Physico-chemical characterisation of the extrapallial fluid of a common tellinnid bivalve *Macoma birmanica* (Philippi) in the mudflats of Sundarbans mangrove, Bay of Bengal

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The chemical composition of the extrapallial fluid of *Macoma birmanica* was examined in order to understand the process of calcification. Concentrations of inorganic ions Na⁺, K⁺, Ca²⁺, Cl⁻ and SO₄²⁻ were found in higher proportion in the pallial fluid than those in the ambient medium. Calcification from pallial fluid was found to be related to the removal of proton from the fluid through the excretion of NH₃. The concentration of total positive charge is 14.1% higher than that of negative charge, indicating the presence of non-diffusible negative ions. From EPR measurements, apparent complexation capacity of Mn in the fluid was in the order of $0.37 \times 10^5 M$ and forms 1:1 chelate with stability constant of $1.1 \times 10^7$ per mole. It was found to be highly undersaturated with respect to the solubility of skeletal aragonite in the pallial fluid and has the capacity to keep high concentration of calcium in solution through the formation of ion pair with HCO₃⁻. Precipitation of aragonite was initiated through the formation of Ca chelates.

The control of the inorganic and organic constituents present in the pallial fluid over the shell formation has been emphasized by several workers. Most of the previous investigation in this area were concentrated on characterizing the mantle and shell. Misogiane & Chasteen have studied the chemical and spectral characteristics of the pallial fluid of a marine species, *Mytilus edulis*. There is a large variation in the chemical composition of the fluid between marine and fresh water species. Such as, the total calcium concentration (10.7 mM) in marine species, *Mytilus edulis* exceeded that of (3.9 mM) fresh water species, *Cristaria plicata*, whereas HCO₃⁻ concentration in *C. plicata* (11.5 mM) is higher than that of *M. edulis* (4.2 mM). Again, the environment of the pallial fluid for the deposition of calcite and aragonite is expected to be different. *Macoma birmanica* secretes aragonite³ and the environment of its pallial fluid for the secretion of aragonite has not yet been studied. This lacuna and differences merit analysis of the pallial fluid of *M. birmanica* living in the variable estuarine environment of Hugli river.

Materials and Methods

The extrapallial fluid was obtained from the samples of *Macoma birmanica* (Philippi) collected every month covering one year (1995) from the lower littoral zone of the exposed mud flats of Chemaguri of Sagar Island (lat, 21°31'N to 21°53'N, long, 88°01'E to 88°15'E), Bay of Bengal during the ebb tide. After opening the valves with a scalpel, the needle of a sterilized hypodermic syringe was inserted between the mantle and nacre of each animal and fluid was withdrawn by gentle suction, yielding approximately 1-2 ml per specimen depending on size. The pH of the withdrawn fluid was measured using Systronics, model 361, micro pH meter (sensitivity ±0.002) and carbonate alkalinity was determined by the measurement of acid neutralizing capacity by pH method. Two successive titrations were accomplished on each sample in a water jacketed cell under controlled temperature. In first titration, 5 ml fluid was titrated with 0.1 N HCl using microburette to an end point pH 3. In second titration, the mixture resulting from the first titration was freed of CO₂ by passing N₂ and again was titrated back with CO₂ free NaOH (0.1 N) to a pH of about 9.6 in order to get the contribution of organic acids. The change of carbonate alkalinity of the pallial fluid was used to measure calcification or dissolution as given by Smith. Na⁺, K⁺ and Ca²⁺ were determined by flame photometric (Systronics, model no. 121, detection limit 0.02-2 ppm) method. Mg²⁺ by atomic absorption spectrophotometer (Perkin-Elmer 380). Cl⁻ by mercurometric method and SO₄²⁻ by turbidimetric (SEICOSPEC, model 200 GL) method. Concentration of chloride was used to calculate salinity.
Carbohydrate and protein was measured by spectrophotometric (SEICOSPEC, model no. 200 GL) method. The apparatus used for the measurement of NH₃ excretion was the same as described by de Voogt and NH₃ concentration was measured using indophenol blue method. The saturation technique was adopted for the determination of solubility product of skeletal calcium carbonate both in the pallial fluid and tidal water. Skeletal calcium carbonate powder (145 mesh) and pallial fluid or tidal water were kept for 24 h at a constant temperature, 25°C. Streptomycin was added to avoid bacterial respiration. The initial alkalinity, pH, concentration of Ca²⁺ and the final pH were used to determine the stoichiometric solubility product (Kₛₚ). Mn complexation capacity of the fluid was studied by EPR spectra (Varian E-4 spectrometer) operating at X-band frequency (9.45 GHz) and modulating frequency 100 KHz.

