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THE FUNCTIONAL SIGNIFICANCE OF THE HAEMOGLOBIN IN A MARINE NEMATODE, *ENOPLUS BREVIS* (BASTIAN)

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SUMMARY

An animal chamber and a simple microspectrophotometer for investigating the *in vivo* oxygenation of the haemoglobin of *E. brevis* are described.

The *in vivo* absorption peaks of this haemoglobin occur at similar wavelengths to those of other nematodes. Mean values, given with their corresponding standard errors, occur at 577·6 ± 0·6 nm, 543·6 ± 0·5 nm and 421·7 ± 1·9 nm for oxyhaemoglobin, and 555·2 ± 0·9 nm and 432·2 ± 1·3 nm for the deoxygenated pigment.

The percentage of oxyhaemoglobin in the pharynx of *E. brevis* decreased at external oxygen tensions of less than 20 Torr, and the pigment was completely deoxygenated at 5 Torr. Stimulation of individuals in aerated sea water for 1–2 min caused a partial deoxygenation of the haemoglobin; the pigment reloaded soon after this period of increased activity had ended. The functional significance of the haemoglobin of *E. brevis* is discussed.

INTRODUCTION

The tissue haemoglobins of nematodes are widely believed to have a respiratory function, but the exact nature of their role has not been clearly demonstrated for any species. However, the haemoglobins present in the perienteric fluid of some nematodes may not function as respiratory pigments; for example, that of *Ascaris lumbricoides* apparently acts as a haematin source, particularly for incorporation into the gametes (Smith & Lee, 1963).

Smith (1969) considered that nematode tissue haemoglobins were analogous to mammalian myoglobin. Nematode haemoglobins may facilitate oxygen diffusion (Lee & Smith, 1965; Ellenby & Smith, 1966; Smith, 1969; Jones, 1972) in a similar way to that described *in vitro* for certain mammalian haemoglobins and myoglobins (Wittenberg, 1959; Scholander, 1960; Hemmingsen, 1963). However, Wittenberg (1966) suggested that facilitated diffusion occurred in those haemoglobins with a rapid rate of oxygen dissociation, and showed that *Ascaris* body wall haemoglobin, with its known slow rate of oxygen dissociation (Gibson & Smith, 1965), did not enhance oxygen diffusion. Nematode tissue haemoglobins could act as a short-term oxygen store, but this is considered to be a minor function of most invertebrate haemoglobins (Jones, 1972). At low oxygen tensions the haemoglobins of *A. lumbricoides*

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(Davenport, 1949), *Nippostrongylus brasiliensis* (= *muris*) (Rogers, 1949), and *E. brevis* (Ellenby & Smith, 1966) do unload *in vivo*. However, this is not a clear indication of a significant function as an oxygen store, because a partial deoxygenation may also occur during facilitation of oxygen by haemoglobin (Wittenberg, 1970).

The marine nematode *E. brevis* used in this work is one of only two free-living nematodes known to possess haemoglobin (Ellenby & Smith, 1966). The pigment is also present in the musculature of the tail region of *E. communis* males, but is found in much higher concentration in *E. brevis* in this region, and in the pharyngeal musculature and hypodermal cords of both sexes. *E. brevis* occurs in a marine mud of a lower and more variable oxygen availability than that experienced by *E. communis* in its habitat amongst *Mytilus* shells on rocky shores. Oxygen tension has a similar effect on the oxygen consumption of both species, and their respiration rates are greatly reduced by low oxygen concentrations (Atkinson, 1973*a*). This work investigates the possibility that the haemoglobin of *E. brevis* could have a significant role in the supply of oxygen to certain tissues in the low and variable oxygen conditions of its niche. This may even occur without a measurable effect on the overall oxygen consumption of the individual.

MATERIALS AND METHODS

The in vivo *absorption spectra of* E. brevis *haemoglobin*

A Schimazdu recording microspectrophotometer was used to give more precise wavelengths for the *in vivo* absorption peaks of the haemoglobin of *E. brevis* than could be obtained with the apparatus described in the main part of this work. Each individual was placed on a cavity slide at room temperature, in sea water containing 0·5% of the narcotic propylene phenoxetol (Ellenby & Smith, 1964). Unfortunately, nitrogen was not available during the experiment, therefore 0·1 M-Na_2SO_3 in sea water was used to give the oxygen-free conditions necessary for the measurement of the *in vivo* spectrum of the deoxygenated haemoglobin of *E. brevis*. This method of obtaining anoxic conditions was not used in the experiments described below.

