THE OXYGEN DISSOCIATION AND ALKALINE DENATURATION OF HAEMOGLOBINS FROM TWO SPECIES OF EARTHWORM

By T. M. HAUGHTON, G. A. KERKUT and K. A. MUNDAY

Department of Physiology and Biochemistry,
The University of Southampton

(*Received* 5 *December* 1957)

INTRODUCTION

Although it has been clear since the work of Fox (1940) and Johnson (1942) that the haemoglobin of *Lumbricus* can take part in oxygen transport in the normal animal, the oxygen dissociation curves of this haemoglobin have not been available. The present paper provides oxygen dissociation curves for haemoglobin from two species of earthworm, *Lumbricus terrestris* (Linnaeus) and *Allolobophora terrestris* (Savigny) forma *longa* (Ude). Temperature has a direct effect on the oxygen dissociation and for this reason the haemoglobin dissociation was recorded at two temperatures approaching the upper and lower limits of the normal earthworm habitat. The results show that the haemoglobins of the two species have different properties and these differences are further confirmed by studies on the rate of alkaline denaturation of the respective haemoglobin solutions.

METHOD

Preparation of blood

The earthworms used in these experiments were *Lumbricus terrestris* (Linnaeus) and *Allolobophora terrestris* (Savigny) forma *longa* (Ude), and they were selected because of their size and availability.

The animals were anaesthetized for a few seconds over chloroform and a lateral slit was made in the body wall in the region of the gizzard; the flaps were pinned down and the coelomic fluid removed by filter-paper. A cut was then made across the ventral blood vessel and the blood collected by insertion of a finely drawn Pasteur pipette. A single *Lumbricus* yielded approximately 30–50 mm.^{3} of blood, while *Allolobophora* yielded less in proportion to size. On removal the blood was immediately frozen and stored over dry ice, and under these conditions remained stable for several days. Each experiment was carried out on a sample of pooled blood obtained on the same day from approximately thirty-six animals. The worms were collected from similar localities at the same time.

When required the blood was brought to the experimental temperature and 0·05 ml. of 3 M phosphate buffer added per ml. of blood (Green, 1933) to give a final pH of 7·3 and a phosphate concentration of 0·15 M. A drop of octyl alcohol was

then added to prevent foaming when the blood was later subjected to low pressures. Finally the blood was centrifuged to remove any particulate matter present.

Determination of the oxygen dissociation curves

The method used had to satisfy the conditions that: (i) only small quantities of blood were required; and (ii) it was applicable to haemoglobin concentrations approximating to those in the intact animal. This latter is necessary since many haemoglobins are known that are only stable at normal physiological concentrations, and furthermore the oxygen affinity of a dilute solution is not necessarily the same as that of haemoglobin *in vivo* (Hill & Wolvekamp, 1936).

The method chosen was based on that of Redfield (1930), the blood being equilibrated at various partial pressures of oxygen and the percentage of dissociation determined spectrophotometrically.

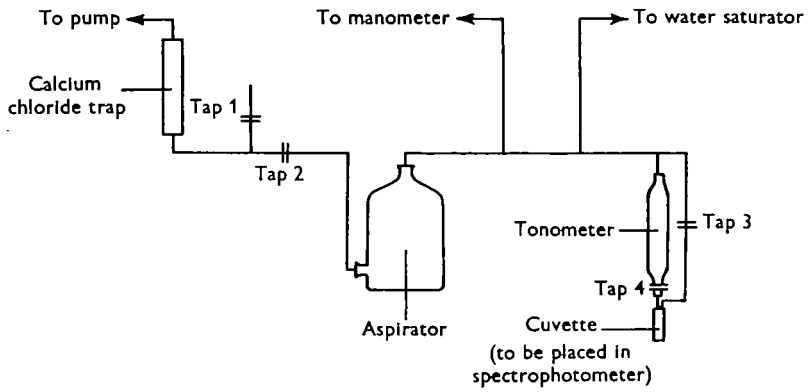

Fig. 1. Block diagram of apparatus used to determine the oxygen dissociation of earthworm haemoglobin. For further details see the text.

