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Mechanisms of intracellular isosmotic regulation: Fate of ^{14}C-labeled serum proteins in the shore crab *Carcinus maenas* after changed environmental salinity

D. SIEBERS

Biologische Anstalt Helgoland (Zentrale); Hamburg 50, Federal Republic of Germany

KURZFASSUNG: **Mechanismen der intrazellulären isosmotischen Regulation: Das Schicksal von ^{14}C-markiertem Serumprotein der Strandkrabbe *Carcinus maenas* nach einem Wechsel der äußeren Salinität.** Individuen der Strandkrabbe *Carcinus maenas*, die zwei Monate in 11 ‰ S gehalten worden waren, wurden innerhalb von 5 Tagen 100 μCi ^{14}C-Glucose und eines ^{14}C-Aminosäuregemisches in physiologischer Lösung ins Hämocoel injiziert. Diese Tiere dienten als Quelle der benötigten ^{14}C-Serumproteinlösung, die anderen, über 2 Monate in 11 ‰ S gehaltenen Strandkrabben ins Hämocoel injiziert wurde. Eine Gruppe dieser Tiere wurde nach der Injektion in 38 ‰ S überführt, die Kontrollgruppe verblieb nach der Injektion in 11 ‰ S. 6 Stunden später wurden die Konzentrationen von Serumproteinen und freien Aminosäuren sowie die im Serum verbliebene Radioaktivität und die Radioaktivität in den gesamten niedermolekularen Intermediärprodukten bestimmt. Aus der signifikanten Abnahme des Serumproteins und der im Serum verbliebenen Radioaktivität sowie aus einer signifikanten Zunahme der Radioaktivität in den osmotisch aktiven Intermediärprodukten wird geschlossen, daß die intrazelluläre niedermolekulare organische Substanz, die während der Osmoregulation bis zu 100 % vermehrt werden kann, zu einem erheblichen Teil durch proteolytische Vorgänge im Serum der Tiere entsteht.

INTRODUCTION

While the osmotic pressure of the body fluids of most marine and estuarine invertebrates is slightly hyperosmotic in relation to the environmental salinity and follows changes corresponding to sea-water concentrations, a variety of invertebrates can maintain the osmotic pressures of their body fluids relatively constant, despite greater fluctuations in the surrounding salinity. The ability of osmoregulation brings about a certain homoiosmoticity of the body fluids.

The common shore crab *Carcinus maenas* belongs to those extremely euryhaline species, which are able to inhabit estuaries with tidal salinity changes. This crustacean can be maintained in salinities between about 8 and 50 ‰ for months. Since the body fluids are not absolutely homoiosmotic, increases in osmotic pressures during increasing external salinities and decreases during decreasing salinities can be realized. Despite little experimental information, intracellular fluids are regarded as being kept

isosmotic to the osmotic pressure of hemolymph, which occupies the largest of the animal's extracellular spaces. The osmotic changes in the body fluids are accounted for by inorganic ions in the hemolymph and to a degree of about 50 % by amino acids in the intracellular fluids. In addition, low molecular neutral carbohydrates, especially trehalose, take part in the increase of intracellular osmotic pressure.

The discussion about changes in the metabolism, inducing increases and decreases of low molecular osmotically active intracellular organic material in relation to environmental salinities has not yet come to an end. Several authors claim a de-novo synthesis of amino acids within the intermediary metabolism, when the intracellular free amino acids are increased by about 100 % after a transfer of animals from a low to a high external salinity. Others claim a considerable participation of proteolytic processes to dispose the increased amount of free amino acids (for further introductory information see: LANGE 1968, FLORKIN & SCHOFFENIELS 1969, SIEBERS 1972, SIEBERS et al. 1972, SIEBERS & LUCU 1973).

In the crayfish *Orconectes limosus,* which inhabits freshwater areas, SIEBERS (1972) established – in a group of animals that had been kept in a salinity of 26 ‰ for 10 days without food – the greatest absolute increase in non-essential amino acids such as alanine, glycine, and proline, but in relation to freshwater controls also all essential free amino acids, which cannot by synthesized by the crayfish, had been increased. Since the animals had not been fed, protein was regarded to be the source of increased free amino acids. This assumption was confirmed by the results, that free amino acids had been increased by about the same amount by which total protein had been decreased in comparison to controls. This amount of hydrolized protein was roughly in the range of that amount, which could be calculated from increased single essential free amino acid levels on the basis of protein composition data.

