Introduction

Most animals live in environments in which important physical and biologic factors vary. The maintenance of internal stability (homeostasis), and indeed the very survival of the individuals, requires appropriately correcting physiologic, biochemical, and behavioural adjustments (Rosenzweig and Farner, 1978). Fishes inhabiting cavern - a state of continuous darkness, reduced oxygen availability, limited food supply and abundance of calcium in the water pockets, at near constant ambient temperature
and relative humidity, must be compelled to bring in specialization in the form of physiological and biochemical adjustments for successful survival. Curiously enough, there is a complete lack of information on changes/alterations (favourable ?) in biochemical parameters of any species of cave fish (also cave animals) attributable to the peculiar (subterranean) mode of life, notwithstanding availability of voluminous literature on a number of epigean fishes relating to biochemical variables and environment (Gus et al., 1978; Samanujam and Sathe, 1980; Bokdawals and George, 1967; Brekkan and Sagi, 1962; Chaskar et al., 1982; Kabe et al., 1980; Noda, 1967; Gordon, 1959).

This study is an attempt to determine a number of biochemical variables, viz., of protein metabolism, such as amino acids, proteins, and activities of transaminases; of carbohydrate metabolism, such as glycogen, lactate, pyruvate and activities of lactate and succinic dehydrogenases; activities of nonspecific phosphomonoesters and acetylcholinesterase; and the tissue levels of certain inorganic cations, such as Na+, K+, Mg++, and Ca++, the latter being particularly relevant in the context of a higher concentration of Ca++ in ambient medium. Levels of biochemical and enzyme activities in the tissue of cave fishes, Nemacheilus eveardi have been compared with levels of corresponding tissues of epigean fish, Lepidocrophalichthys guntla, with a view to make a candid assessment of adaptation.
which could be attributed to living under stress, i.e. under perpetual darkness in cave environment.

Materials and Methods

Fishes were sacrificed by decapitation and muscle, liver, gill and brain were recovered from autopsied fishes. Tissues were stored in deep-freezer at -20°C. Methods used in the determination of levels of several biochemical parameters and enzyme activities are as follows.

1 Total free aminoacid:

Known weights of fresh muscle tissue were homogenized in 2 ml distilled water and transferred to centrifuge tubes; equal volumes of 10% trichloroacetic acid added; the whole was centrifuged at 3,000 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).

Reagents:

1 Hydrindantin:

8 g of ninhydrin (1,23-Indan-tri-oxo-monohydrate, trioxohydrinden monohydrate) was added to 250 ml of distilled water at 90°C; 8 g of ascorbic acid
in 40 ml of water at 40°C was added while stirring. Crystallisation of hydindantin (following reduction of ninhydrin) started immediately; this was allowed to proceed at room temperature for 30 minutes. The hydindantin was filtered off, washed well with water, dried to a constant weight in vacuum desiccator and stored in brown bottle.

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 of distilled water. 12 ml of 0.2 M acetic(a) and 38 ml of 0.2 M sodium acetate solution(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 hydindantin dissolved in 75 ml of methyl collosolve;

25 ml of sodium acetate buffer (pH 5.5) was added and transferred to a dark coloured bottle to prevent interaction with light (ninhydrin being photosensitive). This reagent was prepared fresh.
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 exactly for 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 fresh weight.

2 Total protein

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

Principle

Protein reacts with the Folin-phenol reagent to give a coloured complex. The colour so formed is due to the reaction of copper in alkali with protein and subsequently of copper treated protein with Folin-phenol reagent.
Reagents:

1. 14% Trichloroacetic acid:
   14 g of TCA dissolved in 100 ml of double distilled water.

2. 7% Trichloroacetic acid:
   7 g of TCA dissolved in 100 ml of distilled water.

3. 0.4 N Sodium hydroxide:
   4 g sodium hydroxide (NaOH) dissolved in 250 ml of double distilled water.

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

5. 2% Sodium carbonate solution:
   2 g of sodium carbonate dissolved in 100 ml of 0.1 NaOH (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-sodium-potassium tartrate solution:
   50 mg of copper sulphate dissolved in 10 ml of 1% sodium potassium tartrate solution (6); this solution was freshly made.
Alkaline copper sulphate solution:
50 ml of sodium carbonate solution (5) and 1.00 ml of copper sulphate-sodium-potassium tartrate solution (7) mixed before use.

Commercial Folin-Phenol reagent:
Commercial Folin-Phenol reagent diluted with equal volume of distilled water on the day of use.

Standard protein solution:
20 mg of bovine serum albumin dissolved in 100 ml of distilled water; stored in refrigerator at 4°C.

Procedure:

100 mg of fresh muscle was homogenized in 2 to 3 ml of 0.9% saline and transferred to centrifuge tubes; protein precipitated by adding 2 to 3 ml of 14% TCA to homogenate and the whole centrifuged. Protein was purified as follows: washed twice in 14% TCA; washed in acetone to remove fats; treated with a mixture of methanol: chloroform (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; washed again twice in 7% TCA and finally dried in acetone, followed by ether.

Purified protein was dissolved in appropriate volume of 0.4 N NaOH. 1 ml of aliquot was pipetted in a test tube
and 5 ml of freshly prepared alkaline copper sulphate solution was added to it; 0.5 ml of freshly diluted Folin-Phenol reagent was added after 5 minutes and immediately shaken and incubated at 37°C for 30 minutes. The optical density of the colour developed was read at 750 nm in Spekol against blank of 1 ml of 0.1 N sodium hydroxide which was processed as the test.

Standard graph was prepared by using different concentrations of bovine serum albumin and processing the standard sample as in the case of test sample.

The total protein contents of the test sample was calculated with reference to the standard graph and expressed as mg/100 mg of wet weight.

