THE BIOLOGICAL SIGNIFICANCE OF HAEMOGLOBIN IN NEMATODE PARASITES

II. THE PROPERTIES OF THE HAEMOGLOBINS AS STUDIED IN LIVING PARASITES

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Summary

In the parasites examined, the amounts of water-soluble haematin compounds, of which haemoglobin formed the large part, varied considerably; *Nippostrongylus muris* contained about 6 mg. (as haematin) per g. dry wt. of tissue, *Nematodirus* spp. and *Haemonchus contortus* about 0.8 mg. per g. dry wt. Evidence is presented which indicates that the haemoglobin of *Nippostrongylus muris* may be oxygenated *in vivo*, at least sometimes. The haemoglobin in the living parasites was easily oxygenated and deoxygenated; when the oxygen tension in the medium surrounding the parasites *in vitro* at 37°C. fell below about 13 mm. of mercury (*Nippostrongylus muris*) or 9 mm. of mercury (*Haemonchus contortus* and *Nematodirus* spp.) the oxyhaemoglobin became deoxygenated.

The rate of oxygen consumption by the three species of parasites was not significantly lessened by poisoning the haemoglobins with low concentrations of carbon monoxide at oxygen tensions between 38 and 5 mm. of mercury.

It is concluded that the haemoglobins, though present in sufficient amounts and apparently having suitable properties, are not actively concerned in the transport of oxygen in the tissues of the parasites *in vivo* when the partial pressure of the oxygen in the medium is above 5 mm. of mercury.

I. Introduction

A number of workers (for references see Rogers 1949*c*) have shown that haemoglobin is present in certain nematode parasites. The pigments from *Nippostrongylus muris, Nematodirus* spp., *and Haemonchus contortus* have properties which suggest that they may be effective oxygen carriers at the low partial pressures at which oxygen is available in the normal environments of the parasites (Rogers 1949*c*).

In the present studies attempts have been made to assess the physiological importance of haemoglobins as oxygen carriers in intact parasites. For this purpose, the amounts of haemoglobin in the parasites, the ability of the parasites to deoxygenate the oxyhaemoglobin in their tissues, and the partial pressure of oxygen in the medium at which deoxygenation takes place have been determined. Experiments to determine if haemoglobin of *Nippostrongylus muris* can become oxygenated *in vivo* have also been carried out. Finally, the

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effects of poisoning the parasite haemoglobins with low concentrations of carbon monoxide on the uptake of oxygen at several different partial pressures have been examined.

II. Methods

The methods of obtaining appropriate biological materials have already been described (Rogers 1949*a*).

Oxygen tensions were measured electrometrically by the method of Brink and Davies (1942). The electrodes, the method of calibration, and the method of measuring currents were similar to those used by Rogers (1949*c*). As a rule "open" electrodes were used; as before, they were calibrated immediately before and after use.

Manometric determinations of oxygen uptake were carried out by the direct method of Warburg (1926) with small vessels of about 5 ml. capacity.

Haemoglobin was estimated, as reduced pyridine haemochromogen, by the use of a wedge trough and comparison microscope (Elliot and Keilin 1934).

III. Procedure and Results

(*a*) *The Amount of Haemoglobin in the Parasites*

Frozen parasites were crushed and extracted several times with distilled water and the debris was separated each time by centrifuging. The combined haematin in the extract, which was largely that of oxyhaemoglobin, was then estimated as reduced pyridine haemochromogen. Though it was difficult to extract the haemoglobin from the tissues completely, this method avoided the error caused by the presence of free haematin in the intestines of some of the parasites. The total haematin found in the water extract of the three parasites is shown in Table 1.

Table 1

AMOUNT OF HAEMOGLOBIN FOUND IN THREE SPECIES OF PARASITES

Parasite	Haemoglobin as Haematin (mg. per 100 g. wet tissue)	
	Limits of Variation	Average*
Nippostrongylus muris	112-128	124
Nematodirus spp.	18-24	19
Haemonchus contortus	13-18	16

* Four separate estimations were made with each species.

The amount of haemoglobin in *Nippostrongylus muris* varied somewhat with the age of the parasite. As a rule, the parasites were recovered from the rats on the tenth day after parasitism had been established; figures for the haemoglobin content of such parasites are given in Table 1. Results obtained from the other two parasites varied much more than those from *N. muris*.

