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Studies on Lipids in Anurans II. Positional Distribution of Fatty Acids in Phosphatidyl Choline during Early Development in *Rana nigromaculata* and *Bufo japonicus*

By

Masashi RYUZAKI

Laboratory for Amphibian Biology, Faculty of Science, Hiroshima University, Hiroshima, Japan

(With 6 Text-figures)

INTRODUCTION

Phospholipids are generally one of the major components of the cell membranes in cells and differ from one another in molecular structure among different animals (MARAI and KUKSIS, 1973; MONTFOORT, VAN GOLDE and VAN DEENEN, 1971), organs (KUKSIS, MARAI, BRECKENRIDGE, CORNALL and STACHNYK, 1968), subcellular particles (O'BRIEN and GEISON, 1971), nutritional states (VAN GOLDE, TOMASI and VAN DEENEN, 1967), developmental stages (ABAD, BOSCH, MUNICIO and RIBERA, 1976) and diseases (BERGELSON and DYATLOVITSKAYA, 1973; RUGGIERI and DYATLOVITSKAYA, 1973; WALLACE, 1963). NISHIHARA and KITO (1978) have examined the changes in the molecular species of phospholipids contained in the membranes of dedifferentiating cells of soybean hypocotyl and cotyledone. Other workers have reported on the changes in the fatty acid composition of phosphatidyl choline contained in the brains of rats during the late stage of differentiation (ALLING and KARLSSON, 1973; MARSHALL, FUMAGALLI, NIEMIRE and PAOLETTI, 1966; SKRBIC and CUMINGS, 1970) and in the liver of developing rats (OGINO, MATSUMURA, SATOUCHI and SAITO, 1980). However, there has been no report on changes during early developmental stages in the frog.

The present author attempted to clarify the relationship between the changes in phospholipids and morphogenesis during development. For this purpose, the positional distribution of the constituent fatty acids in phosphatidyl choline molecules was examined by using samples at various stages from the unfertilized egg to the tadpole in *Rana nigromaculata* and *Bufo japonicus* (KAWAMURA, NISHIOKA and UEDA, 1980). The same analysis was made in the liver and brain of some adult frogs in order to compare the results with those obtained from the developing samples.

Present address: Department of Biology, Kitasato University School of Medicine, Sagamihara, Kanagawa 228, Japan

MATERIALS

Rana nigromaculata were collected during the breeding season from the suburbs of Shizuoka City, Shizuoka Prefecture. Eggs were artificially inseminated after ovulation had been accelerated by pituitary injection. For the analysis, samples at the following seven developmental stages were prepared: SHUMWAY stages 1 (unfertilized egg), 3 (two-cell), 9 (late blastula), 17 (early tail-bud), 20 (hatching) and 25 (operculum complete), and TAYLOR-KOLLROS (TK) stage IV (small limb-bud).

Bufo japonicus were collected during the breeding season from the suburbs of Sagamihara City, Kanagawa Prefecture. Fertilized eggs were obtained after natural oviposition by amplexus. Samples at the following three stages were prepared: SHUMWAY stages 3, 10~11 (dorsal lip to mid-gastrula) and 25. They were immediately analyzed or stored at −20°C.

The liver of adult *Rana nigromaculata* and *Bufo japonicus* and the brain of adult *Rana catesbeiana* collected from the suburbs of Ryugasaki City, Ibaragi Prefecture in spring were analyzed. After the animals were pithed, these organs were removed from the body, washed in saline, and analyzed immediately or stored at −20°C.

METHODS

1. Lipid extraction

The lipids were extracted by the method reported previously (FOLCH, LEES and SLOANE-STANLEY, 1957; RYUZAKI, KOJIMA and TAMAI, 1975). Phospholipids were separated by one-dimensional thin-layer chromatography (TLC) on a thin-layer plate of sodium carbonate-impregnated silica gel HR (MERCK, Darmstadt, W. Germany) and developed with chloroform-methanol-acetic acid-water (25: 15: 4: 2 by volume) (SKIPSKI, PETERSON and BARCLAY, 1964). The segment of the silica gel layer containing phosphatidyl choline (PC) was scraped off from the plate. PC was immediately extracted with chloroform-methanol (2: 1 by volume) and then washed with a small volume of distilled water. The amount of PC was measured on the basis of phosphorus content (BARTLETT, 1959) after digestion with 70% perchloric acid. Infrared spectra of PC were taken in a liquid film.

