CHAPTER - III

CARBOHYDRATE METABOLISM IN EMBRYONIC STAGES OF CYPRINUS CARPIO

INTRODUCTION

Lipids, glycogen and adenylic nucleotides constitute the main energy substrates of a mature oocyte. Synthesis and catabolism of these metabolites may help determine certain stages of differentiation. The mature oocyte uses glycogenolysis upto fertilization. After fertilization, the energy demands of egg increase; embryonic respiration continues to increase (Boulekbache, 19 1). Acceleration of respiration during fertilization, gastrulation and organo-

genesis are well known in teleostean embryos (Neyfakh, 1960; Zotin et al., 1967; Milman & Yurowitsky, 1973). How important a contribution does glycolysis make to energy metabolism after fertilization and what programme exists of utilization of different energy substrates stored are interesting questions (Boulekbache, 1981).

First carbohydrate, then protein and last of all, fat are used as energy sources during the development of teleost fish (Seuchar, 1965). Terner (1968*b*), however, opined that though glycogen appeared the endogenous precursor of glucose in trout ova, decline in total carbohydrate between unfertilized and eyed-up stage appeared too small. Total carbohydrate reserve can support endogenous respiration for only a fraction of time needed for development. Since this reserve is maintained throughout development, endogenous carbohydrate cannot be preferentially used substrate and must be preserved at the expense of other endogenous substrates. Terner (1968*b*) considered lipids as the principal endogenous reserve for continuous supply of metabolic energy. Boulekbache et al (1969), however, conjectured glycolysis and TCA cycle to be the main source of ATP and immediate substrates required for biosynthesis during early development. Milman & Yurowitsky (1973) did not observe any change in the activities of glycolytic

enzymes during the period from fertilization to early organogenesis. Neyfakh and Abramova (1974) observed that lycolytic system of oocytes is mainly adjusted to gluconeogenesis and not to glycolysis. Örström and Lindberg (1940), in a pioneer work, too observed that marked utilization of glycogen occours only during 10 minutes which follow fertilization, rate of glycogenolysis, after this initial period, is essentially the same in fertilized and unfertilized eggs for several hours. Shaklee et al (1974, 77) and Phillipp & Whitt (1977) noted only slight alterations in the activities of certain glycolytic enzymes during early development in embryos of Green Sunfish, Warmouth, Medaka and Lake Chubsucker. Boulekbache (1981) himself stated later that enzymes of glycolysis remain at low or moderate levels during early embryogenesis.

Carbohydrate provides little energy and seems to be the main energy source only for a brief period at the time of gastrulation (Daniel, 1947; Nishida & Nakano, 1954). The rest of embryonic and larval periods depend mainly on protein and lipid. Although 40% of protein and 75% of lipid in the egg are used to supply energy and the rest providesmaterials for embryonic synthesis (Hayes, 1949). Yamagami (1960a & b) observed that in the prehatching period mainly phosphoproteins are the source of energy; phosphoproteins contain 40% of the total egg phosphorus

and their level begin to fall after gastrulation. Immediately after hatching, protein and lipid levels of the whole egg fall rapidly (Smith, 1958).

It is thus obvious that considerable divergence exists as to the source that provides energy during the different phases of embryonic development. The present author has attempted to visualize how important is glycolysis in energy metabolism, having described in this Chapter levels of glycogen, pyruvate, lactate and activities of lactate dehydrogenase and succinate dehydrogenase in the different embryonic stages of *Cyprinus carpio*.

METHODS

TOTAL GLYCOGEN:

Glycogen content of the lyophilized samples of each embryonic stage was determined by the method of Seifter, Dayton, Novic and Muntwyler (1950) after extraction with hot alkali.

PRINCIPLE:

Concentrated sulphuric acid hydrolyzes glucosidic bonds of glycogen to give monosaccharides which are further dehydrated to furfural and its derivatives. The furfural reacts with anthrone (1 -keto, 9-10 dihydroanthracene) to give a blue green complex.

REAGENTS:

1. 30% Potassium hydroxide solution: 30 g of potassium hydroxide (KOH) pellets dissolved in 100 ml of distilled water.

