SOME ASPECTS OF THE METABOLISM OF AGEING CELLS

by

DAVID HABESHAW

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Introduction

This thesis was intended as an extension of the work on cell development in roots carried out by R. Brown and various co-workers since 1950. To date most of the work using the root as an experimental system has been concentrated on the initial processes of division, expansion and differentiation and the changes in the more mature cells have not received as much attention. The reason for the neglect of the mature cells is undoubtedly because the seedling root does not provide a simple system for the study of cells after maturity. It does, however, provide a really good system for the study of cells in their division, expansion and early differentiation and the successive segment technique used by Brown makes the investigation of these processes a relatively simple experimental procedure. The changes associated with the period of division and differentiation have been comprehensively studied by Brown and other workers and the results embodied in papers by Brown and Robinson (1952), Heyes and Brown (1965) and Brown and Broadbent (1950).

The main avenues of approach to the problem of cell behaviour in the process of cell development using root systems are as follows:-

1. Analysis at successive segments using the first 1.6 cm of the roots of leguminous seedlings (pea and bean). This technique involves the analysis of batches of successive segments 0.4 - 1 mm in length, measuring a wide range of parameters. The number of cells in each segment is determined and values can be obtained relating the changes in the "average cell" to the process of cell enlargement and early maturity. It has been exploited with success by Brown and the other workers cited above and has proved very profitable in the early stages of cell development after division has ceased.

2. Analysis of changes during the culture of segments from the 2 - 4 mm region of seedling roots. This technique has the value that cell expansion in the isolated segments, when provided with an energy source, is considerable, 50% of that in intact roots. Modifications of the environment can be made and the effects of many variables, inhibitors, growth substances etc., on cell expansion can be observed in the complete absence of cell division. This technique has been used by Robinson and Brown (1954) and Vaughan (1965) and its main value lies in the ability of the technique to follow the changes in cells over a restricted portion of their development. This technique has been applied with great success to the study of cell expansion and the effect of a wide range of factors upon this process.

3. Investigation of the growth of excised root tips grown for considerable periods of time in sterile culture. This work has been primarily used for the investigation of the effect of different culture solutions on growth and the changes in root anatomy and biochemistry during growth in a defined environment, (Street (1964). Also this system has been used to study the effect of various elements and compounds on the process of cell division in root meristems (Brown and Possingham, 1957). This work was invaluable since it provided a compendium of knowledge on the basic problems of root culture which greatly assisted in the design and execution of many of the experiments reported here. It also suggested that the absence of secondary thickening in cultured leguminous roots would provide a simpler system for the study of more mature tissue than the intact root.

4. The analysis of cell development in the root by successive segment techniques associated with histochemical studies of various regions of the developing root. This has been carried out by Jensen (1955) and McGregor and Street (1953) for the early stages of root cell development. The study of the more mature cells of the root where cells in different

tissues will clearly undergo ageing changes at different rates will also require histochemical techniques to define the variation between tissues.

Without going into a comprehensive discussion of all the results and conclusions which previous work produced, it is necessary to raise several points in connection with them for the better understanding of the aims of the present study and the methods used in the investigation. An essential point of the interpretation of these results was that the cells of the root pass through a series of stages of development, each characterised by its own particular enzyme complement. The end of each stage leaves the cell in a position to proceed into any one of several inherent alternative stages of development. The completion of any one of these stages leaves the cell in a position to follow any one of a new set of alternative stages and so on. The developing root system thus becomes more complex as each of the successive stages are passed. Although the developing root system is not an essentially simple system with all the cells in a given region of the root doing the same thing at the same time, (Jensen, 1955). It has enabled valid conclusions to be drawn regarding the development of the hypothetical "average" cell up to the stage at which expansion ceases. After the root cells have reached maturity it is no longer possible to analyse similar segments from intact roots, since secondary changes occur and these are usually restricted to certain cells or tissues. Even cells of the same tissue can have very different functions within the root as the root enters the various stages of its mature development. Since senescence appears to be a progressive process, this makes the interpretation of the average state of cells within a mature segment of the root uncertain unless coupled with a knowledge of the regions of the root segment participating in each of the changes observed in the gross analysis of the root segment. This indicated that for the analysis of mature root segments a histological and anatomical investigation of the root must be

carried out in order to correlate the results of the gross analysis of the segment with the cells involved. This complicated the analysis of the root material and was a departure from the original techniques used for the study of younger root tissues. The observation that all the roots grown in culture for periods exceeding a week produced lateral roots and exhibited an increasing amount of variation in the length of the main root axis during culture was made early in the experimental programme. This observation raised doubts about the ability of the successive segment technique to be extended to cover cultured root tissue of greater age than 8-10 days. In order to test this a series of preliminary experimental runs were made using the successive segment technique to see how useful this technique would be in the study of ageing change over an extended period of culture. The results are given in the appropriate section of the results section of the thesis, but two facts arose from them which necessitated the rejection of a successive segment technique as an experimental system for the study of ageing. These facts were as follows:-

1. The presence of lateral roots and lateral root primordia on the root made it impossible to arrive at any useful estimate of "average cell" behaviour in the segments since a segment may or may not contain such meristematic centres and hence the results can not be applied with certainty to cells undergoing maturation and ageing.

2. The nitrogen content per cell at maturity was found to be dependent on the position of the cell in the root. This was probably because cells formed from the meristem in culture are different from those formed from the meristem in the intact seedling. This does not imply that the development of cultured root cells is abnormal in any large degree. But the fact that the absolute values of certain cell parameters at maturity, of which total nitrogen is an example, were

dependent on the conditions of culture and the age of the meristem at their time of formation means that no basis exists for comparison of the results obtained from different regions of the root.

This showed that in order to fulfil the original purpose of the investigation, which was to complete a survey of the changes in root tissue through maturity to cell death, another experimental approach was necessary. A consideration of the preliminary results and other work with cultured root systems led to the following conclusions. The anatomical and functional heterogeneity of the root must be accepted as an unavoidable consequence of studying mature root tissue. The degree of uncertainty introduced by this must be reduced by carrying out detailed histochemical and anatomical investigations in parallel with the gross analyses of the root tissue. The experimental system utilised must have the following properties. It must have no lateral roots or lateral root primordia and tissues of different ages must be comparable.

There must be no cell division and differentiation should be at a minimum during the period of the experiment. The experimental system described below satisfied most of these requirements and was the one used for the majority of the experiments whose results are presented later in the thesis. In this technique roots were grown using a method substantially similar to that used by Bonner and Addicott (1937) but instead of dividing the roots into a series of successive segments to provide an age series only the basal 1 cm segment was removed from each root and used for analysis. The age series was provided by the repetition of this process for a series of batches of cultured roots separated by successive time intervals. In this way it was possible to provide tissue for analysis which was more or less identical in cell number, anatomy and origin, the only variable being the age of the tissue segment. This system proved to be a good one for the study of ageing changes and an excellent one for the study of the inter-relationships between the older and younger tissues of

the root.

Having by this means produced a system which could be expected to show a series of changes through maturity to death via senescence, a careful search through the available literature was made to establish those parameters most likely to provide useful information on the changes occuring in the root tissue. It is convenient to divide the literature on this topic into several sections.

1. Those dealing with isolated root culture and the various adaptations of the root to different conditions of culture. Fortunately there are several excellent reviews of this topic available, the one referred to most frequently in the design of the experiments was that of Street (1964) other individual papers referred to were those of White (1943), Brown and Possingham (1957) and Bonner and Addicott (1937). Apart from the information on methods and techniques of root culture obtained from these, the only other facts obtained from this section of the literature which had a bearing on the adoption of a cultured root system were those which indicated the absence of any secondary development in cultured root systems. This was important in making the decision to use cultured roots since it enabled a major variable to be removed from the consideration of the mature root system.

2. Those dealing with the development of cells during their differentiation and maturity. The work done in this field seemed to be rather patchy, most attention not unnaturally being devoted to the differentiation and function of the more specialised tissues of the stele and the piliferous layer. The most useful were those of Gahan (1965) on the development of the xylem in root tissue and Crafts (1962) on the development of the phloem sieve tubes. McGregor and Street (1953) also provided evidence of a change in the nature and distribution of acid phosphatases in the tomato root with age. In general it appeared that work on the changes and inter-relationships between mature tissues in

comparable systems had not as yet been very fully investigated although a considerable quantity of work exists which deals with the specialised function of mature cells such as photosynthesis, salt uptake, translocation etc. In fact any cell activity or function can be regarded as typical of a stage in its development but it is obvious that this point of view is not profitable in terms of the production of relevant information so a few papers were selected such as those cited previously which seemed to have a direct bearing on the methodology, experimental design or subject of this work.

3. Those dealing with the subject of senescence. Like the second section this comprises a vast amount of very diverse information. The line separating this and the previous section is of necessity rather arbitrary since there are few changes occuring in plant tissue for which a case cannot be made for considering as part of a predetermined series of changes starting at cell maturity and finishing in death. The bulk of the work on senescence has been carried out on animals rather than on plants but many of the results obtained apply equally well to both. Since a comprehensive survey of the whole topic does not come within the scope of this thesis, only those papers referring to work which had a formative effect on the design of the present experimental work will be cited. A general knowledge of the major theories and experiments in this field was obtained by reference to the publications of Strehler (1963), Sax (1962), Szilard (1959), Leopold (1959) and Comfort (1956). A useful summary of the position in respect of plants was obtained from the reviews by Varner (1961) and Sax (1962). Of more immediate use were those papers which referred to the senescent changes in isolated leaves and fruits which were systems which have several points of similarity with the cultured root system as developed in this work - Yemm (1956, 1950), Sacher (1965), Pirie (1959), Osborne (1962), Chibnall (1954), Chibnall and Wiltshire (1964) and Shaw (1965).

The parameters chosen for the analysis of the basal segments were chosen as follows. DNA, RNA, cell number, oxygen uptake, total nitrogen, protease, dipeptidase, invertase and phosphatase were followed because they had already been used with success to investigate cell development using the successive segment technique. However, some alterations and additions were made to the methods used. DNA was initially measured as a method for estimation of the percentage of live cells in the segment. In fact a very much extended series of observations using microdensitometric and radioautographic techniques coupled with anatomical investigations was undertaken and the final results went far beyond this initial aim. RNA changes were investigated but the problem of extracting suitably clean preparations from the older root tissues was not solved satisfactorily so these estimations were discontinued since other lines of investigation held more promise. Further techniques and parameters were followed as suggested by other work or from a knowledge of the system. For instance, the gross phosphatase analysis was supplemented by a histochemical investigation of this enzyme activity in the hope that the results might follow those of Gahan (1965), and be useful in delineating the cells undergoing senescent change. This technique also enabled living and dead tissue to be distinguished.

The work of Satiro (1962) showed changes in the ability of tissue to reduce tetrazolium salt with increasing age and so an estimation of succinic dehydrogenase activity using tetrazolium salt as the acceptor was added to the range of enzyme estimations. This enzyme was one of those involved in the citric acid cycle and some degree of correlation was expected between this enzyme activity and oxygen uptake. Other enzymes which were also assayed in the ageing root tissue were protease, dipeptidase, invertase and polyphenol oxidase. The first three of these were used because they had already been shown to have quite large changes during the

early stages of cell development and they were followed in this work to confirm or refute the possibility that changes in the protein complement continued into the later stages of cell development. Polyphenol oxidase was included in the enzymes assayed for several reasons. It was thought that since this enzyme was concerned with the synthesis of melaninϕ type compounds and these are in turn implicated in the formation of lignin and the coloured compounds which are formed in older cells it might thus be expected to show changes in activity with age. Also the activity of this enzyme in plant tissue was shown to be increased by bacterial attack on the tissue, Farkas (1964) having shown that there was a marked similarity between the enzyme changes of tissues invaded by certain plant pathogens and the changes produced during senescence and death. This line of reasoning is not so strong as might be supposed since Charles (1954) has shown that some of the changes observed in senescence and normally considered to be due to ageing changes are in fact due to the invasion of the experimental material by bacteria. In spite of this, there were sufficient grounds for including this enzyme in the series of assays carried out on the tissue. Besides the total nitrogen estimations several other nitrogen fractions were determined during the ageing of the root tissue. These included buffer soluble and buffer insoluble nitrogen, amide nitrogen content of the protein and ethanol soluble fractions and the quantity of free ammonia present in the tissues. Electrophoresis of the buffer soluble proteins on acrylamide gels was carried out at intervals during the duration of the experiments to indicate whether there were any gross changes in the protein of this particular fraction. These determinations were supplemented by experiments in which the rate of uptake of C^{14} leucine into protein was measured. This part of the experimental programme was designed to study certain aspects of nitrogen metabolism in senescing tissue which other workers on starving leaves had shown to be very complex. In starving leaves protein synthesis

is maintained to a late stage and the normal respiratory substrate, carbohydrate, becomes depleted and subsequently protein degradation is thought to supply carbon skeletons from amino acids which are utilised in respiration. As a consequence, nitrogen is conserved as amide amino acids, but disorganisation of cell metabolism is an inevitable consequence of unrestricted or unbalanced protein degradation and cell death ensues with a consequent complete breakdown in nitrogen metabolism, especially transamination and free ammonia accumulates. It was hoped that similar processes could be shown to occur in ageing root tissue.

One of the most widespread phenomena in plant senescence is the senescence of a whole plant or plant organ apparently as the result of a stimulus received from the environment or within the plant itself. For instance, the senescence and death of many plants seem related to the production of flowers and fruit. Leopold (1959) has performed experiments which demonstrate clearly that the onset of senescence in soyabean and spinach can be delayed for considerable periods by the removal of flowers and fruits. Lockhart (1961) has shown that this is not the whole story in Pisum sativum and that apical senescence although delayed by deflowering cannot be stopped entirely by this treatment. The senescence and abscission of the leaves of deciduous trees is thought by Osborne (1962) to involve some type of senescence factor providing the stimulus for the senescence and abscission of the leaf. This sort of observation raised the question of whether similar relationships exist in the cultured root system using a basal segment technique. It appeared possible that the rate of growth of the root as a whole might affect the process of ageing in the basal segment especially since other work on cultured roots showed that there was a definite relationship between the meristem and the tissues immediately behind it (Brown and Wightman, 1952). Also there is evidence of a

relationship between the activity of the meristem and the production of lateral roots (Torrey, 1956). Experiments were performed to see if there was any sort of relationship between the physiological age of the whole root as measured by the fresh weight and the condition of the basal segment. These experiments were then compared with the condition of the basal segment analysed on the purely chronological scale of time in culture.

Since in general the roots were grown in a medium which becomes progressively more unfavorable for sustaining growth, the system as developed shows many similarities with the starving leaf system as used by many other workers on senescence.

The growing root system offers several possibilities for studying the effect of various culture conditions on the capacity of the meristem to sustain its rate of division. Under carefully controlled conditions it is possible to keep clones of pea root tissue derived from meristems, growing for several years and these clones do show a type of clonal senescence eventually resulting in the extinction of the cell line. Such clonal senescence is a very long drawn out process taking years rather than months to become apparent, so no attempt was made to carry on research along these lines. If the meristem is left on the root and the whole root is passed through a series of transfers in a specially supplemented medium, division in the meristem ceases at a much earlier time and the size of the cultured root and the frequency of transfer both have an effect on the division in the meristem. Several experiments were undertaken in order to study the effect of medium changes on the growth of the root as a whole and the ageing process in the basal 1 cm segment. These were mainly short term experiments to study the effect of keeping the root in a medium with an optimal sucrose concentration, since initial experiments had shown that the loss of sucrose from the medium was one of the main factors involved in the cessation of growth in roots grown in the

same medium for periods greater than a week. This was not the only deleterious change during culture since experiments on meristem viability showed a progressive decline in the ability of the meristems to sustain division with increasing time in culture even during the initial time where the sucrose concentration was favourable. From observations made during the initial experiments it seemed possible that the pattern of ageing in the basal segment was dependent on the presence of an intact root attached to it. Consequently, the basal segments were amputated and grown for varying periods of time in isolated culture and the changes compared with those occuring in the basal segments of intact roots. This experiment was expected to show that the ageing in the basal segments was under the control of the rest of the root and that the timing of the various ageing changes was controlled by the rate of growth of the root.

Materials and Methods

Section 1. Root culture method.

The method described by Bonner and Addicott (1937) was used for the first experiments. In this technique, peas, var Meteor, were carefully graded for size and those with an average diameter of between $\frac{1}{4}$" and 5/16" were used for experimentation after those with damaged or infected testas had been removed. Before germination the peas were surface sterilised in the following manner. About 100 peas were placed in a 100 ml. wide necked Erlenmeyer flask fitted with a rubber bung. The peas were then soaked for 10 mins. in 80% ethanol, rinsed twice with 50 ml. of distilled water and left to soak for $\frac{1}{2}$ hour in 50 ml. of distilled water, after which, those peas with damaged testas were removed. The soaking facilitated this since peas with damaged testas took up water much more quickly than the normal ones. The peas were then sterilised by placing them for $\frac{1}{2}$ hour in a saturated solution of calcium hypochlorite. The peas were then rinsed with two lots of sterile distilled water and transferred to a sterilised petri dish.

After this sterilisation procedure had been carried out, the peas were transferred to sterile petri dishes, three peas to a dish, containing 20 ml. of distilled water. The dishes were then kept in an incubator at 25°C in the dark for three days. (After this time the peas had germinated and the roots were between 1 and 3 cms. long). All dishes which showed signs of infection were discarded and the apical 1 cm. of the roots with a length of between 1.5 and 2.5 cms. were removed with a sharp scalpel under sterile conditions. Still under sterile conditions these inhocula were transferred to sterilised petri dishes containing 20 ml. of sterilised Bonner and Addicott medium. Three root inhocula were placed in each dish. The dishes were then placed

in an incubator and grown for up to 40 days in the dark at 25 degrees C. Plate 1 shows the amount of growth made by the roots after 27 days of culture.

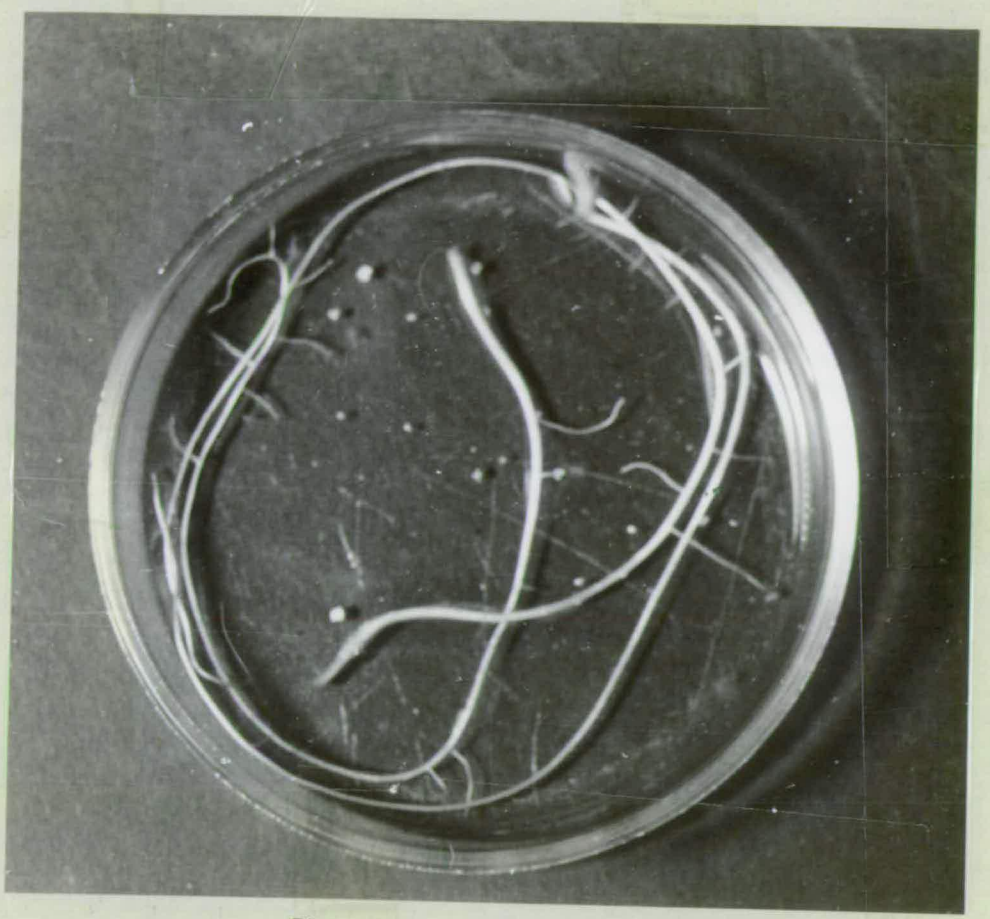


Plate 1

The composition of the Bonner and Addicott medium is given below:-

$Ca(NO_3)_2 4H_2O$	236 mg/litre.
$MgSO_4\ 7H_2O$	36 mg/litre.
KNO_3	81 mg/litre
KCl	65 mg/litre
$K_4H_2PO_4$	12 mg/litre
$Fe_2(SO_4)_3$	1 mg/litre

Made up in 4% Sucrose.

The above procedure was used for all the initial experiments. However, as the size of the experiments increased this method proved to be wasteful in terms of the effort required to produce a given quantity of root material for experimental purposes. Because of this the modification of the method described below was introduced for the remainder of the experiments.

The procedure described previously was followed to the stage at which the sterilised peas were placed in the sterile petri dish prior to planting. The peas were then planted in a sterile pyrex pie dish in moist sterile vermiculite instead of in petri dishes as previously. The water content of the vermiculite was very important since the slightest water stress in the seedling completely stopped the further growth of the root in culture. The optimum water content for germination and strong root growth was 800 ml. of water to every 2 litres of vermiculite. This mixture was sterilised by autoclaving in the pie dishes for 2 hours at 20lbs. pressure. About 100 peas were sown in each pie dish. This modified method of germination provided better roots in larger quantities than the original method. The root inocula were cut and transferred with a special scissor-spatula. The use of this special tool reduced the time and the number of stages spent in the transfer of the root tips to the culture dishes during the setting up of the experiments.

The results given in Section 1b of the Results Section show that the growth of the roots in culture varied considerably within the same experiment. Some of this was due to roots which either did not grow or stopped growing before they should have, or grew in a definitely abnormal manner. Because of this, all roots which were obviously abnormal for one reason or another were discarded before the roots

used for the various estimations were taken. By doing this it was hoped to reduce as far as possible the amount of variation within the experimental material.

Section 2 - Design of experimental systems used

a) Successive segment system.

Roots were grown in the manner described in Section 1, and portions of the root were taken and subdivided into successive segments which were subsequently analysed in the same way as the basal segments from the later experiments. This was an extension of the method used by Brown for younger roots grown in a similar manner. The age series is obtained by selecting roots of different length and selecting different portions of them for analysis. The analysis of the root by this method proved to be unsuitable for roots grown for more than 7 days in culture, so this method was discontinued in favour of an analysis of the basal 1 cm. segment of the root only for the following reasons:-

1) This portion of the root never formed lateral roots and so any changes occurring in the tissue would not be complicated by their presence.

2) There was no cell division in this part of the root and so the cell number remained constant throughout the experiment. (See Results Section). Because the cell number remained constant for this segment the results could be expressed on a per segment basis which allowed the easy comparison of results.

3) The root was at its thickest in this region so there was a maximum yield of material to be obtained by using this segment.

4) All the tissues of this part of the root were fully mature when the culture of the root was started.

This means that this segment contains the oldest root tissues and that these were formed under completely normal conditions.

5) The largest tissue in this part of the root in cell number and bulk is the cortex parenchyma. The cortex parenchyma in this region is thus likely to be the source of the main changes observed in the ageing of this segment.

b) Continuous sampling technique:-

In these experiments 300-350 roots were set up in culture at the same time and samples were taken at intervals and the basal segment only removed for analysis. Apart from the difficulties of setting up an experiment of this size and keeping the infection rate down to an acceptable level this method is liable to an error in sampling. The reasons for this are given in the following discussion.

The roots grown by this method of culture show considerable variation in their rate of growth. From the Figure 1 in Section 1 of the Results Section it can be seen that the variation increases after the lateral roots have begun to form. It is only possible to tell whether a given root is typical of the population as a whole after it has reached its maximum length and fresh weight. The problem arises if the roots taken as samples are chosen on the basis of their closeness to the average growth rate, the method of sampling in effect, increases the population of non-average roots towards the end of the experiment. Because it is not possible to find out what the average growth rate of any batch of roots is going to be before they have completed their growth the average growth rate from another experiment has to be used as a basis for selecting the samples. Unless this is very close to the actual growth rate, the sampling will tend to produce a non-average population

at the end of the experiment. If there is no attempt to select the average roots in the population for use, the results tend to lose their characteristic pattern, because there were not enough roots to allow an increase in the size of the samples taken in order to reduce the variability to a reasonable level. The only way out of this would have been to increase the size of the experiment and this was not possible for various reasons. If the average growth rate on which the roots are selected is higher than the growth rate of the roots that are being sampled, then the percentage of slow growing roots is increased towards the end of the experiment. The reverse applies to a selection on a lower growth rate than that of the experiment. Because the ageing of the basal 1 cm. of the root is controlled by the rate of growth of the root this means that the ageing processes are apparently slowed up at the end of the experimental period. If the reverse is true the ageing processes are apparently speeded up. The method described below was devised to overcome this objection.

c) Batch technique with final sampling at 30 days

This was the method used for the main series of the experiments. In this method ten dishes were set up in culture every three days. Thus at the end of a thirty day period a whole series of roots was obtained each batch of which differed in age by three days from its neighbours in the series. The growth rate over the whole thirty day period was determined by measuring the length of the roots in the successive batches, and the roots required for experimentation were selected on this basis. The only effect of differing growth rates between experiments was to alter the time scale slightly for the different experiments. This can be seen in Fig. 21 in the discussion of variation in this experimental system. The variation produced by this was not as large as that produced by the use of the previous method described in

Section b. The basal 1 cm. segments of all the selected roots of all ages were removed for analysis on the 30th day thus eliminating variation due to slight difference in anglytical technique.

d) Transfer of roots after varying times of culture

A normal experiment was set up as described in Section 2c. After a period of time which depended on the purpose of the experiment, the roots were divided into two identical batches on the basis of the mean growth made in culture. Half of the roots were transferred to fresh medium, the remainder being left in the original medium to act as control. Two experiments of this type were carried out, the first involving a transfer every three days, and the second, one transfer at fifteen days only. The method used in both experiments was the same except for frequency of transfer. In the experiment involving transfer every three days, a selection on the mean growth basis was made for the first transfer only since the act of transfer slowed the rate of growth. The basal segments of the transferred roots were removed for analysis in the normal manner.

e) Isolated basal segment technique

A normal experiment as described in 2c above was set up but of twice the normal size, (20 dishes instead of 10). After 6 days, half of the dishes were taken and the basal 1 cm. amputated from those roots whose growth rate was nearest to the mean growth rate of the whole experiment at that time. The basal 1 cm. segments were replaced in the medium from which the original roots had been taken and allowed to age for the remainder of the experiment. The other half of the roots acted as a control for the experiment. This was repeated for each batch of roots at the 6th day of culture. The basal segments were removed for analysis after 30 days as before.

f) Sucrose supplementation of medium

A double sized experiment was set up as for Section 2e

and allowed to grow for 12 days. The mean rate of growth was then found and the roots divided into two identical batches. One of these batches was supplemented with sucrose and the other left as the control. This process was repeated with each batch of roots as they reached the 12th day of culture. The addition of sucrose was done by adding 5 ml. of 16% sucrose solution to the supplemented batches and 5 ml. of sterile distilled water at the same time to the controls. This addition of water to the controls did not appear to alter their pattern of ageing at all. The basal segments of the roots were analysed in the normal way.

g) Analysis of system on the basis of root growth rather than time in culture

In this type of experiment a whole batch of roots were grown and selected from the 9-30 day period, eliminating all roots which had made no growth at all by 9 days. All roots were mixed together and then weighed individually and divided into groups covering successive ranges of 10 mg. The basal segments from each group were removed and used for analysis in the same way as in the previous experiments. This means that the basal segments from roots of the same chronological age were spread over several weight divisions. This treatment could thus be expected to nullify any change based solely on the time of culture in medium.

3) Analysis of experimental material

a) Measurement of the fresh and dry weight

For the fresh weight determinations the roots were blotted dry between filter paper discs and weighed in a tared watch glass. For dry weight determinations the roots were transferred to tared specimen tubes after the fresh weight determinations and dried to constant weight in an oven at 100°centigrade. The fresh and dry weights of the basal segments were determined in exactly the same manner.

b) Measurement of the length of the main axis of the root

The measurement of the length of the main axis of the root was carried out in two ways depending on the use to be made of the roots subsequently. If it was desired to avoid opening the dish the roots were measured by placing the petri dish on a piece of 1 cm. squared graph paper and counting the number of 1 mm. squares crossed by the root. If the roots to be measured were being harvested for an experiment they were simply stretched out and measured with a ruler.

c) Fractionation procedures used in the analysis of the basal segment.

1) Fractionation procedure 1

This fractionation procedure was used to prepare the ethanol soluble fraction, and other fractions from which the protein nitrogen, protein amide nitrogen, amino acid nitrogen, amide amino acid nitrogen, free ammonia nitrogen and DNA were determined. For this fractionation 6-10 basal segments were used.

The full fractionation procedure is given below including the preparation of the perchloric acid extract for the DNA estimation.

Basal root segments.

Leave for 24 hours at 4°C in 70% ethanol (1 ml. for every 3 segments) decant off the ethanol.

Ethanol extract

Homogenise the extracted segments in 4 mls. of 0.5 M perchloric acid at 0 degrees centrigrade and centrifuge. Discard supernatant.

Resuspend residue in 4 ml. of 0.5 M perchloric acid at 0°C and re-centrifuge. Discard supenatant.

Resuspend in 4 ml. of 35% ethanol at 0°C and re-centrifuge. Discard supernatant.

Resuspend in 4 ml. of 1:1 ether ethanol solution at 0°C and re-centrifuge. Discard supernatant.

Resuspend in 4 ml. of dry ether at room temperature and centrifuge. Discard supernatant.

Residue was allowed to dry at room temperature in the inverted centrifuge tube to give a dry washed pellet. This is resuspended in 1 ml. of 0.5 M perchloric acid, heated to 70°C for 30 mins. and re-centrifuged.

Perchloric acid extract for DNA estimation.

Residue resuspended in 70% ethanol and re-centrifuged. Discard supernatant. Residue resuspended in ether ethanol solution and re-centrifuged. Discard supernatant.

Residue resuspended in dry ether and re-centrifuged. Discard supernatant. Residue was then allowed to dry to give a dry acid-washed pellet

The ethanol extract was then divided into 4 parts for:-

1) Total nitrogen estimation
2) Amide nitrogen estimation
3) Free ammonia nitrogen estimation
4) Chromatography of the ethanol soluble fraction for amino acids.

The dry pellet after the DNA extraction was divided into 2 equal portions for:-

1) Total nitrogen estimation
2) Amide nitrogen estimation.

The total nitrogen in the segment was obtained by the addition of the nitrogen contents of the various fractions. This value was an under-estimation because there was no separate estimation of the nucleic acid nitrogen but this in no case amounted to more than 10% of the total nitrogen value and thus does not alter the significance of the result.

b) Fractionation procedure 2

This was the fractionation procedure used to prepare the polyphenol oxidase enzyme extract but advantage was taken of this to obtain two further protein nitrogen fractions, the buffer soluble and the

buffer insoluble protein. Ten basal root segments were used for this fractionation procedure.

Ten basal root segments

Homogenise in 3 ml. of 70% ethanol at 0°C and centrifuge at 0°C.

Ethanol extract analysed as in Fractionation 1

Resuspend residue in 2 ml. of phosphate buffer pH 6.8, 0.2 M at 0°C and allow the temperature to rise to room temperature and re-centrifuge at room temperature.

Supernatant, the buffer soluble protein fraction. This solution was divided into three equal parts and used for:-

Residue, washed to remove buffer and reduced to a dry pellet in the manner described in Fractionation 1. The total nitrogen content of the pellet was then estimated.

1) Polyphenol oxidase activity estimation.
2) Gel electrophoresis of the soluble proteins.
3) Estimation of the protein nitrogen in the soluble protein fraction. To the solution of soluble protein an equal quantity of 10% TCA was added and the solution cooled to 0°C to precipitate the protein and the solution was then centrifuged. Discard supernatant.

Residue was resuspended in 2 ml. of 70% ethanol and re-centrifuged. Discard supernatant.

Residue was washed and dried in the same way as the other solid fractions in the first fractionation procedue and was reduced to a dry pellet, the nitrogen content of which was then determined.

2) Methods used for the estimation of the nitrogen content of the basal segment and the fractions produced from it by the fractionation procedures.

The basic method used for all the nitrogen estimations was one

of microdiffusion analysis as outlined by Conway, (1962). Two types of estimation were used, one to obtain the total nitrogen content and one to obtain the labile nitrogen content. The free ammonia nitrogen estimation was a modification of the second of these. These methods were used throughout the experimental programme, but the methods of digestion of the various fractions were not the same for every fraction. The changes in technique necessitated by the various fractions are given in the description below.

a) Estimation of the total nitrogen in the basal segment

This was carried out in two ways. The nitrogen content was estimated directly using the whole segment without fractionation or indirectly by the addition of the nitrogen contents of the fractions after fractionation. The first of these techniques was usually preferable and it is described below.

Between 20 and 40 mg. of tissue (2-3 basal segments) were digested in 100 µl of digest acid in a $\frac{1}{4}$" x 3" hard glass test tube heated to 290°C in an aluminium heating block. The digest acid was made up according to the following recipe:-

Analar H_2SO_4 (N.free)	100 g
Selenium	0.2 g
$HgSO_4$	1.0 g
K_2SO_4	6.0 g

The digestion was complete in 2-3 hours depending on the quantity of tissue present. When digestion was complete the tubes were removed from the block and allowed to cool. After cooling, the contents of the tubes were transferred to the outer chamber of a micro-conway unit. This transfer was accomplished by using a pasteur pipette and making three washings of the digest tube with 200-300 µl of distilled water for each washing. These washings were transferred to the outer chamber of the conway unit along with

the acid digest. The centre well was then filled with 0.2 ml. of Boric acid indicator solution ~~(0.2 ml) was added to the centre well~~ and the conway dish cooled on a block of ice (see Conway, 1962). To the outer well of this cooled conway unit 0.5 ml of 50% KOH was added and the dish closed with a glass lid. The solutions in the outer chamber were then mixed by gently swirling the unit. The units prepared in this way were left in the cold room for 24 hours and the ammonia released was titrated directly with 0.001 N HCl using an Agla micrometer syringe. From this titre the quantity of nitrogen present in the original sample was found.

b) Estimation of the labile nitrogen in the whole segment

This again could be done in two ways. The labile nitrogen could be determined directly by the hydrolysis of the whole segment without fractionation or indirectly by the summation of the values for the protein amide nitrogen, the amino acid amide nitrogen and the free ammonia nitrogen. As in the total nitrogen estimations the first of these was the one generally used and the technique for this is described below.

For this method 20-40 mg. of basal segment tissue were placed in a digest tube and 100 µl of 3N H_2SO_4 were added. The tube was then closed with an aluminium foil cap and heated for three hours in a boiling water bath. The tubes were then cooled and their contents transferred to the outer chamber of a cooled conway unit. The tube was then washed with 3 x 100-200 µl aliquots of distilled water and the washings transferred to the conway unit with the acid hydrolysate. 0.2 ml. of indicator solution was then placed in the centre well. To the acid hydrolysate in the outer chamber 100 µl of 2.9 N KOH was added. After this had been mixed with the hydrolysate by gently rocking the conway unit, 0.5 ml of saturated K_2CO_3 was added to the outer chamber and the

unit closed with a glass plate. The solutions in the outer chamber were then mixed by gently swirling the dish and the units were left in the cold room for 18 hours for the ammonia to distil into the boric acid indicator. The ammonia released was titrated directly against 0.001 N HCl as before. The nitrogen determined by this method came from two sources, the amide nitrogen present in the tissue and the free ammonia present in the tissue. This combined value was called the labile nitrogen of the tissue.

c) Methods for the estimations of the nitrogen content of the ethanol soluble nitrogen

1) Estimation of the total nitrogen content of the ethanol soluble fraction.

The digestion was carried out in a 15 ml. centrifuge tube. To this tube was added 0.5 ml if the ethanol extract and 500 µl of digest acid and the tube gently warmed to drive off the water and ethanol. This process was carried out on a Gallenkamp micro-kjeldahl digestion rack specially fitted with a time switch to ensure a steady low heat for this purpose. The relatively large quantity of digest acid used was necessary because the ethanol reacts with the acid and lowers the efficiency of digestion. When all the water vapour had been driven off and the ethanol driven off or partially digested the heat was gradually increased to the normal digestion temperature and the digestion completed. The tubes were allowed to cool and 100 µl of the digest were placed in the outer chamber of a cooled conway unit. This was diluted with 500 µl of distilled water and 0.2 ml of indicator solution was added to the centre well of the unit. To the outer chamber of the unit 0.5 ml of 50% KOH was added and the unit closed with a glass plate. The solutions in the outer chamber were then mixed by gently swirling the unit and the diffusion and titration of the ammonia released were carried out

in the manner previously described.

2) Estimation of the amide nitrogen content of the ethanol soluble nitrogen.

For this estimation 0.5 ml of the ethanol extract was placed in a 15 ml round bottomed centrifuge tube and evaporated to near dryness using gentle heat and a vacuum desicator alternately. The drying was done as gently as possible to minimise the loss of glutamine from the sample. To this dried down sample 100 µl of 3N sulphuric acid was added and the quantity of amide nitrogen present determined in the way described for the labile nitrogen earlier in this section.

3) Estimation of the free ammonia nitrogen in the ethanol extract.

For this estimation 0.5 ml of the ethanol extract was placed directly in the outer chamber of a cooled conway unit. Then 0.2 ml of the indicator solution was placed in the central well of the unit and 1 ml of saturated potassium carbonate added to the outer chamber of the unit. The unit was then closed with a glass plate and the solutions in the outer chamber were mixed by gently swirling the unit. The distillation and titration of the released nitrogen were carried out as described previously.

d) Methods used for the estimation of the total and amide nitrogen contents of the perchloric acid insoluble material.

1) Estimation of the total nitrogen.

The dry pellet produced by the fractionation procedure 1 was left in the 15 ml centrifuge tube in which the washing procedure was carried out and 200 µl of digest acid was added. The pellet was then digested on a micro-kjeldahl digestion rack. The digestion took between 1 and 2 hours depending on the size of the pellet. On

completion of the digestion, the contents of the digest tube were transferred with the washings of the tube to a cooled conway unit and the nitrogen content determined in the same manner as that used for the total nitrogen estimation on the whole segment.

2) Estimation of the amide nitrogen content

To the dry pellet left in the 15 ml centrifuge tube after the washing procedure 200 µl of 3N sulphuric acid were added. The tube was capped with a piece of aluminium foil and heated in a boiling water bath for three hours. The tube was then cooled and the contents transferred to the outer well of a cooled conway unit and the nitrogen content determined in the same way as that previously described for the labile nitrogen estimation. Since the fractionation procedure removes any free ammonia from this fraction this value represents the amide nitrogen content of the fraction which was equivalent to the amide nitrogen of the total protein of the segment.

e) Methods used for the estimation of the buffer soluble protein nitrogen and the buffer insoluble protein nitrogen.

The method used for both these fractions was the same. The sample for estimation was a dry pellet in a centrifuge tube in both cases. The digestion and nitrogen estimation was carried out for both samples in the way previously described for the estimation of the nitrogen content of the perchloric acid insoluble fraction.

3) Estimation of the DNA content

The preparation of the perchloric acid extract for the Dische estimation is outlined in the fractionation procedure given in an earlier part of the materials and methods section. There were two methods used to estimate the DNA content per segment or a related value.

a) Dische reaction

This was a chemical estimation based on the reaction

between deoxyribose and diphenylamine to produce a blue colour, the intensity of which was measured in a spectrophotometer. The method used was a slight modification of the method of Burton (1956) which was itself a modification of the method developed by Dische (1955). The full method for the estimation is given below.

To the 1 ml of perchloric acid extract obtained from the first fractionation procedure, 2 ml of Dische reagent were added and the resultant mixture was incubated in a 15 ml centrifuge tube at 25°C for 18 hours. After this time the OD of the solution was measured in a Unicam SP 500 spectrophotometer at 585 mμ and 650 mμ against a blank consisting of 1 ml of perchloric acid and 2 ml of the Dische reagent. The OD reading at 650 mμ was subtracted from the reading at 585 mμ and the difference between the two readings converted into a quantity of DNA by reference to a standard DNA curve prepared from the OD difference values produced by known quantities of calf thymus DNA. The subtraction of the 650 mμ reading from the 585 mμ reading was adopted as a measure which helped to reduce the interference due to other compounds which react with the reagent and the coloured degradation products of the diphenylamine reagent.

The Dische reagent is made up in the following way:-

1.5 gm of recrystallised diphenylamine was dissolved in 100 ml of glacial acetic acid to which 1.5 ml of analar concentrated sulphuric acid had been added. Just before used 0.1 ml of 2% acetaldehyde was added for each 20 ml of the reagent. This reagent was unstable and was made up fresh each time it was required.

b) <u>Estimation of the DNA content and DNA synthesis using microdensitometry and micro autoradiographic techniques</u>

Preparation of the Feulgen stained sections used for the microdensitometry and radioautography.

All the segments used for both these estimations were labelled with tritiated thymidine and half the sections cut were used for the microdensitometry and the remainder for autoradiography. All the sections were Feulgen stained regardless of their subsequent treatment. Both transverse and longitudinal sections (TS and LS) were cut from the segments but the LS were used only for the autoradipgraphy.

After labelling with tritiated thymidine the basal segments were fixed and embedded according to the method of Johansen (1940) the main details of which are given below.

The material was fixed in a solution containing formalin 5 parts, glacial acetic acid 5 parts and ethanol 95 parts for 12 hours. The material was then washed in 70% ethanol twice and stored in 70% ethanol if necessary. After this the segments were taken up through the following series of tertiary butyl alcohol, ethanol, water solutions spending 2 hours at each stage.

Tertiary butanol (TBA)	Ethanol	Water
70	20	10
85	10	5
90	7	3

The segments were than left for one hour in a solution consisting of TBA 75% and ethanol 25% and then transferred to absolute TBA and left for an hour in each of two changes of absolute TBA. The segments were kept in the final change overnight and then transferred to a mixture of 50% TBA and 50% liquid paraffin and kept in it until they sank to the bottom of the tube. The segments were then transferred to a tube containing a layer of partly melted paraffin wax on top of which there was a layer of the 1:1 TBA liquid paraffin solution. The tube was then placed in an oven at 58°C and the segments allowed to sink into the melted paraffin wax layer and the upper layer of TBA liquid paraffin solution decanted off. The

segments were then transferred through two changes of melted paraffin wax spending 1 hour in each change and finally embedded in fresh paraffin wax.

Sections were cut from the embedded segments using a Cambridge rocking microtome. The TS (were 24 µ thick and) were cut from the middle of the segments the LS (were 12 µ thick and) were cut in such a way as to include the stele in order to get theradial LS. The sections were cut at these thicknesses because the nuclei of the root cortex were orientated with their long axis pointing along the root axis. The nuclei were elipsoids with the major axis three times the length of the minor axis. After the sections had been cut they were transferred to slides which had been specially coated (subbed) in the manner described in the method for the preparation of slides for autoradiography. When the sections had been transferred to the slides they were dewaxed and stained with basic fuchsin using the Feulgen technique as described by Darlington and La-Cour (1960). The reagents used for this reaction were made up as follows:-

1) The reduced basic fuchsin solution

One gm of basic fuchsin was dissolved in a 500 ml conical flask by pouring over it 200 ml of boiling water. The solution was then allowed to cool to 50°C and was then filtered and 30 ml of 1N HCl added to the filtrate. To this acid solution 3.0 g of sodium meta bisulphite were added and the solution allowed to bleach for 24 hours in an air tight bottle in the dark. The solution was then decolourised using 0.5 g of decolourising charcoal which was added to the vigorously shaken solution. After one minute the solution was quickly filtered and stored in a stoppered bottle at 4°C in the dark.

2) Sulphur dioxide water

This was prepared by adding 5 ml of a 10% solution of sodium metabisulphite and 5 ml of 1N HCl to 90 ml of distilled water.

This solution was made up when required since it deteriorated on standing.

The sections on the slides were dewaxed by rinsing for 5 minutes in two batches of xylene and placed for 10 minutes in a 50/50 mixture of xylene and absolute alcohol. After this the slides with the sections on them were taken down through the following ethanol series to distilled water spending 10 minutes in each stage. Absolute ethanol 100%, 95%, 80%, 70%, 50%, 30%, distilled water. The sections were then rinsed in distilled water and hydrolysed in 1N HCl at 60°C for 10 minutes. After this they were rinsed to remove any acid and to stop the hydrolysis. The sections were then stained for 1 hour in the reduced basic fuchsin solution. After this staining, the sections were washed for 10 minutes in 2 lots of sulphur dioxide water to remove any unreduced stain and then in distilled water to remove all trace of the sulphur dioxide. The sections were then dehydrated by passing the slides through the series of alcohol concentrations as previously mentioned. After this the slides were permanently mounted ready for the microdensitometry.

