Free Amino Acids in the Egg Fluid and Embryos of *Pila*

Free amino-acid pools in the course of morphogenesis of *Limnaea* have been studied by BRAHMACHARY and BHATTACHARYA[1,2]. It was shown that the amino-acid content of the *Limnaea* embryos increases considerably at the time of hatching, but the pattern of free amino-acids is constant for a long time and the adult amino-acid pools are resistant to starvation, indicating a stubborn genetic trend. (Later[3], by extracting from a very large number of very young – i.e. a few days old – snails, traces of amino-acids characteristic of adult snails were detected and this all the more emphasized the constancy of the amino-acid pattern.) However, the free amino-acids of the nutrient egg fluid could not be detected.

Although MONROY et al.[4] have recently carried out very interesting studies on the exchange between embryo and nutrient material in fish eggs, even elementary knowledge of the constituents and chemical composition of egg fluids in snails is rather scanty. For example, HESS[5], in his review, sums up the recent findings. Regarding the protein and amino-acid content of egg fluid, probably the only publication is that of JURA and GEORGE[6]. They detected the presence of bound amino-acids by ninhydrin test in the egg fluid of *Succinea putris* and also described some details on the composition of protein in the capsule fluid. We have now worked with *Pila* eggs which are far bigger (and contain much more fluid) than *Limnaea* eggs.

Clumps of eggs were collected from moist places near the edges of ponds and allowed to hatch in shallow vessels containing a little water. The individual eggs of the same clump would show a considerable range of variation; namely, some were at an early stage with no visible embryos while near-hatching snails were found in others.

It has been found[1] that in certain cases direct crushing of the biological material on the whatman paper is preferable to spotting the extraction. Similarly, it was now seen that even a large quantity of fluid, collected by pricking the eggs with pins and then extracting with 70% alcohol, is too poor in amino-acids to be useful for chromatograms, but the egg fluid of only two or three eggs, made to soak directly on the filter paper, yields a recognizable ninhydrin spot. Care should be taken, however, not to allow the wet spot to spread too far. This can be ensured by lifting the egg with the pin almost immediately after stabbing it and then drying the spot with a hot-air blower. By repeating this process two or three times, an egg was made to yield most of its fluid. Thus, by comparing the two ninhydrin spots due to an equal number (four) of eggs at the early stage and at an advanced stage (when the unhatched snail is clearly perceived), one can see the great diminution of intensity (Figure 1).

However, following this method, attempts at developing chromatograms with the solvent, *n*-butanol:acetic acid:water (4:1:1) were a complete failure, since no colour with ninhydrin could be detected. Water-saturated phenol was then used and found to be suitable. Although there was no resolution, by comparing the two streaks from egg fluid and unhatched or newly-hatched snails (crushed on the filter paper) on the same paper, it is obvious that all or nearly all the amino-acids obtainable from the advanced embryo are also present in the capsule fluid, though in a very dilute form. For example, the streak due to fluid from 13 eggs is far less intense than that due to crushing two unhatched snails.

In order to make an approximate estimation of the number of amino-acids in the hatched snails, a very large number of them were crushed and extracted with 70% alcohol. After further concentration of the material and spotting, well resolved ninhydrin spots were found. However, the number of spots varied according to the intensity of spotting. A very intense spotting and direct crushing of a single snail (a few days after hatching) are shown in Figure 2 (solvent – *n*-butanol:acetic acid:water). A comparable number of spots, less resolved but still recognizable, is also seen in a chromatogram with direct crushing of an older snail with the solvent, water-saturated phenol (Figure 3).

Figure 4 shows the comparative chromatograms of starved (1), control (2), and unhatched (3) snails. The

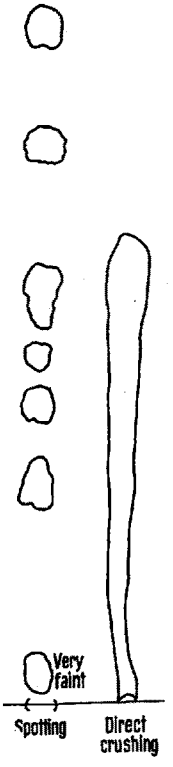


Fig. 2

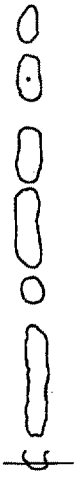


Fig. 3

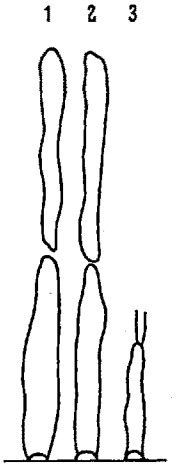


Fig. 4

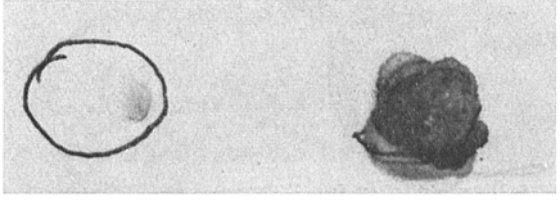


Fig. 1. Left-hand side: early stage.

[1] R. L. BRAHMACHARY and A. BHATTACHARYA, Exper. *19*, 143 (1963).
[2] R. L. BRAHMACHARY and A. BHATTACHARYA, Exper. *19*, 225 (1963).
[3] Unpublished data.
[4] A. MONROY et al., Embryologia *6*, Mangold-Festschrift 151 (1961).
[5] O. HESS, *Fortschritte der Zoologie* (1962), p. 130.
[6] Cz. JURA and J. C. GEORGE, Proc. Kon. Ned. Acad. Wet. *61C*, 590 (1958).

period of starvation was nearly three weeks. These results are in agreement with those of *Limnaea*. Apparently, the free amino-acid pools obtained from catabolic processes (or biosynthesis from precursors in the body) are again forced to assume the original pattern. Thus the pools which defy starvation may be regarded as a stable biochemical index (gene-determined) rather than purely as building blocks for protein. This rather curious finding is, however, in agreement with that of ROBERTS and SIMONSEN[7] with rat tissues[8,9].

