OBSERVATIONS ON THE RESPIRATORY PHYSIOLOGY AND ON THE HAEMOGLOBIN OF THE POLYCHAETE GENUS *NEPHTHYS*, WITH SPECIAL REFERENCE TO *N. HOMBERGII* (AUD. ET M.-EDW.)

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INTRODUCTION

Of the polychaete family Nephthydidae, comprising the single genus *Nephthys*, several species have invaded the intertidal zone (penetrating to about M.S.L.) and may, as burrowers, be locally abundant on sandy shores. The present paper gives the results of a preliminary investigation of the respiratory arrangements in this genus.

Barcroft & Barcroft (1924) reported on the positions of the spectral absorption bands of haemoglobin from *Nephthys* sp., but remarked that haemoglobin solution was much more readily obtainable from *Arenicola*, a fact which may account for the subsequent neglect of *Nephthys* by workers on invertebrate respiratory pigments. Nevertheless numbers adequate for experimental purposes are readily obtainable and the species mentioned in this paper are easy to keep in aquaria with sand in which they may burrow.

In view of the small amount of published information on this genus, some original observations on the mode of life of *Nephthys* have been made, and a summary of the relevant aspects is given below. These facts are essential to the interpretation of the experimental data.

Nephthys is regarded as an errant polychaete. It has well-developed swimming powers but also a pronounced burrowing habit with which is associated no special morphological adaptation except for the enlarged pharynx which is used to great effect in the process of burrowing. When placed in a sand-floored aquarium it immediately burrows and comes to rest eventually with its anterior end at or a few centimetres below the sand surface. Upward excursions are made when necessary to open up the burrow to the overlying water. I have never seen worms under these conditions leave the sand spontaneously and swim freely in the water.

The walls of the burrow are not at all consolidated with mucus and the whole is very impermanent, the animals moving about from time to time in the sand. They seldom penetrate more than 15 cm. below the surface in the natural habitat.

In all the species studied parts of the body surface are ciliated in the manner described by Coonfield (1931), so as to produce antero-posterior water currents flowing in the space enclosed by the neuro- and notopodia and the lateral wall of the burrow. The segmental gills hang down into this space and there can be little doubt that the current which has been observed in sand-floored aquaria, is of

respiratory significance. For this reason the absence of any mucous lining to the burrows appears to determine the close proximity of the worms to the sand surface (cf. *Nereis diversicolor* and *Nerine cirratulus* whose burrows are substantially lined with mucus and which are commonly found at considerably greater depths than *Nephthys*). The lack of mucous lining also determines the collapse of the mouth of the burrow when the tide ebbs and the isolation of the worm from any oxygen supply outside the sand.

It is these features of the normal habitat which lend interest to the estimation of interstitial oxygen in the sand and to the study of the properties and distribution of the respiratory pigment.

As the unspecialized nature of the adaptation of *Nephthys* spp. to littoral life has become apparent, it has seemed desirable to attempt a comparison of the respiratory arrangements in this genus with those of *Arenicola*. Original observations of the oxygen content of the water remaining in exposed *Arenicola* burrows are given, and the comparison between the genera has been developed in the discussion.

MATERIALS AND METHODS

The animals were collected from three localities on the Yorkshire coast, namely 'The Landing' at Robin Hood's Bay, the north end of Filey Bay and the old harbour at Scarborough. In these places the species found are *Nephthys hombergii*, *N. caeca*, *N. cirrosa* and *N. longosetosa*, identified according to the descriptions of Fauvel (1923). In all the above places *N. hombergii* is the most abundant and has been most closely studied.

Various quantitative techniques have been used. A simple method has been devised for obtaining samples of the interstitial water from the sand, which completely avoids contact between the sample and the atmosphere. Oxygen concentrations of these samples were determined by the micro-Winkler technique of Fox & Wingfield (1938) after pre-treatment of the samples according to Alsterberg's method (1926). The positions of the absorption bands of the haemoglobin spectra were determined by the use of the Hartridge reversion spectroscope, while haemoglobin concentration was determined spectrophotometrically on the pyridine haemochromogen derivative. The method of obtaining the oxygen dissociation curve data was based on those of Hill (1936) and of Allen, Guthe & Wyman (1950). Details of the application of these methods to the present problems are given in the appropriate sections.

OXYGEN CONTENT OF THE INTERSTITIAL WATER

Method

Since *Nephthys* is sealed in the sand when the latter is exposed by the tide, it is obviously very desirable to have a reliable estimate of the oxygen content of the interstitial water. An attempt to measure this factor was made by Borden (1931) in connexion with her work on the respiratory physiology of *Arenicola*. She described a sampling method which involved allowing the interstitial water to drain into a

tube pushed into the sand, and subsequently making an allowance for contamination with the atmosphere after the determination of a control. No steps were taken to deal with reducing substances in the water other than hydrogen sulphide, or to allow for oxygen in the reagents, and her failure to detect oxygen in excess of that likely to have been introduced by contact with the air cannot therefore be regarded as proving that the water was completely oxygen-free. A simple and more direct approach to the sampling problem seemed desirable.