Phospholipase A_2 (from bee venom, SIGMA Chemical Co., St. Louis, Mo.) was used to remove the fatty acid residues from PC (HARVERKATE and VAN DEENEN, 1965). About 0.3 mg of purified PC was dissolved in 2 m*l* of diethyl ether, and hydrolyzed by 0.1 mg of phospholipase A_2 in 0.5 m*l* of 0.1 M borate buffer (pH 7.0) containing 2.5 mM $CaCl_2$. The whole reaction mixture was vigorously sonicated for 2 hours at 37°C. A small amount of an aliquot was placed on a silica gel HR plate to confirm the completeness of degradation.

2. Fatty acid extraction

Fatty acids from the 2-position of the PC and lysophosphoglycerides were extracted with chloroform-methanol (2: 1 by volume) and separated by TLC on silica gel HR plates, using a solvent system containing chloroform-methanol-acetic acid-water (160: 50: 2: 6 by volume). The isolated fatty acids and lysophosphoglycerides were methanolyzed in absolute methanol containing 3% hydrogen chloride at 100°C for three hours.

The fatty acid methyl esters were analyzed at 185°C by SHIMADZU GC-5A unit equipped with a 3.0 m × 3 mm glass column packed with 25% EGS on Celite 545 HMDS. They were also analyzed on a gas liquid chromatography-mass spectrometer, SHIMADZU-LKB GCMS-9000 (Kyoto). A 2.0 m × 3 mm glass column packed with 2% DEGS on Chromosorb W (60~80 mesh) was used with helium as a carrier gas (flow rate, 20 m*l*/min). The temperature of the helium separator and the ion source was 250°C and 270°C, respectively. The ionization energy was set at 70 eV.

OBSERVATION

I. Changes in PC content and positional distribution of fatty acids in PC at different developmental stages in Rana nigromaculata

Except for some minor variations, the PC content in total phospholipids remained unchanged throughout the first 300 hours after fertilization (Fig. 1), being about 10%.

At all stages from SHUMWAY stage 1 to TAYLOR-KOLLROS stage IV, the main constituent fatty acid at the 1-position of PC was palmitic acid $C_{16:0}$ (Fig. 2).

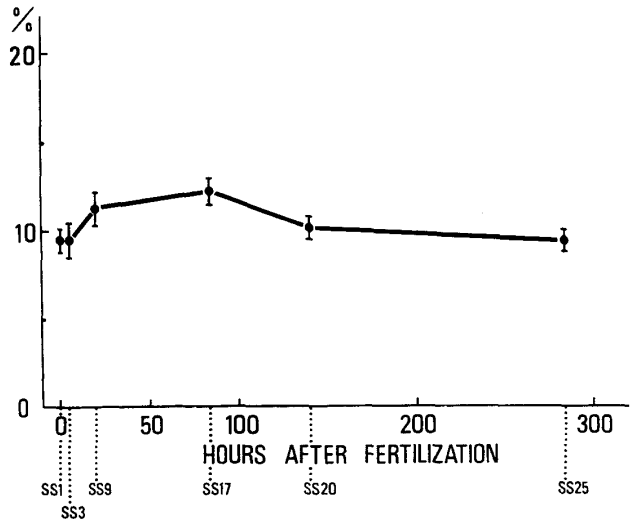

Fig. 1. Quantitative changes of phosphatidyl choline in total lipids during development in *Rana nigromaculata*. The content of phosphatidyl choline in total lipids is shown by percentage.

