THE SYNTHESIS AND PROPERTIES OF RIBONUCLEIC ACID IN DIVIDING PLANT CELLS.

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SUMMARY.

The method described by Yeoman, Dyer and Robertson (1965) for the culture of explants of Helianthus tuberosus tuber tissue was developed to give a system suitable for the study of RNA metabolism in cell division. The system developed is convenient to handle and involves bulk culture of explants in a completely defined liquid medium. About 90% of the cells enter the synchronous first division. Light is a potent inhibitor of cell division. The tissue has a constant response for six months of the year. The system provides a unique opportunity for the examination of the biochemistry of cell division and its induction in higher plant tissues.

Methods were developed for the rapid estimation of the DNA, RNA and protein contents of explants.

Present knowledge of the place of RNA in the control of growth is considered. The temporal and causal relations between RNA and protein synthesis and cell division are reviewed for a variety of organisms. Various aspects of RNA synthesis were examined in the artichoke explants using polyacrylamide gel electrophoresis. Methods for the extraction of RNA from the tissue were investigated with regard to RNA yield, sub-cellular fractionation and degradation of RNA during extraction. Using this knowledge, the synthesis of ribosomal RNA was investigated. Kinetic and base composition data suggest that an RNA of molecular weight 2.07 million is a precursor to both ribosomal RNA molecules. This precursor is compared with precursors in animal cells. A rapid labelling of ribosomal RNA by an unknown mechanism

was also found. Heterodisperse, rapidly labelled fractions were found in the nucleus and cytoplasm. That in the nucleus is heavier than the cytoplasmic heterodisperse fraction, and does not appear to be a simple precursor to it.

Studies of RNA synthesis during the early cell divisions showed stepwise accumulation of ribosomal RNA, with maximum rates of accumulation just before the onset of DNA synthesis and at the time of the first division. Protein accumulation and phosphate uptake also increased at these times.

Various DNA-binding antibiotics, 5-fluoro-uracil, hormone conditions in the medium and light were investigated for their effects on cell division and on DNA, RNA and protein accumulation. It was found that protein accumulation can occur without RNA accumulation, and that protein and RNA accumulation can occur without cell division. The pattern of RNA accumulation remained stepwise when DNA synthesis was inhibited by mitomycin. These results favour an interpretation of the periodicity of early metabolism as a consequence of excision and 2,4-D supply rather than as a consequence of the cell division cycle. Experiments with 5-fluoro-uracil and thymidine showed that a period of RNA synthesis is required for the induction of DNA synthesis and cell division. Later RNA synthesis, not required for the first division, is required for the occurrence of the second and subsequent divisions.

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ABBREVIATIONS.

DNA	deoxyribonucleic acid.	fc	foot candles
RNA	ribonucleic acid.	A_{260}	Absorbance at
r-RNA	ribosomal ribonucleic acid.		260 nm.
t-RNA	transfer ribonucleic acid.	m	million.
m-RNA	messenger ribonucleic acid.	s	sedimentation
D-RNA	DNA-like ribonucleic acid.		co-efficient.
A	Adenylic acid (AMP).		
C	Cytidylic acid (CMP).		
G	Guanylic acid (GMP).		
U	Uridylic acid (UMP).		
T	Thymidylic acid (TMP).		
TTP	Thymidine triphosphate.		
TCA	Trichloroacetic acid.		
PCA	Perchloric acid.		
tris	tris(hydroxymethyl)aminomethane.		
DMSO	Dimethyl sulphoxide.		
EDTA	Ethylenediaminetetraacetic acid.		
TNS	Sodium tri-isopropylnaphthalenesulphonate.		
NDS	Sodium naphthalene 1,5-disulphonate.		
4-AS	Sodium 4-amino salicylate.		
SDS	Sodium dodecyl sulphate.		
2,4-D	2,4-dichlorophenoxyacetic acid.		
POP	2,5-diphenyloxazole.		
POPOP	2-p-phenylenebis(5-phenyloxazole)		
5-FU	5-fluoro-uracil		
FUdR	5-fluoro-deoxyuridine.		
DNase	deoxyribonuclease.		
RNase	ribonuclease.		

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INTRODUCTION.

Little is known of the molecular mechanisms controlling cell division and its onset in previously non-dividing tissues. Existing work points to an important role of ribonucleic acid in these controls. This thesis is an examination of RNA metabolism and its connection with cell division in dividing Jerusalem Artichoke tuber cells.

1. The development of a system for cell division studies.

Growth in micro-organisms is basically cell division combined with the maintenance of cell size. This simplicity has prompted many investigations into cell dvision in these organisms. In higher organisms, growth is more complex, involving cell expansion, cell specialisation and inter-cellular coordination as well as cell division. The study of cell division in higher organisms is therefore facilitated by choosing a situation in which cell division occurs in some degree of isolation from the other growth processes. Some criteria of an ideal system for the study of cell division are:

Uniformity of cell type.

Uniformity of cell behaviour. A high percentage of dividing cells is desirable to eliminate possible side-effects from the metabolism of non-dividing cells.

Synchrony of division permits the determination of temporal relations between cell division and other aspects of metabolism. Systems in which synchrony is inherent or induced mech-

anically are preferable to those in which synchrony is a result of chemical treatment, which might introduce undesirable side effects.

The material must be available in amounts sufficient for biochemical analysis, and should be available for use for most of the year.

Freedom from growth processes other than cell division.

In the case of tissue cultures, a completely defined medium is desirable, to eliminate the chance of side effects from unknown components.

In higher plants, various situations provide partial fulfilment of these conditions.

Root and shoot tips. Meristematic regions containing cells active in division. Culture of excised tips is possible (Gautheret, 1932, 1959; Bonner and Addicott, 1937; Heyes, 1963). However the organ contains a variety of cell types, and products of division quickly embark on an expansion phase of growth. The divisions are not synchronous, though Clowes (1962) has observed a circadian rhythm in mitotic frequency in Zea root tips. Higher degrees of division synchrony have been induced in root tips by treatment with 5-amino-uracil. (Smith, Fursell and Kugelman, 1963; Clowes, 1965; Mattingly, 1966) Root tips have been of most value in studies of the development of the cell from the embryonic state to the mature, differentiated state. (Brown, 1963; Heyes, 1963).

Gametogenesis. In the gymnosperms, many cases of inherently synchronous division occur in the formation of the female gametophyte. These have been reviewed in detail by Erikson

(1964). Little other than descriptive cytological work has been reported for these tissues. They are not suitable for biochemical studies because of the difficulty of obtaining large amounts of material.

The developing microspores of the liliaceous genera Lillium and Trillium provide material with highly synchronous division, which have been the subjects for considerable research. The effects of radiation damage (Sparrow, 1951), changes in respiration (Erikson, 1947) and nucleic acid metabolism (Hotta and Stern, 1963, 1963a; Hotta, Parchman and Stern, 1968) are among the aspects investigated. The disadvantages of this system include the difficulty of obtaining large amounts of material and the long duration of the divisions. Only two divisions occur, of which the first is a reduction division.

Embryological situations. The nuclear divisions occurring during the formation of endosperm have been studied by Bajer (1958, 1958a) and Bajer and Molè-Bajer (1954). Although the divisions are synchronous, the synchrony has not been exploited for biochemical study because of the difficulty of obtaining sufficient material, and because the divisions are dependent on fertilisation.

The growth of the embryo itself is a situation involving cell division. Haber, Carrier and Foard (1961) and Haber (1962) have studied the effects of auxin and kinetin on cell division and cell expansion as separable processes in this location.

Local stimulation of division. Growth may be stimulated locally in previously non-dividing tissues. Micro-organism infection, e.g. root nodules produced by the Rhizobium - legume symbiosis, and crown gall tumours induced by Agrobacterium may

provide especially convenient sites for the study of the induction of cell division. Among aspects of growth already studied is the occurrence of endopolyploidy in the constituent cells of the nodule (Mitchell, 1965).

Renewed cell division leading to callus growth is a common reaction to physical damage in plants. (Haberlandt, 1921, 1922; Noel, 1968). This response forms the basis of most plant tissue culture work. The capacity of highly differentiated plant cells to revert to an embryonic, dividing state is an expression of the high totipotency of plant cells. This is in direct contrast to the situation in higher animal cells. Only rather atypical mammalian cells, such as the tumorous HeLa line (Scherrer, Syverton and Gey, 1953) or the baby hamster kidney line BHK 21 (MacPherson and Stoker, 1962; Russel et. al. 1964) will undergo protracted division and sub-culture satisfactorily.

In plants, it was until recently only tissues from dicotyledons which yielded satisfactory tissue cultures. However it is now possible to establish cultures from monocotyledons such as asparagus. (Wilmar, Hellendoorn and Marja, 1968)

Individual plant cells may be cultured (Muir et. al. 1954; Street and Henshaw, 1963) but cell divisions are infrequent. Eriksson (1966) has induced partial synchrony of division in suspension cultures of Happlopappus cells by 5-amino-uracil or hydroxyurea treatment.

Callus cultures with high levels of cell division have been established using secondary phloem of carrot (Caplin and Steward, 1948), potato tuber tissue (Steward and Caplin, 1951) tobacco stem pith (Silberger and Skoog, 1963) and Jerusalem

Artichoke tuber tissue. (Yeoman, Dyer and Robertson, 1965). Most of the work has been directed to the investigation of the effects of plant growth substances on growth and the development of new roots and shoots. For example, Steward et. al. (1964) described the growth of a complete carrot plant from a single phloem tissue culture cell. One of the few studies directed at the understanding of cell division as such was by Evans (1967) with the artichoke tuber tissue.

Cultures of artichoke tuber tissue have particular advantages for the study of cell division. These include a high reproducibility of response from individual explants (Robertson, 1965) and a year-round supply of adequate amounts of material. The cells are uniformly parenchyma, except for a fraction of one percent of tracheids. (Naik, 1965). Robertson (1965) and Yeoman and Evans (1967) showed that the early cell divisions are inherently synchronous. Adamson (1962) and Mitchell (1967) have shown that the cells of the tuber are all in the 2C state, and Mitchell (1967a) has shown that all cells contain similar amounts of protein and RNA. The synchrony appears to be a result of placing similarly endowed cells in conditions stimulating division at the same time.

There is also a useful background of other work on the tissue. Robertson has investigated the long term growth of callus cultures (Robertson, 1965), and Naik (1965) has studied temperature effects. The influence of hormones on cell division and cell expansion have been considered by Adamson (1962) and Setterfield (1963). Other aspects studied include the effects of auxin on expansion growth and respiration (Bonner, Bandur-

ski and Millerd, 1953; Hackett and Thimann, 1952); the effects of auxin on expansion growth and protein synthesis (Thimann and Loos 1957; Noodén and Thimann, 1963) and carbohydrate metabolism, (Jefford and Edelman, 1963).

Some disadvantages of the sytem used by Yeoman, Dyer and Robertson (1965) for short term division studies included the use of an undefined medium and a culture method which did not permit the growth of large amounts of material. Division activity was low, with only about 40% of the cells entering the first division. Also, the response was subject to seasonal variation.

Section 1 of this thesis is concerned with the following modifications to the system to one more suited to the study of RNA metabolism and cell division:

A. The system as described by Yeoman et. al (1965, 1967) involved the culture of individual explants of tissue (8 mg fresh weight) on agar or in roller tubes. The time required for setting up the cultures by this method limits the size of an experiment to about 150 explants. A liquid medium, bulk culture system was designed which permitted experiments involving 3000 explants.

B. Robertson (1965) showed that long term callus growth depends on the hormones 2,4-D and coconut milk. An examination of the stimulant requirements for short term growth permitted the elimination of coconut milk giving a completely defined medium. The 2,4-D concentration was altered to the optimum for short term growth. These modifications increased the percentage of cells entering the first division from 40 to 65.

The effects of Kinetin, known to affect cell division in this tissue (Setterfield, 1963) were examined briefly.

C. Haberlandt (1921, 1922) recognised the importance of materials from the outer damaged cells in stimulating cell division in potato tuber slices. Many reports record the secretion of materials into the medium by growing tissues, including amino acids (Tulecke and Rutner, 1965), chelating agents (Devillers-Anson, 1961) and peroxidase (Lipetz and Galston, 1959). Robertson, (1965) reported gradual autolysis in the outer cells of artichoke explants during culture.

The accumulation of materials in the medium during culture was examined. An attempt was made to find the significance of the material from the outer, damaged cells for the process of cell division in the tissue.

D. The culture technique used by previous workers with this system involved excision and planting of explants in fluorescent light, followed by culture in total darkness. The effects of light on other non-green tissue cultures are generally to inhibit growth. Zeevaart and Kivilaan (1966) claimed an inhibition of cell division in flax cultures by 900 fc. blue light, but based their measure of division on fresh weights only. Klein (1964) found that green and near ultra-violet light inhibited the fresh weight increases of various tissue cultures and crown gall cultures, while DeCapite (1955) reported a dependence of carrot and virginia creeper growth on light and temperature.

In this investigation of the artichoke, light was found to be a potent inhibitor of cell division. Excision and planting in dim green light raised the percentage of cells entering the first division to 85 - 90.

E. Robertson (1965) and Evans (1967) showed that the tissue

response remained constant for six months after harvesting the tubers in November. After six months, the lag phase before the first division began to lengthen. The seasonal variation in response of the culture system developed in this thesis was examined for a number of factors. This permitted an evaluation of the suitability of the system for year round examination of nucleic acid metabolism and cell division.

2. RNA metabolism and the control of growth.

In bacteria, much is now known of the way in which RNA is involved in the control of growth and response to environmental change. The control mechanism hypothesis of Jacob and Monod (1961) was based on observations on induction and repression of enzyme synthesis resulting from changes in the composition of the culture medium. They postulated that information from the gene for the synthesis of a specific protein is carried to the site of protein synthesis by an unstable messenger RNA. The gene from which the messenger is transcribed is a structural gene. Its activity is controlled by a repressor, the product of a regulator gene, which by binding to an operator gene ajacent to the structural gene prevents transcription. Substances from the environment control the activity of the structural gene by binding with the repressor, either to reduce or to increase its affinity for the operator.

The hypothesis has received considerable experimental verification. Brenner, Jacob and Meselson (1961) and Gros et. al. (1961) demonstrated an RNA fraction with messenger properties in

bacteria. This m-RNA has been shown to stimulate amino acid incorporation into polypeptides in cell free systems. (Nirenberg and Matthaei, 1961; Artman, Silman and Engleberg, 1967). Recently, Gilbert and Müller-Hill have isolated a protein which they claim is the repressor for the Lac genes.

In higher organisms, there are few examples of situations where environmental change leads to specific and completely understood biochemical reaction. Enzyme induction does occur in higher plants, for example the induction of nitrate reductase by NO_3^- (Key and Ingle, 1964) but the phenomenon is rare. There are many situations where environmental change leads to a specific biological response. However the mechanism of this response is often much more complex than the simple case of enzyme induction or repression. Often a complex biochemical or biophysical mechanism is an intermediate between the stimulus and its biological expression. Examples include the phytochrome system in the photoperiodic induction of flowering, and auxin in the tropic response of young shoots towards unilateral light. Yet under different conditions, one intermediate mechanism may have diverse effects. Auxin, for example, may stimulate cell division or expansion in tissue cultures, yet can reduce cell expansion in root tissue and also has effects on abscission of leaves and fruits.

To explain development of cells in a constant environment, it is necessary to postulate internal control of gene action. For example, in the root tip, a cell begins in the dividing state, and progresses through expansion and differentiation phases during root growth. Brown (1963) has proposed that the metabolic state of the cell at any time acts as the inducer for

the succeeding stage of development.

From studies with inhibitors, it is known that RNA and protein synthesis are required for the induction of biological response and the control of development. Key (1964) has shown that RNA and protein synthesis are necessary for auxin induced cell expansion. A requirement for RNA and protein synthesis for the development of respiration in potato tuber slices was shown by Click and Hackett (1963). In the developing root cell system, studies with 8-aza-guanine (Heyes, 1963) and ribonuclease (Yeoman, 1962) have shown that RNA synthesis is necessary for the induction of the succeeding stage in the development.

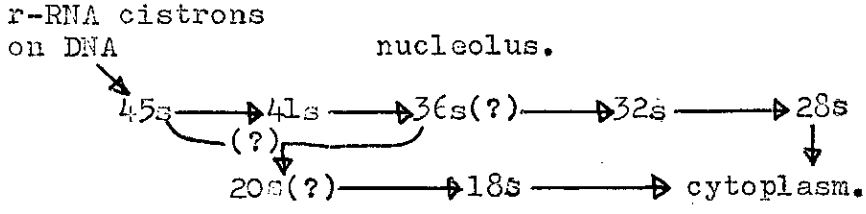
Although the gross involvement of RNA synthesis is well established, the precise mechanisms of gene control and RNA action in higher organisms are unknown. While a control system such as that proposed for the bacterial situation (Jacob and Monod, 1961) might have a part to play in the mediation of higher organism growth and response to environment, it is possible that other control mechanisms are involved, including control at sites other than the gene. Clearly a detailed knowledge of the behaviour and synthesis of RNA in higher organism cells is necessary for a fuller understanding of the control of biological events. Section 2 of this thesis deals with two aspects of RNA metabolism in the artichoke tissue.

Synthesis of ribosomal RNA.

In bacteria, Hecht and Woese (1968) have suggested separate precursors for the 23s and 16s ribosomal RNAs. The precursor to 16s appears to be 130,000 daltons heavier than the 16s; the precursor to 23s 50-100,000 daltons heavier than 23s.

Hecht and Woese note the possibility that the fragments removed during the processing of the precursors might give rise to the 5s ribosomal RNA. This is consistent with the results of Hecht, Bleyman and Woese (1968), which indicate an indirect origin for 5s rather than a direct transcription from DNA.

The synthesis of r-RNA in HeLa cells involves a 'polycistronic' 45s precursor synthesised in the nucleolus. (Perry, 1962; Scherrer, Latham and Darnell, 1963; Penman 1966). This is processed in the nucleolus through a series of intermediates to yield one 28s and one 18s r-RNA molecule, (Weinberg et. al. 1967), which then pass into the cytoplasm. The scheme suggested for the precursor processing is:



In the toad Xenopus, Birnstiel et. al. (1968) have shown that the 18s and 28s cistrons alternate along the DNA. A heavy precursor, with a molecular weight of 2.5m is the first detectable product of these genes.

In plants, Lonberg-Holm (1967) reported a heavy, rapidly labelled RNA, which was not further characterised. Loening (1967) observed a high molecular weight, highly labelled RNA in pea roots which was similar to the ribosomal precursor in animal cells. Murphy and Lovett (1966) reported a heavy, rapidly labelled RNA in Blastocladiella, which decreased in specific activity during the 'chase' incubation while the specific activity of the r-RNA increased. There have been no other reports, and no detailed characterisation of precursors in plant

material.

In Section 2, a heavy RNA fraction is described in artichoke tissue. Analyses of base compositions and the kinetics of labelling of this RNA and of the ribosomal RNAs suggest that the heavy RNA is a precursor to r-RNA, in some ways similar to those reported in animal cells.

Messenger RNA.

According to the hypothesis of Jacob and Monod (1961), messenger RNA should be heterogeneous in weight, should label rapidly and decay quickly, should reflect the base composition of the DNA and should be made in the nucleus and transported to the cytoplasm, there to associate with ribosomes for protein synthesis. All these conditions are satisfied in the bacteria.

In higher organisms, the situation is less well understood. A heavy, rapidly labelled, heterodisperse RNA is produced in the nucleus of animal cells. (Scherrer, Latham and Darnell, 1963). Most of this is broken down in the nucleus. (Scherrer et. al., 1966; Harris, 1964; Bramwell and Harris, 1967; Birnboim, Pene and Darnell,1967). A possible explanation was that this was polycistronic messenger, such as has been found in bacteria (Friesen, 1968), which was broken down selectively so that the messenger for one of the proteins only entered the cytoplasm. While this would account for the lower s values of the cytoplasmic heterodisperse RNA, recent research by Penman, Vesco and Penman (1968) suggests that in HeLa cells, nuclear and cytoplasmic heterodisperse fractions are unrelated in their kinetics of formation.

In the cytoplasm, not all of the heterodisperse RNA appears

to be messenger. Penman et. al. (1968) reported a heterodisperse fraction in the cytoplasm of HeLa cells sedimenting between 10s and 70s which was distinctly different from the heterodisperse RNA associated with the polyribosomes, sedimenting between 8 and 30s. Clearly heterodisperse nature is no indication of messenger function.

The association with polyribosomes might be considered the best criterion of messenger nature. This has focussed research on cytoplasmic messenger on the reticulocyte, which produces about 90% of its protein as haemoglobin. (Lamfron, 1961). But even in this simple situation, confusion exists as to the exact nature of the haemoglobin messenger. The triplet code hypothesis leads to an estimated sedimentation co-efficient of 8s for the messenger. Drach and Lingrel (1965) suggested a homogeneous 16s messenger, while Mathias, Williamson and Huxley (1964) proposed a heterodisperse (14s to 40s) distribution. Gould, Arnstein and Cox (1966) presented data consistent with an 8s messenger which is covalently bound to ribosomal RNA.

The ultimate test of messenger nature is the ability of a specific RNA to stimulate the production of a specific protein in a cell free system from another organism. So far this has only been satisfactorily acheived with viral RNA (Ohtaka and Spiegelman, 1963). Drach and Lingrell (1966) found that reticulocyte 'messenger RNA' did stimulate amino acid incorporation in an E.coli cell free system, but the polypeptides produced resembled E.coli proteins more than haemoglobin. Arnstein and Rahamimoff (1968) have suggested that differences in the method of protein chain initiation between animals and E. coli may prevent transcription of haemoglobin and other animal messengers in the E. coli cell free system.

In plants, RNAs with certain messenger characteristics have been reported. Loening (1962, 1965) found a rapidly turning over fraction in pea roots with a DNA-like base composition. In the soybean, a rapidly turning over, heterogeneous in size RNA with DNA-like base composition was found to be necessary for growth. (Ingle, Key and Holm, 1965; Key and Ingle, 1964; Key, 1966). An association of some of this RNA with polyribosomes has been demonstrated by Lin, Key and Bracker (1966). While these fractions in plants probably contain messenger RNA, they might also include RNAs similar to the nuclear heavy heterodisperse and cytoplasmic non-messenger fractions of HeLa cells. (Penman, et. al. 1968). It is important for the fuller understanding of control systems in the higher plant cell that the nature of the cytoplasmic messenger and its production are understood, and that the significance of the other heterodisperse fractions is determined.

This uncertainty about the nature of m-RNA makes it premature to attempt to explain biological events on the basis of changes in the synthesis of m-RNA. In the artichoke tissue, the investigation of heterodisperse RNA was limited to the demonstration of such fractions in the nucleus and cytoplasm, and a preliminary assesment of their kinetics of labelling.

Techniques.

Various techniques have been employed for the study of RNA metabolism. Autoradiography (e.g. Perry, 1962) has the advantage that it is not necessary to extract RNA from the tissue, and the intracellular location of the newly synthesised RNA may be determined. Woods and Zubay (1965) have developed a method

with Vicia root tips for the selective extraction of t-RNA from sections. This permits the examination of the location of synthesis of known types of RNA

All other important methods for RNA study involve the extraction of RNA from the tissue and its fractionation into different molecular species by various methods. The extraction method is required to give a high yield of RNA, it should not change the nature of the RNA, e.g. by permitting degradation, and it may be required to give a meaningful sub-cellular fractionation. Plant material presents special problems for RNA extraction. The cell wall is tough and must be disrupted. This leads to the danger of damage to the nucleus spoiling the cell fractionation, and damage to the vacuole and other organelles releasing acids and nucleases. Various methods of tissue disruption, sub-cellular fractionation and RNA extraction were tested on the artichoke tissue. Factors influencing the degradation of r-RNA during extraction were studied. From these preliminary investigations, it was possible to arrive at suitable methods for the preparation of RNA from the tissue.

RNA may be fractionated by electrophoresis on polyacrylamide gels (Loening, 1967) or on agar gels (Tsanev, 1965; McIndoe and Munro, 1967). Polyacrylamide gel electrophoresis was the method chosen for the study of artichoke RNA. The fractionation is largely by molecular weight differences. The resolution obtained by gel electrophoresis is much higher than that obtainable by column chromatography on MAK (methylated albumin-kieselguhr) (Mandell and Herschey, 1960) or by density gradient centrifugation, two other commonly used fractionation methods. Polyacrylamide gel electrophoresis is also suitable for the rapid processing of large numbers of RNA samples.

3. RNA synthesis and cell division.

Studies on the relation between RNA synthesis and cell division may be divided into two groups, those which seek to define the temporal relations between RNA synthesis and cell division, and those which seek to interpret the significance of RNA synthesis in the control of cell division.

Clearly, many aspects of metabolism are closely linked to RNA synthesis and cell division. Also, in the present state of ignorance of messenger RNA function and gene transcription control in higher organisms, the study of enzyme synthesis provides the best, though indirect, means of examining m-RNA activity. For these reasons, DNA and protein synthesis as well as RNA synthesis have been considered in the review of dividing systems below and in the experimental work in Section 3.

Studies on temporal relations in the cell division cycle.

Although asynchronously dividing cells may be studied by the appropriate microscopic techniques (e.g. Seed, 1963; Killander and Zetterberg, 1965), the majority of studies in this field have been made with synchronously dividing cells.

Mitosis. During mitosis the nuclear membrane is absent. An abrupt release of RNA from the nucleus to the cytoplasm at the time of disappearance of the membrane has been reported in Amoeba (Rabinowitz and Plaut, 1956) and HeLa cells (Prescott and Bender, 1962).

The nucleolus, known to be the site of ribosomal RNA synthesis (Perry, 1962; Birnstiel, 1967) disappears during mitosis,

and the chromosomes contract. Many reports suggest that this affects their ability to act as templates for RNA synthesis. In the slime mould Physarum, Mittermayer, Braun and Rusch (1964) found a reduced rate of RNA synthesis during mitosis. Mittermayer, Braun, Chayka and Rusch (1966) found a temporary decrease in the proportion of ribosomes present as polyribosomes shortly after mitosis. They suggested that this was a result of reduced m-RNA synthesis during mitosis.

Using the meiotic division during microgametogenesis in liliaceous plants, Hotta and Stern (1963) showed high rates of RNA synthesis from pachytene to diakinesis, and a lower rate at metaphase. Hotta and Stern (1966) reported that isolated leptotene nuclei or chromosomes (uncontracted) supported higher rates of RNA synthesis in vitro than did isolated metaphase nuclei or chromosomes (contracted). An interesting study by MacKenzie, Heslop-Harrison and Dickinson (1967) suggested that most of the ribosomes are destroyed and resynthesised during meiosis.

In synchronised HeLa cells, Scharff and Robbins (1965) showed lower rates of RNA synthesis during mitosis. All types of RNA synthesis appeared to be depressed. Johnson and Holland (1965) reported that when HeLa cells were maintained in metaphase by vinblastine treatment, protein and RNA synthesis were almost completely suppressed, and active polysomes disappeared. The absence of any RNA synthesis in an in vitro system when purified RNA polymerase was added suggests that the contracted DNA was unavailable as a template.

Measurements of gross RNA and protein contents of artichoke explants (Evans, 1967, Mitchell, 1968), show that both

components are increasing rapidly at the time of the first mitosis. Only 40% of cells divided in the system used by these workers, but Mitchell (1967a) has suggested that only dividing cells accumulate RNA.

Interphase. A crucial event in interphase is the replication of the cells DNA. In bacteria growing in log-phase, this occupies virtually the whole of interphase. (Schaechter, Bentzon and Maaløe, 1959). In the vast majority of higher organisms, DNA synthesis occupies only part of interphase. The terminology of Howard and Pelc (1953) is convenient in this connection. The period of DNA synthesis is S-phase, the period of interphase preceeding it is G1 and the period succeeding it is G2. The relative lengths of G1, S and G2 vary greatly in different organisms. (Prescott, 1964). In the artichoke system, a complication is that the cells require a period of adjustment (G0) before they can enter G1. Evans (1967) has calculated the durations of the phases as G0 + G1, 8 hours; S, 14 to 16 hours; G2, 0 to 2 hours.

During interphase, different organisms exhibit diverse patterns of RNA, gross protein and specific enzyme synthesis. Decreased RNA synthesis rates during DNA replication have been observed in slime moulds (Nygaard, Güttes and Rusch, 1960) and during microgametogenesis in Tradescantia, (Moses and Taylor, 1955; Siskin, 1959) and Tulbaghia.(Taylor, 1958). This is thought to be because replicating DNA is unavailable as a template for RNA synthesis. Confirmation of this comes from work on the macronucleus of the ciliate Euplotes. Prescott and Kimball (1961) found RNA synthesis in all parts of the nucleus apart from those

regions making DNA. Various workers with HeLa cells, however, have found no impairment of RNA synthesis during S-phase. (Seed, 1963; Killander and Zetterberg, 1965; Scharff and Robbins, 1965). Reiter and Littlefield (1964) did report a decrease in nuclear RNA synthesis during early S-phase, but this may have been a side effect of division synchronisation by FUdR treatment. Scharff and Robbins (1965) have calculated that less than 5%, and probably only 1% of the HeLa cell genome replicates at one time. Nevertheless it is remarkable that the replication of the 400 ribosomal RNA cistrons, which are grouped together, has no effect on the rate of r-RNA synthesis.

Various organisms show marked periodicity of syntheses during interphase. In the slime moulds, Mittermayer et. al. (1964, 1966) have shown periods of increased RNA and protein synthesis in early and late interphase, with decreased activity in mid-interphase. The DNA replication in this system occurs in early interphase. (Nygaard et. al. 1960) Hotta and Stern (1963, 1963a) have shown a period of thymidine kinase synthesis just before S-phase in microgametogenesis in Lillium. Hotta, Parchman and Stern (1968) have suggested that proteins essential for chromosome pairing and chiasma formation are synthesised between zygotene and pachytene. Robbins and Borun (1967) in HeLa cells, and Prescott and Kimball (1961) with Euplotes observed that histones are synthesised at the same time as DNA.

The bacteria and yeasts provide many examples of periodic enzyme synthesis in synchronously dividing material. (Masters et. al., 1964; Hanawalt and Wax, 1964; Kuempel, Masters and Pardee, 1965). In fission yeast, Bostock et. al. (1966) reported that certain enzymes, such as aspartate and ornithine trans-

carbamylase increase periodically, while others, such as maltase and sucrase increase linearly during the division cycle.

The periodic increases in enzymes in bacteria and yeasts are not reflections of increases in cistron number during DNA replication. Increases may occur at times other than S-phase. (Bostock et. al. 1966) No increase in enzyme activity occurs between the steps. Nevertheless the time of increase in the cell division cycle does appear to depend on the position of the gene on the chromosome. (Halvarson, 1966)

A similar periodicity for RNA synthesis has been reported in E. coli.(Rudner et. al. 1964, 1965). Base composition analyses suggested that the RNA synthesised was predominantly ribosomal. The time of the burst of synthesis was also related to the calculated positions of the genes on the chromosome.

In the artichoke tissue, Evans (1967) and Mitchell (1967a, 1968) have shown a period of increase of RNA and protein just before the beginning of the first S-phase. The RNA and protein contents remained constant during the S-phase and the short G2, and then increased at the time of the first division. In Section 3 of this thesis, the first aim was the description of changes in r-RNA, t-RNA and protein during the first few divisions in tissues with high levels of cell division. All three features showed a marked periodicity of accumulation. In an attempt to relate this to the cell division cycle, changes were examined in explants prevented from dividing by strong light, hormone conditions and mitomycin C. Iyer and Szybalski (1963) have shown that mitomycin C is a specific inhibitor of DNA synthesis in bacteria, with little effect on RNA synthesis. The action appears to be by a cross-linking of the DNA strands.

On discovering that the periodicity of RNA synthesis was independent of DNA synthesis and cell division, an attempt was made to relate the periodicity to the processes of excision and hormone supply.

The significance of RNA and protein synthesis in the control of cell division.

When Tetrahymena is grown in medium deficient in pyrimidines, RNA accumulation is blocked. (Lederberg and Mazia, 1960, Prescott, 1960) Cell division and DNA synthesis continue for extended periods under these conditions. Prescott (1964) has shown that net synthesis of RNA continues, using precursors from degraded RNA.

Most studies on the necessity of RNA and protein synthesis for cell division have involved the use of inhibitors of these syntheses. For example, Morris (1967) found that cycloheximide had an immediate effect on protein synthesis in Chlorella. DNA synthesis and cell division were inhibited after a short delay. Baserga, Estensen, Petersen and Layde (1965) found that actinomycin had an immediate inhibitory effect on RNA synthesis on Ehrlich ascites tumour cells, and that DNA synthesis and cell division were inhibited later.

Cleavage in the sea urchin egg presents a contradictory result. Gross and Cousineau (1963, 1963a) found that actinomycin treatment inhibited RNA synthesis but did not inhibit cell division. Inhibition of protein synthesis by puromycin (Timourian and Uno, 1967) does inhibit cell division. The explanation is that very stable messengers required for the first few divisions seem to be synthesised before fertilisation, and immediately after fertilisation in a period when the egg is imper-

meable to actinomycin. (Gross and Fry, 1966).

Many attempts have been made to find the times in the cell cycle of the periods of RNA and protein synthesis required for cell division. Working with Ehrlich ascites tumour cells, Baserga, Estensen and Petersen (1965) showed by actinomycin-D treatment a period of RNA synthesis during G1 which was necessary for DNA synthesis. Nachtwey and Dickinson (1967) found that division could be inhibited in Tetrahymena by actinomycin-D if applied early enough in the cell cycle. Inhibition of cell division by later treatment was possible only if a higher concentration of actinomycin-D was used.

Investigations with mammalian cells suggest that cells in G2 are highly competent to complete division. Buck, Granger and Holland (1966) failed to inhibit mitosis in HeLa cells with actinomycin-D or puromycin applied up to 2 hours before mitosis. Kishimoto and Lieberman (1964) found that actinomycin-D applied during G2 did not inhibit cell division in cultured kidney cortex cells. Puromycin did inhibit division but p-fluorophenylalanine (FPA) did not. They suggest that this may indicate that G2 is characterised by the synthesis of structural proteins required for division, which can function adequately when containing FPA. Hotta, Parchman and Stern (1968) have shown that protein synthesis is essential during the early stages of meiosis in microgametogenesis.

Systems in which cell division is induced in previously non-dividing cells provide a unique opportunity for the study of the necessity of RNA and protein synthesis for the induction of DNA synthesis and cell division. These systems include the

regeneration of rat and mouse liver after partial hepatectomy, the culture of mammalian kidney cortex cells and the culture of explants from the quiescent artichoke tuber, the system used in this thesis.

When kidney cortex cells from rabbit are cultured, RNA and protein increase 1.5 to 2 fold before DNA synthesis begins. (Lieberman and Ove, 1962) Inhibition of either RNA or protein synthesis prevents DNA synthesis. Lieberman, Abrams and Ove (1963) have shown a stepped doubling of the rate of RNA synthesis during the pre-DNA syntheis period. Low concentrations of actinomycin-D had no effect on the initial rate of RNA synthesis, but inhibited the rise in rate of synthesis and the subsequent DNA synthesis.

In the regeneration of liver, Bollum and Potter (1958) have shown a several-fold increase in DNA polymerase activity before S-phase, and Tsukada and Lieberman (1964) detected an increase in RNA polymerase activity in the nucleolus. The pattern of RNA and protein accumulations, and the dependence of DNA synthesis on preceeding RNA and protein synthesis are similar to the events in cultured kidney cells, (Fujioka, Koga and Lieberman, 1963). Church and McCarthy (1967, 1967a) have shown that more of the DNA becomes available for hybridisation with labelled RNA in hepatectomised liver than in normal liver. Comparing the cytoplasmic and nuclear heterodisperse fractions, Church and McCarthy (1967b) showed increases in both cytoplasmic and nuclear hybridisable RNA. They claim that some of the nuclear heterodisperse stimulated by partial hepatectomy remains in the nucleus, but that some RNA normally confined to the nucleus appears in the cytoplasm in the regenerating liver. In view of the lack of

knowlege of the nature of the heterodisperse RNA and the lack of specificity of the hybridisation reaction, it is perhaps an over-simplification to claim the production of new cytoplasmic messengers and a general de-repression of genes.

In the artichoke tissue, Evans(1967) and Mitchell (1967a, 1968) have shown increases in RNA and protein prior to S-phase. In Section 3, studies with RNA synthesis inhibitors suggest that RNA synthesis is required for the induction of cell division in this tissue. Inhibition of RNA synthesis after the first division inhibited the later divisions.

Inhibitors. The investigation of the dependence of cell division on RNA synthesis involved the use of various types of inhibitors.
A. DNA-binding antibiotics inhibiting both DNA and RNA synthesis. Proflavine (Lerman, 1961;Peacocke and Skerret, 1965), ethidium and daunomycin (Ward, Reich and Goldberg, 1965) inhibit by intercalation of the flat, polycyclic molecule between adjacent base pairs on the DNA. The resulting distortion of the double helix inhibits its use as a template.
B. Base-specific DNA-binding antibiotics, inhibiting RNA synthesis. It was originally hoped to differentiate between the significances of different classes of RNA for cell division. Actinomycin-D, at low concentration has been shown to inhibit r-RNA synthesis preferentially in mammalian cells and bacteria. (Perry, 1962; Perry, Srinivasan and Kelley, 1964; Samarina 1964; Harel et. al., 1964) Key (1966) also found this effect in soybean hypocotyls, but higher concentrations also inhibited D-RNA synthesis and growth. Other examples of actinomycin-D inhibition of messenger-type RNA synthesis include the studies on rat-liver polyribosome

breakdown by Stahelin, Wettstein and Noll (1963) and Scherrer, Latham and Darnell's (1963) observations on unstable nuclear RNA in HeLa cells.

An explanation for the preferential inhibition of r-RNA synthesis might come from the base binding specificity of actinomycin-D. The binding is to the minor groove $-NH_2$ group of guanine residues. (Kersten, 1961; Ward, Reich and Goldberg, 1965) Müller and Crothers (1968) have suggested that the actinomycin chromophore is intercalated between DNA bases at this point. Ribosomal RNA has a higher G + C content than messenger type RNA. Its parent DNA would therefore be expected to have more binding sites available for actinomycin-D. Differences in the sites of the cistrons, those for r-RNA being nucleolar and those for m-RNA on the chromatin (Perry, 1962, Matsuda and Siegel, 1967) might also influence this effect.

In this study, the effects of actinomycin-D and two other G-specific binding antibiotics, chromomycin A_3 and olivomycin (Kersten, 1961; Ward, Reich and Goldberg, 1965) were examined. In an attempt to obtain preferential inhibition of m-RNA synthesis, the antibiotic nogalamycin was tested. This binds preferentially to A-T rich DNA. (Bhuyan and Smith, 1965). The possibility of differential action derived from different base-binding behaviour is considered in the discussion in the light of the effects of these drugs and base compositions of RNA species determined in Section 2.

C. Another attempt to inhibit selectively the synthesis of r-RNA involved the RNA base analogue 5-fluoro-uracil. Key (1966) found that the analogue was incorporated into all classes of RNA in soybean hypocotyls. Ribosomal RNA synthesis was inhibited, but

D-RNA and protein synthesis were not. This suggests that the function of D-RNA as a template for protein synthesis was not inhibited by its 5-FU content. Levin (1966) has presented evidence for an active messenger RNA in bacteria containing another base analogue, 8-aza-guanine.

Key (1966) also reported an inhibition of DNA synthesis by 5-FU. In bacteria, this inhibition is known to operate through an inhibition of the enzyme thymidylate synthetase, (Cohen et. al., 1958; Aronson, 1961a, Heidelberger, 1963.), leading to a shortage of TTP for DNA synthesis. The direct inhibition of DNA synthesis in bacteria may be relieved by a thymidine supply. A similar effect of thymidine on 5-FU action on DNA synthesis was sought and found in the artichoke tissue. This permitted the simpler interpretation of the consequences of 5-FU inhibition of RNA synthesis for cell division.

For much of the study of the relation between RNA and protein synthesis and cell division, it was necessary to process large numbers of samples. The later experiments were therefore confined to estimation of total quantities of RNA, DNA and protein. Appendix 1 deals with the development of methods for making such measurements in the artichoke tissue.

SECTION 1.

This section describes experiments to adapt the artichoke tuber tissue culture system of Yeoman, Dyer and Robertson (1965) for use in the study of RNA metabolism and cell division. A liquid bulk culture system was developed, using a completely defined medium and yielding 85 - 90% of cells entering the first division. An inhibitory effect of light on cell division was discovered.

MATERIALS AND METHODS.

Tubers. A clone of Jerusalem Artichoke, Helianthus tuberosus L. var. Bunyard's Round was maintained at the Department of Botany, King's Buildings, Edinburgh. Tubers were harvested in November and stored in damp sand in polythene bags at 4°C. Sprouting of buds commenced in May of the following year.

Glassware. All glassware was immersed in 50% methylated spirit, N NaOH for 24 hours, washed with hot water, immersed in N/10 HCl for 24 hours and rinsed with distilled water before drying at 95°C.

Chemicals. All chemicals were B.D.H., 'Analar' grade where obtainable.

Culture media. Culture was in a liquid medium based on that of Bonner and Addicott (1937) as modified by Yeoman, Dyer and Robertson (1965). The pH was 5.5

Basic composition	sucrose	4.0g
Solution A	$FeCl_3$	2.0mg
	$Ca(NO_3)_2$	236.0mg
Solution B	KH_2PO_4	12.0mg
Solution C	KCl	65.0mg
	KNO_3	81.0mg
	$MgSO_4.7H_2O$	36.0mg
	Water to	1.0 litre.

Stock solutions of A, B and C were made up at 100 times the final concentration and stored at $0^{\circ}C$.

In addition, various combinations of the following were included:

2,4-D. 2.2mg dissolved / ml ethanol, and included in the medium to final concentrations of 10^{-8} to 10^{-3} M.

Kinetin. Dissolved in warm water to a concentration of 5×10^{-4}M and included in the medium at concentrations of 5×10^{-8} to 5×10^{-5} M.

Coconut milk. Milk was extracted from fresh Ceylon coconuts, filtered, autoclaved and stored at $-20^{\circ}C$. Milk was thawed, filtered and included in the medium at a concentration of 20% v/v.

Sterilisation. Culture medium, in the vessels to be used for culture, instruments for the planting process and assembled Millipore filters were sterilised by autoclaving for 15 minutes at 15 lb / sq. inch pressure.

Tubers were scrubbed to remove soil and surface sterilised by immersion for 30 minutes in a 10% v/v solution of sodium hypochlorite. Any tubers showing signs of surface damage or infection were rejected.

Preparation and planting of explants. The surface sterilised tubers were rinsed twice with sterile water. A slice 3 cm long was cut transversely from the middle of the tuber. From the slice, cores of secondary xylem parenchyma were cut using a 2 mm diameter cannula. The long axis of the core was parallel to that of the tuber. The cores were cut into explants 2.4 mm long using a cutter holding 10 parallel razor blades. Explants

were transferred to the culture vessels. 100 ml conical flasks, 2.5 l Thomson bottles and various sizes of petri dish were used. All planting operations were performed in an ultra-violet light sterilised room. This was illuminated by fluorescent light for early experiments, and by a dim green safelight later. (Ilford No. 909, 25 watt bulb, 50 cm from working surface)

Incubation was in total darkness at 25 ± 0.5°C. The cultures were agitated on a 'Hearson' reciprocating shaker operating at 50 cycles / minute with a 5 cm stroke.

Sampling. During the incubation, samples were removed aseptically in fluorescent, and later in dim green light. The following determinations were made:

Fresh weight. Explants were blotted lightly on tissue paper and weighed to the nearest 0.1 mg.

Dry weight. Explants were dried for 48 hours at 95°C, cooled in a dessicator and weighed to the nearest 0.01 mg.

RNA, DNA and protein contents were measured as described in Section 3.

Cell number. 5 explants were placed in 2 ml 5% chromic acid. After 24 hours at room temperature, the tissue was macerated by drawing the fluid into a Pasteur pipette and expelling it rapidly five times. Cell number was derived from this macerate by two methods.

1. The haemocytometer technique. (Brown and Rickless, 1949) Using a Fuchs-Rosenthal haemocytometer slide, the cell numbers in six grids, each containing 3.2 µl of the suspension were counted. Knowing the total volume of macerate and the number of

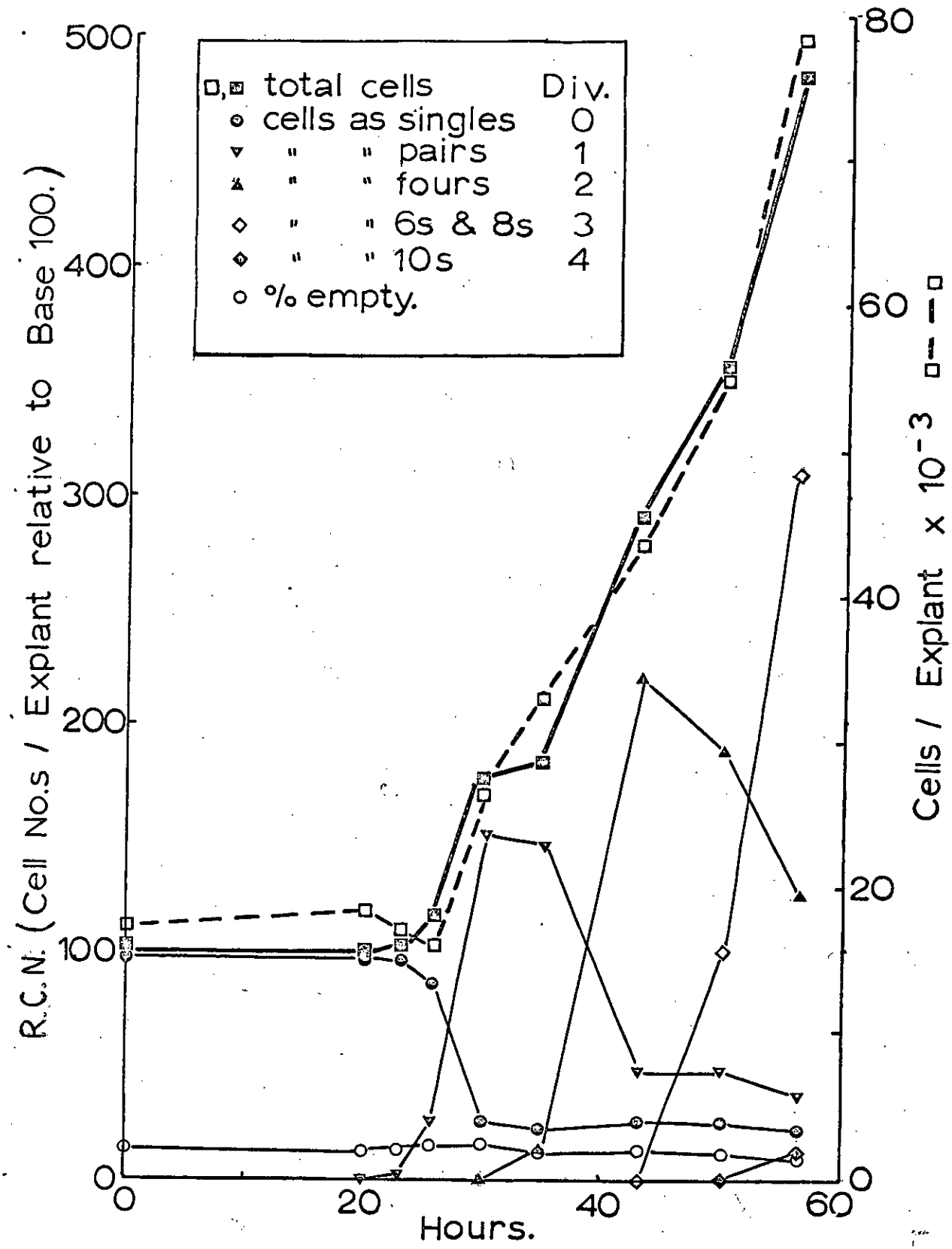


Figure 1.1 Legend opposite.

explants, the cell number per explant was calculated.

2. The incremental method. Evans (1967) reported that after cell division, the daughter cells remained together during maceration, forming a 'pair' of cells separated by a recognisably thin wall. Similarly, the second and third divisions gave rise to recognisable 'fours' and 'eights' in the macerate. From the examination of a sample of cells in a macerate, and counts of total cells (T), pairs, fours, etc, the increment in cells in that sample during culture is

$$I = 1 \times \text{pairs} + 3 \times \text{fours} + 7 \times \text{eights} \ldots\ldots$$

The number of cells in that sample at the beginning of culture is:

$$X = T - I.$$

The number of cells present in the macerate per 100 original cells is:

$$T/X \times 100$$

This value is defined as the Relative Cell Number (RCN) and is a measure of the amount of division. For example, a RCN of 400 represents a four fold increase in cells during culture.

