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Haemoglobin of *Gastrophilus* Larvae. Purification and Properties

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(*Received* 25 *May* 1946)

The object of this paper is the study of the haemoglobin of the larvae of *Gastrophilus* (horse bot fly) which live as parasites in the stomach of the horse. As the haemoglobin occurs only in certain stages of the development of this insect and is localized in highly specialized cells, a brief account will be given of the life history of *Gastrophilus intestinalis* and the structure of the cells harbouring the pigment.

Our knowledge of the life history of these flies is based partly upon an extensive study of the parasite in its normal host and partly upon its behaviour in small experimental animals (see Dinulescu, 1932). The latter applies especially to the stages immediately following the emergence of the larvae from their eggs. The life history of *Gastrophilus* thus reconstructed can be summarized as follows (Keilin, 1944):

'The female fixes its eggs to the hairs of the horse, usually those of the legs, breast and abdomen. In about 5–6 days the larvae are completely formed but they emerge only on contact with the tongue of the host and penetrate under its epithelial layer, forming a superficial gallery. They migrate towards the pharynx, undergo the first moult, leave this gallery and become attached to the mucosa of the pharynx, especially under the epiglottis. They stay in this position only for a short time and then migrate into the stomach where they are found attached by means of their mouth hooks to the mucosa of the cardiac region. The larvae undergo a second moult and pass into the third stage which is usually reached within 45–60 days after the first moult. The larva grows rapidly and full-grown larvae are found already between October and November. They remain, however, in a quiescent stage at least 7–8 months, until May or June, and during this long period of quiescent life they feed very little and their respiratory activity is at its lowest. The larvae finally leave the alimentary tract, burrow themselves into the soil or crevices in the ground, and pupate. The adult fly emerges from the pupa within 20–30 days.'

During the first period of life until the larva reaches the stomach its respiratory requirements are well satisfied by the rich oxygen supply in the surrounding medium. During the next 7–8 months of life within the stomach of the host the larva is immersed in a semi-fluid medium deficient in oxygen and depends on an intermittent contact with air bubbles mixed with ingested food. The respiratory system of the larva is, nevertheless, highly developed and specialized for the aerobic mode of life. It exhibits several important features which enable the larva to overcome the difficulties inherent in its peculiar surroundings. One of those features is the presence of an intracellular haemoglobin.

The young larvae of *Gastrophilus* are of a uniformly red colour which, owing to the great transparency of the cuticle, shows that the haemoglobin is not restricted to one kind of tissue. In fact, on dissection the larva shows the presence of haemoglobin in the whole of the fat body, parietal musculature and hypoderm. As the larva grows the haemoglobin becomes localized in special cells forming a large red mass almost entirely occupying the posterior third of the body. These cells, which are known as tracheal cells, form an integral part of the respiratory or tracheo-spiracular system of the larva. Their distribution in the body, their connexion with the tracheal system, their structure and development were dealt with in papers by Enderlein (1899), Prenant (1900), Portier (1911), Kemnitz (1916), Dinulescu (1932) and Keilin (1944). Of the five pairs of tracheae which originate from postabdominal spiracles, four pairs are exceptionally wide at their base but rapidly decrease in diameter, thus acquiring a distinctly conical shape. All along their surface they give off numerous branches of smaller tracheae which ramify and finally break up into numerous tufts of intraprotoplasmic tracheoles filling the large tracheal cells (Fig. 1 A, B). The latter vary somewhat in size but the majority of them reach about 350–400 μ. in diameter, thus being visible to the naked eye. These cells are of a distinctly red colour owing to the presence of oxyhaemoglobin and are known as haemoglobin or tracheal cells. They probably originate from fat-body cells by gradual differentiation and invasion by tracheae (Dinulescu, 1932). In addition to haemoglobin, these cells are

very rich in glycogen and have a strong succinic-cytochrome system. In a much lower concentration haemoglobin is also present in cells of the fat body, muscles and proventriculus. All these tissues in very young larvae contain a much higher concentration of haemoglobin than in the older larvae. The haemoglobin does not, however, seem to disappear from these cells, but since its amount remains the same per cell, its concentration gradually diminishes in proportion to the growth of these cells.

formed, together with a large amount of glycogen liberated from the tracheal cells, makes further purification of the haemoglobin practically impossible.

Fresh blood of the larva, obtained by a small incision in its integument, is usually a clear yellowish solution almost free of haemoglobin. A full-grown larva of *Gastrophilus* weighing 0·37–0·44 g. contains between 0·1 and 0·13 ml. of blood. The blood of several hundred larvae collected in a vessel darkened

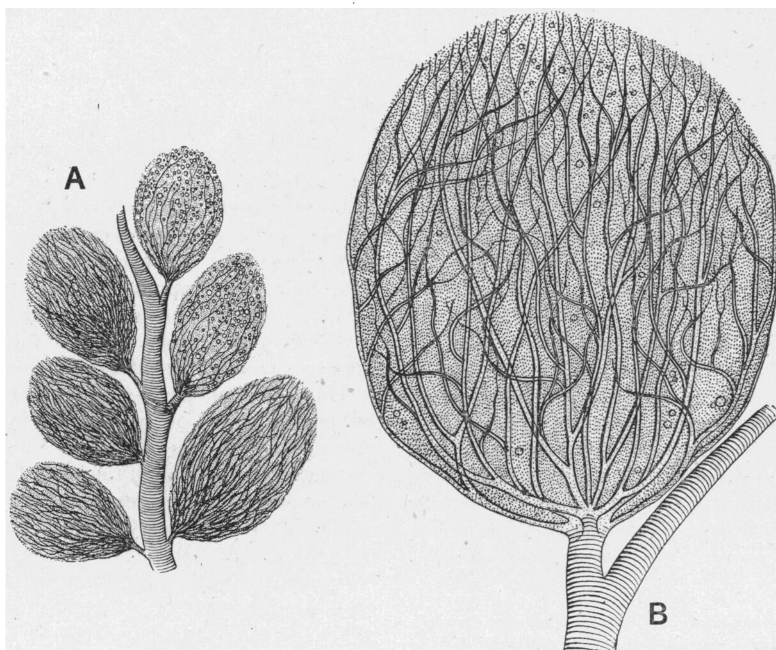

Fig. 1. Haemoglobin cells of a full-grown larva of *Gastrophilus intestinalis*. A, a bunch of red tracheal haemoglobin-containing cells, ×85; B, one tracheal cell showing more clearly the intraprotoplasmic tracheoles filled with air, ×200. The single central nucleus of the cell is not represented.

only on the surface, whereas the yellowish solution below the surface remained pale straw in colour. Later it gradually became opalescent and ultimately formed a very heavy flocculent precipitate.

The tracheal cells containing haemoglobin enable the larva to make better use of an intermittent contact with air bubbles by taking up each time a much larger amount of oxygen than the amount which could be dissolved in the peritracheal tissues, were they devoid of haemoglobin.

