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FREE AMINO ACIDS AS REGULATORS OF OSMOTIC PRESSURE IN AQUATIC INSECT LARVAE

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SUMMARY

Microanalytical techniques are used to show that organic molecules play an important role is osmoregulation. Changes in the inorganic ion composition of haemolymph from fourth instar larvae of *Aedes aegypti* are correlated with the changes in the concentration of organic ions. Free amino acids have a significant role in regulating haemolymph osmotic pressure with respect to the osmotic pressure of the water in which the animal lives. Mechanisms by which amino acid levels could respond to changes in salt concentration are discussed.

INTRODUCTION

Organic components of animals' body fluids, and their regulation, have been studied in less detail than inorganic components. A notable exception has been the study of the use of urea by the crab-eating frog *Rana cancrivora* to increase internal osmotic pressure and thus prevent dehydration in a hypertonic environment (Gordon, Schmidt-Nielsen & Kelly, 1961).

This work stems from the preceding study of ionic regulation in the haemolymph of the fourth instar larvae of the yellow fever mosquito *Aedes aegypti* (Edwards, 1982*b*). The sum of the osmotic effects of the major inorganic ions falls well short of the measured osmotic pressure. Even taking potassium, calcium and magnesium into account a significant gap, which increases with salinity, is found. The residual osmotic pressure is due to organic components in the haemolymph, which are the subject of this study.

MATERIALS AND METHODS

Mosquito larvae were reared from eggs in artificial sea-water solutions ranging between 0 and 30% sea water in concentration. The composition of artificial sea water was as in Edwards (1982*a*). The larvae were fed with desiccated liver powder. Larvae were used for experimentation after they had been in the fourth instar for 72 h. Samples of haemolymph were taken by puncturing the animals and drawing the exuded fluid into a micropipette. The composition of the samples was measured. Osmotic pressure was measured with a Clifton cryostat biological nanolitre osmometer. Sugars and

