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# Studies on Lipids in Anurans I. Changes in the Composition of Four Kinds of Lipids during Development in *Rana nigromaculata*

By

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(With 15 Text-figures)

## INTRODUCTION

While there are abundant reports on experimental embryology of frogs, those concerning the relationship between development and lipids are very scarce. YIAMOUYIANNIS and DAIN (1968) have merely reported on the developmental changes in gangliosides during the early development of the frog, *Rana pipiens*. In the other hand, it is well known that complex lipids are used in forming membranes of cells in mammals. The major lipid constituents of membranes are generally phospholipids, glycolipids, gangliosides and sterols in eukaryotic cells.

Many biochemical studies on lipids during embryogenesis in animals other than frogs have been performed on gangliosides. Gangliosides are generally known to be concentrated in neural plasma membranes (WOLF, 1961; WHERRET and MCILWAIN, 1962; EMMELOT and BOS, 1966), but their biological functions are yet obscure. One approach which has been used to shed some light upon the biological role of gangliosides is to study their changes during development. Several investigations have subsequently been published on the changes in gangliosides from mammalian brains at various developmental stage (SUZUKI, 1965; SANDHOFF, HARZER and JATZKEWITZ, 1968; VANIER, HOLM, ÖHMAN and SVENNERHOLM, 1971; MERAT and DICKERSON, 1973). DREFUS, URBAN, EDEL-HARTH and MANDEL (1975) have reported on the developmental patterns of gangliosides and phospholipids in chick retinas and brains. It is generally known that phospholipids are important constituents of membranes in cells. Several investigators have measured the amount of phospholipids in the brain and liver from mammalian embryos at the fetal stage (CRAWFORD and WELLS, 1979; OGINO, MATSUMURA, SATOUCHI and SAITO, 1979, 1980). It has been found that the phospholipid pattern of mammalian brain and liver does not remarkably change during the embryonic life.

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Galactosyl ceramide belonging to one of the glycosphingolipid group is found only in the brain (KISHIMOTO and RADIN, 1959; DEVRIES and NORTON, 1974) and kidney (KARLSSON and MÅRTENSSON, 1968) alone. In contrast to the kidney, the brain contains a large amount of this substance (SUOMI and AGRANOFF, 1975). The results of many studies have shown that galactosyl ceramide is, indeed, the best marker for nervous system differentiation in mammals (CUZNER, DAVISON and GREGSON, 1965; LEWIN and HESS, 1965; LANDOLT and HESS, 1966). HAUSER (1968) and WELLS and DITTMER (1967) have measured monoglycosyl ceramides including galactosyl ceramide and determined the rate of monoglycosyl ceramide in the total lipids from the brains of rat embryos at various developmental stages.

The present author analysed four kinds of lipids, that is, neutral lipids, phospholipids, galactosyl ceramide and ganglioside-like substance, contained in *Rana nigromaculata* at various developmental stages in order to clarify the relationship between these lipids and organ differentiation.

## MATERIALS

Mature male and female *Rana nigromaculata* were collected from rice fields in the suburbs of Shizuoka City during the breeding season. Some of them were immediately used for chemical analysis, while others were used in producing offspring. Female frogs were induced to ovulate by injecting *Rana catesbeiana* pituitaries. Eggs were artificially fertilized and kept at about 18~22°C. Embryos, tadpoles and juveniles raised from the fertilized eggs were removed at definite developmental stages, placed on dry ice to suspend their development and then stored at −20°C. After freeze-drying for 72 hours, they were weighed to obtain their dry weight. The description of developmental stages followed that of *Rana pipiens* established by SHUMWAY (1940) and TAYLOR and KOLLROS (1946).

Some organs and tissues of adult frogs as well as the whole bodies of embryos, tadpoles and juveniles were subjected to chemical analysis. Extraction and analysis of lipids from embryos and tadpoles were made at six developmental stages of SHUMWAY (SS), that is, 1 (unfertilized egg), 3 (two-cell stage), 9 (late blastula), 17 (early tail-bud embryo), 20 (hatching), and 25 (operculum complete) and at four developmental stages of TAYLOR and KOLLROS (TK), that is, V (the length of the hind limbs is twice the diameter), XV (the proximal toe pads appear), XX (one or both of the fore-legs protrude) and XXV (tail stub disappears). Extraction and analysis of lipids from adult frogs were made on seven kinds of organs and tissues; skeletal muscle, stomach, kidney, liver, sciatic nerve, spinal cord and brain.

