

THE FUNCTION OF HÆMOGLOBIN IN INVERTEBRATES WITH SPECIAL REFERENCE TO PLANORBIS AND CHIRONOMUS LARVÆ. By I. LEITCH, B.Sc., *Carnegie Scholar of Aberdeen University.*

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WINTERSTEIN, in his *Handbuch der vergleichenden Physiologie*, in the chapter on Molluscs (p. 88), is of opinion that the presence of hæmoglobin in some Lamellibranchiates and its absence in other nearly related species is completely obscure; and this may well be taken as an expression of the general verdict regarding the function of hæmoglobin in the Invertebrates.

Let us first collect the information as to its occurrence. The presence of hæmoglobin is first definitely established in worms. Many Chætopods, some Nemertines, Hirudinea and Gephyrea have hæmoglobin either in solution or in blood corpuscles. In Molluscs some Lamellibranchs and one Gastropod, namely Planorbis, possess hæmoglobin. A few Crustacea belonging to the Brachiopoda, Ostracoda and Copepoda: the larva of *Musca* and many species of *Chironomus* complete the list. It will be evident at once that relationship has no part in determining the presence of hæmoglobin, and that, if there be any common factor, it must be sought for from the physiological and not from the morphological standpoint.

Regarding the function of hæmoglobin Winterstein quotes with reference to worms, many experiments to demonstrate the greater power of resistance to lack of oxygen of those possessing respiratory pigments: for example, that Pütter has kept *Hirudo* for ten days in pure nitrogen without injury. He goes on: "A great number of worms are mud-dwellers. That their great power of resistance to lack of oxygen is correlated with their life in this badly oxygenated medium can scarcely be doubted. Bunge has expressed the suggestive idea that, only because of their having passed through the 'preparatory school' of oxygen-want as mud-dwellers are worms capable of living quite

anaerobically in the oxygen-free alimentary canals of higher animals. On the other hand, Lankester has tried to correlate the normal habitat of worms with the formation of respiratory pigments, and for example, expresses the view that only its wealth of hæmoglobin enables the Annelid, *Tubifex*, to live at the bottom of the polluted water of the Thames. Probably, in fact, the two apparently contradictory aspects can be referred to one cause, namely that the adaptation to a medium poor in oxygen led, on the one hand, to a high capacity for anaerobic life, and on the other, to oxygen-binding pigments, the possession of which must be of great advantage under just these circumstances." With reference to Molluscs he quotes similar experiments and observations by Lankester and Miall, Cuénot and Schneider, to show that these are very resistant to lack of oxygen, and indicates a parallel with worms in that their normal habitat is stagnant water or mud. He refers again to Lankester's and Miall's suggested interpretations. Miall's suggestion in his *Natural History of Aquatic Insects* is as follows: "A list of the many animals of all kinds which contain hæmoglobin shows that for some reason or other each of them requires to use oxygen economically. Either the skin is thick, or the respiratory surface limited, or they are enclosed in a shell, or they burrow in earth or mud." He goes on to say that, however, not all animals that have in this way limited facilities for respiration, possess hæmoglobin. "The fact is that the respiratory needs, even of closely allied animals, vary greatly, and further, there are more ways than one of acquiring and storing up oxygen in their bodies." And he continues: "Either the storage capacity for oxygen of the *Chironomus* larva is considerable, or it must be used very carefully, for the animal can subsist long without a fresh supply."

As the sum of this we have, then, one fact, viz. that the presence of hæmoglobin among invertebrates is in all cases correlated with a habitat deficient in oxygen: and one suggestion, viz. that the hæmoglobin acts as a store of oxygen. The fact leads to the first question, the truth of which one is tempted to assume, since we are constantly discovering and re-discovering the exquisite economy of Nature,—the question of whether the hæmoglobin is of use. In the second place there is the question of how the hæmoglobin is used. Does it function as Miall suggests as a store, or how?

Before going on to the experiments by which I have tried to answer these two questions, let me recapitulate the chief points in what we know of hæmoglobin from mammals. The summary is made from the

first five chapters of Barcroft's *The Respiratory Function of the Blood*.