In vivo *oxygenation of* E. brevis *haemoglobin and oxygen tension*

Apparatus

The *in vivo* oxygenation of the haemoglobin within the pharynx of single individuals of *E. brevis* was measured using a recording microspectrophotometer (Fig. 1 A) built by a colleague, L. Smith. Basically, a pencil of light of rapidly changing wavelength was passed through the nematode for a short period, and the required part of the spectrum was scanned before the animal responded to the light source. Changes in the intensity of the light passing through the animal were detected by a photomultiplier and were plotted using an ultraviolet recorder.

Ten adult *E. brevis* were placed in a chamber (Fig. 1 B) containing a turntable, so that each individual was within a separate, transparent unit at the circumference of this rotating disc. Gentle revolution of the axis of the turntable allowed each individual to be selected for the region of the light path. The chamber was fixed to the micrometer stage of the microscope therefore; the position of the nematodes could be moved precisely within a horizontal plane at right angles to the light beam. A water jacket

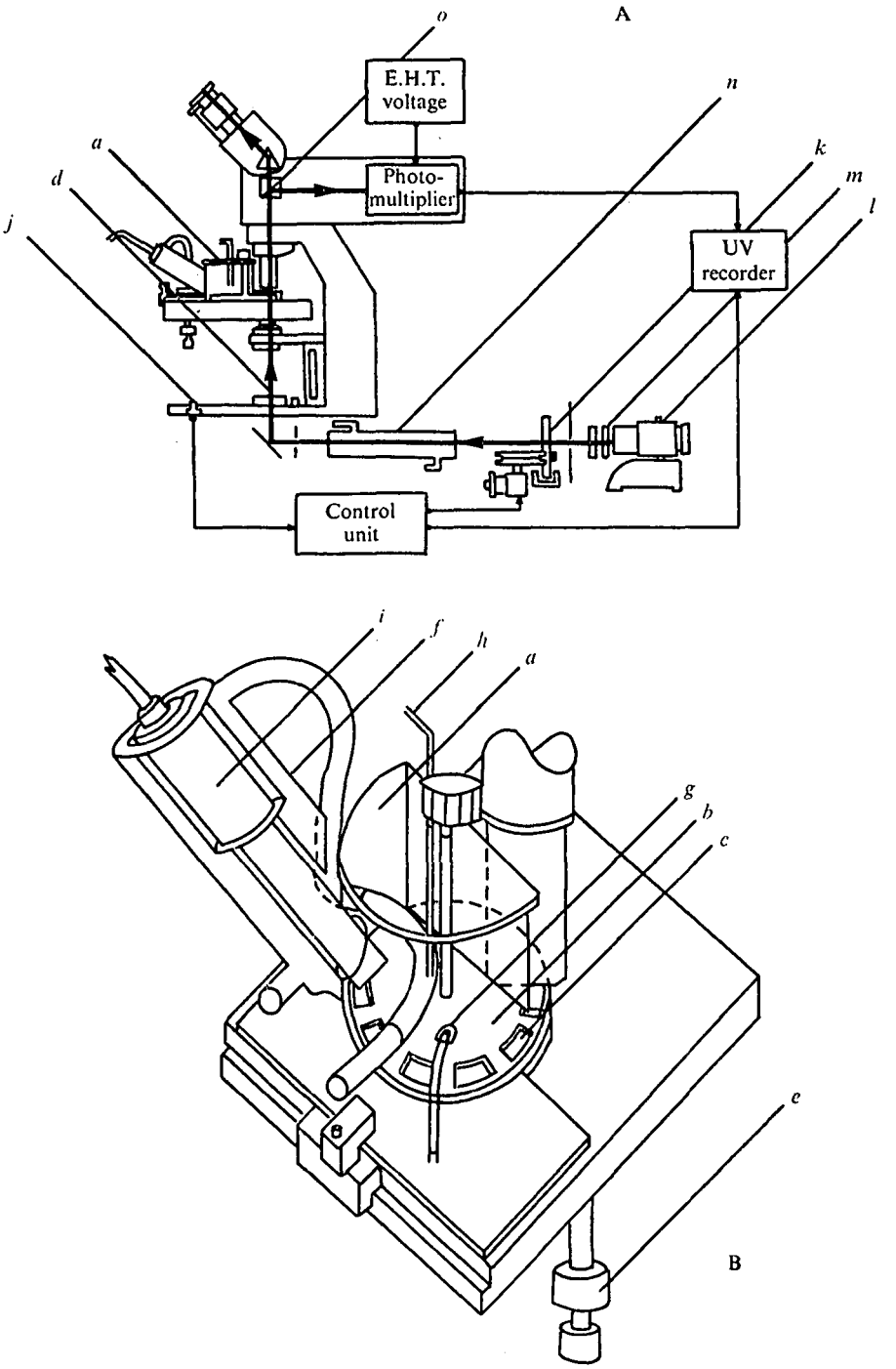

Fig. 1 A, Simplified diagram of the microspectrophotomer. B, Detail of the animal chamber. *a*, Animal chamber; *b*, turntable; *c*, transparent cell; *d*, light path; *e*, micrometer stage; *f*, water jacket; *g*, thermistor; *h*, O_2/N_2 mixture inlet; *i*, oxygen electrode; *j*, switch; *k*, Zeiss running filter; *l*, high intensity tungsten lamp; *m*, 1 of 2 glass heat filters; *n*, water heat filter; *o*, beam splitter.

maintained the chamber and oxygen electrode at a constant temperature of 15 °C, and this was recorded by a shunted thermistor connected to a bridge circuit. An adjustable mixture of oxygen and nitrogen was bubbled through the chamber, and the resultant constant low oxygen tension monitored, using the Radiometer oxygen electrode and ancillary equipment as previously described (Atkinson & Smith, 1973). The microscope and its condenser were adjusted so that the image of the closed field diaphragm had a smaller diameter than the nematode, and both were in the same focal plane. This only occurs when the distance between the condenser and the nematode is extremely small, therefore the lower window of the chamber and floor of the turntable were made of coverslip glass.

A Zeiss running filter was used to vary the wavelength of the light passing through the animal. The beam was split in the microscope head so that a part went to the eyepieces and the remainder was deflected to the window of an E.M.I. 9663B photomultiplier. This was maintained at an E.H.T. of 1000 V, and gave an S_{10} spectral response. Changes in light intensity were measured as changes in the potential difference across the anode load resistor, using the ultraviolet recorder.

