Studies on the growth and development of the excised embryo of *Elaeis guineensis* in culture

Hilary Brenda Pritchard

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ABSTRACT

The aim of this project was to establish the growth and cytological characteristics of the oil palm, (Elaeis guineensis) in culture using the excised embryo as a primary explant system, and then to apply the obtained knowledge to study the induction and differentiation of the callus system. A method was developed for the culture of the excised oil palm embryo. The development of the excised embryo in culture was studied, paying particular attention to the measurement of cell cycle times, by cell number determinations, DNA cytophotometry and autoradiography. Anatomical, morphological, and biochemical changes were followed, together with a detailed investigation of the first twenty-four hours of growth, establishing that a population of nuclei undergo nuclear division without DNA synthesis. Variation in the rate of development of the embryo presented problems, but it could not be reduced by pre-treatments. The development of the embryo under callus-inducing conditions was also investigated. It was found that the callusing embryo is much less variable in rate of development than the excised embryo, but the variation was not reduced as a direct consequence of the pre-treatment or incubation medium. A brief anatomical and morphological study was made of a number of established callus lines, together with an analysis of the DNA content of nuclei and an estimate of cell size. The slow or normal callus cultures may be distinguished from the rapid cultures by the paucity of organised cell areas, whereas the callus which readily differentiates has many meristems throughout the tissue.

It was established that the excised embryo is the best primary explant available and the results of the study of excised embryo growth were discussed with respect to the data obtained from growth under callus-inducing conditions.

CONTENTS

CHAPTER I
INTRODUCTION

The oil palm, Elaeis guineensis, is a cocoid palm the fruits of which are an important commercial source of vegetable oil (Purseglove 1972). The oil, which contains a high proportion of saturated long chain fatty acids (Boatman and Crombie 1958, Opute 1975 and Yampolsky 1922) is present in both the mesocarp and the kernel. The commercial importance of this high yielding plant has stimulated considerable interest in developing breeding programmes for this very heterozygous monoecious species. These were started at the beginning of the twentieth century but as the time interval between the production of seeds in successive generations is approximately seven years the rate of reduction of variability by breeding has been very slow.

At the initiation of the project, the aim was to produce clones of a certain type of palm. This palm was designated "elite" and was characterised by very high yields of fruits and, hence, oil. However, it has since been established that an "elite" palm is not high-yielding due solely to genetic potential, for the large crops of fruit are at least partly due to environmental factors, such as spacing and better

fertiliser regimes. However, the plant must also, obviously, have the genetic potential for high fruit bearing capacity. Therefore it is still of considerable advantage to the grower if clones of oil palms can be produced, as this will allow the setting up of breeding programmes using material of known genetic potential.

Conventional means of vegetative propagation of the oil palm are impossible as the plant only reproduces sexually. As it is a monocotyledon, the meristematic regions are found associated with the apical meristem, which is generally found near ground level in plantation palms, or at the base of the leaves, which are associated with the "trunk". So far it has not been possible to "strike" roots from these leaves, or parts of the plant other than the apical meristem. Tissue culture techniques can provide a method of producing clones of oil palms. Explants from the designated palm can be induced to produce callus when placed in an appropriate nutrient situation and the resultant callus may be maintained and sub-cultured to accumulate large amounts of material. Plants can then be differentiated from the callus by alteration of the balance of growth hormones in the medium, although, these manipulations are only occasionally successful and apparently out of the control of the investigator. Such an experimental approach should allow the production of vegetative clones of oil palm.

This is the overall aim of the project undertaken in the Unilever Laboratories at Colworth House, and at Edinburgh. Since this investigation was started one vegetative clone has been established at Colworth and the plants differentiated from this clone have been transferred to a Malaysian plantation.
A series of major problems remain. Ideally the explant should be from vegetative parts of specified high yielding trees so that the genetic potential of the explant is known. Secondly, callus which grows rapidly should be routinely induced from the explant so that a lot of callus tissue may be accumulated quickly. Thirdly a method of routine differentiation of plants from the callus must be elucidated.

The choice of material for use as a primary explant is important. Primarily, the explant should be of known genetic composition, so that plants produced from the callus will have a known potential. This necessarily will limit the source of the explant to vegetative parts, ideally, the apical meristem, or leaf and root tissue. Tissue from the mature fruit (ie the embryo) is very unlikely to have the same genetic potential as the parent palm, because of the inherent heterozygosity and the almost obligate out-breeding nature of the palm. The explant should be uniform in structure and in response to culture and must be readily obtainable in sufficient quantities for definitive work to be carried out. It must also be sterile or capable of successful sterilisation so that axenic cultures may be established.

There are basically three sources of explants (a) from vegetative material of the mature palm, (b) from vegetative material of aseptically germinated seedlings (both consisting of either roots, leaves, or the apical region) and (c) embryos contained in the mature seed.

(a) The mature palm is a good source of material in the countries where palms are grown in profusion. However, supplies of such material are strictly limited in the United Kingdom. It should also be noted that the successful sterilisation of root and leaf tissue presents the investigator with considerable difficulties. The apical meristem of the shoot is naturally sterile and will remain so if the dissection of the meristem from the surrounding tissue is carried out under aseptic conditions. The cell type of the apical meristem is fairly uniform and the apical meristem provides an ideal explant system. However, removal of the terminal shoot meristem will destroy the palm and unless the production of clonal material can be guaranteed, this is not an economical source of explant material.

(b) Vegetative material can be readily obtained from aseptically germinated seeds. There is no sterilisation problem associated with this procedure , but there is a wide range of cell types, within the seedling. Here again the apical meristem is obviously the best source of material, but removal of the apical meristem will inevitably destroy the plant. Furthermore, the genetic potential of the seedling will not be known. The adventitious roots, radicle and leaves are of mixed cell type, and also of unknown, but identical, genetic potential which will not be useful for production of clones.

Another disadvantage is that the germination of the intact oil palm seed is very variable. This is probably an ecological adaptation for survival of the species, but makes the assembly of large amounts of tissue, at a similar stage of development, very difficult.

(c) In the United Kingdom the only regular source of vegetative oil palm material is the seedling from imported fruit. It is, however, more convenient and quicker to use excised embryos in place of sterile seedlings. The embryo is fully mature, sterile and 'non-dormant' in the ripe seed and so excision and culture of the embryo in a defined medium provides an excellent explant system. The tissue of the embryo is rather more uniform than that of the germinating seedling and the embryo is amenable to culture. Seeds are available in adequate supplies for study and these can be stored for limited periods of time. The only disadvantage in the use of the excised embryo as an explant system is that the genetic potential of the embryo is unknown, and the genetic variability between individuals which is a source of variation in all investigations with seeds and seedlings.

Taking all these factors into account, the best possible explant system that can be used in the UK is the excised embryo. As stated before, germination of the intact oil palm seed is very variable. In the plantations it is commercially desirable that a large proportion of the seed should germinate in a period of perhaps two weeks, so that seedlings at similar stages of development may be planted into nursery beds from each seed batch. Smith and Toovey (1938) considered that a good seed batch was one in which more than 60% of the seeds

germinated within six months and the germination percentage appeared to be genetically determined. Modern seed batches, that are sold commercially for restocking plantations, have a guaranteed 95% germination rate (Green pers. comm.) but the period over which germination takes place is not specified. The plantations have employed a number of methods of stimulating germination, including soaking techniques (Curtler 1926), heating in a greenhouse (Milsum 1927), the use of 'Biologically' heated germination beds (Galt 1953), charcoal heated germinators (Ferwerda 1956), barrels (Desneux 1959) and electrically heated germinators (Rees 1959a, 1962, Labro, Guénin & Rabéchault 1964). Much work has been carried out investigating various combinations of periods of heating and cooling. Pech, de Bilderling & Henry (1947) showed that seeds maintained at 37°C at maximum humidity and then placed for 24 hours into ambient temperatures (about 30°C) every two weeks after an initial period of 2 months at 37°C produced the highest rate of germination. Rees (1959a) showed that seeds maintained at high temperatures (39.5°C) germinated sporadically over a period of 3 months. However, if the seeds were maintained at 39.5°C for up to 70 days and then cooled to 27°C 50% would germinate within about 4 days. He also noted that the moisture content must be greater than 18% for germination (Rees 1959a) and if the seed was maintained as wet as possible, but without superficial moisture the germination was usually excellent (Rees 1959b). Labro, Guénin & Rabéchault (1964) extended the range of Rees' work. They maintained the moisture content of the seeds at around 21% but found that incubation at constant high temperatures (45-60°C) would not allow the seeds to germinate. However if the seeds were incubated at 60°C for up to 12 hours and then cooled, about 40-50% germination would follow.

Many of the problems of variability in germination of the intact seed may be overcome by excision of the embryo. Excision of the embryo also speeds up the rate of growth by removing the embryo from the effect of the inhibitor present in the endosperm. Thus the embryo has been used to study the growth of oil palm tissue in culture. The knowledge obtained from these studies can then be applied to study the growth of the embryo under callus-inducing conditions, and subsequently to study the growth of mature palm tissue, induction and growth of callus in mature palm tissue and finally the differentiation of plants from callus cultures.

One of the earliest papers relating to successful embryo culture was by Van Tieghem in 1873. He grew embryos of Mirabilis jalapa, replacing the endosperm with a paste of endosperm taken from other seeds of the same species or from the seeds of other species. He also showed that the paste made from the endosperm could be replaced by a starch paste which also contained mineral salts and phosphate. This work established that synthetic media could be used to replace endosperm and hence formed the basis of modern embryo culture. Hánnig (1904) made the first systematic attempts at embryo culture using synthetic media. These media contained sugars, mineral salts, plant extracts, amino acids, gelatin and degradation products of protein. With these, he succeeded in growing sterile cultures of Raphanus and Cochlearia embryos.

Dietrich (1924) noted that embryos not sufficiently developed for normal germination in the seed, could germinate in culture.

However, these cultured embryos tended to omit several normal stages of embryogenesis observed in the developing intact seed. Tukey (1938) reported that embryos which were excised from seeds which were not fully formed generally underwent abnormal development. He showed that the degree of abnormality was related to the age of the embryo, very young embryos being the most abnormal in culture. Rijven (1952) distinguished two forms of embryo growth in culture. He defined pregerminal cultures, in which the excised embryos are still undergoing embryogenesis requiring very specific growth media and conditions, and post-germinal cultures, where the excised embryos are fully developed and may be readily cultured on much less exacting media. Raghavan & Torrey (1964) consider that this distinction is not very clear and the definition of any culture ought to be based on physiological responses.

Cox, Stotzky & Goos (1960) excised immature embryos of the wild banana (Musa balbisiana Colla) and were able to culture these to the mature plant. They considered that this technique could be applied to the culture of commercially important varieties of banana.

Embryo culture has been employed extensively where interspecific breeding crosses are not viable because insufficient endosperm develops to support embryo growth. The immature embryo of these crosses may be excised and cultured on a suitable medium, which will take the place of the endosperm, and the embryo may be reared to a normal mature plant. Monocotyledons which are represented in the literature as being successfully reared by this method include Iris (Werkmeister 1934), Lilium (Skirm 1942),

Triticum durum X *Elymus arenarius* (Iwanowskaya 1946), maize (Uttaman 1949), *Hordeum jubatum* X *Secale cereale* (Brink, Cooper & Ausherman 1944), *Hordeum* (Davies 1960), paddy (Niles 1951) and rice (Butany 1958 and Nakajima & Morishima 1958). This technique obviously has great potential for the plant breeder, as it enables crosses, which were previously non-viable, to be grown and examined for combined improved characteristics.

Carew & Schwarting (1958) cultured rye embryos and initiated callus in these. The aim of their work was to re-initiate embryo-like structures and eventually plants from the callus. Rao (1963) gives many other examples of this technique.

The investigation described in this thesis falls into four main sections. The first section (Chapter III) is concerned with the problem of variability in and between seed batches, with respect to seed kernel and embryo size, rates of development, together with studies aimed at reducing variation by pre-selection and treatment of embryos.

The second section (Chapter IV) involves preliminary and more detailed studies on the growth and development of the excised embryo as a primary explant system in culture. This investigation is, mainly, concerned with the determination of cell cycle components using cell number, DNA, and tritiated thymidine labelling experiments, It also is concerned with morphological, anatomical, and biochemical changes which accompany the 'normal' development of the embryo.

The third section (Chapter V) is concerned with the growth of the excised embryo under callus-inducing conditions. Analysis is made of the cell cycle components by cell number, DNA, and tritiated thymidine labelling experiments and these are compared with the data obtained for the 'normally' developing embryo.

The fourth section (Chapter VI) involves a very brief study of a number of established callus lines with respect to DNA content, morphology, anatomy and cell size as a preliminary study towards the elucidation of the reason why different callus lines have varying and especially long cell cycle times.

The results determined in each main section are discussed (Chapter VII) with respect to each other and the relevant literature.

CHAPTER II

Materials

The material used throughout this investigation was that of the Oil Palm, Elaeis guineensis Jacq. Fig 2.1 shows a number of seeds.

Seeds used were from dura x pisifera crosses and were from various Unilever Ltd Plantations (Table 2.1.) The mesocarp was removed and the kernels packed in heavy guage polythene bags for transport by air to Edinburgh. The seeds were not heat-treated. As soon as the seeds were received they were stored in glass jars with screw lids, to slow down water loss, at 25°C. Temperatures below 15°C, or dry conditions for periods longer than a few days, render the seeds non-viable.

Seeds of different crosses were not mixed in any experiment, because of the variability between batches.

Callus cultures of various ages were kindly supplied by Unilever Laboratories at Bedford. These cultures had differing histories,(table 2.2,) but were all originally obtained from parts of seedlings which had been germinated and grown under aseptic conditions.

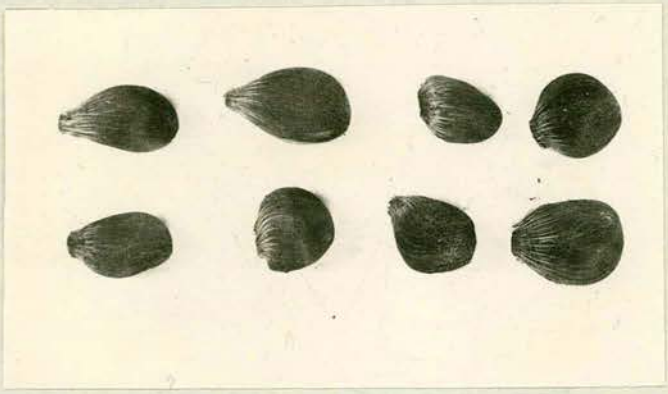


Fig. 2.1 Oil Palm 'seeds'

Fig. 2.2 McCartney bottle

Table 2.1 Origins and History of Seed Batches

Batch No.	Source	Viability Infection	Dates Used	Heat Treatment	Harvest Date
6336 2/3028 x 2/8607	Bedford	-	Nov 73	✓	?
6382A 2/9806 x 2/11222	"	Very good	Dec 73-Jan 74	x	?
6362. 2/8612 x 2/11222	"	-	?	x	?
6378A 2/1314 x 2/0114	"	Very good	Jan-Mar 74	x	?
M08656A 2/7923 x 2/0114	?	-	May-June 74	x	?
7406/0	Uni-Benin Nigeria		Aug 74	x	?
08588659292	Unilobe	Very good	Jan-Mar 75	x	?
2/5616 x 2/11605	"	Bad	Mar 75	x	4.10.74
2/5742 x 2/11605	"	"	July 75	x	31.1.75
2/10414 x 2/11605	"	-	Aug 75	x	
2/5523 x 2/8607	"	Good	Nov 75	x	16.9.75
2/5703 x 2/11605	"	-	Feb 76	x	23.12.75
2/5610 x 2/8607	"	-	Jan 76		18.11.75

Table 2.2 Origins and history of the established callus cultures supplied by Unilever Laboratories

Culture P114L derived from P07321 17.8.70 normal

History

Date	Medium	CM	SUC	CA	2,4-D	NAA	BAP
17.8.70	A.M&S	5%	2%	1000 mg	10 mg	-	-
26.5.71	"	"	"	"	-	5 mg	-
29.3.72	"	"	"	"	5 mg	-	-
28.6.72	"	"	"	"	-	3 mg	-
8.9.72	"	"	"	"	3 mg	-	-

Culture P114M31 derived from P114L 14.11.75 normal

History

Date	Medium	CM	SUC	CA	2,4-D	NAA	BAP
14.11.75	A.M&S	5%	2%	1000 mg	3 mg	-	-

Culture T003A27 derived from P123R/3 13.9.74 rapid

History

Date	Medium	CM	SUC	CA	2,4-D	NAA	BAP
13.9.74	L.M&S	5%	2%	1000 mg	3 mg	-	-
21.4.76	A.M&S	"	"	"	"	-	-

Cultures P182A49 and P182A65 derived from P123R/3 13.9.74 through T003A27. Rapid.

History

Date	Medium	CM	SUC	CA	2,4-D	NAA	BAP
16.12.75	L.M&S	5%	2%	1000 mg	3 mg	-	-
8.1.76	A.M&S	-	4%	1000 mg	-	5 mg	0.1 mg
5.3.76	A.M&S	-	5%	500 mg	-	2 mg	"

Culture P171D67A from kernel grown on tap water agar with the methyl ester of p. hydroxybenzoic acid. Rapid

History

Date	Medium	CM	SUC	CA	2,4-D	NAA	BAP
21.2.75	A.M&S	-	4%	500 mg	5 mg	-	-
28.5.75	"	-	5%	"	-	2 mg	0.1 mg
23.7.75	"	-	"	"	-	"	"
3.9.75	"	-	"	"	-	0.5 mg	0.05 mg
11.11.75	"	-	"	"	-	2.0 mg	0.1 mg

Culture P171D 97D-A from P171D 67A

History

Date	Medium	CM	SUC	CA	2,4-D	NAA	BAP
19.1.76	A.M&S	-	5%	500 mg	-	2 mg	0.1 mg
13.4.76	A. Nitches	-	4.5%	1000 mg	-	-	0.1 mg

SUC sucrose
CM coconut milk
CA caesamino acid
BAP benzyl amino purine

MS Murashige, Skoog
L liquid
A agar

Methods

I. General Methods

A. Preparation of Materials and equipment

1. Equipment

Cross-contamination of material is a problem in sterile culture, particularly so in the oil palm where more than 50% of a batch of seeds may have endogenous infection. To prevent cross-contamination a large number of small containers are required for the incubation of individual excised embryos. These must be transparent, so that light can reach the embryo and must be sterile or capable of withstanding sterilisation. 100 ml conical flasks are too large because of limited incubation space and boiling tubes require support to hold the tubes at a reasonable angle for the most efficient use of incubator space. 3 cm sterile petri dishes dry out too quickly at the high incubation temperatures. McCartney bottles are a convenient size, stand without support and can be resterilised (see Fig 2.2). The rubber discs inside the lids must be removed as they contain a substance inhibitory to plant growth which is washed down into the medium during sterilisation. These bottles can contain 5 or 10 ml of medium without reducing the air volume to levels which limit normal growth-Smith (pers.comm).

All glass ware was thoroughly cleaned before use. The McCartney bottles were scrubbed inside with detergent such as teepol, rinsed twice with running tap water, once with distilled water and dried in a hot air oven.

All the equipment and media used was autoclaved at 15 pounds per square inch for 20 minutes.

2. Sterilisation of Materials

The sterilisation procedure described by Rabechault (1962) was used on seeds where the embryo was to be excised. This procedure assumes that the viable embryo will be inside sterile endosperm, hence the sterilisation technique is to remove infection from the outside of the kernel and to prevent it being introduced into the embryo. See Fig 2.3 and Fig 2.4 for fruit structure.

The shells were cracked with a hammer and those which were damaged, or obviously infected, discarded. The surface of the kernel was sterilised by three minutes immersion in sodium hypochlorite (BDH) (12% free chlorine) before being transferred to sterile distilled water. Kernels that floated in distilled water were discarded, as they were always infected when broken open. Embryos were then excised from the 'stock' of kernels in sterile distilled water.

Seeds which were to be germinated with the embryo still inside the endosperm, but with the operculum removed, were sterilised by a second method. The shells were cracked and any seeds which were infected, or where the testa was damaged, were discarded. The kernels were soaked in 100 volume hydrogen peroxide for 1 hour at room temperature. Hydrogen peroxide is an efficient sterilising agent and decays rapidly to non-toxic oxygen and water. There is some indication that the hydrogen peroxide treatment could stimulate germination by the oxidation of an inhibitor in the endosperm (Smith pers. comm) and make it more uniform. Disposable plastic gloves were used in the handling of the sterilised kernels. The kernels were held between the thumb and first finger and the

Fig 2.3

Fruit Structure (minus mesocarp)

Fig 2.4

Position of embryo in fruit

fp	fibre plug	gc	germination channel
o	operculum	t	tigellum
em	embryo	h	haustorium
ec	endocarp	es	endosperm
te	testa		

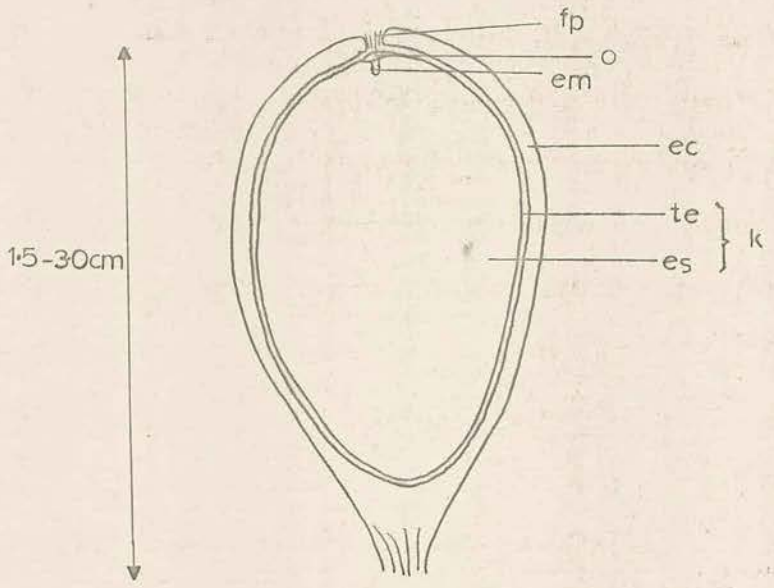


Fig. 2.3

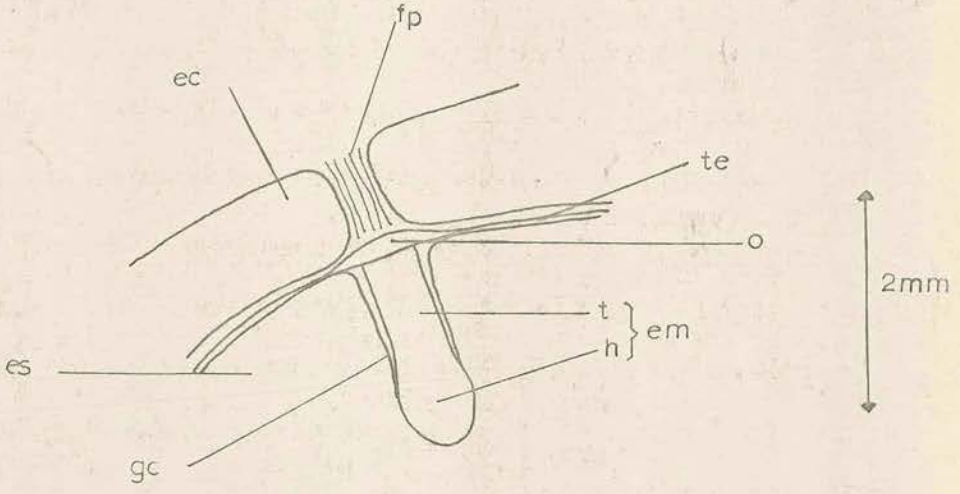


Fig. 2.4

operculum lifted off using a sterile single edged razor blade.

3. The use of the sterile culture room and sterile techniques

Sterilisation of kernels, excision, inoculation and weighing of tissue took place in a sterile room. The room was sterilised by two ultra-violet lamps and bench surfaces were swabbed with alcohol before use. The air in the room was filtered and maintained at a slightly positive pressure to prevent inflow of air. Conventional flaming techniques were used in all procedures.

B. Culture Medium

1. Basic medium

Comparisons of various media had been made by Rabechault, Guenin and Ahee (1970) and Jones and Dethan (1973) under various light and temperature regimes. The results indicated that a high salts medium such as Murashige and Skoog's was required to support optimum growth of excised embryos. Bufford-Morel (1968) and later Dethan (unpublished) established that the optimum sucrose content for Murashige and Skoog's medium is 30 gml^{-1}. Jones and Dethan (1973) established that 1000 mgl^{-1} caesin hydrolysate was required to supply an adequate amino acid mixture. It would be possible to spend months or years defining the perfect medium for the growth of excised Elaeis embryo without obtaining any details of actual growth patterns. It was therefore decided to accept the work already carried out on the media as a base level from which to extend knowledge of the growth of Elaeis.

In all experiments Murashige and Skoog's medium 'A' salts were used modified by the addition of 1000 mgl^{-1} caesin hydrolysate (table 2.3) (Murashige and Skoog (1962)). (Sigma enzymatic)

Table 2.3 Murashige and Skoog "A" salts

$MgSO_4 \cdot 7H_2O$	370 mgl^{-1}
$CaCl_2 \cdot 2H_2O$	440
KNO_3	1900
NH_4NO_3	1650
KH_2PO_4	170
$FeSO_4$	27.8
$MnSO_4 4H_2O$	22.3
KI	0.83
$CoCl_2 \cdot 6H_2O$	0.025
$ZnSO_4 \cdot 7H_2O$	8.6
$CuSO_4 \cdot 5H_2O$	0.025
H_3BO_3	6.2
$Na_2MoO_4 2H_2O$	0.25
Sucrose	30 gml^{-1}
Caesin hydrolysate	1000 mgl^{-1}
EDTA disodium salt	37.3

2. Additives

For normal embryo growth the organic constituents of Murashige and Skoog's medium (Table 2.4) were added to the 'A' salts. Indole acetic acid (IAA) was supplied at 1mgl^{-1} and kinetin at 0.5 mgl^{-1}. These concentrations had been established as optimum for excised embryo growth, by Dethan (unpublished). The media used for the induction of callus was modified by the further addition of 5 mgl^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D).

Table 2.4 Additives

	Glycine	2.0 mgl^{-1}
	Myoinositol	100
	IAA	1.0
Aneurine HCL,	Vitamin B_1	0.1
Pyridoxine,	Vitamin B_6	0.5
	Nicotinic acid	0.5
	Kinetin	0.5
[2,4-D]		[5.0] [2.0]

The medium was adjusted to pH 5.7 to 5.8 using M potasium hydroxide.

3. The use of solid or liquid medium

Preliminary experiments were carried out using Murashige and Skoog's medium, which had been solidified by the addition of 8 gml^{-1} Difco agar. Balanced growth of excised embryos was obtained but there was a tendency for browning to occur on the haustorium. Decreasing the agar concentration to o.5% in many cases made the browning worse.

Work carried out by Baliga and De Guzman (1971) on coconut showed that excised embryos could be successfully cultured on liquid medium and subsequently transferred to solid medium.

Following this suggestion, oil palm embryos were cultured in liquid Murashige and Skoog's medium. Balanced plants were produced, visible germination being about 24 hours earlier than on solid medium, and with much less associated browning of tissue. The majority of experiments were carried out using liquid Murashige and Skoog's medium.

4. Medium for the culture of kernels

Where embryos were germinated while still in the endosperm but with the operculum removed they were incubated on 0.6% tap water Difco agar in McCartney bottles. All the nutrients required for germination are supplied by the endosperm.

5. Medium for the culture of established callus

The mature callus cultures supplied by Unilever Ltd, were grown on Murashige and Skoog's medium or Nitche's medium (half strength Murashige and Skoog 'A' salts) modified by the addition of 2,4-D, NAA or BAP at various concentrations specified in table 2.2.

6. The addition of tritiated (^{3}H)-thymidine

In experiments where the incorporation of ^{3}H-thymidine into the DNA was to be followed the label was added to the liquid Murashige and Skoog's medium before autoclaving to give a final concentration of 1μCi ml^{-1}. The ^{3}H-6-thymidine, obtained from the Radiochemical Centre at Amersham, was supplied with a specific activity of 5.0 Curies/m mol (20.7 mCi/mg) and a radioactive concentration of 1.0 mCi/ml.

C. Culture Techniques

1. Excision

Various methods of excision were attempted. The most rapid was that of Rabechault (1962) and when modified became the most accurate. Disposable plastic gloves were worn. The sterilised kernel was held between the thumb and first finger of the left hand and the operculum lifted off using a sterile single edged razor blade. The endosperm was then cut away around the tigellum until the complete embryo was exposed and could be lifted off the endosperm into a petri dish of sterile water.

2. Inoculation

a) Normal embryo germination

The embryos were weighed (see Analytical Methods) and put into Murashige and Skoog's medium in McCartney bottles using sterile forceps and conventional flaming techniques. The lids were screwed down firmly to prevent too rapid evaporation of the medium. No aeration was supplied as the air volume in the bottles is sufficient for the duration of the experiment (Smith pers.comm.).

b) Callus induction

The embryos were weighed then immersed in 10 volume hydrogen peroxide (Prelim. expts. see chapter 3 section E 2d on variability) for 5 minutes before being put into Murashige and Skoog's medium, modified by the addition of $5mgl^{-1}$ 2,4-D, in McCartney bottles using sterile forceps and conventional flaming techniques.

3. Incubation

Work at the Unilever Laboratories indicated that high incubation temperatures are required for the growth of excised

embryos ,33°C appears to be the optimum. Temperatures above 40°C for prolonged periods are lethal and temperatures below 27°C support only slow embryo growth which is frequently unbalanced.

Preliminary experiments showed that light is not required for the first part of embryo development, but is required for the chlorophyll synthesis and subsequent normal balanced development. The components of the light regime do not appear to be important, so a 12 hour light : 12 hour dark regime was chosen as it is similar to the in vivo situation.

Thus the conditions were established as 33°C, in a 12 hour light : 12 hour dark regime. These were maintained at 4000 lux in a Gallenkamp incubator, which was static. Shaking the embryo tended to produce deformed plants which had not undergone normal development. The established callus cultures were maintained under continuous light conditions at 25°C in a growth room.

4. Colchicine treatment

Preliminary experiments were carried out to establish what concentration and exposure length was optimum for excised embryos. This established 0.01% colchicine as optimum when exposed for between 6 and 12 hours. At higher or lower concentrations and for greater exposures the arrested cells could break through the inhibition. For shorter exposures only small numbers of mitotic figures were found.

Colchicine was pipetted into the McCartney bottles, which

contained the incubated embryos, to give a final concentration of 0.01% (5×10^{-5}M) and left for an exposure of 6 to 12 hours before the embryos were sampled and fixed.

D. Sampling

At the appropriate sample time embryos or callus induced from embryos were taken at random from the incubator. The sample was drained on a piece of tissue paper to remove surface moisture and was weighed to the nearest 0.1 mg on a torsion balance. If the sample was to be used for an estimation of cell number it was immersed in 5% chromic acid at least overnight. If the sample was to be used for the measurement of any other growth parameter or for an anatomical investigation it was fixed in FAA for 12 to 24 hours before being transferred to 70% ethanol for storage.

In preliminary and the callus induction experiments the embryo was studied as a whole. In later experiments it was obvious that the haustorium and tigellum had differing roles, and the embryo was divided into the tigellum and haustorium at the time of sampling in order to follow the different paths of development. The division between the two portions is clearly visible and the cut can be made accurately with a razor blade.

II Analytical Methods

A. Fresh Weight.

The measurement of fresh weight is a convenient growth parameter to assay. It is useful in the study of the growth of the oil palm as it is non-destructible and can be measured

repeatedly on the same individual.

Fresh weights were established under sterile conditions using a torsion balance which had a scale of 0 to 250 mg by 0.5 mg divisions. The balance was kept in the sterile room under the two ultra violet lamps. The pan of the balance was swabbed with ethanol at the beginning of each set of weighings.

The tissue was removed from the McCartney bottle using sterile forceps and conventional flaming techniques. It was blotted dry on tissue (Kleenex medical wipes) and lifted onto the balance pan using forceps. The weight was determined, estimating to the nearest 0.1mg. The tissue was rinsed in sterile distilled water before being returned to the McCartney bottle.

Fresh weight determinations of populations at various sampling times were carried out by the same method but under non-sterile conditions and omitting the rinse in sterile water.

B. Cell Number

The measurement of cell number in tissues uses a destructive technique that gives clear indications of when cell division is taking place.

Cell numbers were estimated by the method of Brown and Rickless (1949). Each embryo or piece of callus tissue was immersed in 2ml of 5% aqueous chromic acid at least overnight at room temperature and up to 6 days in a refridgerator. A cell suspension

was produced by gently pumping the tissue in and out of a Pasteur pipette. If required, the suspension was diluted with distilled water to a counting level of about 200 to 250 cells per field. This level of dilution gives a statistically significant count on a haemocytometer. A mean value of cell counts was obtained from six grid counts and the cell number per piece of tissue calculated by the formula:

$$\frac{\text{Mean cell count} \times 3.2}{\text{Vol. of cell suspension (ml)}}$$

C. Microdensitometric measurements

The Barr and Stroud integrating microdensitometer can be used to measure the relative absorption of stains in sections or squashes of tissue at fixed wavelengths. In this investigation deoxyribosenucleic acid (DNA), total nucleic acid (TNA) and protein components were estimated in oil palm tissue using Feulgen, gallocyanin and 2,4 —dinitrofluorobenzene (DNFB) stains respectively. Feulgen and DNFB stains can be used quantitatively on the same section or squash (Mitchell (1967)).

1) Feulgen Staining

This was carried out by the method shown in Table 2.5.

The Feulgen staining technique can be considered quantitative if this method is closely adhered to. The reaction is dependant on the acid hydrolysis of purine deoxyriboside bonds of the DNA releasing free aldehyde groups which accept the Schiff reagent and produce the characteristic purple colouration. However, DNA is destroyed by acid hydrolysis and hence there must be a compromise between the

length of hydrolysis causing the maximum release of aldehyde groups and the least destruction of the DNA. It must be noted that aldehyde groups other than on the DNA will be released by prolonged hydrolysis.

Preliminary experiments were carried out to establish the optimum hydrolysis time. Embryos were excised and stained by the Feulgen staining procedure detailed in table 2.5 - but varying the length of step 2 from 6 to 16 minutes. The slides were examined by the light microscope. Slides with hydrolysis times of 6 and 8 minutes hardly stained, whereas hydrolysis times of 14 and 16 minutes had clear cytoplasmic staining. The optimum time of hydrolysis can be distinguished using the integrating microdensitometer. The DNA content of 40 nuclei was estimated in the samples which were hydrolysed for 8, 10 and 12 minutes (Fig 2.5). These clearly show that at 8 minutes not all of the possible aldehyde groups have been released for staining and at 12 minutes there is more breakdown of the DNA by acid hydrolysis. This establishes 10 minutes as the optimum hydrolysis time in oil plam embryos using M Hydrochloric acid at 60°C.

A major problem was the comparison of DNA values between various staining batches due to the infrequency of mitotic figures which are generally used as standards. To counteract this, the timing of the staining schedule was strictly adhered to and a standard tissue such as Jerusalem artichoke tuber, was stained in the same batch. Bags of nylon net were used so that up to 15 different sets of tissue could be stained at exactly the same time. This made movement of the tissue between treatments easier than when using staggered starts as had to be done with sections on slides.

Table 2.5 Feulgen staining procedure.

1.	Transfer tissue from 3:1 ethanol:acetic acid fixative to 70% ethanol then to 50%, 30%, distilled water, 10 minutes each.
2.	Place tissue in preheated N HCl($60^{\circ}C$) for 10 minutes exactly.
3.	Transfer to distilled water.
4.	Transfer to Feulgen reagent for 2 hours. (Schiff reagent from BDH chemicals.)
5.	Transfer to distilled water for 1 hour.
6.	Transfer to distilled SO_2 water for $\frac{1}{2}$ hour. (5gm sodium metabisulphite + 5ml N Hydrochloric acid made up to 100ml with distilled water.)
7.	Place tissue on a slide and tap it out using a brass or teflon rod.
8.	Place coverslip on top of tissue and squash.
9.	Place slide on dry ice block until tissue freezes.
10.	Lift off coverslip with a razor blade.
11.	Air dry slide.
12.	Mount coverslip in Canada Balsam.

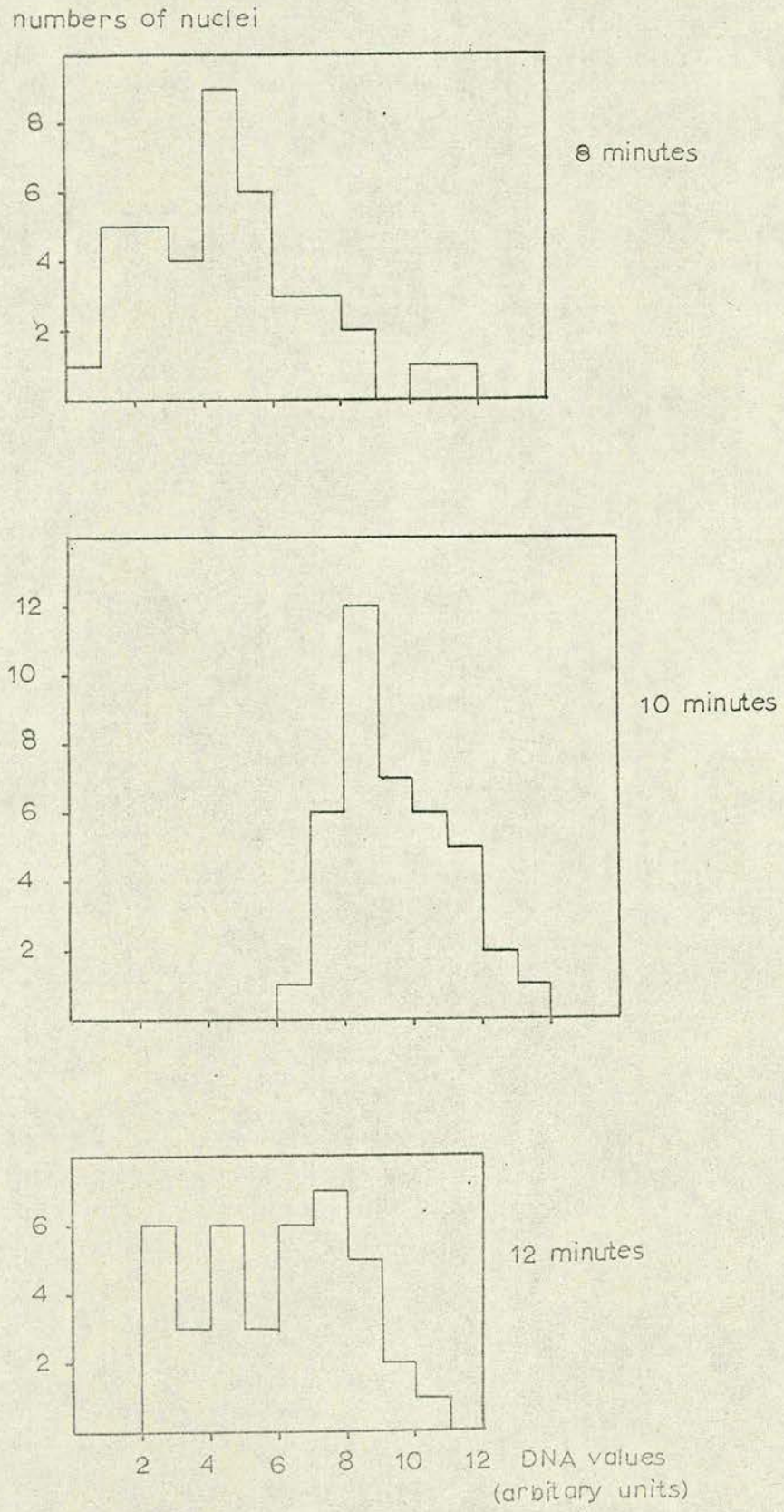
Table 2.6 Dinitrofluorobenzene staining technique for protein estimation

1.	Embed tissue in paraffin wax.
2.	Cut 10µm sections and mount on slides.
3.	Bring sections to water.
4.	Place slides in DNFB reagent for 24 hours at room temperature.
5.	Wash slides in 95% ethanol. 3-4 washes each 2-3 minutes.
6.	Rinse in distilled water.
7.	Place sections in 5% sodium hyposulphite for 40 minutes at $40-45^{\circ}C$.
8.	Rinse in distilled water.
9.	Carry out Feulgen staining procedure steps 2 to 6 inclusive.
10.	Dehydrate tissue and mount.

Table 2.7 Gallocyanin staining technique for total nucleic acid estimation

1.	Embed tissue in paraffin wax.
2.	Cut 10µm sections and mount on slides.
3.	Bring sections to water.
4.	Stain in gallocyanin-chromealum for 24 hours.
5.	Rinse for 1 minute in distilled water at pH1.6 (acidified with HCl).
6.	Repeat rinse until no further dye can be removed.
7.	Dehydrate in alcohol, clear and mount.

Fig. 2.5 Microdensitometry of nuclei hydrolysed for varying periods



The relative DNA content of nuclei was established by measuring the absorption of light at 565nm using x 100 magnification oil immersion lens of the Barr and Stroud Integrating microdensitometer. The aperture size used was the area just larger than that of the nuclear area. Areas of cytoplasm next to each nucleus were measured, using the same aperture size, as background values. Measurements were taken along random transects of sections and squashes.

When sections were used care was taken to ensure that only intact and isolated nuclei were measured. Each nucleus to be measured was examined and those where there was any doubt as to whether it was intact were discounted. Any nuclei which were overlapping throughout the whole thickness of the specimen were also discounted as the microdensitometer is capable of registering readings for nuclei which are out of focus, although the readings will not be optimal. The results obtained using squashes were equivalent to those from sections.

2. Protein Measurements.

The staining procedure is described in table 2.6.

Sections that were to be stained for protein estimation were dried down onto slides that had been scrubbed with Vim, rinsed in distilled water and alcohol. Section adhesive was not used because either it contained protein (glycerin albumen) or it was hydrolysed by HCl (starch).

Measurements of the absorption of DNFB at 400nm were made using x45 magnification oil immersion lens with an aperture of just over

3,000μm^2. The background value was taken as a reading given by the slide alone. Adjacent fields of view were measured so that the total value for each embryo could be calculated, or maps of the distribution of various absorption values could be plotted (see Fig 2.6).

3. Total nucleic acid measurements

The staining procedure is described in table 2.7.

Measurements of the absorption of gallocyanin were made at 575nm using the same technique as for the measurement of protein.

D Radioactive Techniques

Autoradiography

Tissue was pulse and continuously labelled using tritiated thymidine added as detailed in the section on culture techniques. Autoradiographic techniques were used to establish that tritiated thymidine (^{3}H-thymidine) is incorporated into the DNA.

The tissue was stained using the Feulgen technique and then was either embedded in paraffin wax for sectioning or was squashed on slides. The slides had been scrubbed with Vim then rinsed in distilled water and absolute alcohol and dipped in a chrome alum solution (5gm gelatin, 0.5gm chrome alum made up to 1 litre with distilled water) which had aged at least overnight. 10μm wax sections were cut and floated onto the slides. They were allowed to dry down for at least three days on a heated tray before the wax was removed by immersion in xylene. The sections were hydrated through a decreasing series of alcohol and left to air dry. Squashes were allowed to air dry, and both sections and squashes were used in the liquid emulsion

Fig 2.6

Diagram to show the method of measurement of adjacent fields of view over a section of an embryo for gallocyanin and DNFB assessment

Fig 2.7

Two worked examples of probit analysis. Abscissae DNA values (arbitrary units). Ordinates right-probit value

left-number of nuclei per class

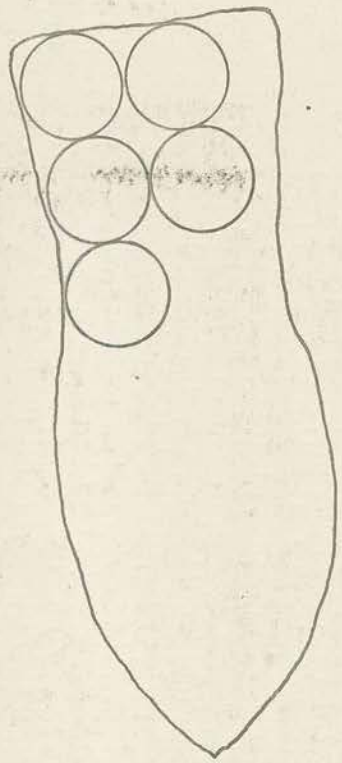
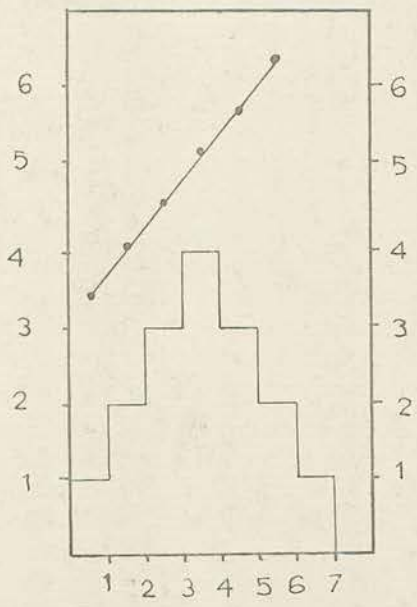
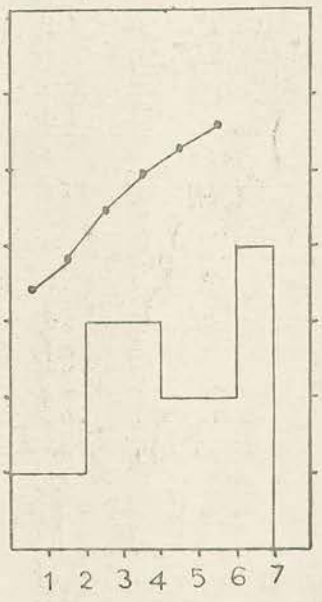


Fig. 2.6

Fig. 2.7





technique of autoradiography. All operations were carried out using an Ilford K904 (dark brown) safelight. The technique is as follows:-

A glass specimen tube was $\frac{2}{3}$ filled with the gel form of Ilford K2 emulsion. The emulsion was melted in a waterbath at 55°C and diluted with half its volume of distilled water. The slides were individually dipped into the emulsion for 30 seconds, drained on filter paper, placed on a drying tower and dried using a hairdrier. When dry the slides were placed in a light-tight box containing silica gel and exposed for 14 days at 4°C. The emulsion was developed using Ilford ID19 developer for 6 minutes and fixed with Ilford Hypana fixer for 6 minutes. The slides were washed with running tap water for two hours and allowed to air dry before mounting in Canada Balsam.

The percentage of labelled nuclei on each slide was estimated by counting the number of labelled and unlabelled cells along random transects using a x 50 magnification oil immersion lens.

E. The Measurement of colchicine-induced mitotic indices

When the embryos had been exposed to colchicine for 6 to 12 hours they were removed from the culture medium, fixed in 3:1 ethanol:acetic acid for 12 to 24 hours and then transferred to 70% ethanol. The tissue was Feulgen stained and either embedded in paraffin wax for cutting 10µm sections, or squashed. The squashes were divided into tigellum and haustorium. The percentage of arrested mitotic figures in each embryo was estimated by counting a population of nuclei along random transects of the sample.

F. Anatomy

1. Anatomy of the developing embryo system and the developing callus system was followed. Samples were taken at various times during the period of development and fixed in FAA for 12 to 24 hours before embedding in paraffin wax. 10µm wax sections were cut using a hand microtome and stuck on slides using glycerin albumen adhesive.

Sections were stained by the method in table 2.8.

Photographs were taken using a x 40 magnification lens of a Zeiss microscope.

2. Scanning Electron Microscopy

The fresh specimen was surface dried, placed on an aluminium stub and glued to it using a gelatin solution. The whole stub was immersed in liquid nitrogen until specimen was frozen solid, when it was ready for insertion into the scanning electron microscope.

G. Probit Analysis

Probit analysis is a form of assessment by which means, on probability grounds alone, population data can be examined and sub-populations within the total distinguished. If there is one population of values in given data a normal distribution curve will be constructed. If there is more than one population of values within the total data the curve will deviate from normal. Probit transformation converts a normal distribution curve into a straight line. The probit value of 5.0 will correspond to the overall mean of the population. The reciprocal of the slope is equal to the standard deviation of the population, hence the smaller the angle of slope from the vertical the

Table 2.8 Safranin and Light Green Staining Schedule

1.	2 washes in xylene.
2.	1 wash in absolute alcohol 5-10 minutes.
3.	1 wash in 95% alcohol 5-10 minutes.
4.	1 wash in 80% alcohol 5-10 minutes.
5.	1 wash in 70% alcohol 5-10 minutes.
6.	Stain in safranin 12 minutes.
7.	1 wash in 70% alcohol.
8.	1 wash in 95% alcohol.
9.	1 wash in absolute alcohol.
10.	Stain in Light Green 1 minute.
11.	Clear in Clove Oil.
12.	Wash in xylene.
13.	Mount in balsam.

more uniform the population.

Any deviation from a normal population curve will produce a more horizontal line in the probit straight line, thus sub-populations will be distinguished by steps. Two worked examples are given as Fig 2.7 and examples 1 and 2.

Example 1. Normal Distribution

DNA Value	Number of Nuclei	% of Total	Cumulative %	Probit Value
1	1	6.25	6.25	3.4658
2	2	12.50	18.75	4.1129
3	3	18.75	37.50	4.6814
4	4	25.00	62.50	5.3186
5	3	18.75	81.25	5.8872
6	2	12.50	93.75	6.5340
7	1	6.25	100.00	∞

Example 2. Non-normal Distribution

DNA Value	Number of Nuclei	% of Total	Cumulative %	Probit Value
1	1	6.25	6.25	3.4658
2	1	6.25	12.50	3.8497
3	3	18.75	31.25	4.5183
4	3	18.75	50.00	5.0000
5	2	12.50	62.50	5.3168
6	2	12.50	75.00	5.6745
7	4	25.00	100.00	∞

Values obtained for the DNA content of nuclei in oil palm tissues were subjected to probit analysis to distinguish populations with varying amounts of DNA changing with time. The DNA values at each time interval were taken as a population, and scored into DNA unit classes. The cumulative percentage of the classes of DNA values were calculated and converted to probits using probit conversion tables (Fisher and Yates 1953). The probit values were then plotted against the DNA values.

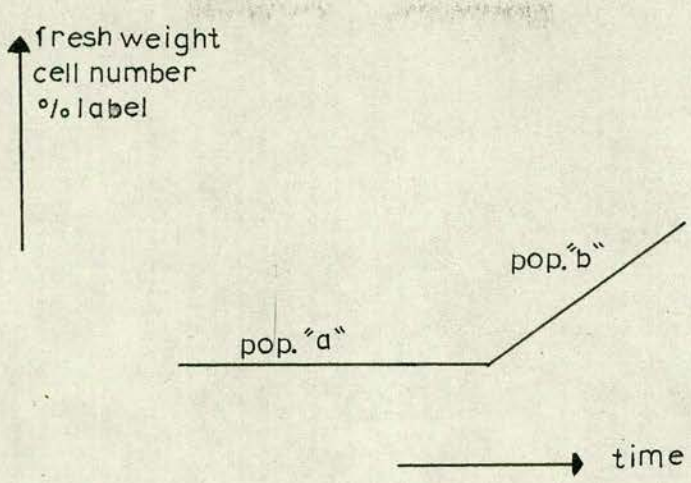
H. Statistical analysis of cell number, fresh weight, and percentage labelling data

The population data concerning cell numbers, fresh weight and percentage labelling changes were treated by statistical analysis using a Canon, Canola F20P calculator. For ease of explanation the data, when plotted as graphs, can be divided into two populations, "a" and "b" (Fig 2.8).

Fig 2.8

A division of data of cell number/fresh weight/ percentage label into populations "a" and "b" for analysis.

Fig. 2.8



The analysis is based on 2 assumptions.

1. The cell number/fresh weight/% label values up to just before the estimated increase (ie population "a") have a normal distribution. That is, these data, plotted as a distribution curve produce a normal curve. This is true in all cases.

2. There is no difference between the distribution of data before the estimated increase (population "a") and after the estimated increase (population b). That is, the total data have a normal distribution.

The mean, standard deviation and standard error of population "a" are calculated and the mean and standard deviation plotted on each graph. Extrapolating these values above the time limits of population "a" will show whether or not population "b" is statistically the same as population "a". If it is the same as population "a" all the values of population "b" (within confidence limits) will fall within the range of standard deviation of population "a". If population "b" is not

statistically the same as population "a", few, if any, of its points will fall within the standard deviation limits of group "a".

If the two populations have been established as different a 'line of best fit' may be calculated for population "b" by the "least squares" method using the Canon calculator. It is valid to ignore values falling within the standard of deviation range of population "a" because these are due to embryos which can be considered not to be viable.

If the line of best fit is extrapolated towards zero, the point where it crosses the S.D. value of population "a" can be considered as the point in time when the increase in fresh weight/cell number/ percentage labelling is initiated.

CHAPTER III

Experimental Work and Results

Variability

A. The problem of Variability

Throughout this investigation variability has been a major problem. Primarily there is variability between seed batches due to genetic variation, the source of supply and the age of the material. Elaeis guineensis is a very heterozygous species, and cultivars, in the true sense of the word, do not exist. The palms are still highly heterozygous despite the breeding programmes, which aim to reduce heterozygosity by repeated inbreeding. However, the time from seed production of one generation to seed production of the next generation is long, a minimum of 7 years is required even for the new fast growing palms, so there is a time restraint on the rate at which inbreeding can take place.

The palm is monoecious, and generally cross-pollinated. Controlled cross-pollination is now carried out on the plantations, but the fruit of any controlled cross will still be variable due to the variation in pollen and female flower genotype.

The number of seeds obtained from any cross depends on the size and number of the inflorescences and the number of fruit that set on each bunch. Bunch size varies between 600 and 2,000 depending on the palm and location. The palm produces about 15 bunches of fruit a year. Inflorescences are produced at the rate of one every 3 weeks but a proportion of these will be male. One of the main factors being bred into the palms is a high female to male inflorescence ratio so that

the bunch number can be increased. Because of these factors and the logistics of transporting the seed to the UK, there is a finite size to any experiment which can be carried out on the progeny of any one cross at any point in time.

It is of obvious ecological advantage to Elaeis that the degree of variation in the fruit should be high, especially in germination. Seeds shed from the palm and left, without interference by man, germinate sporadically any time from four months to two years from when the fruit obtains its maximum size. This delayed germination is probably an adaptation to avoid germination during the hot dry season of West Africa, which lasts for about two months. Economically it is important that germination should be more uniform so that a high proportion of any seeds that are germinated can be planted into nursery beds at the same time. Methods involving heat treatments and artificial water supplies in incubators can reduce the range of germination time of seeds, without the mesocarp, to a period lasting 15 to 20 days, during which over 80% of the seeds will germinate. This germination period is short enough for the plantation situation, but is too long for meaningful experimental work to be carried out on germination. Therefore the variability must be reduced as far as possible by experimental techniques.

The aim of the investigations detailed in this section is to show the extent of variation between and within seed batches and to reduce the variability in development rates by pre-selection of viable, uniform embryos or by pre-treatment of the population to produce a more uniform germination pattern.

B . Variability between seed batches

It was stated in the section on materials that seed from a number of batches and origins was used in the experiments detailed in this thesis. This is due to the time span of the work involved being greater than the viable storage life of the seed. Individual batches vary with respect to seed weight, endocarp thickness, kernel and embryo weight as well as the numbers of seed with 2 or 3 kernels or with endosperm destroyed by fungal infection (generally Penicillium sp. Aspergillus sp. and Mucor sp.). A comparison was made of these parameters between 4 seed batches as an indication of the "base" level of variation within these experiments.

Fig 3.1 shows the range in fresh weight of individual seeds, that is the fresh weight of endocarp, kernel and embryo. Batch 2/5742Dx 2/11605P has the greatest range of weights between 2.3 and 7.6 gm. Batches 2/10414D x 2/11605P and 2/5523D x 2/8607P have similar ranges in weight (1.9-5.2 and 2.7-6.2 gm) but the former hasamuch lower mean value, showing that the seeds are smaller. Batch 7406/0 is the product of an uncontrolled cross. It does not have as large a range as batch 2/5742D x 2/11605P (1.2-5.8 gm) and is more uniform , the wide range being due to 2 large weight seeds.

Endocarp thickness is a characteristic determined by a single gene pair (Beirnier and Vanderweyen 1941), and is the basis of the main classification of varieties of Elaeis into dura, tenera and pisifera types. All the seeds used in these experiments were of the tenera type, that is the F_1 hybrid of dura x pisifera. Table 3.1 shows the variation in endocarp thickness between batches. The endocarp is always of quite constant thickness of 2 mm in controlled cross batches.

Fig 3.1

The range in fresh weight of individual seeds of four seed batches.

Fig 3.2

The range in kernel fresh weight in four seed batches.

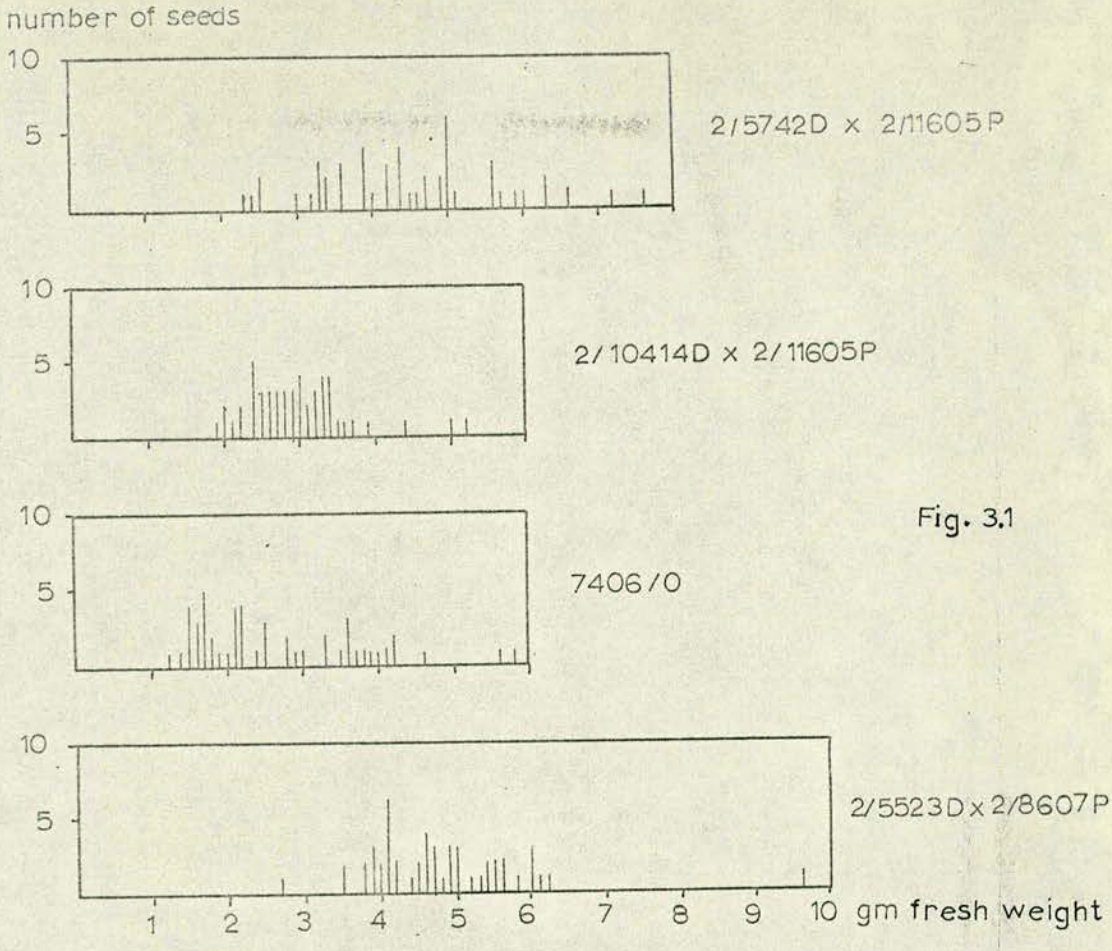

number of seeds
10
5
2/5742D x 2/11605P
10
5
2/10414D x 2/11605P
10
5
7406/0
10
5
2/5523D x 2/8607P
1 2 3 4 5 6 7 8 9 10 gm fresh weight

Fig. 3.1

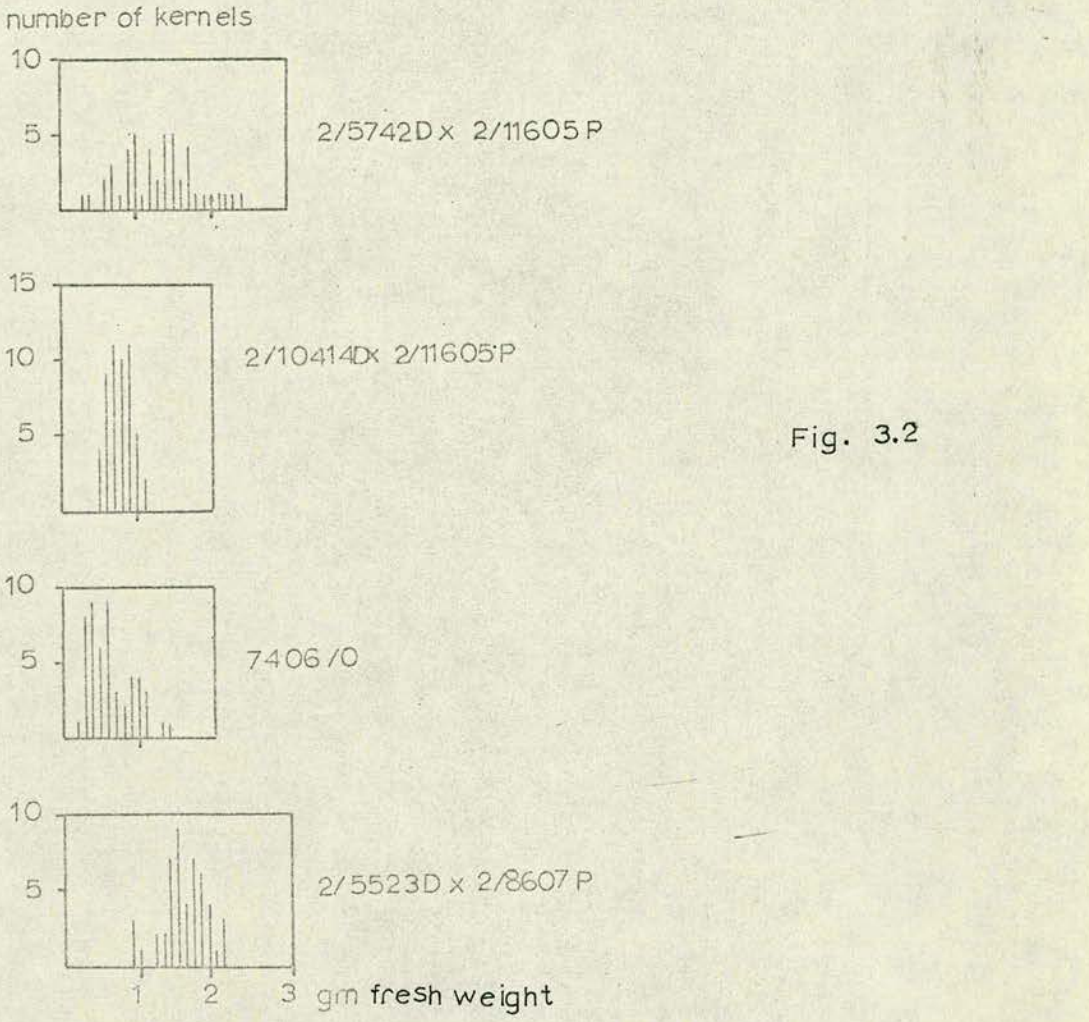

number of kernels
10
5
2/5742D x 2/11605P
15
10
5
2/10414D x 2/11605P
10
5
7406/0
10
5
2/5523D x 2/8607P
1 2 3 gm fresh weight

Fig. 3.2

The uncontrolled cross, batch 7406/0 has a variation of ± 1mm from 2mm thickness. This variation is not sufficient to classify the thinner shells as pisifera type, nor the thicker shells as dura type.

The food reserve of the embryo is the endosperm, and so it is important for it to be of sufficient size to support embryo growth until the embryo becomes autotrophic. A large kernel is also commercially desirable as the source of palm kernel oil, and attempts have been made to increase the kernel size by breeding.

Fig 3.2 shows the range in kernel weights in 4 seed batches. Batch 2/5742 x 2/11605 has the greatest range (0.3-2.4 gm) and batch 2/10414 x 2/11605 has the least range (0.5-1.1 gm) of fresh weights. Batch 7406/0 has the smallest kernels of the 4 batches but has the same range of weights (1.2 gm) as batch 2/5523 x 2/8607 which has a higher mean and hence large kernels.

Fig 3.3 shows the ratio of seed fresh weight to kernel fresh weight. This ratio can be used to determine the proportion of endocarp in each batch. The range of the ratio in batch 2/5742 x 2/11605 is the highest, although the extended range is due to 2 seeds with abnormally low kernel weights. One of these kernels was sterile. The range of batch 2/10414 x 2/11605 is also extended due to a single sterile seed. Batch 7406/0 has a number of kernels which are very light in comparison with the endocarp and these give high ratios. Batch 2/5523 x 2/8607 is very uniform with respect to seed and kernel weight. This ratio of seed to kernel, and the

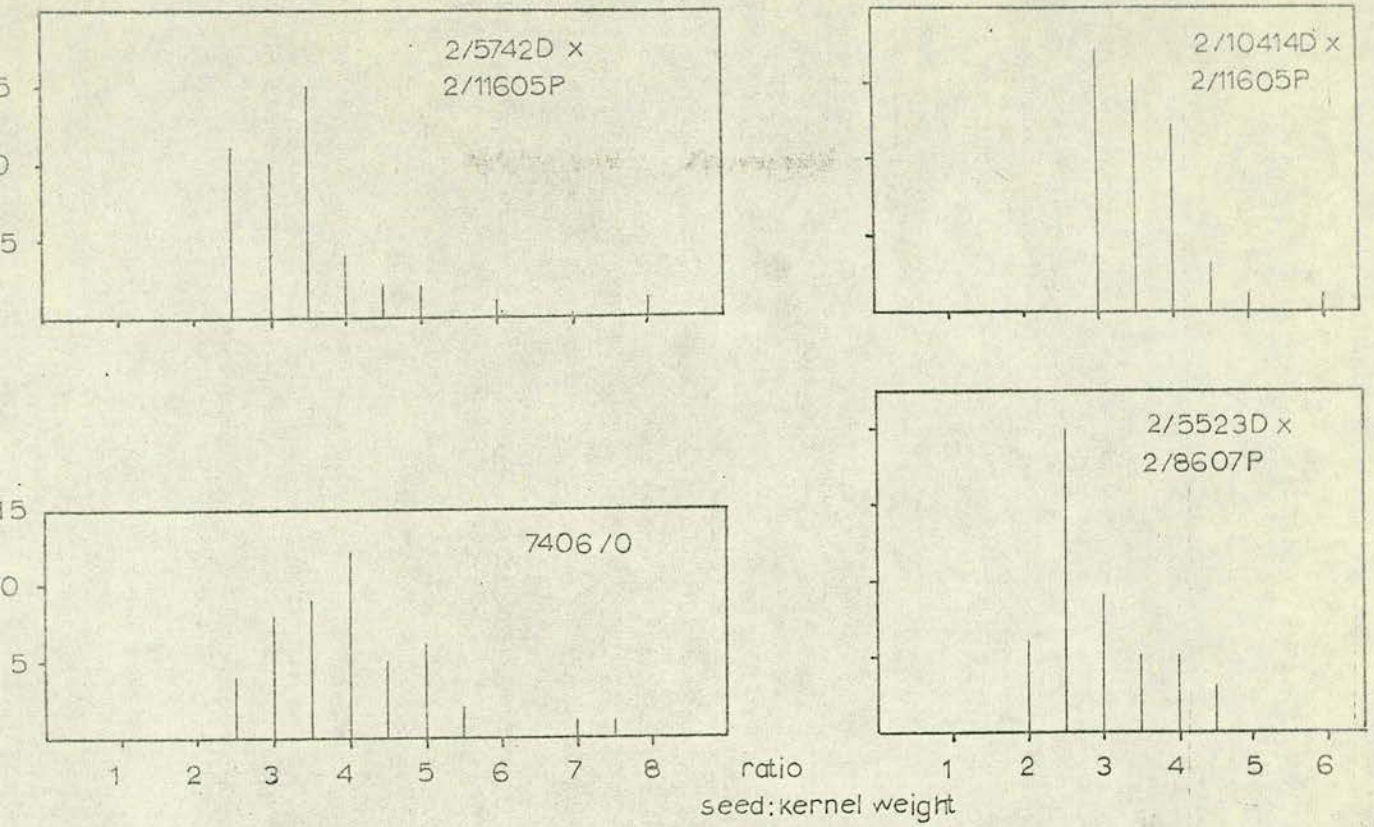
Fig 3.3

The ratio of seed fresh weight to kernel fresh weight in four seed batches.