We know that hæmoglobin forms a chemical compound with oxygen, oxyhæmoglobin, in the proportion of 32 gr. oxygen to 56 gr. iron. But in the circulation it is never united with as much oxygen as it could be. There are present oxygen, oxyhæmoglobin and hæmoglobin and (at any given constant temperature) the relative proportions of oxyhæmoglobin and hæmoglobin will vary according to the tension of oxygen present. A dialysed solution of hæmoglobin, in the presence of different tensions of oxygen, will give different percentages of oxyhæmoglobin and hæmoglobin, which plotted with the relative tensions of oxygen give a rectangular hyperbola, the dissociation curve of hæmoglobin. For each different temperature there will be a different hyperbola, such that a rise in temperature will mean a lower percentage saturation at the same oxygen tension.

The presence of salts causes aggregation of the hæmoglobin molecules, altering the equation to the dissociation curve, and making the curve S-shaped instead of a rectangular hyperbola. The result of this is obviously, that hæmoglobin in blood, which is a solution of salts, will take up oxygen more slowly than dialysed hæmoglobin, but will part with it more readily.

The presence of carbon dioxide, or other acids, *e.g.* lactic acid, lessens the effective concentration of the hæmoglobin: that is to say, in the presence of CO₂ the blood will be less saturated at the same oxygen pressure. In the absence of CO₂ the blood will take up oxygen more quickly than in its presence, and in its presence it will give up oxygen more quickly than in its absence.

My experiments have been made on *Planorbis* corneus, and on *Chironomus* larvæ. *Planorbis* is too well known to need any general description. I used a variety without pigment in the skin. Let me recall the following points regarding its anatomy. It breathes by a lung, which, since it lives in water, can be opened and closed at will. The opening formed by an ingrowth of the mantle edge to meet a similar ingrowth from the neck, is protrusible and projects above the surface-film when the animal breathes. On the inner surface of the mantle lies the two-chambered heart and a rich net of blood vessels. From the body generally, the blood is collected in a large vein which lies along the mantle edge beside the rectum, and from which numerous branches spread over the surface of the mantle. Here the blood becomes arterial and is collected by the vena pulmonis going to the auricle.

The blood from the heart is distributed by two main aortæ, the one supplying the head, pharynx, stomach, etc., and giving off an artery to the foot: the other supplying the viscera generally. The blood vessels form ridges on the mantle surface, and the epithelium over them is ciliated.

Chironomus is one of the commonest of insect larvæ, abounding in ditches and dirty streams, feeding on decaying vegetable matter, living in burrows in the mud lined with earth and leaves glued together by the secretion of their salivary glands. They are conspicuous by their bright red colour, and the surface of the mud where they burrow is as easily recognisable, dotted over with small holes about 1.5 mm. across and each with a little raised rim round the edge. At times the larvæ push their tails out and wave them about, possibly, as Miall says, "as a help to respiration." While they are completely concealed within their tubes an undulatory movement is kept up which no doubt helps to renew the water. According to Miall again, the last segment but one bears two pairs of flexible tubes filled with blood which is constantly renewed by the pulsation of the heart. The heart lies near the posterior end and dorsally. The blood enters by two valves and is driven by the contraction of the heart along a narrow dorsal vessel to the head. Here it escapes into the body-cavity and ultimately returns to the heart. The tracheal system is rudimentary and completely closed.

It has been established by several observers that, in well aerated water, Planorbis and other Limnæidæ have no need to renew the supply of air in the lung. "Skin respiration" is sufficient. To settle therefore, whether on the whole Planorbis uses its hæmoglobin, and if it be used when and how it is used, I performed experiments on the following plan. There were two series of experiments: a series in which the snails were placed in a flask half-filled with water through which air could be passed at a convenient rate, and with a known tension of oxygen, together with a parallel series where to the air was added 1% carbon monoxide. In the presence of this gas the hæmoglobin is rapidly converted to carboxyhæmoglobin, ceases to be of any use as a respiratory pigment and we have Planorbis, physiologically considered, without hæmoglobin. It will at once be clear, that, all other things being equal, if there be a difference in the behaviour of the snails between the two cases, it must be due to the active presence of hæmoglobin in the former. In the one case the amount of oxygen available for respiration will be the amount physically dissolved in the

blood, plus the amount chemically bound by the hæmoglobin: in the other, only the amount physically dissolved.

