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Histochemical Studies of Wound Periderm Formation

VII. Some Considerations on Probable Conditions for the Initiation of the Cell Divisions during the Wound Periderm Formation

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The problem of cell division is one of the fundamental problems in biology. Indeed, it has been studied in detail from morphological view points, but rather a little is known as to the physiological conditions which bring about the cell divisions in plant tissues. Though it has been reported that the cell divisions are induced by some active substances or physical agents (HABERLANDT, 1923; GURWITSCH, 1926; HAMMETT, 1929; BONNER and ENGLISH, 1938; THIMANN, 1952; and others), the causal factors or conditions necessary for the initiation of the cell divisions are not confirmed yet.

In the storage tissues of some plant species, permanent tissue cells recover their meristematic activity when the tissues are cut, while this does not occur in other species. Based on this fact, it seems to be possible to analyse the conditions necessary for the initiation of cell division by checking various histochemical changes that occur in the tissues after cutting. A series of reports along this line has already been published by the present author (BABA, 1953, 1955, 1956, 1958, 1960 a and b). The present investigation is aimed at, in the first place, a determination of the mitotic index after the cutting, and in the second place, a study to see whether anaerobiosis or some chemicals may cause any changes in the mitotic index, and finally, it offers some considerations on probable conditions for the initiation of the cell divisions during the wound periderm formation.

Materials and Methods

Tubers of *Solanum tuberosum* and *Helianthus tuberosus*, and roots of *Raphanus sativus* were used as the materials for the study of mitotic index during the wound periderm formation in the same manner as used in the previous studies. (BABA, 1953, 1955, 1956, 1958, 1960 a and b). Materials, cut with a sharp knife, were kept in air saturated with moisture at a temperature of 30°C for 24, 48, 72 and 96 hours. From these tissue blocks as well as from a freshly made

tissue block, thin sections of the injured tissues were made rectangular to the cut surface. Sections were fixed with a NAVASHIN's fluid or a BOUIN's fluid and stained with a acetocarmine fluid. The permanent preparations thus prepared were observed under a microscope. Parenchymatous cells located within the range of 0.5 mm in thickness from the cut surface inward were observed exclusively, and, among these cells, those approximately recognizable as either in the resting stage or prophase, or metaphase, or anaphase, or telophase, were counted separately. Mitotic index was expressed by the ratio of dividing cells number to the total cells number in percentage.

As tubers of *Solanum tuberosum*, among the materials used so far, show a remarkable recovery of meristematic activity by cutting, they are suitable to study the effects of some chemicals on the occurrence of cell division during the wound periderm formation (*cf.* BABA, 1953, 1955, 1956, 1958, 1960 a and b). Therefore, they were used as the present material. Just prior to the treatment with the chemicals, each tuber was cut into halves. One piece was used as a test sample and another as a control one.

To study the effect of anaerobiosis, N_2 gas was used. Immediately after the cutting, the test sample was placed in a container filled with 100% N_2 gas saturated with moisture at a temperature of 30°C and was kept for 96 hours in this condition. The control sample was placed and kept in air saturated with moisture under the same conditions for the same period.

For the study of the effects of chemicals, the sample was given the treatment described below immediately after cutting, and subsequently they were maintained in air saturated with moisture for 96 hours at a temperature of 30°C. Chemicals used in this experiment are respiratory inhibitors such as potassium cyanide, sodium diethyldithiocarbamate and sodium fluoride, and enzyme substrates such as pyrocatechol, *l*-ascorbic acid and cytochrome C. Concentrations of the solutions of these chemicals are shown in Table 2. Each chemical was dissolved in a M/30 phosphate buffer solution at pH 6.0, and the pH values of the solutions of these chemicals with the exception of potassium cyanide and *l*-ascorbic acid were pH 6.0. As for potassium cyanide and *l*-ascorbic acid, their pH values at final concentration in the M/30 phosphate buffer solution were pH 7.0 and pH 5.0 respectively. Immediately after the cutting, the test samples were dipped in one of the above solutions and were placed under a vacuum of about 3 mm Hg for 5 minutes so as to have the solution penetrated into the injured cells on the cut surface and into the intercellular space near the surface. After the vacuum was broken, the samples were picked up out of the solutions and were placed in air saturated with moisture for 96 hours at a temperature of 30°C. The control samples were similarly treated in the M/30 phosphate buffer solution, and subsequently, they were kept in the same condition as in the case of the test sample. In all these samples thus prepared, the mitotic index was determined in the manner described in the previous paragraph.

