes had a slightly lower fluidity than pellicles. The observed difference in the transfer rate between cilia and the other membranes was consistent with the difference in the membrane fluidity. However, the transfer to microsomes was faster than that to pellicles in spite of the slightly lower fluidity of the microsomal membranes.

Comparison with the transfer rate obtained by a usual method

Using radioisotope technique, we measured the amount of phosphatidylcholine transferred by incubation at 37°C for 30 min between ^{32}P-labeled microsomes and non-labeled pellicles. After the incubation, the two kinds of membranes were separated by centrifugation and their lipids were extracted and developed in a thin layer chromatograph. The analysis of microsomal lipids indicated that the amount of the transferred phosphatidylcholine was 7% of the total phospholipids. The same analysis of lipids from pellicles showed that 6% of total phospholipids was transferred. These values are quite similar, which supports the theory that the transfer of phosphatidylcholine between these membrane fractions occurs by exchange of phosphatidylcholine rather than by fusion of membranes. Moreover, they were in a good accord with that obtained

by an analysis of ESR spectral change (5% of total phospholipids).

We performed another experiment with electron microscopy to confirm that the changes in the ESR spectra occur by lipid exchange, not by fusion. The electron micrographs before and after the incubation of microsomes and pellicles at 37°C for 30 min were essentially the same and did not give positive evidence showing the fusion of these organelles.

Discussion

Subcellular organelles are known to have different distribution patterns of their constituent lipids and fatty acids. It is an interesting problem how the patterns are controlled. The lipid transfer between organelles may be an important factor affecting them, and transfer or exchange of phospholipids between membranes has been a subject of much research. A catalytic exchange has, in fact, been observed between microsomes and mitochondria from various tissues, such as thyroid [14], potato and cauliflower [15] and liver [16—18]. In this report, we have examined non-catalytic transfer of spin-labeled phosphatidylcholine in model membranes and subcellular fractions of a thermotolerant strain of *Tetrahymena* (NT-1), with special attention to the membrane fluidity.

A definite lipid transfer was observed between sonicated vesicular membranes of phosphatidylcholine in the liquid crystalline state. The transfer rate was dependent on the fluidity of the membrane and the activation energy was about 20 kcal/mol. The transfer rate was very small below the phase transition temperature. These intermembrane motions of phospholipid may be related to the flip-flop motion of the molecules in the bilayer membranes. It is interesting therefore to note that the activation energy was in the same order of magnitude as that for the flip-flop motion of phospholipid in egg yolk phosphatidylcholine vesicles (approx. 20 kcal/mol) [19]. The lateral motion of phospholipids also increases with temperature. The activation energy for the motion was, however, much smaller (approx. 5 kcal/mol) [20].

The transfer of lipids between biological membranes was shown by experiments using various organelle membranes taken from *T. pyriformis* cells grown at different temperatures to be faster to more fluid membranes. The transfer between microsome membranes was faster at higher temperatures. The activation energy for the transfer (17.5 kcal/mol) was quite similar to that for phospholipid bilayer membranes. The second line of evidence comes from experiments using organelle membranes isolated from the cells grown at different temperatures. The change in the growth temperature caused alteration in the membrane fluidity as a result of change principally in unsaturation of lipid side chains [4]. Since the other conditions such as morphology, surface charge of proteins and lipids, and their distribution in the membranes must be the same, the results obtained with this system give the most conclusive evidence for the dependence of the transfer upon fluidity. Finally, the differences in the transfer between different organelle membranes also support the general tendency. However, the results on the pellicles and microsomes suggest importance of the other factors, in addition to the fluidity, in the transfer reactions between membranes. Some evidence showed that the lipid transfer occurred

by exchange of lipids rather than fusion of membranes.

Whether some phospholipid exchange proteins are present in Tetrahymena cells is an interesting question. In our preliminary experiment, the pH 5.1 supernatant from the cells did not show any stimulation of the lipid transfer. There is a possibility that phospholipid exchange proteins in primitive cells, if there are any, may have different properties from those of animal cells and work along this line is in progress in this laboratory.

Acknowledgements

The investigation was supported in part by the grants 5R070 from Japan Society for the Promotion of Science and No. 158054 to N.Y. from the Ministry of Education, Japan.

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