Figure 1.1 (Opposite) Changes in cell number during the culture of explants, determined by the haemocytometer (--□--) and incremental (—■—) methods. Changes in numbers of pairs, fours etc. derived by the incremental method are also shown. Macerates of 5 explant samples. Incremental data from counts of 500-cell samples.

The incremental method depends on the daughter cells from divisions remaining together during maceration; any separation would lead to an underestimation of cell number increase. Figure 1.1 compares the data derived by the haemocytometer and incremental methods from macerates made during a culture. The close agreement between the two curves shows that no separation of daughter cells occurred.

The incremental method has three advantages:

1. The division of a few percent of cells can be detected with accuracy. Changes of less than 10% in cell number determined by the haemocytometer method are not statistically significant (Evans, 1967).

2. The numbers of cells present as singles, pairs, fours etc. may be calculated and plotted as in Figure 1.1.
e.g. Numbers of cells as fours (i.e. second division products) per 100 original cells, $N = \frac{4 \times n}{X} \times 100$, where n is the number of fours in a sample with cell number X at the start of culture. Calculations of this type have proved useful in giving a more detailed analysis of division in the system than simple cell number. For example, in Figure 1.1 they show that cells which did not enter the first division did not divide later; the singles count remained constant after division 1.

3. The time taken to make a count by the incremental method is about 1/5 th of that required for a count by the haemocytometer method.

The incremental method has two disadvantages:

1. No absolute number of cells per explant is obtained. If this absolute count is desired, the incremental method could be calibrated by haemocytometer counts on any of the macerates

2. The method is restricted to the early divisions, as after four divisions the cell groups become too dense to examine.

Evans (1967) and other workers with this tissue have always counted empty cells and substantial wall fragments as single cells, allowing for the loss of cell contents during maceration. This leads to a lower estimate of cell division by either method of counting.

Figure 1.1 shows that the count of empty cells plus substantial fragments remained constant at 11 during culture. Examination of the culture medium showed that it contained no detached empty cells or fragments.

When the explant is excised from the tuber, the outermost cells are damaged. Calculations from the cell size data of Robertson (1965) and explant dimensions suggest 18 damaged cells per 100 undamaged cells. Of this 18, half will be large enough to count as empty cells or substantial wall fragments. This estimate is based on a perfectly cylindrical explant with only the outermost layer of cells damaged. Irregularities of shape and slight damage to the underlying cell layers shown in anatomical studies by Robertson (1965) will increase the number of damaged cells slightly.

The calculated figure for cells damaged at excision (9) agrees with the observed counts of empty cells and wall fragments (11). Thus there is no loss of cell contents during maceration, and the exclusion of empty cells from the counts is justified.

For most of the following experiments, cell division was measured by the incremental method, ignoring empty cells and wall fragments in the counting. 300 - 600 cells were counted in each macerate.

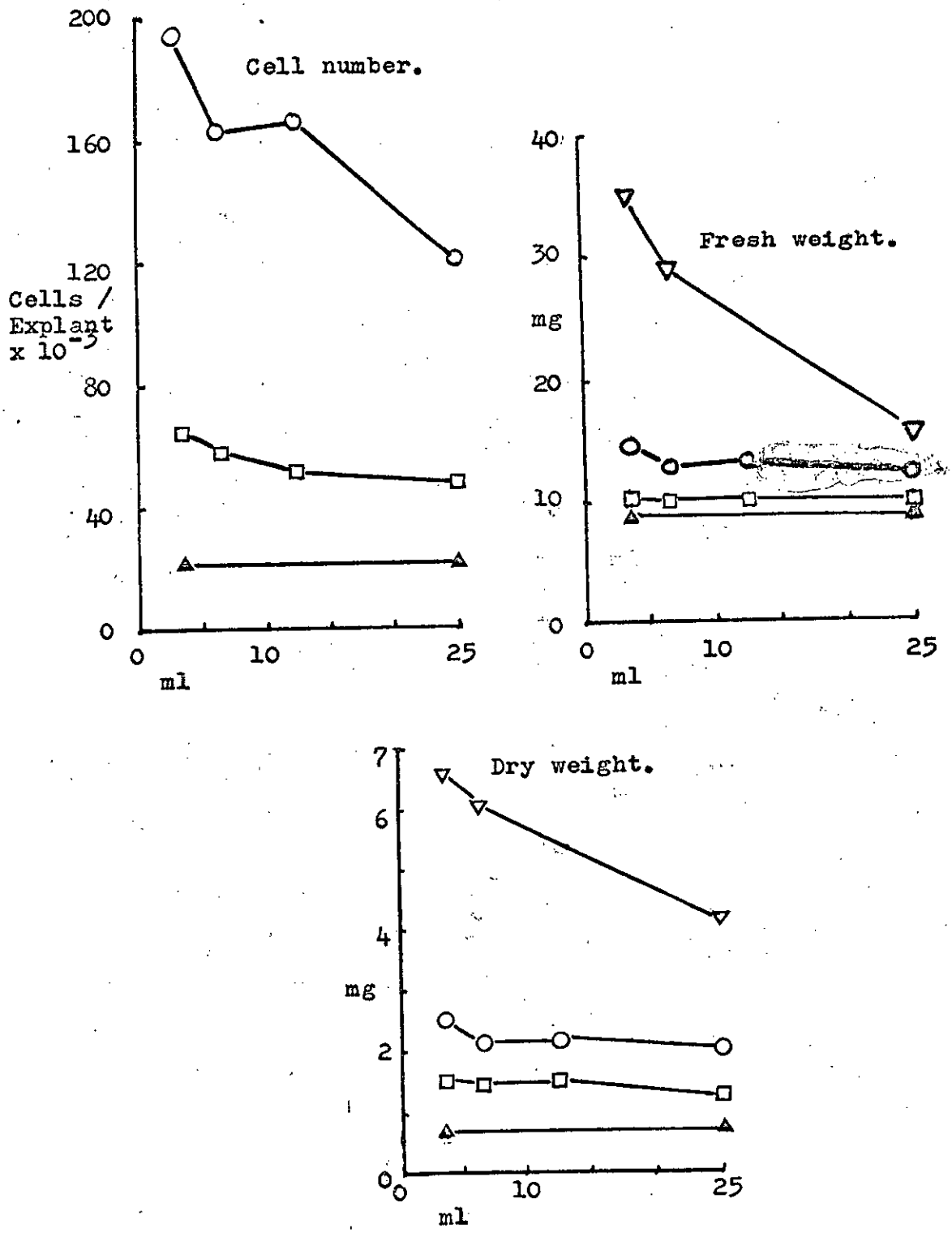


Figure 1.2 Fresh weight, dry weight and cell number per explant after culture for 0 (△), 49 (□), 96 (○) and 336 (▽) hours with various volumes of medium / 20 explants. Weight data from samples of 10 explants, cell number data by counts of macerates of 5 explants by the haemocytometer method.

RESULTS.

1. The establishment of a liquid medium bulk culture system

A. Effects of medium volume per explant.

Cultures of 20 explants were established in 100 ml conical flasks with 3.5, 6.5, 12.5 or 25.0 ml medium. The medium included 10^{-6} M 2,4-D and 20% coconut milk. Planting was in fluorescent light. 3.5ml of medium was the smallest volume which would move the explants during shaking, desirable for the elimination of possible polarity effects.

Figure 1.2 shows that the greatest increases in fresh weight, dry weight and cell number occurred with the smaller volumes of medium. The high weights of the explants grown with 3.5 ml medium at 336 hours prove that there was no shortage of nutrients with this treatment.

For all subsequent experiments, the conditions of the 3.5 ml flask were followed, namely 0.175 ml medium and 0.75 cm^2 surface area of medium per explant.

B. Effects of vessel size and shape.

Cultures were established in medium containing 10^{-6} M 2,4-D and 20% coconut milk, in fluorescent light, in three types of culture vessel:

100 ml conical flask closed by a glass cap. 20 explants, 3.5 ml medium.

9 cm petri dish. 85 explants, 15 ml medium.

2.5 l Thomson bottle lying on its side, the neck closed by a cotton wool plug. 400 explants, 70 ml medium.

Age, hours		0	27	49	76	96	336
	CM%						
Fresh wt /	20	8.5	8.7	9.8	10.7	12.9	29.0
explant. mg	0	8.5	8.6	10.0	11.4	11.6	15.1
Dry wt. /	20	0.68	1.20	1.43	1.76	2.16	6.00
explant. mg	0	0.68	1.20	1.35	1.75	1.85	3.18
Cell No. /	20	22.6	32.3	59.6	113.1	164.1	-
explant $x10^{-3}$	0	22.6	30.0	60.8	103.3	110.6	-

Table 1.1 Fresh weight, dry weight and cell number per explant after various times of growth in medium containing 10^{-6}M 2,4-D, with and without 20% coconut milk (CM). Fresh and dry weights from 15 explant samples, cell numbers by haemocytometer counts of macerates of 5 explants.

Age, hours.	2,4-D concentration. (M)					
	0	10^{-7}	10^{-6}	10^{-5}	10^{-4}	10^{-3}
30	112	127	143	161	128	100
48	128	186	207	261	228	100

Table 1.2 Relative cell numbers after growth for 30 and 48 hours in media with various 2,4-D concentrations. Data from macerates of 5 explants by the incremental method.

Kinetin conc. M.		0	$5x10^{-7}$	$5x10^{-6}$	$5x10^{-5}$
2,4-D conc. (M)	Age, hours.				
0	30	106	107	103	104
	48	106	104	107	102
10^{-7}	30	107	106	110	106
	48	121	111	112	119
10^{-6}	30	136	141	134	122
	48	183	151	181	170
10^{-5}	30	161	147	136	109
	48	216	202	184	161

Table 1.3 Relative cell numbers at 30 and 48 hours after growth in media with various concentrations of kinetin and 2,4-D. Data from macerates of 5 explants by the incremental method.

Analysis of fresh weight and relative cell number at 24 and 48 hours showed that the growth of explants was identical in all vessels.

For most subsequent experiments, petri dishes of 9, 12 and 15 cm diameter were used. This provided adequate flexibility of culture size, and ease of planting and sampling.

2. Effects of medium composition.

A. Omission of coconut milk.

To investigate the feasibility of a completely defined medium, cultures were established in sucrose and salts medium containing either 10^{-6}M 2,4-D or 10^{-6} M 2,4-D and 20% coconut milk. The explants were planted by fluorescent light in 100 ml conical flasks.

The results in Table 1.1 show that initially, no differences existed between the treatments. After 76 hours, by when cell number had increased 4 to 5 fold, all three parameters were lower in the absence of coconut milk. 2,4-D alone, while unsuitable for long term growth studies, equally maintained the early growth, including the early synchronous divisions.

B. Determination of optimum 2,4-D concentration.

Explants were planted in 100 ml conical flasks by fluorescent light. The medium contained sucrose, salts and 2,4-D at 0, 10^{-7}, 10^{-6}, 10^{-5}, 10^{-4} or 10^{-3} M.

Table 1.2 shows that the highest division performance was with a 2,4-D concentration of 10^{-5} M, and that division was completely inhibited at 10^{-3} M. Small amounts of division occurred in the absence of 2,4-D.

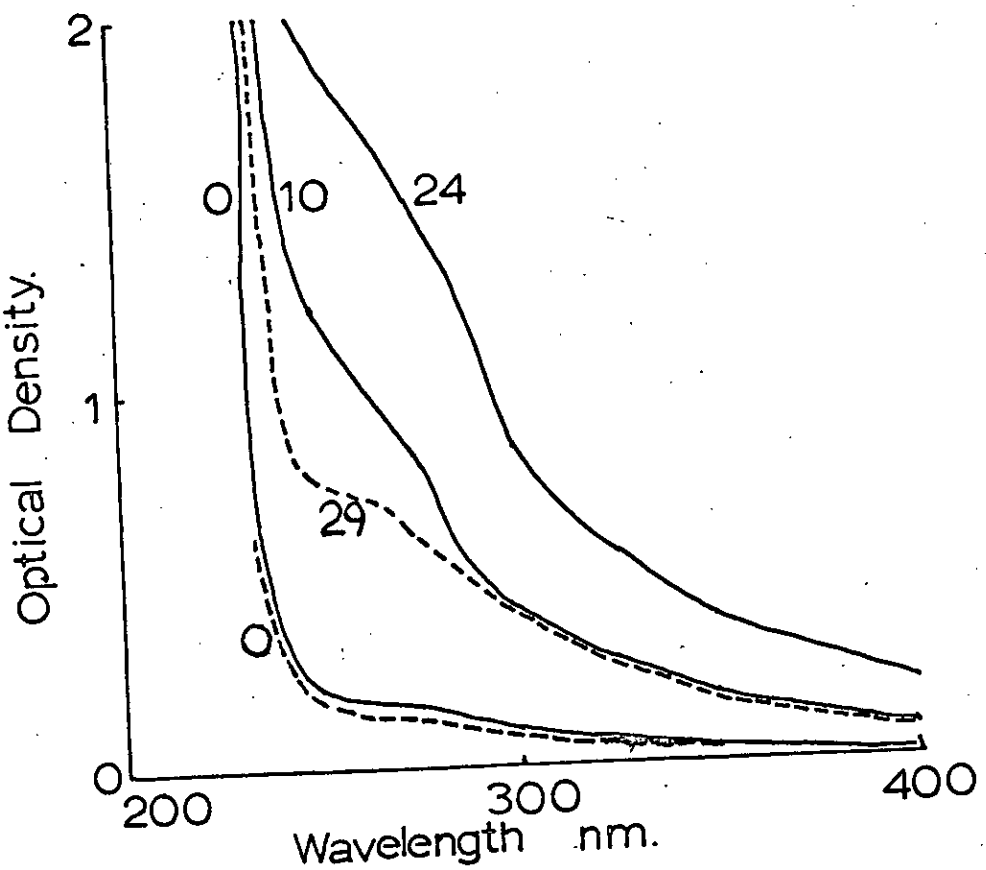


FIGURE 1.3 Ultra-violet absorption spectra of culture media. 20 explants were grown in 3.5 ml medium. Continuous lines: medium containing 10^{-6} M 2,4-D and 20% coconut milk, measured at 0, 10 and 24 hours. Broken line: medium containing 10^{-5} M 2,4-D measured after 29 hours of growth.

C. Effects of kinetin.

Cultures were established by fluorescent light in 100 ml conical flasks. The medium contained sucrose, salts and various combinations of 2,4-D at 0, 10^{-7}, 10^{-6} or 10^{-5} M and kinetin at 0, 5×10^{-7}, 5×10^{-6} or 5×10^{-5} M.

Results presented in Table 1.3 show that in the absence of 2,4-D, kinetin had no stimulatory effect on division. With 2,4-D at 10^{-7} and 10^{-6} M, kinetin at all concentrations used had little effect on the amounts of division. With 2,4-D at 10^{-5} M, kinetin produced an inhibition of cell division, increasing with concentration. The possibility that still lower concentrations of kinetin might have had a stimulatory effect on cell division was not investigated.

3. Materials from the outer, damaged cells and cell division.

A. Changes in the ultra-violet absorption spectrum of the medium.

Explants were grown in two types of culture medium:
Sucrose, salts, 10^{-6} M 2,4-D, 20% coconut milk.
Sucrose, salts, 10^{-5} M 2,4-D.
After various times of growth, samples of medium were centrifuged for 5 minutes at 2000 r.p.m. The ultra-violet absorption spectra were determined in the SP800 recording spectrophotometer.

Figure 1.3 shows that during culture, there was an increase in the ultra-violet absorbance of both types of medium. The increase was greater with the medium containing coconut milk. This may have been due in part to the breakdown of solid materials such as fragments of nuclei in the coconut milk in the course of culture.

Prewash. Additions to sucrose and salts medium	Age, hours.	None	Sucrose + salts medium	Trypsin	Pronase
None	28	118	113	112	110
	48	131	115	119	113
2,4-D	28	152	147	147	142
	48	192	183	189	178
coconut milk	28	140	140	145	137
	48	148	150	161	158
coconut milk + 2,4-D	28	153	149	152	152
	48	210	217	203	209

Table 1.4 Changes in relative cell number after various prewashes and culture in various media. Data from counts by the incremental method on macerates of 10 explants.

	Standard Explants.		Disc Explants.	
Age, hours.	30	48	30	48
Mean % empty cells	17.3	-	14.5	-
Culture medium : sucrose + salts +				
10^{-4} M 2,4-D	128	228	108	170
10^{-5} M 2,4-D	161	271	135	215
10^{-6} M 2,4-D	143	207	124	190
10^{-6} M 2,4-D + 20% CM.	137	204	150	212

Table 1.5 Relative cell numbers of standard and disc explants after culture for 30 and 48 hours in media including various 2,4-D concentrations, with and without coconut milk (CM). Data derived from counts by the incremental method on macerates of five explants or ten discs.

B. Enzyme prewashes and medium composition.

Cultures were established by fluorescent light in 100 ml conical flasks after the following pretreatments:

1. None. Explants planted after excision.

2. Explants washed for 15 minutes in 25 ml sucrose and salts medium.

3. Explants washed for 15 minutes in 25 ml trypsin solution.

4. Explants washed for 15 minutes in 25 ml pronase solution.

Both enzymes were dissolved in sucrose / salts medium adjusted to pH 7.4 with K_2HPO_4, and sterilised by passing through a 0.22u Millipore filter. Explants from pretreatments 3 and 4 were rinsed twice with sterile sucrose and salts medium before planting to remove adhering enzyme.

Explants were grown in sucrose and salts medium with the following supplements:

1. None

2. 10^{-6} M 2,4-D.

3. 20% coconut milk.

4. 10^{-6} M 2,4-D and 20% coconut milk.

Table 1.4 shows that in the absence of 2,4-D and coconut milk, all three prewashes effected a reduction in the small amount of cell division occurring. The proteolytic enzymes had no greater activity in preventing division than medium alone. No significant effects of washing on division appeared when either or both of 2,4-D and coconut milk were present.

At 28 hours, 2,4-D and coconut milk had separately stimulated division almost as much as when together. By 48 hours, stimulation by coconut milk alone was falling off, while stimulation by 2,4-D alone was still as effective as when both

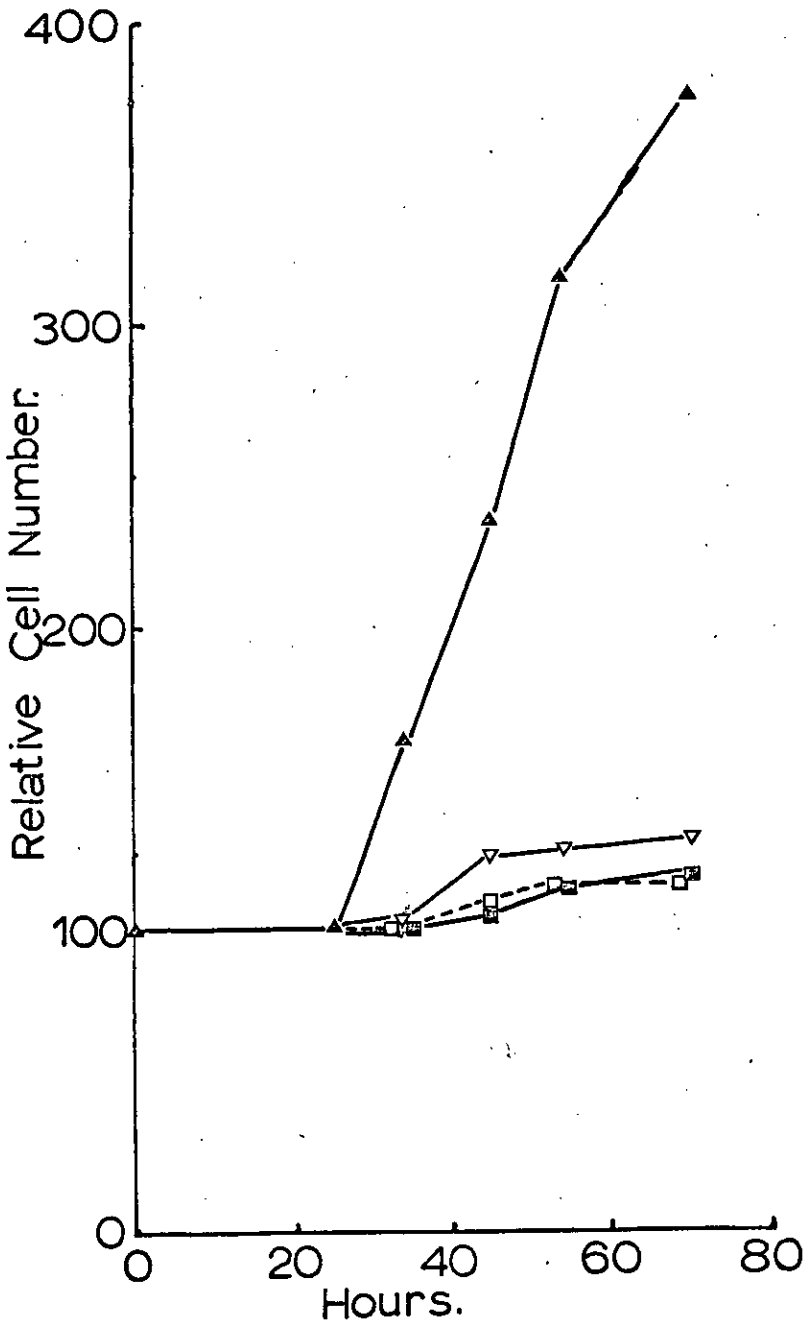


FIGURE 1.4 Changes in relative cell number with time for cultures grown with sucrose and salts medium (□--□), after a 30 minute prewash (▽—▽) with tissue homogenate added to the medium (▽—▽) and in medium includind 10^{-5} M 2,4-D (△—△) Data from counts by the incremental method on macerates of five explants.

stimulants were present.

C. Effects of prewashing and added tissue homogenate.

Cultures were established by dim green safelight in 15 cm petri dishes, each containing 240 explants and 40 ml medium. Four treatments were applied.

1. Explants planted in sucrose and salts medium.

2. Explants prewashed for 30 minutes in sucrose and salts medium before planting in sucrose and salts medium.

3. 20 explants were homogenised in 3 ml sucrose and salts medium using a glass in glass homogeniser. The homogenate was added to a petri dish containing 240 explants and 37 ml sucrose and salts medium.

4. Explants were planted in sucrose and salts medium containing 10^{-5} M 2,4-D.

The data presented in Figure 1.4 show that division was much reduced in the absence of 2,4-D. In none of the cases without 2,4-D did any second division occur. The tissue homogenate caused a doubling of the number of cells entering the first division in the absence of 2,4-D. In this case, prewashing did not reduce the number of cells dividing.

D. Effects of explant size.

Robertson (1965) observed that the first cell divisions are localised in the periphery of the explant. To test the suggestion that this is a consequence of oxygen starvation or CO_2 accumulation in the inside of the explant, two sizes of explant were used.

1. Standard explants, 2mm diameter, 2.4mm long, maximum

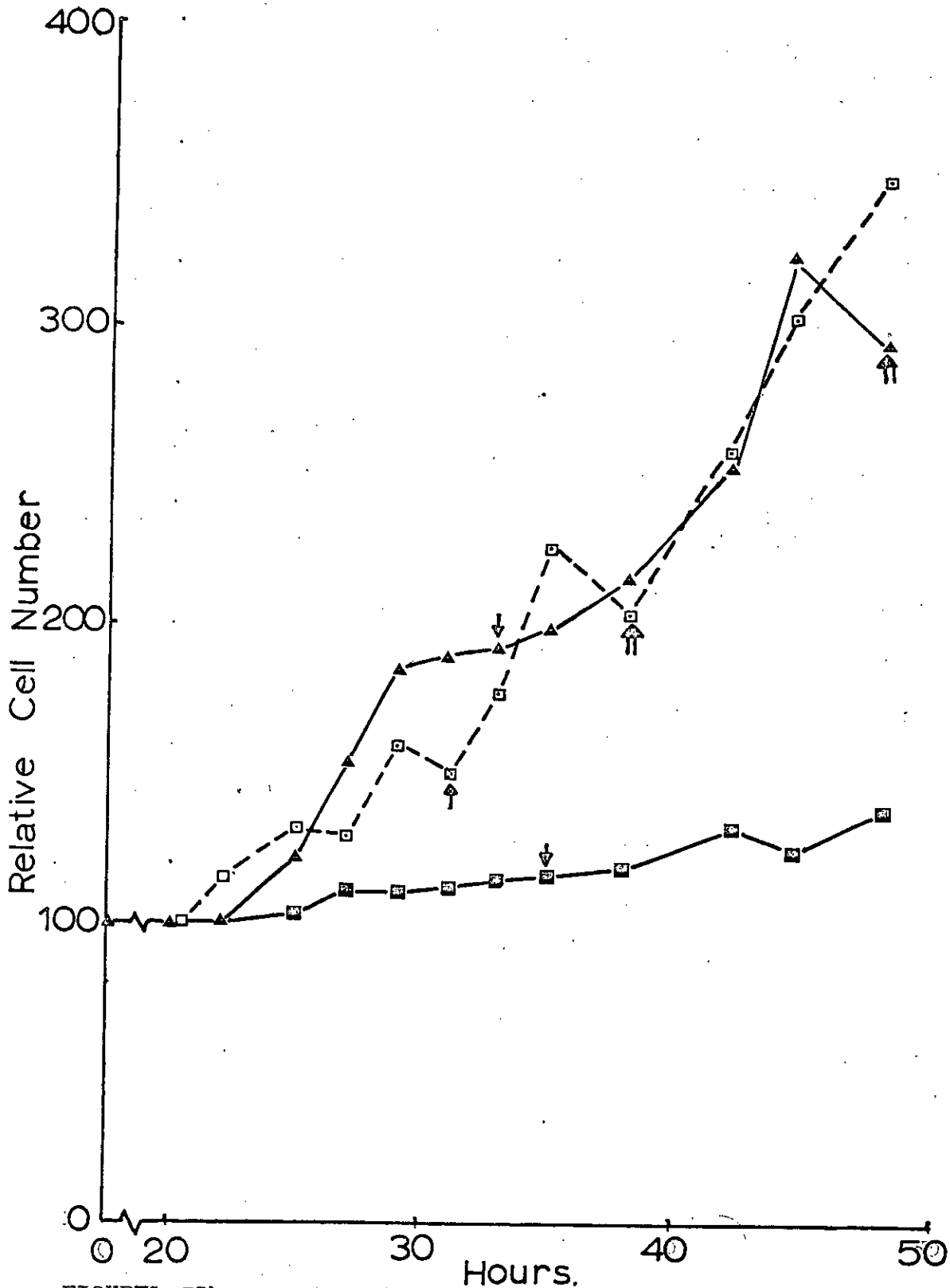


FIGURE1.5Changes in relative cell number with time for explants grown in total darkness (△—△), 120 fc. (□--□) and 450 fc. (□—□). ⇑ First appearance of second division products (fours). ⇑ First appearance of third division products (eights). Data from counts by the incremental method on macerates of five explant samples.

diffusion path 1mm.

2. Disc explants. 2mm diameter, 1.4mm long. Maximum diffusion path 0.7 cm. The discs were cut from the usual 3 cm x 2 mm cores using the gel-chopping apparatus (Section 2). The chopper was sterilised by rinsing with alcohol and drying under ultraviolet light.

Both types of explant were cultured in 100 ml conical flasks, with 20 standard or 35 disc explants in 3.5ml medium. The medium was sucrose and salts with 10^{-4}, 10^{-5} or 10^{-6} M 2,4-D or 10^{-6} M 2,4-D and 20% coconut milk. Planting was in fluorescent light.

The results presented in Table 1.5 show that the disc explants divided less than the standard explants when 2,4-D alone was present. The presence of coconut milk with 2,4-D had no effect on the division of the standard explant, but caused a marked stimulation of division in the disc explants.

4. The effects of light on cell division.

A. Continuous illumination.

Cultures were established in 9 cm petri dishes containing sucrose and salts medium with 10^{-5} M 2,4-D. Illumination during excision and planting was by dim green safelight. The explants were then grown in total darkness or under light intensities of 120 and 450 foot candles. Both light sources were mixed tungsten and fluorescent.

It is clear from the results presented in Figure 1.5 that continuous light had a strong depressive effect on cell division. With 120 fc illumination, the numbers of cells at the ends of the first and second divisions were smaller than in the dark

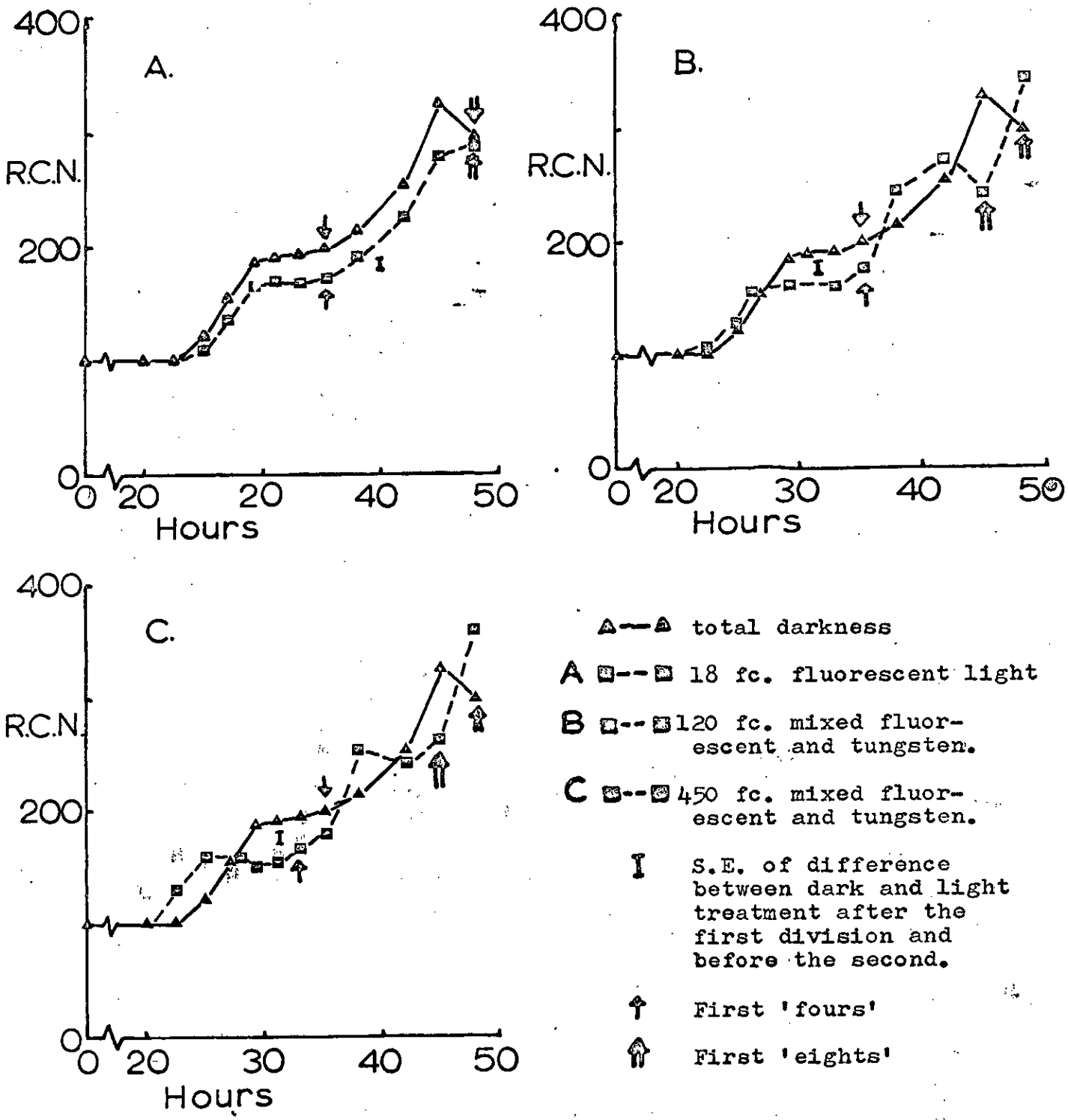


FIGURE 1.6 Changes in relative cell number with time for explants excised in dim green light, exposed to various light conditions for the first 30 minutes of culture and then cultured in total darkness. Data from counts by the incremental method on macerates of five explant samples.

treatment, although the net rate of cell accumulation was the same in both treatments. The divisions occurred more quickly with 120 fc illumination.

B. Initial illumination.

The results of the above experiment suggested that the practice of excising and planting explants by fluorescent light might have an inhibitory effect on cell division. To test this, explants were excised in dim green safelight and the exposed for 30 minutes to one of the following light regimes:

Control. Total darkness.

A. 18 fc fluorescent light. (the sterile room illumination, previously used for excision and planting.).

B. 120 fc)
) Mixed fluorescent and tungsten.
C. 450 fc)

Subsequent incubation was in total darkness.

In the dark control explants, 90% of cells entered the first division, compared with 65% for the fluorescent light treatment and 50% after 120 and 450 fc treatments. (Figure 1.6) Clearly the period immediately following excision is extremely sensitive to light inhibition of cell division. Thus the practice of excising and planting by fluorescent light leads to a significant decrease in the number of cells entering the first division.

Figure 1.7 presents a more detailed analysis of division in the control and treatment C. With the 30 minute initial light treatment, there was a second increase in 'pairs' and a corresponding decrease in 'singles' at the time of the second division at 30 hours. This second wave of division of

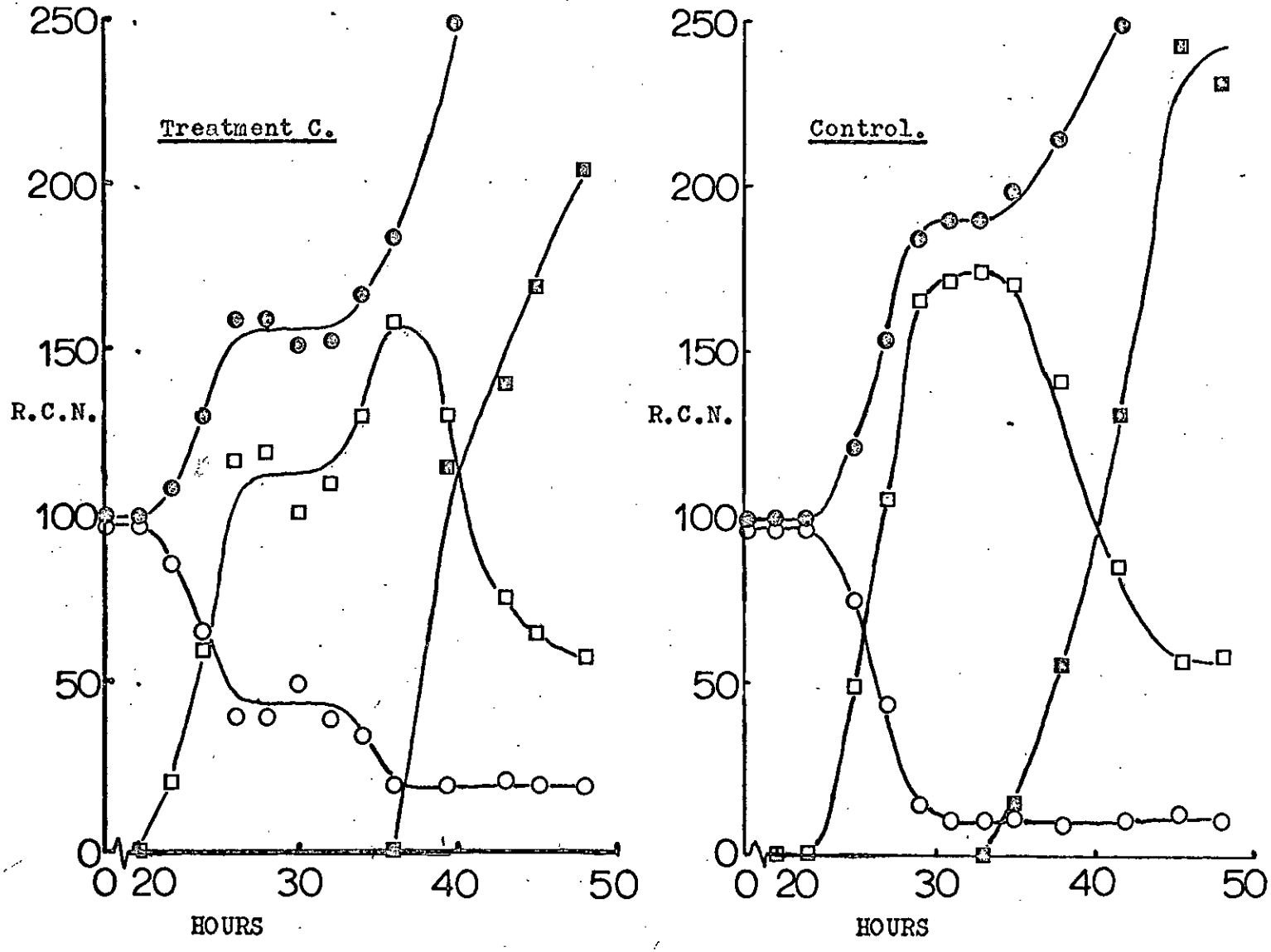


FIGURE 1.7 Changes with time in R.C.N. (⊛); and in numbers of cells as singles (○), pairs (□) and fours (▣) per 100 original cells, for explants grown in the dark after receiving 450 fc (Treatment C) and dark (Control) for the first 30 minutes after excision.

previously undivided cells is a feature of treatments where substantial numbers of cells fail to divide at the time of the first division. A second wave of 'pair' formation at the time of the second division has also been observed by Evans (1967) and Mitchell (1967a) in cultures grown with 10^{-6} M 2,4-D and 20% coconut milk. Under their conditions, 40% of cells enter the first division proper.

5. Seasonal variations in tubers and growth response.

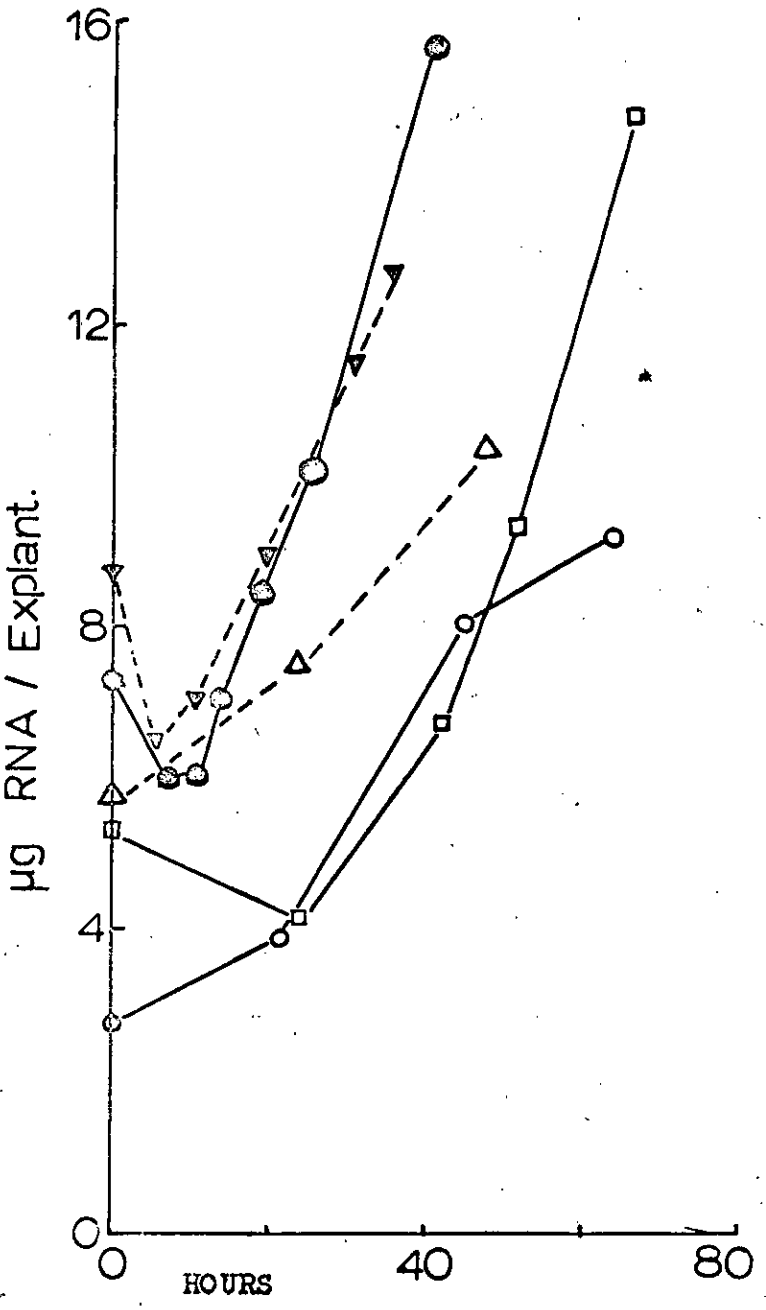
A. Seasonal changes in tubers.

In table 1.6 are shown DNA, RNA and protein contents of freshly cut explants from various tuber types. The immature tubers were from plants grown out of season in a greenhouse, the mature tubers from plants grown outside and harvested in November.

DNA content per explant was higher in the small, immature tubers, probably a consequence of a larger number of smaller cells per explant. The DNA content of explants from mature tubers remained constant during storage at 1 µg / explant. RNA and protein contents were both low in explants from immature tubers. Both reached peak values just after harvest, and showed declines with prolonged storage of the tubers.

B. Seasonal variation in response.

The lag phase before the first division remained constant at 22 hours from harvesting until the following April. Immature tubers and tubers stored for longer periods had longer lag phases. (Table 1.6) The amount of division occurring was inde-



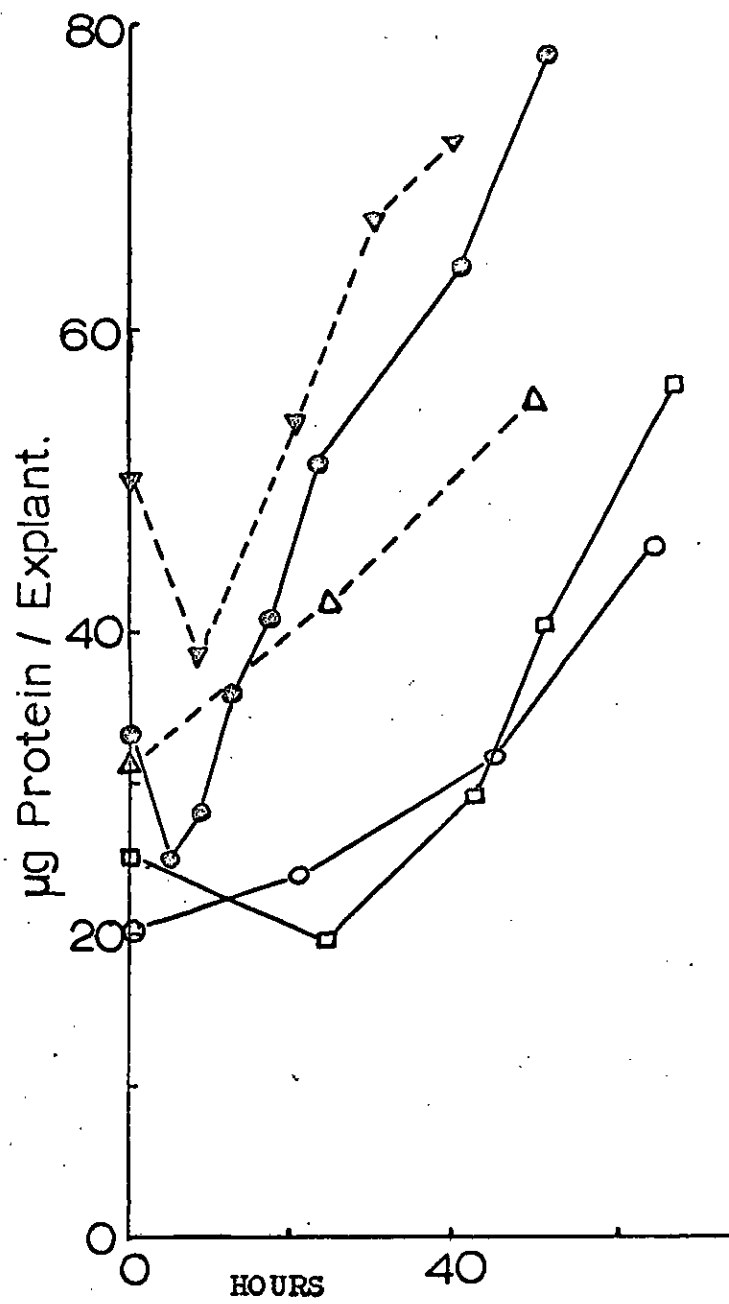


FIGURE 1.8 Changes in RNA and protein per explant with time, for explants from immature tubers (○ 1.5cm dia., △ 2.5cm dia.) and from mature tubers (5cm dia) stored for 0(●), 1 (▽) and 8 (□) months after harvest in November. Derivation of data as for Table 1.6

pendent of the length of the lag phase.

Figure 1.8 shows the time courses of RNA and protein accumulation in explants from immature and stored mature tubers. Long-stored tubers showed longer lag phases before accumulation commenced. The rates of accumulation subsequent to the lag phases were similar in all mature tubers. Immature tubers showed no lag phase but lower rates of accumulation.

Tuber diameter cm.	1.5	2.5	5	5	5	5
Month	-	-	Nov.	Dec.	April	July
µg RNA / explant	2.8	6.0	7.5	8.5	7.0	5.4
µg DNA / explant	1.4	1.5	0.94	1.10	0.98	1.15
µg protein / explant	20	32	33	51	-	25
lag phase, hours	40	30	22	22	22	45
% first division.	85	-	85	80	-	85

Table 1.6 DNA, RNA and protein contents of freshly cut explants, lag phase before first division and percentage of cells entering the first division, for explants from immature tubers and mature tubers after various times of storage. All data from experiments reported in Section 3. All cultures planted in green light in medium containing 10^{-5} M 2,4-D.

DISCUSSION.

1. Factors affecting cell division.

Stimulants. Considerable changes occurred in the ultra-violet absorption spectrum of the medium during culture. (Figure 1.3) Measurements of RNA and protein per explant showed a drop during the first few hours of culture. (Figure 1.8). Robertson (1965) has observed an increase in phosphatase activity, a symptom of autolysis, in the cells immediately underlying those damaged during excision during the early part of culture. There is therefore a loss of materials from the outer regions of the explant to the medium.

These materials appear to act as a stimulant to cell division. When explants were cultured in a medium containing only sucrose and mineral salts, a small amount of division occurred. This amount was reduced by prewashing the tissue, and increased by the addition of tissue homogenate to the medium. The higher growth rates observed when explants were cultured in smaller volumes of medium may reflect the higher concentrations of damaged cell material maintained in the medium.

The inclusion of either or both 2,4-D and coconut milk in the medium promoted more vigorous division. 2,4-D alone would support a 5 - 10 fold increase in cells (Table 1.1); coconut milk alone rather less (Table 1.4). For long term callus growth, involving a 100 fold increase in cells, Robertson (1965) has shown that both stimulants must be present.

Haberlandt (1921, 1922) observed a 'wound hormone' from damaged cells of potato tuber slices, which together with a

'leptohormone', a diffusable chemical from the phloem, would promote cell division. Coconut milk is the product of endosperm breakdown, and hence bears similarities to the damaged cell products. It is possible that division stimulants arising from the damaged cells might compensate for the absence of coconut milk in medium containing only 2,4-D as stimulant, but become depleted after the 5 to 10 fold increase in cell number. Coconut milk itself has been shown to contain a number of components which stimulate division. (Pollard, Shantz and Steward, 1961; Steward and Mohan Ram, 1961). Possible some of these acted in the place of 2,4-D to promote some division when 2,4-D was not present in the medium, but were insufficient for protracted division.

Some confirmation of the similarity between coconut milk and the damaged cell material comes from the experiment with disc explants. (Table 1.5). The discs showed a smaller proportion of damaged cells than the standard explants, probably a result of the different cutters used. The small amount of division in the disc explants compared to the standard explants when only 2,4-D was supplied may have been the result of a smaller amount of 'wound hormone' derived from the damaged cells. The equal amounts of division in the two types of explant when coconut milk and 2,4-D were supplied could then be because the coconut milk eliminated the difference in wound hormone supply.

Gaseous exchange. The influence of gaseous exchange in determining the amount of division is difficult to examine, since any attempt to increase the surface area / volume ratio will result in an increase in the proportion of wounded tissue.

