

THE COMPOSITION OF HAEMOLYMPH IN AQUATIC INSECTS

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INTRODUCTION

Several surveys have been carried out on the distribution of the major cations in insect haemolymph. The first was that by Boné (1944), who estimated the concentrations of sodium and potassium in twenty-seven species, drawn from eight orders and including five aquatic species. This was followed by the larger survey of Duchâteau, Florkin & Leclerq (1953), referred to hereinafter as Duchâteau *et al.* These authors estimated calcium and magnesium as well as the monovalent ions, but very few aquatic insects were examined. Clark & Craig (1953) also extended our knowledge on the occurrence of calcium and magnesium in insects, and the literature on these two ions was reviewed recently by Clark (1958). A comprehensive review of both the inorganic and organic components in insect haemolymph is given by Wyatt (1961).

From the results of his investigation Boné (1944) concluded that there is a correlation between diet and the ratio of sodium to potassium in haemolymph. Both then and later (Boné, 1947), he was aware that this correlation requires several major qualifications, and Duchâteau *et al.* provided evidence that, in fact, it is not generally applicable. Instead, Duchâteau *et al.* proposed that a high concentration of sodium and relatively little potassium occurs in primitive groups of the Insecta, and that this 'ancestral' type of haemolymph is modified in later, more advanced insects. A considerable amount of further information is required before this hypothesis can be evaluated.

The above surveys were concerned only with estimates of cations. Very little is known about the distribution and nature of anions in insect haemolymph and hence there have been few attempts to describe cation-anion balance in quantitative terms. Moreover, these attempts are concerned with terrestrial insects (e.g. Bishop, Briggs & Ronzoni, 1925; Levenbook, 1950; Asperen & Esch, 1956; and in Buck, 1953). A quantitative study has been made of one aquatic insect, the larva of *Sialis lutaria* (Beadle & Shaw, 1950; Shaw, 1955).

Since the aquatic insects have been largely ignored, the present survey was carried out to provide information on the distribution of ions in these insects. The concentrations of calcium and magnesium were not determined, but the results of other investigations have been drawn from the literature and are included in estimates of cation-anion balance. The total concentration of free amino acids, and the freezing-point depression of haemolymph (haemolymph Δ), were also determined in order to assess the con-

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tribution of both inorganic and organic components to the total concentration of the haemolymph. The investigation was extended to include terrestrial insects and some other arthropods, so that the information given here could be discussed further with regard to the hypotheses of Boné and Duchâteau *et al.* This part of the investigation will be presented elsewhere (Sutcliffe, 1962).

MATERIALS AND METHODS

'It is becoming increasingly clear that for the analysis of insect haemolymph to have any biological meaning, it is essential to know the age of the insect, its physiological state, and the environmental conditions under which it was kept prior to analysis' (Levenbook, 1950). In this survey the main object was to reveal major differences between species, and it was not possible to obtain all of these in the same physiological state. In order to provide some information on this point, the stage reached in the life history of each species investigated is given in an Appendix, together with the locality and date of collection. Fortunately, a number of taxonomic keys to the larval and adult stages of British aquatic insects have recently appeared, and it is now possible to give the specific identity in the majority of cases. The authorities employed for identification are also given in the Appendix.

In most cases haemolymph was withdrawn from insects within 24 hr. after collection in the field. Larvae of *Limnephilus stigma*, *Anabolia nervosa*, *Tipula montium*, *Aeschna grandis* and *A. cyanea* were sometimes kept in dishes of tap water for periods of up to 2 weeks before haemolymph was withdrawn. During this time the caddis larvae were fed on decaying leaves. However, the haemolymph Δ , sodium, potassium and chloride concentrations of the above insects were obtained within 3 days after collection in the field.

The insects were rapidly killed or asphyxiated in chloroform vapour, washed in distilled water, blotted dry and then totally immersed in liquid paraffin. A small puncture was made in a suitable position, depending on the kind of insect, and removal of haemolymph was usually assisted by gentle pressure on the abdomen. This treatment occasionally ruptured the gut wall. However, in nearly every case the gut fluid of these insects is dark brown in colour, and when the gut wall was ruptured the haemolymph turned noticeably dark and opaque. These samples were rejected. Haemolymph was collected behind a column of paraffin in fine Pyrex glass pipettes, and was transferred immediately into small Pyrex tubes, sealed at one end and also filled with paraffin. The samples were centrifuged for 1-2 min., sufficient to throw down particulate matter (mainly fatty tissue) but no attempt was made to ensure complete removal of blood cells from the supernatant. The latter was used for all analyses. In some instances it was necessary to pool haemolymph from several individuals to obtain a sample for the estimation of a single component. In these cases the usual practice was to pool haemolymph from a larger number of individuals and then estimate several components in the sample. All pooled samples are indicated in footnotes to the tables. With large species, e.g. Odonata, Trichoptera and *Dytiscus*, sufficient haemolymph was obtained from a single individual for estimation of several components in the sample. Mean values for each component are given in the tables, followed by the number of samples, in parentheses, and the extent of the standard deviation from the mean.

The concentrations of various components of haemolymph were estimated by the following techniques. Haemolymph Δ (micro-cryoscopy), sodium (flame photometry), chloride (micro Volhard titration) and conductivity; as described previously (Sutcliffe, 1961*a*). Potassium was estimated against a series of standard potassium chloride solutions in the same manner as for sodium, accurate to ± 0.5 mM./l. potassium.

Bicarbonate was estimated by the addition of N sulphuric acid to a small sample of haemolymph and absorption of liberated carbon dioxide in N/20 sodium hydroxide containing phenolphthalein as indicator (Shaw, 1955). Accurate to ± 5 mM./l. (as sodium bicarbonate).

Inorganic phosphate was estimated by the method of King (1932), adapted as follows. Haemolymph was centrifuged in ice-cold paraffin. About 20 μ l. of haemolymph supernatant was immediately transferred quantitatively into a test-tube containing an ice-cold solution of 2.5 ml. de-ionized water and 1.5 ml. 25% trichloroacetic acid. After mixing thoroughly by inversion, the protein precipitate was centrifuged down. 3.0 ml. supernatant was added to 4.5 ml. de-ionized water, followed by 0.72 ml. 60% perchloric acid, 0.60 ml. of a 5% ammonium molybdate solution and 0.3 ml. aminonaphtholsulphonic acid solution. Total acid-soluble phosphate was estimated by adding the perchloric acid to 3.0 ml. supernatant and hydrolysing for 24 hr. at 100° C. De-ionized water and other reagents were then added as above. Haemolymph samples were compared against a series of blanks and standard potassium dihydrogen phosphate solutions prepared in the same manner. The results on haemolymph samples were accurate to within ± 50 mg. P/l.