Results

i) *Mitotic index.*

Changes in mitotic indices within 96 hours after the cutting in *Solanum*, *Helianthus* and *Raphanus* are shown in Table 1.

In *Solanum*, dividing cells were not observed in the material immediately after the cutting, but a few cells in prophase stage were found at 24 hours after the cutting (*cf.* Figs. 3–4), showing a mitotic index of 0.3%. In the materials at 48 hours and 72 hours after the cutting, dividing cells in the prophase, metaphase and anaphase stage (*cf.* Figs. 3–8) were observed in the greatest number and those in telophase stage (*cf.* Figs. 9–10) were also observed, and the mitotic index reached as high as 5.8% in both cases. In the material at 96 hours after the cutting, though the dividing cells in prophase, metaphase and anaphase stage were somewhat less than those at 48 and 72 hours, those in telophase stage were in the greatest number, and the mitotic index was 4.9%. In *Helianthus*, no dividing cells were observed in the material within 24 hours after the cutting. In the material at 48 hours after the cutting, dividing cells in prophase and metaphase stage (*cf.* Figs. 12–14) were observed for the first time. Subsequently, with the lapse of time after the cutting, increasing numbers of dividing cells in prophase, metaphase, anaphase and telophase (*cf.* Figs. 12–16) were observed. In the material at 96 hours after

Table 1. Changes in mitotic index of tissue cells within 96 hours after the cutting in *Solanum tuberosum*, *Helianthus tuberosus* and *Raphanus sativus*.

Material	Hours after the cutting	*Mitotic index $\frac{b+c+d+e}{a+b+c+d+e}\times 100\%$	Distribution of mitotic stage					
			Resting	Dividing				
			(a)	Prophase (b)	Metaphase (c)	Anaphase (d)	Telophase (e)	Total (b+c+d+e)
Solanum	0	0	4150	0	0	0	0	0
	24	0.3	4792	13	0	0	0	13
	48	5.8	4930	189	28	38	49	304
	72	5.8	6087	244	18	31	83	376
	96	4.9	5179	132	12	26	98	268
Helianthus	0	0	2200	0	0	0	0	0
	24	0	3630	0	0	0	0	0
	48	0.4	4143	15	1	0	0	16
	72	1.9	5907	68	7	10	31	116
	96	2.5	5030	72	8	15	34	128
Raphanus	0	0	2120	0	0	0	0	0
	24	0	1978	0	0	0	0	0
	48	0	1820	0	0	0	0	0
	72	0	2096	0	0	0	0	0
	96	0	1872	0	0	0	0	0

* The mitotic index is shown as the ratio of dividing cells per total cells.

the cutting, the mitotic index was found to be 2.5%.

Mitotic figures found in the wounded tissue in the course of wound periderm formation in *Solanum* and *Helianthus* are shown in Figures 1–16.

Contrary to the cases of *Solanum* and *Helianthus*, no recognizable figure of cell division was observed in *Raphanus* within 96 hours after the cutting.

ii) *Effects of anaerobiosis and some chemicals on mitotic index.*

Mitotic indices at 96 hours after the cutting in *Solanum* treated with some chemicals immediately after the cutting are shown in Table 2.

As shown in Table 2, cell division was not observed at all in the materials maintained in an anaerobic condition with N_2 gas, while a number of cell divisions were observed in the control materials, showing a mitotic index of 4.5% (*cf.* Figs. 17 and 18).

A potassium cyanide solution at the concentration of M/1,000[1] did not show any effect on mitotic index as compared with the control. When treated with a solution of M/50, the material showed a lower mitotic index as compared with the control one (*cf.* Figs. 19 and 20).

In the case of sodium diethyldithiocarbamate, the mitotic index in the treated sample showed a slightly lower values as compared with that in the control one at the concentration of M/300[1], and it showed a definite decrease at the concentration of M/100 (*cf.* Figs. 21–22).

Sodium fluoride caused a rotting of the wound region in the test sample at the concentration of M/100[1]. At the concentration of M/1,000, hardly any difference was found in the mitotic index between the test and control samples (*cf.* Figs. 23–24).

