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Studies on the Regulation of *Spirostomum ambiguum* EHRBG.

By

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(With 36 figures in the text.)

I. Introduction.

Spirostomum ambiguum is admirably suited for researches on regulation and regeneration, both on account of its large size and on the presence of a long, moniliform macronucleus. But while it has been made the subject of several physiological studies, it appears that only one zoologist, SOKOLOFF, has made use of it in experiments on regeneration. GRUBER (1886), it is true, wished to use this particular form in his experiments on regeneration in the protozoa, but did not succeed in doing so, for as he says: ". . . I never succeeded in multiplying *Spirostomum*, . . . artificially, as it is very difficult to keep isolated in small quantities of water, and even when uninjured soon perishes."

SOKOLOFF (1913, 1924), in his researches, was mainly concerned with the minimum volume of *Spirostomum* necessary for regeneration, and with the speed of regeneration in pieces cut from different parts of the body, and in pieces of different sizes. He showed that this minimum volume lies between $^1/_{53}$ and $^1/_{69}$ of the original volume of the animal. The minimum volume is not the same in pieces taken from different regions of the body. Thus in pieces cut from the middle $^1/_3$ of the individual, it is $^1/_{69}$ of the total volume, while in pieces cut from the anterior or posterior $^1/_3$ of the individual, it lies between $^1/_{50}$ and $^1/_{57}$ of the total volume. The absolute size of the piece appears to have no influence on these results.

In the case of his experiments on the speed of regeneration, he found that if pieces of the same size are cut from the anterior and posterior ends of the animal, the speed of regeneration is much the same in both kinds of pieces. But the speed of regeneration of different sized pieces is not the same. The larger the piece, the greater the speed of regeneration. SOKOLOFF found, however, that this rule held only for those pieces with a volume of more than $^3/_4$ or less than $^1/_{30}$ of the original volume of the animal.

SOKOLOFF was also able to demonstrate that segments without any nuclear material were incapable of regeneration.

From this short summary of SOKOLOFF'S work on *Spirostomum*, it is evident that our knowledge of the phenomena of regeneration in this form is by no means complete, and the present studies are an attempt to remedy this deficiency.

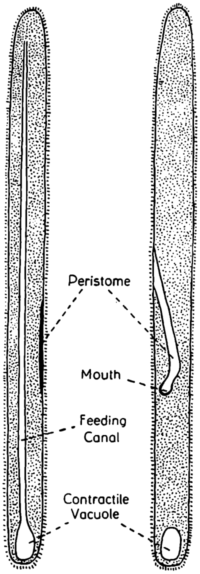
I wish to express my thanks to Professor E. S. GOODRICH for helpful criticism, and to Dr. G. R. DE BEER for his advice and encouragement throughout this work.

2. Material and method.

One of the characteristics of *Spirostomum ambiguum*, as of certain other Heterotricha, is the possession of a moniliform macronucleus, that is to say the macronucleus is greatly elongated and has the shape of a string of beads. It is generally agreed, that if a piece of a ciliate is without a portion of the macronucleus, it will not regenerate completely, though it may continue to live for some time. Owing to the shape of the macronucleus of *S. ambiguum*, extending as it does from nearly end to end of the organism, any piece which is cut from an individual, other than the extreme anterior or posterior ends, will contain a portion of the macronucleus and will thus be capable of regeneration. This makes *S. ambiguum* much more satisfactory material from the point of view of experiments on regeneration than *Spirostomum tieres*, in which the macronucleus is short and bean shaped.

The structures around which the greater part of this work centred were firstly the contractile vacuole and its feeding canal, and secondly the peristome and the mouth.

The contractile vacuole, which is relatively of large size, is situated right at the posterior end of the body, and leading from it is a long, narrow feeding canal which stops short of the anterior end (Fig. 1). In most of the individuals examined, this was the only vacuole present, but occasionally the feeding canal may be swollen out at more than one place into a vacuole. In some individuals vacuoles are visible in the endoplasm quite separate from the vacuolar feeding canal.



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Figs. 1—2. *Spirostomum ambiguum*, showing the positions of the peristome, mouth, vacuole and vacuolar feeding canal.