The total concentration of free amino acids was estimated by the colorimetric method of Folin (1922) as modified by Danielson (1933) and adapted for use with small samples. 4–5 μ l. of haemolymph was transferred quantitatively into 15 μ l. 10% trichloroacetic acid in a small tube. The protein precipitate was centrifuged down and 15 μ l. of supernatant added to 5 ml. de-ionized water. 1 ml. aliquots of reagents were used as detailed by Danielson. Haemolymph samples were compared against blanks and standard solutions of glycine and glutamic acid prepared in the same manner, accurate to ± 5 mM./l. The method gives quantitative results for the α -amino group of most amino acids (Folin, 1922; Danielson, 1933; Frame, Russell & Wilhelm, 1943) and therefore provides information on the molar concentration of free amino acids in haemolymph.

Estimations on each component were made in duplicate, chloride titrations in triplicate.

RESULTS

(a) *Odonata*

The concentrations of sodium and chloride in larvae of both Anisoptera and Zygoptera (Table 1) are very similar to those reported by earlier workers (Table 2), and the results firmly establish that both are generally present in high concentrations in the Odonata. Nevertheless, sodium is always in excess of chloride, and this excess ranges from about 10% in *Enallagma* up to 30% in *Aeschna*.

The concentration of potassium in larvae was always very low and generally did not exceed about 10 m-equiv./l. However, there is a distinct possibility that the potassium concentration is higher in the adult stage. In the case of *Agrion virgo* (Table 1) adults

were taken in June, and although both the sodium and chloride concentrations in haemolymph were very similar to those found in *Aeschna* larvae, the potassium concentration at 27 m-equiv./l. was three times greater. In the following September the potassium concentration in larvae of *Agrion virgo* (obtained from the same locality as the adults) was only 8-9 m-equiv./l., whereas the sodium and chloride concentrations were remarkably similar to those found in the adults. The possibility of changes in some components of the haemolymph at the time of metamorphosis must therefore

Table 1. *The haemolymph Δ and concentrations of sodium, potassium and chloride in Odonata*

Species	Stage	Haemolymph Δ °C. (mean values)	Haemolymph Δ as a NaCl soln. (mM./l.)	Concn. in m-equiv./l.		
				Na	K	Cl
Anisoptera						
<i>Aeschna grandis</i>	Larvae	0.735	206 (13) \pm 23	145 (7) \pm 5	9 (6) \pm 2.3	110 (14) \pm 11
<i>A. cyanea</i>	Larvae	0.701	195 (6) \pm 5	142 (6) \pm 5.5	8 (6) \pm 1.7	112 (6) \pm 12
Zygotera						
(a) <i>Enallagma cyathigerum</i>	Larvae	0.620	174 (8) \pm 5.5	139 (7) \pm 5.5	14 (7) \pm 2	127 (6) \pm 10
(b) <i>Agrion virgo</i>	Adults	0.790	221	145	27.5	109
(c) <i>A. virgo</i>	Larvae	—	—	140, 145	8, 9	112, 112

Haemolymph from: (a), 1 or 2 larvae in each sample; (b) 7 adults in one sample; (c) 7 larvae in each of two samples.

Table 2. *The concentrations of four cations and chloride from other investigations on haemolymph of the larvae of Odonata.*

(Concentrations in m-equiv./l.)

Species	Na	K	Ca	Mg	Cl	Source
<i>Aeschna</i> sp.	135	5	8	20	91*	} Duchâteau <i>et al.</i> (1953)
	179	5	20	12	—	
	143	4	16	—	—	
<i>Libellula depressa</i>	178	4	18	12	77*	} Duchâteau <i>et al.</i> (1953)
<i>Libellula</i> sp.	152	—	16	—	—	
—	—	—	8	5	—	Clark & Craig (1953)
<i>Agrion (Calopteryx)</i> sp.	158	9	—	—	—	Boné (1944)
						*Schoffeniels (1950)

Table 3. *Further analyses on haemolymph of Aeschna larvae*

Species	Conductivity as a NaCl soln. (mM./l.)	Total acid-sol. P (mg./l.)	Inorganic P (mg./l.)	HCO ₃ (m-equiv./l.)	Free amino acids as glycine (mM./l.)
<i>A. grandis</i>	157 (7) \pm 5.5	115 (3)	73 (3)	15 (6) \pm 2.5	39 (11) \pm 14
<i>A. cyanea</i>	—	123 (7) \pm 65	86 (4)	—	34 (10) \pm 13

be borne in mind, and it is perhaps significant that the potassium concentration of an adult *Aeschna cyanea* was 21 m-equiv./l. (sodium = 120 m-equiv./l.). This specimen emerged in the laboratory, and the haemolymph was removed about 1 hr. later.

Measurements of haemolymph conductivity in terms of a solution of sodium chloride (Table 3) indicate that the haemolymph contains a high concentration of ionized salts, with cations and anions each contributing very roughly 157 m-equiv./l.

Now although the chloride concentration is always high, it is insufficient to maintain electro-neutrality with the cations sodium and potassium. Estimates of bicarbonate and phosphate in haemolymph of *Aeschna* larvae (Table 3) showed that both may contribute a small amount to the anion pool. The total acid-soluble phosphate concentration is very low, and it appears to be largely inorganic in nature. For practical purposes it is therefore assumed that the phosphate fraction consists of salts of ortho-phosphoric acid, where each mole of acid probably bonds with 3/2 equivalents of base (the pH of insect haemolymph usually lies between 6.0 and 7.5, see Buck, 1953). Cation-anion balance in haemolymph of *Aeschna* larvae may therefore be represented as shown in Table 4.

Table 4. *The distribution of cations and anions in haemolymph of Aeschna larvae*

(Concentrations in m-equiv./l.)