The treatment with pyrocathechol solution brought about a slight increase in the mitotic index in the treated sample as compared with the control at the concentration of 0.0227 M. This is the concentration at which SIMONDS *et al.* (1953) demonstrated the stimulation of wound healing action by these chemicals. In the case where a solution of M/500 was used, the mitotic index showed a higher value than in the case of 0.0227 M (*cf.* Figs. 25–26).

By treating the test sample with the M/300 *l*-ascorbic acid solutions, the mitotic index showed a slightly higher value as compared with that of the control. The mitotic index treated with a M/100 solution was of a higher value than that of the sample treated with a M/300 solution (*cf.* Figs. 27–28).

In the case of M/2,000 cytochrome C solution, the mitotic index of the test sample did not show a remarkable difference as compared with that of the control (*cf.* Figs. 29–30).

Figures 17–30 show the effects of anaerobiosis and some chemicals on the wound periderm formation described above.

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1) This is the same concentration as used in the previous study for respiratory inhibition (BABA, 1960 a).

Table 2. Effects of anaerobiosis and some chemical substances on mitotic index at 96 hours after the cutting in *Solanum tuberosum*.

Substances	Mitotic index $\frac{b}{(a+b)}\%$	Distribution of mitotic stage		
		Resting stage (a)	Dividing stage (b)	Total cells (a+b)
100% nitrogen gas	0	685	0	685
Control (air)	4.5%	698	33	731
M/1,000 potassium cyanide	2.0%	295	6	301
Control	1.9%	309	6	315
M/50 potassium cyanide	1.0%	764	8	772
Control	1.9%	767	15	782
M/300 sodium diethyldithiocarbamate	1.5%	319	5	324
Control	2.0%	292	6	298
M/100 sodium diethyldithiocarbamate	1.1%	716	8	724
Control	2.1%	696	15	711
M/100 sodium fluoride	—	Rot.	Rot.	Rot.
Control	1.9%	305	6	311
M/1,000 sodium fluoride	2.0%	342	7	349
Control	2.2%	400	9	409
0.0227M pyrocatechol	2.6%	266	7	273
Control	2.0%	287	6	293
M/500 pyrocatechol	3.3%	527	18	545
Control	2.1%	515	11	526
M/300 *l*-ascorbic acid	2.4%	283	7	290
Control	2.0%	296	6	302
M/100 *l*-ascorbic acid	2.9%	473	14	487
Control	2.1%	476	10	486
M/2,000 cytochrome C	1.9%	363	7	370
Control	2.1%	371	8	379

Discussions

In multicellular organisms, the cell multiplication is the fundamental in many biological phenomena such as growth, ontogenesis, morphogenesis or cellular differentiation. In this sense, cell division is one of the most fundamental problems in biology. Though, this phenomenon has been studied very well by numerous investigators from the morphological point of view in the

fields of cytology and karyology, the investigations on this phenomenon have been carried out only by a small number of investigators from the angles of causal analysis of the initiation of the cell divisions. Concerning the initiation of the cell divisions in plants, it has been pointed out that the cells have a potency necessary for the cell divisions, however, they do not enter into the cell divisions unless they obtain the suitable "Faktoren" (*cf.* GURWITSCH, 1926; BÜNNING, 1953). From the results of the inhibitory studies in animal cells, on the other hand, it has been found that an aerobic "process" is necessary for the cells to enter mitosis (BULLOUGH, 1949; and others). Though it has been definitely shown by the experimental studies in plant tissues that some active substances or physical agents induce the cell divisions (HABERLANDT, 1923; GURWITSCH, 1926; HAMMETT, 1929; BONNER and ENGLISH, 1938; THIMANN, 1957; and others), the causal factors or conditions necessary for the initiation of the cell divisions in higher plants are not clearly confirmed yet.

In higher plant cells after injury, the recovery of the mitotic activity of the cell divisions in parenchymatous tissues is observed in the storage tissues of some plants, while, such recovery is not observed in those of other plants. By comparing the physiological and cytochemical changes after the cutting between the former storage tissues and the latter ones and by investigating those changes in advance of and accompanying the initiation of the cell divisions during the wound periderm formation in the former case, it is probably possible to analyze the probable physiological conditions of the initiation of the cell divisions. Judging from this point of view, mentioned above, some considerations on probable conditions of the initiation of cell divisions during the wound periderm formation will be made below.

