Chapter III

BIOCHEMICAL CLOCK MECHANISMS

Introduction

Rhythmic oscillation has been unequivocally regarded as a fundamental property of life (Scheving et al., 1978; Scheving, 1976). Rhythms of varying frequencies (circadian and/or circannual etc.) have been detected in several physiological and metabolic variables, viz., cholesterol, glucose, triglycerides, phospholipids, free fatty acids, corticosterone, testosterone, insulin, thyroxine, 5-hydroxytryptamine, sialic acid, uric acid, urea, phosphate, sodium, potassium, calcium, bilirubin, protein, albumin, globulin, alkaline phosphatase, transaminases, lactic dehydrogenase.
GPT, etc., in plasma and/or serum; glycogen, G6P, GPT, α-HBDH, acid phosphatase, LDH, tyrosine transaminase, DNA, RNA, etc., in liver and many other tissues (Reinberg, 1974; Halberg and Ahlgren, 1980; Halber, et al., 1971; Halberg, 1974; Smoewing et al., 1973; Makersbach, 1976; Halberg et al., 1980). However, most of the studies are confined to mammals including human beings. In nonmammalian and other lower vertebrates reports on rhythmic variations in the physiological and metabolic correlates are relatively limited (Schwassmann, 1971; Brown, Jr., 1973). However, rhythmic motor activities in fishes have been studied extensively (Thorpe, 1978). Rhythms (circadian and/or circannual etc.) in carbohydrate, lipid, erythropoiesis, oxidative, endocrinological, and reproductive parameters have been well documented in fishes (Fernandez and Planas, 1980; Dallahunya et al., 1980; Simpson, 1976; Yamashita, 1969; Gillard and Greton, 1973; Jonas and Bilinski, 1969; Leach and Taylor, 1977; Valtonen, 1974; Larsson and Frange, 1977). But the manner in which the periodicities of these cycles have been determined is based mostly on visual reading of the macroscopic chronograms. Furthermore, not a single piece of report is available on rhythms of cave fishes (Barr, 1976).

In the present chapter, an attempt has been made to understand properties of some of the biochemical clocks of a cave fish. The author has employed the latest available
mathematical model to validate circadian rhythms for glycogen and lactate contents in muscle, lactate in liver, alkaline phosphatase and acetylcholinesterase enzyme activities in gill, and acetylcholinesterase activity in brain tissue.

Materials and Methods

Twenty four fishes, approximately of same body weight and length, were selected from the stock aquarium (40 litre capacity) and were transferred to another smaller aquarium. They were acclimated to light-dark photoperiod, with 12 hours of light, alternating with 12 hours of darkness (light switched on at 08.00 and off at 20.00 h) for twenty one days. The temperature of the experimental room remained near constant (26 ± 4°C). Four fishes were sacrificed by decapitation at each of the following time points, viz., 09.00, 13.00, 17.00, 21.00, 01.00, and 05.00 h. Muscle, liver, gill and brain were recovered from autopsied fishes, frozen at -20°C in a deep-frigidaire until utilised in biochemical assay.

Levels of glycogen and lactate in muscle, lactate in liver; activity of alkaline phosphatase & acetylcholinesterase in gill and acetylcholinesterase in brain were assayed according to methods as follows:

Glycogen

Glycogen content of the muscle tissue was determined by the method of Jeiffler et al. (1950) after hot alkali extraction.
Principle

Concentrated sulphuric acid hydrolyses glycosidic bond to give monosaccharides which are further dehydrated to furfural and its derivatives. The furfural reacts with anthrone (10-keto, 9-10 dihydroanthracene) to give a blue green complex.

Reagents

1. 30% Potassium hydroxide solution

30 g of potassium (KOH) pellets dissolved in 100 ml of distilled water.

2 1% Sodium sulphate solution

1 g of sodium sulphate (Na$_2$SO$_4$) dissolved in 100 ml of distilled water.

3 95% Ethanol

95 ml of absolute ethanol made to 100 ml with distilled water.

4 Anthrone reagent

0.2 g of anthrone dissolved in 100 ml of 95% sulphuric acid (H$_2$SO$_4$). This reagent was freshly prepared.