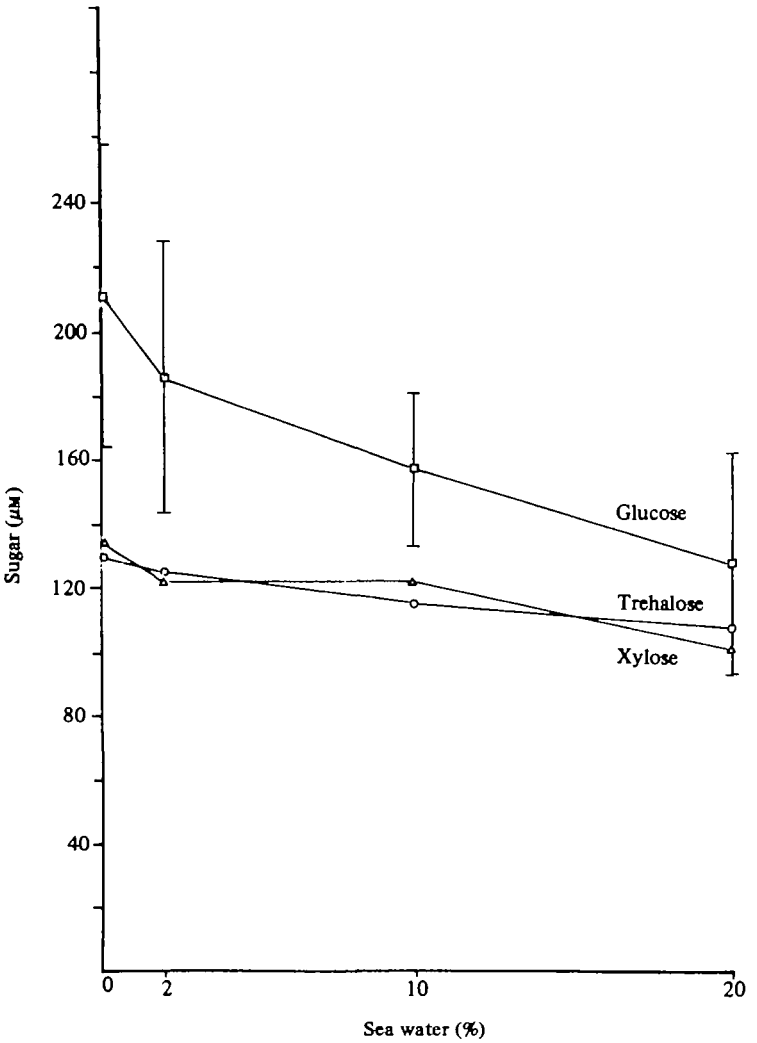

Fig. 1. Total haemolymph sugars as a function of environmental salinity calibrated against glucose, xylose, trehalose. S.E. of mean is greatest in glucose calibration and is plotted. $n = 6$.

amino acids were initially assessed qualitatively using thin layer chromatography and appropriate staining reactions. Subsequently, sugars were measured quantitatively by their charring reaction with concentrated sulphuric acid (Diamond & Denman, 1973). Quantitative amino acid measurements were made using ninhydrin staining and a spectrophotometer. Amino acid analyses of haemolymph samples were run on a Rank high pressure amino acid analyser and a Durrum amino acid analyser. Urea was measured by a miniaturization of the method of Fawcett & Scott (1960).

Osmotic fluxes across the body wall were determined by the method described by Nicolson & Leader (1974).

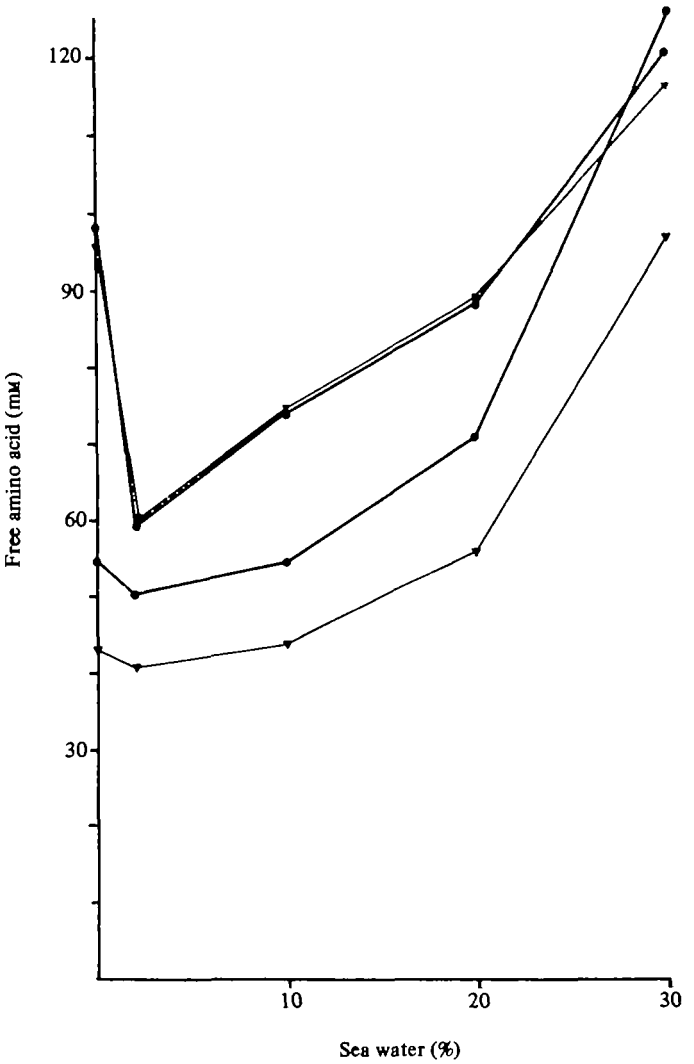

Fig. 2. Free amino acids measured in the haemolymph of larvae raised at salinities between 0 and 30% S.W. ...., Optical density measured at 570 nm; ▼, O.D. measured at 410 nm; ●, Upper two lines are for a fed culture, the lower two for a starved culture. Samples were pooled from 7 to 8 larvae.