Chromatographic analysis of amino acids:

a) Sample for free amino acids:

50 mg of fresh muscles were homogenized in 2 to 3 ml of 70% ethanol and left for 12 hours at 4°C for complete precipitation of protein. Homogenates were centrifuged at 3000 rpm for 15 minutes and supernatants were used for analysis.

b) Sample for protein hydrolysate:

Protein was extracted from 100 mg fresh muscle and
purified as described earlier. The purified protein was hydrolyzed in 6 N HCl at 115°C for 12 hours and hydrolysate was evaporated to dryness in a vacuum evaporator. Residue was dissolved in distilled water and again evaporated to remove traces of acids and finally dissolved in 5 ml of 70% ethanol for chromatographic analysis.

Qualitative analysis:

Qualitative analysis of free and protein amino acids was done by unidimensional 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 found to be n-Butanol : acetic acid : water (4:1:1.4 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:

The amino acids in each sample were analysed by the following technique: A drop of the sample was placed at a spot exactly 6 cm from both edges of a corner of a sheet of whatman paper no. 1 (46 x 57 cm). After thorough air-drying an additional drop was added to the same spot and allowed to
dry. This was done repeatedly until desired concentration of the sample was obtained on the paper. Chromatogram was developed in a standard chromatography chamber (Biswa and Co., Calcutta). One edge of the paper was immersed in first solvent system and allowed to run in an ascending manner along the longitudinal axis of the paper in machine direction until 2/3rd of the length of paper. The paper was then removed and dried in hot air oven. After the first run the paper was turned at right angle to the initial direction and again immersed in second solvent system for development. The solvent was allowed to run to 2/3rd of the length of the paper and then removed from the chamber. After thorough drying the paper was sprayed with 0.2% ninhydrin reagent (in acetone) and allowed to dry in an oven at 60°C for 30 minutes. Amino acids thus developed were identified by comparing their Rf values (Smith and Seakins, 1976) with those of authentic samples developed under identical conditions.

Thin layer chromatography:

Thin layer chromatography was carried out on glass plates (20 x 20 cm), using microcrystalline cellulose. A slurry was prepared by mixing microcrystalline cellulose in appropriate volume of distilled water. Slurry was spread over the glass plates with the help of an applicator and allowed to dry at room temperature. Before applying
the samples, plates were activated at 60°C for 30 minutes. Samples were applied with the help of a micropipette following the same procedure as described for paper chromatography. Two dimensional chromatogram was developed in a rectangular glass jar (25 x 25 x 10 cm) using the same solvent system (as described in paper chromatography) for first and second direction respectively. Amino acids were located by spraying 0.2 ninhydrin (in acetone) and were identified by comparing their Rf values with those of authentic amino acids developed under identical conditions.

Quantitative analysis:

Amino acids in free pool and in protein hydrolysates were separated by two dimensional paper chromatography. Spot of each amino acid was cut out from the chromatogram and eluted in 3 ml of 70% ethanol in a test tube. Standard chromatogram of leucine, with different known quantities, were developed under identical conditions and eluted in 3 ml of 70% ethanol. These served as standard series. A portion of paper, without a spot, was eluted in 3 ml of 70% ethanol to serve as a blank. Optical densities were measured at 570 nm in Zeiss Spekol and quantitation of the amino acids was done with the help of standard leucine curve. Percentage composition of each amino acid was calculated. The values were expressed as percentage composition of the pool, excluding proline.
Glutamate-oxaloacetate transaminase G.O.T.;

(L-Aspartate : 2 oxoglutarate aminotransferases, EC 2.6.1.1)

Fresh tissues were homogenized in ice cold 0.25 M sucrose solution to approximately 5% w/v. The homogenate was centrifuged for 15 minutes at 3000 rpm and the supernatant was used for enzyme assay. The enzyme activity was estimated by the colorimetric method of Leitner and Frankel (1957) as described by Sorogyen and Bernt (1974).

Principle:

L-Aspartate and 2-oxoglutarate react to form pyruvate according to equation

\[(\text{L-Aspartate} + 2\text{-oxoglutarate} \xrightarrow{\text{transaminase}} \text{oxaloacetate} + L\text{-glutamate})\]

and

\[(\text{oxaloacetate} \xrightarrow{\text{decarboxylation}} \text{pyruvate})\]

Pyruvate formed reacts with 2,4-dinitrophenylhydrazine to give 2,4-dinitrophenylhydrazone which gives colour with alkali. Intensity of colour is determined colorimetrically.

Reagents:

1. Buffer substrate solution (0.1 M phosphate buffer, pH 7.4, 0.1 M aspartate; 2 mM-oxoglutarate);
2. 1.50 g dipotassium phosphate \((K_2HPO_4)\), 0.20 g of
monopotassium phosphate ($K_2HPO_4$), 30 mg of 2-oxoglutaric acid and 1.33 g of L-aspartic acid were dissolved in 70 ml of double distilled water; pH adjusted to 7.4 with 0.4 N NaOH solution and the solution made to 100 ml with distilled water.

2 Chromogen (1 ml 2,4-dinitrophenylhydrazine):
20 mg of 2,4-dinitrophenylhydrazine dissolved in 1 N HCl and made to 100 ml.

3 0.4 N sodium hydroxide.
4 g of sodium hydroxide dissolved in 25 ml of distilled water.

4 Standard pyruvate (2 ml):
22.5 mg sodium pyruvate dissolved in 100 ml distilled water; stored at 4°C in a brown bottle.

Procedure:

Test:

1 ml of buffer substrate and 0.2 ml homogenate were taken in a test tube. The reaction mixture was incubated for 60 minutes at 37°C. The reaction was stopped by adding 1 ml of 2,4, dinitrophenyl hydrazine solution.
Control:

Towards the end of the incubation period, a control sample was prepared taking 1 ml of buffer substrate and 1 ml of 2,4, dinitrophenyl hydrazine solution; 0.2 ml of homogenate was added to it.

Blank:

1 ml of buffer substrate, 0.2 ml distilled water and 1 ml of 2,4, dinitrophenyl hydrazine solution mixed together.

