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EXTRACELLULAR ACCUMULATION OF PROLINE, SERINE AND TREHALOSE IN THE HAEMOLYMPH OF OSMOCONFORMING BRACKISH-WATER MOSQUITOES

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SUMMARY

1. Larvae of *Culex tarsalis*, a mosquito, are capable of surviving and developing in dilutions of sea water ranging from 0 mosmol l^{-1} to 700 mosmol l^{-1}. In waters more dilute than 400 mosmol l^{-1}, the larvae osmoregulate, whereas in those more concentrated than 400 mosmol l^{-1}, the osmotic strength of the haemolymph parallels that of the medium, i.e. the larvae osmoconform. Over the full range of external concentrations tested, the larvae regulate the levels of Na^+, K^+, Mg^{2+}, Ca^{2+} and Cl^- in the haemolymph.

2. Analyses of haemolymph samples from larvae adapted to media of 50 mosmol l^{-1} or 600 mosmol l^{-1} indicate that the increase in haemolymph osmotic concentration observed in media above 400 mosmol l^{-1} is due to the accumulation of organic compounds, particularly proline, serine and trehalose.

INTRODUCTION

Aquatic larvae of insects from various orders can survive and develop in saline waters (Cheng, 1976), and those capable of osmoregulating in saline waters have been studied in considerable detail (reviewed by Bradley, 1985). In saline-water mosquitoes of the genus *Aedes*, a hyperosmotic urine is formed in the rectum by fluid secretion (Bradley & Phillips, 1975, 1977). Bicarbonate excretion occurs by HCO_3^-/Cl^- exchange in the rectum (Strange, Phillips & Quamme, 1984) while Mg^{2+} and SO_4^{2-} elimination involves inducible ion transport mechanisms in the Malpighian tubules (Phillips & Maddrell, 1974; Maddrell & Phillips, 1975).

Other saline-tolerant aquatic insect larvae can osmoregulate in dilute media (<400 mosmol l^{-1}) but osmoconform in more concentrated ones (Sutcliffe, 1961; Scudder, Jarial & Choy, 1972; Shaw & Stobbart, 1974). The physiological mechanisms underlying this type of osmoregulatory strategy in insects have not been described. Garrett & Bradley (1984*a*) coined the term 'brackish-water larvae' for osmoconforming species of mosquitoes and demonstrated that these differed from

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osmoregulating saline-water larvae on the basis of saline tolerance, rectal ultrastructure and haemolymph osmotic response to increased salinity.

We report here results of analyses of the major organic and inorganic osmolytes in the haemolymph of the brackish-water mosquito *Culex tarsalis*. We demonstrate that increased haemolymph osmotic concentration in saline media is the result of the accumulation of amino acids and trehalose.

MATERIALS AND METHODS

Experimental animals were taken from a colony of *Culex tarsalis* obtained originally from Dr William Reisen, Arbovirus Research Laboratory, Bakersfield, California. Adults were maintained in 24 cm × 24·5 cm × 31·5 cm cages at 27 °C, in a relative humidity of 70–80 %, under a 12 h: 12 h light: dark cycle. Larvae for colony propagation were fed pulverized rat chow (Purina) and maintained in shallow pans containing 500 ml of 10 % sea water at 27 °C, under a 12 h: 12 h light: dark cycle.

Larvae used for experimental purposes were acclimated to increasing concentrations of sea water diluted with distilled water, by stepwise increases of 100 mosmol l^{-1} at 2-day intervals. The osmotic concentration of the medium was determined using a Wescor vapour pressure osmometer. Larvae were acclimated to the final concentration for 3 days prior to heamolymph sampling. Haemolymph samples were obtained by rinsing larvae in distilled water, drying on filter paper, and exsanguinating on Parafilm. For osmometry, blood samples were rapidly drawn into a micropipette, and introduced into sample holders filled with oil in a Clifton freezing point depression nanolitre osmometer. Both osmometry systems were calibrated with the same standard solutions. Control experiments with standard salines handled identically showed changes in osmotic concentrations of less than 5 % due to evaporation. Sodium, potassium, calcium and magnesium were measured by atomic absorption spectrophotometry (Bradley & Phillips, 1975). Chloride analyses were performed on haemolymph samples pooled from four larvae. Chloride was measured using a Buchler digital chloridometer (Garrett & Bradley, 1984*a*).

