CHAPTER - IV

LIPID METABOLISM IN EMBRYONIC STAGES OF *CYPRINUS CARPIO*

INTRODUCTION

Very little is known of the biochemical morphology of the egg (Nakagawa and Tsuchiya, 1972). Nakagawa and Tsuchiya (1969, '71, '72) described 3 different fractions of lipids in rainbow trout eggs as a result of centrifugation, namely, 1, the oil globule (rich in carotenoids; phosphorus absent; cholesterol esters and higher fatty acids); 2, LDF (triglycerides; cholesterol; cholesterol esters; higher fatty acids; phospholipids and traces of carotenoids) and 3, HDF (50% of which are phospholipids, constituted of lecithin, cephalin, sphingomyelin and lysolecithin; triglycerides; cholesterol and its esters and free fatty acids). LDF and HDF

fractions of the lipoproteins together constituted the yolk globule. The lipoproteins of the yolk globule contained 22.8% of lipids. The oil globule differed from yolk globule in the absence of phospholipids and in having lipids in free form. Yolk globule, on the other hand, had all its lipid in bound form and contained substantial amounts of phospholipids.

Nakagawa and Tsuchiya (1972, '76) did not find any change in oil globule either in lipid composition or in fatty acid composition. They, however, reported increase in oil globule fraction during embryonic development owing to addition of carotenoids. They did not find any difference in the absolute amount of triglycerides per egg yolk during the embryonic period. While Williams (1967) and Terner et al (1968) considered that the embryo absorbed triglycerides as such, Nakagawa and Tsuchiya (1976) opined that transport mechanisms of yolk lipids to embryo were very complicated.

Nakagawa and Tsuchiya (1976) observed further that only bound lipid of HDF decreased rapidly during embryonic stages leading to great changes in fatty acid composition. However, the kinds of lipid protein complexes in HDF, namely, lipoprotein, glycoprotein and phosphoprotein underwent proportionate quantitative losses maintaining their initial ratio of 83: 11: 5.

Earlier, Hayes (1949) implicated 75% of lipid in Salmon egg to supply energy needs of embryo. Terner (1968b) opined that lipids were the principal endogenous reserves for continuous supply of metabolic energy in telolecithal eggs. In addition to supplying fatty acids for oxidation, and thus sparing carbohydrate reserve, the glycolipids on lipolysis were said to liberate glycerol, which then could be converted, through enzymatic action of fructose diphosphatase and glucose-6-phosphatase, ten times more readily than pyruvate, into glucose. This kind of gluconeogenesis (from glycerol) served admirably as replacement of carbohydrates utilized as energy source. Terner (1968b) considered the store of glycerolipids very important in telolecithal eggs, not supplied with external carbohydrate. Carbohydrates seemed to be the main source of energy for only a brief period at the time of gastrulation (Daniel, 1947; Nishida & Nakano, 1954).

Ohman (1944) observed decrease in total lipids of sea urchin eggs during the first seven hours after fertilization. Monroy (1956, '57) had attributed to lipids the property of activation of the egg. He conceived of splitting of lipoprotein complex of the egg at fertilization following sperm penetration, liberating phospholipids which were

then degraded to lysophosphatides which eventually caused egg activation.

Pasternak (1973) suggested stimulation of phospholipid synthesis at fertilization for assembly of new membranes at cleavage and that this was sensitive to inhibition of DNA synthesis. Kim (1976) described depletion of phospholipids and cholesterol in *Cyprinus carpio* with onset of cleavage, and while cholesterol levels stabilized at mid gastrula, phospholipid levels could be stabilized only at eyed up stage.

Recent work clearly establishes the importance of lipids, more so of phospholipids, in embryonic development of teleostean eggs. Work on lipid metabolism during embryonic development has so far been done in detail only in trout *Salmo gairdneri irideus* (Ando, 1962; Terner, 1968$_{a+b}$;Terner et al, 1968; Nakagawa and Tsuchiya, 1969, '71, '72, '76). This is far from adequate. The present author has studied lipid metabolism, including total lipids, total triglycerides, cholesterol and phospholipid levels in different embryonic stages of *Cyprinus carpio* in addition to activities of certain enzymes related to lipid metabolism and has tried to assess the role of lipids in energy metabolism during embryonic development of *Cyprinus carpio*.