Standard:

Different volumes of standard pyruvate taken in test tubes are made to 1.2 ml with buffer substrate; 1 ml of 2,4, dinitrophenyl hydrazine was then added.

Tubes containing test, control, blank and standard solutions were allowed to stand at room temperature for 2 minutes. 1 ml of 0.4 N sodium hydroxide solution was added to each of the five tubes and mixed. Optical density was measured after 5 minutes against blank at 345 nm in spectrophotometer. The values are expressed in μg pyruvate formed/mg protein/hour.
Protein content of the homogenate was determined by the method of Lowry et al. (1951), as described earlier (p 116).

Glutamate pyruvate transaminase G.P.T.:

\[ \text{L-alanine:2-oxoglutarate aminotransferase, EC 2.6.1.2} \]

Homogenate was prepared as described for G.O.T.

Enzyme activity was assayed by the method of Böltzman and Frankel (1957), as described by Bergmeyer and Bernt (1974).

Principle

L-alanine and 2-oxoglutarate react to form pyruvate according to equation

\[ \text{L-alanine} + 2\text{-oxoglutarate} \xrightarrow{\text{G.O.T.}} \text{pyruvate} + \text{L-glutamate} \]

Pyruvate so formed reacts with 2,4-dinitrophenyl hydrazine to give 2,4-dinitrophenyl hydrazone which gives colour with alkali. 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 2-oxoglutarate):

- 1.50 g dipotassium phosphate (K₂HPO₄), 0.25 g monopotassium phosphate (KH₂PO₄), and 9 g of 2-oxoglutarate.
acid and 1.73 g DL-alanine were dissolved in 70 ml of distilled water; pH adjusted to 7.4 with 0.4 N NaOH solution and made to 100 ml with distilled water.

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

3 0.4 M sodium hydroxide:
4 g of sodium hydroxide dissolved in 250 ml of distilled water.

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

Procedure:

Test:
1 ml of buffer substrate and 0.2 ml of pyruvate were taken in a test tube. The reaction mixture was incubated for 60 minutes at 37°C. The reaction was stopped by adding 1 ml of 2,4-dinitrophenyl hydrazine solution.

Control:

During the end of incubation period, a control sample was prepared by mixing together 1 ml of buffer substrate,
1 ml of 2,4-dinitrophenyl hydrazine solution and 0.2 ml of homogenate.

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

Standard:
Different volumes of standard pyruvate solution were made to a final volume of 1.2 ml with buffer substrate; 1 ml of 2,4-dinitrophenyl hydrazine solution was then added.

Tubes containing test, control, blank and standard solutions were allowed to stand at room temperature for 20 minutes. 1 ml of 1.4 M sodium hydroxide solution was added to each of the above tubes and mixed. Optical density was measured after 5 minutes against blank at 545 nm in a spectrophotometer. The values are expressed as µg pyruvate formed/mg protein/hour.

Protein content of the homogenate was determined by the method of Lowry et al. (1951) as described earlier (p 115).
Pyruvic acid:

Known amounts of fresh muscle were homogenized in 3 ml of cold distilled water and the whole transferred to centrifuge tubes. Protein was precipitated in each tube by adding equal volumes of 10% trichloroacetic acid; the tubes were then centrifuged at 3000 rpm for 15 minutes. Protein-free supernatant was used as test sample for estimation of pyruvic acid by 2,4-dinitrophenylhydrazine method of Edelmann and Haapa (1943).

Principle:

The protein-free filtrate was treated with 2,4-dinitrophenylhydrazine to form 2,4-dinitrophenylhydrazone, which reacts with a strong alkali. The pyruvic hydrazone can preferentially be extracted with sodium bicarbonate which is added progressively.

Reagents:

1. 10% Trichloroacetic acid: 1.0 g of TCA dissolved in 1.0 ml of distilled water.

2. 2,4-dinitrophenylhydrazine: small volume of 2 N HCl plus to 1.0 mg of 2,4-dinitrophenylhydrazine and made to 1.0 ml, filtered and stored in cold.
3 10% Sodium carbonate:

10 g of sodium carbonate dissolved in 100 ml of distilled water.

4 1.5 N Sodium hydroxide:

5 g of sodium hydroxide (1N) dissolved in 100 ml of distilled water.

5 Stock standard pyruvic acid solution:

1.87 mg pyruvic acid dissolved in 0.1 N sulphuric acid and made to 100 ml.

6 Working standard pyruvic acid solution:

1 ml of solution (b) diluted to 100 ml with distilled water. This gave 0.01 mg of pyruvic acid per ml.

Procedure:

1 ml of 4-hydroxy-3-methylphenylhydrazine was added to 3 ml of protein free filtrate and 1 ml of working standard solution (b) in separate tubes. 3 ml of xylene was added to each of the above tubes; 3 ml of air was bubbled into each for 2-3 minutes, allowed to settle; lower layer was removed from each tube by tilting the tube.

6 ml of 10% sodium carbonate was then added to each and the solutions titrated to faint pink color for
2 minutes. 5 ml each of lower solution was taken in fresh tubes and 5 ml of 1.5 N sodium hydroxide solution was added to each. The tubes were left for 5-10 minutes.

Optical density was measured at 520 nm, against a blank, prepared by mixing 5 ml of 10 % Na₂CO₃ and 5 ml of 1.5 N sodium hydroxide. Pyruvate content was calculated with reference to standard and expressed as μg pyruvate/100 mg fresh weight.

Lactate dehydrogenase:
(L-lactate : NAD oxidoreductase, EC No. 1.1.1.27)

Muscle was homogenized in ice cold 0.25 M sucrose solution to approximately 5 % (w/v) homogenate. The homogenate was centrifuged at 3,000 rpm at 4°C for 5 minutes. The clear supernatant was used for enzyme assay by the method as described in Sootton (1974).

