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METABOLIC CHANGES DURING CALLUS DEVELOPMENT IN TISSUE ISOLATED FROM JERUSALEM ARTICHOKE TUBERS

by

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INTRODUCTION

The area of interest to which this study belongs is that of developmental physiology. This has proved a complicated subject of investigation, whether the experimental material employed be whole organisms or just excised organs. In contrast, if explants of uniform tissue are removed from organs of homogeneous tissue and then cultured, the development that ensues is relatively simple. For this reason, tissue culture has been increasingly used in studies of development. It has the additional advantages that cultures may be raised in an easily controlled environment, employing what may often be genetically equivalent material, with the possibility of examining fundamental processes without some of their normal developmental adjuncts. Tissue culture appears to offer, therefore, a tool whereby particular developmental processes may be dissected out from their natural concomitants, thus laying these processes open to experimental examination.

This trend in tissue culture work has proceeded from the classical root cultures of White (1934) involving whole organs, through the culture of increasingly uniform tissues which form proliferating callus (Gautheret, 1939; Nobecourt, 1939; White, 1939) to include the single cells or groups of cells cultured by Bergman, (1960). Latterly clonal lines of callus derived from single cells have proved of interest (Arya, Hildebrandt and Riker, 1962; Blakely, 1964). During this time tissue culture has become a versatile tool, and has been variously employed in diverse enquiries.

However, one of the major difficulties involved in tissue culture work has been the variability in growth response. Portions taken from established proliferating callus vary enormously in their response to subculture and it was for this reason that Caplin (1947) and other workers usually returned to the intact plant to establish cultures anew from relatively uniform initial material. With these workers mature parenchyma has frequently been the material of choice, be it

from stem pith of tobacco (Skoog, 1944; Caplin, 1947) or the ground tissue of storage organs (Steward and Caplin, 1952; Gautheret, 1939). Unfortunately variation could still be as high as 300 per cent after 28 days of culture (Caplin and Steward, 1952).

Most tissue culture work has used medium solidified with agar to support growth. This method has certain limitations. Firstly, only one area of an explant is in contact with the agar surface. It is likely, therefore, that as culturing proceeds, there will be certain inequalities in growth response arising from those gradients in nutrients, which will be set up in both the explant and the medium. Similarly, there may be gradients in the exchange of respiratory gases due to the occlusion of the base of the explant by moisture. Gradients of what may be toxic waste products are also a possibility (Steward and Caplin, 1952; but see Caplin, 1963). Secondly in this laboratory it has proven difficult to prepare agar medium without permitting some variation in the amount of moisture which condenses on the agar surface (which inevitably dries out progressively with time during the culture period). This fact is thought to be critical because the exchange of gases via a film of moisture must be less free than when the explant surface is bathed directly by the atmosphere. Thirdly, there is the practical consideration that an experiment using agar medium takes several days longer from conception to inception. Thus, in agar culture, growth and development is subject to polarisation by gravity, by gradients in nutrients, including oxygen and perhaps in waste products including carbon dioxide, and perhaps also by variation of incidental light. In contrast, in liquid culture the only polarity there can be is any inherent polarity depending upon already existing inter-relations at the time of excision. Therefore only three major effects may be invoked to explain any orientation in the callus growth response.

(i) Nutrients, including oxygen will become available to the superficial cells first.

(ii) Cells damaged by excision are confined to the periphery of the explant.

(iii) The cell shape is such that the long axes usually lie across one diameter of the explant.

Accordingly the available methods of liquid culture were examined. All methods involving stationary supports (Heller and Gautheret, 1949; White, 1953) were not considered as they do not prevent the induction of polarity. The two outstanding methods which have proved successful have both been developed by Caplin and Steward (Caplin and Steward, 1949; Steward, Caplin and Miller, 1952). In both their "tumbling tubes" and "auxophyton" they have solved the problem of adequate aeration admirably with elegant but elaborate glassware and machinery. It has been part of the purpose of this study to develop a similar method of liquid culture, using standard equipment if possible, without sacrificing any of the important advantages of liquid culture. A method has been developed employing glass rod, bacteriological tubes and the conventional roller culture apparatus (Section I A).

The second major factor involved in the experimental method concerns the choice of material. Steward and his co-workers, (e.g. Steward and Caplin, 1952, 1954; Steward, Mapes and Mears, 1958; Steward, Shantz, Pollard, Mapes and Mitra, 1961; Steward, 1961) in an outstanding series of papers, selected carrot phloem. This was, in some ways, a surprising choice because the spectacular growth promotion - a fifty-fold increase in fresh weight after fourteen days of culture - is obtained at the expense of the limitation that "one should only draw comparisons between treatments which are tested on a population of explants from the same carrot". This is because the response from one carrot to another can vary as much as 300 per cent (Caplin and Steward, 1952). A crop of carrots is of course the product of cross-fertilisation and this particular root is also very sensitive to soil and to climatic conditions. A second drawback of carrot phloem as experimental material is that the growth response is continuously variable amongst explants taken from along the length of the root axis and that there is a four-fold gradient in response across a 5 mm

radius of secondary phloem. From this steep gradient of response Steward and Caplin take their standard explants. This fact severely limits the size of the population from which comparable explants may be withdrawn. Thus the use of carrot phloem offers a very large yield at the price of a small quantity of comparable material and of prohibitively poor replication from one carrot to the next. The fact that this lack of comparability is not only quantitative but also qualitative, as evinced by Caplin's (1956) data on casein hydrolysate supplements (in which some carrots are shown to improve their response by at least 100 per cent in the presence of casein while others are unaffected) is a particularly potent reason for avoiding carrot tissue for the work envisaged in this study.

In contrast, the parenchymatous tissue found in the storage tuber of the Jerusalem artichoke, Helianthus tuberosus, has been found to yield a less dramatic but more consistent response to culture. In this Department, Yeoman (1962) had demonstrated that a four-fold increase in fresh weight and a ten-fold increase in cell number was regularly achieved using agar cultures of tissue taken from the variety "fuseau". The tissue is uniformly parenchymatous containing scattered rays of xylem traces which can often be avoided in the selection of explants. Finally the artichoke material has been grown from a single clone and is therefore genetically a pure line, barring chance somatic mutations.

The initial aim of this study therefore, was to develop a method which would satisfy these provisions. The culture technique must be relatively simple, give a high yield and of course be aseptic. Section IA describes the development of such a technique. Secondly, the growth response must be reproducible and obtainable at all times of the year. Fortunately, with appropriate storage of dormant tubers, explants of artichoke material proved to have these features and Section II B describes this aspect of the method. Ultimately all that is required is that the system grows in the desired fashion and thereafter the problems tackled tend to be limited

to those permitted by the characteristics of the method rather than vice-versa. However, it can be said in favour of the system developed that the yield is high and predictable within certain limits, (Section I B) that experiments may be performed at all seasons, and that contamination is a rare event.

Developmental Studies.

It was hoped that by developing such a method of culture, it might be possible to examine various aspects of development in this simplified situation. Yeoman had already shown (1962) that the development that proceeds, using agar cultures, may be divided into two phases: a dedifferentiation phase followed by a redifferentiation phase. Particular attention was then paid to analysis on a cellular basis (Yeoman, Dyer and Robertson, 1965) as such an approach seemed valuable when it was found that extensive changes occur in the pattern of cell divisions. Concerning cell division, liquid culture simplified the experimental situation still further by removing several possible sources of polarity. Thus the process, prerequisites, position, and polarity of division all become examinable using this system with short periods of culture. Similarly, the organisation of development in a simplified tissue can be followed by examination of longer term culture of up to 28 day duration. During this time considerable expansion and cellular differentiation occur. The first aim of the developmental studies reported here was to define the time-course of changes that occur during this course of development by determining the changes in such parameters of growth as fresh weight, dry weight, total cell number, number of differentiated tracheid cells, total nitrogen content, alcohol insoluble nitrogen content and ribonucleic acid content. Such a description in physiological units serves as a framework on which developmental changes may be pinned and Section II A reports these changes.

Changes of this kind, however, must depend upon more subtle changes in the

metabolic machinery. The contrast between the quiescent cells in a dormant storage organ and the vigorous and sometimes spectacular growth in cultured callus tissue must depend upon radical changes in the vast array of specific macromolecules which constitute the component parts of the machinery of each cell. As a first step towards elucidating such changes, a scan of the proteins from one fraction of the cultured material (the supernatant component derived from homogenisation and differential centrifugation) was obtained by disc-electrophoresis. This demonstrated quantitative and qualitative changes in the protein pattern. Thereafter it was logical to examine particular enzymes and to contrast the changes in different enzymes. Accordingly enzymes from widely differing areas of metabolism were chosen in the expectation that widely differing patterns of change might be observed. This proved to be the case and the results are reported in Section II B. In order to try and correlate changes in particular enzyme systems with particular aspects of development, a beginning was made to the localisation of changes in the activity of some enzymes by the methods of histochemistry. These proved fruitful and the interpretation of changes involved in morphogenesis derive largely from these observations. It would be useful, too, if such changes in enzyme activity could be correlated with the rise or fall of particular proteins observed in the electrophoretic scan. The histochemical methods are not yet definitive enough to realise such an objective. Ultimately it may be possible to demonstrate, without reference to nucleic acids, the synthesis of particular enzymes in the protein scan, to show that it has been recently synthesised by use of tracer-carbon, and to show where in the tissue (and perhaps even in the cells) that particular enzyme is at work. Thereafter the involvement of nucleic acids may be investigated. Such a possibility awaits considerable refinement in many of the techniques involved but is by no means impossible.

The Serendipity of Synchrony.

During the longer term studies it was learnt that cell division in this

system is not a random affair. On the contrary it is now known (Evans, 1965) that between 30 and 50 per cent of the cells in a population of 20,000 will proceed through mitosis in the space of five hours. The remainder of cells appear not to divide during the subsequent six hours. This would appear to be the first system, using higher plant tissues (apart from reproductive tissue) which offers the possibility of examining partially synchronised divisions (Erickson, 1964). Attempts are in progress to improve the degree of synchrony (Evans, 1966). It may now be possible to follow the changes involved in the cell cycle and thereafter to employ inhibitors, and to vary the cultured conditions, in order to learn more about the process itself and indeed Section II C reports preliminary results concerning this cell cycle. The fine structural changes involved are under examination (MacKinnon, 1966) and the nucleic acid metabolism is being explored (Fraser, 1966).

In some ways the purpose of this study has been to explore the possibilities of artichoke tuber tissue as experimental material and to examine the general changes in metabolism without becoming too involved in a particular area of especial interest. However, while examining the "metabolic changes during development" enough has been discovered to promote enthusiasm for further study.

EXPERIMENTAL MATERIAL

The plant used throughout this investigation was the Jerusalem artichoke Helianthus tuberosus L. Clonal material of the cultivar. Bunyard's Round was kindly grown at the Royal Botanical Garden, Edinburgh.

Tubers were lifted on the first convenient dry, mild day after the early frosts had caused the haulm to wither. Single plants usually yielded more than six tubers each weighing over 40 g. Each tuber was placed in a small polythene bag containing slightly damp sand and numbered according to the plant of origin. All the bags were assembled and buried in damp sand in several plastic bins and kept at 3 - 5°C in a cold room. These storage conditions made available a supply of tubers for growth experiments throughout the year. During the first year of storage (1962-3) tubers were placed, unwrapped, in sand, in bins, in a cold shed whose temperature was not controlled. Under these conditions, sprouting began during April and subsequently blackrot infection spread through many, though not all, of the tubers.

EXPERIMENTAL METHODS

A CULTURE PROCEDURE

1. Aseptic Inoculation of Explants

The medium, contained in 3 ml aliquots in metal-capped pyrex test-tubes, was autoclaved at 15 lb per sq in, for 15 minutes. Instruments and glassware, including mounts, were dry sterilised at 330°C for 2 hours in a hot air oven. The sterile transfer room was thoroughly sprayed with 60 per cent v/v alcohol and the fablon covered tables swabbed with alcohol three times as was the manipulation cabinet. This measured 30 x 30 x 30 cm, had a glass top, two sides painted with a high gloss finish, and a glass base raised 7 cm above the working surface. Instruments were flamed with alcohol before each manipulation.

The selected tuber was thoroughly scrubbed with a stiff brush to remove all dirt and much of the periderm. The stem was cut back and the tuber surface sterilised for 30 minutes in a solution of sodium hypochlorite containing 2 - 3 per cent v/v available chlorine. After rapid rinsing in two changes of sterile water the tuber was cut into three or four pieces producing one, or two, segments that were about 2.5 cm long. From each segment a succession of 2 mm diameter cores were removed (using a stainless steel canula with plunger) and cut into 2·4 mm lengths using the mass cutter (p. 10). The explants were rapidly transferred onto a sterile disc of tufnol in a petri dish. The disc provided a dark background which helps the next manipulation. From it, each individual explant was impaled, along its axis, upon a spike drawn from the edge of a solid glass rod, and dropped into a test tube containing 3 ml of Medium A. During this operation, each glass rod was held by an aluminium foil sheath to ensure asepsis. The tube mouth was flamed with the loosely fitting aluminium cap in position.

The mounts, of pyrex glass, had these dimensions:

Rod diameter	6 - 8 mm.
Rod length	54 - 56 mm.
Spike length	3 - 8 mm.

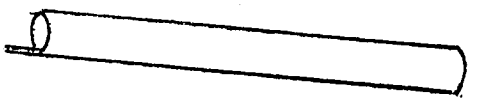


Figure 1a. Illustration of a mount.

Test-tubes were 20 ml, 150 mm x 15 mm, round-bottomed, lipless bacteriological tubes. The aluminium (Oxoid) caps were 20 mm x 17 mm diameter.

The mass cutter consisted of 10 stainless steel Personna razor blades separated by 2.4 mm brass spacers. The design of these (Figure 1b) ensured rigidity of the blades.

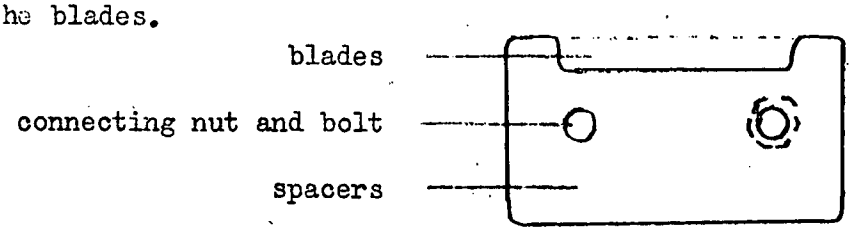


Figure 1b. Illustration of one of the eleven brass spacers.

2. Cultural Conditions and Medium

The culture room was maintained at 24° ± 1°C, and remained dark except while operators mounted withdrew or observed cultures. Thus cultures were exposed to low illumination for, at most, 5 minutes each day. Klein (1964) has shown that in Sunflower (H. annuus L.) crown gall cultures, that up to 2,000 foot candle hours over a period of 28 days produces only 5 per cent supression over dark grown controls. He further stated that this response "follows the Bunsen-Roscoe Reciprocity Law in that the biological effect of the product of duration and intensity of irradiation was a constant within the biological variation of the system". He also showed that "upon returning tissues to darkness, the growth rate returned to control levels".

Humidity was not controlled, but presumably was maintained at a high and fairly constant level within the culture tubes.

Medium A, which was used throughout except in several treatments described in Section I (p. 54) is that developed by Bonner and Addicott (1937) supplemented by 20 per cent coconut milk (Cocos nucifera) and 2:4 dichlorophenoxyacetic acid (2, 4-D) at 10^{-6}M. The full list of constituents was as follows:-

Coconut milk	200 ml/l	
Sucrose	40 g/l	
2, 4-D	0.221 mg/l	
$Ca(NO_3)_2$	236 mg/l	solution A
KH_2PO_4	12 mg/l	solution A
$Mg(SO_4)\ 7H_2O$	36 mg/l	solution B
KNO_3	81 mg/l	solution B
KCl	65 mg/l	solution B
$Fe_2(SO_4)_3$	2 mg/l	solution C

Stock solutions of A, B and C were made up at 100 times the strength required and 10 ml of each used for each litre of medium as this was required. The 2-4D was added as the free acid in 1 ml of alcohol in which it is soluble.

Coconuts were obtained on two occasions. The first were harvested in Ceylon and shipped to this country by October of 1962. A second similar batch arrived in October 1963.

The liquid endosperm from these nuts was filtered through glass wool, autoclaved at 15 lb per sq in for 15 minutes, and allowed to settle to sediment precipitated protein. The supernatant was stored in bulk in plastic bottles at -20°C in the deep freeze.

Thus the milk has been twice autoclaved by the time it is used.

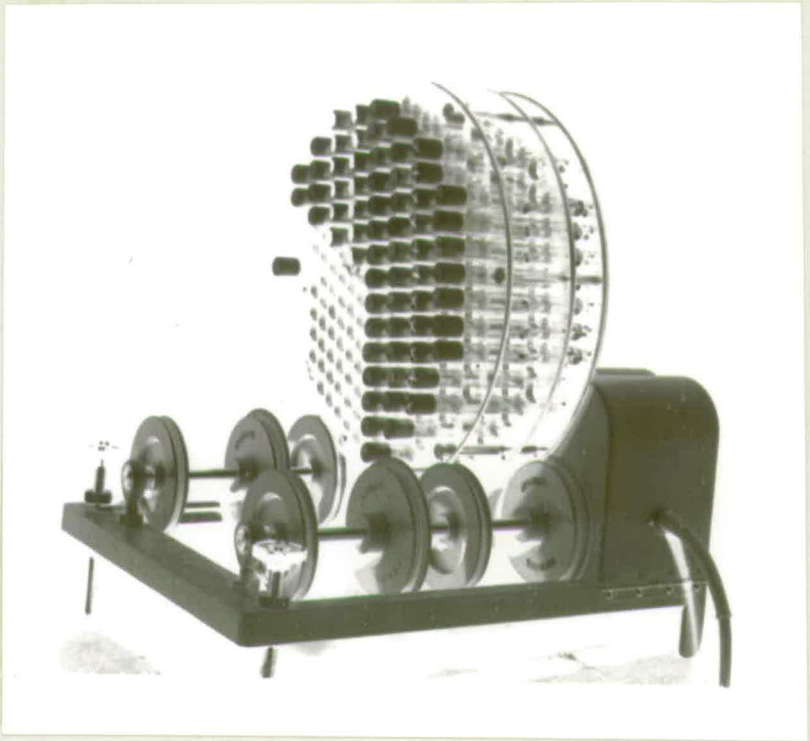


Figure 2. A conventional roller culture apparatus.

Up to 400 inoculated tubes can be mounted in two circular drums which are placed on the two revolving axles of a conventional roller culture apparatus. (Fig 2) The whole unit is racked up to an angle of 10° from the horizontal to ensure that the medium in the test tubes is maintained at the required level. This level is such that the explants dip into and out of the medium by virtue of their eccentric relation to the revolving mounting rod.

Each drum revolves at 0.125 r.p.m. and thus each mount revolves at 0.25 r.p.m.

3. Harvesting of Explants.

At selected time intervals, a group of cultures were withdrawn from the drum, each callus was separated from its mount, rinsed in a little distilled water, and its fresh weight recorded. In allotting explants from any one harvest to a particular group for assay, a standard method of distribution was followed. In, for example, the seasonal experiments (series 3, p. 88) twelve calli were harvested at each interval. The two explants whose fresh weights were closest to the mean value were used for cell number estimates. Three explants close to the mean were used for phosphatase assay. The two largest and the smallest were used for dry weight determination and the remaining three were used for nitrogen determinations. In experiments where samples were examined for anatomy or for the localisation of enzyme activity, the callus whose fresh weight was closest to the mean value was taken for these purposes.

Due to the time it necessarily takes to mount, for example, 180 explants, these were cut and impaled in three "batches", whose time of mounting may differ by 30 minutes per batch. It may be noted that the cultures in each batch remain upright while the total number is accumulated and thus all members of one batch contact the medium at the same time when the drum is actually placed on the

culture apparatus. Thus although the treatment (in most experiments) is uniform, the time of exposure to it may differ by 60 minutes. Conversely the tuber slice may rest at room temperature for 60 minutes longer before the later cores are taken. To remove any bias from these two factors, equal groups are taken from each batch, in any given harvest. Randomisation within each batch was achieved during the mounting of explants from the disc of tufnol. Any departures from this method will be described in the experiments concerned.

4. Modifications of the Procedure

The methods described above employ two modifications introduced during the course of this study. In early experiments (Experiment 2-10) hollow glass tubes were used as mounts until it was learnt that solid rods were more suitable. Similarly a 10 per cent calcium hypochlorite solution was made up for each experiment by filtering a freshly prepared saturated solution. Later it was found that sodium hypochlorite was equally effective and much simpler to use.

Finally it was discovered that an unidentified sporeforming motile bacillus could resist sterilisation with the hypochlorite solution. Thus cores were subsequently removed all within minutes of slicing the tuber, and groups of cores were preserved in a humid atmosphere in a petri dish containing a moist piece of tissue until they could be cut and mounted. This routine for cutting cores prevented spores, which apparently germinated in about 20 minutes, from being able to cause large scale losses. With this treatment any spores that are carried onto the segments of tuber as the scalpel cuts each portion are segregated to the ends of isolated cores, which are rejected before the spores can germinate and migrate to all the incipient explants.

B ANALYTICAL PROCEDURES

1. Determination of Fresh Weight

Calli were placed on two thicknesses of tissue paper which absorbed most

of the surface moisture, lightly rolled over, and weighed to the nearest tenth of a milligram. Subsequently each was transferred to one or another vessel for further analysis.

2. Determination of Dry Weight

Groups of calli were dried at 95°C for 48 hours or more in corked 5 x 2·5 cm specimen tubes and cooled to room temperature. The uncorked bottle was then weighed before and after knocking out the calli and the difference recorded to the nearest tenth of a milligramm.

3. Estimation of Cell Number

The total number of cells present in an explant at any given time was estimated by the method of Brown and Rickless (1949) as modified by Brown and Cartwright (1950). A number of calli were macerated in a known volume of 5 per cent chromic acid. This procedure dismembers the tissue into individual cells, or groups of cells which are sufficiently small for their exact number to be counted. The density of the cell suspension was then determined by the application of normal haemocytometer techniques. The suspension was made as homogeneous as possible by withdrawing and expelling the bulk of the macerate 5 times through a 5 ml syringe fitted with a 1 mm bore, oblique-tipped canula, prior to rapid transfer of a drop of macerate to the haemocytometer slide. For each of 6 such transfers the number of cells over a Fuchs-Rosenthal grid were counted and the number of cells present in the original volume of acid was computed from the mean of these six estimates. In a few cases, when large numbers of cells were present, only four counts were necessary. While the total number of cells was recorded using a tally counter, a mental count of the number of tracheids was also taken. In older calli it was found that more prolonged maceration resulted in better separation. However, after five days in a corked tube cells began to disintegrate.

Brown and Rickless (1949) reported that the mean of two counts do not differ by more than 5 per cent in estimates of cell numbers in pea root segments, while Amoore (1961) recorded a standard deviation of 14·1 per cent for the same material. The nine sets of counts involved in the May Seasonal Experiment have a mean standard deviation of 11·7 per cent. As the callus becomes older, groups of cells tend increasingly to withstand maceration. However, in all cases the groups which separate remain small enough to be accurately counted. The cell counts of macerates which have an increasing proportion of cells in groups would be expected to have an increasing standard deviation and this was found to be so. However, the increase in sample size with increase in age tended to counteract this effect, by reducing the standard deviation. Also, the changes in cell number in the later stages of culture are increasingly large and so this larger spread of counts makes little difference to the comparative value of results.

The number of calli in each macerate was never less than two and was always chosen so that cell counts would be greater than 100. As the number of cells per explant increased with culture, the number of calli per estimate was reduced or the volume of acid was increased from a minimum of 2 ml to a maximum of 10 ml. Where the cell counts for two calli in 10 ml exceeded 400, only half the haemocytometer grid was scanned.

4. Determination of Nitrogen

(a) Introduction

The total quantity of nitrogen in a sample was estimated after digestion in acid. This converted organic nitrogen to ammonium sulphate which was then estimated by one of two methods. The "total nitrogen" was usually estimated. On some occasions this "total nitrogen" was divided into "alcohol soluble nitrogen" and "alcohol insoluble nitrogen", by extracting the sample in two successive volumes of 5 ml boiling ethanol for 10 minutes each. The alcoholic extract was

concentrated by further boiling, to about 0.1 ml. The subsequent procedure for digestion of nitrogen was the same for all these parameters. It would perhaps have been preferable to have employed a perchloric acid or trichloracetic acid precipitation method for separating the high and low M.Wt. nitrogenous compounds but it proved difficult to retain the pellet from such small quantities of material. Realising the drawbacks of alcoholic extraction, the procedure was rigidly, though arbitrarity, standardised so that every determination would at least be comparable.

Samples (1 - 4 whole calli; concentrated alcoholic extract of 1 - 4 calli; or the alcohol insoluble residue from 1 - 4 calli) were then digested with 0.3 ml of "digest acid" for 60 minutes at c.200°C in hard-glass round-bottomed centrifuge tubes set on a micro-Kjeldahl digestion rack. The digest acid was 36 N nitrogen-free sulphuric acid containing the equivalent of 0.2 g/l of $CuSeO_3$ (derived from $CuSO_4$ + Na_2SeO_3). Within 30 minutes the digest is clear and Table A shows that after 30 minutes digestion is complete and that up to 70 minutes of digestion does not result in any losses. 60 minutes is, therefore, a suitable digestion period.

The samples were cooled, diluted to 10 ml and 1 ml aliquots taken for nitrogen estimation. Three methods of estimation were employed.

(b) The Method of Conway

To estimate the quantity of nitrogen in digested samples the micro-diffusion method developed by Conway (1962) was at first used (Experiments 10 and 14), with these modifications.

1. In the procedure for cleaning vessels, a 5 minute boil in lissapol detergent helped to remove vaseline, and final drying in a 90°C oven obviated the need for a vaseline wall, because after this treatment the two reagents added

to the outer well remained as discrete drops until they were mixed by tilting the vessel.

2. A different concentration of indicator was found to be more easily distinguishable as it passed from green to pink through a definite colourless phase.

Accordingly, the method consisted of adding 0.2 ml of indicator (0.0016 per cent bromo cresol green plus 0.0002 per cent methyl red) in boric acid to the centre well whose rim had been painted with melted vaseline. A 1 ml aliquot of diluted digest plus a discrete drop of 40 per cent potassium hydroxide were placed in the outer chamber of the No. 2 (Gallenkamp) microdiffusion vessel. Delivery of sample was through a slow straight-tube pipette. The ground glass lid was made airtight with vaseline and the reactants were mixed by tilting and rotating the vessel. Ammonia is released from the alkaline solution and is absorbed into the weakly acidic solution. The distillation is essentially complete in a few hours. After standing overnight the lid was removed and the dissolved ammonia titrated until colourless against N/100 sulphuric acid using an Agla microburette. The subsequent pink colour confirmed that the end point had been passed.

In Table 1 the results of a preliminary experiment are presented. These show that sodium and potassium hydroxide are equally effective as the alkali and that the concentration of alkali may vary from 10 to 40 per cent. It also shows that the vaseline is free of nitrogen and, as this barrier to "alkali creep" is simpler to apply than paraffin wax, it was used throughout.

Treatment	Titres (Agla units)				Mean
Standard Vaseline barrier	10.90	10.65	10.45	10.20	10.55
Standard Paraffin barrier	10.90	10.55	10.55	10.20	10.55
Standard + 10% NaOH	10.65	10.55	10.45	10.30	10.50
Standard + 40% KOH	10.75	10.70	10.55	10.55	10.65

Table 1: Comparison of 4 variants of the Conway method of estimating nitrogen using 1 ml of ammonium sulphate (12.3 mg/ml) as Standard. 5 Agla units = 1 µl.

The recovery of 105 per cent of the standard suggested that, as Conway has warned, the most critical aspect of the method is pipetting errors. Subsequently a Grade A pipette was obtained and at room T° 99.5 per cent recovery was recorded. This value was also checked by using the Agla burette for delivering the sample and again 99.5 per cent recovery was obtained.

This satisfactory figure, and the fact that the normality of the sulphuric acid was checked by titration against sodium carborate solutions (Vogel, 1951) allowed confidence to be placed in the delivery pipette and in the modified determination of end point. Determinations were carried out in triplicate to allow for biological variation.

This method is very accurate but timeconsuming and, since the biological variation can be up to 20 per cent, a second method was used for routine analysis.

(c) Nesslerisation

The alternative method is Nesslerisation in which nitrogen can be estimated with an error of less than 3 per cent (Johnson, 1941). This depends upon the ability of Nessler reagent (Na_2HgI_4) to give a yellow product ($NH_2Hg_2I_3$) in proportion to the amount of ammonium ion present at a slightly alkaline pH. The method is modified from that of Johnson, and was as follows.

Nessler reagent was prepared by dissolving 5g of potassium iodide in 5 ml of water, and adding concentrated mercuric chloride until a permanent red ppt. remained. 20 ml of 5N. NaOH were added, and the solution made up to 200 ml.

Before use, this was centrifuged to remove any precipitate.

Estimation involved adding 1 ml of Nessler reagent to 2 ml of 0.5N NaOH followed by a 1 ml aliquot of diluted digest, and reading the colour developed at 410 mμ in a Unicam Spectrophotometer, using a blank cuvette containing 2 ml NaOH + 1 ml Nessler reagent + 1 ml aliquot of 0.3 ml digest acid in 10 ml water. A calibration curve using standard dilutions of $(NH_4)_2\ SO_4$, equivalent to 14, 28, 56 and 112 μg N respectively, was constructed for each batch of Nessler reagent. The colour was always read after 2 - 5 minutes although it is stable for up to 15 minutes. The absorption peak of the reaction product occurs at 410 mμ.

(d) The Method of Lowry

Finally a third method was employed on one occasion to estimate the protein in the soluble fraction after fractionation of homogenates by differential centrifugation. This was the Lowry method using the Folin Ciocalteu Reagent to estimate the sum of the biuret reaction for protein and the phospho-molybdic

phosphotungstic reaction with tyrosine or tryptophane, and was employed as described by Colowick and Kaplan (1957).

5. Measurement of Gaseous Exchange

The uptake of oxygen and evolution of carbon dioxide were measured by Warburg's Direct Method, (Umbreit Burris and Stauffer, 1959). The rates of exchange of these gases were recorded by the interval uptake method. The volume of the medium in the Warburg flasks (1 or 2 ml) and the number of explants in each flask (6 - 20) are reported in the experiments concerned as is the method of adding the inhibitor dinitrophenol.

6. Measurement of the pH of the Medium

The pH of the bulked medium was measured to the second decimal place, within 20 minutes of harvesting, using a Model 23A Direct Reading pH Meter (Electronic Instruments, Richmond, Surrey).

7. Estimation of Nucleic Acids

(a) A Consideration of the Literature

The estimation of total ribonucleic acid (RNA) and of deoxyribonucleic acid (DNA) is at present a technique which is continually being improved. In order to find the best method, a critical survey of the literature was necessary. In addition communication amongst workers in this field was thoroughly discussed with various members of this laboratory.

Certain facts gradually emerged. There are two major difficulties to be overcome, namely (1) the clean separation of RNA and DNA and (2) the elimination of interfering substances. A variety of methods are available, and, while some are better than others, no single method can be applied to all tissues. It is obvious that, with different tissues there will be different interfering substances. It is especially true that many methods adequate for

animal tissues are quite inadequate for plant tissues.

The determination may be divided into four stages, namely, extraction, hydrolysis, purification and estimation.

Extraction

The preliminary extraction is designed to remove phospholipids with organic solvents, and to remove such acid soluble substances as phosphates, sugars and polysaccharides, and nucleotide coenzymes (Hutchison and Monro, 1961). Cold 5 or 10 per cent perchloric acid (PCA) or trichloracetic acid (TCA) is suitable for the removal of acid solubles and various combinations of organic solvents are satisfactory lipid solvents (see e.g. Smillie and Krotkow, 1960; Millikan and Pickett, 1964). For plant material it is important to include a hot ethanolic wash to remove, amongst other things, pectins (per. com. Heyes).

Hydrolysis

This step involves the conversion of polynucleotide molecules to soluble nucleotides and it is during this process that DNA is separated from RNA. Advantage is taken of the fact that in RNA the 2' hydroxyl of the ribose group will form a cyclic diester with the hydroxyl of the 3'5' phospho-diester linkage which, under alkaline conditions will result in cleavage of the RNA chain at the 5' link. DNA, having no 2' hydroxyl on its pentose residue, remains an acid precipitable polymer.

The extracted mixture of RNA and DNA may be subjected to either acid hydrolysis (Ogur-Rosen, 1950; Schneider, 1945) or to alkaline hydrolysis (Schmidt Thannhauser, 1945). There are several important criticisms of acid hydrolysis.

(1) The purine riboside linkage is liable to acid hydrolysis. This

results in conversion of some DNA to apurinic acid after which separation of RNA from DNA is not possible, (Loring, 1955).

(2) Solubilisation of RNA is not complete. Deken-Grensen and Deken (1959) give evidence of this and Heyes (1960) has shown that the amount solubilised varies with the age of the tissue concerned.

(3) Up to 10 per cent of the pyrimidines may be lost (Heyes, 1960).

For these reasons acid hydrolysis was avoided.

The alternative, alkaline hydrolysis, has certain drawbacks, of which the major one is that it is difficult to remove interfering substances, especially protein. The standard procedure for plant material (Schmidt-Thannhauser, 1945; Heyes, 1960; Ingle, Beevers and Hageman, 1964; Millikan and Pickett, 1964) is treatment with N alkali at 25°C or 0.3N alkali at 37°C for 1 - 18 hours. This treatment yields acid soluble ribotides and acid precipitable DNA polymer. Chilling prior to acidification reduces the amount of solubilised protein.

Purification

The acid soluble product of alkaline hydrolysis will still contain some interfering protein and most of this can be removed by adsorption onto Dowex II anion exchange resin (Deken-Grensen and Deken, 1959) leaving an acid eluate containing purified ribonucleotides. Dowex I will remove any DNA by the elution of ribonucleotides with 0.12N HCl, thus leaving DNA on the column (Millikan and Pickett, 1964).

DNA cannot easily be measured by ultraviolet spectrophotometry because of contaminating protein etc. Use must therefore be made of one of the pentose colour reactions. However, care must be taken to remove any keto sugars which will also react with these reagents. In most plant tissues keto sugars are

negligible but in some genera of the Compositae, fructose in various polymeric forms, is the major storage carbohydrate. Helianthus, unfortunately, is one such genus. A preliminary treatment with hot alkali before the liberation of the purine bound deoxypentose may remove this hazard.

Estimation

Estimation of ribonucleotides by ultraviolet absorption at 260 mμ is now accepted practice as is calibration using native purified RNA or purified yeast RNA. Measurement of the 260/230 mμ ratio gives some indication of the purity of the sample. Deken-Grensen and Deken (1959) find that a good ratio for difficult material (tobacco leaves) is of the order of 0.7 which improves to 1.7 after Dowex II purification. Similarly green Euglena ribonucleotides improve from 0.7 to 2.4. Values between 2.5 and 3.0 are generally accepted as "clean".

The most useful estimation of DNA is by the diphenylamine reaction. Hot perchloric acid will cleave purine ribosidic linkages yielding 2-deoxyribose which can be measured by the diphenylamine reaction of Dische (1930) as modified by Burton (1956). If careful checking for interfering sugars is carried out (by an examination of the full absorption spectrum) then this method is always satisfactory.

In conclusion, to determine the amount of RNA and of DNA from the same sample of tissue, it would be necessary to demonstrate

(1) That DNA is not present in the ribonucleotide extract

(2) That RNA is not present in the DNA extract

(3) That the ribonucleotide preparation has minimal contamination from proteins, pectins and polyphenols. Good 260/230 absorbancy ratios would verify this last condition. The contamination hazard may change with the age of the tissue (Heyes, 1960) and this would require to be checked.

(4) That ketosugars and polysaccharides are not interfering with the 2-deoxypentose estimates.

(b) Experimental Results

In order to evaluate the amounts of interfering substances in callus tissues a preliminary experiment was run. This consisted of taking cultured explants, which were harvested after the number of days specified, and submitting them to the Schneider modification of the Schmidt-Thannhauser procedure, and then estimating RNA by u/v absorption and DNA by the diphenylamine reaction.

On the day of harvest, explants were weighed and placed in the deep freeze, where they were allowed to accumulate until the nineteenth day. On that day, after thawing, each group of three explants was homogenised in a Potter-Elvehjem ground glass homogeniser in 2 ml of half normal perchloric acid, the homogeniser was then rinsed with 2 further volumes of 2 ml of acid and the bulked volumes were centrifuged at 500 x gravity for 15 minutes. The precipitate was washed with 2 ml of the above acid, and the acid-soluble fraction rejected. To remove lipid the residue was washed in the following:-

75 per cent ethanol	(twice)
Absolute "	(")
1:1 alcohol:ether	(")
Diethyl ether	(")

The pellet was air dried and alkaline hydrolysis performed for 70 minutes in 1 ml of N NaOH, at room T°. DNA was precipitated with 3 ml of N HCl. The solution was then centrifuged, the ribotides removed in the supernatant, and their extinction measured, after dilution, at 260, 232 and 240 mμ in the Unicam spectrophotometer using a blank cuvette containing 1 ml N NaOH and 3 ml N HCl. DNA was solubilised in hot (70°C) half-normal perchloric acid

during 25 minutes. After cooling to room temperature the protein was spun down and the supernatant, containing degraded DNA, collected. This DNA solution was incubated at 30°C for 18 hours with two volumes of diphenylamine reagent containing acetaldehyde (Burton, 1956). This reagent was made up by adding 2.75 ml of concentrated H_2SO_4 to a 1 per cent solution of recrystalised diphenylamine in 100 ml glacial acetic acid.

Acetaldehyde was present at a final concentration of 0.1 per cent. Employing an incubated blank containing 1 volume of 0.5N perchloric acid plus 2 volumes of diphenylamine reagent, extinctions of the colour developed were measured at 595 and 650 mμ.

This procedure was thought to be the best possible after a consideration of the extensive review of Hutchison and Monro (1961).

Day	RNA absorption					DNA absorption		
	O.D. at					O.D. at		
	260	240	232	$260/240$	$260/232$	595	650	△
0	0.03	0	0.006	-	-	0.005	0.0025	+.0025
1	0.075	0.035	0.063	2.15	1.10	0.003	0.0010	+.002
2	0.173	0.113	0.160	1.53	1.07	0.0015	0.0015	.000
3	0.250	0.166	0.210	1.51	1.19	0.0110	0.006	+.005
5	0.356	0.250	0.315	1.42	1.13	0.013	0.006	+.007
6	0.725	0.525	0.66	1.38	1.10	0.040	0.010	+.03
7	0.770	0.58	0.732	1.33	1.05	0.043	0.015	+.03
10	1.10	0.88	1.11	1.25	1.00	0.073	0.020	+.05
14	0.882	0.71	0.882	1.24	1.00	0.050	0.017	+.03

Legend is on p 26

Table 2 : 3/3/65. Absorbancies and absorption ratios in the estimation of RNA and DNA content of groups of three explants cultured for various lengths of time. Extraction procedure was the Schneider modification of Schmidt-Thannhauser (p. 24) and u/v absorption of soluble ribonucleotides was measured at three wavelengths, while the intensity of colour of the Dische reaction product was measured at 2 wave lengths.

From the results presented in Table 2 it can be seen that there is not enough RNA present at Day 0 and Day 1 to bear significant comparison. From the remaining data it can be seen that:

(1) Contamination increases with age of culture. This is shown by the decreasing ratios both of 260/230 and of 260/240.

(2) Assuming that the absorption by contaminants is the same at 240 and 260 mμ in any given sample, (it is unlikely to be less) and that a "clean" ratio would be 2.5 it appears that about 50 per cent of the absorption at 260 mμ can be attributed to contaminants on Day 3. This value increases to about 70 per cent by Day 14, which confirms the increase in contamination with age.

(3) On Day 3 the 260/230 value compares favourably with those given by Deken-Grensen and Deken (1959) for Euglena extracts and tobacco extracts, while the 260/240 ratio is very similar to the Euglena value. This would suggest, not surprisingly, that the interfering substances in Artichoke have

different absorption characteristics, compared with those of Euglena.

(4) Within the limits of this method the best estimate of RNA is given by the difference between 260 and 240 mμ absorption values.

(5) The DNA values suggest that there is a sugar present that contributes enough colour at 650 mμ to balance the colour at 595 mμ. This also changes progressively with the age of the tissue. An increase in DNA and/or a decrease in fructose (during culture) would give the kind of results tabulated in Table 2.

It was concluded from this experiment that the problems in obtaining an accurate estimate of both RNA and DNA from the same sample are considerable. However, a rough estimate of RNA was obtained from the (260-240) mμ values. It appears that separate estimates of RNA and DNA might be necessary using separate samples of tissue. Such a procedure circumvents the difficulties of separating DNA from RNA at the expense of sacrificing a large amount of tissue. This approach is now being investigated by Mr. Evans in this department.

(c) Microdensitometric Method

Explants were fixed in 1:3 acetic alcohol, dehydrated through a tertiary butyl alcohol series, and embedded in paraffin wax. Sections were cut at 5Oμ, hydrolysed for 12 - 15 minutes, and stained in Feulgen Reagent for 1 hour, before mounting in Canada balsam on thin glass slides. The intensity of staining was estimated at 5,700°A using a Barr and Stroud integrating microdensitometer.

This procedure was carried out by Dr. Mitchell and has been described in full (Mitchell, 1965).

8. Investigation of the Anatomy of Explants

Samples were fixed in formalin acetic alcohol (50 per cent ethyl alcohol:glacial acetic acid:40 per cent formaldehyde in the proportions of 18:1:1). After dehydration in a tertiary butyl alcohol/ethanol series (Jensen, 1962) sections were embedded in paraffin wax, cut at 15μ, hydrated and stained in 1 per cent Delafield's haemotoxylon. Serial sections were mounted in Canada Balsam on thick glass slides.

9. Analysis of Proteins Using Disc Electrophoresis

(a) Fractionation Procedure

From each harvest explants were homogenised in 1 ml of grinding medium at pH 7.5. This medium was designed to maintain the integrity of cell particles as far as possible and contained: 0.5M sucrose
30 mM tris-2-amino 2(hydroxymethyl) propane 1:3 diol.
24 mM hydrochloric acid
1 mM magnesium acetate
50 mM potassium chloride.

Homogenisation was achieved using a motor driven teflon head in round-bottomed hard-glass centrifuge tubes. While artichoke material required prolonged homogenisation, care was taken not to allow the appearance of brown polyphenol compounds which signify oxidase activity in material which has been ground for too long.

A full fractionation schedule (Fig. 3) was followed, although only the supernatant of the final 140,000 x gravity spin was taken for protein analysis and this was stored in the deep freeze.

Fraction		speed (r.p.m.)	x gravity	duration
I	Cell wall and nuclear	2,500	500	15 m
II	Mitochondrial	11,000	12,500	15 m
III	Fraction X	17,000	30,000	30 m
IV	Free ribosomal	40,000	140,000	90 m
V	Supernatant			

Figure 3 : Schedule of fractionation procedure for callus homogenates.

Although fractions II, III and IV were used for a variety of preliminary studies not reported in this thesis, it may be recorded that while there was no detectable pellet of II, III or IV in fresh material, there were distinct pellets of mitochondrial, fraction X, and ribosomal fractions in homogenates of 1 day old tissue and in all older tissues.

(b) Electrophoretic Technique

The separation of proteins was performed by the method of Davis and Ornstein (1961) which has been developed by Miss Williams in this laboratory and full details will appear in her thesis. In brief, a small volume, 0.1 ml, of the protein solution was layered on top of two concentrations of acrylamide gel which had been chemically polymerised one on top of the other, in a length of glass tubing. In 0.5 cm of spacer gel (5 per cent acrylamide) the proteins sort themselves out according to their electrophoretic mobility, and become concentrated into discs. During electrophoresis these discs become further separated by being distributed along 4.5 cm of fine pore gel (15 per cent acrylamide) by virtue of their differential charge and their differential ability to move through a fine pore molecular mesh. Thus the proteins are

separated by a combination of their electrophoretic properties and their molecular size. After about 30 minutes, by which time a tracker disc of the dye bromophenol blue, which was layered onto each gel after the protein solution, has reached the end of the gels, the gels are removed from their glass tubes and stained in methanol acetic water (5:1:5) which contains 0.01 per cent Amido Black. This stain visualises the protein bands and the background stain is removed by de-staining in methanol acetic water (5:1:5).

(c) Visualisation of Enzyme Activities

Enzymes, provided they are not inactivated during extraction or separation, may be located along the protein scan by incubating the unstained gels with an appropriate substrate and a suitably insoluble coupling dye.

The position of acid phosphatase activity (and therefore of acid phosphatase enzyme) can be visualised if the gels are incubated at room temperature in a buffered mixture of 1 mg/ml of sodium α-napthyl phosphate and 1 mg/ml tetrazotised o-anisidine for 18 hours. Prolonged exposure to sunlight alters the dye colour so gels must be stored in the dark. Acetic acid, a common storage fluid for gels, extracts phosphatase activity from the gels, and so these gels were stored in distilled water.

Similarly, if gels are incubated with each of the different acids of the tricarboxylic acid cycle in the presence of 1 mg/ml of the dye Nitro BT, the appropriate dehydrogenase activities can be analysed. These enzymes are much more sensitive to elevated temperatures and to storage even at -20°C, and the visualisation of their activity was much less reproducible. This may also be due to the fact that these enzymes are normally located in the mitochondria and any activity found in the supernatant fraction must depend on the homogenisation procedure damaging the mitochondria. Nevertheless some activity was demonstrated

and the intensity of staining for both dehydrogenase and for phosphatase was recorded graphically by a scan of the colour intensity along the gels using a Joyce-Loebl Chromoscan.

10. Estimation of Enzyme Activities in Tissue Homogenates

(a) Phosphatases

The method used for the estimation of phosphatase activity in Jerusalem artichoke tissue was a modification of the technique used for bean root tissue by Robinson and Brown (1952). In principle the technique is to disrupt explants by freezing or by homogenising and then to estimate the amount of inorganic phosphate released by the incubation of homogenates with an appropriate substrate. The inorganic phosphate released was estimated by a modification of Allen's technique (1940) and depends upon Osmond's original (1887) observation that a blue colour, proportional to the molybdate dissolved, is produced by phosphomolybdic acid under reducing, acidic conditions. This method has since been developed by a number of workers (Taylor and Miller, 1914; Bell and Doisy, 1920; Fiske and SubbaRow, 1925; Martland and Robinson, 1926; King, 1932).

In detail, the technique was to take 3 or 9 explants from each harvest. Estimations were always performed in triplicate on one or three explants each. Explants were either homogenised on harvesting, (Experiments 26, 30 and 50) or were frozen at -20°C and allowed to accumulate until the 21st day after inoculation and then their activity was estimated on that day, (Experiment 33 and series 3). Each explant or group of 3 explants were homogenised in a ground glass Potter-Elvehjem homogeniser in 2 ml of buffer. The homogeniser was rinsed with a further 2 ml of 0.2M tris-maleate buffer of pH 6.2 and the two volumes were incubated at 25°C together with 1 ml of 1 per cent sodium β-glycerophosphate as substrate. The reaction was stopped after 60 or 90 minutes by the addition of 0.8 ml of 60 per cent perchloric acid. In addition to stopping enzyme activity and helping

to remove protein, the perchloric acid also brings the reaction mixture to the correct pH. The debris of the homogenate was then centrifuged down and the supernatant diluted to 15 ml with distilled water. Thereafter, 0.4 ml of 8.3 per cent ammonium molybdate was added followed by 0.8 ml of amidol reducing agent. Amidol reagent consists of a fresh preparation of 2g sodium metabisulphite plus 0.1g of amidol (2:4 diamino-phenol hydrochloride) in 10 ml of distilled water. Amidol, ammonium molybdate and phosphate complex to form a blue solution. The intensity of the blue colour was read using the Tungsten lamp and red photocell of the Unicam Spectrophotometer, at 725 mμ. Estimations were zeroed using a blank containing all the reactants except the homogenate. This optical density value was converted to μg of inorganic phosphate released per plant per hour by reference to a calibration curve.

In order to adapt the method for artichoke material several conditions had to be established and these will be dealt with in the sequence in which they appear in the method. The method of estimating phosphate is exceedingly sensitive and so the minimum amount of tissue which may be usefully employed depends only upon taking a large enough sample to allow for the natural variation amongst explants. To estimate the extent of this variation, 15 explants were assayed individually and, while the coefficient of variation in fresh weight was 4 per cent, the coefficient for phosphatase activity was found to be 10 per cent. Accordingly, it was concluded that, at any one time, the mean of three individual assays would provide a good estimate of the phosphatase activity in a population of explants.

The effects of freezing and thawing are not fully understood. Bieleski (1964) has shown that plant acid phosphatase can still function at -28°C in the frozen state, and also in 40 per cent methanol. However, this activity is only of the order of 1 per cent of that found at 25°C. It is well known that in animal tissues, acid phosphatase is commonly found in the lysozome fraction of

homogenates (de Duve, 1959) and indeed in lysosomes (Novikoff, 1963). Freezing and thawing tissues will release the acid phosphatase by rupturing the lipid membrane of the lysozome thus permitting substrates and enzymes to meet. However no fractionation data has come to our notice suggesting that lysozomes extend to the plant kingdom although Gahan (1965) has described them from histochemical data and Holcomb, Hildebrandt and Evert (1965) have described round bodies called "spherosomes" which have acid phosphatase activity. Table 3 shows the results of an experiment using fresh explants to examine the effect of a single freeze and thaw cycle on the phosphatase activity.

TREATMENT	ACTIVITY	
	(a) O.D. per 3 explants	$=^{t}$(b)µg P_i per hour per explant.
Fresh	0.55	1.5
Frozen for 60 minutes	0.50	1.3
" " 24 hours	0.47	1.3
" " 14 days	0.32	0.9

Table 3: 22/10/64. A comparison of the phosphatase activity in fresh explants with that in explants frozen for various lengths of time at +15° to -22°C. Values depend upon triplicate estimates using groups of 3 homogenised explants. P_i = inorganic phosphate.

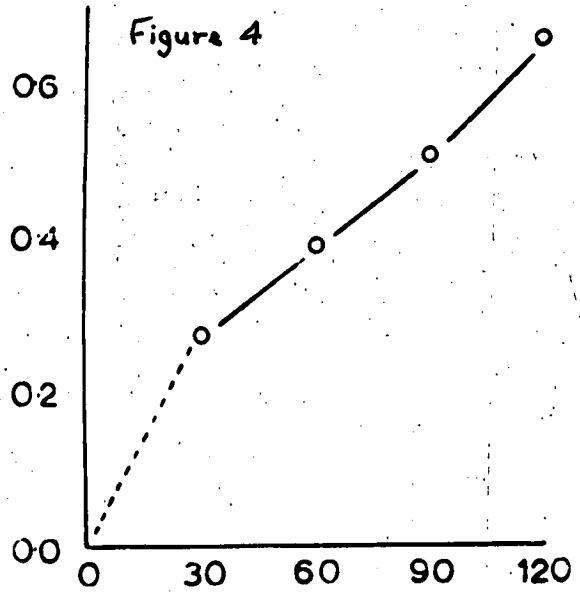
The results presented in Table 3 suggest that there was no "latent" activity and that prolonged freezing reduced the activity by some 40 per cent.

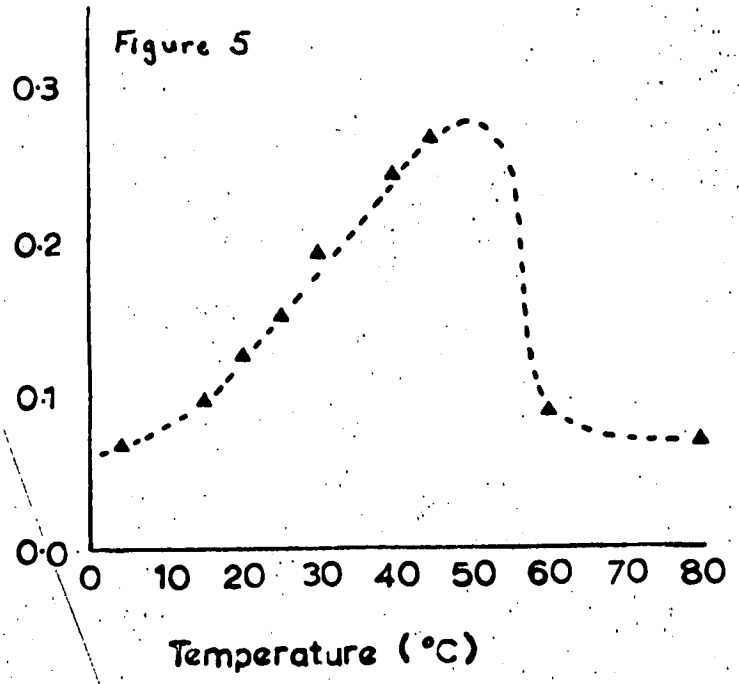
Thus, either an assay must be performed on the day of each harvest or some compromise must be found. Two compromises were chosen. Firstly, explants were assayed fresh at each harvest and the determination carried through to the point where the enzyme preparation was killed at the end of incubation and then all the samples were preserved at room temperature until the final harvest date. On this day all the estimations were completed using a common set of reagents, controls and blank solutions. This is the most accurate method but is still time consuming. Secondly, since the activity had been found to decrease during freezing, any remaining increases might be expected to be significant if explants are allowed to remain frozen for between 7 and 21 days and then assayed at the same time on the 21st day after inoculation. This compromise is less satisfactory because the effects of cold storage on different ages of callus have not been ascertained but it was thought reasonable to use this simplified method to demonstrate the seasonal trends in phosphatase activity (series 3, p.88).

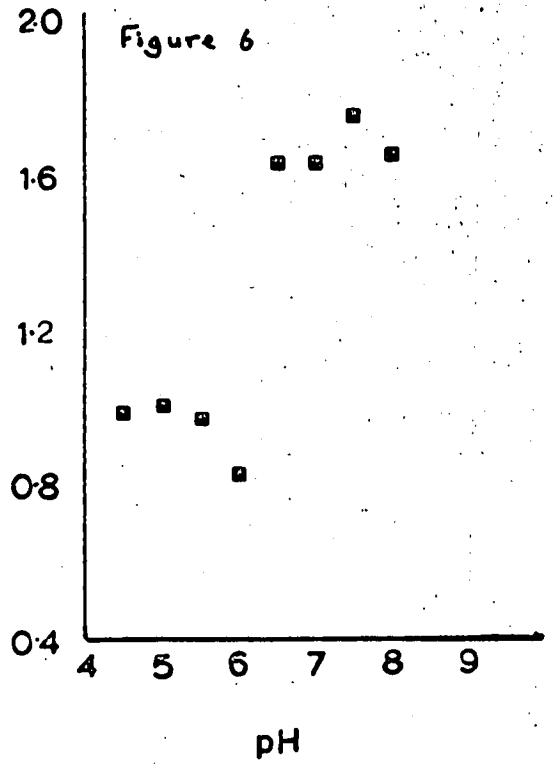
Since homogenisation takes some time it is convenient if explants can remain thawed for various lengths of time. Thawed explants held at room temperature for up to 210 minutes showed little change in activity.

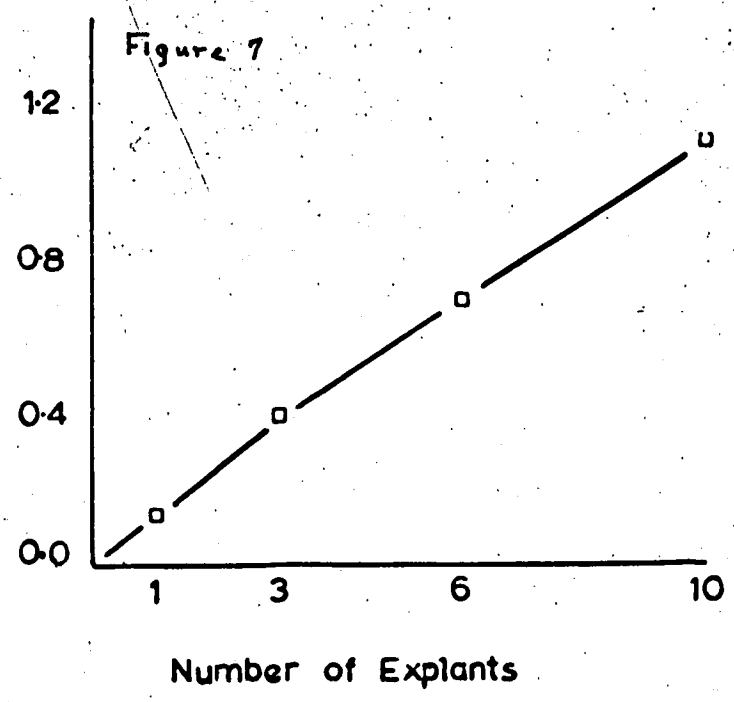
Because silicon dioxide will also complex with molybdic acid to give a blue colour it was necessary to check that no such particles were produced by the grinding process. No increase over controls was found.

Although Robinson and Brown (1952), following the practice of Bottelier, Holter and Linderstrom-Lang (1943) found it unnecessary to homogenise their material, it was found that with fresh explants homogenisation gave a large increase in activity over intact explants. Moreover as the callus increases in size during the experimental period the length of the diffusion path between the enzyme and substrate will change and the characteristics of membranes of the cell









Figures 4 to 7. All units of the axis are Optical Density values. The figures show the relationship between phosphatase activity and

(i) the duration of incubation of the reaction mixture (Figure 4),

(ii) the temperature of incubation (Figure 5),

(iii) the pH of the reaction mixture (Figure 6),

(iv) the amount of tissue in the mixture (Figure 7).

Values were determined using phthalate buffers for the four lower values and tris maleate buffers for the four higher values.

organelles may also alter, so it was thought desirable to homogenise all calli. The fact that fresh, whole, 8 mg, explants have a 15 per cent higher activity per mg of tissue than do fresh 35 mg explants supports this contention. However, in certain preliminary experiments whole explants were used as this is simpler, and the objections cited above do not apply to comparisons amongst explants of uniform age and of uniform size.

By estimating the phosphate present before and after grinding in the absence of substrate on both fresh explants and mature calli it was shown that there is no significant release of phosphate from fresh tissue on homogenisation. There was a measurable appearance of phosphate in calli during development but since this was less than 5 per cent of the phosphate released during the enzyme assay, it did not interfere with the estimation of phosphatase activity. The sample of β-glycerophosphate used throughout these experiments had 0.2 per cent inorganic phosphate and this standard amount of impurity was used as an artificial zero for all estimations and also served as a check that the assays were uniform from one series to the next. It was found that standard solutions of glycerophosphate increased in inorganic phosphate percentage unless the solution was stored in a refrigerator.

In order to establish that enzyme activity is proportional to the duration of incubation, triplicate groups of three explants were frozen, thawed, homogenised, and incubated for a variety of times. The results, plotted in Figure 4, show that for periods of inoculation between 30 and 120 minutes the increase in incubation is proportional to the enzyme activity. Both 60 and 90 minute periods were used according to which time was most convenient.

Similarly triplicate groups of 1, 3, 6 and 10 explants were frozen, thawed and homogenised, and incubated for 60 minutes. The results plotted in

Figure 7 show that the amount of activity recorded is proportional to the amount of tissue. Thus there is a quantitative recovery of activity for a ten-fold difference in the quantity of tissue assayed.

In addition, phosphatase activity was estimated at many temperatures from 4° to 80°C, using a 60 minute incubation period. For the 4°C incubation chilled test tubes were returned to a cold room, incubators at 25° and 30°C were employed, and the remaining values were determined using constant temperature water baths. It may be seen from the results plotted in Figure 5 that there is appreciable activity at both 6° and 80°C, and there is an optimum between 45° and 60°C. Because this enzyme is known to increase in activity with temperature and yet to be denatured increasingly with time at elevated temperatures, it will be appreciated that the temperature optimum will therefore decrease with more prolonged incubation times. For this reason, having confirmed that the phosphatase activity being measured here is a typical hardy example of that group of enzymes, the physiological temperature of 25°C was chosen for all incubations, especially as it had been shown that at this temperature after 90 minutes of incubation there is no decrease in activity (Fig. 4).

The pH optima of phosphatases vary with the substrate and with the specific source of the enzyme. It had been shown in this laboratory that the pH optimum for several substrates, using pea root tissue is 6.2 (Dixon, 1962). An attempt to define a pH optimum for artichoke tissue gave the results recorded in Figure 6. Here it can be seen that the values estimated using tris-maleate buffers are approximately double those using pthalate buffers irrespective of the pH. Using pthalate buffer Brown and Robinson (1952) measured the activity at pH 4.0. However, because of the higher activity found using tris-maleate buffer, and because there appears to be considerable activity at all the hydrogen ion concentrations examined, it was decided to estimate the activity at pH 6.2 which is

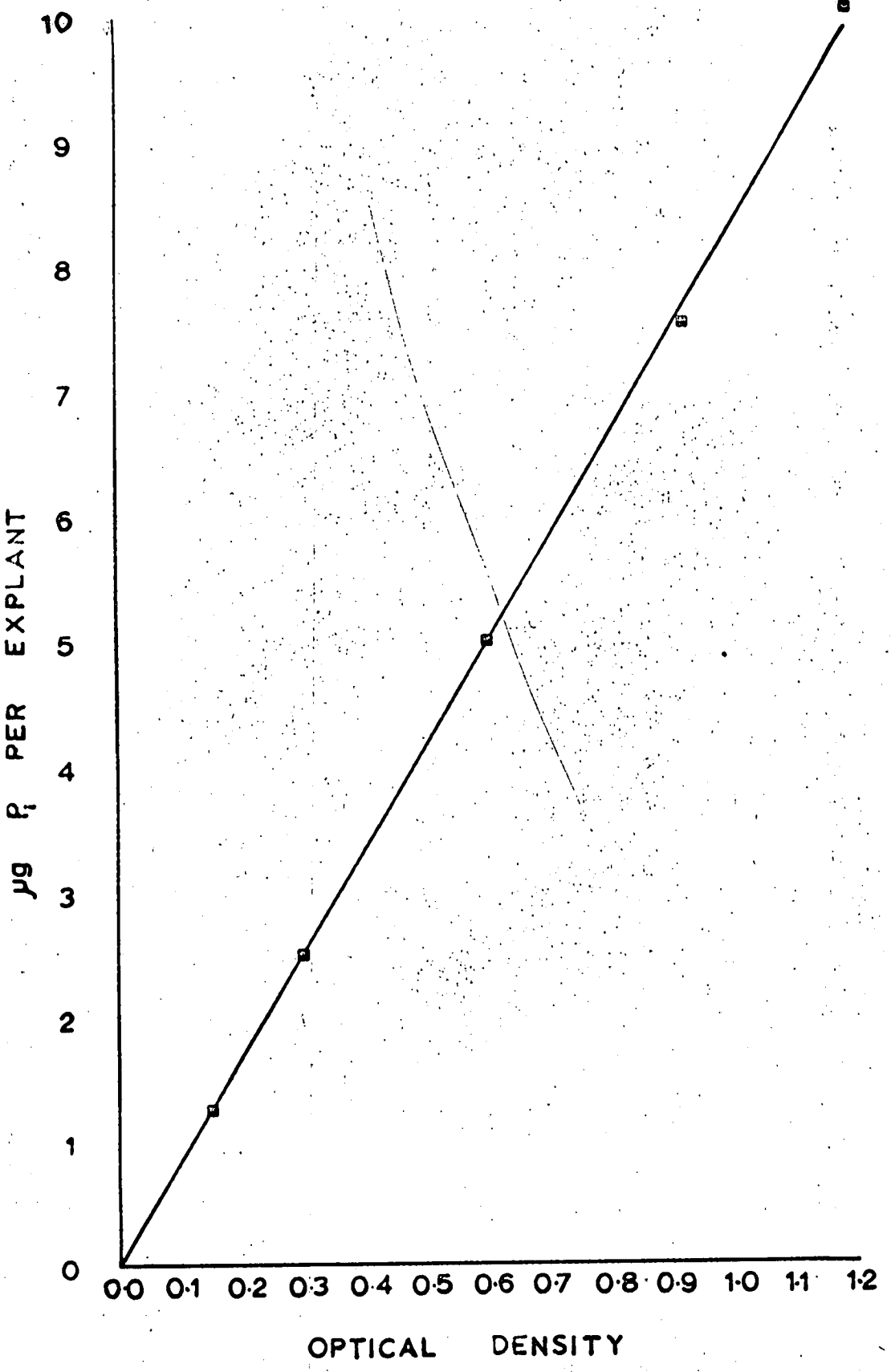


Figure 8. Calibration curve showing the Optical Density of standard solutions of inorganic phosphate (P_i) over the range 0 to 10 µg.

also the average pH of the culture medium.

In order to define a calibration curve for the inorganic phosphate concentrations produced by hydrolysis of the phosphate ester bond, standard potassium dihydrogen phosphate was diluted appropriately at concentrations between 0 and 10 µg per ml. There is linear proportionality over an Optical Density range of 0 - 1.2 units (Fig. 8). Values per ml were read off and converted to values expressed as µg per explant per hour of incubation. The results presented in Table 4 show that the colour developed is stable between 10 and 40 minutes after the time of mixing the reagents. It also shows that the greater the activity being assayed the sooner the stable colour begins to deepen, but the time limit given permits up to 100 mg of tissue to be assayed accurately.

TIME (m)	½	1	1½	2	4	6	8	10	20	30	40	50	60	120
3 explants				14	14	14	14	15	15	15	15	15	15	15
5 "	21	28	28	29	30	30	30	30	30	30	30	30	30	31
10 "		53		58	58	58	59	59	59	59	59	60	60	62

Table 4. A comparison of the rate of colour development using different quantities of tissue, showing that the colour is stable between 10 and 40 minutes. All values are in O.D. units x 100.

It was established initially that controls of the boiled tissue and of the perchloric acid killed homogenate showed no activity. In Experiment 26, which had harvests at 0, 2, 5, 7, and 14 days, triplicate controls which lacked substrate

and also controls which lacked substrate and were not incubated, were carried out and this showed that there is a slight amount of inorganic phosphate accumulated in the callus as it develops and incubation of the homogenate releases a further small amount. These amounts are negligible compared with the phosphate released by enzyme activity. In routine assays these controls could not be applied because this would require very large numbers of cultures, and so triplicate determinations were recorded without controls.

(b) Ribonuclease

The activity of this enzyme was measured by a modification of the method of Robinson and Cartwright (1958). This involves incubating whole explants with a substrate of yeast nucleic acid and measuring, by u/v spectrophotometry, the acid soluble nucleotide fragments produced.

The procedure was to freeze and thaw whole calli, rinse them three times in distilled water, and then incubate single explants in triplicate with 1 ml of 0.5 per cent dialysed yeast nucleic acid in 0.02M phosphate buffer at pH 6.0 for an hour at 40°C. The reaction was stopped, by the addition of an equal volume of 5 per cent trichloracetic acid (TCA), containing 0.5 per cent uranyl acetate as coagulant, to the chilled centrifuge tubes. This reagent (McFadyen, 1934) also precipitates the large molecules of yeast nucleic acid, leaving the hydrolysed products of ribonuclease activity in solution. The precipitate was removed by centrifugation and the soluble nucleotides diluted to 10 or 20 ml for their Optical Density (OD) to be measured at 260 mμ using the u/v lamp of a Unicam spectrophotometer. As TCA has an appreciable absorption at this wavelength, control solutions which did not contain calli were taken through the whole assay procedure and this value was subtracted from each determination to give the O.D. attributable to the nucleotide fragments released by ribonuclease activity.