Evans (1967) observed similar amounts of division in 1 and 2 mm diameter explants and in hollow explants, but lower amounts in 3 mm explants. Yeoman, Naik and Robertson, (1968) have grown explants under higher partial pressures of oxygen, with no increase in the proportion of cells dividing. However, both these experiments were conducted with explants planted by fluorescent light, with 10^{-6} M for the 2,4-D concentration. With the possibility that division was restricted by light and sub-optimal 2,4-D, gaseous exchange may not have been a limiting factor.

The approach adopted in the development described in this section was to maximise opportunity for gaseous exchange, by using the minimum amount of medium and loosely closed vessels. The 90% division obtained in the fully developed system proves that gaseous exchange rates do not limit cell division. The poorer growth with larger volumes of medium (Figure 1.2) may have been a result of slower gaseous exchange caused by the longer diffusion path in the liquid phase.

Light. Clearly light is a potent inhibitor of cell division in this system. Davidson (1968) has confirmed the effect for longer periods of culture, and has shown that light stimulates expansion growth in the later part of culture. Apart from the high levels of division obtained by planting in dim green safe-light, there is a further advantage in that division of previously undivided cells at the time of the second division becomes impossible. This may be considered as an improvement to the synchrony of the system.

The 10% of cells which did not divide when explants were

planted in dim green light are likely to have been the cells near the outside crushed during the excision of the explant. No. The autolytic nature of metabolism in these cells (Robertson, 1965) suggests that it may not be possible to stimulate them to divide.

Explants planted by fluorescent light in medium containing 10^{-6} M 2,4-D and 20% coconut have been shown to contain two regions of non-dividing cells, the autolysing region referred to above, and a central core. It is probable that explants planted by fluorescent light in medium containing 10^{-5} M 2,4-D, in which 40% of cells failed to divide, also contained such a non-dividing core. The paradoxical situation then arises that cells in the centre, where the light intensity was lowest, were most inhibited from division by light. Some sort of interaction, in which light partially destroys a division stimulating factor arising from the outer regions may be involved.

The distribution of dividing cells, the wavelengths of light involved and other aspects of the light effect are being investigated by Davidson in this department.

2. The suitability of the system for cell division studies.

The system developed here goes some way to satisfying the criteria of an ideal system set out in the introduction (Page 1). It provides a tissue of uniform cell type which may be handled conveniently, with a high percentage of cells entering synchronous division. The medium is completely defined.

Synchrony. The first division in this tissue lasts for 6 to 8 hours. (Figures 1.1, 1.7). Evans (1967) has calculated the time taken by an individual cell to undergo mitosis as 3 hours. The synchrony is therefore not complete; it also lasts only for a few divisions, each division being less synchronous than the previous one.

Figures 1.1 and 1.7 show that under conditions in which 80 to 90% of cells enter the first division, cells which did not enter into any of the divisions failed to divide later. The capacity to undergo repeated division appears therefore to be localised in the individual cell rather than in the tissue as a whole.

Other growth processes. Figure 1.2 shows that a 9.5 fold increase in cell number was accompanied by a 1.7 fold increase in fresh weight. There is therefore very little expansion growth of cells during the early growth of the tissue.

A process of loss of differentiation, or de-differentiation, accompanies the cell divisions. The cells change from the highly vacuolate storage parenchyma type of the tuber to smaller, non-vacuolate meristematic or embryonic types. This is a reversal of the trend in primary growth of the intact plant. No redifferentiation of cells to new specialised cell types occurs, but Robertson (1965) has reported the formation of 'nodules', resembling the root tip meristem in structure, after much longer periods of culture with coconut milk.

During storage at $4^{\circ}C$, the tubers may be assumed to be relatively quiescent. Excision and 2,4-D treatments, while stimulating cell division, might also stimulate other, unconn-

ected processes. The de-differentiation of cells and the possible side effects of excision make the behaviour of the system more complex than simply cell division. Caution is therefore required before interpreting any observation as having any causal relation with cell division. In section 3, the relation between observed events and cell division is considered in this light.

Seasonal variation. With immature tubers, or those stored for long periods, the lag phase before the first division was longer than in freshly harvested tubers. Similar results have been obtained by Robertson (1965) and Evans (1967) for explants cultured in medium containing 10^{-6} M 2,4-D and 20% coconut milk. Mitchell (1967, 1967a) has shown that the S-phase remains constant at 14 - 16 hours and G2 at 0 - 2 hours at all times of year. The increase in pre-division lag phase therefore represents an increase in the duration of the pre-S-phase.

The high DNA / explant content of tissue from immature tubers (Table 1.6) suggests that these tubers were growing by cell expansion as well as by cell division. The long-stored tubers were breaking down to support the developing buds (Jefford and Edelman, 1961) as is evident from their declining RNA and protein contents. Both expanding and breaking down conditions appear to require longer periods of adjustment before becoming competent to synthesise DNA. This variation in starting point for a process of differentiation to the dividing state may provide a useful tool for the study of the induction of cell division.

The low RNA and protein contents of explants from immature

tubers suggests that RNA and protein accumulations occur during the growth of the tuber. This may explain the lack of lag phase before RNA and protein accumulation in explants from immature tubers. The short and long stored mature tubers, which are not accumulating RNA and protein, again require a period of adjustment before accumulation may begin.

For the period November to April, the nucleic acid and protein contents of the tubers, and the performance of explants in culture remains constant. While it would have been desirable to restrict experimental work to this period, this is clearly impossible. The seasonal variation has two disadvantages. The comparison of experiments conducted at different times of year is difficult, and the designing of experiments when the pre-division lag phase is increasing becomes uncertain. Yeoman (1967) has grown artichoke plants out of season in a greenhouse, and hopes to secure a year round supply of constant response material by this method.

Despite the constant characteristics and response of tubers and explants from November to April reported above, other workers have found that certain fundamental changes occur in the tuber during this period of storage. Jefford and Edelman (1961, 1963) found that storage at 2°C magnified the conversion of fructosans to oligosaccharides. There was also a change in the dormancy of buds. After less than 7 weeks of storage, when the tubers were placed in higher temperatures, daughter tubers were produced. After 7 or more weeks at 2°C, the bud sprouted when the tuber was placed in a higher temperature. These results have been confirmed by Yeoman (1967). Thus during the early part of storage, the

tubers become vernalised. Effects of this change on the tissue cultures have not yet been detected.

3. Conclusion.

With the above reservations, the system provides a unique opportunity for the study of the induction of cell division and the events accompanying cell division in higher plant tissue.

The culture conditions developed in Section 1 and used for most experiments in Sections 2 and 3 are listed:

1. Explants are excised and planted in dim green safelight, and cultured in total darkness.
2. Culture is in liquid medium in petri dishes or conical flasks.
3. The culture medium contains sucrose, salts and 10^{-5} M 2,4-D. It is fully defined and does not contain coconut-milk.
4. Under these conditions, 80 to 90% of cells will take part in the first division.

SECTION 2.

RNA synthesis was examined in artichoke explants by polyacrylamide gel electrophoresis. The extraction of RNA from the tissue, and the degradation of ribosomal RNA during extraction were investigated. The synthesis of ribosomal RNA was found to involve two heavy precursors. Heterodisperse labelled RNAs were found in nuclear and cytoplasmic fractions.

MATERIALS AND METHODS.

Tissue. Explants were cultured by the method developed in Section 1. Cell number was estimated routinely after the first division to check that culture conditions were correct.

Chemicals. Acrylamide (Eastman Kodak) was recrystallised from chloroform, and methylene bisacrylamide (Koch-Light or B.D.H.) was recrystallised from acetone, as described by Loening (1967). Sodium naphthalene 1,5 disulphonate (NDS) and sodium tri-isopropyl naphthalene sulphonate (TNS) were obtained from Eastman Kodak, N, N, N', N'-tetramethylethylenediamine (TMED) from Koch-Light. Tris was B.D.H. specially purified or Sigma 'Trisma'. Deoxyribonuclease was Sigma electrophoretically purified. Sodium dodecyl sulphate (SDS) was B.D.H. specially purified grade. m-cresol was redistilled. All other chemicals were B.D.H., 'Analar' grade when obtainable.

Glassware. Non-radioactive glassware was cleaned as described in Section 1. Radioactively contaminated glassware was cleaned and decontaminated in a 2% v/v solution of Decon 75 for 24 hours.

Labelling of explants with 32-P. 15 - 30 explants were 'pre-incubated' in 10 ml of culture medium containing sucrose, salts, 10^{-5} M 2,4-D, but no phosphate, for 1 minute. This treatment was to remove residual phosphate-containing meduim from the intercellular spaces.

For the 'pulse' incubation, high specific activity 32-P

phosphate (25 to 97 c/mg P, Radiochemicals Centre, Amersham) was dried under vacuum to remove HCl. 32-P was taken up in phosphate-free culture medium to give an activity of 1 mc per 6 to 25 ml medium. 3 ml of pulse solution were used for each batch of explants.

The effects of the period of exposure to phosphate-free medium on RNA labelling were checked. Explants were pre-incubated in phosphate-free medium for 60 minutes before labelling. The resulting RNA labelling pattern and amount were similar to those obtained with a 1 minute phosphate-free pre-incubation. This suggests that no detrimental effects to RNA synthesis arise from the short exposure to phosphate-free medium.

The 'chase' incubation was in 10 ml standard culture medium (KH_2PO_4 concentration 12 mg / l.)

For the labelling, explants were placed in a 50 ml 'Spinco' cellulose acetate centrifuge tube with a perforated base. The explants were lifted in this between the incubation solutions, which were held in 100 ml 'Spinco' tubes. This arrangement also permitted agitation and aeration of the solutions, by raising and lowering the inner tube.

Sterile precautions. Generally no attempt was made to maintain absolute sterility during labelling. Autoclaved medium and phosphate-free medium were used to prepare the incubation solutions, which were stored at - 20°C and brought to 25°C just before required.

In one experiment, investigating the breakdown of ribosomal RNA to particles similar in size to bacterial r-RNA, additional sterile precautions were observed to prevent any

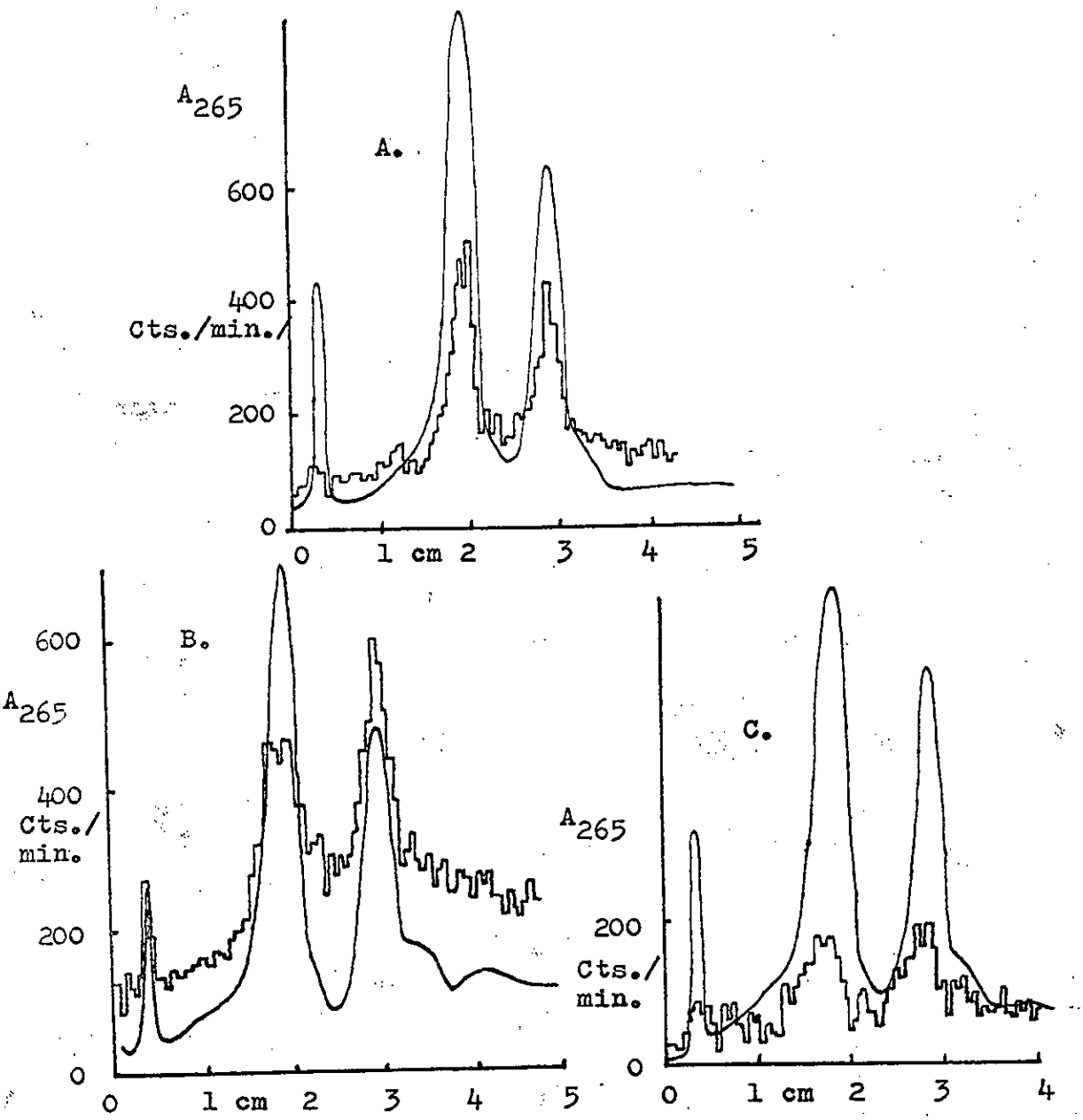


FIGURE 2.1 Electrophoresis of whole tissue RNA, prepared from explants exposed to three light regimes;

A. Culture and radioactive incubation in total darkness, handling operations in daylight.

B. Culture and incubation in 450fc. mixed fluorescent and tungsten light.

C. Culture and radioactive incubation in total darkness; handling operations by dim-green safelight.

complication of results by bacterial RNA synthesis. 32-P concentrated solution, assumed to be self-sterilising, was dried in a sterile beaker covered by sterile tissue. Cellulose acetate tubes were exposed to ultra-violet light for 24 hours after rinsing with alcohol. Sterile aluminium foil caps covered the tubes during the incubation.

Light effects. Radioactive incubation was in an incubator in the dark at 25°C. Transfer of explants to and between incubation solutions resulted in a few minutes exposure to daylight or fluorescent light. The effects of this light on the RNA labelling were checked.

Explants were grown and labelled under three light conditions:
A. Culture and radioactive incubation in total darkness, but handling operations in daylight.
B. Culture, incubation and handling in 450 fc mixed fluorescent and tungsten light.
C. Culture and radioactive incubation in totoal darkness, handling operations by dim green safelight.

29 hour old explants were labelled by a 60 minute pulse and a 1 minute chase. Total nucleic acid was prepared by homogenising and method 1 extraction (see below). RNA was fractionated by electrophoresis on 2.4% gels for 90 minutes.

The optical scans of Figure 2.1 show similar total amounts of ribosomal RNA for all treatments. Results presented in Section 3 confirm that light has no effect on the gross rate of RNA accumulation. The labelling pattern was similar in each case, but the amount of labelling was greater in the two light treatments than in the dark treatment. This suggests that light

might either increase the rate of turnover of RNA or increase the uptake of 32-P into the tissue.

To distinguish between these two possibilities, explants cultured and labelled under the three conditions were crushed on planchets and counted:

Treatment	counts / 100 sec / explant.
A.	452×10^3
B.	513×10^3
C.	171×10^3

These results show that 32-P uptake was stimulated even by the short exposure to daylight during transfer of explants to the incubation solutions. While it would have been best to eliminate the possibility of unknown light effects on the RNA metabolism by handling in dim green safelight, it was not practical to handle radioactive materials in the darkroom as a routine measure.

Tissue disruption. Two methods were used to break the tissue prior to extraction of nucleic acids:

A. The explants were frozen with solid CO_2 to the platform of the gel-slicing apparatus described below, and chopped at 50 μ intervals. As the cells are on average 100 μ long (Robertson, 1965) this ensured that every cell was broken. The material was then suspended in extraction medium. This method will be referred to as 'chopping'.

B. The explants were homogenised in a glass-in-glass homogeniser, motor driven, at full speed, for 20 seconds, in the appropriate extraction medium. This method will be referred to as 'homogenisation'.

Various other methods of tissue disruption were tried, including homogenisation with a 'Teflon' homogeniser and grinding in a pestle and mortar with solid CO_2. These methods were incapable of breaking up the tissue in a reasonable length of time.

Sub-cellular fractionation and RNA extraction. Two methods were used for the extraction of RNA, based on the fractionation of the tissue into cytoplasmic and nuclear fractions by mechanical or chemical means.

A. Method 1. A mechanical separation into crude nuclear (chromatin and cell walls) and cytoplasmic fraction was effected by the technique used by Loening (1965) with pea root tissue. 15 - 30 explants (120 - 240 mg fresh weight) were homogenised in 2 ml homogenising medium at 0°C, or were added to the medium after chopping.

Homogenising medium:	Sucrose	0.3M
	tris	30mM
	$Mg(CH_3COO)_2$	0.1mM
	KCl	50mM
	Cleland's reagent	5mM
	HCl to pH 7.6 at 0°C.	

This medium differs in two respects from that used by Loening (1965). The sucrose concentration was reduced from 0.5M to 0.3M to ensure separation of the aqueous and phenol phases during deproteinisation (see below). Cleland's reagent (dithiothreitol, Cleland, 1964) was necessary to prevent browning of the homogenate, probably a result of oxidation of polyphenols.

In an attempt to prevent nuclease activity during homogenisation leading to degradation of RNA, tissues were also homogenised in homogenising medium containing 0.5% NDS.

The homogenate was separated into cytoplasmic (supernatant) and nuclear (debris) fractions by centrifuging at 2000 r.p.m. for 5 minutes at 0°C. The nuclear fraction was resuspended in 2 ml homogenising medium. When no fractionation to cytoplasmic and nuclear was required, total nucleic acid was extracted from the entire homogenate.

TNS and 4-aminosalycylate (4-AS) (Kirby, 1965) were added to the fractions to final concentrations of 1 and 4% respectively. Deproteinisation was effected by emulsifying with an equal volume of phenol / cresol mixture at 5°C.

Phenol / cresol:	Phenol	500 g
(Kirby, 1965)	m-cresol	70 ml
	water	about 150 ml
	8-hydroxy-quinoline	0.5 g

The organic and aqueous phases were separated by centrifugation at 3000 r.p.m. for 15 minutes. The phenol layer was discarded and the aqueous phase and interfacial precipitate re-extracted with a further phenol / cresol treatment. The aqueous phase was made to 3% NaCl and extracted for a third time with phenol / cresol. Nucleic acids were precipitated by mixing the aqueous phase with 2 volumes of ethanol, and storing for 12 hours at - 20°C.

This method of extraction and fractionation will be referred to as 'method 1'.

B. Method 2. A separation of cytoplasmic and nuclear materials may be based on the finding that nuclear DNA and at least some

nuclear RNA are not released by phenol extraction in the absence of detergents. (Georgiev et. al. 1960, 1963; Kirby 1965; Hastings and Kirby, 1966).

15 to 30 explants were homogenised in 4 ml NDS buffer with 4 ml phenol / cresol mixture, or were mixed with these after chopping. All operations were at 5°C. The mixture was emulsified thoroughly and centrifuged at 10,000 x g for 15 minutes. The 'cytoplasmic' RNA was then in the aqueous phase, and the 'nuclear RNA and DNA in the interfacial precipitate and phenol / cresol layer. Nuclear fraction nucleic acids were released by mixing the interfacial precipitate and phenol layer with 4 ml Kirby medium, and centrifuging for 15 minutes at 3000 r.p.m. TNS to 1% and 4-AS to 5% were added to the cytoplasmic fraction, which was then emulsified with an equal volume of phenol / cresol and centrifuged at 3000 r.p.m. for 15 minutes.

NDS buffer:	NDS	0.5%
	NaCl	50mM
	tris	10mM
	HCl to pH 7.4 at 0°C.	
'Kirby medium'	TNS	1%
	phenol / cresol	6%
	4-AS	6%
	NaCl	0.15M

The nuclear and cytoplasmic fractions were made to 0.5M NaCl and extracted once more with equal volumes of phenol/ cresol. Nucleic acids were precipitated from the final aqueous phase by mixing with 2 volumes of ethanol and leaving at 0°C for 12 hours.

Where no fractionation to nuclear and cytoplasmic RNA was required, total DNA and RNA was extracted by homogenising the tissues in 4 ml 'Kirby' medium, followed by phenol / cresol treatments as above.

This method of extraction and fractionation will be referred to as 'method 2'.

Precipitated nucleic acids from both methods of extraction were pelleted by centrifugation at 0°C for 5 minutes at 2500 r.p.m. A rough estimate of 32-P uptake by the tissue was obtained by measuring the radioactivity of the alcoholic supernatant. 3 x 50 µl samples of the supernatant were dried on Whatman 3MM filter paper, mounted on planchets and counted. Values were corrected for supernatant volume and explant number.

Further purification of RNA. To remove 32-P containing impurities, the nucleic acid precipitate was dissolved in 0.5ml of SDS / acetate mixture.

SDS / acetate: SDS 0.5%

sodium acetate, pH 6, to 0.15M

Reprecipitation was effected by addition of 2 volumes of ethanol and overnight storage at -20°C. The re-precipitation procedure was repeated once or twice.

DNase treatment. After one SDS / acetate reprecipitation, nucleic acids were washed twice with 80% alcohol to remove SDS, and dried briefly under vacuum to remove alcohol. The nucleic acids were dissolved in 0.2 ml buffer at 0°C:

tris 50mM

magnesium acetate 2.5mM Acetic acid to pH 7.15, 20°C.

The buffer was added through a millipore filter to prevent RNase from contaminating bacteria from degrading the RNA. DNase was stored at - 20°C at a concentration of 500 µg / ml in the same buffer including 10% DMSO. The DNase solution was thawed at 0°C without shaking and added to the nucleic acid solution to a final concentration of 50 µg / ml. After 20 minutes digestion at 0°C, nuclease action was terminated by the addition of 0.8 ml SDS / acetate mixture. The solution was extracted once with phenol / cresol and RNA was precipitated by 2 volumes of ethanol at -20°C for 12 hours. This was followed by another SDS / acetate reprecipitation.

Preparation of RNA for electrophoresis. RNA or nucleic acids were washed twice by resuspending in 80% ethanol. The pellet was dried under vacuum and dissolved in 0.1 to 0.2 ml of 1/5 th strength electrophoresis buffer containing 5% sucrose. To obtain a measure of the total nucleic acid content of the preparation, 10 µl were diluted to 0.5 ml with distilled water. The relative nucleic acid content was estimated from the absorbnace at 260 nm.

Preparation of gels. A stock solution of acrylamide was prepared:

Acrylamide	15%
bis acrylamide	0.75%

This solution could be stored at 5°C in the dark for 3 months. Gels of final acrylamide concentrations of 2.2 and 2.4% were prepared:

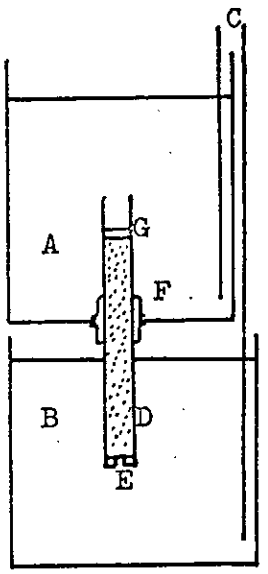


FIGURE 2.2 Section of gel electrophoresis apparatus. Scale X 1/2

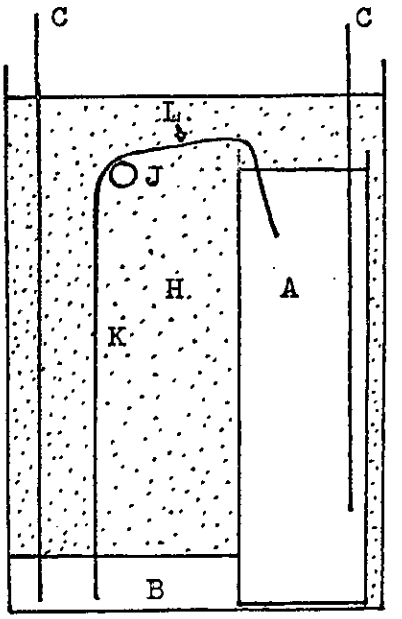


FIGURE 2.5 Nucleotide electrophoresis apparatus. Scale X 1/10

A Top buffer compartment. B Bottom buffer compartment. C Platinum electrode. D Perspex gel tube. E Gel retaining ring F Grommet G RNA sample. H White spirit. J Paper support bar, glass rod. K Whatman 3MM paper. L Base line.

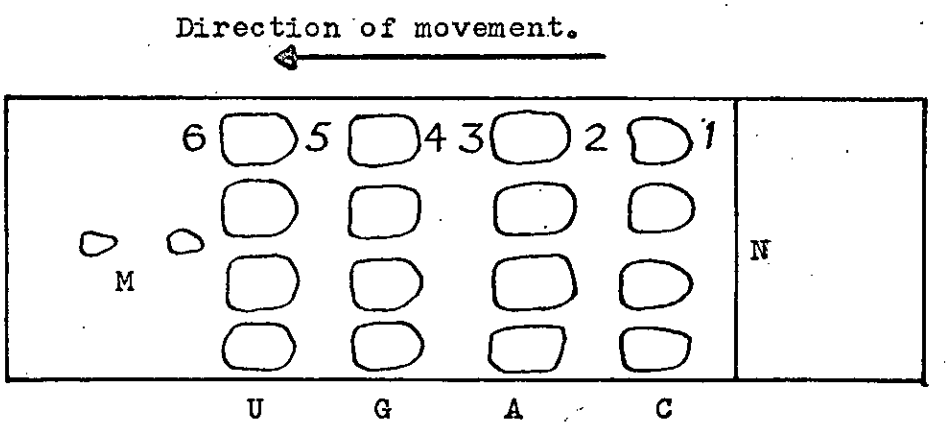


FIGURE 2.6 Separation of nucleotides obtained by high-voltage electrophoresis. Scale x 1/5.
M Marker dye spots. N Base line. 1 - 6 blanks. C,A,G,U nucleotide spots visualised in ultra-violet light.

Final Acrylamide concentration	Stock Acrylamide solution	Electrophoresis buffer	Water.
2.2%	5 ml	6.8 ml	22.0 ml
2.4%	5 ml	6.25 ml	19.7 ml

Electrophoresis Buffer.	tris	36mM
	NaH_2PO_4	30mM
	$EDTANa_2$	1mM
	pH 7.6 to 7.8 at room temperature.	

Mixing the buffer with acrylamide and water to make the gels diluted it to a strength equal to that of the running buffer.

The mixture of acrylamide, buffer and water was degassed under vacuum for 20 seconds. 25 µl TMED and 0.25 ml of a freshly prepared 10% solution of ammonium persulphate were added. The solution was mixed, avoiding aeration, then pipetted immediately into the gel tubes to within 1.5 cm of the top. Water was carefully layered onto the top of the mixture to obtain a flat gel surface. The gels were allowed to set for 30 minutes at 25°C.

The gel tubes were of perspex, 0.25 inch internal diameter, and 2.5 inches long. Into the bottom end was inserted a ring of PVC tubing, about 2 mm thick, which prevented the gel from sliding out of the tube during electrophoresis. While the gels were being cast and set, the hole in the ring was plugged by a piece of glass rod.

Electrophoresis. 6 or 8 gel tubes were mounted vertically in the electrophoresis tank as shown in Figure 2.2. The running buffer was the electrophoresis buffer diluted five times and

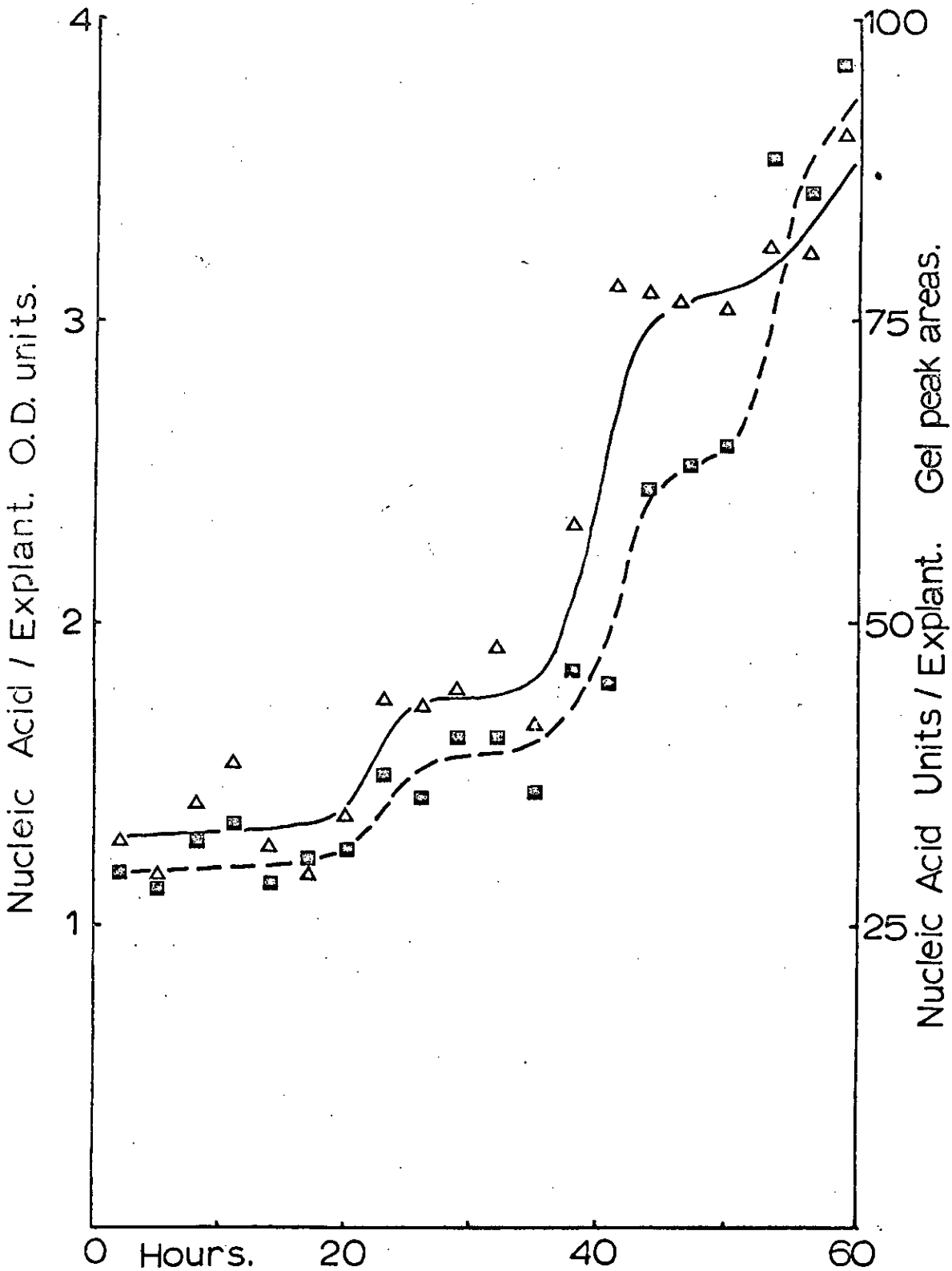


Figure 2.3 Changes in total nucleic acid per explant with time of culture, measured by absorbance at 260 nm of solution applied to gels (▣--▣) and by summation of gel peak areas.(Δ-Δ)

including 2 g / l SDS. About 200 ml were placed in each buffer compartment. The buffer was used once only. The immersion of the gel tubes in the buffer over most of their length prevented overheating during electrophoresis. Care was taken to exclude bubbles from the holes in the gel retaining rings.

The gels were 'pre-run' for 30 - 60 minutes to remove polymerisation catalysts and unpolymerised monomer, and to allow SDS to enter the gel. The current was 5 ma per gel at 50 V at room temperature. 10 to 100 µl of the RNA solution, containing up to 100 ug of RNA was layered on top of the gel. Electrophoresis was continued for 1 to 2½ hours depending on the degree of separation required.

Optical scanning. Gels were scanned for absorbance at 265 nm in a Joyce Loebl Chromoscan. This was fitted with a mercury vapour lamp, a slit about 0.8mm x 0.05mm, and interference (265 nm) and liquid filters. The liquid filter was a solution of para dimethylaminobenzaldehyde, 10 - 12 mg / 100 ml methanol

The gel was held in a parallel sided quartz cuvette. The machine was set to expand the scanned length of the gel 3 times. Four full scale deflection values for absorbance were available to suit the RNA concentration of the gel. Of these, the highest, 2 O.D., gave a non-linear response with concentration, while the others, O.D.s 1, 0.5 and 0.33 gave a roughly linear response. In the latter cases, the area of a scan peak was linearly proportional to the concentration of the RNA in the peak. Ingle (1968) has shown that an estimate of the amount of an RNA species present may be obtained by measuring its optical peak area. This was done by weighing a tracing of the peak. Figure

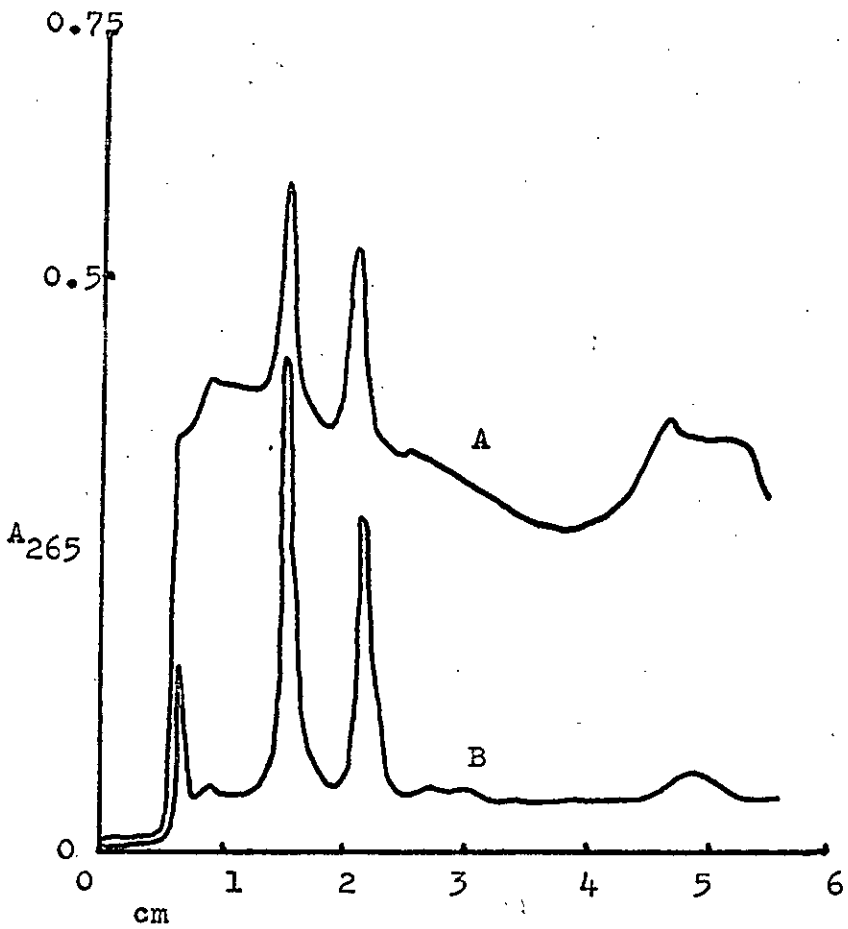


Figure 2.4 Electrophoresis of cytoplasmic fraction RNA prepared by method 1. Scan A. gel scanned immediately after electrophoresis. Scan B. gel washed for 4 hours in distilled water before scanning. 0 - 0.5cm - baseline set for distilled water.

2.3 shows the total nucleic acid per explant estimated in two ways, by the gel peak area method and by the absorbance at 260 nm of the RNA solution prepared for electrophoresis. The two methods gave very similar curves for the change in total nucleic acid per explant during culture.

It was frequently found that gels had a high background absorbance, often localised near the top of the gel. The amount seemed to increase with prolonged storage of the gels before electrophoresis. It was removed by soaking gels in distilled water for 2 - 4 hours before scanning. Figure 2.4 shows the reduction of background absorbance obtained by a four hour wash. Burns (1968) has calculated that no ribosomal RNA is lost during washing, but that 20% and 50% of transfer RNA is lost after 4 and 24 hours washing.

Radioactivity scanning. The gel was frozen in an aluminium trough lying horizontally on solid, powdered CO_2. No CO_2 was allowed to touch the gel directly, as this caused distortion during freezing. The frozen gel was frozen to the platform of a chopper developed by Loening (1967), and similar to the McIlwain tissue slicer (McIlwain and Buddle, 1953). The gel was allowed to thaw slightly and was cut into transverse slices 0.5 mm long. These were dried on adhesive labels, or on every second frame of the non-emulsion side of 16 mm cine film. Adhesive label mounted slices were stuck to planchets and counted in the Beckman 'Lowbeta' low background gas flow counter. Film mounted slices were counted with an IDL scaler. This was fitted with a geiger tube in a lead castle, a sample changer derived from a cine camera film magazine, and print out and control units.

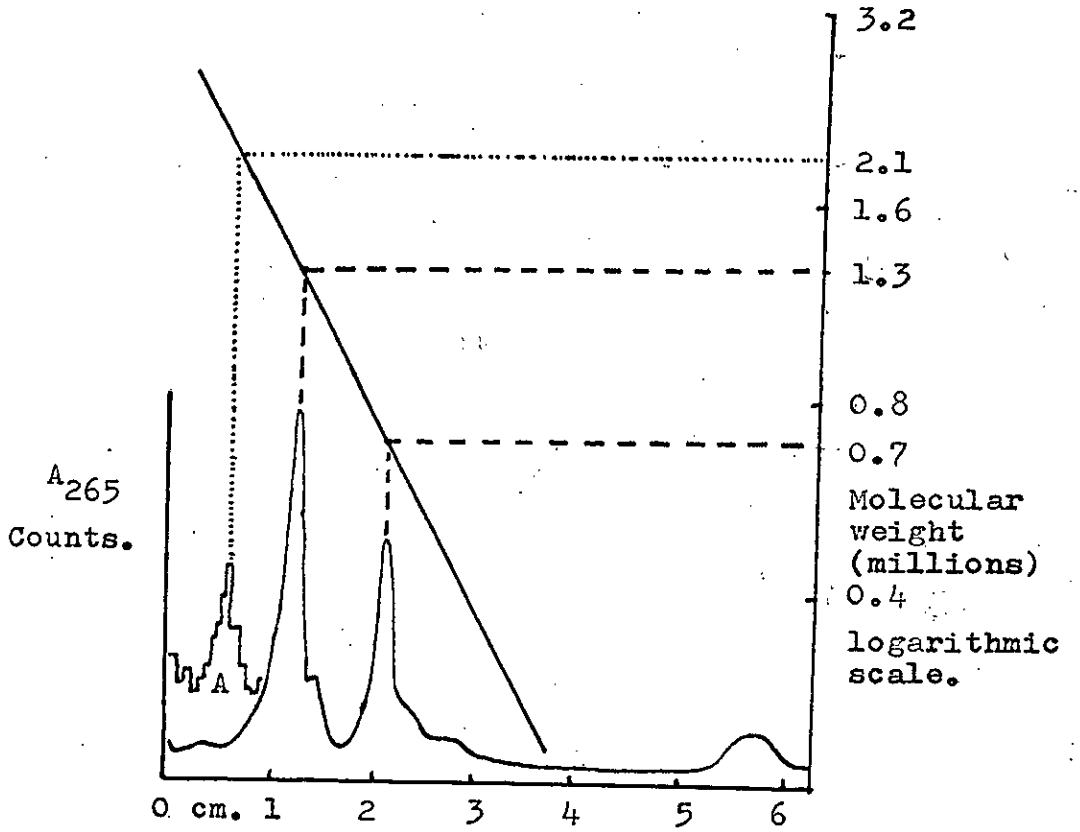


Figure 2.7 Method of calculating molecular weights of gel peaks. The straight line is a log. plot of molecular weight against length, established by reference to the 1.3 and 0.7m ribosomal RNA peaks. The dotted line shows the derivation of a molecular weight of 2.1m for the radioactivity peak marked 'A'.

Two methods were used to ensure accurate alignment of radioactivity and optical scans:

A. Before optical scanning, two fine India ink marker lines were injected into the gel at right angles to the long axis. The gel was frozen with a stopper against the top end to preserve its shape and with the bottom end free. The markers were readily identifiable in the optical scan and in the dried gel slices. The region between the markers on the optical scan was divided into the counted number of slices between the marked slices, and the same scale was extended beyond the markers.

B. The length of the unfrozen gel was determined from the optical scan. The gel was held at the same length during freezing by stoppers at both ends. The length of gel which was not cut was subtracted from the total. The remainder was divided into as many sections as slices were cut.

Molecular weight determination. Bishop, Claybrook and Spiegelman (1967) and Loening (1968) showed a linear relationship between log. molecular weight and electrophoretic mobility. Loening has determined the molecular weights of higher plant ribosomal RNA as 1.28m and 0.70m, by comparison with mammalian r-RNA (Taken as 1.75m and 0.70m from Hamilton, 1967) and with E. coli r-RNA (1.08m and 0.56m, Stanley and Block, 1965).

Figure 2.7 shows how a plot of log molecular weight against distance was established for a gel by reference to the r-RNA positions and known weights, and demonstrates the determination of a molecular weight for another peak.

Base compositions. Gel slices were removed from the film and

and incubated with 2 ml of:

Piperidine	10%
$EDTANa_2$	1mM
Yeast RNA	0.25 mg / ml.

for about 48 hours at 60°C in a tightly closed bottle.

The supernatant, containing RNA hydrolysed to mononucleotides, was removed and the gel slices extracted for 1 hour with 2 ml piperidine solution without added yeast RNA. The supernatants were combined and evaporated to dryness at 60°C. The nucleotides were dissolved in a few drops of buffer:

Acetic acid	7.5%
pyridine	0.75%
pH 3.5	

The nucleotides were applied to a sheet of Whatman 3MM paper as a streak 1.5 cm long.(Figure 2.6) A marker spot of 0.5% orange G, 0.5% acid fuchsin was also applied. The remainder of the paper was soaked with buffer applied so as to sharpen the nucleotide bands. After blotting off surplus buffer, the paper was placed in the electrophoresis tank (Figure 2.5) and a current of 60 ma at 1200 V was applied for about 1 hour. By this time, the marker dyes had almost reached the lower buffer region.

The paper was air dried overnight. The added yeast RNA allowed nucleotide spots to be marked out by inspection in ultraviolet light. (Figure 2.6). Nucleotide spots and blanks were cut out and mounted on planchets for counting in the Lowbeta counter, or were immersed in scintillation fluid:

Toluene	1000 ml
PPO	5 g
POPOP	0.03 g

for counting with the packard 'Tri-Card' liquid scintillation spectrometer. A minimum of 1000 counts, and normally more than 2000 counts above background were collected for each nucleotide. (95% confidence limits of counts less than 5% of count) Separations in which the blank counts were more than 10% of the nucleotide count were rejected. Base compositions were calculated as percentages of the total nucleotide count.

The amount of radioactivity in an RNA species was determined from the number of counts in its radioactivity scan peak. A correction was made for the background of heterodisperse label.

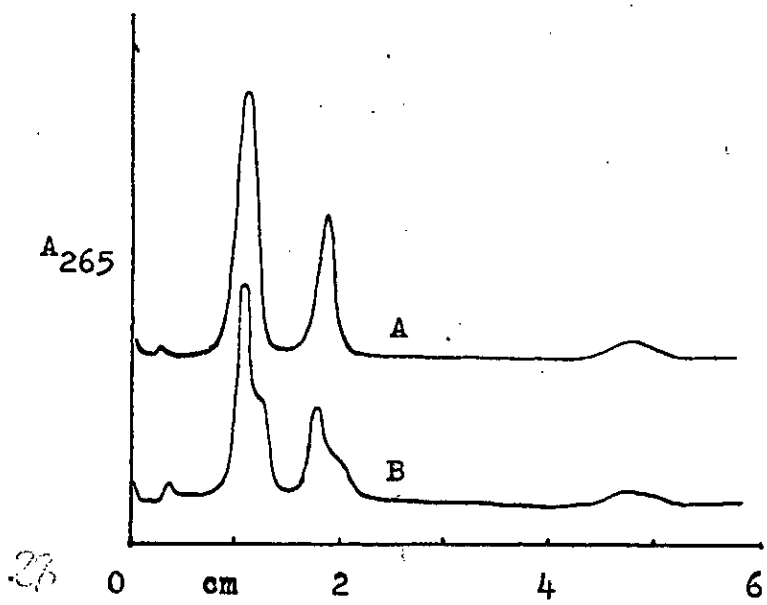


Figure 2.8 Electrophoresis of cytoplasmic fraction RNA prepared by method 1. A. Homogenate processed quickly at 0°C. B. Homogenate exposed to room temperature for 5 minutes.

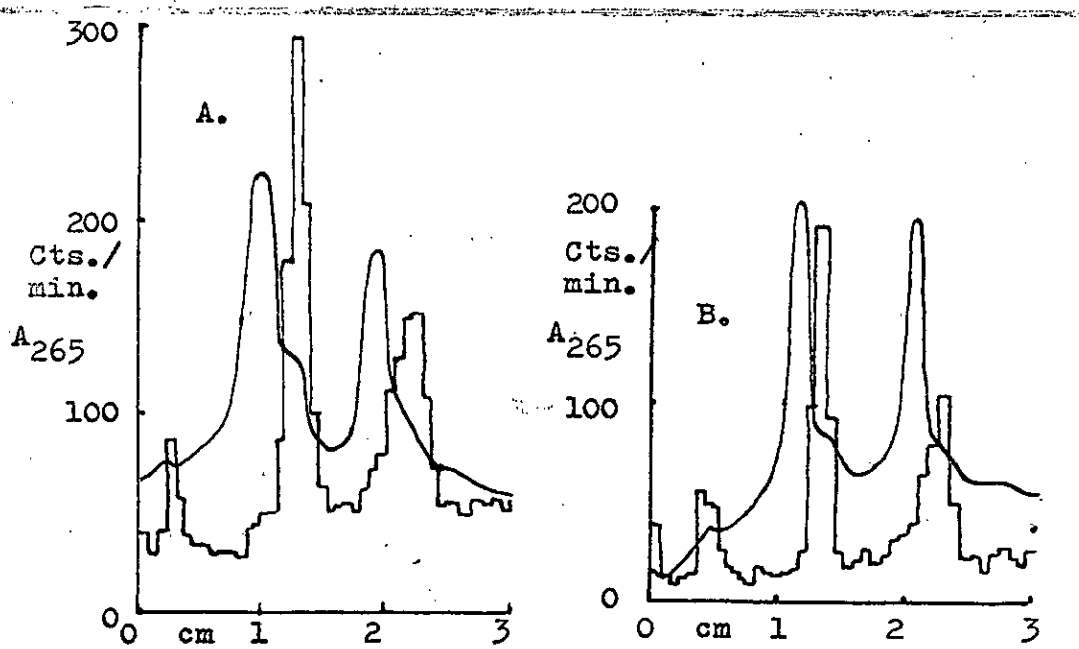


Figure 2.10 Electrophoresis of cytoplasmic fraction RNA prepared by method 1. A. Homogenised in homogenising medium. B. Homogenised in homogenising medium containing 0.5% NDS.

RESULTS.

1. Ribosomal RNA breakdown.

A. Detection.

Cytoplasmic fraction RNA was prepared by homogenisation and method 1. One sample was exposed to room temperature (25°C) for 5 minutes after homogenisation, the other was processed quickly at 0°C. Electrophoresis of the RNA was on 2.4% gels for 90 minutes.

Ribosomal RNA from the 0°C treatment occurred as two symmetrical peaks (Figure 2.8). The 25°C treatment resulted in the appearance of additional shoulders of molecular weights about 1.1m and 0.6m.

It was frequently observed that in experiments involving large numbers of samples, hence a delay in processing, such lower molecular weight shoulders increased at the expense of the 1.28m and 0.70m ribosomal peaks, even though the samples were stored on ice between homogenisation and addition of detergent. Thus during the early stages of method 1 extraction, ribosomal RNA appears to be susceptible to degradation to slightly smaller units.

B. Breakdown in old and newly synthesised r-RNA.

Explants from freshly harvested tubers were cultured for 27 hours before labelling by a 30 minute pulse in 32-P followed by 30, 90 or 210 minute chases. Labelling was under sterile conditions. RNA was prepared by homogenisation and method 1. Electrophoresis of the RNA was on 2.4% gels run for 110 minutes.