The measurement of the density of the Feulgen staining in the nuclei of the sections was carried out using an integratingmicrodensitometer (Barr and Stroud model GN2). The average density of the Feulgen staining per nucleus was found by determining the densities of 100 nuclei from TS's of each age of root segment in each of the areas of the root under investigation. The average value obtained from these readings was qualitatively the same as the value of DNA content per segment found from the chemical estimation. No attempt was made to relate the two types of determination quantitatively because of the difficulties of technique involved. These investigations were carried out on TS's only since the nuclei on the LS's were not so suitable for this type of investigation by reason of their shape. Because time on the densitometer was limited it was not possible to increase the number of nuclei measured beyond about

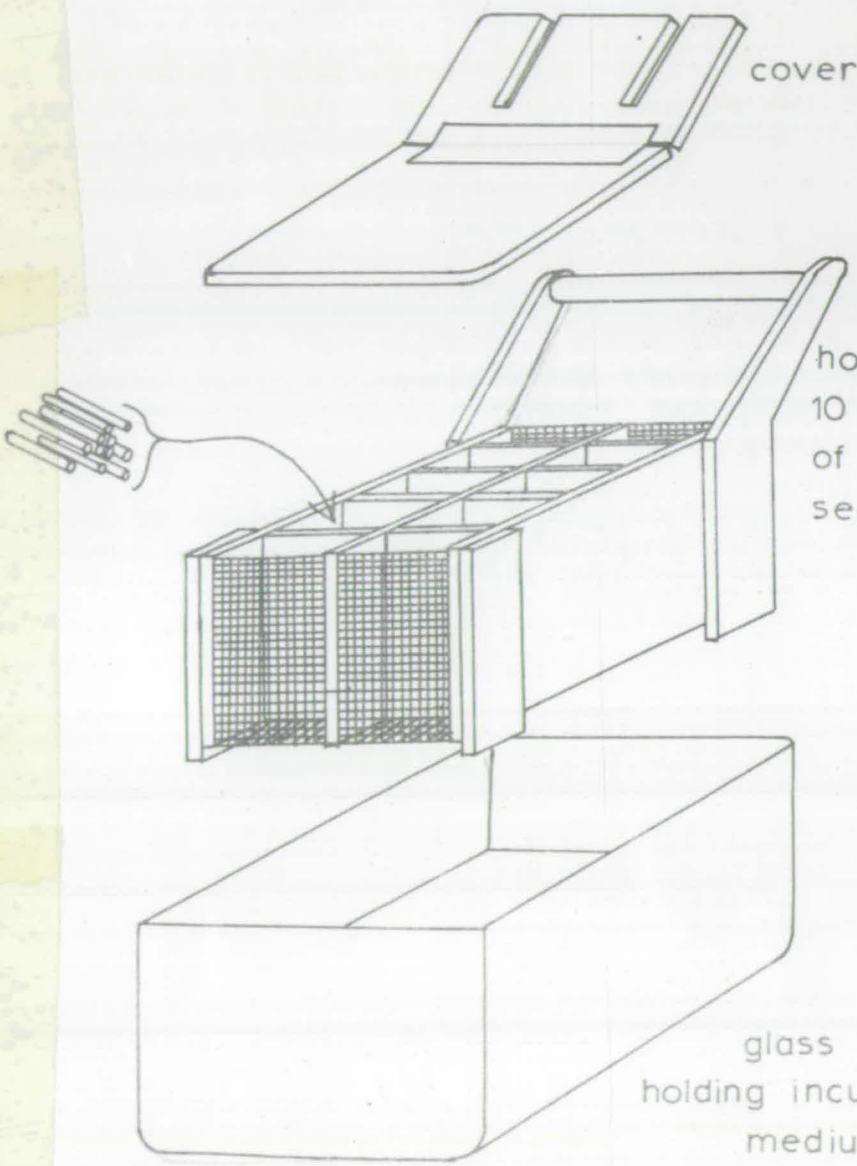
400 for each age of segment. This was considered sufficient to determine the general trend of rise and fall in the DNA content especially since the counts of Feulgen staining nuclei and the results of the phosphatase histochemistry confirm the changes indicated by the Feulgen densitometry.

As well as the microdensitometric investigations of DNA content per nucleus carried out as described above the number of Feulgen staining nuclei in various regions of the cortex was taken. This was done by counting the number of nuclei in the appropriate regions for at least 10 serial TS's of 3 different sections from each age group the average number of nuclei per TS for each region of the root was then calculated from these figures.

c) Method of labelling the nuclei of the basal segment with tritiated thymidine and the autoradiographic technique used.

The object of this type of experiment was to confirm the results of the Feulgen microdensitometry which indicated the position and time of DNA synthesis in the basal segment of the root. The method of labelling the roots was as described below. The root segments of ages between 3 and 30 days were harvested in the normal manner which gave 10 batches of root segments separated by successive intervals of three days. After harvest they were placed in the special holder (Diagram 1) and the holder placed in a dish of incubation medium which consisted of the normal culture medium containing 1.5 μc of tritiated thymidine per 50 ml of medium. The pH of this incubation medium was adjusted to 6.4 to minimise the shock of transferring the root segments to the incubation medium. Before use the whole apparatus and all the incubation media and chase media were sterilised by autoclaving or swabbing with ethanol. Aseptic conditions were maintained during the harvest of the segments and their transfer to the

Apparatus for incubation of basal root segments



Dia 1

incubation dish and the subsequent incubation and chase. The roots were labelled for 6 hours at a temperature of 25°C and this was followed by a chase in an identical medium which contained an equivalent amount of cold thymidine. This lasted for 6 hours and was carried out under sterile conditions. The segments were then fixed for 12 hours and sectioned as was described in the previous section.

All slides used in the autoradiographic and the Feulgen densitometric techniques were subbed by the method recommended by Kodak as being ideally suited to the preparation of glass surfaces to receive a coat of photographic emulsion. The technique was one originally used by Wall (1929). The slides are cleaned by soaking in a solution of:-

Potassium dichromate	100 g
Sulphuric acid (conc)	100 ml
Water to make	1000 ml

When the slides are clean enough to be perfectly wetted by tap water they are well washed in distilled water and then dipped bodily into the following solution which was maintained at 25°C

Gelatin	5.0 g
Chrome alum	0.5 g
Water to make	1000 ml

Without further treatment the slides were placed in a rack to drain and dry. Care was taken to ensure that the slides were not touched with the bare hand or dried in a dusty place. This treatment ensures that the sections and the photographic emulsion stick well to the glass and that the emulsion spreads evenly on the surface of the slide. When the sections have been cut, mounted on slides and stained in the manner previously described and have reached the point in this procedure at which the sections were washed in distilled water after Feulgen staining the slides to be prepared for autoradipgraphy were selected and treated in

the following manner.

This method was derived from the improved method of Kopriwa and Lebond (1962). Certain alterations were made to allow for the use of a different emulsion (Ilford K2) and plant instead of animal tissue. The final method developed and described below employed as many of the points of technique known to reduce the background of the emulsion without destroying the basic simplicity of the technique. The whole dipping and drying of the slides was carried out in a darkroom using a Wratten series 1 (red) filter. In the darkroom a flat bottomed 3" x 1¼" specimen tube was supported in a water bath maintained at a temperature of 55°C. Shreds of the Ilford K2 emulsion were added slowly to this tube and allowed to melt. This was continued until the tube was about 1/3rd full of the melted emulsion when an equal quantity of distilled water was added and the mixture gently stirred to mix the diluted emulsion to an even consistency. The stirring was done with a very clean glass rod. The slides which had been kept in distilled water since the completion of the Feulgen staining, were taken one at a time, drained by touching the bottom edge of the slide on a piece of clean blotting paper and placed in the diluted emulsion. The slides were allowed to remain in the diluted emulsion for 30 seconds and were then removed and placed upright in a rack with their bottom edges in contact with a piece of clean blotting paper and the excess emulsion allowed to drain off. After the slides had drained for 10 minutes they were placed in a current of clean dry air for ½ hour to dry. They were then placed in racks in light tight aluminium boxes (50 slides to a box) together with a paper sack containing 5 g of silica gel to dry the air in the box when it is sealed for exposure. The boxes were then sealed with black adhesive tape and stored in a refrigerator at 4°C for an exposure time of 14 days. After this the boxes were returned to the dark room and the slides were developed in the boxes,

which were water tight, using Kodak D19 developer with a development time of 6 minutes at 21°C. After fixing and washing the, the slides were dehydrated and permanent mounts made of the autoradiographs.

Both LS's and TS's were prepared and the average number of labelled nuclei on each type of section in each age group was found by taking the number of nuclei per section.for 10 successive serial sections for several roots in each age group and averaging the results to arrive at an average figure for the group.

There were several points of interest arising from this technique which require further explanation:-

Scrupulous cleanliness must be observed at all times since if the slides were dirty there was poor adhesion between the slides and the emulsion and the emulsion coat was not uniform.

The consistency of the emulsion must be such that an even layer over the slide is obtained by dipping the slide in the emulsion. By altering the dilution of the emulsion it was possible to regulate the thickness of the film to some extent. The 50:50 emulsion water mixture was found to give the best results for this system. In practice it was found that this emulsion dilution gave an even thickness of emulsion over the whole slide except for a ridge of emulsion at the bottom end of the slide. This was impossible to avoid but could be kept to a reasonably small area of the slide with a good fluid emulsion and careful drainage of the slide after dipping.

Slow even drying of the emulsion was very important in reducing the level of background on the slides. Fast drying causes stresses in in the emulsion which lead to the production of silver grains on development of the slides. Contamination of the slides with chemicals also leads to an increase of the background of the autoradiographs.

The use of the Feulgen staining method to show the positions

of the nuclei introduces a loss of the ~~labelled thymidine~~ tritium from the sections during the hydrolysis. This loss of radioactive label has been shown by Lang and Maurer (1965) to amount to 18% using the type of Feulgen staining technique to which the sections were subjected. This loss was considered to be justifiable in view of the superiority of the Feulgen staining technique over the alternative methods available. The loss of label was proportional to the length of the hydrolysis so the loss would have no effect on the relative numbers of thymidine labelled nuclei observed. The main effect of this hydrolytic loss of label would be to reduce the degree of labelling per nucleus in all ages by 18%. This would not affect the conclusions drawn from the experimental results since only the relative number of nuclei per section and the positions in the root where the DNA synthesis occurred were discussed and neither of these would be affected in their essentials by a loss of radioactivity of this type.

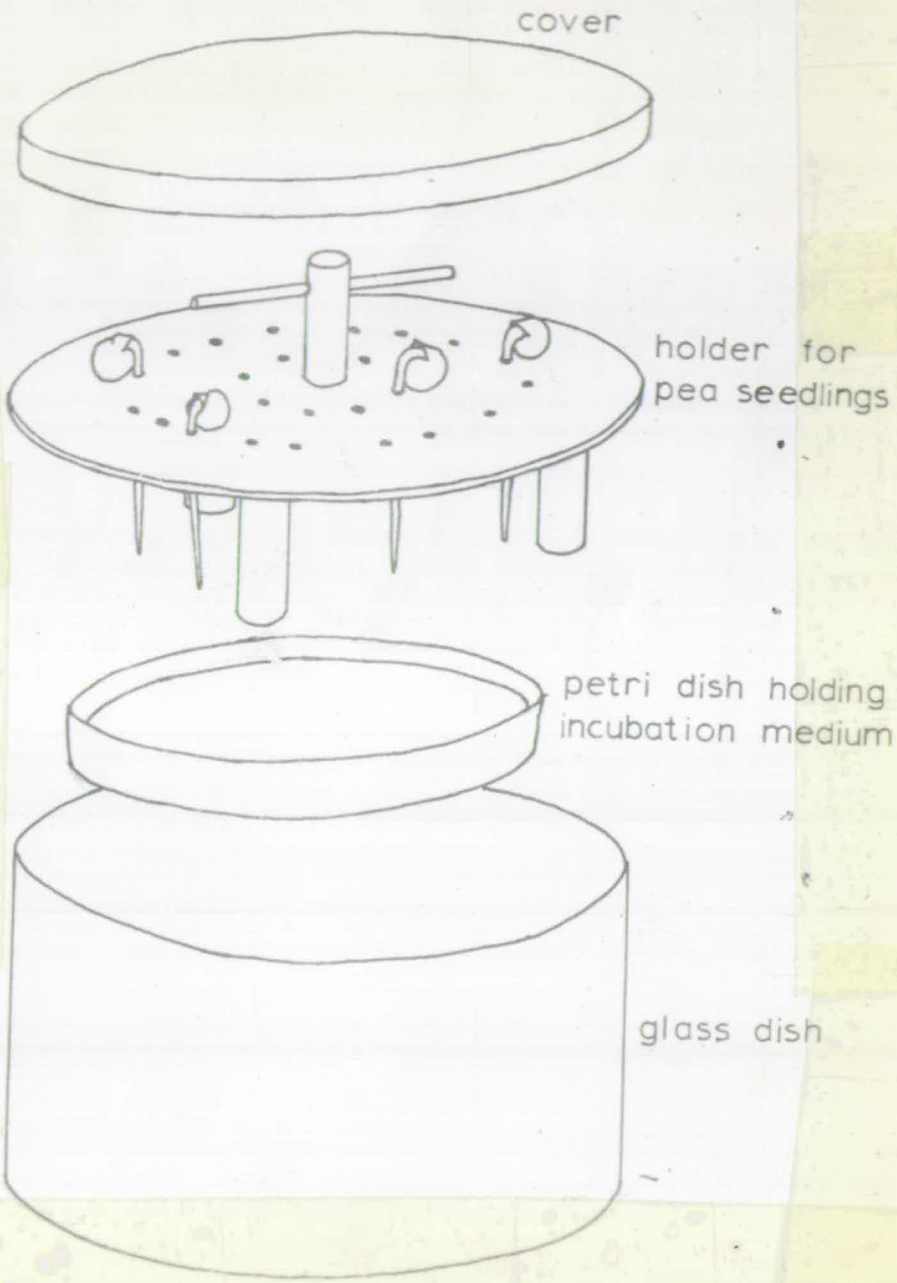
4) Methods involving labelling the basal root segment with C^{14} leucine.

a) Labelling the root segment prior to culture and the method of analysing the movement of radioactivity out of this labelled region during growth of the root.

The apparatus shown in Dia. 2 allows the apical 1 cm. portion of a sterile 3 day old seedling to dip into a solution (20 ml) containing inorganic salts (Bonner and Addicott medium minus sucrose) and 0.05 µc per ml of C^{14} leucine. The roots were left to incorporate C^{14} leucine from the medium for 3 hours and then the apical 1 cm. tips were removed and transferred to petri dishes for culture in the normal manner. No chase with non-radioactive leucine was given and an analysis of the segments after labelling showed that 80% of the radioactivity incorporated

Dia 2

Apparatus for C14 leucine incubation of pea seedling root tips



was not extractable with hot 70% ethanol. After this labelling prior to culture the roots were allowed to grow in the normal manner which they did in spite of the increased amount of handling during the setting up of the experiment. The rate of growth of the labelled roots was the same as that of a batch of normal roots grown as a control.

To analyse the spread of the radioactivity incorporated in the basal region to other parts of the root and medium during culture single roots were taken at various time intervals from the start of culture and cut up into successive 1 cm segments. These segments were dried onto 1 cm filter paper discs in an oven at 60°C and when completely dry were transferred to 7 ml of scintillation counting fluid in a glass counting vial together with the disc of filter paper. The samples were then counted for 100 minutes in a Packard scintillation counter. This gave the radioactivity along the root in counts per minute per cm. A considerable amount of radioactivity was associated with the root cap cells but since the root cap was often sloughed off into the medium or left as a collar round the growing root the activity due to this source was ignored in presenting the results. The loss of activity to the medium was followed by transferring 10 µl of the medium to a 1 cm filter paper disc after filtration to remove cell debris and counting this in a scintillation counter after drying in an oven at 60°C. The activity of the medium was expressed as cpm per root per ml of medium.

The whole experiment was set up under aseptic conditions. The apparatus and incubation medium were sterilised by autoclaving or swabbing with ethanol and every attempt was made to ensure the sterility of the cultures derived from the labelled root tips. With the apparatus shown in the Diagram up to thirty roots could be labelled in one experiment.

b) Method of investiating the uptake of C14 leucine by basal 1 cm segments during ageing.

The root segments were harvested and prepared for incubation in the way described for the labelling of the basal segments with tritiated thymidine. Ten basal segments of each age were used and the apparatus used for the labelling is shown in Diagram 1. The incubation medium consisted of the normal Bonner and Addicott medium adjusted to a pH of 6.4 and containing 0.1 μc of C14 leucine per ml. Strict asepsis was observed during the whole incubation procedure and all apparatus and media used were sterilised beforehand. The incubation in the C14 leucine medium lasted 2 hours and the root segments were then transferred to another medium containing cold leucine for 30 minutes for chasing. When this was completed the tissue was fractionated using the fractionation procedure 1 outlined previously.

After fractionation 50 μl aliquots of the buffer insoluble residue suspension, the buffer soluble protein solution and the ethanol soluble fraction were transferred to filter paper discs which were then dried and washed with cold TCA to coagulate the protein in the pores of the filter paper. The filter paper discs were then washed with cold TCA, hot TCA, 35% ethanol, ethanol/ether, and dry ether to remove any leucine not incorporated in protein. The activity in the washed discs was measured in a Beckman low background counter and the results expressed as counts per minute incorporated per segment per 2 hours.

5) Measurement of oxygen uptake

This was carried out using ten segments of each root age using the Warburg manometric technique as described in Umbreit, Burriss and Stauffer (1959).

6) Methods of determining enzyme activities in the basal segment

a) Polyphenol oxidase

The method used was a spectrophotometric estimation based on the oxidation of adrenalin tartrate to adenochrome by

this enzyme in the presence of hydrogen peroxide. The method is a modification of one used for the estimation of adrenaline oxidase in blood plasma developed by Jasinski and Wkodzimiertz (1961). An anzyme extract was prepared using the scheme given in fractionation procedure 2. This extraction procedure was a modification of that used by Dawson (1949). The reagents used in the estimation of the enzyme activity were:-

The enzyme extract which was a protein solution in 0.01 M phosphate buffer pH 6.8.

The adrenaline tartrate solution. This consisted of 0.015 g of adrenaline tartrate dissolved in 50 ml of 0.01 M phosphate buffer pH 6.8 containing 5 ml of 1/50 N HCl. This solution deteriorates on storage but was usable for about a week if kept in the dark at 4°C.

A 6% solution of hydrogen peroxide.

The reaction was carried out in a 3 ml glass cuvette with a 1 cm light path in a Unicam SP 500 spectrophotometer the progress being followed by the increase in OD at 500 mμ. The blank and reaction cuvettes were made up as follows:-

Reaction cuvette	Control cuvette
2 ml of adrenaline solution	2 ml of adrenaline solution
1 drop of 6% peroxide	1 drop of 6% peroxide

Both cuvettes were now stirred and the OD determined. This value should be zero or only a little removed from it. To the tubes was then added:-

0.2 ml of enzyme extract	0.2 ml of distilled water

Both cuvettes were then stirred and measurements of the OD difference at 500 mμ were taken every 1 minute for 10 minutes. The gradient of the OD rise over this period was taken as a measure of the activity of the enzyme. The activity was expressed in terms of rate of increase in OD at 500 mμ in the initial 10 minutes of reaction per segment, and is referred to in the

results section as arbitrary units.

Table 1 gives the results of experiments carried out to determine the time course of the reaction and to see if the activity of the enzyme was proportional to the quantity of root material used for the preparation. The results in this table show that the time course of the reaction is linear for the first 15 minutes and the enzymic activity is proportional to the quantity of root tissue used.

b) Alanylglycine dipeptidase

This method was a modification of the method described by Jensen (1962) first used by Linderstrom (1932). The method was scaled up somewhat to suit the tissue and apparatus available. In this method 50 µl of 30% glycerol 70% 0.15 M phosphate buffer pH 7.4 were placed in a ¼" x 3" test tube and 20-25 mg of basal root segment tissue was added. In this type of experiment all the basal root segments of a given age were pooled and cut up into 4 equal pieces. Four of these pieces were selected at random and used for the enzyme estimation. After the tissue was added to the glycerol buffer solution the tubes were well stirred to disperse the tissue in the buffer and were left for 1 hour at room temperature. Then 50 µl of substrate solution consisting of 0.2 M alanylglycine adjusted to pH 7.4 with NaOH was added and the tube well shaken and stoppered with a rubber bung. The tubes were then kept at 25°C for two hours. After incubation the tubes were unstoppered and the reaction stopped by adding 20 µl of 0.05 N HCl in 95% ethanol. After this 500 µl of acetone containing 2 mg per ml of napthyl red was added and the solution shaken and titrated to the orange red end point with 0.05 N HCl in 95% ethanol. The end point colour was the colour of the acetone napthyl red solution. The controls consisted of tubes in which the reaction was stopped by the addition of the 20 µl ethanol HCl solution at the beginning of the incubation period. A

Table 1 Polyphenol oxidase time course and proportionality

10 root tips		20 root tips		30 root tips	
Time in mins.	OD at 500 mμ	Time in mins.	OD at 500 mμ	Time in mins.	OD at 500 mμ
1	-	1	0.001	1	-
2	-	2	0.015	2	0.017
3	-	3	0.032	3	0.055
4	-	4	0.050	4	0.100
5	0.014	5	0.070	5	0.150
6	0.030	6	0.089	6	0.213
7	0.032	7	0.111	7	0.259
8	0.041	8	0.136	8	0.299
9	0.049	9	0.174	9	0.358
10	0.079	10	0.207	10	0.425
11	0.088	11	0.229	11	0.450
12	0.099	12	0.241	12	0.503
13	0.103	13	0.260	13	0.545
14	0.109	14	0.300	14	0.582
15	0.153	15	0.388	15	0.630
Gradient		Gradient		Gradient	
0.011		0.024		0.050	

control was provided for each of the ages of root segment tissue examined. The control titre was subtracted from the experimental titre and the quantity of substrate hydrolysed by the enzyme was calculated. This quantity was used as a measure of the enzyme activity.

The experiments reported below were carried out to test the time course of the reaction and to determine whether the enzyme activity was proportional to the quantity of root tissue used in the experiment. For the proportionality experiment the quantities of the solution used were scaled up to allow for the much larger quantities of tissue used. In both experiments the tissue used consisted of 2.5 mm segments obtained by dividing the basal 1 cm segment of a 3 day cultured root into 4 parts. Five of these segments were used for the time course of the experiment and 5, 10 and 15 for the proportionality experiment. The results of these experiments are given in Table 2. The activity in this table is given in the form of the increase in the titre over the control in units of 0.02 ml after 2 hours incubation. In the results section the activities are represented by the quantities of substrate hydrolysed per segment per 2 hours.

The variation in the results of the time course experiment was large probably because of the fact that this was a micromethod which used only a very small quantity of root material for each determination which increases the probability that the tissue selected for each determination was not typical of the segments in each age batch. Since the enzyme methods were only required as indicators of change in the basal segment this method was used as described since it was thought on the basis of the above results to be accurate enough for this purpose where only large changes in enzyme activity would be considered as significant.

c) Phosphatase.

This method was based on the Allen (1940) amidol

Table 2 Dipeptidase activity time course and proportionality

Time in mins.	Titre Difference	Number of root segments	Titre Difference
30	1.809	5	2.780
60	1.287		
90	2.677	10	6.301
120	2.905		
150	2.914		
180	4.430	15	8.076

colourimetric method and was precisely the same as that used by Vaughan (1965) in this department. The assay was carried out at a pH of 6.8 since the pea root tissue enzymes were known to have a high activity at this pH. No attempt was made to study the acid and alkaline phosphatases separately since the object of this assay was to obtain a general idea of the overall activity of these enzymes in the segment rather than a detailed picture of the relative activities of the types of enzyme present.

The reagents used in the assay technique were:-

1) Calibration stock solution consisting of 4.3 g of KH_2PO_4 in 100 ml of distilled water.
2) An 8% solution of ammonium molybdate in distilled water.
3) A 60% solution of perchloric acid in water.
4) Amidol solution: (2,4 dinitrophenol hydrochloride) consisting of 0.1 g of amidol added to 2.0 g of sodium metabisulphite in 8 mls of distilled water and the whole made up to 10 mls with distilled water after filtration.
5) Incubation buffer which was 0.2 M Tris buffer at pH 6.2 containing 0.5 M sodium glycerophosphate as a substrate.

The tissue for assay was frozen overnight and then slowly thawed and homogenised in 2 ml of buffer substrate solution. Four root segments were used and substrate lacking and enzyme lacking controls were run in parallel with the experimental tubes. The tubes were then incubated at 25°C for one hour. After this time the reaction was stopped by the addition of 0.8 ml of 60% perchloric acid. The tubes were then centrifuged to remove the cell debris and the supernatant decanted off and diluted to

10 mls with distilled water. To this solution was added 0.4 ml of the molybdate solution and 0.8 ml of the amidol solution. The tubes were then shaken and left for $\frac{1}{2}$ hour at room temperature. After this time the OD at 725 mμ was taken using a Unicam SP 500 spectrophotometer with the substrate only control as the blank.

As in the case of the previous enzyme methods the time course of the assay and the proportionality of the extraction of activity were tested by experiment. The results of this are given in Table 3.

Ten 1 cm basal segments cut from three day old cultured roots were used for each time estimation in the time course experiment and the time of incubation for the proportionality experiment was one hour. The units in which the enzyme activity was expressed here and in the results section were μg phosphate released per segment per hour. The conversion of the spectrophotometer readings to phosphate concentrations was effected by reference to a graph prepared from a series of dilutions of the stock phosphate solution run in parallel with the experimental determinations.

d) Invertase

This method was based on the ability of reducing sugars to reduce ferric ion to ferrous ion. The quantity of ferric ion reacting in this way was found by a back titration technique involving the liberation of iodine by the ferric ion from potassium iodine and its titration with sodium thiosulphate. The method used to follow the hydrolysis of the sucrose in the culture medium was very similar to the one used for the enzyme determination. The reagents used in the assay procedure are listed below:-

1) Substrate buffer solution consisting of 0.1 M phosphate buffer at pH 6 containing 2% sucrose.

2) Potassium ferricyanide solution 0.005 M $K_3Fe(CN)_6$ in 0.1 M sodium carbonate.

3) Potassium iodide solution 2.5% and zinc sulphate

Table 3 Phosphatase activity time course and proportionality

Time in mins.	µg phosphate released	Number of root segments	µg of phosphate released
20	2.7	1	1
40	5.1	2	3.5
80	9.6	3	4.5
360	14.0	4	6.0

solution 5%. These solutions were made up separately and mixed just before use.

4) A 15% acetic acid solution

5) A 0.005 M solution of sodium thiosulphate solution.

6) A 5% solution of starch for use as an indicator.

The controls in this experiment consisted of tubes in which the enzyme had been rendered inactive by heating in a boiling water bath for half an hour. Two to four root segments were used depending on the amount of tissue available. This tissue was homogenised in the buffer substrate (1 ml of buffer solution for every segment used). The control tubes were then boiled and the remainder incubated in a water bath at 25°C for one hour. After this time 10 mls of ferricyanide solution was added to each tube and the tubes then heated in a boiling water bath for 15 minutes after being thoroughly shaken. After heating 5 ml of the potassium iodide zinc sulphate solution was added and the tubes were again shaken and allowed to cool to room temperature. Then 1 ml of the acetic acid solution was added and the contents of the tubes well mixed by shaking them. The contents of the tubes were then titrated against the thiosulphate solution using the starch solution as indicator. This method of using the production of reducing sugar as an index of invertase activity was the same as the method used by Brown (1952) for the study of the enzyme in cultured roots and the method of estimating the reducing sugar concentration was developed by Hagedorn and Jensen (1923).

The method used for the estimation of the reducing sugar present in the medium after various periods of culture is presented here since it was basically the same as the method for the enzyme estimation given above differing only in that more concentrated solutions were used. The method was a repeat of the Hagedorn reducing sugar estimation as outlined in the method for invertase determination using 1 ml portions of the medium from

the culture dishes and solutions of ferricyanide and thiosulphate 100 times as concentrated as those used in the invertase estimation. The medium of ten dishes of the same time in culture was pooled and made up with distilled water to 200 ml and the 1 ml aliquots for reducing sugar estimation were taken from this. The results were expressed as grammes of sucrose remaining unhydrolysed per 100 ml of culture medium.

Proportionality and time course experiments were run for this enzyme estimation as for all the other enzyme assay techniques. The results of these experiments are given in Table 4.

The enzyme activities in the results section were expressed in µM sucrose hydrolysed per segment per hour.

e) Succinic dehydrogenase

This method was based on the ability of the succinic acid dehydrogenase to reduce triphenyltetrazolium chloride to the coloured tetrazonium salt. The method was a modification of one originally developed for plant tissue by Kun and Abood (1949). The general background information about the application of this method to plant tissues in general was provided by the work of Sitiro (1962). Two types of control were required for this method of estimating the succinic acid dehydrogenase activity, these were:-

1) A substrate lacking blank consisting of homogenised root tissue and the tetrazolium salt. This was necessary since the tissue has the ability to reduce the tetrazolium salt in the absence of the substrate (sodium succinate). This ability was especially marked in the meristematic regions and the younger tissue of the root.

2) A tissue absorption blank which consisted of the homogenised tissue and a known quantity of the tetrazolium salt reduced in situ by the addition of a few crystals of sodium dithionite

Table 4 Invertase activity time course and proportionality

Time in mins	µM sucrose hydrolysed per 10 segments	Number of roots	µM sucrose hydrolysed per hour
20	15.5	1	3.2
40	30.0	2	5.5
60	38.0	3	12.5
		4	17.0

(sodium hydrosulphite). This blank allowed the efficiency with which the reduced salt was extracted from the tissues by acetone preparatory to the measurement of the OD of the colour produced in a spectrophotometer. After trials with this type of control it was found that the efficiency of extraction did not vary much with the ages of the tissue used and so this control was omitted from the final experimental procedure.

In its final form the enzyme assay was carried out as described below, the reagents used were:-

1) A 0.1% solution of triphenyltetrazolium chloride
2) A 0.1 M phosphate buffer solution pH 7.4
3) a 0.2 M solution of sodium succinate.
4) Dry acetone.

Each age of tissue was represented by an experimental tube and a substrate lacking control tube. Three segments were used for both of these. The basal root segments were homogenised in 0.5 ml of buffer solution and transferred to a 10 ml conical centrifuge tube. To these tubes 0.3 ml of the substrate solution was added to each of the experimental tubes and an equal quantity of buffer was added to the control tubes. Then 0.6 ml of the tetrazolium salt solution was added to all the tubes. All the tubes were then incubated for 30 minutes at 25°C. After incubation, 2.5 ml of acetone were added to each tube. All the tubes were then stoppered and shaken for 5 minutes on an automatic shaker. The tubes were then centrifuged to remove all the debris and the supernatant water acetone solution removed and its absorption at 420 mμ measured in a 3 cm light path microcell in a Unicam SP 500 spectrophotometer. The blank against which the experimental tubes were measured was the appropriate substrate lacking control tube. The quantity of tetrazolium salt reduced was determined by

Table 5 Succinic dehydrogenase, time course and proportionality

Time in mins.	µg TPC reduced per segment	Number of root tips	µg TPC reduced per half hour
20	10.1	1	8.0
40	21.1	2	22.0
60	28.0	3	26.0
		4	42.0

reference to a graph of the OD produced by the reduction of known quantities of tetrazolium salt with crystals of sodium dithionite. This calibration graph was prepared in parallel with the experiment. Proportionality and time course experiments were carried out using this enzyme determination and the results of this are given in Table 5. The tissue used for these experiments consisted of 1 cm root tips cut from three day old pea seedlings. The time course experiment was set up using 2 of these root tips and the incubation time for the proportionality experiment was 30 minutes.

The units used in the experimental results section for succinic dehydrogenase activity were µg of tetrazolium salt reduced per segment per hour.

f) Protease

This method was one derived from the casein digestion method for the estimation of trypsin activity used by Laskowski (1955). This in turn was based on a method originally used by Kunitz (1947). The reagents used in this assay were:-

1) Phosphate buffer 0.2 M pH 7.4

2) A 1% solution of casein made up in the buffer solution. If the casein solution has a high UV absorption left in the supernatant after precipitation with TCA then it may be necessary to dialyse the casein solution before use. For most of the estimations the casein used was pure enough to make this precaution unnecessary.

3) A 10% solution of TCA.

The method of estimation was carried out as follows. Six root segments of each root age were taken and divided into two portions. Three roots were used to set up the experimental assay tube and three were used for the control tube thus there was one control tube for each age of tissue. In each case the three segments were homogenised in 1 ml of the

buffer solution and to this homogenate was added an equal quantity of the casein buffer solution. The solution was then placed in a 10 ml conical centrifuge tube and incubated in a water bath at 25°C for an hour. The reaction in the control tubes was stopped before incubation by the addition of an equal quantity of 10% TCA solution. Apart from this the control tubes were treated in exactly the same way as the experimental tubes. After the incubation period was complete the reaction in the experimental tubes was stopped by the addition of an equal quantity of 10% TCA. The control and experimental tubes were then left to stand for half an hour to let the precipitate coagulate and then centrifuged. The supernatant from each tube was then removed and the OD difference between the experimental tube and the control tube at 280 mμ was determined using a Unicam SP 500 spectrophotometer. This difference in the OD at 280 mμ was taken as a measure of the enzyme activity of the tissue. The activity was thus expressed in units of OD at 280 mμ per segment per hour.

As in the case of the other enzyme techniques used the time course and the proportionality of the enzyme extraction were determined. The methods used in these experiments and the results from them are given below. In the time course experiment 5,1 cm root tips were used in the experiment and the control for each time examined. The incubation time in the proportionality experiment was half an hour apart from these changes the methods used were those described above. The results of the two experiments are given in Table 6.

As well as these experiments other experiments were run to establish the distribution of the protease enzyme between the soluble and insoluble protein fractions. The results of these showed that 1.5 to 3.5 % of the total activity of the mature root tissue is present in the debris fraction after the extraction of the soluble protein with buffer. These quantities are small enough to be accounted for by contamination

Table 6 Protease, time course and proportionality

Time in mins.	OD difference at 280 mμ per segment	Number of segments	OD difference at 280 mμ per half hour
30	0.024	5	0.029
60	0.065	10	0.340
90	0.095	15	0.605
120	0.139		

of the debris by the soluble protein of the supernatant during the separation of the two fractions after the homogenisation. This showed that the protease activity of root tissue was present in the buffer soluble protein fraction.

9) Methods used in the chromatography of the ethanol soluble fraction.

The main components of the ethanol soluble extract were amino acids and the chromatography of the extract was carried out to identify the components of the amino acid pool and their relative abundances at different times of culture. In the method used 20 µl of the ethanol extract was applied to a 14" square piece of Whatman No. 4 filter paper and chromatogramed in two dimensions using the following solvent systems. First direction phenol (60 g) water (20 g), second direction either, N-butanol (9 vols), acetic acid (1 vol), and water (2.5 vols) or, tertiary butanol (120 vols), methyl ethyl ketone (50 vols), water (40 vols), and 98% formic acid (14 vols). The chromatographic "maps" produced by these two solvent systems were very similar but the tertiary butanol has the advantage of being more stable than the other second solvent and can be stored more easily. Both of the second solvent systems were used during the period of experimentation and produced the same results. The chromatograms were developed by dipping the dried chromatogram in an 0.1% solution of ninhydrin in acetone and keeping the dipped chromatogram in an oven at 80°C in the dark for 25 minutes. After this time the chromatograms were removed from the oven and kept for a further 30 minutes in the dark at room temperature to allow the full ninhydrin colour to develop. The spots corresponding to the various amino acids were then identified by their colour and reference to a standard chromatogram of known amino acids run at the same time. After the amino acids present had been identified and marked the spots on the chromatogram were fixed by dipping the chromatogram in a solution of the

following composition; saturated copper nitrate 10 mls, 10% nitric acid 10 mls, made up to 100 ml with acetone. When these chromatograms are dried in the oven at 80°C the amino acid ninhydrin spots develop a light stable, pinkish-red colour.

For the estimation of the relative quantities of the amino acids on the chromatograms the amino acid spots were cut out from the paper and after being cut up into smaller pieces were placed in a tube and the ninhydrin copper complex extracted with 3 ml of methyl alcohol. The colour was satisfactorily extracted by the methanol if the paper was left in it in a stoppered tube for three hours. After the red colour was eluted out of the paper into the methanol the density of the red colour at 500 mμ was measured in a Unicam SP 500 spectrophotometer using as a blank a methanol extract of an equivalent area of the chromatogram which did not contain any amino acid. This OD value at 500 mμ gives a rough idea of the relative quantities of the amino acids present on the chromatogram.

Most of the amino acid in the extract was in the form of homoserine and it was not possible to get large enough quantities of the other acids on the chromatogram for analysis by this method without causing the homoserine spot to streak on the chromatogram and interfere with the other spots around it. This method was not sensitive enough to deal with the very small quantities of amino acid in the relatively faint spots produced by the amino acids other than homoserine. The three amide amino acids were pooled and the colour produced by the combination of these was determined. The homoserine and amide amino acids between them accounted for 90% of the colour on the chromatograms and most of this was due to the homoserine. This method provided a rough quantitative check on the patterns of change during ageing found for the alcohol soluble nitrogen and the alcohol soluble amide nitrogen.

8) Method used for the estimation of the calcium content of the root segments.

This method was a modified form of that described by Jensen (1962) and originally developed for plant tissues by Tyner (1948). It was a colorimetric method based on the fact that calcium will combine with chloranilic acid to produce an insoluble precipitate. The tissue to be analysed was blotted dry and placed in a test tube ($\frac{1}{4}$" x 3"). The tubes were then placed in an aluminium heating block and the tissue ashed by heating in a muffle furnace at 450°C for 5 hours. After ashing the tubes were slowly cooled and one drop of distilled water was added (0.5 ml) together with 100 µl of dilute HCl (1 part anlar HCl to 3 parts of distilled water) and the ash dissolved. The samples were then evaporated to dryness in an oven at 100°C. The resulting residue was then taken up in 50 µl of 0.1 N acetic acid and warmed gently for ten minutes without allowing the solution to boil. To the warm samples 200 µl of distilled water and 100 µl of chloranilic acid (0.1% solution) were added and the tubes allowed to stand overnight at room temperature. Following this the tubes were centrifuged to remove any precipitate and the OD of the supernatant measured at 500 mµ in a Unicam SP 500 spectrophotometer. The blank for the spectrophotometer measurement consisted of 200 µl of distilled water, 100 µl of chloranilic acid, and 50 µl of 0.1 N acetic acid. The OD difference between the blank and the sample gives the quantity of chloranilic acid used by the calcium which was converted into the calcium content of the sample by reference to a standard graph prepared using known calcium concentrations against the same blank.

9) Method used for the phosphatase histochemistry

The method used was based on a technique of Grogg and Pearse (1952) and is based on the coupling of the α-napthol released from α naphthyl phosphoric acid by the phosphatase with orthoanisidine tetrazonium

chloride to form a purple water insoluble dye which deposits at the site of the phosphatase activity in the cell. The basal root segment tissue used was frozen overnight in the deep freeze and thawed just before use. It was found that this treatment increased the phosphatase activity of the tissue and thus reduced the incubation times necessary for the development of the colour produced by the reaction. The tissue was hand sectioned in transverse section and the sections transferred directly to drops of the reaction medium on a slide. The drops of the reaction solution and the TS's were then covered with a coverslip to reduce the evaporation from the reaction drop. The colour was then allowed to develop by placing the slides in an air tight plastic box lined with damp filter paper which was kept at 25°C in an incubator. Under these conditions the diffuse activity associated with cell death was easily visible in the cells of the section in half an hour. The localised activity characteristic of living cells was slower to develop and took about an hour to develop properly. The reaction mixture was made up of two solutions which were mixed just before use. The solutions were 0.3 g of α-napthylphosphoric acid in 100 ml of 0.1 M acetate buffer pH 4.5 and 0.5 g o-anisidine tetrazonium salt also dissolved in 100 ml of acetate buffer pH 4.5. The solutions will keep for a month after mixing if they are stored at 4°C in the dark and for even longer periods if they are stored unmixed. Two types of control were carried out, a substrate lacking control and incubation in the presence of fluoride ions. In the first control there was complete inhibition of the reaction in the second there were indications of a very slight activity after prolongued incubations. In both the experimental and control sections there was a considerable quantity of brownish yellow or greenish coloured breakdown products of the orthoanisidine tetrazonium salt which stained the cell walls of the dead cells of the section. This staining of the dead cell walls was very useful since it allowed an accurate delineation of the

areas of dead tissue in the root cortex. It did however make the interpretation of the sections difficult wherever there was a tissue composed of dead and dying cells mixed up together because in this case the brown staining of the dead cells tended to obscure the purple staining from the phosphatase reaction. The only time that this was a difficulty was during the death of the piliferous layer between 6 and 12 days. Apart from this portion of the root at this time there was no possibility of confusing the brown or green staining of the cell walls by breakdown products with the purple colour produced by the phosphatase reaction.

10) Measurement of number of cells per segment.

The number of cells per segment was determined by the method of Brown and Rickless (1949), as modified by Brown and Broadbent (1950).

Five segments were left in 5 ml of a 5% solution of chromium trioxide in water for 24 hours at room temperature. The tissue was then macerated by forcing the segments in and out of a glass pippette.

The number of cells in a known volume of suspension was counted under the high power of a microscope using a Fuchs-Rosenthal bright line haemocytometer with a counting chamber of 0.2 mm depth.

The number of cells per segment was calculated from the average values of counts made on five samples of each suspension.

c) Measurement of medium changes

1) Measurement of the medium volume per dish and sucrose concentration.

Ten petri dishes from each age group were selected choosing if possible those dishes which contained the roots chosen for the analysis of their basal segments. After the removal of the roots the contents of the culture dishes were pooled in a measuring

cylinder and the average volume of medium per dish was found by dividing the resulting volume in the measuring cylinder by ten. This culture medium was then made up to 195 mls with distilled water to bring the volume up to the day zero value. One ml aliquots of this solution from each age of medium were taken and their reducing sugar content was found by using the same technique as that used in the invertase estimations but with solutions 100 times more concentrated. This gave the reducing sugar content of the medium in g per 100 ml and from this the amount of sucrose hydrolysed to provide this was calculated. This value was then subtracted from the quantity of sucrose present at the start of culture to give the quantity of sucrose remaining in g per 100 ml. The results of these determinations were checked qualitatively by a chromatographic analysis of the carbohydrates in the culture medium which was carried out in the following manner. An aliquot of 25 µl from the diluted culture medium of each age group was spotted onto an 18" x 24" sheet of Whatman No. 4 chromatography paper, the sheet was then run as a descending chromatogram using iso-propanol (160), water (40) as the solvent. The day zero medium was used as a reference since it was known to contain only sucrose and could thus be used as a standard. After running the chromatograms they were dried and cut into strips each strip corresponding to one age of culture medium. These strips were then dipped in a solution containing analine, 1%; diphenylamine, 1%; dissolved in acetone (10 vols) and 85% phosphoric acid (1 vol). This developing solution produced brown spots on the paper for the sucrose and the fructose and a blue spot for the glucose when the chromatogram was heated gently. The results from these chromatographic were in agreement with the results from the reducing sugar estimations carried out on the medium.

2) Measurement of medium pH

This was carried out on the pooled medium prior to dilution using a Vibret laboratory pH meter.

Results Section

1) Preliminary results

These preliminary results are from experiments in which an attempt was made to extrapolate the successive segment technique developed by Brown and Broadbent (1951) to roots grown for up to 30 days in culture. The results given in this section refer to roots of 7 and 27 days in culture. Later results show that the rate of increase in main axis length is not constant throughout the period of culture so segments of the same length do not represent equal periods of time. This is an inevitable consequence of extending the period of culture beyond 6 days and is most marked for the 27 day old roots. The rate of growth as expressed by the extension of the main axis of the root is discussed later in the results section but in Diagram 1 the relationship between the length and age of root tissue for the 7 and 27 day old roots is given together with an indication of the morphology of the two types of root. Diagram 1 also shows the average position and number of lateral roots in the 27 day old root. The production of lateral roots is another complication attendant on the prolonged period of culture.

For the analysis of the 7 day old root system ten roots were used their average length being 6.1 cms. The 5 cms. of the root shown by arrows in Diagram 1 were taken for analysis and analysed for total nitrogen, Labile nitrogen and Calcium content. The Fresh weight and Cell Number of all the segments were also taken. The results are presented in Table 1.

