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Biochemical and Genetic Studies on
Development in the Amphibian

by

Mary Mes-Hartree

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at the University of Ottawa, School of Graduate Studies.

January 1979

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Embryological Study of
the p Mutant in
Ambystoma mexicanum

ABSTRACT.

A recessive lethal mutation p (premature death), which arrests development at stage 37 in the Mexican axolotl, was studied through the use of embryological manipulations. These consisted of parabiosis, telobiosis, ectopic and reciprocal gill transplants and ectopic limb and eye transplants. Both parabiosis and telobiosis failed to rescue the mutant embryo. Mutant gill transplants also failed to develop on normal embryos. However, mutant limbs and eyes can develop on normal embryos. This indicates that the p mutation is not an autonomous cell lethal mutation, as previously reported but an organ or tissue specific mutation.

INTRODUCTION

The Mexican axolotl (Ambystoma mexicanum) has proven to be an excellent experimental animal for the purpose of genetic studies. The ease with which eggs can be obtained, and their large size, facilitates embryological manipulations. There are over 30 known mutant genes which affect development from oogenesis to maturity (Humphrey, 1975). These genes have been grouped in five categories according to the manner in which they are expressed (Briggs, 1973, Malancinski & Brothers, 1974).

The first group are known as the maternal effect genes. These produce defects in the egg cytoplasm, and are powerful tools for the study of interactions, during development, between the egg cytoplasm and the nuclei. The best studied of this group is the ova deficient mutant (o). Female axolotls homozygous for the o gene produce eggs that are arrested at gastrulation (Humphrey, 1966). These eggs can be corrected by injecting plasm from normal oocytes or eggs (Briggs & Cassens, 1966). The presence of the o^+ substance, produced during oogenesis in the egg cytoplasm, is essential for normal activation, during blastula, of the nuclear genes required for gastrulation and organogenesis (Brothers, 1976).

The second group of genes involved with early development are the nucleolar mutants. All three genes in this group act to reduce the size of the nucleolus. The molecular basis for the smaller size is unknown, but this group may prove useful for the study of nucleolar function (Carroll, 1972).

The third group involves organogenesis and produces defects affecting a specific organ or tissue. Two of these genes, e for eyeless and c for cardiac nonfunction, are particularly useful in studying induction (Briggs, 1973). The c mutation exerts its effect on the anterior endoderm, which induces heart formation (Jacobson, 1960) and not on the mesoderm itself (Humphrey, 1972). This provides an excellent system to investigate the molecular basis of the specific inductive interactions. The e mutant on the other hand, shows its effect on the induced tissue, the ectoderm which forms the eye, and not on the inducing component, the chorda mesoderm (Van Deusen, 1971). Therefore, the competence of the ectoderm is in question here. Many genes in this group show a wide range of abnormalities which often makes it difficult to pinpoint their exact action. In the case of the c lethal the most obvious abnormality is the pear shape of the body. This shape is due to the accumulation of fluids resulting from renal nonfunction, which in turn is linked to the lack of circulation caused by the non-functioning heart (Humphrey, 1972).

The fourth group are the genes affecting pigment cell differentiation, and/or pattern formed by pigment cells. Wild-type axolotls have three types of pigment cells which give them their mottled dark green color. These pigment cells are melanophores, which produce black pigment, xanthophores, which produce yellow pigment and iridiophores, which produce iridescent white pigments. The four genes in this category are white (d), albino (a), melanoid (m) and axanthic (ax). The

white axolotl has reduced numbers of both melanophores and xanthophores. The mutation affects the skin and prevents migration or differentiation of the prepigment cells (Dalton, 1949). The velvety black appearance of the melanoid is caused by an increase in melanophores and a decrease in the number of xanthophores. The albino axolotl has a yellow appearance and is defective in melanogenesis. This gene was introduced into the axolotl from the tiger salamander (Ambystoma tigrinum) through a brilliant series of experiments performed by Dr. R. Humphrey (1967). The axanthic animals lack xanthophores and iridiophores. Not only are these mutations beneficial to the study of pigmentation but they also serve as genetic markers.

The last and largest group of genes control cell function and are known as the autonomous cell lethals. The cell lethals are defined as genes whose lethal effects cannot be corrected by parabiosis of mutant embryos to normal ones, or by grafting of mutant tissue to normal embryos (Chung & Briggs, 1975). The effect of these genes are intrinsic to the cell in the sense that they cannot be corrected by being placed in a healthy environment. These genes show their effect as early as pre-hatching or as late as feeding larval stages. The ability of these mutants to differentiate functional cells of many types and to develop to advanced embryonic stages suggests that substances stored in the egg become limiting factors in later development when additional input of these substances are required for further growth. An example of this is the gill lethal, recognized by abnormal gills and slow yolk metabolism, whose

cell membranes show a reduced capacity for binding enzymes (Tompkins, 1970).

This thesis concerns itself with the earliest autonomous cell lethal, the premature death mutant (p), described by Trottier and Armstrong (1977). Morphologically the mutants cease to grow at stage 37 and are recognizable by their lateral curvature and their simple branched gill rudiments which frequently develop a small bulb at the distal end. The heart beats feebly but there is no circulation. The blood islands remain distinct until death. The mutants can be stimulated to move their tails feebly, but exhibit no righting reflex or swimming movements. Mutants can survive for about a week without growth after which time disintegration of the superficial tissues begins.

Histologically, the mutants show a complex group of abnormalities. Although the heart appears normal, the cephalic portions invariably contain a plug of undifferentiated cells. The major veins remain as thin-walled undifferentiated sinuses and the blood cells remain in the blood islands. The pharyngeal endoderm only forms closed fingerlike projections instead of the normal outpocketing which lead to normal gill pouch formation. The liver remains in a primitive state, the midgut does not develop and the myotome structure remains less differentiated in the mutant embryos. Only the nervous system is free of abnormalities at the light microscopic level.

Many of the observed abnormalities can be attributed to the lack of circulation, which could be caused by the plug in

the heart. The other abnormalities are all endodermally derived (gut, liver and gill pouches) or else endodermally induced (heart and muscles). These observations are the bases for the hypothesis that the mutant has a defective endoderm.

If this hypothesis is correct, then the mutant phenotype should be corrected by either parabiosis or organ transplants. Then the p mutant could no longer be classified as an autonomous cell lethal and would have to be placed in the tissue specific category where it would then be useful in the study of the role of the endoderm in the development of the embryo.

METHODS

Mature females were injected intramuscularly with 80 I.U. of follicle stimulating hormone and intraperitoneally with 50 I.U. luteinizing hormone twelve hours prior to mating. Just before mating, males were placed in an ice bath for 30 minutes, after which males and females were placed together. The resulting eggs were kept in 100% Holtfreter's saline and incubated at 10°C until they reached the desired stage. This procedure facilitated the timing of stages and assured that all embryos were at the same stage.

Operations on the embryos were carried out in sterile plastic petri plates, lined with 2% agar (DIFCO), which contained suitable depressions for the embryos. The operations were performed in calcium-free sterile 100% Holtfreter's saline and embryos were allowed to develop in sterile 100% Holtfreter's saline containing 400 mg/l streptomycin and 400 mg/l Penicillin G.

Parabiosis and telobiosis were performed according to the method described by Rugh (1962) on embryos at stages 23-24 of the Screckenberg and Jacobson (1975) series, after being dejellied manually using watchmaker's forceps.

Gill transplants were performed on embryos at stages 30-31; both ectopic and reciprocal transplants were done by the method of Rugh (1962).

Ectopic limb transplants were performed at stages 33-34 as previously described by Slack (1977). Eye transplants were also performed at this stage.

Materials:

Follicle-stimulating hormone was obtained from Burns Veterinary Supply (Hamilton, Ont.). Luteinizing hormone was purchased from Schwartz/Mann (Orangeburg, N.Y.). Penicillin G, streptomycin and MS 222 were from the Sigma Chemical Co. (St. Louis, Mo.).

RESULTS

Parabiosis as reported by Trottier and Armstrong (1977) had proven unsuccessful and the mutant invariably caused the death of the normal twin. However, the number of parabiosis of the desired combination was limited (four) so more operations were performed. All of the parabiotic combinations of normal to mutant that were obtained showed the same characteristics as previously described. The normal half of the pair developed well until they lost their red blood cells to the mutant and thus both animals died. A common circulation was never established and it appeared as though the cells were trapped in the mutant's blood islands which remained distinct. Dr. R. Humphrey suggested that the proximity of the mutant blood islands caused the red blood cells of the normal twin to be trapped. Telobiosis were performed in the hope of overcoming this problem, but all combinations of normal to mutant exhibited the same characteristics as the parabiotic twins.

Table I shows the total number of operations performed and the number of normal and mutant combinations. The percentage of normal to mutant for these experiments, is 21 which is within the range predicted for a Mendelian recessive trait ($p = .25$). This rules out the possibility that some of the mutants were phenotypically normal as a result of fusion with a normal embryo.

TABLE I

Results of Parabiosis and Telobiosis between sibs and non-sibs, where one embryo was the progeny of a heterozygous p mating. Operations were performed at stages 23-24.

Sibs ¹	Number of Combinations		Number of Combinations		
	Normal-Normal	Normal-Mutant	Non-Sibs ¹	Normal-Normal	Normal-Mutant
Telobiosis	18	11	Telobiosis	6	6
Parabiosis	8	2	Parabiosis	18	4
Total	26	13	Total	24	10

Number of embryos from heterozygous p animals 112

Number of p/p mutants 23

1 Sibs are the result of a heterozygous $+/p$ matings where $\frac{1}{4}$ of the progeny are expected to be p/p . Non-sibs mean that one of the pair is the progeny of a heterozygous mating, while the other is from a wild-type mating.

