Int. J. Speleol. 6 (1974), pp. 249-254.

Free Amino Acids in a Cave Beetle *Darlingtonea kentuckensis* Valentine (Coleoptera: Carabidae)

by

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INTRODUCTION

There have been few studies investigating the physiology and biochemistry of terrestrial invertebrate troglobites. This is primarily because of difficulty in rearing, unavailability of specimens and small organism size. The cave beetle, *Darlingtonea kentuckensis* Valentine, is readily available from caves along the Cumberland Plateau in Kentucky. Because of its availability and since insects are noted for a high concentration of amino acids in their hemolymph this beetle was chosen for the analysis of its free amino acids. Dietary habits and pigmentation can be discussed in relation to the presence or absence of specific amino acids.

MATERIALS AND METHODS

Specimens

Specimens of *D. kentuckensis* were collected from Sloans Valley Cave, Pulaski County, Kentucky. Beetles were placed in small vials filled with silt and kept cool during transportation. In the laboratory the vials were emptied into a petri dish which was then flooded with carbon dioxide. The anesthetized beetles were identified and sexed. Individual beetles were kept at 13°C. in plastic petri dishes containing a base of 50% charcoal-50% plaster of Paris covered with a fine layer of cave silt. The substrate was kept moist with the periodic addition of distilled water. Groups of petri dishes were stored in covered plastic boxes with distilled water in the bottom to maintain a saturated atmosphere. Each beetle was fed three to four *Drosophila melanogaster* larvae per week.

Preparation of Samples

In experiments 1 to 4 the beetles were used directly; in experiments 5 and 6 beetles were first killed by placing one beetle in each test tube and heating the tube for 10 minutes in a boiling water bath. Two drops of distilled water saturated with phenylthiourea were added to each tube; the beetle was crushed with a glass stirring rod. The homogenate was allowed to stand for a few minutes and the entire supernate was spotted on a chromatography plate.

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Preparation of Plates and Standards

Thin-layer chromatography plates (20 cm x 20 cm) were prepared with a 250 micrometer layer of Silica Gel G (Stahl, 1965).

Standards were applied at the following concentrations: 10 micrograms each of cystine and methionine; 25 micrograms each of alanine and tyrosine; 50 micrograms each of β-alanine, α-amino-butyric acid, arginine, aspartic acid, glutamic acid, glycine, histidine, hydroxyproline, isoleucine, leucine, lysine, phenylalanine, proline, serine, threonine, tryptophan and valine.

Development and Detection

Solvent system 1 of chloroform-methanol-17% ammonia solution (40:40:20 v/v/v) took about 75 minutes to travel a 15 cm distance. The plate was air-dried and then developed in solvent system 2 composed of n-butanol-glacial acetic acid-water (60:20:20 v/v/v). Development time was about 150 minutes for a 15 cm distance.

Plates were sprayed with ninhydrin (Stahl, 1965) and heated for 30 minutes at 55°C. The spots were marked with a dissecting needle and numbered immediately upon being removed from the oven. After 30 minutes the plates were checked again and any additional spots which had appeared were marked and numbered.

RESULTS

The results are given in Table 1. The identified spots were present when the plates were removed from the oven, while unidentified spots # 15 to 21 appeared within the next 30 minutes. All spots appeared less intense than the standards; indicating that the concentration of amino acids per beetle is lower than the amounts listed for the standards.

Thirteen free amino acids were identified from *D. kentuckensis*. The amino acid present as the largest spot was lysine. Histidine, proline, glumatic acid, serine, alanine, valine, and isoleucine and/or leucine, were present as moderate spots. Glycine, threonine, tyrosine, tryptophan and phenylalanine were present as small spots. β-Alanine, α-amino-butyric acid, arginine, aspartic acid, cystine, hydroxyproline and methionine were not detected.

Cystine may have been present as unidentified spot # 18; however, identification was not positive. Arginine which has a low limit of detection (0.06 micrograms) was absent. It is unlikely that lysine and arginine did not separate in the experimental runs, since separation was obtained with the standards. It is possible that these basic amino acids were bound to an inorganic ion (Wyatt, 1961). Histidine is very effective in forming complexes with divalent metals; this may account for its variable R_f observed in solvent system 1. The identity of unidentified spots # 15 to 21 is unknown. Suspect compounds include modified amino acids, compounds in the urea cycle, small peptides and amino sugars (Wigglesworth, 1965; Wyatt, 1961; Patton, 1963; Chen, 1971).

