CHAPTER - II

PROTEIN METABOLISM IN EMBRYONIC STAGES OF CYPRINUS CARPIO

INTRODUCTION

Growth of fish embryos is dependent upon the quantity of nutrient (particularly protein and energy) stored in yolk, since the membrane is impermeable to amino acids and nucleic acids (Neyfakh and Baramova, 1974). Successful ontogenesis results from conversion of yolk material to tissues of the embryo (Zeitoun et al., 1977). In fish, translocation of yolk protein to blastoderm is the ultimate feature of growth. One of the most interesting phenomena of developing eggs is how the yolk proteins are converted into proteins of embryo (Yoneda, 1977). As the size of blastoderm increases, the amount of protein in yolk decreases, at the

expense of yolk degradation, migration of amino acids in blastoderm and protein synthesis in embryo cells. In loach blastoderm, protein is one-tenth of total egg protein; near end of gastrulation this value doubles and by the time of hatching it is 3 to 3.5 fold the initial value of protein. In early larva about two-third of proteins are constituted by yolk proteins (Abramova and Vasilyeva, 1973). Nakagawa and Tsuchiya (1972) distinguished three fractions, namely, lipoprotein, phosphoprotein and glycoprotein in yolk proteins. They maintained that although there was a marked decrease in protein content of the yolk during embryogenesis, all three fractions of proteins were equally converted to low molecular products, hence ratio of the three kinds of proteins was maintained constant.

Aside this, yolk protein is also the main source of energy to growing embryo. 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 and Nakano, 1954). The rest of the embryonic and larval period depends mainly on protein and lipid as the energy source. In the prehatching period it is mainly the phosphoprotein, constituting about 40% of the total egg phosphorus in salmon, which is the source of energy and its level begins to fall soon after

gastrulation. Nakagawa and Tsuchiya (1972) further affirmed that only bound lipid of MDF is utilized by embryo for its energy requirement and not the free globule.

Two main periods of protein synthesis were identified in sea urchins, namely fertilization through cleavage and late blastula or early gastrula (Chen, 1967). In studies on protein metabolism it is of considerable value to have data on amino acid component in the egg (Nakano and Yamamoto, 1972). Thus, it is obvious that metabolism of proteins stored in yolk is of vital importance to the growing embryo, primarily as a source for macromolecules needed in protein synthesis and additionally as an energy source.

Besides study of free and protein amino acids in different embryonic stages, activity of transaminases and glutamate dehydrogenase also become very much relevent for the study of protein metabolism. Both glutamate oxaloacetate and glutamate pyruvate transaminases play important roles in protein metabolism. They not only promote synthesis and degradation of amino acids, but also provide a reversible link between carbohydrate and amino acid metabolism. Another important enzyme related to protein metabolism is glutamate dehydrogenase. It

provides a means for the net incorporation of ammonia into amino acids and a reversible link between carbohydrate and amino acid metabolism. By coupling with transamination reactions the glutamate dehydrogenase can bring about the general oxidative deamination of diverse amino acids thus enabling them to enter into energy metabolism (via Kreb's cycle).

The present chapter deals with the studies on total free amino acids, total protein, percentage composition of amino acids in free and protein pools, activities of glutamate oxaloacetate transaminase, glutamate pyruvate transaminase and glutamate dehydrogenase in the different embryonic stages of Cyprinus carpio.

METHODS

TOTAL FREE AMINO ACIDS:

Known weights of lyophilized samples of different embryonic stages were homogenized in 2 ml of distilled water and transferred to centrifuge tubes; equal volumes of 10 % trichloroacetic acid added and the whole centrifuged at 3000 rpm for 15 minutes; supernatants were used for the estimation of total free amino acids by the method of Moore and Stein (1954) as described by Plummer (1971).

PRINCIPLE:

Ninhydrin (triketohydrindene hydrate), a powerful oxidizing agent, reacts with all α-amino acids between pH 4.0 and 8.0 to give a purple coloured compound. This reaction, known as ninhydrin reaction, is modified to quantitate total free amino acids colorimetrically at 570 nm.

REAGENTS:

1. Hydrindantin: 8 g of ninhydrin (1, 2, 3 indantrion-monohydrate, trioxohydrinden monohydrate) was added to 200 ml of distilled water at 90°C; 8 g of ascorbic acid in 40 ml of water at 40°C was added while stirring; crystallization of hydrindantin (following reduction of ninhydrin) started immediately; this was allowed to proceed at room temperature for 30 minutes. The hydrindantin was filtered off, washed well with water, dried to a constant weight in vacuum dessicator and stored in brown bottle. Yield of hydrindantin was 75 to 80 gms.

2. 0.2 M Sodium acetate buffer (pH 5.5):

   (a) 0.2 M acetic acid: 12 ml of 99.5% glacial acetic acid diluted to 100 ml with distilled water.

(b) 0.2 M Sodium acetate: 1.64 g sodium acetate dissolved in 100 ml distilled water. 12 ml of 0.2 M acetic acid (a) and 88 ml of 0.2 M sodium acetate (b) when mixed gave 0.2 M sodium acetate buffer (pH 5.5).

3. Ninhydrin Reagent: 2 g of ninhydrin and 0.3 g of hydrindantin dissolved in 75 ml of methyl cellosolve; 25 ml of sodium acetate buffer (pH 5.5) was added and transferred to a brown coloured bottle to prevent interaction with light (ninhydrin being photosensitive). This reagent was prepared fresh before use.