**Results and Discussion**

The pH of pallial fluid of large (length ≥ 7 cm, breadth ≥ 4.4 cm), medium (length ≥ 6 cm, breadth ≥ 3.8 cm) and small (length ≥ 5 cm, breadth ≥ 3 cm) was found to be 7.74 ± 0.16, 7.76 ± 0.15 and 7.76 ± 0.11, respectively. A comparison of concentration of inorganic ions present in the ambient medium and extrapallial fluid (Table I) revealed that in marine bivalve, *Mytilus edulis*, the difference in Ca²⁺, Mg²⁺, Na⁺ and K⁺ are small, whereas, in estuarine bivalve *Mytilus bairdii*, these ions and HCO₃⁻ are greatly increased in the pallial fluid than those of ambient medium. The Donnan ratio of these ions as obtained from their average concentrations are not equal as required quantitatively by the Donnan principle. The concentration of total positive charge is 14.1% higher than that of negative charge, indicating the presence of non-diffusible negative ions. Considering K⁺ ion activities in extrapallial fluid and ambient water the transepithelial potential was calculated to be -12.6 mV at 37°C using Nernst equation. Similar result for *Laminaea stagnalis* was found to be -15.1 mV. The values of equilibrium concentration calculated using this membrane potential and activities in the pallial fluid and ambient medium indicates that the gradient favours passive diffusion of ions out of the pallial fluid. Again, ionic ratios with chloride were higher than those of the ambient medium. Thus it indicates that Na⁺, K⁺, Ca²⁺, Cl⁻ and SO₄²⁻ ions must be actively taken up from the tidal water; which is necessary for the regulation of acid-base balance, ionic composition to provide a favourable environment for enzyme activity, to extrude toxic substances and metabolic products. In order to isolate and characterize distribution of different form of calcium, extrapallial fluid was centrifuged at 8000 g for 30 min, then dialysed against 300-fold excess of 50 mM Tris-HCl buffer at pH 7.5 for 48 h at room temperature, using spectra per membrane tubing (MW cut off 6000-8000 D). The resultant solution is labelled as the centrifuged dialysed fluid. The particulate portion was digested with perchloric-nitric acid mixture for 12 h at 70°C and then analysed for calcium. Results are given in

<table>
<thead>
<tr>
<th></th>
<th>H⁺</th>
<th>Na⁺</th>
<th>K⁺</th>
<th>Ca²⁺</th>
<th>Mg²⁺</th>
<th>HCO₃⁻</th>
<th>CO₃²⁻</th>
<th>Cl⁻</th>
<th>SO₄²⁻</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Mytilus edulis</em></td>
<td>442</td>
<td>9.5</td>
<td>10.7</td>
<td>58.0</td>
<td>4.2</td>
<td>-</td>
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<tr>
<td>(Marine)</td>
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<td></td>
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<tr>
<td>Seawater</td>
<td>427</td>
<td>0.1</td>
<td>0.2</td>
<td>3.0</td>
<td>0.5</td>
<td>-</td>
<td>-</td>
<td>-</td>
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</tr>
<tr>
<td><em>Macoma bairdii</em></td>
<td>1.78×10⁶</td>
<td>272.5</td>
<td>7.74</td>
<td>7.63</td>
<td>25.9</td>
<td>5.56</td>
<td>-</td>
<td>0.18</td>
<td>270.0</td>
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<tr>
<td>(estuarine)</td>
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<tr>
<td>Tidal water</td>
<td>8.3</td>
<td>190.5</td>
<td>2.0</td>
<td>13.6</td>
<td>0.26</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Donnan ratio</td>
<td>2.05</td>
<td>1.28</td>
<td>1.61</td>
<td>0.995</td>
<td>0.87</td>
<td>8.2</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Equilibrium</td>
<td>2.68×10⁶</td>
<td>65.6</td>
<td>1.48</td>
<td>0.29</td>
<td>1.31</td>
<td>0.07</td>
<td>-</td>
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<td>concentration</td>
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<td>Ratio with</td>
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<td>chlorinity</td>
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<tr>
<td>Pallial fluid</td>
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<td></td>
<td></td>
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<tr>
<td>Tidal water</td>
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</tbody>
</table>