The mouth, with the peristome leading into it, is shown in Fig. 2. The position of the mouth varies very considerably in different individuals, though probably in the majority of forms it occupies a position about $^1/_3$ of the length of the individual from the posterior end.

The *Spirostomum* used during the first part of this work were obtained from a stock kept in a tank in the Department of Zoology and Comparative Anatomy at Oxford. Later a fresh stock was cultured in a large dish in the laboratory. The animals were kept in this dish for a period of several months by feeding them on the faeces of guinea-pigs [1]).

The pieces and whole individuals used in the experiments were kept in solid watch-glasses. The medium used was pond water to which had been added a small quantity of guinea-pig faeces. In such a medium, *Spirostomum* can be kept alive in small watch-glasses for more than a fortnight, though if pond water alone is used, death is comparatively rapid.

3. Results of Experiments.

A. Regulation of Form.

a) After complete removal of a piece.

When a *Spirostomum* is cut into two pieces it sometimes happens that these pieces disintegrate very rapidly. The endoplasm streams out from the cut end, and in the space of about a minute not a sign of the piece is left. Part of the macronucleus can frequently

[1]) SPECHT (1935) has recently published a paper on the culture of *Spirostomum ambiguum*. The medium he uses is a hay and wheat infusion, to which has been added fresh cow manure.

be seen in such cases protruding from the cut end of the piece. Such rapid disintegration occurs mainly in small pieces.

This phenomenon is widely known in the Infusoria and, according to Verworn, it is particularly evident in those forms, the protoplasm of which is especially rich in water (see Verworn, 1909, p. 387). An added factor in the case of *Spirostomum* bringing about this so-called 'kornigen Zerfall', is the instability of the superficial layer of its ectoplasm.

In most cases, however, the exposed endoplasm is covered over at once by ectoplasm. At first the cut end may be of various shapes, but usually within the space of about twenty-four hours, the cut end or both cut ends have rounded off completely, and it is difficult if not impossible to distinguish them from the extremities of normal individuals.

b) After transverse incision.

In one experiment in which an effort was being made to cut an individual into two pieces, the scalpel only went part of the way through. This individual was set aside, and the injury was seen to become steadily less until it disappeared altogether.

This occurred also in a later experiment. Here however, the cut went very far through, so that the two pieces were only connected by a very narrow strand of protoplasm (Fig. 3). When the next observation was made some twenty-four hours later, the two semi-detached pieces had fused together.

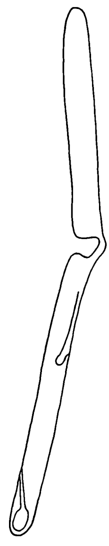


Fig. 3. A *Spirostomum* after transverse incision.

Similar lateral incisions were made on other individuals, and only in the event of the incision being a very deep one, so that the connecting link between the anterior and posterior pieces was extremely narrow, did these pieces separate from each other.

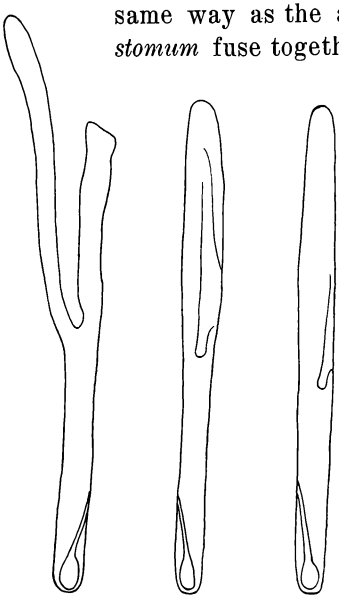
c) After longitudinal incision.

Spirostomum ambiguum is so narrow that it is not possible to make an anterior or posterior incision without first causing the animal to expand. If a *Spirostomum* is placed in a very small quantity of water, it contracts in the longitudinal axis and expands in the transverse, and a longitudinal incision can then be made.

This technique entails a very heavy mortality, for the incision of an expanded *Spirostomum* leads, in the majority of cases, to a rapid disintegration.

