1 The authors are grateful to Mr C.T. Luk for his skilled technical assistance.
2 Friedman, G.D., Siegelaub, A.B., and Seftzer, C.C., New Engl. J. Med. *290* (1974) 469.
3 World Health Organization Technical Report, Series No. 568, Geneva 1975.
4 Jarvis, L.R., and Whitehead, R., Gastroenterology *78* (1978) 1488.
5 Moody, P.M., Griffith, R.B., and Averitt, J.H., Proceedings of the University of Kentucky Tobacco and Health Research Institute, Conference Reports, Kentucky, 1972.
6 Ageel, A.M., Parmar, N.S., and Tarig, M., Life Sci. *34* (1984) 751.

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Age-dependent changes of rat liver plasma membrane composition

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Summary. The chemical composition of liver plasma membrane was studied in Wistar rats aged between 3 and 24 months. Results obtained indicate a significant age-dependent positive correlation of both the protein:phospholipid and cholesterol:phospholipid ratios, whereas the protein:cholesterol ratio seems to remain unaffected. Phospholipid analysis of liver plasma membrane reveals that only the phosphatidylcholine content has a significant negative correlation with age; all other phospholipid species remain basically unchanged.
Key words. Liver plasma membrane; aging; phospholipids; cholesterol.

A number of cellular functions which involve, at least partially, the participation of the plasma membrane show age-related modifications[2,3]. Whether aging affects the gross plasma membrane composition, leading in turn to the observed modifications, is still under debate since the characteristics of many membrane-bound proteins, including enzymes and receptors, are largely influenced by the physicochemical properties of the membrane microenvironment[4].

Data to be reported here deal with some major modifications occurring in rat liver plasma membrane composition over the post-maturation period of life, spanning from 3 to 24 months. As it is well known, the hepatocyte plasma membrane is a complex organelle consisting of three functionally and morphologically distinct domains: i.e. the blood sinusoidal front, the bile canalicular front and the lateral surfaces, accounting respectively for about 72%, 13% and 15% of the total surface area[5]. As a first approach, and in order to obtain membrane preparations representative of all three domains, we chose to follow the isolation procedure recently developed by Hubbard and coworkers[5] which gives a yield of about 50% sinusoidal front membranes, whereas previous methods seem to favor an enrichment of bile canalicular and lateral membranes[5]. The present work indicates that liver plasma membrane composition is indeed affected by aging, in a fashion which is suggestive of a possible decrease of membrane lipid fluidity.

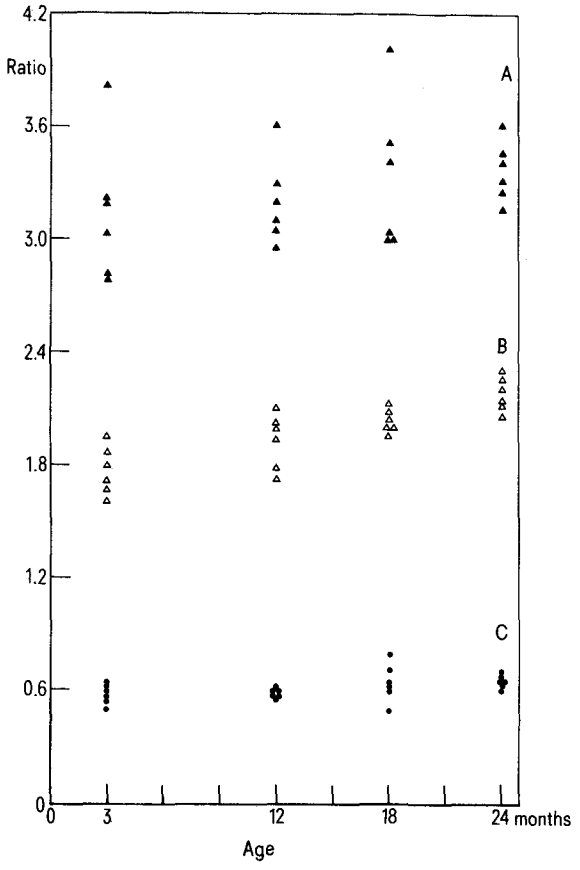
Material and methods. Male Wistar rats, aged between 3 and 24 months, fed with a standard diet, were starved 16 h before experiments. Before sacrifice, animals were anesthesized by i.p. injection of sodium pentobarbital (12 mg/kg b.wt). The average b.wts of animals employed were as follows (mean ± SD): 140 ± 35 g (3 months); 440 ± 65 g (12 months); 465 ± 40 g (18 months); 505 ± 45 g (24 months). Each age group consisted of six animals.