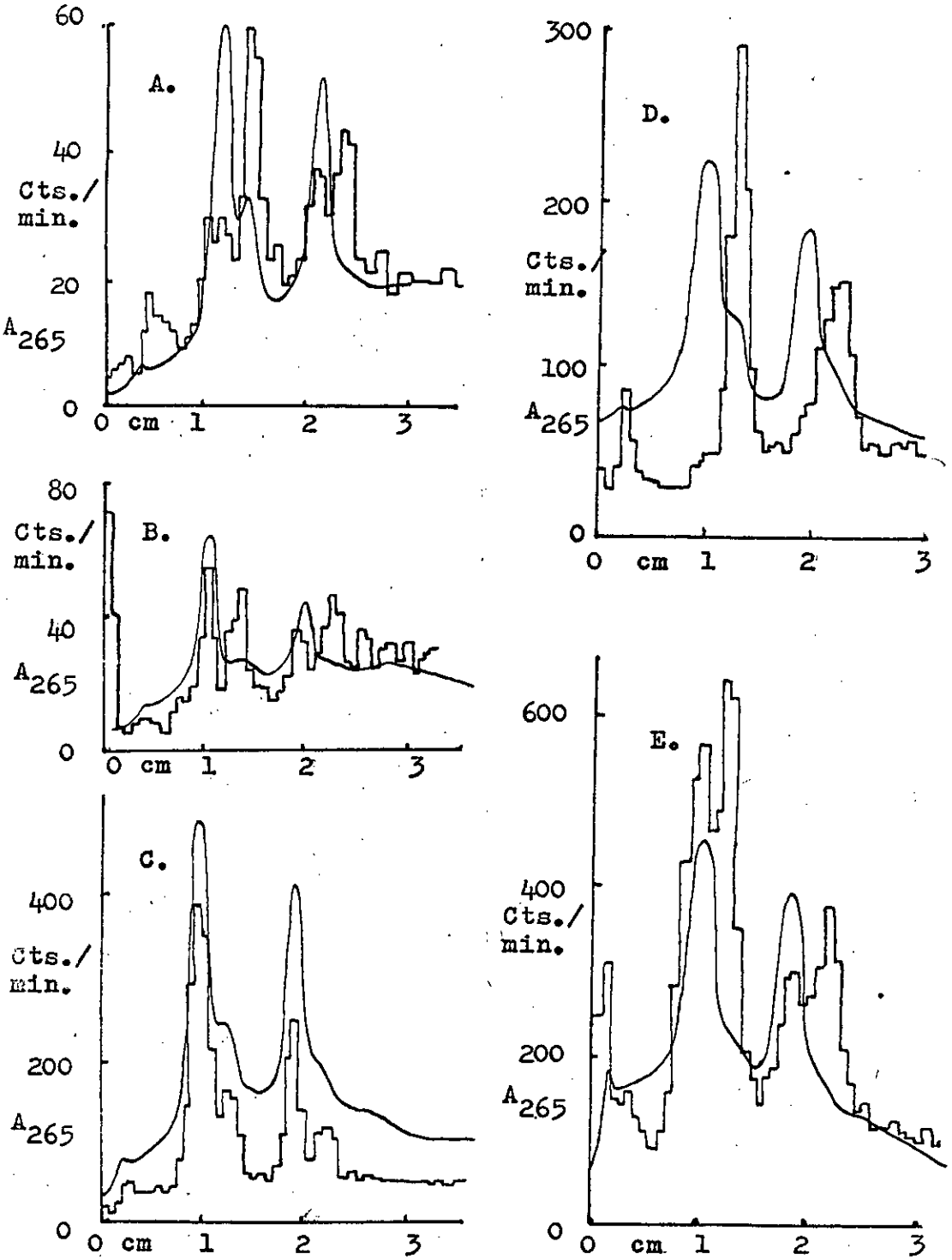


Figure 2.9 Electrophoresis of cytoplasmic fraction RNA prepared by method 1 from explants from freshly harvested tubers (A,B,C) and from tubers stored for 12 months (D,E). Labelling was by a 30 minute pulse followed by chases of 30 min. (A,D) 90 min (B) or 210 min. (C,E)

In figure 2.9,A,B,C, optical scans for all samples show shoulders on the two ribosomal RNA peaks. About 5% of the total r-RNA occurred in the degraded form. The radioactive scan for the 30 minute chase shows about 70% of the labelled, i.e. newly synthesised RNA, in the degraded form. With longer chase timings, this percentage dropped, until after 210 minutes chase, only 10% of label was in the degraded form.

Newly synthesised r-RNA appears therefore to be more susceptible to degradation than older RNA.

C. Effect of tuber age on breakdown.

Explants from tubers stored for 12 months were cultured, labelled and used for RNA preparation at the same time and in the same was as described in B above.

The optical scans (Figure 2.9, D,E) show amounts of degradation of r-RNA similar to those obtained with new tubers. The amount of breakdown of the newly synthesised RNA was much higher with the old tubers than with the new, for all chase timings.

D. Effects of NDS in the homogenising medium.

27 hours old explants from 12 months-stored tubers were labelled by a 30 minute pulse and a 30 minute chase. RNA was prepared by method 1. Homogenisation was either in homogenising medium or in homogenising medium containing 0.5% NDS. Electrophoresis of the RNA was on 2.4% gels for 110 minutes.

Figure 2.10 shows that in both cases, 5% of the total r-RNA, and 100% of the labelled r-RNA were in the 1.1m and 0.6m degraded forms. NDS therefore had no inhibiting action on the degradation of r-RNA.

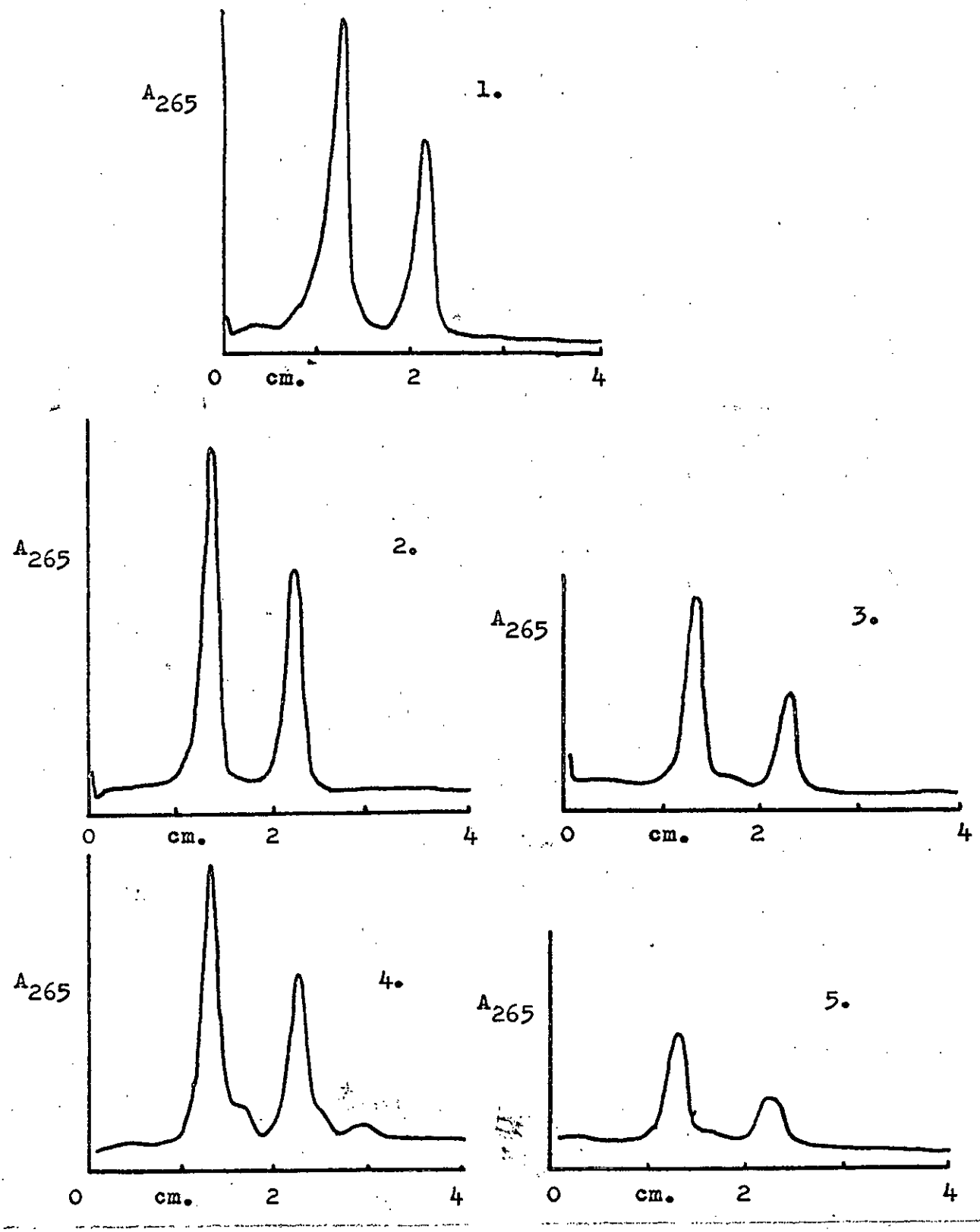


Figure 2.11 Electrophoresis of RNA, prepared by method 1 (4 - cytoplasmic fraction, 5 - nuclear fraction) or method 2 (1 - whole tissue preparation from homogenisation in Kirby medium. 2 - cytoplasmic fraction and 3 - nuclear fraction from homogenisation in NDS / phenol

E. Further effects of RNA preparation method on degradation.

RNA was prepared from freshly excised explants by three methods:

A. Total RNA was extracted by homogenisation in Kirby medium.

B. Cytoplasmic and nuclear fraction RNAs were extracted by homogenisation and method 2.

C. Cytoplasmic and nuclear fraction RNAs were extracted by homogenisation and method 1.

All samples were DNase treated and analysed by electrophoresis on 2.2% gels run for 90 minutes.

With RNA preparation by method 2 or by homogenisation in Kirby medium, no breakdown of ribosomal RNA was detectable in the optical scans. (Figure 2.11, 1,2,3) With method 1, no breakdown was visible in the nuclear fraction, but the cytoplasmic fraction showed breakdown products with molecular weights of 1.1m and 0.6m as before and a third peak with a weight of 0.5m.

r-RNA is therefore susceptible to degradation during extraction by method 1 but not during extraction by method 2.

F. Plastid ribosomal RNA.

The 1.1m and 0.6m labelling peaks detected above are similar in size to the plastid ribosomal RNAs found by Loening and Ingle (1967). A check was made on the artichoke tissue to find if plastid ribosomal RNA was synthesised under any conditions.

Explants were grown for 67 hours under various hormone conditions (2,4-D 0 to 10^{-5} M, coconut milk 0 or 20%), various light conditions (dark, 50fc, 450 fc, continuous or for the first 30 minutes of culture only) and various combinations of light and hormone treatments. RNA was extracted quickly by method

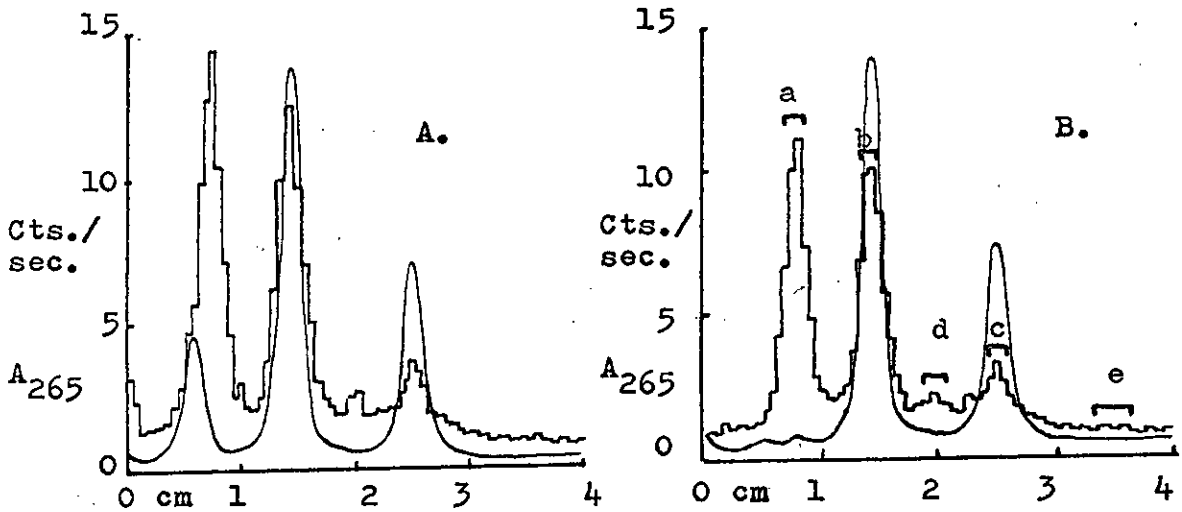


Figure 2.12 Electrophoresis of nuclear fraction RNA and DNA, prepared by homogenisation and method 1. A - not DNase treated B - DNase treated. a - e indicate regions for which base composition was determined (Table 2.1)

Sample	M.Wt	Base Composition - % counts in				$\frac{G+C}{A+U}$
		C	A	G	U	
a	2.1m	21.3	26.8	33.0	18.9	1.19
b	1.3m	20.6	28.7	32.0	18.7	1.11
c	0.7m	19.3	28.3	29.3	23.1	0.95
d.		20.4	30.4	26.9	22.2	0.90
e		17.5	33.4	25.0	24.1	0.74

TABLE 2.1 Base compositions of RNA fractions from gel regions shown in Figure 2.12

1 and examined on 2.4% gels. In no case was any evidence of 1.1m and 0.6m RNA found, and none of the treatments caused the explants to turn green. Plastid ribosomal RNA synthesis does not therefore appear to occur in the cultured artichoke explant.

G. Conclusion.

Ribosomal RNA may be extracted without degradation to 1.1m and 0.6m species by method 2, and by method 1 provided that the homogenisation and centrifugation are carried out rapidly at 0°C.

2. The efficiency of extraction of ribosomal RNA.

The aim of this experiment was to examine the yields of RNA, and the nature of the sub-cellular fractionation given by the two methods of tissue disruption and the two RNA extraction procedures.

Explants were cultured for 24 hours, and labelled by a pulse of 15 minutes. The chase incubation was for 75 minutes. Tissues were disrupted by homogenisation or by chopping while frozen with solid CO_2. Sub-cellular fractionation to 'cytoplasmic' and 'nuclear' fractions, and extraction of nucleic acids were by method 1 or method 2. Each sample was divided into two equal parts, one of which was DNase treated. The RNA was fractionated by electrophoresis on 2.2% gels for 90 minutes.

A. Features of gels.

Figure 2.12 shows representative scans. The optical scans show DNA at 0.6 cm, and ribosomal RNA at 1.4 and 2.5 cm. DNase treatment removed most of the DNA, but disclosed a small optical

RNA prep. method.	Fraction	DNase.	Totals			Radioactivity.		
			1.28m	0.70m	DNA	2.07m	1.28m	0.70m
Homogenisation, method 1.	C	-	208	110	0	6.7	31.8	17.8
	C	+	229	125	0	9.5	44.3	27.6
	N	-	80	35	33	61.5	58.5	5.6
	N	+	72	32	3	34.3	52.8	7.3
Chopping, method 1.	C	-	102	40	0	4.4	8.6	4.8
	C	+	92	44	0	3.2	8.5	7.6
	N	-	102	54	30	52.7	52.7	6.9
	N	+	104	49	4	53.8	54.0	12.2
Homogenisation, method 2.	C	-	194	114	0	2.8	24.0	20.4
	C	+	192	96	0	2.9	17.2	16.6
	N	-	24	12	3	0.9	5.6	1.8
	N	+	20	9	0	3.3	7.6	2.2
Chopping, method 2.	C	-	154	80	0	4.3	18.9	15.3
	C	+	158	88	0	3.2	20.8	15.9
	N	-	15	7	1	1.2	6.1	1.9
	N	+	20	7	0	3.4	8.9	1.4

Table 2.2 Quantities of 1.28m and 0.70m ribosomal RNA and DNA/30 explant, measured by gel peak areas (Arbitrary units); radioactivity of 2.07m, 1.28m and 0.70m peaks on gels (Counts /400 sec/ 30 explants x 10^{-3}), after tissue disruption by chopping or homogenisation and extraction by method 1 or method 2, with and without DNase treatment. C-cytoplasmic fraction, N-Nuclear fraction.

optical peak at 0.8cm.

Radioactivity peaks were associated with both ribosomal optical peaks. A small peak of radioactivity at 2.0 cm between the two ribosomal peaks was found in cytoplasmic and nuclear fractions from all methods of preparation. The large radioactive peak at 0.8cm corresponds to a molecular weight of 2.07m. It had a higher mobility than the DNA optical peak, and was DNase resistant.

Examination of base compositions (Figure 2.12, Table 2.1) shows that the 2.07m peak had a ribosomal type base composition, with a high G + C content. Base compositions of the small peak at 2.0 cm and of the polydisperse labell at 3.5 cm were high in A + U content.

B. Comparison of extraction methods.

Total yields:

DNA. The yield of DNA from method 2 was about 1/10 th of that obtained by method 1. (Table 2.2) DNase treatment removed over 90% of the DNA, and had no effects on the RNA recoveries.

r-RNA. Chopping of the tissue gave lower recoveries of r-RNA than homogenising, as expected of the visibly less efficient tissue breakage. Method 2 extraction gave lower recoveries than method 1. The ratio of the amounts of 1.28m to 0.70m r-RNA components was close to the theoretical 1.85 with all methods of preparation.(Table 2.3)

Distribution between fractions. The distribution of extracted r-RNA between cytoplasmic and nuclear fractions varied greatly. Relatively more RNA was obtained in the nuclear fration with method

RNA prep. method.	DNase	C / N ratio		1.28m / 0.70m ratio.	
		total	counts	total	counts.
Homogenisation	-	2.7	0.45	1.99	3.88
Method 1.	+	3.4	0.86	1.92	2.79
Chopper,	-	0.91	0.16	2.17	5.24
Method 1	+	0.89	0.16	2.11	3.16
Homogenisation	-	8.5	5.69	1.73	1.34
Method 2	+	9.9	2.80	2.02	1.32
Chopper,	-	11.1	4.18	1.94	1.46
Method 2.	+	9.2	2.91	1.87	1.72

Table 2.3 Ratios of optical amounts of 1.28m + 0.70m and radioactivities of 2.07m + 1.28m + 0.70m in cytoplasmic and nuclear fractions (C / N); ratio of optical amounts or counts in 1.28m to 0.70m. (1.28m / 0.70m) Data from electrophoresis of RNA prepared by two extraction methods and two tissue disruption methods, with and without DNase treatment.

1 than with method 2. The method of tissue disruption made no difference to the distribution when method 2 extraction was used. With method 1 however, chopping the tissue resulted in a higher proportion of r-RNA in the nuclear fraction than homogenisation. This again is consistent with the less efficient tissue breakage by chopping, and with greater adhesion of cytoplasm to the nuclear debris.

Labelled RNA yields:

2.07m As in the case of DNA, the labelled 2.07m component was obtained in much greater amounts by method 1 than by method 2. (Table 2.2) DNase treatment had no effects on the amount of 2.07m label except in the homogeniser / method 1 treatment, where the amount of 2.07m label was halved by DNase treatment. This finding is contrary to all other experience, and is considered the result of some experimental error.

r-RNA. Recoveries of 1.28m r-RNA label were also higher with method 1 extraction than by method 2. Yields of 0.7m r-RNA were similar with both methods of extraction. With method 1 extraction, homogenisation gave higher label recoveries in the cytoplasmic fraction than did chopping. Yields in the nuclear fraction were similar with both methods of tissue disruption. With method 2 extraction, the method of tissue disruption did not affect the yield of r-RNA label.

The ratios of label in 1.28m to label in 0.70m were in the range 3 to 5 with method 1 and 1.3 to 1.7 with method 2. The ratio did not depend on the method of tissue disruption.

Distribution between fractions. Generally, the proportion of the RNA label recovered in the nuclear fraction was higher than the

RNA Prep. method	Fraction.	DNase.	Specific Activity of: 2.07m	1.28m	0.70m	fraction.	tissue.
Homogenisation. Method 1.	C	-	21	153	162	177	420
	N	-	535	731	160	1092	
	C	+	27	193	221	230	384
	N	+	330	733	228	746	
Chopper. Method 1.	C	-	31	84	120	125	436
	N	-	338	517	128	720	
	C	+	24	92	173	142	479
	N	+	349	517	249	779	
Homogenisation. Method 1.	C	-	9	124	179	153	161
	N	-	25	233	150	231	
	C	+	10	90	173	127	157
	N	+	113	380	244	452	
Chopper. Method 2.	C	-	18	123	191	165	186
	N	-	55	407	271	417	
	C	+	13	132	181	162	196
	N	+	126	445	200	507	

Table 2.4 Specific activities of 1.28m and 0.70m r-RNA, of 2.07m expressed relative to total ribosomal amount; mean specific activities of sub-cellular fractions and whole tissues, expressed as total counts in 2.07m, 1.28m and 0.70m / total amount of 1.28m and 0.70m in the fraction or tissue. Units of specific activity counts/400 sec./gel peak area unit. RNA extracted using two methods to disrupt tissue, two methods for RNA extraction, and with and without DNase treatment.

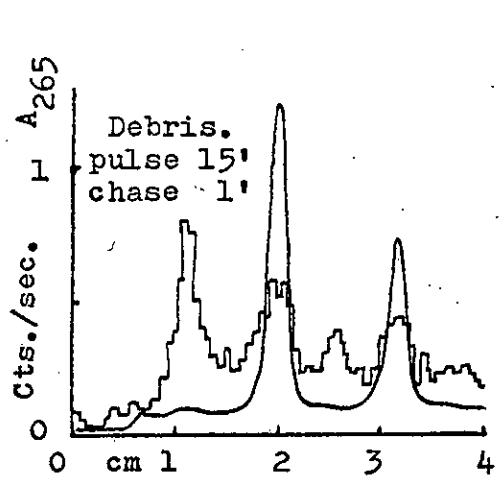
proportion of the total RNA. With method 1 extraction, the vast majority of the 2.07m label was recovered in the nuclear fraction. Method 2 extracted a higher proportion of total label in the cytoplasmic fraction than in the nuclear; method 1 extracted a higher proportion of the total label in the nuclear fraction than in the cytoplasmic. The failure of method 2 to extract 2.07m label and nuclear 1.28m label was the major cause of this difference. Chopping resulted in a higher proportion of label in the nuclear fraction than homogenisation with method 1 extraction, but made no difference with method 2 extraction. Thus the effects of tissue disruption method on label distribution were similar to those on total amount distribution.

Specific activities (Table 2.4)

2.07m The specific activity of the 2.07m labelled RNA was calculated with reference to the total amount of r-RNA present, because of the impossiblity of measuring the area of the 2.07m optical peak with accuracy. With method 1 extraction, the 2.07m specific activity was much higher in the nuclear fraction than in the cytoplasmic fraction.

r-RNA. The 1.28m specific activity was greater in the nuclear fration than in the cytoplasmic fraction in all cases. 0.70m specific activities were similar in both fractions. For the tissue as a whole, method 1 extraction yielded RNA of 2.5 times the specific activity of that from method 2 extraction. Tissue disruption method had no effects on specific activity.

C. Conclusion. For the study of 2.07m and r-RNA synthesis reported below, homogenisation and method 1 were chosen because


Debris.
pulse 15'
chase 1'
Cts./sec.
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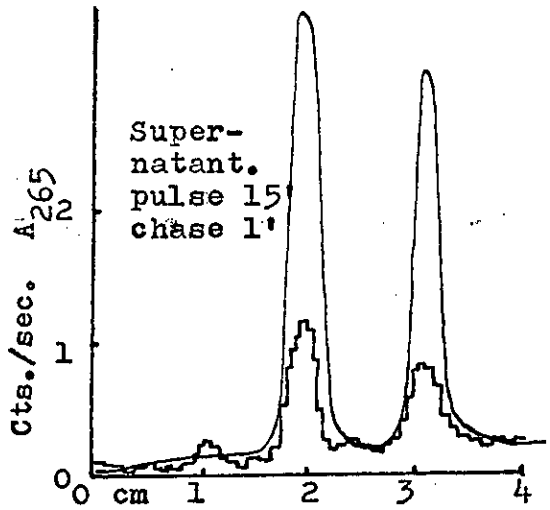
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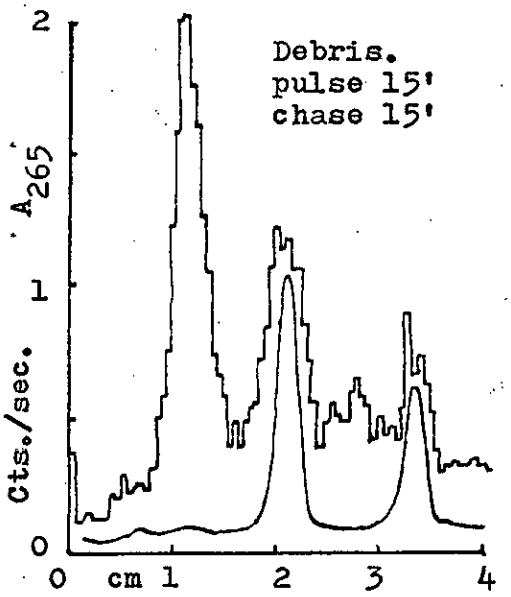
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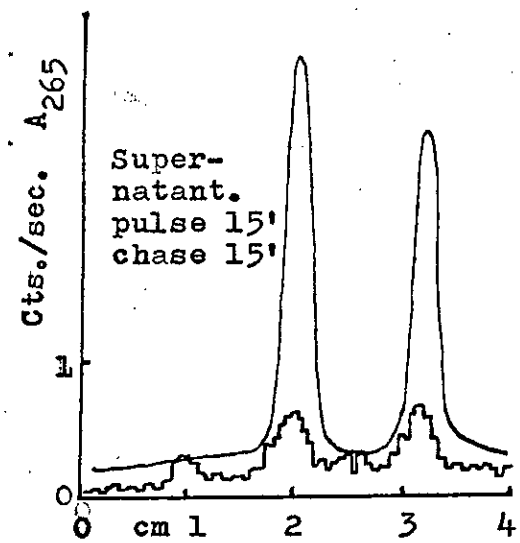
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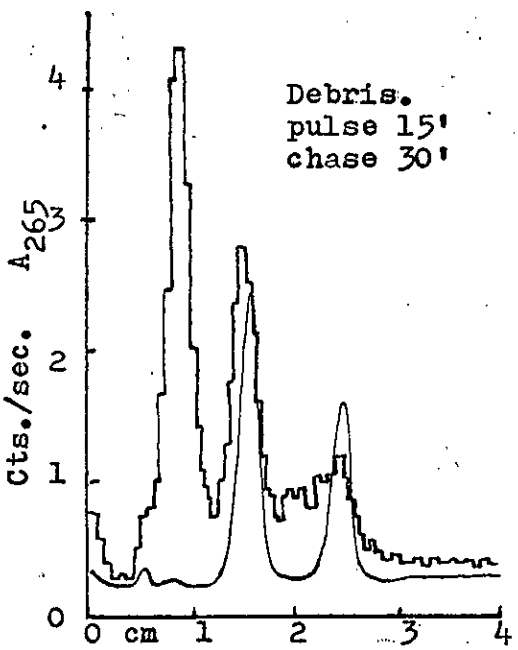
Debris.
pulse 15'
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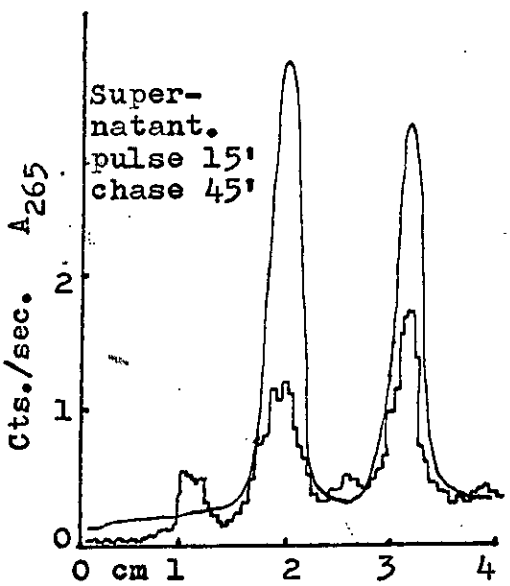
Super-
natant.
pulse 15'
chase 45'
Cts./sec.
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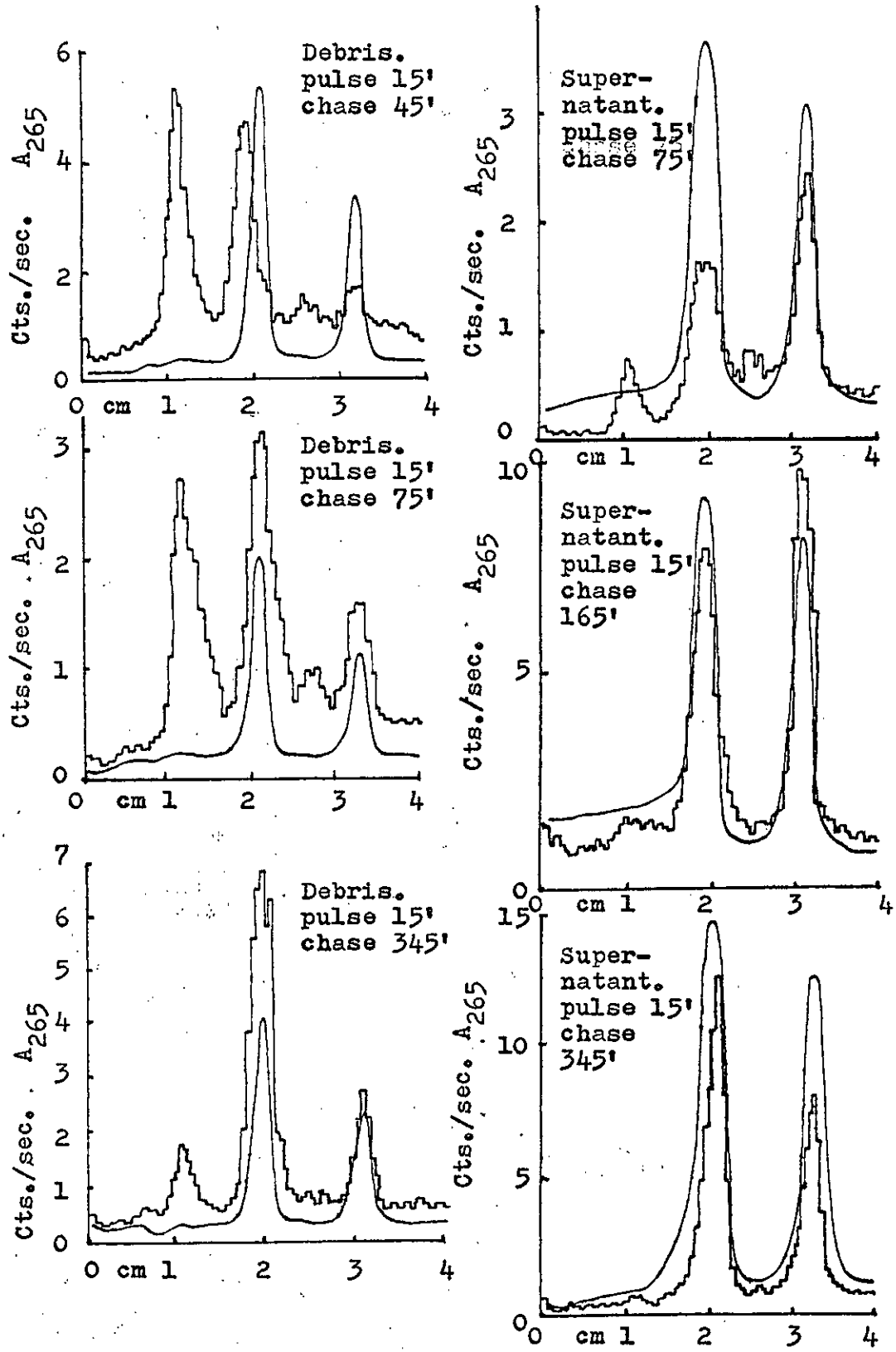


Figure 2.13 Electrophoresis of cytoplasmic and nuclear fraction RNAs prepared by method 1 after various pulse and chase timings.

of the high recoveries obtained. The quality of the subcellular fractionation given by the various methods is considered in detail in the discussion.

3. The synthesis of ribosomal RNA.

The ribosomal-type base composition of the 2.07m labelled RNA peak found in the previous experiment, and its high specific activity suggested that it might be a precursor to ribosomal RNA similar that that reported in HeLa cells (Scherrer et.al. 1963, Weinberg et. al.1967). This experiment was designed to investigate the kinetics of labelling of the 2.07m and ribosomal RNAs.

Explants were cultured for 40 hours. The pulse incubation was for 15 minutes. Chase incubations were for 1, 15, 30, 45, 75, 165 and 345 minutes. RNA was extracted by the homogeniser / method 1 procedure. Care was taken throughout to keep the fractions at $0^{o}C$ and to start the detergent extraction as soon as possible to eliminate the chance of r-RNA degradation. The experiment was carried out twice; in one case all samples were DNase treated.RNA was fractionated by electrophoresis on 2.2% gels, which were run for 2 hours.

A. 32-P 'uptake'. The 32-P content of the alcohol soluble fraction decreased after the end of the pulse. (Figures 2.15, 2.19).

B. DNA. Labelling was apparent by 15 minutes. (Figure 2.17) and remained constant after 45 minutes.

C. Total RNA labelling. This was measured as the sum of the

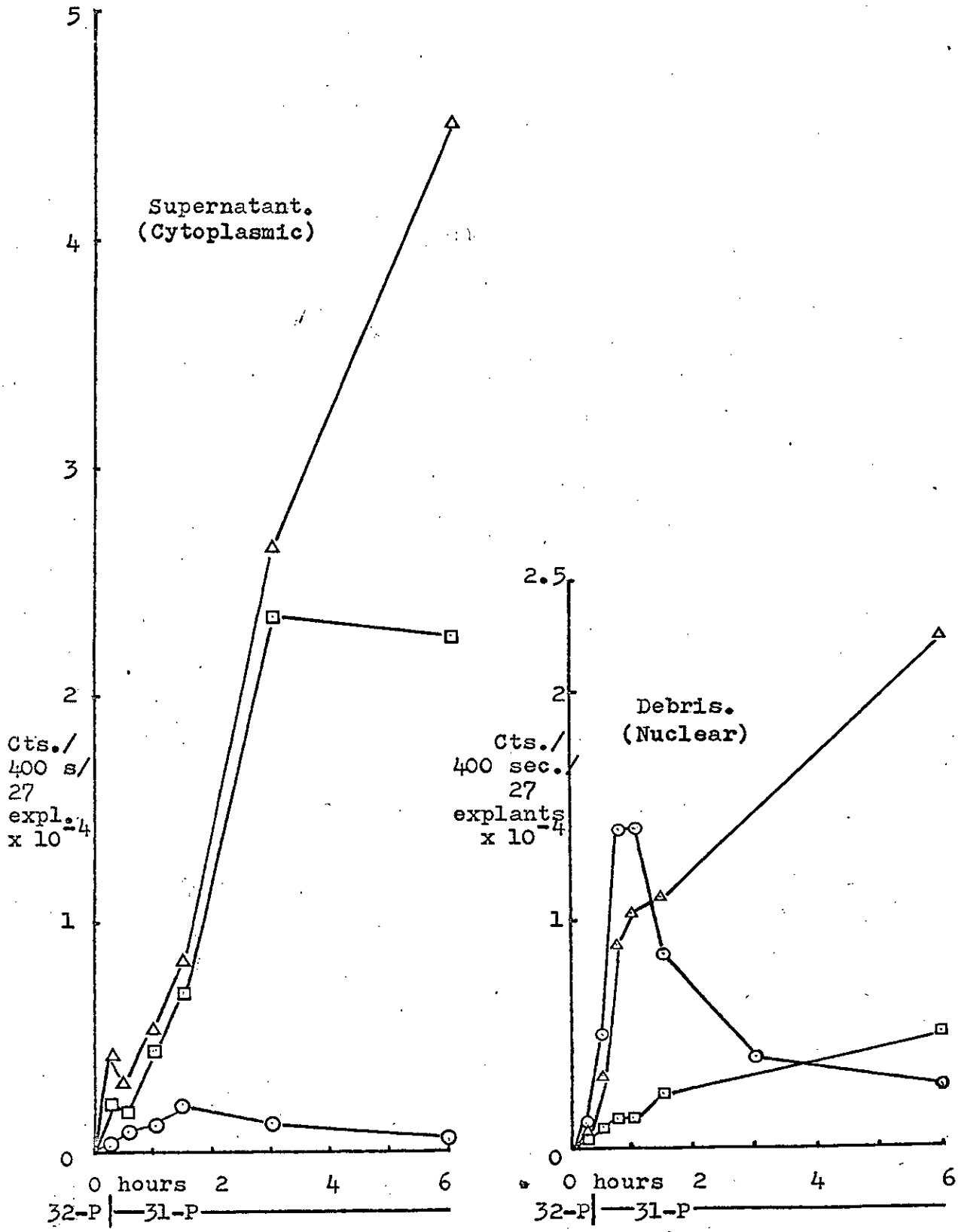


FIGURE 2.14 Changes in radioactivity of 2.1m (O——O), 1.4 + 1.3m (△——△) and 0.7m (□——□) RNA in debris and supernatant fractions, from tissues pulse incubated for 15 minutes followed by various chase timings. Data derived from electrophoresis scans of Fig 2.13

label in the 2.07m, 1.4m, 1.28m and 0.70m peaks. No account was taken of polydisperse label between the peaks. The polydisperse 'background'labelling in the peaks was estimated and subtracted from the total label in the peak. 'Total' RNA labelling therefore represents label in the ribosomal RNA molecules and their precursors.

Total RNA labelling increased steadily for 3 hours (Figure 2.19). In Figure 2.15, a decrease in rate of labelling became apparent after 3 hours.

D. 2.07m labelling. The 2.07m label peak in the nuclear fraction increased until 45 minutes, and then decreased. (Figure 2.14) The small amount of this label in the cytoplasmic fraction followed a similar pattern.

Determinations of molecular weight from 16 scans gave the mean value for this peak as 2.07m. All individual values were within ± 0.04 of the mean.

E. Ribosomal RNA labelling.

Scans. The electrophoresis scans shown in Figure 2.13 show that in the supernatant fractions, the labelling peak associated with the 1.28m ribosomal RNA optical peak was coincident with it for all pulse-chase timings. In the nuclear fraction, coincidence of the optical and radioactive peaks was observed for chases up to 30 minutes, and with chases lasting 75 minutes and longer. But with a 45 minute chase, the radioactivity peak was distinctly heavier than the optical peak. Examination of 6 scans gave a mean molecular weight value of 1.40m for the heavy label peak. All individual values were within ± 0.03m of

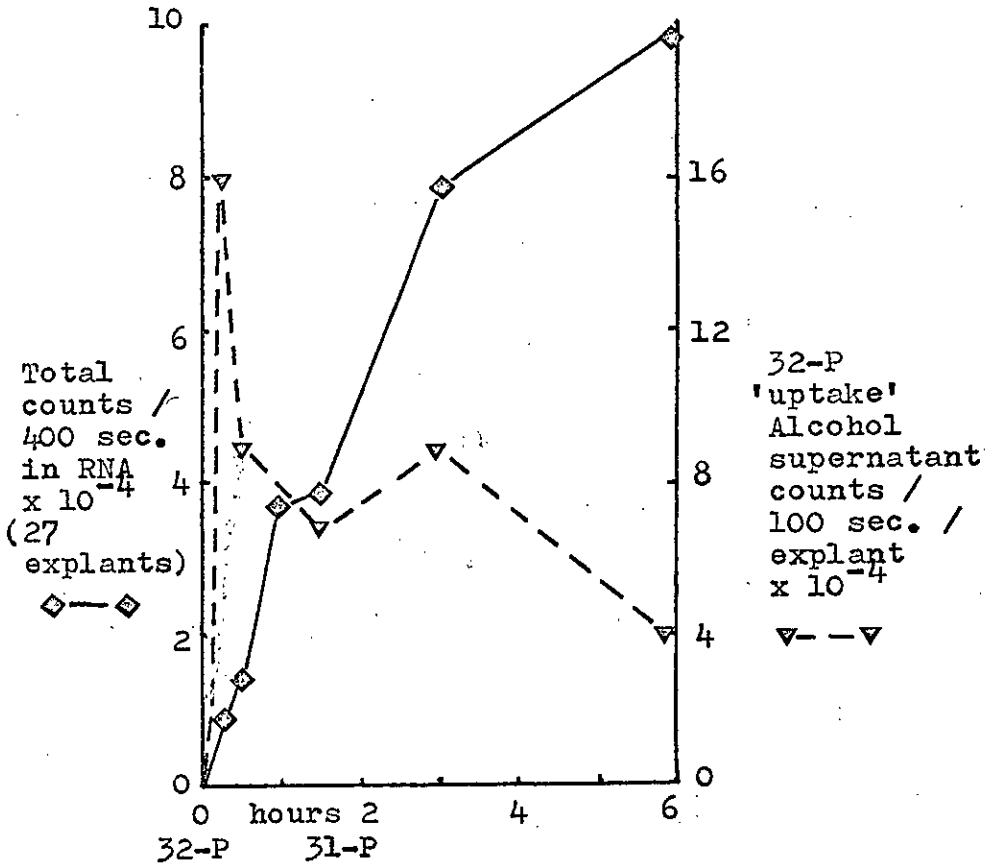


FIGURE 2.15 Changes in 32-P content of alcohol supernatant (32-P 'uptake') with time of incubation, and total label in 2.1, 1.4, 1.3 and 0.7m RNA fractions- data derived from Fig. 2.14

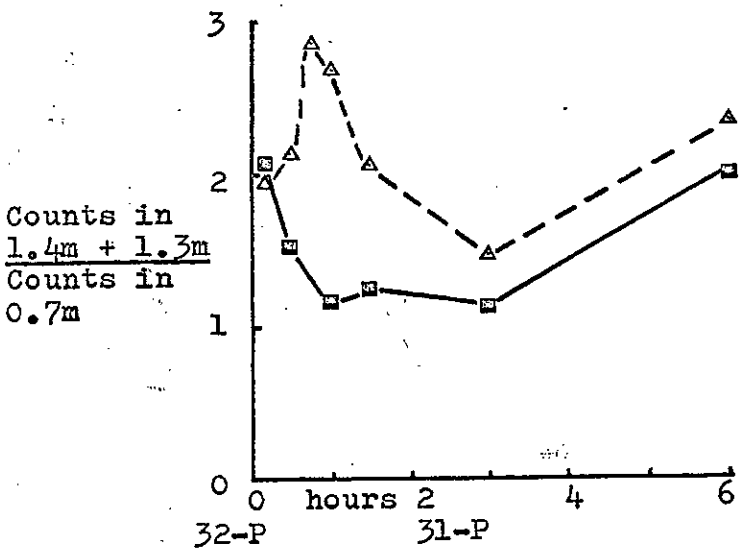


FIGURE 2.16 Changes in ratio of 32-P-label in 1.4 + 1.3m to 0.7m in the entire tissue (△——△) and cytoplasm only (▣——▣) with time of incubation. Data derived from Fig. 2.14

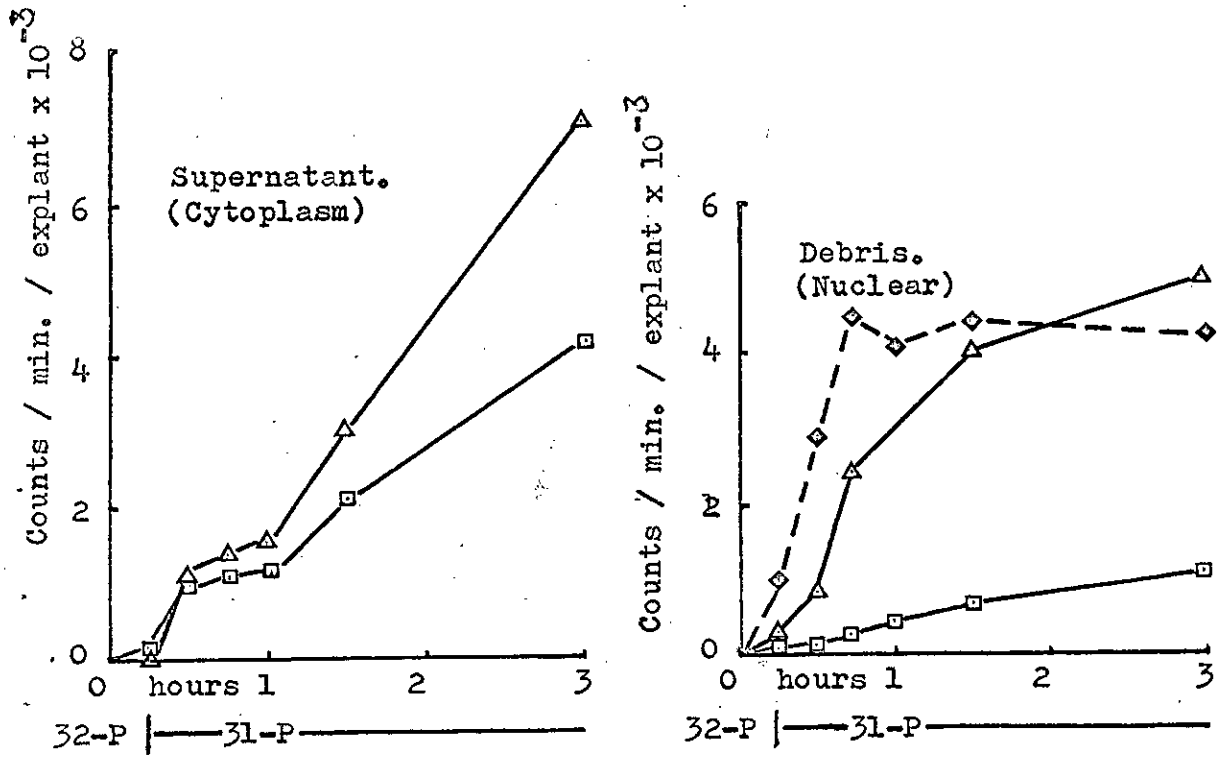


FIGURE 2.17 Changes in radioactivity of 1.4 + 1.3m (△——△) and 0.7m (□——□) RNA in debris and supernatant fractions, and in DNA (◇- - -◇), after a 15 minute pulse and various lengths of chase.

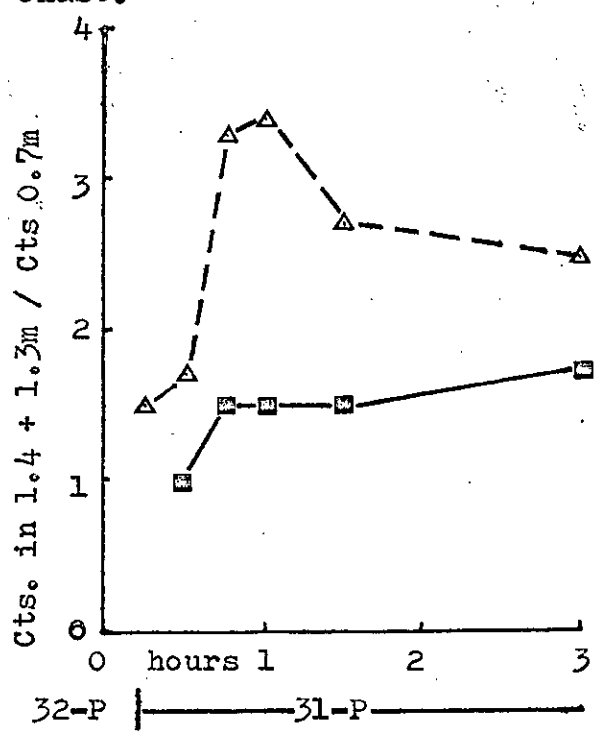


FIGURE 2.18 Changes in ratio of 32-P label in 1.4 + 1.3m to 0.7mM in the whole tissue (△——△) and in cytoplasm only (□——□) with time of incubation. Data derived from Fig. 2.17

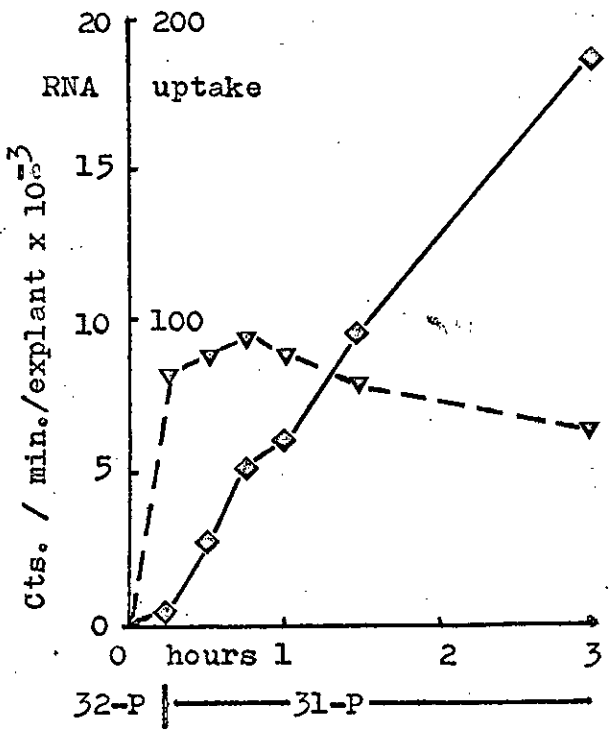


FIGURE 2.19 Changes with time of incubation in 32-P content of alcohol supernatant (32-P 'uptake') (▽——▽) and total label in 1.4, 1.3 and 0.7m RNA fractions (◇——◇) - data derived from Fig. 2.17

the mean. The 1.40m radioactivity peak was constantly observed in nuclear fractions after comparable radioactive incubation timings.