METHODS

Extraction and purification of haemoglobin

One of the greatest difficulties in the extraction and purification of haemoglobin from the tracheal cells is the presence in the perivisceral fluid or the blood of the larva of a strong phenol oxidase and its chromogen. On crushing the tissues of the larva in presence of blood, the pulp gradually darkens and the haemoglobin undergoes rapid oxidation to methaemoglobin. The melanin which is ultimately

Extraction. A successful extraction of haemoglobin from the tracheal cells can be obtained only when the latter are well washed of all traces of the blood which surrounds them. For this purpose the larvae are cut open longitudinally with a small pair of scissors from the anterior end to a point about 3–4 mm. from the posterior end of their body; care must be taken during this manipulation to avoid crushing the tracheal cells. The blood or perivisceral fluid of the larvae is allowed to drain off on a funnel and the bodies are washed by suspending them in physiological saline solution. Each larva is then stretched open and the red cluster of haemoglobin cells attached to their tracheae is cut out as one mass and dropped into physiological saline solution. The red masses of tracheal cells, usually attached by means of their tracheae to the postabdominal spiracles, are carefully washed free

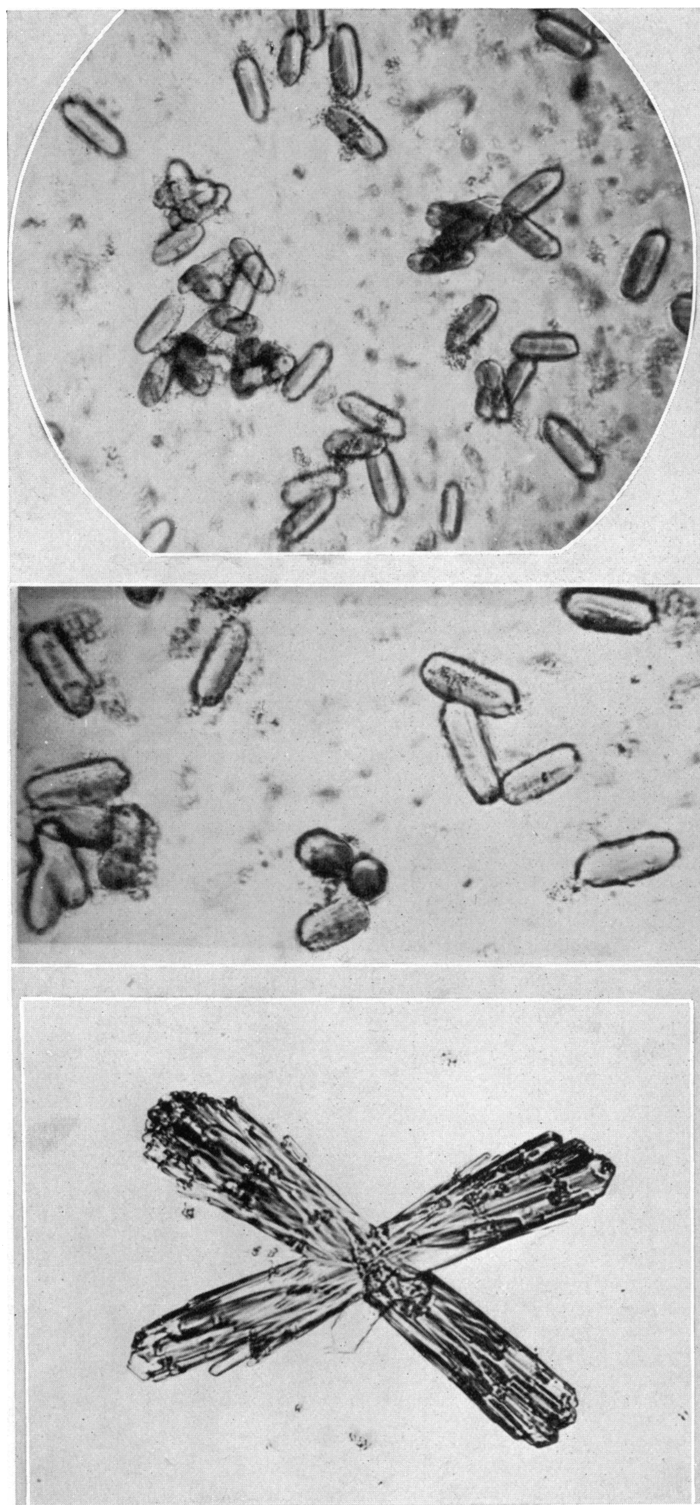

Fig. 2. Two types of crystals of *Gastrophilus* methaemoglobin. A, ×300; B, similar crystals, ×425; C, ×145.

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from the blood by several changes of saline solution and finally washed once in distilled water.

The washed cells are ground in a mortar with a little distilled water and the turbid red fluid strained through muslin. The residue in the muslin is returned to the mortar and extracted with more water, the manipulation being repeated three or four times until the residue is practically colourless. All the extracts are combined and saturated with CO, which tends to protect the haemoglobin from autoxidation to methaemoglobin. On centrifuging, the fluid shows a thin top fatty layer followed by the main bulk composed of bright red opalescent fluid and a thin layer of brownish deposit composed of cellular debris containing the complete cytochrome-succinic system and other intracellular enzymes. The opalescent red fluid is sucked off, saturated again with CO and treated with saliva in order to digest the glycogen. Within a few hours at room temperature the fluid becomes clarified, and this stock solution of haemoglobin was used for further purification by fractionation with ammonium sulphate. The yield of purified haemoglobin and the ease of preparation depend to some extent on the state of the tracheal cells which varies with the time of year. The best yield of pure haemoglobin is obtained in February or March when the tracheal cells contain very little fat. Later in the year the number of fat droplets is gradually increased and the material becomes less suitable for the preparation of pure haemoglobin. However, for spectrophotometric investigations or the study of the O_2 dissociation curve, haemoglobin purified to only 70 or 80% is more suitable than the completely pure pigment, since the latter has a tendency to undergo oxidation to methaemoglobin.

Fractionation with ammonium sulphate. The stock solution of haemoglobin was treated with solid $(NH_4)_2SO_4$ to 0·7 saturation and brought with N-NaOH to about pH 7. The mixture was cooled in ice and the precipitate when properly aggregated was filtered off and discarded. The filtrate was brought with $(NH_4)_2SO_4$ to 0·85 saturation. The precipitate was filtered off, dissolved in the minimum amount of water saturated with CO and dialyzed against running water until free from $(NH_4)_2SO_4$. If any precipitate was formed, it was filtered off and discarded. If the solution was not optically clear, the treatment with $(NH_4)_2SO_4$ was repeated and the fraction between 0·7 and 0·85 saturation again collected. The purity of haemoglobin thus obtained varies from 65 to 80%. The purity of different fractions was determined from their haemin content estimated as pyridine haemochromogen, assuming that the best crystalline preparations, which were free from amorphous material and contained 3·92% haemin, were 100% pure.

In other preparations of purified haemoglobin no precautions, such as CO treatment, were taken to avoid its oxidation to methaemoglobin. From 1200 larvae of *Gastrophilus*, a haemoglobin solution was obtained containing an equivalent of about 28 mg. haemin. The purity of the sample was about 76%. Such a sample was fractionated again with $(NH_4)_2SO_4$, giving the three fractions (A, B and C) shown in Table 1. Fractions A and B were combined, dialyzed, treated again with $(NH_4)_2SO_4$ to 0·7 saturation and neutralized. After being kept in the cold overnight, the precipitate was filtered off and discarded. The solution was brought to 0·75 saturation, and the precipitate from this fraction was collected and dialyzed to give a solution of about 99% purity, practically all in the form of methaemoglobin and containing altogether about 3·9 mg. haemin. A second fraction precipitated between 0·75 and 0·80 saturation reached 93% purity and contained 4·4 mg. haemin.