## METHODS

### 1. Lipid extraction

As shown in Fig. 1, the total lipids were extracted by the method described by

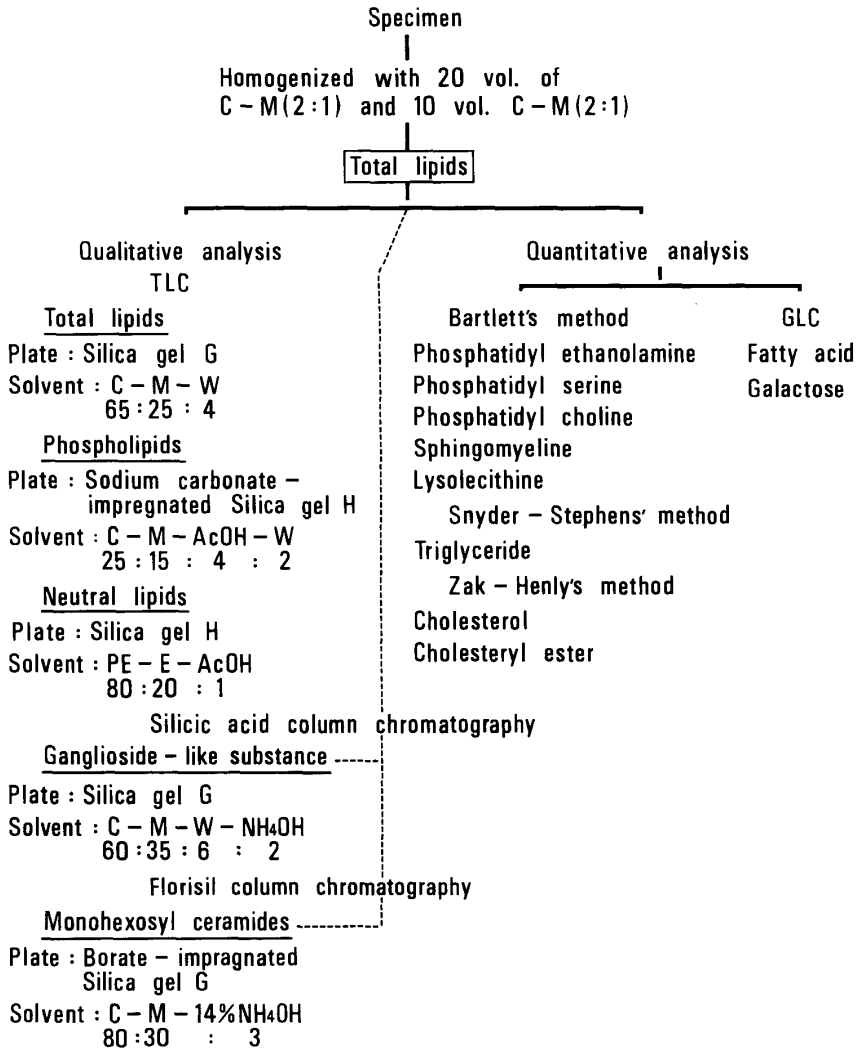


Fig. 1. The method of analysis.

C, chloroform M, methanol W, water PE, petroleum ether E, diethyl ether AcOH, acetic acid TLC, thin-layer chromatography GLC, gas liquid chromatography

FOLCH, LEES and SLOANE-STANLEY (1957). The samples were homogenized with 20 volumes of chloroform-methanol (2: 1 by volume) by the use of an ultra-homogenizer, UH-1 type, NISSEI, Tokyo. The extracts were filtered through a BÜCHNER funnel. The residual substance was similarly treated with 20 volumes of chloroform-methanol (1: 2 by volume) and the residues were again homogenized with 20 volume of chloroform-methanol (2: 1 by volume) in the same way. The extracted solutions were combined and concentrated to dryness by a rotary evaporator. The substance thus obtained was dissolved in a suitable volume of chloroform-methanol (2: 1 by volume) and was filtered through a glass filter (GA-100, TOYOROSHI, Tokyo). The soluble material was partitioned with distilled water as described by FOLCH, LEES and SLOANE-STANLEY (1957). Each of the upper and lower phases thus obtained was adjusted to a suitable volume, re-

spectively, and subjected to chemical analysis. The lipid contained in the lower phase was dried and weighed.

### 2. Thin-layer chromatography

a. Preparative plate

Thin-layer plates were prepared according to routine procedure. Precoated thin-layer plates (Kieselgel 60 F254, MERCK) were also used.

b. Boric-acid-impregnated plate

A borate-impregnated silica gel G plate was made by a slight modification of YOUNG and KANFER's method (1968) as described previously (RYUZAKI, KOJIMA and TAMAI, 1975). After fractionation by a silicic acid and florisil column chromatography as described later, the glycolipid fraction was separated on a borate-impregnated silica gel G plate with solvent system C described below.

c. Sodium carbonate-impregnated plate

A sodium carbonate-impreganted plate was made by the method of SKIPSKI and PETERSON (1964). The lower phase lipid was concentrated to dryness and dissolved in a small quantity of chloroform-methanol (2: 1 by volume). The soluble substance containing phospholipids was separated on a sodium carbonate-impregnated plate with solvent system D described below.

Samples were applied in bands about 1 cm in width at the orgin and chromatographed by the ascending method with the following solvent system. A, chloroform-methanol-water (65: 25: 4 by volume); B, chloroform-methanol-water-28% ammonia (60: 35: 6: 2 by volume); C, chloroform-methanol-14% ammonia (125: 45: 4.5 by volume) (KEAN, 1966); D, chloroform-methanol-acetic acid-water (25: 15: 4: 2 by volume); and E, petroleum ether-diethyl ether (80: 30 by volume).

Phospholipids were located with DITTMER and LESTER's reagent and glycolipids with anthrone-sulfuric acid. Ganglioside-like substances (sialic acid bound to lipids) were detected with BIAL reagent.

### 3. Analytical methods

a. Phospholipid classes

Each kind of phospholipids was isolated by the use of sodium carbonate-impregnated plate, and the silica gel layer segment containing each phospholipid was scraped off from the plate. The amount of each phospholipid was determined on the basis of the phosphorus content by the method of BARTLETT (1959).

b. Neutral lipid classes

Neutral lipids were separated by one-dimensional thin-layer chromatography on a Kieselgel H plate with solvent system E. The bands of neutral lipids were visualized with iodine vapor. The segment of the silica gel layer containing each neutral lipid was scraped off and the amount of the latter was measured.

i) Triglyceride The amount of triglyceride was determined by the method of SNYDER and STEPHENS (1959).

ii) Cholesterol and cholesteryl ester These were determined according to the method of ZAK-HENLY (1957).

