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Characterization of the Extracellular Haemoglobins of *Artemia salina*

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The following factors were measured for extracellular haemoglobins of *Artemia salina*: a minimal molecular weight of globin chain per haem group (based on the iron and haem contents), the absorption coefficients, the absorption spectra of various derivatives and the amino acid compositions. These were compared with those of the haemoglobins of other invertebrates. Three *Artemia* haemoglobins (I, II and III) had similar molecular structures, constructed from two-globin subunits of 122000–130000 mol.wt. Since the minimal mol.wt. was determined to be 18000, this suggests that one globin subunit was bound by seven haem groups, and hence one haemoglobin molecule (240000–260000 mol.wt.) should contain 14 haem groups. A successful identification of this high-molecular-weight subunit required first the denaturation of haemoglobin in 1% sodium dodecyl sulphate before sodium dodecyl sulphate gel electrophoresis. Denaturation by prolonged incubation (12–36h) at room temperature in the presence of 0.1% sodium dodecyl sulphate [Bowen, Moise, Waring & Poon (1976) *Comp. Biochem. Physiol.* **B55**, 99–103] was accompanied by extensive proteolysis, resulting in low recovery of the stainable protein and heterogeneous gel patterns. Regardless of which electrophoretic system was used, the high-molecular-weight subunit was always present provided that 1% sodium dodecyl sulphate was present during denaturation. These results contrast with those obtained by Bowen *et al.* (1976). However, preferential cleavage of the globin subunit (α) seemed to occur *in vitro* when standard conditions were used, producing two specific fragments having mol.wts. of 80000 (β) and 50000 (γ).

Although the extracellular haemoglobins of invertebrates are generally giant molecules having sedimentation coefficients between 33S (molluscs) and 61S (annelids) (Svedberg, 1933; Rossi-Fanelli *et al.*, 1970; Waxman, 1971; Swaney & Klotz, 1971; Shlom & Vinogradov, 1973; David & Daniel, 1974; Wood & Mosby, 1975; Andonian & Vinogradov, 1975; Wood *et al.*, 1976), crustaceans seem to have much smaller haemoglobin molecules (11–17S) (Svedberg & Eriksson-Quensel, 1934; Hoshi *et al.*, 1967; Ar & Schejter, 1970; Waring *et al.*, 1970; Moens & Kondo, 1976*b*; Bowen *et al.*, 1976). The only exception appears to be those of *Chironomus* species, where the extracellular haemoglobins exist as monomers (16000 daltons) and dimers (32000 daltons) (Svedberg & Eriksson-Quensel, 1934; Braun *et al.*, 1968).

We have shown previously that the extracellular haemoglobins of the brine shrimp, *Artemia salina*, contain globin chains having relatively high mol.wts. (50000, 80000 and 122000–130000) (Moens & Kondo, 1976*b*,*c*). Further, since the major polypeptide (mol.wt. 122000–130000) could be cleaved to give two minor polypeptides (mol.wts. 50000 and 80000) *in vitro* the largest polypeptides have been suggested to be the subunit globin chains of *Artemia* haemoglobins (L. Moens & M. Kondo, unpublished work). Bowen *et al.* (1976) suggested that low-mol.wt. polypeptides (13000–17000 daltons) are the subunits of *Artemia* haemoglobins. These subunits were detected by SDS†/polyacrylamide-gel electrophoresis of the denatured haemoglobins in a discontinuous buffer system. Therefore we have re-examined this problem by conducting SDS/polyacrylamide-gel electrophoresis under two conditions. The results indicate that the high-molecular-weight subunits could be detected in both electrophoretic systems, as long as the haemoglobins were denatured in the presence of 1% SDS, but not when 0.1% SDS was used.

Experimental

Preparation of Artemia haemoglobins

Artemia salina was cultivated and crude haemoglobins were prepared as described previously (Moens & Kondo, 1976*a*,*b*). Crude preparations were further purified by several cycles of stepwise and gradient elutions with NaCl in 50mM-Tris/HCl (pH7.5) on DEAE-Sephadex A-50 (2.5cm × 8cm and 2cm × 20cm respectively). Separated haemoglobins I,

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† Abbreviation: SDS, sodium dodecyl sulphate.

II and III were finally chromatographed on Sepharose 6B (1cm×45cm) in 100mM-NaCl/50mM-Tris/HCl (pH7.5), and concentrated by ultrafiltration (Amicon, UM-10 membrane).

Analytical methods

Spectral analysis was done with a Brückl S.E.M. HRS 4001C spectrophotometer for samples dissolved in either 5mM-Tris/glycine (pH8.3) or 25mM-barbital buffer (pH8.6), as indicated in the legends to Figures. Iron and haem were determined by the formation of ferrous *o*-phenanthroline (Cameron, 1965, 1970), and pyridine haemochromogen (Swaney & Klotz, 1971) respectively by using human haemoglobin and crystalline haematin as controls. The iron content was verified by atomic absorption spectroscopy at 248.3 and 372nm with a Perkin–Elmer 503 spectrophotometer.