5 Standard glucose solution

20 mg of glucose dissolved in 100 ml of distilled water and diluted to 1 litre. 5 ml of this solution contains 100 μg of glucose.
Procedure

Weighed samples of muscles were taken in test tubes containing 3 ml of 30 % KOH solution and digested in boiling water bath. After cooling the tubes, 0.5 ml of 1 % Na₂SO₄ and 3.5 ml of 95 % ethanol were added to each tube to precipitate glycogen.

The contents in each tube were stirred with a glass rod and the rod was washed in the tube itself with a small quantity of ethanol. The tubes were gently heated, cooled and allowed to stay overnight for complete precipitation of glycogen at room temperature. The next day the tubes were centrifuged at 3,000 rpm for 15 minutes. The supernatant was discarded and the residue was dissolved in about 3 ml of distilled water and reprecipitated with 2.5 ml of 95 % ethanol. It was centrifuged again and supernatant was discarded. The precipitate was finally dissolved in 50 ml of distilled water. Estimation of glycogen was carried out as follows: 5 ml of aliquot was transferred to a wide mouth boiling tube. This served as a test sample. 5 ml of standard glucose solution containing 10.0 μg of glucose was taken in another boiling tube. This served as standard. 5 ml of glass distilled water was taken in a third test tube. This served as a blank.

The tubes containing test and standard solutions and blank were placed in ice cold water. Dry and clean glass beads were used to prevent bumping following addition of anthrone
reagent. 10 ml of freshly prepared anthrone reagent (4) was added to each tube with a fast flowing pipette. The contents were mixed by shaking. The tubes were then transferred to a boiling water bath for exactly 30 minutes and then cooled to room temperature. The optical density (O.D.) of the test and standard solutions were read against blank at 630 nm in a spectrophotometer.

The amount of the glycogen was calculated from the equation:

\[ \mu g \text{ of glycogen in } 5 \text{ ml of aliquot} = \frac{100 \times O.D.}{1.11 \times 5} \]

where, \( O.D. \) = O.D. of test solution
\( S = O.D. \) of standard solution

1.11 = Conversion factor of glucose to glycogen.

Lactic acid

Known weights of muscle tissue were homogenized in 10% trichloracetic acid (TCA) and the homogenates were centrifuged at 3000 rpm for 15 minutes. The supernatants in each case were used for the estimation of lactic acid by the method of Fiske and Subbarow (1941).

Principle

Glucose and other interfering materials of protein free extract are removed by Van Slyke-Salkowsi method of
treatment with copper sulphate and calcium hydroxide. The solution is then heated with concentrated sulphuric acid. Lactic acid is thus converted to acetaldehyde, the latter is then determined by reaction with p-hydroxydiphenyl in the presence of copper ions.

Reagents

1. 20% Copper sulphate solution
   200 g of CuSO₄ · 5H₂O dissolved in 750 ml of water with gentle heating; the solution was cooled and later diluted to 1 litre with distilled water.

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

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

4. Ca(OH)₂ powder.

5. Concentrated sulphuric acid (H₂SO₄):
   Reagent grade, ion free H₂SO₄ with low nitrate content was used.

6. p-Hydroxydiphenyl reagent:
   1.5 g of p-hydroxydiphenyl was dissolved in 10 ml of
5% NaOH 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.

Stock standard lactic acid solution:

0.215 g of pure lithium lactate dissolved in 100 ml of distilled water in 1 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.

Tracking 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 solution gave 0.01 mg 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% copper sulphate 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 1
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 4% CuSO4 (2) was added to each tube. Subsequently 6 ml of chilled analar H2SO4 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. Precipitating reagent was dispersed again throughout the solution quickly and uniformly by shaking. All the tubes were incubated at 30°C ± 1°C for 90 seconds and were allowed to cool to room temperature. The optical density was measured against blank at 570 nm in Spectol. The values are expressed as μg lactate per 100 mg fresh weight of muscle.

Non-specific phosphomonoesterases (Alkaline phosphatase E.C. No. 3.1.3.1 and Acid phosphatase E.C. No. 3.1.3.2)

Homogenates of fresh tissues were prepared in 0.25 M ice cold sucrose solution to approximately 5% w/v; centrifuged at 3000 rpm at 4°C for 15 minutes and supernatant was used for enzyme assay.
Principle

Enzyme extract, on incubation with buffered sodium \( \beta \)-glycerophosphate, releases inorganic phosphate by enzymatic hydrolysis. The inorganic phosphate reacts with an acid molybdate solution to form phosphomolybdic acid. The phosphomolybdic acid is reduced by addition of 1,2,4 amino-neptholsulphonic acid reagent to produce blue colour (Fiske and Subba Rao, 1925). The intensity of the colour developed is directly proportional to the amount of inorganic phosphate, which in turn indicates the enzymatic activity of phosphatase.