A Dionics D-500 ninhydrin-based amino acid analyser was used to measure haemolymph amino acids. Pooled haemolymph samples were centrifuged to remove haemocytes. 2 µl of the supernatant was combined with 100 µl of citrate loading buffer (pH 2·2). This solution was placed in a capped, polyethylene microcentrifuge tube, frozen in liquid nitrogen, and either used immediately or stored at −70 °C in a freezer. Thawing and any necessary further dilutions occurred just prior to injection into the amino acid analyser. Trehalose was identified by infrared spectroscopy (Crowe, Crowe & Chapman, 1984). Pooled haemolymph samples were diluted in 80 % ethanol and centrifuged in a microfuge to remove cells and proteins. Concentrations of trehalose were determined by comparison with standards using high-pressure liquid chromatography (HPLC) with an index of refraction peak detector.

RESULTS

Larvae of *Culex tarsalis* can survive in media ranging from 0 to 700 mosmol l^{-1}. In media more concentrated than 700 mosmol l^{-1}, growth and pupation are inhibited. In artificial sea water of salinities below 400 mosmol l^{-1}, larvae of *Culex tarsalis* regulate the osmotic concentration of the haemolymph (Fig. 1). At salinities above 400 mosmol l^{-1}, the larvae osmoconform, i.e. larval haemolymph osmotic concentration is essentially identical to that of the external environment.

To determine which compounds accumulate in the haemolymph to produce the pattern of osmoconformation observed in Fig. 1, we analysed numerous inorganic and organic solutes in larvae acclimated to 50 mosmol l^{-1} and 600 mosmol l^{-1}. The osmotic concentrations of haemolymph samples taken from animals raised at 50 and 600 mosmol l^{-1} were 275 ± 25 mosmol l^{-1} (mean ± S.D., $N = 6$) and 595 ± 44 mosmol l^{-1} (mean ± S.D., $N = 7$), respectively, and the inorganic and organic components are given in Table 1. The concentrations of Na^+ and Cl^- were lower in the haemolymph of animals acclimated to 50 mosmol l^{-1} sea water than in those acclimated to the more concentrated medium. The concentrations of Mg^{2+}, Ca^{2+} and K^+ did not change. The slight increase in the ionic activity observed does not account for the total increase in the osmotic activity of the haemolymph. Therefore, other osmotically active solutes must account for the increase in haemolymph osmotic concentration.

Trehalose is a major haemolymph sugar in insects (Wyatt, 1967). In animals raised in 50 mosmol l^{-1} solution, the haemolymph contained relatively high levels

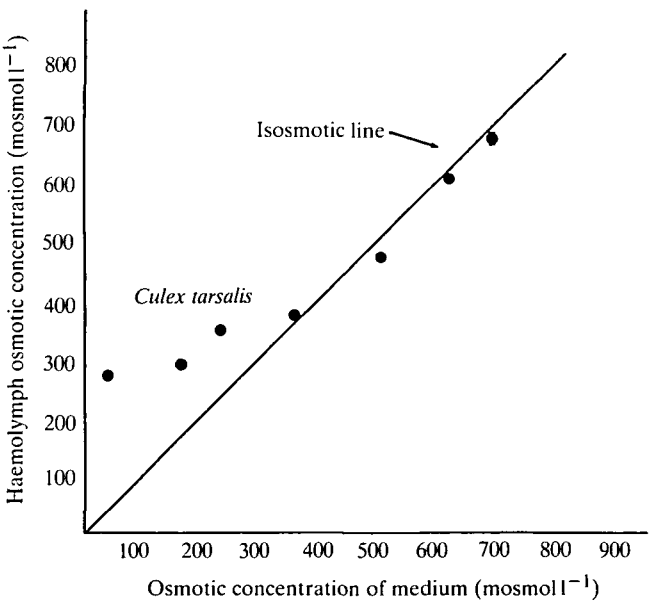

Fig. 1. The relationship between the mean osmotic concentration of the haemolymph of larvae of *Culex tarsalis* and the osmotic concentration of the external medium to which they were acclimated for a minimum of 3 days. One standard deviation of the mean is smaller than the size of the points illustrated. Sample size is six or greater for each point.

(8 mmol l^{-1}) of trehalose, and after acclimation to 600 mosmol l^{-1}, the level increased more than four-fold. This increase accounts for only 7 % of the total increase in the osmotic activity of the haemolymph.