Table 1. *Properties of the three fractions of* Gastrophilus *haemoglobin separated by* $(NH_4)_2SO_4$ *precipitation*

Fraction	$(NH_4)_2SO_4$ saturation	Haemin (mg.)	Purity (%)
A	0 –0·75	10·3	84
B	0·75–0·78	6·0	85
C	0·78–0·85	4·8	75

A sample of the most highly purified haemoglobin when examined in the Tiselius apparatus was found to be a practically homogeneous protein with less than 5% of impurities.

Crystallization. Crystallization of *Gastrophilus* haemoglobin was achieved by two slightly different methods:

(1) Solid $(NH_4)_2SO_4$ was added to a concentrated sample of purified haemoglobin until the solution showed a fine precipitate. A few drops of water were added just to dissolve the precipitate, and the vessel, loosely covered, was placed in an ice-chest. Crystals of methaemoglobin appeared a fortnight later. Larger crystals were produced by redissolving these crystals in the minimum amount of water and seeding the fluid with the above crystals (Fig. 2 A, B).

(2) A salt-free concentrated solution of purified haemoglobin was dialyzed against a saturated solution of $(NH_4)_2SO_4$ until precipitation was almost complete. The suspension was centrifuged, the precipitate collected, transferred to a small cellophan tube and dialyzed in a small beaker against 0·6 saturated $(NH_4)_2SO_4$. The beaker was placed in a small desiccator containing P_2O_5 and kept in the ice-chest. A few days to several weeks later large crystals of haemoglobin appeared in the dialysis sac (Fig. 4 C).

RESULTS

Properties of Gastrophilus *haemoglobin*

Haematin content and equivalent weight. The haematin content of *Gastrophilus* haemoglobin was determined on four purified preparations, two of which were composed of crystalline material. These samples were dialyzed against distilled water until completely free from $(NH_4)_2SO_4$, the slight precipitate which appeared after dialysis being filtered off and discarded. The dry weights of the samples were determined in the usual way, and their haematin contents were estimated as pyridine haemochromogen either spectrophotometrically or by means of a microspectroscope and the double-wedge trough, using as standard a solution of pyridine haemochromogen prepared from pure haemin. Estimated in this way, the haemin contents of the above samples were 3·65, 3·73, 3·85 and 3·92%, the highest value being probably that of the pure preparation. These results indicate that the equivalent weight of *Gastrophilus* haemoglobin is about 17,300, which is very near the values obtained for blood haemoglobin and myoglobin of vertebrates.

The molecular weight of *Gastrophilus* haemoglobin as shown by Adair, Johnston & Ogston (1946) is 34,000 ± 3000. It is very near the molecular weight of haemoglobin of two other invertebrates, the bivalve mollusc *Arca* and the larva of *Chironomus* which, according to Svedberg & Pedersen (1940), are 33,500 and 31,500 respectively. The molecule of *Gastrophilus* haemoglobin is therefore half the size of that of the blood of its host and contains two instead of four haem nuclei.

Stability. The stability of the haemoglobin was determined by mixing a salt-free solution of the pigment with equal volumes of 0·1 M buffer solutions of different pH. For the alkaline range, borax and NaOH-Na_2HPO_4, and for the acid range sodium acetate-acetic acid buffers were used. The solutions were allowed to stand at room temperature and examined spectroscopically at intervals. The spectroscopic observations were carried out either directly or after the addition of $Na_2S_2O_4$. In the latter case the intensity of the absorption bands of the haemochromogen determines the degree of denaturation of the pigment. It was found in this way that the haemoglobin is stable between *p*H 4 and 9·5. Outside that range it gradually undergoes denaturation, and at pH 11 a considerable degree of denaturation is obtained within a short period of time.

Isoelectric point. The isoelectric point of the haemoglobin was determined in a Michaelis electrophoresis cell. The haemoglobin solution was dialyzed for at least 24 hr. against 0·01 M-phosphate or acetate buffers of different pH, and the same buffer solutions were used for filling the upper arms of the cell. A current of 0·7–1·2 mA. at 220 V. was used, the observations being recorded every 30 min. for about 2 hr. Owing to certain irregularities in the arms of the cell and the fact that the experiments were carried out at room temperature, the results obtained cannot be considered as strictly accurate. However, the isoelectric point obtained was 6·20 ± 0·05 (Table 2).

Table 2. *Migration of* Gastrophilus *haemoglobin in electrophoresis cell at different* pH

pH	Migration towards	pH	Migration towards
5·44	Cathode	6·22	Anode
5·96	„	6·35	„
6·12	„	6·73	„
6·16	„	6·94	„
		7·32	„

Absorption spectra of Gastrophilus *haemoglobin and its derivatives*

The absorption spectrum of the oxyhaemoglobin was determined on samples of about 83% purity since more highly purified preparations of haemoglobin showed a tendency to oxidize to methaemoglobin. The measurements were carried out with a Hilger-Nutting visual spectrophotometer, the solutions of haemoglobin being placed in a Thunberg tube fitted with a 2 cm. optical cell. The haematin content of the preparation was estimated as pyridine haemochromogen. The results of these measurements

Table 3. *Position of absorption bands of* Gastrophilus *haemoglobin and its derivatives*

Haemoglobin and derivatives	α-band (mμ.)	β-band (mμ.)
Oxyhaemoglobin	581	545
CO-haemoglobin	571·5	540·5
Haemoglobin	558	
Methaemoglobin	637	

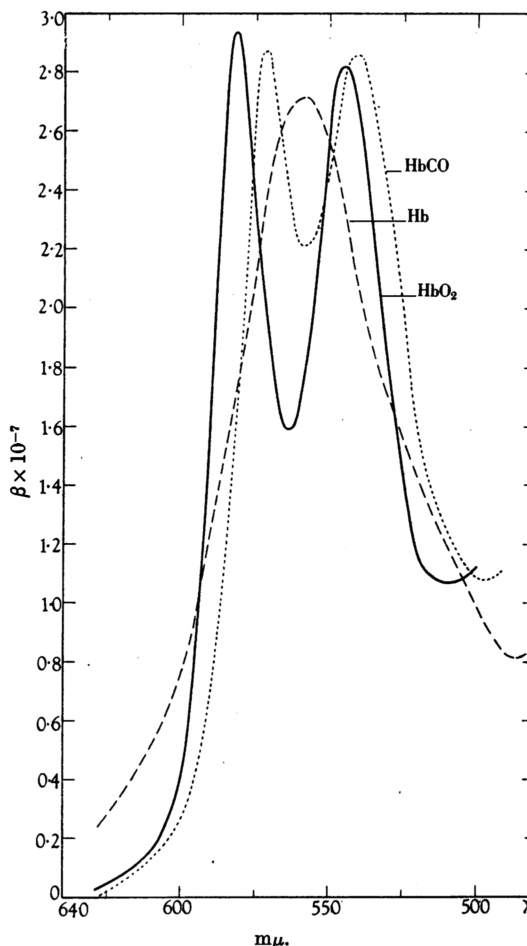


Fig. 3. Absorption spectra of *Gastrophilus* haemoglobin, oxyhaemoglobin and CO-haemoglobin. $\beta = \frac{1}{c \times l} \log_e \frac{I_0}{I}$, where c = concentration of haemoglobin in g.-atom Fe/ml., l = length of vessel in cm. and I_0 and I = intensities of incident and transmitted light respectively. Intervals between readings 1–10 mμ.

are shown in the curves of Fig. 3, and the positions of the maxima of the absorption bands in the visible region of the spectrum are shown in Table 3.