Reagents

1 Alkaline phosphatase substrate (pH 10.0): 0.5 g of sodium \( \beta \)-glycerophosphate and 0.424 g sodium diethyl barbiturate dissolved in 100 ml of distilled water and stored under a layer of petroleum ether in a refrigerator.

2 Acid phosphatase substrate (pH 5.0):

0.5 g of sodium \( \beta \)-glycerophosphate, 0.242 g sodium diethyl barbiturate and 5 ml of 1 N acetic acid dissolved in 100 ml of distilled water and stored under a layer of petroleum ether in a refrigerator.
3  30% Trichloroacetic acid (TCA):
30 g of TCA dissolved in 100 ml of distilled water.

4  5% Trichloroacetic acid:
5 g of TCA dissolved in 100 ml of distilled water.

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

6  Stock standard phosphate solution:
0.351 g of pure dry monopotassium phosphate dissolved in distilled water in a 1 litre volumetric flask; 10 ml of 10 N H₂SO₄ added and the whole diluted to 1 litre with distilled water. This solution contains 0.4 mg of phosphate in 5.0 ml.

7  Working standard phosphate solution:
6.25 ml of stock phosphate solution (6) taken in a 100 ml volumetric flask; 16.7 ml of 30% TCA added and the whole diluted with distilled water to 100 ml. This solution contains 0.04 mg of phosphate in 8 ml of 5% TCA.

8  Ammonium molybdate solution:
5.0 g of ammonium molybdate dissolved in 40 ml of distilled water; 60 ml of 10 N H₂SO₄ added to it
and the whole diluted to 200 ml with distilled water.

9 15 % Sodium bisulphite solution:
15 g of sodium bisulphite dissolved in 100 ml of
distilled water.

10 20 % Sodium sulphite solution:
20 g of sodium sulphite dissolved in 100 ml of
distilled water.

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

Procedure:

Test:
9.00 ml of buffered substrate of required pH and
1.0 ml of extract were shaken in a test tube. The
reaction mixture was incubated for 30 minutes at
37°C. The reaction was stopped by adding 2 ml of
30 % TCA.
Control:

Control sample was prepared with 9.00 ml of buffered substrate, 2.0 ml of 30% TCA and 1.0 ml of extract in that sequence.

Both test and control samples were centrifuged at 3000 rpm for 15 minutes. 8.0 ml each of test sample, control sample, working standard phosphate solution (7), and 5% TCA as blank were taken in four test tubes graduated to 10 ml; 1.0 ml of ammonium molybdate added to each tube and mixed thoroughly; 0.4 ml of aminonaphthol-sulphonic acid reagent then added to each tube and mixed thoroughly. The whole then diluted to 10 ml with distilled water. The tubes were left for 5 minutes and optical density was read against blank at 660 nm in Specol. The values are expressed as µg inorganic phosphate released/mg protein/hour.

Protein content of the extract was determined by the method of Lowry et al. (1951) as described in Chapter IV.

Acetylcholinesterase (E.C. 3.1.1.7):

Fresh tissues were homogenised in ice cold 0.25 m sucrose solution to approximately 5% w/v; centrifuged at 3000 rpm at 4°C for 15 minutes; supernatant was used for enzyme assay by the colorimetric method of Molotil (1951), as modified by Kurali Krishnadass (1967).
**Principle**

Acetylcholinesterase catalyzes the following reaction

\[
\text{CH}_2 - O - COH + \text{CH}_2 \text{OH} \xrightarrow{\text{HCl, } N - (CH_3)_3} \text{HCl, } N - (CH_3)_3 + \text{CH}_2 \text{OH}
\]

Acetylcholine chloride  
Choline

Removal of acetylcholine per unit time is measured by comparison of its initial concentration in a reference tube with the final concentration in experimental tube. Acetylcholine reacts with hydroxylamine to form corresponding acylhydroxamic acid, which forms a strongly coloured ferric hydroxamate with ferric salts.

