ABNORMAL PROTOPLASMIC PATTERNS AND DEATH IN SLIGHTLY HYPERTONIC SOLUTIONS

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PLATES 5 TO 7

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A variety of experiments indicates that the life processes of the cell depend to a large extent on the behavior of the protoplasmic surface. It is therefore important to utilize all available means to follow changes in this surface.

It is of much interest from this point of view to find that alterations in the protoplasmic surface of *Nitella* due to plasmolysis are accompanied by striking changes in the chloroplasts. Such changes have apparently not been seen in other cells but in *Nitella* they are very obvious. Slightly hypertonic solutions of sugars and of electrolytes may cause contraction of the chloroplasts. The clear spaces between them enlarge in certain regions and assume characteristic patterns (Figs. 1 to 6). Such changes are irreversible and are soon followed by death.

A study of this behavior may throw light on the nature of the protoplasmic surface and on the properties of protoplasmic gels as well as on the process of death. An understanding of the mechanism involved may help to explain the action of hypertonic solutions in other cases as, for example, in the artificial parthenogenesis of marine eggs.

The protoplasm in which these changes occur consists of a layer not over 15 microns thick. The outer portion of the protoplasm is a gel[1] in which the ellipsoid chloroplasts are arranged in longitudinal rows (Fig. 1). As we focus down through the cell to the vacuole we encounter only a single layer of chloroplasts so that an incident ray of light coming from above never passes through two chloroplasts in succession before reaching the vacuole. The inner part of the protoplasmic layer is liquid and normally shows constant streaming. Inside this layer of protoplasm is the large central vacuole filled with sap.

When cells[2] are placed in a slightly hypertonic solution of sucrose (0.25 to

[1] This is shown by the behavior in the centrifuge where the outer part of the protoplasm may split up into long straight rods, each containing a single row of chloroplasts. Curved structures of this sort are seen in Fig. 5.

[2] The observations were made on *Nitella flexilis*, Ag. The cells were freed from neighboring cells and observed at once or kept in the laboratory in Solution A (*cf.* Osterhout, W. J. V., and Hill, S. E., *J. Gen. Physiol.*, 1933–34, **17**, 87) at 15°C. ±1°C. An hour before use the temperature was raised to about 25°C. Cells about 8 cm. in length were used.

Some cells were resistant and did not develop splits as the result of slight plas-

0.4 M) so that slight plasmolysis[3] occurs in 5 minutes or less we usually see immediate changes in the ellipsoid chloroplasts. They shrink somewhat and their shape may become more nearly spherical; eventually they may round up into spheres[4] whose diameter is less than that of the smallest diameter of the original ellipsoid. This evidently involves a loss of volume.

These alterations increase the amount of clear space between the longitudinal rows of chloroplasts. A further increase may be caused by the displacement of chloroplasts toward the interior of the cell so that they come to lie on a different level. We may then encounter, as we focus down into the cell, 2 or 3 successive layers of chloroplasts instead of a single layer as is normally the case. This occurs more often in the later stages of the process.

Soon after the contraction of the chloroplasts begins we see that in certain places the narrow clear spaces between the longitudinal rows of chloroplasts become wider (Figs. 2 and 3). Since these widened clear areas look like splits this term will be used for convenience in description.

The splits appear as clear areas entirely free from chloroplasts (Figs. 2 to 6); they run lengthwise, tapering off at both ends.[5] In a single cross-section of the cell there may be as many as a dozen such splits. Some of them steadily increase in size but their development does not follow a regular pattern. Several small splits may appear at about the same time in the same region, some developing much faster than others (Fig. 5). Sometimes there is only a single split in a given region and this may increase so as to occupy nearly half of the circumference of the cell.[6] The formation of splits may thus involve a considerable increase in the amount of clear space between the longitudinal rows of chloroplasts.

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molysis. In former experiments with plasmolysis (Osterhout, W. J. V., *J. Gen. Physiol.*, 1943–44, **27**, 139) all the cells were resistant and recovered if the plasmolysis had not gone too far. In these cells no splits were observed.

[3] In some cases considerable loss of water results in a flattening of the cell (change in shape of the cross-section) rather than in a retraction of the protoplasm from the cellulose wall.

[4] It might be suggested that the appearance of shrinkage is brought about by a shift in the position of the chloroplast by which its long axis ceases to be parallel to the surface of the cell. This does not appear to be the case when starch grains in the chloroplast enable us to see differences in the opposite ends of the chloroplast.