The spectrophotometric curve shows a somewhat low value for the height of the α-band of oxyhaemoglobin. Since the sample contained at least 2 % of methaemoglobin the real height of the α-band would reach about 3×10^7. The distance between the maximum absorption of the bands of oxyhaemoglobin and CO-haemoglobin, in other words, the 'span' (Anson, Barcroft, Mirsky & Oinuma, 1924) of this haemoglobin, is approximately 95·5 A. (Fig. 3 and Table 3). This is an unusually high value, met with so far only in the case of the haemoglobin in the nodules of leguminous plants (Keilin & Wang, 1945). In all other haemoglobins the span varies from about 48 to 56 A. (Anson *et al.* 1924). According to Roche (1932) and Theorell (1934*a*, *b*), on the other hand, the span of myoglobins has an exceptionally low value of about 32 A.

Determination of the oxygen dissociation curves

Since the amount of *Gastrophilus* haemoglobin available was always limited, the manometric or gasometric methods for the study of the oxygen dissociation were unsuitable and had to be replaced by a spectroscopic method. The method we adopted was based on that described by Hill (1936) and by Hill & Wolvekamp (1936), with certain modifications due to the somewhat different type of apparatus we used. The principle of this method consists in matching the absorption spectra of a solution of haemoglobin kept in a Thunberg tube and equilibrated at different pressures of O_2 with the absorption spectra of optical mixtures of oxyhaemoglobin and reduced haemoglobin kept separately in the two compartments of a double-wedge trough.

The solution of oxyhaemoglobin was completely deoxygenated by evacuation of the Thunberg tube, and definite amounts of O_2 were introduced into the tube in the form of an oxyhaemoglobin solution of the same concentration but saturated with air. In this way, whilst keeping the concentration of haemoglobin in solution constant, very small and perfectly measurable amounts of O_2 could be repeatedly introduced into the tube.

Description of apparatus. The oxygen dissociation curves were determined both for strong and weak solutions. The concentration of haemoglobin in a strong solution was about 1×10^{-3} g.-atom Fe/l., which is 50–75 % of the concentration in the tracheal cells, whereas its concentration in a weak solution was about one-tenth of that of the strong solution. To make possible the accurate spectroscopic examination of a very thin layer of the strong solution of haemoglobin, we used a modified Thunberg tube with an internal diameter of 1·5–2 cm. in the upper part and 1 cm. in the lower part (Fig. 4). The tube is provided with a glass plunger filled with distilled water and sealed. The plunger is 4 cm. long and about 0·88 cm. in diameter. It is slightly wider at both ends which are ground to fit closely the lower portion of the Thunberg tube. Two longitudinal grooves are cut in the surface of the enlarged portions of the plunger, thus allowing it to slide easily in and out of the terminal portion of the tube, displaçing the fluid which escapes through the grooves. The middle portion of the plunger should be of uniform

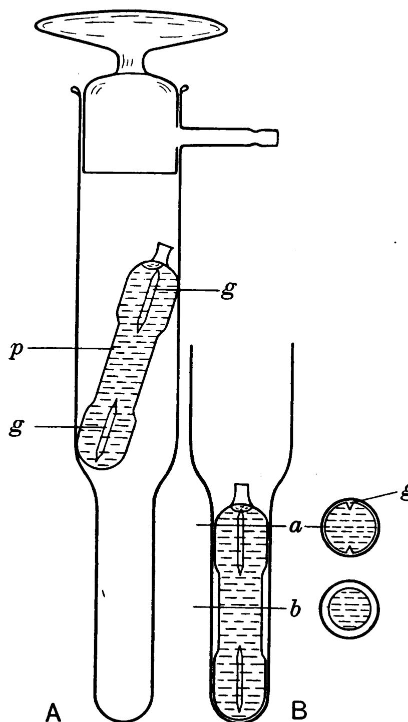


Fig. 4. Modified Thunberg tube for study of the oxygen dissociation of *Gastrophilus* haemoglobin. A, complete tube with the plunger (*p*) within the wider portion of the tube; B, lower portion of the tube with the plunger in the position for spectroscopic examination; *a* and *b*, sections of this portion of the tube at two levels of the plunger; *g*, the side grooves in the end portions of the plunger.

diameter for at least 1 cm. of its length. Plungers with different external diameter of the middle portion were available for use according to the strength of the haemoglobin solution under investigation. The total capacity of the Thunberg tube with the plunger in position is about 14 ml.

Dilute solutions of haemoglobin were studied in a Thunberg tube, to the end of which was fused a 2 cm. cell with optical glass end-plates.

The Thunberg tube of either of these two types used in our experiments is immersed vertically in a water-bath fitted with glass side windows and kept at 39–40°. To avoid any accidental displacement, the tube is supported by a well-fitting collar (Fig. 5 A). The spectroscopic measurements are carried out by means of a microspectroscope ocular fitted into a microscope (Fig. 5 B). A double-wedge trough 15 cm. long and 2·3 cm. wide (inside measurements) is placed close to the aperture (*d*) on a brass platform *c* which is attached to

the microscope by means of the ring b. The exact position of the trough in relation to the aperture d is read on the scale fixed to the brass platform and covered by a sheet of glass upon which the trough slides easily. The trough is illuminated by means of an 8 V. bulb (f) with a compact filament. The objective of the microscope was removed, and the clarity of the absorption spectra was much improved by inserting into the microscope tube a Ramsden ocular, after removing its attachment ring (i, Fig. 5 B).*

Procedure. The concentrated solution of haemoglobin (0·5 ml.) is introduced into the Thunberg tube, and the plunger is slid down into its lower portion. The height of the solution is marked with a diamond on the outer surface of the tube. A separate portion of this haemoglobin solution is diluted 10 times with phosphate buffer pH 7·3 and introduced into one compartment (e_2) of the double-wedge trough, the other compartment (e_1) being filled with water. While keeping the illumination of the two spectra equal by means