Total amino acid concentrations in the haemolymph of larvae raised at 600 mosmol l^{-1} are enriched five-fold (Table 1). Proline and serine show the largest increases. Together, these two amino acids account for approximately 30 % of the osmotic concentration of the haemolymph in larvae reared at 600 mosmol l^{-1}.

The total inorganic ions measured in the haemolymph of animals acclimated to 600 mosmol l^{-1}, taking into account expected ionic dissociations, was 209 mmol l^{-1}. Upon addition of the trehalose and amino acids, the total concentration of osmotically active compounds was 505 mmol l^{-1}. Assuming that each molecule is dissolved and fully osmotically active this should yield an osmotic concentration of 505 mosmol l^{-1}. The compounds measured, therefore, account for 505 mosmol l^{-1} of the 595 mosmol l^{-1} measured in the haemolymph of these larvae. If similar calculations are carried out for larvae reared at 50 mosmol l^{-1}, the osmolytes measured account for 197 mosmol l^{-1} of the 275 mosmol l^{-1} actually present in the haemolymph. Osmolytes which were not measured (e.g. organic acids, proteins,

Table 1. *Analysis of inorganic and organic components in the haemolymph of larvae of* Culex tarsalis

Osmolyte	5 % seawater-adapted larvae	60 % seawater-adapted larvae
Na^+	78 ± 9 (8)	98 ± 12 (8)
K^+	10 ± 3 (8)	10 ± 3 (8)
Cl^-	46 ± 3 (4)	96 ± 26 (4)
Mg^{2+}	2 ± 0·4 (10)	2 ± 0·5 (10)
Ca^{2+}	3 ± 0·5 (10)	3 ± 1·1 (10)
Trehalose	8 ± 2 (6)	37 ± 13 (6)
Amino acids	(N = 10)	(N = 11)
Ala	4·1 ± 0·6	15·7 ± 3·0
Arg	1·0 ± 0·3	2·0*
Asp	0·3 ± 0	1·7 ± 0·1
Cys	—	4·6 ± 0·7
Glu	1·1 ± 0·2	5·0*
Gly	1·1 ± 0·2	3·9 ± 1·7
His	4·9 ± 1·0	6·9 ± 5·0
Iso	0·6 ± 0·2	3·8 ± 1·7
Leu	15·2 ± 3·0	23·1 ± 5·0
Lys	0·8 ± 0·2	1·6 ± 0·2
Met	0·3 ± 0	5·8*
Pro	8·1 ± 2·0	123·7 ± 30·0
Ser	8·4 ± 0·7	53·7 ± 16·0
Tyr	3·4 ± 0·1	2·9*
Val	0·5*	3·8*

Values expressed as mean (mmol l^{-1}) ± S.D. (N).

Values for all haemolymph components compared between groups were statistically significantly different at the $P < 0{\cdot}005$ level, with the exceptions of Mg^{2+}, Ca^{2+}, histidine and tyrosine.

* These amino acids had values below the resolution of the amino acid analyser in eight samples.

etc.) therefore account for 78 mosmol l^{-1} of activity in the haemolymph of animals reared at 50 mosmol l^{-1}, and 90 mosmol l^{-1} in the haemolymph of animals reared at 600 mosmol l^{-1}. We propose that these haemolymph components are essentially identical in the two groups of larvae and that the major osmotically active haemolymph components have been identified. This suggestion is supported by examination of HPLC fractions of haemolymph from larvae utilizing ultraviolet and index of refraction detectors. No large unidentified peaks were observed. In particular, our methods revealed no substantial concentrations of glucose, organic acids or polyols.

The above calculations rest on the assumption that the inorganic and organic solutes measured possess an osmotic activity close to the ideal osmotic activity. To test this assumption, we made a solution containing 123 mmol l^{-1} proline, 54 mmol l^{-1} serine, 23 mmol l^{-1} leucine, 56 mmol l^{-1} trehalose, 5 mmol l^{-1} sodium citrate, 10 mmol l^{-1} KCl, 80 mmol l^{-1} NaCl and 10 mmol l^{-1} Hepes buffered with NaOH to pH 7·0. This solution has a total solute concentration of 469 mmol l^{-1} and a measured osmotic concentration of 436 mosmol l^{-1}. This indicates that, at this concentration, the solutes possess 93 % of the ideal osmotic activity.