The apparatus chosen for equilibration of the blood consisted of a 100 ml. cylindrical separating funnel which served as a tonometer. A range of oxygen pressures was obtained by evacuating the air of the tonometer to the required degree. The tonometer was connected in series to an Edwards rotary vacuum pump through a 5 l. aspirator and a calcium chloride trap (Fig. 1). Between the aspirator and tonometer were two side connexions, one leading to a mercury manometer, the other leading to a 100 ml. vessel containing water so placed as to keep the system saturated with water vapour, thus minimizing evaporation from the blood. Between the calcium chloride trap and the aspirator was a tap 2, which separated the system from the pump during equilibration. Air could be admitted through tap 1.

The water-vapour pressure was constantly checked by a wet-bulb thermometer placed in the aspirator. In general, on initial pumping the vapour pressure decreased slightly, but when the pump was cut off and tap 2 closed the system rapidly became saturated with water vapour. The internal pressure of the tonometer was calculated

by subtracting the recorded manometric pressure and the appropriate vapour pressure at that temperature from the barometric pressure. The partial pressure of oxygen then was calculated by multiplying this pressure by the fraction of oxygen present in dry air.

The optical density of the blood was measured at 660 mμ (Roddie, Shepherd & Whelan, 1956) in a Unicam S.P. 600 spectrophotometer, and for this purpose a special cuvette was made from selected pieces of microscope slide, the edges being sealed with Apiezon wax. The cuvette had a thickness of 1·1 mm. and held approximately 0·55 ml. of blood. It was fitted with two tubes, a delivery tube terminating at the bottom of the cuvette (to avoid formation of air locks) and an outlet tube at the top (Fig. 2). The delivery tube was connected through tap 4 to the tonometer while the outlet tube was connected by pressure tubing through tap 3 to the vacuum line.

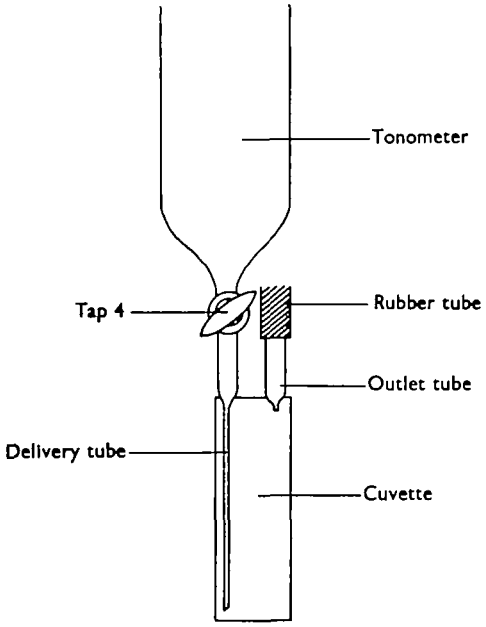

Fig. 2. Tonometer and cuvette. The cuvette was made from cut pieces of microscope slides. Note that the delivery tube ends near the base of the cuvette; this prevents the formation of airlocks when the blood is transferred from tonometer to the cuvette.

In operation the tonometer was detached from the vacuum line and with tap 4 closed the blood sample was introduced. The tonometer was then reconnected to the vacuum line and the pressure reduced to the required level. The blood was then equilibrated in the tonometer at each partial pressure for 15 min. During this time the tonometer was gently agitated by hand in the near-vertical position with tap 4 closed, thus minimizing loss of solution by spreading on the walls. When equilibration was complete tap 4 was opened and the blood flowed into the cuvette.

The cuvette with its attached tubes was then placed in the Unicam cell holder and held in position by a Perspex holder which allowed the passage of tubes to the cuvette. The holder with cuvette was then placed in the spectrophotometer and the optical density measured, in a series of determinations in which the partial pressure of oxygen was progressively reduced.