In the present work the samples were drawn into 20 ml. all-glass syringes through a special cannula attached to a standard Luer hypodermic needle mount. The cannula was a 20 cm. length of 15 s.w.g. stainless steel tubing, and attached just below and projecting a few millimetres beyond the tip was a small pointed cap, in shape somewhat like a miniature candle snuffer. This protected the tip of the cannula while it was being pushed into the sand and also carried down immediately in advance of the tip a small pellet of cotton-wool. In this way the cannula could be inserted into the sand without becoming choked and the syringe could be filled with water by gently withdrawing the plunger. At first a small quantity of water was drawn in to displace the air-bubble in the dead space. Next the cannula was re-inserted into the sand to the required depth and the full sample of 10 or 20 ml. taken. The cannula was then removed and the syringe sealed without air bubbles by means of a short piece of rubber tubing and a piece of glass rod.

The first samples taken by this method and analysed by the technique of Fox & Wingfield (1938) gave titres significantly below the value for the blanks (as determined by Krogh's method (1935)), representing the oxygen dissolved in the reagents. From this it was concluded that the sample contained reducing substances which interfered with the reagents, and accordingly Alsterberg's (1926) modification of the Winkler method was adopted. The pre-treatment with Alsterberg's reagents was carried out in the sampling syringe, and a smaller (1·5 ml.) sample was subsequently withdrawn into the syringe-pipette for the normal Fox & Wingfield procedure.

The same techniques have been used to obtain and analyse samples of the water which remains in the burrows of *A. marina* after the sand is exposed by the tide, using polythene instead of steel tubing for the sampling-syringe cannula. Inserted into the tail shaft the polythene cannula readily followed the natural configuration of the burrow, and a 10 ml. water sample was easily withdrawn.

Results

Interstitial water samples from depths of 7·5 and 15 cm. were obtained at Filey, Scarborough and Robin Hood's Bay, in the latter instance at various intervals after the exposure of the sand by the tide. The results of the determination of oxygen content of these samples (Table 1) in each case indicate the presence of a definite though small concentration of oxygen, the values ranging from 0·11 to 0·35 ml. of oxygen per litre. No steady drift of oxygen tension with exposure time is evident; neither is there a significant difference between the samples from depths of 7·5 and 15 cm.

Table 1. *Concentration of dissolved oxygen in the interstitial water of the sand*

Stations: A, 'The Landing', Robin Hood's Bay; B, Filey sands, close to the Brig; C, The Old Harbour, Scarborough. All about M.S.L.)

Station	Hours after exposure	Depth (cm.)	Dissolved oxygen (ml./l.)	Station	Hours after exposure	Depth (cm.)	Dissolved oxygen (ml./l.)
A	$-\frac{1}{4}$	7·5	0·31	B	4	7·5	0·27
	$-\frac{1}{4}$	15·0	0·27		4	7·5	0·33
	0	12·5	0·20		4	15·0	0·22
	0	12·5	0·13		4	15·0	0·24
	0	7·5	0·22				
	0	15·0	0·35				
	3	12·5	0·22	C	4	7·5	0·35
	5	7·5	0·26		4	7·5	0·27
	5	15·0	0·20		4	15·0	0·24
	6	12·5	0·26		4	15·0	0·33
	6	7·5	0·15				
	6	15·0	0·11				

Mean of all samples = 0·25 ml./l., corresponding to 6·7 mm. tension of oxygen at 15° C.

Table 2. *Concentration of dissolved oxygen in the residual water of* Arenicola *burrows*

(All samples from burrows on 'The Landing', Robin Hood's Bay at about M.S.L.)

Hours after exposure	Dissolved oxygen (ml./l.)	Hours after exposure	Dissolved oxygen (ml./l.)
0	1·48	2	0·43
0	0·76		
		$3\frac{1}{2}$	0·67
$\frac{1}{4}$	0·85	$3\frac{1}{2}$	0·49
$\frac{1}{4}$	1·03		
		5	0·43
2	0·45	5	0·56

Mean of the two 5 hr. samples = 0·50 ml./l., corresponding to 13·4 mm. tension of oxygen at 15° C.

The oxygen dissolved in the reagents, which is allowed for by deducting the value of a blank titration, accounts for more than half of the oxygen determined in every case. However, since replicate blank determinations agree within ±4%, the variation in the results is almost certainly real and representative of slight local fluctuations in the density of interstitial micro-organisms. When the tide ebbs *Nephthys* is therefore sealed in a medium in which the oxygen concentration corresponds to a partial pressure of less than 10 mm. of Hg. The mean value of all the determinations corresponds to a partial pressure of 6·7 mm at 15° C. It is interesting to note that the values for the Scarborough samples, where conditions appear to be 'fouler', fall within the range of values for samples from the cleaner sands at Robin Hood's Bay and Filey.*

* The substrate in Scarborough harbour contains much higher proportions of silt and organic matter than those at Robin Hood's Bay or Filey and the water is polluted by fishery activities. The *Nephthys* population consists entirely of *N. hombergii*, whose tolerance of silt has been noted by various authors (e.g. Southward, 1953). The population is also remarkable for a high concentration of coelomic haemoglobin; the absence of other *Nephthys* spp. may be connected with respiratory problems.

