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Micrurgical studies on virus-infected plants

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[Plates 28]

Introduction

Micromanipulative methods have been used very seldom for the study of plant cells chiefly because glass instruments which are the only ones available are usually either too brittle or too flexible to penetrate the tough cell walls. A few plant cells (e.g. the staminal hairs of *Tradescantia*) are suitable for micrurgical studies, but these are of little use for virus work. Eleven different viruses were inoculated into each of three species of *Tradescantia* without any infections resulting.

By the use of specially shaped needles it has been found possible to work with many of the cells of Solanaceous plants. The cutinized hair cells of *Solanum nodiflorum*, which can rarely be penetrated through the side walls, can be successfully operated on if the protoplast is approached through the transverse septum. For many operations these cells are particularly favourable, as diffusion of the mounting medium into the cell is very gradual, most of the large surface being protected by the thickened wall. Livingstone and Duggar (1934) have compared the virus content of different parts of the infected tobacco cell and of cells containing and cells devoid of inclusions. In this paper various properties of infected cells, including their virus content, will be described.

Description

The pH of the cell contents

Many determinations have been made by indicators of the *p*H of the cell contents, but every method described is open to the grave criticism that it involves considerable injury to the cell and a lapse of time before any reading is obtained. Injury to the cell is known to increase the hydrogen-ion concentration. A method suggested by Professor Péterfi,

although not entirely free from the possibility of error, is the most accurate yet defined. A little dry indicator is dropped on to a spot of vaseline, from which it is picked up on the tip of a micro-needle. The tissue to be examined is arranged in a hanging drop and the cell is punctured with the needle bearing the dye. The indicator dissolves at once in the cell sap and the colour is immediately noted. Indicators which change their colour and not merely their depth of colour are used. A possible source of error lies in the wounding of the cell, but this error should not be great as the wound is small and the reading is taken very quickly.

Various tissues from healthy plants of *Solanum nodiflorum*, from the same species infected with aucuba mosaic virus (Henderson Smith 1928), from healthy *Hyoscyamus niger* and from *H. niger* infected with *Hyoscyamus* virus 3 (Hamilton 1932) were examined. The object was primarily to compare virus-free and virus-diseased tissue but the following results accrued.

(1) The *p*H of the contents of the hairs of the two species, in both healthy and diseased plants was approximately the same (*p*H 6·8).

(2) The contents of the epidermis and the palisade tissue were in all cases slightly less acidic than the hair cells, the *p*H lying between 6·8 and 7·2.

(3) No differences were observed in the hydrogen-ion concentration between healthy and diseased plants in any type of cells.

The stability of the intracellular inclusions

The amorphous inclusion bodies. The vacuolated amoeboid inclusions of tobacco mosaic disease have been described as very fragile structures which break down on contact with the micro-needle but withstand considerable disturbance in the cytoplasm in their immediate vicinity (Livingstone and Duggar 1934). In aucuba mosaic disease, inclusions are produced by the gradual aggregation and fusion of minute particles which circulate in the cytoplasmic stream a few days after inoculation (Sheffield 1931). These inclusions are similarly frail, very much more so than is the cell nucleus. Slight mechanical pressure on the outside of the cell destroys the inclusion immediately, although the cell may not be sufficiently crushed for any other injury to be visible. Puncturing the inclusion with a very fine needle causes its immediate disintegration. The three pictures 1 *a*, *b* and *c* (Plate 28) were obtained by working with a Leitz Makam camera attached to the microscope, the operations being watched through the side observation tube of the camera. After the first picture (1*a*) was taken the plate was

exchanged and the dark slide removed in readiness for a second exposure, so that fig. 1*b* could be taken with the shortest possible delay after the inclusion was punctured. In the first photograph of the series, the needle is seen to have penetrated the transverse septum and to have passed well into the vacuole. The inclusion, not having been touched, is quite unaffected. Then the inclusion body was punctured and the needle rapidly withdrawn. 15 sec. after the puncture the inclusion is seen to have changed its consistency and to have formed a mass of clear bubbles amongst which a few granules are visible (fig. 1*b*). These granules are morphologically similar to those which went to form the body. After a further 10 min. the bubbles have disappeared and a few scattered granules only remain (fig. 1*c*). Figs. 2 and 3 (Plate 28) are comparable to fig. 1*b* showing stages in a similar disintegration following injury of the inclusion body. On injury an inclusion may disintegrate with the formation of a few granules without first forming a vesicle. If two inclusions are present in a cell and one only is pierced, that one disintegrates, the other remaining quite unchanged.