Fig 3.4

The range in embryo fresh weight in four seed batches.

Fig. 3.3


2/5742D x
2/11605P
2/10414D x
2/11605P
2/5523D x
2/8607P
7406/0
1 2 3 4 5 6 7 8
ratio
1 2 3 4 5 6
seed: kernel weight

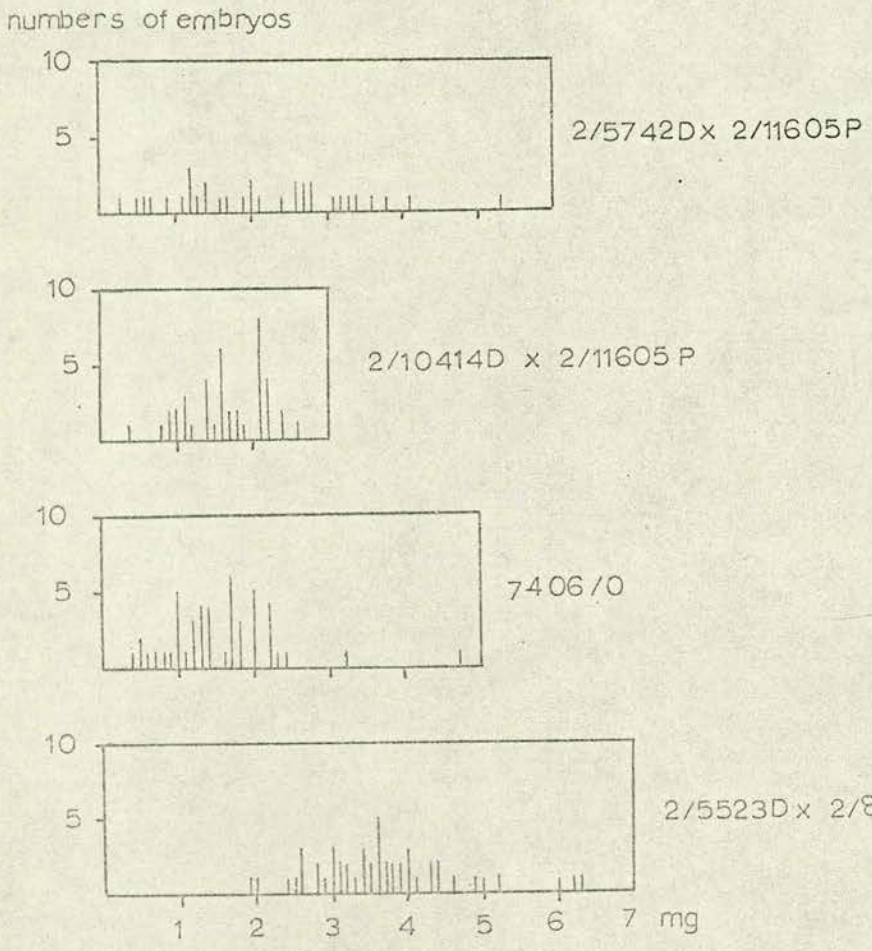

numbers of embryos
10
5
2/5742D x 2/11605P
10
5
2/10414D x 2/11605P
10
5
7406/0
10
5
2/5523D x 2/8607P
1 2 3 4 5 6 7 mg

Fig. 3.4

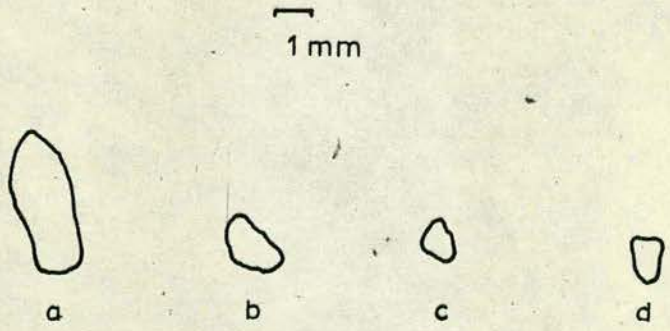
endocarp thickness data taken together imply that the endocarp forms a fairly constant proportion of the seed weight, this proportion varying with the seed batch. It is unlikely then that the thickness of the endocarp is a source of variation in the rate of development of the embryo.

Embryo size is an important factor in germination and in successful embryo culture. Embryos with low fresh weight (less than 2 mg) are frequently not perfectly formed, for example the haustorium may be highly reduced, and will show very low viability in culture. Fig 3.5 shows a number of ill-formed embryos.

Fig 3.4 shows a comparison of embryo fresh weight in 4 seed batches. Batch 2/5742 x 2/11605 embryos have a wide range of fresh weight, with no distinct mean value, whereas batch 2/10414 x 2/11605 has a quite distinct peak with little variation. Batch 7406/0 has1embryo with low fresh weight and 2 with very high fresh weights, but there is a distinct peak between 1 and 2 mg. Batch 2/5523 x 2/8607 had a large range of values with a distinct peak at about 3.5 mg. There are 3 embryos with high fresh weights increasing the range of values. There is little evidence that high fresh weight embryos germinate more readily than average fresh weight embryos, and so these cannot be selected for or against. (see table 3.2)

Table 3.1 shows that the number of multiple kernels in each seed batch varies between 1 and 2. The occurence of multiple kernels depends on the fertilisation of more than one egg cell in the tricarpellate ovary. Each flower has the potential for producing 3 kernels but in most cases 2, or more rarely, 1 abort. In only one example of the 200 seeds examined, was there a seed with 3 fully

Fig. 3.5



Drawings of abnormal excised embryos

a) normal embryo

b) small and mis-shapen embryo

c) embryo with greatly reduced haustorium

d) perfectly formed embryo, but too small to develop normally in culture

Table 3.1

A comparison of 4 seed batches showing shell thickness, multiple, infected or sterile kernels in a sample of 50 seeds each.

	2/5742D x 2/11605P	2/10414D x 2/11605P	7406/0	2/5523D x 2/8607P
Endocarp Thickness	2mm	2mm	1:2:2 ratio of 1:2:3 mm	2mm
Number of Multiple Kernels	1	1	2	1
Number of Sterile Kernels	15	8	1	0
Number of Infected Kernels	12	4	2	2

developed fertile kernels (batch 7406/0). In most cases the endosperm of an aborted embryo does not develop but, in batch 2/5742 x 2/11605 many (15) sterile kernels had developed, but these were all below average in weight. In batch 2/10414 x 2/11605, 8 sterile kernels were found, whereas batch 7406/0 had one and 2/5523 x 2/8607 had none. The number of kernels which have been destroyed by fungal infection varies between batches. 2/5742 x 2/11605 has many (12) infected kernels, 2/10414 x 2/11605 had 4 and 7406/0 and 2/5523 x 2/8607 had 2 each.

In conclusion these results show that the proportion of embryos that will be useful for excision varies between batches because of the proportion of multikernel seeds and the number of sterile or fungal infected kernels.

C . Variability in the germination of the intact kernels

The germination of the intact kernel is as variable as the seed with the mesocarp removed, with the added problem of more rapid water loss when the protective endocarp is removed. The removal of the operculum from the kernel can, however, reduce the variation in the germination period (Hussey 1958). This must be done under sterile conditions, and the kernel incubated aseptically, as the removal of the endocarp and operculum increases the susceptibility of the kernel to infection.

The operculum appears to inhibit imbibition and extension of the embryo through the germination channel, and thus must be removed if rapid germination is to be effected.

Kernels of seed batch 2/10414 x 2/11605 were sterilised using hydrogen peroxide, the opercula removed and the kernels inoculated onto tap water agar in McCartney bottles. Hussey (1959) has shown that high oxygen levels can stimulate germination of seeds, probably by increasing the available oxygen to facilitate the oxidation of inhibitor present in the endosperm. This is a known irreversible oxidative process associated with germination, described by Rees (1959). The hydrogen peroxide treatment here sterilises the surface of the kernels and could also act as an oxidising agent to inactivate the inhibitor. The kernels were incubated under standard conditions and the embryos were sampled at 48 hour intervals for changes in fresh weight and cell number.

Fig 3.6 shows that the mean fresh weight of the embryos increased from the time of excision until day 6. Between day 6 and day 8 the fresh weight remained steady and then began to increase rapidly after day 8. The range of weights at each sample time is low at excision, but increases with incubation.

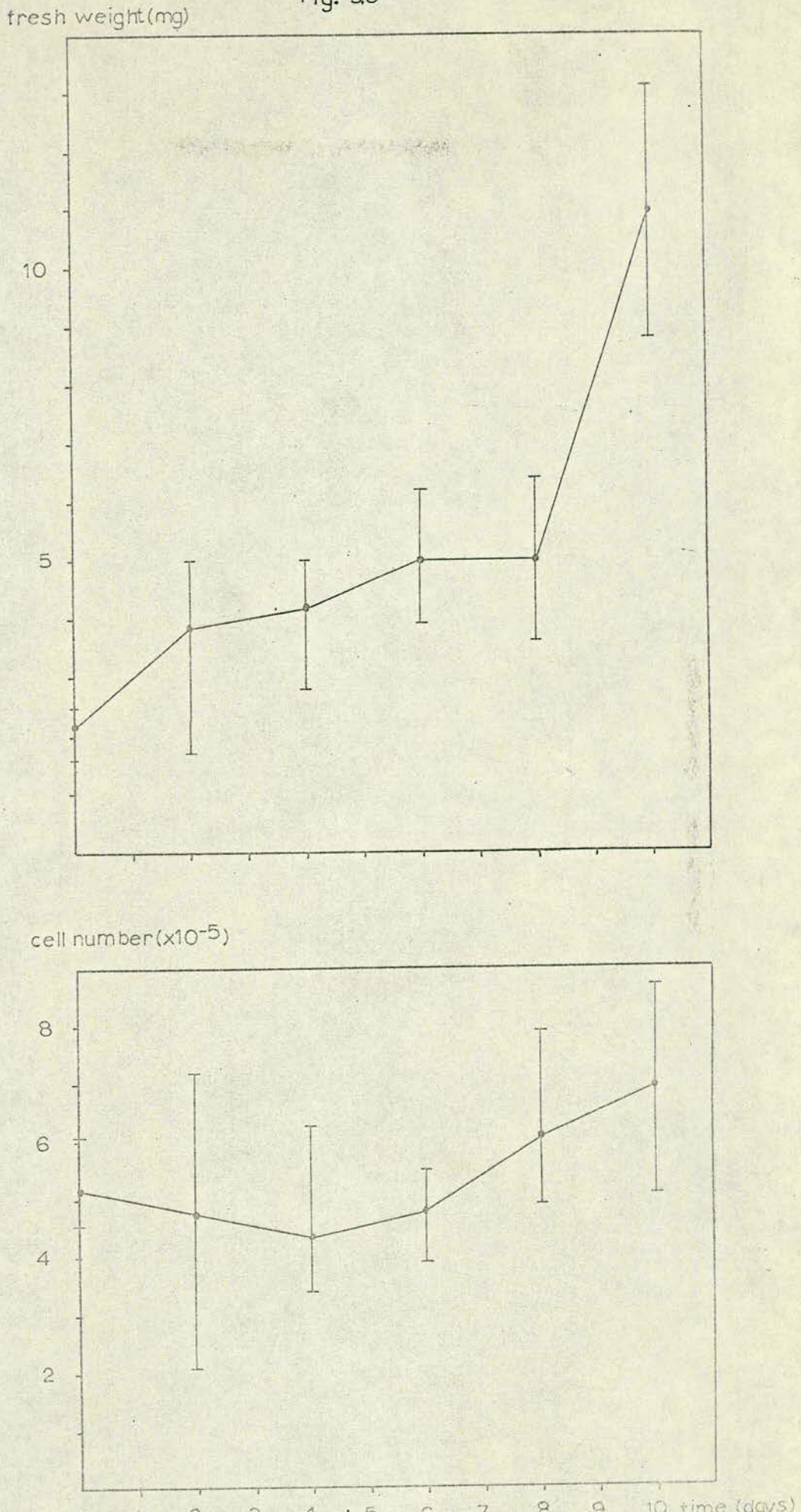
The mean cell number decreases from excision until 4 days of incubation but as the range of values is large the decrease is not statistically significant. The mean cell number increases from day 4 to day 10 but the range of results remains similar, and there is no significant increase because of the wide range. Further samples were not taken after 10 days of incubation because no more kernels showed any signs of development. However, the kernels were left under the standard incubation conditions for a further 4 weeks, after which time they were examined. A further 10 embryos were at various stages of development, 3 had turned green, and one of these was at the "hammer" stage of development. This shows that the development of embryos

Fig 3.6

Changes in mean fresh weight with time of excised embryos.

Changes in cell number with time in excised embryos.

Fig. 36


fresh weight (mg)
10
5
cell number (x10⁻⁵)
8
6
4
2
1 2 3 4 5 6 7 8 9 10 time (days)
range

attached to the endosperm is very variable.

D. Variability within batches.

1.) Correlations between excised embryo fresh weight at excision, after 24 hours and germination.

The development of the intact kernel with the operculum removed is very variable, but Hussey (1959) has shown that the variability can be reduced by the excision and culture of the embryo alone. In order for meaningful work to be carried out with the excised embryo, the extent of this variability within seed batches must be established. This can be done by studying various physical parameters, such as fresh weights and the buoyancy of embryos within seed batches.

Embryos at excision have a lower fresh weight than those which have been incubated for 24 hours. This increase in fresh weight is almost entirely due to imbibition. It was postulated, that by examination of the fresh weight data from a population of embryos it might be possible to correlate the potential of the embryo to germinate with the ratio of the fresh weights at excision and after 24 hours of incubation. If there is a correlation the embryo which would not germinate normally could be distinguished and discarded after 24 hours of incubation and the sample size to be taken at subsequent intervals could be reduced, as variability would be decreased and germination of the selected embryo would be ensured.

Twenty embryos of seed lot 7406/0 were excised and incubated under the usual conditions. They were weighed under sterile conditions at the time of excision and after 24 hours. After 12 days it was determined whether or not each embryo germinated. The percentage increase in fresh

weight during the first 24 hours of incubation was determined and the ratio of the fresh weight at excision to fresh weight after imbibition. Table 3.2 shows that not all embryos which have greater than 50% increase in fresh weight during imbibition will germinate. Some embryos which increase by less than 50% germinate and some do not. Similarly there is not a correlation between the ratio of fresh weights at excision and after imbibition, and germination.

Thus there is no correlation between the initial excision weight, the fully imbibed weight and germination, and these combined parameters cannot be used to detect variability within experiments at an early stage.

2) Floating or sinking of embryo

In preliminary experiments using liquid culture medium it was readily apparent that some embryos immediately fell to the bottom of the medium whereas others remained buoyant, just below the meniscus. It was considered that there could be a correlation between the buoyancy of the embryo and its germination capacity. If this is true it should be possible to select embryos that would germinate early in the incubation period.

Twenty embryos of seed batch 7406/0 were excised and cultured for 12 days under the usual conditions. It was noted at 24 hours whether or not the embryos were floating.

Table 3.3 shows that embryos which float for more than 77% of the incubation period invariably germinate. Embryos which float for less than 77% of the time vary in their responses to whether they will

Table 3.2 The percentage increase in fresh weight during the first 24 hours of incubation of excised embryos.

Fresh weight (0 hr)	Fresh weight (24 hr)	FW0/FW24	% inc	Germ
3.0 mg	5.1 mg	0.59	70	-
4.8	7.7	0.62	60	-
3.6	5.6	0.64	56	✓
3.2	5.0	0.64	56	✓
3.5	5.3	0.66	51	✓
4.0	6.0	0.67	50	-
4.5	6.5	0.69	44	✓
4.3	6.2	0.69	44	✓
4.2	6.0	0.70	43	✓
4.5	6.2	0.72	38	-
4.2	5.8	0.72	38	✓
4.7	6.5	0.72	38	✓
4.3	5.9	0.73	37	✓
4.6	6.3	0.73	37	✓
5.0	6.8	0.74	36	✓
4.0	5.4	0.74	35	-
3.7	5.0	0.74	35	-
4.5	5.8	0.78	29	-
3.5	4.5	0.78	29	-
3.5	4.5	0.78	29	-

Table 3.3

Floating or sinking of embryos over a period of 12 days and the germination capacity of the embryo. F = floating - not floating.

	0 day	1	2	3	4	5	6	7	8	9	10	11	12	% time floating	Germination
1.	F	F	F	F	F	F	F	F	F	F	F	F	F	100	✓
2.	F	F	F	F	F	F	F	F	F	F	F	F	F	100	✓
3.	F	F	F	F	F	F	F	F	F	F	F	F	F	100	✓
4.	-	F	F	F	F	F	F	F	F	F	F	F	F	92	✓
5.	-	F	F	F	F	F	F	-	F	F	F	F	F	85	✓
6.	-	F	-	F	F	F	F	F	F	F	F	F	-	77	✓
7.	F	F	F	F	F	-	F	-	F	-	-	F	-	62	x
8.	F	F	F	F	F	F	F	-	F	-	-	-	-	62	✓
9.	F	F	F	F	F	F	-	F	-	-	-	-	-	54	x
10.	F	F	F	F	F	-	F	-	-	-	-	F	-	54	✓
11.	F	F	F	F	-	F	F	-	-	-	-	-	-	46	x
12.	F	F	F	-	-	-	-	F	F	-	-	F	-	46	x
13.	F	F	F	-	F	-	-	F	-	-	-	-	-	39	x
14.	-	F	F	F	F	F	-	-	-	-	-	-	-	39	✓
15.	F	F	F	F	-	-	-	-	-	-	F	-	-	39	✓
16.	F	F	F	-	-	F	F	-	-	-	-	-	-	39	✓
17.	F	F	-	-	F	-	F	-	-	-	-	F	-	39	x
18.	F	F	F	-	-	-	-	-	-	-	-	-	F	31	✓
19.	-	F	F	F	-	-	-	-	-	-	-	-	-	23	x
20.	-	F	-	F	-	-	-	-	-	-	-	-	-	15	x

germinate, for example embryos 7 and 8 both float for 62% of the time but only embryo 8 germinated. The same can be said in comparing the germination of embryos 13 and 17 with embryos 14, 15 and 16, all of which floated for 39% of the time.

Using data presented in this table it would be difficult to extrapolate the floating capacity of an embryo from data of the first 5 days to the rest of the incubation period and by this means obtain a method of selecting viable embryos early in the incubation time. For example embryo 6 would be selected as not capable of normal germination and embryo 7 would be selected as capable of normal germination. Thus the technique of distinguishing embryos with the capacity to germinate normally from those that will not, by means of this buoyancy data, is of little use in a short term experiment where samples are taken daily as it would be impossible to build up data as shown in Table 3.3. However, if there was to be a preliminary incubation period of, for example, 6 to 8 days before sampling was due to take place, the capacity for floating could be used as a simple distinguishing factor for selecting viable embryos.

E . Methods of counteracting variability

The problem of variability has been realised and steps have been taken to reduce it as far as possible. These precautions fall into two main categories.

1) Selection by physical characteristics.

This form of selection has been carried out routinely in all experiments. It eliminated the small and ill-formed embryos which are least likely to grow normally. All embryos less than 1mm long or weighing less than 2mg and all embryos which are ill-formed, for example

when the haustorium has not developed in proportion to the tigellum, are discarded. The larger embryos are not eliminated because early experiments have shown that these do not necessarily have a higher growth rate than the average embryo.

In a normal healthy excised embryo the tigellum is pale green in colour and the haustorium is cream. Non-viable embryos become cream or white all over, and in some cases, translucent. Abnormally coloured embryos are discarded at excision.

A normal embryo is generally turgid at excision and always after 24 hours of culture. Embryos which become flaccid or decline in fresh weight at any time during the culture period will not germinate normally and can be discarded.

2) Selection by pretreatments

It has been shown that germination can be speeded up by the excision of the embryo from the endosperm, that is by removal of the non-dormant embryo from the effects of the inhibitor that is present in the endosperm. However, germination of a population of embryos is not uniform and it is possible that a 'trigger' must be released for germination to begin. This 'trigger' could be the production of a certain enzyme and/or the inactivation of a compound that inhibits germination, reaching a certain critical level so that the active process of germination is embarked upon and it becomes impossible to return to the previous quiescent state. In a non-uniform population of embryos this 'trigger' is released at various times, relative to each individual embryo. It was anticipated that a physiological shock, such as low or high temperature, or a chemical shock, such as hydrogen peroxide

treatment, could be given to the population to activate the 'trigger' more uniformly throughout the population. This would have the effect of reducing the variation in germination rates within the population.

It was decided that the reaction to each pre-treatment could be assessed by measuring the changes in fresh weight in individual embryos by means of sterile re-weighings, and comparing these weights with those of control embryos that were maintained under standard growing conditions. Fig 3.7 shows the changes in fresh weight of the control population with time and the mean of this curve is also shown in Figs 3.8 to 3.11.

Fig 3.7 shows a sharp increase in fresh weight and range of weights between excision and day 1, this is followed by little increase in either weight or range of values between day 1 and day 4. At day 4, an exponential increase in fresh weights begins, associated with a very rapid increase in the variation in the population which reaches a peak at day 12 when the range extends from 4.8mg to 45.5mg.

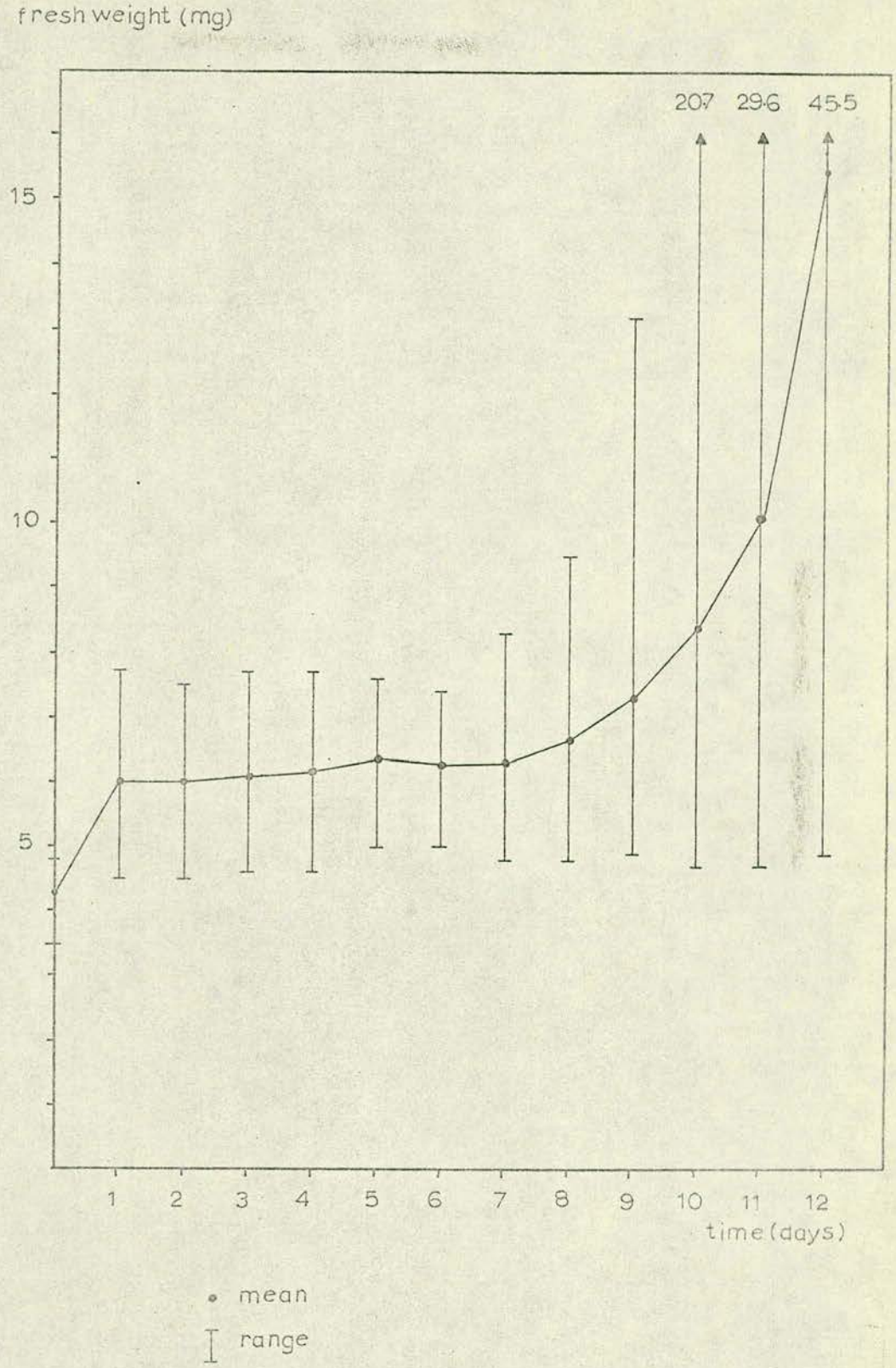
a) Sub-optimal temperature treatment

Henry (1959) showed that growth of excised embryos at 17.5°C is extremely slow and that the rate of growth, measured by leaf extension doubles between 20 and 25°C. Thus growth is possible at lower temperatures but is optimal at 33°C. It was postulated that a period of incubation at sub-optimal temperatures could enable the embryo to reach a certain stage or 'barrier' in development, after which the higher optimum temperature is required for the embryo to activate the 'trigger' and for development to begin. If all the embryos in a population had reached the 'barrier' the transfer to optimal growth temperature

Fig 3.7

Changes in fresh weight established by sterile re-weighings in a control population of embryos.

Fig. 3.7


fresh weight (mg)
20·7
29·6
45·5
15
10
5
1
2
3
4
5
6
7
8
9
10
11
12
time (days)
mean
range

might 'trigger' a population at a uniform stage of development to begin the germination process together, and thus the variation in a population would be reduced.

Embryos of batch number 2/5523 x 2/8607 were excised, weighed and inoculated into McCartney bottles by the usual methods. They were incubated at 25°C in continuous light for 72 hours before transference to the standard incubation temperature of 33°C for the remaining 216 hours of the experiment. The embryos were weighed under sterile conditions at 24 hour intervals. Table 3.4 shows that imbibition followed the normal pattern, the fresh weight increased by a half within 24 hours. However, none of the embryos increased in fresh weight after 24 hours of incubation.

The variation of the population remained constant and no embryos germinated.

Embryos of batch 2/5523 x 2/8607 were excised, weighed and inoculated into McCartney bottles by the usual methods. They were incubated at 25°C in continuous light for 24 hours before transference to the standard temperature of 33°C for the remaining period of the experiment. The embryos were weighed at 24 hour intervals under sterile conditions. Two of the 10 embryos developed endogenous fungal infections. The mean values of non-germinating embryos are shown in Fig 3.8 together with the actual weights of 3 germinating embryos; 2 of which germinated rapidly and one slowly. All embryos imbibed normally between excision and day 1 in the period when they were at 25°C. The rapidly germinating embryos increased by nearly

Table 3.4. Changes in fresh weight in a population of embryos with time after incubation at a sub-optimal temperature (25°C) for 4 days

Time	Mean FW (mg)	Max FW/sample	Min FW/sample
0	3.0	4.3	2.6
1	4.5	5.8	3.4
2	4.4	5.7	3.2
3	4.5	5.6	3.2
4	4.4	5.5	3.3
5	4.4	5.5	3.4
6	4.4	5.5	3.3
7	4.2	5.3	3.1
8	4.3	5.5	3.3
9	4.2	5.3	3.1
10	4.6	5.8	3.4
11	4.3	4.8	4.1
12	4.5	4.9	4.2

Fig 3.8

Changes in fresh weight, established by sterile re-weighings, in a population of embryos incubated for 24 hours at sub-optimal temperatures before transference to normal incubation temperatures.

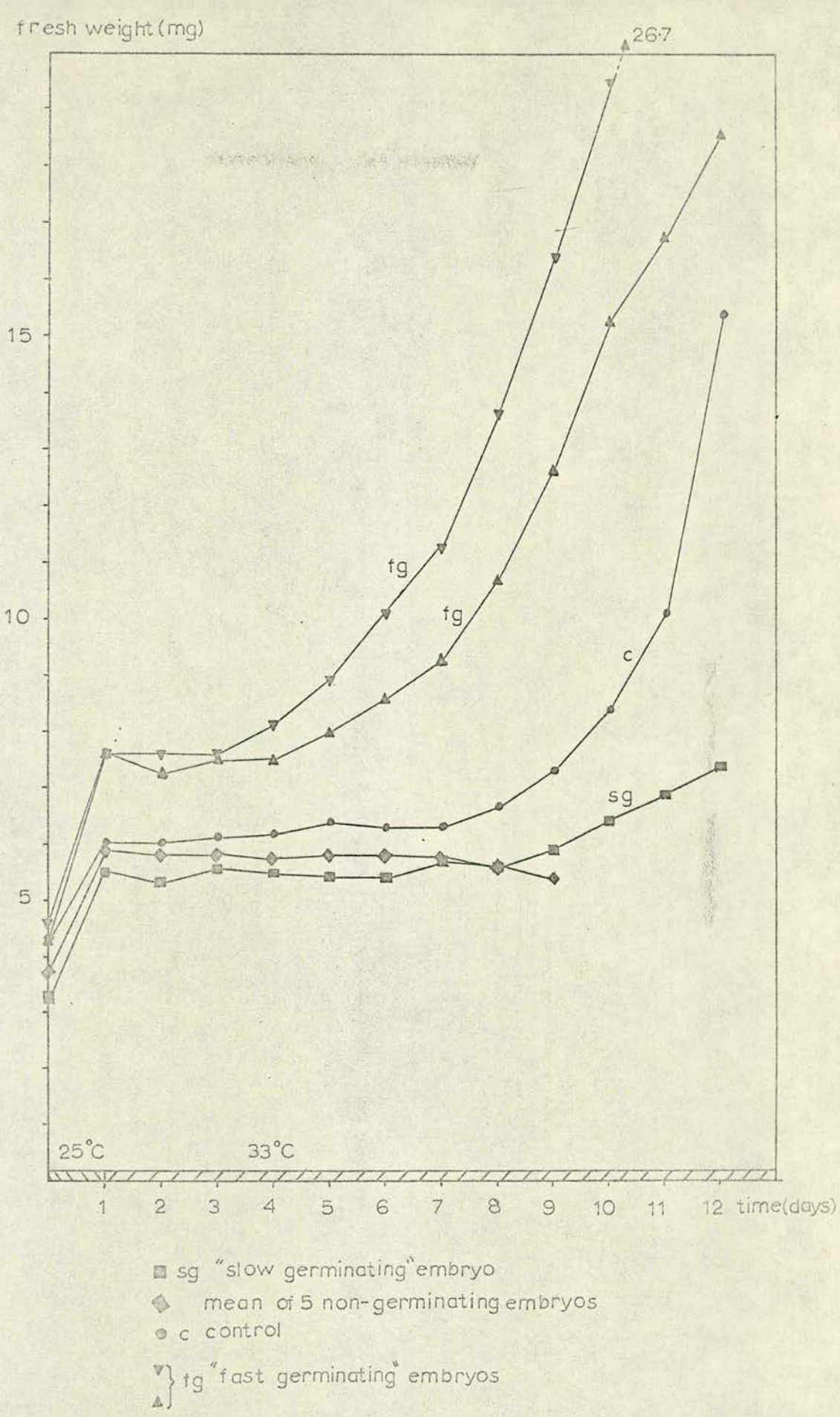

fresh weight (mg)
26·7
15
10
5
fg
fg
c
sg
25°C
33°C
1
2
3
4
5
6
7
8
9
10
11
12
time(days)
sg "slow germinating" embryo
mean of 5 non-germinating embryos
c control
fg "fast germinating" embryos

Fig. 3.8

100% in fresh weight whereas the non-germinating, and slow germinating embryos increased by only 50%. The rapidly germinating embryos maintained a fresh weight of about 7.5mg until 3 or 4 days of incubation and then began to increase in fresh weight by day 12. The slow germinating embryos remained fairly constant in weight between 1 and 8 days and then began to increase linearly from day 8 to day 12. The non-germinating embryos maintained the imbibed weight of about 5.7mg until day 7 and then began to decrease. **Thus a low temperature treatment does not reduce variability.**

b.) High temperature treatment

It has been described by Rees (1959) that germination of the intact seed can be speeded up by maintaining the seed, with the mesocarp removed, at 39.5°C for 70-80 days and then cooling to 28°C and watering. 50% germination of a batch can be completed within less than 5 days from cooling. Rees considers that this heat treatment inactivates the inhibitor of germination and allows germination to begin more rapidly. An extrapolation of this heat treatment could be applied to excised embryos. Trace amounts of the inhibitor could be associated with the embryo and so pre-treatment at high temperatures could inactivate the inhibitor and allow the embryo to begin germination as soon as it is transferred to optimal incubation conditions.

Embryos of batch 2/5523 x 2/8607 were excised, weighed and inoculated into McCartney bottles by the usual methods. They were incubated at 39°C for 24 hours in darkness being being transferred to the standard conditions at 33°C. The embryos were weighed under sterile conditions at 24 hour intervals. Five of the embryos developed endogenous fungal infections. Fig 3.9 shows that one embryo germinated normally. This embryo imbibed in the first 24 hours increasing in fresh weight by 50%. Little change in weight occured until day 5. After day 5 an exponential increase in weight occurred lasting until day 10 and slowing to a linear increase between day 10 and day 12. Four embryos imbibed normally increasing by almost 50% in the first 24 hours of incubation. The mean values and range of weights are shown in Fig 3.9. The fresh weight of the population remained steady until day 5 when there followed a non significant decrease in fresh weight.

Embryos of batch number 2/5610 x 2/8607 were excised, weighed and inoculated into McCartney bottles by the usual methods. They were incubated at 36°C in complete darkness for 72 hours, and then transferred to the usual conditions at 33°C. The embryos were weighed aseptically at 24 hour intervals and the results plotted as Fig 3.10. One embryo developed an endogenous fungal infection and was discarded. The remaining 9 embryos increased in fresh weight by nearly 70% by imbibition between 0 and 24 hours. Between day 1 and day 4 the mean fresh weight remained steady and then between day 4 and day 9 the mean fell. Between day 9 and day 12 there was an increase in fresh weight but it was not statistically significant. None of the embryos germinated. These data show that an initial incubation period at a high temperature does not reduce variability.

Fig 3.9

Changes in fresh weight, established by sterile re-weighings, in a population of embryos incubated at 39°C before transference to normal incubation temperatures.

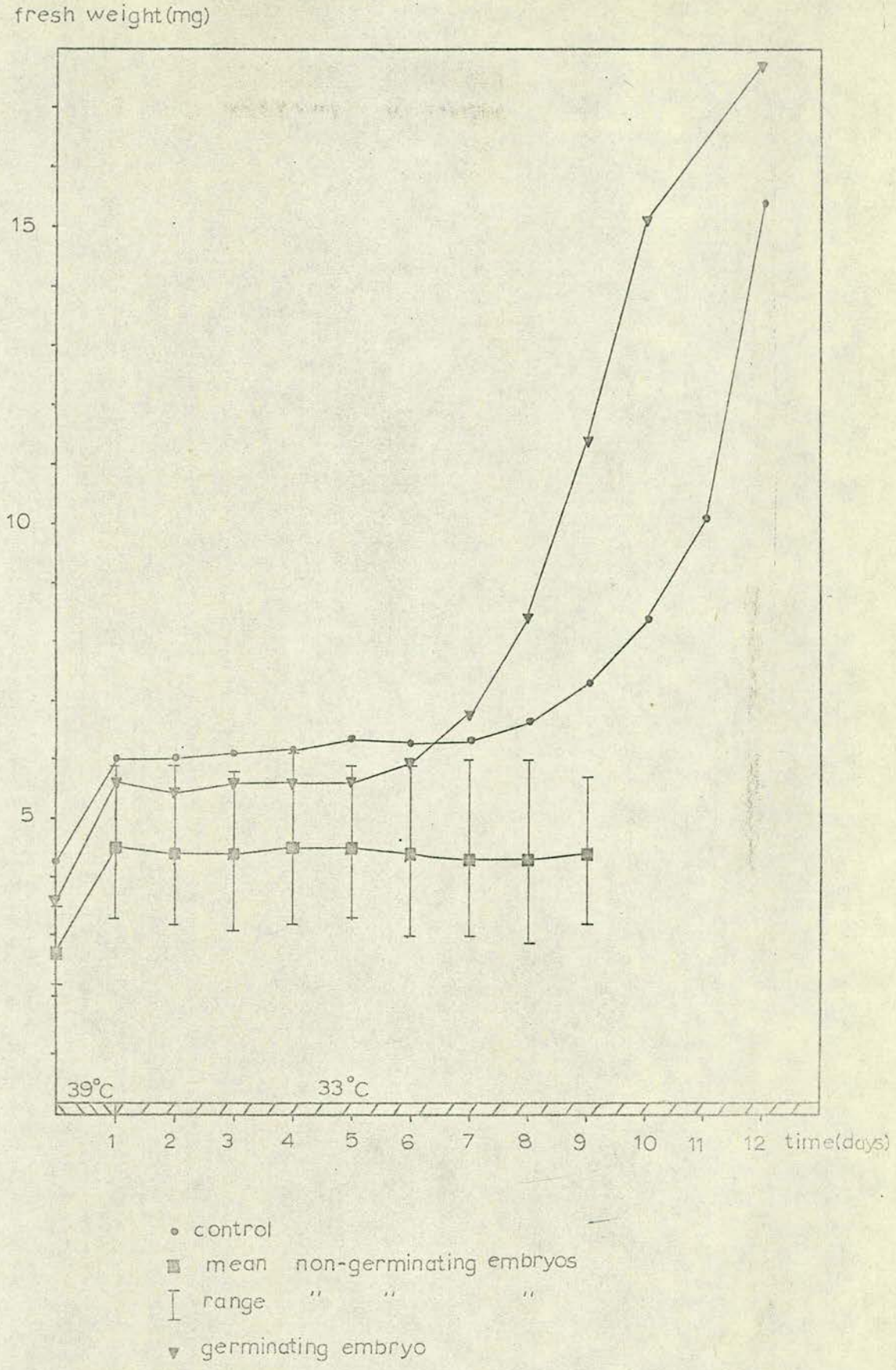

fresh weight (mg)
15
10
5
39°C
33°C
1
2
3
4
5
6
7
8
9
10
11
12
time (days)
control
mean non-germinating embryos
range " " "
germinating embryo

Fig. 3.9

Fig 3.10

Changes in fresh weight, established by sterile re-weighings, in a population of embryos incubated at 36°C before transference to normal incubation temperatures.

Fig 3.11

Changes in fresh weight, established by sterile re-weighings, in a population of embryos which were transferred to fresh medium daily for the first five days of incubation.

Fig. 3.10

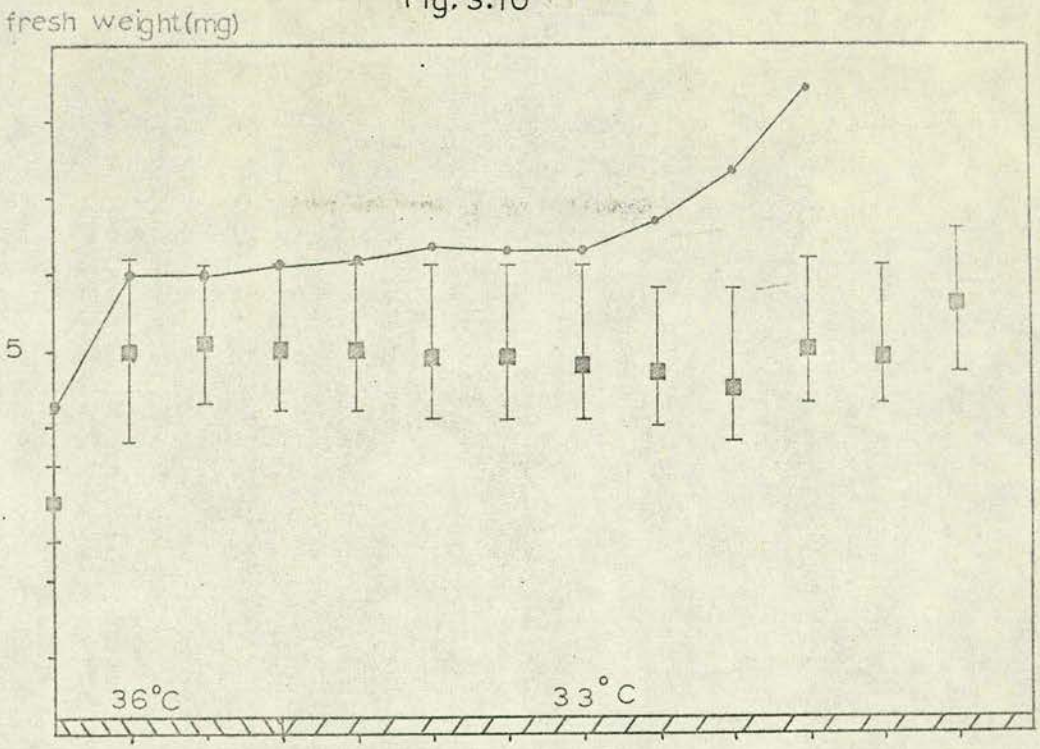
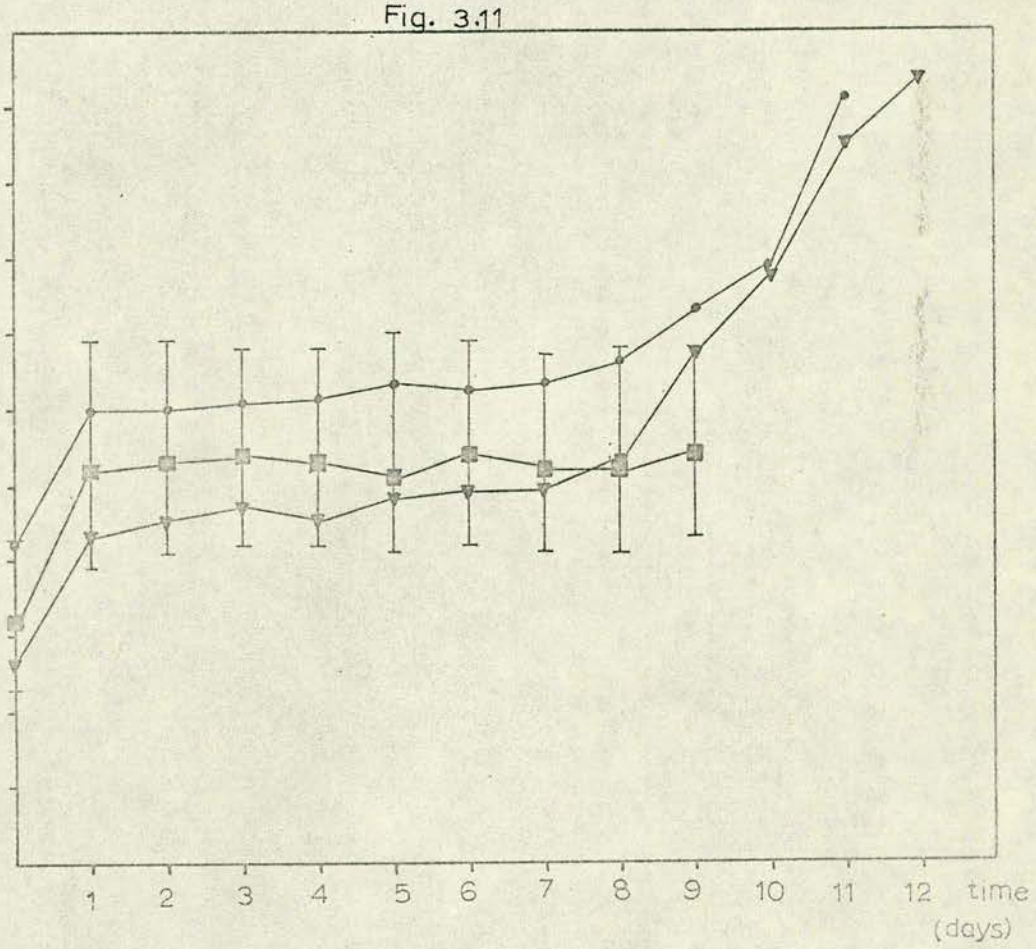


Fig. 3.11



- control
- mean non-germinating embryos
- range " " "
- germinating embryo

c) Leaching of substances out of the embryo

It is possible that some of the inhibitor present in the endosperm could also be found in the excised embryo. The inhibitor could leach out of the embryo into the medium down a concentration gradient. If the medium was changed at intervals it might be possible to remove the inhibitor so that germination could proceed at a faster rate.

Embryos of batch 2/5523 x 2/8607 were excised, weighed and inoculated into McCartney bottles. These were incubated under the usual conditions at 33°C. The embryos were weighed, aseptically, at 24 hour intervals and transferred to fresh medium each day for the first 5 days of incubation.

Fig 3.11 shows that all embryos imbibed normally, increasing 100% in fresh weight and did not germinate. One embryo continued to increase slightly until day 7 and then increased exponentially as it germinated. Thus changing the medium does not reduce variability.

d) Pretreatment with hydrogen peroxide

Work by Hussey (1959) has shown that the germination period can be speeded up in the whole seed by flushing the seed incubator with oxygen. As the excised embryo is not dormant, this implies that the inhibitor of germination is destroyed by oxidation and an oxidising treatment could provide the 'trigger' for germination. Hydrogen peroxide is an ideal oxidising agent as it decays to harmless water and oxygen.

Embryos of batch number 2/5523 x 2/8607 were excised, weighed and immersed in 10 volume hydrogen peroxide for 10 minutes, before being inoculated into McCartney bottles and incubated under the standard conditions. During the 10 minute pretreatment period a lot of oxygen was evolved, collecting as bubbles around the embryo. All the embryos imbibed normally but none germinated. Many embryos had areas of brown tissue on the surface of the tigellum and in some cases the whole embryo

blackened. This indicates that the hydrogen peroxide treatment was damaging the tissues in some way. This experiment was repeated but the period of immersion in hydrogen peroxide was halved to 5 minutes. Six embryos developed endogenous fungal infections, the remaining embryos imbibed normally but did not germinate. However, in two instances callus tissue developed and so this pretreatment has suggested a method of initiating callus formation as a wounding response, where the damage to the surface has been produced by H_2O_2.

Despite the ability of the hydrogen peroxide to initiate callus formation it does not have any effect in reducing variability in the excised embryo populations.

F. Summary

Variability in the oil palm is high despite organised breeding programmes in commercial plantations. Variation has not been eliminated because of the almost obligate requirement for cross-pollination and the length of the breeding cycle of the palm with respect to the length of the breeding programmes. The variability of seed germination is probably an ecological adaptation to the environment to avoid the dry season of West Africa

The fresh weights of intact seed (minus mesocarp), endosperm and embryo vary in and between batches, as do the numbers of sterile or infected seed.

There is more variation in the germination of the embryo while still in the endosperm, but with the operculum removed, than in the excised embryo. The operculum appears to inhibit imbibition and extension of the embryo through the germination channel.

Variation within seed batches was studied to see if correlations between physical parameters and germination existed. There is no correlation between the non-imbibed weight of an embryo and germination, so this cannot be used as a means of reducing variability in germination. It has also been shown that variability cannot be reduced by the selection of floating or non-floating embryos.

Variability can be reduced by routine elimination of embryos that are small, ill-formed, flaccid or of abnormal colouration.

Various chemical and physical pre-treatments were attempted, all of which reduce viability and did not give more uniform germination.

A pre-treatment with hydrogen-peroxide induced a tendency to callus formation.

CHAPTER IV

Growth of the Excised Embryo. The Control System

An essential pre-requisite to this investigation was the establishment of a primary explant system (see Chapter I). It is important that such a system should possess certain essential characteristics. It should be easily cultured under completely sterile conditions and readily available in large quantities. The explants should respond in a uniform manner and should if possible have a known, uniform genetic potential. It would appear from preliminary studies here and at Colworth House that the only suitable candidates are embryos excised from sterilised fruits, despite the limitation of unknown genetic potential. Accordingly a study was made of the structure of the excised embryo and the behaviour of the embryo in culture. Particular attention was paid to the cytological characteristics of the embryo and the kinetics of cell division over a period of development, designated germination. The techniques of cell counting, labelling and autoradiography using ^{3}H-thymidine, and microdensitometry were employed to investigate the mode and time of cell division with respect to the cell cycle, and changes in DNA, protein and total nucleic acid content.

A. Changes in morphology associated with the development of excised embryos.

The embryos used in these experiments were fully developed, having reached maturity about 60 days from fertilisation and about 90 days before harvesting (de Poerck 1950). The embryos have well developed apical and root meristems, two fully differentiated leaves and the rudiments of a third. The embryos are not dormant while in the seed,

but germination is delayed by an oxidisible inhibitor present in the endosperm (Rees 1959). Many examples have been cited by Raghavan (1966) where the excision of the embryo breaks the seed dormancy and the oil palm should be included among these (Hussey 1959).

An excised embryo in culture exhibits marked changes in morphology as development proceeds during the first 12 days of incubation. Fig 4.1 shows a range of embryos in which morphological changes associated with germination can be seen. Group B embryos are also drawn as Fig 4.2 to assist in description; these were incubated under a 12 hour light: 12 hour dark photoperiod.

Embryo 1 of figure 4.2 is a freshly excised embryo. It is torpedo shaped, the blunt end is the tigellum and the pointed end the haustorium. Embryos, at excision, have a cream coloured tigellum and a paler, almost white, haustorium.

Embryo 2 has been incubated for 6 days. A 'hammer' shape has been formed at the side of the tigellum, as the root and shoot meristems increase in volume. By day 8 a leaf sheath has been formed, in the position of the 'hammer' head, but it has not yet developed much chlorophyll. By day 9 a significant amount of chlorophyll is present and the first leaf has emerged through the leaf sheath.

Root development is slower than the leaf, but there is a clear protrusion which marks the site of radicle extension. The haustorium

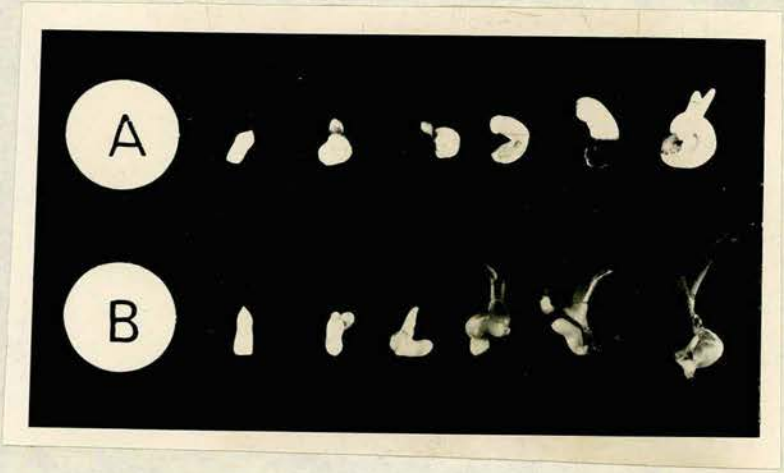
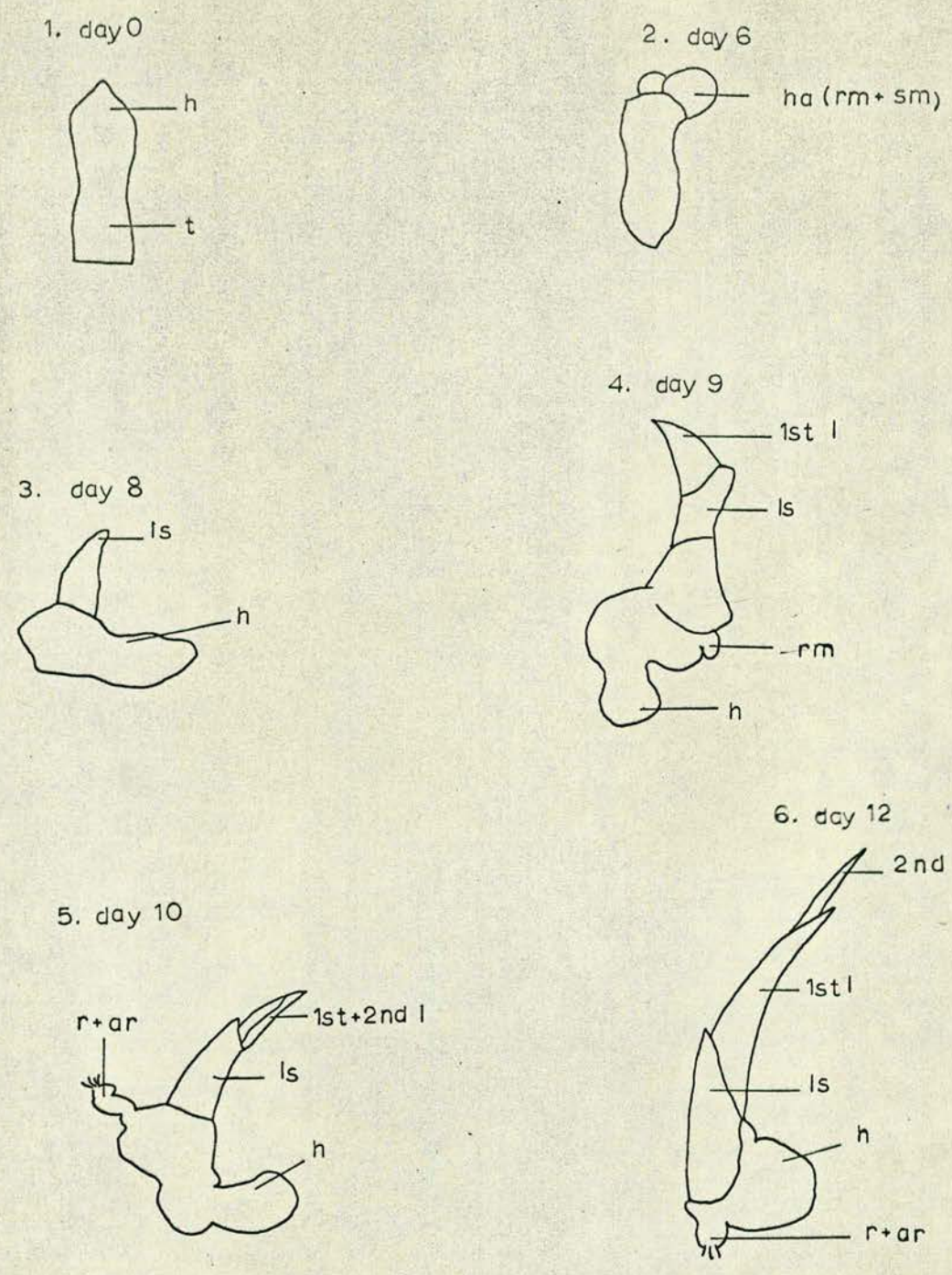


Fig. 4.1

Morphological changes associated with germination



t - tigellum
h - haustorium
ha - hammer
ls - leaf sheath
rm - root meristem
sm shoot meristem
r radicle
ar adventitious roots
1st l 1st leaf
2nd l 2nd leaf

Fig. 4.2 Morphological changes in excised embryos associated with normal development. Drawn from Fig. 4.1

is beginning to increase in volume and its surface has become deeply ridged. By day 10 a second leaf has emerged and the radicle and associated adventitious roots are clearly discernible. The haustorium has again increased in volume. By day 12 the excised embryo has two distinct green leaves, a leaf sheath, a radicle and some adventitious roots. The haustorium has increased in volume and has become deeply ridged. In the intact seed the haustorium expands, digesting the endosperm extracellularly, until it completely fills the endocarp. In vivo, it is never photosynthetic, but in culture it will turn green when exposed to light.

The embryos designated group A in Fig 4.1 were incubated in complete darkness, a situation similar to the condition in the intact seed. The first four embryos, numbering from the left, show the formation of the 'hammer' shape (2 and 3) and the expansion of the haustorium (4) from the freshly excised embryo (1). Embryo 5 is abnormal as the 'hammer' shape has not been formed and the haustorium has become brown in colour. In embryo 6 two leaves have penetrated through the leaf sheath and the radicle is about to increase in length. This is the embryo, equivalent to embryo 4 in group B, showing that development is accelerated in embryos grown in the light. Chlorophyll has not been produced in dark grown embryos and the haustorium has increased proportionately more in volume in the dark.

B. Changes in Anatomy associated with germination

It can be seen that there are marked morphological changes associated with the development of an excised embryo and it is anticipated that these will be associated with clear anatomical changes. Accordingly embryos undergoing development were examined for anatomical changes

particularly to define and characterise the growth centres in the excised embryo.

Embryos at various stages of germination were fixed, embedded in wax, sectioned at 10 µm and stained with safranin and light green (see Chapter II FI 1).

Longitudinal sections of the embryos were photographed using a Zeiss microscope with an overall magnification of ×40. The complete section was recorded by taking a series of overlapping photographs. These composite pictures are shown as Figs 4.3 to 4.8.

At excision (Fig 4.3) the oil palm embryo has a leaf sheath, two developed leaves and the rudiments of a third at the apical meristem, an associated root meristem in the tigellum, and an undifferentiated haustorium. There is little or no vascular tissue between the tigellum and haustorium, and apart from the meristematic regions in the tigellum, the cells are similar in both portions of the embryo. At day 4 (Fig 4.4) the shoot and root meristems are beginning to increase in volume. Together with the leaf sheath they eventually form the characteristic 'hammer' shape, beneath the epidermis. Vascularisation of the tissue between the tigellum and haustorium is beginning to take place. The surface of the haustorium is becoming furrowed.

By day 8 (Fig 4.5) a lateral outgrowth of the tigellum forms the characteristic 'hammer' shape. This section has been cut through part of the leaf (leaving a gap in the section), showing the well developed vascular tissue. The vascular tissue in the haustorium is developing along the centre of the section. Fig 4.6 shows an embryo after 11 days of incubation, cut through the haustorium, the root and shoot

Figs. 4.3 – 4.8

Changes in anatomy associated with germination

t tigellum
h haustorium
sm shoot meristem
rm root meristem
ha hammer
fu furrows
vt vascular tissue
vb vascular bundle
ls leaf sheath
r radicle
2l 2nd leaf
3l 3rd leaf

Fig . 4. 3

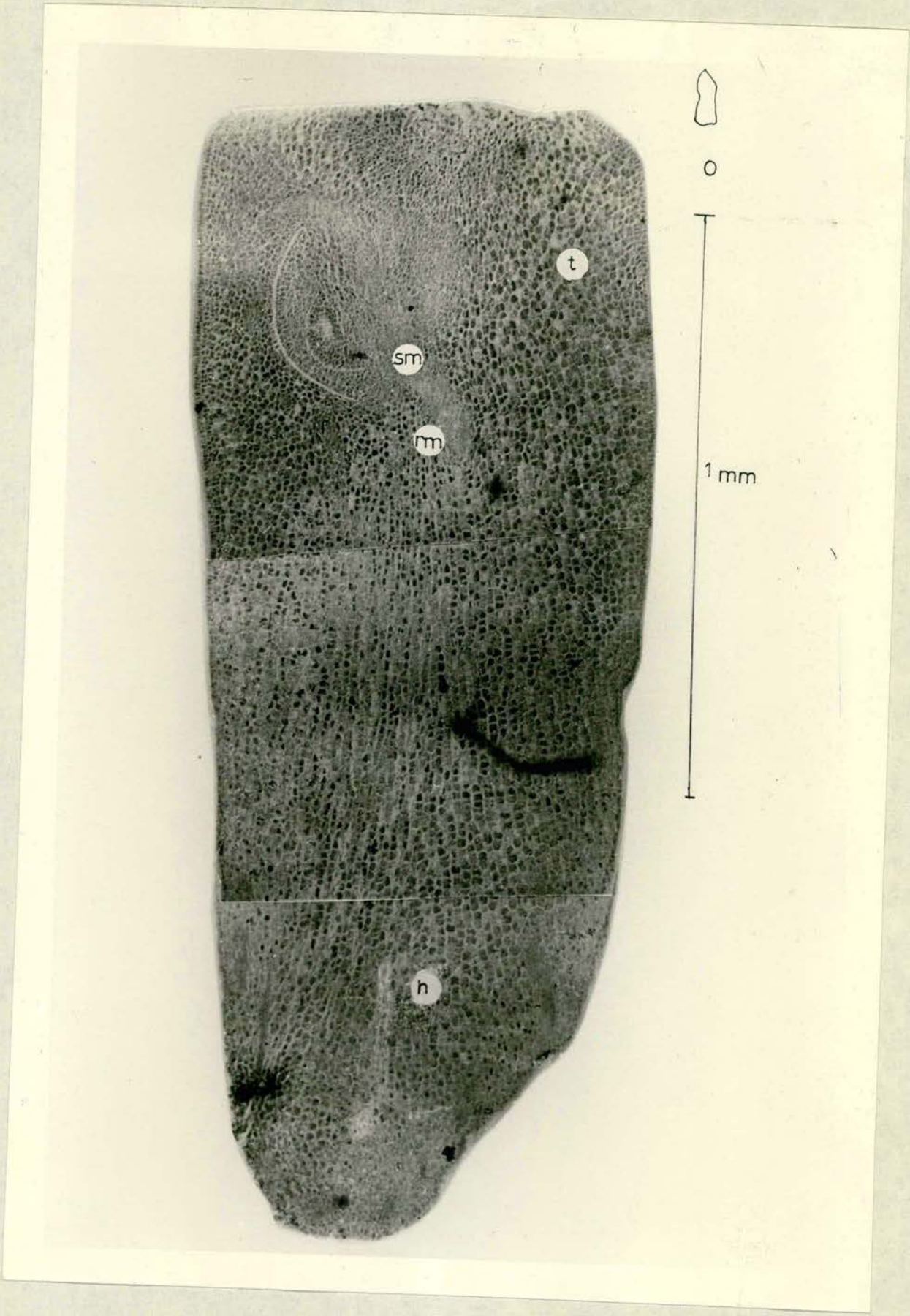


Fig. 4.4

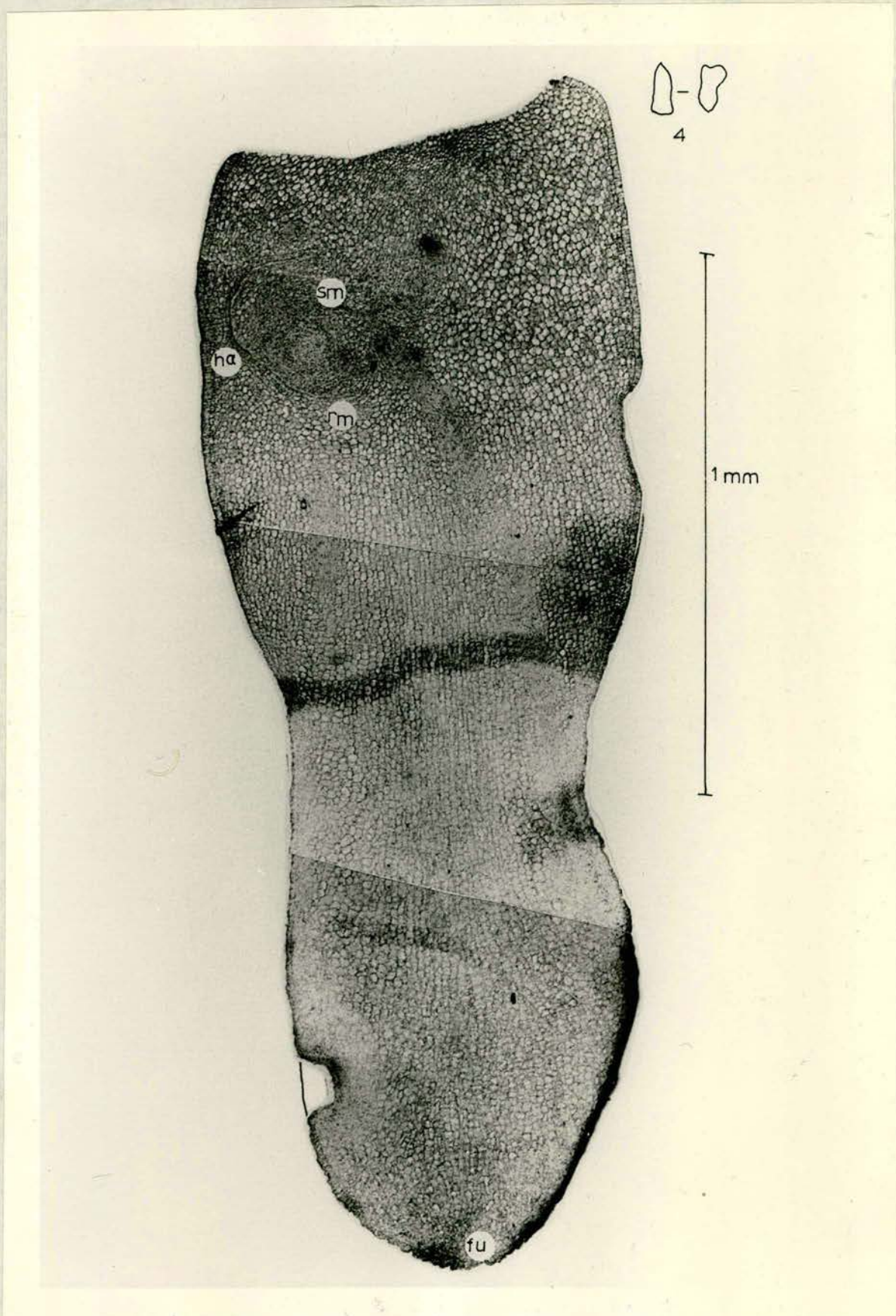


Fig.45

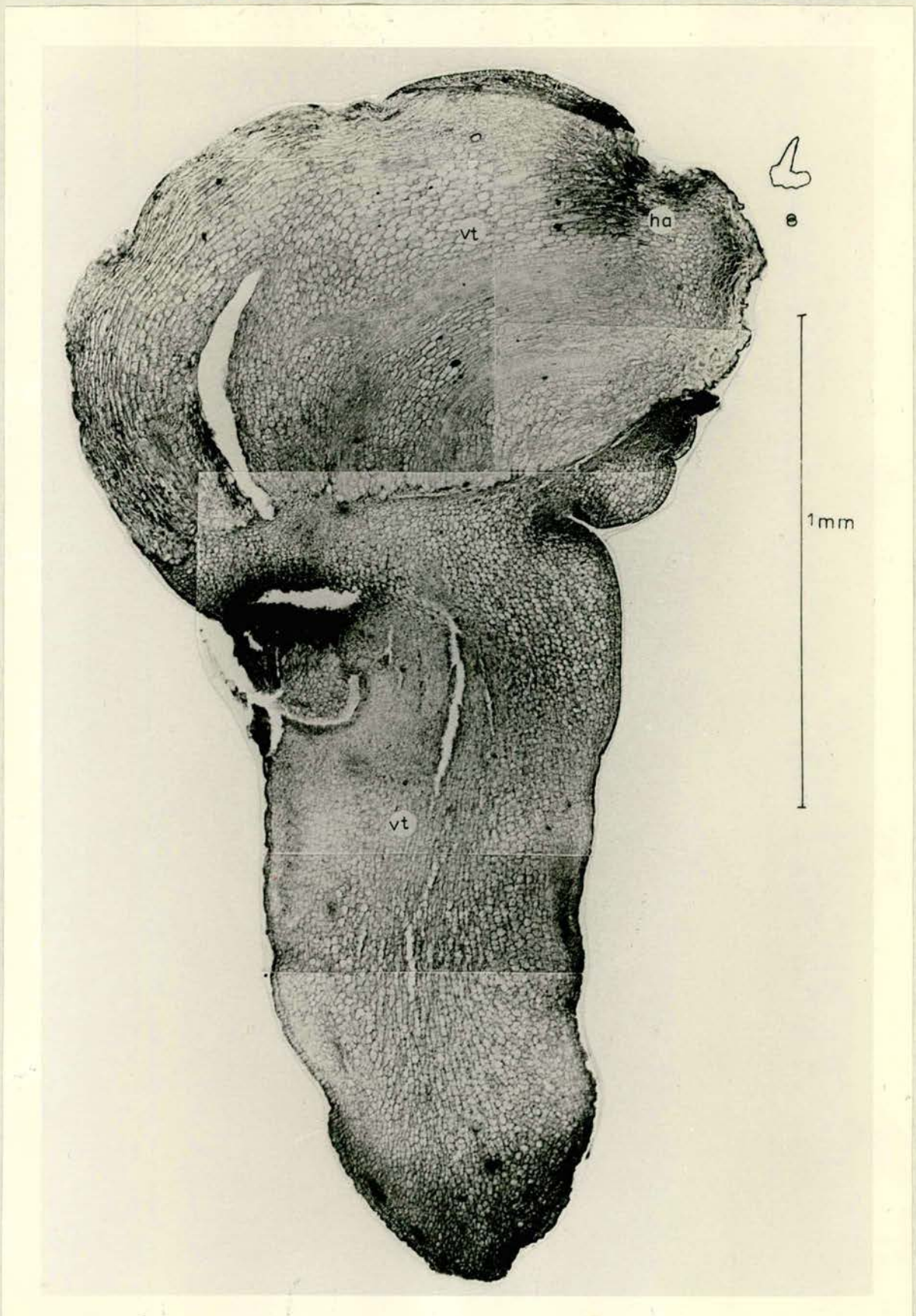


Fig. 4.6

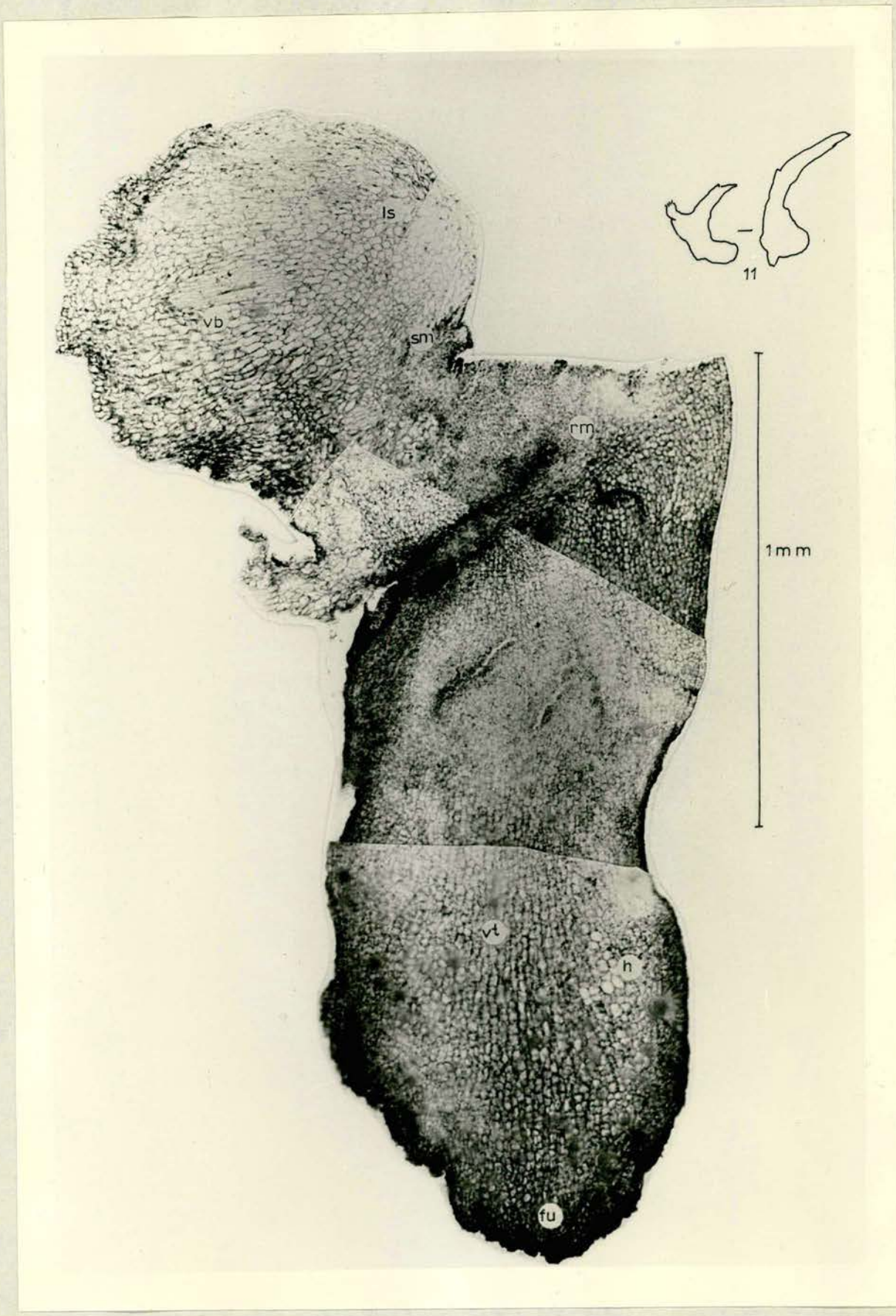


Fig. 4.7

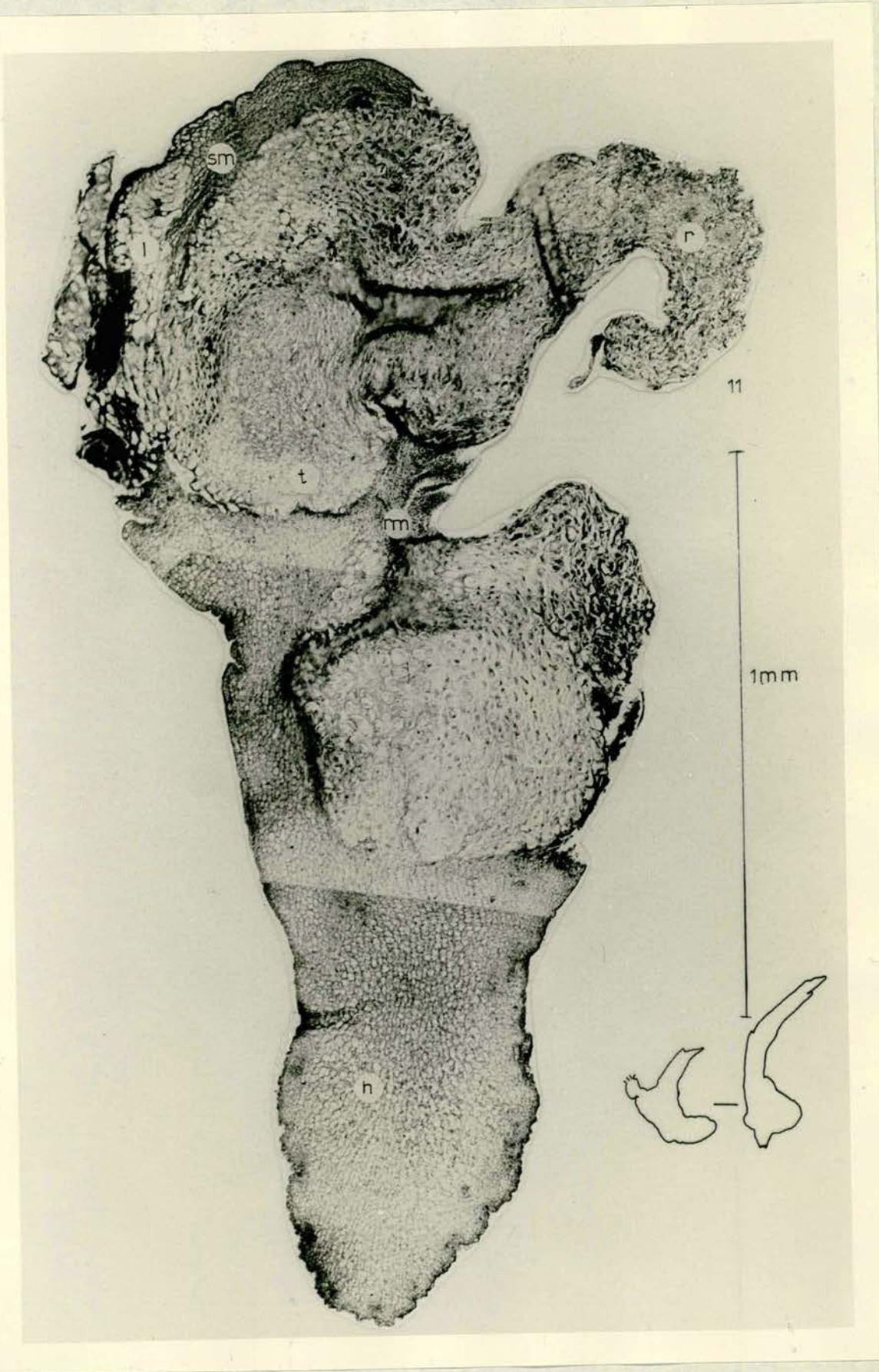


Fig. 4.8

meristems and part of the leaf sheath. The leaf sheath consists of parenchymatous cells with small vascular bundles. The meristematic region, which has been partially cut, has increased in size from that of the newly excised embryo. The haustorium has developed vascular tissue with secondary thickening and its surface area has been greatly increased by folding of the epidermis. Fig 4.7 shows an embryo after 11 days of incubation cut through the haustorium and radicle. The haustorium which appears to be greatly reduced in size due to the angle of cut of the section, is quite distinct in cell type from the tigellum which in this example consists of mainly tissue of the 'hammer'. The radicle is to the right of the section and has an associated meristematic region at the proximal end, from which the adventitious roots will emerge. The leaf apical meristem is at the top of the section and part of the leaf tissue extends to the left of it.