(*b*) *The Examination of* Nippostrongylus muris *Haemoglobin* in vivo

Parasitized rats which had been fasted for 6 hours were anaesthetized with "Nembutal," 40 mg. per kg., given subcutaneously. The rat was tied to a small board fitted with a clamp and so arranged that it could be placed on a microscope stage. Hair was removed from the belly of the rat and a 2 cm. incision made in the abdominal wall over the portion of the gut to be examined. About 5 cm. of small intestine containing the parasites was drawn out of the abdominal cavity and held in a clamp between two warmed glass slides. The haemoglobin in different parts of the exteriorized intestine was then examined with a spectroscope mounted in the microscope. The small clumps of worms, as seen through the intestinal wall, showed more intense oxyhaemoglobin bands than the parts of the gut where no parasites were present.

When the parasitized ether-anaesthetized rats were killed by asphyxiation with nitrogen-carbon dioxide, and the abdomen left unopened for 5-10 minutes, the parasites showed a dark colour, like that of haemoglobin, immediately after the intestine was opened. The haemoglobin became oxygenated very quickly. On the other hand, when intestines of rats were opened immediately after the animals had been killed with chloroform, the parasites showed the normal bright oxyhaemoglobin colour.

These experiments, though open to criticism, suggest that, *in vivo,* the haemoglobin of *Nippostrongylus muris* is oxygenated at certain times at least. This would be expected if the oxygen in the host gut fluids reached a partial pressure of about 15 mm. of mercury (see later in this paper). The experiments carried out by Rogers (1949*c*) suggest that the oxygen tensions in the contents of the small intestine of the rat may sometimes reach a partial pressure of 30 mm. of mercury.

(*c*) *The Deoxygenation of Oxyhaemoglobin in Intact Parasites*

Parasites were placed in a cell containing physiological saline at 37°C.; an "oxygen" electrode, a capillary bridge from a 0.15M sodium chloride half-cell, and a small coil heater were placed in position in relation to the cell, as shown in Figure 1. The heater was designed to hold the temperature of the cell and its contents between 36 and 38.5°C. The cell and heater were then placed on the axis of a microscope adjusted horizontally and from which the stage had been removed as shown in Figure 2. The arrangement was such that a beam of light could be passed through the condenser and through holes in the heater so that the contents of the cell could be examined with a low power objective and eyepiece. By this means the cell and electrodes were so adjusted that the microscope was focused on a small area just where the tip of the "oxygen" electrode was in close proximity to the parasites on the bottom of the cell. The microscope eyepiece was then replaced by a micro-reversion spectroscope. This arrangement allowed the simultaneous spectroscopic examination of the haemoglobin in the parasites and the determination of the oxygen tension in the saline adjacent to the parasites under examination.

The respiratory activity of the parasites led to the removal of oxygen from the saline in their vicinity faster than it could be replaced by the influx of oxygenated saline. As a result, the oxygen tension at the electrode tip fell, as did the current recorded by the valve microammeter. At certain oxygen

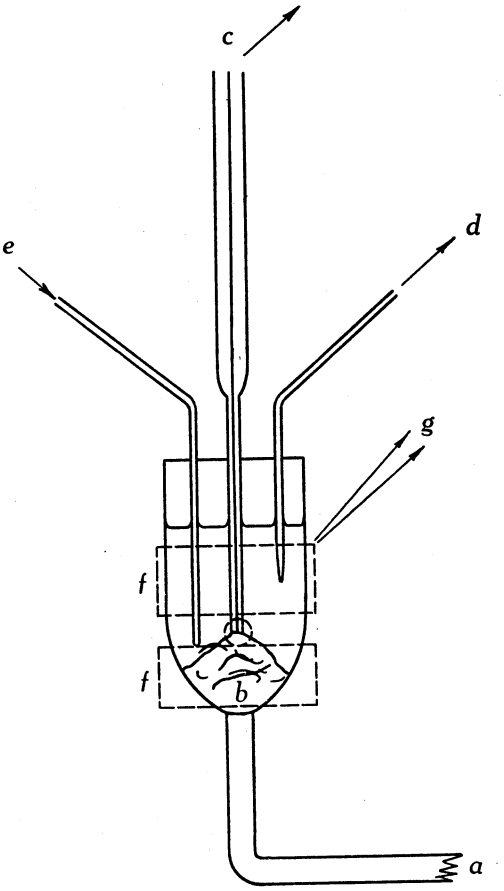

Fig. 1.—Arrangement of electrodes in the cell used for studying the deoxygenation of oxyhaemoglobin in living parasites. The cell was supported by means of the solid glass rod (*a*); the parasites (*b*) were allowed to fall to the bottom of the cell and the tip of the "oxygen" electrode (*c*) placed on the parasites in the centre of the field of the microscope (indicated by a circle). A capillary (*d*) made connection to a 0.15M NaCl calomel half-cell; when necessary, gas mixtures could be passed into the medium in the cell through the tube (*e*). The cell contents were held at a temperature of 36-38.5°C. by the perforated coil heater (*f*) connected (*g*) to a suitable power supply.