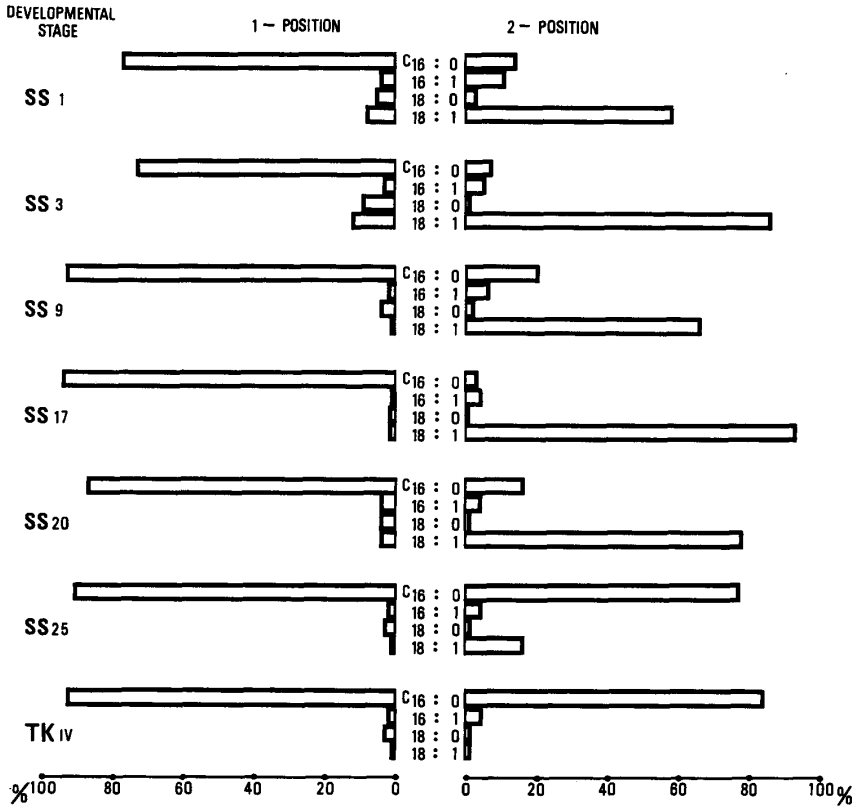

Fig. 2. Changes in positional distribution of fatty acids in phosphatidyl choline during development in *Rana nigromaculata*.

$C_{16:0}$, $C_{16:1}$, $C_{18:0}$, $C_{18:1}$, kinds of fatty acids.

While fatty acid $C_{16:0}$ represented 75~79% of the total fatty acid content in unfertilized and fertilized eggs, it was 89~94% in embryonic and tadpole stages. In contrast to embryos and tadpoles, the eggs contained a relatively large amount of stearic acid $C_{18:0}$ and oleic acid $C_{18:1}$ at the 1-position of PC, although the gross pattern of the constituent fatty acids did not change throughout the stages examined. The fatty acids at the 2-position of PC remarkably differed from those at the 1-position in that there was a drastic change in the main kind of fatty acid between SHUMWAY stages 20 and 25 (Fig. 2). While the main constituent fatty acid at the 2-position of PC contained during the period between SHUMWAY stage 1 and 20 was $C_{18:1}$, that contained at SHUMWAY stage 25 and TAYLOR-KOLLROS stage IV was $C_{16:0}$.

II. Positional distribution of fatty acids in PC at different developmental stages of Bufo japonicus

At the 1-position of PC, the main constituent fatty acids were $C_{16:0}$ and $C_{18:1}$, although the former was remarkably larger in content than the latter (Fig. 3). The gross pattern showed no remarkable variations among SHUMWAY stages 3, 10~11 and 25 (Fig. 3). In contrast with the fatty acids at the 1-position, those at the 2-position were not constant in content, although fatty acids $C_{16:0}$ and