RESULTS

Of the organic molecules in insect haemolymph, sugars or amino acids seem most likely to be involved in osmoregulation, for example amino acids form up to 52·8% of the osmolar concentration in some Lepidoptera (Sutcliffe, 1963). TLC plates of haemolymph samples from *Aedes aegypti* larvae in a range of salinities indicated that the sugar levels were constant while those of amino acids increased. These results were quantified as follows.

Sugars were measured by their charring reaction with concentrated sulphuric acid

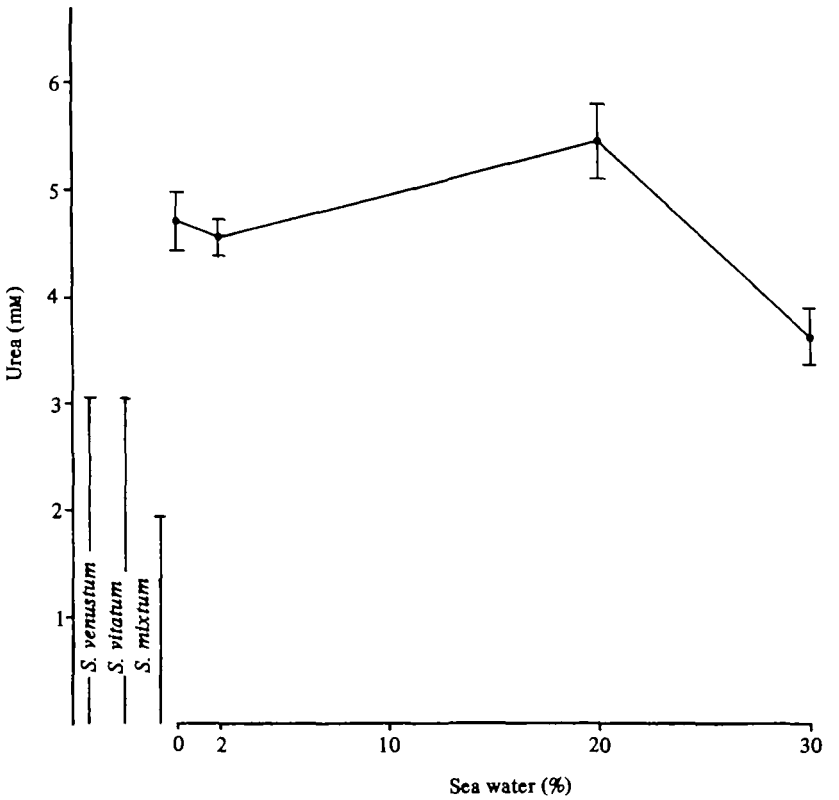

Fig. 3. Changes in haemolymph urea content with salinity compared to the haemolymph urea content of larval blackflies. S.E. of mean indicated. $n = 6$.

and were found to be present in the haemolymph at between 100 and 250 μm (Fig. 1) Therefore, sugars are at too low a level to be responsible for the osmotic pressure changes observed in the preceding paper (Edwards, 1982*b*).

Colorimetry in association with ninhydrin staining was used to quantify the free amino acids plus urea in the haemolymph. Larvae raised in low salinity medium had low levels of amino acids plus urea while those from high salinities had high levels. In 2% sea water, concentrations as low as 40 mM were recorded while in 30% sea water the figure was as high as 125 mM (Fig. 2). Changes of this magnitude are sufficient to explain the observed changes in osmotic pressure (Edwards, 1982*b*). These changes were not due to changes in urea concentration, because urea was found to be present at between 4 and 6 mM and was not affected by salinity (Fig. 3). Thus, whereas urea is used to regulate the osmotic pressure of the blood in some vertebrates (Gordon, Schmidt-Nielsen & Kelly, 1961), free amino acids are the changing organic component in the haemolymph of *Aedes aegypti* larvae.

