PROTEIN DEGRADATION AND ^{14}C AMINO-ACID INCORPORATION RATES INTO THE FOOT MUSCLE PROTEINS OF POND SNAIL *PILA GLOBOSA* DURING AESTIVATION

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ABSTRACT

Protein degradation and ^{14}C amino-acid incorporation rates in the foot muscle proteins of the pond snail, *Pila globosa* were studied with reference to aestivation. Lysosomal enzymes like: cathepsin, acid phosphatase except β-glucoronidase showed a decrease in activity on aestivation. Cathepsin activity showed an elevated temperature optimum on aestivation. Decreased proteolysis and autolysis on aestivation indicated a lowered turnover of proteins. To test this ^{14}C amino-acid incorporation rates were examined. Total proteins, myosin, actin, actomyosin, and tropomyosin did not exhibit any change in their incorporation rates. Sarcoplasmic proteins and collagen fraction decreased significantly in contrast to paramyosin on aestivation. It was concluded that aestivation resulted in changes in heterogeneous turnover of certain protein molecules.

INTRODUCTION

A TRANSLATIONAL compensation of protein synthesis in gold fish skeletal muscle during thermal acclimation has been demonstrated (Das and Prosser, 1967). Das (1967) showed that sub-cellular fraction from muscles of 5° C adapted gold fish incorporated ^{14}C–leucine into protein faster than those from 25° C adapted fish. This augmentation was greater in microsomal than in nuclear, mitochondrial or soluble fractions. It is well known that cold-adaptation brings forth an increase in protein synthesis (Rao, 1967; Prosser, 1967) but, this increase is far smaller in muscle tissue as compared to other tissues (Das, 1965). Cold-adaptation results in a higher turnover

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of proteins besides the quantitative and qualitative differences that account for conformational changes in the protein molecule (Prosser, 1967). For a greater turnover, degradation as well as synthetic rates must be balanced. During warm adaptation a greater degradation by the augmentation of lysosomal enzymes occurs and synthetic activity in cells is reduced (Hazel and Prosser, 1970). Such studies, however, were not extended to the muscle tissues of poikilotherms. Amino-acid incorporation rates during thermal accilimation of muscle proteins are known (Das and Krishnamoorthy, 1969). While studying the amino-acid incorporation, these authors compared the yields of contractile, sarcoplasmic and collagenous protein fractions and the levels of ^{14}C incorporation in the different muscles of cold and warm-acclimatized gold fish, *Carassius auratus*.

That aestivation brings about adaptation in protein synthesis of *Pila globosa* was indicated in the present study, by differences in the extractabilities of the various muscle proteins (Vijaya Brahmanandam, 1972). To confirm and extend these findings, investigations were made on the amino-acid incorporation, the rates of protein degradation by lysosomal proteases and the activities of other lysosomal enzymes.

MATERIAL AND METHODS

Gastropod snails of the species *Pila globosa* were collected from fresh-water ponds in and around Bangalore and brought to the laboratory, where they were kept in aquaria. As they are voracious vegetable feeders, the aquaria were provided with cabbage slices, and *Hydrilla* plant on which they normally feed. After one week, when they got used to aquarium conditions, a batch of them were made to aestivate by embedding them in dry mud in large wooden boxes $24'' \times 14'' \times 6'$ for required period. Before burying, the snails were allowed to crawl in glass troughs for overnight to dispel mantle water. The temperature in the wooden box was maintained at $35 \pm 2^{\circ}$ C by the heat generated through burning an electric lamp. Care was taken to maintain darkness by covering the bulbs with tin foil. At a time about 50 to 100 snails were aestivated, starting a batch every month, so that the aestivated snails were always at hand for analysis. In most cases, animals aestivated for six months were selected for the investigation unless otherwise mentioned.

Actively feeding snails from aquaria were used as controls.

Since the foot muscle was very tough, all homogenizations were done in a mortar, by grinding muscle slices in acid-washed sand.