2. 1% Sodium sulphate solution: 1 g Sodium sulphate (Na_2SO_4) dissolved in 100 ml of distilled water.

3. 95% Ethanol: 95 ml of absolute ethanol diluted to 100 ml with distilled water.

4. Anthrone Reagent: 0.2 g of anthrone (Merck) dissolved in 100 ml of 95% sulphuric acid (H_2SO_4). This reagent was prepared just before use.

5. Standard glucose solution (0.02 mg/ml): Accurately weighed 20 mg glucose dissolved in 100 ml of distilled water and solution diluted to 1 litre. 5 ml of this solution contains 100 µg of glucose.

PROCEDURE:

Lyophilized samples of each stage were weighed accurately and taken in test tubes containing 3 ml each of 30% KOH solution. The tubes were placed in boiling water bath and kept till the contents were digested; cooled thereafter and added

0.5 ml of 1% Na_2SO_4 and 3.5 ml of 95% ethanol to each tube, while shaking, to precipitate glycogen.

The contents in each tube were stirred with a glass rod and the rod washed with a small quantity of ethanol each time. The tubes were gently heated, cooled and allowed to stand overnight for complete precipitation of glycogen at room temperature; they were centrifuged at 3000 rpm for 15 minutes; supernatant decanted and adhering ethanol removed by heating the tubes in a water bath. Precipitated glycogen in each tube was dissolved in 3 ml of distilled water and reprecipitated with 95% ethanol. It was centrifuged again and the supernatant discarded. Finally, the precipitate was dissolved in distilled water and the solution made to 50 ml in a volumetric flask.

Glycogen was estimated colorimetrically as follows:

5 ml of aliquot was transferred to a wide mouth boiling tube. This served as a test solution. 5 ml of standard glucose solution, containing 100 µg of glucose, was taken in another boiling tube. This served as standard. 5 ml of glass distilled water was taken in a third tube to serve as a blank.

The tubes containing blank, standard and test solutions were placed in ice cold water. Dry

and clean glass beads were used to prevent bumping following the addition of anthrone reagent. 10 ml of freshly prepared anthrone reagent (4) was added to each tube with a fast flowing pipette and the contents were mixed by vigorous shaking.

The tubes were then transferred to a boiling water bath, kept for exactly 30 minutes and then cooled to room temperature. The optical densities(O.D) of the test and standard solutions were read against blenk at 620 nm in spekol (Carl Zeiss, Jena).

The amount of glycogen was calculated from the equation:

$$\mu g \text{ of glycogen in 5 ml of aliquot} = \frac{100 \times U}{1.11 \times S}$$

where	U	=	O.D. of test solution
	S	=	O.D. of standard solution
and	1.11	=	conversion factor of glucose into glycogen (as given by Morris).

Glycogen content was expressed in mg/100 mg dry wt. of eggs.

PYRUVIC ACID:

Known amounts of fresh samples of different embryonic stages were homogenized in 2 ml of cold distilled water and the whole transferred to centrifuge tubes. Protein was precipitated by adding

equal volumes of 10% trichloroacetic acid and the tubes centrifuged at 3000 rpm for 15 minutes. Protein free supernatant was used as test sample for estimation of pyruvic acid using 2,4-dinitrophenylhydrazine by the method of Friedemann and Haugen (1943).

PRINCIPLE:

Protein free filtrate when treated with 2,4-dinitrophenylhydrazine forms 2,4-dinitrophenylhydrazone, which reacts with strong alkali to form a reddish compound that is estimated colorimetrically. Other ketoacids also react to form similar hydrazones but the pyruvic hydrazone can be preferentially extracted with sodium carbonate.

REAGENTS:

1. 10% Trichloroacetic acid: 10 g of TCA dissolved in 100 ml of distilled water.
2. 2,4-dinitrophenylhydrazine: 100 mg of 2,4-dinitrophenylhydrazine was dissolved in small volumes of approximately 2 N HCl and then diluted to 100 ml with acid. Filtered and filtrate stored in cold.
3. 10% Sodium Carbonate: 10 g of Sodium carbonate (Na_2CO_3) dissolved in 100 ml of distilled water.