iii) Free fatty acid Free fatty acid isolated by the preparative thin-layer chromatography was esterified with 3% hydrogen chloride-methanol at 100°C for three hours. The fatty acid methyl esters were extracted with petroleum ether from the reaction mixture and washed with distilled water. The fatty acid methyl esters were analyzed at 160°C by SHIMADZU GC-4BM unit equipped with a 1.5 m × 3 mm glass column packed with 15% ethylene glycol succinate on Celite 545 HMDS. Methyl heptadecanoate (Applied Science Laboratories Inc., Lot 1933, Penna.) was used as the internal standard.

c. Galactose in total lipids

For the determination of galactose bound to lipids, the lower phase lipids were concentrated to dryness and then methanolyzed in absolute methanol containing 3% hydrogen chloride at 100°C for three hours. After exhaustive methylation of methyl glycosides, the content of galactose was determined by gas-liquid chromatography at 135°C by the use of SHIMADZU GC-5A unit equipped with a 1.5 m × 3 mm glass column packed with 4% OV-1 on Celite 545 HMDS. Mannitol was used as the internal standard.

## 4. Column chromatography

The lower phase lipid was concentrated to dryness and dissolved in a small quantity of chloroform and chromatographed on a silicic acid column (100 mesh, MALLINCKRODT Chemical Works) according to the method of VORBECK and MARINETTI (1965). Glycolipids were eluted with two kinds of chloroform-methanol mixtures, 4: 1 and 7: 3 by volume. The two fractions were then mixed and evaporated up to dryness. The glycolipid thus obtained was dissolved again in a small quantity of chloroform-methanol (9.5: 0.5 by volume) and chromatographed on a florisil column (60 ~ 100 mesh, Floridin Co.) according to the method of RADIN, BROWN and LAVIN (1956). Monoglycosyl ceramides were eluted with two kinds of chloroform-methanol mixtures, 4: 1 and 7: 3 by volume. The two fractions of monoglycosyl ceramides were mixed and evaporated up to dryness by a rotary evaporator. The substance thus obtained was dissolved in a small quantity of chloroform-methanol (2: 1 by volume) and subjected to thin-layer chromatography.

The upper phase lipid was concentrated to dryness and dissolved in a small quantity of chloroform-methanol (9.5: 0.5 by volume). The soluble substance was chromatographed on a silicic acid column (100 mesh, MALLINCKRODT Chemical Works). Ganglioside-like substance (sialic acid bound to lipids) was mainly eluted with two kinds of chloroform-methanol mixtures, 6: 4 and 4: 6 by volume. Then the two fractions of ganglioside-like substance were mixed and concentrated to dryness by a rotary evaporator. The substance thus obtained was dissolved

again in a small quantity of chloroform-methanol (9.5: 0.5 by volume) and rechromatographed on a silicic acid column (100 mesh, MALLINCKRODT Chemical Works). The ganglioside-like substance was eluted with two kinds of chloroform-methanol mixtures, 4: 1 and 7: 3 by volume. The two fractions of ganglioside-like substance were mixed and concentrated to dryness by a rotary evaporator. The substance thus obtained was dissolved and subjected to thin-layer chromatography.

## OBSERVATION

### *I. Dry weight of specimens and lipids*

The changes in dry weight of a group of 50 specimens and their lipids at each of six developmental stages are shown in Fig. 2. The percentage of lipid weight to specimen weight ranged from 25 to 30% in all the stages. While the specimen weight did not significantly change until the early tail-bud stage (SS17), it gradually decreased after this stage. In contrast to the specimen weight, the lipid weight scarcely decreased until the hatching stage (SS20), that is, only changed from 18.0 mg into 17.6 mg, although there was a transitory decrease between the unfertilized and the two-cell stage. After this stage, the lipid weight gradually

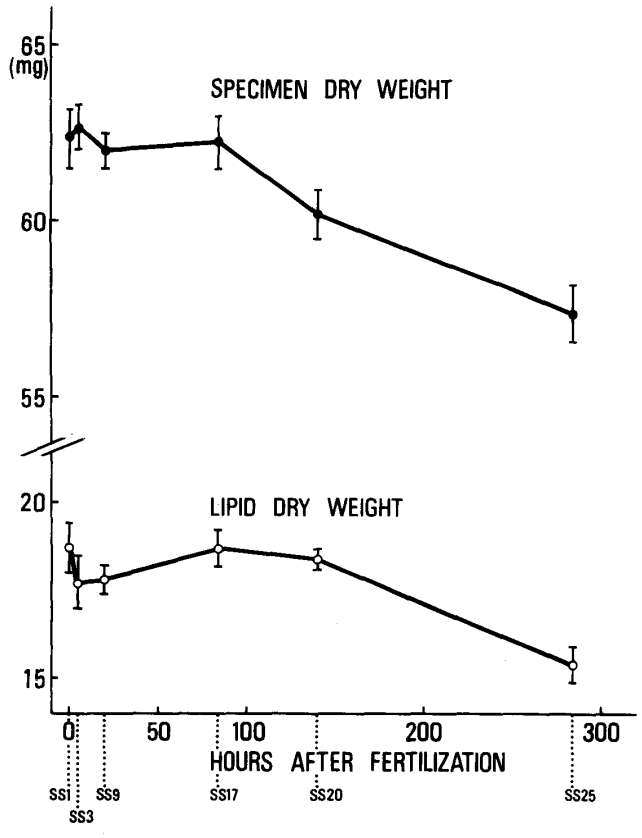


Fig. 2. Changes in weight of fifty freeze-dried eggs, embryos or tadpoles and of lipids extracted from them.

decreased to reach 15.6 mg at SHUMWAY stage 25.

