PHOSPHORUS FRACTIONS OF CRAYFISH HAEMOLYMPH, SERUM AND HAEMOCYANIN*

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1. Different amounts of haemolymph proteins participate in clot formation in Astacus leptodactylus and Orconectes limosus (20% and 10%, respectively), although in both species the content of proteins in haemolymph is similar.

2. In both species the content of total phosphorus in the clot was similar (0.33-0.49%, w/w) but it was about 6 times lower in serum of O. limosus than in serum of A. leptodactylus (2.2 and 14.8 mg/100 ml, respectively). An even greater difference in phosphorus content was found in the protein precipitated from serum.

3. In both species lipid phosphorus was predominant (77% of total haemolymph phosphorus in A. leptodactylus and 52% in O. limosus). Phospholipids were found mainly in serum.

4. Only traces of phosphorus (0.005%) and small amounts of fatty acids were found in purified haemocyanin preparations.

Fresh waters in Poland are inhabited by four crayfish species. Three of them are native: Astacus astacus (L) and Astacus leptodactylus (Esch.), both spread all over Poland; the third one, Astacus torrentium, was found only in the region of Sudety mountains (Grabda, 1973). The fourth species, Orconectes limosus Raf., commonly called American crayfish, was brought at the beginning of this century from Pennsylvania (Delaware river), (Kossakowski, 1966). This species is very mobile and seems to be more resistant to stress conditions.

In consideration of a possibility that all crayfish species come from a common ancestor, it is of interest to compare some physico-chemical

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properties of native species and the American crayfish which had lived so
long under different ecological conditions.
It has been found (Gondko et al., 1981) that the haemolymph of
A. leptodactylus and of O. limosus differ in protein content, clotting time
and in concentration of some cations. On the other hand, no significant
differences in the amino acid composition of haemocyanin were found
(Adamska & Gondko, 1983).
In the present paper the content of phosphorus in the whole haemolymph,
clot, serum and serum protein, obtained from three crayfish species was
compared. Special attention was paid to the presence of phosphorus and
phospholipids in haemocyanin. It has been suggested (Gondko & Aleksiuk,
1981; Gondko & Michalak, 1981) that haemocyanin contains a lipid component.
Zatta (1981) reported the presence of non-dialysable lipids (1.5%) associated
with Carcinus maenas haemocyanin; these are mainly phospholipids containing
phosphatidylcholine. To check whether phospholipids constitute an integral
part of haemocyanin molecule, phosphorus content in various haemocyanin
preparations was compared.

MATERIALS AND METHODS

The animals were caught in July and stored in an aquarium for at
least 5 weeks, with occasional feeding.
Haemolymph from at least a dozen animals (the two sexes together
or separated) was quickly collected and analysed individually or pooled.
A part of the haemolymph was used for immediate analysis of protein
and phosphorus content. Another part was left for clotting. The clot
was centrifuged and washed with distilled water with stirring for about
2 h. Thereafter, the moist clot was lyophilized or dried (50°C) to constant
weight. The serum obtained was treated with 10% trichloroacetic acid (1:1)
and the protein precipitate (containing 95% of haemocyanin) was centrifuged.
The sediment was washed with 5% trichloroacetic acid, then with water,
and dried. In particular fractions protein, total P and lipid P were determined
according to Scheme 1.
Haemocyanin preparations. Crude haemocyanin was obtained by ultra-
centrifugation of serum after clotting. It was purified by chromatography
on Ultrogel AcA-22 column (2.5 × 80 cm, eluent: 0.1 M-Tris/HCl + 40 mm-
-CaCl₂, pH 7.5). During chromatography the blue haemocyanin fraction was
separated from two red-yellowish fractions. After rechromatography on the
same column the haemocyanin preparations showed the A₂₈₀/A₃₄₀ ratio
of about 4. The crude and purified haemocyanin preparations were
immediately lyophilized.
Crude haemocyanin of A. astacus as well haemocyanins of A. leptodactylus
and O. limosus purified on Ultrogel AcA-22 column were extracted to remove
lips and phosphorus compounds. The extraction with a chloroform/methanol mixture (2:1, v/v) for 8 h in a Soxlet apparatus led to delipidation of haemocyanin. Delipidated haemocyanin preparations were washed three times (10 min) with 0.2 M-HClO₄. Subsequently haemocyanin precipitates were treated with 5% trichloroacetic acid at 93°C for 10 min and with Triton X-100. Phosphorus content in the preparations was determined after each step of the extraction procedure.

Scheme 1: Determination of protein and phosphorus in haemolymph fractions

*Phosphorus estimation.* Phosphorus was estimated by the method of Bartlett with a slight modification (Gondko et al., 1985). For both mineralization and colour development 5 M-H₂SO₄ was used. After mineralization the solution was neutralized with ammonia against p-nitrophenol. Lipid phosphorus in the whole haemolymph and serum was estimated in the extracts prepared according to Folch et al. (1957).