tensions, the removal of oxygen from the oxyhaemoglobin by the metabolic activities of the parasites was faster than the rate of oxygenation of haemoglobin caused by the diffusion of oxygen from the oxygen-depleted medium.

Under such conditions the oxyhaemoglobin became deoxygenated and the prominent bands of oxyhaemoglobin were replaced by the faint and diffuse band of haemoglobin. The actual fading of the oxyhaemoglobin usually took place over a period of about 30 seconds, but it was possible, by means of the reversion spectroscope, to select an approximate end-point which could be recognized without difficulty. The accuracy with which the end-points could be determined was, indeed, relatively high compared to that with which the

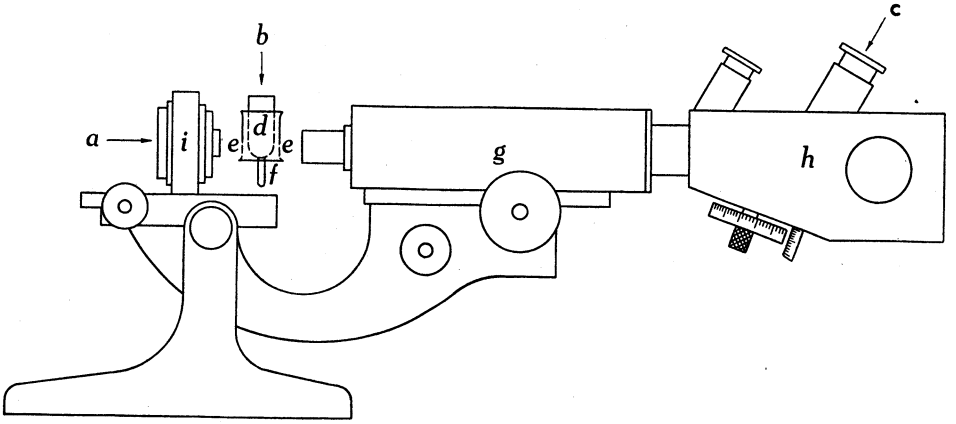

Fig. 2.—Apparatus used for studying the deoxygenation of oxyhaemoglobin in the living parasites. A strong light (*a*) was passed through the cell (*b*) and the spectrum was observed at (*c*). The cell, which was surrounded by a heater coil (*d*) perforated at (*e*), was supported by means of the solid glass rod (*f*). The micro-reversion spectroscope (*h*) was fixed to the barrel of the microscope (*g*). Light intensity was controlled by means of the condenser and diaphragm (*i*).

oxygen tension could be determined because the rate of fall of the oxygen tension in the medium close to the parasites was not steady. This was largely due to the active movements of the parasites themselves, which led to an irregular flow of medium past the tip of the "oxygen" electrode. The rise and fall in the electrode current was sometimes as much as 15 per cent. and only within such limits of accuracy was it possible to determine the oxygen tension in the saline, close to the parasites, at the time when the oxyhaemoglobin became deoxygenated. After a determination was made, the contents of the cell were well mixed with a stream of air; when the parasites had settled at the bottom of the cell the position of the electrode was readjusted and the determination repeated. The results obtained by examining several different lots of the three species of parasites are shown in Table 2.

(*d*) *The Efficiency of the Parasite Haemoglobins as Oxygen Carriers*

The proportions of carbon monoxide in all the gas mixtures shown in Table 3 were found to be sufficient to cause the formation of carboxyhaemoglobin within the parasites. These gas mixtures were therefore used to study the uptake of oxygen by parasites in which the haemoglobin was not functioning as an oxygen carrier. To decrease the possibility of carboxyhaemoglobin

dissociation during the experiments, the Warburg vessels were covered by small black bags. As a routine, at the end of each experiment in which carbon monoxide was used, the parasites were examined to make sure that the haemoglobin was largely saturated with carbon monoxide.