### *II. Thin-layer chromatograms of lipids in organs and tissues of adult frogs*

The thin-layer chromatographic patterns of total lipids, neutral lipids and phospholipids in each of the skeletal muscle, stomach, kidney, liver, sciatic nerve, spinal cord and brain are shown in Figs. 3, 4 and 5. By comparing these chromatographic patterns, it was found that phospholipids and neutral lipids were commonly contained in all the organs and tissues examined, while a large amount of monoglycosyl ceramides (galactosyl ceramide) was detected only in nervous organs as shown in Fig. 3. A large amount of ganglioside-like substance was only found in the brain of adult frogs (Fig. 12).

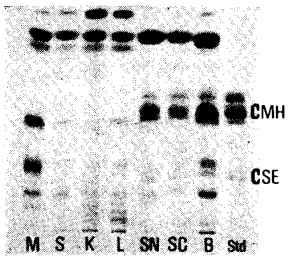


Fig. 3.

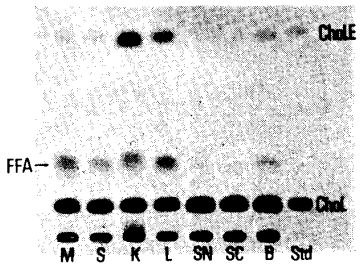


Fig. 4.

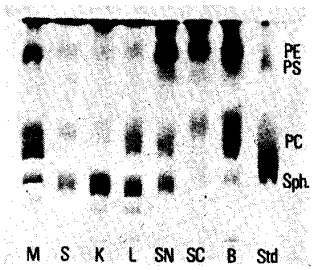


Fig. 5.

Fig. 3. Thin-layer chromatograms of total lipids from various organs and tissues of adult frogs.
M, muscle S, stomach K, kidney L, liver SN, sciatic nerve SC, spinal cord B, brain CMH, a mixture of glucosyl ceramide from GAUCHER's spleens and galactosyl ceramide from bovine brains CSE, sulfatide from bovine brains Std, authentic standard

Fig. 4. Thin-layer chromatograms of total neutral lipids from various organs and tissues of adult frogs.
Chol., cholesterol Chol. E, cholesteryl ester FFA, free fatty acid Std, authentic standard

Fig. 5. Thin-layer chromatograms of total phospholipids from various organs and tissues of adult frogs.
PC, phosphatidyl choline PE, phosphatidyl ethanolamine PS, phosphatidyl serine Sph, sphingomyelin Std, authentic standard

### *III. Thin-layer chromatograms of lipids in eggs and developing specimens*

Fig. 6 shows thin-layer chromatographic patterns of lipids extracted from about 200 eggs, embryos or tadpoles at each of five stages, SHUMWAY stages 3 to 25, 30～50 tadpoles at TAYLOR-KOLLROS stage V and 30～50 metamorphosing (TK stage XX) and metamorphosed (TK stage XXV) frogs. An anthrone sulfuric acid positive band, which migrated to the same position as the authentic standard of monoglycosyl ceramides, appeared first in tadpoles at SHUMWAY stage 25.

Thin-layer chromatograms of phospholipids obtained from the same materials as those used in examining lipids are shown in Fig. 8. Phospholipids separated into bands of phosphatidyl ethanolamine, phosphatidyl serine, phosphatidyl

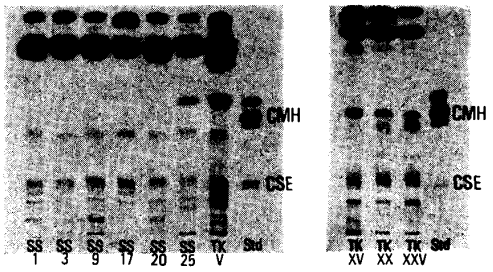


Fig. 6. Thin-layer chromatograms of total lipids from eggs, embryos and tadpoles in *Rana nigromaculata.*

CMH, a mixture of glucosyl ceramide from GAUCHER's spleens and galactosyl ceramide from bovine brains CSE, sulfatide from bovine brains Std, authentic standard

choline, sphingomyelin and lysophosphatidyl choline at all the developmental stages. Lysophosphatidyl choline was detected in a much larger amount in SHUMWAY stages 1 to 25 than that in TK stages XV~XXV.

Thin-layer chromatograms of neutral lipids obtained from the same materials as those used in examining lipids are shown in Fig. 7. Neutral lipids separated into bands of cholesterol, cholesteryl ester, triglyceride and free fatty acid. The band densities of free fatty acid and cholesteryl ester were remarkably stronger at the stages from SHUMWAY stage 1 to TK stage V than those at the later stages. On the other hand, the band of triglyceride appeared as only a trace at stages from SHUMWAY stage 1 to TK stage V, except for SHUMWAY stage 17. However, triglyceride was a major component in neutral lipids at TK stages XV and XX.

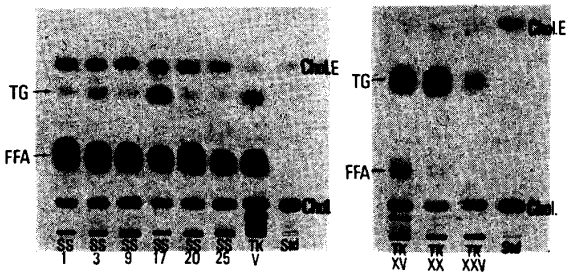


Fig. 7. Thin-layer chromatograms of total neutral lipids from eggs, embryos and tadpoles in *Rana nigromaculata.*

Chol., cholesterol Chol. E, cholesteryl ester FFA, free fatty acid TG, triglyceride Std, authentic standard

