METHODS OF STUDYING THE RESPIRATORY EXCHANGE IN SMALL AQUATIC ORGANISMS, WITH PARTICULAR REFERENCE TO THE USE OF FLAGELLATES AS AN INDICATOR FOR OXYGEN CONSUMPTION.

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The results described below are a sequel to those already published in this *Journal* on the spontaneous aggregation of flagellates.[1] In that publication it was shown that a flagellate, *Bodo sulcatus*, forms aggregations in regions where the concentration of dissolved oxygen is an optimum for it. This optimum is less than the saturation concentration of oxygen dissolved in water under atmospheric partial pressure. The flagellates move out of regions where the oxygen concentration is above or below the optimum to gather into the optimal regions. They are positively chemotactic to a certain concentration of dissolved oxygen.

This behavior of the flagellates can be made use of to indicate changes in the concentration of dissolved oxygen due to the respiration of an aquatic organism present in the water. For if the organism under investigation be kept motionless in a suspension of the flagellates in water saturated with oxygen at the atmospheric partial pressure, the flagellates will collect into those regions where the oxygen concentration is lowered through the respiratory activity of the organism. The sizes of the aggregations of flagellates thus formed will show the relative amounts of oxygen absorbed by the different parts of the surface of the organism.

The following experiment will show how the method is applied. A small fresh water invertebrate, such as a *Chironomus* larva, is

[1] Fox, H. M., An investigation into the cause of the spontaneous aggregation of flagellates and into the reactions of flagellates to dissolved oxygen, *J. Gen. Physiol.*, 1920–21, iii, 483, 501.

placed on a slide in a few drops of liquid from a *Bodo* culture. A cover-glass, supported at its four corners by wax feet, is placed over the liquid and is pressed down so that the larva is just prevented from moving, without being injured by the pressure (Fig. 1). The flagellates in the neighborhood of the larva swim in to its surface,

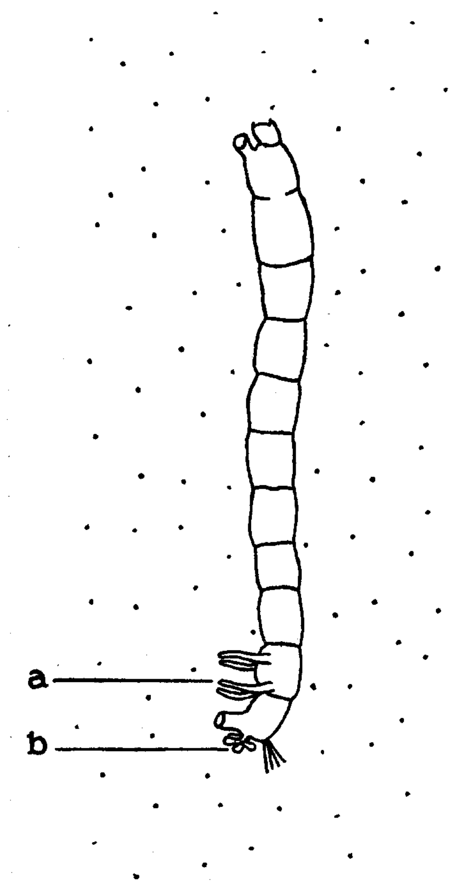

FIG. 1.

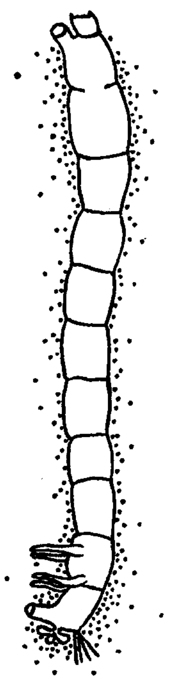

FIG. 2.

FIG. 1. *Chironomus* larva which has just been placed in a suspension of flagellates. *a*, ventral gills; *b*, anal gills. In all diagrams the density of the dots represents the density of distribution of the flagellates.