Fig 4.8 shows a section through part of the 'hammer' of an embryo incubated for 8 days. To the right of the section is the radicle meristem with well developed vascular tissue. To the left of the picture is the part of the tigellum from which the 'hammer' has extended, which is above the haustorium (see inset). The shoot apex is surrounded by two developed leaves encased in the leaf sheath. These leaves are well developed with vascular bundles. At the left side of the meristem there is the primordium of a third leaf. The vascular tissue connecting the leaf and root tissue to the tigellum is well developed. Fig 4.9 shows an area of vascular tissue taken from this region and magnified x 100. Fig 4.10 shows a shoot apical dome (x 100) and part of a differentiated leaf taken from a freshly excised embryo (Fig 4.3). The surface of the meristem is not smooth and there are no nuclei in mitosis. These examinations show that growth occurs mainly in the meristematic regions of the root and shoot.

Fig. 4.9

Section through leaf tissue showing vascular bundle. from mag x100

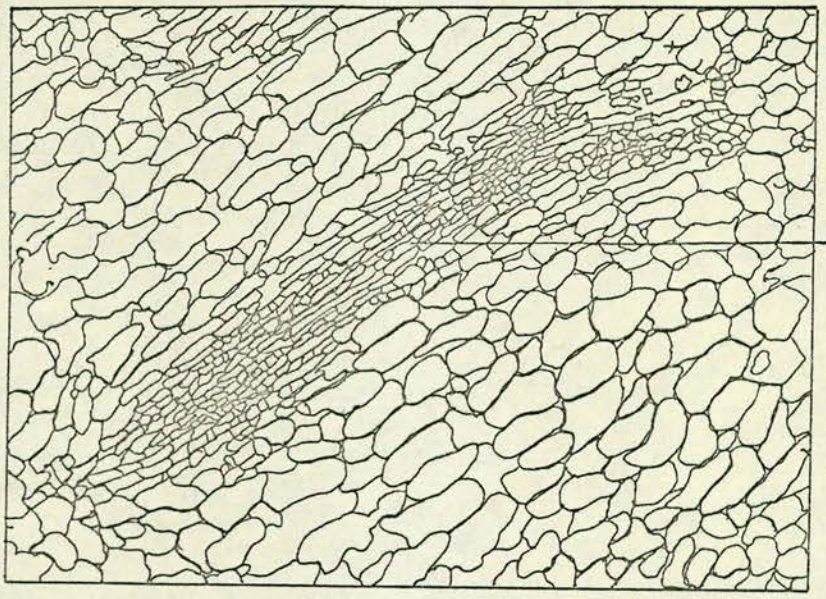
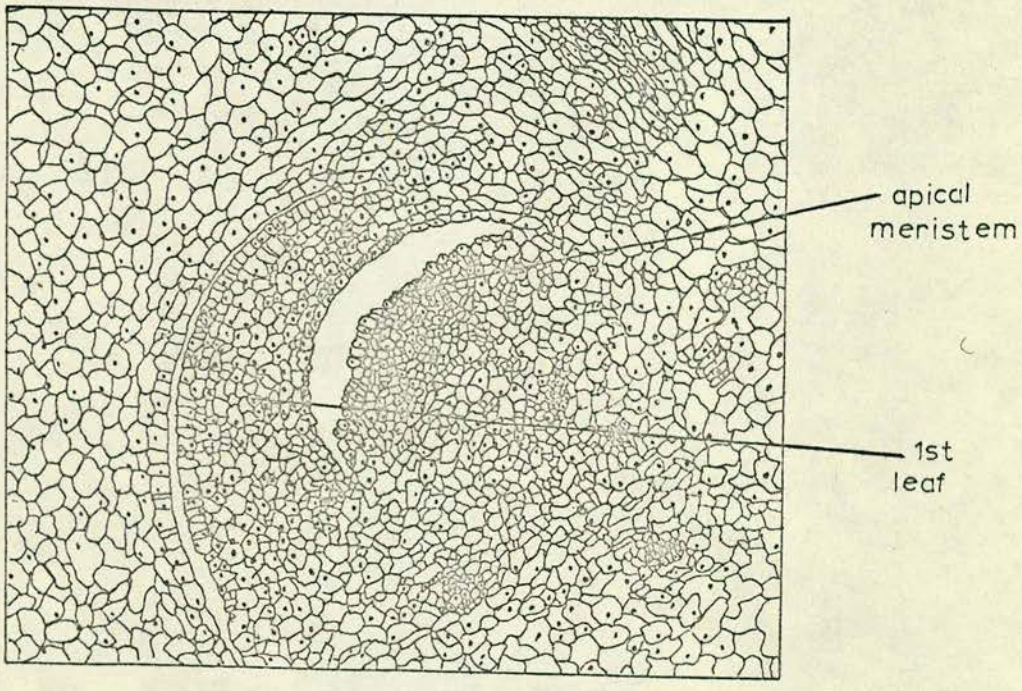


Fig. 4.10

Section through apical meristem. from mag x100



C. Preliminary experiments

The studies of morphological and anatomical changes in the primary explant system show obvious cytological differences associated with the development of the embryo. Preliminary experiments were carried out to establish changes in cell number, DNA, protein and TNA content as well as the amount of labelling with tritiated thymidine so that growth could be characterised and estimates could be made of the timing of the component phases of the cell cycle.

1) Determination of fresh weight by a reweighing procedure.

Growth can be defined as an irreversible increase in mass. If this definition is accepted, the best method of measuring growth would be by the measurement of dry weight changes. The estimation of dry weight, however, is a destructive process, but if care is taken, so that variation due to surface moisture and the humidity of weighing conditions is minimised, the increase in fresh weight of an embryo can be considered to be due to growth and could parallel the increase in dry weight. This would not be true in the period of imbibition where rapid water uptake increases the fresh weight at a much higher rate than the increase in dry weight, or for a structure accumulating large amounts of organic reserves such as starch.

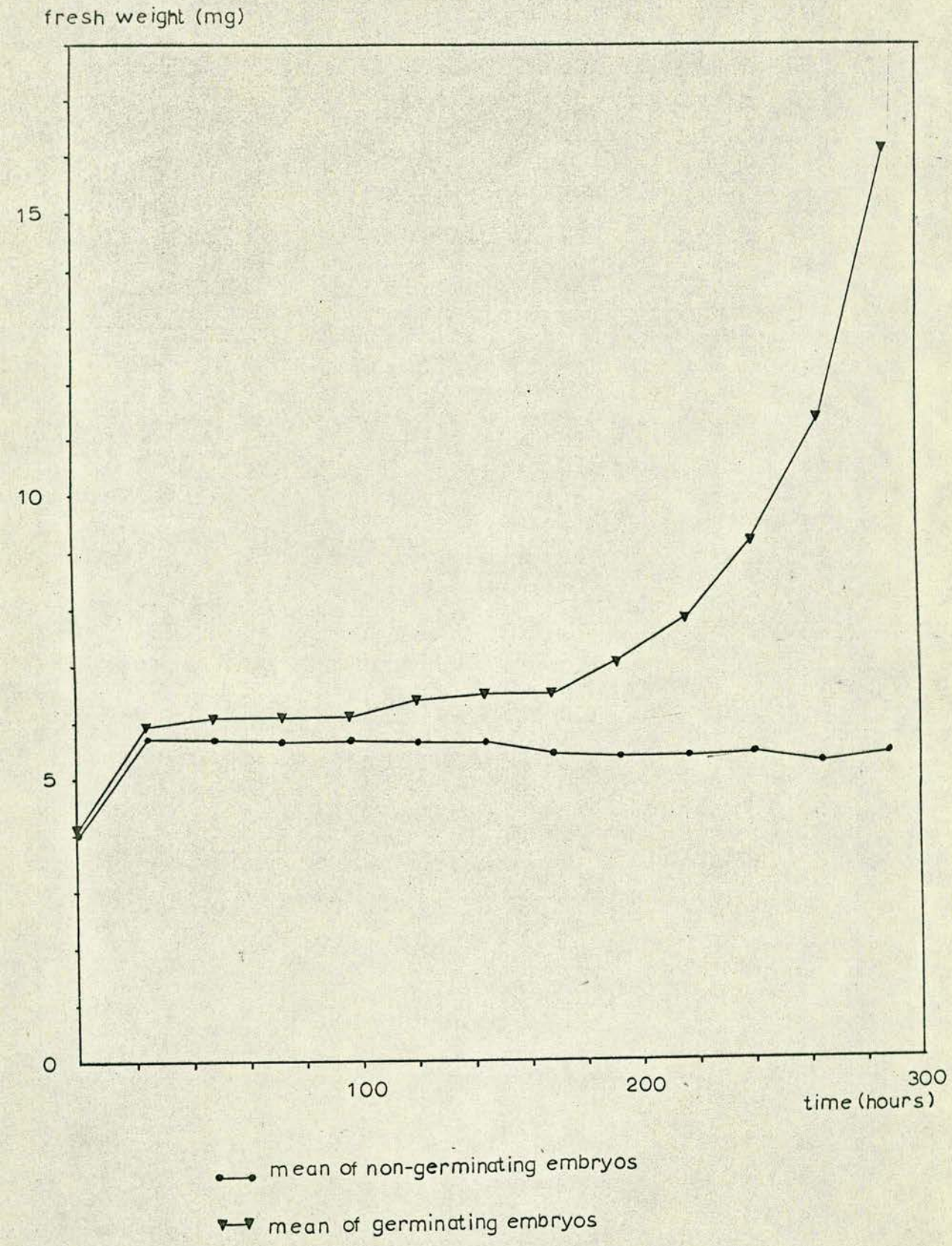
The determination of fresh weight can thus be considered a convenient parameter to follow growth patterns taking into consideration the disadvantages already mentioned. As this assay is non-destructive it can be used repeatedly on the same individual. Care must be

taken to maintain standardised weighing conditions and to reduce variation in surface moisture by uniform surface drying treatments. Repeated removal from the medium and weighing has no effect on the growth of the excised embryo.

Embryos of seed batch 7406/0 were excised, weighed and inoculated into McCartney bottles containing the defined Murashige and Skoog's medium (Materials Chapter II section B1 and 2). They were incubated under standard conditions and were weighed at 24 hour intervals. Fifteen of the total of 20 embryos, at the end of the experiment, were considered to have germinated and 5 were considered not to have germinated. The mean value of the germinated and non-germinated populations were calculated and plotted as Fig 4.11. In germinating embryos there is an approximately 50% increase in fresh weight within 24 hours from excision. The mean value of the population remains steady, at about 6 mg, until after 96 hours incubation, when a rapid increase in fresh weight begins. This increase continued until the experiment was terminated.

The fresh weight of the non-germinating embryos increased by almost 50% during the first 24 hours of incubation. The mean fresh weight remained steady between 5.8 and 5.5 mg until 144 hours and then fell, and continued to decrease until the end of the experiment.

Fig. 4.11 Changes in fresh weight, with time, in a population of excised embryos.



It can be seen that fresh weight changes in viable embryos follow three distinct phases. The initial rapid increase lasts for 24 hours or less and is associated with imbibition. The second phase is where little or no change in weight occurs, and the mean length of this phase in this experiment is 72 hours. The length of the second phase varies with individual embryos and can last up to 240 hours. The third phase is the period of exponential increase in fresh weight which is associated with the more visible signs of germination, such as greening, and radicle and shoot extension. In non-germinating embryos the first phase of fresh weight change is also found but the third phase probably never occurred due to the extension of the second phase.

Changes in fresh weight in an explant system are generally associated with growth and cell division, apart from periods of imbibition. Growth may also be determined by following cell number changes.

2) Changes in Cell number

The measurement of cell number provides a simple method of determining the onset of cell division in an embryo and establishing the pattern of change in cell number with time. However, the technique of macerating the embryo in chromic acid is destructive and so must be carried out on populations of embryos to obtain successive measurements.

A population of embryos of batch 2/7923 x 2/0114 M08656A were excised, inoculated into the defined Murashige and Skoog's medium and incubated under standard conditions. At excision and subsequently at 24 hour intervals 4 embryos were sampled, macerated in chromic acid and the cell number of each embryo estimated.

The cell numbers were statistically analysed using a Canon Canola calculator as detailed in the section on methods (Chapter II H) Fig 4.12 shows that there is no statistically significant increase in cell numbers until between 140 and 170 hours of incubation from excision. The variation in cell numbers during the period up to 160 hours is lower than after 160 hours. A line of best fit, calculated by the least squares method, for the values between 168 hours and 288 hours has a very low regression coefficient of 0.33. Using this regression line it could be estimated that cell number doubling occurs after about 216 hours of incubation. Figure 4.12 shows that there appear to be two distinct phases in cell number changes in a population of embryos incubated for up to 12 days. The initial phase has no cell number increase and in the second phase the cell numbers increase but variation in the population makes it impossible to detect whether the increase is linear, exponential or stepped.

3) Microdensitometric measurements on 10 µm wax sections of embryos to determine changes in DNA, protein and total nucleic acid with time.

The cell numbers of excised embryos do not increase immediately on incubation but only after a lag period of about 160 hours.

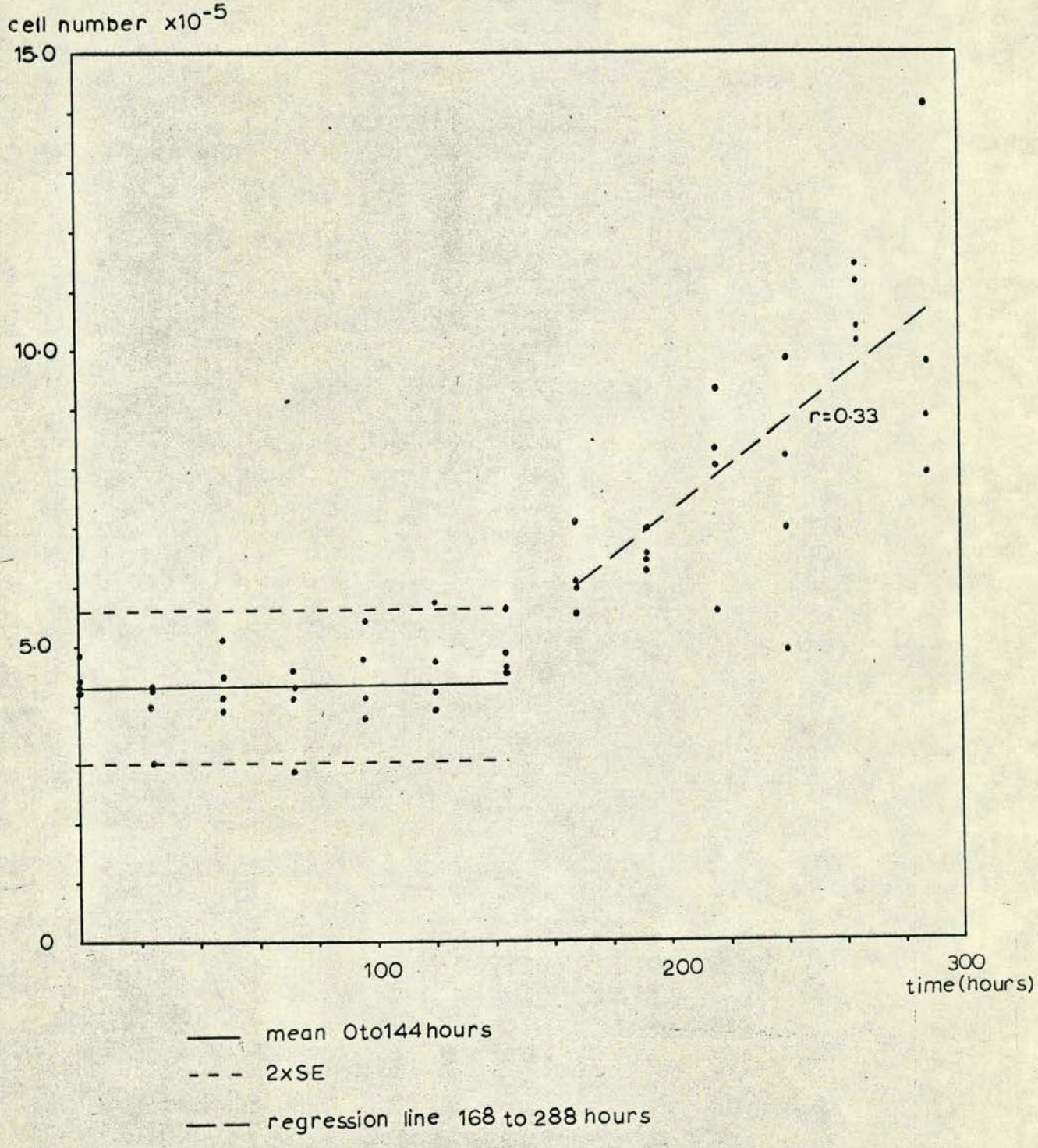


Fig. 4.12 Cell number increases, with time, in a population of excised embryos.

Measurement of the DNA content of nuclei in developing embryos will show when DNA synthesis occurs before cell division and will form a basis for subsequent cell cycle analysis. Various other cytological characteristics would also be expected to change with development of the embryo, especially protein and total nucleic acid content which can both be readily assayed.

Nucleic acids and total protein could have been measured chemically on individual embryos, however this would not have established where changes were taking place in the embryo, for example if the changes were in the tigellum or in the haustorium. Nucleic acids, protein and, more specifically, DNA were assayed by microdensitometric examination of sections of embryos. By this method the actual site or nucleus, where the nucleic acid or protein content is changing, was established. This information was used to establish the cytological characteristics of the developing embryo.

A population of embryos of batch number 2/1314 x 2/014 6378A was excised, inoculated into the 'standard' Murashige and Skoog's medium and cultured. Samples were taken at the time of excision and at subsequent recognisable stages that were defined in the section on morphology (figs 4.1 and 4.2). Embryos were chosen from these clearly recognisable stages of development so that variability within the experiment could be reduced to a tolerable level, and that any differences in DNA, TNA and protein contents would be due to differences at the time of sampling and not to individual variation.

A sample was also taken at 96 hours, as experiments using tritiated thymidine (Chapter IV section C3d) showed that variability between samples at this time is low and it is at approximately this time that the first labelled nuclei can be distinguished.

The samples were embedded in wax, sectioned and stained either for total nucleic acid estimation (gallocyanin) or for DNA and protein estimations (Feulgen and dinitroflurobenzene, DNFB).

a) DNA measurements

These were taken from sections through the meristematic regions such as shown in Fig 4.3. Changes in the DNA content of a nucleus are generally associated with cell division. The DNA content of a nucleus varies depending on which stage the nucleus has reached in the cell cycle. The DNA content is at a minimum value before the start of the period of synthesis ('S') and reaches a maximum, double the content of the minimum value, at the end of 'S'. This high level is maintained until metaphase. After metaphase the nuclear DNA content is halved and remains at this level until the beginning of the next 'S' period. If the DNA levels at the highest or lowest points of the cell cycle are known, measurement of the DNA content of any nucleus will give an estimate of the stage the nucleus has reached in the cell cycle. However, mitotic figures were not found at any stage in this experiment and so only an overall picture can be obtained from the histograms plotted from the readings.

Fig 4.13 shows that this freshly excised embryo has nuclei with little variation in DNA content, ranging from 9 to 18 units with the mode at 12 units. The distribution of values is not quite normal as there is a very small secondary peak around 18 units, suggesting a small population with a high mean value. DNA synthesis has taken place by 96 hours of incubation, (Fig 4.14) as the mode of the main peak has risen to 21 units with a secondary peak at about 26 units and the range has altered to between 11 and 33 units.

Fig 4.15 shows that after 144 hours of incubation there is a backward movement of the main peak to around 17 units, however there is a higher number of nuclei at the 20 unit class interval. This distribution of DNA values is skewed to the higher values. There are few nuclei with low values within the overall range between 11 and 27 units, indicating that DNA synthesis has taken place. In Fig 4.16 after 192 hours of incubation, there is a substantial proportion of nuclei with a low DNA content skewing the graph to the left. The range of values has extended again to between 8 and 32 units, showing a less uniform population. The main peak of the population is between 21 and 23 DNA units although few nuclei are in the 22 unit class. After 216 hours of incubation (Fig 4.17) the population of nuclei with low DNA content has declined and is mirrored by an increase in nuclei with a high DNA content, indicating that DNA synthesis has occurred. The range of DNA levels has decreased to between 12 and 32 units because of the loss of the nuclei with a low DNA content. The distribution of nuclei within the population has

Figs 4.13 to 4.18

Changes in DNA levels in nuclei of excised embryos, with time.

Abscissa: DNA content (arbitrary units)

Ordinates: Number of nuclei at each class interval

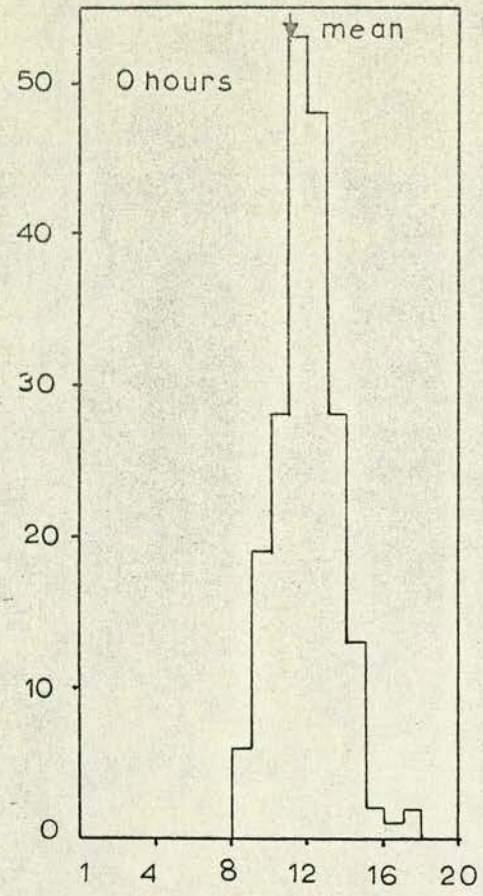


Fig. 4.13

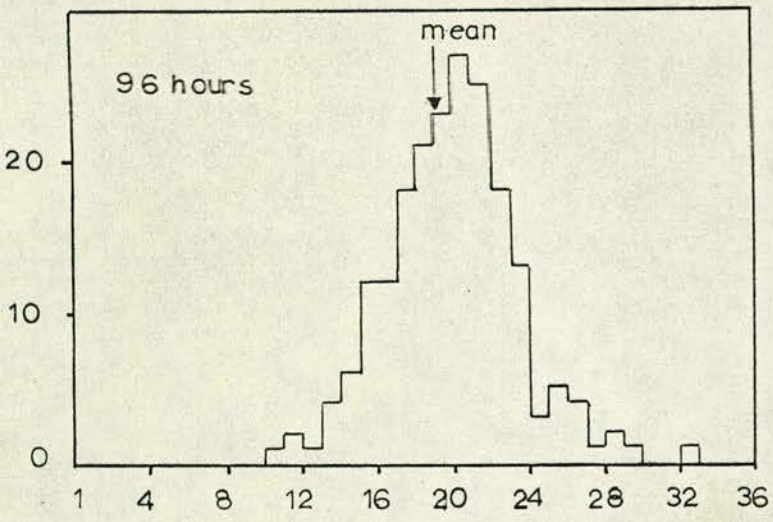


Fig. 4.14

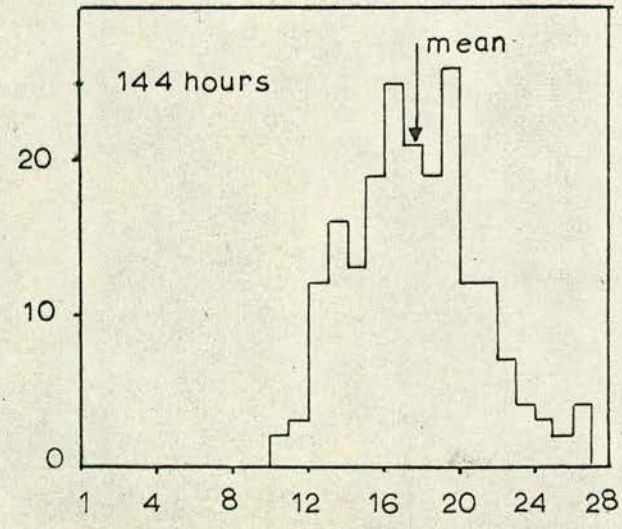


Fig. 4.15

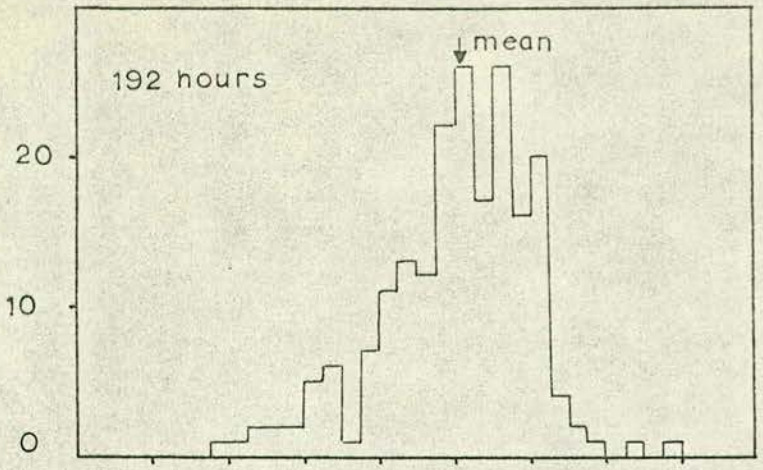


Fig. 4.16

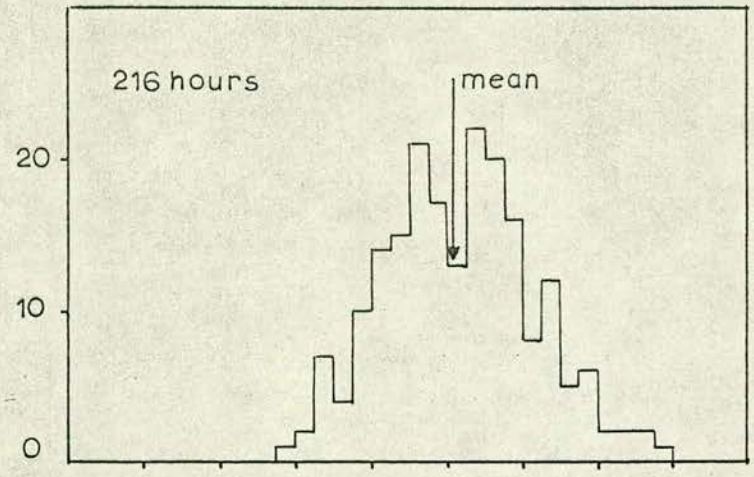


Fig. 4.17

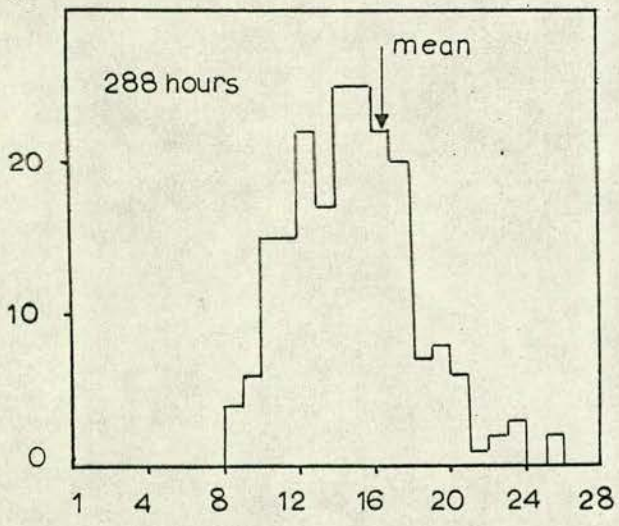


Fig. 4.18

become less uniform and may be bimodal, with peaks at around 19 and 22 units. This implies that there could be at least two populations of nuclei at different stages in the cell cycle, and hence with different DNA contents, within the embryo and each population is dividing with some degree of periodicity, but not in phase with each other. After 288 hours of incubation (Fig 4.18) the main peak of the population has shifted to between 15 and 16 DNA units, although there is a suggestion of secondary peaks at 13,20 and 24 units. The range of values is between 9 and 26 units.

Taken overall, the DNA content of nuclei of excised embryos changes during germination and becomes much more variable than the small range of DNA levels found in the newly excised embryo. There may be some degree of periodicity of nuclear division in the embryos because of the discrete peaks at differing DNA levels. This possibility is further supported by a general lack of mitotic figures. The absence of mitotic figures could mean that either mitosis is very short or that mitotis is synchronous and of short duration so that the samples have been taken at times when mitosis is not occurring. The first explanation is unlikely because some mitotic figures, however few, should have been observed. The second explanation is perhaps more likely as it also accounts for the changing peaks of the DNA measurements. Clearly these results are only suggestive and require clarification.

b) Protein measurements

Protein measurements were made on the same sections as the DNA measurements and covered the whole embryo. Proteins are essential components of living organisms, required structurally and as enzymes. It is expected that germination of the oil palm will be associated with rapid and considerable changes in protein type and concentration. Synthesis and catabolism of proteins takes place in different parts of the excised embryo, and hence measurement of the protein content by areas would show any variations over the embryo which would be missed by gross protein estimation and anyway the total amount of protein in each section can be determined by totalling the protein readings.

It must be noted that the number of fields of view measured in each embryo varies with the size of the embryo, but the mean value of protein content per field of view at each time interval has been calculated (Table 4.1).

Fig 4.19 shows the distribution of protein content of a freshly excised embryo. The values range from 18 to 74 units with a main peak around 28 units and secondary peaks at about 36, 52 and 56 units. After 96 hours of incubation (Fig 4.20) the range of values extends from 10 units to 72 units and the distribution has become separated into two distinct peaks, a lower one around 17 units with a subsidiary peak at 25 units and a higher one around 46 units with secondary peaks at about 43, 53 and 57-58 units. Thus after 96 hours of incubation there are distinct areas either with lower protein content or higher protein content and no areas with intermediary values.

Figs 4.19 to 4.24

Changes in protein content, with time, in excised embryos.

Abscissa: Protein content (arbitrary units)

Ordinate: Number of fields of view in each class interval.

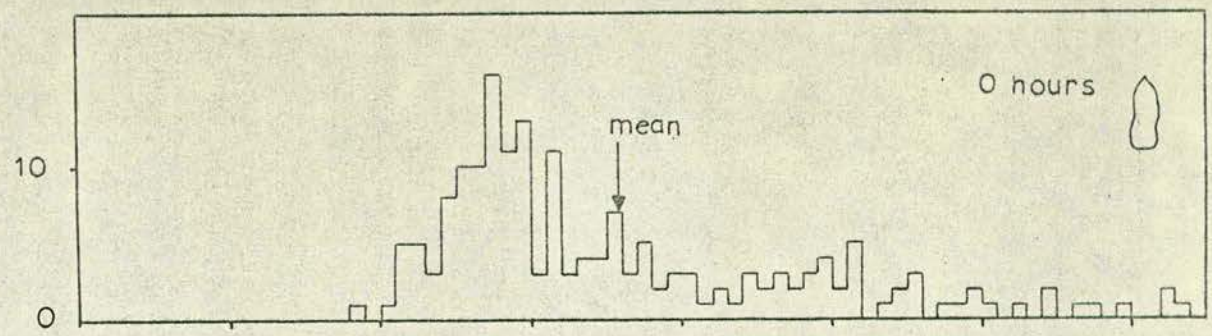


Fig. 4.19

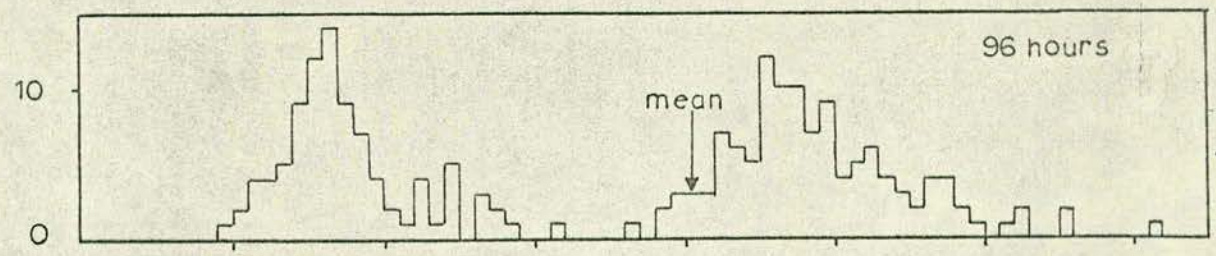


Fig. 4.20

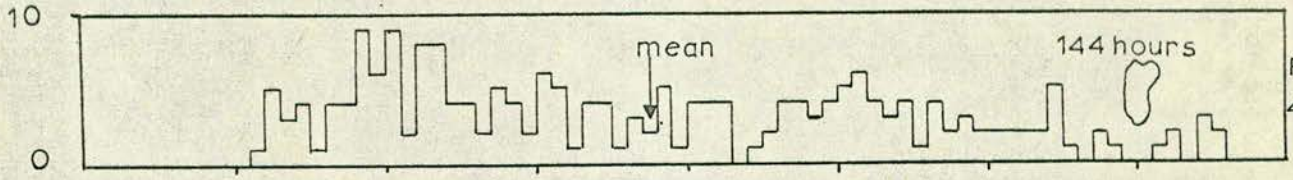


Fig. 4.21

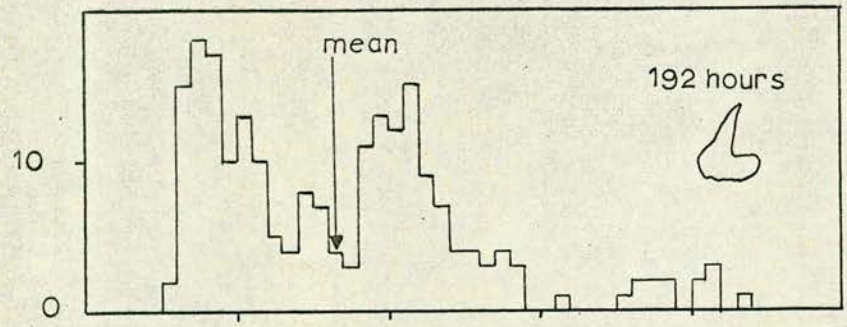


Fig. 4.22

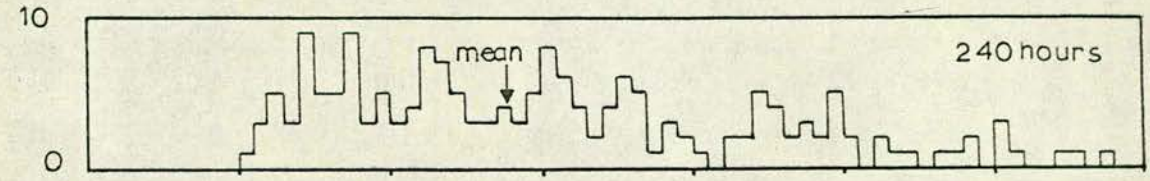


Fig. 4.23

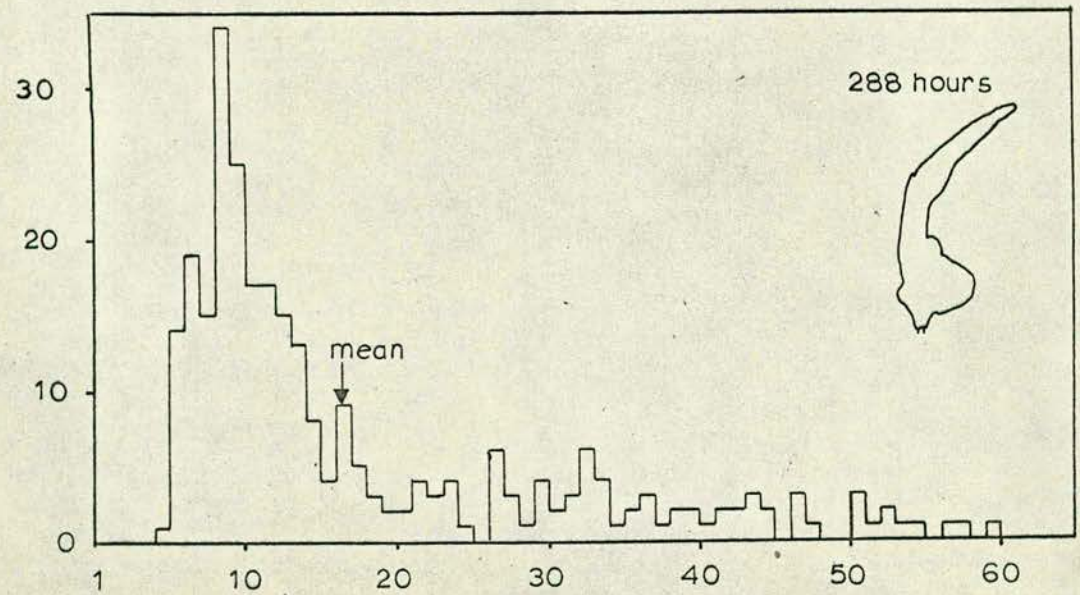


Fig. 4.24

After 144 hours of incubation (Fig 4.21) the range of protein values extends from 12 to 76 units and there is no major peak at any value. There is a break in the distribution of values at 44 units. Fig 4.22 shows the distribution of protein values after 192 hours of incubation. The range is much less than at any other time interval, extending from 6 to 44 units. There are three distinct peaks at 8, 22 and around 40 units with subsidiaries at 15 and 28 units. Fig 4.23 shows the distribution of protein values after 240 hours of incubation. There are no distinct peaks over the range of 11 to 68 units although there are more areas at the lower end of the range. After 288 hours of incubation (Fig 4.24) the range of values decreases again to between 5 and 60 units and there is only one major peak at 9 units. This compares with the freshly excised embryo (Fig 4.19) but the peak is at a much lower level.

Table 4.1 shows that the mean protein content per field up to 144 hours of incubation remains similar to the freshly excised level. The mean value increases above this at 96 and 144 hours and then halves by 192 hours. This is reflected in Figs 4.21 and 4.22 where there is a decrease in the number of high protein content fields, probably due to cell vacuolation. At 240 hours of incubation the mean protein content per field increases again shown by the increase in number of areas with high levels of protein and subsequently decreases at 288 hours.

The distribution of protein in the embryo is uneven over the section and the high protein areas cannot be associated with any structures, or tissue regions, which indicates that protein levels are changing over the whole of the excised embryo.

Table 4.1 Changes in the mean value of protein units per field of view and fresh weight with time.

Time (hours)	Number of fields measured	Total protein units (arbitary)	Mean protein/field	Fresh weight (mg)
0	179	6511	36.37	4.1
96	180	7555	41.97	5.3
144	206	7839	38.97	8.0
192	215	3710	17.26	9.6
240	177	5529	28.57	12.7
288	281	5057	18.00	20.6

c) Total nucleic acid measurements

Previous sections have established changes in DNA and protein, associated with visible changes, as the excised embryo develops. Measurement of TNA changes by fields of view will show where changes in nucleic acid are occurring within the embryo.

Gallocyanin is a quantitive stain of total nucleic acid, ie deoxyribose and ribose nucleic acids, in the nucleus and cytoplasm.

Measurements of total nucleic acid content were made on 10 µm longtitudinal wax sections cut through the meristematic regions of excised embryos, and are plotted as histograms. The number of fields of view varies with the size of each embryo and so it is useful to compare the mean value per field in each embryo (Table 4.2).

Table 4.2 shows that the amount of nucleic acid per field of view decreases slightly between excision and 96 hours of incubation, and this would be due to cell expansion without proportional nucleic acid synthesis. Between 96 and 144 hours the nucleic acid level trebles and this period is associated with one of rapid DNA synthesis as shown by the ^{3}H-thymidine labelling experiments Chapter IV E5. The nucleic acid content decreases between 144 and 192 hours, which is a period of rapid increase in cell number, where cell expansion would be taking place. The subsequent doubling and halving of nucleic acid content between 192 and 288 hours would also be associated with cell expansion.

Table 4.2. Changes in the mean value of nucleic acid units per field of view and fresh weight with time.

Time (hours)	Number of fields measured	Total nucleic acid units (arbitary)	Mean nucleic acid per field	F.W. (mg)
0	449	1953	4.35	4.3
96	317	1078	3.40	5.5
144	420	4463	10.63	7.4
192	736	4620	6.28	9.0
240	548	6497	11.86	13.8
288	363	2066	5.69	19.6

At excision (Fig 4.25) the range of nucleic acid values is quite large (1-26 units) with a single main peak at 3 units thus producing a very skewed graph due to a few high values. Fig 4.26 shows that after 96 hours of incubation the range of nucleic acid values decreases (1 to 17 units), but the peak remains in the same position at 2 units. After 144 hours of incubation (Fig 4.27) the range increases (1 to 36 units) and the main peak at 5 units is associated with a series of smaller peaks at 16, 22 and 27 units. At 192 hours of incubation (Fig 4.28) a single peak of values is again found at 5 units, and the distribution is skewed to the right within a range of 1 to 21 units. At 240 hours of incubation (Fig 4.29) the range of values has reached its maximum (1 to 40 units) and the main peak has reached 7 units. There are subsidiary peaks at 19, 24, 34 and 40 units. At 288 hours (Fig 4.30) the main peak has moved back to 4 units and the secondary peaks are at 8 and 16 units. The range of values extends from 1 to 29 units but is extended above the main population range by two single fields of view measured at 23 and 29 units.

The high values of mean nucleic acid content per field are due to areas of high nucleic acid content associated with the meristematic regions and these regions would be where cell division is taking place.

4) Labelling experiments with tritiated thymidine

Microdensitometric measurements have shown that the DNA content of nuclei changes with the development of the excised embryo but does not specify, very accurately, the proportion of nuclei synthesising DNA. Using tritiated thymidine (^{3}H-thymidine) as a label it was anticipated that the nuclei which

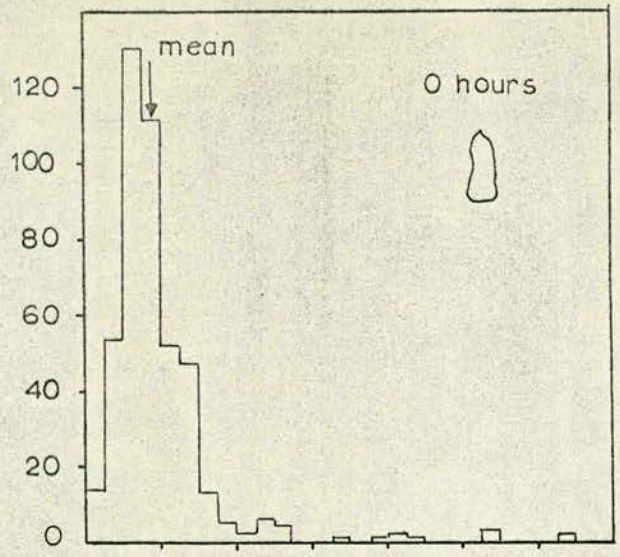


Fig.4.25

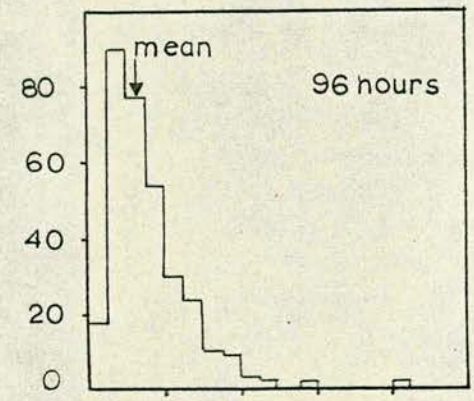


Fig. 4.26

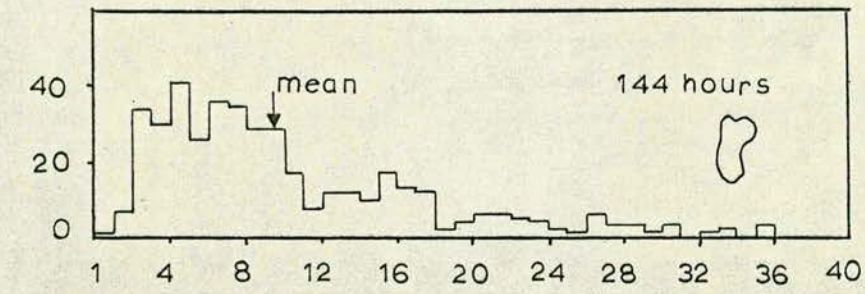


Fig. 4.27

Figs. 4.25 to 4.30 Changes in total nucleic acid content of embryos, with time.

Abscissa Total nucleic acid (arbitrary units)

Ordinate Number of fields of view in each class interval.

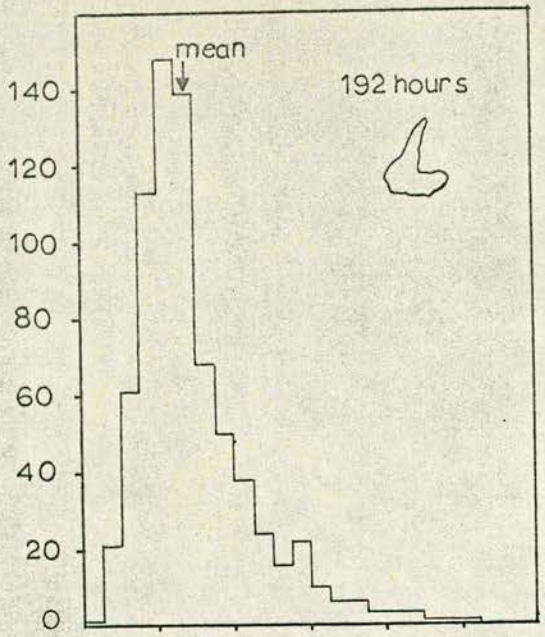


Fig. 4.28

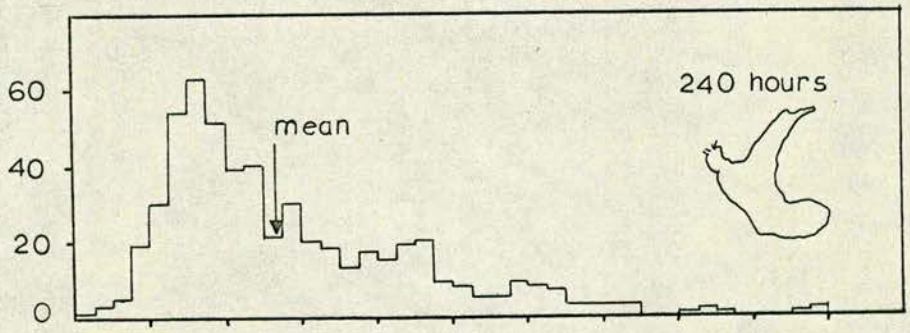


Fig.4.29

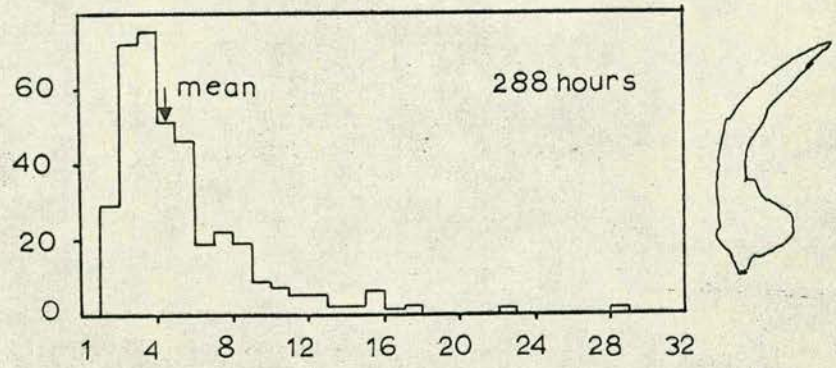


Fig. 4.30

were synthesising DNA in the excised embryos could be identified and some determination of cell cycle times could be made. Embryos synthesising DNA would incorporate the supplied labelled thymidine into the DNA and these nuclei can subsequently be distinguished by nuclear staining and autoradiography. The preliminary measurements of DNA changes in excised embryos had suggested some degree of periodicity in nuclear division and this ruled out the possibility of 'flash' labelling procedures for timing the phases of the cell cycle. It was therefore decided to use a continuous label of ^{3}H-thymidine. A continuous label of tritiated thymidine, when supplied to a population of cells will be incorporated only when the cells are synthesising DNA and will be cumulative. If the cells are periodic then the percentage of labelled cells will increase sharply from zero during S phase, and remain steady during G_2 and M. If the cells are randomly distributed throughout the cell cycle the increase in labelled nuclei will take place gradually over the whole cell cycle.

Embryos from batch number 2/7923 x 2/0114 Mo 8656A, were excised, inoculated into McCartney bottles containing 5 ml of Murashige and Skoog's medium and tritiated thymidine (^{3}H-6-thymidine) at an activity of 1 µCi/ml. At 12 hour intervals two embryos were removed, fixed in 3:1 ethanol:acetic acid, Feulgen stained, embedded in wax and sectioned at 10 µm.

These sections were brought to water, dipped in photographic emulsion and exposed in darkness at 4°C for 14 days. At the end of the exposure period the slides were developed and the percentage of labelled nuclei determined by counting along random transects using an eyepiece graticule as a measuring line.

The embryos in this experiment did not germinate well, and this was reflected in the simultaneous cell counts and in the labelling indices. The percentage of labelled nuclei are shown in Fig 4.31.

The earliest labelling was detected at the 60 hours sample when less than 0.5% of the nuclei on only one slide were labelled. Therefore 60 hours or less can be considered as the duration of 'G_1' in this population of embryos. The percentage of labelling increases to over 60% by 276 hours. It is not possible to determine the shape of the curve because of the small sample size, the poor germination and the variability of the seed batch. However, it shows that labelling of DNA increases with time and that the labelling is associated with the meristematic regions of the tigellum and the developing vascular connections between the haustorium and tigellum.

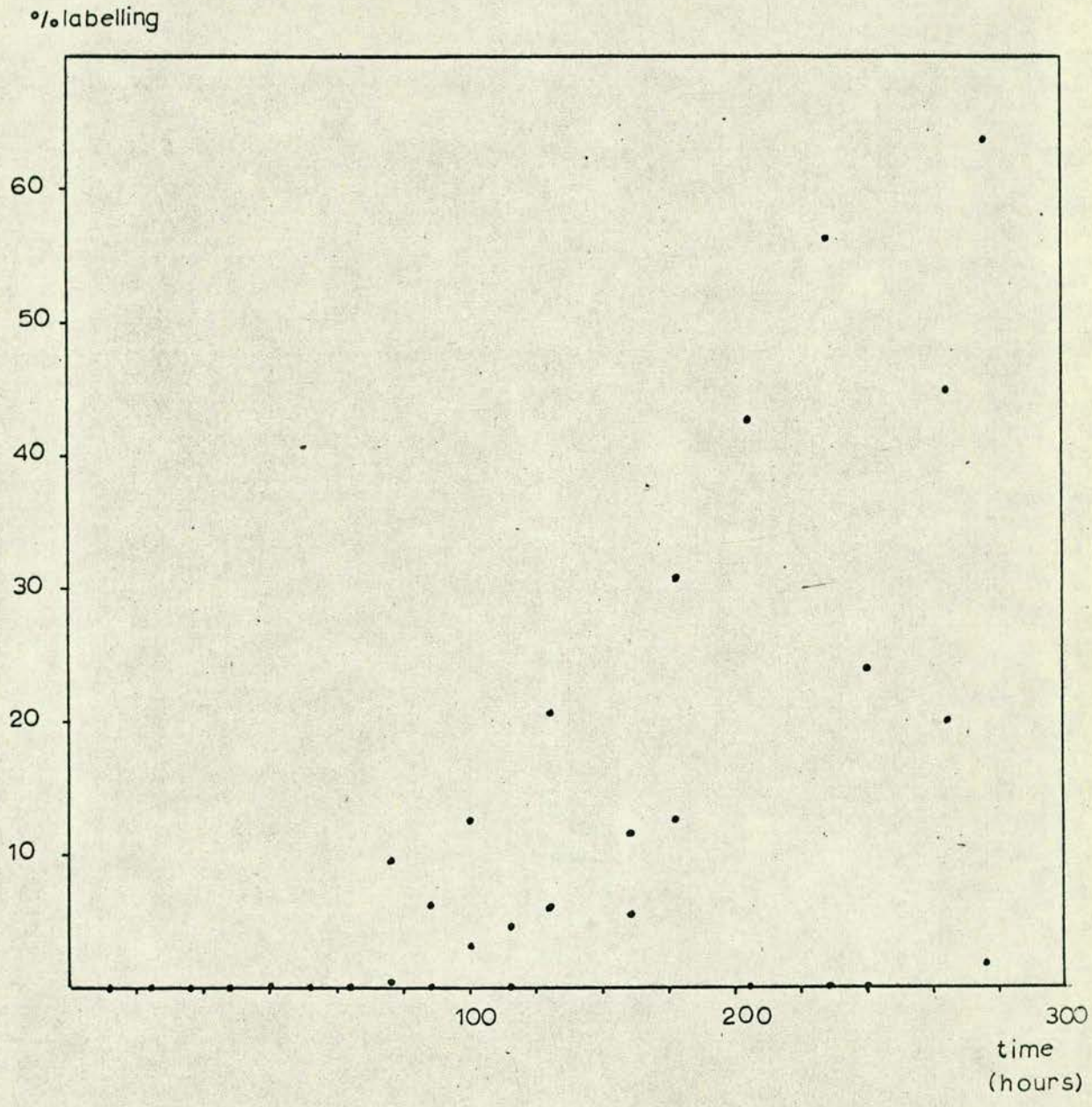


Fig. 4.31 Changes, with time, in the percentage of nuclei labelled with tritiated thymidine.

D. The sub-division of the excised embryo into tigellum and haustorium

The results from preliminary experiments in which morphological, anatomical and cytological changes were followed, clearly showed that the tigellum and haustorium have differing roles in the development of the embryo. The haustorium and tigellum appeared to develop at different rates during germination, they have different colours and developed different structures. Anatomically they are different. The tigellum has the root and shoot meristems, some vascular tissue and some parenchyma, whereas the haustorium consists mainly of parenchyma and developing vascular tissue.

Division of the embryo into its two components after various periods of incubation would allow each to be assayed separately and when taken as part of a population could show the growth characteristics of each portion. The embryos were only divided at the time of sampling so that the tigellum and haustorium developed as a single unit.

1) Changes in fresh weight

Embryos from seed batch 2/5742 x 2/11605 were excised, inoculated into McCartney bottles and incubated under standard conditions. At 24 hour intervals between 2 and 4 embryos were sampled, divided into tigellum and haustorium using a razor blade, weighed and immersed in 2 ml of chromic acid for cell counts as described in (Chapter II Methods D).

In Fig 4.32 the tigellum shows a doubling in fresh weight from about 1 to 2.0 mg in the first twenty-four hours of incubation. The fresh weight increases very slowly between 24 hours until 120 hours and then, despite increasing variation the fresh weights increase in all except one sample. One tigellum weighed over 30 mg by 240 hours of incubation.

Fig 4.32 shows that the haustorium also doubles in weight from about 0.75 to 1.5 mg in the first 24 hours of incubation. The fresh weight increases steadily but slowly until 144 hours of incubation. Subsequently only one individual increased rapidly.

The haustorium does not increase in fresh weight as much as the tigellum, the maximum weight, attained after 240 hours, is 29.5 mg, and the rate of increase is always lower. The tigellum is generally heavier than the haustorium and this difference is accentuated during the developmental period in the intact fruit when the haustorium attains a maximum size, filling the endocarp.

2) Changes in cell number

The embryos used in section 1 above were macerated in chromic acid and the cell numbers determined. The cell numbers are presented in Figs 4.34-4.35. Little significance change in cell number occurs in the tigellum up to 120 hours of incubation, after which there is an approximately linear

Fig 4.32

Changes in fresh weight, with time, in the tigellum.

Fig 4.33

Changes in fresh weight, with time, in the haustorium.

Fig 4.34

Changes, with time, in cell number in the tigellum.

Fig 4.35

Changes, with time, in cell number in the haustorium.