Gill transplants:

The gill transplants included the endoderm of the pharynx as well as the overlying ectoderm and mesoderm. Both donor and recipient animals were maintained in the same petri plate and examined daily to note the progress of the transplants. An ectopic transplant of normal gill tissue to a mutant recipient gave the startling result of the normal gill developing to a further stage than the mutant gill (Fig. 1). The normal gill almost reached hatching stage while the mutant embryo was still at stage 36. There was no circulation in the gill and the animal soon disintegrated after this photo was taken. To show that the site of the normal gill was not the contributing factor to the gill's success, reciprocal transplants were performed on non-sibs. Again the normal gill develops better on the mutant embryo than does the mutant's own gill (Fig. 2b). The mutant gill on the other hand does not develop on the normal embryo (Fig. 2a); the normal embryo has reached stage 40 while the mutant gill is still at stage 36 and retains the characteristic bulb at the distal end. A circulation was never established in the mutant gill. The mutant gill was absorbed with time until the normal embryo only had one remaining gill (Fig. 3).

Limb and eye transplants:

Since homozygous p mutants die before limb development, reciprocal transplants would be futile. Instead the presump-

Figure 1. Ectopic gill transplant of normal gill tissue
to the belly region of a p mutant axolotl.



Figure 2. Ventral view of wild-type (A) and *p* mutant (B) axolotls. The left gills were reciprocally transplanted; that is the left gill of embryo A was placed in the left gill area of embryo B and the left gill of embryo B was placed in the left gill area of embryo A.



B



A





Figure 3. Dorsal view of a normal melanoid axolotl approximately 3 months old whose left gill, which is now absorbed, was taken from a p mutant.





tive limb area from a stage 33 embryo from a heterozygous $+/p$ mating was ectopically transplanted into the flank region of an embryo resulting from a normal mating. The area consisted of ectoderm and underlying mesoderm. Donor and recipient were maintained in the same petri plate and carefully examined. The wound resulting from the removal of the tissue healed before stage 37 at which time donors could be classed as p mutants. When donors reached this stage, they were preserved in formalin and carefully cross-labelled with the recipient. These embryos were independantly scored as to their phenotype.

Grafted limbs of p/p mutants can develop normally on a normal recipient (Fig. 4a). Figure 4b shows the mutant who donated the limb area, to the embryo in 4a; this embryo shows all the characteristics of a p mutant and also the bulbs at the distal end of the gills. Ectodermal flank tissue from a p mutant was also successfully transplanted onto a normal white embryo resulting in a white animal with a dark patch of skin due to the heavily pigmented flank tissue of the p mutant (Fig. 5).

Using the same procedure as for limb transplants we have had success with eye transplants. In this instance the entire optic vesicle and overlying ectoderm was carefully removed using iridectomy scissors. A region of ectoderm and mesoderm was removed next to the spine and the eye material was carefully placed in this depression. The animal's recovery was closely monitored and a transplanted eye from a p mutant was successfully implanted in a melanoid animal. There is no photo of

Figure 4. Ventral view of a wild-type axolotl (A) with an extra limb on its left side which was donated by the p mutant in B.



Figure 5. Normal white embryo showing a dark patch of skin behind the left forelimb. This patch of skin originated from the flank tissue of a heavily pigmented p mutant.



* this animal since the darkly pigmented eye is barely visible against the black background of the melanoid animal.

DISCUSSION

The parabiosis and telobiosis experiments confirm the observations of Trottier and Armstrong (1977), that fusion of the p mutant to a normal embryo does not alter the p mutant development. Parabiosis, however, does not provide conclusive evidence that a mutant is an autonomous cell lethal. In this instance, parabiosis could have failed for a number of reasons, the most obvious being the lack of circulation in the mutant. The disappearance of the red blood cells from the normal embryo is puzzling. One explanation is that these cells get trapped by the mutant at the site of joining; another is that the mutant produces a substance that immobilizes or destroys the red blood cells.

We can safely rule out a toxic substance as the cause of death of the mutant, since gill transplants of normal gills to mutant embryos develop to a further stage than do the mutant gills. The fact that the mutant gills will not develop in a normal environment could indicate that the gill itself is defective. A point to remember here is that the gill pattern is not laid down in the ectoderm but in the endoderm and that gills fail to develop after removal of the endoderm (Severinghaus, 1930). In our transplants, endodermal tissue was also transplanted, and thus the failure of the mutant gills in the normal embryo may be related to the incompetence of the anterior endoderm, as previously suggested (Trottier & Armstrong, 1977).

The limb and eye transplants certainly place this mutant in the organ or tissue specific category. The limb is dependent on the lateral mesoderm and the overlying ectoderm for its proper development (Balinsky, 1975). The endoderm has some role to play since in endodermless embryo two pair of forelimbs frequently develop (Nieuwkoop, 1946). In our case the forelimbs were normal presumably because of the normal endoderm present in the recipient embryo.

The eye is induced early in development, by the roof of the archenteron which acts on the neural plate and subsequently on the eye cup rudiment. This structure along with the head mesoderm induces lens formation. The fact that both the limb and the eye are relatively independent of endoderm for normal differentiation further supports the hypothesis that the anterior endoderm is not functioning properly in p/p embryos.

A method to further test this hypothesis would be to perform reciprocal endoderm transplants between embryos from a normal mating and embryos from a mating between two heterozygote adults carrying p . If the embryos from the heterozygous mating all develop normally and some of the embryos from the normal mating develop p characteristics then this would be conclusive evidence that the endoderm is at fault.

Any mating that will produce p/p embryos must be between heterozygotes and only $\frac{1}{4}$ of the embryos are of the desired p/p genotype. These do not reveal themselves until stage 36 which is at a later stage than most embryological manipulations. This means that the transplants are performed with normal ani-

mals in the hope that some will be of the desired type, that is normal/mutant combinations. To overcome this difficulty one could, by gonadal transplantation, produce normal axolotls whose germ cells would be of the p/p genotype. This way all the embryos from a mating between such animals would be of the p/p genotype thus facilitating embryological experiments. Such a mating would also permit biochemical studies to be started on this mutant. Various regions of the endoderm could also be tested in the hopes of pinpointing the location of the defect. The possibility also exists that the syndrome may be corrected through injections of tissue extracts as in the 'o' mutant (Briggs & Justus, 1968). This would then permit the characterisation of the chemical substance involved.

The discovery that the p mutant is not an autonomous cell lethal but a tissue specific defect opens the field to future research that will undoubtedly expand our knowledge of the mechanisms involved in development and differentiation.

REFERENCES

- Balinski, B.I. 1975. An Introduction to Embryology 4th edition. W.B. Saunders Company, Toronto.
- Briggs, R. 1973. Developmental Genetics of the Axolotl. Symp. Soc. Dev. Biol. F.H. Ruddle (Ed). Academic Press, New York. Vol. 31: 169-199.
- Briggs, R. and Cassens, G. 1966. Accumulation in the oocyte nucleus of a gene product essential for embryonic development beyond gastrulation. P.N.A.S. 55: 1103-1109.
- Briggs, R. and Justus J.T. 1968. Partial characterization of the component from normal eggs which corrects the maternal effect of gene 'o' in the Mexican axolotl. J. Exp. Zool. 167: 105-116.
- Brothers, A.J. 1976. Stable nuclear activation dependent on a protein synthesised during oogenesis. Nature. 260: 112-115.
- Carroll, C. 1972. Genetic and Experimental Studies in the Mexican axolotl. PhD Thesis, University of Indiana.
- Chung, H.M. and Briggs, R. 1975. Experimental studies on a lethal gene 'e' in the Mexican axolotl. Ambystoma Mexicanum. J. Exp. Zool. 191: 33-48.
- Dalton, H.C. 1949. Developmental analysis of genetic differences in pigmentation in the axolotl. P.N.A.S. 35: 277-283.

- Humphrey, R.R. 1966. A recessive factor (o, for ova deficient) determining a complex of abnormalities in the Mexican axolotl (Ambystoma mexicanum). Dev. Biol. 13: 57-76.
- Humphrey, R.R. 1967. Albino axolotls from an albino tiger salamander through hybridization. J. Hered. 58: 95-101.
- Humphrey, R.R. 1972. Genetic and experimental studies on the mutant gene (c) determining absence of heart action in embryos of the Mexican axolotl. Dev. Biol. 27: 365-375.
- Humphrey, R.R. 1975. The axolotl, Ambystoma mexicanum. Handbook of Genetics, R.C. King (Ed). Plenum Press, New York. Vol IV: 3-17.
- Jacobson, A.G. 1960. Influences of ectoderm and endoderm on heart differentiation in the Newt. Dev. Biol. 2: 138-154.
- Malancinski, G.M. and Brothers, A.J. 1974. Mutant genes in the Mexican axolotl. Science. New York. 184: 1142-1147.
- Nieuwkoop, P.D. 1946. Experimental investigations on the origin and determination of the germ cells and on the development of the lateral plates and germ ridges in urodeles. Part I. Arch. Neerland Zool. 8: 1-36.
- Rugh, R. 1962. Experimental Embryology 3rd edition. Burgess Publishing Company, Minneapolis, Minnesota.
- Schreckenber, G.M. and Jacobson, A.G. 1975. Normal stages of development of the axolotl, Ambystoma Mexicanum. Dev. Biol. 42: 391-400.
- Severinghaus, A.E. 1930. Gill development in Ambystoma punctatum. J. Exp. Zool. 56: 1-29.

- Slack, J.M.W. 1977. Determination of anteroposterior polarity in the axolotl forelimb by an interaction between limb and flank rudiments. *J. Embryol. exp. Morph.* 39: 151-168.
- Tompkins, R. 1970. Biochemical effects of the gene g on the development of the axolotl Ambystoma mexicanum. *Develop. Biol.* 22(1): 59-83.
- Trottier, T.M. and Armstrong, J.B. 1977. Experimental studies on a mutant gene (p) causing premature death of Ambystoma mexicanum embryos. *J. Embryol. exp. Morph.* Vol 39: 139-149.
- Van Deusen, E.B. 1971. Studies on embryonic determination: effects of gene 'e' on forebrain ectoderm in the axolotl. *Am. Zool.* 11: 678.