Experimental procedure

Individuals were collected and stored as previously reported (Atkinson, 1973*a*), except that in these experiments all individuals were stored in one 250 ml pot of filtered sea water. Adult females as well as males were used, because a large number of animals was required.

Ten *E. brevis* adults were placed in the chamber, one in each 7 by 4 mm unit of the turntable. The animals were approximately 4 mm in length, with a diameter of about 0·2 mm, and therefore had sufficient space for movement. The nematodes were maintained for three hours at a constant low oxygen tension before measurement. Rotation of the turntable brought the first *E. brevis* chosen for the experiment into the region of the optical path. The micrometer stage was adjusted until the edge of the animal, at the selected part of its pharynx, was just within the light beam. A further adjustment was made so that the individual lay just to one side of the light. Measurements were always taken in the pharyngeal region just behind the onchial musculature; this part of the animal has the highest haemoglobin content. With experience this position could be located rapidly without disturbing the inactive nematode. A quick adjustment of the micrometer stage in the opposite direction moved the nematode back into the light beam. Immediately, a microswitch was operated to start the Zeiss running filter and drive the chart of the ultraviolet recorder. Wavelengths between 500 and 620 nm were scanned within two seconds before the animal responded to the light. In this way, a trace for the spectrum of the haemoglobin was obtained for each of the 10 individuals at a known low oxygen tension within the chamber. In all cases, the height of the α peak of the haemoglobin in millimetres was expressed as a percentage of the value obtained for the same position on each animal at the end of the experiment. These second readings were recorded after the nematodes had been narcotized for 30 min by 0·5% propylene phenoxetol in sea water, at an atmospheric oxygen tension. On average, the height of the α peak on the recorder chart was approximately 15 mm for narcotized animals. The method described has many limitations,

d cannot give absolute values for the oxygenation of the haemoglobin. However, the apparatus is capable of estimating, with sufficient accuracy for this work, any change in the *in vivo* oxygenation of the pigment.