<i>A. grandis</i>				<i>A. cyanea</i>			
Na ⁺	145	Cl ⁻	110	Na ⁺	142	Cl ⁻	112
K ⁺	9	HCO ₃ ⁻	15	K ⁺	8	HCO ₃ ⁻	15
		PO ₄ ³⁻	6			PO ₄ ³⁻	6
Totals	154		131		150		133

It appears that in both species some 20 m-equiv./l. on the anion side is unaccounted for. This deficit may be even larger, since other workers have found small amounts of calcium and magnesium in haemolymph of anisopterous larvae (Table 2). In *Aeschna* each of these ions could contribute about 15 m-equiv./l. to the cation pool, although in fact the contribution may well be considerably less due to binding with proteins and organic acids. For example, Breen & Freeman (1961) found that in human plasma approximately 50% of the calcium is bound to proteins. Nothing is known about ion-binding in insect haemolymph, except that in *Telea polyphemus* larvae (Lepidoptera) some 15-20% of the calcium and magnesium is bound to macromolecules which did not pass ultrafilters (Carrington & Tenney, 1959).

The total concentrations of free amino acids in larvae of *A. grandis* and *A. cyanea* are shown in Table 3. The estimates are very similar to that of Duchâteau & Florkin (1958) using a microbiological assay technique. These authors quantitatively estimated fifteen amino acids in *Aeschna* larvae and found a total concentration of 399 mg./100 ml. plasma. The eight commonest amino acids in their list have a mean molecular weight of 120; hence the total molar concentration is roughly 33 mM./l. compared with the present estimates of 39 mM./l. in *A. grandis* and 34 mM./l. in *A. cyanea*. Schoffeniels (1960) gives lower values, equivalent to 8-9 mM./l. (as leucine) in *Libellula* larvae and 10 to 11 mM./l. in *Aeschna* larvae. However, it seems likely that the total concentration of free amino acids varies considerably between individuals (or in different larval instars?), since the range of individual variation found in the present investigation was quite large, e.g. from 12 to 53 mM./l. in *A. cyanea* larvae.

It is tempting to suggest that the anion deficit of at least 20 m-equiv./l. can be accounted for by part of the free amino acid fraction. However, the isoelectric points of most amino acids are very close to the slightly acid pH values of insect haemolymph, and therefore they are probably present largely as 'zwitterions'. Moreover, as Wyatt

(1961) has already pointed out, the free amino acids in insect haemolymph may be regarded as frequently making a net contribution to cations rather than anions, since free aspartic and glutamic acids usually occur in low concentrations (in contrast to their amides) whereas arginine, lysine and histidine are generally more abundant. Now in *Aeschna* larvae the concentration of free aspartic and glutamic acids is about 280 mg./l. and the concentration of the basic amino acids is about 550 mg./l. (Duchâteau & Florkin, 1958). Hence both the acidic and the basic amino acids each contribute only about 4 m-equiv./l. to the total ion pool. Moreover they balance each other out. It appears, then, that the free amino acids must be regarded as contributing mainly to the 'non-electrolyte' fraction of the haemolymph.

Consider now the osmotic effects of the known components in *Aeschna* haemolymph. Here, and in most other exopterygotes, the haemolymph contains relatively high concentrations of sodium and chloride. Therefore, to obtain a rough estimate of the total osmotic activity of the known components, these were regarded as if they were a simple solution of sodium chloride. Thus all concentrations were expressed in mm./l. (amino acids) or mg. ions/l. (inorganic ions) and were added together. The total, S, was treated as though derived from a fully dissociated monovalent salt, whose concentration, expressed in mm./l., would be S/2. In order to convert this to m-osm./l. the correction factor appropriate for a NaCl solution was used. An approximate correction factor (osmotic coefficient) was obtained from the relationship that for

Table 5. *The major osmotic components of haemolymph in Aeschna grandis larvae*

Free amino acids	39 mm./l.
Na	145 mg. ions/l.
K	9
Ca	7.5
Mg	7.5
Cl	110
HCO ₃	15
PO ₄	4
Total	337 reckoned as mg. ions/l.
Total $\times \frac{1.92}{2} =$	324 m-osm./l.
Osmolar concn. of haemolymph (from Δ)	395
Total osmolar concn. of components	324
Osmotic deficit	71 m-osm./l.

171 mm./l. NaCl $\Delta = 0.61^\circ$ C. found empirically in this investigation. By extrapolation, the freezing-point depression of a 1 molar NaCl solution is 3.567° C. Assuming that for a 1 molar solution of ideal non-electrolyte $\Delta = 1.86^\circ$ C., then the osmotic coefficient is $3.567/1.86 = 1.92$. Hence, in this paper, S mg. ions/l. corresponds to $S \times 1.92/2$ m-osm./l. The total osmotic activity of the haemolymph (m-osm./l.) was obtained from the freezing-point depression of the haemolymph, employing the relationship 1 osmole/l. = $\Delta 1.86^\circ$ C.

In *A. grandis* larvae the osmolar concentration of the known components is 324 m-osm./l. (Table 5). Since the total activity of the haemolymph is equivalent

to 395 m-osm./l. it appears that 71 m-osm./l. remain unaccounted for. This osmotic deficit might be attributed to the presence of proteins, peptides, carbohydrates, and other large molecules. Of these, the concentration of protein-nitrogen is low in *Aeschna* larvae (Raper & Shaw, 1948; Shaw & Beadle, 1949) and will exert a negligible osmotic effect. The concentrations of other large molecules in *Aeschna* larvae is unknown, but in larvae of *Gastrophilus intestinalis* (Diptera), where they occur in high concentrations, there is still a large osmotic (and anion) deficit (Levenbook, 1950). As an alternative, most of the osmotic deficit could be due to the presence of components with relatively low molecular weights, such as non-amino carboxylic acids. These are known to occur in insect haemolymph, often in very high concentrations. For example, the total concentration of known carboxylic acids in haemolymph of *Gastrophilus* is 123 m-equiv./l., and similar concentrations are reported in lepidopteran larvae (see Wyatt, 1961). Citrate occurs in a variety of insects, with a concentration of 32 mM./l. in larvae of *Bombyx mori* (Levenbook & Hollis, 1961). These acids could account for not only the osmotic deficit but also the anion deficit in *Aeschna* haemolymph. In fact, current investigations employing ion-exchange resins (unpublished) suggest that haemolymph in *Aeschna* larvae contains roughly 20-30 m-equiv./l. 'strong' acid, including non-amino polycarboxylic acids. This is sufficient to account for all of the anion deficit in *Aeschna* larvae, and lends further support to the conclusion reached above that the free amino acids largely contribute to the 'non-electrolyte' fraction of the haemolymph.