Livers were perfused in situ with 300 ml of cold 0.15 M NaCl before starting the plasma membrane isolation procedure according to Hubbard and coworkers[5]. Isolated liver plasma membranes were first washed with 0.015 M EDTA (pH 7.4), then resuspended and washed with 0.05 M Tris-HCl buffer (pH 7.5) containing 0.1 M NaCl, and finally resuspended in twice distilled water at a final concentration of 1.5 mg protein/ml. The purity of our plasma membrane preparations was checked by the distribution of marker enzymes (Na^+–K^+)-ATPase and 5'-nucleotidase, assayed as previously reported[6]. The average enrichment factor of specific activity in the plasma membrane fraction with respect to homogenate was 24 and 19 for (Na^+–K^+)-ATPase and 5'-nucleotidase respectively. In order to rule out a possible contamination of plasma membrane preparations by endocellular membranes, routinary assays of cytochrome oxidase, acid phosphatase and glucose-6-phosphatase were carried out following already reported procedures[7]. The average enrichment factors were 0.3, 1.0 and 0.9 for cytochrome oxidase, acid phosphatase and glucose-6-phosphatase respectively. Proteins were estimated by the method of Lowry[8] using bovine serum albumin as a standard.

Total phospholipids were extracted according to Bligh and Dyer[9] all steps being performed under nitrogen. Individual phospholipid species were separated by thin layer chromatography using silica gel G plates (Merck, Darmstadt, FRG) activated for 1 h at 120°C as previously reported in detail[10]. The average recovery was 85%, a result which remains constant with aging. Total cholesterol was determined by the cholesterol oxidase method using a high performance 'Monotest' kit (Boehringer, Mannheim, FRG).

Results and discussion. Figure 1 shows three major ratios of plasma membrane components. Protein:cholesterol ratio (fig. 1, A) remains fairly constant with aging, whereas protein:phospholipid (fig. 1, B) as well as cholesterol:phospholipid ratio (fig. 1, C) show a positive correlation with age, increasing significantly between 3 and 24 months; it is worth mentioning that an increase of the latter ratio with age has been reported so far only in human erythrocyte plasma membrane[4,11]. In particular a t-test for means obtained from data reported in figure 1, B, gave the following results: 3 months vs 12 months, n.s.; 3 months vs 18 as well as 24 months, $p < 0.001$; 12 months vs 18 months, n.s.; 12 months vs 24 months, $p < 0.01$; 18 months vs 24 months, $p < 0.01$. For data reported in figure 1, C, only the comparison between age groups 3 and 24 months gave a significant ($p < 0.001$) t-test. The phospholipid pattern of liver plasma membranes in aging Wistar rats is reported in figure 2. The phosphatidylcholine level (fig. 2, A) shows a significant negative correlation with age, whereas phosphatidylserine + phosphatidylinositol (fig. 2, B), phosphatidylethanolamine (fig. 2, C) and sphingomyelin (fig. 2, D) levels do not seem to be significantly related to age. The comparison between age groups for means obtained from data reported in figure 2, A gave the following results as for a t-test evaluation: 3 months vs 12, 18 and 24 months, $p < 0.001$; all other differences being not significant.

Our results represent, to our knowledge, the first report of the



phospholipid composition of rat liver plasma membrane during aging. Actually, a report was presented years ago[12] showing no significant change of liver plasma membrane composition with age, but the comparison with our data is somewhat difficult because of the different rat strain employed and the unclear indication of the age of the animals, which were only divided according to their weight.

As far as young adult animals (3 months) are concerned, the results reported in figures 1 and 2 are in good agreement with published literature[12-17]. These results represent, so far, the only available characterization of the phospholipid pattern of lipid plasma membranes obtained following the Hubbard's procedure[5]. Moreover, our results strongly support the morphological and enzymatic characterization reported by Hubbard[5], being in close agreement with results published by Evans and coworkers[15] concerning the lipid composition of sinusoidal front liver membranes, thus suggesting that Hubbard's method does indeed give an appreciable enrichment of this fraction of plasma membranes.

It has been proposed that the decrease of phosphatidylcholine content[18,19] and/or the increase of protein:phospholipid and cholesterol:phospholipid ratios[18,20] can be considered among factors leading to the decrease of membrane lipid fluidity. On such a basis, the results reported in the present communication indicate that a significant decrease of liver plasma membrane

Figure 1. Protein:cholesterol ratio (A, mg/μmole); protein:phospholipid ratio (B, mg/μmole); and cholesterol:phospholipid molar ratio (C) were determined on rat liver plasma membranes isolated from animals of various ages. Regression analysis of reported values gives the following correlation coefficient (r): A, $r = 0.33$ (n.s.); B, $r = 0.82$ ($p < 0.01$); C, $r = 0.46$ ($p < 0.05$).