Immediately after the cutting, nadi-oxidase, pyrocatechol oxidase, *p*-cresol oxidase, dopa-oxidase and ascorbic acid oxidase show a positive reaction in *Solanum* and *Helianthus*, whereas, they do not show a positive reaction in *Raphanus* (BABA, 1953, 1955, 1958 and 1960b). In these investigations, though the cell divisions are observed in the former two materials during the wound periderm formation, they are not observed in the latter one.

Before the occurrence of the cell divisions during the wound periderm formation in *Solanum* and *Helianthus*, the following remarkable changes are detected (BABA, 1953, 1955, 1956, 1958, 1960a and b): The increase of the activities of nadi-oxidase, pyrocatechol oxidase, ascorbic acid oxidase, peroxidase, catalase and dehydrogenases, the increase of the respiratory activity, the lowering of pH value, the decrease of the amount of ascorbic acid. Among these changes, however, the changes in the activities of peroxidase, catalase and dehydrogenase and the change of respiratory activity show the similar tendency to those of *Raphanus*, in which cell divisions are not observed after the cutting.

In advance of and accompanying the occurrence of the cell divisions

during the wound periderm formation in *Solanum* and *Helianthus*, some remarkable changes are detected in the wounded tissues. They are those of the activities of nadi-oxidase, pyrocatechol oxidase and ascorbic acid oxidase, decrease of the amount of ascorbic acid, and the lowering of pH values (BABA, 1953, 1955, 1956, 1958 and 1960b).

Comparing the previously reported facts cited above with the changes in mitotic index after the cutting, the following consideration are obtained. In the first place, the phenomena which are considered to have little parallelism with the mitotic index are the changes in the activities of *p*-cresol oxidase, dopa-oxidase, catalase and dehydrogenase, and those of respiratory activity. In the next place, the phenomena which show roughly parallel relations with the mitotic index and hence considered to be closely related to it are the changes of the activities of nadi-oxidase, pyrocatechol oxidase and ascorbic acid oxidase, the decrease of ascorbic acid and the lowering of pH values.

As to the phenomena belonging to the former group, the lack in parallelism between these phenomena and the mitotic index does not necessarily mean that these phenomena have nothing to do with the occurrence of cell division in the wounded tissues, because, if only a small part of the total activity takes part in the occurrence of cell division, the activity measured as a whole may fail to show the parallelism with the mitotic index.

On the other hand, as to the phenomena belonging to the latter group, the increase of the activities of nadi-oxidase, pyrocatechol oxidase and ascorbic acid oxidase, and the lowering of pH value always occur just in advance of an increase in the mitotic index (at 24 hours after the cutting). These facts seem to indicate that the increase of such enzyme activities as mentioned above and the lowering of pH value in the tissues may give favorable conditions for causing cell division, if these changes may not be regarded as a direct cause for the initiation of the cell divisions. From the present investigations, it also seems to indicate that an aerobic process is necessary for the initiation of the cell divisions during the wound periderm formation. As evidences to support this view, the following descriptions or considerations may be mentioned.

1) In the present investigation, any cell divisions during the wound periderm formation are not observed at all in anaerobiosis, that is to say, in pure N_2 gas. This fact strongly supports the view that an aerobic process is necessary for the initiation of the cell divisions during the wound periderm formation. This view is quite in accordance with the evidence that the initiation of mitosis is, strictly speaking, an aerobic process, which have been recently accepted by many researchers (*cf.* BULLOUGH, 1950; and others).

2) As stated in the descriptive part of this paper, the treatment of the cut tissue by potassium cyanide and sodium diethyldithiocarbamate caused a decrease in the mitotic index of wounded tissue. It is known that potassium

cyanide and sodium diethyldithiocarbamate are the inhibitors for nadi-oxidase, pyrocatechol oxidase and ascorbic acid oxidase (*cf.* KUBOWITS, 1937; KING, 1939; WARBURG, 1949; JAMES and GARTON, 1952). This fact indicates that the inhibition of the activity of these enzymes results in the inhibition of the cell divisions during the wound periderm formation, and supports the view mentioned above.