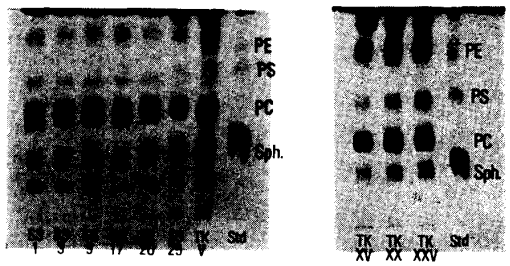


Fig. 8. Thin-layer chromatograms of total phospholipids from eggs, embryos and tadpoles in *Rana nigromaculata.*

PC, phosphatidyl choline PE, phosphatidyl ethanolamine PS, phosphatidyl serine Sph, sphingomyelin Std, authentic standard

## *IV. Quantitative data of lipids in eggs and developing specimens*

The quantitative data of total phospholipids, free fatty acid, triglyceride and total cholesterol obtained from seven stages, SHUMWAY stages 1, 3, 9, 17, 20 and 25 and TK stage V, are shown in Fig. 9. The dry weight of each lipid is shown in the rate of total lipids.

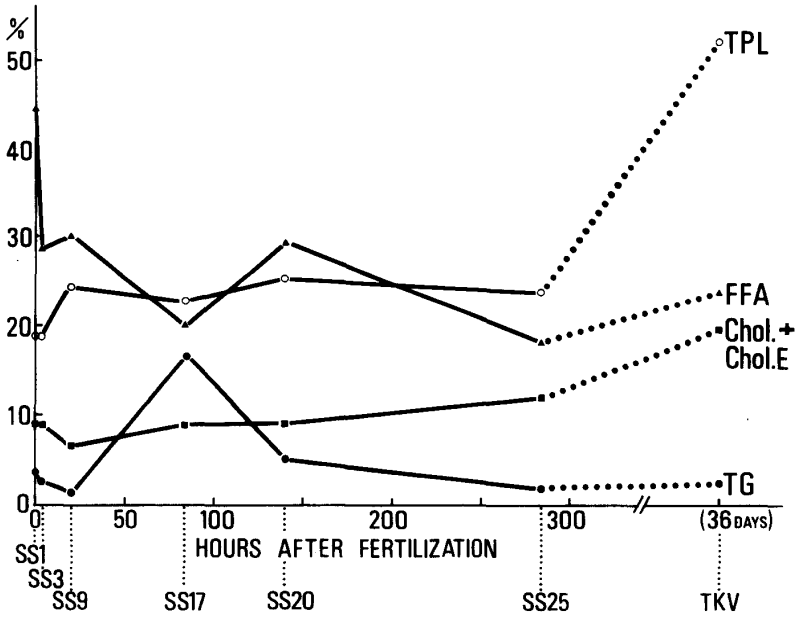


Fig. 9. Changes in the rates of total phospholipids, total cholesterols (cholesterol+cholesteryl ester), free fatty acid and triglyceride to total lipids from eggs, embryos and tadpoles in *Rana nigromaculata*.

TPL, total phospholipids FFA, free fatty acid Chol.+Chol. E, cholesterol+cholesteryl ester

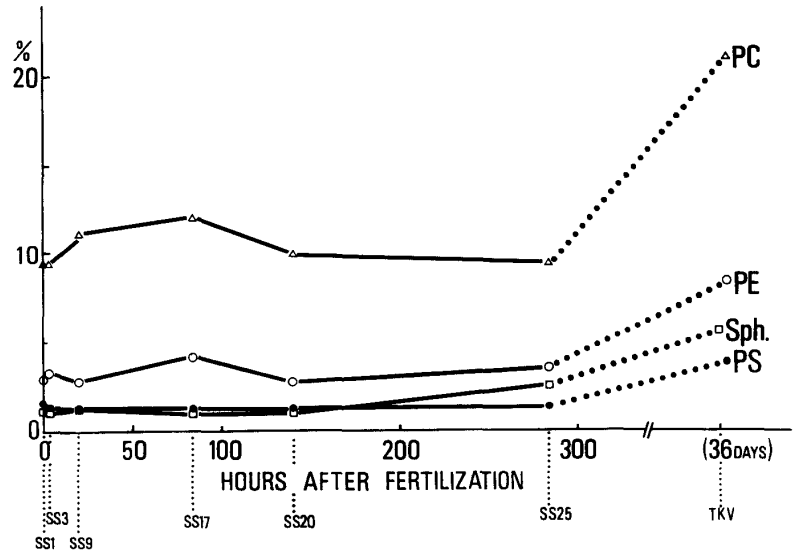


Fig. 10. Changes in the rates of phosphatidyl choline, phosphatidyl ethanolamine, phosphatidyl serine and sphingomyelin to total lipids from eggs, embryos and tadpoles in *Rana nigromaculata*.

PC, phosphatidyl choline PE, phosphatidyl ethanolamine

PS, phosphatidyl serine Sph, sphingomyelin

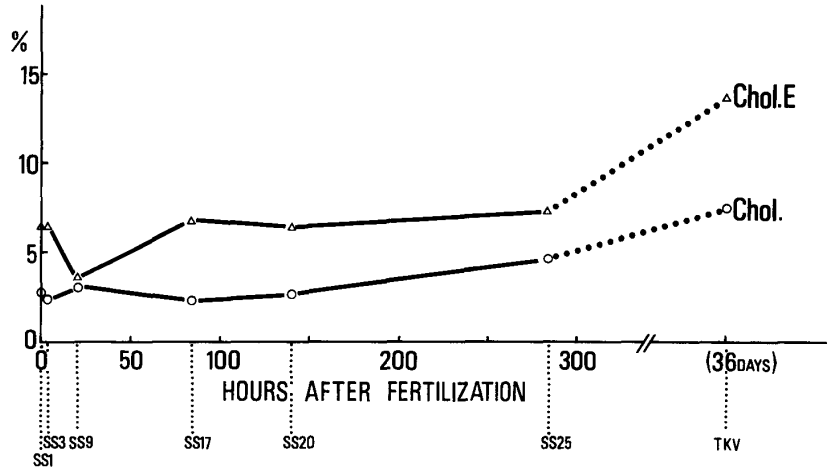


Fig. 11. Changes in the rates of cholesterol and cholesteryl ester to total lipids from eggs, embryos and tadpoles in *Rana nigromaculata*.
Chol., cholesterol Chol. E, cholesteryl ester