Statistical analyses of the data were done according to the methods suggested by Croxton (1953).

The foot muscle tissues from active and aestivated *Pila globosa* were used for the following studies.

(1) In vitro *Autolytic and Proteolytic Activity*

The entire foot muscle was excised after breaking open the shell, immediately weighed, minced and homogenised in 0·25 M sucrose with 1 g of acid-washed sand. The homogenisation was carried out in cold. A few drops of 0·1% Triton X – 100 (Sigma Chemical) was added during extraction. A 5% (w/v) homogenate was thus prepared and centrifuged at −4° C for 10 minutes at 4,000 × g and the supernatant was used for experimentation. Most of the soluble proteins of the muscle were solubilized, whereas the contractile proteins being insoluble in 0·25 M sucrose settled down as a precipitate. 1 ml aliquots of the supernatant were transferred into test-tubes and incubated for 1 hour at the desired temperature in a water-bath. The protein from each of the samples was then precipitated with an equal volume of 10% trichloro-acetic acid (TCA). An unincubated TCA denatured homogenate served as the control. After centrifugation, the supernatant was assayed for total amino-acids by the method of Moore and Stein (1954). Autolysis was expressed as μg total amino-acid released per 1 hour by 1 mg of initial homogenate protein, estimated by micro-Biuret method (Itzaki and Gill, 1964).

(*a*) *Cathepsin activity.*—The clear 5% homogenate prepared as indicated above was mixed with an equal volume of 0·2 M phosphate buffer and incubated for 3 hours in a thermostat water-bath at different temperatures. After the incubation period, the protein was precipitated by the addition of 10% TCA and the amino-acid content of the supernatant was measured by the ninhydrin colorimetric method (Moore and Stein, 1954). An unincubated TCA denatured homogenate served as the control. The pH activity curves were determined over a wide range of pH (2 to 10) using acid-pthalate, phoshate and *tris* buffers (Gomori, 1955).

(*b*) *Proteolytic activity.*—The clear 5% homogenates were dialysed at 4° C for 2 hours and then used for assay. 10 ml of the clear 5% homogenate was dialysed against 500 ml of 0·25 M sucrose, at −4° C for 2 hours. The assay mixture contained 1 ml of the dialysate, 1 ml of 1% casein (w/v) on 0·005 M sodium bicarbonate and 1 ml of 0·1 M calcium chloride. The mixture was incubated at the desired temperature in a water bath for 3 hours. At

the end of the incubation period 2 ml of ice cold 10% TCA was added to stop the reaction. After centrifugation the amino acid content of the supernatant was estimated as described above. Specific activity was expressed as μg amino-acids released per mg initial protein per hour.

(2) *Lysosomal Enzyme Activities*

(*a*) *Acid phosphatases.*—The entire foot muscle excised from the snail was homogenized in 15 ml of 0·15 M sucrose and few drops of 0·1 Triton X–100 was added to the homogenat . After standing for 1 hour in the cold, the homogenate was centrifuged at 7,000 rpm at 0° C in an International PR 6 model centrifuge. The supernatant was collected and the enzyme was assayed according to Bodansky (1932) using β–glycerophosphate as substrate.

(*b*) *β-glucoronidase.*—The foot muscles were homogenised in distilled water as described in the earlier paragraphs and triturated for 1 hour in cold. The homogenate was then centrifuged at 4° C and the enzyme in the supernatant was assayed according to Fishman *et al.* (1948) using phenolphthalein glucoronidate as the substrate.

(3) *Analyses of Proteins*

(*a*) *Total protein.*—Samples of 0·05 g foot muscle were homogenized in ice cold distilled water and the proteins were precipitated with 10% TCA. The precipitate was collected after centrifugation and used for the determination of protein content by Biuret method (Layne, 1957) or micro–Kjeldhal method. The same precipitate in some estimations was washed twice with alcohol, twice with a mixture of ethanol and ether (3:1 v/v) and finally twice with ether. The washed samples were oven–dried at 80° C to remove ether and weighed in an electrical balance to determine the protein content gravimetrically.