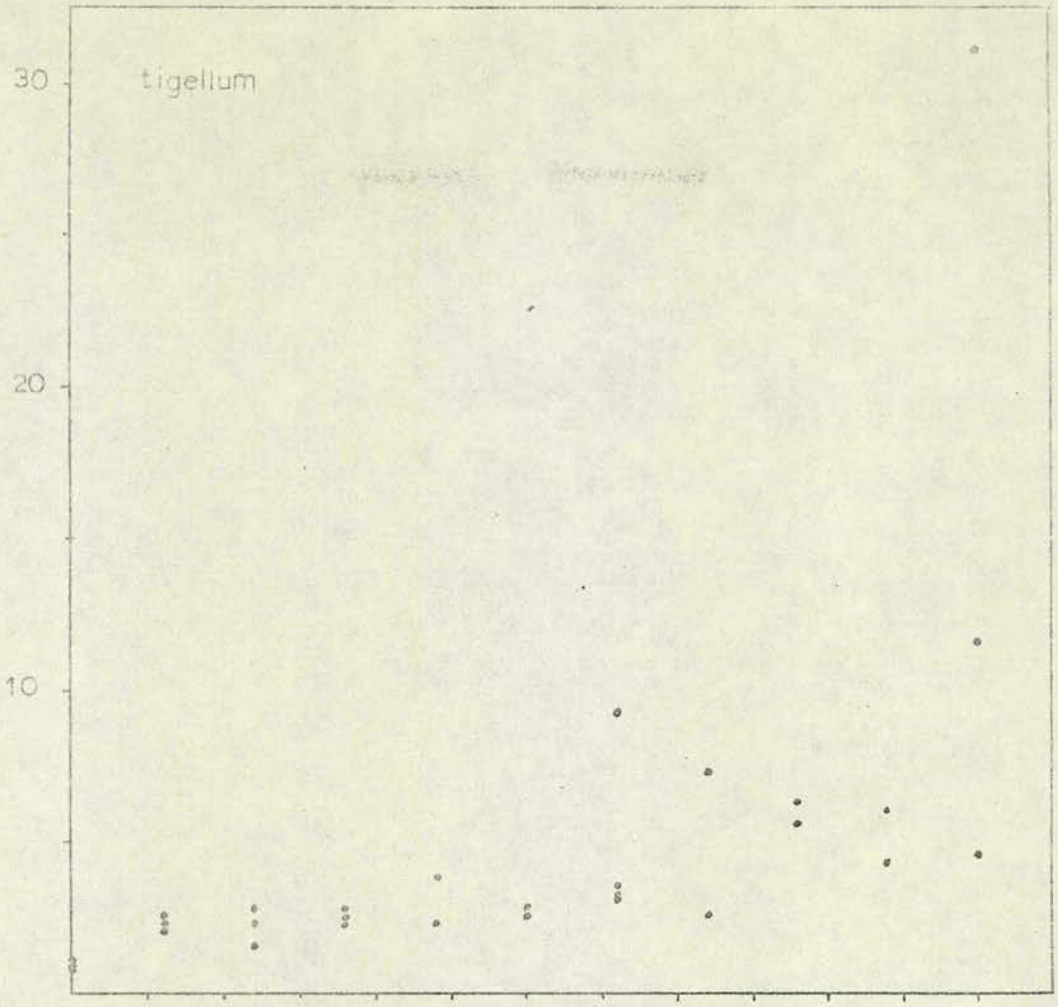


Fig. 4.32

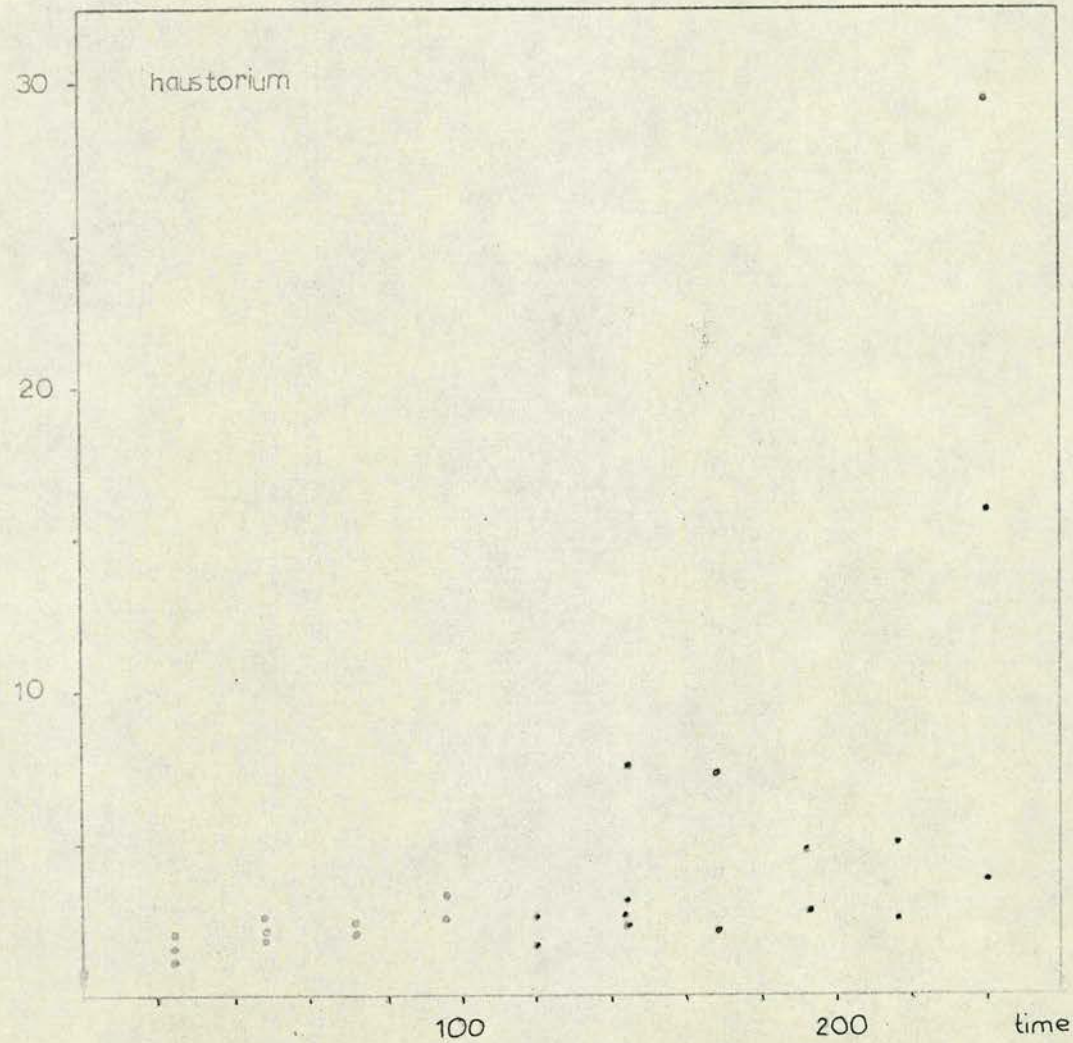


Fig. 4.33

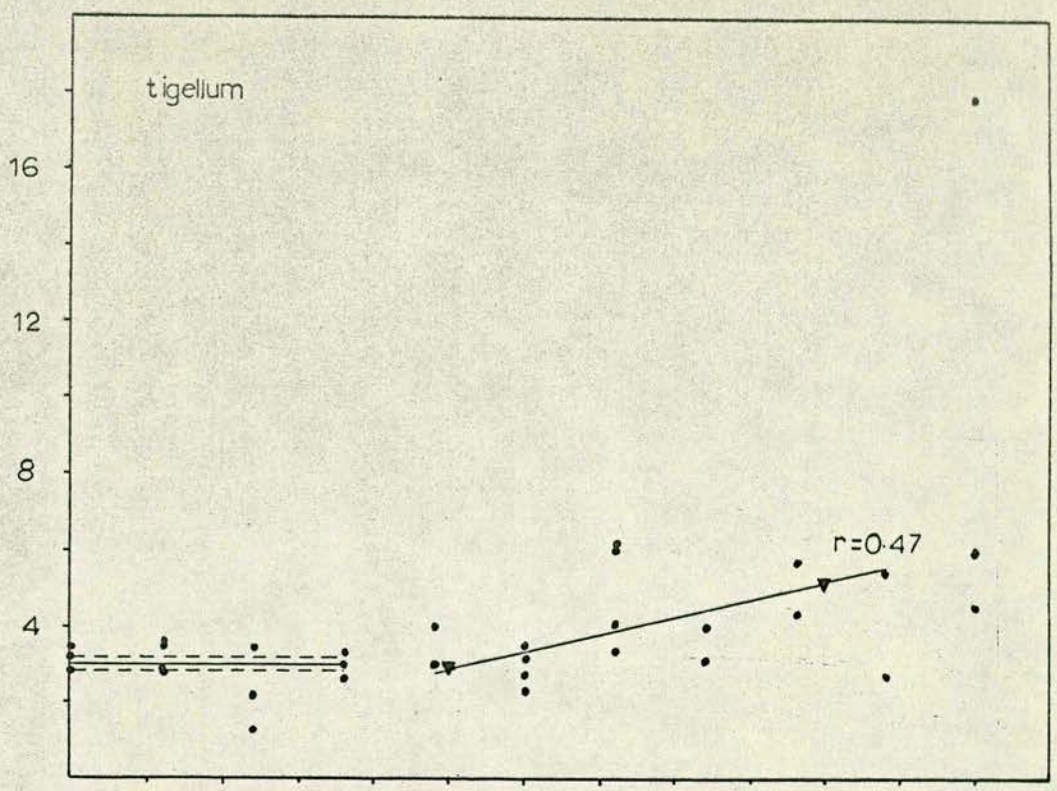


Fig. 4.34

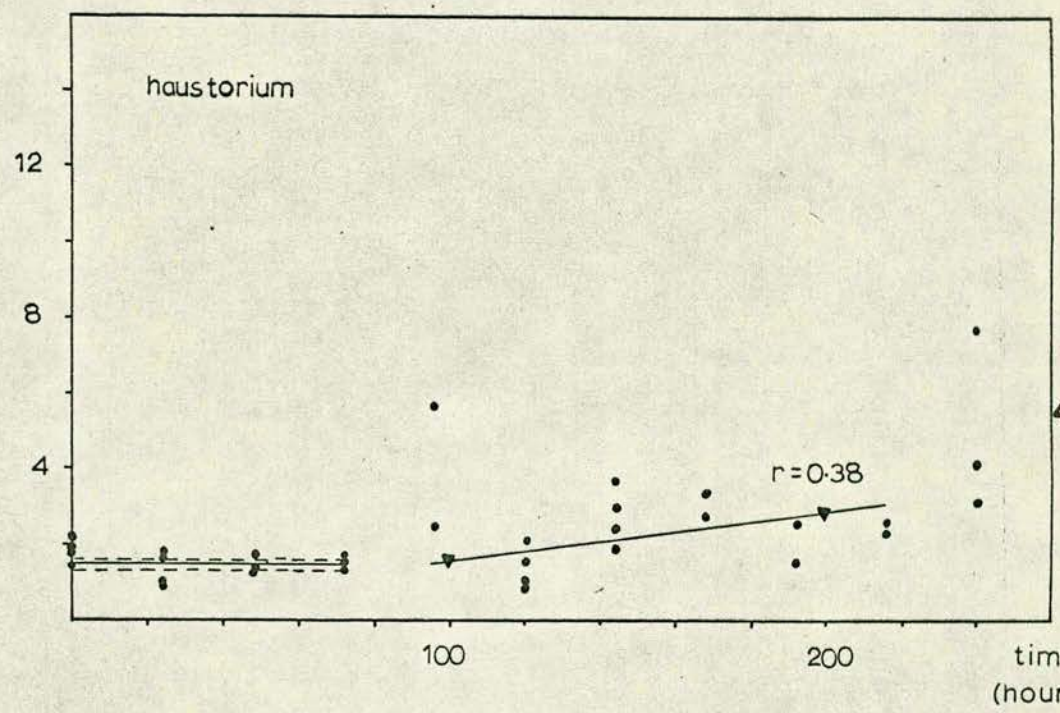


Fig 4.35

mean & SE 0 to 72 hours

regression line 96 to 216 hours

increase. The cells of the haustorium appear to undergo division slightly earlier at about 96 hours and again the subsequent increase in cell number is approximately linear, but at a lower rate than in the tigellum. This can be seen from the calculated regression lines, which show that the haustorium is more variable than the tigellum, after a period of incubation of 100 hours.

3) Changes in mean fresh weight per cell

The results of sections 1) and 2) above can be combined to calculate the mean fresh weight per cell per embryo. Cells undergoing division will halve in fresh weight between the initiation of mitosis and the completion of cytokinesis as two daughter cells are formed from one parent. The mean value of fresh weight per cell for each embryo was calculated and the overall mean at each time interval presented in Fig 4.36.

Fig 4.36 shows that the mean fresh weight per cell in the tigellum trebles between the time of excision and after 48 hours of incubation. Between 48 and 96 hours there is apparently a small decrease, and this is followed by a further approximate doubling up to 168 hours. There is then a second small decrease at 192 hours and this is followed by a further increase up to 240 hours.

The mean fresh weight per cell of the haustorium increases between 0 and 96 hours of incubation. Initially (0 to 24 hours) this increase is rapid but the rate decreases between 24 hours

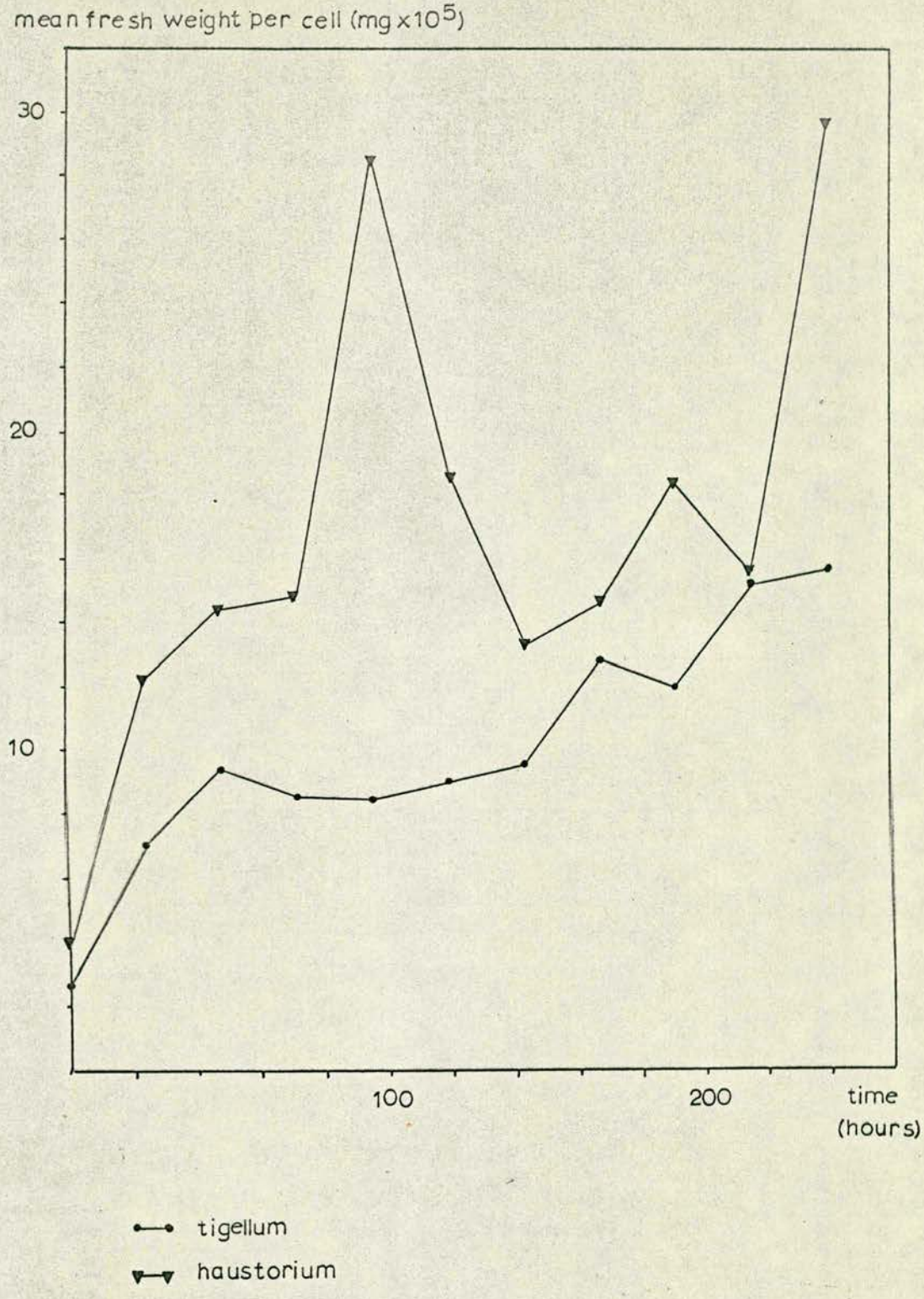


Fig. 4.36 Changes in mean fresh weight per cell, with time, in the tigellum and the haustorium.

and 72 hours so that at 72 hours the average cell is two and a half times the original weight at excision. The increase in fresh weight between 72 and 96 hours is very rapid and is an approximate doubling of the 72 hour weight, from about 14.6×10^{-5} mg to 28.3×10^{-5} mg. During the next 48 hours there is a halving of the fresh weight per cell from about 28.3×10^{-5}mg to 13.2×10^{-5}mg. Between 144 and 192 hours of incubation there is a 50% increase in mean fresh weight per cell but this is not maintained at 216 hours. Between 216 and 240 hours there is a doubling of fresh weight per cell from 15.3×10^{-5} mg to 29.6×10^{-5} mg.

Thus the tigellum shows an overall maintained increase in mean fresh weight per cell, indicating that the cells are generally getting larger. The observed pattern of doubling and subsequent halving of the mean fresh weight per cell in the haustorium suggests that a large population of cells are undergoing cytokinesis within a very short period. This period coincides with the first detectable increase in cell number at 96 hours. This period of cytokinesis is followed by another, with a smaller population of cells, between 168 and 216 hours, and the beginning of a third, equal in magnitude to the first, at 216 hours. These results would suggest a mean cell generation time of 144 hours for the main population of cells in the haustorium.

It is perhaps interesting to note that the haustorial cells are always heavier than the tigellal cells, and can be up to three times heavier (at 96 hours).

E. A time course experiment to determine the kinetics of the cell cycle in a population of embryos.

Changes in the basic growth parameters of fresh weight and cell number together with anatomical, morphological and cytological changes have been described in a series of preliminary experiments which due to availability of seeds were carried out using a number of different batches. These experiments have established that DNA synthesis, nuclear and cell division take place in the developing embryo, and that the developmental patterns of the tigellum and haustorium are different. The preliminary experiments were carried out using a number of different seed batches, and because there is established variability between batches (see Chapter III) it was decided to confirm the preliminary results investigating the cell cycle by repeating the experiments using a population of embryos from the same seed batch. Using a single seed batch it was hoped that the time of initiation of DNA synthesis could be directly related to the time of the first increase in cell number and the changing patterns of nuclear DNA content.

The embryos used in this time course experiment were from seed batch number 0588659292. They were incubated, under the standard

conditions, in the defined Murashige and Skoog's medium containing 1 μCi/ml of tritiated thymidine. Each embryo was weighed at the time of excision and at sampling to establish changes in fresh weight in the population. In order to obtain 'maximum data potential' from this experiment it was decided to sample embryos at 6 hour intervals for a period of 12 days. This would mean that large numbers of embryos would be used to counter variability and to make the experiment statistically viable. The experiment was set up using 4 sets of embryos, excised at 6 hour intervals so that sampling was carried out at one time each 24 hour interval (see Table 4.3).

Each sample consisted of six embryos, three of which were macerated in chromic acid for the determination of cell numbers and three were Feulgen stained and used for autoradiography. At each 24 hour interval two embryos were sampled for Feulgen staining and squashed to establish the DNA content of nuclei by microdensitometry.

1) Changes in fresh weight

The weights at each sample time are presented in Fig 4.37. The results were analysed stastically as described in the methods Chapter II section H. The fresh weight increases between 0 and 24 hours due to imbibition. It can be seen that no significant increase in fresh weight occurs between 24 and 120 hours of incubation. If a regression line is calculated for the population from 150 hours to 250 hours (ignoring values within two standard deviations of the non-increasing population) and then extrapolated,

Table 4.3 Timing of the start of 4 sets of embryos to sample at 24 hour intervals.

Set number	Excision time	Sample time	Experimental time (hours)
1	9 am day 0	9 am day 1	24
2	3 pm day 0	"	18
3	9 pm day 0	"	12
4	3 am day 1	"	6
1	9 am day 0	9 am day 2	48
2	3 pm day 0	"	42
3	9 pm day 0	"	36
4	3 am day 1	"	30

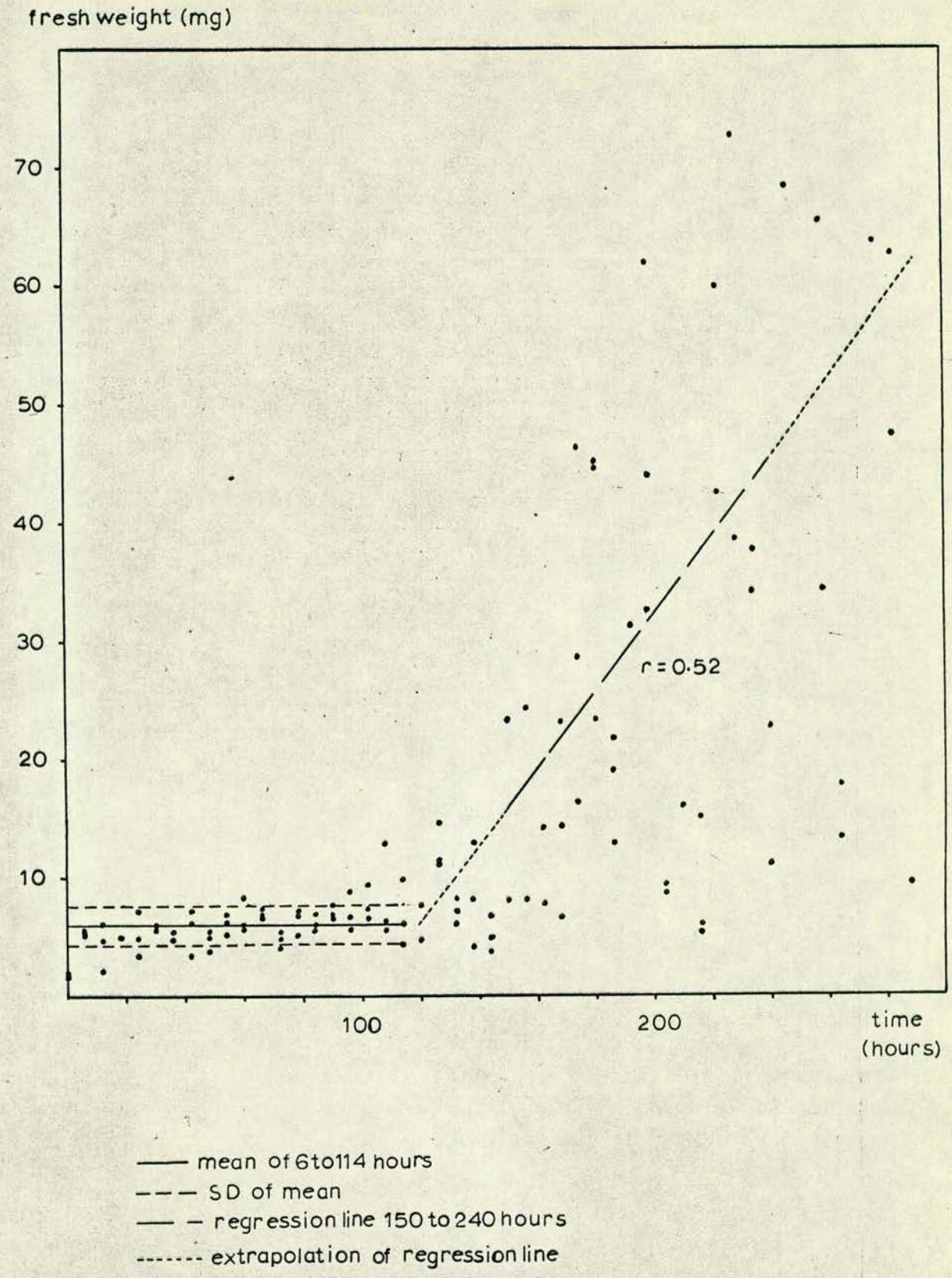


Fig. 4.37 Changes in fresh weight, with time, in a population of excised embryos.

the first increase in fresh weight occurs about 124 hours from excision. Fresh weight doubling takes place at imbibition and by 140 hours, 178 hours and 254 hours by estimation from the regression line. This shows that the rate of increase is falling offrapidly and that the increase probably follows a curve that will become S shaped. Initially, variation within the population is low, increasing slightly following imbibition, but it increases rapidly from about 110 hours as shown by the low regression coefficient of 0.52, which suggests a straight line fit is unlikely.

2) Changes in cell number

These are presented in Fig 4.38. The mean and standard deviation of the population from 0 to 126 hours of incubation were calculated and regression lines were fitted for incubation times between 150 hours and 186 hours and between 222 and 282 hours. Extrapolating the first fitted regression line it can be said that cell numbers probably begin to increase at c.152 hours. The rate of increase in cell numbers from 152 hours until 288 hours is not continuous, as there is a break in the rate of increase. This could imply a degree of periodicity in cell division. The first doubling in cell number takes place by 184 hours. The second doubling takes place by 290 hours. It must be noted however that the co-efficient of regression is low (150-186 hrs = 0.49 and 222 to 282 hrs = 0.37) expressing the high degree of variability, higher than that of the fresh weight data.

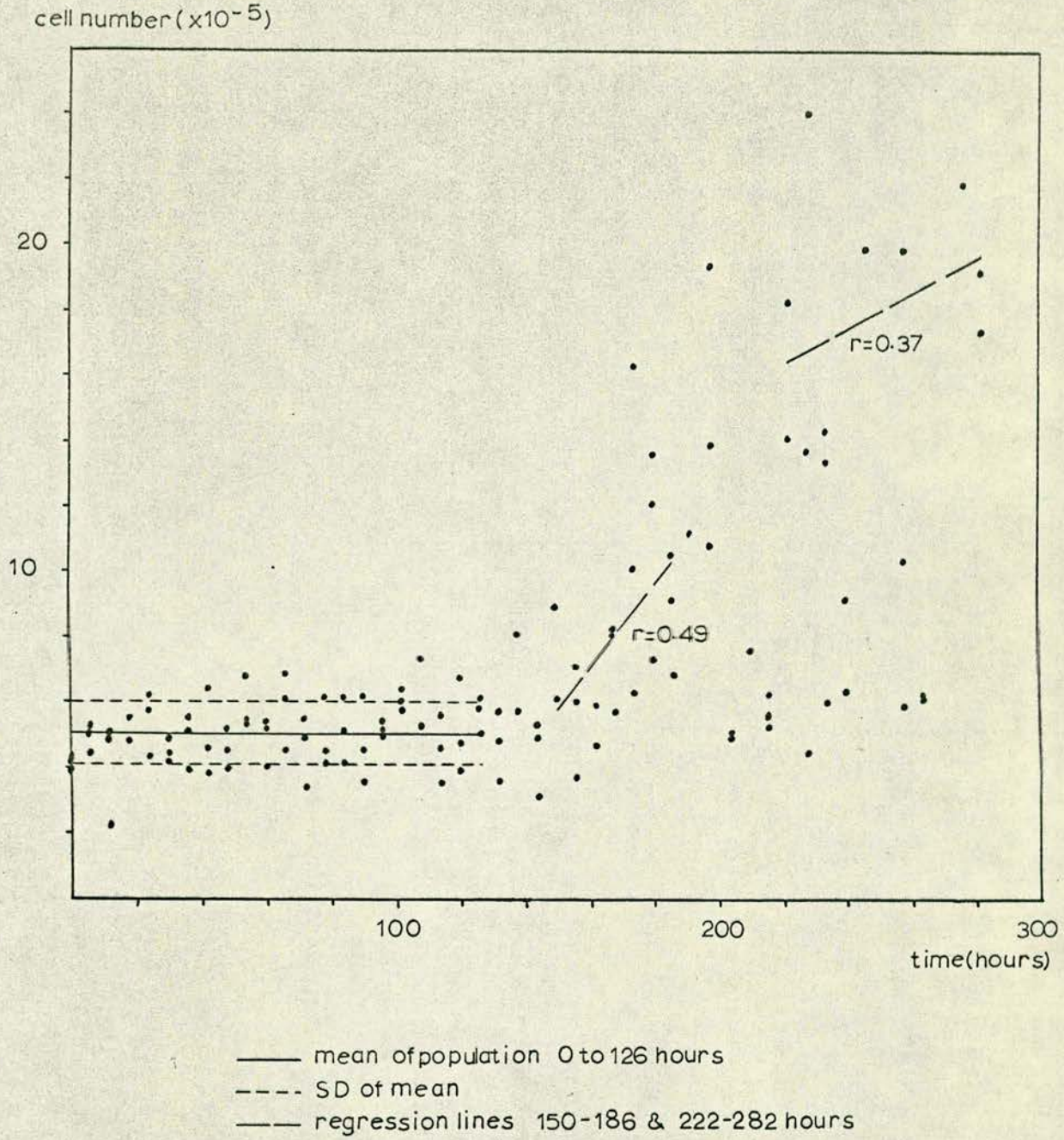


Fig. 4.38 Changes in cell number, with time, in a population of excised embryos.

There is a suggestion (not statistically significant because of the sample size) from these data that there could be an increase in cell number within the first 24 hours of incubation from excision. Further evidence for this is at presented later in the microdensitometric measurements (Chapter IV section 5d) and in a further more detailed study of the first twenty-four hours of growth (Chapter IV section 6).

3) Changes in mean fresh weight per cell.

The mean fresh weights per cell have been calculated from the data obtained from cell numbers and fresh weight and are presented as Fig 4.39, where it can be seen that the mean fresh weight per cell doubles within the first 6 hours of incubation due to imbibition.

The variation in and between samples increases with the length of the experimental period and so it is not possible to define the form of increase. However all samples after the period of imbibition are heavier than the freshly excised embryo, showing that all the embryos imbibe substantially.

4) Changes in DNA content

Samples were taken at 24 hour intervals, Feulgen stained in batches using uniform Jerusalem artichoke tuber tissue as a standard (see Chapter IIC), divided into tigellum and haustorium and each portion squashed separately on slides. Measurements were made on 200 nuclei in each squash along random horizontal transects using the

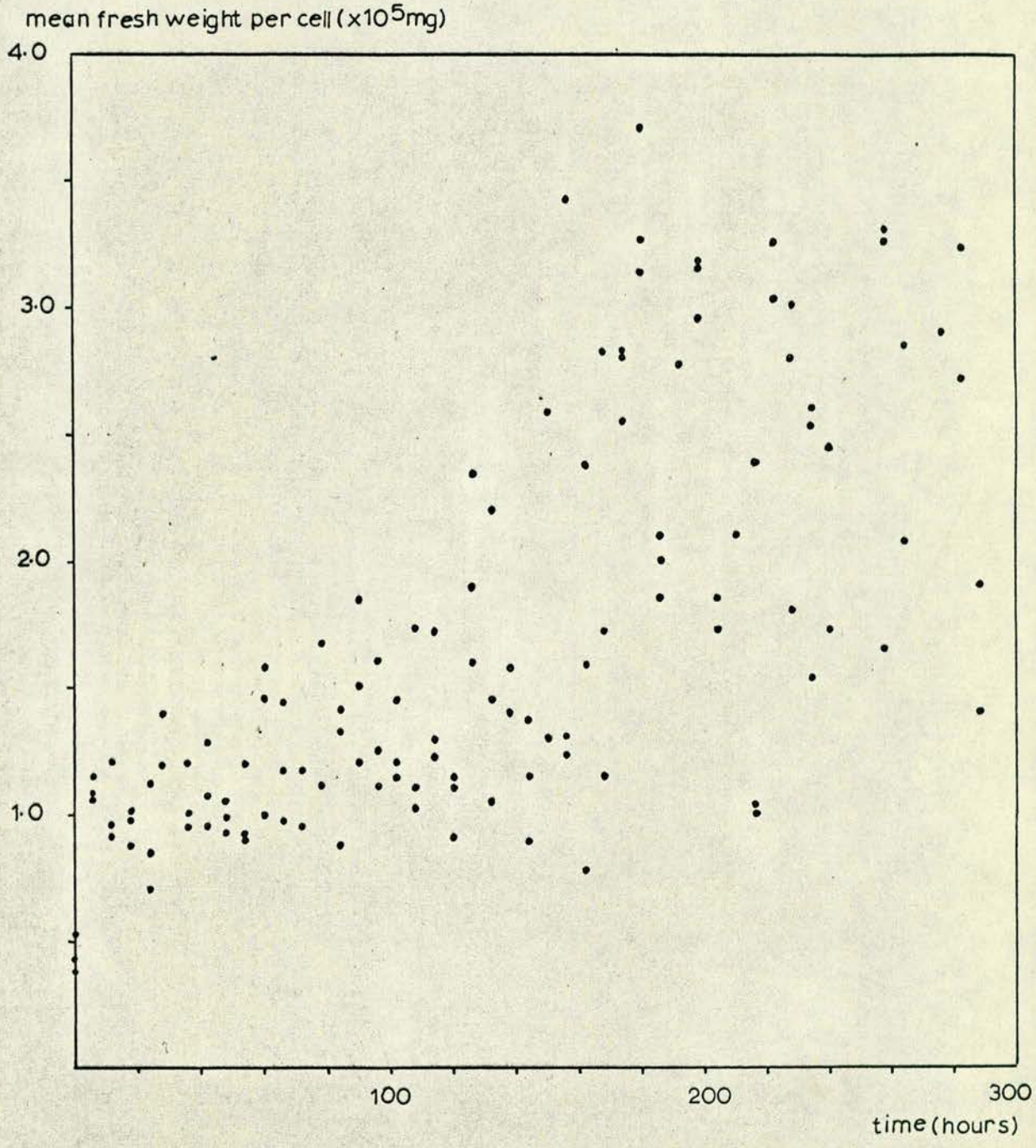


Fig. 4.39 Changes in mean fresh weight per cell, with time, in a population of excised embryos.

microdensitometer. One complete set of tigellum and haustorium readings at each time interval covering the duration of the experiment was obtained before measurement of the duplicate batch was attempted. While the second set of measurements was being made the microdensitometer developed a progressive fault which reduced the absorption values. However it is possible to correct the fault by calculating the ratio of the values of corresponding first and second set values and multiplying the second (low) set by that ratio. Table 4.4 shows the calculated conversion.

Table 4.4 Conversion of 2nd set readings to rectify machine fault.

Time	Mean of Population				Ratio 1:2		corrected means Set 2 values	
	Set 1 tigellum	haustorium	Set 2 t	h	t	h	t	h
0	22.80	16.60	20.18	21.60	1.13	0.77	22.80	16.66
24	19.05	18.65	15.14	14.53	1.26	1.29	19.08	18.74
48	19.90	18.89	15.07	13.28	1.32	1.42	19.89	18.86
96	23.37	20.33	15.57	17.60	1.50	1.16	23.34	20.42
144	21.46	14.86	12.05	11.13	1.78	1.34	21.45	14.91
168	19.55	19.03	12.37	12.28	1.58	1.55	19.54	19.03
264	21.43	16.93	12.62	12.14	1.70	1.39	21.45	16.87

The figures showing the second set of readings (Fig 4.48-4.54 and 4.63-4.69) are plotted from the uncorrected values with the uncorrected mean indicated and the corrected mean in parenthesis. Throughout this section the uncorrected values of set 2 are used

with the corrected values in parenthesis. The corrected values can be used in direct comparison with the values obtained in the first batch.

Probit analysis may be used to examine the distribution of a population of values and to assess the probability of the distribution deviating from normal (see Chapter IIG). The analysis can be used as a convenient method of comparing the nuclear DNA contents of the tigellum and haustorium in and between the two sets of measurements by distinguishing populations of nuclei with the same DNA content.

Tigellum measurements - set 1.

Figure 4.40 shows the distribution of DNA values in the tigellum of a freshly excised embryo. The range of values is between 13 and 29 units and the mean and mode are both at 22 units. The probit analysis shows that the distribution is not even with sub-populations between 14 and 16 units, 17 and 21 units, and 21 and 29 units, and that there is a greater proportion of high value nuclei than low value nuclei.

After 24 hours of incubation (Fig 4.41) the range of values increases greatly but the mean and mode fall to 19 and 18 units respectively. The range, however, is increased to between 7 and 38 units by a small number of high DNA content nuclei forming isolated populations. There are a number of sub-populations with peaks at 15 and 21 and the isolated populations at 29 to 31 units, 33 to 34 units and 37 to 38 units. The fall in the mode and mean values suggest a decrease in the number of nuclei with high values due to nuclear division.

Figs 4.40 to 4.47

Changes in DNA content of nuclei in tigella.

Set 1 (T1)

Figs 4.48 to 4.54

Changes in DNA content of nuclei in tigella.

Set 2 (T 2)

Figs 4.55 to 4.62

Changes in DNA content of nuclei in haustoria

Set 1 (H1)

Figs 4.63 to 4.69

Changes in DNA content of nuclei in haustoria.

Set 2 (H 2)

Abscissa: DNA content (arbitrary units)

Ordinate: Right-probit value

Left - numbers of nuclei per class.

Prophase and metaphase figures.

Anaphase and telophase figures.

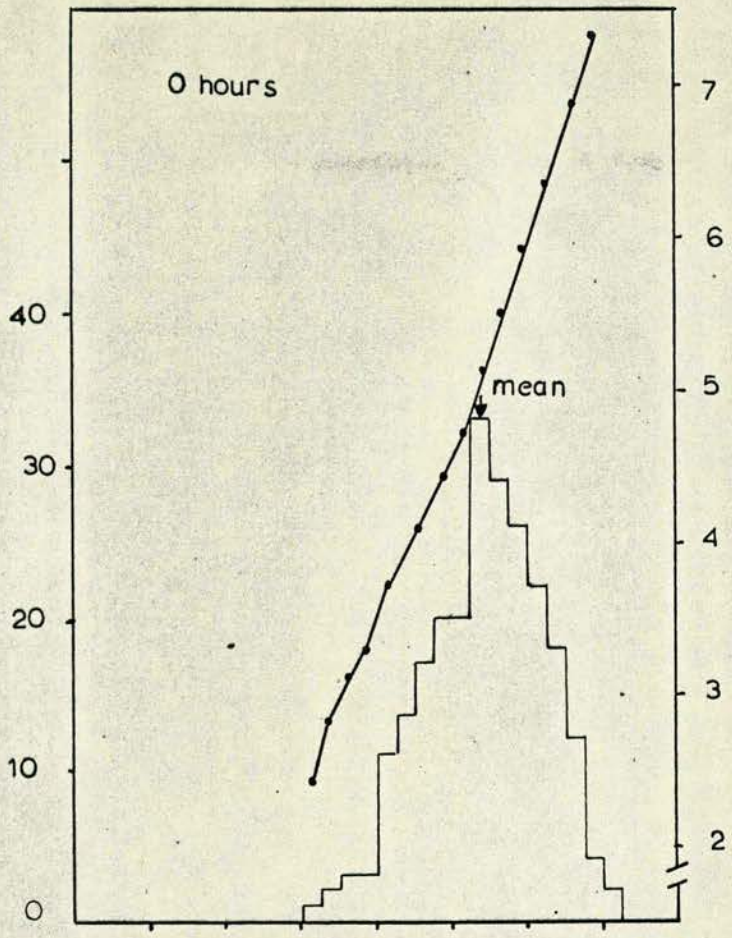


Fig. 4.40
(T1)

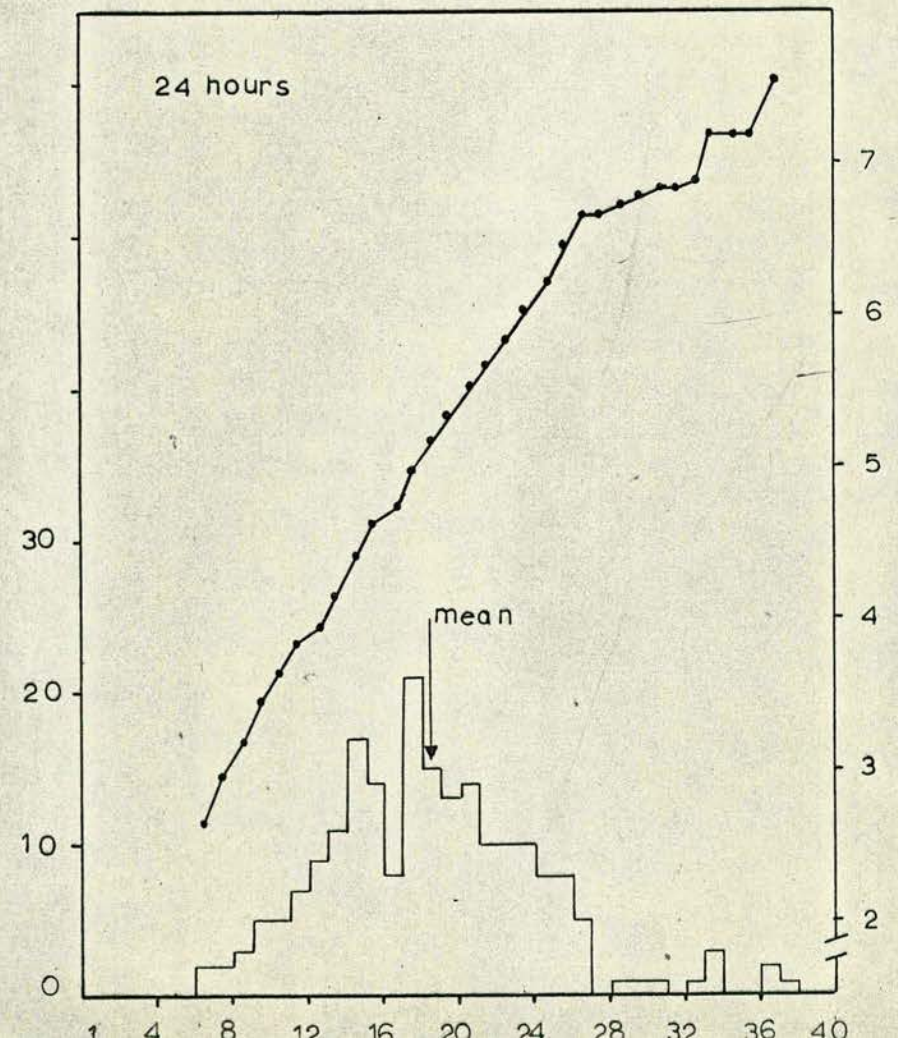


Fig.4.41
(T1)

After 48 hours of incubation (Fig 4.42) the range of values is reduced to between 11 and 36 units, but the mode increases to 22 units while the mean remains at 19 units. There are a number of secondary peaks shown by probit analysis to be at 12, 18 and 35-36 units.

After 96 hours of incubation (Fig 4.43) the range of values has increased to between 8 and 45 and although the mean increases to 23 units the mode is maintained at 22 units. The distribution of values is uneven and probit analysis distinguishes a number of sub-populations, and the low number of values at 21 and 28 units divides the main population of values into 3, apart from the 5 isolated populations at 8, 36, 39, 41 and 45-46 units. The increase in number of nuclei with high values indicates that DNA synthesis is taking place and this is confirmed by the labelling experiments using tritiated thymidine.

After 144 hours of incubation (Fig 4.44) the range of results is increased again to between 8 and 47 units but the mode and mean both fall to 21 units suggesting that nuclear division has taken place. This is confirmed by the estimated changes in cell number which begin to increase between 140 and 160 hours of incubation. The distribution of values is very uneven within the total range especially at the high DNA values. Probit analysis distinguishes populations with peaks at 10, 15, 18, 21, 24, 29, 31 and 34 units with isolated groups of nuclei at 39, 42 and 47 units.

After 168 hours of incubation (Fig 4.45) the range of values is reduced to between 8 and 27 units as the nuclei with a high DNA content have disappeared, presumably due to division. The reduction in the number of nuclei with high values is reflected in the reduction of the

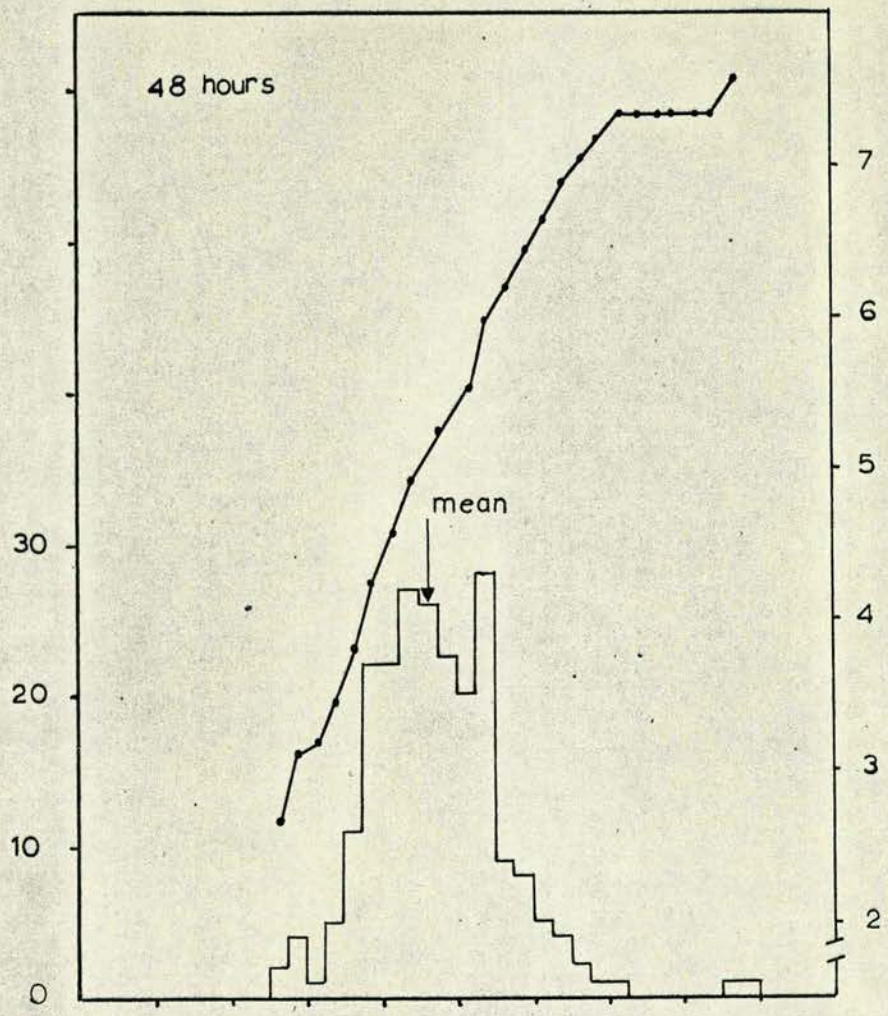


Fig. 4.42
(T1)

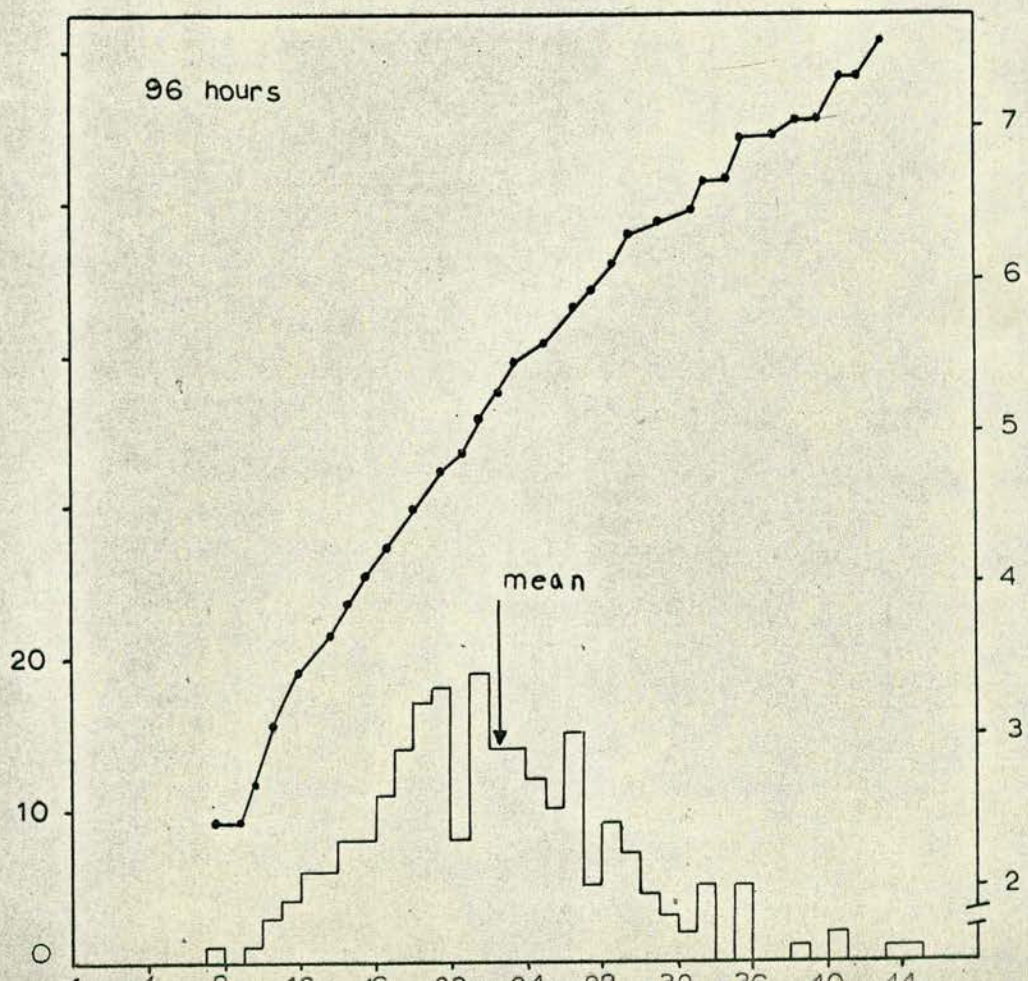


Fig.
4.43
(T1)

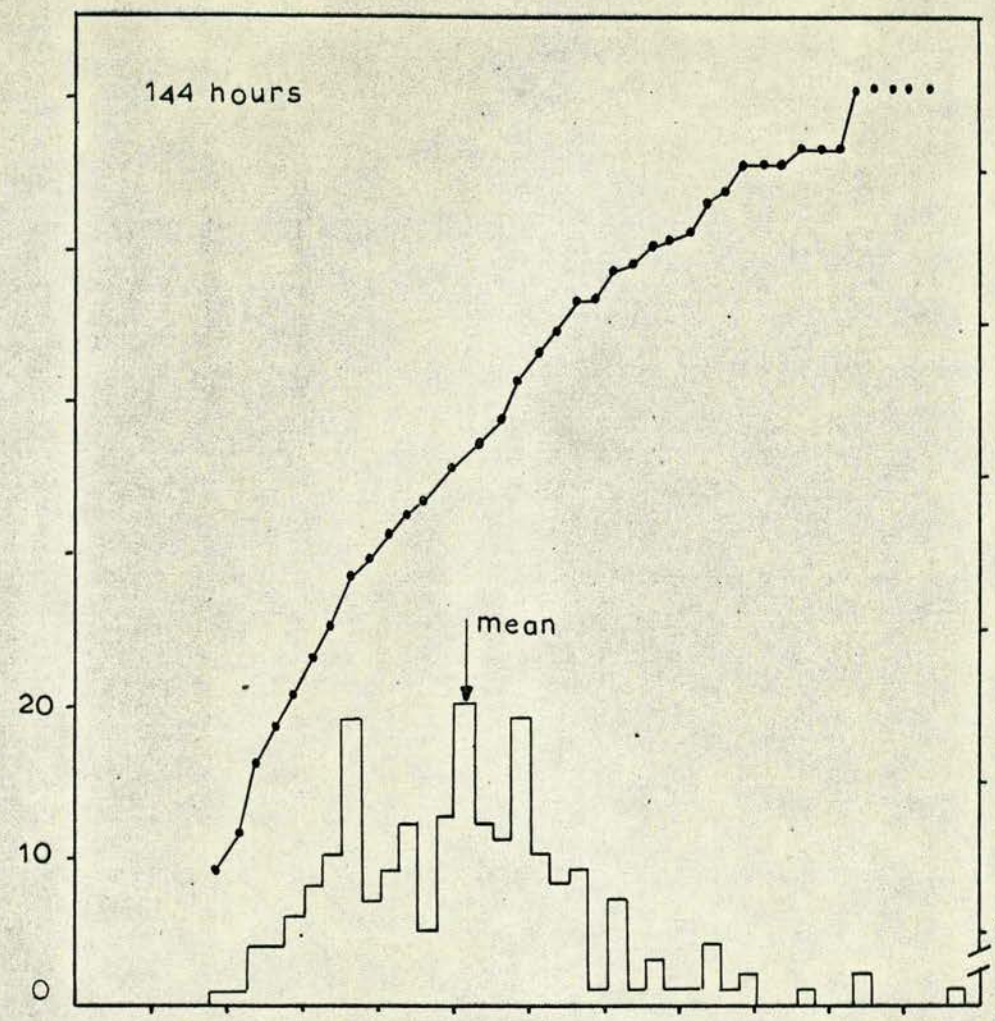


Fig. 4.44 (T1)

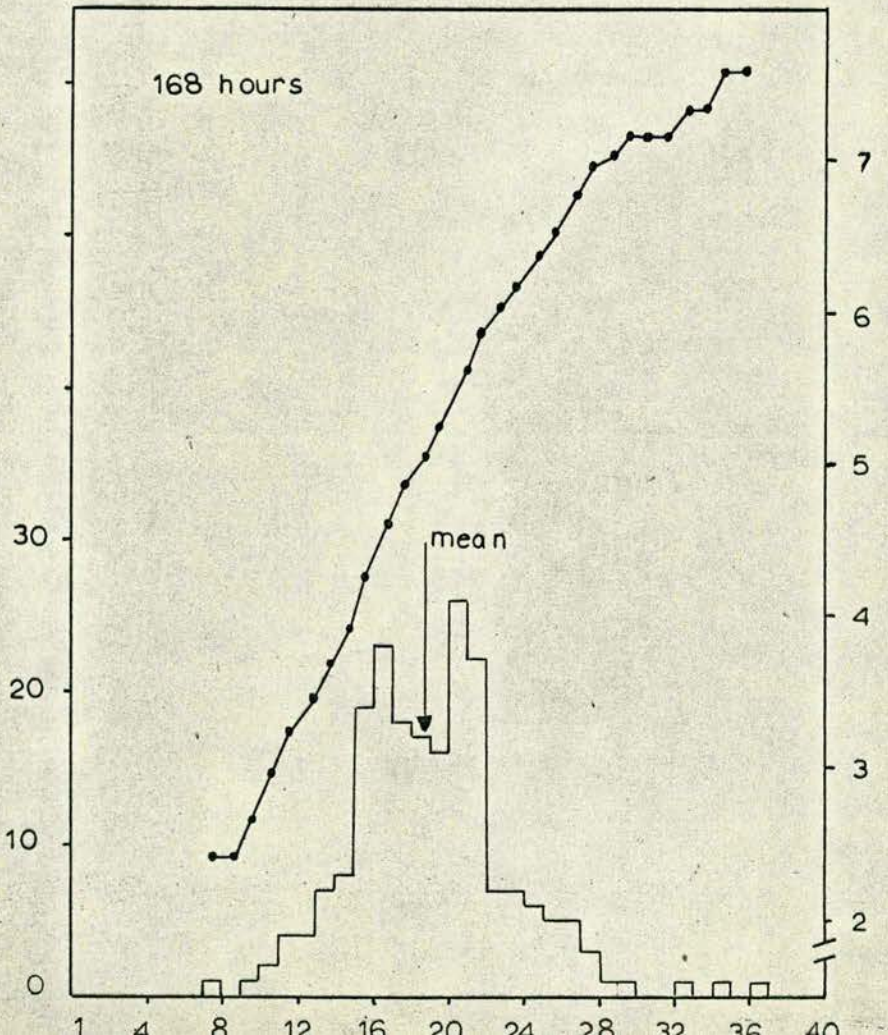


Fig. 4.45 (T1)

mean to 19 units, although the mode remains at 21 units. Probit analysis shows that the population of nuclei at 168 hours is more uniform than at 144 hours, although there are a number of sub-populations with peaks at 12, 17, 21 and 27 units and isolated individuals at 8, 33, 35 and 37 units.

After 240 hours of incubation (Fig 4.46) the sample population becomes much less uniform with a wide range of values between 7 and 53 units. The mean increases to 22 units, showing that the number of high value nuclei has increased, and the mode remains at 21 units. The number of sub-populations increases with peaks at 11, 15, 17, 23, 27, 29, 33 and 35 units with isolated groups around 40, 48, 51 and 53 units. These high DNA content groups, when compared with the values of DNA content of mitotic figures in the second group of measurements suggest that these nuclei may be polyploid.

After 264 hours of incubation (Fig 4.47) the distribution of DNA values becomes even less uniform than at 240 hours and the range extends to between 6 and 55 units. The mean falls slightly to 21 units but the mode decreases to 15 units. This, together with the shape of the histogram, suggests that a population of nuclei in the region of 16 to 28 units at 240 hours of incubation has either divided, to give the high mode value of 15 and 13, or has synthesised DNA to increase the number of nuclei with a high DNA content. The distribution of nuclei is very uneven with peaks at 10, 13, 15, 18, 20, 23, 25, 28 and 30 units and with isolated populations around 32, 36, 40, 43, 45, 48 and 55 units.

Fig.4.46 (T1)

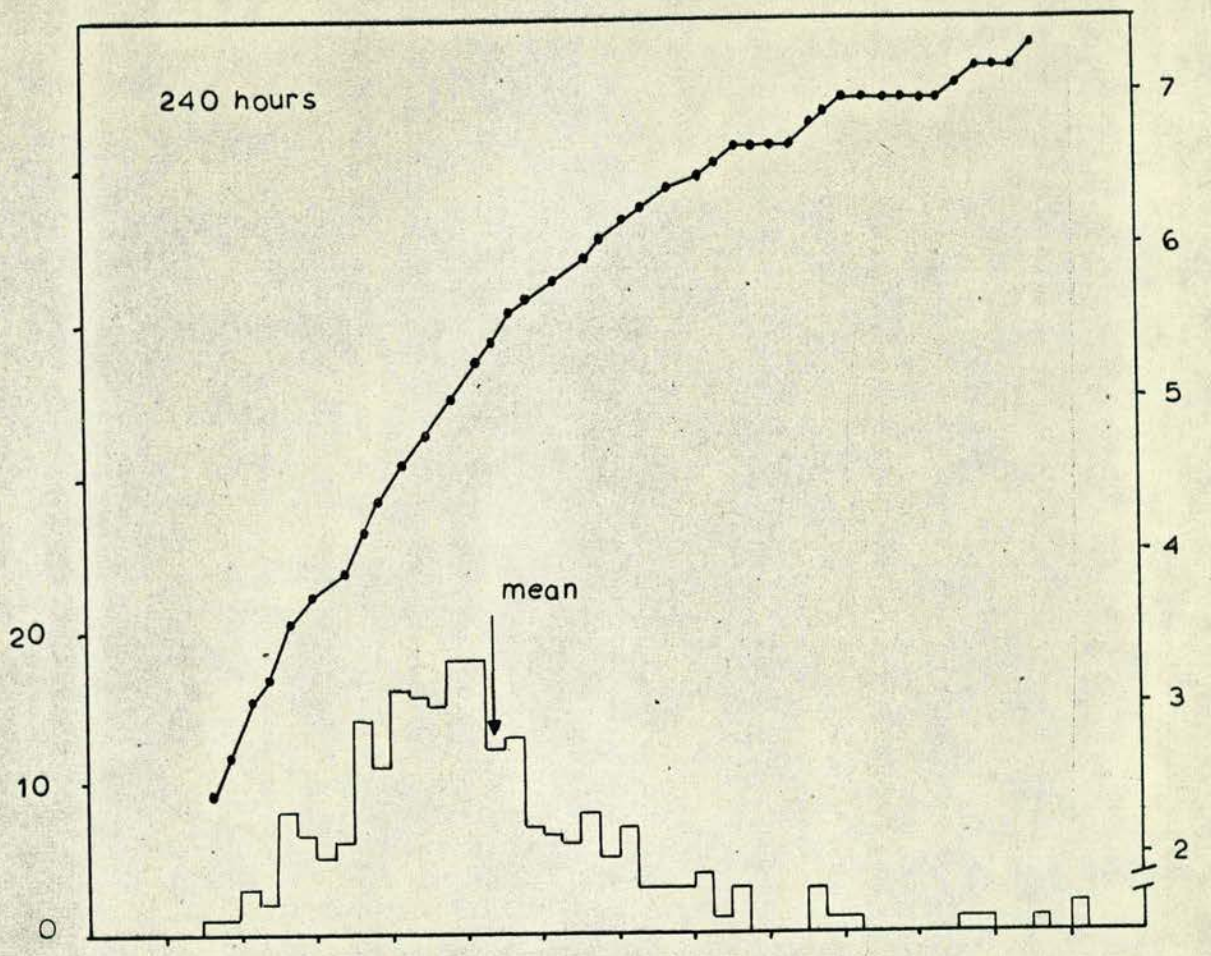
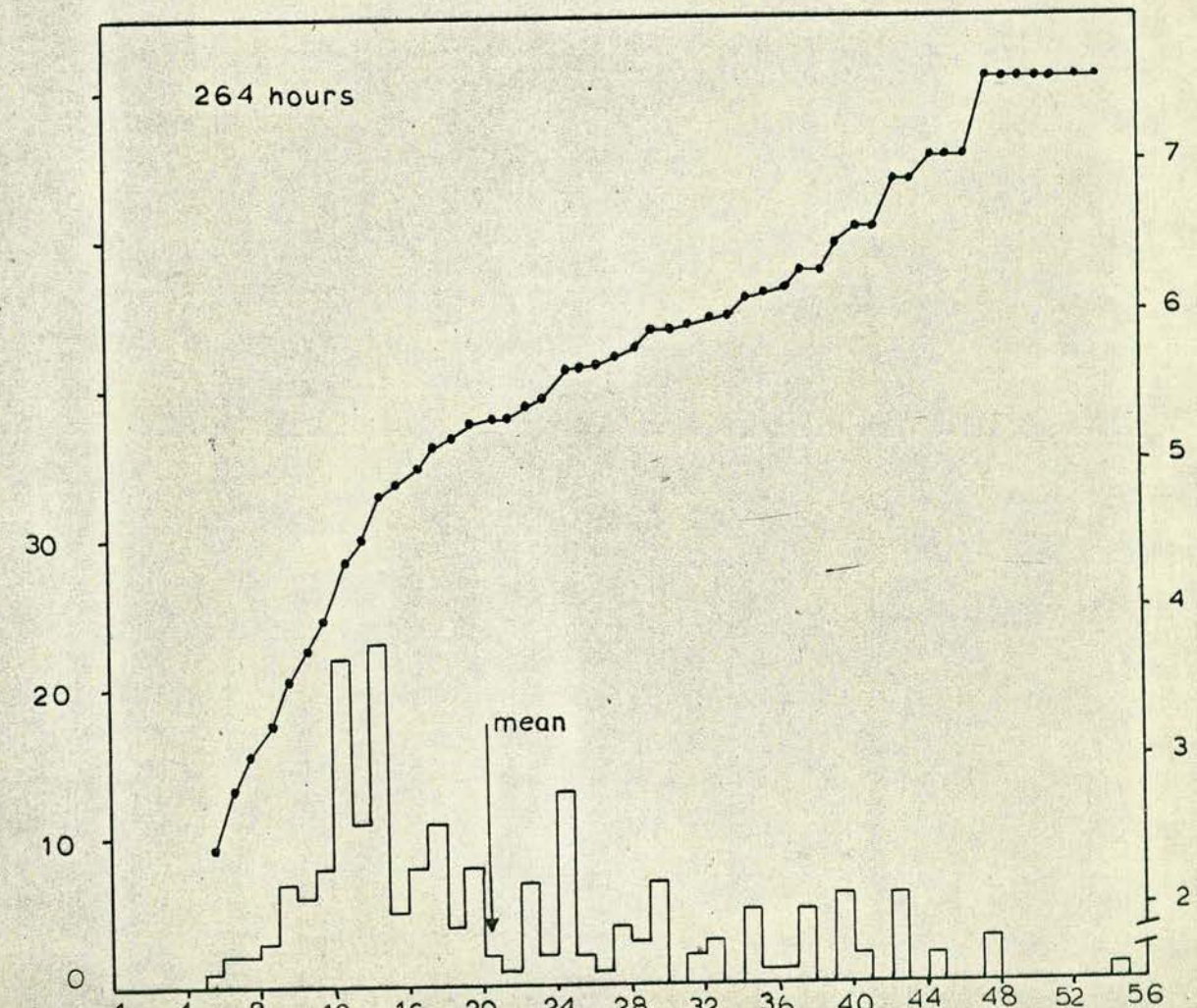


Fig. 4.47 (T1)



Tigellum measurements set 2.

Fig 4.48 shows that at excision this tigellum consists of a number of populations within the overall range of 13(14.69) to 26(29.38) units. The mean of the total population is at 21(23.73) units and the mode at 20(22.6) units. Probit analysis distinguishes populations between 14(16.8) and 17(19.21) units and 19(21.47) and 23(25.99) units. Despite these internal sub-populations, the overall population is quite uniform, as shown by the angle of slope of the probit analysis, and compares closely with the equivalent measurements of the first set (Fig 4.40).

After 24 hours of incubation (Fig 4.49) the distribution of nuclear content has altered, the mean shifting down to the same position as the mode at 15(18.9) units, and the range increases from 8(10.08) to 24(30.24) units. This implies that a proportion of the nuclei with a high DNA content have divided and now constitute the low DNA content population, shown by probit analysis to be between 8(10.08) and 11(13.86) units. There are secondary peaks of nuclei around 12(14.4), 20(25.2) and 22(27.72) units, the last two of which correspond to the high value population at excision and may be the remainder of this population which is slow at undergoing division. These measurements are similar to those of the first set, Fig 4.41, but have a smaller range and there are no isolated populations.

Fig 4.50 shows that after 48 hours of incubation the mode of the population decreases to 14(18.48) units while the corrected mean increases to 19.8 units although uncorrected it remains at 15 units. The range decreases to between 10(13.2) and 21(27.72) units and probit analysis divides the distribution into sub-populations between 10(13.2) and 12(15.84), 13(17.16) and 17(22.44) and 18(23.76) and 21(27.72) units. This tigellum has more sub-populations and a smaller range of values

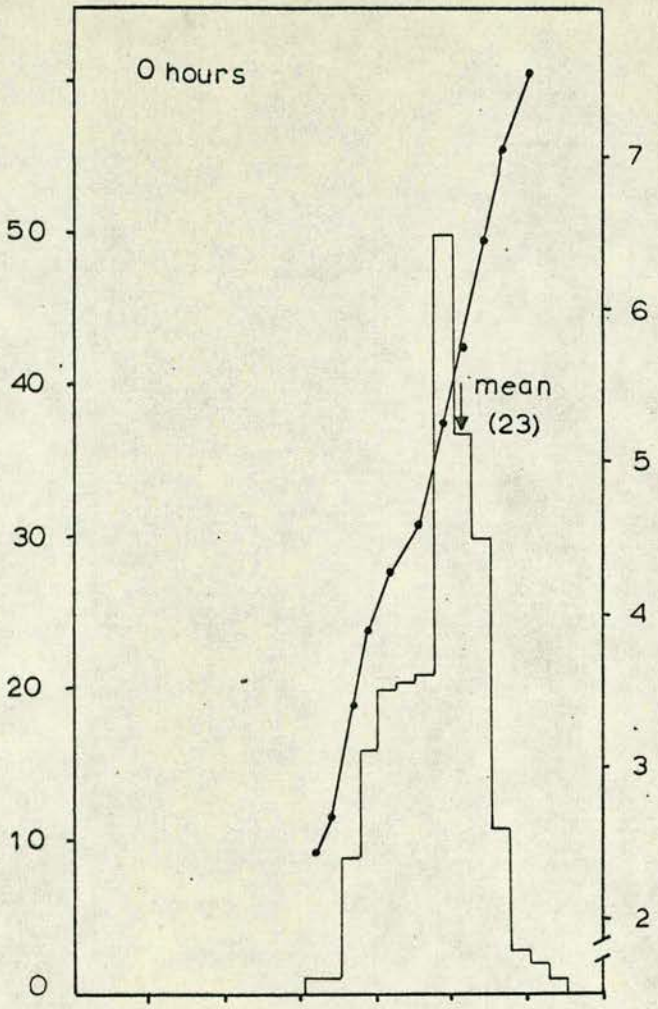


Fig. 4.48
(T 2)

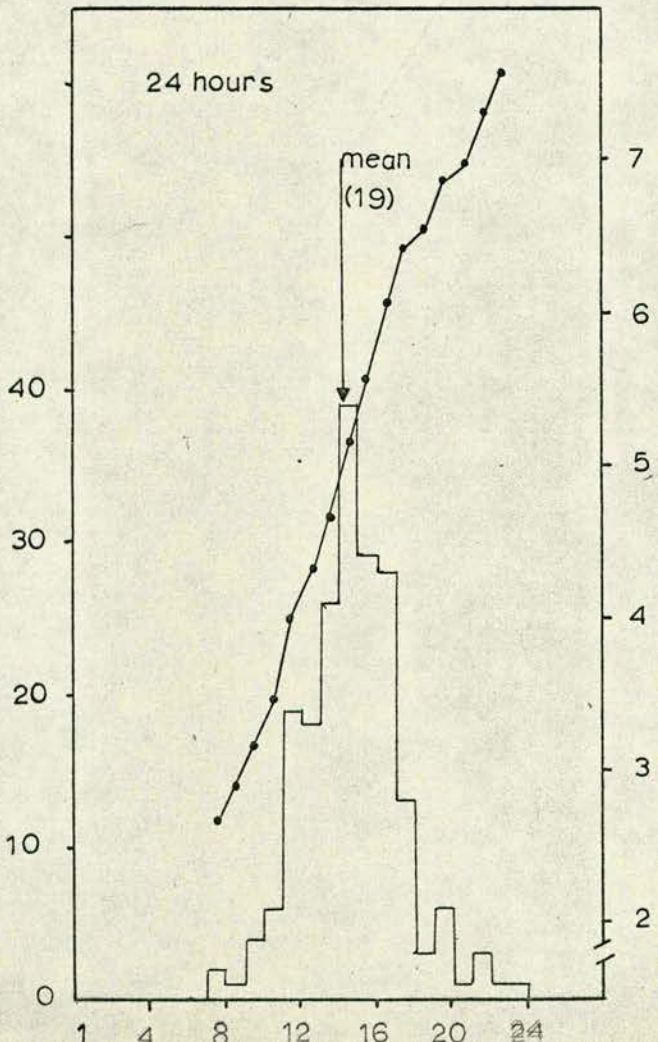


Fig. 4.49
(T 2)

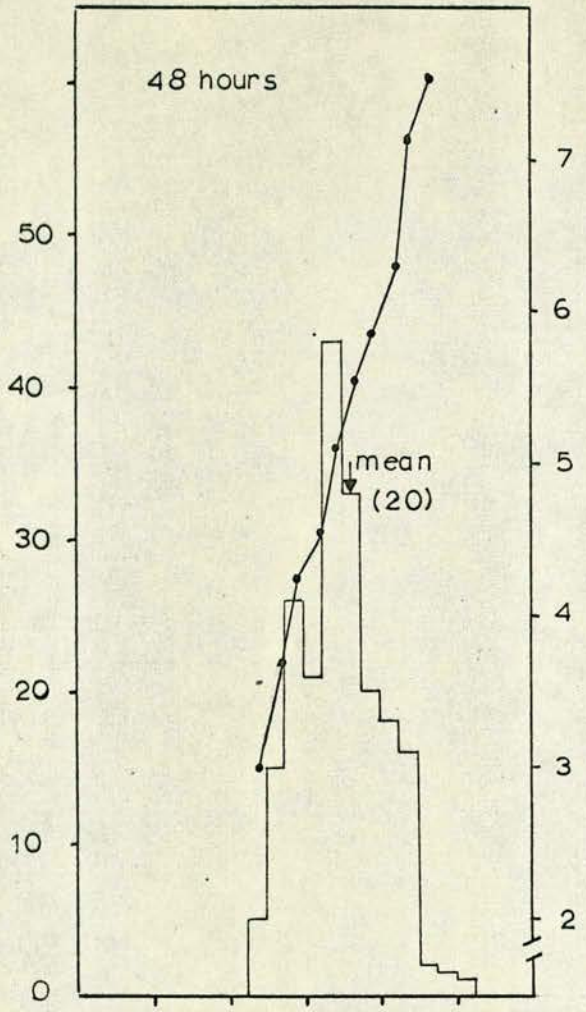


Fig. 4.50
(T2)

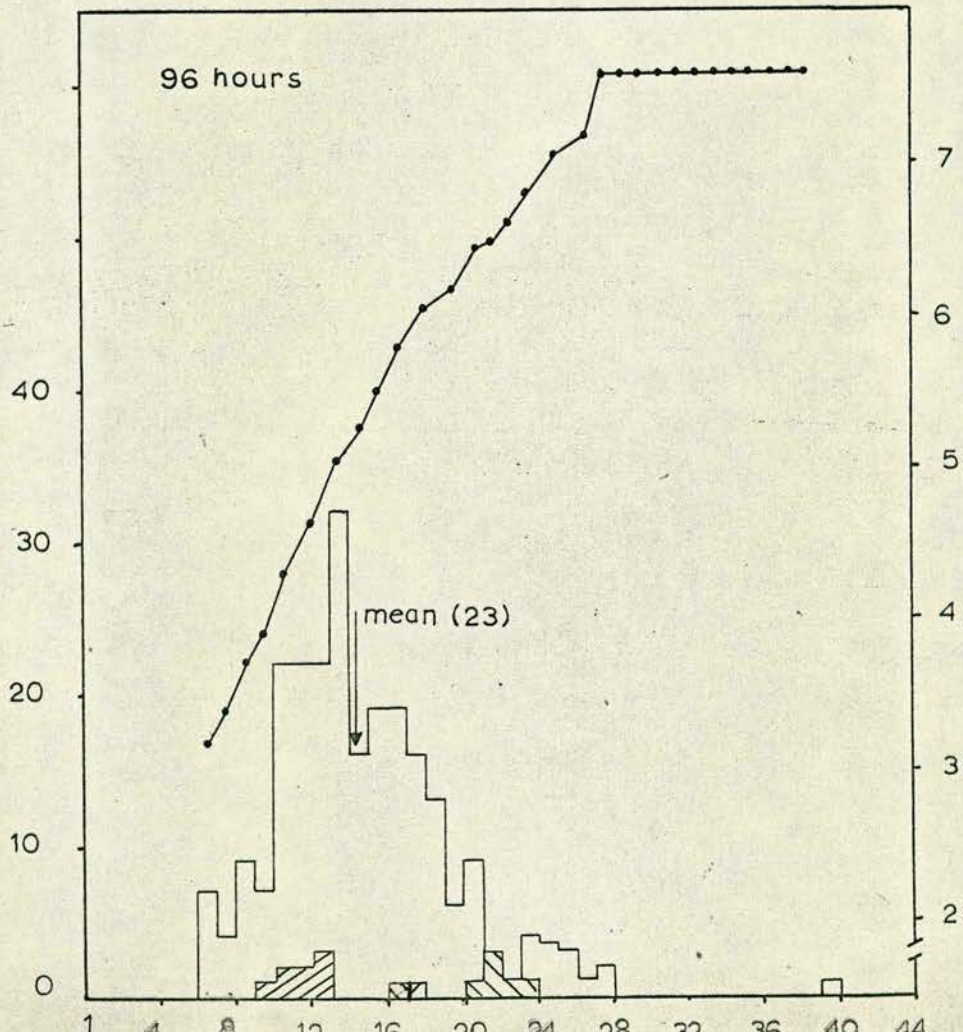


Fig. 4.51
(T2)

than the equivalent tigellum of the first set of measurements (Fig 4.42).