For the analysis of the 27 day old root system, ten roots were used their average length being 19.8 cms. The 14 cms. of root shown by arrows in Diagram 1 were taken for analysis after the removal of all lateral roots, and divided into 1 cm. segments. The same parameters were

Diagram 1

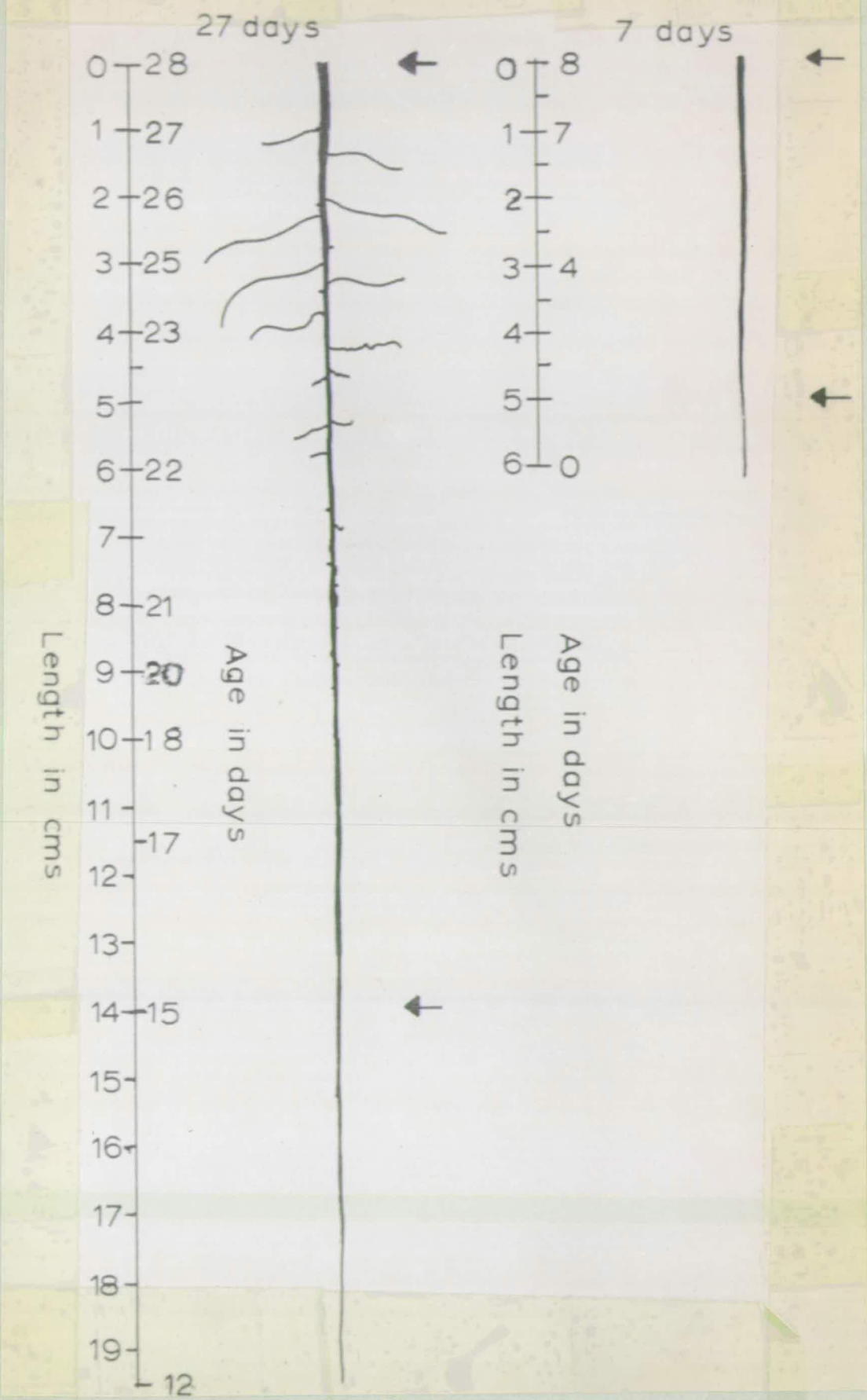

27 days
7 days
Length in cms
Age in days
Length in cms
Age in days
0 28
1 27
2 26
3 25
4 23
5
6 22
7
8 21
9 20
10 18
11
17
12
13
14 15
15
16
17
18
19
12
0 8
1 7
2
3 4
4
5
6 0

Table 1 Analysis at successive 5 mm. segments numbered from the base at 7 day old cultured roots.

Seg.	Fr. Wt/Seg. mgs.	Cell No./Seg. x10^{-3}	Total Nitrogen µg /mg Fr	Total Nitrogen µg/C x10^{-3}	Labile Nitrogen µg/mg F	Labile Nitrogen µg/C x10^{-3}	Ca content µg/mg F	Ca content µg/C x10^{-3}	Age in Days
1	16.0	3.7	2.4	10.0	0.1	0.4	0.2	0.7	8.0
2	11.1	4.4	3.2	8.1	0.1	0.3	-	-	7.6
3	9.3	3.5	-	-	0.1	0.3	0.4	1.0	7.3
4	5.0	2.1	1.9	4.5	0.2	0.5	-	-	7.0
5	2.8	1.4	3.2	6.2	0.3	0.6	0.6	1.1	5.8
6	2.1	1.3	4.6	7.4	0.6	1.0	-	-	4.6
7	1.3	0.7	5.1	9.5	0.5	0.9	1.1	2.00	4.0
8	0.8	0.7	5.4	9.2	0.9	1.0	-	-	3.3
9	0.6	1.0	14.6	8.8	1.8	1.1	-	-	2.8
10	0.6	1.3	14.7	6.8	1.3	0.8	-	-	2.5

Table 2 Analysis of successive 1 cm. segments numbered from the base at 27 day old cultured roots.

Seg.	Fr. WT./Seg. mgs.	Cell No./Seg. x 10^{-3}	Total Nitrogen µg/mgF x 10^{-3}	Total Nitrogen µg/C x 10^{-3}	Labile Nitrogen µg/mgF x 10^{-3}	Labile Nitrogen µg/C x 10^{-3}	Ca content µg/mgF x 10^{-3}	Ca content µg/C x 10^{-3}	Age in Days
1	21.9	8.7	1.5	3.8	0.2	0.5	0.01	0.05	28.0
2	9.9	6.4	1.8	2.8	0.4	0.6	0.04	0.10	26.0
3	8.0	6.3	1.8	2.3	0.7	0.9	-	-	25.0
4	6.6	3.9	3.3	5.6	0.9	1.5	0.10	0.20	23.0
5	7.9	3.1	2.8	7.1	0.4	1.0	-	-	22.5
6	9.0	3.1	2.7	7.8	0.2	0.6	0.12	0.4	22.0
7	8.0	2.4	3.6	12.0	0.9	3.0	-	-	21.5
8	6.6	3.8	5.8	10.0	1.3	2.3	-	-	21.0
9	5.5	2.4	-	-	1.3	3.0	0.42	0.01	20.0
10	3.5	1.9	7.9	14.5	1.6	3.0	0.37	0.70	18.0
11	3.5	2.7							
12	2.8	2.8							
13	3.1	2.7							
14	2.6	2.6							

measured for these segments as for the 7 day system. The four apical segments were used for Fresh weight and Cell Number determinations only. The results are presented in Table 2.

The results of Table 1 show the changes during the initial period of ageing up to 8 days after cell expansion has been completed. This initial period shows a decline in total nitrogen, labile nitrogen and calcium content per cell. This is compatible with the results obtained from similar but younger systems (Heyes personal communication) as far as the nitrogen fractions are concerned but is at variance with the data on the accumulation of calcium with age reported by Lansing (1942) ~~for Elodea~~. From these experiments it would seem that calcium accumulation plays no part in the ageing of pea root tissue. There is in fact a decline in the calcium content per cell with age.

The data obtained from the 27 day old roots show similar trends but a comparison of the data in Tables 1 and 2 show a marked discontinuity in the nitrogen values per cell between the 8th and the 18th day. The presence of lateral roots also makes all values based on cell number unrepresentative of the progress of an average cell thus the method of analysis using successive segments can only be applied to cultured root systems in which the rate of growth is constant and there is no lateral root formation. These conditions can be satisfied for cultured roots for the first week of culture but are not compatible with the longer periods of culture necessary for the full cycle of senescence and death to be completed.

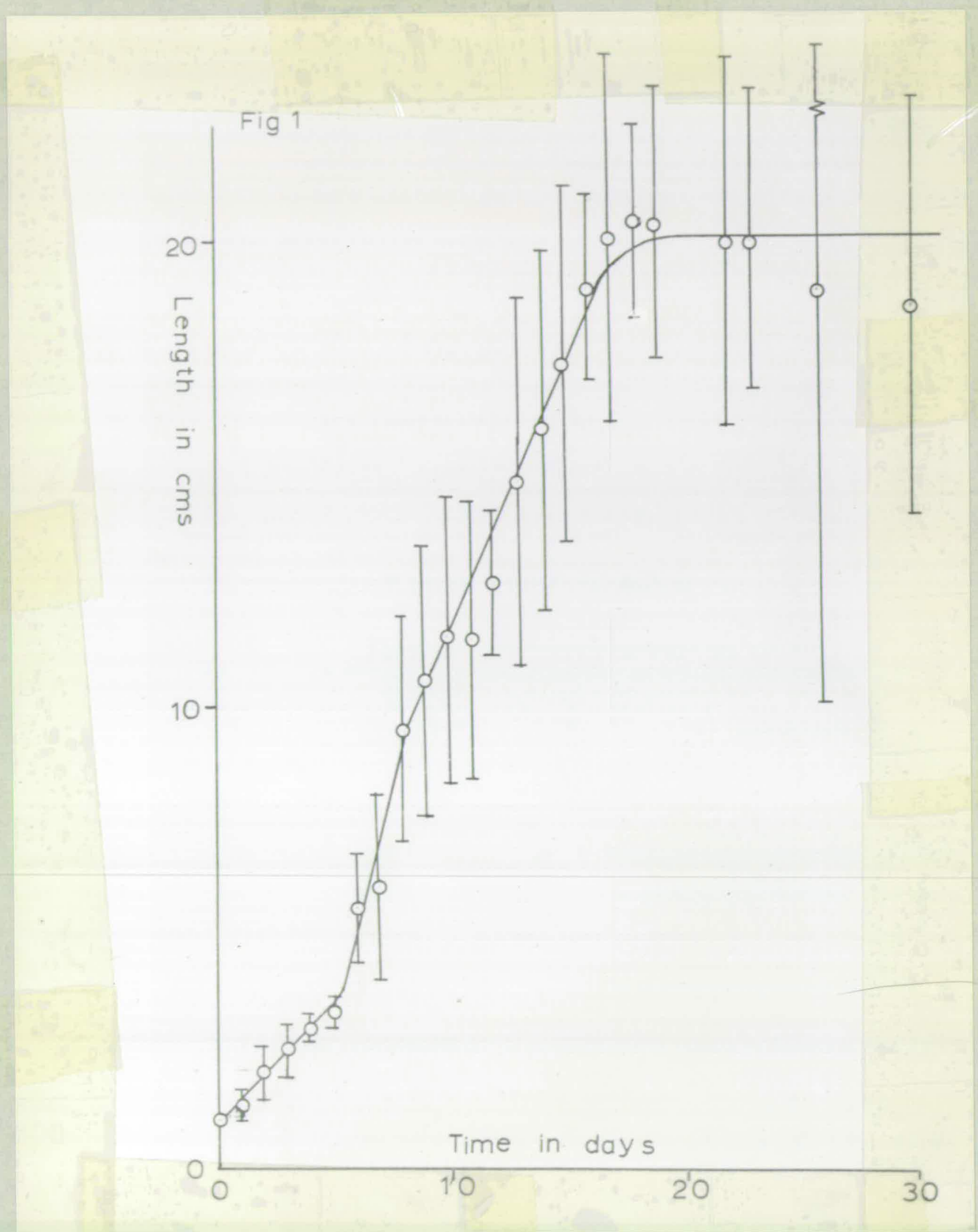
2) Growth of the root in culture

a) Increase in the length of the main axis

The results are given in Table 1 and plotted in Figure 1 and are the average values from several experiments and some 350 individual roots. The 95% confidence limit is given for each of the average results

Table 1 Growth in length of cultured 1 cm. root tips

Time of culture in days	Length of root in cms.	Time of culture days	Length of root in cms.	Time of culture days	Length of root in cms.
1	1.4 ± 0.2	11	11.4 ± 1.6	21	-
2	2.0 0.4	12	12.7 3.9	22	19.9 ± 4.0
3	2.6 0.5	13	14.8 3.9	23	19.9 ± 3.6
4	3.1 0.3	14	15.9 3.8	24	-
5	3.4 0.2	15	17.4 2.2	25	-
6	5.6 1.2	16	19.0 2.4	26	18.9 ± 8.8
7	6.1 2.1	17	20.1 2.3	27	-
8	9.5 2.4	18	20.4 3.1	28	-
9	10.5 2.8	19	20.4 3.4	29	-
10	11.4 3.0	20	-	30	18.6 ± 4.4



Increase in the length of the main axis of the root with time

in Table 1 and Figure 1.

The points arising out of these results are:-

1) The increase in length ceases at 15 to 18 days. This implies that the meristem of the main axis ceases to divide at this time.

2) The period between 1 and 18 days can be divided into two parts. The first, covering the period 0 to 5 days is characterised by a constant rate of increase of 0.5 cm. day and a low variability as measured by the 95% confidence limits. The second, between the 5th and the 18th days has a higher rate of increase in length (1.3 cm/day) and a steadily increasing variability. There is some evidence that this second period consisted of two periods of differing growth rate. A short initial period between the 5th and 8th days with a very high growth rate of 1.8 cms/day and a longer period with a rather slower growth rate of about 1 cm/day between the 8th and 18th day of culture. The high variability of the results during this period of culture means that no real significance can be attached to this although it was quite marked in the results of individual experiments. The graph in Figure 1 shows that the second period with two different growth rates but a straight line representing a uniform growth rate over this period is equally possible in view of the high variability.

3) The changes in the growth rate as measured by the increase in the length of the root were associated with the formation of lateral roots.

Later results will show that the root takes some time to acclimatise to the culture medium so initially the growth rate of the root was somewhat below the maximum possible rate. This acclimatisation lasts for 3 to 6 days. Towards the end of this period the rate of growth increases as the acclimatisation is completed. However, this faster rate

Table 2 Production of lateral roots on cultured 1 cm. root tips

Time of culture in days	% of roots with laterals	Time of culture in days	% of roots with laterals
1	0	11	62
2	0	12	89
3	0	13	100
4	0	14	100
5	0	15	100
6	6	16	100
7	15	17	100
8	18	18	100
9	39	19	100
10	55	20	100

of growth slows as lateral roots appear. The appearance of lateral roots was associated with a decrease in the growth rate of the root. The decrease in the rate of growth of the root was roughly proportional to the number of lateral roots present. Roots with a large number of laterals were consistantly shorter in length than roots of the same age with only a few laterals present. The number of laterals per root varied between 1 and 30 and this is either a cause or a consequence of the high variability shown by the results in Table 1.

Table 2 shows the percentage of roots showing lateral roots or lateral root primordia at various times in culture.

On the basis of the interpretation given, these results indicated that a decrease in growth rate could be expected between 9 and 10 days. The data of Table 1 shows such a change in rate but the higher variability reduces its significance.

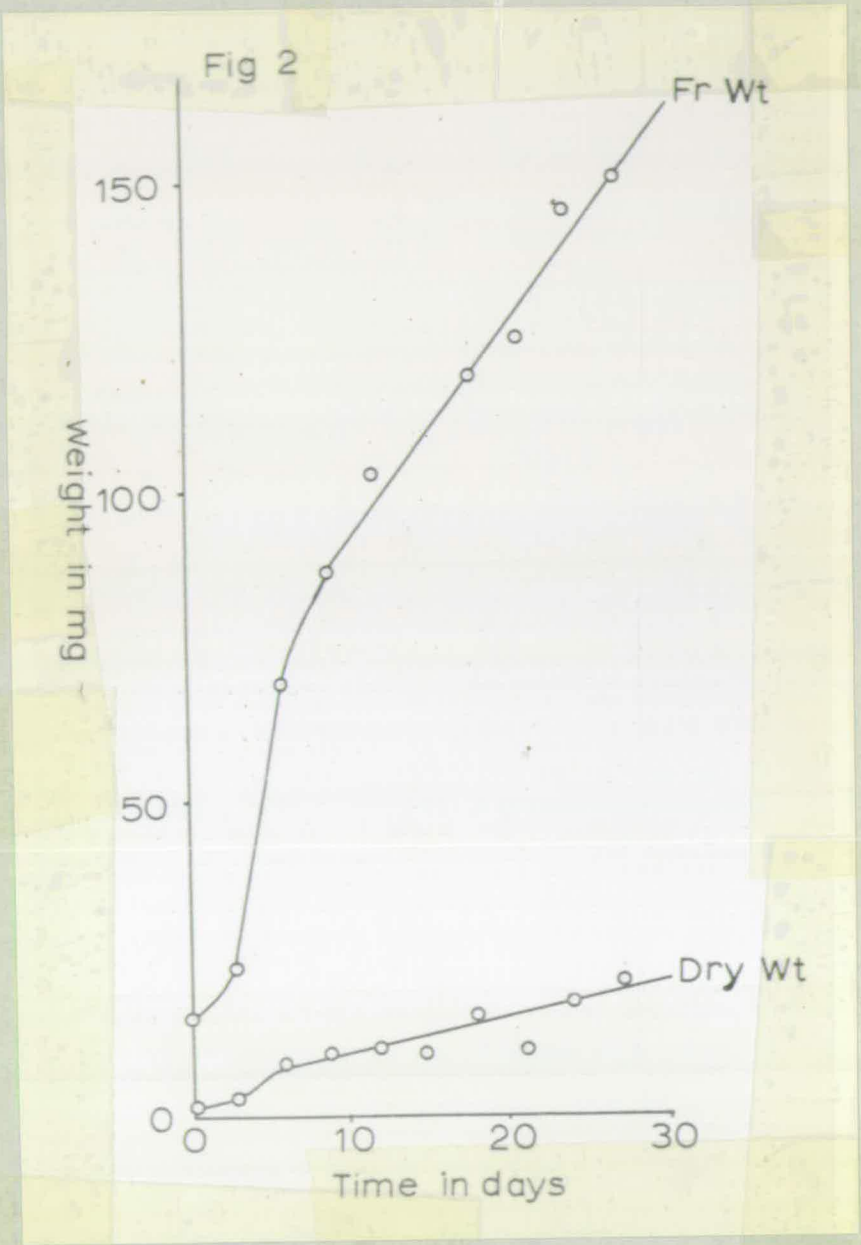
b) Increase of the Fresh and Dry Weights of the root

The results given in Table 3 represent an average from 10 roots.

These results are presented graphically in Figure 2. The form of these graphs is very similar to the root length curve of Figure 1 the only difference being that both the fresh and dry weights continue to increase after the root has ceased to increase in length. There was the same decrease in the growth rate associated with the formation of lateral roots but for these growth parameters it was much more marked. The slowing of the rate of increase in weight associated with the onset of lateral root formation shows that the numerous lateral root primordia do not compensate for the reduction in the activity of the main meristem. It was not known whether the decline in the growth of the primary meristem was a cause of the formation of lateral roots or whether the formation of lateral roots was a cause of the decline in the growth rate but the results of Tables 1, 2 and 3 show that the two factors are correlated.

Table 3 Fresh and dry weight increase during growth of 1 cm. root tip in culture.

Time of culture in days	Fresh Weight in mgs.	Dry Weight in mgs.
0	15.1	1.6
3	23.4	2.2
6	68.5	7.5
9	85.8	9.0
12	103.0	10.8
15	102.3	8.7
18	117.8	16.1
21	125.3	9.7
24	146.6	17.3
27	151.1	21.3



Fresh and dry weight increase during growth of 1cm root tip in culture.

c) Changes in the medium during the growth of the root

The volume, pH and sucrose concentration of the medium were followed in an experiment lasting 30 days. The results are given in Table 5.

These results show that the pH is initially unfavourable for root growth and that the roots bring the pH up to the optimum value for growth (pH 6.4) in the first 6 days. This result explains the slow initial growth shown in Figures 1 and 2.

The decrease in volume is due to evaporation from the medium. Most of the liquid evaporated condenses on the lid of the petrie dish and may run back into the medium during the handling of the dishes. This probably explains the poor fit of the results to what should have been a straight line relationship of volume against time.

The very rapid hydrolysis of the sucrose in the medium was confirmed by chromatography. After 15 days no sucrose was detected on chromatograms although plenty of glucose and frutose was present up to the end of the 30 days of culture. The results show that the hydrolysis of the sucrose proceeds at a faster rate than the products of the hydrolysis can be used by the root.

These results show that the medium becomes progressively more unfavourable to growth as regards both osmotic and carbohydrate milieu. The pH of the medium was constant after the first 6 days and changes could be neglected after the initial period of culture was complete. It was decided that in spite of the deleterious changes of volume and carbohydrate composition the culture method would be used unchanged in future experiments since there was the likelihood that ageing would be accelerated by such environmental stress.

d) The effect of repeated transfer on the growth of the root

Three batches of roots were used in this experiment:-

(a) A control consisting of roots grown in the normal way

(b) An experiment in which the whole root was transferred every three days to fresh medium.

(c) An experiment in which only the apical 1 cm. was transferred to fresh medium every three days.

The roots in (a) and (b) were measured at 3 day intervals and

Table 5 Changes in the culture medium during the growth of 1 cm. root tips in culture.

Time of culture in days	pH	Volume/Dish in mls.	Sucrose g/100 ml.
0	4.3	19.0	4.0
3	5.9	17.5	2.8
6	6.4	17.3	1.3
9	6.4	17.0	0.3
12	6.4	14.8	0
15	6.4	15.5	
18	6.1	15.0	
21	6.2	15.5	
24	6.2	12.5	
27	6.4	11.5	

their lengths recorded. In (c) the length of root produced since the first day of culture was recorded. The results are given in Table 5 and Figure 3. Ten roots were used for each batch.

These probable results show that the transferred roots did not grow as well as the control roots. The reason for this was that the repeated transfer of the root caused it to be continually in medium with a sub-optimal pH. Comparison of the results for (b) and (c) also show that the amputation of the older root tissues had no effect on the rate of growth of the apical 1 cm.

e) Viability of fresh and aged root tips in fresh and used medium.

In these experiments root tips(1 cm. apical portion) were cut from roots which had been grown for various times in culture and transferred to fresh media. Ten root tips were used from each age of root and they were all grown for three days in the fresh medium. After three days growth the roots were measured and the average length and the percentage of roots showing growth were recorded for each batch. To reduce the time taken for the pH of the fresh medium to be raised to the optimum for growth the root tips were sown 10 to a dish so one dish was used for each age of root. The other half of the experiment consisted of placing fresh 1 cm. tips in the spent medium used to grow the roots for the first half of the experiment. The root tips were placed 3 to a dish and 4 dishes were set up for each age of spent medium. After three days the roots were measured and the average length and the percentage showing growth in each of the different media were recorded. The results are given in Table 6.

These results show that there was a decline in both the viability of the meristem with age and the ability of the medium to support growth during the growth of 1cm. root tips in culture. The increased rate of

Table 4 The effect of repeated transfer of cultured roots to fresh medium.

Day of culture	Average length of root in cms. Control (a)	3 day transfer of whole root (b)	3 day transfer of apical 1 cm. (c)
0	1.0	1.0	1.0
3	2.0	2.0	2.0
6	3.9	3.0	2.7
9	6.0	3.8	3.4
12	8.0	4.2	3.8
15	11.5	4.4	3.9

Effect of repeated transfer of cultured roots to fresh medium

Fig 3

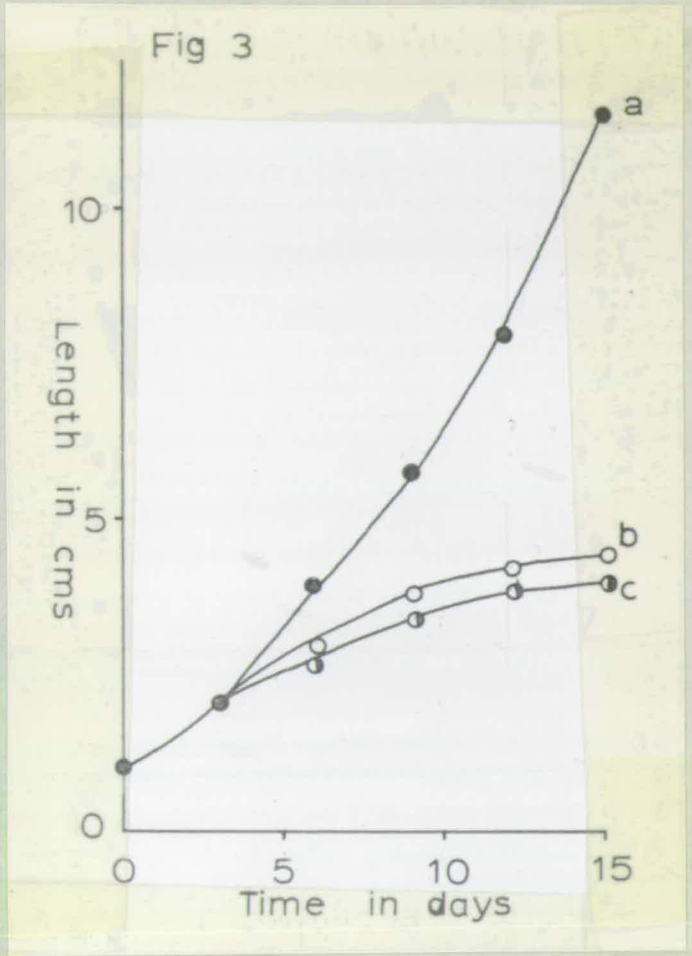


Fig 4a

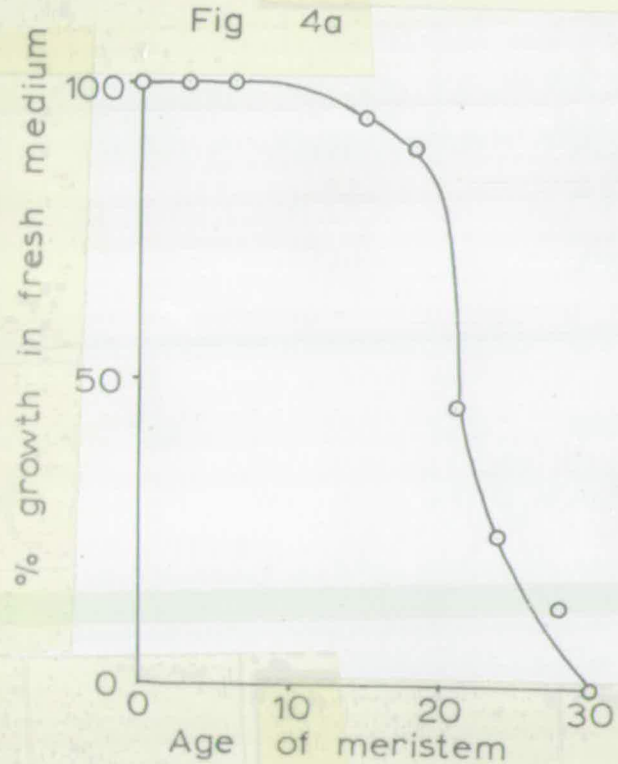
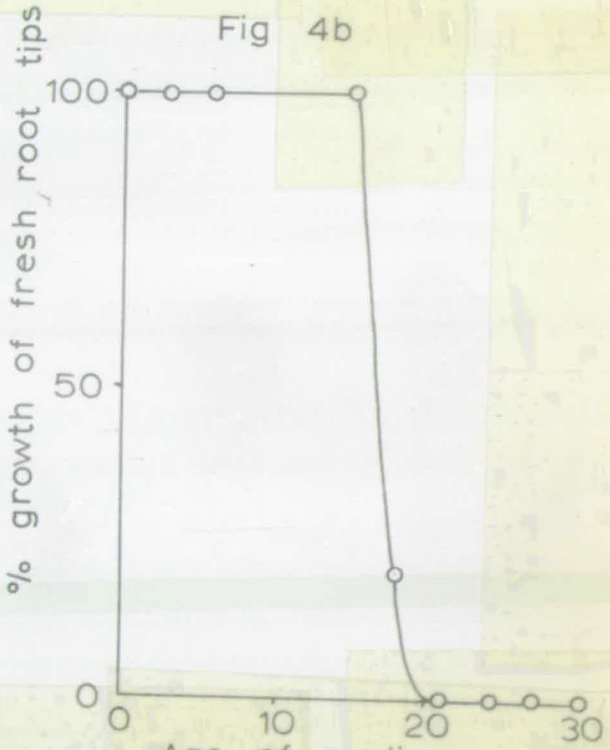


Fig 4b

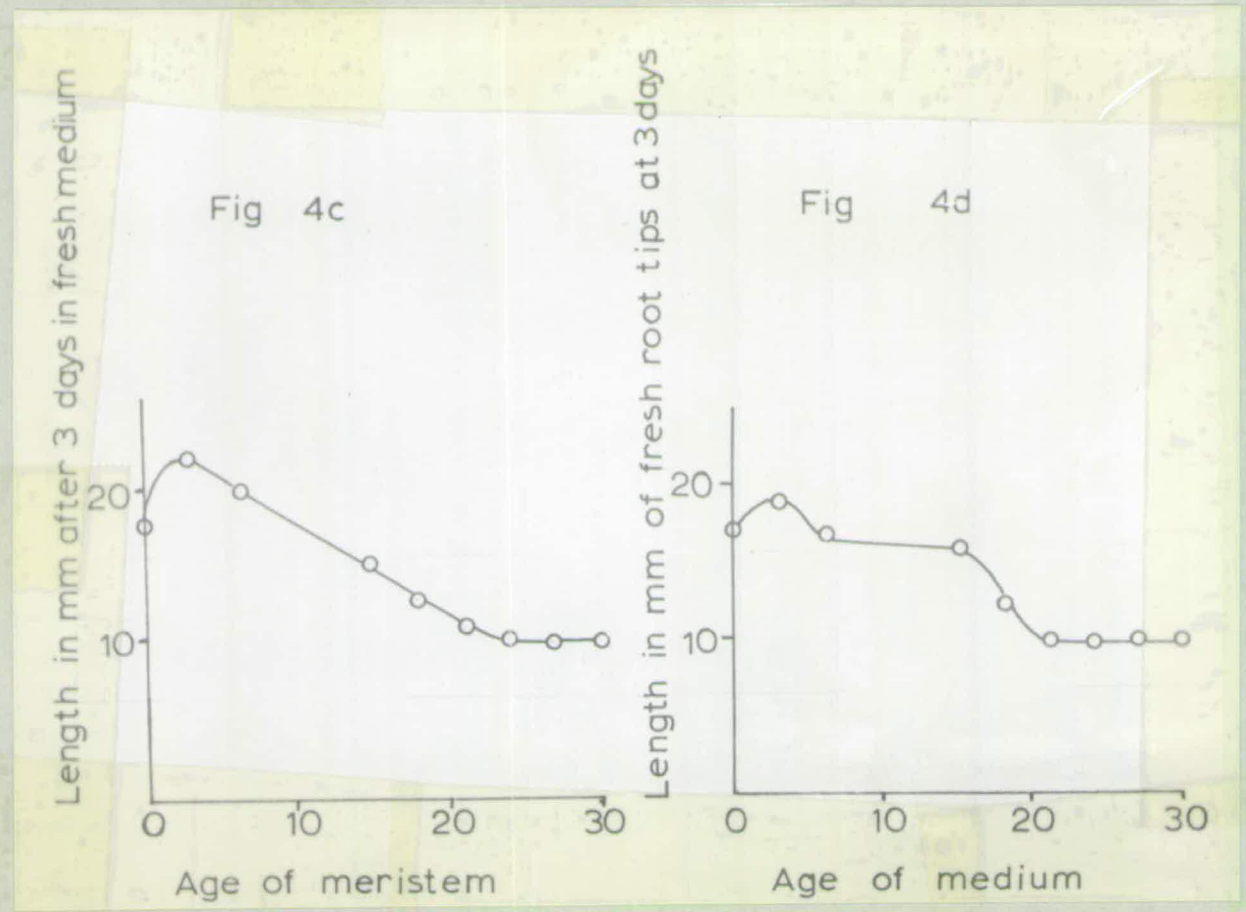


Effect of root age and medium age on meristem viability

Table 6a Effect of Growth of 1 cm. root tips in aged media and growth of 1 cm. root tips cut from cultured roots in fresh media Table 6b

Age of Medium in days	Average length fresh root tips in aged medium in mm. at 3 days	% fresh root tips growing in aged medium	Age of root ~~medium~~ in days	Average length old root tips in mm. at 3 days	% old root tips growing in new medium
0	17	100	0	17	100
3	19	100	3	22	100
6	17	100	6	20	100
15	16	100	15	15	94
18	12	20	18	13	88
21	10	0	21	11	46
24	10	0	24	10.5	25
27	10	0	27	10	10
30	10	0	30	10	0

Effect of meristem age and medium age on root growth.



growth shown by the fresh material in the 3 day old medium was due to the pH effect noted in the previous section and provides additional evidence for it. The increased rate of growth of the 3 dayold root tips in fresh medium probably means that these roots were to some extent adapted to growth at a sub-optimal pH. The results show that both the root meristems and the culture showed ageing changes but that these changes were not of the same form. The root meristem showed a straight line relationship between root age in culture and the division activity of the meristem as measured by increase in length, if the first three days were ignored. The medium showed an ability to support growth until the 15th day at which point the growth declined very quickly.

These results support the interpretation that the cessation of growth in length was due to changes in the medium since the growth in length ceased abruptly as was shown in Figure 1. At the same time there was the gradual decline in meristematic activity. This can be easily seen by refferance to Figure 4a - d.

3) Changes in the basal 1 cm. segment during the growth of the root in the ageing medium

a) Fresh Weight

The results given in Table 7 were the average results of two experiments and each age of segment was represented by 30 to 40 segments. The 95% confidence limits are given in Table 7. The results are presented graphically in Figure 5.

The considerable variation in the results was due to the presence of root hairs on the root segments. A root segment having turgid root hairs on it was not easy to blot dry and so would tend to have a higher fresh weight than a root segment in which the root hairs were all dead. Since it was possible to have a root segment with both living and dead root hairs on it in practically any proportion on the

Table 7 Change in Fresh weight of basal 1 cm. segment during culture.

Age of Basal Segment in days	Fresh Weight (mg.)
0	13.1 ± 4.1
3	15.2 6.4
6	16.5 3.9
9	17.5 6.7
12	17.6 4.5
15	19.3 5.0
18	20.6 5.6
21	18.9 9.4
24	17.3 4.8
27	17.4 4.7
30	15.8 ± 4.7

Table 8 Change in number of cells in basal 1 cm. segments during culture

Age of the Basal segment in days	Cell Number ($\times 10^{-3}$)
0	18.75 ± 0.08
3	11.10 ± 0.22
6	8.72 ± 0.24
9	8.62 ± 0.24
12	8.28 ± 0.18
15	7.66 ± 0.38
18	-
21	-
24	-
27	-
30	8.94 0.58

Fresh weight of basal segment

Fig 5

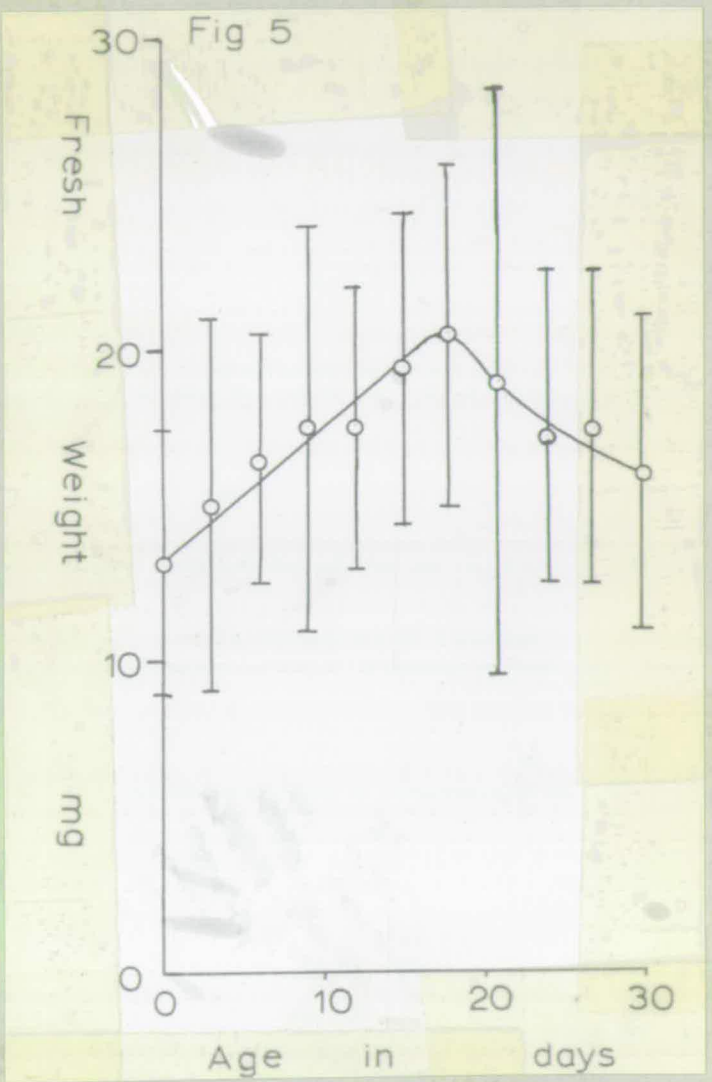
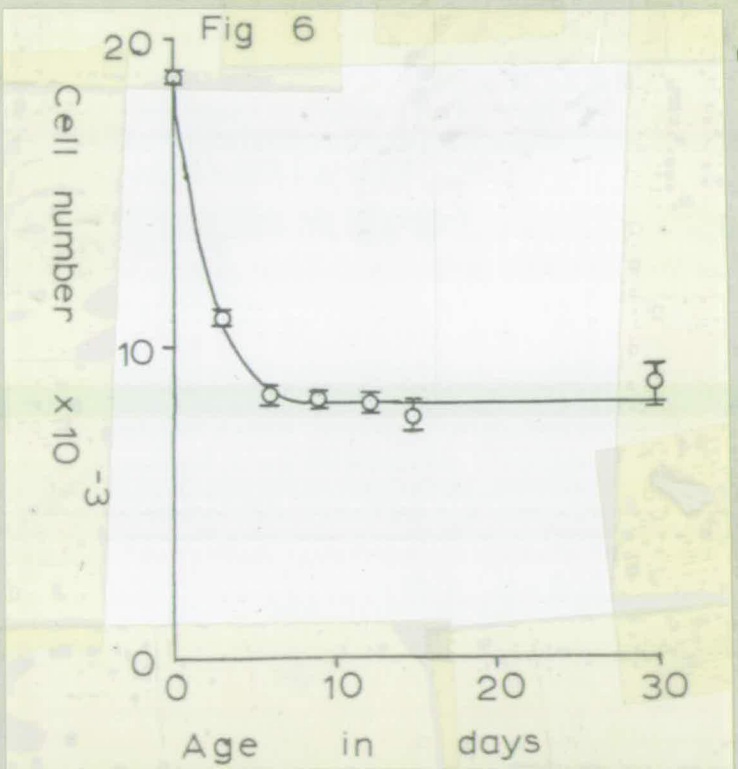


Fig 6



Cell number of basal segment

variability in weight as measured by the 95% confidence limits was inclined to be high especially in the middle of the experimental period.

b) Cell Number

The method used was not entirely successful in macerating the vascular tissue of the older root segments and so was not carried out as a routine procedure for all experiments. The results given in Table 8 were from one experiment in which the maceration technique was at least partially successful.

The results of this and other experiments show that the cell number per segment remained constant within the limits of error from the 6th to the 30th day of culture. This showed that there was no cell division in the basal segment. The 95% confidence limits are given in Table 8. The results themselves are plotted in Figure 6 together with the confidence limits.

c) Histology of the basal segment

1) Anatomy of the segment

Changes in the anatomy of the basal segment were observed on longitudinal and Transverse sections from three sources:

1) The safranin light green stained Transverse sections (T.S.) cut specifically for this purpose.
2) The hand cut T.S. cut for the phosphatase histochemistry.
3) The Feulgen stained transverse and longitudinal sections used for autoradiography and DNA microdensitometry.

Plates 1 and 2 show the general appearance of the root in T.S. in the basal segment region at 9 and 27 days.

The anatomy of the root was completely normal except for the

Plate 1 Haematoxylin stained TS of a 9 day old basal segment

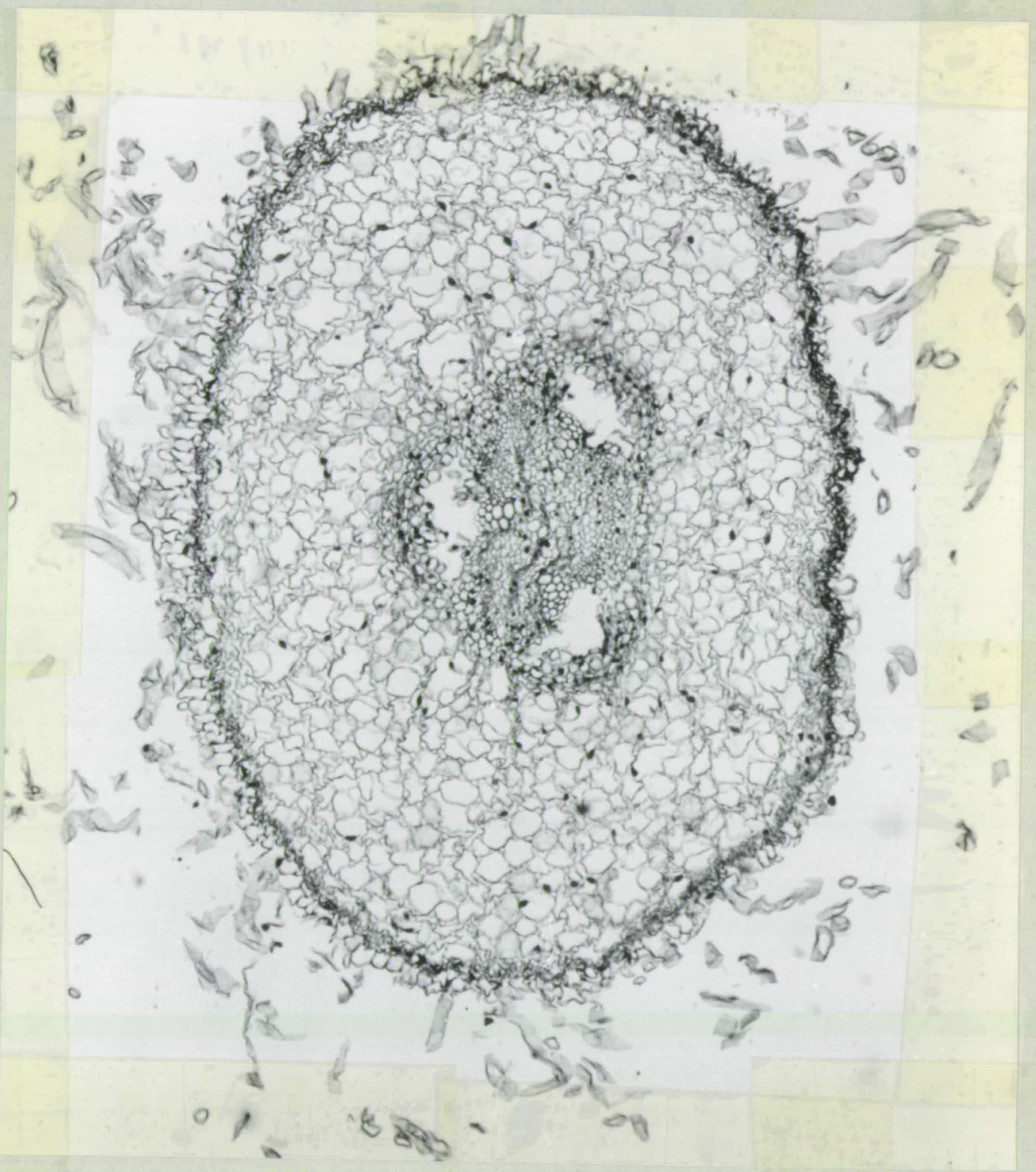
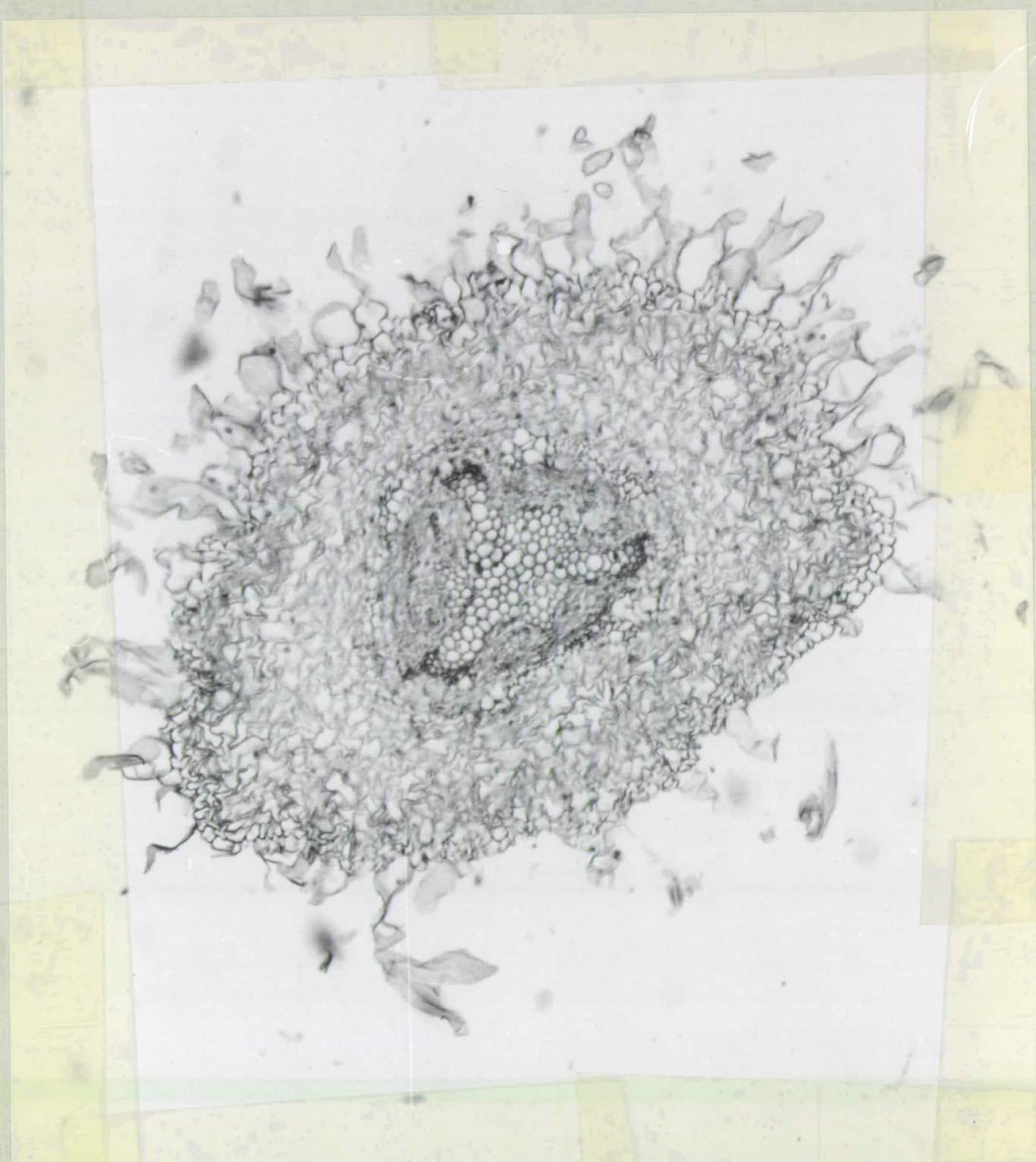


Plate 2 Haematoxylin stained TS of a 30 day old basal segment



relatively poor degree of lignification in the vascular system and the absence of any secondary thickening in the stele or cortex. Both of these points have been observed frequently in other work on cultured roots. They both seem dependent on the auxin concentration and the lignification seems dependent on the sucrose concentration also (Street, 1952; Hughes, 1960; White, 1943). Examination of transverse sections (T.S.) of the basal segment stained with safranin and light green did show that a few divisions had taken place in the vascular system but these were too few to have any effect on the cell number which could be detected with the method used for cell number estimation. Feulgen stained sections (both T.S. and L.S.) were examined but no nuclei were ever found in the course of division. Any cell division which does occur is very limited and occurs only in the stele during the first three days of culture.