The amino acid composition of hydrolysed and unhydrolysed cell-free samples of haemolymph were determined with amino acid analysers. Insufficient data was collected to relate the overall changes in free amino acids to specific amino acids in the haemolymph. However, the measurement of total amino acids at three different

Table 1. *Amino acid analysis of hydrolysed cell-free haemolymph*

Culture (% s.w.) ...	0	2	20	
Amino acid	mM	mM	mM	$\sigma n-1$
Asparagine	17·668	13·317	16·199	1·476
Threonine	11·382	8·755	10·024	1·783
Serine	16·073	11·080	16·981	4·269
Glutamic acid	65·468	57·910	64·069	11·803
Proline	10·354	7·915	9·049	1·515
Glycine	20·605	16·095	21·159	4·575
Alanine	18·762	14·533	19·111	3·689
Valine	10·019	6·969	8·773	1·807
Methionine	0·797	0·707	1·357	—
Isoleucine	7·357	6·083	6·747	1·165
Leucine	12·725	9·821	10·354	2·917
Tyrosine	4·487	3·319	4·319	1·176
Phenylalanine	7·482	5·311	6·791	1·380
Histidine	51·966	46·846	40·667	5·621
Lysine	11·374	8·510	9·060	1·633
Arginine	16·286	13·059	17·960	1·369
Ammonia	43·152	39·687	42·794	9·657
Sum of acids + Ammonia	325·957	269·949	306·326	—

0 and 2% figures are an average of two figures; 20% figures are the mean of four measurements. Standard deviations are given for the 20% figures except methionine which was only detected in one sample – that value is given.

Table 2. *Amino acid analysis of unhydrolysed cell-free haemolymph*

	mM
Glutamine	9·10
Glycine	0·84
Alanine	1·19
Leucine	14·40
Phenylalanine	0·16
Histidine	8·74
Lysine	1·35
Arginine	3·37
Ammonia	2·76

The sample comes from 2% larvae and was run twice at two different concentrations.

Table 3. *Net osmotic flux (J_{os}) measured between 0 and 15% sea water*

s.w. (%)	J_{os} (ml/g h^{-1})	$\sigma n-1$
0	$6{\cdot}35\times10^{-3}$	$1{\cdot}51\times10^{-3}$
5	$7{\cdot}00\times10^{-3}$	$1{\cdot}08\times10^{-3}$
10	$6{\cdot}62\times10^{-3}$	$1{\cdot}42\times10^{-3}$
15	$5{\cdot}72\times10^{-3}$	$0{\cdot}86\times10^{-3}$

$n = 5$.

salinities (Table 1) and the free amino acids in haemolymph from animals kept in 2% sea water (Table 2) will provide a useful baseline for future studies.

Osmotic fluxes of water across the body wall were almost constant over the measured range, 0–15% sea water, suggesting either that body wall permeability can be varied with salinity or that the osmotic gradient is maintained constant. The latter appears

to be the case as the rise in osmotic pressure of the haemolymph roughly parallels the rise in concentration of the external medium.

DISCUSSION

This study has shown significant changes in amino acid levels in the physiological range (0–30% sea water). Under 'normal' conditions (2% sea water) there are 40–60 mM free amino acids in the haemolymph, contributing about 20% of the total osmotic pressure. With increasing salinity amino acid levels rise steeply and may reach 120 mM in the haemolymph of larvae maintained in 30% sea water. The larva is hyperionic to the external medium; as the osmotic pressure of the external medium is increased, so the amino acids increase thereby acting as an osmotic buffer. Urea remains constant at a level comparable with other freshwater dipteran larvae (Gordon & Bailey, 1976). Inorganic cations are kept constant but the gradient to the outside is also maintained, therefore the osmotic influx of water is unaltered. There is little change in the osmotic flux at different salinities.