Ten anterior and eight posterior longitudinal incisions of various lengths were successfully carried out.

The result of such an experiment is not always the same. In some cases the two 'heads' or 'tails' may fuse together, in just the same way as the anterior and posterior pieces of a *Spirostomum* fuse together after lateral incision. In Figs. 4—6, a case of fusion after an anterior incision is illustrated, and in Figs. 7—9 the result of a posterior incision is shown.



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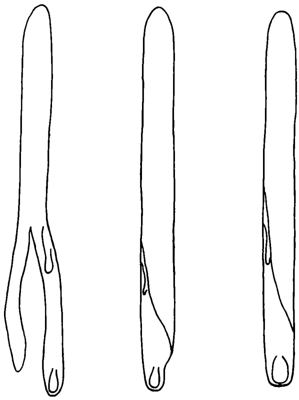
Figs. 4—6. Three stages in the regulation, by 'fusion', of a *Spirostomum* after anterior longitudinal incision.



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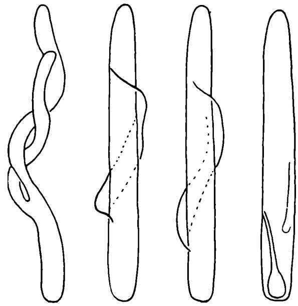
Figs. 7—9. Three stages in the regulation, by 'fusion', of a *Spirostomum* after posterior longitudinal incision.

Figs. 10—13 illustrate a further case of fusion, differing somewhat from the above. In this case the two anterior limbs twined round each other directly after the operation and as the drawings show, they gradually fused together; so that, at the end of four days, complete regulation had occurred. This process also occurred in another case, but here regulation was complete after twenty-four hours.

Fusion of the two limbs is not, however, always the method of regulation. In some cases one of the limbs was gradually absorbed into the other (Figs. 14—19).

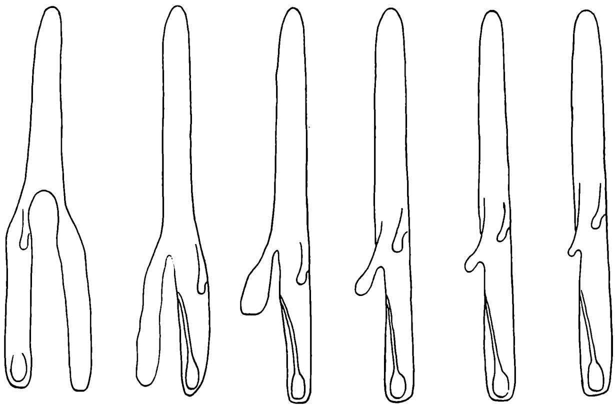
The third method of regulation may be termed separation, an example of which is illustrated in Figs. 20—23. Here the two limbs become separated from each other and each undergoes a regulation of its own.

Fusion took place in six cases, absorption in three and separation in six. The regulation was not perfect in every case. In three cases of separation, one of the pieces failed to regulate. Three out of the eighteen individuals operated on failed to regulate at all.



Figs. 10—13. Four stages in the regulation, by 'fusion', of a *Spirostomum* after anterior longitudinal incision.

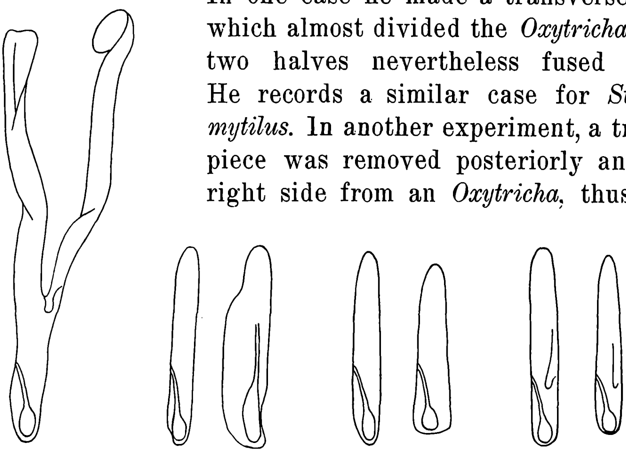
Regulation after transverse and longitudinal incision has been observed in other ciliates. Gruber (1886), for example, carried out longitudinal incisions on *Stentor coeruleus*. He stated that usually the two limbs separated. Balbiani (1891 a) described in detail the result of an experiment in which the mouth, peristome and adoral zone of a *Stentor* were removed and a deep longitudinal posterior incision performed. Fusion



Figs. 14—19. Six stages in the regulation, by 'absorption', of a *Spirostomum* after posterior longitudinal incision.

of the 'tails' did not begin until the ninth day after the operation, but complete regulation eventually occurred.