4. 10% Trichloroacetic acid: 10 g TCA dissolved in 100 ml of distilled water.

PROCEDURE:

2 ml of ninhydrin reagent was added to 1 ml of supernatant in test tubes; they were stoppered and kept in a boiling water bath for exactly 6.5 minutes and cooled immediately thereafter. The contents were made to 10 ml with distilled water. The colour was read against blank at 570 nm. The amount of ninhydrin positive substances was calculated from the standard graph for leucine. The amino acid content was expressed as mg/100 mg dry weight.

TOTAL PROTEIN

Total protein content of lyophilized egg samples was precipitated in 14% trichloroacetic acid and purified by the method as described by Taylor and Haynes (1966). Purified proteins were dissolved in known volumes of 0.4 N sodium hydroxide and estimated by the Folin-phenol method of Lowry et al (1951).

PRINCIPLE:

Protein reacts with the Folin & Ciocalteau's phenol reagent to give a coloured complex. The colour so formed is due to the reaction of alkaline copper with protein and subsequently of copper treated protein with phosphomolybdate of Folin & Ciocalteau's phenol reagent.

REAGENTS:

1. 14 % Trichloroacetic acid (14% TCA): 14 g of TCA dissolved in 100 ml of distilled water.
2. 7 % TCA: 7 g of TCA dissolved in 100 ml of distilled water.
3. 0.4 N Sodium hydroxide solution: 4 g sodium hydroxide (NaOH) dissolved in 250 ml of distilled water.

4. 0.1 N Sodium hydroxide solution: 1 g NaOH dissolved in 250 ml of distilled water.

5. 2% Sodium carbonate solution: 2 g of sodium carbonate (Na_2CO_3) dissolved in 100 ml of 0.1 N NaOH solution (4).

6. 1% Sodium potassium tartrate solution: 1 g of sodium-potassium tartrate dissolved in 100 ml of distilled water.

7. 0.5% Copper sulphate – Na-K tartrate solution: 50 mg of copper sulphate dissolved in 10 ml of 1% Na-K tartrate solution (6). This solution was freshly prepared.

8. Alkaline copper sulphate solution: 50 ml of Na_2CO_3 solution (5) and 1 ml of copper sulphate-Na-K tartrate solution (7) mixed just before use.

9. Folin and Ciocalteau's phenol reagent: Commercial Folin & Ciocalteau's phenol reagent diluted with equal volume of distilled water on the day of use. This reagent is a solution of sodium tungstate and sodium molybdate in phosphoric and hydrochloric acids.

10. Standard protein solution: 20 mg of bovine serum albumin dissolved in 100 ml of distilled water in a volumetric flask and stored in refrigerator.

PROCEDURE:

A. Precipitation and purification of proteins:

50 mg of lyophilized egg samples powdered in a pestle-mortar; homogenized in 2-3 ml of Hank's saline and transferred to centrifuge tubes; protein precipitated by adding 2 to 3 ml of 14% TCA to homogenate and the whole centrifuged. Precipitate of protein was purified as follows: washed twice in 14% TCA; washed in acetone to remove fats; treated with a mixture of chloroform:methanol (1:1 v/v) at 55°C to remove phospholipids; washed in ether to remove remaining fats; treated with 7% TCA at 90°C for 20 minutes to remove nucleic acids and finally, washed again twice in 7% TCA and dried in acetone, followed by ether.

B. Protein estimation:

Purified protein was dissolved in appropriate volume of 0.4 N NaOH; transferred to 10 ml volumetric flask with washings and made up to the mark with distilled water. 0.5 ml of aliquot of total protein was added to 5 ml of alkaline copper sulphate solution (3) in a test tube; 0.5 ml of 0.1 N NaOH was added to 5 ml of alkaline copper solution in another tube to serve as blank. Different concentrations of standard albumin solution (10) were added to a series of tubes each containing 5 ml of alkaline copper sulphate solution.

Tubes were then shaken and 0.5 ml of freshly diluted Folin-Ciocalteau's phenol reagent was added to each after 5 minutes. The tubes were shaken again and incubated at 37°C for 30 minutes. Optical densities were read against blank at 750 nm.

Total protein content of the test sample was calculated with reference to the standard graph and expressed as mg/100 mg of dry weight.

CHROMATOGRAPHIC ANALYSES OF FREE AND PROTEIN AMINO ACIDS

A. Preparation of samples:

(1) Free amino acids' extraction:

100 mg each of lyophilized egg samples were taken, powdered in a pestle mortar, homogenized in 2 to 3 ml of 70% ethanol and transferred to centrifuge tubes. Homogenates were kept for 12 hours at 4°C to allow complete precipitation of proteins; contents then centrifuged at 3000 rpm for 15 minutes; supernatants used for chromatographic analysis of free amino acids.

(2) Protein hydrolysis:

(a) Acid hydrolysis: Proteins were extracted from 50 mg of lyophilized eggs and purified (procedure as on page 36). Acid hydrolysis of this purified protein was then carried out in 6 N HCl at 115°C for

about 12 hours. The hydrolysate was evaporated to dryness in vacuum evaporator. It was redissolved in a small volume of distilled water and again evaporated to dryness to remove traces of acid. Finally, it was dissolved in 3 ml of 70% ethanol for chromatographic analysis.