[5] These are quite different from the irregular areas devoid of chloroplasts which sometimes appear in cells not subjected to plasmolyzing solutions and which may possibly result, in part at least, from manipulation in collecting and separating the cells. But such areas sometimes develop along with the splits.

[6] Even where there is little contraction of the chloroplasts the clear space between some longitudinal rows may diminish as the splits increase so that the clear space in the splits increases at the expense of the clear space between rows where no splits exist.

The splits may continue to develop for an hour or more. When the process reaches a certain stage death ensues, as shown by the entrance of acid fuchsin,[7] and by the exit of chlorides[8] (as demonstrated by adding $AgNO_3$ to the external solution).

The development of splits appears to be irreversible from the start. If the cell is transferred to distilled water as soon as the splits begin to appear they may continue to develop very much as though no change had been made in the external solution.[9] When the plasmolyzing solution is replaced by water the protoplasm expands but the chloroplasts do not, and this creates more clear space which is incorporated into the splits. But in many cases this alone does not appear adequate to explain the increase in the size of splits after the cell is returned to water and this increase may be aided by shrinkage of the chloroplasts and their displacement toward the interior of the cell.

If the cell shows protoplasmic motion just before it is returned to water the motion usually stops soon after the return to water and death soon follows, as shown by the entrance of acid fuchsin and the exit of chlorides. The turgor may be temporarily regained after the return to water but it soon disappears. The protoplasm usually contracts soon after this (false plasmolysis) and may shrink to half its normal diameter. In certain cases, however, if the exposure to the plasmolyzing solution has been less than a minute and retraction of the protoplasm occurs without any contraction of the chloroplasts or formation of splits the cell may live for some hours after the return to water.

In most cases splits do not form in solutions of sucrose which are not able to produce evident plasmolysis.

In some cases the formation of splits begins just before the retraction of protoplasm from the cellulose wall (the retraction may occur only in certain parts of the cell but the splits may appear everywhere.

With these facts in mind we may consider the conditions under which the splits arise. The outer layer of the protoplasm is a stiff gel in which the chloroplasts are arranged in longitudinal rows, each row in a rod of gel.[10] When plasmolysis occurs it is not surprising that as these rows become displaced the longitudinal arrangement of the chloroplasts is preserved. The shrinkage of

[7] The acid fuchsin (National Aniline Co.) is used in 1 per cent solution in phosphate buffer (at pH 8) which has a cationic concentration of 0.006 M. If the dye is merely dissolved in water the pH is about 2.6 which is injurious.

[8] *Cf.* Osterhout, W. J. V., *J. Gen. Physiol.*, 1922–23, **5**, 709.

[9] If plasmolysis begins without formation of splits and if the cell is then placed in water the protoplasm may expand without development of splits but usually the cell does not live long after this. During this expansion of the protoplasm the normal motion may continue for a time.

[10] This is shown by subjecting the cells to centrifugal force which causes the protoplasm to split up into rods each of which contains a single row of chloroplasts.

the chloroplasts adds to the clear space between the rows but we do not know why the clear space increases chiefly in certain places to form splits.

It might be suggested that splits are due to movement of water, as, for example, when the plasmolyzing solution is replaced by water and there is a sudden rush of water into the protoplasm followed by the development of splits. But when this mechanical disturbance is lessened by transferring the cell from the plasmolyzing solution in gradual steps[11] splits appear as before.

It is evident, however, that if a mechanical disturbance of the protoplasm is great enough it may produce splits. For example, when a cell is placed on a slide without a coverglass we may cover a region *A* at one end with water separated by a vaseline barrier from the rest of the cell[12] (called *B*). If we place 0.5 M sucrose at *A* water enters at *B* and a rush of sap in the vacuole toward *A* is observed. After half an hour the 0.5 M sucrose at *A* is replaced by water. We then see a rush of sap in the vacuole from *A* to *B*. This occurs because the previous exit of water at *A* has left behind osmotically active substances which cannot pass out through the protoplasm so that the osmotic pressure in the sap at *A* has increased.[13] When the sucrose at *A* is replaced by water the latter enters at *A*, rushes along the vacuole[14] to *B* and passes out at *B*. The splits which sometimes develop at *B*[15] might be attributed to the mechanical disturbance of the violent outgoing current at *B*.

In some cases splits develop at *A* but in order to produce splits and irreversible contraction of the chloroplasts the ingoing current must be more violent than that described in a former paper[16] where no splits were produced by the ingoing current and the contraction of the chloroplasts could be reversed by an outgoing current.

