AN INVESTIGATION INTO THE CAUSE OF THE SPONTANEOUS AGGREGATION OF FLAGELLATES AND INTO THE REACTIONS OF FLAGELLATES TO DISSOLVED OXYGEN.

PART I.

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(Received for publication, September 21, 1920.)

The Phenomena of Spontaneous Aggregation and Band Formation.

It is well known that many flagellate and ciliate Protista form spontaneous aggregations. That is, when a drop of water containing the organisms is mounted on a slide for examination under the microscope the flagellates or ciliates are frequently seen to collect into clumps or masses. The center of such a collection may be a piece of some solid present in the water, or there may be no such visible focus. It is the second of the two cases that is here termed spontaneous aggregation, and the present investigation was undertaken with the object of finding out the cause of this phenomenon. The discovery of the cause then led on to the more general question of the relation of the organisms to varying amounts of dissolved oxygen.

Throughout the investigation the same species of flagellate was used; namely, Bodo sulcatus. It was originally described by Mereschkowsky. For the present investigation the flagellate was obtained by taking grass from the garden behind the laboratory at Plymouth and steeping it in tap water. The best material was obtained from 6 day old cultures. The flagellates were then most abundant without being too much mixed with other organisms. In cultures older than 6 days there were too many bacteria present and, still later on, too many ciliates.

The phenomenon of spontaneous aggregation is best studied as follows: A square cover-glass supported by wax feet at its four corners, or, better still, by short pieces of glass rod previously cemented to the corners, is placed on a dry slide. Some liquid from a *Bodo* culture is then run in from a pipette beneath the cover-glass until it just fills the space between the latter and the slide. At first the flagellates are evenly scattered throughout the preparation (Fig. 1) but they do not remain so for an indefinite time. At the end of an interval which may be 2 minutes or 2 hours they begin to collect into one or more groups towards the center of the cover-glass, the size of these aggregations increasing until they contain most of the *Bodo* present in the liquid (Fig. 2). The flagellates in the aggregations are in intense movement. There is no solid body forming the center of a collection and indeed the presence of any such object is purposely avoided by filtering the suspension of *Bodo* through fine bolting-silk before making the preparation. Under a square cover-glass measuring $\frac{3}{8}$ inch $\times$ $\frac{7}{8}$ inch, which was the size used in the
experiments, one central aggregation is usually formed but sometimes there are several, always near the center.

After an aggregation has been in existence for a short time a clear space free from flagellates appears at its center. The central space enlarges until the flagellates come to lie in a circular band around it, this band being easily visible to the naked eye (Fig. 3). The increase in size of the clear area goes on steadily, and as the band of flagellates surrounding it approaches the edges of the cover-slip the sides of the band become flattened (Fig. 4) and then, still enlarging, the band gradually comes to form a square, the sides of which are parallel to the sides of the cover-glass. When this square-shaped band of flagellates has reached a certain distance from the edges of the preparation, it becomes stationary, the central clear area no longer increasing in size (Fig. 5). The distance of the final position of the band from the edge of the cover-slip depends on the height of the latter above the slide; the band comes to lie the nearer to the edge the lower the cover-slip. The great majority of the flagellates present in the preparation are in the band, but nevertheless there are always a few swimming in the region between the band and the edge of the cover-glass.
A grain of sand lying in the path of the band of *Bodo* as it advances from the center towards the edges of the preparation has no effect on the band: the latter approaches and passes the grain of sand without being deflected. An air bubble, however, keeps the band at a distance from it. If the bubble lies sufficiently far in from the edge of the cover-glass the advancing band of flagellates becomes bent inwards to form a bay enclosing the bubble (Fig. 6). As the main band continues to move outwards the horns of the bay meet so that an inner ring of flagellates is left behind surrounding the bubble (Fig. 7). The bubble, then, behaves to the band just as the water-air surface at the edges of the preparation does: it keeps the flagellate band at a certain distance from it. But there is this difference between the two cases, that whereas the main band comes to a halt and remains stationary at a certain distance inside the edges of the preparation, the band encircling the bubble slowly approaches the latter. This continues until the flagellates come into contact with the surface of the bubble itself (Fig. 8), where they remain for a short time and then dissipate, swimming out to join the main band.
If a preparation such as has been described, in which the square-shaped band of Bodo is established with its sides parallel to the edges of the cover-glass, is kept in a moist chamber and examined again on the following day, it will be found that the band has retreated somewhat from the edges of the cover-slip. On the following day again the band will be further in still and will have taken on a circular instead of a square form. Later on all the flagellates will be clumped in one mass at the center of the slide, after which they will gradually dissipate to become evenly scattered throughout the liquid again. In fact the Bodo band goes through the same series of changes which