Robinson and Cartwright (1952), using segments of bean root tissue, showed that the rate of reaction remains constant with time and that reaction rate is proportional to enzyme concentration. The justification for freezing and thawing as a means of destroying the integrity of the tissue has been discussed (Robinson and Brown, 1952, see p. 31). In this case it would have been preferable to homogenise each explant for the reasons discussed in connection with the assay of phosphatase activity, but it was found that not only did the presence of homogenate debris alter the efficiency of the precipitation of substrate by TCA, but also that this alteration changed with the age of the culture. This was perhaps due to the increase with time in the amount of debris but may also involve changes in connection with the maturation of the callus. An example of this effect which is representative of the whole range of harvests from 0 - 21 days is shown in Table 5.

No of calli per value	Treatment		Triplicate values (O.D. units)			Mean Value
	Incubation time	% of substrate				
0	0	0	.89	.88	1.06	0.94
3	0	0	.79	1.09	1.10	1.00
0	0	0.5	.84	.82	-	.83
0	60	0.5	.84	.82	.81	.82
3	60	0	1.13	1.14	1.13	1.13
3	60	0.5	1.04	1.03	1.02	1.03

Table 5: D7 values from Experiment 29.
16/1/64. Assay for ribonuclease activity.
Calli were homogenised and incubated with yeast nucleic acid as substrate. The blank cuvette contained water.

It may be seen from the results presented in Table 5 that the absorption due to trichloracetic acid is 0.94 and that, while the presence of unprecipitated material from the homogenate may increase this blank value, the removal of some TCA from solution by precipitation with the excess substrate reduces the blank value. Incubation of the substrate does not change this reduced value. However incubation of the homogenate in the absence of substrate does increase the value suggesting that native ribonucleic acid may be being hydrolysed. Finally, if substrate and homogenate are incubated together, the effect of reduction due to removal of TCA and increase due to solubilisation of nucleotides result in an increase of 0.2 over the blank TCA value.

Because so little is known about the changing concentration and state (e.g. bound or unbound) of the native ribonucleic acid this could not be used as an assay substrate, and because of the undefinable nature of the interplay of the two contributions to absorption described above, it was concluded that it was not possible to devise suitable control assay conditions using homogenised explants.

It may be emphasised that, because the tissue at Day 0 is relatively inactive and quiescent, very small amounts of activity require to be measured and therefore a highly critical aproach has to be adopted. Activities in, for example, pea root tissues, were found to be of the order of 10 - 100 times greater than that of fresh artichoke explants. As a result many of the exigences, which can be ignored for active tissues because the activity due to the enzyme is very high, must be carefully investigated for artichoke material.

Having found that effective precipitation of excess substrate is vital to this assay, the optimal conditions were sought. At the concentration recommended by Robinson and Cartwright (1952) which was a final concentration of 1.25 per cent TCA, the blank value was found to increase in proportion to the concentration of the

substrate added. This is shown in Table 6.

Water ml	1% YNA ml	2.5% TCA ml	Final Conc^{n}. of TCA	Final Conc^{n}. of YNA	O.D.
1.75	0.25	2	1.25	0.006%	0.18
1.50	0.5	2	1.25	0.013%	0.55
1.0	1.0	2	1.25	0.025%	1.23
0.0	2.0	2	1.25	0.050%	2.58

Table 6. A comparison of O.D. obtained with four concentrations of substrate. In all cases uronyl acetate was present at 0.125 per cent. The blank cuvette contained 2 ml of 2.5 per cent TCA plus 2 ml of water.

If, however, the percentage of TCA in the reaction mixture is varied, it can be seen, from the results presented in Table 7, that the recorded O.D. decreases as the percentage TCA increases.

Water ml	1% YNA ml	2.5% TCA ml	Final Conc^{n}. of TCA	Final Conc^{n}. of YNA	O.D.
1	1	0.5	0.50	0.040	2.75
1	1	1.0	0.83	0.033	1.98
1	1	2.0	1.25	0.025	1.28
1	1	4.0	1.66	0.017	0.65

Table 7. A comparison of the O.D. obtained with four different concentrations of TCA and of YNA. Uranyl acetate was present at a concentration of one tenth that of TCA. Control values in the

absence of YNA were subtracted from the final O.D. given in column 6.

It was found that dialysis of the substrate against equimolar buffer reduces the blank values, and also that the precipitation characteristics vary with the sample of substrate YNA. Nevertheless an extrapolation to zero of the four values found in Table 7 suggest that 2.5 per cent TCA is the best final concentration of TCA. Using dialysed substrate, and a final concentration of 2.5 per cent McFadyen's Reagent, satisfactory low blank values were obtained and this method was therefore used. Standing at room temperature followed by centrifugation for 15 minutes at 2,500 r.p.m. was found to be ideal for maximum precipitation.

The pH optimum for ribonuclease activity is given by Robinson and Cartwright as 6.0 and this was confirmed in a preliminary experiment with artichoke tissue.

In defining a curve of activity at various temperatures it was found that TCA hydrolyses an alarming amount of substrate and that this amount increases with elevated temperatures. These results are shown in Table 8.

Temperature of incubation	O.D. of blank	O.D. of samples	O.D.
25°	.250	.250	.000
45°	.285	.300	.015
60°	.315	.370	.055
75°	.330	.390	.060
90°	.395	.415	.020

Table 8. A comparison of the O.D. recorded at 5 temperatures of incubation when the TCA is added

to the samples at their incubation temperatures.

Blank values consist of the complete assay, carried out without explants.

A subsequent experiment in which the blank values in Table 8 were repeated and compared with blanks which were chilled for 2 minutes in the deep freeze prior to addition of cold TCA gave, for each temperature, minimum blanks of approximately 0.19 O.D. units.

The results in Table 8 therefore confirm that ribonuclease is another hardy enzyme with an optimal activity during a 60 minute incubation, of between 60° and 75°C. In all subsequent experiments TCA was added, cold, to chilled incubation mixtures.

Fresh explants and mature calli, which were boiled for 2 - 5 minutes, were found to have no activity, but this control could not be carried out in the time course experiment due to the paucity of material.

(c) Invertase

The activity of invertase was measured by incubating the tissue with 2 per cent sucrose buffered at pH 6.0 and assaying for reducing sugar by the method of Hagedorn-Jensen (Strepkov, 1937). The excess alkaline ferricyanide remaining after reaction with reducing sugars was measured by back-titration of potassium iodide with sodium thiosulphate.

This method has been used routinely in this department (e.g. Robinson and Brown, 1952; Vaughan, 1966) and has been found satisfactory using a variety of tissues. A preliminary experiment measuring the activity present in pea root tissue confirmed that the method was efficient.

(d) Inulase

The activity of this enzyme was measured by the substitution of 0.5 per cent inulin for 2 per cent sucrose as substrate in the method for determining invertase activity described above. Buffer was ommitted and the activity estimated at approximately pH 6.0 (Green, 1888).

(e) Succinic dehydrogenase

The activity of this enzyme was measured by what is basically the method of Hiatt (1961) which had been modified from that of Ells (1959). It depends upon the reduction of 2:6 dichlorophenolindophenol by a preparation of the enzyme in the presence of phenazine methosulphate (PMS), a compound which couples electron transfer between reduced pyridine nucleotides and 2:6 dichlorophenolindophenol (Ells, 1959). The loss of colour in the dye may be followed spectrophotometrically (Price and Thimann, 1954).

The experimental cuvette contained the following constituents in the final volume of 3 ml.

Solution A:
- 5×10^{-2} M potassium phosphate buffer, pH 7.4
- 4×10^{-2} M sodium succinate as substrate
- 3×10^{-5} M sodium 2:6 dichlorophenolindophenol
- 1×10^{-2} M potassium cyanide

Cyanide, according to Hiatt (1961) continuously reduces the disulphide groups formed by the action of hydrogen peroxide on the thiol groups of the dehydrogenase and so increases the recorded activity by some 60 per cent. The peroxide arises from the reoxidation by oxygen of some of the reduced N-methyl phenazonium sulphate.

Solution B: 0.3 mg/ml of methyl phenazonium sulphate added separately to the cuvette.

Solution C: 1 ml of crude enzyme extract added finally to start the reaction.

Solution A was stored cold at 3 times the strength indicated. Solution B was freshly made up each time at 3 times the strength indicated. Solution C was freshly prepared for each harvest. The experimental cuvette contained 1 ml of each of the three solutions.

The enzyme extract was prepared by taking the supernatant fraction containing mitochondria and all lighter particles and debris after a homogenate of three explants had been centrifuged, cold, at 2,500 r.p.m., for 15 minutes to remove the nuclear and cell wall fraction.

The homogenate was obtained by grinding explants in a ground glass homogeniser in 4 ml of the following grinding medium:-

0.4 M sucrose

0.1 M phosphate buffer pH 7.4

0.02 per cent bovine serum albumin (Heyes, 1963)

0.01 mM magnesium

This medium was designed to maintain as far as possible the integrity of the particulate organelles in the homogenate.

The loss of colour in the experimental cuvette was automatically recorded during a period of 2 minutes using a Joyce-Loebl Chromoscan with appropriate filters to measure the colour intensity at 595 mμ.

Although Hiatt (1961) found it possible, using a purified enzyme extract, to measure the activity at room temperature, this temperature was found to rapidly inactivate the crude extract. This is shown by the results presented in Table 9. The extract, in a cold centrifuge tube, was allowed to stand at room temperature for 30 minutes and activity was measured on aliquots on three occasions.

Time at room T° since fractionation	Dehydrogenase Activity
3 m	11
15 m	4
30 m	0

Table 9. The effect upon enzyme activity of standing the preparation at room temperature for up to 30 minutes. Activity is recorded as the change in Optical Density recorded by the Chromoscan between 15 and 45 seconds after mixing the reagents. The extract was obtained from a single 42 day old callus.

It was concluded from the results in Table 9 that there is a rapid loss of activity by the extract if it is allowed to stand at room temperature.

A similar experiment was performed to see if activity is also lost when the extract is allowed to stand at 0°C. The results of this experiment are found in Table 10.

Time at 0°C since fractionation	Dehydrogenase Activity
2 m	18
15 m	15
30 m	5

Table 10. The effect upon enzyme activity of standing the preparation at 0°C for up to 30 minutes. (P.T.O.)

Activity is recorded as the change in Optical Density recorded by the Chromoscan between 30 and 60 seconds after mixing the reagents.

The results presented in Table 10 show that there is some slight loss of activity during the first 15 minutes due to oxidation of the enzyme preparation during standing, and that this loss was greatly increased during the subsequent 15 minutes. For this reason the activity of all preparations was estimated within 15 minutes of fractionation.

Initially all reagents were stored cold and used cold, but this arrangement still gave some inconsistent results as both the reaction cuvette and control cuvette adjusted to room temperature during the recording of results. Finally the reaction solutions, (A and B) were brought from cold storage to 25°C using a water bath and the enzyme preparation (C) was taken from an ice jacket and added, cold, under standard conditions. This was found to give consistent and reproducible results. A blank experimental cuvette using these conditions in the absence of enzyme extract showed no change in Optical Density during 5 minutes. Before the activity in each experimental cuvette was recorded, such a blank cuvette was used to zero the Chromoscan pen at a convenient position on the recording paper.

Hiatt (1961) established that the enzyme activity of his purified extract was insignificant when succinate or PMS was ommitted from the reaction cuvette; that the rate of reduction of 2:6 dichlorophenolindophenol was proportioned to enzyme concentration over the range used in his studies; and that no activity was obtained with a boiled enzyme extract.

In order to confirm Hiatt's finding that the reduction of the dye is

proportional to the enzyme activity, four, 70-day old calli, weighing 100 mg each, were homogenised and serial dilutions of the fractionated preparation were assayed for activity. The results are presented in Table 11.

Enzyme Concentration =t mg tissue/cc	Dehydrogenase Activity
50	26.5
25	13
12.5	5.5
6.3	4.5
3.1	0

Table 11. The effect of increasing enzyme concentration upon the activity recorded. Activity is measured by the change in Optical Density units for the interval 15 - 45 seconds after adding the extract.

The results presented in Table 11 demonstrate that the activity found in these crude extracts is proportional to the amount of tissue from which they were fractionated. In this experiment boiled enzyme controls showed zero activity as did control reaction mixtures which ommitted PMS. A control which lacked substrate from Solution A gave some activity which was rather variable, suggesting that the extracts contained a small amount of native succinate (cf. Jensen, 1962).

In order to define the range of variation found in the activity of this enzyme, fresh explants were homogenised and their activity measured. Groups of 1 or 3 explants showed no measurable activity but a group of 10 gave a change in

O.D. of 4 units. This value is too low for accurate comparisons so four 70 day calli were homogenised individually and their succinic dehydrogenase activity measured. The results are presented in Table 12.

F. Wt. of 70 day callus	Replicates of Dehydrogenase Activity	Mean of Replicates
98.2	-19	-19
89.0	-19, -19	-19
94.9	-12, -17, -20	-16
84.3	-16, -16, -19	-17

Table 12. A comparison of the activity found in four explants of similar fresh weight. Where possible, three replicate values were determined. Activity is recorded as the change in O.D. for the interval 15 - 45 seconds after adding the extract.

From the results shown in Table 12 it was concluded that replicates of single explants would be adequate for these determinations provided the explants showed activity sufficient to give a measurable change in O.D. units.

11. Localisation of Enzyme Activities in Sections by Histochemical Techniques

(a) Phosphomono-esterase II - Gomori Technique for Acid Phosphatase

The method used was that of Gomori (1952) cited in Jensen (1962). It depends upon the ability of acid phosphatase to remove a phosphate group from the

substrate β-glycerophosphate. This phosphate combines with lead ions and the lead phosphate is converted to insoluble black deposits of lead sulphide in the presence of ammonium sulphide. The validity of this procedure when localising activity within the cell is controversial but when used at the tissue level, it has gained wide acceptance (Jensen, p.336, 1962).

Sections 100μ thick were cut from explants using a freezing microtome. Within three minutes the sections, floating in a 2 per cent calcium chloride solution to minimise diffusion of the enzyme, were transferred to an incubation medium at 25°C. This medium consisted of 0.2M acetate buffer of pH 5.0, containing 1.32 mg/ml of lead nitrate (0.04M) and 0.32 mg/ml of the substrate β-glycerophosphate. After various periods of incubation a section was removed, rinsed, and the reaction product developed in dilute ammonium sulphide, rinsed again and the section finally mounted in glycerin jelly. Because the precipitate of lead phosphate will only form after a given concentration of free phosphate has been liberated, the optimum incubation time varied with the enzyme activity, and so a series of sections were examined at intervals. The optimum length of incubation (as shown by the maximum contrast between test and control section) varied with the age of the tissue. Thus fresh explants failed to give a reaction after 20 hours of incubation while 5 day old callus gave a heavy deposit after 2 hours and a distinct reaction could be detected after only 20 minutes. Jensen (1962) recommends 30 minutes to 18 hours at 37°C.

The controls consisted of:-

(a) Tissue incubated in medium minus substrate

(b) Tissue incubated in medium containing 0.01M sodium fluoride

(c) Tissue placed in ammonium sulphide solution without being placed in substrate solution.

(d) Heat killed tissue, which had been boiled 3 minutes, and was then carried through the whole procedure.

When these controls were applied, in every case a yellow-brown deposit was found which suggested the presence of some free phosphate in the explants and perhaps also in the substrate. This is, of course, paralleled by the biochemical data (p.). Thus, the sites of acid phosphatase activity are shown by regions of black deposits of lead sulphide in contrast to the ubiquitous diffuse yellow precipitate found in all the control sections.

(b) Phosphomonoesterase II - Azo-Dye coupling technique.

In this alternative method for locating the regions of acid phosphatase activity, the method of Grogg and Pearse (1952) was followed. It is based on the liberation from an artificial substrate, of a phosphate group which is immediately coupled with a diazonium dye which then precipitates in situ. Many combinations of substrate and of dye are possible but in this case, following Grogg and Pearse (1952), sections were immersed for various periods in a solution of 0.5 mg/ml α-napthyl phosphate and 0.3 mg/ml of diazotised o-anisidine in 10 ml of 0.2M acetate buffer at pH 5 and 25°C. The deep purple colour indicating a positive reaction contrasts sharply with the pale yellow breakdown products of o-anisidine.

The controls consisted of:-

(a) tissue incubated in medium minus substrate.

(b) tissue incubated in medium containing 0.01M sodium fluoride.

(c) tissue placed in tetrazotised o-anisidine without first putting them in α-naphthyl phosphate.

(d) heat killed tissue, boiled for 3 minutes, and taken through the whole procedure.

In each case the yellow colour appeared but no purple reaction product. Fluoride, however, was not found to be entirely inhibitory.

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(c) Phosphomono esterase I - Azo Dye coupling Technique.

The localisation of alkaline phosphatase activity was performed using the method for acid phosphatase but substituting 0.2M glycine -NaOH as buffer at pH 9.4 and using longer incubations. The reaction product was an orange colour and required careful comparison with control sections before a positive result could be affirmed.

(d) Succinic De-hydrogenase - Reduced Tetrazolium Salt Technique.

The method used for this enzyme is that of Nachlas Tsow De Souza Cheng and Seligman (1957) described by Jensen (1962). Hand-sections from fresh tissue were incubated "in air" at 37°C in a few drops of a solution of 0.2M phosphate buffer containing 0.2M potassium succinate as substrate and 1 mg/ml of Nitro BT (2'2' diphenyl nitro phenyl 5, 5' diphenyl 3, 3 (3 3' dimethoxy 4, 4 biphenylene) ditetrazolium salt) as an electron acceptor. This tetrazolium salt forms an insoluble coloured formazan in its reduced form.

Although this reaction was found to be satisfactory using hand sections of young pea roots, the level of activity was found to be considerably less in callus tissue. In comparable sections of callus tissue little reaction could be observed when material was immersed in the incubation mixture at 25°C. However, when such sections were incubated at 37°C in only a few drops of reaction mixture which was renewed from time to time, reproducable results were obtained. The drying out and replenishment of incubation mixture alters the concentration of the reactants but, because all the tissue in any given section receives the same treatment, and because the feature of interest here is the localisation of some cells with more succinic dehydrogenase activity than others, this variation in reactant concentration was admitted as justifiable.

The absence of substrate was found to make little difference to the

reaction which confirms the report of Jensen (1962) that plant material may contain a supply of succinate within its tissues.

A deep blue colour indicated regions of high succinate dehydrogenase activity, while on occasion (in pea-root sections, but not in callus tissue) the pink monoformazan colour was also observed.

12. Electron Microscopy.

The preparation of electron micrographs was kindly carried out by Mr. Tulett of this Department. Fresh explants, or cultures grown for 72 hours, were fixed in cold 6 per cent glutaraldehyde in phosphate buffer at pH 6.7, post-fixed in either 2 per cent osmium tetroxide or 2 per cent potassium permanganate and embedded in Araldite (Luft, 1961). Sections were cut at 0.8 μ on a Huxley microtome and the osmium treated material was stained with uranyl acetate and lead citrate according to the procedure of Reynolds (1963). Grids were examined in an AEI EM6 microscope.

EXPERIMENTAL RESULTS SECTION I

THE METHOD OF CULTURE

SECTION IA - The development of the Method.

Introduction.

The object of the experiments reported in this section was to develop a method of liquid culture for whole explants, isolated from plant material, which would satisfy the following provisions:-

1. The experimental material must be such that a large amount of uniform clonal tissue is available for each experiment.

2. The experimental material must be able to withstand prolonged storage of up to twelve months and still provide a comparable growth response to a given set of conditions.

3. The technique must be aseptic and yet rapid enough to permit a large number of explants, of the order of 200, to be set up in a short space of time.

4. The yield of cultured tissue must be high.

5. Variation amongst cultures, within each experiment, must be low.

Throughout this work, the experimental method was precisely the same except for one modification, and it will be shown later that this change did not have a significant effect upon the growth response of the cultures. Therefore in this and the succeeding experimental sections, "The Standard Procedure" will refer to a particular method of inoculation, culture and harvesting, which has been set down previously (p.9-13). In every experiment which involved a variety of treatments, a control employing the Standard Procedure was carried out. The various treatments

will be described with the experiments concerned.

In this first Section, single features of the system were taken and their variation and yield examined by comparing the fresh weight achieved after 14 days of culture. This length of time was chosen as a standard for all comparisons because data of Yeoman (1962, published in Yeoman, Dyer and Robertson, 1965) had shown that, at Day 14, cultures were emerging from their most rapid period of growth and significant differences between treatments could best be recognised at this time. During the course of these early experiments results were recorded and experience gained which had some bearing upon the first three requirements of the method and so these will be considered first.

Provisions 1 and 2: Choice of material.

The starting point of this study was that Yeoman (loc. cit.) had shown, in the course of his growth experiments, that explants from tubers of Helianthus tuberosus L. var. fuseau grow satisfactorily at 26°C on a medium solidified with 1 per cent agar. The medium was that described as Medium A in this study.

It was immediately recognised however that tubers of this variety seldom weigh more than 30 g and that in order to withdraw 200 uniform explants, it would have been necessary to use more than one tuber. Although clonal material is genotypically the same, each tuber may be expected to have some phenotypic differences which may result in differences in growth potential. This, on "a priori" grounds, was undesirable, especially as Steward and Caplin (1952*) had shown that phloem explants taken from various carrot roots could vary as much as 300 per cent in their response to culture. For this reason, H. tuberosus cultivar. "Bunyard's Round" was tested and found to give a similar response to that of var. fuseau. Many tubers of this variety can be collected which yield more than 300 explants and clonal material had been raised since the early (1962) experiments of Yeoman, and

so this cultivar. Bunyard's Round was used exclusively to satisfy the first provision of the method.

An additional reason for rejecting var. fuseau was that in Experiment 1 a red pigment was observed in a proportion of the explants after 5 days of culture. This proportion increased with time. Exhaustive tests were carried out to see if this was due to infection by a micro-organism, but crushed red explants showed no infection in the medium nor when plated onto potato dextrose agar of yeast difco agar. When a red explant was cultured in physical contact with a normal white one there was no infection. Neither fungal hyphae nor bacteria could be detected in sections or smears of explants stained with Cotton Blue or Gram's Stain.

A similar pigment is mentioned briefly by a number of authors (Caplin and Steward, 1949; Hackett and Thimann, 1952; Steward and Blakely, 1965; and Bourne, 1962) but has not yet been thoroughly examined. However, the unexplained nature of its origin made it an undesirable feature of the growth system.

The second provision is concerned with the length of time during which the stored material remained responsive to culture.

First Method of Storage (p. 8)			Second Method of Storage (p. 8)		
Experiment	Date 1962-3	F. Wt. (mg) on D 14	Experiment	Date 1963-4	F. Wt. (mg) on D 14
4	5 December	22.0	23	16 October	28.3
6	10 January	23.2	25	29 October	26.7
10	6 February	22.5	26	18 November	26.6
11	1 March	20.0	29	8 January	20.9
12	6 March	24.7	31	24 February	29.0
14	10 April	23.5	32	18 March	23.1
15	15 May	17.0	33	28 April	26.3
17	23 May	14.3	35	1 June	30.6
18	June	10.0	October	14 October	27.3

Table 1. 1. A comparison of the control fresh weights obtained at Day 14 (D 14) in 18 experiments. Each method of storage is represented by 9 control values.

From the results presented in Table 1. 1. it can be seen that the growth response under the original set of conditions (p. 8) shows a decline starting at some time after mid-April. This decline coincided with sprouting of tubers in the storage bins. After sprouting, black-rot infection spread through most of the tuber apices. For this reason in October 1963, an improved method of storage (p. 8) was tried and it transpired that this method preserved the potential for growth throughout the succeeding twelve months. Finally it can also be seen from the values recorded in Table 1. 1. that the growth response in individual experiments can be compared by using the control values of each experiment as a standard of reference.

These considerations, longevity of response and comparability of the growth obtained, satisfied the second provision of the method and confirms the choice of H. tuberosus cultivar. Bunyard's Round as the experimental material.

Provision 3: Simplicity with Asepsis.

This provision is concerned with the technique of culture. It was necessary to find a simple and rapid method which remained aseptic throughout the experiment. Earlier methods of liquid culture described by Steward and Caplin (1948, 1952), although very efficient, required complicated glassware and machinery. The conventional roller culture technique used for animal tissues (Gey, 1933; Lewis, 1935) depends upon the fact that animal tissues can adhere to glass surfaces. In this technique the inoculum is stuck to the inside of the roller tube with a drop of blood plasma and thereafter, as the mounting drum revolves, the inoculum dips into and out of the culture fluid. The subsequently proliferating tissue adheres firmly to the glass of the roller tube. However, plant tissues do not normally adhere to glass surfaces and therefore in order to adapt this technique for the culture of higher plant tissues, it was necessary to find a method of holding the tissue in place. Pyrex glass is a suitably inert material and it was found that if a thin spike was drawn from the edge of a length of glass tubing (or rod), small explants could be impaled and held in the desired position relative to the medium.

It would have been ideal if these spikes had identical length, thickness, position, degree of taper and angle with respect to the rod axis. Initial variation in these features, however, resulted in variation in the precise position in which each explant was held with respect to the medium and this might be expected to give rise to variation in the growth achieved during culture. During Experiments 1-17, increasing skill in manufacturing these mounts resulted in what were virtually standard mounts. Losses, due to explants falling from their mounts

and mounts screwing out of the medium, were also reduced from up to 30 per cent to less than 5 per cent during this time.

By this method of mounting explants in liquid medium (p. 9), each explant could be mounted in 20 seconds and 200 cultures can be set up in the space of two hours. Contamination of cultures by micro-organisms was generally below 1 per cent.

An initial difficulty was that hand-cutting of tissue cores into appropriate lengths was prohibitively slow and gave rise to considerable contamination, and so a tool was designed (p. 10) which cut up to 90 explants from excised cores at one time.

Accordingly, the technique developed was simple, aseptic, and yet allows large experiments to be set up rapidly.

Provisions 4 and 5: Yield and Variation.

Single features of the system which might contribute towards a high yield or a low variation amongst individuals were examined in turn. It was necessary to consider these two provisions together because a high yield might have been inseparable from a high variation. For this study, the compromise of a high yield associated with a workable variation was sought.

The first factor to be considered was that of nutrient medium. From the literature, four popular defined media were selected. The constituents are listed in Appendix A (p.188) and are taken from the recipes of White, and of Gautheret (Paul, 1959), Nitsch and Nitsch (1956) and of Bonner and Addicott (1937). In using White's medium, Steward and Caplin (1951) had found that the addition of the synthetic auxin 2:4 dichlorophenoxy-acetic acid (24D) had promotive effects for certain tissues, and so this medium was tested in both the presence and the absence of 24D. In this laboratory Yeoman, following the practice of Caplin and Steward (1948,

1952 etc.) in employing coconut milk as a supplement, had confirmed that the growth response of artichoke tissue is greatly increased if 20 per cent coconut milk (a complex mixture of nutritive organic substances) is included in the medium. Steward and his co-workers (Shantz and Steward, 1956) have since partially defined the chemical constituents of the milk of Florida coconuts, and have concluded that no single constituent plays a dominant role in the growth promotion by coconut milk but that the balanced additive and synergistic effects of the many chemicals present corporately provide the stimulus. For this reason Bonner and Addicott's medium was tested in the presence and absence of 20 per cent autoclaved coconut milk. In this experiment a power failure curtailed the run on the eighth day so values are tabulated for Day 8 by which time the cultures showed significant differences which might be expected to be magnified by Day 14.

Medium	F. wt at Day 8 (mg)	No. of explants harvested	S.D. of F. Wt.
Bonner & Addicott + 20% coconut milk + 24D at 10^{-6}M	26.4	10	3.6
Bonner & Addicott	12.7	7	2.5
White + 24D at 10^{-6}M	10.0	7	2.8
White	10.6	6	6.3
Nitsch	14.2	8	8.4
Gautheret	5.5	7	0.3

Table 1. 2: Experiment 2: 31/10/62. A comparison of the growth promoting properties of six different media using the Standard Procedure.

From the results presented in Table 1. 2. it can be seen that with none of the defined media was the yield high, nor did the addition of 24D to White's medium enhance its growth promotion. The high standard deviation for the treatments with White's medium and that of Nitsch was a reflection of the fact that in these treatments a few explants increased in fresh weight while the majority remained near their initial values. Such a variable response is undesirable. Gautheret's medium, without supplement, was inhibitory to growth. On the other hand, Bonner & Addicott's medium supported some consistent growth which was greatly increased by the addition of coconut milk.

It was concluded from this experiment that it would not be possible to use a fully defined medium without a time-consuming investigation to develop one, and that therefore the most suitable medium was that employing Bonner and Addicott's recipe with a coconut milk supplement.

The possibility remained, however, that the promotive effect of coconut milk would be more effective on defined media other than that of Bonner and Addicott. This was briefly examined in Experiment 3 by comparing its addition in the presence of 24D to both Bonner and Addicott's and White's media. On this occasion final cell number was estimated in addition to recording final fresh weight.

Medium	F. Wt on Day 14 (mg)	S.D. of F. wt.	Cell No. on Day 14
A: Bonner & Addicott + 24D (10^{-6}M) + 20% coconut milk	33.7	4.8	750,000
B: White + 24D (10^{-6}M) + 20% coconut milk	22.5	2.5	300,000

Table 1.3: Experiment 3: 18/11/62. A comparison of the fresh weight and cell number obtained with two media, using the Standard Procedure.
Fresh Weight at Day 0 = 8 mg; Cell number at Day 0 = 20,000.

From the results presented in Table 1.3. it can be seen that Medium A supports a higher yield than Medium B but has a higher variation. As this amount of variation was tolerable, Medium A was preferred for this study because of the high yield it supported. The fourfold increase in fresh weight and the almost fortyfold increase in cell number was considered satisfactory and therefore further search for a better medium was deferred as being unlikely to justify the expenditure of time.

Having decided upon Medium A for all further experiments, it was necessary to find the optimum volume of medium to be used in the roller tubes. In Experiment 4, four treatments, using 1 ml, 2ml, 3ml and 4ml of medium were compared.

Volume of Medium per Culture tube	F. Wt. (mg) on D 14	S.D. of F. Wt.	No. of Explants Harvested
1	22.9	6.4	7
2	22.8	4.7	9
3	22.0	4.9	9
4	19.7	3.3	9

Table 1.4: Experiment 4: 5/12/62. A comparison of 4 treatments, using different volumes of Medium A, employing the Standard Procedure.

The results from Experiment 4, presented in Table 1.4. show that growth promotion was the same using 1ml, 2ml and 3ml of medium. This suggests that the nutrients required for this amount of growth are present in excess even in 1ml of medium and that the explants are growing at the maximum intrinsic rate possible for Medium A (although physical features of the cultural environment may also be limiting growth). The reduced yield obtained with 4ml of medium is perhaps due to the less favourable conditions for gaseous exchange resulting from the explant only breaking the surface of the medium for a very short portion of its cycle of rotation. An alternative reason for a reduced yield could be that "staling products" (sensu Steward and Caplin, 1952) may be released by the explants (but see Caplin, 1965). However, the effect of staling products might be expected to be more potent in the smaller volumes of medium and so the results of Experiment 4 would suggest, in agreement with Caplin (1965) that staling products do not influence the growth of calli in this system.

It was decided therefore that either 2 or 3ml of medium would be a suitable volume. 1ml is rejected because of the high variation in response. Indeed, some explants actually died. 4ml is rejected because the growth achieved is significantly below (at the 10 per cent level) that of 3ml. Although the fresh weight values for 2 and for 3ml are not significantly different, morphological examination showed that in 2ml of medium there is a tendency for increased, unorganised proliferation on the inner surface (centripetal with respect to the mount) of the explant. This suggests an undesirable asymmetrical effect upon growth due to the different conditions for gaseous exchange to which this surface is exposed. The inner surface is never submerged in 2ml of medium although it is wetted by surface capillarity. In 3ml of medium all surfaces are at some time submerged and for this reason 3ml is selected as the most uniform treatment and therefore as the best volume of medium.

An additional reason for preferring 3ml of medium is that in more prolonged cultural periods the larger volume is less likely to limit growth through the exhaustion of critical nutrients.

The next aspect of the culture methods to be examined was the size of the inoculum. It was necessary to find the smallest viable explant which would give a high yield with low variation. This would permit the maximum number of explants to be taken from each tuber.

Length of Explant (mm)	Number of explants harvested	F. Wt. on day 14 (mg)	S.D. of F. Wt.	F. Wt./mm length
1.2	11	8.5	0.8	6.8
2.4	9	18.9	2.0	7.6
3.5	9	28.1	2.0	8.0
5.0	6	40.3	4.0	8.1

Table 1.5. Experiment 7: 16/1/63. A comparison of the growth obtained using the Standard Procedure with explants of standard 2 mm diameter and four different lengths (Standard Deviations are not comparable as the lengths were derived from different methods of cutting).

It can be seen from the results presented in Table 1.5. that if the yield is expressed per unit original length, then only in the smallest size of explant was the amount of growth greatly reduced. Therefore the smallest size which gave a high yield with low variation was the explant 2.4 mm in length.

Similarly in Experiment 8, three canulas of inside diameter 1.2, 2 and 4 mm were used to provide standard length (2.4 mm) explants of three different

diameters.

Diameter of Explant	Number of explants harvested	F. wt. (mg) on Day 21	S.D. of F. Wt.	Original F. Wt. on Day 0
1.2 mm	7	18.8	14.0	3.0
2 mm	14	59.5	32.0	8.8
4 mm	13	79.4	38.0	30.2

Table 1.6. Experiment 8: 24/1/63. A comparison of the growth obtained using the Standard Procedure with standard 2.4 mm long explants of three different diameters. Explants were harvested on Day 21.

Of the 1.2 mm explants only 50 per cent survived and these, as the S.D. indicates, had a very variable response. The 4mm canula invariably split the segment of tuber during excision which made it impossible to take many explants from a single tuber. The growth achieved by these 4 mm explants was under three fold and the variation was high. On the other hand, the 2 mm explants gave a high yield of almost seven fold with a similar variation and so this diameter of explant was retained for future experiments.

These two experiments served to confirm that the size of explant originally selected, namely 2.4 mm in length and 2 mm in diameter, is the most suitable for this study.

In order to improve the method of mounting inocula, an experiment to compare the mounts made from glass rod and from glass tubing was carried out. There were several reasons why the solid rod might have been expected to reduce the variation in growth response. These are listed as follows:-

1. The rods are heavier and therefore less likely to screw out of the medium (as up to 30 per cent of the tube mounts tend to do). Most explants which screw clear of the medium dry up or are culled, but those which screw only a short way and then fall back might not be culled and could give rise to an increased fresh weight.

2. In the tube mounts, it is possible that a portion of the nutrient is trapped within the mount and is unable to contribute to the bathing of the culture with fresh nutrient. This might reduce the fresh weight achieved.

3. The greater surface area of the tube mount visibly alters the characteristics of the meniscus of medium around the explant and this may alter the surface tension and the diffusion characteristics for both nutrient uptake and waste produce release especially with regard to gaseous exchange.

It was also thought necessary to check the need for any mount at all, although previous unpublished results of Yeoman (1962) had shown that after 28 days of culture there was a large difference in yield.

Mount	Number of explants harvested	F. Wt. on Day 14 mg	S.D. of F. Wt.	F. Wt. Increment as a % of Day 0.
Tube	13	20.9	2.0	145%
Rod	14	20.0	1.7	135%
None	11	17.1	1.0	100%

Table 1.7: Experiment 11: 1/3/63. A comparison of the growth obtained using the Standard Proceudre, employing 3 methods of mounting the explants.
F. Wt. at Day 0 = 8.5 mg; S.D. = 0.4.

From the results recorded in Table 1.7, it can be seen that there is no significant difference between the two types of mounting material, and also that it would indeed be desirable to employ a mount, especially as the difference recorded between treatments with and without mounts at Day 14 might be expected to have increased considerably by the 28th day.

The results presented in Table 1.7 suggest that none of the factors listed above play a significant part in the variation produced but since mounts made from glass rod are easier to make, are less fragile, and give rise to fewer losses, they were used for all future experiments.

It can also be concluded from this experiment that because the growth obtained after 14 days is not significantly different in the two treatments that therefore experiments using either of these mounts may be directly compared, over a 14 day growth period.

The Adoption of the Standard Procedure.

In the preceding experiments a method of liquid culture for plant tissues has been developed and as a result what has been described as the Standard Procedure was provisionally adopted. With the aim of improving this method the succeeding experiments in this section were designed to examine the nutritional components of the medium to see whether any simple adjustments might enhance the growth yield. A subsidiary aim was to learn something of the contribution which each constituent of Medium A makes to the total growth changes.

It has been shown by Caplin (1956) that casein hydrolysate, a complex mixture of amino acids and perhaps vitamins, will increase the growth response of carrot cultures over that achieved using a modified White's medium supplemented with coconut milk. Pollard, Shantz and Steward (1961) also suggested that several

hexitols, including inositol, contributed to the total stimularoty effect of coconut milk. For this reason Experiment 9 was designed to find whether or not either of these two supplements would have any effect upon the growth response produced by the particular conditions of the Standard Procedure. Four treatments were tested in addition to the control using Medium A, and these treatments and their results are presented in Table 1.8.

Treatment	Number of explants harvested	F. Wt. (mg) on D 14	Level of Sig. Diff.
Medium A	10	33.1	
Medium A + inositol	14	34.0	-
Medium A + casein hydrolysate	12	29.8	**
Medium A + cas. + inositol	13	30.2	**
Medium A + cas. + inos. - CM	11	25.8	***

Table 1.8: Experiment 9: 1/2/63. A comparison of the growth obtained using the Standard Procedure with five different media. Casein hydrolysate (cas.) is present at 110 p.p.m. and inositol (inos.) at 20 p.p.m. CM = 20 per cent coconut milk.

It was concluded from the results presented in Table 1.8. that, at the concentrations used, inositol has no effect, and casein hydrolysate has a depressing effect upon the growth achieved using Medium A. The fact that, in the absence of coconut milk and the presence of both additives, the fresh weight increment is 70 per cent that of the control may be attributed either to stimulation by the additives or by the remaining components of the control medium. Results will later

be presented to suggest that this latter is the case (p. 71). Accordingly casein hydrolysate and inositol were not considered useful additives to Medium A under the conditions specified.

In order to find the optimal concentration of coconut milk six treatments were compared, employing concentrations of autoclaved coconut milk ranging from 0 to 25 per cent.

Per Cent Coconut Milk	Number of Explants Harvested	F. Wt. (mg) on D 14	S.D. of F. Wt.
0	13	19.0	2.6
5	14	22.4	3.2
10	15	25.0	2.4
15	12	25.1	2.3
20	12	25.2	2.0
25	12	25.3	3.2

Table 1.9. Experiment 12: 6/3/63. A comparison of the growth obtained using the Standard Procedure, with six concentrations of coconut milk employed in Medium A.

From the results presented in Table 1.9. it is clear that concentrations from 10 through 25 per cent are equally effective. As high percentages were not inhibitory, it was thought that a high percentage would help to "blanket" out any variations that may occur in subsequent batches of milk and also the variable response from tubers. Accordingly no change was made in the Standard Procedure.

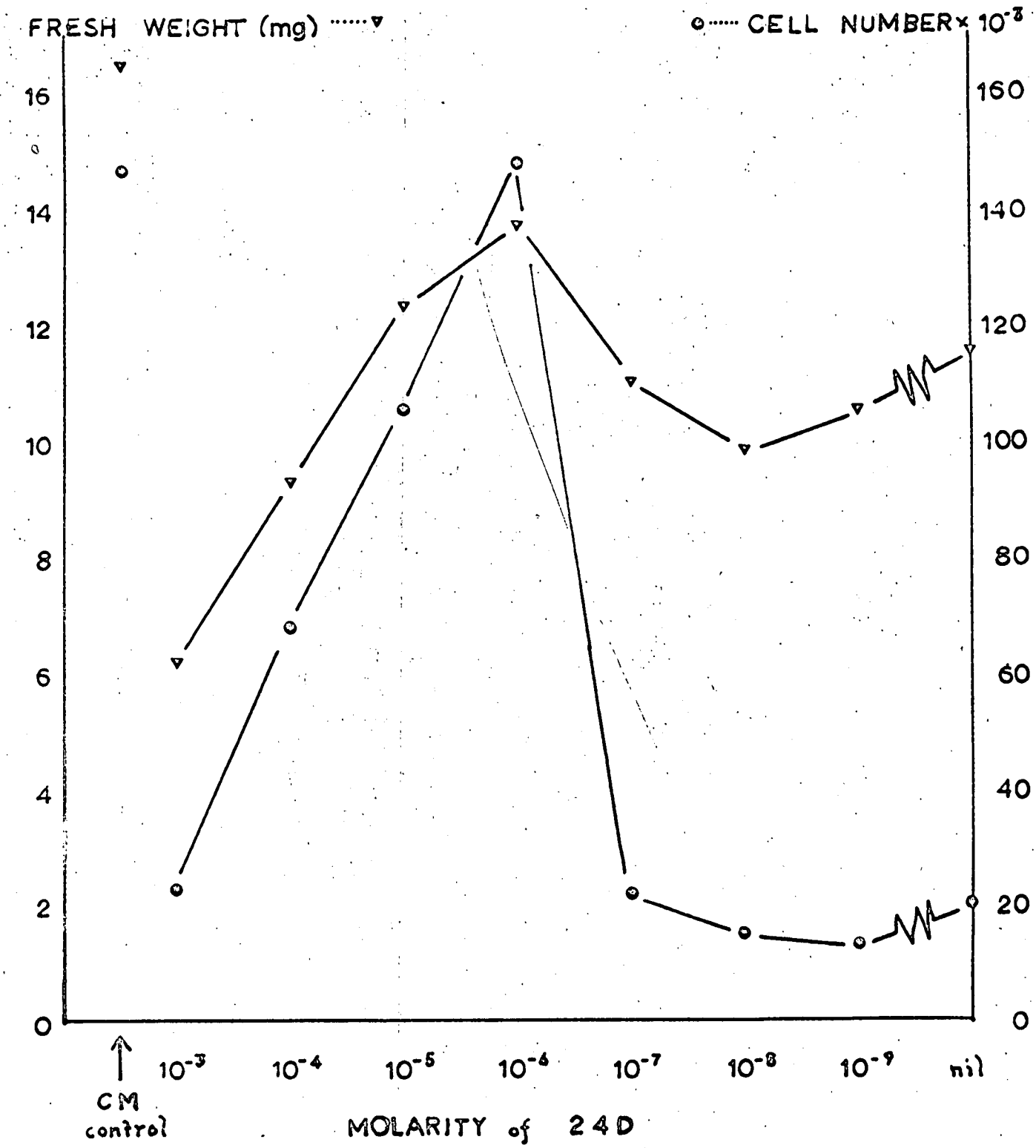


Figure 1.1. Experiment 22. The growth response over 14 days to culture on full Medium A (CM control) and to media lacking coconut milk and containing 8 molarities of 24D (2:4 dichlorophenoxyacetic acid). The fresh weight of 10 replicates were measured.

Similarly it was of interest to find the optimum concentration of 24D in the absence of coconut milk. Therefore, in Experiment 22 the four concentrations listed in Table 1.10 were tested as supplements to Bonner and Addicott's salt mixture in the presence of 4 per cent sucrose.

Molarity of 24D	No. of explants Harvested	F. Wt. (mg) on D 14	S.D. of F. Wt.	Cell No. on D 14
10^{-8}	10	10.2	0.7	9,000
10^{-7}	10	10.3	0.9	13,000
10^{-6}	8	18.1	2.0	129,000
10^{-5}	10	13.6	1.7	96,000
Control	10	23.0	3.4	330,000

Table 1.10: Experiment 28:27 9/10/63. A comparison of the growth obtained using the Standard Procedure in the absence of coconut milk employing four molarities of 24D. The medium used in the control was Medium A.

The results presented in Table 1.10 show the response after 14 days of culture in terms of final fresh weight and of final cell number. It can be seen that both parameters have an optimum value at 10^{-6}M 24D, but that both fall short of the control values.

Following this experiment it was decided to investigate the growth response to a full range of 24D concentrations and the results of Experiment 27 are presented in Figure 1.1. Because 24D is sparingly soluble in water, all molarities were made up individually in absolute ethanol or by serial dilution from

2.2 mg per ml of absolute ethanol.

Apart from confirming that the optimal concentration for growth promotion in terms of fresh weight and cell number is 10^{-6}M, several other observations can be made. Molarities of 10^{-8} and 10^{-9} reduced the estimated cell number to a value below the initial value of 20,000. This must involve the sloughing off of cells, dead or alive, and no explanation of this phenomenon can at present be offered. Similarly, molarities of 10^{-9}, 10^{-8}, 10^{-7}. 10^{-4} and 10^{-3} all resulted in less fresh weight increase than was obtained in the total absence of 24D. It would appear therefore that, at all molarities tested, 24D exerts a controlling influence upon the process of tissue expansion.

Experiment 27 also showed that, at least on one occasion, an equal cell number increase was supported by Medium A both lacking and also containing coconut milk. The question then arises whether coconut milk is necessary for the stimulation of cell division and also for the promotion of an increase in fresh weight.

Experiment	F. Wt. (mg) on Day 14, Medium A.	F. Wt. (mg) on Day 14, Medium A-CM	$\frac{A-M}{A} \times \frac{100}{1}$ for F. Wt.	$\frac{A-M}{A} \times \frac{100}{1}$ for Cell No.
9	(33.1)	(25.8)	70%	-
12	25.2	19.0	71%	-
20	28.7	10.0	7%	0%
22	23.0	18.1	66%	c 40%
27	16.5	13.7	71%	c 100%

Table 1.11: A comparison of the growth obtained employing Medium A, and Medium A lacking coconut milk (A-CM) on five separate occasions. Experiment 9 was harvested on Day 21. A = growth increment over Day 0 on Medium A. A-M = growth increment on Medium A lacking coconut milk.

The relevant data from five experiments has been assembled in Table 1.11. This shows a consistent response, with the fresh weight increase in Medium A lacking coconut milk being about 70 per cent that in full Medium A, with the singular exception that in Experiment 20 there is very little increase at all in the absence of coconut milk. The response in terms of cell number varies from 0 to 100 per cent. In Experiment 20 there was no division, in Experiment 22 there was about half that achieved by the controls and in Experiment 27 both treatments gave the same cell number. Possible explanations for this will be discussed, (p. 98), but it was nevertheless concluded that in order to obtain a uniform response it remained necessary to include coconut milk in Medium A.

Conversely, it was also of interest to know whether the three other major components of Medium A are required to support growth using the Standard Procedure. Accordingly explants were cultured on five media, in each of which except the control, one of these major components was lacking.

Medium	No. of explants harvested	F. Wt. on day 14	Estimated Cell number on Day 14	D. Wt. on Day 14
Medium A	14	16.5	147,000	2.0
A - sucrose	14	16.5	153,000	2.0
A - salts	13	16.5	-	2.0
A - 24D	14	18.5	94,000	3.9
A - coconut milk	15	13.7	148,000	2.0

Table 1.12: Experiment 28: 5/12/63. A comparison of the growth obtained using the Standard Procedure employing five treatments in which Medium A lacks, in turn, each one of the four components of the full medium.

The results presented in Table 1.12 show that if 24D is omitted from Medium A then the cell number increase is reduced by almost 40 per cent while the fresh and dry weight is increased. The omission of sucrose however has no significant effect upon either fresh weight, dry weight or the estimated cell number obtained, and although cell number was not recorded for this treatment the effect of omitting Bonner and Addicott's salt mixture appears to be similar.

The next experiment was designed to answer the question "Are the conditions for gaseous exchange exerting a limiting effect upon the growth of cultures?" This possibility had been suggested by an observation concerning those explants whose mounts had screwed clear of the bulk of the medium in their culture tube and yet had not dried out. It will be realised that a medium containing 20 per cent coconut milk and 4 per cent sucrose is a very sticky one. It is perhaps for this reason and also because of the particular shape of each individual

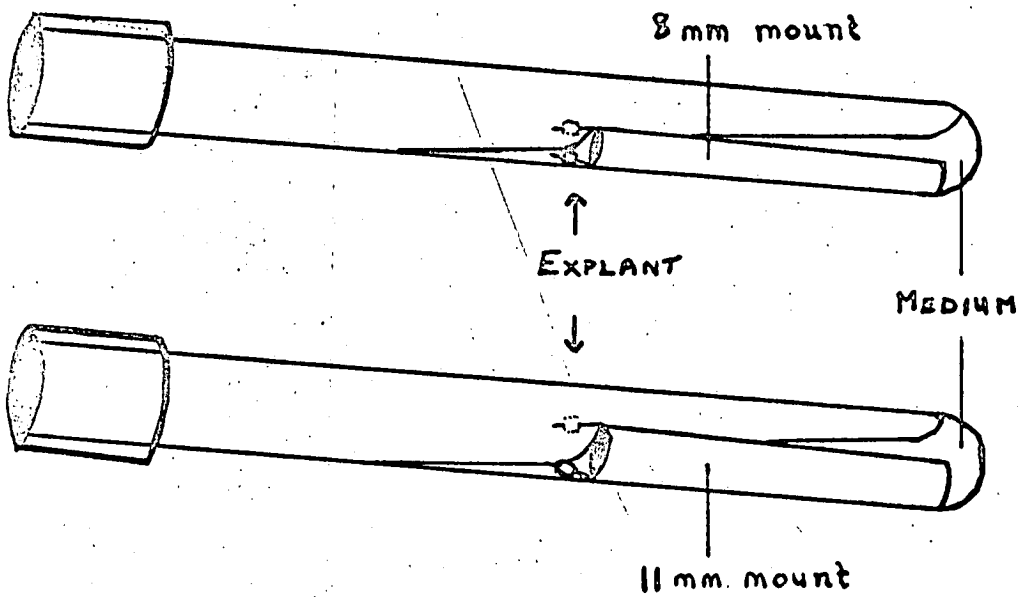


Figure 1.2. An illustration of the mounts used in Experiment 6. Explants impaled on the lower mount will be carried clear of the medium during a greater proportion of each revolution of the respective mounts.

mount that occasional mounts were seen to ride up the slope of the tube and out of the medium. These have been dubbed "crawlers". Most crawlers continued to crawl up the tube until they pushed the cap off the culture tube and fell out or were culled. Some, however, crawled only a short distance and there remained stuck fast to the tube walls. These crawlers frequently received enough medium by capillary creep along the interface between mount and culture tube to allow the explant to grow. Those that did survive invariably became very much heavier than the controls and on harvesting were found to have very much higher cell numbers. This suggested either that excess nutrients are inhibitory (this is not so, p. 63) or that increased aeration has a stimulatory effect.

Secondly the bilateral symmetry of cultures grown in 2 ml of medium discussed above (p.63) had also brought to light this possibility. However, an alternative interpretation of this latter observation could be that the growth on the outer (centrifugal with respect to the mount) surface of the explant became modified due to its repeated contact with the containing culture tube. This possibility provoked a second question, namely, "Would the proliferation noted above occur over the whole surface if the explant never rested against the tube during the revolution of the mount?"

It was thought that contrasting the growth response supported by two kinds of mount might answer both questions. These mounts are drawn in Figure 1.2 The larger 11 mm mounts hold the explants for a greater proportion of their cycle in air, than do the standard 8 mm mounts. The angled spikes on the 11 mm mounts ensure that explants never contact the tube walls during culture. The precise angle of the spikes was rather variable which is doubtless the reason for the high variation recorded in Table 1.13.

Mount diameter	F. Wt. on Day 14 (mg)	S.D. of F. Wt.
8 mm	23.2	1.9
11 mm	31.5	8.9

Table 1.13: Experiment 6: 10/1/63: A comparison of the growth obtained using the Standard Procedure employing the two types of mount described in Fig. 1.2

The results presented in Table 1.13 show that the larger mounts support an increased growth response, and the high variation amongst the explants held by these mounts suggest that growth promotion is very sensitive to small changes in the conditions of aeration. This would suggest either that excess medium is deleterious (Experiment 4, Table 1.4 showed that this is not the case) or that improved conditions for gaseous exchange promote an increase in growth of cultures.

This suggestion was taken up in Experiment 34 which was designed to examine further the effects of more favourable conditions for gaseous exchange than obtain in the Standard Procedure. This experiment was performed much later as it took a considerable length of time to receive the glass rod of unusual diameters and to construct the modified culture drum. This drum was capable of holding 70, 25 mm diameter culture tubes. Mounts of 5, 8 and 11 mm diameter were constructed. These diameters were chosen so that explants could be subjected to three contrasting conditions.

Condition A was defined as the control condition employing the Standard Procedure, with 15 mm tubes containing 3 ml of medium. Conditions B, C and D used

25 mm tubes and 5 ml of medium. Condition B (5 mm mounts) was such that each explant while rotating as usual, was always submerged in the medium. Condition D (11 mm mounts) was such that the explants, while revolving, were suspended clear of the medium longer than they were submerged in the medium. Because conditions B, C and D all employed 5 ml of medium, held in 25 mm tubes, condition C (8 mm mounts) was chosen to provide conditions as nearly comparable as possible with Condition A. With respect to gaseous exchange, therefore, Condition A and C lie in a position between B and D. It will be recalled (p. 63) that an increase in volume of medium, per se, makes no significant difference to the fresh weight increase over 14 days. In addition to fresh weight values, the dry weight of explants and an estimate of their total cell number and tracheid number was recorded. The results of these four treatments are presented in Table 1.14 and tests for significant differences are summarised in Table 1.15.

Treatment	F. Wt. on Day 14 (mg)	D. Wt. on Day 14 (mg)	Cell No. on Day 14	Tracheid No. on Day 14
A	23.3	1.9	265,000	25,000
B	27.0	2.4	388,000	13,000
C	31.2	2.5	591,000	9,000
D	36.4	3.3	690,000	5,000

Table 1.14: Experiment 34: 28/5/64. A comparison of growth obtained using the Standard Procedure modified in the manner described for Conditions A, B, C & D. Four different parameters of growth are recorded.

Test	Degrees of freedom	Variance Ratio	Level of Significance
A cf B	22	7.66	*
B cf C	19	11.36	* *
C cf D	20	13.97	* *
A cf C	22	34.73	* * *

Table 1.15: Summary of tests for significant differences between treatments A, B, C & D in Experiment 34.

The difference between Conditions A and C was found to be highly significant. In these treatments the major variable which had not yet been investigated, was the 3-fold difference in the area of interface between the medium and the atmosphere. The effect of reducing the period of revolution by half was not tested but was regarded as negligible. It would seem, therefore, that the increased surface area for exchange of gases between the air and the medium had a significant effect upon the growth achieved by the explants.

A second means of making the conditions more favourable was by reducing the proportion of time during which the explant lies submerged in medium.

This reduces the mean diffusion path of gases between the atmosphere and the explant. Even when an explant, during rotation of its mount, is brought clear of the medium, the whole surface remains moistened by adherent medium. However, the longer the medium remains in air, the more medium will drain off and the more moisture will evaporate off. This will cut down the diffusion path which gases must necessarily traverse. Therefore, in comparing treatments B, C and D, progressively more felicitous conditions for gaseous exchange were being examined, and

the differences brought out in Table 1.15 indicate that the conditions for gaseous exchange are critical in the cultural procedure employed in this study. Clearly, it would be advantageous to use Condition D for further experiments, however 70 explants per drum is not enough for the experiments envisaged so this method was set aside for the present time.

From the additional data presented in Table 1.14 it can be seen that, correlated with increased fresh weight increment, there was also an increase in dry weight, in total cell number, and a decrease in the number of tracheids. These changes therefore are also correlated with the more favourable conditions for gaseous exchange.

SECTION IB - Sources of Variation in the Method.

Introduction.

In this section it is proposed to examine and assess all the major sources of variation in the system evolved in Section IA. This system is a particular method of liquid culture for whole explants, employing the "Standard Procedure", in which samples are harvested at random from a population at intervals over a period of up to 28 days, and subjected to a variety of analytical procedures in order to define a time-course of changes, during culture, which may be of physiological significance.

In the course of a series of experiments which are to be related one to another, it is necessary to recognise and account for the major sources of variation. Thus, two batches of coconut milk and two methods of storage will be compared. The variation amongst tubers, the variation within a single tuber, and the variation due to the length of storage of the tubers will be examined.

Most of these sources of variation are assessed on the basis of their response to culture over a period of fourteen days. However, as many of the workers with this material have ignored the effects of storage upon their source of material, (Steward and Berry, 1934; Reinders, 1942; Goris, 1948; Caplin, 1965; Sperling and Laties, 1963) while others have simply reported the method and duration of storage, (Hackett and Thimann, 1952; Nitsch and Nitsch, 1956; Macey, 1965; Masuda and Yanagishima, 1965) it was thought that a consistent survey of the growth potential during storage was necessary. Accordingly, during the 1964-5 season, bimonthly experiments were carried out to define any seasonal changes in growth response that may take place.

Total Variation.

In Table 2.1, all the control values of fresh weight on the fourteenth day of culture (Day 14) have been assembled, in order to compare the variation in the yield in all the experiments performed in this study.

The second column of the results presented in Table 2.1 show how remarkably consistent the control fresh weight values have been over the 32 experiments considered. The coefficients of variation for each experiment are a measure of the variation within that experiment and these are given in column 6 of Table 2.1. The average of these coefficients is 12 per cent. If the mean values are grouped then their coefficient of variation is less than 16 per cent.

It was concluded that the variation from tuber to tuber is as low as can be hoped for in a biological system and the variation within a population of explants drawn from a single tuber is especially favourable.

Source of Variation	Mean of pooled experimental means	Number of ~~explants~~ experiments concerned
Storage I, 1962-3	23.1	10
Storage II, 1963-4-5	25.5	21
1962 artichokes	23.9	10
1963 "	24.9	14
1964 "	26.5	7
Coconut Milk, Batch I	24.7	13
" " " II	25.2	18

Table 2.2 Some possible sources of variation tabulated with the mean values of the pooled experimental means from the experiments concerned with that particular source of variation. Artichokes lifted on three occasions (7/11/62, 11/11/63, & 16/11/64), coconut

(Legend continues on p. 83)

TABLE 2.1

Experiment	F. Wt. on Day 14	No. of Replicates	S.D. of the mean of F. Wt.	Date of the Experiment	Coef. of Var. = $\frac{SD}{mean} \times \frac{100}{1}$ per cent
3	33.9	6	4.8	19/11/63	14
4	22.7	9	4.9	5/12/63	22
6	23.2	9	1.9	10/ 1/63	8
7	18.9	11	2.0	16/ 1/63	11
8	(27.1)	6	4.8	24/ 1/63	18
9	(33.1)	16	5.0	1/ 2/63	15
10	22.5	11	1.7	6/ 2/63	7
11	20.0	14	1.7	1/ 3/63	9
12	25.1	12	2.0	6/ 3/63	8
13	22.3	21	2.1	19/ 3/63	9
14	23.5	19	2.0	10/ 4/63	9
15 (& 16)	17.3	10	1.7	15/ 4/63	10
17	14.3	11	1.2	23/ 4/63	8
20	28.7	10	3.4	26/ 8/63	12
21	29.8	26	3.1	2/ 9/63	10
22 (& 24)	23.0	10	3.4	9/10/63	15
23	28.3	10	6.0	16/10/63	21
25	26.7	21	2.9	29/10/63	11
26	26.6	22	4.0	18/11/63	15
27 (& 28)	16.5	14	2.0	5/12/63	12
29	20.9	20	5.3	8/ 1/64	25
30	18.0	18	1.5	13/ 1/64	8
31	(29.0)	27	1.3	24/ 2/64	5
32	23.1	20	3.1	18/ 3/64	13
33	26.3	30	3.1	28/ 4/64	12
34	23.3	13	3.5	28/ 5/64	15
35	30.6	14	2.8	5/ 6/64	9
Oct. 63	27.3	12	2.0	14/10/64	7
Oct. 64	29.9	12	2.5	14/10/64	8
Nov.	26.6	12	4.3	11/11/64	16
41	24.5	4	2.0	30/11/64	8
Jan.	26.7	12	1.7	13/ 1/65	6
50	26.0	16	3.1	12/ 2/65	11
March	25.8	12	2.6	17/ 3/65	10
May	26.3	12	2.0	27/ 5/65	8

TABLE 2.1

The mean fresh weight values at Day 14 (Day 14), the number of replicates harvested, and the Standard Deviation of these replicates from their mean values, are assembled for all the experiments performed in this study. These control values are tabulated according to the chronological order of the experiments. Experiments 3 - 17 involved tubers lifted in 1962 and stored under the first set of conditions, while the remainder were lifted in 1963 (Experiment 20 - Oct. 63) or in 1964 (Oct. 64 - May 64) and used the second method of storage.

The medium for Experiments 3 - 22 incorporated Batch I coconut milk and the remainder contained Batch II.

Although Experiment 5 was lost due to contamination there were in fact no Experiments 18 and 19 and after Experiment 36 the majority of experiments involved a shorter period of culture than 14 days. Therefore all possible data have been assembled, without selection.

Experiments 8 and 9 were harvested after 21 days and Experiment 31 was carried out at 29°C and so these values were not included in the derivation of secondary data.

The mean value of all the figures in column 6 is 12 per cent.

milk received on two occasions (August 1962 - Batch I and October 1963 - Batch II), and two sets of storage conditions (p. 8) are all considered.

In Table 2.2 three possible sources of variation are examined. It will be realised that these are not easily separable one from another, as, for example, the first storage condition was confined to artichokes lifted in 1962, while the second involved tubers lifted both in 1963 and 1964. However, it is clear that there was very little difference between any of these "treatments", and it was concluded that experimental values from any combination of them may be directly compared, because any variation due to these treatments will be negligible in comparison with the inherent biological variation in response described above (column 6, table 2.1).

Concerning the coconut milk, however, there must be added one proviso that follows from Experiment 24 which was set up to compare the two batches of coconut milk. On Day 14, Batch I gave a fresh weight value of 23.0 mg while Batch II yielded 40.2 mg. It was concluded that Batch II for some unknown reason promoted a much greater response, and reluctantly (because Batch I was running out) Batch II was used thereafter. However, as may be seen from the subsequent Day 14 values in Table 2.1, the control response returned to "normal" that is, of the order of 25.0 mg. The most obvious explanation is that there are compounds in autoclaved coconut milk which break down over a period of a few days and that if the milk is incorporated into culture medium before this period has elapsed, then there remain undegraded compounds which can double the growth response. In Experiment 24, it was recalled that the milk had been autoclaved only two days prior to its final autoclaving for use in Medium A on the following, third, day. This possibility has not been further tested (because a third batch of milk has not

been necessary) but it would be of some interest to examine the growth response to unautoclaved and recently autoclaved coconut milk.

A comment can also be made about the low means recorded in Experiments 27 and 30. In an attempt to obtain a large number of explants, a particular kind of tuber was used for these experiments, which until this time had been avoided. These were the very large rather amorphous tubers which are found at the base of the stem and from which many of the lateral tubers arise. Although the hypothesis has not been tested, it is possible that these morphologically distinct tubers may have also a distinct physiological character.

Variation amongst explants from a single tuber.

In order to examine the variation within one particular tuber, Experiment 48 was planned with statistical analysis in view. Before inoculation the tuber was carefully cut into three segments of about 2 cm depth. This allowed 2 cm of apex and 2 cm of base to be rejected. The three segments were designated A (basal), B (median) and C (distal). Two groups of explants were withdrawn from each segment. The first, designated "outer" was taken from approximately 2 mm within the cambial ring, and the second, designated "inner", was taken from approximately 6 mm within the cambial ring. These relationships are described in Figure 2.1. The six groups so derived were randomised on the culture drums in blocks of six. After 7 days each explant was harvested and their fresh weights recorded. Explants of each group from blocks 1 & 2, 3 & 4, 5 & 6, etc., were paired and macerated together in 5 ml of chromic acid for the estimation of cell numbers.

The fresh weight data has been processed to elucidate significant differences. The primary data is presented in Table 2.3, the statistical analysis is ~~collected in Appendix and~~ summarised in Table 2.4. Four missing values were calculated by the method of Yates (Cochrane & Cox, 1957). Comparison of A, B & C

is defined as the longitudinal effect and comparison of Outer and Inner as the depth effect. It was found that differences in treatments were significant and that the interaction between the longitudinal and depth effects was highly significant. Five breakdown analyses showed that differences are increasingly significant towards the distal segment and that these are emphasised at the inner depth. However, breakdown analyses amongst the four treatments pertaining to segments A and B proved that, at a given depth, the differences between A and B are insignificant.

Without resorting to analysis of the cell number data, it is clear that the situation is very similar to that of the fresh weight increase, with one important difference, In five of the treatments, the mean cell weights calculated from the primary data and recorded in Table 2.3, are all very similar. This would suggest that the increase in cell number follows the same trend as that of fresh weight increase. The inner position of the distal, apical segment, has, however, a 30 per cent greater cell weight, which suggests that the large increase in expansion displayed by explants from this region is not supported by an equivalent increase in cell division. These results were, of course, very encouraging for experiments which are primarily concerned with the process of cell division. However, although in the other five treatments it would seem that the processes of expansion and of cell division are balanced, it is still true that explants from different depths will give different increases in cell number.

This experiment demonstrated that as long as the distal portion of the tuber is avoided, the critical factor is the depth of the origin of the explants. It showed that if explants have to be taken from two depths as they must in large experiments, the randomisation of explants is necessary. In fact, on common sense grounds, this method of approach had already been adopted, but in experiments which

TABLE 2.3.

TREATMENT		Outer	Inner	Outer	Inner	Outer	Inner
Segment	Block	D7 F.Wt. mg.	D7 F.Wt. mg.	D7 Cell No. x 10^{-3}	D7 Cell No. x 10^{-3}	D7 Cell Wt. µg	D7 Cell Wt. µg
Segment A (proximal)	1	16.7	13.7	277	318		
	2	16.1	27.1			.059	.064
	3	15.7	16.6	237	242		
	4	16.9	14.4			.069	.064
	5	15.2	20.3	224	234		
	6	15.5	16.5			.069	.079
	7	15.2	19.6	179	253		
	8	14.2	17.1			.082	.073
	9	15.6	17.0				
	10	16.6					
Mean:-		15.8	18.0	229	262	.070	.070
Segment B (median)	1	14.5	17.9	252	228		
	2	15.2	16.8			.059	.076
	3	14.4	14.5	223	210		
	4	13.7	16.1			.063	.073
	5	17.7	18.7	238	247		
	6	17.0	26.9			.073	.092
	7	15.2	17.6	210	300		
	8	15.5				.073	.059
	9	16.2					
	10	16.0					
Mean:-		15.5	18.4	231	246	.067	.075
Segment C (distal)	1	18.8	26.8	297	250		
	2	18.8	23.8			.063	.101
	3	18.4	25.7	238	285		
	4	15.7	29.2			.072	.096
	5	17.6	25.0	258	312		
	6	24.7	28.1			.082	.085
	7	16.2	28.9	212	290		
	8	15.9	24.4			.076	.092
	9	15.9	24.9				
	10	17.6	27.4				
Mean:-		17.9	26.4	251	284	.073	.094

Table 2.3. Experiment 48. 26/1/65. Primary data concerning the fresh weight and cell number of explants cultured for 7 days to compare the growth response from 6 distinct regions of one tuber. These regions are illustrated in Figure 2.1.

