CHANGES IN THE PATTERN OF ENZYME
ACTIVITIES DURING THE CELL DIVISION CYCLE

by

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SUMMARY

The initial aim of this investigation was to examine the patterns of activity for a number of different enzymes during the cell division cycle in a higher plant tissue. The system employed in this study was cultured tissue isolated from the tubers of the Jerusalem artichoke (Helianthus tuberosus. L.). Explants cultured in the presence of a mineral salts medium containing sucrose and the synthetic auxin 2,4-dichlorophenoxyacetic acid (2,4-D) exhibit a cell population which divides synchronously for two or three divisions. For cell cycle experiments, all cultures were prepared in low intensity green light and grown in liquid culture in darkness, in order to obtain the maximum percentage cell division.

The enzymes chosen for this study were all associated with deoxyribonucleic acid (DNA) metabolism and included enzymes of biosynthesis and degradation. Prior to investigations of enzyme patterns during the cell division cycle, quantitatively valid assay methods were established for each individual enzyme.

Cell cycle experiments were confined to the first synchronous cell division of the artichoke system and the activity of each enzyme was measured with particular reference to the onset and extent of DNA synthesis as indicated by the rate of incorporation of labelled thymidine into DNA. Characteristic increases in the levels of DNA, total nucleic acid, acid resistant protein and cell number during the cell cycle, confirmed that the system was behaving as reported by previous workers.

The activities of two deoxyribonuclease enzymes with substrate affinities for native and denatured DNA respectively, and the suppressed activity of thymidine monophosphate phosphatase, showed characteristic patterns during the culture period which were not associated with cell division but were a property of freshly excised tissue in culture. The biosynthetic enzymes DNA polymerase, thymidine (TdR) kinase and thymidine monophosphate (dTMP) kinase however, showed characteristic patterns of activity associated with cell division and in each case the first major increase in enzyme activity was coincident with, or subsequent to the onset of DNA synthesis.

The increased activities of TdR and dTMP kinases during 'S' were neither due to activation of pre-existing enzyme nor to the removal of inhibitors. FUdR, an inhibitor of DNA synthesis prevented these enzyme increases during 'S', which suggests that the increases occur as a result of DNA synthesis.

The results of these investigations have shown that the artichoke system is suitable for an extensive examination of enzyme patterns during the cell cycle.

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CHAPTER 1

INTRODUCTION

During recent years our knowledge of metabolic events which take place during the cell division cycle has been considerably enriched as a result of investigations with numerous synchronously dividing systems. For cell cycle studies, any degree of synchrony in a system immediately bestows upon it untold advantages when compared with asynchronous systems, as a large amount of material at the same 'stage' of division at any time during the cell cycle, is provided. This in effect amplifies changes taking place at the cellular level so that they become characteristic of a tissue mass and this situation is ideal for a study of the cytological and physiological changes, which take place in the life of a cell as it proceeds from one division to the next.

When a cell divides there is a need for energy, replication of organelles and the synthesis and accumulation of various macro-molecules, including deoxyribonucleic acid (DNA), ribonucleic acid (RNA), and protein. In the majority of plant and animal cells, increases in the levels of such macromolecules are confined to definite periods of the cell cycle and are therefore described as periodic. In view of these observations, many workers have suggested that the control mechanisms for these and other changes, might be mediated via periodic increases in the activities of the various enzymes controlling these processes.

Recently, the patterns of activity of many different enzymes during the cell division cycle, have been described for a large number of bacterial, animal and lower plant synchronous systems.

Before describing the higher plant synchronous system which was employed in this investigation, it is important to consider other systems for which enzyme data is available in relation to the various ways in which synchrony was induced, as such a consideration aids the interpretation of enzyme results with respect to the timing of the events of the cell division cycle. A list of the enzymes which have been assayed in the following systems and the appropriate references, has been compiled in Appendix 1.

There are two important bacterial systems which employ the species Escherichia coli and Bacillus subtilis. In bacteria, the induction of synchrony involves preparation of a stationary culture, inoculation of filtered cells into fresh medium, followed by incubation at 37°C on a shaker (Nagata 1963). Under these conditions the mean generation time for B. subtilis is only 70 minutes. The bacterial cell cycle differs markedly from that of the majority of organisms in that the 'S' period, during which DNA synthesis takes place, occupies over 70% of the cell cycle.

The concept of inducibility or potential for synthesis of enzymes, applies to bacteria and to certain members of the plant kingdom including yeasts, algae and higher plants. In these organisms, enzymes which are always present in the cell are known as constitutive, whilst inducible enzymes can be produced only when the need arises. There are various mechanisms of induction (or derepression). For instance an enzyme can be induced by the presence of its substrate. When enzyme appearance is caused by removal of a repressor substance (often the product of reaction)

then enzyme synthesis has been 'derepressed'.   In the bacterial
system which employs B. subtilis, an abundance of genetic data
derived from the use of mutants in enzyme studies, has enabled
close correlations to be made between patterns of enzyme increase
during the cell cycle and genetic maps (Masters and Pardee 1965).

Two different types of experimental synchronous system exist
for animal cells.   The first of these includes suspension cultures
of unicellular organisms, of which the best known is the protozoan
Tetrahymena pyriformis.   Although this organism has been used
extensively for cell cycle studies, no enzyme data is available.

The second type of animal system includes tissue cultures
of various mammalian cell types.   The regenerating rat liver system
was much used by early workers although recently, as better systems
have become available it has been found that this system is less
satisfactory since less than 10% of the cells take part in the
burst of cell division following partial hepatectomy.   In this
system, enzymes involved in DNA synthesis are present in greater
amounts than in normal liver and these enzymes gradually decrease
to their normal levels on the cessation of DNA synthesis (Bollum
and Potter 1959).   A similar system exists which employs rabbit
kidney cells (Lieberman et al. 1963) and in both of these systems
the mean generation time exceeds 70 hours.

Many of the remaining animal systems rely on the use of a
variety of potent metabolic inhibitors for the induction of
synchrony.   These systems may be less reliable than systems
synchronized by other methods, because of possible harmful effects
on the cell population.

The compound 5-fluoro,2-deoxyuridine is supposedly a specific inhibitor of DNA synthesis, as it prevents the enzymic formation of thymidine monophosphate (dTMP).   This inhibition can be reversed by supplying thymidine (TdR) which becomes converted to dTMP by the enzyme thymidine kinase.   This property has been used by Littlefield et al. (1963) to induce synchrony in mouse fibroblasts (L cells).   Firstly, FUdR is added to the culture to halt DNA synthesis.   When TdR is added 16 hours later, DNA synthesis occurs for 6 - 8 hours followed by a partially synchronous cell division.

A second method used to induce synchrony in cultures of L cells (Turner et al. 1968) and Hela cells (Stubblefield and Mueller 1965) makes use of the nucleic acid synthesis inhibitor, amethopterin (methotrexate).   Due to its similarity in structure to folic acid, amethopterin interferes with the folic acid reductase reactions essential to the formation of the pyrimidine ring structure (Porter and Wiltshaw 1962).   Hence the unavailability of thymine residues causes DNA synthesis to cease.   Reversal occurs as in the previous system, by transferring the culture 16 hours later to a fresh medium, in which amethopterin is replaced by thymidine.   The mean generation time for systems synchronized with FUdR or amethopterin is in the region of 20 hours for L cells and 12 hours for Hela cells, but as DNA synthesis begins immediately after TdR reversal there is no well defined G_1 (or pre-'S') period associated with the first division cycle.

For cultures of Don C chinese hamster fibroblasts, the induction of synchrony often employs the inhibitor colcemid which has the effect of blocking cells in metaphase. After a culture in the exponential phase of growth has been treated with colcemid for 2½ hours, the cells are washed with fresh medium and DNA synthesis begins after a lag phase of 2 hours (Stubblefield and Murphree 1967). Using this method the mean generation time is in the region of 12 hours.

A second method of inducing synchrony in Hela cell cultures which has the advantage that it does not involve the use of metabolic inhibitors, involves the preferential detachment of mitotic cells from monolayer cultures in the presence of a calcium deficient medium. This technique relies upon the inability of cells during mitosis to adhere to a glass surface.

Synchronous systems of plant cells are numerous and employ a wide variety of different organisms. Synchronous cultures of the myxomycete Physarum polycephalum have been used extensively for cell cycle studies (e.g. Sachsenmaier and Ives 1965). Synchrony is achieved by the transfer of a single micro-plasmodium from a submerged culture to a surface culture. The first mitosis occurs 5-7 hours after fusion of the microplasmodia and subsequent mitoses occur at intervals of 9-12 hours. For enzyme experiments, the interval between the second and third mitoses was used as the experimental period. An unusual characteristic of this system is the absence of a well defined G_1 (or pre-'S') phase, and the

existence of a prolonged G_2 phase, so that the events of mitosis are not well separated from the onset of subsequent DNA synthesis.

There are several important synchronous systems which employ species of yeasts (ascomycetes). In cultures of Saccharomyces cerevisiae, synchrony is achieved by starvation of the cells for 10 days followed by resuspension in a succinate and mineral salts medium at $25^{\circ}C$ (Gorman et al. 1964). The mean generation time for all but the first division cycle is approximately 200 minutes. In this and other species of Saccharomyces the abundance of genetic data derived from the use of mutants in enzyme experiments has led to important correlations for eucaryotic cells, between enzyme changes during the cell cycle and replication of the genome (Tauro and Halvorson 1966).

In cultures of the fission yeast Schizosaccharomyces pombe synchrony is achieved by sedimentation of cells by centrifugation in a linear glucose gradient (Mitchison and Creanor 1969). This method relies upon a relationship between the size of the cells and their 'stage' with respect to the cell cycle. For this species of yeast, the absence of genetic data has prevented inter-pretation of enzyme results with respect to replication of the genome.

Cultures of the green photosynthetic algae Chlorella pyrenoidosa and Euglena gracilis, are synchronized by alternating light and dark periods. Both systems have a mean generation time of about 14 hours and have been used by a number of different workers for enzyme studies on the cell cycle [e.g. Knutsen (1965) for

<u>Chlorella</u>, and Walther and Edmunds (1970) for <u>Euglena</u>.]

For the few existing higher plant systems, synchrony has been induced in a number of different ways and the following systems have been employed recently for cell cycle studies. Root meristem tissue has been partially synchronized following treatment with 5-amino uracil (Mattingly 1966). A similar system in which synchrony was induced in pea root meristems by prolonged carbohydrate starvation coupled with use of the inhibitor FUdR was described by Kovacs and Van't Hof (1970). The partial synchronization of cultures of <u>Haplopappus gracilis</u> using a number of inhibitors including 5-amino uracil and hydroxy-urea, was reported by Erikson (1966).

Suspension cultures of <u>Acer pseudoplatanus</u> have been synchronized by inoculating cells from a stationary culture into a fresh culture medium (Street 1968). Roberts and Northcote (1970) have also employed a sycamore callus system synchronized using the plant growth substances kinetin or 6-benzyl amino purine. The majority of the above systems have the disadvantage that there is only a small percentage cell division.

Microsporogenesis is a naturally occurring phenomenon and provides the only higher plant systems for which enzyme patterns have been related to the cell division cycle. The best known of these is the developing microspore system of <u>Lilium longiflorum</u> [Stern (1960) and (1961), Hotta and Stern (1961)(1963a and b) and (1965)]. The interphase between the first and second meiotic divisions occupies a period of 20-22 days and the developmental
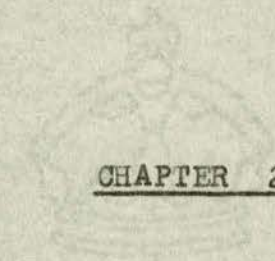
stage can be recognised by measuring the length of the flower bud from which the anther was removed.   Enzyme estimations have been carried out using extracts prepared from whole anthers, and in enzyme induction studies whole anthers were excised from the plant and placed in the culture medium, as it was impossible to culture the microspores without the surrounding anther tissue.   This system is not ideal for a study of the changes accompanying cell division as it is not representative of a typical mitotic situation and secondly, the microspore is an extremely specialized type of cell and therefore is not representative of a typical higher plant cell.

The system used in the current investigation employed tissue from the tubers of the Jerusalem artichoke (Helianthus tuberosus. L, Var. Bunyards Round).   When explants isolated from the storage parenchyma region of the tuber are placed in a mineral salts medium containing sucrose and the synthetic auxin 2,4-dichlorophenoxyacetic acid (2,4-D), cell division is initiated.   The first synchronous division occurs after an initial lag phase of about 24 hours (Yeoman, Evans and Naik 1966; Yeoman and Evans 1967).   The length of the lag phase of the first division cycle increases with the length of storage of the tubers but the lag phase of the second division cycle is constant at 12 hours.   After the first two divisions the system becomes increasingly asynchronous and eventually, after a long culture period a callus is formed (Yeoman, Dyer and Robertson 1965).

The cells which divide within the explant are confined to the cell layers near to the surface, but do not include the layers of damaged cells in immediate contact with the culture medium. Provided that the explants are excised under low intensity green light and cultured in darkness, the proportion of cells per explant which divide in the first synchronous division can exceed 60% (Fraser et al. 1967). In contrast to the suspension cultures of higher plant cells previously mentioned, the artichoke system is typical of the _in vivo_ higher plant situation in that each individual explant represents an organised tissue which permits interactions to take place between individual cells. Another advantage of the artichoke system over other systems is that synchrony occurs naturally, although division is induced. The ease with which cultures of artichoke explants can be set up under sterile conditions is also advantageous as the system obtained is free from microbial contamination and therefore ideal for experiments which involve the feeding of isotopes.

Although a variety of different enzyme activities have been studied in other systems (Appendix 1), the group of enzymes chosen for this investigation in the artichoke system, were all associated with DNA metabolism and included enzymes of biosynthesis and degradation. The initial aim of this series of experiments was to determine patterns of activity for a number of different enzymes during the cell division cycle with particular reference to the timing of possible periodic changes with respect to the onset and extent of DNA synthesis.

CHAPTER 2

EXPERIMENTAL METHODS

SECTION A

EXPERIMENTAL MATERIAL

The higher plant tissue used in this investigation was isolated from the tubers of Jerusalem artichoke (Helianthus tuberosus, L. var. Bunyards round.)   Clonal material of this variety was grown in the garden of the Botany Department, Kings Buildings, Edinburgh.

Tubers were usually harvested during November when they had reached their maximum size.   Usually 15 - 20 large tubers were obtained from each plant and these were placed in groups of 4 or 5, in labelled polythene bags together with some damp sand.   The bags were placed in a large polythene bin, covered with more damp sand and stored in a cold room at $4^{\circ}C$.   Under these conditions of storage the tubers were prevented from sprouting until May or June of the following year.

SECTION B

CULTURE PROCEDURES

1. Composition of the culture medium.

The culture medium used throughout this investigation had the following composition:-

$MgSO_4.7H_2O$	36 mg.)	
KNO_3	81 mg.)	Solution A
KCl	65 mg.)	
KH_2PO_4	12 mg.	Solution B
$Ca(NO_3)_2$	236 mg.)	
$FeCl_3$	1 mg.)	Solution C
Sucrose	40 gm.	
2,4-D	0·22 mg.	

Distilled water to a total volume of 1000 ml.

[For solid media, 10·0 gm. agar was included].

Stock solutions of A, B and C were freshly made up at intervals at 10 x the above concentration and stored in a refrigerator until required. A stock solution of 2,4-D was made up by dissolving 2·2 mg. 2,4-D in 10 ml. of ethanol. The complete medium was stored in a deep freeze at -20°C.

The sucrose + mineral salts + 10^{-6}M 2,4-D medium described above, was used in all experiments where 'dividing' cultures were required. In experiments in which 'non-dividing' cultures were required, a medium containing sucrose + mineral salts without 2,4-D was used.

2. <u>Aseptic preparation of flask cultures and the cultural conditions.</u>

Instruments, glassware and paper tissues were sterilized inside tin boxes in an oven at 150°C for at least 3 hours. The instruments and paper tissues were wrapped in aluminium foil during sterilization.

Each 100 ml. conical flask contained 15 ml. of medium and a magnetic stirrer coated with heat resistant PTFE., and was plugged with non-absorbent cotton wool. Flasks of distilled water and medium were autoclaved at a pressure of 15 psi. for 15 minutes.

Artichoke tubers of uniform shape and similar size were selected from the same plant and any small, broken, diseased or irregularly shaped tubers were discarded. The selected tubers were scrubbed to remove surface soil and were surface sterilized by immersion in 4% v/v sodium hypochlorite for 25 - 30 minutes.

The aseptic preparation of cultures was carried out inside a specially designed 'sterile room' containing two ultra-violet lamps which were permanently switched on when the room was unoccupied. This room was maintained at a slightly higher air pressure than its surroundings so that when the sliding door was opened, the air flow was outwards, thus preventing the entry of air-borne micro-organisms from the outside.

After surface sterilization, the tubers were rinsed 2 or 3 times with sterile water and transferred to the sterile room. Individual tubers were wiped free of surface moisture with a sterile tissue and each transferred to a spirit pot.

The following procedure was carried out in low intensity green light (Ilford Bright green safe light < 1 ft.-c, Filter No. 909). All instruments were flamed after immersion in meths. before use.

The ends of each tuber were removed with a scalpel and discarded. A series of cores approximately 3 cm. long and 2 mm. in diameter were removed from the storage parenchyma region of the tuber using a metal canula. These cores were cut into $2 \cdot 4$ mm. lengths with a specially designed cutter. After the explants had been transferred to the flask, the neck of the flask was flamed.

Flasks were placed inside a black box with a light tight lid, which was then placed on top of a bank of magnetic stirrers which revolved at 250 rpm., in a dark growth room maintained at $25^{\circ}C$. During growth, the cultures were observed using low intensity green light only.

For cell cycle experiments a series of cultures were prepared in 100 ml. conical flasks, each containing about 160 standard explants (2 mm. x $2 \cdot 4$ mm.) in 15 ml. of culture medium. The cultures were always prepared in low intensity green light and grown in the dark.

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Culture in roller bottles.

During preparation of labelled DNA (Ch.3) another culture procedure was adopted to avoid contaminating a large number of magnetic stirrers with radioactive medium. In addition to the usual constituents, the culture medium contained 200 ml. of coconut milk/litre of medium. [Coconut milk was obtained from fresh coconuts, filtered through glass wool, autoclaved at 15 psi. for 20 minutes and stored in a deep freeze at -20°C. Before addition to the medium the coconut milk was separated from precipitated protein by centrifugation].

Cultures were prepared in roller bottles (600 ml. capacity glass jars with straight sides and a narrow neck). The 15 ml. of culture medium in each roller bottle also contained in this instance 50 μCi ^{3}H-thymidine (specific activity 22·8 Ci/m mole) and 15 μg. of unlabelled thymidine. Because of the radiation hazard the aseptic preparation and inoculation of explants was performed in the light. Each bottle culture containing 160 explants was placed on its side and revolved at 2 rpm. on a special apparatus designed for the purpose, in a growth room at 25°C. The explants adhered to the walls of the bottle and passed through the culture medium once every revolution.

Other culture methods.

For all cell cycle experiments, flask cultures were prepared using the conditions previously described. In the development of enzyme assay methods suitable for use with artichoke tissue (Ch.3),

other methods of culture were often used. For example, flask cultures which contained coconut milk in the culture medium (in the concentration given above for roller bottle culture) were often used when explants were cultured for more than 4 or 5 days. [Coconut milk is required for prolonged culture of artichoke tissue. (Yeoman 1969)].

During the summer season, when liquid cultures were prone to serious microbial contamination, 10 - 20 isolated explants were often cultured in a petri dish on top of a solid medium containing 1·0% agar (Robertson 1966). This procedure reduced the spread of microbial contamination and simplified the identification of contaminated tissue.

3. Estimation of the degree of contamination of the culture medium by micro-organisms.

A number of 1·0 ml. capacity blow-out pipettes containing loosely packed cotton wool in the mouthpiece end, were placed inside a metal drum and sterilized either in the autoclave at 15 psi. for 20 minutes, or in an oven at $150^{\circ}C$ for 3 hours. A series of plastic petri dishes containing 10 ml. sterile 2% nutrient agar (Oxoid) medium were prepared.

At each sampling time, a culture was transferred from the growth room to the sterile room. After removal of the cotton wool plug, the mouth of the flask was flamed and 0·5 ml. of culture medium withdrawn and placed on top of agar in a petri dish. The 0·5 ml. of medium was spread over the agar surface using a bent

glass rod which had previously been sterilized.

The agar plates were incubated at room temperature for 2 - 3 days and the number of colonies of micro-organisms counted. Assuming that 1 micro-organism gave rise to 1 colony, the number of micro-organisms present per ml. of culture medium at each sampling time was equal to twice the number of colonies.

It must be emphasized that this test is designed to determine the number of micro-organisms present in the culture medium and not in the tissue. Although nutrient agar is capable of supporting growth of the majority of micro-organisms this test only accounts for those micro-organisms able to grow on this medium.

Estimations of the microbial contamination of the culture medium for 3 different cell cycle experiments are shown in Appendix 2.

For the experiment described in Appendix 2a, approximately one third of all cultures examined were completely devoid of micro-organisms. Although the remaining flasks showed some contamination, this was not significantly large to constitute a serious contamination hazard. When incubation at room temperature was extended from 2 to 3 days, a larger increase in colony number generally occurred in the more highly contaminated cultures suggesting self contamination. For this reason, the incubation period used, never exceeded 3 days.

Previous workers in this laboratory have noticed that cultures prepared at the end of the 'artichoke season' were often more prone to microbial contamination than cultures prepared at the beginning

of the season.   Although both experiments were carried out late in the season, the sterility of the experiment described in Appendix 2b is much improved over that described in Appendix 2a, due to the fact that the surface of all tubers had been freed of surface moisture using sterile tissues, during preparation of the cultures. As this procedure was found to reduce contamination it was adopted in all subsequent experiments.

The experiment described in Appendix 2c was carried out at the beginning of the artichoke season and shows very little microbial contamination.

As no serious contamination was observed in the three experiments for which data is available, it was considered un-necessary to carry out these tests for every experiment.

4. Cleaning of glassware.

In order to maintain the inside surface of the culture flasks in a smooth condition, each flask was used only twice in between a rigorous washing procedure, designed to add a fresh layer of glass to the surface (Paul 1959).   The washing procedure was as follows:-

1. Remove any surface debris from the glass vessel by brushing in running water.

2. Boil for 30 minutes in a cleaning solution (1 ml. of a stock solution containing 80 gm. sodium metasilicate + 9 gm. Calgon/litre, diluted to 100 ml. with water.

3. Rinse thoroughly in tap water.

4. Leave glassware to soak in $\frac{N}{100}$ HCl for 4 hours.

5. Rinse thoroughly in tap water.

6. Leave glassware in distilled water for at least 3 hours.

7. Rinse in distilled water and dry in a hot air oven.

All other glassware such as test tubes etc. was occasionally subjected to the above procedure although normally they were washed in a detergent solution containing teepol and rinsed in tap water and distilled water.

Radioactive glassware was soaked for at least 2 days in a solution containing 20 ml. Decon 75/litre of tap water, followed by thorough brushing in a hot solution of teepol and thorough rinsing in tap water and distilled water.

SECTION C

STANDARD ANALYTICAL PROCEDURES

1. Cell number estimation.

The technique which was used to estimate the total cell number per explant was first described by Yeoman, Dyer and Robertson (1965) and is a modification of the method of Brown and Rickless (1949).   Five explants were placed in a sample tube containing 2 ml. of 5% chromic acid, which was then stored in a refrigerator for at least 24 hours.   The tissue was macerated by repeatedly drawing all the liquid into a syringe rapidly and then expelling it. The macerate was mixed to prevent cells from settling and a small volume of liquid placed beneath a coverslip on a haemocytometer slide.   All the cells covering the grid were counted with the aid of a tally counter.   Within the macerate, single intact cells were counted as 1, pairs of cells counted as 2, and broken cells were only counted if more than half intact.

Six determinations were made for each macerate and the mean value obtained.

Cell number was determined using the equation:-

$$\text{cell number} = \frac{\text{total volume of macerate } (\mu l.)}{\text{*Volume of fluid on the grid}} \times \frac{\text{average count}}{\text{no. of explants}}$$

(*Volume of fluid on the grid = 3·2 μl. for a standard slide)
For an average calculation

$$\text{Average cell number/explant} = \frac{2000 \times x}{3·2 \times 5}$$

(where x = average count)   or $125 \times x$

This cell counting technique was also used to determine the timing of cell division. For a sample taken 2 - 4 hours before division commenced, many of the cells on the grid appeared to have blackened nuclei. The onset of division could be recognised even though the cell number had not substantially increased if either mitotic figures (nuclei in the process of division), or cell pairs (the 2-cell product of a single division which is not separated by this technique) were present.

The end of division was recorded when the average cell number per explant ceased to increase.

2. Measurement of total nucleic acid.

This estimation was carried out using the method developed by Evans (1967).

Two or 3 samples of 15 explants were fixed by placing them in sample tubes containing methanol and storing these in a deep freeze ($-20^{\circ}C$) for at least 3 hours. The explants were transferred to conical centrifuge tubes and subjected to the following extraction procedure which was designed primarily to remove from the cells, substances which absorb ultra-violet light and may confuse the total nucleic acid determination and also substances which may interfere with the Burton method for DNA. The following procedure was carried out in a cold room at $4^{\circ}C$ using cold solvents.

5 ml. of 5% trichloro-acetic acid (TCA) for 20 minutes.
Repeat.
5 ml. of 0·05M formic acid in methanol (15 minutes)
Repeat twice.

5 ml. of 80% ethanol (15 minutes)

5 ml. of 100% ethanol (at least 15 minutes)

5 ml. of 50% ethanol/ether (15 minutes)

5 ml. of 100% ether (15 minutes)

The tubes were then inverted and the explant pellets dried in air to remove all traces of ether, before replacing them in the conical centrifuge tubes.

The total nucleic acid was removed from the explants by hydrolysis at $70^{\circ}C$ using 0·5N perchloric acid (PCA). A total of 0·1 ml. of 0·5N PCA per explant was used (i.e. 1·5 ml. for 15 explants) and 3 consecutive extractions were carried out using 0·5 ml. of 0·5N PCA. The hydrolysates were then combined. At least 2 blanks (no explants) were subjected to this procedure. [The remaining explants were washed in 5 ml. of 70% ethanol (15 minutes) and stored in 5 ml. of 100% ethanol in sealed tubes in the cold room until required for estimation of acid resistant protein].

In order to measure total nucleic acid, 0·2 ml. samples of hydrolysate were diluted to 0·8 ml. with 0·5N PCA. The optical density at 260 mμ was measured on the SP.500 spectrophotometer using quartz micro-cuvettes. A typical absorption spectrum for the total nucleic acid hydrolysate from 28 hour explants is shown in Fig. 2.1. and was obtained using an SP.800 spectrophotometer.