Fig 4.51 shows the distribution of nuclei after 96 hours of incubation. A number of mitotic figures are present, showing that nuclear division is taking place. The range of the population has increased to between 7(10.5) and 40(60) units although the range is extended from 28(42) units to 40(60) units by a single nucleus. The mode is at 14(21) units and the mean at 15(22.5) units, both having increased from the 48 hour sample.

Probit analysis distinguishes a number of small sub-populations 7(10.5) to 10(15), 11(16.5) to 14(21), 15(22.5) to 17(25.5), 20(30) to 21(31.5), 22(33) to 25(37.5) and 27(40.5) to 28(42). A group of prophase and metaphase configurations is present between 21(31.5) and 24(36) units a single prophase figure at 17(25.5) units and a group of anaphase and telophase configurations can be seen between 10(15) and 13(19.5) units and at 18(27). This overall distribution is similar to the first set (Fig 4.43).

After 144 hours of incubation (Fig 4.52) the range of results has decreased to between 6(10.68) and 26(46.28) units, and it is skewed towards the higher values. The mean and mode are at 12(21.36) units, both having decreased from the level of the 96 hour sample. Probit analysis shows secondary peaks around 9(16.02) units, around 16(28.48) and around 20(35.6) units. Prophase and metaphase configurations are found at 19(33.8), 21(37.38), 22(39.16) and 24(42.72) units. Anaphase configurations are found at 7(12.46) and 11(19.58) units. The range, mean and mode of this sample correspond closely to those of the first set sample but the distribution in this sample is more even.

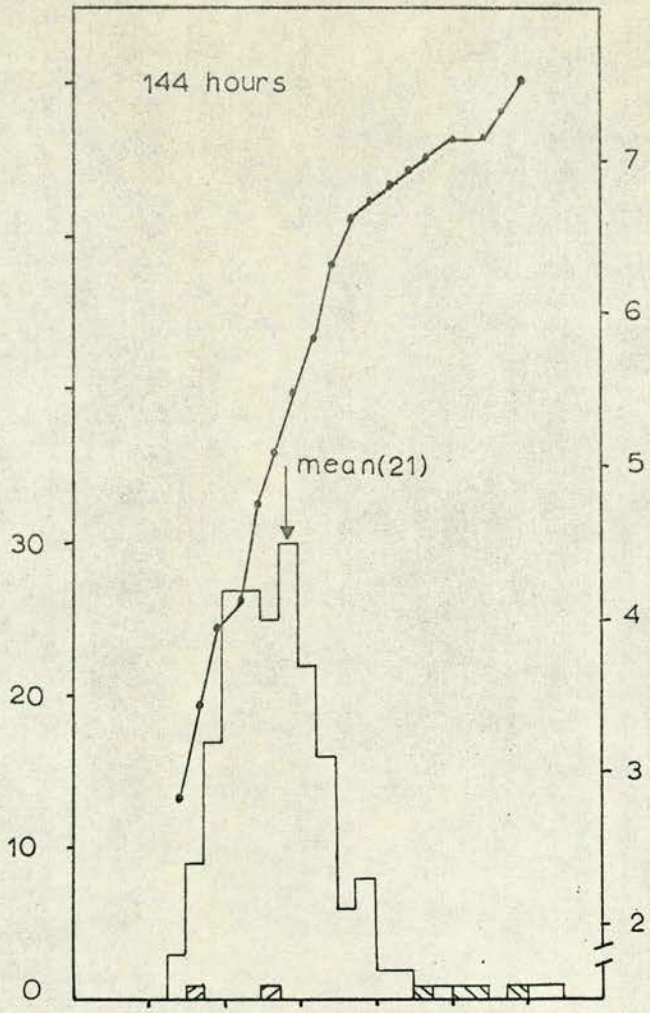


Fig.4.52
(T 2)

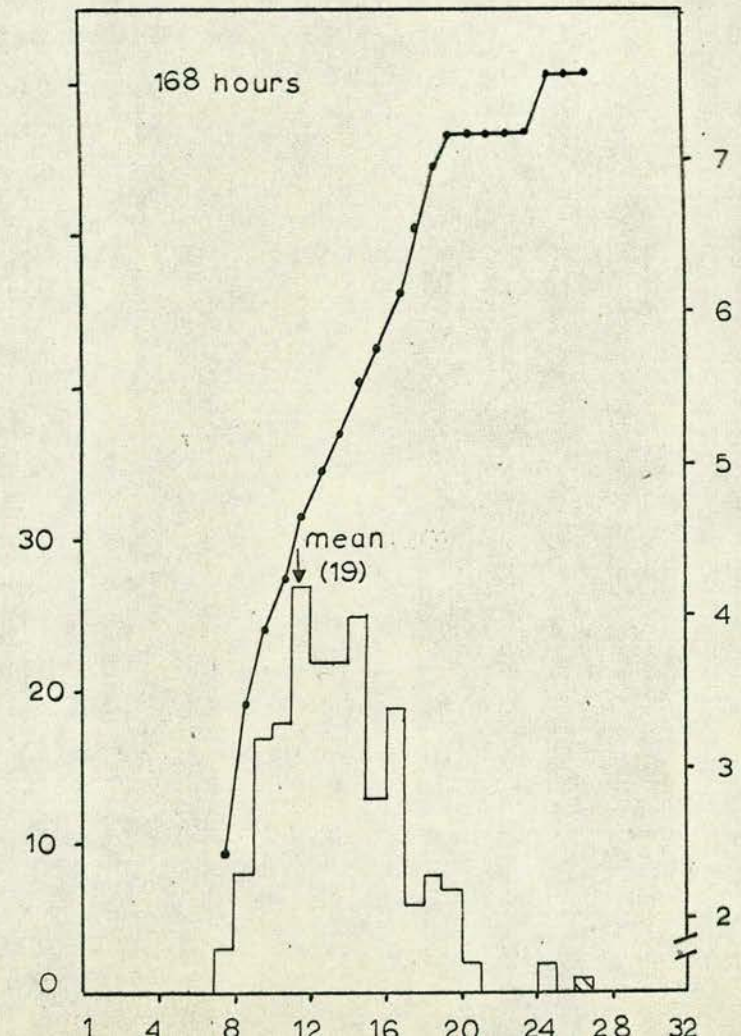


Fig. 4.53
(T 2)

Fig 4.53 shows the distribution of DNA values after 168 hours of incubation. The range is from 8(12.64) to 27(42.66) units, the same magnitude as at 144 hours but at a higher level. The mode and mean are both at 12(18.95) units, lower than at 144 hours showing that there is a greater proportion of low DNA content nuclei at this time. There are secondary peaks at 15(23.7), 17(26.85) and 19(30.02) units, shown by an uneven distribution in the probit analysis. A prophase configuration is found at 27(42.66) units. The distribution of this sample is similar to that of the 168 hour sample of the first set (Fig 4.45).

Fig 4.54 shows the distribution of DNA values after 264 hours of incubation. The range of 8(13.6) to 24(40.8) units is less than at 144 and 168 hours of incubation whereas the mode at 12(20.4) units is higher and the mean at 11(18.7) units is slightly lower. Probit analysis while showing that the total population is more uniform, distinguishes sub-populations between 8(13.6) and 10(17.0), 11(18.7) and 15(25.5), 15(25.5) and 17(28.9) and 17(28.9) and 20(34.0) units with 2 isolated nuclei at 24(40.8) units. This sample is much more uniform than the sample at 264 hours in the first set of measurements (Fig 4.47).

Haustorium measurements set 1.

The sample taken at excision in this set (Fig 4.55) is the only sample out of many which shows much variation. There is a wide range of values between 5 and 30 units, the mean is at 16 units and the mode at 15 units. Probit analysis distinguishes a number of sub-populations with peaks at 9, 11, 17, 21 and 29 units. An explanation of the non-uniformity of this population could be that the haustorium has begun to develop while within the intact seed. The development could not have

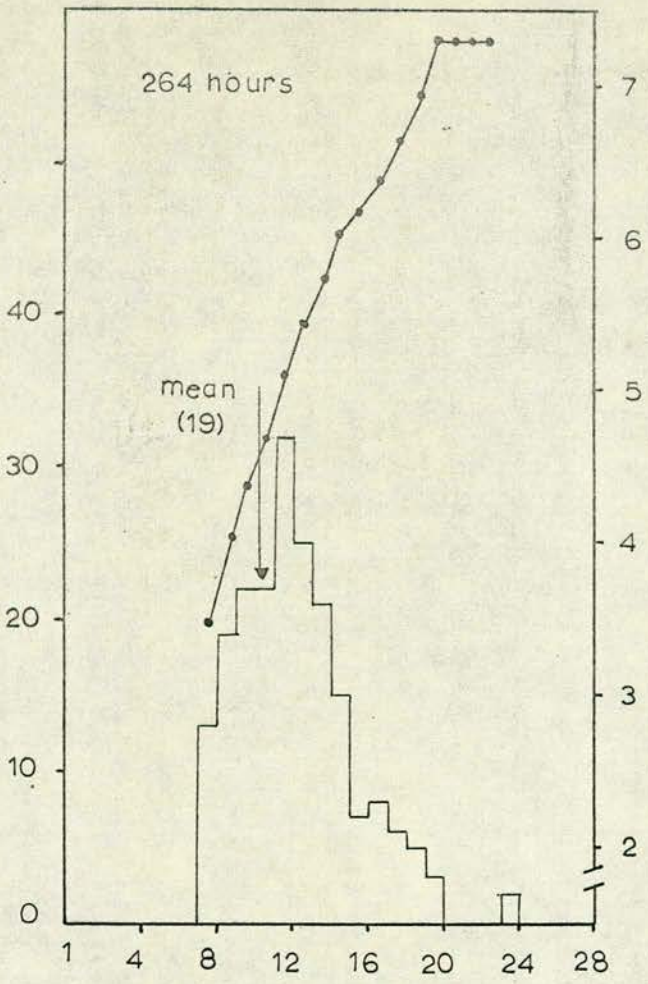


Fig. 4.54
(T 2)

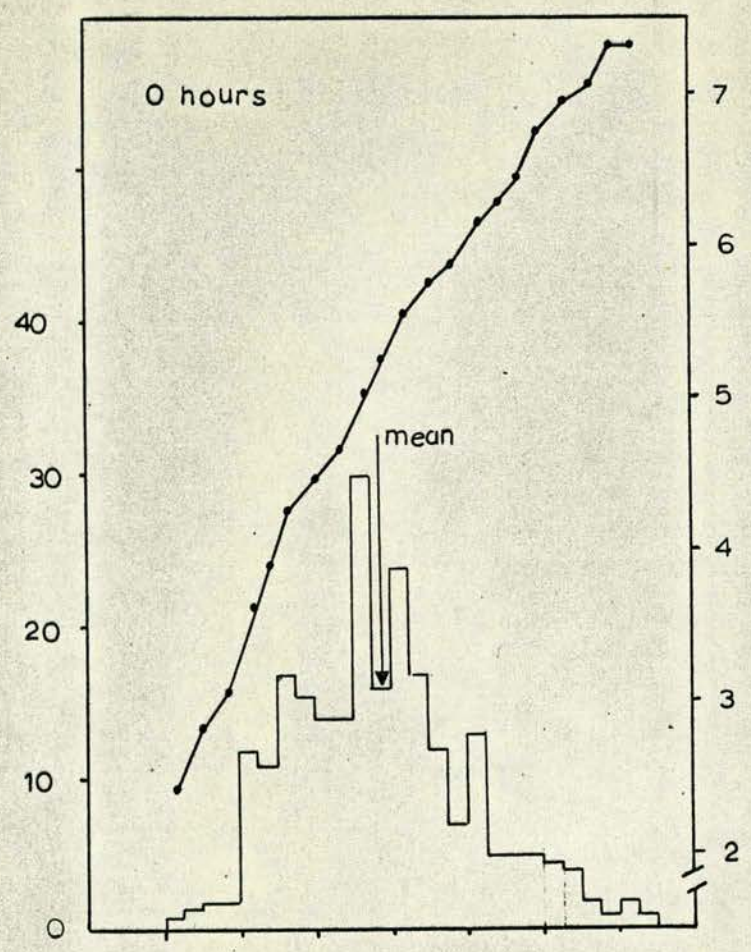


Fig. 4.55
(H1)

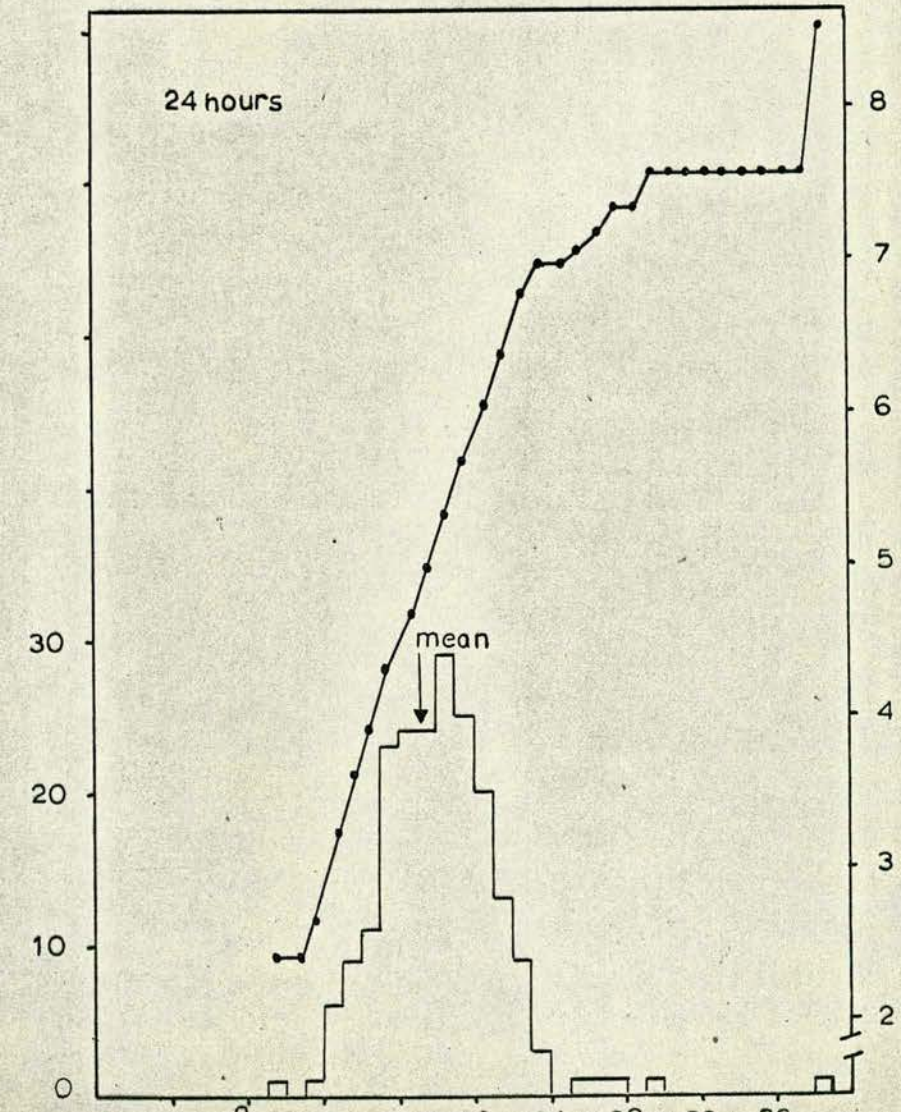


Fig. 4.56
(H1)

proceeded very far because the tigellum (Fig 4.40) is quite uniform.

The sample taken after 24 hours of incubation (Fig 4.56) is much more uniform than the sample at excision. The range however is wider, between 10 and 39 units, extended by 4 isolated populations around 10, 28, 30 and 39 units. The mean of the population is 18 units and the mode 19 units. The high value nuclei could be about to divide.

Fig 4.57 shows that after 48 hours incubation the range of DNA content in the haustorium has decreased to between 12 and 33 units, and the mode has decreased to the same value as the mean at 18 units. Probit analysis distinguishes sub-populations around 12, 16 and 24 units, and isolated populations around 27, 29 and 33 units.

After 96 hours of incubation (Fig 4.58) the population has become much more variable with the range increasing to between 9 and 39 units and the mode and mean increasing to 19 and 20 respectively. There are 4 isolated populations around 29, 32, 35 and 39 units suggesting that these nuclei are synthesising DNA. This suggestion is supported by the labelling experiments using tritiated thymidine which show that DNA synthesis is occurring at this time. Probit analysis distinguishes sub-populations with peaks around 12, 15 and 26 units.

After 144 hours of incubation (Fig 4.59) the range of values is increased to between 7 and 47 units by a number of individual nuclei at 27, 29, 32, 36, 39 and 47 units. The main population has become almost bimodal in distribution with peaks at 13 and around 22 units, and the overall mean and mode have decreased to 14 and 13 units respectively.

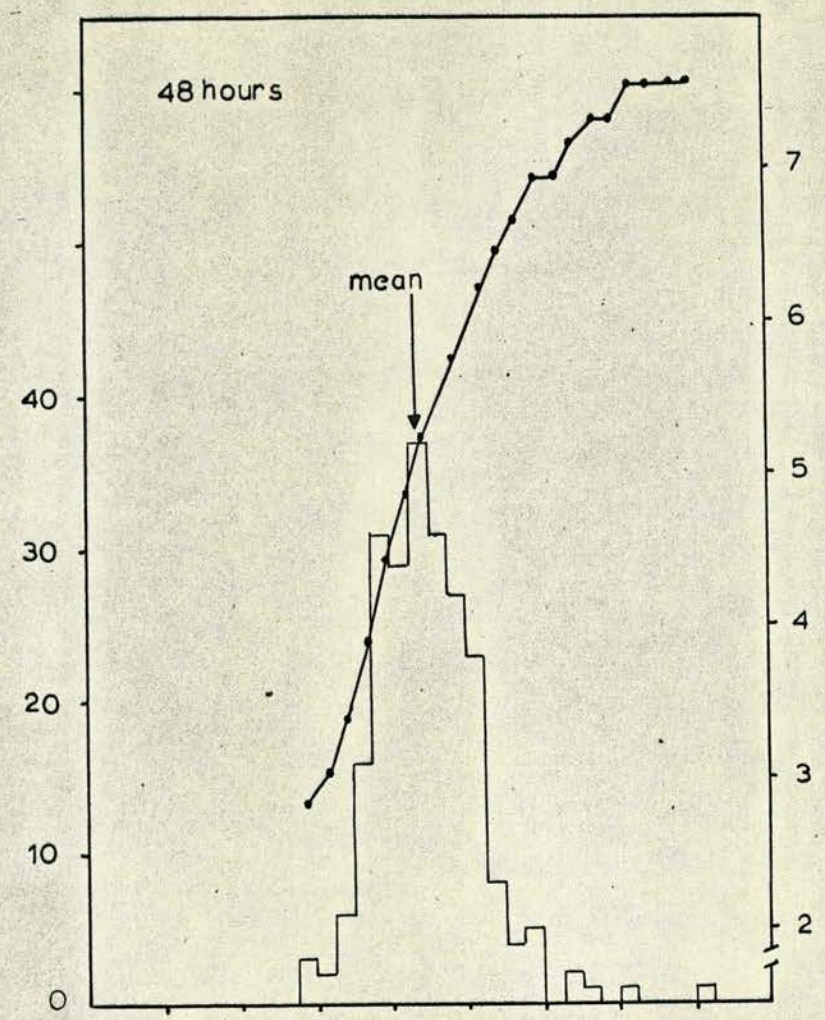


Fig. 4.57
(H1)

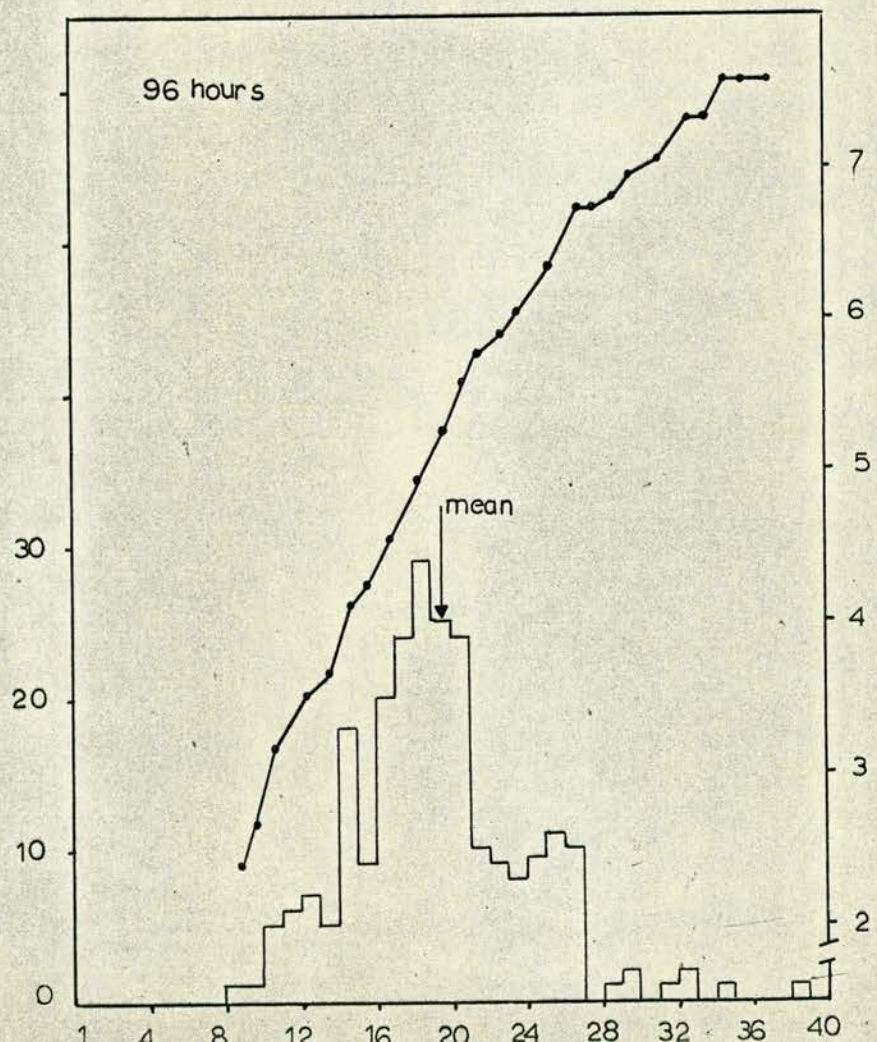


Fig. 4.58
(H1)

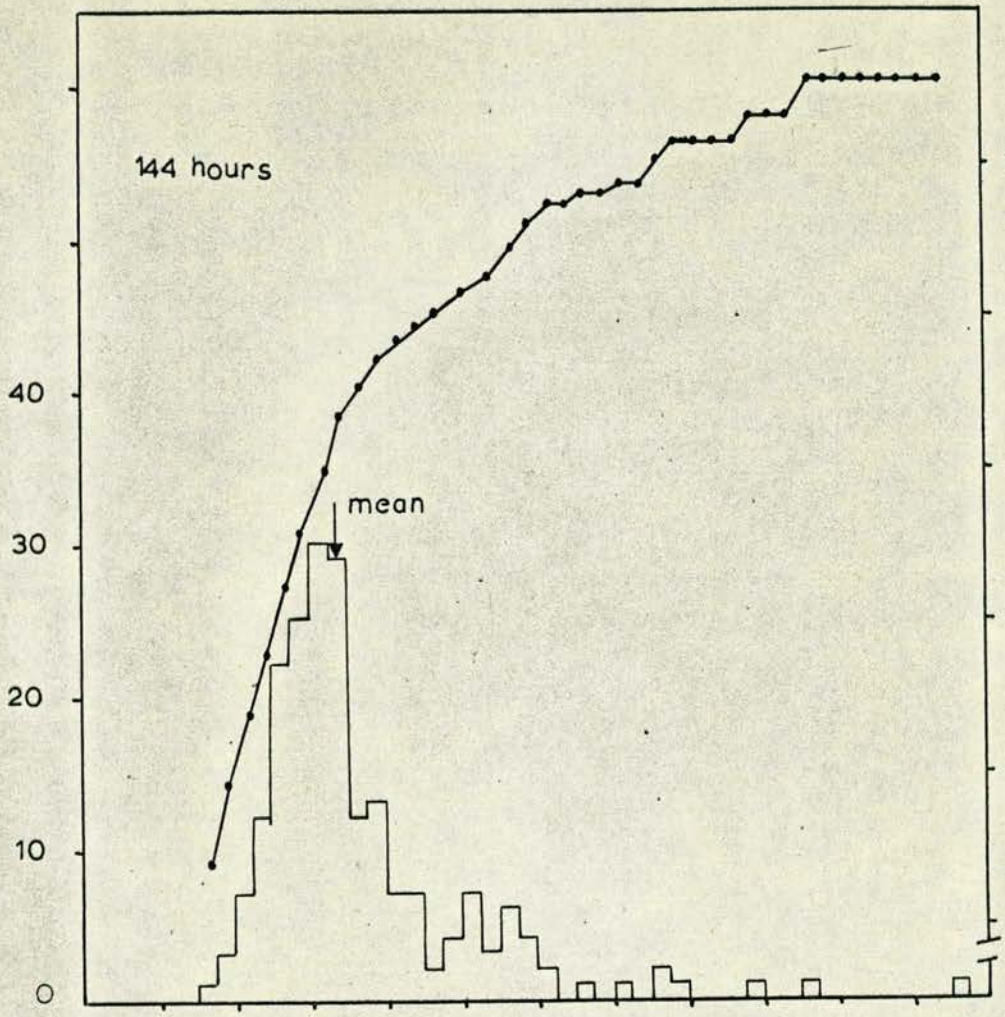


Fig.4.59
(H1)

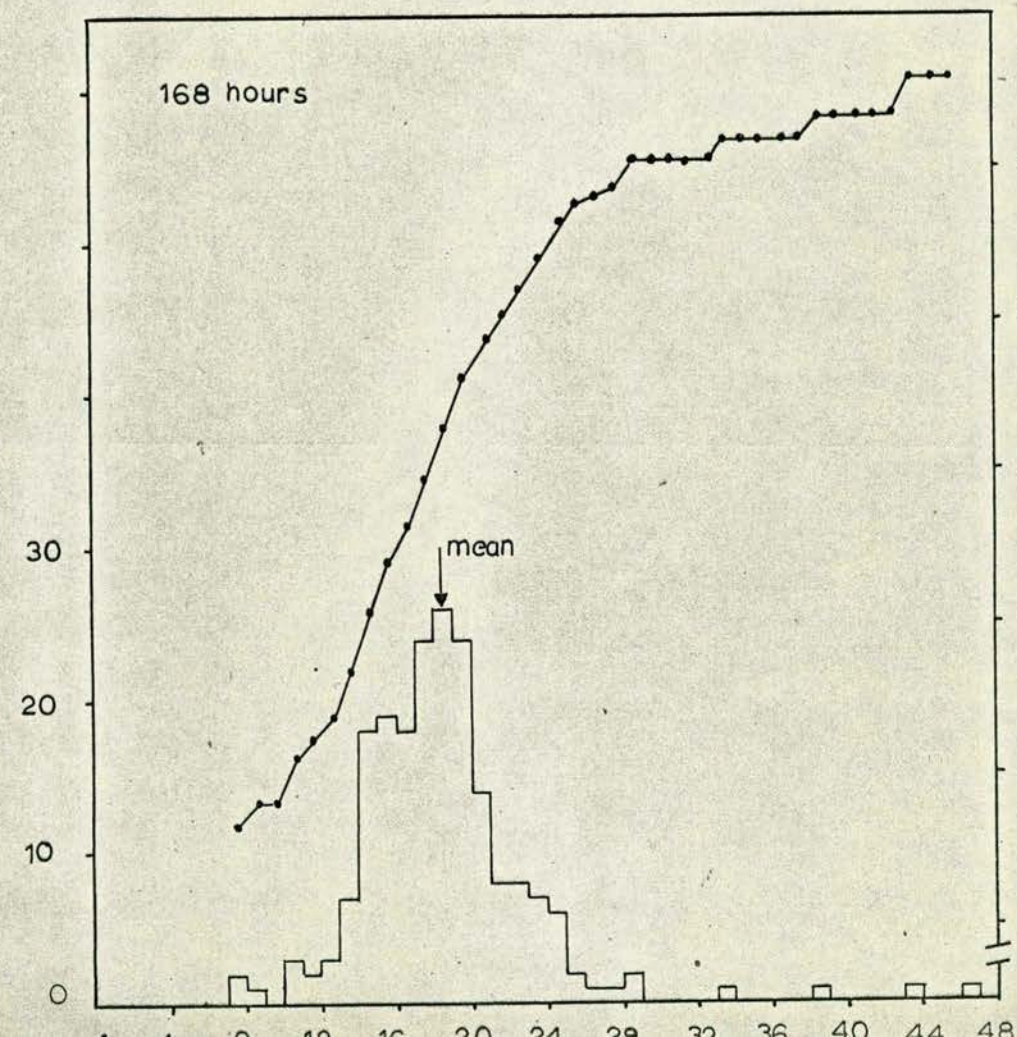


Fig.4.60
(H1)

After 168 hours of incubation (Fig 4.60) the mean and mode of the population increase to 19 units. Although the range remains very similar, between 8 and 47 units, to that at 144 hours, the distribution has altered greatly. The main peak of values has moved up to the position of the trough of the bimodal distribution at 144 hours, indicating that DNA synthesis is occurring. Probit analysis distinguishes sub-populations around 8, 11, 16, 25 and 28 units with individual nuclei at 34, 39, 44 and 47 units.

Fig 4.61 shows that at 240 hours of incubation the range of values is extended to between 5 and 54 units by a single nucleus at 54 units, whereas the main population extends between 5 and 36 units. The mean and mode have decreased to 17 and 14 units respectively suggesting that nuclear division has taken place. Probit analysis shows that there are a number of sub-populations within the total population with peaks around 5, 11, 20, 27, 29, 32 and 36 units.

Fig 4.62 shows that at 264 hours of incubation, although the overall range has decreased to between 6 and 38 units the bulk of the population has a greater proportion of nuclei with DNA content similar to the mean value of 16 and mode of 13 units. Probit analysis shows that the population is more uniform with smaller numbers of sub-peaks than at 240 hours. These peaks are around 6, 13, 15, 20, 22 and 25 units.

Haustorium measurements set 2.

Fig 4.63 shows the distribution of DNA values in the haustorium of a freshly excised embryo. The range of values is between 14(10.78) and 27(20.79) units with the mean and mode at 21(16.17) units. Probit

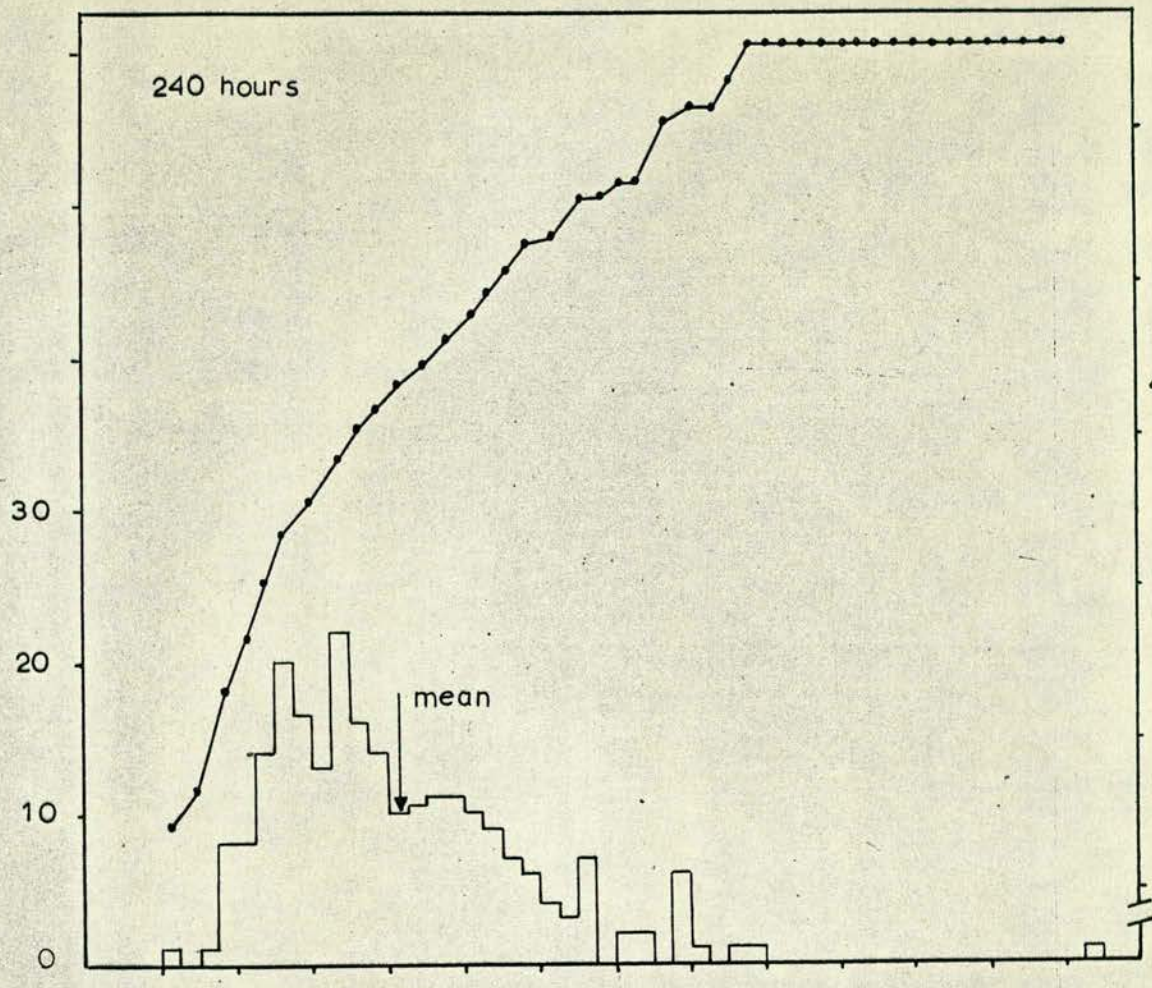


Fig 4.61 (H1)

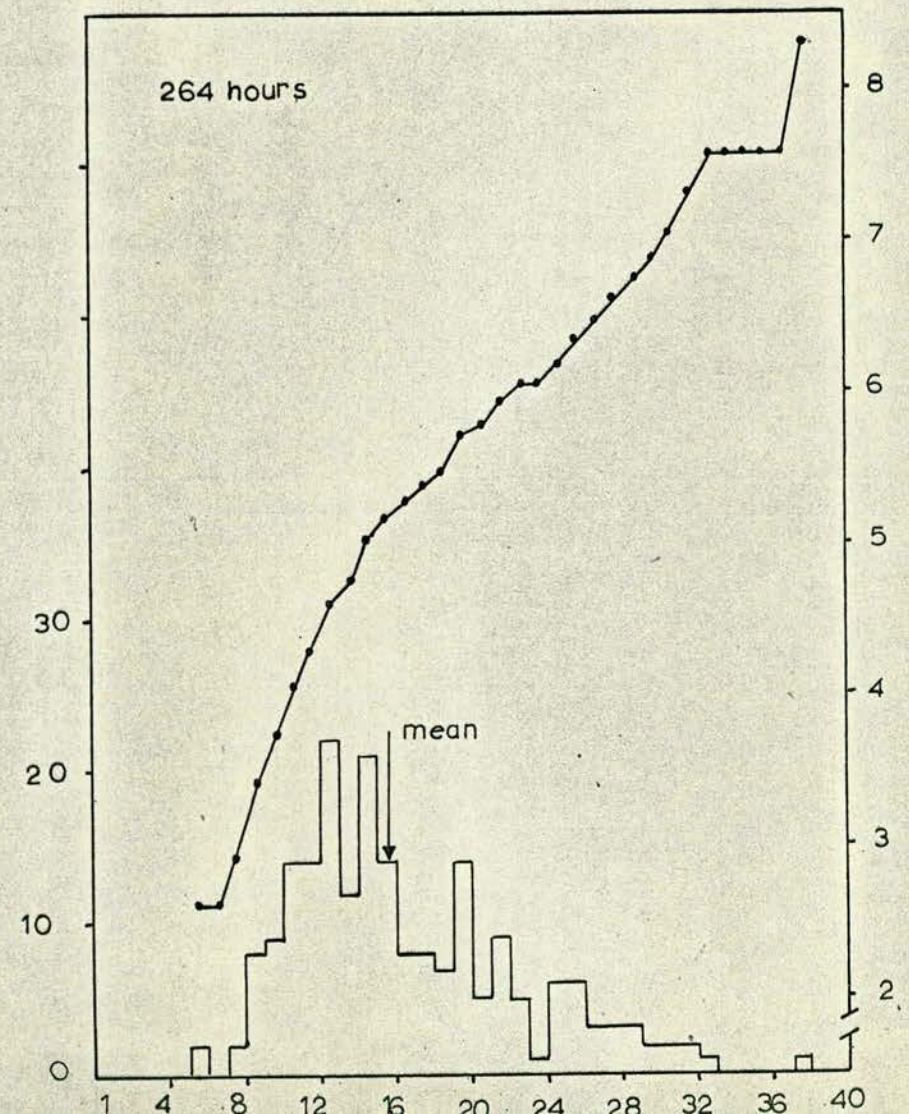


Fig. 4.62
(H1)

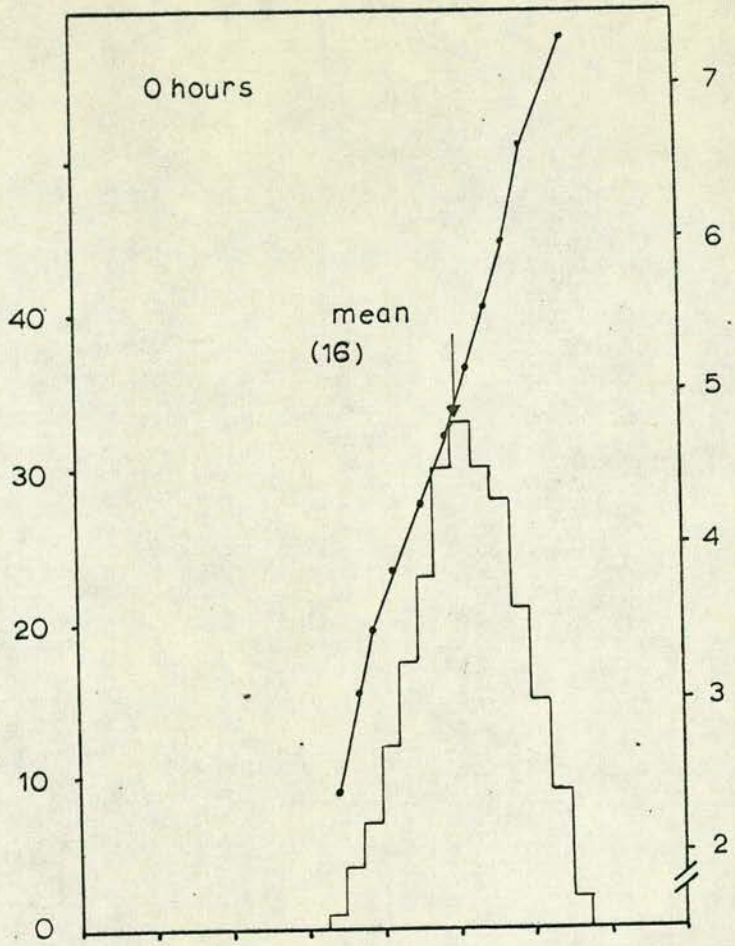


Fig. 4.63

(H 2)

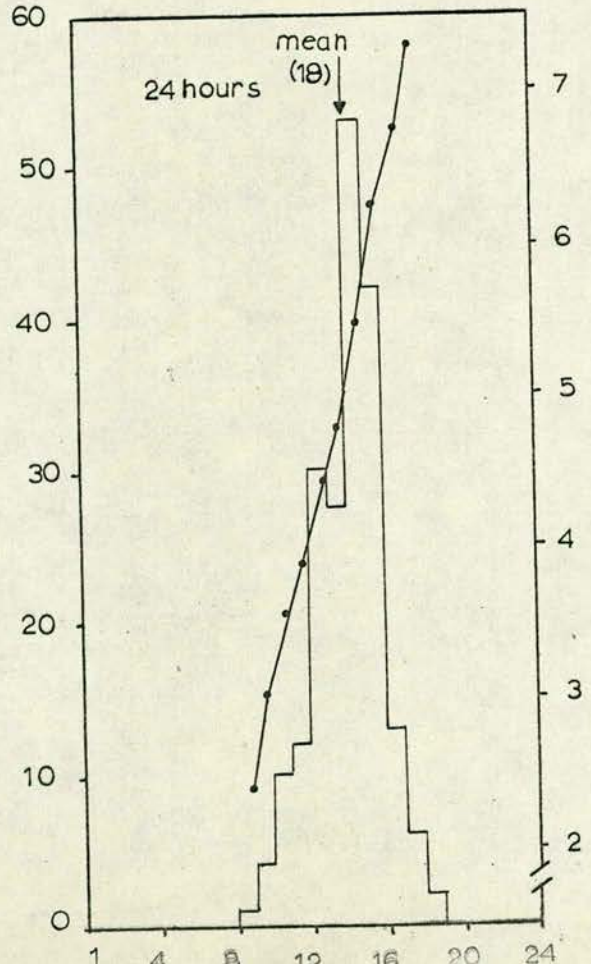


Fig. 4.64

(H 2)

analysis shows that the total distribution can be divided into a number of populations, 2 main ones at 14(10.78) to 17(13.09) units and 17(13.09) to 23(17.71) units and a number of smaller populations between 23(17.71) and 27(20.79) units. This haustorium is much more uniform than that measured in the first set.

After 24 hours of incubation (Fig 4.64) the range has increased to between 9(11.61) and 19(24.51) units and the mean and the mode have both increased to the same value of 15(19.35) units. Probit analysis shows that the total population is much more uniform than at excision but with three sub-populations at 9(11.61) to 10(12.9) units, 10(12.9) to 16(20.64) units and a mixed population between 16(20.64) and 19(24.51) units. This sample is more uniform than the equivalent sample of the first set of readings, although the distribution is similar.

After 48 hours of incubation (Fig 4.65) the range of values is the same as at 24 hours, between 7(9.94) and 16(22.72) units, but at a lower level, and the mean and the mode decrease to 13(18.46) units. Probit analysis shows that there are only two sub-populations in the overall distribution, a major one between 8(11.36) and 16(22.72) units and a minor one between 7(9.94) and 8(11.36) units.

After 96 hours of incubation (Fig 4.66) the distribution of DNA values becomes less uniform and the range is extended to the higher levels by individual or pairs of nuclei. The range is from 10(11.6) to 32(37.12) units, with a mean of 17(19.72) units and a mode of 19(22.04) units. Secondary peaks can be distinguished by probit analysis at 11(12.76), 15(17.40), 27(31.32), 29(33.64) and 32(37.12)

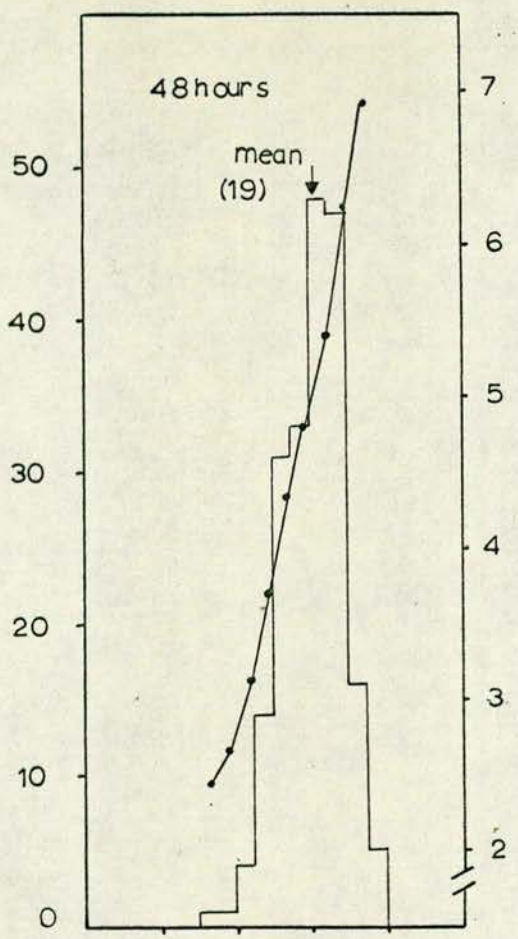


Fig. 4.65

(H2)

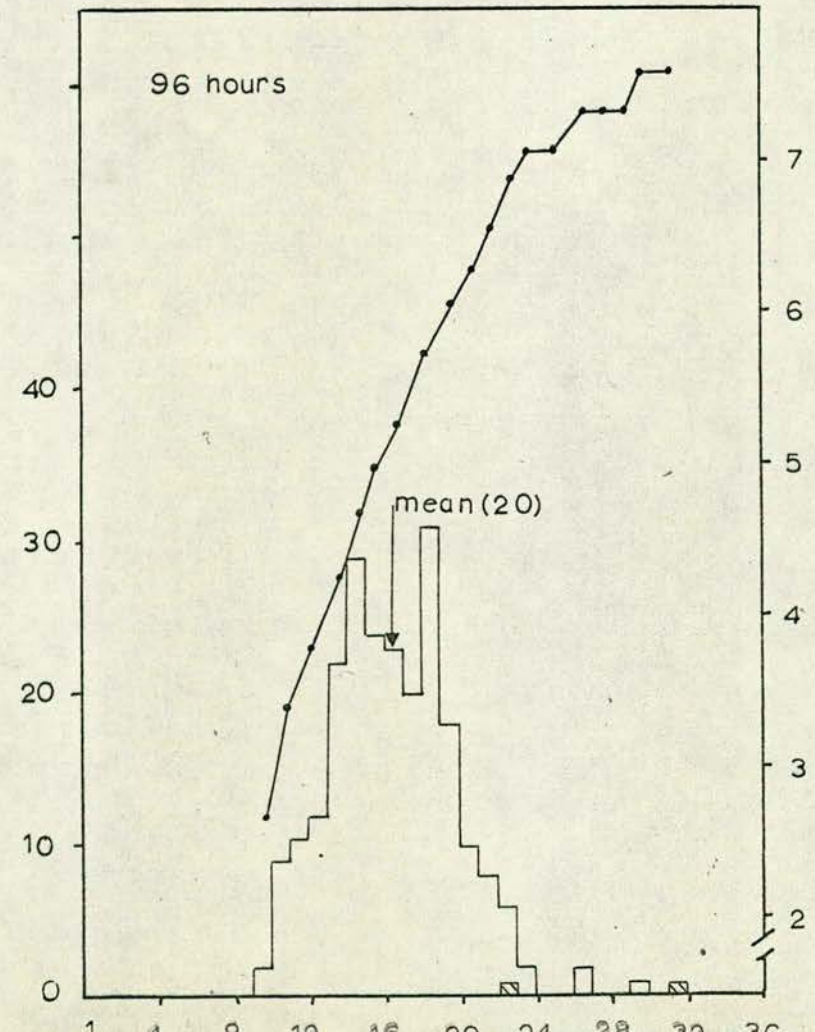


Fig. 4.66

(H2)

units. Prophase configurations were found at 32(37.12) units and 23(26.68) units.

Fig 4.67 shows that after 144 hours of incubation the DNA content of the haustorium becomes less uniform. The range of values increases to between 5(6.7) and 28(37.52) units which shows an increase in the number of nuclei with a lower DNA content. The mode of the distribution is reduced to 8(10.72) units and the mean falls to 11(14.72) units. Probit analysis shows that the distribution is normal between 5(6.7) and 8(10.72) units, but it then becomes non-uniform and a number of sub-populations can be distinguished. Prophase configurations were found at 19(25.46), 23(30.82) and 28(37.52) units, and an anaphase configuration was found at 13(17.42) units. This overall distribution is very similar to that of the haustorium of the first set.

After 168 hours of incubation (Fig 4.68) the range is extended to between 7(10.85) and 29(44.95) units. The mode has increased to 10(15.5) units, and the mean has increased to 12(18.60) units. Probit analysis shows that the whole population is much less uniform, consisting of a large number of sub-populations. Prophase configurations are found at 20(31.0), 23(35.65), 25(38.75) and 29(44.95) units. The distribution is more even than that of the equivalent haustorium from set 1 (Fig 4.60).

Fig 4.69 shows the distribution of DNA content after 264 hours of incubation. The range of values is increased at the lower end of the scale, to between 4(5.56) and 31(43.09) units. Correspondingly the mode has decreased to 9(12.51) units and the mean to 12(16.68) units.

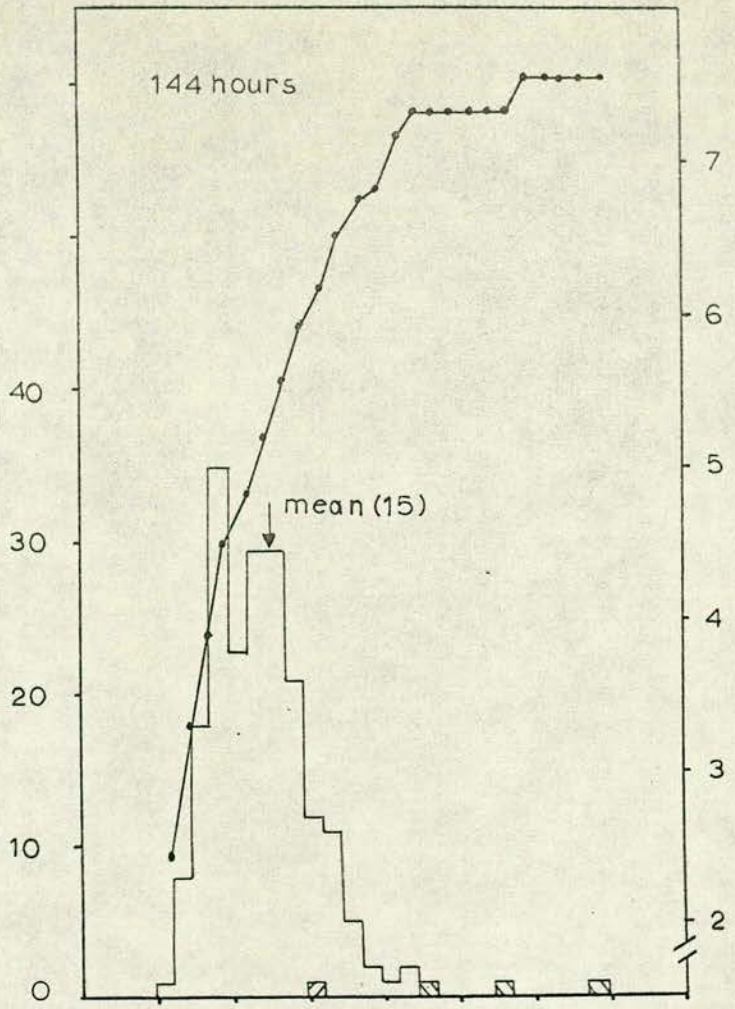


Fig. 4.67

(H2)

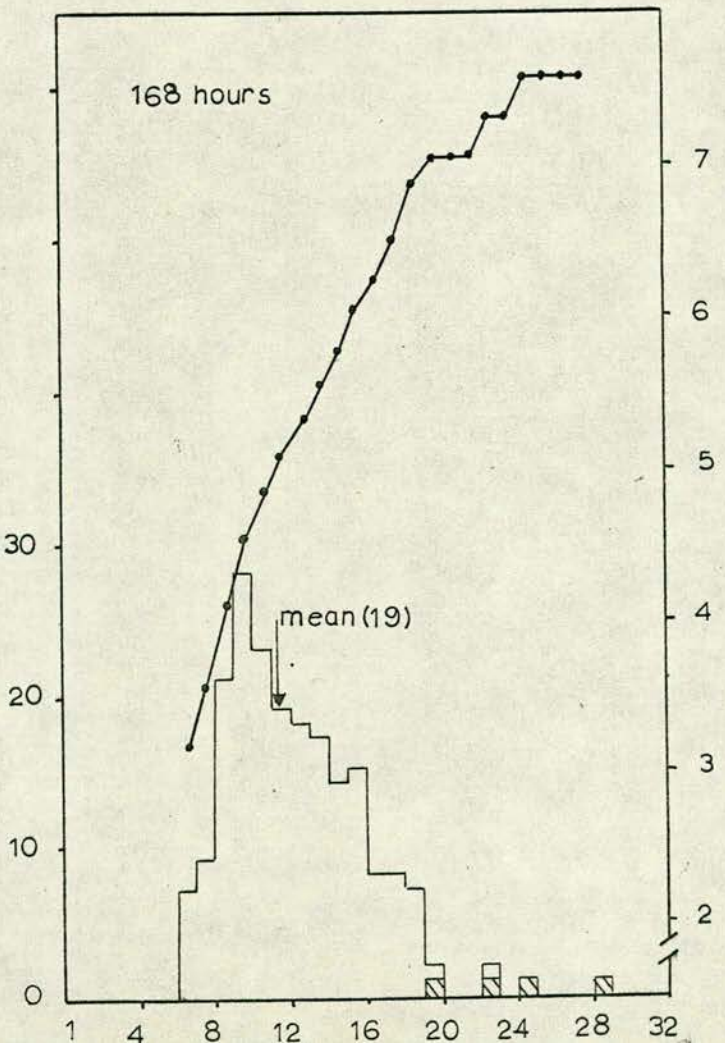


Fig. 4.68

(H2)

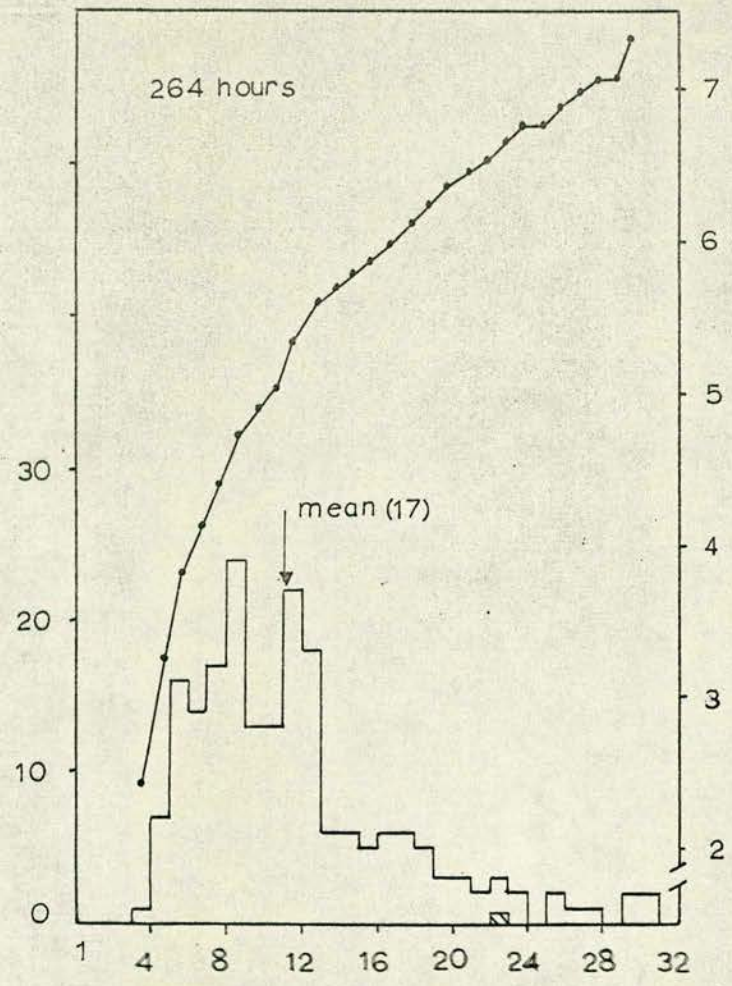


Fig. 4.69

(H2)

Probit analysis shows that the population is not uniform and there are a number of sub-populations around 6(8.34), 9(12.51), 12(16.68), 17(23.63), 23(38.87), 26(36.14) to 28(38.92) and 30(41.70) to 31(40.09) units. A prophase configuration was found at 23(38.87) units. The distribution of DNA values in this haustorium is similar to that of the equivalent in set 1 (Fig 4.62).

Overall it can be seen that there are changes in the DNA content of both the tigellum and haustorium of excised embryos, when they are incubated under conditions favourable to growth. Duplicate measurements confirm these changes, although, due to individual variation, the samples are not identical. The haustorium and tigellum differ in their response to incubation, and in the majority of examples the haustorium is more uniform than the tigellum. Tables 4.5 and 4.6 summarise the data.

Probit analysis distinguishes a number of sub-populations within the overall distribution and the presence of discrete peaks which change position suggests that there is some degree of periodicity in nuclear division. Further evidence for this is suggested by the fact that in many samples no mitotic figures are found, whereas in others there are many. (Tables 4.5 and 4.6.)

There is evidence of nuclear division in the period between excision and 24 hours of incubation, suggested by the backward shift in mean values and range of values at 24 hours. However, there is little subsequent change after 24 hours until 96 hours of incubation, when DNA synthesis has obviously taken place and nuclear division begins, shown by the presence of mitotic figures. The suggested nuclear

Table 4.5
Summary of DNA data (tigellum)

time (hours)	MEASURED DNA VALUES: population mean	population mode	% observed mitotic figures	prophase	metaphase	anaphase	telophase	probit populations major	probit populations minor
[1] O_1	22·8	22	–					3	1
[1] O_2	22·8	22·6	–					2	2
24_1	19·05	18	–					3	4
24_2	19·08	189	–					3	3
48_1	19·90	22	–					2	3
[2] 48_2	19·89	18·48	–					3	1
96_1	23·37	22	–					3	5
96_2	23·34	21	7·0	25·5, 31·5–36		15, 19·5, 27		6	1
144_1	21·46	21	–					8	3
[3] 144_2	21·45	21·36	3·0	42·72	33·8, 37·38, 39·16	12·46, 19·58		3	2
168_1	19·55	21	–					4	4
168_2	19·54	18·95	0·5		42·66			4	2
240_1	22·00	20–21	–					8	4
264_1	21·43	15	–					9	7
264_2	21·45	20·4	–					4	1

Table 4·6 Summary of DNA data (haustorium)

time (hours)	MEASURED DNA VALUES							probit populations	
	population mean	population mode	% observed mitotic figures	prophase	metaphase	anaphase	telophase	major	minor
[1] 0_1	16·60	15	-					5	-
[1] 0_2	16·66	16·17	-					2	3
24_1	18·65	19	-					3	4
24_2	18·74	19·35	-					1	3
48_1	18·89	18	-					3	3
48_2	18·86	18·46	-					1	2
[2] 96_1	20·33	19	-					3	4
96_2	20·42	22·04	1·0		26·68, 37·12			2	4
144_1	14·86	13	-					2	6
144_2	14·91	10·72	2·0	30·82, 37·52	25·46	17·42		2	5
[3] 168_1	19·03	19	-					5	5
168_2	19·03	16·60	2·0	31, 35·65, 38·75, 44·95				2	7
240_1	17·00	14	-					5	3
264_1	16·93	13	-					5	2
264_2	16·87	12·51	0·5		38·87			4	3

division during the first 24 hours of incubation, followed by a lag period of 48 hours before any further change in the DNA content, can be investigated more fully using tritiated thymidine as a label to establish the onset of DNA synthesis.

5. Continuous labelling of embryos using tritiated thymidine.

Embryos were incubated in Murashige and Skoog's medium containing 1μCi/ml tritiated [^{3}H]-thymidine and prepared for autoradiography using the methods described in chapter IID. After 14 days exposure the slides were developed and the percentage of labelled nuclei determined by counting along random horizontal transects using an eyepiece micrometer as a guide. The percentage of labelled nuclei in tigellum and haustorium are shown as Fig 4.70 and 4.71.

In the tigellum no labelled nuclei are found until after 72 hours of incubation. As the percentage of labelling increases so does the variation within the population, so it is difficult to determine the pattern of increase.

In the haustorium, some labelling is detected at 48 hours (less than 1%). From 72 hours onwards there is clear labelling, the percentage of which increases up to 174 hours. A regression line calculated by the least squares method has been plotted. It has a coefficient of regression of 0.61 and shows a linear increase between 102 and 156 hours. After 160 hours there is an increase in variability, the fitted regression line has a very low coefficient of regression showing that the points do not fit on a straight line which suggests there is no linear increase in percentage labelling.

Figs 4.70

Changes in the percentage of labelled nuclei in tigella incubated with tritiated thymidine.

Fig 4.71

Changes in the percentage of labelled nuclei in haustoria incubated with tritiated thymidine.

Fig. 4.70

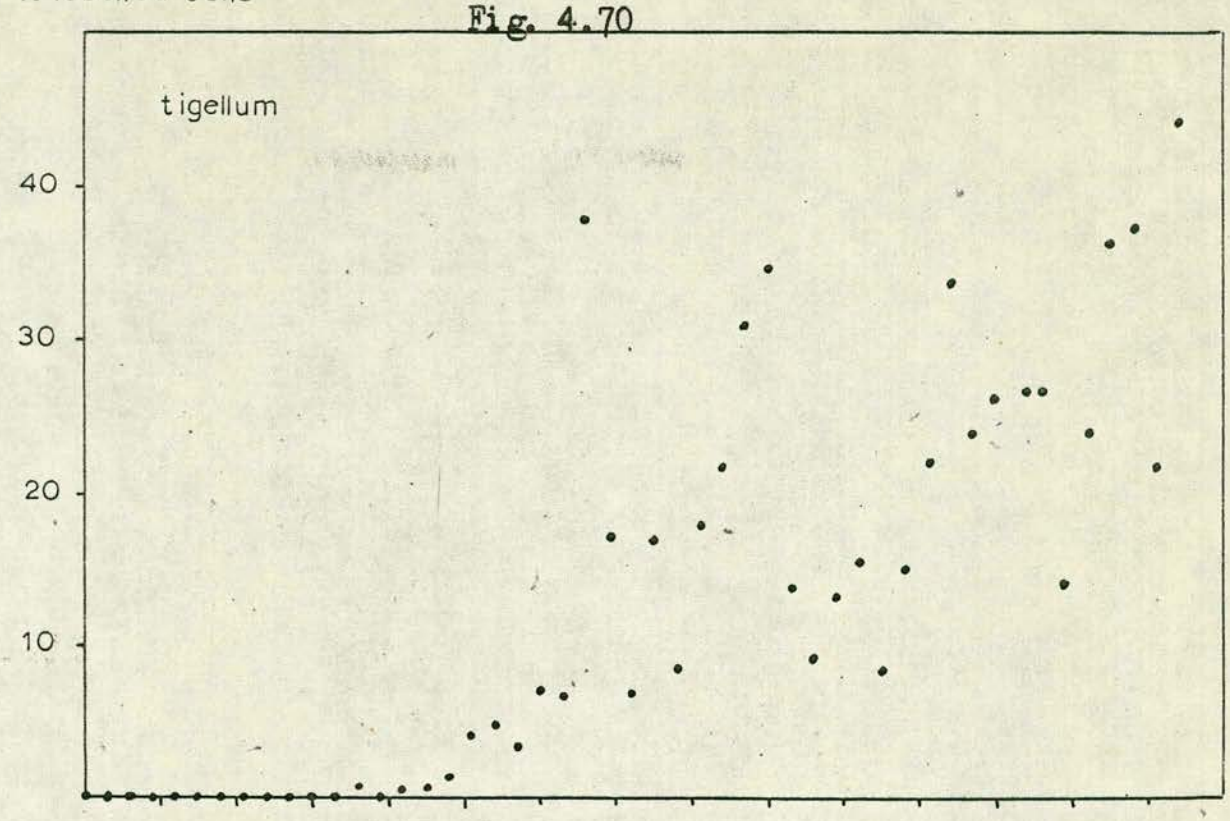
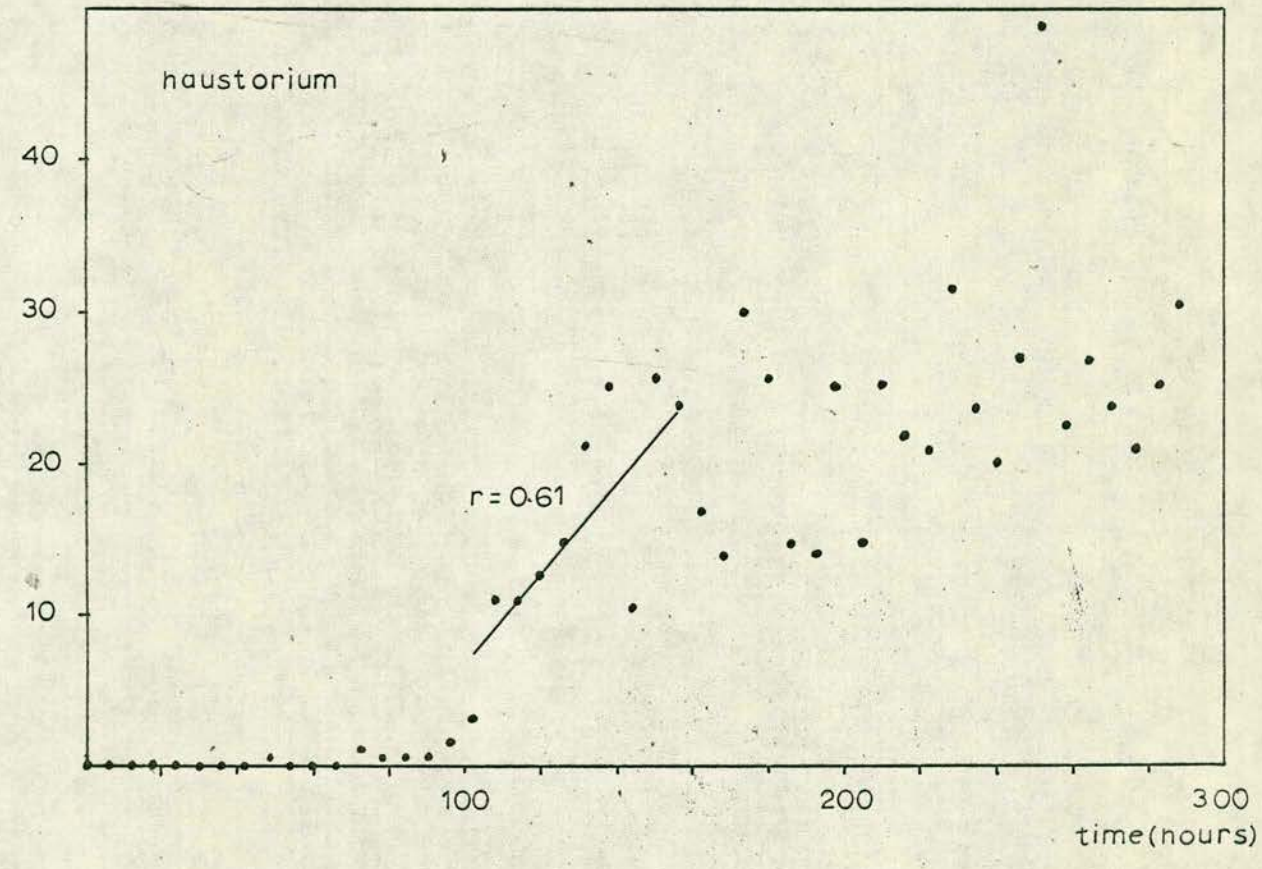


Fig. 4.71



• mean

—— regression line 102-156

From this it may be estimated that G_1 lasts for 48 to 72 hours. The variability makes it impossible to distinguish S, G_2 and M, but together they extend from 72 (or 48) hours to 150 hours and the second G_1 follows. The single high value of about 48.0% at 252 hours indicates that this individual embryo had completed the second G_1 and was in the second phase of S G_2 and M.

