SEASONAL VARIATION IN THE RESPIRATORY ACCLIMATIZATION OF THE LEECH *ERPOBDELLA TESTACEA* (SAV.)

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In a preliminary study of the oxygen consumption of leeches (Mann, 1956), it was shown that *Erpobdella testacea* has an oxygen consumption which is independent of the oxygen concentration in the water, over the range 2–6 ml./l., provided that it has been acclimatized overnight to the oxygen concentration of the experiment. The determinations in these experiments were made by enclosing the leeches in glass-stoppered bottles for periods of about 1 hr. Such experiments are unsatisfactory for a study of acclimatization because: (i) the oxygen concentration is constantly changing, (ii) the duration of the experiments is limited, and (iii) the closing of the bottles and the shaking which is necessary to ensure proper mixing of the water make it difficult to keep the animals in a quiescent state. An apparatus was therefore devised to enable the animals to be placed in a slow stream of water of any desired constant oxygen content, so that their oxygen consumption could be measured over a period of days. In this way it was possible to obtain a true resting state, and to investigate acclimatization in differing oxygen concentrations at different times of year.

APPARATUS AND METHODS

The dropping mercury electrode was used for determining the oxygen concentrations in the water. The difficulties which have to be overcome when using this method in flowing water for long periods are of two kinds. First, the speed of the water flowing past the electrodes influences the readings obtained, independently of the oxygen concentration, so it is necessary to obtain a constant, minimal flow in order to calibrate accurately. Secondly, mucus produced by the leeches tends to accumulate on the capillary and make the rate of dropping of the mercury uneven. This in turn produces erratic readings for a given oxygen concentration. The first difficulty was overcome by abandoning rubber bungs and rubber tubing as far as possible in the construction of the apparatus, and using standard glass cones and sockets for all joints, thus eliminating leaks and facilitating input and removal of experimental animals. A specially designed fine-control glass tap was used to produce a constant rate of flow (Fig. 2b) and the water was slowed in the vicinity of the electrodes by making the electrode chamber of relatively large dimensions. It was then found that reproducible readings could be obtained for any given oxygen concentration and rate of flow. The second difficulty, caused by the clogging

of the capillary, was overcome by adopting the wide-bore dropping mercury electrode of Briggs, Davies, Dyke & Knowles (1957) which delivers the mercury upwards at an angle of 45° through a capillary of 0·8 mm. internal diameter. The head of mercury is reduced to 15 cm. and the flow is further restricted by a middle section of capillary tubing, between the reservoir and the capillary tip, about 34 cm. long and 0·2 mm. internal diameter, bent as shown in Fig. 1.
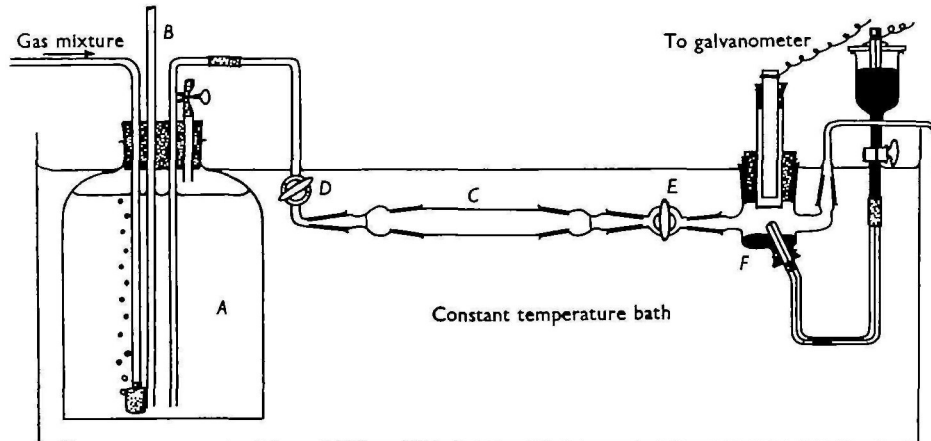