In experiments lasting several days in solutions of sucrose too dilute to produce plasmolysis cells do not live so long as those kept in distilled water, indicating toxic action due to the sucrose solution (or to organisms growing in it); but this does not appear to play a part in plasmolytic experiments since in the dilute solutions death occurs without production of splits.

Turning now to the chloroplasts it may seem surprising that the contraction

[11] *E.g.* from 0.4 M sucrose to 0.25 M, then to 0.1 M, then to 0.05 M, and then to water.

[12] *A* and *B* are each about 30 mm. long. *Cf.* Osterhout, W. J. V., *J. Gen. Physiol.*, 1946–47, **30,** 439.

[13] The hydrostatic pressure does not become greater at *A* than at *B* but the osmotic pressure may do so. *Cf.* Osterhout, W. J. V., *J. Gen. Physiol.*, 1946–47, **30,** 439.

[14] If the sucross at *A* is replaced by water the rush toward *B* may be so violent that some of the chloroplasts near the vaseline barrier in *A* may be dislodged and carried along the cell toward the end wall.

[15] The ingoing current causes the chloroplasts to contract as described in a former paper (Osterhout, W. J. V., *J. Gen. Physiol.*, 1946–47, **30,** 229); they expand again in the outgoing current.

[16] Osterhout, W. J. V., *J. Gen. Physiol.*, 1946–47, **30,** 229.

of the chloroplasts which accompanies plasmolysis occurs in an outward current of water because it was stated in a former paper[16] that contraction occurs in an ingoing but not in an outgoing current. But in the former experiments only part of the cell was covered with the sucrose solution and water could enter freely into the rest of the cell (which was covered with water) and there was no indication of plasmolysis at any point. In the present experiments the plasmolyzing solution covered the entire cell and the resulting plasmolysis appears to be necessary for the production of splits except when the outgoing current is violent as described below.

It should be noted, however, that contraction of the chloroplasts may occur before the loss of water becomes great enough to produce visible retraction of the protoplasm from the cellulose wall.

In this connection the following experiment is of interest. A region at one end of the cell about 25 mm. long, designated as *A*, is covered with 0.5 M sucrose, separated by a vaseline barrier from the rest of the cell, *B*, which is covered with water. Water then enters at *B*, there is a rush of liquid in the vacuole toward *A*, and water comes out at *A*. If the outgoing current is strong enough[17] splits may occur at *A* and at their edges there may be some contraction of chloroplasts extending to 3 or 4 rows on each side of the split. These chloroplasts appear to be in much the same condition as the small rounded chloroplasts on each side of the "white line" in normal cells. These white lines resemble splits in some respects. They occur under normal conditions at the two edges of an imaginary plane which runs lengthwise through the center of the cell and separates the protoplasmic current flowing in one direction from the current in the opposite direction. Along the two lateral edges of this plane we see two white lines, *i.e.* well-marked clear spaces devoid of chloroplasts on opposite sides of the cell, running along its entire length. We usually see particles moving in opposite directions on opposite sides of the white line. As a rule none of the particles crosses the white line.

It is an interesting fact that when splits are produced by sucrose the normal white lines usually widen.[18]

If we use a higher concentration of sucrose (0.5 M or 0.6 M) in this experiment water may come out at *A* faster than it can be supplied through the vacuole from *B* and as a result there may be slight plasmolysis at *A*.

Results resembling those described here were obtained in preliminary experiments with glycerol, glucose, mannitol, raffinose, NaCl, $CaCl_2$, and sea water.

[17] To ensure this it may be necessary to increase the concentration of the sucrose. If it is raised to 0.5 M or 0.6 M plasmolysis may occur at *A* even though water is entering at *B*.

[18] Owing to the slightly spiral course of the longitudinal rows of chloroplasts the white lines on opposite sides of the cell appear to cross at certain places as we focus down through the cell.

A variety of solutions too dilute to produce plasmolysis may cause death without producing splits. Among these are solutions of $HgCl_2$, iodine, acetic acid, oxalic acid, and hexylresorcinol.

If the effects hitherto described were due merely to loss of water (without retraction of the protoplasm from the cellulose wall) they might be expected when the loss of water is brought about by evaporation. In order to test this cells were placed on slides without water and watched while evaporation went on. As a rule only one or two large splits developed but there was little or no retraction of the protoplasm and no appearance of the characteristic pattern of numerous small splits.