Changes in the in vivo *oxygenation of* E. brevis *haemoglobin with stimulation*

For these experiments a single animal was used on each occasion. The nematode was placed in a chamber without a turntable, and held in a microclamp made of two strips of nylon. One piece of nylon was cemented to a glass coverslip, and the second placed parallel to it but fastened to the glass by two fine lengths of rubber. The two pieces of nylon just touched, but could be separated with a needle. Immediately before an experiment the microclamp was held open and a nematode was placed between the two pieces of nylon. When the needle was removed, the grip of the clamp on the broadest part of the animal was just sufficient to prevent its escape. However, the pressure did not alter the body shape, and the nematode was still capable of lateral movement of its narrower, pharyngeal region. The animal was stimulated into activity by square wave electrical impulses for 0·5 sec every 2 sec for 1–2 min. The current was passed between two KCl/Agar bridge electrodes 1 cm apart, at each side of the microclamp. A potential difference of 90 V between the electrodes and 40 V across the sea water was required to cause a definite response by the animal. At lower voltages, the intensity of the current through the animal was inadequate, because the relatively large volume of sea water around the small nematode caused severe electrical shunting. During stimulation, the animal worked vigorously and attempted to escape from the restraining groove. Estimates for oxygenation of the haemoglobin were obtained before stimulation, immediately after stimulation, and at 1 min intervals for the following 5 min. Again, these values were expressed as percentages of the value obtained for each individual after 30 min in sea water containing 0·5% of the narcotic propylene phenoxetol.

RESULTS

The in vivo *absorption spectra of* E. brevis *haemoglobin*

Mean values for the *in vivo* absorption peaks of this haemoglobin, given with their standard errors, occur at 577·6 ± 0·6 nm, 543·6 ± 0·5 nm and 421·7 ± 1·9 nm for oxyhaemoglobin; the corresponding values for deoxyhaemoglobin are 555·2 ± 0·9 nm and 432·2 ± 1·3 nm. These means are based on measurements for the haemoglobin in the pharynx of 12 individuals of *E. brevis*. Similar readings were obtained for the haemoglobin of the tail musculature of this species and *E. communis*. The values in the visible region of the spectrum are similar to those compiled for a number of animal-parasitic nematodes by Lee & Smith (1965). Average wavelengths calculated from their data are 577·6 nm and 547·7 nm for the oxygenated state, and 555 nm for the deoxygenated pigment. Clearly, the pigment in certain tissues of *E. brevis* and *E. communis* is a haemoglobin.

In vivo *oxygenation of* E. brevis *haemoglobin and oxygen tension*

Mean values for the percentage oxyhaemoglobin were estimated at 15 oxygen tensions between 5 and 43 Torr for a total of 131 individuals (Fig. 2). Regression analysis, assuming a linear relationship, has been carried out by the method of least

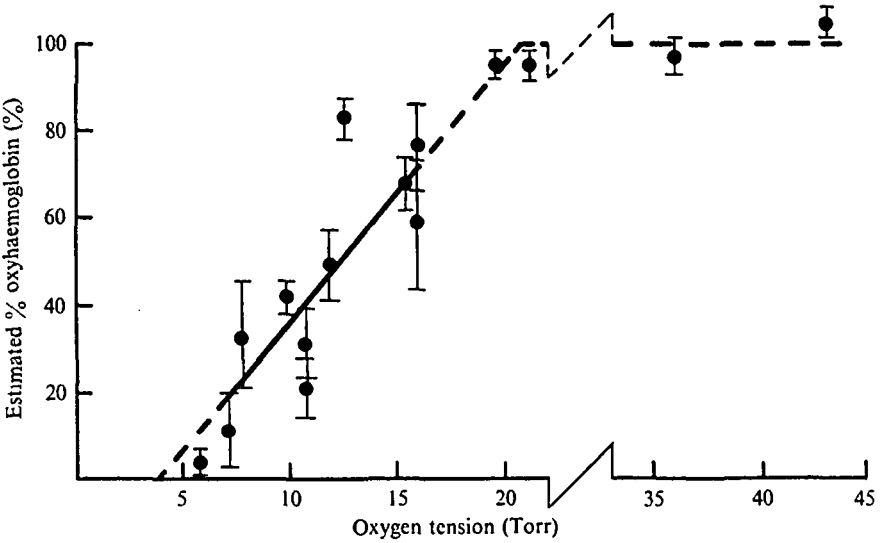

Fig. 2. Estimated percentage of oxyhaemoglobin in the pharynx of *E. brevis* at low oxygen tensions. Each value is the mean of approximately 8 individuals. Limits of the standard error of the mean, ⊢.