Fig. 1. Diagram showing general arrangement of the polarographic respirometer. For explanation of lettering see text. The reservoir *A* is drawn to a smaller scale than the rest of the apparatus, and is in fact a 10 l. aspirator.
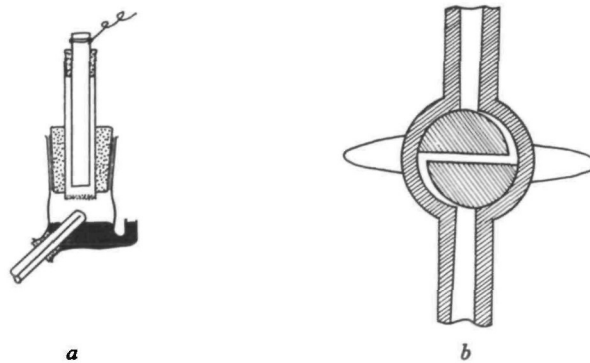

Fig. 2. (*a*) Section through the electrode chamber (*F* in Fig. 1) approximately at right angles to the plane of Fig. 1. (*b*) Section through the fine-control tap (*D* in Fig. 1) showing the tapering grooves on the key.

The general arrangement of the apparatus is also shown in Fig. 1. Water in the reservoir *A* is equilibrated with the gas mixture from a cylinder, or with air from a pump, and after equilibration the gas outlet from the reservoir is closed and a head of water built up in the tube *B*. This drives the water through the respiration chamber *C*, which has a piece of platinum gauze at each end to contain the animals. The tap *D* is the fine-control tap illustrated in Fig. 2*b*, and the effect of the fine

grooves cut in the key is to give a controlled rate of flow at any point in the range 50–500 ml./hr. The tap E is a standard one which may be used as a coarse control of the rate of flow, but it was also found useful because when partly closed it produced a jet of water which caused the water to be thoroughly mixed before reaching the electrode chamber. This eliminated fluctuations in the readings due to imperfect mixing of the water in the respiration chamber. The electrode chamber, F, is essentially a hollow glass ball with five apertures, as shown in Figs. 1 and 2a. The largest aperture is that at the top for receiving the zinc reference electrode. It is fitted with a 32 mm. ground glass cone and socket to facilitate opening the chamber for cleaning the capillary and the inner walls, the rubber bung being fitted into the glass cone. The other two apertures fitted with ground glass joints (10 mm.) are for the inflow and outflow of water. The tip of the capillary is led through a rubber bung. It lies in a plane at right angles to the direction of flow of water and is directed at 45° to the horizontal, with its tip in line with the inflow. The fifth aperture is for mercury overflow and is constructed so that water cannot leave by that route, even when under slightly more than atmospheric pressure. The dropping mercury electrode was kept at 0·4 V. negative with respect to the zinc electrode. The latter generates 1·0 V., so that the apparatus is working on the second step of the oxygen polarographic curve, where there is no polarographic maximum. It is not therefore necessary to add a suppressor and the apparatus can be used with any type of water. Reading tap water was used throughout the experiments, care being taken to avoid water which had passed through copper pipes as this was lethal to the leeches. Chlorine was added at a concentration of 1 p.p.m. to prevent growth of micro-organisms. The polarographic current was measured with a Cambridge Voltamoscope and the rate of flow of water was measured at the outflow using a stop-watch and a measuring cylinder.

The procedure in any experiment was first to pass the water through the apparatus with no animals present, until a steady reading was obtained over a period of several hours. Leeches were then added, and the respiration chamber wrapped in black paper. Under these conditions the leeches distributed themselves well in the chamber, and were never seen to undulate their bodies, even in water of low oxygen concentration. All experiments were carried out at 20 ± 0·1° C.

