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The Production of Auxin by Autolysing Tissues

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Summary. Autolysing plant tissues are known to produce auxin when extracted with ether. It has been shown that autolysing plant, yeast and rat liver tissues produce auxin *in vitro;* this suggests that relatively unspecific mechanisms are involved. Furthermore, sterile plant and animal tissues which have been killed by freezing and thawing induce nodules of differentiated cells in a previously undifferentiated callus of *Phaseolus vulgaris*. The callus tissue is known to differentiate in response to applied gradients of auxin. Plant and animal tissues killed by boiling were considerably less effective in inducing differentiation in the tissue. The evidence indicates that auxin is a normal product of autolysing cells. It is suggested that dying cells are an important source of auxin in the plant.

Introduction

It is well known that plant tissues under continuous extraction with wet ether, in the cold, release or produce auxin for many months. Only the auxin extracted in the first few hours is considered to represent the free auxin in the tissue. Since the continued release of auxin is abolished by boiling the tissue it is probably a result of enzymic activity, and the kinetics of release also point to this conclusion. The addition of proteolytic enzymes to unboiled and to boiled tissue results in a release of auxin, but the amount released from boiled tissue is considerably less than that produced from unboiled tissue (LINK and EGGERS, 1941; THIMANN, SKOOG and BYERS, 1942). These results suggest that with autolysing tissue auxin is produced both by its release from a protein-bound form and by its formation from a precursor which is available in greater amounts in the presence of exogenous proteolytic enzymes. Tryptophan can be converted to indolyl acetic acid (IAA) by many plant tissues (KULESCHA, 1952; GORDON, 1961) and is also released from proteins as they are hydrolysed: tryptophan could therefore be one of the sources of the auxin produced by autolysing tissues under the conditions of ether extraction.

The production of auxin by plant tissues during ether extraction suggests that in the plant dying and dead cells may be a normal source of auxin. Both by direct estimation of auxin production and by using a model system which employs the technique of sterile tissue culture we have obtained evidence which supports this conclusion. The model

system is based on the experiments which have shown that differentiation can be induced in certain undifferentiated callus cultures by applied gradients of auxin (WETMORE and RIER, 1963; JEFFS and NORTHCOTE, 1966, 1967). We have investigated whether autolysing tissues can substitute for auxin in experiments of this type.

Materials and Methods

Extraction and Estimation of Auxin. The methods used were based on those described by LARSEN (1955). The tissue homogenates were acidified to pH 3 using methyl orange as an internal indicator and then extracted twice with peroxide free ether for 2 hours at 0° C. The ether extract was concentrated, and the acidic auxins separated by shaking with sodium bicarbonate solution, which was then acidified to methyl orange and re-extracted with peroxide free ether. This ether extract, containing acidic ether-soluble substances, was evaporated to dryness, taken up in a small volume of ethanol and applied to chromatograms on Whatman paper No. 1. These were developed in a descending system with isopropanol: ammonia: water (10:1:1 v.v). Marker strips were run with IAA the position of which was revealed by the ferric chloride-perchloric acid spray (LARSEN, 1955). The zones of the paper chromatograms corresponding to the marker IAA were eluted with water and diluted to 5 mls with phosphate buffer (final concentration 0.01 M; pH 5). These solutions were placed in specimen tubes for the bioassay. Standard solutions were made using IAA (0.01—2.0 μg).

Seeds of *Avena sativa* var Padarn (Ison Ltd., Cambridge) were grown on sand in dim red light for 3 days before being used. The top 2 mm of the seedlings was removed and sections 10 mm long were prepared using a special cutting instrument. The leaves were left inside the coleoptiles (BENTLEY and HOUSLEY, 1954). All operations were carried out in red light. Ten sections were floated on each test solution and left in the dark at 25° C for 20 hours. They were then removed, placed on a glass plate in a photographic enlarger, and photographic images of them were prepared, magnified 6 times. The lengths of the sections on these photographs were measured. Standard curves showed a linear relationship between extension and the log of the IAA concentration over the range used (0.01—2.0 μg).

Tissue Homogenates. Pea leaves (25 g) from plants of *Pisum sativum* (var. Laxton superb) grown in a greenhouse for 3 weeks were homogenized in a blendor with 0.1 M phosphate buffer (50 ml) at pH 8. The homogenate was filtered through muslin and samples of equal volume were placed in boiling tubes together with toluene and stoppered with cotton wool, covered by aluminium foil. They were incubated at 37° C in a water bath with constant shaking.