The root segment has an anatomy precisely that of a young normally grown pea root before secondary thickening has started. The segments stay in this 'juvenile' state throughout the whole period of culture. The root when cut in T.S. consists of the usual triarch stele, cortex and piliferous layer. The stele remained unchanged throughout the period of culture except for a slight increase in the lignification of the larger xylem vessels and the deposition of staining products (SUBERIN) in the endodermis. The piliferous layer and the cortex gradually died as the age of the root segment increased. This necrotic process did not proceed at a uniform rate throughtout the experiment but occurred in a series of discrete stages which are listed below.

a) <u>Formation and death of the root hair and the piliferous layer</u>

Root hairs have already begun to differentiate from the piliferous layer in the oldest tissue of the 1 cm. apical segment

used to inoculate the culture dishes at the start of the culture. The root hairs were fully developed at the basal end of the root segment by the third day of culture and by the 6th day of culture had died and lost their turgidity. The root hairs at the other end of the root segment were fully mature at about the 9th day of culture and had died completely by the 12th day of culture. It took about a week for this necrosis of the piliferous layer to pass the length of the segment. This gradual nature of the various processes occuring in the basal segment illustrated by this observation is unavoidable so the results given in the later sections must be interpreted as the average state of the segment. They may only reflect the state of affairs in a small percentage of the whole segment.

The dead root hairs and piliferous cells were not lost into the medium but were retained as a layer of collapsed cells around the cortex which is still alive at this time.

b) Necrosis of the outer cortex

This region of the cortex was defined as the layer of parenchyma cells 3-4 cells thick which lies immediately beneath the piliferous layer. The necrosis began as soon as the necrosis of the piliferous layer was complete and progressed along the root behind it. The necrosis is slow and continues until the 21st day of culture.

c) Necrosis of the middle cortex

This region of the cortex consists of the large parenchyma cells which make up the bulk of the cortical tissue. The necrosis of the middle cortex was rapid, far faster than the necrosis of the outer cortex. Necrosis started on the 21st day and had spread throughout the middle cortex by the 24th. Not only was this necrosis more rapid than those previously described but it was also spread evenly throughout the length of the segment. This stage of the necrosis was

thus homogeneous for the segment.

d) Necrosis of the inner cortex

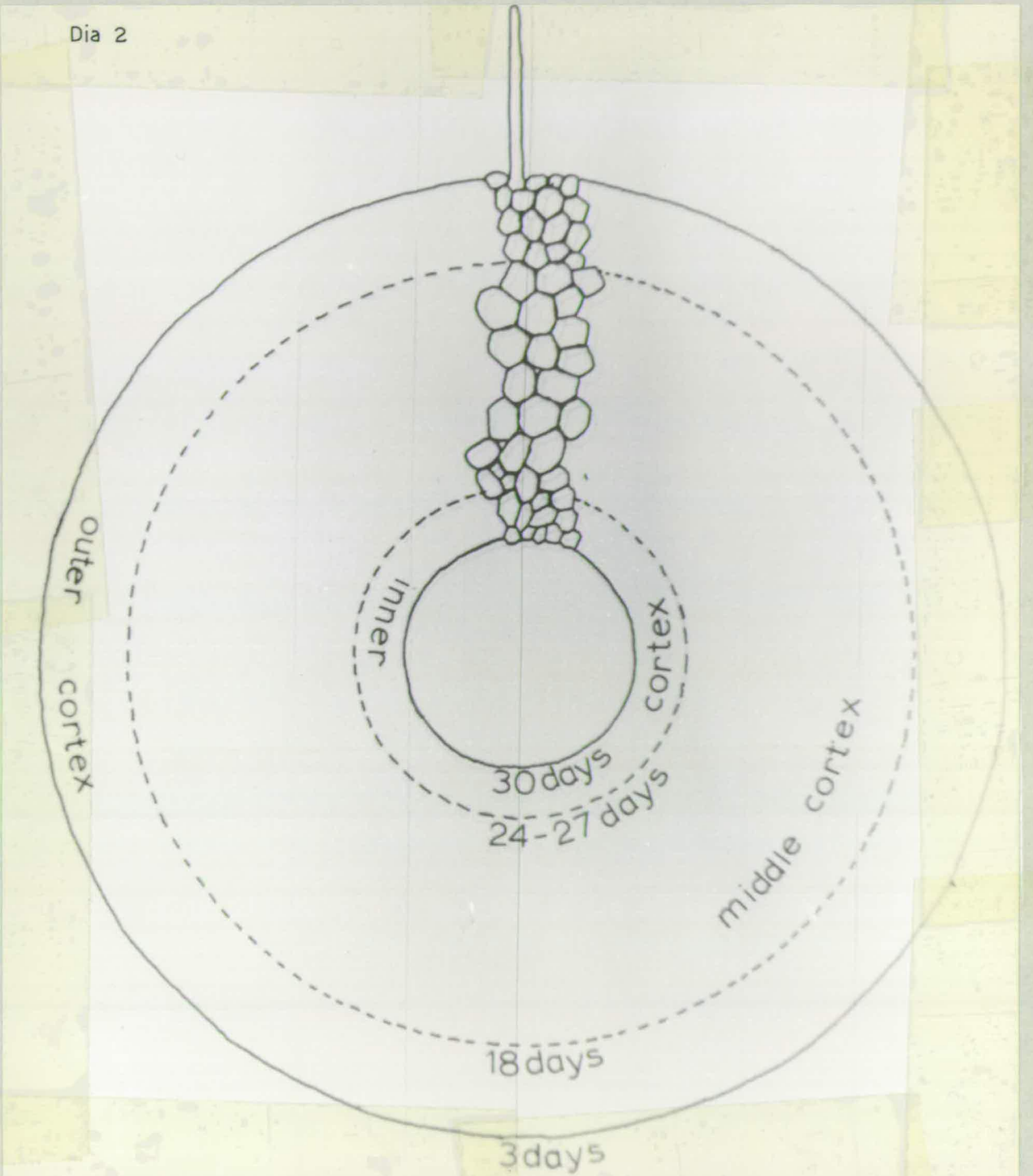
This region comprises a layer of smaller parenchyma cells about 2-3 cells thick immediately external to the endodermis. This layer remains unaffected by the necrosis of the middle cortex and begins to die only after the cortex external to it is dead. The process of necrosis begins between the 24th and the 27th days and is complete by the 30th day. See diagram 2

The only other features of this necrotic process which were noted concerned the timing of the several periods of necrosis in the various regions. The two stages a) and b) above were discussed under two separate headings although in fact this was an arbitrary division of what appeared to be a continuous process, the necrosis of the outer cortex following the necrosis of the piliferous layer immediately. In the histochemical preparations for phosphatase determination it was noted that there often appeared to be a pause between the completion of the necrosis of the outer cortex and the start of the necrosis of the middle cortex.

Changes in the anatomy of the basal segment were observed on transverse and longitudinal sections (T.S. and L.S.). The observations were drawn from three sources; the safranin light green stained sections referred to previously, the sections on which the phosphatase histochemistry was carried out (this will be discussed in the following section) and the Feulgen stained T.S's and L.S's used for estimations of DNA content by microdensitometry.

2) Phosphatase histochemistry

This technique was used primarily to investigate the distribution of the acid phosphatase enzymes in the cells of the root. Its usefulness lay entirely in the fact that it could be used as a


Dia 2
outer cortex
inner cortex
middle cortex
30 days
24-27 days
18 days
3 days

sensitive indicator of the dead, the dying and the still living tissues in the root cortex. There are three important points which give this technique its usefulness. Firstly the phosphatase activity changes from a pattern of highly localised activity in granules within the cytoplasm to one of diffuse general activity spread throughout the cytoplasm of the cell as it died. Secondly there was a large increase in the activity of the enzyme during this change. The death of the cell was thus marked by a change in the activity and the distribution of the activity within the cell. Finally it was noted that the cell walls of autolysed cells took up some of the breakdown products of the reaction mixture and stained a green colour which changed to a yellowish brown on standing. Cell walls of living cells and cell walls of cells which had lost their contents during the sectioning did not take up this stain. These points are illustrated by the following four plates with their explanations.

Plate 3

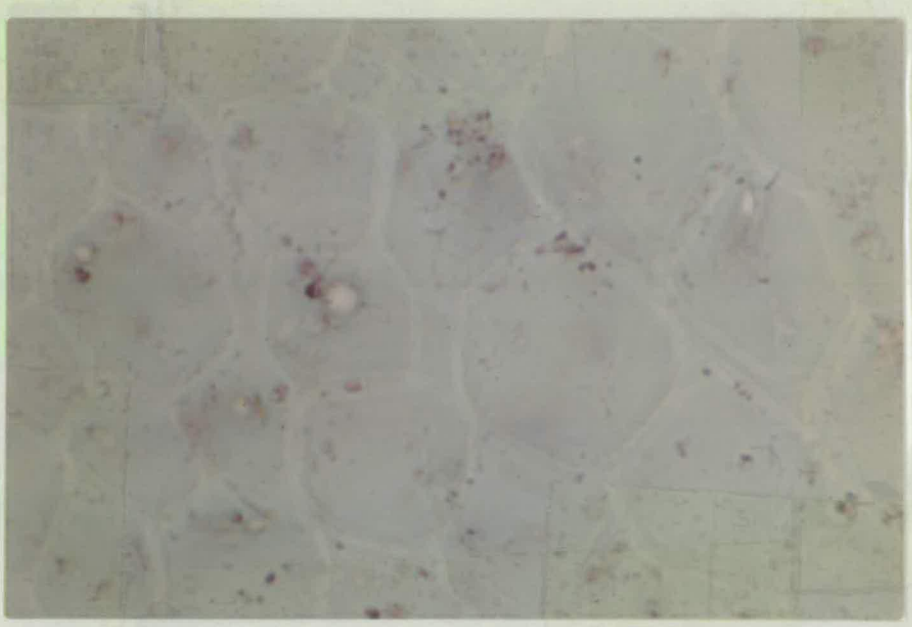
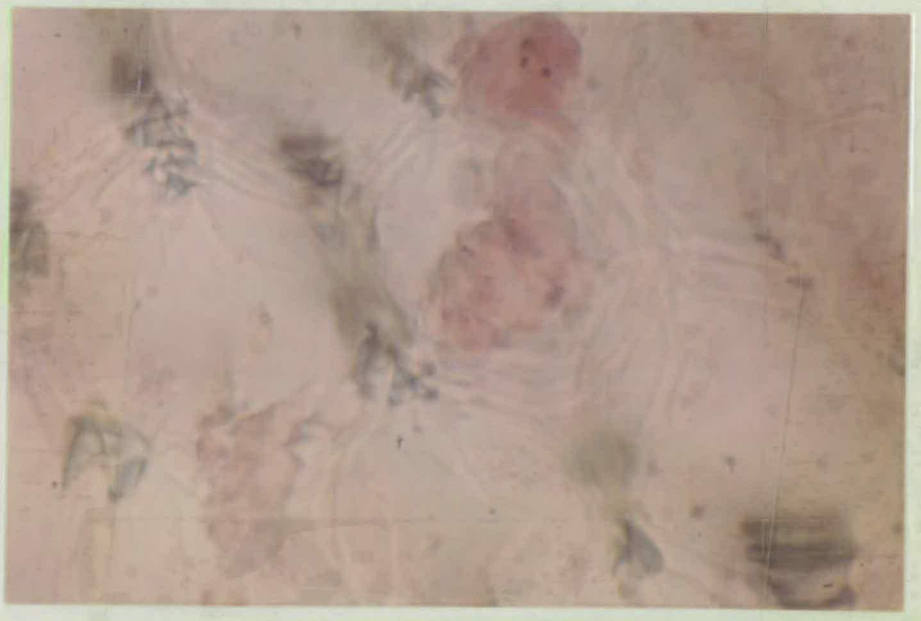


Plate 3 shows the granular activity in living pith parenchyma cells stained for one hour. These cells show the granularity of the activity rather better than the cells of the cortical parenchyma because they have more cytoplasm in relation to their bulk. The living cortical cells show a similar but less pronounced pattern which is hard to photograph successfully as can be seen from Plate 4.

Plate 4

This section was only stained for half an hour and shows the appearance of a living parenchymatous cell in the cortex.

Plate 5



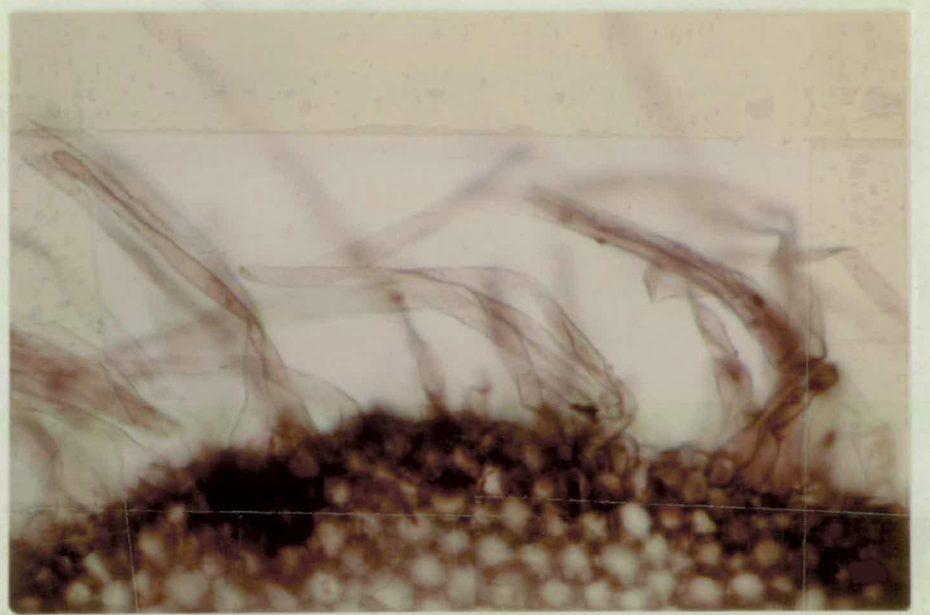
This shows the phosphatase activity in some dying cells from the same section as the live cells of Plate 4. The activity is spread throughout the cytoplasm and the level of activity is much higher. Staining time was ½ hour.

Plate 6

This plate shows living and dead cells. The cell walls of the dead cells are stained a yellowish brown colour those of the live cells are unstained.

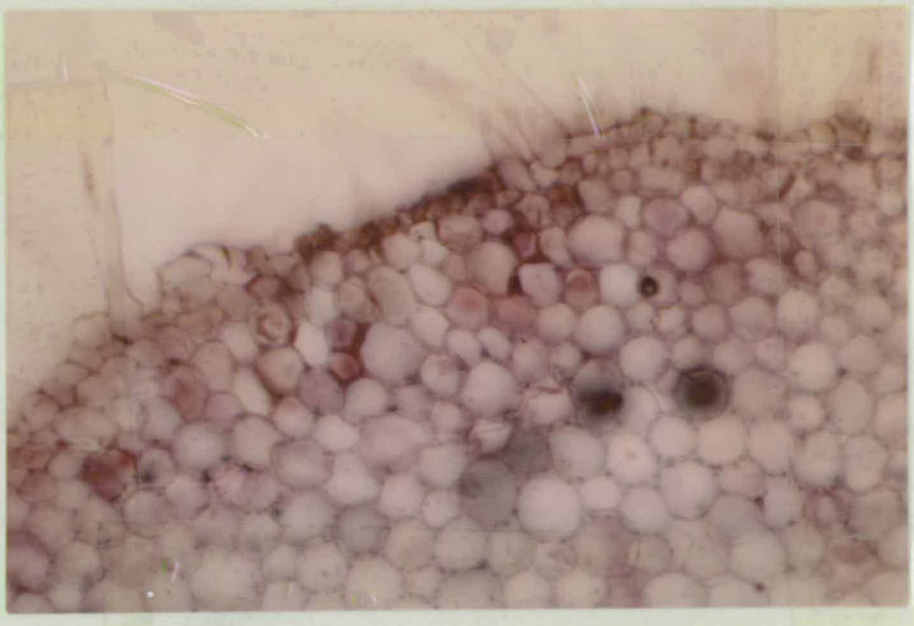
Plates 7 - 10 show the appearance of the cortex at various ages when stained to show phosphatase activity. These show the stages of necrosis in the cortex with increasing age.

Plate 7



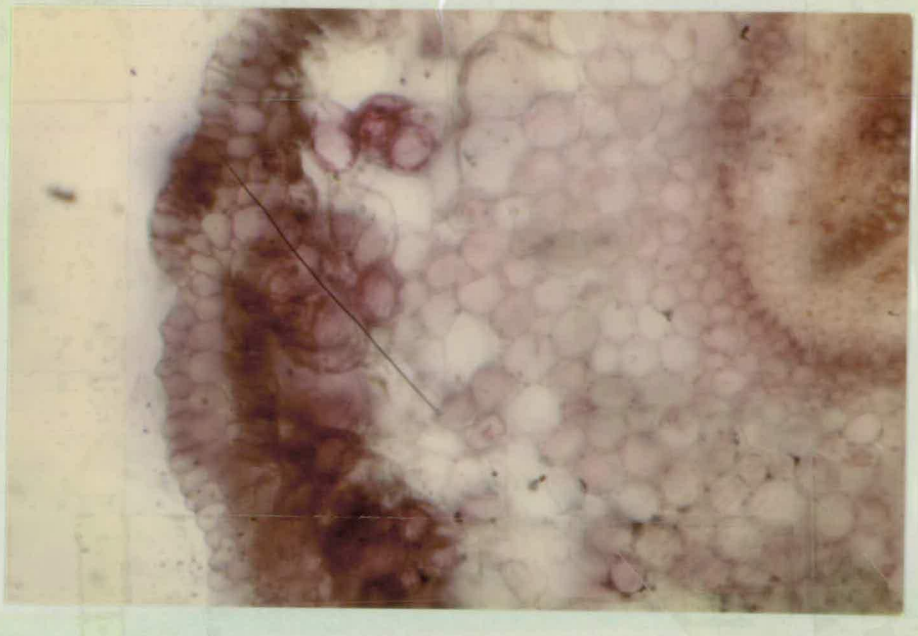
This is a T.S. from a 9 day old segment of the root and shows the activity in the piliferous layer and the outer cortex and the brown staining of the dead

Plate 8



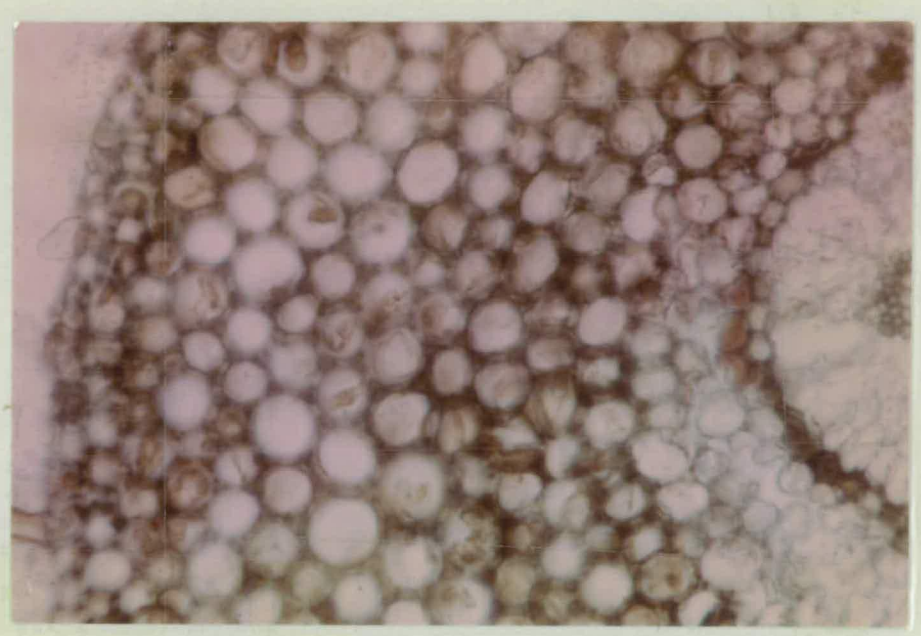
This is a T.S. from an 18 day old segment. The activity and stained dead cells show the process of necrosis in the outer cortex. There is little activity in the middle and inner cortex.

Plate 9



This plate is from a T.S. from a 27 day old segment and shows the spread of the diffuse type of activity associated with cell death to most of the middle and inner regions of the cortex.

Plate 10



This is a T.S. of a 30 day old root and the heavy staining due to the breakdown products shows that the entire cortex is dead except for a layer of cells outside the endodermis which show the diffuse type of activity.

d) Changes in the nitrogen containing fractions.

1) The Total nitrogen content of the basal segment

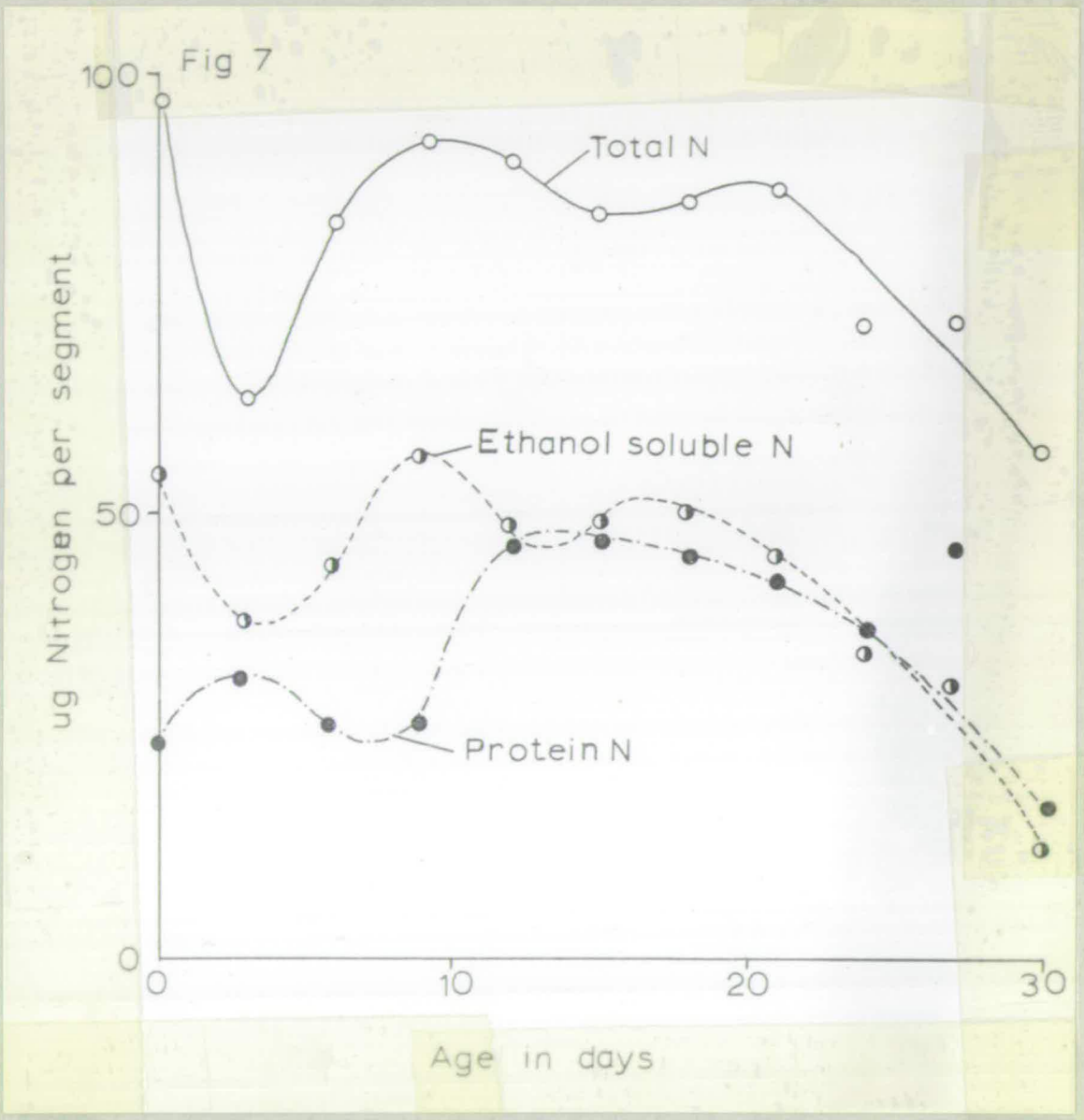
The results presented (in Table 9 and Figure 7) are the average values of the results from 6 experiments.

2) The nitrogen content of the perchloric acid precipitated fraction

The results presented (in Table 9 and Figure 7) are the average values of the results of three individual experiments. This parameter was approximately equal to the protein nitrogen content of the segment. The results are presented in Table 9 and Figure 7.

Table 9 Changes in the nitrogen content of the basal 1 cm. segment during culture

Age of Segment in days	Total nitrogen (µg/seg)	Protein nitrogen (µg/seg)
0	97.3	24.6
3	64.1	32.0
6	83.5	26.8
9	92.1	26.5
12	90.0	46.4
15	84.8	47,2
18	86.1	45.3
21	87.5	45.6
24	71.8	36.5
27	71.9	46.3
30	57.8	16.6



Changes in the nitrogen content of the basal segment during culture.

The graphs of Total nitrogen and Total protein nitrogen content are presented in Figure 7 together with the results for the Ethanol soluble nitrogen content from Table 13. The double peak shape of the total nitrogen curve can be seen to be a combination of the Ethanol soluble nitrogen (equivalent to the amino acid pool) and the Protein nitrogen. The Ethanol soluble nitrogen and the Protein nitrogen are the two major nitrogen fractions in the cell and they do not seem to be related to one another in any simple way. The Total nitrogen is simply the sum of these two fractions plus a little nitrogen from the other nitrogen containing fractions of the cell. The more complicated relationship of the Ethanol soluble nitrogen to the Protein nitrogen is discussed later, but it is necessary at this point to correlate some of the features of the changes shown in Figure 7 to the stages of cortical necrosis in the basal segment discussed in the presentation of the histology results. The decline after day 21 in all the nitrogen fractions in Figure 7 is most probably due to the death of the cells in the middle and inner cortex. The decline in total nitrogen and Ethanol soluble nitrogen in the 0-3 day period is due to the loss of the meristematic cells from the basal segment at this time. The period from 6-12 days cannot be so easily interpreted because several processes involving both necrosis and differentiation are occuring during this period. The rise in the protein content and the fluctuations of the amino acid pool are the result of an interaction between several processes one of which is possibly ~~almost certainly~~ the initiation of a process of cell division in the middle and inner cortex which is stopped short of actual division.

The protein nitrogen fraction was divided into two further fractions; the buffer soluble nitrogen and the buffer insoluble nitrogen. The first of these was equivalent to the soluble protein nitrogen or 'supernatant' protein fraction and the second to the insoluble 'structural' protein of the segment.

3) Buffer soluble protein nitrogen

a) Nitrogen content of this fraction

The results presented were from one experiment only and are given in Table 10 and presented in graphical form in Figure 8a.

b) Electro-phoresis of the buffer soluble protein

The pattern of protein bands produced by the electrophoresis of the soluble protein of 0, 3, 18 and 27 day old root segments on acrylamide gels is shown in Figure 9a - d. The graphs in Figure 9 are tracings of the patterns produced by the scanning of the stained cells after electro-phoresis.

c) Incorporation of C14 Leucine into this fraction

Only one experiment was carried out to investigate the incorporation of C14 Leucine into this soluble protein fraction. The results of this experiment are given in Table 10 and as a graph in Figure 8b.

4) Buffer insoluble nitrogen

This was approximately equivalent to the insoluble protein nitrogen of the segment. Again only one experiment was carried out for each of the nitrogen content and C14 Leucine incorporation estimations on the segments.

a) Nitrogen content of the insoluble fraction

The results are given in Table 11 and graphically in Figure 8a.

b) Incorporation of C14 Leucine into this fraction

The results are presented in Table 11 and in Figure 8b.

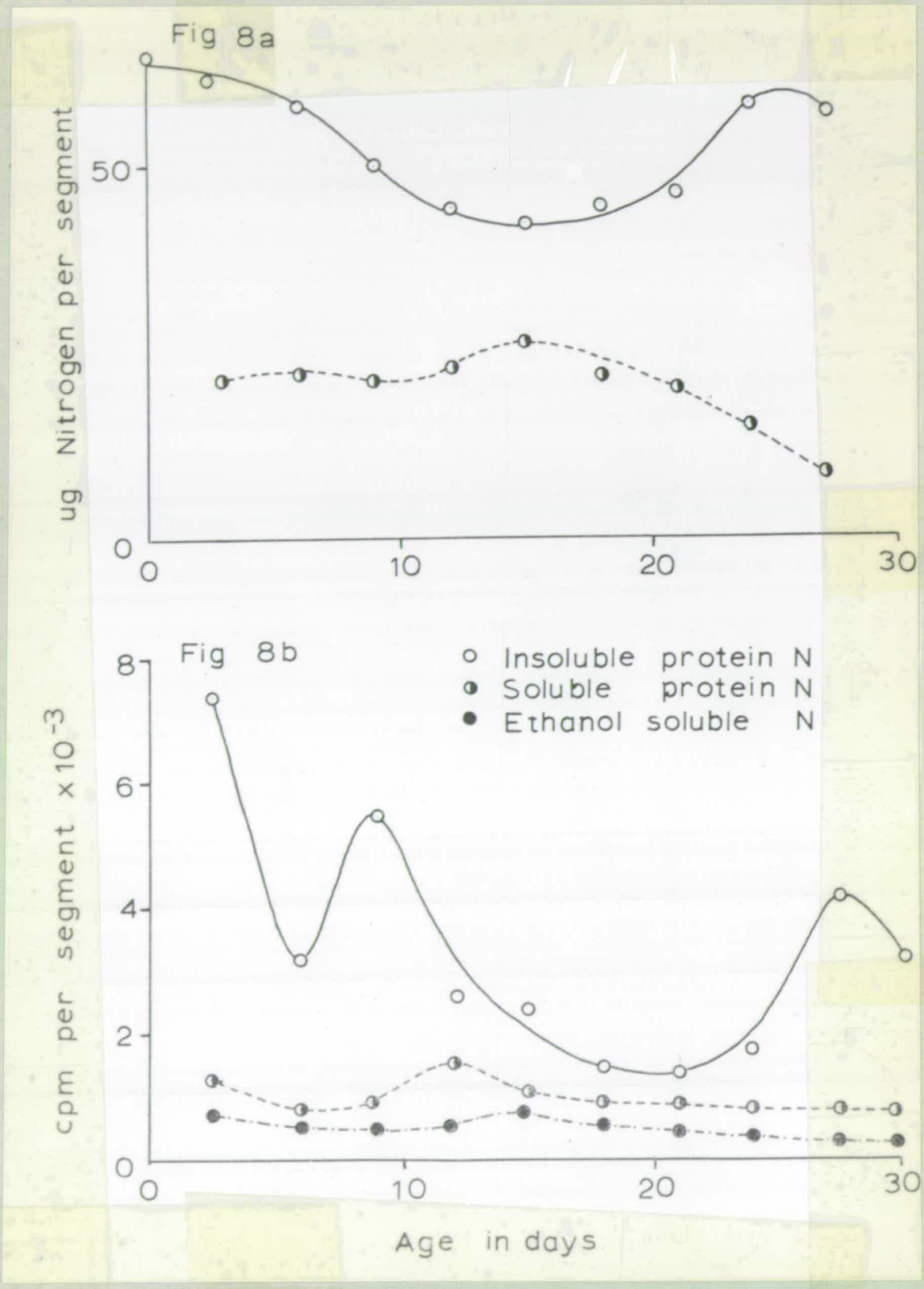
Figure 8a shows the breakdown of the Protein nitrogen curve in Figure 7 into two component curves representing the Soluble protein

Table 10 Buffer soluble Nitrogen content of the basal 1 cm. segment during culture and C14 Leucine incorporation into this fraction.

Age of Segment in days	Soluble protein nitrogen content µg/seg	Soluble protein C14 incorporation cpm/seg
0	-	-
3	21.0	1296
6	23.4	800
9	21.3	856
12	22.8	1520
15	26.7	932
18	21.3	-
21	21.0	892
24	15.3	776
27	8.7	800
30	-	720

Table 11 Insoluble protein nitrogen content and C14 Leucine incorporation into this nitrogen fraction of the basal 1 cm. during culture

Age of Segment in days	Insoluble protein nitrogen content µg/seg	Insoluble protein C14 incorporation cpm/seg
0	64.9	-
3	62.3	7,400
6	56.9	3,200
9	49.8	5,480
12	44.4	2,560
15	42.6	2,460
18	44.6	1,440
21	46.5	1,520
24	56.4	1,780
27	55.3	4,240
30	-	3,200



8a Protein nitrogen fraction changes in the basal segment during culture

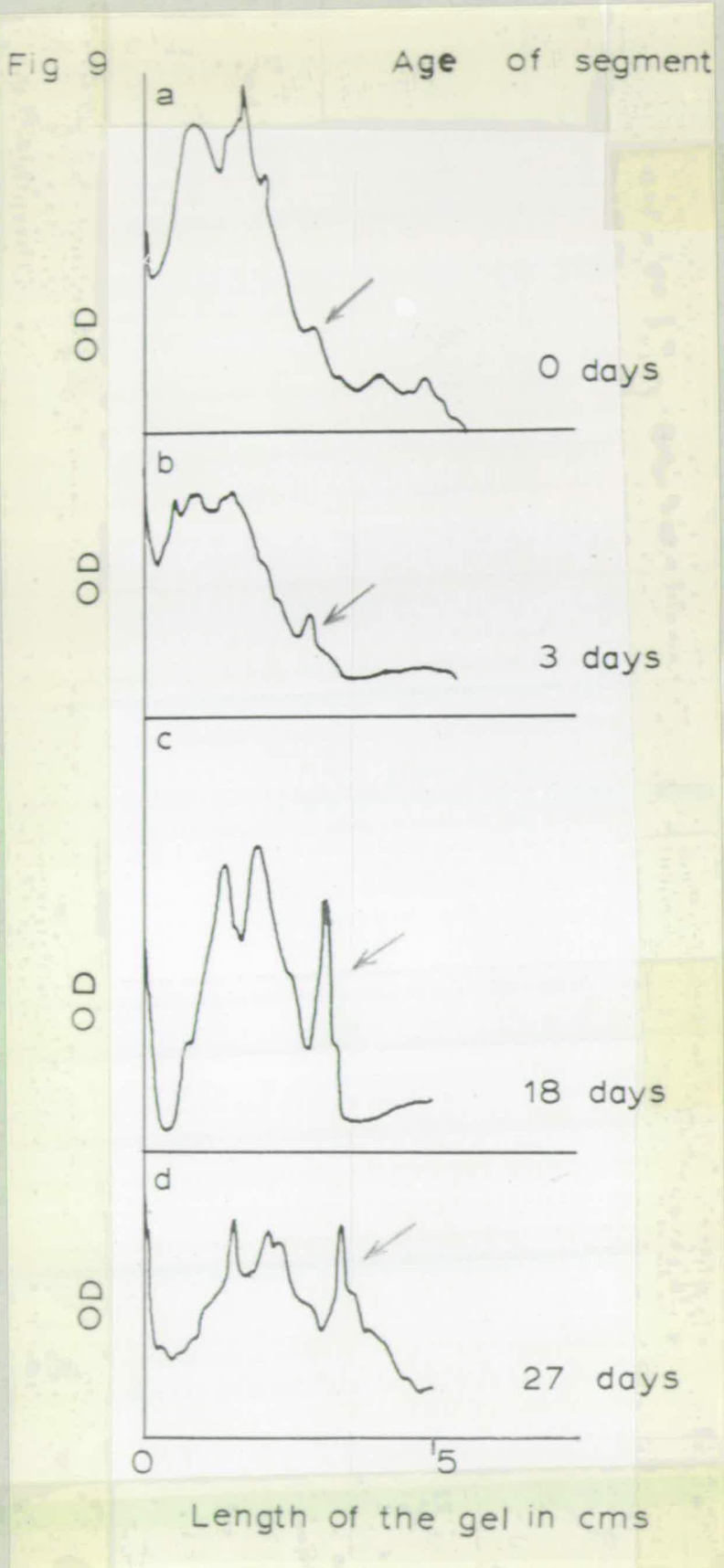
8b Changes in rate of C14 leucine incorporation into protein and amino acid fractions of the basal segment during culture.

nitrogen and the Insoluble protein nitrogen. The sum of these two curves approximates to the shape of the Protein nitrogen curve of Figure 7 but the rise in the Protein nitrogen at 12 days is not as marked. This may be attributed to the fact that the Protein nitrogen results in Table 9 are an average of three sets of experimental results and hence can be expected to differ from the results of a single experiment such as those in Figure 8a. At least one of the sets of experimental results agreed closely with the values for Protein nitrogen obtained by the addition of the curves in Figure 8a. The rise at 12 days is entirely confined to the soluble protein nitrogen fraction and the fall in this fraction after 15 days is paralleled by a rise in the Insoluble protein nitrogen fraction.

Figure 8b shows the C14 Leucine incorporation into the fractions shown in Figure 8a together with that of the Ethanol soluble fraction of Figure 7 and Table 13. The incorporation for the two protein nitrogen fractions parallels the nitrogen content curves for these two fractions in Figure 8a. If this is a true incorporation into Protein it indicates a remarkably high level of Protein synthesis in cells well into their senescent decline. The relatively low values of activity in the amino acid pool may indicate that Leucine is not a major constituent of the pool which would be in agreement with the results of the chromatography of the Ethanol soluble fraction but it could also be a result of the chase with non-radioactive Leucine given after the incubation with C14 Leucine.

Figure 9 shows the chromoscan traces of 4 gel electrophoresis patterns of the soluble protein fractions at different ages. These show that besides the changes in the quantity of protein in this fraction with age there is a difference in the type of protein present at different ages. This point is brought out in Figure 9 by the appearance of a small

Fig 9



Electrophoresis patterns from the soluble proteins of the basal segment

band of protein which increases in size between 3 and 18 days and then begins to decline by the 27th day. This peak is arrowed in Figures 9a-d. Other less spectacular changes exist but it is sufficient to note that the overall appearance of the scans differ very considerably and there is no doubt that the scans at 3 and 18 days differ sufficiently for it to be assumed that a change in the protein composition has occurred in the intervening period.

5) Labile nitrogen content

The "Labile" nitrogen of the root segments and the fractions derived from them was defined as the nitrogen released as ammonia from the various fractions by excess alkali after hydrolysis at 100°C in 3N HCl. The labile nitrogen was made up of contributions from; the free amino acid amides, the amide groups of protein and any free ammonia present in the tissues. The contribution from each of these sources is given in the results.

a) Total labile nitrogen content

This is an average result taken from 6 sets of experimental results. The results are presented in Table 12 and in Figure 10.

b) Amide nitrogen content of the total protein

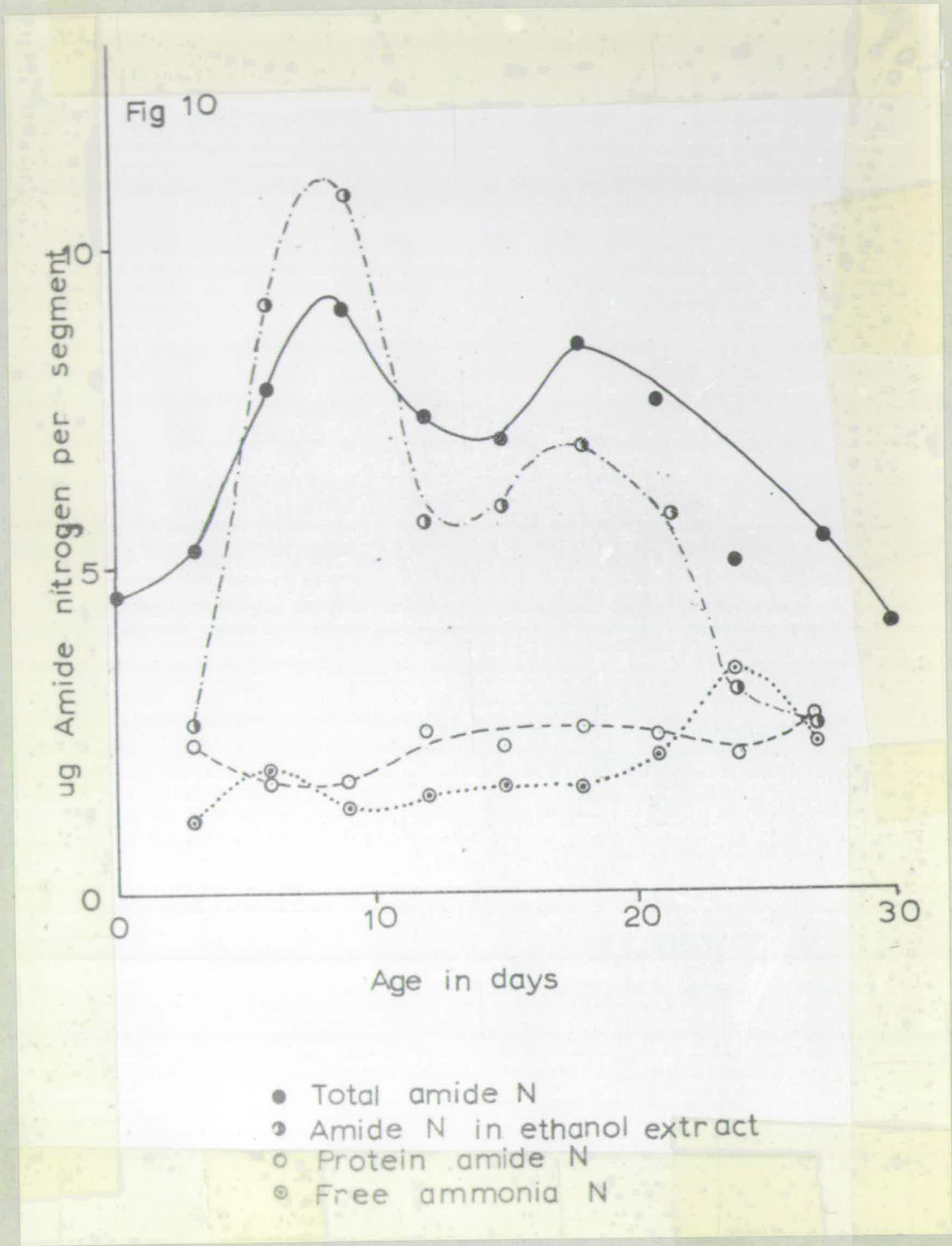
This is the result of one experiment from which all the results concerning the distribution of the labile nitrogen were obtained. The results are presented in Table 12 and in Figure 10.

c) Amide nitrogen content of the ethanol soluble fraction

The Ethanol soluble fraction contains the amide amino acids and the other constituents of the amino acid pool. The amide content of this fraction thus represents the quantity of amides present in the pool. The results are given in Table 12 and Figure 10.