When it was required to render functionless the haemoglobin of the blood, the leeches were placed in the dark in water containing carbon monoxide at a concentration one-sixth that of the oxygen, and left until all the haemoglobin had been converted to carboxyhaemoglobin. The time required varied from 3 hr. for small leeches to 6 hr. for large ones, and in practice the latter were left in the treated water overnight in order to be ready for experiments the next day. The method used to observe the carboxyhaemoglobin was to compress a leech between a well-slide and a plain slide, in a solution of sodium hydrosulphite, and examine the preparation under a microscope with a hand spectroscope attached. The presence of two dark bands which did not fade even in these deoxygenated conditions indicated the presence of carboxyhaemoglobin. Checks of this type were made before and after each experiment.

RESULTS

A. Oxygen consumption in relation to feeding and starvation

In previous work (Mann, 1956) it had been shown that leeches collected from the field and subsequently starved showed a reasonably constant oxygen uptake for at least a week. Unfortunately it was not possible to obtain locally sufficient *E. testacea* for the whole series of experiments, and supplies were sent by post from the Windermere laboratory of the Freshwater Biological Association. The leeches were given a meal of *Tubifex* on arrival, and kept in well-aerated water until their oxygen uptake had reached a steady level. The pattern of oxygen consumption after feeding is shown in the first part of the curve in Fig. 3. There is a threefold increase in oxygen uptake after a meal, a decline over a period of 4 days, followed by a steady level which is maintained for several days.
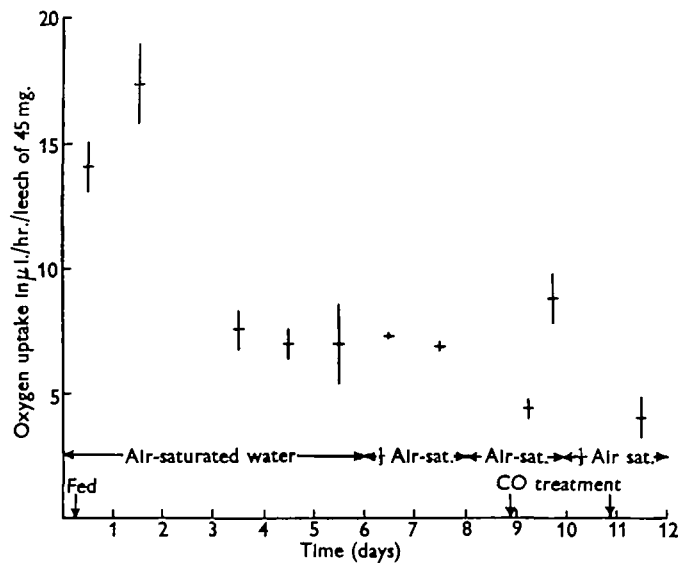

Fig. 3. Mean oxygen consumption of twenty-two *Erpobdella testacea* of average weight 45 mg. subjected to various treatments as described in the text. Lines are drawn to represent twice the standard error above and below each point.

B. Acclimatization to low oxygen concentration in the water

For the first series of experiments the water in the reservoir was equilibrated with a gas mixture of 11 % oxygen and 89 % nitrogen, so that the oxygen content was about 50 % of air-saturation. Leeches, which had been fed and then starved for at least 4 days while being kept in well-aerated conditions, were placed in the apparatus and given time to settle down. In early experiments it was not always easy to obtain a steady rate of flow of water, together with a constant dropping rate for the mercury, and as readings were only taken when these conditions obtained, the number of readings which could be taken in a day was limited. In Table 1 is

Table 1. *Oxygen consumption in µl./hr. of* Erpobdella testacea *in one-half air-saturated water*

Date of start	Day 1		Day 2		Day 3		Uptake in air-saturated water	Weight (mg.)
	n	Mean and S.E.	n	Mean and S.E.	n	Mean and S.E.		
5. vi. 56	9	12·1 ± 0·4	13	14·8 ± 0·3**	7	14·9 ± 0·7	15·1	92
26. vii. 56	8	15·7 ± 2·4	11	22·7 ± 0·9*	4	20·2 ± 0·2	16·5	105
22. viii. 56								
Hours 1–4	—	2·1 ± 0·4	—	—	—	—	—	—
Hours 5–7	—	8·1 ± 1·2	8	6·9 ± 0·2	10	8·2 ± 0·4	9·5	55
5. ix. 56	11	6·5 ± 0·7	12	7·2 ± 0·6	7	8·4 ± 0·6	9·0	50
11. ix. 56	12	5·7 ± 0·6	9	4·8 ± 0·4	—	—	6·5	33