FIG. 2. The same larva shortly afterwards. The flagellates collect on the surfaces of the larva which are absorbing oxygen.

collecting there in ever increasing numbers (Fig. 2). This collection is not a surface energy phenomenon which might be caused by any solid object in the suspension, for if a larva which has just been killed by being dropped into hot water is used in place of the living larva, there is no aggregation of flagellates at its surface. The *Bodo* are simply attracted towards the surface of the living larva because in this neighborhood the respiratory activity of the insect has lowered the concentration of oxygen dissolved in the water. The reason for the collection is the same as that for the spontaneous aggregation previously described.[1] The flagellates move into a region of lower oxygen concentration caused, in the case of spontaneous aggregation, by their own respiration, in this experiment by the respiration of the larva. Here, of course, the aggregation of flagellates takes place much more rapidly than in spontaneous aggregation for the larva consumes relatively much more oxygen than the flagellates. If spontaneous aggregation occurred rapidly, it would interfere with the collection of the flagellates on the respiratory surfaces of the larva. The danger of spontaneous aggregation is, however, averted by a preliminary filtering of the *Bodo* suspension through bolting-silk. This not only removes fragments of debris from the culture jar but the exposure of the water to the air during filtration allows it to become saturated with oxygen. This greatly retards subsequent spontaneous aggregation.[1]

More flagellates collect on some parts of the insect's surface than on others. These are the most actively respiring surfaces. When the aggregation has reached certain dimensions, a clear space free from flagellates appears between it and the surface of the larva (Fig. 3). This is due to the same cause as the appearance of an area free from flagellates in the center of a spontaneous aggregation; the dissolved oxygen has been reduced to a certain low concentration at which the flagellates leave the region. The aggregation of flagellates on the surface of the larva now gradually becomes a band separated from the surface by a clear space, this happening first at those surfaces which are most actively respiring. Eventually the flagellates move out everywhere from the surface of the larva and come all to lie in a band encircling it (Fig. 4). In the case of an animal, such as a *Simulium* pupa, which respires only in one part, namely through the walls of certain filamentous appendages, no oxygen at all being absorbed by the rest of the body surface, the aggregation and band of flagellates have the forms shown in Fig. 5. In all cases the band gradually enlarges, moving out to a stationary equilibrium position just within and parallel to the edges of the cover-glass. This is the same phenomenon as the final stage of spontaneous aggregation and band formation.

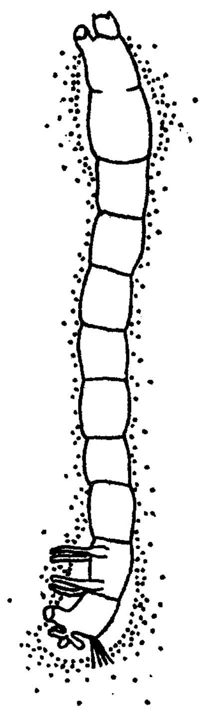

FIG. 3.

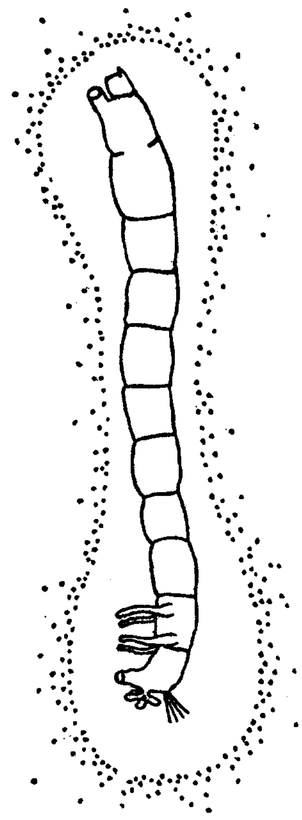

FIG. 4.

FIG. 3. The same larva a little later. The flagellates leave those surfaces of the larva which by their more active respiration have first reduced the concentration of dissolved oxygen below the optimum for the flagellates.

FIG. 4. The same larva. The flagellates have all left the surface of the larva and lie in a gradually spreading band in the zone of optimum oxygen concentration.