Table 6. The haemolymph Δ and concentrations of sodium, potassium and chloride in Ephemeroptera, Plecoptera and Hemiptera-Heteroptera

Species	Stage	Δ °C. (mean values)	Haemolymph Δ as a NaCl soln. (mM./l.)	Concn. in m-equiv./l.		
				Na	K	Cl
Ephemeroptera						
(a) <i>Ephemera danica</i>	Larvae	0.504	141 (7) \pm 11.5	103 (7) \pm 6	18 (6) \pm 2.5	77 (7) \pm 5.5
(b) <i>Ecdyonurus dispar</i>	Larvae	0.529	148 (7) \pm 16	—	—	110 (6) \pm 8
Plecoptera						
(c) <i>Perla bipunctata</i>	Larvae	0.644	180 (7) \pm 28	127 (7) \pm 6	12 (6) \pm 4	106 (12) \pm 17
(d) <i>Dinocras cephalotes</i>	Larvae	0.583	163 (5) \pm 7	117 (11) \pm 14.5	10 (8) \pm 1.5	111 (8) \pm 11.5
Hemiptera-Heteroptera						
(e) <i>Corixa punctata</i>	Adults	0.600	168 (8) \pm 16.5	112 (8) \pm 5.5	31 (7) \pm 5	75 (9) \pm 10
(f) <i>Notonecta obliqua</i>	Adults	0.756	212 (8) \pm 2.5	155 (8) \pm 15	21 (8) \pm 10	121 (5) \pm 15

Haemolymph in each sample obtained from: (a) 5-8 larvae; (b) 2-3 larvae for Cl, Δ on individuals; (c) 2-3 larvae, K on individuals; (d) 1-3 larvae, Δ on individuals; (e) 6-10 adults; (f) 4-6 adults.

(b) Ephemeroptera, Plecoptera and Hemiptera-Heteroptera

The results of analyses on representatives of these aquatic exopterygotes are given in Table 6. It is immediately clear that, as in the Odonata, the concentrations of both sodium and chloride are very high. Nevertheless, sodium again exceeds chloride, and the excess varies from 5% in larvae of *Dinocras cephalotes* up to 50% in adult *Corixa punctata*. Compared with sodium and chloride the concentration of potassium is low, although from Table 6 it might appear that the potassium concentration is generally relatively high in the aquatic Heteroptera. However, in adult *Gerris najas* the

potassium concentration is no greater than that in *Aeschna* larvae, since Boné (1944) found only 8 m-equiv./l. (sodium = 142 m-equiv./l.).

Measurements of haemolymph conductivity indicate that inorganic salts are largely ionized in haemolymph of *Perla bipunctata* (Table 7). Hence the cations sodium and potassium exceed chlorine anions by 16 m-equiv./l. in *Dinocras cephalotes* and by 33 m-equiv./l. in *Perla bipunctata*. Some of this excess is balanced by phosphate, which contributes roughly 9 m-equiv./l. (Table 7), and a further contribution may be made by bicarbonate. As in the Odonata, free amino acids probably form part of the 'non-electrolyte' fraction in the haemolymph.

Table 7. *Further analyses on haemolymph of plecopteran larvae*

Species	Conductivity as a NaCl soln. (mm./l.)	Total acid-sol. P (mg./l.)	Inorganic P (mg./l.)	Free amino acids as glycine (mm./l.)
<i>Perla bipunctata</i>	140 (8) ± 15*	178 (6) ± 104	—	35 (6) ± 3*
<i>Dinocras cephalotes</i>	—	174 (5) ± 58	130 (3)	39 (4)

* Haemolymph from 2-3 larvae in each sample.

Table 8. *The major osmotic components of haemolymph in two plecopteran larvae*

	<i>Perla bipunctata</i> (concn. in mm./l. or mg. ions/l.)	<i>Dinocras cephalotes</i>
Free amino acids	35	39
Na	127	117
K	12	10
Cl	106	111
PO ₄	6	6
Totals	286	283
	Concn. in m-osm./l.	
Osmolar concn. of haemolymph (from Δ)	346	313
Total osmolar concn. of components	275	272
Osmotic deficit	71	41

The calculated contribution of the known components to the total osmolar concentration of plecopteran haemolymph is shown in Table 8. Calcium, magnesium and bicarbonate probably also contribute a small amount to the total osmolar concentration. Nevertheless, it appears that, as in *Aeschna* larvae, organic non-amino acids may be present in relatively high concentrations in plecopteran larvae. Relatively low concentrations of these acids may also exist in larvae of Ephemeroptera. On the other hand, very high concentrations of both amino and other organic acids possibly occur in adult Heteroptera. For example, in *Corixa punctata* sodium, potassium and chloride contribute only 218 mm./l. in a total haemolymph concentration of 323 m-osm./l., and in *Notonecta obliqua* the same ions contribute 297 mm./l. in a total haemolymph concentration of 406 m-osm./l. A small part of this large unknown fraction may be accounted for by divalent ions, since Clark & Craig (1953) found 31 m-equiv./l. calcium and 19 m-equiv./l. magnesium in *Notonecta kirbyi*, but only 8 and 4 m-equiv./l. respectively in *Hesperocorixa larigata*.

(c) Aquatic endopterygotes

The results of analyses on representatives of various endopterygotes are given in Table 9. The most striking feature, present in all of these insects, is the low chloride concentration compared with that found in the exopterygotes. Thus the highest mean value for chloride, 60 m-equiv./l. in *Colymbetes fuscus* larvae, was less than the lowest mean value for chloride in the exopterygotes, 75 m-equiv./l. in *Corixa punctata*. The chloride concentration in haemolymph of other aquatic endopterygotes is also low, e.g. 31 m-equiv./l. in *Sialis lutaria* larvae (Shaw, 1955) and 52 m-equiv./l. in *Aedes aegypti* larvae (Wigglesworth, 1938). Moreover, in some species the chloride concentration was extremely low, no greater than 10 m-equiv./l. in larvae of *Tipula montium*, *Anabolia nervosa* and *Limnephilus stigma*. Boné & Koch (1942) found only 7 m-equiv./l. in larvae of *L. flavicornis*. The potassium concentration was also low, varying from 29 m-equiv./l. in larvae of *Nymphula nymphæta* down to 7 m-equiv./l. in *Phryganea* larvae. Thus the range of potassium concentrations is the same as that found in the exopterygotes. In comparison the sodium concentration is apparently always high, and this is supported by the results of others. Some of the latter are shown in Table 10. Also, from Tables 9 and 10 it appears that the range of sodium concentrations in haemolymph of aquatic endopterygotes is similar to that found in aquatic exopterygotes, with the outstanding exception of *N. nymphæta*. In this lepidopteran larva the mean sodium concentration was only 40 m-equiv./l.

