PHYSIOLOGY OF LOW TEMPERATURE ACCLIMATION IN TROPICAL POIKILOTHERMS

III. Quantitative Changes in the Bound and Free Amino Acids in the Earthworm, *Lampito mauritii*

BY S. RAGHUPATHIRAMIREDDY AND KANDULA PAMPAPATHI RAO

(Department of Zoology, Sri Venkateswara University, Tirupati, India)

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INTRODUCTION

EARLIER papers in this series (Pampapathi Rao, 1962; Saroja, 1962; Pampapathi Rao, 1963 *a*, *b*) have shown that acclimation of earthworms and freshwater mussels to low temperature resulted in a decrease in the free amino acid content of body fluids and an increase in the protein nitrogen of tissues indicating increased protein synthesis during cold acclimation. These investigations therefore suggest changes in the free amino acid and protein contents of poikilotherms on temperature acclimation.

The present investigation is an attempt to understand the qualitative as well as the quantitative changes in the free and bound amino acid patterns in the metabolism of the earthworm during thermal acclimation.

MATERIAL AND METHODS

Earthworms of the species *Lampito mauritii* were collected from the outskirts of Tirupati and acclimated to 36° C. and 20° C. for 15 to 20 days in glass troughs, containing water just sufficient to keep the worms submerged. These worms were fed on blotting paper throughout the period of acclimation, and water in the glass troughs was changed every day. A third set of animals, similarly kept for 15–20 days at laboratory temperature ($28 \pm 1°$ C.) and fed on blotting paper, served as the control animals.

After acclimation the worms were carefully blotted and weighed. Then each worm was cut into two halves, and weighed separately. In each case, the anterior half was used for the analysis of bound amino acids and the posterior half for the free amino acids.

The anterior half of the earthworm was subjected to acid hydrolysis a 110° C. for 20 hours using 6 N HCl (Fruton and Simmonds, 1961). After repeated washing and evaporation, the hydrolysate was extracted with 96% alcohol and used for chromatographic analysis. The posterior half of the animal was ground in 96% ethanol and centrifuged. The supernatant was chromatographed for free amino acids.

Circular filter paper (Whatman No. 1 circles of 32 cm. diameter) chromatography, as described by Giri and Rao (1952), was employed using butanol: acetic acid: water (4: 1: 1) as the solvent system. Good resolution of the amino acids was obtained by running the chromatogram twice with the same solvent system. Identification of the amino acids was done by running parallel controls spotting the known and unknown side by side. The chromatograms were developed with 0·2% ninhydrin solution.

For quantitative estimation each amino-acid band was carefully cut and eluted with 75% ethanol and 0·1% copper sulfate solution. The optical density of the eluate was measured in a Spectrophotometer (Hilger Watts, England) at 510 mμ. The concentration of the amino acid in the test solution was evaluated from the standard curve prepared for each amino acid.

RESULTS

The total free amino acid content decreases on cold acclimation and increases on warm acclimation of the earthworms (Table I). On the other hand the total bound amino acids increase on cold acclimation, but decrease on warm acclimation. The decrease in free amino acid content on cold acclimation (43%) is approximately twice the increase in free amino acid content on warm acclimation (24%). The increase in bound amino acid content on cold acclimation (9·2%) is roughly twice the decrease in bound amino acids on warm acclimation (4·7) (Table I).

Cystine, lysine, serine and/or flycine, glutamic acid, and alanine are recorded as free amino acids in normal and cold and warm-adapted earthworms. Free arginine was recorded only in normal- and warm-adapted earthworms, but not in cold-adapted ones. Good separation of the band corresponding to free serine and glycine could not be achieved even after the double run. But the separation was, however, better with bound serine and glycine. As serine was already reported to be a part of the free amino acid pool of earthworms (Rosenberg and Ennor, 1961) the band in the free amino acid chromatogram was considered in this case as serine and/or glycine. An amino acid, whose identity is not clear, but which has a slightly

