STUDIES ON THE DEVELOPMENT OF THE GRAFT UNION IN

LYCopersicon Esculentum, Mill.

by

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DECLARATION.

I declare that this thesis was composed by myself and the work described therein to be my own.

Maria Kiedzybodzka.
This study was concerned with establishing the changes in pattern of protein synthesis associated with graft formation in *Lycopersicon esculentum* Mill., using a dual labelling technique.

The mechanical strength of the graft was used as a parameter of development. Mechanical strength was found to be related to both cell number and number of tracheary elements for breaking weights up to 130 g after which it was related to tracheary element number alone. The transition between two stages occurred 3-4 days after grafting.

The ability of a graft to form in isolation was investigated. It was found that a graft could develop in culture for at least twenty-four hours after excision without significant change in the pattern of development.

Two approaches were used in an attempt to produce a grafting situation in the absence of micro-organisms:

1. Surface sterilisation of the grafted internode.
2. Grafting of sterile plants.

Ungrafted internodes could be kept sterile with ease but grafted internodes always had considerably higher levels of contamination. Attempts to impose sterility were abandoned because it was essential to have comparable levels of contamination as protein synthesis in grafted and ungrafted tissue was being compared.

Comparison of the pattern of protein synthesis in grafted and ungrafted tissue, using the dual labelling technique, was made on soluble and insoluble protein fractions for 3 twenty-four hour periods, 2, 3 and 4 days after grafting. Many differences were found for both fractions during all time periods studied. Regeneration between two active surfaces (grafting) was compared to regeneration against an inert surface (wounding) using the same technique and certain proteins were seen to be synthesised preferentially as a result of grafting rather than wounding.

This technique was applied to putative plasma membrane fractions and changes in the pattern of protein synthesis were associated with fractions containing PaUP + vesicles.

Specificity of PaUP as a stain for plasma membrane was investigated, using a variety of species and modifications of the method, and found to be unreliable as the sole criterion for establishing
the identity of the plant plasma membrane. It was tentatively concluded that such P3CP + fractions must play a major role in the development of the graft.
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SECTION A:

INTRODUCTION.
INTRODUCTION.

Grafting, in a botanical context, is the process by which pieces of plants are joined together in such a manner that they will unite and continue their growth as a single individual. Graft formation occurs occasionally in nature where plants grow in very close proximity. Naturally occurring root grafts are commonly found in forests where the roots of trees interweave and may eventually fuse together. Vascular continuity can then be established between the two partners of the graft. Occasionally this occurs between branches of trees or between the stems of climbing plants. This property has been exploited as a horticultural practice for many centuries. Indeed grafting was a well established practice, described by Aristotle (384-322 BC). Since then grafting has become an important technique used to propagate plants which have desirable characteristics that cannot be transmitted unaltered through the seed (Summers, 1930), and which cannot be easily propagated with other techniques. Grafting can also be used to change the stature or form of a plant, (e.g. dwarfing) to adapt plants to wider climatic ranges, to prevent disease, to secure better root systems (e.g. rose roots which will not succour), to transmit virus variegation effects, and for many other purposes (Roberts, 1949). However, not all grafts are successful, there are many well documented cases where grafting fails to produce an integrated unit. The terms compatible and incompatible are used to denote partners which will or will not form a successful graft. Compatibility in the horticultural sense refers to a graft union which survives throughout the life of the plant and any breakdown of the graft prior to the death of the plant indicates that the combination was incompatible (Garner, 1970). Roberts, (1949) proposed that the term compatible should be applied to the long term success of a graft and that anything less should be referred to as incompatibility, partial incompatibility or delayed incompatibility. Therefore compatibility would be considered as any inter-influence between stock and scion rather than be restricted to effects arising directly from the union. This definition is useful in a horticultural context where the long term fate of the graft is important however, for studies of the mechanism of grafting this presents problems if the experiment must encompass the lifetime of the plant. The cellular events which accompany the formation of the graft
union are still not completely understood. One of the difficulties overcome by Roberts and Brown (1961) was the lack of a quantitative subsequently means of assessing the development of a graft. In 1961, Roberts and Brown devised an important new method to follow graft formation, which required measurement of the increase in mechanical strength of the graft union. The test was applied to a simple graft, made by cutting across an internode of a tomato seedling and rejoining the separated portions. The mechanical strength of the graft was measured by pulling apart the stock and scion and determining the force required to break the graft. A modified beam balance was used to measure this force. With the balance in equilibrium, the graft was used to anchor one balance arm to an immovable base. The force applied to the other balance arm was increased at a uniform rate by pumping water into a reservoir attached to this balance arm. When the weight of water on one side of the balance just exceeded the strength of the graft, the graft would break and the pump was stopped automatically. The balance was returned to equilibrium to determine the weight of water necessary to break the graft. The weight required to do this was equal to the breaking strain of the graft which was a measure of the mechanical strength of the graft. This weight is referred to as the breaking weight of the graft. Roberts and Brown, (1961) found that the force required to break the graft increased, for up to eleven days after grafting, until the graft was as strong as the surrounding tissue. This provided an easily manipulated experimental system in which the state of development of a graft can be assessed rapidly. The study of the development of the tomato autograft by this method provides an ideal experimental system for the study of graft formation. Subsequently, this technique was further developed and applied to both autografts and heterografts of various members of the Solanaceae (Yeoman and Brown, 1976). However, it is the events accompanying the formation of an autograft which must be fully understood before the development of heterografts can be considered.

The freshly cut surfaces of stock and scion are held together when the graft is constructed. Initially there is contact over the complete surface of both faces but shrinkage and collapse of cells occurs within a few hours. This shrinkage is not uniform and occurs to a greater extent in the peripheral region of the stem, in the cortex
and around the vascular bundles than in the pith. This results in partial separation of stock and scion and, for the first two days, cohesion between stock and scion is only in the region of the pith. At the same time cells in and around the vascular bundles are induced to divide and begin to fill the gap between stock and scion. Eventually cells from the two surfaces will meet and join and differentiation of vascular elements occurs rejoining the vascular system of stock and scion. It is the re-establishment of a continuous vascular system between stock and scion which characterises the successful development of a graft (Hartman and Kester, 1961). Lindsay et al., (1974) investigated the relationship between the increase in breaking weight of a graft and the anatomical events occurring at the same time. Both Roberts and Brown, (1961) and Lindsay et al., (1974) showed that the increase in breaking weight of the graft occurred in two stages. Initially the increase in breaking weight was slow and then increased rapidly during the later stages of development of the graft. Lindsay et al., (1974) also studied the relationship between both cell division and vascular differentiation and breaking weight, and suggested that the initial slow increase in breaking weight occurred whilst the cells of the two surfaces were dividing and that, the large increase in mechanical strength of the graft was due to the establishment of vascular continuity across the graft union. Yeoman and Brown, (1976) showed that in incompatible grafts the very large increases in mechanical strength did not occur and that vascular union was never achieved.

The establishment of vascular continuity across the union determines the success of a graft. This follows the initial cohesion between the stock and scion which occurs in all grafts whether compatible or incompatible. The initial cohesion of the grafted surfaces involves production of new cells which span the gap between the stock and scion in the periphery of the stem.

After these cells have come into contact, the vascular system of stock and scion are joined by differentiation of the intervening cells. The initial contact between the cells occurs before the development of the graft is arrested in incompatible cases. It is suggested that an interaction occurs at this stage between the cells of stock and scion which determines whether vascularisation will occur between the two graft partners.
This developmental stage, immediately prior to vascularisation, was studied in detail during this investigation. In particular the patterns of protein synthesis associated with this phase of development of the graft were investigated. The pattern of protein synthesis was investigated because this was a general parameter of development and the patterns of protein synthesised at different stages in the development of the graft could be compared with those of the ungrafted plant. This would distinguish between the pattern of protein synthesis which would occur in an undisturbed tomato internode and the pattern due to the many diverse events involved in the formation of the graft union. These would be associated with proliferation of new cells in the grafted region, redifferentiation into new vascular tissue and any events which are particular to the grafting process. Such events might arise from the interaction of cells of the stock and scion when they are brought together. Proteins have been implicated in cell interactions in other plant systems studied. An example of this is the pollen-stigma interaction (Heslop-Harrison, 1978) where proteins and glycoproteins have been implicated as factors controlling the success of pollination. However, before any investigation can be made as to whether proteins are involved in cell interactions during the development of the graft it is necessary to determine which proteins are synthesised during the grafting process and to see whether some of these are confined to a situation where interactions between two surfaces occur. This may be done by comparison with a "wounded" situation. This question cannot be resolved completely by investigation of the events accompanying autograft formation but these must be studied before any further investigations of more complex heterografts are attempted. This study will be confined to an investigation of tomato autograft formation.

Initially the investigation will confirm the findings of Roberts and Brown, (1961) and of Lindsay et al., (1974) and determine the timing of the transition between the two developmental stages observed by Lindsay et al., (1974), by investigation of the relationship between the mechanical strength of the graft and the increase in both number of cells and tracheary elements. The pattern of protein synthesis characteristic of the transition between the two stages of graft development was investigated during these stages. A dual labelling
These G2-related events could not correlate their development.

It is suggested that the study of reaction in formation in the culture is an added all of the developmental process, in the first phase, in the intact plant. It is not necessary to form a system for the study of G2-related reactions in formation in the intact plant.

In the study of a system for the study of G2-related reactions in formation in the intact plant, the possibility of a correlation between the G2 events by the G2 reaction, etc., the direct application of the comparison and to the G2 reaction, etc., this study more direct correlation between the two. The technique is therefore a direct correlation between the comparison of both the G2-related tissue and unrelated tissue as one sample and make the comparison of the reaction of the same reaction. It was possible to extract and enhance the expression from the related reactions. Because the reaction of the A2-related reactions are a response to G2-reaction, the G2-reaction is due to the G2-related reactions. As a result, the A2-related reactions are a comparison of the same G2-related reactions. In this situation, it is important to determine the same activity of both the G2-related tissue. In the same reaction, the reaction of the G2-related tissue can be determined. The expression, the reaction of the G2-related tissue due to each factor can be determined. The expression, the reaction of the G2-related tissue due to each factor can be determined.
The strategy used in this study was to remove grafts from the plant at different stages of development and to analyse their subsequent development. In this way at least the critical stages of graft development could be studied in isolation to determine whether there was significant divergence from the pattern of development of a graft in the "intact" plant. At the same time the role played by the rest of the plant was investigated. Attempts were made to increase the amount of development which occurred after excision of the grafted internode by supplying exogenous growth factors and also by increasing the size and complexity of the explant. An "isolated" grafting system was developed which would undergo the same pattern of development as a graft in an "intact" plant for at least twenty-four hours after excision. This provided a system in which the graft could be studied for twenty-four hours, after excision at various times after graft construction.

Another problem which must be recognised when studying the incorporation of radioactively labelled amino acids into protein is the possible contribution of the large numbers of bacteria and micro-fungi which are present on the surface of the plant. These may affect the results obtained in two ways. These organisms might directly affect the development of the graft but as grafting is always carried out in the presence of such micro-organisms, this is unlikely. However, it cannot be ruled out that micro-organisms which can incorporate the same amino acids into proteins as plants, might interfere with the patterns of synthesis obtained. The possibility of eliminating the presence of such contaminants during the development of the graft was investigated. Two approaches were assessed in this study. The first attempted only the sterilisation of the grafted region and maintenance of acceptable levels of contamination in this region throughout the period studied. The second attempted to produce totally "sterile" plants, to graft these and maintain them in a sterile condition throughout their development. However, if attempts to impose sterility on the tissue being studied resulted in dissimilar levels of contamination between grafted and ungrafted tissue then this was likely to affect the results. Clearly, if the levels of contamination in an unsterilised system were similar then it was less likely that any difference in pattern of synthesis found between grafted and ungrafted tissue was due to microbial contamination and more likely that it was due to the activity of the plant.
This series of experiments provided a system to which the dual labelling technique could be applied to study the pattern of protein synthesis associated with the critical stages of graft formation. Initially this technique was used to study the total pattern of protein synthesis associated with graft changes. The pattern was determined for soluble fractions comparing grafted and ungrafted tissue. The analysis was made for 3 twenty-four hour periods surrounding the critical stages of graft formation. Analysis of the pattern of protein synthesis was also made for a wounded situation. A graft cannot be constructed without wounding of the plant and so inducing a wound response. In an attempt to distinguish between the pattern of protein synthesis arising from the wound response, intrinsic in the grafting process, and that due to the interaction between stock and scion, a situation was devised in which the same wound was made but the stock was allowed to regenerate against an inert surface. The pattern of activity as a response to a "wound healing" was compared with that found as a response to graft formation.

If cell interaction plays a major role in determining the compatibility of a graft then such an interaction is most likely to occur at the surface of the cells of the union. Yeoman and Brown, (1970) suggested that such an interaction occurs at the point at which the cells touch and determines whether vascular continuity is achieved.

The plasmalemma and cell wall would be involved in such interactions so the pattern of synthesis of the proteins of the plasmalemma was of particular interest. The dual labelling technique was applied to putative plasma membrane fractions prepared using the best existing plasma membrane techniques available (Hodges et al., 1972). Identification of the plasma membrane was made using the periodic acid, chromic-phosphotungstic acid technique (PACP) of Littlefield and Bracker, (1972). This was the generally accepted method of identification available at the time.

The use of PACP technique as the sole criterion for identification of the plant plasma membrane was investigated. In particular its specificity as a stain for plasma membrane was investigated using tissue sections derived from the same source as the membrane fractions. The results of the application of the dual labelling technique to putative plasma membrane fractions were evaluated taking into account...
the results of investigation of the specificity of the PACP technique.

This study does not attempt to provide evidence for a protein based recognition system playing a major role in the development of a successful graft but attempts to provide a background of information concerning the development of the tomato autograft. The work can subsequently be extended to heterografts both compatible and incompatible and the situation found there can be analysed in context comparing the graft induced protein synthesis to that of the autograft.

This study attempts to provide a background of information concerning the development of autografts which may be subsequently applied to the study of heterografts. The patterns of protein synthesis during graft formation are studied. The study was confined to the transition between the two stages of development, the latter of which is characteristic of successful graft formation and is absent in incompatible grafts. Attempts were made to distinguish between the patterns of protein synthesis of an intact internode and a grafted internode and also to distinguish between "wounding" and grafting. The pattern of synthesis of proteins of a putative plasmalemma fraction was also investigated.
SECTION B:

MATERIALS AND METHODS
B.1. **MATERIALS**

Most of this investigation was confined to a study of an autograft situation using seedling tomato plants (*Lycopersicon esculentum* Mill. cultivar "Ailsa Craig"). The techniques employed have been described previously by Roberts and Brown, (1961) and Lindsay, (1972).

In addition to the experiments using tomato plants some preliminary studies were carried out to evaluate the use of a cucumber hypocotyl autograft. The material used was the germinated seedling of *Cucumis sativus* L. cultivar "Stockwood Ridge".
B.2. PROCEDURAL METHODS

B.2.1. Growth of material

a. Lycopersicon esculentum Mill.

The experimental material was obtained by raising the plants from seed using the following procedure. Seeds were evenly distributed over seed trays containing John Innes No. 1 compost (7 parts sterilised loam, 3 parts peat, 2 parts sand and 4 oz. John Innes base fertiliser and \(\frac{1}{4}\) oz. lime per bushel) and then thinly covered with the same. After watering the seeds were allowed to germinate in a greenhouse.

Conditions in the greenhouse were those of normal daylight intensities supplemented by mercury vapour lights to give a sixteen hour daylength irrespective of season. The temperature in the greenhouse was 16 to \(\pm\) 25°C and watering was carried out daily.

When the cotyledons were fully emerged and expanded the seedlings were transferred individually to 3" pots containing the same compost and returned to the greenhouse. When the plants were five weeks old they were transferred to growth room conditions. At this time the plants had between five and six emerged though not fully expanded leaves. The length of the primary internode was by this time sufficient (approx. 4 to 5 cm long) to allow easy manipulation. Although this procedure does not produce an absolutely uniform population it does enable, an extremely uniform group of plants for experimental purposes, to be selected from a larger population.

The growth rooms were maintained at a temperature of \(24^\circ C \pm 2^\circ C\) with a sixteen hour day length during which light was applied by warm white fluorescent tubes giving an intensity of 900 foot candles at plant level the humidity of the room was normally maintained at between 40 to 50%.

b. Cucumis sativus L.

Seeds were imbibed overnight in distilled water at \(4^\circ C\) and then sown in \(3\frac{\text{i}}{2}\)" pots containing moistened perlite. Four seeds were planted in each pot. The seeds were left to germinate under growth room conditions as described above. A mineral supplement of 100 ml of Hoagland's solution was applied to each pot after germination had occurred.
Table B.1. Composition of Hoagland's solution (Hoagland and Arnon, 1938)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>KNO₃</td>
<td>5.0 mM</td>
</tr>
<tr>
<td>Ca(NO₃)₂.4H₂O</td>
<td>5.0 mM</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>1.0 mM</td>
</tr>
<tr>
<td>MgSO₄.7H₂O</td>
<td>2.0 mM</td>
</tr>
<tr>
<td>E.D.T.A. Fe/Na salt</td>
<td>3.4 µM</td>
</tr>
<tr>
<td>H₃BO₃</td>
<td>45.0 µM</td>
</tr>
<tr>
<td>MnCl₂.4H₂O</td>
<td>9.0 µM</td>
</tr>
<tr>
<td>ZnSO₄.7H₂O</td>
<td>0.8 µM</td>
</tr>
<tr>
<td>CuSO₄.5H₂O</td>
<td>0.3 µM</td>
</tr>
<tr>
<td>H₂MoO₄</td>
<td>0.6 µM</td>
</tr>
</tbody>
</table>

The plants were left to grow, watered as required, until the cotyledons had fully emerged and expanded when they were considered large enough to be grafted (nine to twelve days after planting).
B.2.II. **Grafting of tomato plants**

The type of graft used throughout this investigation was an autograft in which the plant was cut in two, midway between the cotyledons and the first foliage leaf and then reassembled in its original situation. The primary internode was cut horizontally in two using a sharp razor blade. The plant was then reassembled with the vascular tissues of stock and scion aligned. This was simply accomplished by holding the plant up to the light. The scion was then held in place with a strip of silicone rubber (approx. 1.5 x 3.0 cm) which gave it a rigid, transparent and inert support. The silicone rubber strip was wrapped around the grafted region and secured between the jaws of a bulldog clip (3.0 cm long) supported by a glass rod (Fig. 1). The plants were watered after grafting.

The grafted plants were immediately returned to conditions of high humidity (90%) and left in the dark for twenty-four hours then kept under decreased light intensity (100 foot candles) for a further two days by placing them under the growth room bench. Subsequently the plants were restored to the original level (40 to 50%) forty-eight hours after grafting.

These procedures were used in an attempt to deter wilting. Although wilting did still occur, usually some time after the initial dark period had finished, it was not of a very serious nature and complete recovery occurred within the next two days. Plants treated according to this procedure invariably yielded successful grafts which were mechanically strong. Watering of grafted plants was carried out at intervals to maintain the soil in a damp but not waterlogged state.
Figure 1

Diagram to show grafting method
B.2.III. Grafting of cucumber plants

The construction of cucumber autografts was carried out using the same procedures as for tomato autografts with a few modifications. Because the epicotyl remained unexpanded in the experimental material the large hypocotyl of the cucumber seedling was used for grafting rather than the first internode of the tomato seedling. The graft was made midway between the perlite level in the pot and the cotyledons. Alignment of vascular tissue was facilitated because the hypocotyl is square in cross-section. The graft was supported as before with a silicone rubber strip but clamped with a smaller bulldog clip (2.0 cm long).

Plants were watered after grafting and immediately placed in conditions of high humidity (90%) with no light. After twenty-four hours the plants were returned to full light intensity (900 foot candles) and the humidity was allowed to return to normal levels over forty-eight hours, this being the time taken for the large amount of water, which accumulates under the high humidity conditions, to disappear. As the grafted seedlings are very sensitive to waterlogging they were kept on the bench top, which does not collect water, as this provides better drainage. If waterlogging is allowed to occur then the stock rots and the grafted seedlings are no longer suitable for experimental purposes. Watering of grafted plants was carried out at regular intervals to maintain the perlite in a damp but not waterlogged state. Otherwise conditions were the same as used for tomato autografts and yielded "successful" grafts in nearly all cases. The problem of rotting was never totally eliminated but by grafting large numbers of plants it was possible to obtain sufficient healthy grafts for experimental purposes.
B.2.IV. Sampling Procedures

a. "Grafted tissue"

For the purposes of this investigation "grafted tissue" was defined as the region 1 mm on either side of the graft. This region contains all the tissue formed during grafting and pre-existing surrounding cells which may also be affected by graft formation.

The 2 mm region to be sampled was always marked onto the plants at the beginning of the experiment using indian ink. Two small marks were made 2 mm apart midway between the cotyledons and the primary leaves and the graft was made between these two marks. Samples were taken by excising the region between these two marks at times determined by experimental protocol. Subsequent treatment varied depending on the type of investigation in progress and will be outlined at the appropriate place. This method of marking ensured that increases in volume of the tissue being studied did not interfere with the results obtained as the same region was always sampled even if it increased in size.

b. Ungrafted tissue

Ungrafted tissue was used as a control making it possible to assess whether the experimental observations made were peculiar to grafted tissue or merely a result of the normal activity of the tissue in question. Ungrafted tissue was removed from plants similar to those used for grafting with a 2 mm region marked as described above. These intact plants were then left to develop under the same conditions as the grafted plants. Control plants were given a silicone rubber support identical to that used with the grafted plants so that the results of constriction or pressure applied to the tissue by the support were not confused with grafting phenomena. Sampling procedures were identical to those used for grafted tissue.

Modification of the ungrafted tissue samples was necessary in some experiments to reduce the number of plants needed to manageable proportions. For these purposes tissue was taken from fewer internodes than used for the grafted sample and a greater amount of tissue was taken from the centre of each internode. A section not more than 10 mm long was cut out from the middle of the internode and cut up into thin slices (0.5 mm). Tissue from four internodes was pooled and the slices mixed up, then an amount of tissue was selected at random.
to equal the weight of the grafted sample. These procedures will be
described in more detail at the appropriate point in the experimental
section.

c. **Wounded tissue**

To distinguish changes in grafted tissue which are due to
wounding rather than grafting a further control was used in some
experiments. Wounded tissue was obtained by treating plants in the
same way as when grafting except that the plant was not reassembled
after cutting and the scion was replaced by a section of P.T.F.E. rod
of the same diameter and held in place in the same way as the scion in
normal grafts. 1 mm was then removed at the appropriate time in the
same way as used for grafted samples. This gives an indication of
what happens when tissue is wounded and allowed to regenerate with the
similar mechanical limitations and conditions of a normal graft.
B.2.V. Culture of excised grafted internodes

a. Excision of grafted internode

The graft containing internode was excised at time intervals determined by the experiment from plants as previously described. Unless otherwise specified the grafted internode was excised so that no axillary buds were left on the tissue (see Fig. 2). The internodes were excised using a sharp single edged razor blade. A horizontal cut was made below the primary node and above the axillary buds of the cotyledonary node. The internode was supported by its silicone rubber strip throughout the excision and transfer procedures so that mechanical disturbance of the graft was minimised. The bulldog clip was then removed freeing the graft from the supporting glass rod and a paper clip was used to secure the edges of the silicone rubber strip providing the graft with rigid support. Use of the support was maintained because it reduced disturbance of the graft during transfer. Also if the graft produces more callus in one part of the graft then the two faces of the graft can be pushed apart unless they are firmly held in contact across their entire width.

Care must be taken to ensure that the size of the rubber strip is not so large as to occlude a large part of the internode. This tends to cause the tissue to soften probably as a result of limiting the extent of water loss and gaseous exchange. At least 10 mm of both stock and scion should be exposed to the atmosphere.

The grafted internode was transferred to the incubation medium by holding the scion with curved forceps and pushing it into the agar medium a sufficient distance to support it in an upright position but not far enough to cause occlusion of the scion. These procedures cause a minimum of breakage or disturbance in transfer and the tissue is maintained in the same orientation as in the intact plant.

b. Culture medium

The culture medium consisted of a simple mineral salts solution as used for culture of artichoke explants (Yeoman and Davidson, 1971). No growth substances or organic compounds were added to the medium as these would encourage the growth of contaminants.
Diagram to show position of cuts for excision of grafted internode
The medium consists of:

<table>
<thead>
<tr>
<th></th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>KNO₃</td>
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</tr>
<tr>
<td>KCl</td>
<td>869.0 µM</td>
</tr>
<tr>
<td>MgSO₄·7H₂O</td>
<td>146.0 µM</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>88.0 µM</td>
</tr>
<tr>
<td>Ca(NO₃)₂·4H₂O</td>
<td>1.0 mM</td>
</tr>
<tr>
<td>FeCl₃·6H₂O</td>
<td>3.7 µM</td>
</tr>
</tbody>
</table>

Solution A

Solution B

Solution C

Solutions A, B and C were stored at 10X concentration at 4°C and 10 ml of each added to 1L of medium to give the above concentrations. 1% agar was added to solidify the medium. This also acts as a support for the tissue. The medium was then autoclaved at 121°C for 20 minutes before use.

c. Culture vessels and conditions

Excised internodes were cultured in glass bottles (2 oz. or 4 oz. Beatson’s depending on size of tissue) sealed with foil caps. These bottles are large enough to accommodate the internodes with ease and because of their wide necks do not interfere with introduction and removal of internodes. They are of a sufficient volume to maintain adequate levels of humidity and air. The bottles were always sterilised before use by autoclaving at 121°C for 20 minutes. They were then filled with 15 ml of sterilised agar and allowed to set. The agar was dispensed with sterile syringes. After excision and transfer of the grafted internode (or control tissue) to the culture vessel they were resealed with a double layer of sterile aluminium foil and returned to the growth room conditions used for grafted plants. These procedures were modified for some particular experiments but these modifications will be described at the appropriate place.
B.2.VI. Method for the application of growth substances to excised grafts

The growth substances used were either IAA or the synthetic auxin 2,4-D applied to the top cut surface of the excised internode as a lanolin paste. Different concentrations ranging from 0.01% to 10% (w/w) were used. These were obtained by dissolving the appropriate weight of growth substance in 1 ml absolute ethanol and this was added to the appropriate weight of molten lanolin. On addition to lanolin the ethanol boiled vigorously and stirring of the solution ensured even distribution of the auxin in the paste and also helped drive off all the ethanol. The lanolin was then allowed to solidify. Control treatments with no growth substances were made by application of a lanolin paste made by addition of 1 ml of absolute ethanol to the same amount of molten lanolin. The solidified paste was liberally applied to the cut surface of the internode with a wooden toothpick.
Initially radioactivity labelled amino acids were applied basally from the agar medium used for culturing the excised internodes but under these conditions the levels of incorporation were found to be too low to allow the examination of the pattern of incorporation into different proteins. It was thought that if the diffusion path was limited and the isotope applied from a solution then higher levels of incorporation would be obtained. So the agar was left out of the medium and the volume of medium used was reduced from 15 ml to 0.2 ml so that nearly all the medium was taken up by the internode during the labelling period. 0.2 ml was found to be a sufficient volume to supply the internode over a twenty-four hour period without drying out completely, maintaining availability of precursors over the period of study, but was a small enough volume to ensure that a substantial part of it was taken up into the internode. The medium was contained in small glass vials (9 mm in diameter, 20 mm in height). This also served as a support for the grafted internode and the silicone rubber strip could be dispensed with when transferring to these bottles. This glass vial was stuck down onto the bottom of the 2 oz. glass Beatson bottle previously described using araldite as this would withstand successive periods of autoclaving.

2 ml of water was put into the bottom of the larger bottle to raise the humidity within the bottle to prevent drying out of the tissue and evaporation of the radioactive solution. The top of the container was sealed with aluminium foil.

The incubation vessels were sterilised by autoclaving prior to use and the appropriate radioactive solution was applied immediately before transfer of the grafted internode.

At intervals internodes were excised as before but the silicone rubber strip was removed at this point and the internodes transferred to the small glass vials using curved forceps which supported the graft and minimised disturbance during transfer. The internodes were then returned to the growth room for twenty-four hours after which they harvested.

The labelling medium contained either 5 μCi of $^{35}$S-methionine or 100 μCi of $^3$H-methionine were supplied by the Radiochemical Centre, Amersham, at the following specific activities and concentrations.
$^{35}$S-methionine

Specific activity - 150 Ci/mmol and an initial radioactive concentration of 4.0 mCi/ml from which one has to calculate the activity on the day of usage allowing for the 81 day half life of $^{35}$S. This was done using the decay tables provided by Amersham.

$^{3}$H-methionine supplied as L-$[2(n)^{3}$H] methionine specific activity 1.0 Ci/mmol with radioactive concentration of 1.0 mCi/ml.

Isotopes supplied at these activities and concentrations yielded comparable incorporation levels into protein. Higher activities of $^{3}$H-methionine are supplied as lower incorporation is found which is probably due to the difference in specific activity. This ensures that the ratio of $^{3}$H/$^{35}$S activity in an identical situation will be approximately 1.0.
B.2.VIII. Sterilisation procedures

a. Sterilisation of equipment

All glassware used in experiments requiring aseptic conditions was sterilised by autoclaving at 121°C for 20 minutes. Containers were sealed prior to sterilisation with either cotton wool stoppers covered with foil or with foil alone. Test tubes were closed with aluminium caps (Oxoid cap-o-test). Glass-teflon homogenisers were sterilised, after wrapping the separate components with foil, by autoclaving as above. Where it was necessary to use the homogenisers for more than one sample they were resterilised by washing with absolute alcohol and then rinsed repeatedly with sterile distilled water to remove any traces of alcohol before a fresh sample was introduced. These were dried by inverting over sterile tissues. This was sufficient treatment to prevent significant cross-contamination as no evidence of this could be seen when sterile water was introduced and then assayed in the same way as samples of tissue. The pestle was resterilised where necessary by flaming with ethanol.

All instruments and paper tissues were wrapped in foil and sterilised in a hot air oven at 150°C for 5 hours. The instruments were flamed with ethanol in between each manipulation according to conventional "sterile techniques". Razor blades were individually wrapped in foil and sterilised in an oven as above and either used once or flamed with ethanol before re-use. Where possible fresh razor blades were used for each individual manipulation but if the number of samples made this impossible a fresh one was used for different samples to eliminate any cross-contamination between samples.