= Data not available or calculated.
Table 2. Amount of particulate calcium was 10.28-18.06 µg/ml out of the 257.4-262.32 µg/ml of total calcium. The amount of calcium diffusing out was 202.59-204.82 µg/ml i.e. 78.1-78.6% of the total calcium in the fluid is not strongly associated with macromolecular components. Had that amount formed strong chelate with soluble macromolecule (MW > 8000 D) present in the pallial fluid, it would have not diffused out through the membrane. 36.73-47.22 µg/ml of calcium remained in the dialysed fluid. This indicates that the pallial fluid contains some soluble macromolecular component (MW > 8000 D), which forms strong chelate with that amount of calcium. Major portion of this particulate fraction was carbohydrate type of compounds. The total carbohydrate and protein in pallial fluid varied between 6.38 and 103.74 µg/ml; 368 and 459 µg/ml, respectively. The UV absorption spectra of the soluble macromolecular component present in the pallial fluid showed λ max at 230 and 262.5 nm and the optical density ratio at those wave lengths was 1.61. Pure proteins have absorption ratio of about 1.8 at 230 nm and 260 nm.

Expected concentrations of calcium are calculated from the stoichiometric solubility constant value at in situ salinity which ranged between 4.25 and 35.01 x 10^-3 and temperature between 26 and 35°C using the relation for aragonite 20. Observed calcium concentrations are 9 to 15 times higher than its expected concentrations (Fig. 1). Again, the calcium concentrations in pallial fluid are 1.15 and 1.6 times higher than those of ambient medium for marine and estuarine species, respectively.

High concentrations of calcium in the pallial fluid were found during premonsoon (mid February—mid June) when the pH of the pallial fluid are low and 76.6% decrease of calcium concentration in the pallial fluid was noticed during monsoon (September). Attempt has been made to examine how this excess calcium is present in the pallial fluid.

The variable, excess calcium had been singled out to examine its joint relation with the other variables.

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Different form of calcium and carbohydrate in the extrapallial fluid of <em>M. binnana</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Percentage of total</td>
<td>Calcium</td>
</tr>
<tr>
<td>Free and bound to small chelates or soluble micro molecular component</td>
<td>78.1-78.6</td>
</tr>
<tr>
<td>Bound to soluble macro molecular component</td>
<td>14.0-18.04</td>
</tr>
<tr>
<td>Bound to particulate matter</td>
<td>3.91-7.01</td>
</tr>
</tbody>
</table>

HCO₃⁻, carbohydrate, Mg²⁺ and SO₄²⁻ by the application of multiple regression analysis. All data of excess Ca²⁺ (mM/l), CO₃²⁻ (mM/l), carbohydrate (µg/ml), Mg²⁺ (mM/l) and SO₄²⁻ (mM/l) were found to fit the following equations:

**For large size specimen:**

\[
\text{[Excess Ca]} = 0.4890 [\text{HCO}_3^-] + 0.0118 [\text{carbohydrate}] + 0.0487 [\text{Mg}^{2+}] + 0.2046 [\text{SO}_4^{2-}] - 0.888866 (R^2 = 89.0%).
\]

**For medium size specimen:**

\[
\text{[Excess Ca]} = 0.9620 [\text{HCO}_3^-] + 0.01135 [\text{carbohydrate}] + 0.2019 [\text{Mg}^{2+}] + 0.0232 [\text{SO}_4^{2-}] - 4.71176 (R^2 = 88.2%).
\]

**For small size specimen:**

\[
\text{[Excess Ca]} = 0.3966 [\text{HCO}_3^-] + 0.0068 [\text{carbohydrate}] + 0.1519 [\text{Mg}^{2+}] + 0.0945 [\text{SO}_4^{2-}] - 0.77769 (R^2 = 91.37%).
\]

HCO₃⁻ and Mg²⁺ played the predominant role for the occurrence of excess calcium in the pallial fluid.

Experimental results of the solubility product determined at 25°C and salinity between 11.49 and 13.07 x 10^-3 are given in Table 3. The solubility of aragonite in the pallial fluid of all the three size classes showed higher values than that of estuarine water. From the ratio of ionic product and estimated solubility product it is found that pallial fluid is highly under-saturated which varied between 0.03 and 0.15%. Similar under-saturated condition was also reported in the pallial fluid of *M. edulis* containing 9.5 mM calcium.