** Significantly different from preceding column, $P = 0·01$; * $P = 0·05$.

recorded for each day of each experiment the number of readings, their mean, and the standard error of the mean. For reasons given in the previous paper, the oxygen uptake is recorded in µl. of oxygen consumed per leech of a stated weight, per hour, rather than µl./g. On the right-hand side of Table 1 is given the oxygen consumption of a leech of the same weight in air-saturated water, calculated from the results of earlier work. In the experiment of June 1956 there was a highly significant increase in oxygen consumption on the second day compared with the first, and the same result was obtained in July, except that the significance of the increase was somewhat obscured because there was a marked increase in oxygen consumption towards the end of the first day. In August the same thing happened as in July, and this has been emphasized in the table by calculating separately the mean and the standard error for the readings obtained in the first 4 hr. and those obtained later in the same day.

In the experiments of early September there is a slow increase of oxygen consumption on each of 3 days, but none of these changes is statistically significant at the 5 % level of probability. By the third day the uptake is close to that obtained by enclosing the leeches in bottles containing air-saturated water, and the difference is probably accounted for by the lower level of activity in the respirometer. It seems likely, therefore, that while in the first three experiments the leeches showed an initial low level of oxygen consumption, rising as the animals became acclimatized until their uptake was comparable with that found in air-saturated water, in the fourth experiment the leeches may have been partly acclimatized before the experiment began, possibly because of exposure to water of low oxygen content before collection.

In the experiment of 11 September there was a decrease in oxygen consumption on the second day, and there is no conclusive evidence that acclimatization occurred at all. This result led to the idea that acclimatization might be more marked in early summer than at other times of the year, so another series of experiments was planned, running from winter to summer of 1957. Two changes were made in the procedure. The water was equilibrated with a gas mixture of 7 % oxygen and 93 % nitrogen, so that the dissolved oxygen was equal to about one-third air-saturation, and immediately before each experiment in water of low oxygen content, the oxygen

consumption of the leeches was measured in air-saturated water. The results are summarized in Table 2.

Table 2. *Oxygen consumption of* Erpobdella testacea *in one-third air-saturated water*

Date of start	Uptake in air-saturated water		n	Mean and S.E.	n	Mean and S.E.	n	Mean and S.E.	Weight (mg.)
	n	Mean and S.E.							
7. ii. 57	10	11·6±0·9	10	4·3±0·7**	4	2·2±0·6	5	3·5±0·01	92
9. iv. 57	4	7·2±0·6	15	5·4±0·2*	6	4·6±0·2	—	—	40
14. v. 57	15	8·6±0·4	12	7·7±0·5	—	—	—	—	61
31. v. 57	9	7·0±0·3	8	7·3±0·01	4	6·9±0·07	—	—	42
3. vi. 57	7	4·8±0·4	8	2·7±0·02**	13	3·6±0·07**	—	—	31

** Significantly different from preceding column, $P = 0.01$; * $P = 0.05$.

In February the rate of oxygen consumption in oxygen-poor conditions is very low indeed, and there is no suggestion at all of acclimatization. The same is true of the April experiment, except that there is a higher level of uptake in the oxygen-poor water, even at the beginning. In May the level of oxygen uptake is approximately the same in the low oxygen concentration as in the high. Apparently we again have the condition where acclimatization may have occurred prior to the start of the experiment. It seemed possible that this might have occurred during the journey in the post, so on 3 June determinations were made with animals collected locally. Here was seen once again a low uptake on the first day, with a highly significant increase on the second, indicating progressive acclimatization.