The percentage of labelled cells in the tigellum is higher than in the haustorium, so more nuclei are synthesising DNA but variability in rates of synthesis is much higher in the tigellum. The duration of G_1 is less than that of the lag period before significant cell number increases occur, as is expected. However, DNA measurements suggest that there is nuclear division within the first 24 hours of incubation not associated with any nuclear labelling. This suggests that either the added label of tritiated thymidine is not being incorporated into the DNA due to lack of thymidine kinase activity at this stage, or that nuclear division is occurring without DNA synthesis. This is further investigated and discussed in the next section.

F. An investigation into the growth of excised embryos during the first 24 hours of incubation.

It would appear from earlier experiments in this study that the first 24 hours of incubation of the excised embryo is a critical period in the development of the embryo, as shown by the increase in fresh weight due to imbibition (Chapter 4 5a), the changes in DNA content of the nuclei (Chapter 4 5d), and the suggestion, although not statistically significant, of a cell number increase (Chapter 4 5b). It has also been shown that fresh weight increases will occur, irrespective of whether normal development follows.

When embryos are incubated with tritiated thymidine, no labelling of nuclei can be detected until 72 hours of incubation, but there is some evidence for nuclear division within the first 24 hours of incubation, from which it can be inferred that either the label is not being incorporated into the DNA or that nuclear division is taking place without DNA synthesis.

The microdensitometric measurements of DNA suggest that the observed fall in the mean DNA value is due to a decrease in nuclei of high DNA content and this is parallelled by an increase in nuclei with a low DNA content. This observation would imply that nuclear division is occurring without DNA synthesis. Presumably the nuclei have synthesised this DNA in a period of embryo development prior to excision and have remained in G_2 until excision and culture triggers the onset of mitosis in these nuclei.

In most cell counting experiments a small increase in cell number can be distinguished during the first 24 hours of incubation, although there is no good statistical evidence for this because the sample sizes were always too small. Mitotic figures were not observed, which could mean that a small population of cells was dividing with some degree of periodicity or that mitosis is very short in comparison with the length of the rest of the cell cycle.

In this experiment attempts were made to determine whether cell division occurs during the first 24 hours of incubation from excision, using a statistically significant sample size. It was anticipated that nuclear division could be observed using colchicine to arrest mitosis and that the DNA content of the dividing nuclei could be measured and

compared with non-dividing nuclei in order to establish the DNA content of dividing nuclei.

1) Changes in cell number.

Embryos from batch number 2/5523 x 2/8607 were excised, weighed, inoculated into a defined Murashige and Skoog's medium and incubated under standard conditions. Ten embryos were sampled at 0, 6, 12 and 24 hours, weighed and macerated in chromic acid overnight at room temperature, and for up to 6 days at 4°C.

The cell numbers of each embryo were determined and plotted as Fig 4.72.

The standard errors for each time interval were calculated on a Canola calculator. There is no significant difference between the population of 0 and 6 or 12 hours. However there is a statistically significant difference between the cell numbers at 0 and 24 hours and it can be seen that cell division occurs within the first 24 hours of incubation and a small increase in cell number occurs (about 11%).

2) The use of colchicine to arrest mitosis and determine the proportion of dividing cells.

Although there is a significant increase in cell number during the first 24 hours of incubation mitotic figures were not observed. This would indicate either that mitosis is very short in comparison with the rest of the cell cycle or that there is a high degree of periodicity in division.

Colchicine interferes with mitosis by preventing spindle formation

Fig 4.72

Cell number changes in embryos during the first 24 hours of incubation.

Fig 4.73

Changes, with time, in the percentage of nuclei in the pro-metaphase configuration.

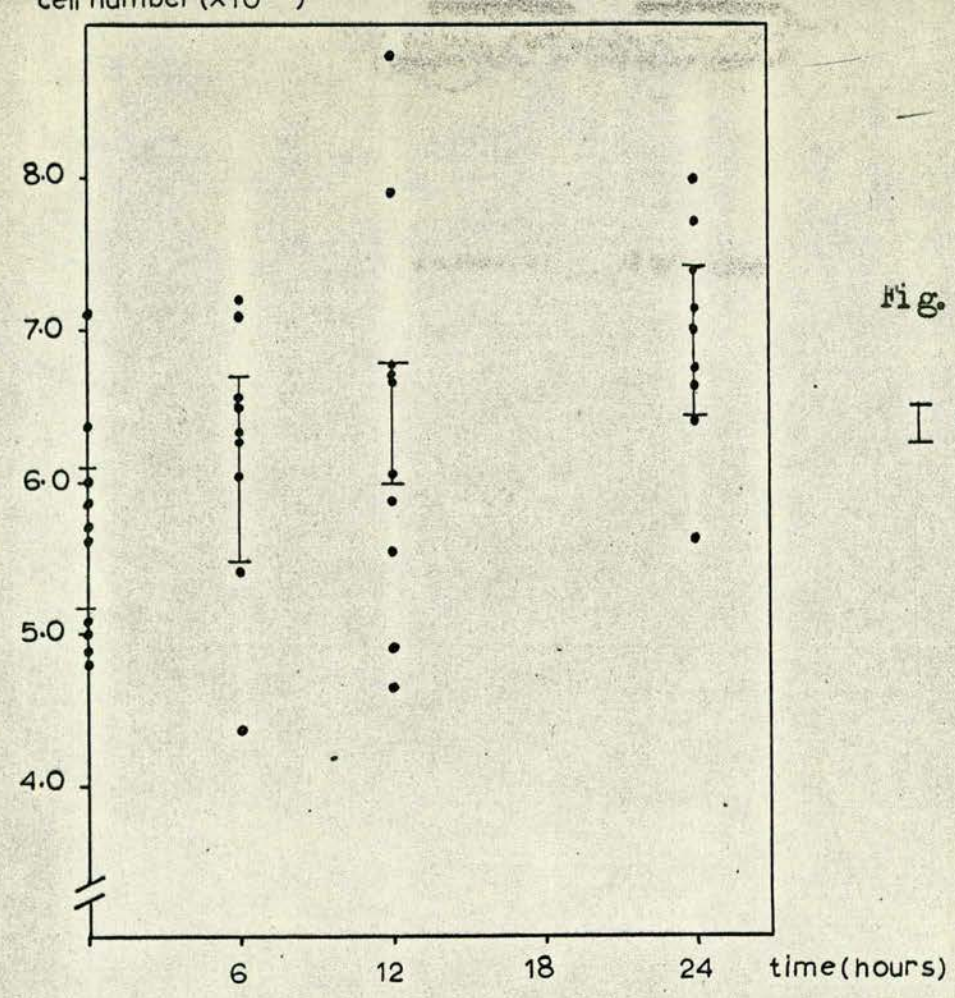
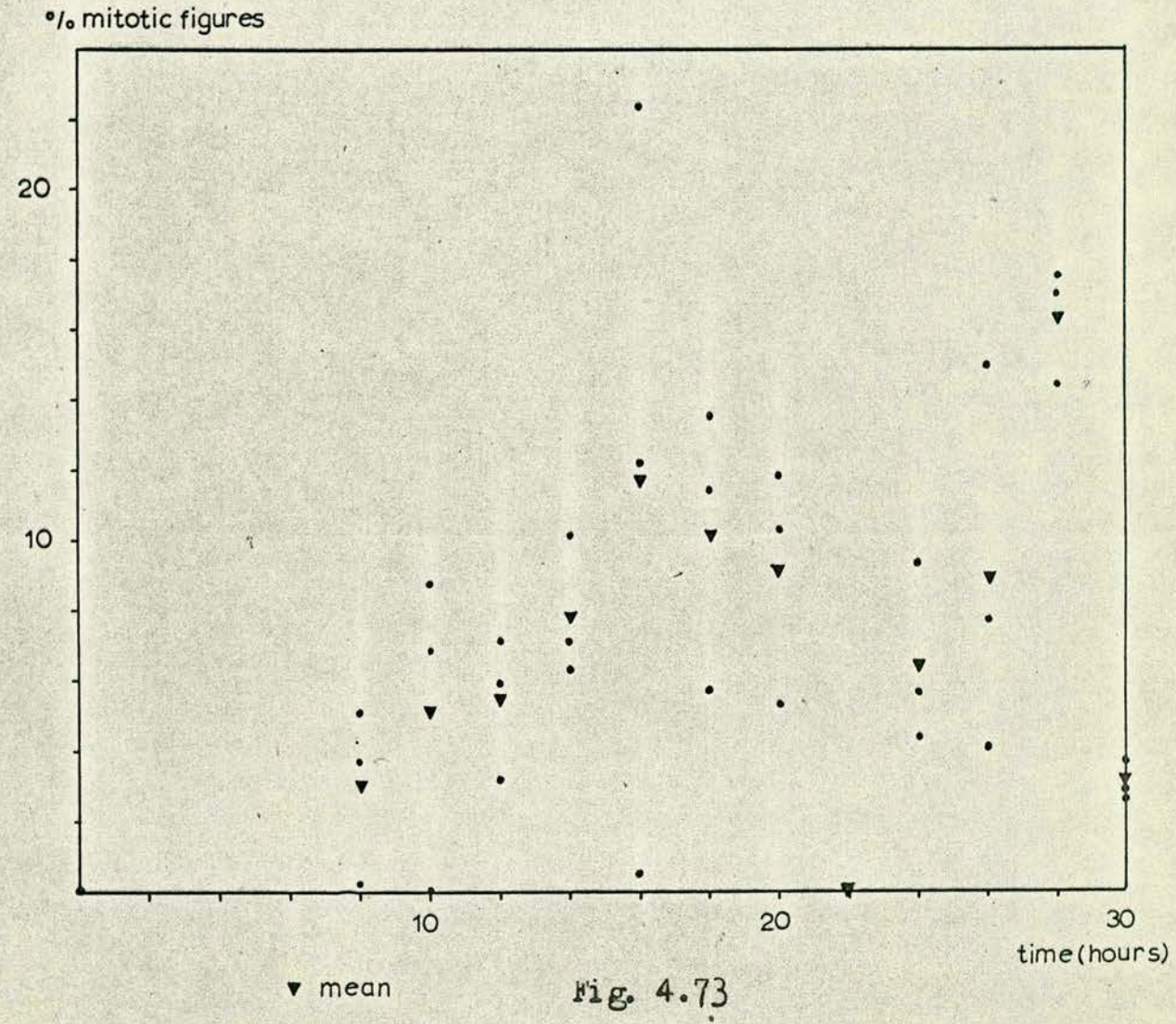


Fig. 4.72

I standard error



▼ mean

Fig. 4.73

so that, generally, cells undergoing mitosis will be halted in the mitotic cycle at metaphase.

Embryos were cultured so that they were exposed to colchicine for the determined optimum period of 6 hours before sampling. Hence all cells undergoing mitosis during that 6 hours should be halted at metaphase. Colchicine was added to the 0-6 hour sample immediately on excision and to subsequent samples at 2 hourly intervals for 24 hours. Thus any mitosis taking place over the first 30 hours of incubation should be observed, after Feulgen staining and squashing.

No conventional metaphase plates were found and all the mitotic figures were those of the pro-metaphase type described by Eigsti and Dustin (1955)

Changes in the percentage of mitotic figures found are plotted as Fig 4.73. The data are plotted at the time when the sample was taken, not when the colchicine was added.

The percentage of pro-metaphase configurations reaches a peak of 11.7% 16 hours from excision, that is in embryos cultured for 10 hours before six hours exposure to colchicine, followed by a second discrete peak of 16.3% at 28 hours. This bimodal distribution suggests 2 separate populations of cells are dividing each with a degree of periodicity. The first peak occurs within the 24 hours of the start of the investigation and at approximately 11% agrees with the calculated significant 11% increase in cell number during that period. The second peak, after 24 hours of incubation, could be due to division of nuclei of a different ploidy series which are also in G_2 at excision (see CVII for an explanation

of the ploidy series).

3) Microdensitometric measurement of the DNA content of nuclei treated with colchicine.

It has been established previously that cell division occurs during the first 24 hours of incubation of an excised embryo. However, no tritiated thymidine is incorporated into the DNA of the embryo until at least 48 hours of incubation. This could either be due to the non-incorporation of the ^{3}H-thymidine because of the cell lack thymidine kinase, or to the dividing population of cells already having synthesised the required DNA and are resting in G_2 of the cell cycle. The second possibility can be tested by the measurement of the DNA values of the dividing nuclei and comparing them with the DNA content of non-dividing nuclei. As no mitotic figures have been found in routine examination of embryos, possibly due to the periodicity of division, colchicine must be used to distinguish dividing nuclei by arresting division at metaphase.

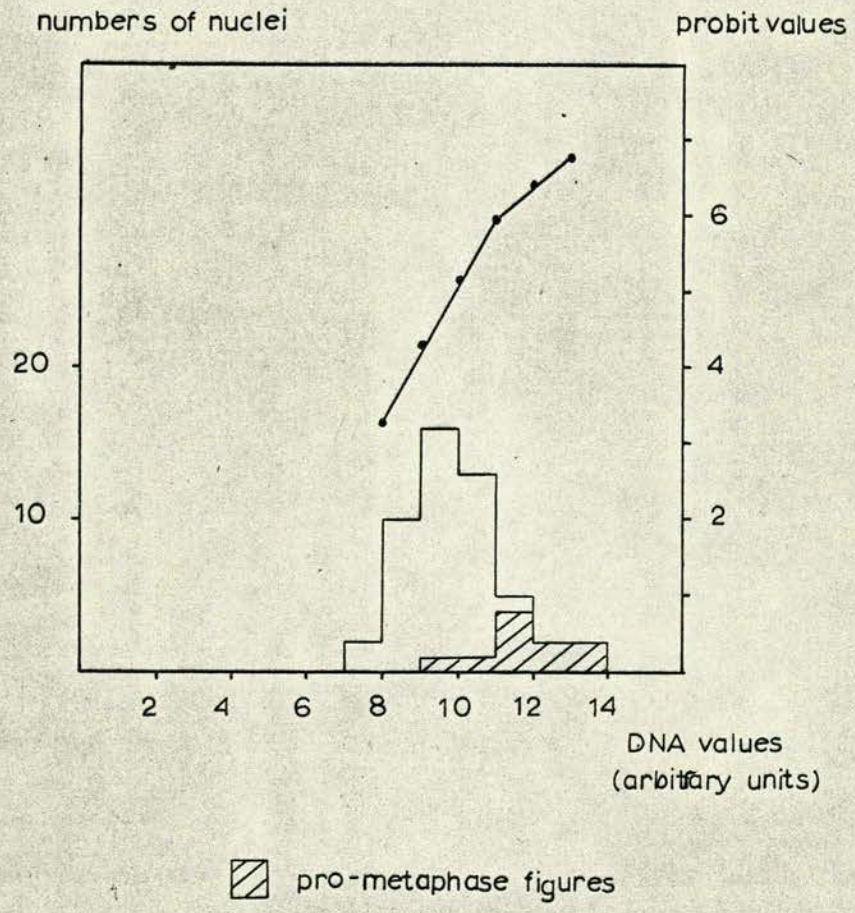
Embryos were incubated for 12 hours under standard conditions and colchicine was then added to the medium. The embryos were incubated for a further six hours before they were sampled, Feulgen stained and squashed. This incubation period was chosen as the previous experiment (F2) had shown it to produce many mitotic figures.

The slides were used for the microdensitometric determination of the DNA content of nuclei, noting at the same time, whether the nucleus was in interphase or mitosis. The values were subjected to probit analysis and these are presented in Fig 4.74.

Only pro-metaphase nuclei were found to have a high DNA value.

Fig. 4.74

The distribution of DNA values in an embryo treated with colchicine at 24 hours of incubation.



The nuclei with a high DNA content could be separated, by probit analysis, from the main, more uniform population. The mean of the nuclei with high DNA values was about twice that of the mean of the majority of the population. This establishes that the nuclei dividing in the first 24 hours of incubation have synthesised the DNA required for nuclear division before excision of the embryo from the seed, and have halted in G_2. Excision and incubation of the embryos under favourable conditions triggers the onset of nuclear and cell division in 11% of the cells in the embryo which are in G_2. This produces an 11% increase in cell number which is parallelled by a decrease in the number of high DNA content nuclei and an increase in the number of low DNA content nuclei.

G. Summary.

Excised embryos rapidly change in shape on incubation. The haustorium enlarges and a 'hammer' shape is produced from the tigellum. The leaves and radicle emerge from the hammer head. Chlorophyll develops between day 7 and day 8 in embryos grown in the light. The embryo develops a vascular system to absorb nutrients, from the endosperm, through the haustorium to the developing meristematic tissue of the shoot and roots.

Preliminary experiments designed to follow changes in fresh weight of excised embryos by repeated sterile weighings of a population showed three phases in weight change. The first phase is due to imbibition and lasts 24 hours, in the second, lasting between 72 and 240 hours, little change occurs. The third phase is a rapid increase in fresh weight associated with the visual signs of germination, that is greening, the formation of the hammer shape and leaf, radicle and haustorium expansion.

Preliminary experiments performed to follow increases in cell numbers in excised embryos showed that no statistically valid increase in cell number occurs until between 140 and 160 hours of incubation. Cell number doubling occurs at about 216 hours from excision.

Microdensitometric measurements of DNA, protein and total nucleic acid on 10 μm sections of embryos showed changing distributions with time. The DNA content of nuclei of freshly excised embryos is quite uniform. The range in variation of DNA content increases with time, and sub-populations with differing levels of DNA are formed with extended incubation times. Freshly excised embryos have few areas of high protein content. Incubation changes the distribution so that a bimodal distribution of high and low protein areas are found. However, the high protein areas cannot be associated with any specific region of the embryo. If the value of mean protein content per field of view is calculated, it can be shown to halve between excision and 192 hours of incubation.

Total nucleic acid measurements made from 10 μm sections show an increase in the range of amount of nucleic acid with time. The mean amount of nucleic acid per field increases with incubation up to 144 hours and then halves and increases with subsequent 48 hour intervals.

Preliminary labelling experiments with tritiated thymidine established G_1 as having a duration of 60 hours. By 276 hours over 60% of the nuclei had synthesised DNA, and these cells were associated with the meristematic regions of the tigellum and the developing vascular connections between the haustorium and tigellum.

The embryo of the oil palm has two distinct portions. The tigellum contains the meristematic regions of the shoot and root systems and the haustorium is the site of synthesis and secretion of enzymes for the absorption of endosperm. The tigellum and haustorium develop along distinct pathways.

Experiments designed to follow changes in fresh weight show that the tigellum and haustorium imbibe at the same rate, but, while the haustorium continues to increase in fresh weight in the period 24 to 96 hours, the tigellum does not. Between 120 and 144 hours both begin to increase in fresh weight exponentially, the tigellum at the greater rate. The tigellum is generally heavier than the haustorium and the difference is increased on germination as the haustorium soon attains a maximum size, while the tigellum continues to increase.

The haustorium undergoes cell division 24 hours earlier than the tigellum at 96 hours, but the subsequent rate of increase in cell number is slower. The tigellum, from the measured population of embryos, is more variable in cell number than the haustorium.

The mean fresh weight per cell, calculated from the fresh weight and cell number data, of the haustorium is greater than that of the tigellum. The haustorium has two populations of cells, which may be distinguished by the mean fresh weight per cell data and these divide synchronously but separately from each other. The larger synchronous population of cells has a mean cell generation time of 144 hours.

In a time course experiment it was established that after an imbibition period of 24 hours duration no increase in fresh weight occurs

until 124 hours of incubation. This is comparable to the result obtained in the preliminary experiment using embryos which were re-weighed. Cell numbers show a statistically significant increase after 152 hours of incubation. A regression line, calculated by the least squares method has a low coefficient of regression due to the high degree of variability. The calculated mean fresh weight per cell doubles within 6 hours of incubation and can be said to double again by 150 hours of incubation. However the variability of the population is high.

Microdensitometric measurements of the DNA content of nuclei in the tigellum and haustorium showed that the two portions of the embryo, although similar early in incubation, had different developmental patterns. The DNA values of nuclei at excision formed a single peak with little variation. Incubation increased the variation and the number of peaks. Probit analysis of the data distinguished sub-populations of nuclei of similar values.

The tigellum of embryos cultured with medium containing tritiated thymidine had labelled nuclei after 72 hours of culture. The percentage of labelled nuclei in the tigellum increases with incubation time, but less than 50% are labelled by 288 hours. The variation in the percentage of labelling increases with time.

Autoradiography of the haustorium shows that labelling of the DNA begins as early as 48 hours from excision in one, possibly abnormally advanced, embryo. The percentage of labelled nuclei increases rapidly from 72 to 150 hours. Variation within the population increases rapidly from 130 hours of incubation. G_1 can be considered to extend from excision (0 hours) to 48 or 72 hours, and S, G_2 and M from 72

(or 48) hours until 150 hours. It is impossible to distinguish S, G_2 and M because of variation. In the haustorium less than 32% of the nuclei are labelled, this is a lower level than that of the tigellum. On the basis of a mean value of 46% labelled nuclei at 252 hours of incubation, the haustorium of that group of three embryos can be considered to have completed the second G_1 phase and to be in the second (S G_2 and M) phase. This mean value serves to emphasise the variability of the population despite the other more uniform values of the haustorium.

Investigations into the first 24 hours of incubation of excised embryos established that cell division takes place, with an 11% increase in cell number. Colchicine was used to determine that the largest number of dividing nuclei is found between 10 and 18 hours of incubation from excision. Microdensitometric measurements of the DNA in the nuclei established that the cells dividing in the first 24 hours of incubation were in G_2 of the cell cycle in the excised embryo.

Overall, the excised embryo is a useful primary explant with which to study the growth of the oil palm, despite the disadvantage of unknown genetic potential. Its growth has been clearly defined by a number of parameters, which can later be used to compare with the induction of callus in the excised embryo.

Chapter V

Callus Induction in the Excised Embryo

In the previous chapter a detailed study was made of the growth and development of the excised embryo in conditions which promoted 'normal' growth. Despite the high degree of variability encountered in this part of the investigation, a growth analysis of the excised embryo was produced and a detailed account obtained of the cytological characteristics of the cultured embryo, the primary explant system. In this chapter the normal developmental pattern of the embryo is disturbed by two treatments, a short exposure to hydrogen peroxide and subsequent culture in contact with a medium containing 2,4-D. These treatments induce changes in the embryo which ultimately give rise to callus tissue.

The growth of the developing callus tissue was followed paying particular attention to the changing cytological characteristics and kinetics of cell division. The mode and the time of cell division, with respect to the cell cycle in the developing callus tissue, were investigated using the techniques of cell counting, labelling and autoradiography using ^{3}H-thymidine, and microdensitometry.

A. Changes in morphology associated with callus induction

When an excised embryo is pre-treated with hydrogen peroxide and then incubated under callus inducing conditions it undergoes morphological changes as callus tissue is initiated. Fig 5.1 shows the freshly excised embryo, a characteristic torpedo shape with the tigellum, the blunt end,

Figs 5.1 to 5.13

Morphological changes in excised embryos, associated with callus-induction.

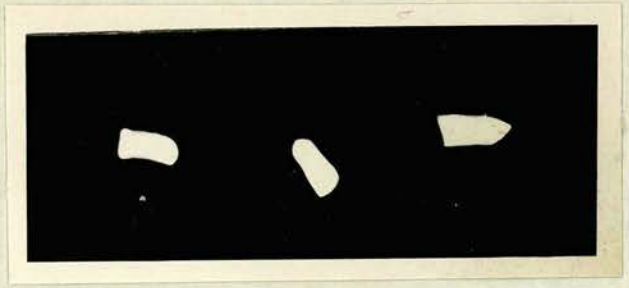


Fig. 5.1

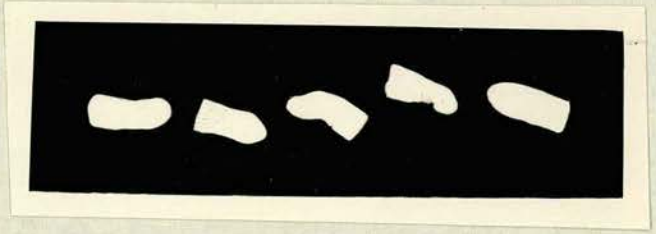


Fig. 5.4

Fig. 5.7

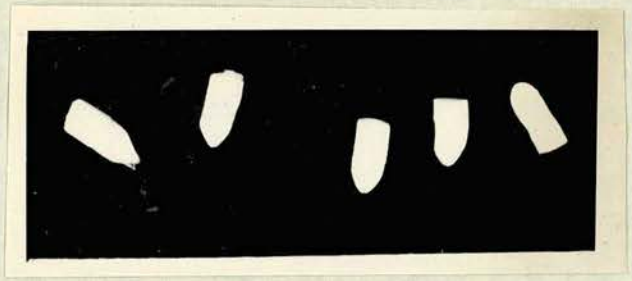


Fig.5.2

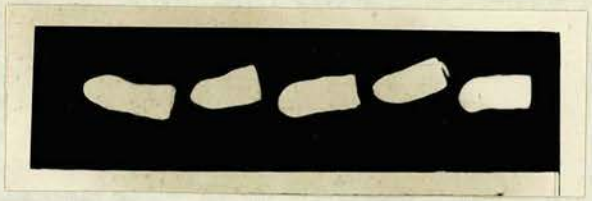


Fig. 5.5

Fig. 5.8

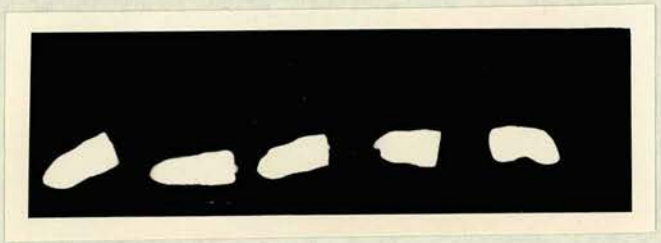


Fig. 5.3

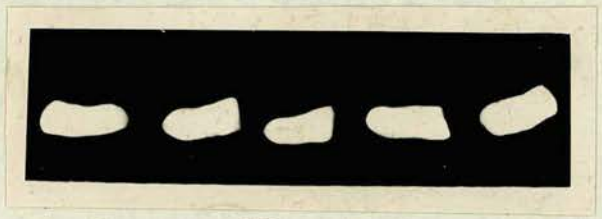
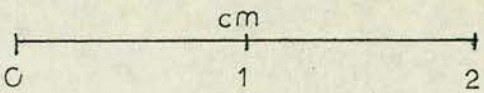


Fig. 5.6



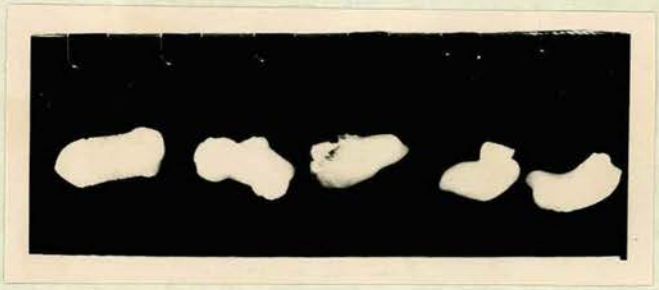


Fig. 5.9

Fig. 5.10

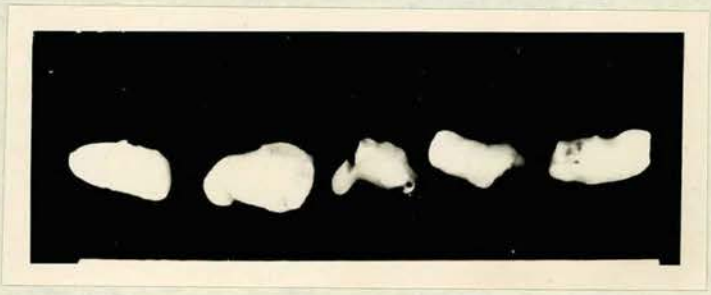


Fig. 5.11

Fig. 5.12

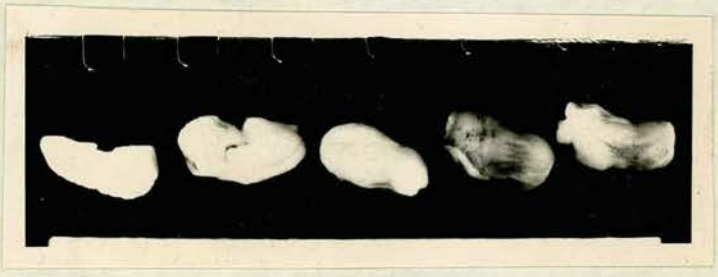
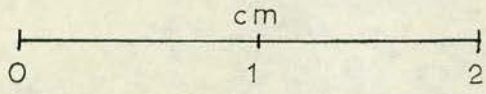


Fig. 5.13



separated by a slight constriction from the haustorium which is the pointed end. After 24 hours of incubation (Fig 5.2) there is little change in shape although the embryos have increased in volume, due almost entirely to imbibition. By day 2 (Fig 5.3) the embryos appear to have elongated slightly and this increase is maintained at 72 hours (Fig 5.4) and 96 hours (Fig 5.5). By 120 hours (Fig 5.6) the first and second embryos from the right have lost the very smooth outline and the constriction between the tigellum and haustorium. This swelling is exaggerated by 144 hours (Fig 5.7) when the embryos become misshapen but without any sign of the 'hammer' formation associated with germination. The swelling and alteration of shape continues throughout the incubation period (Fig 5.8 to 5.13) so that by 288 hours of incubation (Fig 5.13) the embryo has at least doubled in volume and length from its size at excision.

Greening of the embryo is not invariable but begins at 144 hours (Fig 5.7) and many embryos become brown, shown as dark areas (Fig 5.9) from 192 hours onwards.

All of the embryos grown under callus inducing conditions float in the medium, and are much more buoyant than embryos germinating under non-callusing conditions.

B. Anatomical changes associated with callus induction

Callus induction in excised embryos is associated with profound anatomical changes.

Fig 5.14 shows that little anatomical change has taken place in an embryo incubated under callus inducing conditions for 30 hours in comparison with the freshly excised embryo of Fig 4.3. After 48 hours of incubation (Fig 5.15) there is also little change. By 144 hours of incubation (Fig 5.16) the shoot meristem, although still visible, has hardly developed from the stage characteristic of the freshly excised embryo. The area between the tigellum and haustorium appears to break down due to cell fracture. There is a distinct area in the centre of the haustorium which has begun to develop callus while the outside tissue remains similar to that of the haustorium under non-callus inducing conditions. At 168 hours of incubation this effect is greatly exaggerated as shown in Fig 5.17. The splitting of the de-differentiated cells in the centre of the embryo has increased and the area of normal haustorial cells at the edge has decreased. There is no obvious increase in the surface area of the haustorium by furrowing, a general feature of the germinating embryo. The shoot meristem has not developed, but directly below it, and appearing to originate from it, four areas of very small cells have developed in the site where, in the normal germinating embryo, vascular tissue would have developed. By 192 hours of incubation (Fig 5.18) these areas of small cells have increased in number and in distribution, so that they are found throughout the embryo. The splits in the tissue have also become more obvious after 216 hours (Fig 5.19) the majority of the embryo has dedifferentiated except for a small remaining band of embryo cells just below the epidermis. The areas of small cells have increased and the cells are distributed across most of the tigellum and down one side of the haustorium. These are splits between the

Figs. 5.14 – 5.20

Anatomical changes associated with callus induction in excised embryos.

sc small callus cells
lc large callus cells
et embryo tissue
ec embryo cells
sm shoot meristem
rm root meristem
t tigellum
h haustorium

Fig . 5.14

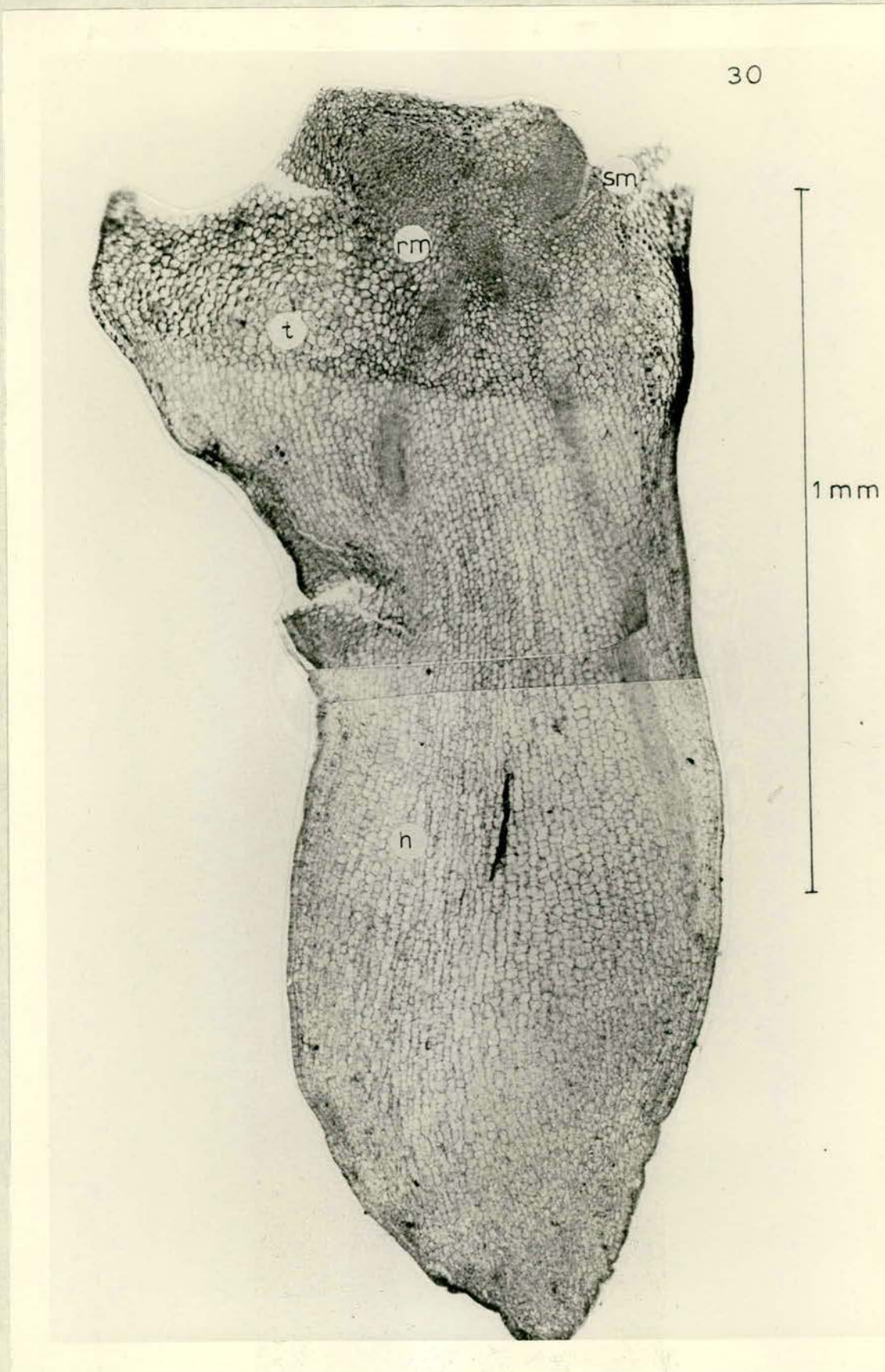


Fig. 5.15

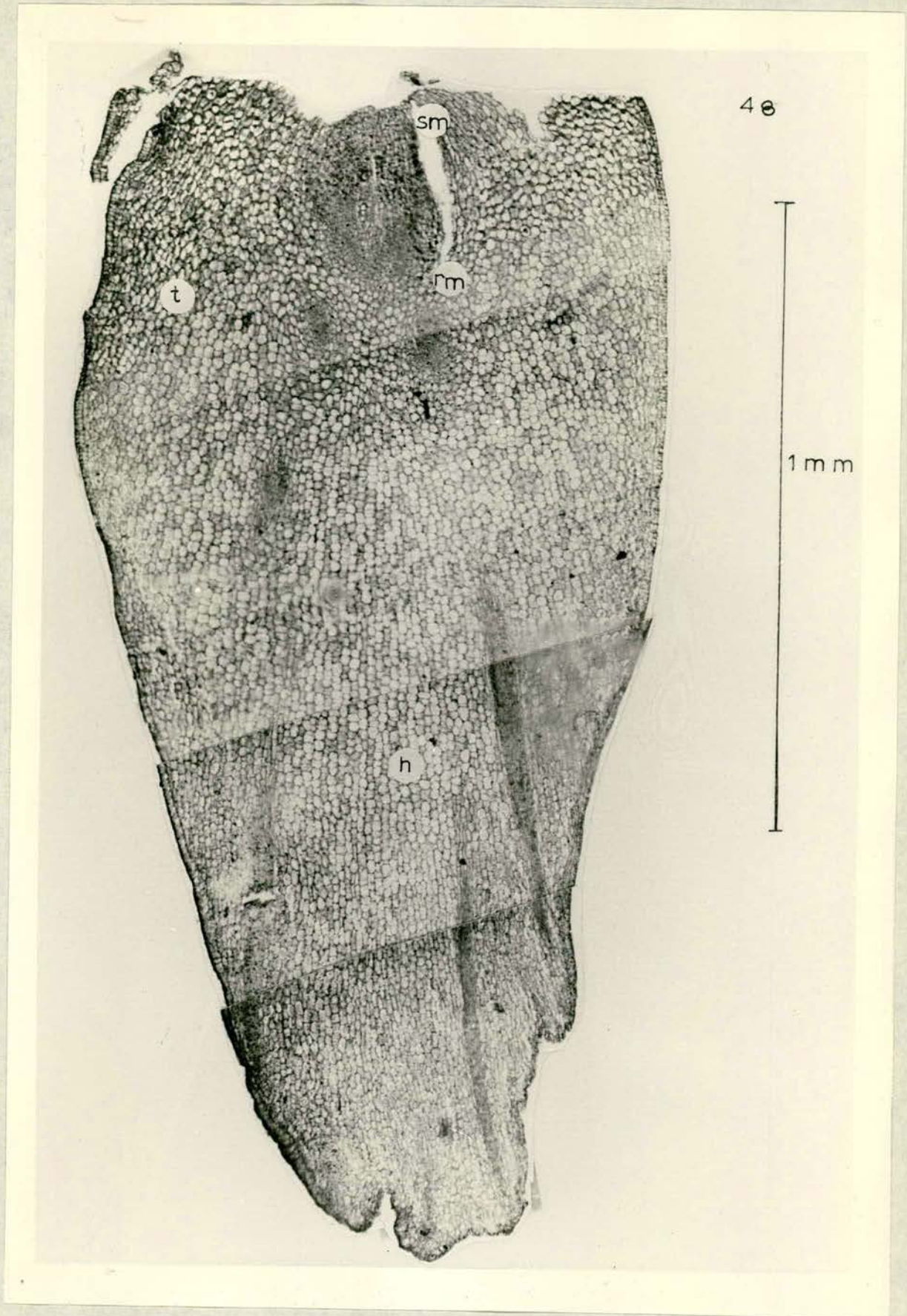


Fig. 5.16

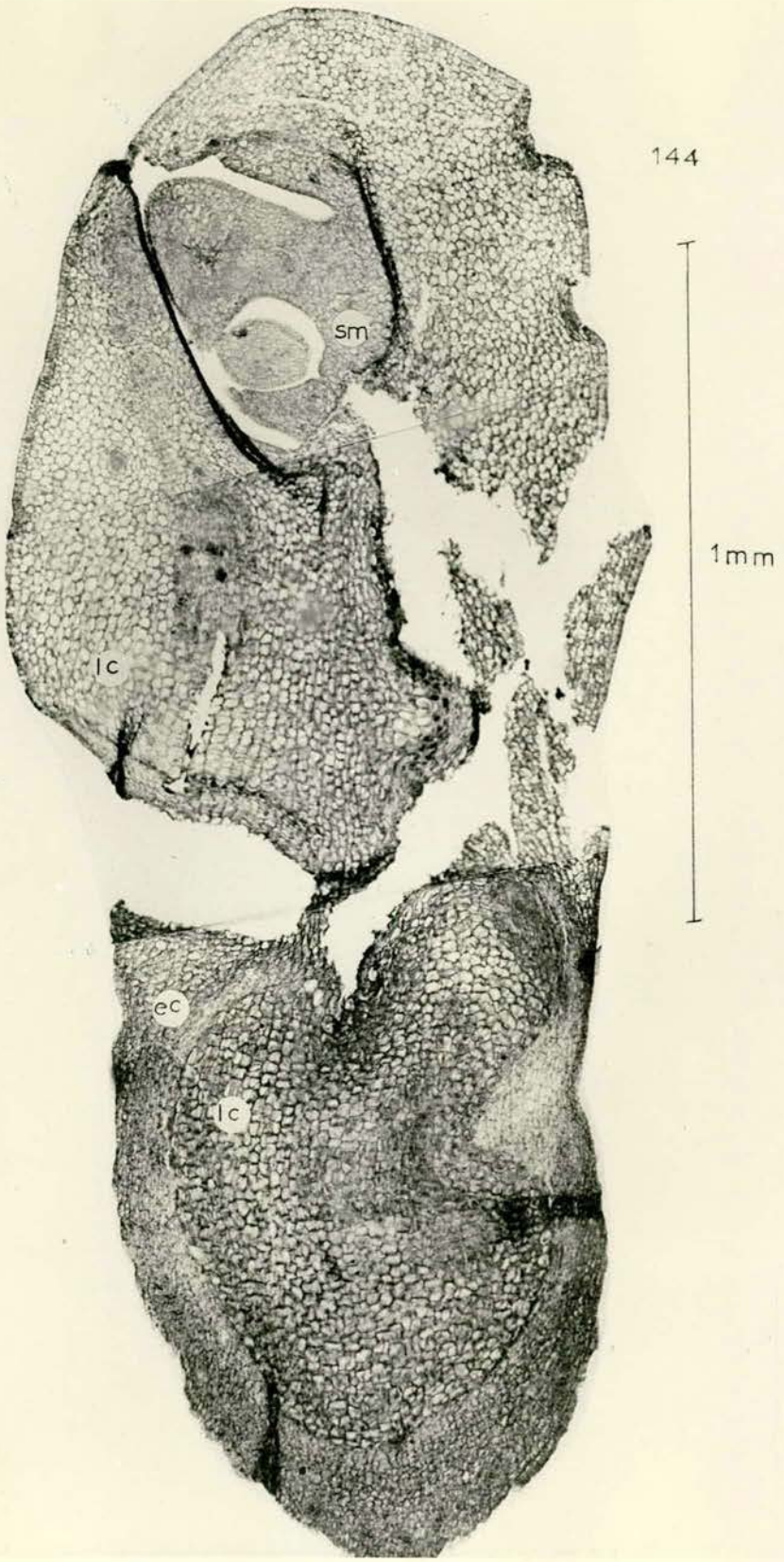


Fig. 5.17

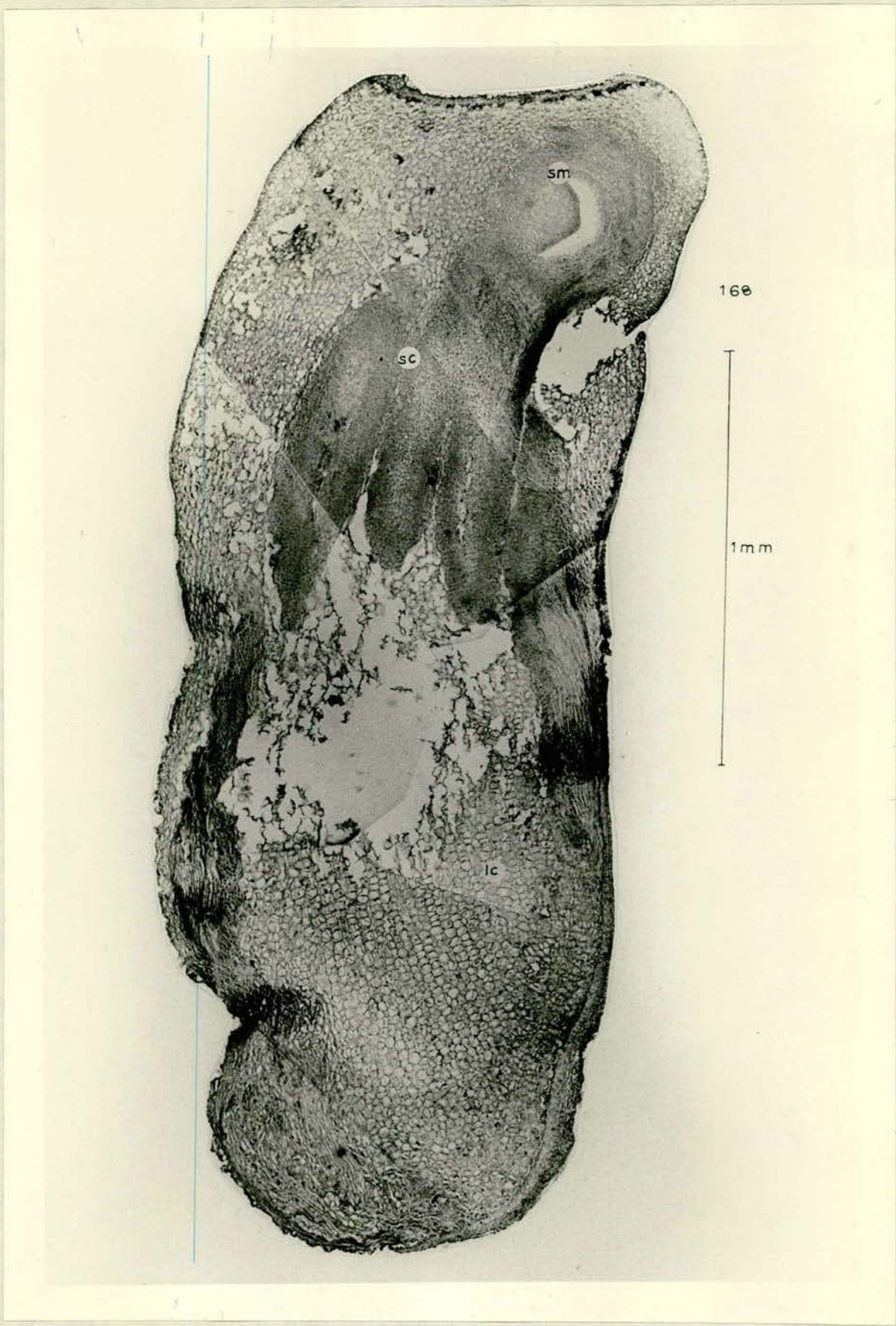


Fig. 5.18

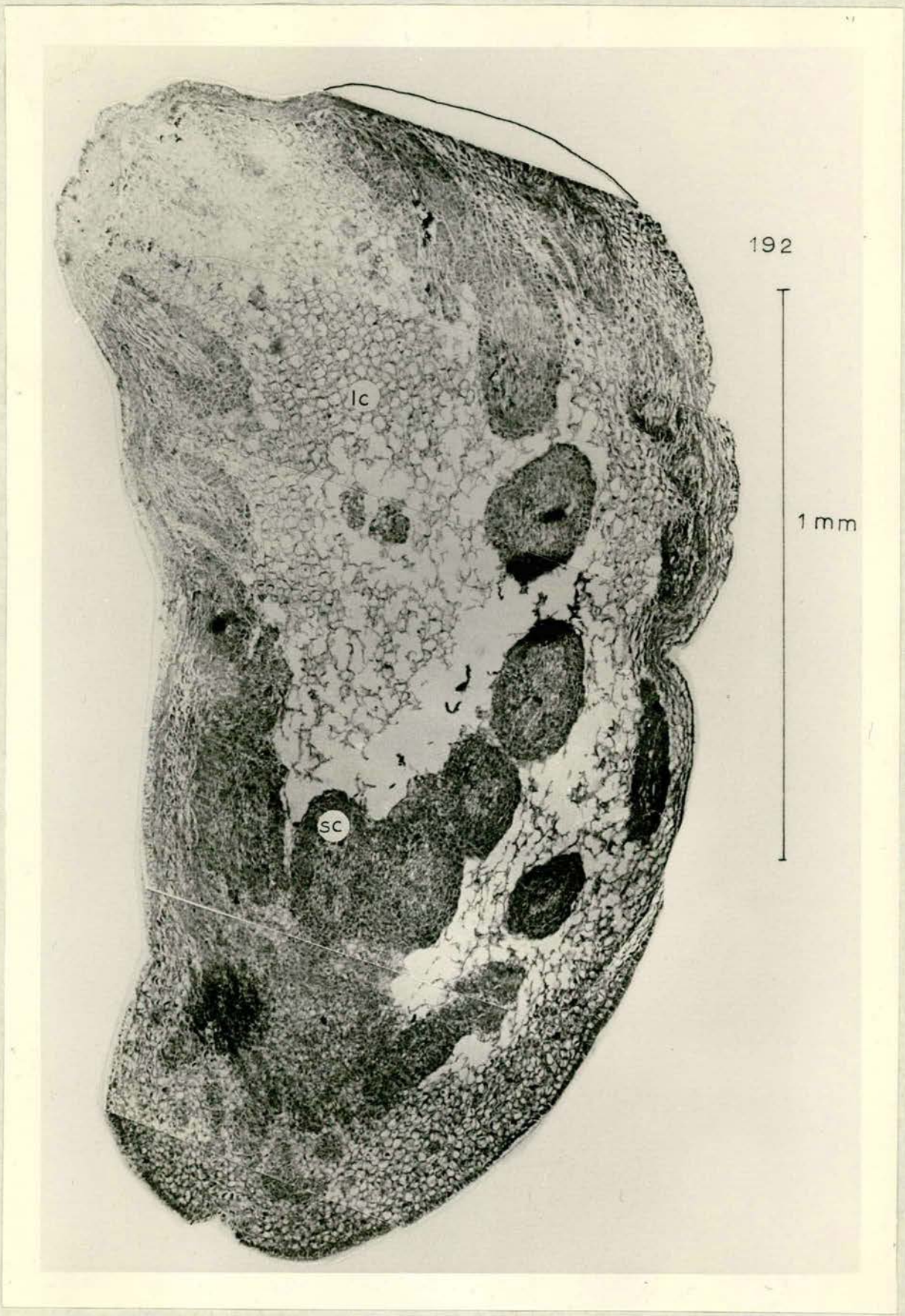


Fig. 5.19

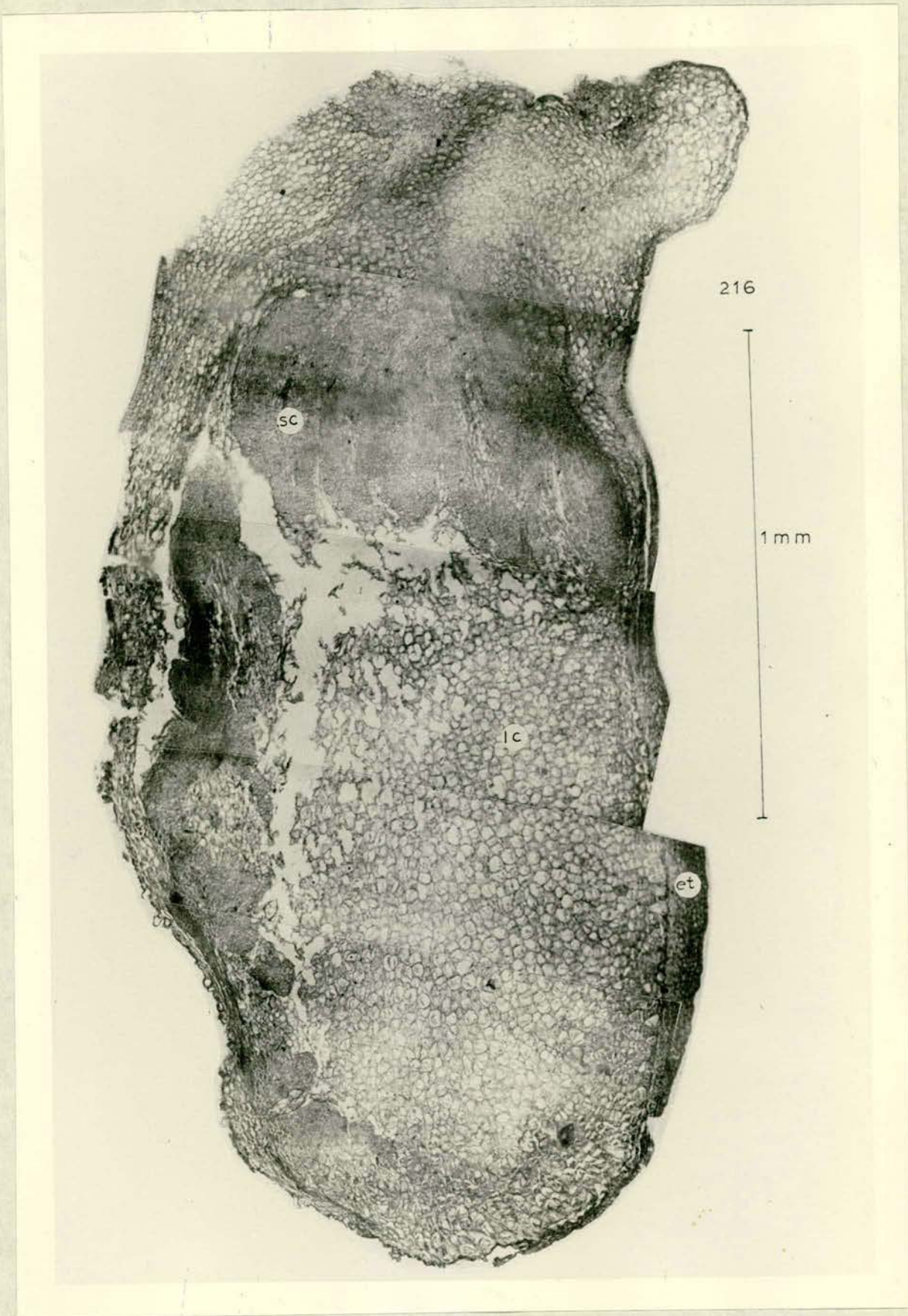
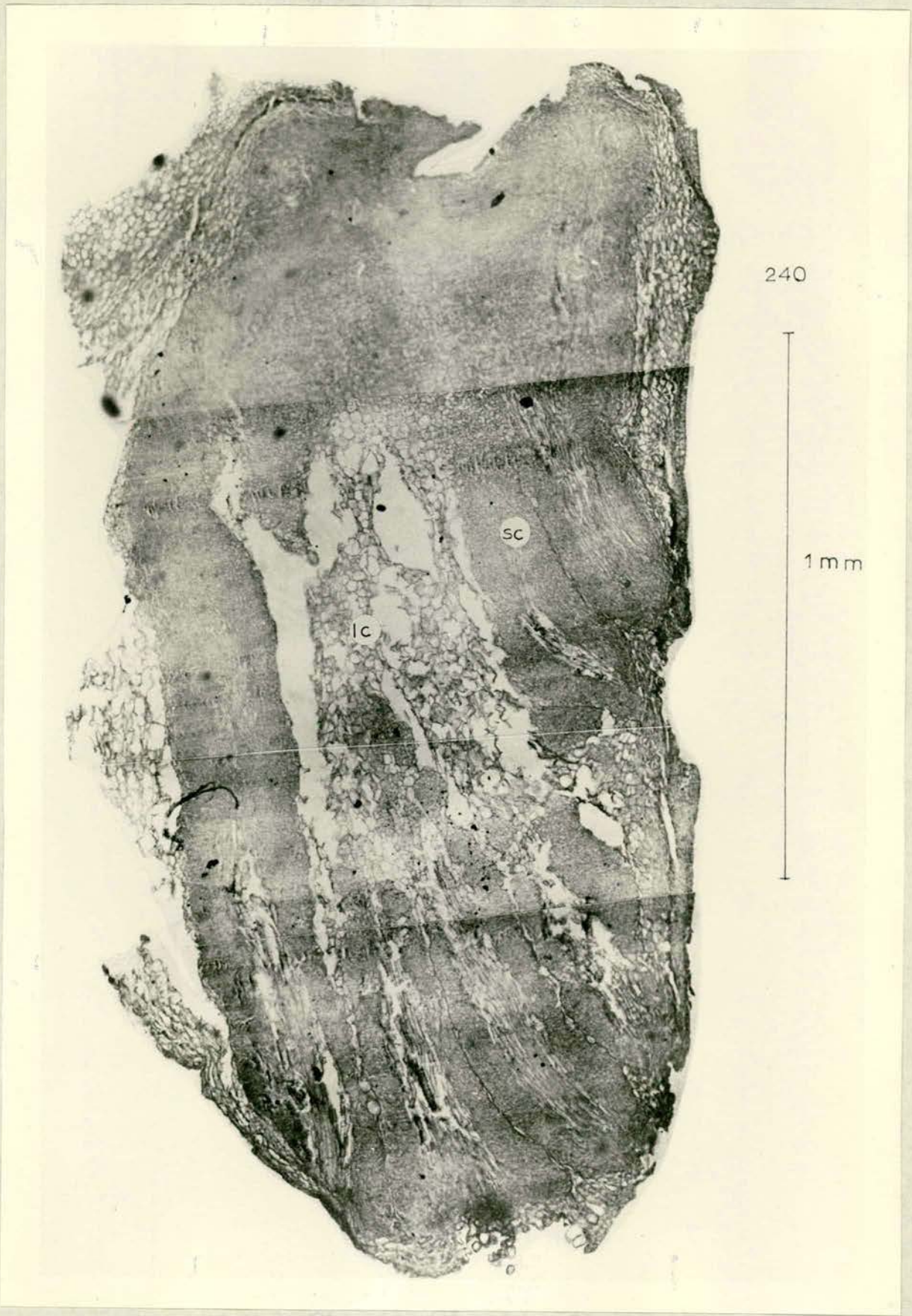


Fig. 5.20



remaining embryo cells and the callus cells and especially in the adjoining region of the small and large callus cells.

After 240 hours of incubation (Fig 5.20) the whole of the embryo has become callused with mainly small cells, but also larger ones which give rise to splits in the embryo. The splits in the embryos would account for the distortion in the shape of the embryo and explain why the callused embryos are so buoyant, as noted in the previous section.

C. Determination of fresh weight by reweighing procedures

The established morphological and anatomical changes in the embryo undergoing callus-induction are associated with growth. Growth can be further assayed in individual embryos by the measurement of fresh weight. The changes in fresh weight of embryos can be followed by repeated weighings of individual embryos under sterile conditions. If the weighing conditions and degree of surface moisture are closely controlled the majority of changes in fresh weight will be directly proportional to changes in dry weight. The notable exception to this is the rapid increase in fresh weight associated with imbibition, where there is no parallel increase in dry weight. Hence the assay of fresh weight, if treated with care, will give a measured parameter of growth as an irreversible increase in mass.

Embryos of batch number 2/5703 x 2/11605 were excised, weighed, pre-treated with hydrogen peroxide and inoculated into standard callus-inducing Murashige and Skoog's medium. The embryos were incubated under standard conditions apart from a short period each day when they were weighed. The individual weight changes are shown in Figs 5.21 and

Figs 5.21 to 5.22

Changes in fresh weight in individual embryos, determined by sterile reweighing techniques.

Fig 5.23

Mean value and range in fresh weight of the embryos described in Figs 5.21 and 5.22.

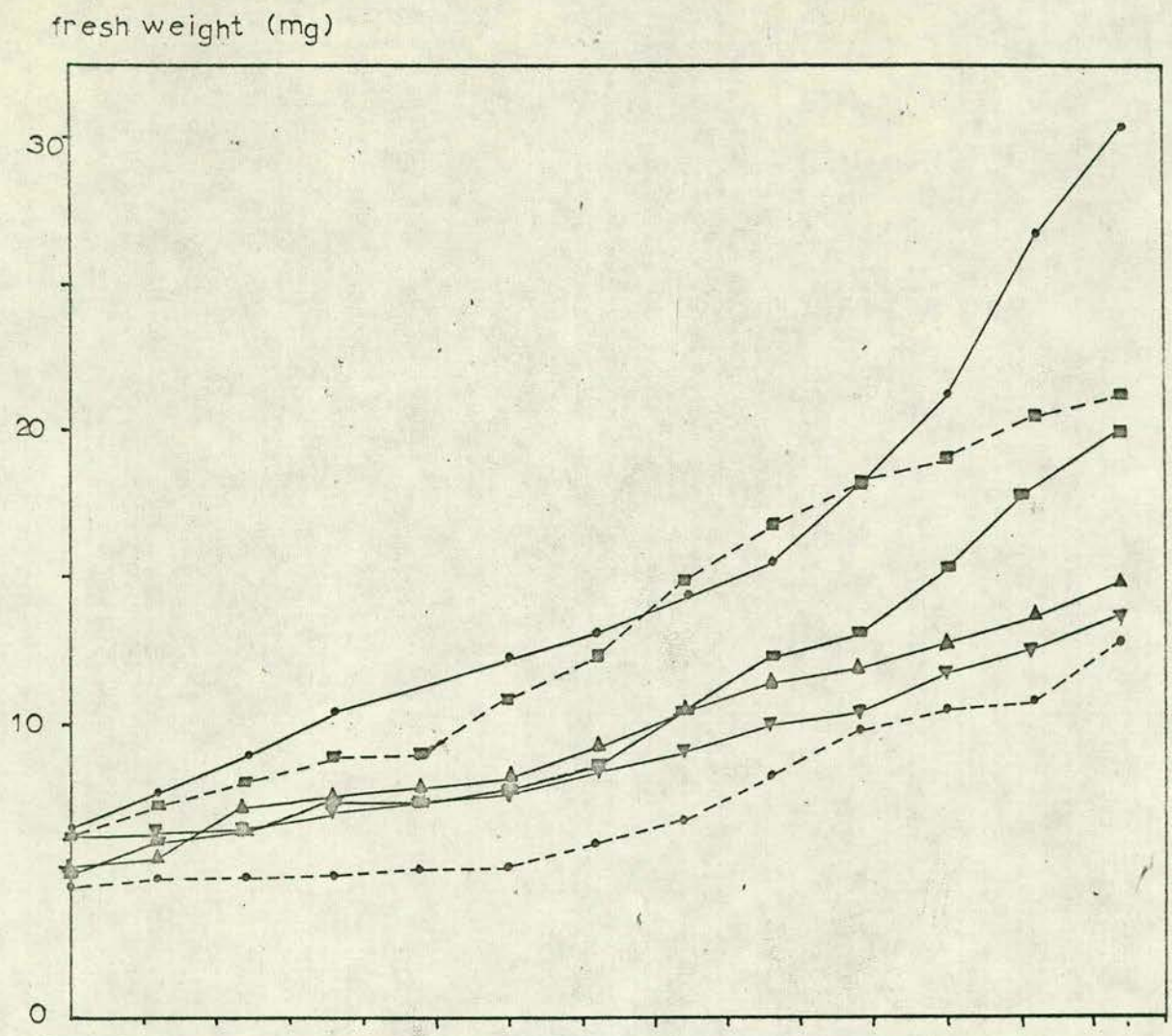


Fig. 5.21

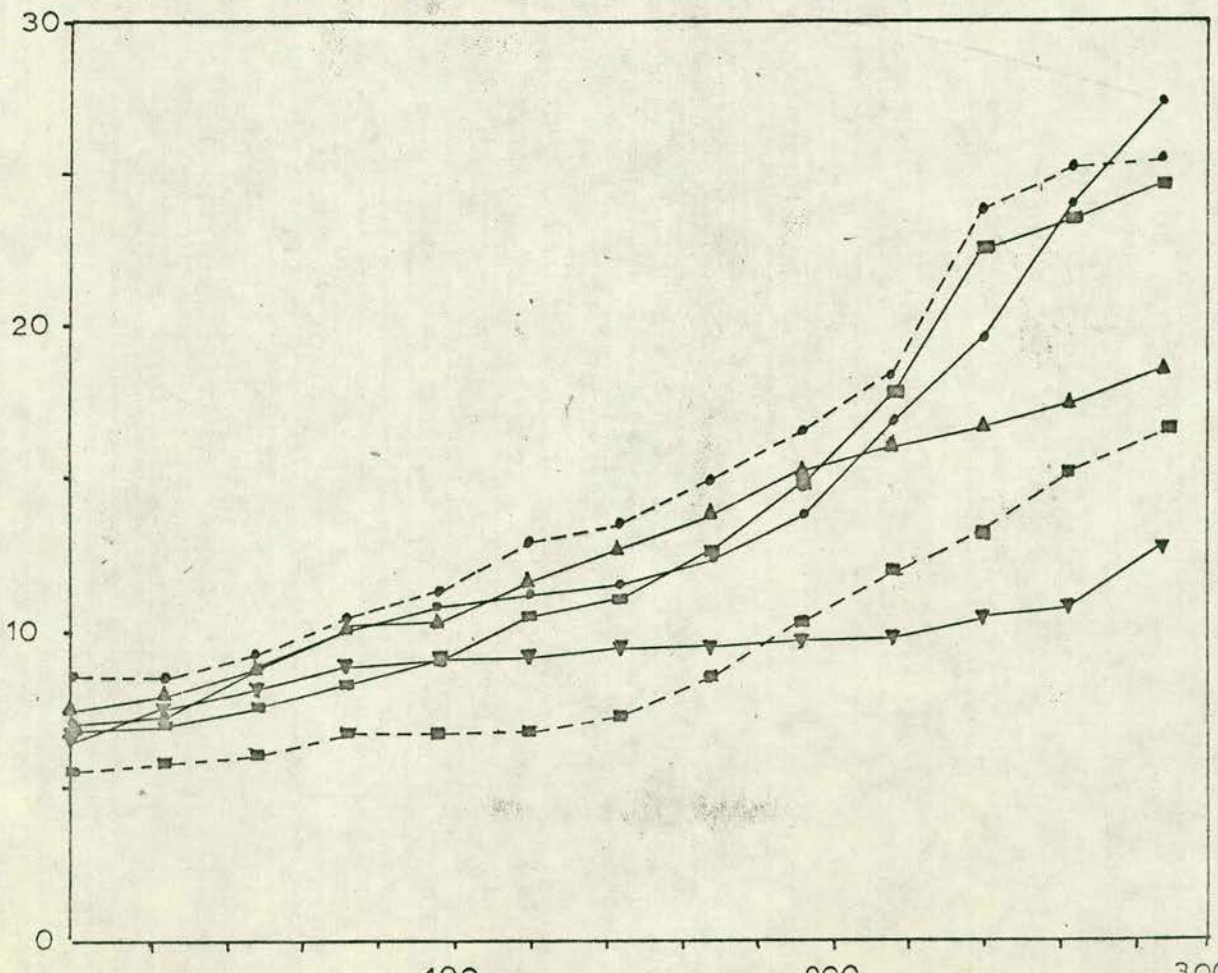


Fig. 5.22

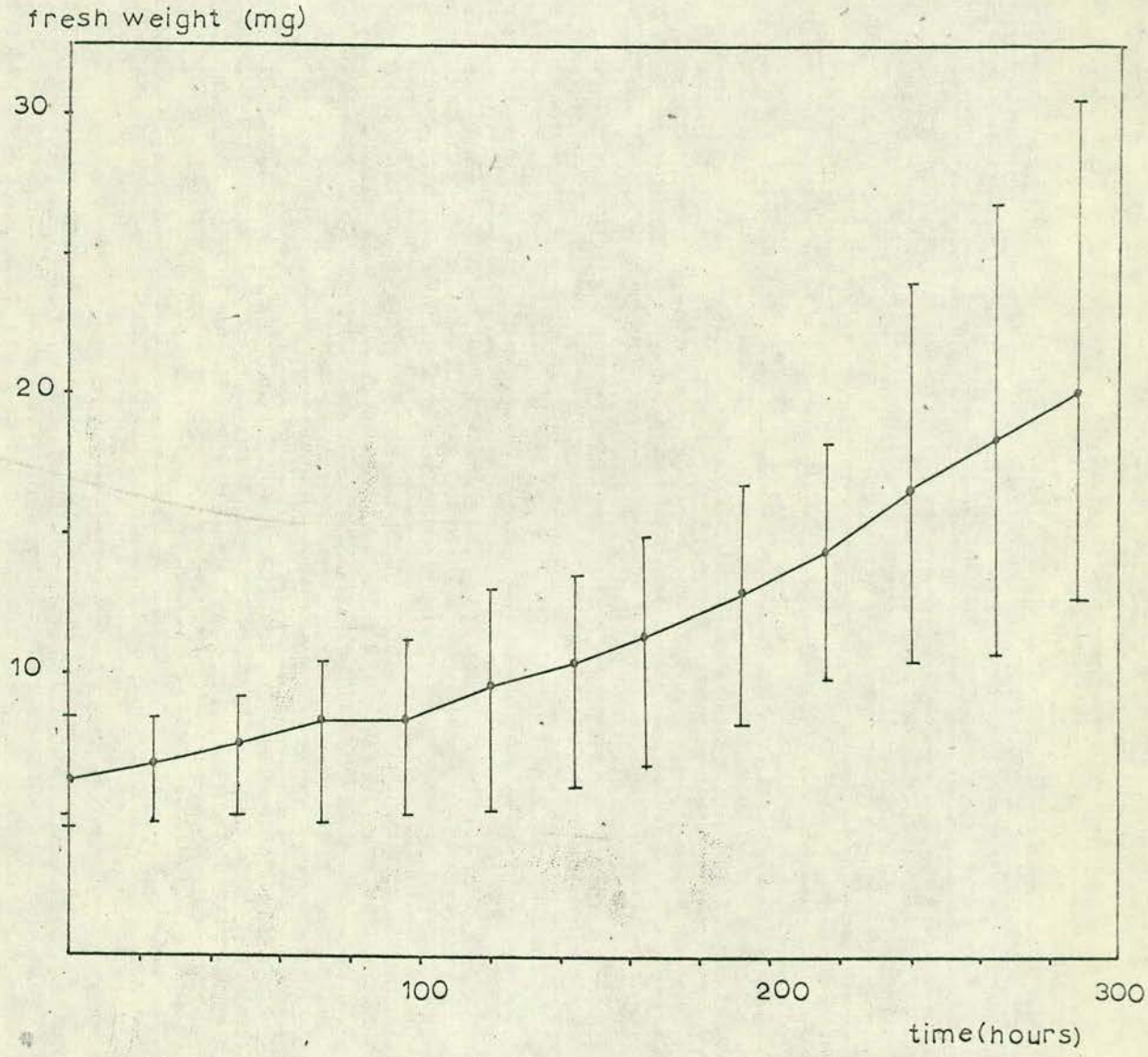

fresh weight (mg)
30
20
10
100
200
300
time(hours)
mean of population
range " "

Fig. 5.23

5.22 because the number of samples is too great to be shown clearly in one figure. Figure 5.23 shows the mean values and range of measurements in the total population. Figs 5.21 and 5.22 show that in all except one example the fresh weight of these embryos, under callus-inducing conditions increases between the start and 24 hours of incubation. After 24 hours this increase is maintained, until the end of the experiment, but the increase cannot be distinguished as either linear or exponential. Fig 5.23 shows that the range of values is low initially but increases with the incubation period after between 72 and 96 hours of incubation. The mean values suggest that the initial increase in fresh weight, between excision and 72 hours is linear and subsequent to a 'pause' between 72 and 96 hours there is a second linear increase followed by another higher rate of increase starting around 216 hours of incubation.