Increased solubility of calcium carbonate in the pallial fluid was mainly due to the ion pair formation of HCO₃⁻ with Ca and Mg, respectively. Inspite of their inhibitory effect the precipitation of calcium carbonate took place from the pallial fluid.
Table 3 — Comparison of solubility product of aragonite in pallial fluid and ambient medium

<table>
<thead>
<tr>
<th>Specimen size</th>
<th>Salinity (x$10^3$)</th>
<th>Temperature (°C)</th>
<th>Extrapallial fluid</th>
<th>Extraneous water</th>
<th>Calculated$^{19}$</th>
<th>Ionic product in pallial fluid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Large</td>
<td>11.49</td>
<td>25</td>
<td>3.33$x10^4$</td>
<td>1.91$x10^7$</td>
<td>1.96$x10^7$</td>
<td>3.33$x10^7$</td>
</tr>
<tr>
<td>Medium</td>
<td>11.93</td>
<td>25</td>
<td>7.29$x10^4$</td>
<td>1.93$x10^7$</td>
<td>2.04$x10^7$</td>
<td>5.42$x10^7$</td>
</tr>
<tr>
<td>Small</td>
<td>13.07</td>
<td>25</td>
<td>3.19$x10^4$</td>
<td>2.97$x10^7$</td>
<td>2.22$x10^7$</td>
<td>4.86$x10^7$</td>
</tr>
</tbody>
</table>

The conditions for the formation of chelates with Ca$^{2+}$ ($^{t^2_g,e^0}$) can be compared with the high spin Mn$^{2+}$ ($^{t^2_g,e^3}$) chelates having no ligand field stabilisation energy. For example, chelates of Ca$^{2+}$ and Mn$^{2+}$ with EDTA at 20°C and ionic strength, 0.1 have almost same stability ($\log K_{Ca\text{-EDTA}}=10.7$ and $\log K_{Mn\text{-EDTA}}=13.8$)$^9$. Manganese complexation capacity of the fluid was studied by Electron Paramagnetic Resonance Spectra (EPR). Figure 2 shows a typical example of EPR spectra of $5\times10^{-5}M$ manganese solution at room temperature (25°C) in native fluid and diluted seawater.

Salinity of both native fluid and diluted seawater were maintained at 7.02$x10^{-3}$. The six line first derivative spectrum was the characteristic of free Mn$^{2+}$ ($I=5/2$ and non-degenerate ground state = $^6S$) and decrease of EPR signal intensity indicated the complexation of Mn$^{2+}$ in the native fluid. The spectroscopic splitting factor, g, for Mn$^{2+}$ ($^6S_{5/2}$) was calculated to be 2, using the equation,

\[ g = \frac{3/2 + S(S+1) - L(L+1)}{2(L+1)} \]

Again, $g = hv/\beta H$ where, v is the frequency of radiation and $\beta$ is the Bohr magneton. The g value was calculated to be 1.99 from the known values of microwave frequency (v), $9.45 \times 10^9$ cycles/sec and the resonance field (H), 3400 gauss. Because of the non-degenerate ground state of Mn$^{2+}$ ($^6S_{5/2}$), the g value nearly equals to the free electron, small deviation was due to spin-orbit coupling$^{21}$. By comparing the signal intensity of the fluid with that of standard solution, the total Mn$^{2+}$ concentration in the fluid was found to be $5 \times 10^{-7}M$. To further examine the Apparent Complexation Capacity of manganese (ACC$_{Mn}$), a series of Mn$^{2+}$ solutions in diluted seawater (salinity = $7.024 \times 10^{-3}$) and native fluid of similar concentrations ranged between $1 \times 10^{-5}$ and $8 \times 10^{-5}M$ were prepared, and from the decrease of EPR signal intensity free and bound Mn were calculated. A plot similar to that described by Scatchard$^{22}$ was used to verify the chelation capacity and to determine the stability constant, K, for the
metal-ligand association. The concentration of bound Mn\(^{2+}\) was determined by the relationship \([\text{Mn}^{2+}]_{\text{bound}} = (\text{Mn}^{2+})_{\text{added}} - (\text{Mn}^{2+})_{\text{free}}\), where, \((\text{Mn}^{2+})_{\text{free}}\) was measured directly from the EPR intensity. If there is one class of binder sites then the data should obey the equation:

\[
\frac{(\text{Mn}^{2+})_{\text{bound}}}{(\text{Mn}^{2+})_{\text{free}}} = K[\text{Mn}^{2+}] + n[\text{L}]_{\text{total}}
\]

where, \(n\) is the ligand-to-metal ratio in the complex, \([\text{L}]_{\text{total}}\) is the total concentration of ligand, and \(K\) is the stability constant for the metal ligand association. In this case \(n = 1\) (vide supra) and \([\text{L}]_{\text{total}}\) is, therefore, the chelation capacity. \(K\) and \(n\) were computed from the intercept on the abscissa and the slope of the line depicted in Fig. 3, being \(0.37 \times 10^{-5}\) and \([L]_{\text{total}} = 1.1 \times 10^4\) per mole, respectively. The chelation capacity and the stability constant of the chelates in the pallial fluid of \(M. edulis\) were found to be \(9 \times 10^{-5}\) and \(1.7 \times 10^5\) per mole, respectively. Therefore, for \(M. birmanica\) both chelation capacity as well as stability constant of the chelate are found to be low.

Since the formation of CaCO\(_3\) from Ca and HCO\(_3\) results in the release of proton, therefore, the removal of proton is necessary for the continued deposition of shell crystals. Proton removal by carbonic anhydrase catalysis and by ammonia are the two possible mechanisms for eliminating ammonia from the extrapallial fluid. Therefore, the possible mechanism may be:

\[
\text{Ca}^{2+} + \text{HCO}_3^- + \text{NH}_4^+ \rightarrow \text{CaCO}_3 + \text{NH}_4^+^+
\]

A positive linear correlation between pH of the extrapallial fluid and amount of \(\text{NH}_4^+\) (\(\mu\text{g} / \text{gm dry wt.}\)) excretion was observed.

For medium size \(\text{NH}_4^+\) = 27.65 pH – 187.0

\[(r = 0.52, p < 0.1)\]

For small size \(\text{NH}_4^+\) = 26.62 pH – 178.3

\[(r = 0.69, p < 0.02)\]

One typical example of two successive titrations of pallial fluid are given in Figs 4 and 5. The end point of each titration was obtained by using Gram method for estuarine water\(^{24}\). \(F_1\) values were calculated by using the relation, \(F_1 = \left(v_o + v_a\right) \times 10^{pH}\) where \(v_o\) and \(v_a\) are the volume of the pallial fluid and added acid, respectively. Contribution of carbonate alkalinity and conjugate base of the metabolic acids towards total alkalinity were calculated from first and second titration end point. Data are given in Table 4. Considerable proportion of metabolic acid or non-carbonate alkalinity was found to be present in the pallial fluid and the contribution of basic anions whose pKa values varied between 4.25 and 5.4 was found between 11.7 and 14.4%.

Monthly variations of carbonate alkalinity of the pallial fluid is shown in Fig. 6. Change of carbonate alkalinity of pallial fluid in one month interval, considering chlorinity as a conservative index was used to calculate the amount of CaCO\(_3\) precipitation or dissolution (Table 5). Increase of shell radius was observed in post-monsoon and pre-monsoon when CaCO\(_3\) precipitation took place.

Therefore, higher proportion of inorganic constituents in pallial fluid than that in ambient

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**Fig. 4** — The pH titration curve of extrapallial fluid of *Mconomilla birmanica* collected in August with 0.1 N HCl at salinity, 17.28x10\(^3\).

**Fig. 5** — Back pH titration curve of acidified pallial fluid of *Mconomilla birmanica* with 0.1N NaOH.
medium was noticed throughout the study period and seasonal variations of constituents of pallial fluid were observed. It was found low in monsoon and high in pre-monsoon. Occurrence of excess inorganic cations over anions was found necessary for the presence of anionic organic constituents, which again accelerated the precipitation of thermodynamically unstable aragonite from the undersaturated pallial fluid through the chelation followed by the formation of spherulites. Occurrence of large number of microscopic spherulites were observed in the native fluid. These spherulites were found soluble when pH of the native fluid was lowered to 3.4-3.1. Calcium binding organic chelates could be possible nucleator of CaCO₃ through the formation of organic spherules, followed by the formation of aragonite crystallites which develop into spherulites that finally constitute a complete shell layer.

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