Lipid Changes
During Development of
Xenopus laevis Embryos

ABSTRACT

Xenopus laevis embryos were analyzed for their lipid content and composition from fertilization to feeding stages. Neither the total lipid content, nor the relative amounts of the major lipids changed until after hatching. By feeding stages the amounts of phosphatidylcholine (PC), phosphatidylethanolamine (PE) and sterol ester (SE) had increased twofold, but the amount of triglyceride per tadpole remained constant. Prior to hatching, ^{14}C -acetate label was incorporated primarily into the fatty acids of PE. After hatching, increasing amounts of label were found in other lipids. The most marked changes occurred just before the onset of feeding, and pulse-chase experiments suggest that non-lipid reserves are mobilized at this stage for de novo lipid synthesis. We believe that membrane biogenesis during early embryogenesis involves mobilization of lipid reserves accompanied by some transacylation of PE at the inner surface of cell membranes. Synthesis of sterols and glyceride backbones may begin about hatching, but the onset of most de novo biosynthesis occurs abruptly about 24 hr prior to the onset of feeding.

List of common abbreviations:

TLC Thin-layer chromatography
MG Monoglycerides
DG Diglycerides
St Sterol
TG Triglycerides
SE Sterol esters
Sph Sphingomyelin
PC Phosphatidyl choline
PI Phosphatidyl inositol
PS Phosphatidyl serine
PE Phosphatidyl ethanolamine

INTRODUCTION

The amphibian egg is characterized by a self-sufficiency with respect to metabolic interactions with its environment. Development in this 'closed system' must depend on rearrangement of stored materials from maternal origins (Cohen, 1954). The energy required for development is supplied by carbohydrates, which are the first energy-rich component to be used by the dividing egg (Williams, 1965). Lipids also constitute an important energy depot (Wallace, 1963). Both of these substances are found in yolk-platelets, whose major components are protein (78%), carbohydrate (14%) and lipid (2%). Lipids are also present in fatty yolk, or lipochondria (Holtfreter, 1946). These are irregularly shaped bodies whose homogenous material is bounded by a thin protein coat, which is not a true membrane (Wischnitzer, 1964). Lipochondria as such are difficult structures to study biochemically since they remain scattered after centrifugation and adhere to the heavier yolk platelets.

Studies on the ultra structural changes in the developing egg have shown that the yolk platelets are resorbed in a definite pattern, with the most actively differentiating regions losing their yolk platelets first (Karasaki, 1963, Follett & Redshaw, 1974). Long structural units become detached from the crystalline main body of the yolk and produce fine particles which subsequently appear to change into a variety of membranous components. The appearances of these membranous components coincides with a greatly increased demand for plasma membranes required for rapid cellular multiplication.

Morrill and Kostellow (1964) have studied the lipid gradients which exist at blastula stage in the amphibian embryo. They separated the ectoderm, endoderm, and mesoderm and measured the amount of lipid and phospholipid in each germ layer. They found that there exists a phospholipid gradient going from ectoderm, to mesoderm, to endoderm, and a concurrent negative gradient for the neutral lipids. Therefore, the cells with the highest metabolic activity have the lowest phospholipid content and the highest amount of neutral lipid. The phospholipid gradient follows the same pattern as the yolk platelet gradient.

Studies on the lipid content in amphibian embryos (Bufo arenarum Wensel) reveal that there is no change in the lipid content as embryos develop (Kutsky, 1950). There is also no change in the phospholipid content, or the individual fatty acids associated with them, from oocyte to neurula (Barassi & Bazan, 1974 a). The two phospholipids studied, phosphatidyl choline and ethanolamine, remain unaltered from oocyte to gill circulation (Barassi & Bazan, 1974 b). This is a surprising result, since, from oocyte to later stages, phospholipids are in great demand for membrane formation.

When oocytes are prelabelled with P^{32} (by injecting P^{32} into females along with pituitary extracts) there is an increase in the specific activity of the P^{32} in phospholipids and a decrease in the acid soluble pool (Crupkin et al, 1973; Pechen & Barassi, 1974). This increase could indicate de novo synthesis of phospholipids, or increased turnover of the phosphorylated moiety. Since the phospholipid content of the embryo

does not change, de novo biosynthesis seems unlikely. The turnover theory sees the phospholipids arriving at the nascent membrane site, being dephosphorylated to facilitate entry into the membrane and then being rephosphorylated from the labelled pool. This line of research uses the embryonic development period as a model for the study of membrane biogenesis.

Amphibian embryos also incorporate C^{14} -acetate into lipids, with the greatest labelling into triglycerides, phosphatidyl choline and ethanolamine (Miceli & Brenner, 1976). The incorporation into lipids is very small (2% of the total uptake) and one can dispute whether this truly reflects de novo synthesis of lipids. It was also shown that C^{14} -glycerol is not incorporated in vivo or in vitro in developing embryos, and that pre-labelled oocytes show no change in specific activity of the C^{14} -glycerol in lipids during development (Pechan & Bazan, 1977). This seems to rule out de novo biosynthesis of lipids at least until the gill circulation stage and further supports the hypothesis that lipid biosynthesis occurs during oogenesis, and that these lipids are stored in yolk and are transported to membrane sites when needed.

The constancy of phospholipid composition and content during development may be easier to understand if one views phospholipids, not as macromolecules themselves, but as constituents of macromolecules, as are amino acids, nucleotides or sugars. Therefore, the phospholipids themselves do not change but they are utilized as 'building blocks' for multimolecular structures and it is these that change as yolk is utilized to.

make embryonic membranes (Pasternak, 1977).

The radioisotope studies mentioned above have either dealt with prelabelled embryos, cell-free homogenates or else very short radioactive pulse period. All of these approaches have inherent drawbacks. Prelabelling embryos is akin to running pulse-chase experiments where the pulse period is restricted to the oocyte; the preparation of a cell-free homogenate is an extremely disruptive process and the short radioactive pulses invariably incorporate too little radioactivity into lipids to be of much use. To overcome these drawbacks we decided on a longer pulse of twelve hours, hoping that this would permit sufficient label to enter the embryo and to be incorporated into lipids so that individual lipids could be examined. By using sequential twelve hour pulses, a more complete picture could be formulated of the lipids at various stages. Because little was known about the lipid composition and content of Xenopus laevis from oocyte to hatching, preliminary studies were necessary to measure these parameters.

MATERIALS

Animals:

Mature Xenopus were obtained from C.W. Fletcher (Hampstead, Maryland) and The Amphibian Facility, University of Michigan (Ann Arbor) and maintained in accordance with the principles of Care of Experimental Animals - A Guide for Canada (published by the Canadian Council on Animal Care).

Spawning was induced, in mature Xenopus, by injecting both males and females with 150 I.U. chorionic gonadotropin in the dorsal lymph sac 24 hours prior to mating. Just before being placed together, the females were reinjected with 500 I.U. chorionic gonadotropin. The females began to lay eggs 12-18 hours after being placed together with males. The eggs were staged according to staging tables of Niewkoop and Faber (1956). A brief description of stages referred to in the text is given in Appendix III.

Jelly layers were removed chemically with a solution of 1.75 gr. Cysteine and 0.6 gr. TRIS in 50 mls water, pH adjusted to 7.8. Eggs were then rinsed in 10% modified Holtfreeters saline (Trottier & Armstrong, 1975).

Lipid extraction and identification:

Lipids were extracted from a known number of embryos (usually 50) according to the procedure described by Kates (1972) for cell fractions, blood cells and plasma. Dry weight, for total lipids, was determined as described by Kates (1972):

dry weight for individual lipid classes was determined using a dichromate oxidation procedure (Dittmer & Wells, 1969). Total phosphorous was measured by the modified microprocedure (Kates, 1972). Lipids were separated by TLC on silica gel H (Merck) and on silica gel-0 prepared plates (Analabs). TLC plates were sprayed with 50% sulfuric acid or exposed to iodine vapours for nonspecific visualization. Dittmer-Lester reagent (Dittmer & Lester, 1964) was used to detect phosphate, ninhydrin (Skipski et al, 1962) was used to indicate presence of a free amino group, and Dragendorff reagent (Wagner et al, 1961) was used to detect choline.

Various solvent systems were employed with the view of finding a suitable system for routine work (c.f. Appendix 1 and 2). For routine separation of polar lipids, the solvent system containing chloroform - methanol - acetic acid - water (75:45:12:4) was found to be most suitable. Prior to running the samples in this solvent, the plates were prewashed with acetone - petroleum ether (75:25) to run the neutral lipids to the top of the plate and this also provides better separation of the polar lipids. The neutral lipids were separated by the method of Freeman and West (1966) which employs two solvent systems. The first contains ethyl ether - benzene - ethanol - acetic acid (40:50:2:0.2) and the second contains ethyl ether - hexane (6:94).

Identification of the phospholipids involved chromatography with standards. Identification was also based on cochromatography on paper of the deacylated products of standards

and unknown phospholipids. After preparation (Kates, 1972), the deacylated products were run in phenol-water, 100:38 (v/v). Spots were detected by treatment with the glycol spray of White and Freeman (1967). Plasmalogens of PE, PC and PI were detected as minor components using a mild acid hydrolysis (Dittmer & Wells, 1969).

Identification of fatty acids:

Fatty acid methyl esters were prepared according to Kates (1964). Samples were analysed in a Hewlett Packard model 402 gas-liquid chromatograph equipped with a flame ionization detector. The fatty acids were identified by comparison of retention-times relative to palmitate with those obtained for standards, as well as published values (Kates, 1972). Most analyses were done on columns packed with 10% butanediol succinate on Chromosorb W (Chromatographic Specialties, Brockville, Ontario) and run at 180°C. As an aid to the identification of the fatty acids, many of the samples were also run on 10% Apiezon L on Chromosorb W at 190°C. Weight percentages were computed according to the peak height times retention time method.