The length of time after feeding, sex and treatment (Table 2) did not affect the results. In the laboratory *D. kentuckensis* can be kept up to two months without

Table 1. Separation of Ninhydrin Positive Compounds from *Darlingtonea kentuckensis*

Compound	Experiment Number[a]						R_f in solvent system[b]	
	1	2	3	4	5	6	1	2
Alanine (0.05)[c]	+	+	++	++	++	++	0.39 ± 0.06	0.30 ± 0.02
β–Alanine (0.06)	–	–	–	–	–	–	0.17	0.29
α–Amino-butyric acid	–	–	–	–	–	–	0.17	0.29
Arginine (0.06)	–	–	–	–	–	–	0.12	0.15
Aspartic acid (0.2)	–	–	–	–	–	–	0.23	0.28
Cystine	–	–	–	–	–	–	0.40	0.16
Glutamic acid (0.4)	++	++	++	++	++	++	0.27 ± 0.04	0.31 ± 0.02
Glycine (0.006)	+	+	+	+	–	+	0.32 ± 0.03	0.27 ± 0.02
Histidine (0.5)	++	++	+++	++	++	++	0.42 ± 0.06	0.10 ± 0.01
Hydroxyproline (0.1)	–	–	–	–	–	–	0.27	0.17
Isoleucine and/or Leucine	++	+	+	+	++	++	0.60 ± 0.04	0.46 ± 0.02
Lysine (0.03)	+++	+++	+++	+++	+++	+++	0.11 ± 0.01	0.09 ± 0.01
Methionine (0.4)	–	–	–	–	–	–	0.61	0.42
Phenylalanine (0.2)	+	+	+	+	+	+	0.68 ± 0.06	0.48 ± 0.02
Phenylthiourea ?	+	+	+	+	+	+	0.91 ± 0.05	0.56 ± 0.04
Proline (0.5)	++	++	++	++	++	++	0.32 ± 0.04	0.22 ± 0.02
Serine (0.1)	++	++	++	++	++	++	0.38 ± 0.06	0.24 ± 0.02
Threonine (0.1)	+	+	+	+	+	+	0.45 ± 0.06	0.28 ± 0.02
Tryptophan (0.5)	+	+	+	+	+	+	0.61 ± 0.04	0.51 ± 0.03
Tyrosine (0.1)	+	+	++	+	+	+	0.47 ± 0.06	0.46 ± 0.02
Unknown # 15	–	+	+	+	+	+	0.25 ± 0.04	0.20 ± 0.02
Unknown # 16	+	+	+	–	+	–	0.29 ± 0.05	0.14 ± 0.02
Unknown # 17	–	–	–	–	+	+	0.57 ± 0.03	0.16 ± 0.01
Unknown # 18	–	+	–	–	+	–	0.42 ± 0.07	0.18 ± 0.01
Unknown # 19	–	+	–	–	+	–	0.44 ± 0.08	0.34 ± 0.02
Unknown # 20	–	–	–	–	+	–	0.43	0.69
Unknown # 21	+	–	–	–	–	–	0.20	0.13
Valine (0.2)	++	++	++	+	+	+	0.51 ± 0.04	0.39 ± 0.02

[a] Symbols refer to intensity of spot (+++ = strong, ++ = medium, + = weak, – = absent)
[b] Numbers indicate solvent system used for chromatography. R_f values are one standard deviation of the mean.
[c] Limit of detection in micrograms (Stahl, 1965).

feeding. The food is stored in the crop and is passed into the midgut at a slow rate (Sperka, unpublished data). Animals starved for two weeks could have a food reserve in their crop. Any free amino acids present in the crop were included in the analysis.

Table 2. History of Specimens before Homogenization

Experiment	Sex	Time after feeding	Treatment
1	M	2 weeks	none
2	F	2 weeks	none
3	M	3 days	none
4	F	3 days	none
5	M	3 days	10 min. at 100° C
6	F	3 days	10 min. at 100° C

DISCUSSION

Although essential amino acids are not known for *D. kentuckensis*, insects generally require the same ten essential amino acids as mammals (Gilmour, 1965). All of these were detected except for arginine and methionine.