The results of oxygen determinations on water samples from exposed *Arenicola* burrows on the 'Landing' at Robin Hood's Bay are shown in Table 2. In spite of the limited number of samples it is clear that even after 5 hr. exposure the water in the burrow may contain twice as much oxygen as the interstitial water in the adjacent sand (cf. Tables 1 and 2).

The partial pressure of oxygen corresponding to the mean of the two 5 hr. samples is 13·4 mm. at 15° C. The two samples taken from separate burrows (as were all the duplicates) at zero time indicate already a sharp fall from full saturation (5·8 ml./l. at 15° C.) and there is a large difference between them. This presumably reflects the respective amounts of activity by the two worms since the occasion on which each performed its last previous bout of respiratory irrigation.

THE RESPIRATORY PIGMENT

Nephthys has a closed blood vascular system containing a red pigment but possesses also a red pigment in the coelomic fluid. Attention was drawn to the latter by the conspicuous pink colour of the everted pharynx of *N. hombergii* from Scarborough. This was due to the inflow into the extrovert during protrusion, of a coelomic fluid which in these worms contained a relatively high concentration of pigment. The presence of the pigment in the coelomic fluid was later confirmed in worms from Robin Hood's Bay and Filey and in all the four species.

Practically all the coelomic fluid can easily be removed by inserting a long fine pipette in the region of the 20th segment between the body wall and the dorsal side of the gut. This can be done without visible injury to the blood vessels. By centrifuging the extracted fluid a perfectly clear pink solution can be obtained, the deposited debris consisting largely of gametes.

The collection of blood is more tedious because of the small size of the blood vessels. The major part of the coelomic fluid having been removed, the worm is pinned out on a dry wax block and the body cavity opened dorsally from head to tail. The remaining coelomic fluid is removed with filter-paper. The dorsal blood vessel and the longitudinal vessels serving the extrovert are then punctured, taking great care not to damage the gut. The animal is set on one side for 10 min. or so, during which time the blood gently oozes into the body cavity from which it can be collected with a fine pipette. This technique yields but a fraction of the total blood, in one case 0·35 ml. from thirty-two *N. hombergii* aggregating 49·3 g. The purity of blood samples obtained in this manner may be questioned, but provided the body cavity is adequately dried with filter-paper after opening the worm and the gut wall is not perforated, the degree of contamination with other fluids is very small and any solid material collected can be removed by centrifuging.

Microscopic examination of fresh blood and coelomic fluid and of smear preparations failed to show any pigmented corpuscles, although there was often an abundance of non-pigmented cells in the coelomic fluid. There was no separation of 'plasma' and pigment on standing nor could the pigment in either case be concentrated by centrifuging the fresh fluid at 5000 r.p.m. It was therefore concluded that it is in free solution in both the coelomic fluid and the blood.

Both the blood and the coelomic fluid from *N. hombergii* and *N. caeca* change to a more bluish hue on deoxygenation. In each case the colour reverts to the original on shaking with air. Spectroscopic examination of both fluids shows: (*a*) a pair of fairly sharp absorption bands in the yellow-green region in the oxygenated state; (*b*) a single diffuse band of intermediate position in the de-oxygenated state. Both pigments are therefore characterized as haemoglobins. The positions of the absorption bands of the oxyhaemoglobins of both blood and coelomic fluid from *N. hombergii* and *N. caeca* have been determined with the Hartridge reversion spectroscope. In each case the readings were compared with alternate readings on sheep haemoglobin of similar dilution. The mean wave-length values obtained are set out in Table 3.

Table 3. *Positions of spectral absorption bands of* Nephthys *oxyhaemoglobins*

(Determined with the Hartridge reversion spectroscope —Å. units)

Species	Vascular		Coelomic	
	α	β	α	β
N. hombergii	5751 ± 3	5387 ± 5	5750 ± 3	5389 ± 8
N. caeca	5755 ± 3	5391 ± 7	*5757 ± 5	5396 ± 6
Sheep	5764 ± 3	5402 ± 3		

* This figure agrees with that given by Barcroft & Barcroft (1924) for coelomic haemoglobin of *Nephthys* sp.

It is apparent that the α-bands of both the vascular and coelomic haemoglobins are shifted towards the blue end compared with sheep haemoglobin, and thus resemble the α-band of *Arenicola* oxyhaemoglobin (5746 Å., Barcroft & Barcroft, 1924), although the shift in the case of *Nephthys* haemoglobin is not so great. The differences between vascular and coelomic haemoglobins in each species of *Nephthys* and the differences between the species are not significant.