![Fig. 7. Main flagellate band reaches its equilibrium position having left behind an inner ring surrounding air bubble.](image)

![Fig. 8. Flagellates around air bubble advance to its surface.](image)

it originally underwent in its formation but in the reverse order. Through all the series of changes the organisms continue in full motile activity. The retiral of the band from the outside may be hastened by placing the slide in an ice chest. In the course of a few hours only, the square band will have become a circular band situated near the center of the preparation. The swimming activity of the Bodo is, incidentally, not noticeably decreased by the low temperature. If, on the other hand, the moist chamber containing the preparation is kept at a higher temperature than that of the laboratory, the band retires from the edges much more slowly, if at
The Cause of Aggregation and Band Formation.

In seeking for the causes of these phenomena, there are three questions to be asked.

1. What is the cause of the aggregation of the flagellates at the center of the preparation?
2. Why does an aggregation become a band surrounding a region clear of flagellates, which continuously increases in size?
3. Why does the central clear region cease to increase in size when the band of flagellates bordering it has reached a certain distance inside the air-water surface at the edge of the cover-slip?

The different condition which arises at the center of the preparation, attracting thither the flagellates, must be due to some volatile substance in solution, which at the free edges of the liquid is effecting an exchange with the atmosphere, either going into or out of solution. The only gases in solution which would be changed in amount by the living organisms are oxygen and carbon dioxide; the amount of the former present in solution in the water must continuously decrease and the amount of the latter increase. These changes in the concentrations of dissolved oxygen and carbon dioxide will take place more rapidly in the central region of the liquid than at its edges; for at the edges the oxygen used up by the flagellates will be replaced from the atmosphere, while the carbon dioxide produced by them in this region will go out of solution into the atmosphere as soon as its tension in solution rises above the value corresponding to its partial pressure in the

all. Furthermore, a flagellate band which has been caused to retire inwards by keeping the preparation at a low temperature will move outwards again as soon as the slide is replaced in the higher temperature of the laboratory.

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atmosphere. In the center of the preparation no exchange with the air can take place, so that here the concentration of dissolved oxygen will decrease and that of carbon dioxide increase most rapidly.

It seems probable, then, that the flagellates collect in the central region because they are attracted either (a) into a region of higher hydrogen ion concentration, or (b) into one where the concentration of dissolved oxygen is lower. Which of these alternative explanations is the correct one? It was attempted to answer this question by isolating the two possible causes and allowing each to act separately.

To test suggestion (a) a long cover-glass was supported over a slide by wax feet placed beneath its four corners. Some filtered suspension of *Bodo* was then let in under the cover-glass from one end, so as not to fill completely the space between the cover-glass and the slide. Immediately afterward from the other end of the cover-glass some water which had been saturated with carbon dioxide was let in (Fig. 9). If such a solution had an attractive influence on *Bodo*, the flagellates would have collected in the region where the two liquids merged. This they did not do.

To test suggestion (b) a similar preparation was made but in place of carbonic acid, reduced indigocarmine was introduced beneath one end of the cover-slip. Indigocarmine (sodium sulfinidigoate) was reduced in the absence of oxygen (in a stoppered bottle) by a solution of 1 per cent glucose containing 1 per cent caustic potash. By this means the yellow leuco base is formed. As much of a concentrated solution of indigocarmine was used as the glucose would turn from blue to yellow in 1 hour in the stoppered bottle. In the presence of oxygen the yellow leuco base reoxidizes instantane-
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ously to blue indigocarmine. This takes place either when the leuco-base is exposed to the air or when it comes into contact with water containing oxygen in solution. In our experiment under the cover-glass the dissolved oxygen was abstracted from a zone of the Bodo suspension bordering the drop of reduced indigocarmine. It was found that all the flagellates from the neighboring region of the suspension collected rapidly in this zone, forming there a crowded band (Fig. 10). Thus the Bodo are attracted into a region whence the dissolved oxygen has been removed. As control tests, indigocarmine alone, glucose alone, and caustic potash alone were substituted for the mixture of the three but in no case was there any aggregation of Bodo. This experiment was repeated with precisely the same result using in place of the reduced indigocarmine either