4. 1.5 N Sodium hydroxide: 6 g of Sodium hydroxide (NaOH) pellets dissolved in 100 ml of distilled water.

5. Standard stock pyruvic acid solution: 107 mg Lithium pyruvate dissolved in 0.1 N sulphuric acid and diluted to 100 ml with 0.1 N sulphuric acid.

6. Standard working pyruvic acid solution: 1 ml of stock solution (5) diluted to 100 ml with distilled water. This solution gave 0.01 mg of pyruvic acid per ml.

PROCEDURE:

1 ml of 2,4-dinitrophenylhydrazine was added to 3 ml of protein free filtrate and allowed to react for 5 minutes at room temperature. 3 ml of working standard solution was taken in a separate test tube. 3 ml of xylene was added to each of the above tubes; steam of air was bubbled into each for 2 minutes and allowed to settle; lower layer was removed from each tube by a capillary tipped dropper and discarded. 6 ml of 10% Na_2CO_3 was then added to each tube and again mixed by bubbling air through the mixture for 2 minutes. After settlement of mixture, 5 ml each of lower solution was taken in fresh tubes and 5 ml of 1.5 N sodium hydroxide solution added. The tubes were left for 5-10 minutes.

Optical densities were measured at 520 nm against blank, prepared by mixing 5 ml of 10% Na_2CO_3 and 5 ml of 1.5 N sodium hydroxide.

Pyruvic acid content was calculated with reference to the standard graph and expressed as µg/100 mg fresh weight of eggs.

LACTIC ACID:

Known amounts of fresh samples of the different embryonic stages were homogenized in 10% trichloroacetic acid (TCA) and the homogenates transferred to centrifuge tubes. They were centrifuged at 3000 rpm for 15 minutes. The supernatants were used for estimation of lactic acid by the method of Barker and Summerson (1941).

PRINCIPLE:

Glucose and other interfering materials of protein free homogenate are removed by Van Slyke-Salkowski method of treatment with copper sulphate and calcium hydroxide. On heating with concentrated sulphuric acid, lactic acid is converted to acetaldehyde, which is then determined by reaction with p-hydroxydiphenyl in the presence of copper ions.

REAGENTS:

1. 20% Copper sulphate solution: 400 g of $CuSO_4 \cdot 5H_2O$ dissolved in 1 litre of water with gentle

heating; solution cooled and later diluted to 2 litre with distilled water.

2. 4% Copper sulphate solution: 1 volume of 20% copper sulphate solution (1) diluted to 5 volumes with distilled water and mixed thoroughly.

3. 10% Trichloroacetic acid: 10 g of trichloroacetic acid dissolved in 100 ml of distilled water.

4. Calcium hydroxide powder: Anhydrous calcium hydroxide ($Ca(OH)_2$) powder was used.

5. Concentrated sulphuric acid (H_2SO_4): Ion free H_2SO_4 with low nitrate content was used.

6. p-Hydroxydiphenyl reagent: 1.5 g of p-hydroxydiphenyl reagent dissolved in 10 ml of 5% sodium hydroxide solution; 2 ml distilled water added with slight warming and stirring; the solution then made to 100 ml with distilled water and stored in brown bottle.

7. Stock standard lactic acid solution: 0.215 g of pure dry lithium lactate dissolved in 100 ml of distilled water in one litre volumetric flask; 1 ml of concentrated sulphuric acid added; the whole made to one litre with distilled water and thoroughly

mixed. This gave 1 mg lactic acid in 5 ml of solution and was stable for a long time when kept in refrigerator.

8. Working standard lactic acid solution: 5 ml of stock standard solution (7) diluted to 100 ml in glass stoppered volumetric flask with distilled water and mixed thoroughly; this gave 0.01 mg of lactic acid per ml and was prepared fresh daily.