## 1. Neutral lipids

As shown in Figs. 9 and 11, large amounts of free fatty acid, triglyceride and cholesteryl ester were detected. The rate of free fatty acid to total lipids was about 45% at SHUMWAY stage 1 and decreased rapidly until about 28% at SHUMWAY stage 3 about 2 hours after fertilization. This decrement continued until SHUMWAY stage 25. The rates of free fatty acid and triglyceride changed dramatically at SHUMWAY stage 17. While the rate of free fatty acid decreased rapidly, that of triglyceride increased rapidly at this stage. At SHUMWAY stage 20, the former rate increased rapidly, while the latter rate decreased rapidly. After this stage, the rate of both substances gradually decreased. Such changes in the rate of free fatty acid and triglyceride were also found in thin-layer chromatographic pattern, as shown in Fig. 7.

The rate of cholesterol to total lipids was approximately 2.7% at SHUMWAY stage 1 and 5% at SHUMWAY stage 25. A large amount of cholesteryl ester was detected at each of all the SHUMWAY stages from 1 to 25, the rate of cholesteryl ester to total lipids being about 6%, although there was a sharp dip at SHUMWAY stage 9.

## 2. Phospholipids

The quantitative data of phospholipids are given in Figs. 9 and 10. The rate of phospholipids to total lipids was 19% at SHUMWAY stage 1. This rate gradually increased and attained 24% at SHUMWAY stage 25. Phosphatidyl choline and phosphatidyl ethanolamine were the major components of phospholipids. Phosphatidyl choline occupied about 50% of total phospholipids, while phosphatidyl ethanolamine occupied 20~25% at most of SHUMWAY stages 1~25. The rates of phosphatidyl choline and phosphatidyl ethanolamine to total lipids were relatively stable. The rate of sphingomyelin to total lipids did not

change during the period from SHUMWAY stages 1 to 20, while afterward it increased slightly until SHUMWAY stage 25. The rate of phosphatidyl serine to total lipids did not significantly change from SHUMWAY stages 1 to 25.

### *V. Thin-layer chromatograms of ganglioside-like substance in eggs and developing specimens*

Thin-layer chromatograms of ganglioside-like substance (sialic acid bound to lipids) are shown in Fig. 12. The arrows indicate violet bands stained with BIAL reagent. These bands were not found at SHUMWAY stages 1~9. A single violet band which indicated the existence of ganglioside-like substance was detected for the first time at SHUMWAY stage 17, while several bands stained similarly appeared at SHUMWAY stage 25.

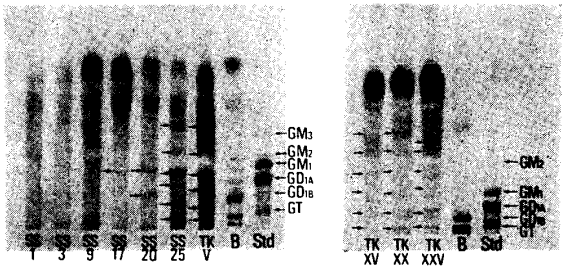


Fig. 12. Thin-layer chromatograms of ganglioside-like substance from eggs, embryos and tadpoles in *Rana nigromaculata.*

$GM_3$, $GM_2$, $GM_1$, $GD_{1A}$, $GD_{1B}$, GT, gangliosides B, brain Std, authentic standard

The total of these bands is called ganglioside-like substance in the present report, although in Fig. 12 each of them is given a symbol in accordance with the nomenclature used by SVENNERHOLM (1964).

### *VI. Thin-layer chromatograms of monoglycosyl ceramids in eggs and developing specimens*

Thin-layer chromatograms of monoglycosyl ceramides are shown in Fig. 13.

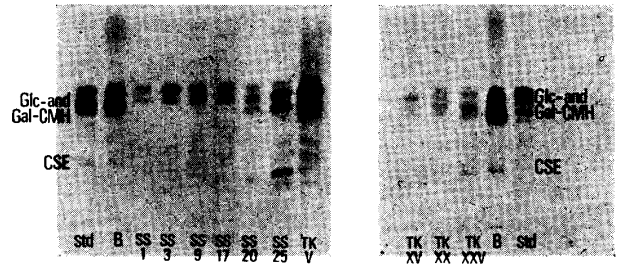


Fig. 13. Thin-layer chromatograms of monoglycosyl ceramides from eggs, embryos and tadpoles in *Rana nigromaculata.*

Glc- and Gal-CMH, a mixture of glucosyl ceramide from GAUCHER's spleens and galactosyl ceramide from bovine brains CSE, sulfatide from bovine brains B, brain Std, authentic standard

These chromatograms at SHUMWAY stages 1 to 17 were characterized by the existence of two bands, while those at SHUMWAY stages 20 and 25 and probably in later stages were characterized by existence of three bands.