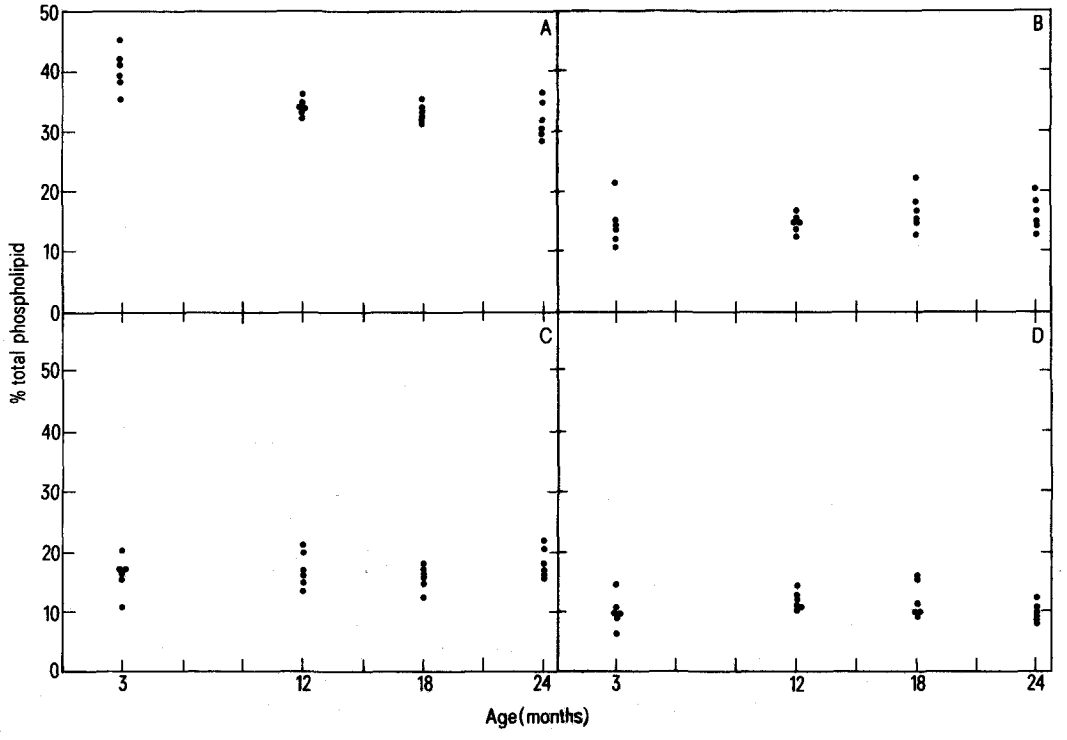


Figure 2. Phosphatidylcholine (A); phosphatidylserine + phosphatidylinositol (B); phosphatidylethanolamine (C) and sphingomyelin (D) were determined on liver plasma membranes isolated from animals of various ages. Regression analysis of reported values gives the following correlation coefficient (r): A, $r = 0.79$ ($p < 0.01$); B, $r = 0.28$ (n.s.); C, $r = 0.18$ (n.s.); D, $r = 0.03$ (n.s.).

lipid fluidity might take place during the aging process, thus suggesting that a variety of membrane-linked phenomena, such as enzymatic activities or hormone responsiveness[2-4], are in some way modulated by changes of plasma membrane microenvironment.

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2 Roth, G.S., Mech. Ageing Dev. *9* (1979) 487.
3 Robert, L., in: Mammalian Cell Membranes, vol. 5, p. 220. Eds G.A. Jamieson and D.M. Robinson. Butterworths, London 1977.
4 Grinna, L.S., Gerontology *23* (1977) 452.
5 Hubbard, A.L., Wall, D.A., and Ma, A., J. Cell Biol. *96* (1983) 217.
6 Luly, P., Barnabei, O., and Tria, E., Biochim. biophys. Acta *282* (1972) 447.
7 Scapin, S., Autuori, F., Baldini, P., Incerpi, S., Luly, P., and Sartori, C., Comp. Biochem. Physiol. *73B* (1982) 779.
8 Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J., J. biol. Chem. *193* (1951) 265.
9 Bligh, E.G., and Dyer, W., Can. J. Biochem. Physiol. *37* (1959) 911.
10 Piacentini, M., Spinedi, A., Beninati, S., and Autuori, F., Biochim. biophys. Acta *731* (1983) 151.
11 Araki, K., and Rifkind, J.M., Life Sci. *26* (1980) 2223.
12 Rubin, M.S., Swislocki, N., and Sonenberg, M., Proc. Soc. exp. Biol. Med. *442* (1973) 1008.
13 Emmelot, P., Bos, C.J., Van Hoeven, R.P., and Van Blitterswijk, W.J., Meth. Enzym. *31* (1974) 75.
14 McMurray, W.C., and Magee, W.L., A. Rev. Biochem. *41* (1972) 129.
15 Kremmer, T., Wisher, M.H., and Evans, W.H., Biochim. biophys. Acta *455* (1976) 655.
16 Schroeder, F., and Soler-Argilaga, C., Eur. J. Biochem. *132* (1983) 509.
17 Solyom, A., and Lauter, C.J., Biochim. biophys. Acta *298* (1973) 743.
18 Shinitzky, M., and Barenholz, Y., Biochim. biophys. Acta *515* (1978) 367.
19 Hirata, F., and Axelrod, J., Science *209* (1980) 1082.
20 Singer, S.J., and Nicolson, G.L., Science *175* (1972) 720.