Principle:

Lactate dehydrogenase (LDH) is an enzyme of almost universal distribution and catalyses the reversible transformation of pyruvate to lactate. Pyruvate is reduced to lactate by incubation with homogenate in the presence of coenzyme NAD+. The reaction is stopped by adding dinitrophenyl hydrazine solution which reacts with the
remaining pyruvate forming 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 isoenzymes 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):

3.445 g of disodium hydrogen phosphate (Na$_2$HPO$_4$) and 0.675 g potassium dihydrogen phosphate (KH$_2$PO$_4$) 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 dinucleotide (NADH):
10 mg of NADH dissolved in 10 ml of phosphate buffer. This was prepared fresh for each batch of tests.

6 2,4-dinitrophenyl hydrazine (2 mM):
40 mg of dinitrophenyl hydrazine 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 0.4N sodium hydroxide solution:
16 g of sodium hydroxide dissolved in 1 litre distilled water.

Procedure:

Test:
1 ml of buffered substrate (4) and 0.1 ml homogenate each 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 dinitrophenyl hydrazine 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 dinitrophenyl hydrazine solution (6) taken in test tube.

Blank:

1.2 ml phosphate buffer (2) and 1 ml of dinitrophenyl hydrazine solution (6) in another test tube.

Tubes containing test, control and blank solutions 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 Spekol. The values are expressed as \( \mu \text{mol pyruvate reduced/mg protein/hour} \).

Protein content of the homogenate was determined by the method of Lowry et al. (1951), as described earlier on page 116.

Succinate dehydrogenase:

(Succinate (Acceptor) oxidoreductase ; EC No. 1.3.99.1)

Fresh muscle was homogenized in ice cold 0.1 M phosphate buffer (pH 7.7) to approximately 5 \%(w/v) homogenate homogenates centrifuged at 3000 rpm at 4°C for 5 minutes; supernatants used for enzyme assay by the method of Nachlas et al. (1960).
Principle

\[
\text{Succinate} + \text{E FAD} \rightarrow \text{Fumarate} + \text{E} + \text{FADH}_2
\]

\[
\text{FADH}_2 + \text{Phenazine} \rightarrow \text{FAD} + \text{Reduced PMS}
\]

\text{Reduced PMS} + \text{Methosulphate} \rightarrow \text{Formazan} + \text{Phenazine}

\text{Tetrazolium salt} + \text{Methosulphate}

FADH\textsubscript{2} formed in first reaction, in the presence of phenazine methosulphate (PMS), reacts with 2-p-iodophenyl 3-p-nitropheryl 5-phenyltetrazolium chloride (INT) to form strongly coloured formazan (reduced tetrazolium salt). Phenazin 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 intensity in aqueous solution.

Reagents:

1. 0.2 M Monopotassium solution:
   
   2.721 g KH\textsubscript{2}PO\textsubscript{4} dissolved in 100 ml of distilled water.

2. 0.2 M Potassium hydroxide solution:
   
   1.002 g K\textsubscript{2}CO\textsubscript{3} 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.

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

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-phenyl tetrazolium chloride dissolved in 100 ml of distilled water.

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

8 Phenazine methosulphate solution:
80 mg of phenazine methosulphate dissolved in 10 ml of distilled water; solution stored at 5°C in brown bottle.
Phenazine methosulphate (for calibration curve):
100 mg of phenazine methosulphate dissolved in
10 ml of 0.1 M phosphate buffer kept at 37°C

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

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

Procedure:

Test:
1.5 ml of phosphate buffer, 0.5 ml of sodium succinate
solution, 1.0 ml of INIT solution, 0.5 ml gelatin
solution and 0.5 ml of homogenate were taken in test
tube; 0.5 ml of phenazine methosulphate solution
was immediately added to above. 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 with mixing.

Control:
1.5 ml of phosphate buffer, 0.5 of INIT sodium
succinate solution, 1.0 ml of INT solution, 0.5 ml gelatin solution and 0.5 ml of 0.25 M hydrochloric acid were taken in a test tube; 0.5 ml homogenate and 0.5 ml of phenazine methosulphate added to above.

Blank:

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.

Succinate dehydrogenase activity was calculated from the standard graph and expressed as μg of formazan formed/mg protein/minute. Protein content of the homogenate was determined by the method of Lowry et al. (1951), as described earlier on page 116.

Inorganic cations:

Known weights of muscles were digested with concentrated nitric acid and processed for the analysis of inorganic ions by atomic absorption spectrophotometry. Water samples collected from the spot were also analysed by this method.
Principle:

In atomic absorption spectrophotometry, a light beam is directed, through the flame in to a monochromator and on to a detector that measures the amount of light absorbed by the atomized element in the flame. The amount of light absorbed at a wavelength characteristic to a particular element is proportional to the concentration of element.

Reagents:

A stock solution (Vogel, 1961):

Standard stock solutions of Na⁺, K⁺, Ca⁺⁺ and Mg⁺⁺ were prepared as follows:

1 Sodium solution:
   2.5418 g of anhydrous NaCl dissolved in 1 litre of deionized distilled water; this solution contains 1000 µg of sodium/ml.

2 Potassium solution:
   1.909 g of KCl dissolved in 1 litre of deionized distilled water. The solution contains 1000 µg of potassium/ml.

3 Calcium solution:
   2.4973 g calcium carbonate dissolved in dilute
HCl and the whole solution made to 1 litre with
deionized distilled water. The solution contains
1000μg of calcium/ml.

4 Magnesium solution:

10.0135 g of magnesium sulphate (MgSO₄·7H₂O) 
dissolved in 200 ml of deionized distilled water; 1 ml of concentrated nitric acid was then added and 
the whole made to 1000 ml with deionized distilled 
water. One ml of this solution contains 1000 μg of 
magnesium.

B Working standard solution:

Working standard solutions of Na⁺, K⁺, Ca²⁺ and 
Mg²⁺ containing different concentrations (10, 25, 50, 75 
and 100 μg/ml) were prepared by diluting appropriate 
volumes of stock solutions.