Radioactive labelling:

The embryos were labelled with C^{14} -acetate. Fifty-five embryos were incubated for 12 hours at room temperature in 10 mls media D (Hampel et al, 1975) adjusted to pH 7.2 and 10 μ C C^{14} sodium acetate. After incubation, the embryos were rinsed in cold water. Five embryos were digested using 1 ml tissue

solubilizer (NCS). Lipids were extracted from the remaining embryos. Radioactivity was counted in a Beckman liquid scintillation counter using Biofluor as the cocktail for the lipid samples, and Econofluor for the digested samples. All values were corrected for background and counting efficiency. Known aliquots of lipid extract were applied to TLC plates and run in neutral and polar solvents. Each lipid spot was identified using iodine vapour and then scraped into vials and counted. Any radioactivity not associated with lipid spots was checked by autoradiography. The recovery of activity using this procedure was greater than 80%.

Chemicals:

Chorionic gonadotropin and lipid standards were obtained from the Sigma Chemical Co. (St-Louis, Mo.). Silica gel-O plates, Biofluor, Econofluor and C^{14} -acetic acid sodium salt (56 mCi/mmol) were purchased from New England Nuclear (Boston, Mass.).

Technical grade chloroform was redistilled before use. Methanol was spectro analysed grade from Can-Lab. All other chemicals used were reagent grade.

Autoradiograms were done using Kodak Medical Blue-Brand film and developed with Kodak Developer and Fixer purchased from Picker Nuclear (Ottawa, Ont.).

RESULTS

Lipid content:

Total dry weight determinations of the embryos at various stages are reported in Table I. Variation in the lipid content of eggs from different females was slight and there was no significant change in the total amount of lipid from fertilization to hatching. The total lipid samples were also analysed for phosphorous content. Values ranged from 1.0 to 1.5 $\mu\text{g}/\text{egg}$ (average $1.27 \pm 0.29 \mu\text{g}$) and again no trend was apparent. The amount of individual lipids was determined by the dichromate oxidation procedure of Freeman and West (1966) using separate standard curves for polar and neutral lipids. This procedure was used to quantitate the major lipid components, that is, PC, PE, SE, TG, but lacked the necessary sensitivity for the minor lipids. The results showed no change in the amount of PC, PE, TG, SE up to hatching. However, just prior to feeding, at stages 40-41, there is a marked increase in the amount of PC, PE and sterol esters with no change in the triglyceride levels (Table II). Analysis of the fatty acid composition of the total lipids also revealed no change during development (Table III). The most common long-chain fatty acids were found to be the polyunsaturated linoleic (18:2) and arachidonic (20:4) acids.

C¹⁴-acetate labelling:

A preliminary experiment was done to see if cold acetate would increase the efficiency of uptake of labelled acetate.

TABLE I

Lipid content of X. laevis embryos

Stage ¹	Lipid per embryo, ug
(St. 3)	112 ± 1 ²
(St. 7)	125 ± 8
(St. 9)	108 ± 7
(St. 11)	109 ± 11
(St. 16)	110 ± 10
(St. 35)	109 ± 19

¹ Stages in parentheses were determined from the normal tables of Niewkoop and Faber (1956).

² Means ± SE based on determinations from eggs from three different females (five in the case of stage 9 embryos).

TABLE II
Content of individual lipid classes of X. laevis embryos

Stage	Lipid class per embryo, μg				SE
	PC	PE	TC		
Pre-hatching (10-34)	28 \pm 2 ¹	14 \pm 2	56 \pm 3	16 \pm 2	
Post-hatching (35-39)	30 \pm 3	14 \pm 2	59 \pm 3	13 \pm 1	
Pre-feeding (40-41)	63 \pm 2	28 \pm 1	58 \pm 4	25 \pm 8	

1 Means \pm Standard error based on determinations from 150 embryos.

TABLE III
Fatty acid composition of total lipids from *X. laevis* embryos

Fatty acid	Stage of development							
	3	7	9	11	16	35		
Palmitic	20.7	21.3	21.2	21.4	20.8	21.3		
Palmitoleic	10.3	11.1	11.3	10.6	10.9	9.4		
C ₁₇ cyclo	1.3	1.3	1.4	1.4	1.2	1.3		
Stearic	6.3	6.2	6.6	6.2	6.4	6.3		
Oleic	27.3	26.9	26.2	26.8	27.4	27.6		
Linoleic	11.4	11.9	10.8	12.7	11.2	12.7		
Linolenic	--	--	1.1	--	--	--		
Arachidic	1.0	--	1.5	--	--	--		
U1	1.3	1.1	2.2	1.2	--	--		
U2	1.9	2.0	2.0	2.0	1.9	2.1		
Arachidonic	9.4	9.2	9.3	10.3	9.9	9.9		
U3	--	--	1.4	1.0	--	1.8		
U4	1.7	1.8	1.5	1.6	1.4	--		

Note: Values are expressed as weight percentages, and are averages from at least two different animals. Variation from one animal to another was negligible. Only values above 1.0% are reported. The relative retention times of the unidentified fatty acids denoted by U1-U4 to that of palmitic acid on a BDS column at 180°C were 4.32, 5.04, 6.40 and 6.98, respectively. Stages were determined from the normal tables of Niewkoop and Faber (1956).

The results indicate (Table IV) that the addition of cold carrier decreases the uptake of labelled acetate. Autoradiograms of the separated lipids on TLC plates also verified this trend.

Sequential labelling of the embryos from early cleavage to stage 41 consisted of 12 hour pulses. The total incorporation into embryos, lipids and fatty acids was measured; from these percent incorporation of label into lipid as compared to total uptake by embryos was calculated. In the same manner percent incorporation into fatty acids as compared to total uptake by lipids was also calculated. The data is presented in Figure 1. Percent incorporation into lipids ranges from 3.9 to 12.1. The percent incorporation of the fatty acids shows a decline from 88 to 48 percent as development proceeds.

The incorporation into different lipid classes was also measured by separating the lipids on TLC plates and then counting the radioactivity in each lipid spot. The lipid profiles are presented in Table V in the form of percent incorporation into individual lipid classes as compared to the total lipid. The neutral lipids show a steady rise from 7.4 percent to 23.3 percent up to hatching after which there is a sudden drop down to 10.8 percent followed by a steady rise up to 25.6 percent. Most of the radioactivity (72.2 percent in early stages) is found in the PE fraction. This value drops to 18.7 percent by stage 41 having taken a sudden drop at stage 40. The other phospholipids show a steady increase.

The incorporation of labelled material has been reported to be stimulated by the presence of intact jelly coats (Hampel

TABLE IV

Effect of cold acetate on the uptake of C^{14} -acetate by *X. laevis* embryos. Embryos were pulsed for 12 hours with 1 $\mu C/50$ embryos.

Cold acetate (M)	D.P.M. (50 embryos)
0	52,599
10^{-5}	50,287
10^{-4}	25,155
10^{-3}	9,577
10^{-2}	108

Figure 1. Labelling of intact *X. laevis* embryos.

Incorporation into lipids and fatty acids following a 12-hour C^{14} -acetate pulse. The results are presented as percent incorporation into the lipid fraction as compared to the total uptake and the percent incorporation into the fatty acids of these lipids. The stages mentioned represent the stage at the end of the pulse period.

● fatty acids

○ lipids

I - standard error

I^x - range

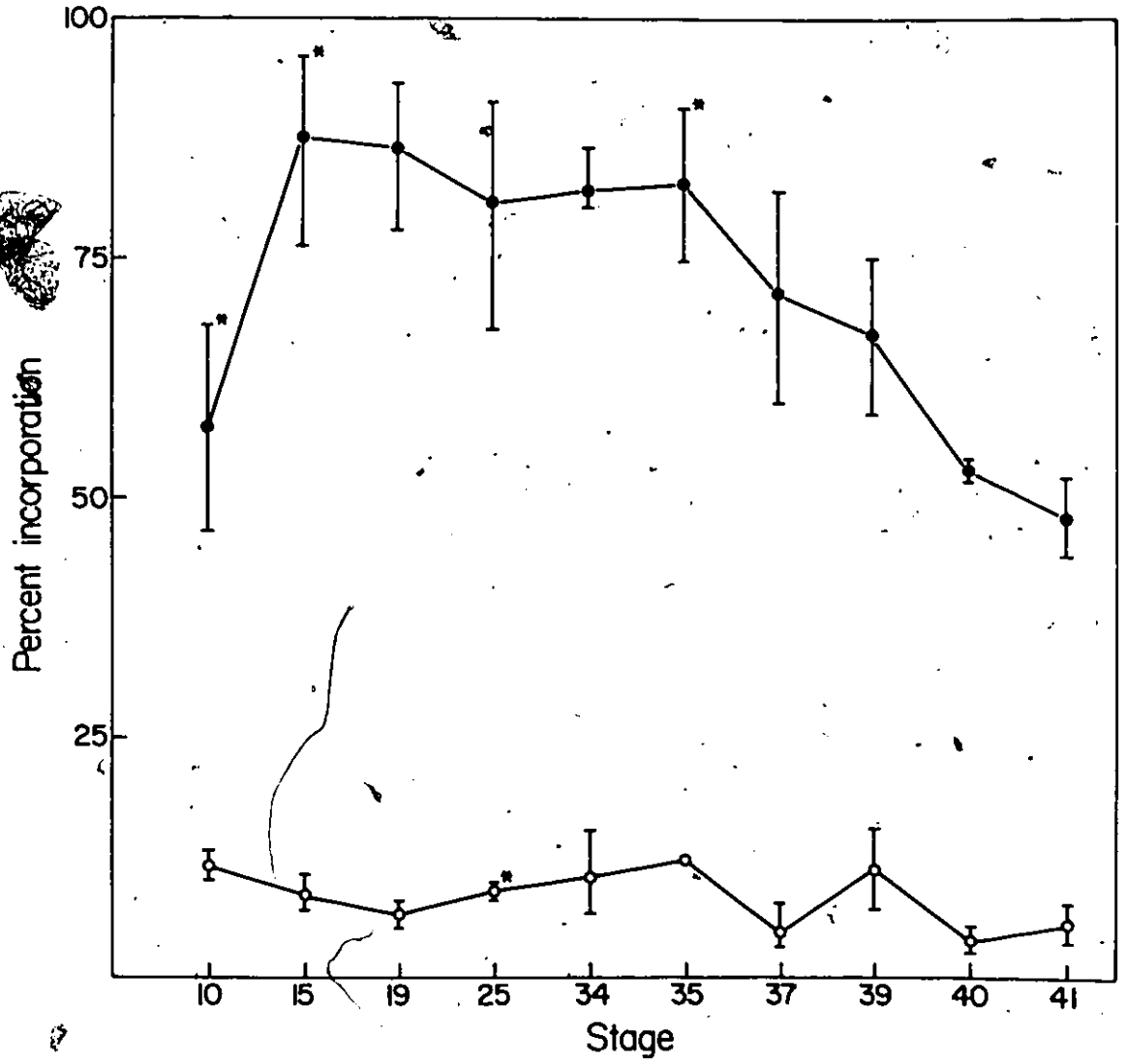


TABLE V

Lipid profile of C¹⁴-acetate labelled lipids following a 12-hour pulse of intact X. laevis embryo at sequential stages. Results are presented as percent incorporation into individual lipid class as compared to total lipid incorporation.