Table 12 Changes in Labile nitrogen in the basal 1 cm. in culture

Age of Segment in days	Total Labile nitrogen µg/seg	Amide nitrogen in protein µg/seg	Amide nitrogen in amino acids µg/seg	Nitrogen in free ammonia µg/seg
0	4.6	-	-	-
3	5.3	2.3	2.6	1.1
6	7.8	1.7	9.1	1.8
9	9.1	1.7	10.8	1.3
12	7.4	2.5	5.8	1.5
15	7.0	2.2	6.0	1.6
18	8.5	2.5	6.9	1.6
21	7.6	2.4	5.8	2.1
24	5.1	2.1	3.1	3.3
27	5.4	2.7	2.6	2.3
30	4.1	-	-	-



Changes in the "labile"nitrogen fractions of the basal segment during culture.

d) Free ammonia in the ethanol soluble fraction

These results are presented in Table 12 and Figure 10 as nitrogen from free ammonia.

The relationships between the 'Labile' nitrogen fractions is most clearly seen by reference to Figure 10. Firstly it must be noted that the Total labile nitrogen figures are average figures from several experiments and so do not match up quantitatively with the other three sets of figures which are the results of a single experiment. In spite of this the qualitative fit of the results is good and shows that the main contribution to the labile nitrogen is made by the amino acid amides with the contribution from the protein amides being much less and following the protein content. The contribution of the protein amide to the total labile nitrogen was only about a third of the amino acid amide contribution. The free ammonia content was relatively small until after 21 days, when it increased sharply.

6) Ethanol soluble nitrogen

The main constituents of this fraction as far as nitrogen containing compounds are concerned were amino acids.

a) Total nitrogen content

The results presented in Table 13 and in Figure 7 are the average results of four experiments.

b) The composition of the ethanol soluble fraction as shown by paper chromatography

The following amino acids were identified in the ethanol soluble fraction, Homo-serine, glycine, serine, alanine, arginine, glutamine, aspargine, glutamic acid and aspartic acid. Quantitative estimations of the amounts of homo-serine and amide amino acids were made and the results presented in Table 13 and Figure 11 show that the bulk of the amino acid pool was made up of homo-serine and that the

Table 13 Ethanol soluble nitrogen content and C14 Leucine incorporation together with the OD of amino acids ninhydrin complexes from chromatograms of Ethanol extract of 1 cm. basal segment during culture.

Age of Segment in days	Alcohol sol nitrogen µg/seg	Alcohol sol fraction C14 incorporation cpm/seg	Homo-serine OD at 500 mu	Amino acid amides OD at 500 mu
0	54.9	-	-	-
3	37.9	608	1.20	-
6	43.5	528	1.35	0.33
9	56.7	432	1.80	0.33
12	48.2	560	0.79	0.24
15	48.7	696	0.70	-
18	50.8	600	0.84	0.20
21	44.0	440	0.82	0.39
24	34.1	400	0.35	0.21
27	31.9	344	-	0.12
30	13.8	264	-	-

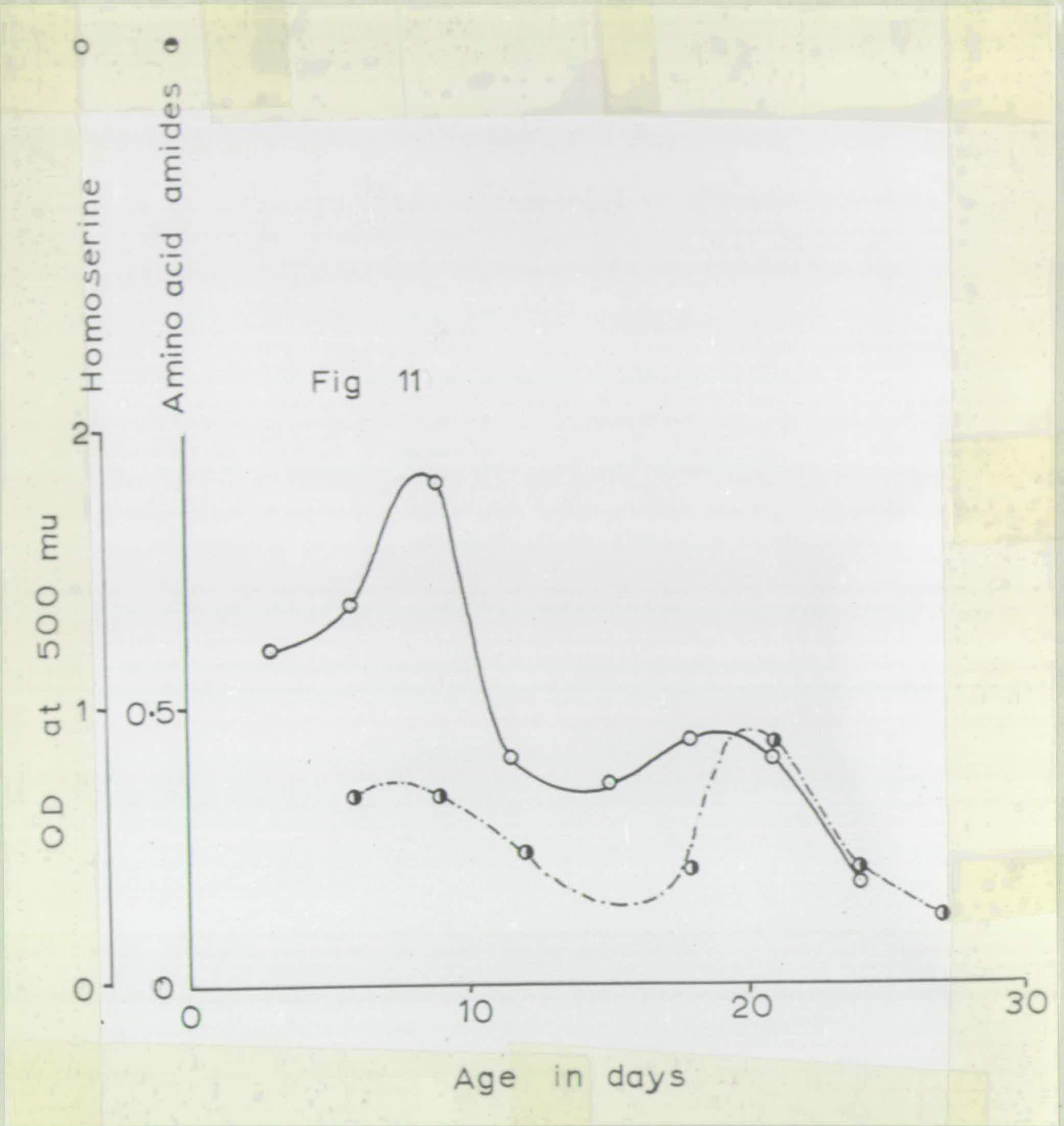


Fig 11

Changes in the concentration of amino acids in the basal segment as shown by a chromatographic method of estimation.

graphs in Figure 11 were qualitatively very similar to the graphs of ethanol soluble and amino acid amide nitrogen in Figures 7 and 10 respectively.

c) Uptake of C14 Leucine into the Ethanol soluble fraction

The results are given in Table 13 and graphed in Figure 8b. The unexpectedly low value for the size of the Leucine pool as shown by the activity incorporated into the ethanol soluble fraction can be explained by the chase incubation of cold Leucine which was given.

The results of this section show that changes in the quantity and distribution of nitrogen occur in the root tissue as ageing proceeds and a general discussion of these results is presented later in the thesis.

7) C14 Leucine mobility in the root

The fall of the protein content of the segment with age shown by the previous results indicates that the protein must be hydrolysed and the products of this hydrolysis lost from the basal segment. The products of protein breakdown can pass into the medium or to other parts of the root. The results given in Tables 13 a-b show the movement of radioactivity within the growing root and the medium after an initial incorporation of C14 Leucine into the apical segment of a seedling root prior to excision and culture.

Table 13 a shows the movement of the radioactivity in the root as it grows in culture.

Table 13b shows the loss of C14 to the medium as the basal segment dies.

The results in Table 13a are shown as histograms in Figure 12. These results indicate that after 27 days about 15% of the C14 taken up as C14 Leucine remains at the original site of incorporation. About 70% of the total activity originally taken up by the root is lost to the medium and 15% is moved to other parts of the root. There is no loss

Table 13a Spread of activity along the root during its growth in culture

3 day old root 3 cms. long cpm/cm	15 day old root 10 cms. long cpm/cm	24 day old root 14 cms. long cpm/cm	27 day old root 19 cms. long cpm/cm
204	144	176	200
98	140	70	130
68	23	30	62
	7	17	42
	0	6	15
	0	7	27
	0	0	12
	0	0	4
	0	0	10
	0	0	6
		0	7
		0	11
		0	7
		0	0
			0
			7
			11
			6
			11

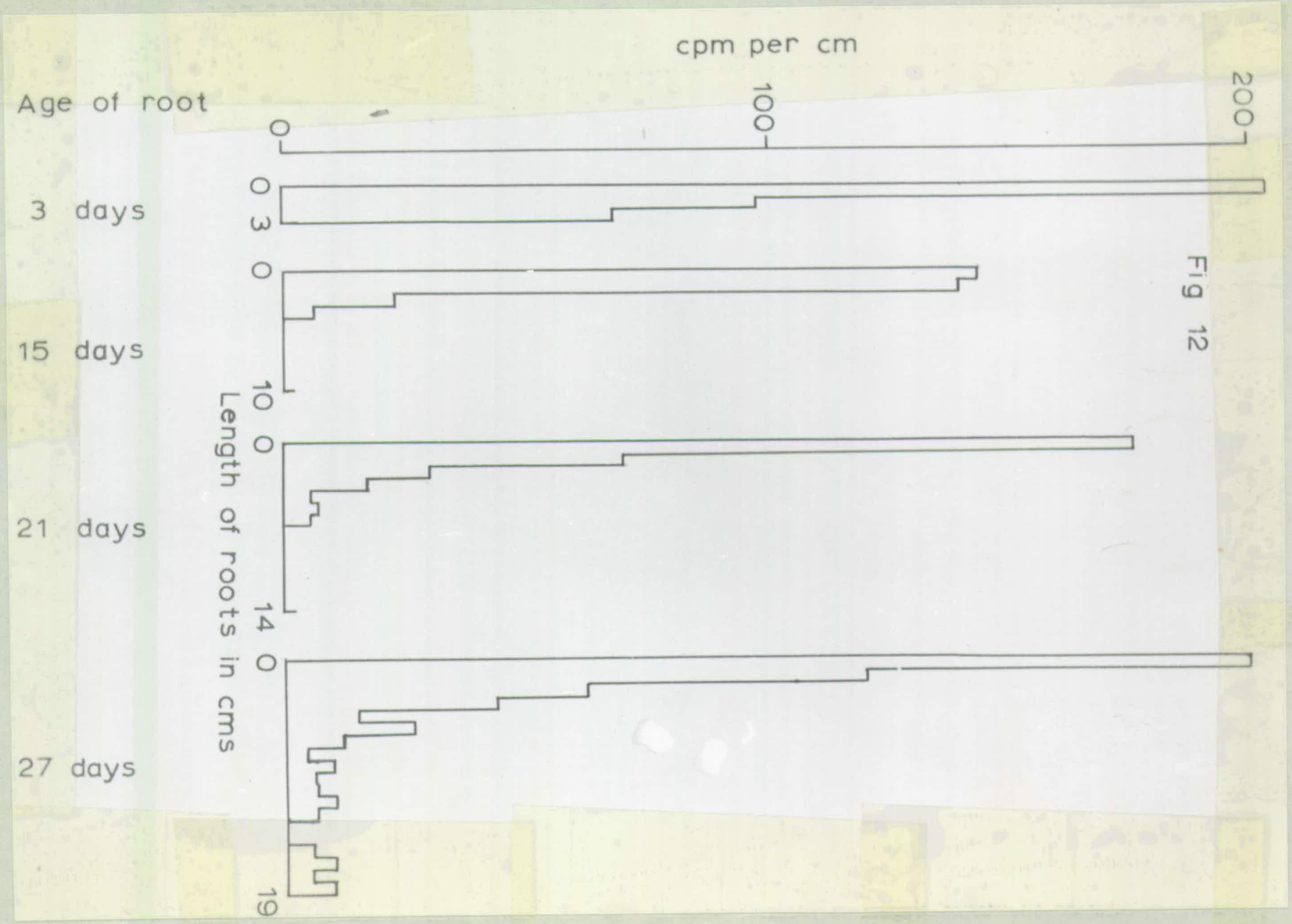


Fig 12

Movement of C14 leucine incorporated into inoculum of 1cm root tips during subsequent growth in culture

Table 13b Radioactivity lost to medium during culture of 1 cm. root tips

Age of root days	Number of counts in medium cpm/ml/root
3	none
15	none
21	120
27	450

to the medium before the 15th day of culture and little movement of activity within the root before this time. Movement of the radioactivity out of the original site of incorporation does not begin until the senescence and death of the cortex has begun. The uptake of C14 Leucine was not uniform for all the seedling roots so anything more than a qualitative comparison between the roots was not possible. The results of this type of experiment agree with those of the preceding experiments in showing a loss of protein from the basal segment during the senescence and death of the cortex.

e) Changes in the enzyme activity with the age of the segment

Two of the six enzymes whose activity was determined during the ageing of the basal segment were only determined by one experiment each. These two were phosphatase and succinic acid dehydrogenase. The results for the other enzymes are those which were most typical of the pattern of change shown by two or more experiments. The results presented in Table 14 are those for all six enzymes. The results are presented graphically in Figures 13a-f in the following order: 13a alanylglycine dipeptidase, 13b protease, 13c succinic acid dehydrogenase, 13d phosphatase, 13e polyphenol oxidase and 13 f invertase.

If the Figures 13a-f are studied they show that there were large changes of activity in all the enzymes in the basal segment which were followed during the culture of the root. These large variations support the inference made from the results given in Figure 9 that the composition of the soluble protein fraction in the basal segment varies continuously during the culture of the root. The protease and the dipeptidase activities show large increases at the 12th day which parallel the increase in protein content which occurs at the same time. The other enzyme activities show the same two peak distribution as has

Table 14 Enzyme activities per 1 cm. basal segment during culture

Age in days	Polyphenol oxidase in Arbitrary Units	Dipeptidase µg Dipeptide hydrolysed /seg	Invertase uM Sucrose hydrolysed /seg	Protease OD 280 mu /seg	Phosphatase µg P/seg
0	4.5	1.8	3.3	-	13.0
3	2.2	5.5	3.5	0.000	14.5
6	6.3	7.6	4.3	0.029	16.5
9	7.4	0.7	5.0	0.000	13.3
12	6.5	1.7	3.5	0.000	12.5
15	4.6	12.5	4.3	0.113	12.5
18	7.0	5.9	4.7	0.100	10.6
21	7.3	4.4	4.7	0.091	11.0
24	3.5	5.5	-	0.046	11.5
27	2.3	6.3	5.3	0.054	12.5
30	-	4.3	-	-	-

	Succinic acid dehydrogenase µg tetrazolium salt reduced/seg
0	8.0
3	10.0
6	8.0
9	10.0
12	-
15	41.0
18	55.0
21	41.0
24	32.0
27	28.0
30	-

Fig 13a Dipeptidase

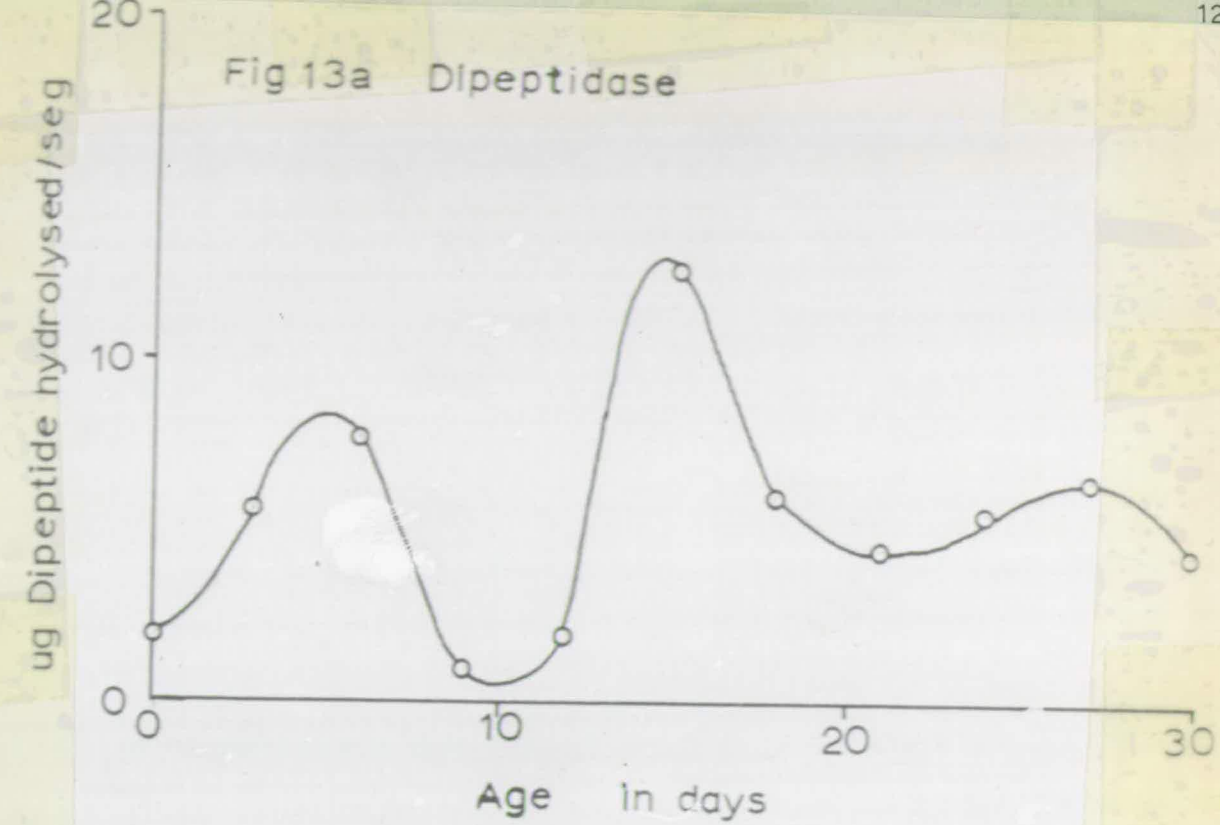
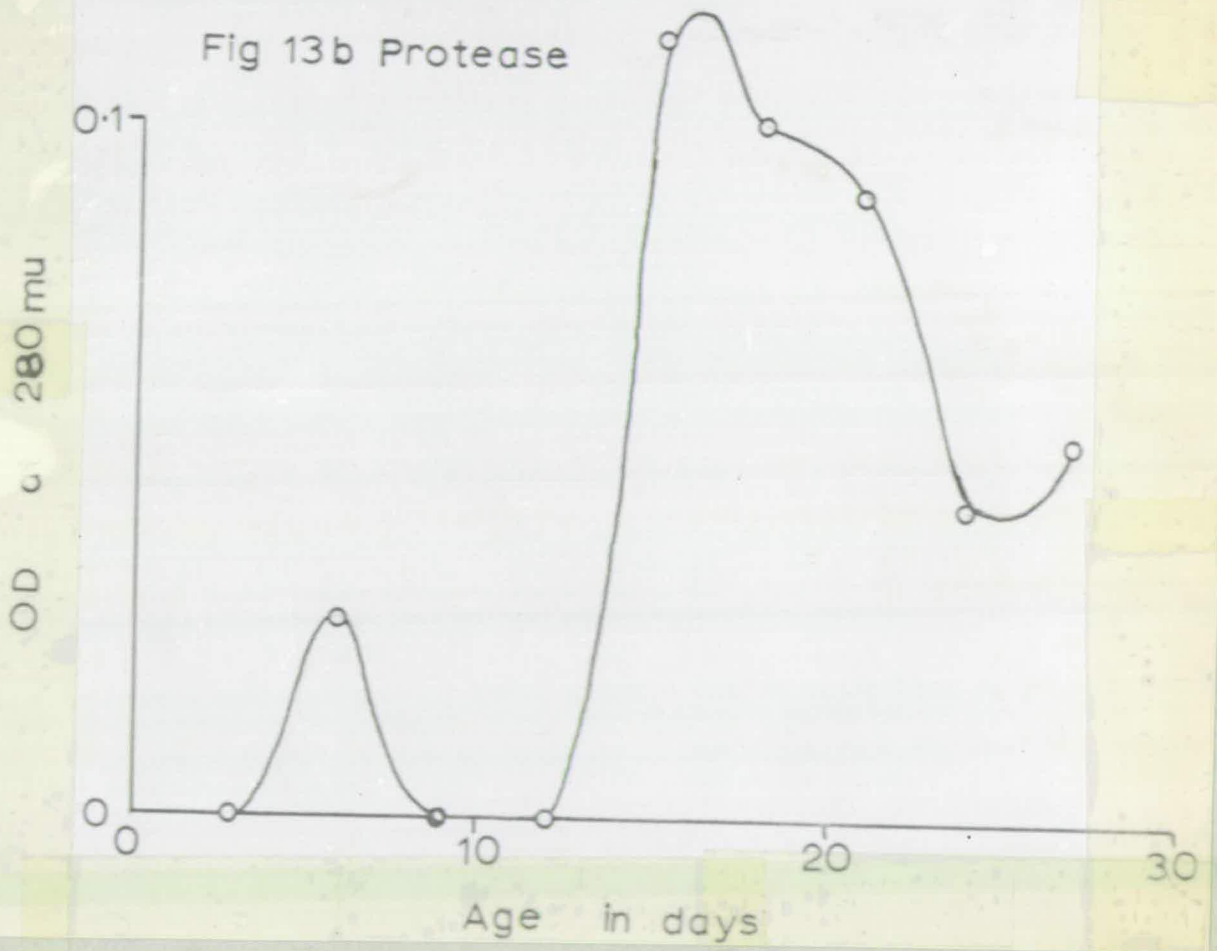


Fig 13b Protease



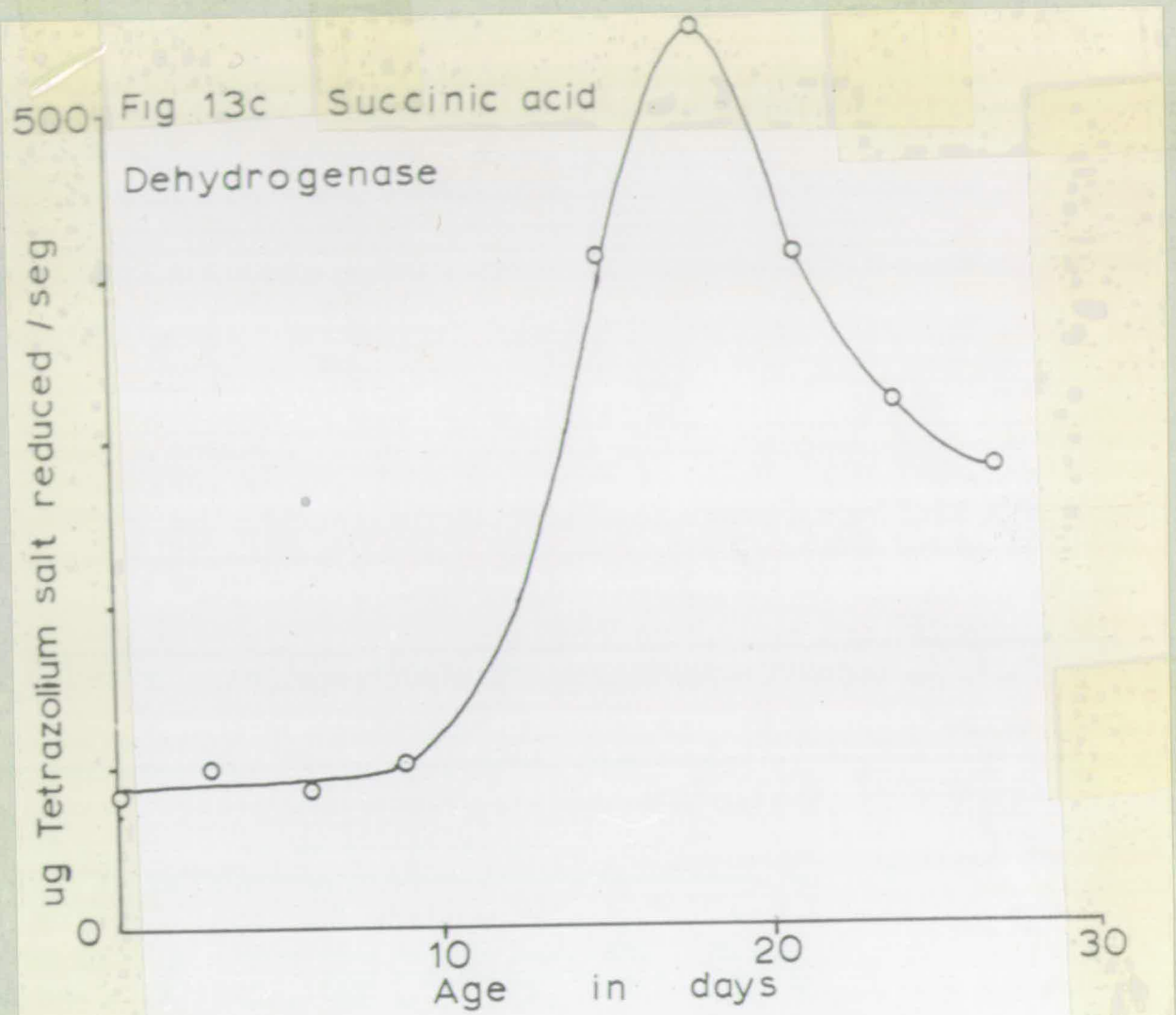


Fig 13c Succinic acid
Dehydrogenase
500
0
ug Tetrazolium salt reduced / seg
10
20
30
Age in days

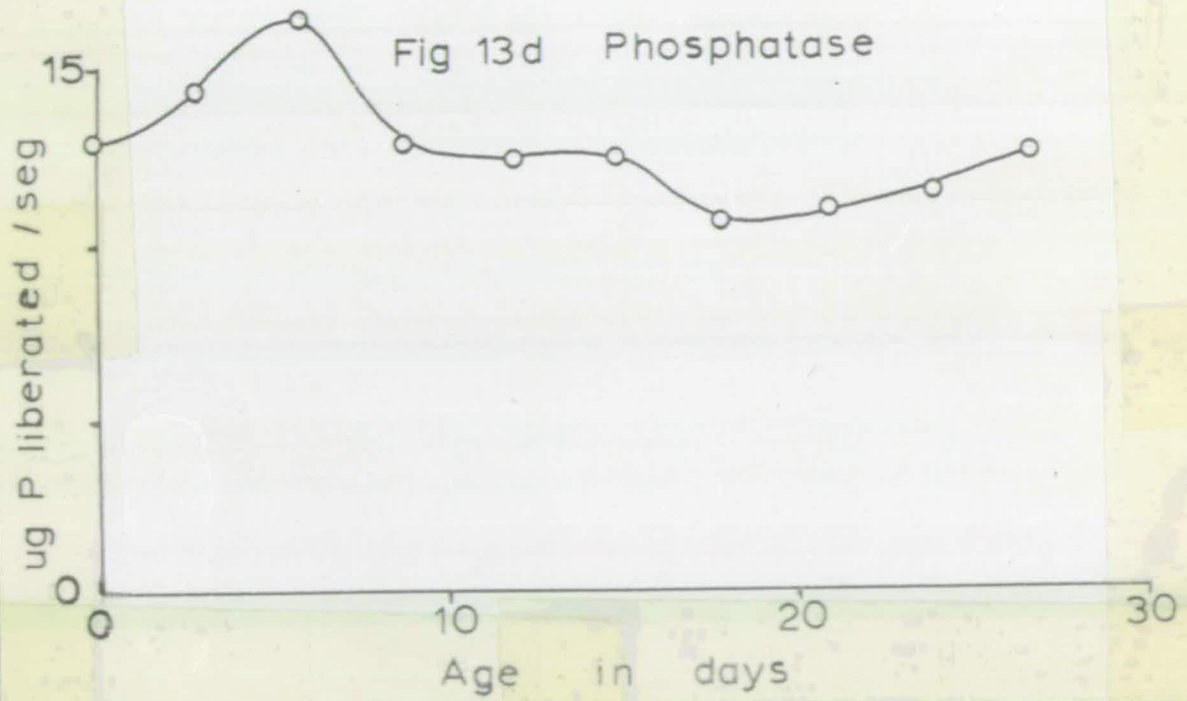


Fig 13d Phosphatase
15
0
ug P liberated / seg
0
10
20
30
Age in days

Fig 13e Polyphenol oxidase

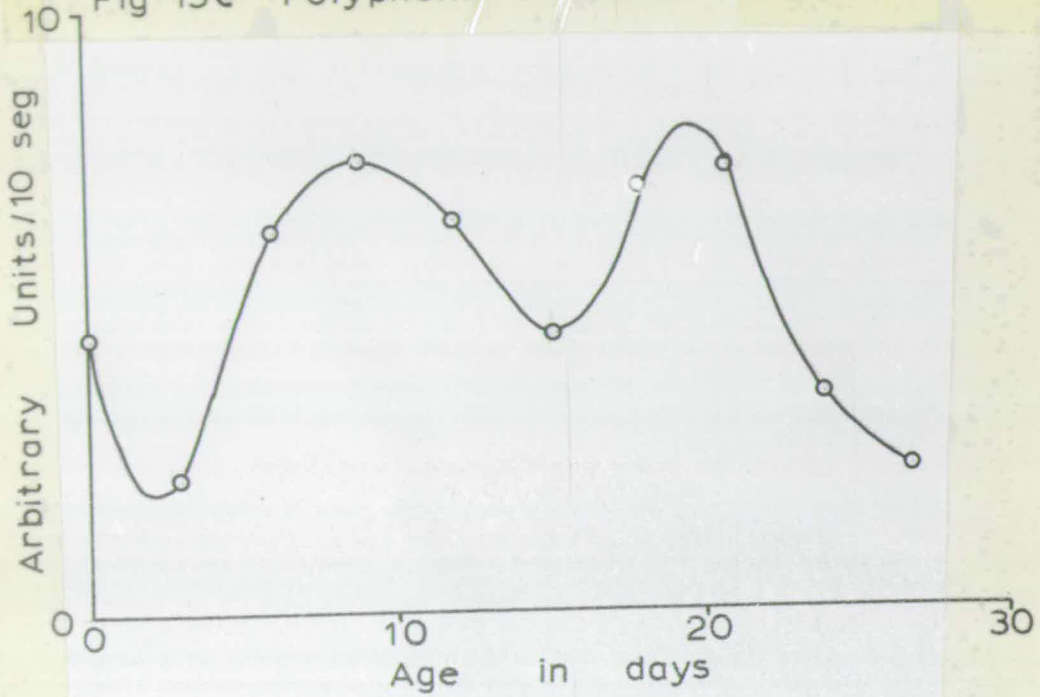
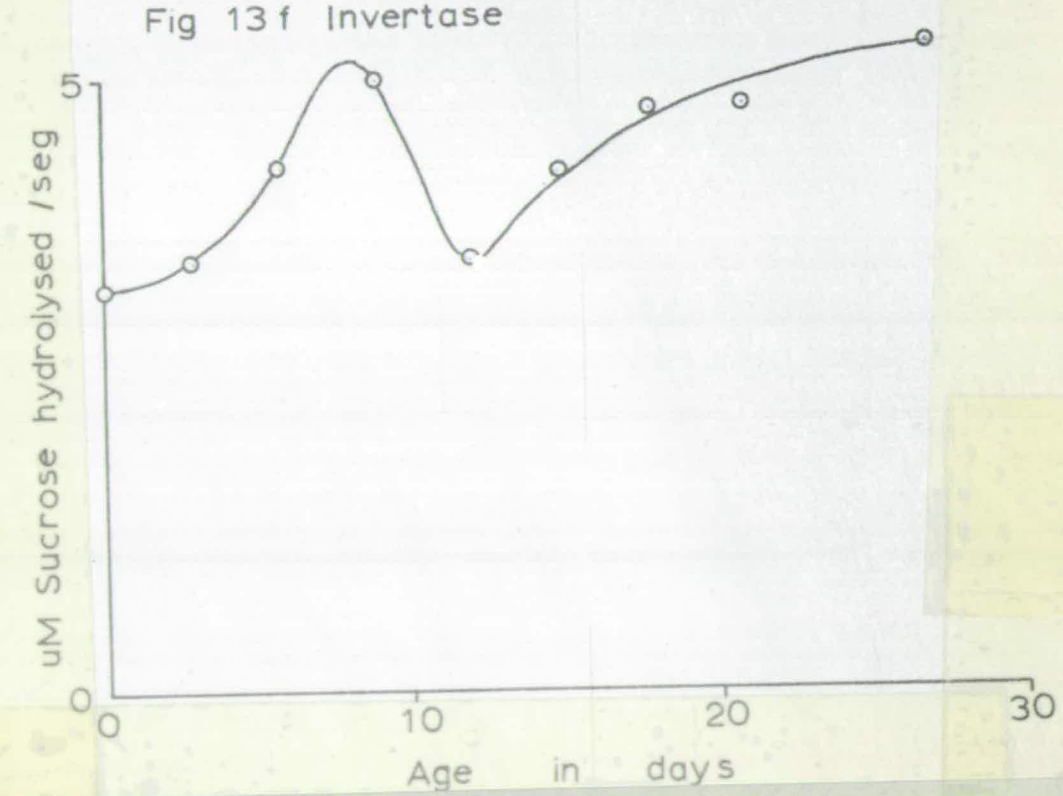


Fig 13 f Invertase



already been presented in several other parameters such as the amino acid nitrogen and the labile nitrogen. The succinic acid dehydrogenase was the only enzyme which did not show more than one peak of activity. This may be due to the method used for the enzyme assay rather than the lack of a high activity in the initial period of the experiment. The younger segments have a very high ability to reduce the tetrazolium salt used in the determination in the absence of the succinic acid substrate whereas the older segments have very little of this ability. The method ignores this non substrate reduction of the tetrazolium salt which gives the low figures for the succinic acid dehydrogenase activity in the first 12 days of the experiment. It is therefore possible that the results for this period are unrepresentative of the level of succinic acid dehydrogenase activity. The results given in the series of Figures 13a-f, show that the ageing of the basal 1 cm. segment is a process of considerable complexity involving change in the enzyme complement and periods of high metabolic activity even at quite late stages in the senescent decline of the tissue.

f) Respiration (oxygen uptake)

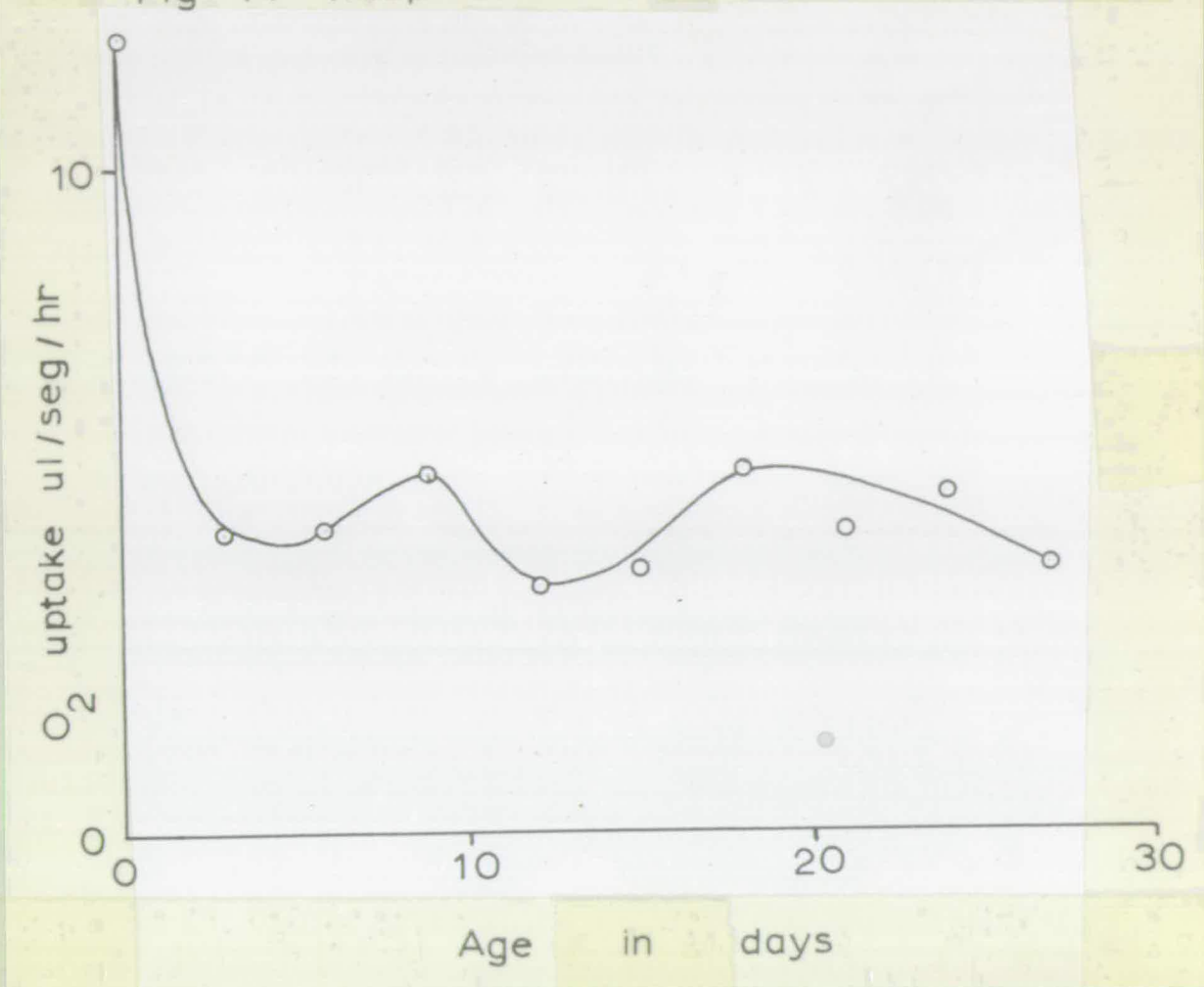
Several determinations of the rate of oxygen uptake were made all of which produced substantially the same results. The results given in Table 15 and Figure 14 are typical of those obtained.

Table 15 and Figure 14 show an initial rapid fall in the oxygen uptake as the meristem grows out of the basal segment during the 0-3 day period. Thereafter the results follow the same sort of two peak rise shown by the majority of the parameters analysed. Although the second rise in the oxygen uptake occurs at a time immediately prior to the death of a considerable region of the cortex it was not considered to be a 'climacteric' rise of the sort observed in some other systems. The evidence from the other results points to its being associated with a

Table 15 Oxygen uptake per 1 cm. basal segment during culture

Age in days	O_2 uptake in ul/seg
0	11.9
3	4.5
6	4.6
9	5.4
12	3.6
15	4.0
18	5.4
21	4.5
24	5.1
27	4.0
30	-

Fig 14 Respiration



period of increased biosynthetic activity in a region of tissue which dies from other causes a short time afterwards. This is discussed at length later in the thesis.

g) DNA content

The relevant results can be presented under four headings; 1) The DNA content/segment determined by a chemical technique. 2) The DNA content/nucleus as determined by microdensitometry. This technique gave the average DNA content/nucleus which was not the same thing as the DNA content/segment but which was sufficiently close to it to allow a useful qualitative comparison to be made. This technique also allowed a comparison of the DNA content in various regions of the root to be made. 3) Counts of the numbers of nuclei in various regions of the cortex. 4) Labelling of nuclei in the root with tritiated thymidine which fixed the times and positions at which the synthesis of DNA took place.

The results of 1) showed the pattern of change in the DNA content of the segment. The results of 2), 3) and 4) provided results which could be interpreted in connection with the stepwise necrosis of the cortex shown in section 2 c and the periods of synthesis shown in part 4 of this section.

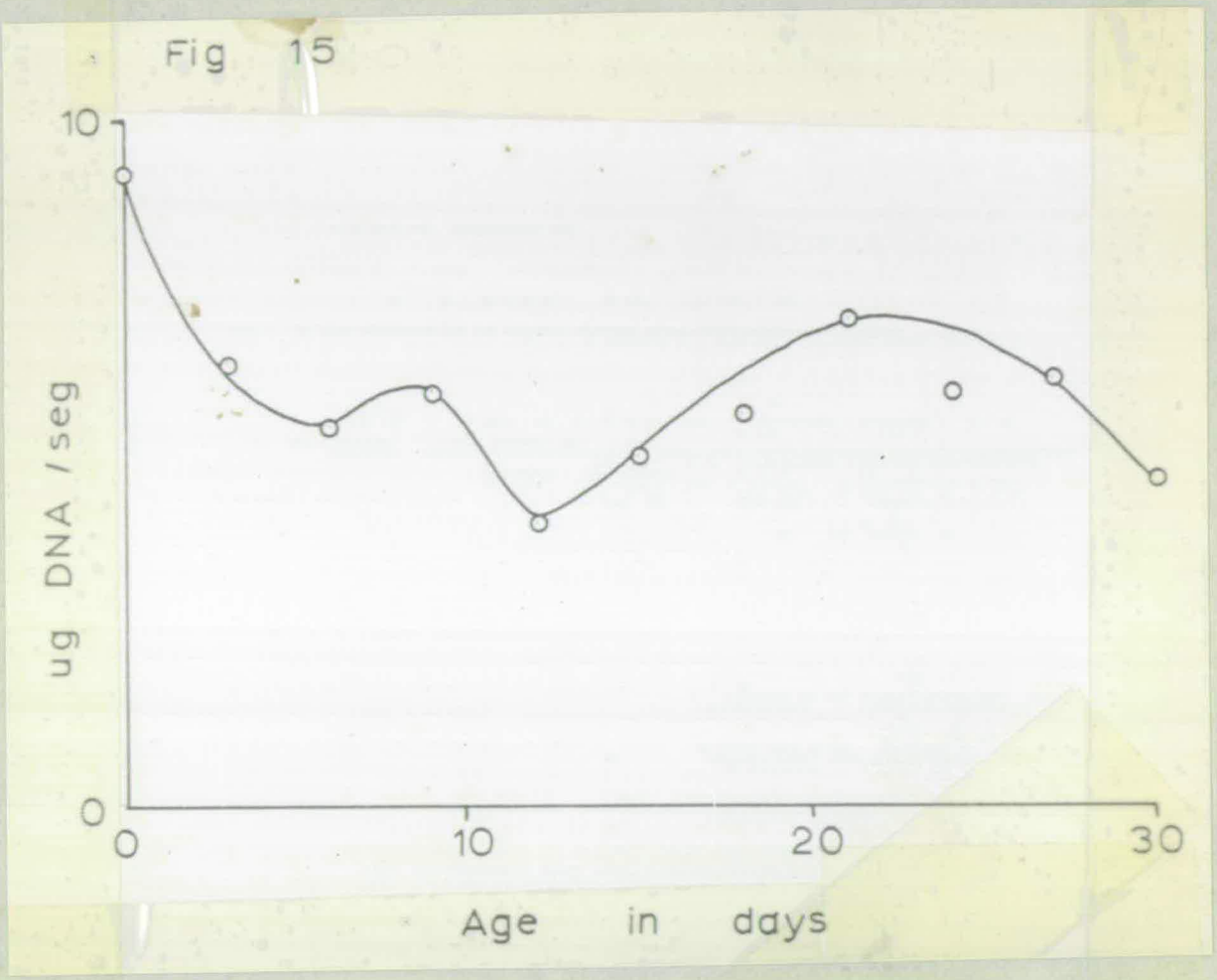
1) DNA content/segment by the Dische method

The results presented in Table 15 are the average results of several experiments and are presented graphically in Figure 15.