Rats (Wistar strain) were killed by decapitation, their liver tissue (20 g) was rapidly removed, placed in water (40 ml) and homogenized in a blendor. The homogenate was filtered through muslin and incubated with toluene at 37° C as described above.

Bakers' yeast was suspended in distilled water and samples were incubated with toluene at 37° C as described above.

Samples from all the experiments were taken at various times and stored in the deep freeze.

Tissue Culture Experiments. The callus of *Phaseolus vulgaris,* the media on which it was grown, and the sterile methods employed, have all been described by JEFFS and NORTHCOTE (1966, 1967). The *Phaseolus* callus was normally grown on a full nutrient medium but for the experiments described here on the induction of

differentiation a maintenance medium was used which contained only salts and vitamins, and naphthylacetic acid (50 μg/l) and sucrose (0.5%) (JEFFS and NORTHCOTE, 1966).

In the experiments of JEFFS and NORTHCOTE an approximate 1 cm cube of callus had a groove cut in the top and a wedge shaped piece of agar gel containing the substances to be tested was placed in this groove. In the experiments reported here, killed tissues were placed in a similar groove in the top of the callus block and a small amount of 1% agar gel was used to cement them into the groove.

The tissues tested for their ability to induce differentiation were placed in sterile boiling tubes and killed either by being placed in a boiling water bath for 10 minutes or by being frozen twice to —30° C and then thawed. Microscopic examination of similarly treated tissues showed that they were killed by these methods.

The tissues tested were: (a) *Phaseolus vulgaris* callus. The killed samples were taken from the same callus growth that was used for the experimental induction of differentiation. (b) *Parthenocissus tricuspidata* crown gall callus. This callus has been maintained in culture in this laboratory for several years on a medium containing no growth factors. The culture was originally supplied by Dr. KASSANIS of Rothamstead Experimental Station. (c) Rat Liver tissue. Rats were killed by decapitation, the skin was swabbed with alcohol and liver tissue was rapidly removed using sterile instruments.

After the experimental cultures had been set up they were left for 1 month at 25° C. They were then fixed, dehydrated, embedded in wax, sectioned and stained with safranin and picric-analine blue and mounted as described by JEFFS and NORTHCOTE (1966). They were examined on an optical microscope (Zeiss Ultraphot); polarized optics were used to detect xylem cells and ultraviolet fluorescence microscopy of aniline blue stained sections was used to detect phloem (CURRIER, 1957).

For each test system at least five callus blocks were examined, and the experiments were carried out on three separate occasions.

Fresh sections of callus blocks from each experiment were also examined; they were stained with phloroglucin/HCl (JENSEN, 1962). Microscopical examination of these enabled an estimate to be made of the relative amounts of differentiation which had been induced by the various treatments. The tracheids in each nodule of differentiated tissue stained red and could clearly be seen when the section was examined at low magnification.

Results

IAA Production by Autolysing Tissues. The amounts of IAA in tissues which had been autolysing for various times at 37° C are shown in Fig. 1, for pea leaf tissue, yeast and rat liver. In all cases the amount of IAA increases with time. This production of IAA cannot be attributed to bacterial growth since all samples were incubated in the presence of toluene.

Induction of Differentiation in Phaseolus vulgaris Callus by Autolysing Tissues

1. Parthenocissus Crown Gall. Frozen and thawed crown gall tissue induced the differentiation of numerous groups of tracheids. They were sometimes surrounded by an organized cambial zone (Fig. 2) but no phloem was detectable when the tissue was stained with dilute aniline