(*b*) The analyses of different protein fractions like sarcoplasmic, myosin and actin were followed according to the basic principle described by Barany *et al.* (1965). Collagens were extracted according to the method of Jackson and Cleary (Glick, 1966) and estimated by determining the hydroxyproline content after hydrolyzing the protein in 6 N HCl, colorimetrically by Woessner's method (Glick, 1966).

Tropomyosin was extracted by the method of Bailey (1948) and estimated by Biuret method (Layne, 1957).

Paramyosin and actomyosin were extracted by the method of Johnson *et al.* (1959) and estimated by Biuret method (Layne, 1957).

4. ^{14}C-*Amino-Acid—Incorporation into Muscle Proteins*

Uniformly labelled ^{14}C algal hydrolysate obtained from the Atomic Erengy Establishment, Government of India, was injected into the foot muscle of *Pila globosa*, at the dose of 2 μc in 0·2 ml of the hydrolysate per snail. The injection was done with a hypodermic syringe which was introduced through a hole made very close to the mouth of the snail. The injected animals were kept at their respective environments for the desired length of time before they were sacrificed for analysis.

Fractionated proteins as described above were precipitated with ice-cold 5% TCA and dehydrated with alcohol, chloroform and ether. The dried powder (1 to 2 mg) was weighed in a Mettler balance (Zurich, Swiss made, type S5 cap. 160 g, No. 47031) in glass vials and dissolved in 0·5 ml of hydroxide and 5 ml of dioxane based scintillation fluid (200 g) of Naphthalene, 10 g of POP, 0·25 g of POPOP (dimethyl) dissolved in 1 litre of diosane (BDH). A Packard Tricarb scintillation counter model 2002 was enployed for counting the radioactivity. The specific activity of radioactive incorporation was expressed as counts min^{-1} mg^{-1} protein residue, after applying necessary correction for the background counts and the self-absorption by protein.

Results

The *in vitro* autolytic activity in the foot muscle of active and aestivated *Pila globosa* is presented in Table I. At 25° C the active muscle did not show significant variation from the aestivated muscle with reference to the autolytic activity. However, at 36° C the aestivated muscle showed 50% reduction in the autolytic activity when compared to the active one. 36° C is the habitat temperature for the aestivated snails and 25° C for active snails. Rates of incorporation of aestivated muscle at 25° C however was 3-fold greater than at 36° C. Thus, in *Pila* as in other poikilotherms it is the environmental or habitat temperature that influences the metabolic activity. The increase in autolytic rate of active muscle homogenate at 36° C indicates greater thermal sensitivity to autolysis.

Table II presents the data on cathepsin activity of the muscle homogenates. The temperature optimum for cathepsin activity was low for aestivated snails

TABLE I

Autolytic activity in the foot muscle of Pila globosa *aestivated for* 6 *months measured at habitat temperatures*

Snails		Specific activity = μg a-acids released/hr/mg initial protein	
		25° C	36° C
Active	..	24 ± 5	158 ± 28
Aestivated	..	18 ± 4	68 ± 16
Incidence of change on activation	No change t = 2·095	P > 0·05	Decrease < 0·001 t = 6·232

Values are mean ± S.D. of 3 observations.

TABLE II

Cathepsin activity in the homogenates of foot muscle of Pila globosa *aestivatod for* 6 *months measured at the optimum as well as habitat temperatures*

Snails		Specific activity mg a-acid/mg homogenate protein/hr		
		at optimum	at habitat temperature	
Active	25°C	0·38 ± 0·04	22 - 25° C	0·37 ± 0·05
Aestivated	12°C	0·26 ± 0·04	34 - 37° C	0·08 ± 0·02
Incidence of change on aestivation	Decrease t = 37·98	P < 0·001	Decrease t = 63·29	P < 0·001

Values are mean ± S.D. of 3 observations.