Stevens (1903) produced double monsters in *Stentor* by a somewhat different technique. In his experiments both fusion and separation occurred. Finally the work of ISHIKAWA (1913) on double monsters may be noticed. In the case of anterior cuts through the peristome of *Stentor*, regulation was brought about by fusion. ISHIKAWA also made use of *Oxytricha fallax* in his experiments. In one case he made a transverse incision which almost divided the *Oxytricha*, but the two halves nevertheless fused together. He records a similar case for *Stylonychia mytilus*. In another experiment, a triangular piece was removed posteriorly and on the right side from an *Oxytricha*, thus leaving



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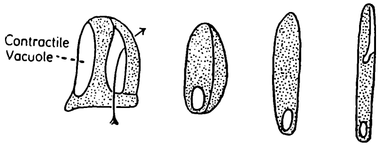
Figs. 20—23. Four stages in the regulation, by 'separation', of a *Spirostomum* after anterior longitudinal incision.

a piece projecting on the left. This was absorbed. Longitudinal incisions carried out on *Stylonychia* resulted in both fusion and separation.

The three types of regulation observed in *Spirostomum* occur therefore in other Infusoria. They show that in the Infusoria the process of form regulation is extremely highly developed.

d) After internal injury.

In two cases, an operation on a *Spirostomum* which was followed by a certain amount of disintegration, resulted in a piece being obtained with an internal injury in the form of a cavity. One of these cases is illustrated in Figs. 24—27.



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Figs. 24—27. Four stages in the regulation of a piece of a *Spirostomum* after internal injury.

A small piece was cut out of a *Spirostomum* in the region of the mouth. This piece began to disintegrate, and in the region of the mouth and peristome a hole was formed right through it, owing to the piece being thinner here than elsewhere. As shown in Fig. 24, there was obtained a ring of protoplasm encircling an open cavity, the arm containing the new contractile vacuole being the widest. Subsequently this hole was filled in, the vacuole was reduced in size, the shape of the piece became that of a normal *Spirostomum*, and finally a new mouth and peristome were regenerated.

The second case was a far less striking example of regulation. The piece was nearly normal in shape, but there was a hole through its extreme posterior end. This internal injury was effaced in less than twenty-four hours.

B. Regulation of the Vacuolar System.

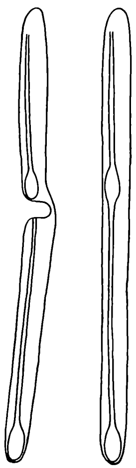
The contractile vacuole, as noted earlier, is situated at the extreme posterior end of the body, and from it the long vacuolar feeding canal runs forward. If a *Spirostomum* is cut in half, a contractile vacuole is formed at the posterior end of the anterior half by an expansion of the posterior end of the vacuolar canal of this half. This reformation of a vacuole is completed within the space of about five minutes and can be easily observed under the microscope.

This new vacuole can be formed at any level of the body, wherever the cut has been made. Even if a very small anterior tip is cut off, a vacuole soon forms at the posterior end of the piece, though the feeding canal, owing to its being extremely narrow in this region, cannot be made out.

The formation of this new vacuole is not dependent simply on the cutting of the feeding canal. If a *Spirostomum* is cut in half, a new vacuole forms at the posterior end of the anterior piece, but never at the anterior end of the posterior piece. If a piece is cut from the middle of the body, a vacuole forms at the posterior end of the piece, though directly after the operation the canal is open at both ends. On the other hand it is necessary for the vacuolar canal to be severed before a vacuole will form, as will be shown below.