The absence of α-amino-butyric acid and hydroxyproline in *D. kentuckensis* may be explained by its natural diet. In nature *D. kentuckensis* is a carnivore feeding on the eggs and young of another carnivore, *Hadenoecus subterraneus*. These feeding habits prevent it from obtaining α-amino-butyric acid and hydroxyproline directly from plant material. α-Amino-butyric acid can also be formed by the degradation of methionine (Gilmour, 1965). The pathway for the biosynthesis of α-amino-butyric acid from methionine may exist in *D. kentuckensis*; however, methionine was not detected. In the honey bee, *Apis mellifera*, the occurrence of hydroxyproline in the hemolymph is believed to be from the free hydroxyproline in ingested pollen (Pratt, 1950). The biosynthesis of hydroxyproline from proline has been reported in the chironomid midge, *Acricotopus lucidus* (Chen, 1971). The pathway for the biosynthesis of hydroxyproline from proline appears to be absent in *D. kentuckensis*.

In *D. kentuckensis* the sclerotization and darkening of the cuticle is a very slow process taking approximately three months to reach normal hardness and pigmentation (Marsh, 1969). Recent work has indicated that β-alanine is involved in the normal process of sclerotization in the fruit fly, *Drosophila melanogaster* and the fleshfly, *Sarcophaga bullata* (Hodgetts, 1972; Bodnaryk, 1971). In *Drosophila* those mutants which failed to sclerotize their pupal cases were found to have a defective β-alanine metabolism (Hodgetts, 1972). However, those mutants have a darker

cuticle than the wild type, suggesting that when sclerotization is retarded there are more sites available for the darkening reaction. Ross and Monroe (1972) have shown that aspartic acid and uracil are the precursors of β-alanine in the housefly, *Musca domestica*; however, aspartic acid was not detected in *D. kentuckensis*. Since the cuticle does undergo hardening over a relatively long period of time the pathway for the synthesis of β-alanine may not be entirely blocked.

Marsh (1969) has suggested that the darkening of the cuticle with age is due to the deposition of pterines. Most pteridines are colorless and xanthopterin (yellow) and erythropterin (red) are possible suspects for the gradual change in pigmentation. Pteridines have been found but that they are totally responsible for the color change seems unlikely, since the cuticle retains pigment after extraction with hot methanol (Sperka, unpublished data). This pigment is probably melanins derived from tyrosine.

The long time required for the darkening of the cuticle of *D. kentuckensis* suggests that the conversion of tyrosine to melanin proceeds at a very slow rate. This may be the result of a defective or very low level of the enzyme polyphenoloxidase. Observations that hemolymph and cell fragments in a capillary tube exposed to air at a high relative humidity do not darken over a period of two weeks further suggests that polyphenoloxidase is defective (Sperka, unpublished data). Tyrosine was present in the homogenate; this suggests that the abnormal pigmentation in this troglobitic insect is due to a defect in the enzymatic conversion to melanin.

ACKNOWLEDGEMENTS

I wish to thank Dr. Thomas C. Barr, Jr., Biological Sciences, University of Kentucky and Dr. Douglas L. Dahlman, Department of Entomology, University of Kentucky for the use of laboratory equipment and facilities and Mr. David P. Beiter, Kidder, Kentucky for assistance on collecting trips.

KEY WORD INDEX

Free amino acids, cave beetle, *Darlingtonea kentuckensis*, Coleoptera, Carabidae, troglobite.

SUMMARY

Free amino acids of *Darlingtonea kentuckensis* were investigated by two-dimensional, thin-layer chromatography on Silica Gel G. Thirteen amino acids which could be identified (alanine, glutamic acid, glycine, histidine, isoleucine and/or leucine, lysine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine and valine) and seven unidentified ninhydrin-positive spots were found. β-Alanine, α-

amino-butyric acid, arginine, aspartic acid, cystine, hydroxyproline and methionine were not detected.

No difference was observed in the free amino acids with respect to sex of beetle, time after feeding and method of sample preparation.

RESUME

Les acides aminés libres du Coléoptère troglobie *Darlingtonea kentuckensis* ont été recherchés par chromatographie à deux dimensions sur couche mince de gel de silise G. Treize amino-acides ont pu être identifiés (alanine, acide glutamique, glycine, histidine isoleucine et/ou leucine, lysine, phénylalanine, proline, sérine, thréonine, tryptophane, tyrosine et valine), mais sept taches positives à la ninhydrine n'ont pu l'être. La β-alanine, l'acide α-aminobutyrique, l'arginine, l'acide aspartique, la cystine, l'hydroxyproline et la méthionine n'ont pas été décelés.

Dans les acides aminés libres, on n'a pas observé de différence relative au sexe du Coléoptère, au temps écoulé depuis la prise de nourriture et à la technique de préparation de l'échantillon.

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