A number of spectrophotometric determinations of coelomic haemoglobin have been made using the pyridine haemochromogen derivative. Coelomic fluid from a number of worms was pooled until a large enough sample was obtained to make a single determination. Between six and twelve worms were needed to give 0·5–1·0 ml. of coelomic fluid after centrifuging. The volume of clear solution having been carefully measured it was diluted to exactly 5 ml. with distilled water and poured into a centrifuge tube. To this 0·5 ml. of normal sodium hydroxide was added, and the mixture was then heated in a water-bath for 3 or 4 min., to denature the globin and oxidize the haem. After a thorough shaking, 1 ml. of pyridine was added and the contents of the tube shaken again. The solution was then centrifuged until a perfectly clear supernatant fluid could be decanted. A trace of pure sodium hydrosulphite was finally added to reduce the haematin and bring about the formation of the pyridine haemochromogen. The second centrifuging was necessary because the sodium hydroxide brought down a flocculent white precipitate. Since some of the haematin might be adsorbed on to this precipitate, the clear haemochromogen solution was decanted and the precipitate resuspended in distilled water

with a little sodium hydroxide, pyridine and a trace of hydrosulphite. When this suspension was examined with a hand spectroscope no absorption bands were seen, so it was concluded that all the haem from the original sample of coelomic fluid was present in the decanted supernatant fluid in the form of pyridine haemochromogen.

A figure representing the concentration of pyridine haemochromogen and hence the concentration of haemoglobin was obtained by measuring the light absorption of the solution in a 1 cm. cell in the Hilger Biochem Absorptiometer. The absorption was measured over the wave-length range transmitted by Chance glass filters OG 1 and OY 2 which give a maximum transmission in the region of 550–555 mμ, corresponding to the principal absorption band of the haemochromogen. Absolute values for haemoglobin concentration expressed in terms of oxygen capacity (μl. per ml.) were obtained by reference to a calibration curve. This curve was made by plotting the measured light absorption of haemochromogens prepared from known dilutions of sheep haemoglobin whose oxygen capacity had been determined with the Van Slyke apparatus. Knowing the degree of dilution of the original coelomic fluid in preparing the haemochromogen, the original oxygen capacity of the coelomic fluid could be calculated. From the results of these determinations, (Table 4), it appears that the concentration of coelomic haemoglobin in the Scarborough population is some $2\frac{1}{2}$ times that of the pigment in the Robin Hood's Bay worms. There is a considerable variation between specimens in each population, which is unfortunately masked to some extent by the need to pool the coelomic fluid from a number of worms in order to get a determination.

Table 4. *Oxygen capacity of coelomic haemoglobin solution from* Nephthys hombergii

(Pooled samples from six to twelve worms)

Locality	O_2 capacity (μl./ml. of fresh coelomic fluid)					Mean
'The Landing' R.H.B.	1·66	1·60	1·26	2·78	2·06	1·88
Scarborough harbour	—	3·40	4·98	6·16	—	4·85

Table 5. *Oxygen-combining potential of coelomic fluid per gram (wet weight) of worm*—Nephthys hombergii

Locality	Wt. of worm (g.)	Vol. coel. fl. (ml.)	Total O_2 (μl.)	O_2 (μl.) per g.
R.H.B.	21·6	3·3	6·6	0·31
Scarborough	27·5	4·0	17·9	0·65

In a number of cases worms were weighed (after drying on filter-paper) before the coelomic fluid was removed. It is thus possible to make a few rough calculations of the oxygen-combining potential of the coelomic fluid per g. of worm. These data grouped according to the locality from which the worms were taken, are shown in Table 5.

An attempt has also been made to estimate the total haemoglobin content of *N. hombergii* from Robin Hood's Bay. Worms were cut into small pieces with scissors and ground in a mortar with clean sand and a little sea water. The total contents of the mortar were then transferred to a tube and centrifuged at about 5000 r.p.m. for 20 min. The supernatant fluid was not quite clear, but it was decanted and treated with sodium hydroxide and pyridine. In three cases it was possible to get perfectly clear solutions after the final centrifuging, and in each of these cases a little haemochromogen could be detected with the hand spectroscope on resuspending the solid material from the bottom of the centrifuge tube. However, when a final clear solution was obtained it was decanted, its volume measured, some hydrosulphite added and the light absorption measured. Having read off the oxygen capacity of the extract from the calibration curve and knowing its total volume, the total oxygen-combining potential of the whole extract could be calculated. In Table 6 this is related to the wet weight of the worms from which the extract was made. These figures are minimal estimates of total haemoglobin since some of the pigment is lost in each of the two centrifuging processes. However, it is unlikely that the true values are more than 50% higher.

Table 6. *Oxygen-combining potential of whole worm extracts of* Nephthys hombergii—*all from Robin Hood's Bay*

Wt. of worm (g.)	Vol. extract (ml.)	O_2 capacity (μl./ml.)	Total O_2 (μl.)	O_2 (μl.) per g. of worm
2·7	5·5	2·04	11·2	4·1
2·9	11·6	0·76	8·8	3·0
1·7	5·6	0·78	4·4	2·6

The oxygen consumptions of a number of *N. hombergii* have been determined at 15° C. by means of a multiple dropping-mercury electrode. (The design and use of this instrument will form the subject of a separate paper.) Worms were enclosed singly in small glass bottles containing sea water and allowed to deplete the dissolved oxygen. The oxygen concentration in the bottles was determined at half-hourly intervals over a period of 4–5 hr. It was then possible to calculate the oxygen consumption of individual worms at a number of different oxygen concentrations. As the oxygen concentration of the medium fell from 4·5 to 1·25 ml./l. the mean oxygen consumption fell from 80 μl./g. (wet weight)/hr. to 20 μl./g./hr.