PROCEDURE:

2 ml of protein free supernatant, 5 ml of freshly prepared lithium lactate standard solution and a reagent blank (5 ml of distilled water) were taken in three separate centrifuge tubes; 1 ml of 20% $CuSO_4$ solution was added to each and then made to 10 ml with distilled water. One g of powdered $Cu(OH)_2$ was added to each tube with a spatula. They were stoppered and shaken vigorously until uniformly dispersed. The tubes were kept for an hour with frequent shaking and later centrifuged at 3000 rpm for 15 minutes. 1 ml of supernatant was taken from each of above tubes in separate tubes with an internal diameter of 18 to 20 mm. 0.05 ml of $CuSO_4$ solution was added to each tube. Subsequently, 6 ml of chilled analar H_2SO_4 was added, initially drop by drop, with uniform mixing of the contents, to each tube. All the tubes were kept in a boiling

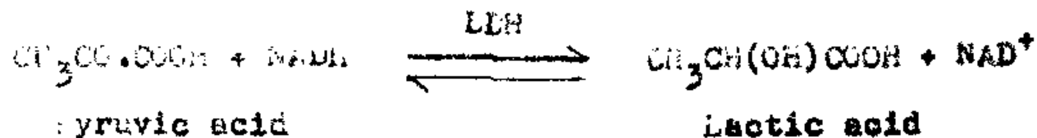
water bath for 5 minutes and cooled to 20°C or below in running cold water. 0.1 ml of p-hydroxydiphenyl reagent was then added drop by drop to each tube. Precipitate of reagent was dispersed again through out the solution quickly and uniformly by shaking. All the tubes were incubated at 30 ± 1°C for 90 seconds and were allowed to cool to room temperature. The optical density was measured against blank at 570 nm in spekol. The values are expressed as µg lactate/100 mg fresh weight of eggs.

LACTATE DEHYDROGENASE:

(L-lactate: NAD oxidoreductase, E.C. No. 1.1.1.27).

Eggs were homogenised in icecold 0.25 M sucrose solution to approximately 10% (w/v) homogenate. The homogenate was centrifuged at 3000 rpm at 4°C for 5 minutes. The clear supernatant was used for enzyme assay by the method as described by Wootton (1974).

PRINCIPLE:



Lactate dehydrogenase (LDH) is an enzyme of almost universal distribution and catalyzes the reversible transformation of pyruvate to lactate. Pyruvate is reduced to lactate by incubation with

homogenate in the presence of coenzyme NADH. The reaction is stopped by adding dinitrophenylhydrazine solution which reacts with remaining pyruvate to form a hydrazone. The amount of unreacted pyruvate is found by measuring the brown colour produced when the hydrazone is made alkaline. The determination is performed at 25°C, since the electrophoretically slower isozymes of LDH are known to be very sensitive to heat.

REAGENTS:

1. 0.25 M Sucrose solution: 8.557 g of sucrose dissolved in 100 ml of distilled water.

2. Phosphate buffer (pH 7.4): [illegible].445 g of disodium hydrogen phosphate (Na_2HPO_4) and 0.675 g potassium hydrogen phosphate (KH_2PO_4) dissolved in 250 ml of distilled water, adjusting pH to 7.4.

3. Stock sodium pyruvate solution (37.5 mM): 415 mg of sodium pyruvate dissolved in 100 ml of phosphate buffer (2). This solution was stored at - 20°C in a deep frigidaire.

4. Working sodium pyruvate buffered substrate (0.75 mM): 1 ml of stock pyruvate (3) solution diluted to 50 ml with phosphate buffer (2). This was prepared fresh.

5. Reduced nicotinamide adenine dineucleotide (NADH): 10 mg of NADH dissolved in 10 ml of phosphate buffer. This was prepared fresh for each batch of tests.

6. 2,4-dinitrophenylhydrazine (2 mM): 40 mg of dinitrophenylhydrazine dissolved in 8.5 ml of concentrated hydrochloric acid (HCl). This was made to 100 ml with distilled water and stored in a brown bottle.

7. 0.4 N sodium hydroxide solution: 16 g of sodium hydroxide dissolved in 1 litre of distilled water.