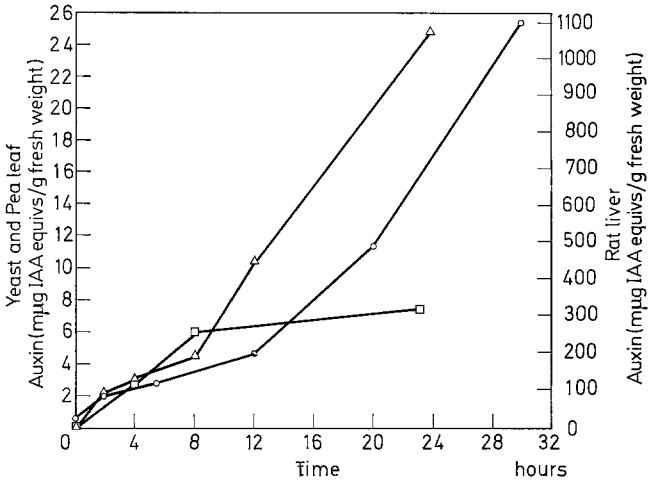


Fig. 1. Auxin production by pea leaf homogenate incubated at 37° C, □—□; Auxin production by autolysing yeast incubated at 37° C, ○—○; Auxin production by rat liver homogenate incubated at 37° C, △—△

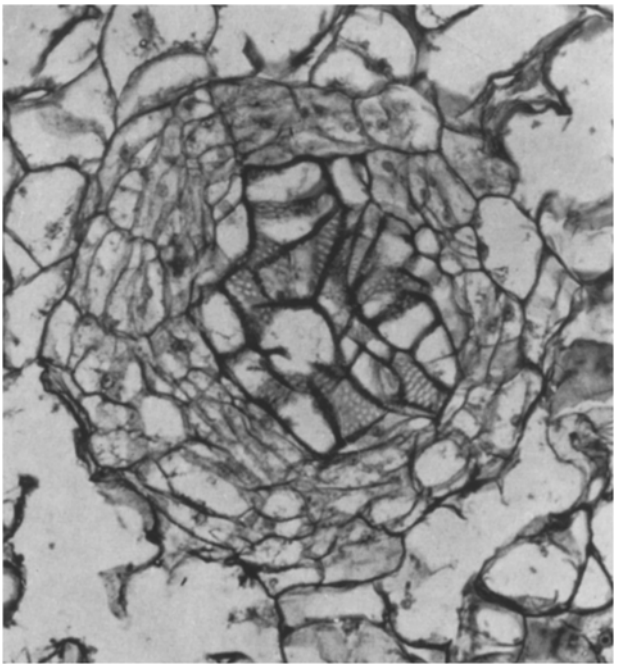


Fig. 2. Nodules of differentiation induced in *Phaseolus vulgaris* callus by frozen and thawed *Parthenocissus* crown gall tissue. Note the central group of reticulately thickened tracheids surrounded by a cambial zone. × 225

blue and viewed under the UV fluorescence system (Fig. 5). Boiled crown gall tissue also induced the differentiation of groups of tracheids in most of the callus blocks that were examined. However there were roughly one third to one quarter as many as when frozen and thawed tissue was used.

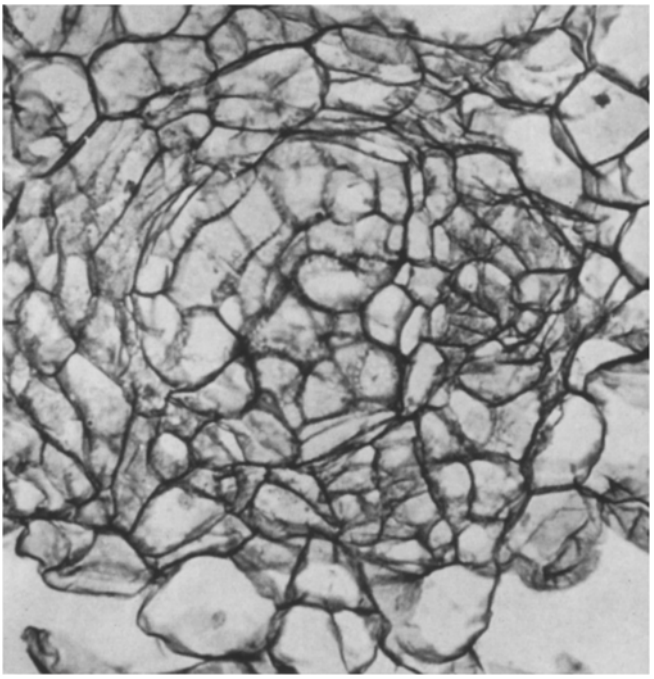
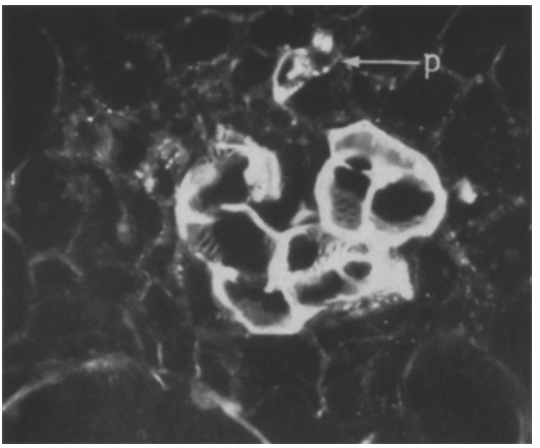