All serial dilutions were performed using sterile disposable pipettes (Sterilin) and sterile disposable petri dishes (90 mm in diameter) were used for storing and cutting of samples, also for incubation of contaminants or germination of seeds. These disposable items are supplied sealed in bags and have been sterilised by irradiation. All manipulations in experiments requiring asepsis were carried out inside a "sterile room" which is maintained at positive pressure continually and is irradiated with ultra violet light for a minimum of 30 minutes before use. The air supply to the room is filtered to exclude contaminants. Standard aseptic manipulation procedures were always employed to minimise contamination.
b. Application of liquid sterilising agents to tomato internodes

A variety of sterilising agents were tested in this situation and these will be discussed later but the same general method of application was always used and this will be outlined here. The time of application varied and will be described in the appropriate place. In order to apply a liquid over a fairly long period of time to only one part of the plant the primary internode was wrapped in absorbent tissue and the sterilising solution was applied to this with a syringe until the tissue was saturated (approximately 0.3 ml). The internodes were then left to allow the sterilising agent time to act. Times varying, depending on experiment. Then the tissue was removed and the internode covered with sterile silicone rubber in the same manner as for grafting. No attempt was made to remove the sterilising agent. In some cases the plants were grafted at this stage whilst in others sterilisation was attempted after grafting. More details of these procedures will be given in Section C.3.

c. Sterile grafting method

All equipment used for grafting (bulldog clips, silicone rubber strips, razor blades, glass rods, etc.) were sterilised either by autoclaving or in a hot air oven as detailed previously. Standard precautions for maintaining sterility were observed throughout all grafting procedures. The grafting technique employed was similar to the non-sterile method. It was necessary to cut and rejoin to stock and scion manually using sterile forceps or gloved hands. Wherever possible handling of surfaces to be sterilised was avoided. For example, if only the internode to be grafted was required to be sterile then the plant would be held by the non-sterile parts whilst it was cut and rejoined. The silicone rubber strip was then wrapped around the internode ensuring that the inside of the strip was never handled. When sterile plants were used these were handled in regions as far away from the graft as possible. To avoid contamination of the plants at this stage plastic gloves were worn at all times and were repeatedly sterilised by immersion in ethanol. All operations were carried out in a sterile room.

d. Sterilisation of growth chambers

Commercially available propagators were used as growth chambers
for the raising of sterile cucumber seedlings. These consisted of a tray (420 x 220 x 80 mm) with a tall clear plastic cover (420 x 220 x 140 mm) which could be sealed together. No provision was made for an outside supply of air or water and the ventilation holes were permanently sealed. For the duration of the experiments sufficient air and moisture were present for the maintenance of the cucumber seedlings. The propagators were sterilised by washing out the inside of the boxes with 95% ethanol and the boxes were left open to allow the ethanol to evaporate under ultraviolet light in a sterile room. The boxes were sealed after one hour and only opened momentarily for the transfer of plants. As the chambers were sealed the plants were maintained in conditions of high humidity so no external supply of water was necessary.

e. Growth media

1. For the detection of contaminants

This was prepared by mixing 13 g of nutrient broth (Oxoid C21) with 1L of distilled water and 10g of agar. This mixture was then autoclaved at 121°C for 20 minutes and poured into 90 mm disposable sterile petri dishes under sterile conditions.

Oxide nutrient broth consists of:

- "Lab-Lemco" Powder (Oxoid L29) 1.0 g. l⁻¹
- Yeast Extract (Oxoid L20) 2.0 g. l⁻¹
- Peptone (Oxoid L37) 5.0 g. l⁻¹
- Sodium chloride 5.0 g. l⁻¹
- pH 7.4

This is a general purpose nutrient medium suitable for the growth of most micro-organisms. Some micro-organisms do have special growth requirements but for the purposes of this investigation it was considered adequate to estimate the number of micro-organisms growing on this medium as an indication of the sterility of the tissue.

2. Medium for germination and screening of cucumber seeds

A growth medium was required which would not interfere with the germination of the cucumber seeds and would enhance the growth of microbial contaminants. This would enable the elimination of any
contaminated seeds early in procedure before cross-contamination could occur. 

Cucumber seed medium consists of:

- Normal strength Hoagland's solution (Section B.2.I.)
- Sucrose 2%
- Agar 1%

This was sterilised by autoclaving at 121°C for 20 minutes and poured into 90 mm petri dishes as with nutrient agar. Addition of 2% sucrose to the medium was sufficient to allow rapid proliferation of contaminants so that after 4 days all contaminated seeds could be discarded.

3. **Medium for growth of sterile cucumber seedlings**

Square plastic pots ($3_{2}''$) were filled with perlite and a mineral supplement of 100 ml of standard Hoagland's solution was added to each pot. The perlite was then moistened and the pots sealed individually with foil and autoclaved for 30 minutes at 121°C (15 lb/sq. in.). This was the time taken for autoclave tape in the centre of such a pot to blacken. This was further checked by plating samples of the perlite onto nutrient agar plates and incubating under standard conditions. No colonies were observed under these conditions so that the growth medium and its container were considered to be sterile. The pots were individually opened only immediately prior to planting of germianted, sterile seeds.

f. **Sterilisation and growth of cucumber seeds**

Unimbibed seeds were immersed in absolute ethanol for two minutes, rinsed in sterile distilled water, submerged in a 2% solution (w/v) of sodium hypochlorite for 30 minutes (Yeoman, 1973) then after repeated washing in sterile distilled water the seeds were allowed to imbibe overnight in a sealed sterile container at 4°C. The imbibed seeds were then screened for contamination by allowing them to germinate on a sterile growth medium (Cucumber seed medium) to encourage the growth of bacteria and fungi. Six seeds were placed on each petri dish (to minimise cross-contamination) and standard sterile precautions were maintained throughout. The seeds were allowed to germinate in the
light at 25°C for 4 days. This time was considered ample for any contaminating organisms to grow and produce easily detectable colonies. In this time unsterilised seeds produced ample evidence of contamination. Uncontaminated seedlings were then selected and planted into sterile pots using flamed forceps and sealed into propagating chambers. These were then transferred to the growth room and the plants were left undisturbed until needed for experimental manipulation.
B.2.IX. Fixation and embedding procedures for electron microscopy

All material for electron microscopy was processed in the same way unless specified otherwise in the experimental section. Fixation was carried out overnight in 3% gluteraldehyde in 0.1 M sodium cacodylate buffer pH 7.1 at 4°C. All traces of gluteraldehyde were then removed by repeated washing in 0.1 M sodium cacodylate buffer pH 7.1. The tissue was then subjected to post-fixation in 1% OsO₄ for two hours. (This step was sometimes omitted.) After further washing in cacodylate buffer the tissue was dehydrated by taking it through an alcohol series. When completely dehydrated it was transferred to propylene oxide and after several changes to a mixture of propylene oxide and araldite. This was to ensure complete penetration of the araldite. The tissue was left overnight in a mixture of propylene oxide: araldite 1:3 and finally transferred to pure araldite which was allowed to polymerise. The tissue was now embedded in a solid araldite block and could be sectioned using a glass knife and L.K.B. ultratome as required.
B.3. ANALYTICAL METHODS

B.3.I. Determination of breaking weight

The strength of a graft was measured by a determination of the weight required to break it using an apparatus similar to that described by Lindsay et al., 1974. (See Fig. 3.) The apparatus consists of a beam balance at one end of which there is a tank into which water is pumped at a constant rate from a reservoir. The other end of the balance bears a crocodile clip into which the top of the graft is introduced whilst the bottom of it is held by a second clip secured to an immovable base. Thus as the amount of water on one side increases, the force on the graft increases until it exceeds its mechanical strength and the graft breaks. As the graft breaks the swing of the balance beam activates a mercury switch which cuts the power to the water pump. The weight needed to return the balance to equilibrium represents the breaking weight of the graft. This does not give the actual weight required to break the graft because the weight is not applied in one instant but the weight applied increases steadily with time. The value will in fact be proportional to the actual breaking strain and as long as the same conditions are used in all determinations then a relative measure of the strength of the graft is obtained which is comparable for different samples.

The grafted internode was cut from the plants to be sampled and held by the original silicone rubber supporting strip. This ensures minimum disturbance of the graft. The balance was brought to equilibrium and the graft introduced into the top clip and the silicone rubber strip released. The stock was then lowered gently into the bottom clip until the beam was horizontal. At this point the pump was switched on manually and was subsequently switched off automatically. In some cases the graft could not be broken by the machine because a big enough force could not be applied (the reservoir being full) or the tissue pulled out of the clips, or the break did not occur at the union. In these cases the breaking weight value obtained was recorded with a + indicating that the true value was in excess of that measured. This usually only occurred in the final stages of graft development and did not interfere unduly with the results obtained. Measurements were always made on a minimum of six replicates and in some cases ten.
Figure 3

Diagram of breaking weight apparatus

a. Balance pan
b. Beam
c. Graft under test
d. Clip
e. Fulcrum
f. Pump
g. Feed tube
h. Load
i. Reservoir
j. Mercury switch
b.3.II. Estimation of the number of cells and tracheary elements

The number of cells and/or tracheary elements in the graft was estimated using the method of Brown and Kickless, (1949) with a modification to increase the efficiency of maceration. The method relies on the maceration of the tissue into single cell units or aggregates of a few cells in a known volume of macerating medium. Samples of this were introduced onto a haemocytometer and the cells contained above the grid counted using a microscope and tally counter. The number of cells in the tissue were then calculated using the following formula.

\[
\text{Number of cells in tissue} = \frac{\text{No. of cells counted} \times \text{Volume of macerate} \times \text{Dilution factor}}{\text{Volume of haemocytometer grid (3.2 µl)}}
\]

Haemocytometers with a counting volume of 3.2 µl were used. The frequency of tracheary elements was estimated at the same time as total cell number.

The premarked 2mm internodal segments were cut from the grafted internodes (or equivalent) after the breaking weight had been determined. These were softened in N HCl at 60°C for ten minutes, the hydrochloric acid removed, 2.0 ml of 5% chromium trioxide in water added and the samples were left at room temperature (ca 22°C) for two days. Maceration was completed by drawing the sample in and out of a Pasteur pipette until a uniform suspension was obtained. Samples of this were drawn into the counting chamber from a capillary tube. For each sample six grids were counted and the mean of these was taken as the value for the sample and a minimum of three replicate grafts was used for each determination. This treatment gave a more uniform suspension with the tomato internode tissue than chromic acid alone but it was never possible to eliminate all clumps of cells. More rigorous maceration tended to cause considerable amounts of cell damage making it difficult to obtain accurate estimates of cell number. The treatment chosen was such that the size of the clumps was as small as possible without increasing the amount of cell damage significantly. This presents a reasonable compromise and makes it possible to examine at least the substantial changes that occur.
Tracheary elements were taken to be any cells which show signs of lignification. This can be easily seen due to the difference in refractance of the cell wall.
B.3.III. Estimation of microbial contamination in experimental tissue

a. Tomato

The tissue sample was equivalent to that used in labelling experiments so that an assessment could be made of whether or not there was an appreciable contribution by microbes to the observed incorporation patterns.

In order to obtain a measure of the level of microbial contamination present in any sample ten 2 mm segments were cut using sterile razor blades, observing standard aseptic handling procedures. These were homogenised in a sterile glass teflon homogeniser with 1.0 ml sterile water. The viable count was determined for these samples using the standard microbiological serial dilution technique maintaining strict asepsis throughout. Four ten-fold dilutions were used and 0.1 ml triplicate samples of each dilution were then plated onto nutrient agar plates (see section B.2.VIII.e.) and incubated for two days at 37°C.

The number of colonies per plate were counted and the number of viable contaminants in each of the original samples was calculated using the following formula.

\[
\text{No. of viable contaminants} = \frac{\text{No. of colonies counted} \times \text{dilution factor} \times \text{volume of homogenate}}{\text{volume plated out}}
\]

All instruments and media used were sterilised by autoclaving at 121°C for fifteen minutes and flamed in ethanol before use. All manipulations were carried out in a sterile transfer room. This gives an estimation of the number of viable contaminants which might interfere with the incorporation patterns.

b. Cucumber

Initial sampling was made on a non-quantitative basis as it was not possible to sample the hypocotyl of the ungerminated seed. The purpose of these samples was to eliminate any seeds with contaminants that had survived the sterilisation procedure. In these cases whole seeds were simply transferred to a medium containing sucrose and mineral salts (see section B.2.VIII.e.) and incubated for two days at 25°C. The number of contaminated seedlings in each sample was recorded.
Later sampling was carried out on a quantitative basis by taking 2 mm lengths from the hypocotyl. The hypocotyls were cut aseptically using an "artichoke explant cutter" (see Fig. 4) which gives uniform cylinders 2 mm long. Segments containing the graft or a corresponding region in ungrafted samples were then homogenised and the number of contaminants in the sample determined by the method used for tomato.
Figure 4.

Diagram of the artichoke explant cutter

Razor blades set 2mm apart

grooves to hold cucumber hypocotyl

hinge
B.3.1v. Extraction of protein from experimental material

A sample of at least ten 2 mm segments including the graft union was used for each protein extraction. These were obtained as described in Section B.2.4., and frozen in aluminium foil packets on dry ice. The graft which had been incubated with radioactively labelled methionine, was weighed and an equal weight of ungrafted tissue, which had been incubated in methionine labelled with the other isotope, was added and the tissue pooled.

The same procedure was carried out on wounded controls so that protein was extracted from a one to one (w:w) ratio of grafted and ungrafted tissue at different times and of a one to one (w:w) ratio of wounded and ungrafted tissue.

The combined tissue was homogenised using an ice-cold pestle and mortar with 0.5 ml of extraction buffer for every gram of tissue. Extraction buffer was composed of:

- Sucrose: 10% (w:v)
- β-mercaptoethanol: 1% (v/v)
- Tris: 50.0 mM
- pH: 7.5

The homogenate was then centrifuged at 10,000 g for 10 minutes to remove whole cells, nuclei, mitochondria, chloroplasts as well as some endoplasmic reticulum, cell wall fragments and large fragments of plasmalemma. The supernatant contained ribosomes, lysosomes and fragments of nuclear, mitochondrial, chloroplast and plasma membrane, and tonoplast as well as all the soluble proteins. The pellet contained a large amount of membrane bound protein so in order to release this for analysis it was solubilised with 2% sodium dodecyl sulphate (SDS).

Further extraction of the pellet was carried out after washing the pellet three times with a large volume of extraction buffer. Then the pellet was resuspended in extraction buffer containing 2% sodium dodecyl sulphate (0.5 ml per g of original tissue). This was heated at 95°C for five minutes to render soluble all membrane bound complexes (this will also dissociate proteins to their basic sub-unit structure). After cooling this was centrifuged 10,000 x g for 10 minutes at room
temperature because S.D.S. will precipitate out at low temperatures. This yielded a pellet which was discarded and a supernatant which contained the solubilised proteins.

This means that two fractions were obtained, the 10,000 x g supernatant which is the soluble fraction, and the 10,000 x g pellet the insoluble fraction.

These fractions were either stored in a deep freeze or analysed immediately.
Electrophoretic separation of proteins

The patterns of incorporation of radioactive amino acids into proteins were compared after polyacrylamide-SLS gel electrophoresis of the appropriate samples carried out by a modification of the method described by Laemmli, (1970). Polyacrylamide gels were produced by polymerisation of acrylamide (the monomer) and a cross linking agent Bis-acrylamide (N,N'-methylene-bis-acrylamide). The polymerisation was initiated by the presence of free radicals produced by natural light in the presence of ammonium persulphate. TEMED (N,N',N'-tetramethyl ethylenediamine) was added to improve gelation. Sodium dodecyl sulphate was employed as it forms complexes with the proteins, surrounding the protein molecule so eliminating charge difference of the protein, so that the separation is on a basis of molecular weight. Addition of SLS also prevents aggregation during electrophoresis because of its capacity to split hydrogen and hydrophobic bonds. A discontinuous buffer system in two phases was used (Ornstein, 1964; Davis, 1964; Stewart and Barber, 1964). The initial stacking gel utilised a pH 6.8 buffer whilst the separating gel utilised a pH 8.8 buffer. The stacking gel has large pores and enables the sample to be concentrated, after the components have contracted to small zones they enter a small pore separating gel which holds back the components according to their size and allows the formation of discrete bands after a suitable time interval. In all cases 10 cm slab gels were used as these enabled ten to fourteen samples to be analysed simultaneously under exactly the same conditions as they are all running through the same gel and electrical field.

Constituents of the electrode buffer

- Tris (hydroxymethyl) aminomethane: 0.05 M
- Glycine: 0.38 M
- Sodium dodecyl sulphate: 0.1%
Constituents of sample buffer

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Stacking gel consists of:

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<td>Bis-acrylamide</td>
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<tr>
<td>TEMED</td>
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<tr>
<td>pH</td>
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Separating gel consists of:

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</tr>
<tr>
<td>(10.00%)</td>
<td></td>
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<tr>
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</tr>
<tr>
<td>Sodium dodecyl sulphate</td>
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</tr>
<tr>
<td>TEMED</td>
<td>~ 0.06%</td>
</tr>
<tr>
<td>Ammonium persulphate</td>
<td>0.05%</td>
</tr>
</tbody>
</table>

The two gel mixes were made up from the appropriate proportions of the following stock solutions:

- Acrylamide stock: 30% Acrylamide, 0.2% Bisacrylamide
- Separating gel buffer stock: 1.875 M Tris, pH 8.8
- Stacking gel buffer stock: 0.600 M Tris, pH 6.8
- SDS-stock: 10% SDS
The plates are clamped in the indicated positions after making sure that the upright spaces are in good contact with the lower.

The vacuum grease can be seen as a continuous film across all the spaces on both sides if the seal is good.

b.

Introduce stacking gel mix. to give wells for introduction of sample.
The slab gels were prepared by fixing two glass plates together separated by a 1 mm space maintained by perspex strips along the edges. All surfaces were coated with vacuum grease except those in contact with the space into which the gel was to be poured. The plates were clamped together using large bulldog clips (see Fig. 5) which allows the separating gel solution to be introduced and prevents its leakage during the polymerisation process. It is important that the grease is not allowed to come into contact with the gel as it will interfere with the polymerisation and may cause inconsistencies in the gel.

After clamping the glass plates together the assembly was balanced upright on the bulldog clips. A plain perspex former was lowered 2 cm into the slot and clamped into position. This allows room for the stacking gel to be poured on top and also ensures a straight edge for the interface between the gels. The former was slightly narrower than the 14.5 cm slot into the glass plates so that a Pasteur pipette could be introduced into the space to allow the gel mix to be poured into the space provided.

At this stage the separating gel mix was made up. All the ingredients except the SDS and the TEII were added and the solution degassed to exclude any oxygen dissolved in the stock solutions which might inhibit polymerisation. The SDS and the TEII were then added, the solution mixed by vigorous shaking and immediately introduced between the glass plates. This must be done carefully so that no bubbles are introduced as these will affect the subsequent running of the gel. The assembly was left undisturbed until the polymerisation process was complete, usually 20-30 minutes at 20°C. The former was then removed and the top of the gel washed with distilled water. A tooth shaped former was then introduced (see Fig. 5.b.) and clamped in position with the lower edge at least 5 mm away from the top of the separating gel. This is to give the samples enough room to concentrate as they run into the stacking gel. The stacking gel mix was then made up and poured onto the top of the separating gel and up around the teeth of the former. This was allowed to polymerise after which the comb was removed and the wells were filled with electrode buffer.

Both top and bottom reservoirs of the gel tank were then filled with electrode buffer, the bottom former removed and any remaining vacuum grease wiped off using filter paper and electrode buffer. The gel was lowered into the bottom tank making sure that no bubbles were
trapped at the bottom of the gel. The gel was then clamped to the back plate of the tank which connects the top and bottom reservoir making sure the cut away top is facing the top tank. This was sealed below the level of the top tank with a line of vacuum grease so that there was no leakage of buffer between the two tanks and so that electrical continuity between the two reservoirs was solely through the gel. The top tank was then filled until the level of buffer was up to the top of the two gel plates and the top of the gel was below the surface of the buffer in the top reservoir.

Between 50 and 100 μl of sample was now introduced into each well using a Hamilton syringe. The 10% sucrose in the sample buffer makes it sink to the bottom on the well. Tracker dye (Bromophenol blue) was added to the end wells. This runs very close to the solvent front and allows one to see when this has reached the end of the gel to ensure that none of the proteins run off the end of the gel. An electrical potential was then applied across the two tanks and the current was maintained at a constant 17 mA whilst the voltage increased from an initial 40 V to 120 V in a typical run. The run is complete in about 4½ hours. When the run has been completed the gel was removed from between the plates and stained with a mixture of 1% Coomassie Brilliant Blue in 50% methanol and 5% acetic acid for 1 hour then destained in 50% methanol and 5% acetic acid. The pattern of protein sub-units can now be seen as blue bands against a clear background.
B.3.VI. Analysis of radioactive incorporation patterns

After staining the gels were cut into strips (each strip contained the pattern for one sample) usually derived from grafted and control tissue labelled with different isotopes but extracted and run as one sample. The strip was then frozen with dry ice and cut into 1 mm slices using a Joyce-Loebel automatic gel slicing machine. Each slice was put into a scintillation vial and solubilised in 30% H$_2$O$_2$ (0.6 ml) at 80°C for 3 hours. After cooling 5 ml of scintillation fluid was added.

The scintillation fluid was Toluene based with butyl PBD as the scintillant and Triton to emulsify any water produced by the decomposition of the H$_2$O$_2$. The scintillant fluid had the following composition:

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Toluene</td>
<td>1000 ml</td>
</tr>
<tr>
<td>Triton-X-100</td>
<td>500 ml</td>
</tr>
<tr>
<td>Butyl PBD</td>
<td>6.1 g</td>
</tr>
</tbody>
</table>

The radioactivity of each slice was then determined using an Intertechnique Scintillation counter with dual channel counting facilities which enable the activity of two different isotopes to be separated on the basis of the difference in energy emission. Each isotope produces a particular pulse amplitude spectrum in a scintillation counter. As long as these spectra have a reasonable separation then by setting different upper and lower counting limits on different counting windows one can simultaneously assay the activity of two isotopes in one solution. $^{35}$S and $^3$H labelled compounds were used as the $\beta$ particles emitted by $^3$H have a much lower and narrower spectrum of energies than the $^{35}$S so that these can be separated by setting counting limits as illustrated in Fig. 5. So although this is not a measure of the total activity of either isotope the values obtained will be proportional to the total activity and will thus be comparable. The diagram also shows that some of the counts in A are in fact due to B so that it is necessary to allow for this by calculating the percentage spillover between channels, between the two channels after which the ratio of counts due to $^{35}$S and $^3$H can be obtained. The $^3$H/$^{14}$C channels on the scintillation counter were used because the emission spectra of $^{14}$C and $^{35}$S are similar enough not to make a
Figure 6

Diagram to show how the Liquid Scintillation Spectrometer distinguishes between different isotopes by setting of appropriate counting limits.

No. of pulses per minute per unit pulse amplitude interval

--- Total spectra

--- spectrum due to isotope A.

--- spectrum due to isotope B.

$L_1 \rightarrow U_1$ counting channel for A.

$L_2 \rightarrow U_2$ counting channel for B.

Pulse amplitude
significant difference to the counting efficiency. The percentage spillover between channels was calculated by vials containing only $^3$H or $^{35}$S on both channels and finding the percentage of the $^3$H counts which register on the $^{35}$S channel and the percentage of the $^{35}$S counts which register on the $^3$H channel. The spillover of $^3$H counts into the $^{35}$S channel was found to be 0.4% which is insignificant and can be ignored. However, the spillover of $^{35}$S counts into the $^3$H channel was 2.8% and this must be taken into account when calculating ratios. The 2.8% spillover of $^{35}$S counts into the $^3$H channel means that 2.8% of the counts due to $^{35}$S (i.e. counts registered on $^{35}$S channel) also register on the $^3$H channel and must be subtracted from the counts registered on the $^3$H channel to find the counts due to $^3$H alone.

The ratio of counts due to $^3$H and $^{35}$S can be calculated according to the following formula.

$$\frac{C_{^3H}}{C_{^{35}S}} = \frac{(cpm_{^3H} - bg_{^3H}) - (cpm_{^{35}S} - bg_{^{35}S})(\% \text{ spillover of } cpm_{^{35}S} \text{ into } ^3H \text{ channel})}{(cpm_{^{35}S} - bg_{^{35}S})}$$

$cpm_{^3H}$ = counts per minute registered on $^3$H channel

$cpm_{^{35}S}$ = counts per minute registered on $^{35}$S channel

$bg_{^3H}$ = background on $^3$H channel

$bg_{^{35}S}$ = background on $^{35}$S

$c_{^3H}$ = counts due to $^3$H

$c_{^{35}S}$ = counts due to $^{35}$S
B.3.VII Method of the fractionation of membranous material including the plasmalemma

The supernatant obtained by centrifugation as described in Section B.3.IV, and referred to as the soluble fraction can be further separated into different fractions using the method of Hodges et al., (1972) which is outlined below. The tissue was homogenised using an ice-cold pestle and mortar and 4 volumes (v/w) of homogenising medium was added. (All further operations were carried out at 4°C or on ice.) The homogenising medium consists of:

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sucrose</td>
<td>0.25 l</td>
</tr>
<tr>
<td>EDTA</td>
<td>3 ml</td>
</tr>
<tr>
<td>Tris-MES</td>
<td>25 ml</td>
</tr>
<tr>
<td>pH</td>
<td>7.2</td>
</tr>
</tbody>
</table>

(EDTA = ethylene diamine tetra acetic - this is added to prevent aggregation of membranes.)

(MES = morpholino ethane sulphonylic acid.)

The homogenate was then centrifuged at 13,000 g for 15 minutes to give a pellet containing unbroken cells, cell walls and large pieces of membranous material, which is discarded. The supernatant was centrifuged at 80,000 g for 30 minutes yielding a supernatant containing soluble protein and a pellet containing the membranous material. This was resuspended in the homogenising medium and resedimented under the same conditions twice more to remove any soluble protein from the pellet. Finally the pellet was resuspended in up to 5 ml gradient buffer with 18% sucrose, and then layered onto a discontinuous sucrose density gradient. The gradient was made by layering sucrose solutions of different densities beginning with 45% sucrose and ending with 20% (see Fig. 7). The sample was evenly split between 2 and 3 separate gradients and the fractions were pooled after collection.
Fig. 7

The sucrose solutions were obtained by mixing the appropriate quantities of a stock sucrose solution and gradient buffer solution.

<table>
<thead>
<tr>
<th>Volume of stock sucrose solution</th>
<th>Volume of gradient buffer</th>
<th>% sucrose solution (w/v)</th>
</tr>
</thead>
<tbody>
<tr>
<td>18.0 ml</td>
<td>2.0 ml</td>
<td>45%</td>
</tr>
<tr>
<td>15.2 ml</td>
<td>4.8 ml</td>
<td>38%</td>
</tr>
<tr>
<td>13.6 ml</td>
<td>6.4 ml</td>
<td>34%</td>
</tr>
<tr>
<td>12.0 ml</td>
<td>8.0 ml</td>
<td>30%</td>
</tr>
<tr>
<td>10.0 ml</td>
<td>10.0 ml</td>
<td>25%</td>
</tr>
<tr>
<td>8.0 ml</td>
<td>12.0 ml</td>
<td>20%</td>
</tr>
<tr>
<td>7.2 ml</td>
<td>12.8 ml</td>
<td>18%</td>
</tr>
</tbody>
</table>

**Stock sucrose solution**

- Sucrose: 50% w/v
- Tris-HES: 1 ml
- EDTA: 3 ml
- pH: 7.2

**Gradient buffer**

- Tris-HES: 1 ml
- EDTA: 3 ml
- pH: 7.2
The gradients were then subjected to centrifugation at 25,000 rpm in a 3 x 25 ml swing-out rotor for 2½ hours. The gradients were then pumped out by pumping 60% sucrose into the bottom of the tube so forcing the gradient up into a tube from the top of the gradient and fractions were collected from the interfaces by monitoring the absorbance of the gradient at 254 nm using an LKB uvicord (type 4701A).

Analogous fractions from different gradients were pooled at this time and the membranes were sedimented out at 80,000 g for 30 minutes. The supernatants were discarded and the pellets were either fixed for electron microscope study or taken up in gel sample buffer to allow their analysis on polyacrylamide gels.
B.3.VIII. Method for identification of plasmalemma containing fractions

Most work involving the preparation of plant plasma membranes makes use of a so-called specific staining technique first described by Roland, (1969) and Roland and Vian, (1971). Modification of this technique for use with membrane vesicles obtained by fractionation as described by Hodges et al., (1972) was described by Littlefield et al., (1972) and this was the method used for tentative identification of plasmalemma containing fractions obtained by the method described in the previous section.

After obtaining a series of pellets corresponding to different fractions from the gradient these were fixed in 3% glutaraldehyde in sodium cacodylate buffer pH 7.4 and post-fixed with 1% OsO₄ and thereafter processed through an ethanol acetone series and finally embedded in araldite. After embedding thin sections were cut (60-90 nm) using an LKB ultratome. These were collected using a gold loop and transferred onto the surface of the staining solution. Staining was carried out in a series of small wells containing the appropriate solution by floating sections onto the surface of the solution in the well and transferring at the appropriate time from well to well with a small loop made from gold wire which enabled the section to be transferred on a small drop of liquid. Care must be taken to prevent the sections sticking to the sides of the wells and to the loop. The sections can be seen by shining a light obliquely at the surface of the staining solutions. After the last staining solution the sections were mounted onto copper grids with a formvar film and then to protect the sections from damage from the electron beam they were carbon coated. The sections were then examined under the electron microscope.

The staining method uses periodic acid followed by a combination of chromic and phosphotungstic acid and is referred to as the PACP staining method. Listed below is a summary of the staining method as described by Littlefield et al., (1972) and also two other modifications of the technique which were used on intact tissue when checking the specificity of the stain.

PACP stain after Littlefield and Bracker, (1972)

1. Float thin sections onto 1% Periodic acid (w/v) for 30 minutes.
2. Wash in distilled water 10 x 5 minutes.
3. Stain in 1% Phosphotungstic acid (w/v) in 10% Chromic acid (w/v) for 15 minutes.

4. Wash in distilled water 10 x 5 minutes.

PACP stain after Roland, Lemui and Horré, (1972)

1. Float thin sections onto 1% Periodic acid (w/v) for 30 minutes.
2. Wash in distilled water 10 x 5 minutes.
3. Stain in 1% Phosphotungstic acid (w/v) in 10% Chromic acid (w/v) for 5 minutes.
4. Remove excess stain with distilled water 10 x 5 minutes.

PACP after Roland and Vian, (1971) and Rambourg, (1967)

1. Float thin sections directly onto 1% Phosphotungstic acid (w/v) in 10% Chromic acid (w/v) for 2 minutes.
2. Remove excess stain with distilled water 10 x 5 minutes.

These different methods will be discussed in more details in Section 0.6.
B.3.IX. Statistical Analysis.

Statistical analysis of results was confined to Section C.1 and C.2. The degree of variation associated with breaking weight, cell number and tracheary element number was indicated by the standard error of the mean. These were calculated according to Bailey, (1959) and are shown in the appropriate figures.

In Section C.1, the relationship between these parameters was investigated using linear regression analysis of individual pairs of values for five experiments. This function was calculated according to Bailey, (1959).
SECTION C:

RESULTS
C.1. CHARACTERISATION OF THE DEVELOPMENT OF THE GRAFT UNION

This chapter is devoted to characterisation of the development of the graft union in the tomato autograft system. The establishment of a graft can be examined by studying various parameters. In this chapter the development is analysed in terms of mechanical strength, cell division and vascular differentiation. The mechanical strength of a graft is reflected by its breaking weight which gives an indication of the extent to which the stock and scion have developed to become a single unit; the mechanical strength of which is comparable to that of the original tissue. Thus an indication of the degree to which the tissues have reintegrated and a measure of the grafting process can be obtained. Measurements of changes in cell and tracheary element number were made so that the role of cell division and vascular differentiation in the development of the graft could be determined. Both these processes are essential for the formation of a graft. It is apparent from simple observation that new cells are required to replace those which have been destroyed when the tissue is cut and to bridge the gap between stock and scion. The re-establishment of a continuous vascular system is a major factor in the development of a successful graft, contributing to the restoration of mechanical strength and a conducting system for the transport of water, ions and metabolites necessary for the growth and development of the plant.

Therefore vascular differentiation was examined by following the increase in tracheary element number as these elements are constituents of the vascular system which are easily identified. These lignified elements also contribute a large part to the mechanical strength of the plant.

