fluxes of sodium are mediated by a single cell type. In the intestine, it has been proposed that secretion is a function of the crypt cells and absorption a property of the villus epithelium[2]; it appears to us to be more likely[14] that each cell possesses both transport processes but their relative capacities change during maturation. The present observations with the kidney appear to justify the assumption that a single cell can harbour both absorptive and secretory mechanisms.

1 We are grateful to Mrs P. Ganguillet, S. Henriot, Miss M. Augstburger and D. Mettraux technical skilful technical assistance, and to the Fonds National Suisse for financial support.
2 M. Field, Gastroenterology *66*, 1063 (1974).
3 J.W.L. Robinson, V. Mirkovitch and F.V. Sepúlveda, Pflügers Arch. *371*, 9 (1977).
4 F. Ganguillet, V. Mirkovitch, J.W.L. Robinson and Sz. Gomba, Pflügers Arch. *341*, 171 (1973).
5 Z.S. Agus, J.B. Puschett, D. Senesky and M. Goldberg, J. clin. Invest. *50*, 617 (1971).
6 G. Fülgraff and A. Meiforth, Naunyn-Schmiedebergs Arch. Pharmac. *283*, 596 (1978).
7 E. Espinosa-Meléndez and M. Levy, Can. J. Physiol. Pharmac. *56*, 596 (1978).
8 R.M. Friedler, S. Tuma, A. Koffler and S.G. Massry, Proc. Soc. exp. Biol. Med. *159*, 48 (1978).
9 J.W. Strandhoy, G.W. Doyle and T.E. Zook, J. Pharmac. exp. Ther. *210*, 410 (1979).
10 J.L. Kinzie, J.A. Ferrendelli and D.H. Alpers, J. biol. Chem. *248*, 7018 (1973).
11 J.W.L. Robinson, V. Mirkovitch, F.V. Sepúlveda and H. Menge, J. Physiol., Paris *73*, 5 (1977).
12 I.W. Weiss, K. Morgan and J.M. Phang, J. biol. Chem. *247*, 760 (1972).
13 R.A. Podevin and E.F. Boumendil-Podevin, Biochim. biophys. Acta *375*, 106 (1975).
14 J.W.L. Robinson, B. Winistörfer and V. Mirkovitch, Res. exp. Med. *176*, 263 (1980).

Significance of the variation in haemolymph copper-protein ratio in the crab *Scylla serrata* (Forskal) during different hours of the day

M. Arumugam and M.H. Ravindranath

Department of Zoology, University of Madras, Madras - 600005 (Tamil Nadu, India), 28 January 1980

Summary. In *Scylla serrata,* haemolymph copper is bound to protein and it can be more reliably determined by the 2,2′ biquinoline method than by other spectrophotometric methods. Ionic or free copper is absent from the haemolymph. The lack of significant time-of-day variation in copper concentration and the occurrence of variation in total protein concentration and copper-protein ratio, indicate fluctuations in copper-free proteins, which may either be periodically sequestered or released by the tissues during different hours of the day.

A number of authors have shown that factors that influence the haemolymph protein concentration of decapod also affect the haemolymph copper concentration[1-3]. Since the amount of ionic copper in the haemolymph of decapods is negligible[4,5] a strong association of haemolymph copper with protein is indicated. The copper-bound proteins have been shown to range from 17% to 93% of the total haemolymph protein concentration[1-3,6,7]. The copper-bound proteins are also shown to vary under the influences of various physiological conditions like moulting and starvation[1,3], suggesting that the haemolymph copper/protein ratio is not a constant feature under these conditions. The variations in copper/protein ratio would thus indicate the relative fluctuations in copper-bound proteins and copper-free proteins under the respective conditions. Therefore, the haemolymph copper/protein ratio was studied at different times of day in *Scylla serrata,* in which the haemolymph protein has been shown to vary in relation to the time of day[8,9], after selecting a reliable and consistent method for determination of total haemolymph copper concentration.