Fig. 3. Nodule of differentiation induced in *Phaseolus vulgaris* callus by frozen and thawed liver tissue. A group of tracheids surrounded by a cambial zone can be seen. × 284

2. Phaseolus Callus. A portion of the callus was removed from the callus mass used for the differentiation experiment, killed either by freezing and thawing or by boiling and then replaced in the callus block. With the frozen and thawed tissue a number of nodules of tracheids appeared, sometimes associated with phloem cells (Fig. 4). When boiled tissue was used, similar areas of differentiation appeared, but usually there were not so many as with frozen and thawed tissue.

3. Rat Liver. The rat liver was removed rapidly with sterile instruments and killed by freezing and thawing or boiling. When grafted into the callus, most of the samples remained sterile. A few were infected, and these were discarded. Frozen and thawed rat liver induced large numbers of nodular groups of tracheids often surrounded by a fairly tightly



Figs. 4—6. Differentiation in *Phaseolus vulgaris* callus stained with dilute aniline blue and viewed with ultraviolet light

Fig. 4. A nodule of differentiation induced by frozen and thawed *Phaseolus* callus. The group of tracheids fluoresces and so does the callose in the walls of a phloem cell (*p*). × 245

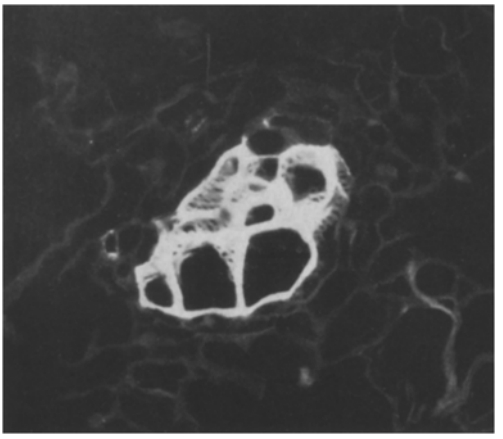


Fig. 5. A nodule of tracheids which fluoresce induced by frozen and thawed *Parthenocissus* crown gall tissue. No phloem can be seen. × 205

organised cambial zone (Fig. 3). No phloem could be seen by the fluorescence technique (Fig. 6). Examination of serial sections of the callus also revealed nodular areas of cells in division which did not have tracheids associated with them. The boiled tissue had little or no effect on about half the callus blocks examined, and in the others induced a few nodules containing tracheids.

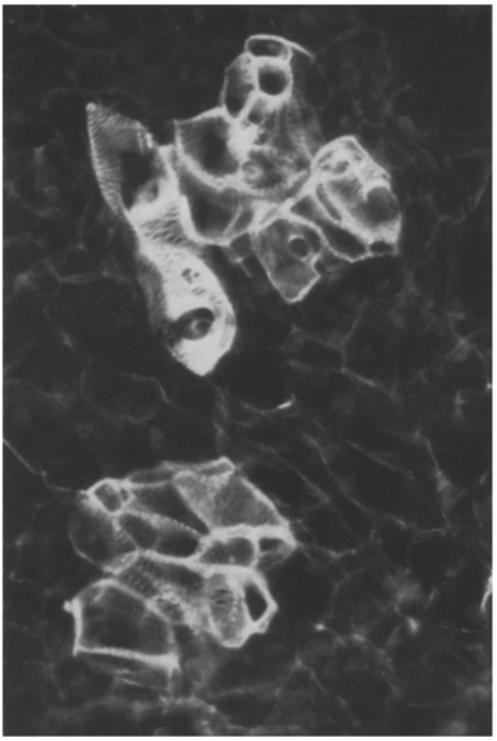


Fig. 6. Two nodules of tracheids induced by frozen and thawed rat liver tissue. No phloem can be seen. × 220