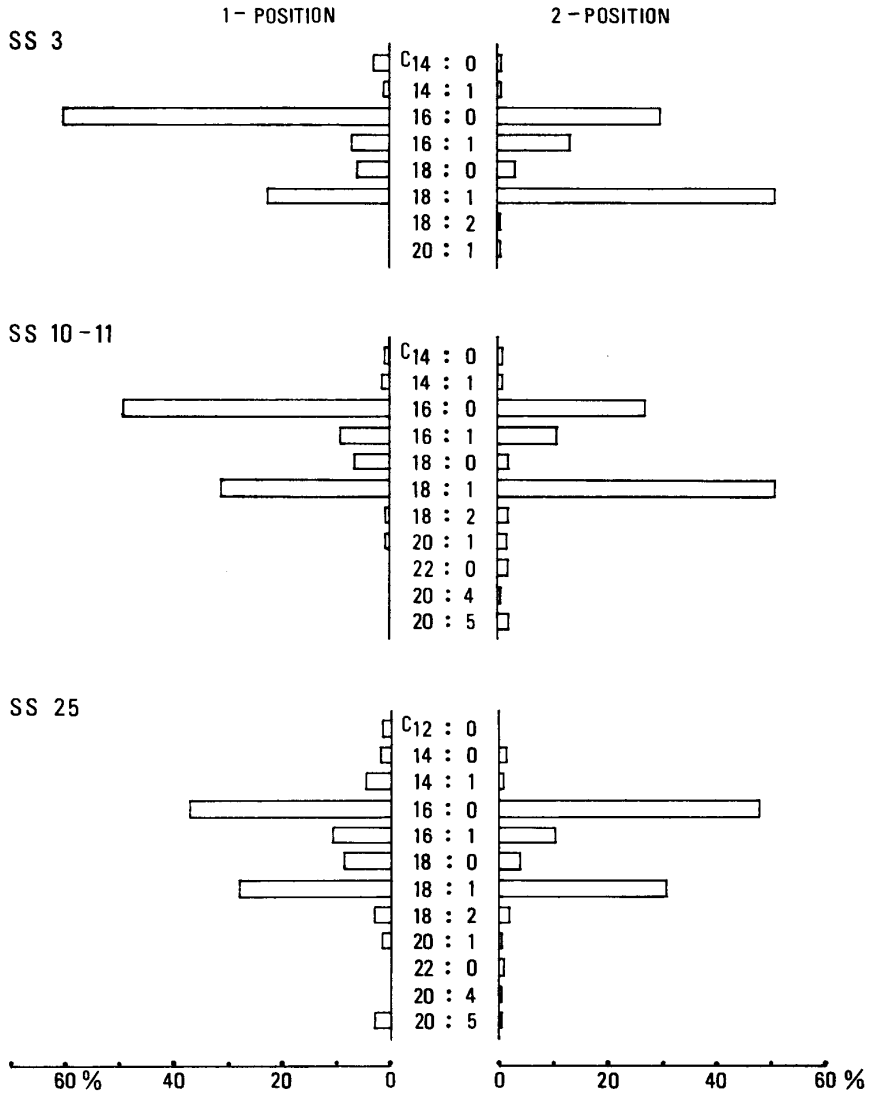

Fig. 3. Changes in positional distribution of fatty acids in phosphatidyl choline during development in *Bufo japonicus*.

$C_{12:0}$, $C_{14:0}$, $C_{14:1}$, $C_{16:0}$, $C_{16:1}$, $C_{18:0}$, $C_{18:1}$, $C_{18:2}$, $C_{20:1}$, $C_{22:0}$, $C_{20:4}$, $C_{20:5}$, kinds of fatty acids.

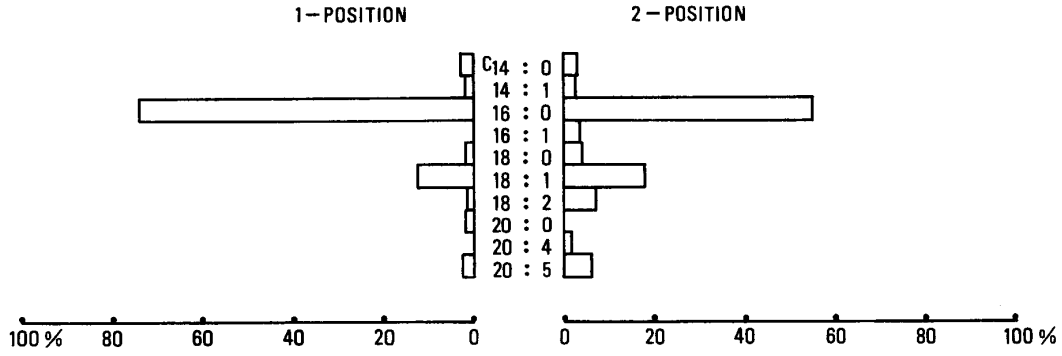

Fig. 4. Positional distribution of fatty acids in phosphatidyl choline from the liver of adult *Rana nigromaculata*.