(12° C). In addition, the specific activity was significantly lower in aestivated muscle than in the active muscle, both at optimum and habitat temperatures. The shift in the temperature optimum for proteolytic activity on aestivation is shown in Fig. 1, indicating that active and aestivated *Pila* foot muscles are characterized by different types of proteases, cathepsins probably, since the cathepsins are known as intracellular proteases. The multifold decrease in cathepsin activity on aestivation is noteworthy, serving, perhaps, to lower the rate of degradation of intracellular proteins.

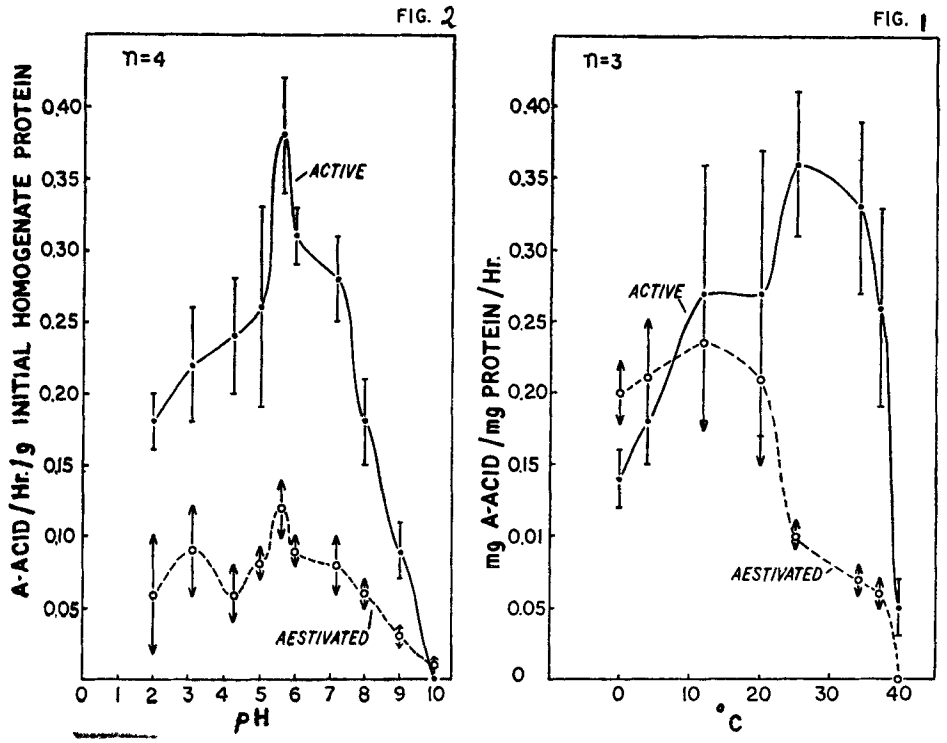


FIG. 1. Influence of temperature in the proteolytic activity in the foot muscle homogenates of *Pila globosa*.

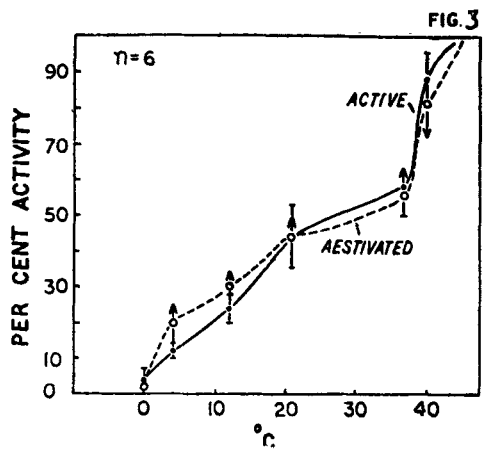
Plots are mean ± S.D. of 3 observations.

FIG. 2. Influence of pH on the cathepsin activity in the foot muscle homogenates of active and aestivated *Pila globosa*.

Plots are mean ± S.D. of 4 observations.