Discussion

The ability to produce indolyl acetic acid is not confined to plants. It is made by a wide variety of organisms, especially when tryptophan is supplied. Whatever its route of formation, there is little to suggest that any very specific enzyme systems are involved in animals, bacteria, fungi or higher plants. The wide-spread presence of enzymes which lead to the production of IAA from tryptophan means that the formation of IAA may be the result of an increase in the availability of tryptophan, such as might be expected when cells die and autolyse: any free compartmentalized tryptophan would be released, and it would presumably also be produced from proteins as they are hydrolysed. If any protein-bound IAA is present it too might be released by the same process. If we can accept the idea that IAA production in animals is relatively unspecific, then it does not seem implausible to suggest that it might be formed by a similarly unspecific mechanism in plants, especially as plants produce IAA if anything less efficiently than animals. Animal, yeast and other

plant tissues all produce IAA as they autolyse, and the processes involved may be very similar.

Differentiation can be induced in *Phaseolus vulgaris* callus by applied gradients of auxin (JEFFS and NORTHCOTE, 1966). Although no doubt many substances are released by autolysing tissues, the simplest explanation of the observed induction of differentiation by autolysing tissues is that they act as a source of auxin. That crown gall tissue should have this effect is not surprising: crown gall tissues are known to produce and contain auxin (KULESCHA, 1952). Some of the effects of killed *Phaseolus* callus could also be due to the release of the synthetic auxin, 2.4 dichlorophenoxy acetic acid stored in the cells in some form, since it had previously been grown on a medium containing this auxin. But in both these cases the role of enzymic activity in producing auxin is implied by the fact that tissues killed by freezing and thawing were more active in inducing differentiation than boiled tissues. The same is true of the rat liver tissue, but here the difference between boiled tissue, which had little or no effect, and unboiled autolysing tissue was much more pronounced.

Auxin may thus be a normal product of autolysing cells. Since the death of cells is a normal and important feature of plant growth and differentiation, dying cells may therefore be important sites of auxin formation in the plant. They could, indeed, account for most of the known sites of auxin production (SHELDRAKE and NORTHCOTE, 1968a, b).

The production of auxin by damaged, dying cells might also help to explain the wound response. Damaged cells provide substances which stimulate cell division (BLOCH, 1941), and apart from observations on the wound response following physical wounding, there are many others which also suggest that dying cells release such substances. In his studies on embryo development, NUTMAN (1939) observed that "the initiation of each new phase of development occurs by the degeneration of some previously formed tissue". In many virus diseases, cells adjacent to the necrotic area undergo hyperplasia and hypertrophy (ESAU, 1938). In cell aggregates in tissue cultures, cell division is found adjacent to areas of necrosis (BLAKELY and STEWARD, 1961). In groups of tobacco callus cells grown in microculture, growth takes place in surges; when some of the mature cells become senescent and die, some of the smaller cells are stimulated to divide and produce cells which enlarge and differentiate (JONES, HILDERBRANDT, RIKER and WU, 1960). The association between cell division and areas of necrosis has also been reported in genetic tumours (HAGEN, GUNCKEL and SPARROW, 1961) and in crown gall (BANFIELD, 1935). Since cell division, studied in tissue culture, has been found to be triggered off by a combination of auxins and kinins (SKOOG and MILLER, 1957) all these phenomena, which seem to be variations

on the wound response, might be explicable in terms of a release of auxins and also kinins from dying cells. Dying cells might produce kinins as the nucleic acids break down (SHELDRAKE and NORTHCOTE, 1968a). They may also liberate auxin, and there is evidence that auxin really is formed at wound surfaces (HEMBERG, 1943). The idea of auxin being a normal product of dying cells enables the wound response and most of the major sites of auxin production in the plant to be understood as different aspects of the same phenomenon.

The hypothesis that much of the auxin in the plant is formed as a consequence of cell death does not mean that auxin production by this mechanism would be haphazard and uncontrolled. Cells within the intact plant do not normally die at random: cell death is itself controlled. The amounts and availability of auxin produced by dying cells would be regulated by enzymes bringing about its destruction and by the auxin transport system.

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