For this purpose normal, intermoult, male specimens of the crab *Scylla serrata* (Forskal) of 175–200 g were used. Haemolymph was collected directly into a micropipette after cutting the tip of the dactylus. The sodium diethyl dithiocarbamate (SDDC) method[10], the oxalyl dihydrazide (ODH) method[11]and the 2,2′ biquinoline (BQ) method[12] were compared for determination of total copper in the haemolymph. In the first 2 methods copper was liberated from haemolymph protein by incubating in 6 N HCl[13]. Results presented in table 1 reveal that the ODH method gave poor results. With the SDDC method the absorbance after adding amyl alcohol-ether was highly variable. The BQ method was precise, consistent and reliable. This method has also previously been employed by a number of crustacean hematologists[2-5]. The haemolymph copper concentration determined in 12 crabs ranged from 35.0 to 153.3 µg/ml. This is in accordance with the range reported for other decapods[2,3,16]. No ionic copper is found in the haemolymph, confirming earlier reports[4,5]. The haemolymph protein concentration, determined by Biuret method[17] ranged from 27 to 150 mg/ml.

To study the effect of time of day on haemolymph copper and protein concentrations, the animals were kept tied and placed in plastic buckets containing 3 cm of water. Soon after haemolymph collection, the wound was cauterized. After 2 h, another appendage was cut; the same amount of haemolymph sample was collected 5 times at intervals of 2 h from one and the same animal. 5 animals were used to collect the data. The experimental animals were alive and active after the experiments. Table 2 shows the results of measurements of time-of-day variation in haemolymph copper, protein concentration and copper/protein ratio.

The copper concentrations remained somewhat constant throughout, whereas the protein concentration increased from an initial value of 53.2 ± 8.7 mg/ml at 10.30 h to 86.2 ± 7.4 mg/ml at 12.30 h and declined steadily thereafter. The pattern of variation in haemolymph protein concentration confirms an earlier report on *Scylla serrata*[9].

Analysis of variance of the results pertaining to copper and protein concentration showed that the influence of the time of day on haemolymph copper is insignificant. The influence on haemolymph protein concentration is, however, significant at $p = 0.05$ level, confirming earlier reports on *Scylla serrata*[8,9]. The significant variation in the haemolymph protein concentration could be due to injury and/or the loss of haemolymph. To what extent the protein concentration as well as the copper concentration were influenced by injury and/or the loss of haemolymph was

Table 1. Haemolymph copper concentration in *Scylla serrata* as determined by 3 different methods (in μg/ml)

SDDC method[10]	ODH method[11]	BQ method[12]
64.9±1.2 (9); 5.2%	57.2± 2.1 (9); 11.1%	53.5±1.6 (9); 8.6%
40.2±1.2 (9); 8.9%	62.8± 2.3 (10); 9.9%	53.6±1.3 (5); 5.5%
48.9±1.2 (9); 7.5%	66.3± 3.9 (6); 14.5%	48.1±1.7 (9); 10.7%
34.0±1.3 (9); 11.4%	100.8±10.7 (9); 32.9%	35.0±0.5 (10); 4.9%
119.1±4.5 (8); 10.7%	-	107.7±1.2 (8); 3.0%

The values represent mean ± SE and coefficient of variation. The number of aliquots of haemolymph is given in parenthesis.

Table 2. Summary of the changes in haemolymph copper, protein and copper/protein ratio (× 100) in the experimental (I) and control (II) crabs during time of day

	Time of day 10.30 h	12.30 h	14.30 h	16.30 h	18.30 h	Anova f-value
Copper concentration (μg/ml)						
I Range	78.4–139.5	56.5–144.1	74.2–152.7	63.5–110.3	44.6–78.9	
Mean ± SE	102.5±12.8	95.4±13.7	110.8±13.2	85.2±7.6	67.4±6.8	2.15
(N)	(5)	(5)	(5)	(5)	(5)	
II Range	48.0–145.3	74.7–140.0	78.7–146.7	66.6–153.3	46.7–74.67	2.7
Mean ± SE	86.88±17.04	89.9±11.93	104.2±11.1	108.2±13.5	72.7±15.8	
(N)	(6)	(6)	(6)	(6)	(6)	
Protein concentration (mg/ml)						
I Range	34.0–57.0	65.0–100.0	44.0–93.0	27.0–79.0	29.0–72.0	3.16*
Mean ± SE	53.2±8.7	86.2±7.4	72.6±9.8	55.2±10.1	49.6±8.5	
(N)	(5)	(5)	(5)	(5)	(5)	
II Range	48.0–116.0	68.0–150.0	55.0–88.0	42.0–96.0	28.0–52.0	13.3**
Mean ± SE	79.8±14.3	98.67±12.9	74.4±6.2	75.8±12.1	38.6±4.8	
(N)	(5)	(5)	(5)	(5)	(5)	
Copper/protein ratio (× 100)						
I Range	0.144–0.319	0.089–0.144	0.101–0.179	0.121–0.235	0.082–0.232	12.3**
Mean ± SE	0.203±0.03	0.100±0.02	0.152±0.01	0.171±0.02	0.145±0.03	
(N)	(5)	(5)	(5)	(5)	(5)	
II Range	0.087–0.144	0.057–0.137	0.104–0.267	0.118–0.159	0.09–0.267	9.67**
Mean ± SE	0.119±0.008	0.086±0.013	0.159±0.024	0.135±0.007	0.159±0.029	
(N)	(6)	(6)	(6)	(6)	(6)	