(b) Alkali hydrolysis: Purified protein was hydrolyzed in 5 N NaOH for 6 to 8 hours at 120°C (Smith and Seakins, 1976) for the detection of tryptophan, which is destroyed during acid hydrolysis.

Chromatographic techniques for qualitative and quantitative analysis of amino acids were performed as given by Smith & Seakins (1976).

B. Qualitative Analysis:

Qualitative analysis of free and protein amino acids was done by uni and two dimensional ascending paper and thin layer chromatography, using whatman filter paper No. 1 and microcrystalline cellulose respectively. The best solvent systems under laboratory conditions, for both paper and thin layer chromatography, were n-butanol : acetic acid : water (4 : 1 : 1.6 v/v) and n-butanol : pyridine : water (1 : 1 : 1 v/v) as first and second solvent systems respectively. 0.2% ninhydrin solution in acetone was used as locating reagent.

PAPER CHROMATOGRAPHY:

Two dimensional paper chromatography for the analysis of amino acids in each sample was done by the following technique: A drop of the sample was placed exactly at 8 cm distance from both edges of a corner of whatman paper No. 1 (46x57 cm). The spot was allowed to dry with the help of a hair drier. Additional drops were added to the same spot with continuous air drying until the desired concentration of the sample was obtained on the paper. Chromatogram was developed in a standard chromatography chamber (Biswas & Co., Calcutta). Edge of the paper near the spot was dipped in a boat containing n-butanol : acetic acid : water (4 : 1 : 1.5 v/v) and allowed to run in ascending direction, with the longitudinal axis of the paper in machine direction, for 20 hours. Paper was removed after 20 hours and dried thoroughly. It was again developed in the same manner, but for 30 hours. Paper was taken out of the chamber and dried thoroughly in hot air using a heavy duty oven. Paper was then turned at right angles to the initial direction of run and immersed in second solvent system of n-butanol : pyridine : water (1 : 1 : 1 v/v). After about 24 hours of development, the paper was removed and dried thoroughly in hot air to remove the traces of solvent completely. The paper was then sprayed with 0.2 % ninhydrin (in acetone) reagent.

spots of amino acids were developed after keeping the paper in an oven at 60°C for 30 minutes. Amino acid spots were identified by comparing their Rf values (Smith and Seakins, 1976) with those of authentic amino acid solutions developed under identical conditions.

THIN LAYER CHROMATOGRAPHY:

Thin layer chromatography was done on glass plates (20x20 cm) coated with a thin layer of microcrystalline cellulose. Adequate quantity of microcrystalline cellulose was mixed with appropriate amount of distilled water to form a smooth,paste-like slurry. It was spread over clean and dry glass plates with the help of an applicator. Plates were dried at room temperature. Before applying the samples, they were activated at 60°C for 30 minutes. The required amount of sample was applied about 3 cms above the lower edge of the plate, drop by drop, with simultaneous drying as described for paper chromatography (p. 39). Plates were developed in rectangular jars (25x25x10 cm) using n-butanol : acetic acid : water (4 : 1 : 1.6 v/v) for first dimensional run and n-butanol : pyridine : water (1 : 1 : 1 v/v) for second dimensional run respectively. Amino acid spots were located by spraying 0.2 % ninhydrin (in acetone), followed by drying in

an oven at 60°C for 30 minutes. Identification of amino acid spots was done by comparing their Rf values with those of standards developed under identical conditions.

C. Quantitative Analysis:

Amino acids in free pool and in proteins were separated by two dimensional paper chromatography. After identification, each amino acid spot was cut from the developed chromatogram and allowed to elute in 3 ml of 70% ethanol. Different concentrations (10 to 50 µg) of leucine were also spotted on chromatographic sheets and developed under identical conditions, followed by elution in 3 ml of 70% ethanol. These served as standards. A portion of the same paper, without a spot, was similarly eluted in 3 ml of 70% ethanol, which served as a blank. Optical densities were measured against blank in spekol at 570 nm. Amino acids were quantitated with reference to the standard leucine graph and percentage of each amino acid in the total pool was calculated. Proline could not be quantitated, hence percentage composition of each amino acid was calculated excluding proline.

Mean values of three observations were included in the quantitative data of amino acids.

GLUTAMATE OXALOACETATE TRANSAMINASE (GOT)

(L-aspartate:2 oxoglutarate aminotransferase, E.C. 2.6.1.1.)

Fresh samples of each stage were homogenized in icecold 0.25 M sucrose solution to approximately 10% (w/v) homogenate. It was centrifuged for 10 minutes at 3000 rpm and the supernatant taken for enzyme assay. GOT activity was estimated by the method of Reitman and Frankel (1957) as described by Bergmeyer and Bernt (1974).

PRINCIPLE:

Glutamate oxaloacetate transaminase catalyzes the reaction -

$$\text{L-aspartate} + \text{2 oxoglutarate} \xrightleftharpoons{\text{GOT}} \text{oxaloacetate} + \text{L-glutamate}$$

Oxaloacetate formed, spontaneously decarboxylates to pyruvate, which reacts with 2-4 dinitrophenyl hydrazine to give 2-4 dinitrophenyl hydrazone in alkaline medium. The characteristic colour of pyruvate hydrazone can be measured colorimetrically at 500-550 nm.