Absorption coefficients for haemoglobin II were determined with a Zeiss PMQ 3 spectrophotometer. The samples were dialysed for 25h against quartz-distilled water before examination. The dry weight of protein was determined by weighing with a Cahn electrobalance *in vacuo* after the sample had been freeze-dried and dried for 48h at 60°C *in vacuo*.

An apparent molecular weight of *Artemia* haemoglobins and their subunits was estimated by gel filtration in Sepharose 6B (Marrink & Gruber, 1969) and in Sepharose 4B in the presence of 6M-guanidine hydrochloride (Mann & Fish, 1972) respectively. A Sepharose 6B column (1cm×45cm) was equilibrated at 4°C with 10mM-sodium barbital buffer (pH7)/100mM-NaCl and 1.15ml fractions were collected with a flow rate of 7.4ml/h. A Sepharose 4B column (0.8cm×50cm) was equilibrated at 4°C with 50mM-Tris/HCl (pH7.5)/6M-guanidine hydrochloride and 0.43ml fractions were collected with a flow rate of 1.8ml/h for the reduced and alkylated haemoglobin samples.

Polyacrylamide-gel electrophoresis

The method of gel electrophoresis in 7% polyacrylamide gel (0.6cm×10cm) was described before (Moens & Kondo, 1976*b*). The gels were stained with 0.2% benzidine in 0.5% acetic acid and 0.06% H_2O_2 for 30min by the method of Clarke (1964). Benzidine was handled with disposable plastic hand-gloves in a hood.

Continuous polyacrylamide-gel electrophoresis in the presence of 0.1% SDS was carried out in 7.5% gels by the method of Kamen *et al.* (1972). The protein was denatured by heating to 95°C for 3min and then 10–40μg of protein was dissolved in 200μl of 5mM-Tris/glycine (pH7.5)/1% SDS/1% 2-mercaptoethanol/10% (v/v) glycerol/0.01% Bromophenol Blue. After electrophoresis at a current of 8mA per gel, all gels were cut at the bottom edge of the tracking-dye band and stained with 0.1% Coomassie Brilliant Blue/50% methanol/7% acetic acid, followed by destaining in the same solvent without dye.

The method of discontinuous polyacrylamide-gel electrophoresis in the presence of 0.1% SDS (Bowen *et al.*, 1976) was performed in 7.5% gels (0.6cm×10cm) overlaid with 2.5% stacking gels (Waring *et al.*, 1970). Electrophoresis was carried out at 0.1–1mA per gel for 24h.

Protein samples were denatured as described by Bowen *et al.* (1976), as follows. The sample was incubated at room temperature (approx. 20°C) either for 12h in 40mM-Tris/HCl (pH7.5)/1% 2-mercaptoethanol in the presence of 0.1 or 1% SDS (as indicated in the legend to Fig. 3) (procedure 1), or for 36h in 10mM-sodium phosphate buffer (pH7.0)/1% 2-mercaptoethanol in the presence of 0.1 or 1% SDS, followed by heating at 95°C for 60min (procedure 2). In both procedures the samples incubated with 1% SDS were finally dialysed for 12h against the corresponding incubation buffers with 0.1% SDS, 1% 2-mercaptoethanol and 10% glycerol; 10–40μg of protein (as indicated) in 200μl was placed on the gels.

Amino acid analysis

Globin was prepared by the method of Teale (1959) and dialysed against quartz-distilled water, followed by concentration by ultrafiltration. A protein sample was hydrolysed at 110°C in redistilled 6M-HCl, which had been deoxygenated with N_2 gas, in an evacuated tube for 24, 48 or 96h. Amino acid analysis was carried out with a JEOL J.L.C. 6AH automatic amino acid analyser by the method of Spackman *et al.* (1958). Half-cystine was determined as cysteic acid (Hirs, 1956) and tryptophan was measured spectrophotometrically (Goodman & Morton, 1946). The values for threonine and serine were obtained by extrapolation to zero time of hydrolysis, and valine, isoleucine and histidine were calculated from the values of 96h hydrolysis.

Results and Discussion

Induction of haemoglobin de novo in swimming nauplius

The absorption spectra of the partially purified haemoglobin samples of embryos at 0, 5 or 10h, the swimming nauplius and the adult are illustrated in Fig. 1. Only samples from swimming nauplius and adult exhibit a typical spectrum for the presence of haemoglobin, having absorption maxima at 412, 539 and 574min (Table 1). The same haemoglobin samples were examined by electrophoresis in 7% polyacrylamide gels, stained with benzidine/H_2O_2 (Fig.