The experiment was varied by removing adhering drops of water from the cell by means of filter paper and then covering it with a thin layer of light paraffin oil (for medicinal use). A slow evaporation of water then occurred through the oil. This produced much the same effect as evaporation in air.

DISCUSSION

These experiments reveal a novel set of reactions. Slight plasmolysis causes the chloroplasts to shrink and shift so that the space between them increases and assumes a characteristic pattern with numerous clear areas running lengthwise in the cell.

The process is irreversible. Once started it continues even when the plasmolyzing solution is replaced by water. Eventually the sap in the vacuole passes out through the dying protoplasm which may become a compact elongated mass with no appearance of vacuole.

The contraction of a chloroplast involves a loss of volume[19] but this is not necessarily the case with the protoplasm as water escapes from the vacuole.

Whether the shift in the position of the chloroplasts is due to changes in the chloroplasts or in the surrounding protoplasm or in both is not clear. It is possible that local changes in the thickness of the gel portion of the protoplasm might push the chloroplasts into the pattern seen in the splits. But it is also possible that this pattern might arise as the result of an active movement of the chloroplasts. Active movements of chloroplasts occur in many plants but are usually determined by the direction and intensity of the incident light.

It might be suggested that the normal arrangement of the chloroplasts is due to a mutually repulsive action. In the red alga *Griffithsia*[20] this may be the case for when all the chloroplasts are collected in one end of the cell under the action of centrifugal force they may return to their normal positions when the centrifugal force ceases to act. We do not know to what extent mutual repulsion plays a part in the normal arrangement of the chloroplasts in *Nitella* or in the production of splits.

[19] See Osterhout, W. J. V., *J. Gen. Physiol.*, 1946–47, **30**, 229.

[20] Osterhout, W. J. V., *Proc. Nat. Acad. Sc.*, 1916, **2**, 237.

In seeking the cause of these phenomena we may consider (1) chemical effects, and (2) mechanical action.

1. It does not seem that chemical effects due to loss of water are the chief cause of the numerous small splits since loss of water can occur without producing the characteristic pattern seen in plasmolysis. For example, when a cell is placed on a slide and water is removed by means of filter paper and the cell is then observed while evaporation goes on we usually see a single large split and the cell may flatten to a thin ribbon, but there is no separation of the protoplasm from the cellulose wall and no development of the characteristic small splits resulting from plasmolysis in hypertonic solutions.

It seems improbable that the splits are caused by the chemical action of sucrose for they are not produced by prolonged exposure to solutions too dilute to plasmolyze, although death may ensue after an exposure of several days to such solutions.

2. It would therefore seem probable that the splits are due chiefly to mechanical causes. Apparently splits are associated with retraction of the protoplasm from the cellulose wall although it is not necessary that the retraction should occur at the exact point where the splits occur and the splits may come a little sooner than the visible retraction. It seems possible that injury initiated at one point may spread along the cell as in the marine alga *Griffithsia*.[20]

In harmony with this view is the fact that an ingoing current of water (p. 294) or an outgoing current (p. 294) may, if sufficiently strong, produce splits. This might well be a mechanical effect although chemical changes due to the removal of substances by the current of water are not excluded.[19]

On this view the loss of water would be important chiefly as causing the mechanical changes accompanying retraction of the protoplasm from the cellulose wall.

How can the retraction of the protoplasm from the cellulose wall produce injury? It must be remembered that the non-aqueous film which covers the surface of the protoplasm is a very delicate structure too thin to be visible under the microscope. The experiments indicate that the normal processes of the cell depend on the integrity of this film. It would seem that such a structure might be easily damaged in the process of protoplasmic retraction. And it seems possible that such injury may occur even without visible retraction of the protoplasm if the cellulose wall is penetrated by delicate threads of protoplasm such as have been described for various plant cells.[21] Some of these extensions are just at the limit of visibility. If there are protoplasmic extensions in *Nitella* as attenuated as the non-aqueous surface layer of the protoplasm they may be invisible. If the process of retraction begins with the withdrawal of such extensions from the cellulose wall irreversible changes may occur before any visible

[21] *Cf.* Küster, E. Die Pflanzenzelle, Jena, G. Fischer, 1935, 103.

retraction of the main body of protoplasm takes place. Such irreversible injury might conceivably involve production of splits. Slight plasmolysis at the top or bottom of the cell might escape detection.