If a *Spirostomum* is laterally incised so that the anterior and posterior parts are only connected by a very narrow bridge of protoplasm, it usually happens that the vacuolar canal is severed. It was observed that in the space of a few minutes a new vacuole was formed at the posterior end of the anterior portion, though no such formation occurred at the anterior end of the posterior portion. Such individuals, therefore, have two vacuoles, one at the posterior end of the body, the other at whatever part of the body the incision was made (Fig. 28).

Now as already noted, the anterior and posterior portions of such individuals frequently join up in such a way that a normal individual is again obtained. It was found that when such a fusion had occurred, the posterior part of the vacuolar canal had joined up with the anterior vacuole, so that the anterior and posterior parts of the severed canal were once more in communication by way of this anterior vacuole (Fig. 29). These facts were confirmed on several individuals.



28 29

Figs. 28—29. Two stages in the regulation of the vacuolar system of *Spirostomum*.

It is not particularly surprising that on fusion of the two portions of the individual the posterior part of the feeding canal should join up with this anterior vacuole, since when the cut edges fuse, their material will presumably occupy the same position as before the operation, and the posterior part of the canal will be brought automatically into contact with the anterior vacuole.

In one individual which was not incised very deeply so that fusion occurred very quickly, a second vacuole was formed, but it was very small, indicating that the rate at which fusion occurs influences the size of this new vacuole.

That the formation of this new vacuole is due to the severing of the canal and not merely to the effect of the incision was shown by incising an individual without severing the canal. In this case no vacuole was formed.

Certain individuals in which two vacuoles had been obtained in the above manner, were observed again some hours after complete fusion of anterior and posterior parts had taken place. It was found that the anterior vacuole had completely disappeared, and that the canal had regained its normal form. In a later experiment this process of reduction was observed under the microscope, the whole process being completed in twenty-five minutes. Other experiments confirmed the fact that the reduction of an anterior vacuole takes much longer than the formation of a new vacuole at the posterior end of a piece.

C. Regulation of the Mouth and Peristome.

It has already been mentioned that the position of the mouth varies considerably in different individuals. Probably the commonest position is somewhere about $^1/_3$ of the length of the individual from the posterior end[1]). But in many *Spirostomum* the mouth may be as much as $^1/_2$ from the posterior end, or even a short distance in front of this. On the other hand in some specimens the position of the mouth was found to be no more than $^1/_4$ from the posterior end, and in a few cases even less than this[2]). The position of the mouth may therefore be said to vary between $^1/_2$ and $^1/_4$ from the posterior end. If, in exceptional cases, it exceeds these limits it is only to a very slight extent. Thus the mouth never lies quite close to the anterior and posterior extremities of the animal.

To study the regulation of the mouth and peristome in *Spirostomum*, a number of specimens were cut transversely in two, the cuts being made at various levels. If a specimen is cut through just in front of the mouth, a long anterior piece is obtained which is, of course, devoid of a mouth. (It will however possess a portion of the peristome.) In most cases a new mouth is regenerated in such pieces within the space of twenty-four hours. In fourteen *Spirostomum* which had been operated on in the above fashion, the position of the regenerated mouth was either $^1/_4$ from the posterior end or less than a $^1/_4$.

The regeneration of the mouth was also examined in small anterior pieces, the cut passing only a short distance behind the anterior end. In these pieces also the position of the regenerated mouth was far back. Thus twenty-one out of twenty-four pieces had the mouth $^1/_4$ or less from the posterior end. In only three did it appear to be further forward than $^1/_4$ and then not more than $^1/_3$.

The position of the regenerated mouth in short posterior pieces was usually, however, considerably further forward. In fourteen out of twenty-five pieces it was as far forward as $^1/_2$ from the posterior end. In the remaining pieces it was further back, but in only four was it as far back as $^1/_4$ from the posterior end.