The 0.70m radioactivity and optical peaks were coincident at all times in both fractions.

Nuclear fraction. The rate of 1.40m + 1.28m labelling increased for 45 (Figure 2.17) or 60 (Figure 2.14) minutes, followed by a decrease in the rate of labelling. During the first 60 minutes of incubation, the rate of 1.28m label increase in the nuclear fraction was greater than the rate in the cytoplasm. 0.70m labelling was low in the nuclear fraction at all times, about 1/5th of the 1.4m + 1.28m labelling

Cytoplasmic fraction. Considerable changes in the rates of accumulation of 1.28m and 0.70m labelling in the cytoplasmic fraction occurred during the early stages of incubation. In Figure 2.14, the labelling of the 1.28m component was at twice the rate for the 0.70m for the first 15 minutes. There was then a slight fall in the amounts of both types of label, before both components increased at the same steady rate until three hours. Thereafter no further increase in 0.70m labelling occurred; the 1.28m labelling increased until 6 hours.

In a separate experiment, (Figure 2.17), a similar pattern was shown. Labelling was at a high rate between 15 and 30 minutes; the rate of 1.28m increase again being greater than that of 0.70m. Between 30 and 60 minutes, both components increased little in radioactivity. After 60 minutes, higher, constant rates of labelling were maintained until the experiment ended at 3 hours. During this last period of labelling the 0.70m labelling rate was lower than that for 1.28m.

2.07m radioactivity peak. Nuclear fraction.

Pulse	chase	C	A	G	U	No. Detns.
15	1	22.7	29.7	30.4	17.2	1
15	15	21.2	27.3	31.7	19.9	2
15	30	20.9	27.6	31.0	20.6	2
15	40 - 345	21.3	26.8	30.8	21.3	5

1.40m radioactivity peak. Nuclear fraction.

Pulse	chase	C	A	G	U	No. Detns.
15	45	20.7	29.2	30.3	19.9	2

1.28m radioactivity peak.

Pulse	chase	Nuclear fn. C	A	G	U	Det.	cytoplasmic fn. C	A	G	U	Det.
15	1	18.2	33.1	27.7	21.0	1	21.8	26.9	33.0	18.3	1
15	15	19.8	29.2	30.5	20.4	2					
15	30	21.0	29.3	30.4	19.3	2					
15	45						20.1	29.6	32.0	18.4	1
15	75-345	21.4	27.6	31.6	19.4	4	21.8	28.4	31.4	18.5	5

0.70m radioactivity peak.

Pulse	chase	Nuclear fn. C	A	G	U	Det.	cytoplasmic fn. C	A	G	U	Det.
15	1	18.7	29.1	26.9	25.3	1	19.9	26.4	30.4	23.2	1
15	30	19.0	31.6	26.3	23.1	1					
15	45-345	20.7	27.7	27.5	24.1	3	20.4	27.3	28.6	23.7	6

Region between the 1.28m and 0.70m peaks.

Pulse	chase	C	A	G	U	Detns.
15	1-15	15.9	33.0	26.4	24.7	1
15	30	21.5	30.1	25.3	23.1	1
15	45-345	20.5	28.5	27.9	23.0	3

Region lighter than 0.70m.

Pulse	chase	C	A	G	U	Detns.
15	30-75	19.2	32.8	25.2	22.9	2 (Nuclear)
15	165	19.0	36.7	23.2	21.1	1 (cytoplm.)

Table 2.5 Percentage base compositions of RNA fractions from gels shown in Figure 2.13, and number of individual determinations made for each base composition.

(1.40m + 1.28m) / 0.70m labelling ratio. In both experiments, the ratio for the whole tissue rose from a value close to 2 at 15 minutes to about 3 by 1 hour. (Figures 2.16, 2.18) During the remainder of the incubation, the ratio fell again to values closer to 2.

The ratio of label in the cytoplasmic fraction was 2 at 15 minutes, fell rapidly to just over 1, then rose to 2 again by 6 hours. In the other experiment (Figure 2.16) the ratio followed a similar pattern, rising from 1 to 1.6 between 30 minutes and 3 hours.

F. 1.0m labelling peak. The small radioactivity peak between the two ribosomal RNA peaks was visible in all cytoplasmic and nuclear fraction scans (Figure 2.13), though less distinct with longer chase timings. Because of its comparatively low radioactivity, it was not possible to obtain measurements for kinetic data.

G. Base compositions. (Table 2.5) The 1.28m and 0.70m RNA labelled in the nuclear fraction after 1 and 15 minute chases had slightly lower G + C contents than similar peaks from longer chase timings. Otherwise there were no significant changes in the base compositions of 2.07m, 1.28m and 0.70m RNAs with chase time. There was no difference between the base compositions of the 1.40m and 1.28m peaks in the nuclear fraction. 1.28m base composition was similar in the nuclear and cytoplasmic fractions. The nuclear 0.70m had a slightly lower G + C content than the cytoplasmic 0.70m.

In confirmation of the results in Table 2.1, the G + C content of the 2.07m peak (Mean 52%) was higher than that of the 1.28m (Mean 50%) which was higher than that of the 0.70m (Mean 48%).

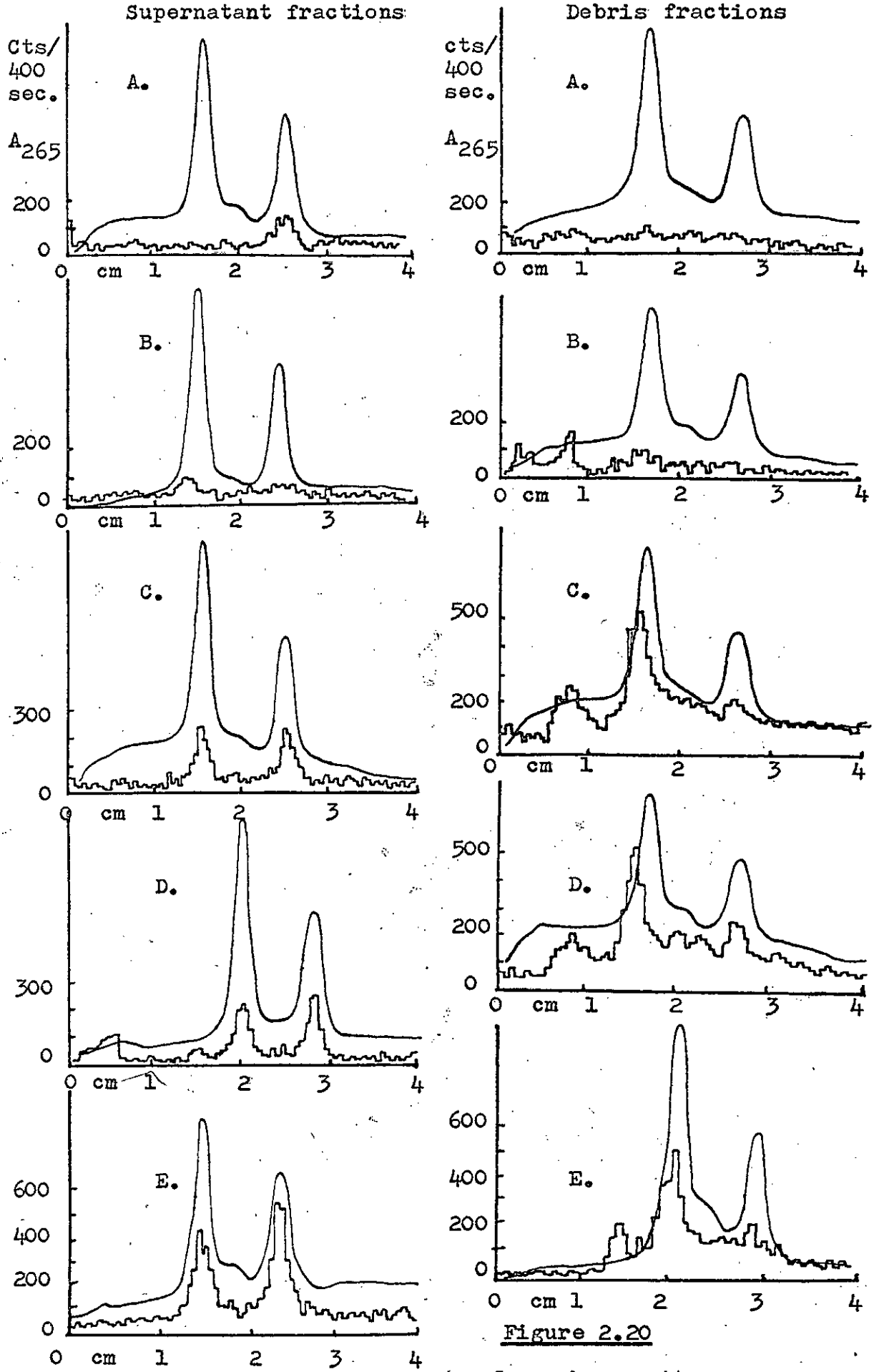


Figure 2.20

Legend opposite.

The 1.0m peak base composition increased in G + C content from an initial 42% to 50% by 6 hours. The base composition of the RNA lighter than 0.70m was high A, low G + C content.

4. Effects of 5-fluoro-uracil on r-RNA synthesis.

5-fluoro-uracil was added to a concentration of 1 mM after various times of culture. The radioactive incubation pre-wash, pulse and chase solutions also contained 1 mM 5-FU. A control treatment received no 5-FU. After a 15 minute pulse and a 75 minute chase, RNA was prepared by homogenisation and method 1. All samples were DNase treated.

All treatments showed similar values for the 32-P content of the alcohol supernatant. 5-FU therefore had no effects on 32-P uptake.

The control treatment (Figure 2.20, E) showed 2.07m, 1.28m and a small 0.70m labelling peaks in thenuclear fraction, and and 1.28m and 0.70m labelling peaks in the cytoplasmic fraction. When 5-FU was present from 0 hours, no label peaks were visible

Figure 2.20 (Opposite) Electrophoresis of cytoplasmic and nuclear fraction RNA. Nuclear fraction E and cytoplasmic fraction D were on 2.2% gels, all others on 2.4% gels. 5-fluoro-uracil was supplied at a concentration of 1 mM from 0 (A), 17 (B), 21 (C) and 22 (D) hours during culture. Labelling commenced at 22 hours. E - control, no 5-FU treatment.

in the nuclear fraction (Figure 2.20, A). The cytoplasmic fraction showed a label peak coincident with the 0.70m optical peak. Very small 1.28m and 0.70m radioactivity peaks were visible in the cytoplasmic fraction when 5-FU was added 5 hours before incubation. (Figure 2.20, B) The 2.07m peak was the only one labelled in the nuclear fraction.

When 5-FU was added 1 hour before incubation or at the start of incubation, (Figure 2.20, C, D), 1.28m and 0.70m labelling peaks were detectable in the cytoplasmic fraction, though in smaller amounts than in the control. In the nuclear fractions, amounts of 2.07m labelling greater than in the control were apparent. The labelling associated with the 1.28m optical peak was of the heavier, 1.40m form, in contrast to the 1.28m label of the control.

5. Polydisperse labelled RNA.

A. Extraction.

Freshly cut explants were labelled by a pulse of 120 minutes followed by a chase of 60 minutes. RNA was extracted by three methods.

A. Extraction of total RNA by homogenisation in Kirby medium.

B. Extraction of cytoplasmic and nuclear fraction RNAs by homogenisation and method 2.

C. Extraction of cytoplasmic and nuclear fraction RNAs by homogenisation and method 1.

All samples were DNase treated. 2.2% gels were loaded with 1/4 of the RNA yield from 30 explants for treatments A and B, and

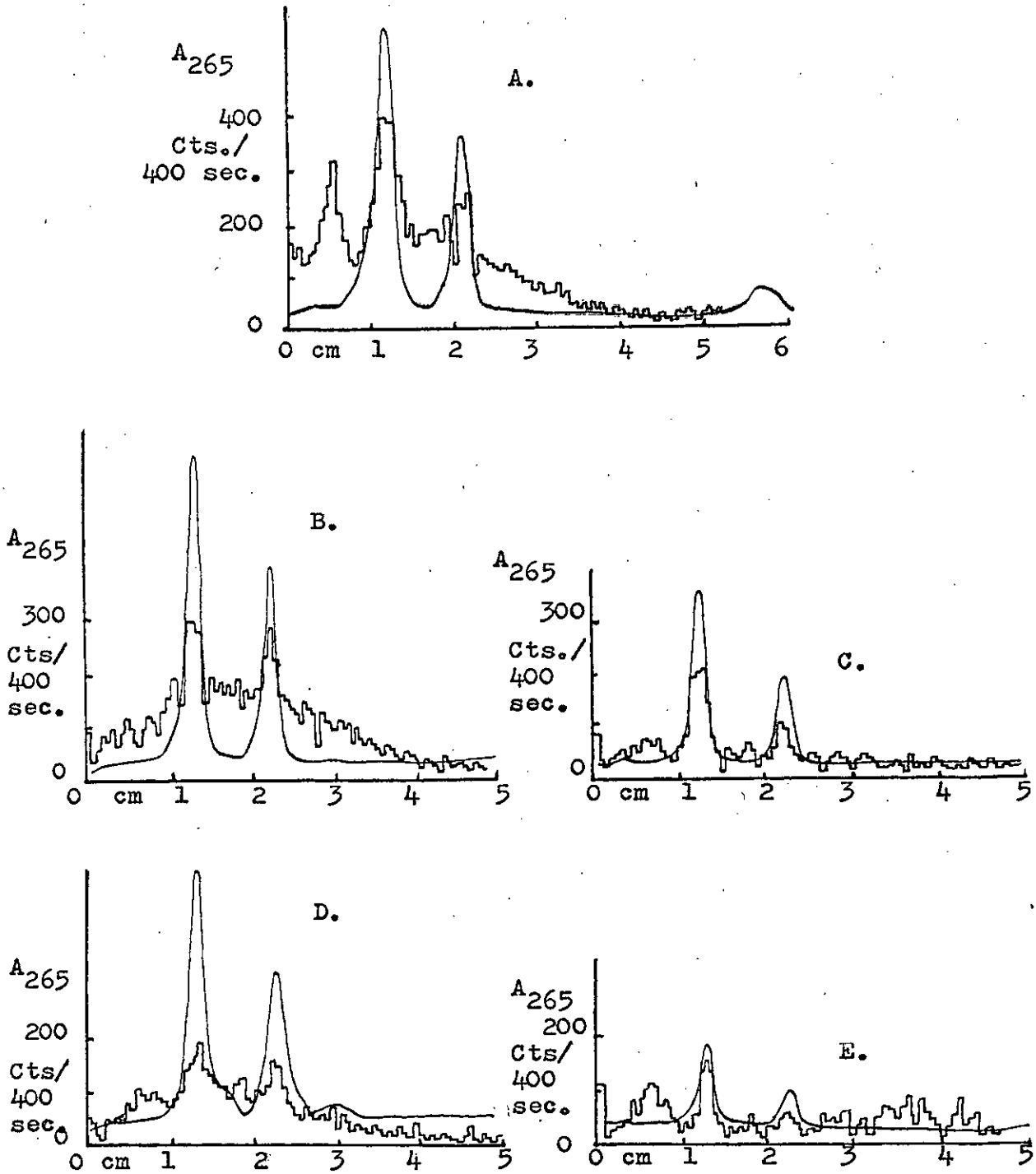


FIGURE 2.21 Electrophoresis of Artichoke RNA. A. Whole tissue preparation, homogenised in Kirby buffer. B. cytoplasmic and C. nuclear fractions from homogenisation in NDS buffer and phenol. D. supernatant and E. debris fractions from homogenisation in homogenising medium. (A,B,C - method 2; D,E -method 1)

with 1/6 th for treatment C. Electrophoresis was for 90 minutes.

With treatment A, the radioactivity scan shows 2.07m, 1.28m and 0.70m labelling peaks on a background of polydisperse labelling between the origin and 4 cm. (Figure 2.21 A) Both methods of tissue fractionation resulted in the appearance of this polydisperse label in the cytoplasmic fraction. (Figure 2.21, B, D). Little polydisperse label was detectable in the nuclear fractions. (Figure 2.21, C, E). The amount of polydisperse labelling extracted was similar with all three methods allowing for the differences in the amount of RNA loaded onto the gels.

B. Kinetics of labelling.

This experiment was carried out with explants cultured for only 1 hourto reduce the amount of r-RNA and r-RNA precursor labelling. Figure 3.1 shows that the incorporation of 32-P into r-RNA is very low in the early part of culture.

Explants were labelled by a 60 minute pulse, followed by 1, 30 and 120 minute chases. RNA was prepared by homogenisation and method 1. In an attempt to reduce cytoplasmic contamination of the nuclear fraction, the debris from the homogenate was resuspended in 1 ml homogenising medium and re-sedimented. The supernatant was added to the cytoplasmic fraction. Electrophoresis was for 90 minutes on 2.4% gels.

Little ribosomal RNA labelling occurred in this young tissue, and the rate of appearance of labelled r-RNA molecules was slower than with the material cultured for longer periods. (Figure 2.13) After 61 and 90 minutes labelling, a small 2.07m

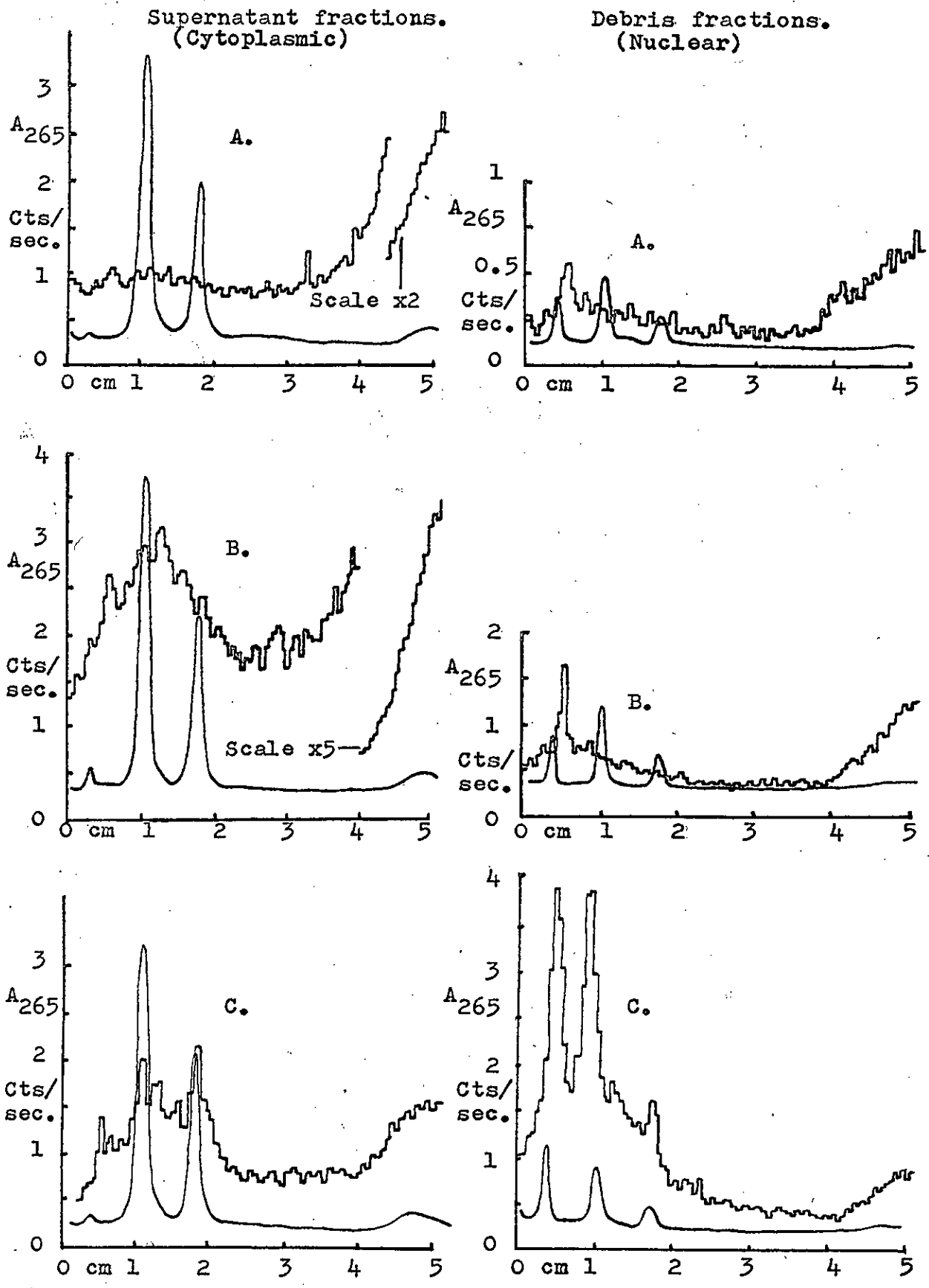


FIGURE 2.22 Electrophoresis of supernatant and debris fraction RNA. Pulse incubation 60 minutes, chase incubations 1 min. (A), 30 mins. (B) and 120 mins. (C). RNA prep. by method 1.

peak was visible in the nuclear fraction. (Figure 2.22) By 180 minutes, 2.07m and 1.40m labelling peaks were apparent in the nuclear fraction, and small amounts of 1.28m and 0.70m had appeared in the cytoplasmic fraction.

Polydisperse labelling, with a maximum at 1.4 cm, was most marked in the cytoplasmic fraction after 90 minutes incubation, and declined with the longer chase. In the nuclear fraction, a polydisperse region of lower mobility than that of the cytoplasm (maximum less than 1 cm) was visible after 90 minutes, and was most prevalent at 180 minutes.

The low molecular weight 32-P containing contaminant, beyond 4 cm, was most common in the cytoplasmic fractions. It decreased with longer chase incubation.

DISCUSSION.

1. RNA extraction.

Yields.

Polydisperse labelled RNA was extracted equally by method 1 and by method 2.

Method 2 gave lower yields of DNA and of the larger ribosomal RNA molecules - 2.07m and 1.28m - from the nuclear fraction than did method 1, i.e. the Kirby medium failed to extract these molecules from the phenol and interfacial precipitate. Figure 2.21 shows that when tissues were homogenised directly in Kirby medium to extract total nucleic acid, a yield of 2.07m equal to that obtained by method 1 extraction was obtained. The preliminary extraction of cytoplasmic RNA in method 2 by NDS buffer / phenol therefore reduces the ease of subsequent extraction of high molecular weight r-RNA and DNA by Kirby medium.

This might be a result of denatured proteins binding to the nucleic acids, as was considered by Kirby (1965). Burns (1968) found that r-RNA was difficult to extract from fern gametophyte material when a phenol-detergent method was used, but that a preliminary mild deproteinisation with chloroform/ octanol resulted in higher yields.

No effects of temperature (Georgiev and Mantieva, 1962) on extraction were examined with the artichoke material, though in view of the protection from nuclease action and the more satisfactory sub-cellular fractionation (see below) with method 2, this could still be worthwhile.

Degradation of r-RNA during extraction.

It is evident that while method 1 of RNA preparation gives high yields, it exposes the RNA to the danger of degradation, probably by endogenous nuclease action. Explants from old tubers appear to have a higher RNase content than those from freshly harvested tubers. This is consistent with the state of breakdown in the old tubers to support the developing buds. The higher susceptibility to degradation of newly synthesised RNA (Figure 2.9) implies that a measure of protection from RNase attack is conferred by the addition of ribosomal proteins to the RNA. Burns (1968) has shown that treatment of homogenates of higher plant tissue with trypsin for short periods leads to extensive degradation of ribosomal RNA.

The persistence of degradation when 0.5% NDS was added to the homogenising medium used for method 1 extraction (Figure 2.10) suggests that this hydrophilic salt alone is not an inhibitor of RNase. Protection from nuclease action was obtained with the NDS buffer / phenol homogenisation of method 2, (Figure 2.11), a consequence of the phenol rather than the NDS. Homogenisation in Kirby medium also prevented nuclease action (Figure 2.11). The TNS component of this medium is known to be a strong nuclease inhibitor. (Hastings and Kirby, 1966; Parish and Kirby, 1966).

Apart from inhibition by detergents such as TNS, various other methods have been suggested for the inhibition of nuclease action during RNA extraction. These include the addition of proteins (Hallaway, 1965) or polyvinyl sulphate (Clark et. al., 1964) to the homogenising medium to prevent rupture of RNase-containing lysosomes, binding proteins to

bentonite, providing inhibitory concentrations of zinc or magnesium ions (Holden and Pirie, 1955) and extracting at high pH, where nucleases are less active. (Click and Hackett, 1966) In general, these methods have to be used for cell fractionation, but are less effective than inhibition by anionic detergents.

The specific breakdown of r-RNA to two distinct radioactive peaks (1.1m and 0.6m) or to two or three optical peaks (1.1m, 0.6m and 0.5m) suggests that there may be particularly exposed or sensitive sites on the molecules, or extremely specific ribonucleases. Ingle (1968) has observed similar breakdown to specific molecules in bean tissue, including breakdown of plastid r-RNA. He concludes that at least some of this breakdown may be by a natural process in vitro. In view of the results in Figure 2.8, the breakdown in artichoke RNA seems to be entirely after homogenisation.

Sub-cellular fractionation.

Combinations of the two methods of tissue disruption and the two methods of RNA extraction gave various distributions of the RNA between the 'nuclear' and 'cytoplasmic' fractions. (Table 2.3). The 10:1 cytoplasmic:nuclear ratio for r-RNA obtained with method 2 is similar to the values quoted by McLeish (1963) from chemical determinations of RNA on cells and isolated nuclei.

The lower ratios obtained by method 1 suggest cytoplasmic contamination of the nuclear fraction. This was especially high when the chopping method of tissue disruption was used, consistent with the less efficient tissue breakage.

In method 1, there was also evidence for a small amount of nuclear fraction contaminating the cytoplasmic fraction. About 1/10 th of the tissues 2.07m labelling appeared in the cytoplasm, as well as a little DNA. This could be a result of damage to a proportion of nuclei during tissue disruption.

2. Ribosomal RNA synthesis.

In the cytoplasmic fraction, the labelling of 1.28m and 0.70m seemed to occur in two stages. (Figures 2.14;2.17).

1. An early labelling during the pulse, which stopped or declined in the early stages of the chase.

2. A progressive increase in label, beginning early in the chase, and continuing for some hours.

It is proposed that this later labelling was a labelling involving complete synthesis of the r-RNA molecules and complex precursors, and that the early labelling was by some means not involving complete synthesis or precursors. There is further circumstantial evidence for a dual labelling of r-RNA:

1.The ratio of 1.28m to 0.70m labelling in the tissue was greater than the theoretical 1.85:1 during the early stages of the incubation. (Figures 2.16; 2.18) This is inconsistent with a labelling by complete synthesis of the molecules, where labelling in proportion to molecular weight is expected. Later in the chase, when molecules labelled by synthesis were presumably present, the ratio fell towards the theoretical value.

2. In the nuclear fraction, 1.28m labelling was present with chases of 1 to 30 minutes, was absent at 45 minutes, and was present again with chases of 75 minutes or longer. This

suggests an early labelling of 1.28m which was either degraded or exported to the cytoplasm, followed after a gap by labelling resulting from de novo synthesis.

3. In the pea root, Loening (1968) did not detect labelled 0.70m and 1.28m in the cytoplasm until 40 and 60 minutes respectively. These times are similar to those shown in Figure 2.17 for the start of the second increase in cytoplasmic labelling.

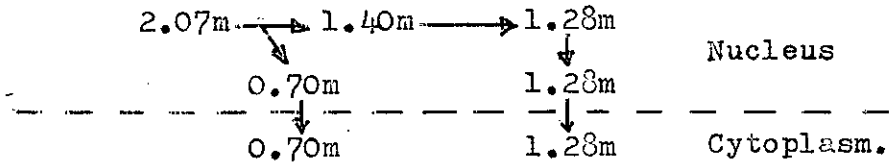
Labelling involving the synthesis of new molecules.

(In this part of the discussion, the early labelling behaviour of r-RNA is disregarded.)

The 2.07m peak in the nuclear fraction labelled faster than the other r-RNA peaks during the pulse and early chase. Later in the chase, it decreased in radioactivity, while the labelling of the 1.28m and 0.70m peaks increased. The high specific activity of the 2.07m peak suggests a rapid turnover. Its base composition was similar to that of ribosomal RNA. All this evidence supports the hypothesis that the 2.07m peak is a precursor to ribosomal RNA.

In the nuclear fraction after a 45 minute chase, there was little radioactivity associated with the 1.28m r-RNA optical peak. (Figure 2.13), but a radioactivity peak of molecular weight 1.40m was present. After longer chases, 1.28m label was evident, but there was no 1.40m label. The restriction to the nuclear fraction, ribosomal-type base composition and appearance after the 2.07m labelling suggests that the 1.40m peak is an intermediate in the processing of the 2.07m precursor. The scheme proposed for the processing of the 2.07m

precursor may be represented as:



It will be noted that in this scheme, the entry of 1.28m into the cytoplasm is later than the 0.70m, because of the extra stage involving the 1.40m precursor. This is consistent with the 1.28m:0.70m labelling ratio in the cytoplasm, which was below the theoretical 1.85:1 in the early part of the chase, and increased later. (Figures 2.16; 2.18).

Loening (1969) has found a similar pair of precursors in the pea root.

The measurement of the molecular weight of the first precursor, 2.07m ± 0.04m is 0.09m greater than the combined molecular weights of the 1.28m and 0.70m r-RNAs. This implies that part of the precursor molecule is not conserved during processing. Clearly there is a loss of weight in the stage from 1.40m ± 0.03m to 1.28m, which is within the limits of measurement error of the calculated total weight lost in 2.07m precursor processing. But there is also evidence from the base composition data of Table 2.5 for a loss of weight during the initial splitting of the 2.07m molecule. The mean G + C content of the 2.07m is 52%, of the 1.40m and 1.28m 50% and of the 0.70m 48%. This suggests the loss of a piece of RNA of high G + C content. No correction for the low G + C content heterodisperse RNA (Table 2.5) was made in estimating the base compositions of the precursor and ribosomal RNA, as the amount of heterodisperse RNA relative to the label in the peaks was small (Figure 3.13), and Figure 2.21 shows that heterodis-

disperse contamination was likely to affect all peaks to similar degrees.

While the base composition data suggest a loss of RNA in the first split of the 2.07m precursor, the molecular weight determinations do not show any loss of RNA. The weight of the portion lost might be within experimental error, or the weight of the 2.07m precursor might have been underestimated for two reasons. A higher G + C content might increase the amount of folding in the molecule, making it more compact and hence more mobile in the gel. Alternatively, the layering of water over the top of the gel before polymerisation might lead to a reduced gel concentration at the top, resulting in initially greater mobility of the 2.07m. It has been noticed that when gels were run for 1 hour, the calculated molecular weight of the precursor is less than 2.07m.

Studies with animal cells also suggest a loss of RNA with a high G + C content during the processing of the precursor. In HeLa cells, Weinberg et. al (1967) conclude that up to 50% of the weight of the 4.6m precursor is lost during processing, from measurements of molecular weights by gel electrophoresis. In the base composition studies of Willems et. al. (1968), the 45s (4.6m) precursor was found to have 70% G + C, and the 28s + 18s 64%. They concluded that the discarded portion was 57 - 77% G + C. Jeantur et. al (1968) have found se sequences of non-ribosomal type, with very low A content, in the 45s and 32s intermediate. They concluded that 30 to 50% of the weight of the 45s is lost during processing.

In the study of the r-RNA cistrons of Xenopus DNA by Birnsteil et. al. (1968), it is concluded that the 28s and

18s templates alternate along the DNA, separated by lengths of DNA with 85% G + C content. The precursor in this case is of 2.5m molecular weight, with a higher G + C content than the final r-RNAs. The loss of RNA during processing is about 0.3m.

The discarded high G + C content portion was not detected in the artichoke tissue or in the animal cells, suggesting a rapid degradation. The significance of the high G + C content regions is open to question. Presumably they must be capable of exerting considerable influence on the secondary structure of the precursor molecule. It is perhaps relevant that the amount of discarded, high G + C material increases with increasing size of the final r-RNA molecules. (Loening, 1969).

	r-RNA molecular weights.	proportion of precursor not conserved.
Artichoke	1.28m; 0.70m	c. 5%
Xenopus	1.5m 0.7m	c.12%
HeLa	1.75m 0.70m	30-50%

One function for the high G + C regions might be to hold the ribosomal RNA in certain configurations during the addition of ribosomal proteins. This 'folding function' might be required to a greater extent with larger r-RNA molecules.

5-FU and r-RNA synthesis.

5-fluoro-uracil when applied 5 or more hours before labelling completely inhibited the labelling of r-RNA and 2.07m precursor. The effect when the inhibitor was added just before incubation with 32-P was to reduce the amounts

of label in 1.28m and 0.70m, while increasing 2.07m labelling and preventing the conversion of 1.40m to 1.28m. (Figure 2.20). Willen and Stenran (1966) reported an accumulation of presumed heavy precursors of r-RNA in rat liver after injection of 5-FU. Other conditions may lead to anaccumulation of precursor, including poliovirus infection (Weinberg et. al. 1967), phosphate starvation and the inhibition of protein synthesis by cycloheximide (Willems et. al., 1968)

The early labelling of r-RNA.

Various explanations may be considered to account for the early labelling behaviour of the r-RNA. The label is unlikely to represent the precursors to bacterial r-RNA (Hecht and Woese, 1968) in view of the sterile precautions taken. The 1.28m r-RNA of the artichoke is also significantly heavier than the precursor to bacterial 23s r-RNA. Plant plastid r-RNA has been shown to have molecular weights similar to bacterial r-RNA. (Loening and Ingle, 1967). It is not yet known if the plastid r-RNA follows a similar precursor pattern to the bacteria. Even if this were the case, it is unlikely that plastid ribosomal RNA precursors would be preoduced in a non-green tissue which does not show any finished plastid r-RNA.

The early labelling might be a consequence of precursor breakdown by nuclease action following on homogenisation, rather than by a slower in vivo processing of the 2.07m. The high early 1.28m:0.70m labelling ratios argue against this interpretation.

The possibility that the early labelling is a result of

messenger RNA fractionating with the r-RNA is discounted by the ribosomal type base composition of the early 1.28m and 0.70m labelled peaks. (Table 2.5)

The possibility that non-nucleic acid phosphate might be contributing to the labelling of the peaks is ruled out by the normal recoveries of total counts in the nucleotides in base composition determination.

A further possibility is that already synthesised r-RNA molecules might be labelled in some way. This cannot be by the turnover of only a few terminal nucleotides as occurs in transfer RNA (Ingle, Key and Holm,1966), as this would give a distorted base composition, whereas that observed was close to normal ribosomal. The high 1.28m:0.70m labelling ratio observed during early incubation suggests that the larger r-RNA molecule might have more of such labelling sites per unit weight than the smaller r-RNA.

Thus the machanism of this early labelling remains unresolved.

3. Heterodisperse labelling.

Two polydisperse labelled fractions were detected in the artichoke tissue, a heavier one in the nuclear fraction and a lighter one in the cytoplasmic fraction. (Figure 2.22) The fact that the cytoplasmic was already beginning to chase out by the time of maximum accumulation of the nuclear labelling suggests that the nuclear material cannot be simply a precursor to the cytoplasmic, In this respect the situation is similar to that found in HeLa cells by Penman, Vesco and

Penman,(1968).

Although an extensive analysis of base compositions was not carried out, the determinations from the light heterodisperse regions of other gels (Figure 2.13; Table 2.5) showed a high A content similar to the messenger fraction from pea roots described by Loening (1965) and to the D-RNA and polyribosome-associated RNA of soybean roots. (Lin, Key and Bracker, 1966)

The polydisperse RNA of the artichoke exhibits three characteristics proposed for messenger RNA: Heterogeneity of weight, base composition and in the cytoplasm a high rate of turnover. But without further information on their behaviour and synthesis, no definite functional significance can be assigned to these fractions.

SECTION 3.

A detailed study of RNA synthesis during the first few cell divisions was made using polyacrylamide gel electrophoresis. The remainder of the Section is concerned with an examination of the relation between RNA, DNA and protein syntheses and cell division. Conditions known to inhibit cell division, such as light, hormone conditions and mitomycin, were used to test the dependence of RNA and protein changes on cell division. Inhibitors of nucleic acid synthesis were examined for their effects on the tissue, and used to determine the necessity of RNA synthesis for cell division.

MATERIALS AND METHODS.

Culture conditions. Explants were obtained, planted and cultured as described in Section 1. Except where otherwise specified, planting was by dim green light and culture in total darkness. The medium contained sucrose, aslts and 2,4-D at 10^{-5} M. Culture was in various sizes of petri dish or 100 ml conical flasks.

Chemicals. All chemicals were B.D.H., 'Analar' grade when obtainable. Antibiotics were a gift from Dr. K. Jones. Antibiotics and 5-fluoro-uracil were dissolved in water to a concentration 10 times that required in the culture medium. Addition of the antibiotics to the culture medium was made aseptically through a Millipore filter.

Gel electrophoresis. 32-P labelling of explants, RNA preparation, electrophoresis and derivation of data from scans were carried out as described in Section 2.

Cell number was estimated by the incremental method described in Section 1, on samples of 3 - 5 explants.

DNA, RNA and protein estimations. The development of the methods used for quantitative estimation of DNA, RNA and protein is described in Appendix 1.

Explants were stored in methanol at $0^{\circ}C$ between harvesting and analysis. Washing to remove contaminating materials was by the method of Holdgate and Goodwin (1965). The explants were processed without homogenisation for the washings and for

nucleic acid and protein extraction.

Nucleic acids were extracted by the procedure of Schneider (1945). 8 - 10 explants were incubated with 1 ml 0.5N PCA for 20 minutes at 70°C. The tissue was washed with 0.5ml PCA. RNA was calculated from the difference in absorbance at 260 and 315 nm of the combined supernatant and wash. SP500 and SP800 spectrophotometers were used.

The explants were extracted a further three times with 0.5 ml aliquots of 0.5N PCA for 20 minutes at 70°C. All PCA extracts were combined and used for the measurement of DNA by the diphenylamine colour reaction for deoxyribose. (Burton, 1956). The colour intensity was taken as the difference in absorbances at 600 and 650 nm, measured using 40 mm cells in the SP500 spectrophotometer.

Protein was extracted into 1 ml N NaOH at room temperature for 24 hours, and measured by the colour produced with Folin and Ciocalteau's reagent in the Lowry method (Lowry et. al. 1957)

Absolute amounts of DNA, RNA and protein were calculated using B.D.H. calf thymus DNA sodium salt, B.D.H. yeast RNA, sodium salt and Calbiochem bovine serum albumin, grade B as standards. The DNA and RNA standards were found to be only 75% pure; the absolute values reported in the results section are therefore 25% high.

All DNA, RNA and protein measurements quoted in the results section are the means of three individual determinations. Almost all individual values were within $\pm$ 10% of the mean.

RESULTS.

1. Description of RNA synthesis during two cell divisions.

Cultures of 30 explants were established in 100 ml conical flasks with 3.5 ml medium. Planting was carried out five times at intervals of 12 hours. For each planting, one large tuber was used. All tubers in the experiment came from the same plant.

Following the final planting, one flask from each planting was used for RNA preparation. This was repeated at three hourly intervals for twelve hours. This procedure made it possible to sample a total culture time of 60 hours at three hourly intervals. A check that all plantings were dividing at the same time was made by withdrawing explants for cell number determination at times during culture.

From each flask, 3 explants were used for cell number determination and the remainder for RNA preparation. Labelling was by a 30 minute pulse followed by a 60 minute chase. Total RNA was extracted by homogenisation and method 1. The homogenising medium contained 0.5% NDS. The RNA was fractionated on 2.2% gels for 90 minutes.

In this series of cultures, the first division lasted from 35 hours to 42 hours. (Figure 3.1) The second division commenced at 47 hours. DNA labelling began some time before 30 hours, though it was difficult to distinguish DNA label from the radioactive 2.07m r-RNA precursor. This also explains the erratic nature of the DNA labelling curve. DNA quantity

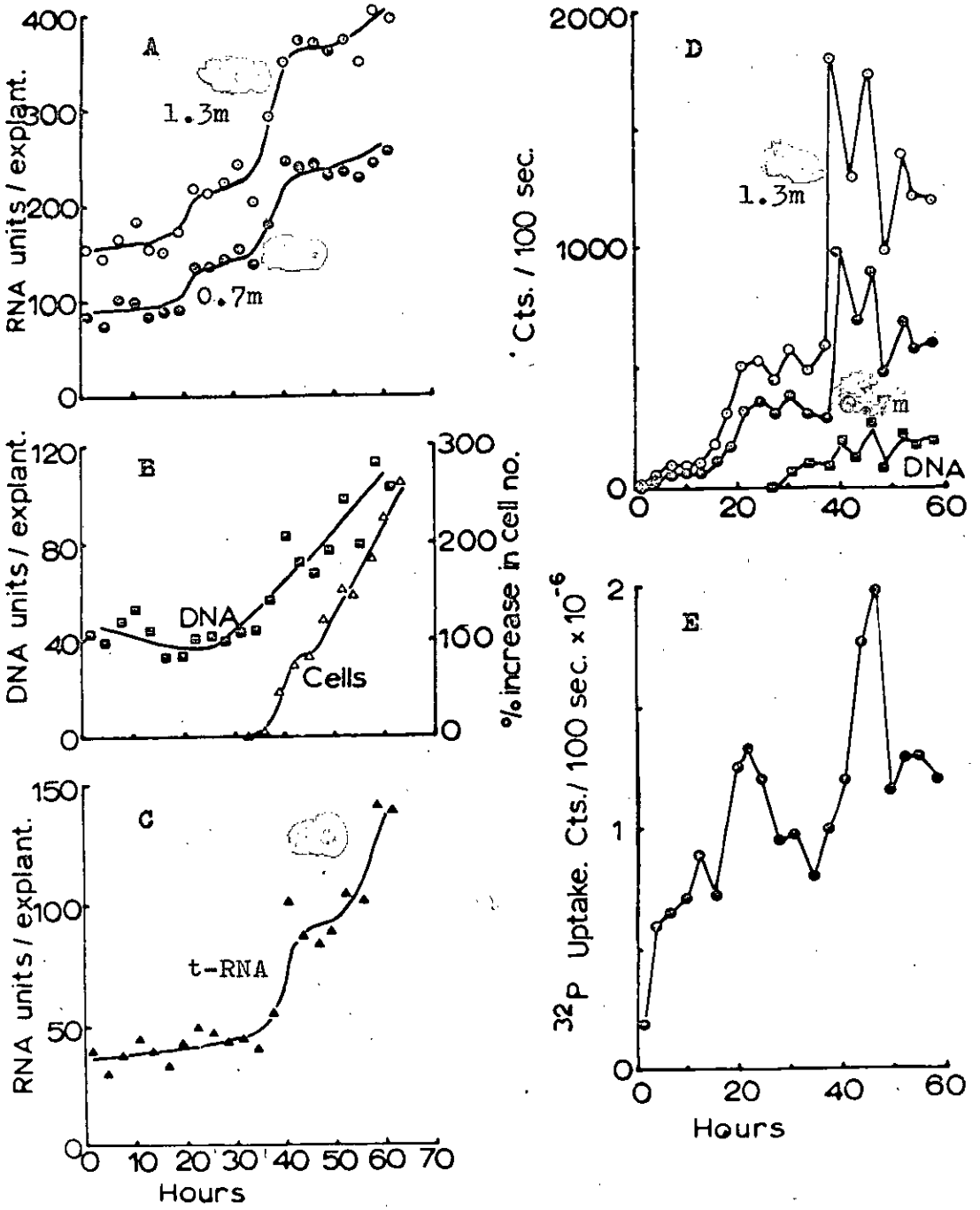


FIGURE 3.1 Changes in amounts of 1.4 and 0.7 m ribosomal RNA per explant (A); DNA per explant (B); and transfer RNA per explant (C) measured from gel peak areas; percentage increase in cell number (B); radioactivity incorporated into 1.3 and 0.7 m ribosomal RNA and DNA in a 30 minute 32-P pulse / 60 minute 31-P chase (D) and 32-P content of the alcohol soluble fraction '32-P uptake' (E) with time of culture.

increased after about 20 hours. From these data, and from measurements of the length of S-phase and G2 by Mitchell (1967) and Evans (1967), it may be concluded that the first S-phase occurred between 20 and 34 hours.

For the first 16 hours, little change occurred in the amounts of r-RNA or t-RNA, although the 32-P incorporation curves show that some synthesis of RNA was occurring, even in the earliest sample. Just before the beginning of S-phase, there was a rise in the rate of 32-P incorporation into r-RNA, and a rise in the amount of r-RNA, though not of t-RNA. The rate of 32-P incorporation into r-RNA remained steady between 20 and 35 hours, i.e. virtually for the duration of S-phase. During this period the r-RNA totals increased at a slow rate. A second rise in the rate of 32-P incorporation into r-RNA began at about the same time as the first division. The rate of total r-RNA accumulation increased at the same time, and t-RNA accumulation commenced. Towards the end of the first division, 42 hours, the rate of 32-P incorporation into r-RNA began to decline, as did the rate of accumulation of total r-RNA. t-RNA accumulation also slowed down at this point, but resumed the previous higher rate of accumulation at 48 hours. After 50 hours, the rate of 32-P incorporation into r-RNA declined, the accumulation of total r-RNA was at a low rate, but t-RNA and DNA accumulations and cell division continued unimpaired.

The 32-P content of the alcohol supernatant ('32-P uptake') showed an increase with time, marked by two distinct peaks at the times of rising incorporation of 32-P into r-RNA, i.e. at 20 and 45 hours.