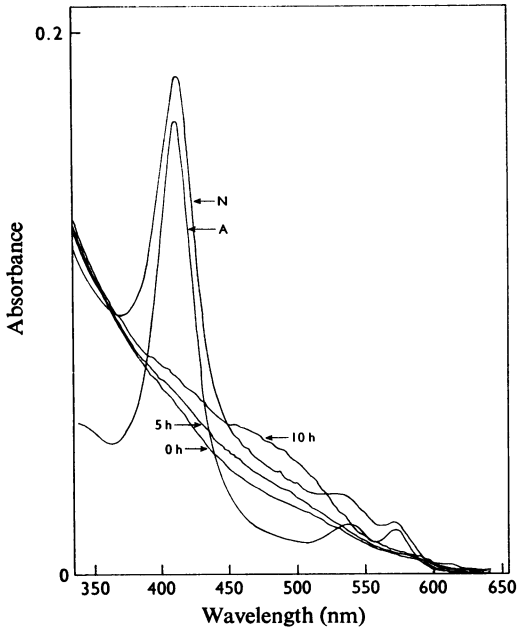

Fig. 1. *Spectral analysis of the partially purified extracts of Artemia salina during development*

Extracts in 5mM-Tris/glycine (pH8.3) were prepared from the animals indicated and the absorption spectrum was measured without purification. A, adult; N, nauplius; 0h, 5h and 10h indicate encysted embryos developed for these hours under standard conditions.

2). The benzidine-positive bands are clearly detected only in nauplius and adult samples, but not in the earlier developmental stages.

Three electrophoretically distinct haemoglobins (I, II and III) could be induced during a life cycle of *Artemia salina* (Moens & Kondo, 1976*b*) and their ontogeny has been studied in detail (Heip *et al.*, 1977; J. Heip, L. Moens & M. Kondo, unpublished work). The first haemoglobin to be induced in the swimming nauplius is haemoglobin II, which is detected within 4h after hatching, and within 24h the second haemoglobin, haemoglobin III, can be identified (Heip *et al.*, 1977).

Spectral analysis

Table 1 summarizes the absorption maxima of three purified *Artemia* haemoglobins and their various derivatives, together with those of human and several known branchiopod haemoglobins. The identical absorption spectra of pyridine haemochromogens obtained from *Artemia* haemoglobins and human haemoglobin A as well as crystalline haematin implies the presence of iron protoporphyrin IX as the prosthetic group of *Artemia* haemoglobins (Paul *et al.*, 1953). Under various conditions these haemoglobins all exhibit similar spectral patterns, the exceptions being recognizable shifts with the reduced haemoglobin of the conchostracan crustacean, *Cyzicus* (Ar & Schejter, 1970).

Absorption coefficients, $A^{1\%}_{1cm}$, of the purified *Artemia* haemoglobin II were determined and compared with those reported for other invertebrate extracellular haemoglobins (Table 2). Our measurements are virtually identical with those reported by Bowen *et al.* (1976).

Minimal molecular weight of Artemia haemoglobin

The minimal mol.wt. per haem group of *Artemia* haemoglobin was calculated from the iron and haem contents (0.299 and 3.60%, w/w, respectively; Table 3), to be 18600 ± 1700 and 17200 ± 1700 respectively. Thus an average mol.wt. per haem group from these values is about 18000, which is in a fair agreement with the independent determination (19000) by Bowen *et al.* (1976). In Table 4 are compared the published values for minimal molecular weights of various extracellular haemoglobins. Most of these minimal mol.wts. are in the range 18000–26000. It has been suggested that in annelid haemoglobins more than one small polypeptide chain shares one haem group (Waxman, 1971, 1975; Shlom & Vinogradov, 1973; Andonian & Vinogradov, 1975; Vinogradov *et al.*, 1975; Wood *et al.*, 1976), whereas in mollusc haemoglobins many haem groups are bound to one high-molecular-weight polypeptide chain (Waxman, 1975; Wood & Mosby, 1975).

In crustacean haemoglobins, we have shown previously that *Artemia* haemoglobins contain three relatively high-molecular-weight polypeptides (Moens & Kondo, 1976*b*,*c*). Moreover, two minor β and γ polypeptides (80000 and 50000 daltons) were shown to be derived from the major α polypeptide (122000 daltons) by a specific proteolytic cleavage produced *in vitro*, and the α polypeptide was proposed to be the real subunit globin chain of *Artemia* haemoglobins (L. Moens & M. Kondo, unpublished work). Thus a native *Artemia* haemoglobin molecule is constructed from two such subunit polypeptide chains. Further, on the basis of the determined molecular weight per haem group (Table 3), we estimate that seven haem groups are bound to one subunit globin chain, making this haemoglobin similar to those found in mollusc haemoglobins (Waxman, 1975).