After determination of the optical density the tonometer and cuvette were removed from the spectrophotometer and held in a horizontal position with the delivery tube on the lower side of the cuvette. The blood was then returned to the tonometer by slightly raising the pressure of the system with tap 4 closed and tap 3 open, followed by lowering the pressure with tap 4 open and tap 3 closed. The blood was then ready for re-equilibration at another partial pressure of oxygen.

During equilibration, provided that frothing is controlled, little or no denaturation of the protein occurs. The final measurements for the completely reduced solution were obtained by the addition of a trace of sodium dithionate.

The percentage saturation of the haemoglobin, at any given partial pressure, was calculated from the recorded optical density at 660 mμ by linear interpolation between the values for the fully oxygenated and for the fully deoxygenated condition.

RESULTS

The experiments on the dissociation of the blood were carried out in constant temperature rooms at 7° and 20° C. Each experiment was performed on a pooled blood sample adjusted to pH 7·3 as described earlier.

Fig. 3 shows the points of a dissociation curve for the blood of *Lumbricus terrestris* at these two temperatures. Each curve shows the points from two different samples of blood collected some 2 weeks apart, and as can be seen there is good reproducibility. The points for the lower temperature (7° C.) lie on a less sigmoid curve and to the left of those for the higher temperature (20° C.). The partial pressure of oxygen at which the blood is 50 % saturated is equivalent to 2 mm. Hg at 7° C. and 8 mm. Hg at 20° C., and the partial pressure of oxygen at which the blood is 95 % saturated is 9 mm. Hg at 7° C. and 22·5 mm. Hg at 20° C.

Fig. 4 shows a similar series of points for the blood of *Allolobophora terrestris*. Here too the points at the lower temperature lie on a less sigmoid curve and to the left of those at the higher temperature. The partial pressure of oxygen at which the blood is 50 % saturated is 0·7 mm. Hg at 7° C. and 6 mm. Hg at 20° C. and the blood is 95 % saturated at 3·75 mm. Hg at 7° C. and 17·5 mm. Hg at 20° C.

These results are in general agreement with those obtained by Barcroft & King (1909) and Macela & Seliskar (1925) for the blood of man, tortoise, frog and *Planorbis*. These authors found that when all other factors, such as ionic strength, pH, and protein concentration were kept constant, a change in temperature brought about a shift in the dissociation curves. At lower temperatures the curves became less sigmoid and moved to the left of the graph, i.e. the haemoglobin had a greater oxygen affinity at lower temperatures.

The figures also show that there is a difference between the dissociation of the blood of *Lumbricus* and *Allolobophora*, and this difference is more clearly seen in