In artichoke tissue, the total nucleic acid consists of approximately 90% RNA and about 10% DNA (Mitchell 1967), hence measurement of total nucleic acid is essentially a measurement of RNA.
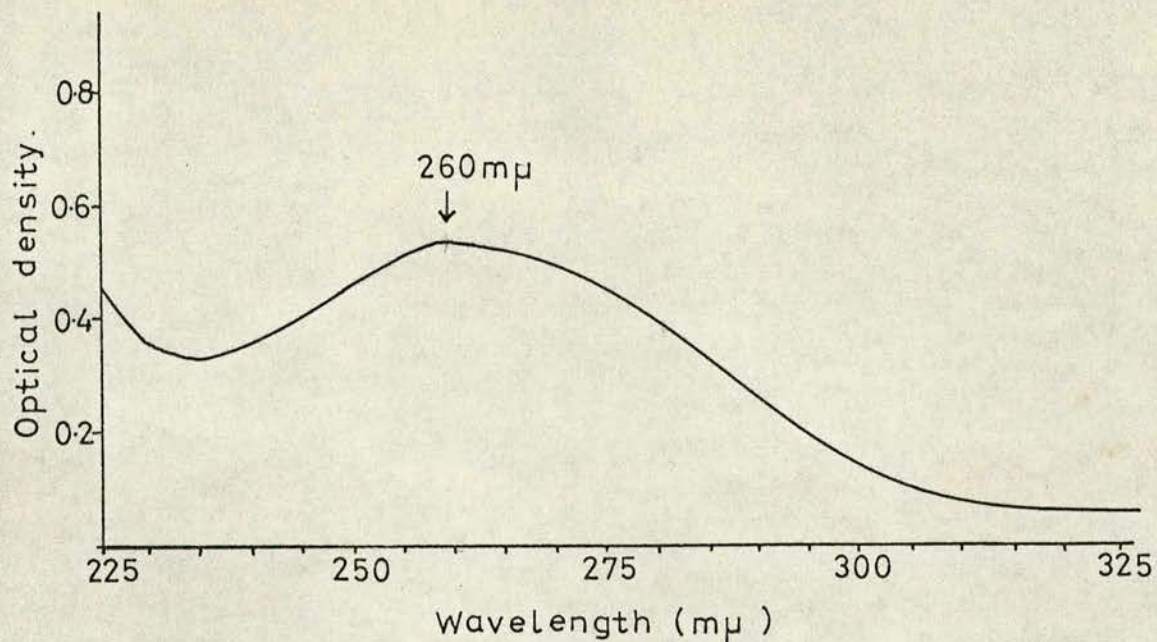
FIG. 2 . 1   Absorption spectrum of a total nucleic acid
hydrolysate from 28 hour explants.
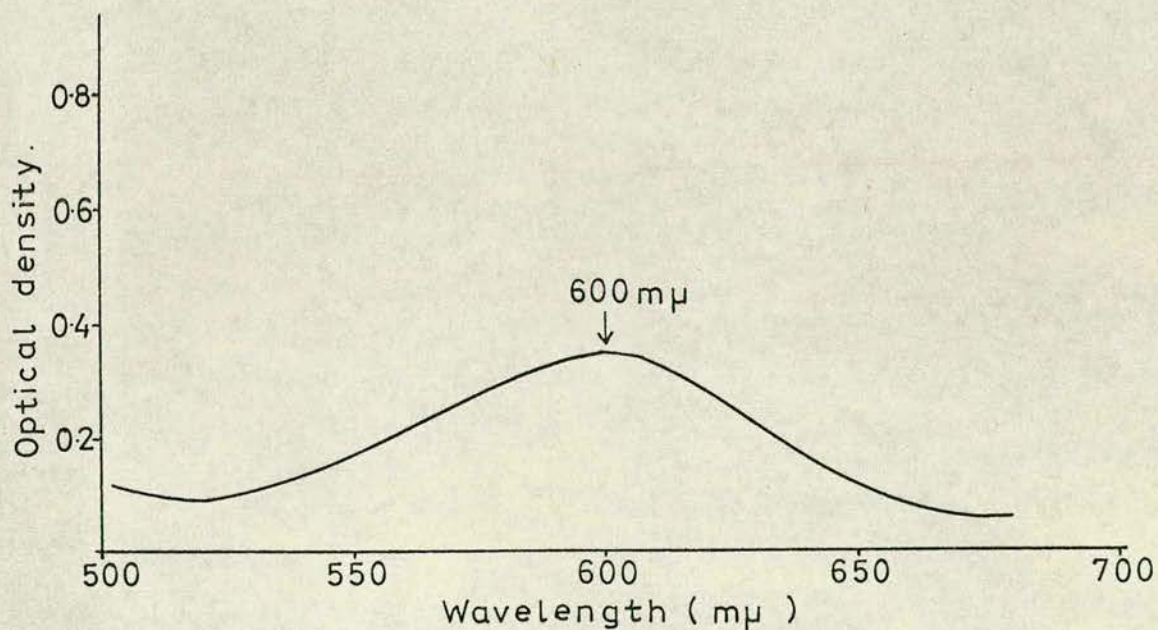
FIG. 2.2 Absorption spectrum of the product of the
diphenylamine reaction for a hydrolysate prepared
from 32 hour explants.

3. Measurement of DNA.

a. Burton's chemical method.

The 1·3 ml. of PCA hydrolysate remaining from the total nucleic acid estimation described above, was used for this determination.

The method used was basically that of Burton (1956), and although this method as described by him is supposed to minimise the effect of substances which interfere with the diphenylamine reaction, the series of solvent extractions described for the total nucleic acid procedure was an essential prerequisite for the accurate determination of DNA by this method (Evans 1967).

For this determination, Burton's reagent contained

  2 gm. diphenylamine (Analar)

  1·5 ml. conc. H_2SO_4 ("   )

  8 mg. acetaldehyde ("   )

  glacial acetic acid (analar) to a total volume of 100 ml. This reagent was made up on the day required.

Twice the volume of Burton's reagent (2·6 ml.) was added to one volume of hydrolysate (1·3 ml.) in a hard glass tube.   The tubes were sealed with parafilm and placed in a water bath at $30^{o}C$ for 18 hours.

The intensity of the blue colour, which was developed in the presence of DNA hydrolysis products, was measured using the SP.500 spectrophotometer and 4 cm. light path cells.   The optical density at 600 and 650 mµ was recorded for each sample and the difference ($\Delta O.D_{600-650}$) found.   A typical absorption spectrum (obtained using the SP.800 spectrophotometer) is shown in Fig. 2.2. for a
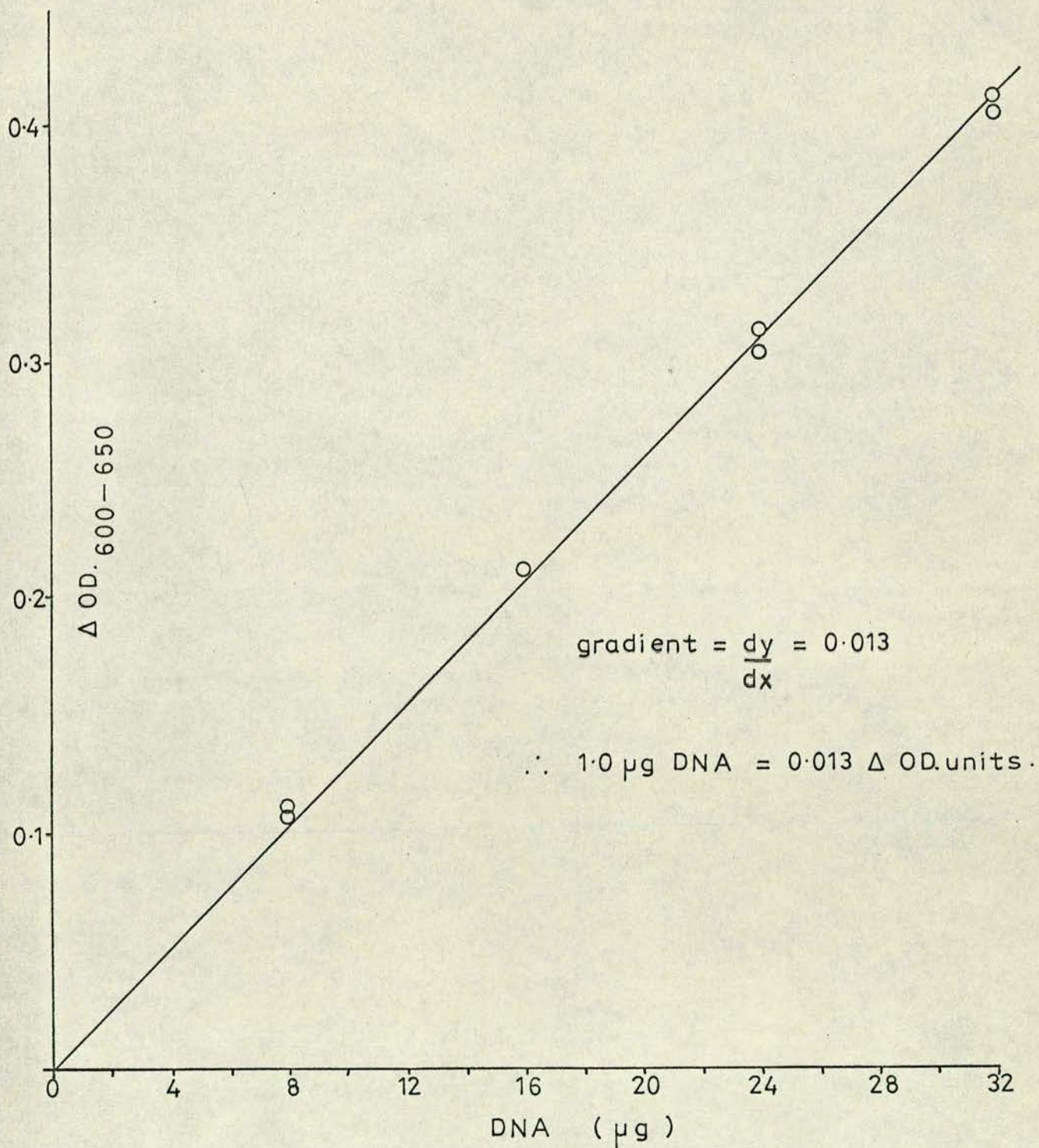
FIG. 2.3   DNA calibration curve for Burton's method.

32 hour sample.

In order to calibrate this method in terms of μg. of DNA, a stock standard solution containing 40 mg. of calf thymus DNA per ml. of 0·5N PCA, was prepared and stored in a refrigerator. Standards containing 8, 16, 24 and 32 μg. of DNA per 1·3 ml. of 0·5N PCA were prepared in duplicate and were also subjected to the above procedure on each occasion.

Using the gradient of a standard calibration curve values for Δ O.D.$_{600-650}$ were converted to μg. of DNA. A typical calibration curve is shown in Fig. 2.3.

b. The Feulgen microdensitometric method.

This procedure was carried out by Dr. Jackson using the method of Mitchell (1967). Samples of 5 explants were required for this determination and these were fixed in acetic acid/ethanol (1:3) and stored in the deep freeze. After the staining procedure had been carried out, the relative absorptions of the Feulgen stained nuclei were measured at 5,700 Å using a Barr and Stroud integrating microdensitometer.

For the data presented in Experiment 4 (Alias 9) of Ch. 4, each value is the result of readings taken on 232 individual nuclei. In contrast to the results of Mitchell (1967) for estimations using similar tissue, the values have not been corrected for the number of nuclei which do not divide, so that the results represent accumulation of DNA in all 232 nuclei examined. Results were expressed as 'mean relative absorption as per cent 2C value'.

4. <u>Estimation of the rate of DNA synthesis and associated parameters.</u>
a. <u>Rate of DNA synthesis.</u>

The rate of DNA synthesis was estimated by measuring the rate of incorporation of ^{3}H-thymidine (^{3}H-TdR) into DNA.

Thymidine labelled with tritium in the 6 position, at a specific activity of 20 - 25 Curies/m mole, was obtained from Radio-chemicals, Amersham.

The rate of DNA synthesis was first measured for this tissue by Evans (1967). In a preliminary time course experiment, this worker had found that for tissue of various ages, the rate of incorporation was linear for pulses of between 30 and 60 minutes duration. Subsequently, a pulse of 45 minutes duration was adopted as a standard procedure.

The method used in this investigation was a modification of the method of Evans (1967) and although it differed in several respects, the pulse used was also of 45 minutes duration.

A sample of 16 explants was transferred from each culture flask to a 25 ml. conical flask containing 5 ml. of culture medium, a magnetic stirrer and ^{3}H-TdR at a concentration of 3 μCi/ml. The flask was placed under standard cultural conditions for 45 minutes and then the explants and stirrer were transferred to a 100 ml. conical flask containing 8 ml. of 3×10^{-4}M unlabelled TdR in distilled water, for a further 15 minutes. The explants were fixed in 4 ml. of methanol and the sample tubes were stored in a deep freeze at -20°C.

Each sample of 16 explants was then subjected to the solvent extraction procedure as described for total nucleic acid estimation, and at the ether dry pellet stage, each sample of 16 explants was divided into two samples of 8 explants.

Each sample of 8 explants was hydrolysed at $70^{o}C$ using a total of 1·4 ml. of 0·5N PCA. This involved 3 consecutive extractions, each of 20 minutes duration using 0·5, 0·5 and 0·4 ml. of 0·5N PCA. The hydrolysates were combined.

The acid hydrolysate was neutralized with approximately 0·1 ml. of 5N KOH, and the tubes were cooled in ice to accelerate the precipitation of potassium perchlorate. A 0·5 ml. aliquot of the clear solution was mixed with 15 ml. of dioxane scintillator and counted in a scintillation counter. After correcting for the background counts, the results were expressed as counts/minute/explant (cpm) of ^{3}H-TdR incorporated into DNA.

Because two values were obtained from each sample of 16 explants an average value could be calculated. In experiments which include estimations of the rate of DNA synthesis during the cell cycle, the average value is most often quoted but the duplicated values are always shown.

b. Rate of uptake of ^{3}H-TdR into the tissue.

The rate of uptake of ^{3}H-TdR into the tissue was obtained by adding the counts removed in the methanol fixative and in the first and second washes with 5% TCA., to the average counts incorporated into DNA. The radioactivity of these solutions was estimated by mixing 0·5 ml. of the appropriate solution with 15 ml. of dioxane

scintillator and counting for 10 minutes.   For each solution the counts/minute for each explant were calculated before the values were added to give the rate of uptake of ^{3}H-TdR into the tissue.

c. Percentage of the total added counts which were incorporated into DNA.

In several instances, the percentage of the total added counts incorporated into DNA was estimated.   The radioactivity of the original pulse medium containing ^{3}H-TdR was estimated by counting a 0·5 ml. aliquot of this solution using dioxane scintillator as previously described.   From the number of counts per minute available to each explant and the rate of DNA synthesis, it was possible to calculate what percentage of the total added counts was incorporated into DNA.

d. Counts left in the residue.

In experiments 9 and 10 (see Ch. 4), in addition to measurements of the rate of DNA synthesis a further procedure was included to determine whether any ^{3}H-TdR remained in the explant residue after the nucleic acid had been removed by PCA hydrolysis.

The tissue remaining from the hydrolysis procedure was stored in 70% ethanol in the cold room until required.   The explants were then washed with 5 ml. of 50% ethanol (20 minutes), 5 ml. of 30% ethanol (20 minutes) and 5 ml. of distilled water (20 minutes).

In order to obtain a cell suspension from which a quantitative estimation of the remaining radioactivity could be made, 4 explants from each sample of 8 were blotted to remove excess moisture and

placed in a scintillation vial containing 0·5 ml. of 0·05M EDTA (adjusted to pH 9·0 with N NaOH). The scintillation vials were placed so that the explants were completely immersed in the solution and were heated in a water bath at $60^{o}C$ for about 18 hours. The scintillation vials were then agitated to fragment the explants using a microid flask shaker.

The resulting alkaline cell suspension was neutralised with 0·2 ml. of 0·5M PCA and mixed with 15 ml. of dioxane scintillator. Samples were counted for 10 minutes and the results expressed as counts/minute/explant.

In Experiment 10 (Ch. 4), the above estimation was carried out only on one of the duplicated samples of 8 explants. The other sample was subjected to a more stringent hydrolysis procedure, designed to remove any remaining DNA residues from the explants.

The second hydrolysis procedure which was carried out directly after the first, involved 3 consecutive extractions at $70^{o}C$, each of 1 hour duration, with a total volume of 3 ml. of 1·0N PCA. The residue was then treated as described above.

5. Estimation of acid resistant protein.

The acid resistant protein remains in the residue after the total nucleic acid has been removed by the standard PCA hydrolysis. After PCA extraction, the remaining explants were washed in 5 ml. of 70% ethanol for 15 minutes and stored in 5 ml. of 100% ethanol in a cold room at $4^{o}C$. The explants were dehydrated by treatment with 50% ethanol/ether and absolute ether and dried in air to

remove all traces of ether. The ether dry pellets were transferred to a pyrex test tube together with 3 or 4 small pieces of carborundum (to prevent 'bumping' of the solution during the subsequent digestion). A 0·3 ml. aliquot of digest acid (36N 'nitrogen free' H_2SO_4 containing the equivalent of 0·2 gm./l $CuSeO_3$) was added to each sample and the tubes were heated in micro-Kjeldahl racks in a fume cupboard until the solution became colourless, and subsequently for a further 30 minutes. Two blanks containing digest acid only were treated in the same way. After cooling, 9·7 ml. of distilled water was added to each sample.

Total nitrogen content of the 'digest' was estimated using the method of Conway (1962).

For this estimation, Conway indicator contained

    10 gm. boric acid

    200 ml. ethanol

    20 ml. stock indicator [0·033% bromocresol green and 0·066% methyl red in absolute alcohol]

    Adjusted to pH 5·1, and made up to 1 litre with distilled water.

The rims of both outer and inner walls of Conway dishes were coated with vaseline and two vaseline barriers were placed across the floor of the outer well to divide it into two compartments. A 0·2 ml. aliquot of Conway indicator was placed in the centre well and a 0·5 ml. aliquot of 40% NaOH placed in one of the outer compartments. A 0·5 ml. aliquot of sample was placed in the other compartment. The Conway dishes were sealed with ground glass lids

and cooled by placing on ice.   Cooling was essential so that
when the solutions in the outer compartments were mixed, the
resultant heat of neutralization would be absorbed by the glass,
instead of causing a pressure within the dish which was high enough
to break the seal.   The solutions in the outer well were mixed
carefully by rotating the dish, and the Conway dishes were left in
the cold room at 4°C for 20 - 24 hours.   The indicator solution in
the central well was titrated against $0 \cdot 01N$ H_2SO_4 using a Beckman
microtitrator, until the colour changed from green to pink.

The Conway method was calibrated in terms of μg. of nitrogen
using a standard ammonium sulphate solution which contained $2 \cdot 64$ mg.
$(NH_4)_2SO_4$ and 3 ml. digest acid made up to 100 ml. with distilled
water.   Standard solutions containing $1 \cdot 4$, $2 \cdot 8$ and $4 \cdot 2$ μg. nitrogen
were placed in Conway dishes in duplicate and treated as above.
In this way the titration value was related to μg. of nitrogen.   A
typical calibration curve is shown in Fig. 2.4.

In order to express the results in terms of total protein, the
value for μg. nitrogen per explant was multiplied by the standard
protein conversion factor of $6 \cdot 25$.

The Conway dishes were cleaned by removing excess vaseline
with paper tissues and boiling both dishes and lids for 30 minutes
in a strong solution of Decon 75.   The dishes were then brushed
in a hot solution of teepol, rinsed in tap water and distilled
water, and soaked in distilled water overnight before drying in
a hot air oven.
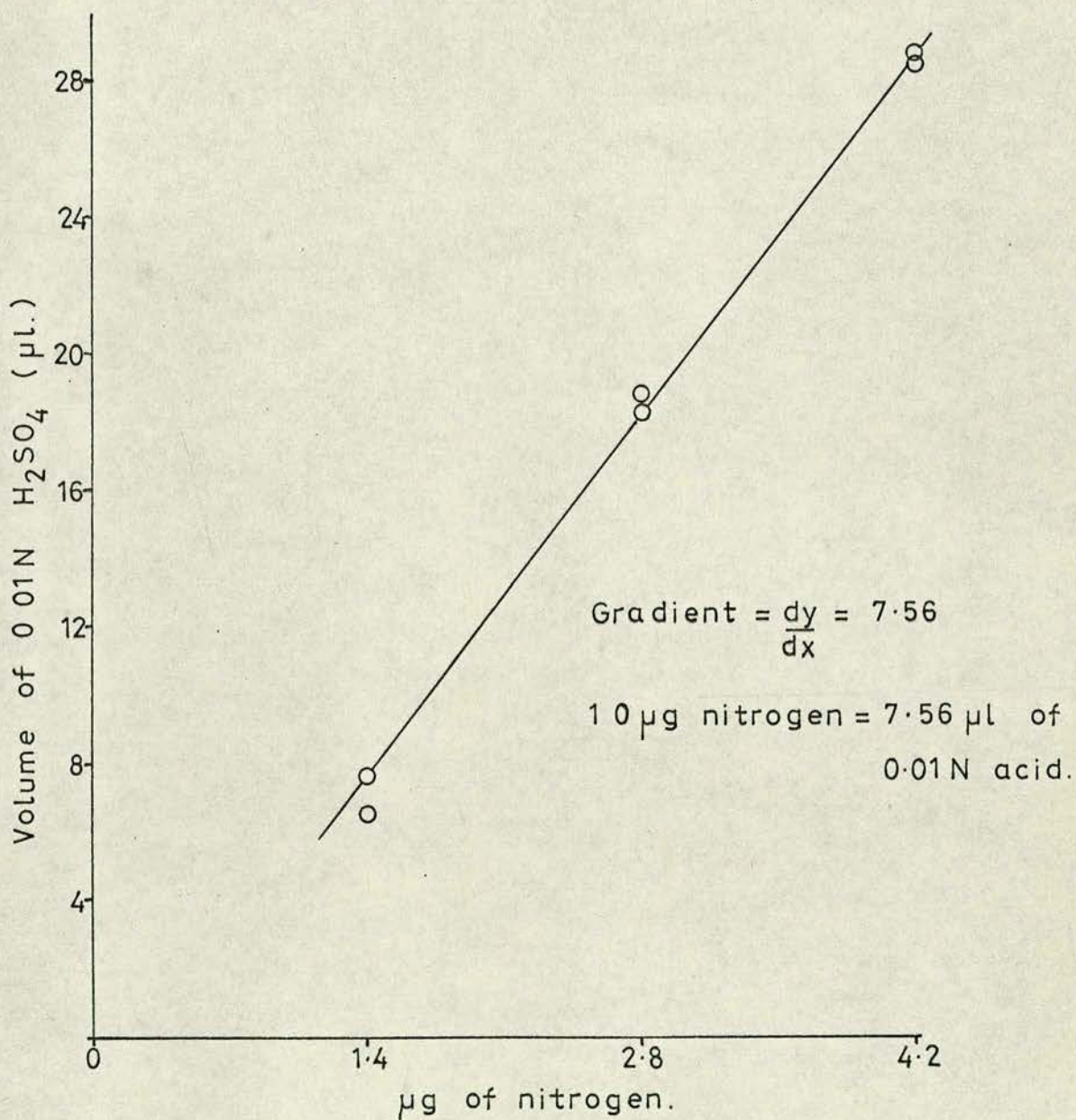
FIG. 2·4   Calibration of Conway's method using

standard $(NH_4)_2SO_4$.

SECTION D

ENZYME ASSAY METHODS

1. 'Native'-DNAase.

The term 'native'-DNAase refers to the deoxyribonuclease which hydrolyses native artichoke DNA.

a. Preparation of extract.

After removal of the growth medium, the explants from each culture flask were washed 3 times with distilled water and retained on muslin. Groups of 100 explants were placed on Whatman No. 1 filter paper to remove the bulk of surface moisture and then transferred to a cold mortar for 5 minutes. The grinding medium was 1·5 ml. of 0·01M tris-maleate buffer pH 7·2 containing 0·5% β-mercaptoethanol (Et.SH) and 400 µg./ml. bovine serum albumin (BSA). After preliminary maceration of the tissue using a pestle and mortar, further homogenisation was achieved using a Kontes hand glass homogeniser. Finally the homogenate was centrifuged at 2200 g. for 10 minutes at 0°C in a plastic tube, and the supernatant fraction was retained for use as the enzyme extract. Assays were carried out immediately following the preparation of extracts.

b. Enzyme assay.

All assays were carried out using 15 ml. pyrex conical centrifuge tubes. The native artichoke DNA labelled with ^{3}H-thymidine which was used as substrate was prepared as described in Ch. 3. Substrate solutions made up to a concentration of 100 µg./ml. in 0·01M tris buffer pH 8·0, were stored in a deep

freeze at -20°C in cellulose nitrate tubes covered with parafilm.
A fresh sample of substrate solution was removed from the deep
freeze for each experiment.   The reaction mixture which had a
total volume of 0·16 ml. contained

      10 µl. native ^{3}H-DNA (1 µg. of DNA in 0·01M tris buffer pH 8·0
                           containing approx. 6000 counts/minute)

      0·05 ml. 0·2M tris maleate buffer pH 5·6 containing 0·015M $MgCl_2$

      0·1 ml. extract (added last).

Incubation was carried out for 10 minutes in a shaking water bath at
30°C and the reaction was stopped by the addition of 0·1 ml. of
carrier DNA (Sigma type V calf thymus DNA at a concentration of
2 mg./ml. of 0·01M tris buffer pH 8·0) followed by 0·2 ml. of 20%
(w/v) perchloric acid (PCA).   The tubes were transferred to the
cold room for 5 minutes to aid the precipitation of excess and
carrier DNA followed by a centrifugation at 0°C for 10 minutes at
1000 g.   The supernatant fraction which contained the acid soluble
products of the reaction was decanted into a scintillation vial,
neutralised with 0·1 ml. of 0·5N NaOH and mixed with 15 ml. of
dioxane scintillator.   Three replicate 1 minute counts were
obtained for each sample.   For each experiment, 2 controls were
prepared in which the extract of the assay medium was replaced by
grinding buffer.

    In order to measure the total possible hydrolysis, the extract
component of the assay mixture was replaced by 0·07 ml. grinding
buffer + 0·03 ml. DNAase I (a 1 mg./ml. solution of DN-C DNAase I
from bovine pancreas, made up in 0·01M tris buffer pH 8·0).

Duplicate samples were incubated at 30°C for 30 minutes to ensure complete hydrolysis of the substrate.

Enzyme activities were expressed as percentage conversion of labelled DNA to acid soluble products/10 minutes/0·1 ml. extract, where each average count had been corrected for the control and expressed as a percentage of the total possible hydrolysis.

The final composition of the 'native'-DNAase assay medium is shown in Table 2.a. The above method was used to assay 'native'-DNAase for all cell cycle experiments described in Ch. 4.

2. 'Denatured'-DNAase.

The term 'denatured'-DNAase refers to the deoxyribonuclease which hydrolyses denatured artichoke DNA.

a. Preparation of extract.

The procedure was identical to that used for 'native'-DNAase with the following additions:-
A 50 fold dilution was prepared by adding a 0·05 ml. aliquot of the supernatant fraction to 2·45 ml. of cold grinding buffer. The diluted extract was thoroughly mixed.

b. Enzyme assay.

The denatured artichoke DNA used as substrate was prepared as follows. A sample of native ^{3}H-DNA was removed from the deep freeze and transferred to a pyrex conical centrifuge tube. Denaturation was effected by heating the sample in a water bath at 100°C for 5 minutes during which time the water loss by evaporation was minimised by placing a glass marble on top of the tube to act as

Materials Present	Amounts Present in a total volume of 0·16 mls.	Molarity within incubation mixture
Native artichoke DNA	1 µg.	
Tris maleate buffer pH 5·6	10 µ moles	62·5mM
Tris maleate buffer pH 7·2	1 " "	6·25mM
$MgCl_2$	0·75 " "	4·67mM
β-mercaptoethanol	4·5 " "	28·1mM
Bovine serum albumin	40 µg.	

Table 2.a.   Composition of the assay medium for 'native'-DNAase.

a condenser whilst at the same time blowing cold air onto the sides of the tube. The tube was then plunged into an ice bath.

This treatment has the effect of separating the double stranded DNA molecules into single strands. The denatured-DNA substrate may not be completely single stranded however, as some reannealing may have taken place despite rapid cooling, either between parts of the same strand or between two different strands.

The reaction mixture which had a total volume of 0·16 ml. contained

10 μl. denatured-DNA (1 μg. of denatured DNA in 0·01M tris buffer
pH 8·0 containing approx. 6000 counts/min.)

0·05 ml. 0·2M tris maleate buffer pH 6·4 containing 0·015M $MgCl_2$

0·1 ml. diluted extract (added last).

Following a 20 minute incubation period in a shaking water bath at $30^{\circ}C$, the procedure was as described for 'native'-DNAase. Duplicate estimations of control values and total possible hydrolysis using denatured-DNA were carried out for each individual experiment.

The final composition of the 'denatured'-DNAase assay medium is shown in Table 2.b.