### *VII. Borate-impregnated thin-layer chromatograms of monoglycosyl ceramides in eggs and developing specimens*

The chromatograms of monoglycosyl ceramide extracted from brains of 50 adult frogs are shown in Fig. 14. It was found by comparison with the authentic standard that the monoglycosyl ceramide is galactosyl ceramide. Galactosyl ceramide appeared for the first time as a single band at SHUMWAY stage 9 and increased slightly in intensity until the hatching stage. From SHUMWAY stage 25, the band of this substance began to increase rapidly in intensity. Glucosyl ceramide which is the other kind of monoglycosyl ceramides was obtained at SHUMWAY stages 1 to 25 and TK stage V. The band of glucosyl ceramide appeared to consist of two or more subbands, as compared with the authentic standard.

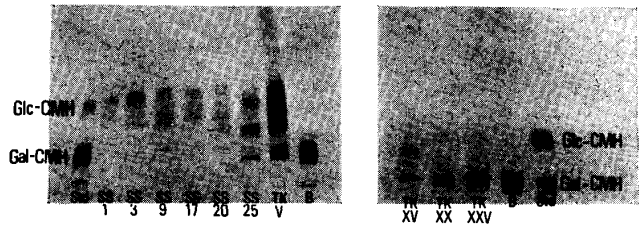


Fig. 14. Thin-layer chromatograms of monoglycosyl ceramides from eggs, embryos and tadpoles in *Rana nigromaculata.*

Cal–CMH, galactosyl ceramide from bovine brains Glc–CMH, glucosyl ceramide from GAUCHER's spleens B, brain Std, authentic standard

### *VIII. Galactose bound to lipids in eggs and developing specimens*

A change in the content of galactose bound to lipids at SHUMWAY stages 1 to 25 and TK stage V is shown in Fig. 15. While galactose bound to lipids was not detected at SHUMWAY stage 1, a trace of this substance appeared at SHUMWAY stage 3. Galactose bound to lipids increased significantly at SHUMWAY stage 9, where the band of galactosyl ceramide was detected for the first time by thin-layer chromatography and then it levelled off at least up to the tail-bud stage. While there was a slight increase at the hatching stage, a significant increment occurred at SHUMWAY stage 25, when the tadpoles were growing rapidly. This increment continued thereafter during the tadpole stage, as shown in Fig. 15.

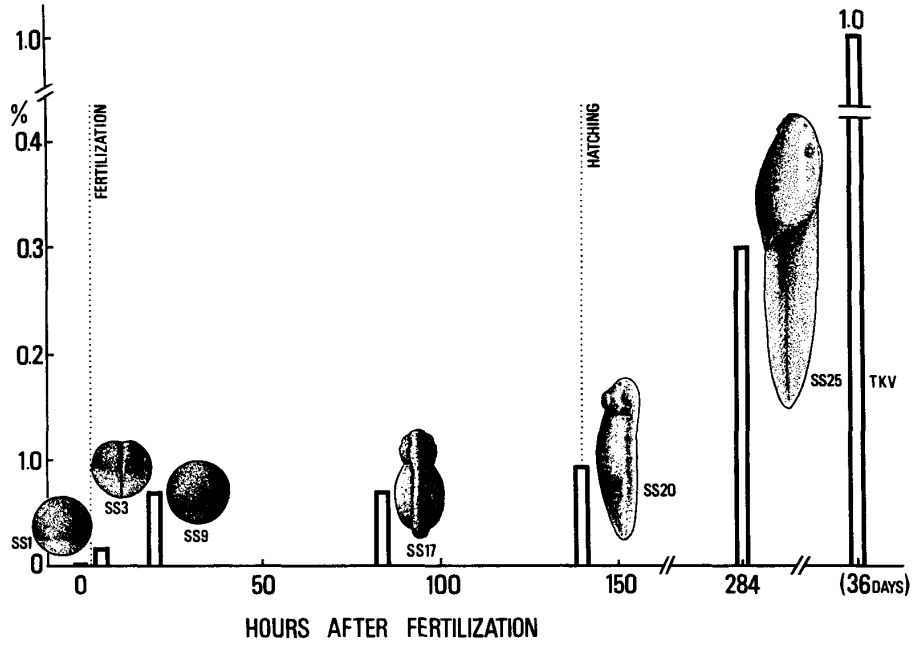


Fig. 15. A change in the content of galactose bound to lipids during development in *Rana nigromaculata*. The content of galactose in total lipids is shown by percentage.

## DISCUSSION

In living systems the structures of cells are based on large molecules, such as proteins, polysaccharides and complex lipids, whilst the organization of the systems appears to be the function of nucleic acids. Complex lipids are used to form membranes of cells. The major lipid constituents of membranes are generally phospholipids, glycolipids, gangliosides and sterols in eukaryotic cells.

The yolk of an amphibian egg contains the energy source and structural substance for development of the embryo. It has been found that the formation of the yolk is organized by mitochondria (WISCHNITZER, 1966). According to the study of LØVTRUP (1953) and URBANI (1973) on frog embryos, carbohydrate is used as the predominant energy source at the early stages of development and lipids at later stages. However, it was found in the present study that free fatty acid rapidly decreased between SHUMWAY stages 1 and 3, and subsequently it decreased slowly. This seems to indicate that free fatty acid is used as an energy source in the earliest stage of development. At SHUMWAY stage 17, early tail-bud embryo, a compensatory relationship was found between triglyceride and free fatty acid, as shown in Fig. 9. Triglyceride increased rapidly at this stage and then diminished rapidly at SHUMWAY stage 20, while free fatty acid decreased rapidly at SHUMWAY stage 17 and recovered swiftly at SHUMWAY stage 20. This phenomenon seems to indicate that morphogenesis at the tail-bud stage requires triglyceride at the cost of free fatty acid. This stage agrees with that when the yolk mass begins to disappear speedily by changing the shape. On the other hand, both free fatty acid and triglyceride decreased remarkably during the period from SHUMWAY stages 20 to 25. Such a decrement may be a result of

consumption of energy sources.