DISCUSSION

Proline and trehalose are known to act as non-toxic osmolytes which accumulate to high concentrations in bacteria, plants and animals subjected to desiccation, freezing or high salinity (Measures, 1975; Treichel, Brinckmann, Scheitler & von Willert, 1984; Yancy *et al.* 1982; Florkin & Jeuniaux, 1974). In certain insects, high levels of amino acids and organic acids occur in the haemolymph (Coutchie & Crowe, 1979*a*,*b*). The majority of these observations has been made on terrestrial insect species, particularly in the orders Lepidoptera, Coleoptera and Hymenoptera (Shaw & Stobbart, 1974; Yancy *et al.* 1982). Edwards (1982) measured high levels of amino acids which increased with salinity in *Aedes aegypti*, a freshwater mosquito. The present report is the first to demonstrate the use of these compounds as major extracellular osmolytes during adaptation to high salinity in a brackish-water mosquito larva.

The mechanisms underlying the accumulation of the amino acids and trehalose in the haemolymph of *Culex tarsalis* remain obscure. Since different osmolytes show distinct degrees of accumulation (e.g. proline increases 15-fold, trehalose only four-fold), water loss during saline adaptation cannot be the sole mechanism underlying the increase in solute concentration in the haemolymph. This suggests an active mechanism for the accumulation or proliferation of these osmolytes, e.g. *via* osmotically or ionically activated enzymes (Bishop, Greenwalt & Burcham, 1981), or substrate-regulated enzymatic pathways (Madin, Loomis & Crowe, 1985).

Euryhaline marine invertebrates from a number of phyla osmoconform to the external aquatic medium (Fyhn, 1976; Gilles, 1979; Deaton, 1981). Marine invertebrates concentrate organic osmolytes intracellularly in response to increasing salinity, avoiding the toxicity associated with the accumulation of salts (Pierce &

Greenberg, 1973; Baginski & Pierce, 1975, 1977; Hillbish, Deaton & Koehn, 1982; Schoffeniels, 1976). Amino acids, amino acid derivatives and some sugars (e.g. trehalose) are the organic solutes most commonly accumulated for the purpose of regulating intracellular osmotic activity and cell volume (Bishop *et al.* 1981; Crowe, 1981; Gilles, 1979; Gilles & Pequeux, 1981; Pierce, 1982; Zurburg & De Zwann, 1981). During periods of decreasing salinity these are released (Gilles & Pequeux, 1981; Pierce, 1982).

By contrast, we have shown that in larvae of *Culex tarsalis* amino acids and trehalose accumulate extracellularly in the haemolymph. Presumably, the tissues are also isosmotic with the haemolymph and the external medium, probably due to high intracellular levels of organic compounds. The osmotic concentration in the cells and the intracellular osmolytes present have not yet been examined in osmoconforming insects.

Waters of marine origin have relatively fixed ionic ratios, even during periods of dilution or concentration. Osmoconforming marine invertebrates allow the ions in the haemolymph to conform to the external environment (Gilles, 1979). In the marine environment their haemolymph is therefore always rich in NaCl. By contrast, larvae of *Culex tarsalis* allow the haemolymph to osmoconform while controlling its chemical composition. Such a strategy is particularly useful for exploiting athallasohaline waters of terrestrial origin which can have very diverse ionic compositions, including waters rich in $MgSO_4$ or $NaHCO_3$ (Bradley & Phillips, 1977). It is perhaps for this reason that these larvae inhabiting athalassohaline waters have evolved an osmoregulatory strategy distinct from that of the marine invertebrates.

The osmotic and ionic responses of the haemolymph of *Culex tarsalis* to changing concentrations in the medium are identical to those described for *Culiseta inornata* (Garrett & Bradley, 1984*a*). Electron microscopic studies have shown that both species possess a rectum with a single rectal segment and cell type (Garrett & Bradley, 1984*b*; and unpublished observations). For these reasons, larvae of both species fall into the physiological category of 'brackish-water larvae', as distinct from freshwater larvae which have a lower saline tolerance and saline-water larvae which have a two-part rectum and exhibit osmoregulation in highly saline media (reviewed by Bradley, 1987).

At present, it is not known whether larvae of *Culiseta inornata* accumulate the same organic compounds as is reported here for *Culex tarsalis*. If the same organic compounds are accumulated by both species, it may indicate a shared brackish-water ancestor. Alternatively, it may be a result of convergent evolution, perhaps reflecting the unusual capabilities of high concentrations of proline and trehalose to protect proteins and membranes from denaturation (Yancey *et al.* 1982; Crowe *et al.* 1984).

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