Stages during pulse	Total	Neutral	MG	ST	TG
4 - 10	7.4(5.7,9.2)	¹	2.1[1.5,2.6]	0.3(0.2,0.4)	2.6(2.1,3.1)
10 - 15	11.3(8.6,15.7)		3.5[1.9,5.5]	0.6(0.5,1.1)	4.1(3.1,4.6)
15 - 19	16.2(11.8,21.2)		3.4(2.7,4.2)	0.6(0.5,0.8)	3.7(3.1,4.4)
19 - 25	15.7(11.4,20.4)		3.2[2.8,3.6]	0.6(0.5,0.8)	3.3(3.1,3.5)
25 - 34	21.1(14.1,29.0)		1.7	2.2[1.1,3.7]	6.1(3.5,9.4)
34 - 35	23.3(21.7,25.0)		0.4	2.2	5.6
35 - 37	10.8(8.8,12.8)		2.0[1.7,2.2]	2.1[2.1,2.0]	5.3(4.5,6.2)
37 - 39	13.3(10.7,16.1)		3.0(2.2,3.9)	2.0[1.2,3.1]	5.8(4.8,6.9)
39 - 40	15.5(13.5,17.7)		5.7[5.1,6.3]	4.6(3.8,5.4)	10.6(7.9,15.7)
40 - 41	25.6(21.0,30.3)		3.2[2.8,3.7]	4.8(4.2,5.3)	14.8(10.0,20.0)

1 Standard Error denoted by curved bracket ()

2 Range denoted by square brackets []

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TABLE V (continued)

Stages during pulse	PC	PI+PS	PE
4 - 10	0.9(0.8,1.2)	3.2(3.0,3.4)	72.7(62.7,81.8)
10 - 15	1.2(1.2,1.2)	3.2(2.9,3.5)	68.8(58.4,78.3)
15 - 19	2.1(1.9,2.2)	3.0(2.2,3.8)	59.6(45.5,72.9)
19 - 25	2.4(2.2,2.6)	3.4(2.9,3.9)	56.1(41.9,69.8)
25 - 34	10.0(5.2,16.2)	4.1[4.8,3.5]	43.8(32.3,55.7)
34 - 35	9.4	4.9	44.4
35 - 37	8.0(5.7,10.6)	7.5(6.1,9.1)	40.7(34.6,47.6)
37 - 39	9.2(6.3,12.5)	5.8(4.4,7.5)	52.2(45.4,58.9)
39 - 40	19.7(15.9,25.3)	21.2(19.6,22.9)	19.7(16.7,23.0)
40 - 41	15.9(14.6,17.2)	14.0(13.1,14.9)	18.7(14.8,22.9)

et al, 1975). The previous experiments were performed with intact jelly coats which remained on the embryos during the homogenization procedure. Several experiments were performed to test for possible uptake by the jelly coat, and the stimulation of uptake provided by the jelly coat. When embryos are pulsed for 12 hours without the protective jelly coat, they incorporate an order of magnitude less label than the intact embryos. The profile of uptake is presented in Table VI and is not much different from the profile with intact jelly coats, except for the sudden rise, during the stage 16-26 interval, of incorporation into lipid. This rise could have been masked by the jelly in the previous experiment. To test the action of the jelly, jelly coats alone were incubated with C^{14} -acetate. Though there was some incorporation of radioactivity into lipid associated with the jelly, when these lipids were separated on a TLC plate the pattern of labelling was not that of the previous experiments with intact embryos. Most of the label was found at the origin (34%) or at the solvent front (57%).

Pulse-chase experiments:

Pulse-chase experiments consisted of a 12 hour pulse for all embryos in a given run, then these were rinsed and 55 embryos were taken for sampling every 12 hours (c.f. Methods). The loss of activity as compared to the original pulse (time 0) was measured for stages from gastrula to hatching, since the pulse period was from early cleavage stages to gastrula. Post-hatching stages were not measured since once the embryos hatched

TABLE VI

Incorporation pattern and lipid profile of dejellied embryos pulsed for 12 hours with C¹⁴-acetate.

Stages during pulse ¹	% incorporation			Lipid class ³			
	Lipid ²	Fatty acid ³	Neutral	TC	PC	PI+PS	PE
4 - 10	7.8	55.2	10.8	6.7	5.1	34.3	30.6
10 - 18	11.0	65.2	7.0	4.1	3.0	1.7	86.2
16 - 26	36.0	70.8	12.3	3.8	4.1	3.0	73.8
26 - 34	6.0	64.1	20.1	14.3	19.0	8.1	29.6

1 Values reported are for the stage at the end of the pulse period.

2 Percent incorporation of total uptake by embryos.

3 Percent incorporation of total uptake by lipids.

4 Means of two determinations.

from their jelly coats, the radioactivity of post-hatching embryos increased over the level of pre-hatching embryos; this could be due to the ingestion of radioactive label associated with the jelly coat, which gets released upon hatching. The results show (Figure 2) that little radioactivity is lost until the time of hatching at which time a drastic drop occurs. The fatty acids show an increase until hatching when the level of radioactivity drops. The lipid profile, given in Table VII, shows an increase in the PI and PS fraction at stages 33 to 36 and a decrease in PE at these stages.

A similar experiment was run on embryos without their protective jelly coats and these were followed to post-hatching stages. The results in Figure 3 show a sudden drop in label as soon as the chase period begins and then the label remains steady until stage 40 at which time the lipid fraction contains more label than during the pulse period. The level of incorporation was too low to permit a lipid profile to be done.

When embryos are pulsed for 12 hours at the time of hatching, and then allowed to develop without label for 4 days, the label in the lipid fraction shows a significant increase at stage 41 (Figure 4) followed by a decline to stage 46 and then a gradual rise until stage 48. The lipid profile remains steady except for a high percentage of label (27%) in the sterol fraction of stage 46 embryos (Table VIII).

When the embryos are pulsed and fed simultaneously and then allowed to develop without label for 48 hours, the radioactivity in the embryos remained stable after the initial loss

Figure 2. Pulse-chase of intact *X. laevis* embryos.

Twelve hour C^{14} -acetate pulse at gastrula followed by 36 hours chase (until hatching). Results are shown as percent activity of the embryos, lipids and fatty acid fractions, taking time 0 to be 100%.