Despite their fragility these inclusions can, if certain precautions are taken, be removed intact from the cells containing them (fig. 4, Plate 28). It is necessary, besides selecting a cell which can be operated on whilst avoiding all mechanical pressure in the region of the inclusion, to use also a suitable mounting medium.

The stability of the body in the mounting medium seems to depend on the osmotic pressure. Bodies were removed into solutions of NaCl, KCl or $CaCl_2$ of 0·01–0·1 M. In a 0·1 M solution the body persists; at 0·07 M disintegration is gradual; below 0·05 M it is very rapid.

The effect of the *p*H of the mounting medium on the inclusions was also tested. In a 0·01 M solution, they broke down at all degrees of acidity from 7 to 2·2. In a 0·1 M solution the bodies remained intact over the same range of *p*H values. The bodies thus appear to be almost unaffected over a wide range. The only effect noticed was that in the more acid solutions the bodies withstand maltreatment more readily than in a neutral solution.

The facts that the stability of the body depends on the osmotic pressure of the medium containing it and also that it often forms a vesicle on breaking down, at first sight appear to be arguments in favour of the existence of a membrane around the inclusion. Its mode of formation, its appearance and extreme fragility do not suggest this.

Striate material. Tobacco mosaic disease produces in infected cells, in addition to the amoeboid *X*-bodies, flat plate-like crystals which were named striate material because cross-striations become visible under the

action of acids (Iwanowski 1903; Goldstein 1926). These striations are present always, being visible in polarized light. They are merely emphasized by reducing the *p*H value when the plate may actually break down into long needle-like fibres. Beale (1937) has drawn attention to the morphological similarity between precipitated virus and the needles produced from the striate material.

Similar plate-like crystals are produced by the breaking down of the large non-crystalline inclusions of aucuba mosaic disease. Enation mosaic (Ainsworth 1937) produces in tomato *X*-bodies and striate material visually indistinguishable from those of tobacco mosaic. The striate material of tobacco mosaic and of enation mosaic have been examined by micromanipulative methods.

The application of slight mechanical pressure to the exterior of the cell in the vicinity of the plate results in its immediate breakdown into striae. Pricking with a micro-needle causes a similar formation of needle-like fibres. This is contrary to the results of Livingstone and Duggar (1934), who describe the formation of a granular mass on pricking.

Attempts were made to isolate these plate-like crystals. The medium used was a 0·1 M solution of potassium phthalate buffered to a *p*H of 3·4. In every case the plate broke down into long needles on being removed from the cell. In a few cases the crystal was taken from the cell completely surrounded by cytoplasm. Then it remained apparently intact although its striate structure was clearly visible (fig. 5, Plate 28). Immediately an attempt was made to remove the cytoplasmic envelope the plate broke down into fibres. It is improbable that these crystals could be successfully isolated even in a less acid mounting medium, as the drop in *p*H of the cell contents resultant on injury would probably cause the disintegration of the plate before it reached the medium.

As the crystals could not be isolated it was impossible to test their virus content as was done in the case of the non-crystalline inclusions of aucuba mosaic disease.

The virus content of the cell

Since intracellular inclusions were first observed much discussion has arisen as to their origin and nature. They have been variously described as organisms causing the disease and as mere symptoms of the disease producing them. The demonstration of the mode of formation of the inclusions of aucuba mosaic (Sheffield 1931) suggested that they are formed by the host cell as a response to the stimulation of the virus. The question

still remained as to whether the inclusions contain the virus, and if so, whether the virus is confined to the inclusions or whether it is also dispersed throughout the cytoplasm.

It was intended to pick up small pieces from different parts of the cell contents and to compare the amounts of virus present. This involved having a ready test for the presence of minute quantities of virus: it was imagined that inoculation would provide such a test, but this soon proved totally inadequate. It was then thought that the amounts injected were too small to contain the minimal dose necessary to cause infection. But when quantities of virus, certainly sufficient to produce the diseased condition, were injected by micro-pipette directly into cells, in no case was more than one-tenth of the expected number of infections obtained (Sheffield 1936). The inoculation test was therefore temporarily abandoned, and a serological test similar to that designed by Matsumoto (1935) was tried. This too proved unsatisfactory. In order to carry out such a test with minute quantities of antigen it was necessary to watch the reaction under dark-ground illumination where it was found impossible to distinguish with certainty between an antiserum-antigen precipitate and the various granules and globules of the cytoplasm. A ring test was then tried in the micro-pipette, a drop of antiserum being picked up after the antigen. The end of the pipette containing the ring was broken off and mounted. No ring was visible by transmitted light, and it was very difficult to make pipettes of the exact size to give a mount of the thickness necessary for dark-ground examination. It was then decided to abandon the finer experiments planned, to collect larger quantities of extracted material, and to inoculate these into leaves which would show local lesions. Purified virus was to be used as a standard with which to compare the extracted material. This method was applied to *Solanum nodiflorum* infected with aucuba mosaic disease.