Both the timing and the relationship of these parameters to breaking weight were looked at. These measurements may also provide additional parameters which can be used to compare the development of the graft under different conditions. For the study of some processes with respect to grafting it may be necessary to change the experimental conditions. It may not be possible to check if the process being studied is unaffected by the change from the usual conditions. Clearly, it is by the observation of these parameters under differing conditions that an assessment can be made as to whether the experimental conditions affect the development of the graft.
C.1.I. Analysis of the development of the graft union: breaking weight increase

Grafts were made according to the procedures described in Methods (B.2.II.) and the breaking weight of a sample of six grafts determined at daily intervals. No samples were taken on day one as the degree of cohesion was too small to permit accurate measurement of breaking weight and the graft was frequently broken or weakened significantly by the manipulations required to introduce the graft into the breaking weight apparatus. Samples were denoted by the time which had elapsed since grafting. The day on which the plants were grafted was designated Day 0 and the following days numbered from 1 to 7 accordingly. Samples were taken until day seven by which time the breaking weights were such that either, the tissue was not broken at the graft union because the strength of the graft was at least as great as the strength of the internodal tissue or, the machine fails to break it altogether, pulling the tissue out of the clips, so that the exact breaking strain of the graft cannot be determined. The breaking weight determinations for a single experiment are shown in Fig. 1.1.

The mechanical strength of the graft can be seen to increase slowly over the first three to four days and then more rapidly, reaching a maximum measurable value on the seventh day. During the first four days the mean breaking weight does not exceed 100 g but over the next three days it increases to a mean value of ca. 650 g.

The variation found in any one experiment is too high to permit detailed analysis of development using a single set of data and the sample number in any one experiment is of necessity rather low because of the number of different samples needed. A higher degree of replication for all stages of development was obtained by accumulating data from a number of experiments carried out in the same way. The mean breaking weights plotted against time together with the standard errors of the mean can be seen in Fig. 1.2. Each point represents a mean of thirty values obtained from five experiments. The same general course of development can be seen but the standard errors are smaller allowing interpretation of the patterns of development to be made with more certainty. As time progresses after the assembly of the graft the breaking weight does not increase in a linear fashion. The rate of increase in breaking weight tends to increase with time,
Figure 1.1:
Increase of breaking weight with time for a single experiment.
Figure 1.2
Increase of breaking weight with time – mean of five experiments.
so that the breaking weight of a graft probably increases as an exponential function. Initially the rate of increase is slow with the mean breaking weight increasing from 0 to just over 50 g in three days. During the next day it increases to over 100 g and over the next three days to about 600 g. If a semi-logarithmic plot is constructed (Fig. 1) with these data it can be seen that over the first five days there is an exponential increase in breaking weight and after this period the rate of development begins to slow down as the grafting process nears completion. This confirms the overall picture of development as previously found by both Roberts and Brown, (1961) and Lindsay, (1972) and gives a reproducible sequence of events describing the establishment of the graft which can be further used as a basis for further studies.
Figure 1.3

Log breaking weight plotted against time

Days

Log breaking weight

1.0

2.0

3.0
C.1.II. Analysis of the development of the graft union in cell and tracheary element number increase.

After measurements of the breaking weight of a graft a region 1 mm on either side of the graft union was removed, macerated and the cell number determined according to a modification of the method of Brown and Rickless, (1949) as outlined in Methods (B.3.II.). This 2 mm segment was marked on all the internodes before grafting so that any increases in volume within this region would not interfere with the measurement. Marking of the internode before grafting ensures that even if expansion occurs within this region the same tissue will be sampled. In addition samples were taken from ungrafted plants and treated in the same way so that it was possible to determine the change in cell number occurring in ungrafted specimens. Whilst the number of cells in a sample was being scored a note was also made of the number of tracheary elements, so that it was possible to see when any increase in the vascular tissue occurred. Fig. 1.4 shows the total cell number changes for a single experiment. Clearly, the increase in cell number in grafted plants is very large compared to the increase in the corresponding region in ungrafted plants.

The increase in cell number in ungrafted tissue is approximately two-fold whereas in the corresponding grafted tissue the increase is in the order of nine-fold. The variation seen can be reduced by combining the data obtained from five experiments as before (Fig. 1.5). The mean increase in cell number is now of the order of six-fold with grafted plants and less than two-fold in the case of ungrafted plants, showing that there is a substantial increase in cell number due to grafting.

Cell number increases slowly at first but the rate of increase rises until Day 6 when the process stops and no further increase is seen. This coincides with the fall in the rate of increase of breaking weight. The pattern of increase in cell number seems to resemble the increase in breaking weight to a certain extent, although cell division appears to stop whilst breaking weight is still increasing at a reduced rate.

A more detailed examination of the relationship between these two parameters will be made later. It is difficult at this stage to say whether the initial increase in cell number is linear or exponential.
Figure 1.4
Increase in cell number of the grafted region with time for a single experiment.

G = Grafted
U = Ungrafted
Figure 1.5

Increase in cell number of the grafted region with time mean of five experiments.

G = Grafted
U = Ungrafted
Any plot. 1. A. shows the result of plotting the mean breakage weight for a number of experiments with the result that the mean breakage weight is greater than the mean breakage weight for any other day after the strength of the data. 2. The data also shows that there is a definite relationship between these two variables.

The mean breakage weight for any one day is greater than the mean breakage weight for any other day after the strength of the data. 2. The data also shows that there is a definite relationship between these two variables.

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Figure 1.6
Log cell number plotted against time
Figure 1.7
Increase in tracheary element number with time for a single experiment

G = Grafted
U = Ungrafted
Figure 1.8
Increase in tracheary element number with time
mean of five experiments

G = Grafted
U = Ungrafted
Figure 1.9

Log tracheary element number plotted against time
particular point in time against the corresponding mean cell number, the data are drawn from a series of experiments as before.

Where breaking weight values are low during the first stages of development there is a fairly good correlation with cell number but as breaking weight increases the scatter of points becomes larger indicating that there is probably no relationship over the latter part of development.

If linear regression analysis is applied to the data some idea can be obtained as to the likelihood of whether or not there is a simple relationship between these two parameters. Fig. 1.10 also shows a series of regression lines together with their respective correlation coefficients. These lines were obtained by first treating all the data as a whole and then by dividing the data into two and analysing both parts separately to see if there was any change in the relationship with time.

When linear regression analysis is applied to all the data a line is obtained with a regression coefficient of 0.009 and a correlation coefficient of 0.560 which is significant at \( P = 0.01 \). If the values are divided at a breaking weight of 130 g which represents the stage of development reached approximately four days after grafting where the increase in breaking weight is becoming very rapid then two regression lines are obtained. The regression line calculated for points with breaking weight values below 130 g has a regression coefficient of 0.031 and a correlation coefficient of 0.701 which is again significant at \( P = 0.01 \). However, for values above 130 g another line is produced with different regression and correlation coefficients. These are 0.004 and 0.202 respectively and in this case the correlation is not significant at even \( P = 0.05 \). This indicates that it is unlikely that there is a correlation between breaking weight and cell number when breaking weights are over 130 g and the stage of development where a rapid increase in breaking weight is reached. It would appear that there is a correlation between these two factors before this point and that the overall correlation is probably influenced by the high initial correlation which brings the value of the overall correlation coefficient to significant levels in spite of the insignificant correlation where breaking weight exceeds 130 g. It can be concluded that there is initially a relationship
Figure 1.10

Linear regression analysis of the relationship between breaking weight and cell number of the grafted region.
between cell number and breaking weight which breaks down as development progresses. This does not necessarily mean that the initial breaking weight increase is dependent on cell number increase but simply emphasises the concurrence of these events. This is also consistent with the studies of Lindsay, (1972) who also found that the development of the graft union could be looked at in two parts.

The same analysis was repeated on the data for tracheary element number and graft development which was available from the same series of experiments. Fig. 1.11 was obtained by plotting mean tracheary element number against mean breaking weight and also shows the regression lines obtained after the same analysis was applied to the data as was for the cell number data. When linear regression analysis is applied to all the data a line is produced with a regression coefficient of 0.006 and a correlation coefficient of 0.748 which is significant at P = 0.01. When the data is divided the line calculated on data with breaking weight values up to 130 g has a regression coefficient of 0.012 and a correlation coefficient of 0.728 which is again significant at P = 0.01. The line produced using the remainder of points has a regression coefficient of 0.005 and correlation coefficient of 0.507 which is lower than that of the other two lines. However, when the critical values of r (correlation coefficient) are looked at, this is still seen to be significant although only at P = 0.05. This differs from the situation found with the cell number data because there is a significant correlation throughout the period of study whichever way the data is treated. The conclusion may be drawn that initially whilst mean breaking weight is increasing slowly it is related to both mean cell number and mean tracheary element number, but in the latter stages of development there is only a significant correlation with tracheary element number. It is not possible to say from this type of evidence whether a causal relationship exists between these parameters but simply that certain parameters increase at the same time and that both the cell and tracheary element number increases might be responsible initially for the increase in mechanical strength of the graft as measured by breaking weight and that in the latter stages this is influenced by tracheary element number alone.

Now that the changes in these numbers have been characterised and related to the development of the graft union it is possible to use these as parameters for comparison of the development of the graft
Linear regression analysis of the relationship between breaking weight and tracheary element number of the grafted region.
under different experimental conditions. The change in the pattern of development which occurs when its breaking weight reaches 130 g also coincides with the divergence in patterns of development between compatible and noncompatible grafts (Yeoman and Brown, 1976). The events surrounding the change in the pattern of development will be further discussed in the following chapters. The patterns of protein synthesis during this part of the development of the autograft will be studied to provide further information about this critical period in the development of a successful graft union. To facilitate these studies attempts were made to isolate the development of the graft union from the influence of the rest of the plant. An isolated grafting system should facilitate the application of exogenous substances to the graft union and offers the only real possibility of the study being made without the interference of the bacteria and fungi which live in a close relationship with every plant. The validity of any in vitro system will be assessed by comparison of the development of the graft union under these conditions with its development within the plant. The comparison will be made in terms of increases in breaking weight, cell number and number of tracheary elements.
C.2. PRODUCTION OF A GRAFT IN A CULTURE SITUATION.

Studies on grafts in "intact" plants are restricted by a number of limitations and it is clear that further investigation of the grafting process would be facilitated by the development of a system in which the graft union was allowed to form in isolation. The application of inhibitors or labelled precursors could be made directly to the graft union avoiding the competition for these substances by other centres of activity. In such an in vitro situation the development of the graft would be under greater control and the effects of other parts of the plant would be removed. The absence of micro-organisms from the surface of the plant tissue would eliminate competition for precursors between plant and microbe and simplify the interpretation of the patterns of protein synthesis, where it is difficult to decide if the pattern obtained is a result of the action of the plant's synthetic system or the result of protein synthesis by micro-organisms or an interaction between the two.

Previous attempts at establishing an in vitro grafting system as described by Lindsay, (1972) have had limited success. In these studies the internode to be grafted was excised at the beginning of the experiment and then the graft was made in the excised internode and allowed to develop standing upright with one end embedded in a solid culture medium (Hoagland's solution with 2% sucrose and 1% agar). Under these conditions the success rate of graft formation was low, and very variable, and full development of the graft was never achieved. The highest breaking weight values observed were low and there was no evidence of re-establishment of vascular continuity.

Roberts (1960) reported that regeneration of xylem would occur after excision from the plant 48 hours after initial wounding suggesting an inductive effect, as the vascular strands did not complete differentiation until 72 hours after wounding. In order to determine whether there was a similar inductive effect in the re-establishment of vascular continuity across the graft union, developing grafts were excised at various times.
C.2.I. Comparison of final breaking weights of unexcised grafts with cultured excised grafts.

The approach used in this study was to construct the graft in the plant, then to remove the internode with the graft to a culture situation at various times along a developmental time axis. Using this approach it was possible to determine how long the graft had to remain in the intact plant before it could develop in isolation and also whether graft development would continue, and for how long, in the absence of the parent plant.

Populations of grafted plants were set up and allowed to develop as in Section B.2.II. At daily intervals the grafted internodes were excised and transferred to a glass bottle containing a mineral salts medium solidified with agar (see Section B.2.V. and Fig. 2.1.a.). Full details of the procedure are described in Section B.2.IV. After excision the grafted internodes were left to develop alongside unexcised grafts with which their development was compared. Seven days after grafting the breaking weights of both sets of grafts were determined in the usual way (Section B.3.I.) and the breaking weight of the excised graft was expressed as a percentage of the value obtained for the unexcised grafts. This enabled comparison of the treatments to be made in which the best treatments produced the highest percentages, and eliminated differences between experiments. (The amount of development undergone by the excised graft is compared with that of a graft made at the same time but allowed to develop undisturbed.) The values represented here are derived from a mean of ten cultured grafts composed with a mean of ten unexcised grafts.

Fig. 2.2 (a-m) shows results from two experiments where grafts were excised on successive days up to five days after grafting. There is a lot of variation between experiments despite attempts to standardise the results. Only one treatment showed a large amount of development this being excised five days after grafting. In general the trend was for a greater amount of development to occur in treatments where a longer time had elapsed between grafting and transfer to culture. This however, may be solely a result of the amount of development which has occurred prior to excision. A more detailed study of the whole period of development would resolve this question. With the exception of graft transferred five days after grafting none of the excision
Figure 2.1

Diagram to show the position of the cuts made to produce different explants.
Fig. 2.2. (A-M)

a. Grafted internode excised and transferred to culture immediately after grafting (Day 0).
b. Grafted internode excised and transferred to culture 1 day after grafting.
c. Grafted internode excised and transferred to culture 1 day after grafting.
d. Grafted internode excised and transferred to culture 2 days after grafting.
e. Grafted internode excised and transferred to culture 2 days after grafting.
f. Grafted internode excised and transferred to culture 2 days after grafting.
g. Grafted internode excised and transferred to culture 2 days after grafting.
h. Grafted internode excised and transferred to culture 3 days after grafting.
i. Grafted internode excised and transferred to culture 3 days after grafting.
j. Grafted internode excised and transferred to culture 4 days after grafting.
k. Grafted internode excised and transferred to culture 4 days after grafting.
l. Grafted internode excised and transferred to culture 5 days after grafting.
m. Grafted internode excised and transferred to culture 5 days after grafting.
Figure 2.2(A-M)

Comparison of mean breaking weight on day 7 of control and excised grafts
conditions were able to produce a similar amount of development under
in vitro conditions as in the intact plant. Grafts cultured from five
days onwards would not yield much information about the development of
the graft union. By this stage development is almost complete and the
most interesting stages of the process have occurred. It appears that
it is not sufficient to allow the graft to develop for a short inductive
period in the plant before isolation. It may be that a continuous
supply of growth factors is needed throughout the development of the
graft rather than exposure for a short inductive period after which
development is independent of the effects of the rest of the plant.
C.2.II. Investigation of the role of leaves, buds, cotyledons and exogenously applied "auxins" in the development of the graft union in an excised internode.

The regeneration of vascular tissue is implicit in the development of a successful graft. The cultured grafts must have this regenerative capacity. There are extensive reports in the literature of factors which affect vascular differentiation and regeneration. The role of auxin in this phenomenon was reported by Wetmore and Sorokin, (1955); Wetmore and Rier, (1963); Wetmore et al., (1964); Sachs, (1968); and Jacobs, (1952). Wetmore showed xylogenesis in callus as a result of treatment with auxin. The work of Sachs, (1968) on grafting of excised roots showed that apical application of 1% I.A.A. from a lanolin paste was effective in allowing regeneration of vascular elements across the graft. The work of Jacobs and his co-workers investigated the relationship between the application of exogenous auxin and the number of vascular elements regenerated after wounding (Jacobs, 1970). This work established the involvement of I.A.A. in this regeneration phenomenon. A further set of experiments was conducted using excised internodes of tomato plants (Thompson and Jacobs, 1966) and regeneration levels in excised internodes were restored to control values (unexcised) by apical application of 0.1% I.A.A. Although in this work only a limited number of vascular elements were severed, whereas the tomato autograft vascular system was completely severed, the addition of apical application of exogenous auxins or the supply of endogenous auxins from leaves or axillary buds may be effective in allowing the graft to develop completely in an excised internode.

The aim of this experiment was to determine if retention of leaves, axillary buds or cotyledons, or the supply of exogenous auxins would enable the graft to develop in isolation and if the restoration of vascular continuity was analogous to the regeneration of vascular tissue in a limited wounding situation as studied by Thompson and Jacobs, (1966).

The same approach was used as in Section C.2.I. The investigation was limited to excision of grafted internodes two days after grafting. This was the earliest time at which the graft had sufficient strength to enable manipulation with only a small number of breakages. Excision of the grafted internodes was performed as before but the part of the plant excised was varied as shown in Fig. 2.1. (b-e). Excision of the
grafted internode along the lines shown produced the following treatments.

b. Grafted internode with one primary trifoliate leaf.
c. Grafted internode with two cotyledons and associated axillary buds.
d. Grafted internode with two cotyledons and axillary buds and one primary trifoliate leaf.
e. Grafted internode excised with the axillary bud of the primary trifoliate leaf.

In addition to this, grafts were excised so that all the leaves and axillary buds were removed but the apical meristem remained. In all cases the root system was removed. Also two days after grafting a series of internodes excised with no leaves, buds or cotyledons (Fig. 2.1.a.) were treated with either I.A.A. or a more stable auxin 2,4 dichlorophenoxyacetic acid (2,4-D) by a single application to the apical end of the grafted internode, from a lanolin paste. The concentration of I.A.A. was 0.1% as used by Thompson and Jacobs, (1966) whereas 2,4-D was used at a wide range of concentrations from 0.01% to 10% to determine the most effective level.

The results of all the treatments are shown in Fig. 2.2 (n-z). These have been expressed as a percentage of the control breaking weights so that comparisons can be made between treatments. A value of 100% would be obtained if the excised graft developed fully so that its breaking weight was equal to that of the control unexcised grafts which were allowed to develop at the same time. The values presented here are derived from a mean of ten cultured grafts compared to a mean of ten unexcised grafts.

Fig. 2.2 (n-s) shows the results of treatments where two days old grafts were excised as shown in Fig. 2.1 (b-e). In general the retention of more of the plant appeared to increase the amount of development over that seen where only the grafted internode was excised (Fig. 2.2 ,d-g). The most successful treatments would appear to be p and q where the cotyledons and the buds of the cotyledonary internodes were retained. In treatment p the leaf was also present. The high variation found between identical treatments makes interpretation of the results difficult.

Fig. 2.2 (t-z) represents internodes excised two days after grafting and treated with either I.A.A. or 2,4-D in a lanolin paste or just
Fig. 2.2 (N-Z)
n. Grafted internode with one primary trifoliate leaf excised and transferred to culture 2 days after grafting.

o. Grafted internode with one primary trifoliate leaf excised and transferred to culture 2 days after grafting.

p. Grafted internode with 2 cotyledons excised and transferred to culture 2 days after grafting.

q. Grafted internode with one primary trifoliate leaf and two cotyledons excised and transferred to culture 2 days after grafting.

r. Grafted internode with axillary bud of primary internode excised and transferred to culture 2 days after grafting.

s. Grafted stem with all axillary buds and leaves excised and transferred to culture 2 days after grafting.

t. Grafted internode excised, treated with 0.1% I.A.A. (lanolin paste) and transferred to culture 2 days after grafting.

u. Grafted internode excised, treated with lanolin paste and transferred to culture 2 days after grafting.

v. Grafted internode excised, treated with 0.01% 2,4-D (lanolin paste), and transferred to culture 2 days after grafting.

w. Grafted internode excised, treated with 0.1% 2,4-D (lanolin paste) and transferred to culture 2 days after grafting.

x. Grafted internode excised, treated with 1.0% 2,4-D (lanolin paste) and transferred to culture 2 days after grafting.

y. Grafted internode excised, treated with 10% 2,4-D (lanolin paste) and transferred to culture 2 days after grafting.

z. Grafted internode excised, treated with lanolin paste and transferred to culture 2 days after grafting.
Figure 2.2 (N-Z)

Comparison of mean breaking weights on day 7 of "control" and excised grafts.
lanolin paste. Again none of these treatments restores development to control levels. Treatments x and y where high levels of 2,4-D were used seemed to inhibit the development of the graft. The grafts treated with lanolin paste alone developed more than these. The levels of 2,4-D used however, are very high approaching herbicidal concentrations. Treatment with 0.01% and 0.1% 2,4-D increased the percentage development over untreated internodes, but the values were still below 20%. Clearly treatment with 2,4-D did not restore development of the excised graft to control levels and cannot be used as a culture system. Treatment with I.A.A. was less effective. In fact the untreated internode excised at the same time developed to a greater extent. This lack of very real positive effect was surprising considering the success of Thompson and Jacobs, (1966). Even if it were not possible to restore control levels of development an increase in the amount of development might be expected over the untreated excised internodes. I.A.A. induced vascular regeneration might require the presence of some intact vascular strands. None of the treatments studied (with the exception of grafts transferred five days after grafting) were able to achieve more than 50% of the development observed under in vivo conditions. Factors supplied by the rest of the plant would appear to be necessary for the complete development of the graft. The retention of more of the plant did increase the degree of development. None of the explants employed possessed roots and it might be these that contribute some significant factor to the development of the graft. The retention of roots in a cultured situation would make the achievement of sterility difficult. The differences seen in the percentage development occurred after excision because these were all excised at the same time. The small amount of development in the excised graft may be due to either an extremely slow overall rate of development, or similar initial rates of development until endogenous supplies of factors necessary for the development of the graft provided by the rest of the plant are used up. This question can only be resolved by comparison of the development of both excised and control grafts in detail for the whole seven day period. If the excised graft can develop, for even a short time, at a similar rate to that of the graft in the "intact" plant then this may provide a basis for a culture system where at least parts of graft development might be studied.
C.2.III. Time course of development of grafted internodes excised two, three and four days after grafting.

The following experiment was conducted to determine whether the failure of the graft to fully develop in isolation was due to a slower overall rate of development, or a similar rate of development for only a short period, following excision of the grafted internode. The development was followed in detail examining not only the development of breaking weight but also the increase in cell number and number of tracheary elements. Clearly it is important to determine whether vascular regeneration is occurring in the isolated graft. The development of the isolated graft was directly compared with the development of the graft union in the "intact" plant. The development of graft union in excised internodes was studied by determining breaking weight, cell number and number of tracheary elements in the grafted region along a time axis. Samples were taken daily and the complete course of development was compared with unexcised grafts. Grafted internodes were excised without axillary buds, leaves or cotyledons as shown in Fig. 2.1.a. After excision these were transferred to culture vessels and allowed to develop. The grafts were harvested each day, until seven days after grafting, as described in Section B.2.IV and B.2.V. Grafts were excised two, three and four days after grafting. These times were chosen because they encompass the stages at which the changes in pattern of development described in Section C.1. occur and are probably the stages of most interest in this study.

A comparison of breaking weights of excised and unexcised grafts is shown in Fig. 2.3 - 2.8. The results of two experiments are shown. The graph shows the mean breaking weight on six consecutive days determined from a sample of six grafts together with the standard error of the mean. Where the error bars overlap, the bar to the left of the line represents the excised graft. Fig. 2.3 and 2.4 show a comparison of the breaking weights with grafts excised two days after grafting. The development of the graft was very similar in both experiments. The breaking weight of the excised graft never increased by the same amount as the unexcised graft, however there was a slow increase throughout the period studied. For the first two days after excision the rates of increase of breaking weight were very similar although the divergence from the control breaking weight curve increased with time. For the
Figure 2.3
The development of breaking weight of excised grafts compared with grafts in the "intact" plant, Day 2 excision.
Figure 2.4
The development of breaking weight of excised grafts compared with grafts in the "intact" plant, Day 2 excision.
first twenty-four hour period the rate of increase was very similar.

Figs. 2.5 and 2.6 show the comparison of the increase in breaking weight of unexcised grafts and grafts excised three days after grafting. Again although the rate of increase in breaking weight slowed down with time and the final breaking weight was well below that of the control grafts, for the first twenty-four hour period at least, the increases in breaking weight were very similar. Both experiments show this initial similarity in development which begins to slow down and significantly diverges between twenty-four and forty-eight hours after excision and culture.

Figs. 2.7 and 2.8 show two experiments in which the breaking weights of unexcised grafts are compared to grafts excised four days after grafting. In one experiment (Fig. 2.8) the development of the excised graft follows the development of the control graft closely until the end of the experiment. The variation in the excised material, however, was very great, particularly for days six and seven. In the other experiment, although the final breaking weight was higher than the breaking weight of grafts excised two and three days after grafting, the rate of increase in breaking weight was slower. However, as before, the initial development of the excised graft in culture is very similar to the development of the graft in the plant.

A clearer picture of the development of the graft can be seen by studying the increases in cell number and the number of tracheary elements accompanying the increase in the mechanical strength of the graft. Figs. 2.9 - 2.14 show the increase in cell number with time in both excised and unexcised grafts. Each point represents a mean of 3 values determined as described in Section B.3.II. and is presented together with the standard error of the mean. Error bars follow the same convention as used for the breaking weight data. Figs. 2.9 and 2.10 show the increases in cell number in grafts excised two days after grafting and allowed to develop in culture. The results of the two experiments are not exactly the same. In the first, the increase in cell number was very similar to the control values for the first twenty-four hour period in culture and then, the development appeared to slow down and cease. In the second experiment, the increase in cell number persists for a longer time. There is no significant difference between the cultured graft and the control graft until after
Figure 2.5
The development of breaking weight of excised grafts compared with grafts in the "intact" plant, DAY 3 excision.
Figure 2.6:
The development of breaking weight of excised grafts compared with grafts in the "intact" plant, Day 3 excision.
The development of breaking weight of excised grafts compared with grafts in the "intact" plant, Day 4 excision.
Figure 2.8
The development of breaking weight of excised grafts compared with grafts in the "intact" plant, Day 4 excision.
Figure 2.9

Increase in cell number of the grafted region of excised grafted internodes compared with that of the "intact" plant, Day 2 excision.

G = Grafted
U = Ungrafted
Figure 2.10
Increase in cell number of the grafted region of excised grafted internodes compared with that of the "intact" plant, Day 2 excision.

G = Grafted
U = Ungrafted

CELL NUMBER X 10^5

DAYS

2 3 4 5 6 7

UNEXCISED

EXCISED
three days in culture. Subsequently there was a large drop in cell number followed by a rise, this was probably due to variations in the material, but the general trend showed a decrease in the rate of development. The pattern of increases in cell number was similar to the increases in breaking weight. For at least the first twenty-four hours, after transfer to culture the development of a graft excised two days after grafting followed the development of a graft allowed to develop undisturbed. The cell number in ungrafted internodes both excised and unexcised are also shown and there was no difference between the two. In fact neither show a great increase in cell number.

Figs. 2.11 and 2.12 show a comparison of the increase in cell number in the grafted region of internodes, excised three days after grafting, with that of unexcised grafts. Again the increase in cell number was similar at first but diverged as the time in culture was prolonged. In both experiments there was no significant difference, between the development in culture and the development of the unexcised graft, for forty-eight hours after excision. After this the rate of development in culture slowed down. The corresponding ungrafted internodes showed no significant differences between the control and the cultured internode for the whole period of development.

The comparison of the increase in cell number in the grafted region of internodes, excised four days after grafting and cultured for the remaining three days, with that of unexcised grafts is shown in Figs. 2.13 and 2.14. Although the overall increase in cell number of the cultured grafts was different from the control, the increase in cell number was not significantly different from the control graft twenty-four hours after excision and transfer to culture. The same pattern of cell number increase in culture emerges as with grafts excised at earlier stages of development. The graft appeared to be able to develop in isolation in the same way as the graft developed in the plant, for a short length of time, after which development slows down and may even cease altogether. Although the sample number was small and the experimental variation was large, pattern of development in the two experiments was similar. Again there was no significant difference between the corresponding region of cultured and non-cultured ungrafted internodes.

The increases in cell number in culture after excision show that development occurs as in the whole plant for a short time no matter
Figure 2.11
Increase in cell number of the grafted region of excised grafted internodes compared with that of the "intact" plant, Day 3 excision.

G = Grafted
U = Ungrafted
Figure 2.12

Increase in cell number of the grafted region of excised grafted internodes compared with that of the "intact" plant, Day 3 excision.
Figure 2.13

Increase in cell number of the grafted region of excised grafted internodes compared with that of the "intact" plant, Day 4 excision.

\[ G = \text{Grafted} \]
\[ U = \text{Ungrafted} \]
Figure 2.14

Increase in cell number of the grafted region of excised grafted internodes compared with that of the "intact" plant, Day 4 excision.

\[ G \text{ = Grafted} \]
\[ U \text{ = Ungrafted} \]
when the graft is excised. After a time, which in every case exceeds the first twenty-four hours in culture, the rate of development slows down and may even cease altogether.

The successful establishment of a graft involves the regeneration of the vascular system. It is essential that the culture system is able to regenerate vascular elements in the same way as the graft in the plant. It may be possible to study part of the development of the graft in isolation if the production of tracheary elements in culture proceeds in the same way as in the unexcised graft for a time. The number of cells and tracheary elements in each grafted region was determined simultaneously (Section B.3.II). A comparison of the numbers of tracheary elements in the grafted region of cultured grafted internodes excised two, three and four days after grafting with that of the corresponding region of grafted plants is shown in Figs. 2.15 – 2.20. Figs. 2.15 and 2.16 show the number of tracheary elements produced in culture by grafts excised two days after grafting. The overall increase in number of tracheary elements was very small in both cases. The increase in number of tracheary elements, in the control graft, was greatest in the latter part of development by which time the development of the cultured grafts had ceased. During the initial stages of development, when the increases in the control grafts were small, then a similar level of increase was seen in cultured individuals. For at least the first twenty-four hours, after excision and transfer to culture, the production of tracheary elements, in culture, did not differ significantly from their production in the unexcised graft. The question of whether significant vascular regeneration can occur in culture cannot be answered by analysis at this stage of development because even in the unexcised graft there is very little vascular regeneration. The inability of a graft, excised two days after grafting, to fully develop, might be due to an inability to produce a significant increase in tracheary elements or, simply due to the fact that the excised tissue was unable to produce sufficient metabolites, to sustain the final development of the graft union.

Figs. 2.17 and 2.18 show the increase in number of tracheary elements in culture after excision of the grafted internode three days after grafting. The first experiment (Fig. 2.17) does not show a great increase in number of tracheary elements, however, for the first
Figure 2.15

Increase in tracheary element number of the grafted region of excised grafted internodes compared with that of the "intact" plant, Day 2 excision.

G = Grafted
U = Ungrafted

UNEXCISED

G

EXCISED

G

U

UNEXCISED

DAYS

TRAČEARY ELEMENT NUMBER X 10^4
Figure 2.16
Increase intracheary element number of the grafted region of excised grafted internodes compared with that of the "intact" plant, Day 2 excision.

G = Grafted
U = Ungrafted
Figure 2.17
Increase in tracheary element number of the grafted region of excised grafted internodes compared with that of the "intact" plant, Day 3 excision.

G = Grafted
U = Ungrafted
Figure 2.18

Increase in tracheary element number of the grafted region of excised grafted internodes compared with that of the "intact" plant, Day 3 excision.

G = Grafted
U = Ungrafted
twenty-four hours the number of tracheary elements was identical to that of the unexcised graft. In the second experiment there was an increase in number of tracheary elements, but after forty-eight hours the number decreased. This decrease can also be seen in Fig. 2.12 and was probably due to sample variation rather than a real decrease in the overall cell number. The sample size was small and in addition to this the determination of tracheary element number was not very accurate due to the small numbers actually counted (see Section C.1.). The second experiment shows that there was no significant difference between the behaviour of the graft in the plant and in culture for the first forty-eight hours after excision. Again the excised internode in culture appeared to be able to maintain the development of the graft for at least twenty-four hours after excision from the plant.