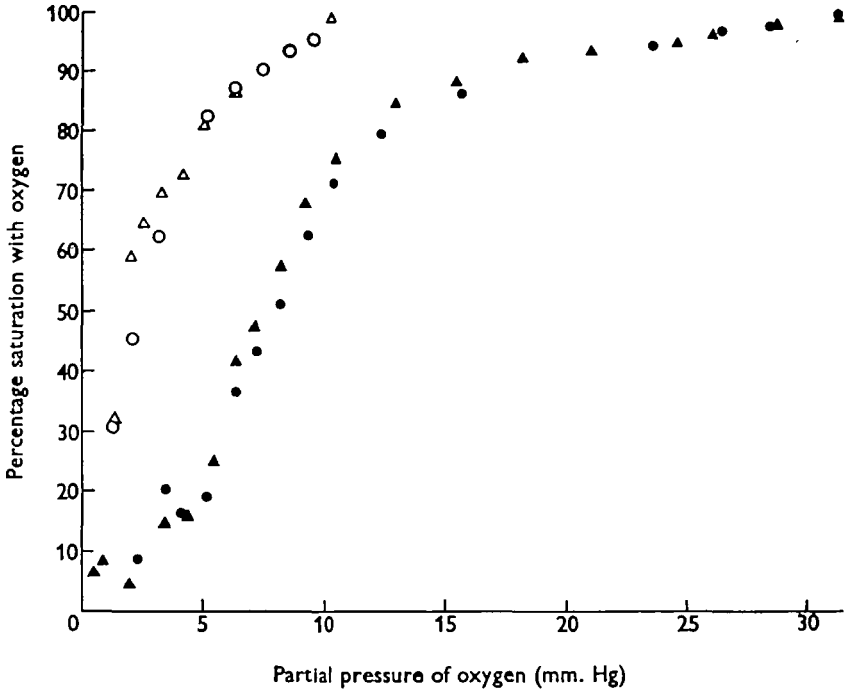

Fig. 3. Oxygen dissociation curves of haemoglobin from *Lumbricus terrestris*. The open symbols refer to 7° C., the black symbols to 20° C.

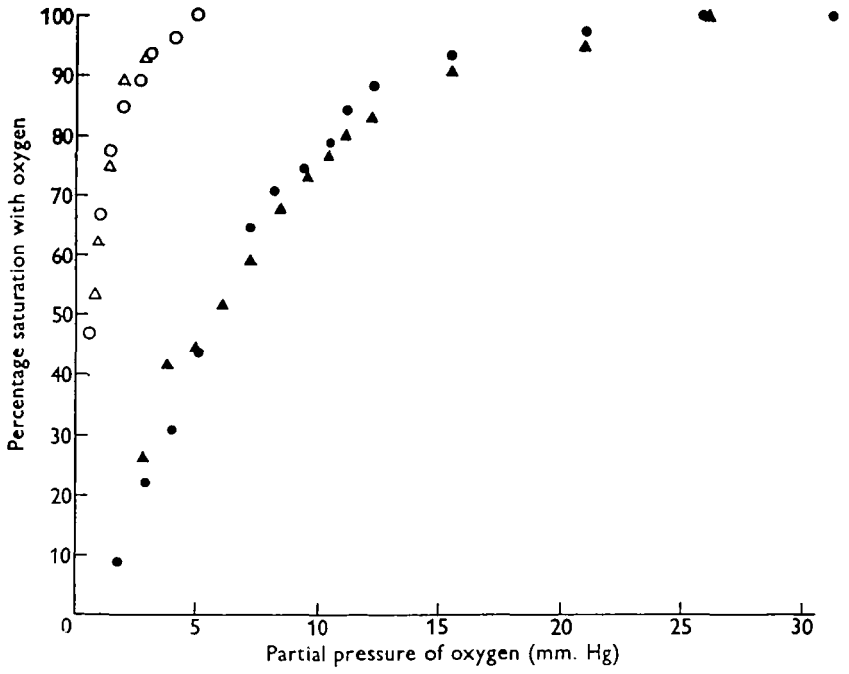

Fig. 4. Oxygen dissociation curves of haemoglobin from *Allolobophora terrestris*. The open symbols refer to 7° C., the black symbols to 20° C.

Fig. 5 where the four curves are plotted together. The curves for *Allolobophora* blood at a given temperature lie to the left of those for *Lumbricus* at the same temperature. This indicates that the haemoglobin of *Allolobophora* differs from that of *Lumbricus* and has a higher oxygen affinity.