PROCEDURE:

Test: 1 ml of buffered substrate (4) and 0.1 ml homogenate of each sample were taken in test tubes and kept in a water bath at 25°C. Reaction was started after a few minutes by addition of 0.1 ml of NADH solution to each tube; incubation was done for exactly 15 minutes. Tubes were then removed and reaction was checked by adding 1 ml of dinitrophenylhydrazine solution (6) to each tube while mixing.

Control: 1 ml of buffered substrate (4), 0.2 ml of phosphate buffer (2) and 1 ml of dinitrophenylhydrazine solution (6) were taken in a test tube.

Blank: 1.2 ml phosphate buffer (2) and 1 ml of dinitrophenylhydrazine solution (6) were taken in another test tube.

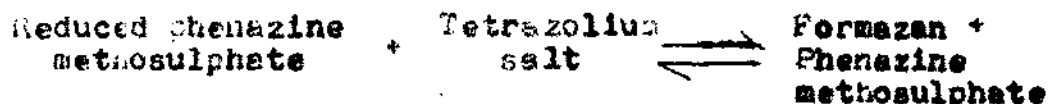
Tubes containing test, control and blank solution were allowed to stand at room temperature for 20 minutes. 10 ml of 0.4 N sodium hydroxide solution was added to each of the above tubes and mixed. Optical density was measured after 10 minutes against blank at 510 nm in spekol. LDH activity was expressed in terms of μ moles pyruvates reduced/mg protein/hr. Protein content of the homogenate was determined by the method of Lowry et al (1951) as described earlier on page 34 .

SUCCINATE DEHYDROGENASE:

(Succinate (acceptor) oxidoreductase;
E.C. No. 1.3.99.1)

Fresh eggs were homogenized in icecold 0.1 M phosphate buffer (pH 7.7) to approximately 5% (w/v) homogenate; homogenates centrifuged at 3000 rpm at $4^{o}C$ for 5 minutes; supernatants used for enzyme assay by the method of Nachlas et al (1960).

PRINCIPLE:



$FADH_2$ formed in first reaction, in the presence of phenazine methosulphate (PMS), reacts with 2-p-iodophenyl, 3-p-nitrophenyl, 5 phenyltetrazolium chloride (INT) to form strongly coloured formazan (reduced tetrazolium salt). Phenazine methosulphate serves as an intermediate electron carrier between this dehydrogenase and INT. Addition of simple protein (gelatin) to the reaction mixture keeps formazan dispersed finely enough to permit determination of the colour density in aqueous solution.

REAGENTS:

1. 0.2 M monopotassium phosphate solution: 2.721 g KH_2PO_4 dissolved in 100 ml of distilled water.
2. 0.2 M Potassium hydroxide solution: 1.002 g KOH dissolved in 100 ml of distilled water.

3. Phosphate buffer (pH 7.7): 50 ml of solution (1) and 44 ml of solution (2) were mixed and the pH was adjusted to 7.7. Total volume was then made to 100 ml with distilled water. Stored in cold.

4. 0.2 M sodium succinate solution: 5.403 g of sodium succinate dissolved in 100 ml of double distilled water. Stored in cold.

5. Gelatin solution (0.1 %): 100 mg of gelatin dissolved in 100 ml of distilled water.

6. Stock INT solution (0.2%): 200 mg 2-p-iodophenyl, 3-p-nitrophenyl, 5 phenyltetrazolium chloride dissolved in 100 ml of distilled water. Stored in cold.

7. Working INT solution (0.2 mg/ml for calibration curve): 1 ml of stock INT solution (6) diluted to 10 ml with distilled water. Stored in cold.

8. Phenazine methosulphate solution (8 mg/ml): 80 mg of phenazine methosulphate (PMS) dissolved in 10 ml of distilled water. Kept at 5°C in brown bottle. It was prepared fresh before use.

9. Phenazine methosulphate (PMS) solution (10 mg/ml for calibration curve): 100 mg of phenazine methosulphate (PMS) dissolved in 10 ml of

0.1 M phosphate; kept at 37°C for 15 minutes; prepared fresh.

10. 0.25 M HCl: 0.935 ml concentrated hydrochloric acid diluted to 100 ml with distilled water.