The virus content of the inclusions was first examined. It was essential first to determine the number of bodies necessary to give any appreciable number of lesions. The figures of Bawden and Pirie (1937) indicated that, if the comparison were to be of any value, the dilution of virus should not be greater than 1 in 10^8. Assuming the inclusions to be spherical and of average diameter 20μ or (2×10^{-3}) cm., the volume of each would be $4(10^{-3})^3$ c.c. For such an approximation, the specific gravity can be assumed to be 1. The weight of each inclusion is then $4(10^{-9})$ g. If each inclusion contains as much virus proportionately as the fibres, then to obtain a 1 in 10^8 suspension it is necessary to suspend ten inclusions in 4 c.c. of liquid.

To obtain a number of inclusions of as uniform a size as possible they were taken always from the largest hairs and always from a cell in the same position in the hair. For technical reasons the cell next to the basal one was chosen. To remove a body a hair was selected in which the inclusion of the chosen cell lay near the base but preferably not actually on the cross-wall. The whole hair was arranged in a hanging drop of 0·1 M potassium phthalate solution buffered to a *p*H of 3·4. The hair was held at the base by a large needle or by forceps so that it was flat against the cover-glass. The hair was then divided into two pieces by a cut just below the top wall of the selected cell. The upper part of the hair was pushed away and attention was confined to the portion held by the forceps. This now consisted of a basal cell by which it was held and the cell from which the top wall was already cut and from which the inclusion was to be removed. This was done either by exerting a very gentle pressure at the base of the cell or by replacing the cutting needle by a pipette and removing the body from the cell by suction. The needles were then replaced by two pipettes both of sufficiently large diameter to admit the inclusion body and one of which contained phthalate buffer solution. The inclusion was then picked up in the empty pipette and placed on a clean part of the cover-glass. It was washed several times by flooding with liquid from the one pipette and removing the liquid with the second. Finally, the inclusion was picked up in the pipette which had supplied the liquid for washing and was dropped into a tube containing a very small quantity of liquid. Usually water was chosen, as in this medium the inclusions disintegrate and the substance composing them would presumably become evenly dispersed. Until a sufficient number of inclusions was collected, the tube was stored in a refrigerator. The suspension was then diluted to the desired extent, allowed to stand for some hours and then inoculated by rubbing on to leaves of *Nicotiana glutinosa*.

For each of the first experiments ten inclusions were isolated, suspended in 4 c.c. of water and rubbed into the leaves. A 1 in 10^8 solution of purified aucuba mosaic virus was used as a standard for comparison, and a water control was also used. This experiment was repeated four times, and the results are summarized in the first column of figures in Table I.

The second column of figures of this table shows the results of a similar but more detailed experiment, comparisons having been made at two different dilutions. Twenty inclusions were isolated and suspended in 1·6 c.c. water. 0·3 c.c. of this suspension were diluted to 1·5 c.c. to give a concentration the same as that used in the previous experiments. These two suspensions were used as inoculum. The standards for comparison

were a 2×10^{-7} and a 10^{-8} dilution of aucuba mosaic fibres. A water control was also employed making a total of five different treatments. Inoculations were made on randomized half-leaves of *N. glutinosa*, each treatment being replicated twelve times.

TABLE I. THE INFECTIVITY OF INCLUSION BODIES INDUCED BY AUCUBA MOSAIC VIRUS

Inoculum	No. of lesions	
	60 leaves	12 half-leaves
Water control	0	0
Virus fibres (1 in 10^8)	19	1
,, (1 in 2×10^7)	—	4
Inclusions (10 in 4 c.c.)	15	1
,, (10 in 0·8 c.c.)	—	7

The figures obtained from such experiments must inevitably be very small. It was the work of several days to isolate the ten bodies used in each of the first series of experiments. To obtain figures which could be subjected to statistical analysis, many hundreds of inclusions would need to be isolated. The results obtained indicate quite definitely that virus is present in these inclusions.

The lesions obtained were few in number, but none appeared in the water-inoculated plants. The numbers of lesions caused by the suspension of inclusions were in each case of the same order of size as those produced by the standard dilution of fibres with which the inclusions were compared, suggesting that the inclusions contain a considerable proportion of virus.