METHODS

TOTAL LIPIDS:

Lyophilized samples of different embryonic stages were weighed accurately; homogenized in chloroform-methanol (2:1) mixture and proceeded for the estimation of total lipids by the method as described by Mishra (1968).

PRINCIPLE:

Lipids were extracted and dissolved in pure lipid solvents, purified and estimated gravimetrically using a dry thimble.

REAGENTS:

1. Chloroform methanol mixture (2:1 v/v): 200 ml of pure chloroform added to 100 ml of pure methanol.
2. Chloroform-methanol-water mixture: 96 ml of solution (1) mixed with 4 ml of distilled water.
3. Normal saline (0.9%): 90 mg of anhydrous sodium chloride (NaCl) dissolved in 100 ml of distilled water.

Weighed eggs were homogenized in chloroform-methanol (2:1) mixture (1). After temperature equilibration and final volume adjustment (10 ml) the

homogenate was filtered through a fat free filter paper into a glass stoppered tube. Residue was re-extracted with 10 more volumes of chloroform-methanol mixture (1) for 2 hours and filtered. Filtrate was evaporated to dryness in vacuum at 45°C in rotatory evaporator; the dried residue was dissolved in chloroform-methanol-water mixture (2) to liberate protein bound lipids and evaporated as before. Dried residue was redissolved in chloroform-methanol mixture (1) and layered with 1/5th volume of normal saline (3) in a separating funnel; the two were mixed gently and allowed to stand at room temperature until the two phases separated clearly. Chloroform phase (lower) was collected and evaporated to dryness at 40° to 50°C.

Dried residue was dissolved in small volume of chloroform and transferred to accurately weighed glass thimble. Chloroform was evaporated completely and the extract dried at 105°C to constant weight.

The difference between the initial and final weights of the thimble gave the weight of total lipids, which was expressed as mg lipids/100 mg dry weight of eggs.

CHOLESTEROL:

Cholesterol was estimated from the total lipid extracts of each embryonic stage by the method

of Kenny (1952) as modified by Plummer (1971).

PRINCIPLE:

Acetic anhydride, in the presence of concentrated sulphuric acid, reacts with cholesterol in a chloroform solution to produce a characteristic blue green colour. In this reaction, known as Liebermann-Burchard reaction, coloured complex is formed possibly due to esterification of hydroxyl group at 3 position, as well as to other rearrangements in the molecule.

REAGENTS:

1. Chloroform: Highly pure grade chloroform was used.
2. Acetic anhydride-sulphuric acid mixture(30:1): 30 ml of acetic anhydride mixed carefully with 1 ml of concentrated sulphuric acid. This reagent was prepared just before use.
3. Standard cholesterol solution: 20 mg of cholesterol dissolved in 10 ml of chloroform in a volumetric flask. Stored in cold.
4. Working cholesterol standard solution: 1 ml of standard cholesterol solution (3) diluted to 10 ml with chloroform. 1 ml of this solution gave 0.2 mg cholesterol. This solution was prepared just before use.

PROCEDURE:

Dry and clean glassware were used for cholesterol estimation. Total extracted lipids were dissolved in chloroform and diluted to a known volume in a volumetric flask; this served as an aliquot of total lipids. 2 ml of aliquot of each sample was taken in 10 ml volumetric flask. A standard series containing different concentrations of cholesterol (4) and a blank containing 2 ml of chloroform were also taken in 10 ml volumetric flasks. 2 ml of acetic-anhydride:sulphuric acid mixture (2) was added to each of them, mixed thoroughly and kept immediately in dark. Volumes were made to 10 ml with chloroform and optical densities measured against blank at 680 nm in Zeiss Spekol.

Cholesterol content was calculated with reference to the standard and expressed in terms of mg/100 mg dry weight.

PHOSPHOLIPIDS:

Phospholipids were estimated from the total lipid extracts of each embryonic stage, by the method of Youngburgs (1930) as described by Oser (1965) in terms of total lipid phosphorus.

PRINCIPLE:

The extracted lipids are oxidized with sulphuric acid and hydrogen peroxide; phosphate released is determined colorimetrically by the method of Fiske and Subbarao (1925).