$C_{14:0}$, $C_{14:1}$, $C_{16:0}$, $C_{16:1}$, $C_{18:0}$, $C_{18:1}$, $C_{18:2}$, $C_{20:0}$, $C_{20:4}$, $C_{20:5}$, kinds of fatty acids.

$C_{18:1}$ were the main constituents. While fatty acid $C_{18:1}$ was larger in amount than fatty acid $C_{16:0}$ at SHUMWAY stages 3 and 10~11, the latter was larger in amount than the former at SHUMWAY stage 25.

III. Positional distribution of fatty acids in PC from the liver and brain of frogs or toads

In the liver of *Rana nigromaculata*, the most abundant fatty acids at the 1- and 2-position was $C_{16:0}$, followed by $C_{18:1}$ (Fig. 4). In the liver of *Bufo japonicus*, the positional distribution of fatty acids of PC was similar to that in the liver of *Rana nigromaculata* (Fig. 5). A similiar pattern of fatty acid constituents was also found in PC extracted from the brain of *Rana catesbeiana*, although there was a third abundant fatty acid which was $C_{16:1}$ at the 1- and 2-position (Fig. 6).

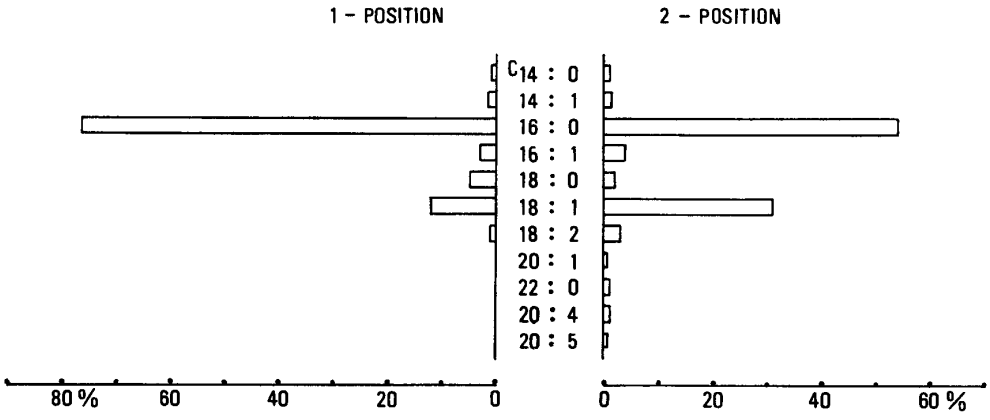

Fig. 5. Positional distribution of fatty acids in phosphatidyl choline from the liver of adult *Bufo japonicus*.

$C_{14:0}$, $C_{14:1}$, $C_{16:0}$, $C_{16:1}$, $C_{18:0}$, $C_{18:1}$, $C_{18:2}$, $C_{20:1}$, $C_{22:0}$, $C_{22:4}$, $C_{20:5}$, kinds of fatty acids.

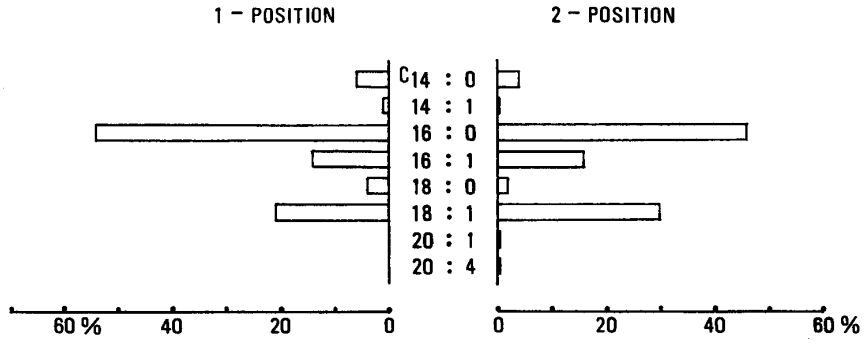

Fig. 6. Positional distribution of fatty acids in phosphatidyl choline from the brain of adult *Rana catesbeiana*.

$C_{14:0}$, $C_{14:1}$, $C_{16:0}$, $C_{16:1}$, $C_{18:0}$, $C_{18:1}$, $C_{20:1}$, $C_{20:4}$, kinds of fatty acids.