The above method was used to assay 'denatured'-DNAase in Experiments 1, 2, and 5 of Ch. 4.

In Experiment 3, however, the preparation of the extract was modified so that the activity of dTMP kinase could be measured in the same experiment. 100 explants were macerated in 1·0 ml. grinding buffer (0·2M sodium phosphate buffer pH 8·0 containing 0·5% EtSH. and 400 μg./ml. BSA) as previously described. After the

Materials Present	Amounts Present in a total volume of 0·16 ml.	Molarity within incubation mixture
Denatured artichoke DNA	1 μg.	
Tris maleate buffer pH 6·4	10 μ moles	62·5mM
" " " pH 7·2	1 μ moles	6·25mM
$MgCl_2$	0·75 " "	4·67mM
β-mercaptoethanol	4·5 " "	28·1mM
Bovine serum albumin	40 μg.	

Table 2.b.   Composition of the medium used to assay

'denatured'-DNAase.

standard centrifugation procedure, the supernatant fraction was
diluted 30 fold with 0·1M tris maleate buffer pH 6·4.   The assay
mixture, which was similar in its final composition to that used
in other experiments contained

    10 µl. denatured-DNA,

    0·05 ml. of 0·015M MgCl$_2$,

    0·1 ml. of diluted extract.

Incubation was carried out for 20 minutes in a shaking water bath
at 37°C.

3. DNA Polymerase.

This enzyme assay which was carried out by Dr. Jackson, was
similar to that of Wever and Takats (1970).

a. Preparation of extract.

The extract was prepared as described for 'native'-DNAase
except that prior to the final centrifugation, 0·2 ml. of homogenate
prepared using the hand homogeniser was retained for the assay of
DNA polymerase.   Extracts prepared in this way were used in
Experiments 1, 2 and 5 of Ch. 4.

In Experiment 2, results were also compiled using a
2000 g./5 minutes supernatant fraction as the enzyme extract.   In
Experiment 4, alias (9), which also included assays for TdR kinase
and dTMP kinase, 100 explants were macerated in the usual way in
1·0 ml. of grinding buffer (0·2M sodium phosphate buffer pH 8·0
containing 1·0% EtSH and 400 µg./ml. BSA) and a 2200 g./10 minute
supernatant fraction was used as the enzyme extract.

b. Enzyme assay.

The radioactive substrate was Schwarz 2-^{14}C-deoxythymidine triphosphate (dTTP) obtained as a 10 μCi/ml. solution in 50% ethanol at a specific activity of 45·5 mCi/m mole.

The assay mixture contained 0·2 ml. of 'medium A' (the composition of which is shown in Table 2.c.), 0·1 ml. of denatured calf thymus DNA (200 μg. DNA) and 0·1 ml. extract. 'Medium A' contained various substrates, protectors and cofactors essential to the reaction and the denatured DNA which was present as a template, was denatured using the method described in the 'denatured'-DNAase assay.   The final composition of the reaction mixture is shown in Table 4.d.   After a 30°C incubation for 30 minutes, the reaction was stopped by addition of 0·6 ml. of 7% perchloric acid (PCA). The following procedure was designed to remove the excess labelled dTTP which had not been incorporated into DNA.

1. Stand mixture in ice for 10 minutes.

2. Add 2 ml. of cold water and mix.

3. Centrifuge at 2000 g. for 5 minutes.   Discard supernatant.

4. Dissolve pellet in 0·3 ml. of 0·2N NaOH.

5. After 1 hour, add 0·6 ml. of 7% PCA and repeat entire procedure until 4 precipitations with PCA have been carried out.

Finally, the pellet was dissolved in 1 ml. of 2N NH_4OH, plated on an aluminium planchet, and counted for 20 minutes using a Beckman low Beta II gas flow counter.

Control values were obtained by incubating a sample without

Materials added	Volumes added (ml.)
0·1M β-mercaptoethanol	1·0
1·0M tris buffer pH 8·0	2·5
0·1M $MgCl_2$	2·5
9·4mM dATP	1·0
9·3mM dGTP	1·0
9·7mM dCTP	1·0
86·3mM ATP	0·5
0·3mM 2-[14]C dTTP*	0·62
Total volume	10·12 ml.

Store in deep freeze compartment of refrigerator.

*0·15 ml. Schwarz 2-^{14}C-dTTP was taken to dryness over a fine jet of air. Then 0·6 ml. H_2O was added followed by 0·02 ml. 8·2mM dTTP to give a final concentration of 0·3mM dTTP.

Table 2.c.   The composition of 'medium A' used in the DNA polymerase assay.

Materials Present	Amount Present	Final Molarity
*Bovine serum albumin	40 µg. protein	-
*Tris-maleate buffer pH 7·2	1·0 µ mole	2·8mM
*β-mercaptoethanol	6·48 " "	18·5mM
β-mercaptoethanol	1·98 " "	
Tris buffer pH 8·0	49·5 " "	141·4mM
MgCl$_2$	4·95 " "	14·1mM
ATP	0·43 " "	1·23mM
dATP	0·19 " "	0·54mM
dGTP	0·19 " "	0·54mM
dCTP	0·19 " "	0·54mM
2-^{14}C-dTTP	3·6 n mole	10·3µM
DNA and extract to a total volume of	0·35 ml.	

Table 2.d.   Final composition of the medium used to assay DNA
         polymerase.

[* present in the grinding medium]

enzyme for 30 minutes.   Extract was then added and the reaction stopped immediately.

To calculate enzyme activities the counts from duplicated reactions were added, corrected for the control, and converted to p moles dTTP incorporated/30 minutes/0·1 ml. of extract.

c. DNAase I control

In order to show that the product of enzyme action was susceptible to attack by deoxyribonuclease (e.g. in Experiment 5, Ch. 4), a standard enzyme assay was carried out, but instead of stopping the reaction with PCA, a heat treatment at $60 - 65^{\circ}$C for 10 minutes was used.   Following the addition of 25 µg. of DNAase I (0·01 ml. of a solution containing 0·25 mg./ml. of Sigma DN-C DNAase I from bovine pancreas) a second incubation was carried out for 30 minutes at 37°C.   Addition of 0·6 ml. of 7% PCA was followed by the standard washing procedure.

4. Thymidine kinase (TdR kinase)

a. Preparation of extract.

1. Explants from one culture flask were filtered through muslin to remove the growth medium and groups of 100 explants were placed on filter paper to remove the bulk of surface moisture.   The explants were transferred to a cold mortar for 5 minutes prior to the addition of 1 ml. of grinding medium (0·2M sodium phosphate buffer pH 8·0 containing 0·5% EtSH and 400 µg./ml. BSA) and a pinch of silver sand.   After adequate maceration, the homogenate was transferred to a plastic centrifuge tube and centrifuged at 2200 g.

for 10 minutes at 0°C. The supernatant fraction was used as the enzyme extract and was stored in ice until required. Assays were always carried out immediately following preparation of the extract. Extracts prepared as above were used in Experiments 7, 8 and 11 of Ch. 4.

2. A second method of extract preparation which was used in Experiments 13 and 14 was a minor modification of the above method. After preliminary maceration of the explants in a pestle and mortar, without silver sand, further homogenisation was achieved using a Kontes hand glass homogeniser followed by the standard centrifugation procedure. In Experiment 9, the second method of extract preparation was used, but in this instance the concentration of EtSH within the grinding buffer was 1%.

b. Enzyme assay.

The radioactive substrate was thymidine-6-T (i.e. TdR labelled with ^{3}H in the 6 position), supplied at a specific activity of 20 - 30 Curies/mM from Radiochemicals, Amersham. All reactions were carried out in pyrex conical centrifuge tubes. As the TdR kinase reaction is dependent upon a continued supply of ATP, an ATP generating system was included, in this case the breakdown of phospho-enol pyruvate (PEP) by PEP kinase. Two different enzyme assay procedures were used in the cell cycle experiments.

METHOD 1. In Experiments 7, 8 and 11 of Ch. 4, the assay mixture contained 0·1 ml. of 'Medium B' (the composition of which is shown in Table 2.e.), 0·025 ml. of pyruvate kinase (a 10 mg./ml solution of

Materials Added	Amounts Added
0·4M phosphate buffer pH 8·0	2·5 ml.
0·1M MgCl$_2$	0·025 ml.
ATP	6·2 mg.
phospho-enol pyruvate	8·4 mg.
^{3}H-thymidine	0·5 mCi
unlabelled thymidine	42 µg.

Distilled water to a total volume of 5 ml.

Final concentration of phosphate buffer is 0·2M

Table 2.e.   The composition of 'medium B' used
              in the thymidine kinase assay.

Sigma Type I pyruvate kinase from rabbit skeletal muscle) and
0·1 ml. of enzyme extract (added last).   The final composition
of the reaction mixture is shown in Table 2.f.   After a 20 minute
incubation in a shaking water bath at $37^{\circ}C$, the reaction was stopped
by addition of 0·4 ml. of 95% ethanol.   Protein denaturation was
continued by placing the tube in a water bath at $100^{\circ}C$ for 2 minutes.
The supernatant fraction containing excess substrate (TdR) and
product (dTMP) of the reaction was separated from precipitated
proteins by a 1000 g. centrifugation for 5 minutes at $0^{\circ}C$, and
decanted into a specimen tube.   The pellet was re-extracted with
0·2 ml. of 70% ethanol, recentrifuged, and the supernatants combined.
Sample tubes were stored in a deep freeze at $-20^{\circ}C$.   The product
of the reaction (dTMP) was separated from excess substrate (TdR) by
subjecting a 50 µl. aliquot of the ethanolic supernatant to cellulose
acetate electrophoresis for 4 hours in the cold room ($4^{\circ}C$) at a
potential difference of 200 volts in 0·05M ammonium formate buffer
pH 3·5.   A detailed description of the cellulose acetate electro-
phoresis procedure is given at the end of this method.   After
drying in air, the electrophoresis papers were cut into 22 ½-cm.
strips each of which was immersed in 10 ml. of toluene scintillator
and counted for 1 minute in a scintillation counter.

Blank estimations were carried out by replacing the extract
component of the reaction mixture with grinding buffer.

The expression of enzyme activity is described with reference
to an example after the detailed description of the cellulose acetate
electrophoresis procedure.

Materials Present	Amount Present	Final Molarity
*bovine serum albumin	40 μg. protein	-
*β-mercaptoethanol	4·5 μ mole	20mM
*sodium phosphate buffer pH 8·0	20 " "	))177mM
sodium phosphate buffer pH 8·0	20 " "	)
$MgCl_2$	0·05" "	0·2mM
ATP	0·2 " "	0·8mM
phospho-enol pyruvate	0·5 " "	2·0mM
phospho-enol pyruvate kinase	0·25 mg. protein	-
unlabelled thymidine	3·47 n mole	) 0·017mM) specific) activity
thymidine-6-T	0·4 " "	) 25·3 μCi/n) mole
Extract to a total volume of	0·225 ml.	

Table 2.f.   Final composition of the assay medium for
          thymidine kinase.
             (* present in the grinding medium)

METHOD 2. In Experiments 9, 13 and 14 of Ch. 4, which also included assays for thymidine monophosphate (dTMP) kinase, the TdR kinase assay was modified for convenience to resemble the dTMP kinase assay. The assay mixture which was similar in final composition to that previously described contained 50 µl. of 'medium B', 10 µl. pyruvate kinase (10 mg./ml) and 50 µl. of extract. The incubation was carried out as previously described but in this case the reaction was stopped by placing the reaction tubes in a water bath at 100°C for 2 minutes. After cooling in ice, a 1000 g. centrifugation for 5 minutes at 0°C was carried out to separate the supernatant containing substrate and product of the reaction, from precipitated proteins. The reaction tubes were stored in the deep freeze at -20°C. As a 10 µl. aliquot of the supernatant fraction was required for cellulose acetate electro- phoresis a better separation of TdR and dTMP was obtained compared with the previous method.

These two slightly different methods of TdR kinase assay gave comparable results in cell cycle experiments. The second method is preferable as it is shorter and therefore more convenient than the first. It is also possible that the second method is more sensitive due to improved separation of substrate and product by cellulose acetate electrophoresis.

c. Cellulose acetate electrophoresis.

The electrophoresis buffer was a solution of 0·05M ammonium formate (3·393 gm./l.) adjusted to pH 3·5 with formic acid.

Complete dissociation of phosphate groups occurs at pH 3·5 and during subsequent electrophoresis these negatively charged molecules tend to move towards the anode.

Oxoid cellulose acetate electrophoresis strips 20 x 5 cm., were trimmed to 19 x 5 cm. and a baseline was drawn in pencil 6 cm. from one end. The strips were wetted by placing them in a tray of buffer and blotted with filter paper (Whatman No. 1). Solutions were applied to the baseline using a 10 μl. pipette and the spot was allowed to dry.

A diagram of the electrophoresis tank in side view is shown in Fig. 2.5. The perspex electrophoresis tank was subdivided into two main compartments separated by a perspex barrier which on the upper surface bore prongs to support the middle of the electrophoresis strips. The two main compartments were further subdivided into outer and inner compartments by perspex barriers, each of which contained 3 round holes plugged with absorbent cotton wool. Each inner compartment was furnished with a platinum wire electrode which was connected to the terminals of a power pack.

Cold buffer was placed in each compartment of the tank, up to the level of the cotton wool plugs, so that there was liquid continuity between the outer and inner compartments. It was important that an equal level of buffer was maintained in all compartments. The ends of the electrophoresis strip rested on perspex supports which slotted into the sides of the tank. The middle of the strip was supported by perspex prongs. The ends of the strip were connected to the buffer by means of paper wicks
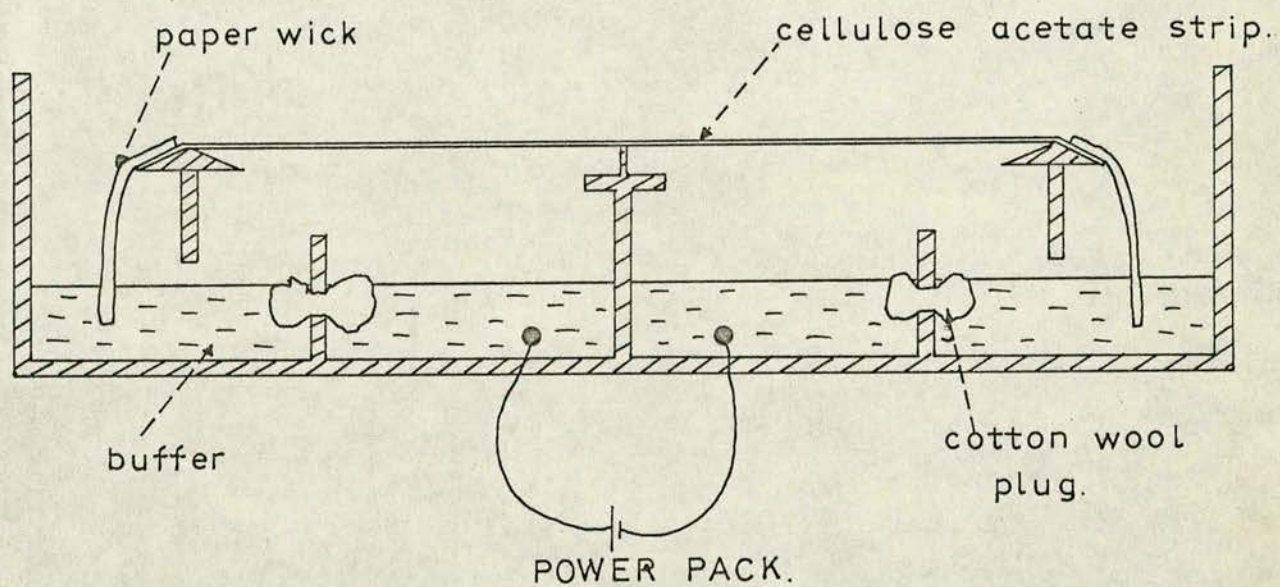
FIG. 2.5 Diagram of the cellulose acetate electrophoresis tank in side view.

(16 x 5 cm. strips of Whatman No. 3 chromatography paper soaked in buffer).   Each electrophoresis tank could accommodate 3 electro- phoresis strips placed side by side, and the strips were placed so that the baseline was closest to the cathode.   A plastic lid was placed over the tank and a potential difference of 200 volts was applied to the terminals.

The electrophoresis procedure was carried out in a cold room at $0 - 4^{\circ}C$.   The progress of the electrophoresis of standard solutions was observed using ultra violet (UV) illumination.   After adequate separation of the UV absorbing bands, the power pack was switched off, the strips were withdrawn from the tank and dried in air.   The strips were observed in UV light and the UV absorbing bands were ringed with pencil.

d. Expression of enzyme activity.

After separation of the radioactive substrate (TdR) and product (dTMP) of the TdR kinase reaction, each cellulose acetate strip was cut into 22 $\frac{1}{2}$ x 5 cm. bands and each band was counted for 1 minute in toluene scintillator.   The electrophoretic separation of a typical radioactive assay which was carried out using 26 hour artichoke tissue and the second method of assay is shown in Fig. 2.6. The results of a control estimation (no enzyme) are shown as a dotted histogram in the dTMP region only of the electrophoretic separation.   The results for the 26 hour assay show that the product of the reaction (dTMP) is well separated from the substrate (TdR) which remains on the baseline.   The TdR kinase activity of this sample was estimated as follows.   For the 26 hour assay the

FIG. 2.6 Separation by cellulose acetate electrophoresis of the radioactive substrate and product of the TdR kinase assay for 26 hour tissue.
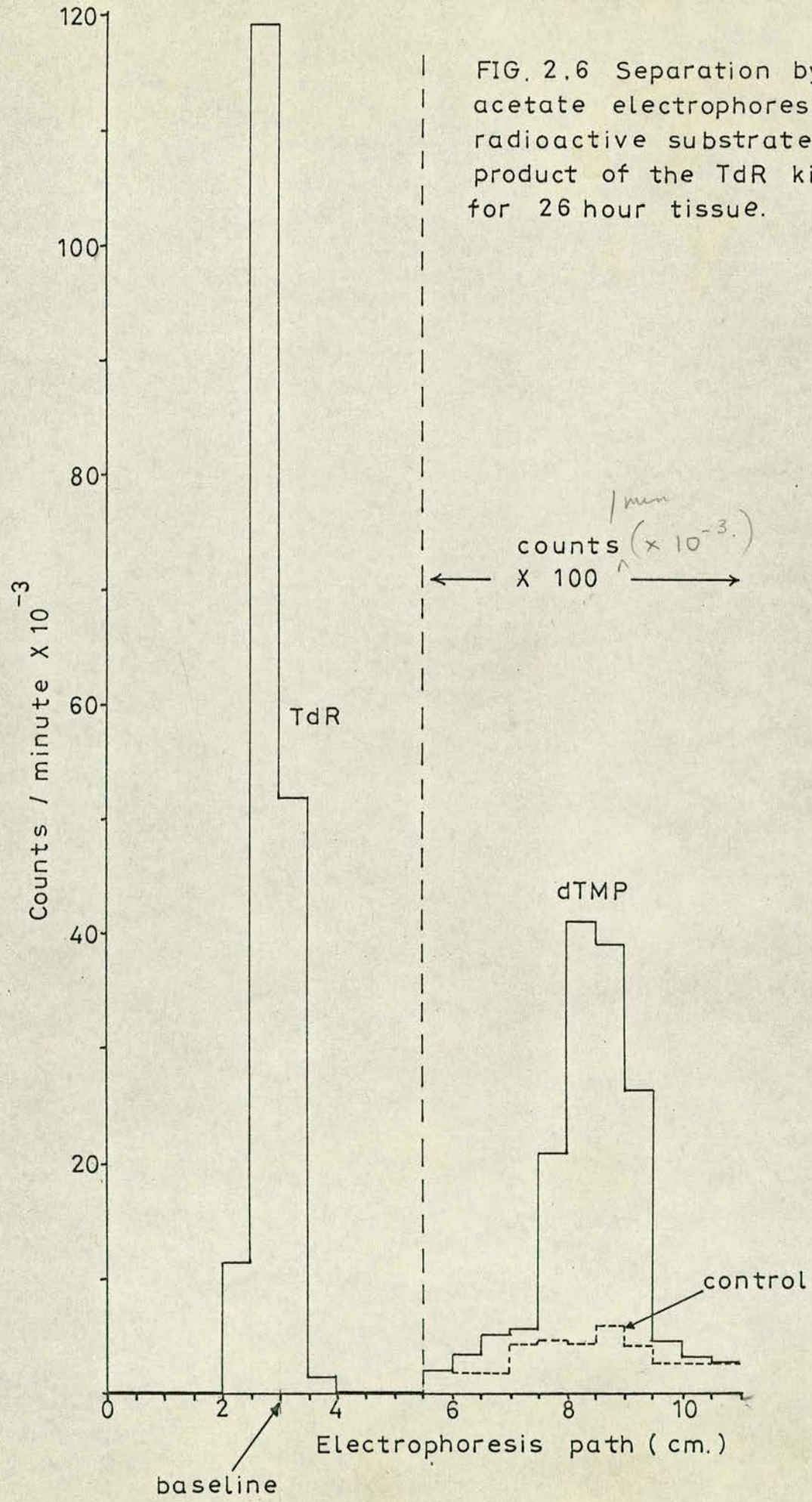
percentage of the total counts in the dTMP peak was estimated:-

	counts/minute	% of total counts in dTMP
TdR (strips 1 - 10)	183,962	
dTMP (strips 11 - 22)	1,620	0·87
Total counts	185,582	

For the control assay the percentage of the total counts in the same position as the dTMP peak was estimated:-

	counts/minute	% of total counts in region of dTMP
TdR (strips 1 - 10)	127,896	
dTMP region (strips 11 - 22)	405	0·32
Total counts	128,301	

When the 26 hour conversion is corrected for the control, the following activity is obtained 0·87 - 0·32 = 0·55%. Hence the TdR kinase activity of this preparation represents 0·55% conversion of TdR to dTMP.

It is possible to express this value in terms of p moles of TdR converted/20 minutes/0·1 ml. of extract using the relationship

$$p \text{ moles} = x \times \frac{\% \text{ conversion}}{100}$$ (where x = total number of p moles of TdR originally present in the reaction mixture. This includes unlabelled TdR).

For the 26 hour sample, each individual assay originally contained 3930 p moles of TdR. Therefore the TdR kinase activity of this example is $\frac{0·55}{100} \times 3930 = 21·6$ p moles of TdR converted/ 20 minutes/0·1 ml. of extract.

This expression of enzyme activity was used in all the cell cycle experiments (Ch. 4). Although several TdR kinase assays were carried out using 0·05 ml. of extract instead of 0·1 ml., the activity in terms of p moles was always expressed in terms of 0·1 ml. of extract to enable direct comparisons to be made between the different experiments.

At this point it is relevant to note that the control sample shown in Fig. 2.6. also exhibits a small peak in the region of dTMP. The percentage of the total counts which this small peak represents tends to increase on storage of the radioactive medium. Therefore it was important that control estimations were included in every experiment. According to Evans and Stanford (1963), aqueous solutions of TdR tend to decompose at a rate of 1 - 2% per month. These workers found that after storage of an aqueous TdR solution (specific activity 0·26 Ci/mM) for 39 months at -40°C, the final composition of the solution was 35% thymidine-T (tritiated), 55% thymine-T and 10% 2-deoxyribose-T.

The identity of the substance observed in the same position as dTMP in control samples is uncertain. As thymine remains on the baseline during cellulose acetate electrophoresis (see Ch. 3) and 2-deoxyribose is unlikely to occupy the same position as dTMP, the identity of the contaminant remains a mystery.

5. <u>Thymidine monophosphate kinase</u> (dTMP kinase)

a. <u>Preparation of extract</u>.

The extract was prepared as in the second method of extract preparation quoted for TdR kinase.　This method was used for Experiments 10, 12, 13 and 14 of Ch. 4.　In Experiment 9 however, the procedure was identical except that the grinding buffer contained 1% β-mercaptoethanol.

b. <u>Enzyme assay</u>.

The radioactive substrate was thymidine (methyl-T)-5' mono-phosphate (i.e. dTMP labelled with ^{3}H in the methyl position), supplied at a specific activity of 1000 mCi/m mole.

The dTMP kinase reaction is dependent on a continued supply of ATP and in addition to supplying excess ATP, an ATP generating system was used.　The ATP generating system employed in this case was the same as that used for the TdR kinase assay, i.e. the break-down of phospho-enol pyruvate by pyruvate kinase.　All reactions were carried out in pyrex conical centrifuge tubes.

The assay mixture contained 50 μl of 'Medium C' (the composition of which is shown in Table 2.g.), 10 μl. of pyruvate kinase (10 mg./ml) and 50 μl. of enzyme extract (added last).　The final composition of the assay mixture is shown in Table 2.h. After a 20 minute incubation in a shaking water bath at 37°C the reaction was stopped by placing the reaction tubes in a boiling water bath for 2 minutes.　After cooling in ice,a 1000 g. centri-fugation for 5 minutes at 0°C was carried out to separate the

Materials added	Amounts added
0·4M phosphate buffer pH 8·0	2·5 ml.
0·1M $MgCl_2$	0·025 ml.
ATP	24·8 mg.
phospho-enol pyruvate	8·4 mg.
dTMP-methyl-T	0·5 mCi

Distilled water to a total volume of 5 ml.

Final concentration of phosphate buffer is 0·2M

Table 2.g.   Composition of 'Medium C' used in the
dTMP kinase assay.

Materials Present	Amount Present	Final Molarity
*Bovine serum albumin	20 μg. protein	-
*β-mercaptoethanol	2·25 μ mole	20·5mM
*sodium phosphate buffer pH 8·0	10 " "	)))181·8mM)
sodium phosphate buffer pH 8·0	10 " "	
$MgCl_2$	0·025 " "	0·2mM
ATP	0·41 " "	3·7mM
Phospho-enol pyruvate	0·25 " "	2·3mM
dTMP-methyl-T	0·5 n mole	) 4·5 μM) specific) activity) 1 μCi/n mole
phospho-enol pyruvate kinase	100 μg. protein	-
Extract to a total volume of	110 μl.	

Table 2.h. Final composition of the medium used to assay
dTMP kinase.

supernatant from precipitated proteins.   The reaction tubes were stored in a deep freeze at -20°C.

The product of the reaction (dTDP) was separated from excess substrate (dTMP) by high voltage electrophoresis of a 30 μl. aliquot of the supernatant.   Electrophoresis was carried out for 1 hour at a potential difference of 1000 volts on Whatman No. 3MM chromatography paper using 0·05M ammonium formate buffer pH 3·5. The high voltage electrophoresis procedure is described in more detail at the end of the method.   Each electrophoresis path was cut into 24 strips each 1 cm. wide.   Each 1 x 6 cm. strip was cut into two (1 x 3 cm.), each half folded, and both halves completely immersed in 10 ml. of toluene scintillator.   Each sample was counted for 1 minute using a scintillation counter.

The expression of enzyme activity is described with reference to an example after the detailed description of the high voltage electrophoresis procedure.

Thymidine monophosphate phosphatase (dTMP phosphatase)

On counting the electrophoresis papers from the dTMP kinase assay it was found that a certain proportion of the counts in dTMP had been converted to thymidine (TdR).   This activity, although suppressed as far as possible by the use of a high concentration of phosphate buffer at a high pH in the reaction mixture, was quantitatively valid (see Ch. 3. Sect. C), and could be measured together with the activity of dTMP kinase.   It must be emphasised however that due to the suppressive effect of the buffer, dTMP

phosphatase was not being assayed under optimal conditions.

High voltage electrophoresis.

The electrophoresis buffer was 0·05M ammonium formate adjusted to pH 3·5 with formic acid. Complete dissociation of phosphate groups occurs at pH 3·5 and during subsequent electrophoresis, these negatively charged molecules tend to move towards the anode.