From the various groups of data presented above it is now possible to make a very rough estimate of the value of the combined vascular and coelomic haemoglobins in providing a potential oxygen-store to meet the needs of the worm at times of oxygen lack. Assuming the lower level of metabolism quoted above, it will be seen that the overall oxygen-combining potential (Table 6) of the order of 2·5–4·0 μl. of oxygen per g. of worm will not meet the needs of the animal for more than 7–12 min. The contribution of the coelomic haemoglobin in the case of the Robin Hood's Bay worms will suffice for about 1 min., while in the case of the Scarborough worms this figure may be increased to about 2 min. (see Table 5).

Whatever role haemoglobin may play in the life of *N. hombergii* it is quite clear

from the above considerations alone that it cannot usefully act as an oxygen store in the sense that it can materially help the animal over the period of exposure by the tide. Neither can the superior coelomic haemoglobin concentration in the Scarborough population be concerned with providing additional oxygen storage capacity.

THE OXYGEN DISSOCIATION CURVES OF *NEPHTHYS* HAEMOGLOBINS

Method

The data for the construction of the oxygen dissociation curves of coelomic and vascular haemoglobin from *N. hombergii*, were obtained by the use of Hill's method (1936). In the case of the vascular haemoglobin, which was available in much smaller quantities of an initially more concentrated solution, Hill's tonometer was replaced by one of the form described by Allen *et al.* (1950), and the method of adding oxygen to the system was changed accordingly.

Freshly collected coelomic fluid and blood, usually obtained from the same batch of worms, were diluted fourfold with M/20 phosphate buffer mixture to give pH 7·4, and then centrifuged. The perfectly clear haemoglobin solutions were decanted and either used immediately or stored in the deoxygenated state in contact with pure nitrogen at 4° C. No solutions older than 4 days were used, and at this age repeat determinations gave the same results as did the initial ones. Two samples, one of coelomic and one of vascular haemoglobin, were rebuffered after a set of measurements at pH 7·4, by adding extra potassium dihydrogen phosphate to give pH 7·0. All determinations were carried out at 15° C.

Because of the limited amounts of *Nephthys* pigment available sheep haemoglobin solution was used in the comparison cups of Hill's apparatus. The absorption spectra of sheep and *Nephthys* haemoglobin solutions of equal concentration are sufficiently alike for matching to be carried out satisfactorily. The oxygen capacity of the sheep haemoglobin solutions was determined with the Van Slyke apparatus and provided a standard from which the oxygen capacities of the *Nephthys* samples could be derived. Hill's tonometer was modified slightly by extending the bottom of the Thunberg tube so that it had the form of an inverted T; this increased the thickness of the solution and made it easier to work with the rather dilute coelomic haemoglobin solutions.

Results

The percentage saturations and the calculated equilibrium oxygen tensions for both coelomic and vascular haemoglobin samples are set out in Table 7. The dissociation curves constructed from these data are shown in Fig. 1.

The first point of interest about the dissociation curves is the absence of a point of inflexion which characterizes the majority of dissociation curves which have been determined. If each set of data is plotted in the form log HbO_2/Hb against log pO_2 the result should be a straight line whose slope represents the value of 'n' in Hill's equation. The data for the coelomic samples give, on this basis, a very close approximation to a straight line of slope = 1; i.e. the coelomic dissociation curves appear to be rectangular hyperbolae (the form of curve given by Hill's equation when 'n' = 1). The data for the vascular haemoglobin samples give a pair of curves

which depart from slope = 1 at the higher oxygen tensions and in fact give a much better fit to a line representing 'n' = 1·2.

Table 7. *The dissociation of* Nephthys *oxyhaemoglobins*

(Percentage saturations and corresponding equilibrium oxygen tensions (in mm. of Hg) of samples of vascular and coelomic haemoglobin from *Nephthys hombergii* at pH 7·4 and 7·0 and at 15° C.)

Pigment	pH	Dissociation equilibrium data							
Coelomic	7·4	% sat.	18·0	27·5	33·5	45·0	52·5	62·5	78·5	90·0
		pO_2	1·84	3·25	4·62	6·12	8·45	11·9	17·5	29·8
		% sat.	16·5	25·0	42·5	62·0	77·0			
		pO_2	1·90	3·40	5·46	9·88	23·0			
		% sat.	12·5	20·0	30·5	40·0	50·0			
		pO_2	0·84	1·96	3·46	5·65	11·8			
		% sat.	15·0	27·0	40·0	60·0	75·0			
		pO_2	1·15	2·82	5·54	8·90	15·8			
	7·0	% sat.	17·5	30·5	42·5	54·0	71·5	86·0		
		pO_2	1·11	2·26	3·85	6·31	10·5	19·7		
Vascular	7·4	% sat.	30·0	38·0	52·5	59·5	66·0			
		pO_2	2·80	3·97	5·12	6·30	8·63			
		% sat.	17·5	25·0	37·5	44·0	50·0	58·5		
		pO_2	1·17	2·35	3·53	4·71	5·89	7·06		
		% sat.	25·0	47·5	60·0	70·0	80·0			
		pO_2	2·31	4·62	6·94	9·26	11·6			
	7·0	% sat.	12·5	22·5	40·0	55·5	65·0	75·0		
		pO_2	1·18	2·37	4·74	7·11	9·48	11·9		