A comparison was then made between the virus content of the inclusions and that of the cytoplasm of the cell. Hair cells of uniform size in which the inclusion lay against the cross-wall were selected. Either that half of each cell which contained the inclusion or the half devoid of an inclusion was isolated. When twenty of each had been collected, each set was suspended in 0·8 c.c. of water. 0·15 c.c. of this suspension was later diluted to 0·7 c.c. As standards for comparison suspensions of aucuba mosaic fibres of concentration 1 in 10^7 and 1 in 5×10^7 were used and also a water control. Inoculations were made on randomized half-leaves of *N. glutinosa*. Each of the seven treatments was replicated six times.

In those cells used in the first experiment the inclusions were recently formed. No appreciable difference was found between the virus content

of the two halves (Table II). A fortnight later the experiment was repeated, using cells 2 weeks older than those used in the first series. The virus content of those parts of the cell devoid of inclusions seemed to have decreased in comparison with that of the virus-containing parts.

TABLE II. COMPARISON OF VIRUS CONTENT OF HALF CELLS CONTAINING INCLUSIONS WITH THAT OF THE REMAINING HALVES OF THE CELLS

Dilutions		Total nos. of lesions per six half-leaves			
Virus	Half-cells	Water	Virus	Half-cells devoid of inclusions	Half-cells containing inclusions
		Younger cells			
1 in 5×10^7	3 in 0·6 c.c.	0	1	1	1
1 in 10^7	15 in 0·6 c.c.	0	4	3	5
		0	5	4	6
		Older cells			
1 in 5×10^7	3 in 0·6 c.c.	0	2	0	1
1 in 10^7	15 in 0·6 c.c.	0	8	2	6
		0	10	2	7

It would appear from these experiments that a relatively large quantity of virus is necessary to produce an infection. The size of the virus particles is not accurately known, but the amount required obviously represents a large number of particles. As all our present methods of inoculation are relatively crude no significance can yet be attached to this. Woodruff and Goodpasture (1929), when testing the infectivity of isolated inclusions of fowl pox, obtained far fewer than the expected number of infections. This failure was due to technical difficulties.

Previous results (Sheffield 1931, 1934) had suggested that the non-crystalline inclusions of aucuba and tobacco mosaic viruses "consisted of material precipitated or coagulated from the cell sap or cytoplasm due either to the action of the virus itself or to some substance or a change in physical conditions produced by unbalanced metabolism resultant on virus infection". The body of aucuba mosaic was conceived as consisting of tiny particles of altered cytoplasm which for purely mechanical reasons became aggregated together. The body was regarded as probably containing some virus, as, if the latter were dispersed throughout the cytoplasm, it would be reasonable to expect some of it to be swept into the body. Results of these micrurgical studies necessitate a slight modification of this view. The amorphous inclusions of aucuba mosaic contain relatively

large amounts of virus. But they are not homogeneous as chondriosomes can be demonstrated within them (Sheffield 1933). The stability of the inclusion over a wide range of *p*H values indicates that the virus they contain does not exist in the same state as that isolated in the test-tube (Bawden and Pirie 1937) which has an isoelectric point of 3·4 and is soluble at the *p*H of expressed sap. The state of the virus within the inclusion and the possible nature of the striate material will be fully discussed elsewhere (Bawden and Sheffield 1939).

I am greatly indebted to Professor T. Péterfi of Copenhagen for much invaluable advice in matters of technique when this study was first commenced.

I must also thank Mr W. G. Cochran of the Rothamsted Statistical Department for assistance in designing some of the experiments and also my colleague, Mr F. C. Bawden, for kindly supplying the purified virus used.

Summary

Cells of virus-infected plants were examined by micromanipulative methods.

The *p*H of the cell contents was found to be the same in diseased and in healthy plants.

The non-crystalline intracellular inclusions of aucuba mosaic disease of tomato disintegrate immediately on slight mechanical pressure or on pricking. They are almost unaffected by acids from *p*H 7 to 2·2. They break down if the osmotic pressure is reduced below 0·07 M but can be isolated into solutions of 0·1 M. These inclusions contain virus but virus may also be dispersed through the cell.

The striate material of tobacco and enation mosaics cannot be isolated, as immediately it is touched with a micro-needle it breaks down into needle-like fibres.