Electrophoresis papers 18 x 5½ in. were prepared using Whatman No. 3MM chromatography paper. A baseline was drawn in pencil 7½ in. from one end and the paper was folded 6½ in. from the same end. Solutions were applied to the baseline with a micropipette and the spots or streaks were dried with hot air. A 7 μl. marker dye spot was often included. Using a pasteur pipette each spot was ringed with buffer to concentrate the spot, and the rest of the paper was wetted in a tray of buffer, blotted between sheets of Whatman No. 1 paper and placed in the electrophoresis tank. Solutions which had been streaked onto the baseline were concentrated onto the baseline by initial parallel applications of buffer to either side of the streak.

A diagram of the electrophoresis tank in side view is shown in Fig. 2.7. The apparatus consisted of a large outer and a small inner glass tank. The small tank was elevated from the base of the large tank by means of glass supports. The large tank contained a small quantity of buffer (2 in. depth) and the small tank was
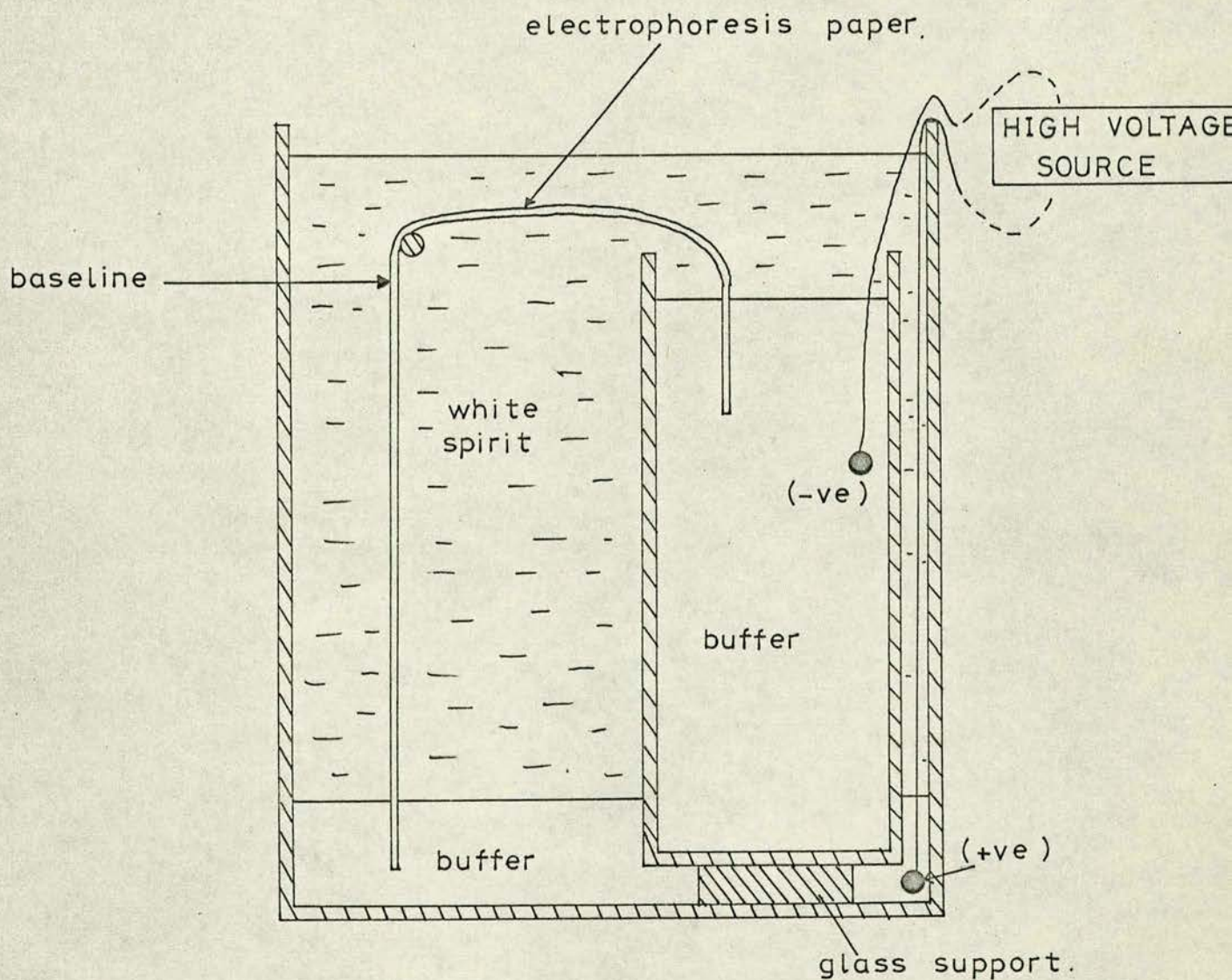
FIG. 2.7 Diagram of the high voltage
electrophoresis apparatus in
side view.

almost filled with buffer. The intervening space was filled with white spirit, which was non conductive and also absorbed the heat generated during electrophoresis. Each buffer compartment was furnished with a platinum wire electrode. The electrophoresis paper was placed in the tank with the fold supported by a glass rod, and with the baseline nearest to the cathode (small tank), so that there was approximately a 2 in. length of paper in each buffer compartment.

The tank, which was set inside a specially designed fume cupboard was connected to a high voltage power source which was also connected in parallel to a circuit in the window of the fume cupboard, so that the full circuit operated only when the fume cupboard was closed. The electrophoresis was carried out at room temperature at a potential difference of 1000 volts and the progress of electrophoresis was observed by the separation of the marker dye. After the power had been switched off, the paper was removed from the tank, dried in air and observed under ultra violet (UV) illumination. Any UV absorbing standard spots were ringed with pencil.

Expression of enzyme activity.

After separation of the radioactive substrate (dTMP) and product (dTDP) of the dTMP kinase reaction by high voltage paper electrophoresis, each electrophoresis path (6 cm. wide) was cut into 24 1 x 6 cm. strips and counted as previously described. [Each electrophoresis paper, approximately 14 cm. wide, could accommodate two 6 cm. streaks of different samples on the baseline

separated by a 2 cm. gap]. The radioactive separation by high voltage electrophoresis of a typical radioactive assay which was carried out using 22 hour artichoke tissue is shown in Fig. 2.8. The results of a control estimation (no enzyme) are shown by a dotted histogram in the dTDP/dTTP region, and indicate that the original preparation of dTMP contained a very small percentage of a contaminant which is probably dTTP. The original preparation also contained a small percentage of TdR which was presumably a consequence of the enzymic preparation of dTMP from TdR by Radio-chemicals.

The product of the dTMP kinase reaction in this sample and in all other samples prepared from artichoke tissue was exclusively dTDP. The dTMP kinase and dTMP phosphatase activities of this sample were estimated as follows:

For the 22 hour sample the percentage of the total counts in the dTMP and TdR peaks was estimated:-

	counts/minute	% of total counts in dTDP/dTTP or TdR
TdR (strips 1 - 4)	8,419	5.79 in TdR
dTMP (strips 5 - 13)	135,665	
dTDP/dTTP (strips 14 - 24)	1,322	0·91 in dTDP/dTTP
Total counts	145,406	

For the control assay, the percentage of the total counts which were in the same position as dTDP/dTTP were added and expressed as a percentage of the total counts. This procedure was repeated for the TdR peak.
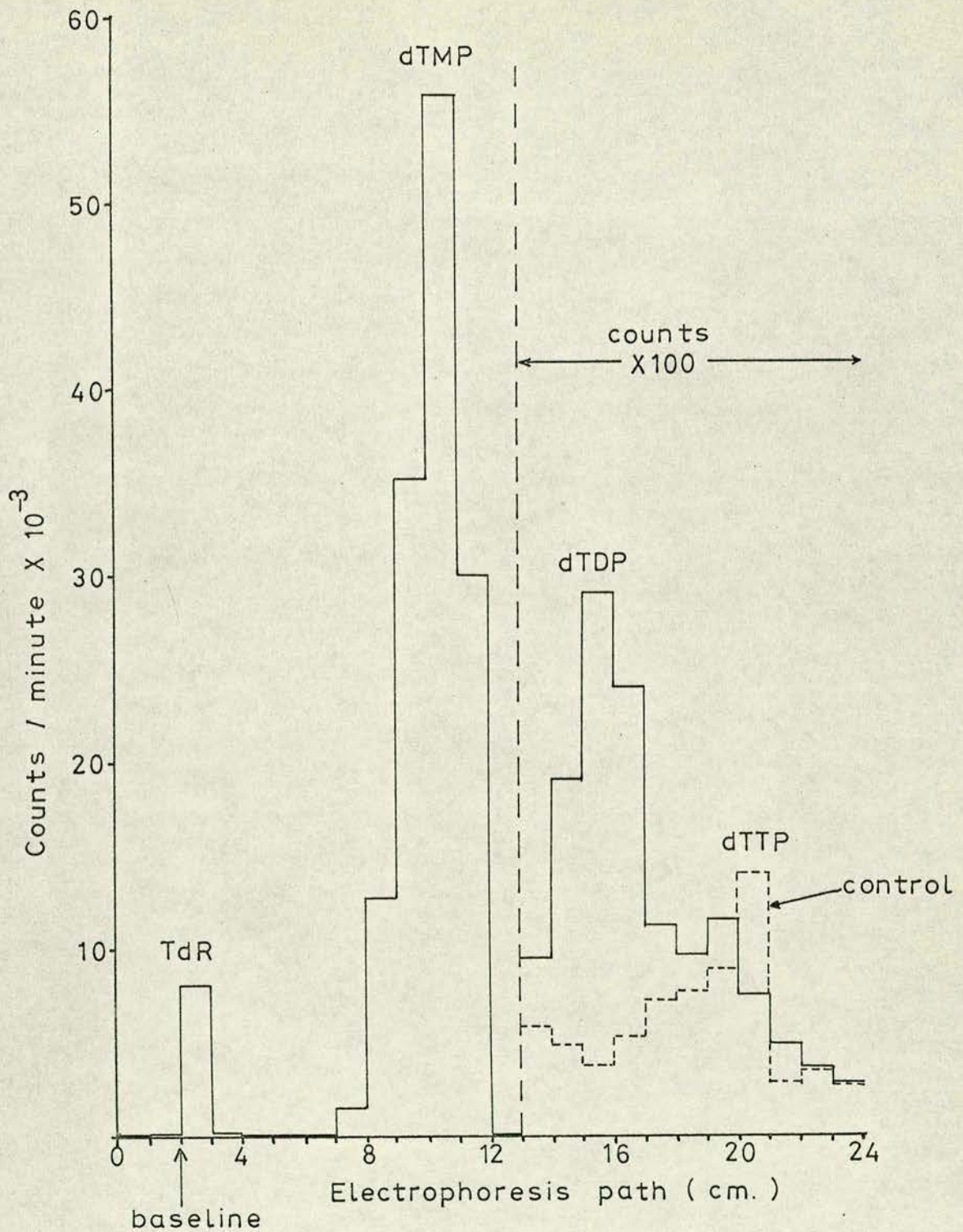
FIG. 2.8 Separation by high voltage paper electrophoresis of the substrate and products of the radioactive dTMP kinase (and dTMP phosphatase) assay for 22 hour tissue.

	counts/minute	% of total counts in dTDP/dTTP or TdR regions
TdR (strips 1 - 4	5,440	3·38 in TdR
dTMP (strips 5 - 13)	155,048	
dTDP/dTTP (strips 14 - 24)	679	0·42 in dTDP/dTTP
Total counts	161,167	

When the 22 hour conversion is corrected for the control, the following activities are obtained.

dTMP kinase   0·91 - 0·42 = 0·49% conversion of dTMP

dTMP phosphatase   5·79 - 3·38 = 2·41%   "   "   "

It is possible to express these activities in terms of p moles of dTMP converted/20 minutes/0·05 ml. of extract using the relationship

$$\text{p moles} = x \times \frac{\% \text{ conversion}}{100}$$ (where x = the total number of

p moles of dTMP originally present in the reaction medium).

In this case, each individual assay originally contained 5000 p moles of dTMP.   Therefore the following activities were calculated.

dTMP kinase $= 5000 \times \frac{0·49}{100} = 24·5$ p moles dTMP converted/20 min./
0·05 ml. of extract.

dTMP phosphatase $= 5000 \times \frac{2·41}{100} = 120·5$ "   "   "   "   "   "

This expression of enzyme activity was used in all the cell cycle experiments (Ch. 4).

Although the control value did not change on storage of the

substrate as it did for TdR, blank estimations were carried out for each individual experiment in case of variations in the pattern of electrophoresis.

SECTION E

SCINTILLATION COUNTING

The weak β emission from compounds labelled with tritium (^{3}H) can be most efficiently measured using scintillation counting techniques.   As ^{3}H labelled compounds were frequently used in enzyme assays and for measurement of the rate of DNA synthesis, this method was employed extensively.   The two types of scintillation fluid used in this investigation were toluene scintillator and dioxane scintillator.

Toluene scintillator.

Toluene scintillator containing 5 gm. of 2,5-diphenyloxazole (PPO) and 0·3 gm. 1,4-bis-[2-(4-methyl-5-phenyloxazolyl)]-benzene (POPOP) per litre of toluene, was used to estimate radioactivity of non-aqueous samples such as cellulose acetate strips (e.g. TdR kinase assay) and paper strips (e.g. dTMP kinase assay).

For counting, non-aqueous samples were always completely immersed in the scintillator solution as the results of a preliminary experiment counting cellulose acetate strips had shown that when a 5 x 0·5 cm. strip (as in the TdR kinase assay), impregnated with dTMP is counted 'standing up' (i.e. with 2/3 of its length projecting above the solution), that a 15·5% increase in the average value of 10 repeated 1 minute counts is obtained on complete immersion.

Average count/min. standing up (mean of 10 readings) = 695·8
    "   "   " completely immersed (" " " ") = 804·0
Percentage increase in efficiency of counting   = 15·5%

When the radioactivity of cellulose acetate or paper strips
is counted using scintillation fluid there is inevitably some loss
of counts (quenching) due to absorption of light by the cellulose
acetate or paper.   This quenching factor was maintained at a
constant value in the TdR kinase and dTMP kinase assays by using
cellulose acetate or paper strips of a standard size and by placing
these into the scintillation fluid in a similar configuration each
time.

e.g. for the ThR kinase assay

cellulose acetate strip
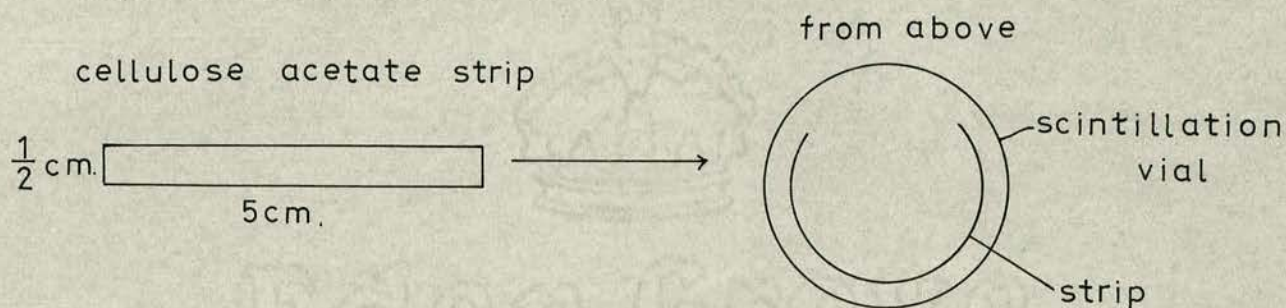
e.g. for the dTMP kinase assay.

two paper strips
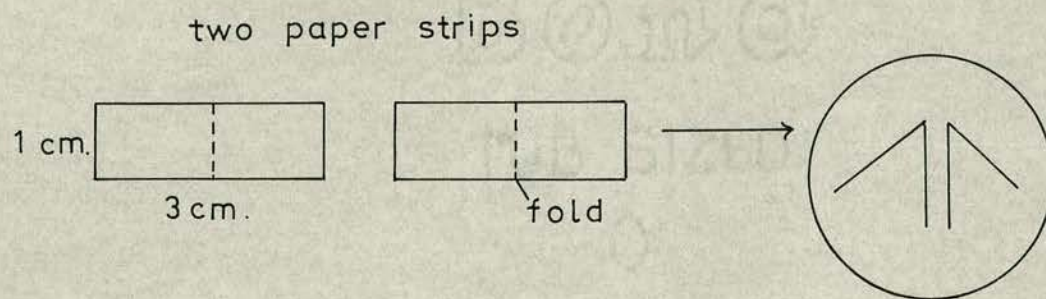
Dioxane scintillator.

Dioxane scintillator was used to estimate the radioactivity
of solutions and had the following composition:-

For 1 litre

> 386·6 ml. toluene
>
> 386·6 ml. 1,4 Dioxane
>
> 233   ml. methanol
>
> 80·6 gm. naphthalene
>
> 5·0 gm. PPO
>
> 0·086 gm. POPOP.

In all instances 0·5 ml. of the aqueous solution to be counted was mixed with 15 ml. of dioxane scintillator.

Loss of counts by quenching can occur in a number of different ways using this scintillator, but the major problem encountered in this investigation was that of acid or alkali quenching. For this reason, all acid or alkaline solutions were neutralised prior to addition of scintillator. As the degree of quenching could not be easily measured using a scintillation counter without an external standard, an attempt was made to overcome this problem by ensuring that the degree of quenching was similar in all samples by neutralizing with a standard volume of acid or alkali. A further safeguard was present in that very acid solutions often produced a green colour in the scintillator, whilst very alkaline solutions produced a cloudy solution.

As the count rate in dioxane scintillator is affected by light the samples were always placed in the refrigerated counter in the dark for 10 minutes before counting ensued, to minimise this effect.

The scintillation counter.

The scintillation counter used in this investigation was a

Packard tricarb model 3003 liquid scintillation spectrometer.

^{3}H was counted at a window setting of 50 - 1000 and a gain of 60%.

^{14}C "   "   " "   "   "   " 50-1000 and a gain of 6%.

Using the above settings for ^{3}H, and an ^{3}H standard in toluene scintillator, it was estimated that this particular counter was approximately 34·5% efficient for counting ^{3}H and 80·8% efficient for counting ^{14}C.

To compare the efficiencies of counting ^{3}H in dioxane scintillator, on standard cellulose acetate strips in toluene scintillator and on standard paper strips in toluene scintillator, a standard ^{3}H-TdR solution was prepared containing approximately 0·001 mCi/10 µl. 10 µl. samples of this solution were spotted in triplicate directly into scintillation vials, onto cellulose acetate strips and onto pairs of folded paper strips.

10 ml. dioxane scintillator was added to the vials containing solution and when the cellulose acetate and paper strips had dried, these were immersed in 10 ml. toluene scintillator as previously described.

The following results were obtained.

	counts/minute			
	Sample 1	Sample 2	Sample 3	Average
Dioxane	404628	391952	392106	396228
Cellulose acetate	326503	331534	313425	323821
Paper	67224	77379	73757	72787

If the counting efficiency of dioxane scintillator is given a value of 100 then in comparison, the efficiency of counting on cellulose acetate strips is $81 \cdot 8\%$ and the efficiency of counting on paper is $18 \cdot 4\%$.   Therefore on cellulose acetate strips, ^{3}H can be counted approximately $4 \cdot 45$ x more efficiently than on paper. This figure is important as it indicates the relative counting efficiencies used in the TdR kinase and dTMP kinase assays.

Decontamination of scintillation vials.

After removal of cellulose acetate or paper strips, vials containing toluene scintillator were recounted and if the count rate was less than 35 counts/minute the vials were retained for reuse.   Contaminated toluene vials, or vials containing dioxane scintillator were decontaminated by washing several times with water (radioactive sink) before immersion in a solution of Decon 75 (20 mls./litre tap water) for at least 2 days.   The vials were rinsed thoroughly in tap water and distilled water before drying in a hot air oven.   Lids of the scintillation vials containing plastic inserts were also treated as above and after rinsing were left to dry on paper towels.

CHAPTER 3

EXPERIMENTAL RESULTS (I)

THE DEVELOPMENT OF QUANTITATIVE ENZYME ASSAY METHODS
SUITABLE FOR USE WITH ARTICHOKE TISSUE

A vital prerequisite to an investigation into changes in enzyme activities during the cell division cycle is the development of quantitatively valid assay methods for each of the enzymes to be studied.

In this chapter, experiments culminating in the successful establishment of quantitative assay methods for the enzymes 'native'-DNAase, 'denatured'-DNAase, TdR kinase and dTMP kinase are described. It is considered important also to include methods which although successful with other plant tissues were shown to be unsuitable for artichoke tuber tissue, because it demonstrates the importance of relating the method of enzyme assay to the selected tissue.

The method which was used to assay DNA polymerase activity was shown to be quantitatively valid in artichoke tissue by Jackson (1970), and the complete method was described in Chapter 2.

SECTION A

DEOXYRIBONUCLEASE

Deoxyribonuclease (DNAase) is responsible for the enzymic breakdown of DNA by tissue extracts. However, the complete breakdown of the DNA molecule often relies upon a combination of two different enzymes with different substrate specificities. Enzymes which attack the ends of the molecule releasing individual nucleotides are known as exonucleases, whilst enzymes which attack at sites which are remote from the ends of the molecule releasing oligonucleotides are known as endonucleases. Some enzymes may possess both of these capacities.

The majority of enzyme assay methods for DNAase depend on the release of acid soluble products and hence are directed towards the measurement of exonuclease action. For example, Srivastava (1968) used a method based on the direct measurement of ultra-violet absorbing reaction products for the estimation of DNAase activity in barley leaves, whilst Sheldrake and Northcote (1968) used a similar method to estimate DNAase activity in xylem sap. Stern (1961) however, employed a colorimetric determination of the products of DNAase action in Lilium anther tissue, whilst Walther and Edmunds (1970) used a radiochemical estimation of acid soluble products to measure DNAase activity in Euglena.

This section describes experiments leading up to the successful development of a radiochemical assay method suited to the measurement in artichoke tissue of the enzymic breakdown of 'native' -

DNA by 'native'-DNAase and the breakdown of denatured -DNA by 'denatured'-DNAase.   Before this successful radiochemical method was developed however, two non-radiochemical enzyme assay methods were shown to be unsuitable for the measurement of DNAase activity in artichoke tissue.

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by 'denatured'-DNAase.   Before this successful radiochemical
method was developed however, two non-radiochemical enzyme
assay methods were shown to be unsuitable for the measurement of
DNAase activity in artichoke tissue.

DNAase Assay (According to Allfrey & Mirsky (1952))

Assay medium

0·5 ml. substrate (4 mg./ml. Sigma type V, highly polymerised calf thymus DNA made up in 0·2M acetate buffer pH 5·2)

1·5 ml. 0·2M acetate buffer pH 5·2.

1·0 ml. enzyme extract.

3·0 ml. total volume

The reaction mixture was allowed to equilibrate at 35°C for 5 minutes prior to addition of the extract. After a 2 hour incubation at 35°C the reaction was stopped by the addition of 0·8 ml. of 3·6M trichloro-acetic acid (TCA), to precipitate excess DNA. After a 3000 g. centrifugation at 0°C for 30 minutes, the supernatant fraction which contained acid soluble reaction products was decanted into fresh tubes.

Control

Tubes containing buffer + extract only were incubated for 2 hours, followed by the addition of 0·5 ml. of substrate and 0·8 ml. of 3·6M TCA at the same time.

The diphenylamine reaction

3·0 ml. diphenylamine reagent (freshly made up by adding 1 ml. of conc. H_2SO_4 to 40 ml. 1% diphenylamine in glacial acetic acid)

1·0 ml. supernatant from the enzyme assay

0·5 ml. distilled water.

Tubes were placed in a water bath at 100°C for 20 minutes and then allowed to cool at room temperature. Optical density at 600 mμ and 650 mμ was measured against a reagent blank (1·5 ml. H_2O + 3 ml. diphenylamine reagent) using an SP.500 spectrophotometer and 4 cm. light path cells.

Calibration of the method

The diphenylamine reaction was calibrated in terms of μg. of DNA, using a stock solution containing 15 mg. calf thymus DNA/100 ml. of buffer.

1. The Dische diphenylamine method.

Deoxyribonuclease (DNAase) activity has been measured in anther tissue of <u>Lilium longiflorum</u> by Hotta and Stern (1961) using the method of Allfrey and Mirsky (1952). This method is based on the determination of the acid soluble deoxyribose products of the enzyme reaction using Dische's reagent and is dependent on the development of a blue colour in the presence of diphenylamine. Before attempts were made to adapt this method to artichoke tissue, preliminary tests for DNAase activity were carried out using pea root tips and wheat embryos because meristematic tissues are known to be a rich source of hydrolytic enzymes.

DNAase activity in pea root and wheat embryo tissue.

Peas of var. Meteor, were planted in a pie dish in damp vermiculit (vermiculite:water approx. 2·5:1), covered with a glass lid and left for 48 hours at 25°C in the light. After harvesting the roots were washed and the first 5 mm. of the root tip removed. Root tips were homogenised with a pestle and mortar, at a concentration of 40 mg. of fresh weight tissue/ml. cold 0·2M acetate buffer pH 5·2. The homogenat was filtered to remove debris and the clear solution retained as the enzyme extract.

Wheat seeds (var. Procter) were placed in batches of 50 in petri dishes lined with 3 filter papers, each containing 7 ml. of distilled water, and placed in the dark at 25°C for 48 hours. Embryos were removed after harvesting the seedlings and were homogenised as describe above. The final filtrate was retained as the enzyme extract.

The DNAase assay (according to Allfrey and Mirsky (1952)) is shown opposite. In this experiment, 3 different concentrations of

extract were used (0·5 ml., 1·0 ml., and 1·5 ml.) and controls were carried out for the 1·0 ml. concentration of pea extract only. Optical density at 600 and 650 mμ was recorded for each extract and in both cases the OD. 650 mμ was very much lower than the OD. 600 mμ. The relationship between OD. 600 mμ and volume of extract is shown in Fig. 3.1. for the pea and wheat extracts. This relationship is approximately linear in both cases, and as a low control value of 0·034 was obtained for pea extract, this method provides a valid estimation of the DNAase activity of pea extracts.

The absorption spectrum (obtained using the S.P. 800 spectro-photometer) of the colour developed in both reactions is shown in Fig. 3.2. for the most concentrated extracts. For pea extract, the blue colour exhibits an absorption maximum at 600 mμ whilst the colour developed using wheat extract has a violet tinge and an absorption maximum at about 582 mμ. From the results of this experiment, it was concluded that DNAase activity was present in pea extract, but that some other substance present in the extract from wheat, which was not DNA, was interfering with the development of the colour.

Preliminary assay for DNAase activity in artichoke tuber tissue.

