Changes in protein-calcium association during different hours of a day in the haemolymph of the crab *Scylla serrata* (Forskal)

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Summary. In *Scylla serrata,* the haemolymph free calcium and bound calcium showed fluctuations during the different hours of the day. Whenever bound calcium decreased or increased in the haemolymph, the free calcium increased or decreased respectively; the significance of this is discussed. Besides, the suitability and reliability of Webster's chloranilic acid method, compared with other methods for the determination of haemolymph free and bound calcium, are empirically assessed.

In body fluids, in general, calcium exists in the ionic state, in a state bound to citrates, carbonates, phosphates and organic anions, and in a state bound to colloids[1]. The functional role of calcium differs in these 3 states[2-4]. In the haemolymph of crustaceans a major proportion of the calcium is shown to be bound to protein[5-8], although it is possible that calcium may also occur bound with acidic lipids[9-12] and acid mucopolysaccharides[13,14]. If calcium were to be bound to haemolymph proteins, then it might vary with the protein level of the haemolymph, as is known in human plasma[15]. Haemolymph protein concentration in *Scylla serrata* has been shown to vary with the time of day[16]. Therefore the calcium-protein relationship during different hours of the day was studied in *Scylla serrata,* after selecting a reliable and consistent method for the determination of haemolymph free and bound calcium.

For this purpose, normal, intermoult, male specimens of *Scylla serrata* of carapace width of 80-122 mm were used. Haemolymph was collected directly into a micropipette after cutting the tip of the dactylus. Haemolymph was transferred immediately to the tube containing deproteinizing agents, for any rise in pH caused by loss of CO_2 will favour binding of more calcium to protein[17,18]. A flame photometric method, a permanganometric titration method and Webster's spectrophotometric method using chloranilic acid were compared for the determination of calcium in the haemolymph. The mean total haemolymph calcium values obtained with the 3 methods do not differ much from one another (table 1). However, taking the percentage co-efficient of variation into consideration, Webster's method showed the smallest co-efficient of variation, indicating consistency in the performance of the method.

For determining protein-bound calcium the haemolymph was deproteinized using 4% and 20% TCA and 80% ethanol as deproteinizing agents. With the permanganometric method, the 80% ethanolic and 4% and 20% TCA supernatants contained 26.2±2.1, 13.5±1.9 and 13.4±5.1 mg% of calcium respectively. With the chloranilic acid method, the ethanolic supernatant of the same sample of haemolymph showed a value of 93.4±6.6 mg% whereas 4% and 20% TCA supernatants showed 14.2±3.9 and 7.0±2.8 mg% of calcium respectively. Thus the values obtained for the ethanolic supernatant employing the titrimetric method are lower than those with the spectrophotometric method. In the titrimetric method 2 ml of ethanol is added to 0.2 ml of haemolymph (10 times dilution) whereas for the spectrophotometric method, 0.2 ml of ethanol is added to 0.2 ml of haemolymph.

Whether the difference in the degree of dilution has a bearing on the free calcium value was tested using Webster's method. If samples were diluted 15-20 times the values were 82-92% lower than those for samples diluted

Table 1. Heamolymph calcium concentration in *Scylla serrata* (Forskal) as determined by 3 different methods. Values are expressed in mg/100 ml

Size (mm)		Flame photometry[19]	Permanganometric method[20]	Chloranilic acid method[21]
97	Mean ± SE	146.44 ± 2.45 (10)	146.60 ± 4.39 (10)	146.38 ± 3.14 (9)
	Coefficient of variation (%)	9.83	9.47	6.44
134	Mean ± SE	100.00 ± 2.56	145.33 ± 4.13 (10)	142.44 ± 1.54 (10)
	Coefficient of variation (%)	16.16	8.48	3.42
119	Mean ± SE	135.20 ± 1.46 (5)	127.80 ± 4.03 (9)	147.30 ± 5.58 (10)
	Coefficient of variation (%)	2.48	9.98	10.05
111	Mean ± SE	130.20 ± 0.56 (10)	135.27 ± 2.77 (11)	152.09 ± 3.12 (9)
	Coefficient of variation (%)	1.36	6.80	6.16

Sample number is given in parenthesis.