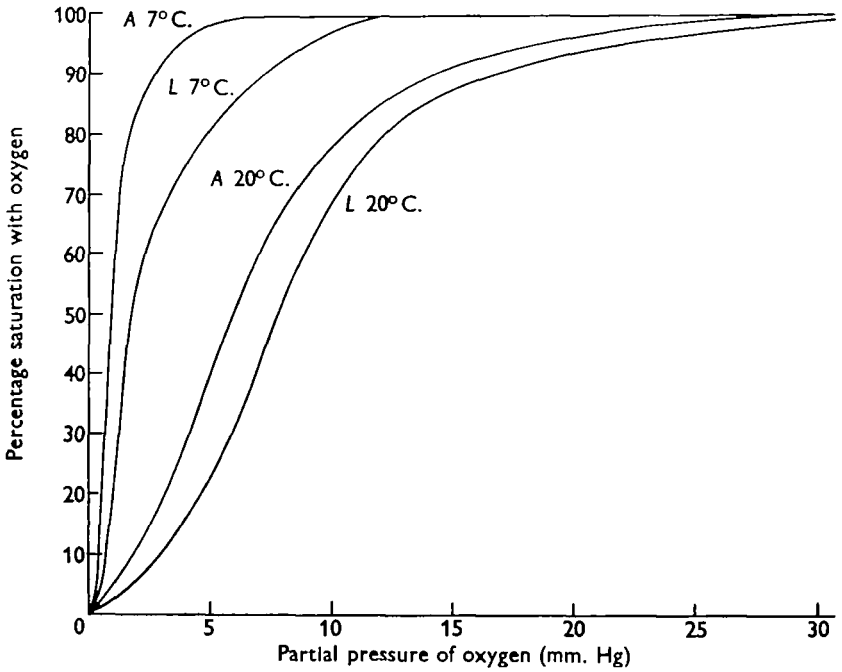

Fig. 5. Superimposed oxygen dissociation curves of haemoglobin from *Lumbricus* and *Allolobophora*. The curves for *Allolobophora* lie to the left of those for *Lumbricus* at the corresponding temperatures. *A* refers to *Allolobophora*, *L* to *Lumbricus*.

Alkaline denaturation of earthworm haemoglobin

Another method by which differences between haemoglobins have been previously demonstrated makes use of the difference in the rate of alkaline denaturation (Brinkman & Jonxis, 1937; Ramsey, 1941). Alkaline denaturation rates were therefore studied on blood samples from these two species of earthworm.

In these experiments the oxyhaemoglobin is converted under controlled conditions to alkaline globin haemochromogen (Lemberg & Legge, 1949) and the reaction is followed spectrophotometrically. The earthworm blood was diluted with distilled water to bring it to a concentration of 0·12 g. haemoglobin/100 ml. of solution. 2·5 ml. of the diluted blood was placed in a 1 cm. cuvette and the cuvette placed in a constant temperature holder at 25° C. The holder was inserted into a Unicam S.P. 600 spectrophotometer and 0·5 ml. of glycine buffer (Vogel, 1945) was added to the cuvette to bring the final pH to 12·7. The extinction coefficient at 541 mμ. was measured at intervals of 30–60 sec. until the readings became constant. From these results the percentages of unchanged oxyhaemoglobin remaining in the solution could be calculated, and were then plotted against time. Fig. 6 shows representative

curves of blood from the two species. It will be seen that the blood of *Allolobophora* denatures much more rapidly than does that from *Lumbricus*, *Allolobophora* blood being denatured in just under 6 min. as compared with 23 min. for *Lumbricus*. We have found that three other species of earthworm have specific denaturation times: namely, *Lumbricus rubellus* 2·5 min., *Eisenia foetida* 5 min., and *Lumbricus festivus*, 11·5 min. Sufficient numbers of these other species were not available for determination of the oxygen dissociation curves.

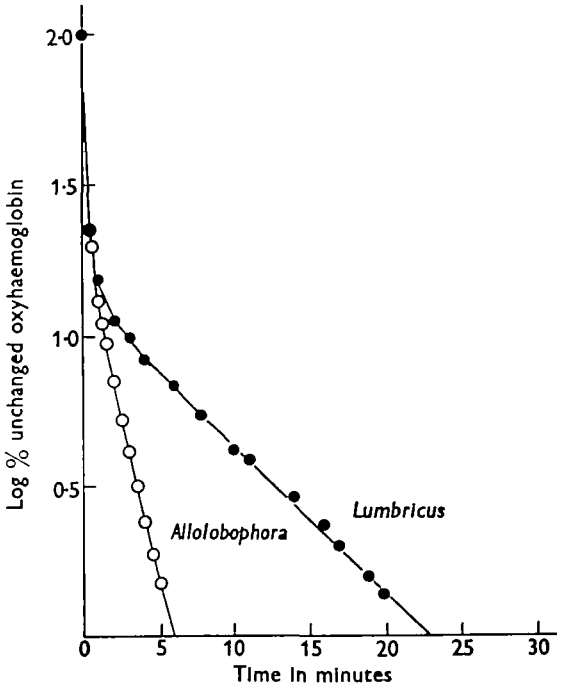

Fig. 6. Rate of alkaline denaturation of haemoglobin solutions from *Lumbricus* and *Allolobophora*. *Allolobophora* haemoglobin denatures more rapidly than does that of *Lumbricus*.