In an attempt to detect DNAase activity in artichoke tissue, a similar experiment to the one described above was carried out using extracts prepared from fresh artichoke tissue (0 hour) and from asynchronously dividing tissue which had been cultured for 5 days. The extracts were prepared at a concentration of 40 mg. fresh weight tissue/ml. buffer using a glass homogeniser instead of a
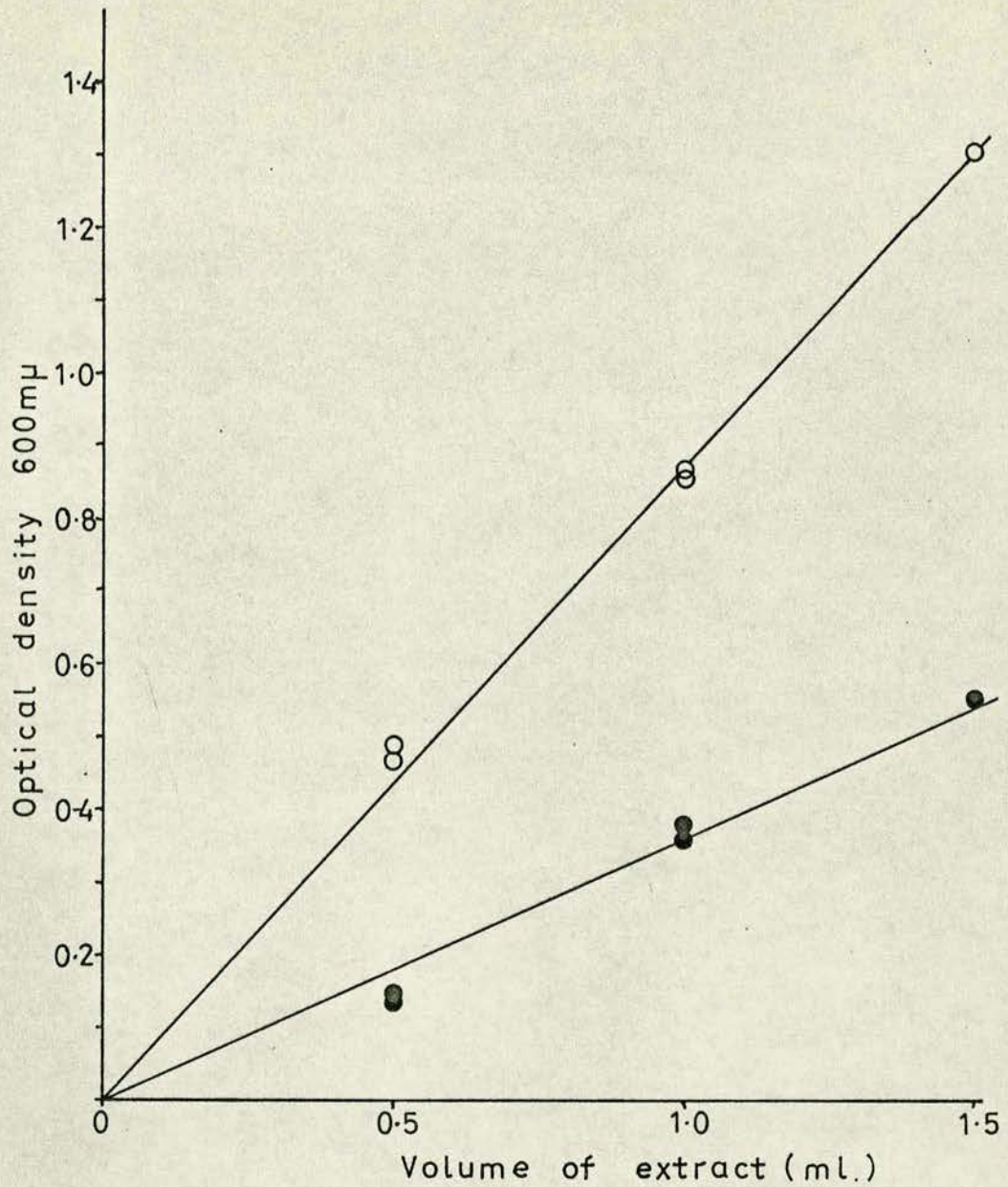
FIG. 3 .1   The relationship between OD. 600mμ and volume of wheat (O) and pea (●) extract for the DNAase assay based on the diphenylamine reaction.
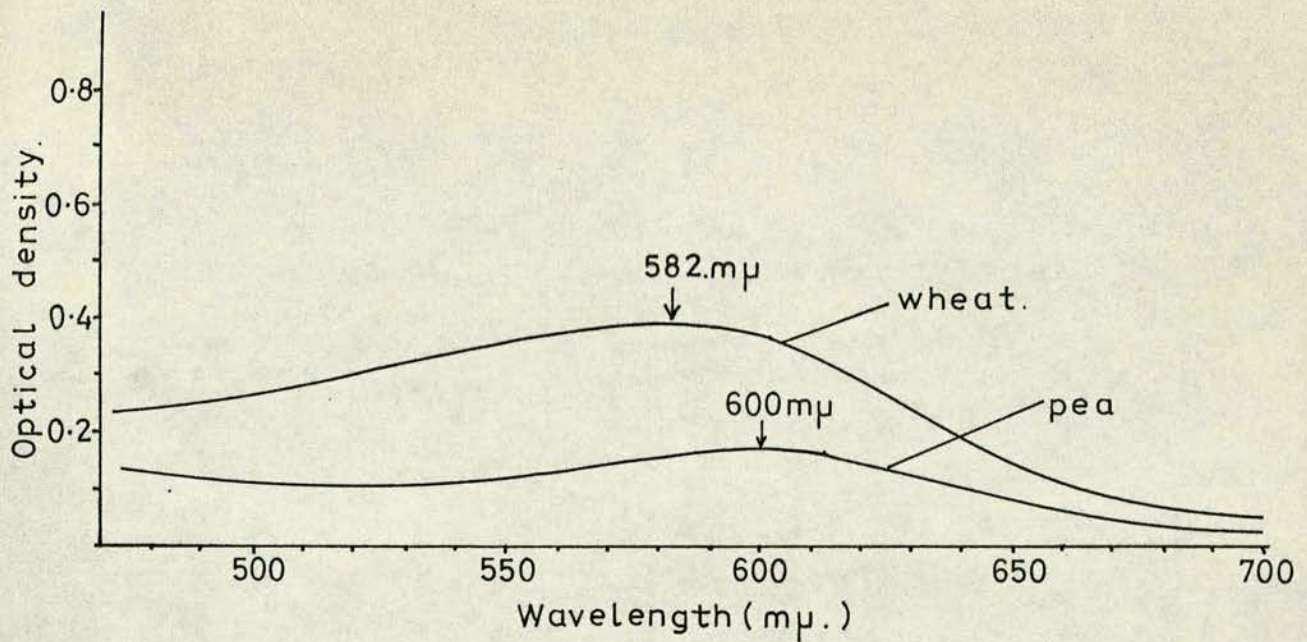
FIG. 3.2 Absorption spectrum of the colour developed with diphenylamine reagent for DNAase assay of pea and wheat extracts.
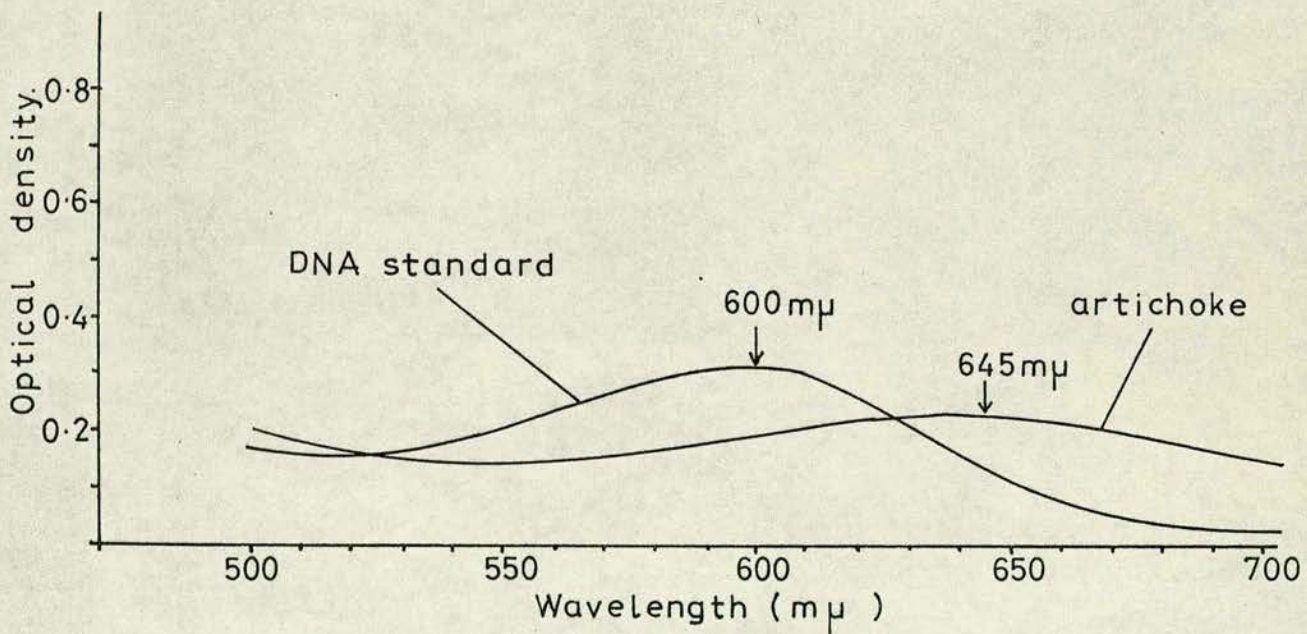
FIG. 3.3 Absorption spectrum of the colour developed with diphenylamine reagent for a DNA standard and for DNAase assay of 0 hour artichoke extract.

pestle and mortar, and the homogenates were filtered to remove debris. As in the previous experiment, 3 different concentrations of extract were used and optical densities recorded at 600 and 650 mμ. The results for the 0 hour extract, are shown in Table 3.a. The results for the day 5 extract were similar. From these results it is immediately apparent that the optical density at 650 mμ is greater than the optical density at 600 mμ and that the activity of the samples containing 1·0 ml. extract is not significantly different from the corresponding control.

From the results of this experiment, it was concluded that the presence of a contaminant (probably fructose) was interfering with the diphenylamine reaction.

In a similar experiment in which 0 hour artichoke tissue was homogenised using a pestle and mortar at a concentration of 200 mg. tissue/ml. buffer similar results were obtained. The absorption spectrum of a reaction carried out using 2·5 ml. of this extract is compared with that of a DNA standard in Fig. 3.3. and exhibits an absorption maximum in the region of 645 mμ.

An attempt was made to minimise the effect of this contaminant by employing a freezing technique similar to that used by Robinson and Brown (1952) for the assay of acid phosphatase in root sections. Explants were immersed in buffer in a petri dish and placed in a deep freeze at -20°C for 1 hour. During this treatment and the 3 subsequent washes with distilled water, it was anticipated that soluble substances such as the contaminant would diffuse out of the cells into the surrounding medium. The reaction was directed

Volume of extract (ml.)	Optical Density SP.500 (each value is the mean of 2 individual readings)	
	600 mμ	650 mμ
0·5	0·030	0·034
	0·021	0·046
1·0	0·102	0·107
	0·059	0·091
1·5	0·128	0·144
	0·200	0·192
1·0 (control)	0·050	0·074
	0·056	0·078
1·0 (unincubated control)	0·057	0·084

Table 3.a.   The effect of varying the concentration of 0 hour
artichoke extract on the optical density at 600
and 650 mμ of the colour developed with
diphenylamine reagent.

towards the assay of bound DNAase by placing a number of intact frozen explants in a 25 ml. conical flask containing the usual reaction medium, and after a 2 hour incubation in a shaking water bath at $35^{\circ}C$ a $1 \cdot 0$ ml. aliquot of solution was tested using the diphenylamine reaction.   Although the optical density at 600 mμ was proportional to the number of explants used the coloured solution still had an absorption maximum in the region of 650 mμ.

Anticipating that an increased level of DNAase activity might exist in explants which had been cultured for a number of days, a time course experiment was carried out using Day 13 explants. Incubations of 1, 2 and 3 hours were carried out using samples of 3, 6 and 9 frozen explants.   The direct results of this experiment are shown in Fig. 3.4. and the 2 hour control values are also shown. A zero time control was carried out only for the 6 explant series. For the 6 explant series, the OD. 600 mμ increases substantially between 0 and 1 hour but as the 2 hour control value is similar to the experimental value the pattern shown is probably due to release of a soluble contaminant into the reaction medium during the incubation.

The diphenylamine method was finally abandoned due to interference from contaminating substances.

2. The optical density 260 mμ method.

This method relies upon the release of acid soluble products from DNA.   These products which are separated from residual DNA absorb ultra-violet (UV) light at 260 mμ, and this method has been
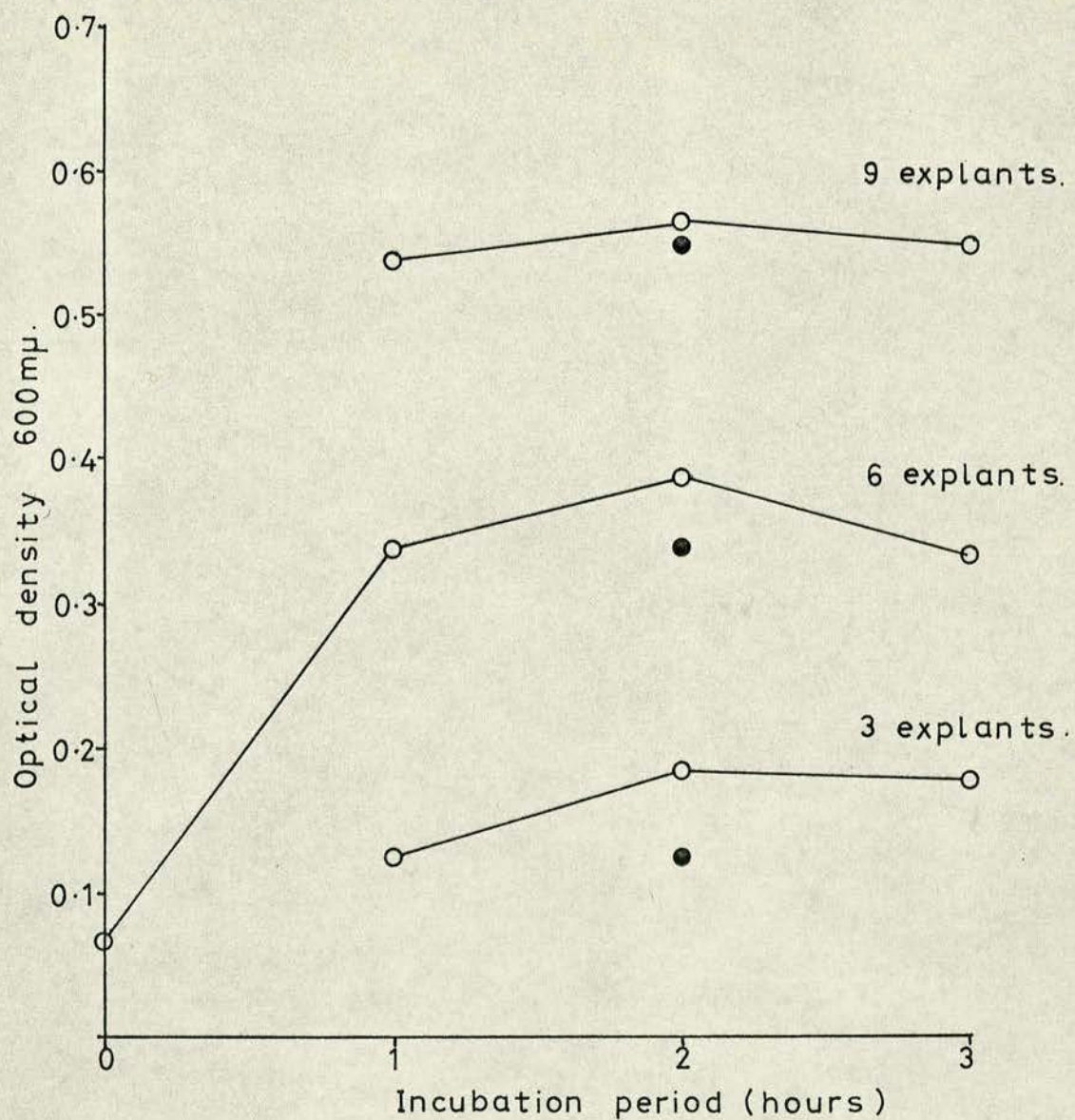
FIG. 3·4 Time course for DNAase activity measured using the diphenylamine method for series of 3, 6 and 9 explants, in relation to the 2 hour control values (●).

used to assay DNAase activity in rat liver tissue by De Duve et al. (1955) and in xylem sap by Sheldrake and Northcote (1968). This method is not as specific for DNAase as the diphenylamine method.

In a typical reaction using plant extract, DNAase action on the DNA substrate yields acid soluble products, but if ribonuclease (RNAase) is also present within the extract a further release of acid soluble products may occur from the breakdown of endogenous RNA. However, this method can be specific for DNAase, provided that a pure DNA substrate is used and that controls incubated without DNA are carried out for every concentration of extract that is used. This procedure corrects for endogenous RNAase activity and other UV absorbing substances present in the extract.

Basically, this method was similar to the previous method but it differed in the mode of stopping the reaction. As TCA has a considerable UV absorption, the reaction was stopped by addition of an equal volume of 10% perchloric acid (PCA). This was followed by a 10 minute period in the cold room to aid precipitation of excess DNA, and the standard centrifugation procedure.

A preliminary experiment was carried out to test the effectiveness of the PCA precipitation. This involved preparation of the following solutions:-

a. 6 ml. 10% PCA (Blank)
b. 0·5 ml. DNA (4 mg./ml.) + 5·5 ml. buffer (0·2M acetate buffer pH 5·2).
c. 0·5 ml. DNA + 2·5 ml. buffer + 3 ml. cold 10% PCA. This solution was treated as described above and the supernatant fraction retained.

The ultra violet absorption measured on the SP.800 spectrophotometer is shown for all 3 solutions in Fig. 3.5., and it is estimated that at 260 mμ, the precipitation of DNA is about 94% efficient. This also indicates that a small proportion (about 6%) of the DNA preparation is acid soluble under these conditions, hence it is important that a control of this type is carried out for every different concentration of DNA that is used.

Because the $OD._{260}$ of artichoke extracts is known to be rather high it was decided to extend the use of the frozen explant technique in order to reduce control values to a minimum.

Preliminary test for DNAase activity in asynchronously dividing tissue.

A preliminary experiment was carried out using groups of 10 frozen Day 18 explants. Two 25 ml. flasks containing 2·5 ml. of buffer (0·2M acetate pH 5·2), 10 frozen explants and 10 glass beads (to increase the depth of solution by displacement) were allowed to equilibrate at $35^{o}C$ for 5 minutes before the reaction was started in one of the flasks by the addition of 0·5 ml. of DNA (4 mg. DNA/ml. 0·2M acetate buffer pH 5·2). After a 2 hour incubation in a shaking water bath, the flasks were transferred to the cold room where DNA was added to the control flask and the contents of both flasks were poured through muslin (to retain explants and glass beads) into plastic centrifuge tubes. After addition of 3·0 ml. of cold 10% PCA, the tubes were allowed to stand in the cold for 10 minutes followed by a 3000 g. centrifugation at $0^{o}C$ for 30 minutes. The optical density of the supernatant fraction at 260 mμ was
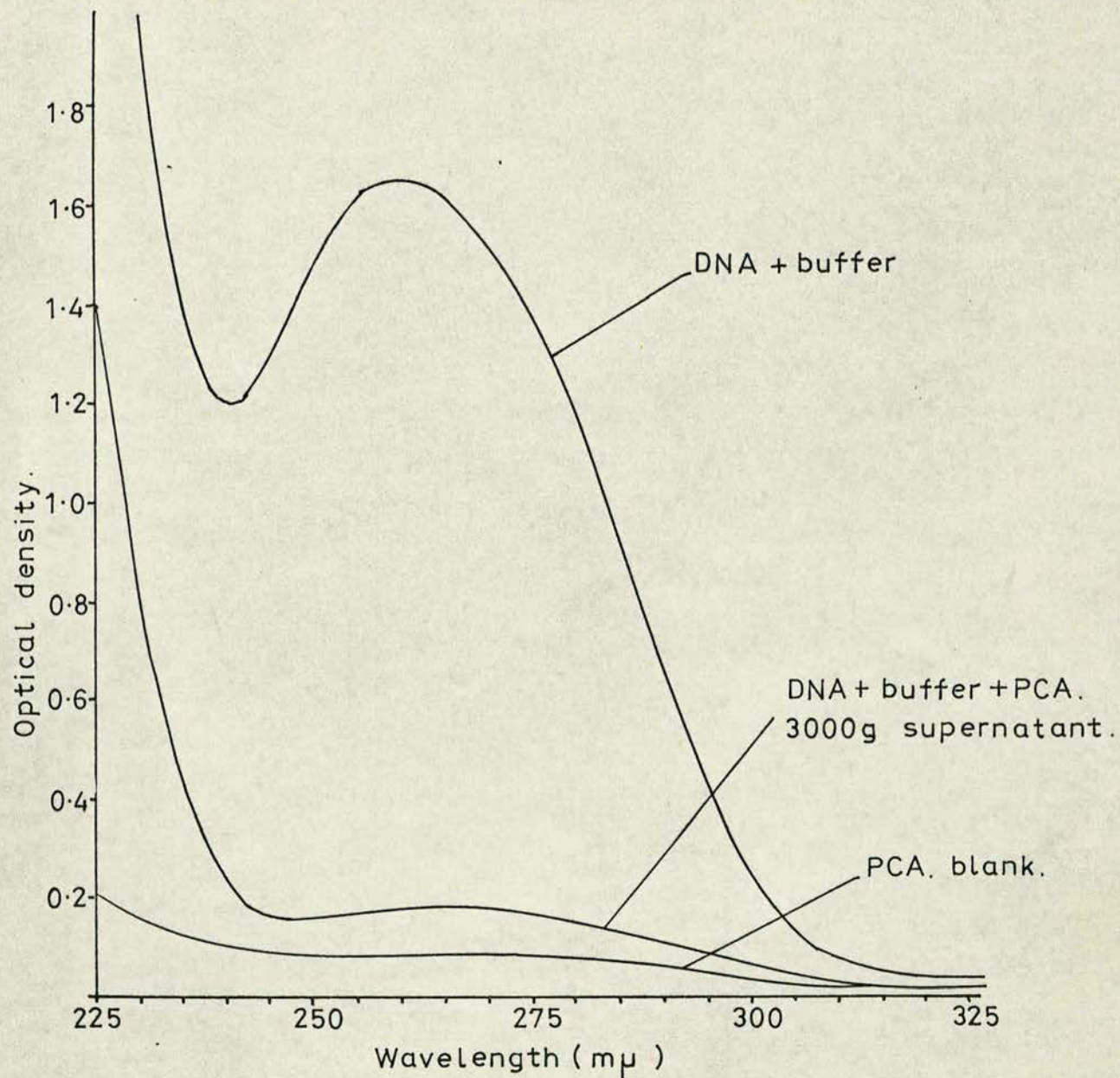
FIG.3.5 The effectiveness of DNA precipitation
using 10 % aq. PCA.

measured using an SP.800 spectrophotometer.

The uncorrected results are shown in Fig. 3.6. and it is evident that some DNAase activity has been detected although the control for endogenous RNAase activity and other contaminants is also high.

Robertson (1966) has shown that RNAase activity of this tissue increases with the length of the culture period and is negligible during the first 3 days of culture. Anticipating that control values might be reduced if fresh artichoke tissue was used, the experiment was repeated using frozen Day 0 explants. A reasonable DNAase activity equivalent to an OD_{260} value of 0·31 was obtained using samples of 10 frozen explants but the control value remained high.

Attempts to reduce the control value included the use of β-mercaptoethanol (EtSH) and the preincubation of frozen explants with a preparation of RNAase in order to reduce the endogenous RNA content.

The treatment with EtSH was designed to prevent the browning of explants often observed during the freezing treatment, but as this substance has a high ultra-violet absorption and could not be removed from the explants by normal washing procedures, it produced even higher control values than before.

Frozen explants which had been preincubated at $35^{\circ}C$ for 30 minutes in a 1 mg./ml. solution of RNAase in buffer, were used for the DNAase assay after 3 washes with buffer. Higher control values than normal were produced. This observation can be explained on
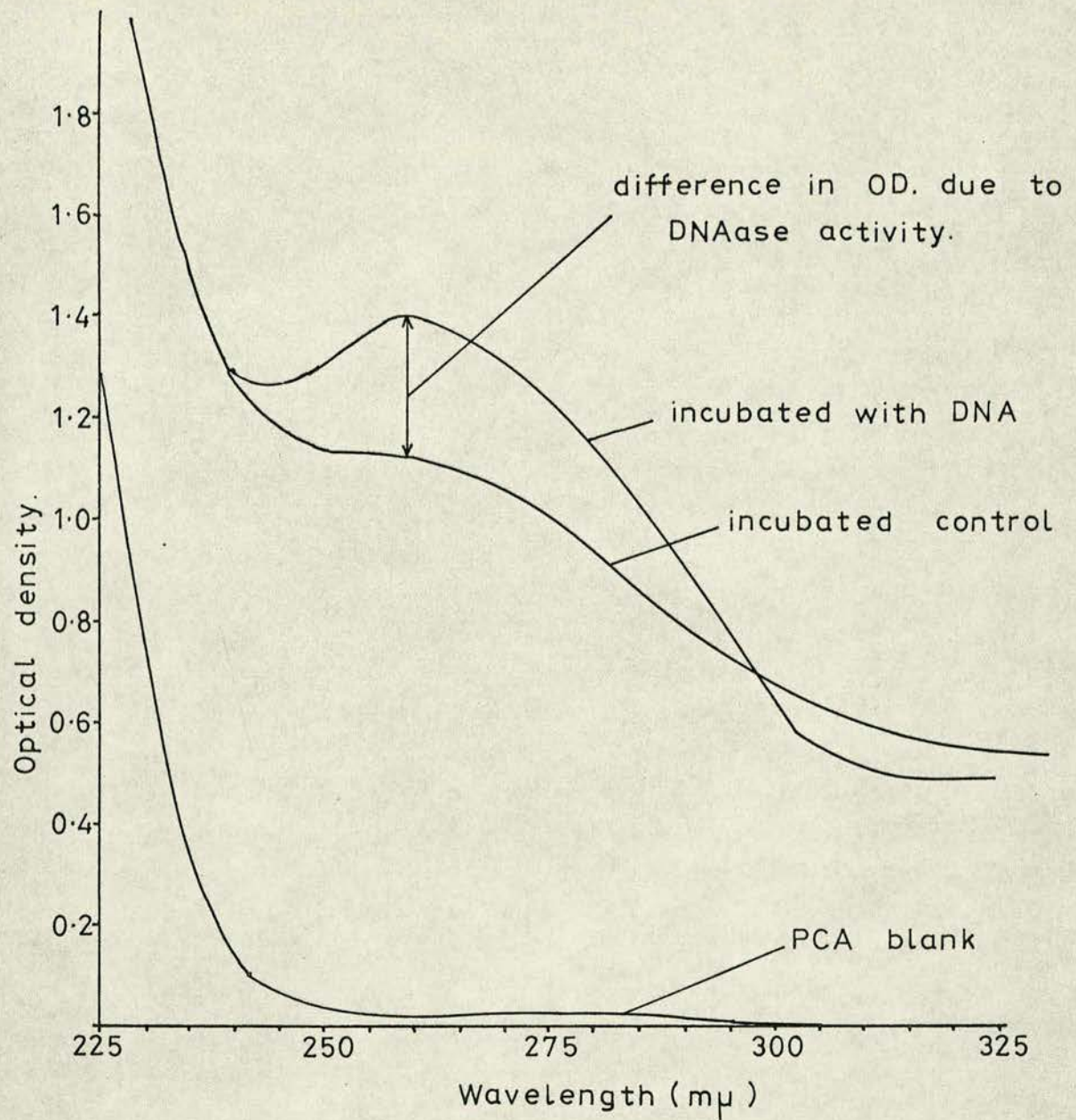
FIG. 3·6   DNAase activity of a sample of 10
day 18 explants.

the basis that the RNAase was retained within the explants and continued to hydrolyse endogenous RNA during the DNAase incubation.

Using the original method, a time course experiment was carried out using groups of 10 frozen Day O explants.   The results of this experiment which are shown in Fig. 3.7. indicate that this method is not sufficiently sensitive to measure accurately the activities obtained for incubation periods of less than 2 hours.

Further attempts to obtain a linear time course using samples of 50 frozen Day O explants and a number of different substrate concentrations, were also unsatisfactory due to increased control values.

Finally, an attempt to obtain more adequate precipitation of DNA using 10% PCA in ethanol (as used by Srivastava, 1968, in barley leaves) resulted in the complete loss of DNAase activity, although this solution was more effective in precipitating DNA than 10% aqueous perchloric acid.