- △ fatty acids
- lipids
- embryos

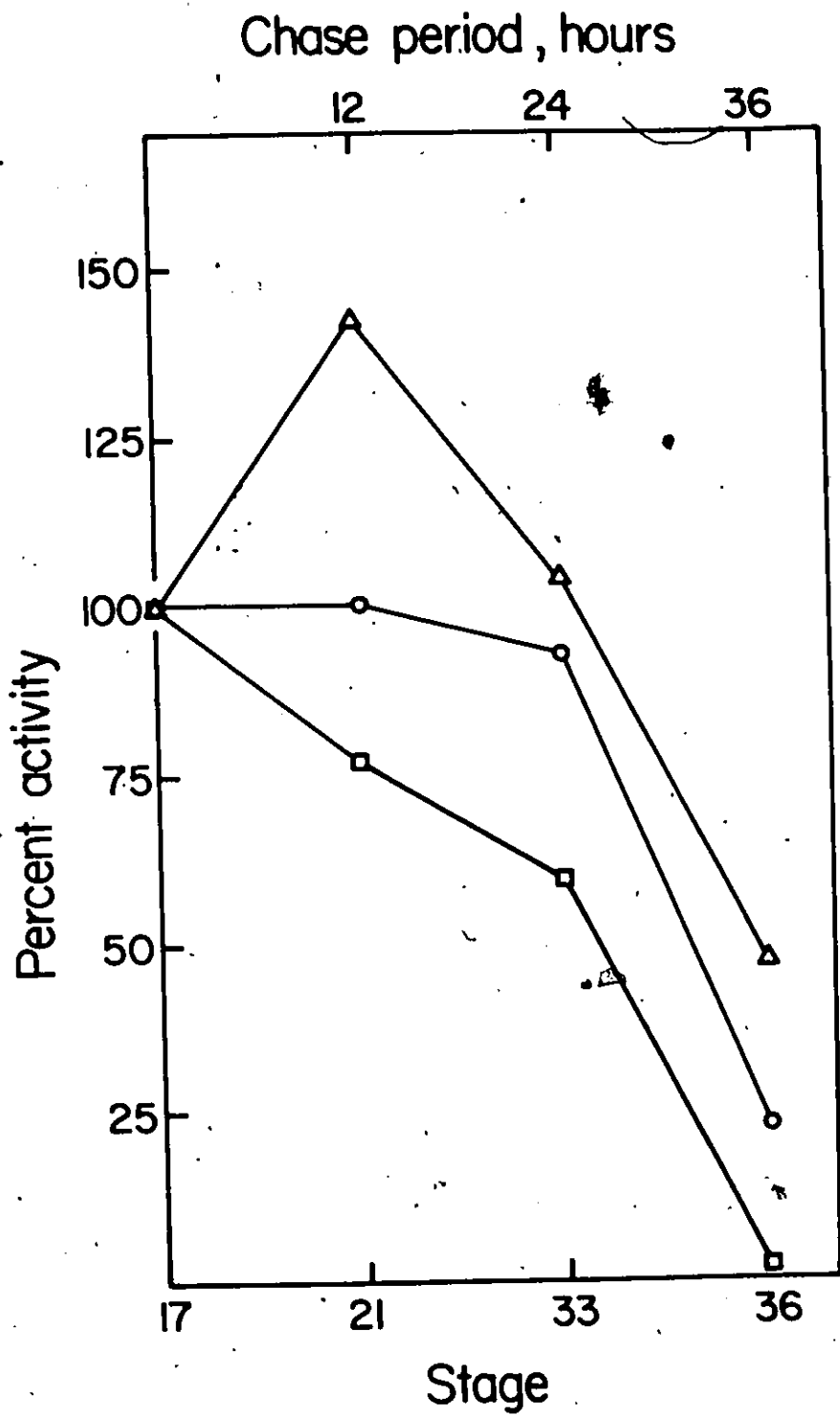


TABLE VII

Lipid profile of C¹⁴-acetate labelled lipids following a 12 hour pulse of intact embryos at gastrula and then a 36 hour chase. The results are presented as percent incorporation into specific lipid classes as compared to total uptake into lipids.

Chase period (hours)	Stage	Total Neutral	MG	ST	DG	TG	SE	Sph	PC	PI+PS	PE
0	17	16.3	3.0	0.9	0.4	2.0	0.8	0.8	5.4	3.1	55.1
12	27	8.8	2.0	2.1	0.6	3.3	0.9	1.7	2.6	7.0	64.1
24	33	17.2	0.9	3.0	1.1	2.9	0.8	1.2	3.7	22.2	49.8
36	36	14.0	-	4.5	1.7	3.3	1.6	2.3	4.7	19.4	46.5

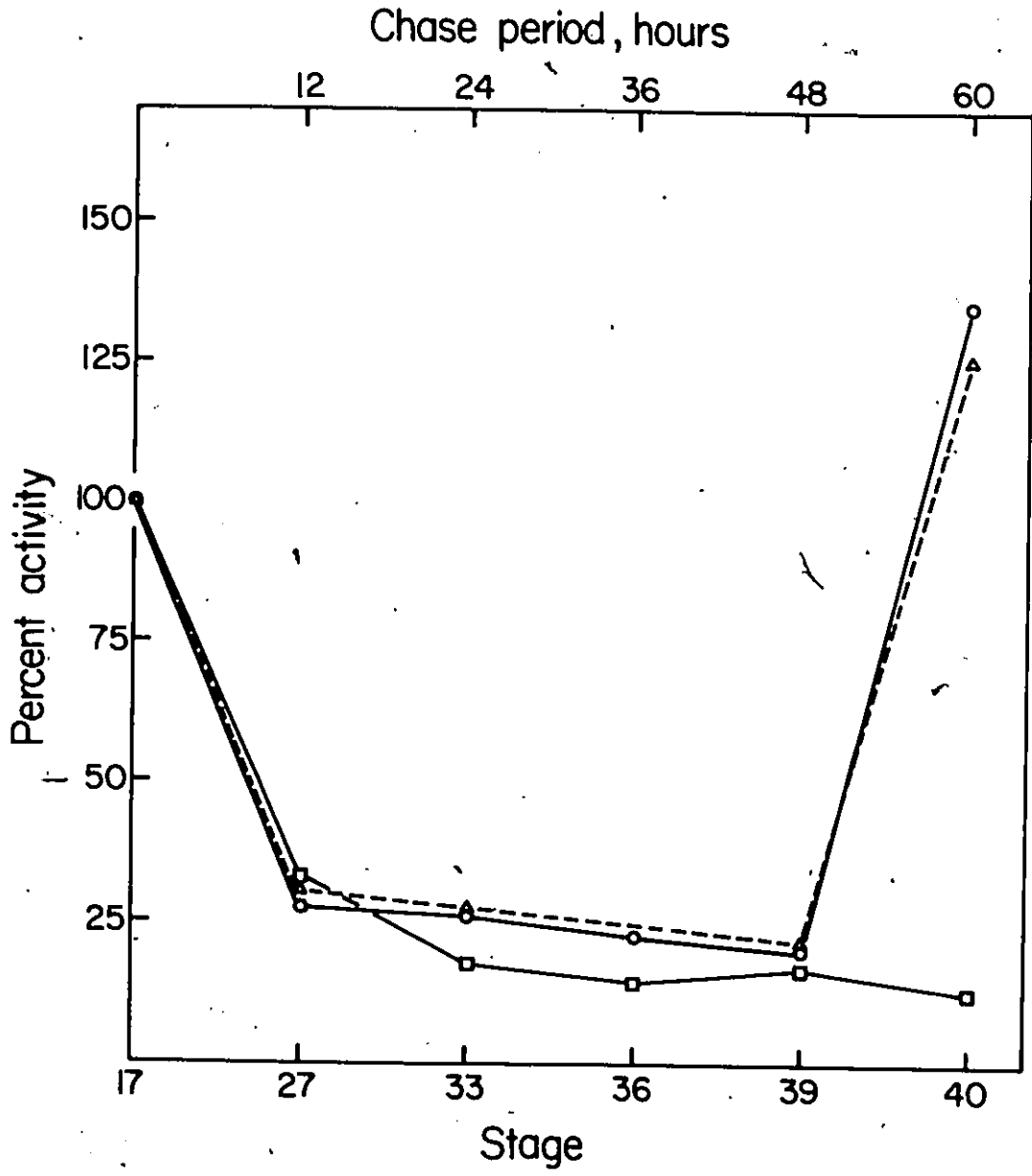
Figure 3. Pulse-chase of dejellied *X. laevis* embryos.

Twelve hour C^{14} -acetate pulse at gastrula followed by 60 hours chase. Results are shown as percent activity taking time 0 to be 100%.

△ fatty acids

○ lipids

□ embryos



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Figure 4. Pulse-chase of hatched *X. laevis* embryos.

Twelve hour C^{14} -acetate pulse at hatching followed by 96 hours chase period. The results are presented as percent activity taking time 0 to be 100%.

△ fatty acids

○ lipids

□ embryos

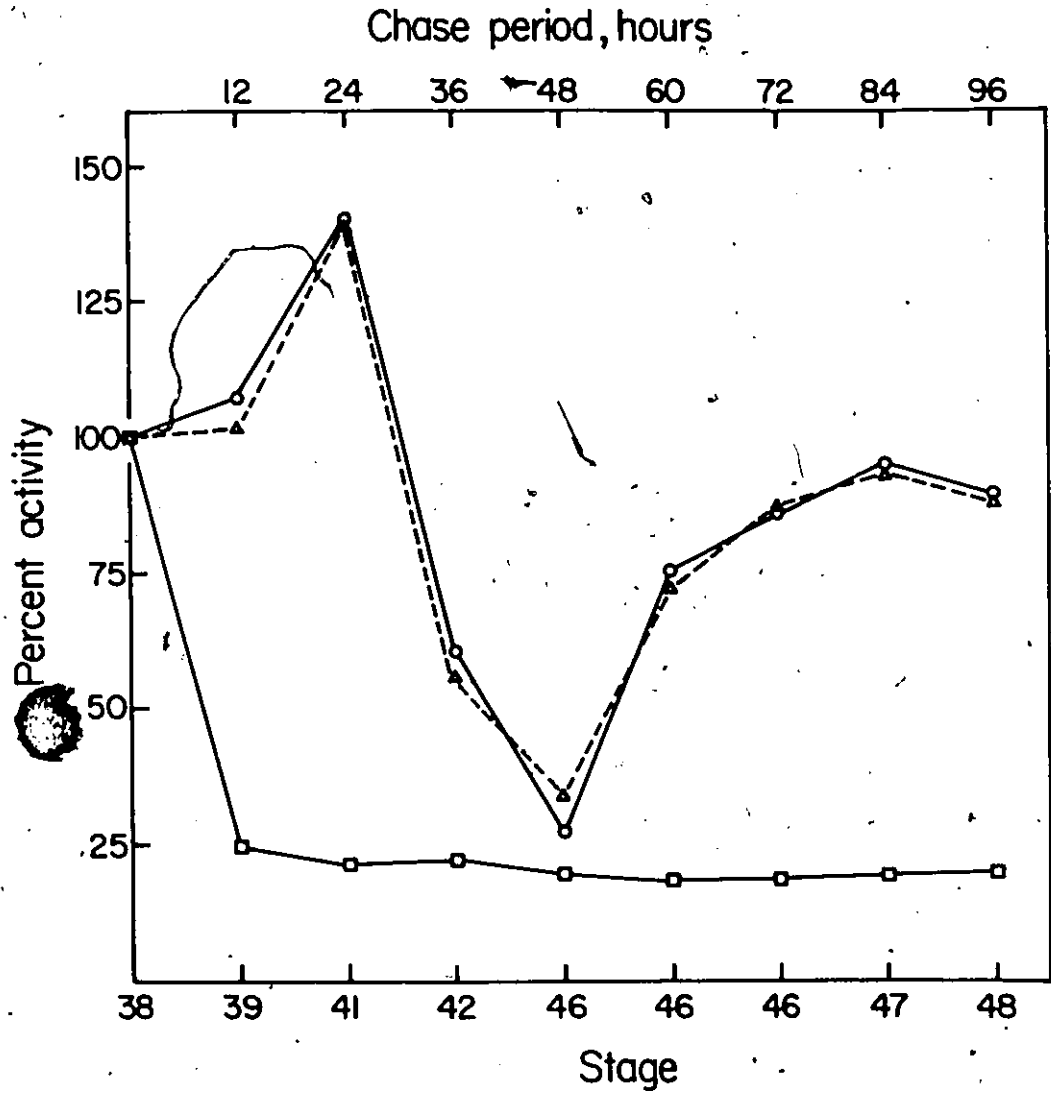


TABLE VIII

Lipid profile of C¹⁴-acetate labelled lipids following a 12 hour pulse at hatching followed by a 96 hour chase period. The results are presented as percent incorporation into individual lipid classes as compared to the total uptake by all lipids.