Effect	Level of Significance
Six Treatments	*
Depth effect, A, B & C	* *
Depth effect, in Segment A	*
" " " " B	* *
" " " " C	* * *
Longitudinal effect at Outer Depth	* *
" " " Inner "	* * *
Longitudinal effect of A cf B, Outer	-
" " " A cf B, Inner	-

Table 2.4. Experiment 48. Summary of analysis for significant differences in the growth potential of explants from six regions of a single tuber.

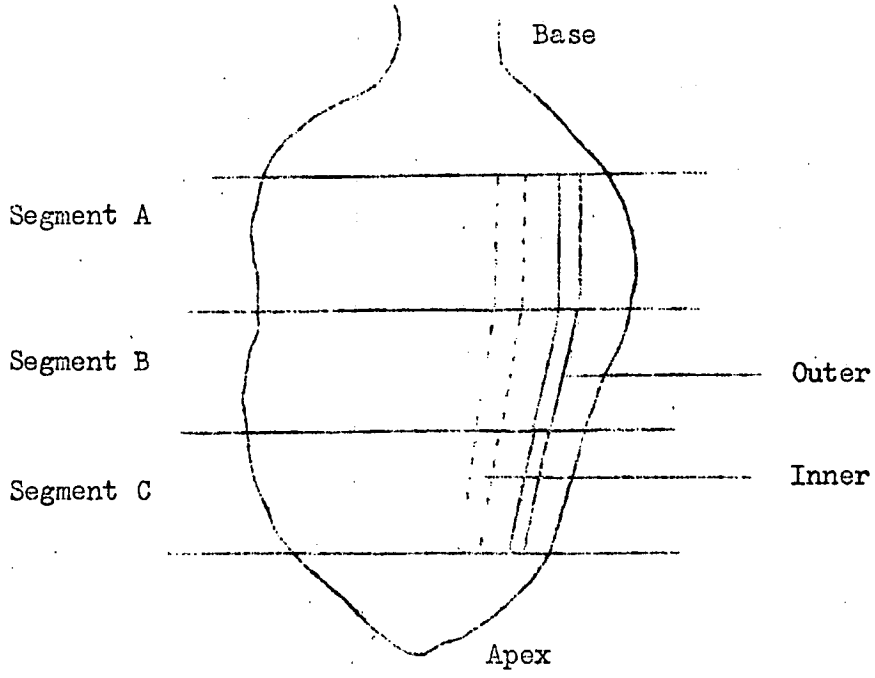


Figure 2.1. Explants for Experiment 48 were taken from the 6 regions indicated in this L.S. of an artichoke tuber.

were envisaged concerning the induction of division (Section II C, p.138) it had become necessary to define more accurately the requirements for minimal variation, so that the maximum number of explants, with a lower potential variation than had hitherto been used, could be confidently inoculated.

Seasonal Variation.

Edelman (1963) has shown that artichoke tubers during storage, undergo certain changes in their carbohydrate complement. Amongst other things there is an increase in the concentration of low polymer inulin molecules with a corresponding decrease in the longer chain molecules. It might be expected that the different states of preparedness, due to progressive changes in both raw materials (carbohydrate and protein reserves, etc.), and also in control molecules (those chemicals, known and unknown, which dictate the physiological changes connected with dormancy, sprouting, vernalisation, etc.), during prolonged storage would result in differences in response during the culture of explants. Although, a high, "blanketing" concentration of coconut milk is supplied to Medium A to obviate this effect, it remained necessary to examine any variation in response to culture that may be attributable to the length of storage which the tubers undergo prior to culture. Accordingly, a series of what were defined as "seasonal" experiments were carried out at bimonthly intervals, during the 1964-65 academic year. In October 1964, two experiments were run in parallel. One employed explants taken from a tuber which had been stored at $4^{\circ}C$ for twelve months subsequent to lifting in October 1963. Explants for the second experiment were taken from two small tubers lifted in October 1964 and kept at $4^{\circ}C$ for two days prior to use. These latter tubers, weighing 17 and 19 g, were still in the process of swelling in the ground when they were lifted, and had not yet been exposed to frost. Sufficient explants were obtained from these two tubers to permit harvesting unto the fourteenth day. For all subsequent seasonal experiments tubers weighing more than

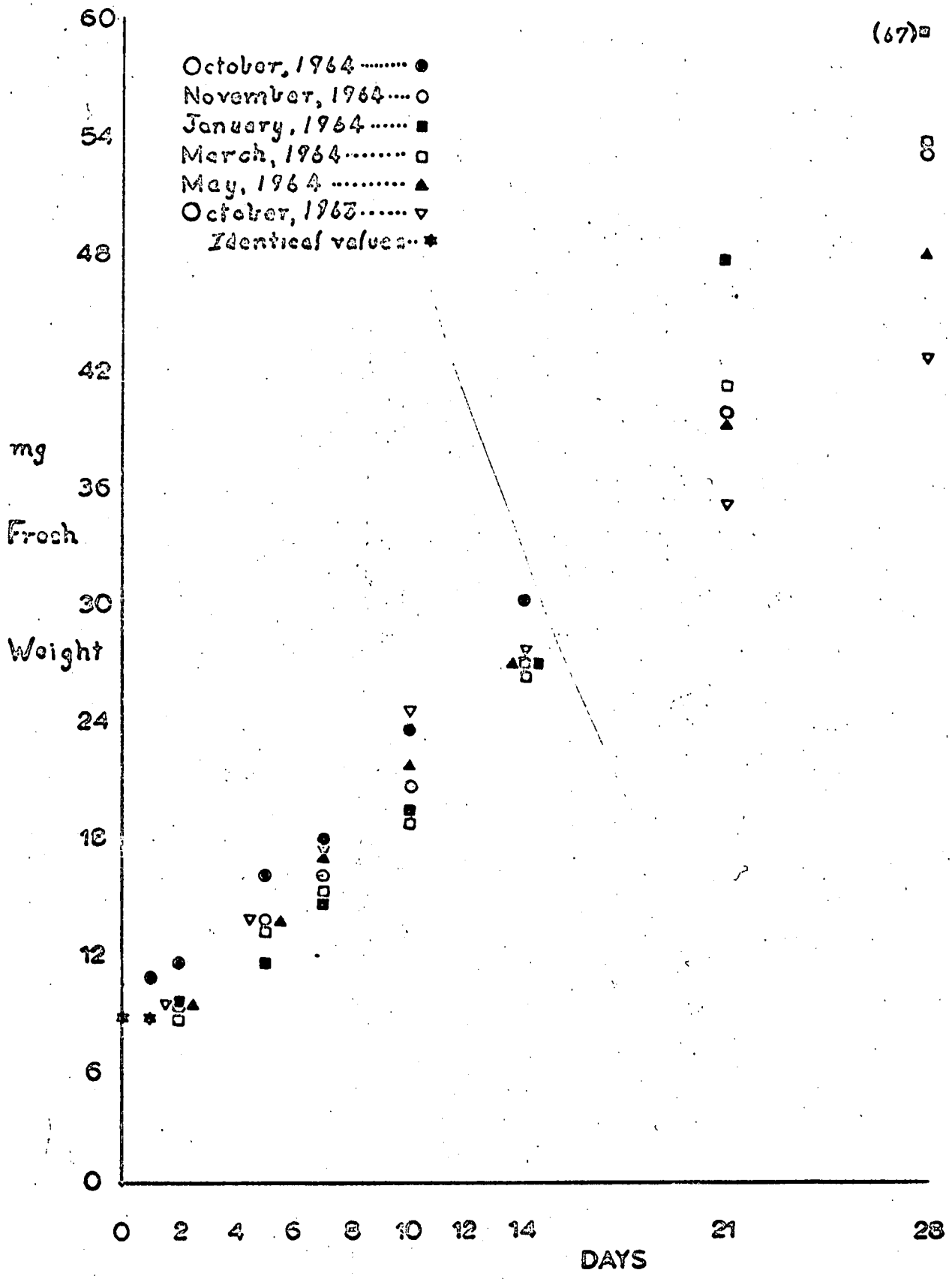


Figure 2.2. Experiments 60 - 65. A scatter diagram of the changes in fresh weight during 28 days of culture. The six experiments were initiated using tubers of increasing "age". Each point represents 12 replicates.

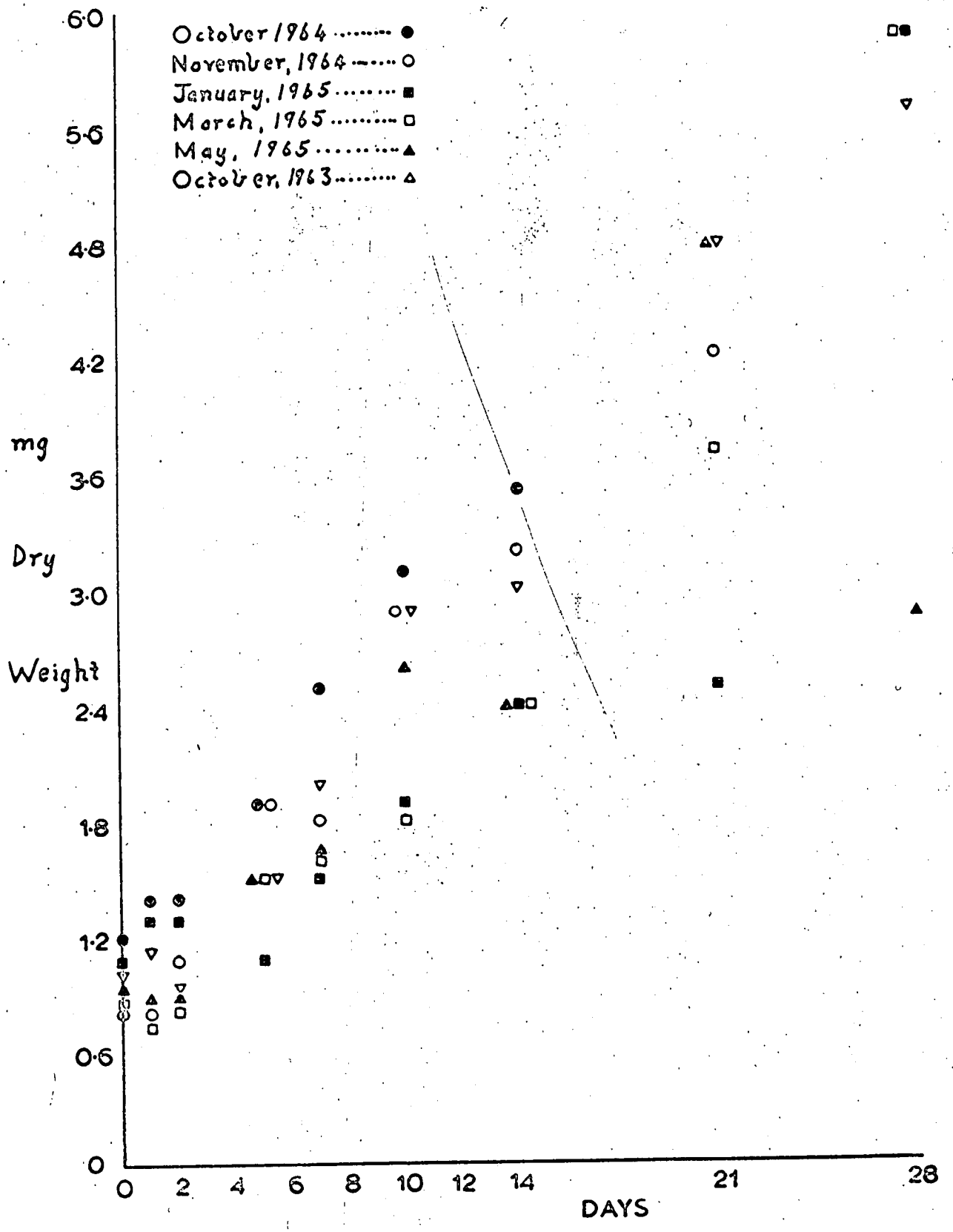


Figure 2.3. Experiments 60 - 65. A scatter diagram of the changes in dry weight during 28 days of culture. The six experiments were initiated using tubers of increasing "age". Each point represents the mean for 3 calli.

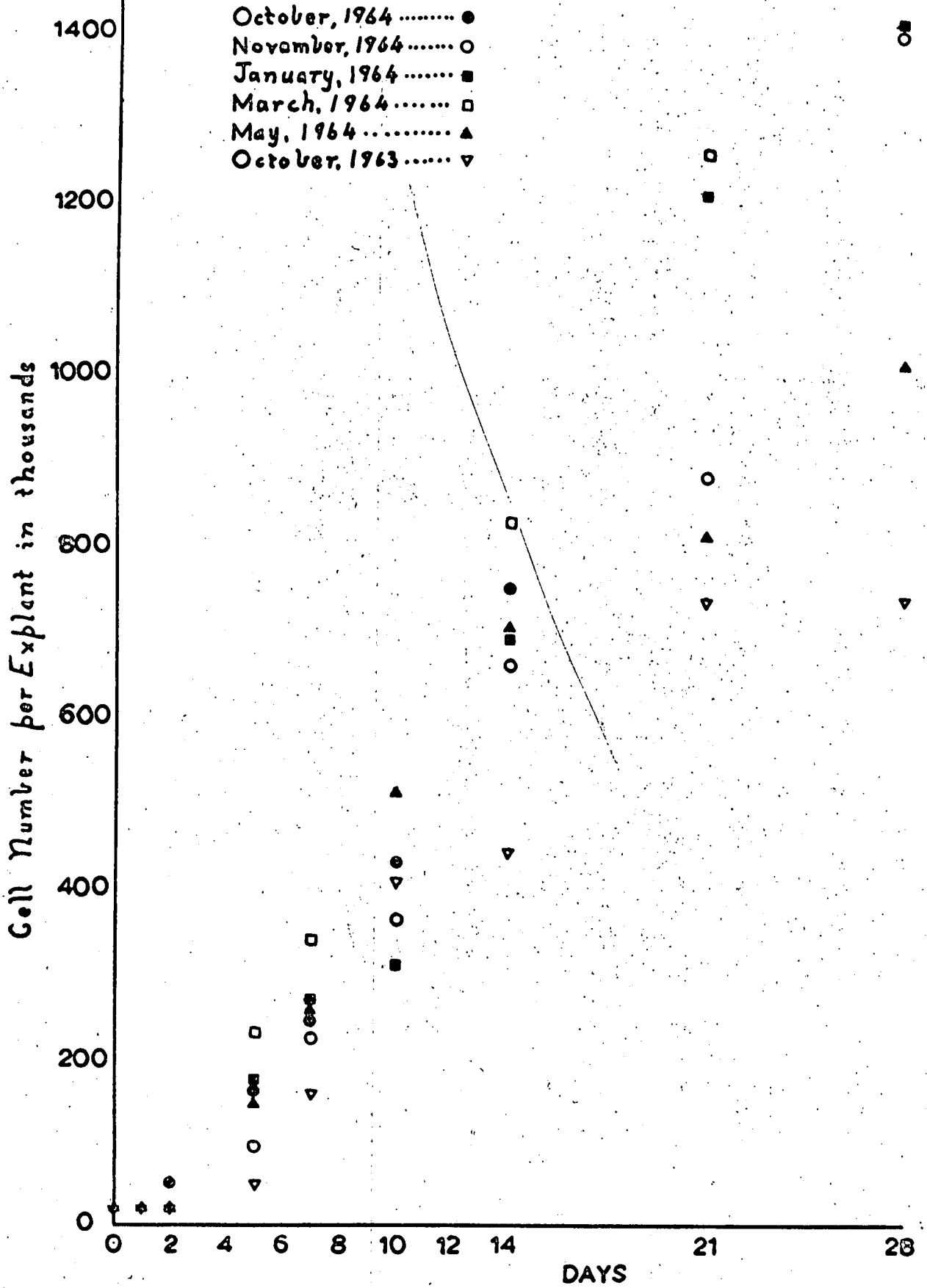


Figure 2.4. Experiments 60 - 65. A scatter diagram of the changes in cell number during 28 days of culture. The six experiments were initiated using tubers of increasing "age".

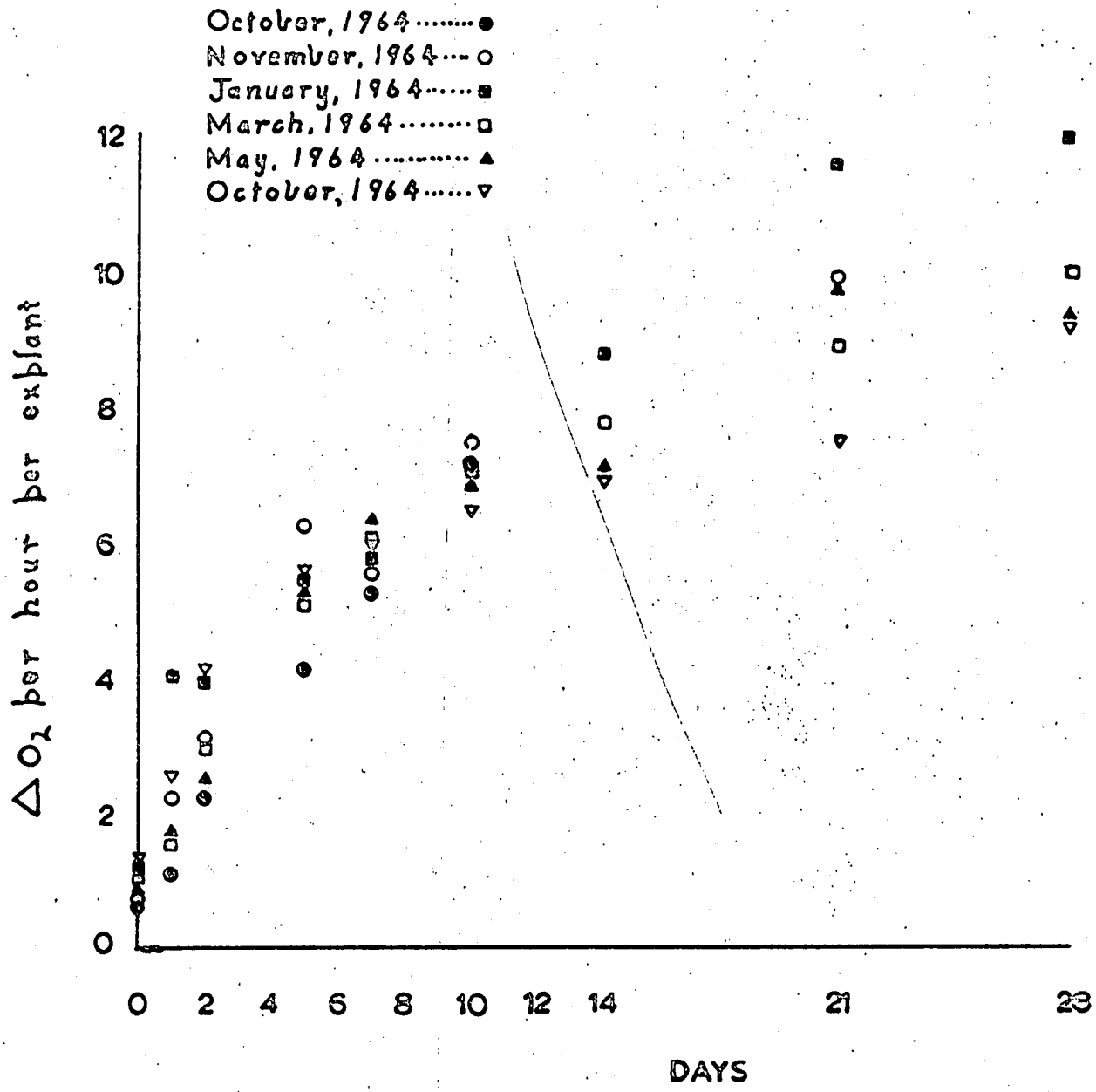


Figure 2.5. Experiments 60 - 65. A scatter diagram of the changes in the rate of oxygen uptake during 28 days of culture. The six experiments were initiated using tubers of increasing "age". Each point is the mean of duplicate determinations.

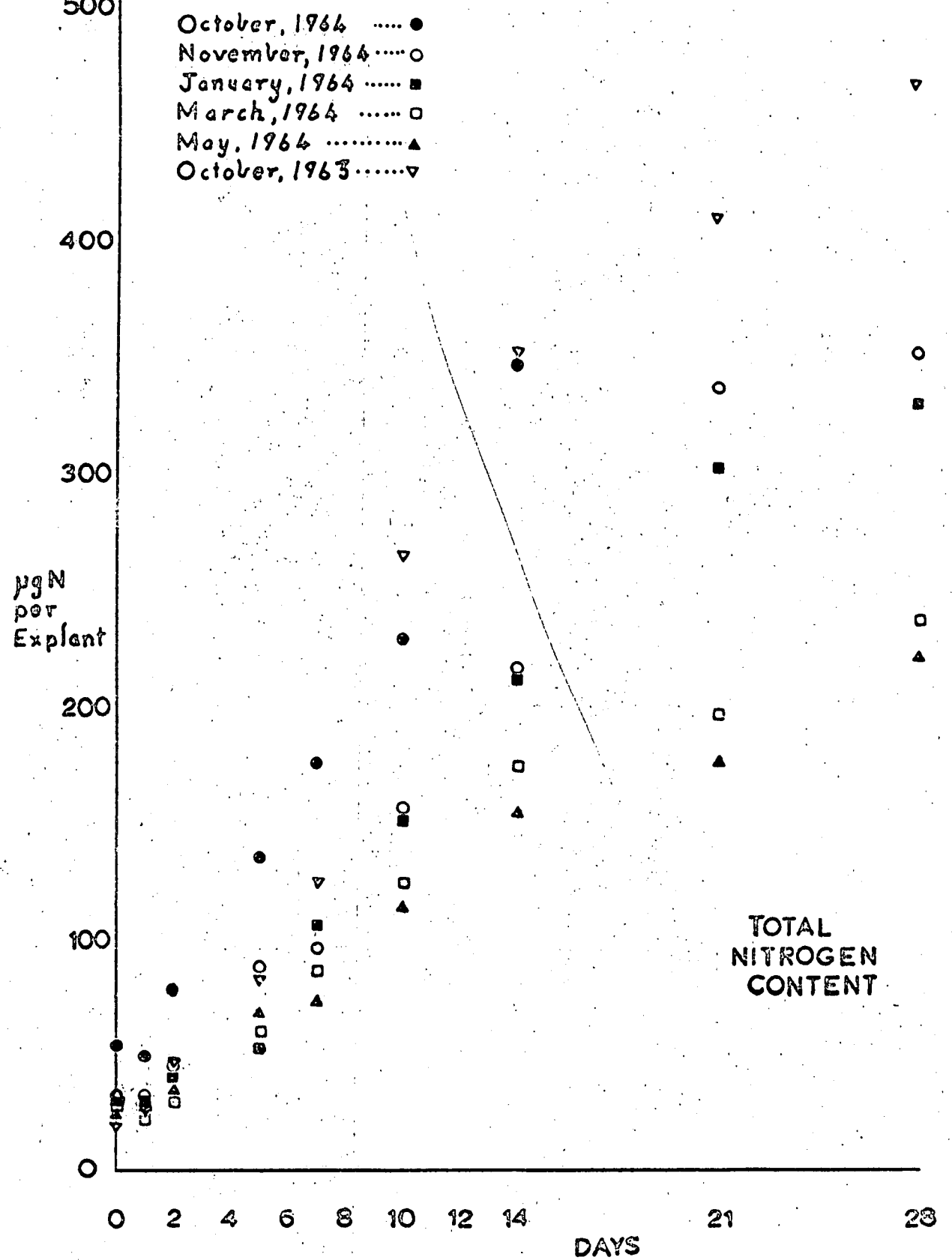


Figure 2.6. Experiments 60 - 65. A scatter diagram of the changes in the total nitrogen content during 28 days of culture. The six experiments were initiated using tubers of increasing "age". Each point represents triplicate aliquots from the digest of a single group of explants.

50 g and having a similar morphology with few lateral buds, were taken from storage at 4°C at the appropriate intervals. The major variable, therefore, was the length of storage which these tubers had undergone. The time in months since storage began will be referred to as the "age" of the tubers.

The responses to the cultural conditions of the Standard Procedure after periods of up to 28 days are set out in the scatter diagrams of Figures 2.2 - 2.6 It can be seen that the trends of change in the parameters of fresh weight (Figure 2.2), dry weight (Figure 2.3), cell number (Figure 2.4) and of oxygen uptake (Figure 2.5) are very similar, especially over the first fourteen days. There is some divergence in the fresh weight values for Day 21 and 28 which may be connected with the age of the tubers. There are consistent differences in the total nitrogen values (Figure 2.6) and in the total cell numbers. However, the main point which emerges from the scatter diagrams is the striking similarity of response in all the parameters measured, and it is for this reason that experiments performed with tubers of various ages may be usefully compared, provided that the following points of difference are borne in mind.

The values for October 1964, show two major departures from the normal trends. Firstly, there was a marked increase in fresh weight and in cell number during the first two days which was unique. Secondly, while oxygen uptake values were the lowest observed, the increases in fresh weight, dry weight and in total nitrogen were consistently larger than in the other seasonal experiments. The tubers for this experiment were clearly in a different physiological state from stored tubers because they had not been exposed to frost or to prolonged cold storage and may still be in the process of advancing towards the state of dormancy. The cells of these tubers cannot, therefore, be regarded as quiescent. For this reason, the experiment is only included for the purpose of comparison and the mean

values (Figures 3.10-14) do not include values from October 1964.

However, one obvious seasonal difference in response is shown by the total nitrogen values, (Figure 2.6). Until Day 7 the values for each experiment are similar, but in each of the subsequent harvests the highest value is recorded by the earliest, November, experiment and the other values decrease progressively as the age of the tuber, from which the explants were started, increases.

The second important seasonal variation in response is shown by the cell number values presented in Table 2.5.

	Estimated Cell number in thousands.						
Day	Oct. 64	Nov. 63	Jan. 63	March 63	May 63	Oct. 63	Mean
0	28	20	23	-	24	18	23
1	30	20	21	20	30	18	23
2	54	24	27	30	30	26	32
5	158	95	175	230	150	50	143
7	240	222	263	337	252	156	245
10	430	364	310	-	509	408	404
14	740	656	684	822	703	441	674
21	-	875	1,200	1,250	810	730	973
28	-	1,338	1,400	-	1,010	730	1,120

Table 2.5. Using the Standard Procedure, harvests were used for various estimates of which cell number is presented here. Six experiments were performed starting in the months indicated and the mean value for each harvest is presented in the last column and includes the October 1964 value.



Figure 2.27. A group of cells found in a cell macerate. Two cells are seen within the original parenchyma cell.

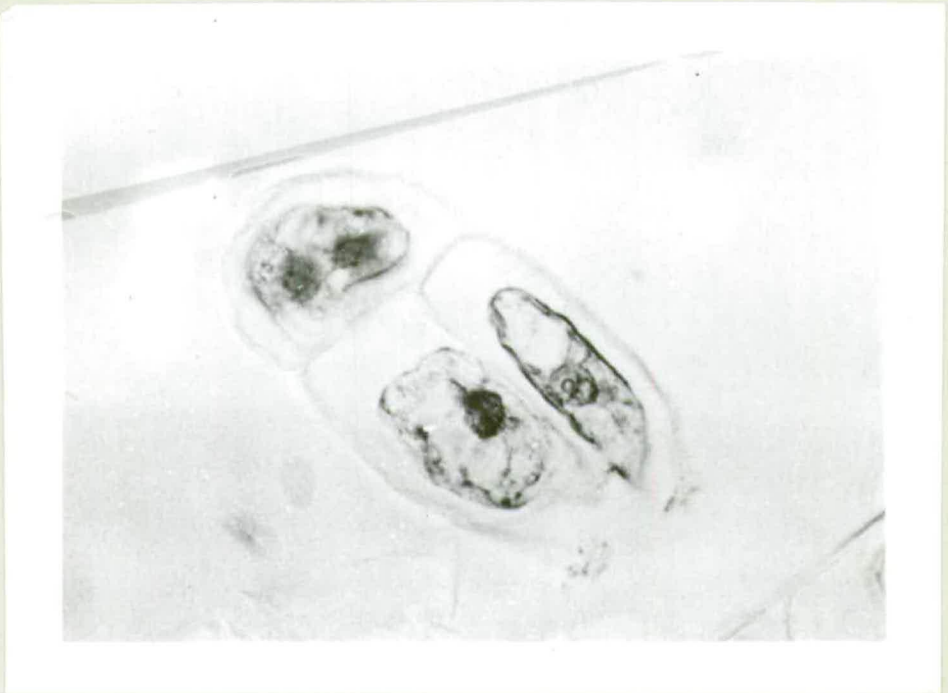


Figure 2.28. A group of cells found in a cell macerate. Four cells are seen within the original parenchyma cells.

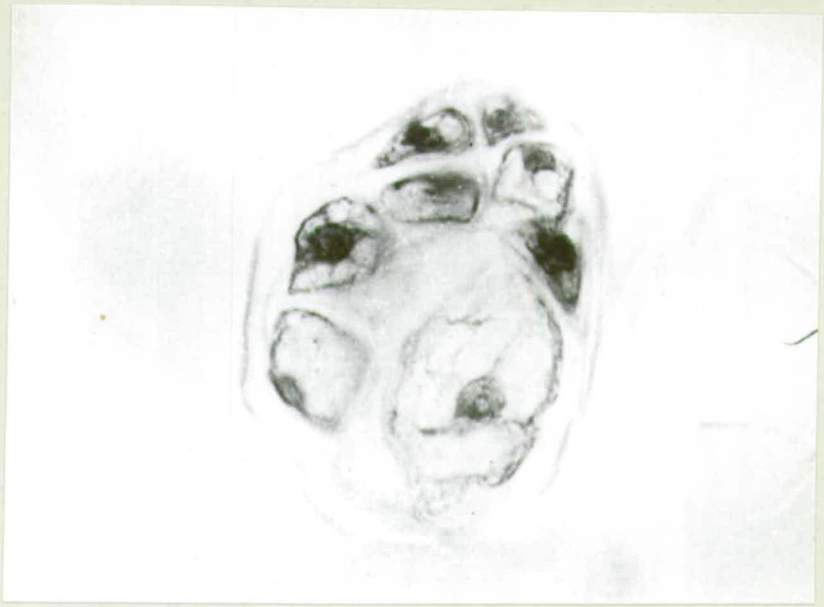


Figure 2.29. A group of cells found in a cell macerate. Eight cells are seen within the original parenchyma cell.



Figure 2.30. A group of cells found in a cell macerate. In one cell there are eight nuclei (not all at optimum focus) and in the other there are at least four.

In the bimonthly values from Day 5 to Day 28, the cell number value increases from November through to March and then decreases through May to October. It is possible these differences are a function of the initial cell number of the explant, which, probably by coincidence, also tend to follow this pattern. These latter differences are much too small however, to be significant statistically.

Finally, the fresh weight responses on Day 21 and Day 28 tend to show a similar pattern to that of cell number except that the highest values occur in the January experiment.

There are certain other observations of a qualitative nature which have a bearing on the seasonal variation. In Experiments 41 and 46 set up on 30/11/64 and 14/12/64 respectively, unusual groups of cells were observed in the chromic acid macerate. Chromic acid maceration tends to separate the original cells of the explant and to retain the newly formed daughter cells within the containing wall of the parent cell. Thus, two cell and four cell groups are often seen within the original parenchyma cell walls. However, after 48 and 60 hours of culture in Experiments 41 and 46, various groups of cells in the macerate had the appearance of embryos. Groups which can be likened in shape though not in size to two, four and eight cell stages are shown in Figures 27-9. Their organisation is quite different from the normally equational divisions observed at other times in the cell macerates.

In the May seasonal experiment chromic acid macerates from the 48 hour harvest showed many binucleate cells and many daughter cells which had only a very thin crosswall. At later harvests occasional four to eight-nucleate cells were noticed. This observation tallies with similar records of binucleate cells from 48 hr. harvests in, for example, Experiment 32 which was set up in March 1964. It would appear that at this time of year, the formation of primary cell wall is in some way impeded or reduced. An example of both a four and an eight-nucleate cell is shown in Figure 2.30.

When these last two phenomena, namely the presence of embryo-like stages, and the presence of binucleate cells after 2 days of culture, are related to the age of the tubers, a striking regularity in the chronology of the data emerges. This is summarised in Table 2.6.

Experiment	Date	Nature of the first wave of divisions
Seasonal	October 13	Binucleate
E 57	" 20	Very thin walls, multiple divisions
Seasonal	November 11	Normal, thin walls
E 40	" 13	Normal
E 26	" 19	Normal
E 41	" 30	Embryo-stages at 48 hrs., 60 hrs.
E 44	December 7	Normal up to 36 hrs.
E 46	" 14	Embryo-stages at 48 hrs.
Seasonal	January 13	Normal
E 50	February 12	Normal
Seasonal	March 17	Normal
E 33	April 28	Binucleate
Seasonal	May 27	Binucleate
E 17	June 3	Binucleate
E 36	July 7	Binucleate
Seasonal	July 13	Binucleate

Table 2.6. A comparison of the morphological expression of the first wave of divisions with the "age" of the tubers from which explants were taken.

Age of Tuber in months	Experiment	Date of Day 0	Time of 30% increase in number, in hours.
0	Seasonal	14/10/64	24
2	45	8/12/64	29
6	54	1/ 4/65	36-48
6	55	19/ 4/65	39-41
8	34	6/ 7/64	48-60

Table 2.7. Data collected from five experiments to illustrate the advancing time of the first increase in cell number correlated with the age of the tuber. In the seasonal experiments 24 hours is the time at which many division stages were seen and not the time of 30% increase. The 30% increase must, however, be within 4 hours of this time.

A final effect of the age of the tuber is described in Table 2.7. It can be seen from the fragmentary data collected from experiments designed for various other purposes, that the time of the first major increase in cell number advances with the age of the tuber. Systematic data collected by Mr. Evans in this department has subsequently confirmed and enlarged upon this observation.

DISCUSSION

The first objective of this study was to develop a suitable method of culturing whole explants for use in growth studies. The disadvantages of cultures supported on solid media have been mentioned earlier (p.2-3), the most serious being the inevitable induction of polarity in the growth of the explant. While resting on agar, a callus is subjected to gravity, to unequal nutrient and waste product gradients, perhaps to unequal illumination, and certainly to selective waterlogging along its region of contact with the medium. The anatomy of calli grown on agar display the results of these unequal stimuli. Unequal proliferation is a feature of such calli (Nitsch and Nitsch, 1956) and the manifestation of polar transport in explants of stem pith of Jerusalem artichoke has been described by Sevenster and Karstens (1955). The klinostat effect of the revolving mount and the continual motion of the explant through the medium rule out the polarising effects of all the above stimuli. As a result the only polarity that can be invoked to explain orientation in calli grown in liquid culture, must depend upon inherent interrelations that exist at the time of inoculation.

For example: (1) There is a marked difference between the cut, exposed surface and the intact, protected interior. The superficial layers of cells will first receive nutrients, including oxygen, and the interior tissue will receive them last, if at all.

(2) Most cells have their long axis across one diameter of the explant. Some damaged cells whose long axis is at right angles to a tangent at the circumference of the explant may therefore extend further into the explant. This may result in the wound effect of the damaged cells on the periphery extending further into the callus in particularly two regions.

It has been shown that, if explants are taken from widely different positions in the tuber then there is an increased growth response in explants from

the apical region (p. 84 - 88). This might be ascribed to a polar distribution of a growth stimulating substance. This substance could be a gibberellic acid since the studies of several workers (Brüinsma, 1962; Okazawa, 1960) have implicated this growth hormone in both the tuberisation and in the spring burst of the apical bud of potatoes. It might therefore be argued that within each explant there may be a polar distribution of growth hormones. However the experiment discussed above, also showed (p. 88) that there is no manifestation of this effect, if present, amongst explants taken from more similar but still considerably different positions within the tuber.

(3) Lastly is remains possible that within each excised explant endogenous substances or substances absorbed from the nutrient medium are subjected to polar transport (Caplin, 1947; Sevenster and Karstens, 1955; Jablonski and Skoog, 1954).

It is of course true that most methods of liquid culture overcome the major effects of polarity observed with agar cultures. However, the method developed for this study offers a number of additional advantages. Some of these depend upon the choice of the experimental material while others depend upon the experimental technique. The material, storage parenchyma from Jerusalem artichoke tubers has the following advantages.

(1) The average variation in growth response, from within a population of up to 300 explants taken from a single tuber, is as low as 12 per cent.

(2) The variation amongst the means of 32 experiments, using 32 different tubers, is only 16 per cent.

(3) There is low variation in response to culture throughout twelve months of storage which permits experimentation all the year round.

(4) The growth response has been much the same from year to year over a period of three years.

(5) In addition, there is a considerable amount of published information about the chemistry and growth potential of this tissue (e.g. Goris, 1948; Nitsch and Nitsch, 1956, 1960; Adamson and Adamson, 1957, Adamson, 1962; Masuda and Yanagishima, 1965; Bacon et al. 1951, 1952, 1960, 1961; Edelman et al, 1960, 1961 a, b. 1963 a-f).

It can be seen therefore that one major difficulty, the variability of response to culture, which has beset the interpretation of results based on tissue culture work, has been overcome using this material. The experiments performed by this method of culture may be quantitatively compared one with another, and qualitative differences may be ascribed to real effects and not to those unavoidable differences which occur along the wide spectrum of varied response to be expected in many tissue culture systems. This reduces the need for repetition and replication and transfers some of the conclusions in this study, from the realm of informed opinion to that of statistically qualified probability.

Having selected artichoke tubers as the experimental material, the technique offers these advantages:-

(1) Contamination by micro-organisms is less than 1 per cent in routine experiments, and, as each explant is cultured individually, such contamination involves only single explants.

(2) Cultures may be set up at the rate of 100 per hour.

(3) The yield is high especially in regard to the cell number increase. There is a five fold increase in fresh weight and a concomitant fifty-fold increase in cell number.

(4) The system uses simple, standard equipment.

It was decided that the characteristics enumerated above justified the adoption of the Standard Procedure (p. 54) as a suitable method of pursuing the growth studies which are the topic of this thesis.

Having adopted this Procedure, certain aspects of it have a bearing upon the growth studies to be described in Section II. Firstly the medium, when it was provisionally chosen was simply one which worked (i.e. promoted the kind of growth desired) although it did not necessarily have optimum concentrations of each component, and still less did it represent the optimal balance between each component. However it has since been learnt that the presence of sucrose and of the basic salt mixture does not have a measurable beneficial nor detrimental effect upon the fresh and dry weight increases and that sucrose does not affect the cell number increase. These components are retained in Medium A simply to permit valid comparisons to be made amongst all the experiments performed. It has been shown that 2,4D is present at close to the concentration which is optimal in the absence of coconut milk, and that the growth promotion in the presence of a range of 2,4D concentrations which lack coconut milk, shows a curve with a well defined maximum concentration at 10^{-6}M. This form of curve is characteristic of the curve of hormone action. In contrast, coconut milk is increasingly effective in promoting growth at concentrations up to 10 per cent v/v and is then equally effective over the range of 10 - 25 per cent. This would suggest that the dominant effect is one of satisfying a requirement for substances which may be present in excess without detrimental effects upon growth promotion. This is a convenient feature because it permits the use of excess of this component which is calculated to "blanket out" some of the variations which might be expected to be inherent in tubers of differing sizes, shapes and ages. Thus it is employed as a kind of physiological buffer which

may keep the growth response within certain limits.

Setterfield (1963) and Adamson (1962) have discussed the competition or balance between the processes of tissue expansion and of cell division. It has been shown (p.71-2) that when the growth response on medium lacking coconut milk and that lacking 24D are compared to that in full Medium A, the presence of coconut milk promotes a relative increase in fresh weight and the presence of 24D promotes a relative increase in cell number. The balance of these two components must be critical and the mere coincidence of their separate optima does not necessarily point to optimum growth conditions. However the particular combination arrived at results in the kind of growth that is required for this study and so the concentrations as listed for Medium A have been retained throughout the experimental work.

An additional nutrient which is not listed above as one of the components of Medium A, is oxygen. It is abundantly clear that in certain unusual circumstances (e.g. "crawlers", p. 74 ; large 11 mm mounts, p. 75 ; condition D in Experiment 34, p. 76) the increased facility for gaseous exchange promotes an increase in the fresh weight and cell number achieved during 14 days of culture. A critical experiment (Experiment 34, p. 76) compared the conditions of the Standard Procedure (Condition A) with three other conditions in which common to all three there was an increase in the diameter of the containing tube with a resultant increase in the area of medium exposed to the atmosphere. Within these three treatments however, mounts were constructed so that Condition C was in all other respects closely similar to the standard Condition A while Conditions B and D were placed at the two extremes of facility for gaseous exchange. In Condition B explants remained submerged in the medium during their rotation, in Condition C they dipped equally in and out of the medium, and in Condition D explants were only

submerged for a very short period of time in each rotation. From the graded growth response increasing from B, through C to D, it seems likely that the conditions of the Standard Procedure (which apart from the factor mentioned above is closely comparable with Condition C) occur at a point along a gradient of oxygen supply or of carbon dioxide removal. It will be recalled that all three Conditions B, C and D showed significant increases over Condition A. Both these effects suggest that the facility for gaseous exchange may be exerting a limiting effect upon the net growth capacity of each explant.

The surprising contrast between Conditions A and C and more especially between A and B would suggest that the most critical feature of gaseous exchange is not the proportion of time which the explant spends clear of the bulk of the medium but rather the area of interface between atmosphere and medium available for the solution of oxygen and perhaps for the dissolution of carbon dioxide.

Seasonal Variation.

The essential similarity of the growth response of tubers of various ages has been described, and it was concluded that for most purposes, experiments performed with tubers of different ages may be usefully compared.

However, despite the blanketing effect of the medium, certain seasonal Changes have been recorded.

1. Explants from tubers which have not received cold treatment have a different response to culture from those which have received cold treatment.
2. The older the tuber from which explants are taken, the less total nitrogen content there is in calli especially during the later stages of culture.
3. For the first six months the amount of cell division increases with the age of the tuber and thereafter it decreases.

4. The length of the lag period in cell number increases with the age of the tuber.

5. Perhaps during the first month of storage and certainly after 6 months of storage the ability of cultured explants to complete the first cytokinesis tends to be impaired.

In this section, the major features of a new method for the liquid culture of whole explants have been defined. This method permits small explants from storage tissue to be cultured under standard conditions in a complex nutritive medium. Some knowledge has been gained of the effects of the various components of this medium in order that the system may, in the future, be modified to provide different but comparable modes of growth and development. Certain changes in response due to the "age" of the tuber have been described.

The growth response which has been observed under the chosen conditions is highly reproducible, and as a result, many aspects of development can be usefully investigated using this basic technique. Normal organic development is the orderly progression from a microscopic egg or meristem to a large and fully differentiated structure. Bloch (1961) in discussing development distinguishes 5 processes: (1) Growth (increase in organic mass); (2) Differentiation (diversification and maturation of this mass); (3) Pattern formation; (4) Regressive change (embryonisation and dedifferentiation); (5) Regeneration of patterns (after disturbance or de novo). Development in a callus is the orderly (or disorderly) progression from the initial excised state through a succession of changes, which may take a variety of courses according to the conditions imposed, until development is arbitrarily terminated. It will be shown in Section II that all these processes may be observed, in simplified circumstances, during callus development using the system evolved in Section I.

EXPERIMENTAL RESULTS - SECTION II

THE TIME-COURSE OF CHANGES DURING CALLUS DEVELOPMENT.

Introduction.

The first section has described the development of a suitable method of culturing the chosen material and the kind of variation in fresh weight which the method is liable to exhibit. These have been necessary preliminaries to the study of growth and development in this work. In considering growth, a definition of terms is also necessary. Bloch (1961) has stated that "Growth is a fundamental property of protoplasm, synthesis of new protoplasm takes place through the activity of that already present". This synthesis of protoplasm is registered by "a more or less permanent increase in organic mass". Inorganic mass being negligible, this is tantamount to citing the index of dry weight. Another generally accepted criterion of growth is that of an irreversible increase in volume, which may be measured by the increase in fresh weight. The growth of a tissue mass will usually involve the processes of cell division and of cellular expansion. Both contribute to the increase of the mass.

In order to study "metabolic changes during callus development" it is necessary to have a system which can be carefully controlled and easily manipulated, and which exhibits those aspects of growth and development which are relevant to the project in hand. The "system" used here is a particular pattern of changes made manifest when explants from the storage parenchyma of the Jerusalem artichoke tuber are cultured over a period of 28 days under the conditions described previously as the "Standard Procedure" (p. 54). The object of the experiments reported in this section was to define in quantitative terms, certain changes in the developing metabolism of a typical callus. These changes are indicated by such

well used parameters of growth as fresh weight, cell number, total nitrogen content, dry weight, and oxygen uptake.

In describing the changes in these parameters it will be realised that, being a biological system, there is considerable quantitative and qualitative variation amongst individuals. Therefore, when "the cell number on Day 7" or "the change in fresh weight during the first seven days" is referred to, it can only mean "the average cell number for the explants assayed from the harvest on the seventh day" or "the difference between the mean fresh weights recorded on Day 0 and on Day 7". Thus a "typical" callus may be imagined which has all the average properties of the parameters measured. It will be realised that no such callus can exist but the concept is a useful one in considering the typical changes involved in a study of this kind.

Experimental Design.

The procedure used to define these typical changes has been to start the culture of a large number of explants on a given day (Day 0) and to harvest at random from this population, appropriate numbers of explants on selected days throughout the time-course of the culture period. The number of explants inoculated, the number withdrawn at each harvest, and the selection of the harvest intervals depended upon the particular design of each experiment.

For each harvest a minimum of twelve explants was taken, although in some experiments rather more were required in order to have enough explants to assay, for example, the gaseous exchange of explants. If the measurement of gaseous exchange was included in an experiment then the harvested explants were first transferred to Warburg flasks for three hours and their fresh weights were recorded subsequently. Otherwise the fresh weight of each explant was determined immediately on harvesting and the harvest divided into groups for a variety of

assays (see p. 12).

The choice of harvest intervals followed one of three plans. The first, Plan 1, was to harvest on Days 0, 2, 5, 7, 14, 21 and 28 in order to define the broad changes associated with the growth and development of the callus. A series (Series 1) of six experiments employing this plan were completed. Using these intervals, but drawing data from several later experiments as well, changes in the rate of gaseous exchange, (both of oxygen uptake and of carbon dioxide release), in dry weight, in alcohol insoluble nitrogen and in total nitrogen will be described.

Having completed this broad survey of the changes over 28 days, interest was concentrated on the first seven days of culture, which is the time when cell number increase is most rapid and when morphological changes are least complex. To investigate this rapid increase more closely, another series of six experiments used daily harvest intervals until Day 7 followed by harvests on Day 10 and Day 14. These intervals (Plan 2), and data from these six experiments only (Series 2), are used to record changes in the fresh weight and cell number of the "typical" callus. Certain other data from these experiments will be considered in Section II B, (p.117).

The third plan involved shorter intervals, designed to investigate the changes associated with the induction of cell division during the first days of callus development and will be described in Section II C, (p.138).

Finally, at intervals throughout the course of experiments in Series 1 and Series 2, data were gathered for the six seasonal experiments described in Section I B, (p. 79). These, for convenience, have been given the alternative Experimental Numbers of 60 - 65 and called Series 3. In order to compare these experiments with those of Series 1 and 2, the values from the seasonal experiments have been averaged and are presented graphically in Figures 3.10-13. It has

already been pointed out (p. 89) that some of the variations in the primary data seem to be due to progressive changes in the growth capacity of tubers of different ages, while others appear to be random. Accordingly, these curves (and all the secondary data derived from them) are presented, not as a mean curve of a number of replicated experiments, but as the typical growth response obtained (despite the progressive changes related to the age of the tubers) when artichoke explants are cultured using the Standard Procedure, through a period of eight months.

Choice of Experimental Data for Presentation.

Because these three plans of approach have been adopted, a full mean of all values from every experiment at each harvest would not be meaningful. This is because, in Plan 1, certain values (Days 1, 3, 4, 6 and 10) are missing. If, as is the case, the mean curves for the two series differ slightly, then the plots for these latter values would be biased in favour of the Series 2 results and thus distort the pattern of changes. Therefore, each parameter of growth is presented using the harvest intervals of one or the other of the first two plans. Data from the appropriate harvests of the second series of experiments, using Plan 2, can of course be included in the summation from which the mean values based on the format of Plan 1 are derived.

All the data recorded in these two series of experiments are presented without selection in Appendix B (p. 190). It can be seen from the results recorded therein that Experiment 32, in the second series, was very atypical. It had a reduced fresh weight and cell number in the first few days; the dry weight actually decreased during the first five days; the oxygen uptake was double the average value for other experiments. It was decided that if these values were included in the mean for Series 2, then it was likely to obscure the true picture of metabolic changes, and so the results from this experiment were not used. In Series 1,

although values for Day 21 and Day 28 have been recorded, they are not plotted on the growth curves so that the scale of the abscissa may remain comparable with the plots for Series 2. These two points serve only to extend the retardation phase over a longer period, and their omission allows the more dramatic early phases of the growth changes to be emphasised.

In most cases the replicate experiments of Series 1 and 2 tend to show the same trends of change and so the data is presented as a simple mean curve for all the experiments in which data were recorded for the index of growth in question. The scatter of points is however less than the scatter found in Series 3 (p.89). For the estimation of ribonucleic acid only two experiments were carried out and so both sets of values are plotted (Figure 3.6). In the index of alcohol soluble nitrogen, because the data is rather disperse the values are given both as a mean and in the form of a scatter diagram (Figure 3.5). For the index of total nitrogen, as there appear to be two distinct trends, all the experimental values are plotted in company with a mean curve (Figure 3.4).

Data for Series 3 are all presented as mean curves derived from the values presented as scatter diagrams in Section I B (p. 89).

The results in this section will be described in three parts - II A, II B and II C.

S E C T I O N II A

CHANGES IN THE MAJOR INDEXES OF GROWTH.

Introduction.

In this section, growth changes of two kinds will be considered. These are the changes in the amount of growth: the increase in a parameter during a given time; and the changes in growth rate: the increase in a parameter over a period of time in relation to the amount present at the start of that period. In the former case, changes will be described in terms of a lag phase, if present, and an increase or a decrease in the amount of growth. In the latter case, the terminology of Monod (1949) is perhaps the most apposite, since it was devised for the analysis of systems using bacterial cultures in which growth is directly related to cell division. Thus the process of cell division may be logically examined using these definitions of Monod's.

1. Lag phase, growth rate null
2. Acceleration phase, growth rate increasing
3. Exponential phase, growth rate constant
4. Retardation phase, growth rate decreasing.

In growth studies, many criteria of growth have been used. It would seem that the particular index selected varies with the material used and with the purpose of the study. No single criteria has universal application, but in this study most of the common parameters have been followed. Each will be described in turn, without being committed to any one index as the most accurate or relevant indication of growth changes.

Fresh Weight changes.

These changes are plotted in Figures 3.1 (Series 2) and 3.10 (Series 3).

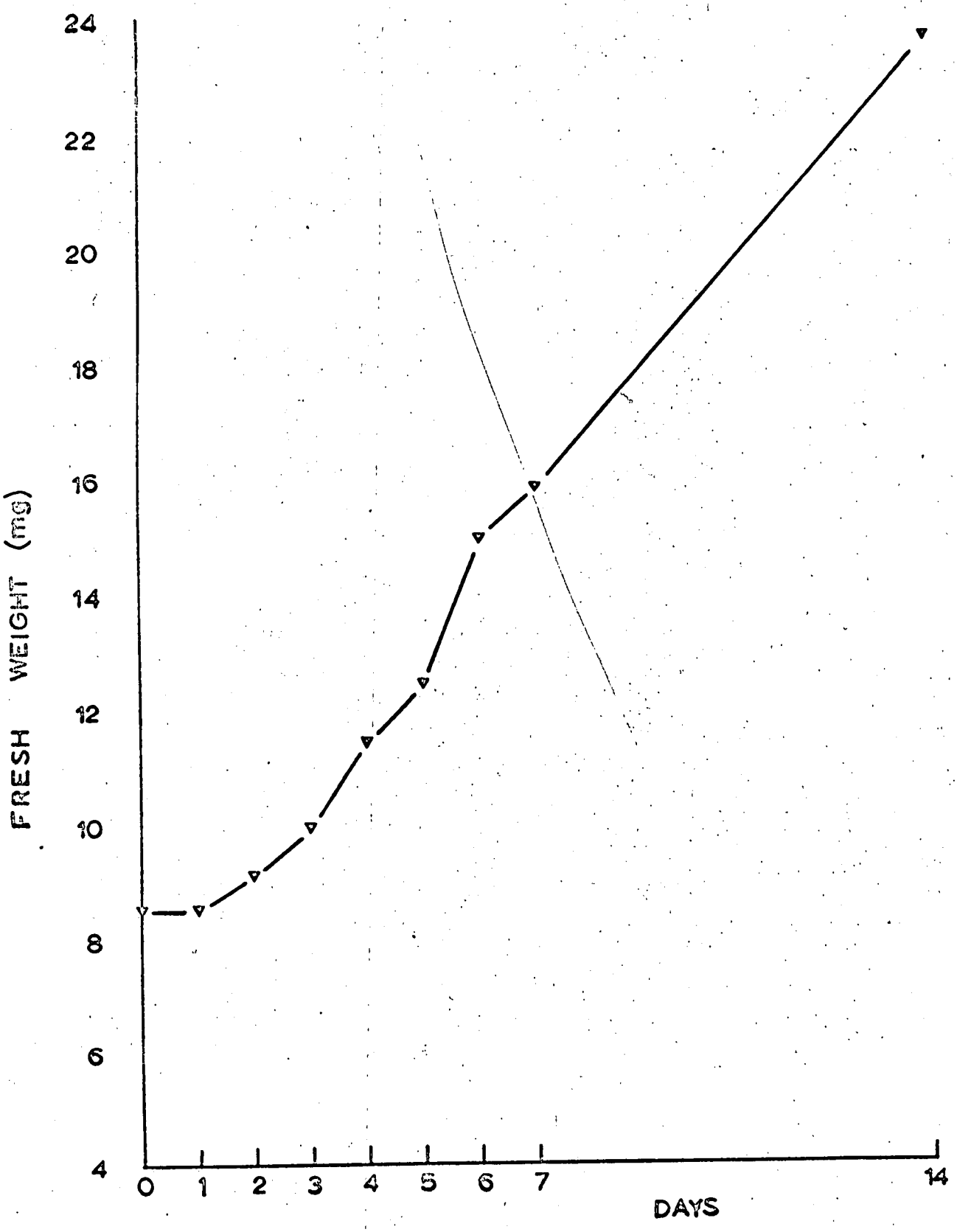


Figure 3.1. Series 2. Changes in the fresh weight of explants during 14 days of culture. Each point is the mean of 60 values from 5 experiments.

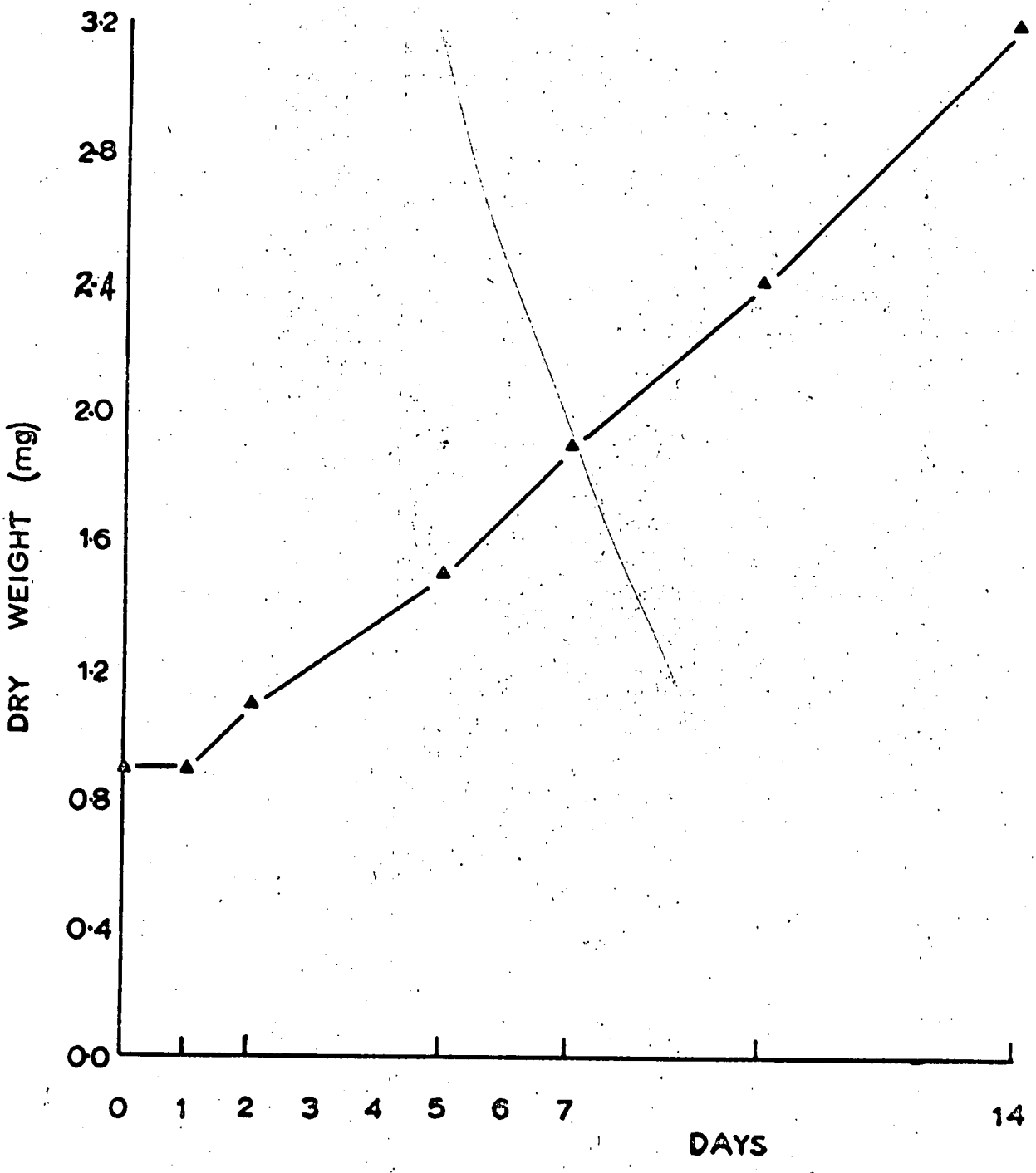


Figure 3.2. Series 1 and 2. Changes in the dry weight of explants during 14 days of culture. Each point is the average of 6 experimental values.

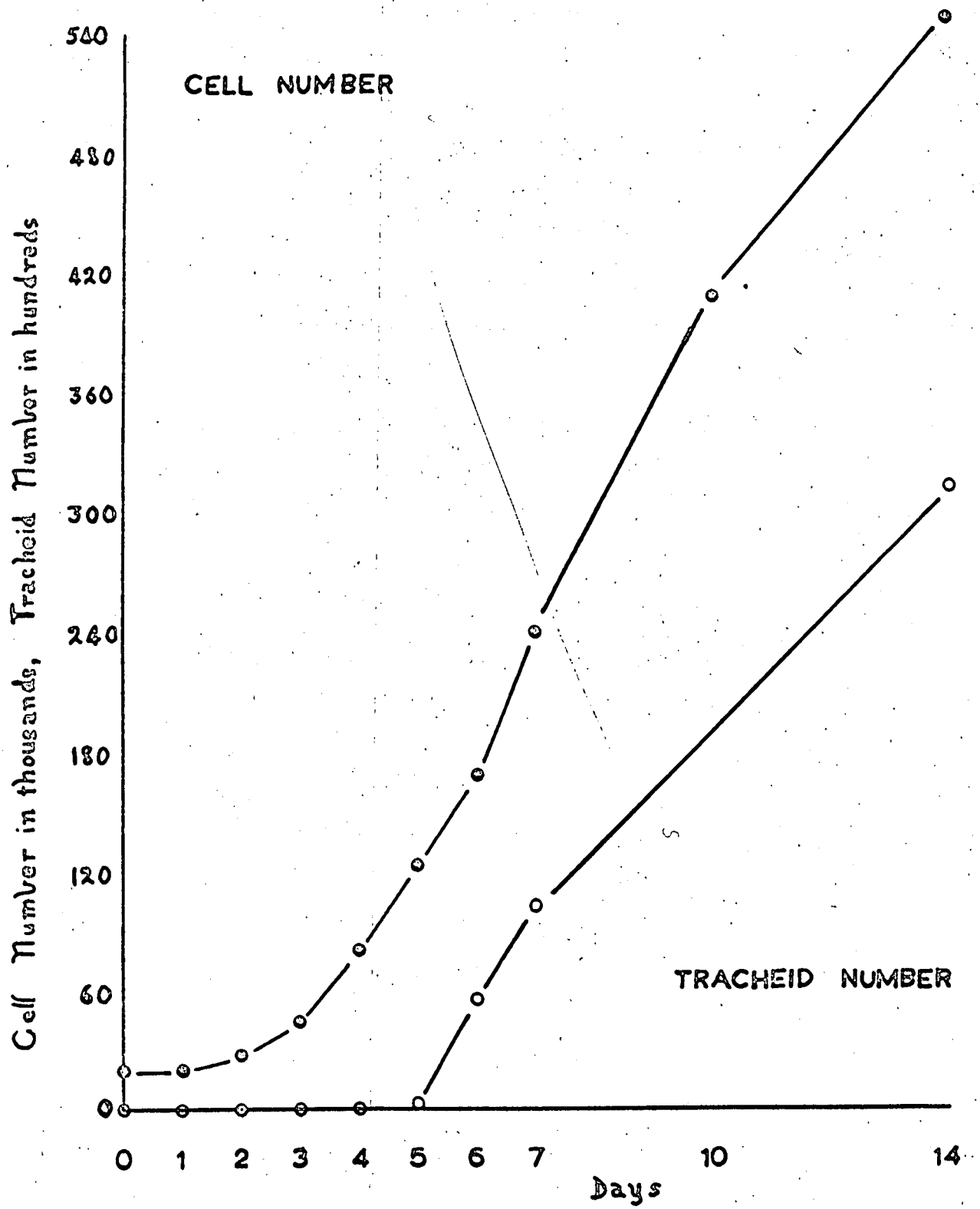


Figure 3.3. Series 2. Changes in the cell number and tracheid number of explants during 14 days of culture. Each point is the average of 6 experimental values.

In both series there was a lag phase of 1 day, followed by a more or less linear increase (corr. coeff =) until the end of the plot. This represents an almost constant amount of growth throughout the culture period after the lag phase. In Series 2, the closer harvest intervals showed that there was an inflection in the growth plot at Day 6 and when these values are plotted on a logarithmic basis two exponential phases may be distinguished by a slight change of rate at about Day 7, (Figure 3.14)(p109)

Dry Weight Changes.

These are plotted in Figures 3.2 (Series 1 and 2) and 3.10 (Series 3)(p109) Again, in both sets of data there is a distinct lag phase of 1 day, followed by a linear increase (corr. coeff. =) until the end of the plot.

This indicates a constant amount of growth throughout the period of culture after the lag phase. Although there is no inflection on the numerical curve, a log plot of these values (Figure 3.15) p109 again suggests a slight decrease in rate at about Day 7 (Series 1 and 2) or Day 10 (Series 3). It can also be seen from Figure 3.10 that in Series 3 the ratio between dry weight and fresh weight is almost constant.

Changes in Cell Number.

The changes in total cell number are plotted in Figures 3.3 (Series 2) and 3.11 (Series 3). p109 Here also there is a lag phase of about 1 day, but thereafter there is a typically sigmoid growth curve. There is no exponential phase, barring one exceptional case, but there are distinct phases of acceleration and of retardation. The transition between these two phases at about Day 5 coincides with the appearance of an appreciable number of tracheids in the cell population (Figure 3.3). These differentiated cells increase in number throughout the retardation

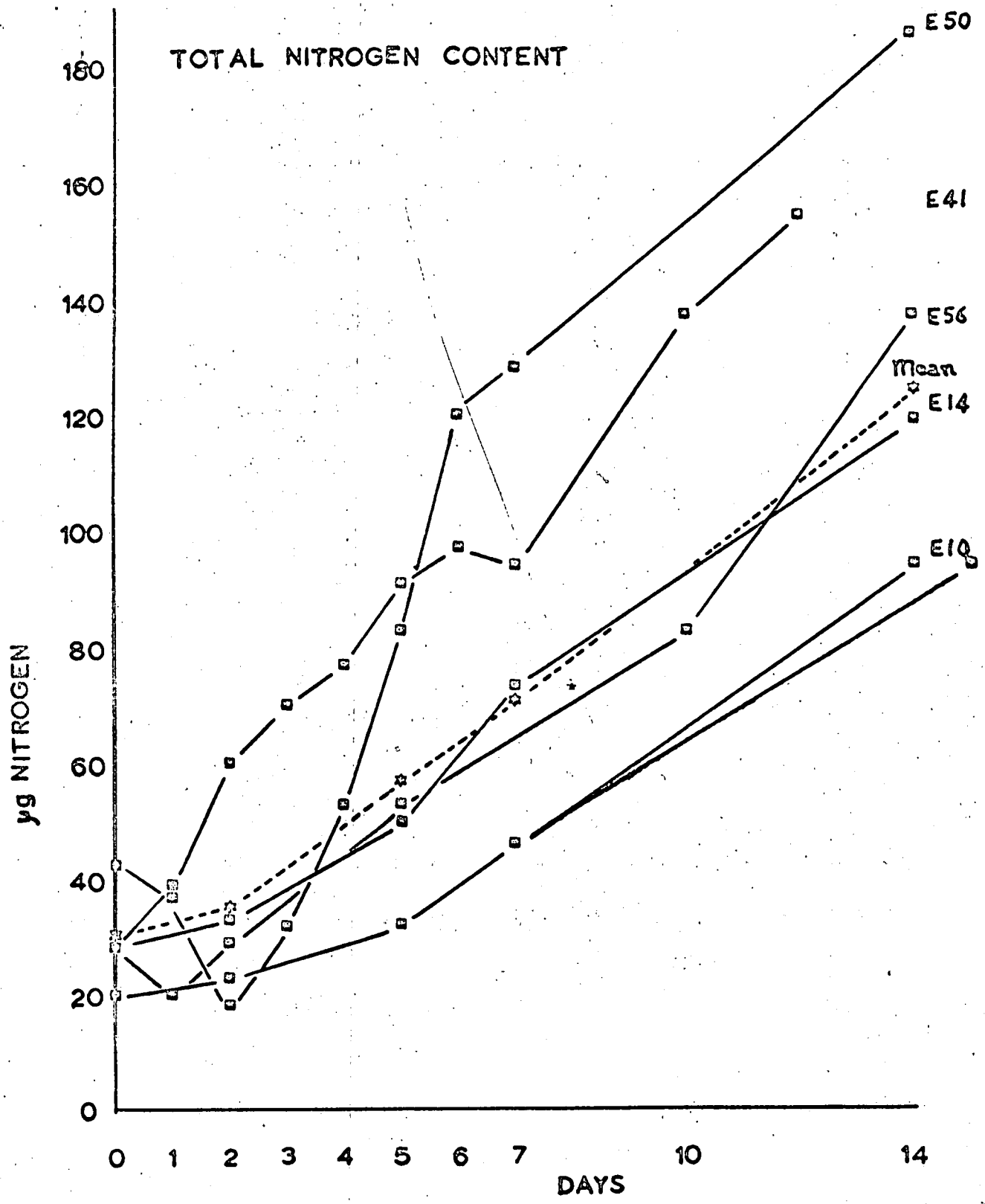


Figure 3.4. Series 1 and 2. Changes in the total nitrogen content of explants during 14 days of culture. Each point is the mean from three replicates. Data from 5 individual experiments (e.g. E 50 etc.) are plotted. Mean values according to Plan 1 are indicated by the dotted line.

phase.