Further experiments (SIEBERS et al. 1972) established that in *Carcinus maenas* serum proteins decrease from 4.0 to 2.8 g/100 ml within 12 h after a transfer of crabs from 11 to 38 ‰ S. These facts had already been published by DRILHON-COURTOIS (1934) after investigating other crustacean species: in freshwater crustaceans such as *Astacus astacus* and *Telphusa fluviatilis* the protein content of the blood decreases greatly when the animals are transferred to various concentrations of sea water.

The serum protein decrease in *C. maenas* is accompanied by a decrease in free serum amino acids. The supposition arose that especially serum proteins participate in the corresponding increase of intracellular free amino acids by hydrolytic processes. This supposition was confirmed by results on the extracellular space in *C. maenas,* which does not differ significantly in response to the external salinity (10–50 ‰ S), so that serum protein concentrations (g/100 ml) also represent real serum protein amounts in the animal, because the volumes of distribution did not change (SIEBERS & LUCU 1973).

The present investigation was designed to obtain a ^{14}C-labeled *Carcinus* serum protein solution, to inject this solution into the hemolymph of crabs, which had remained for more than 2 months in 11 ‰ S, and to elucidate the fate of the injected labeled serum protein after a transfer of crabs from 11 to 38 ‰ S in relation to controls remaining in 11 ‰ S after the injection.

MATERIALS AND METHODS

Missing knowledge about the existence of differences in serum proteins in relation to sex and age-dependent body weight was the reason to use only male crabs of nearly equal fresh weights (40 ± 5 g fresh weight). The crabs were collected near the island of Helgoland, North Sea, at the beginning of February 1974, and kept at 11° C and 11 ‰ S for 2 months. The animals were fed daily with small pieces of common shrimp *Crangon crangon* until 3 days before the experiments started. The water was filtered in circuit. Neither mortality nor moulting occurred.

Two crabs were chosen to provide ^{14}C-labeled serum material. The crabs were kept separately under conditions equal to the rest with the exception that they were not fed. Twice daily over a period of 5 days they were injected with 0.5 ml of a solution containing 50 μCi of a protein hydrolysate-C14 (U) with the specific activity of 57 mCi/mg-atom carbon and 50 μCi of glucose-C14 (U) with the specific activity of 335 mCi/m mol (The Radiochemical Centre, Amersham), which were dissolved in 10 ml of physiological *Carcinus* saline (Pantin 1934), previously diluted 1:2 with distilled water. The dilution of the original physiological saline provided a saline of an osmotic pressure equal to the hemolymph of the crabs maintained in 11 ‰ S. 2 days after the last injection, hemolymph from these highly uniformly labeled crabs was withdrawn from the base of a walking leg by a hypodermic needle. Hemolymph was always handled at 0° C (ice-bath). After centrifugation (0° C, 15 min, 15 000 rpm) the supernatant ^{14}C-labeled serum was stored at – 26° C until use. Since the serum of *C. maenas* consists of about 4000 mg of protein, 20 mg of free amino acids and 6 mg of low molecular carbohydrates per 100 ml, the radioactive label of low molecular substances was also regarded as negligible in relation to protein. The resulting serum contained 54.7 mg protein and 187 000 dpm per ml, the specific activity is 3400 dpm per mg of protein.

The experiment started with the injection of 163 μl of this ^{14}C-labeled serum protein solution (containing 8.9 mg of protein) into the hemocoel of crabs by means of a hypodermic syringe attached to a fixed micrometer screw, by which the steel plunger could be controlled exactly. The crabs' serum protein was increased by the injection by about 2 %.

After the injection one half (n = 13) of the crabs remained in 11 ‰ S, the other half (n = 13) was transferred to 38 ‰ S.