Bowen *et al.* (1976) reported that they were able to identify, by their discontinuous SDS/polyacrylamide-gel system, two polypeptide chains (bands A and B) having apparent mol.wts. in the range 13000–17000. These were claimed to be the conformational isomers of the same molecular weight. Indeed, the molecular weight of this polypeptide should agree

Table 1. *Absorption characteristics of haemoglobins and their derivatives*

Purified *Artemia* haemoglobins and fresh human haemoglobins dissolved in 25mM-barbital buffer (pH8.6) were used for the spectral measurement of oxyhaemoglobin after proper dilution in the same buffer. Deoxyhaemoglobins were prepared by the addition of solid dithionite immediately before measurement. Carbonmonoxyhaemoglobins were prepared by bubbling CO gas for 25min through a haemoglobin solution. Cyanomethaemoglobins were prepared by the method of Drabkin & Austin (1932), methaemoglobins by oxidation with $NaNO_3$, and pyridine haemochromogens by the method of de Duve (1948), modified by Swaney & Klotz (1971).

	Absorption maxima (nm)									
	Artemia									
Haemoglobin derivative	Hb-I	Hb-II	Hb-III	Human Hb A	*Cyzicus**	*Lepidurus**	*Chirocephalus**	*Moina*†	*Daphnia*‡	*Simocephalus*‡
Oxyhaemoglobin	574	574	574	575	573	576	577	578	577	578
	539	539	538	539	538	540	536	542	543	542
	412	412	412	413	416	—	416	415	—	—
	342	342	342	344	—	—	—	—	—	—
Deoxyhaemoglobin	555	556	556	554	566	559	562	559	—	—
	428	429	428	427	422	—	428	429	—	—
Carbonmonoxy-haemoglobin	567	568	568	568	568	—	—	570	573	574
	536	536	537	536	536	—	—	539	540	539
	417	417	417	418	420	—	—	417	—	—
	342	342	342	342	—	—	—	—	—	—
Cyanomethaemo-globin	536	536	536	538	—	—	—	—	—	—
	417	417	418	417	—	—	—	—	—	—
Methaemoglobin	630	630	630	630	629	630	—	630	—	—
	502	504	502	502	499	500	—	500	—	—
	404	404	405	404	407	—	—	405	—	—
Pyridine haemo-chromogen	554	554	554	554	—	—	—	—	—	—
	523	523	524	522	—	—	—	—	—	—
	474	474	474	474	474	—	—	—	—	—

* From Ar & Schejter (1970).
† From Hoshi *et al.* (1968).
‡ From Hoshi (1957).

perfectly with the estimated minimal mol.wt. per haem group (18000–19000). Therefore, we have systematically re-examined this point by repeating the experiments under both conditions, and using the partially purified *Artemia* haemoglobin II sample. Fig. 3 shows the results obtained when the continuous (Fig. 3*a*) and discontinuous (Fig. 3*b*) electrophoretic systems were used for haemoglobin II. Three treatments were tested: (1) incubation in 40mM-Tris/HCl (pH7.5) for 12h at room temperature (procedure 1 of Bowen *et al.*, 1976); (2) incubation in 10mM-sodium phosphate buffer (pH7.5) for 36h at room temperature, followed by heating at 95°C for 1h (procedure 2 of Bowen *et al.*, 1976); (3) direct heating at 95°C for 3min in the presence of 1% 2-mercaptoethanol and 1% SDS (our normal procedure, based on Kamen *et al.*, 1972). In addition, the SDS concentration was varied (0.1 or 1%) during incubation of the sample.

When samples were incubated in the presence of 1% SDS by either procedure of Bowen *et al.* (1976), three known polypeptides of *Artemia* haemoglobins could be recovered (α, β and γ, Moens & Kondo, 1976*b*,*c*; L. Moens & M. Kondo, unpublished work), regardless of the electrophoretic system used (Fig. 3, gels 2, 4, 8 and 10). Similar results were found even after incubation for 36h and 1h heating at 95°C (Fig. 3, gels 4 and 10), whereas in the presence of 0.1% SDS generally an extremely heterogeneous gel pattern was produced (Fig. 3, gels 1, 3, 7 and 9). Moreover the quantity recovered in the stainable bands on the gels was significantly decreased when the sample was treated with 0.1% SDS (Fig. 3, gels 1, 3, 7 and 9), strongly indicating that extensive proteolytic degradation took place at low concentration of SDS (0.1%), but this was decreased at the high concentration of SDS. In the standard continuous electrophoretic system, in which cytochrome *c* (mol.wt. 13400) and soya-bean trypsin inhibitor (mol.wt. 21500) were reasonably well separated (Fig. 3, gel 6), only a very faint band could be recognized migrating a little faster than the cytochrome *c* marker (Fig. 3, gels 1–5). On the other hand in the discontinuous electrophoretic system, where no separation of these two markers was achieved (Fig. 3, gel 12), because a sharp

pH boundary formed at the electrophoretic front decreased resolution, a distinct band migrating approximately with these markers was observed. This was enhanced in the samples incubated for 36h and heated for 1h (Fig. 3, gels 9 and 10), and might be equivalent to the band observed by Bowen *et al.* (1976). Thus this sharp band seems to occur near the electrophoretic front by collecting a heterogeneous population of polypeptides having a relatively wide range of molecular weights.