As the DNA preparation used obviously contained a small percentage of short chain molecules in addition to high molecular weight DNA, a simple dialysis test was carried out to investigate the possibility that this preparation of DNA was susceptible to RNAase action.   2 ml. of a 4 mg./ml. solution of Sigma type V DNA in 2 x SSC (SSC = standard saline citrate = 0·15M NaCl, 0·015M sodium citrate, pH 7·2) and 1 ml. of 1 mg./ml. RNAase in 2 x SSC (which had been treated at 80°C to inhibit DNAase) were placed inside a dialysis sac inside a test tube containing 12 ml.
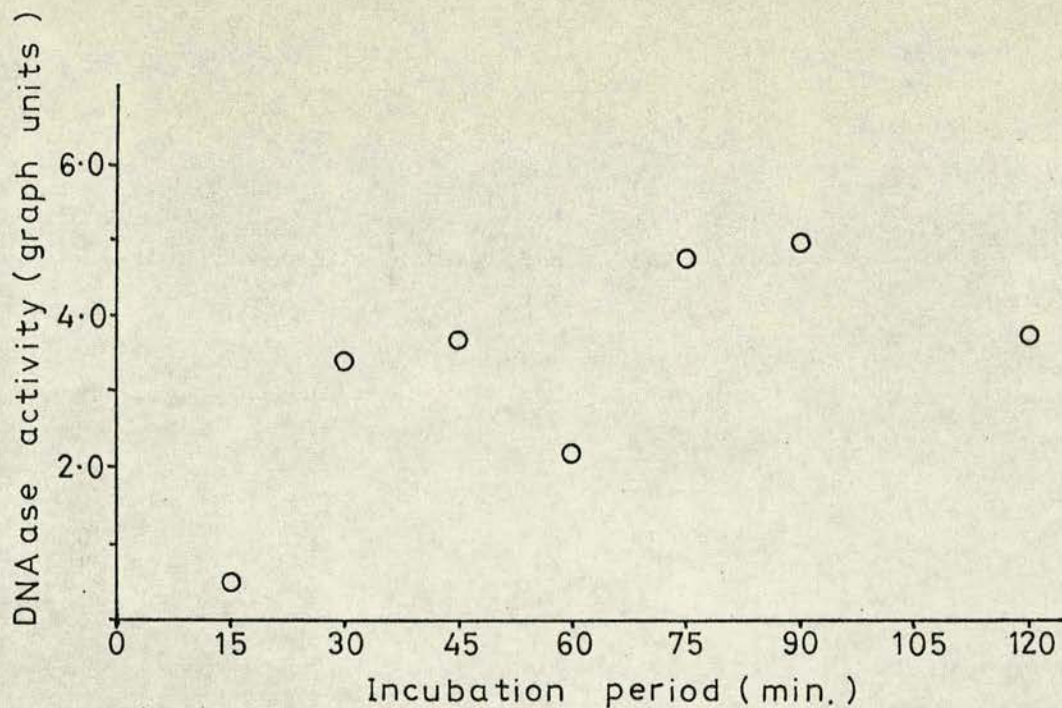
FIG. 3.7   Time course for DNAase activity using
the 'OD. 260 mμ' assay method for samples
of 10, day 0 explants.
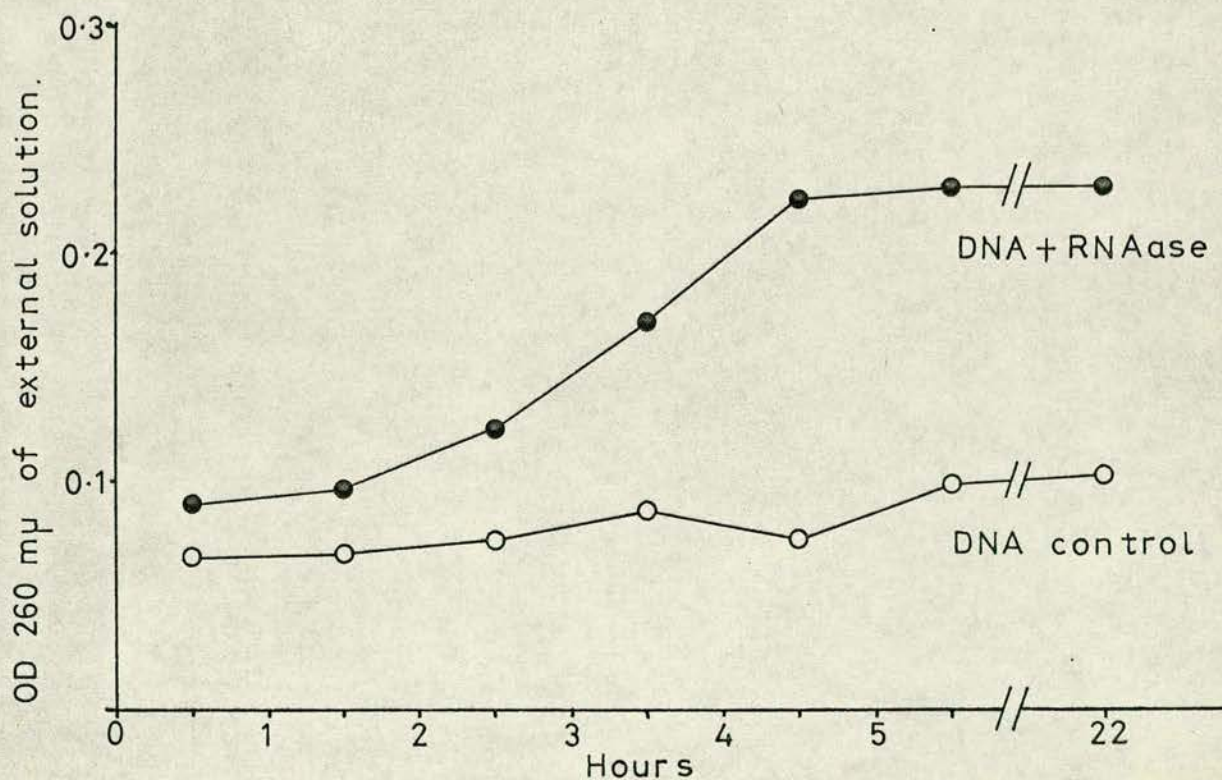
FIG. 3.8   Dialysis of the DNA preparation in the
presence and absence of RNAase.

of 2 x SSC.   A control was prepared without RNAase.   At intervals,
the OD.$_{260}$ of the external solution was measured against a blank
of 2 x SSC using the SP.500 spectrophotometer.   From the results
presented in Fig. 3.8, it is evident that the increase in OD.$_{260}$ in
the presence of RNAase is significantly larger than that observed
for the control.

Although data is not available for a control containing RNAase
only, the above increase may be consistent with the breakdown of
some component of the DNA substrate by RNAase.   From the final
OD.$_{260}$ value of 0·230 and the value of 2·64 for the OD.$_{260}$ given by
2 ml. of the DNA solution in 15 ml. of 2 x SSC, and assuming that
the RNAase preparation is clean, it is estimated that 8·7% of the
DNA preparation is susceptible to RNAase action.

The 'optical density at 260 mμ' method of measuring DNAase
activity was eventually abandoned for a number of reasons:-

1. The failure to obtain a linear time course.

2. The use of frozen explants must inevitably be unsatisfactory
   due to penetration difficulties encountered when a high
   molecular weight substrate such as DNA is used.

3. Although enzyme activity could be measured using this method
   for incubation periods of 2 hours or more, this period is
   considerably longer than that used in conventional enzyme
   assays and the possibility of enzyme denaturation increases
   with the time of incubation.

4. The use of controls for each reaction imposes a further
   source of error on this method as the DNAase activity

represents the difference between two values which are both liable to variation.

5. The purity of the DNA substrate is in doubt and as the accuracy of this method relies on the provision of a pure substrate the validity of this method is also in doubt.

3. The radiochemical assay method.

Radiochemical assay methods are more sensitive than any other type of enzyme assay method and as they are not affected by inter-fering substances they can be used for crude tissue extracts.

Reliable methods are available for the isolation and preparation of high molecular weight DNA from plant tissues and by employing the artichoke tissue culture system grown in the presence of ^{3}H-TdR, it was anticipated that a preparation of high molecular weight artichoke DNA labelled with ^{3}H, could be obtained fairly easily.

a. Preparation of the radioactive substrate.

Ten roller bottle cultures each containing 160 explants were prepared in a medium containing ^{3}H-TdR, as described in Ch. 2. B. The ten radioactive cultures and two control (non-radioactive) cultures were incubated at 25°C for $4\frac{1}{2}$ days. After this period, all the explants from the radioactive cultures (approx. 1600 explants $\simeq$ 32 gm. fresh weight) were washed into a plastic sieve, rinsed with distilled water and blotted with a paper towel. A sample of 5 explants was placed in 2 ml. of 5% chromic acid for determination of cell number. Cell number determinations were also carried out

using explants from the control flasks.   The results were as
follows:-

	Total cell number/explant
Control	109,375
+ ³H-TdR	97,500

These two estimations are similar, almost within the 10% error limits
allowed for this counting technique.   Therefore, neither the
concentration of TdR nor the intensity of the radiation has
seriously affected the extent of cell division.

The DNA extraction which was carried out with the help of
Dr. Ingle, was essentially that described by Wells and Ingle (1970).

The explants were transferred to a large pestle and mortar
containing 50 ml. of grinding buffer.   The grinding buffer
contained

1% (w/v) triisopropylnaphthalene sulfonate (TNS))
) detergents
6% (w/v) p-amino-salicylate (PAS)   )

10mM EDTA (pH 7·4)   - inhibits DNAase activity

6% (v/v) n-butanol   - dissolves the detergents

50mM NaCl   )
) dissolves DNA
10mM tris (pH 7·4)   )

5mM Clelands reagent   - SH protector.

A gentle maceration was carried out, to avoid destroying DNA and to
avoid dissolving too many impurities.   The salt concentration was
increased to 0·5M by the addition of 5 ml. of 5M NaCl and an equal
volume of chloroform-3-methyl butanol-1 (24:1, v/v) was added.

The mixture was shaken vigorously for 10 minutes inside sealed plastic tubes and then centrifuged at 2500 g. for 10 minutes. The upper aqueous layer was retained and further deproteinised by shaking with an equal volume of phenol mixture (phenol saturated with 10mM tris (pH 7·4) containing 10% (v/v) m-cresol and 0·5% (w/v) 8-hydroxyquinoline). After centrifugation at 2500 g. for 10 minutes, the aqueous layer was decanted and the DNA was precipitated by the addition of 2 volumes of ethanol followed by overnight storage at $4^{\circ}C$. After centrifugation (as above), the DNA pellet was dissolved in 0·1 x SSC (SSC = standard saline citrate = 0·15M NaCl, 0·015M sodium citrate, pH 7·2) and the solution was made up to 1 x SSC. The DNA was purified by digestion of the preparation with 50 μg./ml. of DNAase free RNAase (heat treated at $80^{\circ}C$ for 10 minutes (Marmur, 1961)) for 30 minutes at $37^{\circ}C$, followed by digestion of the preparation with 400 μg./ml. pronase for 1 hour. The preparation was stored overnight at room temperature. The DNA was recovered by centrifugation for 18 hours at 39,000 rpm in a Spinco 40.2 rotor at $15^{\circ}C$, and dissolved in 0·1 x SSC. Solutions made up to a density of 1·720 g. cm.$^{-3}$ with CsCl were centrifuged to equilibrium at about 40,000 rpm at $25^{\circ}C$ for 3 days. The UV absorbing fractions were collected, diluted 5 fold and centrifuged for a further 1 day using dilute CsCl.

The DNA preparation contained approx. 28,000 counts/min./20 μl. and the UV absorption spectrum is shown in Fig. 3.9. Assuming that 1·0 optical density units is equivalent to 50 μg. DNA/ml. (Ingle, 1970) it was estimated that the preparation contained approximately
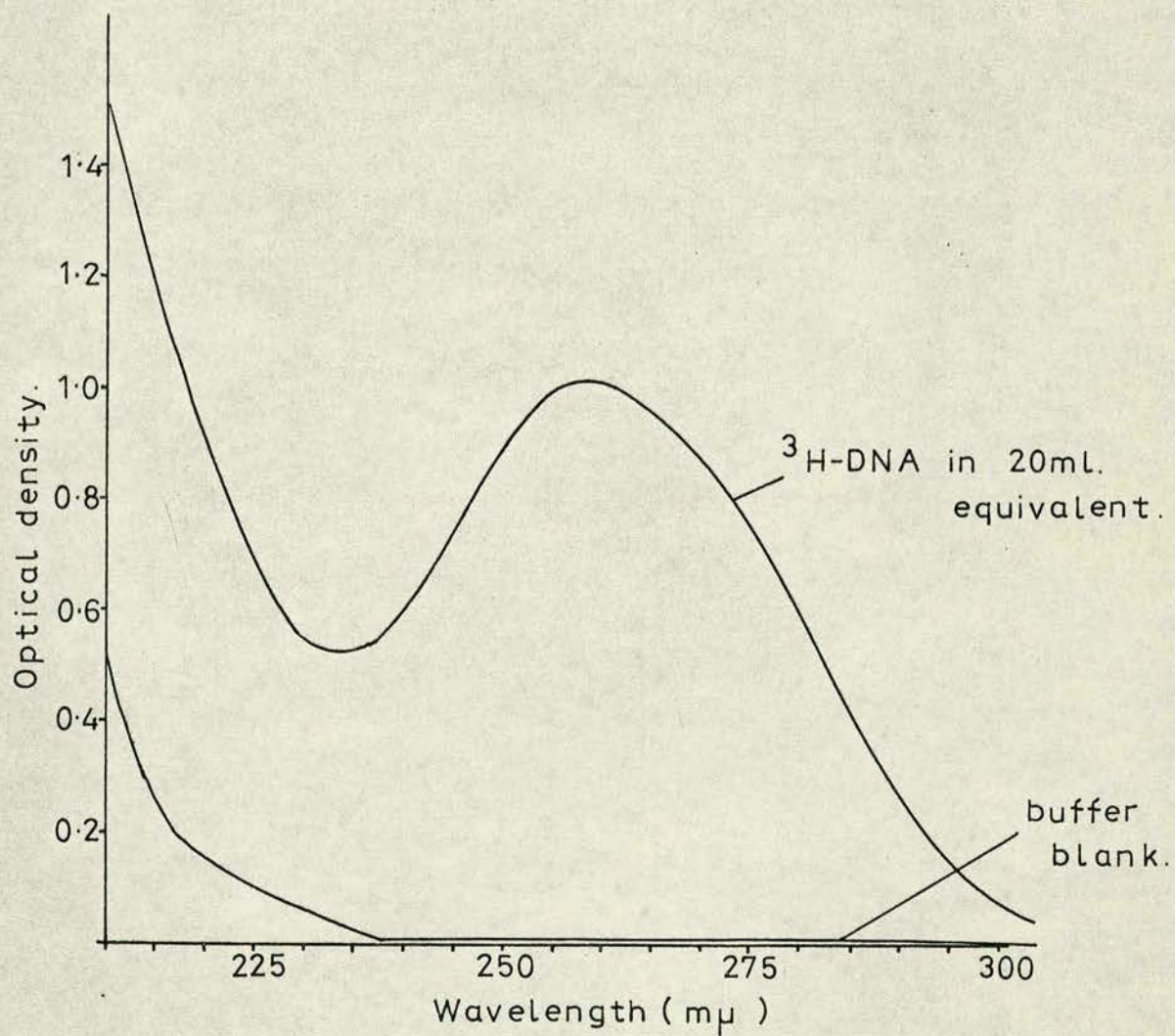
FIG. 3.9 Ultra-violet absorption spectrum for the ^{3}H-DNA preparation.

1 mg. of DNA.   The preparation was diluted to 10 ml. with 0·01M
tris buffer pH 8·0 and stored in 0·5 ml. aliquots in a large
number of cellulose nitrate tubes sealed with parafilm in a deep
freeze at -20°C.

b. Enzyme assay.

The following enzyme assay procedure was adapted from the method
of Dimitrijevic and Launay (1966).   The complete incubation mixture
initially contained

10 µl. ^{3}H-DNA (approx. 1 µg. in 0·01M tris pH 8·0)

0·1 ml. extract

0·05 ml. 0·1M acetate buffer pH 5·7 containing 0·015M $MgCl_2$.
After incubation in a shaking water bath at 35°C for 20 minutes the
reaction was stopped by the addition of 0·1 ml. of carrier DNA
(2 mg. calf thymus DNA/ml. of 0·01M tris pH 8·0) followed by 0·2 ml.
of 20% PCA.   After cooling in ice for 5 minutes, to aid precipitation
of excess and carrier DNA, a 1000 g./10 minutes centrifugation was
carried out at 0°C and the supernatant fraction containing the acid
soluble products of DNAase action was decanted into a scintillation
vial.   Following careful neutralization using approx. 0·1 ml. 5N
NaOH, the solution was mixed with 15 ml. of dioxane scintillator
and counted for 1 minute in a scintillation counter.

The efficiency of precipitation of the ^{3}H-DNA preparation.

Using this method, a preliminary experiment was carried out
to investigate the efficiency of precipitation of the ^{3}H labelled
substrate.   Four experimental tubes were set up, each containing

10 μl. of DNA, 0·1 ml. of grinding buffer, 0·1 ml. of buffer
containing $MgCl_2$ and 0·1 ml. of carrier DNA.   To two of the tubes
(A_1 and A_2), 0·3 ml. distilled water was added whilst the other
tubes were treated with 0·2 ml. 20% PCA followed by the precipitation
procedure, including neutralisation with NaOH.   The radioactivity
of the solutions was as follows:-

	Sample	Counts/minute
A_1)		7455
	DNA controls	
A_2)		5817
B_1)		37
	PCA treatment	
B_2)		33
Blank (Dioxane)		27

Comparing the PCA precipitation values with the blank it is evident
that the precipitation is extremely efficient under these conditions.
However, in samples A_1 and A_2, the DNA (carrier + ^{3}H-DNA) tended to
be precipitated by the scintillator, hence in future determinations
of the total added counts, the DNA was hydrolysed using a pancreatic
DNAase preparation and the total acid soluble counts estimated
using the PCA procedure.

Linn and Lehman (1965a and b), working with Neurospora crassa,
described the existence of two different DNAase enzymes.   The
first, which was capable of hydrolysing native-DNA (double stranded
DNA) and denatured-DNA (single stranded DNA lacking an ordered
structure) had a pH optimum in the region of 5·6.   The second,
which was specific for denatured-DNA, had a pH optimum of 7·5.

A preliminary experiment was carried out to test the possibility that a similar situation might exist with artichoke extracts.

Day 4 artichoke explants grown in the presence of coconut milk, were harvested, washed and blotted and 2 samples each consisting of 700 mg. fresh weight tissue were macerated in a pestle and mortar with silver sand as an abrasive, using the following grinding buffers:-

1. 1 ml. of 0·05M tris pH 7·5 + 0·5% EtSH + 400 µg./ml. bovine serum albumin (BSA)

2. 1 ml. of 0·05M acetate pH 5·7 + 0·5% EtSH + 400 µg./ml. bovine serum albumin (BSA).

Enzyme reactions were carried out for both extracts using both native and denatured-DNA as substrate.

The following results were obtained

	Activity (counts/min.)	
	pH 5·7	pH 7·5
'native'-DNAase	332	76
	371	-
'denatured'-DNAase	4134	2492
	5307	3575

From these results it is apparent that Day 4 artichoke extracts exhibit a higher 'denatured'-DNAase activity.   In addition, both enzymes (if these are two different enzymes) exhibit higher activities at pH 5·7 compared with pH 7·5.   In subsequent experiments, assay methods were developed for both enzymes.

1. 'Native'-DNAase.

In a preliminary experiment, the activity of a 0 hour extract

was determined.   The extract was prepared as previously described using 100 day 0 explants (approx. 700 mg. fresh weight) per ml. of grinding buffer (0·05M acetate pH 5·7 + 0·5% EtSH + 400 µg./ml. BSA).   The control value (no extract) and the total possible hydrolysis of a sample of 'native'-DNA were also determined.   The control value was estimated as described previously.   The total possible hydrolysis was determined by substituting for the extract component of the incubation mixture, 0·07 ml. grinding buffer + 0·03 ml. DNAase (1 mg. DN-C DNAase I from bovine pancreas/ml. 0·01M tris buffer pH 8·0).   After an extended incubation period of 30 minutes, the total acid soluble counts were measured as previously described.   The results were as follows:

	counts/minute		'Native'-DNAase activity (% conversion to acid soluble products)
	Actual values	Average	
Control	86 122	104	0
Total hydrolysis	6288 6437	6363	100
Extract	1127 733	930	13

Since the average activity of a Day 0 extract prepared as above was in the region of 13% conversion for an incubation of 20 minutes duration, the following time course experiment was carried out using identical conditions.

Time course for 'Native'-DNAase.

Using Day 0 extract prepared as described in the previous

experiment, the relationship between enzyme activity and time was investigated for incubation periods varying from 10 to 60 minutes. The results which are shown in Fig. 3.10 indicate that the rate of reaction remained linear even after a 60 minute incubation (approximately 40% conversion to acid soluble products).   Values for the total possible hydrolysis, the unincubated controls and controls which had been incubated for 60 minutes were as follows:-

	counts/min.
Total possible hydrolysis	6638 6977 (Av. 6756)
unincubated controls	38 67 (Av. 52)
Incubated (60 min.) controls	58 78 (Av. 68)

As the control values were not significantly different there was no evidence of non-enzymic hydrolysis during the 60 minute incubation period.

pH dependence of 'native'-DNAase.

Day 0 extracts prepared as previously described were used for this determination with the exception that a more dilute grinding buffer (0·01M tris-maleate buffer pH 7·2 containing 0·5% EtSH + 400 µg./ml. BSA) was used.

The reaction mixture contained

10 µl. native-DNA

0·05 ml. 0·2M tris-maleate buffer (pH 5·2 - 8·4) + 0·015M $MgCl_2$

0·1 ml. extract.

By adding a strong buffer of the appropriate pH to separate reaction
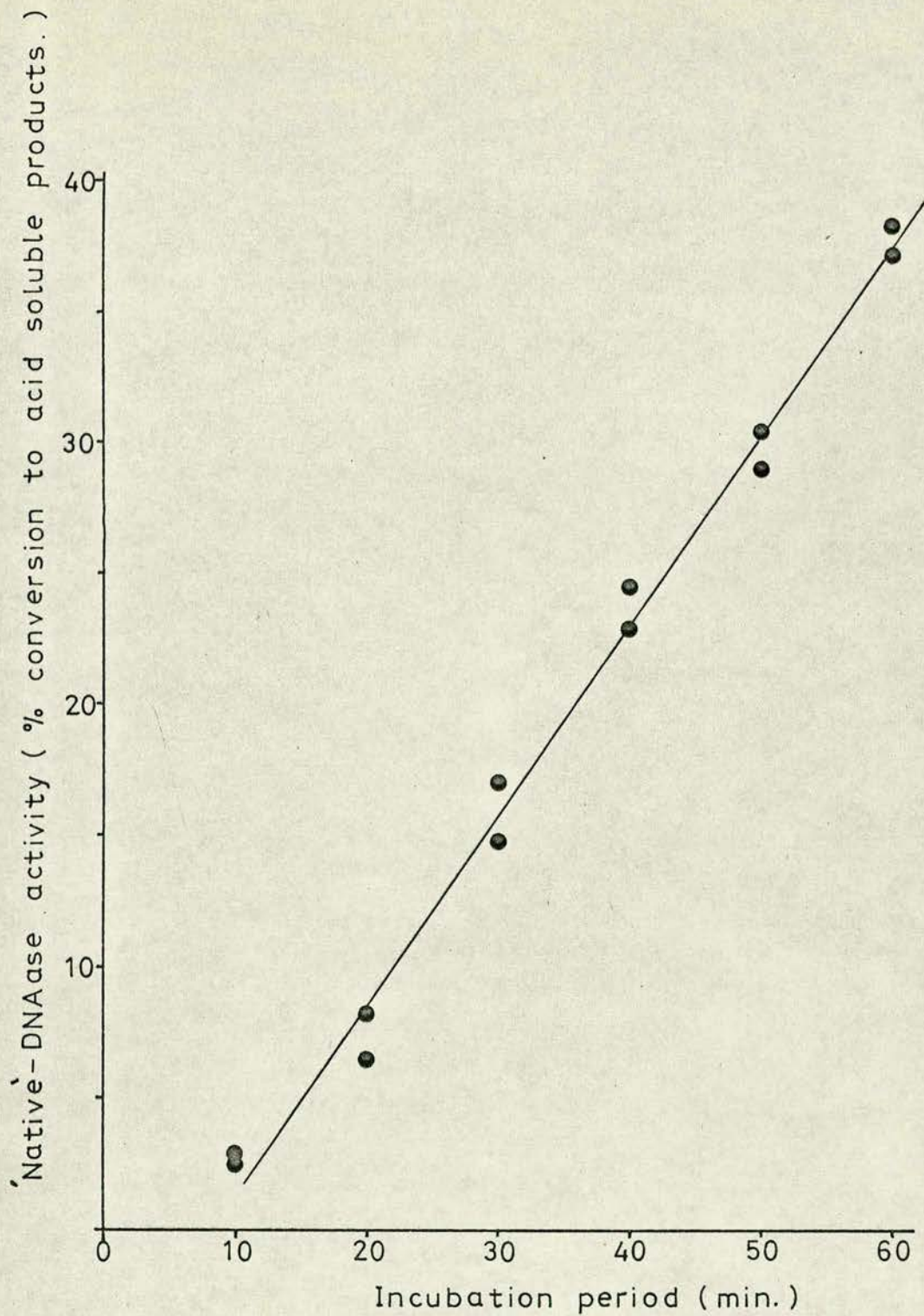
FIG. 3.10 Time course for 'native'-DNAase activity
using the radioactive assay, for day 0
artichoke extract.

tubes the pH of the incubation medium was varied, and the same extract was used for all determinations.   Controls and total possible hydrolysis (at pH 5·6) were also carried out.   All tubes were incubated in a shaking water bath at $35^{\circ}C$ for 30 minutes.

The results of this experiment are shown in Fig. 3.11. from which it is apparent that the optimum pH for this enzyme is in the region of pH 5·6.   From the shape of this pH curve it is evident that small changes in the pH value markedly affect the activity of this enzyme.

2. 'Denatured'-DNAase.

A preliminary experiment was carried out to determine the control value (no extract) and the total possible hydrolysis of a sample of denatured-DNA.   A 0·2 ml. aliquot of ^{3}H-DNA was removed from the deep freeze and denatured by heating in a pyrex tube in a boiling water bath for 5 minutes.   The tube was then placed in an ice bath.

The unincubated control value was obtained as previously described except that in this case, a 10 μl. aliquot of denatured-DNA was used.   The total possible hydrolysis for denatured-DNA was also determined as previously described using a pancreatic DNAase preparation.