The exceptional case mentioned is that of Experiment 10 which was an early experiment employing mounts made from glass tubing. Although it has been shown that the net fresh weight increment achieved over 14 days of culture is very similar with this mount and with those made from glass rod, it remains possible that the growth curve is slightly different. In order to illustrate the exponential nature of the cell number increase in Experiment 10, the log plot for cell number is given in Figure 3.16 (p 109)

Changes in Total Nitrogen Content.

These changes are rather variable and have been shown (Section I B, p. 90) to be modified by the age of the tuber. However, this does not account for all the variability, since it is apparent that in some cases there is an initial unexplained inability to achieve a net synthesis of protein. From the results of Series 1 and 2 (Figure 3.4), two distinct trends can be observed. In the majority of cases, the several curves show general trends of increase which may be variously interpreted as weakly sigmoid, loosely exponential or almost linear. In the remaining two cases, there is a decrease until Day 1 (Experiment 56) or Day 2 (Experiment 50) which is followed by the same general increase shown by the other curves. The data of Series 3 are much more consistent and here the mean growth curve (Figure 3.11) p 109 is very similar to the mean curve for cell number increase, except that there is no lag phase. These changes could also be interpreted as two logarithmic phases with a change of rate between Day 7 and Day 14. The ambiguity of possible interpretations is deliberately emphasised to illustrate the difficulty (and perhaps futility) of fitting a mathematical interpretation to data derived from what is essentially a heterogeneous system (see also Steward, 1958). Extreme caution is therefore necessary when seeking simple explanations of these complex functions of growth.

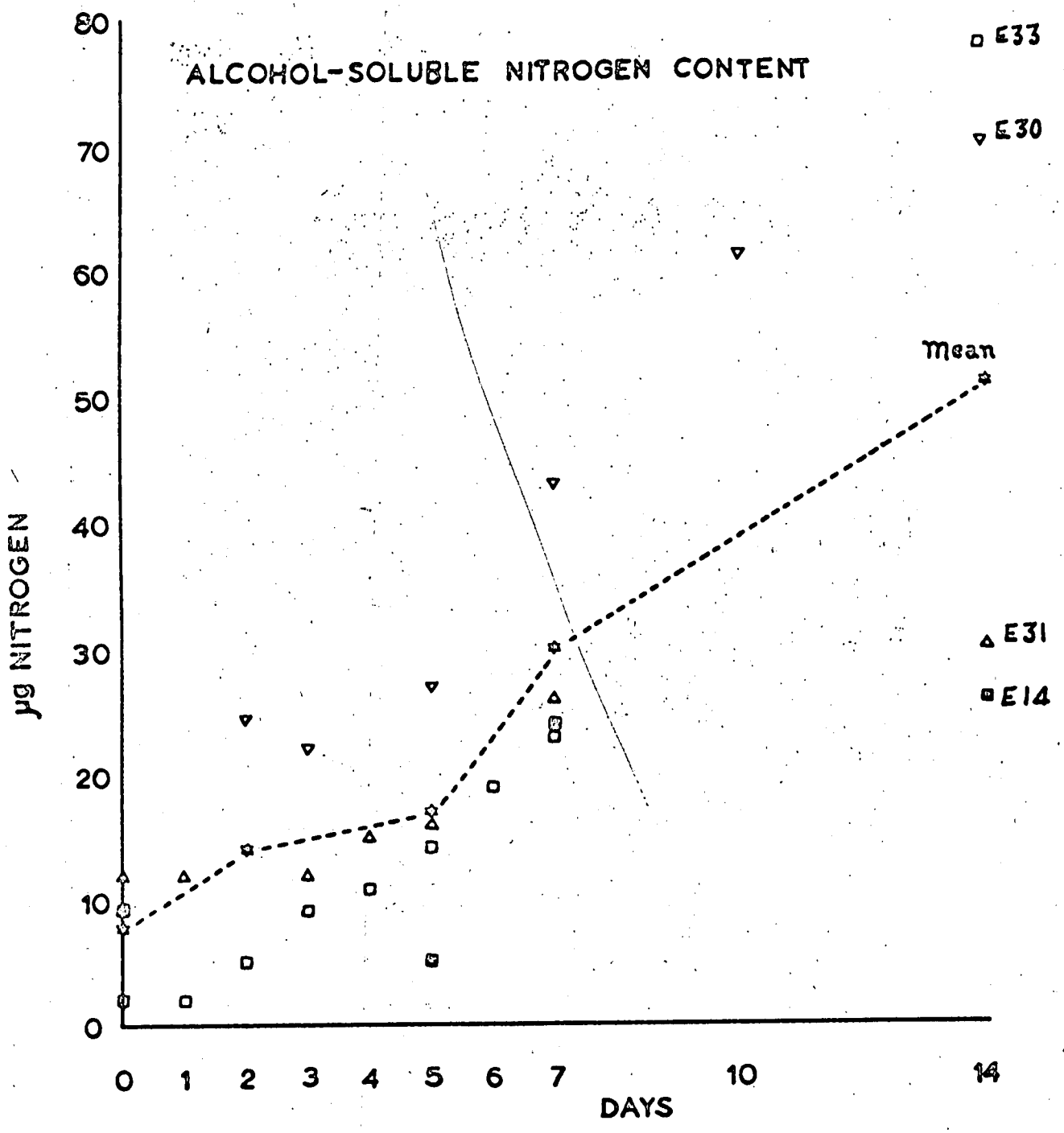


Figure 3.5. Series 1 and 2. Changes in the alcohol-soluble nitrogen content of explants during 14 days of culture. Each point is the mean of three replicates. Data from four individual experiments (e.g. E 33 etc.) are plotted as a scatter diagram. Mean values according to Plan 1 are indicated by the dotted line.

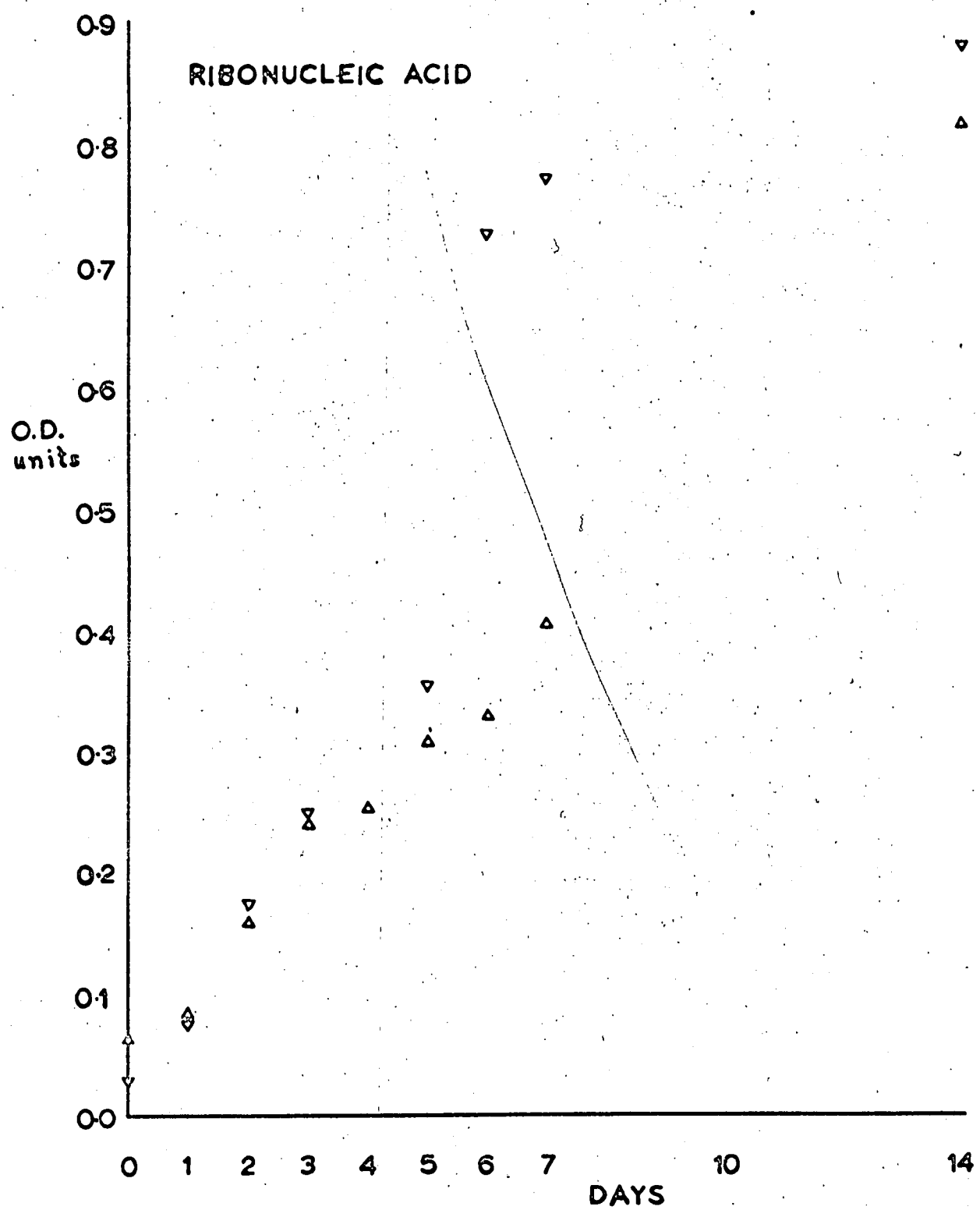


Figure 3.6. Series 2. Changes in estimated ribonucleic acid content of explants during 14 days of culture. Each point represents a single experimental value.

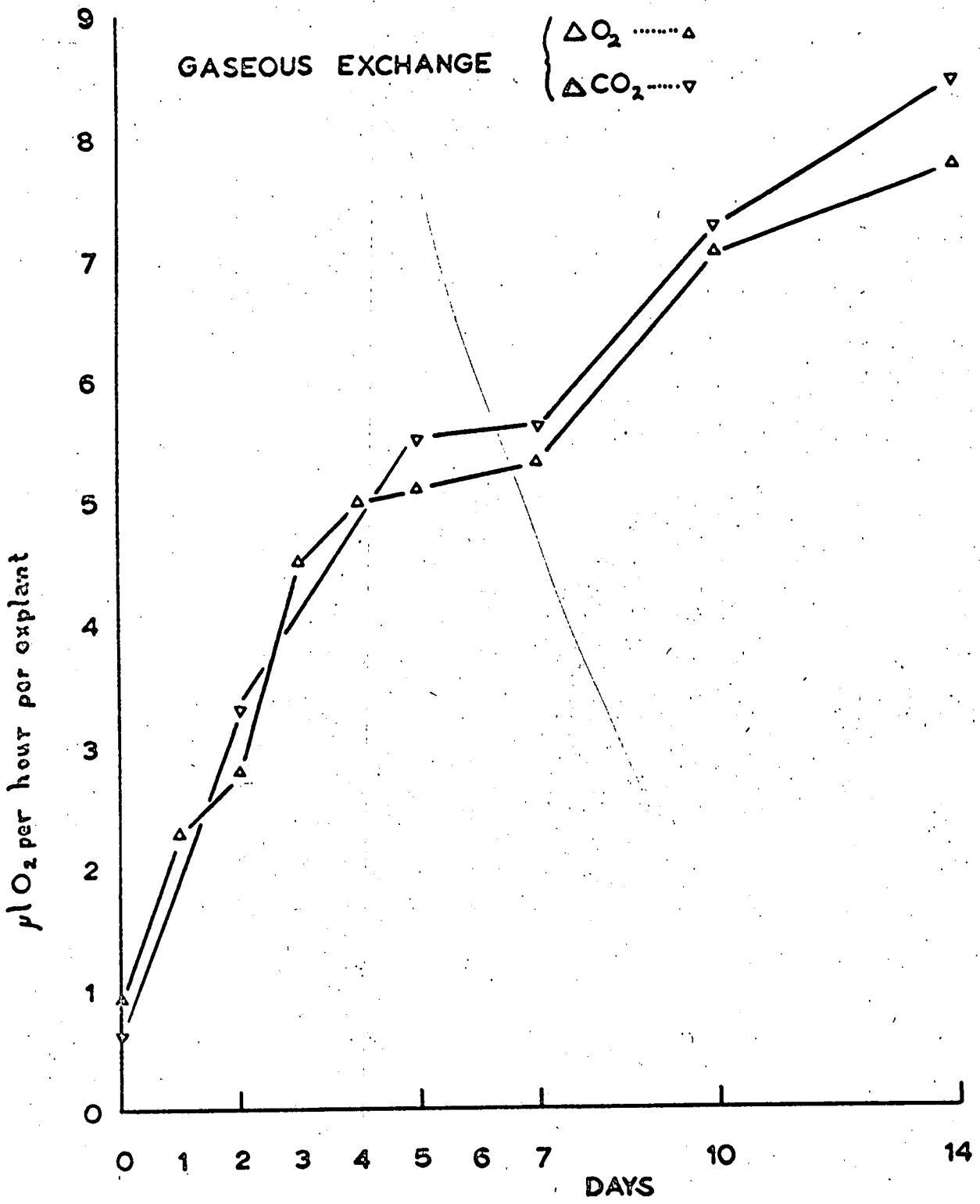


Figure 3.7. Series 1 and 2. Changes in the uptake of oxygen and release of carbon dioxide in explants during 14 days of culture. Oxygen uptake values derive from 4 experiments and carbon dioxide release values from 3 experiments. Each experiment gave duplicate values for each determination.

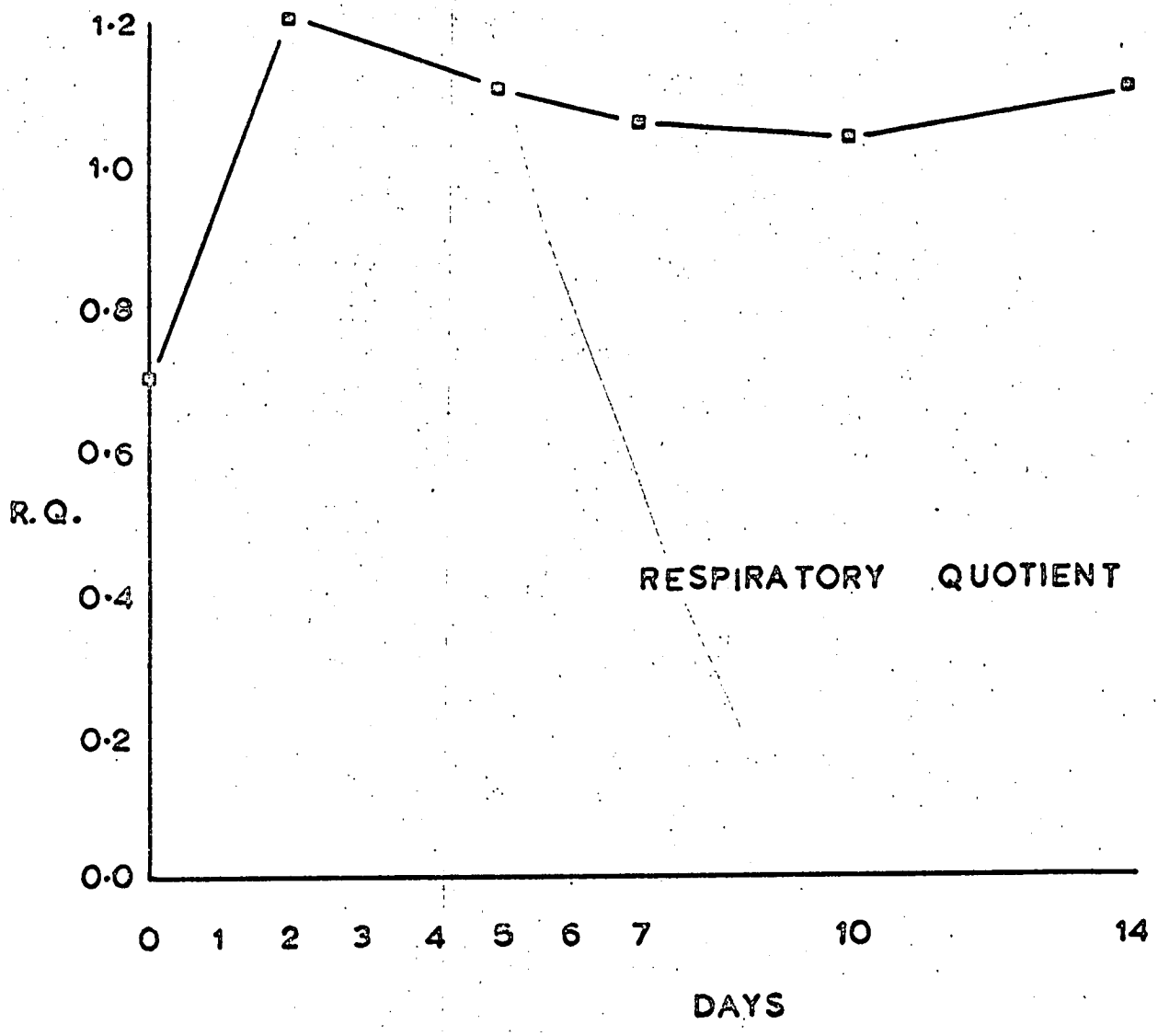


Figure 3.8. Series 1. Changes in the Respiratory Quotient of explants during 14 days of culture. Each point is the mean from 3 experimental values.

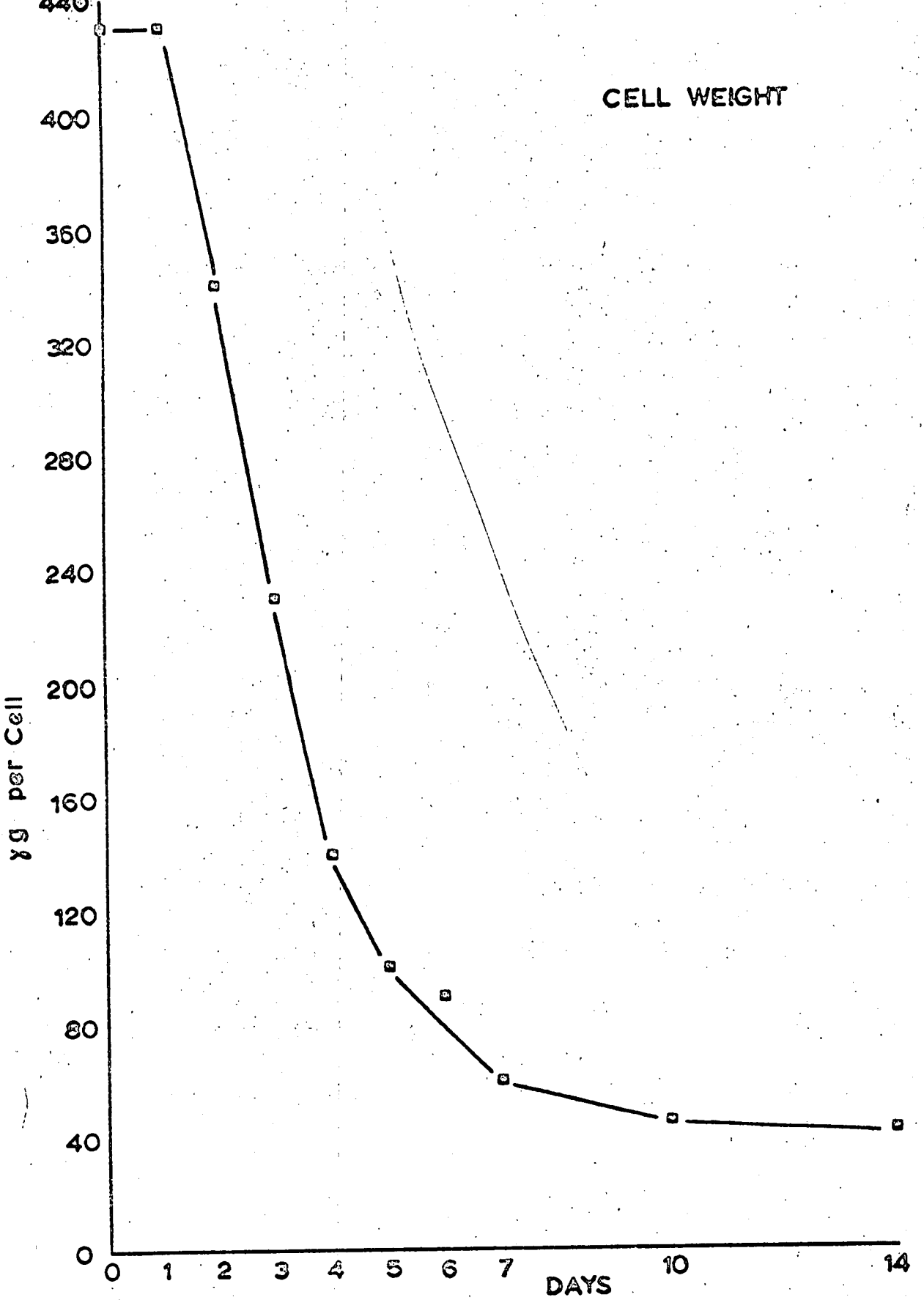


Figure 3.9. Series 2. Changes in cell weight of explants during 14 days of culture. Each value is the mean from 5 experiments.

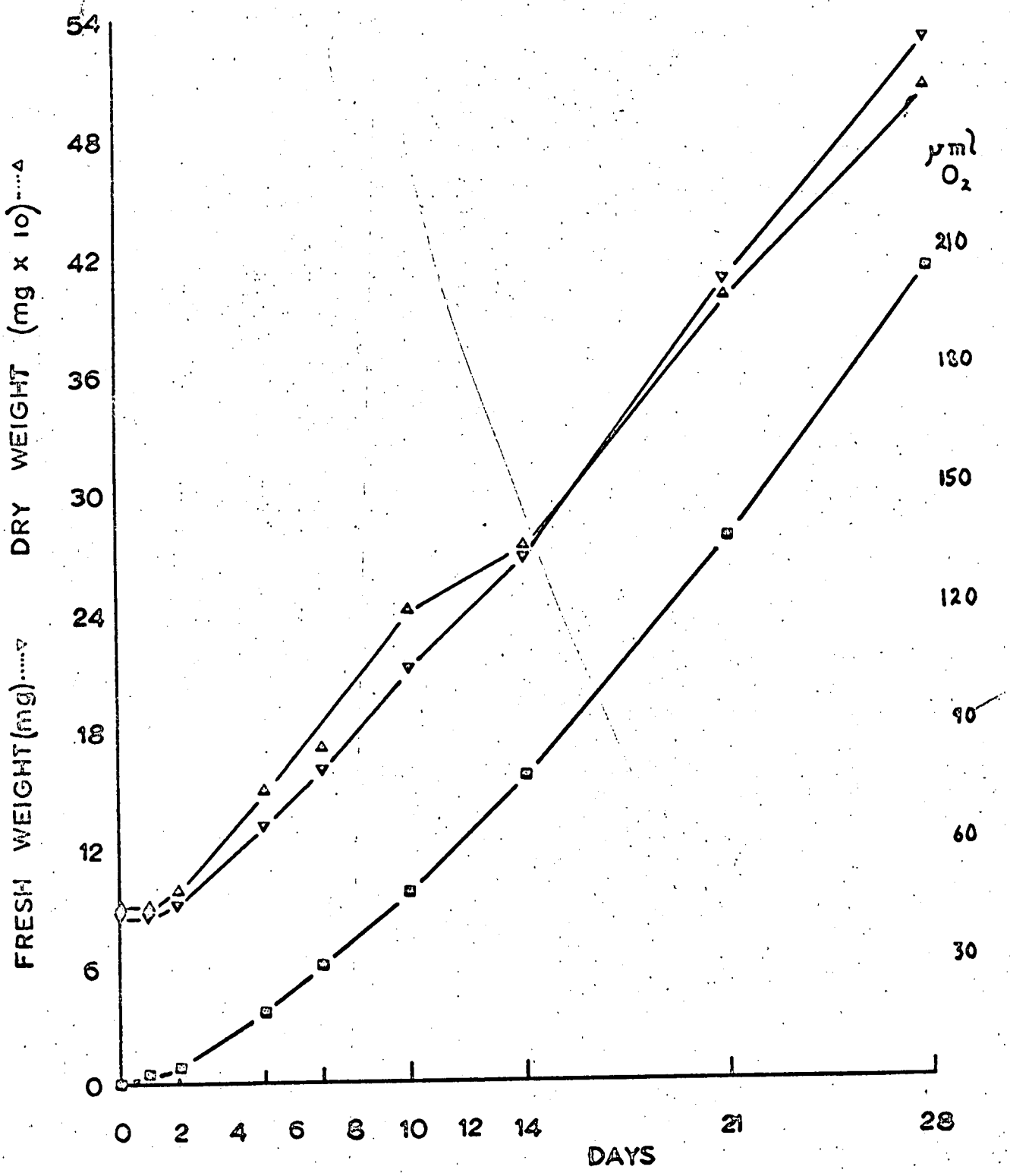


Figure 3.10. Series 3. Changes in the net oxygen taken up since excision, the fresh weight and the dry weight of explants during 28 days of culture. Each value is the mean from 5 experiments (E 61 - 65).

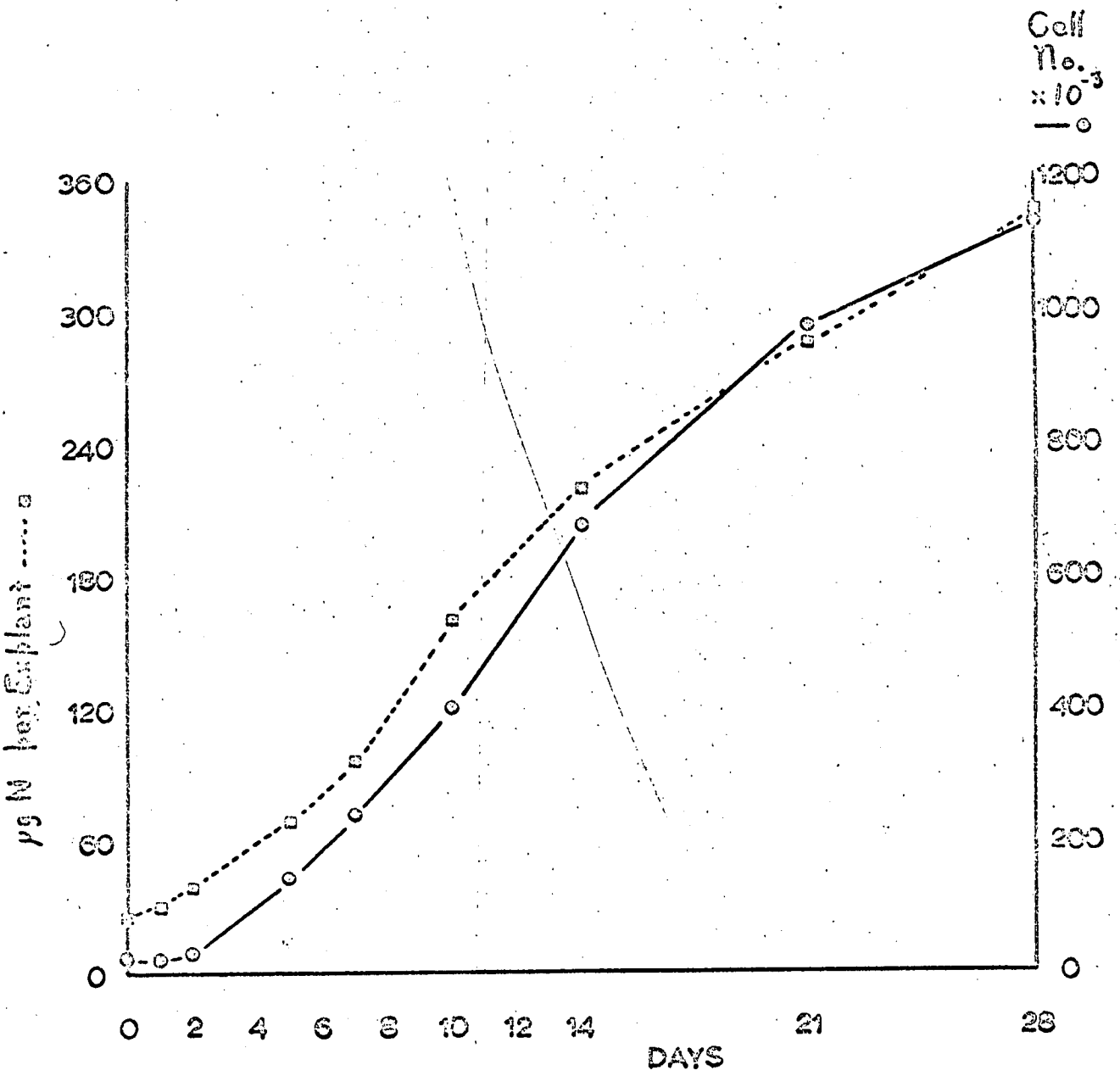


Figure 3.11. Series 3. Changes in cell number and total nitrogen in explants during 28 days of culture. Each value is the mean from 5 experiments (E 61 - 65).

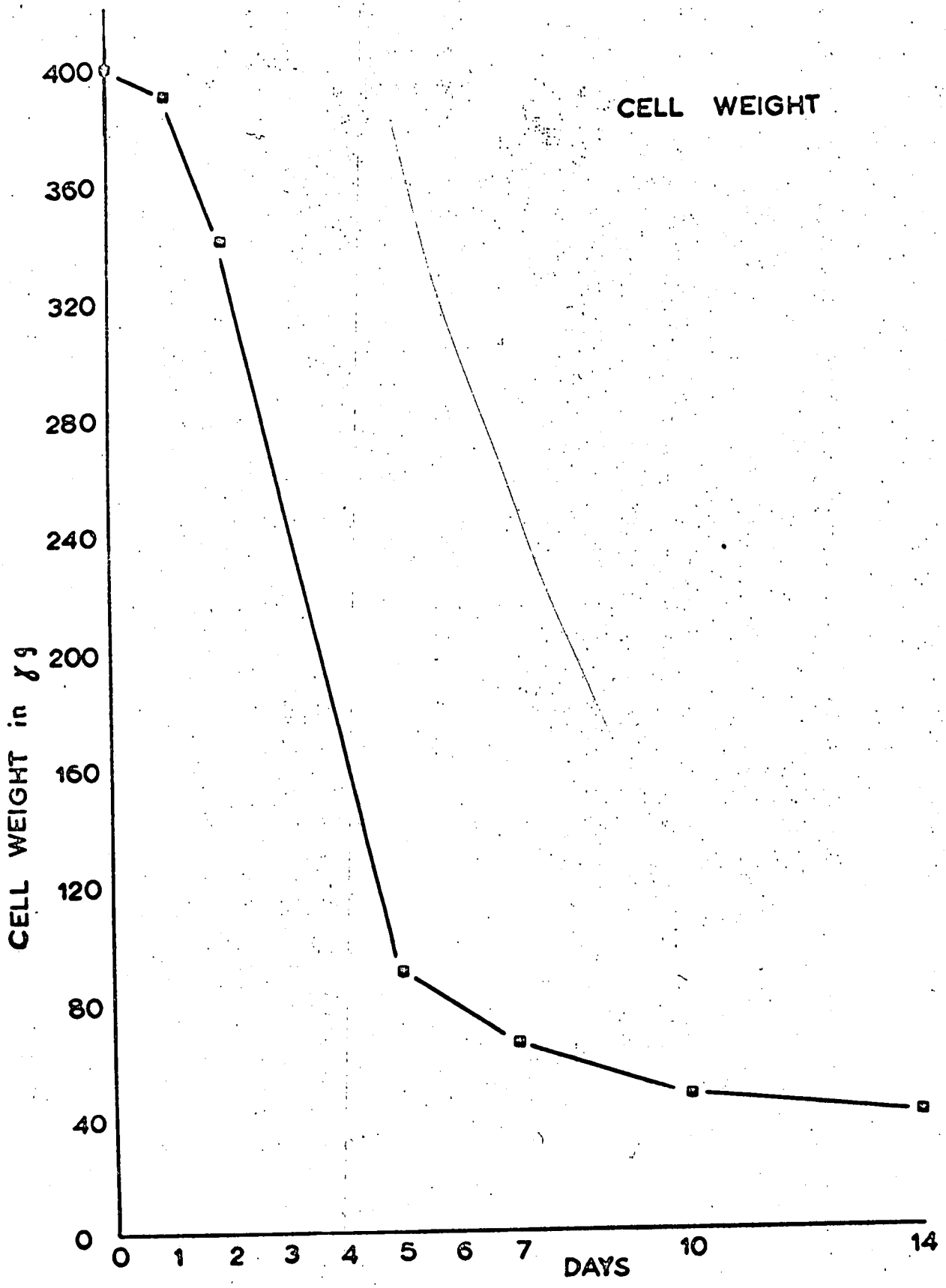


Figure 3.12. Series 3. Changes in cell weight of explants during 14 days of culture. Each value is the mean of 5 experiments (E 61 - 65).

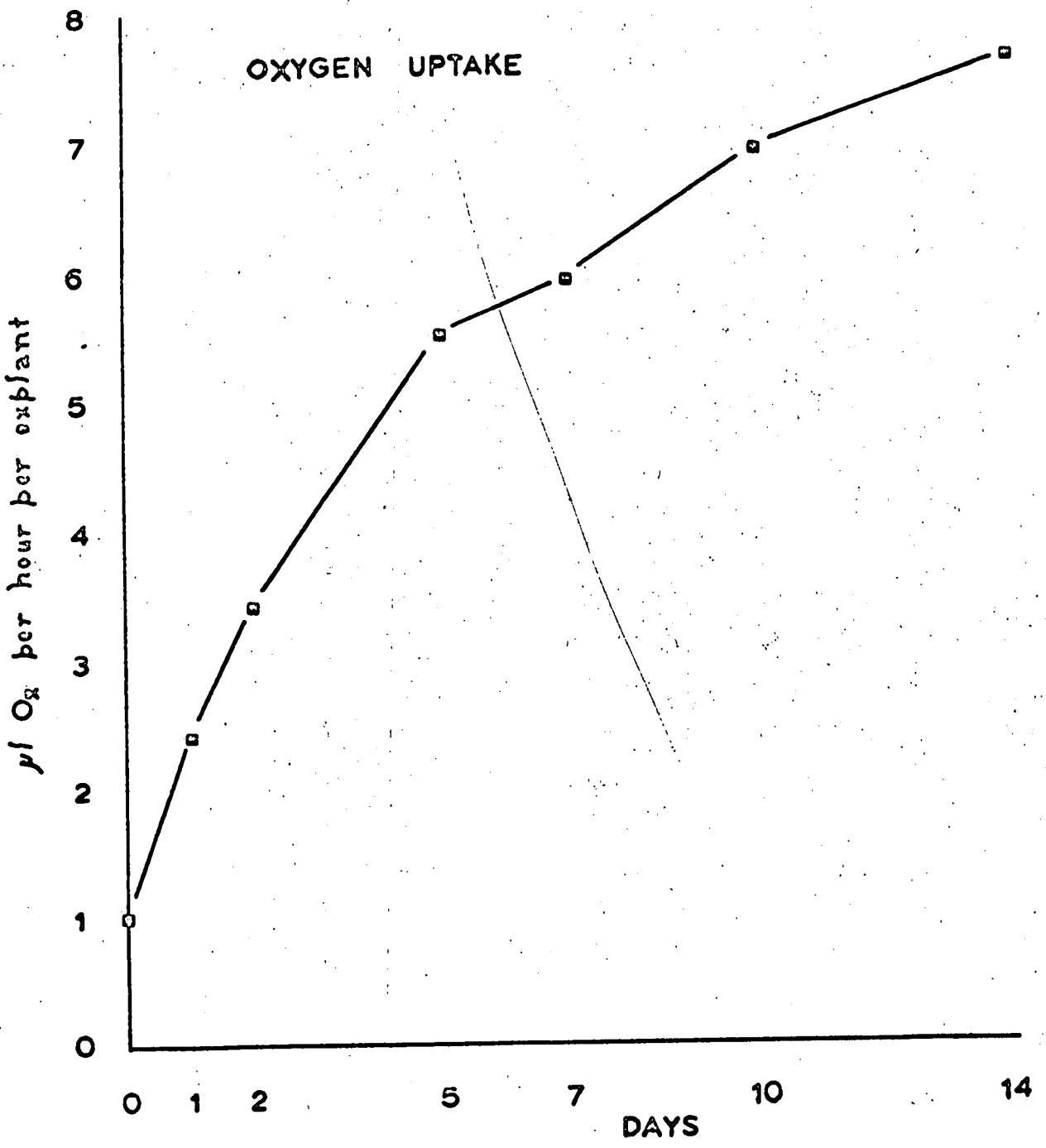


Figure 3.13. Series 3. Changes in oxygen uptake of explants during 14 days of culture. Each value is the mean of 5 experiments (E 61 - 65).

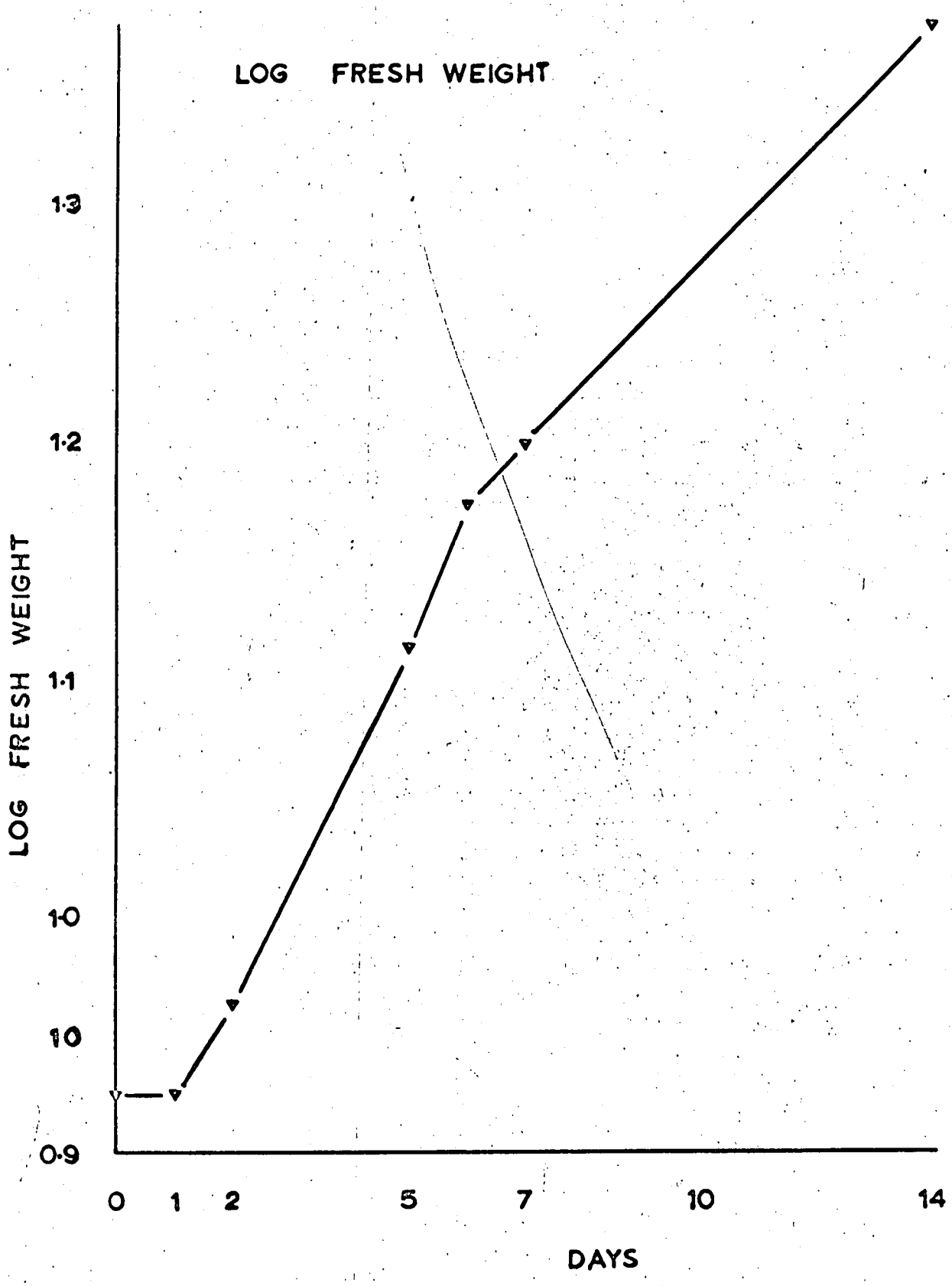


Figure 3.14. Series 2. Changes in fresh weight of explants during 14 days of culture. This is the data from Figure 3.1 plotted on a logarithmic basis.

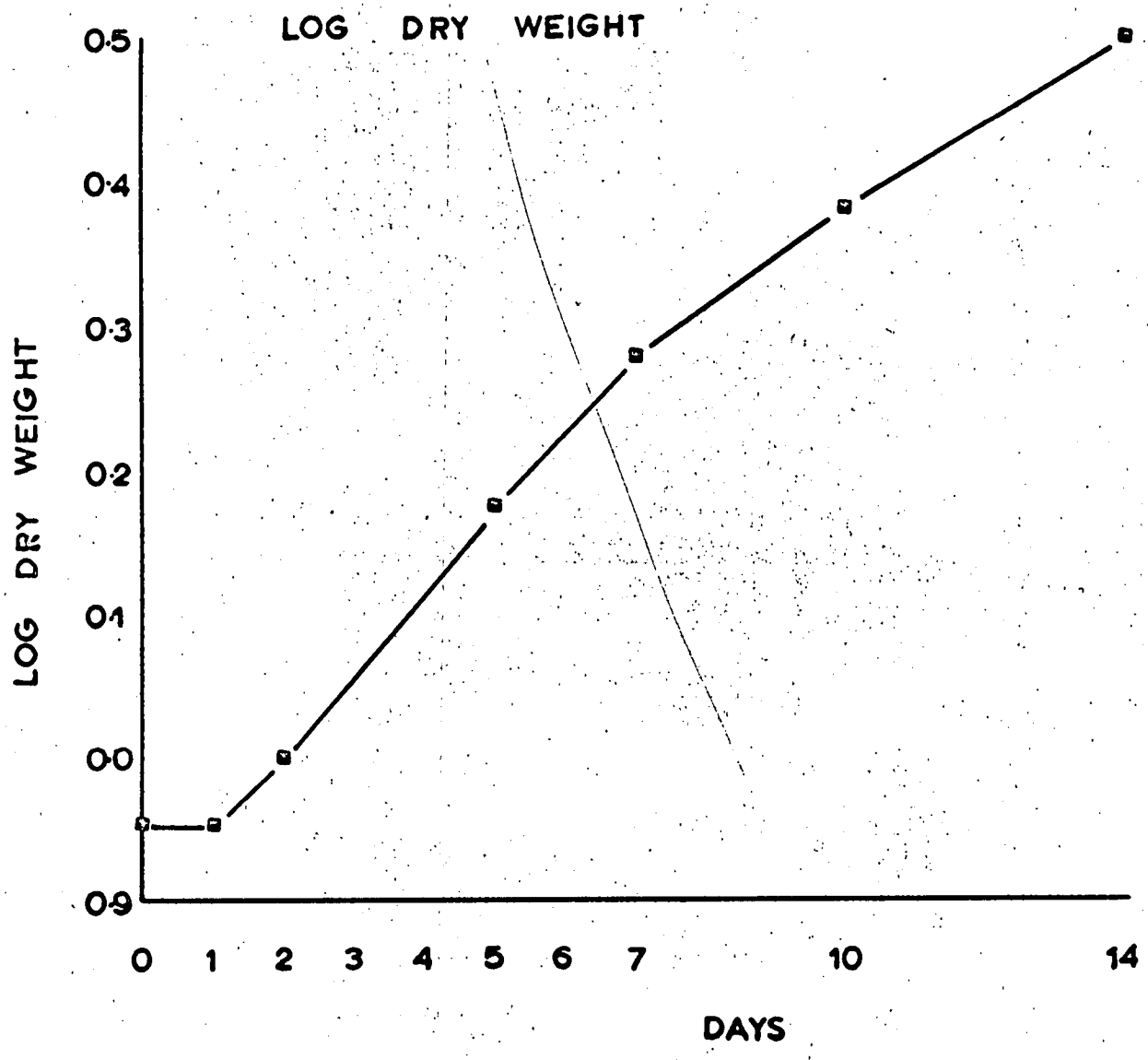


Figure 3.15. Series 1 and 2. Changes in dry weight of explants during 14 days of culture. This is the data of Figure 3.2 plotted on a logarithmic basis.

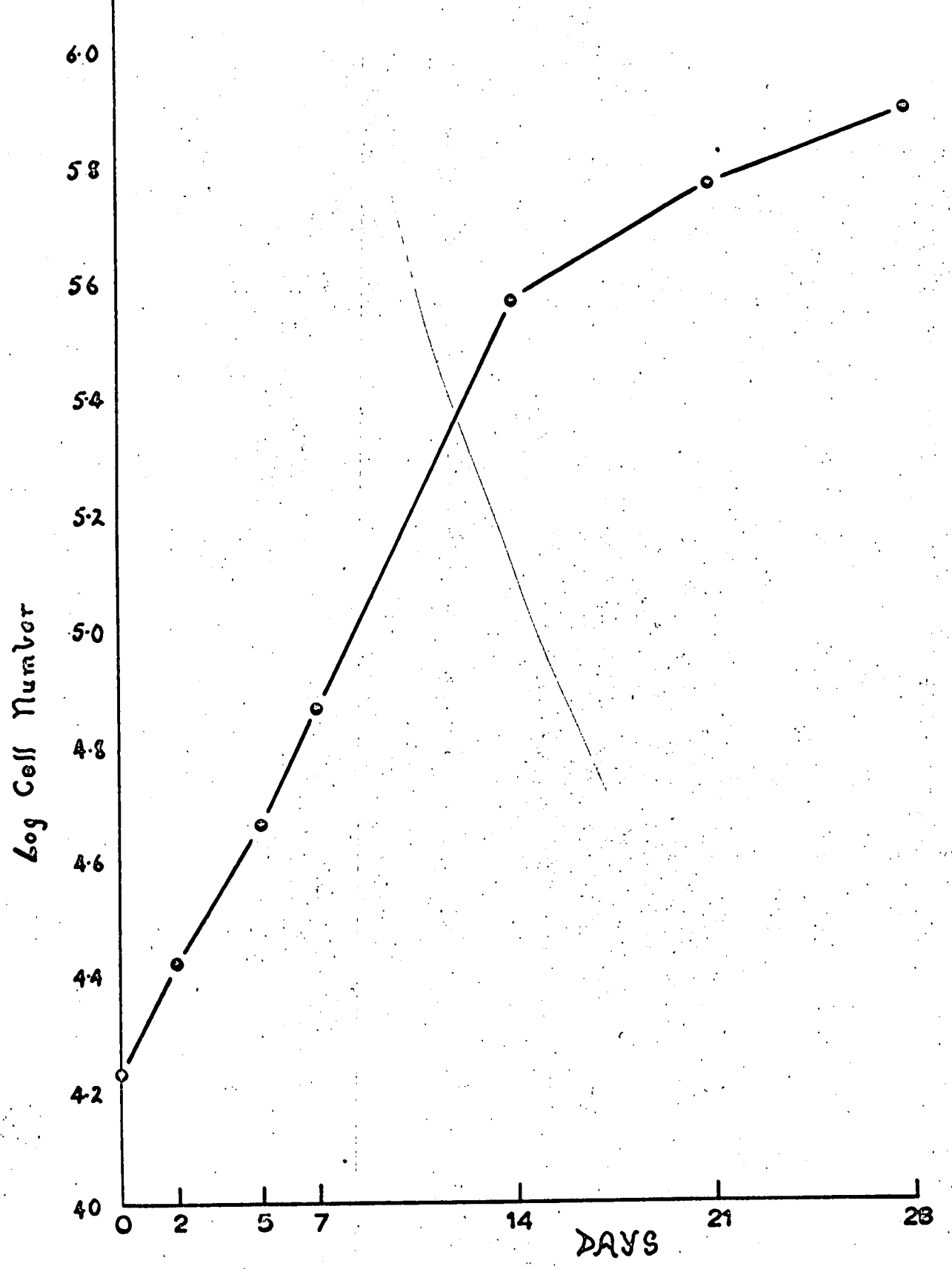


Figure 3.16. Experiment 10. Changes in cell number of explants during 28 days of culture. Data is plotted on a logarithmic basis to illustrate an exceptional case suggesting an exponential increase.

Changes in Alcohol Insoluble Nitrogen.

From the scatter diagram in Figure 3.5 it can be seen that the alcohol insoluble nitrogen shows again a general increase, but that individual experiments have various specific responses. For example, Experiment 33 has a lag phase of 1 day followed by a linear increase until Day 7 and a rapid rise to Day 14, while Experiment 31 has a 3 day lag phase followed by a similar increase to Day 7 but a slow subsequent rise to Day 14. The two other experiments show a wide scatter of points, and the mean curve reflects this scatter while describing a slow upward trend.

Changes in Ribonucleic Acid.

The changes in ribonucleic acid are plotted in Figure 3.6 and show a more or less linear increase throughout the experiment. The linear plot is similar to the plot for fresh and dry weight, although there is no lag phase.

Changes in Gaseous Exchange.

The changes in the rate of gaseous exchange are plotted in Figure 3.7. It will be noted that these values are a measure of the rate of gaseous exchange at each harvest and not a measure of the total amount of uptake or release since culture began. There is here no lag phase but on the contrary, the oxygen uptake on Day 1 is more than double that on Day 0. During the first two days the Respiratory Quotient (Figure 3.8) changes from less than unity (0.8) to greater than unity (1.2). The rapid initial increase in rate for both carbon dioxide release and for oxygen uptake, decreases throughout the experimental period except for an increase between Day 7 and 10 caused by an inflection in the curve at Day 7. In Series 3 the curve for oxygen uptake (Figure 3.13) p109 is very similar and also shows an inflection at Day 7.

By measuring the area under the curve between values in Figure 3.13 a plot can be made of the total amount of oxygen taken up by a callus since culture began. This is shown in Figure 3.10 and it can be seen that the curve for the increase in oxygen consumed bears some similarity to the curves for fresh and dry weight increases, although, in fact, the slope of the curve is constantly increasing.

Changes in Mean Cell Volume.

Since the specific gravity of hydrated tissue is close to unity, the mean cellular volume can be calculated from a knowledge of the cell number and the fresh weight. Values of the fresh weight per cell are shown in Figure 3.9 (Series 2) and 3.12 (Series 3). There is a lag phase of about 1 day, followed by a rapid decrease until, at about Day 10, the volume arrives at a relatively constant value of between 40 and 50 g per cell. Clearly the process of cell division predominates over the process of cellular expansion between Day 1 and Day 7 despite the fact that almost 100 per cent tissue expansion has been achieved during this time. Thereafter the two processes appear to strike a balance such that the cell volume stays constant or decreases only slowly.

Changes in Anatomy.

The repeated suggestion of some kind of hiatus after Day 7 led to an examination of the anatomical changes within the developing callus. Although the anatomy of developing callus tissue has been described both in artichoke material (Sevenster and Karstens, 1955; Gautheret, 1953; and Yeoman, Dyer and Robertson, 1965) and in many other tissues (e.g. White, 1939; Caplin, 1947; Steward, Caplin and Shantz, 1955; Reinert, 1962), the precise development differs with the precise conditions of culture. It is especially necessary to have a knowledge of the anatomical changes in this case because few accounts have been published using systems of liquid culture (Steward and Shantz, 1955; Blakely and Steward, 1964).

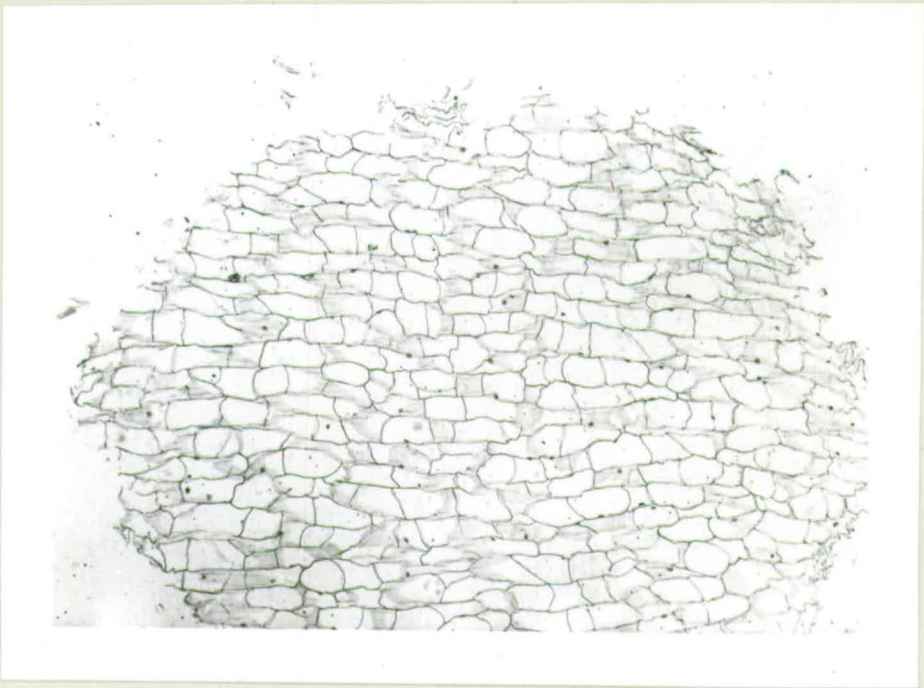


Figure 3.17. Callus anatomy, transverse section, Day 0.
Mag. = x16. 50

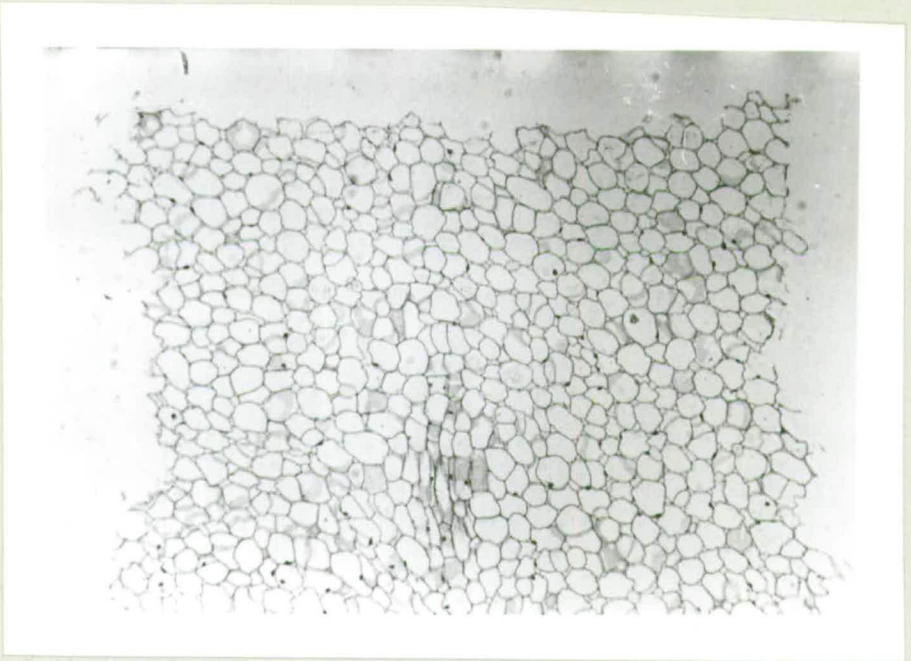


Figure 3.18. Callus anatomy, longitudinal section, Day 0.
Mag. = x16. 50

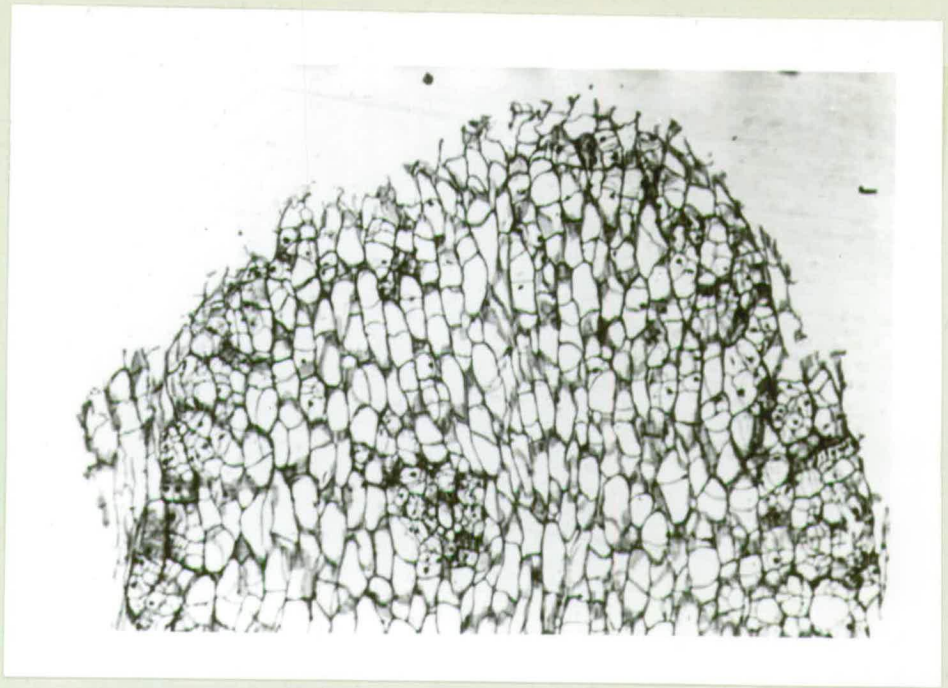


Figure 3.19. Callus anatomy, transverse section, Day 4.
Mag. = x20.5 60

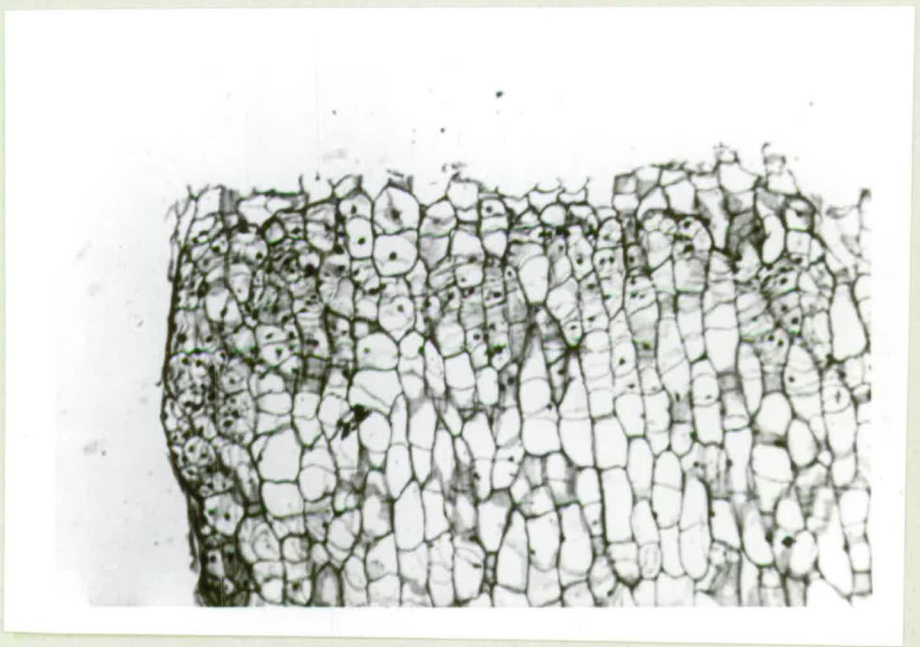


Figure 3.20. Callus anatomy, longitudinal section, Day 4.
Mag. = x20.5 60

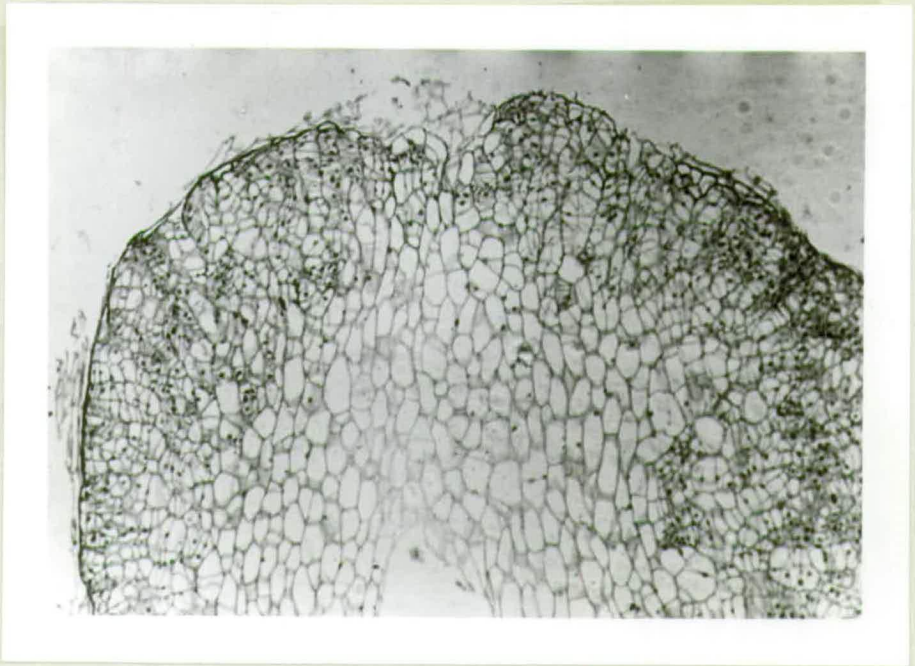


Figure 3.21. Callus anatomy, transverse section, Day 7.
Mag. = x16. 50

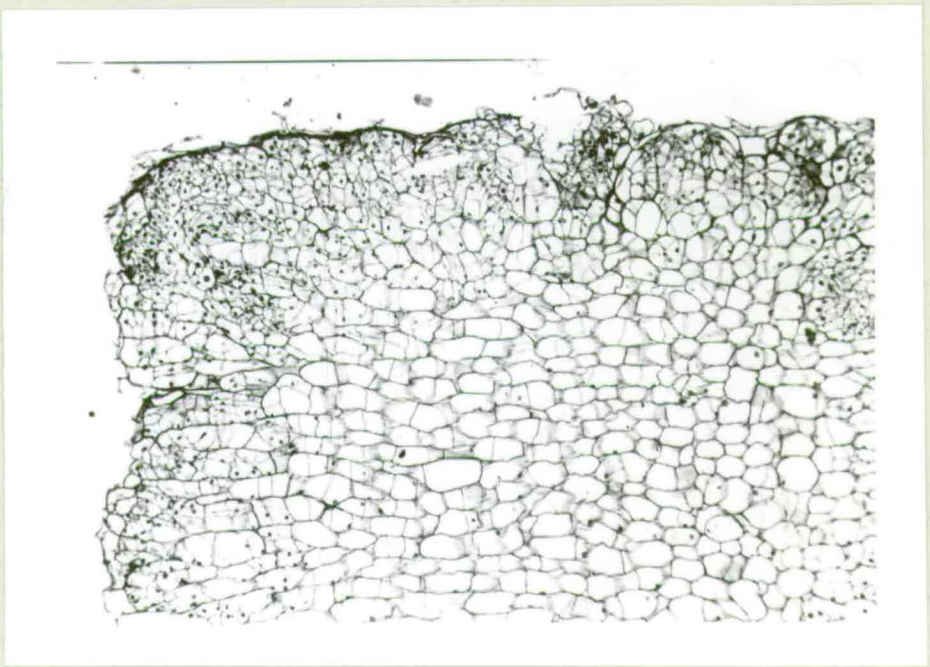


Figure 3.22. Callus anatomy, longitudinal section, Day 7.
Mag. = x16. 50

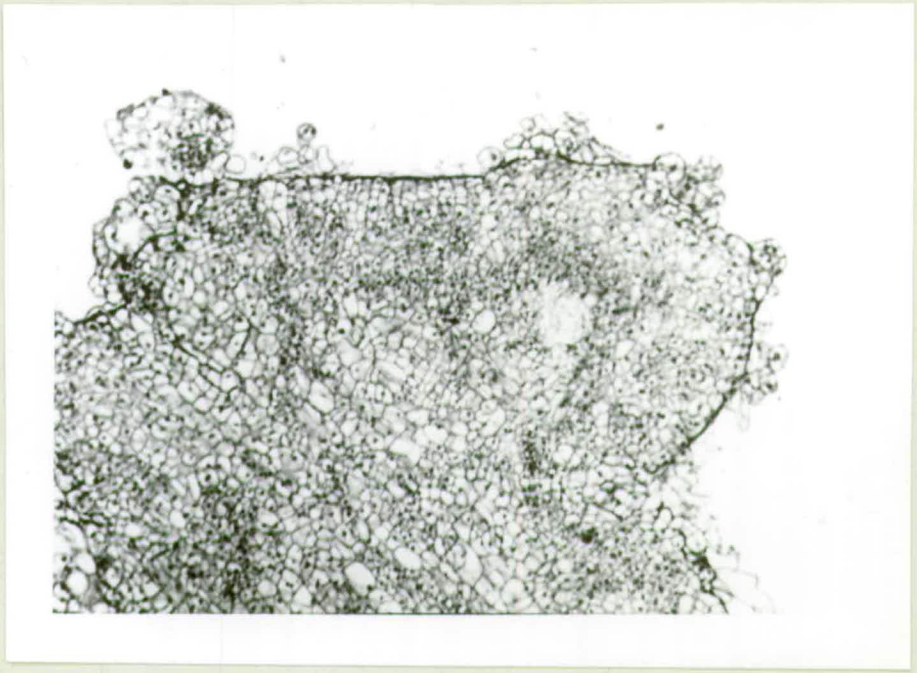


Figure 3.23. Callus anatomy, longitudinal section, Day 14.
Mag. = x32. 100

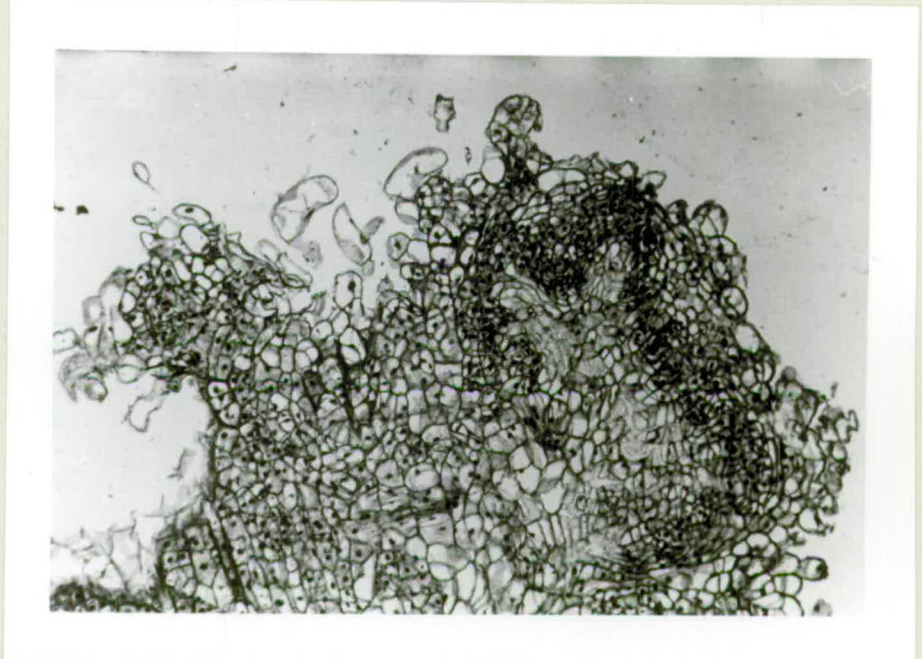


Figure 3.24. Callus anatomy, longitudinal section, Day 14.
Mag. = x32. 160



Figure 3.25. Callus anatomy, longitudinal section, Day 14.
Mag. = x32. 100

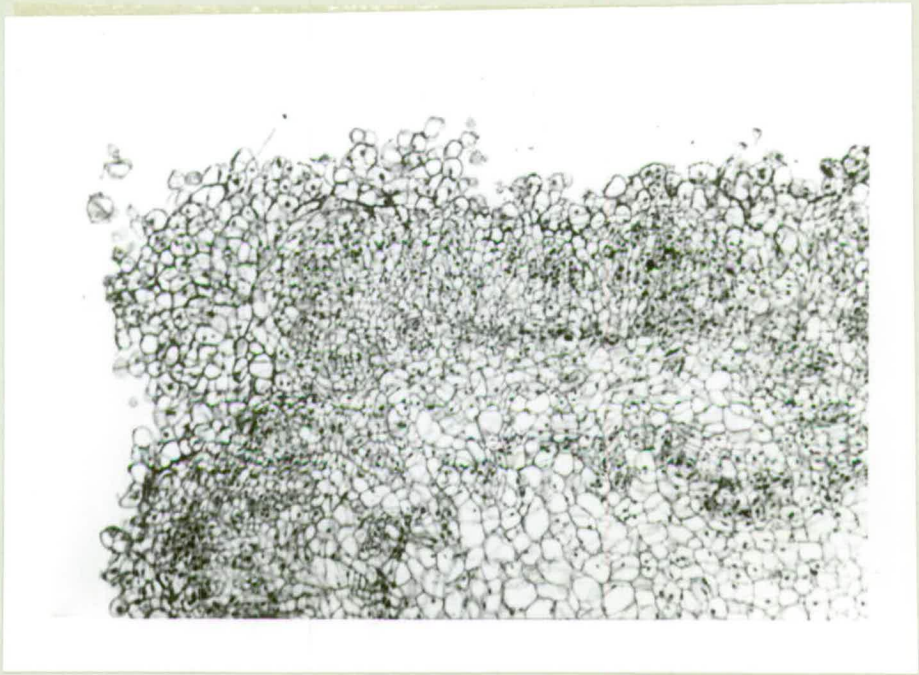


Figure 3.26. Callus anatomy, transverse section, Day 14.
Mag. = x32. 100

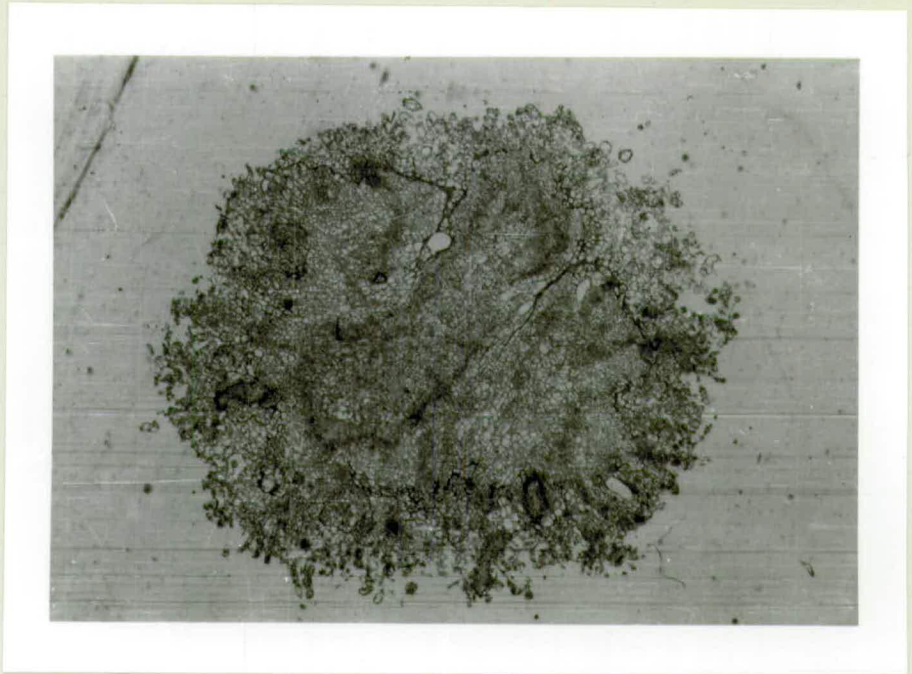


Figure 3.27. Callus anatomy, transverse section, Day 28.
Mag. = x5. 15

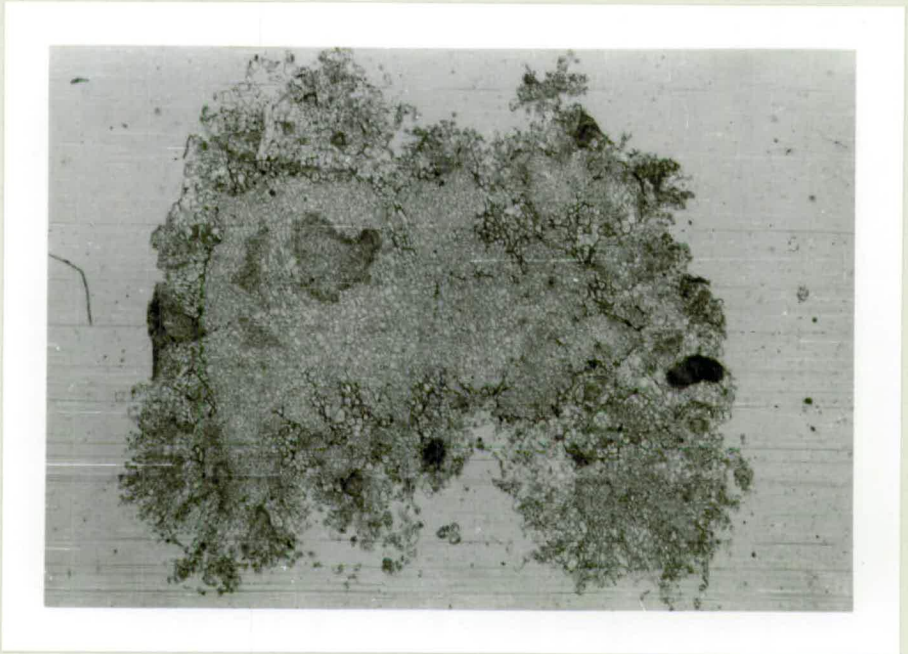


Figure 3.28. Callus anatomy, longitudinal section, Day 28.
Mag. = x5. 15

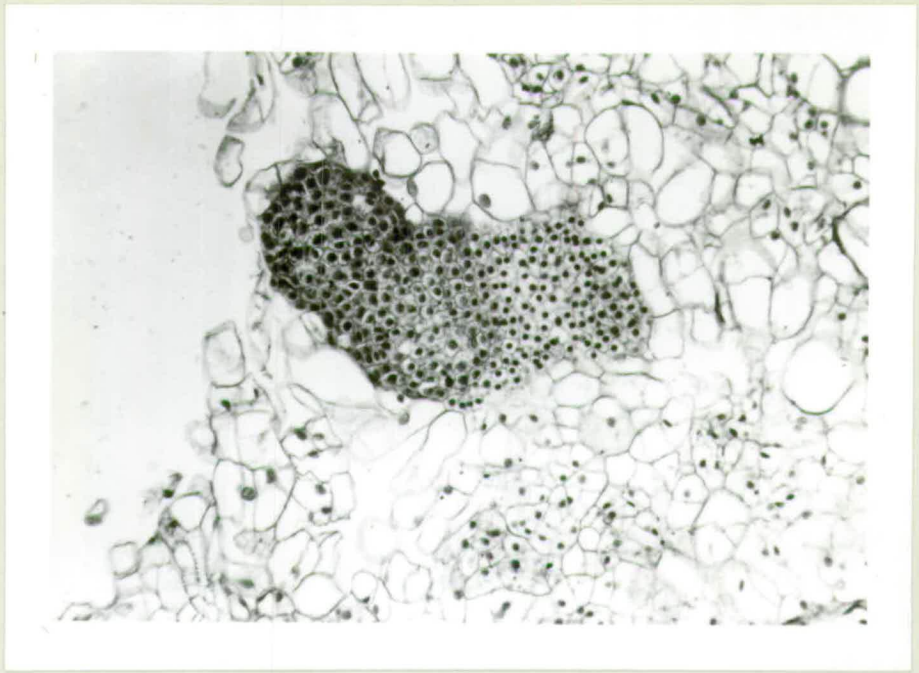


Figure 3.29. Callus anatomy, longitudinal section, Day 28. Mag. = x120. Note the isolated group of small cells embedded in the proliferating periphery.