In conclusion, our electrophoretic experiments were not able to reproduce the results of Bowen *et al.* (1976), but confirm our earlier observations (Moens & Kondo, 1976*b*,*c*). Moreover, it seems clear that in the absence of proteolytic inhibitors 0.1% SDS is not able to protect *Artemia* haemoglobin from proteolytic hydrolysis during a prolonged incubation at room temperature. In this connexion, Bowen *et al.* (1976) noted that multiple bands of high molecular weights were observed when haemoglobin III was incubated for 36h in high concentrations of SDS

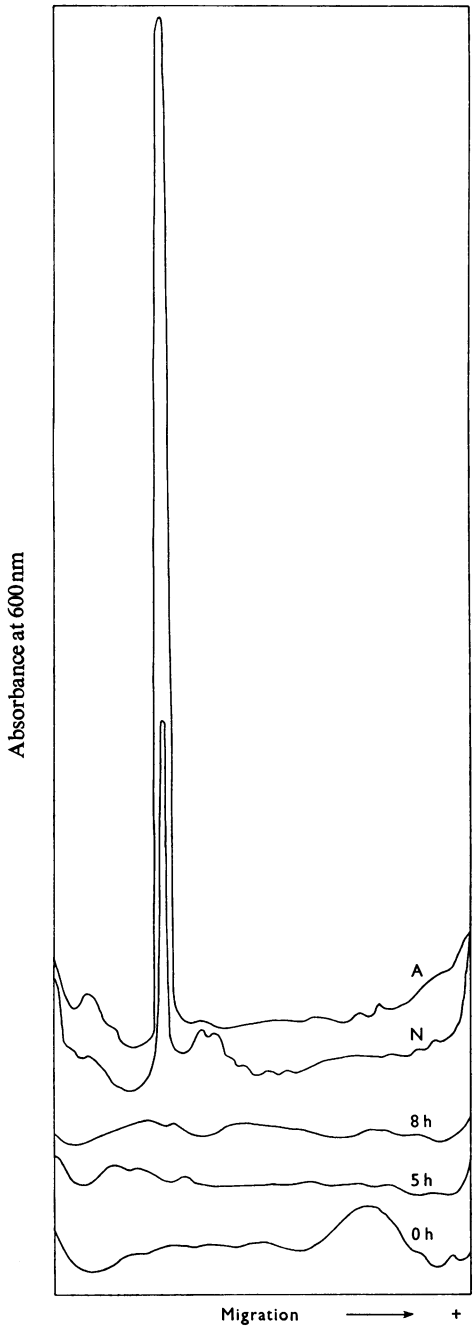

Table 2. *Extinction coefficients of invertebrate extracellular haemoglobins*

Organism	Wavelength (nm)	$A^{1\%}_{1cm}$
Artemia salina	260	15.27
	280	17.09 (17.0)*
	412	47.71 (48.5)
	540	4.87 (5.0)
	575	4.30 (4.5)
Pista pacifica†	280	22
Cirraformia grandis‡	280	22.1
	415	52.2
	539	6.59
	574	6.38
Lumbricus terrestris§	283	22.7
	417	49.0
	542	5.75
Haemopsis sanguisuga‖	280	22.2
Biomphalaria glabrata¶	280	23.8

* From Bowen *et al.* (1976).
† From Terwilliger *et al.* (1975).
‡ From Swaney & Klotz (1971).
§ From Rossi-Fanelli *et al.* (1970).
‖ From Wood *et al.* (1976).
¶ From Figueiredo *et al.* (1973).

Fig. 2. *Benzidine staining of the partially purified extracts in polyacrylamide gels*

The same extracts as those used in Fig. 1 (except that the 10h sample was replaced with an 8h one) were electrophoresed in 7% polyacrylamide gels for 5h at room temperature; 750μg of protein was loaded on gels 0, 5, 8 and N, and 210μg of protein on the gel A. After staining with benzidine/H_2O_2 the gels were scanned at 600nm with a Gilford type 240 spectrophotometer equipped with a linear transport as described in the Experimental section.

Table 3. *Iron and haem contents of Artemia haemoglobin II*

The amount of protein was determined by a Cahn electrobalance as described in the Experimental section. Results of two separate experiments are shown.

Amount (mg)	Iron content (mM)		Haem content (mM)	Minimal mol.wt.	
	Ferrous *o*-phenanthroline*	Atomic absorption†	Pyridine haemochromogen*	Per iron atom	Per haem group
7.377	0.393	0.397	0.426	18700	17300
7.133	0.383	0.389	0.418	18500	17100
Mean				18600	17200

* Each consisted of five separate determinations.
† Each was performed in duplicate.