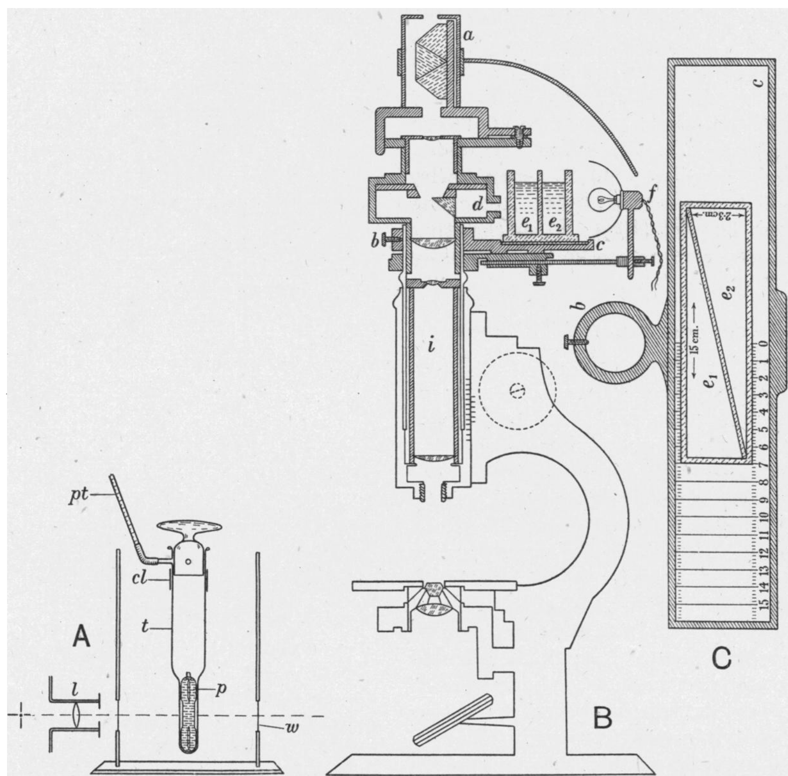


Fig. 5. Complete outfit for the spectroscopic study of the oxygen dissociation of haemoglobin. A, water-bath with the Thunberg tube (t) in position; cl, collar within which the tube can be rotated; p, the plunger; pt, pipette attached to the external outlet of the Thunberg tube; l, microscope lamp and its lens. B, microscope with the spectroscope ocular (a) and Ramsden ocular (i) inserted into microscope tube; b, the ring which attaches the horizontal platform (c) upon which slides the double-wedge trough showing its two compartments e_1 and e_2 in front of the side opening d of the spectroscope. C, face view of platform c and two compartments (e_1 and e_2) of the double-wedge trough.

The bath with the Thunberg tube in a fixed position is placed in front of the microscope. The beam of light from the microscope lamp, rendered parallel by means of a lens, passes through an adjustable vertical slit on to the middle portion of the plunger within the Thunberg tube. This pencil of light is reflected from the concave mirror of the microscope and passes through the substage condenser into the micro-spectroscope ocular. The intensities of light of both the microscope lamp and the side bulb are controlled by two independent rheostats.

of rheostats, the trough is moved along the platform towards the left and the position at which the two spectra match is read off from the scale. From several readings taken while the Thunberg tube is turned within the fixed collar the mean value (a) is obtained. The solution of oxyhaemoglobin in the compartment e_2 of the trough is now diluted until the absorption spectrum of the pigment in the Thunberg tube matches that of the maximum width (2·3 cm.) of the trough; in other words, when the reading of the trough is at 15. The dilution factor $y = a/l$, where a is the mean value of previous readings and l is the internal length of the trough (= 15 cm.). The water in compartment e_1 is removed and both compartments of the trough (e_1 and e_2) are filled with freshly diluted oxyhaemoglobin. The absorption spectrum of the Thunberg

* A similar outfit was previously used for the study of other problems connected with blood pigments (Keilin, 1933; Keilin & Hartree, 1946).

tube will now match that of the trough in any of its positions upon the platform.

The oxyhaemoglobin in the compartment e_1 of the trough is now completely deoxygenated by the addition of a little $Na_2S_2O_4$. The Thunberg tube is removed from the water-bath, 4 drops of 0·25 M-phosphate buffer pH 7 are added to its content and the tube is evacuated until the oxyhaemoglobin is completely deoxygenated and the height of the solution, with the plunger in its original position, drops to the 0·5 ml. mark. The stopper of the Thunberg tube is turned through 180°, and a short length of pressure tubing is attached to the outlet tube and filled by means of a fine pipette with well-oxygenated haemoglobin of the same concentration as that in the tube, taking special care to exclude all air bubbles. A well-graduated 1 ml. pipette filled with the same solution of oxyhaemoglobin is fixed into the rubber tubing, and by carefully turning the stopper of the Thunberg tube a known amount of oxyhaemoglobin solution is forced into the tube by the atmospheric pressure. After each addition of oxyhaemoglobin, the plunger is dislodged from the narrow to the wide portion of the tube which is detached from the pipette and fixed horizontally to a rotating drum immersed in a water-bath kept at 39° and rotated for 10 min. at the rate of 80 r.p.m. After equilibration, the plunger is allowed to slide down and the tube is returned to its original position in front of the microscope. The concentration of oxyhaemoglobin in the tube is then estimated by matching its absorption spectrum with that of the optical mixture of oxygenated and deoxygenated haemoglobin in the two compartments of the trough. In cases of less highly purified haemoglobin solutions several lots of oxyhaemoglobin can be added in succession to the same sample of haemoglobin in the Thunberg tube, thus giving several points on the oxygen dissociation curve, but for more highly purified haemoglobin which has a tendency to oxidize to methaemoglobin this procedure is not suitable. In the latter case, after each determination the Thunberg tube is opened to the air, the haemoglobin fully oxygenated, and from the redetermination of oxyhaemoglobin the amount of methaemoglobin formed during the evacuation and equilibration can be easily calculated. The Thunberg tube is then emptied, carefully dried, and filled with another sample 0·5 ml. of haemoglobin. The experiment is repeated as described above, the amount of oxyhaemoglobin introduced from the side tube being, however, varied each time. In this way a series of points covering the oxygen dissociation curve is obtained.

The pressure of oxygen in the Thunberg tube in mm. Hg corresponding to the percentage of oxyhaemoglobin determined as described above can be calculated from the following equation given by Hill (1936):

$$O_2 \text{ pressure in mm. Hg} = P \times \frac{v_1[O_2] - (v_1+v_2)[HbO_2]}{(v_1+v_2) + A\,(v_0 - v_1 - v_2)},$$

where $[O_2]$ = total concentration of oxygen in the added fluid (= dissolved O_2 + that present as HbO_2).

$[HbO_2]$ = concentration of oxyhaemoglobin in the Thunberg tube.

v_0 = volume of the tube with plunger in place (in ml.).

v_1 = volume of fluid added from the pipette (in ml.).

v_2 = volume of fluid initially present in the tube (= 0·5 ml.).

A = quotient of the concentration of O_2 in gas phase divided by the concentration of O_2 dissolved in liquid and is independent of pressure.

P = the factor for converting a concentration of dissolved oxygen into the equivalent pressure in mm. Hg.

The constants A and P can be calculated from the solubility data.

For the determination of the oxygen dissociation curves of haemoglobin in dilute solutions the same method is applied as for the concentrated solution except that the Thunberg tube used in this case is provided with a 2 cm. optical cell fused to its lower end. If the original haemoglobin solution contains a significant amount of methaemoglobin, the concentration of the latter can be estimated spectrophotometrically by converting oxyhaemoglobin to CO-haemoglobin before and after the addition of $Na_2S_2O_4$ which reduces the methaemoglobin to haemoglobin. From the difference in the densities of the spectra at a suitable wave-length the concentration of methaemoglobin can easily be calculated.