The stage of development after which the major increase in tracheary elements might be expected is about four days after grafting when the breaking weight is increasing rapidly. Grafts excised at this point show a significant overall increase in the number of tracheary elements. Figs. 2.19 and 2.20 show the increase in the number of tracheary elements in culture, after excision four days after grafting, compared to that of unexcised grafts. As expected both experiments show that if grafts are transferred to culture at this late stage there was a significant overall increase in the number of tracheary elements. The overall increase was not as large as the increase in the number of tracheary elements in the unexcised graft. For at least the first twenty-four hours the increase of tracheary elements, in culture, was not significantly different from the increase in unexcised internodes, as time in culture progressed the rate of development appeared to slow down, as with all other excision times.

The increase in tracheary element number, in ungrafted internodes, was very small and in no treatment was there any significant difference between the cultured internode and the unexcised internode.

The development of the graft in culture can be summarised as follows. No matter whether development was considered as an increase in breaking weight, cell number or number of tracheary elements, the pattern of development appears to have been similar. The overall development of the graft union in culture was affected by the time of excision although if the amount of development in culture was compared with the concurrent development of the graft in the plant then this
Figure 2.19
Increase in tracheary element number of the grafted region of excised grafted internodes compared with that of the "intact" plant, Day 4 excision.

G = Grafted
U = Ungrafted
Figure 2.2
Increase in tracheary element number of the grafted region of excised grafted internodes compared with that of the "intact" plant, Day 4 excision.

G = Grafted
U = Ungrafted
suggested that exacted expectad intermediates can be used synthetically. This means that exacted intermediates are considered as potential candidates for further reaction and were subsequently synthesized. Any substrates or reagents controlled to the endogenous one, for at least twenty-four hours after extraction using the endogenous

The exacted exacted can develop in a manner way as an unexpected

intermediate because this expected the growth of microtubule components

considered, if not present, for reassembly or reassembly of essential components, that only the presence of the work of Thompson and Jacob, (1966) suggested that only the presence of the exacted

supplied by extacted only be responsible for the ultimate amount

in the development of vacuolar ete mutants as well.

In the development of vacuolar ete mutants as well the role of extacted intermediates may be increased by extaction may be increased by extaction as a surface extracellular of bacterial extracellular with the nature of these substances is unknown.

not introduced (E. coli). Some of these factors may be supplied by the plant

although, their role of production may be limited to such an extent that some of these factors may be supplied by the plant
cellulose intermediates can never develop to the same extent as the exacted

consisting with each other as intermediates, the presence of such intermediates

extracted together with yeast, and/or oxidation or extraction, this is the

intermediate may be extracted or deleted to such a level that further

growth affected by selected intermediate s representing the whole plant. The supply of these substances within

reduced. The decrease in rate of development of the exacted

slow down and differences between the exacted and unexacted

effect, that the rate of development

difference was reduced. The exacted exacted will develop at the same
to study the synthesis or accumulation of compounds at different times during the development of the graft union, over a twenty-four hour period in culture. In order to study graft development using the system described here a large number of plants would have to be grafted at the beginning of the experiment and then excised and transferred to culture at different times. The direct application of radioactively labelled amino acids to the grafted internode could be made by simple addition to the medium. Their incorporation could be studied during the following twenty-four hour period. Different stages of development could be studied by excising grafted internodes at various times after grafting, and studying incorporation over the following twenty-four hours. Grafts transferred two, three and four days after grafting can be examined in this way. This provides a system in which the patterns of protein synthesis during the critical stages of graft formation may be studied.
C.3. PRODUCTION OF A "STERILE" GRAFTING SITUATION.

In natural situations all plants live together with other organisms. Large numbers of bacteria and microfungi are present, not only in the soil in which the plants grow, but also on the surface of the plant itself (phyllosphere, rhizosphere). Most of these micro-organisms do not exert any harmful effect on the plant, indeed their presence may be beneficial. However, in culture situations where there is a large readily available carbon source, such as sucrose, these harmless micro-organisms will multiply rapidly and usually prevent further growth of the explant. Clearly the presence of large numbers of micro-organisms with an ability to metabolise a wide range of chemical compounds is undesirable, especially where the processes being studied are the primary metabolic pathways which have similar precursors in both plant and micro-organism. It is inevitable that the fate of the added precursors will be influenced by the presence of these organisms. Such influences may be as a result of direct competition or a result of the modification of the precursor by the micro-organisms which is then utilised by the plant.

A variety of methods for obtaining plant tissues free of micro-organisms are available but most are not suitable for the grafting situation. It is possible to grow sterile plants from surface-sterilised seeds under rigorously aseptic conditions. Here it would be necessary to grow and maintain the sterility of large numbers of tomato plants for some five weeks before grafting and then maintain sterility through all the subsequent manipulations for a further week. This is possible but the probability of introducing contaminants is very high considering the number of plants which would have to be treated (in excess of 100 plants) and the number of manipulations which must be performed. Also tomato plants grown under completely sterile conditions are weak and unsuitable for grafting.

An alternative approach to the use of completely sterile tomato plant is to surface sterilise the internode before grafting. This should prove effective as it is this internode which is put into the labelling solution and even if absolute sterility is not achieved the degree of contamination should be reduced to an acceptable level. Methods of surface sterilisation employ compounds ranging from
non-specific antiseptics to antibiotics which are specific for a particular group of prokaryotes. Ultraviolet radiation will kill bacteria. However, the effects of radiation on the plant are undesirable. It must be borne in mind that antiseptic compounds will eliminate the microbial contaminants but will have an effect on the outer layers of the plant especially if used at a high concentration for an extended time period. Accordingly the choice of antiseptics must be confined to those which are not particularly vigorous in their action and can be easily and rapidly removed so minimising any effects on the plant tissue.
C.3.1. Use of non-sterile plants - surface sterilisation of the grafted internode.

As a preliminary to the study of treatments designed to reduce or eliminate the microflora present on the surface of tomato plants, the number of micro-organisms present on the plant material was determined under the experimental conditions to be used in grafting. The experiments which will be most affected by the presence of micro-organisms are likely to be those in which the pattern of protein synthesis is determined using radioactive amino acids. Because micro-organisms can incorporate the same amino acids into their proteins and some of these may be extracted along with the proteins in the plant, this makes it difficult to decide whether the pattern obtained is due to the plant or its microflora.

a. Assessment of the level of contamination.

Grafted and ungrafted internodes were excised and transferred to culture bottles using the same procedure described for incorporation studies (Section B.2.V. and B.2.VII.). Transfer to incubation conditions was carried out two and three days after grafting and samples were taken twenty-four hours later.

Samples consisted of ten 2 mm segments (see Section B.2.IV.) similar to those used for the extraction of protein for incorporation studies. Sampling procedures were identical except that aseptic techniques were maintained throughout and homogenisation was performed in a sterile glass-teflon homogeniser with sterile water. The viable count was determined on these samples using the microbiological serial dilution technique maintaining strict asepsis throughout (Section B.3.III.a.).

An estimation of the numbers of viable contaminants present in 10 x 2 mm segments containing the graft or an equivalent region of ungrafted tissue is shown in table 3.1.
Table 3.1.

<table>
<thead>
<tr>
<th></th>
<th>Time of transfer to incubation conditions</th>
<th>Time of sampling for analysis</th>
<th>Viable count per sample (10 x 2 mm seg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grafted</td>
<td>2 days after grafting</td>
<td>3 days after grafting</td>
<td>4.0 x 10^6</td>
</tr>
<tr>
<td>Ungrafted</td>
<td>2 days after grafting</td>
<td>3 days after grafting</td>
<td>8.0 x 10^5</td>
</tr>
<tr>
<td>Grafted</td>
<td>3 days after grafting</td>
<td>4 days after grafting</td>
<td>1.2 x 10^6</td>
</tr>
<tr>
<td>Ungrafted</td>
<td>3 days after grafting</td>
<td>4 days after grafting</td>
<td>1.5 x 10^5</td>
</tr>
</tbody>
</table>

The number of contaminants present on the grafted tissue was greater than that on the ungrafted. However, the viable count on the ungrafted tissue taken on successive days shows a difference which is probably a good indication of variation due to the method of determination rather than a real variation with time. The actual number of contaminants is not really important rather their order of magnitude. In both grafted and ungrafted tissue there is a considerable population of micro-organisms present. The population was fairly diverse but did not include fungal colonies so that the major source of contamination was bacteria.

Clearly the level of contamination in both grafted and ungrafted tissue was high. The number of bacterial cells present on the segment analysed was in the same order of magnitude as the number of plant cells in a similar segment. Such a large population of contaminants could seriously interfere with the results of incorporation studies.

Elimination of these contaminants from this region was attempted using a variety of agents.

b. Sterilisation to eliminate or reduce bacterial contamination to levels suitable for incorporation studies.

A variety of factors must be satisfied whilst establishing an effective sterilising procedure.
1. Levels of contamination are such that any incorporation by the contaminants would not significantly contribute to the results.

2. That the agent/method used does not interfere with process being studied e.g. if using antibiotics are these interfering with the protein synthesis of the plant.

3. That the agent/method used does not interfere with the establishment of the graft union.

Of the variety of antiseptic agents available hydrogen peroxide was considered to have the properties of a good surface sterilising agent. It is less harmful than most other sterilising agents and breaks down rapidly to oxygen and water (Yeoman, 1973) neither of which is toxic.

Antibiotics may also be used as sterilising agents and have both advantages and disadvantages. The main advantage is the specificity of action toward prokaryotes rather than eukaryotes. The specificity of action of antibiotics may also confer a disadvantage because antibiotics tend not to be effective against all prokaryotes but only a definite spectrum of bacterial species within which there may exist a number of resistant strains. Even with broad-spectrum antibiotics the probability of eliminating all contaminants is remote but it may be possible to reduce the bacterial population. Antibiotics may also have effects on plants (Allende, 1969), After a study of the effects of various antibiotics on plants he concluded that although they had an effect on various processes the concentrations effective against prokaryotes and eukaryotes tend to be significantly different. Therefore it is necessary to establish that the antibiotic used does not have a significant effect on the plant so causing any interference with the results obtained. Only broad spectrum antibiotics were used in this study because one would expect a wide variety of organisms present.

These sterilisation procedures were tested using ungrafted tomato plants.

1. Application of 12% $\text{H}_2\text{O}_2$ for twenty-five minutes (Yeoman, 1973).
2. Irradiation with ultra-violet light for 10 or 20 minutes.
3. Application of antibiotic solutions for a period of 3 hours.

All solutions were applied to absorbant tissue surrounding the internode at saturation levels.
The antibiotic combinations tested are shown in table 3.2 with the amount applied shown in brackets.

Table 3.2.

a. D-threo-chloramphenicol (1.5 mg)
b. Streptomycin/penicillin (38.4 mg, 30.0 mg)
c. Tetracycline/oxytetracycline/streptomycin/chloramphenicol (2.1 mg, 6.9 mg, 1.1 mg, 3.9 mg)

The plants were treated in exactly the same way as for grafting except that the internode was not cut, but simply covered with a sterile silicone rubber strip.

The sterilising procedures employed are shown in Fig. 3.1. Prior to sterilisation the 2 mm region, in which the graft was normally made, was marked (Section B.2.II.) so that an equivalent region to that used for protein extraction could be sampled and the level of contamination assessed. The internode was then treated with dilute detergent (0.001%) to act as a wetting agent so that the sterilising agent could be brought into intimate contact with the internode, the surface of tomato is covered with hairs and tends to be hydrophobic (Yeoman, 1973). The detergent was then washed off with water and the various sterilisation procedures applied (Fig. 3.1). After sterilisation all manipulations were carried out using standard sterile techniques (see Section B.2.VIII.). Samples were taken at the times shown in Fig. 3.1 and the degree of contamination was assessed using the techniques described in Section B.3.III.

Samples were taken so that the level of contamination could be assessed throughout the procedures. The general appearance of the plants was observed throughout the procedures so that any treatment which damaged the plant could be eliminated.

The levels of contamination at different stages in the procedure are shown in table 3.3. All samples were given a sample code shown in Fig. 3.1. The results of the analysis of the level of contamination for each sample are shown in table 3.3 alongside their sample code.

Some treatments may be discarded without any further consideration because of the damage done to the plant by the treatment. Treatment
Figure 3.1
experimental protocol

Non-sterile 5 week old tomato plants (2 mm, sampling region marks as for cell counts etc.)

- Each internode in 0.02% detergent
- Wash internode in H2O

- Remove internode in tissue

- Immerse internode for 2 mins.
- Irrigate internode for 0.3 ml
- Irrigate internode for 0.3 ml
- Irrigate internode for 0.3 ml

- Treat internode as for grafting using sterile supports etc.
- But do not actually graft

- Return to growth room

- Day 0

- Day 1

- Day 2

- Day 3

- Day 4

- Day 5

- Day 6
<table>
<thead>
<tr>
<th>Sample Code</th>
<th>Method of Sterilisation</th>
<th>No. of 2mm. segments per sample</th>
<th>No. of viable contaminants per sample</th>
<th>No. of viable contaminants per 2mm Segment</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>12% H₂O₂</td>
<td>5</td>
<td>10⁵</td>
<td>10⁵</td>
</tr>
<tr>
<td>1</td>
<td>12% H₂O₂</td>
<td>5</td>
<td>10⁶</td>
<td>10⁶</td>
</tr>
<tr>
<td>2</td>
<td>12% H₂O₂</td>
<td>5</td>
<td>10⁵</td>
<td>10⁵</td>
</tr>
<tr>
<td>3</td>
<td>12% H₂O₂</td>
<td>5</td>
<td>10²</td>
<td>10²</td>
</tr>
<tr>
<td>4</td>
<td>12% H₂O₂</td>
<td>5</td>
<td>10²</td>
<td>10²</td>
</tr>
<tr>
<td>A₁</td>
<td>Ultra-violet irradiation (10 mins.)</td>
<td>5</td>
<td>3.2 x 10²</td>
<td>6.4 x 10³</td>
</tr>
<tr>
<td>A₅</td>
<td>Ultra-violet irradiation (20 mins.)</td>
<td>5</td>
<td>1.6 x 10⁴</td>
<td>3.2 x 10³</td>
</tr>
<tr>
<td>B₁</td>
<td>Ultra-violet irradiation (20 mins.)</td>
<td>5</td>
<td>3 x 10³</td>
<td>6 x 10³</td>
</tr>
<tr>
<td>B₅</td>
<td>Ultra-violet irradiation (20 mins.)</td>
<td>5</td>
<td>10⁵</td>
<td>10⁵</td>
</tr>
<tr>
<td>C₁</td>
<td>Antibiotic A i.e., Chloramphenicol for 3 hours</td>
<td>5</td>
<td>10²</td>
<td>10²</td>
</tr>
<tr>
<td>C₃</td>
<td>Antibiotic A i.e., Chloramphenicol for 3 hours</td>
<td>5</td>
<td>4 x 10²</td>
<td>8 x 10</td>
</tr>
<tr>
<td>C₅</td>
<td>Antibiotic A i.e., Chloramphenicol for 3 hours</td>
<td>5</td>
<td>1.8 x 10³</td>
<td>3.5 x 10³</td>
</tr>
<tr>
<td>C₆</td>
<td>Antibiotic A i.e., Chloramphenicol for 3 hours</td>
<td>5</td>
<td>3.7 x 10⁴</td>
<td>7.4 x 10³</td>
</tr>
<tr>
<td>D₁</td>
<td>Antibiotic B Streptomycin/ Penicillin for 3 hrs.</td>
<td>5</td>
<td>10³</td>
<td>10³</td>
</tr>
<tr>
<td>D₃</td>
<td>Antibiotic B Streptomycin/ Penicillin for 3 hrs.</td>
<td>5</td>
<td>4 x 10³</td>
<td>8 x 10</td>
</tr>
<tr>
<td>D₅</td>
<td>Antibiotic B Streptomycin/ Penicillin for 3 hrs.</td>
<td>5</td>
<td>2.4 x 10³</td>
<td>4.7 x 10³</td>
</tr>
<tr>
<td>D₆</td>
<td>Antibiotic B Streptomycin/ Penicillin for 3 hrs.</td>
<td>5</td>
<td>5.6 x 10³</td>
<td>1.4 x 10³</td>
</tr>
<tr>
<td>E₁</td>
<td>Antibiotic C Tetacycline Oxetetracycline Streptomycin Chloramphenicol for 3 hrs.</td>
<td>5</td>
<td>10²</td>
<td>10²</td>
</tr>
<tr>
<td>E₃</td>
<td>Antibiotic C Tetacycline Oxetetracycline Streptomycin Chloramphenicol for 3 hrs.</td>
<td>5</td>
<td>3.0 x 10²</td>
<td>8 x 10</td>
</tr>
<tr>
<td>E₅</td>
<td>Antibiotic C Tetacycline Oxetetracycline Streptomycin Chloramphenicol for 3 hrs.</td>
<td>5</td>
<td>5.8 x 10²</td>
<td>1.4 x 2</td>
</tr>
<tr>
<td>E₆</td>
<td>Antibiotic C Tetacycline Oxetetracycline Streptomycin Chloramphenicol for 3 hrs.</td>
<td>5</td>
<td>7 x 10³</td>
<td>7 x 10³</td>
</tr>
<tr>
<td>F₁</td>
<td>12% H₂O₂ for 20 minutes</td>
<td>5</td>
<td>3 x 10³</td>
<td>6.0 x 10²</td>
</tr>
<tr>
<td>F₃</td>
<td>12% H₂O₂ for 20 minutes</td>
<td>5</td>
<td>8 x 10²</td>
<td>1.6 x 10²</td>
</tr>
<tr>
<td>F₄</td>
<td>12% H₂O₂ for 20 minutes</td>
<td>5</td>
<td>10³</td>
<td>10³</td>
</tr>
<tr>
<td>F₅</td>
<td>12% H₂O₂ for 20 minutes</td>
<td>5</td>
<td>2.6 x 10³</td>
<td>5.3 x 10²</td>
</tr>
<tr>
<td>F₆</td>
<td>12% H₂O₂ for 20 minutes</td>
<td>5</td>
<td>5.5 x 10³</td>
<td>1.1 x 10²</td>
</tr>
</tbody>
</table>
with hydrogen peroxide, ultra-violet irradiation and the final antibiotic solution containing tetracycline, oxytetracycline, streptomycin and chloramphenicol all caused very obvious damage to the plant. The effects seen ranged from blackening or shrivelling of the stem at the point of application, to distortion of the leaves or chlorosis, all of which would probably mean that grafting could not be carried out and also that one could not discount the probability of the treatment interfering with the process being studied.

Sterilisation by chloramphenicol on its own (treatment C) or the combination of streptomycin and penicillin did not have a visible effect on the plant. Treatment with chloramphenicol reduced the level of contamination of ungrafted internodes to acceptable levels and maintained this level for sufficient time for grafting experiments to be carried out. The level increased with time probably due to the multiplication of bacteria which are outwith the spectrum of action of chloramphenicol. The increase could also be due to migration from unsterilised areas although the internode is closely covered by a silicone rubber strip. Treatment with streptomycin/penicillin also reduced the number of viable bacteria by at least a hundred fold and these reduced levels remained fairly constant through the remainder of the experiment. Higher levels of sterility may be obtainable with both these agents with modification of the procedure. Chloramphenicol was selected for further experiments because it was able to reduce the levels of contamination significantly when used alone and does not affect plant systems at concentrations which are inhibitory to bacterial growth. The use of chloramphenicol as a sterilising agent was further investigated.

The concentration of chloramphenicol used in the preliminary experiment was very high (5 mg/ml) and is at a level which affects eukaryotic systems (Leaver and Edelman, 1965; Halvor and Freeman, 1968). Clearly it is essential to determine the minimal concentration at which chloramphenicol is effective.

**c. Determination of the minimal effective concentration of chloramphenicol for sterilisation.**

Ungrafted plants were taken through the same procedure as in the previous experiment (b) and treated with chloramphenicol solutions (0.3 ml) for three hours as shown in Fig. 3.2. The concentrations
Figure 3.2

Unsterile tomato plants
 wounds region marked

↓
wash with 0.02% detergent

↓
wash with H2O

↓
wrap internode in tissue

↓
apply 0.5ml of 0.3ml of 0.3ml of 0.3ml of
5000μg/ml 200μg/ml 100μg/ml 50μg/ml
chloramphenicol chloramphenicol chloramphenicol chloramphenicol
for 3 hrs

↓
remove tissue treat as for grafting
but do not cut sterile procedures

↓
sample

↓
remove to growth room

sample

↓
remove to growth room

sample

↓
remove to growth room

sample

↓
remove to growth room

DAY 0

DAY 1

DAY 2

DAY 3

DAY 4

sample

↓
remove to growth room

sample

↓
remove to growth room

sample

↓
remove to growth room

sample

↓
remove to growth room
<table>
<thead>
<tr>
<th>Sample Code</th>
<th>Method of sterilisation</th>
<th>No. of 2 mm. segments per sample</th>
<th>No. of Viable Contaminants per sample</th>
<th>No. of Viable Contaminants per 2mm Segment</th>
</tr>
</thead>
<tbody>
<tr>
<td>A₀</td>
<td></td>
<td>5</td>
<td>$10^3$</td>
<td>$10^2$</td>
</tr>
<tr>
<td>A₃</td>
<td>5000 µg/ml Chloramphenicol</td>
<td>5</td>
<td>$2.4 \times 10^3$</td>
<td>$4.9 \times 10^2$</td>
</tr>
<tr>
<td>A₄</td>
<td></td>
<td>5</td>
<td>$5.0 \times 10^3$</td>
<td>$1 \times 10^3$</td>
</tr>
<tr>
<td>B₀</td>
<td></td>
<td>5</td>
<td>$10^3$</td>
<td>$10^2$</td>
</tr>
<tr>
<td>B₃</td>
<td>200 µg/ml Chloramphenicol</td>
<td>5</td>
<td>$7.9 \times 10^3$</td>
<td>$1.6 \times 10^2$</td>
</tr>
<tr>
<td>B₄</td>
<td></td>
<td>5</td>
<td>$3.6 \times 10^3$</td>
<td>$6 \times 10^2$</td>
</tr>
<tr>
<td>C₀</td>
<td></td>
<td>4</td>
<td>$1.7 \times 10^3$</td>
<td>$2.4 \times 10^2$</td>
</tr>
<tr>
<td>C₃</td>
<td>100 µg/ml Chloramphenicol</td>
<td>5</td>
<td>$4 \times 10^4$</td>
<td>$8 \times 10^3$</td>
</tr>
<tr>
<td>C₄</td>
<td></td>
<td>5</td>
<td>$1.5 \times 10^3$</td>
<td>$3 \times 10^2$</td>
</tr>
<tr>
<td>D₀</td>
<td></td>
<td>5</td>
<td>$4 \times 10^2$</td>
<td>$8 \times 10$</td>
</tr>
<tr>
<td>D₃</td>
<td>50 µg/ml Chloramphenicol</td>
<td>5</td>
<td>$2 \times 10^3$</td>
<td>$4 \times 10^2$</td>
</tr>
<tr>
<td>D₄</td>
<td></td>
<td>5</td>
<td>$2 \times 10^3$</td>
<td>$4 \times 10^2$</td>
</tr>
</tbody>
</table>
employed were 5 000 µg/ml, 200 µg/ml, 100 µg/ml and 50 µg/ml. Samples were taken as before at the points indicated in Fig. 3.2. The levels of contamination found are shown in table 3.4. All concentrations of chloramphenicol reduced the level of contamination to around 10^2 organisms per 2 mm region of stem and these levels were maintained for at least four days after sterilisation. Clearly the lowest concentration, 50 µg/ml is the most useful as it will have the least effect on the plant. All subsequent sterilisation treatments were performed with a concentration of 50 µg/ml. So far, all of these assessments of sterilisation procedures have for convenience been carried out on ungrafted plants so that it still remains to ascertain whether or not the actual procedure of grafting will introduce more contaminants or enable those which are present to multiply in the wound area. It is also important to determine whether the sterilisation procedure will allow the normal development of the graft union, this will be investigated first because any effect of chloramphenicol on graft development would automatically rule out the use of this sterilisation procedure.

To determine whether the sterilisation procedure involving chloramphenicol did not affect the development of the graft union two populations of grafts were set up. One population was set up as described in Section B.2.II. and the other was grafted using sterile procedures involving essentially the same steps as shown in Fig. 3.2 treatment D except that the graft was constructed after the sterilisation procedure. This is described in more detail in Section B.2.VII.c. The breaking weight on Day 7 (i.e. the time by which graft formation will be complete) of the sterilised and control (untreated grafts) were determined and compared. No significant difference was found in the breaking weight of the two populations so it may be concluded that sterilisation has no marked effect on the development of the union. This sterilisation method can be used in conjunction with grafting experiments provided it is effective in maintaining sterility in grafted internodes.

d. Determination of the effectiveness of the sterilisation method after grafting and labelling procedures.

To determine whether sterilisation with 50 µg/ml chloramphenicol is effective when applied to the grafting situation the internodes to
be grafted were sterilised as in Section C.3.I.c (treatment D). One half of the plants were grafted using sterile procedures described in Section B.2.VIII. and the other half were treated in a similar way but not grafted. The procedures are summarised in Fig. 3.3. After this some plants were left undisturbed whilst others were taken through the procedures employed for incubation in labelled amino acid solutions to determine whether the levels of contamination could be kept low during labelling procedures. It is during these procedures that the levels of contamination must be kept very low to minimise the incorporation of labelled amino acids into micro-organisms. Sampling procedures have been previously described and the results obtained are shown in table 3.5.

The samples taken on the final day (C_7 and B_7) from the populations of ungrafted and grafted plants left undisturbed after sterilisation and "grafting" procedures had been completed, exhibited differing levels of contamination. The ungrafted sample showed a low and acceptable level of contamination but the level of contamination in the grafted sample was ten times greater. In the case of internodes taken through procedures employed in labelling with amino acids the levels of contamination were higher (see B_t and C_t). The levels of contamination of grafted internodes taken through these procedures (B_t) are certainly too high, there having been an increase in contamination during the incubation and transfer process of up to a hundred-fold. The bracketed values represent contamination levels determined in two separate experiments. The ungrafted internodes do not show such a large increase in contamination level and the levels are acceptable. The cause of the increase in contamination in the grafted internodes during incubation must be ascertained and eliminated.

It is possible that the contaminants are introduced during the excision and transfer process. If the contamination was solely as a result of introduction of contaminants during excision and transfer procedures a similar rise in the contamination levels of ungrafted tissue would be expected. The difficulty in handling the grafted internode aseptically is greater than handling of the ungrafted internode, so this may lead to additional contamination. The other alternative is that the increase in the level of micro-organisms was due to multiplication of contaminants already present from initial low levels to higher levels after incubation. Reduction of the increase
Figure 3.3
experimental protocol

Unsterile tomato plants
sampling area marked

- wash in 0.05% detergent
- wash in H2O
- wrap internode in tissue
- apply sterilising solution
  50μg/ml chloramphenicol (0.5ml)
  leave for 3 hrs.

Graft 1st internode

Graft plants after removing tissue using sterile procedures

DAY 0

DAY 1

DAY 2

DAY 3 sample A3 sample B3

DAY 4 sample A4

DAY 5

DAY 6

DAY 7 sample A7 sample B7

UNSTERILE GRAFTED STERILE GRAFTED STERILE UNGRAFTED
<table>
<thead>
<tr>
<th>Sample Code</th>
<th>Method of sterilisation (grafted/ungrafted)</th>
<th>No. of 2 mm. segments per sample</th>
<th>No. of viable contaminants per sample</th>
<th>No. of viable contaminants per 2 mm segments</th>
<th>Time of sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>50 μg/ml Chloramphenicol</td>
<td>5</td>
<td>1.2 x 10^3</td>
<td>2.4 x 10^2</td>
<td>After sterilisation</td>
</tr>
<tr>
<td>A_3</td>
<td></td>
<td>5</td>
<td>2.0 x 10^4</td>
<td>4 x 10^3</td>
<td>3 days after grafting</td>
</tr>
<tr>
<td>A_4</td>
<td>grafted-untreated</td>
<td>5</td>
<td>1 x 10^5</td>
<td>2 x 10^4</td>
<td>4 days after grafting</td>
</tr>
<tr>
<td>A_7</td>
<td></td>
<td>5</td>
<td>4 x 10^5</td>
<td>8 x 10^4</td>
<td>7 days after grafting</td>
</tr>
<tr>
<td>B_0</td>
<td></td>
<td>4</td>
<td>3.75 x 10^2</td>
<td>9 x 10</td>
<td>After sterilisation and grafting</td>
</tr>
<tr>
<td>B_3</td>
<td>50 μg/ml Chloramphenicol</td>
<td>5</td>
<td>(3.6 x 10^3)</td>
<td>(7 x 10^2)</td>
<td>3 days after</td>
</tr>
<tr>
<td>B_4</td>
<td>grafted</td>
<td>5</td>
<td>(2 x 10^4)</td>
<td>(5 x 10^3)</td>
<td>After transfer and incubation</td>
</tr>
<tr>
<td>B_7</td>
<td></td>
<td>5</td>
<td>(8 x 10^4)</td>
<td>(1 x 10^5)</td>
<td>7 days after</td>
</tr>
<tr>
<td>C_0</td>
<td></td>
<td>5</td>
<td>2.7 x 10^4</td>
<td>5 x 10^3</td>
<td>After sterilising etc.</td>
</tr>
<tr>
<td>C_3</td>
<td>50 μg/ml Chloramphenicol</td>
<td>5</td>
<td>(4.0 x 10^3)</td>
<td>(8.0 x 10^2)</td>
<td>3 days after</td>
</tr>
<tr>
<td>C_4</td>
<td>ungrafted</td>
<td>5</td>
<td>(1.4 x 10^3)</td>
<td>(3 x 10^2)</td>
<td>After transfer and incubation</td>
</tr>
<tr>
<td>C_1</td>
<td></td>
<td>5</td>
<td>(2 x 10^3)</td>
<td>(4 x 10^2)</td>
<td>7 days after</td>
</tr>
</tbody>
</table>
in contamination in either case might be achieved if the time of application of the sterilisation agent were changed.

e. Determination of the most effective sterilisation time.

Sterilisation prior to the construction of the graft makes it necessary to maintain low levels of contamination over a long period of time and throughout many manipulations. If the sterilisation agent was applied immediately prior to the labelling procedure then both this time period and the number of these manipulations would be reduced.

To minimise mechanical disturbance of the graft the silicone rubber support was lined with filter paper to which the chloramphenicol was added as before. Details of the procedure are shown in Fig. 3.4. Samples were taken before and after sterilisation, and also after excision and transfer to incubation bottles and finally after twenty-four hours in the incubation bottle. Samples were taken at these times to ascertain the effectiveness of the procedure, showing the times at which contamination was introduced and also whether adequate levels of sterility could be maintained throughout similar procedures as used for labelling studies. A set of ungrafted controls was also taken through the procedure. The results are presented in table 3.6. The level of contamination three days after grafting in unsterilised plants was in the order $10^5 - 10^6$ micro-organism per segment, but slightly lower in ungrafted plants. After application of the sterilisation agent this was reduced to $10^3$ contaminants per segment, however, after excision and transfer to the incubation bottle this number rose to $10^4$ and after twenty-four hours incubation the number on the grafted segment was in the region of $10^6$ to $10^7$, whilst on ungrafted internodes it had risen to $10^5$. These levels are unacceptable. A considerable amount of protein synthesis will inevitably accompany the increase in number of contaminants causing interference in the study of incorporation of labelled amino acids. It appears that unless the levels can be substantially reduced either prior to grafting or incubation then the numbers, which either multiply from resistant bacteria present on the internode, or migrate from the rest of the plant, or are introduced during manipulation, will increase to unacceptable levels during the course of the experiment.