**Reagents**

1. 0.15 M phosphate buffer (pH 7.4)

16.72 g of disodium hydrogen phosphate (Na$_2$HPO$_4$) and
2.72 g of monopotassium dihydrogen phosphate (KH₂PO₄) dissolved in 100 ml of double distilled water.

2 0.004 M acetylcholine solution.
Stock solution (3.04 M) was prepared by dissolving 0.7466 g of acetylcholine chloride in 100 ml of HCl of pH 4.0. Stock solution was diluted with 9 volumes of phosphate buffer (1) before use.

3 2 M Hydroxylamine hydrochloride:
13.9 g of hydroxylamine hydrochloride dissolved in 100 ml of distilled water.

4 3.5 N sodium hydroxide:
14.00 g of sodium hydroxide (NaOH) dissolved in 100 ml of distilled water.

5 Alkaline hydroxylamine solution:
equal volumes of hydroxylamine hydrochloride solution(3) and 3.5 N NaOH solution (4) were mixed together before use.

6 Hydrochloric acid (1:1):
Concentrated (s. p. gravity 1.13) diluted with equal volume of distilled water.
7 0.37 M Ferric chloride solution
10 g of ferric chloride dissolved in 100 ml of 0.1 M HCl.

8 0.25 M sucrose solution
8.56 g of sucrose dissolved in 100 ml of distilled water.

Procedure

1 ml of extract was added to 1 ml of buffered substrate solution; incubated for 30 minutes at 37°±1°C; reaction was stopped by the addition of 2 ml of alkaline hydroxylamine hydrochloride. The tubes were shaken thoroughly and 1 ml of HCl (1:1 HCl:H2O) was added with further shaking and the whole was centrifuged. 2.5 ml of supernatant was taken in a test tube and 0.5 ml of ferric chloride solution was added. The contents were again thoroughly mixed. Zero time controls were maintained by adding 2 ml of alkaline hydroxylamine hydrochloride prior to the addition of the enzyme extract. Colour of the hydroxamic acid formed was read at 540 nm against the blank. The blank contained 1 ml of buffer instead of buffer substrate mixture.

The activity of the enzyme was determined by the amount of unreacted acetylcholine chloride left after 30 minutes of
incubation. The intensity of the colour at the end of the incubation gives the amount acetylcholine left unhydrolysed. On the basis of difference between initial substrate concentration and substrate concentration left unhydrolysed, the actual level of the enzyme activity was estimated and expressed in moles of acetylcholine chloride hydrolysed per mg protein per hour. Protein content of the extract was determined by Lowry et al. (1951).

Statistical methods

Data (mean ± 1 SE) were plotted by the conventional way against clock hour. Different features of time-dependent variables were assessed. Statistical analysis for comparison of groups of data were carried out by one way analysis of variance followed by Duncan's multiple-range test (Bruning and Kintz, 1977). Single cosinor was employed to determined rhythm parameters, viz., mesor, amplitude, and acrophase (Halberg et al., 1967, 1972; Nelson et al., 1979).

Results

Muscle glycogen

The macroscopic chronogram (Figure 15) illustrates data plots of means (± 1 SE) as a function of circadian time scale. Data were subjected to analysis of variance. Results revealed statistically significant time effect (P<0.05; Table 13).
The muscle glycogen content was found to be highest at 01.00 h. The muscle had lowest amount of glycogen stores at 13.00 h (Table 17). Both the time points are exactly 12 hours apart from each other. When both highest and lowest means were compared, a statistically significant difference was witnessed (Duncan's multiple-range test, Table 17). The percent change from lowest to highest mean was 28%.

Results of the cosinor analysis of data reveal detection of statistically significant circadian rhythm in the muscle glycogen content of cave fishes (Table 19; Figure 18). The zero-amplitude hypothesis was rejected at 2.9% level. The acrophase was located at -16° (01.07), with 95% confidence intervals between -339° (22.6 h) and -53° (03.53 h)(Table 19). The mean was of 0.31 (mg/100 mg), with an amplitude 0.04 (mg/100 mg).

Muscle lactate

Results of analysis of variance reveal statistically significant time effect (P < 0.05) on the content of lactate in the muscle tissue (Table 18).