Results. Fig. 6, which summarizes the results of these experiments, shows that the oxygen dissociation curve of a strong solution of *Gastrophilus* haemoglobin at 39° is not S-shaped as is usually the case in the haemoglobins both of vertebrate and invertebrate origin; it is a rectangular hyperbola

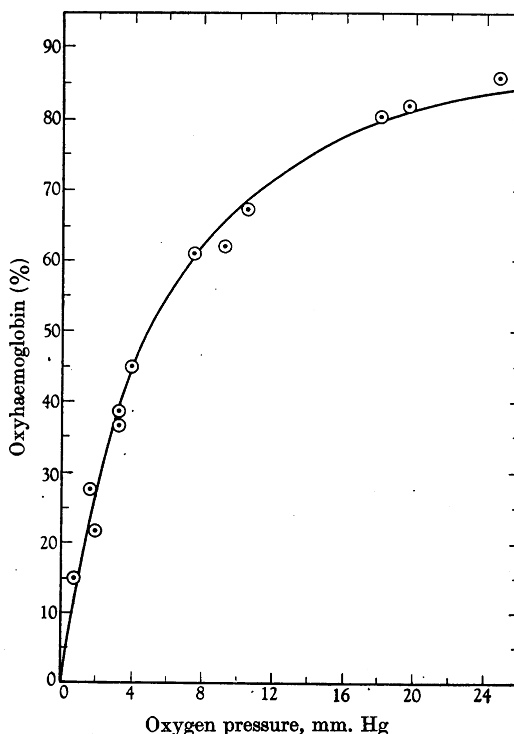


Fig. 6. Oxygen dissociation curve of a strong solution (1×10^{-3} g.mol. haematin/l.) of *Gastrophilus* haemoglobin at 39°.

such as is given by the blood haemoglobin of the eel (see Redfield, 1933) or by myoglobin (Hill, 1933, 1936; Theorell, 1934*c*). In the reversible reaction of oxygenation given by *Gastrophilus* haemoglobin at 39° and expressed by the usual equation

$$Kp^n = [HbO_2]/[Hb],$$

where p is the pressure of oxygen in mm. Hg, the dissociation constant $K = 0{\cdot}195$ and $n = 1{\cdot}035$. The oxygen pressure (p_{50}) corresponding to 50% oxyhaemoglobin for the strong solution of the pigment at 39° is 4·9 mm. Hg. The small difference between the dissociation curve obtained experimentally and the rectangular hyperbola is well within an experimental error, as shown by the value of n and Fig. 7, where log [HbO_2/Hb] is plotted against

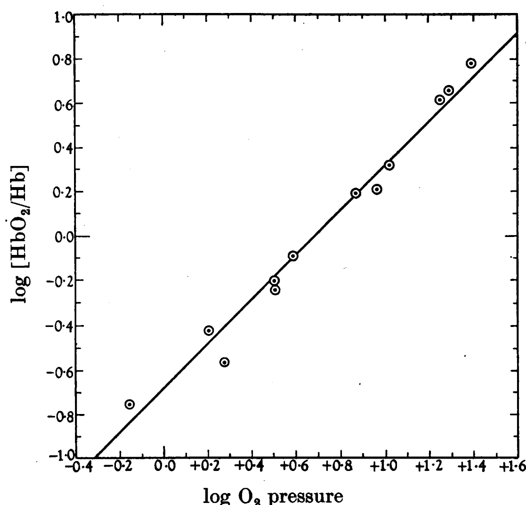


Fig. 7. Oxygen dissociation of *Gastrophilus* haemoglobin; $\log \frac{[HbO_2]}{[Hb]}$ plotted against log pressure of O_2 in mm. Hg.

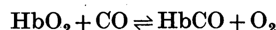
log mm. O_2 pressure. As the molecule of *Gastrophilus* haemoglobin contains two prosthetic groups it would appear that both haem nuclei have the same affinity for oxygen.

The p_{50} for the dilute solution of this haemoglobin ($0{\cdot}84 \times 10^{-4}$ g.-atom Fe/l.) was difficult to determine owing to its very great affinity for oxygen. The approximate value of the oxygen pressure obtained from several experiments was less than 0·02 mm. Hg at 39°. In this respect *Gastrophilus* haemoglobin occupies an intermediate position between the usual types of haemoglobins and that of *Ascaris lumbricoides*, the intestinal parasite of pigs.

A. lumbricoides, as was previously shown (Keilin, 1925), has two different haemoglobins, one in the body wall, the other in the perienteric fluid, differing from each other and from the haemoglobin of the host in the position of their absorption bands. These two haemoglobins were recently reinvestigated by Davenport (1945), who found that they have an exceptionally high affinity for oxygen. He found it impossible to obtain complete deoxygenation by boiling them *in vacuo* at 20°. What is, however, more important, is that $Na_2S_2O_4$, which rapidly removes all traces of oxygen dissolved in a haemoglobin solution, produces only a very slow deoxygenation of the *Ascaris* haemoglobins. Thus *in vacuo* at pH 7 and at 8°, $Na_2S_2O_4$ produces half-dissociation of oxygen from the body-wall haemoglobin in 250 sec., and from the perienteric haemoglobin in about 1000 sec. According to Davenport, oxygen dissociates from perienteric haemoglobin 10,000 times and from the body-wall haemoglobin 2500 times more slowly than it does from sheep haemoglobin.

Relative affinity of haemoglobin for O_2 *and* CO

For the reversible reaction



$$HbO_2 + CO \rightleftharpoons HbCO + O_2$$

the equilibrium constant

$$K = [HbCO] \times pO_2/[HbO_2] \times pCO$$

was determined spectrophotometrically. For this purpose a well-aerated oxyhaemoglobin solution was introduced into a Thunberg tube fitted with a 2 cm. optical cell, and the concentration of the solution so adjusted as to give a maximum density reading of about 1·5 in the region of the α-band. The densities at the wave-lengths 565 and 581 mμ. were carefully determined. The air in the Thunberg tube was replaced by pure CO which was well equilibrated with the solution, and the densities at the same wave-lengths were redetermined. The CO was then completely removed and replaced by a mixture of CO and O_2 of known composition. After careful equilibration the densities at the same wave-lengths were determined again.

Having obtained these values the ratio of the concentration of HbCO and HbO_2 can be calculated from the following equation:

$$[HbCO]/[HbO_2] = (D_1E_2 - D_2E_1)/(D_2F_1 - D_1F_2),$$

where D_1 and D_2 = densities of the mixtures of HbO_2 and HbCO at wave-lengths 565 and 581 mμ. respectively;

E_1 and E_2 = densities of pure HbO_2 and

F_1 and F_2 = densities of pure HbCO at the same wave-lengths; densities = $\log_{10}(I_0/I)$, where I_0 and I = intensities of incident and transmitted light respectively.

Using the gas mixture consisting of 21% O_2 and 21% CO in nitrogen, the equilibrium constant K was found to be 0·67 at pH 7·3 and 25°. This value of K is very low when compared with those of the blood haemoglobins of vertebrates which vary from 125 to 550 (Table 4). It is interesting to note that the

highest value (500–550) of this constant was found for the blood haemoglobin of the horse which is the host of the *Gastrophilus* larva. The lowest hitherto known values of K are 28–51 for myoglobin (Theorell, 1934*b*; Roche, 1933), and about 37 for the haemoglobin in the root nodules of leguminous plants (Keilin & Wang, 1945).