The curves showing the accumulation of total r-RNA have

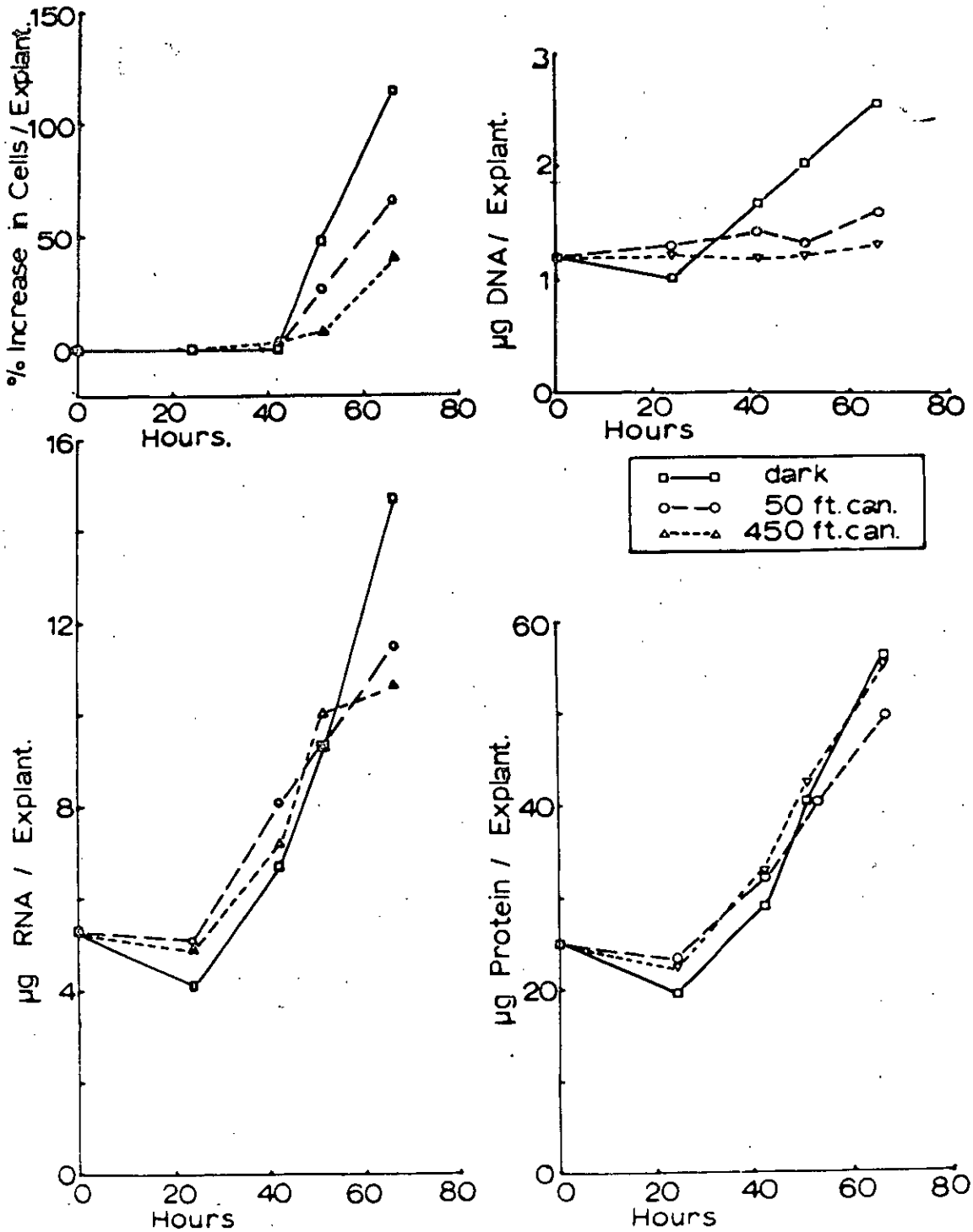


FIGURE 3.2 Changes in percentage increase in cell number, DNA, RNA and protein per explant with time of culture under three light regimes.

been analysed by computer by Mr. A. G. Hill. A stepped line, as drawn, was found to be a better fit than a straight line by a factor of 1000.

It was not possible to obtain data for isotope incorporation into t-RNA. 32-P containing contaminants obscured the labelling of this region on the gels, although they could have been removed by further SDS/acetate reprecipitations. 32-P may also be incorporated into existing t-RNA molecules by end-group turnover.

2. Effects of conditions known to affect cell division on RNA, DNA and protein accumulations.

A. Light.

In Section 1, initial or continuous illumination was found to be a potent inhibitor of cell division.

Explants were excised and planted in dim green safelight. Light treatments were applied as follows:

1. Subsequent culture in total darkness.

2. Culture in 50 fc. continuous illumination.

3. Culture in 450 fc. continuous illumination.

4. Exposed to 50 fc. light for 30 minutes after planting, then cultured in total darkness.

5. Exposed to 450 fc. light after planting for 30 minutes, then cultured in total darkness.

Both light sources were mixed tungsten and fluorescent.

Continuous illumination (Figure 3.2) supressed DNA synthesis and cell division, but had no effects on protein accumulation. RNA accumulation was initially unimpaired, but after

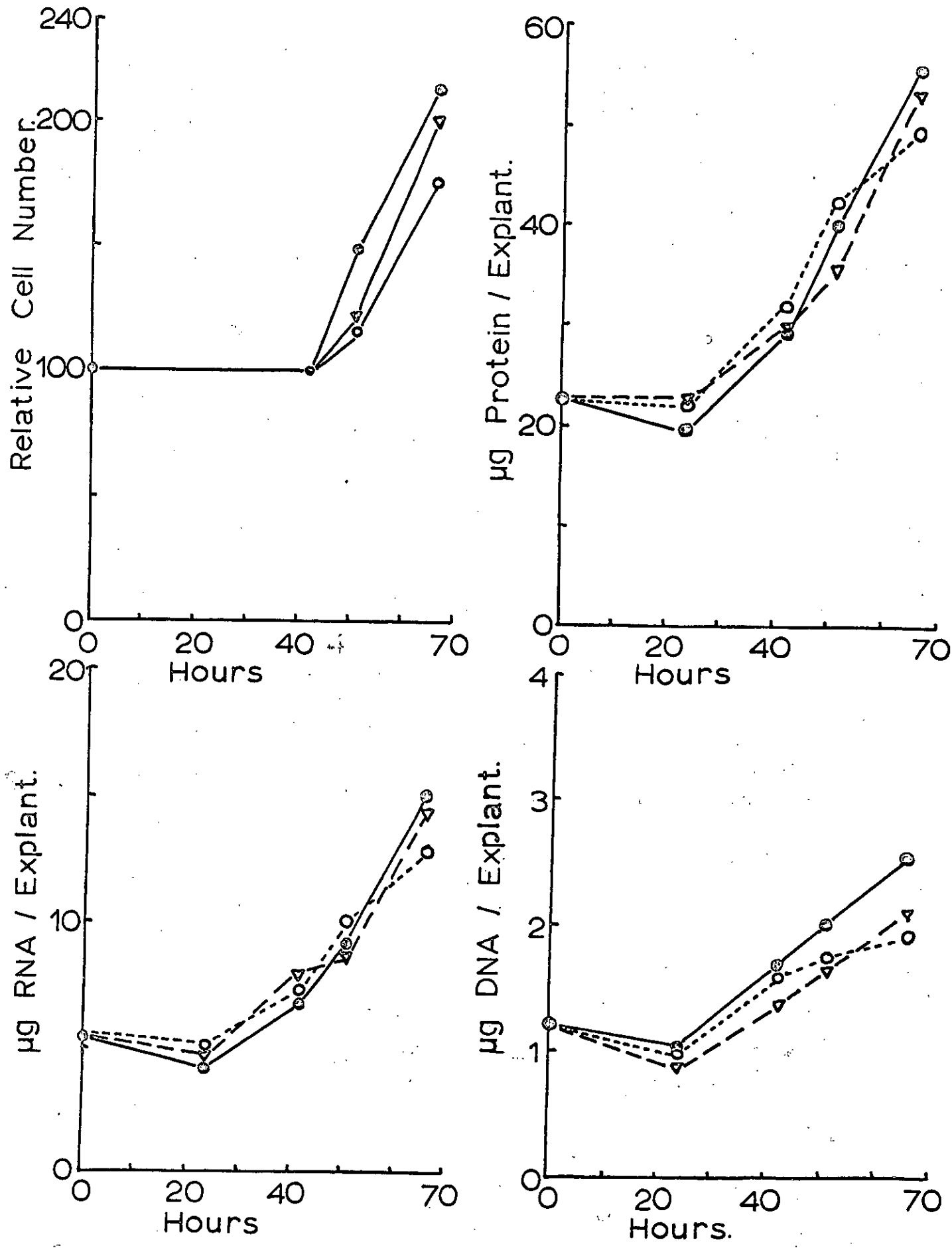


Figure 3.3 Legend opposite.

50 hours, by which time a 2 fold increase had occurred, both light intensities gave values lower than the dark treatment.

Initial illumination (Figure 3.3) had slight inhibitory effects on DNA synthesis and cell division, but no significant effect on RNA or protein accumulation.

B. 2,4-D concentration and coconut milk.

In section 1 it was established that cell division was dependent on the concentration of 2,4-D in the medium, but not on the presence of coconut milk.

Cultures were established in media containing 2,4-D at 0, 10^{-8}, 10^{-6} and 10^{-5} M, and at 10^{-6} M with 20% coconut milk.

Figure 3.4 shows results with various 2,4-D concentrations. The lower the concentration of 2,4-D, the lower the amounts of DNA synthesis and cell division. Protein accumulation was equal at all 2,4-D concentrations until 50 hours; thereafter slightly lower rates of accumulation occurred with 0 and 10^{-8} M 2,4-D. After an initial decrease, probably a result of the loss of cytoplasm from the outer, damaged cells, RNA accumulation occurred in all treatments. The rate of increase until 40 hours was lower in the absence of 2,4-D. After 50 hours, no further accumulation of RNA occurred in explants grown with 0 or 10^{-8} M

Figure 3.3 (opposite). Changes in relative cell number, DNA, RNA and protein per explant with time. Explants cultured in darkness after exposure for the first 30 minutes to dark (—●—), 50 fc light (—▽—) and 450 fc light (—○—)

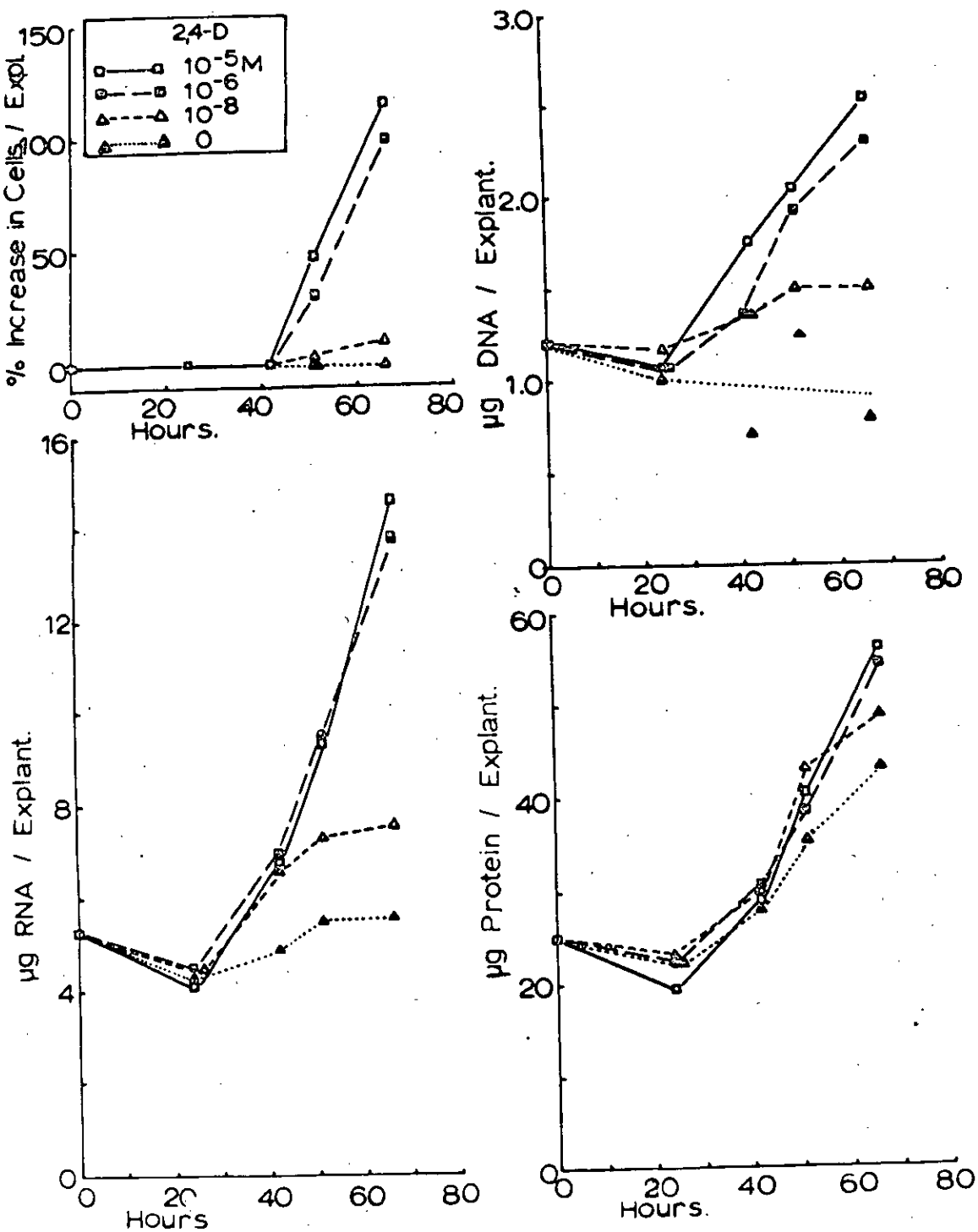


FIGURE 3.4 Changes in percentage increase in cell number, DNA, RNA and protein per explant with time of culture in media of various 2,4-D concentrations.

2,4-D; explants with 10^{-6} M or 10^{-5} M 2,4-D showed similar, continuing accumulations.

coconut milk.	R.C.N.		µg DNA / explant.		µg RNA / explant.		µg protein / explant.	
	0	20%	0	20%	0	20%	0	20%
0 hours	100		1.20		5.3		25	
24 hours	100	100	1.05	0.78	4.6	4.7	22	22
42 hours	100	100	1.33	1.13	6.9	6.3	31	29
51 hours	131	120	1.90	1.41	9.4	9.2	38	39
67 hours	205	160	2.31	1.78	13.8	12.4	54	49

Table 3.1 Changes in relative cell number, DNA, RNA and protein per explant with time for cultures grown with 10^{-6} M 2,4-D, with and without 20% coconut milk.

The inclusion of 20% coconut milk in medium containing 10^{-6} M 2,4-D had no effect on the rates of RNA or protein accumulation. Cell division and DNA accumulation appeared to occur slightly later when coconut milk was present, but the rates of increase were similar to those in cultures without coconut milk.

C.Explant pre-washing and added tissue homogenate.

Two experiments were conducted to find what significance the material from the outer, broken cells has in the DNA, RNA and protein accumulations. The experiment also tested the effects of prewashing with medium containing dimethylsulphoxide (DMSO), which was subsequently used in an attempt to aid penetration of inhibitors.

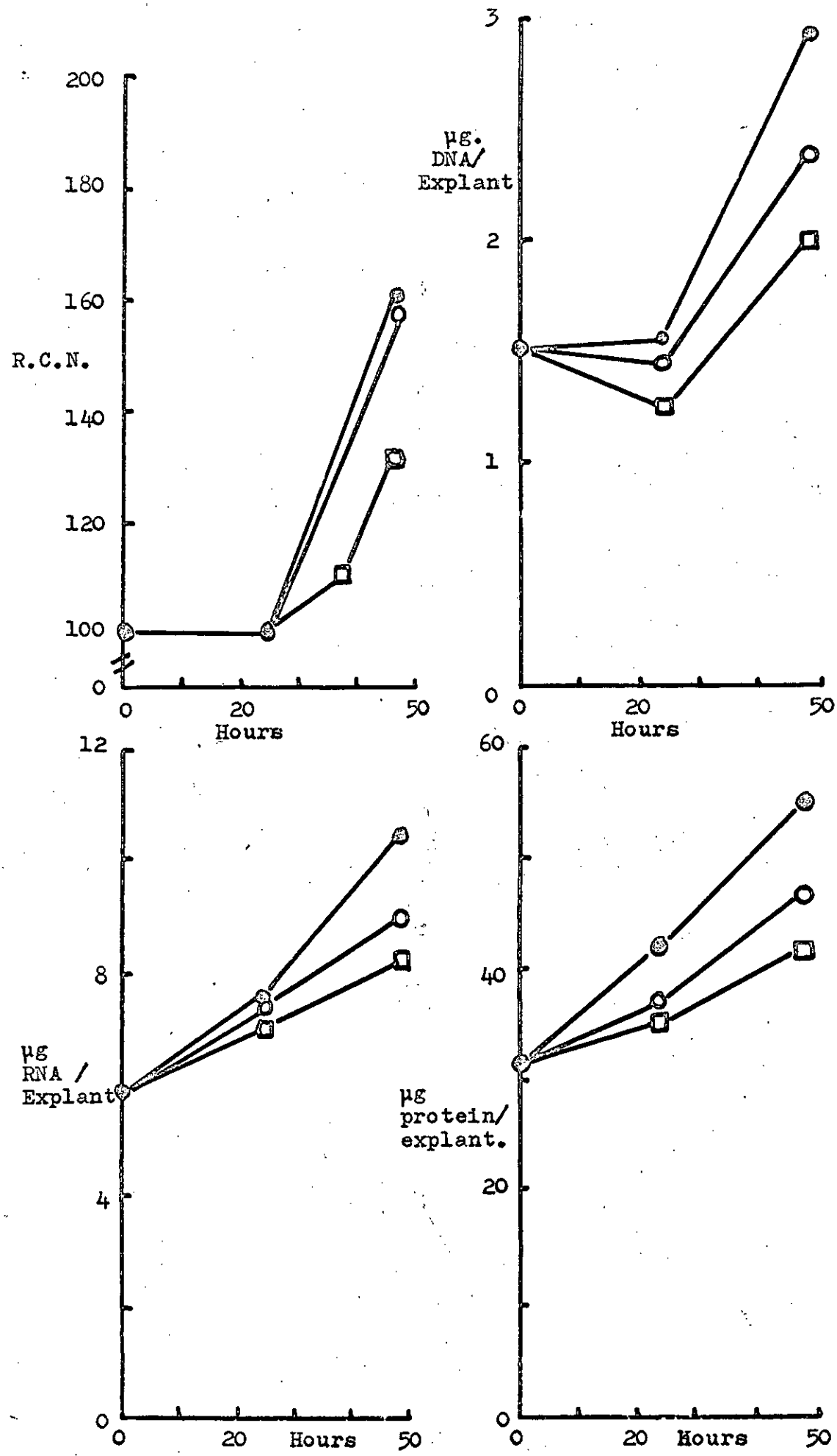


Figure 3.5 Legend opposite.

(i). Explants from small, immature tubers, 1.5 cm in diameter, were treated as follows:

1. Control, planted after excision.

2. Explants pre-washed for 30 minutes in culture medium before planting.

3. Explants washed for 30 minutes in medium including 10% DMSO before planting in DMSO-free medium.

The culture medium contained sucrose, salts and 10^{-5} M 2,4-D.

Prewashing with medium had no effects on cell division (Figure 3.5), confirming the results of Section 1, Table 1.4. RNA, DNA and protein accumulation rates were slightly lower after prewashing. DMSO in the prewash produced a further reduction of these rates of accumulation, and also a reduction or delay of cell division.

(ii) This experiment tested the ability of broken cell products to stimulate RNA accumulation and cell division in the absence of 2,4-D. Explants were treated as follows:

1. Control. Grown in medium containing 10^{-5} M 2,4-D.

2. Grown in medium containing sucrose and salts only.

3. Prewashed for 30 minutes in sucrose and salts medium; cultured in sucrose and salts medium.

4. 20 explants were homogenised in 3 ml sucrose and salts

Figure 3.5 (Opposite) Changes in relative cell number, DNA, RNA and protein per explant with time. (—●—) Control. (—○—) Explants prewashed 30 minutes in culture medium. (—□—) Explants prewashed 30 minutes in culture medium containing 10% DMSO.

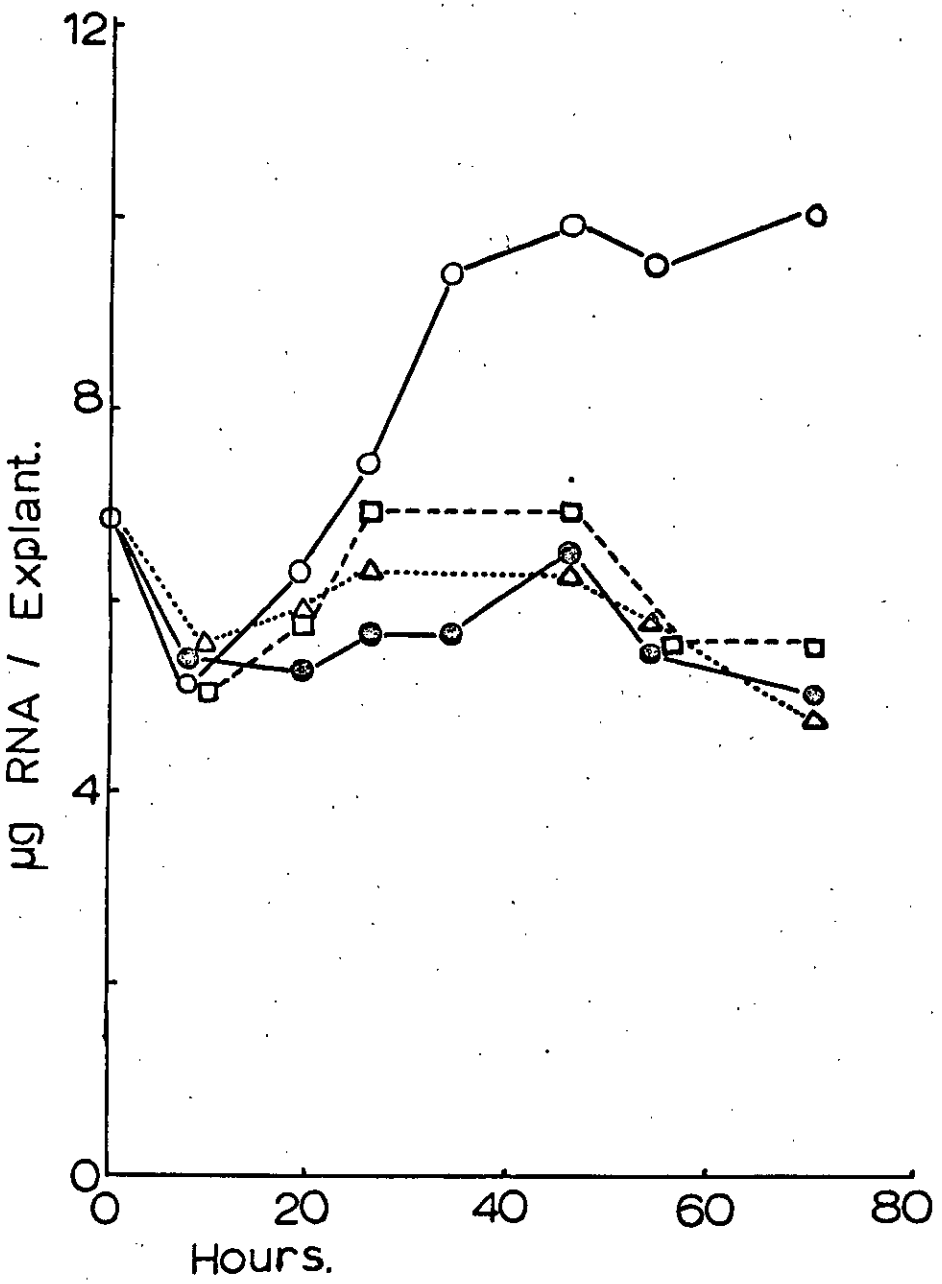


Figure 3.6 Changes in ug RNA / explant with time of culture in medium containing 10^{-5} M 2,4-D (O—O) or in medium without 2,4-D (●—●); after prewashing (Δ---Δ) and with tissue homogenate added to the medium. (□--□)

medium. The homogenate was added to a culture of 240 explants in 37 ml sucrose and salts medium.

As shown in figure 1.4, a little cell division occurred in the treatments without 2,4-D. Prewashing did not reduce the amount of division. Tissue homogenate doubled the amount of division occurring in the absence of 2,4-D.

Each treatment without 2,4-D showed a small rise in RNA, that with tissue homogenate being greatest. Prewashed and normal tissues without 2,4-D showed similar accumulations, though that in the prewashed tissue occurred faster. (Figure 3.6)

Thus in the absence of 2,4-D, the material from broken cells appears to promote small amounts of cell division and RNA accumulation. As noted in Section 1, prewashing does not appear to remove material from the cut surface of the explant efficiently.

D. The effects of mitomycin and light on the patterns of RNA and protein accumulation.

The results in Figure 3.1 indicated that the early accumulation of RNA occurs in a stepwise manner. This conclusion is confirmed by the results of Evans (1967) and Mitchell (1967a, 1968) who have shown that protein accumulation is also stepwise. To test for a relation between these steps and DNA synthesis and cell division, two conditions were chosen which allow RNA and protein accumulation while inhibiting DNA synthesis and cell division. The conditions were treatment with mitomycin (Figure 3.8) and strong light (Figure 3.2).

Explants were excised from mature tubers stored for 1

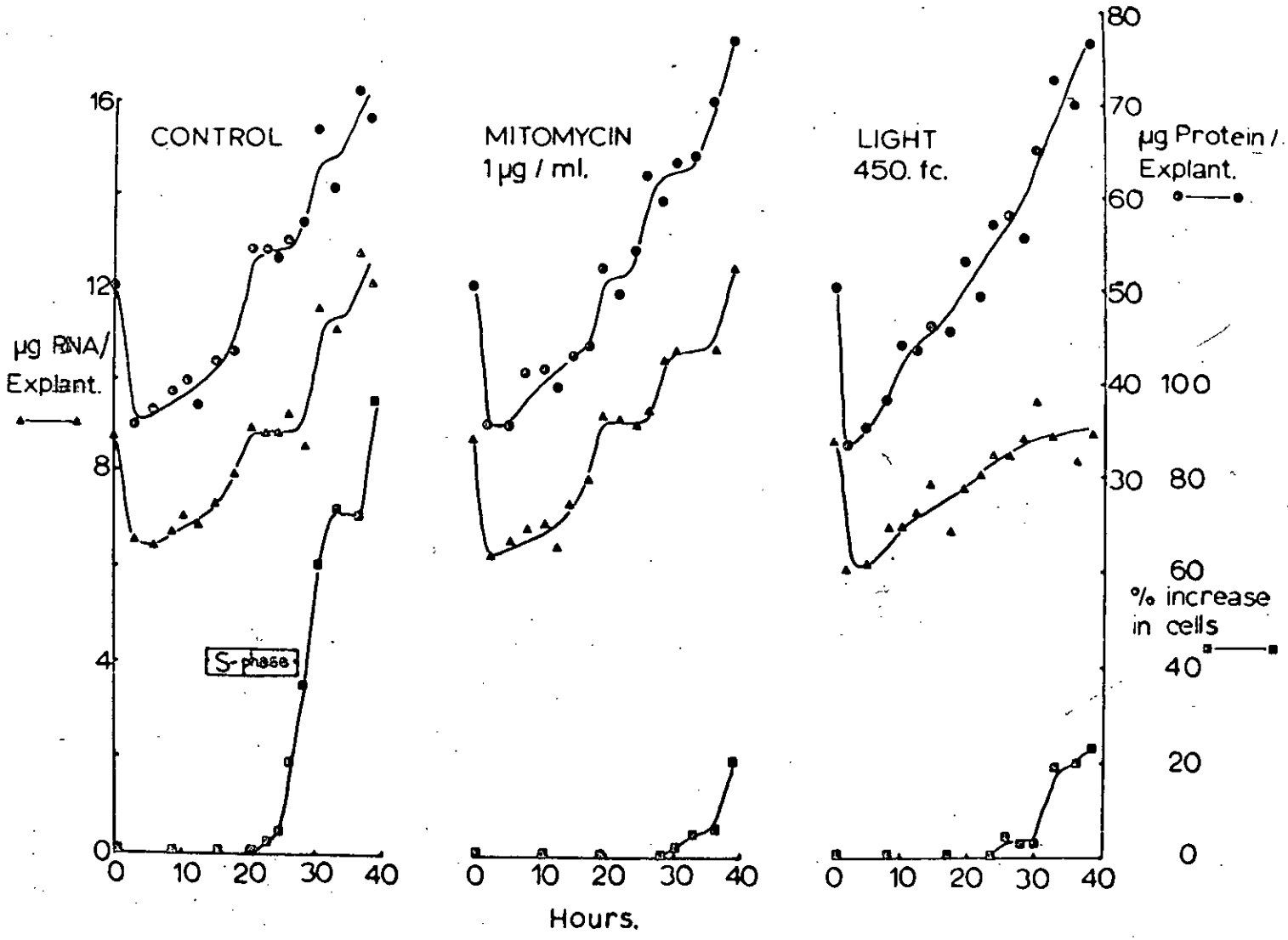


Figure 3.7 Legend opposite.

month and planted in dim green light in medium containing 10^{-5} M 2,4-D. The following treatments were applied:

1. Control. Cultured in darkness.

2. Mitomycin was added to the medium to a concentration of 1 µg / ml. Culture was in darkness.

3. Cultured in mixed fluorescent and tungsten light of 450 fc intensity.

With the control treatment, (Figure 3.7) division commenced at 22 hours, and some 80% of cell entered the first division. RNA and protein totals showed the initial drop associated with the loss of material from the cut cell on the surface of the explant. Thereafter, increases in both occurred, with plateaux between 20 and 27 hours.

In the presence of mitomycin, cell division was delayed until 30 hours, and much reduced in amount. The RNA accumulation showed a definite plateau between 20 and 27 hours. Until just after 30 hours, the pattern of RNA accumulation was identical to that in the control. The second plateau in the RNA accumulation curve with mitomycin was probably a consequence of inhibition of RNA accumulation by mitomycin: the results for 57 hour old cultures presented in Figure 3.8 show a 50% inhibition of RNA accumulation by mitomycin by this time. The curve for protein accumulation in the presence of mitomycin

Figure 3.7 (Opposite) Changes in percentage increase in cell number, RNA and protein contents of explants with time, for control cultures incubated in darkness, cultures incubatedin darkness with 1 µg / ml mitomycin, and cultures incubated in 450 fc light.

showed a gross rate of accumulation similar to that in the control. More data would be required to establish whether the protein accumulation was stepwise or not in the presence of mitomycin.

Cell division in cultures grown in the light was delayed and reduced compared to the control. RNA accumulation was at a lower gross rate than in the control, and the curve was not stepped. Protein accumulation occurred at the same overall rate as in the control, but the accumulation curve was linear, not stepped.

Thus the periodicity of RNA accumulation during early growth, and possibly also of protein accumulation, is unrelated to DNA synthesis and cell division. The periodicity is destroyed by strong light treatment, but not by mitomycin.

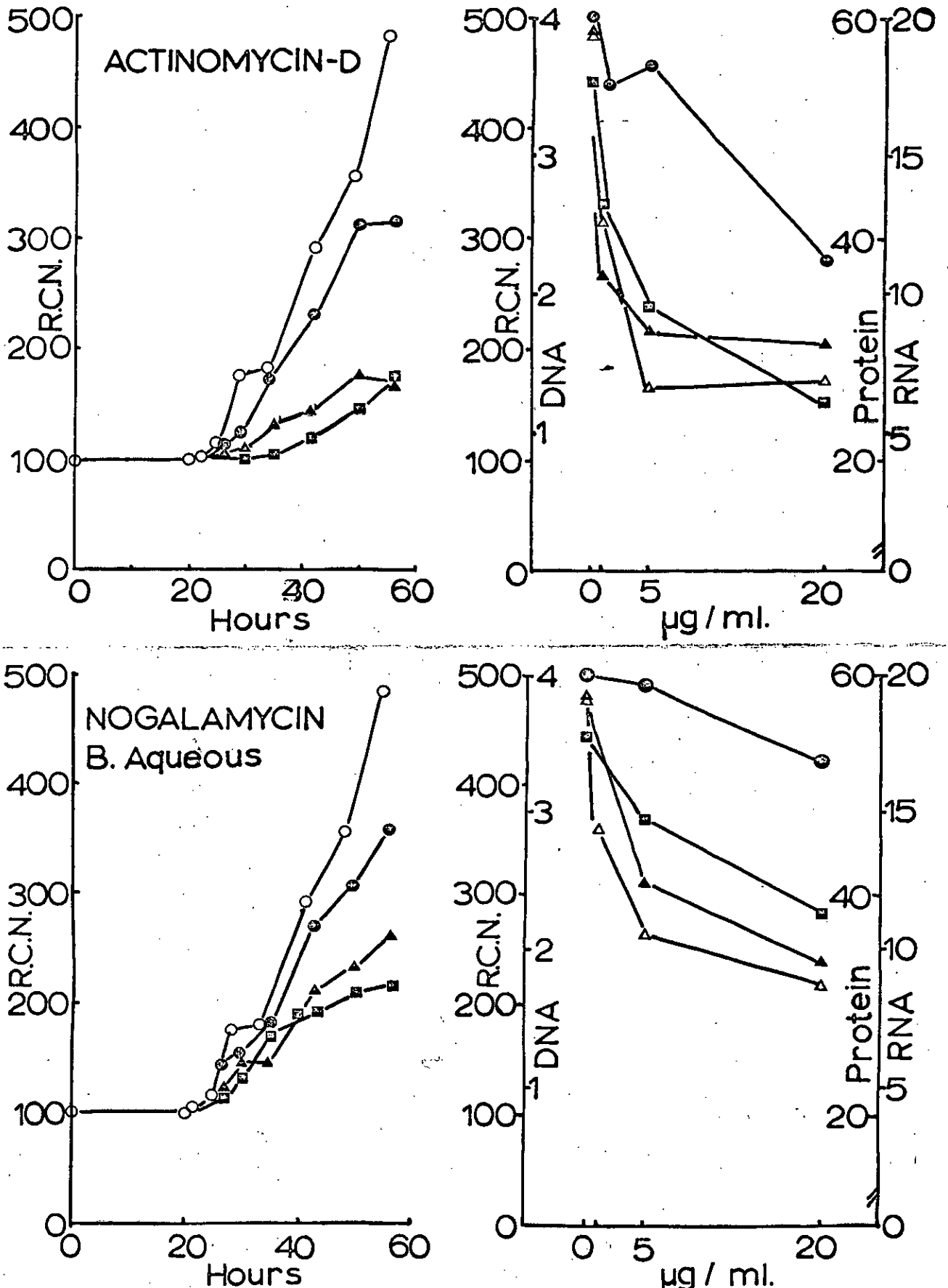

ACTINOMYCIN-D
R.C.N.
Hours
DNA
Protein
RNA
µg / ml.
NOGALAMYCIN
B. Aqueous
R.C.N.
Hours
DNA
Protein
RNA
µg / ml.

Figure 3.8

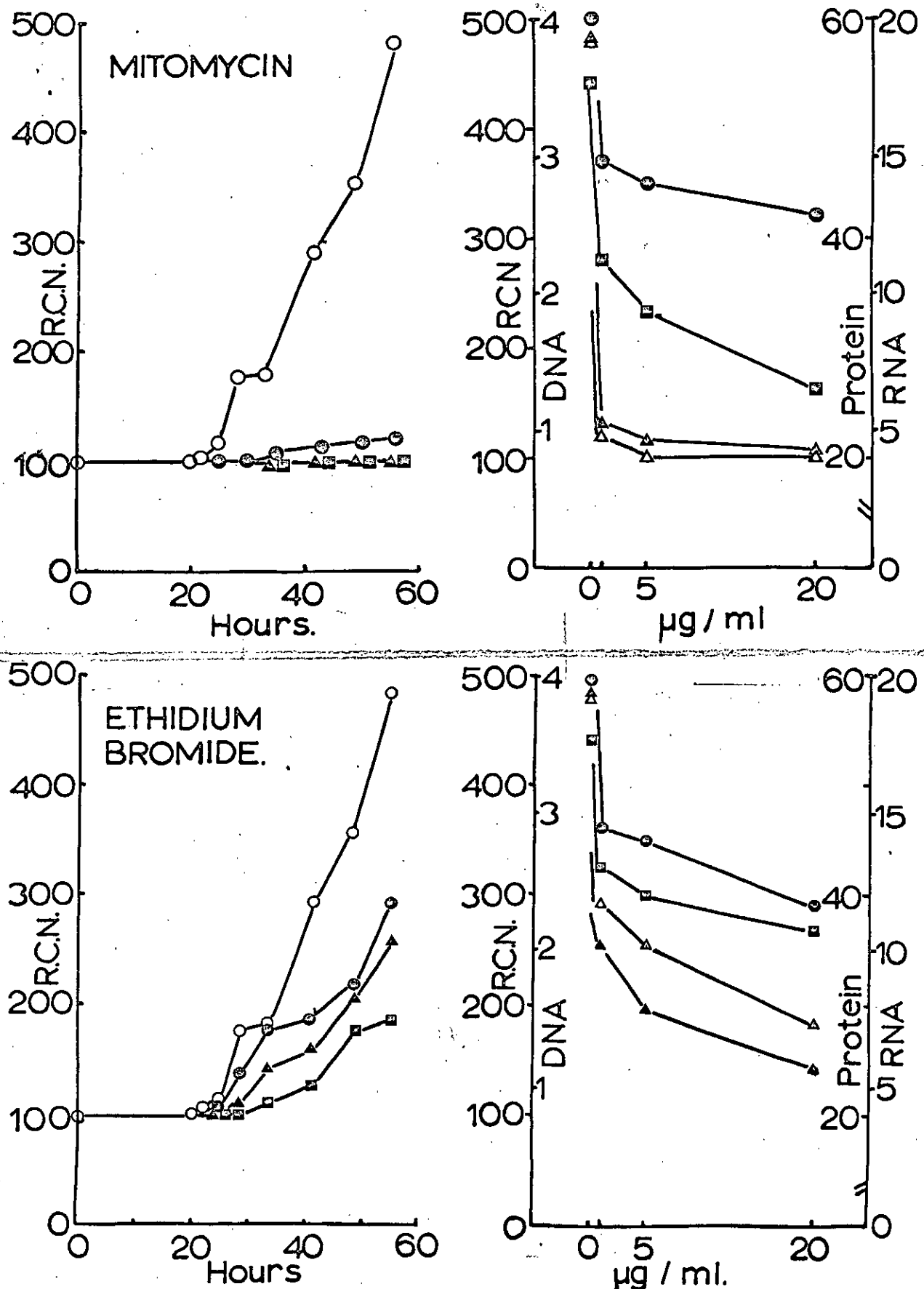


Figure 3.8

Legend overleaf.

3. Effects of inhibitors of nucleic acid synthesis.

A. Examination of inhibition by various antibiotics.

Various DNA-binding antibiotics were screened for effects on cell division, DNA, RNA and protein accumulation. The antibiotics tested were actinomycin-D, Chromomycin A_3, olivomycin, nogalamycin, proflavine hemisulphate, daunomycin, ethidium bromide and mitomycin-C, at concentrations of 1, 5 and 20 µg / ml in the culture medium. It was not possible to dissolve nogalamycin completely in either water or absolute alcohol. Aqueous and alcoholic solutions were centrifuged to remove insoluble residues and used at nominal concentrations of 1, 5 and 20 µg / ml; the true concentrations being somewhat lower.

Cell number was determined during the growth of cultures. RNA, DNA and protein contents of explants were determined at 57 hours.

Figure 3.8 shows results for four of the antibiotics. With the exception of mitomycin, all antibiotics showed very similar effects. Cell division and DNA synthesis were the parameters most inhibited. RNA accumulation was slightly less inhibited by the

Figure 3.8 (Opposite) Changes in relative cell number with time for explants grown with 0 (—○—), 1 (—●—), 5 (—▲—) and 20 (—■—) µg / ml antibiotic in the medium.

Relative cell number (—△—), µg DNA / explant (—▲—), µg RNA / explant (—■—) and µg protein / explant (—●—) after 57 hours growth in media containing various concentrations of antibiotics.

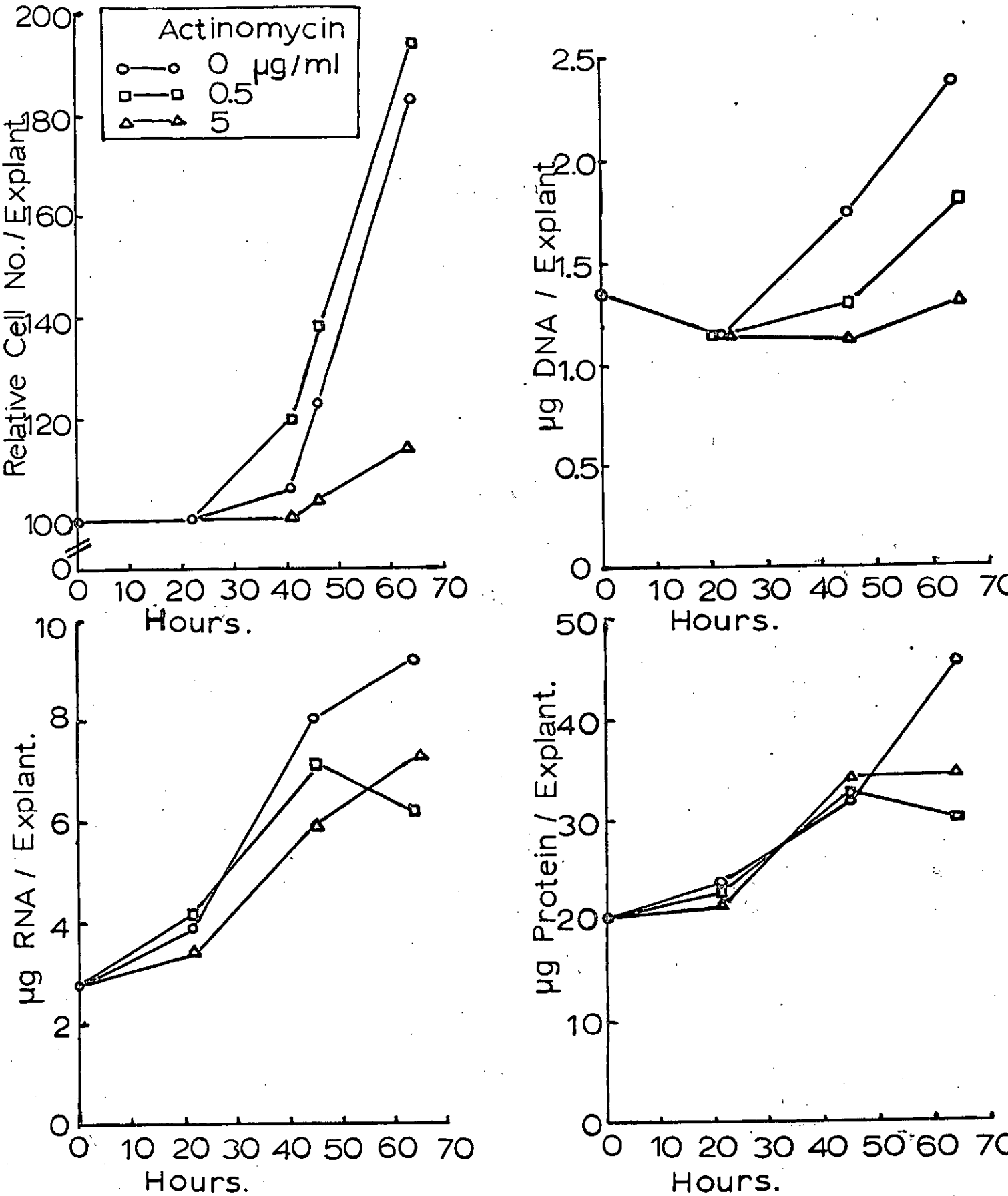


Figure 3.9

Legend opposite.

three intercalating agents, daunomycin, ethidium and proflavine, than by the antibiotics binding to specific bases. In all cases protein accumulation was the factor showing least inhibition. The alcoholic and aqueous solutions of nogalamycin were closely similar in effects, though the alcoholic solution had slightly stronger inhibitory effects on RNA and protein accumulation than the aqueous.

The effects of mitomycin on RNA and protein accumulation were similar to those obtained by the other antibiotics, but mitomycin was a more potent inhibitor of DNA synthesis and cell division than any other antibiotic.

Actinomycin-D and mitomycin-C were selected for further study.

B. The effects of actinomycin-D.

Explants were excised from small, immature tubers. Culture was in media containing actinomycin-D at 0, 0.5 or 5 µg / ml.

Cell division and DNA synthesis were both strongly inhibited by 5 µg / ml of actinomycin-D. With an antibiotic concentration of 0.5 µg / ml, there was no immediate effect on cell division. The marked inhibition of DNA accumulation by this concentration suggests that inhibition of cell division might occur after longer periods of culture. (Figure 3.9) In the experiment re-

Figure 3.9 (Opposite). Changes in relative cell number, DNA, RNA and protein per explant with time of culture in media containing various concentrations of actinomycin-D.

ported in Figure 3.8, an actinomycin-D concentration of 1 µg / ml had little effect on cell division until 50 hours, and then caused complete inhibition.

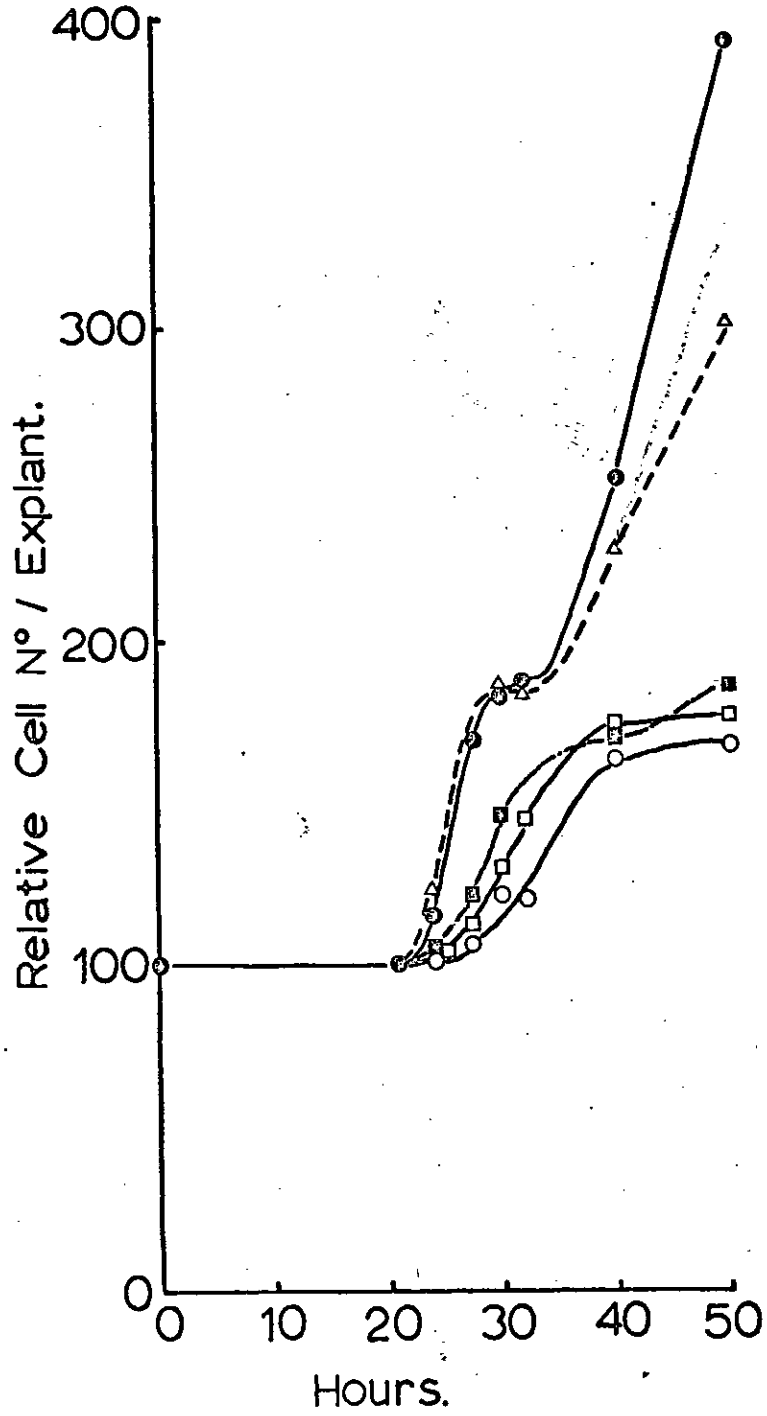
Until 41 hours, protein accumulation was uninhibited by actinomycin-D, and RNA accumulation was only slightly inhibited. After 41 hours, both protein and RNA accumulation ceased in the presence of actinomycin-D.