By this method the relative amounts of oxygen absorbed by different surfaces of a small aquatic organism can be estimated very delicately. Most oxygen is taken in at those places where most flagellates accumulate and where, later on, the aggregation first moves away from the surface in the form of a band. The use of the flagellates as an indicator in such work has the more value in that they can be cultivated either in fresh or in salt water. Throughout the present investigation the flagellate employed was *Bodo sulcatus*. It was obtained by steeping grass from the garden behind the laboratory at Plymouth in tap water.

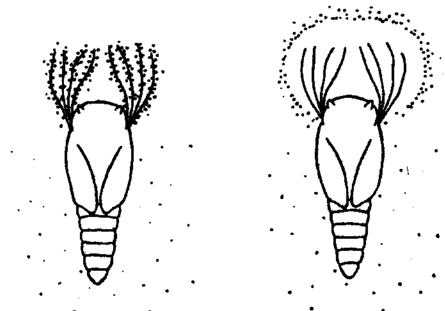

FIG. 5. *Simulium* pupa in a suspension of flagellates. A, the flagellates collect on the surfaces which are absorbing oxygen; B, later, the flagellates leave the region where the oxygen concentration has fallen below the optimum for them.

The use of flagellates for detecting the intake of oxygen by the surfaces of organisms is complementary to Engelmann's well known method of demonstrating the output of oxygen in photosynthesis by means of bacteria.[2]

The flagellates have been used so far to test the respiratory activities of several types of aquatic insect larvæ, but the work so far has been done mostly from the point of view of perfecting the methods. In the future it is proposed to examine systematically all available types of larvæ with the particular object of settling the functions of the several kinds of so-called gills. To make the meaning clearer, the work done with the red *Chironomus* larva will now be outlined.

[2] Engelmann, T. W., Neue Methode zur Untersuchung der Sauerstoffausscheidung pflanzlicher und thierischer Organismen, *Arch. ges. Physiol.*, 1881, xxv, 285.

These larvæ have on the ventral surface of the eighth abdominal segment four tube-like hollow outgrowths, in which the blood circulates. On the dorsal surface of the last abdominal segment there are four shorter hollow outgrowths, also with a blood stream through them. All these processes are thin-walled and have a thin cuticle. It is usually assumed that the chief respiratory gas-exchange takes place through the walls of these outgrowths, the so-called ventral and anal gills. When the larvæ are tested with the *Bodo* suspension, it is found that oxygen is absorbed by the whole of the general body surface of the larva except the head which has a very thick cuticle. The intensity of absorption is usually greatest in the posterior abdominal segments, but this is not invariably so. The most actively respiring segments vary from one individual to another, and when a single individual is tested a number of successive times the most actively respiring regions may vary in position each time. However, the remarkable result is the behavior of the so-called gills. No more oxygen is found to be absorbed by the "anal gills" than by the general body-surface and no oxygen at all is absorbed by the "ventral gills." The latter may be seen to project through and beyond the collection or band of flagellates without influencing it (Figs. 2 and 3).

This result can be confirmed by quite another mode of experimentation. The blood of these larvæ contains hemoglobin dissolved in it. When a larva is examined with a microspectroscope the whole body shows the oxyhemoglobin absorption bands. If a larva is now placed in water in a hollow-ground slide and covered with a cover-slip, the edges of which are sealed down with vaseline, at the end of about 20 minutes the whole body of the larva shows the reduced hemoglobin absorption spectrum. The larva does not die when this has occurred. It will remain alive with heart beating for many hours beneath the sealed cover-slip. If a small bubble of air is now allowed to enter beneath the cover-slip, so that it comes to rest up against the surface of the larva, an examination with the microspectroscope shows that oxyhemoglobin first appears inside the body of the larva at a point nearest to the bubble and thence it spreads over the rest of the body. Thus the part of the body-surface nearest to the bubble first absorbs the oxygen, and not the "gills." But a more striking result than this can be obtained. If by chance the bubble comes to rest in contact with the "ventral gills," the first appearance of oxyhemoglobin is inside the abdominal segment nearest to the bubble and not within the "gills" themselves. This fully bears out the result obtained with the flagellates. Whatever be the function of the "ventral gills," it is not that of absorbing oxygen.