The maximum concentration of lactate in the muscle was recorded at 13.00 h and minimum at 10.00 h (Table 17). Both the mean values differed statistically significantly
...tically significant circadian rhythm in muscle lactate concentrations was detected (Table 19; Figure 13). The probability that the amplitude of this rhythm is not equal to zero was 99.7% (Table 19). The acrophase was located at -162° (10.8 h) with a confidence intervals between -133° and -191°. The mean and amplitude were 71.96 (μg/100 mg) and 24.4 (μg/100 mg) respectively.

Liver lactate

Analysis of variance was performed on 24 h data. The time effect was not statistically significant (Table 18).

However, chronogram demonstrates highest recorded mean value at 05.00 h and that of lowest at 17.01 h (Figure 15). Both the time points were apart from each other by 13h. Statistically significant variation between points were also not witnessed, when Duncan's multiple-range test was employed. The change from lowest to highest recorded mean amounted to 86 μg/l only.

A statistically significant circadian rhythm in the lactate concentrations of liver tissue was not validated
with the help of cosinor rhythmometry. However, the
acrophase was witnessed at -48° (03.2 h) with a mesor
18.2 (μg/l 100 mg), and an amplitude 2.56 (μg/l 100 mg)
(Table 19).

Gill alkaline phosphatase

Data averages (± 1 S.E.) have been plotted as a function
of time (Figure 16). Results of analysis of variance did
not reveal statistically significant time effect (Table 21).

The peak enzyme activity was found at 13.00 h and that
of nadir at 17.00 h; statistically significant between-group
variations were also not observed with the help of Duncan's
multiple-range test. However, the change from lowest to
highest recorded mean was 42 μ.

Cosinor analysis of the data also failed to validate
a circadian rhythm for the alkaline phosphatase activity in
the gill (Table 22). However, the acrophase was detected
at -167° (11.13 h), with a mesor of 2.24 (μg pi/mg/h), and
an amplitude 2.56 (μg pi/mg/h).

Gill acetylcholinesterase

Analysis of variance was performed on 24 h data and
statistically significant time effect was observed (Table 31).
Maximum enzyme activity was noticed at 17.00 h and minimum at 09.00 h. The lowest mean value significantly differed from that of the highest (\( P < 0.01 \) ; Table 20). The percent change from the lowest to highest recorded mean was 70.

A statistically significant circadian rhythm was also obtained with the help of the cosinor analysis of the data (Table 22 ; Figure 19). The hypothesis of zero-amplitude test was rejected at 1.7 \( \pi \) level. The circadian acrophase was detected at \( -250 \) (13.37 h), with 95% confidence intervals between \( -351 \) and \( -321 \). The mean and amplitude were 1.904 (\( \mu \)mol \( \alpha \)-ch/\( \mu \)g protein/h) and 0.332 (\( \mu \)mol \( \alpha \)-ch/\( \mu \)g protein/h) respectively (Table 21).

Brain acetylcholinesterase

Analysis of variance was employed to analyze 24 h data on brain acetylcholinesterase enzyme activity. However, statistically significant time effect was not noticed (Table 21).

Highest enzyme activity was noticed at 17.00 h and lowest activity was recorded at 09.00 h. Both the time points were closely interlinked from each other (Table 20). When compared, they differed from each other at \( \pi \) level. The change from the lowest to highest recorded mean amounted to 31.3.
A statistically significant circadian rhythm was validated for the acetylcholinesterase activity in the brain tissue with the help of cosinor analysis of the data (Table 22; Figure 19). The assumption of zero-amplitude hypothesis was rejected at 3.8% level. The circadian acrophase was detected at $-271^\circ$ (18.07 h), with 95% confidence intervals between $-230^\circ$ and $-312^\circ$ (Table 22). The mesor was of 2.9 ($\mu$mol ACh/mg protein/h) with an amplitude 0.312 ($\mu$mol ACh/mg protein/h).