It is of interest to note that if we accelerate the process sufficiently by using a higher concentration of sucrose so as to produce rapid retraction of the protoplasm there may be no appearance of splits and protoplasmic motion may continue much longer than with slight plasmolysis. This may be true even when the cross-section of the protoplasm has been reduced by 25 per cent in rapid plasmolysis. But when such cells are replaced in water splits occur and death follows.

If the splits result from mechanical disturbance of the non-aqueous film at the outer surface of the protoplasm it would seem that slow retraction of the protoplasm is more effective than rapid and expansion is effective as well as retraction (as seen when the cell is returned to water after plasmolysis). But the rapid plasmolysis usually withdraws more water and this may play a part.

It might be suggested that the changes observed in hypertonic solutions bear some resemblance to those seen in syneresis.

The injurious effects of plasmolysis are shown by contraction of the chloroplasts and by the formation of splits. But the formation of splits seems to be more significant since, as shown in former papers,[22] contraction is a reversible process when brought about by lead acetate or by an ingoing current of water.

These effects on *Nitella* are of interest in connection with the action of hypertonic solutions in artificial parthenogenesis, particularly in view of Loeb's suggestion that this involves an injurious action which is checked before it has gone too far.

The processes leading to the formation of splits throw additional light on properties of protoplasmic gels which have recently been discussed[16, 23] in connection with *Nitella* and *Spirogyra*. Further studies in this field are desirable.

It would seem that if we understood the mechanism which produces splits we should have a much better picture of the death process and this in turn would throw light on the mechanism of life processes.

SUMMARY

Some interesting properties of protoplasm are revealed when slightly hypertonic solutions of sugars or of electrolytes are applied to *Nitella*.

The chloroplasts contract and the space between them increases and forms a characteristic pattern consisting of clear areas extending lengthwise along the cell and tapering off at both ends.

The development of these areas is irreversible from the start. If the cell is

[22] Osterhout, W. J. V., *J. Gen. Physiol.*, 1945–46, **29**, 73; 1946–47, **30**, 229.

[23] Osterhout, W. J. V., *J. Gen. Physiol.*, 1945–46, **29**, 181.

returned to water after plasmolysis begins these areas continue to enlarge in much the same fashion as when no change is made in the external solution. The cell soon dies whether returned to water or left in the plasmolyzing solution. Similar results are obtained with other sugars, with NaCl, $CaCl_2$, and sea water.

Similar reactions are also brought about by strong ingoing or outgoing currents of water. This suggests that mechanical action may be chiefly responsible for the result and this idea is in harmony with other facts. It seems possible that the retraction of the protoplasm from the cellulose wall may disturb the delicate non-aqueous film which covers the outer surface of the protoplasm and thus produce injury. Such an effect might take place even without visible retraction if the injury occurred in protoplasmic projections extending into the cellulose wall.

A study of this behavior may throw light on the nature of the protoplasmic surface and on the properties of protoplasmic gels as well as on the process of death. An understanding of the mechanism involved may help to explain the action of hypertonic solutions in other cases as, for example, in the artificial parthenogenesis of marine eggs.

EXPLANATION OF PLATES

PLATE 5

FIG. 1. Normal cell showing the ellipsoid chloroplasts arranged in longitudinal rows (in many cells these rows follow a slightly spiral course).