Table 4. *Minimal molecular weights of extracellular haemoglobins of invertebrates*

Percentages in parentheses are calculated from the reported minimal molecular weights per haem group. Mean values ± S.D. are shown.

Organism	Iron (%, w/w)	Minimal mol.wt.	No. of determinations	Haem (%, w/w)	Minimal mol.wt.	No. of determinations	Reference
Crustaceae							
Artemia salina	0.299	18600±1700	14	3.60	17200±1700	10	Present study
	0.29	19000	—	—	—	—	Bowen *et al.* (1976)
Cyzicus cf. *hierosolymitanus*	0.307	18690	—	(3.41)	18070	—	Ar & Schejter (1970)
Annelida							
Pista pacifica	(0.299)	24400±1800	24	(2.03)	30500±2500	14	Terwilliger *et al.* (1975)
Cirraformia grandis	—	—	—	3.08	20100±1800	—	Swaney & Klotz (1971)
Arenicola cristata	(0.214)	26100±400	3	(2.37)	26100±400	3	Waxman (1971)
		—	—	(2.05)	30100±1000	2	Waxman (1975)
Nereis sp.	—	—	—	(2.35)	26200	1	Waxman (1975)
Myxicola infundibulum	—	—	—	(2.80)	22000	1	Waxman (1975)
Lumbricus terrestris	—	—	—	2.67	23200±300	10	Rossi-Fanelli *et al.* (1970)
	0.221	25400±1100	18	2.78	22200±1100	14	Shlom & Vinogradov (1973)
Limnodrilus gotoi	—	—	—	2.21	28000	—	Yamagishi *et al.* (1966)
Haemopsis sanguisuga	—	—	—	2.49	24800	—	Wood *et al.* (1976)
Dina dubia	0.22	25400±2500	—	2.92	21200±2300	—	Andonian & Vinogradov (1975)
Mollusca							
Astarte sp.	—	—	—	(3.67)	16800	1	Waxman (1975)
Planorbis sp.	—	—	—	(3.28)	18800±800	4	Waxman (1975)
Planorbis corneus	—	—	—	(2.76)	22300±1800	—	Wood & Mosby (1975)
Biomphalaria glabrata	0.315	17700	—	—	—	—	Figueiredo *et al.* (1973)

(0.15–5%), whereas these bands disappeared when it was incubated in the presence of 0.1% SDS and electrophoresed in the continuous buffer system. We interpret this observation as being due to an extensive proteolytic hydrolysis of the globin polypeptides in low concentrations of SDS (0.1%).

Amino acid compositions

All *Artemia* haemoglobins have amino acid compositions similar to that of the conchostracan crustacean, *Cyzicus* cf. *hierosolymitanus* (Ar & Schejter, 1970), having high proportions of aspartic acid, glutamic acid, alanine, valine and leucine (Table

5). The amino acid compositions of *Artemia* haemoglobins I, II and III are virtually identical. The only possible differences are in the proportion of tyrosine and arginine, namely one more tyrosine residue in haemoglobins II and III than haemoglobin I, and one more arginine residue in haemoglobin II than in haemoglobins I and III (Table 5). The amino acid analysis of *Artemia* haemoglobins reported by Bowen *et al.* (1976) is comparable with our analysis, except for the absence of a cysteine residue in their analysis. This very low amount or absence of cysteine in crustacean haemoglobins resembles that found in mollusc extracellular haemoglobins, some annelid cellular haemoglobins and human haemoglobin A (Table 5). On the other hand, the extracellular haemoglobins of annelids generally contain several cysteine residues (Table 5), and disulphide bonds are known to be involved in the molecular arrangement of haemoglobins (Waxman, 1971, 1975; Shlom & Vinogradov, 1973), whereas *Artemia* haemoglobins do not seem to contain any disulphide links (Moens & Kondo, 1976*b*; L. Moens & M. Kondo, unpublished work), resembling the vertebrate haemoglobins.

The formula of Harris & Teller (1973) has been used for the comparison of relatedness among haemoglobins by using the amino acid compositions shown in Table 5. The results are summarized in Table 6. Three *Artemia* haemoglobins were extremely similar to each other, as indicated by the low *D* values: 0.0163 (I/II), 0.0143 (I/III) and 0.0173 (II/III). *Artemia* haemoglobin II appears to be related to the haemoglobin of another crustacean, *Cyzicus* ($D = 0.0631$) and is generally close to the extracellular haemoglobins than to the coelomic haemoglobins of annelids (Table 6). Mollusc haemoglobins appear to be less related to *Artemia* haemoglobin II than to annelid extracellular haemoglobins, although the number of the known amino acid compositions of mollusc haemoglobins is not sufficient for a serious comparison. It should be noted that *D* (0.1650) for the coelomic haemoglobin of the annelid *Glyceria dibranchiata*, whose complete amino acid sequence has been determined (Imamura *et al.*, 1972), is exceptionally high, comparing favourably with that (0.1087) of human haemoglobin A. This is caused by