TABLE I

No.	amino acid	Free amino acids		Bound amino acids	
		% change on cold acclimation	% change on warm acclimation	% change on cold acclimation	% change on warm acclimation
1.	Unknown amino acid (near cystine)	No change	No change	..	..
2.	Cystine	−48	− 6	..	..
3.	Lysine ..	−36·6	− 33·1	+ 2	− 5·8
4.	Arginine ..	..	+126·5	− 6·7	−40·6
5.	Aspartic acid	−30·5	+ 15	+37·4	+26·0
6.	Serine ..	− 0·9	+178·0	+35·0	−50
7.	Glycine ..	..	..	+ 6·5	− 8·25
8.	Glutamic acid	−49	−18·5	+37·6	+13·2
9.	Alanine ..	−33·4	+ 33·5	+ 5·1	+10·4
10.	Tyrosine ..	..	..	+ 6·0	+20
11.	Methionine	..	..	+ 6·3	− 0·87
12.	Valine ..	..	..	+ 7·0	− 7·9
13.	Phenylalanine	..	..	+15	+14·00
14.	Leucine and isoleucine	..	..	0	− 3·8
	Total ..	−43	+ 24	+ 9·2	− 4·7

\+ denotes increase.
− denotes decrease.

lower Rf value than cystine, has been recorded in all the three groups of animals. Resolution of this amino acid from cystine was possible only by the double run. This unknown amino acid appears to remain unchanged in quantity with acclimation, as judged by the optical density values.

Lysine, arginine, aspartic acid, serine, glycine, glutamic acid, alanine tyrosine, methionine, valine, phenylalanine, leucine and isoleucine were recorded as bound amino acids in all the three sets of earthworms. Cystine, histidine, aspargine, threonine, proline and tryptophane were not recorded as bound amino acids. The position of proline could not be established as the faint yellow colour which proline gives with ninhydrin merged with the general yellowish background of the bound amino acid chromatogram. Threonine and tryptophane are probably destroyed in the course of acid hydrolysis (Fruton and Simmonds, 1961). Cystine which was present in free condition, was not detected as bound amino acid. To verify whether cystine and histidine are also broken down during acid hydrolysis, a mixture of cystine, histidine, threonine and tryptophane was subjected to the same conditions in which hydrolysis of tissues was carried out. All the four amino acids were found to be destroyed. Aspargine is probably oxidized to aspartic acid during acid hydrolysis (Carlson, 1961).

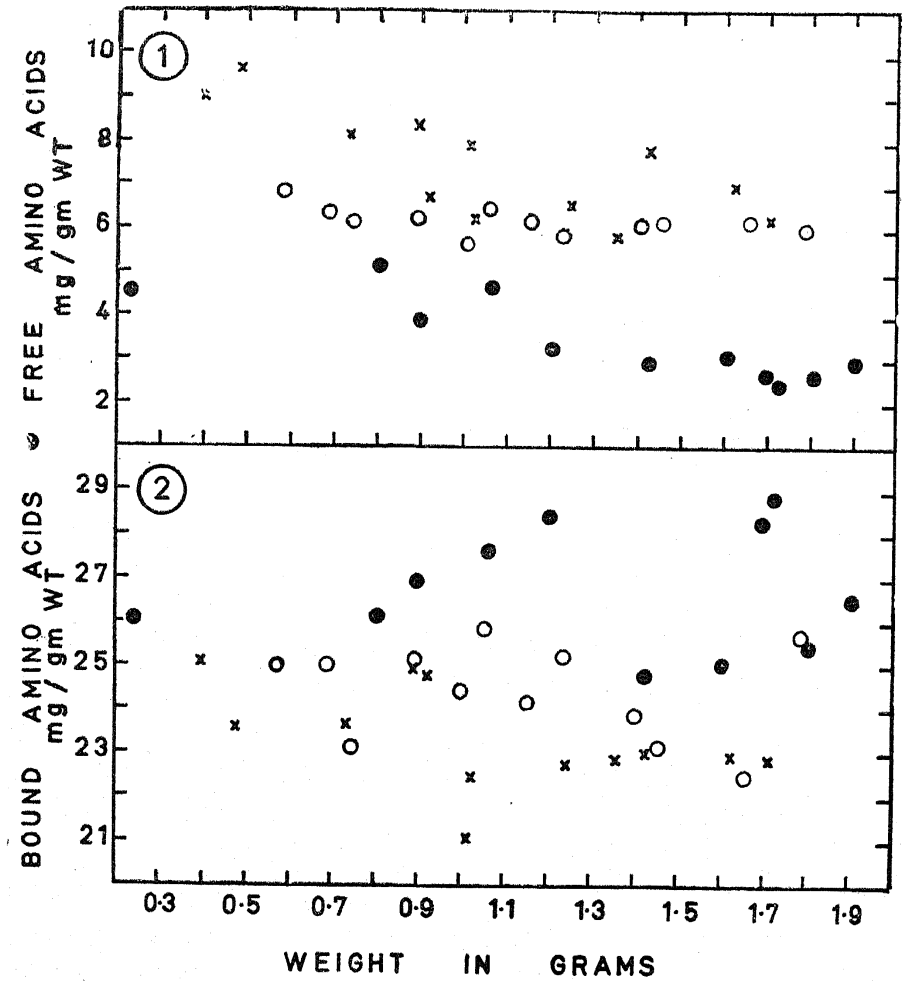
There is a 48% decrease in free cystine on acclimation to low temperature. Free lysine decreases more or less by the same percentage (36·6–33·1%) in both cold- and warm-adapted animals. There is not much difference in the bound lysine in the three sets of earthworms. Free arginine increases considerably (126·5%) on warm acclimation, but completely disappears on cold acclimation. Bound arginine decreases considerably (40·6%) on warm acclimation, but does not change on cold acclimation. Free aspartic acid shows a 30·5% decrease on cold acclimation and a 15% increase on warm acclimation, whereas bound aspartic acid increases on both cold and warm acclimations, the magnitude of increase, however, being greater in the former (37·4%) than in the latter (26%).