Fig. 10. Flagellates beneath a long cover-glass attracted by an oxygen absorber introduced at one end. a, oxygen absorber.

an alkaline solution of pyrogallic acid or of hematoxylin. Both of these solutions take up oxygen rapidly. In the case of the hematoxylin the best procedure was found to be to color the Bodo suspension lightly with the dye, which does not injure the flagellates, and then to introduce some of this suspension under one end of a long, supported cover-slip. Under the other end 0.1 N NaOH was let in. In the zone where the two liquids meet, the hematoxylin rapidly oxidizes in the presence of the alkali, abstracting oxygen from solution in the water. The flagellates in the neighboring region collected in a crowded band in this zone of reduced oxygen concentration. Controls in which the alkali was replaced by water and in which the hematoxylin was omitted gave no aggregation of flagellates.

In this way it can be demonstrated that the flagellates will collect into a region where the concentration of dissolved oxygen is reduced.
but that they will not react to a region of greater hydrogen ion concentration caused by dissolved carbon dioxide. The aggregation then in the center of the liquid beneath a cover-slip must be due to the attractive influence of this central region where the respiratory activity of the organisms has reduced the amount of dissolved oxygen.

The second question—why does an aggregation become a ring surrounding an ever growing clear area—has now to be answered. Do the *Bodo* leave the central area (a) because the concentration of dissolved oxygen has fallen here below an optimum value for them or (b) because the hydrogen ion concentration has increased too much?

If (b) is the cause, the flagellates should leave the central area sooner—that is, they should form a ring sooner—in a preparation which was originally more acid than in one originally less acid. For in the former the critical concentration of H ions which would drive out the flagellates would be arrived at earlier. This test was made. One sample of a culture of *Bodo* was given a concentration of H ions such that when tested with rosolic acid it gave the same yellow color as did tap water saturated with carbon dioxide. A second sample from the same *Bodo* culture was given a concentration of H ions showing a pink with rosolic acid. These changes in the H ion concentrations of the two samples had no effect on the activity of the flagellates as judged under the microscope. If the cause of the formation and spreading of the band is the accumulation of carbonic acid at the center of the liquid, the occurrence must take place sooner in a preparation made from the first sample than in one made from the second. The experiment showed, however, that the band was formed and spread simultaneously in the two preparations.

It must be mentioned here that in this and all other experiments when the times of aggregation or band formation were to be compared in two preparations, the cover-glasses were supported by short pieces of thin glass rod cemented to their corners, not by wax feet. By using pieces of glass having the same thickness it was ensured that the cover-glasses were at the same height above the slides. This is necessary since the height of the cover-glass influences the rate of aggregation and the distance of the equilibrium position of the band from the edge.
It is not carbonic acid, then, that drives out the *Bodo* from the center and we must conclude that, while the organisms collect into a region where the oxygen concentration is lower than the saturation concentration for the atmospheric partial pressure, yet when the oxygen in this region falls below a certain limiting value the organisms are forced to move away again. They remain in a band surrounding the central area of lowest oxygen concentration, this band representing the optimum concentration of oxygen for them. It lies between the central exhausted region and the outside liquid into which oxygen continually dissolves from the air to replace that used up. Further, since in the crowded band the *Bodo* consume the available oxygen more rapidly than it can be replaced from outside, the size of the central area of lowest oxygen concentration continually increases, forcing the band to approach nearer and nearer to the edges of the cover-slip.