The method of experimentation was this. A two-litre bottle containing air with the desired oxygen tension was placed in connection with, on the one hand, a similar bottle with water, and on the other, the flask, half-filled with water containing the snails. The air was first bubbled rapidly through and the flask shaken well to ensure that the oxygen tension in the water of the flask should be the same as that of the mixture. The bottles were then reversed, and the mixture started again to bubble through at such a rate as to continue for approximately a day. At the end of that time, if further observations were desired, the bottles were reversed again with care not to admit atmospheric air during the reversing. During the experiments the snails moved about on the surface of the glass, and on applying a spectroscope over the extended foot against a strong gas light, the oxidation or reduction of the blood could at once be observed.

It appeared then that, for the normal snails,—that is without carbon monoxide,—down to an oxygen tension of 7.7% the blood of the snails is always saturated. They seldom or never came to the surface to breathe. At a tension of 7.2 the blood, as observed in the foot, is reduced and from that point down to a tension of 3% they move about a great deal, and come more and more frequently to the surface to renew the air in their lungs. When the tension falls below 3% they remain steadily at the surface, moving little and breathing frequently. There is no further change to 1%. In the parallel series with carbon monoxide, that is, where the snails are functionally without hæmoglobin, the behaviour is similar down to 3%. From 7% to 3% the animals are very active, much at the surface, and breathe frequently. But below 3% there is a difference. Here the snails become more and more sluggish, move very little and come to the surface slowly, at long intervals. At 2% they were not observed to come to the surface, and at 1% they are extended from their shells and float about in the water.

From these data it is evident that at tensions down to 7% *Planorbis* never makes use of its hæmoglobin: the hæmoglobin is always saturated. Further it does not require to use its lung: diffusion through the surfaces exposed to the water is sufficiently rapid to supply the needs of the animal. Below 7% however, the oxygen available by physical solution in the blood is no longer sufficient: the hæmoglobin is reduced in the foot, that is it is constantly in use, and, to accelerate

the acquisition of oxygen, the lung is frequently filled with air. It is clear that, so long as the hæmoglobin is constantly saturated, there is no advantage to be had in renewing the air in the lung: but, so soon as the blood becomes reduced, it is of the greatest importance for its rapid oxidation that the lung with its rich net of veins and comparatively great facilities for diffusion should be repeatedly filled with fresh air.

Between 3% and 1%, in the presence of hæmoglobin as well as in its absence, the snails become less active: but whereas by decreasing their activity, remaining at the surface and breathing with great frequency, they can with the aid of hæmoglobin maintain a normal condition, in the absence of hæmoglobin they are at 3% already incapable of doing so and go into a more or less latent condition. At a tension of 1% they cannot, without the aid of hæmoglobin, supply even the small amount of air required in such a latent condition and float about in an obviously abnormal state. It is evident of how great importance to *Planorbis* the possession of hæmoglobin is, since in the stagnant pools which it normally inhabits oxygen tensions from 7% downwards are common.

For the *Chironomus* larvæ a similar method of procedure was impossible because the aim of such a series of experiments would be to produce given tensions in the mud in which they live, but to draw any conclusion as to the oxygen tension in the mud from the oxygen tension of a mixture over it seemed too dubious a course, and where any doubt could exist under conditions as marginal as these, the experiments would be without value. An altogether different method had therefore to be adopted, offering, it is true, a picture less complete than that just presented for *Planorbis*, but still easily interpreted in ecological terms. The larvæ were placed in small glass cells with just enough water to prevent their being dried up, but not enough to allow much movement. The cells were made air-tight with a cover-glass as lid, and a current of air of known composition was passed through them.

To determine the amount of saturation of the blood, an Engelman micro-spectroscope was used where the spectrum of the larval blood (the larvæ, on account of their transparency, are easily examined directly) could be compared with superposed spectra of oxyhæmoglobin and hæmoglobin. The oxyhæmoglobin and hæmoglobin were in a trough divided diagonally so as to give two identical wedges of which the thick end of the one coincides with the thin end of the other. The

spectrum from the blood of the larvæ was matched with a point in the spectrum from the trough, which graduates from oxyhæmoglobin at the one end to reduced hæmoglobin at the other, and from a graduated scale the percentage saturation read off. This method and others to be mentioned are due to Prof. Dr August Krogh and will be fully described by him. The method was found by control experiments to be correct to 2%.