The striking point about the results for the DNA content per segment given in Figure 15 is their resemblance in form to the results already given for the other parameters given in the preceding parts of this section. This resemblance is especially marked for the oxygen uptake results given in Figure 14 and is the principle reason for considering the second peak of the oxygen uptake as the result of increased synthetic

Table 15 DNA content per segment of 1 cm. basal segment during culture

Age in days	DNA content µg/seg
0	9.2
3	6.4
6	5.6
9	6.1
12	4.2
15	5.1
18	5.7
21	7.1
24	5.9
27	6.2
30	4.7



Changes in the DNA content of the basal segment during culture.

activity rather than a 'climacteric' rise. The steep fall in the DNA content per segment in the 0-3 day period is no doubt for precisely the same reason as the decline over the same period in the oxygen uptake namely the loss of the meristematic cells from the basal 1 cm. segment.

2) DNA content/nucleus by Feulgen microdensitometry

Several sets of results are presented in Table 16 all of them derived from the same experimental batch of material.

All these results are the averages of the results from transverse sections cut from three different root segments. The results in the first three columns of results in Table 16 are given as graphs in Figure 16a and the last two are presented graphically in Figure 16b.

The results given in Table 16 and Figures 16a-b confirm the results given by a different method in Table 15 and Figure 15. Besides confirming these previous results they show the contributions made to the DNA content by the various regions of the root and the regions of the root where DNA was being lost from the nuclei presumably as a result of cell death and autolysis. The results also indicate the times at which DNA synthesis takes place. The deductions made from these results were;

1) No DNA synthesis occurs in the outer cortex at any time and the loss of DNA from the nuclei in this region proceeds steadily from the third day.

2) The middle and inner cortex nuclei make a relatively greater contribution to the DNA synthesis during the second period of synthesis than they do to the first.

3) Loss of DNA occurs from the outer cortex nuclei only between the 3rd and 21st days and from the rest of the cortex nuclei only after the 21st day.

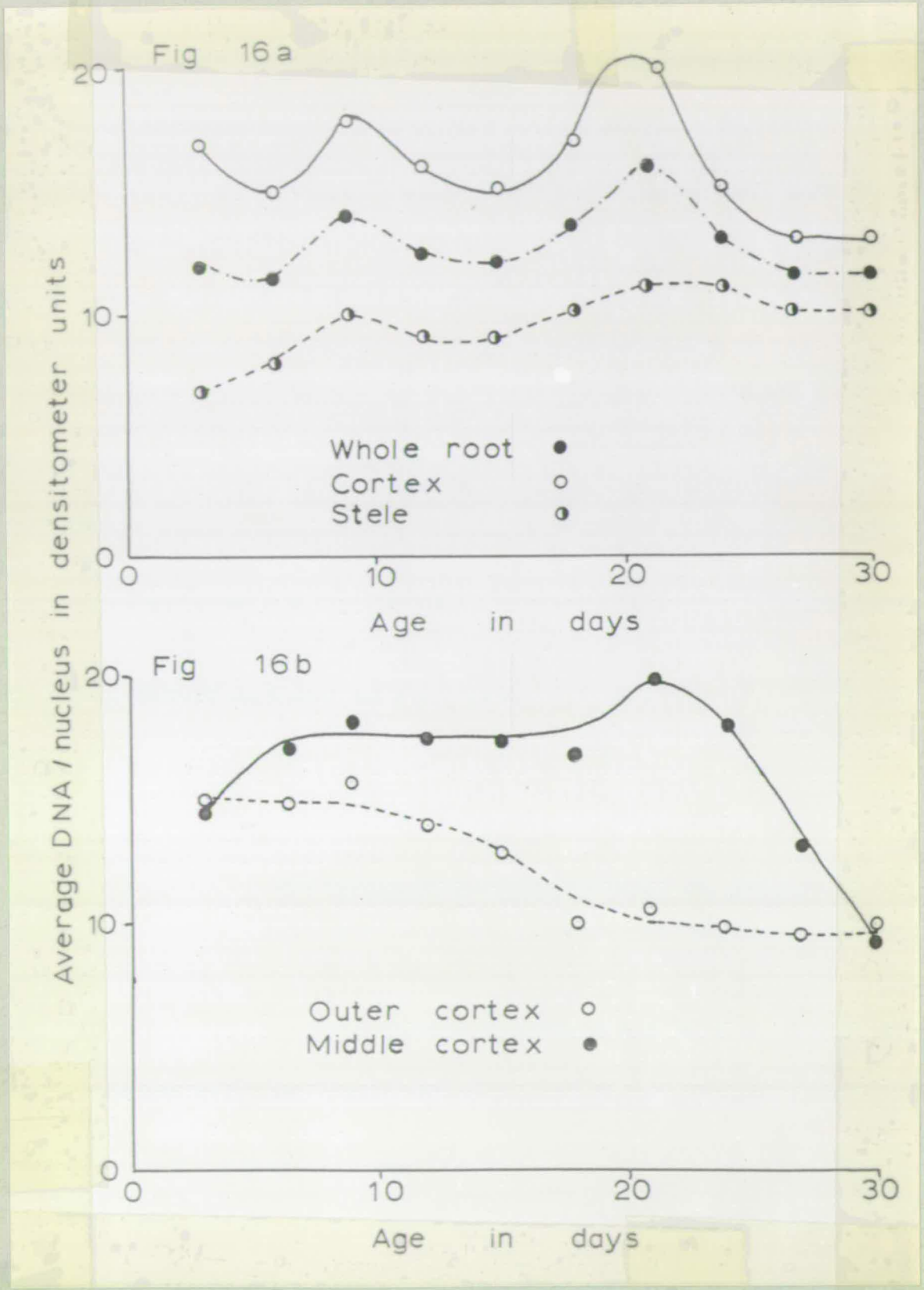
4) After 9 days the gains and losses of DNA in the stele nuclei are relatively unimportant.

3) Counts of the number of nuclei in root T.S.

A series of serial T.S.'s from several root segments

Table 16 Average density of Feulgen staining per nucleus in various regions of T.S.'s cut from the centre of the basal 1 cm. segment during ageing

Age in days	Average density /nucleus whole T.S.	Average density /nucleus cortex	Average density /nucleus stele	Average density /nucleus outer cortex	Average density /nucleus middle cortex
0	-	-	-	-	-
3	12.0	17.0	7.0	15.2	14.5
6	11.5	15.0	8.0	15.0	17.2
9	14.0	18.0	10.0	15.7	18.2
12.	12.0	16.0	9.0	14.0	17.6
15	12.0	15.0	9.0	13.0	17.5
18	13.5	17.0	10.0	10.0	17.0
21	16.0	20.0	11.0	10.7	20.0
24	13.0	15.0	11.0	10.0	18.2
27	11.5	13.0	10.0	9.7	13.2
30	11.5	13.0	10.0	9.3	9.9

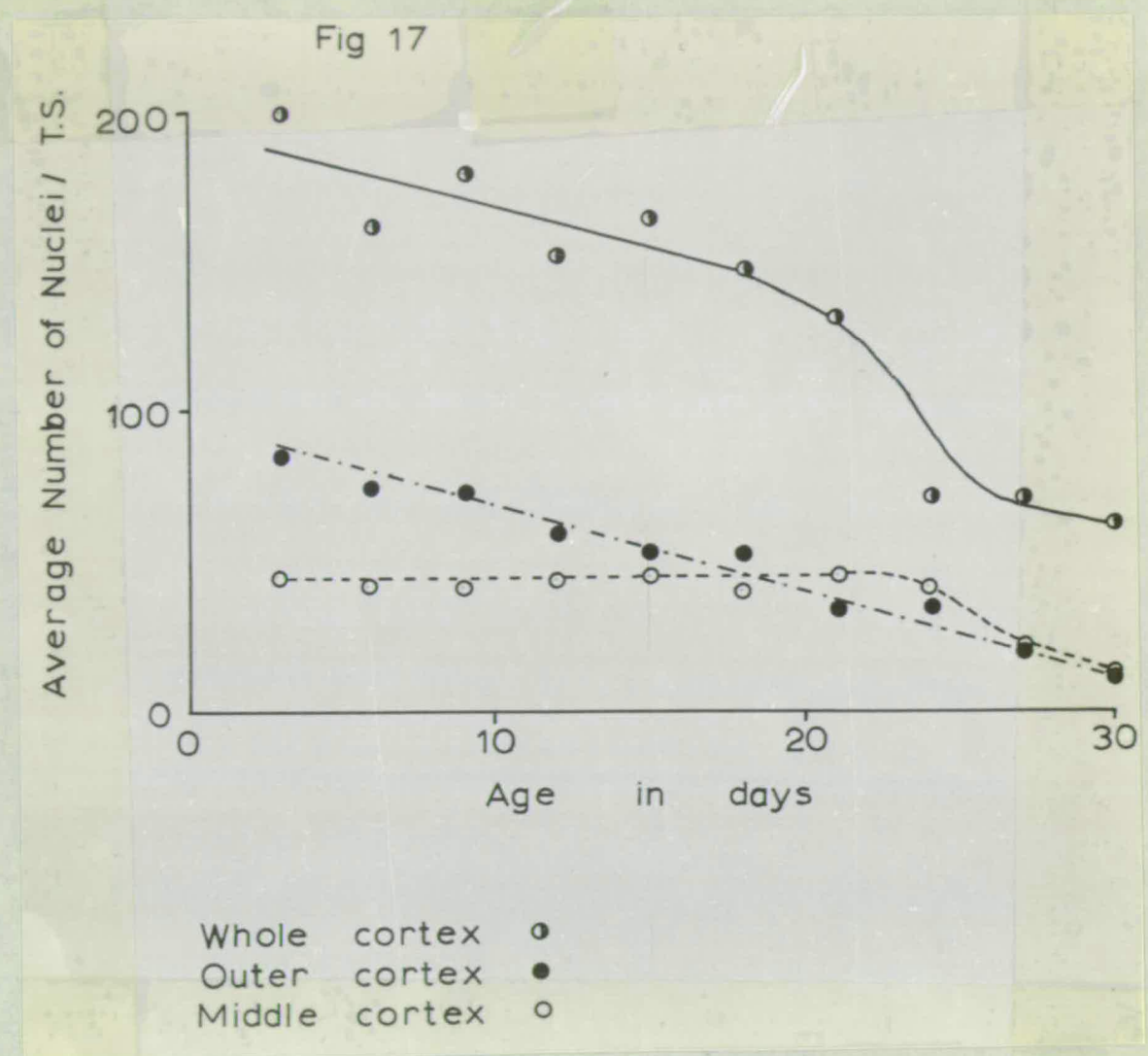


Feulgen densitometric estimation of the DNA content of nuclei in the basal segment during culture.

Table 17 Average number of Feulgen staining nuclei per T.S. of 1 cm. basal segment during culture

Age in days	No of Nuclei whole cortex/ T.S.	No of Nuclei outer cortex/ T.S.	No of Nuclei middle cortex/ T.S.
3	199	85	45
6	162	76	41
9	179	72	41
12	151	58	44
15	163	52	45
18	147	52	39
21	131	36	44
24	71	34	42
27	72	20	18
30	63	11	12

Fig 17



Counts of the numbers of nuclei in TS cut from the basal segment during culture.

were prepared and the number of nuclei in each were counted and the average value for the series was found. These average values are presented in Table 17 and Figure 17.

The counts of the nuclei in the various regions of the root cortex given in Table 17 and Figure 17 correlate well with the results of the Feulgen microdensitometry and the phosphatase histochemistry in indicating the areas of the cortex of the 1 cm. basal segment that are dead or dying at different times during the experiment providing the assumption is made that the loss of the nucleus from a cell is sufficient cause to regard the cell as dead or dying.

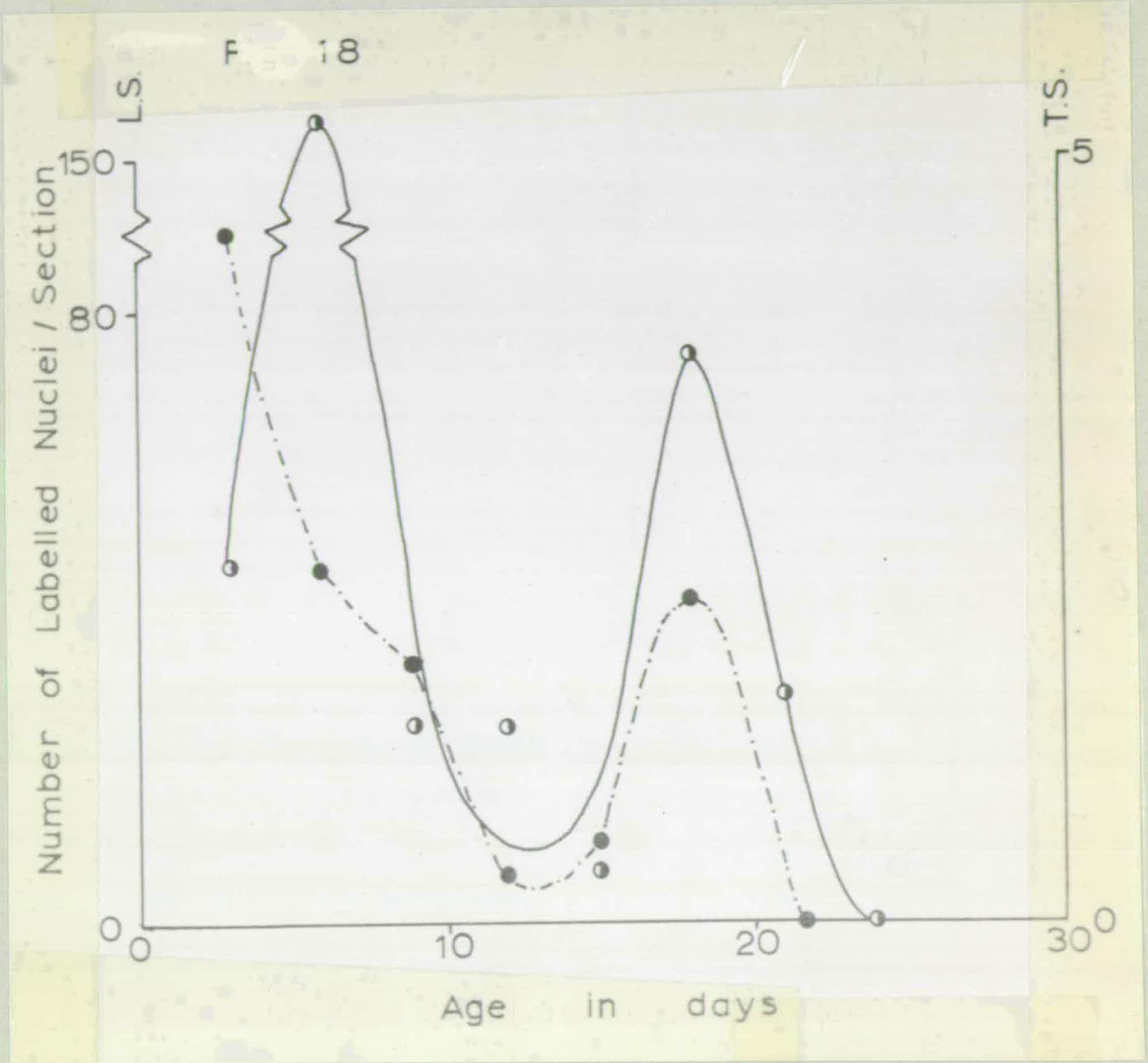
4) Labelling with tritiated thymidine

The number of clearly labelled nuclei per section were counted for T.S.'s and L.S.'s. The results given in Table 18 and Figure 18 are the averages of counts of several sections.

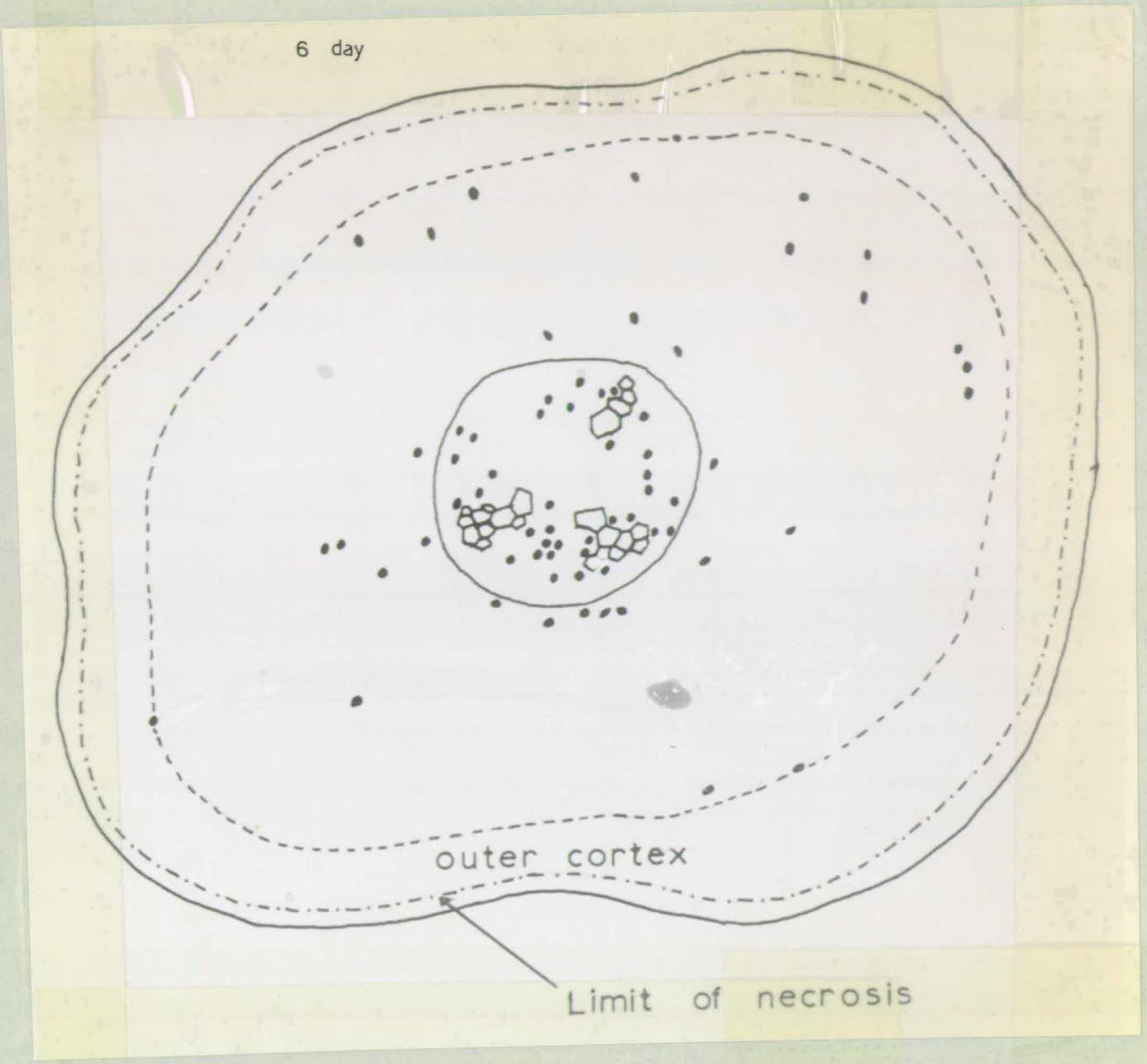
The results of counts of thymidine labelled nuclei and their position in a T.S. of the basal 1 cm. segment given in Table 18, Figure 18 and Diagrams 3a-b fix the time and position of the two periods of DNA synthesis. These confirm the deductions made from the previous results (see section 3g, section 2). The counts of labelled nuclei in T.S. and L.S. show that there are two periods of DNA synthesis reaching maxima at 6 and 18 days. The apparent discrepancy between the T.S. and L.S. values at 6 days given in Figure 18 can be resolved if it is understood that the L.S. samples the whole length of the basal 1 cm. segment, while the T.S. can sample only a small and possibly unrepresentative section of the root even if a number of serial sections are taken. This being so the occasional high or low result is much more likely to turn up in the T.S. counts than in the L.S. counts. The high value of the result at day 3 in the T.S. graph is probably due to this especially as the basal segment is very heterogeneous as regards the distribution of the various metabolically active tissues at this time.

Table 18 Counts of the number of cortical nuclei showing labelling with H^3 thymidine in T.S. and L.S. from the basal 1 cm. segment during culture

Age in days	Number of labelled nuclei/section	
	L.S.	T.S.
3	48	4.5
6	155	2.3
9	26	1.7
12	26	0.3
15	7	0.5
18	74	2.1
21	30	0.0
24	0	0.0
27	0	0.0
30	0	0.0

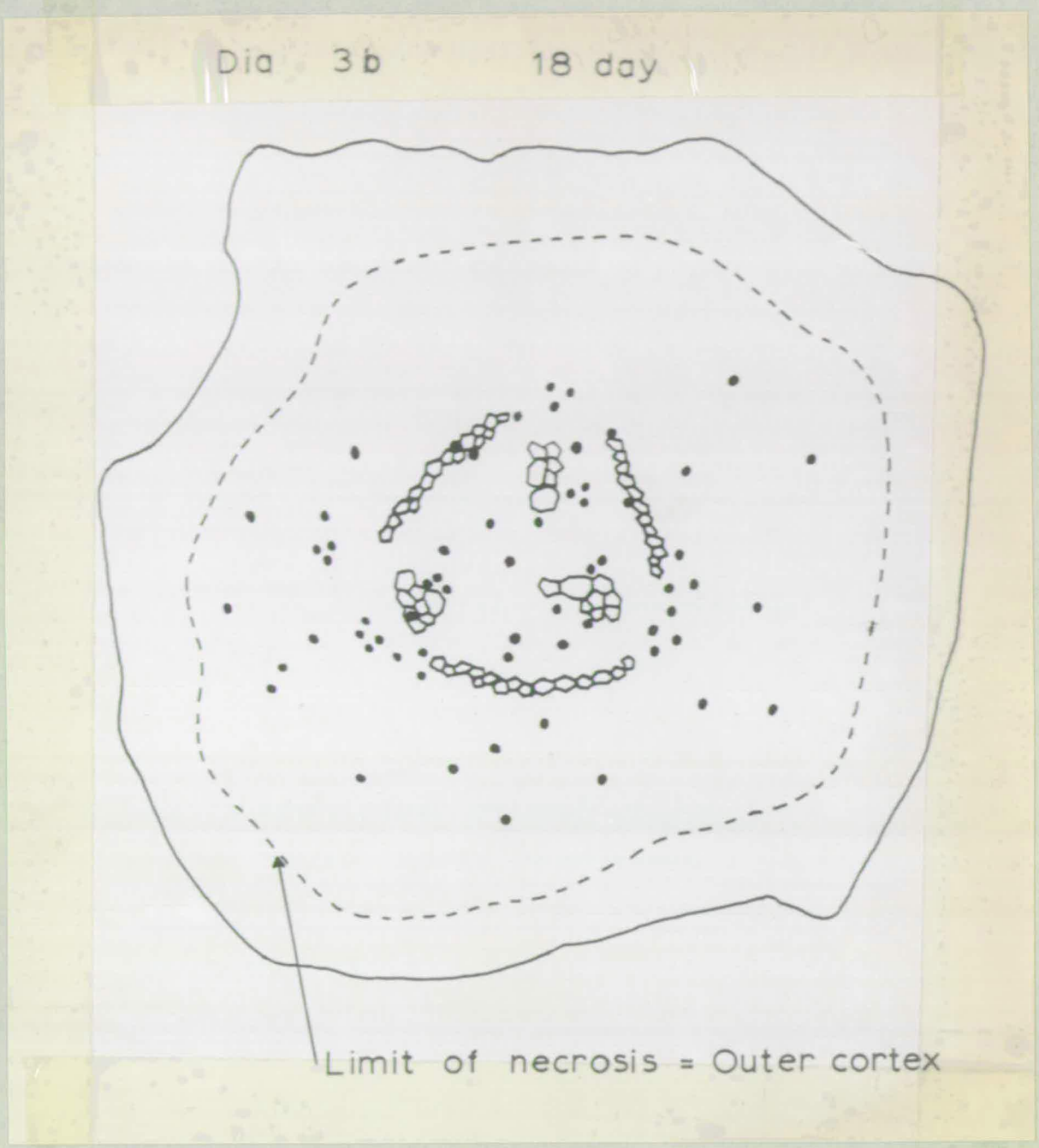


Times of DNA synthesis in the basal segment during culture as shown by the number of nuclei which have incorporated tritiated thymidine.



Dia 3a

Dia 3a and 3b show the distribution of labelled nuclei in TS for the basal segment at 6 and 18 days


Dia 3b 18 day
Limit of necrosis = Outer cortex

The distribution of the labelled nuclei at the two periods of maximum DNA synthesis is given in Diagram 3a-b. This shows the position of labelled nuclei in a series of thirty serial T.S.'s of a root segment when projected onto an outline of the root T.S. These diagrams show:-

1) There is no synthesis at any time in the outer cortex.

2) There is a difference in the percentages of labelled nuclei in the stele and cortex at the two times of synthesis. The figures are;

	% of labelled nuclei in stele	% of labelled nuclei in cortex
6 day	52	48
18 day	43	57

These DNA results complete the results section which deals specifically with the changes in the basal 1 cm. segment during the ageing process.

4) <u>The effect of excision and isolated culture on the pattern of ageing in the basal segment</u>

Two experiments were carried out and the ageing changes in the isolated segments were followed. Both experiments gave substantially the same results. The results from both experiments are given in Table 19. The results given for the DNA, Total nitrogen and labile nitrogen are the average results from the two experiments.

The results of this analysis of the basal segment in isolated culture can be divided into three groups.

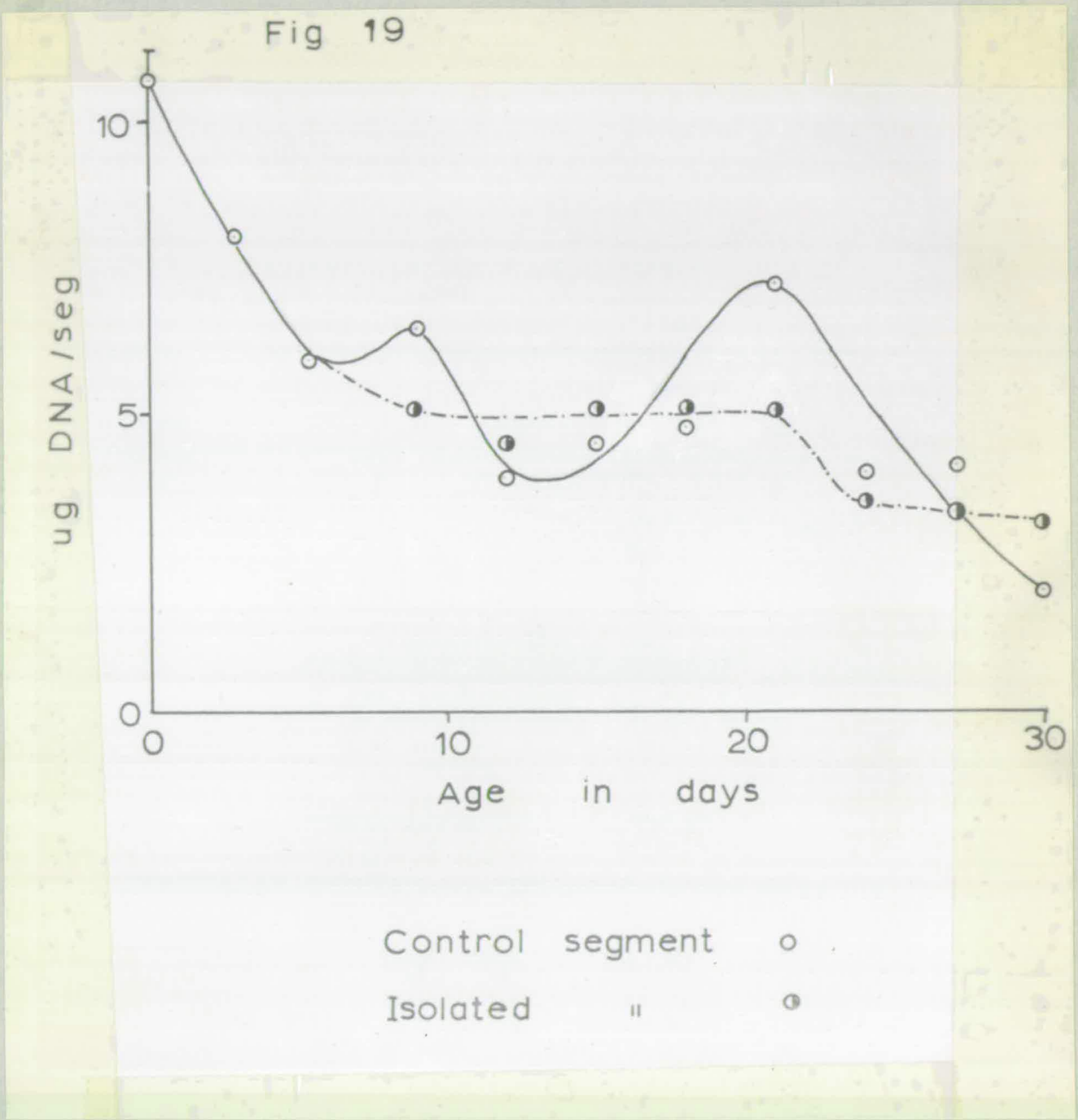
1) The parameters which show a definite change as compared with the control results. These are DNA content, Ethanol soluble, Nitrogen and Labile nitrogen.

2) The parameters which show slight changes from the control values. These are Protein nitrogen, Dry and Fresh Weights and Succinic acid dehydrogenase.

Table 19 Changes in the basal 1 cm. of a cultured pea root. (a) Intact control and (b) after excision at 6 days and returning to culture medium during 30 days.

	DNA	Total N	Eth Sol N	Protein N	Labile N	Cell No	Dry Wt	Fresh Wt	Phos	Invtase	SDH	Medium: Sucrose	Medium: pH
Control													
0	10.8	62.2	-	26.9	6.9	18.75	0.0	16.0	13.0	3.3	80	4.0	4.3
3	8.0	54.2	41.8	22.6	6.2	11.10	1.0	16.7	14.5	3.5	100	2.8	5.9
6	6.0	56.4	36.9	18.6	5.9	8.72	2.1	17.1	16.5	4.3	80	1.3	6.4
9	6.4	53.3	47.4	15.1	6.7	8.62	1.8	17.5	13.0	5.0	100	0.3	6.4
12	3.9	66.8	41.3	17.1	6.3	8.28	2.4	17.7	12.5	3.5	-	0.0	6.4
15	4.5	86.8	37.1	20.5	8.2	7.66	3.1	17.8	12.5	4.3	410	-	6.4
18	4.8	74.7	38.0	19.6	6.9	-	4.0	18.0	10.6	4.7	550	-	6.1
21	7.2	86.3	29.2	19.0	10.9	-	3.2	18.0	11.0	4.7	410	-	6.2
24	3.9	59.6	32.3	26.6	5.6	-	2.9	18.1	11.5	-	320	-	6.2
27	4.0	65.8	36.2	24.5	8.4	8.94	3.9	18.1	12.5	5.3	280	-	6.4
30	2.0	52.6	-	-	4.9	-	2.2	-	-	-	-	-	-
Isolated segment													
9	5.1	44.7	42.1	23.6	6.2	8.24	2.3	18.4	11.5	4.7	480	1.8	6.4
12	4.5	65.1	55.8	21.6	11.3	8.60	2.7	19.0	13.5	4.3	410	-	6.3
15	5.1	81.1	65.4	15.5	11.9	8.94	2.7	19.3	12.5	4.3	-	0.4	5.5
18	5.1	91.0	60.8	27.6	8.2	-	5.4	19.2	13.0	5.0	220	0.3	6.1
21	5.0	78.0	38.8	30.0	8.3	7.84	3.5	19.7	11.0	-	-	0.1	5.6
24	3.4	80.9	68.5	30.0	6.4	-	2.5	19.9	12.6	4.3	270	0.0	5.4
27	3.3	84.3	72.0	21.9	-	-	2.1	19.9	11.5	4.7	300	-	5.1
30	3.0	66.6	39.7	-	5.9	-	2.7	-	-	-	-	-	-

Fig 19



Changes in the DNA content of basal 1cm segments grown in isolated culture

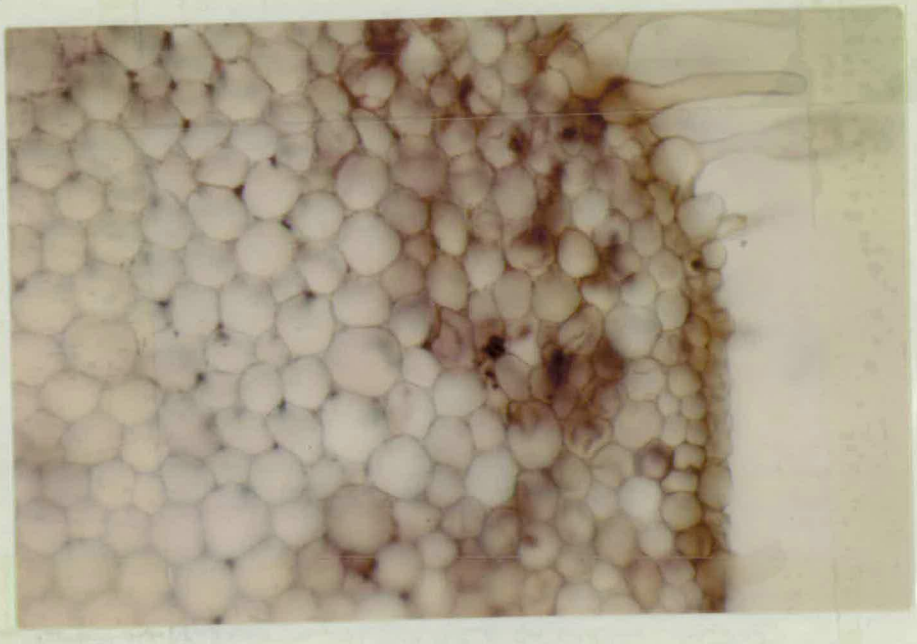
3) The parameters which show no significant change from the control values. These are the Cell number, Phosphatase and Invertase.

The changes in the medium are relatively slight. There is no change in the rate of water loss from the culture dish. The rate of sucrose hydrolysis is lower so the sucrose concentration falls more slowly. The pH falls more sharply than in the control in the later stages of the experiment. These medium changes were those expected in view of the smaller amount of root tissue in the dish.

The change in the DNA content of the isolated segment is presented graphically in Figure 19. This shows that two effects occur in the segment after amputation. Firstly all synthesis of DNA was stopped by removing the segment from the root. This is shown by the reduction in size of the 9 day peak and the complete suppression of the 21 day peak. Secondly the fall of DNA content between the 9th and the 15th day was not as large as in the control. This implies that the necrosis of the outer cortex of the root had been slowed down or delayed by the excision of the segment from the root. Confirmation that this was so was obtained from a Phosphatase histochemical examination of an 18 day old isolated segment root. The Plate 9 shows the appearance of a T.S. cut from an isolated 18 day old root segment when stained for Phosphatase activity. The appearance of this section is typical of the pattern of necrosis shown by a 'normal' root segment at about 12 days. This can be seen by comparing Plate 19 with Plate 5 (9 day old root) and Plate 6 (18 day old root). Plate 19 is much closer to the Plate 5 (9 day old root) in appearance than it is to the 18 day root shown in Plate 6.

The large increase in the ethanol soluble and labile nitrogen can be discussed together since they both represent the changes in the

Plate 11



This shows the appearance of a T.S. of an 18 day old root grown since the 6th day of culture as an isolated segment, after staining to show acid phosphatase activity.

size of the amino acid pool. These two results therefore indicate that one of the major changes produced in the basal segment by the amputation of the rest of the root was a large and immediate increase in the free amino acid in the basal segment. The simplest interpretation of this result is to assume that this represents an accumulation of amino acids which in the intact root would have been translocated to the younger parts of the root and used for protein synthesis. The accumulation of amino acid is then explained because of the lack of the sink normally present in the form of the remainder of the root allows the accumulation of amino acids at the site of synthesis. The presence of a large quantity of amino acids also explains the rather higher protein nitrogen content of the isolated basal segments if it is assumed that the limiting factor in protein synthesis in the basal segment is the availability of amino acids.

The higher protein content itself is sufficient to explain the delay in the ageing process which is a feature of the isolated basal segment system since it is known that the maintenance of a high level of protein per cell delays the onset of senescent decline in many of the systems used for the study of senescence.

The slightly higher fresh weight of the isolated basal segments may simply be a reflection of the higher number of living cells in the segment as compared with the control segment which is due to the delay of the ageing process by the treatment given. No meaningful pattern in the dry weight results is obvious except that the variation in this parameter in the isolated segments seems higher than that shown for the control segments.

5) <u>The effect of transferring the root to fresh medium after 15 days of culture</u>

The results of the one experiment carried out are given in Table 20.

These results show that apart from a slight increase in the Fresh weight of the root the effect of the transfer on the root was very small most of the parameters show little or no difference from the changes occurring in the basal segment of a normally cultured root.

6) <u>The effect of added sucrose on the twelth day of culture on the growth of the root and the ageing of the basal 1 cm. segment</u>

The results from the experiment carried out are given in Table 21.

These results seem to indicate that the added sucrose has delayed the onset of the later stages of senescence possibly by increasing the period of time that the root can grow in the medium.

Table 20 Changes in the Nitrogen and DNA fractions produced in the basal 1 cm. segment by transfer of whole roots to fresh medium at 15 days.

Age in Days	DNA µg/seg	Total Nitrogen µg/seg	Labile Nitrogen µg/seg	Ethanol sol N µg/seg	Fresh weight of whole root in mg.
3	4.2	69.6	4.5	33.2	17.7
6	5.5	63.9	10.4	35.8	23.8
9	5.5	82.1	13.6	32.6	74.5
12	1.7	88.0	8.9	40.4	78.2
15	2.3	50.5	8.3	41.6	98.5
18	9.0	53.3	13.4	39.8	100.0
21	9.0	46.9	5.1	30.4	116.0
24	7.6	60.0	4.4	15.0	122.1
27	6.8	88.2	3.8	15.8	136.4
30	1.4	77.6	3.6	27.6	154.2
Transferred root segments					
18	7.0	71.5	12.6	33.4	107.0
21	7.3	75.6	6.3	23.2	122.1
24	9.8	78.9	7.4	25.2	135.7
27	5.3	69.0	-	26.8	145.6
30	8.3	68.5	2.9	17.4	176.5

Table 21 Changes in the DNA, Fresh Weight, Total Nitrogen and Labile Nitrogen in the 1 cm. basal segment of sucrose supplemented roots as compared with a set of control roots grown in normal medium.

Age in days	Fresh Weight whole root in mg	DNA µg/seg	Total Nitrogen µg/seg	Labile Nitrogen µg/seg
Control				
3	-	3.1	66.1	4.9
6	-	1.8	98.7	10.8
9	-	3.9	119.0	12.5
12	-	4.9	159.5	8.3
15	67.0	2.5	153.6	8.2
18	76.5	2.2	154.6	9.4
21	79.9	2.1	145.7	8.2
24	95.3	5.6	101.5	5.2
27	104.5	4.2	106.9	5.3
30	-	-	-	-
Sucrose supplemented roots				
15	61.5	3.1	59.0	8.5
18	65.7	3.5	-	7.5
21	90.6	3.4	115.2	6.2
24	125.5	3.4	94.2	9.8
27	144.4	7.0	147.1	11.7

7) Division of the roots into 'age' groups on the basis of a growth parameter (whole root Fresh weight) rather than the length of time in culture

The purpose of this experiment was to determine whether physiological age, as measured by the amount of growth in culture, was more relevant than the concept of age as time in culture to the elucidation of the change taking place in the basal segment of the root. The results of this experiment are given in Table 22a-b.

The protein nitrogen and protease activity are presented graphically in Figure 20.

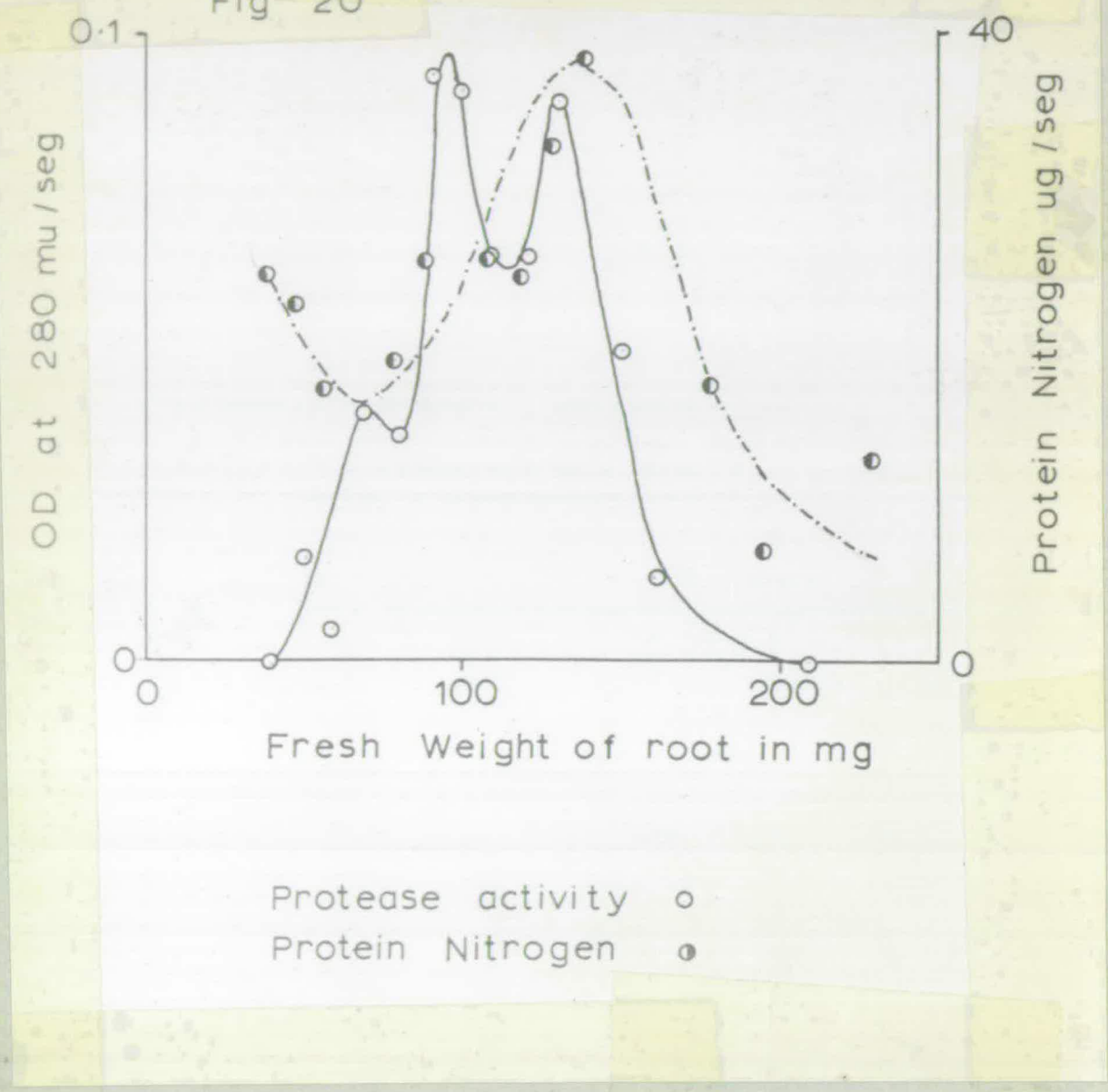
Table 22a

Fresh Weight of whole root in mg	DNA in µg/seg	Ethanol soluble AN µg/seg	Protein N µg/seg
30 - 40	3.5	5.1	24.6
40 - 50	1.0	4.7	22.6
50 - 60	3.5	8.7	17.3
60 - 70	-	13.4	-
70 - 80	2.0	5.0	18.8
80 - 90	5.5	4.4	25.2
90 - 100	4.5	3.9	-
100 - 110	2.7	4.0	25.8
110 - 120	1.5	3.0	24.6
120 - 130	2.5	3.5	32.4
130 - 140	7.5	6.9	38.1
150 - 160	7.6	6.5	-
170 - 180	10.0	6.4	17.3
180 - 190	4.0	10.6	6.8
210 - 220	-	5.3	-
220 - 230	-	7.5	12.6

Table 22b

Fresh Weight whole root in mg	Protease OD at 280 mu per segment
30 - 40	0.000
40 - 50	0.017
50 - 60	0.005
60 - 70	0.039
70 - 80	0.036
80 - 90	0.093
90 - 100	0.091
100 - 110	0.065
110 - 120	0.065
120 - 130	0.088
140 - 150	0.050
150 - 160	0.013
200 - 210	0.001

Fig 20



Changes in the protein nitrogen content and the protease activity of the 1cm basal segment as the root increases in size and fresh weight.