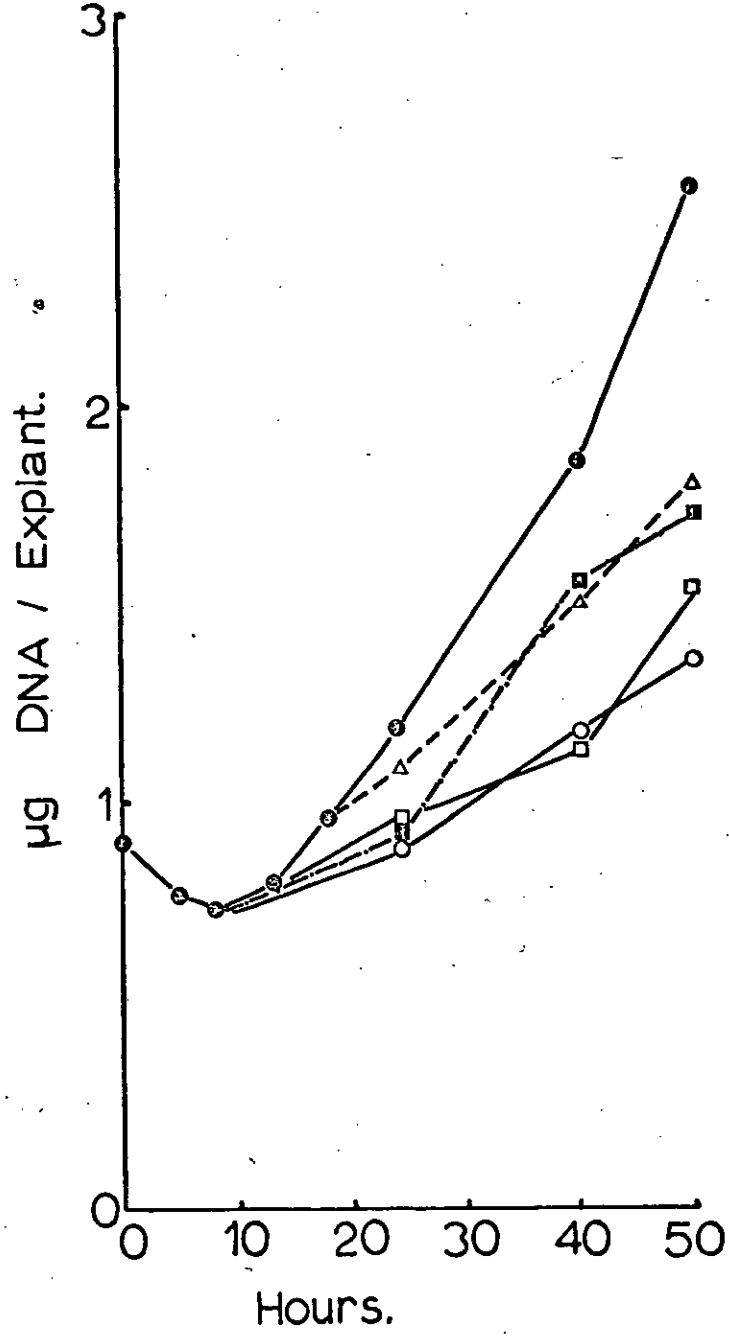
The long delay observed before inhibition of RNA and protein accumulation in this experiment suggested that the uptake of actinomycin-D into the cells might be delayed. The effects of DMSO on the inhibitions produced by actinomycin were investigated on explants from the same tuber type, by prewashing explants in medium containing 10% DMSO for 30 minutes. (DMSO is known to aid the penetration of the skin by certain substances, and has been used in place of injection.)

Little difference in the effects of actinomycin-D on RNA, DNA and protein accumulations showed after DMSO prewashing. Either DMSO did not speed up the penetration of the cells by actinomycin-D, or a delay in the uptake of the antibiotic is not responsible for the delay in the inhibition of RNA and protein accumulation.

C. The effects of adding actinomycin-D after various times of culture.

Explants were obtained from mature, freshly harvested tubers. Actinomycin-D was added to the medium to a concentration of 5 µg / ml after 0, 5, 8 or 18 hours of culture. A control treatment received no actinomycin-D.


400
300
200
100
0
Relative Cell N° / Explant.
0
10
20
30
40
50
Hours.


3
2
1
0
µg DNA / Explant.
0
10
20
30
40
50
Hours.

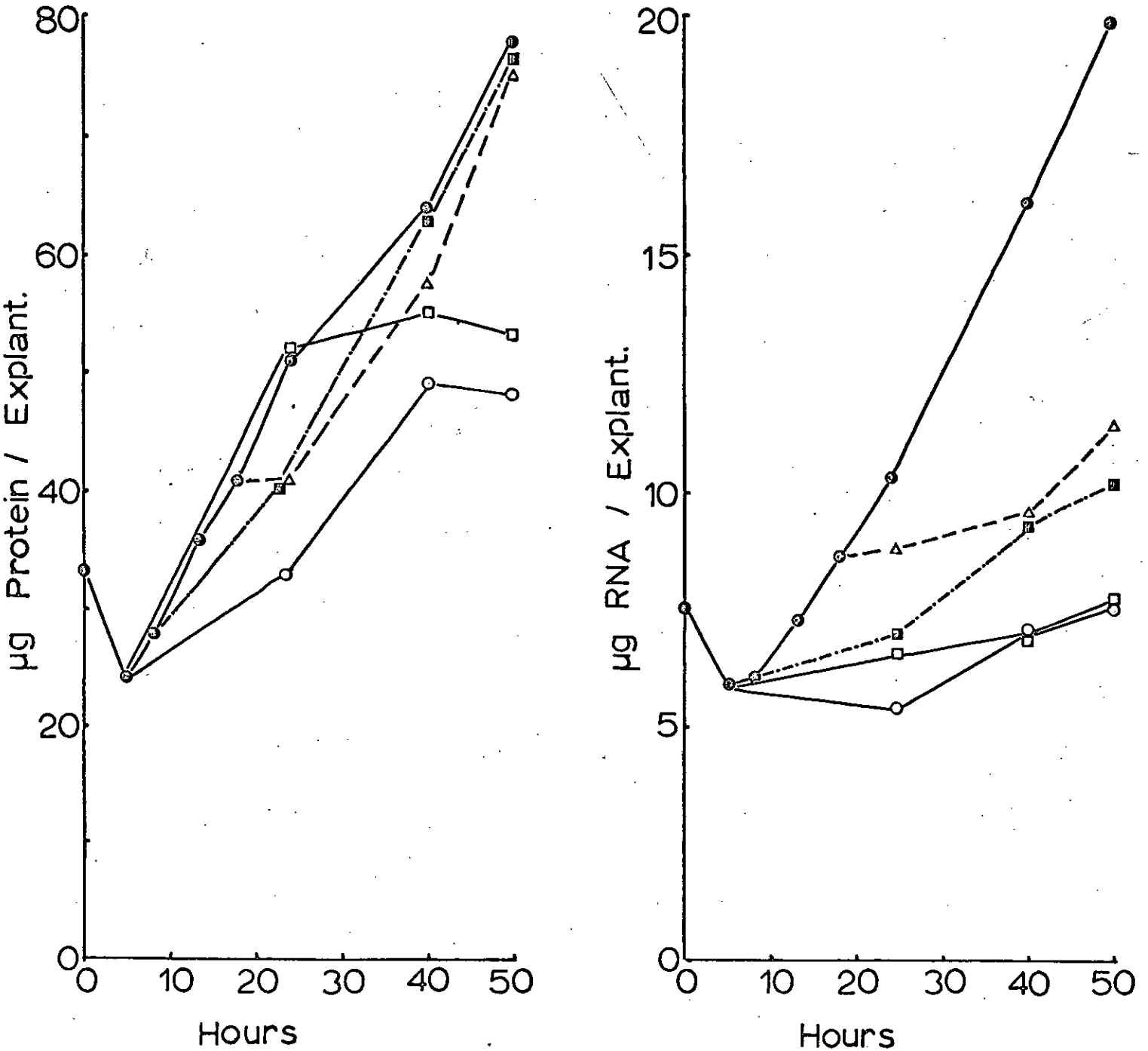


Figure 3.10 Legend overleaf.

Division in the control treatment commenced commenced at 22 hours (Figure 3.10). From the results of Mitchell (1967) and Evans (1967), it follows that S-phase began at about 8 hours. Addition of actinomycin-D to cultures at 0, 5 or 8 hours led to a delayed first division, though with almost as many cells eventually dividing as in the control. The second division was either inhibited or delayed until after the end of the experiment. Cultures to which actinomycin-D was added at 0 or 5 hours showed no 'fours' (i.e. second division products) in the macerates at 50 hours. A small number of 'fours' was observed in the macerate of 50-hour old explants to which actinomycin-D had been added at 8 hours. Actinomycin-D added at 18 hours, 4 hours before the beginning of the first division, had no effect on the first division. The second division was slightly inhibited. Examination of the macerate at 50 hours showed that 'eights' were present, i.e. some third division occurred.

DNA accumulation was inhibited by actinomycin-D added at all times, generally the earlier the addition the greater the inhibition.

RNA accumulation was strongly inhibited. The inhibition appeared immediately after the addition of actinomycin-D. This finding contrasts with the delay before inhibition reported in

Figure 3.10 (Opposite). Changes in relative cell number, DNA, RNA and protein per explant with time in cultures to which actinomycin-D was added to a concentration of 5 µg / ml after 0 (—●—), 5 (—□—), 8 (-·-■-·-) or 18 (--Δ--) hours. (—⊙—) control, no actinomycin-D.

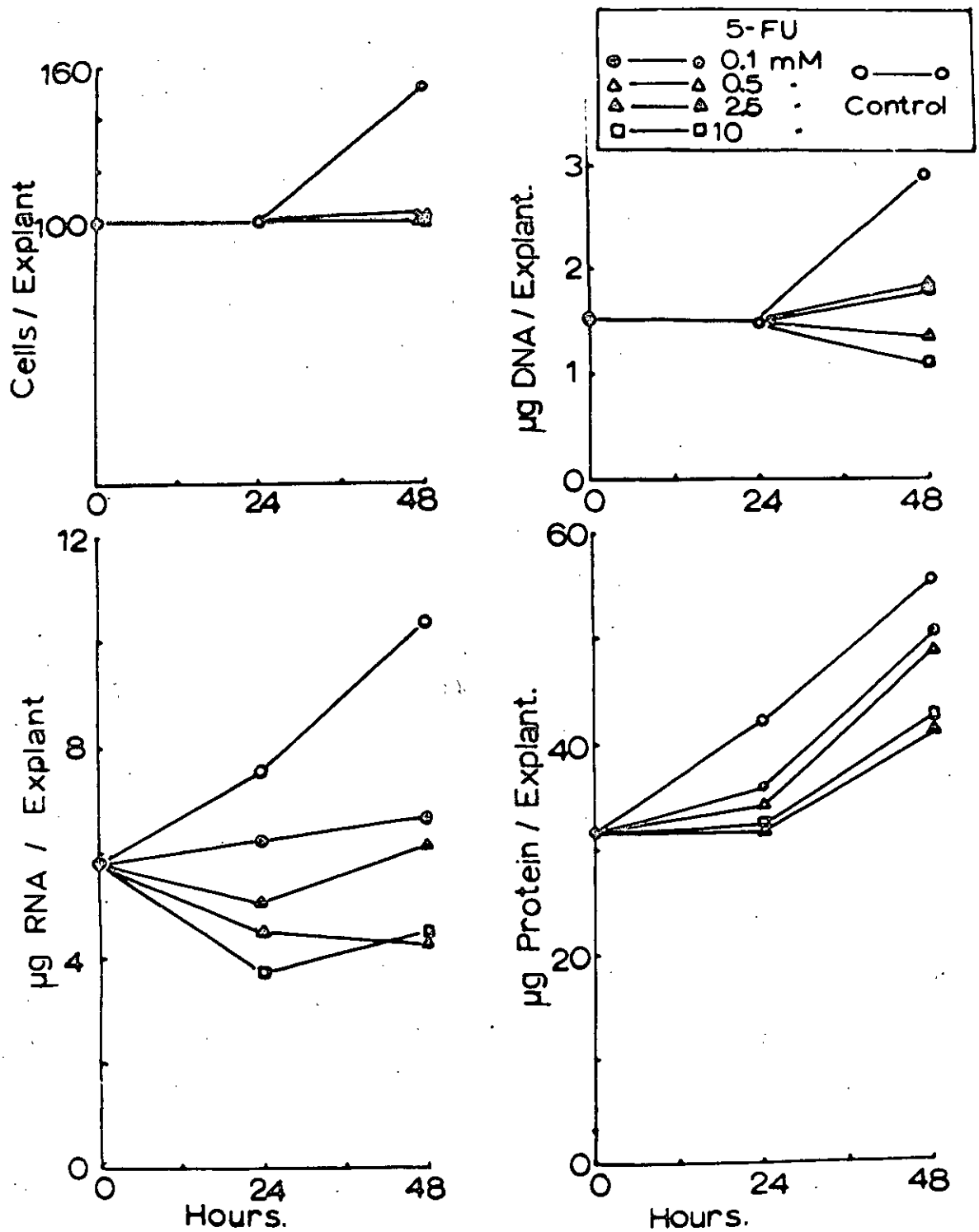


Figure 3.11 Changes in cell number, DNA, RNA and protein per explant with time of culture in media containing various concentrations of 5-fluoro-uracil.

Figure 3.9. With actinomycin-D addition at 0 hours, no RNA accumulation occurred until after the first division had commenced.

When actinomycin-D was added at 0 hours, protein accumulation was at a reduced rate, and ceased by 40 hours. Addition of actinomycin-D at 5 hours caused no immediate effect on the rate of protein accumulation, but accumulation ceased after 20 hours. Addition of the drug at 8 or 18 hours had little effect on the subsequent rate of protein accumulation.

D. The effects of 5-fluoro-uracil.

Explants were obtained from small, immature tubers. 5-FU was added to the culture medium to final concentrations of 0, 0.1, 0.5, 2.5 or 10 mM.

5-FU inhibited cell division completely at all concentrations, and had also very strong inhibitory effects on DNA and RNA accumulations. (Figure 3.11). Protein accumulation was inhibited, but to a lesser extent than RNA and DNA.

Prewashing for 30 minutes in medium containing 10% DMSO and 5-FU had no effects on the subsequent inhibitions by 5-FU.

E. The effects of 5-fluoro-uracil with and without thymidine, when added after various times of culture.

The experiment was conducted in two parts.

(i) Explants were excised from small, immature tubers. Thymidine and 5-FU were added to the medium at the beginning of culture as follows:

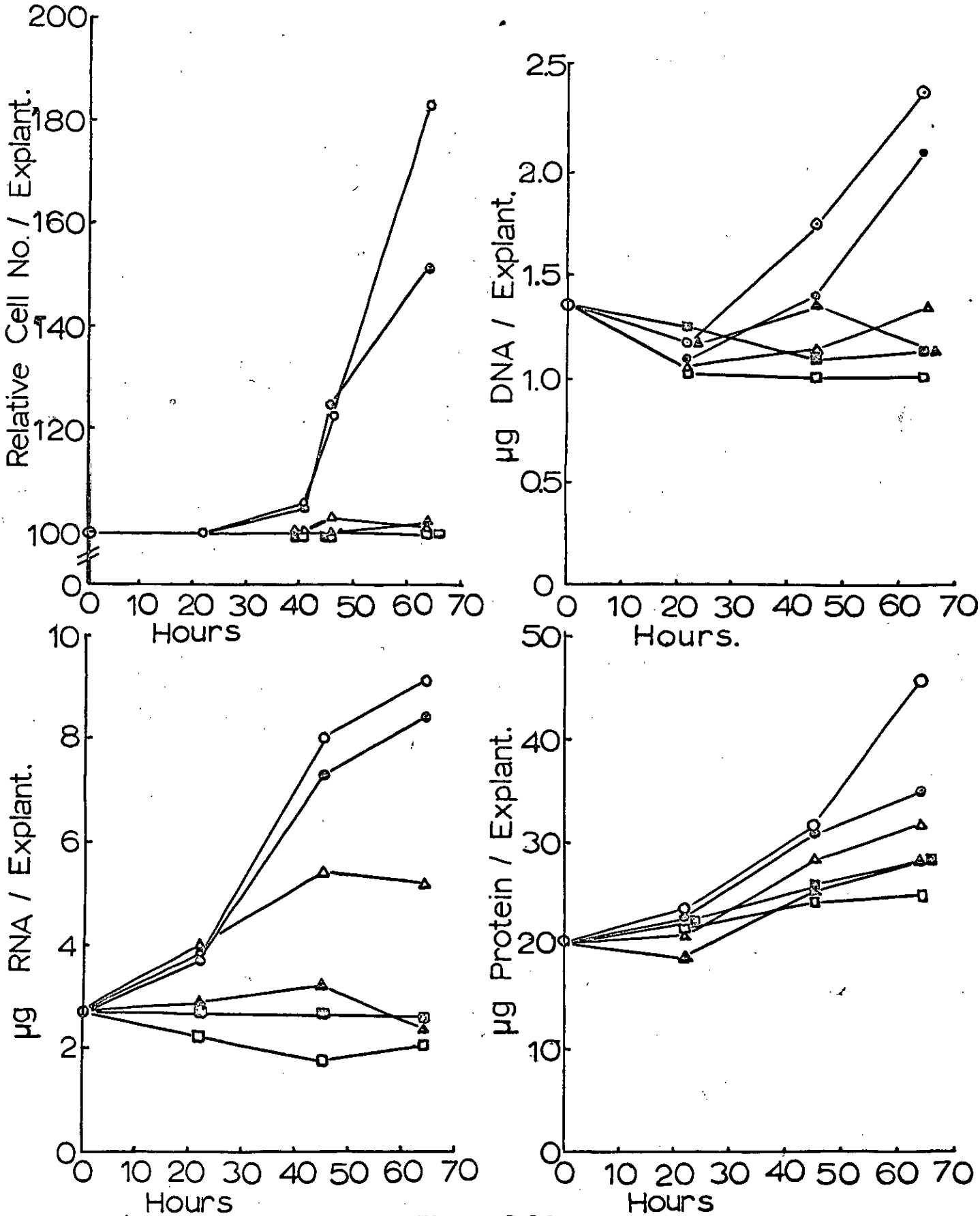


Figure 3.12

Legend opposite.

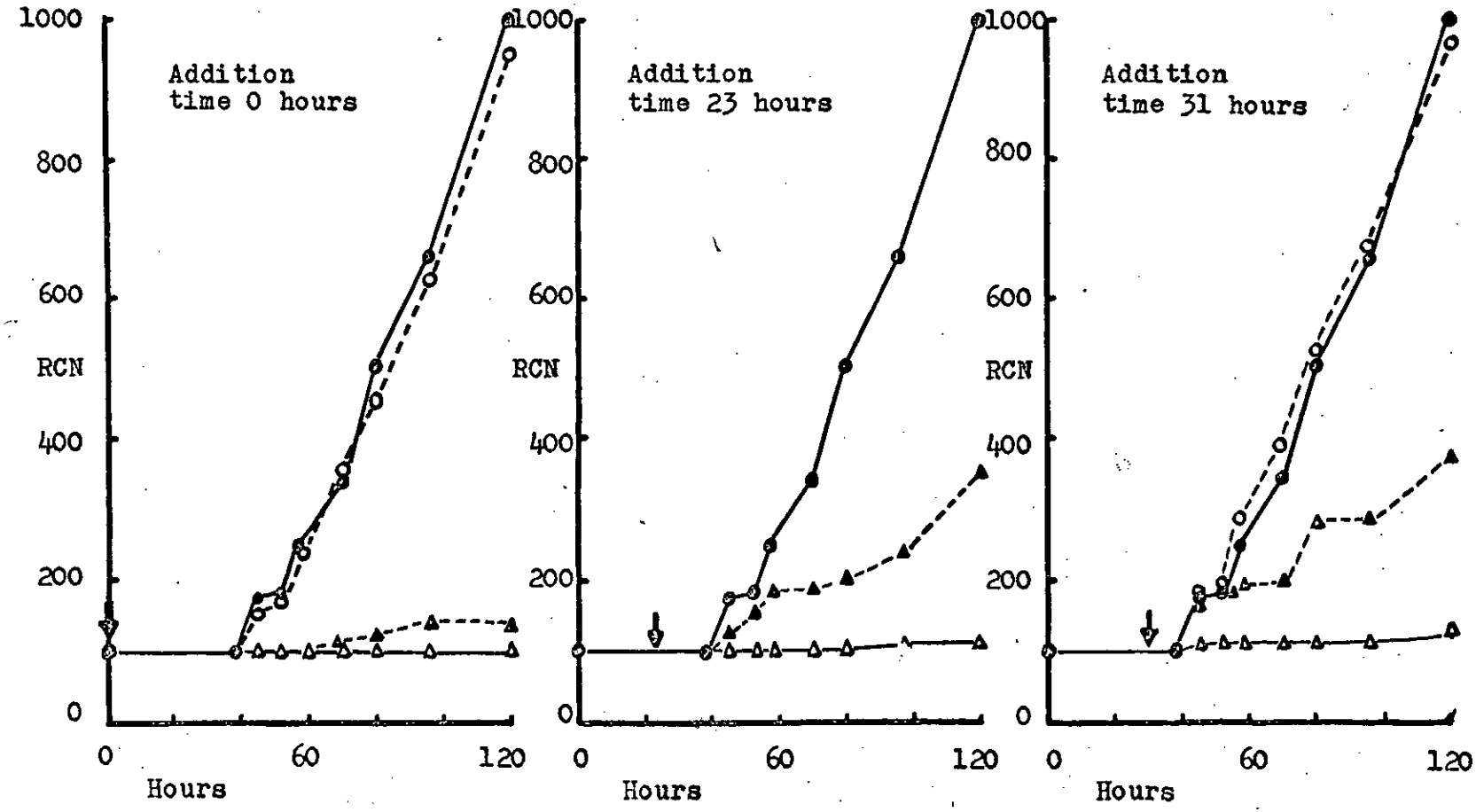
Treatment.	5-FU	Thymidine.
1.	0.1 mM	
2.	0.1 mM	0.33 mM
3.	0.5 mM	
4.	0.5 mM	0.33 mM
5.		0.33 mM
6.	Control. No additions.	

The concentration of thymidine was calculated to give 100 times the amount required for DNA synthesis for a 90% first division. Treatment 5 tested for any effects of thymidine on cell division. Excess thymidine (2.5 mM) blocks DNA synthesis in HeLa cells (Xeros, 1962; Rao and Engelberg, 1966).

(ii) Explants were obtained from a mature tuber. 5-FU (0.1 mM) with and without thymidine (0.33 mM) was added after 0, 23, 31, 45 or 57 hours of incubation. Thymidine alone was added to other cultures after 0 or 31 hours. Control cultures received no additions.

Figure 3.12 (Opposite) Changes in relative cell number, RNA, DNA and protein per explant with time of growth in media containing 0.1 mM 5-FU (—△—); 0.5 mM 5-FU (—□—); 0.1 mM 5-FU + 0.33 mM thymidine (—▲—); 0.5 mM 5-FU + 0.33 mM thymidine (—■—); 0.33 mM thymidine (—●—); control, with neither 5-FU nor thymidine (—○—).

Figure 3.13


Addition
time 0 hours
Addition
time 23 hours
Addition
time 31 hours
RCN
Hours
0
200
400
600
800
1000
60
120

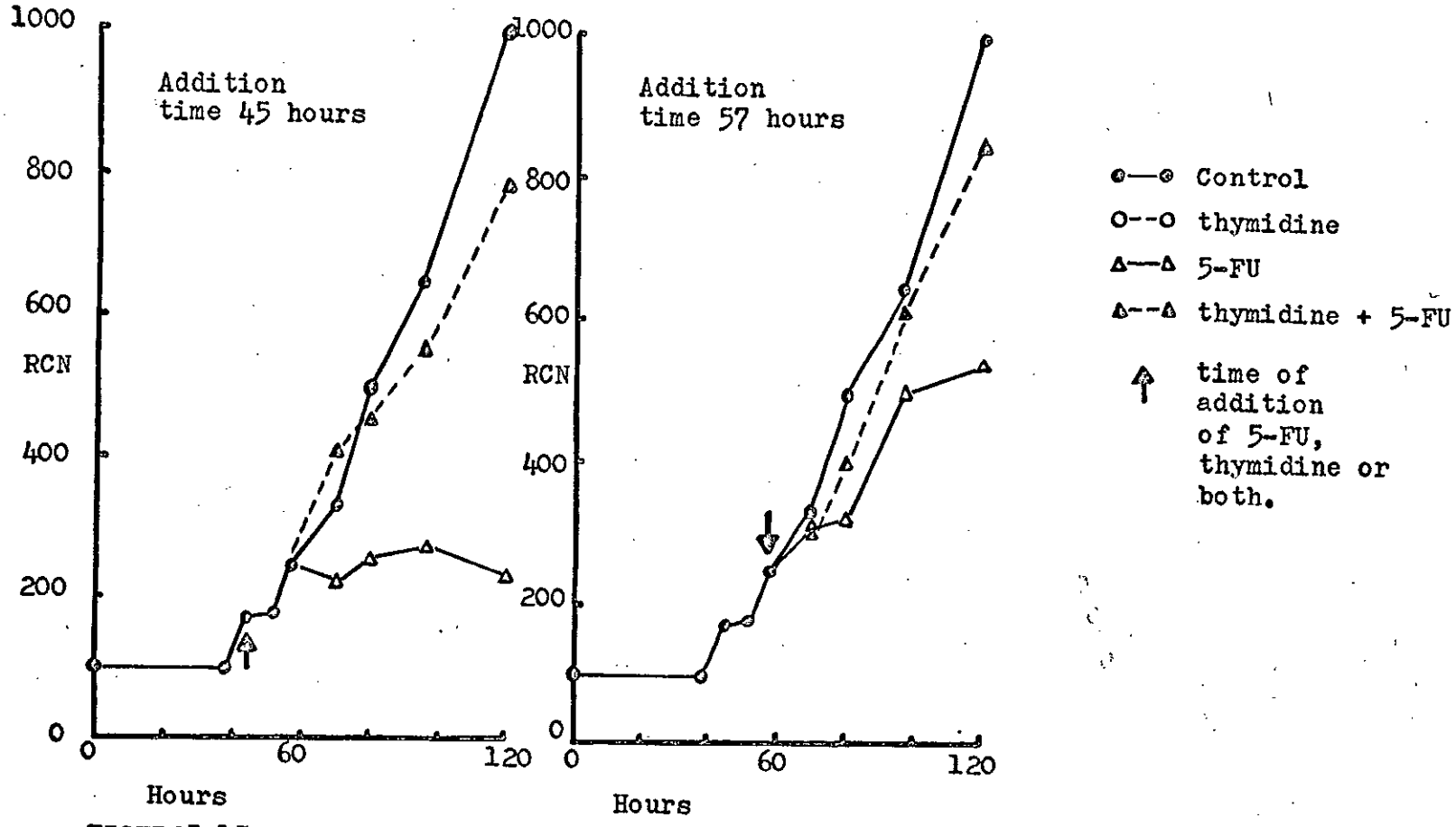


FIGURE 3.13 Changes in relative cell number with time for cultures to which thymidine (to a concentration of 0.33 mM), 5-fluoro-uracil (to a concentration of 0.1 mM) or both thymidine and 5-FU were added after various times

In the first part of the experiment, thymidine alone had no effect on the rate of RNA accumulation. (Figure 3.12) DNA accumulation was at a slightly lower rate than in the control, consistent with the slower cell division rate with thymidine. Protein accumulation was slightly inhibited after 50 hours.

The addition of 5-FU alone to cultures caused inhibitions similar to those in the previous experiment. (Figure 3.11) The addition of thymidine to cultures with 5-FU did not affect the 5-FU inhibitions of cell division, or of DNA and protein accumulation. Thymidine had no effect on the 5-FU inhibition of RNA accumulation when the 5-FU concentration was 0.5 mM. With a 5-FU concentration of 0.1 mM, the addition of thymidine appeared to intensify the 5-FU inhibition of RNA accumulation.

In the second part of the experiment (Figure 3.13), the first division in the control treatment commenced at 39 hours. The onset of S-phase therefore occurred at about 26 hours. (Mitchell, 1967; Evans, 1967).

Thymidine alone added at 0 or 31 hours had no effects on cell division.

Addition of 5-FU alone at 0, 23 or 31 hours resulted in complete inhibition of the first cell division. When added at 45 hours, just after the first division, some second division occurred before complete inhibition of division by 57 hours. Addition of 5-FU during the second division, at 57 hours, gave a lower rate of cell number increase, with complete inhibition of division after 96 hours. Thus whenever 5-FU was added during culture, inhibition of cell division followed, though the lag before complete inhibition of division increased with the age

of the culture at the time of addition.

Considering the treatments where both 5-FU and thymidine were added:

Thymidine had little effect in relieving the inhibition of cell division by 5-FU when the addition was made at the start of culture. By 120 hours, only 40% of the cells had divided.

Addition at 23 hours, just before the time calculated for the beginning of S-phase, permitted the same percentage of cells as in the control to complete the first division, but the division was delayed. The second division was very much inhibited and delayed.

Addition at 31 hours, i.e. during S-phase, allowed an unimpaired first division. The second division was reduced and delayed.

When the addition was made at 45 or 57 hours, subsequent rates of division were a little lower than in the control.

DISCUSSION.

The results presented in Section 3 show that considerable changes in the DNA, RNA and protein contents of explants occur during the first few divisions. The first part of this discussion considers whether these changes are related to the cell division cycle or whether they occur independently. The second part of the discussion analyses the opposite question, the dependence of cell division on RNA synthesis.

1. The dependence of DNA, RNA and protein changes on the cell division cycle.

DNA. In all the treatments applied in Section 3, DNA synthesis preceeded cell division. This is consistent with Mitchell's (1967) finding that all cells in the freshly cut explant are in the 2C state. With no treatment did DNA synthesis occur without subsequent cell division. Mitchell (1967) has found that no endopolyploidy occurs.

DNA synthesis is therefore intimately linked with cell division.

Protein. In the dividing tissue, the protein content per explant doubles between excision and the end of the first division. The accumulation is stepwise, with periods of rapid accumulation just before S-phase and at the time of the first division (Figure 3.7).

When explants were incubated with mitomycin (Figure 3.7) or in a high light intensity, (Figures 3.7; 3.2) protein

accumulation was unimpaired, while DNA synthesis and cell division were strongly inhibited. Protein accumulation does not therefore depend on the occurrence of cell division.

When explants were cultured in medium containing no 2,4-D, (Figure 3.4), and also in medium containing actinomycin-D and 2,4-D, (Figure 3.10), considerable protein accumulation occurred with reduced or absent RNA accumulation. Protein accumulation therefore does not rely on a concurrent increase in ribosomes. Electron microscope studies by Bagshaw (1968) on the artichoke tuber show that the freshly cut explant has a high content of ribosomes.

Although net increase in protein may occur in the absence of RNA accumulation, DNA accumulation or cell division, it cannot be claimed that this increase is of the same kind of proteins as occurs in the dividing, control system. This problem might be examined by characterisation of protein complements for tissues grown under different conditions by polyacrylamide gel electrophoresis.

RNA. In dividing explants, RNA content has doubled by the end of the first division. The early accumulation is in a stepwise manner, with periods of rapid accumulation, and increases in the rate of 32-P incorporation into r-RNA just before the beginning of S-phase and at the time of the first division. (Figure 3.1). While it might be argued that the increased labelling of the r-RNA is merely a reflection of the increased 32-P uptake at these times, the close correspondence between r-RNA amounts and labelling suggests that both labelling and uptake were manifestations of increased metabolic activity in

the tissue at these times.

The rate of 32-P incorporation into r-RNA remained constant during S-phase. (Figure 3.1) Any unavailability of DNA for transcription while engaged in replication, and any effect of the doubling of cistron number during S-phase therefore have no effect on the rate of r-RNA synthesis.

The maximum rates of RNA accumulation and r-RNA labelling which occur at the time of the first division are in contradiction to the known lower rates of RNA synthesis during metaphase in other cell types. However the artichoke tissue lacks complete synchrony. Evans (1967) has shown that division in the individual cell lasts for 3 hours; in the whole tissue division lasts for 6 to 8 hours. A high rate of RNA synthesis close to mitosis could obscure any reduction in rate during metaphase.

When explants were grown in strong light (Figures 3.2; 3.7), or with mitomycin (Figure 3.7), RNA accumulation occurred at almost the same rate as in the control, but in the virtual absence of DNA synthesis and cell division. Not only is the occurrence of RNA accumulation independent of DNA synthesis, but the periodicity of accumulation is also independent of DNA synthesis and the cell division cycle. (Figure 3.7, mitomycin treatment).

A non-dependence of RNA accumulation on DNA synthesis has been reported for other organisms. Kanazir and Errera (1954) and Klein and Forssberg (1954) have reported in E. coli and Ehrlich ascites tumours respectively that temporary elimination of DNA synthesis by irradiation did not interfere with the rate of RNA accumulation. Mitchell (1967a) on the

other hand has concluded that in the artichoke explant only cells which synthesise DNA can accumulate RNA. The lower 2,4-D concentration used in the culture medium by Mitchell - 10^{-6} M, might explain the contradiction in results, as 2,4-D stimulates RNA accumulation. (Figure 3.4). Key (1963), Key and Shannon (1964) and Izawa (1961) among others have reported stimulation of RNA synthesis in plant material by applied auxins. Setterfield (1963) recorded a stimulation of RNA synthesis by auxin and kinetin treatment of artichoke tuber discs. Chrispeels and Hanson (1962) and Key, Lin, Gifford and Dengler (1966) have suggested that the 2,4-D stimulation of RNA accumulation in soybean involves mainly ribosomal RNA.

It is possible that there is seasonal variation in the duration of RNA accumulation in the cultured explant. The tissues used for figures 3.1 and 3.6 showed a declining rate of RNA accumulation by the time of the second division; both were using tubers stored for 6 months. Experiments using tubers stored for shorter periods, e.g. Figures 3.7; 3.10, showed no decline in the rate of RNA accumulation.

Conclusion. The early growth of the explants is characterised by two periods of intense metabolic activity, involving high rates of RNA and protein accumulation and 32-P uptake (Figure 3.1) and occurring just before the beginning of S-phase and at the time of the first division. Evans (1967) has shown that the uptakes of thymidine and uridine have peaks at times similar to those for phosphate uptake. Yeoman (1967) has shown that the rate of respiration increases during these two periods, and Evans (1967) and Mitchell (1967) have shown that DNA syn-

thesis for the first and second divisions follows closely on the first and second periods of intense activity respectively.

The demonstration that the periodicity of RNA (and perhaps protein accumulation) is independent of DNA synthesis and cell division suggests that these periods of intense metabolic activity are not the result of a rhythm based on the cell division cycle. The question then arises what causes this periodicity of metabolic activity. The following explanation is proposed for the periodicity of RNA accumulation:

When explants were grown in the absence of 2,4-D, a slight rise in RNA content occurred, at the same time and of the same size as the first increase in RNA in explants grown with 2,4-D (Figure 3.4). Yeoman (1967) has found that the addition of 2,4-D to explants grown for a period without 2,4-D causes a further rise in RNA. These observations lead to the hypothesis that in explants grown with 2,4-D, the first rise might be stimulated by the broken cell materials and autolysis products, the second rise in RNA by the 2,4-D. The stimulation of RNA accumulation by tissue homogenate added to medium containing no 2,4-D is consistent with this explanation. A sensitive test of the similarity between the rise in RNA in the absence of 2,4-D and the first rise in the presence of 2,4-D would be provided by measuring changes in the amounts of r-RNA and t-RNA on gel electrophoresis separations: Figure 3.1 shows that the first rise with 2,4-D present involves only r-RNA.

The hypothesis may be extended in an attempt to explain the destruction of the periodicity of RNA accumulation by strong light. (Figure 3.7). Light treatment causes explants

to turn brown during culture, probably by some effect on the damaged and autolysing cells of the outside. Light also increases the rate of 32-P uptake. (Section 2). Thus the way in which light destroys the periodicity of RNA accumulation, and also changes the timings of the cell divisions (Figure 1.5) might be through an influence on the supply and uptake rates of stimulants.

A study of changes in solute uptake rates, respiration etc. in tissues inhibited from division by mitomycin would determine whether this hypothesis can be extended to explain other aspects of the two peaks of metabolic activity in the early growth of explants.

2. The dependence of DNA synthesis and cell division on RNA synthesis.

The inhibitors chosen for this investigation were selected in the hope of obtaining selective inhibition of specific classes of RNA synthesis. The extent to which this aim was realised will be considered first, and then the effects of inhibiting RNA synthesis on cell division will be discussed.

A. The effects of inhibitors on RNA, DNA and protein synthesis.

5-fluoro-uracil. This was a potent inhibitor of DNA and RNA accumulation. Protein accumulation was inhibited to a lesser extent. (Figure 3.11). The inhibition of cell division, and therefore of DNA synthesis, was eliminated when thymidine

was supplied with 5-FU just before the beginning of S-phase, while 5-FU alone supplied at this time still inhibited division. This strongly suggests that a mechanism similar to that in bacteria (Cohen et. al., 1958; Aronson, 1961a) is in operation in artichoke tissue, with thymidine 'short circuiting' the inhibitory effect of 5-FU on thymidylate synthetase. But in the absence of DNA totals for the experiment reported in Figure 3.13, especially for the treatment where 5-FU alone was added at 23 hours, the mechanism by which thymidine relieves the 5-FU inhibition of cell division is not conclusively proved.

The inhibition of protein accumulation recorded in Figure 3.11 is in contradiction to the results of Key (1966) with soybean, who found that 5-FU had no effect on protein synthesis although it was incorporated into the messenger-like D-RNA. In the artichoke tissue, the 5-FU may either inhibit messenger synthesis, or the incorporation of 5-FU into m-RNA may inhibit its function. In bacteria, Aronson (1961) has suggested that the inhibition of protein synthesis by 5-FU is indirectly through the production of abnormal ribosomes. Andoh and Chargaff (1965) have comfirmed the production of abnormal ribosomes in E. coli, and have shown that up to 70% of the uridylic acid may be replaced by the fluoro- derivative. An inhibition of protein synthesis in the artichoke tissue by abnormal ribosome production is considered unlikely, in view of the rapid and complete inhibition of r-RNA synthesis by 5-FU. (Figures 3.11, 2.20) and the high content of ribosomes of the freshly excised explant. (Bagshaw, 1968). It is, however, a possibility that the ribosomes existing at the time of excision are in some way less capable of

Base composition %	r-RNA				'm-RNA'			
	C	A	G	U	C	A	G	U
	21	26.5	30.5	22	19	34	24	22
Base composition of template DNA (Double stranded)	G	T	C	A	G	T	C	A
	26	24	26	24	22	28	22	28

Antibiotic	Calculated maximum % of bases available for antibiotic binding.	
G-specific (Actinomycin-D etc.)	26	22
A-T specific (Nogalamycin)	48	56

Table 3.2 Percentages of DNA bases capable of binding G-specific and A-T specific antibiotics, calculated from mean base compositions of 2.07m r-RNA precursor and heterodisperse RNA lighter than 0.70m reported in Table 2.5.

supporting protein synthesis than those synthesised during culture.

Antibiotics. With the exception of mitomycin, all the DNA-binding antibiotics tested caused very similar amounts of inhibition of DNA, RNA and protein accumulation and cell division. This is surprising in view of the different modes of action and the known differences in effect in other situations. For example, in bacteria, Hurwitz et. al. (1962) have found conditions in which actinomycin-D inhibited RNA synthesis by 80% and not DNA synthesis, and in which proflavine inhibited RNA synthesis by 30% but DNA synthesis by 85%.

Considering the possibility of differential action of antibiotics on r-RNA and m-RNA synthesis on the basis of differences in base-binding specificity, Table 3.2 shows the calculated percentages of bases in r-RNA and 'm-RNA' cistrons available for binding G-specific or A-T specific antibiotics. On this interpretation, actinomycin-D, a G-binding antibiotic, would be expected to inhibit r-RNA synthesis preferentially, while the A-T-binding nogalamycin would inhibit m-RNA more. However the differences in available binding sites are small. It should also be noted that the ribosomal RNA of artichoke tissue is of lower G + C content (50%) than r-RNA of tissues in which r-RNA synthesis is preferentially inhibited by actinomycin. For example, Willems et. al. (1968) have quoted a G + C content of 64% for the r-RNA of HeLa cells. This suggests that the differential effect of actinomycin-D in artichoke tissue might be

less clear cut than in HeLa cells.

The two experiments with actinomycin in the artichoke tissue showed some variation in effect. With immature tubers, cell division and DNA synthesis were most inhibited, while RNA and protein accumulations were only inhibited after a delay of 45 hours. It is unlikely that DNA synthesis was more inhibited than RNA synthesis in view of the highly selective action of actinomycin-D on RNA synthesis in bacteria. (Hurwitz et. al. 1962). A possible explanation of the effects is that certain RNA synthesis, necessary for cell division, was more sensitive to actinomycin-D than that required for RNA accumulation and net protein synthesis.

There is evidence in animal tissues which supports the hypothesis that induced enzyme or RNA synthesis is more sensitive to actinomycin inhibition than is normally occurring enzyme or RNA synthesis:

Lieberman, Abrams and Ove (1963) found that low concentrations of actinomycin-D, which did not affect the initial rate of RNA synthesis in explanted kidney cells, prevented the rise in RNA synthesis rate which was necessary for the induction of DNA synthesis. Holtzer et. al (1964) found that actinomycin inhibited the rise in deoxycytidylate deaminase activity following partial hepatectomy of rats, but had no effect on enzyme activity over long periods in intact liver. Garren, Howell, Tomkins and Crocco (1964) found that actinomycin supressed the rise in tryptophan pyrrolase activity produced by hydrocortisone injection, but had no effects on the enzyme activity at its basal level in non-injected rats.

Applying this hypothesis to the situation in the arti-

choke, DNA synthesis is an induced event, but the data of figure 1.8 and Table 1.6 suggest that net protein and RNA accumulations are occurring in the immature tuber, and are not induced by excision and culture.

With freshly harvested mature tubers, the inhibitions of protein accumulation, DNA synthesis and cell division were similar to those obtained with immature tubers, but the RNA accumulation was immediately and strongly inhibited. (Figure 3.10). The data in this case suggest a preferential inhibition of r-RNA synthesis. They are also consistent with the above hypothesis. In the mature tuber, net RNA accumulation has ceased. (Table 1.6). This then takes r-RNA synthesis into the category of events induced by excision and culture, and allows of a stronger inhibition by actinomycin. Protein synthesis appears to continue for some time after the maturation of the tuber (Table 1.6). This may explain why net protein accumulation remained relatively uninhibited, while the presumed enzyme synthesis necessary for DNA synthesis was strongly inhibited.

In conclusion, neither actinomycin nor 5-FU provided a definite selective inhibition of any class of RNA synthesis.

B. The effects of the inhibitors on cell division.

Although it has not proved possible to conclude anything about the significance of particular RNA types for cell division, the experiments with inhibitors do imply that certain periods of RNA synthesis are necessary for the occurrence of cell division.

When 5-FU (Together with thymidine to eliminate the direct inhibition of DNA synthesis) was supplied from the beginning of culture, no cell division occurred. (Figure 3.13). Supply of 5-FU + thymidine just before the beginning of S-phase allowed the first division to occur. It is concluded that there is a period of RNA synthesis before S-phase which is necessary for the first division. Results with actinomycin-D support this conclusion, although actinomycin-D was a less effective inhibitor than 5-FU. Addition of actinomycin-D at any time before the beginning of S-phase cused a delay in the first division, but addition at the end of S-phase had no effect on the division. One observation with actinomycin-D (Figure 3.10) suggests that although r-RNA synthesis occurs during the pre-S-period (Figure 3.1) this is not required for cell division. With actinomycin-D present from the beginning of culture, no increase in total RNA occurred until the first division was under way.

Although a normal number of cells completed the first division when 5-FU + thymidine were added to cultures just before the beginning of S-phase, the division was delayed. Addition of 5-FU + thymidine in the middle of S-phase permitted the first division to occur at the same time as in the control. Thus RNA synthesis during the first half of S-phase is involved in the control of the first division.

The failure of actinomycin-D added near the end of S-phase (Fig 3.10) or of 5-FU + thymidine added in the middle of S-phase to affect the first division suggests that cells are already competent to complete the first division towards the end of S-phase.

Addition of the inhibitors in the later part of the first S-phase did, however, affect the second division. Actinomycin-D

slowed the rate of division, and 5-FU + thymidine strongly inhibited the second division. Thus RNA synthesis occurring late in the first S-phase but before the end of the first division is essential for the occurrence of the second division.

In the experiment involving addition of 5-FU + thymidine at the beginning or middle of the first S-phase, it might be argued that the inhibition of the second cell division was because thymidine supply was running low after being used for DNA synthesis for the first division. This possibility can be discounted by comparing the effects of adding thymidine and 5-FU at 23 and 45 hours. In the latter, thymidine permitted a relative cell number increase of at least 600; thymidine was required to support a relative cell number increase of only 250 with addition at 23 hours.

Addition of 5-FU + thymidine at the end of the first division or in the middle of the second resulted in subsequent rates of cell division only slightly lower than in the control. This need not necessarily imply a reduced requirement for RNA synthesis by cell division. The exponential nature of cell number increase in this system implies that later in culture, larger absolute numbers of cells are competent to divide. The effects of inhibition of essential RNA synthesis would not appear until this increase in cell number had taken place.

From these experiments with inhibitors, it is concluded that RNA synthesis is necessary for the induction of cell division, and that later RNA synthesis, not required for the first division, is required for subsequent cell divisions.

APPENDIX 1.

The quantitative estimation of DNA, RNA and protein.

INTRODUCTION.

In their review of methods for determining nucleic acids in biological materials, Hutchison and Munro (1961) conclude that different methods must be tested to find the most suitable approach for each tissue. The aim of this investigation was to test and develop satisfactory methods for the determination of DNA, RNA and protein contents of artichoke explants.

Several features were required of the method:

1. It must be possible to store samples until a time convenient for analysis.
2. The process must be rapid enough to permit the analysis of large numbers of samples.
3. The determinations must be accurate. The extraction of substances interfering with final determinations must be effective.
4. DNA, RNA and protein must not be lost during the procedure.

Washing. The initial step in published schemes for analysis of nucleic acid content involves the removal of substances interfering with the final determinations. Acid washing in the cold removes small, soluble molecules such as free nucleotides. This is followed by organic solvent treatment to remove lipids and phospholipids.

The commonly used procedure of Schmidt and Thannhauser (1945) involves the washes: 10% TCA, 3 times; ethanol, 2 times; all at 0°C; ethanol / ether 3:1, 60°C, ether. Several authors have questioned this procedure. Marko and Butler (1957) reported that two washes with cold ethanol were insufficient

to remove all TCA, which subsequently caused degradation of DNA to apurinic acid in the hot ethanol / ether extraction. They prevented this by saturating the first ethanol wash with sodium acetate, which effectively removes the TCA. A similar conclusion was drawn for plant material by Holdgate and Goodwin (1965), who substituted two washes with 90% ethanol saturated with sodium acetate for the first ethanol wash. As an additional precaution, the hot ethanol / ether treatment was replaced by room temperature ethanol / chloroform washes, as first suggested by Berenblum, Chain and Wheatley (1939).

Part of this investigation compared the yields of nucleic acids and protein with the two methods of washing outlined here. For convenience they will be referred to as 'standard' washing (Schmidt and Thannhauser, 1945; ethanol; hot ethanol / ether) and 'Holdgate' washing (Holdgate and Goodwin 1965; sodium acetate / ethanol; ethanol / chloroform).

Extraction. Both procedures lead to an ether dry residue from which nucleic acids are extracted for measurement. Three main methods have been developed for this extraction:

The method of Ogur, Erickson, Rosen, Sax and Holden (1951) is based on a claimed difference in solubility of RNA and DNA in cold perchloric acid. Many workers have questioned the separability of DNA and RNA by this method. (Heyes 1960, Kessler and Engleberg 1962). It was not investigated here.

Schneider (1945) suggested the removal of DNA and RNA together by hot PCA. This method has the disadvantage that DNA and RNA must be measured in the same solution.

The procedure of Schmidt and Thannhauser (1945) is based on

the differential solubility of DNA and RNA in alkali. The RNA is hydrolysed to acid soluble fragments in KOH, the DNA precipitated by lowering the pH at low temperatures and subsequently hydrolysed by hot PCA.

Many authors have found neither the Schneider nor the Schmidt-Thannhauser methods as such to be completely satisfactory. Holdgate and Goodwin (1965) were unable to obtain satisfactory ultra-violet absorption spectra, orcinal colour reactions for ribose or total phosphate values without further purification. Ingle (1963) was unable to measure RNA on Schneider extracts of corn embryos. He found the Schmidt-Thannhauser extraction suitable for RNA measurement only after further purification of the KOH extract, but unsuitable for DNA estimation. De Deken-Grenson and De Deken (1959) have also found purification of KOH extracts of RNA necessary for valid RNA measurements in plant material.

In this investigation, the procedures of Schmidt and Thannhauser (1945) and of Schneider (1945) were tested on the artichoke material.

Determinations. The estimation of nucleic acids in extracts may be based on three properties: the ultra-violet absorption of the bases, colour reactions for ribose or deoxyribose and measurement of phosphate. All may suffer from errors arising from contaminating substances, and discrepancies of 100 fold between determinations by different methods on the same sample have been reported by Ingle (1963).