Glutamic acid decreases in free condition, but increases in bound condition on acclimation to both warm and cold temperatures. The magnitude of decrease of free glutamic acid in cold (49%) is approximately three times the decrease in warm (18·5%) and the increase in bound glutamic acid in cold (37·6%) is approximately three times the increase in warm acclimation (13·2%). Free alanine decreases (33·4%) on cold acclimation to the same magnitude as it increases on warm acclimation (33·5%), but bound alanine increases only by 5·1% on cold acclimation whereas it decreases by 10·4% on warm acclimation. Free serine and/or glycine does not show any change on cold acclimation, but shows a conspicuous increase (178%) on warm acclimation. Bound serine shows a 35% increase on cold acclimation and a 50% decrease on warm acclimation. Bound glycine shows only slight changes with acclimation temperature, which may not be of any significance.

Bound tyrosine shows a 20% increase on warm acclimation and shows only 6% increase on cold acclimation. Phenylalanine appears to increase on warm as well as cold acclimations more or less to the same magnitude (14–15%). Methionine, valine and the leucines do not show any significant changes with temperature acclimation. These amino acids, namely, tyrosine, phenylalanine, methionine, valine and leucines were not recorded in the free condition.

Discussion

The decrease of free amino acids and increase of bound amino acids on cold acclimation and increase of free amino acids and decrease of bound amino acids on warm acclimation (Figs. 1 and 2) probably indicate a shift of amino acids between free and bound conditions during thermal acclima-



Figs. 1–2. Milligrams of total *bound* amino acids per gram weight of tissue in cold-acclimated (closed circles); warm-acclimated (crosses) and normal (open circles) earthworms.

tion. Cold- and warm-adapted animals appear to show size dependence in their free amino acid content. Smaller worms have higher free amino acid content than larger worms on both cold and warm adaptations (Fig. 1). Smaller worms appear to be more sensitive to high temperature than larger worms, whereas larger worms appear to respond more to low temperatures than smaller worms.

The increase of bound amino acids in cold acclimation may be due to incorporation of free amino acids into proteins. Thus aspartic acid, serine and glutamic acid decrease in free condition and increase in bound condition indicating incorporation. Phenylalanine, although absent in free condition, increases in bound state suggesting biosynthesis of this amino acid and its subsequent incorporation into protein. Though there is a decrease in the levels of free lysine, arginine and alanine in cold acclimated worms, they show no appreciable increase in the bound state suggesting a selective breakdown of these amino acids. A potential arginase system probably operates during cold acclimation.