The results on larvae of *Dytiscus* and *Colymbetes* (Table 9) require further comment. In both, the variation in sodium concentration of haemolymph from individuals (*Dytiscus*) and pooled samples (*Colymbetes*) was far greater than that found in all of the other aquatic insects investigated. For example, the sodium concentration in *Dytiscus* larvae ranged from 80 to 145 m-equiv./l. There was also considerable individual variation in the chloride concentration of *Dytiscus* larvae. It is possible that the results reflect large changes in haemolymph composition, depending on whether or not these carnivorous larvae had recently fed, although marked differences of this kind were not found in *Rhodnius* (Ramsay, 1952). However, some of the variation may be due to contamination of haemolymph with fluid from one or more regions of the alimentary system. In contrast with the other aquatic insects, it was very difficult to obtain more than a small quantity of haemolymph from one individual, even from a large *Dytiscus* larva. During the collection of haemolymph, the gut wall of some larvae was clearly ruptured. These samples were rejected, but slight contamination in other cases may not have been detected. The results on adult *D. marginalis* (Table 9) are very similar to those of other investigators (Table 10). Barrat & Arnold (1911) found a haemolymph Δ of 0.77° C. in adult *D. marginalis* and 0.647° C. in adult *Hydrophilus piceus*.

The total concentration of free amino acids in several species is shown in Table 11. These concentrations are similar to the total molar concentration of free amino acids in *H. piceus* (30–60 mM./l.) and in some terrestrial insects, e.g. *Locusta* (about 50 mM./l.), *Carausius* (25–35 mM./l.) and some lepidopteran larvae (data from Duchâteau & Florkin, 1958; Florkin, 1959); they are also very similar to the concentrations found in larvae of Odonata and Plecoptera (Table 3). The high concentration in larvae of *Sialis lutaria* (85 mM./l.) is interesting since in this species Beadle & Shaw (1950) found an average non-protein nitrogen content of 258 mg. %. Hence,

if each molecule of the non-protein nitrogen components contained one atom of nitrogen, the total concentration of molecules would be 184 mm./l. This is double the total concentration of free amino acids now found in *S. lutaria*, but at least some of the excess nitrogen atoms were probably derived from basic amino acids. The remainder may be present in peptides, which are not estimated quantitatively by Folin's method (Frame *et al.* 1943).

Table 9. *The haemolymph Δ and concentrations of sodium, potassium and chloride in some aquatic endopterygotes*

Species	Stage	Δ °C. (mean values)	Haemolymph Δ as a NaCl soln. (mm./l.)	Concn. in m-equiv./l.		
				Na	K	Cl
Coleoptera						
<i>Dytiscus marginalis</i>	Adults	0.745	208 (2)	126 (4)	14 (3)	44 (5) \pm 7
<i>Dytiscus</i> sp.	Larvae	0.629	176 (6) \pm 24.5	115 (5) \pm 28	20 (2)	52 (6) \pm 20
(a) <i>Colymbetes fuscus</i>	Larvae	—	—	127 (5) \pm 19	19 (5) \pm 5.5	60 (5) \pm 4
Trichoptera						
<i>Limnephilus stigma</i>	Larvae	0.380	106 (6) \pm 8	83 (7) \pm 4.5	14 (6) \pm 3.5	10 (6) \pm 4.5
<i>Anabolia nervosa</i>	Larvae	0.405	113 (6) \pm 10*	101 (6) \pm 3*	17 (7) \pm 3.4	9 (6) \pm 2.4
<i>Phryganea</i> sp.	Larvae	0.455	127 (9) \pm 8	69 (12) \pm 10	7 (5), 6-8	37 (11) \pm 7
(b) <i>Philopotamus montanus</i>	Larvae	0.480	134 (8) \pm 5	109 (7) \pm 2	21 (6) \pm 1.6	37 (6) \pm 2.7
Lepidoptera						
<i>Nymphula nymphæta</i>	Larvae	0.552	154 (7) \pm 10	40 (7) \pm 9	29 (7) \pm 6.5	31 (6) \pm 4
Diptera						
<i>Tipula montium</i>	Larvae	0.443	124 (6) \pm 4	115 (6) \pm 8	7 (6) \pm 1.8	8 (6) \pm 1.5

Haemolymph from: (a) 3 larvae in each sample; (b) 4 larvae in each sample, Δ on individuals.

* Data from Sutcliffe (1961b).

Table 10. *The concentrations of the four major cations from other investigations on the haemolymph of aquatic endopterygotes*

Species	Stage	(Concentrations in m-equiv./l.)				Source	
		Na	K	Ca	Mg		
Coleoptera							
<i>Dytiscus marginalis</i>	Adults	165	6	23	38	Duchâteau <i>et al.</i> (1953)	
		140	5	—	—		Ramsay (1953b)
		133	10	—	—		Boné (1944)
<i>Hydrophilus piceus</i>	Adults	124	4	25	47	} Duchâteau <i>et al.</i> (1953)	
		121	14	23	44		
Trichoptera							
<i>Chaetopteryx villosa</i>	Larvae	64	9	—	—	Boné (1944)	
<i>Phryganea</i> sp.	Larvae	92	7	14	51	} Duchâteau <i>et al.</i> (1953)	
Diptera							
<i>Chironomus</i> sp.	Larvae	104	2	11	15		
<i>Aedes aegypti</i>	Larvae	87	3	—	—	} Ramsay (1953b)	
Tabanid	Larva	151	5	—	—		