REAGENTS:

1. Buffer substrate solution (0.1 M phosphate buffers; pH 7.4; 0.1 M aspartate; 2 m M oxoglutarate): 1.50 g dipotassium hydrogen

phosphate (K_2HPO_4), 0.20 g of mono potassium hydrogen phosphate (KH_2PO_4), 30 mg of 2-oxo-glutaric acid and 1.32 g of L-aspartic acid were dissolved in 70 ml of distilled water; pH adjusted to 7.4 with 0.4 N NaOH and the solution diluted to 100 ml with distilled water.

2. Chromogen solution (1 m M 2,4-dinitrophenyl hydrazine): 20 mg of 2,4-dinitrophenyl hydrazine dissolved in 1 N HCl and made to 100 ml with the acid.

3. 0.4 N sodium hydroxide: 4 g of sodium hydroxide dissolved in 250 ml of di tilled water.

4. Standard pyruvate solution (2 m M): 22.0 mg sodium pyruvate dissolved in 100 ml of distilled water in a volumetric flask.

All the reagents except NaOH solution were stored at 4°C in brown bottles.

PROCEDURE:

Test: 1.0 ml of buffer substrate and 0.2 ml of enzyme extract were taken in a test tube. The reaction mixture was incubated for 60 min. at 37°C. The reaction was stopped by adding 1.0 ml of 2,4-dinitrophenyl hydrazine solution.

Control: At the end of incubation period, a control sample was prepared. 1 ml of buffer substrate and 1 ml of 2,4-dinitrophenylhydrazine were taken in a tube and mixed thoroughly. 0.2 ml of the enzyme extract was then added to it.

Blank: 1 ml of buffer substrate, 0.2 ml of distilled water and 1 ml of 2,4-dinitrophenyl hydrazine solution were mixed in a tube.

Standards: Different known concentrations of standard pyruvate were taken in a series of tubes and made up to 1.0 ml with buffer substrate. 0.2 ml of distilled water was then added to each tube, followed by the addition of 1 ml of 2,4-dinitrophenyl hydrazine solution.

All the tubes were allowed to stand at room temperature for exactly 20 minutes. Colour was produced by adding 10 ml of 0.4 N NaOH to each tube. Optical densities were measured after 5 minutes against blank at 545 nm in spekol. Activity was expressed in terms of µg pyruvate formed/mg protein/hour. Protein content of the extract was determined by the method of Lowry et al (1951) as described earlier (p. 34).

GLUTAMATE PYRUVATE TRANSAMINASE (GPT)

(L-Alanine : 2-oxoglutarate aminotransferase E.C. 2.6.1.2.)

Enzyme extract was prepared by the method as described for AST (p. 42). Activity was assayed by the method of Reitman and Frankel (1957) as described by Bergmeyer and Bernt (1974).

PRINCIPLE:

Glutamate pyruvate transaminase catalyzes the following reaction -

L-Alanine + 2 oxoglutarate $\xrightleftharpoons{GPT}$ Pyruvate + L-glutamate

Pyruvate formed thus, reacts with 2,4-dinitrophenyl hydrazine to give 2,4-dinitrophenyl hydrazone, which gives colour in alkaline medium. Intensity of colour is determined colorimetrically.

REAGENTS:

1. Buffer substrate solution (0.1 M phosphate buffer, pH 7.4, 0.2 M DL-alanine; 2 m M 2-oxoglutarate): 1.50 g dipotassium hydrogen phosphate(K_2HPO_4), 0.20 g monopotassium hydrogen phosphate (KH_2PO_4), 30 mg of 2-oxogluteric acid and 1.78 g DL-Alanine were dissolved in 70 ml of distilled water; pH adjusted to 7.4 with 0.4 N NaOH solution and diluted to 100 ml with distilled water.

2. Chromogen (1 m M 2,4-dinitrophenyl hydrazine): 20 mg of 2,4-dinitrophenyl hydrazine dissolved in 1 N HCl and diluted to 100 ml with the acid.

3. 0.4 N Sodium hydroxide: 4 g of NaOH pellets dissolved in 250 ml of distilled water.

4. Standard pyruvate (2 m M) solution: 22.0 mg of sodium pyruvate dissolved in 100 ml of distilled water.

All the reagents, except NaOH solution, were stored at 4°C in brown bottles.

PROCEDURE:

Test: 1 ml of buffer substrate and 0.2 ml of enzyme extract were taken in a test tube, thoroughly mixed and incubated for 60 minutes at 37°C. Reaction was stopped by adding 1 ml of 2,4-dinitrophenyl hydrazine at the end of incubation period.

Control: Towards the end of incubation period, a control sample was prepared by mixing 1 ml of buffer substrate with 1 ml of 2,4-dinitrophenyl hydrazine before the addition of 0.2 ml of enzyme extract.

Blank: 1 ml of buffer substrate was mixed with 0.2 ml of distilled water and 1 ml of 2,4-dinitrophenyl hydrazine solution.

Standards: Different known concentrations of standard pyruvate solution were taken in a series of tubes and made up to 1 ml with buffer substrate. 0.2 ml of distilled water was added to each tube, followed by the addition of 2,4-dinitrophenyl hydrazine.

All the tubes were allowed to stand at room temperature for exactly 20 minutes. 10 ml of 0.4 N NaOH was added to each tube and mixed. Optical densities were read after 5 minutes against blank in a spekol at 545 nm. GPT activity was expressed as µg pyruvate formed/mg protein/hour. Protein content of the extract was determined by the method of Lowry et al as described earlier (p. 34).

GLUTAMIC DEHYDROGENASE:

(E.C. 1.4.1.2-4).