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Explanation of Plate 28

The photographs were taken with a Leitz Makam camera. A Leitz 6L or a Leitz 2 mm. objective was used in combination with a Leitz 10 × periplanat ocular, giving magnifications of × 450 or × 900. *Contact prints have been reproduced with a slight reduction.*

The micro-instruments were controlled by a Janse and Péterfi (Zeiss) micro-manipulator.

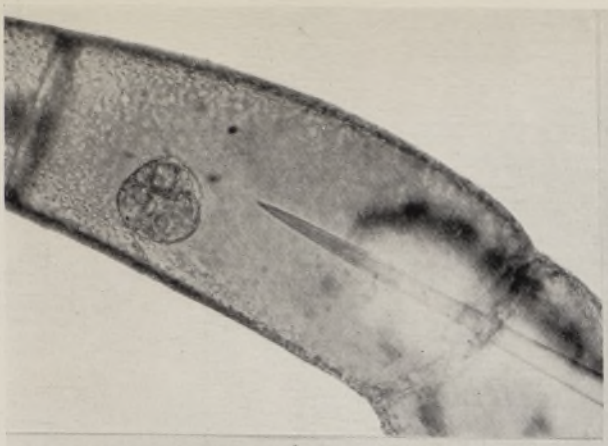
Figs. 1 *a–c*. Cell from hair of *Solanum nodiflorum* infected with aucuba mosaic disease. *a*. A needle has been pushed through the transverse septum well into the cell. Despite the injury to the cell the inclusion body was quite unaffected. (× 383.) *b*. The inclusion was punctured by the needle which was immediately withdrawn again. The body rapidly changed its consistency, forming a mass of clear bubbles in which a few granules were visible. (× 383.) (This photograph was taken about 15 sec. after the body was pricked.) *c*. After 10 min. the inclusion had almost disappeared, a few scattered granules only being visible. (× 383.)

Fig. 2. Similar to fig. 1*b*. About 30 sec. after pricking, the inclusion body had formed a single hyaline vesicle somewhat larger than itself. Further disintegration, as in fig. 1*c*, occurred later. (× 383.)

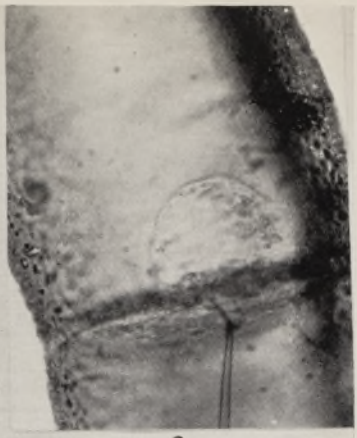
Fig. 3. Similar to fig. 2. About 30 sec. after mechanical injury the inclusion had broken down into a clear hyaline material on the surface of which were a number of granules. Later the appearance of the cell was as in fig. 1*c*. (× 383.)

Fig. 4. An inclusion body produced in *S. nodiflorum* by aucuba mosaic virus was isolated from the cell. This photograph was taken prior to washing the body. (× 765.)

Fig. 5. Striate material produced by enation mosaic virus in tobacco was excised from the cell. The crystal formed needle-like fibres which were held together by an envelope of cytoplasm. Directly this envelope was broken, the fibres separated. (× 383.)



1a



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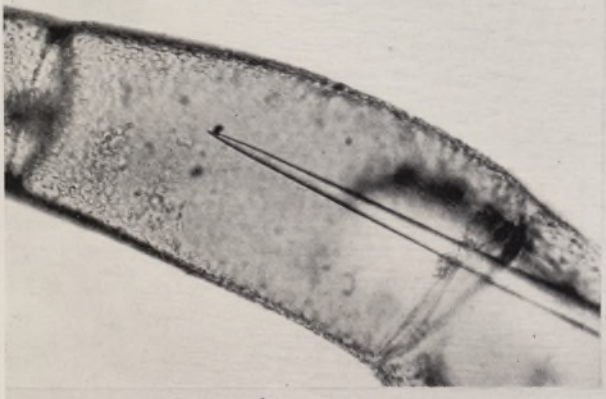
1b

3



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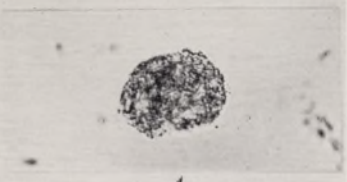

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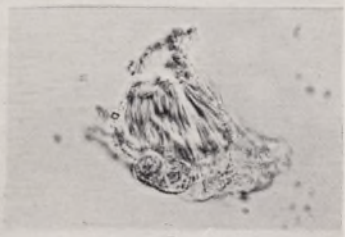

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(*Facing p.* 538)