We will now consider the problem of cation-anion balance in the haemolymph of these endopterygote insects. First of all, it is clear that the chloride concentration is grossly insufficient to maintain electro-neutrality with sodium and potassium. Thus the contribution of chlorine anions ranges from only 49% in *Phryganea* larvae down to a mere 7% in larvae of *Tipula montium*. In fact the excess of cations over chloride

may be even greater, since quite high concentrations of divalent cations, particularly magnesium, have been reported (Table 10). The concentrations of phosphate and bicarbonate in several species is shown in Table 11. In larvae of *Limnephilus stigma* and *Anabolia nervosa* the total concentration of acid-soluble phosphate is high, equivalent to about 15 and 20 mM./l. respectively, assuming that each molecule of the components in the phosphate fraction contains one atom of phosphorus. The phosphate concentration in *Sialis lutaria* larvae is also high, and similar values have been reported from other insects (Buck, 1953; Wyatt, 1961). It also appears that about 70–80% of the phosphate in the caddis larvae is inorganic in nature, and therefore makes a major

Table 11. Further analyses on haemolymph from larvae of aquatic endopterygotes

Species	Conductivity as a NaCl soln. (mM./l.)	Total acid-sol. P (mg./l.)	Inorganic P (mg./l.)	HCO ₃ (mM./l.)	Free amino acids as glycine (mM./l.)
<i>L. stigma</i>	88*	473 (7) ± 95	366 (3)	10 (3)	41 (6) ± 6
<i>A. nervosa</i>	108*	635 (6) ± 51	454 (5) ± 43	—	29 (6) ± 4
<i>Phryganea</i> sp.	93 (11) ± 13	—	—	—	58 (15) ± 17
<i>T. montium</i>	—	100 (3)	50 (3)	—	47 (10) ± 7
<i>S. lutaria</i>	152†	(a) 393 (3)	—	15†	85 (7) ± 13

(a) Haemolymph from 3–4 larvae in each sample.

* Data from Sutcliffe (1961b).

† Data from Shaw (1955).

Table 12. The distribution of cations and anions in haemolymph of two endopterygote larvae

(Concentrations in m-equiv./l.)

<i>Sialis lutaria</i> *				<i>Limnephilus stigma</i>			
Na ⁺	109	Cl ⁻	31	Na ⁺	83	Cl ⁻	10
K ⁺	5	HCO ₃ ⁻	15	K ⁺	14	HCO ₃ ⁻	10
Ca ²⁺	15	PO ₄ ³⁻ †	15			PO ₄ ³⁻	18
Mg ²⁺	38	P†	6			P†	6
Totals	167		67	Totals	97		44

* All data except phosphate from Shaw (1955)

† Organic 'phosphate', see text.

‡ Probable maximum contribution from inorganic fraction.

contribution to the anion pool. The distribution of anions in the organic phosphate fraction is difficult to assess, as insect haemolymph contains a wide variety of phosphorylated compounds (see Wyatt, 1961). However, the contribution of this fraction to the anion pool is not likely to be very great, since only about 3 mg. atoms/l. phosphorus are involved in the case of *L. stigma*, and 6 mg. atoms/l. phosphorus in *A. nervosa*. Also, it may be noted that high concentrations of phosphate are not necessarily characteristic of insects with a low chloride concentration, since the total concentration of acid-soluble phosphate in *T. montium* larvae is only about 3 mM./l. Buck (1953) provides a number of other examples. The concentration of bicarbonate in *L. stigma* larvae is similar to that found in larvae of *A. grandis* and *S. lutaria*, and it seems unlikely that this anion contributes more than about 15 m-equiv./l. in the haemolymph of aquatic insects.

The possible distribution of known ions in haemolymph of two typical aquatic endopterygotes is shown in Table 12, where organic phosphate is estimated as representing a contribution of 6 m-equiv./l. *S. lutaria* is useful as an example because Shaw (1955) has estimated the concentrations of the four major cations. It is clear that the latter are greatly in excess of the anions. It is quite possible that the cation pool may be substantially decreased by complexing with proteins, free amino acids and other organic acids, particularly in the case of the divalent cations, but this remains to be demonstrated. On the other hand, measurements of haemolymph conductivity, in terms of a solution of sodium chloride (Table 11), suggest that at least most, if not all, of the sodium and potassium is ionized and therefore in excess of the known inorganic anions. Hence, it appears that the major part of the anion fraction is not yet accounted for, and the possibility that this anion deficit is met by the free amino acids must now be examined.

From a comparison of Tables 3 and 7 with Table 11 it appears that the total concentration of free amino acids is roughly the same in both the exopterygotes (i.e. Odonata and Plecoptera) and the endopterygotes (i.e. Trichoptera and *Tipula*) with the exception of *S. lutaria* larvae. However, if the concentrations of free amino acids are expressed in terms of their contribution as non-electrolytes to the total osmolar concentration of the haemolymph, this is 10–13% in the above exopterygotes. On the other hand, in the above endopterygotes the contribution is 20–25% except in *A. nervosa* larvae, where it is only 14%. Since the free amino acid fraction is relatively greater in the endopterygotes, it might be inferred that the increase is related to the larger anion deficit. But this is not necessarily so. Some of the difficulties involved in accepting amino acids in the major role of anions were discussed above in the case of *Aeschna* larvae. Furthermore, if acidic amino acids do maintain electro-neutrality with inorganic cations in the endopterygotes, then, from Table 12, it follows that free aspartic and glutamic acids must be present in very high concentrations. For example, in *L. stigma* larvae at least 53 m-equiv./l. acid is required, or a concentration approaching 53 mM./l. amino acids. But the mean total concentration of free amino acids in *L. stigma* is only 41 mM./l. (Table 11) and it seems very unlikely that these consist almost exclusively of free acidic amino acids, particularly as the latter are generally present in extremely low concentrations in other endopterygotes (see Florkin, 1959), where the anion deficit is also large. Finally, further information can be obtained from rough estimates of the osmotic effect of the known components in haemolymph. The osmotic activity of the components was calculated as for *Aeschna* larvae, i.e. as if the components were a solution of sodium chloride. This is certainly not the case in endopterygotes, and the osmotic effect of the summed components may be less than the calculated values given here, particularly as no allowance was made for ion-binding, which may occur to a considerable extent in some cases.