Fresh samples of each stage were homogenized in ice cold 0.1 M phosphate buffer to give approximately 5% homogenates; homogenates centrifuged at 3000 rpm for 5 minutes at 4°C; supernatents used for enzyme assay by the modified method of Nachlas et al (1960).

PRINCIPLE:

L-Glutemate + NAD^+ $\xrightleftharpoons{GDH}$ 2-oxogluterate + NADH + H^+

NADH + Phenazine methosulphate + H^+ $\rightleftharpoons$ NAD^+ + Reduced phenazine methosulphate

Reduced phenazine methosulphate + Tetrazolium salt (INT) $\rightleftharpoons$ Phenazine methosulphate + Formazan (Reduced INT)

NADH formed in the first reaction, in the presence of phenazine methosulphate (PMS), reacts with 2-p-iodophenyl, 3-p-nitrophenyl, 5-phenyl tetrazolium chloride (INT) to form strongly coloured formazan (reduced INT). PMS serves as an intermediate electron carrier between this dehydrogenase and INT. Addition of simple protein (gelatin) into the reaction mixture keeps the 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 the solution(1) and 44 ml of the solution(2) were mixed; pH

adjusted to 7.7 with diluted KOH solution; diluted to the mark in a 100 ml volumetric flask; stored in cold.

4. 0.2 M Sodium glutamate solution: 3.74 g of sodium glutamate dissolved in 100 ml of distilled water; stored in cold.

5. Gelatin (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-phenyl tetrazolium chloride dissolved in 100 ml of double distilled water.

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

8. Phenazine methosulphate solution (8 mg/ml): 80 mg of PMS dissolved in 10 ml of distilled water; prepared fresh before use.

9. Phenazine methosulphate solution (10 mg/ml) for calibration curve: 100 mg of PMS dissolved in 10 ml of 0.1 M phosphate buffer kept at 37°C for 15 minutes. This was prepared just before use.

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

11. 15 m M NAD solution: 10.27 mg of NAD was dissolved in 1 ml of 0.1 M phosphate buffer. Solution was freshly prepared.

12. NADH solution: 50 mg of NADH dissolved in 30 ml cold distilled water. This solution was prepared just before use.

PROCEDURE:

Test: 1.4 ml of phosphate buffer, 0.5 ml of sodium glutamate, 1 ml of INT solution (2 mg/ml) 0.5 ml gelatin solution and 0.5 ml of enzyme extract were taken and mixed in a test tube. 0.1 ml of NAD solution was added to start the enzyme reaction, followed by the addition of 0.5 ml of PMS (4 mg/ml) solution. Contents were mixed and incubated at 37°C for exactly 15 minutes. Reaction was then stopped by adding 0.5 ml of 0.25 M HCl solution.

Control: 1.4 ml of phosphate buffer, 0.5 ml of sodium glutamate, 1.0 ml of INT (2 mg/ml), 0.5 ml gelatin and 0.5 ml 0.25 M HCl were taken and mixed in a tube. 0.5 ml of extract, 0.1 ml NAD and 0.5 ml PMS solution were added and shaken well.

Blank: Blank was prepared as control, except that equal volume of water was taken in place of INT and the enzyme extract.

optical density was read against blank at 540 nm in spekol. The GDH activity was calculated with reference to the standard graph and expressed as µg formazan formed/mg protein/hour. Protein content of the extract was determined by the method of Lowry et al (1951) as described earlier (p. 34).

RESULTS:

Quantification of total free amino acids and total proteins is given in Tables 2 & 3; Fig. 7. Total free amino acids steadily decreased after fertilization until morula stage; they increased to initial level during blastula and gastrula; they decreased again at closing of blastopore but became almost double and even more during comma and eye stages and rose exceedingly (more than 5 times) just prior to hatching (Fig. 7). Total proteins, on the other hand, remained constant throughout the embryonic development, except during closing of blastopore, when their level rose considerably and just before hatching, when their level depleted sharply (Fig. 7). Thus, while initial decrease in free amino acids was not accompanied with any rise in total protein, depletion later, i.e. at closing of blastopore, was accompanied with significant rise in protein, and further, spurt in total free amino acids at prior to hatching stage was accompanied with a sharp depletion in protein. Qualitatively,

there was no difference in the amino acids of free and protein pools except that while methionine could not be detected in free pool, α-amino-n-butyric acid was absent in proteins of the different embryonic stages (Fig. 8 to 28).

Data on quantification of individual free and protein amino acids have been included in Table 4 & 5. When amino acids were classified according to their charge and the concentrations of different groups (neutral, acidic and basic) compared, neutral amino acids constituted the bulk of both free and protein pools of all stages (Table 6). Further, while the neutral, basic and acidic amino acids remained relatively constant in proteins, they fluctuated widely in the free pool. The fall and rise in the levels of neutral amino acids correspond with those of total free amino acids until comma stage (Figs.7 & 29). During eye stage, however, while the neutral amino acids decreased, total free amino acids showed continued rise due to increase in acidic and basic group of amino acids (Figs. 7 & 29).