D. A time course experiment

This experiment was designed to be equivalent to that described in the previous section on embryo growth, so that a direct comparison of the growth of a germinating excised embryo could be made with the growth of an excised embryo under callus-inducing conditions.

Embryos were excised from batch number 2/5523 x 2/8607 and weighed before the pre-treatment with hydrogen peroxide and inoculation into standard Murashige and Skoog callus medium containing 2,4-D. A sub-population of embryos was inoculated into Murashige and Skoog callus medium supplemented with ^{3}H-thymidine. The embryos were incubated under the standard conditions. Samples were taken, at excision and subsequently at 24 hour intervals. Each sample consisted of 11 embryos,

of these three were macerated in chromic acid for cell number determinations, two were fixed in 3 parts ethanol to 1 part acetic acid for microdensitometric analysis of the DNA content and three were fixed in FAA (formaldehyde, acetic acid and alcohol) before embedding in wax and staining with safranin and light green for anatomical investigations. Three embryos of the sub-population incubated in the medium containing 1μCi/ml of tritiated thymidine were sampled fixed in 3:1 alcohol acetic acid for Feulgen staining, squashed and dipped in photographic emulsion for autoradiographic determination of the numbers of nuclei synthesising DNA. All 11 embryos were weighed at the time of sampling to determine changes in fresh weight.

1) Changes in fresh weight

The changes in fresh weight in the population with time are shown in Fig 5.24 where the individual and mean values are plotted.

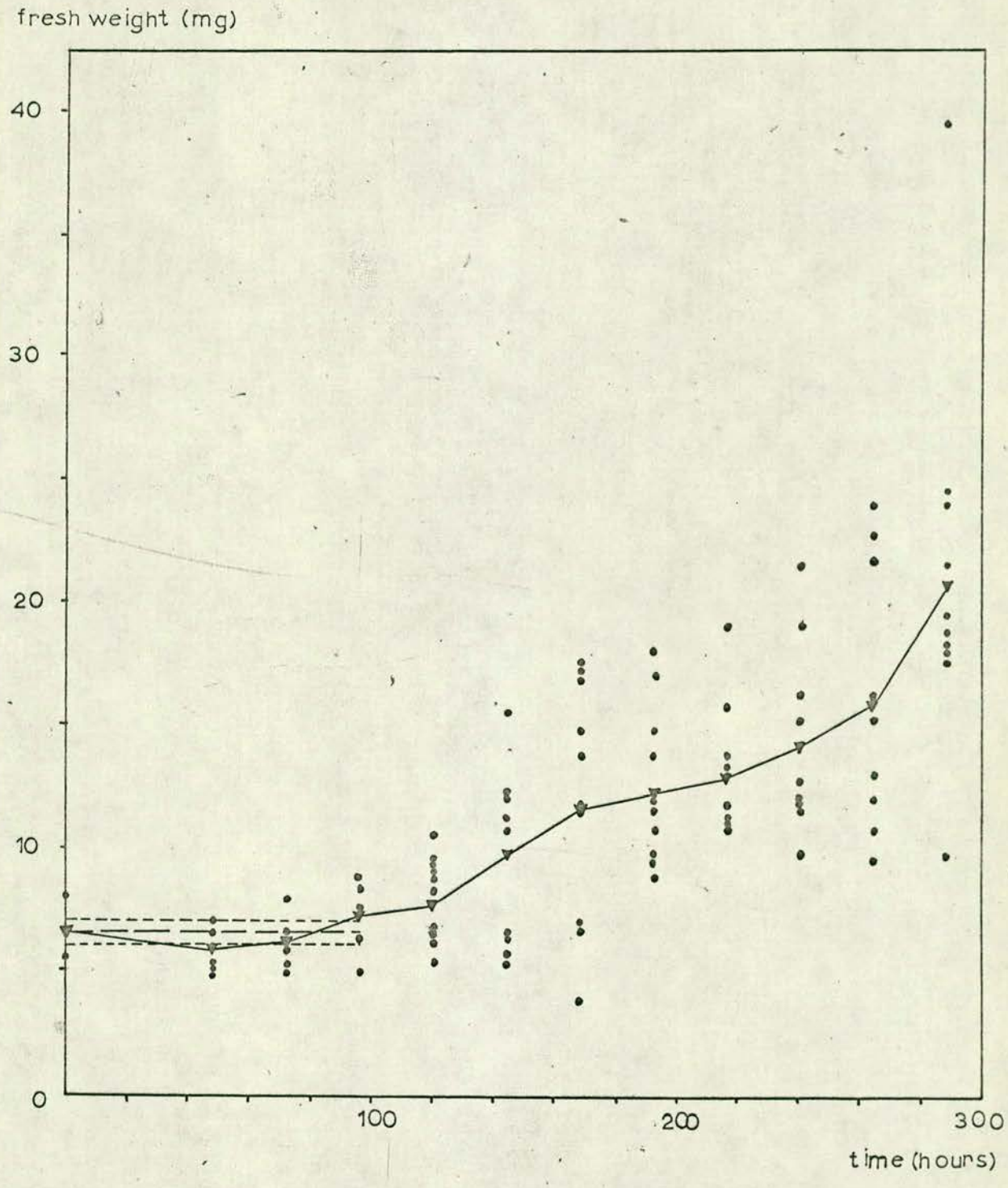
No statistically significant increase in fresh weight occurs between excision and 96 hours of incubation, as shown by the mean value and calculated standard error of this population. After 120 hours of incubation there is a rapid increase in fresh weight until 168 hours of incubation, followed by a slower increase between 168 and 264 hours. After 264 hours of incubation the rate of increase in fresh weight becomes more rapid.

The first doubling in fresh weight occurs after 200 hours of incubation, and the second doubling occurs by 310 hours, so the rate of increase in fresh weight is accelerating.

Fig 5.24

Changes in fresh weight in a population of excised embryos incubated under callus-inducing conditions.

Fig. 5.24



▼ mean of sample

— — mean of 0-96 hour population

--.--.-- S.E. " " " "

2) Changes in cell number

Cell number determinations were carried out to establish the time of onset of cell division and to determine whether there was any form of periodicity in the cell accumulation of embryos grown under callus-inducing conditions.

The calculated changes in cell number in the population are shown as mean values for each sample time in Fig 5.25. Apart from an increase, significant at the 95% level, of 40% between excision and 24 hours of incubation, there is no statistically significant increase in cell number until after 96 hours of incubation. After 96 hours of incubation the cell numbers increase linearly until the termination of the experiment at 288 hours.

A regression line was calculated for the actual values of cell numbers between 96 hours and 288 hours by the "least squares" method. This line has a regression coefficient of 0.75 showing that the cell number in this part of the curve can be considered to be undergoing a linear increase. The first cell number doubling occurs by 182 hours and the second, by extrapolation of the curve at 370 hours. This shows that the rate of cell division is constant over this period of culture.

3) Calculation of the value of mean fresh weight per cell

The value of the mean fresh weight per cell can be calculated from the combined fresh weight and cell number data and will give an indication of the changes in average cell size. These changes are shown in Fig 5.26.

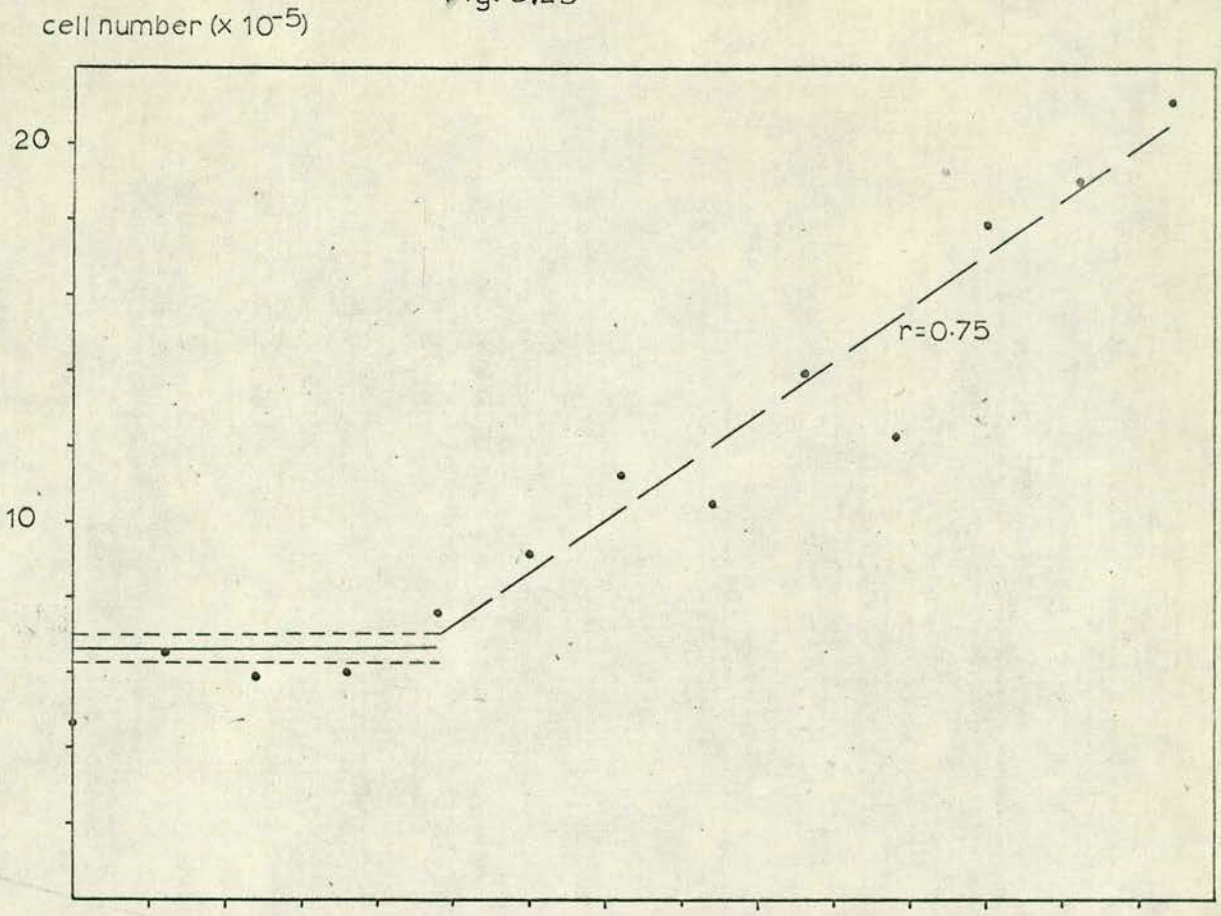
Fig 5.25

Changes in cell number in a population of embryos incubated under callus-inducing conditions.

Fig 5.27

Changes in the percentage of labelled nuclei in a population of embryos incubated under callus inducing conditions.

Fig. 5.25



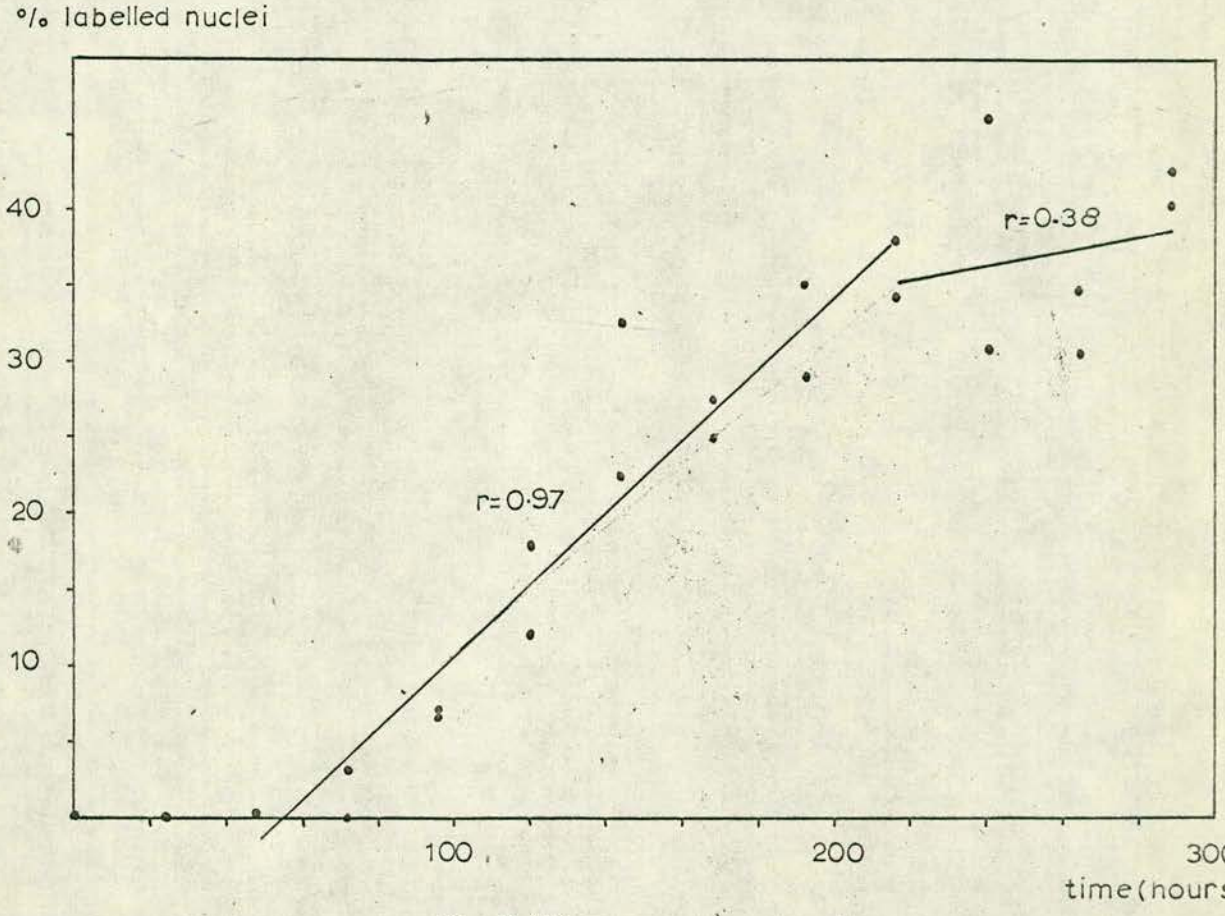
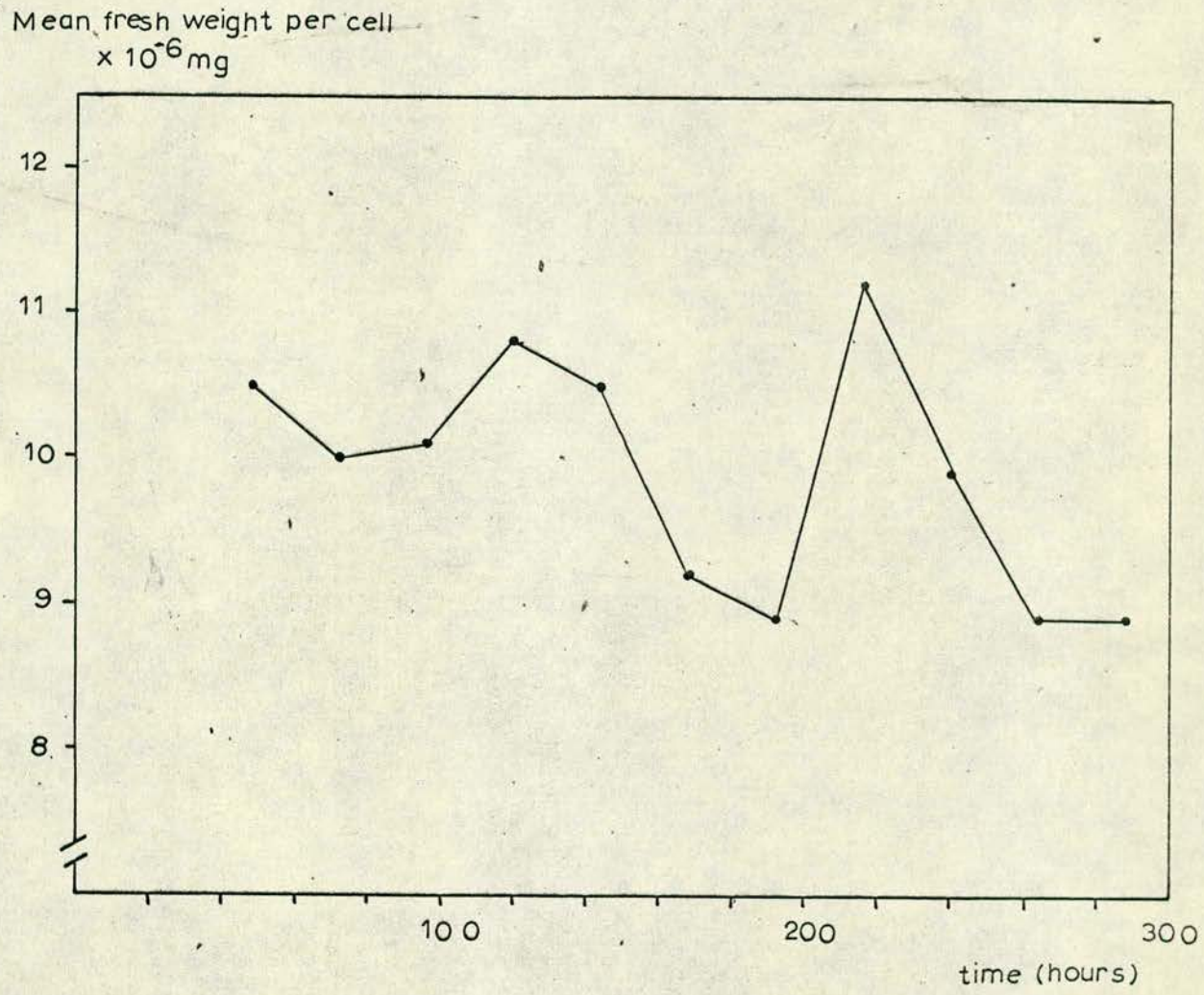


Fig. 5.27

Fig 5.26

Changes in the mean fresh weight per cell in a population of embryos under callus-inducing conditions.



It can be seen in Fig 5.26 that the mean fresh weight per cell does not alter much over the period of the experiment. It reaches a peak at 120 hours of 1.08×10^{-5} mg and of 1.12×10^{-5} mg at 216 hours. The first peak is 60 hours before the first cell number doubling time and the second peak is 16 hours after the first fresh weight doubling time.

The two small peaks in average cell weight suggest there may be some degree of periodicity in cell division but as the magnitude of the peaks is small this would indicate that the majority of cells are dividing asynchronously, with only a proportion of cells dividing with any degree of periodicity.

4) Changes in the percentage of nuclei labelled with tritiated thymidine

It has been suggested from the mean fresh weight per cell data that there may be some degree of periodicity in cell division in the excised embryo incubated under callus inducing conditions, and hence if changes in the percentage of labelled cells are to be followed the label must be supplied continuously in the medium.

All cells that are synthesising DNA, while in contact with tritiated thymidine, will incorporate the label into the newly synthesised DNA and thus it will be possible to distinguish, by autoradiographic techniques, all the nuclei which are synthesising DNA as they become labelled.

Changes in the percentage of nuclei labelled with ^{3}H-thymidine were established by counting labelled versus unlabelled nuclei along random horizontal transects of embryo squashes, and these changes are shown in Fig 5.27.

No labelling is detected until after 48 hours of incubation, and this amount is very low at 0.2%. The amount of labelling at 72 hours has increased to 0.7% and between 96 and 240 hours the percentage of labelled nuclei increases linearly, reaching a maximum value of 46% at 240 hours. A regression line was calculated for the values between 48 and 216 hours, using the least squares method and this line had a regression coefficient of 0.97 which shows a very high probability that the values lie on a straight line, which suggests that the increase in labelling is linear.

From 216 hours of incubation until the termination of the experiment the fitted straight line has a very low coefficient of regression which suggests that the linear increase in the percentage of labelled cells has stopped, and may perhaps plateau between 30 and 40%. From these changes in labelling content of the nuclei it can be established that G_1 extends from 0 to just before 48 hours of incubation and that S, G_2 and M extend from the end of G_1 until 240

hours of incubation.

5) Microdensitometric measurements of changes in the DNA content of nuclei in excised embryos grown under callus-inducing conditions.

Sections 2 and 3 of part D of this chapter have shown that cell division and nuclear replication occur when the excised embryo is incubated under callus-inducing conditions. In this section the pattern of DNA replication was investigated using Feulgen microdensitometry and probit analysis in an attempt to discover the nature of the cell populations participating in the formation of a callus.

The DNA contents of 200 nuclei, selected along random transects, were measured at excision and subsequently at 24 hour intervals throughout this time course experiment.

At excision (Fig 5.28) two populations of nuclei can be distinguished by probit analysis. The range of DNA measurements lies between 4 and 14 units. The mean and mode are both at 9 units. This distribution is very similar to all previous measurements of freshly excised embryos (see Figs 4.13, 4.40, 4.48 and 4.63), but the actual numerical values obtained in separate experiments are not directly comparable because the embryos were stained in different batches and measured at different times.

Figs 5.29 and 5.30 show duplicate measurements of nuclei in embryos incubated for 28 hours. Fig 5.29 has a distribution between 5 and 16 units, slightly larger than in Fig 5.28, with the mean at 10 units and the mode between 10 and 12 units. Probit analysis shows that the population with a high DNA content has decreased in size to become a small group of

Figs 5.28 to 5.47

Changes in DNA content of nuclei in embryos incubated under callus-inducing conditions.

Abscissa: DNA content (arbitrary units)

Ordinate: Right: probit value

Left: numbers of nuclei per class interval.

 prophase and metaphase figures

 anaphase and telophase figures.

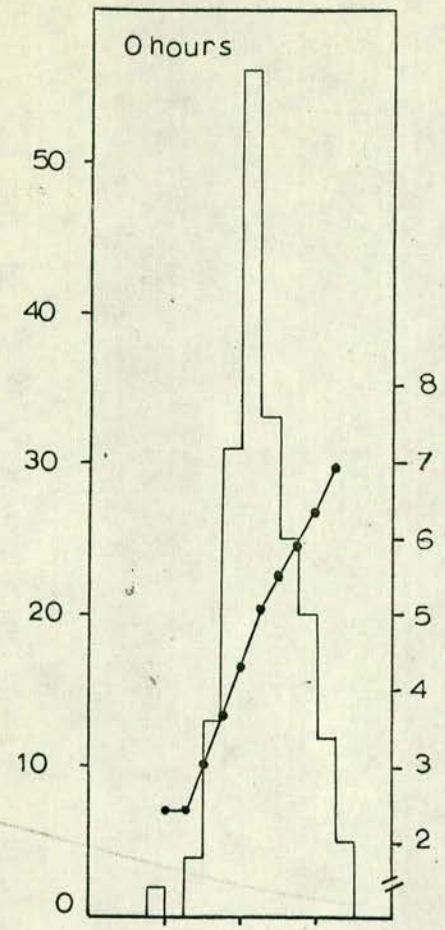


Fig. 5.28

Fig. 5.29

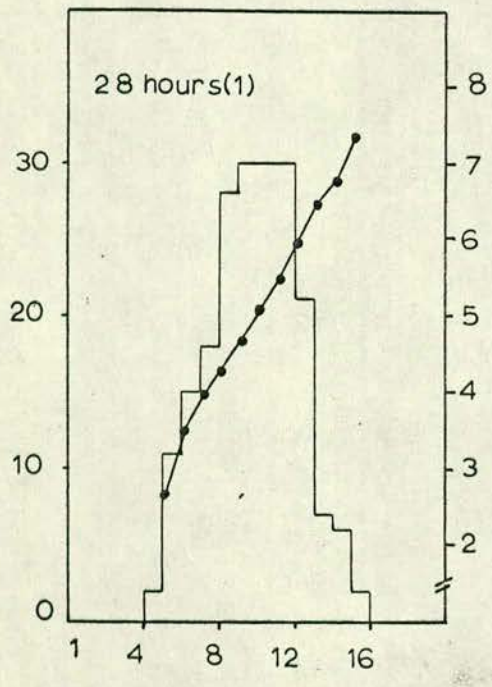
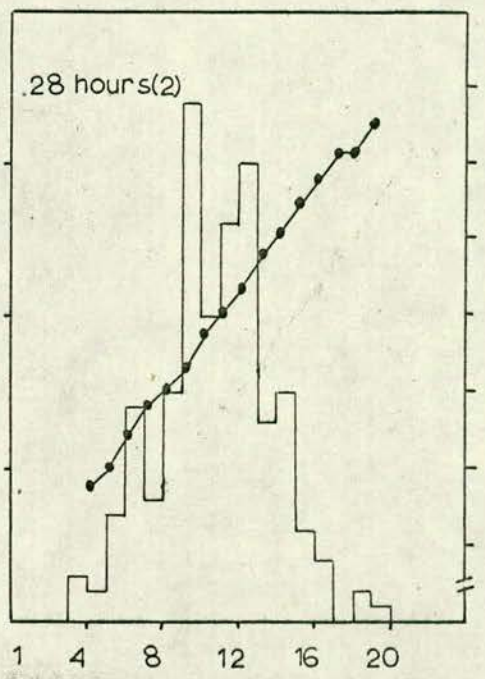


Fig. 5.30



nuclei around 13, 14, 15 and 16 units. After 28 hours of incubation the mode of the population has increased to a value above that found in the freshly excised embryo, but there is an increase in the proportion of low DNA content nuclei, suggesting nuclear division and cytokinesis. This is supported by the observation that a statistically significant increase in cell numbers occurs during the first 24 hours of incubation.

Fig 5.30 shows a much wider distribution of values than the duplicate sample, ranging from 4 to 20 units with the mode at 10 units and the mean at 11 units. Probit analysis distinguishes 4 major and 4 minor populations within the overall distribution, the modes of the major peaks being around 7, 10, 13 and 15 units. This suggests that the embryo has begun to develop before the start of the experiment, that is, prior to excision. This embryo may be similar to that described in Fig 4.55.

After 48 hours of incubation (Fig 5.31) the range of DNA values lies between 5 and 14 units with the mean at 9 units and the mode at 10 units, showing that a greater proportion of the nuclei now have a lower DNA content than at excision or after 28 hours of incubation. Probit analysis distinguishes populations between 5 and 9, 9 and 11 and 11 and 14 units. Fig 5.32 also shows the distribution of nuclear DNA content of an embryo incubated for 48 hours under callus-inducing conditions. The range of values is between 3 and 16 units with the mean at 9 units and the mode at 11 units. This shows that, as in Fig 5.31, there is a higher proportion of nuclei with a lower DNA content. Probit analysis distinguishes 3 major populations between 4 and 6, 8 and 10 and 10 and 14 units and 2 minor populations around 3 and 16 units.

Fig. 5.31

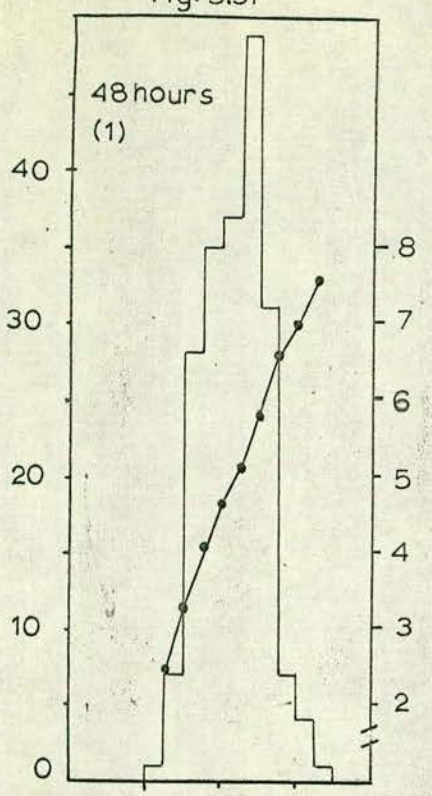


Fig. 5.32

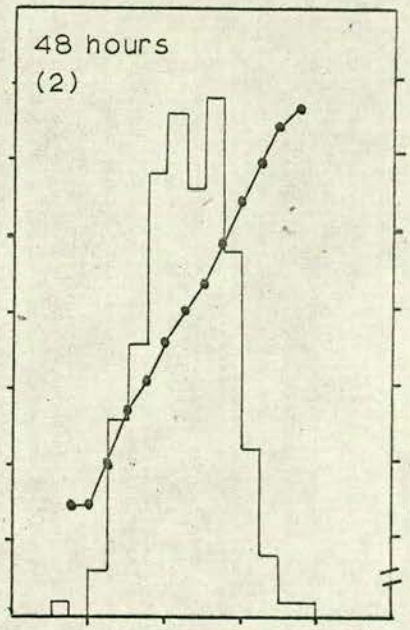


Fig. 5.33

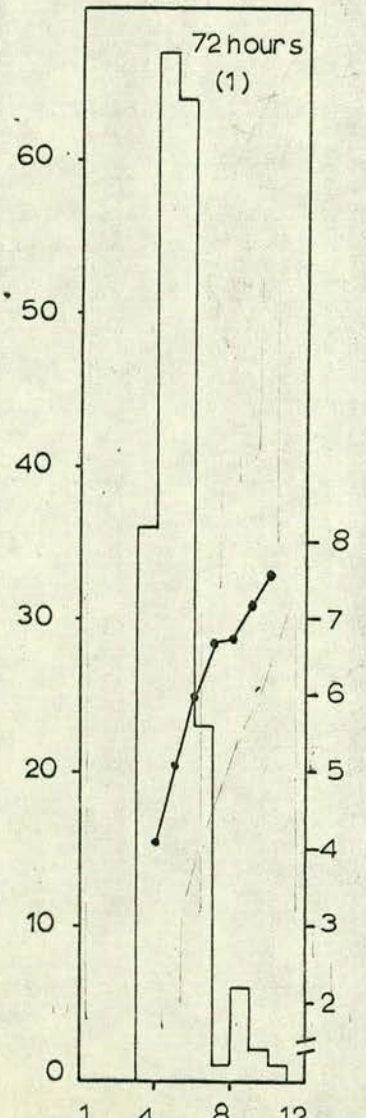
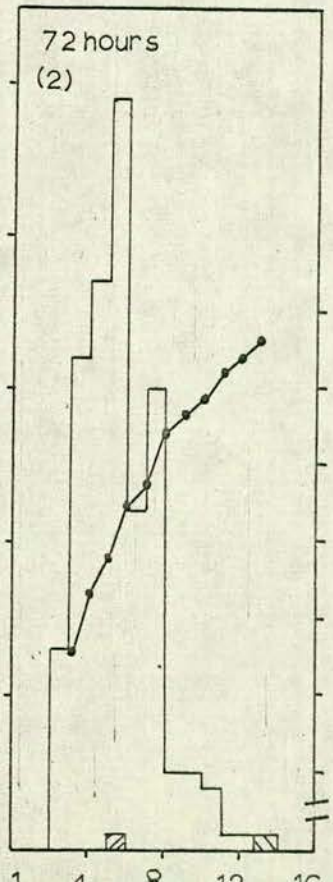


Fig. 5.34



The small populations of high DNA content nuclei in Figs 5.31 and 5.32 suggest that DNA synthesis is taking place. This is confirmed by the presence of labelled nuclei in embryos incubated for 48 hours in a medium containing tritiated thymidine. Examination of Figs 5.31 and 5.32 in direct comparison with Fig 5.29 suggest a greater proportion of low DNA content nuclei present after 48 hours of incubation. This trend is continued in the 72 hour samples of Fig 5.33 and 5.34. Fig 5.33 shows a sample after 72 hours of incubation with two distinct populations of nuclei the larger with a mode of 5 and the smaller with a mode of 9. The overall mode and mean are both 5 units and the overall range is between 4 and 11 units. The presence of such a distinct peak around 5 units, and the smaller peak, constituting less than 5% of the population, suggests that nuclear division has taken place. This is confirmed by the presence of mitotic figures at 6 and 14 units in Fig 5.34. However, it is to be noted that there is no significant increase in cell number until 96 hours. Fig 5.34 has a slightly wider range than Fig 5.33, between 3 and 14 units and the mean and mode are both at 6 units. Two major and 4 minor populations may be distinguished by probit analysis, the main peaks being at 6 and 8 units. Fig 5.35 shows that after 120 hours of incubation the range of values increases to between 5 and 19 units and the mean and mode also increase to 8 units. Fig 5.36 shows that the range of values in the duplicate 120 hour sample also increases to between 2 and 25 units, the mean to 9 units and the mode to 10 units. Probit analysis, of both sets of results, shows that the number of populations has increased, especially those with a higher DNA content. The presence of mitotic figures at 14, 16 and 18 and 8, 9, 10, 13, 19, 20 and 25 units shows that nuclear division is taking place. Cytokinesis must also be occurring as cell numbers are also increasing. There are also nuclei with a high DNA

Fig. 5.35

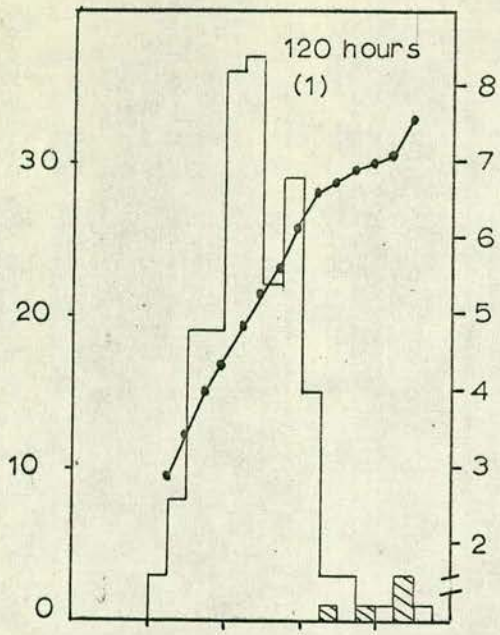
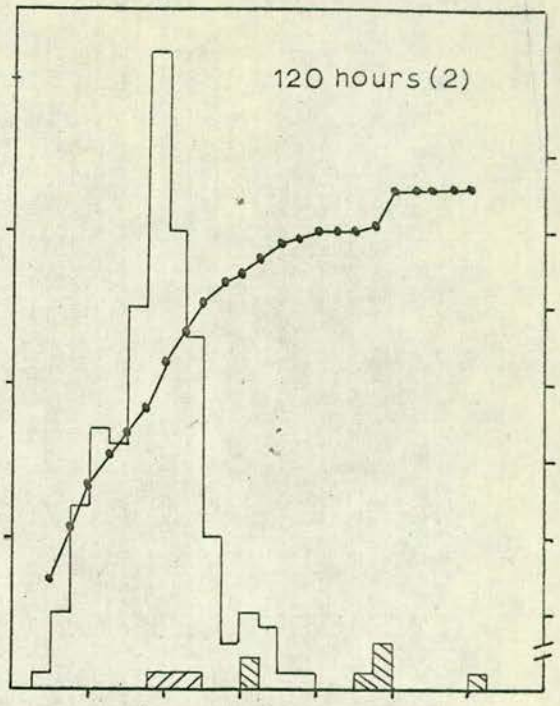


Fig. 5.36



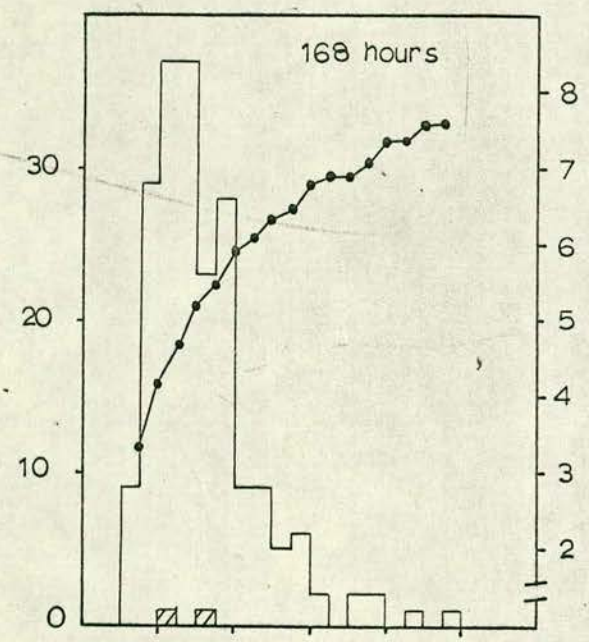


Fig. 5.37

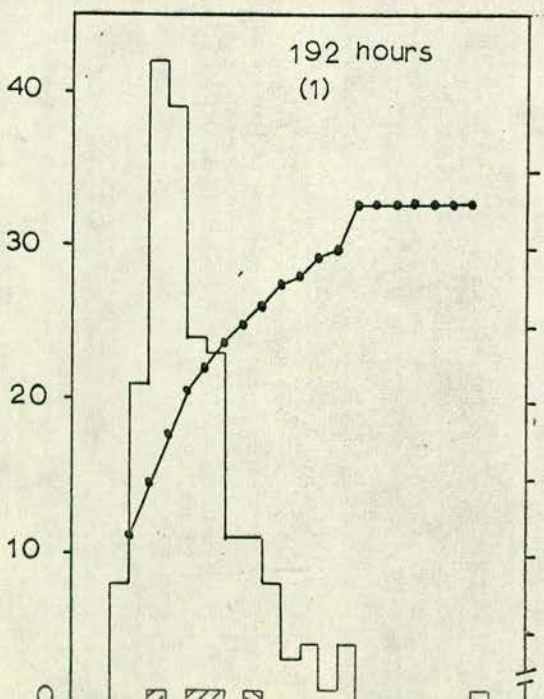


Fig.5.38

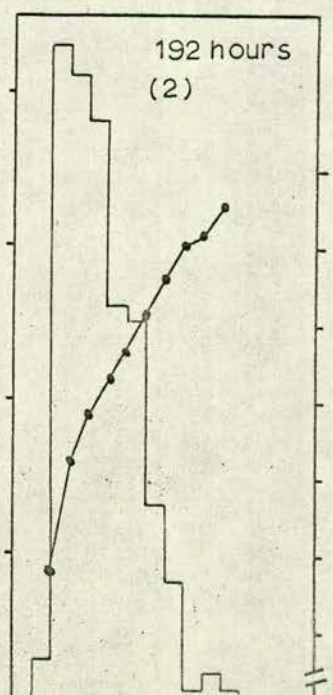


Fig. 539

content confirming the labelling experiments which show that DNA synthesis is occurring.

Fig 5.37 shows a sample after 168 hours of incubation. The range of values lies between 3 and 20 units with the mean at 6 units and the mode at 5 units. This shows that the population is skewed with a higher proportion of nuclei with low DNA content. Probit analysis distinguishes a large number of sub-populations, especially those with higher DNA values which suggests that DNA synthesis has taken place. Two anaphase values are shown at 5 and 7 units, which taken in conjunction with the probit analysis suggest that nuclear division is taking place.

After 192 hours of incubation (Fig 5.38) the mean and mode are the same as the 168 hour sample at 6 and 5 units respectively and the range has increased slightly to between 3 and 22 units. Probit analysis shows that the population is slightly more uniform than at 168 hours, the higher values being less spread out. Anaphase configurations are found at 5, 7 and 8 units together with a metaphase configuration at 10 units. Fig 5.39 shows a duplicate sample of 192 hours of incubation. This sample is much more uniform with a range between 2 and 12 units, the mean at 5 units and the mode at 3 units. The lack of mitotic figures, and the uniformity of the sample, in comparison with other figures, suggests that this embryo is not typical of this time interval.

Figure 5.40 shows the DNA content of an embryo incubated for 216 hours under callus inducing conditions. The range of values is between 3 and 25 units with the mean and mode both at 9 units.

Fig 5 40

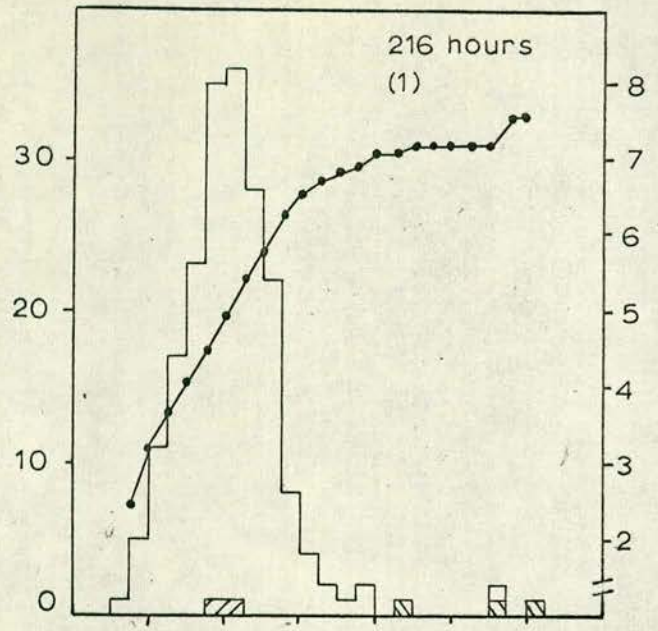


Fig. 5.41

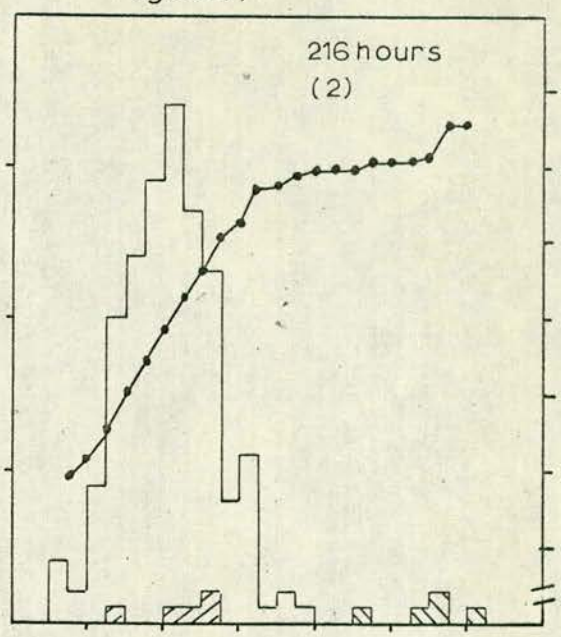


Fig. 5.42

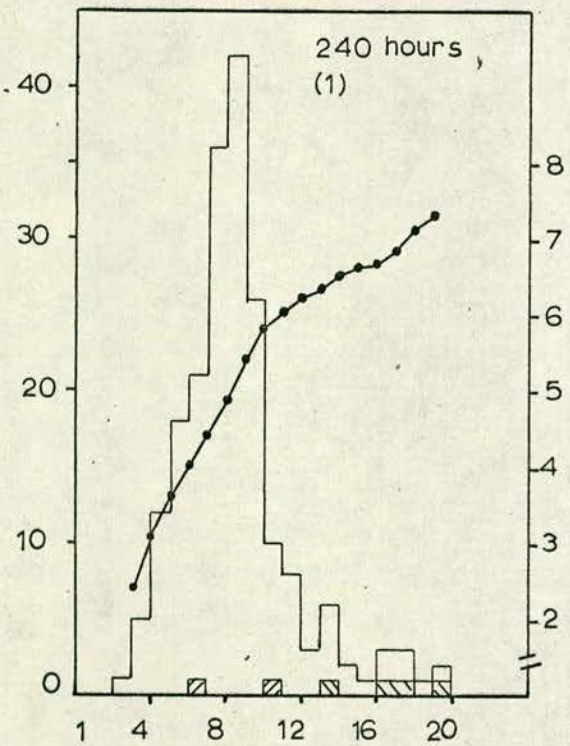
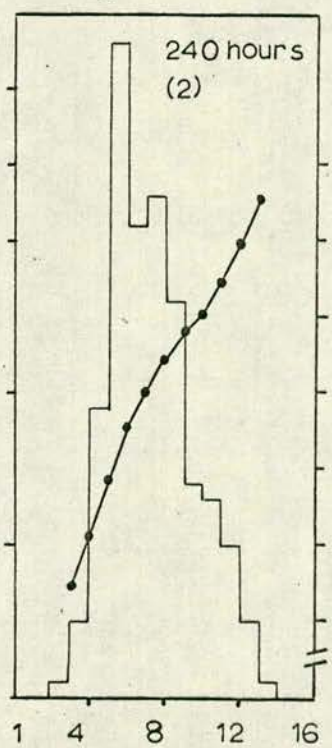


Fig. 5.43



Probit analysis distinguishes a main population between 4 and 11 units and then a number of small populations, a proportion of which are individual values. Anaphase configurations are found at 8 and 9 units, a prophase figure at 18 units and metaphase figures at 23 and 25 units. These figures show that nuclear division is taking place but it may be that at this stage DNA synthesis is occurring at a greater rate than nuclear division, and it is because of this that the mean and mode have increased above the 192 hour values. Fig 5.41 shows a duplicate 216 hour sample that is very similar to Fig 5.40. The range is also between 3 and 25 units, with the mean and mode at 9 units. Probit analysis distinguishes a main population between 5 and 11 units with a number of smaller ones, again where a proportion are individual values. The distribution of mitotic figures is slightly different. There is an anaphase configuration at 9 units, telophase values at 6, 10 and 11 units and prophase figures at 19, 22, 23 and 25 units.

Fig 5.42 shows a sample taken after 240 hours of incubation. The range of values extends from 3 to 20 units, which is less than the 216 hour samples, but the mean and mode both remain at 9 units. Probit analysis distinguishes 3 major populations with means around 6, 9 and 11 units and 6 other minor populations. Prophase figures are found at 14, 17 and 20 units and single metaphase, anaphase and telophase figures at 18, 7 and 11 units respectively. Fig 5.43 shows a duplicate sample taken after 240 hours of incubation. The range of values is reduced to between 3 and 14 units and the mean and mode are reduced to 7 and 6 units respectively. Probit analysis distinguishes 3 major populations with mean values around 4 to 5, 7 and 12 units with 2 minor

populations between 8 and 10 units. No mitotic figures were found in this sample and this, taken with the low amount of variation in the distribution in comparison with Figs 5.40, 5.41 and 5.42, suggests that the stage of development of this sample may be much earlier than the other examples.

Fig 5.44 shows the distribution of DNA values in nuclei after 264 hours of incubation. The range of values extends between 3 and 17 units, the mean is at 8 units and the mode at 7 units. Probit analysis distinguishes 4 major populations with means around 4 to 5, 7, 12 and 15 units, with 3 minor populations between 8 and 11 units. Prophase configurations are found at 13 and 17 units, with a metaphase figure at 16 units. Fig 5.45 shows a duplicate 264 hour sample to that in Fig 5.44. The range extends to between 3 and 16 units with the mean and mode at 7 and 6 units respectively. Four major populations may be distinguished by probit analysis with mean values around 4, 8, 10 to 11 and 13 units with 3 minor populations interspersed. A prophase configuration is present at 16 units and metaphase figures are found at 15 and 16 units.

Fig 5.46 shows the distribution of DNA values in an embryo incubated for 288 hours. The range of values is between 4 and 19 units, the mean at 8 units and the mode at 6 units, showing that there is a large proportion of low DNA content nuclei in this sample. Probit analysis distinguishes 3 major populations with mean values around 5, 7 to 8 and 16 units, with 4 minor populations between 9 and 15 units. Prophase figures are found at 14, 15 and 18 units, metaphase figures at 17 and 19 units and two anaphase figures at 8 and 12 units. The

Fig. 5.44

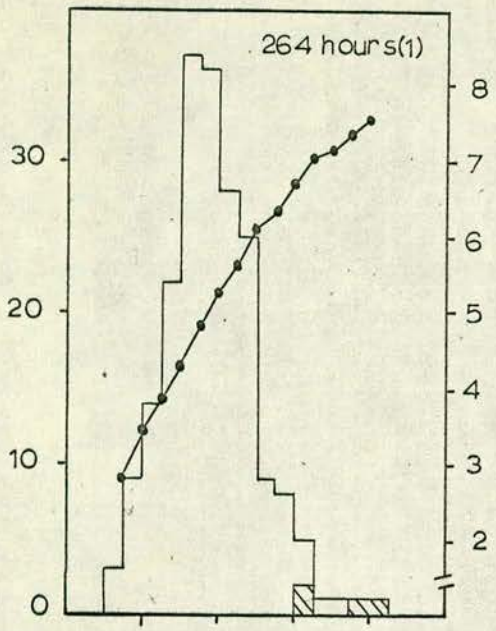


Fig. 5.45

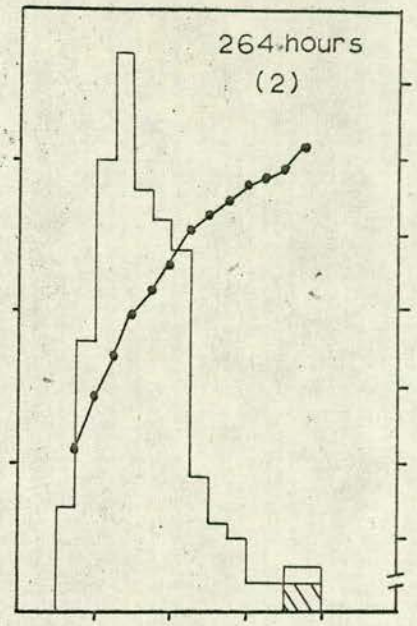


Fig. 5.46

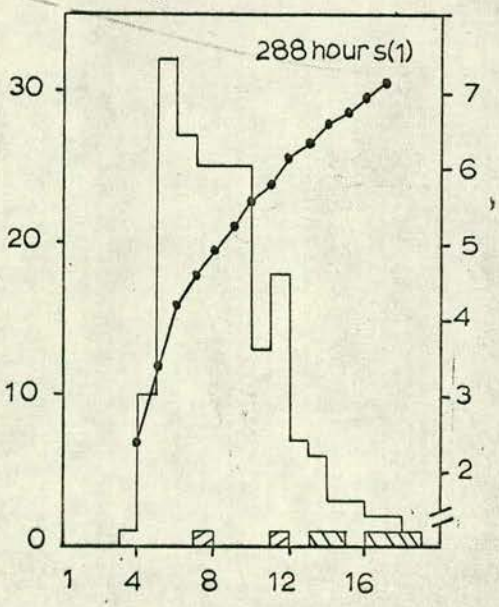
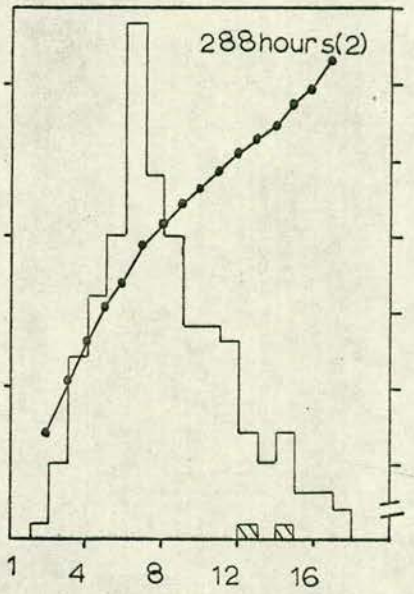


Fig.5.47



duplicate 288 hour sample (Fig 5.47) has a range between 2 and 18 units with the mean and mode values at 9 and 7 units respectively. Probit analysis distinguishes 2 major populations with mean values of around 3 to 4 units and 11 units. A number of minor populations can be distinguished. A prophase figure is found at 13 units and a metaphase configuration at 15 units.

Table 5.1 summarises the data obtained by microdensitometric analysis of duplicate samples. It shows how similar the majority of measured samples are, with the notable exceptions of the 28, 192 and 240 hour samples, with respect to the mean and mode values, the number of major populations distinguished by probit analysis, and the agreement of measured values of mitotic figures.

The exceptions of the second 28 hour sample and the second 192 and 240 hour samples could be explained by the first having undergone precocious development and the last two being delayed.

E. Summary

The morphology and anatomy of excised embryos change dramatically when they are incubated under callus inducing conditions, which includes a pre-treatment in hydrogen peroxide.

The embryos swell and the increase in volume is associated with internal splitting of the tissue so that the embryos become less dense and thus more buoyant. Greening of the tissue, which is variable in occurrence takes place by 144 hours of incubation and is followed by browning in some embryos. The production of callus cells is noticeable

time (hours)	MEASURED DNA VALUES							probit populations	
	population mode	population mean	% observed mitotic figures	prophase	metaphase	anaphase	telophase	major	minor
[1] 0_1	9	9·71	–					2	2
28_1	10–12	10·30	–					2	3
28_2	10	11·26	–					4	4
48_1	10	9·19	–					3	1
[2] 48_2	11	9·61	–					3	2
72_1	5	5·59	–					2	0
72_2	6	6·09	1·0	14		6		2	4
[3] 120_1	8	8·30	2·0	14,16,18	18			4	7
120_2	10	9·98	5·0	13,19,20,25	13, 20, 20	8,9,10		4	3
168	5–6	6·80	1·0			5, 7		1	11
192_1	5	6·93	2·0		10	5, 7, 8		3	3
192_2	3	5·15	–					2	4
216_1	9	9·12	2·5	18	23, 25	8, 9		2	4
216_2	9	9·13	5·0	19,22,23,23,25		9	6,10,11,11	2	8
240_1	9	9·27	3·0	14, 17, 20	18	7	11	3	6
240_2	6	7·80	–					3	2
264_1	7	8·04	2·0	13,13,17	16			4	3
264_2	6	7·24	2·0	16	15,15, 16			4	3
288_1	6	8·93	3·5	14,15,18	17, 19	8, 12		3	4
288_2	7	9·03	1·0	13	15			2	5

[1] 1st cell no. increase 0-24 hours
[3] 2nd " " " 120 hours
[2] 1st labelling detected

Table. 5.1 Summary of DNA data

by 144 hours, both large and very small cells are visible underneath the epidermis. The epidermis remains similar to that in the tigellum of the germinating embryo, although no increase in surface area occurs in the haustorium.

A number of embryos that were weighed, aseptically, at 24 hour intervals increased in fresh weight in two phases, both of which were linear, separated by a period between 72 and 96 hours where no increase occurred. Variability within the population increases with time.

A time course experiment carried out with a large population of embryos showed that little increase in fresh weight occurred between excision and 96 hours of incubation. Subsequent to this the fresh weight increases rapidly until the termination of the experiment, except for a slight slowing down in the rate between 168 and 240 hours. Fresh weight doubling occurred after 200 hours of incubation, and subsequently the rate of increase accelerated.

Cell numbers increased by 40% during the period between excision and 24 hours. No further increase occurred until 96 hours of incubation and then the increase in cell number was linear until the end of the experiment. The cell number doubling time from the mean level between 24 and 96 hours is 182 hours.

The mean fresh weight per cell of callusing embryos changes little over the experimental period, varying from 0.89 to 1.12 x 10^{-5}mg, but there are two small peaks at 120 and 216 hours indicating that there may be some degree of periodicity in cell division.

No incorporation of thymidine was detected in embryos incubated with tritiated thymidine until 48 hours of incubation, thus G_1 extends from 0 to 48 hours. After 72 hours of incubation the increase in mean percentage labelling at each time interval rose linearly between 7.6 and 40%. This shows that S, G_2 and M extend from 48 to 240 hours. The highest percentage labelling reached is 46%. The level of labelling, after 240 hours, remained steady, which indicates that G_1 is reached at 240 hours.

Microdensitometric measurements of nuclear DNA contents showed that the individual callusing embryos are not as variable in DNA content as germinating embryos.

In general the embryos incubated under callus inducing conditions have markedly different growth patterns to embryos that are undergoing normal development, and are also much more uniform in their growth responses.

CHAPTER VI

Preliminary studies on the morphology and cytological characteristics of established callus lines

Callus may be induced with remarkable consistency from excised embryos treated briefly with hydrogen peroxide and grown subsequently in a nutrient medium with the addition of 2,4-D. In the previous chapter a study was made of some of the changes which accompany the induction of callus, with particular reference to the overall growth and cytological characteristics of the constituent cells of the embryo. Lack of time has prevented the establishment and characterisation of callus lines from these excised embryos and such an investigation must wait. However, it was decided that a preliminary study on the composition of callus established at Colworth House would produce a logical extension of this present study, and perhaps permit a wider discussion of the results already accumulated. Accordingly two callus lines were investigated. These were supplied by Colworth House laboratories and are either 'rapid' in their growth, with a cell number doubling time of about 20 days or slow, termed 'normal', in their growth rate with a cell number doubling time of about 6 to 8 weeks.

The limitations and difficulties of such a comparative approach are clear and must be taken into consideration when examining the results. However it should be possible to determine the fundamental differences between two quite different and established stable callus lines and to discover why one is slow growing while the other is fast.

The cultures supplied by Colworth House are identified by code numbers and letters which relate to the original callus explant and the number of subsequent sub-cultures. This method of labelling cultures is comprehensive but complex and does not lend itself to the rapid identification of callus type. Thus, throughout this chapter simpler labels have been employed, which indicate whether the callus is rapid (R) or normal (N) in growth form. Table 6.1 shows the Colworth House labelling together with the labelling used in this thesis.

A. External appearance of the callus cultures

The cultures vary in external appearance, depending on the constituents of the medium in which they have been cultured. All samples were taken from cultures growing on a medium solidified with agar, although some had been cultured in a liquid medium in Steward flasks prior to sub-culture onto solid medium.

The cultures N2 and N3 were grown on Murashige and Skoog's medium containing 3mg/1 2,4-D (Fig 6.1 and 6.2). They are fairly friable, have a moist surface, and bear dark brown patches.

Figure 6.3 shows culture R1, growing on Murashige and Skoog's medium containing 3mg/1 2,4-D. The callus is creamy white in colour and is very moist and soft probably because it was recently transferred from liquid medium in Steward flasks where it had been maintained for 18 months. However, this callus is probably heterogenous, as portions removed from it (Fig 6.4) have either retained the very moist appearance of the parental callus, or have become drier and more friable with an associated browning of the tissue, probably due

Table 6.1 - Simplification of callus code numbers

Colworth House Code	Simplified Code	Growth form
P114L18	N1	Normal
P114M11	N2	Normal
P114M31	N3	Normal
T003A26	R1	Rapid
T003A27	R1a	Rapid
P182A49	R2	Rapid
P182A65	R3	Rapid
P171D67A	R4	Rapid
P171F97D-A	R5	Rapid

Fig. 6.1

Fig. 6.2

Figs. 6.1-6.7 Established callus cultures

Fig. 6.3

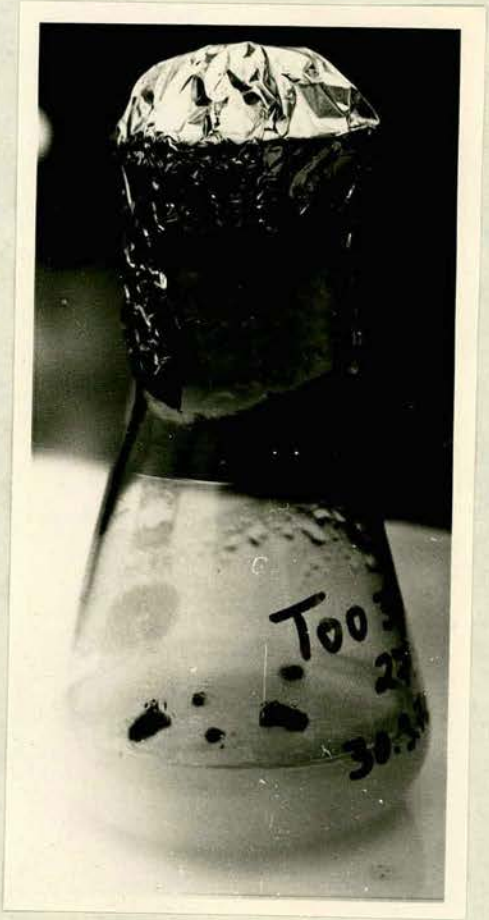


Fig. 6.4

Fig. 6.5

Fig. 6.6

Fig. 6.7

to the formation of polyphenolic compounds.

R3 (Fig 6.5) is a friable tissue when cultured in the presence of NAA, pale cream in colour and there appears to be no variation in the sub-cultures. R4 (Fig 6.6) is a very friable culture, which has a lot of brown colouration. R5 (Fig 6.7) grown on Nitchs' medium containing NAA is a sub-culture of R4 which readily differentiates meristematic regions and subsequently roots and shoots.

Overall the callus is very friable and many parts of it are very brown, but the meristematic regions show up as white shiny hemispherical areas at intervals over the surface of the tissue.

It would appear that cultures grown on media containing NAA are much more friable and drier than cultures grown on media containing 2,4-D.

B. Anatomy of the callus cultures

The marked morphological differences observed between the cultures presumably reflect basic anatomical differences. Pieces of callus were fixed, embedded in wax, sectioned at 10µm and stained with safranin and light green for examination under the light miscroscope. A problem encountered during the embedding process was the loss of cells from the surface particularly from the more moist cultures such as R1a. It was impossible to prevent the loss of cells and so it must be noted that there is a disproportionate amount of firmer tissue in these sections.

Fig 6.8 shows callus N2 to be disorganised with a lot of air

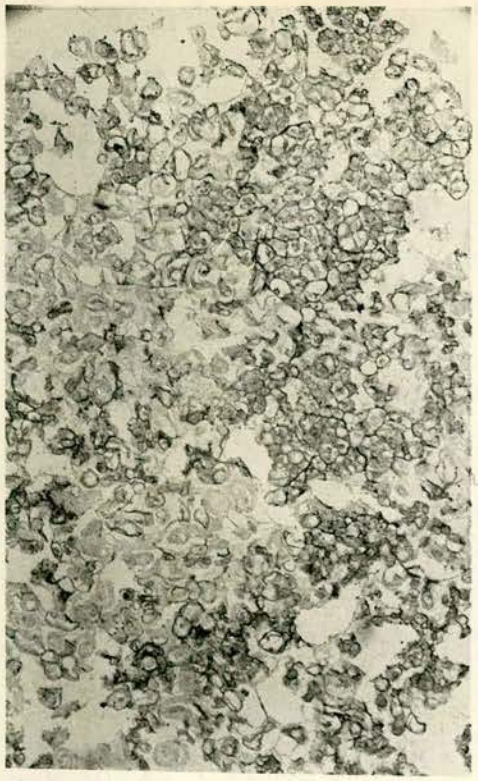


Fig. 6.8 P114M11 N2 (x100mag)

Fig. 6.9 TOO 3A27 R1a (x100mag)

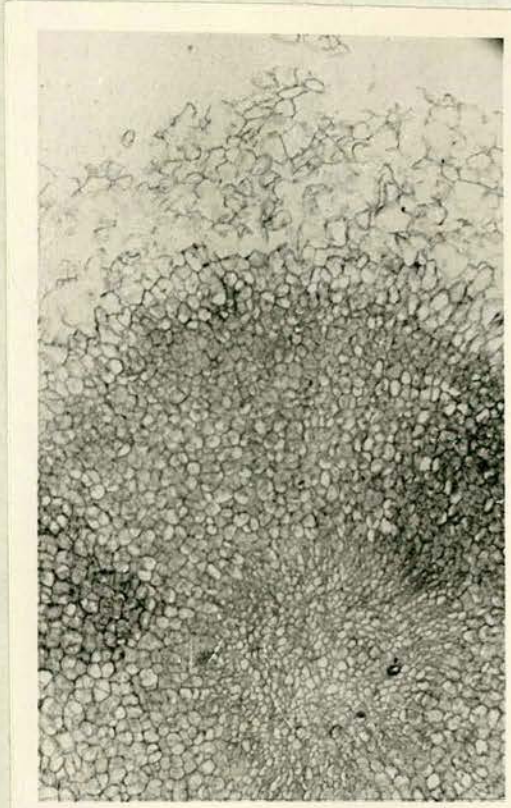


Fig. 6.10 TOO 3A27 R1a (x100mag)

Fig. 6.11 P182A49 R2 (x100mag)

spaces within the tissue, most of which is composed of quite large cells. There are also two areas where cell division appears to be occurring in a disorganised manner. There is no recognisable organisation within the tissue. There is also no clear tissue organisation shown in Fig 6.9 which is part of the callus R1a. The cells in this tissue are not large and generally form only small aggregates, and many of the cells appear to be non-viable. This section was taken through an area of very moist tissue and it must be remembered that for 18 months prior to this sub-culture onto a medium solidified with agar, it had been maintained in liquid culture. Fig 6.10 shows a section through the same callus (R1a) but in an area where organised cell division has taken place within a surrounding tissue of disorganised and, in many cases, apparently non-viable cells.