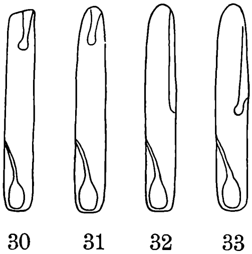
The regeneration of the mouth in a piece cut out of the body of a *Spirostomum* just in front of the mouth (i. e. a piece with neither head nor tail) also occurs within twenty-four hours. In three

[1]) In future, for purposes of brevity, the position of the mouth will be referred to as $^1/_3$, $^1/_2$, or $^1/_4$ from the posterior end.

[2]) It must be understood that these lengths are only approximate, since they were judged by eye and not measured. There is no difficulty in judging the difference in the position of the mouth in two specimens when it is as far forward in one as $^1/_2$ from the posterior end and as far back in the other as $^1/_4$, and a position roughly intermediate can be designated as $^1/_3$ from the posterior end. But to attempt to describe the position of the mouth in terms of further intermediate fractions would be too inaccurate to be of any value.

such pieces the position of the regenerated mouth was about $^1/_4$ from the posterior end.

It was pointed out at the beginning of this section that in *Spirostomum ambiguum* which have not been injured the mouth is very rarely found further anterior than $^1/_2$ from the posterior end, and then only a very short distance. Now it is perfectly simple to cut a *Spirostomum* just in front of the mouth so that a posterior piece is obtained with the mouth and a very short stretch of peristome situated right at the anterior extremity. Similarly we can cut a *Spirostomum* just behind the mouth and so obtain a piece with the mouth at the extreme posterior end. In both these cases, then, pieces are obtained with a mouth situated in a position in which it is never found in normal *Spirostomum*. The question arises, will there be any change in the position of the mouth in such pieces?



Figs. 30—33. Four stages in the regulation of the mouth in a piece of a *Spirostomum* with the mouth at the anterior end.

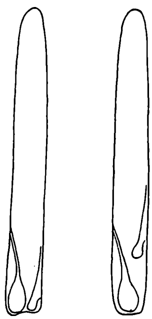
Eight posterior pieces with the mouth at the anterior end were studied. Figs. 30—33 illustrate the regulation of the mouth in these pieces. On the day following the operation there was no change in the piece apart from the rounding off of the anterior end (Fig. 31). Twenty-four hours later it was found that the small piece of peristome and sharply outlined mouth had completely disappeared from the anterior end, while a shallow depression, reaching back to about $^1/_2$ from the posterior end, had been formed (Fig. 32). Two days later a clearly defined mouth had been formed at the end of the peristomial depression, being situated about half way down the body (Fig. 33).

Similar results to these were obtained in the case of four more pieces which had been operated on in the same manner. The regulation of the mouth in all these five pieces took more than twenty-four hours, but this is probably to be explained by the fact that the *Spirostomum* used in these experiments had been kept in small tubes for several days. In a later experiment three posterior pieces with the mouth at the anterior end were cut from *Spirostomum* taken straight from the stock tank, and in these pieces the mouth was got rid of in twenty-four hours. In one of these pieces the new mouth, situated posteriorly, was already formed, and in the other two was beginning to form.

The regulation of the mouth in anterior pieces in which the mouth was at the extreme posterior end was equally striking. Such a piece is illustrated in Fig. 34. When this piece was again examined twenty-four hours later, the mouth was found to have moved a considerable distance further forward (Fig. 35).

This anterior movement of the mouth was studied in a large number of anterior pieces [1]). Only in eight out of sixty-six pieces was there any doubt that a forward movement had not taken place. This movement probably rarely takes as long as twenty-four hours. In one case it occurred within five hours.

In some of these pieces there was no doubt that two anterior movements of the mouth took place, and in one case this second forward movement was observed. The mouth in this piece (Fig. 36), had already undergone the first forward movement, but situated a little way in front of it was a second mouth, the two mouths being joined together by a short stretch of peristome. Later this piece of peristome and the posterior mouth disappeared.



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Figs. 34—35. Two stages in the regulation of the mouth of a piece of a *Spirostomum* with the mouth at the posterior end.

Fig. 36. A stage in the regulation of the mouth of a piece of a *Spirostomum* with the mouth at the posterior end. Second forward movement of the mouth, with two mouths present in a single piece.

These forward movements of the mouth, then, appear to take place by the formation of a new mouth further up the peristomial groove, which is followed by the obliteration of the old mouth and of that part of the peristomial groove which lies between them.