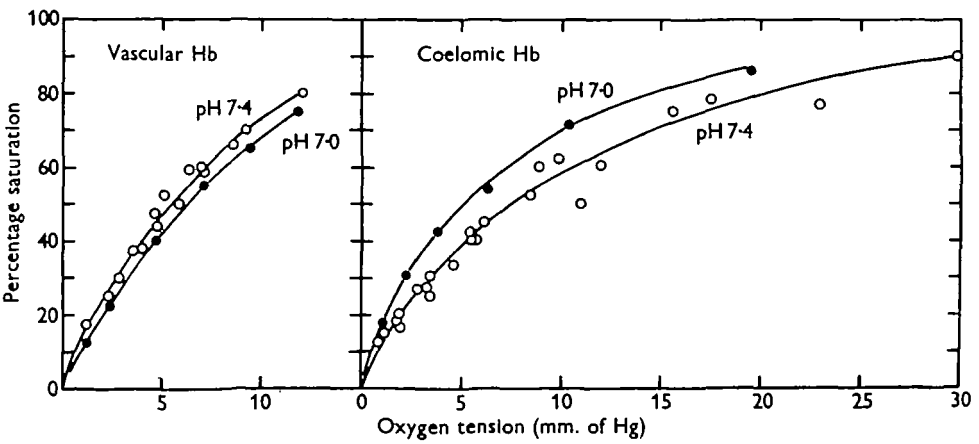

Fig. 1. The oxygen dissociation curves of the vascular and coelomic haemoglobins of *Nephthys hombergii* at pH 7·4 and 7·0 and at 15° C.

It is possible that both the shapes of the dissociation curves and the oxygen affinities of the pigments (relative positions of the curves along the abscissa) may have been affected by diluting the original solutions. Hill & Wolvekamp (1936), who studied the dissociation equilibria of a number of mammalian haemoglobin solutions, found that at dilutions of 1 in 200 all the curves were markedly shifted to the left (oxygen affinity increased) compared with whole blood. In these cases

they were studying in solution pigments whose normal sites are intracellular; the displacement of the curves of dilute suspensions of intact corpuscles was less marked. On the other hand, comparison of the dissociation curves of *Arenicola* haemoglobin obtained by Barcroft & Barcroft (1924), using very dilute blood, and by Wolvekamp & Vreede (1941), using whole blood, clearly indicates that neither the shape of the curve nor the oxygen affinity of this pigment is influenced by dilution. It therefore seems unlikely that in the present examples the moderate dilution (1 in 4) would have appreciably altered the positions of the curves or their shapes. However, it should be borne in mind that the true dissociation curves of *Nephthys* coelomic and vascular haemoglobins may be slightly to the right of those shown.

Although both vascular and coelomic haemoglobin dissociation curves at pH 7·0 are based on a small number of points, there appears in each case to be a small but significant Bohr effect. It is remarkable, however, that in the case of the vascular pigment the effect of increased acidity is to shift the curve to the right in the usual manner, while for the coelomic pigment the effect is reversed. Reversed Bohr effects are very unusual among haemoglobins at least above pH 6·5; the blood of the tadpoles of *Rana catesbiana* (McCutcheon, 1936) and the perenteric haemoglobin of *Ascaris lumbricoides* (Davenport, 1949) are the only examples I have come across in the literature. As a result of this curious situation the oxygen affinities of the two *Nephthys* pigments are comparable at pH 7·0, but at pH 7·4 the vascular pigment has a substantially higher oxygen affinity than the coelomic haemoglobin, the oxygen tensions for 50% saturation being 5·5 and 7·5 mm. respectively.

DISCUSSION

It appears from my observations on the mode of life of *Nephthys* that at least in the littoral species here considered, the greater part of the animal's life is spent below the surface of the sand. The burrow which is of a very impermanent nature can be irrigated with well-aerated sea water while the sand is covered by the sea. But when the sand is exposed to the air the mouth of the burrow collapses and the worm is sealed in. The dissolved oxygen in the interstitial water of the sand corresponds to a tension of about 7 mm. of Hg throughout the period of exposure. It is clear that intertidal species of *Nephthys* are regularly exposed to conditions of poor oxygen supply, and that the respiratory arrangements may be of considerable interest.

The four species of *Nephthys* which have been examined have haemoglobin in free solution in both the blood and the coelomic fluid.* It might be suggested that the freely dissolved haemoglobin in the coelom of *Nephthys* has simply leaked out of the vascular system. It should be emphasized, however, that its occurrence here is a constant feature of the populations examined on the Yorkshire coast. Further, the coelomic haemoglobin differs from the vascular pigment in having (*a*) a reversed

* The existence of a respiratory pigment in both the blood and the coelomic fluid is rare (Romieu, 1923, mentions only *Terebella lapidaria* and *Travisia forbesii*), but an extracellular coelomic pigment appears to be unique. This is neatly correlated with the presence in *Nephthys* of protonephridia and blind coelomostomes (Goodrich, 1945).