C. The effect of rendering functionless the haemoglobin of the blood

After the experiments of 9–10 April when no acclimatization had occurred, and the level of oxygen consumption was $4.6 \pm 0.2\,\mu l.$ per leech per hour, the same leeches were placed in one-third air-saturated water containing carbon monoxide. Next morning they were transferred to the respirometer, which now contained about $0.5\,\text{ml./l.}$ of carbon monoxide in the water to prevent dissociation of the carboxyhaemoglobin. The oxygen consumption was measured at intervals over the next 7 hr., and the mean of ten readings was $3.6 \pm 0.01\,\mu l./\text{hr.}$ per leech of 40 mg. This is a highly significant fall in oxygen consumption. A similar experiment was carried out after the experiment of 31 May 1957, in which the leeches had been acclimatized before the start of the experiment. The mean of eight determinations was $5.0 \pm 0.4\,\mu l./\text{hr.}$ per leech of 42 mg. This is again significantly lower than the value obtained for untreated leeches, and suggests that at one-third air-saturation the oxygen uptake is reduced by about 25 % if the haemoglobin is rendered functionless.

A third carbon monoxide experiment was carried out in May 1957 on leeches which had been kept in well-aerated conditions for several days before the experiment. They were treated with carbon monoxide overnight and then transferred to the respirometer which was charged with water containing oxygen at one-third

air-saturation and carbon monoxide at a concentration of 0·5 ml./l. The oxygen consumption was low at first but rose steadily through the day, as shown in Table 3.

Table 3. *Oxygen consumption of* Erpobdella testacea *in one-third air-saturated water, after treatment with carbon monoxide*

Consumption before treatment	0–1·5 hr.	1·5–4·0 hr.	4·0–6·5 hr.
8·6 ± 0·4	4·1 ± 0·01**	5·5 ± 0·02*	7·5 ± 0·01*

** Significantly different from preceding column, P = 0·01; * P = 0·05.

After a careful check to ensure that the carboxyhaemoglobin had not dissociated it was concluded that the rise in oxygen consumption during the experiment represented an acclimatization effect, which was taking place in spite of the haemoglobin having been rendered functionless. The next step was to investigate whether acclimatization occurred in response to the oxygen shortage induced by the carbon monoxide, or whether it was a response to the low oxygen concentration in the water. Two experiments were devised. In the first, the leeches were treated with carbon monoxide overnight, and next morning their oxygen uptake was measured in air-saturated water. In the second, untreated leeches were placed in the respirometer in air-saturated water and their oxygen uptake was measured while they were receiving carbon monoxide treatment, the appropriate amount of carbon-monoxide saturated water having been added to the reservoir. The result of the first experiment was that the oxygen uptake of the leeches was the same after treatment as before. In the second, the oxygen uptake dropped sharply as shown in Table 4. This suggests that although treatment with carbon monoxide causes a fall in oxygen consumption of about 45 % in air-saturated water, leeches which have been overnight in contact with carbon monoxide have become acclimatized, and are able to take up as much oxygen when the haemoglobin is inactivated as when it is normal.

Table 4. *Consumption of* Erpobdella testacea *in air-saturated water, during treatment with carbon monoxide*

Consumption before treatment	1–2 hr.	2–3 hr.	3–4 hr.
1·8 ± 0·1	1·0 ± 0·1**	0·8 ± 0·1	1·0 ± 0·0