The following results were obtained:-

	counts/minute
Control	92
	170
Blank (dioxane	27
Total possible hydrolysis	12,355
	11,952
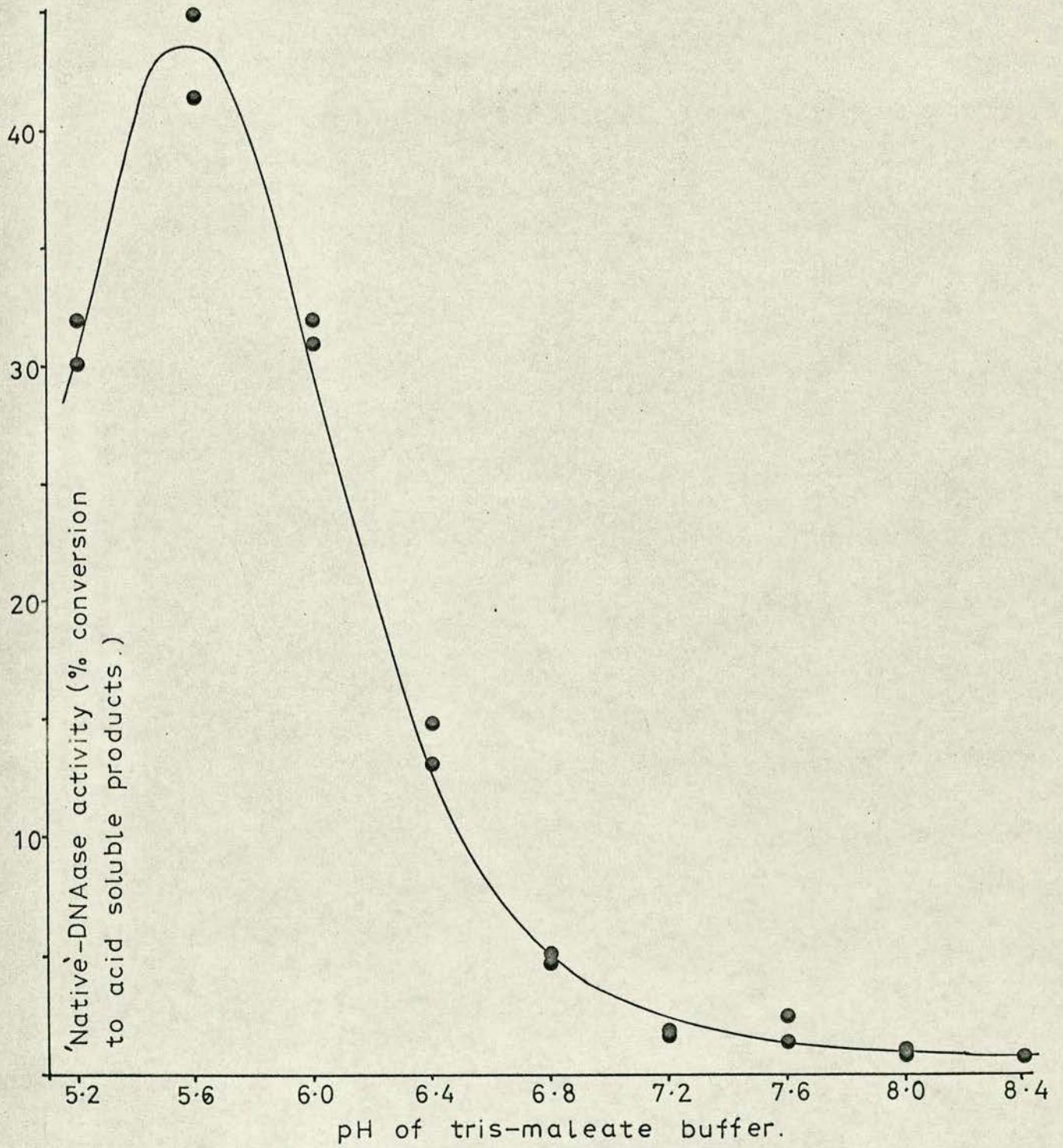
FIG . 3 .11   pH dependence for 'native'-DNAase activity of day 0
artichoke extract.

After the denatured-DNA preparation had been standing in an ice bath for 4 hours, a control value of 710 counts/min. was obtained. After a preparation had been standing at room temperature for 6 hours a control value of 1840 counts/min. was obtained. The latter observations indicate the instability of denatured-DNA which is more marked at higher temperatures. For this reason, a fresh sample of DNA was removed from the deep freeze and denatured prior to each experiment. The total possible hydrolysis figure of about 12,000 counts/min. obtained in this experiment is considerably higher than the values of 6000 counts/min. obtained from samples of native DNA. This anomaly can be explained on the grounds that during the denaturation procedure the volume of the ^{3}H-DNA sample had been considerably reduced due to evaporation. Subsequent experiments included attempts to reduce evaporation loss by 1) placing a glass marble on the mouth of the test tube to act as a condenser, and 2) by directing a blast of cold air onto the sides of the tube during the boiling treatment. Under these conditions the total hydrolysis obtained was usually in the region of 6000 counts/min. but as this value could vary depending on the water loss due to evaporation, a total hydrolysis estimation was carried out for every sample of denatured-DNA prepared.

As the 'denatured'-DNAase activity of day 4 artichoke extracts was rather high in the experiment previously described, extracts prepared using only half the weight of tissue were used in the next experiment.

Time course for 'denatured'-DNAase.

1.   For this experiment, 100 day 0 explants (approx. 700 mg. fresh weight) were macerated in 2 ml. of grinding buffer (0·05M acetate pH 5·7 containing 0·5% EtSH and 400 μg./ml. BSA).   The incubation period was varied from 10 - 60 minutes and the results indicated that complete hydrolysis had taken place after an incubation period of only 10 minutes.   In order to obtain a linear time course for this enzyme it is evident that an even lower extract concentration must be used.   This experiment also included estimations of total possible hydrolysis, unincubated controls and controls which had been incubated for 60 minutes.

	counts/minute.	
unincubated controls	288 134	(Av. 196)
incubated controls	452 172	(Av. 312)
Total hydrolysis	6070 6214	(Av. 6142)

As the above control values are not significantly different, non-enzymic hydrolysis was negligible for the 60 minute period.

Before attempting another time course experiment a Day 0 extract was prepared as above, diluted 10 times with grinding buffer and the activity determined for a 20 minute incubation period.   As a 66% conversion to acid soluble products was achieved this enzyme concentration was used in the subsequent time course experiment.

2.   A time course experiment was carried out in which the incubation period varied from 10 to 50 minutes.   The results of this experiment

are shown in Fig. 3.12, from which it is apparent that the rate
of reaction begins to fall after an incubation period of 20 minutes
when values of 60% conversion of substrate to product have been
exceeded.   In subsequent experiments a reaction time of 20 minutes
was retained, but the concentrations of extract used were such that
the percentage conversion never exceeded 30%.

pH dependence of 'denatured'-DNAase.

An extract was prepared using 100 day 0 explants per ml. of
grinding buffer (0·01M tris-maleate pH 7·2 containing 0·5% EtSH
and 400 µg./ml. BSA).   From the supernatant fraction, a x 50
dilution of enzyme was prepared using grinding buffer.
The reaction mixture contained

10 µl. denatured-DNA

0·05 ml. 0·2M tris-maleate buffer (pH 5·2 to 8·4) + 0·015M $MgCl_2$

0·1 ml. diluted extract.

An incubation period of 20 minutes was used.   From the results of
this experiment which are shown in Fig. 3.13, it is apparent that
the pH optimum for this enzyme is in the region of pH 6·4.

In subsequent cell cycle experiments the activities of 'native'-
DNAase and 'denatured'-DNAase were often assayed in conjunction
with DNA polymerase (pH optimumiin the region of pH 8·0).   In order
to accommodate the pH requirements of all 3 enzymes, extracts were
prepared using a weak grinding buffer of intermediate pH as
described in the pH optimum experiments.

Immediately prior to the first cell cycle experiment, a
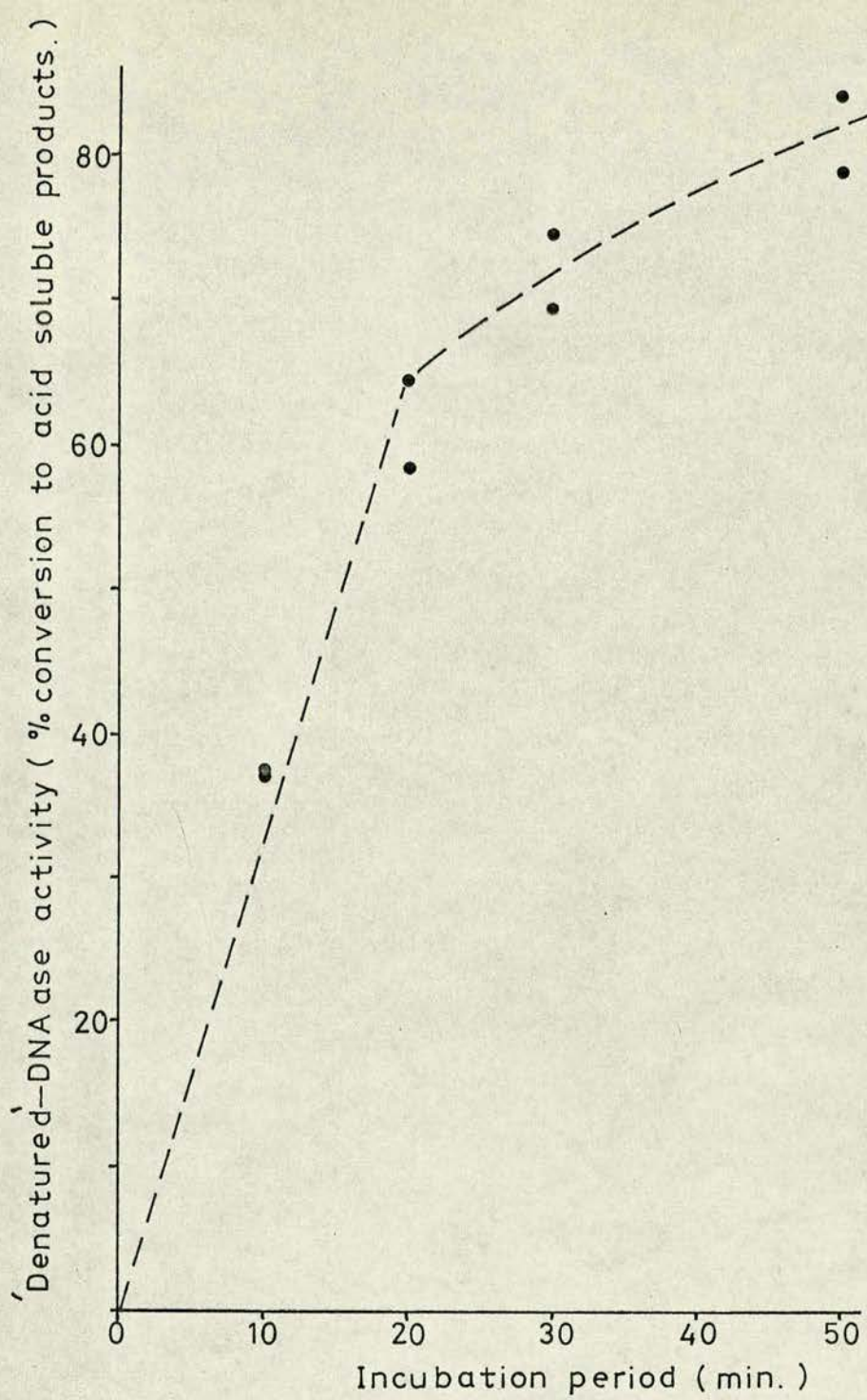
FIG. 3.12 Time course for 'denatured'-DNAase activity of day 0 artichoke extract.
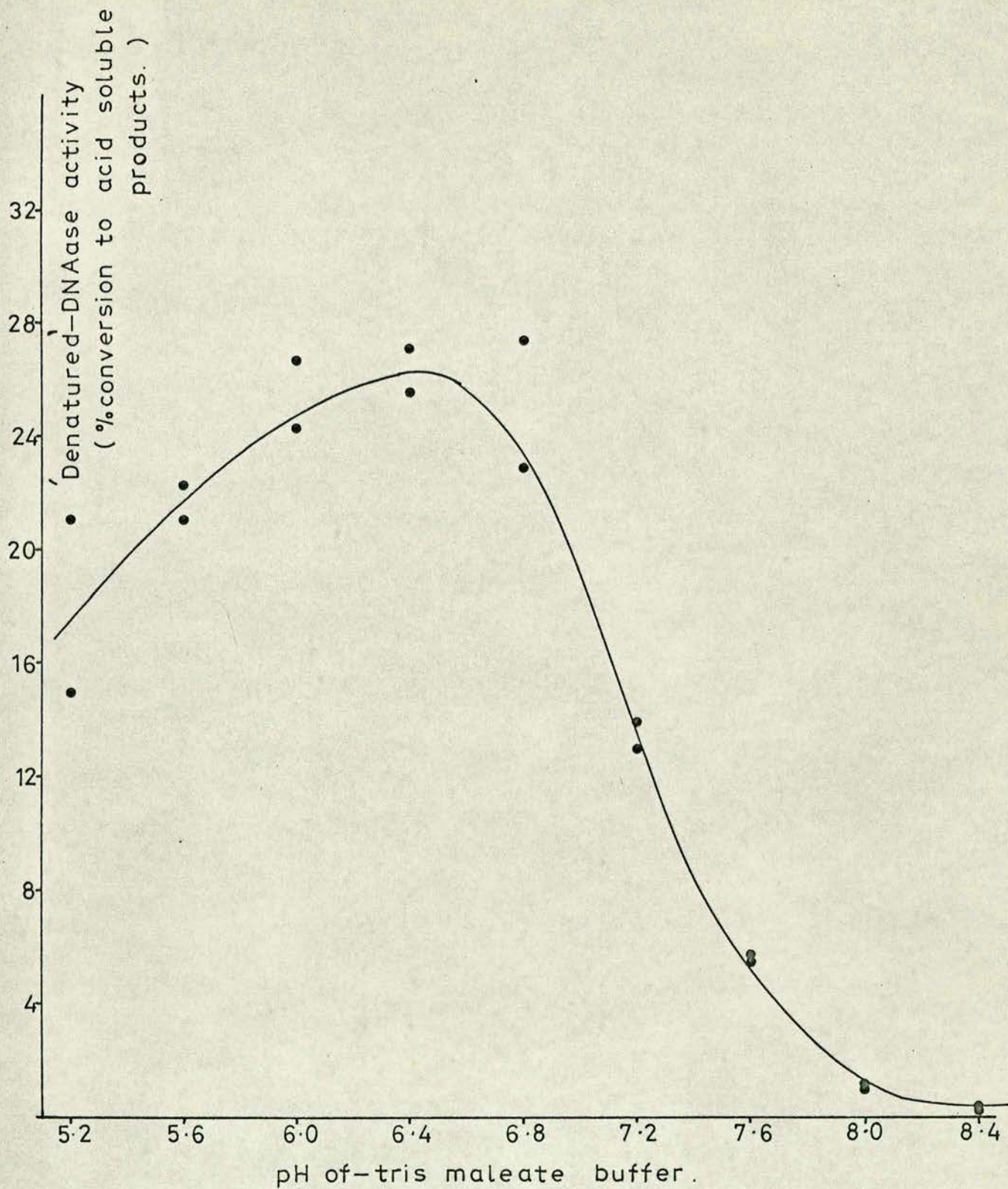
FIG. 3.13   pH dependence for 'denatured'—DNA ase activity of day 0 artichoke extract.

preliminary experiment was carried out to determine the levels of 'native' and 'denatured'-DNAase activity in freshly excised tissue, using the method of extract preparation to be used in the cell cycle experiment.   100 day 0 explants were macerated in a pestle and mortar with 1·5 ml. grinding buffer (0·01M tris-maleate pH 7·2 + 0·5% EtSH + 400 µg./ml. BSA).   No silver sand was included in the pestle and mortar treatment and further homogenisation was achieved using a Kontes hand homogeniser.   The supernatant fraction from a 2200 g. centrifugation at 0°C for 10 minutes was used to assay 'native'-DNAase.   An aliquot of the supernatant fraction was diluted x 50 with grinding buffer, and this diluted extract was used for the 'denatured'-DNAase assay.

Using an incubation period of 20 minutes at 35°C the following conversions were obtained

	DNAase activity (% conversion)
'Native'-DNAase	33·13
	32·06
'Denatured'-DNAase	3·49
	3·20

Anticipating that increases in 'native'-DNAase activity may occur during the cell cycle, a conversion of over 30% is rather high for an initial rate.   Thus, in the final method used in the cell cycle experiments (See Ch. 2. D. 1.) the initial conversion was reduced to a value of less than 10% by a) reducing the incubation period to 10 minutes and b) reducing the reaction temperature to 30°C.

The 'denatured'-DNAase activity of less than 4% obtained in this

preliminary experiment is suitable for use in the cell cycle experiments. In cell cycle experiments the incubation period of 20 minutes was maintained but the incubation temperature was also reduced to $30^{\circ}C$ so that both reactions could be incubated in the same water bath.

So far in this investigation there have been several indications that the enzymes 'native'-DNAase and 'denatured'-DNAase are different enzymes. Firstly the rate of breakdown of denatured-DNA is considerably faster than the breakdown of native-DNA in the same extract. Secondly, the two enzymes have different pH optima and their pH dependence curves differ markedly from each other in shape.

In view of the fact that these may be different enzymes, an investigation of their respective patterns during the cell division cycle may yield interesting results.

Summary of methods

This section describes how successful assay methods have been developed for the measurement of 'native'-DNAase and 'denatured'-DNAase activity in artichoke tissue.

The diphenylamine method, which was used by Stern (1961) to measure DNAase activity during microsporogenesis in Lilium longiflorum, was not suitable for artichoke tissue due to the interfering effect upon the diphenylamine reaction, of some soluble component of the extract.

The $OD._{260}$ mμ method was also unsuitable for the measurement of DNAase activity in artichoke tissue for a number of reasons. The breakdown of high molecular weight artichoke DNA labelled with ^{3}H-thymidine was the basis of a successful DNAase assay. This method was used to measure activities of both 'native' and 'denatured'-DNAase. Because both of these enzymes exhibited a linear reaction during incubation, this method was considered suitable for the assay of DNAase activity during the cell division cycle.

SECTION B

THYMIDINE KINASE

The enzyme thymidine kinase (TdR kinase) catalyses the conversion of thymidine (TdR) to thymidine monophosphate (dTMP) according to the following equation (Dixon and Webb 1958)

Thymidine + ATP $\longrightarrow$ Thymidine-5'-phosphate + ADP.
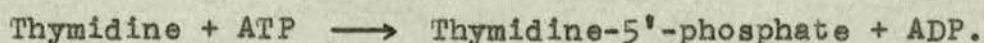
The activity of this enzyme has been measured in a range of tissues, for example Hotta and Stern (1965), in wheat and Lilium, Stubblefield et al. (1965) in chinese hamster, Sachsenmaier and Ives (1965) in Physarum polycephalum, Okazaki and Kornberg (1964) in Escherichia coli, and Brent et al. (1965) in Hela cell cultures. The basis of the assay system in all these communications is the measurement of the conversion of ^{3}H or ^{14}C labelled TdR (substrate) to the phosphorylated product dTMP. The accurate measurement of enzyme activity depends firstly on the successful separation of substrate and product by paper chromatography or paper electrophoresis and secondly on the estimation of radioactivity of the product using various counting techniques. It would appear from the literature that separations involving paper chromatography are popular but subsequent measurement of the radioactive product depends on time-consuming elution procedures or on the use of specialized counting equipment such as a windowless chromatogram scanner.

In view of this a more rapid technique was evolved using cellulose acetate electrophoresis coupled with the direct measurement

of the product on the paper by an efficient scintillation counting technique.

This section describes experiments leading up to the successful development of a TdR kinase assay system for artichoke tissue, and as the cellulose acetate electrophoresis procedure is considered to be a critical part of the method, the development of this technique is also described at this point.

Cellulose acetate electrophoresis.

Using this technique, preliminary tests were carried out in order to establish suitable conditions for the adequate separation of phosphorylated thymidine derivatives from the TdR substrate. A number of standard solutions were prepared at a concentration of 4 mg./ml. of 0·001N formic acid.   These included an adenosine series comprising adenosine, AMP, ADP and ATP and a thymidine series comprising thymine, TdR, dTMP and dTTP.

Standard solutions were applied to the baseline in 2 µl. aliquots.   Two different standard solutions were often applied to one strip.

In a preliminary electrophoresis for $1\frac{1}{2}$ hour at 200 volts
Adenosine moved 19 mm. towards the cathode

AMP	"	9 mm.	"	"	anode
ADP	"	26 mm.	"	"	"
ATP	"	28 mm.	"	"	"

TdR remained on the baseline.

In a 1 hour electrophoresis at 200 volts

Adenosine moved 17 mm. towards the cathode

TdR remained on the baseline

dTMP moved 24 mm. towards the anode

dTTP "   28 mm.   "   "   "

Thymine remained on the baseline.

A mixture of the TdR, dTMP and dTTP standards (0·2 ml. of each 4 mg./ml. solution) was applied in 20 µl., 50 µl. and 100 µl. aliquots onto the baseline of 3 separate electrophoresis strips.   These were subjected to a 2 hour electrophoresis at 200 volts.

An ultra violet photograph of the separation of the 50 µl. mixture is shown in Fig. 3.14.   This was obtained by placing a strip of Kodak No. 2 film paper beneath the electrophoresis strip on top of a curved metal plate.   The papers were held in place by small magnets.   A brief exposure of about 1 second from a UV. light held approximately 3 ft. above the paper was used.   The film was developed, fixed and glazed.   From Fig. 3.15 it is apparent that an adequate separation of TdR from phosphorylated derivatives has been obtained.   Although the dTMP and dTTP bands were beginning to separate, a complete separation of these was not required for the TdR kinase assay.   In the separation of the 20 µl. mixture the dTTP band was not visible.   The separation of the 100 µl. mixture was similar although less well defined, than that of the 50 µl. mixture.
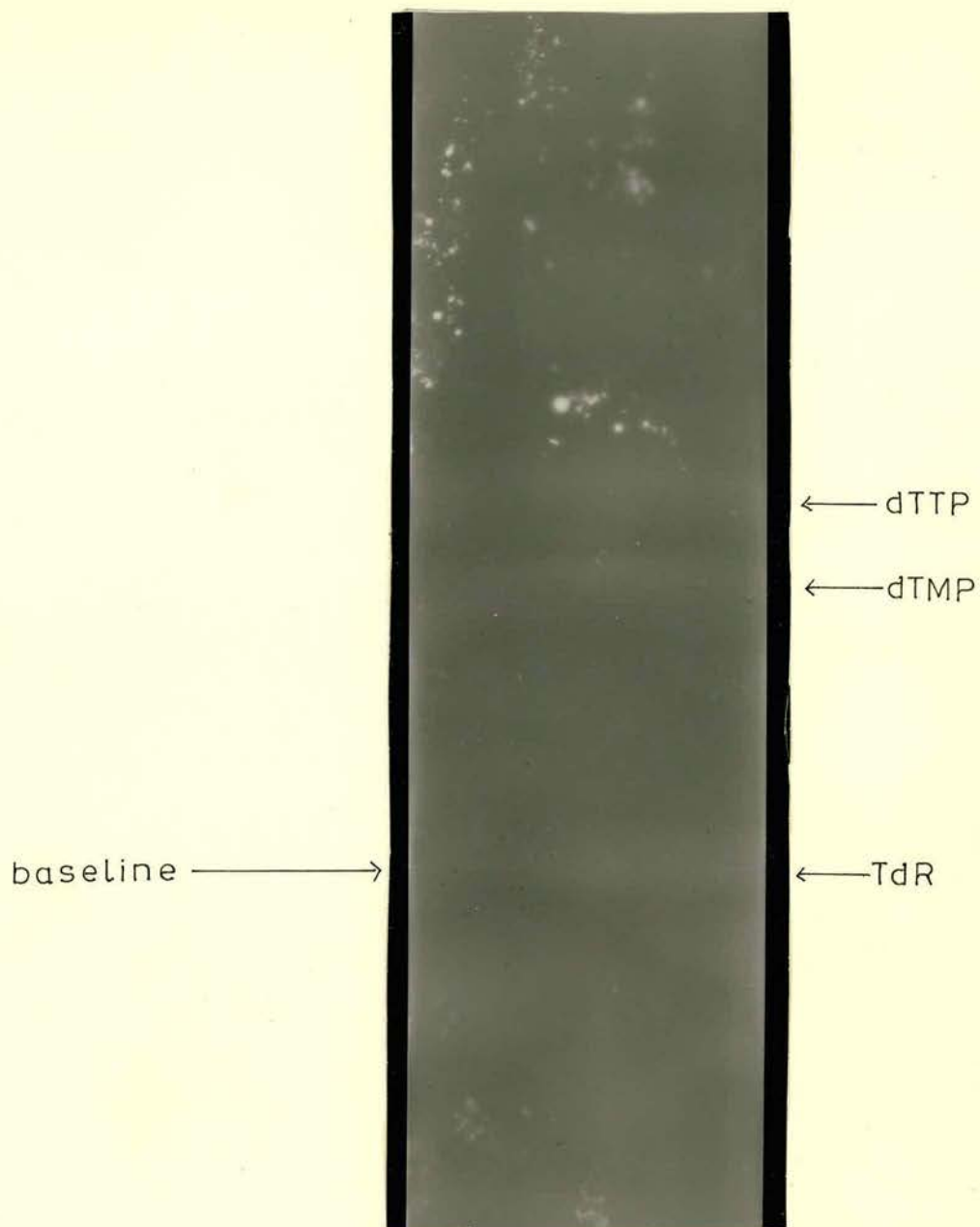
FIG. 3.14 Separation of a mixture of standards by
cellulose acetate electrophoresis.
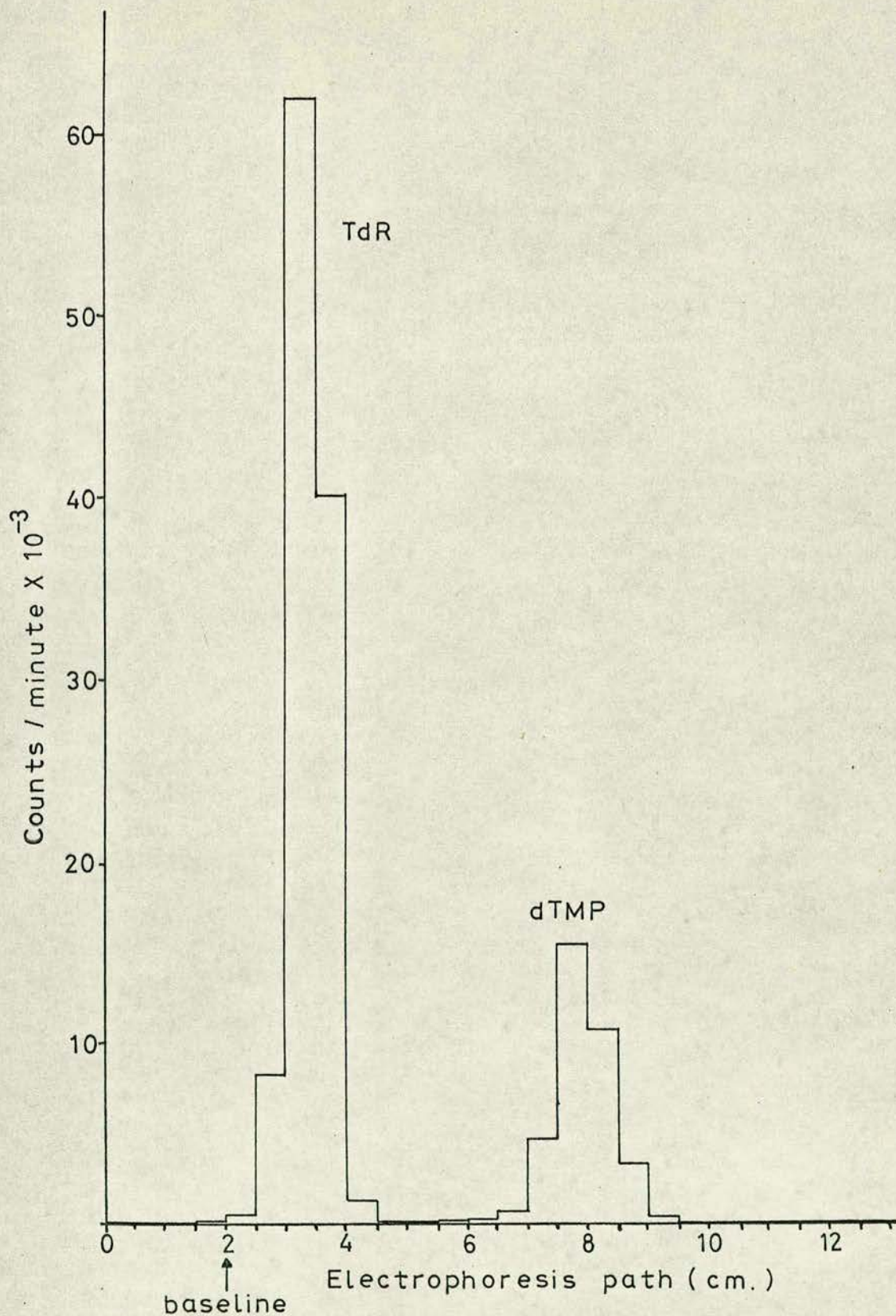
FIG. 3.15   Cellulose acetate electrophoretic separation
of the substrate and product of the TdR kinase
assay, for wheat embryo extract.