3) It has been found in the previous studies that the activities of nadi-oxidase, pyrocatechol oxidase and ascorbic acid oxidase increase in advance of and accompanying the occurrence of the cell divisions during the wound periderm formation and it has been concluded that the increase of the activities of these enzymes has a close connection with the occurrence of the cell divisions during the wound periderm formation (BABA, 1955, 1956, 1958 and 1960b). Then, it may be expected that the addition of the substrate of the enzymes mentioned above accelerates the increase of the respective enzyme activity and hence stimulates the occurrence of the cell divisions. In the present investigation, as it is expected, the treatment of cut tissue with pyrocatechol and *l*-ascorbic acid solution causes an increase in the mitotic index. Therefore, it is highly probable that the increase of the activities of pyrocatechol oxidase and ascorbic acid oxidase in the tissue provides one of the favorable conditions for the initiation of the cell divisions. This conclusion is supported by SIMONDS *et al.* (1953) who have stated that catechol is the most effective compound for stimulating wound healing in the tubers of *Solanum*. Contrary to our expectations, the addition of cytochrome C did not show any marked effects on the mitotic index. This may possibly be due to the oxidation of cytochrome C by external O_2, or possibly because the molecules of cytochrome C are too large to penetrate into the cells.

4) SMALL (1955) has stated that the pH value of the phellogen cells are lower than that of the parenchymatous cells. In the previous paper (BABA, 1956), it has been stated that a lowering of pH value in the tissue is observed in advance of the occurrence of the cells divisions during the wound periderm formation and that the lowering of pH value in the tissue have something to do with the occurrence of the cell divisions. The results stated above may possibly substantiate the view that the lowering of pH value in the tissues provides one of the favorable conditions for the initiation of the cell divisions.

From the above considerations, the present author has arrived at the following conclusions on favourable conditions for the initiation of cell divisions during the wound periderm formation. An aerobic process is necessary for the initiation of the cell divisions and furthermore, an increase of the activities of nadi-oxidase, pyrocatechol oxidase and ascorbic acid oxidase, and a lowering of pH value in the tissue provide some favorable conditions for the initiation of the cell divisions.

Summary

1) Mitotic index was calculated using the tubers of *Solanum tuberosum* and *Helianthus tuberosus* and the roots of *Raphanus sativus* immediately and at 24, 48, 72 and 96 hours after the cutting.

2) Mitotic index was 0 immediately after the cutting both *Solanum* and *Helianthus* and also at 24 hours in *Helianthus*. In *Solanum*, the mitotic index increased gradually with the lapse of time after the cutting and showed the maximum value at 48 and 72 hours. In *Helianthus*, it increased gradually with the lapse of time after the cutting and showed the maximum value at 96 hours. While, in *Raphanus*, the mitotic index was 0 in the course of this experiment.

3) Effects of some chemicals on the mitotic index at 96 hours after the cutting were observed in the tubers of *Solanum tuberosum*. Nitrogen gas, potassium cyanide and sodium diethyldithiocarbamate inhibit the cell divisions to more or less extent, whereas, pyrocatechol and *l*-ascorbic acid brought about a stimulation on the cell divisions, while, sodium fluoride and cytochrome C showed little effect on the mitotic index.

4) From the results of the present and the previous investigations, it is concluded that an aerobic process is necessary for the initiation of the cell divisions, and furthermore, that an increase of the activities of nadi-oxidase, pyrocatechol oxidase and ascorbic acid oxidase, and a lowering of pH value provide favorable conditions for the initiations of cell division.

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Explanation of Plates I–III.

Figs. 1–10. All these figures are photomicrographs showing the stages of nuclear division in parenchymatous cells during the wound periderm formation in the tubers of *Solanum tuberosum*.

In Figs. 1, 3, 5, 7 and 9 (each magnification, 300×), the surface which lies lengthwise on the left side shows the cut surface. Figs. 2, 4, 6, 8 and 10 (each magnification, 600×) are partly twice enlarged photographs of respective Figs. 1, 3, 5, 7 and 9.

Figs. 1 and 2. Resting stage.—Figs. 3 and 4. Prophase.—Figs. 5 and 6. Metaphase.—Figs. 7 and 8. Anaphase.—Figs. 9 and 10. Telophase.

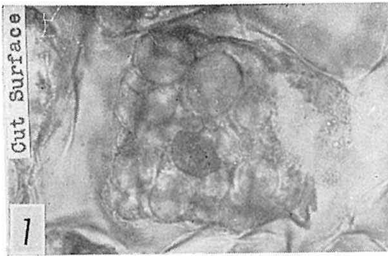
Figs. 11–16. All these figures are photomicrographs showing the stages of nuclear division in parenchymatous cells during the wound periderm formation in the tubers of *Helianthus tuberosus*. (Magnification, 600×).