Wallengren, Balbiani and Johnson have all recorded cases of the regulation of the mouth and peristome in other Ciliata.

[1]) It was not always possible to cut off an anterior piece in such a way that the mouth was right up against the posterior cut surface, but no pieces were used in these experiments unless the mouth was extremely close to this surface.

WALLENGREN (1902), working on *Holosticha rubra*, observed that behind the peristome of some apparently uninjured individuals a new one was laid down, and to the right of it new ventral cilia, which also occurs during transverse fission. But this peristome was much longer than that which is formed during transverse fission. This new peristome was later pushed forward, while the old peristome, together with the anterior end of the individual, degenerated.

BALBIANI'S observations (1891b) were made on *Stentor coeruleus*. He found that a replacement of the mouth and peristome may sometimes occur. The old mouth and part of the adoral zone of cilia are absorbed, and the new mouth and peristome, which have been formed behind the old, move forward into their position. The new adoral zone fuses on the right with the remains of the old adoral zone, so that the final structure is a mixture of the old and the new. The same is true of the peristome, part of the old peristome remaining. This peculiar regeneration is probably necessary on account of the oral structures becoming worn out quicker than the rest of the body. According to BALBIANI this regeneration is a normal process in *Stentor coeruleus*.

JOHNSON (1893) completely confirmed BALBIANI'S results. In one case he even found a double replacement occurring. The new mouth and peristome were already formed, the old structures having nearly disappeared, when yet another mouth and peristome developed and their predecessors rapidly became eliminated.

Recently HETHERINGTON (1932) has denied the existence of such 'physiological' regeneration. He thinks that what has been called 'physiological' or 'spontaneous' regeneration of the mouth and peristome of Stentor might really be reparative, a consequence of injury. SCHWARTZ (1935), however, cites further cases of 'spontaneous' regeneration in *Stentor coeruleus*.

These results show that regulatory regeneration of the oral structures may take place in other ciliates besides Spirostomum, though, as far as I am aware, the experimental induction of such regulatory regeneration has not been recorded in other forms.

4. Discussion.

The present studies cannot be considered as much more than a preliminary survey of the ground. The experiments on form regulation have shown that this phenomenon is well developed in *Spirostomum*, as in the majority of other Ciliata. But there is little to be gained, now, from this line of inquiry. We have at the present time a very large number of observations on the form regulation of animals, both invertebrates and vertebrates, and while these may possess a certain intrinsic interest, it cannot be said that they help us to understand the mechanics of regeneration. On the other hand it seems possible that further experiments on the regulation of the mouth and peristome might help in the search for the factors responsible for regulation and regeneration.

A conclusion of some importance which may be drawn from the present studies is that in any given *Spirostomum* some agency is at work which determines at what level the mouth shall be situated. This conclusion follows from the experiments in which pieces of *Spirostomum* were obtained with the mouth in a position in which it is never found in a normal *Spirostomum*. This 'incorrectly situated' mouth degenerated, and a new mouth was regenerated at the 'correct' level for the piece.

There is now, of course, a very large body of evidence which shows that in the early stages of animals a physiological agency is at work which determines at what level the various organ systems shall arise, and that in the adult stages of the lower forms, in which a considerable amount of regeneration is still possible, this agency persists and is responsible for the nature and position of the regenerated structures. The experimental facts have necessitated the assumption that this physiological agency is a graded one, and have given rise to the well known theory of 'Axial Gradients'.

Further experiments could certainly be devised with a view to demonstrating the presence of an axial gradient in *Spirostomum*, which is responsible for fixing the position of the mouth[1]). In the present studies there is an indication that the position of the regenerated mouth in pieces cut from the anterior region of the body is not the same as in pieces cut from the posterior region of the body. The number of pieces used was, however, too small to allow a definite conclusion to be drawn. But if it can be shown, in a further series of experiments, that the position of the regenerated mouth is, in fact, different in anterior and posterior pieces, then the assumption that an axial gradient is present in *Spirostomum* would be greatly strengthened, for the following reason. Experiments on head-frequency regeneration in Planaria have led to the conclusion that the 'steepness' of the gradient in anterior and posterior pieces