REAGENTS:

1. 10% Trichloroacetic acid: 10 g of TCA dissolved in 100 ml of distilled water.

2. Aminonaptholsulphonic acid reagent: 97.5 ml of 15% sodium bisulphite solution taken in a glass stoppered measuring cylinder; 0.25 g of 1,2,4-aminonaphthol sulphonic acid and 2.5 ml of 20% sodium sulphite added to it; the whole was shaken until the powder dissolved and then transferred to a brown glass bottle and stored in cold.

3. Standard phosphate solution: Accurately weighed 0.357 g of pure dry monopotassium phosphate dissolved in distilled water in a 1 litre volumetric flask, 10 ml of 10 N H_2SO_4 was added; the whole was diluted to 1 litre mark with distilled water. This solution gave 0.4 mg phosphate in 5 ml.

4. Ammonium molybdate solution: 5 g of ammonium molybdate dissolved in 40 ml of distilled

water; 60 ml of 10 N H_2SO_4 added to it and the whole diluted to 200 ml with distilled water.

5. 10 N sulphuric acid: 450 ml of concentrated H_2SO_4 added to 1300 ml of distilled water.

6. 5 N sulphuric acid: 10 N sulphuric acid (5) diluted with equal volume of distilled water.

7. 30% hydrogen peroxide: Highly pure and phosphate free hydrogen peroxide was used.

8. Alcohol ether mixture: 3 volumes of 95% ethanol mixed with 1 volume of pure ether.

PROCEDURE:

1 ml of the aliquot of total lipids (dissolved in chloroform) was transferred to a wide mouth graduated glass stoppered tube containing 18 ml of alcohol:ether mixture; solutions were mixed, boiled and subsequently cooled; total volume made to 20 ml with alcohol ether mixture.

10 ml of the above solution was transferred to a pyrex glass tube containing a dry silica pebble; the solution was evaporated to dryness; 2.5 ml of 5 N H_2SO_4 added to the residue and heated until the contents became brown or black; a drop of hydrogen peroxide was added to the digestion mixture and

continued heating until the contents became colourless; a few drops of water added and boiled for a few moments. Contents were then cooled and transferred to 25 ml volumetric flask, washing the tube repeatedly with distilled water. Blank (2 ml of 10% TCA) and standard (0.5 ml of solution (3) and 2 ml of 10% TCA) solutions were also processed exactly as the test solution.

2.5 ml of ammonium molybdate solution and 1 ml of aminonaphthol sulphonic acid reagent were added, while shaking, to each of the above flasks and volume made to 25 ml with distilled water. Optical densities were determined against blank at 660 nm in spekol. Total lipid phosphorus was expressed in terms of mg/100 mg dry weight.

TRIGLYCERIDES:

Triglycerides were estimated from the total lipid extracts of each embryonic stage by the method of Laurell (1966), as described by Wootton (1974).

PRINCIPLE:

Triglycerides are converted to glycerol, following hydrolysis with sulphuric acid; glycerol is then oxidized to formaldehyde, which is measured colorimetrically after treating with chromotropic acid. Phospholipids and glucose may interfere by

producing their own glycerol, hence silicic acid is used to absorb these compounds.

REAGENTS:

1. Silicic acid (100 mesh): Activated silicic acid (by heating at 120°C for two hours).

2. 33% Potassium hydroxide: 33 g of KOH pellets dissolved in 100 ml of distilled water.

3. Alcoholic potassium hydroxide: 1 ml of 33% KOH mixed with 19 ml of absolute alcohol just before use.

4. Sulphuric acid (0.6 N): 17 ml of concentrated sulphuric acid diluted to 1 litre with distilled water.

5. Potassium metaperiodate (0.02 M): 42 mg of potassium metaperiodate dissolved in 10 ml of distilled water.

6. Sodium arsenite (0.2 M): 260 mg of sodium arsenite dissolved in 10 ml of distilled water.

7. Chromotropic acid (4,5-dihydroxy-2,7 naphthalene disulphonic acid): 300 ml of concentrated sulphuric acid cautiously added to 150 ml of distilled water; 1 g of chromotropic acid was dissolved in 100 ml of water; sulphuric

acid solution was slowly added to the chromotropic acid solution and stored at 4°C in a brown bottle.