Table 4. *Relationship between the partition constant* $K=\frac{[HbCO]\times pO_2}{[HbO_2]\times pCO}$ *of different haemoglobins and their 'span', the distance in A. between the α-bands of* HbO_2 *and* HbCO

Origin of haemoglobin	$K=\frac{[HbCO]\,pO_2}{[HbO_2]\,pCO}$	Span (A.)	$y=\frac{\log K}{\text{span}}$	Observers
Blood of vertebrates	125–550	43–56	0·043–0·050	Anson *et al.* (1924)
Muscles of vertebrates	28–51	31–36	0·045–0·050	Roche (1932, 1933), Theorell (1934*a*, *b*)
Root nodules of leguminous plants	37	100	0·016	Keilin & Wang (1945)
Gastrophilus larvae; tracheal cells	0·67	95	−0·17	Keilin & Wang (present paper)

DISCUSSION

Although haemoglobin is very widely distributed in nature its presence is general only among vertebrates. In invertebrates its distribution is very irregular, almost haphazard, the pigment being either absent in all representatives of large phyla or present in one or few species of a large phylum and absent or replaced by another oxygen carrier such as haemocyanin in other and often closely allied forms.*

The range of the distribution of haemoglobin was recently widened by the unexpected discoveries of this pigment in a ciliate *Paramaecium* among Protozoa (Sato & Tamiya, 1937) and in the root nodules of leguminous plants (Kubo, 1939; Keilin & Wang, 1945). The presence of haemoglobin in root nodules is of especial interest considering that it represents the first case of the occurrence of this pigment in plants and that 'neither the plant cells alone nor the symbiotic micro-organism (*Rhizobium*) cultivated separately are capable of synthesizing haemoglobin. It is only when root cells are invaded by a specific symbiotic micro-organism and begin to proliferate that haemoglobin is formed. *Rhizobium* not only induces growth and multiplication of cells, but also supplies these proliferating cells directly or indirectly with a factor necessary for synthesis of haemoglobin' (Keilin & Wang, 1945).

Since every cell of aerobic organisms, including bacteria, moulds and higher plants, is capable of synthesizing different haematin catalysts such as the components of the cytochrome system and catalase, every cell appears to be a potential carrier of haemoglobin. The limiting factor in the distribution of this pigment in nature cannot therefore be due to the synthesis of the haem nucleus but to the fact that not all haem-producing cells are capable of synthesizing the highly specific proteins which, when combined with haem, form compounds possessing the remarkable property of reversible oxygenation.

Among insects, which form by far the largest group of invertebrates and which exhibit the greatest variety of respiratory adaptations, haemoglobin is known in only a few species belonging to Diptera and Hemiptera. Thus among Diptera it is present in the perivisceral fluid of larvae of certain Chironomidae (midges) and the tracheal cells of larvae of *Gastrophilus*. Among Hemiptera, haemoglobin was found in backswimmers such as *Buenoa margaritacea* Bueno (Hungerford, 1922; Bare, 1928) and *Anisops producta* Fiel (Poisson, 1926), where it is localized in large masses composed of tracheal cells similar to those of *Gastrophilus* larvae. It was found also in the large water boatman, *Macrocrixa geoffroyi* Leach, where it fills the lumen of accessory glands of the male genital system (Haviland-Brindley, 1929).

In *Gastrophilus*, haemoglobin is present only during a certain period of its development, the first-stage larva on hatching from the egg, the pupa and the adult insect being devoid of this pigment. Haemoglobin is, however, present in the larva during the whole of its second and third stages, lasting more than 8 months, during which the larvae live attached to the mucous membrane of the stomach of the horse. This part of the life cycle represents the longest period of the insect's life. In the young larva haemoglobin is equally distributed in cells of different tissues. As the larva grows older, the haemoglobin becomes more concentrated and localized in the tracheal cells. This is mainly due to the fact that the cells of the larva do not divide but grow in proportion to the size of the larva, and that only the tracheal cells keep up the ability to synthesize haemoglobin in pace with their growth, whereas

* Throughout this paper the term haemoglobin is used for all haem-protein oxygen carriers irrespective of their origin. The cumbersome and unnecessary name of erythrocruorin is purposely avoided. This point as well as a few other points concerning the nomenclature of haemoglobin and its derivatives will be discussed elsewhere.

the formation of haemoglobin in other cells lags far behind their growth.

A full-grown larva of *Gastrophilus* weighing 0·45–0·5 g. was found to contain approximately 2·1 mg. haemoglobin which can fix about 2·7 cu.mm. O_2. This amount of oxygen, according to the respiratory activity of the larva, may be used up in 0·5–4 min.*

Since *Gastrophilus* larvae live as parasites in the stomach of the horse, one may ask whether there is any relationship between the haemoglobin of the parasite and that of its host. Table 5, which summarizes the main properties of these two haemoglobins and of myoglobin, clearly shows that they differ markedly in their absorption spectra, span, relative affinities for O_2 and CO, oxygen dissociation curves, ease of autoxidation and molecular weight. In certain properties *Gastrophilus* haemoglobin somewhat resembles the myoglobin of horse or other mammals.

Relation of span to equilibrium constant

It was shown by Anson *et al.* (1924) for the blood of thirteen different species of vertebrates that there is a certain interrelationship between the 'span' (the distance separating the α-bands of HbO_2 and HbCO expressed in A.) and the equilibrium or partition constant $K=[HbCO]\times pO_2/[HbO_2]\times pCO$. Thus by plotting the span against log K they found that the points fell upon one of the two approximately straight lines which could be expressed by an equation $\log K = y \times S$, where S is the span in A. and y is equal to 0·043 or 0·050.

Since the variation in log K denotes the change in the free energy of the system, the linear relationship between log K and the span suggested that the displacement of the α-band of haemoglobin is accompanied by a definite and predictable change in the free energy. The difficulty in accepting this view was the uncertainty as to the linear relationship between log K and the span. In fact, it has already been mentioned by Barcroft (1928, see p. 49) that 'No great stress must be laid on the straightness of the central line. It is very short and over so short a range portions of many shallow curves or S-shaped curves might easily appear straight; but it seems difficult to doubt that a relation of some sort exists.'

The problem was, however, reinvestigated by Theorell (1934 *a*, *b*), who found that in the case of horse myoglobin $K=28$ and the span = 32 A. In this case, log K plotted against the span (S) gives a

Table 5. *Properties of haemoglobin of the* Gastrophilus *larva compared with those of the blood haemoglobin and myoglobin of the horse—its host*

Properties	*Gastrophilus* haemoglobin	Horse blood haemoglobin	Horse myoglobin
Position of α-band in A.: HbO_2	5810	5767	5820
HbCO	5715	5711	5790
$K=\frac{[HbCO]\times pO_2}{[HbO_2]\times pCO}$	0·67	550	28
Span (A.)	95·5	56	30
$y=\frac{\log K}{\text{span}}$	−0·17	0·043	0·045
O_2 dissociation curve	Rectangular hyperbola	S-shaped	Rectangular hyperbola
Autoxidation	Fairly rapid	Slow	Fairly rapid
Mol. wt.	About 34,000	67,000	18,000

Table 6. *Different values of partition constant* $K=\frac{[HbCO]\times pO_2}{[HbO_2]\times pCO}$ *of haemoglobins compared with those of oxidizing enzymes*

Haemoglobin of vertebrates	Myoglobin of mammals	Haemoglobin of root nodules	Haemoglobin of *Gastrophilus*	Phenol oxidase	Cytochrome oxidase
120–550	28–51	37	0·67	0·25–1	0·1

One of the most striking properties of *Gastrophilus* haemoglobin is its very low affinity for CO, the partition constant $K=[HbCO]\times pO_2/[HbO_2]\times pCO$ being only about 0·67. In this respect it differs markedly from all other haemoglobins (Table 6) and shows some resemblance to oxidizing enzymes such as cytochrome oxidase with a partition constant of about 0·1 and polyphenol oxidase, a copper-protein compound, the partition constant of which, as was previously shown (Keilin, 1929), varies from 0·25 to 1.