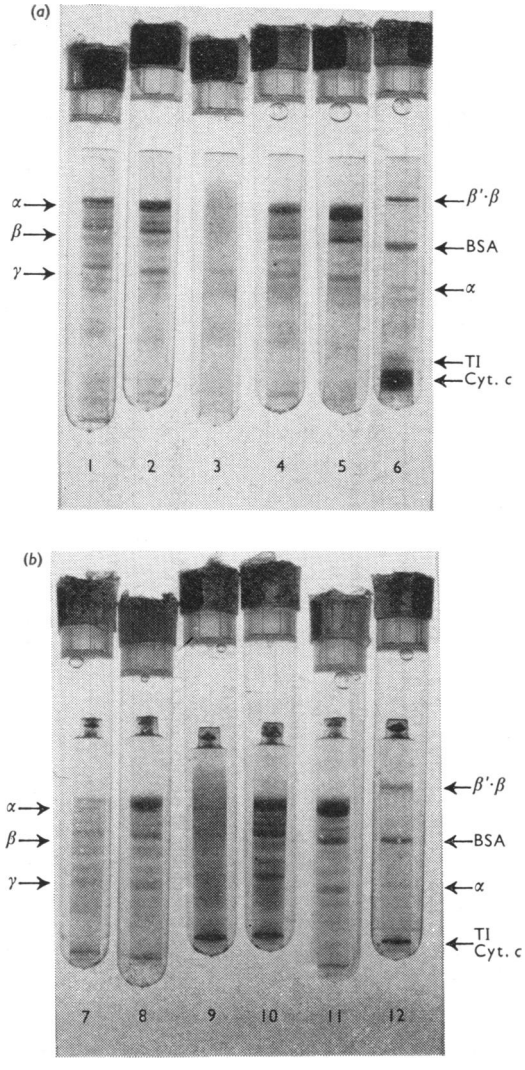

Fig. 3. *Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of Artemia haemoglobin II under various conditions*

All samples were run in 7.5% gels (0.6cm×10cm) in either continuous (*a*) or discontinuous (*b*) buffer systems. The samples for gels 1, 2, 7 and 8 were incubated for 12h in 40mM-Tris/HCl (pH7.5) at room temperature in the presence of either 0.1% (gels 1 and 7) or 1% (gels 2 and 8) SDS; those for the gels 3, 4, 9 and 10 were incubated for 36h at room temperature in the presence of either 0.1% (gels 3 and 9) or 1% (gels 4 and 10) SDS, followed by heating at 95°C for 60min; and those for the gels 5, 6, 11 and 12 were heated at 95°C for 3min in the presence of 1% SDS: 20μg of haemoglobin II was placed on gels 1 and 2, and 40μg on gels 3–5 and 7–11. Gels 6 and 12 contained marker proteins (mol.wts. in parentheses), Combithek (Boehringer Mannheim GmbH; Mannheim, Germany) including the β′ (165000), β (155000) and α (39000) subunits of *Escherichia coli* RNA polymerase and soya-bean trypsin inhibitor (TI; 21500) and horse heart cytochrome *c* (cyt. *c*; 13400). BSA, bovine serum albumin, (68000).

Table 5. *Amino acid composition of Artemia and other haemoglobins*

[a], Brine shrimp (present study); [b], clam shrimp (Ar & Schejter, 1970); [c], lugworm (Waxman, 1971); [d], marine polychaete worm (Swaney & Klotz, 1971); [e], marine polychaete worm (Terwilliger *et al.*, 1975); [f], marine polychaete worm (Garlick & Terwilliger, 1975); [g], earthworm (Shlom & Vinogradov, 1973); [h], horse leech, (Wood *et al.*, 1976); [i], snail (Wood & Mosby, 1975); [j], snail (Almeida & Neves, 1974); [k], bloodworm (Terwilliger *et al.*, 1976); [l], bloodworm (Imamura *et al.*, 1972); [m], marine polychaete worm (Terwilliger & Koppenheffer, 1973); [n], marine polychaete worm (Garlick & Terwilliger, 1974). ND, not determined.