8. <u>Triglyceride stock standard</u>: 200 mg of highly pure tripalmitin dissolved in 100 ml of isopropyl ether.

9. <u>Triglyceride working standard</u>: 1 ml of stock standard (8) diluted to 50 ml with extraction mixture. Prepared fresh.

10. <u>Extraction mixture</u>: Distilled isopropyl ether was stored over KOH pellets at 4°C; mixed 95 ml of absolute ethanol; prepared fresh before use.

<u>PROCEDURE</u>:

Dry and clean glass stoppered test tubes were used for the estimation of triglycerides.

<u>Test</u>: 0.5 g of silicic acid and 1 ml of extraction mixture (10) taken in a tube; 1 ml of total lipid extract and 5 ml of extraction mixture were added subsequently in that order; tube stoppered firmly and shaken vigorously for 30 seconds and intermittently thereafter for 30 minutes. Tubes were allowed to stand for one hour.

<u>Standard</u>: 0.5 g of silicic acid, 1 ml of extraction mixture, 1ml of water and 5 ml of working

triglyceride solution (9) were taken and mixed in a stoppered tube.

Blank: 0.5 g of silicic acid, 1 ml of water and 6 ml of extraction mixture were mixed in a stoppered test tube.

4 ml of supernatant from each tube were transferred to another set of tubes; 0.6 ml of alcoholic KOH solution was then added to each tube, stoppered, mixed and heated at 55°C for 15 minutes to saponify the triglycerides; cooled and 1.5 ml of 0.6 N sulphuric acid added; stoppered again, mixed and allowed to stand for complete separation of the phases.

0.5 ml of lower fraction (containing glycerol) was then transferred to another set of clean tubes; 0.2 ml of potassium metaperiodate solution was then added to each and mixed; 2 ml of sodium arsenite solution was then added to each tube after 10 minutes and, 2 to 4 minutes later, 3 ml of chromotropic acid reagent was added to each and mixed; tubes were stoppered and heated in a boiling water bath for 30 minutes. Care was taken not to expose the tubes following heating to bright sunlight; they were then cooled. Optical densities were read against blank at 570 nm in a spekol.

Triglyceride content was expressed in terms of mg/100 mg dry weight of eggs.

LIPASE (E.C. 3.1.1.3)

Fresh intact eggs of each embryonic stage were homogenized in icecold 0.25 M sucrose solution. The homogenate was kept throughout at 0° to 4°C and centrifuged at 3000 rpm for 15 minutes. The supernatants were used to determine lipase activity by the titrimetric method of Cherry and Crandall (1932) as described by Oser (1965).

PRINCIPLE:

The crude enzyme extract, on incubation with olive oil emulsion, liberates fatty acids. The fatty acids so liberated over a unit period of time are estimated by the titration with sodium hydroxide solution.

REAGENTS:

1. 5% gum acacia: 5 g of gum acacia was suspended in 100 ml of warm distilled water containing 0.2 g sodium benzoate. The contents were mixed and allowed to stand overnight.
2. Olive oil emulsion: Equal parts of pure olive oil and 5% gum acacia containing 0.2% sodium benzoate were mixed thoroughly and stored in refrigerator.

3. Buffer solution: 4.7 g of anhydrous disodium hydrogen phosphate (Na_2HPO_4) and 1.4 g of monopotassium hydrogen phosphate (KH_2PO_4) were dissolved in 100 ml of distilled water.

4. 1% Phenolphthalein: 1 g of phenolphthalein dissolved in 100 ml of 95% alcohol.

5. 0.05 N Sodium hydroxide: 40 g of NaOH was dissolved and diluted to 1 litre with distilled water. This solution was cooled and allowed to stand for two days. This gave 1 N NaOH, which was standardized against the acid of known strength. 5 ml of this solution when diluted to 100 ml gave 0.05 N NaOH.

PROCEDURE:

Control and test samples were prepared as follows:

Control: 3 ml of distilled water and 1 ml of crude enzyme extract were taken in a test tube and placed in boiling waterbath for 5 minutes to inactivate the enzyme. 0.5 ml of buffer solution (3) and 2 ml of olive oil emulsion (2) were added and the contents incubated at $37^{o} \pm 1^{o}C$ for 24 hours.