Procedure:

Fresh muscles were washed thoroughly in deionized distill 
water to remove adhering substances and weighed. Clean muscular 
pieces were digested overnight in 2–4 ml of concentrated 
nitric acid. Digested samples were diluted appropriately 
with deionized distilled water. 5 ml aliquot were taken;
1 ml of lithium (for $Li^+$ and $K^+$) or 1 ml of $La^{++}$ sodium (for $Ca^{++}$ and $mg^{++}$) was then added and diluted to 10 ml with deionized distilled water.

The samples were aspirated and atomized in a flame and absorbance measured by Atomic Absorption Spectrophotometer (Double beam, AA-575 series).

Ionic concentration in muscle and water were expressed as mg/100 mg fresh weight and mg/1000 ml respectively.

Methods for determining the following parameters, viz., glycogen, lactate, nonspecific phosphomonoesterases, and acetylcholinesterase have been described, in detail, earlier (Chapter III).

Results
Total free amino acids and total protein:

Levels of total free amino acids and total protein in the muscle tissue of cave fish were found to be higher, when compared with epigean fish. While the level of total free amino acids ($P < 0.05$), differed statistically, that of total protein did not (Table 23).
Qualitative analysis of free and protein amino acids:

Chromatographic analysis revealed qualitative differences in the free amino acid profiles of cave and epigean fishes in the absence of phenylalanine and methionine in the cave fish and cystine in the latter. However, proline and lysine were absent from free pool of amino acids of both cave and epigean fishes (Plates 1 and 2; Table 24). Protein amino acid profiles of cave and epigean fishes, however, were found alike. In all, 19 amino acids were located in protein hydrolysates of both cave and epigean fishes (Plates 2 and 3; Table 25).

Percentage composition of free and protein amino acids:

When percentage composition of individual amino acids in the free pool were computed, isoleucine, valine, a-amino-butyric acid, glutamic acid, glycine, serine, histidine and ornithine were each found to be statistically significantly less in cave fish as compared with epigean fish (Table 26). However, the percentages of alanine and aspartic acid were strikingly higher in cave fish (Table 26).

Percentage composition of all amino acids of protein except phenylalanine, threonine and serine, nearly corresponded in cave and epigean fish (Table 27).
G.P.T. and G.O.T.: 

Activity of GPT, irrespective of tissue (liver, brain muscle or gill) was found statistically significantly higher in cave fish as compared with epigean fish (Table 29). GOT activity, likewise, was found statistically significantly higher in liver, muscle, and gill tissues of the cave fish as compared with epigean fish (Table 29).

Acid phosphatase:

The average (± SE) values of activity of acid phosphatase in different tissues are shown in table (30). There was no statistically significant difference, irrespective of tissues (viz., liver, gill, muscle), in acid phosphatase activity of cave fish as compared with that of epigean fish (Table 30).

Alkaline phosphatase:

Of the three tissues, liver, muscle and gill, alkaline phosphatase activity of gill alone was found statistically significantly higher in cave fish in epigean fish (Table 31).

Acetylcholinesterase:

Activity of acetylcholinesterase of both brain and gill of cave fish was found statistically significantly
higher than corresponding values of epigean fish (Table 32).

Glycogen:

Levels of muscle glycogen in cave and epigean fish averaged $0.29 \pm 0.017 \text{ (mg/100 mg)}$ and $0.37 \pm 0.02 \text{ (mg/100 mg)}$ respectively, the difference in average concentration being statistically significant (Table 33).

Lactate:

Lactate levels of muscle of cave and epigean fishes were found to be $52.13 \pm 2.23 \text{ (\mu g/100 mg)}$ and $30.35 \pm 2.60 \text{ (\mu g/100 mg)}$ respectively and the difference was statistically significant (Table 33).

Pyruvate:

Pyruvate level of muscle of epigean fish was significantly higher, being $5.32 \pm 0.40 \text{ (\mu g/100 mg)}$ as compared from $2.45 \pm 0.30 \text{ (\mu g/100 mg)}$ in cave fish (Table 33).

Lactate dehydrogenase:

LDH activity of muscle of cave and epigean fish was found to be $6.90 \pm 0.50 \text{ (\mu g_{PO}_4/\text{mg h})}$ and $4.73 \pm 0.35 \text{ (\mu g/\text{mg h})}$ respectively and the difference in activity was statistically significant (Table 34).
Succinic dehydrogenase:

SDH activity of muscle of epigean fish, on the other hand, was found to be significantly higher, being \(2.70 \pm 0.20\) (\(\mu\)g formazan /mg/h) as compared from \(1.76 \pm 0.12\) (\(\mu\)g formazan /mg/h) (Table 34).

Inorganic cations:

The averages (\(\pm SE\)) of levels of inorganic ions, viz., Na\(^+\), K\(^+\), Ca\(^{++}\) and Mg\(^{++}\) in muscle are shown in table(35). Whereas Na\(^+\) was significantly higher in cave fish, Ca\(^{++}\) was found higher in epigean fish. Potassium and magnesium levels, however, although higher in cave fish as compared from epigean fish, the difference was not statistically significant.

Ionic composition of water inside the cave was found higher in respect of Na\(^+\), K\(^+\), Ca\(^{++}\) and Mg\(^{++}\) as compared from water in epigean environment (Table 36).

Discussion

Information on the free amino acid content of fish muscle is voluminous. Reports include studies on this tissue from a number of fishes, viz., Lemon sole (Jones, 1959), Mackerel (Sakaguchi and Simidu, 1965), Anchovy (Wakaki and Suyama, 1966), Carp (Sakaguchi and Kawai, 1970) and
Alaskan Pollak (1960, 1973). The amino acid content of deep water fish of the Bering Sea has also been studied (Nasedkina and Mezhcheryakova, 1966). Siddiqui and Siddiqui (1969), Partmann and Schiasszus (1973) and Suyama and Yoshizawa (1973) studied free amino acids in the muscle of six species of North Indian fresh water fishes, 13 species of fresh water and marine fishes, and 13 species of migratory fishes respectively. However, the present results provide a first hand account of total amino acids of protein, and free and protein amino acids of a cave fish, Neocarchius eyzardi. Data on epigean fish, L. guntia, belonging to the same family, allow comparison.