** Significantly different from preceding column, P = 0·01; * P = 0·05.

D. A composite experiment

To demonstrate each of the effects described in the preceding three sections, twenty-four leeches received in a batch from Windermere were given a meal of *Tubifex*, after which their oxygen consumption was followed until a steady state had been reached. They were then transferred from air-saturated to one-third air-saturated water and their uptake followed for 2 days. Next, after a period in air-saturated water, they were treated with carbon monoxide overnight and their

uptake measured in air-saturated water. Finally, the carbon monoxide experiment was repeated in one-third air-saturated water. The results are shown graphically in Fig. 3. There is no significant difference between the figures of the 4th, 5th, and 6th days, so $7\,\mu$l./hr. may be taken as the normal consumption in air-saturated water. There is no significant change when the animals are transferred to one-third air-saturated water, presumably because the animals are already acclimatized. When transferred to air-saturated water and then treated with carbon monoxide, the level of oxygen consumption fell sharply at first, but rose rapidly after 2 hr. to a level significantly higher than normal. On the last day of the experiment, when the leeches were in low oxygen concentration and had been treated with carbon monoxide, the uptake was at a low level throughout the day.

DISCUSSION

In the earlier paper on leech respiration (Mann, 1956) it was shown that *Erpob-della testacea* may have an oxygen consumption which is independent of, or dependent on, the oxygen concentration of the water, according to whether or not there has been an opportunity for acclimatization. The experiments on which this finding was based were carried out in September. It is now shown that whereas at certain times of the year this leech may become independent after acclimatization, at other times of year it does not do so. If this state of affairs is at all widespread among aquatic invertebrates it will be necessary to accept with caution the distinction which is often made (Zeuthen, 1955; Bishop, 1950) between animals having a dependent type of respiration and those having the independent type. Walshe-Maetz (1953) showed that when *Chironomus plumosus plumosus* L. is acclimatized overnight to the oxygen concentration of the subsequent experiment, its metabolism at oxygen concentrations greater than 45 % air-saturation changes from dependent to independent. Hyman (1929) showed that the degree of independence shown by *Planaria* depends on the amount of acclimatization allowed, and similar results were obtained with various species of arthropod by Hiestand (1931). Acclimatization to low oxygen concentrations has been demonstrated in young speckled trout by Shepard (1955).

Walshe-Maetz was also able to show that for *Chironomus* adaptation to lower oxygen concentrations consists of an increase in the amount of respiratory irrigation in the larval tube, but van Weel, Randall, and Takata (quoted in Zeuthen, 1955) found that the Hawaiian crustacean, *Podophthalmus vigil*, was able to regulate its metabolism in conditions of falling oxygen tension without any corresponding change in ventilatory activity. Shepard (1955) thought that the acclimatization of the speckled trout was due to a change in the oxygen capacity of the blood. In considering what might be the mechanism of acclimatization in *Erpobdella testacea*, one must take into account the fact that no ventilatory activity was seen when the leeches were in the respirometer, although in the earlier experiments in which the leeches were enclosed in bottles (Mann, 1956) undulations of the body were frequently observed. A possible explanation is that in still water a leech builds up

an oxygen gradient round its body, and undulation results in a better oxygen supply, but that in the flowing water of the respirometer the oxygen gradient is broken down and no benefit is derived from body undulation, which is therefore discontinued. Whatever the explanation, the fact remains that the acclimatization observed in the present series of experiments was not brought about by undulatory activity but by some internal mechanism.

In considering the probable nature of this mechanism it is necessary to assess the importance of the haemoglobin. The experiments in this section can only be regarded as exploratory, but results to date indicate that when there is no acclimatization carbon monoxide reduces oxygen uptake by about 25 % in one-third air-saturated water, and by about 45 % in air-saturated water, so we may conclude that between one-quarter and one-half of the oxygen carried by the blood is carried by the haemoglobin. This is comparable with the results obtained by Johnson (1942) for the earthworm. She found that the haemoglobin of the blood was responsible for supplying 22-40 % of the respired oxygen, according to the oxygen pressure of the atmosphere. At times of year when acclimatization to low oxygen concentrations in the water is occurring there is also evidence of acclimatization to carbon monoxide treatment. In the experiment of Table 4, when the leeches were in air-saturated water at all times, treatment with carbon monoxide was followed by a drop in oxygen consumption; but if the animals were left in carbon monoxide overnight their consumption by morning had returned to the same level as before treatment. Again, in the experiment of section D, treatment with carbon monoxide resulted in an initial fall in oxygen consumption, followed by a sharp rise. The experiments in which the leeches were subjected to both carbon monoxide and a low oxygen concentration in the water suggested that while the leeches might at times show signs of acclimatization, they were never able to compensate for the two factors, so that oxygen uptake after treatment never reached the same level as before treatment.