Test: 0.5 ml of buffer and 2 ml of olive oil emulsion were added to test tubes containing

3 ml of distilled water and 1 ml of enzyme extract. Tubes were shaken thoroughly and incubated at $37^{\circ} \pm 1^{\circ}C$ for 24 hours.

At the end of incubation period, 3 ml of 95% ethanol was added to each of the control and test samples to stop the reaction. 2 drops of 1% phenolphthalein (4) were added, mixed well and the contents titrated with 0.05 N NaOH till a permanent pink colour was obtained.

Lipase activity in the extract was calculated by the following equation:

ml of NaOH used for the test sample	-	ml of NaOH used for the control sample	=	Lipase activity in lipase units per ml of extract

The activity was expressed as units/mg protein/24 hours. Protein content of the extract was measured by the method of Lowry et al, as described earlier (p. 34).

RESULTS

Total lipids were found to deplete steadily after fertilization, until late morula stage, rose to near initial level at blastula and again depleted gradually at gastrula but sharply at closing of blastopore; the level increased again at comma stage and thereafter fluctuated in a narrow range (Table 15; Fig. 37).

TABLE - 12

Total lipids (mg/100 mg dry weight) in unfertilized egg and in different embryonic stages of *Cyprinus carpio*

S.No.	Stages									
	Unfertilized egg	Blastodisc	Early morula	Late morula	Blastula	Gastrula	Closing of blastopore	Comma	Eyed	Prior to hatching
1	19.84	18.36	14.96	13.12	20.32	18.00	13.84	15.52	16.16	16.08
2	19.12	17.76	15.80	13.76	19.20	18.24	13.04	15.75	16.08	16.88
3	19.10	18.50	15.92	14.56	20.00	18.40	13.12	15.84	13.84	16.32
4	20.40	-	18.16	17.52	18.96	17.44	12.48	15.84	14.08	15.44
5	20.16	-	19.12	18.16	17.68	17.36	12.02	15.92	14.16	15.52
6	20.14	-	19.28	18.64	18.40	-	-	15.96	14.88	15.84
Mean ± SD	19.79 ±0.56	18.21 ±0.59	17.37 ±1.78	15.96 ±2.42	19.09 ±0.98	17.89 ±0.47	12.90 ±0.69	15.80 ±0.16	14.87 ±1.05	16.01 ±0.54

TABLE - 16

Lipase activity (units/mg protein/24 hrs) in unfertilized eggs and in different embryonic stages of *Cyprinus carpio*

S.No.	Stages									
	Unfertilized egg	Blastodisc	Early morula	Late morula	Blastula	Gastrula	Closing of blastopore	Comma	Eyed	Prior to hatching
1	0.026	-	-	-	0.054	0.061	0.150	0.156	0.191	0.089
2	0.028	0.122	0.090	0.100	0.057	0.077	0.150	0.156	0.161	0.101
3	0.028	0.113	0.030	0.095	0.057	0.046	0.150	0.160	0.191	0.119
4	0.034	0.209	0.030	0.072	0.028	0.077	0.114	0.165	0.161	0.134
5	0.036	-	0.151	0.043	0.067	0.077	0.114	0.165	0.161	0.134
Mean ± SD	0.035 ±0.01	0.148 ±0.05	0.075 ±0.05	0.078 ±0.02	0.057 ±0.01	0.066 ±0.01	0.136 ±0.02	0.162 ±0.004	0.173 ±0.01	0.115 ±0.02

TABLE - 17

Total phospholipids (mg/100 mg dry weight) in unfertilized eggs and in different embryonic stages of <u>Cyprinus carpio</u>

S.No.	Stages									
	Unfertilized egg	Blastodisc	Early morula	Late morula	Blastula	Gastrula	Closing of blastopore	Comma	Eyed	Prior to hatching
1	9.64	4.95	4.09	1.90	1.75	3.35	3.59	5.42	4.96	4.53
2	9.34	5.11	3.50	2.04	1.90	3.50	3.80	5.72	5.11	4.38
3	9.49	5.26	3.94	2.19	1.61	3.65	3.94	5.57	5.25	4.67
4	9.78	5.40	4.23	2.34	2.04	3.79	3.55	6.30	5.40	4.82
Mean ± SD	9.56 ±0.19	5.18 ±0.19	3.94 ±0.32	2.12 ±0.19	1.82 ±0.18	3.57 ±0.13	3.72 ±0.19	5.64 ±0.19	5.18 ±0.19	4.60 ±0.19