The only other obvious alternative is the use of "sterile" plants under sterile conditions throughout their life time. This is not
Figure 3.4
experimental protocol

Day 0
Grafted tomato plants
allow graft to
develop for 3 days

Ungrafted tomato plants

Day 3
Sample

Г 3

apply 0.3ml of 50μg/ml chloramphenicol for 3 hrs
to saturate filter paper around grafted internodes

Sample

Г 3s

excise and transfer grafted internodes to incubation bottles observing aseptic procedures throughout

Sample

Г 3st

allow to develop for 24hrs

Day 4
Sample

Г 4

as for grafted internodes

Sample

Г 3st

as for grafted internodes

Sample

Г 3s

as for grafted internodes

Sample

Г 4
<table>
<thead>
<tr>
<th>SAMPLE CODE</th>
<th>SAMPLE DESCRIPTION</th>
<th>NO. OF Viable CONTaminants PER SAMPLE</th>
<th>NO. OF Viable CONTaminants PER 2mm. SEGMENT</th>
</tr>
</thead>
<tbody>
<tr>
<td>G₃</td>
<td>day 3 grafted unsterilised</td>
<td>$10^6$</td>
<td>$10^5$-$10^6$</td>
</tr>
<tr>
<td>G₃ₘ</td>
<td>day 3 grafted sterilised</td>
<td>$1.42 \times 10^4$</td>
<td>$2.8 \times 10^3$</td>
</tr>
<tr>
<td>G₃ₘₜ</td>
<td>day 3 grafted sterilised excised and transferred to incubation bottles.</td>
<td>$4.7 \times 10^5$</td>
<td>$2.4 \times 10^4$</td>
</tr>
<tr>
<td>U₃</td>
<td>day 3 ungrafted unsterilised</td>
<td>$2.1 \times 10^6$</td>
<td>$4.2 \times 10^5$</td>
</tr>
<tr>
<td>U₃ₘ</td>
<td>as G₃ₘ but ungrafted</td>
<td>$1.2 \times 10^4$</td>
<td>$2.4 \times 10^3$</td>
</tr>
<tr>
<td>U₃ₘₜ</td>
<td>as G₃ₘₜ but ungrafted</td>
<td>$6.2 \times 10^4$</td>
<td>$1.2 \times 10^4$</td>
</tr>
<tr>
<td>G₄</td>
<td>day 4 grafted sterilised. After 24 hrs. incubation</td>
<td>$10^6$-$10^7$</td>
<td>$10^5$-$10^6$</td>
</tr>
<tr>
<td>U₄</td>
<td>as G₄ but ungrafted</td>
<td>$1.1 \times 10^6$</td>
<td>$2.2 \times 10^5$</td>
</tr>
</tbody>
</table>
feasible where the plant must be at least 5-6 weeks old before grafting and then maintained in a sterile condition for a further seven days. If the age at which the plant could be grafted were reduced then this approach might be more feasible. Tomato plants less than 5 weeks old are not amenable to grafting because the plants are too small and their internodes are too short. Also tomato plants grown under sterile conditions are weak and unsuitable for grafting.
C.3.II. **Assessment of the use of "sterile" plants raised in a sterile environment for the study of incorporation patterns in graft formation.**

The conditions necessary to make the use of "sterile" plants possible for grafting experiments are:

1. Short growth period prior to grafting.
2. Reasonably large and robust stem/hypocotyl for grafting.
3. Rapid development of the graft union.
4. Ability to grow on a sterile growth medium.
5. High and uniform germination and growth rate yielding large uniform populations.

Another group of plants with a range of abilities to form heterografts are certain members of the Cucurbitaceae. The grafting relationships of *Cucumis sativus*, *Cucumis melo* and *Cucurbita ficifolia* (Garner, 1970) suggest combinations of compatibility similar to the tomato-*Datura-Nicanera* system and might be used as an alternative. Initial experiments were confined to *Cucumis sativus*, the cucumber, to assess the feasibility of producing sterile plants which can be grafted and kept sterile through formation of the graft union. The cucumber was selected as a likely candidate because it is possible to produce a large robust seedling in a relatively short period of time and it also exhibits a high and uniform germination rate making it possible to produce large uniform populations.

The basic strategy employed was to surface sterilise the dry seeds and after imbibing the seeds, to germinate them on a medium which would screen for any remaining contaminants. Then the uncontaminated seedlings were selected and transplanted into a sterile growth medium and maintained in sealed boxes which were only opened up for the taking of samples to assess the levels of contamination or for grafting of the seedlings.

A summary of the procedures involved in the raising and grafting of sterile plants can be seen in Fig. 3.5. Details of these procedures are given in Section B.2.VIII.c-f. Section B.3.III.b gives details of how estimation of the contamination levels of the cucumber was carried out. The times at which samples were taken are shown in Fig. 3.5. The contamination levels can be seen in table 3.7. The initial
Figure 3.5
experimental protocol

Unsterilised cucumber seed

- allow seeds to germinate as for sterilised ones

DAY 0-1
- submerge in absolute ethanol for 5 mins
- rinse in sterile distilled water
- submerge for 30 mins in 2% sodium hypochlorite (10% of commercial solution)
- 6X5 mins washes in sterile distilled water
- imbibe seeds overnight in sterile sealed container in sterile distilled water at 0°C

DAY 0
- place seeds 6/ plate onto mineral salts/sucrose agar and allow seeds to germinate for 4 days in the light at 23°C
- select uncontaminated seedlings
- place plate in 4" pot containing sterile growth medium
- 4 seedlings/pot
- place pot in propagator (sterilised)
- repeat till propagator full and seal

A sample by placing seedling onto surface of nutrient agar plates
- transfer to growth room and allow seedlings to grow

DAY 6
DAY 7
Day 8
Day 9
Day 10
Day 11

B sample (TY17) cyn segments

DAY 12

C sample (TY10) cyn segments

- graft plants observing sterile techniques

DAY 13

D sample (TY10) cyn segments around grafted region

(5 days after grafting)

E sample (TY10) cyn segments around grafted region

Day 14
Day 15
Day 16
Day 17
Day 18
<table>
<thead>
<tr>
<th>Sample Code</th>
<th>No. of 2mm segments per sample</th>
<th>No. of Viable Contaminants per sample</th>
<th>No. of Viable Contaminants per 2mm Segment</th>
<th>No. of Contaminated seedlings out of a sample of 5 seedlings</th>
<th>Sample Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>F</td>
<td></td>
<td></td>
<td></td>
<td>5 5 5</td>
<td>Presterilisation 3 x 5 seeds</td>
</tr>
<tr>
<td>A</td>
<td></td>
<td></td>
<td></td>
<td>0 0 4</td>
<td>Seeds which have been germinated and planted (3 x 5 seeds)</td>
</tr>
<tr>
<td></td>
<td>a</td>
<td>10</td>
<td>20</td>
<td>2</td>
<td>8 days of growth</td>
</tr>
<tr>
<td></td>
<td>b</td>
<td>10</td>
<td>0</td>
<td>0</td>
<td>(2 mm from centre of hypocatyl).</td>
</tr>
<tr>
<td>B</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>12 days growth. Just before grafting</td>
</tr>
<tr>
<td></td>
<td>a</td>
<td>10</td>
<td>20</td>
<td>2</td>
<td>Immediately after grafting</td>
</tr>
<tr>
<td></td>
<td>b</td>
<td>10</td>
<td>30</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Six days after grafting</td>
</tr>
<tr>
<td></td>
<td>a</td>
<td>10</td>
<td>30</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>b</td>
<td>10</td>
<td>300</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>a</td>
<td>10</td>
<td>$10^5$</td>
<td>$10^4$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>b</td>
<td>10</td>
<td>$5 \times 10^5$</td>
<td>$5 \times 10^4$</td>
<td></td>
</tr>
</tbody>
</table>
sterilisation was effective in reducing the number of contaminated seeds to the odd one or two which were discarded prior to transplantation. The seeds were also sampled in a semi-quantitative way after harvesting and in most cases there was no evidence of contamination, however, in one sample there appears to have been some introduced contamination. More quantitative results were obtained once the plants had grown into reasonably sized seedlings. These show very low levels of contamination with only a few individual contaminants on each segment. This level was maintained for up to 12 days after which the plants were grafted. Sampling immediately after grafting shows a slight rise in the level of contamination in one replicate but not in the other, this indicates the difficulty of preventing contaminants being introduced during the grafting process. However, the levels in both cases would not seriously interfere with the experiments which would be performed using this system. After allowing the graft to develop for six days further samples were taken and these show in both cases unacceptably high levels of contamination. The degree of contamination has increased by a thousand-fold over this period of six days, whereas before grafting, even over a period of 12 days, no more than the odd contaminant was present and these did not increase in this time. However, once the plant was grafted even if there was no serious contamination at the time of graft construction, the act of grafting appears to enable the number of contaminants to increase rapidly. Severing of the vascular tissue during the act of grafting releases large amounts of sugars and other nutrients from the plant and makes them directly available to the contaminants thus allowing them to multiply by a thousand-fold in a period of six days.

At the same time an examination of the development of the hypocotyl graft was made to determine whether the duration of the graft formation was suitable for experiments performed under sterile conditions. The examination of the cucumber hypocotyl graft was made in terms of breaking weight alone. Grafting procedures are described in Section B.2.III. Breaking weight was determined in the same way as for tomato autografts (Section B.3.I). The results are shown in Fig. 3.6. Values of breaking weight are recorded initially, due to adhesion as soon as the graft is constructed. In the tomato autograft a breaking weight of 0 g is found at zero time but with cucumber values of the order of 30 g have been recorded for grafts which have only just been assembled.
Figure 3.6.

Increase in breaking weight of cucumber hypocotyl grafts with time.
Although the initial breaking weight was high it did not increase very rapidly. After seven days the breaking weight did not exceed 100 g. Over the next thirty days a slow increase in breaking weight was seen, rising to 500 g, and if grafts were left undisturbed for seventy days, a mean breaking weight of 709 g was recorded, with individual values in excess of 900 g. This showed that the cucumber hypocotyl graft was not intrinsically weaker than the tomato autograft but that its development was a great deal slower taking 5-6 weeks to develop to the same extent as the tomato autograft does in 7 days.

The slow rate of development of the cucumber hypocotyl graft means that although it need only be maintained sterile for 9 days before grafting, it must be maintained in this condition for a number of weeks after grafting. As the number of contaminants in the grafted region can multiply by a thousand-fold over six days use of the cucumber system for incorporation studies must be ruled out.

No further attempt was made to impose sterile conditions on grafting experiments. The dual labelling technique allows direct comparison of the pattern of incorporation of labelled amino acids into protein by grafted and ungrafted tissue. As it proved impossible to maintain complete sterility in grafted tissue even using totally sterile plant, and attempts to do so ensured a considerable increase in the level of contamination under conditions similar to those used for incorporation studies, it was felt that attempts at sterilisation were increasing the amount of synthetic activity of the contaminants. The same procedures applied to ungrafted tissue resulted in much lower levels of contamination, by the end of the procedures there was also a large difference between the levels of contamination present on grafted and ungrafted tissue. Direct comparison of both tissues after attempted sterilisation would reflect the differences in bacterial population, as well as the differences in the activity of the plant tissue. If the bacterial populations were similar in grafted and ungrafted tissue, then direct comparison of the incorporation patterns would reflect the differences in the activity of the plant tissue. This assumes that the bacterial population was not induced to behave differently as a result of grafting, an assumption impossible to test at present.

As attempted sterilisation tends to lead to a greater difference between the contamination levels of grafted and ungrafted tissue no attempt will be made to impose sterile conditions on the tissue used
for incorporation studies. The possibility that part of the pattern obtained was due to the bacterial population cannot be ruled out. However, certain protein fractions allow more confidence in the results obtained. It was unlikely that many bacteria could be disrupted by the comparatively low shearing forces used in extraction of plant tissue. The insoluble protein fraction (Section B.3.IV.) was most likely to contain protein derived from the micro-organisms present on the surface of the plant material because intact bacteria will sediment under a 10,000 g force applied for ten minutes. The soluble fraction is unlikely to be contaminated with protein derived from these micro-organisms. Clearly the results obtained by comparison of the soluble fractions are unlikely to be due to the action of such micro-organisms. For the same reason comparison of the patterns of incorporation into protein from membrane fractions can be regarded with confidence as being due to the activity of the plant rather than any bacterial contamination as these were obtained from the supernatant of a 10,000 g centrifugation. The following chapters investigate graft induced incorporation of labelled amino acids into protein without any attempt at imposing sterility.
Previous chapters have been devoted to the development of a grafting system which can be manipulated to study the biochemical events which accompany the establishment of a graft. Interest in the role of proteins in the development of the graft was first aroused after observations of the grafting relationships which exist between various members of the Solanaceae (Yeoman and Brown, 1976; Yeoman et al., 1978) which suggested that structural incompatibility or release of toxic substances could not account for the relationships found. This raised the possibility that during graft formation a form of cell recognition might take place which involved protein-based cell interactions akin to that postulated for pollen-stigma interactions (Knox, 1973; Knox and Heslop-Harrison, 1973; Knox et al., 1976; etc.). Such proteins might accumulate early in the development of the graft and influence the subsequent development of the union preventing (incompatible) or encouraging (compatible) the integration of stock and scion. While it is realised that a recognition phenomenon, if one exists, would be easier to investigate in compatible and incompatible heterografts, it is necessary in a preliminary study to ascertain the complex changes which accompany autograft formation. For this reason the following experiments have been confined to the tomato autograft to provide a background against which further investigations can be conducted.

The pattern of protein synthesis accompanying graft formation can be determined by comparison of the pattern of synthesis by grafted tissue, with that of ungrafted tissue. The pattern of incorporation of amino acids into protein from ungrafted tissue reflects the "normal" spectrum of protein synthesis of the tomato stem. Not all the proteins synthesized by grafted tissue are a result of graft formation, however by comparison of the patterns obtained from grafted and ungrafted tissue it is possible to determine the pattern of synthesis occurring as a result of graft formation. Changes in the patterns of protein synthesis were studied by following the incorporation of radioactively labelled amino acids into protein. A dual labelling procedure was employed.

Cysteine, labelled with two different isotopes ($^{35}$S and $^{32}$H), was supplied to the grafted and ungrafted internodes. After incubation for twenty-four hours the tissue was extracted and the proteins separated using polyacrylamide-$S$-$S$ slab gel electrophoresis. This separates the
polypeptides according to their molecular weight. The incorporation of the labelled amino acid into proteins separated on the gel was measured by counting gel slices in a liquid scintillation spectrometer. The two isotopes supplied have sufficiently different spectra of emission that the liquid scintillation spectrometer was able to distinguish the emission due to each isotope. This allows the extraction and analysis of grafted and ungrafted material as one sample. The radioactivity of the proteins of the two tissues can be distinguished during the counting procedure. From the point of extraction the two tissues are subjected to exactly the same conditions so the results obtained are directly comparable. This eliminates error due to:

- Differences in extraction procedure.
- Differences in separation of the protein on the gel.
- Differences in positioning of gel slices.
- Differences in counting conditions.

Elimination of the above, as possible causes of differences in the pattern of protein synthesis, increased confidence that the results obtained were a result of grafting.

It was not possible to eliminate differences in incubation conditions but, it was the relative distribution through the different proteins and not the actual amount of incorporation that was being studied, so these were not important.

The study was confined to three consecutive, twenty-four hour periods which cover the time during which vascular continuity was achieved. Incidentally it is over this period that the differences first appear between compatible and incompatible heterografts (Yeoman and Brown, 1976). The earliest point in the development of the graft union at which it is strong enough to withstand manipulation occurs forty-eight hours after grafting. From this time onwards, for the next three successive, twenty-four hour periods, i.e. the period between Days 2 and 3, Days 3 and 4 and Days 4 and 5, the incorporation patterns of amino acids into protein were examined. In developmental terms this is the period during which the graft progresses from an initial tenuous adhesion probably due to the deposition of pectins and other substances which hold cells together (Lindsay, 1972), to the stronger final breaking weights consequent on the establishment of vascular continuity.
The study was conducted under clean rather than sterile conditions. Previous experiments had shown (Section C.3.) that in attempting to obtain sterile grafts large differences were generated in the contamination levels between grafted and ungrafted internodes. Using clean conditions without sterilisation the number of micro-organisms was high but similar in grafted and ungrafted individuals. In these circumstances it is not possible to attribute with certainty any change, solely to the activity of the plant but it should be remembered that plants do not grow in isolation and that microles are always present during graft formation.
C.4.I. Comparison of incorporation patterns of radioactively labelled methionine in grafted and intact internodes.

The protocol used in this experiment is shown in Fig. 4.1. Plants were grafted as described previously in Section B.2.II. and left to develop undisturbed. After two days ten grafted internodes were excised and incubated in \(^{35}\)S-methionine for 24 hours (Fig. 4.1). Simultaneously four ungrafted internodes were incubated in \(^{3}H\)-methionine. Grafts in excised internodes are able to continue developing normally for twenty-four hours after excision (see Section C.2.II.). After twenty-four hours the premarked grafted region and the equivalent ungrafted region were excised, frozen on solid \(\omega_2\) and stored in a deep freeze (ca \(-20^\circ C\)). The same procedure was repeated on Day 3 and again on Day 4. This technique ensures that the labelled precursors are applied directly to the grafted internode or the equivalent ungrafted internode.

When all the tissue had been harvested then the fresh weight of the tissue from the grafted region was determined and an equal weight of ungrafted tissue (labelled with \(^{3}H\)-methionine) was removed from the region nearest the centre of the internode. The mixture of grafted and ungrafted tissues was extracted and analysed together. The extraction, electrophoresis and counting techniques have been described earlier (Section B.3.IV - B.3.VI.). Both the "soluble" and "insoluble" protein fractions, as defined in Section B.3.IV., were analysed separately. (Use of this system means that competition for label by other parts of the plant is eliminated so increasing the incorporation levels.)

The results are presented as graphs (Figs. 4.2 - 4.7) showing the ratios of counts per minute (c.p.m.) due to \(^{35}\)S and \(^{3}H\) against the relative mobility of the proteins. The relative mobility was calculated by expressing the position of a gel slice as a percentage of the distance from the top of the gel to the solvent front (i.e. as a percentage of the maximum distance the protein could have run). This makes the patterns derived from different gels more comparable. The ratios of radioactivity due to \(^{35}\)S and \(^{3}H\) were calculated by correcting the values obtained from the scintillation counter for background activity and spillover between channels, then calculating the ratio of the corrected activities as described in Section B.3.VI. Where the
**Figure 4.1**

Experimental protocol

**DAY 0**

**GRAFT PLANT**

- First trifoliate leaf: cut 2 mm

- Cotyledon: cut

**DAY 2, 3, 4**

- Excise grafted internode and transfer to incubation bottles

- Incubation bottles contain 0.2 ml mineral salts medium +5 μCi ^35^S-methionine grafted

- Or 100 pCi ^3H^-methionine ungrafted

- Ungrafted controls subject to the same procedures except they are not grafted.

**DAY 3, 4, 5**

- Harvest tissue and put on ice

**GRAFTED**

- Cut out region immediately on either side of graft

- Discard

**UNGRAFTED**

- Slice up and take tissue to equal the fresh weight of the grafted tissue, from 1 cm in the middle of the internode

- Pool tissue

 carry out extraction procedures on the mixture of grafted and ungrafted tissue
ratio of $^{35}$S activity: $^3$H activity was less than 1 then it is presented as the inverse ratio, to avoid the crowding of these values into the part of the graph between 0 and 1. Otherwise, the full extent of the pattern, where activity can increase and decrease on either side of unity depending on increases and decreases in activity of either grafted or ungrafted tissue, would not be seen. All ratios are presented as numbers greater than 1 lying on the appropriate side of the horizontal axis as either $^{35}$S: $^3$H or $^3$H: $^{35}$S. The numerical values of the ratios do not have any significance on their own as the differences in incorporation of the two isotopes due to differences in specific activity of the label and uptake mean that a ratio of activities with a numerical value of one did not necessarily reflect equal metabolic activities. Significance was only attached to regions where the ratio of activities differed greatly from the overall pattern across the gel. Great care must be taken when comparing patterns obtained from different initial tissues and then run separately on different gels which are sliced separately because these will not be directly comparable for the reasons already stated. So the patterns obtained from different samples can only be compared with reservations and significance should not be attached to numerical values but to changes in distribution of relative activity.

a. Soluble protein fractions.

Fig. 4.2 shows the incorporation pattern obtained by comparing the activity of grafted and ungrafted tissue for the period from 2-3 days after grafting. Most ratios across the gel fluctuate around a value of one but there are five areas which stand out markedly from the rest. These are just past the top of the gel (beyond the interface) two peaks around 25% of the mobility, one at 55% and one at 70%. These peaks are due to a greater incorporation of radioactive precursor into proteins (or subunits) by grafted rather than by ungrafted tissue.

In Fig. 4.3 the incorporation pattern for the period 3-4 days after grafting can be seen. The ratios fluctuate widely in different regions of the gel but two regions can be seen to indicate greater relative activity in the ungrafted tissue, due to protein subunits migrating through about 20% and 65% of the gel. There are many peaks representing greater activity of grafted tissue particularly in the top half of the gel where proteins of higher molecular weight are
Figure 4.2
Comparison of incorporation patterns into soluble protein of grafted and ungrafted tissue 2-3 days after grafting.
Figure 4.3
Comparison of incorporation patterns into soluble protein of grafted and ungrafted tissue 3-4 days after grafting.
located. Proteins found in the slices representing \(16\%\), \(23\%\), \(27-32\%\), \(35\%\), \(40\%\) and \(45\%\) mobility appear to differ from the rest of the pattern although this is not so obvious as in Fig. 4.2 because of the greater number of fluctuations found. Clearly it becomes extremely difficult to decide whether there is an overall baseline of ratios in which variation is small and nearly all the proteins behave differently in relation to each other or the background variation is very high.

The incorporation pattern four to five days after grafting is shown in Fig. 4.4. Again there are large fluctuations across the gel but some peaks in particular stand out from the rest. These represent protein subunits with mobilities of \(25\%\), \(27\%\), \(33-35\%\), \(45\%\), \(77\%\) and \(87\%\) together with smaller peaks from which it is difficult to assess the significance. In most regions the ratio indicates greater relative activity on the part of the grafted tissue except at the extreme ends of the gel.

Considering the three periods of time studied the activity of the grafted tissue differs from the ungrafted tissue more and more as time progresses with differences in incorporation being seen in a larger proportion of the gel as time progresses. This suggests that, as the graft develops, the pattern of soluble proteins synthesized diverges increasingly from the control situation. It is difficult to assess the significance of the actual numerical values of the ratios as these will be dependent on the amount of label supplied and taken up by different tissue but if the pattern of these ratios is taken in conjunction with the ratios observed in the insoluble protein fraction from the same sample this should clarify matters. For example, the range of numerical values of the ratios on Days 4-5 is much greater than those from preceding samples which if mirrored by the insoluble protein may be attributed to the supply of precursors rather than a real effect of the different activities of the experimental tissues.

b. **Insoluble protein.**

The patterns of incorporation for the insoluble protein fraction are shown in Figs. 4.5, 4.6, 4.7. These show the pattern of activity for the periods between 2-3, 3-4 and 4-5 days after grafting respectively. During the first twenty-four hour period there are a few larger peaks which stand out from the background. In particular there are two peaks of activity by grafted tissue representing a
Figure 4.4

Comparison of incorporation patterns into soluble protein of grafted and ungrafted tissue 4-5 days after grafting.
Figure 4.5

Comparison of incorporation patterns into insoluble protein of grafted and ungrafted tissue 2-3 days after grafting.
mobility of 25% and 55% and one which represents greater activity on the part of the ungrafted tissue at 32%. However, even these peaks are encompassed by the background fluctuation found with the soluble protein fraction for the same tissue (Fig. 4.2).

For the period 3-4 days after grafting (Fig. 4.6) the pattern is of many areas of greater relative activity on the part of both the grafted and ungrafted tissue. The greater activity due to the grafted tissue is localised in three areas, 20-35%, 45-55% and 65-75%, each of which is divided into three or four peaks. Each of these peaks is not representative of one slice of the gel and almost certainly represents many protein subunits. There are also three areas with greater relative activity due to the ungrafted tissue at 10-15%, ≥35% and 62-64% mobility. Compared with the soluble protein pattern the differences in particular protein groups are more easily seen whilst the ratios lie in the same range. The patterns of incorporation at the top of the gel in soluble and insoluble fractions are very different. In the soluble protein fraction there appears to be more activity due to the ungrafted tissue. Also towards the bottom of the gel 62-64% mobility, there is an area of greater relative activity on the part of the grafted tissue which is not apparent in the equivalent soluble protein fraction.

Fig. 4.7 shows the incorporation pattern produced during the period between 4-5 days after grafting. The pattern can be divided into two parts. Most peaks of relative activity are confined to the top part of the gel whereas the bottom part contains only minor fluctuations. There are distinct peaks at 4 and 7% mobility followed by a similar one at about 20%, three peaks increasing in height from 28-35%, and a very large peak at 40%, all of which represent greater relative activity on the part of the grafted tissue. The very large peak at 40% is due to virtually no $^3$H counts in that region of the gel rather than increased $^{35}$S counts or activity of grafted tissue. Therefore in this region of the gel the incorporation due to ungrafted tissue does not differ markedly from that of adjacent regions but because there is little or no incorporation by the ungrafted tissue compared with that of adjacent regions the large value for the ratio is produced. This must not be taken to indicate large incorporation by grafted tissue into the proteins of this region of the gel compared to the rest of the proteins in grafted tissue, but simply as a big difference between the incorporation of grafted and ungrafted tissue.
Figure 4.6

Comparison of incorporation patterns into insoluble protein of grafted and ungrafted tissue 3-4 days after grafting.
Figure 4.7

Comparison of incorporation patterns into insoluble protein of grafted and ungrafted tissue 4-5 days after grafting.
in this region. The ratios obtained on Days 4-5 are considerably larger numerically than on the preceding times in both soluble and insoluble protein fractions. But this is due to lower incorporation of $^3$H by the ungrafted control tissue than on preceding days which might well be due to a discrepancy in supply or uptake of the $^3$H-methionine by this sample. Therefore it is not possible to attach any significance to the high ratios produced on this day although consideration of the relative incorporation pattern within these extracts is still valid.

The experiment was repeated to ascertain whether the results obtained were reproducible. Fig. 4.8 shows a comparison of the breaking weights from both experiments so that the two experiments can be compared taking into account any difference in the rate of development of the graft union. It is obvious that in the first experiment the development was slower by about twenty-four hours than in the second. This must be taken into consideration when comparing the incorporation data, where apart from the reasons already outlined, one should not expect close similarity in patterns obtained from the two experiments. Better correlation between the two experiments might be found if the comparison is made as follows:

<table>
<thead>
<tr>
<th>Experiment I</th>
<th>Experiment II</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 2-3</td>
<td>may be comparable to Day 2-3</td>
</tr>
<tr>
<td>Day 3-4</td>
<td>may be comparable to Day 3-4</td>
</tr>
<tr>
<td>Day 4-5</td>
<td>may be comparable to Day 3-4</td>
</tr>
</tbody>
</table>

b. Soluble protein.

Fig. 4.9 shows the incorporation pattern obtained for soluble protein 2-3 days after grafting. There is a small basal variation with a few regions in which the ratios are markedly different, these occur in the region representing 25-35% mobility where there appears to be more relative activity due to ungrafted tissue, whilst greater relative activity of grafted tissue is seen in regions representing 5-10%, about 55% and 80% mobility. If this is compared with the first experiment then comparison with Day 2-3 (Fig. 4.2) shows a certain amount of similarity between the two patterns although the very striking peaks found on the first run of the experiment are not seen. There is a striking difference in the region representing
Comparison of the rate of development of the graft union between experiments 1 and 2.
Comparison of incorporation patterns into soluble protein of grafted and ungrafted tissue 2-3 days after grafting.
25-35% mobility where there is a large peak of greater activity due to
grafted tissue in Fig. 4.2 whilst in Fig. 4.9 there is a smaller peak
of greater relative activity due to ungrafted tissue. If Fig. 4.9 is
compared to Fig. 4.3 (i.e. to Days 3-4 after grafting in the first
experiment) then there are certain similarities which can be seen
especially in this 25-35% region. Again although the patterns are not
identical, particularly in the latter part of the gel, the patterns
follow each other to a large extent. If there was a difference in
timing of the developmental sequence between the two experiments but
the difference was not an exact twenty-four hour period coincident with
the labelling periods then similarities with both times might be
expected as the experiments record net activity over the period in
question.

Fig. 4.10 shows the pattern obtained 3-4 days after grafting. In
this case there is a fairly easily recognised basal ratio with obvious
regions differing from this. Areas representing greater relative
activity on the part of the grafted tissue occur between the top of the
gel and 12%, at 17%, 35%, 43%, 49%, 60-65%, 80-85% mobility whilst
there is a broad region about 20-30% where the ungrafted tissue has a
greater relative activity, with the exception of a few peaks. On
comparison with the same time period in the previous experiment
(Fig. 4.3) similarities can be seen in the pattern particularly from
the top of the gel up to about 20% and also at the bottom end, from
65% downwards, although the size of the peaks varies between the two
experiments, the pattern rises and falls at the same time. Throughout
the rest of the gel there are many major peaks which are in agreement
but also a few which are not. Peaks in agreement occur at 35%, 45%
and 50% whilst there is a discrepancy in the region of 30%. Comparison
with Fig. 4.4 shows some areas of similarity in the pattern but also
some large regions of discrepancy notably from the top to 5%, 17-20%,
40%, 50-70% and 85-90%.

The pattern obtained between 4-5 days after grafting is shown in
Fig. 4.11. Relative activity of the grafted tissue is greater in the
following regions, 15-20%, 25%, 30%, 40%, 47-52%, 62% and round about
90%. In addition to this there are a few obvious areas of greater
relative activity on the part of the ungrafted tissue at 23%, 35%,
55% and 75% and also two less obvious regions one at the top of the
gel and the other at 30%. Comparing with Fig. 4.4, i.e. Day 4-5, for
Figure 4.10

Comparison of incorporation patterns into soluble protein of grafted and ungrafted tissue 3-4 days after grafting.
Figure 4.11
Comparison of incorporation patterns into soluble protein of grafted and ungrafted tissue 4-5 days after grafting.
the previous experiment areas of agreement and disagreement can be seen. Allowing for differences in slice position (as explained earlier) wide areas of pattern agreement occur in the following regions: top - 17\%, 45-65\% and from 70\% onwards. Very obvious discrepancies occur at 22\%, 35\% and between 65 and 70\%. In the region of 25-45\% it is more difficult to decide whether or not there is agreement in the pattern.

d. Insoluble protein.