Activities of GOT and GPT transaminases have been shown in Tables 7 & 8 respectively and Fig. 30. Activity of GOT was found to be much higher than GPT throughout the embryonic development. Activity of GPT was observed to increase steadily following fertilization, through early morula - late morula -

Table - 2

Total free amino acids (mg/100 mg dry weight) in unfertilized eggs and in different embryonic stages of Cyprinus carpio

S.No.	Stages								
	Unfertilized egg	Early morula	Late morula	Blastula	Gastrula	Closing of blastopore	Comma	Eyed	Prior to hatching
1	0.305	0.242	0.294	0.447	0.535	0.504	1.032	1.240	2.730
2	0.400	0.447	0.205	0.800	0.400	0.207	1.247	1.400	4.510
3	0.480	0.258	0.176	0.611	0.376	0.517	1.035	1.240	4.047
4	0.353	0.117	0.205	0.211	0.376	0.258	0.511	0.587	1.694
5	0.517	0.329	0.294	0.305	0.564	0.400	0.558	0.750	3.011
6	0.847	0.558	0.647	0.634	0.705	0.800	1.550	1.730	4.510
7	0.682	0.564	0.558	0.400	0.729	0.423	0.682	1.110	3.380
Mean ± SD	0.500 ±0.19	0.379 ±0.19	0.339 ±0.18	0.524 ±0.25	0.540 ±0.15	0.452 ±0.20	0.980 ±0.35	1.151 ±0.38	3.411 ±1.03

T. No. - 3

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

S.No.	Stages								
	Unfertilized egg	Early morula	Late morula	Blastula	Gastrula	Closing of blastopore	Somite	Eyed	Prior to hatching
1	35.91	39.18	34.28	28.57	26.12	35.10	33.47	32.24	23.57
2	31.83	34.28	29.79	29.10	37.55	36.73	28.57	30.20	23.38
3	29.79	27.75	28.57	23.57	31.83	24.38	30.53	31.83	28.16
4	35.10	29.38	32.24	37.55	31.42	35.91	41.63	39.18	30.20
5	32.24	34.28	37.55	40.00	33.47	41.63	35.91	36.73	36.73
Mean	32.97	32.97	32.48	32.98	32.08	35.75	34.02	34.04	29.63
± SD	±2.50	±4.53	±3.59	±6.72	±4.12	±4.37	±5.09	±3.76	±4.70

Table - 4

Percentage composition of free amino acids in different embryonic stages of Cyprinus carpio

Amino acids	Stages								
	Unfertilized egg	Early morula	Late morula	Blastula	Gastrula	Closing of blastopore	Comma	Eyed	Prior to hatching
Leucine + Isoleucine	7.72	9.01	7.39	5.41	4.16	6.18	14.96	11.41	14.32
Phenylalanine	1.40	1.34	1.48	2.03	1.10	1.79	1.30	2.08	2.31
Unidentified spot	-	-	-	-	-	-	-	-	-
Valine	5.26	4.48	4.93	5.41	4.88	5.28	7.52	5.47	11.42
Methionine	-	-	-	-	-	-	-	-	-
Tyrosine	1.79	1.34	0.93	1.35	1.06	1.29	1.59	1.45	1.42
α-amino-n-butyric acid	1.05	1.79	0.98	1.72	1.99	1.79	1.40	1.20	1.98
Proline*									
Alanine	11.03	17.04	11.31	9.41	12.50	13.03	13.06	12.03	23.37
Threonine	3.16	2.24	2.46	3.01	2.63	3.99	3.17	2.52	1.03
Glutamic acid	18.95	22.42	22.66	22.51	17.37	12.58	9.02	15.16	11.72
Glycine	17.19	11.21	12.31	19.07	25.27	18.65	22.54	14.21	13.56
Arginine	3.51	4.03	3.45	3.01	1.99	3.20	1.64	3.56	1.54
Aspartic acid	8.42	12.55	10.34	11.57	10.99	15.45	8.98	5.70	2.68
Serine	5.26	1.79	1.97	2.70	2.54	3.09	2.12	3.40	0.94
Histidine	5.96	5.38	5.91	3.62	6.06	5.28	4.98	7.73	5.03
Lysine + Ornithine	8.41	6.27	12.83	9.10	7.39	8.36	7.17	12.54	7.53
Cystine	T	T	T	T	T	T	T	T	T

* not quantitated; T = Traces ; - = absent.

TABLE - 2

Percentage composition of protein amino acids in different embryonic stages of Cyprinus carpio

Amino acids	Stages								
	Unfertilized egg	Early morula	Late morula	Blastula	Gastrula	Closing of blastopore	Comma	Eyed	Prior to hatching
Leucine + Isoleucine	19.84	23.59	23.38	23.75	25.62	21.00	22.28	22.97	18.85
Phenylalanine	2.15	1.70	1.75	1.32	1.15	2.11	1.77	1.78	1.53
Unidentified spot	1.17	1.31	1.31	1.31	1.40	4.01	1.57	1.74	1.77
Valine	7.36	9.33	11.98	9.53	11.42	11.50	9.67	8.63	10.31
Methionine	2.01	1.99	1.83	1.96	2.01	2.14	1.94	1.83	2.15
Tyrosine	2.11	1.39	1.32	1.42	1.24	1.55	1.71	1.19	1.20
α-amino-n-butyric acid	-	-	-	-	-	-	-	-	-
Proline*									
Alanine	18.23	14.05	14.26	20.24	14.08	13.50	19.05	18.87	15.98
Threonine	4.65	3.67	4.81	4.03	3.08	5.58	4.28	3.91	4.05
Glutamic acid	9.77	8.84	6.83	9.44	8.91	8.74	8.60	8.15	12.67
Glycine	7.52	5.52	5.77	4.52	5.17	5.36	6.64	5.82	7.71
Arginine	4.65	4.90	4.12	3.62	4.94	3.70	4.66	6.34	4.88
Aspartic acid	6.50	4.92	6.64	6.09	5.09	6.99	5.47	5.63	7.04
Serine	1.39	1.26	1.50	1.27	1.52	1.53	1.16	1.26	1.13
Histidine	4.89	3.62	4.91	3.67	4.34	4.87	4.20	4.13	4.35
Lysine+Ornithine	6.35	7.62	9.18	7.17	11.21	6.77	6.67	6.04	5.51
Cystine	0.74	0.44	0.50	0.39	0.71	0.52	0.40	0.66	0.69

* Not quantitated; T = Traces, — = absent.