Table 2. Summary of changes in the concentration of calcium and protein in the haemolymph of *Scylla serrata* (Forskal) during time of day

	Time of day					Analysis of variance
	10.30 h	12.30 h	14.30 h	16.30 h	18.30 h	
Total calcium (mM/l)	37.76 ± 3.47	42.08 ± 4.50	47.46 ± 2.89	39.60 ± 5.72	35.23 ± 4.55	$p > 0.01$
Bound calcium (mM/l)	27.84 ± 4.05	34.03 ± 3.02	43.35 ± 2.70	29.70 ± 3.37	28.55 ± 3.84	$p > 0.01$
'Free' calcium (mM/l)	7.63 ± 1.57	7.28 ± 1.55	3.00 ± 1.20	7.56 ± 1.83	6.68 ± 2.13	$p > 0.01$
Bound calcium/protein ratio (%)	3.49 ± 1.35	2.92 ± 0.72	4.03 ± 0.85	3.36 ± 1.00	3.66 ± 1.33	Not done
'Free' calcium as % of total calcium	20.60 ± 3.41	18.10 ± 3.63	6.56 ± 2.83	20.43 ± 5.28	18.90 ± 4.88	Not done
Protein (g/100 ml)	3.46 ± 0.95	4.86 ± 1.05	4.44 ± 0.98	3.92 ± 0.99	3.66 ± 1.98	$p > 0.01$

Values are given as mean ± SD ($n = 5$).

with an equal volume of ethanol, suggesting that precipitation of protein with ethanol is dependant on the volume of the deproteinizing agent. The values are highly reproducible and reliable after deproteinizing with 20 volumes of ethanol.

For analysis at different times of day a haemolymph sample was collected every 2 h. The cut end was immediately cauterized. Analyses were carried out from 10.30 to 18.30 h at 2-h intervals. The total haemolymph protein concentration was determined using the Biuret method[22].

The haemolymph total calcium ranged from 26.67 to 49.59 mM/l. Bound calcium ranged from 27.84 to 43.35 mM/l. The free calcium ranged from 1.46 to 9.17 mM/l. Protein concentration ranged from 3.46 to 4.86 gm%.

Protein concentration and bound calcium fluctuated identically in that they increased to a maximum at 12.30 h and 14.30 h respectively and declined thereafter (table 2). This finding suggests that Ca in the haemolymph may be complexed predominantly with protein.

It is obvious that the increase in bound-Ca level is due to an increase in protein level. Whatever the source that liberates protein into the haemolymph, it is doing so in such a way that the protein is liberated in the form of calcium proteinate. At 14.30 h, when the protein level in the haemolymph remained constant, about 9.3 mM/l of bound Ca was added in the haemolymph and about 4 mM/l of free Ca disappeared from the haemolymph. Probably the free Ca complexed with the protein at 14.30 h. The proteins may accomodate free Ca by exposing anionic sites, for it is known that soluble proteins change their structure under the influence of pH[23].

The total amount of bound Ca in the haemolymph at 14.30 h is twice that of the free Ca that has disappeared at that hour. It is possible that free Ca from other tissues might have contributed to the increase in bound Ca. It is also likely that some fraction of the bound calcium may represent Ca bound with lipids[9-12] and acid mucopolysaccharides[13].

The fall in bound Ca and haemolymph protein concentration and corresponding increase in free Ca in the haemolymph after 14.30 h argue for the dissociation of Ca from its binding sites; this study stresses the need to investigate variation in the pH of the haemolymph at different times of day because association and dissociation of Ca with protein have been shown to be due to changes in pH[18,24].

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Insect hemocytes: Cells adapted to anaerobiosis

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Summary. Hemocytes from a free-living insect could be grown under strictly anoxic conditions. Their anaerobic metabolism was studied in vitro in such a cellular system.

In most species of insects, oxygen and carbon dioxide are exchanged directly between the cells and the atmosphere through a complex tracheal system. The hemolymph, devoid of respiratory pigment, does not ensure effective respiratory gas exchange, and its oxygen partial pressure and redox potential are low[2-4]. The hemocytes, farthest from the endings of the tracheae, are probably exposed to hypoxic or even anoxic conditions. Actually, the first successful primary cultures of these cells were obtained under a nitrogen atmosphere, containing 3% carbon dioxide and only 1% oxygen[5]. Established hemocyte lines from the American cockroach *Periplaneta americana* L. (HPa strains)[6] gave us the opportunity to characterize in vitro the anaerobic capacities of these cells.

HPa lines were isolated from embryos, nymphs or adults of *P. americana* in the culture medium S20 designed to reproduce the main physico-chemical characteristics of cockroach hemolymph[6]. The concentrations of amino acids and reducing components were high, especially glucose (22 mmoles $\cdot l^{-1}$) and cysteine (4 mmoles $\cdot l^{-1}$). Ascorbic acid (0.015 mmoles $\cdot l^{-1}$) and reduced glutathione (0.05 mmoles $\cdot l^{-1}$) were added.

Normoxia. Cell growth of hemocytes cultured in cotton-stoppered flasks was minimal and the pH of the culture medium rose from 7.4 to around 7.6 (table).

Progressive hypoxia. Cell growth was optimal in rubber-stoppered flasks with a limited initial oxygen store (90 μmoles $\cdot l^{-1}$ for 10^5 cells). In 7-day-old cultures, the oxygen