The pattern of incorporation ratios for insoluble protein during the periods 2-3 days, 3-4 days and 4-5 days after grafting can be seen in Figs. 4.12, 4.13 and 4.14 respectively and should be compared with the patterns in Figs. 4.5, 4.6 and 4.7 which are the corresponding figures for the first experiment. The comparison of Figs. 4.5 and 4.12 is difficult because of the difference in span of the values but by comparing the incorporation patterns of the grafted tissue and the ungrafted tissue separately it is easier to obtain a measure of reproducibility between the two experiments. The difference in the range of ratio patterns is probably in part due to the lower incorporation obtained in the second experiment particularly in the grafted tissue. Therefore taking each period in turn the pattern of incorporation by grafted tissue and ungrafted tissue for the two experiments were compared separately by looking for coincidence in significant areas of increase and decrease of incorporation level, e.g. for the following hypothetical values:

<table>
<thead>
<tr>
<th>Table 4.1.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Expt. 1</td>
</tr>
<tr>
<td>500 1000 1500 1400 1400 200 1000</td>
</tr>
<tr>
<td>increase or decrease</td>
</tr>
<tr>
<td>↑ ↑ ↓ ↓</td>
</tr>
</tbody>
</table>

The agreement and disagreement between the patterns would be recorded as 5 points in agreement out of a total of six or 83\% agreement and 17\% disagreement. In cases where there is a slight but insignificant increase or decrease in incorporation in one pattern but not in the other these are omitted because it is not possible to tell whether or not there is agreement. This is because many such cases
occur where overall incorporation is low and it is difficult to say what is a real difference and what is random variation. Such doubtful areas have been omitted from the calculation.

This comparison was made between the first and second experiment for the three time periods and for grafted and ungrafted tissue. The results are presented in table 4.2 below.

Table 4.2.

<table>
<thead>
<tr>
<th>Time period</th>
<th>Type of tissue</th>
<th>% agreement between experiments</th>
<th>% disagreement</th>
<th>% total that was indeterminate</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-3</td>
<td>Grafted</td>
<td>67</td>
<td>33</td>
<td>22</td>
</tr>
<tr>
<td>2-3</td>
<td>Ungrafted</td>
<td>81</td>
<td>19</td>
<td>13</td>
</tr>
<tr>
<td>3-4</td>
<td>Grafted</td>
<td>78</td>
<td>22</td>
<td>33</td>
</tr>
<tr>
<td>3-4</td>
<td>Ungrafted</td>
<td>68</td>
<td>32</td>
<td>25</td>
</tr>
<tr>
<td>4-5</td>
<td>Grafted</td>
<td>86</td>
<td>14</td>
<td>44</td>
</tr>
<tr>
<td>4-5</td>
<td>Ungrafted</td>
<td>75</td>
<td>25</td>
<td>32</td>
</tr>
</tbody>
</table>

The final column indicates the percentage of the gel slices where it was impossible to decide whether the two experiments were in agreement or not because of the above reason. This column shows that, for both the grafted and ungrafted tissue, the amount of the gel where it is impossible to tell whether the two experiments are in agreement or not, increases with time. This is due to the overall incorporation values for the grafted tissue in the second experiment, particularly in the two later time periods, being lower than in the first experiment. During the final time period in particular there are large areas of the gel where incorporation levels were too low to permit analysis. However, the overall incorporation level in the ungrafted tissue is higher, although this too drops with time, but not to such a great extent as the grafted tissue, so that it is possible to get a better idea of the reproducibility of the method from this tissue.

Considering the pattern obtained for grafted tissue, the degree of reproducibility between the experiments ranges from 67-86%, higher values being obtained, when the percentage of the gel where it is impossible to analyse that pattern increases. However, it is possible
to say that in the regions of the gel where the pattern permits analysis the pattern is reproduced in the two experiments with exceptions scattered throughout the gel, but in all cases outweighed by the similarities. For the period Day 2-3 major differences in relative pattern of incorporation between grafted and ungrafted tissue in the first experiment occurred at 25%, 32% and 55% mobility.

Comparing the two patterns where there are outstanding peaks in the first case (Fig. 4.5) these also occur in the second (Fig. 4.12) but in addition to these, Fig. 4.12 appears to show a large number of peaks the validity of which is suspect and these are probably due to the lower incorporation levels. This emphasises the necessity of having reasonable incorporation levels in both tissues, and the dangers of interpretation where there are large differences in overall incorporation levels between the two tissues being compared. This can produce spurious peaks which reflect more the pattern of incorporation along the gel of one of the tissues being studied rather than a comparison of the two. During the period from Day 3-4, nearly 80% of the grafted patterns are in agreement between the two experiments ignoring the 33% in which it is not possible to determine the pattern. 70% of the ungrafted patterns are in agreement, ignoring the 25% of the gel where it is not possible to determine the pattern. Comparing the patterns of incorporation ratios of grafted and ungrafted tissue from the two experiments (Figs. 4.6 and 4.13) the pattern of ratios obtained is fairly similar with only small exceptions. These occurring at about 12-13%, 35-37% and 57-60%, the radioactivity recorded from 85% onwards is so low that little reliance can be placed on the validity of the pattern. The areas of the gel where there is greater relative activity in favour of the grafted tissue are common to both experiments. The discrepancies in pattern occur in regions where there appears to be greater relative activity in favour of the ungrafted tissue in the first experiment and the position is reversed in the second (12-13%).

If the incorporation by the grafted and ungrafted tissue is examined in this region, the behaviour of the grafted tissue is very similar with the difference being in the ungrafted tissue. The higher ratio is due not to an increase in activity by the grafted tissue but to a dramatic drop in the activity of the ungrafted tissue. In the first experiment there is a drop in activity in this region but this is not so large. This difference might well be attributed to different
Figure 4.12

Comparison of incorporation patterns into insoluble protein of grafted and ungrafted tissue 2-3 days after grafting.
Figure 4.13

Comparison of incorporation patterns into insoluble protein of grafted and ungrafted tissue 3-4 days after grafting.
positioning of the gel slice.

It is possible by summing the values from the surrounding gel slices to diminish the importance of the positioning of the gel slices. Values of 2.68 and 1.91 ungrafted/grafted are obtained for the two experiments. Although this does not eliminate the difference between the two experiments it diminishes it considerably. The next discrepancy occurs in the region 35-37% where in Fig. 4.6 again there is greater relative activity on the part of the ungrafted tissue whilst in Fig. 4.13 the position appears to be reversed. However, summing the surrounding slices an overall ratio of 2.2 and 2.24 (ungrafted/grafted) are obtained for the two experiments which strongly suggests that the difference here might be due to the position of the slice.

The third major discrepancy was in the region 57-60% where again there is a peak of activity in the second experiment and not the first. Summing of the surrounding slices gives overall ratios of 1.83 and 1.09 (ungrafted/grafted) for the two experiments respectively, which eliminates the peak found for the second experiment suggesting that this may be due in part to the position of the slice. This emphasises the difficulties of comparing different gels where the slices are not necessarily coincident. The apparent discrepancy after 85% is not important as in this region the incorporation levels are too low to permit analysis.

When the final time period is considered in the second experiment the pattern in Fig. 4.14 is obtained. However, the incorporation levels obtained were so low that the pattern is indeterminate for almost all the gel (see table 4.2). Ignoring these points there is a large amount of agreement in the pattern between the two experiments. 86% of the grafted pattern and 75% of the ungrafted pattern are in agreement. But because the incorporation found with the grafted tissue is so low the pattern of ratios of grafted/ungrafted will reflect almost solely the pattern for the ungrafted tissue so that the pattern of ratios obtained in the second experiment cannot be considered with any confidence. For this reason comparison of the two experiments is meaningless for this time period and will not be attempted.

The patterns of incorporation obtained are largely reproducible, allowing for the limitations of the comparison between samples incubated under different conditions and subsequently analysed on a separate gel. Use of the dual labelling techniques eliminates these
Figure 4.14

Comparison of incorporation patterns into insoluble protein of grafted and ungrafted tissue 4-5 days after grafting.
considerations from the comparison of grafted and ungrafted tissue in one sample, but not between samples.

The pattern of protein synthesis which is common to both grafted and ungrafted tissue is eliminated by comparison of the ratios of incorporation into proteins between grafted and ungrafted tissue. The resultant pattern reflects the pattern of protein synthesis due to graft formation. Graft formation can be divided into two phenomena, a response to wounding and the reintegration of the tissues of stock and scion. Comparison of the pattern of incorporation in a "wounded" internode with the pattern of incorporation in the grafted internode should show which differences in the pattern are due to reintegration of the tissues of stock and scion.
C.4.II. **Comparison of the incorporation patterns of radioactively labelled methionine by "wounded" and intact internodes.**

The construction of grafts inevitably produces a wound response. This wounding will have an effect on the overall metabolic activity of the plant which may complicate and confuse the interpretation of the events involved in the formation of the graft union. Comparison of the pattern of incorporation of a wounded stem with that of a grafted stem, allows the changes in the pattern of protein synthesis which are due to the grafting (reintegration) process alone to be determined.

In an attempt to determine the pattern of incorporation due to reintegration of stock and scion rather than wounding, a wounded control was devised which could be treated in the same way as the grafted tissue and the pattern of incorporation of radioactive methionine into protein due to wounding could be seen by comparison with ungrafted tissue. The wounded control is described in Section B.2.IV. and is made in exactly the same way as the graft except that a piece of P.T.F.E. rod is used in place of the scion. These assemblages were then treated in exactly the same way as the grafted tissue and eventually extracted with ungrafted tissue so that the pattern of incorporation due to wounding and regeneration against an inert surface could be determined.

The data obtained was treated as described in Section B.3.VI. and is presented in Figs. 4.15 - 4.17 for soluble protein and Figs. 4.18 - 4.20 for insoluble protein. These will be compared with the Figs. 4.2 - 4.4 / 4.5 - 4.7 which represent the corresponding patterns for grafted tissue.

Fig. 4.15 shows the ratios of incorporation into soluble protein between 2-3 days after wounding. This is compared with Fig. 4.2 which shows the corresponding pattern for grafted tissue. The peaks of greater relative activity of the grafted tissue which have been described previously were checked for concurrence in Fig. 4.15. Two of the areas of greater relative activity found in the grafted tissue, one at 2% and the other at 70% mobility, coincide with greater relative activity in the wounded tissue. The other areas of greater activity do not have corresponding peaks of activity in the wounded tissue suggesting that these are due to the grafting process whilst the other two may be due to a wound response. The overall levels in
Figure 4.15
Comparison of incorporation patterns into soluble protein of "wounded" and ungrafted tissue 2-3 days after wounding.
incorporation in the wounded tissue are considerably lower than in the
grafted tissue. However, at least in the regions looked at, the levels
are sufficient to decide what is happening to the pattern. Only the
areas of greater relative activity by grafted tissue which are also
found in wounded tissue are of interest in this study.

Fig. 4.16 shows the ratios for wounded versus ungrafted tissue
for the period 3-4 days after wounding, and this should be compared
with the peaks of greater relative activity by the grafted tissue in
Fig. 4.3. Of the seven outstanding peaks of greater relative activity
of grafted tissue only those at 27% and 40% coincide with outstanding
peaks of greater relative activity of wounded tissue. In addition
there is also a coincident area of greater relative activity of
ungrafted tissue in the region 62-65%. The region of 18% may also
coincide but, although there is a peak in favour of the wounded tissue
activity, it is not distinguishable from background variation. The
peaks at 25%, 32%, 35% and 45% in Fig. 4.3 do not have corresponding
peaks of greater relative activity in Fig. 4.16 so these might be
regarded as representing activity distinct from a simple response to
wounding.

Fig. 4.17 shows the ratios of incorporation by wounded tissue to
ungrafted tissue (i.e. the pattern due to wounding) for the period
4-5 days after wounding. The major peaks of activity are compared with
those found in Fig. 4.4 to give an indication of which peaks are due
to grafting or to wounding. There is a band of greater relative
activity by the wounded tissue at 34% which coincides with a band of
activity in the grafted tissue. There might also be a slight peak of
activity at 25% consistent with the peak found in Fig. 4.4 but this is
very slight in Fig. 4.17. The rest of the peaks found in Fig. 4.4 are
not apparent in Fig. 4.17 so are probably attributable to grafting
rather than wounding. The data in Figs. 4.15 - 4.17 however, are all
based on rather low incorporation levels particularly the end of
Figs. 4.16 and 4.17 where there are large areas of the gel in which
incorporation is too low to allow proper interpretation. Indeed in
Fig. 4.17 the large peak of activity occurs in such an area of low
incorporation which makes it impossible to say whether the peak is
real or an artifact. Although the incorporation levels for Fig. 4.15
were low they were high enough to show some degree of certainty in
Figure 4.16

Comparison of incorporation patterns into soluble protein of "wounded" and ungrafted tissue 3-4 days after wounding.

% ELECTROPHORETIC MOBILITY

WOUNDED: UNGRAFTED (35S:2H)

% ELECTROPHORETIC MOBILITY

25 50 75 100

UNGRAFTED: WOUNDED (cpm ³H: cpm ³S)
Figure 4.17

Comparison of incorporation patterns into soluble protein of "wounded" and ungrafted tissue 4-5 days after wounding.
the comparison with the grafted tissue. The other two were much more dubious.

Fig. 4.18 shows the pattern of incorporation into insoluble protein due to wounded tissue between 2-3 days after wounding and should be compared to Fig. 4.5 showing the corresponding pattern for grafted tissue. Of the three outstanding areas of differential activity in Fig. 4.5, those at 25% and 55% mobility, do not appear to have corresponding areas of greater relative activity in the wounded tissue, in fact Fig. 4.18 shows greater activity on the part of the ungrafted tissue in the region representing 55%. The peak at 32% in Fig. 4.5 representing greater relative activity by the ungrafted tissue may coincide with that found in Fig. 4.18 if allowances are made for different positioning of slices. So the two peaks of greater activity exhibited by the grafted tissue in Fig. 4.5 can be attributed to grafting (i.e. regeneration between two active surfaces) and not wounding (or regeneration by one active surface against an inert one).

Fig. 4.19 shows the pattern of incorporation by wounded tissue for the period 3-4 days after wounding. Comparison with the major peaks of activity found in Fig. 4.6 will show whether these are attributable to grafting solely or to the more general wounding situation. The three major areas of greater activity of the grafted tissue were not mirrored by outstanding areas of greater activity by the wounded tissue. Indeed the pattern of activity by wounded tissue did not show any obvious regions where incorporation into any particular protein subunit was dramatically different from the overall pattern. Interpretation of this pattern however, was made difficult because of the large difference in incorporation level between the wounded tissue and the ungrafted tissue. Because the incorporation by the ungrafted tissue was considerably higher, the fluctuations seen in the pattern were more heavily influenced by fluctuation in incorporation in the ungrafted tissue rather than the grafted tissue.

The pattern of incorporation due to wounded tissue activity can be seen in Fig. 4.20. This is compared with Fig. 4.7 which shows the corresponding pattern for grafted tissue. As in Fig. 4.7 the major fluctuations in the pattern occur in the top half of the gel. Any of the areas exhibiting greater activity by grafted tissue have coincident peaks of activity in the incorporation pattern of the wounded tissue.
Comparison of incorporation patterns into insoluble protein of "wounded" and ungrafted tissue 2-3 days after wounding.
Comparison of incorporation patterns into insoluble protein of "wounded" and ungrafted tissue 3-4 days after wounding.
Figure 14.20

Comparison of incorporation patterns into insoluble protein of "wounded" and ungrafted tissue 4-5 days after wounding.

% ELECTROPHORETIC MOBILITY
There is a peak of activity around 6% which falls between the two peaks found in Fig. 4.7. Again, for the peak of activity at 20% in Fig. 4.7, there is a peak at 22% in Fig. 4.20. In both these cases the difference in position along the gel was not more than one slice. It was possible that this was due to the position of the slices. It was not possible to say with any degree of certainty that these were unique to one situation. The next areas of interest were in Fig. 4.7 where three peaks occur between 28% and 35%. There were three peaks in Fig. 4.20 in this region but mismatched by one slice again. The very large peak of activity in Fig. 4.7 at 40% does not appear to have a corresponding peak in the pattern in Fig. 4.20, the nearest being at 38% which is two slices away. This is probably unique to the grafted situation. The wounded pattern also shows a peak of activity at 47% which is not present in Fig. 4.7. Although some of the peaks of activity in Fig. 4.7 were found in Fig. 4.20, the size of the peaks were much greater in the case of the grafted tissue, but this was probably a reflection of the higher overall levels of incorporation by grafted tissue as opposed to wounded tissue, which in general exhibits a much lower level of incorporation of the labelled precursors.

In conclusion, it is possible to say that there are differences between the incorporation of radioactive labelled amino acids due to grafting and wounding. The incorporation pattern is different when regeneration occurs against an inert surface and when it occurs between two actively contributing surfaces. However, various limitations of the experiment must be borne in mind. One of the biggest in the comparison of these two situations was the very low overall incorporation found in the wounded tissue which makes it difficult to interpret the results. It may be simply that there is very little synthetic activity in this tissue i.e. that the overall rate of metabolic activity in the wounded tissue is very low or that there is a problem in the uptake of the label in this system. The differences might also reflect differences in gaseous exchange as the top of the wounded intervals is occluded, whereas the grafted internode is not.

There are changes in the pattern of incorporation of labelled methionine into protein which are associated with graft formation. These changes are seen in both soluble and insoluble fractions. The
pattern of incorporation associated with graft formation varies according to the stage of development of the graft. It was suggested that at least some of these changes found occurred only as a result of the reintegration of two active surfaces (i.e. as a result of cell interactions) rather than as a response to wounding. The complexity of the pattern found and the difficulties of comparison between patterns, particularly where the patterns were derived from different tissues, labelled and processed separately, make the interpretation of these results very difficult. In particular it is difficult to provide background information for studies on possible recognition systems from a very general survey of the total protein pattern of the grafted region. Study of a particular set of proteins or of particular subcellular fractions, must prove more valuable, if only by reducing the number of different proteins being studied. The subcellular origin of particular proteins, the synthesis of which is associated with grafting, may suggest a role for such proteins. Cell interactions must involve the surfaces of the cells, in particular the cell wall and plasma membrane. The cell wall is relatively inert so the role of the plasma membrane was studied. Changes in the pattern of incorporation of amino acids into protein, during the transition between the two phases of graft formation, that are associated with the plasma membrane, may be involved in cell interactions occurring at this time. In the next chapter the role of the plasma membrane will be investigated by application of the dual labelling technique to putative plasma membrane fractions.
0.5. PATTERNS OF INCORPORATION OF RADIOACTIVELY LABELLED METHIONINE INTO PROTEINS FROM MEMBRANE FRACTIONS OBTAINED BY DISCONTINUOUS DENSITY GRADIENT CENTRIFUGATION.

In the previous chapter the patterns of incorporation of radioactively labelled methionine into protein from either crude soluble or insoluble fractions were investigated using a double labelling procedure. These two fractions are composed of very diverse subcellular elements, many of which are present in both fractions. However, the results obtained were difficult to interpret, and it was decided to simplify the analysis by further fractionation of the soluble and insoluble components. Techniques were used in which there was a reasonable probability of obtaining a fraction which contained predominantly one species of organelle. The focus of attention in this study was the plasmalemma because of its importance in a situation where cellular interactions are involved. The dual labelling procedure was applied to the study of this organelle.

The fractionation procedure:

The method of subcellular fractionation employed was based on that of Hodges et al., (1972), the best method available at the time, which claimed to produce an enriched plasmalemma fraction. This method relies on a gentle homogenisation of the tissue to break up the cells and release the fragments of membranes which form vesicles. The homogenate was then subjected to low speed centrifugation, to eliminate cell walls and other large fragments, followed by centrifugation at a greater force to bring down all the membranous particles from the supernatant (or soluble fraction). Then membrane particles were layered onto a discontinuous density gradient on which the different fractions will separate, according to their buoyant density and will accumulate at the interfaces between different layers of the gradient. These fractions were then collected and analysed.
C.5.I. Comparison of the incorporation patterns of radioactively labelled methionine into protein by grafted and ungrafted internodes.

The experimental procedure was as described in Section C.4.I. until the stage of extraction of the tissue. The study has been restricted to one time period, 3 to 4 days after grafting. At the beginning of the experiment a large number of tomato plants, in excess of the requirement of the experiment, were grafted as previously described and ungrafted plants were also set up. These were then left to develop in the growth room until Day 3 when labelling of the grafted internodes was carried out. At this stage 100 grafted internodes were transferred to bottles containing 5 μCi $^{35}$S-methionine as described in Section B.2.VII. Forty ungrafted internodes were also transferred to similar bottles containing 100 μCi $^{3}$H-methionine. All the tissue was harvested after twenty-four hours using the procedures described in Section C.1.I. The tissue from the grafted and ungrafted internodes was pooled to give 32 g of tissue. (A minimum of 30 g tissue is required for the procedure.) The pooled tissue was composed of 14 g of grafted tissue (fresh weight) and 19 g ungrafted tissue, giving a tissue ratio of 1 : 1.36, this makes no difference to the pattern of incorporation only to the numerical values of the ratios.

The mixture of tissues was fractionated using the procedure described in Section B.3.VII. Fig. 5.1 shows the optical density profile at 254 nm of the "pump out" of the gradient and the delimitation of the fractions. The membrane vesicles were then sedimented and taken up in gel sample buffer containing SDS to release the proteins from the membranes and also reduce the proteins to their sub-units. The total fractions were run in different slots of the same 10% polyacrylamide-SDS slab gel. After running, the gels were sliced and the activities due to $^{35}$S and $^{3}$H were determined as before. These were then corrected for background and spillover between channels and the ratios of the activities were calculated to give the pattern of incorporation due to grafting during this time. In this experiment direct comparison of the different fractions is possible because they are all derived from the same tissue so that differences cannot be due to differences in uptake or supply. On comparing particular slices it must be borne in mind that the positioning
Figure 5.1
Optical density profile of "pump-out" of density gradient showing the delimitation of the fractions obtained.
of these may not be exactly comparable.

The patterns of incorporation into the different fractions from the gradient are shown in Fig. 5.2 - 5.8. These are all plotted on the same scale to allow direct comparison between patterns. Fig. 5.2 shows the pattern obtained from the protein derived from the top of the gradient, that is the 18-20% interface. The ratios obtained all fluctuate in the region of 2.0 (ungrafted: grafted) with a few small deviations from this. There is only one area where there appears to be a significant deviation from the base line fluctuation and this occurs at 51% mobility. This represents an increase activity of the grafted tissue in this region.

Fig. 5.3 shows the pattern activity of the fraction from the 20-25% interface. Again there is a base level of fluctuation with a few small deviations. These all represents greater relative activity of the grafted tissue. They occur in the regions representing 11%, 19%, 66-72%, 81%, 87 and 92%, and perhaps also between 25 and 35%, but these are fairly small. The size of all the deviations is not very great but they are very different from the variation seen in the previous fraction or the middle of the gel in this fraction.

The pattern obtained from the protein of the membrane fraction collected from the 25-30% interface of the sucrose gradient is shown in Fig. 5.4. In comparison to the previous two figures (5.2, 5.3) there are many more peaks of greater relative incorporation by the grafted tissue. The largest of these occur at 19%, 47%, and 57% and then there is an intermediate set of peaks which occur at 16%, 31%, 40-41%, 50%, 52%, 61-63%, 66% and also some smaller peaks at 6%, 13%, 27%, 36%, 37-39%, 44%, 54%, 63%, 73%, 75%, 83%, 87%, 89% and 94%. Some of the smaller sets of peaks may not be significantly different from the base level of variation seen so far but if the base level of variation is taken as being the smallest variation found in the incorporation pattern these differences may be significant. Later fractions show a smaller basal variation and this should be taken as the base line for comparison. The diverse pattern of incorporation found in this fraction compared with the previous two fractions indicates that this fraction is of importance in the grafting process. Obviously it is necessary to determine which membrane types are found in this fraction, at least some of the membrane types found in this fraction play a large part in
Figure 5.2

Comparison of incorporation patterns into protein derived from the top-20% interface of the gradient of grafted and ungrafted tissue.
Figure 5.3

Comparison of incorporation patterns into protein, derived from the 20-25% interface of the gradient, of grafted and ungrafted tissue.
Comparison of incorporation patterns into protein, derived from the 25-30% interface of the gradient, of grafted and ungrafted tissue.
the grafting process even if only in the accompanying cell division.

The pattern of incorporation of the fraction derived from the 30-34% interface of the sucrose gradient is shown in Fig. 5.5. This fraction also exhibits a large number of differences between the incorporation ratios of ungrafted control tissue and grafted tissue. The peaks of greater relative activity indicate that the grafted tissue is incorporating more label into certain proteins than the ungrafted tissue. This may simply be a reflection of an increase in turnover rather than a net increase in synthesis but nonetheless it indicates that in this fraction the grafted tissue appears to be making certain proteins at a higher rate than the control tissue. The largest of these differences occur at 17%, 23%, 28%, 40%, 42%, 44%, 47%, 56% and 64%, with some smaller differences at 3%, 5%, 7%, 33%, 56%, 53%, 59%, 71%, 74%, 77%, 83%, 86 and 91%. Some of these smaller differences may not be significant. The increases in activity in this fraction associated with grafting are of interest. Many will of course be involved in cell division and differentiation but some might be specific to the grafting process alone. At present this cannot be determined. As the plasma membrane is the membrane in which recognition systems might be expected to reside, establishment of the types of membrane present would facilitate interpretation of the results in the context of the events of grafting.

The pattern of incorporation for the fraction from the 34-38% interface can be seen in Fig. 5.6. Again the pattern shows that there are a large number of differences between ungrafted and grafted tissue although the size of these is not quite so obvious as in the previous two Figs. (5.4, 5.5). There is one large peak with a relative mobility of 15% and several smaller but significant peaks occurring at 3%, 26%, 29%, 34%, 41%, 42%, 47%, 51%, 53%, 56-60%, 62%, 64%, 67%, 71-73%, 77%, 81%, 87% and 93%. These peaks, like those in the previous two Figs. (5.4, 5.5), represent greater incorporation of methionine into protein by grafted tissue and so reflect the pattern of protein incorporated into this fraction of grafted tissue during the period 3-4 days after grafting. Again the proportion of plasma membrane contained in this fraction is of great interest.

Fig. 5.7 shows the pattern obtained by analysis of the fraction from the 38-45% interface of the sucrose gradient. The pattern is simply one of fluctuations about a mean. There are a few small peaks
Comparison of incorporation patterns into protein, derived from the 30-34% interface of the gradient, of grafted and ungrafted tissue.

Figure 5.5

% ELECTROPHORETIC MOBILITY
Comparison of incorporation patterns into protein, derived from the 34-38% interface of the gradient, of grafted and ungrafted tissue.
Figure 5.7

Comparison of incorporation patterns into protein, derived from the 30-45% interface of the gradient, of grafted and ungrafted tissue.
of activity but it is not possible to say whether these represent significant differences from basal fluctuations. If these are real differences they are nonetheless small in comparison with those found in the previous three Figs. (5.4, 5.5, 5.6) and are probably of no interest.

Fig. 5.8 shows the pattern obtained by analysis of the fractions from the bottom of the gradient, i.e. 45% - bottom. Apart from one region there is very little variation at all and this should be taken as the base level of variation in the ratio. The exception is in the region of 52-59% where the pattern shows a decrease in the incorporation of $^{35}S$-methionine by grafted tissue into these in relation to the ungrafted control. This is still fairly small and because it is the differences which represent an increase in the activity of the grafted tissue which are of interest, it will not be dealt with further.

Comparison of all the fractions shows that most differences in pattern occur in three fractions, namely the fractions collected from the 25-30%, 30-34% and 34-38% interfaces of the discontinuous sucrose density gradient (Figs. 5.4, 5.5, 5.6). These also show the largest deviations from basal variation of the pattern.

Of the differences between grafted and ungrafted tissue in those fractions already described, some are common to one or more fractions. Fig. 5.9 shows the position of the peaks of greater relative activity of the grafted tissue derived from Figs. 5.4, 5.5, 5.6, to show which differences are common to the different fractions. As can be seen, only two are common to all three fractions, that is the differences representing 44 and 47% mobility. There are seven peaks of greater relative activity of grafted tissue which are common to the fractions derived from the 30-34% and the 34-38% interfaces, five such peaks which are common to the fractions from the 25-30% and the 34-38% interfaces and three which are common to the fractions from the 25-30% and 30-34% interfaces. The similarities of the pattern with respect to these areas may be because the fractions contain more than one membrane type, and have components in common. As well as the differences which occur in more than one fraction, there are twenty differences which are unique to the 25-30% fraction, ten differences which are unique to the 30-34% fraction and eleven which are unique to the 34-38% fraction.
Comparison of incorporation patterns into protein, derived from the bottom of the gradient, of grafted and ungrafted tissue.
Diagram to show the position of areas of activity in fractions 25-30%, 30-34%, 34-38% (Figs. 5.4-5.6).

(Vertical lines represent areas of greater relative activity by grafted tissue, half lines represent smaller peaks of activity.)
C.5.II. Distribution of PACP positive vesicles in the fractions obtained by discontinuous density gradient centrifugation

Published methods for assessing the purity of plasmalemma fractions have relied exclusively on the identification of this organelle by the use of the PACP stain. This is also referred to as the PTA or PTA stain by some authors. Membrane fractions were prepared as for the double labelling experiment and after the final centrifugation the pellets were fixed in 3% glutaraldehyde in (0.1 M) cacodylate buffer pH 7.2, and processed for electron microscopy. A summary of the procedure is included in Section B.2.IX. After embedding in araldite the pellets were sectioned using a LB ultratome to provide thin sections (ca. 90 nm) which were then either stained with the standard lead citrate-uranyl acetate procedure to show all the membranes particles present, or with the PACP stain according to the method of Littlefield and Bracker, (1972) described in Section B.3.VIII. The sections were then viewed and photographed in an A.E.I. E.M.6 electron microscope. Plate numbers 5.1 - 5.11 show the PACP positive membrane vesicles and the general appearance of the fractions after lead-uranyl acetate staining. These fractions coincide with those studied in the previous section in which the pattern of incorporation of methionine into protein was investigated (Figs. 5.2 - 5.8).

Plates 5.1 and 5.2 show the PACP and lead citrate-uranyl acetate stained sections respectively from pooled fraction derived from the top of the gradient (18-20%) plus the 20-25% interfaces of the sucrose gradient. The PACP stained section shows very few positively stained vesicles. There are also some small stained round particles, probably ribosomes. The section which has been stained with the general stain (Pl. 5.2) shows a predominance of ribosomes and small fragments with a few larger membrane vesicles. Other than that, it is impossible to pick out any identifiable structures.

Plates 5.3 and 5.4 show the PACP and the uranyl acetate-lead citrate stained sections of the pellet from the 25-30% interface of the sucrose gradient, corresponding to the incorporation pattern in Fig. 5.4. The section of membrane pellet stained with the PACP stain shows a few large vesicles positively stained, against a background of some small particles, probably ribosomes, and some smaller vesicles which are also positively stained. Although the number of
Plate 5.1.


Plate 5.2.

The same as Pl. 5.1 but stained with lead citrate and uranyl acetate.

Plate 5.3.

25-30% fraction from the density gradient. Otherwise as for Pl. 5.1.

Plate 5.4.

As for Pl. 5.3 but stained with lead citrate and uranyl acetate.

Plate 5.5.

30-34% fraction from the density gradient. Otherwise as for Pl. 5.1.

Plate 5.6.

As for Pl. 5.5 but stained with lead citrate and uranyl acetate.
vesicles which are definitely PACP positive is not large, there are some PACP positive fragments so that this fraction will be considered to contain PACP positive vesicles (PACP+). No attempt will be made to quantify the proportion of these vesicles except in this very general way. The lead citrate - uranyl acetate stained section shows largely the same picture with small ribosome-like particles and smaller vesicles. There are no particularly large vesicles in this field of view. The fraction appears to be fairly heterogeneous.