DISCUSSION

1) Suitability of the experimental system

The reasons for the adoption of the experimental system finally used were given in the introduction and the results on which this reasoning was based are given in the Preliminary section of the results. This discussion examines the suitability of the system for the examination of the ageing processes in the root. An ideal experimental system for the study of cellular ageing would have the following characteristics:-

1) All the cells in the system should be of the same type.
2) The ageing process should occur in all the cells of the system at the same time.
3) The environment of every cell in the system should be constant. There should be no form of external stimulus applied to the system during the ageing of the system.
4) No cell division, expansion, or differentiation must occur in the cells of the system.
5) The system should show senescent changes in the cells of the system culminating in the death of the cells. This change should be independent of any stimulus from the environment of the cells in the system.

Since the aim of this investigation was to examine the ageing changes at a cellular level rather than those at a tissue or whole organ/whole plant level it is reasonable to use these requirements to judge the usefulness of the basal segment of isolated roots grown in culture as a system for the investigation of cellular ageing.

The basal segment is far from being a homogeneous tissue containing every type of cell except meristematic cells. The bulk of the root is composed of parachymatous cells which are present in greater

numbers than any other cell type. The basal segment thus possesses a degree of homogeneity in the sense that one type of cell predominates. Since the majority of the senescent changes in the basal segment only involve the root cortex, this results in considerably more homogeneity as regards the type of cell involved in the ageing changes observed than would be expected from the anatomical heterogeneity of the tissue.

There was no cell division or cell expansion in the basal segment after the first week of culture and in this period the only developmental process was root hair expansion. The ageing processes in the cortex of the basal segment are not synchronous or homogeneous. However, histochemical techniques employed in the analysis of the segment can define areas of the root within which the cellular ageing processes are at least partly synchronous and homogeneous. This means that the changes shown by an analysis of the whole segment can be correlated with the necrosis and hence the senescent change of a particular group of cells within the basal segment. The results when analysed in this way give reasonably good indications of the process of senescent change in the parenchymatous cells of the root. These departures from the ideal experimental system are not really serious because by taking suitable precautions, and using special techniques they can be allowed for in the interpretation of the results.

The greatest difficulty in the analysis of changes occurring in the basal segment is that it cannot be considered as existing in a uniform environment isolated from external stimuli. The system in this respect is extremely complicated and although some of the difficulties of interpretation can be removed by an understanding of the factors involved, the acquisition of this understanding involves a consideration of the ageing of the root as a whole and its effect on the mature and ageing regions. Thus the major limitation of the basal segment system in the investigation of cellular ageing is the impossibility of considering the basal segment

in isolation from the rest of the root. The results show that the pattern of ageing as regards timing and degree of change are dependent on the growth of the whole root. Since the rate and extent of growth are dependent on the medium, medium changes can affect the timing and degree of the ageing changes.

The general pattern of change in the basal 1 cm segment is, in general, unaffected in its essentials by variations in growth rate and medium composition as long as the basal segment is connected to the growing root. Amputation and culture of the basal segment in isolation alters this basic pattern in some respects, notably by the suppression of DNA synthesis. This indicates that at least some aspects of change in the basal segment are a property of its association with the rest of the root and are not an intrinsic property of the cells making up the basal root segment.

This basal segment technique was possibly not ideal for the study of the changes occuring in the ageing of individual cells but is as good as, or better, than many of the systems previously investigated such as isolated leaves and fruits. Its main drawback which it has in common with most comparable higher plant systems is in some respects an advantage since it provides an excellent system for the study of the control of ageing in one part of the root by other parts of the root. The system proved capable of supplying results comparable with those from other systems in general use and confirmed many of the changes already shown to be characteristic of senescent cells.

2) Variability of the system

For a given characteristic any population of living organisms is liable to show a high degree of variation. The simplest way to produce reliable results from such a system is to carry out a large number of individual measurements and use a suitable statistical analysis to calculate the significance of the mean result obtained

from the system. The drawback inherent in this approach is that the larger the variation in the system, the greater is the number of individuals required for analysis in order that the result obtained has the required level of significance. For the experimental system used in this study of ageing the variation in its most fundamental characteristic, namely growth rate, increased with time. This meant that as the age of the system increased the number of roots required to reach a given level of significance in the results increased also. The large number of roots required to give a reasonable level of statistical significance for the results from the older parts of the system exceeded the number it was possible to provide with the facilities and time available.

A formal statistical treatment of the results was thus ruled out by the difficulty of setting up sufficiently large experiments. It was then decided to attempt to reduce the variability within experiments by a careful selection of the material used in them. Variation was kept to a minimum by careful grading of the seed and selecting only those of a given size and ensuring that conditions of germination, excision and culture were kept as uniform as possible. A further selection of material was made when the roots were harvested. This involved the establishment of a rate of growth in length for each experiment and the selection of the roots in each age group which were nearest the average length for that age. This inevitably meant a reduction in the amount of material available but it did ensure that the material used for analysis was as uniform as possible with regard to the morphology of the root. The replicates of most of the parameters measured on root tissue selected in this way did not as a rule differ by more than 10%.

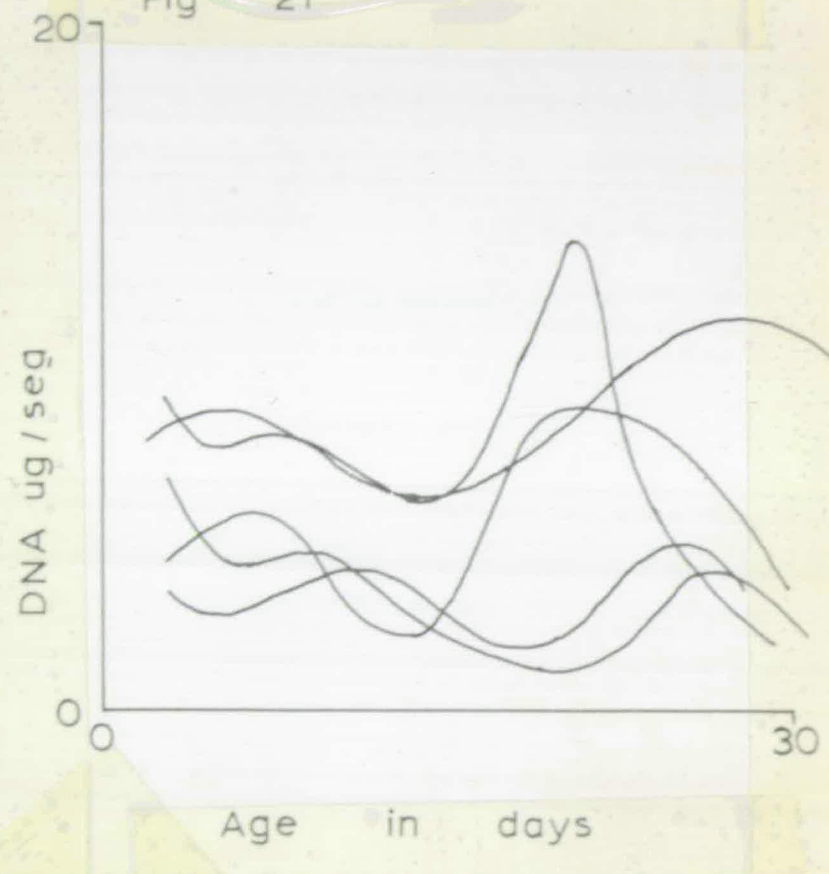
These methods of selection reduced the variation within

experiments to a satisfactorily low level but only at the cost of introducing a larger degree of variation between experiments. The introduction of this increased variation by the procedure used for the selection of the experimental material is discussed after a consideration of other sources of variation between experiments. There were two of these; the first was due to a change in the seed during storage. Old seed was slower to germinate and had a slower growth rate than fresh seed. Secondly, batches of seed obtained from different suppliers at different times of the year also varied with respect to germination time and growth. These two effects ensured that no two experiments had exactly the same growth rate. The selection procedure used tended to accentuate these differences of growth rate by selecting those roots closest to the mean growth rate for each experiment. This means that for two experiments with different growth rates there is a reduced tendency to select roots which are common to both experimental populations as compared with a random sampling technique. A random selection of roots in both populations would reduce the variation between experiments at the expense of increased variation within experiments. Although the variation between experiments was high it was of a form which did not prohibit the making of useful generalisations about the ageing process from sets of experimental results. The results for DNA/segment given in the appendix to the results section are presented in Figure 21. The absolute values of theedifferent sets of experimental results vary considerably but if these are ignored the shapes of the graphs are seen to be very similar. All the experiments show two peaks in the DNA content/segment even though their size and position varies from one experiment to the next.

The variation between the different experiments in the absolute values for the various parameters is not very important for the discussion of the results since no attempt will be made to give more than a cursory discussion on the quantitative nature of the results. The variability

of the results in this respect merely indicates that populations with different growth rates show differences in the quantities and relative amounts of the constituents for which they were analysed. This result is entirely reasonable and was in fact expected. The important fact shown by all the sets of experimental results and typified by the set of DNA results given in Figure 21 is that the same parameters show the same qualitative changes during the ageing of the basal segment regardless of the growth rate. The size and position of the two peaks in the DNA results are dependent on the growth rate but the fact that this pattern appears in all of the experiments indicates that there is a basic series of changes in the root segments which is only modified by the variation in growth rate. Each parameter has a pattern of change during the ageing of the root segment. This pattern of change does not vary in its essentials from one experiment to the next but the sizes and positions of various features of the pattern, such as the two peaks in the DNA results, vary with the variation in the growth rate between experiments. Since the growth rates tend to vary more between experiments in the later stages of the period of culture there is little variation in the position of the first of the DNA peaks and much more variation of position in the second peak. In general the higher the growth rate the closer are the two DNA peaks together (i.e. the time scale for the ageing process in the root segment is contracted) and the lower the growth rate the further apart the two peaks become (equivalent to an extension of the time scale for the process). The interpretation of the results is based on the assumption that the rate of growth determines the timing of the ageing changes in the basal segment. This has been confirmed experimentally and is discussed separately later. Since the assumption holds it was therefore permissible to average each set of results from individual experiments and use the results obtained as a basis for the discussion of the various aspects of ageing. The averaging of the results was in effect a

Fig 21

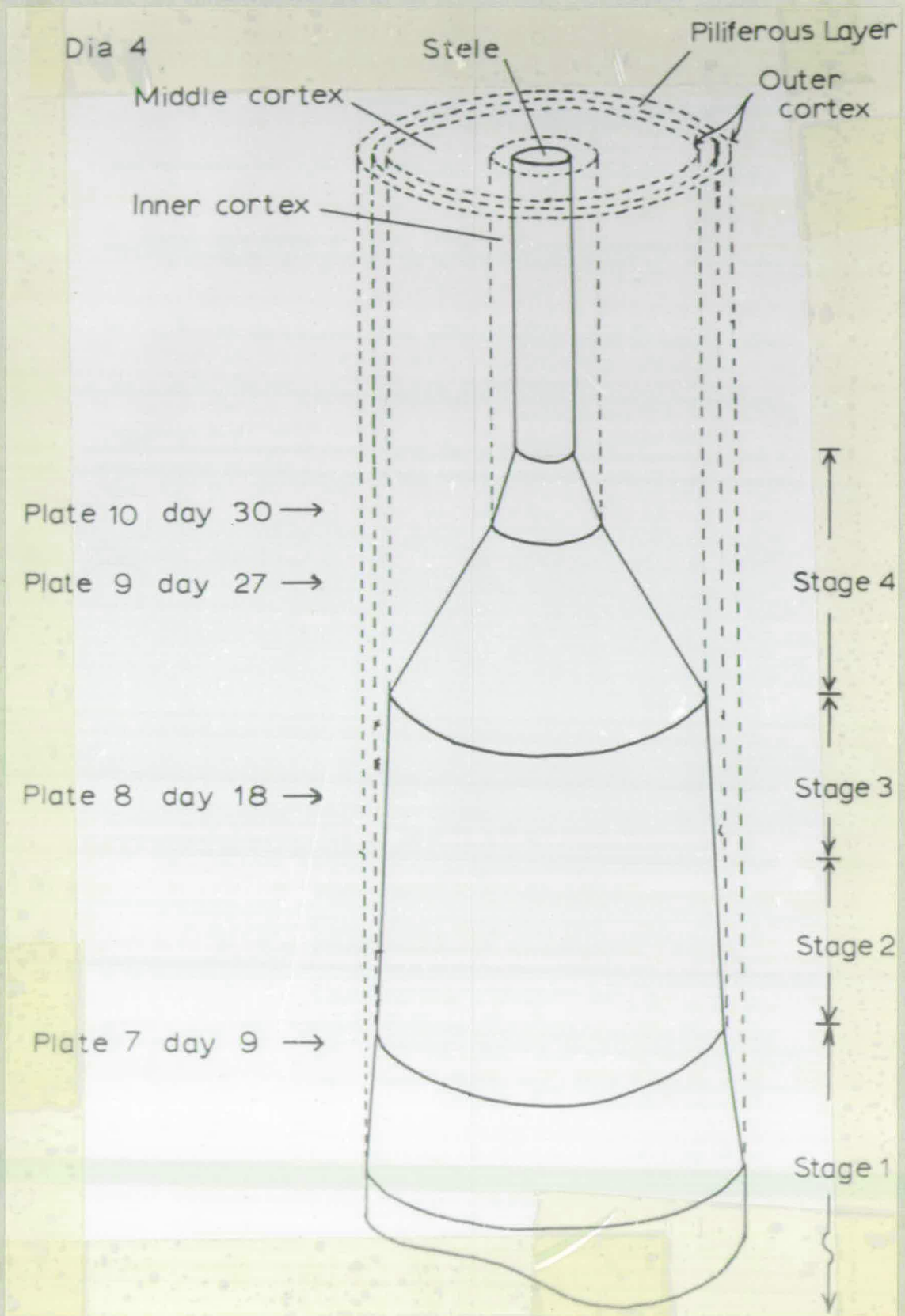


presentation of the various parameters as those to be expected from a batch of experimental material whose growth rate was the average of that of all the individual experiments of the series. That such an average preserves the pattern of change shown in individual experiments is a strong argument for the previously made assumption.

3) Subdivision of ageing changes in basal segment during culture period

Because the bulk of the measurements made were on the whole root segment, it is necessary to have a clear conception of the processes occurring in the basal segment in each of these stages in order to be able to relate the changes to the regions of the basal segment responsible for them. Diagram 4 shows the relationship between the progressive necrosis and the duration of the experiment.

Living tissues are shown outlined with the solid line while the dead tissues are outlined with a dotted line. The areas of the root which correspond to the 4 stages assigned on the basis of the anatomical and biochemical investigations are marked at the right side of the diagram. The vertical distances in the diagram were arbitrarily assigned and do not imply anything about the relative lengths of the various stages in time. This information can be obtained from Figures 22a-d. The arrowed plate numbers to the left of the diagram refer to plates in the results section which show the appearance of TS at these various stages during ageing. The vertical scale of the diagram represents a time sequence of 30 days greatly compressed. By using this diagram the average anatomical state of the basal segment at any of the 4 stages can be seen. The diagram shows that no two portions of the root segment have exactly the same proportions of living, dead and dying tissue. Depending on the time of harvest, the basal segment will contain a mixture of tissues in various parts of the segment which are at different periods of development within one stage or at different


Dia 4
Stele
Piliferous Layer
Middle cortex
Outer cortex
Inner cortex
Plate 10 day 30 →
Plate 9 day 27 →
Plate 8 day 18 →
Plate 7 day 9 →
Stage 4
Stage 3
Stage 2
Stage 1

stages of development. Analysis of the basal segment as a whole therefore provides average values which will only indicate the average degree of necrosis intermediate between the extremes present within the segment at that time. This degree of variation within the segment varies according to the rate at which necrosis occurs in the various tissues.

In figures 22a-d, some of the more important results are presented graphically to emphasise the relationship of the changes observed to each other and to the age of the basal root segment. No vertical scale is given since it is the pattern of change in the parameters shown which will form the basis of the discussion rather than their quantitative relationships. The main nitrogen fractions are shown in Figure 22a. The DNA content and rate of synthesis etc., are shown in 22b, while 22c and 22d show the changes in enzyme activities and the oxygen uptake. In these figures the period 0-30 days has been divided into 4 stages to correspond with the stages of cortical necrosis shown by the histochemical investigations of phosphatase activity and by nucleic counts from Feulgen stained material.

The histochemical and anatomical evidence allows us to divide the ageing process into 3 stages. The biochemical evidence allows us to divide the second of these into 2 parts.

Stage 1 From maturity to the time when necrosis of the piliferous layer is complete.

Stages 2 and 3 The period in which the necrosis of the remainder of the outer cortex occurs. On anatomical and histochemical evidence this is a continuous process but the DNA synthesis at 18 days and the rise in protein nitrogen content show that the second half of this process is distinct from the first half. On this evidence this period is subdivided into two stages - Stage 2 and Stage 3.

Fig 22a

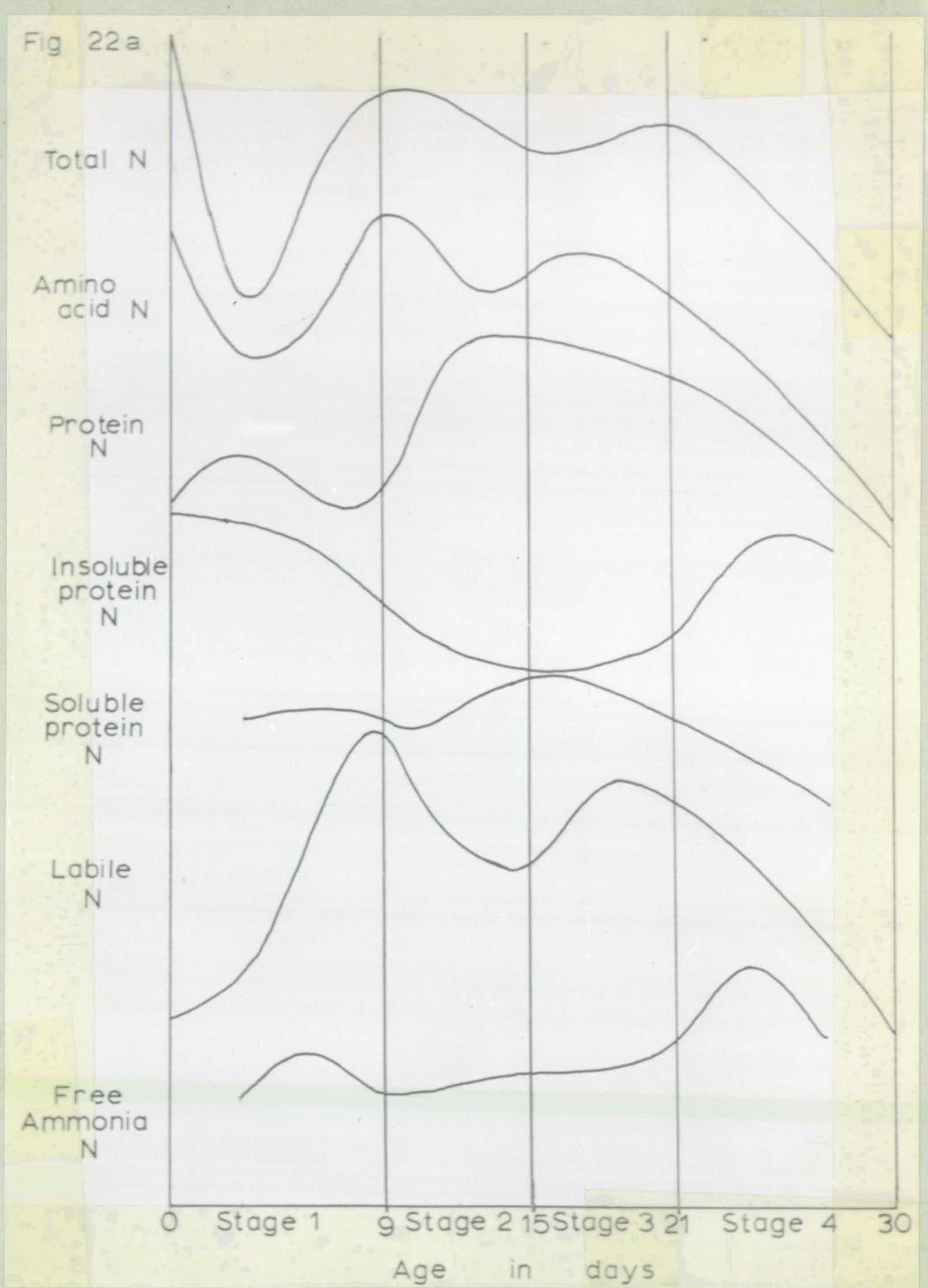

Total N
Amino acid N
Protein N
Insoluble protein N
Soluble protein N
Labile N
Free Ammonia N
0
Stage 1
9
Stage 2
15
Stage 3
21
Stage 4
30
Age in days

Fig 22 b

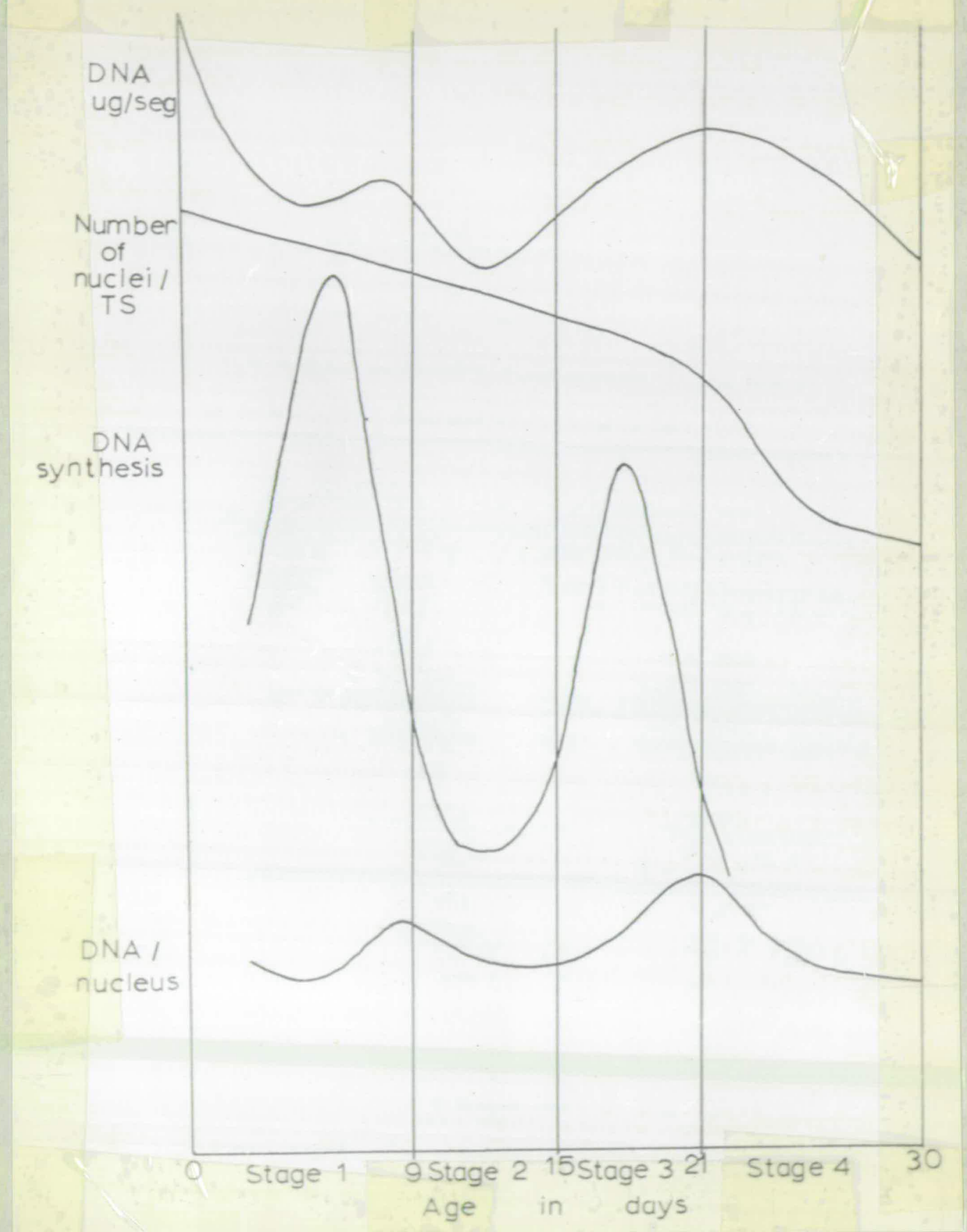

DNA ug/seg
Number of nuclei / TS
DNA synthesis
DNA / nucleus
0
Stage 1
9
Stage 2
15
Stage 3
21
Stage 4
30
Age in days

Fig 22 c

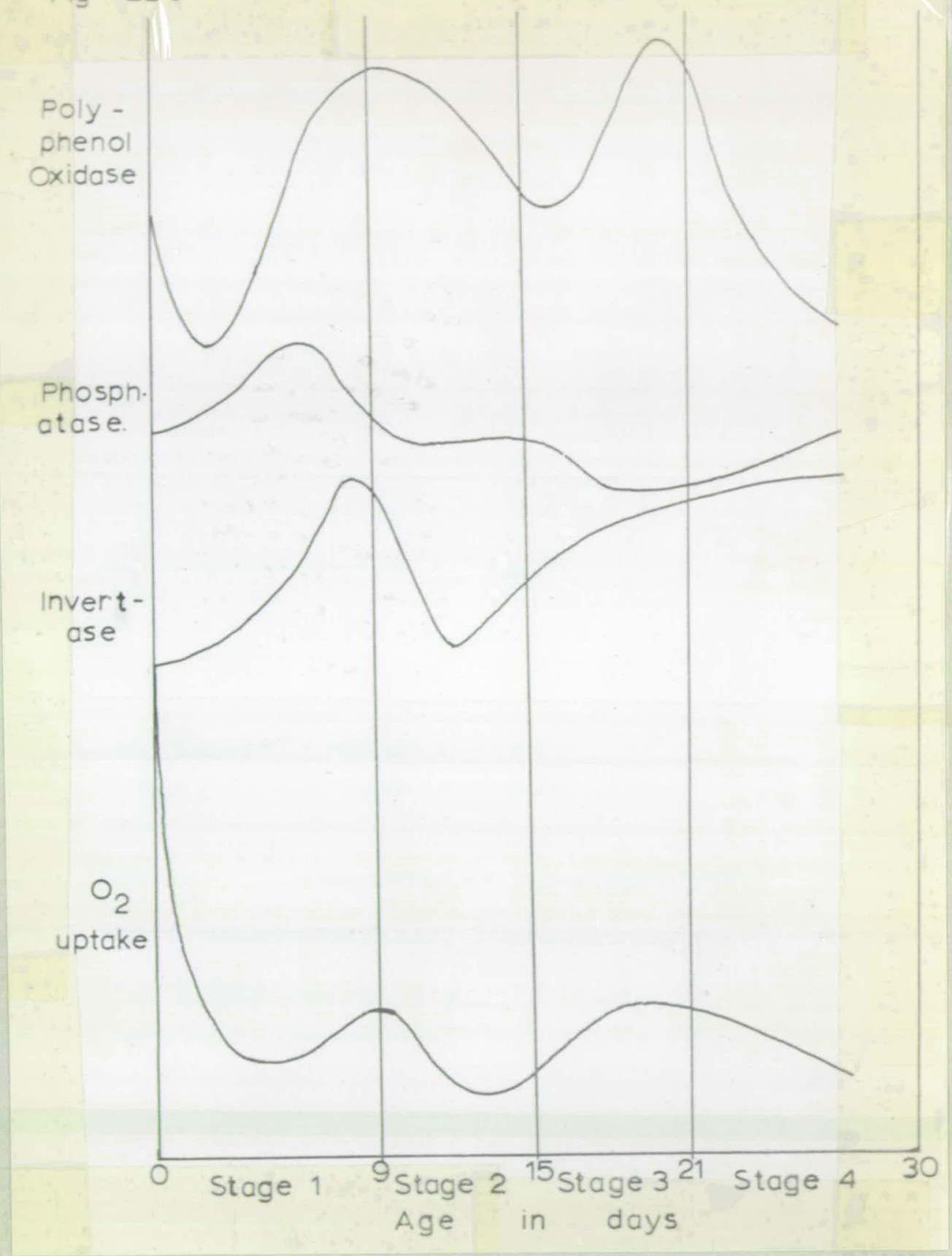
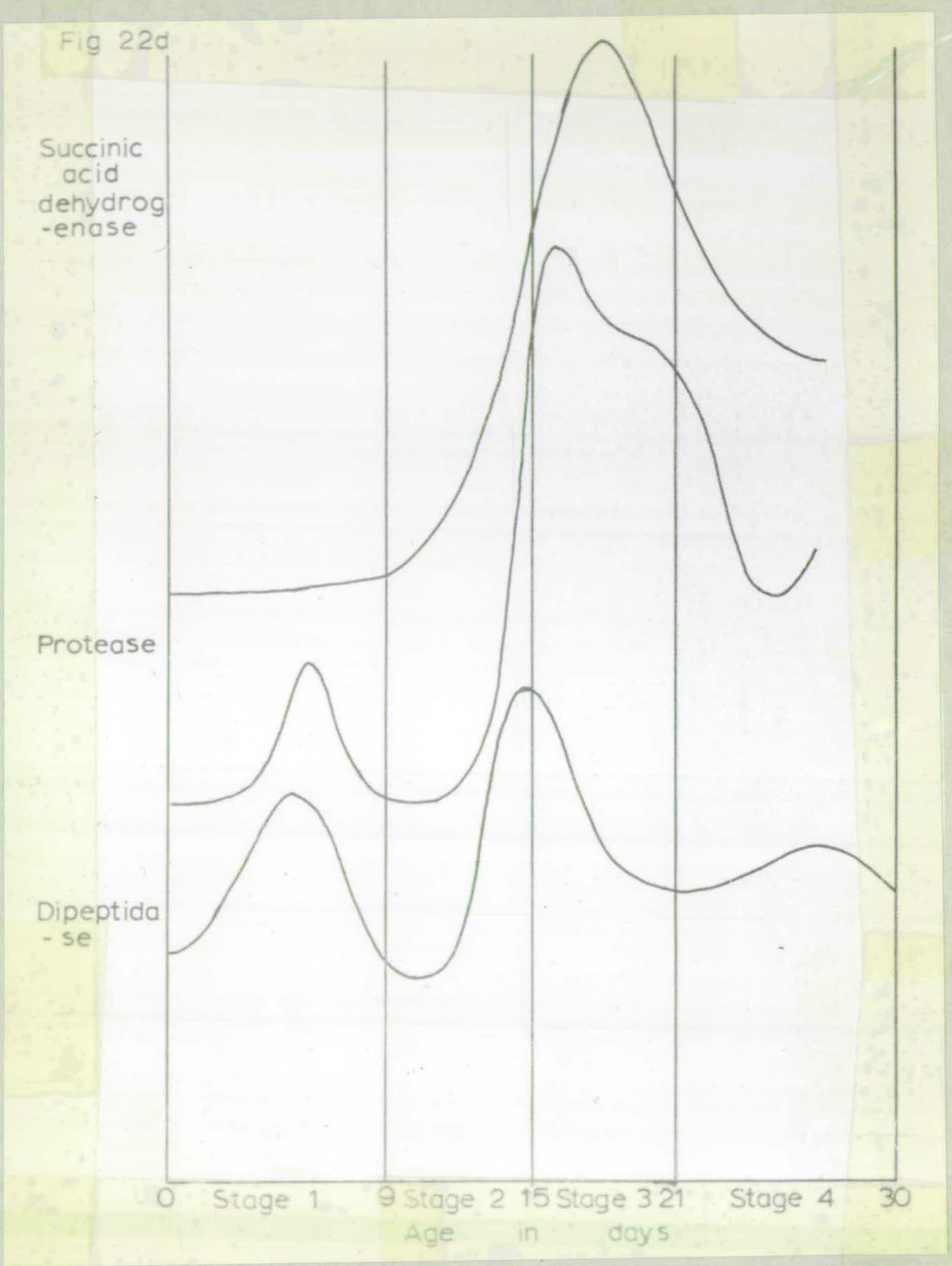

Poly - phenol Oxidase
Phosph- atase.
Invert- ase
O_2 uptake
0
Stage 1
9
Stage 2
15
Stage 3
21
Stage 4
30
Age in days

Fig 22d


Succinic acid dehydrog -enase
Protease
Dipeptida - se
0
Stage 1
9
Stage 2
15
Stage 3
21
Stage 4
30
Age in days

Stage 4 This is associated with the death of the inner and middle cortex.

Throughout all these stages the stele is considered to have little or no change occurring in it. It was not possible to establish this for all parameters, but the DNA content per nucleus in the stele and phosphatase activity in the stele show very little change and there is no detectable necrosis of the stele during the 30 days of the experiment. Those changes occurring in the stele are only those involved with xylem vessel differentiation and lignification and suberisation of the endodermis.

4) Ageing changes in the basal 1 cm segment and their interpretation

a) DNA

The changes in DNA per segment shown in Figure 22b were at first sight difficult to explain since there were three falls and two rises in DNA content to be explained whereas the cell number counts showed a constant cell number per segment after 3 days which of course implied that there was no cell division and no loss of cells. The initial loss in DNA per segment in Stage 1 is clearly a consequence of the decrease in cell numbers per segment as the meristem grows out of the basal segment. An explanation of the subsequent changes is more complicated but may be a consequence (its basis rests on the interaction) of two opposing processes. These processes are the continual loss of cells from the cortex by necrosis and the two periods of DNA synthesis without cell division which occur in the stele and cortex at 6 and 18 days. In Figure 22b the graphs for the two processes of, cell loss (number of cortical nuclei in TS), and DNA synthesis (number of labelled nuclei/LS), are shown together and from these it is obvious that a combination of the two processes can give a

changing DNA content per segment or a DNA content per nucleus. Although the shape of the curve can be explained by a combination of the two sets of results, a more detailed analysis of the DNA synthesis was carried out and this throws some light on the possible causes of the synthesis of DNA and the positions in the root segment in which it occurs.

The fluctuation in the DNA content per segment obtained by using the Dische reaction were not considered significant until an almost identical sequence of changes was obtained for the average DNA content per nucleus using densitometric measurements of Feulgen stained nuclei. Furthermore, the use of the microdensitometric technique allowed the average DNA content per nucleus to be determined for the different regions of the root in TS, and this coupled with nuclei counts and thymidine incorporation allowed a detailed picture of the process to be built up. Firstly, the contribution to the rise in DNA content for the first DNA peak is evenly divided between the stele and cortex.

Secondly, the rise in DNA content which reaches a maximum at 21 days is mainly due to synthesis in the cortex. This is shown by the higher percentage of labelled nuclei in the cortex of the 18 day segment relative to the stele than in the 6 day segment, thus indicating a greater contribution to DNA synthesis from the cortex nuclei in the second DNA peak. The results also show that there is little or no DNA synthesis in the outer cortex of the root during both the periods of DNA synthesis. Feulgen densitometry of the outer and middle cortex shows a gradual decline in the average DNA content of the nuclei in the outer cortex for the whole ageing period whereas the middle cortex nuclei show a rise between 6 and 9 days and another between 18 and 21 days which corresponds exactly withethe DNA synthesis rates shown by the counts of labelled nuclei. An outline of the events affecting the DNA content of the segment in the 4 stages of ageing is given below.

Stage 1 - (0-9 days)

The high DNA content per segment is reduced by the decrease in the cell number per segment as the root meristem grows out of the segment. By 3 days this has ceased to be the main cause of the decline in DNA per segment and the rate of decline slows accordingly. The death of the cells in the piliferous layer begins at 3 days and this is responsible for the loss of DNA per segment until day 6. At day 6 the nuclei of the middle and inner cortex cells and the stele cells begin to synthesize DNA. This does not result in cell division but reverses the fall in the DNA content per segment and causes a rise in the DNA content per segment to a maximum on the 9th day.

Stage 2 - (9-15 days)

By the 9th day the first period of DNA synthesis has more or less ceased and the necrosis of the outer cortex which has been continuing at a steady rate since day 3 results in a fall in the DNA content per segment.

Stage 3 - (15-21 days)

The necrosis of the outer cortex continues at the same rate, but a second period of DNA synthesis in the nuclei of the middle and inner cortex, beginning at 15 days and reaching a maximum at 18 days, causes the DNA content to rise to a maximum at 21 days.

Stage 4 - (21-30 days)

By 21 days DNA synthesis has slowed down and the necrosis of the middle cortex begins. This necrosis of the middle cortex is very rapid and leads to a very rapid decline in DNA.

These deductions about the time and place of the DNA loss and synthesis follow directly from the experimental results, but give little information about the cause of the DNA synthesis and the following discussion presents two possible interpretations of this together with the evidence for them contained in the results. The causes of the two periods of DNA synthesis were probably not the same and they are discussed separately.

First period of DNA synthesis

This period of synthesis was thought to be due to the final stages of cell maturation involving the acquisition by the cells of the middle and inner cortex of their full DNA complement. In a normal root the nuclei increase in volume and average DNA content as the distance from the meristem increases until a constant value is reached. Different regions of the root segment reach this final DNA value per nucleus more rapidly than others. The outer cortex and the piliferous layer are the first to reach this full DNA complement which they do by the third day of culture. This is somewhat later than the equivalent tissue in the normal root because of the check in DNA synthesis imposed by the excision and transfer to the culture medium of the apical 1 cm segment. This deduction is based on the experimental results from the culture of isolated basal segments where amputation of the basal segment was sufficient to stop DNA synthesis occuring in the segment at the time of its excision. Other results on the rate of increase in root length and fresh weight show that the initial rate of growth and by implication the rate of cell division and differentiation is slowed by the excision and placing of the 1 cm basal segment in the culture medium. The outer cortical nuclei have completed their nuclear development at the time of excision as is shown by the fact that there is no further synthesis of DNA in these nuclei after this time. The nuclei in the other regions are still synthesising DNA when the apical 1 cm segment is removed from the root. This process is slowed by removal and transfer of the apical segment to the culture medium but when the root has acclimatised to the new conditions the process is finally completed. This results in the maximum rate of DNA synthesis at 6 days and the maximum DNA content of the segment at 9 days. For these reasons it was considered that the first peak in the DNA content per segment was due to the final stages of nuclear development in the middle and inner cortex and the stele of the root segment. This

distinguishes the first period of DNA synthesis from the second which occurs in cells which are completely mature.

Second period of DNA synthesis

An explanation of the second peak of synthesis is more difficult, and the two put forward in this discussion have much less concrete evidence for them than the explanation of the first peak. The difficulty lies in the fact that the cells in which synthesis occurs are already beginning to show the first signs of senescence i.e. a decrease in total protein and buffer-soluble protein, (see Figure 22a). It is possible to regard it as a real attempt on the part of the cells involved to synthesise DNA prior to cell division, an attempt that is cut short by the death of the tissue before the cells can divide. DNA synthesis may, however, be regarded as a consequence of the disorganisation of the cells during senescence.

1) DNA synthesis as a result of the senescent disorganisation of the tissue.

Allison (1965) has shown that disruption of lysosome-type particles is associated with mitotic activity in some types of cell. Work by Gahan (1965) and Robertson (1966) show that one of the earliest signs of approaching cell death is the disruption of the lysosome-like particles in the cell and the spread of a diffuse type of phosphatase activity in the cytoplasm. In this system it can be argued that the cells in the cortex become senescent and start to lose protein. This loss, which initially occurs from the soluble proteins (enzymes), seems in its initial phase at least, slow and carefully controlled in that the products of this autolysed protein seem to be translocated to the stele and transported to and used by other root cells. One of the first results of this protein loss could be that the cell membranes tend to break down, a wide-spread and well documented ageing phenomena (see Heilbrunn 1959). Lysosome

membranes which have proved to be particularly labile might thus be preferentially disrupted with the consequent liberation of phosphatase enzymes into the cell. If this process of lysosome disintegration were sufficiently slow, it might serve as a stimulus for the commencement of DNA synthesis, which would continue until the release of hydrolytic enzymes produced too great a dislocation of the cell for synthesis to continue. The observed changes in phosphatase activity and DNA synthesis are at least consistent with such a sequence. However, if a process such as the one outlined above were responsible for the DNA synthesis, it should be a general phenomenon of ageing and could be expected to occur in a good many of the systems in which ageing has been investigated. In fact there is no evidence for limited DNA synthesis being a general phenomenon of senescing systems, apart from a reference by Das (1961) who reported an increase in DNA synthesis following exposure to X-rays. Since the ageing of the cells is probably a result of accumulated chromosome damage and X-rays are known to effect the genetic material of the cell, this may be an indication of a connection between ageing and DNA synthesis. Too much emphasis should not be placed on this, since there are many reports of DNA loss following X-ray irradiation. A more convincing argument is, the fact that the outer cortex nuclei, although they are in senescing and dying cells which are of the same type as those showing synthesis of DNA and a similar intensity of phosphatase activity, show no DNA synthesis. This proves that the process is not a general one even for the cortex of the root and shows that DNA synthesis is not an intrinsic property of ageing cells as the above theory would indicate.

2) DNA synthesis as a result of an external stimulus to initiate cell division

It is far from clear what initiates cell division in quiescent tissues in plants, but it is generally agreed that the stimulus

is of a chemical nature, and possibly due to the presence of several compounds each at an optimum concentration. The results of work on artichoke callus cultures, Yeoman (in press), give results which indicate that a gradient of concentrations of these various stimulatory compounds across the quiescent tissue may also be important in deciding which cells of the tissue divide. There is a considerable amount of circumstantial evidence from the preceding results in favour of a theory of this kind. The first result which lends weight to this hypothesis is that there is a rise in the soluble protein of the cells which later show DNA synthesis (Figure 22a). This rise in protein is not a feature of a senescing system, but it is what would be expected from a system in which a change in the type of cellular activity is about to occur. Moreover, the gel electrophoretograms of the soluble protein fraction show that there is a change in the composition of this fraction as well as a rise in the quantity of protein in it. This again points to the fact that this changed soluble protein complement may support a different type of activity. In view of the subsequent DNA synthesis in these cells, it is not unreasonable to suppose that this activity is cell division.

The results of the phosphatase histochemistry and the nuclei counts show that prior to and during this rise in soluble protein content, there is a region of tissue outside the middle and inner cortex which is in the process of autolysis. The translocation experiments using C14 leucine show that at this time no labelled material passes into the medium. This means that the products of the necrotic autolysis of these cells must pass through the middle cortex and into the stele and are translocated to other parts of the root. That this does in fact occur is shown by the movement of radioactivity along the root towards the end of the culture period. This means that there must be a gradient of the products of hydrolysis across the still-living regions of the cortex. If these autolysis products contain compounds which could stimulate cell

division, then this would provide a mechanism which could explain the DNA synthesis and correctly predict the region in which such synthesis would occur. This process of division, once initiated, would proceed until the increasingly unfavourable milieu due to continuing necrosis prevents the cells from reaching the point of division.

This hypothesis has the added advantage of providing an explanation of the lack of DNA synthesis at 18 days in isolated basal segments. The removal of the growing root from the basal segment would remove the sink for the products of outer cortex autolysis and so prevent the establishment of a suitable gradient across the middle and inner cortex. Without the establishment of such a gradient, DNA synthesis would not occur.

This hypothesis interprets the DNA synthesis as a property of cells placed in a particular type of environment rather than a phenomenon specifically associated with senescence. Since few if any of the systems in general use for the study of plant ageing, study the changes in a tissue sandwiched between a region of necrosis and a sink for the products of autolysis, the absence of any reports of this phenomenon is not surprising. The absence of any report supports the interpretation of this phenomenon as a product of the inter-relationship between various parts of the root rather than as a general characteristic of senescing cells.

The DNA synthesis was the only result which differed at all from the general consensus of opinion as to the changes occurring during cellular senescence and death and the adoption of the above explanation places this result in a different category, leaving the rest of the results completely compatible with those found by other workers in a variety of comparable systems.

b) Changes in nitrogen fractions in the basal segment.