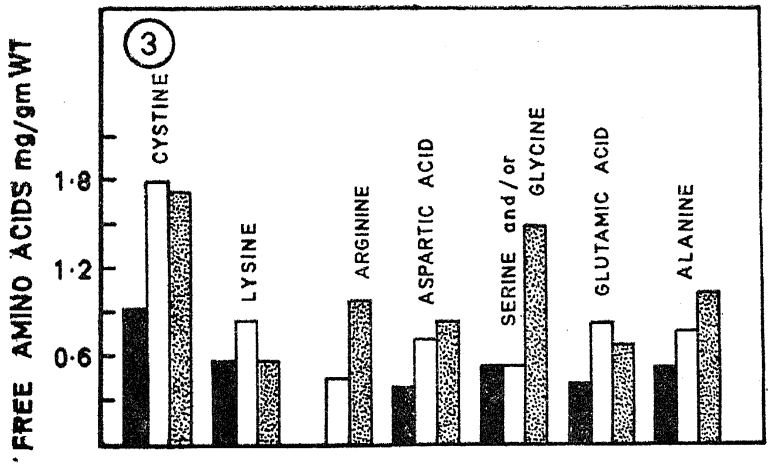


FIG. 3. Histograms for individual *free* amino-acids (milligrams per gram weight of tissue) in normal (open columns), cold (closed columns) and warm (dotted columns) acclimated earthworms.

On warm acclimation, the bound amino acids decrease and free amino acids increase (Figs. 1 and 2) suggesting movement of amino acids from bound to free condition by proteolysis. Thus bound arginine, serine and glycine decrease conspicuously and show a corresponding increase in their free levels. However, bound aspartic acid, glutamic acid, tyrosine and phenylalanine actually increase on warm acclimation. The increase in bound glutamic acid may be due to incorporation of free glutamic acid, which decreases on warm

acclimation. But aspartic acid shows increase in both free and bound states on warm acclimation. Lysine shows a decrease in both free and bound levels on warm acclimation. Lysine is known to yield glutamic acid as a degradation product (Fruton and Simmonds, 1961) and this glutamic acid is probably transaminated to aspartic acid. Both the phenolic amino acids, namely, tyrosine and phenylalanine, increase in bound state in warm acclimation. These amino acids should have been synthesized and subsequently incorporated into proteins, as neither of them form a part of the free amino acid pool in earthworms. The precursors involved in this biosynthesis, however, are not clear. Though there is a slight increase in some of the bound amino acids, the total bound amino acid content shows a net decrease, probably adding to the free amino acid pool on warm acclimation.