Amino acids have an extremely important role in osmoregulation of cells (Schoffeniels, 1973) and haemolymph. An involvement in cellular osmoregulation is seen in crustaceans. Changes in free amino acids brought about by changes in salinity have been shown in the muscles of *Eriocheir sinensis* (Bricteaux-Grégoire *et al.* 1962, Duchateau & Florkin, 1955), *Leander serratus* and *L. squilla* (Jeuniaux *et al.* 1961), *Orconectes limosus* (Siebers, 1972), *Astacus astacus* (Duchateau & Florkin 1961), the isolated nerves of *E. sinensis* (Gilles & Schoffeniels, 1969), and the tissues of *Nereis diversicolor* (Jeuniaux *et al.* 1961). *Eriocheir*, *Astacus*, *Leander* and *Nereis* all show an increase in cellular-free amino acids, thereby raising the cellular osmotic pressure, when the osmotic pressure of the haemolymph is increased by exposing the animal to a more concentrated external medium. Levels of individual amino acids in the haemolymph of crustaceans are known, (Evans, 1972), but changes have note been measured.

Amino acids play an important role in osmoregulation of the haemolymph in insects. The aquatic larvae of *Sialis lutaria* maintain the heamolymph osmotic pressure when changes are made or induced in chloride concentration, by regulation of the 'non-chloride fraction' (Beadle & Shaw, 1950). The freshwater larvae of *Libellula* and *Aeshna* show changes in haemolymph chloride concentration with changes in the external medium (Schoffeniels, 1950) as does the aquatic coleopteran *Dytiscus marginalis* (Schoffeniels, 1960), but 'la pression osmotique du milieu interieur reste practiquement constante'. High levels of free amino acids are found in haemolymph of other insects (Sutcliffe, 1963; Chen, 1966; Florkin & Jeuniaux, 1974). In aquatic nymphs of *Aeshna cyanea*, the haemolymph contains large concentrations of non-protein nitrogen (Raper & Shaw, 1948). The above insects appear to use free amino acids to keep the osmotic pressure of the haemolymph constant against a background of changing ion levels. In contrast the haemolymph of *A. aegypti* follows the crustacean cell pattern, where inorganic ion levels remain constant and free amino acids vary to keep a constant osmotic gradient.

It is not clear whether amino acids involved in osmoregulation of the haemolymph are provided by synthesis, as suggested for *Sialis* (Beadle & Shaw, 1950), or breakdown

protein, as suggested for *Orconectes limosus* (Siebers, 1972). In the isolated nerves of *Eriocheir sinensis* there is evidence that the amino acids are the product of neosynthesis (Schoffeniels, 1973).

The control of haemolymph acids in response to salinity changes could be brought about in a number of ways. Larval mosquitos have a hydrostatic skeleton. At low salt concentrations the animal's body fluids are hypertonic to the medium and water tends to enter the haemolymph. The influx of water is balanced by the loss of urine and the animal's hydrostatic pressure is maintained. If the larva is placed in a more concentrated medium and no regulation occurs, water movement will be reduced, hydrostatic pressure will fall, and the animal will become flaccid. Control of body volume is mediated by a neural mechanism (Ramsay, 1953*a*; Stobbart, 1971*c*). Body volume is monitored by stretch receptors and a hormone could be released from some point in the nervous system, possibly the stomatogastric ganglion. The action of the hormone could be on synthesis of amino acids or on protein catabolism. Alternative to this model is that proposed by Schoffeniels (1968). Energy is supplied through a common pathway to both salt transport and amino acid synthesis. Salt transport is inhibited by the inhibition of a salt-sensitive enzyme at the energy supply step. As salinity increases more energy is directed into amino acid synthesis. The end result is an internal osmotic pressure which rises with the osmotic pressure of the external medium and so keeps the osmotic influx constant.

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