The results obtained were that at 7 mm. oxygen pressure, or about 1%, the blood of the larvæ is little, if at all, reduced. At 5 mm. oxygen tension it was 60% saturated, and at 2.9 mm. only 16.5% saturated, all at a temperature of 17° C. It appears then that down to 7 mm. oxygen tension the larvæ do not use their hæmoglobin at all, but from about 6 mm. downwards it is in use.

In reality, in such putrescent mud as the larvæ inhabit, there will normally be an appreciably high carbon dioxide tension. For analyses of air-bubbles from the sea-bottom tensions of 1.7% and 3.7% carbon dioxide have been found, with oxygen tensions of .2% and .6%, *i.e.* about 1.5 mm. and 4.5 mm. The saturation of the blood was therefore determined at 5 mm. oxygen and 2% carbon dioxide. The result was 48% saturation as contrasted with 60% without carbon dioxide. It is probable then that the larvæ begin to use their hæmoglobin at a slightly higher tension than the 6 mm. determined above, and since the oxygen tension can as quoted be as low as 1.5 mm. with a carbon dioxide tension of 1.7%, it is evident that the possession of hæmoglobin is suited just to the utilisation of these low tensions which exist in such unaerated mud.

From these two series of experiments it must strike one immediately that the hæmoglobin does just that work it is required to do, but that its behaviour is quite different in the two cases. In *Planorbis* the tensions used are from 7% downwards, and in *Chironomus* from 7 mm., or about 1%, down to some unknown point at which limit the larva would probably leave its burrow and go in search of air. This indicates a great difference in the dissociation curves of the two animals, so I set to work to determine them approximately.

The blood of *Planorbis* is best obtained by simply making an incision in the foot. Violent retraction follows and the blood is pressed out. From *Chironomus* it is of course obtained at once by cutting the larva across. The blood was introduced into a small tonometer which could be conveniently examined under the micro-spectroscope and kept at a constant temperature. Air of known composition was passed through

until equilibrium was established and the percentage saturation found, as for the larvæ, by comparison with the superposed spectra of oxyhæmoglobin and reduced hæmoglobin. The experiments gave the following results:

Planorbis.

Temperature	CO ₂ tension	O ₂ tension	Percentage saturation
20° C.	0	10·8 mm.	> 70*
20° C.	0	7·4 mm.	56
20° C.	2%	10·8 mm.	65
20° C.	2%	7·4 mm.	45

* Estimations are accurate only up to 70%.

Chironomus.

Temperature	CO ₂ tension	O ₂ tension	Percentage saturation
20° C.	0	·2 mm.	48
20° C.	0	·17 mm.	50
20° C.	1·25%	·25 mm.	39
20° C.	1%	·17 mm.	38

If reference be made to Barcroft's Fig. 12, giving the curves for dialysed and undialysed hæmoglobin, and Fig. 17 giving the curves for dialysed hæmoglobin at different temperatures, it will be seen that the values for *Planorbis* indicate a curve between the curves III and IV: that is the values for *Planorbis* would fall on the curve for a temperature between 32° and 38°. The figures for *Chironomus* indicate a curve similar to curve I.

Two experiments were made on the blood of *Planorbis* at different temperatures, one at 11·8° C. and one at 6° C. The results were:

Temperature	O ₂ tension	Percentage saturation
11·8° C.	2 mm.	40
6° C.	1 mm.	54

From these experiments it is apparent that the effect of temperature is, as was to be expected, such that a rise of temperature means a fall in percentage saturation at the same oxygen tension.