* Significant at p = 0.05 level; ** Significant at p = 0.01 level.

investigated by taking samples from fresh animals at the same hours of the day (controls). The controls revealed that there is no significant variation in haemolymph copper concentration during different hours of the day. The haemolymph protein concentration showed a peak at 12.30 h as in the animals subjected to repeated injury and bleeding stress. These observations confirm the presence of a time-of-day variation in haemolymph protein concentration, and a lack of variation in copper concentration. These observations also suggest that bound copper may not vary with the time of day. This may be an adaptive means to keep the availability of oxygen constant since protein-bound copper acts as oxygen carrier[14].

The haemolymph copper-protein ratio showed a steep fall at 12.30 h in both control and experimental analyses. The time-of-day variation of the copper/protein ratio is highly significant at the p = 0.01 level in both experimental and control analyses.

The variation in the copper/protein ratio could be due to variation in the copper-free proteins since copper-bound proteins did not vary during different hours of the day. The variation seems not to be due to changes in haemolymph water content, for the latter remained constant at all times of day[18]. Furthermore, when the total protein concentration fluctuated, the copper concentration in the haemolymph remained constant, which would not have been the case if water content were to vary according to the time of day. The variation in the copper/protein ratio during different hours of the day may be due to periodic sequestration or release of copper-free proteins by the tissues; these phenomena are common in arthropods[19–21].

1 E. Zuckerkandl, Annls Inst. Oceanogr. Monaco *38*, 1 (1960).
2 E.C. Horn and M.S. Kerr, Biol. Bull. *125*, 499 (1963).
3 J.S. Djangmah, Comp. Biochem. Physiol. *32*, 709 (1972).
4 G.A. Kerkut, K.M. Moritz and K.A. Munday, Cah. Biol. Mar. *2*, 399 (1961).
5 P. Andrews, Z. vgl. Physiol. *57*, 7 (1967).
6 J.B. Allison and W.H. Cole, J. biol. Chem. *135*, 259 (1940).
7 W. Wieser, J. mar. biol. Ass. U.K. *45*, 507 (1965).
8 M.H. Subhashini and M.H. Ravindranath, Arch. int. Physiol. Biochem. *88*, 47 (1980).
9 K. Kannan and M.H. Ravindranath, Experientia *36*, 965 (1980).
10 S. Ventura and E.J. King, Biochem. J. *48* (1951).
11 E.W. Rice, J. Lab. clin. Med. *55*, 325 (1960).
12 R.J. Guest, Analyt. Chem. *25*, 1484 (1953).
13 C.G. Holmberg, Acta physiol. scand. *2*, 71 (1941).
14 I.M. Klotz and T.A. Klotz, Science *121*, 477 (1955).
15 P. Busselen, Comp. Biochem. Physiol. *37*, 73 (1970).
16 G.W. Bryan, J. mar. biol. Ass. U.K. *44*, 549 (1964).
17 A.G. Gornall, G.J. Bardawill and M.M. David, J. biol. Chem. *177*, 751 (1949).
18 P. Mullainadhan, Thesis, University of Madras, 1979.
19 B.G. Loughton and A.S. West, J. Insect Physiol. *11*, 919 (1965).
20 M. Locke and J.V. Collins, Nature, Lond. *210*, 552 (1966).
21 S.S. Tobe and B.G. Loughton, Can. J. Zool. *45*, 975 (1968).