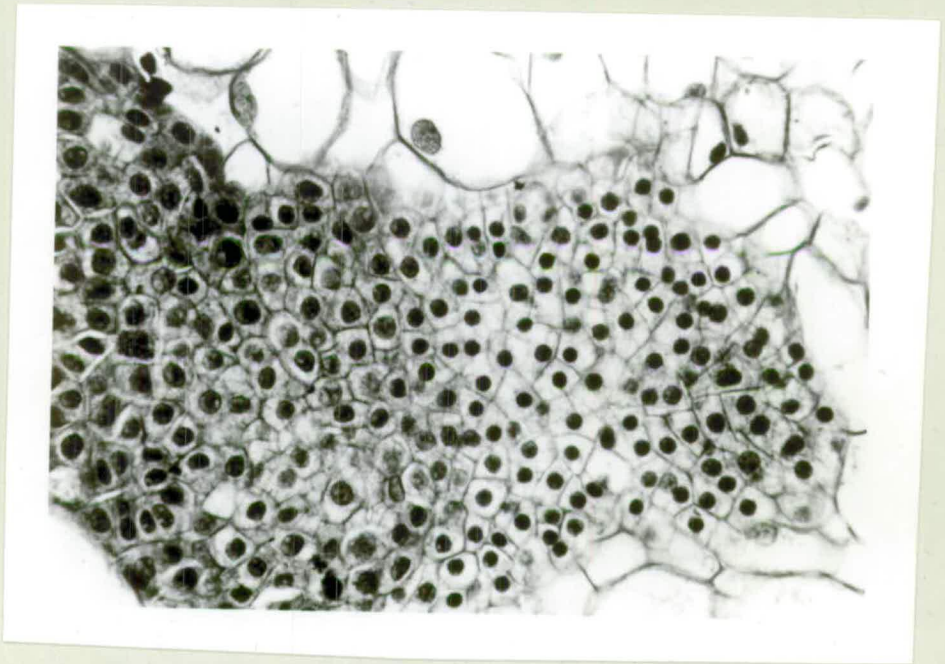


Figure 3.30. Callus anatomy, longitudinal section, Day 28. Mag. = x300. The higher magnification emphasises the sharpness of the separation of the tumourous cells from the surrounding tissue.

Explants were fixed in formalin acetic alcohol, embedded in paraffin wax, cut at 15 μ and stained with Delafield's Haematoxylin (p. 28).

Figure 3.17 shows a transverse and Figure 3.18 a longitudinal section of a fresh, Day 0, explant. Each transverse section of an explant is a portion of a cross-section of a tuber. Therefore it cuts the xylem parenchyma, cells which are laid down with their long axis at right angles to the peripheral cambium, through their long axis. Because the cores of explants cannot always be strictly parallel to the cambium, these sections are usually somewhat oblique to the xylem parenchyma. Any xylem traces are cut transversely (Figure 3.18). Conversely, each longitudinal section of an explant is part of a longitudinal section of a tuber. Figure 3.18 shows one which is a longitudinal tangential section. Here, the section of xylem parenchyma cells is more or less isodiametrical and any xylem vessels will be cut longitudinally (Figure 3.18). From Figure 3.18 it can also be seen that the transverse surface of the explant is less damaged than the circumferal surface. This demonstrates the more brutal tearing motion of the canula as compared with the clean cut of the razor blade. Abundant air spaces can be seen in the L.S. amongst what is remarkably uniform parenchymatous tissue.

Figure 3.19 shows a T.S. of a 4-day old callus. There is a sub-peripheral ring of cells which have divided, some of them several times. Included in this section is a single xylem element around which there are many recently divided cells. The orientation of division in these cells tends to have been radial to the circumference of the element. Similarly the orientation of the new cell walls in the ring of divided cells tends to be tangential to the surface of the callus. Outwith this ring of divided cells is a variable region, 1 - 3 cells thick, of intact cells, subtending the peripheral layer of damaged cells. The region of intact cells may be seen more clearly in the L.S. (Figure 3.20), which also confirms

that the orientation of new cell walls tends to be tangential to the callus surface. It is clear from these sections that cell division is independent of the size of the cell and expansion of a divided cell is not a prerequisite for further division.

In sections of 7-day old callus (Figures 3.21, T.S. and 3.22, L.S.) these features of orientation and localisation of the divided cells, are confirmed. Parallel rows of cells which resemble phellogen tissue can be seen in several places (e.g. Figure 3.21). A considerable amount of expansion must have taken place in the dividing region because the outlines of the original parenchyma cells, which were still noticeable in the 4-day callus sections, have now been obliterated by the proliferating divisions. Limiting this region of division activity is a discontinuous ring of suberised, safranin positive, collapsed cells. Portions of material which have been excluded from the actively growing volume of the callus by this layer, may still be seen loosely attached to the periphery, (e.g. Figure 3.19). It may be seen that the ring of divided cells which was incomplete on Day 4, is now continuous around the circumference of the section, although it is still thinner in the region where the parenchyma cells have their long axis radial to the section. At this time, after 7 days of culture, there is a striking absence of pattern beyond the sharp separation into a peripheral region of divided cells and a central region with very few divided cells. It is noticeable that there have been no divisions around the hole created by the spike of the mount.

By the 14th day of culture there is considerable evidence of both organisation and differentiation, (Figures 3.23 - 3.26). Covering the surface of every callus are nodules of various sizes and shapes. These nodules originate from small groups of cells which may be observed as spherical groups of 8 - 64 cells that remain unseparated by the maceration in chromic acid during the estimation of cell numbers. Although counts have not been made, these groups tend to increase in

size and number between Day 5 and Day 10. From such a centre of organisation a proliferating mass is usually built up. This mass has a well defined pattern with a central region of twisted xylem vessels bearing thickening, an intermediate region of actively dividing cells and an outer region of expanding cells (Figures 3.23 - 3.26). This nodule starts as a tight mass of cells which by the combined processes of division and limited expansion, pushes its way through the suberised skin (Figure 3.23) and thereafter proliferates and differentiates (Figure 3.24) until it begins to "flower" by producing, from its surface, smaller groups of dividing and expanding cells which may fall into the medium (Figure 3.25). The further culture of these cells will be described later (p. 114). In thicker sections, abundant columns of filamentous expanded cells are seen projecting from the surface into the medium.

In sections of 28-day callus (Figures 3.27 and 3.28), although the original form of the 7-day callus with its limiting layer can still be seen, the process of proliferating nodules has repeatedly disrupted the callus surface and a profusion of filaments, minor nodules and loosely packed parenchyma occupies the periphery of the callus.

It is interesting to note in this undifferentiated peripheral region the occasional appearance of very distinctive tumours. These are tight knots of very small non-vacuolate cells with prominent nuclei. They have a very abrupt margin (Figures 3.29 and 3.30) with no gradation of cell size in the adjacent large, vacuolate cells.

Subculture of Viable Cells Sloughed off from Calli.

If culture is prolonged to a six week period, cells and groups of cells are sloughed off into the medium. When the explants were removed and the opaque medium diluted with fresh medium on transfer to 50 ml flasks, these sloughed cells were found to be viable: after shaking the flasks in a Warburg bath at 25°C for six weeks, a number of spheres of tissue had appeared. The largest of these weighed 30 mg and on sectioning was found to be a hollow sphere of loosely packed cells. These cells were largely vacuolate with a circular cross-section and contained amongst them scattered tracheids (~~Figure 3.31, p._____~~) but such organised groups of cells as nodules were not in evidence.

Conclusions.

1. During development in the "typical" callus, a number of trends of change have been established in the given indexes of growth. These trends may be summarised as follows:-

(a) The plots of fresh weight and of dry weight have a lag phase of one day followed by a linear increase.

(b) The plot of cell number has a lag phase of about one day, followed by a sigmoid curve consisting of an acceleration phase and a retardation phase with no recognisable logarithmic phase.

(c) The plot of total nitrogen content is sigmoid and has no lag phase.

(d) On a few occasions the plots for total nitrogen and dry weight show a decrease over the first one to three days followed by the more usual increases.

(e) The plot for ribonucleic acid shows a more or less linear increase with no lag phase.

(f) Gaseous exchange shows a rapid initial stimulation in rate. However the rate of increase in rate decreases throughout the remainder of the culture period.

(g) The Respiratory Quotient changes from about 0.8 to about 1.2 during the first few days, and thereafter remains high.

(h) The plot of net oxygen uptake since the initiation of culture shows a shallow compound interest type of curve.

(i) There is the repeated suggestion of an hiatus in the rates of change, occurring at about Day 7.

(j) The anatomy of development in this system has been described.

2. A comparison of the anatomy with the indexes of growth permits the development of artichoke callus to be divided into three phases.

(a) There is a lag phase during which the cell number remains constant and there is no increase in fresh or dry weight. There is an increase in ribonucleic acid and in both oxygen uptake and carbon dioxide release.

(b) There is a division phase from Day 1 to about Day 7 in which the cell number increases ten fold to about a quarter of a million. During this time the fresh and dry weight doubles, the total nitrogen quadruples and the gaseous exchanges increase eight fold, while the anatomy shows a pattern of regeneration in all but 10 - 20,000 cells situated in the core of the callus. This phase of division is therefore marked by a five fold reduction in cell size.

(c) During the next few days the cell size is further reduced by half and then remains at a more or less constant level of 40 to 50 γg per cell. The remainder of the culture period involves a differentiation phase during which cell number reaches about one million, and there are further increases in all the other parameters measured. The precise beginning of this phase is hard to define: tracheids first appear on about the fifth day, the hiatus in rates of growth changes occurs at about the seventh day while the reduction in mean cell size is finally complete on about the tenth day.

The anatomy shows that the formation of nodules, and therefore the contribution of differentiation to the growing system, begins at about the fifth day and becomes dominant over the regenerative division by the tenth day. Thus the phases of division and differentiation although discontinuous in both time and location, do overlap to some extent when viewed as a whole.

S E C T I O N II B

DEVELOPMENTAL CHANGES ASSOCIATED WITH THE GROWTH OF THE CALLUS.

Introduction.

In Section II A the changes in a variety of parameters of growth have been described and it is clear that the callus traverses a series of developmental stages. From the anatomy of the callus (p. 110) it was seen that the system starts from a simple, uniformly parenchymatous, explant and develops into a more complex, organised and differentiated callus. Organised in this context refers to pattern formation amongst groups of cells and differentiation refers to modification, by various processes including expansion, of those dividing meristematic cells formed during the early stages of callus development.

As the tissue develops so do the individual cells of that tissue and as they pass through different stages of development the pattern of metabolism within both the cell and the tissue is changing (Brown, 1963). The mediators of metabolism are the enzymes, and enzymes are proteins. Proteins are synthesised under the direction of genes and since all the cells in one plant (and indeed, barring chance mutations, all those within a clone) have the same genes, it might be expected that they would have the same proteins. Current thought, however, is that not only is the specificity of the proteins controlled by the genes but also whether or not the proteins are synthesised. If new proteins are synthesised in sufficient quantity in the developing callus their "de novo" appearance might be expected to stand out in any survey of those proteins present during the timecourse of the culture period.

Changes in the Protein Pattern Demonstrated by Disc-Electrophoresis.

A first approach to an understanding of the changes occurring during the developing metabolism of the callus would be to obtain a scan of the changing

proteins. Polyacrylamide gel electrophoresis has recently come into use for the separation of proteins and in this Department Miss Williams has used this method for the separation of proteins from the developing zones of the pea root. An experiment was carried out, (in which Miss Williams kindly carried out the electrophoretic separations) to follow the changing protein pattern during callus development. On harvesting, calli were weighed and consigned to the deep freeze until the 35th day after inoculation. Thus the explants from different harvests had been deep frozen for between 7 and 35 days when the supernatant fraction was prepared by grinding the calli in a medium which preserves as far as possible the integrity of the cell particles (Loening, 1965). The cell walls and organelles were removed by centrifuging, and the resulting supernatant, containing what is now defined as the soluble nitrogen fraction, was returned to the deep freeze to await electrophoresis. An aliquot of the soluble fraction, derived from what was a standard weight (270 mg) of callus tissue was then separated by electrophoresis on polyacrylamide gels and stained with amido-black to differentiate the bands of protein. An estimation of the amount of protein present in this fraction was obtained from another aliquot by the Folin Ciocalteu method (p. 19). It must be emphasised here that although the amido black protein stain and the Folin Ciocalteu reaction are sensitive tests, the amounts of soluble nitrogen fraction used in both determinations were not accurately known. The volume of supernatant recovered after centrifugation varied by $\pm$ 10 per cent and the volume of the fraction applied to the gel was also subject to slight variation as it involves a delicate operation using a simple Pasteur pipette, by hand. Therefore, although the protein pattern obtained faithfully demonstrates qualitative changes, the method does not directly indicate quantitative changes. Relative changes in the size of particular protein bands are, of course, recognisable. Errors due to these volumetric differences are not however thought to be more than 10 per cent and so the estimation of soluble nitrogen still shows the major trends of increase. From each harvest, three explants were withdrawn for the estimation of

total nitrogen and the results of these assays and of the estimation of nitrogen in the soluble nitrogen fraction are presented in Table 4.1.

Day	Total-N per explant µgN	Nitrogen in the Soluble-N Fraction per explant µgN	Nitrogen in the Soluble-N Fraction per 270 mg µgN	F.Wt. mg
0	26	5	150	-
1	20	7	220	-
2	29	10	290	-
5	53	15	450	11.1
10	86	47	650	17.8
15	137	49	610	23.5
21	188	72	605	31.1
28	193	120	690	42.6

Table 4.1. Experiment 56. 26.4.65. Timecourse of changes in three parameters of nitrogen and in fresh weight during 28 days of culture.

From the results presented in Table 4.1 it can be seen that there is a general trend of increase in the nitrogen of the soluble fraction of the developing callus which closely follows the fresh weight trend. However, on a fresh weight basis, the soluble nitrogen fraction increases fourfold during the phase of reduction in cell size (p. 116) and then remains at a fairly constant level during the phase in which average cell volume remains constant. The total nitrogen value decreases on the first day but thereafter shows the same trends of increase as fresh weight and soluble nitrogen fraction.

Visual examination of the stained gels show that certain bands appear or become more intense while others disappear or become less intense. There is of course an increasing total amount of stain. It is clear that there is a

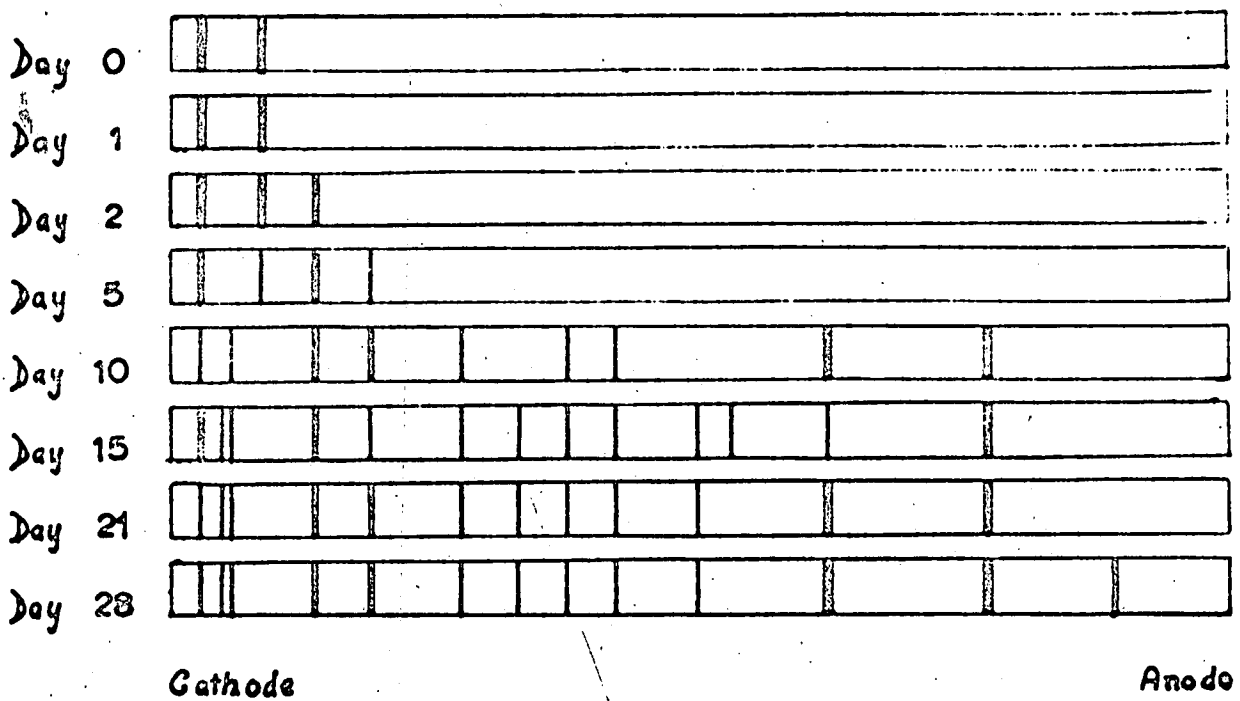


Figure 4.1. The protein pattern of the supernatant fraction obtained after separation by disc-electrophoresis and staining with Amido Black. Each line represents a visible band seen in the gel: thick lines represent more intense or thicker bands. The diagram represents a record of the bands seen on visual examination of the gels. The correspondence in mobility was correlated by a comparison of the bands in the gels with a trace of their intensities recorded on the Chromoscan (Appendix C).

continually changing array of proteins present. In any tissue there are probably hundreds of enzymes present, but only a dozen or so bands appear. These bands may represent an aggregation of several protein types which happen to have the same electrophoretic mobility, or they may be single proteins present in large amounts. There is quite a lot of background staining between the bands, which at times forms an intense plateaux of colour, and this may account for many proteins present in amounts which are too small to appear as separate bands.

A detailed analysis of the bands serves no useful purpose but it may be seen after careful inspection of the gels (Figure 4.1) and comparison with the chromoscan traces (Appendix C,p.198) that for example two prominent bands (13 and 14) appear in the fast running region after the 5th day and become more intense after Day 10. Another prominent band (4) is present initially and disappears (the peak becomes enveloped by background) between the 2nd and 10th day. Two narrow but very sharp bands (2 and 3) appear successively between the 5th and 15th day. It will be realised that while peaks have been given arbitrary numbers this does not imply that in succeeding gels they consist of exactly the same protein or proteins, though this may be the case in many of the bands. However, one band present initially (B) is labelled to illustrate at least one example where a band, (even though the protein(s) it represents may be increasing in amount) sinks into the background and other bands (11 and 12) assume its position as the net level of protein increases still further.

These results demonstrated that, within a context of a general increase in the protein of the soluble fraction, there is considerable change in the proportions and probably in the numbers of the many different proteins represented. Moreover, the appearance and massive increase in intensity, of certain bands (e.g. 2, 3, 13, 14) strongly suggest that rapid synthesis of some of these proteins has been initiated during the progress of development, while the disappearance of a band

(e.g. 4) suggests that synthesis of the protein(s) of this band has stopped. The maintenance of still other bands (e.g. 1) implies that either the rate of breakdown is exceedingly slow or that some protein is turning over with a rate of synthesis equal to the rate of breakdown.

Changes in Enzyme Activities.

Having established that there must be qualitative as well as quantitative changes occurring in the protein pattern which subtends development, it seemed of interest to pin down certain of these changes, and in doing so to choose if possible markers of change which might prove indicative of change in some of the more fundamental processes involved. However, in the first instance, markers were chosen simply to register the fact that changes in particular proteins - more especially, in particular enzymes - do take place; the demonstration of these changes has its own value.

Since the respiration of a tissue involves a considerable array of metabolic processes, the uptake of oxygen and release of carbon dioxide may be taken to represent the sum total of these oxidative processes. The changes in rate of these two indices have been recorded (p.109). There it was shown that there is a very rapid initial increase in the rate of gaseous exchange, whose rate of increase gradually slackens throughout the period of culture.

Changes in Phosphatases.

Attention was then narrowed to one group of enzymes associated with oxidative metabolism, the phosphatases. There are many enzymes which are capable of removing a phosphate group from a large variety of specific substrates. Some have greater specificity than others and even with a single substrate there are often at least two phosphatases capable of hydrolysing the low energy phosphate

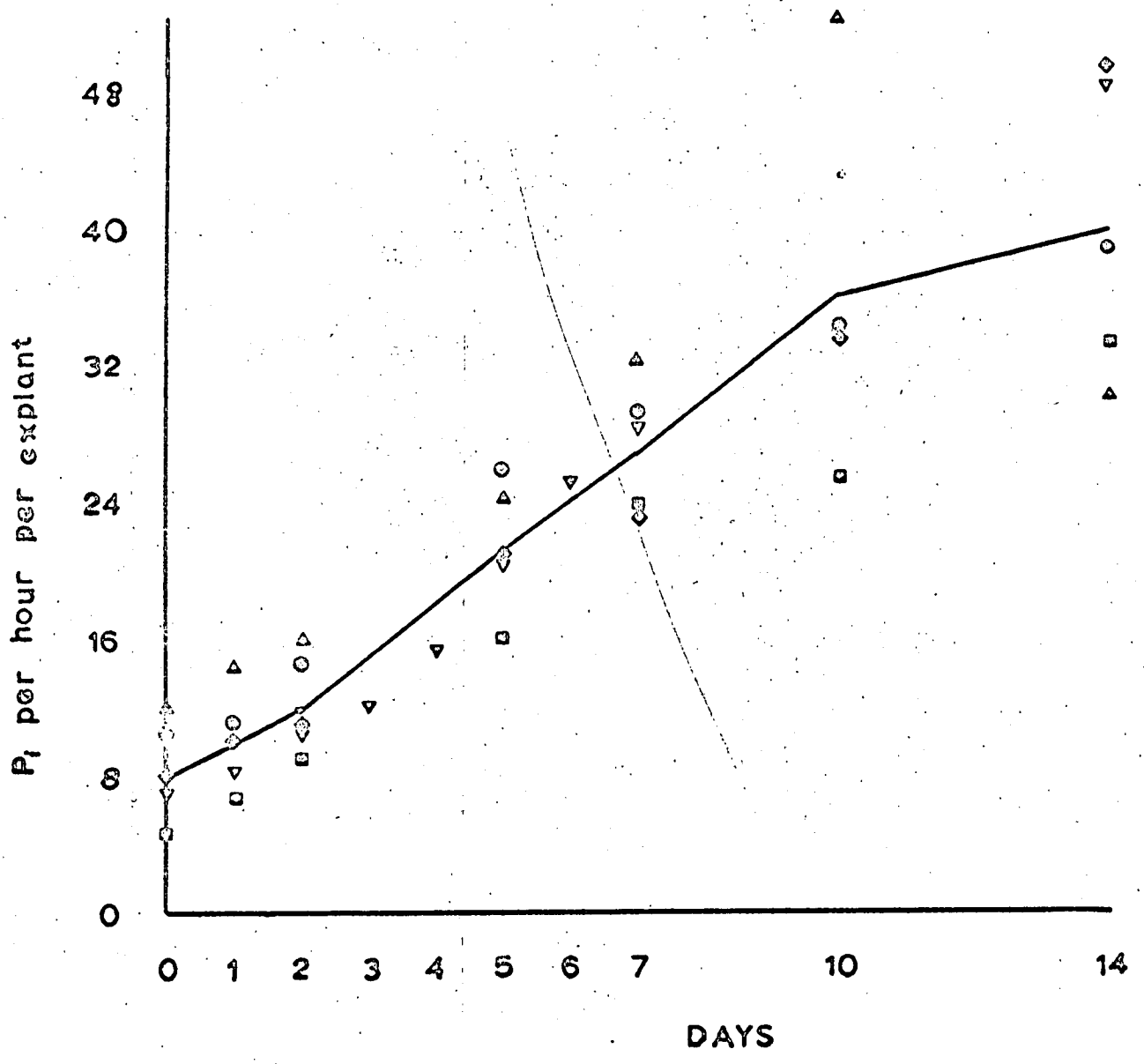


Figure 4.2. Changes in the activity of acid phosphatase at pH 6.2 during 14 days of culture. Each point is the mean of three replicates, and the mean curve for the five experiments is indicated by the solid line. All explants for these determinations were held in the deep freeze until Day 21 and assayed on that day.

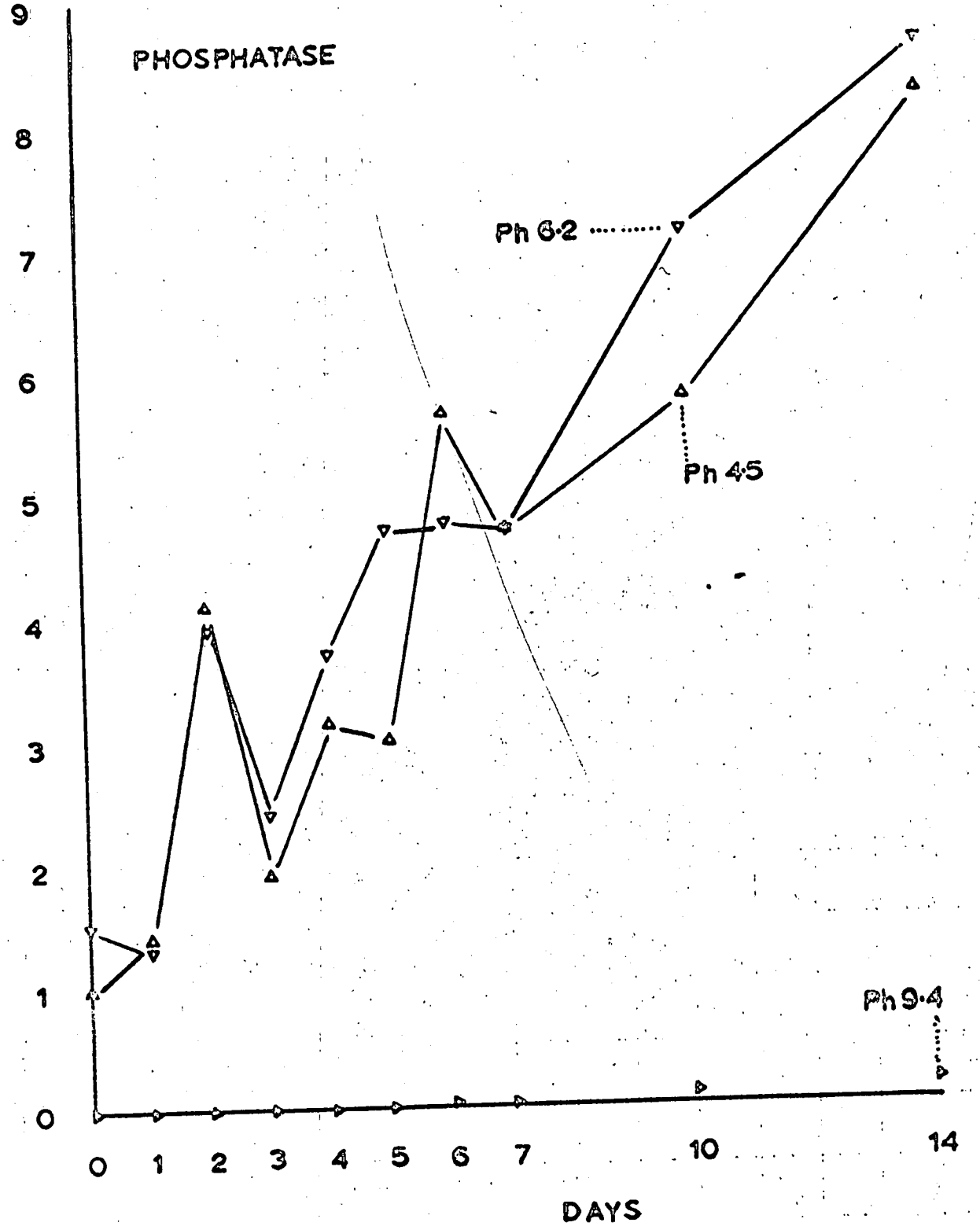


Figure 4.3. Changes in the activity of β-glycerophosphatase at three pH's. There was no detectable activity at pH 9.4. Each point represents 3 replicates. The activity was determined using freshly harvested explants although in each case the amount of phosphate released was measured on the 14th day.

bond at different hydrogen ion concentrations. Such enzymes may be further split into isoenzymes by electrophoretic techniques.

Initially however the ability of homogenates to hydrolyse β-glycerophosphate was measured at pH 6.2. This is the pH of the culture medium and is also the optimum for this enzyme in pea root tissue (Dixon, 1961). Proportionality between enzyme activity and both the amount of tissue used and the length of incubation employed (p. 35) has been established. In test experiments controls of boiled explants, perchloric acid-killed homogenates, homogenates incubated without substrates, and without either substrate or incubation were carried out on both fresh and mature tissues (p.37-8). The controls showed no activity and so, because of the limited amount of explants available, these were not carried out in the routine experiments. Five of these experiments were performed and the values (each of which is the mean of three determinations) for each harvest are plotted in Figure 4.2. The mean values show a shallow sigmoid curve which has no lag phase. There is a phase of acceleration which, between Day 5 and 7, changes to a phase of decline.

Latterly, it was thought necessary to confirm the report that alkaline phosphatase capable of splitting β-glycerophosphate is not present in plant tissues (Jensen, 1962) and so a comparison at three pH's was carried out. In this experiment explants were homogenised on harvesting and incubated immediately. After stopping the reaction, assays were all completed on Day 14 using the same reagents and blank solutions. In this case triplicate substrate blanks were included for each harvest, to allow for the slight increase in inorganic phosphate content of the stored substrate. The results of this experiment 50 are plotted in Figure 4.3 and confirm the absence of detectable activity in this particular alkaline phosphatase and show that the trend of change in activity at pH 4.5 is the same as that at pH 6.2.

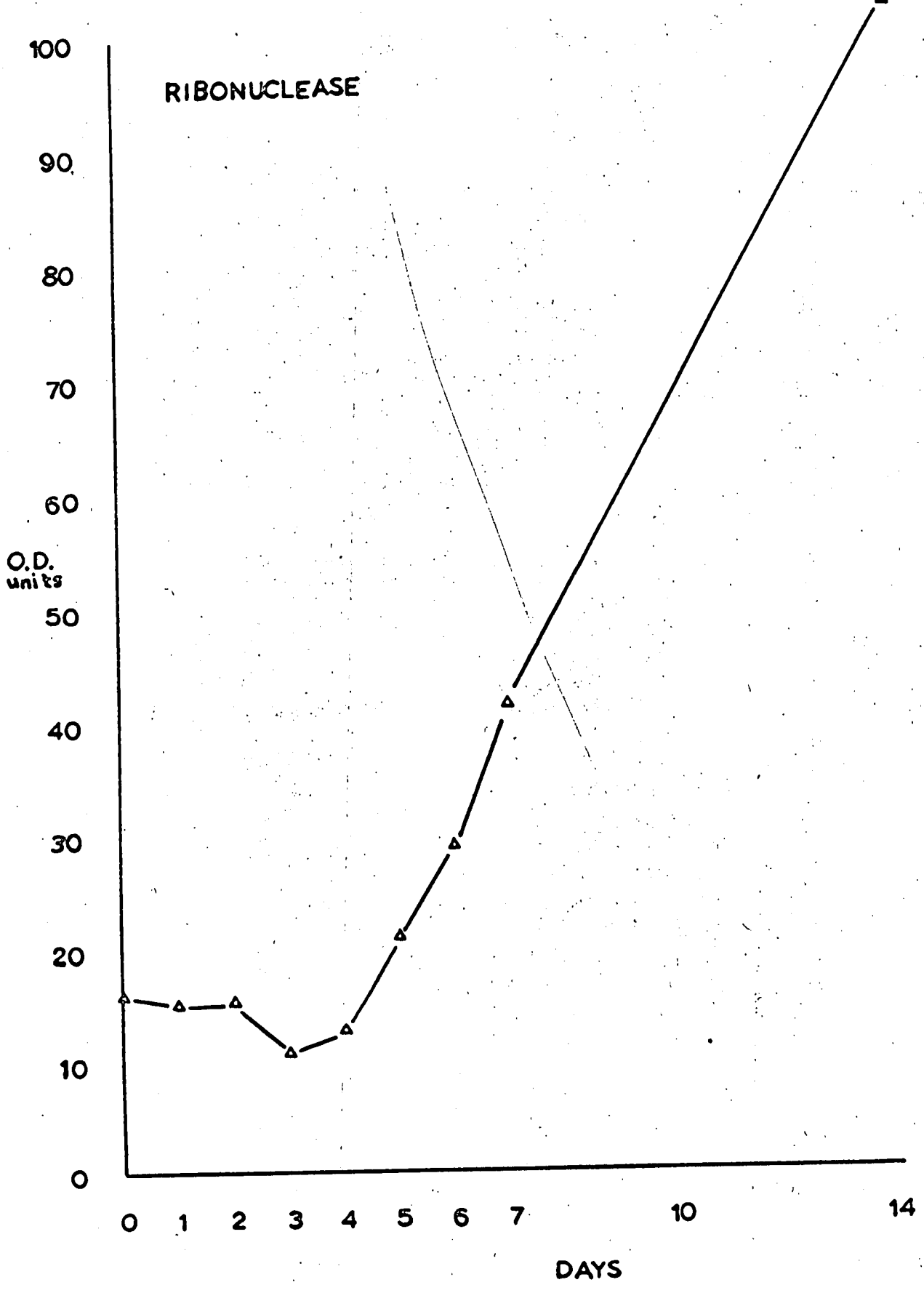


Figure 4.4. Changes in the activity of ribonuclease at pH 6.2. Each point represents 3 determinations of the RNA fragments liberated using arbitrary O.D. units.

Changes in Ribonuclease Activity.

Having defined changes concerned with oxidative metabolism and with hydrolytic activity, attention was turned to another aspect of the general metabolism of the tissue. This was done for two reasons. Firstly, it was of interest to demonstrate a changing pattern of specific enzyme complements to corroborate the changing pattern of protein shown by the electrophoretic separation of proteins of the soluble fraction. Enzymes selected from widely different areas of metabolism would seem most likely to exhibit such differences if these are indeed present.

Secondly, the activity of ribonuclease was measured because this enzyme is concerned in nucleic acid metabolism and may be indicative of the relative intensity of activity in this field, a field which is known to be directly concerned in many aspects of development (e.g. Gifford, 1963; Zubay, 1963; Jensen, 1963).

Robinson and Cartwright (1958) have established proportionality between enzyme activity and both the duration of incubation and the amount of tissue. The optimum pH is reported by them to be 6.0 and both the temperature of the incubation and the method of preparing the tissues has been discussed earlier (p. 38). Explants were harvested and accumulated in the deep freeze until the 21st day after inoculation. On this day samples were thawed, washed and single calli incubated at 40°C with 0.5 per cent of buffered yeast nucleic acid for 60 m. The excess nucleic acid was precipitated with TCA and the concentration of the soluble nucleotides estimated by u/v spectrophotometry.

In Experiment 33 single explants in triplicate were assayed for ribonuclease activity at each harvest time. The results are shown in Figure 4.4.

From the results presented in Figure 4.4 it can be seen that there is a four day lag period during which the value remains low or decreases slightly. On the fifth day there begins a rapid and continuous increase which is maintained until

the end of the experiment. The very low initial value shows a tenfold increase by the 14th day of culture.

Changes in Invertase Activity.

The activity of this enzyme was examined to further demonstrate the relative changes in specific proteins during development of the callus. It was also chosen as a representative of the enzymes of carbohydrate metabolism. The method used is based on the estimation of reducing sugars released by the action of invertase on the substrate sucrose. This method was shown to be effective for pea-root tissue in a preliminary experiment using the Hagedorn-Jensen (Strepkov, 1937) retro-titrimetric method of estimating reducing sugars.

However, activity of invertase could not be detected in artichoke tissue using either whole explants or homogenised tissue. Although untreated explants contain some reducing sugar, no change from this zero value was recorded when the length of inoculation was increased four-fold, or when the substrate was omitted from the reaction mixture. Because this enzyme has been reported to be associated with the cell wall fraction (Newcomb, 1951; Glasziou and Waldron, 1964) explants were homogenised and divided by centrifuging at 500 x gravity into a cell wall fraction and a supernatant fraction. The results of this experiment was presented in Table 4.2.

Tissue	Treatment	Replicates = ^{t} ml N/250 thiosulphate			Mean = ^{t} mg glucose/assay	
Whole explants	No substrate	14.6	13.8	13.4	0.3	
" "	With substrate	14.6	13.6	13.1	0.4	
" "	With substrate, boiled	15.0	14.9			0.15
Homogenised explants	No substrate	13.6	13.3	13.8	0.4	
" "	With substrate	13.2	13.8	13.5	0.4	
" "	With substrate, boiled	15.0	15.1			0.15
Wall-fraction	No substrate	15.5	15.5	15.5		0.15
" "	With substrate	15.3	15.2	-		0.15
	With substrate, boiled	15.4	15.2			0.15
Supernatant fraction	No substrate	13.3	13.0	13.6	0.4	
" "	With substrate	12.6	13.5	12.4	0.5	
" "	With substrate, boiled	15.1	15.2			0.15
Whole explants	With substrate, but without incubation	13.2	13.8		0.4	
Blank		16.1	16.2	16.4		0.0

Table 4.2. 23.6.65. An experiment comparing the activity of invertase using whole, homogenised or fractionated tissue in the presence and absence of sucrose using explants which were previously boiled as one control and explants which were not incubated as a second control. Three replicate values are given with their mean values. Each assay involved 3 explants or a fraction derived from 3 explants.

The results presented in Table 4.2 show that there is no invertase activity in either whole or homogenised explants nor in the wall or supernatant fractions of homogenates separated by centrifugation. This conclusion depends upon the control value for the tissue which was not incubated but assayed directly. The results also suggest that some of the reducing sugar present in explants is to be found in the supernatant fraction. The decrease in value of the controls which were boiled is not understood, it can only be suggested that the fraction of reducing power found in the supernatant fraction is in some way oxidised by boiling. It would appear that there is also a little reducing sugar that is bound to the wall fraction and which is not affected by boiling.

It was thought possible that the activity might be too low to be measured initially but may increase during callus development but the results shown in Table 4.3 show that after 7 days of culture there is still no measurable activity.

Treatment	Mean Titre	$= {}^{t}$ mg glucose/assay
Blank	6.14	0.0
Blank + sucrose	4.38	.25
2 calli	4.95	0.2
2 calli + sucrose + incubation	3.24	0.45
2 calli - sucrose + incubation	5.05	0.15

Table 4.3. A routine assay for invertase activity using 7 day calli. Incubations were for 60 minutes and sucrose was present at 2 per cent. No reducing sugar is released during incubation.

Finally it was also possible that invertase activity may be developed after some time in storage, perhaps correlated with the increasing low polymer fructosan concentration and so the activity was measured on the cell wall fraction obtained during Experiment 57. Although there was an increasing amount of reducing sugar found in this fraction there was no measurable invertase activity.

Activity of Inulase.

Since no invertase activity could be measured, and since the carbohydrate reserve of artichokes is known to be inulin (Rose, 1804) a preliminary experiment was designed to see if there was any measurable activity of an enzyme which releases reducing sugar when inulin is supplied as a substrate.

In 1888 Green "established the presence of a ferment in the germinating artichoke, by whose instrumentality inulin is transformed ultimately into some form of sugar". Further "the ferment is only to be looked for at any moment in cells which are parting with their carbohydrate contents. It cannot be found at all until the young stems begin to emerge from the tuber". Finally, in an experiment with a strikingly modern note (cf. Edelman and Hall, 1965) Green demonstrated that activity may be induced (developed from a "zymogen") by ageing dormant sliced tubers for 24 hrs at 35°C.

Accordingly, groups of 2, 5 and 10 explants from a tuber which was still expanding in the ground, were tested for inulase activity by substituting 0.5 per cent inulin as the substrate in the assay described earlier (p. 43) for invertase activity. Duplicate values showed that there was some reducing sugar present in the explants but that there was no increase in reducing sugar during incubation. It was concluded that there was no measurable inulase activity in fresh tissue of young non-dormant Jerusalem artichoke tubers.

Changes in Succinic Dehydrogenase Activity.

The final enzyme whose activity was estimated was an enzyme of the electron transport system. Since it is a constituent of one of the oxidative pathways of catabolism, the tricarboxylic acid cycle, it is intimately concerned with the supply of metabolic energy which is made available during growth. This enzyme is, therefore, one which might be expected to show a rather different distribution during development from that of the enzyme activities previously considered, since it is involved in yet another facet of metabolism.

Of the methods available for the estimation of the activity of this enzyme, manometry (Honda, 1957) is too crude, as it would require, perhaps, a thousand explants to give a measurable response. The methods based on manual operation (Heyes, 1963) of spectrophotometric apparatus are also too insensitive for this material unless about 100 explants were available for each determination. However, under closely standardised conditions using the recording Joyce-Loebl Chromoscan satisfactory results were obtained using preparations derived from as little as 30 mg of tissue.

Although Hiatt (1961) has established proportionality between activity and enzyme concentration, and defined careful control conditions using purified enzyme extracts, it was necessary to confirm these findings using the crude 500 x gravity supernatant fraction. Since this enzyme is found in the mitochondria, such a supernatant will contain the bulk of the activity of the homogenate. It was shown earlier that the rate of reduction of 2:6 dichlorophenol-indophenol is proportional to the amount of crude extract, that the absence of substrate does not alter the activity and that in the absence of PMS there is no activity (p. 48). The only further conditions necessary were that activity be measured immediately after fractionation and that the extract be kept cold for as long as possible prior to estimation. Presumably this is necessary because there are degradative enzymes

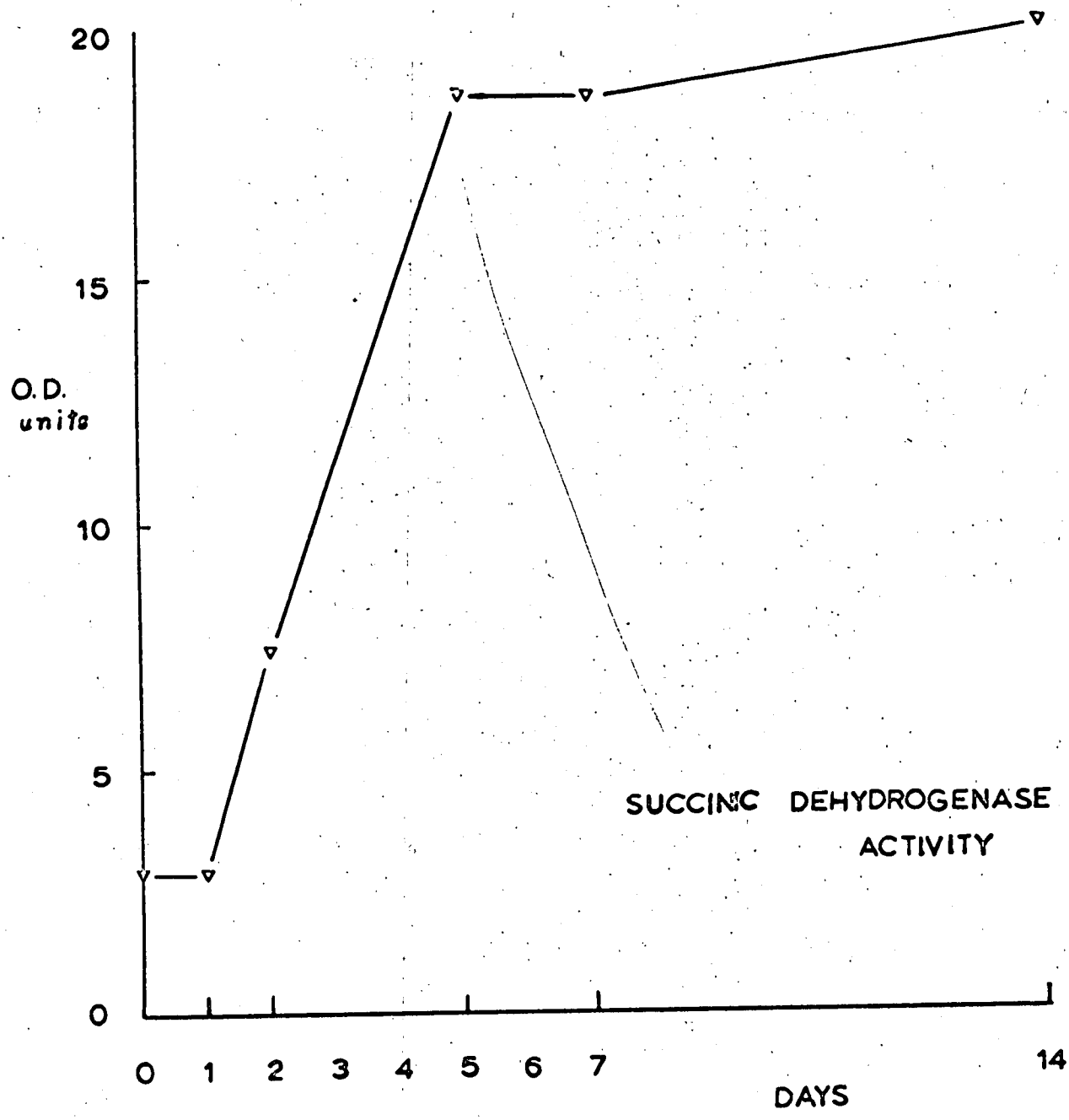


Figure 4.5. Changes in the activity of succinic dehydrogenase at pH 7.4. Each point is the mean of 3 determinations using arbitrary O.D. units which represent the loss of colour of dichlorophenol-indophenol over a standard time interval.

also present in the extract which may denature or interfere with the action of succinic dehydrogenase.

A single experiment has been carried out using this method and the results are presented in Figure 4.5. There is a lag phase of 1 day, a 6-fold increase in rate until the 5th day and activity remains more or less the same thereafter until the end of the experiment. This experiment confirmed preliminary studies which suggested that there was minimal succinic dehydrogenase activity initially but an increasing amount during the first few days of culture.

Localisation of Phosphomonoesterase I and II in relation to Growth Indices.

A large increase in phosphatase activity has been demonstrated by the assay of tissue homogenates. In individual experiments, certain correlations between enzyme activity and cell division activity suggested that the phosphatase activity may be concentrated in the regions of active cell division. In order to test this hypothesis, histochemical methods were employed. Because β-glycerophosphate had been used as the substrate in the biochemical assays, it was logical to use the Gomori lead phosphate reaction (Gomori, 1952) which also depends on the hydrolysis of β-glycerophosphate. However, this is a temperamental reaction (Holt, 1959) and difficult to employ in comparative studies because deposition of reaction product only occurs after a threshold concentration has been reached. Accordingly the azo-dye method of Grogg and Pearse (1952) was also employed to confirm and enlarge upon the results obtained using the Gomori technique.

In Experiment 50, the phosphatase activity in homogenates was measured at pH 4.5, 6.2 and 9.4. In addition both oxygen uptake and carbon dioxide release, and the sensitivity of oxygen uptake to uncoupling with dinitrophenol, were measured. Cell number, total nitrogen content, fresh and dry weight were also recorded. No obvious correlation between phosphatase activities and either cell division or DNP

sensitivity were substantiated and it became clear that the phosphatase visualised by histochemical methods was playing quite another role in the metabolism of the developing callus. All the relevant primary data is collected in Table 4.4 and the progression of phosphatase activity in sectioned material is illustrated by Figures 4.5 - 4.

Day	Fresh Weight	Dry Weight	Total Nitrogen	Cell Number	Tracheid Number	CO_2	O_2	R.Q.	DNP Sensitivity	Phosphate Activity
	mg	mg	µg N.		x 10^{-3}	µl/hr/expl.	µl/hr/expl.		% O_2	P_i/hr/ex.
0	8.4	1.1	43	23,000	-	0.4	1.4	0.3	5	1.5
1	8.5	0.9	37	22,000	-	-	-	-	-	1.3
2	9.5	1.2	18	30,000	-	2.8	3.3	0.8	11	3.9
3	10.5	1.5	32	58,000	-	3.6	4.2	0.9	13	2.4
4	12.7	1.4	53	133,000	-	5.1	5.3	1.0	19	3.7
5	13.8	-	83	222,000	5	6.9	6.2	1.1	48	4.7
6	16.6	1.4	120	288,000	12	7.6	6.4	1.2	5	4.8
7	17.8	1.8	128	488,000	18	8.8	7.4	1.2	4	5.6
10	21.5	2.4	193	600,000	50	9.0	7.6	1.2	16	7.1
14	16.0	3.0	185	860,000	70	-	-	-	20	8.6

Table 4.4. Experiment 50. 12.2.65. Changes in growth indices, gaseous exchange and phosphatase activity during 14 days of culture. The phosphatase activity was measured at pH 6.2. These values together with values for pH 4.5 and 9.4 are plotted in Figure 4.3.

From the results shown in Table 4.4 it can be seen that, while the changes in cell number, respiration and fresh weight are typical of the data summarised in Section II A, the changes in some other parameters are not. Total nitrogen suffers a dramatic fall during the first 48 hours and then rises continuously. Dry weight falls on the first day and then increases in parallel with the fresh weight as usual.



Figure 4.6. Acid phosphatase activity, Day 0.
No activity could be detected.



Figure 4.7. Acid phosphatase activity, Day 1. The reaction product is found in a subperipheral ring (pink or brown colour) while the breakdown products of amisidine (yellow) are found in the superficial, damaged cells.



Figure 4.8. Acid phosphatase reaction. Day 1. An end section which passes through the sub-peripheral zone of the callus.



Figure 4.9. Acid phosphatase reaction. Day 3. Early signs of rind formation.

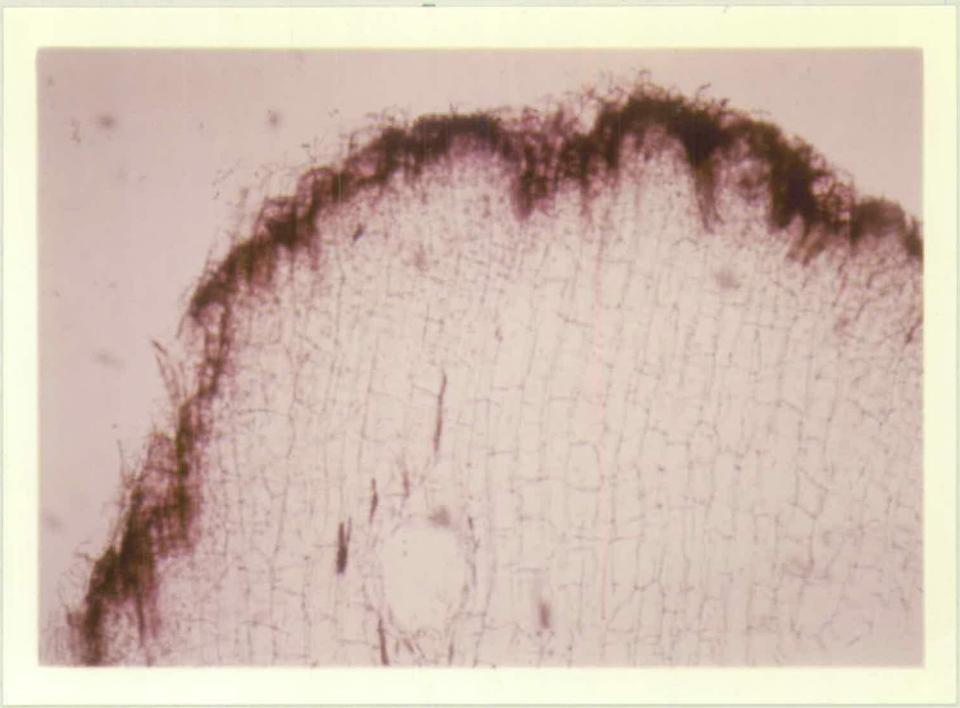


Figure 4.10. Acid phosphatase reaction, Day 4. Note the intense activity in cells which are collapsing to form the rind.

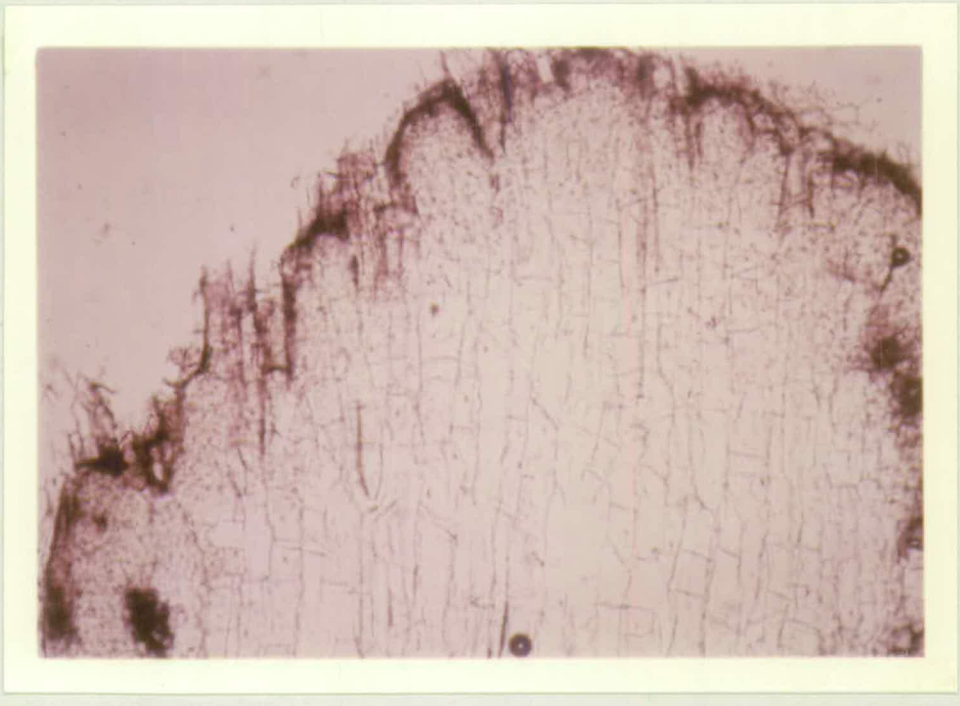


Figure 4.11. Acid phosphatase reaction, Day 5. Note the formation of the rind and the activity in the groups of tracheids.

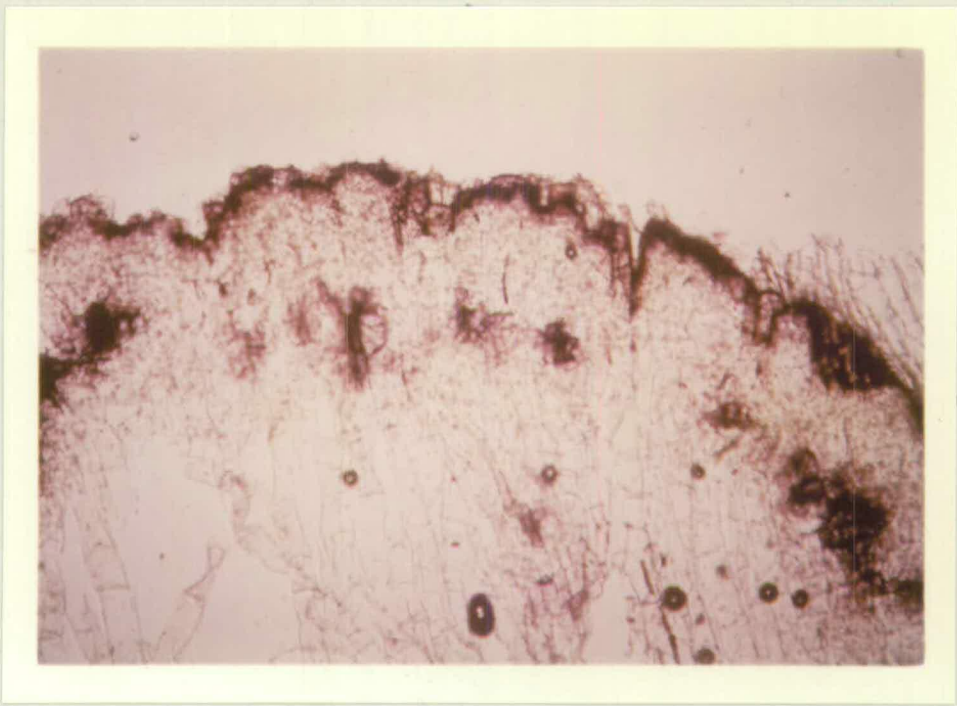


Figure 4.12. Acid phosphatase reaction, Day 7. The completed wind has excluded a raft of cells on the right. Activity is also found in many groups of tracheids.

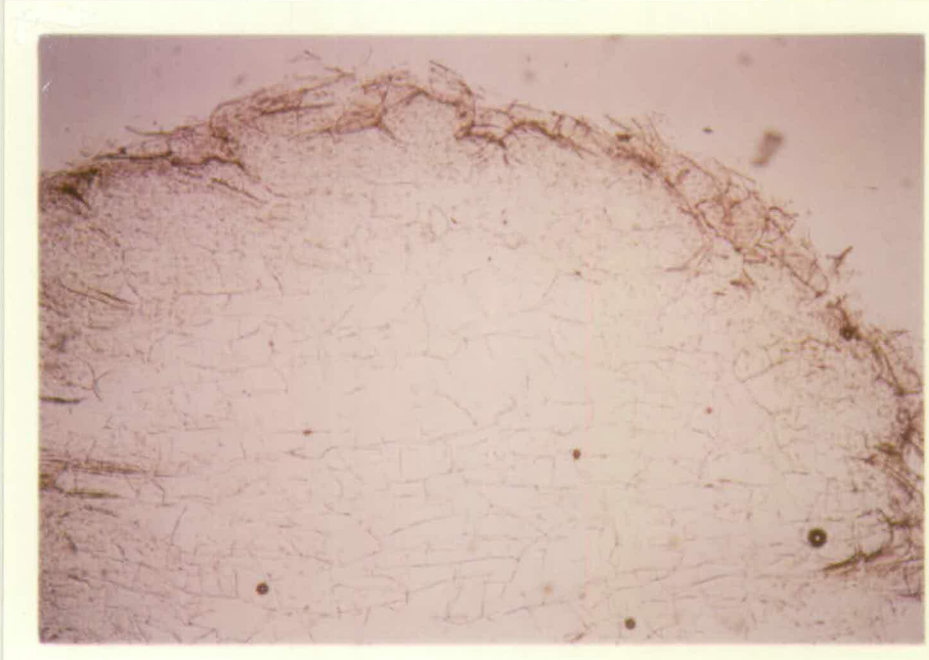


Figure 4.13. Acid phosphatase reaction, Day 6. A control section inhibited with 0.01M fluoride. Note the yellow anisidine breakdown products in the absence of any positive enzyme reaction.