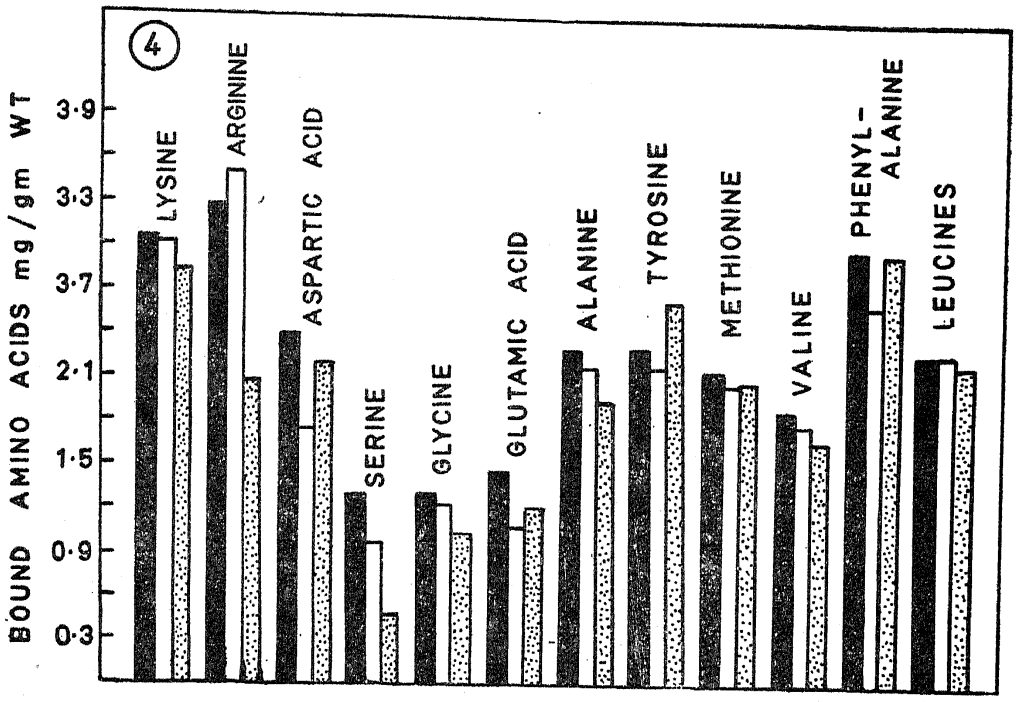


FIG. 4. Histograms for individual *bound* amino-acids (milligrams per gram weight of tissue) in normal (open columns), cold (closed columns) and warm (dotted columns) acclimated earthworms.

The rate of incorporation of free amino acids changes (Jankowsky, 1960), the total free amino acid content decreases (Saroja, 1962) and the tissue protein content increases (Pampapathi Rao, 1962, 1963 *b*; Saroja, 1962) in cold-acclimated poikilotherms. Saroja (1962) reported increase in the protein nitrogen of tissues and decrease in free amino acids of body fluids on cold acclimation and decrease in protein nitrogen of tissues and increase in free amino acids of body fluids on warm acclimation, of the same earthworm,

Lampito mauritii used in the present investigation. The results of the present investigation agree with the findings of Saroja (1962) in the general trend, but differ in that she found the increase in free amino acid content on warm acclimation to be about three times the decrease on cold acclimation, whereas in the present investigation the decrease in free amino acids on cold acclimation is found to be twice the increase on warm acclimation. The increase in the protein synthetic activity of poikilotherms on cold acclimation finds support in the reported increase in RNA content of cold adapted poikilotherms (Pampapathi Rao, 1962, 1963 *c*; Parvatheswara Rao, 1962; Saroja, 1962). The hexosemonophosphate pathway, which operates at a higher capacity in cold acclimation (Ekberg, 1958; Kanungo and Prosser, 1959) probably contributes the pentoses to nucleic acid (Scheer, 1953; Burma, 1960; Fruton and Simmonds, 1961), as " so far attempts to demonstrate the formation of pentoses by other pathways have failed " (Burma, 1960). The decrease in the glucogenic amino acids like glutamic acid, aspartic acid and alanine may also be due to their utilization *via* the HMP.

That the turnover of existing proteins through their depolymerization to peptides or free amino acids may indirectly serve as a partial source for the possible synthesis of new enzyme proteins has been envisaged (Stanier, 1955). There is evidence for adaptive enzyme synthesis in animals (Stanier, 1955; Knox, 1958) and the incorporation of amino acids may be greater into the adaptive enzyme than into other proteins (Gros *et al.*, 1954). The release of amino acids by proteolysis and their reincorporation on thermal acclimation probably results in altered levels of specific enzyme proteins. The patterns of release and reincorporation, however, are not identical in cold- and warm-adapted earthworms, thereby indicating different extents of alteration in the levels of the same or different enzymes, on acclimation to cold and warm temperatures. That such adaptive changes in enzyme systems may be involved in the homeostatic mechanisms compensating for temperature changes in poikilotherms has been envisaged (Prosser, 1958, 1961; Segal, 1961).

SUMMARY

1. A quantitative chromatographic analysis of the free and bound amino-acids of warm-(36° C.) and cold-(20° C.) acclimated earthworms (*Lampito mauritii*) was carried out.

2. Cold acclimation resulted in a decrease in the free amino acids and increase in the bound amino acids, while warm acclimation resulted in an increase in free amino acids and decrease in bound amino acids.

3. It is suggested that there is increased protein synthetic activity in cold acclimation. The turnover of proteins by catabolic release of bound amino acids and resynthesis is envisaged to result in changes in enzyme systems. The patterns of protein turnover appear to be different in cold- and warm-acclimated worms.

4. Changes observed in the individual amino acids like lysine, arginine, aspartic acid, glutamic acid and the phenolic amino acids are discussed in the light of our present knowledge on amino acid metabolism.

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