While muscle content of total free amino acids in cave fish was statistically significantly higher as compared from epigean fish, there was no significant change in the total protein content of the muscle tissue of both fishes. Absence of phenylalanine, proline, lysine and methionine from free pool in cave fish and of proline, lysine and cystine from free pool in epigean fish is striking. The physiological significance of this is difficult to explain at the moment.

The percent composition of alanine and aspartic acid of free pool of amino acids was statistically significantly more in cave fish as compared from epigean fish. These results corroborate observations of Bras et al. (1976) in rainbow trout and Recheh (1966) in carp. Higher amount of
alanine observed in the cave fish might serve as intracellular osmotic effector in the control of the water activity of the cell interior. Similar conclusions were drawn by a number of workers (Schoffeniels, 1976; Camen et al., 1951; Duchateau and Florkin, 1961; Bricteux Gregoire et al., 1962) in epigean fishes and aquatic invertebrates. Statistically significantly higher value of aspartic acid in the muscle tissue of cave fish, along with significantly higher level of GPT activity, is suggestive of extensive transamination. Statistically significantly lower values of isoleucine, valine, $\alpha$-amino butyric acid, glutamic acid, glycine, serine, histidine and ornithine in muscle tissue of cave fish, as compared with epigean fish, were also observed. However, at present it is difficult to explain the physiological significance of such alterations. In protein pool, however, near correspondence between qualitative and percent composition, of amino acids was noticeable in muscle of cave and epigean fishes except a significant decrease in phenylalanine and increase in threonine and serine in the former.

Interestingly, irrespective of tissues (liver, brain, muscle or gill) alanine amino transferase (GPT) activity in cave fish was found to be approximately four times higher than in epigean fish. Would it be that the high concentration of muscle alanine in cave fish is brought about by way of
higher activity of GPT? The GPT activity in muscle tissue of cave and epigean fish, respectively, in the order of $15.69 \pm 3.96$ (µg pyruvate/mg protein/h) and $3.71 \pm 0.67$ (µg pyruvate/mg protein/h), lends support.

Like GPT, GOT activity was also found to be significantly higher in liver, muscle and gill of cave fish. This again is perhaps due to extensive transamination of oxaloacetate into aspartic acid.

Despite high calcium level in water inside cave, *Hemacheilus avunculi* showed much less muscle Ca$^{2+}$ level. Calcium levels in fish blood are known to be relatively constant, irrespective of calcium concentration outside (Jayan et al., 1981). Present results suggest that this may be true also for other tissues. Higher level of sodium in muscle tissue of cave fish, in spite of calcium rich environment, is perhaps due to ionic regulation through gill by active uptake. Significantly higher activity of both acetylcholinesterase and alkaline phosphatase in gill of cave fishes, as compared with epigean fishes, lends testimony to this.

Fishes in the cave environment besides being under photostress, are obviously living under several other stressful conditions including starvation effects.
Statistically significantly lower level of muscle glycogen and pyruvate amply corroborate the starvation effect (Kamalapan and Math, 1980). Further testimony is lent by the very high transaminase activity, both GPT and GSH, and the concomitant high levels of alanine and aspartic acid in the free pool of amino acids. Further, pyruvate and oxaloacetate are being utilized extensively for alanine and aspartic acid biosynthesis. Aside this, the relatively higher level of lactate and corresponding higher LDH activity further substantiate greater dependence of these fishes on glycolysis for energy requirement (Hochackka, 1969). Relatively much lower activity of LDH in cave fish, as compared with epigean fish, further affirms the above contention.
Table 2: Total free amino acids and protein contents in muscle of cave and epigean fish.

<table>
<thead>
<tr>
<th></th>
<th>Cave Fish</th>
<th>Epigean Fish</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>2.44 ± 0.05</td>
<td>0.49 ± 0.05</td>
</tr>
<tr>
<td>Total Protein (mg/100 mg fresh weight)</td>
<td>20.35 ± 1.79</td>
<td>16.07 ± 1.42</td>
</tr>
</tbody>
</table>

Figures in parentheses represent number of fishes in each group.

p < 0.05 statistically significant.
Table 24: Free amino acids in muscle of cave and epigean fish

<table>
<thead>
<tr>
<th>S.N.</th>
<th>Amino acid</th>
<th>Cave fish</th>
<th>Epigean fish</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Leucine</td>
<td>+^b</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>Isoleucine</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>Phenylalanine</td>
<td>_c</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td>Valine</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>Methionine</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>Tyrosine</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>7</td>
<td>a aminobutyric acid</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>8</td>
<td>Proline</td>
<td>=</td>
<td>=</td>
</tr>
<tr>
<td>9</td>
<td>Alanine</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>10</td>
<td>Threonine</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>11</td>
<td>Glutamic acid</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>12</td>
<td>Glycine</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>13</td>
<td>Arginine</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>14</td>
<td>Aspartic acid</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>15</td>
<td>Serine</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>16</td>
<td>Histidine</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>17</td>
<td>Ornithine</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>18</td>
<td>Lysine</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>19</td>
<td>Cystine</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

^b Denotes presence

c Denotes absence
<table>
<thead>
<tr>
<th>S.N.</th>
<th>Amino acid</th>
<th>Cave fish</th>
<th>Epigean fish</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Leucine</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>Isoleucine</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>Phenylalanine</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td>Valine</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>Methionine</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>Tyrosine</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>7</td>
<td>α amino butyric acid</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>8</td>
<td>Proline</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>9</td>
<td>Alanine</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>10</td>
<td>Threonine</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>11</td>
<td>Glutamic acid</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>12</td>
<td>Glycine</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>13</td>
<td>Arginine</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>14</td>
<td>Aspartic acid</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>15</td>
<td>Serine</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>16</td>
<td>Histidine</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>17</td>
<td>Ornithine</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>18</td>
<td>Lysine</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>19</td>
<td>Cystine</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Legend same as Table 24
Table 26: Percentage composition of free amino acid in muscle of cave and epigean fish