As described by PAPAHADJOPOULOS (1973), phospholipids are the ubiquitous and essential components of cell membranes. In this experiment, the changes in phospholipids during the development of frogs were quite similar to those reported by DREYFUS et al. (1975) in the case of chick retinas and brains. Except for some minor variations, there were no significant changes in the distribution of phospholipids during the stages in which phosphatidyl choline and phosphatidyl ethanolamine accounted for 70 to 80% of total phospholipids. Although sphingomyelin was very small in amount, a definite increase was found to take place after the hatching stage. Such an increment in sphingomyelin seems to be related to nerve cell differentiation, because sphingomyelin is one of the major constituents of nerve cell myelin (ANSELL, 1973).

Three kinds of phospholipids, that is, phosphatidyl choline, phosphatidyl ethanolamine and phosphatidyl serine, always existed in unfertilized eggs, cleaving eggs, embryos and tadpoles. The former two substances are known to be the major lipid components of most membranes in animal cells. However, it was noteworthy that phosphatidyl choline always existed in an overwhelming rate among the three phospholipids in contrast to that of adult frog tissues. Such a difference seems to be related to the morphological and physiological changes provoked by metamorphosis in the body. It is very probable that the membranes somewhat differ in minute structure as well as in chemical constitution between the aquatic and terrestrial lives.

YIAMOUYIANNIS and DAIN (1968) have reported that ganglioside-like substances appear at the stage between SHUMWAY stage 11 and 12 in the frog, *Rana pipiens*. In the present study, one spot of ganglioside-like substance was detected at SHUMWAY stage 17, early tail-bud embryo, by the method of thin-layer chromatography. Subsequently, such a spot rapidly increased in number during the embryonic stage up to hatching. As gangliosides are known to be concentrated on the outer surface of cell membranes particularly of nerve cells in mammals (WOLFE, 1961; WHERRET and MCILWAIN, 1962; EMMELOT and BOS, 1966), the increase of ganglioside-like substance during embryogenesis may be related to chemodifferentiation of the nervous system. This chemodifferentiation seems to start at the late gastrula stage, that is, shortly before the formation of the neural folds.

Monoglycosyl ceramides (CMH) were found in two forms, one of which was galactosyl ceramide containing galactose, while the other was glucosyl ceramide containing glucose. In mammals, it has been found that galactosyl ceramide is contained in a large amount only in the neural plasma membranes, while glucosyl ceramide is distributed mainly in the membranes of non-nervous cells or tissues (SUOMI and AGRANOFF, 1965). On the basis of this fact, many investigators have recognized that galactosyl ceramide is the best marker for differentiation of nervous systems in mammals (CUZNER, DAVISON and GREGSON, 1965; LEWIN and HESS, 1965; LANDOLT and HESS, 1966). In the present study it was found that glucosyl ceramide rapidly increased in a number of bands on

thin-layer chromatographic plate during the cleavage, embryonic and early tadpole stage (TK stage V). In contrast with this, galactosyl ceramide which appeared at the late cleavage stage as a single band increased in intensity with further development, although the band always remained as a single band. The early appearance of galactosyl ceramide seems to indicate chemodifferentiation of the nervous system, as found in the case of ganglioside.

A fairly rapid increase in the content of galactose bound to lipids occurred at the early cleavage stage between SHUMWAY stages 3 and 9. After levelling off a remarkable increment occurred when the tadpole began to eat. This increment in the content of galactose bound to lipids agreed with the increased intensity of galactosyl ceramide band obtained by thin-layer chromatography. This seems to indicate that galactose corresponds with the hexose component in galactosyl ceramide.

From the present study it was evident that there was an intimate correlation between the formation of the central nervous system and the increase of ganglioside-like substance, sphingomyelin and galactosyl ceramide. Although the distribution of these substances can not be histochemically observed for the time being, it is believed that they are mostly arranged around nerve cells or their mother cells. There is a possibility that accumulation of these substances induces indifferent cells to become nerve cells by their chemical stimulation during embryogenesis. At any event, it seems evident that chemodifferentiation precedes morphological differentiation in the formation of the nervous system. It is very desirable that the changes in distribution of the above substances during embryogenesis will be histochemically examined in frogs.

## SUMMARY

1. Four kinds of lipids, that is, neutral lipids, phospholipids, galactosyl ceramide and ganglioside-like substance, contained in *Rana nigromaculata* at various developmental stages were analyzed in order to clarify the relationship between these lipids and morphogenesis.

2. Phospholipids and cholesterol were commonly contained in all the organs and tissues examined, while a large amount of galactosyl ceramide and ganglioside-like substance was detected only in nervous organs.

3. Quantitative changes in total phospholipids, free fatty acid, triglyceride, cholesterol and cholesteryl ester were examined at seven developmental stages.

4. The rate of free fatty acid, one of the neutral lipids, to total lipids was about 45% at SS 1 (unfertilized egg) and decreased rapidly until about 28% at SS 3 (two-cell stage). At SS 17 (early tail-bud embryo), there was a compensatory relationship in amount between free fatty acid and triglyceride.

5. Phosphatidyl choline, a major kind of phospholipid, occupied about 50%, while phosphatidyl ethanolamine, another major kind of phospholipid, occupied about 20~25%. These two kinds of phospholipids were nearly constant in percentage from SS 1 to SS 25.

6. Ganglioside-like substance appeared for the first time at SS 17 as a single band on thin-layer chromatography and then as several bands at SS 25. Galactosyl ceramide appeared for the first time as a single band at SS 9 and increased slowly in intensity until the hatching stage. This band began to increase rapidly in intensity from SS 25. A change similar to that in galactosyl ceramide was also found in galactose bound to lipids.

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