DISCUSSION

Phosphatidyl choline is the most abundant phospholipid together with phos-

phatidyl ethanolamine in higher animals, and generally constitutes the major lipid components of the membranes in cells. Their molecules consist of a head-like group of phosphorylcholine and two tail-like chains of saturated or unsaturated fatty acids. Of many molecular species of PC, one with two saturated $C_{16:0}$ chains has so far been found only in rat erythrocytes (VAN GOLDE and VAN DEENEN, 1966), brain (MONTFOORT, VAN GOLDE and VAN DEENEN, 1971), lung (MONTFOORT, VAN GOLDE and VAN DEENEN, 1971; SOODSMA, MIMS and HARLOW, 1976) and liver (OGINO, MATSUMURA, SATOUCHI and SAITO, 1980), although in the rat liver the relative amount of this molecular species decreased rapidly during prenatal development. The present study showed that the PC with two saturated $C_{16:0}$ chains existed most abundantly among PC species in the liver of *Rana nigromaculata* and *Bufo japonicus* and the brain of *Rana catesbeiana*.

Phosphatidyl choline in *Rana nigromaculata* tadpoles at SHUMWAY stage 25 and TAYLOR-KOLLROS stage IV was similar to those in the liver of adult *Rana nigromaculata* and *Bufo japonicus* or the brain of *Rana catesbeiana* in that the most abundant PC species was that with two saturated $C_{16:0}$ chains. In this study, the PC of *Bufo japonicus* tadpoles at SHUMWAY stage 25 was similar to that in the liver and brain of adult frogs or toads. It was noteworthy that the PC found in embryos at the hatching stage completely differed from the above. The most abundant PC species was that with two different chains of fatty acids; one was a saturated $C_{16:0}$ chain and the other was an unsaturated $C_{18:1}$ chain. This pattern was the same in the PC of unfertilized and cleaving eggs and tail-bud embryos. Accordingly, it was evident that the PC in embryos at the hatching stage came from those contained in unfertilized eggs without any distinguishable modification. A similar phenomenon was recognized in the development of *Bufo japonicus*. It is very probable that all the PC of developing frog and toad embryos come from the yolk contained in unfertilized eggs until the embryos become feeding tadpoles. After the tadpoles begin to eat, the unsaturated chain on the 2-position of PC seems to be changed into a saturated $C_{16:0}$ chain. The PC species with two saturated $C_{16:0}$ chains seems subsequently to remain as the most important substance among various molecular species of PC through the lifespan in frogs and toads.

SUMMARY

1. The positional distribution of the fatty acids in phosphatidyl choline (PC) molecules was examined in order to clarify the relationship between the change in phospholipids and the morphological differentiation during development in frogs and toads.

2. At all the stages from the unfertilized egg to the feeding tadpole, the most abundant fatty acid at the 1-position of PC was $C_{16:0}$, while that at the 2-position was $C_{18:1}$ at the stages from the unfertilized egg to the hatching embryo and $C_{16:0}$ at the feeding tadpole stage.

3. The most abundant fatty acid at the 1- and 2-position of PC in the liver

and brain of adult frogs or toads was $C_{16:0}$ as found in the feeding tadpoles.

4. It is assumed that all the PC contained in embryos at the stages before eating come from the yolk contained in unfertilized eggs without making remarkable modification, while they are changed into those of adult type after the tadpoles begin to eat.

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LITERATURE

ABAD, C., M. A. BOSCH, A. M. MUNICIO and A. RIBERA 1976. Age differences in the positional distribution of phosphoglycerides and molecular species of choline phosphoglycerides during development of the chick embryo liver. Biochim. Biophys. Acta **431**: 62–74.

ALLING, C. and L. KARLSSON 1973. Changes in lipid concentrations and fatty acid compositions in rat cerebrum during maturation. J. Neurochem. **21**: 1051–1057.

BARTLETT, G. R. 1959. Phosphorus assay in column chromatography. J. Biol. Chem. **234**: 466–468.

BERGELSON, L. D. and E. V. DYATLOVITSKAYA 1973. Molecular structures of tumor lecithins and their relevance to some properties of tumor cell membrances. Tumor Lipids, edited by R. WOOD. pp. 111–125. American Oil Chemists, Society Press (Champaign, Illinois).