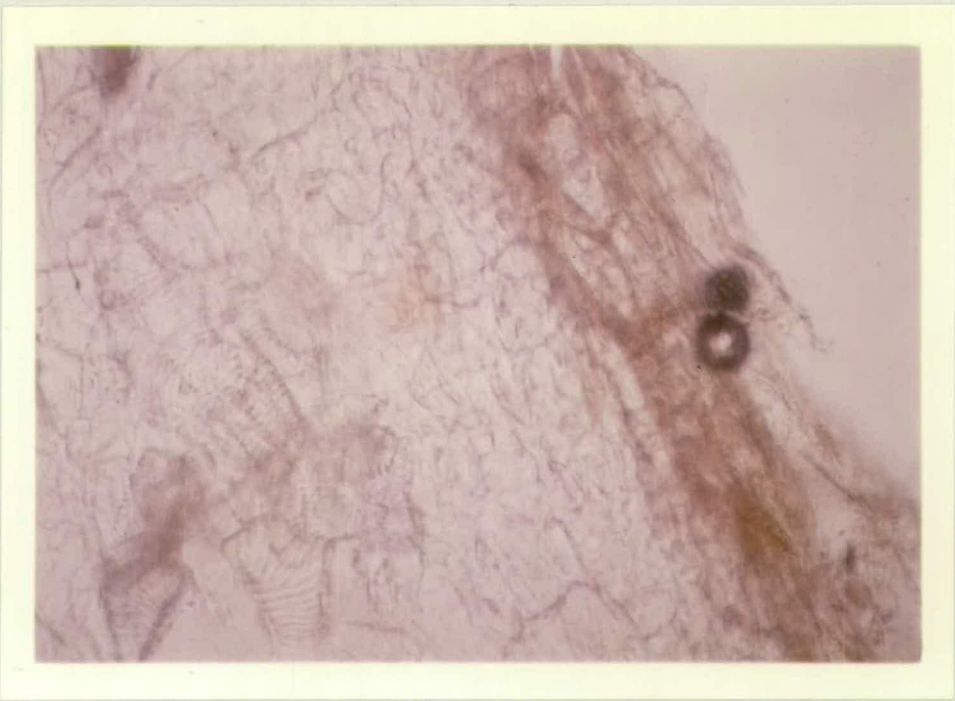


Figure 4.14. A high power view of Figure 4.13 showing the absence of reaction in a group of differentiating tracheids.

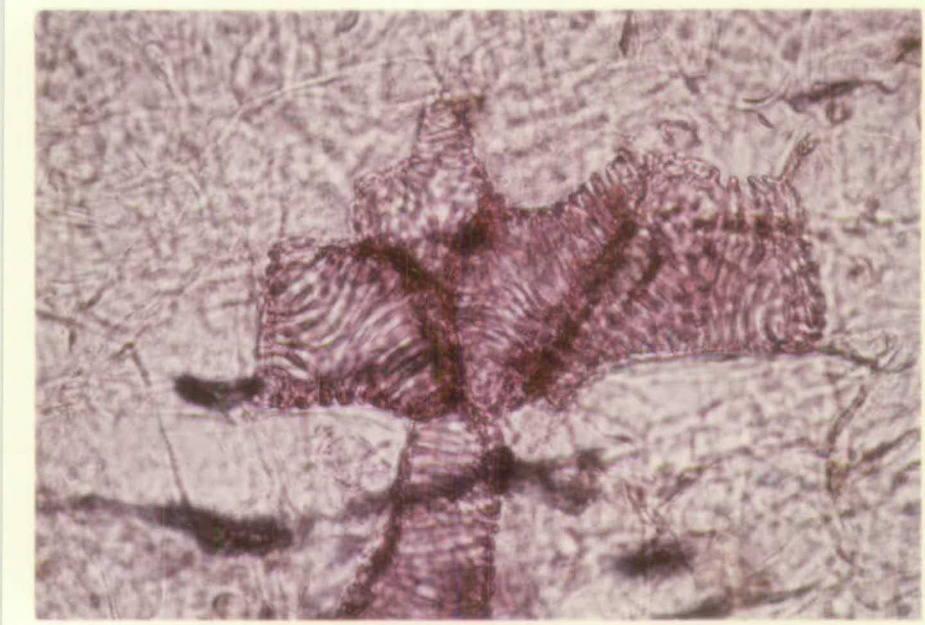


Figure 4.15. A high power view of Figure 4.12 showing the positive reaction in a group of differentiating tracheids.

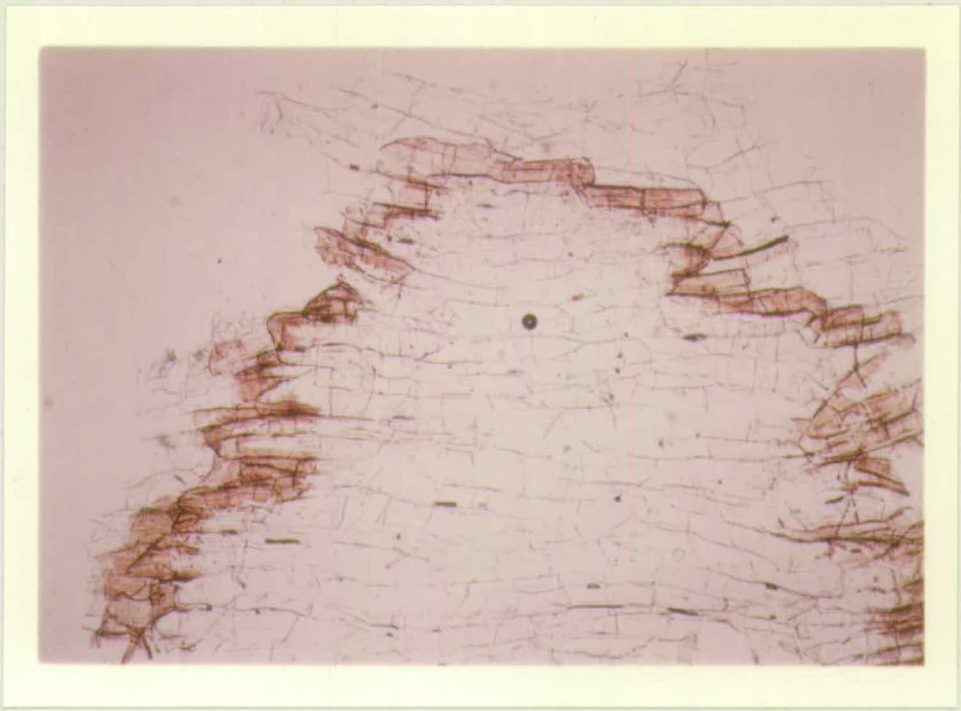


Figure 4.16. Alkaline phosphatase, Day 3. The positive reaction (orange) is clearly confined to a subperipheral layer.

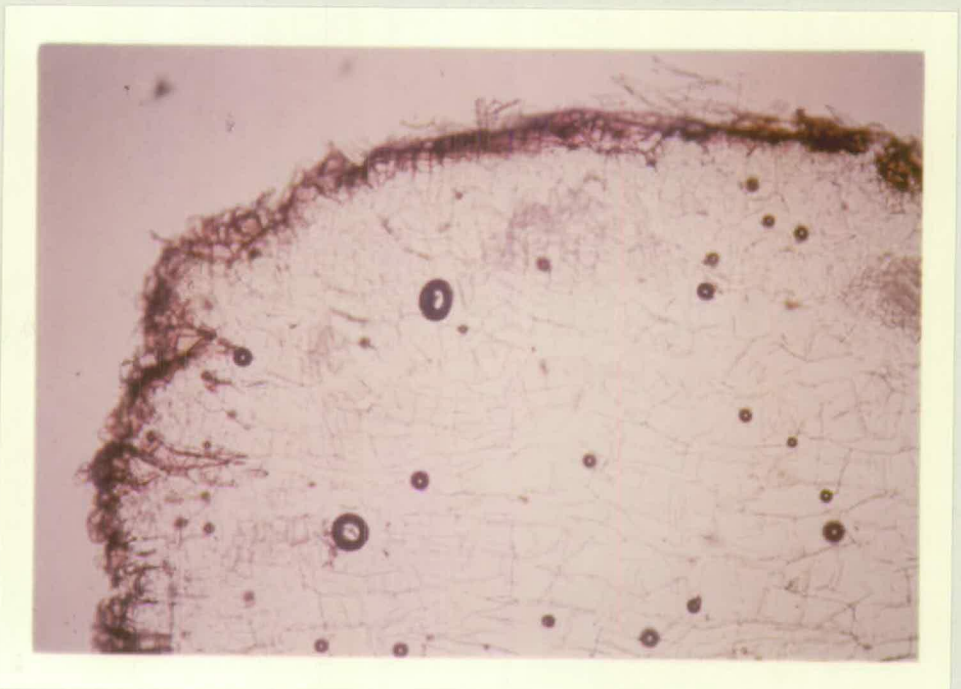


Figure 4.17. Alkaline phosphatase, Day 7. The activity is found in the rind but is absent from the groups of tracheids.

The phosphatase activity on the second day is 3 times that on the first day and the value drops to 2 fold on the 3rd day. It is unlikely that this is a random error because it is the mean of three determinations and a correspondingly high value was also recorded for phosphatase activity at pH 4.5.

The histochemical evidence shows that there is no localised activity in fresh tissue (Figure 4.6) but that prolonged incubation (120 minutes) of 1 day old callus sections shows a distinct ring of activity confined to a sub-peripheral layer of the explant (Figure 4.7). An end-section of this same callus (Figure 4.8) suggests that the whole periphery of the callus contains a region of active phosphomonoesterase II. On the 3rd day (Figure 4.9) this ring has excluded a layer 1 - 3 cells thick and recently divided cells may be seen immediately within the ring of phosphatase activity. No divided cells could be distinguished in the tissue showing a positive reaction for phosphatase activity. By the 4th day (Figure 4.10) the ring has become a skin of collapsed cells which correspond to the safranin-positive skin recorded in the anatomical description (p.112). Tissue outwith this skin tends to be sloughed off but may occasionally still be seen attached to the callus sections (e.g. Figure 4.12). On the 5th day sections show that the skin is beginning to be disrupted by the activity of nodule formation as the actively dividing region pushes outwards by expansion.

Centrifugal to the region of mitotic activity, groups of tracheids have appeared on Day 5, and these are strongly stained for phosphatase activity. They are the knots of tangled tracheids at the centre of nodules which have been described earlier (p.113) and their appearance is confirmed by the tracheid counts (Table 4.4, column 6) of macerates. Their number increases by Day 7 (Figure 4.12). A control section which has been inhibited by 0.01 M fluoride is shown in Figure 4.13. No purple dye is observed although the yellow breakdown products of O-anisidine are seen absorbed onto the broken superficial cells. A high power view

of the control section (Figure 4.14) shows that in a young tracheid there is no staining at all, while in the test section (Figure 4.15) the phosphatase activity is clearly localised in the group of tracheids. No reaction is seen in the nearby divided cells.

The corresponding reaction product of phosphatase activity at an alkaline pH is a deep orange. By altering the buffer to a pH of 9.4 using 0.2 M sodium hydroxide-glycine, the localisation of phosphomonoesterase I can be followed. This was found to be closely similar to phosphomonoesterase II with one important difference. In Figure 4.17, a section of a 7 day callus shows that alkaline phosphatase activity is confined to the superficial ring as in the case of acid phosphatase, but that there is no activity whatever associated with the differentiation of tracheids. A section of a 3 day old callus is shown in Figure 4.16 to demonstrate the similarity at both pH's of the lytic activity found in the sub-peripheral region of the young callus.

The Latency of Acid Phosphatase Activity.

After some explants had been shaken in a Warburg flask for only four hours, considerable phosphatase activity was found by disc electrophoresis of the supernatant proteins and visualisation of these by the azo dye method. This made it necessary to enquire whether such activity depended upon synthesis of the enzyme or merely upon activation of it.

Accordingly explants were tested for "latent" activity by the following treatments on four replicates of ten explants each.

(a) Treatment with an ionic detergent (no nonionic detergent was available for this preliminary experiment).

(b) Five cycles of freezing and thawing.

(c) Pre-incubation for 45 minutes in medium lacking substrate.

Finally, four replicates of ten explants were also homogenised in water and five fractions were separated by centrifugation (Figure 3, p. 29). The results of assay using β-glycerophosphate as substrate are presented in Table 4.5.

(O.D. units - - -)

Treatment	A	B	C	D	Mean
Whole explants	0.83	0.74	0.72	0.72	0.76
Ionic detergent	0.80	0.92	0.88	0.85	0.86
Pre-incubation for 45 m without substrate	0.92	0.92	0.85	-	0.90
Freezing and thawing	0.98	0.96	0.92	0.92	0.94
Fraction I (Debris)	0.21	0.22	0.19	0.22	0.22
" II (Mitochondrial)	0.085	0.082	0.082	0.064	0.08
" III ("x")	0.047	0.047	0.048	(0.098)	0.05
" IV (Ribosomal)	0.177	0.166	0.143	0.123	0.16
" V (Supernatant)	1.58	1.43	1.42	1.36	1.45

Table 4.5. Four replicate values for the phosphatase activity detected in fresh tissue using a variety of treatments. The first four treatments used 10 whole explants each. The latter five employed the fractions indicated from homogenates of 10 explants each.

It will be realised that the homogenisation was very crude but that it would take considerable time to develop a medium and a method of grinding which would preserve any particles that might contain acid phosphatase. Nevertheless there was a 20 per cent increase in activity over the controls. Thus the results presented in Table 4.5 suggest that there may be some latent acid phosphatase activity in artichoke parenchyma tissue. Using this homogenisation procedure about 75 per cent of the enzyme activity was found in the supernatant fraction.

Localisation of Succinic Dehydrogenase.

Because sections cut using the freezing microtome did not give a positive reaction for this enzyme, hand-cut sections were employed. No activity was found during the first few days of culture, but at 5 days, sections, which had been incubated for 30 minutes at 37°C "in air", showed a curious distribution of activity. Perhaps half a dozen single cells appeared to contain succinic dehydrogenase activity. These isolated cells were scattered around the dividing zone. Further incubation resulted in a few more scattered cells showing blue formazan deposits but, significantly, those cells which were originally coloured were now seen to be surrounded by three or four other blue cells. It is tempting to suggest that these isolated cells are those which have the most intense rate of activity of succinic dehydrogenase and that they are surrounded by other cells which have only a slightly lower rate of activity. It would seem that here a metabolic gradient may be manifest where no morphological differences can be observed. These groups of cells occur at a time, and in a locality and distribution that is reminiscent of the origination of nodules. Any connection is purely speculative but may yield to a fine structure, histochemical approach.

With further incubation, or with sections of 7 day explants, the whole region of dividing cells showed a positive reaction for succinic dehydrogenase. Heat treated sections showed no reaction.

Conclusions.

1. Changes in the total nitrogen content of explants have been followed during callus development. Part of this total nitrogen content is contained in the supernatant fraction, obtained by differential centrifugation. The nitrogen content per explant in this fraction increases throughout development. However, when expressed on a fresh weight basis there is an increasing amount during the division phase but a more or less constant amount during the differentiation phase.

2. The soluble proteins contained in the supernatant fraction have been analysed using the technique of disc electrophoresis and a time-course of the changes in the protein of this fraction has been established. With time, there was an increasing amount of staining due to proteins in the gels. Some bands appear and increase in intensity while others decrease in intensity and disappear. There appears to be a continual change in the relative proportions of the proteins present. Some proteins appear to be present throughout the period of culture, while others only appear and increase in amount during the course of development.

3. Homogenates, or in some cases entire explants, have been used to follow the changes in activity of the following enzymes during the development of the callus.

Enzyme	pH	Substrate
Acid phosphatase	6.2	β-glycerophosphate
" "	4.5	β-glycerophosphate
Alkaline phosphatase	9.4	β-glycerophosphate
Ribonuclease	6.2	yeast nucleic acid
Invertase	6.2	sucrose
Inulase	6.2	inulin
Succinic dehydrogenase	7.4	succinate

(a) Phosphatase activity, using β-glycerophosphate, has been assayed at three pH values, 4.5, 6.2 and 9.4. The changes in activity at the two acid pH's were very similar during development, which might suggest that the same enzyme (which has a wide pH range, p. 36) was being measured. There was no detectable activity of alkaline phosphatase at any time during development. The mean plot for determinations of acid phosphatase at pH 6.2 shows a shallow sigmoid curve with no lag phase.

(b) The activity of ribonuclease showed a four day lag period followed by a rapid and continuous increase which was maintained until the fourteenth day. This increase coincides with the onset of differentiation.

(c) Invertase activity could not be detected at any stage of development in explants taken from either young or old tubers.

(d) There was no inulase activity detectable in young non-dormant tubers.

(e) After a lag of one day, the activity of succinic dehydrogenase increased until the fifth day and thereafter remained more or less constant.

4. The sensitivity of oxygen uptake to the inhibitor 2:4 dinitrophenol was found to increase tenfold during the division phase, to return dramatically to a low level on the sixth day, only to increase by fivefold during the differentiation phase.

5. (a) Employing histochemical methods, acid phosphatase (using both β-glycerophosphate and α-naphthyl phosphate) was shown to be localised in a peripheral ring of intact cells which do not divide but collapse by the fourth day to form an enveloping rind of dead cells. It was also localised in differentiating tracheids wherever these were found.

(b) Alkaline phosphatase capable of hydrolysing α-naphthyl phosphate was found in the same peripheral ring of intact cells but never in differentiating cells.

Phosphatase activity would appear to be found in cells undergoing controlled lysis.

6. The supernatant fraction from homogenates, which contains the bulk of the acid phosphatase activity, was analysed by disc electrophoresis and stained for acid phosphatase using α-naphthyl phosphate as substrate. Activity was located in one diffuse and one sharp band.

7. Although the mean curve of phosphatase activity was sigmoid, two experiments (Experiments 26 and 50) showed a dramatic fall in activity between the initial rise and the final rapid increase. The histochemical results suggest a possible explanation. It seems possible that the sigmoid curve has two components, (a) an initial rapid increase (and subsequent decrease) due to the peripheral phosphatase activity followed by, (b) the sharp rise associated with the differentiation of tracheids. If the two processes overlap in time the result will be a sigmoid curve but if peripheral activity is precocious (or tracheid differentiation retarded) then a fall in activity may be observed between the peaks of each process.

SECTION II C.

METABOLIC CHANGES DURING THE FIRST WAVE OF CELL DIVISION.

Introduction.

The results of Section II A (p.101) have demonstrated that the system used in this study falls naturally into three phases: the induction of cell division, the maintenance of cell division (accompanied by some expansion) and a third phase in which cell division, cell expansion and cellular differentiation all contribute to the further organised development of the callus. The future of a particular parenchyma cell which is induced to divide will involve (a) the induction of division (b) the maintenance of division in the daughter cells and (c) the departure from division by the differentiation of cells derived from these daughter cells through such processes as expansion and maturation.

In this Section evidence, much of it of a preliminary nature, will be presented concerning the induction of the first wave of divisions. This includes both the stimulus to increased metabolic activity and also all those changes leading up to the first wave of divisions.

Increase in Cell Number.

In an early experiment it was observed that the cell number changes recorded at daily intervals showed periodic increases. The fact that these increases did not occur uniformly with time suggested the possibility that the division of cells did not occur at random but might occur in waves. This hypothesis was tested by taking harvests at twelve hour intervals and recording fresh weight and cell number. The results are presented in Figure 5.1. For the first 60 hours the number of cells remained more or less constant. Between 60 and 72 hours there was a dramatic rise in the cell number which increased from 20,000 to 34,000 cells. Significantly,

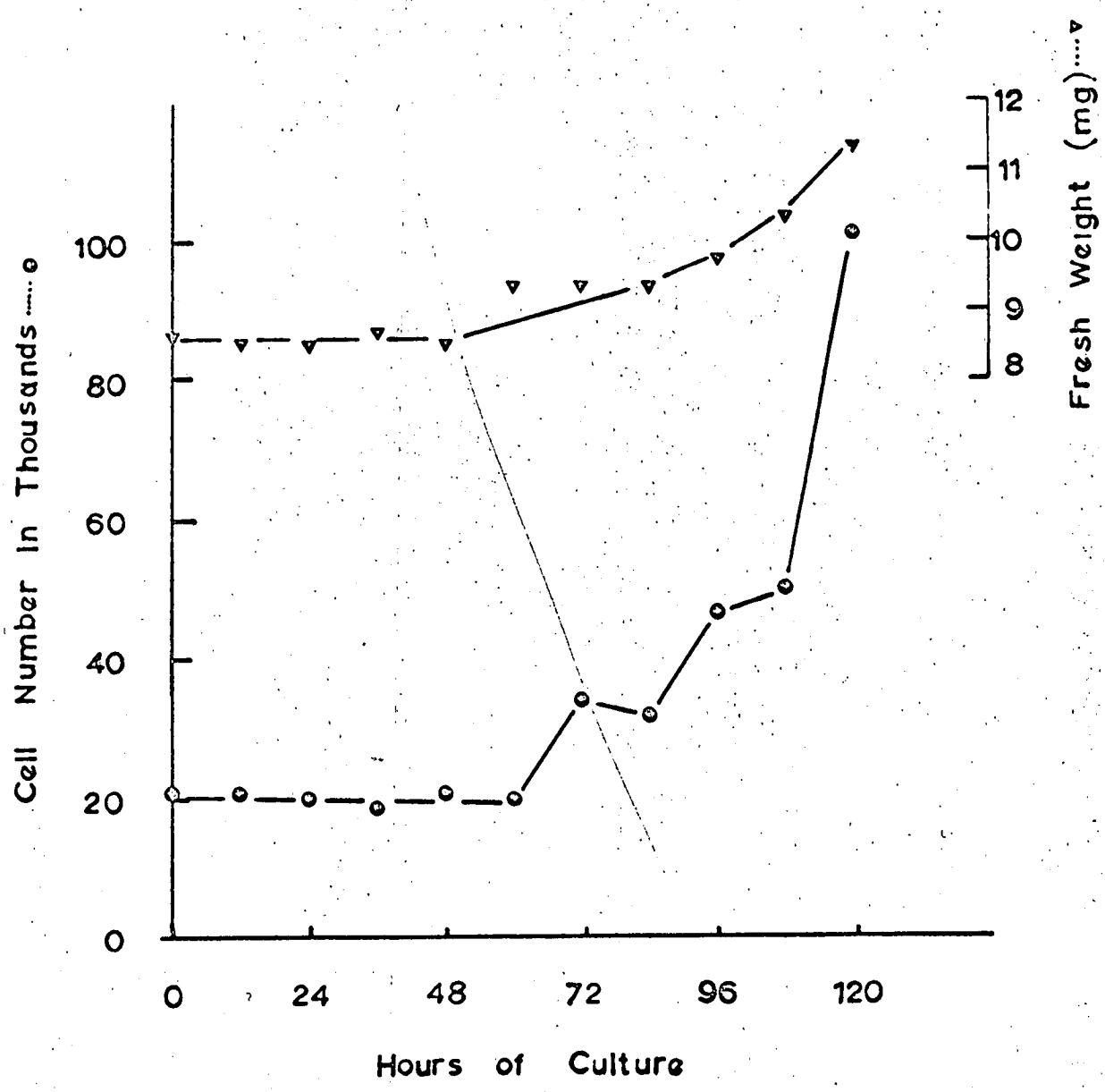


Figure 5.1. Experiment 36. The periodic increase in cell number during five days of culture. Each cell number value represents the mean of six counts from a macerate of 10 explants. Each fresh weight value is also the mean of 10 explants.

during the next 12 hours the cell number did not change. After 96 hours a further stepwise increase of another 70 per cent was recorded and there was again little increase at the subsequent, 108 hour harvest. However, after 120 hours a very large increase had occurred. During this five-fold increase in total cell number there was a gradual increase in fresh weight of about 30 per cent after a 2 day lag phase. This experiment showed that the early mitotic divisions did not occur at random but were to some extent periodic. It may be noted that the first cycle of division was complete by 72 hours but that the second took only 12 to 24 hours and that a further large increase in cell number took place in merely 12 hours. Such a large increase (50,000 cells) must have involved more than one cycle of division and therefore (even if the divisions are now more random than in the earlier waves) it seems likely that some divisions of the fourth cycle are completed in rather less than 12 hours. This would suggest that the duration of the first divisions are not of equal length and division in fact is consummated with increasing rapidity.

When it had been discovered that division activity did indeed occur in waves, two questions immediately required an answer. Firstly, how closely do these initial divisions occur, in other words, to what extent is the mitotic activity synchronised? An investigation into the degree of synchrony is now being carried out by Mr. Evans in this Department.

Secondly, is the timing of the wave of division predictable? Experiment 36 was carried out in July and Figure 5.1 shows the greatly extended lag phase of 60 hours. On surveying the lag phase of earlier experiments, (Table 2.7, p. 93) it appeared that the time at which division occurs might be predictable according to the "age" of the tuber.

It was necessary, however, to confirm that there were waves of division in explants taken from recently stored tubers, because it was possible that the

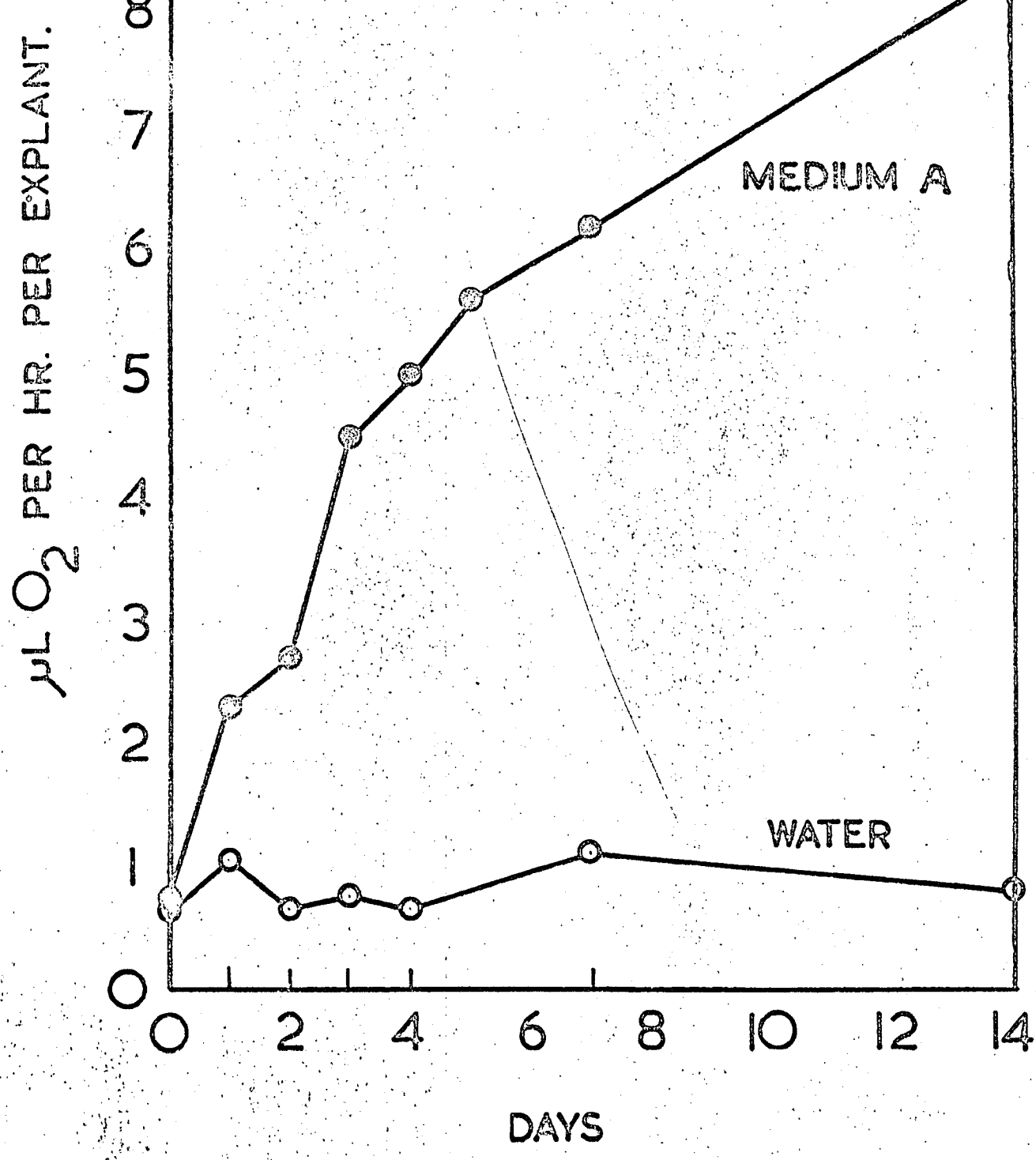


Figure 5.3. Experiment 23. A comparison of the oxygen uptake of explants cultured in Medium A and in distilled water. Cultures were grown as usual in roller tubes and duplicate values of the oxygen uptake were determined using 20 explants per flask for the early values and 5 for the later values. There was no increase in fresh weight or in total cell number in the explants cultured in water.

periodicity of divisions observed in Experiment 36 was a feature of callus development confined to old tubers. A preliminary experiment showed that this was so and the phenomenon has since been amply confirmed (Evans, 1965).

The first cycle of division involves the change from a quiescent state to one which is metabolically active. One of the consequences of this change is the induction of mitotic activity. For this reason the metabolic changes occurring during the induction of this first wave of divisions are of particular interest and were examined by the methods previously developed for the longer term studies. It was known from these studies that there is little fresh weight increase during this time, that dry weight usually increased but might, on occasion (e.g. Experiments 32 and 50) decrease, and similarly the total nitrogen usually increased but sometimes (Experiments 50 and 56) decreased. It seemed unlikely, therefore, that the level of either of these complex entities have a controlling effect upon the first division. More subtle changes would have to be investigated.

Gaseous Exchange.

In contrast to these changes, oxygen uptake invariably increased rapidly over the first few days of culture. However, explants have a large area of superficial, damaged cells and this wounding may be the cause of the initial rise in oxygen uptake. The results of a 14 day experiment to examine the possibility are reported in Figure 5.3. It can be seen that when explants are cultured in water there is only a slight rise in oxygen uptake. On Day 1 there is a 50 per cent increase over Day 0 but thereafter the value returns to a lower figure. Without the nutrients of Medium A no cell division or cellular expansion is accomplished.

In order to examine the oxygen uptake over the first division cycle,

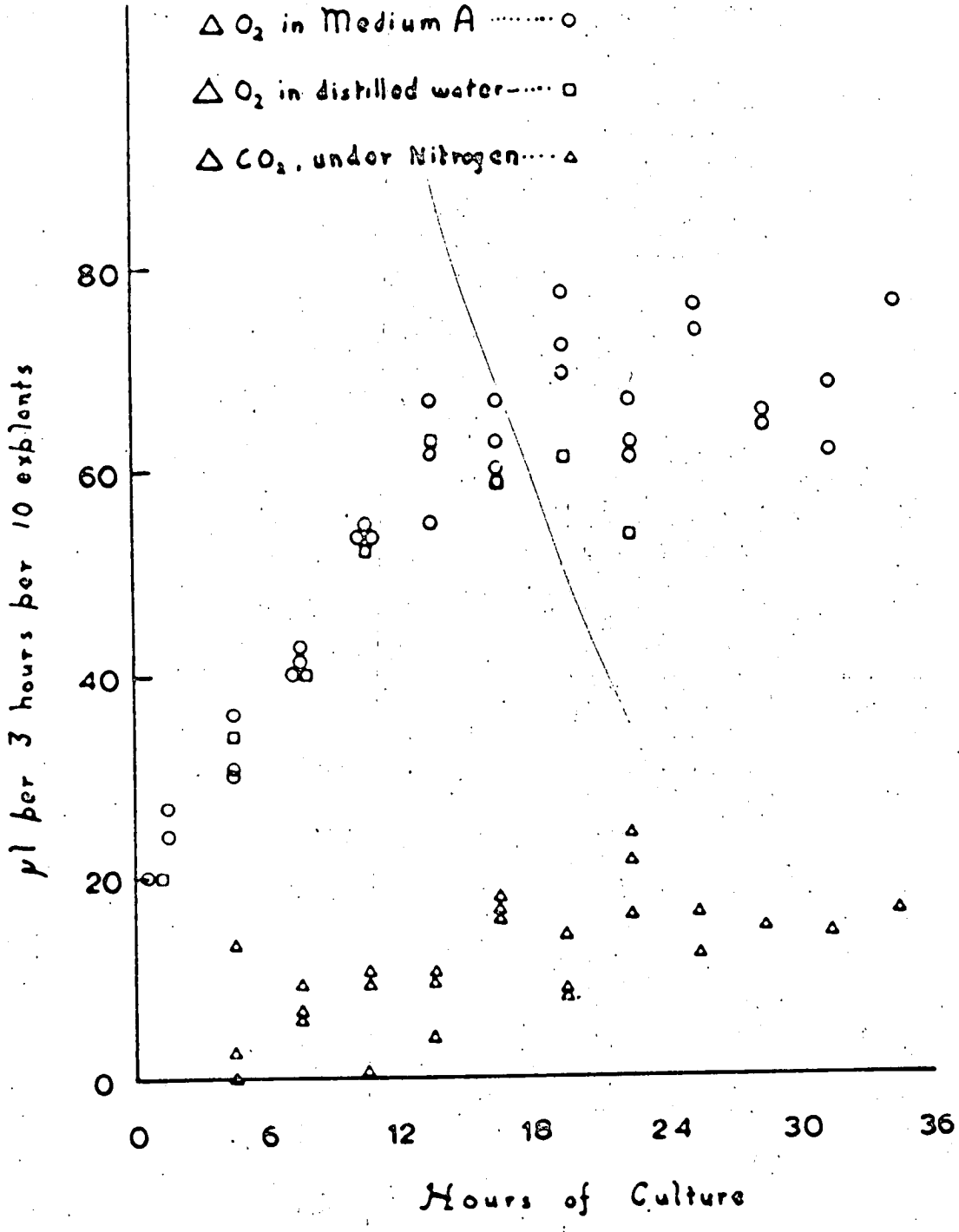


Figure 5.4. A further comparison of the oxygen uptake of explants cultured in Medium A and in distilled water. Some flasks were flushed with nitrogen and their CO_2 output recorded. Their was no difference between the two media under nitrogen. The number of replicates was reduced during the experiment due to sampling for other purposes.

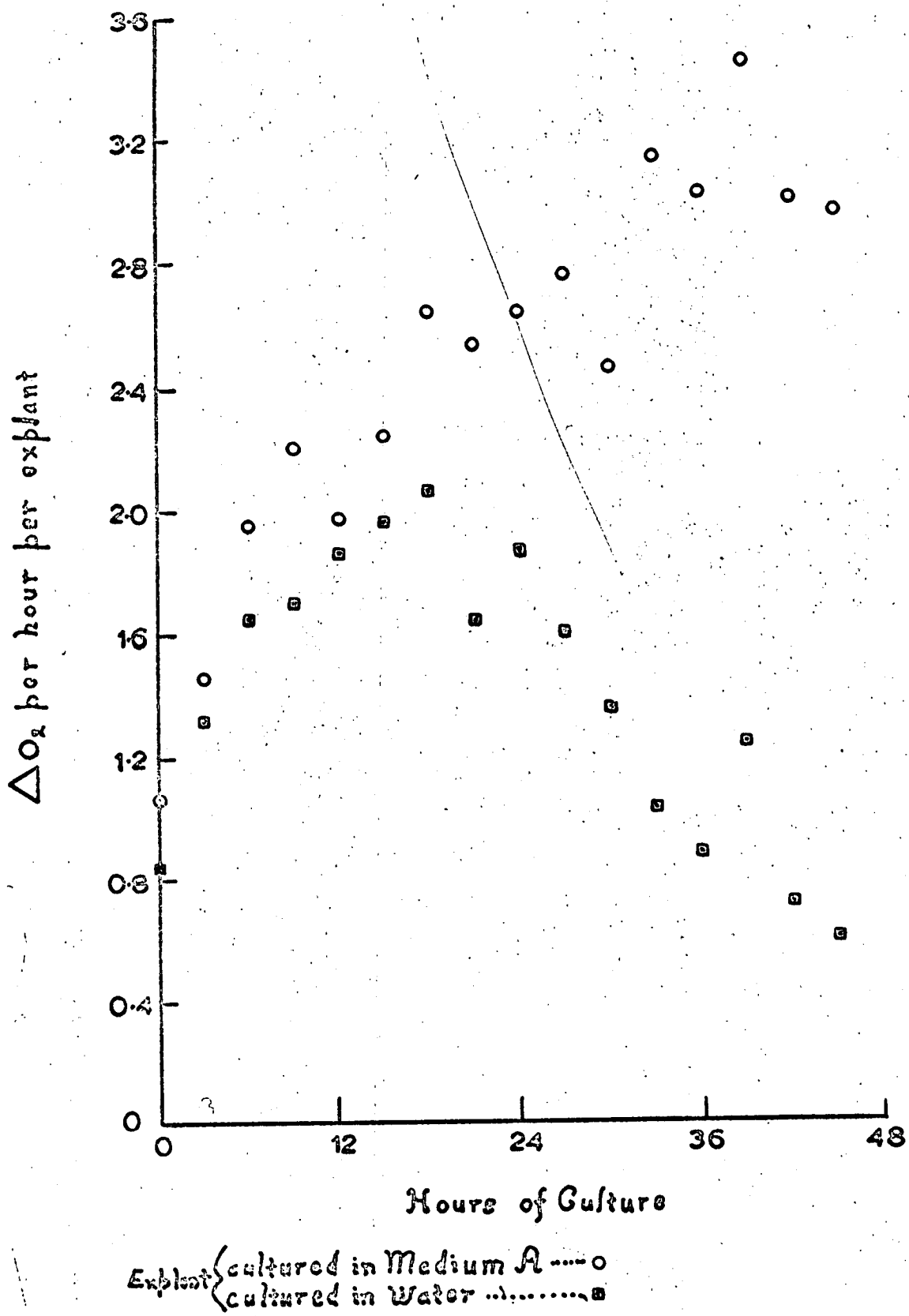


Figure 5.5. Experiment 49. A comparison of the oxygen uptake of explants cultured in Medium A and in water. Each point is the mean from 2 flasks containing 10 explants each. Culture was carried out in the Warburg flasks, which contained 1 ml of medium under sterile conditions.

groups of ten explants were cultured aseptically in Warburg flasks, in 1 ml of Medium A or of water, and their oxygen uptake recorded. The results are plotted in Figure 5.4. It can be seen that both in water and in Medium A there was a rapid rise until about the 15th hour. Thereafter the rate in Medium A remained more or less constant until the 35th hour. At this time a large increase in cell number was observed in macerates of explants taken from one of the three flasks. In these macerates 20 per cent of the cells occurred as recently divided pairs and 1 - 2 per cent had been fixed in telophase prior to cytokinesis. From this evidence it was concluded that the first wave of division was completed after about 36 hours. This was confirmed by examination of sectioned, 36 hour material.

In a preliminary experiment, in which division occurred between 32 and 40 hours, it had been shown that when explants are cultured in water their oxygen uptake decreases after about 16 hours (see Figure 5.5) and the results presented in Figure 5.4 tend to confirm this, although the progress was not followed beyond 24 hours.

A further four flasks were set up as above but were first flushed with nitrogen for 10 minutes to achieve anaerobic conditions (Umbreit, Burris and Stauffer, 1959), and their carbon dioxide output was measured. Three flasks, containing Medium A plus ten explants in each, showed an increasing output of carbon dioxide until about the 16th hour of culture. This was followed by a more or less steady output till the 36th hour, at a rate of 0·5 µl per hour per explant which is comparable to the rate of oxygen uptake in air. The control explants cultured in water showed no output of carbon dioxide under these anaerobic conditions. Hand-cut sections of explants taken from aerobic and anaerobic flasks both showed a positive reaction for acid phosphatase activity, although those cultured in air reacted about twice as quickly. This would suggest that the activity of this enzyme may be to some extent dependent upon increased respiratory activity.

Time (hours)	No. of explants macerated	No. of explants fixed	Cell Number	Pair Number
0	4	3	25,000	0
5		3		
10	3	4	20,000	0
14		4		
16		4		
18		4		
20		4		
21		4		
22		4		
23		4		
24	3	4	20,200	0
25		4	-	-
26		4	-	-
27	3	4	20,100	0
28	3	4	20,000	0
29		4		-
30	3	4	18,000	0
31	3	4	18,000	0
32		4	-	-
33	3	4	22,300	0
34	3	4	23,100	0
35	3	4	23,300	0
36	3	3	20,800	0
37		4	-	-
38	3	3	19,400	0
39	3	4	20,000	0
40		3	-	-
41	3	4	26,700	6,000
42	3	3	24,600	4,400
43		3	-	-
44	3	3	34,800	10,000
45	3	3	35,600	10,000
47	3	3	37,100	10,000
49	3	3	37,700	10,000

Table 5.1. Experiment 55: Details of the harvest times, cell numbers and pair numbers. Columns 2 and 3 show the number of explants which were used for cell number estimates, and the number of explants fixed in acetic alcohol for microdensitometric measurements, at the various harvest intervals.

Having shown that the cell number increase occurs in a step-wise fashion at both the beginning and at the end of the season, it was of interest to find markers which also had a stepped increase. The most obvious possibility was of course deoxyribonucleic acid (DNA).

Experiment 55 was designed to compare cell number with mean DNA content per cell, during 50 hours of culture. This was a joint experiment and all the preparation of sections and microdensitometric measurements were carried out by Dr. J. Mitchell of this Department. The duration of culture and the number of samples withdrawn for each estimate are given in Table 5.1 in company with the cell number estimates.

An attempt was made to define more accurately the number of cells which had undergone mitosis. During the estimation of cell numbers, a count was taken of those pairs of cells which had resisted separation by maceration and which also had a very thin cross-wall. These were taken to be the cells which had recently divided and they were quite distinct from other pairs of cells. Clearly, all the recently divided cells might not be detected by this procedure and so the estimate may be a low one. However, it should record a definite proportion of the daughter cells and reduces the variation due to sampling difficulties in a large population of explants.

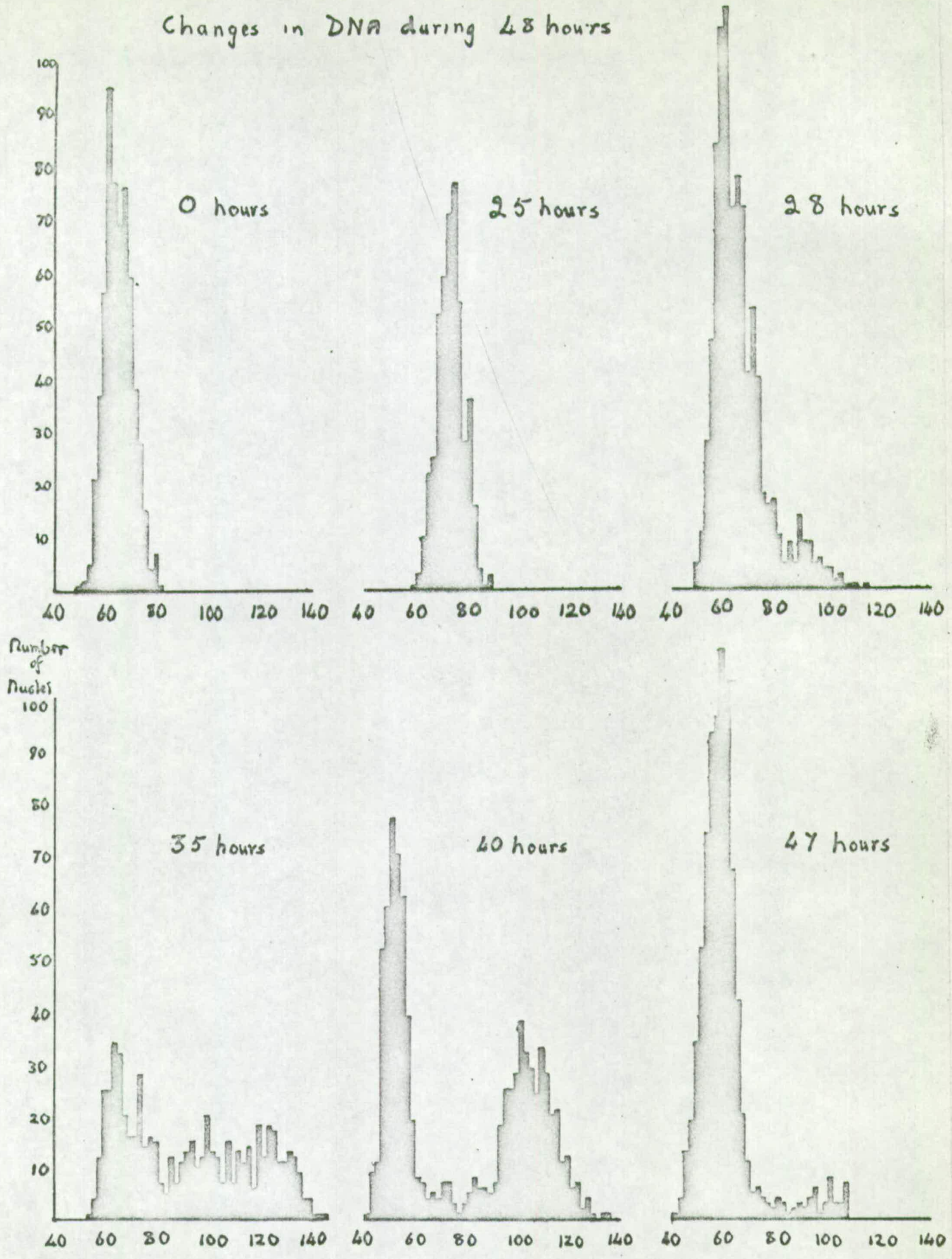


Figure 5.6. Each histogram is a record of the colour density of between 450 and 900 Feulgen-stained nuclei. These nuclei are from sections of explants which were fixed after 6 different periods of culture.

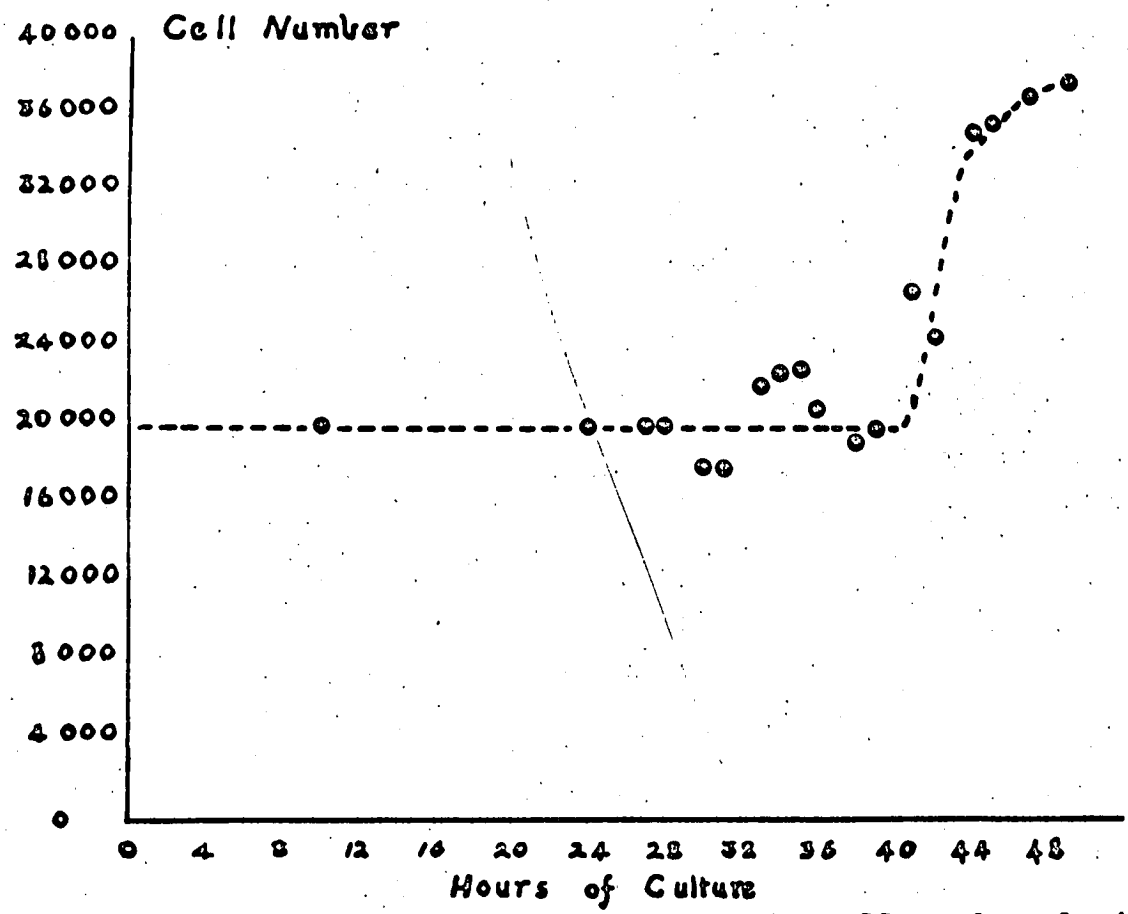


Figure 5.7. Experiment 55. The change in cell number during 50 hours of culture. Each value is an estimation from a macerate of 3 explants.

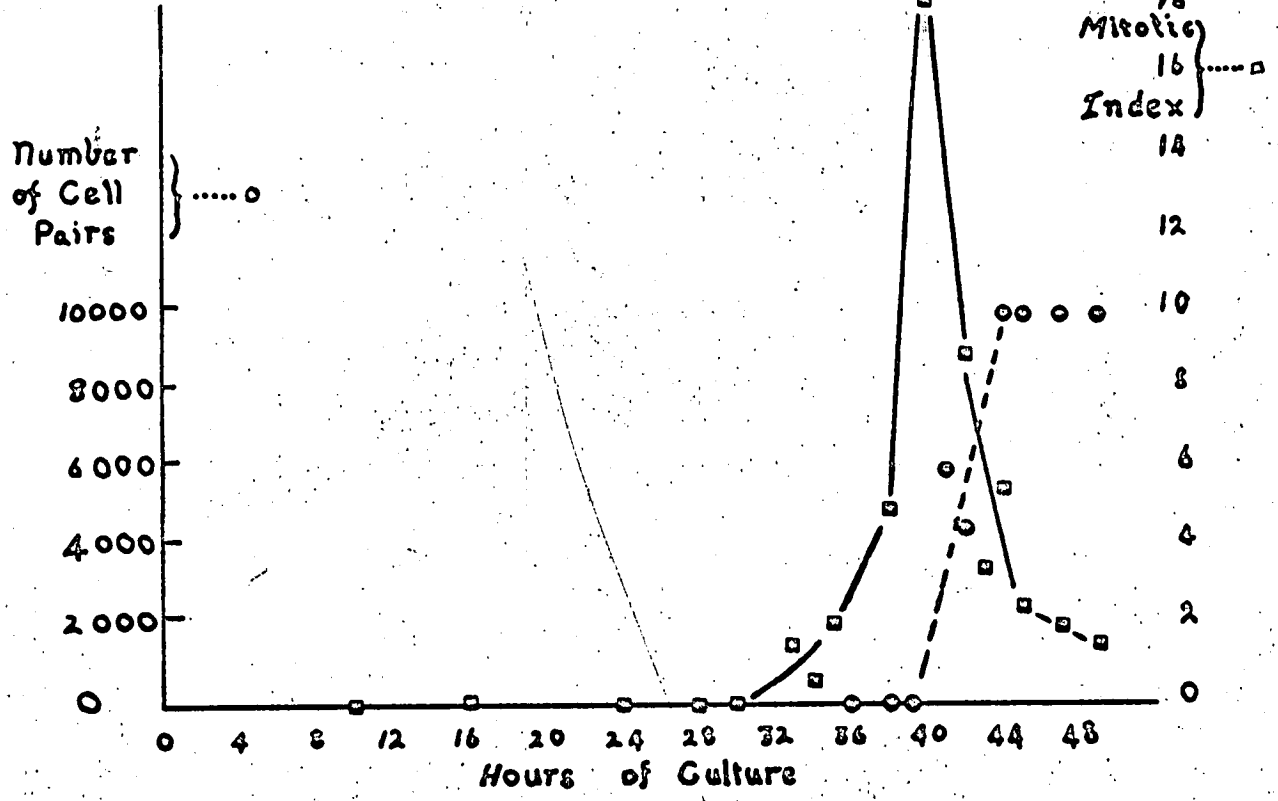


Figure 5.8. Experiment 55. The change in "cell pairs" which are taken to be an index of the recently divided cells, and in mitotic index. The mitotic index was obtained after scanning at least 500 cells of the Feulgen preparations.

Anatomical studies had previously shown (Figure 3.19, p. 111) that division is initiated in a sub-peripheral region, so in order to have comparable material, only tissue sections from the mid portion of explants were examined for DNA determinations. For each time interval the staining intensity of every nucleus in a single section, and in each nucleus in two further half-sections, were recorded and block histograms (Figure 5.6) of the DNA content per nucleus constructed. This involved 400 - 900 measurement at each time interval.

Figure 5.6 shows that initially the nuclear condition of all the parenchyma cells of the explant was found to be normallydistributed about what is taken to be the diploid or 2C value (Partanen, 1959; Adamson, 1962) and it remained so until 25 hours of culture had elapsed. After 28 hours, a proportion of the nuclei had increased in DNA content and were now between 2 and 3C. At 35 hours about 50 per cent of the nuclei had an increased DNA content and their values ranged from 2C up to 4C. However, by 40 hours 50 per cent of the values were found normally distributed about the 4C values of 104 units, while the remainder remained normally distributed about the 2C value. After 47 hours, over 90 per cent of the nuclei were again found to be in the 2C condition. The remaining nuclei may either have been slow to commence synthesis or they may represent the onset of a second wave of synthesis in the newly formed daughter cells.

In Figure 5.7 are plotted the cell number values and in Figure 5.8 the number of divided pairs are recorded with the mitotic indices of the Feulgen stained sections. The pair counts show that a 50 per cent increase in cell number occurred between 39 and 44 hours. The peak in mitotic index immediately precedes at 40 hours, the appearance of cell pairs in the macerates. Both the mitotic indexes and the total cell number values suggest that although the bulk of visible mitoses is confined within a period of five hours, there is nevertheless some spread in the

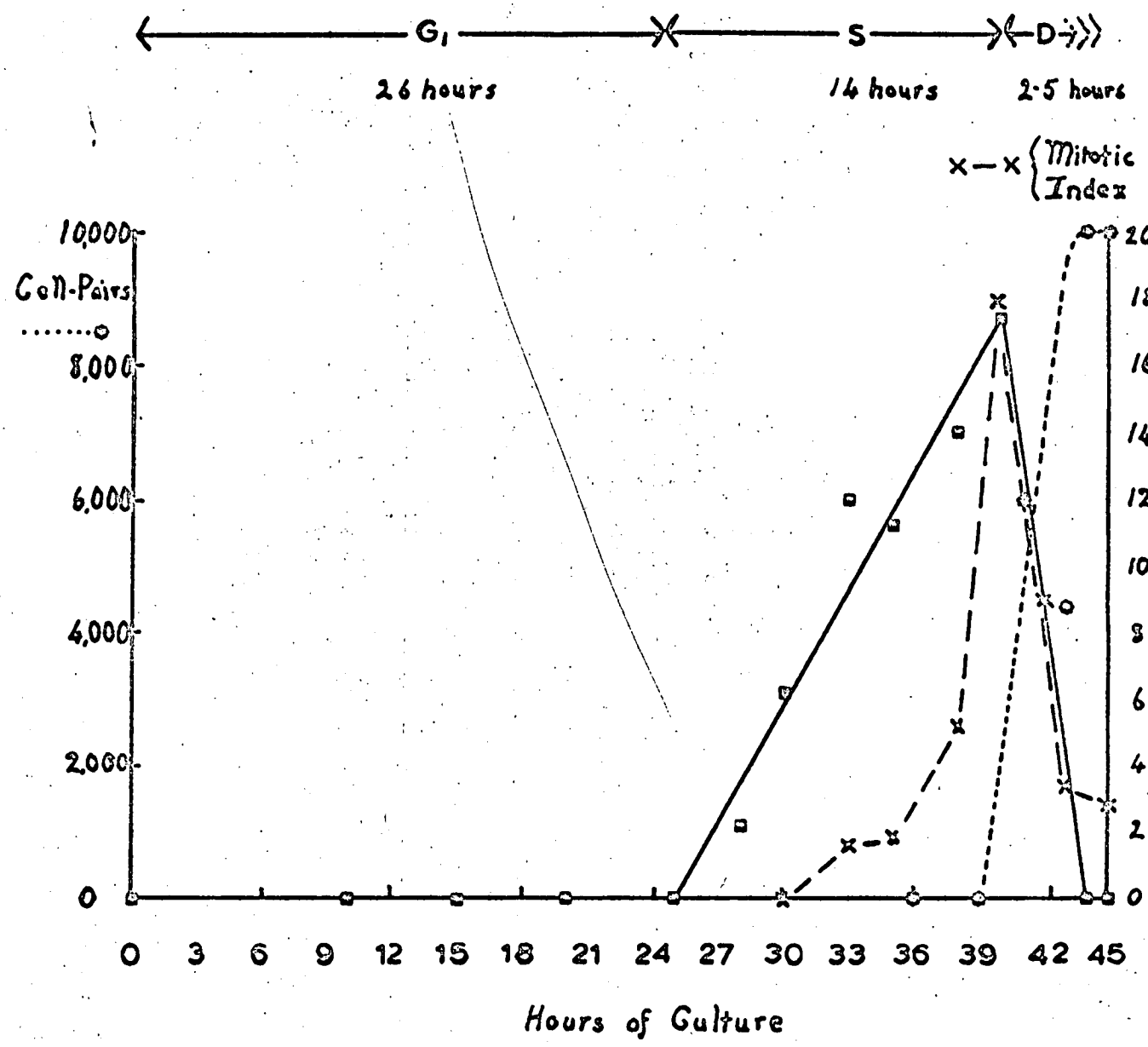


Figure 5.8. A summary of Experiment 55, showing how an estimate of the duration of the G_1, S and D periods may be obtained with reference to DNA synthesis in the first cycle of division. There is no measurable G_2 period.

timing of the remaining 20 - 30 per cent of divisions. The last histogram of Figure 5.6 shows that, within 6 hours of the appearance of daughter cells (41 hours), less than five per cent of the nuclei which have synthesised DNA have failed to divide.

Experiment 55 has been summarised in Figure 5.8 and from the data an estimate can be made of the phases of the division cycle with respect to DNA synthesis. The first, pre-synthesis, phase ("G_1") lasts for the first 26 hours of culture. Presumably in the prehistory of the cells this began in some or all cases several months previously near the beginning of the quiescent storage period. An accurate estimate of the timing and duration of the synthetic ("S") phase can be made. It begins at about 26 hours and is complete by 40 hours giving a length of about 14 hours for this first "S" phase. Division immediately follows DNA synthesis and so there is little or no post synthetic ("G_2") period. The succeeding "G_1" is very much shorter than the first "G_1" and extends for some 6 to 7 hours.

From data collected by Mr. Evans (1965) using "young" tubers it can be shown that the second cycle of division takes about 12 hours. This means that the second period of DNA synthesis must last for less than 6 hours. It is clear, therefore, that the first and second cycles of division show marked differences in the length of the component phases of the division cycle based on observation of DNA synthesis.

Protein Pattern.

After it had been established that both cell number and DNA content per nucleus increase in a stepped fashion it was argued that an examination of particular enzyme activities may give some indication of the contribution such enzymes make to the mitotic process. It is clearly probable that in order to proceed through mitosis not only DNA must be synthesised. Presumably a complement of cell organelles

and constituents must also be synthesised. Because the daughter cells are initially half the size of the parent cells this synthesis may not represent a doubling of every constituent but nevertheless considerable synthetic activity must be initiated to support the division cycle. The change from a quiescent cell prior to culture to an actively proliferating system will also involve gross changes in catalytic activity. It is of interest to demonstrate such changes in specific enzymes.

It has been shown that large changes occur in the protein pattern of the soluble fraction during this time (p. 120). In order to link some of these changes with demonstrable changes in the activity of particular enzymes, a preliminary experiment was carried out in an attempt to apply the staining methods of histochemistry to visualise specific proteins separated by gel electrophoresis. Explants were cultured for up to 60 hours and at various intervals cell numbers were estimated to establish the pattern of cell division over that period. Because cell numbers varied between 17,000 and 28,000 in this experiment, only pair counts are reported. Between 36 and 48 hours 6,000 daughter cells appeared and by 60 hours 13,000 were present, the majority in groups of four. The first wave of division therefore took place after 36 hours and the second wave before 60 hours. The percentage increase was of the order of 25 per cent of the initial number of cells in the explants at each division. At selected intervals 15 explants were harvested, homogenised and subjected to differential centrifugation followed by gel electrophoresis of aliquots of the soluble fraction (p. 117). Table 5.2 is a record of the time of appearance of an appreciable amount of the various fractions.

electrophoretic mobility of the enxyme or to diffusion of reaction product prior to precipitation by the dyes. A linear scan of staining intensity found in each gel was recorded using the Chromoscan.

Acid Phosphatase Activity.

At 0 hours there was no detectable peak of activity along the gel. At twelve hours a major peak had appeared and by 24 hours this had doubled in size. Thereafter there was little change in the peak except that at 32 hours, just previous to the peak in mitotic activity, the activity became very diffuse. At 36 hours the amount had decreased. A second band of phosphatase activity appeared in the spacer gel at about 20 hours and was present thereafter. The area under the curve of the peak in phosphatase activity has been calculated and these results are presented in Table 5.3.

Time (hours)	Area under the curve — Arbitiary O.D. units	
	Phosphatase	Malic Dehydrogenase
0	0	3,800
4	-	2,900
12	170	2,500
20	240	2,900
24	350	1,400
28	360	1,400
32	310	1,100
36	188	

Table 5.3. Phosphatase activity during the first 36 hours. Values are calculated from scans of activity found in gels containing proteins separated by electrophoresis. The values recorded represent the area under the curve of the peak in staining intensity.

It must be emphasised that the procedure has not been standardised enough to give strictly quantitative results. However, the major changes in amount can be demonstrated.

It is clear that there is minimal activity initially, that there is some activity after 12 hours and that this has doubled by 24 hours. If the time of division occurs at about 36 hours and if DNA synthesis takes about 14 hours then this large increase in acid phosphatase activity occurs during the pre-DNA synthesis phase ("G_1"). It is not known whether or not the reduction in activity after 28 hours is in any way related to DNA synthesis or to the mitotic cycle.

Malic dehydrogenase Activity.

In a similar fashion the area under the curve of formazan intensity along a gel which has been incubated with malic acid and Nitro BT electron acceptor has been calculated. These values are also presented in Table 5.3.

It can be seen that activity which was recorded initially remained for 24 hours and then decreased. This change may reflect a genuine change in malic dehydrogenase activity in the callus, but is more likely to represent an alteration in the degree of mitochondrial damage during fractionation. This could result in less mitochondrial protein being found in the supernatant fraction.

Similar analysis for succinic dehydrogenase and for glutamic dehydrogenase showed positive but diffuse and variable staining.

It was concluded that a study of dehydrogenase activities by this method must await the development of a reproducible method for solubilising these proteins from the mitochondrial fraction of homogenates. This is now in progress (Williams, 1966). However, the evidence for dramatic change in the activity of acid phosphatase suggests that this method could be developed for other enzymes found in the supernatant fraction.

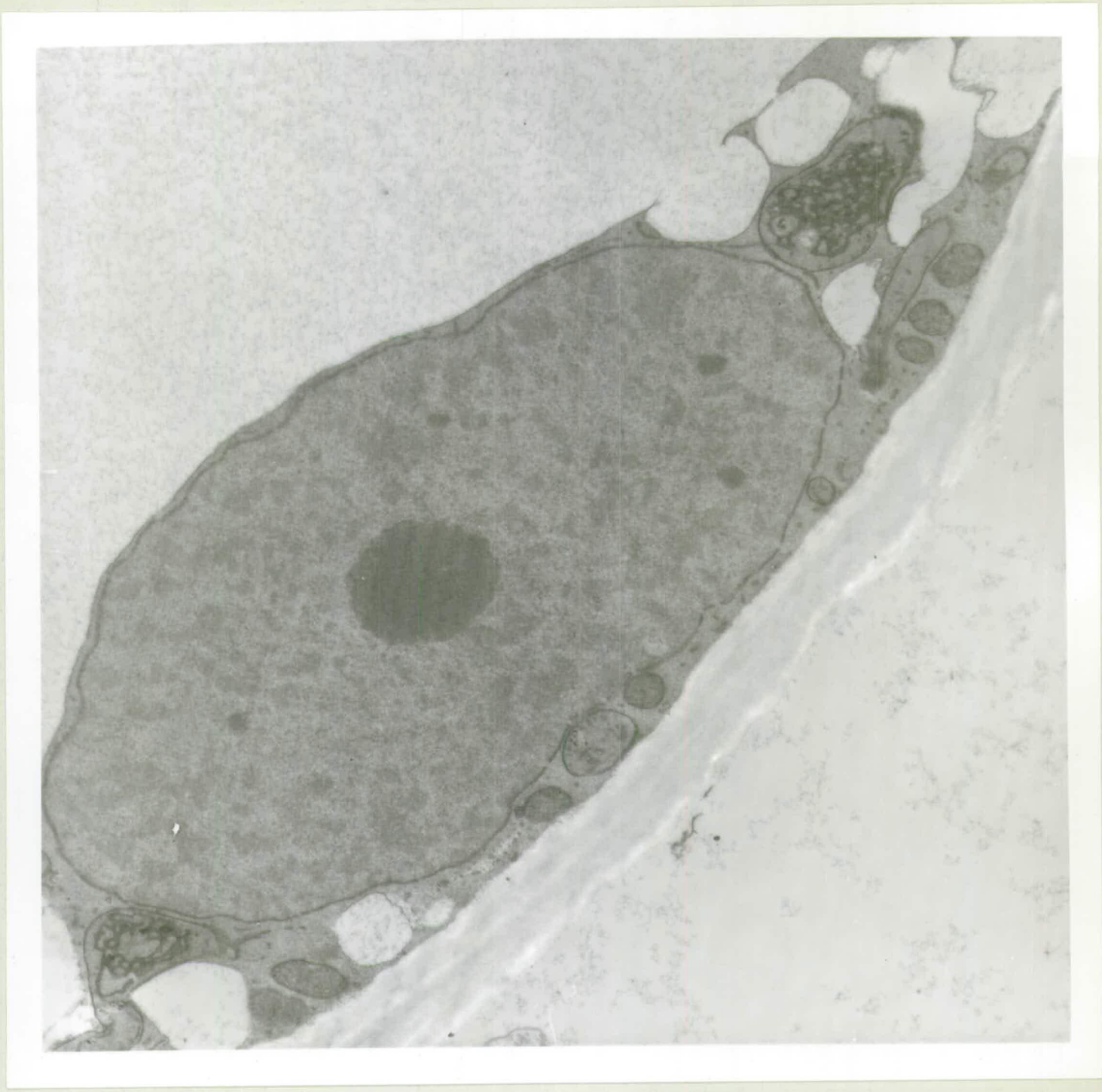


Figure 5.9. Electronmicrograph of fresh tissue. Material was fixed in gluteraldehyde and post-fixed with permanganate. Mag. = 15,000 diam.