TABLE - 18

Percentage fractions of phospholipids, triglycerides and cholesterol in total lipids of unfertilized eggs and in different embryonic stages of <u>Cyprinus carpio</u>

	Stages	Phospholipids	Triglycerides	Cholesterol
I	Unfertilized egg	48.30	12.07	14.80
II	Blastodisc	26.44	14.60	17.95
III	Early morula	22.68	13.76	14.96
IV	Late morula	13.28	18.10	13.98
V	Blastula	9.58	14.40	15.35
VI	Gastrula	19.95	16.49	17.33
VII	Closing of blastopore	26.83	16.20	20.15
VIII	Comma	42.02	13.35	18.98
IX	Eyed	34.83	14.39	16.81
X	Prior to hatching	28.73	12.36	13.74

TABLE - 19

Triglycerides (mg/100 mg dry weight) in unfertilized eggs and in different embryonic stages of *Cyprinus carpio*

S.No.	Stages									
	Unfertilized egg	Blastodisc	Early morula	Late morula	Blastula	Gastrula	Closing of blastopore	Comma	Eyed	Prior to hatching
1	2.27	2.73	2.37	2.91	2.73	3.00	2.16	2.37	2.27	2.00
2	2.55	2.64	2.46	3.00	2.64	2.91	2.00	2.46	2.18	2.18
3	2.09	2.82	2.18	2.55	3.00	2.82	1.91	1.91	2.09	1.64
4	2.64	2.46	2.55	3.09	2.64	3.09	2.27	1.73	2.00	2.09
Mean ± SD	2.39 ±0.25	2.66 ±0.15	2.39 ±0.16	2.69 ±0.24	2.75 ±0.17	2.95 ±0.12	2.09 ±0.16	2.12 ±0.35	2.13 ±0.12	1.98 ±0.24

[illegible]

Cholesterol (mg/100 mg dry weight) in unfertilized eggs and in different embryonic stages of *Cyprinus carpio*

S.No.	Stages									
	Unfertilized egg	Blastodisc	Early morula	Late morula	Blastula	Gastrula	Closing of blastopore	Comma	Eyed	Prior to hatching
1	2.8	3.2	2.6	3.0	2.8	3.2	2.8	3.2	2.4	2.4
2	3.2	3.4	2.4	3.2	3.0	3.0	2.6	3.0	2.6	2.4
3	3.0	3.2	2.6	2.8	2.8	3.0	2.4	3.0	2.4	2.2
4	3.0	3.6	2.6	3.0	3.0	3.4	2.6	3.4	2.8	2.0
5	2.8	3.2	2.8	3.4	3.2	3.2	2.6	2.8	2.6	2.2
6	2.8	3.0	2.4	2.8	2.8	2.8	2.4	2.6	2.2	2.0
Mean ± SD	2.93 ±0.16	3.27 ±0.21	2.60 ±0.18	3.03 ±0.23	2.93 ±0.16	3.10 ±0.21	2.60 ±0.18	3.00 ±0.28	2.50 ±0.21	2.20 ±0.18

Spurt in lipase activity occurred soon after fertilization and later at late gastrula, attaining peak at eyed stage; the rest of stages registered normal activity (Table 16; Fig. 38).

Following fertilization, total phospholipids continuously depleted, almost sharply, until blastula; with onset of gastrulation their levels rose; the accumulation continued until comma stage and depleted again thereafter (Table 17; Fig. 39). Phospholipids constituted the bulk of total lipids at all embryonic stages except at late morula and blastula stages; when their quantitative depletion was utmost (Table 18; Fig. 40).

Triglycerides and cholesterol showed unusual correspondence at each stage of embryonic development, except at the comma stage, when cholesterol level rose sharply. Their levels in different embryonic stages varied only in a narrow range (Table 19 & 20; Figs. 41 & 42).

DISCUSSION

Daniel (1947), Ishida and Nakano (1954) and Terner (1958b) observed in salmonid eggs that carbohydrate provides little energy and seems to be the main energy source only for a brief period at the time of gastrulation. The rest of embryonic and larval period depends mainly on proteins and lipids.