Exactly 6 hours later about 0.5 ml of hemolymph was withdrawn and centrifuged as described. The crabs were stored in air-tight plastic bags at – 26° C. Radioactivity remaining in the serum was counted in a Tracerlab liquid scintillation counter with corrections based on external standard and channel ratio, subsequently followed by internal standardization. Counting vials contained 200 μl of serum, diluted by 10 ml of Insta-Gel (Packard). In the same way 100 μl of the ^{14}C-labeled serum protein injection solution were also counted.

100 μl of serum were used for protein determinations by means of the biuret reaction (Gornall et al. 1949).

For isolation of total intermediary products (amino acids, low molecular carbohydrates, organic acids, nucleotides, and dissolved inorganic salts) the crabs were cut

into small pieces and homogenized in 4 Vol. (w/v) of methanol (Ultra-Turrax, 20 000 rpm, 5 min, room-temperature). Aliquots of the homogenates were centrifuged (10 min, room-temperature, 3600 rpm) and the supernatants were collected. The precipitates were extracted by 80 % methanol (v/v, distilled water), followed by 2 extractions with chloroform/methanol (2:1) and a final extraction with 80 % methanol. All extractions were subsequently followed by centrifugation, the extracts were collected and diluted with distilled water. After centrifugation, the upper aqueous phase, which contains all intermediary products and dissolved inorganic salts, was separated from the lower chloroform-lipid phase, evaporated to dryness, and redissolved in distilled water (SPECK & URICH 1969). The final concentration was: intermediary products of 4 g fresh weight per 5 ml of distilled water.

– 1 ml was taken for the determination of radioactivity after the addition of 10 ml of Insta-Gel. Counting data provided the amount of serum protein that had been hydrolized and transformed within 6 h to fill up the stationary pool of intracellular osmotically active low molecular organic substances.

– 10 μl were used for the determination of total free amino acids by means of the ninhydrin reaction (TROLL & CANNAN 1953). The determination of total amino acids in the crab included also the free amino acids remaining in the hemolymph, but since their amount is less than 1 % of total intracellular amino acids, they are neglected.

All values of individual crabs were calculated for a standard crab of 40 g fresh weight (SPECK & URICH 1969) and compared between the experimental groups by means of the t-test.

RESULTS AND DISCUSSION

The analytical data about the fate of ^{14}C-labeled serum protein in crabs, 6 h after transfer from 11 to 38 ‰ S, are summarized in Tables 1 and 2. Serum protein concentrations decreased from 394 to 330 mg in crabs transferred to 38 ‰ S. They have been calculated for a 40 g crab from serum protein concentrations on the basis of an extracellular space of 17.9 % of body weight (SIEBERS & LUCU 1973). This means a reduction in serum protein of 64 mg/crab within 6 h. Twelve hours after the same transfer, the decrease in serum protein had amounted to 86 mg (SIEBERS 1972).

Table 1

Carcinus maenas. Serum protein, free amino acid concentrations, and specific activity of serum, 6 hours after transfer from 11 to 38 ‰ S in crabs, injected with ^{14}C-labeled *Carcinus* serum protein

	Serum protein concentrations (mg/40 g crab) ± s. e.	Specific activity of serum protein (dpm/mg) ± s. e.	Free amino acid concentrations (mg alanine eq./40 g crab) ± s. e.
Crabs remaining in 11 ‰ S	394 ± 63	50.8 ±12.5	179 ± 25
6 hours after transfer from 11 to 38 ‰ S	330 ± 75	52.4 ± 10.7	185 ± 26
Level of significance	$p < 0.005$	$p = 0.74$	$p = 0.55$

Table 2

Carcinus maenas. Distribution of radioactivity 6 hours after transfer from 11 to 38 ‰ S in crabs, injected with ^{14}C-labeled *Carcinus* serum protein. Percentages of injected ^{14}C-activity are given in brackets

	Injected amount of serum protein ^{14}C-activity (dpm/40 g crab)	Radioactivity remaining in serum (dpm/40 g crab) ± s. e.	Radioactivity detected in the intracellular pool of low molecular intermediary products (dpm/40 g crab) ± s. e.	Calculated radioactivity which has been transformed to CO_2 or other substances than serum protein or intermediary products (dpm/40 g crab)
Crabs remaining in 11 ‰ S	30 400 (100 %)	20 500 ± 4800 (67.4 %)	4900 ± 450 (16.1 %)	5000 (16.5 %)
6 hours after transfer from 11 to 38 ‰ S	30 400 (100 %)	16 900 ± 3500 (55.6 %)	6300 ± 850 (20.7 %)	7200 (23.7 %)
Level of significance		$p < 0.005$	$p < 0.001$	

Free intracellular amino acids do not differ significantly in either group of crabs. This is consistent with an earlier publication (SIEBERS et al. 1972), that the processes of increasing the concentrations of low molecular organic material are slower than those of establishing new osmotic pressures in the hemolymph.