Depending on the pH specificity (Fig. 2) two intracellular cathepsins were detected in aestivated muscle. One of them showed an optimum pH at 5·6 and the other at 3·0. The active muscle showed only one cathepsin

with maximum activity at pH 5·6. Similar pH dependence of cathepsin activity was demonstrated in frog muscles (Krishnamoorthy, 1971).



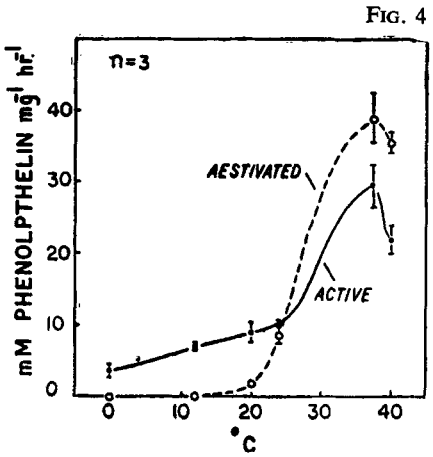


FIG. 3. Influence of temperature on the acid phosphatase activi y in the active and aestivated foot muscle homogenates of *Pila globosa*.

Plots are mean ± S.D. of 6 observations.

FIG. 4. Effect of temperature on the β-glucoronidase activity in the active and aestivated foot muscle homogenates of *Pila globosa*.

Plots are mean ± S.D. of 3 observations.

When the foot muscle homogenate was incubated with casein, amino-acids were released into the supernatant indicating that proteolytic activity was present in *Pila globosa*, as in the vertebrate skeletal muscle. Tables III and IV present the differences in the proteolytic activities of active and aestivated foot muscles. Not only was the temperature optimum lowered on aestivation to 12° C (Table III) but also a considerable reduction in specific activity at the habitat temperature was seen (Table IV).

The enzymatic processes, *viz.*, autolysis, cathepsin activity and proteolysis are localized in lysosomes. Enzymes like acid phosphatase, β-glucoronidase serve as marker enzyme for lysosomes. Thus both the lysosomal marker enzymes acid phosphatases and β-glucoronidase showed lowered activity in the muscle following aestivation.

Acid phosphatase presented (Table V) the same trend as that of cathepsin and proteolysis, in the aestivated muscles; but the temperature optimum was not altered. Both at 45° C (optimum temperature) and at the habitat temperature the aestivated muscle showed about 40% of the activity of the

TABLE III

Proteolytic activity in the foot muscle homogenates of Pila globosa *aestivated for 6 months measured at the optimum as well as at the habitat temperatures*

Snails	Specific activity: mg a-acid/mg homogenate protein/hr			
	at optimum		at habitat temperature	
Active ..	25° C	0·36 ± 0·05	22 – 25° C	31 ± 0·15
Aestivated ..	12° C	0·24 ± 0·02	34 – 37° C	0·065 ± 0·01
Incidence of change on aestivation	Decrease t = 4·441	P<0·005	Decrease t = 35·65	P<0·001

Values are mean ± S.D. of 3 observations.

TABLE IV

Changes in the proteolytic activity of the foot muscle homogenate of Pila globosa *aestivated for six months*

Assay conditions pH 5·7, Casein conc. 5 mg/ml

Temperature of assay °C	Specific activity: mg a-acid/mg homogenate protein/hr	
	Active	Aestivated
0	0·14 ± 0·02	0·20 ± 0·02
4	0·18 ± 0·03	0·21 ± 0·04
12	0·27 ± 0·09	0·24 ± 0·02
20	0·27 ± 0·10	0·21 ± 0·06
25	0·36 ± 0·05	0·10 ± 0·01
34	0·33 ± 0·06	0·07 ± 0·01
37	0·26 ± 0·07	0·06 ± 0·01
40	0·05 ± 0·02	0·0000

Values are mean ± S.D. of 3 observations.

active muscle. The temperature *vs* enzyme activity curves (Table VI and Fig. 3) for acid phosphatase in both the muscles showed the same pattern, but the aestivated enzyme had lower specific activity.