Fig. 11. Resting stage.—Fig. 12. Prophase.—Figs. 13 and 14. Metaphase.—Fig. 15. Anaphase.—Fig. 16. Telophase.

Figs. 17–30. All these figures are photomicrographs showing the effects of anaerobiosis and some chemicals on the wound periderm formation in the tuber of *Solanum tuberosum*. (Magnification, 80×).

The photographs on the right side (Figs. 18, 20, 22, 24, 26, 28 and 30) are the control of those on the left side respectively (Figs. 17, 19, 21, 23, 25, 27 and 29). In all these photographs, the surface which lies lengthwise on the left side shows the cut surface.

Fig. 17. Showing the effect of N_2 gas.
Fig. 19. Showing the effect of M/50 potassium cyanide.
Fig. 21. Showing the effect of M/100 sodium diethyldithiocarbamate.
Fig. 23. Showing the effect of M/1,000 sodium fluoride.
Fig. 25. Showing the effect of M/500 pyrocatechol.
Fig. 27. Showing the effect of M/100 *l*-ascorbic acid.
Fig. 29. Showing the effect of M/2,000 cytochrome C.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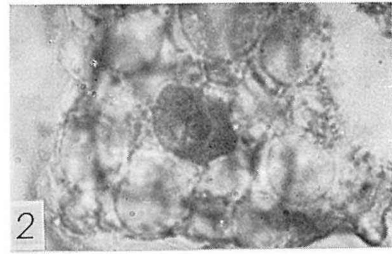
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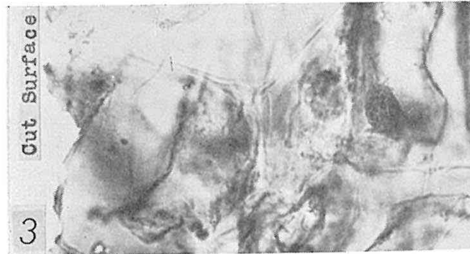
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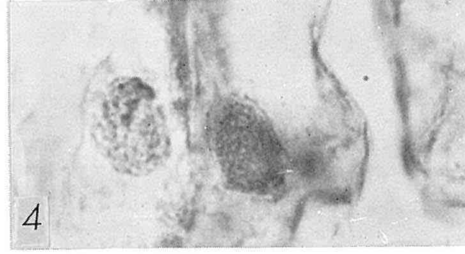
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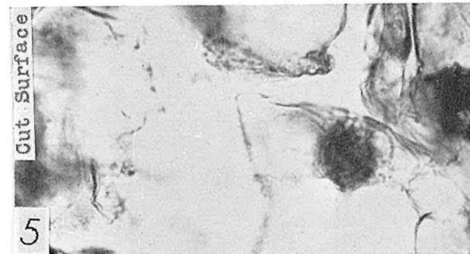
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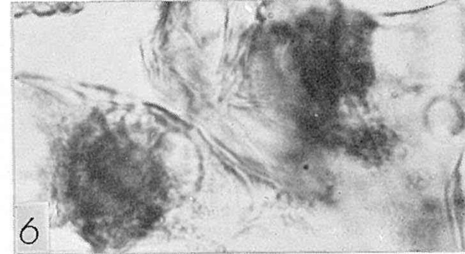
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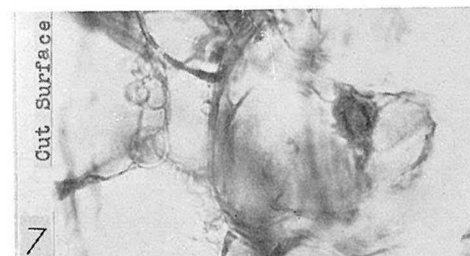
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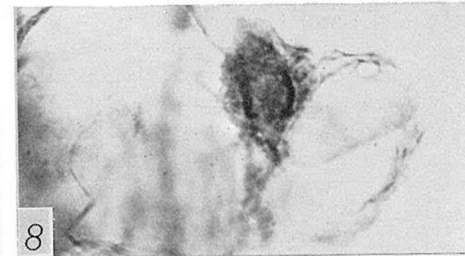
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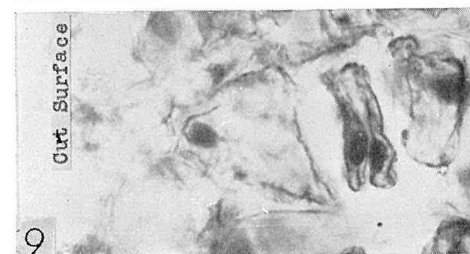
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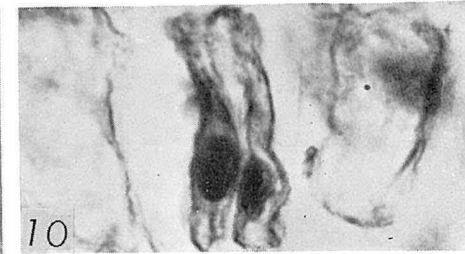
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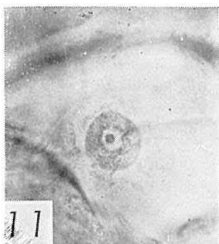