In this investigation, it was possible to obtain satisfactory values for RNA by ultra-violet absorption, and for DNA by

the colour reaction of deoxyribose with diphenylamine (Dische, 1930, Burton, 1956). Evans (1967) was unable to obtain realsitic phosphate determinations from the artichoke tissue. Orcinal determinations of ribose (Bial 1902) and ultra-violet absorption determination of DNA were not possible as the Schneider method of extraction was used. Confirmation of RNA and DNA values obtained was sought from polyacrylamide gel separations and microdensitometry. (Mitchell 1967).

The protein content of a tissue may be measured in several ways. Estimation of amino nitrogen is the basis of many methods, e. g. Conway (1962). These methods involve the time consuming process of converting $-NH_2$ to $(NH_4)_2SO_4$ by acid digestion. The method used here was that of Lowry, Risebrough, Farr and Randall (1957). The protein is extracted into NaOH, and measured by the colour produced by the reaction between Folin and Ciocalteau's reagent and tyrosine residues and a copper-protein complex. No other methods were tested for comparison, as this was possible from the micro-Conway measurements of Evans (1967) and histochemical determinations by Mitchell (1968) on the same tissue.

MATERIALS AND METHODS.

Tissue. Tubers which had been stored for various times were used. Thus some similarly treated experiments conducted at different times have different control values, because of changes in the nucleic acid content of the tubers during storage. Within any one experiment, all explants were drawn from the same tuber. For convenience, experiments were conducted on uncultured explants. Storage of explants between harvesting and analysis, and homogenisation of explants are described in the results section.

Washing. Two methods were used to remove contaminating materials.

1. 'Standard' washing. (Schmidt and Thannhauser, 1945) 10% TCA, 3 times; ethanol, 2 times; all at 0°C. Ethanol / ether 60°C; ether, 20°C.

2. 'Holdgate' washing. (Holdgate and Goodwin, 1965). 10% TCA, 3 times, 90% ethanol saturated with sodium acetate, 2 times; all at 0°C. 95% ethanol, ethanol / chloroform 3:1, twice, ethanol / ether 1:1, twice, ether; all at 20°C.
All washes were of 15 minutes duration. The handling of the material during washing is discussed in the results section.

Extraction. Two methods were used to extract the nucleic acids from the ether-dry tissue residue.

1. The method of Schmidt and Thannhauser (1945). 10 explants were incubated in 1 ml 0.3N KOH for 60 minutes at 37°C. After cooling to 0°C, 0.5N PCA was added to reduce the pH to

2. The precipitate was spun down and the supernatant removed for RNA determination. The precipitate was extracted with 0.5ml 0.5N PCA for 20 minutes at 70°C. The supernatant was removed and the tissue residue extracted a further three times. DNA was determined on the bulked supernatants.

2. The method of Schneider (1945). 10 explants were incubated in 1 ml 0.5N PCA for 20 minutes at 70°C. The supernatant was withdrawn, and the residue washed with 0.5ml cold 0.5N PCA. The two supernatants were combined and used for RNA measurement. The remaining residue was extracted three times with 0.5ml 0.5N PCA at 70°C. All the supernatants, including those used for RNA measurement, were combined and used for DNA measurement.

3. Protein extraction. The tissue residue from the nucleic acid extraction was placed in 1 ml N NaOH for 24 hours at 20°C. The supernatant from this was used for protein determination.

All centrifugation was at 2000 r.p.m. for 5 minutes at 0°C.

Determinations.

1. RNA was measured by the difference in ultra-violet absorption of the extract at 260 and 315 nm. Absorption spectra were obtained using the SP800 Recording Spectrophotometer or the SP500 spectrophotometer. In each case, the absorption at 235 nm was noted as a check on the amount of contamination in the RNA extract. B.D.H. yeast RNA sodium salt was used as a reference standard for the calculation of absolute quantities.

2. DNA was measured by the difference in absorption at 600 and 650 nm of the colour produced by the reaction of deoxyribose with diphenylamine, using Burton's (1956) modifi-

cation of the Dische (1930) method. 40 mm cells were used in the SP500 spectrophotometer. B.D.H. calf-thymus DNA sodium salt was used as the reference standard for the calculation of absolute amounts of DNA in extracts.

3. Protein was measured by the colour produced at 750 nm by the method of Lowry et. al. (1957). Calbiochem bovine serum albumen, grade B, was used as the reference standard.

Chemicals. All chemicals used were B.D.H., 'Analar' grade where obtainable. Highly polymerised calf-thymus DNA (Type 1) was obtained from Sigma.

Storage.	µg. RNA / explant (10 explant samples)			Mean.
A. Fresh explants	5.94	5.71	5.99	5.88
Frozen in water, at -20°C.	6.48	5.84	6.04	6.12
Frozen in 10% TCA at -20°C.	4.88	5.09	5.14	5.04
B. Fresh explants	5.00	4.93	4.93	4.95
Stored in methanol at 0°C.	5.48	4.80	5.32	5.20

Table 4.1. Yields of RNA from freshly cut explants and from explants stored for 2 weeks under various conditions. A. Explants from 4 months stored tubers. B. Explants from 9 months stored tubers.

Homogenisation	µg. RNA / explant. (10 explant samples)			Mean.
A. None	4.03	4.94	4.30	4.42
In 10% TCA, 0°C	4.87	4.58	3.99	4.48
B. None.	5.00	4.94	4.93	4.95
In 85% methanol at 0°C.	2.68	2.05	2.60	2.44

Table 4.2. Yields of RNA from explants washed and extracted with and without prior homogenisation. A. Standard washing, Schmidt-Thannhauser extraction. B. Holdgate washing, Schneider extraction.

RESULTS.

1. Storage of Explants.

Batches of 10 explants were cut and stored for 2 weeks in 2 ml water or 10% TCA at -20°C, or in 2 ml methanol at 0°C. Freshly cut explants were used as controls. The explants, which were not homogenised, were washed by the Holdgate method. RNA was extracted by the Schneider method.

Table 4.1 shows that with the exception of a slightly lower recovery of RNA after storage in TCA, the stored tissues gave similar yields to the freshly cut explants. The cold methanol method was adopted, as tissue frozen in water occasionally turned brown.

2. Homogenisation.

Samples of 10 explants were homogenised in 2 ml medium using a motor driven glass-in-glass homogeniser. The precipitate was resuspended after each step in the washing procedure, and centrifuged for 5 minutes at 2000 r.p.m. to re-pellet.

Evans (1967) using formic acid / methanol washing of artichoke tissue, and Heyes (1967) using pea root tips have observed that these tissues may be washed and extracted without prior homogenisation. To check this, entire explants were immersed for 15 minutes in each washing solution. No centrifugation was necessary.

With standard washing and Schmidt-Thannhauser extraction, homogenised and entire explants gave similar yields of RNA. (Table 4.2) The ultra-violet absorption spectra were satisfactory in both cases. The ommission of homogenisation does not

Washing procedure	'Standard'	'Holdgate'
µg. total nucleic acid ('RNA') / explant.	4.21 4.54 5.09	5.68 5.39 5.07
Mean.	4.61	5.38
DNA / explant. (Diphenylamine Optical density)	0.033 0.044 0.034	0.071 0.070 0.060
Mean.	0.037	0.067
Protein / Explant. (Lowry optical density)	0.415 0.457 0.447	0.510 0.547 0.517
Mean.	0.440	0.525

Table 4.3. Yields of total nucleic acid ('RNA'), DNA and protein per explant after washing by the standard and Holdgate procedures.

restrict the removal of contaminants absorbing in the ultra-violet. (Figure 4.1)

Homogenisation followed by Holdgate washing and Schneider extraction gave RNA yields 50% lower than the unhomogenised explants. The ethanol / chloroform washes of this treatment are so dense that centrifugation at 2000 r.p.m. is unable to sediment the precipitate. Again the ultra-violet absorption spectrum of the extract from the entire explants was satisfactory for RNA measurement.

For all subsequent investigations, washing and extraction were performed on entire explants. The ommission of homogenisation and centrifugation halves the time required. A further saving in time was effected by a rapid wash-changing system. Explants were placed in 3 ml M.S.E. polypropylene centrifuge tubes with narrow slits in the bottom. Batches of these tubes were placed in polypropylene beakers with perforated bases. Changing washing solutions then became simply a matter of lifting the beaker from a trough of one solvent, draining and placing in a trough of the next solvent. This made it possible to change washing solutions for 100 samples in a few seconds, and allow ed simple agitation of the explants during each wash, by raising and lowering the beakers.

3. Washing.

Batches of 10 explants were washed by the standard or Holdgate procedures. Nucleic acids were extracted by the Schneider method, and protein by NaOH for 24 hours.

Washing by the standard procedure gave DNA recoveries 45% lower than the Holdgate washing method. (Table 4.3) These

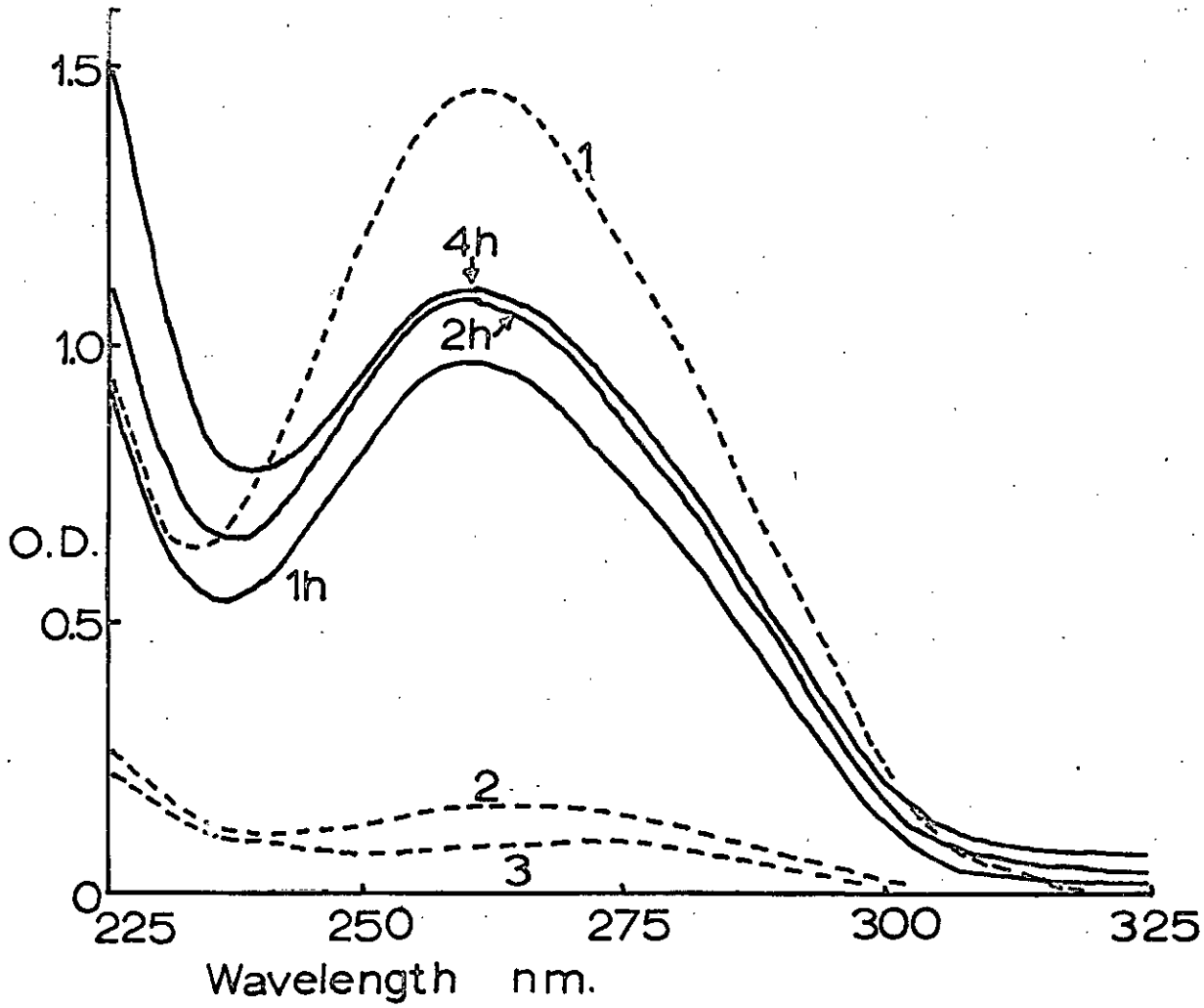


FIGURE 4.1 Ultra-violet absorption spectra of extracts from ether-dry residues of 10 explants by KOH for 1, 2 and 4 hours, (———), and by three successive PCA extractions (-----).

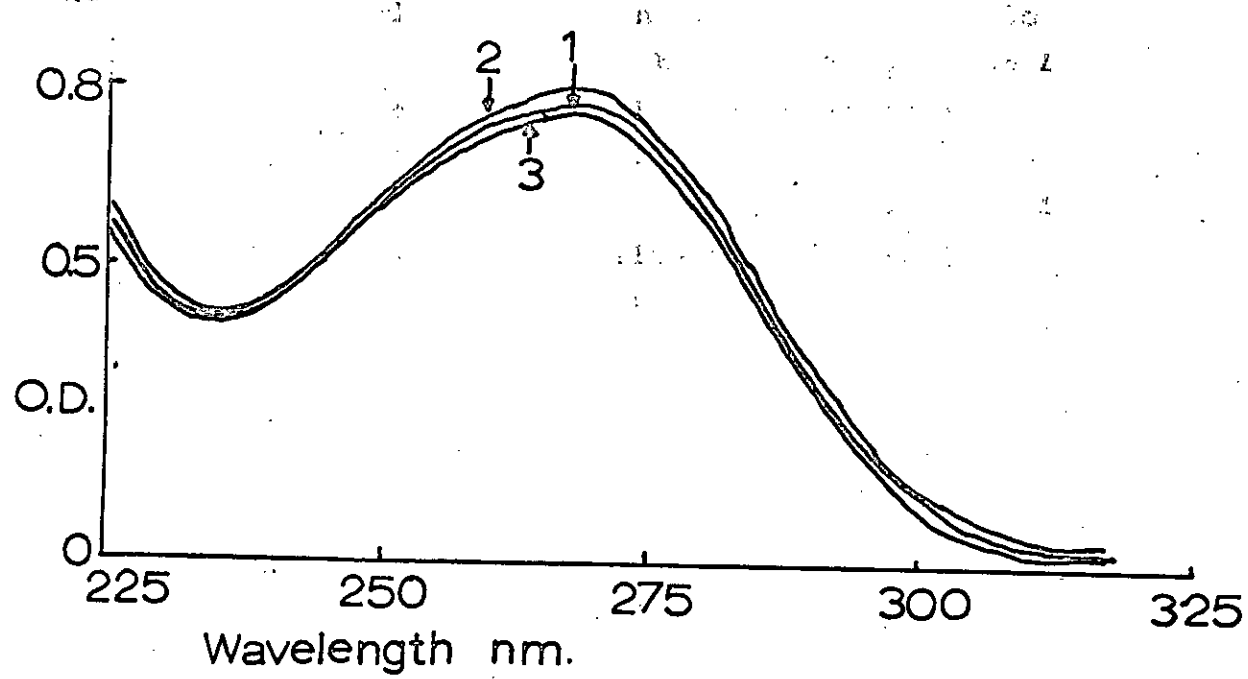


FIGURE 4.2 Ultra-violet absorption spectra of calf thymus DNA after incubation in PCA for 20 (1), 40 (2) and 60 (3) minutes.

findings are similar to those of Marko and Butler (1951), who found a loss of DNA caused by TCA persisting into the hot ethanol / ether extraction of the standard washing procedure. Holdgate and Goodwin (1965) have drawn a similar conclusion for rye tissue.

For most experiments reported below, and for experiments in Section 3, washing was by the holdgate method.

4. Extraction of RNA or total nucleic acid.

A. The procedure of Schmidt and Thannhauser (1945). This involves the extraction of RNA without DNA by KOH. The strength of alkali, time of extraction and temperature have been subject to considerable variation. (See review by Hutchison and Munro, 1961). Holdgate and Goodwin (1965) recommended 16 hours at 37°C with 0.3N KOH for rye tissue.

Figure 4.1 shows ultra-violet absorption spectra obtained by incubating 10 explants in 0.3N KOH for 1, 2 and 4 hours at 37°C. Incubation for more than 1 hour did not increase the amount of RNA extracted, but did increase the extraction of contaminants absorbing in the ultra-violet, as shown by the decreased A_{260}/A_{235} ration (Table 4.5).

B. The Schneider (1945) procedure. DNA and RNA are extracted together by hot PCA. The acid strength, time of incubation and temperature have been varied by many workers. (Hutchison and Munro, 1961).

Figure 4.1 shows absorption spectra of successive extracts of 10 explants with 1 ml 0.5N PCA for 20 minutes at 70°C At least 90% of the total nucleic acid was extracted by the first treatment. The increase in the wavelength of maximum

µg RNA / Explant extracted by KOH, 0.3N, 1 hr., 37°C.	µg total nucleic acid / explant extracted by PCA, 0.5N, 20 min., 70°C
5.48	5.68
4.80	5.39
5.32	5.07
Mean 5.20	Mean 5.38

Table 4.4. RNA or total nucleic acid per explant extracted by the Schmidt-Thannhauser or the Schneider procedure.

Spectrum of	A_{260}/A_{235}	A_{315}/A_{260}
B.D.H. yeast RNA, KOH hydrolysed	2.27	0
Artichoke RNA, KOH extracted for 1 hour	1.89	0.02
2 hours	1.74	0.04
3 hours	1.46	0.07
Artichoke RNA, PCA extracted for 20 mins.	2.37	0.02
60 mins.	1.80	0.03

Table 4.5. A_{260}/A_{235} and A_{315}/A_{260} ratios calculated from ultra-violet absorption spectra of yeast RNA and artichoke RNA extracted by the Schmidt-Thannhauser or the Schneider procedure for various times.

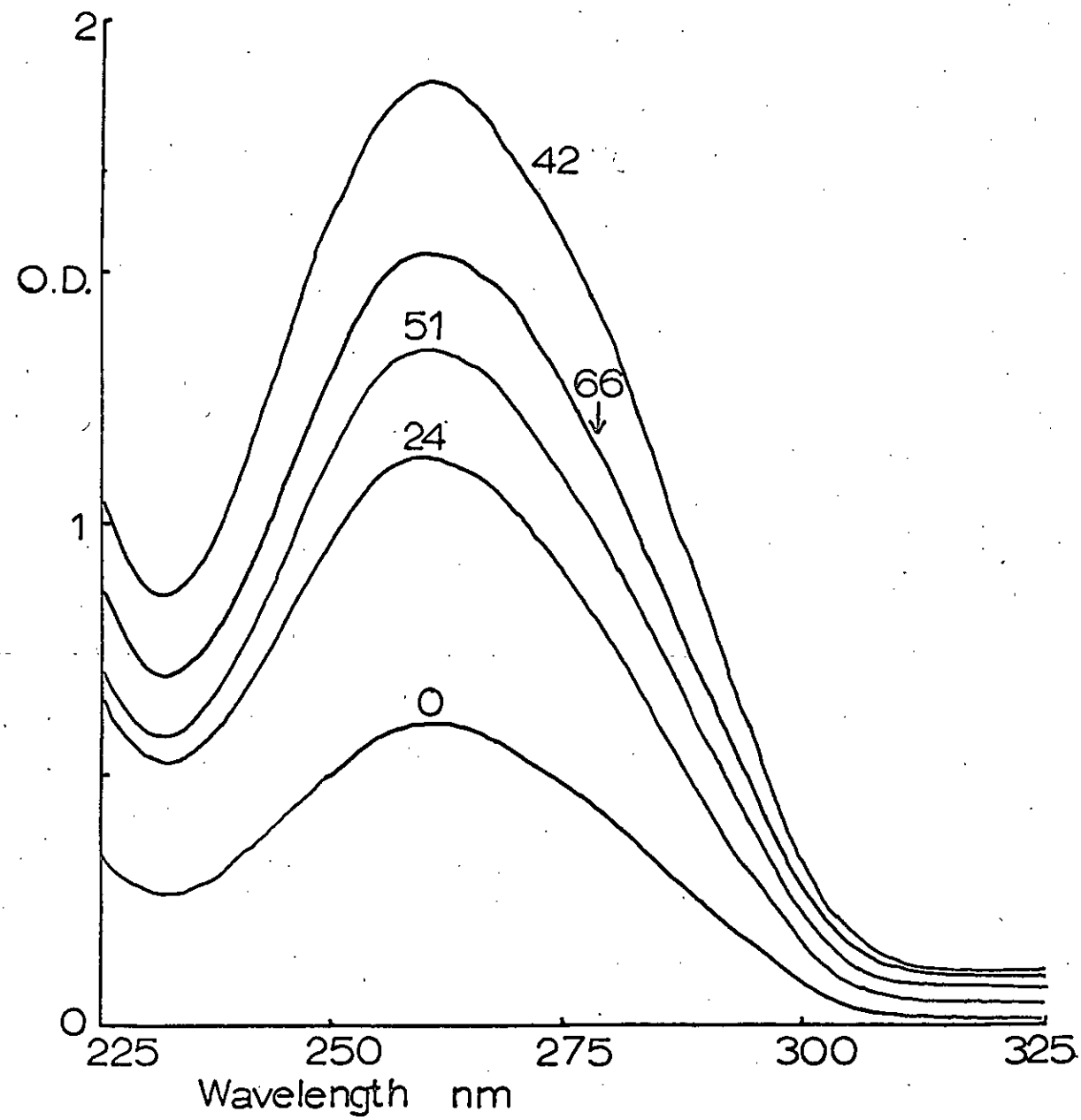


FIGURE 4.3 Ultra-violet absorption spectra of PCA extracts of total nucleic acid from explants cultured for 0, 24, 42, 51 and 66 hours.

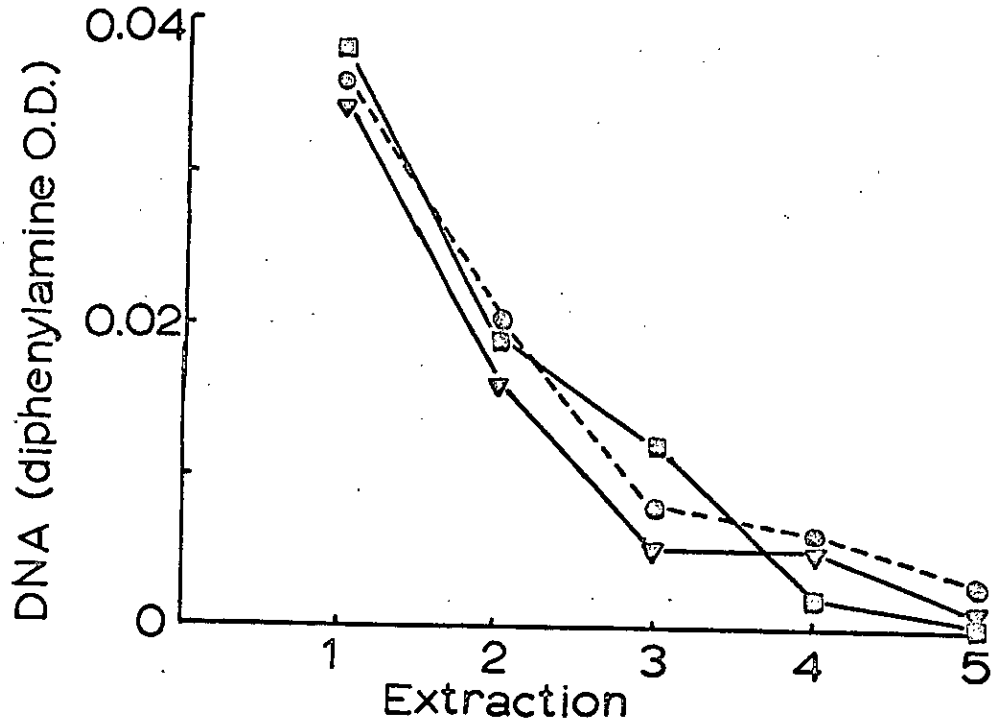


FIGURE 4.4 DNA (A_{600}-A_{650} of diphenylamine colour) in successive PCA extracts of three samples each of 10 explants.

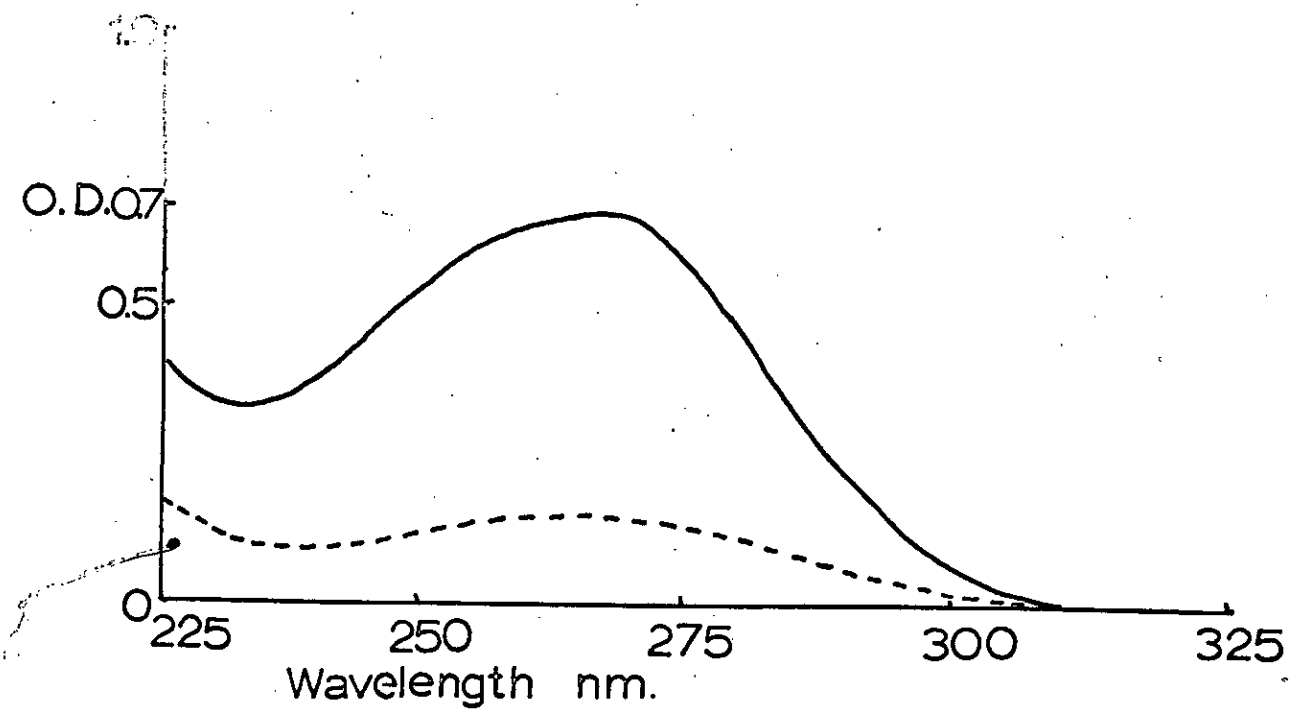


FIGURE 4.5 Ultra-violet absorption spectra of extracts of calf thymus DNA by the Schmidt-Thannhauser procedure. ---- acidified supernatant of KOH extract. —— hot PCA extract of material precipitated by acidification of KOH extract.

absorption in extraction 3 suggests that material other than RNA, probably polypeptide, was being extracted.

C. Comparison of the Schmidt-Thannhauser and Schneider extraction methods. Table 4.4. shows that both methods of extraction gave similar yields of RNA or total nucleic acid. (The absorption spectra of extracts by the two methods in Figure 4.1 provide no measure of absolute amounts extracted.) Table 4.5 compares the ultra-violet absorption spectra of the extracts from the two methods, and from purified yeast RNA as standard. The Schneider procedure extracted the nucleic acid with less contamination of the absorption spectrum, as determined by the A_{260}/A_{235} and A_{315}/A_{260} ratios, than the extraction of RNA by the Schmidt-Thannhauser procedure. Prolonged PCA extraction also resulted in increased contamination.

D. Effects of culturing the explants on spectrum quality. To check for any increase in the contamination of ultra-violet absorption spectra of nucleic acids during the growth of explants, total nucleic acid was extracted by the Schneider procedure from explants grown for 24, 42, 51 and 66 hours. The absorption spectra in figure 4.3 show that no increase in spectral contamination occurred during culture.

5. Extraction of DNA.

A. Schneider Extraction. Figure 4.4 shows the amounts of DNA extracted by successive incubations with 1 ml aliquots of 0.5N PCA at 70°C for 20 minutes. 4 extractions were necessary to extract all the DNA from the tissue.

B. Check for interference from RNA. The diphenylamine method will also produce colours with certain other sugars.

µg calf thymus DNA	µg yeast RNA	Diphenylamine colour
20	0	0.066
40	0	0.135
20	30	0.077
40	10	0.137
0	50	0.008

Table 4.6. Diphenylamine colours ($A_{600}-A_{650}$) produced by mixtures of yeast RNA and calf thymus DNA after hydrolysis in 0.5N PCA for 30 minutes at 70°C.

DNA / Explant Schmidt-Thannhauser extraction.	DNA / Explant Schneider extraction.
0.046	0.060
0.045	0.063
0.048	0.065
0.044	0.063
Mean 0.046	0.063

Table 4.7. Yields of DNA / explant ($A_{600}-A_{650}$ of diphenylamine colour) from explants extracted by the Schmidt-Thannhauser and Schneider procedures.

(Burton, 1956). To check that the ribose of the RNA present in the Schneider extract was not interfering, mixtures of yeast RNA and calf thymus DNA were hydrolysed with PCA and tested with diphenylamine.

Table 4.6 shows that RNA did slightly increase the colour, and that RNA alone gave a very slight reaction. The effect is too small to produce any significant error in DNA measurements. The possibility that the yeast RNA may have contained some DNA must also be allowed for. Burton (1956) reported that ribose or RNA gave less than 0.2% of the colour given by an equal weight of DNA.

C. Check for degradation of DNA. Burton (1956) reported that incubation of DNA in PCA for 60 minutes lead to a drop in the colour intensity of 14%. While it would have been desirable to check this on artichoke DNA, by measuring the diphenylamine colour produced by an extract after various times of incubation, the removal of all solublised DNA at 20 minute intervals was likely to keep degradation to insignificant proportions. The ultra-violet absorption spectra in Figure 4.2 do not show any change in absorbance with time of incubation of calf thymus DNA in PCA at 70°C.

D. Schmidt-Thannhauser extraction. A sample of Sigma highly polymerised calf thymus DNA was extracted by the Schmidt-Thannhauser procedure. Figure 4.5 shows the ultra-violet absorption spectra of the KOH incubation supernatant after addition of PCA to pH2, and of the PCA digest of the precipitate. The presence of considerable ultra-violet absorbing material in the supernatant from the KOH digest suggests incomplete precipitation of DNA by cold PCA. Ingle (1963), working with corn

embryos, concluded that a portion of the DNA became cold acid soluble during KOH extraction.

E. Comparison of Schmidt-Thannhauser and Schneider extraction procedures for DNA. Table 4.7 compares DNA yields from explants extracted by the two procedures. The yield of DNA was lower with Schmidt-Thannhauser extraction, consistent with the partial failure of DNA to precipitate after KOH extraction of RNA.

6. Purity of RNA and DNA reference standards.

The percentage purity of each standard was estimated by comparison of the absorbance of a solution of known concentration (X) with the absorbance calculated for a 100% pure sample (Y). Percentage purity was calculated as X/Y x 100%. Calculations of theoretical absorbances were made on the basis of appropriate solutions of mononucleotides, by the method of Chargaff and Zamenhof (1948), who defined:

$$E(P) = A/C.d$$

E(P) is the absorbance of a nucleic acid solution containing 1 gm atom of phosphorous / litre. A is the absorbance of the sample, C is the concentration in gm atoms of phosphorous / litre, and d is the internal light path of the cell in cm, in this case 1. Therefore the theoretical absorbance, A = E(P).C Percentage phosphorous values were calculated from the molecular formulae of sodium salts of DNA and RNA, assuming an equal frequency of bases. The E(P) value for DNA as mononucleotides was obtained from Beaven, Holiday and Johnson, (1955). The E(P) for RNA as mononucleotides was calculated from molar absorbtivities of individual nucleotides (Beaven,

	RNA	DNA
Observed values.		
concentration ug/ml	31	20
Absorbance at 260 nm	0.86	0.48
Calculated values.		
E(P)	12400	10000
% phosphorous	9.3	9.7
Absorbance at 260 nm	1.14	0.63
% purity of sample	75	77

Table 4.8. Observed absorbances, calculated absorbances and derived percentage purities of DNA and RNA reference standards.

Determination by	RNA	DNA	RNA/DNA
Polyacrylamide gel	306	41	7.5
electrophoresis	274	40	6.9
(Arbitrary units)	334	44	7.6
		Mean	7.3
Chemical	7.5	0.94	8.0
measurement.	8.5	1.10	7.7
(µg / explant)	6.8	0.98	7.0
		Mean	7.6

Table 4.9. DNA and RNA per explant and RNA/DNA ratios determined by chemical measurement and electrophoresis methods for freshly cut explants.

Holiday and Johnson, 1955). All values were correct for pH 1.5 solutions.

Measurements of actual absorbances were made on samples hydrolysed in 0.5N PCA for 20 minutes at 70°C.

The calculated percentage purity figures (Table 4.8) indicate that the DNA and RNA reference standards contained 25% by weight foreign matter, probable salt and absorbed moisture. In confirmation of these results, the B.D.H. Biochemicals Catalogue, 1968, quotes the percentage phosphorous of both standards as 'about 7%', compared with the theoretical values of over 9%.

Thus all absolute values for RNA and DNA quoted in this thesis are 25% too high. Relative differences in DNA or RNA content, and RNA/DNA ratios are not affected.

7. RNA/DNA ratios.

Table 4.9 compares RNA/DNA ratios derived by two methods. Chemical measurements were made on freshly cut explants, using Holdgate washing and Schneider extraction. RNA and DNA amounts were also calculated from optical peak areas of scans of polyacrylamide gel electrophoresis separations of nucleic acids from uncultured explants (Section 3.) 5% was added to the RNA measurements to allow roughly for the polydisperse messenger type RNA not measured in the gel peaks.

There is close agreement between the two methods on a RNA/DNA ratio of 7 to 8. Mitchell (1967a) has arrived at a ratio of 9 from measurements by microdensitometry on gallocyanin stained sections of this tissue.

A. Protein extracted by 30 minutes in N NaOH.	Protein extracted by a further 18 hr. in N NaOH	Total Protein extracted.
0.097	0.222	0.319
0.080	0.209	0.289
0.096	0.200	0.296
0.112	0.156	0.268
0.114	0.187	0.301
Mean 0.100	0.195	0.295

B. Protein extracted by 24 hours in N NaOH.	Protein extracted by a further 24 hours in N NaOH.	Total protein extracted.
0.510	0	0.510
0.547	0	0.547
0.517	0	0.517
0.462	0	0.462
0.515	0	0.515
0.509	0	0.509
Mean 0.510	0	0.510

Table 4.10. Yields of protein (Colour at 750nm in the Lowry reaction) per explant after various times of extraction by N NaOH. A. Standard washing, Schmidt-Thannhauser extraction. B. Holdgate washing, Schneider extraction.

Protein / explant. Schmidt-Thannhauser extraction.	Protein / explant Schneider extraction.	
0.231	0.217	0.291
0.174	0.219	0.264
0.149	0.283	0.245
0.163	0.238	0.257
0.153	0.279	0.270
Mean 0.174	0.256	

Table 4.11. Yields of protein (Colour at 750 nm in the Lowry reaction) per explant after extraction of nucleic acids by the Schmidt-Thannhauser and Schneider procedures. Protein extracted by N NaOH for 24 hours.

8. Extraction of protein.

A. Extraction time. After extraction of nucleic acids, protein was extracted by N NaOH. A brief investigation of the time required for protein extraction is reported in Table 4.10. 24 hours at room temperature extracted all alkali soluble protein. It is not known if this long exposure to NaOH has any degrading action on the protein.

B. Nucleic acid extraction and protein yield. Protein was extracted for 24 hours from explants from which the nucleic acids had been removed by either the Schneider or the Schmidt-Thannhauser procedure. Yields after Schmidt-Thannhauser extraction were lower than after Schneider extraction (Table 4.11) This may be due to loss of protein during the KOH extraction of RNA. This interpretation is consistent with the more contaminated ultra-violet absorption spectra obtained by KOH extraction.

DISCUSSION.

The suitability of storage in methanol, the ommission of homogenisation and the rapid method developed for washing explants fulfil the first two criteria of the method required in the introduction, those of storage and rapid processing of large numbers of samples.

Washing. The standard method of washing to remove contaminants (Schmidt and Thannhauser, 1945) was shown to be less satisfactory than that used by Holdgate and Goodwin (1969). The standard method gave 40% lower yields of DNA than the Holdgate method. This was probable a result of the degradation of DNA to apurinic acid (Marko and Butler, 1957) or to acid soluble fragments by TCA persisting into the hot ethanol / ether wash. Hot TCA will also hydrolyse RNA to acid soluble fragments (Schneider, 1945) which could account for the 15% lower yields of RNA with the standard washing method.

It is, of course, impossible to claim from this evidence that the Holdgate washing procedure does not also involve losses of nucleic acids and proteins. Some check of this could be made by subjecting known amounts of pure nucleic acids and proteins to the washing procedure.

The other possibility for error lies in the inefficient extraction of contaminating materials. Holdgate and Goodwin (1965) reported that no further phosphorous was extracted by a fourth TCA was, and that all lipid phosphorous was extracted by their lipid removal procedure. While the former is a good indication that all mononucleotides have been removed, elim-

inating a possible source of error in the estimation of RNA from 'clean' ultra-violet absorption spectra, they note the possibility of previously insoluble carbohydrates breaking down during hot acid extraction of nucleic acids and interfering with the sugar colour reactions. Plant cell walls contain polymers of galacturonic acid, arabinose and xylulose. Burton (1956) reported that the last two were interfering substances in the diphenylamine reaction. However, for reasons discussed below, the measurement of DNA by diphenylamine in this tissue is thought to be accurate, and does not appear to suffer from interfering substances.

Since none of the determinations used in this investigation depended on phosphate content, it may not have been necessary to remove phospholipids by organic solvent washing. Schneider (1945) noted that ommission of lipid solvent treatment did not affect DNA and RNA measurement in rat liver and brain when the determinations were by colour reactions for ribose and doexyribose. Fleck and Munro (1961) reached similar conclusions for ultra-violet absorption determinations of RNA in rat liver. The lipid extraction procedure was retained here for two reasons, to remove TCA, which absorbs in the ultra-violet, and to remove pigments should they arise during growth. Explants have been observed to turn brown or red if grown in strong light or medium without 2,4-D. These colours were partly removed by the lipid extraction procedure, and do lead to a deterioration in the quality of the ultra-violet absorption spectrum of the Schneider extract.

Since this work was commenced, Evans (1967) has obtained satisfactory results from a washing procedure involving methanol

and formic acid. (Kupila, Bryan and Stern, 1961). Comparisons of yields are difficult, as Evans does not quote absolute values, but changes during culture are of similar size.

Extraction and Measurement. Neither the Schneider (1945) nor the Schmidt-Thannhauser (1945) methods gave completely satisfactory extractions of RNA and DNA. The Schmidt-Thannhauser method was unsuitable for DNA measurement, as some DNA was lost in the extraction of RNA. The ultra-violet absorption spectrum of the RNA extract was more contaminated than that obtained by the Schneider method, probably by polypeptide material. The Schmidt-Thannhauser method also gave lower yields of protein than the Schneider method.

While the Schneider method hydrolysed both DNA and RNA, the ultra-violet absorption spectrum of the first PCA extract is considered to give a good measure of the RNA content for the following reasons:

Firstly, it was observed that the first PCA extraction removed at least 90% of the total nucleic acid (Figure 4.1), but only 40% of the DNA. The DNA content of this tissue represents about 13% of the total nucleic acid (Table 4.9). Thus the first PCA extract contained only a few percent of DNA, insufficient to cause any significant error in the RNA value.

Secondly, the spectra obtained were reasonable free from contaminating materials, with A_{260}/A_{235} ratios of 2.1 to 2.4. Confirmation of RNA values was not sought from the orcinal colour reaction for ribose. (Bial, 1902, Dische and Schwarze, 1937) for various reasons. It was assumed that RNA determinations from clean ultra-violet spectra were accurate. Various authors, in-

cluding Holdgate and Goodwin (1965) and De Deken-Grenson and De Deken (1959) have found plant material especially productive of orcinal-reacting impurities in the RNA extract.

In an attempt to correct for contaminating material in the ultra-violet spectrum, the absorbance at 315 nm was subtracted from the absorbance at 260 nm. Among authors who have tried to correct for contaminating material by taking the difference between absorbances at two wavelengths as the measure of RNA concentration are Warburg and Christian (1942) and Tsanev and Markov (1960). The method requires an exact knowledge of the absorption spectrum of the contaminant for accuracy.

It is fortunate that the artichoke yields RNA extracts suitable for ultra-violet spectroscopy without further purification. Steward et. al. (1964) have obtained RNA extracts with satisfactory ultra-violet spectra from cultured carrot root cells. These findings contrast with reports of highly contaminated RNA extracts from germinating and seedling plants (Ingle, 1963, Holdgate and Goodwin, 1965)

The diphenylamine determination of DNA content could not be checked directly on the extract, as the presence of RNA prevented phosphate and ultra-violet absorption determinations. In view of the similarity of RNA/DNA ratios derived from chemical measurement, polyacrylamide gels and microdensitometry (Mitchell, 1967a), the measurement of DNA by diphenylamine is considered to be reasonably accurate. Although diphenylamine will react with many other substances (Dische, 1955, Burton, 1956) the colours produced are often different from the blue produced with deoxyribose. Reading the absorbance at two wavelengths helps to eliminate errors from spurious colours.

Although developed on freshly cut explants, these methods for RNA and DNA determination are also applicable to cultured material. RNA spectra remained satisfactory over long periods of culture. (Figure 4.3) No increase in non-blue colours with diphenylamine was noted with cultured explants. The DNA quantities determined by the diphenylamine method for growing tissue in Section 3 were generally in good agreement with the amounts of DNA synthesis expected from cell division data.

The investigation into protein measurement reported here leaves certain questions unanswered, notable the possibility of degradation of protein by long exposure to alkali, and the accuracy of the Lowry technique compared with methods for measuring amino nitrogen. It was hoped to check the results against data from the micro-Conway method by Evans (1967), but no absolute values are available for these determinations. Changes in protein content observed during the growth of explants correspond in timing and magnitude to those described by Evans (1967) and also to those found by Mitchell (1968) using histochemical techniques.

The absorption spectra obtained by repeated PCA extraction of nucleic acids (Figure 4.1) show maximum absorption at wavelengths greater than 260 nm. It is probable that this was polypeptide. Thus only the hot-PCA insoluble, alkali soluble fraction of the total protein was measured by the Lowry method. But in view of the agreement with the results of Evans (1967) and Mitchell (1968) for protein changes during growth, the Lowry method provides a reasonably reliable measure of protein content with the virtues of speed and simplicity.

It will be seen from the values quoted for individual determinations of DNA, RNA and protein in this section that most values were within 5% of the mean, and almost all within 10%. The methods selected and developed by this investigation satisfy the four requirements detailed in the introduction, by providing rapid and accurate determinations of the RNA, DNA and protein contents of large numbers of samples.

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Effect of Light on Cell Division in Plant Tissue Cultures

LIGHT strongly influences many aspects of growth in plants. There have, however, been few studies on the effects of light on cell division in non-green plant tissue cultures. In the course of investigating the physiology of cell division in developing callus cultures of *Helianthus tuberosus* it has been observed that light can have an inhibitory effect on cell division.

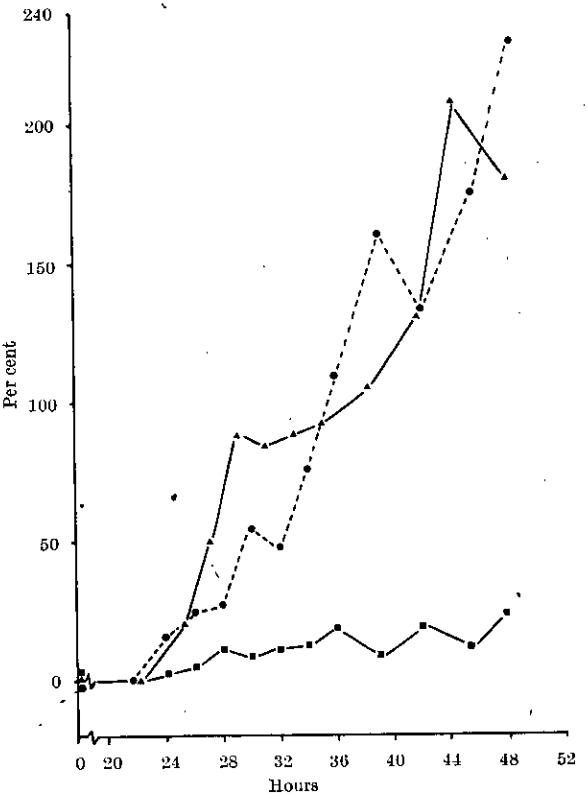

Fig. 1. Percentage increase in cell number per explant with time of incubation. Explants were removed from tubers in green light and cultured in groups of 80 in 12 ml. medium containing 10^{-5} molar 2,4-D, in 9 cm Petri dishes, at 25° C. Agitation was on a reciprocating shaker operating at 50 c/s with a 7 cm displacement. The explants were grown in total darkness (▲), 120 ft.-candles (●) and 450 ft.-candles (■). Both light sources were mixed fluorescent and tungsten. Cell number data were derived from counts on 5 per cent chromic acid macerates of five explant samples.

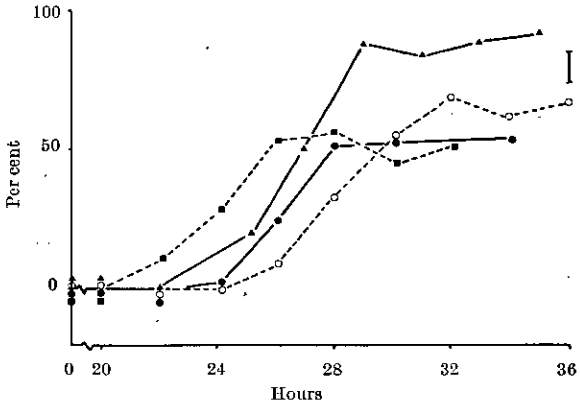

Fig. 2. Percentage increase in cell number per explant with time of incubation. Explants were prepared and cultured as described in Fig 1. For the first 30 min after excision, the explants were exposed to total darkness (▲), 18 ft.-candles fluorescent light (○) and 120 ft.-candles (●) and 450 ft.-candles (■), mixed fluorescent and tungsten light. Subsequent culture was in total darkness. The vertical line represents the *S.E.* of the difference between the dark and light treatments.

Observations[1–3] have shown the course of change in ce number with time in explants taken from tubers of th Jerusalem artichoke and cultured in contact with medium containing 20 per cent coconut milk and 10 molar, 2,4-dichlorophenoxyacetic acid. It has also bee shown[2,3] that the first few divisions are synchronous These observations were made with an experimenta procedure which involved exposure of the tubers and c the excised explants for periods of 30–90 min to abou 18 ft.-candles from a fluorescent source, and exposure t the light from a tungsten lamp when the cultures wer being inspected. Subsequently it has been found that th proportion of cells in the explant that divide at the firs synchronous division increases from 45 to 90 per cent whe the explant is removed in low intensity green light an then cultured in total darkness on a medium withou coconut milk but in the presence of 2: 4-dichlorophenoxy acetic acid (2,4-D) at 10^{-5} moles/l.

Results of two experiments designed to show the ligh effect in division are given in Figs. 1 and 2. The explant were obtained with techniques described elsewhere[1], an grown in bulk culture in a liquid medium.

In the first experiment (Fig. 1), the explants remove in low intensity green light were exposed throughout th period of culture to two intensities of light, and thei growth compared with a control in total darkness. In th second experiment (Fig. 2), the explants were exposed t various light intensities for a period of 30 min afte excision. They were then cultured in total darkness.

It is clear from the results presented in Fig. 1 that the number of cells formed at the first division is significantly greater in the dark than in the light. Continuous light therefore has a strongly depressing effect on cell division.

Preliminary exposure for a short period after excision has a sharply depressing effect on division at all light intensities (Fig. 2). The explants excised in green light and cultured in total darkness form considerably more cells at the first synchronous division than do those exposed to light for the first 30 min after excision. Clearly radiation other than dim green light has a strongly depressive effect on division in this system and most of the effect is exerted in the period immediately after excision. It may be noted that the synchronous pattern is displayed in all conditions in this experimental series.

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