TABLE - 5

ercentage neutral, acidic and basic amino acids in unfertilized eggs and in different embryonic stages of Cyprinus carpio

	Stages	Neutral		Acidic		Basic	
		GAA	MAA	DAA	PAA	GAA	PAA
I	Unfertilized eggs	53.70	67.85	27.37	15.25	17.88	15.89
III	Early morula	49.34	69.90	34.97	13.75	15.69	16.34
IV	Late morula	43.63	66.27	33.00	13.52	22.19	18.21
V	Blastula	48.47	77.00	34.08	15.53	15.73	14.45
VI	Gastrula	54.21	69.41	28.35	14.10	15.44	20.49
VII	Closing of blastopore	53.33	69.43	28.03	15.73	16.85	15.34
VIII	Comma	56.81	70.40	18.00	14.07	13.79	15.53
IX	Eyed	54.11	69.71	20.86	13.78	23.83	16.51
X	Prior to hatching	58.52	69.44	14.40	13.71	15.10	14.85

TABLE - 7

Glutamate oxaloacetate transaminase activity (ug pyruvate/ug protein/hr) in unfertilized eggs and in different embryonic stages of Cyprinus carpio

S.No.	Stages									
	Unfertilized	Blastodisc	Early morula	Late morula	Blastula	Gastrula	Closing of blastopore	Comma	Eyed	Prior to hatching
1	61.99	66.48	74.37	54.80	57.95	57.03	60.56	54.63	47.84	60.53
2	61.61	67.92	78.16	63.69	69.55	56.51	59.24	52.00	59.25	60.53
3	61.99	70.81	71.85	65.18	73.41	71.84	59.24	54.63	52.76	63.11
4	63.37	70.81	70.59	59.25	59.25	72.95	52.66	52.00	54.38	60.53
5	66.12	72.26	75.63	60.73	69.55	60.00	57.93	53.47	59.25	57.95
6	60.61	76.59	70.59	62.21	74.70	61.47	57.93	53.47	57.63	59.24
7	60.61	70.81	68.07	60.73	60.53	65.92	57.93	53.47	54.38	61.84
8	60.61	75.15	65.55	-	61.82	61.47	50.03	50.68	46.26	57.95
Mean	61.99	71.35	71.85	60.94	65.84	62.40	56.94	53.04	53.97	60.21
± SD	±1.95	±3.36	±4.10	±3.35	±6.69	±4.68	±3.64	±1.38	±4.89	±1.79

Table - 4

Glutamate pyruvate transaminase activity (µg pyruvate/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	Comme	Eyed	Prior to hatching
1	4.69	11.00	3.44	10.38	11.98	15.56	12.14	13.87	14.49	17.96
2	6.51	7.33	10.13	12.27	10.26	9.73	13.87	13.87	16.56	16.25
3	6.51	11.00	3.44	8.49	13.68	15.56	13.87	12.14	16.56	17.96
4	-	9.16	3.44	12.27	13.68	13.61	12.14	13.87	16.56	14.54
5	8.37	7.33	3.44	8.49	11.98	11.67	17.34	12.14	20.70	17.96
6	8.37	11.00	10.13	10.38	10.26	19.45	12.14	12.14	18.63	17.96
7	12.35	7.33	10.13	10.38	10.26	15.56	12.14	12.14	8.28	21.39
8	9.88	11.00	11.82	8.49	11.98	11.67	17.34	13.87	18.63	17.96
Mean ± SD	8.09 ±2.53	9.39 ±1.82	9.50 ±1.26	10.13 ±1.56	11.76 ±1.43	14.10 ±3.07	13.87 ±2.27	13.00 ±0.92	16.30 ±3.74	17.75 ±1.93

Table - 2

Glutamate dehydrogenase activity (μ formazan/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	42.77	39.73	48.96	23.08	39.60	25.24	27.12	37.52	79.88	33.52
2	45.24	44.44	48.96	27.44	50.56	25.24	22.60	33.52	56.32	35.96
3	46.24	35.76	48.96	33.10	54.96	16.40	22.60	26.00	51.24	39.28
4	43.08	37.92	48.96	26.88	48.36	15.12	21.12	17.32	45.24	34.68
5	52.00	46.60	46.84	24.56	48.36	15.12	24.12	17.32	39.18	34.68
6	50.84	37.92	48.96	24.56	41.76	13.88	27.12	13.00	39.18	36.96
Mean	41.19	39.73	48.51	27.44	47.27	18.50	24.11	23.61	53.51	36.01
± SD	±3.52	±4.63	±0.85	±4.74	±5.68	±5.28	±2.51	±9.36	±16.40	±2.11

blastula until gastrula, became steady or slightly decreased thereafter during closing of blastopore and comma stages, and showed a spurt of activity during eyed stage, attaining peak just prior to hatching. GOT, on the other hand, showed an initial spurt in activity immediately following fertilization, i.e. at blastodisc, early morula and then again at blastula. In the post blastula stages, however, activity steadily decreased and had subnormal levels at comma and eyed stages. Activity again was found to increase just before hatching (Fig. 30).