Résumé. Dans la capsule des œufs de *Pila* (Gastéropode), le fluide alimentaire contient à peu près tous les acides-aminés libres qui existent aussi dans l'embryon bien développé. L'auteur a déterminé le nombre des acides-aminés libres qui se trouvent dans le corps de ces escargots après l'éclosion. Le modèle-type des acides-aminés libres reste inchangé chez les individus affamés.

R. L. BRAHMACHARY

Research and Training School, Indian Statistical Institute, Calcutta (India), September 12, 1963.

[7] E. ROBERTS and D. G. SIMONSEN, in *Amino Acids, Proteins and Cancer Biochemistry* (Academic Press, 1960), p. 123.

[8] I take this opportunity of thanking Mr. N. CHATTERJEE and Mr. S. K. DE for their indispensable help.

[9] *Note added in proof:* Recently, AWAPARA[10] noted that the amino acid pattern of the land snail *Otala lactea* remains unaltered after a starvation of six months. This result obtained by two-dimensional chromatography is more convincing than my findings.

[10] J. AWAPARA, in *Amino Acid Pools* (Ed. Holden, 1962).

Free Amino Acids in *Limnaea* III

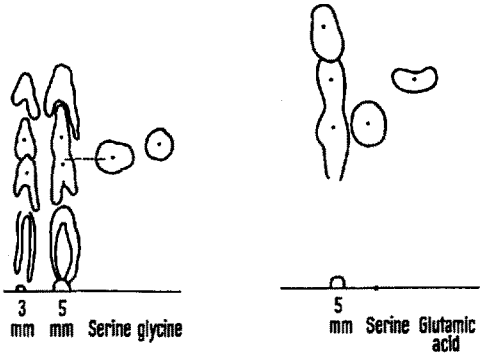
It has been shown in the earlier communications[1,2] inspired by the trail-blazing work of HADORN and MITCHELL[3], that in *Limnaea* there is a constant pattern of amino-acid pools, increasing in intensity with age and size of the snails and that these pools are resistant to prolonged starvation. This constancy and increasing intensity are explicable if all or most of the cells in the growing organism continue to prepare the same type of pools, even during the catabolic process in starvation. Some changes in amino-acid pools in the course of morphogenesis were, however, observed[1].

As, however, the intrinsic amino-acid content of the near-hatching eggs and small snails which have just hatched is very low, the less intense pools escaped notice.

A large number of very small snails (only a few days old), were therefore crushed one after another directly onto the filter paper and chromatographed as earlier[1,2]. The faint band of ninhydrin positive spots having higher Rf values which are characteristic of the adult snails[1] was now revealed. Thus, even at this early stage, practically all the pools are present and the relative intensities are also apparently already fixed. The present finding, therefore, all the more supplements and confirms the view expressed earlier[1]. It is now established that from about the hatching stage onwards only the absolute value of the amino-acid pools increases with the size. The relatively higher intensity of some pools over others, that is constantly maintained either by using up some pools very quickly or by replenishing the more intense pools alone by lysis of older tissue or by a consistent higher rate of biosynthesis, can be regarded as a genetically fixed biochemical index.

In view of the above result it now also seems possible that all the amino-acids of the snail are present even in the prehatching stage and that the two streaks[2] are only four unresolved spots, while the amino-acids with higher Rf values are too small to be detected.

As it would be of some interest to identify the amino-acid pools, pure known amino-acids were chromatographed with the solvent, *n*-butanol:acetic acid:water (4:1:1). With the same degree of caution as expressed by HADORN and MITCHELL[3], it can now be suggested that the four most prominent and clearly resolved amino-acid pools of *Limnaea*[1] are probably histidine or asparagine, serine or glutamic acid, or glycine, threonine and β-alanine. The spots due to pure amino-acids were compared with those due to direct crushing of small snails on the same paper. It is seen from the Figures that direct crushing of 3 and 5 mm long snails yielded streaks with some resolution. Of the four spots due to the 5 mm snail compared with serine and glutamic acid, the second and third are extremely close to the two known acids. In the other Figure also serine is suggestive. (Here the two lowest spots have a very peculiar shape due to the trapping effect of the solid material.)



It may be mentioned that Simpson et al.[4] made a detailed study of free-amino acids in some aquatic invertebrates. They found alanine, β-alanine, glycine, arginine, aspartic acid, glutamic acid, taurine and glutamine in snails. Taurine was found only in the marine snails, and not in fresh water snails such as *Limnaea*.

Résumé. L'auteur a constaté que les acides aminés libres chez les gastéropodes du genre *Limnaea*, immédiatement après l'éclosion, sont les mêmes que chez les adultes. Il a essayé aussi d'identifier chez des sujets adultes les quatre acides-aminés libres les plus caractéristiques. Deux d'entr'eux sont probablement l'acide glutamique et la sérine.

R. L. BRAHMACHARY

Indian Statistical Institute, Research and Training School, Calcutta (India), September 17, 1963.

[1] R. L. BRAHMACHARY and A. BHATTACHARYA, Exper. *19*, 143 (1963).

[2] R. L. BRAHMACHARY and A. BHATTACHARYA, Exper. *19*, 225 (1963).

[3] E. HADORN and H. K. MITCHELL, Proc. Nat. Acad. Sci. *37*, 650 (1951).

[4] J. W. SIMPSON et al., Biol. Bull. *117*, 371 (1959).