<table>
<thead>
<tr>
<th>S.N.</th>
<th>Amino acid</th>
<th>Cave fish</th>
<th>Epigean fish</th>
<th>t</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Leucine</td>
<td>1.23±0.10*</td>
<td>1.60±0.15</td>
<td>2.052</td>
<td>NS</td>
</tr>
<tr>
<td>2</td>
<td>Isoleucine</td>
<td>1.06±0.04</td>
<td>1.27±0.05</td>
<td>3.280</td>
<td>&lt;0.02</td>
</tr>
<tr>
<td>3</td>
<td>Phenylalanine</td>
<td>-</td>
<td>0.63±0.07</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Valine</td>
<td>2.00±0.09</td>
<td>2.78±0.16</td>
<td>4.249</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>5</td>
<td>Methionine</td>
<td>-</td>
<td>0.32±0.05</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Tyrosine</td>
<td>0.33±0.05</td>
<td>0.48±0.04</td>
<td>2.120</td>
<td>NS</td>
</tr>
<tr>
<td>7</td>
<td>α amino butyric acid</td>
<td>1.34±0.15</td>
<td>2.20±0.16</td>
<td>3.921</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>8</td>
<td>Proline</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>Alanine</td>
<td>26.25±1.93</td>
<td>16.59±0.40</td>
<td>4.901</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>10</td>
<td>Threonine</td>
<td>5.59±0.29</td>
<td>6.16±0.54</td>
<td>1.077</td>
<td>NS</td>
</tr>
<tr>
<td>11</td>
<td>Glutamic acid</td>
<td>9.37±0.72</td>
<td>11.43±0.37</td>
<td>2.544</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>12</td>
<td>Glycine</td>
<td>15.83±0.88</td>
<td>13.25±1.10</td>
<td>4.579</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>13</td>
<td>Arginine</td>
<td>11.64±0.98</td>
<td>11.71±0.76</td>
<td>0.056</td>
<td>NS</td>
</tr>
<tr>
<td>14</td>
<td>Aspartic acid</td>
<td>18.44±0.39</td>
<td>10.30±0.82</td>
<td>8.964</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>15</td>
<td>Serine</td>
<td>2.30±0.30</td>
<td>4.00±0.50</td>
<td>2.603</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>16</td>
<td>Histidine</td>
<td>3.73±0.18</td>
<td>5.70±0.34</td>
<td>5.121</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>17</td>
<td>Ornithine</td>
<td>0.79±0.06</td>
<td>2.53±0.23</td>
<td>7.533</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>18</td>
<td>Lysine</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>Cystine</td>
<td>0.22±0.2</td>
<td>-</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

See legend to Tables 3 and 24
Table 27: Percentage composition of protein amino acid in muscle of cave and epigean fish

<table>
<thead>
<tr>
<th>S.N.</th>
<th>Amino acid</th>
<th>Cave fish</th>
<th>Epigean fish</th>
<th>t</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Leucine</td>
<td>8.14±0.40</td>
<td>7.71±0.32</td>
<td>0.839</td>
<td>NS</td>
</tr>
<tr>
<td>2</td>
<td>Isoleucine</td>
<td>4.53±0.27</td>
<td>5.50±0.34</td>
<td>2.234</td>
<td>NS</td>
</tr>
<tr>
<td>3</td>
<td>Phenylalanine</td>
<td>2.16±0.19</td>
<td>3.14±0.20</td>
<td>3.552</td>
<td>&lt; 0.02</td>
</tr>
<tr>
<td>4</td>
<td>Valine</td>
<td>5.35±0.48</td>
<td>5.15±0.27</td>
<td>0.363</td>
<td>NS</td>
</tr>
<tr>
<td>5</td>
<td>Methionine</td>
<td>3.27±0.22</td>
<td>3.55±0.17</td>
<td>0.672</td>
<td>NS</td>
</tr>
<tr>
<td>6</td>
<td>Tyrosine</td>
<td>2.09±0.10</td>
<td>2.12±0.16</td>
<td>0.159</td>
<td>NS</td>
</tr>
<tr>
<td>7</td>
<td>α-amino butyric acid</td>
<td>3.08±0.12</td>
<td>3.63±0.28</td>
<td>0.021</td>
<td>NS</td>
</tr>
<tr>
<td>8</td>
<td>Proline</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>Alanine</td>
<td>9.21±0.75</td>
<td>7.85±0.20</td>
<td>1.752</td>
<td>NS</td>
</tr>
<tr>
<td>10</td>
<td>Threonine</td>
<td>6.50±0.30</td>
<td>5.07±0.18</td>
<td>4.087</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>11</td>
<td>Glutamic acid</td>
<td>13.55±0.43</td>
<td>15.01±0.47</td>
<td>2.292</td>
<td>NS</td>
</tr>
<tr>
<td>12</td>
<td>Glycine</td>
<td>5.24±0.39</td>
<td>4.64±0.17</td>
<td>1.410</td>
<td>NS</td>
</tr>
<tr>
<td>13</td>
<td>Arginine</td>
<td>4.90±0.09</td>
<td>5.57±0.38</td>
<td>1.716</td>
<td>NS</td>
</tr>
<tr>
<td>14</td>
<td>Aspartic acid</td>
<td>8.17±0.38</td>
<td>7.72±0.59</td>
<td>0.641</td>
<td>NS</td>
</tr>
<tr>
<td>15</td>
<td>Serine</td>
<td>7.83±0.17</td>
<td>6.65±0.15</td>
<td>2.999</td>
<td>&lt; 0.025</td>
</tr>
<tr>
<td>16</td>
<td>Histidine</td>
<td>4.68±0.14</td>
<td>5.49±0.45</td>
<td>1.718</td>
<td>NS</td>
</tr>
<tr>
<td>17</td>
<td>Ornithine</td>
<td>7.30±0.18</td>
<td>6.81±0.52</td>
<td>0.890</td>
<td>NS</td>
</tr>
<tr>
<td>18</td>
<td>Lysine</td>
<td>2.90±0.09</td>
<td>4.06±0.67</td>
<td>1.716</td>
<td>NS</td>
</tr>
<tr>
<td>19</td>
<td>Cystine</td>
<td>0.70±0.03</td>
<td>0.83±0.19</td>
<td>0.676</td>
<td>NS</td>
</tr>
</tbody>
</table>