We thus arrive at the tentative conclusion that there is a mechanism of acclimatization which operates in summer but not in winter, and is independent of the haemoglobin of the blood, in the sense that acclimatization can occur when the haemoglobin is inactivated. Apparently the effect of oxygen want produced by placing the animals in water of low oxygen content is comparable with that produced by knocking out the haemoglobin, and both produce an acclimatization response at certain times of year. Perhaps the simplest mechanism which could be envisaged in the light of the known facts is that there is an increase in rate of circulation of the blood in oxygen-poor conditions. This may be combined with body undulations in still water but not in running water. Information on this mechanism is clearly required, but will be difficult to obtain, owing to the deep pigmentation of the tissues.

Finally one may consider the possible significance of this mechanism in the life of the animals. *Erpobdella testacea* has been found by the author in greatest numbers in this country in reed swamps made up of dense growths of *Sparganium* in highly organic mud. In summer the leeches may be collected from the leaf bases, where cocoons are deposited, but in winter they move down into the mud. In both

kinds of situation it is to be expected that low oxygen concentrations will occur. In winter there is apparently no attempt to regulate the metabolism, which must sink to a very low level, but in the spring and summer, when there is active growth and breeding, the leech is able to regulate its metabolism at a level comparable with that found in air-saturated water.

SUMMARY

1. In summer the leech *Erpobdella testacea* becomes acclimatized to a low concentration of oxygen in the water, and can maintain a constant rate of oxygen consumption down to one-third air-saturation. This acclimatization does not occur in winter.

2. The mechanism of acclimatization is such that it can operate when the leeches are resting, and when the haemoglobin has been prevented from functioning by treatment with carbon monoxide.

3. In the course of normal respiration in air-saturated water at 20° C. about 45 % of the oxygen is transported to the tissues by the haemoglobin. In one-third air-saturated water about 25 % is transported in this way.

4. After a meal of *Tubifex* the oxygen consumption increases threefold, and declines to the previous level over a period of 4 days.

5. A polarographic respirometer embodying a wide-bore dropping mercury electrode has been developed for this work. It provides on a galvanometer a constant indication of the oxygen concentration in the water which has passed over the animals.

REFERENCES

BISHOP, D. W. (1950). Respiration and metabolism. In Prosser, C. L. (ed.), *Comparative Animal Physiology*. Philadelphia and London.

BRIGGS, R., DAVIES, F. S., DYKE, G. V. & KNOWLES, G. (1957). Use of wide-bore dropping mercury electrode and zinc reference electrode for continuous polarography. *Chem. Ind.* pp. 223–4.

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

HIESTAND, W. A. (1931). The influence of varying tensions of oxygen upon the respiratory metabolism of certain aquatic insects and the crayfish. *Physiol. Zoöl.* 4, 246–70.

HYMAN, L. H. (1929). The effect of oxygen tension on oxygen consumption in *Planaria* and some echinoderms. *Physiol. Zoöl.* 2, 505–34.

MANN, K. H. (1956). A study of the oxygen consumption of five species of leech. *J. Exp. Biol.* 33, 615–26.

SHEPARD, M. P. (1955). Resistance and tolerance of young speckled trout (*Salvelinus fontinalis*) to oxygen lack, with special reference to low oxygen acclimatization. *J. Fish. Res. Bd Can.* 12, 387–446.

WALSHE-MAETZ, B. M. (1953). Le métabolisme de *Chironomus plumosus* dans des conditions naturelles. *Physiol. Comp.* 3, 135–54.

ZEUTHEN, E. (1955). Comparative Physiology (Respiration). *Ann. Rev. Physiol.* 17, 459–82.