About 40% of the protein of egg and 75% of lipid are used to supply energy (Hayes, 1949). Terner (1968b) affirmed lipids as the endogenous reserves for continuous supply of metabolic energy in telolecithal ova. In addition to supplying fatty acids for oxidation, and thus sparing carbohydrate reserve, the glycerolipids liberate glycerol on lipolysis. Glycerol then can be converted to glucose through fructose diphosphatose and glucose-6-phosphatase. The large store of glycerolipids may play an important role in the nutrition of telolecithal ova. Nakagawa and Tsuchiya (1972) opined the breakdown of lipid moiety in the yolk protein to be more rapid than that of protein moiety.

The present author's observation on levels of glycogen in embryonic stages of Cyprinus carpio affirm that glycogenolysis followed soon after fertilization for a brief period until blastodisc stage and later only at closing of blastopore onward until hatching, vindicating that glycogen could be an energy source only for a limited period. Also, data on levels of total protein and free amino acids revealed a steady decline in levels of total free amino acids after fertilization until late morula and a steep degradation of protein in the prior to hatching stage, suggesting their significance in meeting energy requirements of embryo at these

crucial stages. Protein and lipids thus have both been incriminated in energy metabolism of the growing embryo at periods when glycogen is not the major source.

Depletion in total lipids after fertilization until late morula in *Cyprinus carpio*, as observed by the present author, accompanied with steep decline in the levels of phospholipids during these stages is strongly suggestive of the importance of phospholipids both in energy metabolism as well as assembly of cell membranes at cleavage. In fact, the decline in the level of total lipids is mainly on account of the depletion of its phospholipid fraction. This is in accord with Smith (1958) and Kim (1976) who suggested decline in levels of phospholipids respectively in *Salmo gairdnerii irideus* and *Cyprinus carpio* and their utilization in energy requirement of early embryo, but is clearly at variance with Pasternak (1973) who suggested stimulation of phospholipid synthesis at fertilization in sea urchin eggs. The subnormal levels of phospholipids between late morula and blastula is presumably due to relatively low ebb in activity of embryo having attained the plateau phase of cell lineage. Phospholipid accumulation initiates with the onset of gastrulation, intensifies in the post gastrula period until the comma stage, and depletes during eyed and prior to hatching stages. Yamagami (1960 a,b) observed rapid loss of phospholipids after hatching in *S. gairdnerii irideus*.

Levels of total lipids deplete sharply at gastrula and closing of blastopore stages, in marked contrast with that of phospholipids. Krigsgaber & Neyfakh (1968, '72) found protein synthesis most intensive at onset of gastrulation and maximum at closing of blastopore stages. The present author's study on protein levels during these stages (Fig. 7) corroborate the above. Further, synthesis of glycogen, as observed by the present author (Fig. 32), with onset of gastrulation in C. carpio and by Danilenko (1970) in C. auratus are clear pointers that lipids, the glycerolipids, to be more precise, are the only energy source available during this crucial stage of development.

The present author entirely endorsed Terner's (1968b) contention on the role of glycerolipids in glyconeogenesis and glucose, thus formed, serving to supplement the energy requirement of the embryo.

Lipid accumulation again initiates at comma stage, following prevalence of both glycogenolysis and protein degradation, providing for the needed energy requirements. This is again in order considering that lipid (both lipoprotein and phospholipids) levels are known to deplete very fast after hatching (Yamagami, 1960 a, b; Nakagawa and Tsuchiya, 1972, '76).

Triglyceride and cholesterol vary in a narrow range throughout the embryonic development of

Cyprinus carpio. Lipase activity is found to correspond generally with levels of lipids through out the embryonic development.

This study brings home that lipids, as a source of energy to growing embryo, are even more important than proteins and glycogen. Three distinct phases of their utilization can be identified, namely, after fertilization until late morula stage, when phospholipid degradation is the principal source of energy, at blastula and through gastrula until closing of blastopore stage, when glycerolipids are the most important energy source, and between comma and eyed up stage, phospholipid degradation supplements glycogenolysis as an energy source. After hatching, however, together with protein, their importance in energy metabolism is too well known.