TABLE 2

OXYGEN TENSION (MM. OF MERCURY) IN SALINE MEDIUM, CLOSE TO THE PARASITES, WHEN THE OXYHAEMOGLOBIN WAS JUST COMPLETELY DEOXYGENATED

Parasite	Oxygen Tension (mm. of mercury)	
	Limits of Variation	Average*
Nippostrongylus muris	10.4-16.9	13.3
Nematodirus spp.	6.4-12.8	9.4
Haemonchus contortus	7.2-12.8	9.2

* Five determinations were made with each species of parasite.

The Warburg shaking rates were the same as those used previously (Rogers 1949*b*), which had been shown to be adequate for the type of vessel and the amount of medium used. In each experiment the oxygen uptake of the carbon monoxide-poisoned parasites was compared with that of the normal animals. The results obtained, especially at low partial pressures of oxygen, varied greatly owing to the fact that the oxygen uptake was small and the parasites tended to form clumps in the Warburg vessels at high shaking rates.

TABLE 3

PERCENTAGE INCREASE OR DECREASE IN OXYGEN UPTAKE CAUSED BY POISONING PARASITE HAEMOGLOBINS WITH CARBON MONOXIDE AT SEVERAL PARTIAL PRESSURES OF OXYGEN. EXPERIMENTS WERE CARRIED OUT AT 37°C. FOR 30 MINUTES

Gas Mixture			*Nippostrongylus muris*	*Nematodirus* spp.	*Haemonchus contortus*
O_2	CO	N_2			
5	2.5	92.5	Increased 4%	Increased 4%	Increased 5%
2	2	96	Increased 6%	Decreased 8%	Increased 10%
1	2	97	Increased 14%	Increased 1%	Increased 8%
0.5	2	97.5	Increased 10%	Decreased 6%	Decreased 11%

In Table 3, the effect of poisoning the parasite haemoglobins with carbon monoxide is shown as the percentage increase or decrease in oxygen uptake over that of the normal parasites, which had been examined under the same conditions except that no carbon monoxide was present. The experiments were carried out over a period of 30 minutes. No appreciable inhibition with carbon monoxide was obtained when the experiments with *Nematodirus* spp. were repeated with twice the carbon monoxide concentrations shown in Table 3.

On the whole, then, it would appear that the presence of carbon monoxide caused little change in the respiratory activity of all three species of parasites,

even at low oxygen tensions. It seems difficult, therefore, to accept the parasite haemoglobins as being effective carriers of oxygen under the conditions used in the manometric experiments. The accuracy of the method was such that small inhibitions due to the carbon monoxide might not have been detected. However, there is little doubt that if the haemoglobins were biologically important as oxygen carriers the inhibition would have been much greater, and it seems probable, therefore, that the oxygen transported to the enzyme systems of the parasites is not carried by the haemoglobins in the accepted manner.

As the failure to observe a lowered oxygen uptake in animals with carbon monoxide-poisoned haemoglobin might suggest that the respiration was limited by the availability of oxygen from the medium, in which circumstance carbon monoxide poisoning might not lower the Q_{O_2}, it is perhaps necessary to emphasize that care was taken to ensure an adequate Warburg shaking rate (see Rogers 1949*a*).

IV. Discussion

If the haematin content of the parasite haemoglobin is similar to that of mammalian haemoglobin, it can be calculated from the oxygen consumption rates of the parasite given by Rogers (1949*a*), that the amount of haemoglobin in *Nippostrongylus muris* is sufficient to supply its oxygen requirements when it is respiring at its maximum *in vitro* rate, assuming that the time for half-dissociation of the oxygen from the parasite's oxyhaemoglobin (t_{50}) was less than 0.9 seconds. (The t_{50} for sheep haemoglobin is given by Hartridge and Roughton (1923) as 0.0025 second at 37°C.). With oxyhaemoglobin from *Haemonchus contortus* and *Nematodirus* spp., a t_{50} of less than 0.25 second, or less than 100 times that of the host would be required. However, at the relatively low respiration rates of the parasites *in vivo* (Rogers 1949*a*), the t_{50} values would have to be at least three times those given above before the turnover rate would begin to limit respiration if all the oxygen was transported via oxyhaemoglobin. It was obvious, without detailed examination, that the haemoglobins of the Trichostrongyle parasites had t_{50} values very much less than those of the perienteric fluid of *Ascaris lumbricoides*, 1000 ± 100 seconds, or its body wall, 250 seconds (Davenport 1945), though it could not be ascertained without elaborate experiment what the actual t_{50} values were. As it was found that carbon monoxide poisoning did not reduce the oxygen uptake of the parasites, even at lowered oxygen tensions, when respiration was slow, it seems unlikely that the failure of the haemoglobin to transport physiologically significant amounts of oxygen was due to the slow dissociation of the oxyhaemoglobin.