This piece of the callus appears to contain established regions of fairly organised cell division which are connected only by a matrix of non-dividing cells. Fig 6.11 is very similar to Fig 6.9 although it shows that callus R2 has some cells which are smaller and are found in slightly larger aggregates. There are larger air spaces in this tissue, than in R1a and there is also a proportion of non-viable cells.

Fig 6.12 shows a section through callus R4 where there is an organised area of small rapidly dividing cells concentric with larger dividing cells, surrounded by a matrix of a loose, disorganised cell arrangement. The whole culture consists of these organised areas connected by the larger, also viable, non-organised cells. Many of the cells appear to have become organised into vascular bundles.

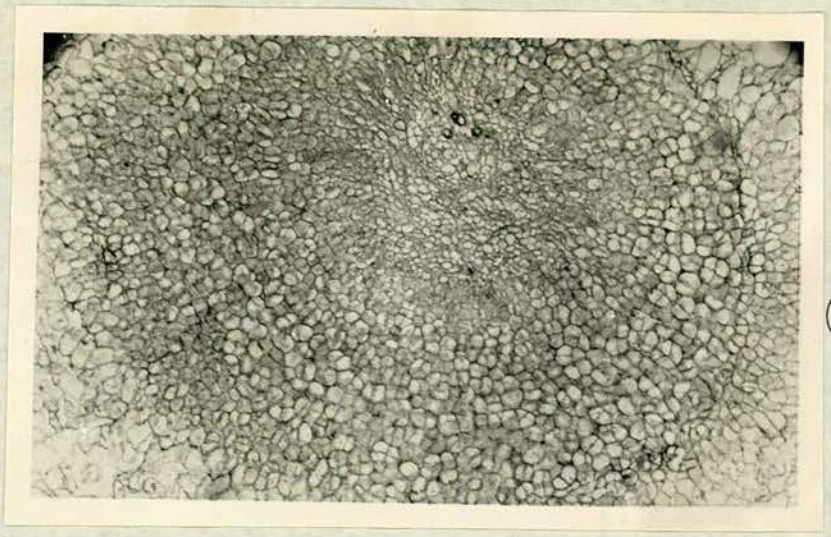


Fig. 6.12
P171D67A
R4
(x100mag.)

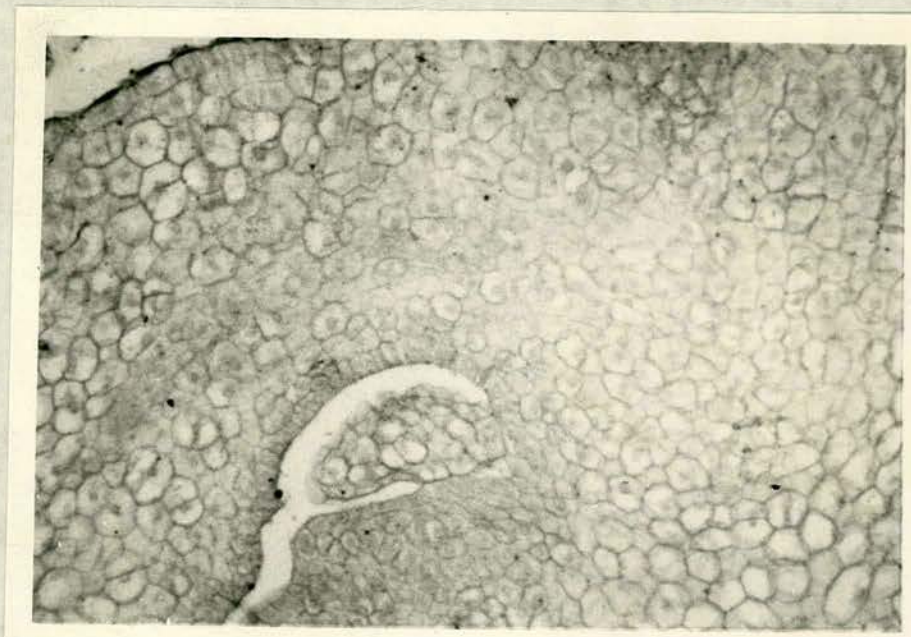


Fig. 6.13
P171F97D-A
R5
(x100mag.)

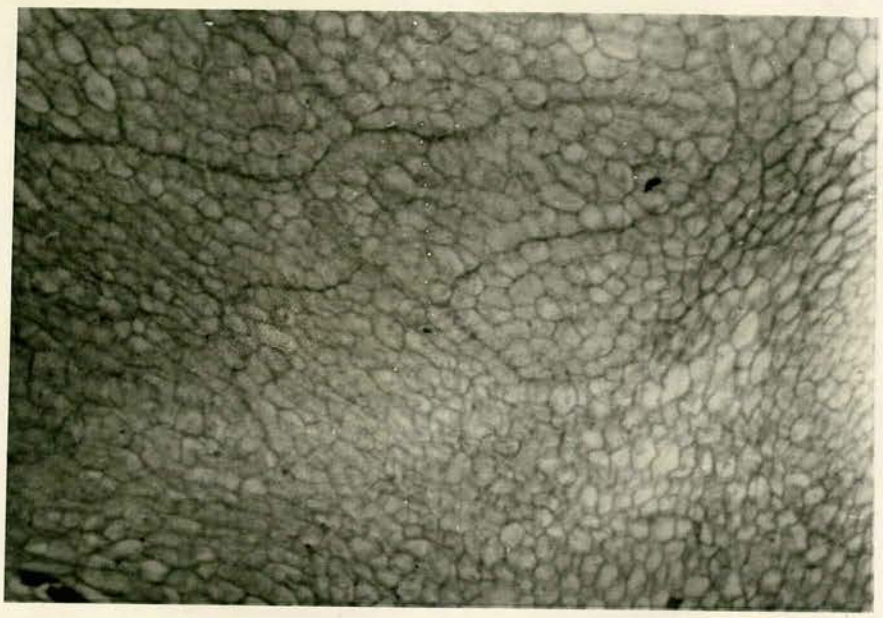


Fig. 6.14
P171F97D-A
R5
(x100mag.)

Fig 6.13 is taken from a section through callus R5, which readily organises into meristematic regions and differentiates roots and shoots. This section is through a meristematic region which was found internally in the callus. The meristem appears to be very similar to the apical dome of the excised embryo, except that there does not appear to be any organisation of the tissue below the meristem and, in this section, there is no germination split for the meristem to develop through as there is in the embryo. The cells are small at the meristem but are of an equivalent size to those in the organised regions in Fig 6.8, Fig 6.10 and Fig 6.12.

Fig 6.14 shows a piece of tissue of R5 where a number of meristems have differentiated. These are randomly distributed and show no clear polarity.

C. Examination of scanning electron micrographs.

Fig 6.15 is a low magnification surface view of a meristematic region of callus R5. This surface has no areas of small nodules and, although the surface is smooth, it is undulating. Fig 6.16 confirms this observation, showing that the surface has clearly defined cells which are randomly arranged. Fig 6.17 shows that the cell surfaces are quite smooth, apart from small regions of pitting which was probably caused by damage to the cells in the freezing process.

These photographs show that the different callus cultures appear to have different surface properties, but there are greater similarities between the surface of the normal and rapid lines (P114L31 and P182A65) than the surface of the meristematic region of callus R5.

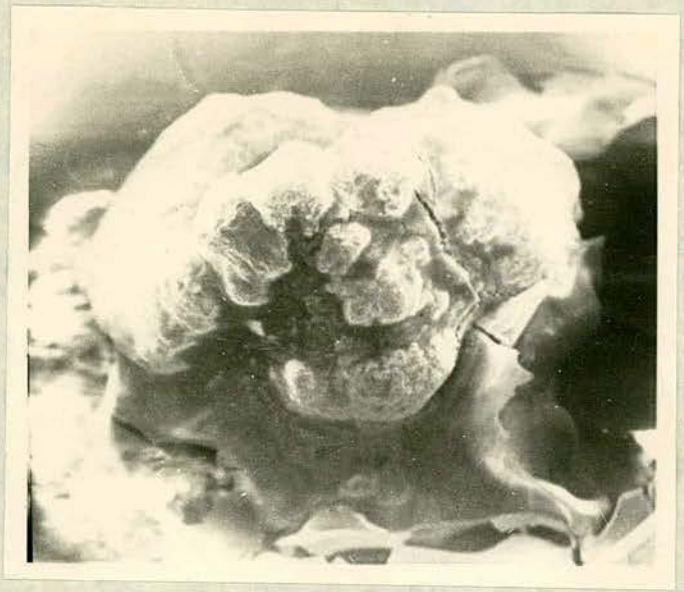


Fig. 6.15
SEM of P171F97 D-A (R5)
(x 25 mag)

Fig. 6.16
SEM of P171F97 D-A (R5)
(x 500 mag.)

Fig. 6.17
SEM of P171F97D-A (R5)
(x 1000 mag.)

D. A comparison of the values of mean fresh weight per cell in established callus cultures.

One of the basic characteristics of a callus culture is the observed range in cell size, as the form of the culture tends to be related to its cellular components. Thus a comparison of cell size made between cultures could be used to characterise the callus.

Samples were taken from a number of callus lines, surface dried and weighed before overnight maceration in chromic acid. The cell number of each sample was determined and the value of mean fresh weight per cell of each culture calculated. These values are shown as Table 6.2.

Table 6.2 shows that the 'rapid' callus cultures R1a, R2 and R3 have a mean cell fresh weight two to three times that of the 'normal' callus cultures N2 and N3. The 'normal' culture P114L18 is of the same line of descent as N2 and N3 but was separated from the common ancestor at an earlier date.

Culture R4 is a 'rapid' callus and is an ancestor of the culture which, when grown under suitable conditions, will readily produce meristematic, regions. All the rapidly growing callus cultures examined in this investigation are derived from a common ancestor. This original callus must have been heterogenous in cell type as all cultures derived from it grow rapidly, but only some sub-cultures have the capacity to generate meristematic regions. This is a common phenomenon (Yeoman 1970). The cell size of R4 is between the low weight cells of the normal growth rate callus and the high weight cells of the rapidly growing callus, which would indicate that cell size is an important diagnostic factor in determining

Table 6.2 The calculated mean fresh weight per cell in a number of Callus Lines

Callus	Code	Growth Type	Mean Fresh Weight per cell x 10^{-5} mg
T003A27	R1a	Rapid	3.0
P182A49	R2	Rapid	3.2
P182A65	R3	Rapid	3.1
P171D67A	R4	Rapid	2.3
P114L18	N1	Normal	2.8
P114M31	N3	Normal	1.3
P114M11	N2	Normal	1.5

culture type.

Cultures R2 and R3 are sub-divisions of the same parental culture and, as expected, are very similar in mean cell fresh weight. Culture R1a has a common ancestor with P182A line (R2 and R3) and so it has, as expected, a mean cell fresh weight very similar to the P182A line.

E. Investigation into the DNA content of samples of nuclei in callus tissue by microdensitometry.

The DNA content of a nucleus can be measured microdensitometrically and the value obtained used to determine which stage of the cell cycle the nucleus has reached. Measurement of the DNA content of a sample of nuclei can show if the nuclei are dividing and whether or not they do so in a synchronous manner. If mitotic figures are present it is possible to determine 2C and 4C values for the DNA content.

Samples of callus tissue were fixed in 3:1, ethanol: acetic acid before staining with Schiff reagent using the Feulgen procedure, and squashing on microscope slides. The DNA content of 200 nuclei was determined using the Barr and Stroud integrating microdensitometer and the results plotted as Figs 6.18 to 6.25.

Fig 6.20 shows the distribution of nuclear DNA content in a sample of callus culture N1. In this culture there is a large range of nuclear DNA contents from 3 to 40 units, although the range is extended from the main population spread of 3 to 25 units by two isolated nuclei at 33 and 40 units. Most of the nuclei have DNA contents at the lower end of the range, with the main peak of readings at 5 units and secondary peaks at

Figs. 6.18 – 6.25

Microdensitometric analysis of DNA of established callus cultures

Abscissa: DNA content (arbitrary units)

Ordinate: numbers of nuclei per class

prophase and metaphase figures

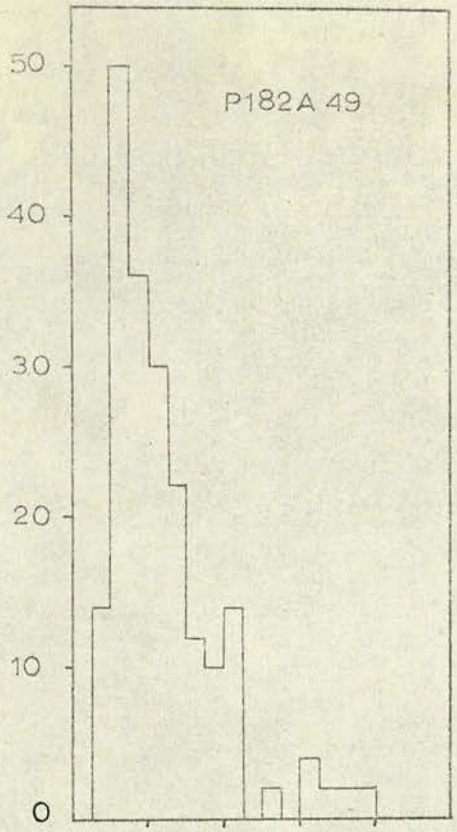
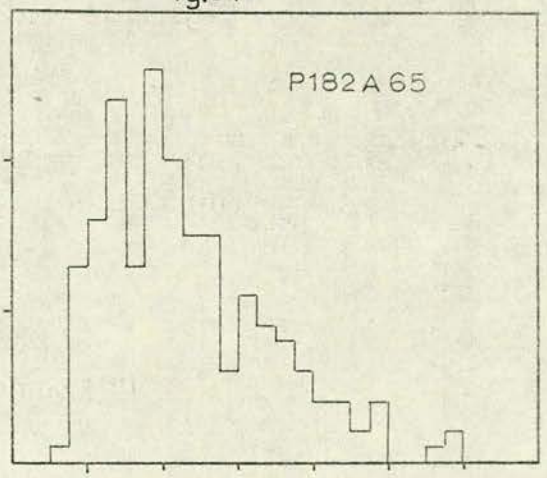


Fig. 6.18

Fig.6.19



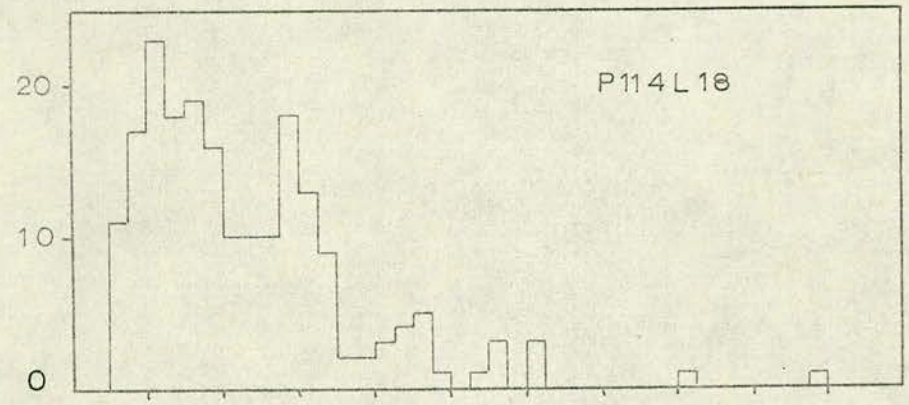


Fig. 6.20

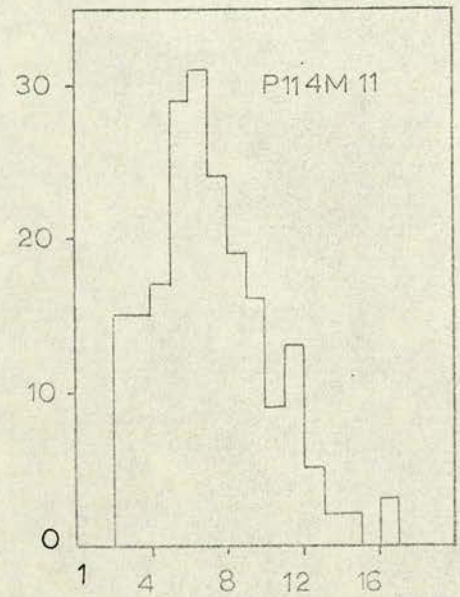


Fig.6.21

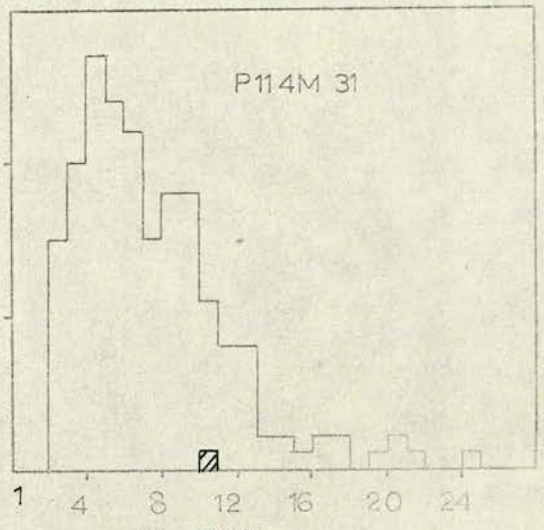


Fig. 6.22

12 and 19 units.

Fig 6.21 shows that culture N2 has a much smaller range of values and hence is more uniform in DNA content than N1, with a range of values between 3 and 17 units. The main peak is around 7 units with secondary peaks at 12 and 17 units. In Fig 6.22 it can be seen that culture N3 has a range of DNA values between 3 and 25 units with the main peaks at 5 units, a secondary peak at 9 and 10 units and a "tail" of values between 13 and 25 units. Two mitotic figures a prophase and a metaphase configuration were found with DNA contents of 11 units. This suggests that the 4C value of DNA at 11 units and so the 2C value will be around 5 or 6 units. The 2C value coincides with the main peak of DNA values, which indicates that a population of nuclei could have just divided synchronously. However, there are a number of nuclei with values less than the 2C value or more than the established 4C value. This would indicate that there are nuclei in this callus that have varying DNA contents and are dividing at different times.

Fig 6.19 shows the distribution of DNA content in a population of nuclei from a sample of the 'rapid' callus R3. The range of DNA content is from 3 to 24 units and this is divided into 4 groups with peaks at 6, 8, 13 and 24 units. The peaks at 6, 13 and 24 units run in a series of C, 2C and 4C, and so could be part of a population which has become polyploid.

Fig 6.18 represents the distribution of a nuclear DNA content in culture R2. The values spread between 2 and 16 units with a main peak at 3 units and an isolated population between 13 and 16 units. Overall,

the DNA content of the nuclei is quite uniform and this could indicate some degree of periodicity in nuclear division.

In Fig 6.24 the range of DNA values in the rapid culture R4 is between 3 and 18 units, but the distribution within the range is not as uniform as in culture R2. There is a main peak at 7 units and a secondary one at 12 units on the skewed part of the distribution. Culture R1a (Fig 6.23) is also less uniform in nuclear DNA content with a range between 3 and 24 units, peaks at 6, 9 and 13 units and an isolated population between 22 and 24 units, with the main population skewed towards the higher values.

Culture R5, when incubated under favourable conditions, will readily differentiate roots and shoots, Fig 5.25 shows that the distribution of DNA content of the nuclei within the range of 4 to 22 units is even, apart from two peaks at 8 and 11 units. This suggests that nuclear division is generally asynchronous as the DNA content of the nuclei form a complete range of values showing that the nuclei are equally distributed in the stages of the cell cycle. However, as there are two peaks of DNA content at 8 and 11 units, there could be two small sub-populations which are dividing periodically and hence there are a number of nuclei at a similar stage of the cell cycle with a similar DNA content.

When examining Figs 6.18 to 6.25 together it must be borne in mind that the cultures may be at different stages in the growth cycle although they have been initiated and sub-cultured at the same time. Thus any differences observed could be due either to the stage of growth that

the individual callus has reached (which should be approximately the same) or to inherent differences between the cultures. All the cultures, except for R5, have a high percentage of nuclei which could be periodic in their nuclear cycle, and this gives rise to a distribution of nuclear DNA contents with a main peak and some secondary peaks, generally skewed towards the higher values. R5 is a rapid callus which differentiates to form roots and shoots, and this culture appears to be mainly asynchronous in nuclear division at this sample time, although there are probably two partly synchronous sub-populations.

There do not appear to be many differences between rapid and normal cultures, with respect to DNA content except that N1 (Fig 6.26) has 5 very high DNA content nuclei, where comparable values are not found in other cultures, and this indicates some degree of polyploidy. Table 6.3 summarises the data presented in this chapter.

Summary

The fast and slow growing callus cultures differ in external appearance and this appears to be at least partly related to the culture medium, as medium containing NAA tends to support the growth of a more friable tissue that tends to turn brown. The normal and rapid callus lines can only be distinguished morphologically when visible organised structures appear on the surface. However, their anatomical structures are quite distinct. Normal callus does not form organised areas of small rapidly dividing cells, as does the rapid callus, but consists of aggregations of cells that are often broken or split. The rapid callus, which does not produce organised structures, has areas of cells that appear to be organised, but these groups are not connected to each other except by cells which do not appear to be viable. Callus which differentiates

Table 6.3 Summary of data presented in Chapter VI

Callus Code	Morphology	Anatomy	Mean FW per cell x 10^{-5} mg	DNA range (arbit units)	Presence Mitotic figs
N1	-	-	2.8	3-40	0
N2	Friable/brown	disorganised	1.5	3-17	0
N3	Friable/brown	-	1.3	3-25	1%
R1	Moist	-	-	-	-
R1a	-	small aggregates	3.0	-	-
R2	-	small aggregates	3.2	2-16	0
R3	Friable	-	3.1	3-24	0
R4	Friable/brown	organised	2.3	3-18	0
R5	Roots/shoots	meristems	-	4-22	0

to form roots and shoots possesses definite meristematic regions, very similar to those seen in the freshly excised embryo.

Scanning electron micrographs show that the culture R5, which readily initiates meristematic regions, has a very smooth surface with distinct cell walls, in comparison with the other rapid lines and the slow callus which have formed nodular areas in which the cells have a similar appearance to xylem vessels with secondary thickening.

The calculated mean fresh weight per cell varies with the callus type. Two of the normal callus cultures have cells one half or one third the weight of the rapid callus cultures. The third normal callus culture examined had a mean fresh weight per cell between the weights of the other normal and rapid callus cultures and the same as the weight of the culture which differentiates.

The DNA content of the nuclei sampled for each callus culture suggests that all the cultures, apart from R5 show some degree of periodicity with respect to nuclear division.

CHAPTER VII

DISCUSSION

The work embodied in this thesis was undertaken as part of a project of which the long term objective is to produce, at will, clones of mature oil palms by the culture of vegetative parts of Elaeis. In the United Kingdom, it is difficult to obtain quantities of mature palm tissue that can be readily sterilised and are amenable for tissue or organ culture. However, relatively large quantities of uniform sterile tissue can be obtained from embryos and these may be used as a primary explant system. One disadvantage of using embryos for a study of this type is that, because of the 'out-breeding' nature of the oil palm, the embryos have an unknown genetic potential. However, this disadvantage is far outweighed by the ready availability and fast growth of the embryo which make this system most suitable for the investigation described in this thesis. Subsequently it is hoped that the information obtained here using the callusing embryo will be applied to other oil palm explant systems.

One of the major problems presented by any tissue culture system is the provision of a suitable medium to support growth. Such a medium must contain a readily available carbon source, minerals and growth factors, (Yeoman 1973), and should be selected and developed for the particular tissue under investigation. Previous to the commencement of this study standard culture media had been modified to support the growth and development of oil plam embryos in culture.

Rabéchault, Guénin and Ahée (1970) compared the growth of embryos on the media of Heller (1953), Hoagland and Arnon, (1938) Knop, (1865) Randolph and Cox (1943), Olsen (1950) and White (1943) using the

same hormones and organic constituents as described by Rabéchault (1962). Rabéchault, Guénin and Ahée (1970) followed the growth and development of embryos on these six media, harvesting the cultures after various incubation times at which they measured fresh and dry weights, leaf number and dimensions of aerial portions of the plants. From these data they concluded that the first three media supported better growth. Martin, Cas and Rabéchault (1972) compared the growth of cultured roots from aseptically germinated embryos on the media of Murashige and Skoog and White and concluded that the medium of Murashige and Skoog supported better growth than that of White. Jones and Dethan (1973) compared the growth of excised embryos on a series of media and came to the conclusion that Murashige and Skoog's medium supported the best growth. From these papers, it would appear that a high salts medium, such as that of Murashige and Skoog, is most suitable for the growth of the embryo. Excised embryos of oil palm will not grow and develop on a simple mineral medium without added organic constituents. Jones and Gopal (1972) showed that the addition of 1000 mg/l of caesin hydrolysate to Murashige and Skoog's medium promoted the normal development of the excised oil palm embryo. It has been shown that IAA is required to stimulate root formation and kinetin promotes the enlargement of the haustorium (Jones and Dethan 1973). They also showed that the optimal concentrations of IAA and kinetin for growth are 0.5 mg/l and 0.1 mg/l respectively. However, later work in the same laboratory claimed that 1.0 mg/l IAA and 0.5 mg/l kinetin were superior (Jones pers. comm.). Bouvinet and Rabéchault (1965) isolated gibberellic acid from oil palm nuts, and concluded that GA was an important growth factor. They substituted IAA with GA_3 at concentrations between 10^{-8} and 10^{-4}gm/l, in an attempt to stimulate growth, but their results were inconclusive and suggested GA_3 had no effect on growth. From this it may be concluded

that caesin hydrolysate, IAA and kinetin are necessary for the balanced growth of embryos in culture.

All cultures require a suitable carbon source. Sugars are generally used, and these may, in some cases, also have a role in osmotic control. Bufford-Morel (1968) examined the effects of glucose, fructose, maltose and sucrose on the growth of excised oil palm embryos. It was established that maltose and fructose inhibit root formation and that the haustorium turns brown in concentrations greaterthan 2%. Bufford-Morel suggested that sucrose was the most suitable carbon source at a concentration between 2 and 3%. Dethan and Jones (1975) used sucrose and sorbitol separately and in mixtures and showed that sorbitol alone affects haustorial growth whereas optimal growth may be supported by sucrose alone or a mixture of the two sugars at a concentration between 3 and 4%. Thus sucrose at a concentration of 3% was chosen for the experiments detailed in this thesis.

Nearly all of the experiments, designed to determine the optimal growth medium for oil palm embryos, have been carried out on media solidified with agar at a concentration of 0.8% (w/v). Many embryos grown on a solid medium turned brown and frequently showed unbalanced development, either producing shoots or roots but not both together. De Guzman (personal communication) showed that excised embryos from coconuts produced balanced growth only when cultured for an initial period in a liquid medium. The "Makapuno" coconut, in which the embryo will not develop <u>in situ</u> but will after excision and culture, is an important commercial variety. Here tissue culture has been employed to produce clones of coconut palms. If the embryo is excised directly on to solid medium the shoot will form before the root (Baliga

and De Guzman (1971) and De Guzman, Del Rosario and Eusebio (1971). If the embryo is excised and grown in a liquid medium throughout the culture period shoot development is retarded. Subsequent transfer to solid medium will promote root growth so that shoot development occurs after root formation.

Excision of the oil palm embryo into a liquid medium prevents the rapid browning associated with development on agar (Bouvinet and Rabéchault 1965) and also accelerates the rate of development so that two leaves are visible by 10 to 12 days of incubation as opposed to 30 to 40 days when the embryos are cultured on agar (Rabéchault 1962). This may be due to the greater freedom of movement of nutrients or metabolites in a liquid medium. In a solidified medium, the establishment of gradients may limit the availability of nutrients or lead to the accumulation of inhibitory substances in contact with the embryo.

The temperature of incubation of any culture is important if balanced plants are to be produced. Van Overbeek, Siu and Haagen-Smit (1944) showed that the optimum temperature for growth of excised Datura embryos is between 27°C and 32°C, much higher than for the mature plant. This finding was supported by Rijven (1952) in Shepherd's purse and Choudhury (1955) in immature tomato embryos. Mauney (1961), cultured cotton embryos, and showed that high temperatures could support higher growth rates, but the resultant plants tended to be very spindly. Henry (1959) followed the growth of oil palm seedlings during the first three months of development. He found that temperatures below 17.5°C supported only very slow growth, mainly of the roots, and that the growth rate doubles between 20°C and 25°C.

Increasing the temperature to 28°C did not increase the growth rate significantly however, humidity is important particularly at the higher temperatures. Having examined data obtained from plantation reports concerning electrically heated germinators it was decided to use an incubation temperature of 33°C. This temperature was later confirmed as optimal for embryo culture by Dethan (Dethan and Jones 1975).

It was decided that when embryos were to be incubated under callus-inducing conditions only minimal alterations should be made to the conditions used to study normal development of the embryo. Accordingly most of the media components were fixed. Callus can be initiated by the addition of NAA (W K Smith 1970, Thomas 1971, Smith, Thomas and Knight 1972, Martin, Cas, and Rabéchault 1972) or 2,4-D (W K Smith 1970, Thomas 1971, Rabéchault, Martin and Cas 1972, Jones 1973, Smith and Thomas 1973). Smith and Thomas (1973) showed that callus may be initiated in excised embryos by any concentration of 2,4-D between 2 and 10 mg/l and will be maintained by concentrations between 0.5 and 5 mg/l. Thus it was decided to use a concentration of 5 mg/l 2,4-D for the initiation and maintenance of callus cultures.

Embryos within a batch of seeds are fairly uniform at excision, with respect to physical characteristics and the attained stage of development (Hartley 1967). The general anatomy of the normal embryo at excision does not vary between batches, there are two fully developed leaves, the rudiments of a third and a root meristem in the tigellum. The haustorium possesses no discernible structures. However, other characteristics, such as fresh weight and cell number, vary within batches and, to a much greater degree, between batches, and it is this variation that

has presented most difficulty in the use of the embryo as a primary explant system.

Hussey (1959) showed that embryos within the seed were not dormant, but a delay in germination was due to an inhibitor in the endosperm. The effect of the inhibitor could be completely nullified by the excision of the embryo from the endosperm. Similar examples of this effect include Iris (Randolph and Cox 1943) and Xanthium (Wareing and Foda 1957). The literature concerning oil palm plantation work and the experiments described in the chapter on variability have shown that variation in the rate of development of the excised embryo in culture is high. Treatments aimed at inactivating any residual inhibitor in the embryo do not appear to reduce this variation and, in the majority of examples, they appear to reduce the viability of the embryos. Thus it must be noted, in the investigations described in this thesis such variation has been substantially reduced by the pre-selection of embryos which are most likely to undergo normal development.

Variation in development between embryos in cultured populations is only apparent some time after excision. The embryos of any given seed batch at excision are very similar in fresh weight, cell number and DNA content, although there is a greater amount of variation between seed batches. This low degree of variability within a batch may be expected as the embryo reaches maturity within the drupe, about 90 days before harvesting (de Poerck 1950), and so within any cross the embryos should be quite similar with respect to physical characteristics. The initial development of embryos appears to be uniform up to a certain point in time, after which it becomes more variable. The first 24 hours of incubation appears to be an exception to this generalisation.

It was noted that, in the majority of embryos examined, the mean DNA content per nucleus decreased between the time of excision and the first 24 hours of incubation, which is suggestive of nuclear division. However, labelling experiments showed that tritiated thymidine was not incorporated into the DNA until at least 72 hours of incubation. More detailed experiments, in which cell numbers were estimated, showed that there is a statistically significant increase of about 11% between excision and 24 hours of incubation. When these results are taken together they suggest that the nuclei which divide without DNA replication were in G_2 of the cell cycle when the embryo was excised. The use of colchicine to arrest mitosis, and the measurement of the DNA content of the pro-metaphase (Eigsti and Dustin 1955) and interphase nuclei established that the pro-metaphase nuclei exhibited DNA contents approximately twice that of the interphase nuclei which is consistent with the observation that these nuclei did not incorporate ^{3}H-thymidine until after 72 hours of incubation. Probit analysis of the DNA content of the population of nuclei at excision indicates that there are two approximately equal groups of nuclei in which the nuclei of one group have a DNA content twice the amount of the other group. However, only 11% divide during the first 24 hours of incubation. This evidence is consistent with the view that the nuclei with a high DNA content are in G_2 of the cell cycle. Therefore it would appear that these nuclei which undergo division, within 24 hours from excision, had reached G_2 in the cell cycle before overall development of the fruit had ceased, and therefore had remained at this stage until the embryo was removed. Stein and Quastler (1963) noted that in maize, the root cap, coleorhiza and scutellar node were the first portions of the embryo to resume DNA synthesis, 30 hours from the commencement of soaking. They observed

that a proportion of the nuclei of the root and shoot underwent mitosis before DNA synthesis was resumed, at about 50 hours and 70 hours respectively from the start of soaking. They concluded that these shoot and root nuclei were at the 4C value of DNA ie the G_2 stage of the cell cycle in the intact fruit. J K Smith (1970) confirmed the observation of the presence of a mixed G_1 and G_2 population in maize embryos but disagreed as to the time of division of the G_2 nuclei. Avanzi et al (1963) noted the occurrence of G_1 and G_2 nuclei in the radicle of dry seeds of Triticum durum Desf, in the proportion of 2 to 1 in the root cap cells, pro-vascular cells and in the cells at the base of the meristem. The presence of mixed populations of G_1 and G_2 cells is not confined to observations within the plant kingdom, Gelfant (1963) has noted separate G_1 and G_2 populations in cultures of mouse epidermal cells and he considers that these are maintained in culture as individual groups with differing cell cycle times. Chang (1963) noted the incorporation of ^{32}P into nuclei of barley embryos did not occur until one round of cell division had already taken place, suggesting that nucleic acid synthesis is not initiated until after the first nuclear division. The increase in cell number and the number of arrested pro-metaphase nuclei in the embryos subjected to colchicine treatment in this study suggest that in the normally developing embryo approximately 11% of the nuclei undergo division, within the first 24 hours from excision without DNA replication. This does not imply that only 11% of the nuclei are in G_2 at excision, as after 24 and 48 hours of incubation in the presence of ^{3}H-thymidine there is still a residual population of unlabelled nuclei with a higher DNA content. The incorporation of tritiated thymidine into nuclei of the embryo was not observed until 72 hours of incubation.

It should be noted that all the oil palm embryos examined undergo imbibition and cell and nuclear division without DNA replication irrespective of whether the embryo would subsequently undergo 'normal' development. These data suggest that although the individual excised embryo is not uniform, the variation in response to excision and culture is low during the initial period of incubation.

The length of the period during which the growth of the population can be considered not to be highly variable depends on the batch of seeds and the measured parameter of growth. Table 7.1 summarises a number of experiments and indicates the variation between individual experiments. In the preliminary experiments the post-imbibitional fresh weight increase occurs after approximately 168 hours of incubation, whereas, in the comprehensive time course experiment, the increase occurred at about 125 hours. At this time the variation between the fresh weights of individual embryos was higher than that of the post-imbibitional period. After this increase it it possible to distinguish three populations of embryos, the first in which the fresh weight increases exponentially after a short lag phase, but following the initial rise due to imbibition. The second population consists of embryos that do not develop significantly and remain at the post-imbibitional level of fresh weight for the duration of the experiment. The third population consists of individuals which remain at the post-imbibitional level of fresh weight for a period longer than that of the first population but which subsequently increase in fresh weight at a lower rate than the first population of embryos (see Fig 3.8). This sub-division into three populations can also be made using the cell number data. The preliminary experiments showed that the cell numbers

Table. 7.1

Summary of results

	preliminary expts.	time course expt.	tigellum	haustorium	callus induction
average fresh weight at excision (mg)	4·0	5·9	0·75	0·5	6·6
max. F.W. at end (mg)	45·5	72·5	31·0	29·5	20·9
time of post-imbibition F.W. inc. (hours)	168	125	120–140	140–160	62
ave. cell no. at excision ($\times 10^{-5}$)	4·15	5·1	3·0	1·4	6·6
max. cell no. at end ($\times 10^{-5}$)	14·5	24·0	17·8	7·7	21·1
time of cell no. inc. (hours)	126	152	100 – 110	90 –100	92
cell no. doubling time (hours)	236	185	240	260	120 –140
first detect. incorp. of thymidine (hours)	60	(48) 72	—	—	48
max °/o labelling	63 5	<50	—	—	46

begin to increase around 126 hours, whereas in the comprehensive time course experiment the increase was delayed until 152 hours. The three distinct populations of embryos undergoing "normal" growth, "slow" growth and "no" growth can be easily distinguished. It is the presence of these sub-populations which makes the variation so high in any sample of embryos. This variation increases with the growth of the population as is shown by the range of values obtained when the proportion of labelled nuclei is determined after the embryos have been exposed to tritiated thymidine. Here, there is little variation in the percentage early in the experiment, but once labelled nuclei are detected the variation between individuals increases.

An explanation of this variation can be obtained by comparing the growth of the tigellum with the haustorium. The haustorium is generally smaller and shows a greater degree of periodicity with respect to cell division than the tigellum (Fig 4.36). The two parts of the embryo increase in fresh weight and cell number at different times and the cell doubling times are not the same. All of these factors, taken together, indicate that the growth of the excised embryo is uniform up to a certain point in time after which the growth of individual embryos proceeds at different rates. It appears that something happens, or does not happen, at this point so that subsequently some embryos undergo normal development whereas others do not. This point in time may vary between embryos giving rise to populations which grow at different rates. Whatever happens, or does not happen, at this time appears to be in some way associated with the onset of DNA synthesis, as it is from this point that the major increase in variation is observed. The population of embryos which does not increase in cell number or fresh weight above the post-imbibitional level does not incorporate tritiated

thymidine, whereas those embryos which constitute the slow developing population only begin to incorporate labelled thymidine after an extended lag period. This suggests that there is an endogenous factor, which initiates DNA synthesis and triggers the subsequent development of the embryos after a period of imbibition.

A further feature of the variation within populations of embryos is shown by the differences observed between the distribution of nuclear DNA contents in duplicate samples. Mitotic figures were not frequently observed, which suggests that mitosis forms a very small part of the cell cycle and/or there is some degree of periodicity in nuclear division. This suggestion of periodicity is supported to some extent by the observation of occasional high values for the mitotic index. Mitotic indices for the comprehensive time course experiment described in Chapter IV are shown in Tables 4.5 and 4.6. The DNA values for these mitotic figures have a wide range. This range is exaggerated because of the use of the conversion factor, (see Chapter IV E4). The measurement of the DNA content per nucleus is by means of a "flying dot" scanner. This is a mechanical device where a small aperture flies across the image in a series of parallel lines and measures the DNA content of a series of areas. The DNA values are then integrated so that an average reading for the nucleus may be calculated. The scanner is therefore constructed to measure uniformly coloured areas. (Barr and Stroud Handbook.) If however, the stain within the nucleus is not uniformly distributed (when the nucleus is pycnotic of during mitosis when the DNA is condensed,) underestimated values will result. This underestimation will be greatest with telophase and metaphase nuclei. The values measured for anaphase and telophase (2C) fall within the range

of 12 to 27 units and the values for prophase and metaphase (4C) fall between 25 and 44 units. The range of these values is very large, perhaps too large to be explained entirely by errors in the mode of measurement. However, there are no distinct groups of values for mitotic figures which could be interpreted as a polyploid series (Fig 7.1) and so, although, there is a suggestion of such a series the data presented to not confirm it. Patau, Das and Skoog (1957) showed that nuclei in tobacco pith cultures had varying DNA contents, within a polyploid series. Murashige (1974) has stated that many plant tissues contain a series of polyploid nuclei. It is also not unusual for cultures derived from plant tissues to exhibit a high incidence of polyploidy, indeed attempts to determine the range of numbers in callus cultures of oil palm suggest this may be true. (W K Smith, pers. comm.). A further problem arising from the DNA analysis is that of nuclei with measured DNA contents lower than 2C. Mitra and Steward (1961) and Partanen (1963) have argued that aneupoloidy and haploidy are more common in plants than had been originally suggested. Thus it may be possible that the oil palm embryo may contain a number of nuclei which have DNA values less than 2C. None of these nuclei with a low DNA content have been observed in mitosis in this study, so it may be assumed that they constitute a small non-dividing population. Such nuclei have not been observed in freshly excised embryos and therefore must arise during culture, their origins are at present obscure.

In table 7.1 a summary of data is presented from a series of experiments in which cell number, nuclear DNA content and the proportion of nuclei labelled with tritiated thymidine were determined during the development of excised embryos in culture. From these data it is possible to estimate the approximate length of the first cell cycle and

some of the component phases. It was shown previously (Chapter IV), from cell number estimations, that the average cell doubling time in the population of embryos was 185 hours, and at this point, the percentage of labelled nuclei was approximately 40%. It may be assumed that the increase in the proportion of labelled nuclei to 40% of the total was due to DNA replication and subsequent division of 20% of the cells in the population. This seems likely as the increase to 40% of the total takes place over a short time interval. It can be seen, from Table 7.1, that the first labelled nuclei are detected 72 hours after the start of the experiment, therefore the minimum duration of G_1 is about 72 hours. In addition, the first significant increase in cell number does not occur until about 152 hours, when about 20% of the nuclei are labelled with tritiated thymidine.

Thus the following estimates of the cell cycle and some of its phases may be made.

Minimum time of cell cycle = 152 hours = $G_1 + S + G_2 + M$

G_1 = 72 hours.

therefore $S + G_2 + M = 152 - 72 = 80$ hours.

As has been stated earlier, cell number doubling occurs at 185 hours. The calculations presented above show that as far as labelled nuclear divisions are concerned only 20% of the cell population is involved in the first round of division and that at 185 hours only about 40% of the nuclei are labelled, these resulting directly from the division of the 20% of labelled nuclei. These values suggest an inconsistency, as they can only account for a 20% increase in cell number at 185 hours whereas the cell number data show a 100% increase at this time. An alternative

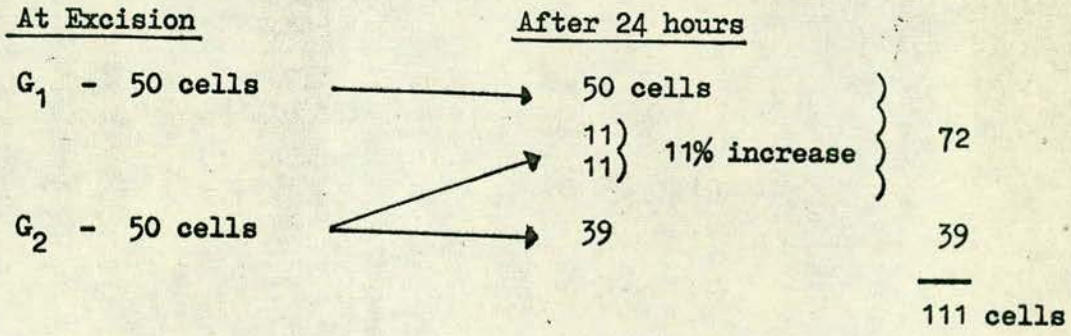
explanation must be sought. An obvious suggestion is that a large increase in cell number can occur from the division of cells which are in G_2 at the time of excision, which will divide without DNA replication and hence will not become labelled with tritiated thymidine. This is not an unreasonable suggestion for evidence presented elsewhere (Chapter IV) from a study of the change in DNA content of the nuclei of cultured embryos has shown that two major populations are present in the embryo at excision (see tables 4.5 and 4.6). Although there is some variation, at excision approximately 50% of the population is in G_1 while the remainder is in G_2.

Fig 7.2 is a model designed to show increases in cell number expressed as actual numbers starting with a sample of 100 cells at excision. These cells are equally divided between G_1 and G_2 cells.

In the 'normally' developing embryo 11 of the cells in G_2 undergo division within 24 hours from excision leaving 39 cells in G_2 and 72 cells in G_1 (50 + 11 + 11) after 24 hours of incubation. This is, of course, within an 11% increase in cell number. At 152 hours 20% of the total cells in the whole embryo are labelled with tritiated thymidine. At 152 hours in the model system there are 111 cells present of which 20% would be labelled. Thus at 152 hours 22.2 cells are labelled, and if all these subsequently divide 44.4 labelled cells will be produced, leaving 49.8 cells unlabelled and in G_1.

After 24 hours of incubation there are 39 cells remaining in G_2. If all of these cells divide, after 152 hours, 78 cells will be produced. This would suggest that the remaining G_2 cells (39 after 11 divide) are "entrained" into division by the G_1 cells that have just replicated DNA.

Fig 7.2 A Model to explain cell number changes in the 'normally' developing embryo.



Immediately before the cell doubling time 20% of the total number of cells become labelled. Thus 20% of 111 cells (22.2 cells) are labelled.

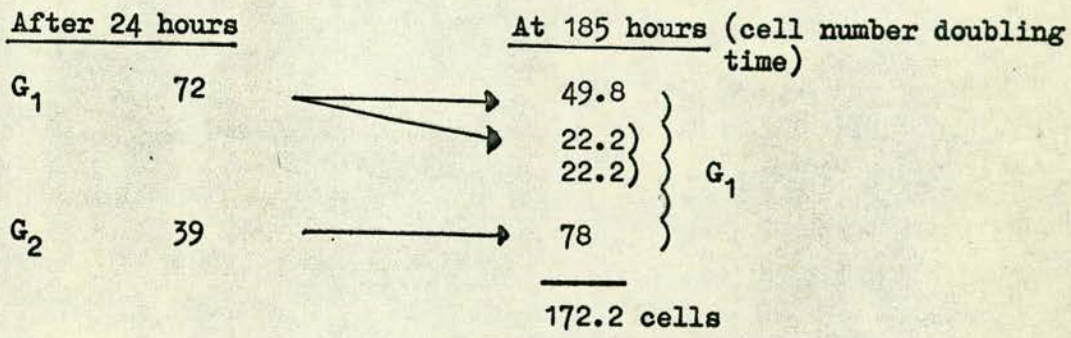
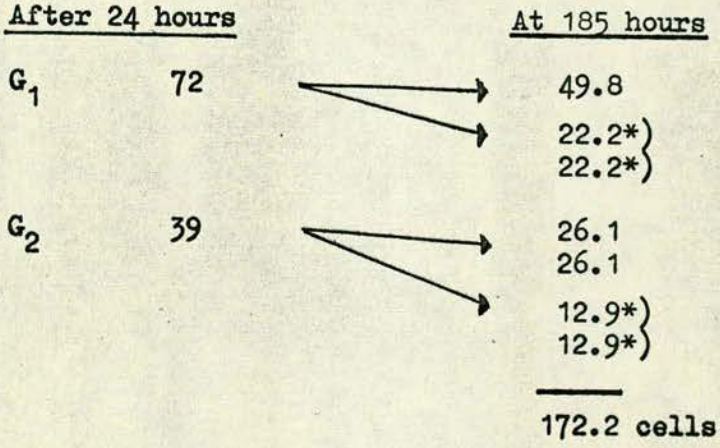


Fig 7.3 A model to explain the number of labelled nuclei



Therefore 102.0 cells are unlabelled 59%
70.2 cells are labelled = 41%

The idea of one population of cells being "entrained" by another population has been suggested by Yeoman and Davidson (1971) working with synchronously dividing artichoke explants. This model will account for a cell number increase of 72.2% from the original cells, and is in fairly close agreement with the observed 100% increase. However it does not account for the increase to 40% of the number of labelled nuclei as the 22.2 G_1 cells which become labelled only constitute about 25% of the final population of 172.2 giving a discrepancy of about 15% or 25.8 cells. Again a further explanation must be sought. Examination of the DNA data shows that around the cell doubling time of 185 hours a number of nuclei have DNA contents which are suggestive of polyploidy. It appears most likely that a proportion of the cells remaining in G_2 after the first 24 hours of incubation synthesise DNA without a prior reduction in DNA content from 4C to 2C and hence become 8C nuclei. This proportion is postulated to be equal to 15% of the final 172.2 cells, that is 25.8 cells and these become labelled after the 20% of labelled cells is observed at 152 hours. Fig 7.3 shows the second part of Fig 7.2, using an asterisk to denote labelled cells.

The increase in cell number of approximately 72% is not far short of a cell number doubling. The remaining inconsistency may be explained by a shortage of tritiated thymidine which will produce reduced proportions of labelled nuclei. This may be true particularly later in the experiment.

These data concerning cell number changes, the increases in labelled nuclei and DNA measurements are consistent within this interpretation and thus it can be seen that meaningful results can be obtained despite the increasing levels of variation towards the end of the experiment.

These experiments have established some of the characteristics of the growth of excised embryos in culture. The second step, in this project of establishing clones of oil palms by tissue culture, was to apply the knowledge obtained from the growth of the excised embryo under conditions which permit 'normal' development, to the growth of the excised embryo under conditions in which a callus is induced (Chapter V). The growth characteristics of callus induction may then be applied to the study of established callus cultures in order to discover why some cultures grow slowly and do not differentiate while others grow rapidly and differentiate readily.

The induction of callus requires repeated cell division before an obvious callus is produced. The cells which synthesise DNA and subsequently divide are always found towards the centre of the embryo, little or no cell division has been observed in the outer layers during the course of these experiments.

It has been established (Chapter IV) that in the freshly excised embryo a proportion of nuclei are in G_2 and therefore it is safe to assume that nuclear division takes place, without DNA synthesis, within the first 24 hours of incubation of an embryo placed in callus-inducing conditions. This assumption is confirmed by the occasional observed increases in cell number of just less than 40% during the first 24 hours of incubation in one or two embryos. However, the measured populations of nuclei at 28 and 48 hours of incubation (Figs 5.29 to 5.32) do not suggest that 40% of the nuclei have divided in these embryos, especially as in these samples there is an increase in the mean and the mode to levels about those of the freshly excised embryo. This would suggest that the

proportion of G_2 nuclei undergoing division varies with the individual embryo when it is under callus-inducing conditions, and that the division period may extend over more than 24 hours. Probit analysis has been used to distinguish populations of nuclei (both major and minor) with similar DNA values. If the major probit population with the highest DNA content (including any minor populations with higher DNA contents) is examined the mean of the highest value population may be estimated. These nuclei will constitute the G_2 population. If the population is assumed to have a normal distribution the mean value is that of the centre of the range. The number of nuclei in that population may be estimated and hence the percentage of the total population of measured nuclei may be calculated. It has been established that 50% of the nuclei are in G_2 at excision (Chapter IV). If the percentage of G_2 nuclei can be estimated at time intervals after excision the proportion of G_2 cells which have divided can be calculated. These percentages are shown as Table 7.2. Examination of a 28 hour sample (Fig 5.29) shows that there is an increase in the number of nuclei with a low DNA content over that at excision, but this is masked in the mean and mode values by the proportion of nuclei with a high value. Probit analysis which estimates the decrease in size of the G_2 population indicates that the proportion of nuclei dividing without replication of DNA in this embryo is about 25%. Similar analysis of the 48 hour population (Fig 5.31) suggests that about 41.5% of the available G_2 population divide whereas in Fig 5.32 the proportion is about 39.0%. The first 72 hour sample (Fig 5.33) suggests that at least 44% of the G_2 nuclei have divided with some degree of periodicity, thus reducing the mean and the mode. As the population is so uniform it could be suggested that DNA synthesis has not begun in this sample, whereas the presence of mitotic figures, together with a proportion of nuclei with a high DNA content in Fig 5.34 suggests that

Table 7.2 - The estimated percentage of G_2 nuclei in samples taken between excision and 72 hours.

Time	% G_2 cells	% division of G_2 cells
0	50	—
28_1	25	25
48_1	8.5	41.5
48_2	11.0	39.0
72_1	6.0	44.0
72_2	2.5	47.5

a large proportion of G_2 nuclei had divided (about 47.5%), DNA synthesis had been initiated and nuclear division has begun. These data are in agreement with the hypothesis that the time and the proportion of G_2 nuclei that divide varies with the individual embryo when it is incubated under callus-inducing conditions, but not under conditions which promote 'normal' development.

The induction of callus in the excised embryo is not accompanied by a large increase in fresh weight during the duration of the experiment (12 days). The gains are about half to one third of those found in embryos undergoing 'normal' development. However, the onset of the post-imbibitional increase in fresh weight occurs after only 62 hours of incubation as opposed to between 120 and 168 hours in embryos cultured in non-callus inducing conditions (see table 7.1). Despite the lower levels of fresh weight attained, cell numbers ultimately increase to a level equivalent to that reached by the 'normally' developing embryos, this shows that the cells formed do not undergo rapid expansion resulting in a large fresh weight increase. The appearance of nuclei labelled with tritiated thymidine at 48 hours precedes the major increase in cell number and fresh weight. This suggests that the 'trigger' mechanism for the initiation of growth and development is activated earlier under callus inducing conditions. The maximum percentage of labelled cells found under callus-inducing conditions is about the same as that found in embryos undergoing 'normal' development.

Table 7.1 shows a summary of the data obtained during studies on the growth of excised embryos under callus inducing conditions. Cell numbers, the DNA content of nuclei and the percentage of labelled nuclei

may be used to estimate the approximate length of the first cell cycle and some of the component phases. The cell number doubling time has been calculated to be 130 hours and at this time the percentage of labelled nuclei was approximately 46%. Similar reasoning to that presented earlier in this discussion concerning 'normal' embryo growth, may be used to devise a model to describe cell division in embryos undergoing callus induction. From table 7.1 it can be seen that the first labelled nuclei are detected 48 hours after the start of the experiment. Thus the minimum duration of G_1 is about 48 hours. The first significant increase in cell number does not occur until 92 hours of incubation, thus the minimum total cell cycle time is approximately 92 hours.

$$T_{min} = 92 \text{ hours} = (G_1 + S + G_2 + M)$$

$$G_1 = 48 \text{ hours.}$$

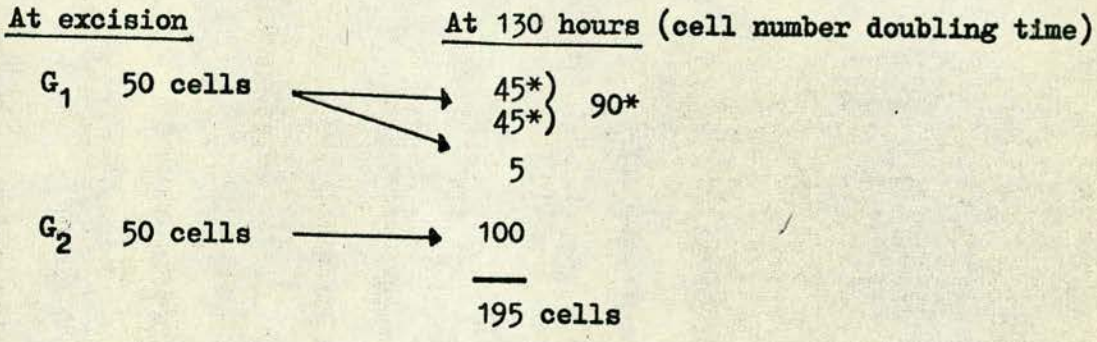
$$\text{therefore } (S + G_2 + M) = 92 - 48 = 44 \text{ hours.}$$

Following the reasoning given previously, cell number doubling occurs around 130 hours which is inconsistent with the observation that approximately 46% of the nuclei are labelled with tritiated thymidine at this time. Again, it is reasonable to assume that the difference between the observed number of labelled nuclei and the observed cell number increase is due to the presence of a substantial proportion of G_2 nuclei at excision, which undergo division without immediate prior replication of DNA and hence will not become labelled. It has been observed that 50% of the nuclei at excision are in G_2.

In callus induction the incidence of polyploidy is low at

130 hours and hence very nearly all the labelled cells will originate from the G_1 population. Fig 7.4 shows a model to explain the cell number and labelling changes using an asterisk to denote nuclei labelled with tritiated thymidine.

Fig 7.4 A model to explain cell number changes in excised embryos undergoing callus induction.



Therefore 105 cells are unlabelled = 54%
90 cells are labelled = 46%

The very small discrepancy of 5 cells (less than 2.5%) may be accounted for by experimental error. Thus the data concerning cell numbers and the number of labelled nuclei are consistent and this system is clearly very amenable to experimental work elucidating cell cycle times.

It is perhaps appropriate at this point to mention the reduction in the observed overall variation between embryos cultured in a callus inducing medium. This medium contains 2,4-D which, unlike IAA is not photo-oxidisible and hence is persistent throughout the culture period. This would suggest that the presence of auxin in the medium over a long period of time may be important in the reduction of variability. Thus

it was possible that the use of low levels of 2,4-D (below that optimum for callus-induction) could reduce the degree of variation within a population. Pilot experiments were carried out growing embryos in Murashige and Skoog's medium with varying concentrations of 2,4-D (from 5.0 mg/l to 0.05 mg/l) with or without pre-treatments with hydrogen peroxide. These experiments showed that callus was induced, in some individuals, at the lowest concentration of 2,4-D and without a pre-treatment with hydrogen peroxide. Therefore low concentrations of 2,4-D cannot be used to reduced variation in a system where normal growth of the excised embryo is being studied as 2,4-D tends to produce callus. It is not possible to determine, from the experiments carried out so far, why callus induction should reduce variability.

A further feature of the reduction of variability is that the range of observed DNA values, in embryos cultured under callus inducing conditions, is smaller than in embryos undergoing 'normal' development, as shown by Fig 7.1. There is a complete range of DNA values for the measured mitotic figures in embryos exposed to callus inducing conditions, between 5 and 25 units apart from values of 11, 21 and 24 units. Again, as with DNA measurements of nuclei in the 'normally' developing embryo, a range of values would be expected for any measured nucleus because of the method of establishing the DNA value. The range of anaphase and telophase values is between 5 and 12 units and the prophase and metaphase values between 10 and 25 units. The 2C values have 2 peaks at 5 and 8 units, whereas the 4C values have suggestions of peaks at 13, 18, 20, 23 and 25 units. These data are consistent with the observed DNA values in the 'normally' developing embryo which suggest that cell populations are present in the culture

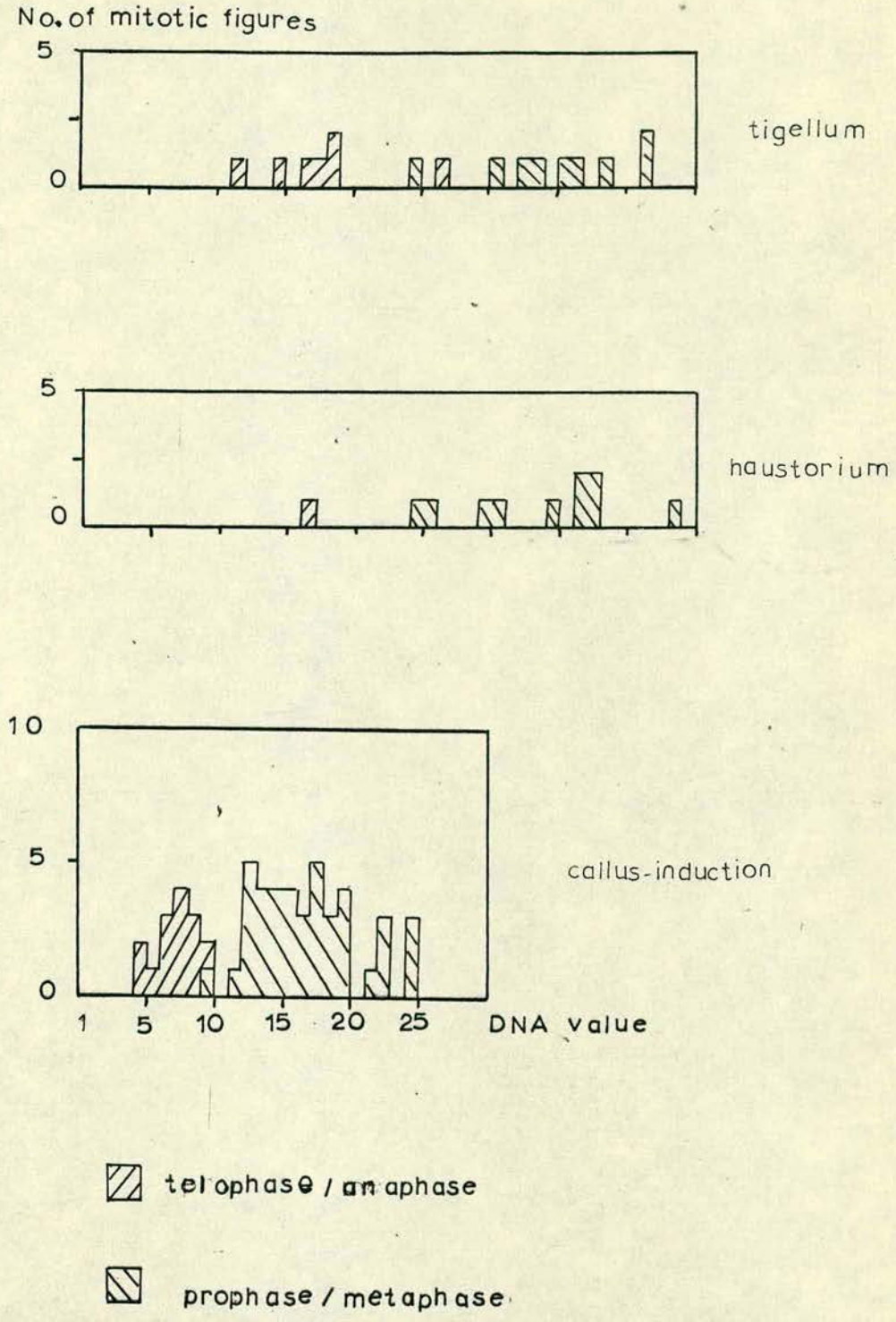


Fig. 7.1

Range of DNA values of measured mitotic figures

with varying chromosome numbers. The DNA status of the embryo at excision and the immediate subsequent changes presented special features which required a more detailed investigation.

A number of characteristics, such as fresh weight, cell number and estimates at cell cycle times, have been established for excised embryos undergoing both 'normal' development and callus induction. These have shown some similarities in DNA regimes, but differences in cell responses to culture and they may now be used as a basis from which to carry out a brief examination of basic characteristics of established callus lines.

The callus supplied had cell doubling times of about 20 days (rapid callus) and between 6 and 8 weeks ('normal' callus), both of which are much longer than that measured for the 'normally' developing embryo and the embryo under callus-inducing conditions. This would suggest that the growth rate of established callus is consistently lower than the growth rate of callus induced from an intact embryo. Although time has not permitted experiments to be performed on the growth characteristics of callus separated and established from embryos in this laboratory it seems likely that a considerable slowing down in the rate of growth accompanies the sub-culture of induced callus from the parent embryo.

The measurement of fresh weight (Chapter V 4C) showed that the rate of increase in fresh weight was accelerating with time, but the measurement of cell number increases suggested that the increase in cell number, once cell division was initiated, was constant over the period of the experiment.

Thomas (1971) states that callus initiation in sterile roots of oil palm is initially rapid for the first three weeks of induction, but then the rate begins to slow down, following an 'S' shaped curve. Henry (1959) had followed growth of the aerial parts of oil palm seedlings, measuring total leaf length. He established that the leaves followed a sigmoid growth curve. The roots, however, when assayed by total root length, followed a biphasic sigmoid growth curve, as the main root became subordinate to adventitious roots. The work of Thomas and Henry would suggest that the growth of the induced callus would eventually slow down to a lower rate following prolonged incubation.

Anatomical investigation of the established cultures suggested an explanation for the varying growth rates. The normal, slow growing, callus consists mainly of large numbers of loosely packed and frequently damaged cells. These cells appear to be generally non-viable. Growth appears to be restricted to a small number of clusters of cells dispersed throughout the culture. It is anticipated that cell cycle analysis of these clusters of cells would indicate that the rate of cell division is comparable to that of the excised embryo or the embryo under callus-inducing conditions. If this is true, it would show that the slow rate of callus growth is due to the large proportion of non-viable cells and not to abnormally extended cell cycle components. Examination of cultures of rapid callus shows that the number of groups of cells which appear viable is higher than that found in the "slow" callus cultures which further confirms the hypothesis raised above. The callus culture which readily differentiates roots and shoots has many meristematic regions throughout the whole of its structure, but also has some areas of dis-organised tissue. The meristematic cells are small and packed with very dense cytoplasm whereas cells of the non-differentiating rapid callus are

not so full of cytoplasm and in some cases are very highly vacuolated. Thus it appears that the rate of growth of established callus cultures is associated with the proportion of viable cells.

Measurement of the DNA content of nuclei also confirms these ideas. In most cultures there is a high proportion of nuclei with a low DNA content. The exception to this is culture R5, a callus which readily differentiates, where, although there are some nuclei with a low DNA value, the proportion is low. It is suggested that the low DNA content nuclei are those of cells which are non-viable and appear as a loosely packed disorganised tissue. The nuclei with higher DNA contents are most probably associated with the organised areas within the callus tissue. All of the cultures except R5, have distributions with discrete peaks at various class intervals. Culture R5, however, (Fig 5.25) has a much more uniform distribution of DNA content, but with a series of peaks at single class intervals which are equal in magnitude. This distribution of DNA values has not been found in the 'normally' developing embryo or in the embryo grown under callus-inducing conditions. This distinct pattern would suggest a means of distinguishing between calluses which are likely to differentiate readily and those which are not. However, the observed differences might relate to the stage of development of the callus.

Overall, these experiments have established some of the characteristics of the growth form of a primary explant system, the excised embryo of Elaeis guineensis. The knowledge obtained from this study of the growth of the embryo has been applied to an investigation into the induction of callus in the embryo. It has been shown that the induced

callus growth is much less variable, as a population characteristic, than that of the 'normally' developing embryo and the rate of cell accumulation is more rapid, initially, due to a shorter cell cycle. The experiments have shown that the growth of the excised embryo, under the two sets of conditions, is different. However, it has not been possible to establish why the variation should be less under callus inducing conditions or what methods may be used to counteract or reduce the variation. Suggestions have been made that the variation in growth begins at the initiation of DNA synthesis.

Brief studies of a number of established callus cultures have shown that there is a distinction between the cytological characteristics of normal and rapid growing cultures.

There are three major problems associated with the development of a clonal system in oil palms derived by tissue culture. The first is the establishment of an explant system from which callus may be routinely initiated without the destruction of the mature palm. The second is the maintenance of active cultures from the explant and the third the regular induction of balanced plants from the callus.

The first problem has been tackled in this thesis using the excised embryo as an explant system with moderate success, the main disadvantage being that unknown genetic potential of any palm differentiated from the callus. The second and third problems may be studied in parallel following the process of callus induction and the development of organised root and shoot systems by changes in histology, cytology and the DNA status.

Growth of the induced callus sytem should be followed by measuring the rate of cell division, and the proportion of dividing cells. This will determine whether it is the rate of division which slows down, by the extension of G_1 and G_2, or whether it is the proportion of dividing cells that decreases. This thesis has established that cell division in the excised embryo undergoing callus induction is neither totally synchronous nor totally asynchronous. There has been some indication that when tritiated thymidine is present continuously in culture, from the time of excision, the supply may become limiting to the number of labelled nuclei towards the end of the experiment (12 days). However if prolonged pulses of thymidine are given for about 4 hours in each 24 hours over an extended period, the proportion of nuclei synthesising DNA at that time may be determined. Repeated exposure of the same callus, combined with grain counts would establish whether only a certain proportion of cells in each round of division subsequently divide or whether the callus consists of a single group of cells which divide repeatedly.

It is well known that it is difficult to differentiate balanced plants from callus lines that have been well established. For example Barba and Nickell (1969) were able to obtain roots and shoots from newly-formed sugar cane callus but were unable to in clones sub-cultured for five years. Up to the present time the induction of plantlets from oil palm callus has only been attempted on well-established callus lines. It would probably be very profitable to attempt to differentiate roots and shoots as soon as a callus has been formed. This work has shown that visible callus is obtained after 6 days culture in a medium containing 2,4-D and so this obtained callus could have been transferred to a different medium after only 4 or 5 days on a callus inducing medium.

Thus it may be possible to differentiate plants before totipotency of the callus cells is lost.

APPENDIX I

GLOSSARY

Seed - fruit with the mesocarp removed, consisting of endocarp and kernel

Endocarp - the shell surrounding the kernel

Kernel - endosperm plus testa and embryo

Endosperm - storage tissue. Food reserve for the embryo

Tigellum - portion of embryo containing root and shoot meristems

Haustorium - portion of the embryo concerned with endosperm hydrolysis and absorption

Rapid callus - callus that has a cell number doubling time of approximately 20 days

Normal or slow callus - callus that has a cell number doubling time of between 6 and 8 weeks

Germination - the period between excision of the embryo and the stage at which the shoot emerges and adventitious roots are formed (normal development)

Abbreviations

2,4-D	2,4-dichlorophenoxyacetic acid
DNA	deoxyribose nucleic acid
DNFB	2,4-di nitrofluorobenzene
GA	Gibberellic acid
G_1 G_2	Gap_1 Gap_2 (in the cell cyle)
^{3}H-thymidine	tritiated thymidine
IAA	3-indolylacetic acid
M	Mitosis (in the cell cycle)

Abbreviations (Contd)

NAA	Naphthaleneacetic acid
S	Period of DNA synthesis in the cell cycle
SEM	Scanning electron microscope
TNA	total nucleic acid

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