11. NADH solution: 50 mg of reduced nicotinamide adenine dinucleotide (NADH) dissolved in 30 ml of cold distilled water. Prepared fresh.

PROCEDURE:

Test: 1.5 ml of phosphate buffer, 0.5 ml of sodium succinate solution, 1.0 ml of INT solution (2 mg/ml), 0.5 ml of gelatin solution and 0.5 ml of homogenate were taken in a test tube and 0.5 ml of PMS solution added immediately. Incubation was done for exactly 15 minutes at 37°C. Tubes were then removed and reaction stopped by adding 0.5 ml of 0.25 M hydrochloric acid while mixing.

Control: 1.5 ml of phosphate buffer, 0.5 ml of sodium succinate solution, 1.0 ml of INT solution, 0.5 ml of gelatin solution and 0.5 ml of 0.25 M HCL were taken in a test tube and 0.5 ml homogenate and 0.5 ml PMS added immediately.

Blank: It was prepared as control, except that equal volume of water was taken in place of INT and homogenate.

Optical density was measured against blank at 540 nm in spekol. The activity of succinate dehydrogenase was calculated from the standard graph and expressed as µg formazan formed/mg protein/hr. Protein content of the homogenate was determined by the method of Lowry et al (1951) as described earlier on page 34 .

RESULTS

Following fertilization, glycogen depletion was observed at blastodisc stage, during late morula and in all stages subsequent to gastrula until hatching. Glycogen accumulation, on the other hand, occurred during early morula, blastula and gastrula stages (Table 10; Fig. 32).

Pyruvate levels increased during early morula, depleted until gastrula and increased steadily thereafter attaining peak at eyed stage. In just prior to hatching stage, however, there was again a decline (Table 11; Fig. 33).

Lactate levels inordinately increased until early morula stage, depleted to subnormal levels at late morula, through blastula and gastrula, until closing of blastopore, but rose again during comma, eyed and just prior to hatching stages (Table 12; Fig. 34).

TABLE - 10

Total glycogen (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	3.87	2.67	3.27	4.00	3.20	2.73	4.40	2.06	2.37	2.27
2	4.00	2.20	3.00	3.60	2.67	2.60	3.83	2.67	2.13	2.13
3	-	2.05	3.47	3.93	3.[illegible]7	3.47	3.40	[illegible].40	2.30	2.00
4	-	2.65	3.89	1.64	3.12	7.10	4.57	4.33	2.34	2.08
5	[illegible].55	1.34	3.[illegible]	2.25	2.56	6.93	[illegible].33	4.50	2.[illegible]	1.94
6	2.97	2.55	4.40	1.84	3.[illegible]7	[illegible].38	4.50	4.71	2.05	2.05
7	3.27	2.85	[illegible].99	2.35	2.85	7.11	[illegible].83	1.39	0.32	0.21
8	1.39	1.50	1.82	0.95	1.82	1.2[illegible]	1.93	1.93	0.85	0.64
9	1.82	1.60	2.14	0.96	1.50	1.60	2.98	1.82	0.35	0.64
10	1.60	2.03	1.93	1.18	1.07	1.39	1.98	4.91	2.35	2.35
Mean ± SD	2.72 ±1.01	2.22 ±0.45	3.18 ±0.93	2.27 ±1.19	2.59 ±0.87	4.11 ±2.58	3.41 ±0.98	3.07 ±1.37	1.81 ±0.83	1.63 ±0.80

TABLE - 11

Pyruvic acid (μg/100 mg fresh 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	2.58	2.58	3.62	4.53	2.52	1.46	1.94	2.22	3.98	-
2	2.76	2.05	3.00	2.50	3.50	2.50	4.13	3.45	3.00	-
3	2.54	2.32	2.32	2.13	2.32	2.94	2.32	3.05	3.05	3.05
4	2.54	2.32	2.32	2.13	2.32	2.45	2.90	2.54	3.55	3.05
5	2.03	2.32	2.90	1.70	2.30	2.45	2.32	3.05	3.55	3.05
6	1.52	2.32	2.90	1.70	2.90	1.95	2.50	3.05	3.05	2.54
Mean ± SD	2.33 ±0.46	2.32 ±0.16	3.13 ±1.01	2.78 ±1.36	2.74 ±0.45	2.33 ±0.45	2.75 ±0.77	2.89 ±0.44	3.36 ±0.39	2.92 ±0.25