Before entering into a discussion of the entire set of results, the results from the C^{14} leucine incorporation experiments

will be discussed separately since a discussion at the appropriate position in the main discourse would break up its continuity. The points requiring explanation are associated with the relatively high rate of incorporation into the insoluble nitrogen fraction and the departure of the incorporation rate from the change in the total quantity of insoluble protein nitrogen at about 9 days. The higher rate of incorporation into the insoluble protein nitrogen fraction relative to the soluble protein nitrogen fraction was probably due to the method of preparation of the fractions. This involved homogenisation in absolute ethanol and it was inevitable that a proportion of the soluble protein present would be rendered insoluble in the subsequent buffer extraction after such a treatment. This denaturation of the soluble protein would be selective and primarily affect the more easily denatured proteins present. This explanation implies that there were two periods in the ageing of the root tissues at which the proportion of this easily denatured protein increased relative to the soluble protein content. The first of these is at around 9 to 12 days and is not an increase in the total quantity of insoluble protein since the insoluble protein nitrogen shows a steady decline during this period. This first increase in C^{14} leucine incorporation must therefore be due to the synthesis of insoluble protein of a high specific activity. This means that the cells of the basal segment must be making one type of protein rather than a large number of other cell proteins. If this were not so and the increase in incorporation general for all cell proteins the C^{14} leucine would be expected to be incorporated into both fractions at much the same rate. The best explanation of this fact in view of the other results is that which assumes that this increase in insoluble protein is due to an increase in the protein synthesising machinery preparatory to the increase in the quantity of soluble protein in the period immediately following. The other results show that immediately after

this increase in the incorporation rate into the insoluble protein there is an increase in the incorporation rate into the soluble protein and a little later still the total protein of the cell increases sharply. This increase was entirely due to an increase of the soluble protein fraction since the insoluble protein nitrogen declines throughout this period. Moreover at this time the composition of the soluble protein fraction as shown by gel electrophoresis changes. This indicates that a period of metabolic change is taking place at this time. In the discussion of the changes in the DNA content it has already been proposed that these changes were due to the start of a process of preparation for division in the cells of the middle and inner cortex. This hypothesis can be developed into an explanation of the results of the C14 leucine incorporation experiment in the following manner. The cells of the cortex receive the stimulus to divide. ~~To do this they need to produce new protein in greater quantities than they have been doing in the immediate past so their first reaction to~~ A consequence of the division stimulus is ~~to produce~~ the production of more polysomes in order to cope with the increased rate of protein synthesis necessary for the cell division. The relatively large (ribosome-polysome) complexes necessary for protein synthesis would be more easily rendered insoluble with the homogenisation in ethanol and this would explain the increase in the incorporation rate into the insoluble protein fraction. This increase in the quantity of protein synthesising material would be relatively insignificant in terms of the total quantity of insoluble protein nitrogen present but would represent a considerable proportion of the C14 leucine incorporated at the time under consideration. At this time both the ribosomes themselves as well as the protein being synthesised from them would be labelled. At the time immediately after this supposed synthesis of ribosomes (after 12 days) the incorporation of C14 leucine into the insoluble protein fraction falls. This can be

explained on the basis that the ribosomes themselves were not being labelled at this time at the same rate and only the protein synthesised on them at this time was labelled. The quantity of C14 leucine incorporated into the soluble protein increases at this time, a result which would not be incompatible with this explanation.

The second rise in the incorporation of C14 leucine into the insoluble protein fraction after 21 days, was of rather a different type. Here the rate of uptake paralleled exactly the increase of the insoluble protein nitrogen. The incorporation of C14 leucine per μg of nitrogen remained much the same. This state of affairs needs a rather different type of explanation than that advanced for the previous observation and is open to two interpretations.

1. The rate of protein synthesis increases during senescence and that most of the protein so synthesised is rendered insoluble during the preparation of the fractions.

2. The uptake of C14 leucine was not a true protein synthesis but was due to an incorporation mechanism of some other type. This cannot be due to a simple hydrogen bonding since the radioactive amino acid was not removed by the washing procedure.

Any explanation of the incorporation in terms other than that of incorporation into protein must explain the fact that the radioactive amino acid was apparently chemically bonded to the insoluble residue. Of these two alternatives the first is the most attractive because it is in agreement with the results obtained by Chibnall and Wiltshire (1954), which indicate that senescing leaf tissue shows a high rate of protein synthesis as measured by the uptake of isotopic nitrogen into protein. The fact that other senescing systems show relatively high rates of protein synthesis well into senescence does tend to support the first alternative and suggestions of how this could come about have already appeared in the literature and a hypothesis combining most of these features has been

proposed by Wulff (1962). This explanation, given in brief below, was proposed to account for RNA changes occuring in senescing tissue but as a consequence of these the protein synthesis would be altered in such a way as to give the pattern of change observed in the ageing of this and other systems. In this hypothesis the ageing changes in the cell were considered to originate from defects in the genetic material of the cell which lead to the production of defective RNA. This in turn leads to the production of enzyme proteins of abnormal function. Because of this there would be an accumulation of substrate molecules with a resultant derepression of RNA synthesis. The result of this would be an increase in protein synthesis and a proportion of this synthesis would result in protein with no function in the cell. This theory requires that a proportion of this protein synthesised in senescence be nonfunctional in the sense that it is synthesised by abnormal RNA. In this case it is not unreasonable to suppose that this nonfunctional protein would be more liable to denaturation than the normal functional enzyme proteins in the senescing cells. This being the case, the rise in the insoluble protein in the initial stage of senescence can be accounted for. This would also fit in with the increasing rate of synthesis as measured by C14 leucine uptake during the initial stages of senescence since the rise in incorporation and presumably synthesis could be explained as an attempt by the cell to produce sufficient useable protein from a system in which a percentage of the m RNA is faulty. This discussion can be summarised as follows. The results of the C14 leucine incorporation show two periods of increased protein synthesis at about 9 days and at 21-27 days. These two periods of increased synthetic activity are fundamentally different and two hypotheses can be advanced to explain them. The 9-12 day peak of activity was explained as a consequence of the increased rate of protein synthesis prior to the DNA synthetic activity shown by the cortex cells. The 21-27 day rise

was explained in terms of malfunctioning of the protein synthetic apparatus of the cell during senescence. The evidence available from this and other work was not incompatible with either of these explanations. In the following discussion these two hypotheses are fitted into the results from other experiments.

The events of the first 6-9 days are primarily those of development and differentiation rather than ageing and senescence. At the end of Stage 1 the outer cortex cells begin to die and there is a rise in the amino acid pool and the free NH_4+ in the root as a consequence of this. Some of this material is utilised by the cortex cells and some is moved to the stele and passed to other regions of the root. As a result of these processes it was supposed that a gradient of the products of the autolysis of the outer cortex was set up across the rest of the cortex in the basal segment. As a result of this, some of the cells of the cortex are stimulated into a new cycle of synthetic activity. This activity was thought to be associated with a preparation for cell division because of the subsequent rise in the DNA content of these cells. The first symptom of this increased activity is an increase in the incorporation of C14 leucine into the insoluble protein fraction of the cell on day 9. After this the rate of incorporation into the soluble protein fraction increases followed by an increase in the quantity of soluble protein present per cell. The activities of the succinic dehydrogenase, protease and dipeptidase enzymes increase sharply at this time and the fall in the polyphenol oxidase, invertase and phosphatase enzymes is slowed or stopped. Towards the end of Stage 2 DNA synthesis begins and the respiration rate (O_2 uptake) which had been falling begins to rise again. At this stage the increased synthetic activity of the cells receives a check. The mechanism of operation of this check cannot be fully determined from

the results but it must be one which is operated by the growth of the rest of the root. This condition follows from the results of those experiments which showed that the development of ageing in the basal segment was dependent on the growth of the root as a whole. The simplest explanation of this condition is one involving an extension of the sink/gradient theory previously proposed to account for the stimulation of protein and DNA synthesis in the middle and inner cortex cells. This mechanism would operate as follows; the dying cortical cells release amino acids which increase the size of the amino acid pool. Some of these amino acids and other compounds are translocated across the cortex to the stele and removed by the rest of the root which acts as a sink. In the process, however, the cortical cells are stimulated to divide and begin to synthesise the increased quantity of protein necessary to sustain this activity. This, however, increases the rate of consumption of amino acids (and other necessary factors) beyond that which can be provided by the outer cortex necrosis and the synthetic capability of the cortex cells, the amino acid pool thus declines and the cortex cells have to compete with the rest of the root for the amino acids available. This would result in a decline in protein synthesis in the cortex cells and eventually the synthetic activity induced in these cells would stop. Given a constant supply of amino acids from cortex cell synthesis and necrosis, this would mean that the size of the sink which is proportional to the rate of growth of the rest of the root would determine how much synthetic activity occured in the middle and inner cortex. In order to keep the amino acid pool at the required level, more amino acids must be made available from the cells of the cortex if the sink remained high. If the demand exceeded the quantity which could be supplied by synthesis, some of the cells would die through lack of available raw materials. On their death the cell contents

would be hydrolysed and then passed to the other cells of the root. The amino acid pool could be kept large enough to supply the sink to the rest of the root only by a continued necrosis of the cortex. If the sink and the synthetic ability of the cortical cells remained fixed this would be an accelerating process since the greater the number of cells dying the larger becomes the synthetic contribution demanded of the remaining live cells and the less they are able to meet it so the result is an increased rate of cortex necrosis. Assuming that this method of control is the one operating in this system the sequence of events can be explained as follows.

The declines in the 0-3 day period shown by parameters such as the ethanol soluble nitrogen, DNA, and O_2 uptake are due to the loss of meristematic cells from the segment. The most significant change occurs at the end of Stage 1 and marks the beginning of the death of the piliferous layer. At this time there is an increase in amino acid nitrogen, amide nitrogen and ammonia. The latter two parameters are changes which can be considered as being typical of ageing systems (Yemm, 1950). The rise in the amino acid pool is large and at first sight appears too large to be accounted for by the limited necrosis occuring at this time. However, the C14 leucine labelling experiment shows that the loss of radioactivity from the basal segment is small during this period so a relatively slow rate of outer cortex necrosis coupled with the synthesis of amino acids in the new mature cortex cells should be sufficient to maintain the rise of the amino acid concentration in the basal segment. This rise of amino acid results in a gradient which is assumed to trigger the change of the mature cells of the cortex to a state of preparation for cell division. The first indication of this is the rise in C14 leucine incorporation into the insoluble protein fraction which is perhaps indicative of a synthesis of the protein synthesis machinery of the stimulated cells previously described. This is followed by an increased incorporation

of C14 leucine into the soluble protein fraction which shows an increased soluble protein nitrogen content a little later. Another result confirming this is the rise in the activity of the protease, dipeptidase and succinic dehydrogenase enzymes. Of these the protease enzymes can be shown to be associated exclusively with the soluble protein fraction. This rise in the soluble protein content is short and occurs in the second half of Stage 2. During this rise the amino acid content falls and this is presumably due to both the loss of amino acids from the segment to the rest of the root and the increased incorporation into the protein in the cortex. This fall slows down protein synthesis in the cortex and the protein content has assumed a steady level by the end of stage 2. The cells concerned with this are those in the middle and inner cortex and at the end of Stage 2 they had reached a state enabling a limited amount of DNA synthesis to occur. ~~of the protein content.~~ This DNA synthesis starts at day 15 and reaches a maximum at day 18. The DNA content reaches a maximum at 21 days. However, the soluble protein nitrogen begins to decline at the start of Stage 3 and although DNA synthesis continues until the end of Stage 3 it is limited in extent because the loss of soluble protein reduces the ability of the cells to sustain DNA synthesis. Apart from the DNA synthesis all the changes from the end of Stage 2 are characteristic of ageing cells in other systems. The only cells concerned in these changes are those of the middle and inner cortex and the first change is a decline in the soluble protein content. This is not due to a decrease in the rate of protein synthesis but to a decreased functional stability of the protein synthesised. The C14 leucine incorporation into the soluble protein although lower than at the time of the nett increase in the second half of Stage 2 is still as high as at the early part of stage 3 which was sufficient to maintain the level of soluble protein in the segment. This indicates that there is sufficient

protein synthesis to maintain the soluble protein level but that an increasing percentage of this is in some way rendered insoluble before or during the extraction procedure and thus appears in the insoluble protein fraction. As a result of this the fall in soluble protein is paralleled by an increase in the insoluble protein and an increase of C14 leucine uptake into the insoluble protein. As well as this shift of protein from one fraction to another there is a decline in the total protein nitrogen of the segment which shows that protein breakdown exceeds protein synthesis. The high rate of C14 leucine incorporation into both fractions shows that there is an increase in the rate of protein synthesis as measured by C14 leucine incorporation. This does not increase or even sustain the protein content of the segment which falls with increasing rapidity during Stage 4. This shows that the loss of protein is due to an increased rate of degradation and not to a reduced rate of synthesis. This finding is similar to that found by Chibnall (1954) for starving leaves. The ethanol soluble nitrogen, amide nitrogen and free ammonia nitrogen results give more information about this process of protein loss. Soon after the total protein nitrogen in the segment begins to fall, the ethanol soluble nitrogen (amino acids) begins to rise. This can be regarded as a consequence of the increased rate of protein breakdown. However, this rise in ethanol soluble nitrogen is not as large as that in Stages 1 and 2 probably for two reasons.

The rate of loss from the basal segment is higher. Radioactivity from C 14 leucine incorporated into proteins in the original innoculum first appears in the medium at this time. This shows that for the first time the products of protein hydrolysis are lost to the medium. Some are also transported to the rest of the root.

The products of protein breakdown are used as energy sources

for cell metabolism. Although the determination of RQ was not carried out, the accumulation of amide nitrogen in the amides of the amino acid pools indicates that there is some utilisation of the carbon skeletons of the amino acids released by protein hydrolysis in respiration and nitrogen released is stored in the form of amino acid amides. The amide concentration per segment reaches a maximum after the amino acid content has begun to decline and falls off more slowly than the decline of the amino acids as a whole indicating an increase in the percentage of amide amino acids in the ethanol soluble fraction. This evidence is supported by the chromatographic estimations which show an increase in the concentrations of amide amino acids in the ethanol soluble fraction. Similar observations have been obtained from senescing leaf systems by (Yemm, 1956), who reached similar conclusions about the nitrogen cycle in barley leaves.

In Stage 4, which is characterised by a very rapid necrosis of the remaining regions of the cortex, there is a rapid decline in all the protein fractions except the insoluble protein nitrogen and the free ammonia. The rise in insoluble protein nitrogen can be put down to an increasing lability of the protein produced at this time which makes it more liable to irreversible precipitation in the fractionation procedure. Together with this there is also the possibility that this increase is due to a malfunctioning of the protein synthesising machinery of the cell which results in a high proportion of the protein produced being unable to fulfil its assigned function thus destroying the ability of the cell to control the processes occuring in it. The rise in the free ammonia at the same time as the rise in insoluble protein nitrogen is evidence that the processes controlling the synthesis and breakdown of protein and amino acids are no longer functioning normally. This rise has also been observed in starving leaves (Yemm, 1950) and has been taken by him as an indication of a breakdown in the regulation of protein metabolism.

The final half of Stage 4 is characterised by a decline of the insoluble protein and the free ammonia nitrogen as the cells finally die and what remains within the cell wall is hydrolysed by the residual activity of the less labile hydrolytic enzymes. The decline in free ammonia nitrogen in the 27-30 day period is probably due to the fact that the cortex by this time is composed of dead inert cell walls and coagulated cell contents which would offer little obstruction to the diffusion of ammonia into the medium.

The pattern of development suggested by these results for the protein nitrogen cycle in senescing cells can be summarised as follows.

In mature cells protein synthesis equals protein hydrolysis, resulting in a stable protein content but protein hydrolysis becomes higher than the rate of synthesis and the protein content begins to fall. At the same time as this is happening there is a change in the type of protein synthesised such as to make it more liable to denaturation and presumably less able to carry out its function in the cell. The rate of protein synthesis then rises but this is reflected only in an increase of the insoluble, easily denatured protein while the soluble protein of the cell continues to fall steadily. The rate of soluble protein loss is greater than the rate of the rise in insoluble protein so there is still a nett loss of protein from the cells. The first sympton of increased protein hydrolysis is an increase in the amino acid pool, the size of which increases for a while but eventually decreases as amino acids are removed from the pool by loss to the medium and to the rest of the root and their utilisation as a substrate for respiration by the senescing cells. The result of this second process is an increase in the amide amino acid concentration since the nitrogen of metabolised amino acids is transferred to these compounds. Thus, as the amino acid content falls, the amide amino acid concentration rises. This rise does not persist for long since the transamination system of the cells becomes faulty and free ammonia is

released by the respiration of the amino acids formed by protein hydrolysis. This free ammonia increases steadily as the cell approaches disintegration but itself falls as the cells die and allow it to escape into the medium. A large percentage of the insoluble protein remains in the cell after death since most of the protein loss of the cells is from the soluble protein but this too declines slightly in the final stages of cell disintegration probably as a result of the unregulated activity of the less labile proteolytic enzymes.

Most of the points raised above have been established as occuring in other ageing systems, especially in detached leaves which have been frequently used to study changes in senescent plant cells. The general changes almost universally observed are:-

1. Decline in protein content supported by the results of Yemm (1950, 1956); Chibnall (1954); Chibnall and Wiltshire (1954); Sacher (1965) and Shaw (1965).

2. Sustained rate of protein synthesis noted by Chibnall (1954).

3. Utilisation of amino acids for respiration with resultant increases in free amides (Yemm 1950, 1956).

c) Changes in enzyme activity in the basal segment

The changes in enzyme activities and respiration are summarised in Figures 22c and d. It must be emphasised that the changes presented in these figures refer to changes in activity only. The increases in activity do not necessarily imply that a greater or smaller quantity of enzyme is present, although the rate of C^{14} incorporation into protein and the change in the electrophoretic pattern of the soluble proteins of the cell make it likely that there is in fact some change in the quantities of enzymes present at different stages of ageing.

The enzyme activities and O_2 uptake were determined for use as "markers" of the physiological state of the root. There was no intention

of extrapolating from an observed enzyme activity to its function in the basal segment. The enzymes investigated were selected as has already been described in the introduction because they were thought likely to show changes during the ageing of the root tissue. In the discussion which follows some surmises are made about the possible reasons for the observed changes in enzyme activity and O_2 uptake but the primary use of the enzyme activities was to confirm that the patterns of change shown in other parameters and by the root segment anatomy were accompanied by changes in the activities of some of the enzymes present.

The enzyme activity changes observed were adequate to provide this confirmation. Each enzyme activity shows a pattern of change which in timing fits in well with one or other of the other parameters. Enzymes of a similar function such as protease and dipeptidase show very similar patterns of activity. If a rapid change in enzyme activity is considered as indicative of a change in the metabolic state of the root segments then the results of the enzyme activities taken as a whole show that there must be two periods of change within the basal segments. The first at the end of Stage 1 between 6 and 9 days and the second from the end of Stage 2 to the beginning of Stage 4, between 13 and 21 days. The enzyme activities thus indicate times of change in the metabolism of the basal segment which agree with the observed changes in other parameters.

Enzyme activities are expressed throughout as activity per segment and although there is no change in the number of cells during culture, the number of live cells decreases steadily. Clearly certain enzymes or enzyme systems will have little activity in dead cells and it is of interest to discuss some of the observed changes in the light of the progressive death of cells in the basal segments.

1) Protease and alanylglycine dipeptidase.

These were both proteolytic enzymes in the sense that alanylglycine dipeptidase is a member of the protease group of enzymes which were measured collectively. The activity change of the dipeptidase was of the same form as that of the protease enzymes generally. The protease activity increases with the rise in soluble protein nitrogen and falls with the soluble protein nitrogen. If we then express the protease activity as a function of the protein content of the cell to get a measure of the specific activity this value would be constant for Stages 1 to 3. If expressed on a live cell basis the results show that over this period the activity per live cell would rise with the rise in protein nitrogen per cell and stay constant during the fall of protein nitrogen during Stage 3 and early Stage 4 since the protein nitrogen loss at this time is due to a loss of cells. However, in the last half of Stage 4 the protease activity stops falling at a time when the protein nitrogen and cell number are still falling. This means that there is a rise in the specific activity and the activity per live cell of the protease enzymes. At this time the protein synthesis activity as shown by C^{14} leucine incorporation has begun to fall which makes it improbable that this rise in specific activity is due to increased synthesis of proteolytic enzymes. Electrophoresis of the soluble proteins shows that there was no change in the pattern of soluble protein after electrophoresis from this age of segment which is slightly in favour of there being no synthesis of new proteolytic enzymes at this time. Since the cells of the root were dying and still losing protein at this time the best interpretation of this result would seem to be that the proteolytic enzymes were more resistant to degradation than the other cell proteins and hence survived in an active form right to the end of the senescence process. This is a reasonable hypothesis in view of their function and the knowledge that proteolytic enzymes from other systems have proved relatively resistant to degradation and denaturation.

2) Succinic dehydrogenase

Interpretation of activity of this enzyme must be made with care since the method used needs careful evaluation. To begin with the low values for the succinic dehydrogenase activity in Stage 1 are probably not due to a lack of activity in the tissue but due to the ability of the tissues at this time to reduce the triphenyl tetrazolium chloride in the absence of added succinate. This was probably a consequence of an adequate supply of succinate in the younger tissues. Apart from this, which if true, indicates a drastic drop in the succinate levels in the root tissue during Stage 2, the specific activity would be expected to show little change in Stages 1 to 4 since the change in activity parallels the change in the protein nitrogen content. In Stage 2 there is a rise in the activity per live cell which was paralleled by a rise in the O_2 uptake over this period. This would seem to indicate that the rise in Stage 2 was not entirely due to the changing succinate concentration at this time but was in part connected with a period of increased respiratory activity.

3) Polyphenol oxidase

The rises and falls in the polyphenol oxidase activity show little relationship to the changes in protein nitrogen content and as a result of this there are large changes in both the specific activity and activity per live cell during the first three stages. In the 4th Stage the activity declines with the number of living cells showing that this enzyme like the succinic dehydrogenase is destroyed during the earlier stages of cell senescence and does not persist during the final stages of cell autolysis. The change in the polyphenol oxidase activity was remarkably similar to those of the O_2 uptake and the DNA content/segment. This similarity could be related to the O_2 uptake in some way since the enzyme is a terminal oxidase but probably bears no relationship to the DNA content beyond the fact that O_2 uptake seems to be

stimulated during DNA synthesis and so may indirectly stimulate polyphenol oxidase activity. However, the relationship of the polyphenol oxidase activity to O_2 uptake and respiration is by no means certain.

4) Invertase

The most important fact for this enzyme activity is the increase in both specific activity and activity per live cell in Stages 3 and 4. If the possibility of increased or preferential synthesis of this enzyme was ruled out, the high level of specific activity can only be explained on the basis of a very high activity per live cell in the cells still living or a resistance to degradation and denaturation of the type proposed for the protease enzymes. Of these two alternatives the latter appears the most likely in view of the "tough" nature of invertase enzymes isolated from other systems and the observation by Dixon (1963) that invertase appears to be concentrated in cell walls, where cell "death" on loss of organisation may not affect its activity.

5) Phosphatase

At first sight the results from this experiment seem to parallel those of the invertase activity in that there is an increasing specific activity in the final two stages of the senescence of the segment. The phosphatase histochemistry, however, shows that in this case there is a vastly increased phosphatase activity in the dying cells which must more than offset the loss of activity due to the loss of cells by death. In the case of this enzyme therefore, the continued high specific activity was due to a real increase in enzyme activity (due to breakdown of lysosomes) in the still "living" cells rather than any undue resistance to denaturation of the enzymes involved.

6) O_2 Uptake

As has already been mentioned the results follow those of the DNA content and polyphenol oxidase activity. The relationship

of the O_2 uptake to these is tenuous but the result needs discussion for the following reasons. Firstly, the increase in the rate of O_2 uptake in Stage 3 is in no way a "climacteric" rise in the sense that this word is usually used in connection with senescence. The rise in O_2 uptake occurs at a time when the system is undergoing a pe riod of activity involving DNA synthesis, when the protein levels are high and the cells are showing no signs of senescent disorganisation (ie. no increased phosphatase activity). During the subsequent decline and autolysis of the root tissue the O_2 uptake per unit protein nitrogen falls steadily as the number of living cells falls. The results obtained thus rule out any burst of respiratory activity of the type associated with the ripening of fruit. The O_2 uptake rise that does occur seems much more likely to be associated with the increased synthetic activity of the cells. As far as this system is concerned senescence proper is associated with a slowly declining O_2 uptake. High respiration rates have been reported in starving leaf tissues but doubt must be cast on these since in many cases the leaves concerned were yellowed and no precaution was taken against bacterial attack. Charles (1954) repeated many of the starving leaf experiments and found that both yellowing and death burst "respiration" were very much reduced by the addition of antibiotics to the leaves. The basal segment of the cultured pea root system was sterile and did not show any large rise in O_2 uptake. Some of this later rise in O_2 uptake may be due to the increased use of amino acids from protein hydrolysis as respiratory substrates.

The most striking changes in specific activity and activity per cell occur in Stages 1 and 2 at a time when the processes occurring in the basal segment tissues are not primarily those of senescent change. These changes indicate a considerable activity in the basal segment at this time and give indications that the processes occurring in the

presenescent mature tissue are complex. Most of the changes in the later half of Stage 2 seem to be associated with the preparation for the limited synthesis of DNA in the middle and inner cortex cells. Apart from this the only two other observed anatomical changes in the basal segment in Stages 1 and 2 were the loss of the meristem in the first three days of culture which produces a decline in many of the parameters which was due to the reduction in the cell number of the basal segment and the expansion and necrosis of the root hairs and the piliferous layer. Some of the changes noted around nine days such as increased amino acid and free ammonia contents in the segment and some of the changes in enzyme activities, notably in the protease and phosphatase enzymes, show similarities to the changes occurring during the later senescent decline of the middle and inner cortex tissue. There is some justification then in assigning the changes observed from later Stage 1 to the middle of Stage 2 to the process of piliferous layer necrosis. However, since most of the root tissue is not involved in this process it is not possible to say unequivocally that these changes are definitely associated with the death of the piliferous layer since it is probable that other processes are occurring at the same time.

The enzyme activities and O_2 uptake during senescence can be divided into groups according to their behaviour during senescence.

a) Those such as succinic dehydrogenase and polyphenol oxidase which show no change of specific activity and activity per live cell during senescence and whose activity thus remains proportional to the number of live cells present in the segment. The O_2 uptake changes in senescence were of this type although the specific O_2 uptake may not fall as fast as the live cell number due to the change of respiratory substrate to amino acids from carbohydrate.

b) Those such as the protease and invertase enzymes which show an increase in specific activity with senescence which was

probably due to the greater resistance to denaturation and degradation during cell autolysis which these enzymes possess.

c) Those such as phosphatase which show an increase in specific activity during senescence which was associated with a very large increase in the enzyme activity of dying cells. The phosphatase histochemistry results show that this was a consequence of a release of presynthesised enzymes from particles in the cytoplasm rather than new enzyme synthesis.

5) Relationship between the ageing processes in the basal 1 cm segment of the root and the growth processes in the rest of the root.

Since different techniques were used to study various aspects of the dependence of senescence upon the remainder of the root, the results will be discussed separately.

a) Culture of the basal segment in isolation

The most important differences observed in this isolated basal segment from the "normal" ageing changes in the basal segment were the absence of any period of DNA synthesis in the segment after amputation and the large increases in amide nitrogen and ethanol soluble nitrogen in the isolated segment. These last two parameters both indicate that the size of the amino acid pool showed a large increase in the isolated segment. Also the form of the DNA change in the isolated segment shows that there was a much reduced rate of loss in Stages 2 and 3 in the isolated basal segment. That this was due to a reduced rate of outer cortex necrosis was confirmed by a comparison of phosphatase-stained TS of the same age from isolated and control segments. The results show that in the isolated basal segment no net DNA synthesis occurs. The second rise in total nitrogen and the rise in protein nitrogen were delayed, and there was no real change in the activities of the enzymes measured, namely phosphatase, invertase and succinic acid dehydrogenase.

The most important result produced by this isolated segment culture experiment was to show that the synthetic activity in Stages 2 and 3 which results in DNA synthesis is dependent on the presence of an intact root attached to the basal segment. The method by which the root as a whole controls the ageing process in the basal segment must be a chemical one of some kind and the simplest type of system which could achieve control is one of the sink/gradient type postulated in the discussion of the DNA synthesis and the nitrogen fraction changes. There are enough facts available to show that some sort of control exists and that it is dependent on the rate of growth of the root as a whole. There is no real evidence to suppose that the control is exerted by a gradient of metabolites passing from the older root cortex to the root growing points via the vascular system; but the c^{14} leucine translocation experiments show that there is a movement of amino acids from the basal segment to the rest of the root and a good case can be made out for the retention of this source/sink metabolite gradient hypothesis at least as a basis for the discussion of the results.

Changes in the basal segment in isolated culture fit well with the hypothesis used in the discussion of the previous results. On amputation, the amino acid pool and presumably the other metabolite pools in the tissue are being fed from two sources, the synthesis of these compounds in the living regions of the cortex, and the liberation of these compounds from autolysing cells of the dying piliferous layer. In the "normal" basal segment this rise is offset by transport from the segment to the rest of the root and by the subsequent utilisation of these compounds in the synthetic processes which are triggered in the basal segment by the source/sink gradient. In the isolated basal segment the sink has been removed so there is no gradient set up across the cortex and so no synthetic activity occurs. With both types of sink removed the amino acid content of the tissue rises. The relative importance of

the two types of sink cannot be determined from the results available, although the results of labelling with C14 leucine seem to indicate that the rate of transport out of the basal segment to the rest of the root is low but too much reliance should not be placed on this since the proportion of the protein labelled during the short (3 hours) exposure to the C14 leucine must be extremely small, and may not be representative.

The increase in the size of the amino acid pool and the loss competition between the cells of the basal segment and the cells of the rest of the root for available metabolites, makes the cell environment less austere and thus increases the survival rate of the cortical cells. Hence the slower rate of cortex necrosis observed in the cortex of the isolated segment. The total nitrogen and protein nitrogen of the isolated segment do rise but at a later time then in the control "normal" segment. This synthesis may be a response to the high level of the amino acid pool or may be a belated start of the "division" process of the control root. The first possibility seems the most likely since the protein and other nitrogen fractions follow a pattern of change not paralleled in the "control" segments.

b) Transfer of whole root to fresh medium

The results of this experiment were indecisive but it was included to show that changes in the medium had very little effect on the pattern of ageing in the basal root segment unless the rate of growth of the root was affected. The growth rate, as shown by the fresh weight increase with time, was not significantly altered by the transfer to fresh medium. The results of the analyses of the basal segments from transferred and the control roots are very similar except for the total nitrogen results but in this case the pattern of change was nearer to the general pattern shown by other experiments on the "control" segments.

c) Sucrose supplementation

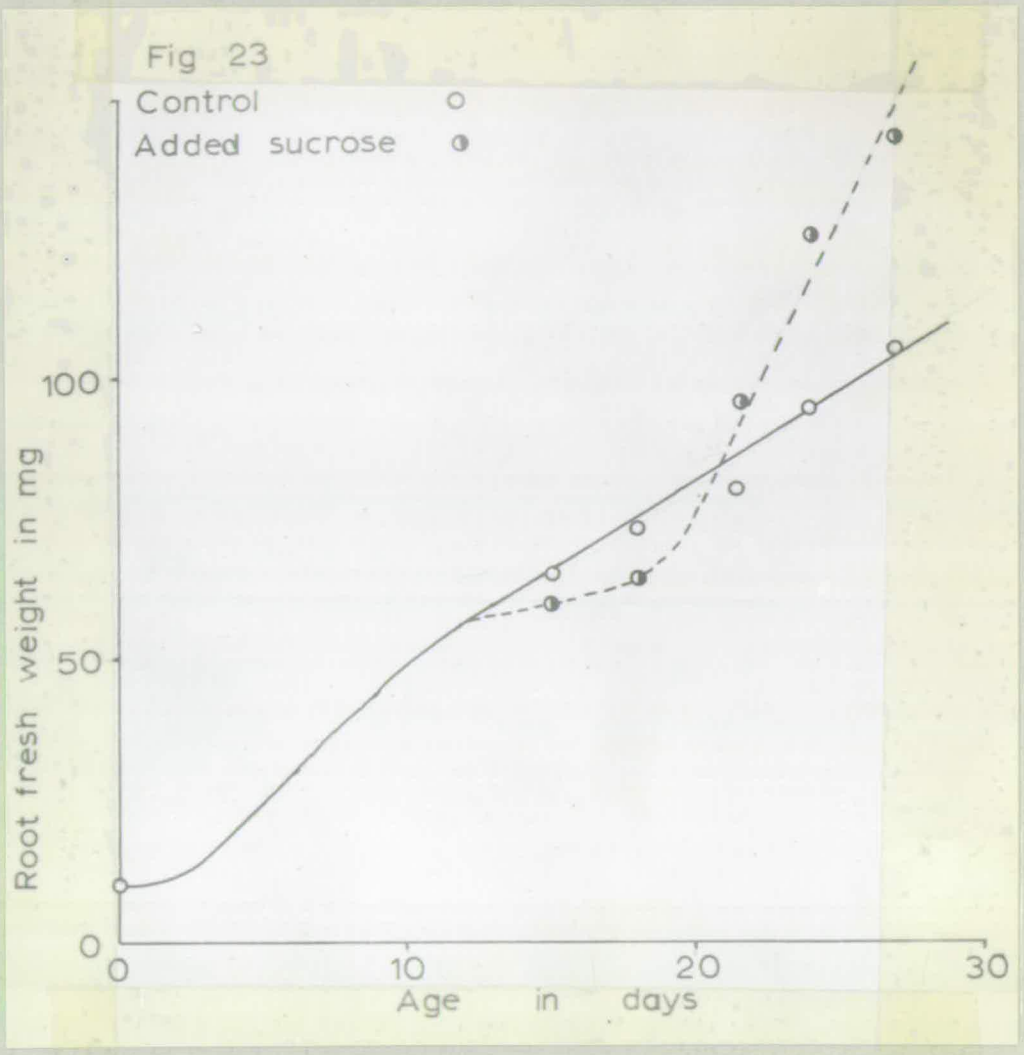
The results of this experiment showed that the sucrose supplementation had a large effect on the rate of growth of the roots treated. The addition of sucrose to the medium increases both the fresh weight and the length of the main axis of the roots. It seems to have no effect on the number or growth of the lateral roots.

The general effect on the other parameters seems to be a delaying of the changes shown by the basal segment of control roots for up to nine days. This delay is most marked for the DNA per segment, the amide nitrogen per segment and the total nitrogen per segment. This is difficult to explain since all previous discussions about the ageing of the basal segment have assumed that the ageing process was related to the rate of growth of the root and the set of results discussed in the next section show that this is substantially correct. If sucrose increases the rate of growth of the root the addition of sucrose would be expected to accelerate the ageing changes in the basal segment, not postpone them. A study of the fresh weight changes (Figure 23) shows that the first effect of the addition to the culture medium was a reduction in the rate of growth of the roots lasting about six days, followed by a rapid increase in growth rate until the treated roots showed a significantly higher fresh weight than the control roots. This lag in growth rate due to the addition of sucrose starts at 12 days which is just at the time that the "synthetic" activity is induced in the basal segment. The addition of sucrose thus produces a check in growth at this crucial period and it is this which slows up the whole series of changes in the segment.

d) Study of changes in the basal segment on the basis of whole root growth

Up to this time it has been tacitly assumed in the discussion that the control of the ageing process was dependent on the

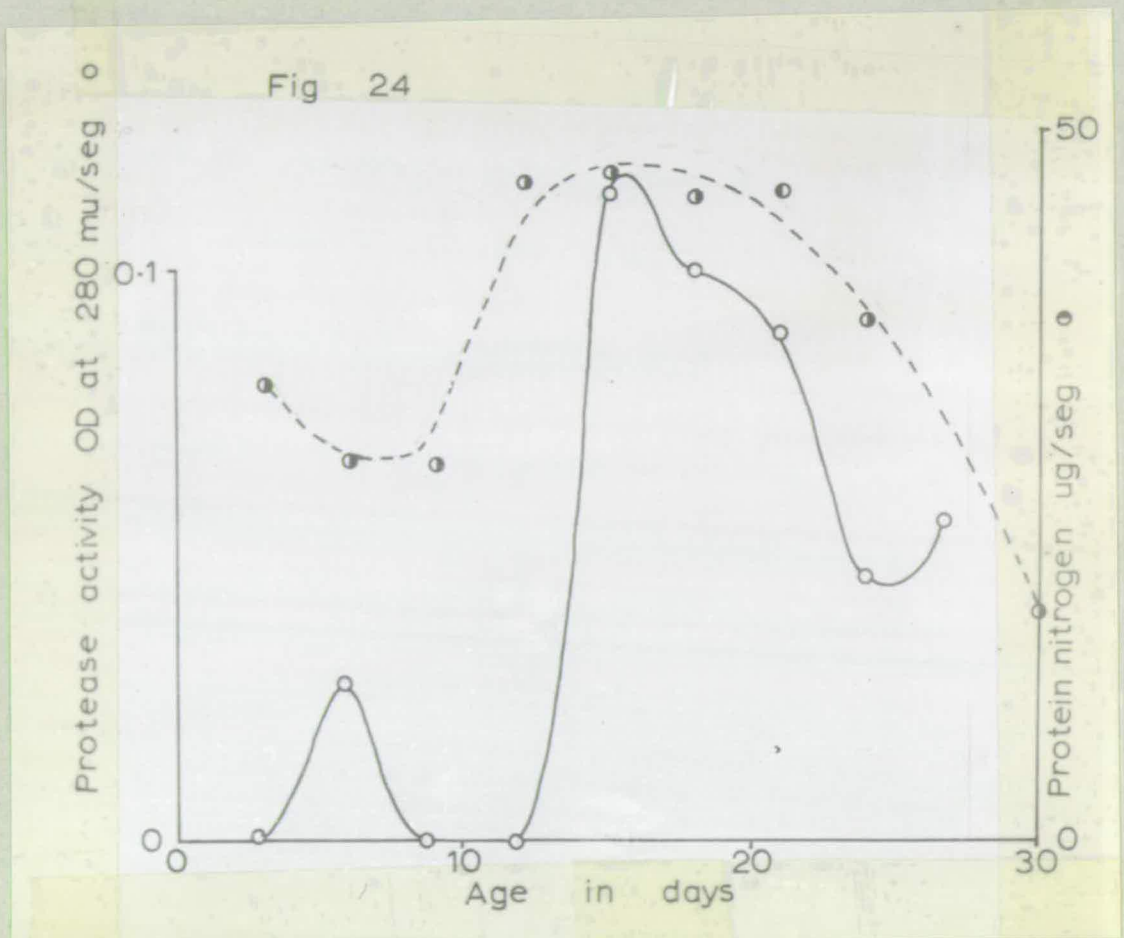
Fig 23


Control
Added sucrose
Root fresh weight in mg
0
50
100
0
10
20
30
Age in days

growth of the root rather than the time the root was grown in culture. This type of experiment was designed to test this assumption without altering the medium or interfering with the roots themselves during culture. The roots from a whole experiment were pooled and divided into groups according to fresh weight rather than time in culture. By this technique, roots of the same "chronological age" could be spread over several of the "physiological age" weight groups. If the changes observed were independent of root growth rate and dependent on time only this treatment would tend to destroy the patterns of change observed or at least to obscure them. No attempt was made to discard roots unless they had calloused or were infected. This was done so as to ensure that the experiment was a real test of the hypothesis that timing and speed of passage through the ageing changes in the basal segment were determined by the rate of growth of the root. If the basic pattern of ageing change shown in the analysis of the basal segments obtained by a "normal" type of selection according to chronological age was reproduced in the analysis of the basal segments selected on the basis of fresh weight then there is a strong case for regarding the control of ageing by the rate of growth of the root as a whole as established. A comparison of the protein nitrogen and protease activity of the two types of experiment (Figure 24) shows that the pattern in both are remarkably similar thus substantiating the hypothesis given above. The division according to fresh weight of the whole root seems to possess greater precision than the "normal" method since it is able to resolve the "shoulder" of protease activity(in the time selected roots) into a third peak of activity.

Taken together, the results of these experiments show that the start of division activity (DNA synthesis) was initiated by some sort of signal for which the presence of the whole root was necessary. The ageing processes in the basal segment were controlled by the rate of growth of the whole root. The medium affected the ageing processes only in so far as it

Fig 24


Protease activity OD at 280 mu/seg
0·1
0
0
10
20
30
Age in days
50
0
Protein nitrogen ug/seg

was able to affect the growth of the root.

The conclusions arising out of the whole discussion can be summarised as follows.

In the discussion of the ageing changes in the root it is necessary to have a firm idea of the regions of the root primarily involved in each stage of ageing - senescence and death. Phosphatase histochemistry and careful anatomical investigation allows the regions of dead, dying and live tissue to be determined. From this it is possible to correlate the changes occurring in the metabolic parameters of the root segments with the senescent changes in particular regions of the root. The pattern of change recorded during cell senescence is very similar to that found in other senescing systems. The complexity of the metabolic changes in the basal segment is due entirely to the heterogeneity of the experimental system. The basal root segment consists of several types of tissue which exhibit different patterns and rates of ageing. It is the interaction between these which produces the complex patterns observed experimentally.

The changes characteristic of senescence, namely fall in protein, DNA, amino acid pools etc., together with transient increases in amide and free ammonia content are typical of changes found in other senescent systems. The continuation of high rates of protein synthesis and the increasing specific activity of enzymes during senescence also have parallels in other work on senescent systems. The only point in which the observed results differ from those generally accepted is the absence of any "climacteric" rise in respiration, but an explanation for this as a consequence of the sterility of the experimental system can be put forward.

DNA synthesis with its associated protein synthesis is not a phenomenon of senescence but rather a consequence of the interaction between the basal segment and the rest of the root. The evidence all points to the fact that this is not a generalised phenomenon of ageing.

The timing of the various processes of necrosis and synthesis in the basal segment system is controlled by the rate of growth of the root as a whole.

An hypothesis for the control of the ageing processes by means of a source/sink gradient between the dying basal segment tissue and the younger tissues of the root is proposed and discussed in the light of the various experimental results.

There is a definite movement of the products of cell autolysis to other regions of the root as shown by the evidence for the transport of amino acids from the basal segment to the younger tissues. During the later stages of senescence the rise in amide indicates an increasing use of amino acids as respiratory substrates and the production of free ammonia indicates a disruption of nitrogen metabolism just before death.

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Appendix to Results Section

	Total Nitrogen / Segment						
	1	2	3	6	5	7	4
0	53.2	42.5	62.2				142.0
1							
2	50.4						
3		44.0	33.2	51.9	69.6	65.0	116.7
4	44.22						
5							
6		66.5	36.6	76.2	63.9	96.9	106.6
7	50.22						
8							
9	48.30	62.6	37.9	68.6	82.1	117.7	123.8
10	42.84						
12		56.5	54.9	78.6	88.0	158.0	104.0
13							
15		50.0		86.8	50.5	152.0	102.5
16	38.56						
18	52.58	75.6	48.6	100.7	53.3	153.0	119.1
21		52.9		86.3	46.9	143.6	107.7
22	86.06						
24		46.3	50.5	68.6	60.0	98.2	107.3
26	46.48						
27	20.54	44.3	45.1	86.5	88.2	104.6	113.8
30		43.3		52.6	77.6	-	-

	Acid precipitated nitrogen			Alcohol Soluble nitrogen			
	3	7	1	4	5	6	7
0	26.9		22.3	54.9			
3	22.6	36.0	37.4	47.4	33.2	41.8	29.0
8	18.6	37.5	24.2	41.9	35.8	36.9	59.4
9	15.1	37.7	26.8	66.9	32.6	47.4	80.0
12	17.1	99.2	23.0	52.2	40.4	41.3	58.8
15	20.5	87.2	34.0	51.0	41.6	37.1	65.0
18	19.6	95.2	21.2	67.4	39.8	38.0	57.8
21	19.0	85.2	32.7	58.3	30.4	29.2	58.4
24	26.6	54.8	28.2	45.8	15.0	32.3	43.4
27	24.5	80.2	34.4	51.5	15.8	36.2	24.4
30			16.6		26.6		

DNA / SEGMENT

	2	23	5	6	7
0	7.65	10.75			
2					
3	8.88	8.79	4.2	7.0	3.1
4					
6	8.84	7.69	5.5	4.2	1.8
7					
9	8.06	7.87	5.5	4.9	3.9
10					
12	6.86	3.72	1.7	4.0	4.9
13			7.0		
15	11.75	6.91	2.3	2.0	2.5
18	7.42	8.32	9.0	1.4	2.2
21	9.34	14.40	9.0	0.8	2.1
24	9.11	6.33	7.6	1.4	5.2
27	11.85	3.26	6.8	4.7	4.6
30	10.58		1.4	2.0	

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