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Morphological and histochemical demonstration of a glycocalyx on the cell surface of *Chattonella antiqua*, a 'naked flagellate'

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Summary. Electron microscopy demonstrated the presence of glycocalyx on the cell surface of *Chattonella antiqua*, which was previously believed to be 'naked'. Histochemical analysis of this structure indicates that the glycocalyx consists of at least two different types of acidic complex carbohydrates, sulfated and nonsulfated, together with a neural carbohydrate-protein complex with 1,2-glycol groups and α-D-mannosyl and α-D-glucosyl residues.
Key words. Glycocalyx; histochemistry; flagellate; Rhaphidophyceae; *Chattonella antiqua*.

Modern microscopic techniques have disclosed a wide variety of cell surface specializations in phytoplankton. Nevertheless, there is a flagellate group called 'naked plankton'[1,2]. *Chattonella antiqua*, noted for its bloom, is a species of this group belonging to Rhaphidophyceae. The extremely delicate structure of these organisms has prevented an approach to the study of their surface morphology by conventional microscopic techniques. Thus, they have long been considered to be bounded simply by the plasma membrane directly exposed to the ambient water[3]. In animal and plant cells, however, recent studies have accumulated abundant evidence that most if not all cell surfaces have a special structure with polysaccharide-rich components[4,5]. Though the terminology of these structures has not been established, 'glycocalyx', proposed by Bennet[6] seems to be most often used in recent papers. In the present study, we attempted to elucidate the surface morphology and histochemistry of *Chattonella antiqua* at electron microscopic (EM) and light microscopic (LM) levels.

Materials and methods. Patches of *Chattonella antiqua* were obtained from Aichi Prefectural Fisheries Experimental Station. The organism was originally isolated by S. Toriumi from Mikawa Bay, Japan in 1972. *Chattonella* cells were grown in Guillard 'f' medium[7], at 20–24 °C, under 3000 lux CW fluorescent light, and a 14:10 h light:dark photoregime. For the present study, cultures in the early-exponential growth phase (at a cell density of about 500/ml) were used. For the EM study, 100 µl of a cell suspension were placed in a centrifuge tube, and 200 µl of 1% alcian blue (AB) solution (pH 2.5) were added to the tube just prior to the use of a fixative (200 µl) consisting of collidine-

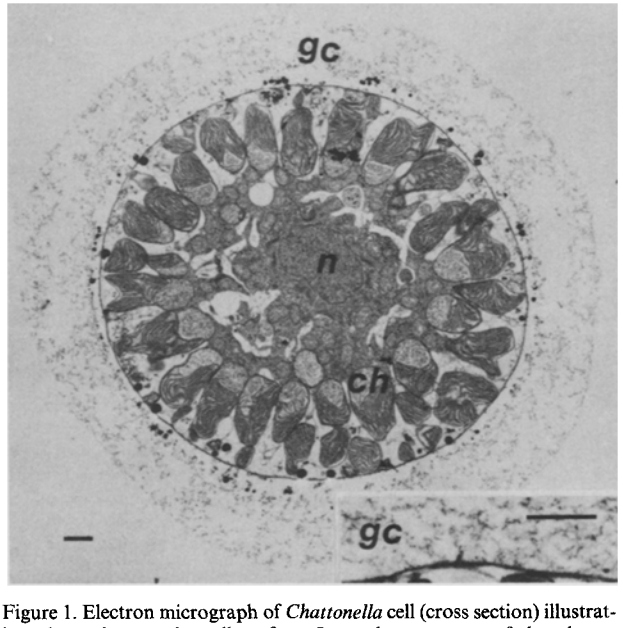

Figure 1. Electron micrograph of *Chattonella* cell (cross section) illustrating glycocalyx on the cell surface. Inset shows a part of the plasma membrane from another cell. Fine filaments appear to be continuous through the plasma membrane. Bars denote 1 µm. gc, glycocalyx; ch, chloroplast; n, nucleus.