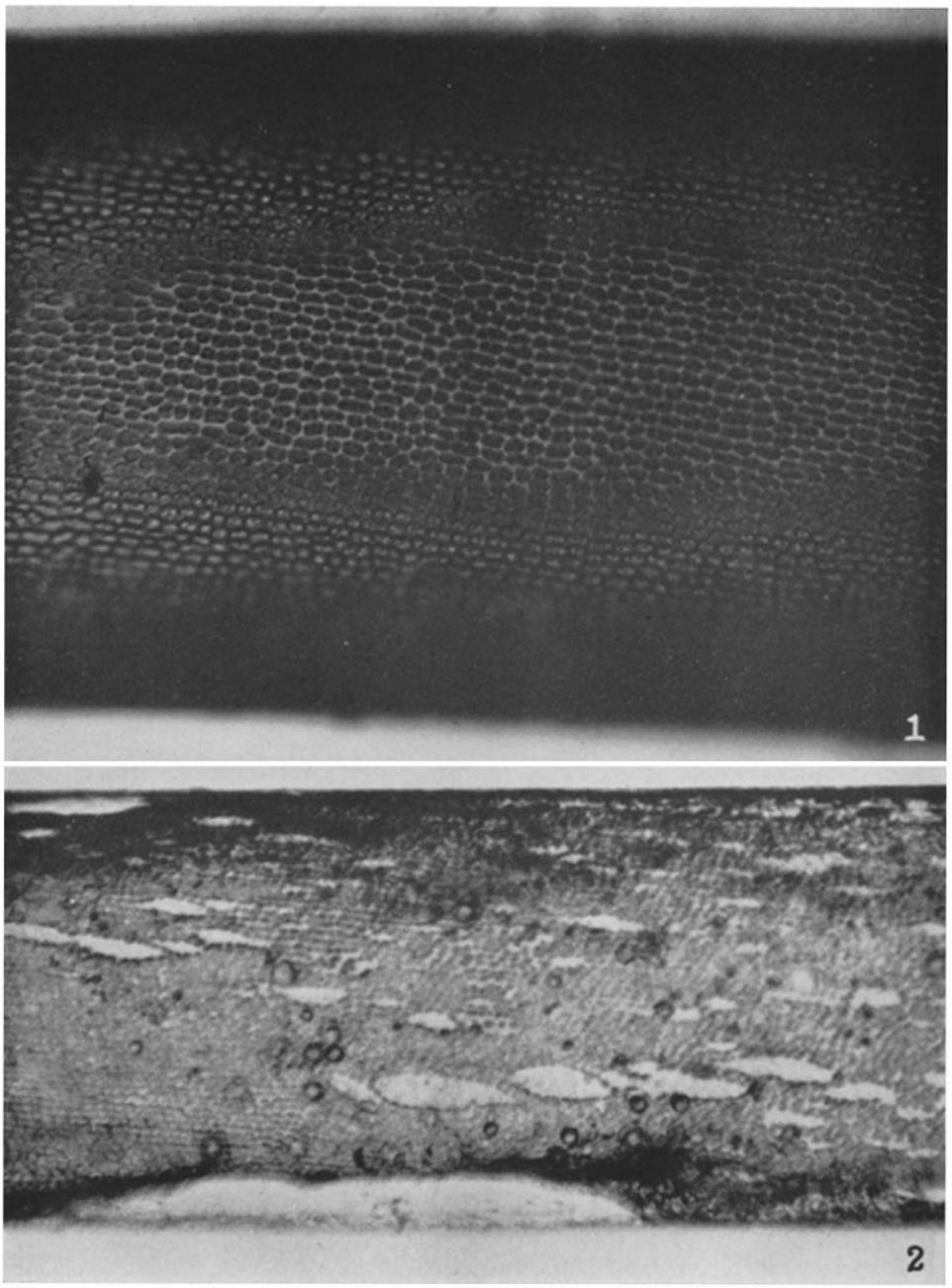
When the microscope is focussed below the center of the chloroplast (as seen in the middle of Fig. 1) the chloroplasts appear darker than the spaces between them because they consist of more highly refractive material than the substance lying between them. In this position of the microscope the chloroplasts seen at the top and bottom of the figure are in a lower plane than those seen in the middle of the figure so that the plane of the focus is above their centers and consequently they appear lighter than the spaces between them. This difference in the position of the chloroplasts is due to the curvature of the surface of the cylindrical cell. ×190.

FIG. 2. In several places the clear space between the longitudinal rows of chloroplasts has widened to form clear areas running lengthwise and tapering off at both ends; these are called for convenience "splits."

The figure shows several stages in the development of splits.

Several small clear circular areas surrounded by heavy dark lines are due to spherical bodies which are commonly found in the sap.

Plasmolysis is shown at the bottom and to a much less extent at the top (extreme left). ×120.