	Content (mol of residue/18 000 g of protein)																
	Extracellular haemoglobin											Coelomic haemoglobins					
Amino acid	*Artemia*[a] I	*Artemia*[a] II	*Artemia*[a] III	*Cyzicus*[b]	*Arenicola*[c]	*Cirratulus formia*[d]	*Pista*[e]	*Thelopus*[f]	*Lumbricus*[g]	*Haemopsis*[h]	*Planorbis*[i]	*Biomphalaria*[j]	*Glyceria robusta*[k]	*Glyceria dibranchiata*[l]	*Pista*[m] 1	*Pista*[m] 2	*Thelopus*[n]	Human haemoglobin Ai
Asp	15.20	15.24	15.25	20.80	19.7	22.3	24.84	22.3	18.0	11.6	20.1	25.3	18.7	14.4	15.7	17.9	16.9	12.3
Thr	7.34	7.10	7.62	8.04	6.6	4.8	8.47	5.9	5.3	3.8	4.0	4.4	8.8	1.38	11.6	9.76	6.8	8.0
Ser	8.42	8.17	8.74	8.16	7.8	8.4	10.49	11.1	7.6	5.9	8.0	11.4	8.6	12.14	9.96	8.95	5.6	8.6
Glu	17.80	17.98	17.88	16.54	18.4	16.0	19.43	19.2	14.3	14.2	13.6	17.8	17.3	12.50	12.1	13.42	14.6	8.6
Pro	5.61	5.46	5.88	4.62	4.6	5.4	6.27	6.6	5.1	4.8	2.9	5.0	6.4	3.85	4.55	7.80	4.5	6.9
Gly	10.67	10.54	11.46	10.73	11.2	12.6	12.29	12.4	8.9	5.8	8.7	9.9	14.3	26.21	10.27	4.76	14.6	10.7
Ala	12.21	11.86	12.04	15.56	13.2	17.4	14.64	12.8	11.1	9.5	12.3	13.9	22.0	34.38	16.0	7.91	21.4	17.7
½Cys	1.14	1.19	1.24	0	4.6	5.1	5.04	4.7	4.9	2.0	0.3	0.5	0.6	0.72	1.61	3.0	1.1	1.0
Val	12.86	12.30	11.56	15.14	12.5	11.0	12.27	11.6	8.1	12.3	0	13.4	11.3	12.50	13.25	10.27	14.6	15.0
Met	2.91	2.84	2.97	1.00	3.9	0	1.39	1.6	0.6	1.5	2.0	1.5	5.2	5.87	3.83	3.30	3.4	1.6
Ile	8.06	8.19	8.52	5.75	5.2	6.4	9.67	8.7	8.0	4.3	5.6	6.0	8.8	9.35	6.49	10.68	3.4	0
Leu	16.26	16.84	16.81	19.01	12.3	14.4	17.53	14.9	14.9	13.6	15.5	17.8	18.2	13.58	12.22	12.41	15.6	17.7
Tyr	2.35	3.46	3.50	2.29	3.2	2.0	3.36	3.5	2.3	3.6	1.4	2.0	4.2	3.76	6.12	3.57	6.8	2.1
Phe	9.19	9.90	9.94	8.47	7.8	9.0	9.64	9.3	6.7	7.8	5.7	7.4	6.4	3.76	9.17	10.17	7.9	7.5
Lys	9.37	10.18	9.37	8.04	7.8	6.2	8.62	8.55	8.2	8.9	8.4	8.9	13.1	12.36	14.2	14.64	16.9	10.7
His	5.16	6.11	5.56	6.20	5.9	2.0	7.87	4.4	8.8	10.2	2.7	2.6	4.6	6.35	3.1	3.0	4.5	8.1
Arg	7.86	9.54	7.64	5.29	9.2	10.0	7.71	8.1	8.8	6.2	6.8	7.9	6.5	3.38	4.89	6.89	3.4	3.1
Trp	1.14	1.31	1.19	1.99	3.2	ND	1.68	2.7	2.4	2.2	2.8	2.5	4.8	2.4	1.0	2.0	2.2	2.1

Table 6. *Relatedness of some of the invertebrate haemoglobins*

The measure of the relatedness was calculated from the amino acid compositions by the formula $D = \sqrt{\sum (X_A - X_B)^2}$ where X_A and X_B were mole fractions of each amino acid of two proteins A and B (Harris & Teller, 1973).

(a) *Artemia* haemoglobins

	$10^2 \times D$		
	I	II	III
I	—	1.633	1.430
II	—	—	1.728

(b) *Artemia* haemoglobin II against other invertebrate haemoglobins

	$10^2 \times D$													
	Extracellular haemoglobins									Coelomic haemoglobins				
	Cyzicus	*Arenicola*	*Cirratulus formia*	*Pista*	*Thelopus*	*Lumbricus*	*Haemopsis*	*Planorbis*	*Biomphalaria*	*Glyceria robusta*	*Glyceria dibranchiata*	*Pista* 1	*Pista* 2	*Thelopus*	Human haemoglobin A
Haemoglobin II	6.31	5.64	8.41	5.55	5.67	5.89	5.98	7.59	8.13	7.19	16.50	8.49	8.12	9.73	10.87

the unusually high contents of glycine (13.6%) and alanine (19%) residues in the monomeric haemoglobin of *G. dibranchiata*, as compared with the general contents of these amino acid residues (6.2–8.9% for glycine and 7.5–12.45% for alanine; Table 5) in other haemoglobins.

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