Chase Period (hrs)	Stage	Total %		Sph	PC	PI+PS	PE
		Neutral	TG				
0	38	25.1	7.5	9.8	9.0	9.8	26.4
12	39	29.3	11.3	7.0	10.4	13.6	18.8
24	41	14.5	12.0	6.9	11.1	9.7	27.1
36	42	32.3	14.6	7.3	11.5	10.6	19.1
48	46	31.9	15.2	9.1	13.4	9.4	14.6
60	46	28	11.4	4.7	11.7	8.3	16.4
72	46	40.9 ²	11.0	4.2	10.7	5.5	10.1
84	47	27.9	11.2	6.1	12.9	9.5	15.6
96	48	23.8	10.9	7.5	18.0	10.3	15.8

- 1 At this stage embryo were fed yeast.
- 2 Most of the radioactivity was present as sterol (26.7%) which was undetectable prior to this stage.

while that in the lipid fraction steadily declined. The lipid profile remained essentially the same throughout the experiment (Table IX).

TABLE IX

Incorporation pattern and lipid profile of *X. laevis* embryos pulsed with C¹⁴-acetate for 12 hours at feeding stage and chased for 48 hours.

Chase period (hrs)	% activity 1			% incorporation 2									
	Stage	Embryo	Lipid acid	Fatty acid	Neutral	St	DG	TG	SE	SPH	PC ^b	PS+PI	PE
0	46	100	100	100	31.5	8.8	2.6	12.7	2.5	7.3	19.9	6.2	18.5
12	46	41.8	81.4	57.4	29.6	10.8	1.6	16.1	3.8	7.8	24.1	9.2	18.1
24	46	41.2	64.6	57.9	38.2	13.6	0.9	10.0	2.1	8.8	15.6	6.5	12.7
36	46	42.2	52.8	75.4	36.1	11.9	1.5	10.0	2.3	9.1	19.2	7.9	15.4
48	47	38.8	60.4	60.0	40.5	12.2	1.3	9.2	2.1	7.9	18.5	7.3	15.3

1 Percent activity is calculated as the loss or gain of counts taking time 0 to equal 100%.

2 Percent incorporation is calculated as the counts in each lipid class as compared to the total counts in the lipid Fraction.

DISCUSSION

Our results for the quantitative analysis agree with other authors (Kutsky 1950, Barassi & Bazan, 1974 a, b, Miceli & Brenner, 1976) in that there is no change in the lipid content or profile from fertilization to hatching. The first evidence of increases in the amount of certain lipids is at stage 40-41, which is just prior to feeding. PC increases from 30 $\mu\text{g}/\text{egg}$ to 63 $\mu\text{g}/\text{egg}$, PE from 14 $\mu\text{g}/\text{egg}$ to 28 $\mu\text{g}/\text{egg}$ and sterol esters from 13 $\mu\text{g}/\text{egg}$ to 25 $\mu\text{g}/\text{egg}$. There was no change in the amount of triglycerides. The lack of biosynthesis of phospholipids before hatching is puzzling since there is a great need, at this time, for phospholipids for membrane formation. This would indicate that the egg already possesses all the lipids for development. Yolk has been implicated as the major storage site of phospholipids (Karasaki, 1963, Follett & Redshaw, 1974) for membranogenesis.

However, Miceli and Brenner (1976) have shown in an in vitro experiment, that palmitic acid is elongated to stearic acid in embryo homogenates. In vivo, they have also shown that both de novo synthesis of fatty acids and desaturation to form myristoleic, palmitoleic and oleic acids occurs in Bufo arena-
rum Hensel embryos. In oocytes they found that 26.9% of the label was incorporated into unsaturated fatty acids, whereas at gastrulation 52.6% of the label was found in the unsaturated fatty acids. Thus in the early stages of embryogenesis there is increased labelling of unsaturated fatty acids. Further work by Pechen D'Angelo (et al) (1977) has shown that PE is the

most highly unsaturated lipid, at ~60% unsaturation, as compared to 55% for PC and 25% for both PI and PS.

When the embryo is labelled with C^{14} -acetate, it is presumed that the acetate goes to acetyl-CoA and is incorporated into fatty acids. The de novo synthesis of saturated fatty acids occurs in the soluble fraction of the cytoplasm by a group of seven enzymes, the fatty acid synthetase complex. Acetyl CoA serves as a primer for the successive additions of acetyl residues from malonyl CoA. The normal end-product of these reactions is the saturated palmitic acid which can be elongated and unsaturated in the mitochondria or in the endoplasmic reticulum (Lehninger, 1972).

These fatty acids can then be incorporated into phosphatic acid which is first converted into 1,2-diacylglycerol, and then in turn utilized in the formation of phospholipids. Acetyl CoA is involved in many other pathways including the tricarboxylic acid cycle, ketone synthesis and cholesterol synthesis; thus C^{14} -acetate is a fairly non-specific label.

Brown (1967) has suggested that the enhancement of labeling seen when embryos remain intact, with their jelly coats, is due to bacterial contamination which readily picks up the label. However, Hampel et al (1975) has shown that this is not the case since ribosome profiles with intact labelled embryos shows that the ribosomal proteins are eukaryotic and not prokaryotic. Other workers have also shown that the intact X. laevis embryos are capable of taking up and incorporating amino acids and nucleosides and that the uptake and retention

is dependent upon the presence of the jelly coat (Knutson & Prahlad, 1971, Robinson et al, 1972). The present work shows a similar enhancement of uptake with jelly coats. However, if eggs are labelled intact and dejellied just prior to analyses, then the level of labelling is similar to embryos which have been labelled without their jelly coats. This suggests that the jelly itself incorporates label. Tests show that this is true but that the profile of uptake of the lipids is not that of intact embryos. A clue to the role of the jelly has been provided by recent work in our lab (Mieke Mes, personal communication) which shows that when embryos are labelled intact and then dejellied either manually or chemically, and the lipids analysed, there is twice as much label in the manually dejellied eggs as in the chemically dejellied eggs. Chemical dejelling is quite a drastic procedure that exposes the egg surface to harsh treatment as opposed to manual dejelling. Thus it appears that the jelly may somehow help to retain the incorporated label. If this is true then the jelly has a far more important role than one of a physical barrier to disease and injury.

Our results with a C^{14} -acetate pulse labelling shows that incorporation into lipids was steady at about 10%. The most notable feature of the pulse labelling experiment is that PE, which is not the major phospholipid, has the highest label associated with it. Does this incorporation of C^{14} -acetate reflect de novo synthesis or chain elongation of fatty acids? If the acetyl- C^{14} -CoA supplies all the carbon atoms, then the

label will be alternately distributed throughout the fatty acid chain of the product formed by the de novo synthesis, whereas if elongation is occurring then the label would be asymmetrically distributed in the fatty acid chain with the label concentrated at the carboxyl end. This would provide a means of verifying whether we are incorporating label by de novo synthesis or by elongation.

The percentage of label in fatty acids is 80-90 up to hatching and declines after this time to 44. The distribution of label within the fatty acid chain has not been determined. The decline in fatty acid labelling after hatching could be due to an increase in synthesis of non-saponifiables. Evidence for this is the increase in labelling of sterols from 2% before hatching to 4-5% after hatching. Although sterols cannot totally explain the decrease in fatty acid labelling probably other lipids in the same class as sterols are being synthesized.

The high level of unsaturation reported for PE (Pechen D'Angelo et al, 1977) could explain why it is so highly labelled in our case. The highest level of PE labelling corresponds to the same stage as the high labelling of unsaturates reported by Miceli and Brenner (1976). Thus the high labelling of PE may reflect transacylation necessary to achieve the correct degree of unsaturation required for the physico-chemical attributes of the membranes. If this is the case then one has to explain the lack of labelling in PC, which is also highly unsaturated. Data obtained by a variety of methods show that in eukaryotic cells the choline containing lipids are preferenti-

ally localized in the outer leaflet of the bilayer and that PE is also asymmetrically distributed with about 70 to 80% in the inner leaflet (Bretcher, 1973, Van Deenan et al, 1976). When membrane biogenesis was studied with isotopic lipids, labelled fatty acids from the cytoplasmic pool were incorporated mainly into the inner leaflet (Renooij et al, 1976). It seems likely that PE, which is in the inner leaflet, is more accessible to transacylation, and this could partly explain the high amount of label in PE.

In the pulse-chase experiment (Fig 3-4) the label in lipid and fatty acids shows a dramatic increase at stage 40. It may seem unusual that the label goes up in a pulse-chase experiment, but this is probably due to mobilization of stored material and a shunting of the acetyl CoA towards fatty acids biosynthesis. The decrease in labelling once feeding begins (Fig. 4) probably reflects the ingestion of unlabelled precursors which go directly to lipid biosynthesis. The increase in the next 48 hours of the chase is difficult to explain but could only reflect more reserved acetate being mobilized.

The evidence seems fairly strong in support of the hypothesis that de novo lipid synthesis begins just prior to feeding at stage 41. The rise in the pulse-chase experiments support this, as do the results of the dichromate experiment where there is a marked rise in the amount of PC, PE and SE. The lipid profile (Table V) of the pulse experiment also supports the fact that some major event is occurring at this time, since the profile changes drastically at this stage. The pro-

file at stage 40 and 41 is similar, although not identical, to the profile on Table IX, which should reflect de novo synthesis since embryos were labelled and fed simultaneously. Thus de novo synthesis only begins to an appreciable extent some 12-24 hours before feeding. The lack of appreciable lipid biosynthesis during embryonic development is easier to understand if one views phospholipids not as macromolecules but as building blocks for membranogenesis (Pasternak, 1977). As such the lack of change in these building blocks is due to the ample storage of these in yolk.