**RESULTS AND DISCUSSION**

From the differences between the protein content in the haemolymph and serum the contribution of protein to the clot formation was calculated (Table 1). About 20% of haemolymph protein takes part in clot formation in *A. leptodactylus* and only 10% in *O. limosus*, although in both species the level of protein in haemolymph is similar. The difference may be even greater because in the case of *A. leptodactylus* the clot was partly dissolved during washing with water, as evidenced by a positive reaction with 10% trichloroacetic acid in washing. Though the total phosphorus content
Table 1

Protein content of the haemolymph and serum, and phosphorus content of the clot

<table>
<thead>
<tr>
<th>Species</th>
<th>Haemolymph protein (g/100 ml)</th>
<th>Serum protein (g/100 ml)</th>
<th>Percentage of protein in the formed clot</th>
<th>Phosphorus content of the clot (mg/g dry weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Astacus leptodactylus</em></td>
<td>2.01 ± 0.26 n = 5</td>
<td>1.64 ± 0.21 n = 5</td>
<td>18.4</td>
<td>4.20 ± 0.5* n = 4 4.95 ± 0.41** n = 6</td>
</tr>
<tr>
<td><em>♀</em></td>
<td>3.00 ± 0.52 n = 5</td>
<td>2.41 ± 0.52 n = 5</td>
<td>19.7</td>
<td>4.27 ± 1.1* n = 4</td>
</tr>
<tr>
<td><em>Orconectes limosus</em></td>
<td>2.47 ± 0.67 n = 11</td>
<td>2.25 ± 0.74 n = 6</td>
<td>8.9</td>
<td>3.32 ± 1.5* n = 4</td>
</tr>
<tr>
<td><em>♀</em></td>
<td>2.09 ± 0.76 n = 9</td>
<td>1.80 ± 0.57 n = 7</td>
<td>13.8</td>
<td>4.26 ± 0.85* n = 4</td>
</tr>
</tbody>
</table>

* Clots from individual animals.
** Clots from pooled samples (from 10-12 animals each).

in the haemolymph was strikingly different in these species (Table 2). The total phosphorus content per dry weight in the clots was constant, irrespective of sex and species of the animal examined (Table 1). This suggests that the clotting protein of Invertebrates, coagulogen, may contain phosphorus. It is well known that the clotting protein of Vertebrates, fibrinogen, is a phosphoprotein (Krajewski & Dmochowski, 1963). However, it should be stressed that while the contribution of protein to the clot formation in *A. leptodactylus* is generally in agreement with other results, the data concerning phosphorus content are widely divergent (Durliat & Vranckx, 1976), an observation which is hard to explain.

The 6-fold difference in the total phosphorus concentration in haemolymph between *A. leptodactylus* and *O. limosus* is related to the difference in phosphorus content in the serum (Table 2). In both species the level of acid soluble phosphorus in serum is similar, but phosphorus content in the protein sediment and lipid fraction is 9-10 times higher in *A. leptodactylus*. In both species lipid phosphorus constitutes the main phosphorus fractions of the haemolymph. The high phosphorus content in the protein sediment, 95% of which is haemocyanin, seems to be due to a contamination by phospholipids. This is suggested by comparison of phosphorus content in crude and purified haemocyanin preparations (Table 3). After extraction with the Folch mixture and other extractions, of the crude haemocyanin preparation from *A. astacus* the phosphorus level decreases to that found in haemocyanin preparations from *A. leptodactylus* and *O. limosus*, purified by column chromatography. In extracts of the purified haemocyanin preparations from *O. limosus* haemolymph, obtained with the Folch mixture
### Table 2

**Content of phosphorus compounds in the haemolymph and serum (mg/100 ml)**

<table>
<thead>
<tr>
<th>Species</th>
<th>Total haemolymph phosphorus</th>
<th>Lipid haemolymph phosphorus</th>
<th>Total serum phosphorus</th>
<th>Lipid serum phosphorus</th>
<th>Acid-soluble phosphorus</th>
<th>Phosphorus in sediment (mg/g dry weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Astacus leptodactylus</td>
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</tr>
<tr>
<td>♂</td>
<td>16.60 ± 4.87 n = 8</td>
<td>12.08 ± 3.65 n = 8</td>
<td>14.59 ± 4.48 n = 7</td>
<td>11.99 ± 3.62 n = 7</td>
<td>0.78 ± 0.22 n = 5</td>
<td>4.67 ± 1.1* n = 7 4.49 ± 0.7** n = 6</td>
</tr>
<tr>
<td>♀</td>
<td>16.35 ± 7.60 n = 5</td>
<td>13.14 ± 6.18 n = 5</td>
<td>14.98 ± 4.34 n = 5</td>
<td>11.24 ± 5.69 n = 5</td>
<td>1.13 ± 0.26 n = 4</td>
<td>3.34 ± 0.9* n = 6 3.13 ± 0.7** n = 3</td>
</tr>
<tr>
<td>Orconectes limosus</td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>♂</td>
<td>3.35 ± 1.90 n = 10</td>
<td>2.02 ± 0.75 n = 8</td>
<td>2.30 ± 0.89 n = 9</td>
<td>1.24 ± 0.47 n = 7</td>
<td>0.96 ± 0.38 n = 5</td>
<td>0.40 ± 0.06* n = 6 0.54 ± 0.05** n = 6</td>
</tr>
<tr>
<td>♀</td>
<td>2.47 ± 1.21 n = 10</td>
<td>1.35 ± 0.43 n = 6</td>
<td>2.10 ± 0.45 n = 10</td>
<td>1.07 ± 0.45 n = 7</td>
<td>0.69 ± 0.06 n = 4</td>
<td>0.49 ± 0.1* n = 6 0.54 ± 0.05** n = 5</td>
</tr>
</tbody>
</table>

* Sediments from individual animals.
** Sediments from pooled samples, 10-12 animals each.
and subjected to methanolsysis, small amounts of fatty acids (mg/g protein) were found: \( C_{16} \), \( C_{18} \) and \( C_{18.2} \) (0.22, 0.10 and 0.17, respectively). Traces of phosphatidylcholine (0.5) were detected in this extract by thin-layer chromatography. It can be concluded from these data that neither phosphorus nor phospholipids appear to be integral components of haemocyanin. Probably under physiological conditions phospholipids are rather loosely associated with this protein.

This paper is dedicated to the memory of the late professor Antoni Dmochowski.

**REFERENCES**


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