FOLCH, J., M. LEES and G. H. SLOANE-STANLEY 1957. A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem. **226**: 497–507.

HARVERKATE, F. and L. L. M. VAN DEENEN 1965. Isolation and chemical characterization of phosphatidyl glycerol from spinach leaves. Biochim. Biophys. Acta **106**: 78–92.

KAWAMURA, T., M. NISHIOKA and H. UEDA 1980. Inter- and intraspecific hybrids among Japanese, European and American toads. Sci. Rep. Lab. Amphibian Biol., Hiroshima Univ. **4**: 1–125.

KUKSIS, A., L. MARAI, W. C. BRECKENRIDGE, D. A. CORNALL and O. STACHNYK 1968. Molecular species of lecithins of some functionally distinct rat tissues. Can. J. Physiol. Pharmacol. **46**: 511–524.

MARAI, L. and A. KUKSIS 1973. Comparative study of molecular species of glycerolipids in sarcotubular membranes of skeletal muscle of rabbit, rat, chicken, and man. Can. J. Biochem. **51**: 1365–1379.

MARSHALL, E., R. FUMAGALLI, R. NIEMIRE and R. PAOLETTI 1966. The change in fatty acid composition of rat brain phospholipids during development. J. Neurochem. **13**: 857–862.

MONTFOORT, A., L. M. G. VAN GOLDE and L. L. M. VAN DEENEN 1971. Molecular species of lecithins from various animal tissues. Biochim. Biophys. Acta **231**: 335–342.

NISHIHARA, M. and M. KITO 1978. Changes in the phospholipid molecular species composition of soybean hypocotyl and cotyledon after dedifferentiation. Ibid. **531**: 25–31.

O'BRIEN, J. S. and R. L. GEISON 1971. The mass distribution of the phosphatidylcholines in subcellular fractions of rat brain. J. Neurochem. **18**: 1615–1623.

OGINO, H., T. MATSUMURA, K. SATOUCHI and K. SAITO 1980. Changes in molecular species of rat liver choline glycerophospholipids with development. Biochim. Biophys. Acta **618**: 431–438.

RUGGIERI, L. D. and E. V. DYATLOVITSKAYA 1973. A comparative study of lecithins from YOSHIDA

hepatoma AH130, MORRIS hepatoma 5123C and host rat livers. Tumor Lipids, edited by R. WOOD. pp. 89–110. American Oil Chemists, Society Press (Champaign, Illinois).

RYUZAKI, M., H. KOJIMA and Y. TAMAI 1975. Study on amphibian lipids—II. Characteristic constituents of monoglycosylceramides from the skin of three frog species. Comp. Biochem. Physiol. **52C**: 81–84.

SHUMWAY, W. 1940. Stages in the normal development of *Rana pipiens*. I. External form. Anat. Rec. **78**: 139–147.

SKIPSKI, V. P., R. M. PETERSON and M. BARCLAY 1964. Quantitative analysis of phospholipids by thin-layer chromatography. Biochem. J. **90**: 374–478.

SKRBIC, T. and J. CUMINGS 1970. Fatty acids of lecithin in subcellular fractions during maturation of brain. J. Neurochem. **17**: 85–90.

SOODSMA, J. F., L. C. MIMS and R. D. HARLOW 1976. The analysis of the molecular species of fetal rabbit lung phosphatidylcholine by consecutive chromatographic techniques. Biochim. Biophys. Acta **424**: 159–167.

TAYLOR, A. C. and J. J. KOLLROS 1946. Stages in the normal development of *Rana pipiens* larvae. Anat. Rec. **94**: 7–24.

VAN GOLDE, L. M. G., V. TOMASI and L. L. M. VAN DEENEN 1967. Determination of molecular species of lecithin from erythrocytes and plasma. Chem. Phys. Lipids **1**: 282–293.

VAN GOLDE, L. M. G. and L. L. M. VAN DEENEN 1966. The effect of dietary fat on the molecular species of lecithin from rat liver. Biochim. Biophys. Acta **125**: 496–509.

WALLACE, R. A. 1963. Studies on amphibian yolk. IV. An analysis of the main-body component of yolk platelets. Ibid. **74**: 505–518.