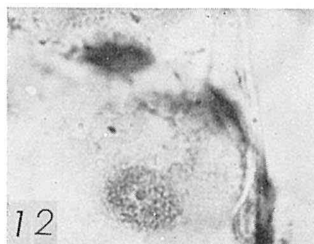

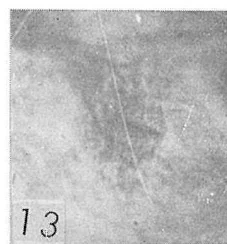

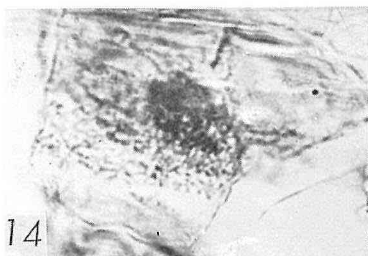

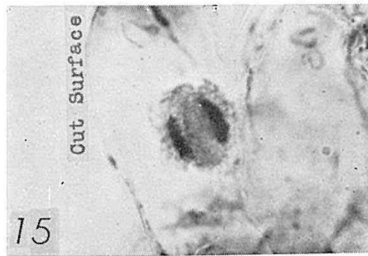

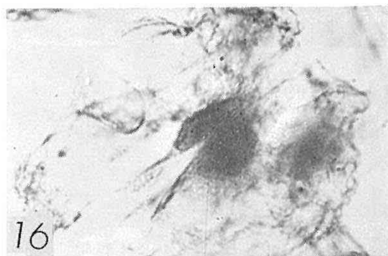

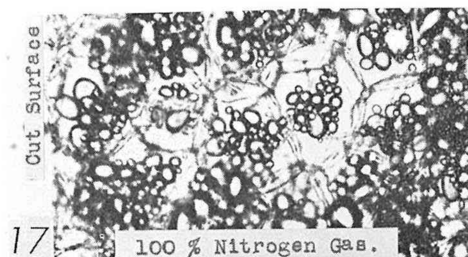

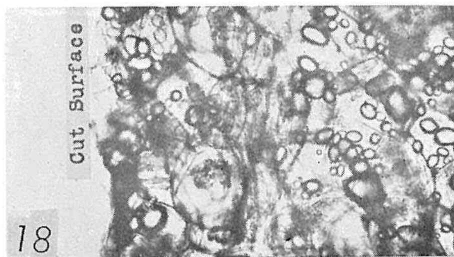

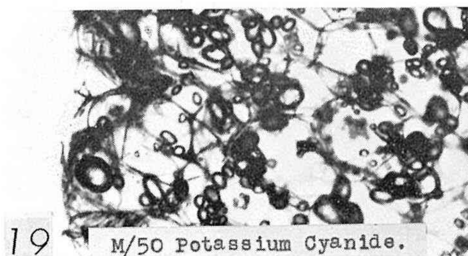

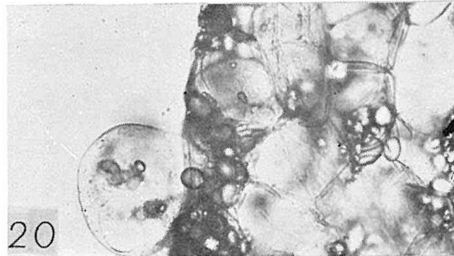

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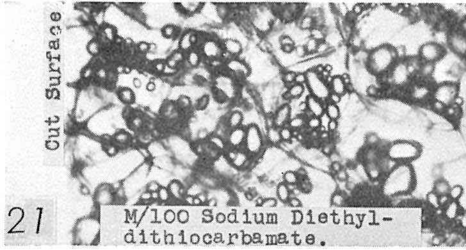
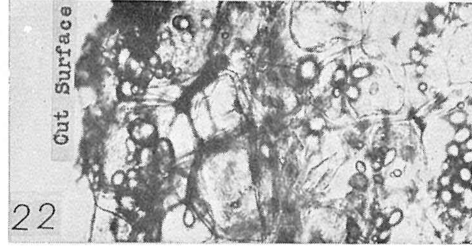


21 M/100 Sodium Diethyl-dithiocarbamate.



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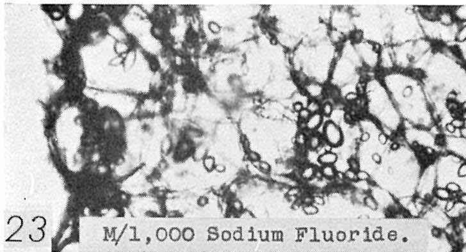