DISCUSSION

The oxygen dissociation curves of earthworm haemoglobin have not previously been determined. The curves described in the present paper agree fairly closely with those recorded for other annelids, though in some cases other workers have used diluted bloods and worked at different temperatures, all of which tend to alter the position of the curves. Some figures that can be roughly compared are as follows. The partial pressure of oxygen necessary for 50% saturation is 2 mm. Hg for *Lumbricus*, 0·7 mm. Hg for *Allolobophora*, 1·5 mm. Hg for *Arenicola*, 0·5mm. Hg for *Tubifex* (Fox, 1945), 5·5 mm. Hg for *Nephthys* (Jones, 1955) and less than 1 mm. Hg for *Alma* (Beadle, 1957). These values are generally appreciably lower than those recorded for vertebrate haemoglobins (Prosser, Bishop, Brown, Jahn & Wulff, 1950).

It is interesting to consider whether the difference in the dissociation curves of *Lumbricus* and *Allolobophora* are related to differences in the physiological behaviour of the two species.

In general, earthworms inhabit the top layers of the soil but we noticed in digging for worms that as the summer drew on *Allolobophora* became more difficult to find, while *Lumbricus* remained quite common. Evans & Guild (1947) showed that during the summer *Allolobophora terrestris* goes into aestivation, curling up into a tight ball 1–2 ft. below the surface of the ground. Baweja (1939) stated that the soil temperature follows the atmospheric temperature down to about 8 in. below the surface, while the temperature is roughly constant throughout summer and winter at a depth of 3 ft. It is possible that the behavioural difference could be due in part to respiratory difficulties, but the difference in the curves for the blood pigments is in fact greater at a lower temperature than at a higher one. Consequently no correlation can be seen between the respiratory pigment properties and the physiological behaviour of the earthworms. It seems possible that the summer aestivation is affected by some other factor such as the availability of water; *Allolobophora* has a different nephridial pattern from that of *Lumbricus* (Goodrich, 1945).

In many animals such as the sheep, goat and cow, the haemoglobin of the foetus has a dissociation curve lying to the left of that for the adult. Brinkman & Jonxis (1937) showed that the foetal haemoglobin was less resistant than adult haemoglobin to alkaline denaturation. Similarly, in earthworms the dissociation curves of *Allolobophora* lie to the left of those of *Lumbricus*, and the haemoglobin is less resistant than *Lumbricus* haemoglobin to alkaline denaturation.

It is not clear whether the alkaline resistance of haemoglobin is directly related to the oxygen affinity of the haemoglobin especially when considered intergenerically. What is reasonably clear is that it does provide a method of distinguishing between types of haemoglobins and in this it has shown that the *Allolobophora* and *Lumbricus* haemoglobins differ.

We hope at a later date to examine the paramagnetic resonance of haemoglobin crystals from *Lumbricus* and *Allolobophora* blood in the hope that this too will throw further light on the different properties of the haemoglobins (Bennett, Gibson, Ingram, Haughton, Kerkut & Munday, 1957).

SUMMARY

1. A method is described for determination of the oxygen dissociation curves of the blood of two species of earthworm, *Lumbricus terrestris* and *Allolobophora terrestris*.

2. The following values have been obtained for the partial pressures of oxygen (mm. Hg) required to saturate the blood:

	50 % saturation		95 % saturation	
	7° C.	20° C.	7° C.	20° C.
Lumbricus	2	8	9	22·5
Allolobophora	0·7	6	3·75	17·5

3. Times for alkaline denaturation at pH 12·7 have been found as follows: *Lumbricus terrestris* 23 min., *L. rubellus* 2·5 min., *L. festivus* 11·5 min., *Allolobophora terrestris* 6 min., *Eisenia foetida* 5 min.