Consider the case of *S. lutaria* larvae, in which the concentrations of both chloride and the free amino acids are high. The total concentration of known cations and anions is 199.5 mM./l. To this we may add 85 mM./l. free amino acid, giving a sum total of 284.5 mM./l. with an osmotic effect of about 273 m-osm./l. Now the total concentration of the haemolymph is 339 m-osm./l. (Shaw, 1955) so that about 66 m-osm./l. remain unaccounted for. Consider also the larvae of *L. stigma*. Here, the sum of known cations and anions is 132 mM./l., free amino acids contribute 41 mM./l., and

the total osmotic concentration of these components is about 166 m-osm./l. Since the total concentration of the haemolymph is 204 m-osm./l., there is an osmotic deficit of some 38 m-osm./l. Similar deficits occur in larvae of *A. nervosa* (33 m-osm./l.), *Phryganea* (21 m-osm./l.) including the concentrations of calcium and magnesium reported by Duchâteau *et al.* in Table 10, and there is a deficit of 49 m-osm./l. in *T. montium*. Where unknown, the concentrations of bicarbonate and phosphate were each assessed at 15 mM./l. It is believed, therefore, that the calculated total osmotic concentrations of the known components are maximum ones in the case of *S. lutaria* and *Phryganea*. Since the osmotic deficits are quite large, particularly in the larvae of *S. lutaria* and *T. montium*, it is inferred that, as in the Odonata, the haemolymph may contain fairly high concentrations of non-amino organic acids. Since the anion deficits are usually greater than the calculated osmotic deficits, it is possible that some of these organic acids are at least di-acidic. In fact, preliminary investigations on the caddis larvae *L. stigma*, *A. nervosa* and *Phryganea* indicate that the haemolymph contains roughly 50 m-equiv./l. non-amino 'strong' acid (excluding chloride).

DISCUSSION

Current opinions on the status of chloride in insect haemolymph hold that this anion is generally present in very low concentrations (Buck, 1953; Wyatt, 1961). This view arises from the fact that the great majority of investigations have been made on haemolymph from Lepidoptera, Hymenoptera, Coleoptera and Diptera, and these are all endopterygote insects. It is now clear that in a number of aquatic insects the chloride concentration is very high. These are all exopterygotes, and since the chloride concentration in haemolymph of a variety of terrestrial exopterygotes is also very high it is suggested elsewhere that this is characteristic of the Exopterygota (Sutcliffe, 1962). It is also suggested that, in contrast, a relatively low chloride concentration is characteristic of the Endopterygota.

Boné (1944) proposed that a correlation exists between the diet of an insect and the ratio of sodium to potassium in haemolymph; in carnivorous insects this ratio is greater than unity whereas in herbivorous insects the ratio is less than unity. Although Boné was aware of inconsistencies in the data, he concluded that the correlation is generally applicable to all insects. In spite of severe criticism by Duchâteau *et al.*, Boné's hypothesis is still widely quoted, so the results of the present investigation are examined from this viewpoint.

Now in all of the aquatic insects so far investigated, the sodium concentration is greater than the potassium concentration. Moreover, with the exception of *Nymphula*, sodium exceeds potassium by a factor of at least five and occasionally by a factor of more than ten. Hence, according to Boné's hypothesis we might expect that all of these insects are carnivorous. This is undoubtedly so in the case of the Odonata, *Dytiscus* and *Colymbetes*. Also, both *Perla* and *Dinocras* are primarily carnivorous, feeding particularly on ephemeropteran and dipteran larvae, but it is worth noting that members of other genera in the Plecoptera are essentially herbivorous (Hynes, 1941; Jones, 1949, 1950). On the other hand, most of the Ephemeroptera are herbivorous (Rawlinson, 1939; Jones, 1949, 1950) and so are the Tipulinae (Alexander, 1920). All of the trichopteran larvae investigated here are essentially herbivorous. Members of the Limnephilidae and *Phryganea* feed mainly on living and dead phanerogams, algae,

diatoms, 'detritus' and possibly bacteria (Lloyd, 1921; Jones, 1949, 1950; Hanna, 1957) and *Philopotamus montanus* feeds on diatoms (Jones, 1949). In the Hemiptera-Heteroptera, *Notonecta* and Gerrids are obviously carnivores, but *Corixa punctata* is omnivorous and feeds on both algae and small dipteran larvae (Poisson, 1935; Sutton, 1951). Larvae of *Nymphula* live on the leaves of *Potamogeton* (Wesenburg-Lund, 1943).

From the above it is quite clear that in the aquatic insects there is no correlation between diet and the ratio of sodium to potassium in haemolymph. In fact there is now good evidence to support the view that the relative proportions of these two ions, together with the distribution of chloride, are related to phylogeny and systematic position within the Insecta, and the exceptionally low sodium concentration found in *Nymphula* larvae is consistent with this view (Sutcliffe, 1962).

Table 13. *The concentrations of the major cations and anions in four bodies of water in the English Lake District**

Source	(Concentrations in m-equiv./l.)						
	Na	K	Ca	Mg	Cl	HCO ₃	SO ₄
Lily Tarn	0.15	0.01	0.10	0.08	0.19	0.03	0.15
Esthwaite Water	0.20	0.02	0.41	0.12	0.20	0.31	0.22
Clay Pond	0.34	0.02	0.59	0.20	0.32	0.47	0.31
Windermere, N. Basin	0.16	0.01	0.27	0.07	0.17	0.17	0.14

* Data from Mackereth & Heron (1953-55).

It is interesting to compare the concentrations of ions in haemolymph of aquatic insects with the concentrations of the same ions in fresh water. Four examples of Lake District waters are given in Table 13, as most of the insects used in the present investigation were collected in these waters (Cunsey Beck flows out of Esthwaite Water, see Appendix). It appears that in the haemolymph potassium is concentrated by a factor of 1×10^3 , calcium and magnesium by about 1×10^2 , and sodium is concentrated by roughly 5×10^2 . Chloride may be concentrated to the same extent as sodium or by a factor of less than 1×10^2 . If the concentrations in the haemolymph are maintained by absorbing these ions from the external medium, then it is clear that at least one, and probably several of the ions must be actively transported against the concentration gradient. Very little is known about the uptake of ions from the external medium, except in larvae of *Aedes aegypti* (Koch, 1938; Ramsay, 1953a; Treherne, 1954a; Stobbert, 1960). These investigators have shown that sodium, potassium and chloride are all taken up against the concentration gradient, and sodium is actively transported. Potassium and chloride may be actively transported, and each ion may have a separate transport system. The site of uptake is largely in the anal papillae, and salt balance is maintained in 'tap water'. According to Krogh (1939) larvae of *Libellula* and *Aeschna* have separate systems for active uptake of sodium (but not potassium) and anions, but the experimental evidence was not published. In *Sialis* larvae (Shaw, 1955) sodium is actively absorbed in the midgut and chloride follows passively from high salt concentrations, but neither of these ions is absorbed from tap water (0.3 mm./l. sodium chloride). There is no external ion-absorbing mechanism in *Sialis* larvae and salts must be gained from the food. *Helodes* larvae must also gain salts from the food since

chloride balance is not maintained in stream water (0.35 mM./l. chloride), although chloride is taken up via the gut and anal papillae (Treherne, 1954*b*). In view of these differences it is clear that we need to know more about ion absorption in aquatic insects. Also, it would be interesting to determine, for each ion, the minimum external concentration required to maintain balance.