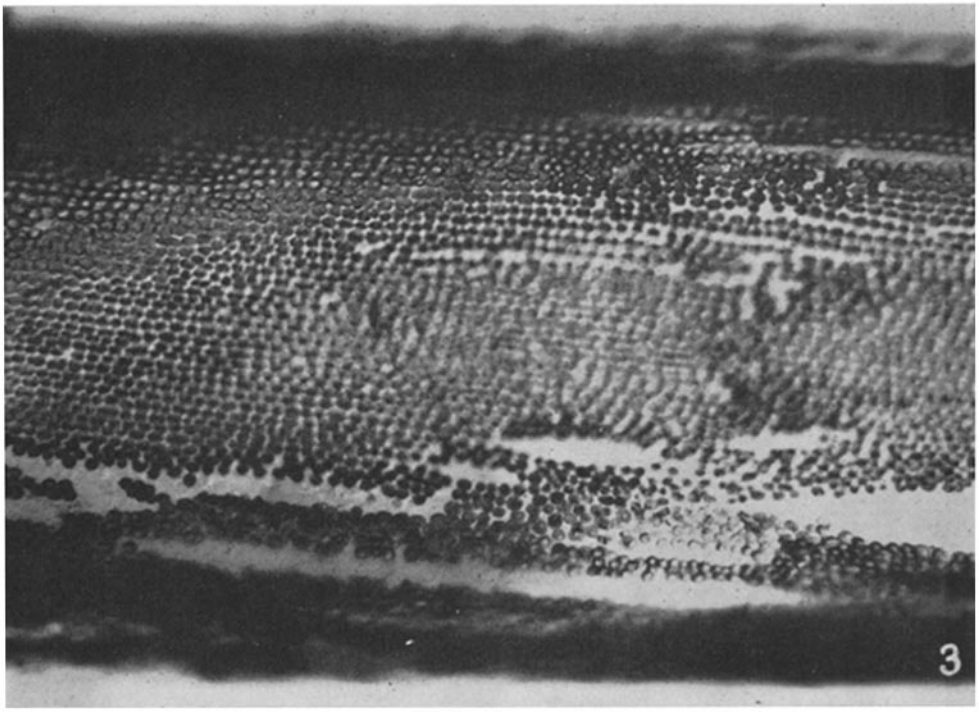
(Osterhout: Abnormal protoplasmic patterns)

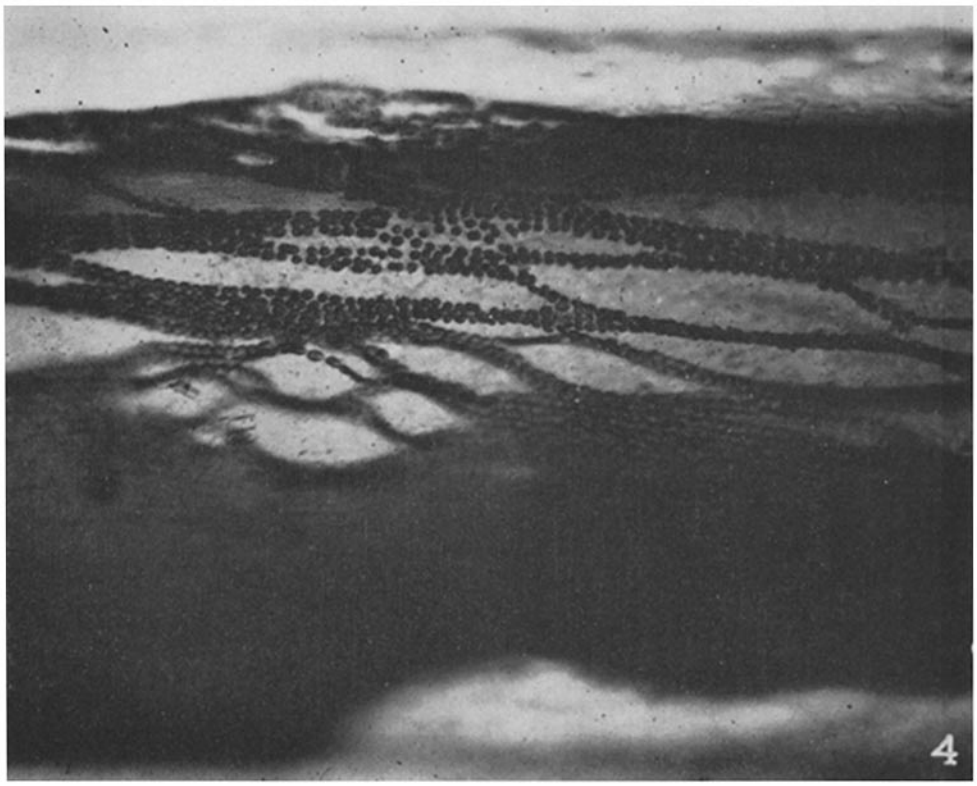
PLATE 6

FIG. 3. Small splits at the top, larger ones at the bottom.