Discussion

Present results clearly demonstrate the presence of circadian rhythm in the contents of muscle glycogen, muscle lactate, and in the acetylcholinesterase enzyme activities of gill and brain tissues of cave fish, *Nemacheilus gvezardi*, when subjected to LD 12:12 photoperiod. Since literature on biological clocks of cave fishes is completely lacking, it is probably the first information of such a kind. However, a statistically significant circadian rhythm either in the content of liver lactate or in the alkaline phosphatase activity of gill tissue was not detected. This clearly indicates that even though fishes live inside the cavern under complete darkness their biochemical clocks could be synchronised by artificial photoperiods. At present
it is not possible to explain why rhythms in liver lactate and alkaline phosphatase activity in gill tissue did not appear following exposure of the fishes to LD 12:12 photoperiod for 3 weeks. Could it be that still longer period of acclimation is required for the expression of rhythm in the above two biochemical parameters? Furthermore, investigations of rhythm in all the above parameters of cave fishes under natural conditions would be of great physiological importance.

The acrophase of the circadian muscle glycogen rhythm appeared at -16° (01:07 h), with 95% confidence interval ranging between -339° and -53°. Over the single transverse circadian time scale there was a 38% variability from lowest to highest recorded means. Although glycogen is found in almost all tissues, muscle and liver tissues are metabolically more important in this regard. Usually glycogen functions as a reserve of glucose and, depending upon the requirements, it is readily broken down into glucose to meet energy demands of the entire organism (Newsholme and Start, 1973). Present results, therefore, suggest that probably there is less glucose utilization in the cave fishes during mid-night which in turn allows maximum accumulation in the form of muscle glycogen during this time of the circadian time scale. Since we do not have any data/information on circadian rhythms either in glycogen synthetase or in glycogen
phosphorylase activities of this cave fish, or any other fish, it is difficult to make any suggestion at present. Possibly, a similar mechanism(s), as in mammals, might be operating leading to exhibition of circadian rhythm in the content of muscle glycogen.

Existence of circadian rhythm in the content of muscle lactate has been reported in snakes (Gratz and Hutchison, 1977). However, the above report consists of observations at two time points only over a circadian scale. Aside this, the authors have not utilized inferential statistics to validate circadian rhythm for muscle lactate concentrations. In the present study, however, lactate content of muscle has been determined at six time points and a rhythm was detected by cosinor analysis of the data. The highest lactate accumulation in the muscle of cave fishes was found to be at 162° (10.8 h). Conversely, the circadian bathyphase with respect to lactate accumulation in the muscle was detected at 342° (22.8 h), with 95% confidence intervals ranging between 313° (22.87 h) and 11° (0.73 h). Surprisingly, there is a higher degree of overlapping between the acrophase timings of muscle glycogen and bathyphase timings of muscle lactate. This suggests that there is an inverse relationship between the accumulation of glycogen and lactate in the muscle tissue, i.e., when muscle glycogen content of the cave fish is maximum, lactate concentration...
is lowest. At the moment it is difficult to correlate
the role of liver lactate in the accumulation of muscle
glycogen since data on liver glycogen have not been
collected.

Circadian rhythm in the concentration of lactate
in the liver tissue of a number of vertebrates has been
known (Mayersbach, 1976; Bratz and Hutchison, 1977).

The absence of rhythm in the liver lactate in the
present case is surprising. However, the acrophase was
detected at $-43^\circ$ (03:3 h), i.e., approximately during the
mid-scotophase.

Alkaline phosphatase is an enzyme which hydrolyses
various phosphate esters and related compounds (Long, 1971).
Although, in mammals, serum alkaline phosphatase has been
known to undergo high amplitude circadian rhythm (Scheving
et al., 1975), in the present study, a statistically
significant circadian rhythm in gill alkaline phosphatase
was not obtained. The fresh water fishes, which make
copious urine, osmotic regulation is effected through
uptake of ions through gills. Higher activity of alkaline
phosphatase, to promote active uptake of ions from ambient
medium, is the natural corollary.

Relatively much higher activity of alkaline
phosphatase in the present cave fishes, as compared from
their epigean relatives (Chapter IV), lends testimony to increased osmotic stress in cave environment and is suggestive of a much higher rate of active uptake of ions to maintain hypertonic regulation. Absence of a circadian rhythm in the activity of alkaline phosphatase is again natural in that the fishes are almost continuously under osmotic stress in the cave environment.

The importance of acetylcholine-acetylcholinesterase in nerve transmission is too well known. Circadian rhythm in the acetylcholinesterase activity in the brain tissue of a number of animals, viz., invertebrates (Pavan Kumar et al., 1979), lizards (Subramanyam et al., 1981; Pavan Kumar et al., 1979) mammals (Mayersbach, 1976; Schiebeler and Meyersbach, 1974) has been well documented. In the present study also, assumption of zero-amplitude hypothesis for brain acetylcholinesterase of cave fishes was rejected, suggestive of occurrence of circadian rhythm.