The fact that the *Bodo* band is a zone of optimum oxygen concentration at once gives the clue to the third question originally asked—why does the advancing band cease to advance when it has reached a certain distance from the edges of the cover-glass? As the band advances towards the water-air surface at the edges of the preparation it must eventually reach a position where there is a state of equilibrium between the oxygen used up by the flagellates and that diffusing inwards from the edges. At this point the band will remain stationary.

That this inward diffusion of oxygen is really the factor which controls the distance of the band of flagellates from the edge can be shown very simply as follows. A preparation with a *Bodo* band which has become stationary is placed in a gas chamber on the stage of a microscope and oxygen is passed through the chamber. Almost immediately the flagellate band commences to retire inwards towards the center of the liquid under the cover-slip. It gets gradually less square and more circular until it forms a small ring and finally becomes one mass of flagellates at the center of the preparation. When now a stream of hydrogen is passed through the gas chamber in place of the oxygen, the band reforms and slowly but continuously increases in circumference. In the first case the oxygen dissolving in the edges of the liquid and diffusing inwards causes the position of opti-
mum oxygen concentration for the flagellates to move inwards. In an atmosphere of hydrogen, on the other hand, the oxygen goes out of solution again around the free edges of the liquid and the position of optimum oxygen concentration for the flagellates again moves outwards.

Thus oxygen is the controlling factor in the position which the flagellates take up: they collect into regions where the concentration of dissolved oxygen is an optimum for them. The behavior of the advancing band in the presence of an air bubble now receives its explanation. The bubble acts at first like the edges of the preparation, the water immediately around the bubble being saturated with oxygen at the atmosphere partial pressure of the gas, so that the flagellates are unable to move right up to its surface. But as the flagellates are continually taking up oxygen, the amount of the latter present in the bubble gradually becomes exhausted. The flagellates are thus enabled to approach closer to the bubble until they touch its surface, where they remain for a short time until nearly all the oxygen in the bubble has gone into solution and been consumed by them. Then the oxygen concentration at the surface of the bubble falls below the optimum for the \textit{Bodo} and they leave the region altogether.

This phenomenon can be imitated as follows. A cover-slip with wax legs is placed on a slide and some of the indigocarmine-glucose-caustic potash mixture let in beneath it with a pipette. The central area of the preparation soon becomes yellow, the indigocarmine being reduced here in the absence of oxygen. This yellow area is square with its sides parallel to the edges of the cover-glass. It is surrounded by a purple band, the region between this purple band and the edge of the liquid being blue. The purple band corresponds exactly to the flagellate band. It is the region of equilibrium between oxygen consumed at the center and oxygen diffusing in from the edge. Any air bubble in the yellow area is surrounded at first by a narrow blue ring, separated from the yellow by a circular purple band. The purple band approaches slowly but continuously to the bubble until it lies on the surface of the latter and then it gradually disappears. This occurs when all the oxygen of the bubble has gone into solution and been used up. We have here an exact parallel to the behavior of a \textit{Bodo} band surrounding an air bubble.
We must now ask, what is the cause of the gradual slow retiral of a *Bodo* band from the edges of a preparation when left over night at room temperature, of its more rapid retiral in the cold, and of its spreading out again when replaced at a higher temperature? These phenomena also can be imitated with an indigocarmine preparation such as has just been described. When the slide with its yellow central region separated from the blue border by a square purple band, the sides of which are parallel to the edges of the cover-glass, is placed in the ice chest the purple band contracts. It retires towards the center of the solution, the corners of the square becoming rounded until it assumes the form of a ring. When the preparation is now replaced at a higher temperature the purple ring expands again, becoming square as it nears the edges of the cover-glass. When left over night in a moist chamber at room temperature the purple band recedes slowly from the edges of the liquid. Now this behavior of the purple band of partially reduced indigocarmine is due to the different solubilities of oxygen at different temperatures. On the ice more oxygen goes into solution and drives the purple band inwards. When the preparation is warmed some of the oxygen goes out of solution and the purple band approaches the edges again. Left at room temperature the concentration of dissolved oxygen at the edges of the preparation gradually rises because the water was not originally saturated with oxygen, so that the band retires slowly inwards. The cause of the similar behavior of a *Bodo* band under the same conditions must be precisely the same. The band of flagellates moves to a position nearer to or further from the edge according to the lesser or greater amount of oxygen going into solution at the different temperatures. An alternative explanation is that the *Bodo* band retires inwards at a low temperature or when left for some time because under these circumstances the flagellates change their oxygen optimum. The suggestion, however, becomes very improbable in view of the parallel behavior of the indigocarmine band, the cause of which is known.