TABLE - 12

Lactic acid (µg/100 mg fresh weight) in unfertilized eggs and in different embryonic stages of *Cyprinus carpio*

No.	Stages									
	Unfertilized egg	Blastodisc	Early morula	Late morula	Blastula	Gastrula	Closing of blastopore	Comma	Eyed	Prior to hatching
1	6.62	17.65	20.23	7.72	9.65	7.35	5.36	8.83	13.45	16.92
2	5.62	19.12	16.02	7.35	9.15	6.25	5.15	9.19	13.45	15.81
3	5.52	18.39	16.75	6.98	8.34	7.35	5.15	8.46	13.03	16.92
4	5.83	18.02	21.33	7.35	9.85	7.35	5.88	8.09	13.45	15.81
5	5.58	17.28	18.75	6.09	9.10	5.62	4.41	8.45	14.29	15.45
6	7.72	18.39	20.96	10.30	12.13	7.35	5.38	7.72	14.71	13.02
7	6.07	-	-	6.07	5.00	7.14	5.07	13.22	15.75	11.79
8	5.72	-	-	5.43	6.07	8.93	5.00	13.93	15.39	10.72
9	6.79	-	-	6.07	6.07	6.43	5.36	14.29	12.46	10.72
10	5.00	-	-	7.14	5.00	7.14	4.64	-	12.09	10.00
Mean ±SD	6.18 ±0.77	18.14 ±0.64	19.57 ±1.35	7.35 ±1.23	8.13 ±2.45	7.19 ±0.74	5.34 ±0.57	10.24 ±2.72	13.81 ±1.20	14.22 ±3.05

TABLE - 13

Lactate dehydrogenase activity (μ moles pyruvate reduced/mg protein/hr) 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	2.00	1.78	1.50	2.00	1.58	2.08	1.95	1.72	2.24	1.76
2	1.88	1.76	1.44	1.94	1.44	1.72	1.52	1.66	2.12	1.56
3	1.88	1.76	1.42	1.94	1.44	1.72	1.48	1.68	2.05	1.60
4	1.88	2.12	1.42	1.92	1.44	1.72	1.52	1.65	2.12	1.60
5	1.54	2.05	2.04	2.15	2.02	2.12	2.00	1.53	2.02	2.32
6	1.64	2.06	2.00	2.24	2.02	2.12	2.00	1.88	1.92	2.40
7	1.64	2.12	2.00	2.12	2.08	2.08	2.00	1.88	2.00	2.40
8	1.64	2.05	2.00	2.15	2.12	2.12	1.96	1.88	2.02	2.44
9	-	-	-	2.08	-	-	-	1.72	2.24	-
Mean ± SD	1.77 ±0.15	1.96 ±0.17	1.74 ±0.29	2.05 ±0.11	1.76 ±0.32	1.96 ±0.20	1.80 ±0.25	1.77 ±0.10	2.08 ±0.11	2.01 ±0.41

TABLE - 14

Succinate dehydrogenase activity (µg formazan formed/mg protein/hr) 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	398.40	433.34	558.60	577.68	425.64	697.95	255.28	354.50	463.40	454.08
2	390.40	455.50	503.92	597.72	425.64	753.80	249.20	354.50	445.56	514.08
3	408.32	455.04	534.12	567.68	425.64	737.40	243.12	359.30	481.20	442.64
4	428.28	455.60	519.00	507.52	464.92	711.38	255.28	354.50	445.56	478.36
5	428.28	475.20	549.20	634.44	445.28	730.20	249.20	370.36	454.44	478.36
Mean	412.34	459.25	612.97	589.00	437.42	740.09	250.42	358.73	458.05	475.50
± SD	±15.10	±16.05	±34.76	±31.50	±17.57	±37.74	± 5.09	± 6.89	±14.91	±26.05

Lactate dehydrogenase activity was found to increase at late morula, gastrula and eyed stages (Table 13; Fig. 35).