Cholinesterase is an enzyme that hydrolyzes acetylcholine to form acetic acid and choline (Malkin, 1976). In other units it has been suggested that the amplitude of acetylcholine rhythm is greater in gill than in other tissues (Subramanyam et al., 1981). However, in the present study, the amplitude of the rhythm in gill is found to be greater than that of the amplitude in the brain. This
definitely suggests the greater importance of acetylcholinesterase in active uptake than in neuro-
transmission system in this cave dwelling species of fish. Acrophases of circadian rhythm in jill and brain
tissues surprisingly overlapped and were detected during
the late light fraction of the LD 12:12 photoperiod.
Similar light-induced increase in the acetylcholine esterase
enzyme activity has been reported for the other animals
including invertebrates as well as mammals (Mayersbach, 1976;
Subramanyam et al., 1981). Could it be that the light induced
response of this enzyme is common throughout the animal
kingdom? Even tissues of highly specialized cave adapted
fishes are not spared. Further studies on this aspect
would be rewarding.

Concluding, it is suggested that cave adapted fishes
retain the ability of synchronization by the photoperiod,
which is supposed to be the strongest "Zeitgeber" of the
environment. However, this is true only for this
troglophilic fish. Extension of this study in a specialized
troglobite would be revealing in clearing several existing
controversies in the field of chronobiology.
Table 1: Circadian variations in the glycogen and lactate content of tissues of cave fish, *euzetard*.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Clock Hour</th>
<th>Glycogen (unit)</th>
<th>Lactate (unit)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>01.00</td>
<td>0.34±0.02</td>
<td>19.5±3.00</td>
</tr>
<tr>
<td></td>
<td>09.00</td>
<td>0.36±0.04</td>
<td>17.0±2.16</td>
</tr>
<tr>
<td></td>
<td>13.00</td>
<td>0.35±0.03</td>
<td>17.5±3.77</td>
</tr>
<tr>
<td></td>
<td>17.00</td>
<td>0.34±0.02</td>
<td>20.5±1.70</td>
</tr>
<tr>
<td>Muscle</td>
<td>01.00</td>
<td>0.32±0.01</td>
<td>16.0±2.90</td>
</tr>
<tr>
<td></td>
<td>09.00</td>
<td>0.34±0.03</td>
<td>17.0±2.30</td>
</tr>
<tr>
<td></td>
<td>13.00</td>
<td>0.35±0.04</td>
<td>19.0±1.90</td>
</tr>
<tr>
<td></td>
<td>17.00</td>
<td>0.36±0.02</td>
<td>20.0±1.30</td>
</tr>
</tbody>
</table>

*Mean ± SE (mean value of four observations)*

<table>
<thead>
<tr>
<th>Variable</th>
<th>Tissue</th>
<th>Clock Hour</th>
<th>Glycogen (unit)</th>
<th>Lactate (unit)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Muscle</td>
<td>01.00</td>
<td>0.32±0.01</td>
<td>16.0±2.90</td>
<td></td>
</tr>
<tr>
<td></td>
<td>09.00</td>
<td>0.34±0.03</td>
<td>17.0±2.30</td>
<td></td>
</tr>
<tr>
<td></td>
<td>13.00</td>
<td>0.35±0.04</td>
<td>19.0±1.90</td>
<td></td>
</tr>
<tr>
<td></td>
<td>17.00</td>
<td>0.36±0.02</td>
<td>20.0±1.30</td>
<td></td>
</tr>
</tbody>
</table>

*Mean ± SE (mean value of four observations)*
and indicate conduct if case third is necessary for study of the topic on change of life.
Table 19: Hysterometric summary based on least-square fitting of 24 h cosine function

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Variable (unit)</th>
<th>Lactate</th>
<th>Glycogen (mg/l)</th>
<th>Lactate (mg/l)</th>
<th>Glycogen (mg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Muscle</td>
<td>No. value limit</td>
<td>24 h</td>
<td>0.19 ±0.29</td>
<td>24 h</td>
<td>1.19 ±0.49</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.00 ±0.02</td>
<td>0.00 ±0.02</td>
<td>0.00 ±0.02</td>
<td>0.00 ±0.02</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.00 ±0.02</td>
<td>0.00 ±0.02</td>
<td>0.00 ±0.02</td>
<td>0.00 ±0.02</td>
</tr>
</tbody>
</table>

Table 17: Legend as in Table 7

Legend:

- Lactate: 24 h
- Glycogen: 24 h

Units: No. value limit and time in hours.