*Confirmatory Facts.*

There are several facts and experiments which strikingly confirm the conclusion that the concentration of dissolved oxygen is the con-
trolling factor in the behavior of the flagellates. Of these I shall mention three. They are: (1) the effect of the original oxygen content of the *Bodo* suspension on the rate of band formation; (2) the reduction of oxyhemoglobin by *Bodo*; and (3) the effect of a green plant in sunlight on the band.

To show the effect of the oxygen content of the suspension of flagellates on the rate of aggregation and band formation two preparations were made on slides in the manner already described. In the first preparation the liquid was let in beneath the supported cover-glass immediately after having been pipetted out of the culture jar. For the second preparation a pipetteful of liquid was taken from the same culture jar, placed in a petri dish, and exposed to the air for a short time before being let in under the cover-glass. A *Bodo* culture always contains less oxygen in solution than plain water in a similar jar at the same temperature. This is of course due to the fact that the flagellates are continually absorbing oxygen in respiration. Consequently the suspension of *Bodo* which had been exposed to the air in the petri dish had acquired a greater oxygen content than that taken directly from the culture jar. It was found that the aggregation of flagellates was much slower in the aerated than in the non-aerated preparation and that the central clear area became established later in the former.

It was to be expected that the central clear area bordered by the band of flagellates would become established later in the aerated preparation. Here it must take longer for the organisms to consume sufficient oxygen to make the central region untenable for them. But it is not at first obvious why the central aggregation should form later in the aerated preparation. It would be expected, rather, that when in the presence of more oxygen than the optimum the flagellates would migrate from any region of higher into any region of lower oxygen concentration. Now a region of relatively lower oxygen concentration due to the respiration of the flagellates must arise as soon in the center of the aerated as of the non-aerated preparation; nevertheless, in the former the organisms are not attracted so soon towards the center as they are in the latter. It seems thus that when the liquid is well aerated the flagellates are not sensible to a region of lower oxygen concentration: it is not until the oxygen content has been
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reduced by a certain amount throughout the slide that the *Bodo* feel the attraction of the central region. They are thus only sensible to this influence when in the presence of an oxygen concentration closely approaching the optimum for them.

This was confirmed by an experiment with an oxygen absorber such as reduced indigocarmine or pyrogallic acid. Two slides were prepared with long cover-slips supported by pieces of glass rod. Under one cover-slip was run in some *Bodo* suspension taken straight from the culture jar and under the other some of the same culture which had been aerated by exposure in a petri dish. The liquid was not allowed in either case completely to fill the place beneath the cover-slip, but a space was left at one end into which the oxygen absorber was to be run. When this had been done, it was seen that the flagellates in the aerated preparation collected much later into the region of reduced oxygen concentration next to the indigocarmine than they did in the non-aerated preparation. In fact whereas in the non-aerated liquid the *Bodo* moved at once into the region next the oxygen absorber, in the aerated liquid they did not do this until by their own respiration they had reduced the oxygen content of the general suspension to a point approaching the optimum for them. The flagellates thus gave no response to lowered oxygen concentration when in the presence of an amount of oxygen much above their optimum.