The validity of the experiments, which were carried out to determine the condition of the haemoglobin in *Nippostrongylus muris* in the intact host intestine, depended among other things on whether the amount of haemoglobin in the parasites was larger than that in the host tissue. Porter (1935) stated that the bright red patches found in the intestines of parasitized host animals were due to excess blood in the villi rather than to the pigment in the parasites

themselves. Though there is no doubt that the villi were dilated where the "worm nests" were situated, the high concentration of oxyhaemoglobin in the parasite tissues was such that, in the present experiments, microscopic examination showed the blurred red tissues of the parasites through the walls of the host intestine. It was therefore possible that the increased density of the oxyhaemoglobin bands where the parasites were situated was due to the parasite haemoglobin. Unfortunately, the general absorption due to the walls of the host intestine made it very unlikely that the faint diffuse band of haemoglobin would have been detected if the pigment in the parasites had been deoxygenated. However, the finding of the bright red colour in the parasites when they were examined immediately after the death of the host, whereas the darker colour of the haemoglobin was present when the host intestine was not opened until some 5 minutes after death by asphyxiation, gave further support to the indication that *Nippostrongylus muris* haemoglobin was oxygenated *in vivo*.

Owing to the thickness of the walls of the sheep intestine and abomasum, and to the low concentration of haemoglobin in *Nematodirus* spp. and *Haemonchus contortus*, the examination of the haemoglobin in these parasites could not be carried out in the live host.

The oxygen tensions at which the oxyhaemoglobin in the living parasites was deoxygenated were much higher than those found when purified oxyhaemoglobin solutions were used (Rogers 1949*b*). This may have been due to the fact that, in the *in vivo* studies, the oxygen tension measured was that of the medium close to the parasites and not that of the actual tissue containing the haemoglobin. In the medium, even close to the parasites, the oxygen tension may well have been much higher than that in the tissues. Further, the oxyhaemoglobin in the tissues of *Nippostrongylus muris* reached a much higher concentration than that used in the solutions, which would have caused an increase in the affinity for oxygen (Hill and Wolverkamp 1936). Other factors which may have been concerned were temperature (16°C. was the temperature used in experiments with the purified pigment), pH, and salt concentration. However, it is clear that, in order to maintain fully oxygenated haemoglobin in the parasites, the partial pressure of oxygen in the host gut fluids, in which the parasites normally live, would have to reach 13 mm. of mercury for *Nippostrongylus muris* and 9 mm. for the sheep parasites. Though oxygen tensions of the order of 13 mm. of mercury probably occur in the contents of the small intestine of the rat close to the mucosa, it is doubtful if pressures of 9 mm. are reached in the contents of the sheep's abomasum or in that part of its small intestine where *Nematodirus* spp. normally live (Rogers 1949*c*).

Many invertebrates, e.g. *Tubifex tubifex, Sabella pavonina,* and *Lumbricus herculeus,* have haemoglobins which have been found to be efficient oxygen carriers at atmospheric pressures (Dausend 1931; Ewer and Fox 1940; Johnson 1942). The haemoglobins of some Chironomid larvae, however, do not transport physiologically significant amounts of oxygen when the surrounding

medium is more than 25 per cent. (*Tanytarsus*) or 44 per cent. (*Chironomus*) saturated with air (Walshe 1947; Ewer 1942). The haemoglobin in *Daphnia,* like that in Trichostrongyle parasites, is not functionally active as an oxygen carrier even at low partial pressures of oxygen (Fox 1948).

Laser (1944) has shown that the nematode *Ascaris lumbricoides* lacks catalase; when this parasite is exposed to high concentrations of oxygen, hydrogen peroxide accumulates in the tissues in toxic amounts. If catalase is generally absent from the tissues of nematode parasites, the possibility that the haemoglobins may have some physiological value as peroxidases might be considered.

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