APPENDIX 1

R_F values of polar lipids in various solvent systems on TLC.

Lipid class	R _F values in solvent							
	1	2	3	4	5	6	7	8
Sphingomyelin	.17	-	.09	-	.23	.19	.23	-
Phosphatidyl choline	.30	-	.23	.04	.38	.35	.34	.13
Phosphatidyl ethanolamine	.72	.14	.69	.22	.57	.46	.46	.49
Phosphatidyl inositol	.51	-	.54	-	.26	.16	-	-
Phosphatidyl serine	.51	-	-	-	-	-	-	-
Lysophosphatidyl choline	.11	-	-	-	-	-	-	-
Lysophosphatidyl ethanolamine	-	-	-	-	.30	.22- .25	.23	-

- Solvent 1 chloroform-methanol-acetic acid-water (75:45:12:4)
 2 chloroform-methanol-acetic acid-water (80:13:8:0.3)
 3 chloroform-methanol-acetic acid-water (65:43:1:3)
 4 chloroform-methanol-acetic acid-water (80:26:4:0.3)
 5 chloroform-methanol-water (65:25:4)
 6 chloroform-methanol-ammonia (70:30:5)
 7 chloroform-methanol-ammonia (65:25:5)
 8 chloroform-methanol-ammonia (60:50:2.5)

APPENDIX 2

R_F values of neutral lipid classes
in various solvent systems on TLC

Lipid class	R _F value in solvent					
	1	2	3	4	5	6
Monoglycerides						.16
Diglycerides		.31	.10, .21		.45	.52
Triglycerides	.69	.57	.47	.27	.74	.70
Sterols	.15	.20	.15	.09	.40	.41
Sterol esters	.84	.74	.71	.90	.80	.81

- Solvent 1 Petroleum ether-diethyl ether-ammonium (75:25:1)
 2 Heptane-isopropyl ether-acetic acid (60:40:4)
 3 Hexane-diethyl ether-acetic acid (80:20:1)
 4 Petroleum ether-ethyl ether-acetic acid (90:10:1)
 5 Isopropyl ether-acetic acid (96:4) then
 Petroleum ether-ethyl ether-acetic acid (90:10:1)
 6 Ethyl ether-benzene-ethanol-acetic acid (40:50:2:0.2)
 then ethyl ether-hexane (6:94)

APPENDIX 3

Brief description of stages commonly referred to in the text (drawn from the staging tables of Niewkoop and Faber (1956)).

Stage number	Length (mm)	External Criteria
4	1.4 - 1.5	four-cell egg
10	1.4 - 1.5	early yolk-plug
15	1.5 - 1.6	early neural fold
17	1.5 - 1.6	late neural fold
25	2.8 - 3.0	beginning of fin formation
27	3.4 - 3.7	fin translucent except for region behind anus
33/34	4.7 - 5.3	beginning of heartbeat
35/36	5.3 - 6.0	hatching
37/38	5.6 - 6.2	gill rudiments nipple shaped
39	5.9 - 6.5	outline of proctodeum and tail myotome forming angle at 135°
40	6.3 - 6.8	mouth broken through
41	6.7 - 7.5	gills broad and flat
42	7.0 - 7.7	beginning of opercular folds
46	9 - 12	hindlimb bud visible
47	12 - 15	hindlimb bud distinct
48	14 - 17	forelimb bud visible, gold-coloured abdomen

REFERENCES

- Barassi, C.A. and Bazan, N.G. 1974 a). Fatty acid distribution in lipids and ^{32}P incorporation into phospholipids during early amphibian development. *Lipids*. 9: 27-34.
- Barassi, C.A. and Bazan, N.G. 1974 b). Metabolic heterogeneity of phosphoglyceride classes and sub-fractions during cell cleavage and early embryogenesis: model for cell membrane biogenesis. *J. Cell Physiol.* 84: 101-114.
- Bretcher, M.S. 1973. Membrane structure: some general principles. *Science*. 181: 622-629.
- Brown, D.D. 1967. Nucleic acid determinations in embryos. In Methods in Developmental Biology, Wilt and Wessells (Eds). T.Y. Crowell, New York, pp. 685-701.
- Cohen, S. 1954. The metabolism of C^{14}O_2 during amphibian development. *J. Biol. Chem.* 211: 237-354.
- Crupkin, M., Barassi, C.A. and Bazan, N.G. 1973. Incorporation of ^{32}P during early amphibian embryogenesis. *Comp. Biochem. Physiol.* 456: 523-528.
- Dittmer, J.C. and Lester, R.L. 1964. A simple, specific spray for the detection of phospholipids on thin-layer chromatograms. *J. Lipid Res.* 5: 126-127.
- Dittmer, J.C. and Wells, M.A. 1969. Quantitative and qualitative analysis of lipids and lipid components. In Methods in Enzymology. Academic Press, New York. Vol. 14: 482-530.

- Follett, B.K. and Redshaw, M.R. 1974. The physiology of vitellogenesis. In Physiology of Amphibian II, Lofts (Ed.). Academic Press, New York. 219-298.
- Freeman, C.P. and West, D. 1966. Complete separation of lipid classes on a single thin-layer plate. J. Lipid Res. 7: 324-327.
- Hampel, A., Prahlad, K.V., Brei, S.T., Ruefer, B. and Mzot, C. 1975. Permeability of Xenopus laevis embryos: specific incorporation of precursors into eukaryotic proteins and nucleic acids. Dev. Biol. 44: 169-177.
- Holtfreter, J. 1946. Experiments on the formed inclusions of the amphibian egg. J. Expt. Zool. 101: 355-405.
- Karasaki, S. 1963. Studies on amphibian yolk. J. Ultra. Res. 9: 225-247.
- Kates, M. 1964. Simplified procedures for hydrolysis or methanolysis of lipids. J. Lipid Res. 5: 132-135.
- Kates, M. 1972. Techniques of Lipidology. North Holland Publishing Co. Amsterdam.
- Knutson, T.L. and Prahlad, K.V. 1971. 3,5,3-triiodo-L-thyronine uptake and development of metamorphic competence by Xenopus laevis embryonic tissues. J. Expt. Zool. 178: 45-58.
- Kutsky, P.B. 1950. Phosphate metabolism in the early development of Rana pipiens. J. Expt. Zool. 115: 429-460.
- Lehninger, A.L. 1972. Biochemistry. Worth Publishers. New York.

- Miceli, D.C. and Brenner, R.R. 1976. Fatty acid biosynthesis during embryogenesis in the amphibian Bufo Arenarum hensel. Lipids. 11: 291-295.
- Morrill, G.A. and Kostellow, A.B. 1964. Phospholipid and nucleic acid gradients in the developing amphibian embryo. J. Cell. Biol. 25: 21-29.
- Niewkoop, P.D. and Faber, J. 1956. Normal Tables of Xenopus laevis (Daudin). North-Holland Publishing Co. Amsterdam.
- Pasternak, C.A. 1977. Lipid changes in membranes during growth and development. In Lipid Metabolism in Mammals, Snyder (Ed.). Plenum Press. Vol. 2: 353-383.
- Pechen, A.M. and Bazan, N.G. 1974. Membrane ³²P-phospholipid labelling in early developing toad embryos. Expt. Cell. Res. 88: 432-435.
- Pechen, A.M. and Bazan, N.G. 1977. Lipid metabolism in early development using labelled precursors incorporated during oogenesis and in cell-free embryo homogenates. Lipids. 12: 131-134.
- Pechen d'Angelo, A.M., Bonini de Rommelli, I.C., Alonso, T.S. and Bazan, N.G. 1977. Composition and metabolism of phospholipids during early stages of vertebrate embryonic development. In Function and Biosynthesis of Lipids, Bazan, Brenner and Giusto (Eds). Plenum Press, New York. 249-256.
- Rennoij, W., Van Golde, L.M.G., Zwaal, R.F.A., Van Deenan, L.L.M. 1976. Topological asymmetry of phospholipid metabolism in rat erythrocyte membranes. Eur. J. Biochem. 61: 53-58.

- Robinson, D., Prahlad, K.V., and Hampel, A.E. 1972. Amino acid uptake by Xenopus laevis embryos: effect of triiodo-L-thyronine. Comp. Biochem. Physiol. 43: 749-754.
- Skipski, V.P., Peterson, R.F. and Barclay, M. 1962. Separation of phosphatidyl ethanolamine, phosphatidyl serine, and other phospholipids by thin layer chromatography. J. Lipid Res. 3: 467-470.
- Trottier, T.M. and Armstrong, J.B. 1975. Hormonal stimulation as an aid to artificial insemination in Ambystoma mexicanum. Can. Jour. Zool. 53: 171-173.
- Van Deenan, L.L.M., de Grier, J., van Golde, L.M.G., Nauta, I.L.D., Renooij, W., Verklei, A.J. and Zwaal, R.S.A., 1976. Some topological and dynamic aspects of lipids in the erythrocyte membrane. In Structure of Biological Membranes, Abrahamson and Pascher (Eds). Plenum Press, New York. 107-118.
- Wallace, R.A. 1963. Studies on amphibian yolk III. A resolution of yolk platelet components. B.B.A. 74: 495-504.
- Williams, J. 1965. Chemical constitution and metabolic activities of animal eggs. In The Biochemistry of Animal Development Vol. I. Weber (Ed.) Academic Press, New York.
- Wagner, H., Hörhammer, L. and Wolff, P. 1961. Dünnschichtchromatographie von phosphatidien und glykolipiden. Biochem Z. 334: 175-184.
- White, D.C. and Frerman, F.E., 1967. Extraction, characterization and cellular localization of the lipids of Staphylococcus aureus. J. Bacteriol. 94: 1854-1867.

Wischnitzer, S. 1964. Ultrastructure changes in the cytoplasm of developing amphibian oocytes. J. Ultra. Res. 10: 14-26.