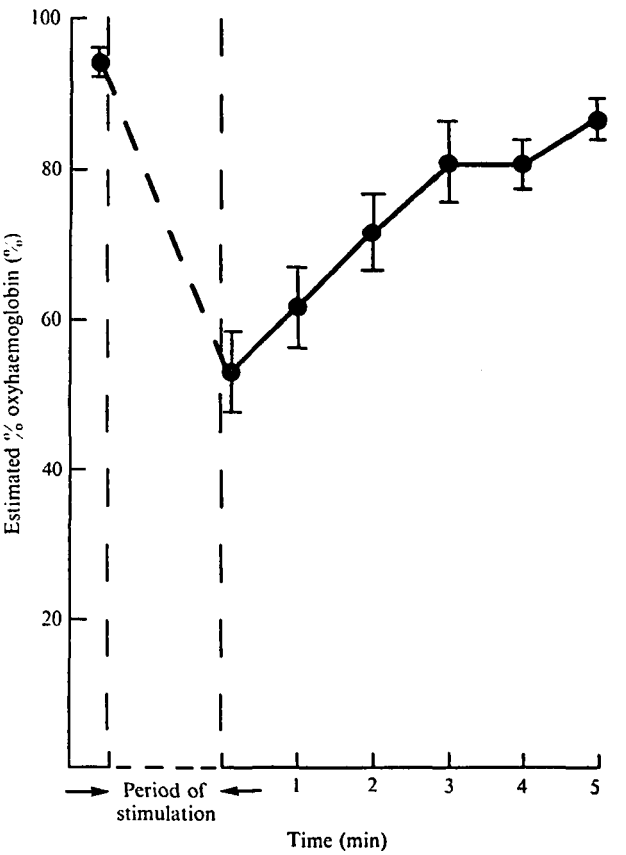

Fig. 3. Estimated percentage of oxyhaemoglobin in the pharynx of *E. brevis* after stimulation. The means are based on values for 22 individuals. Limits of the standard error of the mean, ⊢.

squares on the 10 means showing a partial deoxygenation. The values show a definite increase in the percentage of oxyhaemoglobin at oxygen tensions greater than 5 Torr, the pigment is 50% oxygenated at approximately 12 Torr, and fully loaded with oxygen at 20 Torr.

Changes in the in vivo *oxygenation of* E. brevis *haemoglobin*

The *in vivo* percentage oxygenation of the haemoglobin of *E. brevis* individuals was estimated before electric shock treatment, after 1–2 min of this treatment, and every 60 sec for the following 5 min (Fig. 3). Immediately after the period of hyperactivity caused by stimulation, the mean percentage oxyhaemoglobin was 53%. This is significantly less than the value of 94% recorded before this treatment ($P < 0{\cdot}001$), or the mean of 87% recorded 5 min after stimulation ($P < 0{\cdot}001$). The extent of this deoxygenation, and the time scale of recovery, are likely to be influenced by the availability of oxygen during this period. In the experiment, the environmental oxygen tension was approximately 150 Torr, but the diffusion of oxygen into the nematode was probably influenced by the restraining groove. However, the experiment shows that a period of hyperactivity at a constant ambient oxygen tension did cause a transient dissociation of oxygen; the haemoglobin reoxygenated after the end of this period of increased activity.

DISCUSSION

The haemoglobin of inactive *E. brevis* was not fully oxygenated *in vivo* at oxygen tensions in the surrounding sea water of less than 20 Torr (Fig. 2). This nematode does consume oxygen at low oxygen tensions (Atkinson, 1973*a*), and this probably causes a considerable diffusion gradient across the tissues of the individual. Therefore, the oxygen tension experienced by the partially deoxygenated pigment in the tissues may be appreciably less than that measured in the sea water around the animal. This oxygen gradient may also cause the percentage oxygenation of the haemoglobin to decrease towards the central tissues. Unfortunately, the size of this effect cannot be easily estimated because not all the tissues of *E. brevis* have a similar haemoglobin content (Ellenby & Smith, 1966).

Measurement of the *in vivo* percentage oxyhaemoglobin of individuals after stimulation by a short period of electric shock treatment showed that hyperactivity caused a temporary reduction in the oxygenation of the haemoglobin in the pharynx (Fig. 3). An artificial experiment of this nature requires careful interpretation, but there can be little doubt that the high level of muscular activity caused an increase in oxygen demand which resulted in a partial deoxygenation of the pharyngeal haemoglobin. This transient deoxygenation induced by hyperactivity was recorded at an atmospheric oxygen tension, but would occur more readily at low oxygen tensions. Under these conditions, the quantity of oxygen within the tissues is reduced, and the maximum rate of diffusion is less able to meet an increase in oxygen consumption.