The spectroscopic method is one suited to this particular larva alone; it cannot be applied where hemoglobin is absent. The flagellate method, however, is one of general application.

At the same time that the oxygen intake was studied, the relative output of carbon dioxide by different surfaces of the body was investigated. This was done by mounting the larva on a slide in the same manner as for studying the oxygen absorption, but, in place of a suspension of flagellates, an indicator was used which changes color as carbon dioxide goes into solution, altering the hydrogen ion concentration of the water. The position and extent of the color change indicates the place and amount of the carbon dioxide output. Several indicators are available which change in color about the neutral point of water. Neutral red and rosolic acid were tried but were found unsuitable for these experiments because in the thin layer of solution between cover-slip and slide the colors are not sufficiently intense to show a sharp change. The indicator which was found suitable for use was a solution of hematoxylin with just sufficient alkali added to make it a bluish pink. In regions where carbon dioxide is being given off by the larva into the water the bluish pink color changes through orange to yellow. The color change is sharp even in the thin layer of liquid between cover-slip and slide. It can be more exactly observed when the examination is made under a very low power of the microscope, such as a 2 inch objective. All tests were discarded in which the larva defecated or in which any liquid at all came out through the anus, for the rectal fluid is alkaline.

Using the red *Chironomus* larva it was found by this method: (1) that carbon dioxide is given off by the whole body surface except by the head and the ventral gills; (2) that most carbon dioxide is given off by the posterior abdominal segments, but that the relative amounts given off by different segments vary in different individuals and in the same individual at different times; (3) that carbon dioxide is not always given off by the different surfaces of an individual larva in the same relative amounts that oxygen is absorbed by these surfaces; (4) that the "anal gills" give off no more carbon dioxide than the general body surface; and (5) that the ventral gills give off no carbon dioxide.

It is intended to apply the flagellate method of studying the oxygen intake and the indicator method of studying the carbon dioxide output to small aquatic types of the various groups of the animal kingdom, but first of all a systematic examination will be made of aquatic insect larvæ with special reference to the functions of the so-called blood gills and tracheal gills and to the relation of closed tracheal systems to respiration.

SUMMARY.

1. Flagellates are positively chemotactic to a certain concentration of dissolved oxygen which is lower than that in water saturated with oxygen under atmospheric partial pressure. Consequently, when a small aquatic animal is held motionless between cover-slip and slide in a suspension of flagellates in water saturated with oxygen, the flagellates are attracted to those parts of the animal which are absorbing oxygen. The relative sizes of the flagellate aggregations then show the relative activities of the different surfaces of the animal in absorbing oxygen.

2. Applying this method to the red *Chironomus* larva it was found that the animal respires by the whole body surface except by the head and the "ventral gills" and that the relative intensity of oxygen intake by the different parts of the body varies in different individuals and in the same individual at different times.

3. The absence of oxygen intake by the "ventral gills" was confirmed with the microspectroscope. In oxygen-free water all the hemoglobin of the blood becomes reduced. When an air bubble is now introduced so that it touches the "ventral gills" oxyhemoglobin first appears in the nearest body segment to the bubble, not in the "gills."

4. When a small aquatic animal is held motionless between cover-slip and slide in a solution of an indicator which changes color about the neutral point of water the relative extent of color change at different surfaces of the animal's body indicates the relative amounts of carbon dioxide given off by these surfaces.

5. Using this method with the red *Chironomus* larva similar conclusions were reached for carbon dioxide output as for oxygen intake.

The work was done in August and September, 1919, at the Laboratory of the Marine Biological Association, Plymouth. I wish to thank the Director and staff for their constant kindness and assistance. I wish also to thank the trustees of the Ray Lankester Fund for having nominated me to an Investigatorship and Sir Ray Lankester, K.C.B., F.R.S. for having lent me a microspectroscope.