* The physiological significance of haemoglobin as an oxygen carrier will be discussed in a separate paper devoted to this subject.

point which lies on the straight line obtained by previous workers (Anson *et al.* 1924) and expressed by the equation $\log K = 0{\cdot}045 \times S$. This point lying at an appreciable distance from the cluster of points obtained for the blood haemoglobins of different species of vertebrates and uniting this cluster with the zero point makes, according to Theorell, the existence of a real linear relationship between $\log K$ and the span very probable.

It may be mentioned here that the span of myoglobin of ten different vertebrates was found by Roche (1932) to vary between 31 and 36 A., whereas the value of the equilibrium constant K given by him was about 51. These results, when plotted in the usual way, would give a point lying somewhat above the line obtained by previous workers.

The first marked deviation from this relationship was shown by the haemoglobin of the root nodules of leguminous plants (Keilin & Wang, 1945). Here the span is about 100 A. and K about 37, and in the equation expressing their relationship $\log K = y \times S$, $y = 0{\cdot}016$ (Table 4). The deviation is still more marked in the case of the haemoglobin of *Gastrophilus* which has a span of about 95 Å., $K = 0{\cdot}67$ and $y = -0{\cdot}17$. The last two results, contrary to those obtained by previous workers, rob the linear relationship between $\log K$ and the span of its generalization and therefore of its possible significance.

The haemoglobin of *Gastrophilus* shares with that of the nodules of leguminous plants and with myoglobin the property of a comparative ease of oxidation to methaemoglobin. In fact, it is somewhat difficult in the absence of CO to keep these haemoglobins in the ferrous state, especially when they are separated from the rest of the cells which contain them, and are highly purified. A very slow oxidation to methaemoglobin is a general property of haemoglobin compounds, and the kinetics of this reaction for blood haemoglobin were carefully investigated by Brooks (1935). He found that the rate of oxidation at different oxygen pressures is proportional to the concentration of the unoxygenated haemoglobin and to a function of the oxygen pressure. This confirmed previous observations by Neill & Hastings (1925) who found that the rate of oxidation of haemoglobin by molecular oxygen was greater at a small partial pressure of oxygen. According to Brooks, oxygen affects this reaction in three different ways: (1) it determines the concentration of the other reactant, the unoxygenated haemoglobin, (2) it oxidizes this compound, the rate being proportional to the first power of the oxygen pressure and (3) it acts as an inhibitor of this oxidation which may be 'due to the collision of oxygen molecules with an activated molecule or complex'.

As to the comparative ease of autoxidation of the above-mentioned haemoglobins, it may be due to some slight differences in the mode of union of the prosthetic group with the protein, thus affording less protection against oxidation of the haem iron which, on deoxygenation, may become more reactive. One cannot, however, exclude the possibility that the oxygen dissociating from its union with haem may possess a slightly higher level of reactivity than the ordinary molecular oxygen and that this level may vary with the haemoglobin. However, by no method so far available can any difference be detected between the oxygen dissociating from oxyhaemoglobin and the free molecular oxygen.

The oxidation of haemoglobin to methaemoglobin, even in the case of *Gastrophilus*, is a very sluggish reaction when compared with that of haem or haemochromogen. In fact, it is not the autoxidation of haemoglobin which is difficult to understand, but the unusual slowness of this reaction and the marked resistance of the divalent iron of haemoglobin to oxidation, contrasted with the great ease of the formation of reversible oxygenated compound. This property of oxygenation, which haemoglobin shares with only three other natural oxygen carriers—chlorocruorin, haemocyanin and haemerythrin—so far has no analogy among known natural or artificial compounds. It is conceivable that the oxygenation is the first step in the oxidation reaction of all haematin or iron compounds, but that this oxygenated state is usually of an extremely short duration, being rapidly followed by the valency change of iron. In the haemoglobin molecule, the native globin not only protects the divalent iron from oxidation but also brings about the stabilization of this oxygenated compound. That proteins which protect the haematin iron from autoxidation do not necessarily stabilize the oxygenated compound is clearly shown by the components *a*, *b* and *c* of cytochrome and by alkaline helicorubin which are neither autoxidizable nor capable of undergoing oxygenation. Their oxidation can, however, be catalyzed by another haematin-protein compound, cytochrome oxidase.

The fact that the iron of haemoglobin is protected from oxidation but is capable of reversible oxygenation makes haemoglobin a good oxygen carrier but a very inefficient oxidizing catalyst. On denaturation, haemoglobin loses the property of carrying molecular oxygen but acquires the property of autoxidation and becomes a much better oxidizing catalyst although never reaching the efficiency of the natural haematin protein catalysts. Although the gap separating the haemoglobin compounds from the natural oxidizing catalysts still remains unbridged, the few properties common to haemoglobin of the *Gastrophilus* larva, the haemoglobin of root nodules and to some extent myoglobin, bring somewhat closer the two groups of haem-protein compounds: the oxygen carriers and the oxidizing catalysts.

SUMMARY

1. Larvae of *Gastrophilus* which live in the stomach of the horse contain an intracellular haemoglobin localized mainly within the giant tracheal cells.

2. A method is described for the purification and crystallization of this haemoglobin.

3. Pure *Gastrophilus* haemoglobin behaves in the Tiselius apparatus as a homogeneous protein and has an isoelectric point of pH 6·2. The purest sample contains 3·92 % haemin which corresponds to an equivalent weight of 17,300. The molecular weight of this haemoglobin is about 34,000, i.e. half the molecular weight of the blood haemoglobin of vertebrates. The molecule of *Gastrophilus* haemoglobin therefore contains only two haem nuclei.

4. Its oxygen dissociation curve very closely approximates a rectangular hyperbola.

5. It has a high affinity for O_2; in a strong solution of haemoglobin at 39° the half-saturation with O_2 is reached at an O_2 pressure of 4·9 mm. Hg and in a dilute solution at less than 0·02 mm. Hg.

6. It has a low affinity for CO, the partition constant $K = [HbCO] \times pO_2/[HbO_2] \times pCO$ being about 0·67, which is 800 times lower than that of horse-blood haemoglobin and is of the same order of magnitude as the partition constants of certain oxidizing enzymes which vary from 0·1 to 1.

7. The 'span' or the distance between the α-bands of oxyhaemoglobin and CO-haemoglobin is about 95 A., which is very near that of the root nodule haemoglobin (100 A.) but much larger than the 'span' of other haemoglobins and myoglobins (32–56 A.).

8. Neither the haemoglobin of *Gastrophilus* larvae nor that of the root nodules of leguminous plants supports the view as to the existence of a linear relationship between log K and the 'span'.

9. Haemoglobin of *Gastrophilus* larvae differs from blood haemoglobin of the horse—their host—in many respects, such as (1) the position of absorption bands, (2) the 'span', (3) the relative affinities for O_2 and for CO, (4) the shape of the dissociation curve, (5) the ease of autoxidation and (6) the molecular weight. Since these two haemoglobins have the same prosthetic group, they must differ in the structure and the composition of their proteins. *Gastrophilus* larvae therefore synthesize their own haemoglobin.

10. Certain properties common to the haemoglobin of *Gastrophilus* larvae, the haemoglobin of root nodules and myoglobin, such as (*a*) the high affinity for O_2, (*b*) the low affinity for CO, (*c*) the low partition constant K, (*d*) the comparative ease of oxidation to methaemoglobin and (*e*) the localization within fixed non-circulating cells of the organism, reduce somewhat the gap separating the two groups of haematin-protein compounds, oxygen carriers and certain oxidizing catalysts.

Our thanks are due to Dr R. Markham for the assistance he has given us in this investigation.

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