Radioactivities remaining in serum differ significantly (Table 2). They provide evidence that in crabs, transferred from 11 to 38 ‰ S, serum proteins have been metabolized by about one half, in controls by about one third. The percentages of radioactivity remaining in serum establish that crabs which have been transferred to the high salinity have metabolized 44.4 % of serum protein within the first 6 h after transfer and the controls have metabolized 32.6 %.

The question about the metabolic fate of the hydrolized ^{14}C-amino acids is answered by results on the radioactivity detected in the intracellular pool of low molecular intermediary products (Table 2). In crabs, transferred to 38 ‰ S, 20.7 % of initial serum protein activity can be detected in low molecular organic substances, in controls only 16.1 % ($p < 0.001$). Low molecular organic substances have not been further separated into amino acids, carbohydrates, organic acids, and nucleotides, to obtain the distribution of radioactivity between these classes of substances, because all of them are osmotically active.

Finally, percentages of radioactivity have been calculated, which has been transferred from serum protein to CO_2 or substances other than serum protein or intermediary products, such as non-serum protein, polymers such as polysaccharides and nucleic acids or lipids (Table 2). It becomes obvious, that the total protein metabolism must have been increased, because a greater proportion of serum protein has been transformed via amino acids in the crabs, which had been transferred to 38 ‰ S, than in controls.

Experimental results, however, do not enable the author, to distinguish clearly between hydrolysis of serum proteins and their resynthesis from free amino acids. The presented balance of radioactivities between serum protein and low molecular substances is therefore regarded as a net balance within 6 hours of experiment. However, protein amounts in serum are regarded as constant in controls from the moment of injection to the time of withdrawing hemolymph 6 hours later, a constancy obtained by equal rates of hydrolysis and resynthesis. Specific activities of serum protein (Table 1) have remained constant. Radioactive free amino acids originating from serum protein are diluted considerably within the pool of intracellular free amino acids, and resynthesis takes place from this diluted pool. Thus, the pursuit of label from serum proteins predominantly demonstrates hydrolytic pathways. These results are not in agreement with the assumption of a de-novo synthesis of intracellular free amino acids as proposed in previous publications by FLORKIN & SCHOFFENIELS (1965), FLORKIN et al. (1964), and FLORKIN & SCHOFFENIELS (1969).

SUMMARY

1. Two individuals of the common shore crab *Carcinus maenas*, which had been maintained for 2 months in 11 ‰ S, were injected 100 μCi of ^{14}C-glucose and a

^{14}C-amino acid mixture in physiological saline during 5 days. They were the source of a ^{14}C-labeled serum protein, which was injected into the hemocoel of crabs which had been maintained for more than 2 months in 11 ‰ S. Six hours after injection the distribution of label and concentrations of serum proteins and free amino acids were investigated in 13 crabs, transferred to 38 ‰ S after the injection, and in 13 crabs (controls) which remained in 11 ‰ S.

2. In crabs transferred from 11 to 38 ‰ S, proteolytic processes in serum increased; this was demonstrated by a decrease in serum proteins 6 hours after transfer. This assumption is confirmed by the fact, that ^{14}C-radioactivity in serum originating from serum proteins decreased by 44.4 % within 6 hours after transfer, whereas in controls it decreased by 32.6 %.
3. Serum proteins have been metabolized in favour of low molecular intracellular intermediary products, such as amino acids, sugars, and organic acids, all contributing to the new intracellular osmotic pressure. This is shown by a significant increase in radioactivity of low molecular intracellular intermediary products.

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Author's address: Dr. D. Siebers
Biologische Anstalt Helgoland (Zentrale)
2000 Hamburg 50
Palmaille 9
Federal Republic of Germany