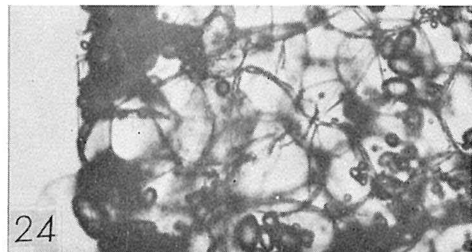


23 M/1,000 Sodium Fluoride.



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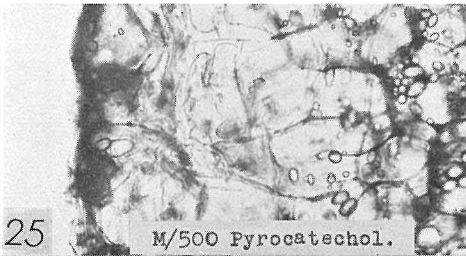
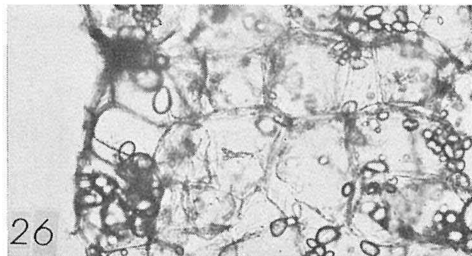


25 M/500 Pyrocatechol.



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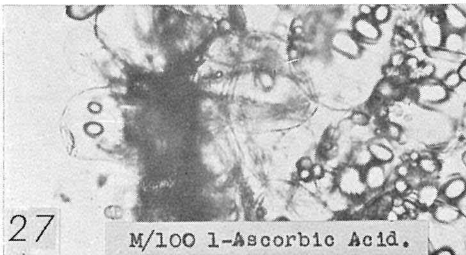
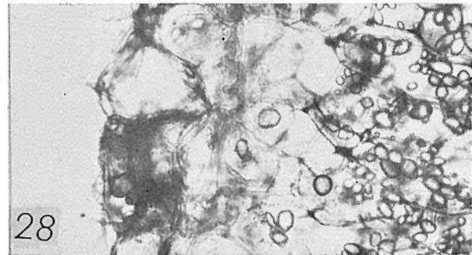


27 M/100 l-Ascorbic Acid.



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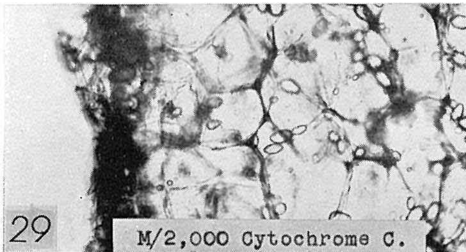
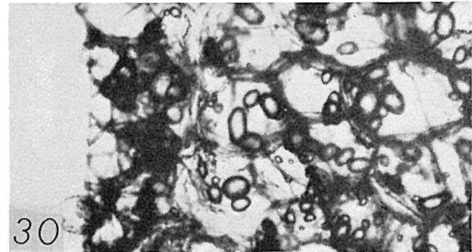


29 M/2,000 Cytochrome C.



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