Many workers (Lee & Smith, 1965; Ellenby & Smith, 1966; Smith, 1969; Jones, 1972) have suggested that nematode haemoglobin might facilitate oxygen diffusion. Hemmingsen (1963) showed that the enhanced flux of oxygen through seal myoglobin was almost independent of the oxygen tension, except that it was abolished by more than a very low oxygen tension at the lower end of the diffusion gradient. Apparently,

facilitation makes its greatest relative contribution to the oxygen supply of a tissue when the oxygen gradient causes the haemoglobin to deoxygenate near the site of utilization but leaves the pigment fully oxygenated at the surface of the tissue (Wittenberg, 1970). Therefore, Fig. 2 would suggest that the contribution of the facilitated flux to the supply of oxygen in resting *E. brevis* would be greatest at oxygen tensions less than 20 Torr. However, since increased levels of activity have been shown to reduce the oxygenation of the pigment at higher oxygen tensions (Fig. 3), the facilitated flux may also be considerable at these times. The presence of haemoglobin may ensure that the oxygen supply to some tissues is maintained during periods of increased oxygen consumption in the low oxygen regime of the habitat. The facilitated flux may also reduce changes in oxygen supply to certain tissues, caused by the movement of the animal in the marked oxygen gradient present in its niche (Atkinson, 1973*b*). Much of the haemoglobin present in *E. brevis* occurs in the pharynx, and this may suggest that the pigment plays a significant role during the muscular activity associated with feeding. The enhanced rate of oxygen diffusion may only occur periodically during pumping by the pharynx, and may not be readily detected in measurements of the overall oxygen consumption of the individual. It is, therefore, of interest that *E. brevis* and *E. communis* have similar rates of oxygen consumption at low oxygen tensions, although the latter contains considerably less haemoglobin (Atkinson, 1973*a*). The difference in the haemoglobin content is most marked in the pharynx of the two species. The pigment probably increases the availability of oxygen to the pharynx of *E. brevis* during feeding in the low oxygen regime of its mud habitat, but this role is unnecessary for *E. communis*, as it lives in a more aerobic niche among *Mytilus* shells.

Unfortunately, it is not certain that nematode haemoglobins do facilitate oxygen diffusion. Wittenberg (1966) reported that the body-wall haemoglobin of *A. lumbricoides* does not facilitate oxygen diffusion, and considered that this was due to its known low rate of dissociation (Gibson & Smith, 1965). Further measurements are required, for few other species have been adequately studied, and it is not certain that all nematode haemoglobins do have low rates of oxygen dissociation.

Haemoglobins that combine reversibly with oxygen act as oxygen stores, but the value of this function is often challenged because of the limited oxygen capacity of the system (Wittenberg, 1970; Jones, 1972). However, it is possible to envisage conditions in which an oxygen store might have a functional significance in *E. brevis*. It is unlikely that the haemoglobin would satisfy all but the lowest oxygen demand for more than a few minutes, as individuals rapidly deoxygenate their haemoglobin in anaerobic conditions. The pigment may act as a short-term oxygen store. Contraction of the pharynx could cause a sudden local fall in the tissue oxygen tension, and the released oxygen might be rapidly utilized. Jones (1972) considered that the presence of haemoglobin in non-muscular tissue was suggestive of a facilitated diffusion function. In general, this is probably correct, but if the haemoglobin in the non-muscular hypodermis of *E. brevis* does deoxygenate slowly, it may also reduce changes in the oxygen tension of this tissue caused by the periodic activity of adjacent working muscles.

Further study is required of the possible facilitation of oxygen diffusion by nematode haemoglobins. Facilitation may increase the rate of oxygen supply possible to specific tissues of *E. brevis* in the low oxygen regimes of its habitat during periods of hyperactivity. In these conditions, oxygen released during a partial deoxygenation of

The pigment may also have a minor, but significant, effect on the oxygen supply to specific tissues.

I wish to thank Mr L. Smith for allowing me to use and modify the microspectrophotometer described in this work. He and Professor C. Ellenby were extremely helpful throughout the course of this investigation.

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