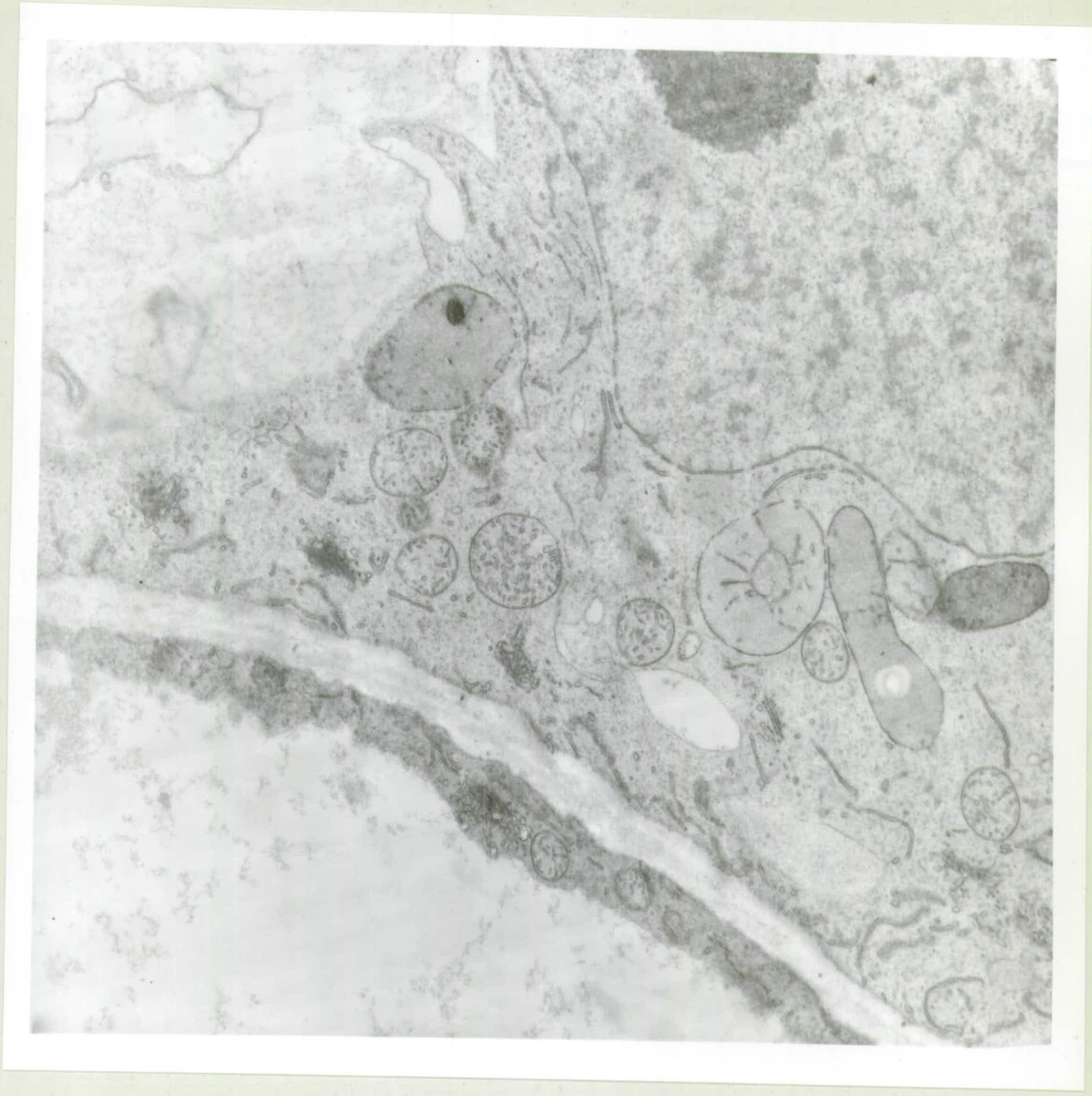


Figure 5.10. Electronmicrograph of 72 hour tissue. Material was fixed in gluteraldehyde and post-fixed with permanganate. Note the large number of Golgi bodies. Mag. = 15,000 diam.

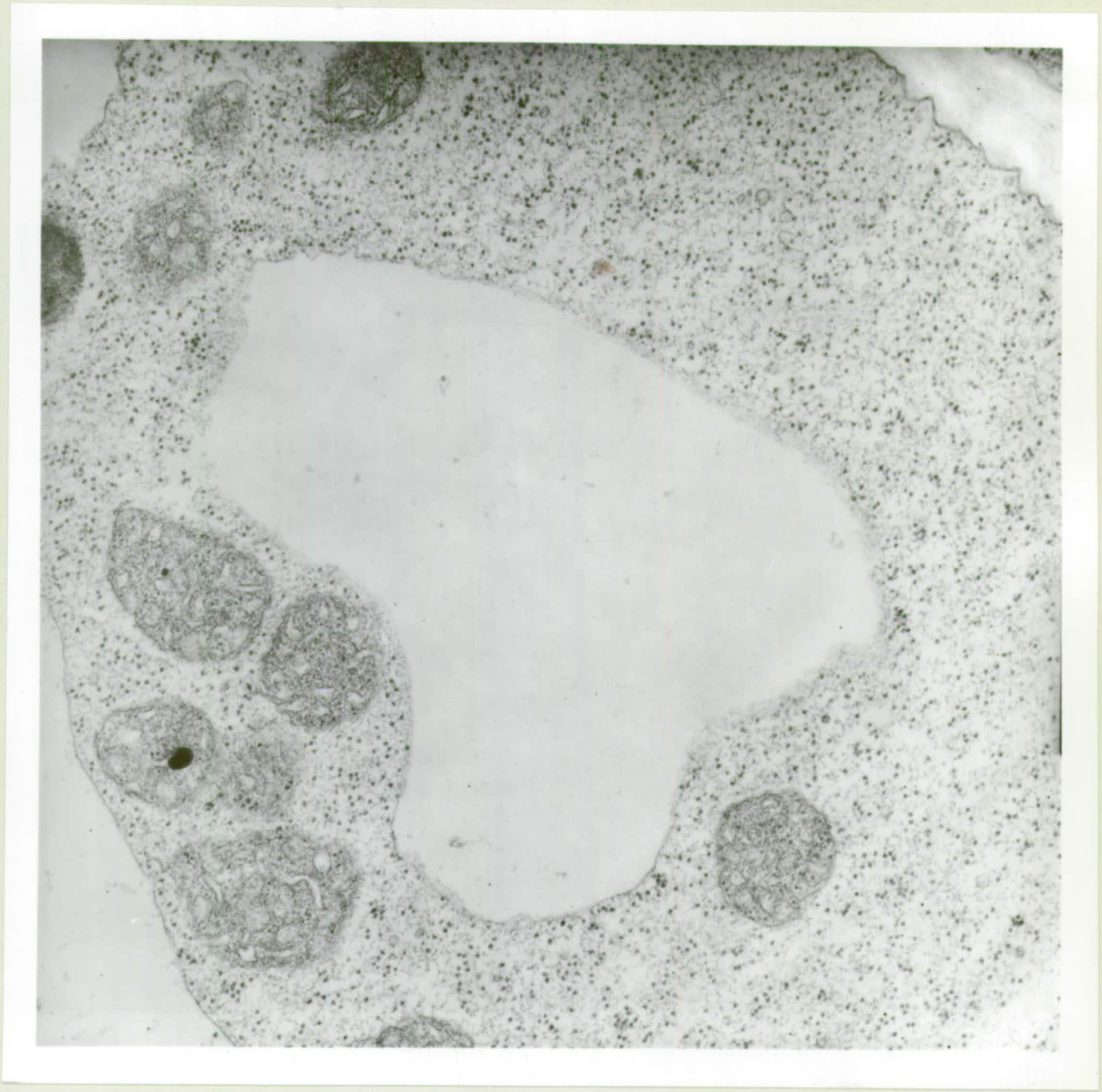


Figure 5.11. Electronmicrograph of fresh tissue. Material was post-fixed with osmium and stained with uranyl acetate and lead. Mag. = 42,000 diam.



Figure 5.12. Electronmicrograph of 72 hour tissue. Material was post-fixed with osmium and stained with uranyl acetate and lead. Mag. = 42,000 diam. Note the spirals of paired ribosomes.

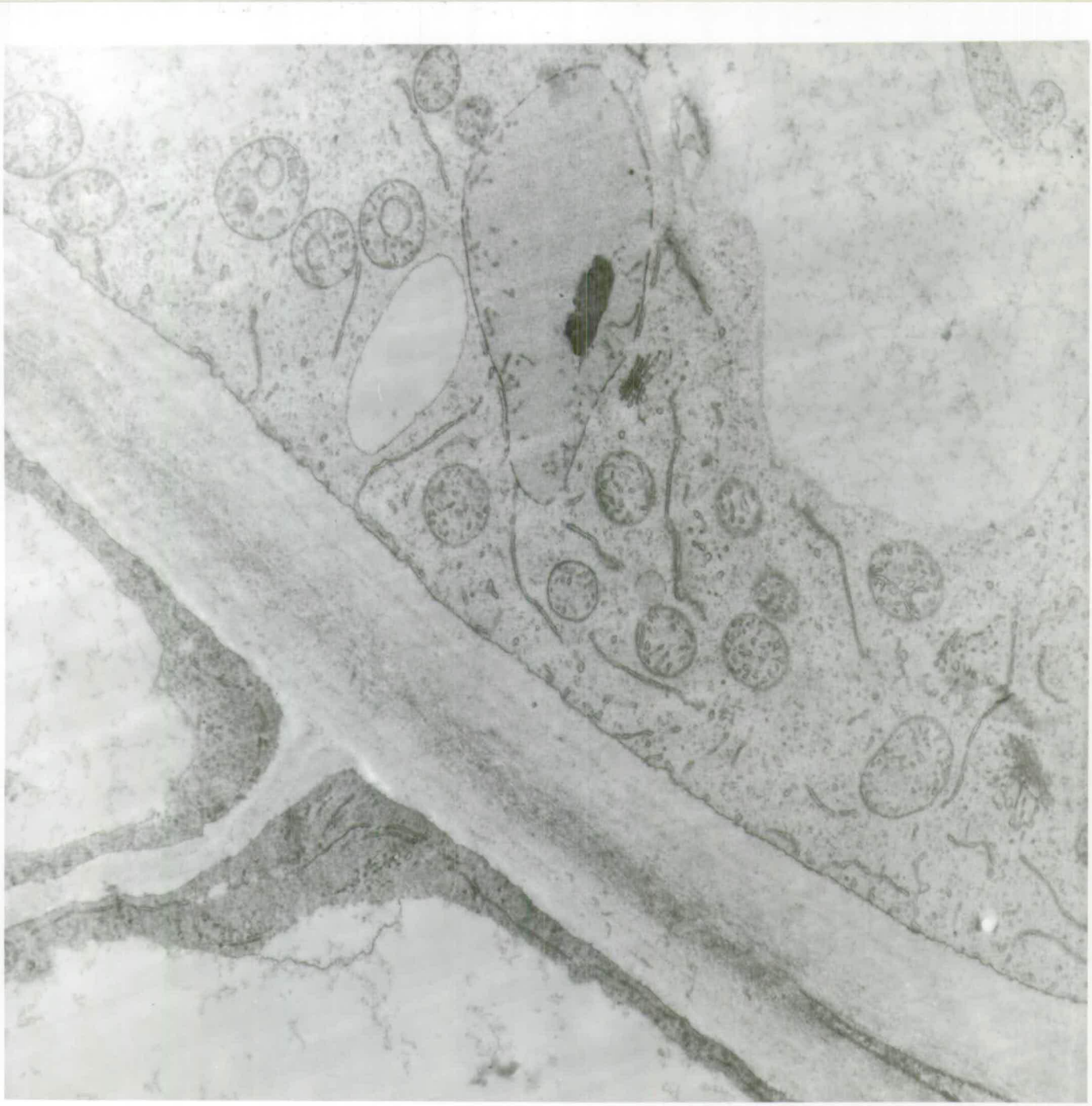


Figure 5.13. Electronmicrograph of 72 hour tissue. Material was fixed in glutaraldehyde and post-fixed with permanganate. Mag. = 13,000 diam. Note the thin cross-wall which has been formed since culture was initiated.

Fine Structure.

A preliminary study of the fine structure of fresh and cultured material brought to light several differences between quiescent and metabolically active tissues. Figures 5.9 - 10 show that after 72 hours of culture there appears to be an increase in the number of Golgi bodies. While no obvious changes can be seen in either the nucleus or in the mitochondria, there is perhaps an increase in endoplasmic reticulum. In the osmium-treated material, (Figures 5.11 - 12), spirals of ribosomes can be found in profusion in some cultured material but not in fresh material. Close examination of these ribosomes show that they are paired. Each pair appears to have a large and a small ~~number~~ me. From their configuration it seems unlikely that these strings of ribosomes lie on membranes. Large unidentified bodies with a variety of structures within can be seen in both fresh and cultured material. Figure 5.13 is presented to illustrate that the newly formed wall can be very thin compared with the original parenchymatous wall.

Conclusions.

1. The earliest divisions occurring during culture are not random but on the contrary proceed in a series of waves. The period of these waves tends to shorten with time.

2. The oxygen uptake of explants maintained on both Medium A and on water is stimulated almost immediately after excision. In water this stimulation is followed by a rapid fall after about 16 hours, whereas in Medium A the stimulation continues throughout culture. The division activity which is supported by Medium A but not by water alone, appears to depend, therefore, upon a sustained stimulation of gaseous exchange.

3. It has been shown that, prior to division, about half of the cells of each explant double their DNA content, during a 14 hour synthetic period, and subsequent to a presynthetic period of approximately 26 hours. The vast majority of those cells that do not divide, do not increase their DNA content during this time. Of the cells which do divide, the majority of divisions were complete within three hours.

4. An acid phosphatase which was not detectable in the supernatant fraction from fresh tissue, appeared in this fraction within 12 hours of the initiation of culture.

5. Results of preliminary studies with the electron microscope suggest that there is an increase in the number of Golgi bodies and polyribosome chains after culture has begun.

D I S C U S S I O N

Development may be defined as a system of complex units in functional harmony proceeding through a series of stages. At one level of organisation the units in question may be cells and at another we may consider the molecular components within the cell. It is a matter of choice how the developmental stages are defined. Until recently descriptions were based primarily upon morphological observations, but increasing attention is now being paid to the biochemical basis of the morphological expression and Brown (1963) has formulated a general scheme based on a consideration of the metabolic states which support each stage of development in the root. This, then, is a complex phenomenon and the integrated development of a callus may be usefully broken down into at least four basic processes. Thus, growth, differentiation, pattern formation and regressive change all interact in their contribution to the developmental changes which have been described in this study. A rigidly controlled "system" has been evolved using parenchyma tissue of artichoke tubers, and the foundation of the data described in Section II depend upon the reproducible and indeed predictable nature of the growth response of such tissue to the conditions selected. On the basis of these descriptions, the course of development of the typical callus may be divided into three stages.

These three developmental stages may be characterised by changes in mean cell size of the population and in the anatomy of the tissue. First, there is a lag phase in which the cell size remains constant. In general this phase varies in length from 20 to 40 hours, increasing with the age of the tuber (Table 2.7 p. 93). Then there is a phase characterised by decreasing cell size which is initiated by the occurrence of division in the sub-peripheral layers of the explant (Figure 3.19 p. 111). During this phase, regressive change, involving a progressive return to a meristematic or ground state in the outer region of the callus, results

in the formation of a particular pattern (Figure 3.19 p. 111). The distinctive feature of this pattern is that division has proceeded throughout the outer region of the callus leaving a core of undivided parenchyma cells within. After about 5 - 7 days, the initiation of a new pattern, that of nodule formation, begins to supersede the regressive changes, and a new course of development proceeds. This new course is marked by increasing cellular differentiation through both the maturation of tracheidal cells and the expansion of other cells. However, this increase in volume is balanced by the continued division and sub-division of parenchymatous cells which still continues throughout this phase. Between 5 and 10 days therefore, there is a transition into the third phase which is characterised by a more or less constant mean cell size. These three phases will be termed the lag phase, division, and differentiation phases respectively. While they are named for the predominant morphological feature of their development, these are not exclusive terms, because both division and differentiation proceed throughout the second and third phases. Growth of the tissue mass accompanies these changes occurring at different rates throughout both differentiation and division phases. The lag phase too, is only a lag phase with respect to mean cell size because, in reality, it is a phase of intense metabolic change.

Although the changes which accompany these three phases have been followed quantitatively for most of the common indexes of growth and despite the fact that they do seem to group into two mathematical forms - either linearity or a sigmoid curve - it must be emphasised that a formula "Which gives a mere coincidence of numbers may be of little use or none, unless it go some way to depict or explain the "modus operandi" of growth" (D'Arcy Thomson). Indeed an example of an apparently sigmoid curve which may well be the sum of two distinct processes has been described for the activity of acid phosphatase (p. 137). Nevertheless the use of biochemical and physiological units helps to define the course of development

more accurately and perhaps more objectively. The consistency of the changes recorded in these growth parameters suggest that the system used in this study may offer certain advantages for investigating the "modus operandi" of growth.

Development has been described as being "ultimately a question of the switching on and off of genes" (Lang, 1965). It may be summarised as follows:-

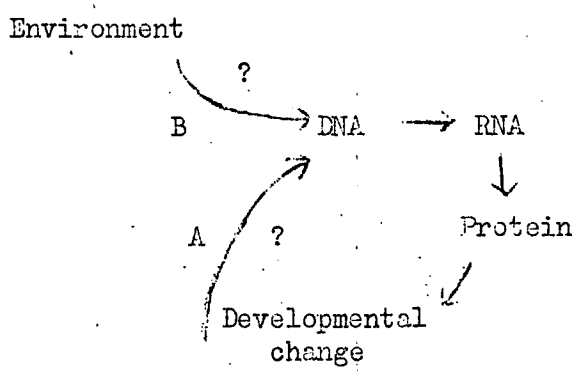


Figure 6.1. A naive molecular model of development. Processes A and B result in some genes being switched on and others being switched off.

In most plant systems DNA is fixed and constant, while RNA is continually changing under the indirect influence of a continually different and heterogeneous development and of a changing environment. The environment is known to be capable of "switching on" chromosomal puffs (Clever, 1963) and the products of development continually feed back to affect the activity of DNA directed development by such processes as end product repression and enzyme induction (Moyed and Umbarger, 1962). In artichoke callus, because of their uniform structure and pre-history all the cells involved in a single experiment are in as closely comparable a state of development as possible. Similarly the environmental conditions have been held constant (nutrients are thought to be present in excess, p. 63 , no evidence has

been found for the modification of growth by "staling" products, p. 63 , and the gaseous exchange of the medium appears to be limiting but constant). It is interesting to note, however, the profound alteration in development caused by the presence of a single xylem trace in an explant (Figure 3.19 p. 111). Here a cluster of orientated divisions has been organised in a region of the callus where no division would normally occur. The fact that the lignified vessels in such a xylem trace consist of dead cells dramatises the delicate balance between two potential courses of development. Nevertheless, since all the cells of the explant have the same genetic endowment and are more or less uniform in their phenotypic prehistory, and further, since the cultural conditions are constant, it is clear that the generation of patterns in a uniform tissue must depend upon the position of individual cells in relation to the explant as a whole. Thus the outcome of the interplay between constant extrinsic and constant intrinsic factors may depend entirely upon this position effect. Although this idea is not new (Vöchting, 1877) the present system is an attractive one for examining in detail such a dictum.

The scheme outlined in Figure 6.1 will be made manifest both at the molecular level and at the cellular level because differences in cellular development results in the genesis of morphologically distinct tissues. In this system, three morphological sequences are distinguishable at the tissue level.

Firstly there is the formation of a superficial ring of collapsed cells which by about the fifth day form an enclosing rind of lysed cells at the margin of the callus.

Secondly there is the formation of a region of divided cells immediately within the confining rind of collapsed cells. This sequence proceeds from the 2nd to about the 7th day and to an ever-lessening extent thereafter.

Finally there is the origination and development of nodules, which becomes dominant over the second phase after 7 - 10 days.

1. Rind Formation.

The excision of explants results in the formation of a surface of broken cells. Within these, a single layer of cells forming a closed cylinder may be distinguished by their high phosphatase activity (e.g. Figure 4.7 p. 131). This layer may be compared with the "überreizten Zellen" of Brieger (1924) and the "überschlagenen Zellen" of Olufsen (1903). The cells of this layer never divide but rather go through a process of controlled autolysis. Control is implied by the fact that when they have finally collapsed, the tonoplast and plasmalemma remain intact (MacKinnon,1966). By contrast, dead cells, which appear to have retained their contents, but have not collapsed, do not show high phosphatase activity. Such cells may be seen to be still loosely attached to the callus surface. By about the fifth day the collapsed cells may be several layers thick and it is not clear whether the original layer is augmented by cells from without or within. During this time a safranin positive substance is secreted which is perhaps equivalent to the "Wundgummi" of Molisch (1921).

The position of this rind and the remarkable spatial contiguity of the cells which exhibit high phosphatase activity suggest that this sequence may be a consequence of wounding. The result of the formation of this rind is to place a barrier between the intact tissue and the external environment. The function, if any, of this barrier is not known but it may be noted that there is some evidence (Homan, 1964) to suggest that an abscission layer can prevent IAA transport while permitting 24D transport, and so it may be surmised that the barrier described above may also be to some extent selective.

The formation of an enveloping rind of lysed cells may or may not influence the course of development. Two distinct influences are however possible. The rind may be a selective barrier to the uptake of some constituents of the external milieu. If this is so, then the physiological situation would be

expected to alter when the growing callus bursts through this limiting layer of necrotic tissue. When the nodules emerge from the dividing region and push through the original surface of the callus they do tend to proliferate by expansion of the superficial cells in a form of development that has not previously been expressed by the callus tissue. These cells thus exposed to the external medium, have only recently been laid down and they are therefore quite able to accommodate expansion growth. In contrast the original cells of the explant which were initially exposed to the medium possessed long established walls and such cells, under normal circumstances in plants, are unable to accommodate much expansion (Lamport, 1964; Ball and Joshi, 1965).

The second influence that may be operating concerns the transport of metabolites. McGregor and Street (1953) have suggested that high phosphatase activity may be connected with the transport of sugar. Intense hydrolytic activity must result in the breakdown of at least some of the constituents of what were otherwise healthy cells prior to excision. Certainly the contents must either diffuse out into the medium or be imported into the sub-peripheral layers of the explant. A degenerately autolytic process might be expected simply to release the degraded products of autolysis, to diffuse where they will. This would result in export of these compounds into the medium. Controlled autolysis, however, in which the limiting membranes of the cytoplasm remain entire, could conceivably have as one of its functions the supply of metabolites preferentially to the adjacent tissues. Nucleotides, amino acids and sugar units may well all be involved in the solute transport suggested by McGregor and Street (1953). During 24 hours of culture there is a large increase in the absorption of the medium especially at 260 mμ but also between 200 and 400 (Fraser, 1966).

Finally, it is of interest that the acid phosphatase activity is, to some extent, "latent". That is, the activity recorded increased by about 20 per cent when explants were submitted to the standard treatments for the detection of latency

(De Duve, 1963). It seems possible that, in a storage tissue whose normal remaining function is the supply of metabolites to the developing bud, that hydrolytic enzymes may be present in some inactive or segregated form, perhaps in particular structures analagous to lysozomes in animal tissues. Investigations of this possibility are in progress (MacKinnon, 1966).

2. The Regenerative Pattern of Divisions.

What has been defined earlier (p. 116) as the division phase is the morphological expression of this regeneration sequence. It concerns the conversion of a tissue of large mature parenchyma cells into a tissue of small meristematic cells. The tenfold reduction in size effects a transition from the parenchymatous state to the meristematic condition through repeated mitotic divisions. This second sequence of activity is at first more intense in the regions where the callus cells have their long axis tangential to the callus surface (e.g. Figure 3.19 p. 111) but by the 7th Day (Figure 3.21) most calli have a complete sub-peripheral ring of meristematic tissue enclosing a central region whose cells have not divided, (Figure 3.22 p. 112). Incidentally the regions of low initial activity appear to be subjacent to the regions of initially incomplete rind formation.

The genesis of this pattern of regeneration has three components.

(a) The induction of division in the sub-peripheral region of the callus. This occurs during the lag phase.

(b) The maintenance of mitotic activity in the divided daughter cells and the increasing involvement of adjoining cells in mitotic activity. This occurs during the division phase.

(c) Thirdly, there is the departure from this contagious mitotic activity. This occurs by the superimposition of the differentiation phase, and involves, initially, a sudden novel orientation of localised divisions.

These three components of the regenerative sequence correspond to the three phases of development. They are concerned with the process of division which now emerges as a dominant feature contributing to the development occurring in this system. One of the characteristics of plant cells is the rigidity of mature cell walls and it appears to be the case that expansion does not usually proceed in mature cells without the intervention of division (Ball and Joshi, 1965). Such division produces cells which have an extensible wall capable of supporting expansion. Conversely, of course, development does not normally proceed unless division is accompanied by cell enlargement (Torrey, 1957). Therefore, division is, here, a pre-requisite of development if only to enable the developing cells break out of the constraining framework of the mature cell walls. The newly formed walls are laid down under the original parenchymal walls (Figure 5.13 p. 150 ; Priestley and Scott, 1939). During enlargement in liquid culture new walls stretch but the old ones do not and these, therefore, tend to be sloughed off (Lamport, 1964; Ball and Joshi, 1965). Although division is not a part of development the consequence of division and of the orientation of division is vital to the course of development. For this reason an enquiry into the process of division in this system of culture is mandatory in order to understand the ensuing development.

(a) The Induction Phase.

Between Day 1 and Day 2, 30 - 50 per cent of the cells of the explant have proceeded through mitosis. This phase may therefore be called the induction phase as far as division is concerned since the phase is at an end when an appreciable number of daughter cells have been formed. One of the essential features of cell division is that the deoxyribonucleic acid is doubled in quantity prior to the separation of chromosomes during mitosis. It has been shown that the cells which undergo division have completed this doubling and that all the cells which do achieve the 4C condition may be expected to divide. There is no evidence for such cells remaining in a

polyploid condition. No abnormalities of division have been observed at this stage. It would appear therefore that during the induction phase, a high proportion of the parenchyma cells are stimulated to carry out all the processes necessary to support normal mitotic division. This constitutes a dramatic change in metabolic activity transforming quiescent cells, ("with their metabolic fires damped down") where energy transformation and utilisation is at a minimum, into active cells which are using energy for diverse synthetic and organisational processes at a greatly increased rate.

During this stimulation there may be some slight increase in fresh weight, perhaps an increase in dry weight and a threefold increase in the uptake of oxygen. In the first 30 hours of culture, the coefficient for oxygen absorption rises from 0.9 µl per hour per explant to 2.4 µl per hour per explant and the coefficient for carbon dioxide release rises from 0.7 µl per hour per explant to 2.6 µl per hour per explant. Thus the respiratory quotient changes from 0.8 to about 1.1 during this time suggesting a particularly intense stimulation of decarboxylation reactions. At the same time the total nitrogen generally increases although on several occasions it dramatically decreased (Figure 3.4 p.108). This would suggest that induction of division may be accompanied by an increase in total protein but in no way depends upon such an increase. For similar reasons it is not dependent upon an increase in dry weight (Appendix B, p.190).

When explants are cultured in water instead of Medium A (Figure 5.3 p.140) there is no induction of division. Induction is dependent therefore upon substances in the medium. Although division may sometimes proceed on the fully defined Medium A which lacks coconut milk, at certain times of the year it cannot do so. Clearly induction depends upon the presence of both an auxin (e.g. 24D) and also upon substances which are present in coconut milk but which may also be present in the artichoke parenchyma at certain stages of tuber development. The

presence of coconut milk in the medium invariably induces division and must therefore contain both these factors. Steward and his co-workers have carried out an exhaustive examination of the substances found in coconut milk without finding any magic division factors (e.g. Shantz and Steward, 1952; Steward and Shantz, 1959; Steward, 1961) and they have concluded that a variety of substances contribute at different stages, in different ways, and to different extents to the overall stimulation of division in carrot tissue. Zwar and Yeoman (1966) have shown that kinetin, in the presence of casein hydrolysate, stimulates greater division than that found using Medium A. A variety of substances therefore can replace coconut milk at certain times of the year, but coconut milk is invaluable because it induces a predictable response at all times of the year.

When the contrast between using Medium A and using water, is examined more closely by comparing their oxygen uptake (Figure 5.4 p. 141) it was shown that for the first 18 hours the stimulation of uptake was comparable but that after this time the value for explants cultured in water began to decrease. Low values had been recorded at 48 hours and at subsequent harvests up to 14 days in an earlier experiment (Figure 5.3 p. 140). On this occasion (Figure 5.4 p. 141) division occurred at about 36 hours in about one third of the cells cultured on Medium A. It seems probable therefore that division activity is dependent upon a sustained intensification of respiratory processes.

Thus the storage parenchyma cells, which would not normally divide again, are induced by culture to do so. The physical process of division requires a certain amount of energy, although Amoore (1963) has calculated that this is, in pea root meristems, a billion times less than the level of energy available. However, a much greater amount of energy must be required to support all the essential synthetic processes upon which division depends. Undoubtedly, in addition to a doubling of DNA content, many other essential constituents of the cell must be

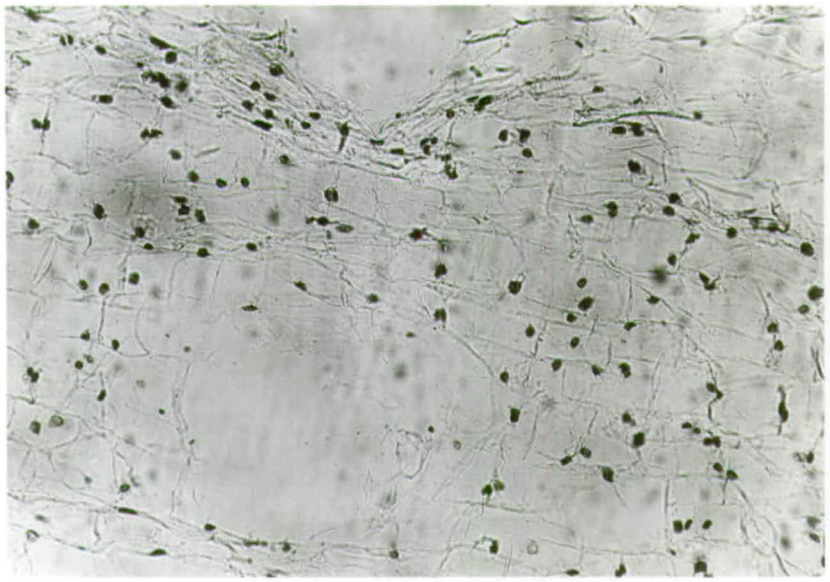


Figure 6.4. Transverse section of a 40 hour callus stained with Feulgen to show the orientation of the crosswalls.

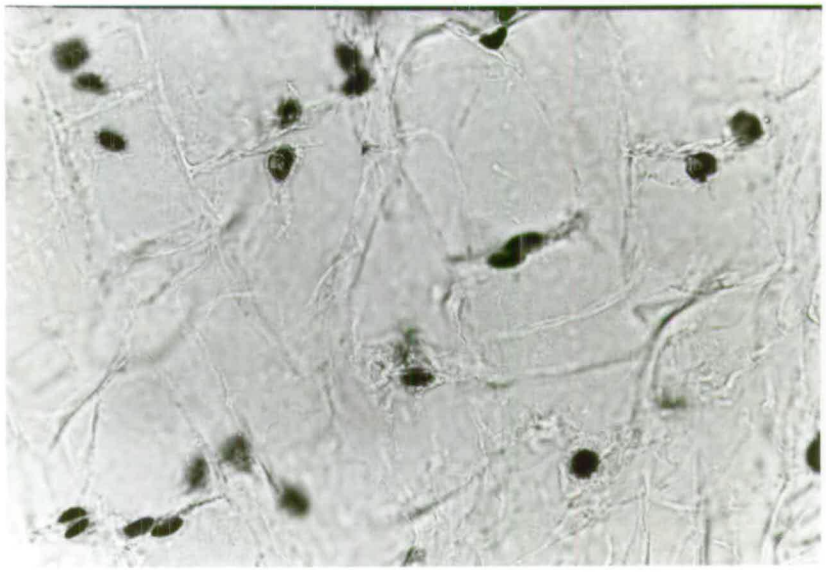


Figure 6.5. A selected field from a 40 hour callus section, which has been stained with Feulgen to show the orientation of new walls. Seven "telophase" figures whose new walls are transverse to the long axis of the parent cell can be seen.

manufactured. Primary wall must be synthesised and laid down, new membrane and mitochondrial material must be synthesised and assembled. Certainly, during fractionation of homogenates, detectable pellets of the mitochondrial fraction, of fraction x, and of the ribosomal fraction each appeared after about 20 hours of culture. Presumably a large increase in the whole gamut of cell organelles must have been effected. Such syntheses would require considerable oxidative activity to provide the energy to drive the production and activation of precursors, the polymerisation of macromolecules, and the assembly of organelles. Thus while the induction phase may be accompanied by increases in total protein and in dry weight, these increases are not a "sine qua non" for division. Yet, in contrast, a sustained increase in respiration would appear to be just as important as a doubling of DNA.

The directing effect of cell division upon development seems to depend largely upon its orientation. An examination of the planes of division in a 40 hour callus whose predominent mitotic figure was that of one or another stage of telophase was illuminating. All the transvacuolar cytoplasmic baffle of the new cell walls (first described by Brieger, 1924) were arranged across the short axis of the cells and cut each cell approximately in half, in accordance with the laws of both Sachs (1887) and Errera (1888). The few exceptions were associated with a xylem trace. This suggests that the orientation of the first division bears no relation to the morphology of the explant although of course the position of the dividing cells is clearly confined to a subperipheral region. Judging from the position of new cell walls, subsequent divisions are much less homogeneous in their orientation, although a pattern of phelloderm-like tissue does emerge after a number of successive divisions. Figures 6.4-5 show the first new walls.

The position of the initial divisions is a puzzling feature of the early development of the callus. It would appear to be a result of the proximity of

either the medium or of the autolysing cells. Classically the response to wounding is the formation of a limiting layer by "gumosis" followed by division to form a cambium in the adjacent cells (e.g. Brieger, 1924). It is clear that without certain undefined substances contained in the medium no division will take place, but it may also be significant that the areas of reduced cell division are subjacent to the areas of incomplete rind formation. The evidence for the complicity of these two factors is suggestive. Brieger, Reihe and Haberlandt all agree that the amount of division is proportional to the amount of "wound stimulating substances" (Brieger, 1924). In tumours formed by incubation of the schizomycete Agrobacterium Tumifaciens onto various hosts by needle puncture, the size of the tumour is related to the size of the wound. Extracts of wound tissue are said to contain "tumour inducing principle" which have been shown to contribute to the induction of tumour divisions. It seems possible that the peripheral lytic activity in the cells comprising the rind may make available a supply of transportable substances whose destination could have a bearing on the location of adjacent division activity. From evidence based on fine structure histochemistry, certain phosphatases (though not the ones measured in this study) have been implicated in transport activity and it may be that both hydrolytic breakdown and a metabolically dependent transport are induced here.

There is one natural feature which modifies the induction of division and that is the age of the tuber. In general, the longer the tuber has been stored the longer the induction period (Table 2.7, p. 93). This has now been adequately confirmed (Evans, 1966). Whether this is due to a rundown of metabolic potential, or the gradual diminution of stored reserve metabolites, or to some regulatory control associated with dormancy or vernalisation, has not yet been investigated. Certainly there is a qualitative change in the potential for induction of division: explants from young and especially old tubers often form

binucleate cells initially, although usually cytokinesis catches up with karyokinesis at a later stage of development. Zwar (1966) has shown that this ability to form cell wall is relieved by the addition of kinetin, in the presence of casein hydrolysate, and these results are in agreement with those of Das Patau and Skoog (1957) who used tobacco pith cultures. This might suggest that continued culture induces the synthesis of a kinetin like substance. Those divisions occurring in explants from "middle aged" tubers are further distinguished by their peculiar form which is reminiscent of embryogenesis. Such scattered observations remain to be investigated more thoroughly but suggest that the more critically the induction of division is examined the more subtle differences in development due to slight changes in the environment are exposed. It is one of the advantages of this system that such small differences produce consistent changes and are therefore examinable.

Thus it is clear that the induction of division in parenchyma explants depends upon a number of factors. There is firstly, a chemical requirement which coconut milk can supply. At certain stages of tuber development this requirement can be replaced by 24D in the presence of salts and of sucrose. Washing of explants prior to culture tends to remove (or in some way render ineffectual) those substances in the explant which are necessary for the induction of division by 24D (Zwar, 1966; Fraser, 1966).

Secondly, excision and culture of the explant has certain consequences. The explants are transferred to a new temperature where metabolism is no longer severely restricted. They are introduced into a new regime where the facility for gaseous exchange is enhanced and without this it is unlikely that the necessary oxidative energy for synthesis could be liberated.

Finally, the consequence of exposing a block of tissue which has broken cells covering the whole exterior to a new chemical and physical environment must be many and diverse. The formation of a subperipheral zone of high phosphatase

activity is one consequence and the changes resulting from this activity may well have a bearing upon the induction of division. Possible selective transport of diffusable substances and translocation of specific metabolites from the cells to the division region cannot be ruled out.

Undoubtedly the combination of these and other influences result in the "switching on" of a course of cellular development that contributes to, and culminates in, a large proportion of the cell population suffering the common fate of mitotic division. Various studies concerning this induction phase are now in progress (Evans, 1964; MacKinnon, 1965; Fraser, 1965).

Maintenance of Division.

Between Day 1 and Day 7 the cell number of a typical explant increases from about 20,000 to about 250,000. By this time the early stages of nodule formation may be observed but as yet this involves only a few cells. In the interim, despite a doubling of fresh weight, cell division has reduced the mean cell size by more than five fold. If the biological equivalent of Newton's first law of Motion hold true, then presumably all that is required to maintain active division is that conditions remain the same. Despite extrinsic factors being constant, such a requirement can never of course be satisfied in this system, because an inevitable ancillary of growth is that novel intrinsic relationships of shape and of size are continually being created. It is, however, of interest that Skoog and Tsui (1948) (amongst others, e.g. Gautheret, 1939; Nobecourt, 1939; White, 1939) find that callus development can be maintained indefinitely in an undifferentiated but proliferating state by subculturing on a medium with a given concentration of growth substances. Manipulation of the hormone balance can induce new courses of development e.g. root and shoot initiation (Skoog and Miller, 1957), or even regeneration of plantlets (Steward, Mapes and Mears, 1958). The orientation of the plane of division in the first wave of mitoses has been described as obeying

the laws of Sachs (1887) and of Errerra (1888) and its discussion properly belongs to the induction phase. The subsequent planes of division are much less regular although they are by no means random. While many four cell stages are seen in cell macerates which have also bisected the parent cell equally and with the minimum area of cross wall, many others are less regular in their division. Consequently, by the fifth or seventh day a layer of divided cells resembling a periderm can frequently be seen especially in a longitudinal section of the callus.

Little is known about the requirements of this maintenance period but it appears that all the parameters measured (including dry weight and total nitrogen) tend to increase rapidly as the cell number rises. This is presumably a reflection of the continued synthesis required to support continued growth.

It has already been mentioned that this periderm-like region usually forms preferentially opposite the regions of lysed cells. It would be of some interest to investigate the cause of this coincidence and perhaps throw some light on the development of phellogen.

The question has also been posed: are meristems to be considered as organiser regions whose functional changes represent changes initiated within the meristem itself, or are they simply plastic regions in which new cells are moulded into organs and tissues in response to stimuli proceeding from other sources (Sussex, 1963). It seems possible that the place of departure from this periderm course of development might be amenable to manipulation by both physical and chemical means. It is known that both temperature (Naik, 1965) and oxygen tension (p. 78) alter the rate of tracheid formation which is the principle index of departure from the regenerative phase. It may be possible to examine the extent of the self-regulatory homeostasis of such a meristematic condition by the judicious administration of various metabolic inhibitors.

As usual, a hotbed for speculation arises where least work has been done.

Departure from Division.

This aspect of development marks the end of the division phase. There is no evidence to suggest that the mitotic activity ceases, although it does appear to do so in agar cultures (Yeoman, Dyer and Robertson, 1965) but rather it may be the case that a new morphogenetic sequence becomes dominant. Nodules arise in the regenerating tissue between the 5th and the 7th day and although regenerative division activity may continue, the orientated divisions associated with nodule formation increasingly predominate in the further development of the typical callus. The orientation of the division spindles which is the first outward sign of this organisation is a problem which cannot yet be examined directly although it is perhaps significant that a similar orientation is seen around the isolated xylem elements (e.g. Figure 3.19 p. 111) which may occasionally be found within a callus. This effect, remarked upon earlier, (p.155) may be due either to the conduction of nutrients, or of oxygen, from the medium through the open tubes of these tracheary elements to the parenchyma cells concerned, or to some chemical stimulation provided by the xylem elements. The former explanation seems unlikely when it is recalled that no divisions have ever been seen surrounding the hole created by the glass spike of the mount. The chemical stimulation from the xylem may originate from these cells or may be a legacy of material which the vessels contained before excision. These two possibilities cannot at present be resolved but both are compatible with data of Jablonski and Skoog (1954) and Wetmore and Sorokin (1955) who have shown that the presence of xylem tends to induce division in tissue cultures.

Differentiation Phase.

The onset of this phase is marked by the initiation of nodules. From this beginning, a new pattern is increasingly superimposed upon the actively dividing region. Eventually the periderm-like tissue is obliterated by the

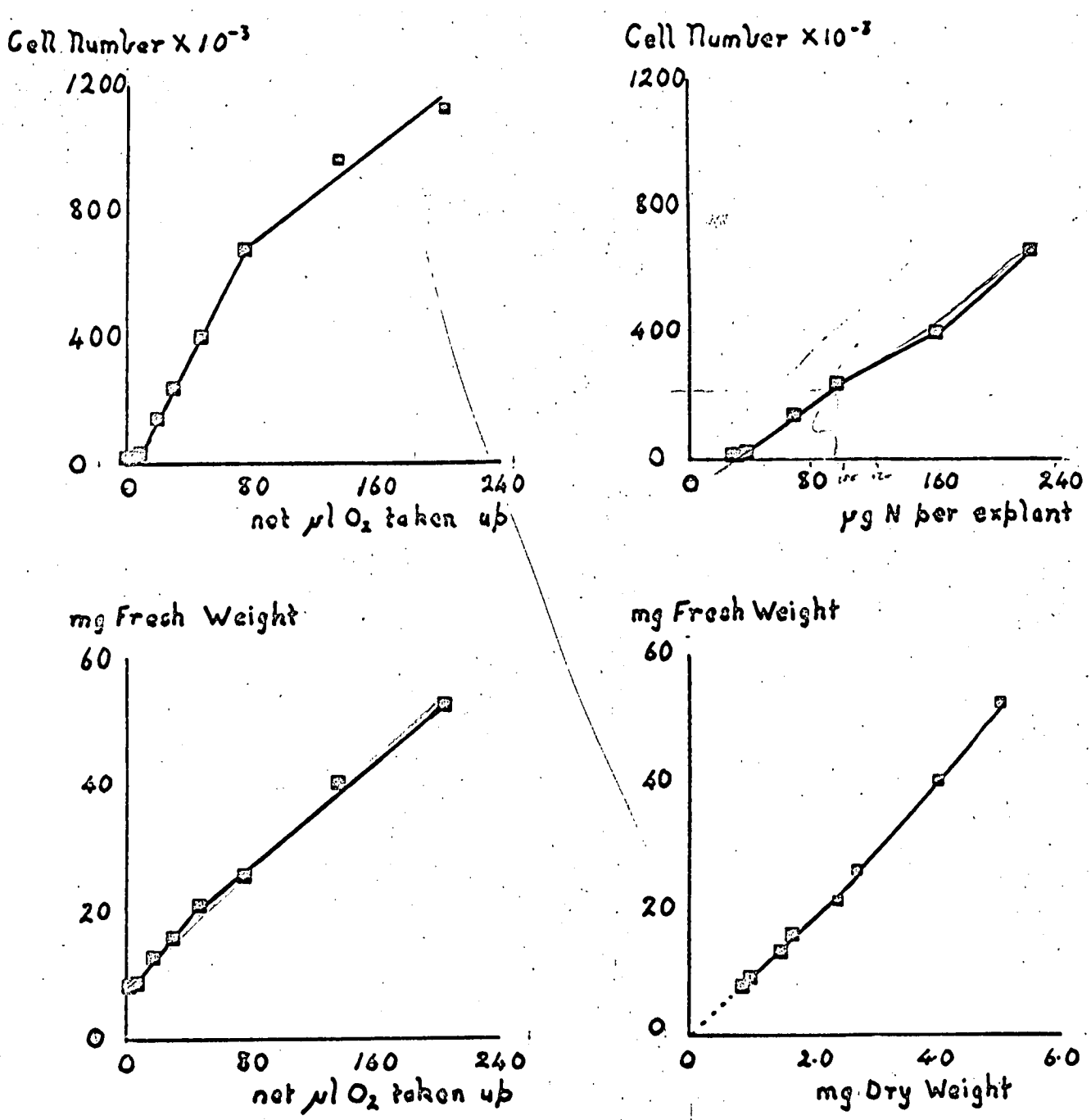


Figure 6.2. Various parameters of growth plotted as ratios of one anoth bring out any consistent trends that may be present.

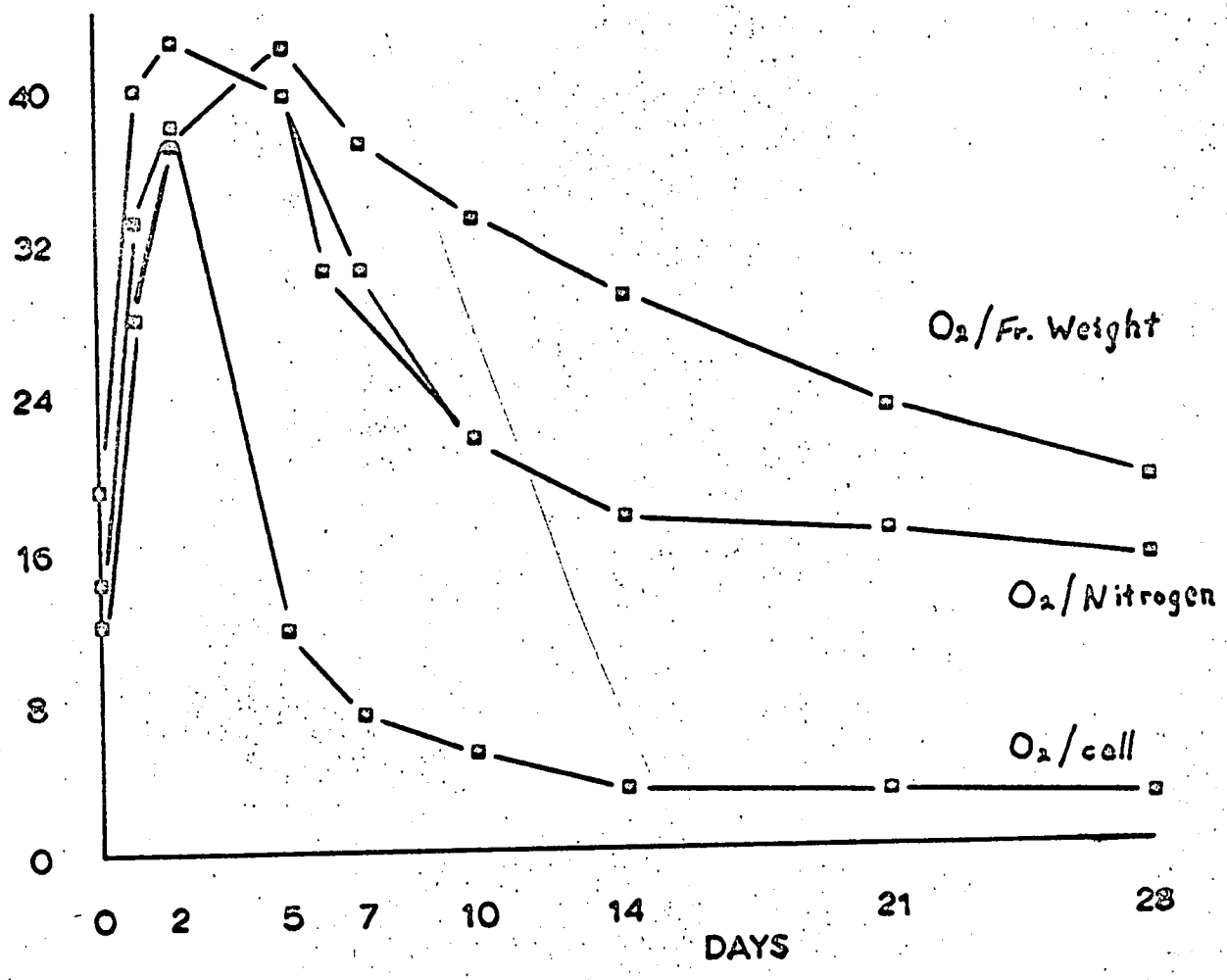


Figure 6.3. A plot of the QO_2 on a fresh weight, total nitrogen and per-cell basis. The data is derived from the mean values of Series 3.

increase in size of the large numbers of nodules found on each callus. The continuing pattern of nodule formation which flows from this original orientation of division involves the formation of large numbers of scalariform tracheids (Esau, 1953). The sequence comprises expansion in some cells and continued division in others, and finally it may involve some kind of homeostatic relation which maintains the mean cell size at approximately the same level. This relationship may of course be entirely fortuitous, although it occurs with remarkable regularity.

During the differentiation phase fresh and dry weights continue to show a linear increase. Values for total nitrogen and alcohol insoluble nitrogen also rise. Much of the secondary data shows that there is a distinct change in relation as development shifts from predominantly division activity to predominantly differentiation activity. When one parameter is plotted against another there is a distinct change in the relative balance of several pairs of growth marker, although there remains a more or less linear relationship (Figure 6.2)

When QO_2 (F. Wt.) or $QO_{2(N)}$ or QO_2 (cell) are plotted against time, (Figure 6.3 p. 168) it can be seen that there is a dramatic change in the relationship at this same time of transition suggesting some sort of interdependence of these factors, after a particular cell size has been reached. As the differentiation phase continues, these secondary markers of growth changes tend to remain more or less constant. This would suggest that, in a multicellular tissue, once a particular average cell size has been reached regenerative change is superceded by a controlled phase of activity involving expansion, division and differentiation. This control can only be attributed to some built-in, intrinsic regulatory mechanism, which is an inherent property of any given mass of coherent cells.

During this phase, the histochemical evidence suggests that the activity of phosphatase measured is not in any direct way associated with either dividing

cells or with expanding cells but solely with the differentiating tracheids. There may also be some remaining activity associated with autolytic cells on the callus surface. The appearance of tracheids in any number are perhaps the most practical indication of differentiation in this system. The total number of tracheids present relates to the total amount of differentiation which has been achieved while the amount of phosphatase positive tracheids would be an index of the amount of differentiation actually proceeding at a given time. It is curious that only the acid phosphatase is concerned in tracheid autolysis, and this fact is again suggestive of a controlled process.

The activity of ribonuclease is entirely different. It remains at a minimal level until the onset of differentiation and then increases markedly. Whether this is a cause or an effect is of course unknown. Several reports have however linked ribonuclease activity with differentiation and it would be easy to postulate causal connections. However, at present little can be added to the coincidence except that a connection has been suggested between RNA and expansion. Giles and Myers (1966) find that these two entities proceed in parallel under several circumstances in pea internodes and also in Lupinus hypocotyls (1964). The scanty data for RNA does parallel the fresh weight increase in this system except that there is no lag phase. It has already been suggested that the old rigid walls militate against any immediate expansion while division liberates the protoplasm from the constraint of old walls. Nooden and Thimann (1965) have shown too that expansion depends upon protein synthesis in artichoke tissue and such synthesis may well depend upon ribosome synthesis in the originally quiescent tissue. New protein is doubtless required for both division activity and for differentiation. The bulk of RNA is of course ribosomal and it has been suggested (Waddington, 1966) that the nucleolus consists largely of ribosomal RNA in transit towards the cytoplasm. Certainly, in this system it has been observed (Naik, 1965) that an early sign of

metabolic activity is the appearance of large swollen nucleoli. Sectioned material would suggest that at later stages the nucleoli are less active. It is possible, therefore, that one link in the chain suggested by A of Figure 6.1, is the destruction of "division" ribosomes and the construction of "differentiation" ribosomes. A large increase in ribonuclease activity may well be expected to be associated with such changes.

Limiting Factors.

In most growing systems, under favourable conditions the growth rate is slow at first, then gains speed and eventually slows down and comes to a halt. The S-shaped curve of growth thus obtained represents the integrated sum of the curves for each growing organ and cell. Environmental conditions may alter growth rates, but do not usually alter the sigmoid form of the growth curve. However, under certain circumstances a linear growth plot may be found. Firstly, there may be a restricted amount of an essential constituent (e.g. a given enzyme or hormone) which is present in the initial material and provided this constituent cannot be synthesised under the conditions prevailing, growth will be linear (e.g. Monod, 1949). Secondly, if there is a constant, but limited, supply of an essential nutrient, the growth may also be linear. In a heterogeneous growth system it is of course possible that the rise and fall of different processes may by chance result in a linear growth plot for a particular index of growth.

The indexes of fresh weight and of dry weight (Figures 3.1 and 3.2) both show a more or less constant growth rate. The most obvious explanation is that there is a limited supply of one or another nutrient. It has been shown that all the nutrients except oxygen are present in excess (p. 63). It has also been shown that improved conditions for gaseous exchange yield an increased fresh weight after 14 days. It seems therefore that one limiting factor in this system of

culture is the limitation of gaseous exchange. It is of particular interest in this condition that increasing the area of interface between the atmosphere and the medium results in an increased growth response. Thus the linear relation might be attributed to a constant limited rate of solution of oxygen in the medium (or of dissolution of carbon dioxide from the medium). It was also shown (p. 78) that manipulation of the facility for gaseous exchange modifies not only the amount of growth but also the development of the tissue. Thus, the better the conditions, the fewer the tracheids formed. If tracheids are an index of the intensity of differentiation it would appear that amelioration of gaseous exchange accentuates the division phase and reduces or delays the differentiation phase. All the data discussed above is also compatible with the idea (Laties, 1962) that an endogenous volatile inhibitor is the controlling gas.

From the foregoing discussion it is clear that, because the tissue is uniform and the growth response to culture is reproducible the development of a mass of originally uniform cells can be examined. The fact that these constituent cells form an interdependent co-operating unit precludes totally unorganised growth of the kind observed when viable cells are sloughed off from the surface of an artichoke callus and permitted to grow. Similarly, smaller explants than those used here tend to produce friable undifferentiated tissue. Thus it would seem that the accident of cells being incorporated into a tissue mass imposes some coherence of development amongst the constituent cells. Such coherent development permits tissue specialisation and the establishment of morphogenetic sequences.

However, development can be examined at another level. A rather radical view of this process described it as "Ultimately a question of the switching on and switching off of genes". Accepting the current dogma concerning the repression and derepression of nucleic acid templates, it might be possible to examine that aspect of development which constitutes the cell cycle. For this purpose it would

be necessary to find a population of developing cells which were all passing through identical stages at the same time. Although such a situation has not yet been found, nevertheless the periodicity of the early mitotic divisions which have been described (p. 139) have made it possible to examine the developmental stages concerned in the induction of the first wave of division and does not exclude the possibility of examining it also in the second wave (Yeoman, Evans and Naik, 1966).

Brown (1963), in examining the development associated with extension growth in pea roots, has formulated a scheme which postulates a progression of changing metabolic states. These states are primarily related to the catalytic protein and the transition from one state to the next is brought about by particular enzyme complements, which arise out of the previous metabolic state. These changes are thought also to involve a changing ribonucleic acid complement.

Howard and Pelc (1953) have defined the cell cycle in terms of the synthesis of deoxyribonucleic acid. These authors divide the cell cycle into a presynthesis period ("G_1"), a period of DNA synthesis ("S"), a post synthetic period ("G_2"), and the period of visible mitotic movement ("D"). In meristematic cells, each cytoplasmic and nuclear component of the cell must undoubtedly go through a phase of increase at some stage during the cell cycle. Thus there are presumably, "G_1", "S", "G_2" and perhaps even "D" stages for, say, mitochondria, plastids, golgi apparatus, endoplasmic reticulum and also polysomes. To distinguish them from the classical symbols based upon DNA synthesis it may be helpful to designate these with an appropriate suffix, e.g. $G_{1\ mit}$, S_{golgi} or $G_{2\ plastid}$. Of course the phases of synthesis of these organelles need not be related to the mitotic cell cycle although e.g. polysomes have been reported as being so related (Johnson and Holland, 1966). It is unlikely that the various "S" periods would coincide and so the shifting metabolic balance during the cell cycle could well be expressed in terms of Brown's (1963) formulation of the metabolic states supporting

extension growth.

It is for this reason that certain biochemical markers of change have been followed during the period up to and including the first wave of divisions in cultured explants or artichoke parenchyma. It is necessary first to define a timecourse of changes in recognised indexes of metabolic development such as DNA ~~control~~ content, protein content, and selected indicative enzyme activities, prior to an attempt to elucidate the mechanism of switching from one state to the next.

It seems likely that in this unusual case of the cell cycle that certain changes precede the synthetic mechanisms that constitute the interphase development of the cell. These changes are changes in both the regulatory control of development and in the support to development furnished by the oxidative, energy-supplying catabolic processes of each cell. Such changes, involved in the transition from a quiescent to more active metabolism, may or may not overlap with the changes involved in cell multiplication.

At present the preliminary data in Section II C (p. 138) do no more than define some changes that occur during the cell cycle. However, the changes illustrated by the electronmicrographs (Figures 6.9 - 6.13 p. 150) demonstrate alterations at the structural level which are doubtless related to the radical alteration in metabolic activity (e.g. in oxygen uptake and in phosphatase activity) and therefore provide scope for a variety of investigations using this system.

SUMMARY

1. Explants isolated from tubers of the Jerusalem artichoke, Helianthus tuberosus, grow vigorously when placed in contact with an agar medium.

2. Using this material, a variety of media, of supplements, of volumes of medium and of sizes and shapes of both glass mounts and of explants were all examined using a conventional tissue culture apparatus and the best available conditions were chosen for a culture period of 14 days. Thus a Standard Procedure was evolved which employed cylindrical explants, of 2 mm diameter and 2.4 mm length, impaled upon glass rod mounts by a thin spike, and these were cultured in 3 ml of Bonner and Addicott's medium supplemented by 10^{-6}M 24D and 20 per cent autoclaved coconut milk. Aseptic cultures were maintained at 25°C in the dark for culture periods of up to 28 days.

3. It was shown that over a 14 day culture period the nutrients are present in excess, that there is no evidence for "staling" products, and that the facility for gaseous exchange may be a limiting factor upon the growth of the callus.

4. It was shown that the response to 14 days of culture was remarkably uniform: the average coefficient of variation for individual experiments was 12 per cent while the coefficient of the means for all 32 long-term experiments was as low as 16 per cent.

5. It was shown that the culture of explants removed from artichokes from three successive annual harvests employing coconut milk obtained from two different annual shipments showed only slight variations in their fresh weights.

6. Certain small changes in response to culture related to the "age" of the

tuber were however discovered. Cultured explants from older tubers tended to have an increased total nitrogen content, while the cell number achieved during culture tended to reach a maximum in explants taken from tubers that had been stored for three months.

7. The onset of division in culture tends to be delayed in explants from "older" tubers. Explants from "older" tubers seem to have some difficulty in forming the first new cell wall, and this may be either very thin or absent. However, cell walls are successfully formed on continued culture.

8. Explants taken from the apical region of the tuber tend to show greater cell expansion but similar cell division to the explants from other parts of the tuber. Those removed from the inner region yield, on culture, an increased fresh weight and higher cell number so that the cell size remains comparable.

9. There is considerable seasonal fluctuation in response to culture in the absence of the coconut milk supplement, although on some occasions the response can be just as large as the control. This emphasises the value of a "blanketing" concentration of coconut milk in the medium.

10. Using the standardised procedure and cognisant of there being a predictable growth response, the timecourse of the typical changes in a number of selected indexes of growth and development was determined in order to provide a sound basis for the examination of some aspects of these processes. Repeated experiments confirmed the following features of the response to culture.

11. There is an immediate stimulation of respiration per explant which continues throughout culture albeit at an ever decreasing rate.

12. The Respiratory Quotient changes from about 0.8 to about 1.2 during the first two days, and thereafter remains above unity.

13. The plot of total nitrogen content is usually sigmoid with no lag phase.

14. The plot of cell number increase is usually sigmoid after a lag phase of one day.

15. The plot of fresh and dry weight has a lag phase of one day which is followed by a more or less linear increase.

16. On occasion the total nitrogen and the dry weight may decrease during the first days of culture.

17. The anatomy of the growth of a callus has been described in order to compare the metabolic changes with the morphological developments.

18. From these trends it became clear that development in this system falls naturally into three phases.

(a) A lag or induction phase of one day where the cell size remains the same but in which the rise in oxygen uptake suggests intense metabolic activity.

(b) A division or regeneration phase of about six days in which the mean cell size is decreasing while all the other parameters of growth are increasing.

(c) A phase of differentiation which originates on about the fifth day, becomes dominant by the tenth day, and is characterised by a more or less constant mean cell size. During this time there is considerable expansion and tracheid formation, and the

development of nodules becomes widespread throughout the callus.

19. A scan of the proteins of the "soluble" fraction obtained by differential centrifugation of homogenates suggested that there are both qualitative and quantitative changes occurring during callus development.

20. The activity of particular enzymes in homogenates of callus tissue have been examined. There was no detectable activity of either invertase or inulase. Acid phosphatase (pH 6.2) showed a sigmoid curve although it is suggested that this results from the fortuitous blending of two distinct maxima, which depend initially upon activity in the superficial layers but latterly upon activity in the differentiating tracheids. No activity could be detected for an alkaline phosphatase (pH 9.4) using β-glycerophosphate as substrate. The activity of ribonuclease at pH 6.2 was very low for about five days and then showed a rapid and continuous rise. The activity of succinic dehydrogenase had a lag phase of a day, followed by a sharp rise to a fairly steady level after the fifth day.

21. The relative changes in these widely differing enzymes show that development in the callus is supported by a constantly changing complement of enzymes.

22. The localisation, by histochemical methods, of enzyme activity proved illuminating. Acid phosphatase is initially confined to a sub-peripheral ring of cells outwith the region of intense cell division. This ring of activity increases in intensity and is found in a rind of collapsed cells which encloses the callus by the fifth day. By this time activity has appeared in immature differentiating tracheids in the body of the callus. The physiological role of this acid phosphatase would appear, therefore, to be concerned in autolysis. The fact that an alkaline phosphatase is also present in the rind but not in the tracheids would

suggest that this might well be a controlled process. It is possible that this activity may be found in organelles analagous to the lysozomes found in animal tissues.

23. There is very tentative evidence that succinic dehydrogenase activity is found in the areas of most intense metabolic activity.

24. The induction phase was examined in greater detail, and the earliest metabolic event which has so far been detected is the rapid stimulation of oxygen uptake.

25. During this stimulation acid phosphatase appears in the proteins of the "soluble fraction" and it seems likely that the intensity of activity of this enzyme is to some extent dependant upon an increase in metabolic activity.

26. It was shown that appreciable pellets of such subcellular fractions (from callus tissue homogenates) as the mitochondrial and ribosomal fraction appear after about 20 hours.

27. Their appearance is followed by an approximately 14 hour period of DNA synthesis which immediately precedes the division of those cells which have doubled their DNA. There is a quiescent or retarded volume of tissue in the core of the callus which does not divide or synthesise DNA at this early stage.

28. The first series of divisions is partially synchronised.

29. The influence of such morphogenetic sequences as the formation of a protective rind and the origination of nodules is emphasised.

30. The potential of the liquid culture of artichoke tissue is indicated for the examination of such topics as: the synchronised cultures of higher plant

tissues; the influence of nucleic acid changes on the induction of division; the fine structural developments associated with the induction of metabolic activity in a quiescent tissue; the scope for a lysozome hunt in plant material; the potential for manipulating the orientation of cell divisions; the effect of artificial inhibitors upon the cell cycle; the effect of storage of tuber tissue upon its potential for growth on deprived culture media.

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APPENDIX A.

1. Bonner and Addicott's Medium.

Nutrients	mg per litre
$Ca(NO_3)_2$	236
$MgSO_4 \cdot 7H_2O$	36
KNO_3	81
KCl	65
KH_2PO_4	12
$Fe_2(SO_4)_3$	2
24D	0.22
Sucrose	4,000
Coconut milk	20% v/v

2. Gautheret's Medium.

Nutrients	mg per litre
$Ca(NO_3)_2$	100
KNO_3	25
$MgSO_4$	25
KH_2PO_4	25
$Fe_2(SO_4)_3$	50
$MnSO_4$	2
KI	0.5
$ZnSO_4$	0.1
H_3BO_3	0.15
$Ti_2(SO_4)_3$	0.2
$NiSO_4$	0.05
$CoCl_2$	0.05
$CuSO_4$	0.05
Glucose	30,000
Cysteine - HCl	10
Thiamine	1
Ca pantothenate	0.1
Biotin	0.1
Inositol	100
Naphthalene acetic acid	0.3

3. Nitsch's Medium.

Nutrients	mg per litre
KNO_3	2,000
KCl	1,500
$MgSO_4 \cdot 7H_2O$	250
$NaH_2PO_4 \cdot H_2O$	250
$CaCl_2$	25
Sucrose	2,000
Indole acetic acid	0.17

4. White's Medium.

Nutrients	mg per litre
$Ca(NO_3)_2$	200
$MgSO_4$	360
Na_2SO_4	200
KCl	80
NaH_2PO_4	16.5
KI	0.75
$Fe_2(SO_4)_3$	2.5
$MnSO_4$	4.5
$ZnSO_4$	1.5
H_3BO_3	1.5
Glycine	3.0
Thiamine	0.1
Niacin	0.5
Pyridoxine	0.1
Sucrose	20,000

APPENDIX B

Mean Fresh Weight Data - Series 2.

Experiment	Harvest							Mean
		30	32	33	36	41	50	
	Day 0	8.8	8.3	8.3	8.7	8.6	8.4	8.5
	1	8.5	8.4	8.7	8.6	8.4	8.5	8.5
	2	9.2	8.8	9.3	8.6	8.7	9.5	9.1
	3	9.6	8.9	10.4	9.3	9.9	10.5	9.9
	4	-	9.5	11.6	9.7	11.7	12.7	11.4
	5	11.7	10.7	13.0	11.3	14.2	13.8	12.8
	6	12.8	12.7	15.3	-	15.8	16.6	14.9
	7	13.6	13.7	16.0	14.0	-	17.8	15.8
	10	15.4					21.5	
	14	18.0	23.1	26.5		24.0	26.0	23.6
Month		Jan.	Feb.	Apr.	July	Nov.	Feb.	
Year		1964	1964	1964	1964	1964	1965	

Values for Experiment 32 are not included in the means (see p.)