Activity of glutamate denydrogenase (Table 9), while it rose appreciably during early morula, blastula and eyed stages, registered significant decline during late morula and remained subnormal during gastrula, closing of blastopore and comma stages. A perceptible decline in its activity was noticeable just prior to hatching (Fig. 31).

DISCUSSION

Monroy et al (1961) observed that fish embryos developed at the expense of yolk protein degradation and liberation of amino acids. Changes in the levels of protein and free amino acids are a measure of metabolic activity during ontogenesis, observed Zeitoun et al (1977) in Salmo gairdneri. Nakagawa and Tsuchiya (1972) found lipoprotein,

glycoprotein and phosphoprotein in the ratio 83:11:6 to compose yolk protein of Salmo gairdneri iridaus and consequent on their utilization during embryogenesis, decrease occurred almost equally in the three fractions. Earlier studies on cyclic changes in the levels of proteins during embryogenesis revealed that protein synthesis is not increased after fertilization (Krigsgaber and Neyfakh, 1968, '72). Zeitoun et al (1977), however, found a sudden decline of free amino acid level after fertilization accompanied by a slight rise in protein and attributed this to be due either to protein synthesis or to decline in protein catabolism. Protein synthesis begins to rise at blastula stage, increase being most intensive at onset of gastrulation, attaining maximum at the end of gastrulation (Williams, 1967; Nakagawa and Tsuchiya, 1972; Krigsgaber and Neyfakh, 1968, '72) Zeitoun et al, 1977).

A steady decrease of total free amino acids following fertilization until morula, without any concomitant increase in total protein levels in Cyprinus carpio, as observed by the present author and also reported earlier (Chetty et al, 1982), is in full agreement with Krigsgaber and Neyfakh (1968, 1972) and suggests further that the free amino acids during this phase are being utilized in energy metabolism. This agrees well with Williams (1967) who

emphasized that proteins and lipids are the energy sources during embryonic development of salmonid eggs, and not carbohydrates, except for a brief period at the onset of gastrulation, when the latter serve as the energy source (Daniel, 1947; Hishida and Nakano, 1954).

Decrease in the total free amino acids level, accompanied with a perceptible rise in total protein at closing of blastopore stage in Cyprinus carpio lends testimony to earlier observations of Krigsgaber and Neyfakh (1968, 1972) that protein synthesis is intensified at onset of gastrulation and is maximum towards the end of it. Further, increase in total free amino acids during comma and eye stages and excessive rise in prior to hatching stage clearly suggests extensive catabolism of proteins just before hatching. However, the nearly equal levels of protein in unfertilized eggs until early gastrula and then again during comma and eyed stage are related to translocation of yolk protein to tissue protein without any significant quantitative loss, as concluded by Williams (1967).

Transaminases provide reversible link between carbohydrate and amino acid metabolism and are involved in biosynthesis and degradation of a number of L-amino acids. In a regenerating system,

Ramachandran et al (1972) incriminated GPT in transamination of pyruvate into alanine and thence to alanine family of amino acids in the first week, when anaerobic break down of muscle glycogen was prominent, and GOT in transamination of oxaloacetate into aspartate family of amino acids, when aerobic oxidation of muscle glycogen became established in the second week of tail regeneration, following autotomy in Mabuya carinata. Although regeration system is different from embryonic development because of systemic involvement in the former, the two situations are comparable with regard to the need for amino acids, protein and nucleic acid biosynthesis since the egg membrane is impermeable to these metabolites.

Relatively greater increase in activity of GPT at gastrula, eyed and just prior to hatching stages is visualized to be on account of transamination of pyruvate into alanine and alanine family of amino acids, so necessary for intensified protein synthesis during these stages, synthesis of glycogen in the pregastrula stage rendering available a carbohydrate source of energy.

Peak activity of GOT at blastodisc and early morula obviously is due to transamination of aspartate into TCA cycle intermediates. Increased GOT activity

during blastula, on the other hand, is visualized to be on account of resumption of protein synthesis (Neyfakh and Abramova, 1974). Spurt in activity of GOT, as observed by the present author, and high GDH activity, as reported by Bhasker and Swarup (1977) in the prior to hatching stage of Cyprinus carpio, synchronizing with extensive catabolism of proteins and concomitant rise in levels of free amino acids, are clearly suggestive of extensive transamination of aspartate into energy cycle substrates at this crucial stage.

Relatively high activity of GDH at early morula falls in line with the general thesis that amino acids are degraded to provide substrates of energy metabolism except during gastrulation. Rise in GDH activity during blastula and the eyed stages is related to protein synthesis, which starts at the former stage and becomes very extensive at the latter stage. GDH catalyzes very specific reaction. It would not be too unreasonable to assume that some of the proteins synthesized at eyed stage possibly may be glutamate rich. Since not much work has been done in developmental physiology of fishes, it would only be safe to conclude that protein metabolism and energy metabolism are geared together in embryonic stages of Cyprinus carpio.