Succinate dehydrogenase, however, increased moderately during early cleavage until late morula, sharply at gastrula and appreciably at comma and eyed stages; activity, however, depleted at blastula and closing of blastopore stages (Table 14; Fig. 36).

DISCUSSION.

After fertilization, energy demands of the egg increase; embryonic respiration continues to increase (Boulekbache, 1981). Acceleration of respiration during fertilization, gastrulation and organogenesis are well known in teleostean embryos (Neyfakh, 1960; Zotin et al, 1967; Milman & Yurowitsky, 1973). Danilenko (1970) observed in Carassius auratus L. that intensity and utilization of glycogen is not the same in different periods of early embryogenesis. He described accumulation at the beginning of division right upto the formation of large cellular morula, a considerable drop at small cellular morula and blastula stages, rise again at gastrula and a sharp decrease at the beginning of embryo formation. Boucher (1965) emphasized that first carbohydrate, then protein and last of all, fat are used as energy sources during development of a teleost fish.

Orström and Lindberg (1940), in a pioneer work, however, found marked utilization of glycogen during the 10 minutes following fertilization. Monroy (1965) corroborated a polysaccharide (glycogen) breaking down in 10 minutes after fertilization.

The present study clearly vindicates occurrence of glycogenolysis soon after fertilization with simultaneous boost in lactate levels (also observed by Milman, [1955] in loach embryos) and LDH activity at the blastodisc stage (in 45 minutes following fertilization). This is soon followed by increase in pyruvate levels during the early morula stage with concomitant rise in LDH activity. The present author considers this phase, from fertilization to early morula, as the first phase of accelerated respiration, glycogen (Fig. 32), free pool of amino acids (Fig. 7) and lipids (Fig. 37) providing energy sources. Higher activities of transaminases, both GOT and GPT, more so GOT (Fig.30), during these stages are highly suggestive of this. This opinion of the author finds further support in that carbohydrate provides little energy and seems to be the main energy source for a brief period at the time of gastrulation (Daniel, 1947; Nishida and Nakano, 1954) and the rest of embryonic and larval period depends mainly on protein and lipid (Hayes,

1949) and further, that lipids are the principal endogenous reserves for continuous supply of metabolic energy (Terner, 1968b).

Rise in glycogen levels from blastodise to early morula, a considerable drop at late morula and blastula stages, rise again at gastrula and a sharp decrease at the beginning of embryo formation i.e. during comma stage, as observed by the present author in Cyprinus carpio, are fully in accordance with Danilenko (1970) in Carassius auratus.

A spurt in SDH activity, with the onset of gastrulation (also observed by Bhaskar & Swarup, 1977), indicates a second phase of accelerated respiration. During this phase, while total free amino acids and lipids are found to deplete, glycogen accumulates. The present author's observation lends full testimony to the general corrollary that proteins and lipids are the energy sources of developing embryos most of the time and not glycogen.

After a brief lull, a third phase of accelerated respiration is initiated with early organogenesis at comma stage. A sharp drop in the level of glycogen and total lipids, accompanied with a concomitant rise in lactate and pyruvate levels and LDH activity, occurs. Glycogen depletion continues, with accompanied rise in lactate and pyruvate levels and LDH and SDH activities, during eyed stage. Just

prior to hatching, however, extensive protein catabolism, accompanied with a tremendous spurt in levels of free amino acids and transaminases' activity marks culmination of this third and final phase of accelerated respiration. SDH activity is very high and obviously protein combustion and transamination become the principal sources of energy at this crucial stage.

This study clearly identifies 3 main phases of accelerated respiration in the development of _Cyprinus carpio_, namely, after fertilization until early morula stage; a second optimum phase, with the onset of gastrulation; and a third phase, during organogenesis, culminating in intense respiratory activity at just prior to hatching stage. Energy needs of the growing embryo are met with principally through protein and lipid metabolism and, to a limited extent only, through glycogenolysis.