[1]) The presence of an axial gradient in susceptibility in *Spirostomum* has been demonstrated by Child using potassium cyanide, potassium permanganate and other agents. (See Child, 1919, p. 139 and 1924, pp. 79, 80.)

is not the same. The expectation is that this difference in 'steepness' of the gradient in these two sorts of pieces will influence the position of the regenerated mouth in these pieces, and BUCHANAN (1927), working on *Phagocata gracilis,* was able to demonstrate that in the regeneration of pieces of this animal the mouth appears more and more anteriorly as the region from which the piece is taken becomes more and more posterior, a result comparable to that indicated in *Spirostomum.*

The formation of a contractile vacuole only at the posterior end of a severed vacuolar canal and never at its anterior end, might also receive its explanation in terms of the gradient hypothesis.

It has already been pointed out that in a random sample of normal Spirostomum, the position of the mouth varies considerably. BISHOP (1923) has shown that this variation in the position of the mouth is related to the process of division.

If the two halves of a newly divided *Spirostomum* are examined, the position of the mouth in each will be found to be about $^1/_4$ from the posterior end. (BISHOP states that the mouth may be situated almost at the extreme posterior end, about level with the middle of the contractile vacuole, but in the newly divided *Spirostomum* I have examined it was never as far back as this.) According to BISHOP growth takes place much more rapidly behind the mouth than in front of it. Thus the mouth appears to move forward gradually, so that in animals about to divide it is roughly central in position. A second mouth then forms towards the posterior end. In this way the variation in the position of the mouth is explained.

Now in the experiments on the regulation of the mouth in anterior pieces in which the mouth was at the extreme posterior end, it was suggested that the forward movements of the mouth took place by the formation of a new mouth further up the peristomial groove, followed by the obliteration of the old mouth and of that part of the peristomial groove which lay between them. In the light of BISHOP's observations, however, it is possible to explain these forward movements of the mouth by growth between the mouth and the posterior end of the piece. Nevertheless, I believe the first explanation to be the correct one, since these forward movements occurred within a relatively short space of time, and since in one case the forward movement of the mouth by the formation of a second mouth further up the peristomial groove was actually observed.

In conclusion we may note one more fact of considerable interest in connection with the mouth. Fifty *Spirostomum* were kept under observation in watch-glasses. In the majority of them the mouth underwent no change in position. But in eight individuals there was a most definite change in its position. They were individuals which at the start of the observations had the mouth $^1/_2$ from the posterior end. In five of them the mouth moved as far back as $^1/_4$ from the posterior end. The other three showed a posterior movement of the mouth, but not quite so marked as in the first five.

It is hoped that the unanswered problems raised by these studies will receive their elucidation in further work in the near future.

5. Summary.

1. The regulation of the form of *Spirostomum ambiguum* has been studied after complete removal of a piece of an individual, after transverse incision, after longitudinal incision, and after internal injury. The power of form regulation was found to be highly developed. After longitudinal incision regulation may take place in three different ways.

2. A contractile vacuole is regulated out of the vacuolar feeding canal at the posterior end of an anterior piece but never at the anterior end of a posterior piece. This regulation is completed in about five minutes, and takes place at whatever level of the body a cut is made. In a piece cut out of the body of a *Spirostomum* the vacuole forms only at the posterior end of the piece.

If a *Spirostomum* is cut nearly in half a vacuole forms at the posterior end of the anterior portion. When the posterior portion fuses up with the anterior, the posterior part of the feeding canal runs into this anterior vacuole, which is then removed.

3. The regeneration of the mouth was studied in long anterior pieces, in short anterior and posterior pieces, and in pieces cut out of the bodies of *Spirostomum*. Pieces were also studied which had been cut in such a way that the mouth was present in a position in which it is never found in a normal *Spirostomum*. Such a mouth was got rid of, and a new one regenerated in the 'correct' position.

4. It is suggested that certain of the facts brought to light by these studies point to the presence of an Axial Gradient in *Spirostomum* which is responsible for fixing the position of the mouth.

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