TABLE V

Acid phosphatase activity in the foot muscle homogenates of Pila globosa *aestivated for six months*

Snails	Specific activity : μM Pi/mg homogenate protein/min			
	at optimum		at habitat temperature	
Active ..	45° C	8·3 ± 1·6	22 – 25° C	3·52 ± 0·81
Aestivated ..	45° C	5·2 ± 0·62	34 – 37° C	1·36 ± 0·28
Incidence of change on aestivation	Decrease, t = 4·412	P<0·001	Decrease, t = 6·178	P<0·001

Values are mean ± S.D. of 6 observations.

TABLE VI

Effect of temperature on the acid phosphatase activity of sucrose solubl fraction of the foot muscle of Pila globosa *aestivated for six months*

Temperature °C	Per cent activity	
	active	aestivated
0	4 ± 3	2 ± 0·2
4	12 ± 2	20 ± 5·1
12	24 ± 4·8	30 ± 4·2
21	44 ± 4·8	44 ± 5·0
37	58 ± 8	56 ± 7·1
40	89 ± 7	82 ± 10·6
45	100	100

Values are mean ± S.D. of 6 observations.

TABLE VII

β-glucoronidase activity of the foot muscle of Pila globosa *measured at the optimum as well as habitat temperatures*

Snails		specific activity: mM phenolphthalein/mg/hr			
		at optimum		at habitat temperature	
Active	.. 37° C	29·0 ± 6·0		22 – 25	9·5 ± 0·5
Aestivated	.. 37° C	39 ± 7·0		34 – 37	39 ± 7·0
Change on aestivation		Increase t = 2·658	P<0·05	Increase t = 17·32	P<0·001

Values are mean ± S.D. of 6 observations.

TABLE VIII

Effect of temperature on the β-glucoronidase activity of sucrose soluble fraction of the foot muscle of Pila globosa *aestivated for* 6 *months*

Temperature	Specific activity: mM phenolphthalein $mg^{-1}hr^{-1}$	
	Active	Aestivated
0	3·5 ± 0·5	0·000
12	7·25 ± 0·7	0·00
20	9·00 ± 1·5	32·25 ± 0·75
24	9·5 ± 0·5	8·000 ± 0·5
37	29 ± 6·0	39 ± 7·0
40	24 ± 4	36 ± 2

Values are mean ± S.D. of 3 observations.

β-glucoronidase activity showed the same temperature optimum (Fig. 4 and Tables VII and VIII) for both active and aestivated muscles. At the optimal temperature both the enzymes showed no appreciable change in the activity (Table VII) but at habitat temperature the aestivated enzyme showed a 4-fold increase in the activity.

The incorporation of ^{14}C amino-acid into the other muscle proteins like myosin, actin and actomyosin and tropomyosin did not vary on aestivation. The incorporation rates were measured at the respective habitat temperatures.

TABLE IX

Amino acid incorporation into different fractions of foot muscle protein in the pond snail Pila globosa *aestivated for six months*

Cpm ^{14}C-algal protein hydrolysate/mg protein residue/hr at habitat temperaure

Protein fraction	Active	Aestivated	Incidence of change on aestivation
1. Total proteins (6)	104 ± 33	74 ± 27	No change .. $t = 1{\cdot}88$ $p > 0{\cdot}05$
2. Sarcoplasmic Protein (6)	180 ± 52	44 ± 16	decrease .. $t = 6{\cdot}12$ $p < 0{\cdot}001$
3. Mysin (6)	20 ± 4	14 ± 7	no change .. $t = 1{\cdot}82$ $p > 0{\cdot}05$
4. Actin (6)	29 ± 17	20 ± 8	no change .. t 1·17 $p > 0{\cdot}25$
5. Actomyosin (3)	9 ± 1	10 ± 2	no change .. $t = 0{\cdot}776$ $p > 0{\cdot}4$
6. Collagen (6)	161 ± 39	72 ± 21	decrease .. $t = 4{\cdot}85$ $p < 0{\cdot}001$
7. Paramyosin (3)	16 ± 3	24 ± 3	increase .. $t = 3{\cdot}26$ $p < 0{\cdot}01$
8. Tropomyosin (3)	± 2	6 ± 2	no change .. $t = 0{\cdot}613$ $p > 0{\cdot}5$

Values are mean ± S.D.