* Denotes not measured

See legend to Table 23
Aspartate aminotransferase (GOT) activity in different tissues of cave and pigean fish.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Cave fish (mg pyruvate/mg protein/h)</th>
<th>Pigean fish (mg pyruvate/mg protein/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart</td>
<td>11.44 ± 0.72</td>
<td>2.58 ± 0.57</td>
</tr>
<tr>
<td>Muscle</td>
<td>8.73 ± 0.72</td>
<td>2.67 ± 0.57</td>
</tr>
<tr>
<td>Brain</td>
<td>11.76 ± 0.72</td>
<td>3.57 ± 0.57</td>
</tr>
<tr>
<td>Liver</td>
<td>5.57 ± 0.57</td>
<td>2.58 ± 0.57</td>
</tr>
</tbody>
</table>

See legend to Table 23.
<table>
<thead>
<tr>
<th>Cave</th>
<th>GPT</th>
<th>Activity (μg pyruvate/mg protein/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.005 &gt;</td>
<td>29.97</td>
<td>1.99 x 10^{-3}</td>
</tr>
<tr>
<td>0.005 &gt;</td>
<td>10.16</td>
<td>3.70 x 10^{-3}</td>
</tr>
<tr>
<td>0.005 &gt;</td>
<td>0.40</td>
<td>9.93 x 10^{-3}</td>
</tr>
<tr>
<td>0.005 &gt;</td>
<td>0.09</td>
<td>5.49 x 10^{-3}</td>
</tr>
</tbody>
</table>

Note: See Table 23 for full details.
See legend to Table 23

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Unit</th>
<th>Cave vs Epigean</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>ml/g</td>
<td>Cave vs Epigean</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

See Table 20: Acid phosphatase activity in different tissues of cave and epigean fish.
Alkaline phosphatase activity in different tissues of cave and epigean fish.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Unit (\mu mol Pi/mg/h)</th>
<th>Cave (\textsuperscript{o}C)</th>
<th>Epigean (\textsuperscript{20}C)</th>
<th>p value Cave vs Epigean</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>0.036</td>
<td>20.0\textsuperscript{o}C</td>
<td>30.0\textsuperscript{o}C</td>
<td>NS</td>
</tr>
<tr>
<td>Gill</td>
<td>0.092</td>
<td>2.468</td>
<td>2.54\textsuperscript{0.3}</td>
<td>NS</td>
</tr>
<tr>
<td>Muscle</td>
<td>0.003</td>
<td>0.555</td>
<td>0.76</td>
<td>NS</td>
</tr>
</tbody>
</table>

See legend to Table 23.
Table 3.2: Acetylcholinesterase activity in different tissues of cave and epigean fish

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Activity (μmol/min/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain</td>
<td>0.399</td>
</tr>
<tr>
<td>Gill</td>
<td>4.805</td>
</tr>
<tr>
<td>Muscle</td>
<td>3.070.26, 2.479.21</td>
</tr>
<tr>
<td>Cave</td>
<td>2.030.22</td>
</tr>
<tr>
<td>Epigean</td>
<td>0.959.046</td>
</tr>
</tbody>
</table>

Note: Data from Table 23.
### Table 33: Glycogen, lactate and pyruvate content in muscle of cave and epigean fish

<table>
<thead>
<tr>
<th>Variable</th>
<th>Cave</th>
<th>Epigean</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycogen</td>
<td>0.29±0.017</td>
<td>0.37±0.007</td>
</tr>
<tr>
<td>Lactate</td>
<td>5.23±0.27</td>
<td>3.02±0.40</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>2.49±0.30</td>
<td>5.74±0.40</td>
</tr>
</tbody>
</table>

*Unit: µmol/g tissue

See legend to Table 23
**Table 34**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Cave</th>
<th>Epigean</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactate dehydrogenase</td>
<td><strong>6.47 ± 0.05</strong></td>
<td><strong>8.73 ± 0.35</strong></td>
</tr>
<tr>
<td>(unit: g protein/mg protein)</td>
<td>6.90 ± 0.50</td>
<td>4.78 ± 0.35</td>
</tr>
<tr>
<td>Succinic dehydrogenase</td>
<td><strong>1.76 ± 0.12</strong></td>
<td><strong>2.74 ± 0.20</strong></td>
</tr>
<tr>
<td>(mg lactate/mg pyruvate)</td>
<td>4.030</td>
<td>2.760</td>
</tr>
</tbody>
</table>

See legend to Table 23
**Table 39**: Levels of certain inorganic cations in muscle of cave and epigean fish

<table>
<thead>
<tr>
<th>Ion</th>
<th>Cave</th>
<th>Spigean</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ca</td>
<td></td>
<td></td>
</tr>
<tr>
<td>K</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Na</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Note: df = degrees of freedom*
<table>
<thead>
<tr>
<th>Ion</th>
<th>Inside water</th>
<th>Outside water</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na⁺</td>
<td>22.00</td>
<td>16.00</td>
</tr>
<tr>
<td>K⁺</td>
<td>10.00</td>
<td>10.00</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>4.00</td>
<td>2.00</td>
</tr>
<tr>
<td>Mg²⁺</td>
<td>3.00</td>
<td>3.00</td>
</tr>
</tbody>
</table>

**Table 36**: Levels of inorganic cations in water inside and outside cave.