Let us return to our starting-point. We have now demonstrated the truth of Lankester's observations that the possession of hæmoglobin is closely connected with the habitat of *Planorbis* and *Chironomus* in badly aerated water. We have demonstrated that the hæmoglobin is present in solutions such that, in each case, it is adapted for the utilisation of just those low oxygen tensions to which the animals are likely

to be subjected. In order to test Miall's suggestion that the hæmoglobin functions as a store of oxygen we require to know first, how much oxygen the hæmoglobin can store, and second, how much the animals use. That is to say, we require an estimation of the oxygen capacity of the blood and of its amount, and a determination of the rate of gas exchange for each of the animals.

Estimations of the hæmoglobin concentration for *Planorbis* by Haldane's hæmoglobinometer were made by Dr Marie Krogh and myself. These estimations we regarded however as unsatisfactory on account of the impossibility of matching the colours exactly, and Dr Krogh showed that this is due to the presence of another, lipochrome, pigment. Our estimations of the oxygen capacity of the blood of *Planorbis* gave 1.5 and 1.2 volumes p.c. An estimation by Barcroft's method gave .9 volumes p.c.—a lower value as we expected. For *Chironomus* the concentration of the blood was estimated in a micro-colorimeter, by comparison under the microscope with solutions of known strengths. The method on testing was correct to 2%. One larva gives enough blood for an estimation. The average oxygen capacity for a large number of larvæ was found to be 6 c.c. p.c.

The quantity of blood was found by weighing the animals intact, and again after removing as nearly all the blood as possible. The results were, for *Planorbis*, that the blood is a third of the weight of the snail, and for *Chironomus* a half, in both cases an enormously high proportion in comparison with vertebrates.

Finally in Prof. Krogh's micro-respiratory apparatus estimations were made of the gas exchange. The figures showed that a snail uses 1.09 c. milligrams of oxygen per gram per minute, and that *Chironomus* uses 2.675 c. milligrams of oxygen per gr. per min. We have now all the data necessary for the consideration of hæmoglobin as a store of oxygen. Let us begin with *Planorbis*.

A snail weighing .9 gr. used .98 c. mgr. of O_2 per min. It possessed 33% of blood with an O_2 capacity of .9 c.c. p.c., *i.e.* to a sufficiently accurate approximation it possessed .3 c.c. of blood which would therefore contain .0027 c.c. of oxygen when saturated. Now it uses .00098 c.c. per min., so that the length of time in minutes for which the total amount of oxygen the blood is capable of bearing can suffice is given by $\frac{.0027}{.00098}$, *i.e.* for approximately three minutes. In this case the utility of hæmoglobin as a store is evidently very small.

For *Chironomus*: ten larvæ weighing .16 gr. used .428 c. mgr. of oxygen in 1 min. *Chironomus* has 50% of blood of an oxygen capacity

of 6 c.c. p.c., *i.e.* these ten larvæ had .08 c.c. of blood whose total oxygen capacity was .0048 c.c. oxygen. So as above the time for which this would suffice as a store is given by $\frac{.0048}{.000428}$, that is to say for about 12 mins. Here owing to the large amount of blood which the larvæ possess and its high concentration, the time is longer, but even so, it is clear that from the point of view of a practical store of oxygen, its usefulness is insignificant. One cannot seriously talk of a "storage capacity" for 3 or 12 minutes. The animals neither store oxygen in the sense in which Miall meant it (he was thinking of his experiment where a larva lived for *five days* in boiled water), nor do they use it carefully.

CONCLUSIONS.

In Planorbis and Chironomus the function of the hæmoglobin consists in making available, by its power of binding oxygen chemically, a quantity of oxygen sufficient for the needs of the animals at oxygen tensions so low that the necessary amount is not supplied by physical solution. In Planorbis the mode of action is as in Vertebrates: it depends not at all on a power of "storing" oxygen, but wholly on the constant circulation of a current of alternately oxidised and reduced blood between the lung and the tissues. In Chironomus it is even simpler, but quite similar: namely, the constant binding of oxygen at the surface of the body and the constant giving up of it in the interior: a continuous mixing and interchange of oxidised and reduced blood kept in motion by the beating of the heart. And the only difference is that in each case the hæmoglobin is present in just such a solution as enables it to utilise these low tensions of oxygen to which it is liable to be subjected.

These experiments were planned by Professor Krogh, and my most sincere thanks are due to him for his unfailing counsel and kindness during the performance of them.