The chloroplasts are contracted and in some cases rounded up to form spheres. Slight plasmolysis. ×170

FIG. 4. Well developed splits separated in some places by single rows of chloroplasts. Plasmolysis is shown at top and bottom. ×200



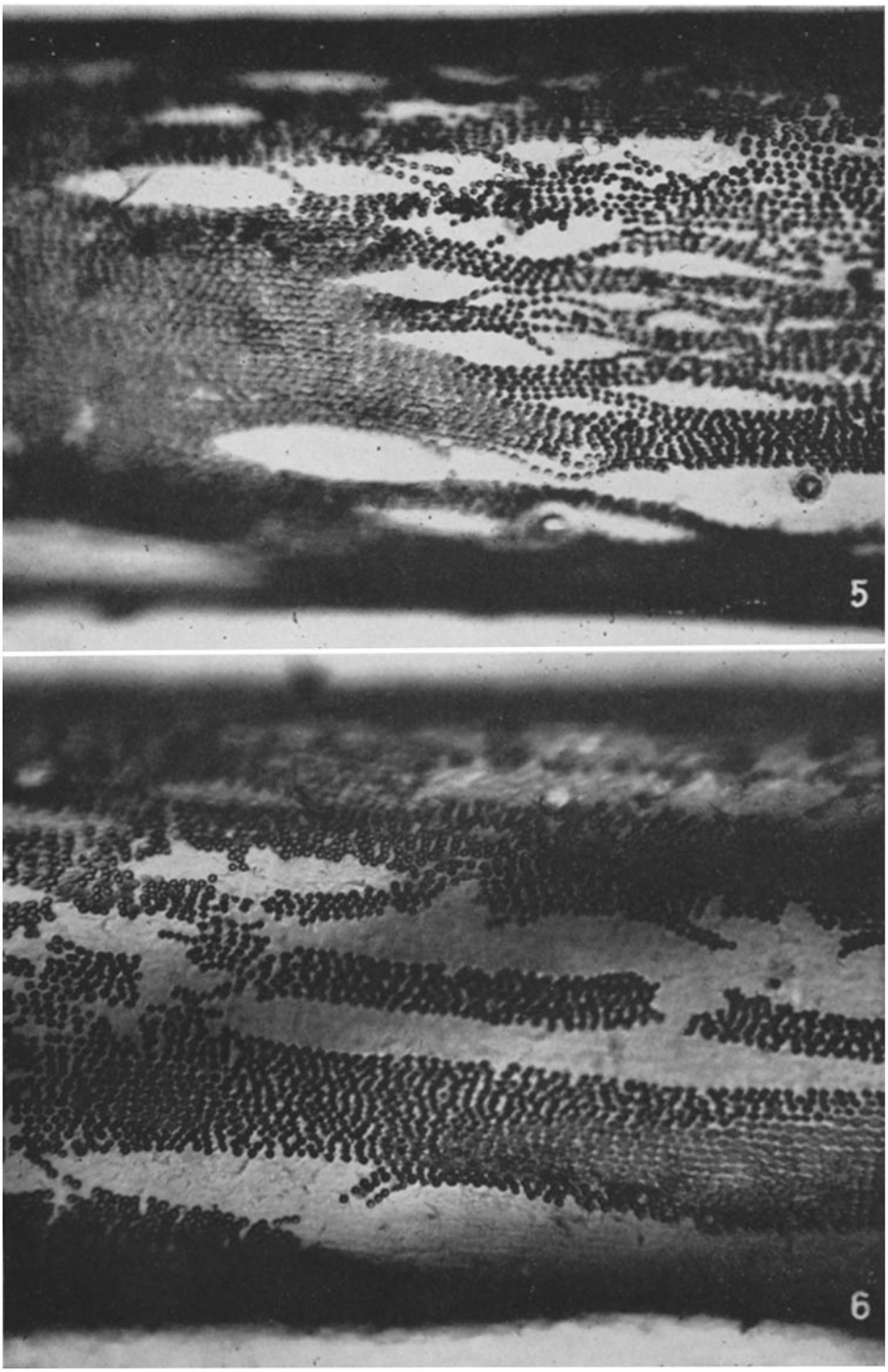


(Osterhout: Abnormal protoplasmic patterns)

PLATE 7

FIG. 5. Splits over the entire cell. In several places single rows of chloroplasts separated off. Plasmolysis is shown in lower left hand corner. In the lower right hand corner a large clear space with a sphere with a light center and a clear zone around it: such spheres often appear in the vacuole. ×180.

FIG. 6. Very large splits. Each of these began as a very small split such as is visible in Fig. 2, upper right hand corner. ×190.



(Osterhout: Abnormal protoplasmic patterns)