Finally, it is possible that non-amino organic acids, as well as the free amino acids, are concerned in osmoregulation. For example, Schoffeniels (1960) gives the concentrations of chloride and free amino acids in haemolymph of *Libellula* and *Aeschna* larvae, and in adult *Dytiscus marginalis*. When these insects were transferred into distilled water the haemolymph osmotic pressure remained fairly constant, the chloride concentration was roughly halved, and the total concentration of free amino acids was doubled. Thus in *Libellula* larvae the chloride concentration fell from 120 to 60 mM./l., whereas the total concentration of free amino acids increased from about 9 to 17 mM./l. (as leucine). It is therefore clear that the increase in concentration of free amino acids is not sufficient to balance the decrease in chloride concentration. This is also the case in the other insects which Schoffeniels investigated.

SUMMARY

1. Analyses were made of haemolymph obtained from aquatic representatives of nine major orders in the Insecta.

2. In the Odonata, Plecoptera, Ephemeroptera and Hemiptera-Heteroptera, the sodium concentration is relatively high and the potassium concentration is low. The chloride concentration is relatively high, but it never exceeds the sodium concentration.

3. In the Neuroptera, Coleoptera, Trichoptera and Diptera the sodium concentration is relatively high and the potassium concentration is low. In *Nymphula nymphaeta* (Lepidoptera) both the sodium and potassium concentrations are low. In all of these endopterygotes the chloride concentration is relatively low.

4. There is no correlation between diet and the sodium/potassium ratio in haemolymph of aquatic insects.

5. The problem of cation-anion balance is discussed. It is suggested that free amino acids contribute little to the anion pool. Bicarbonate and phosphate, largely as inorganic phosphate, contribute a small amount to the anion pool in exopterygotes. Phosphate contributes considerably more to the anion pool in some endopterygotes. Nevertheless, in all of the insects investigated, there is a deficit of anions. This deficit is usually greater in the endopterygotes.

6. The calculated osmotic activities of the known ions and free amino acids are less than the observed osmotic activities of haemolymph. It is inferred that non-amino organic acids must be present. Preliminary investigations indicate that non-amino acids are present in the concentrations required to account for both the osmotic and the anion deficits.

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APPENDIX

During the investigation analyses were made on haemolymph of the species listed below. The following information is given immediately after the name of each species: locality and county where collected; date of collection; stage in the life-history; reference to the authority consulted for identification. Reference 12 provides an indispensable guide to the aquatic insects.

Abbreviations: (L), Lancashire; (N), Northumberland; (W) Westmorland.

Odonata: Anisoptera.

Aeschna grandis (L.). Lily Tarn, Loughrigg (W), Aug. 1960; Black Lough (N), Apr.-May 1961; medium size and large larvae, some in final instar. Ref. 3.

A. cyanea (Müll.). Small pond in Gosforth Park (N), Mar.-May 1961, large larvae, some in final instar. Ref. 3.

Odonata: Zygoptera.

Enallagma cyathigerum (Charp.). Lily Tarn (W), Oct. 1960, medium-size larvae. Ref. 3.

Agrion virgo (L.). Cunsey Beck (L), June 1961, adults (Ref. 8); Sept. 1961, medium-size larvae. Ref. 3.

Ephemeroptera.

Ephemera danica Müll. Windermere (L), Mar. 1961, large larvae, some in final instar. Refs. 11, 13.

Ecdyonurus dispar (Curt.). Windermere (L), June 1961, medium-size larvae. Refs. 9, 13.

Plecoptera.

Perla bipunctata Pict. Tributary of Grizedale Beck (L), Aug. 1960, larvae nearly all in 3rd year (Ref. 5); Wooler Water (N), Apr. 1961, 3rd-year larvae in final instar. Ref. 6.

Dinocras cephalotes (Curt.). R. Alwin (N), Feb.-Apr. 1961, 3rd-year larvae. Refs. 5, 6.

Hemiptera-Heteroptera.

Corixa punctata (Illig.). Clay pond, Wray Castle (L), Sept. 1960, adults. Ref. 10.
Notonecta obliqua Gall. Lily Tarn (W), Clay pond (L), Aug.-Sept. 1960, adults. Ref. 10.

Megaloptera.

Sialis lutaria (L). Black Lough (N), Apr.-May 1961, medium size and large larvae. Refs. 4, 7.

Coleoptera.

Dytiscus marginalis L. Several ponds (L) and (N), Apr.-Sept. 1961, adults. Ref. 1.
Clay pond (L), June 1961, small, medium and large larvae (species unidentifiable).

Colymbetes fuscus L. Small pond on Holy Island (N), Apr. 1961, small, medium and large larvae. Ref. 1.

Trichoptera.

Limnephilus stigma Curt. Small pond in Gosforth Park (N), May 1961, large larvae, many in final instar. This species can be identified by characters given in Ref. 16. Identification confirmed by rearing adults. Ref. 15.

Anabolia nervosa Leach. R. Blyth (N), May 1961, large larvae, many in final instar. Identified as for *L. stigma*.

Philopotamus montanus Don. Small beck in Bark House Wood, Cunsey (L), June 1961, large larvae probably about to pupate. Kindly identified by Mr J. Edington.

Phryganea sp. Lily Tarn (W), Aug. 1960, June 1961. Black Lough (N), May 1961, medium and large larvae.

Lepidoptera.

Nymphula nymphæta L. Hodson's Tarn (L), July 1961, medium and large larvae, some probably about to pupate. Refs. 14, 17.

Diptera.

Tipula montium Egg. R. Coquet (N), Feb.–Mar. 1961, medium and large larvae.
Ref. 2. Identification kindly confirmed by Mr A. Brindle.

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