It was mentioned at the commencement of this account that in a normal cover-slip preparation the *Bodo* ring may become established in 2 minutes or it may take 2 hours to form. A series of experiments demonstrated that this great difference in time is due to different initial oxygen contents of the suspensions. If the suspension to be used is exposed to the air for some time during the preparatory filtering operation it will have a higher oxygen content than liquid taken straight from the culture jar, for the oxygen concentration in the cultures is always considerably lower than the saturation concentration under atmospheric partial pressure, and therefore in the filtering through bolting-silk oxygen is absorbed from the air. Aggregation and ring formation always take place later in a suspension that has been exposed to the air than in one that has not.
The following is an account of four experiments which illustrate this point. The same *Bodo* culture was used throughout. In each experiment two preparations were made; (a) with *Bodo* suspension taken straight from the culture jar, and (b) with a suspension previously aerated. In Experiment 1 the aeration was done by pouring from one watch-glass to another for 15 seconds. The times taken for the band of flagellates to take up its stationary position were; (a) 11 minutes, and (b) 45 minutes. In Experiment 2 the aeration was done in the same way and the times were; (a) 4 minutes, and (b) 46 minutes. In Experiment 3 the aeration was performed by leaving a small quantity of the liquid in a watch-glass for 56 minutes at 18°C., the laboratory temperature being 23°C. The times were; (a) 2 minutes, and (b) 56 minutes. In Experiment 4 for the preparation of Slide (a) 10 cc. of *Bodo* suspension were kept in a watch-glass for \(\frac{1}{2}\) hour at 26° and for (b) 10 cc: were similarly treated at 17°. There was no noticeable difference in activity of the flagellates at the two temperatures. As soon as the liquids were pipetted under the coverslips their temperatures became identical and equal to that of the laboratory so that the different times taken for the bands to become established must have been due to the different quantities of oxygen which had gone into solution at the two temperatures. The times were; (a) 2½ minutes, and (b) 14 minutes.

The consumption of oxygen in the center of developing *Bodo* rings can be demonstrated in preparations containing hemoglobin. For this purpose a pipetteful of liquid from a *Bodo* culture is placed in a watch-glass and one or two drops of blood from a pricked finger are mixed with it. The blood becomes lake. It in no way interferes with the activity of the flagellates. If now a preparation is made in the usual way beneath a supported cover-slip, no sooner does the central aggregation of flagellates become a ring than the bluish color of the region surrounded by the ring shows that it contains reduced hemoglobin. The blue color here is in marked contrast to the scarlet of the oxyhemoglobin outside the ring of flagellates, and the distribution of hemoglobin and oxyhemoglobin is easily verified with the microspectroscope. Thus after the flagellates have used up the available free oxygen dissolved in the water, they extract that which is bound in the oxyhemoglobin. When this too is exhausted at the
center of the slide, a circular band of flagellates is formed surrounding a region of reduced hemoglobin. As would be expected, aggregation and band formation take place much more slowly in the presence of oxyhemoglobin than in plain water for much more oxygen is available.

In a typical case two preparations were made simultaneously from the same culture; (a) without blood, and (b) with blood. During the mixing of (b) with blood, (a) was exposed to the air in exactly the same way as (b). The times for the ring to become established in its stationary position were; (a) 1 hour, 15 minutes, and (b) 3 hours, 40 minutes.

In the preparations containing hemoglobin the circular Bande zone surrounding the central clear area, when once formed, grows in the usual way, becomes square, and takes up its stationary position some distance inside the edge of the cover-slip. The whole central area within the band contains reduced hemoglobin while the edges outside it show the bright scarlet of oxyhemoglobin. The flagellate band itself lies just within the region of reduced hemoglobin.

A demonstration of the effect of oxygen on the equilibrium position of the Bande band can be arranged as follows. Some suspension of Bande is let in with a pipette under a cover-glass supported at its corners, and a piece of some aquatic green plant, such as a frond of moss, is pushed into the liquid from the middle of one side of the cover-glass. The slide is placed in the dark and when the band of flagellates becomes established in its stationary position it will cut straight across the moss (Fig. 11). The preparation is now exposed to diffuse sunlight, so that oxygen is produced by the plant in photosynthesis.

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Fig. 11. Preparation kept in darkness with flagellate band in equilibrium position interrupted by a piece of moss. a, glass rod supporting cover-glass; b, moss.
The edges of the flagellate band which touched the moss on either side immediately move back from it and bend inwards (Fig. 12) and in a few minutes the band has reformed with an indentation to include the moss (Fig. 13). Replaced in the dark the band straightens out again to its original position as the extra oxygen which was produced by the plant in the light is consumed by the flagellates. The two ends of the band in touch with either side of the moss bend slightly outwards towards the edge of the slide (Fig. 11) because the moss is now absorbing oxygen.