No. in parenthesis are the number of observations.

The results in this study support the view, that aestivation as an adaptation to warm temperature shows reduced synthetic rates of muscle proteins However, this reduction is seen only in sarcoplasmic proteins and collagen (Table IX).

Discussion

The activities of lysosomal acid hydrolases have been shown to be augmented in several pathological conditions of muscle like vitamin E deficiency (Zalkin *et al.*, 1962), heriditary muscular dystrophy (Tappel *et al.*, 1962), denervation atrophy and tenotomy (Pollack and Bird, 1968). Similarly, the augmentation of autolysis (Krishnamoorthy *et al.*, 1971) and proteolytic activity (Krishnamoorthy, 1971; Iodica *et al.*, 1966) have been recorded in the denervation-atrophied muscle. Max *et al.* (1971) have demonstrated that the lysosomal enzymes like β-glucosidase, β-galactosidase, arylsulfatase and acid-phosphatase have increased during disuse-atrophy. Although significant dehydration atrophy was recorded in the aestivated foot muscles, Vijaya Brahmanandam (1972) only β-glucoronidase increased in its activity whereas striking reduction was seen in autolytic, proteolytic and lysosomal hydrolases on aestivation.

The decrease in amino acid-incorporation rates in the sarcoplasmic proteins, with the complementary decrease in autolytic and degradative proteolytic activity in the aestivated muscle, suggests that this muscle has a low turnover rate when compared to the active muscle. The non-uniform rates of degradation as well as amino-acid incorporation into different protein fractions would account for accumulation and wastage of different protein fractions (Table IX) on aestivation. For instance, the muscle proteins are not degradable (Krishnamoorthy *et al.*, 1971; Funabiki and Cassens, 1972) but the incorporation rates in them are not altered. Consequently no significant changes in their levels were recorded in the aestivated tissue. In other words, when a fraction shows a high incorproration rate and lesser degradation, it accumulates quantitatively in the muscle. These studies reveal that during aestivation there is reduced turnover of a majority of muscle proteins. A few of them like actin, tropomyosin, myosin and actomyosin (Table IX) because of reduced degradation and normal incorporation, maintain the synthesis at normal level. As a result, these proteins accumulate in the aestivated foot muscle.

Heterogeneous turnover of myofibrillar proteins is well understood. Velick (1956) has estimated that the half-life of actin was 67 days, tropo-

myosin 27 days, heavy meromyosin 80 days and light meromyosin 20 days. Dreypus *et al.* (1960) concluded that the myofibrils display a definite-life span of about 30 days. They also discussed that the myosin exists in a state of inertness and its metabolic behaviour can be compared with that of hemoglobin. Funabiki and Kandatsu (1965) found that actin turnover differs from that of plasma globulin. McManus and Mueller (1966) reported that the half-life for both light and heavy meromyosins was 29 days. Das and Krishnamoorthy (1969) found different turnover rates of proteins in goldfish muscle, which are augmented by the temperature of acclimation. Funabiki and Cassens (1972) observed that the order of degree in relative turnover rate is :

troponin > *a*-actinin > tropomyosin > actin=myosin.

The present results show the existence of heterogeneous turnover of proteins in the snail which is influenced by the environmental adaptation.

Lowered turnover rates of proteins and decreased lysosomal activity are characteristic of warm adaptation of poikilotherms (Hazel and Prosser, 1970). Similar results obtained during aestivation of *Pila globosa* indicate that aestivation and warm adaptation have the same basic metabolic compensatory mechanism.

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