The composition of the membrane pellet from the 30-34% interface is shown in plate 5.5 (PACP) and plate 5.6 (uranyl acetate and lead citrate). The corresponding incorporation pattern can be seen in Fig. 5.5. The PACP stained section has a large number of vesicles exhibiting varying degrees of staining intensity. The size of the vesicles varies considerably and those staining with PACP are not of uniform size. This fraction is considered to be PACP positive. Plate 5.6 is a general view of the section and shows a large diversity in size of vesicles. Some of the vesicles appear to be empty shells whilst others have inclusions, but it is not possible to identify particular sub-cellular fragments. In comparison to the previous fraction (Pl. 5.3) there are more PACP positive fragments and they can be considered to be PACP++. The lead citrate - uranyl acetate stained section shows a similar picture of widely differing shapes and sizes of vesicles, some with and some without inclusions. Again the heterogeneity of the fractions can be seen, and the proportion of large vesicles is increasing. These larger vesicles appear to be PACP positive and one would expect the plasmalemma to form relatively large fragments although the grinding of the tissue may produce wide ranging sizes of vesicles (Galbraith and Northcote, 1977).

Plates 5.7 and 5.8 show the PACP and lead citrate - uranyl acetate stained sections of the membrane pellet derived from the 34-38% internode, corresponding to the incorporation pattern in Fig. 5.6. There are a large number of PACP positive vesicles although in some cases the whole membrane of the vesicles does not stain evenly. There are many small faint vesicles which should be taken as PACP negative but overall there are more PACP vesicles in this section than in the previous fractions and so in general it may be regarded as PACP+++. The lead citrate - uranyl acetate stained section (Pl. 5.8) shows that again the fraction is heterogeneous in composition and is
Plate 5.7.

34-36% fraction from the density gradient. Membrane fractions from tomato stem (see Section B.1. and B.3.VII.). PASP staining procedure (Littlefield and Bracker, 1972. Section B.3.VIII.). Magnification x 16,000. Post-fixed with 1% OsO₄.

Plate 5.8.

As for Pl. 5.7 but stained with lead citrate and uranyl acetate.

Plate 5.9.

38-45% fraction from the density gradient. Otherwise as for Pl. 5.7.

Plate 5.10.

As for 5.9 but stained with lead citrate and uranyl acetate.

Plate 5.11.

45% - bottom of gradient. Otherwise as for Pl. 5.7.

Plate 5.12.

As for Pl. 5.11 but stained with lead citrate and uranyl acetate.
mainly composed of vesicles of varying size both with and without inclusions.

The plates corresponding to the incorporation pattern derived from the 58-45% interface of the sucrose gradient are numbered 5.9 (PACP) and 5.10 (lead citrate - uranyl acetate). The PACP stained section shows only a few small vesicles with very definite positive staining, the rest are very faint and it is difficult to decide whether this staining is positive or not. In comparison to previous fractions this can be regarded as PACP+ but it obviously contains a large proportion of other membrane fragments. The heterogeneity of the fraction may be seen from the lead citrate - uranyl acetate stained section (Pl. 5.10). Most of the vesicles are small with a few large ones interspersed. Again the vesicles cannot be readily identified as being derived from any particular source.

The final fraction consists of those particles which are denser than the range of the gradient which sediment at the bottom. The PACP stained section of this fraction can be seen in Pl. 5.11. There are virtually no PACP positive vesicles in this section and so this fraction can be regarded as PACP negative (PACP-). The lead citrate - uranyl acetate stained section is shown in Pl. 5.12 and an extremely diverse selection of vesicles with a very granular background and some stacks of membranes probably Golgi bodies can be observed. The distribution of PACP positive vesicles throughout the fractions derived from the density gradient is summarised in table 5.1.

Table 5.1:

<table>
<thead>
<tr>
<th>Plate number</th>
<th>Fraction from gradient</th>
<th>Relative proportion of PACP staining vesicles</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.1</td>
<td>18-20% plus 20-25%</td>
<td>-</td>
</tr>
<tr>
<td>5.3</td>
<td>25-30%</td>
<td>+</td>
</tr>
<tr>
<td>5.5</td>
<td>30-34%</td>
<td>++</td>
</tr>
<tr>
<td>5.7</td>
<td>34-38%</td>
<td>+++</td>
</tr>
<tr>
<td>5.9</td>
<td>38-45%</td>
<td>+</td>
</tr>
<tr>
<td>5.11</td>
<td>45% to bottom</td>
<td>-</td>
</tr>
</tbody>
</table>

These are four fractions which exhibit the presence of vesicles which stain with PACP. These are the 25-30%, 30-34%, 34-38% and...
the 38-45% fractions. Arranged in order of the amount of PACP staining in the section, these are as follows: 34-38% then 25-30% and then 38-45% which contains only a few such vesicles. As was seen earlier, the first three of these are the fractions which showed the greatest number of changes in pattern. These changes then can be said to be associated with the presence of PACP staining vesicles which Hodges et al., (1972) claim are vesicles derived from the plasmalemma. However, Hodges et al., (1972) found the PACP positive fractions to be those from the 34-38% interfaces of the gradient whereas here, although the most PACP stain is found in the 34-38% fraction, there are also appreciable amounts in the 30-34% fraction and smaller amounts in the 25-30% and the 38-45% fraction.

These differences may be at least in part accounted for by the initial grinding of the tissue. It is difficult to standardise such grinding which will damage the cellular contents, reducing the characteristic differences in size and density between cellular membrane structures so that reproducible separation will be difficult. Also there may be differences characteristic of the source of the tissue. Hodges et al., (1972) used oat coleoptiles whilst Shore and MacLachlan, (1975) using pea epicotyls and a continuous gradient found the PACP staining fraction corresponded to an approximate buoyant density of 1.13 g/cc which is equivalent to the 30-34% interface on the discontinuous gradient.

The apparent association of the changes in incorporation pattern with PACP positive staining membrane vesicles may suggest that some of these changes are associated with the plasma membrane if the PACP stain is an indicator of its presence. This point will be fully explored in the next chapter. The source of some or all of these changes may be situated in the plasmalemma but, bearing in mind the heterogeneity of these fractions, it is also possible that they might be due to other parts of the fraction which happens to be co-sediment with these vesicles.
C.6. IDENTIFICATION OF THE PLASMALEMA OF PLANT CELLS.

In the previous chapter, membrane vesicles were separated according to their various densities on a discontinuous density gradient. However, it is necessary to identify particular membrane types with certainty so that the composition of the different fractions can be determined. The membrane which was of particular interest in this study was the plasmalemma. All previous studies, with few exceptions, have made use of a "specific staining technique" to identify the plasma membrane (e.g. Horrè et al., 1970; Lembi et al., 1972; Hodges and Leonard et al., 1972, 1973, 1974; Strobel et al., 1974; Cassagne et al., 1976; Leigh et al., 1975; Hardin et al., 1972;). In many cases where attempts have been made to use criteria other than a "specific staining technique" for identification, such as enzyme markers, the proof for the specificity of these markers depended on previous identification using the "specific staining technique" (Van der Wooue et al., 1972; Gardiner et al., 1975). Occasionally the specificity of an enzyme for the plasma membrane was an extrapolation of results with animal tissues. However, many of these markers such as 5'-nucleotidase, Mg-dependent ATPase and lipid with high sphingomyelin were not confined to the plasmalemma in plant tissues (Horrè et al., 1970; Poux, 1967; Hall, 1969). Horrè et al., (1970) compared rat liver plasma membrane preparations with plasmalemma preparations from plant cells and concluded that the characteristics of the surface membranes differed. They were unable to demonstrate that these markers could be used in plant systems. Mg^{2+} dependent ATPase activity has been reported in young vacuoles and dictyosomes and cell walls of plants by Poux, (1967) and Hall, (1969) who showed the presence of ATPases again in young vacuoles and in endoplasmic reticulum, in addition to the plasmalemma.

The heavy reliance on a "specific staining technique" such as PAM as the sole criterion for identification of the plasmalemma necessitates confirmation of the specificity of the stain for all tissues used. There are reports questioning the specificity of PAM stain after application to sections of tissue rather than homogenates (Thom et al., 1975). In this investigation it was claimed that the tonoplast and other structures were stained as well as the plasmalemma. Hall and Flowers, (1976) maintained that in some cases staining of the
plasmalemma was not constant.

Areas of unstained plasmalemma were found in cells adjacent to ones which appeared to show specific staining. In other cells they also found that staining varied within an individual cell. They also observed staining of the tonoplast. Nagahashi et al., (1978) whilst admitting that specificity was not absolute, maintained that the plasma membrane may be distinguished from other cell components such as vacuoles, golgi apparatus, endoplasmic reticulum, nuclear membrane, mitochondria, chloroplast and proplastid membranes. This claim was based on the observation that staining of membrane components other than the plasmalemma was only occasional in tissues studied. These authors claim that lack of specificity was due to technical error (although with their extremely meticulous methodology they still found areas where specificity was not absolute) and that the PALP staining procedure was basically sound. They do however, admit the necessity to check the specificity of the stain for all tissues.

In this section attention has been concentrated on the application of the PALP staining procedure in various forms to both isolated membrane fractions and to sections of a variety of different tissues, to establish the validity of this technique.

The PALP staining technique was first described by Roland, (1969) and by Roland and Vian, (1971). At this stage the technique was applied to tissue which had not been subjected to post-fixation with OsO₄ and staining was simply a matter of floating thin sections onto the meniscus of a 1% solution of phosphotungstic acid in 10% chromic acid. When the technique was adapted to the identification of plasmalemma preparations in the form of vesicles (Morré et al., 1970; Roland et al., 1972) post-fixation with OsO₄ was always carried out. This treatment tended to make any lipid containing structures electron dense, therefore prior to treatment with the PALP stain the sections were "bleached" in 1% periodic acid for 30 minutes and washed in distilled water. The PALP stain (1% phosphotungstic acid 10% CrO₃) was applied for 2-3 minutes and the excess stain removed by repeated washing with distilled water.

The most commonly used method for identification of the plasmalemma after fractionation was as described by Littlefield and Bracker, (1972). This was the method used by Hodges et al., (1972), who devised the fractionation procedure for the plasmalemma, and was a modification of the method that Morré et al., (1970) used with fractions of membrane
vesicles. The staining time was increased to fifteen minutes by Littlefield and Bracker, (1972). The PACP staining techniques and all modifications used are described in Section B.3.VIII.
C.6.I. Application of PACP stain to membrane preparations from tomato stem and melon hypocotyl.

The results of the application of the PACP staining procedure to membrane fractions derived from tomato stem have already been described in Section 5.11. The procedure was applied to membrane fractions, obtained by the method of Hodges et al., (1972) after embedding and sectioning. These procedures are described in Section B.2.IX. The PACP procedure used was that of Littlefield and Bracker, (1972) and is described in Section B.3.VIII. The results can be seen in plates no. 5.1, 5.3, 5.5, 5.7, 5.9, 5.11. The distribution of PACP positive vesicles throughout fractions derived from the density gradient was summarised in table 5.1. This is reproduced here for convenience.

<table>
<thead>
<tr>
<th>Plate No.</th>
<th>Fraction from gradient</th>
<th>Distribution of PACP stain</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.1</td>
<td>18-20% + 20-25%</td>
<td>-</td>
</tr>
<tr>
<td>5.3</td>
<td>25-30%</td>
<td>+</td>
</tr>
<tr>
<td>5.5</td>
<td>30-34%</td>
<td>++</td>
</tr>
<tr>
<td>5.7</td>
<td>34-38%</td>
<td>+++</td>
</tr>
<tr>
<td>5.11</td>
<td>38-45%</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>45-Bottom</td>
<td>-</td>
</tr>
</tbody>
</table>

No fraction derived from the gradient contained a large number of these vesicles and all fractions were very heterogenous. To see if the purity of the fractions with respect to PACP staining could be improved the fractionation technique was applied to melon hypocotyl (*Cucumis melo*) (tissue not containing chloroplasts). The results of the application of the PACP stain to membrane preparations isolated from melon hypocotyls can be seen in plates 6.1, 6.3, 6.5 and 6.7 which represent fractions from the 25-30% interface; the 30-34% interface, the 34-38% interface and the 38-45% interface respectively.

Plates no. 6.2, 6.4, 6.6 and 6.8 are equivalent sections stained with lead citrate and uranyl acetate and show the general composition of the fractions. All the fractions show some evidence of staining although only plate no. 6.5 shows any convincing signs of differential staining. The other plates show similar staining in fairly diverse vesicles or
Plate 6.1.

25-35% fraction from the density gradient. Membrane fraction from melon hypocotyl (5 days old). Prepared as in Section B.3.VII. PA-CP staining procedure (Littlefield and Bracker, 1972) see Section B.3.VIII. Magnification x 16,000. Post-fixed with 1% OsO₄.

Plate 6.2.

As for Pl. 6.1 but stained with lead citrate and uranyl acetate.

Plate 6.3.

30-35% fraction from the density gradient. Otherwise as for Pl. 6.1.

Plate 6.4.

As for Pl. 6.3 but stained with lead citrate and uranyl acetate.

Plate 6.5.

34-38% fraction from the density gradient. Otherwise as for Pl. 6.1.

Plate 6.6.

As for Pl. 6.5 but stained with lead citrate and uranyl acetate.
Plate 6.7.

38-45% fraction from the density gradient. Membrane fraction from melon hypocotyl (5 days old) prepared as in Section B.3.VII. PACP staining procedure (Littlefield and Bracker, 1972) see Section B.3.VIII. Magnification x 16,000. Post-fixed with 1% OsO4.

Plate 6.8.

As for Pl. 6.7 but stained with lead citrate and uranyl acetate.

Plate 6.9.

Tissue section (T.S.) tomato stem (5 weeks old). PACP staining procedure (Littlefield and Bracker, 1972) see Section B.3.VIII. Magnification x 13,000. Post-fixed with 1% OsO4.

Plate 6.10.

As for Pl. 6.9 except magnification x 16,000.

Plate 6.11.

T.S. melon hypocotyl (5 days old) stained with lead citrate and uranyl acetate. Magnification x 6,000. Otherwise as for Pl. 6.12.

Plate 6.12.

T.S. melon hypocotyl (5 days old). PACP staining procedure, (Littlefield and Bracker, 1972) see Section B.3.VIII. Magnification x 50,000. Post-fixed with 1% OsO4.
membrane fragments. The technique does not appear to be very convincing for fractions derived from melon hypocotyl. The difficulty in deciding what was PACP positive (+) and what was not, can be seen here. Plates no. 6.1 and 6.3 contain a large number of stained vesicles whilst plate no. 6.5 contains the heaviest stained vesicles.

The results of the application of the procedure to tomato stem show that a distribution of PACP positive membrane vesicles can be seen in different fractions of the gradient but the result from the melon gradient show how difficult it is to decide whether such staining has occurred at all. This illustrates the necessity of applying the stain to sections of whole tissue so that the stain can be evaluated in a situation where the identity of different membranes is without doubt. Application of the procedure to sections of tissue will also show the intensity of staining which represents a positive result rather than background staining. This will also allow the assessment of the procedure as a criterion for the identification of the plasmalemma.
C.6.II. **Specificity of the PAGP stain.**

In order to assess the specificity of the stain for isolated membrane preparations it is necessary first to use it on sections of tissue from which the membrane vesicles were derived. In this section the staining of the whole range of subcellular structures will be examined and assessed. Only in this way can the usefulness of the stain be evaluated.

Blocks of tissue cut from tomato stem and melon hypocotyl were fixed, embedded and stained using the same procedures as for membrane fractions. Plates 6.9 and 6.10 are typical of the results obtained with sections of tomato stem and 6.12, 6.13 and 6.14 of those obtained with melon hypocotyl sections. In the tomato sections (plates 6.9, 6.10) a heavily staining line appeared next to the cell wall. There was also a vesicle of some sort which was also heavily stained (arrowed). Photographs of melon sections (Pls. 6.12, 6.13, 6.14) show how heavily the middle lamella was stained. In all the cases presented here it was this structure which showed the heaviest staining. Some regions of plasmalemma also appear to stain. It is difficult to distinguish the amount of staining obtained with the tonoplast and other vesicles from that obtained with the plasmalemma. Plate 6.11 shows a low power section of the same tissue subjected to the lead citrate and uranyl acetate staining procedures and is included to help in interpreting the other micrographs. None of these results suggest that this method results in the specific staining of the plasmalemma.

In order to obtain better differential staining a series of modifications were tried. The length of periodic acid treatment was varied up to two hours. The length of the staining period with PTA/CrO$_3$ was decreased to five minutes. Alternative destaining procedures using Cl$_2$ instead of, and in addition to, periodic acid, were also tried. None of these modifications produced any significant difference to the reliability of the stain. However, during the treatments in which the destaining times were increased there was an indication that the lack of specificity might be due to the post-fixation with OsO$_4$, which was likely to stain any lipid present (heavily stained lipid globules could be seen in many of the sections examined). Clearly, this would contribute to the lack of specificity of the procedure as all membranous structures contain lipid and so would tend to show some degree of staining.
Plate 6.13.

T.S. melon hypocotyl (5 days old). PACP staining procedure (Littlefield and Bracker, 1972) see Section B.3.VIII. Magnification x:50,000. Post-fixed with 1% OsO₄.


As for Pl. 6.13 but magnification x 40,000.

Plate 6.15.

T.S. cucumber hypocotyl (5 days old). PACP staining procedure 15 minutes in 1% phosphotungstic acid 10% chromic acid. No post-fixation. Magnification x 20,000.

Plate 6.16.

T.S. cucumber hypocotyl (5 days old). PACP staining procedure (Littlefield and Bracker, 1972) see Section B.3.VIII. No post-fixation. Magnification x 20,000.

Plate 6.17.

As for Pl. 6.16.

Plate 6.18.

As for Pl. 6.16.
In order to investigate this possibility more tissue was fixed and embedded but the post-fixation with $\text{OsO}_4$ was omitted from the procedure. This made the procedure more like the original recipe of Roland et al., (1969, 1971). The tissue used was cucumber hypocotyl because the possibility of using this as an alternative grafting system was being investigated at the time. Two stain procedures were investigated using this tissue.

1. Staining with $\text{PTA/CrO}_3$ for 15 minutes alone. Plate 6.15.

Examination of these micrographs, which are typical, shows that there are a few areas of dense staining of the plasmalemma, but overall, there is no convincing differential staining of the plasmalemma, which can be used to identify the plasmalemma in separate fractions, with certainty. Plates 6.19 and 6.20 show lower power views of sections of the same material generally stained. The plasmalemma does appear to be rather fragmented which might contribute to the poor staining obtained with PAGP.

General lack of success with established staining procedures made it necessary to establish whether it was the tissue that was unsuitable (tomato, melon and cucumber) or whether the technique was at fault. This was done by repeating the procedures described by other workers exactly, using the same species. Two cases were carefully selected because the procedures were described unequivocally and the claims of specific staining were consistent with the evidence presented. This made it possible to compare the results obtained in this study with published photographs. The examples were the work of Roland et al., (1972) on stems of onion plants (Allium cepa) and that of Roland and Vian, (1971) using the root meristems from peas (Pisum sativum, germinated for 4 days) and growing stems of Ballota nigra. Roland and Vian, (1971) used the staining procedure described by Kambourg, (1967). At the same time blocks of tissue from young stems of species used in this grafting study were prepared and simultaneously put through the same staining procedures. Details of the staining procedures are given in Section B.5.VIII.

Plate no. 6.21 shows a photographic reproduction of the results.
Plate 6.19.

T.S. cucumber hypocotyl (5 days old). No post-fixation. Lead citrate and uranyl acetate staining. Magnification x 10,000.

Plate 6.20.

As for Pl. 6.19.

Plate 6.21.

Section of onion stem from Roland et al., (1972). Magnification x 65,000. Post-fixed with 1% OsO₄.

Plate 6.22.

T.S. Datura stramonium stem (4 weeks old). PaCP staining procedure according to Roland et al., (1972) see Section B.3.VIII. Magnification x 8,000. Post-fixed in 1% OsO₄.

Plate 6.23.

T.S. cucumber hypocotyl (see Section B.1.). Otherwise same as Pl. 6.22.

Plate 6.24.

Section from onion stem (Allium cepa). PaCP staining according to Roland et al., (1972). Magnification x 8,000. Post-fixed in 1% OsO₄.
that Roland et al., (1972) obtained with onion stem. The cell wall and plasma membrane show intense staining compared with all other structures in the micrograph. Plate no. 6.24 shows the results obtained when the method of Roland et al., (1972) was repeated using onion stem. No evidence can be seen of specific staining of the plasma membrane. A portion of cell wall can be seen with some membranous structures on the lower side (arrowed). Very little can be seen of cell contents and the section when scanned was composed of pith cells. Roland et al., (1972) appear to have included more meristematic regions which may account for the different results. This raises the question of whether at least some of the anomalies observed are due to PAP stain reacting differently with different cell types. The stain might appear to be specific to the plasma membrane under the reported conditions in one tissue but not in another tissue of the same plant.

Plates 6.22 and 6.23 show Datura stramonium stem and cucumber hypocotyl treated according to the method of Roland et al., (1972). Very faint staining of various membranes can be seen but there does not appear to be an enhanced staining of the plasma membrane.

The second method, that of Roland and Vian, (1971) and Rambour, (1967), using the meristem of pea (Pisum sativum) and B. nigra stem gave the best results. Plates 6.25, 6.26, 6.27, 6.31, 6.32 and 6.33 show sections of B. nigra tissue treated in this way whilst 6.28 and 6.29 are photographs of sections stained with lead citrate and uranyl acetate. Plates 6.35 - 6.38 inclusive show pea meristems subjected to PAP stain using the method of Roland and Vian, (1971) and Rambour, (1967) and plates 6.30 is a view of the same tissue stained with lead citrate and uranyl acetate for comparison. In these micrographs the plasma membrane was heavily stained and can be easily identified in all of the plates. This is the density of staining necessary to be certain that staining has occurred. The procedure used here was simply two minutes staining in 1% PTA, 10% CrO₃ on tissue untreated with OsO₄ and appears to stain the plasma membrane reproducibly.

Plates 6.25, 6.26 and 6.27 show heavily stained plasma membrane adjacent to the cell wall but plate 6.26 shows an equally heavily stained line around the outside of the cell wall where the middle lamella is positioned. Plates 6.31, 6.32 and 6.33 show areas of tissue which contain more organelles so that the specificity of the stain can be assessed. Again there is convincing staining of the plasma membrane but
Plate 6.25.

**T.S. Ballota nigra** stem. PaCP staining procedure (Holand and Vian, 1971; Rambourg, 1967) see Section B.3.VIII. Magnification x 10,000.

No post-fixation.

Plate 6.26.

As for Pl. 6.25 but magnification x 10,000.

Plate 6.27.

as for Pl. 6.25.

Plate 6.28.

**T.S. Ballota nigra** stem. No post-fixation. Stained with lead citrate and uranyl acetate. Magnification x 10,000.

Plate 6.29.

As for Pl. 6.28 but magnification x 16,000.

Plate 6.30.

**T.S. Pisum sativum.** 4 days germinated pea root meristem. No post-fixation. Stained with lead citrate and uranyl acetate.

Magnification x 10,000.
Plate 6.31.

T.S. *Bassia nigra* stem. PCAF staining procedure (Roland and Vian, 1971; Hambourg, 1967) see Section B.3.VIII. Magnification x 16,000. No post-fixation.

Plate 6.32.

As for 6.31.

Plate 6.33.

As for Pl. 6.31 but magnification x 10,000.

Plate 6.34.

Meristematic cells of *Pisum sativum* (4 days germinated pea root meristem) from Roland and Vian, (1972). No post-fixation. Magnification x 45,000.

Plate 6.35.

T.S. root meristem from 4 days germinated peas (*Pisum sativum*). PCAF staining according to Roland and Vian, (1972) and Hambourg, (1967). Magnification x 10,000. No post-fixation.

Plate 6.36.

As for Pl. 6.35 but magnification x 16,000.
plate 6.33 shows a chloroplast with a large starch grain where the thylakoid membranes have stained very clearly and heavily, as well as the external surface of the cell wall. So there remains some doubt as to the specificity of the stain using B. nigra.

An example of the results achieved by Roland and Vian, (1972) with cells from the pea root meristem is shown as photographic reproduction in plate 6.34. These were taken at a very high magnification so it is difficult to assess the specificity. In addition to staining of the plasmalemma (p.e.) some endoplasmic reticulum (r.e.) is also stained.

Results obtained in this study with cells from the pea root meristem are shown in plates 6.35 - 6.38. Plate 6.36 shows a few areas of plasmalemma stained against a very low contrast background. There were however, other membranes (arrowed) which also stained. One of these was probably the endoplasmic reticulum and the other the nuclear membrane. Plate 6.36 also displays areas where double membranes are visibly stained and again these were probably endoplasmic reticulum. Plates 6.37 and 6.38 also show these membranes and in addition to this there were developing vacuoles. These vacuoles had heavily stained areas and in some the whole membrane was significantly stained. Again there was staining of the endoplasmic reticulum.

These were the best results obtained with PACP stain. The results obtained were similar to those reported by Roland and Vian, (1972) allowing for the difference in magnification. There was some evidence for lack of specificity in both this study and also that of Roland and Vian, (1972). In particular, endoplasmic reticulum appears to be stained in both preparations. This establishes that the results obtained in this study were similar to those obtained by other workers, and now it is important to determine whether the staining procedure works equally well on the tissues selected for this study.

The exact procedure was repeated using tissue blocks of cucumber and melon hypocotyl without post-fixation with OsO₄ prior to embedding. Plates 6.39 and 6.40 show PACP stained areas from large and small cells from the cucumber hypocotyl respectively whilst plates 6.41 and 6.42 show comparable tissue stained with lead citrate and uranyl acetate. The level of staining, particularly in the large cells, was not as dense as found previously but some areas of plasmalemma were stained. Again there was consistent heavy staining of the middle lamella. The
Plate 6.37.

T.S. root meristem from 4 days germinated peas (Pisum sativum). PAOF according to Roland and Vian, (1972) and Hamburger, (1967) see Section B.iii.VIII. Magnification x 16,000. No post-fixation.

Plate 6.38.

As for Pl. 6.37.

Plate 6.39.

T.S. cucumber hypocotyl (5 days old). PAOF staining procedure (Roland and Vian, 1972; Hamburger, 1967). No post-fixation. View from area with large cells. Magnification x 16,000.

Plate 6.40.

As for Pl. 6.39 but from area with small cells.

Plate 6.41.

As for Pl. 6.39 but stained with lead citrate and uranyl acetate. Magnification x 10,000.

Plate 6.42.

As for Pl. 6.40 but stained with lead citrate and uranyl acetate. Magnification x 10,000.
small cells showed heavier more continuous staining of areas adjacent to the cell wall. There do not appear to be any other structures which were staining except for the arrowed structure the identity of which is uncertain.

The results obtained with melon hypocotyl tissue show some convincing examples of plasmalemma staining in both small and large cells e.g. plates 6.43, 6.44, 6.46, 6.49 and 6.50. However, again as shown in plate 6.43, structures which were not plasmalemma also appeared to stain. Areas shown in plates 6.47, 6.48 and 6.51 show lead citrate and uranyl acetate stained sections to aid interpretation of the PaCP stained sections.

When evaluating the usefulness and specificity of the PaCP stain, the question which must be asked is whether the plasmalemma is the only structure which stains and whether it is easily distinguishable from other structures simply on the basis of the staining response.

Even in the best preparations other structures stain such as the middle lamella which is heavily stained in all cases and the endoplasmic reticulum (plates 6.36 and 6.38) and the edges of vacuoles. Mitochondria in sections of Ballota nigra tend to show up as ghosts and not stain to the same extent as the plasmalemma in a "good" preparation. However, as was seen in plate 6.33 chloroplasts show staining particularly of the thylakoid membranes.

The variation obtained with this stain suggests that there is a certain amount of tissue specificity if not organ specificity as might be the case with onion stem. The positive results of Roland et al., (1972) might have been obtained with more meristematic areas not present in the preparation of onion shown here.

The preparation of some sections from pea root meristems included the adjacent region of the root cap and this exhibited totally different staining characteristics. Heavy staining was observed throughout the root cap cells giving the appearance of light unstained walls surrounding almost uniformly dark contents with no distinguishable membrane. The adjacent cells of the meristem (plates 6.35 - 6.38 inclusive) show very different staining properties. This strongly suggests that the stain is only applicable to certain tissues or even certain regions of as a differential procedure for use as a marker for the plasma membrane.
Plate 6.43.

T.S. melon hypocotyl (5 days old). PASP staining procedure (Roland and Vian, 1972; Rambourg, 1967) see Section B.3.VIII. No post-fixation. magnification x 10,000 from area with small cells.

Plate 6.44.

As for Pl. 6.43.

Plate 6.45.

As for Pl. 6.43 but magnification x 16,000 and from area of large cells.

Plate 6.46.

As for Pl. 6.45.

Plate 6.47.

As for Pl. 6.45 but stained with lead citrate and uranyl acetate. magnification x 10,000.

Plate 6.48.

As for Pl. 6.43 but stained with lead citrate and uranyl acetate. magnification x 10,000.
Plate 6.42.

T.S. melon hypocotyl (5 days old). PAPF staining procedure (Roland and Vian, 1972; Hambourg, 1967), see Section B.3.VIII. No post-fixation magnification x 10,000 from area with large cells.

Plate 6.50.

As for Pl. 6.49.

Plate 6.51.

As for Pl. 6.49 but stained with lead citrate and uranyl acetate. Magnification x 16,000.
There is little cytochemical basis for the specificity of this stain. Marinozzi, (1968) reported that phosphotungstic acid at low pH has an affinity for polysaccharides. The original use of the stain was in animal tissue where it was used to stain Golgi apparatus (Rambourg, 1967). In a more detailed cytochemical study there was evidence that much of the staining by phosphotungstic acid at low pH was due to the presence of sialic acid residues within surface coat glycoprotein (Dermer, 1973). In another study however, evidence was presented for the binding of phosphotungstic acid to a wide variety of polymers including phospholipids (Scott and Webb, 1975). This evidence suggests that absolute specificity of this stain to one membrane system is unlikely because of the seemingly wide range of compounds which will react with phosphotungstic acid under these conditions.

There are reports in the literature of lack of specificity which has also been confirmed in this work. Hendriks, (1976), Thom et al, (1975) and Hall and Flowers, (1976) all found lack of specificity of the staining method. Amongst structures found to be stained were the tonoplast, outer cell wall and chloroplast membranes. In addition to this, Hall and Flowers, (1976) found that the inner region of the cell wall also stained heavily in some sections where the membrane had come away from the wall. Some of the staining of plasmalemma in other preparations could be due to this. It can be difficult to distinguish the plasma membrane from the inner edge of the cell wall particularly under the conditions found with this staining method where overall contrast is very low. Following the criticisms of the PACP stain Nagahashi et al, (1978) published a report in support of the procedure. They maintained that lack of specificity was due to technical errors and published a very detailed staining procedure which included the use of high temperatures and humidity to increase uniformity of staining. Although they conclude that, with such meticulous precautions, the stain is specific, they admit that even in their hands and using such exhaustive procedures, areas of uncertainty are found! Hall and Flowers, (1976) also tried a similar method to this but maintained that it did not improve the results obtained.

If a staining procedure is to be used as the sole means for the identification of membrane fractions then not only must it clearly stain the plasmalemma, it also must do this consistently throughout all
the cell types present in the tissue. In addition the procedure must
not stain any other structure, particularly the tonoplast as this is a
likely contaminant of plasmalemma rich fractions.