Bohr effect, and (*b*) at pH 7·4, which probably represents the *in vivo* condition, a substantially lower oxygen affinity. The oxygen affinity difference is not likely to be due to the difference in haemoglobin concentration between the blood and the coelomic fluid, because in haemoglobins which do show dilution effects it is the stronger solutions which have the lower oxygen affinities (Hill & Wolvekamp, 1936). Thus the evidence at present available indicates that the coelomic and vascular haemoglobin molecules of *Nephthys* are different.

At present no suggestion can be made regarding the possible significance of the difference between the oxygen affinities of the two pigments. In so far as they have any contact with each other there will be a tendency for oxygen to be transferred from the coelomic fluid to the blood if they are at the same oxygen tension. The relationships between vertebrate haemoglobin and myoglobin and between the haemoglobins of foetal and maternal blood immediately spring to mind, but it would be idle to speculate about the present difference in oxygen affinity and in the Bohr effect, in the absence of detailed knowledge of the anatomy of the vascular system in *Nephthys*. Neither is it possible, at present, to make any suggestion regarding the difference in concentration of haemoglobin in the coelomic fluids of the Scarborough and Robin Hood's Bay populations of *N. hombergii*. Studies of the respective environmental conditions are being pursued.

About the physiological significance of the general shape and position of the *Nephthys* dissociation curve some tentative suggestions can be offered. In Fig. 2 are shown the dissociation curves of the vascular and coelomic haemoglobins of *Nephthys* at pH 7·4 and 15° C. and the dissociation curve of the haemoglobin of *Arenicola marina* at pH 7·5 and 19° C. (from Wolvekamp & Vreede, 1941). At 15° C. the *Arenicola* curve would lie a little further to the left. Also indicated in Fig. 2 (by short vertical lines above the abscissa) are the levels of oxygen tension which have been found in the interstitial water of the sand and in the residual water of *Arenicola* burrows respectively (calculated for 15° C.). Let it be assumed that there will be an oxygen gradient of about 10 mm. pressure between the external medium and the arterial blood. (Fox (1945) found gradients of this order in *Chironomus* larvae and in *Tubifex* at limiting tensions of oxygen uptake, though in a form like *Arenicola* with well-developed gills the gradient may be rather less.) Then it is clear that *Arenicola* blood can be almost fully saturated with oxygen from the residual water in the burrow even after 5 hr. exposure. On the other hand, the only oxygen available to *Nephthys* during the exposure period (that in the interstitial water) is at such a low tension as to be virtually unusable. In these conditions the very steep dissociation curve and high oxygen affinity of the *Arenicola* haemoglobin may be seen as an adaptation which enables the animal to survive the exposure period with the minimum of anaerobic metabolism. Whereas in *Nephthys*, metabolism during the exposure period will of necessity be predominantly anaerobic and there will be no selection pressure leading to the evolution of a pigment with a very steep dissociation curve.

There are other animals which are from time to time exposed to conditions of complete or almost complete oxygen lack and which yet have pigments of very high

oxygen affinity. One example is to be found in larvae of the *Chironomus plumosus* group. Estimates of the oxygen tension for half-saturation in this species are 0·2 mm. (Leitch, 1916) and 0·6 mm. (Fox, 1945), while the pigment remains partly oxygenated *in vivo* down to 13 mm. external oxygen tension (Fox, 1945). Ewer (1942) recorded dissolved oxygen concentrations down to 0·3 ml./litre in ponds in which numerous chironomids were to be found; this corresponds to an oxygen tension of about 7 mm. at 20° C. and the haemoglobin of these animals must have been permanently deoxygenated so long as the oxygen tension in the water remained at this low level. Walshe (1950) has shown that the function of haemoglobin in