All values are the mean of at least twelve samples recorded in mg.

Mean Fresh Weight Data - Series 1.

Experiment	Harvest							Mean
		10	14	23	25	26	29	
	Day 0	7.8	8.4	8.5	7.8		8.5	8.2
	1			9.0				
	2	8.0	8.4		10.3	10.0	9.1	9.2
	5	10.9	12.0	13.5	15.1	14.4	13.5	13.2
	7	13.0	14.9	15.4	16.2	17.9	16.6	15.7
	10				18.8			
	14	22.5	23.5	28.3	26.7	26.6	20.9	24.8
	21	33.7	34.4			32.5	23.2	31.0
	28	41.3	45.8			43.5	23.5	38.5
Month		Feb.	Apr.	Oct.	Oct.	Nov.	Jan.	
Year		1963	1963	1963	1963	1963	1964	

All values are the mean of at least 12 samples recorded in mg.

Dry Weight Data - Series 1 and 2.

Harvest	Experiment							Mean
	10	14	25	26	32	33	50	
Day 0	0.8	0.84	0.8	1.3	1.0	0.92	1.1	0.9
1	-	-	-	-	0.78	0.93	0.9	0.9
2	1.0	0.87	1.3	1.3	0.74	0.97	1.2	1.1
5	1.3	1.2	2.1	2.0	0.64	1.0	0.9	1.5
7	1.6	1.6	1.8	2.5	0.95	1.8	1.8	1.9
10	-	-	2.3	-	-	-	2.4	2.4
14	2.6	2.3	3.4	3.9	1.7	3.9	3.0	3.2
21	3.8	3.5		4.3				3.9
28	4.9	4.4		5.9		5.5		5.2

All values, in mg per explant, are derived from the measurement of the dry weight of at least 3 explants.

Estimated Ribonucleic Acid Data.

Day	0	1	2	3	4	5	6	7	14
Experiment 33	.063	.082	.157	.245	.253	.313	.334	.405	.813
" 50	.03	.075	.173	.250	-	.356	.725	.770	.882

Values are expressed in Optical Density units per explant, measured at 260 mμ. The first experiment used two and the second experiment used three, bulked explants for each determination.

Estimated Tracheid Number.

Harvest	Experiment					Mean
	30	32	33	41	50	
Day 4	0	0	0.4	1.3	0.6	0.5
5	1	2	1.6	1.5	4.6	2.2
6	3		5	2.4	12	5
7	10		11	-	14	12
14	33	5	16	13	65	32

All values are recorded in thousands of tracheids per explant and were estimated from the same macerates as were the total cell numbers. Values for Experiment 32 are not included in the means.

Cell Number Data - Series 1.

Experiment	Harvest							Mean
		10	14	23	25	26	29	
	Day 0	17	14	-	12	16	16	15
	1	-	-	27	-	-	-	
	2	27	18	30	23	28	17	24
	5	52	50	87	(244)	87	103	76
	7	74	207	131	220	166	198	166
	10	-	-	-	270	-		
	14	370	555	550	500	372		469
	21	558	875			810		748
	28	770	1200			1050		1067
Month		Feb.	Apr.	Oct.	Oct.	Nov.	Jan.	
Year		1963	1963	1063	1963	1963	1964	

All values are recorded in thousands of cells per explant and are estimates of the average cell number per explant in a macerate of 2 - 10 explants.

Cell Number Data - Series 2.

Experiment	Harvest							Mean
		30	32	33	36	41	50	
	Day 0	16	15	17	21	18	23	19
	1	16	16	22	20	18	22	20
	2	22	15	27	22	33	30	27
	3	32	27	34	34	64	58	44
	4	-	55	44	46	100	133	81
	5	109	50	72	101	109	222	124
	6	125		92	-	165	288	168
	7	242	107	158	123	189	488	240
	8	253				190		
	9	306				231		
	10	350				261	600	
	12	417				370		
	14	584	444	423		395	863	566
Month		Jan.	Mar.	Apr.	Jul.	Nov.	Feb.	
Year		1964	1964	1964	1964	1964	1965	

All values are recorded in thousands of cells per explant and are estimates of the average cell number per explant in a macerate of 2 - 10 explants.

Total Nitrogen Data.

	Experiment					Mean
	10	14	41	50	56	
Day 0	20	28	28	43	26	26
1			39	37	20	32
2	23	34	60	18	29	33
3			70	32		
4			77	53		
5	32	50	91	83	53	62
6			97	120		
7	46	73	94	128		85
10			137	193	83	
14	94	119		185	137	138
21	108	147			188	
28	135	176			193	

Values are recorded in μg total-nitrogen per explant. The data in Experiments 10 and 14 were determined by the Conway method and the remainder by Nesslerisation.

Alcohol Soluble Nitrogen Data

	Experiment			
	14	33	31	30
Day 0	9	2	12	-
1	-	2	12	-
2	14	5	-	24
3	-	9	12	22
4	-	11	15	-
5	5	14	16	27
6	-	19	-	49
7	24	23	26	43
14	26	78	30	70

Values are recorded in µg of alcohol insoluble nitrogen per explant. Experiment 14 values were estimated by the method of Conway and the other values by Nesslerisation.

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Growth and Differentiation of Plant Tissue Cultures

I. Changes Accompanying the Growth of Explants from *Helianthus tuberosus* tubers

BY

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Botany Department, Edinburgh University

With three Plates and eight Figures in the Text

ABSTRACT

Explants derived from artichoke tubers proliferate rapidly when cultured aseptically on a nutrient medium containing sugar, mineral salts, coconut milk, and 2,4-dichlorophenoxyacetic acid. This rapid growth, which occurs in three distinct stages, is accompanied by massive changes in numbers of cells, fresh and dry weight, and pronounced changes in the rates of gaseous exchange. The very short lag phase is followed by a period of exponential cell division during which the original cellular pattern is partially obliterated. At the end of this growth phase redifferentiation occurs, tracheides are formed in large numbers, and distinctive nodular bodies appear which resemble meristems. Under the conditions of these experiments they do not develop further.

INTRODUCTION

THE present series of observations is part of an investigation in which it is proposed to examine the processes of dedifferentiation and of subsequent differentiation that occur in a cultured callus during its development. The investigation is based on cultures of Jerusalem artichoke (*Helianthus tuberosus*) callus tissue started from explants isolated from tubers, and induced to grow through to the stage at which distinctive morphological structures are formed. In the course of this development, an initial dedifferentiation occurs which involves a partial extinction of the original cellular pattern; subsequently a differentiation ensues which leads to the establishment of distinctive nodular bodies which resemble meristems. It is these two processes which are of primary interest, but their investigation requires some reference to the course of growth and development in the culture as a whole. Observations on certain general features have accordingly been made in a preliminary phase of the investigation and it is the results of these that are reported here. Two cultural series are involved, the first depending on an agar medium and the second on a liquid medium. With both, growth has been analysed in terms of anatomical and cytological changes, and also in terms of protein nitrogen contents. In addition, with the liquid cultures determinations have been made of changes in the gaseous exchange during the cultural period which is normally 28 days.

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Artichoke callus cultures have been used by earlier workers to investigate other problems, for example, Gautheret (1941), Nitsch and Nitsch (1956, 1957), and Setterfield (1963), but, hitherto, detailed data on the growth of this tissue over an extended experimental period have not been published. Carrot cultures, however, have been studied extensively by Steward and co-workers at Cornell (see reviews for references).

Materials and Methods

The general experimental design involved setting up a population of cultures, taking random samples from these at intervals, and making particular observations on the cultures withdrawn. The frequency of sampling varied with the phase of development and each sample normally involved 10–15 cultures. The techniques of the investigation are described below in relation to (i) preparation of the inoculum, (ii) cultural conditions, (iii) anatomical and cytological observations, (iv) the measurement of different criteria of growth, and (v) the analysis of the gaseous exchange.

Preparation of the Inoculum

The inocula were taken from tubers of two varieties of Jerusalem artichokes, Bunyard's Round, and Bunyard's Fuseau. The first was used with liquid and the second with agar cultures. All the explants for a single experiment were taken from a single large tuber.

The tubers were taken from plants grown in the open. They were harvested in late October and stored in damp sand in closed polythene bins. Initially they were stored in groups at 6–8° C, but later it was found that storage of single tubers at 4° C further delayed sprouting and thus extended the period during which the tubers could be used.

Before taking the explants, each selected tuber was washed in running water, wrapped in absorbent tissue paper, and then immersed for 30 minutes in a freshly prepared solution of 10 per cent w./v. calcium hypochlorite. Subsequently the absorbent paper was removed and the tubers washed in three changes of sterile distilled water.

After washing, a selected tuber was cut transversely into slices about 25 mm in length. From these, cylinders were taken from storage parenchyma within the xylem rays. The cylinders were removed with a stainless-steel cannula 2 mm in diameter. About 2 mm at each end of every cylinder was cut away and discarded. After trimming in this way all the cylinders from the same tuber were aligned and cut into 2-mm lengths with a cutter designed for the purpose. This procedure gave 120 explants in a single operation. The explants within the group differed by about 10 per cent in fresh weight.

Cultural Conditions

The medium used throughout was a solution of mineral salts and sugar to which coconut milk and 2,4-dichlorophenoxyacetic acid had been added. The basic medium was that developed by Bonner and Addicott (1937) for the

culture of excised roots. To this, coconut milk was added at the rate of 200 ml/l and 2,4-dichlorophenoxyacetic acid was dissolved in it to give a concentration of 0·22 mg/l. The coconut milk was extracted from fresh Ceylon coconuts, autoclaved, and filtered. It was handled in large quantities, and samples for particular experiments were taken from stocks stored in polythene containers in a deep freeze.

The agar cultures were developed in screw-cap bottles each containing 10 ml. of medium solidified with 1 per cent agar. Each explant was placed on its side in a bottle, all of which were incubated in the dark at 26° C.

The liquid cultures were established in test-tubes containing 3 ml. of standard medium. An explant was impaled on the end of a spike drawn out from the edge of the cut end of a short length of Pyrex glass rod. One spike was placed in each tube, which was then closed with an aluminium cap. All the tubes were then placed at an angle of 10–15° from the horizontal in a conventional roller-tube apparatus placed in the dark at 26° C. The explant with the spike was held just above the surface of the liquid, but as the drum revolved once every eight minutes, the explant dipped in and out of the medium.

Anatomical and Cytological Observations

Anatomical and certain cytological observations were made on sections of material fixed in Randolph's (CRAF) modification of Navashin's fixative, embedded in paraffin wax and cut on a microtome at 10 μ. Sections were stained by Johannsen's quadruple stain method.

Mitotic indexes were determined from Feulgen stained squash preparations. When five-day-old and younger material was being examined a single preparation was made from an entire explant. When older material was being examined two or more preparations were made from the same explant after dissecting it into equivalent segments. Each preparation was examined along a series of random transects, a thousand cells being scored in each case for the presence or absence of mitotic figures. Where an explant was fragmented a mean value was calculated from the determinations from different preparations.

Measurement of Criteria of Growth

Growth was measured as changes in (*a*) fresh weight and dry weight, (*b*) cell number, and (*c*) protein content. For fresh-weight and dry-weight determination, the individual explants were first freed either from bits of agar by brushing, or from drops of liquid nutrient medium by carefully washing with distilled water and then blotting lightly with absorbent paper tissue. The fresh weight was measured by weighing immediately after either of these operations; the dry weight by weighing again after the explants had been dried in an oven at 90° C for up to 48 hours.

Cell numbers were determined by the haemocytometer technique of Brown and Rickless (1949). In each macerate the total number of cells and the number of tracheidal cells were determined simultaneously.

Protein nitrogen was determined as alcohol-insoluble nitrogen, since in preliminary experiments it was found that trichloroacetic acid did not give quantitative precipitations of the protein. Single explants, after extraction with three changes of boiling absolute alcohol, were digested by the normal Kjeldahl procedure, and the ammonia produced was estimated by a Conway microdiffusion technique.

Analysis of the Gaseous Exchange

The gaseous exchange was analysed by the Direct Method of Warburg. Five to twenty explants were used for each estimation, and all measurements were made at 26° C. Coefficients for the gaseous exchange based on fresh weight and respiratory quotient values were calculated from the average hourly gas exchange over a three-hour period. Determinations were only made with tissues cultured in liquid media, since those from agar cultures became waterlogged when placed in the liquid in the annular compartment of the respirometer flask.

Results

Anatomical and Cytological Changes

The inoculum consists of uniform parenchymatous cells with occasional vessel elements from xylem traces (Plate 1*a*). Division begins shortly after the explant is transferred either to the liquid or to the agar medium, and mitotic figures are evident in preparations 24 hours after establishment of the cultures (see Plate 1*b*). At this stage all the mitotic figures are in the outermost layers of cells and the axes of the visible spindles are predominantly parallel to the surface of the explant. From the second to about the seventh day mitotic figures continue to appear, but only in the four or five surface layers. During this phase the axes of the spindles tend to be at right angles to the surface, resulting in rows of cells at right angles to the surface (Plate 1*c*). This pattern continues for about the same time in both agar and liquid cultures, but, whereas in liquid culture the divisions are distributed uniformly over the whole surface, in agar culture they tend to be restricted to the surfaces not in contact with the medium.

On about the fifth day, with both cultural conditions, small areas of random divisions appear below the surface. The cytoplasm of these areas stains deeply with Fast Green. On about the seventh day these areas become prominent (Plate 2*a*), and it is evident that they have enlarged through random divisions. At the same time general surface divisions apparently cease. With liquid cultures the nodules are scattered uniformly over the whole surface, but with agar media they tend to be restricted to the superficial layers immediately above the surface of the medium. Cut cells on the surface of the explant disintegrate and form a layer which stains deeply with safranin (see Plate 1*d*). New cells which arise during the exponential period cease to divide and push through this safranin-positive layer.

Later the nodules enlarge and with increasing size a structural differentiation appears within them. By about the 14th day each nodule has a layer of meristematic cells towards the outside (Plate 2*b*) giving rise to radial rows of cells next to the external surface of the explant. The outer surface of the

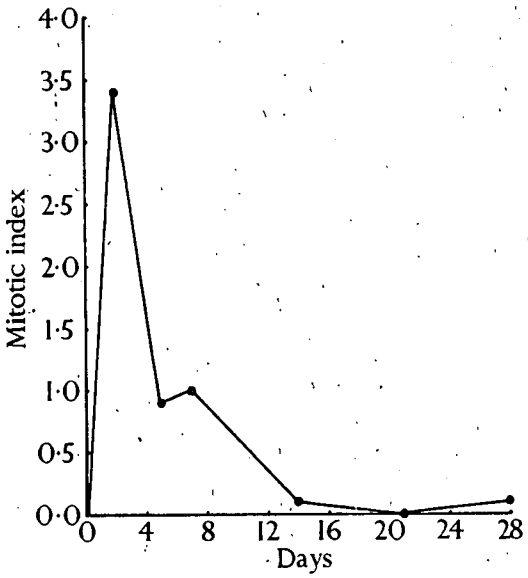


FIG. 1. Changes in the mitotic indexes of artichoke callus tissue cultured on an agar medium.

explant by this time has been ruptured (Plate 2*c*). The cells produced externally from the surface meristem of the nodule become highly inflated and are readily detached from the rest of the system. In the centre of the nodule the cells have enlarged and apparently ceased to divide. The production of the nodular bodies is accompanied by the differentiation of tracheidal elements immediately below them (Plates 2*d* and 3). This differentiation occurs from the parenchymatous cells of the body of the explant. The changes in the distribution of divisions are accompanied by striking changes in the mitotic indexes. These are shown in Fig. 1, from which it is evident that the index is high immediately after division begins within the first 48 hours. Thereafter it falls sharply and particularly between the second and seventh days. From about the 14th to the 28th day the value of the index is less than 0·1.

Cell Number

The change in cell number with time is shown in Fig. 2. At the beginning of the experimental period the number is *c.* 20,000, at the end with agar it is about 1,400,000 and with the liquid medium about 1,250,000. With the first there is about a seventyfold and with the second about a sixtyfold increase. From the data of Fig. 2 there is an indication of a relatively low rate of increase during the first two days. This is again suggested in Fig. 3 which gives

the logarithmic values of the data of Fig. 2 plotted against time. From this figure it is evident that after the initial lag, increase in cell number occurs in the two distinct phases. From the second to the seventh day the increase is exponential with a constant relative rate of increase. During the rest of the experimental period the relative rate of increase decreases with time.

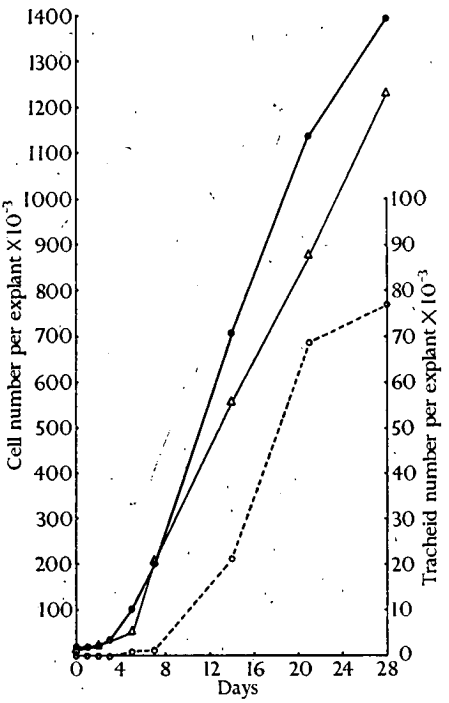


FIG. 2. Changes in the cell (●—● on agar, △—△ in liquid) and tracheide number (○- - -○ on agar) of artichoke callus tissue.

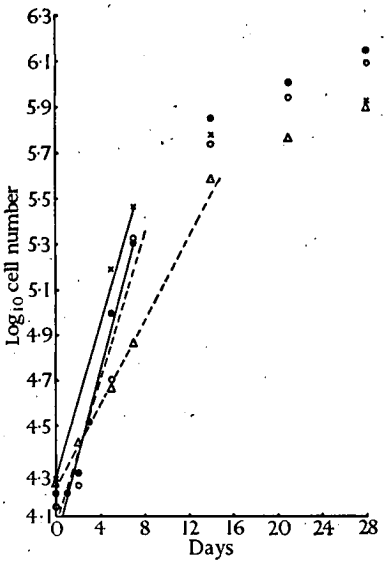


FIG. 3. A plot of the $\log_{10}$ of cell number of artichoke callus tissue against time (●—●, ×—× on agar; △- - -△, ○- - -○, in liquid).

Fig. 2 shows the change in the number of tracheidal cells with time. Evidently during the first seven days there is little or no change, but thereafter the number increases from about 1,000 to about 80,000. The change after seven days is particularly sharp and well defined.

Fresh and Dry Weight

The changes in fresh and dry weight with time in both cultural series are shown in Figs. 4 and 5. The fresh-weight data suggest an initial lag phase extending over the first two days, but this phase, although it may involve dry weight, is not particularly well shown by the dry-weight data. After two days both fresh weight and dry weight increase more or less linearly with time throughout the rest of the experimental period. It is significant that fresh weight and dry weight increases (as indeed also cell number increase) are consistently greater on agar, which indicates that growth on this medium is

not being limited by restricted contact, or by low rates of diffusion of nutrients through the medium to the local area of contact. Average fresh-weight and dry-weight values per cell may be calculated from the cell number and fresh and dry-weight data and these are given in Figs. 4 and 5. These values

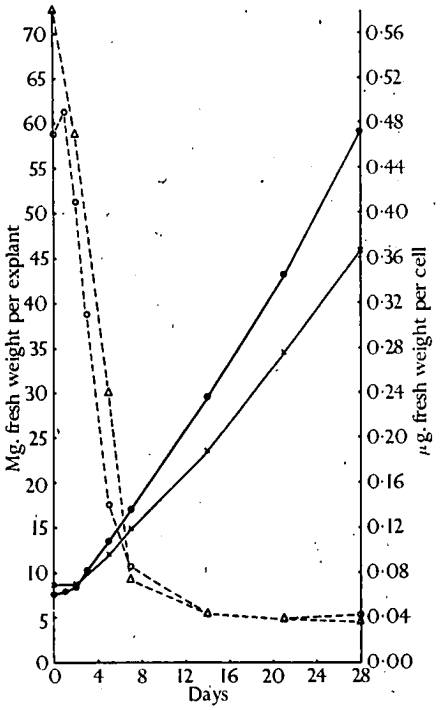


FIG. 4. Changes in the fresh weight (●—● on agar; ×—× in liquid) and average cell fresh weight (○- - -○ on agar; △- - -△ in liquid) of artichoke callus tissue.

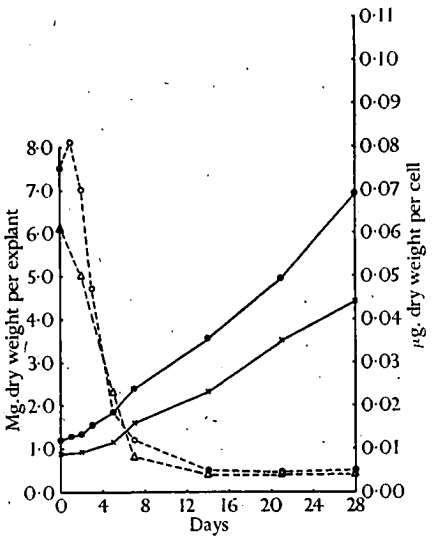


FIG. 5. Changes in the dry weight (●—● on agar; ×—× in liquid) and average cell dry weight (○- - -○ on agar; △- - -△ in liquid) of artichoke callus tissue.

do not of course indicate changes in any particular cellular type, but they do show the relative effects of different quantitative changes with respect to changes in the size of the cellular population. It is evident that the average fresh and dry weights per cell decrease rapidly throughout the first seven days. Thereafter the decrease is slower and after 14 days the average weights are constant. The data indicate that during the first seven days the rate of division is such that neither the fresh nor the dry weights of the parent cell are restored before the next division begins. After seven days either some of the products of division expand and grow or, in the dividing groups, the parental size is consistently restored.

Protein Nitrogen

The change in protein nitrogen with time for both media is shown in Fig. 6. In both cases the increase is more or less linear with time and continues throughout the experimental period. Again the increase is greatest with the

series on agar, and with this series there is some suggestion of a lag phase during the first two days.

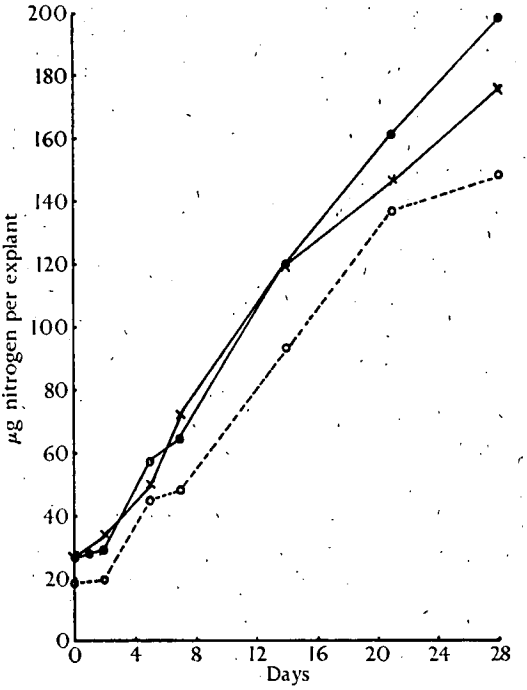


FIG. 6. Changes in the total N content (×—× in liquid) and alcohol insoluble N content (●—● on agar; ○- - -○ in liquid) of artichoke callus tissue.

Gaseous Exchange

The changes with time in the rates of oxygen uptake and carbon dioxide evolution per explant on a liquid medium are shown in Fig. 7. Both rates increase markedly during the first 14 days and thereafter more slowly. The changes in the rates of gaseous exchange shown in Fig. 8 are an expression of changes in intrinsic rates and of changes in the total mass of respiring tissue. Changes in the intrinsic rates may be assessed from the respiratory coefficients based on fresh-weight values. These are shown in Fig. 8, which also gives values for the respiratory quotient at the different stages of development. Evidently the Q_{O_2} and the Q_{CO_2} increase markedly and rapidly during the first seven days. The values recorded show a slight increase between the seventh and 14th days, and a marked continued decrease during the remainder of the experimental period. The respiratory quotient at the beginning of development has the low value of about 0·80; during the first seven days it rises to a value of about 1·20 and thereafter remains more or less constant.

Values for the oxygen absorption and carbon dioxide evolution per cell may be calculated from the cell number and gaseous exchange data and these are given in Fig. 7. These values have the same general significance as the

corresponding values for fresh weight and dry weight given in Figs. 4 and 5. Both oxygen absorption and carbon dioxide production per cell increase noticeably during the first 48 hours. Clearly the relative effect of the activation of respiratory activity in this phase is greater than that of the decreasing

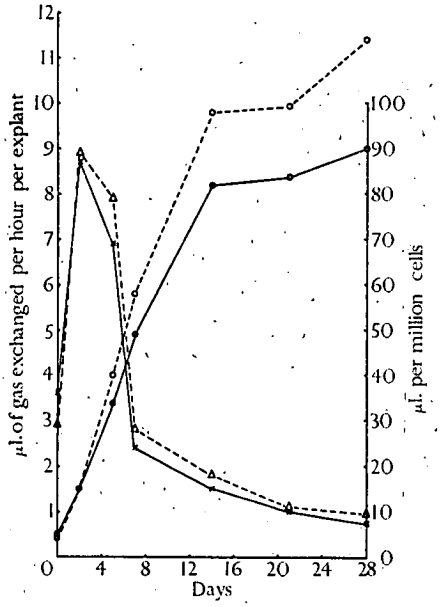


FIG. 7 Changes in the oxygen uptake (●—●) and carbon dioxide output (○- - -○) of whole explants of artichoke callus tissue and in gaseous exchange per cell (×—× O_2, △- - -△ CO_2).

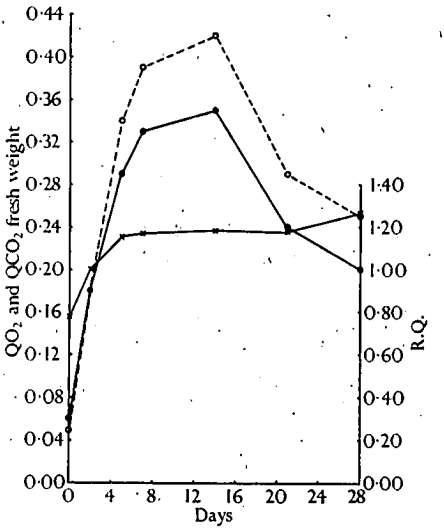


FIG. 8. Changes in the Q_{O_2} fresh weight (●—●), Q_{CO_2} fresh weight (○- - -○) and R.Q. (×—×) of artichoke callus tissue growing in liquid culture.

size of the cell. After 48 hours the continuing decrease in cell size is expressed in sharp decreases of the average values for the exchange during the following five days. After seven days the average values continue to decrease, although more slowly, as a result of decreasing cell size and of decreasing intrinsic respiratory activity.

DISCUSSION

The array of data presented above shows that the growth and development of the system occurs in two phases. The first, which may be characterized as a dedifferentiation phase, occupies the first seven days, and the second, which may be called a differentiation phase, extends over the rest of the experimental period.

The evidence of the two phases is shown particularly strikingly by the respiration data. When the inoculum is excised from the parent tuber intrinsic respiratory activity and the respiratory quotient are low, and the immediate effect of transfer to the culture medium is an activation of the respiratory process which extends over the first seven days. This activation promotes a

rise of the coefficient for oxygen absorption from 0·06 to 0·33 and for carbon dioxide production from 0·05 to 0·39. Simultaneously the respiratory quotient rises from 0·80 to 1·20 suggesting a particularly intense stimulation of decarboxylation reactions.

When the inoculum is transferred to either medium it may be supposed that respiration is then too low to sustain synthetic activity. As a result there is possibly a lag in the synthesis of protein and of other components involved in the accumulation of dry weight. With the activation of respiratory activity, rapid synthesis begins, and after the second day dry weight and protein can increase.

When the inoculum is transferred respiration is also too low to sustain rapid division. This only begins after the second day when respiration has increased. It then continues in those regions of the tissue where aeration is likely to be least restrained and respiration therefore likely to be most active. The regions involved are all the surface layers in a culture in a liquid medium and the surface layers not in contact with the medium in an agar culture. From the second to the seventh day cell number increases exponentially. The simplest interpretation of this situation implies a constant rate of division in a constant proportion of cells in the population. The constant proportion is no doubt secured by a constant relation between surface and volume in an expanding, virtually cylindrical system. It is significant that during the first phase the mitotic index changes considerably. It is highest during the first two days when the rate of division is low and when respiration is low, and it decreases sharply when division is rapid and respiration high. It is possible that the initial high index is a consequence of a rapid progress into mitosis of cells which in the original tuber were in a similar state of preparedness, and it is further possible that it is also consequence of a slow progress through mitosis as a result of a low respiration rate. Brown (1951) has shown that the mitotic index is a measure only of the relative durations of interphase and mitosis. This being the case, a decreasing index in a situation where the rate of division is remaining constant implies a decrease in the relative duration of mitosis with a corresponding lengthening of interphase. Division is clearly not linked to the accumulation of protein or the increase in dry weight. If it were, cell size might be expected to remain constant. In fact it does not and continues to decrease until the phase of rapid division is complete.

Division in the first phase is characterized by the predominance of periclinal divisions. No interpretation can be offered as to the nature of the factors which determine this decisive morphogenetic feature.

The second phase is dominated by the emergence of the nodular meristematic bodies as distinctive morphological entities. The formation of these clearly depends on some factor supplied by the nutrient, since when the explant is on agar they tend to be formed immediately above the point of contact with the medium, where the concentration of nutrients is likely to be highest. With the appearance of the nodules divisions cease over the rest of the surface and become restricted to the nodules. As a result the explant which has

hitherto retained the geometrical form of the inoculum now becomes deformed and irregular in shape, and again as a result of this change the relative rate of increase in the total number of cells decreases with time.

In due course division becomes further restricted to the nodule meristem, with a consequent further reduction in the proportion of dividing cells in the whole explant. This no doubt contributes to the further reduction in the value of the mitotic index that occurs during the second phase. As divisions become restricted to the external surface the central cells of the nodule become vacuolated, and this change is undoubtedly reflected in the values of the fresh and dry weights per cell that are recorded at this stage. Evidently the effect of division in reducing the size of the superficial cells is compensated by an expansion of the central cells of the nodule with a consequent establishment of constant average fresh and dry weights for the whole nodule. One of the most striking features of the second phase is a decrease in the intrinsic rate of respiration. After remaining more or less constant from seven to 14 days, the coefficients for the rates of oxygen uptake and carbon dioxide evolution decline sharply. It is probable that this decline is a consequence and not a cause of the morphological change. When the surface cells cease to divide, ageing begins and this is characterized by a declining respiration rate. Further it has been shown that meristematic tissues are characterized by a low intrinsic respiration, and it is therefore probable that the emergence of these will tend to depress the intrinsic respiration of the explant as a whole.

Finally it may be noted that in the second phase there tends to be a sharp increase in the number of tracheidal cells. It is significant that anatomically these are associated with the bases of the nodular bodies. Wetmore and Sorokin (1955) showed that a bud grafted into a callus promoted the development of tracheidal elements below it. It may therefore be suggested that the increase in tracheides is a direct consequence of the development of the nodular meristematic bodies.

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EXPLANATION OF PLATES

PLATE 1

(*a*) V.S. of the inoculum (DAY 0) showing large vacuolated parenchyma cells, and occasional small xylem traces (arrowed). ×60.

(*b*) V.S. of the developing callus (DAY 1), showing an anticlinal division in a cell of the outer layer. ×480.

(*c*) V.S. of the developing callus (DAY 3), showing radial rows of new cells immediately beneath the outer surface of the tissue. ×600.

(*d*) V.S. of the developing callus (DAY 3). The arrow indicates damaged cells on the outer surface degenerating to form the safranin-positive layer seen in Plate 2. ×480.

PLATE 2

(*a*) V.S. of the developing callus (DAY 7). The arrow indicates a region of small, non-vacuolated cells which have been formed at the surface of the agar. Here the cells stain densely with fast green. As a result of mitotic activity, this region will develop into a meristematic nodule. ×120.

(*b*) V.S. of the developing callus (DAY 14) showing a median section of a meristematic nodule. The divisions are no longer randomly oriented, there being a dome of radially arranged meristematic cells. Cells behind this, in the centre of the nodule, have ceased to divide and are expanding. ×120.

(*c*) V.S. of the developing callus (DAY 14). The same preparation as in Plate 2*b*. As a result of cell division followed by expansion, particularly of the friable mass of large irregular cells formed to the outside of the meristematic dome, the nodule has burst through the surface layer of the culture. ×60.

(*d*) V.S. of the developing callus (DAY 21) showing a nodule in median section. The polarity of the nodule is now very clear, and the meristematic dome has become conical due to the elongation of the cells behind it. To the rear of the nodule, but formed in association with it, a complex system of tracheides has been differentiated (arrow). ×60.

PLATE 3

Median V.S. of an almost fully developed meristematic nodule (DAY 28).

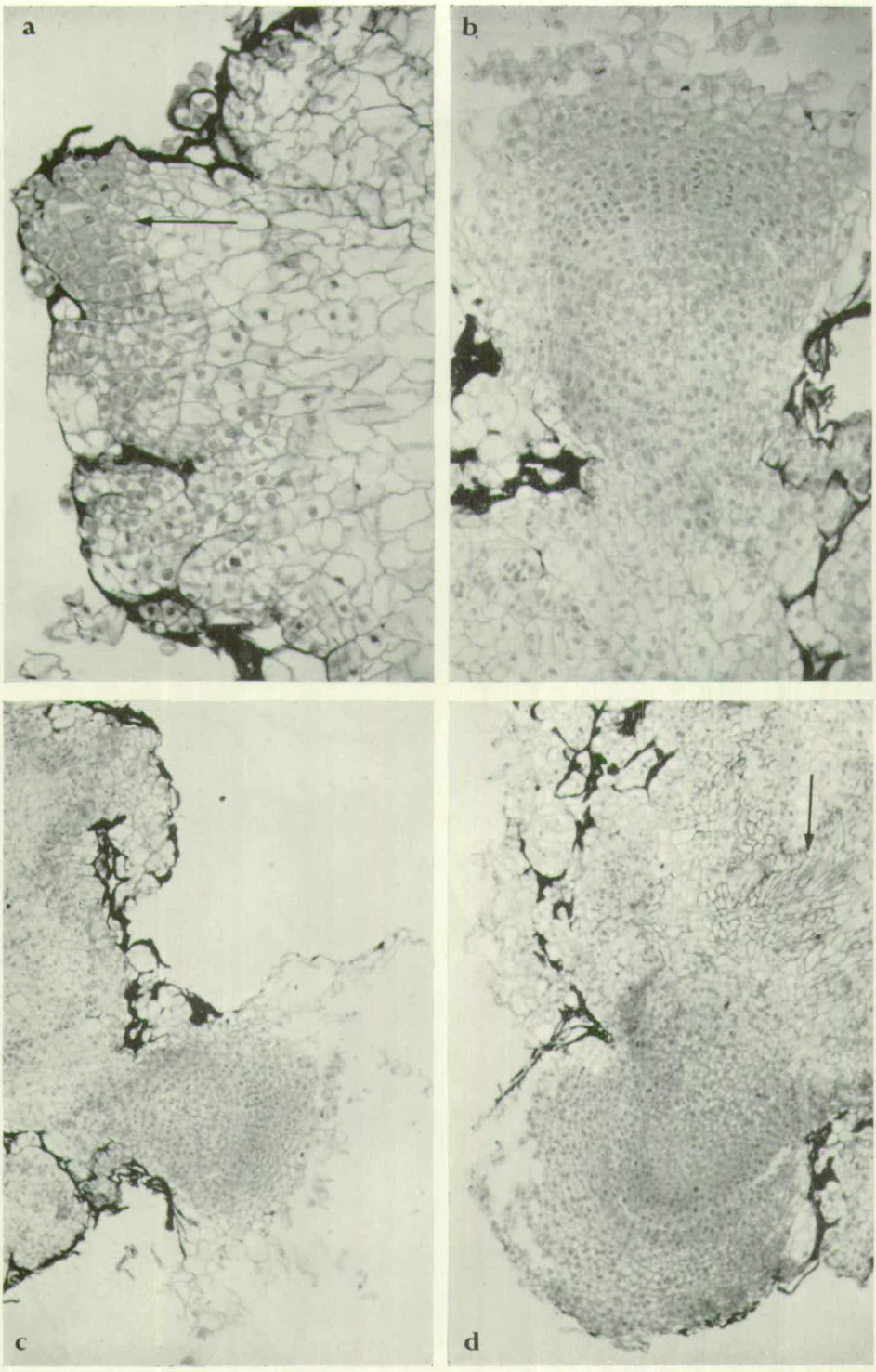
Four distinct regions are visible:

(i) An outer region of cells produced in radial rows from the meristem, expanding at first isodiametrically and then later to form large, irregular cells in a loose friable tissue.

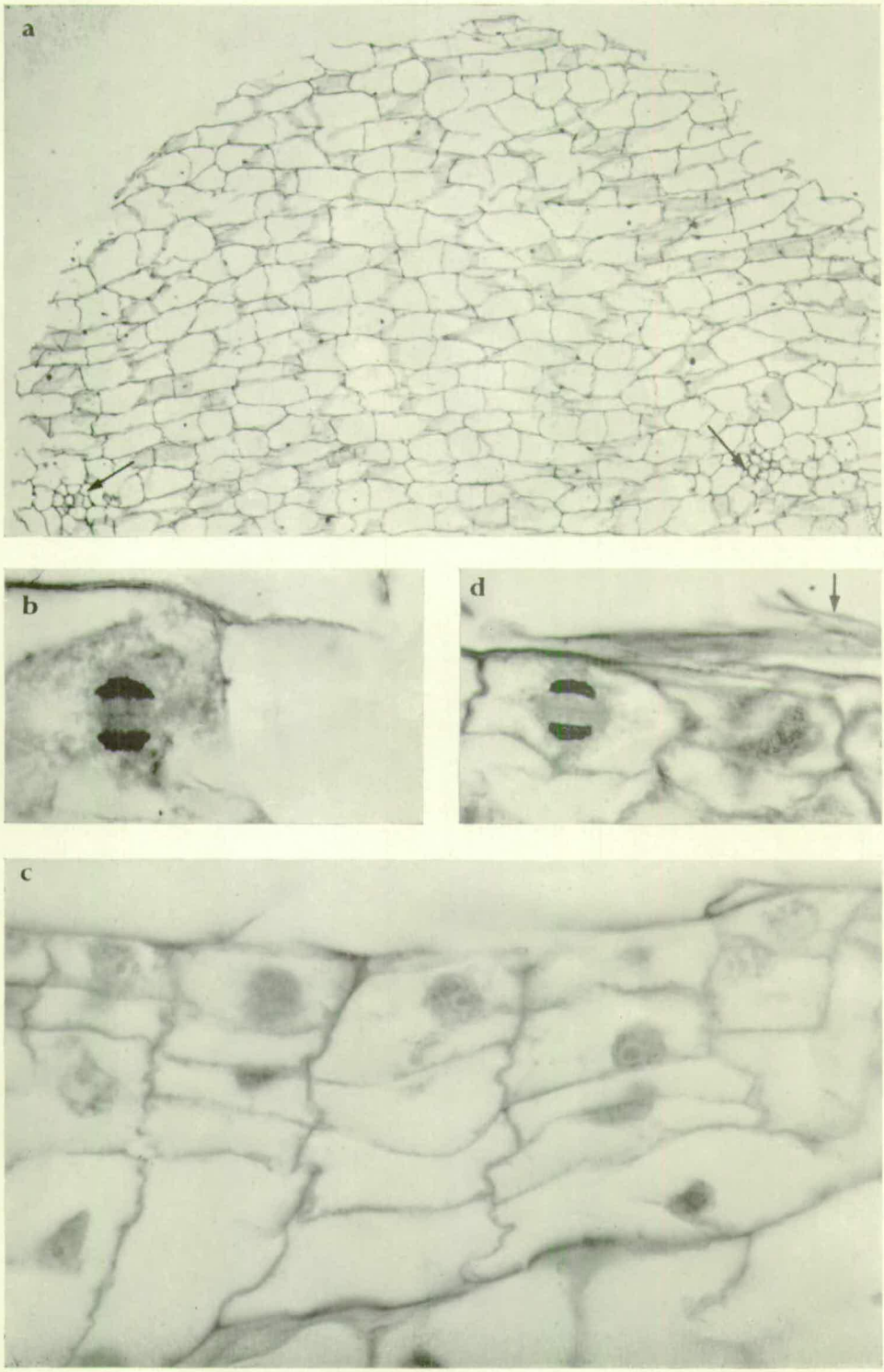
(ii) An elongated meristematic dome, cutting off new cells inside and out.

(iii) Cells becoming increasingly elongated, all in the same direction, by expansion.

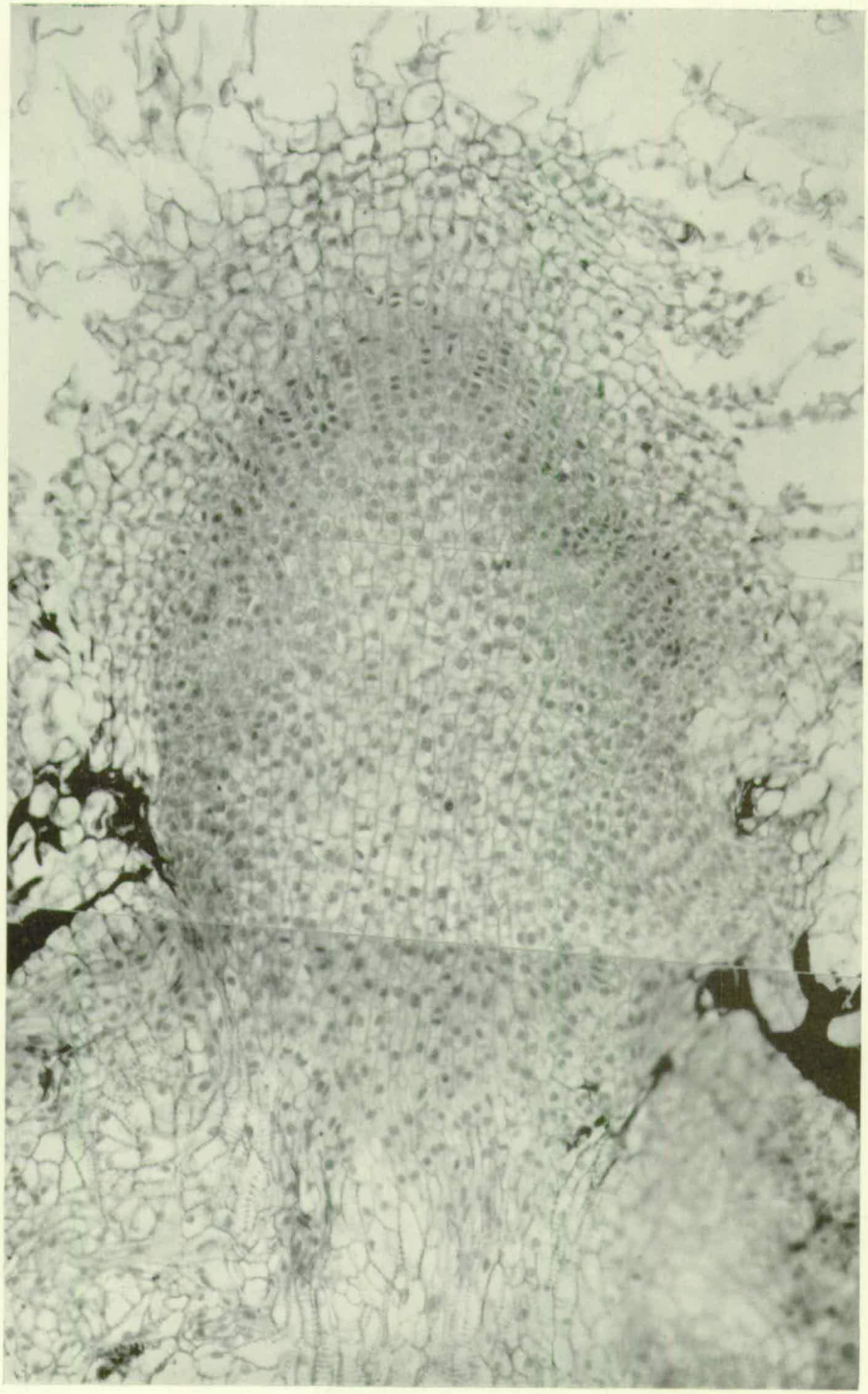
(iv) A region of more or less orientated tracheides, formed in the ground tissue but integrating with the base of the nodule. ×150.



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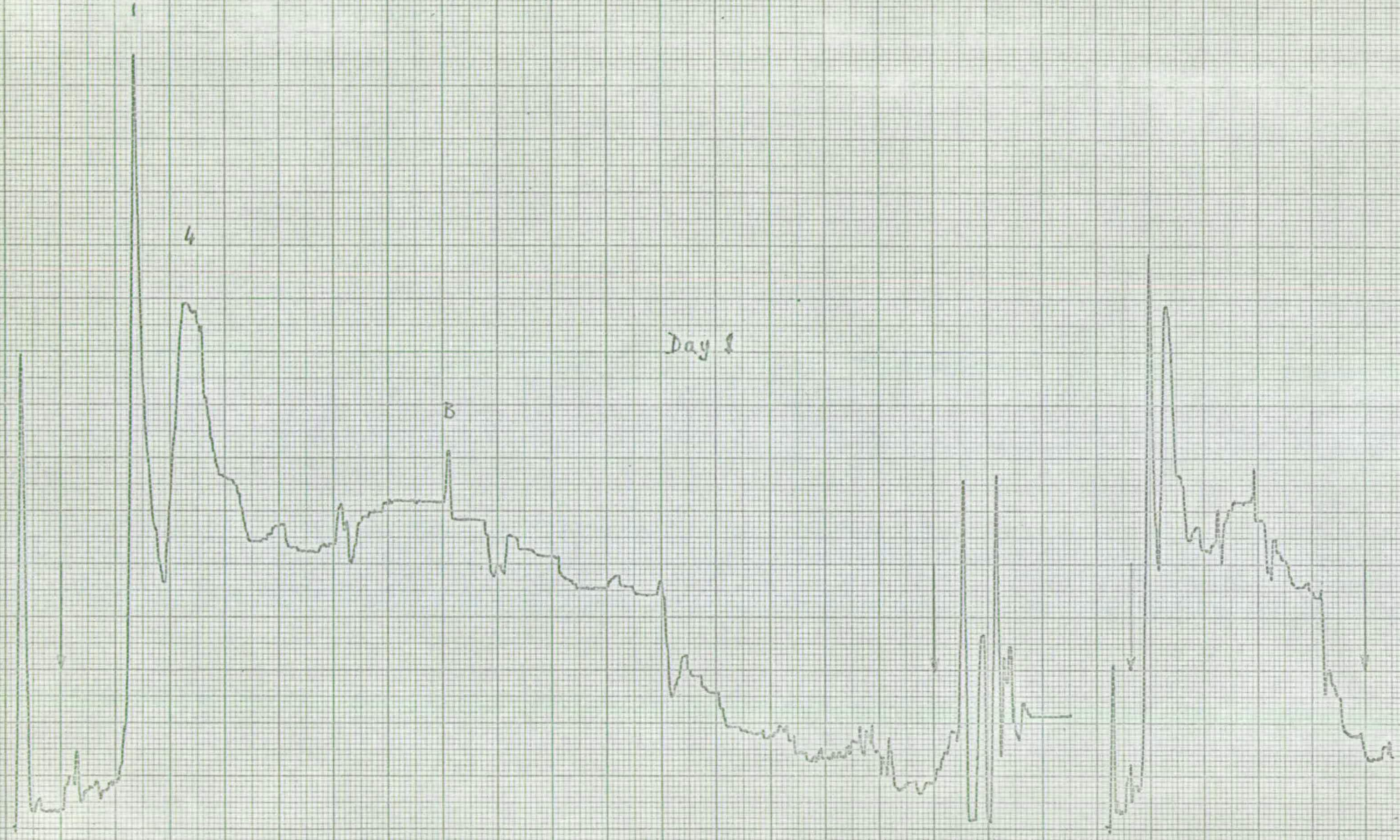


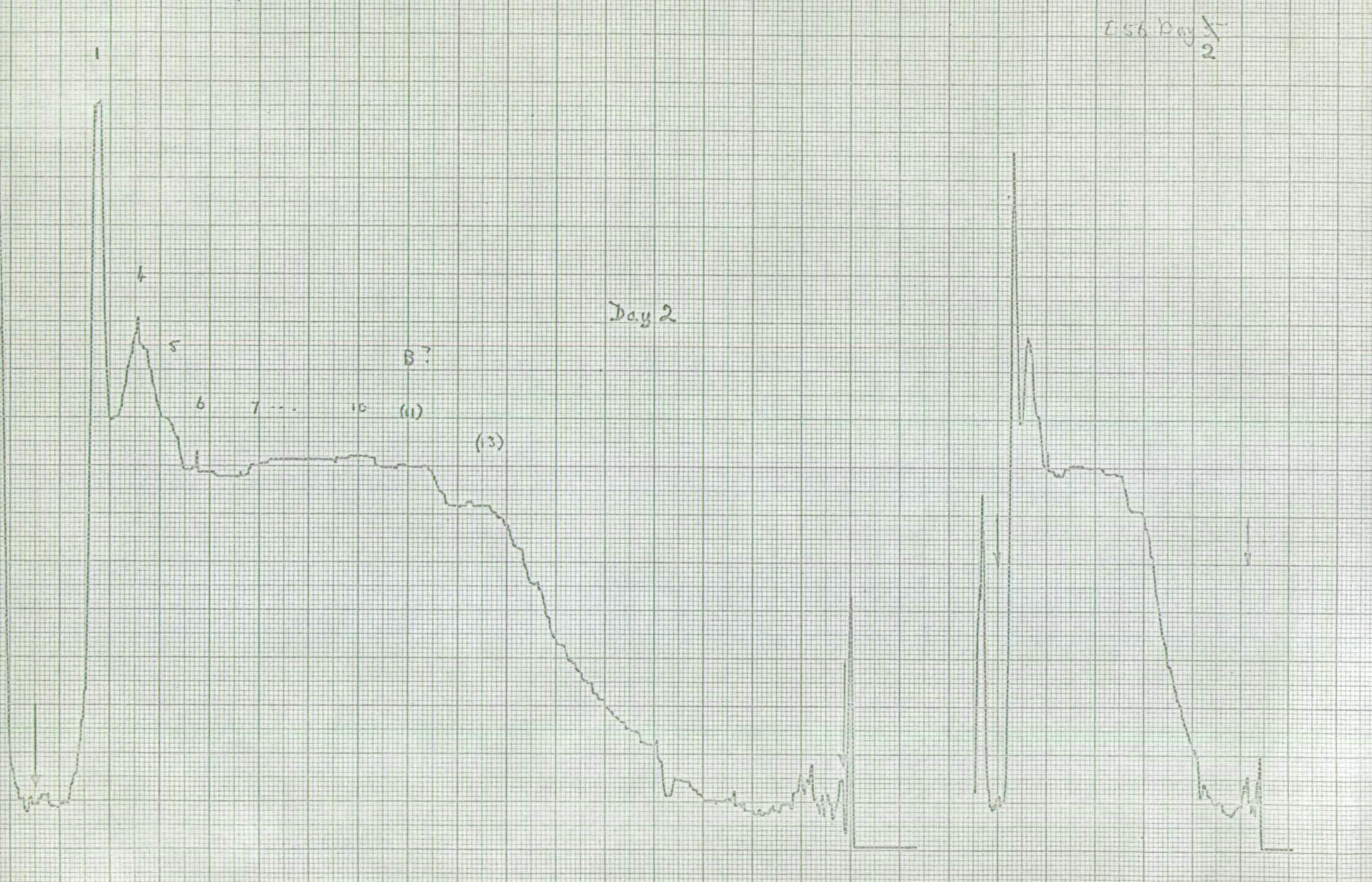
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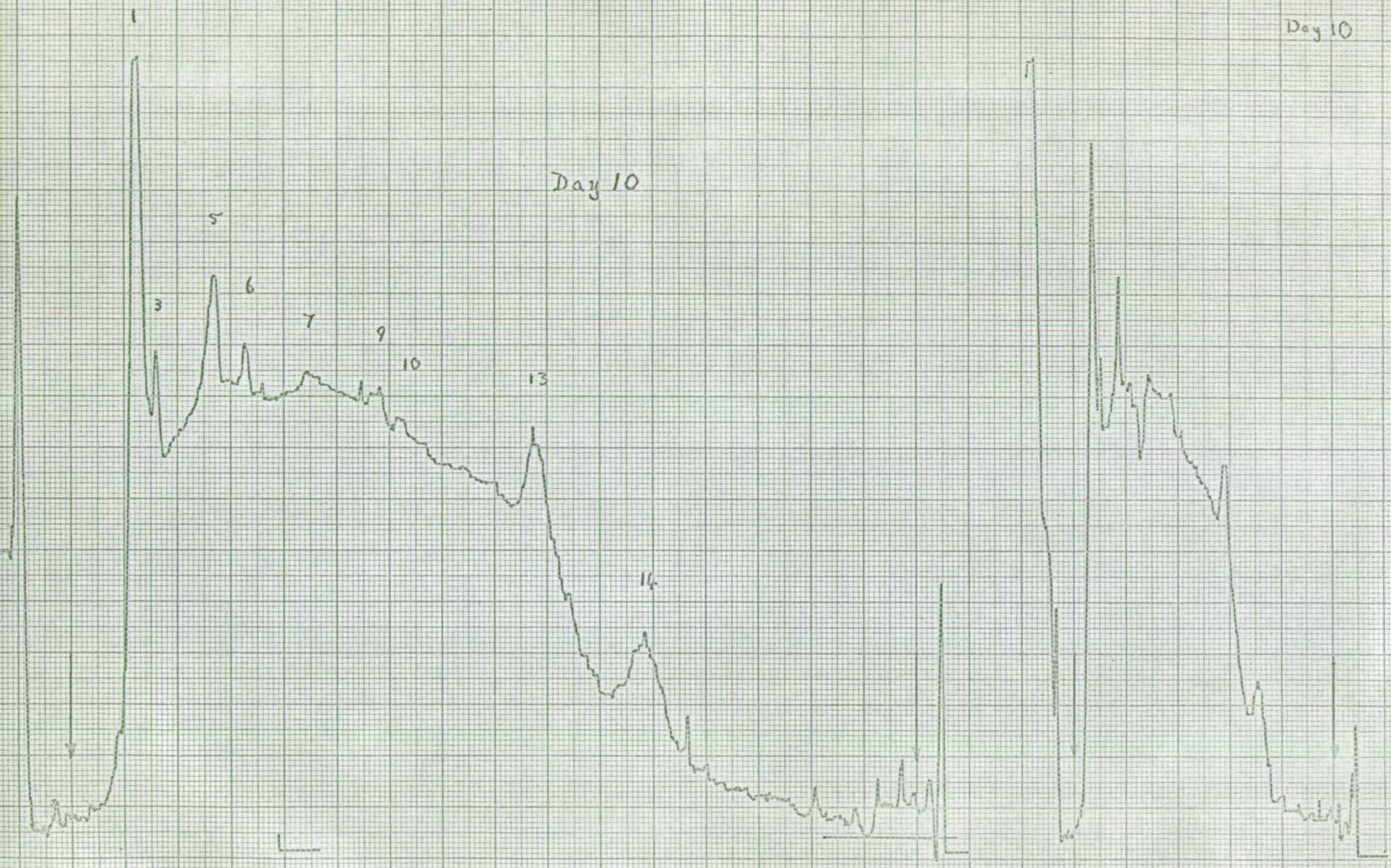
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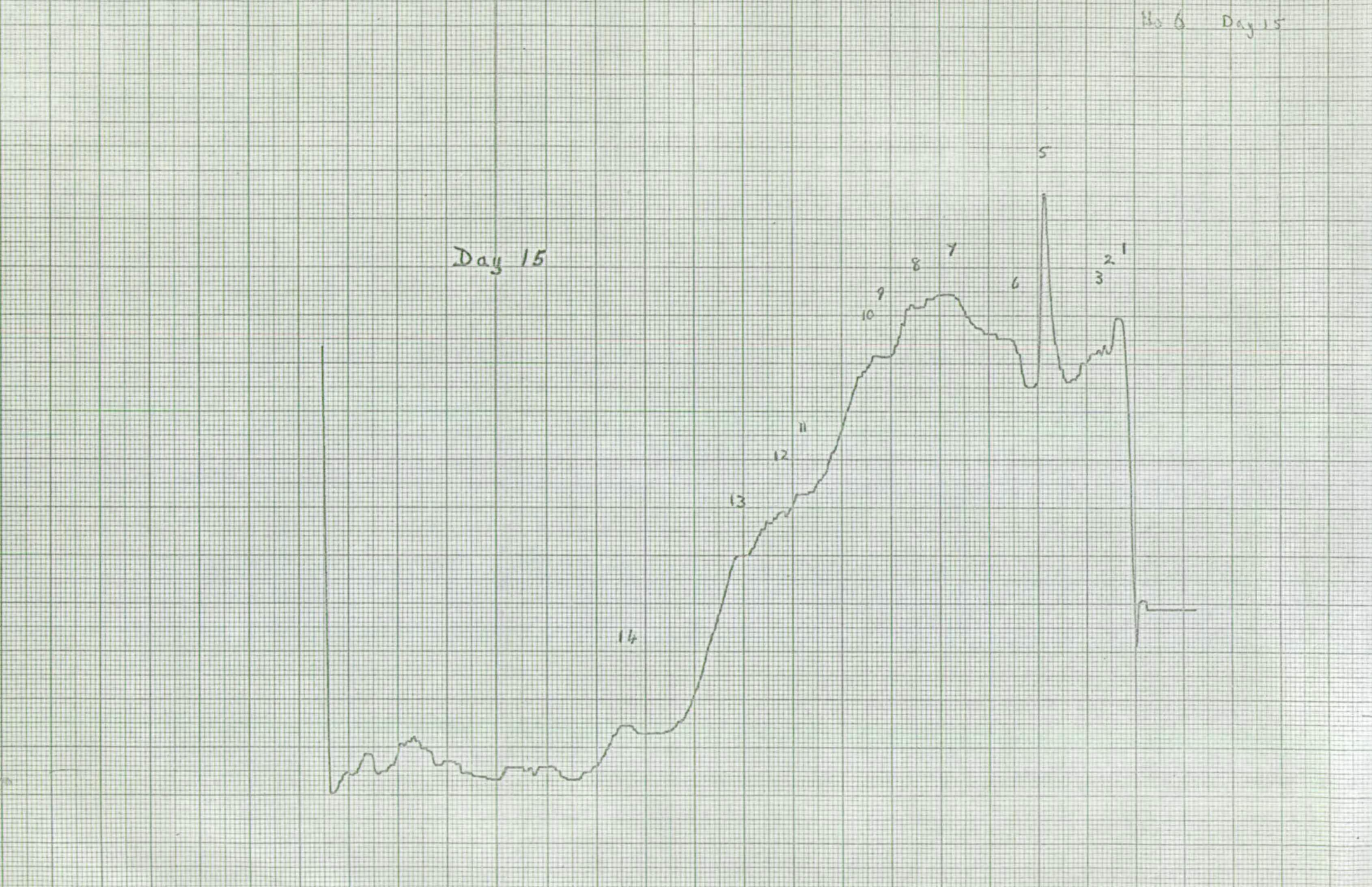
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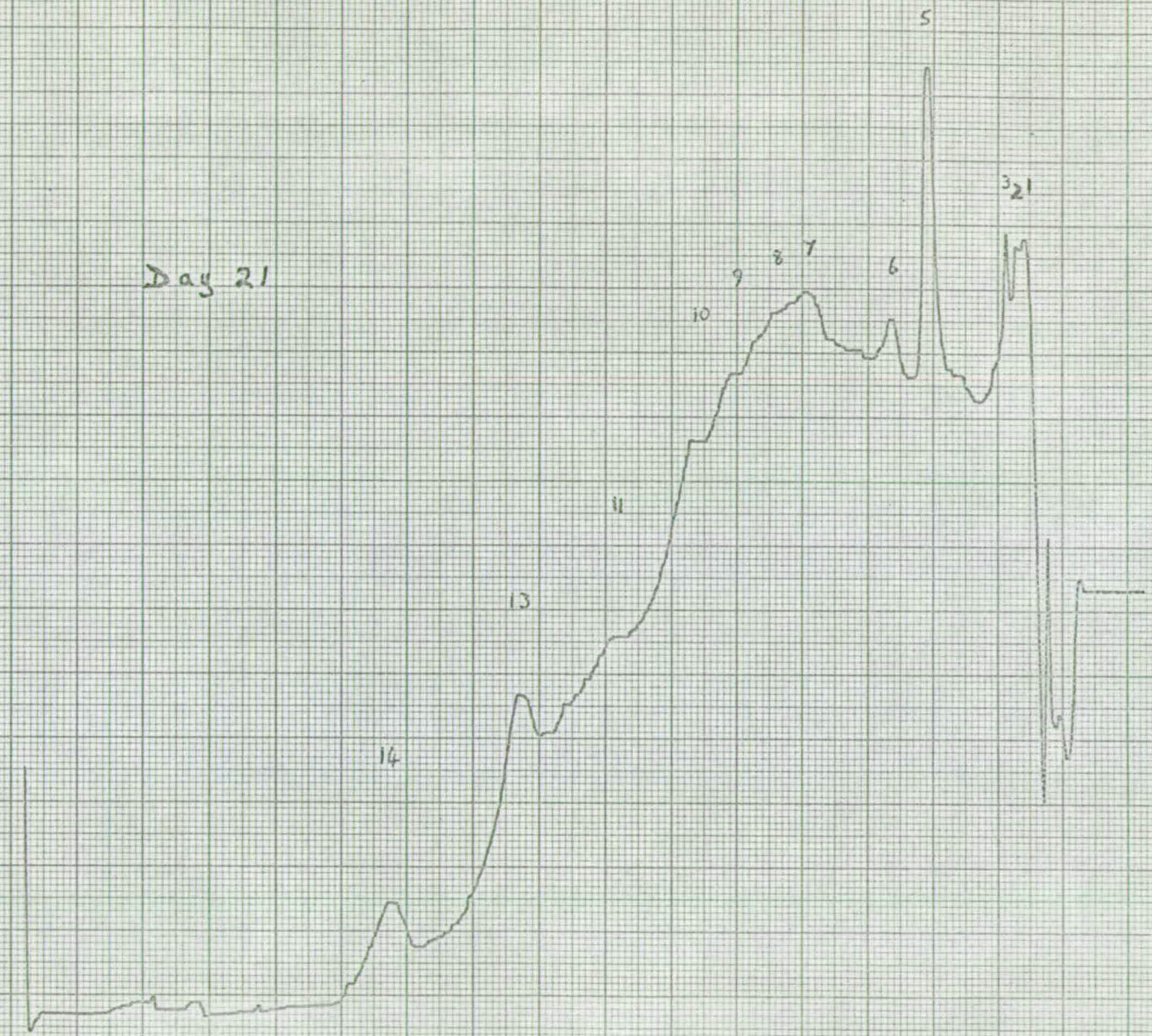


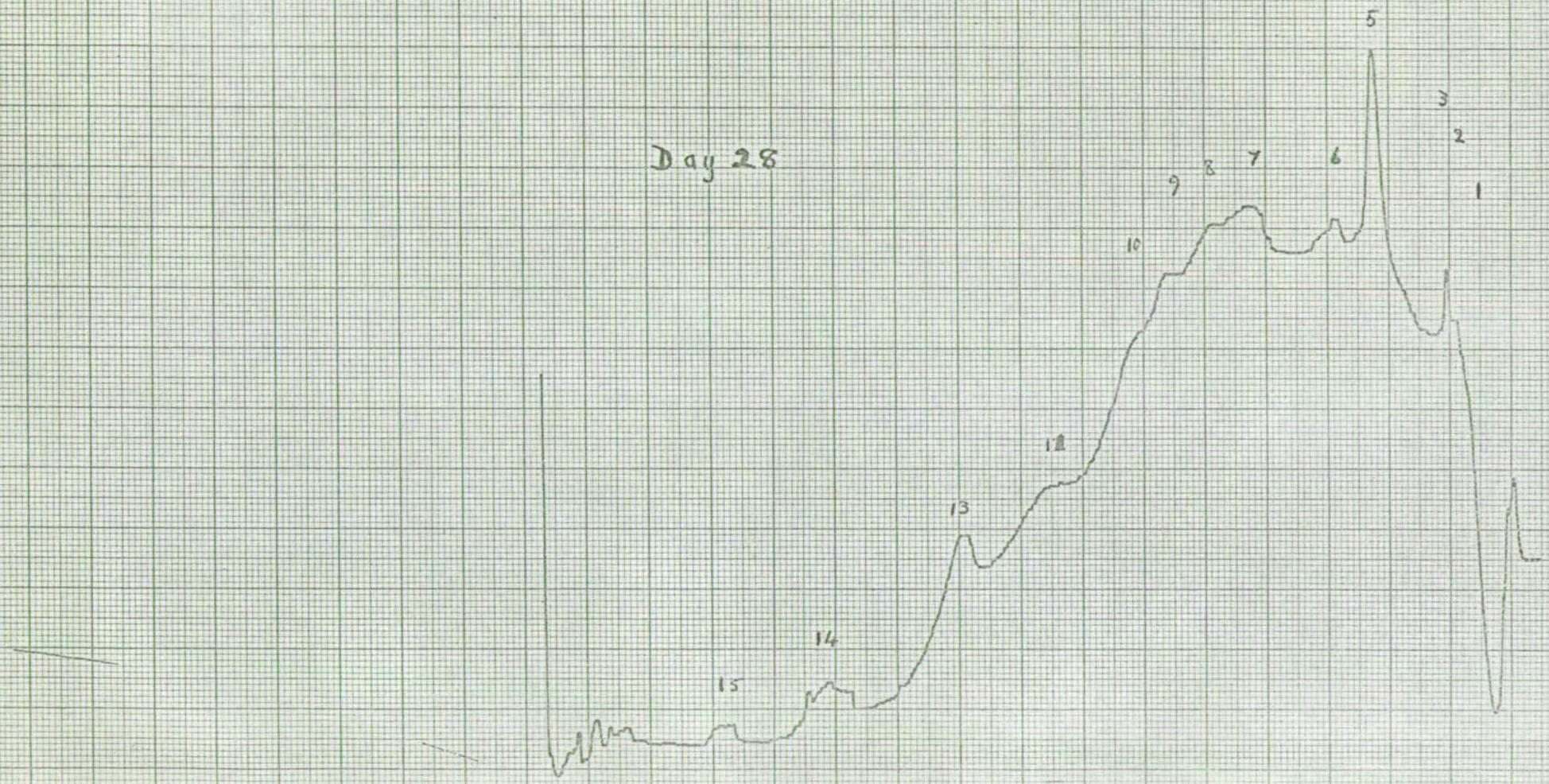
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Day 10 Day 10




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