Because of these grave reservations as to the validity of the PACP
stain as a means for the identification of the plasmalemma it is
impossible to say with any certainty whether any of the fractions
studied in Section C.5 were enriched in plasmalemma. However, the
staining of the gradient fractions with PACP is included so that results
can be compared with published work.

Alternative methods of identification have been tried, these
include lactoperoxidase mediated \(^{125}\text{I}\) labelling of surfaces of maize
coleoptiles (\textit{Zea mays}, Hendriks, 1976). Hendriks, however only compares
results of incorporation from different fractions without any effort to
show localisation of the label in intact tissue. Hall and Roberts (1975)
used this technique with maize (\textit{Zea mays}) but, although the degree of
iodination was greatly reduced in the absence of applied lactoperoxidase,
they found high levels in the 13,000 g pellet and in protein precipitated
from the 30,000 g supernatant suggesting considerable intercellular
activity probably due to endogenous peroxidase activity. They also
obtained very high incorporation in the mitochondrial fraction. They
concluded that this method was not suitable for identification of plant
plasmalemma. This method was also studied extensively in this
laboratory and the same conclusion was reached (Kilpatrick, 1978,
personal communication).

Other methods of labelling plasma membrane surfaces include the
formation of bonds between amino groups and pyridoxal phosphate followed
by reduction with \(\text{NaBH}_4\) to give a tritiated product (Hall and Roberts,
1975), but the results obtained using \textit{Zea mays} roots were unsatisfactory.
Galbraith and Northcote (1977) investigated the use of diazotized
\(^{35}\text{S}\) sulphanilic acid as a specific cell surface labelling agent using
protoplasts of soybean (\textit{Glycine max}). Again analysis of the incorporation
of the diazotized \(^{35}\text{S}\) sulphanilic acid is carried out by comparison
of the distribution of radioactivity in different fractions obtained
from the fractionation procedure. There are some inconsistencies,
namely the relatively high proportion of radioactivity recovered in a
fraction containing unbroken protoplasts, some plasma membrane and cell
wall debris and nuclei. Otherwise the distribution of the radioactivity
was consistent with the pattern one would expect if labelling was
confined to the surface. Any remaining doubts could be removed by looking at the distribution of radioactivity in both intact protoplasts and fractions using autoradiographic techniques. The method is a very promising one except for the limitations imposed because of the use of protoplasts. In the preparation of protoplasts proteins might be lost from the plasmalemma and so a complete picture of incorporation patterns of amino acids into proteins during grafting could not be obtained. However, such a method could be used to characterise the plasmalemma and evolve other methods of distinguishing the plasmalemma from other membranes and then these might be applied to fractions obtained from whole cells and tissues rather than from protoplasts.
SECTION D:

DISCUSSION
DISCUSSION.

Anatomical studies of the development of the graft union have shown that, after the initial cut is made and the two surfaces of the graft are brought into contact, there is first a proliferation of cells forming a callus between the two surfaces after which vascular regeneration occurs. Indeed the successful formation of a graft is characterised by the re-establishment of vascular continuity (Hartman and Kester, 1961). The sequence of events in the formation of stem graft have been described by Krenke, (1933) Ercole, (1965) and Copes, (1969). They reported that the cells killed by the cut, form a brown layer and the living cells beneath became meristematic and form callus tissue. The brown layer eventually disappears and fusion occurs while new vascular tissue and a new cambium develop in the graft callus. In many herbaceous grafts the line of union remains visible, long after grafting as a thickened wall (Mendel, 1936; Yeoman and Brown, 1976; Stoddard and McCully, 1979). The initial adhesion was investigated by Lindsay, (1972). Studies previous to the work of Roberts and Brown, (1961) were limited by the lack of a quantitative measure of the development of the graft. Roberts and Brown showed that a relationship exists between the development of the graft union and the increase in mechanical strength as measured by breaking weight. This relationship established with tomato autografts has been shown to apply to several other grafting situations (Yeoman and Brown, 1976). From extensive studies on tomato autografts Roberts and Brown, (1961) Lindsay et al., (1974) correlated the increase in mechanical strength with the pattern of development of the graft. They proposed a two stage process in which the first stage corresponded to the increase in cell number during callus formation whilst the second stage corresponded to the vascularisation of the union. The results presented in this thesis confirm these earlier studies in which, the breaking weight of the graft increased slowly at first and then increased very rapidly towards the end of the period. Although the total number of cells increases throughout the development of the graft, the increase in the number of tracheary elements is more pronounced in the latter stages of the graft union development. More detailed examination of the relationships of breaking weight with cell number, and breaking weight with the number of tracheary elements also showed that graft development could be
divided into two phases. During the initial phase of development where the mechanical strength of the graft did not exceed 130 g and was still increasing slowly, the breaking weight of the graft correlated with both the overall number of cells in the grafted region and the number of tracheary elements. However, during the later stages of development, when the mechanical strength of the graft was rapidly increasing, there was only a significant correlation between breaking weight and tracheary element number. Anatomical investigation of the tomato graft was carried out by Lindsay, (1972) and he showed that callus tissue was produced early in the development of the union. The production of a certain amount of callus appears to be essential in providing a ground tissue for the differentiation of the cambial and vascular elements without which a successful graft cannot be formed (Sass, 1932; Crafts, 1934; Mendel, 1936; Juliano, 1941; Esau, 1953). Later in the development, the rate of breaking weight increase coincides with the time at which vascular connection first becomes apparent (Lindsay, 1972). These observations are consistent with the relationships of breaking weight with cell number and breaking weight with the number of tracheary elements. The relationship does not appear as clear cut as suggested by Lindsay, (1972) who suggested that the initial increases in breaking weight were correlated with callus production whilst the more rapid increases in breaking weight in the latter part of the development, were correlated with vascularisation. Although in this study there is a correlation between breaking weight and the number of tracheary elements (a measure of the progress of vascular regeneration) during the latter part of development, the increase in breaking weight during the initial stage of development correlates not only with callus formation (or increase in cell number), but also with the increase in the number of tracheary elements.

The most important factor in the establishment of a successful graft union is the re-establishment of vascular continuity (Hartman and Kester, 1961). Yeoman and Brown, (1976) studied the grafting relationships of three members of Solanaceae. They found that compatible heterografts developed in the same way as autografts but that incompatible heterografts never achieved vascular continuity. They suggested that the initial stages of development were common to both compatible and incompatible grafts and the difference was
confined to the later stages. The transition between the first and second phase of development must be vital in determining the success of the grafting process. The transition between the two phases of development occurs between three and four days after grafting and so the time immediately preceding and following the transition will contain the most important events of the grafting process.

The conditions necessary for the establishment of a successful autograft were investigated in part whilst attempts were made to produce a graft which could develop in isolation from the rest of the plant. Lindsay, (1972) in a preliminary study of the development of excised grafts found that these excised grafts although they developed measurable breaking weights, (the maximum ever achieved was usually in the region of 100g after seven days) were never able to undergo the complete development of the union. The breaking weight of an unexcised graft at this stage would be in excess of 700 g. The excised internodes were supplied with mineral salts and sucrose but no growth substances. As the re-establishment of vascular continuity is a characteristic requirement of all successful grafts (Hartman and Kester, 1961) application of factors involved in regeneration of vascular tissue after wounding was investigated.

The experiments performed on excised grafts showed certain similarities with vascular regeneration around a wound. All those experiments were performed using the final day breaking weight as a measure of the development of the graft and comparing this to the development of unexcised grafts. Increased graft development was seen in the presence of leaves, buds and cotyledons. The presence of the primary trifoliate leaf and its axillary bud were able to increase the amount of development of the graft. These results are consistent with the results of studies on vascular regeneration in parenchyma of wounded stems. Von Haan Albeest (1934) demonstrated that leaves apical to the wound produced necessary factors for vascular regeneration. However, the presence of the cotyledons and the buds in the lower axils increased the amount of development to an even greater extent. The application of 0.1% IAA to the apical end of the grafted internode in a similar manner to that employed by Thompson and Jacobs, (1966) gave disappointing results as did application of 2,4-D (0.01 - 1%). Unlike IAA, 2,4-D slightly increased the degree of development. In
contrast, Thompson and Jacobs, (1966) found that 0.1% IAA applied apically to wounded excised tomato stems restored the extent of regeneration to a level found in the intact plant. If the regeneration observed in wounded stems was strictly analogous to that found in the graft some enhancement of the grafting process might be expected even a restoration to control values. The difference between the two situations may simply be a question of scale. In the work of Thompson and Jacobs, (1966) only one vascular bundle was severed and it was this regeneration that was completed to the same extent in excised internodes with 0.1% IAA, as in the intact plant. In the graft all the vascular bundles of the stem are severed so that the amount of regeneration required to restore the vascular system completely is much greater and a simple application of IAA may not be sufficient. Other factors which have been implicated in vascular regeneration are gibberellic acid (Roberts and Foskett, 1966), sucrose (Rubery and Foskett, 1969) and cytokinins (Houck and La Motte, 1977). However there are conflicting reports about the requirement for all of these factors (Jacobs, 1959; Thompson, 1965; Thompson and Jacobs, 1966). In the grafting situation these may be a requirement for sucrose or other growth factors. In addition the synthetic capacity of the excised internode might not be sufficient to provide all the basic building blocks necessary for the regeneration of all the vascular tissue. Another major difference between the two situations was that regeneration occurs across a limited wounded area of the vascular tissue and the translocation system although incomplete is still almost intact. In a graft there is no direct translocation across the union because all of the vascular bundles have been severed and the transport of metabolites is disrupted. Indeed, there is evidence that the major path of IAA movement is through the sieve tubes (Rischnich, 1935) although it can move through parenchyma cells (Jacobs and McCready, 1967). However, it is argued that the auxin responsible for the regeneration of a particular vascular strand, originates from that severed vascular strand and accumulates to induction levels as a result of the severing of the phloem (Jacobs, 1970). The part played by the unsevered strands has not been investigated, these may have a role in supplying the basic metabolites necessary for the construction of the specialised cells of the vascular tissues or in the supply of growth factors. When cotyledons with axillary buds
Fact that the root can continue development for at least twenty-four
weeks after exposure. A clear picture of the root in isolation was provided by the
exposed roots. The regeneration process was already underway when the
exposed roots became evident and the exposed root was not to the development of the
exposed root. The exposed root, however, was not only to the development of the
exposed root, but also to the regeneration of the root in isolation. Such experiments could
produce regeneration across the root union of a certain root to develop an
exposed root in isolation. In the case of the root in isolation, the root, the exposed root was
exposed and the exposed root union, which was not to the development of the
exposed root, was to develop an exposed root in isolation.

When these are referred to with the expected
results. If these are referred to with the expected
results, it is believed that the restoration of the exposed root
union, which is not to the development of the exposed root,
was to develop an exposed root in isolation.

In the case of the root in isolation, the root, the exposed root was
exposed and the exposed root union, which was not to the development of the
exposed root, was to develop an exposed root in isolation.
hours after excision before any significant deviation from the normal pattern of development. This means that the development of the graft can be studied for periods of up to twenty-four hours in isolation from the rest of the plant and provides a system in which the incorporation of radioactively labelled amino acids into protein during graft formation can be studied and manipulated without interference from other parts of the plant.

Another source of potential interference in studies of the incorporation of amino acids into protein are the micro-organisms which co-exist with all plants. The number of micro-organisms found in this study under standard conditions was of the order of $10^5$ to $10^6$ organisms per 2 mm segment surrounding the graft. The possible effect of the microflora on grafting can be seen when it is realised that the size of the population is comparable with the number of plant cells in the region under study. The mechanism of protein synthesis in plants and micro-organisms is similar, both are able to utilise the same precursors but an essential difference is that the ribosomes of bacteria have different characteristics from those of eukaryotic organisms and in particular different sensitivities to antibiotics. Attempts made initially to remove the microflora from a limited area of the plant surrounding the grafted region were partially successful. A variety of sterilising agents were used in the first instance but it was only the antibiotic solutions which did not significantly affect the plant. Use of ultra-violet irradiation or H$_2$O$_2$ was not possible because of the gross detrimental effects seen following their application. Chloramphenicol was chosen as a useful inhibitor of the microbial population. This antibiotic was found to be effective in controlling the levels of contamination in studies of protein synthesis in storage tissue disks (Leaver and Edeleman, 1965). However, the use of chloramphenicol in maintaining sterility in the grafted tomato internodes was not very effective. Only in ungrafted internodes was it possible to reduce the levels of contamination significantly and these levels could only be maintained for a number of days if no further manipulation was required. The construction of a graft after sterilisation of the internode was accompanied by a significant increase in the level of contamination over the duration of the experiment. Indeed the levels present were too high to exclude the possibility
that the contaminating microflora might significantly affect the results. Further modification of the sterilisation procedures were attempted. Sterilisation was left until immediately prior to excision of the grafted internode and its transfer to culture instead of being carried out prior to grafting. This reduced the time over which sterility had to be maintained to twenty-four hours and also the number of manipulations through which the tissue was subjected after sterilisation. Even after this modification the levels of contamination in a grafted internode were of the order of $10^3$ organisms per grafted segment, after transfer to culture the number increased, and after the twenty-four hours culture of the grafted internode, levels were in the order of $10^5$-$10^6$. Such levels are found without sterilisation.

Unfortunately, it was never possible to eliminate the microflora completely by sterilisation and the remaining contaminants multiplied and/or migrated from other parts of the plant. As an alternative approach sterile plants were raised from seed and maintained in sterile conditions throughout their life time. This method was attempted using cucumber seedlings because suitable tomato plants for grafting cannot be raised in aseptic conditions. Cucumber seedlings are large enough for grafting within twelve days of sowing and it was considered that it might be possible to raise cucumber plants in aseptic conditions. The production of sterile seedlings suitable for grafting did not provide any problems. It was possible to grow reasonably large numbers of seedlings with a negligible microflora within twelve days. The degree of contamination did not appear to vary over this time. However, immediately after grafting in some cases, the level of contamination was raised slightly, and after a period of six days the levels had increased significantly. The remaining few micro-organisms must have multiplied rapidly once the graft was made probably because of the release of the contents of the phloem which would provide a rich growth medium for micro-organisms.

Because of these difficulties and the grafting characteristics of the cucumber seedlings it was decided to abandon this approach. No further attempts were made to obtain a "sterile" graft because even with the very rigorous precautions taken with the cucumber grafts it appeared impossible to maintain sterility throughout the development of the graft. Attempts at sterilisation increased the differences
in microflora between the grafted internode and the ungrafted control, because sterility can be maintained with relative ease in ungrafted internodes. The studies of the patterns of incorporation of radioactively labelled amino acids into protein during the development of a graft were conducted by comparing the pattern of incorporation in equivalent regions of grafted internodes with ungrafted ones. It was thought that if attempts at sterilisation increased the differences in levels of contamination of the tissues under study then it was better not to attempt sterilisation at all, in which case the difference between levels of contamination in grafted and ungrafted internodes would be minimised. However, interpretation of the results obtained must take into account the presence of the microflora.

The microflora might have interfered with the incorporation studies in two ways. It may have affected the development of the graft directly or competed for the labelled amino acid and the proteins from these micro-organisms may have been extracted alongside those of the plant. It was unlikely that micro-organisms had directly affected the development of the graft union. In horticultural practice, grafting is carried out without any attempts to exclude micro-organisms from the grafted region. Such grafts rarely become diseased as a result of microbial proliferation after grafting. Observations with the scanning electron microscope have not found large numbers of bacteria within the union. It is likely that the plant can regulate this non-pathogenic microflora to a certain extent and that the microbial population is self-limiting.

The second point to consider is whether the microbes present in the surface of the plant are likely to incorporate large amounts of the radioactively labelled amino acid. The only microbes which could affect this study were those found on the surface of the plant in the grafted region which was analysed. It was unlikely that substantial amounts of labelled amino acid were available to these micro-organisms as they were not in direct contact with the incubation medium. Supply of labelled amino acid to these micro-organisms would have been from the plant. The labelling studies were made only after the transport system of the plant was sealed over and leakage into the grafted region had ceased. Any labelled amino acid incorporated by the micro-organisms would have to be absorbed from the plant cells. It is unlikely that substantial
amounts could be incorporated into bacterial proteins in this way. If there was some incorporation of labelled amino acid into the protein of micro-organisms, this was unlikely to interfere with the incorporation patterns of all the fractions studied. The plant material was homogenised with a pestle and mortar to extract the proteins, which was likely to require the use of considerable force before they ruptured. The preliminary 10,000 g centrifugation would sediment the majority of the micro-organisms. The "insoluble" fraction was the only fraction which (10,000 g supernatant) and the membrane fractions which were all derived from a 10,000 g supernatant are unlikely to contain any bacterial proteins.

The pattern of incorporation of methionine into protein, during the three twenty-four hour periods, encompassing the critical stage of the transport of an ungrafted intermediate, by incubation of the tissue in a solution containing 35S-methionine (granted) or 3H-methionine (ungrafted), for twenty-four hours. After this treatment with an equivalent amount of ungrafted tissue, the soluble and insoluble proteins extracted were separated using polyacrylamide-gel slab gel electrophoresis to show the spectrum of protein sub-units present. Gel electrophoresis to show the spectrum of protein sub-units present, and the pattern of radioactive distribution was then cut into slices. By plotting these different patterns in each case, it can be seen that a large number of changes in incorporation were taking place throughout the stages studied. These changes in the pattern of incorporation could be seen in both soluble and insoluble fractions because of the difficulty of separating what is background variation and what are real differences in a single pattern. There was also a problem in...
comparing different patterns because the overall ratio might be
different due to a difference in the amount of the labelled amino acid
solution supplied to either partner rather than to a difference in the
activity of the tissue.

An analysis was also made in which the pattern of incorporation of
"wounded" and ungrafted tissue was compared. Again a large number of
areas were found with differential activity between the wounded and
ungrafted tissue. Bearing in mind the difficulty of comparing patterns
from different gels, the patterns of incorporation due to wounding were
compared with those due to grafting and some areas of activity were
found which occurred in the grafted tissue, but not the wounded tissue.
The wounded tissue was a graft which instead of having living tissue
as its scion had a piece of inert plastic so that the comparison was
with regeneration taking place against an inert surface as opposed to
regeneration between two living surfaces. The pattern of incorporation
due to grafting was determined by comparison of the incorporation
patterns of grafted and ungrafted tissue. Proteins whose synthesis
was increased as a result of grafting would have a number of functions.
Graft formation is characterised by extensive cell division, vascular
differentiation and wound regeneration as well as interaction between
stock and scion. Proteins synthesised as a result of grafting may be
involved in any one of these processes. Some of the proteins which
were synthesised as a result of grafting were not found in the pattern
of incorporation of "wounded" tissue. These proteins were characteristic
of regeneration and interaction between two living surfaces rather than
regeneration against an inert surface. The difference between the two
situations was that in the second there could be no interaction between
stock and scion so that the proteins which were synthesised as a
result of grafting but not "wounding" might have a role in such cell
interactions. However, it was unlikely that vascular regeneration
occurs in the wounded tissue so these proteins might be implicated in
this process also.

The sub-cellular origin of these proteins may help to determine
what their role was in the grafting process. Both soluble and insoluble
fractions contained material from many different organelles. Also
there was material which was common to both fractions. The source of
the graft induced proteins can be determined by further fractionation
to separate the sub-cellular organelles.
Some of these proteins, the increased synthesis of which was associated with grafting rather than regeneration might have a role to play in compatibility mechanism. If the basis for compatibility in graft formation involves a protein based recognition system as suggested by Yeoman et al., (1978) then it is reasonable to suppose that such proteins would be situated in the outer layers of the cell probably the plasmalemma. It is clearly important to examine the pattern of synthesis of the plasmalemma proteins during the development of the graft, in particular during the critical stage when the transition between two phases of the development of the union. It has been suggested that the initial phase is common to all grafts, compatible and incompatible, whilst the second phase only occurs in compatible grafts and the development of incompatible grafts diverges at this point, this is probably the point in time that such a recognition/compatibility system would be involved.

The soluble fraction was further fractionated, as this fraction was unlikely to be contaminated by bacterial proteins. This fraction can be used to prepare membrane fractions using the method of Hodges et al., (1972). This is the most widely used method for obtaining putative plasmalemma fractions. All attempts at isolation of the plasmalemma from plant sources have founded on its identification after fractionation, when the membrane is no longer recognisable. The most commonly used criterion for establishing the presence of plasmalemma in a membrane fraction is the PAGP staining technique (Hardin et al., 1972; Hodges et al., 1972; Koechler et al., 1976; Lembi et al., 1972; Shore and MacLachlan, 1975; Strobel and Hess, 1974; Van der Woude et al., 1972). The basis for this technique was provided by Roland et al., (1972) who first reported selective staining of the plasmalemma in situ. This has been extrapolated to the identification of plasmalemma fragments in the form of vesicles from fractionated tissue homogenates. The reliability of this method has been questioned on the grounds that staining of other sub-cellular structures has been observed in situ. The reliability of this method was tested in this study using many different tissues and also some modifications of technique but in no case was the staining observed in tissue sections such, that any reliability could be placed on this as the sole criterion for identification of the plasmalemma. In many
cases the staining of the plasmalemma was not very convincing, the intensity of staining being such that there was very little contrast between plasmalemma and other structures and in some cases the staining was uneven. In addition to this, even in the cases where the most obvious staining of the plasmalemma was seen, there was also staining of other structures which was equally convincing. Other structures stained included the tonoplast, endoplasmic reticulum and Golgi bodies. Reports in the literature claim staining of the tonoplast (Thom et al., 1975; Hall and Flowers, 1976), endoplasmic reticulum (Thom et al., 1975), prolamellar bodies, lipid droplets and ribonucleoprotein from ribosomes (Quail and Hughes, 1977). The latter have a recognised propensity to become redistributed and absorbed to membraneous components during homogenisation. All these components have the potential to generate PAP positive profiles in cell free homogenates and would falsify the identification of the plasmalemma. The second criticism of the method concerns the assumption that the basis for the specificity, towards the plasmalemma seen in situ under critically exacting staining conditions, will be preserved unaltered in homogenates (Hendriks, 1976; Jesaitis et al., 1977; Quail and Browning, 1977; Quail and Hughes, 1977).

Nagahashi et al., (1978) argued that if very stringent staining procedures were adhered to, then the stain was selective and could be applied to homogenates, as long as the inclusion of sections from fixed intact tissue in the staining procedure was routinely made alongside the sections of homogenate. However, even they, with their extremely rigorous precautions, did find areas in some sections where non-specific staining was occurring! Because, such non-specific staining could occur in an area of a section which otherwise showed specificity of the procedure, the use of this method as the sole criterion for establishing the identity of the plasmalemma is suspect.

Some attempts have been made to introduce the use of enzymes markers which are associated with the plasma membrane of animal cells. 5'-nucleotidase, alkaline phosphatase and Na^+, K^+ dependent ATPase, have not been shown to be exclusively associated with the plasmalemma of plant cells and in some cases possess negligible activity in plant homogenates (Morré et al., 1970; Hodges and Leonard, 1974). Some enzymes such as glucan synthetase have been associated with putative
plasmalemma fractions from various sources including onion stem (Van der Woude et al., 1972), oat roots (Hodges et al., 1972), sugar cane leaves (Thom et al., 1975) and soybean hypocotyl (Hardin et al., 1972) but there is no evidence that it is exclusive to this membrane system, indeed, it is associated with Golgi apparatus. An association has been shown between a Mg^{2+}-dependent, K^+ stimulated ATPase and putative plasmalemma fraction from oat roots (Hodges et al., 1972) and also sugar cane leaves (Sinensky and Strobel, 1976), but the possibility of its occurrence elsewhere has not been eliminated.

Attempts have also been made to introduce external markers to intact tissue before homogenisation and subsequent fractionation. Lactoperoxidase catalysed iodination of externally exposed proteins is a method used successfully on a wide variety of animal cell types (Phillips and Morrison, 1971; Hubbard and Cohn, 1971), lymphocytes (Karchalonis, 1971), fibroblasts (Hynes, 1973) and slime mould amoebae (Green and Newell, 1974). The technique has been applied to plant tissue by Hendriks, (1976) and Hall and Roberts, (1975) and also in this laboratory (p. comm. Kilpatrick, 1978). The moderately high degree of incorporation they obtained in the absence of lactoperoxidase may have been due to the action of endogenous peroxidases and since these are of widespread occurrence in plant tissues, this may be a problem with plant cells in general.

In the absence of any single way of unequivocally identifying the plasmalemma at present, the composition of the membrane fractions obtained from tomato was assessed by use of the PaCP stain, this being the only criterion available and because it was so widely used. Thus the fractions obtained could be compared with that of other workers.

PaCP positive vesicles were found in various fractions but predominately in the higher density fractions. The presence of PaCP positive vesicles correlates with the greater changes in pattern of protein synthesis. The fractions containing the most PaCP positive vesicles were those in which there were many large differences in the pattern of synthesis between grafted and ungrafted tissue. It was not possible to decide whether these fractions contain plasmalemma with any degree of certainty and the purity of the fractions obtained was poor. It is not inconceivable that the changes observed are associated
with other sub-cellular fragments which are enriched alongside the PACP positive vesicles.

Because this study was confined to the tomato autograft it is difficult to assign a particular significance to the result in terms of compatibility involved in grafting. The study was made to provide a background against which heterografts, both compatible and incompatible, could be studied. Thus it might be possible to locate a membrane component which behaves differently in an incompatible graft. Such a component could then be purified and characterised and its role in grafting elucidated. However, before any such study can be undertaken the methods of purification and identification of the plasmalemma from plant sources must be improved. When a reliable method of purification is available and it is possible to say with certainty that a sub-cellular fraction is derived from the plasmalemma, the behaviour of the proteins in such a fraction will be of great interest in the study of grafting. If it becomes possible to isolate a fraction which is synthesised preferentially in a compatible graft, and is confined to the plasmalemma, but absent from an incompatible graft, then this would be consistent with a recognition role for the plasmalemma. Such proteins however, might not be concerned with the primary recognition step but might be synthesised as a response to the initial recognition. At this stage it is impossible to say what the nature of the recognition system involved in grafting is, but on the basis of the grafting relationships seen (Yeoman et al., 1978) it was suggested the graft compatibility involved a recognition system. The inability of stock and scion to achieve vascular continuity can be due to structural or major biochemical differences between stock and scion. In those members of the Solanaceae studied there was no connection between anatomy and failure to form a successful union. Indeed Lindsay, (1974) reported that in the tomato autograft, deliberate mismatching of the vascular tissue of stock and scion failed to influence the development of the graft, other than slowing it down to a limited extent. There are reports of biochemical incompatibilities between pear scions and quince rootstocks (Gur et al., 1968) and between peach scions and almond rootstocks (Gur and Blum, 1973) where a cyanogenic glycoside descending from scion to stock is hydrolysed by β-glycosidase, releasing cyanide, killing the cells in the union thus preventing
formation of the graft union. It is thought improbable that such a cause exists for the incompatibility found between tomato and Nicandra because such differences would tend to promote necrosis in the union, which have not been observed (Yeoman et al., 1978). This evidence is consistent with another cause for incompatibility, the one suggested was a form of cell recognition with the most likely site for recognition being the point of contact between cells growing out from around and within the vascular bundles. The role of auxin in vascular regeneration is well established and it could be argued that there might be a disparity in the levels of such factors in the incompatible union. However, as tomato will form successful unions with Datura, and Latura will form successful unions with Nicandra but tomato will not form successful unions with Nicandra, it is hard to envisage how differences in response to auxin and such factors would produce such a result. Indeed it is possible to obtain a successful union between tomato and Nicandra by insertion of a bridge of tissue from Datura between the stock and scion. It is doubtful that a bridge, in which the endogenous levels of such fractions would be low, could maintain the environment for the successful union, whilst the rest of the plant is producing much larger amounts. It may be important which surfaces are in contact and that the reason for incompatibility resides in the cells of the union themselves rather than in substances transmitted from the rest of the plant. From this study it was not possible to shed any light on the mechanism of compatibility as the study has been restricted to the tomato autograft.

Studies of changes in pattern of the whole spectrum of proteins in the grafted region were difficult to interpret as the number of changes was so great. During the formation of the graft union there are so many events underway including extensive cell division and differentiation which will only occur to a limited extent in the ungrafted internode. It is probable that if any proteins were involved directly in the determination of compatibility, it would reside in the cell surface either in the plasma membrane or cell wall. So the pattern of changes in the putative plasmalemma fraction was also analysed. It was found that the greatest number of changes in the pattern was associated with the presence of PACP positive vesicles. Taking into account the reservation implicit in the method this suggests that these fractions
do play an important part in the formation of the graft union at the stage of development where any compatibility mechanism will be involved. It must not be forgotten that all these studies were made without any attempt to exclude the micro-organisms which are normally present on the developing plant and some of the changes might be due to the action of the microflora rather than the plant. However, in future studies it will become necessary to resolve this question entirely. Improvement of the method of purification and isolation of the plasmalemma from plant sources is necessary also, before any firm conclusions can be made and such work extended. The use of protoplasts for this has been investigated (Gaibraith and Northcote, 1977) and, although membranes derived from protoplasts may lose some constituents during preparation, these may play a useful role in the long term by providing membrane fractions which can be analysed and properties which are found could be applied to the preparation of plasma membrane from whole cells.

In conclusion this study has shown that the development of the graft union takes place in two stages. During the first stage the increase in breaking weight is dependent on both increase in the total number of cells and also the increase in the number of tracheary elements whilst during the latter phase of development the increase in breaking weight correlates with the increase in the number of tracheary elements alone. Following those observations attempts were made to study the pattern of incorporation of radioactively labelled amino acids into protein during the critical stage, between the two stages of development. The study was made using the isolated grafting system devised but with no attempt made to eliminate the normal microflora of the plant. Many changes in pattern were found both before and after this stage. It was felt that if it were known from which sub-cellular organelles these proteins were extracted then a role for these proteins might be suggested. In particular the role of the plasmalemma was considered to be of great interest. Unfortunately no undisputed method of identification exists for these organelles and it was not possible to state categorically that a particular fraction contained plasmalemma. However, the fractions containing PACP staining vesicles were identified as these provide the only widely used method of identification so that the fractions obtained can be compared to the
work of other people. It was found that most of the changes in patterns of incorporation occurred in fractions which were PAGF positive and it was tentatively concluded that this fraction must play a major role in the development of the graft. The specificity of the PAGF stain was investigated and it was found not to be a reliable means of identification of the plasmalemma so highlighting the need for further work on the identification of plant plasmalemma.
Future work.

Further attempts must be made to produce plasma membrane fractions which can be positively identified. The use of protoplasts should facilitate such work. Once a plasma membrane fraction is obtained this can be characterised and any properties which are specific to the plasma membrane can then be applied to preparations from whole cells so that the fractionation procedures can be improved. Then it will be possible to repeat the dual labelling studies during different stages of development of the graft and also compare the patterns obtained for compatible and incompatible heterografts. If a protein can be found which is confined to the plasma membrane and is characteristic of either compatible or incompatible heterografts then this would be a likely candidate for a recognition protein. Purification of such a protein and raising of an antibody to it would allow study of the location of this protein in the graft union in both compatible and incompatible situations. Presence of such proteins in the interacting surface of a graft would point to a recognition role.

Further attempts to determine the factors necessary for the development of grafts in isolation from the rest of the plant are also necessary. In particular the investigation of the use of more complex growth media and the role of growth factors. In such a way a grafting system which could be manipulated easily and could develop completely in culture might be obtained.
SECTION E:

REFERENCES
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