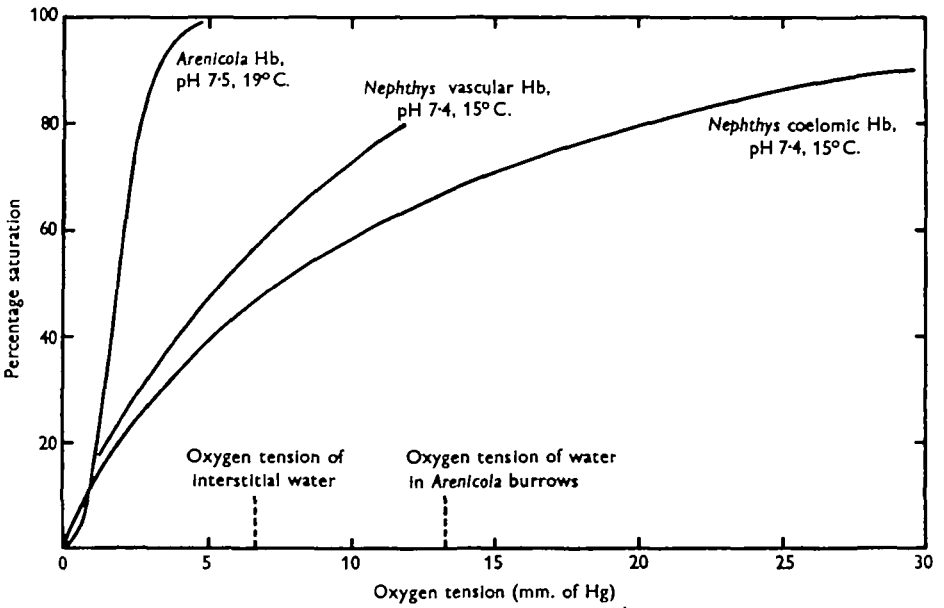

Fig. 2. Comparison of oxygen dissociation curves of the vascular and coelomic haemoglobins of *N. hombergii* at pH 7·4 and 15° C. and of the haemoglobin of *Arenicola marina* at pH 7·5 and 19° C. (*Arenicola* curve from Wolvekamp & Vreede, 1941). The levels of interstitial oxygen tension and of oxygen tension in the residual water of exposed *Arenicola* burrows are indicated by vertical lines above the abscissa.

Chironomus plumosus is to facilitate (*a*) feeding and respiratory irrigation of the burrow during periods of gradual oxygen depletion, and (*b*) recovery, from completely anaerobic metabolism, under conditions of partial oxygen-lack (above 7 % air saturation, i.e. about 11 mm. oxygen tension). Thus for an animal exposed to gradual changes from oxygen sufficiency to complete oxygen lack and vice versa, there are periods (often very extended ones in fresh-water habitats) when oxygen transport at low tensions is both possible and desirable. A respiratory pigment with a steep dissociation curve and high oxygen affinity is then obviously advantageous. On the sea shore an unspecialized burrower like *Nephthys* never has the opportunity for oxygen transport at low tensions because the tension of the available oxygen changes too rapidly from an adequate to a functionally unusable level and vice versa.

Arenicola, on the other hand, with its specialized mode of life can probably obtain oxygen throughout the exposure period by virtue of the conditions in its consolidated burrow and of the adaptation of its respiratory pigment.

The idea of a storage function, originally postulated for *Arenicola* haemoglobin by Barcroft & Barcroft (1924), has been criticized by a number of workers in more recent years (see especially Wolvekamp & Vreede, 1941) on the grounds of the inadequacy of a store calculated to last about an hour. This is a very pressing argument when it is realized that *Arenicola* is often to be found almost as high on the shore as H.W.N.T. (Brady, 1943; Watkin, 1942), and that in this position it will be exposed for over 9 hr. Hecht (quoted by van Dam, 1938) found *Arenicola* in places which were only covered by spring tides. The possibility envisaged in the above speculations, of oxygen transport at low tensions during the period of exposure, offers a satisfactory alternative to the storage hypothesis. The attribution of a storage function to the haemoglobin of *Nephthys* is ruled out by the calculation (see above) that the total combined oxygen in this case would meet the animal's needs for a period of the order of only 10 min.

This speculation on the difference in respiratory arrangements between *Nephthys* and *Arenicola* suggests a number of directions in which further experimental work is needed to test the hypothesis. The use of the CO-method, notably developed by Prof. H. Munro Fox and his school, should confirm (or otherwise) the idea of potential low or high tension oxygen transport systems* in *Arenicola* and *Nephthys* respectively. It is desirable to have determinations of actual oxygen gradients across the body wall in each case under various external oxygen conditions. The need for a low tension oxygen transport system must be further examined in the light of the observations by Wells (1949) of intermittent aerial respiration by *Arenicola* in glass U-tubes containing 'stagnant' water. It would also be most interesting to have an estimate of the oxygen debt incurred in individuals of both species after a given period of exposure in the natural habitat. Experiments are being planned to throw light on these points. If the hypothesis is borne out by further experiments it would appear that despite the similarity of habitat, *Nephthys* and *Arenicola* represent widely different respiratory types, the former unspecialized and the latter highly specialized in its adaptation to littoral life.

SUMMARY

1. Littoral representatives of the genus *Nephthys* are described as burrowing forms, able to irrigate their burrows with well-aerated sea water except when the sand is exposed by the tide. Then they are sealed in and have no access to oxygen outside the sand.

2. The concentration of dissolved oxygen in the sand water corresponds to a tension of about 7 mm. of Hg compared with a value about twice as great in the residual water in *Arenicola* burrows.

* Cf. the work on the respiratory function of chlorocruorin (Ewer & Fox, 1940), the dissociation curve (Fox, 1926, 1932) and the respiratory behaviour (Wells, 1951) of *Sabella pavonina*. This is a case where all three approaches indicate a high-tension oxygen transport system.

3. Extracellular pigments in the blood and coelomic fluid of *Nephthys* spp. are characterized as haemoglobins. The quantity of these pigments is shown to be inadequate as an oxygen store for the exposure period.

4. Dissociation curves for both pigments from *N. hombergii* are found to be approximately hyperbolic and the oxygen affinities relatively low.

5. The significance of the difference in oxygen affinity and the direction of the Bohr effect, between the vascular and coelomic pigments cannot yet be evaluated.

6. It is suggested that the *Nephthys* pigments are unspecialized and may function as a high-tension oxygen transport system only when the sand is covered by the sea. This is contrasted with the possibility of low-tension oxygen transport by the haemoglobin of *Arenicola*.

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