4. These results indicate definite differences in the properties of the haemoglobin of the species examined.

This investigation has been supported by a grant from the Medical Research Council to one of us (K.A.M.).

REFERENCES

BARCROFT, J. & KING, W. C. R. (1909). The effect of temperature on the dissociation curve of the blood. *J. Physiol.* **39**, 374–84.

BAWEJA, K. D. (1939). Studies on the soil fauna, with special reference to the recolonisation of sterilized soil. *J. Anim. Ecol.* **8**, 120–61.

BEADLE, L. C. (1957). Respiration in the African swampworm *Alma emini* Mich. *J. Exp. Biol.* **34**, 1–10.

BENNETT, J. E., GIBSON, J. F., INGRAM, D. J. E., HAUGHTON, T. M., KERKUT, G. A. & MUNDAY, K. A. (1957). The investigation of haemoglobin and myoglobin derivatives by electron resonance. *Physics Med. Biol.* **1**, 309–20.

BRINKMAN, R. & JONXIS, J. H. P. (1937). Alkali resistance and spreading velocity of foetal and adult types of mammalian haemoglobin. *J. Physiol.* **88**, 162–6.

EVANS, A. C. & GUILD, W. J. MCL. (1947). Studies on the relationships between earthworms and soil fertility. I. Biological studies in the field. *Ann. Appl. Biol.* **34**, 307–30.

FOX, H. MUNRO (1940). Functions of chlorocruorin in *Sabella* and haemoglobin in *Lumbricus*. *Nature, Lond.*, **145**, 781–2.

FOX, H. MUNRO (1945). The oxygen affinity of certain invertebrate haemoglobins. *J. Exp. Biol.* **21**, 161–5.

GOODRICH, E. S. (1945). The study of nephridia and genital ducts since 1895. *Quart. J. Micr. Sci.* **86**, 115–392.

GREEN, A. A. (1933). The preparation of acetate and phosphate buffer solutions of known pH and ionic strength. *J. Amer. Chem. Soc.* **55**, 2331–6.

HILL, R. & WOLVEKAMP, H. P. (1936). The oxygen dissociation curve of haemoglobin in dilute solutions. *Proc. Roy. Soc.* B, **120**, 484–95.

JOHNSON, M. L. (1942). The respiratory function of the haemoglobin of the earthworm. *J. Exp. Biol.* **18**, 266–77.

JONES, J. D. (1955). Observations on the respiratory physiology and on the haemoglobin of the polychaete genus *Nephthys*, with special reference to *N. hombergii*. *J. Exp. Biol.* **32**, 110–25.

LEMBERG, R. & LEGGE, J. W. (1949). *Hematin Compounds and Bile Pigments*. New York: Interscience.

MACELA, I. & SELISKAR, A. (1925). The influence of temperature on the equilibrium between oxygen and haemoglobin of various forms of life. *J. Physiol.* **60**, 428–42.

PROSSER, C. L., BISHOP, D. W., BROWN, F. A., JAHN, T. H. & WULFF, V. J. (1950). *Comparative Animal Physiology*. Philadelphia: W. B. Saunders Co.

RAMSEY, H. J. (1941). A comparative study of haemoglobin denaturation. *J. Cell. Comp. Physiol.* **18**, 369–77.

REDFIELD, A. C. (1930). The equilibrium of oxygen with the hemocyanin of *Limulus polyphemus* determined by aspectrophotometric method. *Biol. Bull., Woods Hole*, **58**, 238–55.

RODDIE, I. C., SHEPHERD, J. T. & WHELAN, R. F. (1956). A photometric method for the rapid measurement of blood oxygen saturation and capacity. *J. Physiol.* **131**, 2*P*.

VOGEL, A. I. (1945). *A Textbook of Quantitative Inorganic Analysis*. Longmans Green and Co. Ltd.