Notes:
- Data for muscle is presented in Table 7.
- Data for lactate and glycogen are presented in Table 17.
<table>
<thead>
<tr>
<th>Tissue Variable</th>
<th>Clock Hour</th>
<th>Alkaline Phosphatase (unit)</th>
<th>Acetylcholine Esterase (unit)</th>
<th>Acetylcholine Alkaline Phosphatase (unit)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gill</td>
<td>09:00</td>
<td>2.00</td>
<td>2.00</td>
<td>2.00</td>
</tr>
<tr>
<td></td>
<td>12:00</td>
<td>2.00</td>
<td>2.00</td>
<td>2.00</td>
</tr>
<tr>
<td></td>
<td>15:00</td>
<td>2.00</td>
<td>2.00</td>
<td>2.00</td>
</tr>
<tr>
<td></td>
<td>18:00</td>
<td>2.00</td>
<td>2.00</td>
<td>2.00</td>
</tr>
<tr>
<td></td>
<td>21:00</td>
<td>2.00</td>
<td>2.00</td>
<td>2.00</td>
</tr>
<tr>
<td></td>
<td>01:00</td>
<td>2.00</td>
<td>2.00</td>
<td>2.00</td>
</tr>
<tr>
<td></td>
<td>04:00</td>
<td>2.00</td>
<td>2.00</td>
<td>2.00</td>
</tr>
<tr>
<td>Brain</td>
<td>09:00</td>
<td>2.00</td>
<td>2.00</td>
<td>2.00</td>
</tr>
<tr>
<td></td>
<td>12:00</td>
<td>2.00</td>
<td>2.00</td>
<td>2.00</td>
</tr>
<tr>
<td></td>
<td>15:00</td>
<td>2.00</td>
<td>2.00</td>
<td>2.00</td>
</tr>
<tr>
<td></td>
<td>18:00</td>
<td>2.00</td>
<td>2.00</td>
<td>2.00</td>
</tr>
<tr>
<td></td>
<td>21:00</td>
<td>2.00</td>
<td>2.00</td>
<td>2.00</td>
</tr>
<tr>
<td></td>
<td>01:00</td>
<td>2.00</td>
<td>2.00</td>
<td>2.00</td>
</tr>
<tr>
<td></td>
<td>04:00</td>
<td>2.00</td>
<td>2.00</td>
<td>2.00</td>
</tr>
</tbody>
</table>

* Differences from mean value obtained at 17:00 h; p < 0.05, and p < 0.01 respectively.

Table 2: Variation in the enzyme activities in tissues of cave fish.
A two-way analysis of variance was performed on data illustrated in Table 20.

<table>
<thead>
<tr>
<th>Source of Variation</th>
<th>Value</th>
<th>df</th>
<th>Mean Square</th>
<th>Variance</th>
<th>df</th>
<th>Deviation</th>
<th>df</th>
<th>Mean Square</th>
</tr>
</thead>
<tbody>
<tr>
<td>Within Group</td>
<td>2.975</td>
<td>18</td>
<td>0.169</td>
<td>0.001</td>
<td>18</td>
<td>0.169</td>
<td>18</td>
<td>0.001</td>
</tr>
<tr>
<td>Between Groups</td>
<td>3.345</td>
<td>17</td>
<td>0.197</td>
<td>0.001</td>
<td>17</td>
<td>0.197</td>
<td>17</td>
<td>0.001</td>
</tr>
<tr>
<td>Total</td>
<td>6.320</td>
<td>35</td>
<td>0.180</td>
<td>0.001</td>
<td>35</td>
<td>0.180</td>
<td>35</td>
<td>0.001</td>
</tr>
</tbody>
</table>

In cases of cave fish, no activity.

**Table 20:** Analysis of variance summary for study of time effects on enzyme activity.
Table 20: Statistical summary based on least-square fitting of the cosine function for data extrapolated in Table 22.