The TdR kinase assay

A preliminary assay for TdR kinase activity was carried out using wheat embryos, which are known to be a rich source of TdR kinase activity.   The assay of TdR kinase activity in 48 hour old wheat embryos described by Hotta and Stern (1965) was repeated.

Five petri dishes were prepared each containing 50 wheat seeds placed on top of 2 layers of filter paper moistened with 6 ml. of distilled water.   These were incubated in the dark at $25^{\circ}C$ for 48 hours.   The embryos were removed and 330 mg. of tissue was macerated in 1 ml. of 0·02M phosphate buffer pH 7·2 using a pestle and mortar and silver sand to act as an abrasive.   The homogenate was centrifuged at 2200 g. for 5 minutes at $0^{\circ}C$ and the supernatant fraction was retained as the enzyme extract.   A medium containing various substrates and cofactors essential to the TdR kinase reaction was prepared as follows.

5 ml. of 'the medium' contained:

0·5 ml. 0·2M phosphate buffer pH 7·2
0·025 ml. 0·1M $MgCl_2$
6·2 mg. ATP
8·4 mg. phospho-enol pyruvate (PEP)
0·24 mCi ^{3}H-thymidine (specific activity 22 Curies/m mole)
95 µg. unlabelled thymidine.
Distilled water to a total volume of 5 ml.

For individual assays, the reaction mixture contained

0·1 ml. medium
0·025 ml. pyruvate kinase (10 mg./ml.)
0·1 ml. tissue extract.

The mixture was incubated at 25°C for 20 minutes, and the reaction
was stopped by the addition of 0·4 ml. of 95% ethanol.   The
ethanolic suspension was heated for 1 minute in a boiling water
bath and then centrifuged at 1000 g. for 5 minutes to obtain a
clear supernatant fluid.   The residue was extracted with 0·2 ml.
of 70% (v/v) ethanol, recentrifuged and the supernatant fluids
combined in a sample tube which was stored in a deep freeze at -20°C.

Controls were carried out, in which grinding buffer replaced
extract in the incubation mixture.   A 100 μl. aliquot of the
supernatant was subjected to a 2 hour electrophoresis at 200 volts.
The strips were dried and each was cut into 25 separate ½ cm.
strips, each of which was immersed in toluene scintillator and
counted for 1 minute.

Under these conditions the radioactive TdR and dTMP peaks
were not adequately separated and the electrophoresis period was
increased to 3 hours and finally to 4 hours.   The results of a
4 hour separation for the wheat extract assay are shown in Fig. 3.15.
A 4 hour electrophoresis of a standard mixture containing TdR,
dTMP and dTTP, indicated that the radioactive peaks shown in
Fig. 3.15 were in the same position as TdR and dTMP.

The radioactive separation of the control sample was similar
in shape with respect to the TdR peak, but the dTMP peak was absent.

To obtain a measurement of enzyme activity all the counts in
the TdR peak were added together.   This procedure was repeated for
the dTMP peak.   The counts in the dTMP peak expressed as a

percentage of the total counts gave a value of 24·30%.

	counts/min.	
e.g. strips 4 - 10 (TdR)	112545	
" 10 - 19 (dTMP)	36164	24·30%
	148709	

For this sample the variation due to counting was tested by
repeating the 1 minute count of all the samples. A value of
24·27% was obtained.

When the counts for the control were treated in the same way,
a value of 1·33% was obtained due to the background counts which
occupied the same position as the dTMP peak. For the TdR kinase
reaction which was carried out in triplicate for the wheat extract,
the following results were obtained:-

	% counts in dTMP region	TdR kinase activity (% conversion of TdR to dTMP)
Control	1·33	0
Wheat extract	24·30	22·97
	24·55	23·22
	27·10	25·77

A high percentage conversion of TdR to dTMP was obtained using
wheat extract thus confirming that this method was suitable for
detecting TdR kinase activity in plant extracts.

For wheat extract Hotta and Stern (1965) had shown that a
plot of activity vs. concentration of protein in reaction mixture
was proportional up to 200 μg. of protein, where the protein content
of extracts was measured using the method of Lowry et al. (1951).

Anticipating that the TdR kinase assay might be successful for
artichoke extracts which contained a similar concentration of
protein, an extract was prepared using 1 gm. of freshly excised
explants (approx. 143 explants) in 5 ml. of buffer as previously
described.   The protein content of 0·2, 0·15, 0·10, 0·05 and
0·01 ml. aliquots of extract was determined using Lowry's method
which was calibrated using a standard solution of bovine serum
albumin.   From the average result, it was estimated that
approximately 23 freshly excised explants macerated in 1 ml. of
buffer would produce an extract which contained approx. 100 µg.
protein/0·1 ml. of extract.

Preliminary assays using artichoke tissue.

Experiment 1.

For this experiment, two different extracts were prepared.
1. 25 freshly excised explants (240 mg. fresh weight) were macerated
in 1·0 ml. of 0·02M phosphate buffer pH 7·2 in order to obtain an
extract of soluble protein content approximately 100 µg./0·1 ml.
2. 6 asynchronously dividing explants (approximately 240 mg. fresh
weight of explants over 21 days old which had been grown in a
coconut milk medium) were macerated in 1·0 ml. of the same buffer.

Enzyme assays were performed in triplicate for both extracts
and 3 control estimations were also carried out.   The results
were as follows:-

	% counts in dTMP region	TdR kinase activity (% conversion of TdR to dTMP)
Control	0·82	0
Day 0	0·98	0·16
Asynchronous	1·30	0·48
	1·10	0·28

These activities were not sufficiently high to merit counting all the replicate samples. Instead, an attempt was made in the next experiment to increase activity by increasing the concentration of tissue in the extract.

Experiment 2

A sample of 100 freshly excised explants were macerated in 1·0 ml. of buffer i.e. 4 x the concentration of tissue used in the previous experiment. The standard assay procedure was carried out using 0·1 ml. of extract. The following results were obtained.

	% counts in dTMP region	TdR kinase activity (% conversion of TdR to dTMP)
Control	0·84	0
	1·22	-
Day 0	1·18	0·34
	1·00	0·16
	1·06	0·22

Although activities were corrected for the lowest control value, they showed considerable variation between replicate determinations and the percentage conversion to dTMP was minimal.

In other systems, e.g. Lily, there is an increase in TdR kinase activity associated with cell division.   For this reason it was anticipated that asynchronously dividing artichoke tissue grown in culture might provide a richer source of the enzyme.

Experiment 3

In this experiment, explants were grown on an agar-2,4D medium for 5 days.   Three extracts were prepared using 144, 330 and 660 mg. of explants/1·0 ml. of buffer respectively.   The TdR kinase activities obtained using the 3 extracts were as follows.

Fresh weight tissue/ml.	% counts in dTMP region	TdR kinase activity (% conversion of TdR to dTMP)
0 mg. (control)	1·07	0
144 mg.	1·41	0·34
330 mg.	1·45	0·38
	1·34	0·27
660 mg.	1·67	0·57
	2·00	0·93

Although the activity was roughly proportional to the amount of tissue/ml. of buffer, the conversion to dTMP was less than 1·0%, even for the highest concentration of extract.   The possibility that a further doubling of the concentration of a synchronously dividing explants/ml. of buffer might produce a conversion to dTMP above 1·0% was examined in the next experiment.

Experiment 4

For this experiment, 1330 mg. of Day 7 explants (grown in

liquid culture in the presence of coconut milk) were macerated
in 1·0 ml. of 0·02M phosphate buffer containing 400 µg./ml. bovine
serum albumin (BSA).   BSA was included because Okazaki and
Kornberg (1964a) had shown that this substance protects the enzyme
and therefore enhances TdR kinase activity in Escherichia coli.
Assays carried out using 0·03, 0·06 and 0·1 ml. aliquots of extract
yielded the following results.

Volume of extract	% counts in dTMP region	TdR kinase activity (% conversion of TdR to dTMP)
0·0 ml. (control)	1·35	-
	1·17	0
0·03 ml.	1·27	0·10
	1·21	0·04
0·06 ml.	1·89	0·72
	1·76	0·59
0·10 ml.	2·08	0·91
	2·43	1·26
	2·56	1·39

All activities were corrected for the lowest control value.   The
average activities obtained from assays carried out using 0·1 ml.
of extract exceeded 1·0% conversion.   Results for the 3 different
concentrations of enzyme showed that enzyme activity was proportional
to the quantity of extract used.   For asynchronously dividing
tissue, the results of this experiment were not significantly higher
than predicted from the results of the previous experiment due to
the presence of BSA, therefore this substance was not included in

the grinding medium used in the next experiment.

Experiment 5.

Using the assay method so far established, a preliminary
experiment was carried out to determine the level of TdR kinase
activity during the first part of the cell division cycle. For
this experiment which was carried out on 11/6/69, explants were cut
in low intensity green light and grown in the dark on an agar
medium containing sucrose, mineral salts and 2,4-D. An agar
medium was used in order to reduce the contamination risk which is
prevalent in liquid cultures at this time of the year and each agar
plate contained 20-30 isolated explants. Samples of 100 explants
were withdrawn at intervals up to 29 hours, and extracts were made
using 1·0 ml. of 0·2M phosphate buffer pH 7·2 (final buffer
concentration in reaction mixture $\simeq$ 0·1M). Enzyme assays were
carried out in triplicate for each extract and the incubation was
for 1 hour at 25°C. The results of this experiment are shown in
Fig. 3.16. In a cell number estimation which was carried out at
41 hours, the presence of blackened nuclei indicated that the
cells were about to divide. Therefore the estimated division time
for this experiment was in the region of 45 hours. As the 'S' period
begins approximately 12-14 hours before division in this system (see
Ch. 4) this experiment probably covered most of the pre-'S' period.
A low TdR kinase activity was maintained throughout this period.
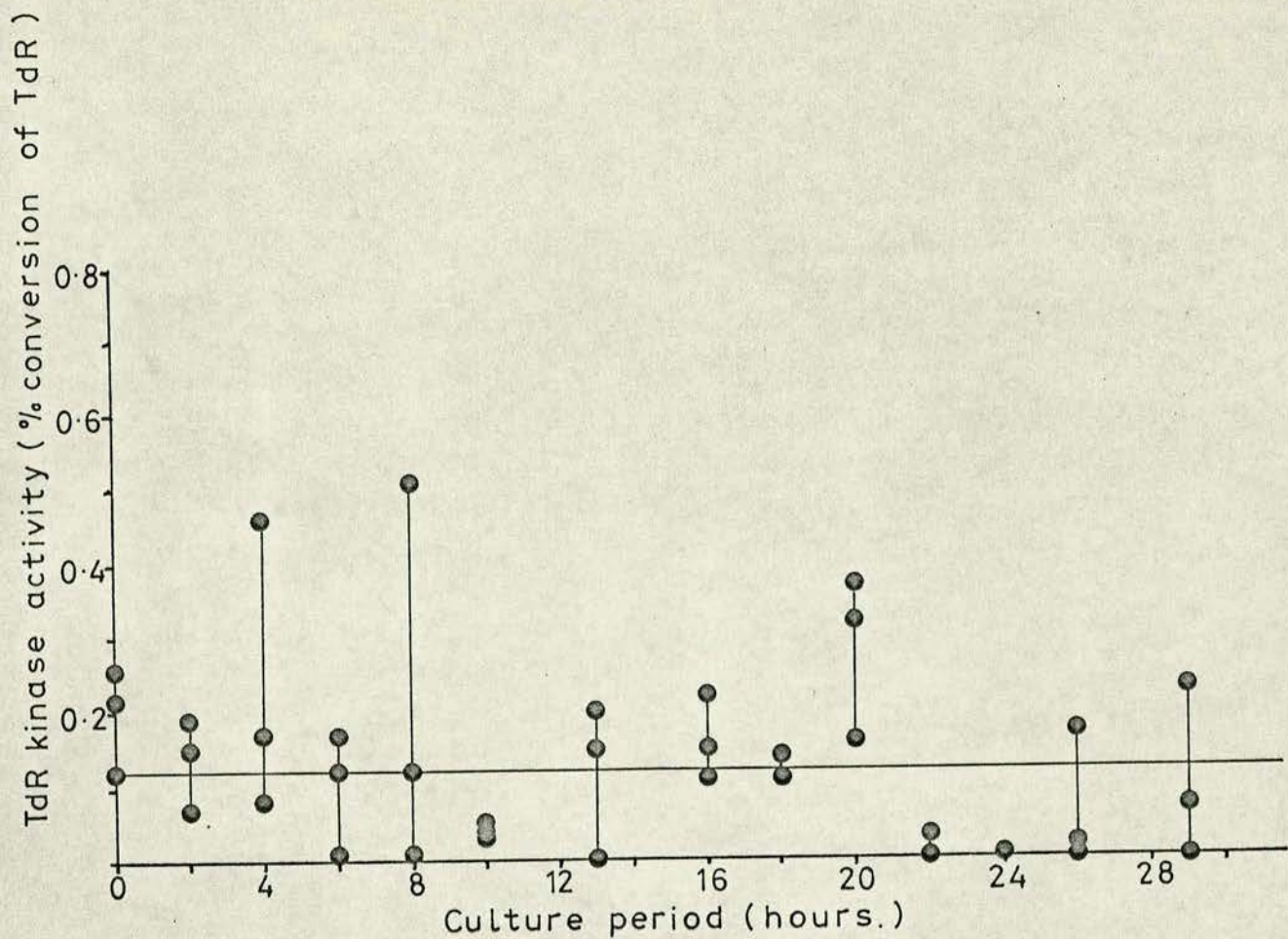
FIG. 3.16   TdR kinase activity during the first part
of the cell cycle (majority of pre -'S' period)

As further experiments on the cell cycle were inpracticable due to the increasing length of the lag phase during the summer season, the period of time up until the next 'artichoke season' (November) was spent on improving the sensitivity of the TdR kinase assay method.

Because of the lack of availability of the large numbers of tubers required to set up asynchronously dividing cultures, and the contamination problem at this time of the year another source of dividing artichoke tissue was sought.

Growth of artichoke tuber buds.

Three different systems of growing artichoke tuber buds were investigated.

1.   The terminal bud of an artichoke tuber was removed and the tuber was placed in a little water beneath an inverted beaker.

2.   Individual tuber buds plus surrounding tissue were placed in petri dishes on top of filter papers moistened with distilled water.

3.   As for 2. except that water was replaced by a solution of sucrose + mineral salts (see Ch. 2).

All systems were placed in the dark at $25^{\circ}C$ for $2\frac{1}{2}$ days.

The best yield of shoot tissue (Shoots approx. 1 cm. long of average weight in the region of 100 mg.) was obtained from the first system and this system was used to provide tissue for further experiments.

Experiment 6.

In this experiment the TdR kinase activity of an extract prepared using 632 mg. of artichoke shoot tissue/ml. of 0·02M phosphate buffer pH 7·2 was determined.   For comparison, the activity of a 0·1 ml. sample of wheat embryo extract prepared as previously described, was also measured.   The activities of 0·03, 0·06 and 0·1 ml. aliquots of artichoke shoot extract were determined. The following results were obtained.

	% counts in dTMP region		TdR kinase activity (% conversion of TdR to dTMP)
Control	1·58		
	1·40	Average 1·50	0
	1·52		
Wheat	11·5		10·00
	9·3		7·80
Artichoke 0·03 ml.	1·69		0·19
	1·62		0·12
0·06 ml.	4·30		2·80
	2·08		0·58
0·10 ml.	3·45		1·95
	2·08		0·58
	2·43		0·93

Although there is considerable variation between replicates, this experiment has shown that for artichoke shoot extracts, TdR kinase activity is very roughly proportional to the volume of extract. The activity of the 0·1 ml. aliquot of extract is similar to that

obtained using a similar extract prepared from 'asynchronous' explants (see Ex. 3).   The TdR kinase activity of wheat extract is considerably higher however, and indicates that this medium (which was also used in the previous experiment) is capable of supporting considerable TdR kinase activity.

Experiment 7.

In a further attempt to increase the sensitivity of the method, the proportion of radioactive substrate molecules in the radio-active medium was increased by preparing a new medium of twice the specific activity of the previous one.   This was achieved by preparing a medium as before except that only half the amount of unlabelled TdR (42 µg.) was added to 5 ml. of medium.   An extract was prepared using 690 mg. of shoot tissue per 1·0 ml. of buffer and the activities of 0·03, 0·06 and 0·10 ml. aliquots of the extract were determined.

	% counts in dTMP region		TdR kinase activity (% conversion of TdR to dTMP)
Control	2·52 2·63	Average 2·58	0
0·03 ml.	2·65 3·11		0·07 0·53
0·06 ml.	3·65 3·46		1·07 0·88
0·10 ml.	3·71 4·27		1·13 1·69

These values are on average higher than those obtained in the previous experiment and the enzyme activity is approximately proportional to the volume of extract.

Experiment 8.

In this experiment the specific activity of the radioactive medium was again increased by doubling the amount of ^{3}H-TdR added to 5 ml. of medium. In this instance 5 ml. of medium contained 0·5 mCi of ^{3}H-TdR. In addition to the use of a high specific activity medium, increased incubation temperatures of 30°C (Wanka and Poels 1969) and 37°C (Brent et al. 1965) were used. All tubes were shaken initially and at 5 minute intervals during the incubation, to ensure adequate mixing of the reaction mixture. The extract was prepared using 689 mg. of shoot tissue/ml. buffer. The following results were obtained.

	% counts in dTMP region		TdR kinase activity (% conversion of TdR to dTMP)
Control (no enzyme)	2·84 2·52 2·66	Average 2·67	0
30°C	4·87 5·68 4·84		2·20 3·01 2·17
37°C	5·34 5·66 5·55		2·67 2·99 2·88

Although the conversion was over 2% at both incubation temperatures, the values at 37°C were on average higher than the 30°C values. For this reason, an incubation temperature of 37°C was used in all subsequent experiments.

Experiment 9.

In previous experiments, all extracts prepared either from cultured artichoke explants or from artichoke shoots were brown in colour which was presumably due to endogenous peroxidase and oxidase activity.   In this experiment an attempt was made to prevent browning by including 0·5% β-mercapto ethanol (EtSH) in the grinding medium as a protector of sulphydryl groups.   The effect of including this substance and the effect of including BSA as a protein protector (for reasons quoted in Ex. 4) on enzyme activity were compared with the use of the normal grinding medium.

3 extracts were prepared each containing 693 mg. of artichoke shoots/ml. of grinding medium using a) 0·02M phosphate buffer, b) 0·02M phosphate buffer containing 400 µg./ml. BSA and c) 0·02M phosphate buffer containing 0·5% v/v EtSH.   The incubation was carried out at 37°C, but in contrast to the previous experiment the tubes were not shaken during the incubation.   The following results were obtained.

	% counts in dTMP region	TdR kinase activity (% conversion of TdR to dTMP)
Control (from Ex.8)	2·67	0
a) Buffer only	4·24	1·57
	3·50	0·83
	3·41	0·74
b) + BSA	4·52	1·85
	4·72	2·05
	4·41	1·74
c) + EtSH	4·50	1·83
	4·25	1·58
	4·16	1·49

The activities obtained in the presence of BSA and in the presence of EtSH are on average higher than the activities obtained in their absence. The extract prepared in the presence of EtSH was yellow in colour whilst all other extracts were brown. Because of the possible stimulatory effect on the activity of TdR kinase of both BSA and EtSH, and the action of EtSH in preventing browning of extracts, both these substances were included in the grinding medium used in all subsequent experiments.

Although this experiment was performed using similar conditions to those used in Experiment 8, the TdR kinase activity of the extract prepared using 0·02M phosphate buffer pH 7·2 was markedly lower in this experiment. A possible explanation of this discrepancy is that the assay tubes were unshaken during incubation. This would suggest that a shaking treatment during incubation may increase the apparent enzyme activity, therefore a shaking water bath was used for all subsequent incubations to ensure adequate and uniform mixing of the assay media.

Experiment 10.

In view of the substantial percentage conversions obtained using this assay, a time course experiment was carried out using the method so far established. An extract was prepared using 690 mg. of artichoke shoots/ml. of grinding medium (0·02M phosphate buffer pH 7·2 + 0·5% EtSH and 400 μg./ml. BSA). Incubations of 0, 10, 20 and 30 minutes duration were carried out in triplicate using a shaking water bath at 37°C. The results shown in Fig. 3.17
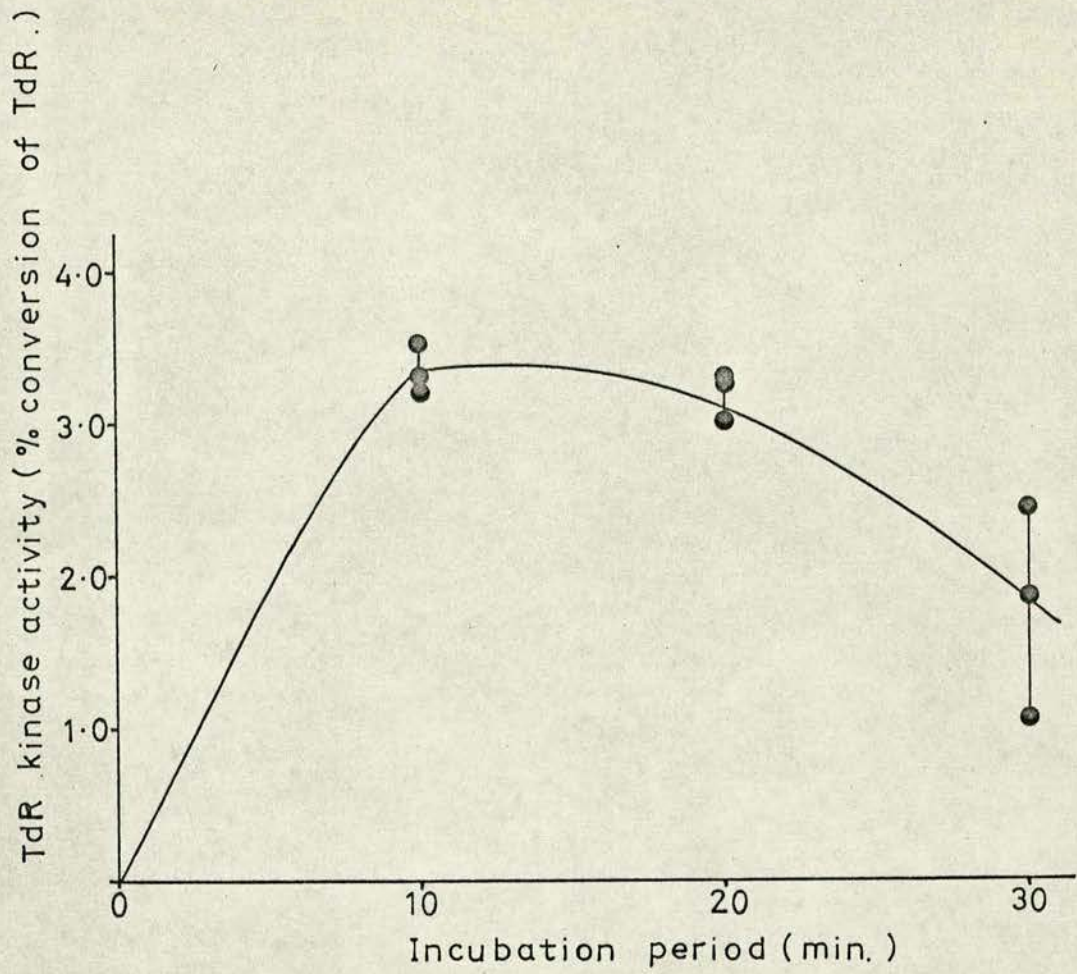
FIG. 3.17 Time course for TdR kinase activity
of artichoke shoot extract.

indicate a lower level of the dTMP product of the reaction after 20 and 30 minutes incubation. This indicates that breakdown of dTMP has occurred.

There are several possible fates of the dTMP product of the TdR kinase reaction.

a) further phosphorylation i.e. dTMP $\longrightarrow$ dTDP $\longrightarrow$ dTTP.

b) dephosphorylation i.e. dTMP $\longrightarrow$ TdR mediated by phosphatase. To test the first possibility the conversion of counts to dTDP or dTTP was investigated. The remainder of the cellulose acetate strip from one of the 30 minute incubations was cut into $\frac{1}{2}$ cm. strips and counted. No evidence of a radioactive dTDP or dTTP peak was detected.

Hotta and Stern (1961) have shown the breakdown by phosphatase action, of the dTMP product of the TdR kinase reaction in anther tissue. In this system they showed that the breakdown of added ^{3}H-dTMP to ^{3}H-TdR by the extract, was completely suppressed in the presence of 0·2M phosphate buffer.

Similar tests were carried out in the next experiment to determine whether a similar situation existed in the artichoke shoot assay under the present assay conditions and if so, whether the phosphatase activity could be suppressed by the use of high molarity phosphate buffers.

Experiment 11.

For this experiment two different extracts were prepared, the first using 706 mg. artichoke shoots/ml. of 0·02M phosphate buffer

pH 7·2 and the second using 696 mg. artichoke shoots/ml. of 0·2M phosphate buffer pH 7·2.

^{3}H-labelled dTMP was prepared using the standard wheat assay of TdR kinase activity as previously described. Several 100 μl. aliquots of the supernatant were subjected to a 4 hour electrophoresis and the dTMP band was located by counting one of the strips in the usual way. For the one strip that was counted it was estimated that a 27·1% conversion of substrate to product had yielded a 2 cm. dTMP band containing a total of 71,000 counts/minute. A 2 cm. band in the same position was removed from replicate electrophoresis strips and each band was subdivided into 6 equal strips, i.e.
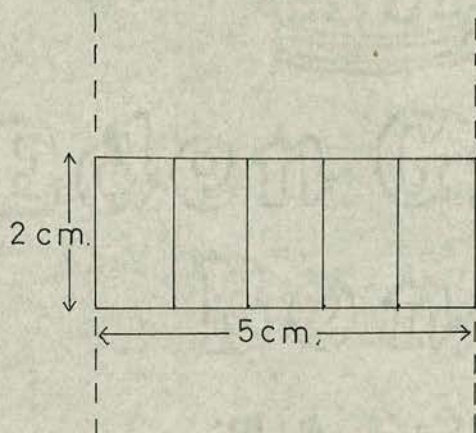
Each individual strip, which contained approximately 12,000 cpm. ^{3}H-dTMP was cut into quarters and placed in the bottom of a conical centrifuge tube. A 0·5 μl. aliquot of 0·1M MgCl$_2$ was added to each tube (as phosphatases may require the presence of Mg^{++} ions). The six tubes were then treated as follows.

	Additions	Final molarity

a) 0·1 ml. 0·02M buffer + 0·1 ml. 0·02M extract   0·02M

b)   " H_2O +   "   0·2M extract   approx.0·1M

c)   "   0·2M buffer +   "   "   "   0·2M

d)   "   0·4M buffer +   "   "   "   approx.0·3M

e) 0·2 ml. 0·02M buffer (Blank)

The electrophoresis strips containing [3]H dTMP were eluted for 30 minutes with the buffer component prior to the addition of extract.   After a 1 hour incubation in a shaking water bath at $37^{\circ}C$ the reaction was stopped as for the TdR kinase reaction except that the second extraction with 70% ethanol was omitted.   100 µl. aliquots of the supernatant fraction were subjected to the standard 4 hour electrophoresis procedure.   The strips were counted as for the TdR kinase assay and results were expressed as percentage of the total counts present in TdR.   For the blank sample, the absence of counts in TdR indicated the purity of the dTMP preparation.

The extent of dephosphorylation is compared with the molarity of phosphate buffer in the assay medium, in Fig. 3.18.   The approximately linear relationship indicates that dephosphorylation of dTMP is suppressed with increasing molarity of phosphate buffer but the suppression is not complete, even at 0·3M.   In all subsequent experiments a final phosphate buffer concentration in the TdR kinase reaction medium of 0·2M was used, as the use of 0·3M or 0·4M phosphate buffers was subject to severe crystallization problems on cooling.   A further attempt was made to suppress
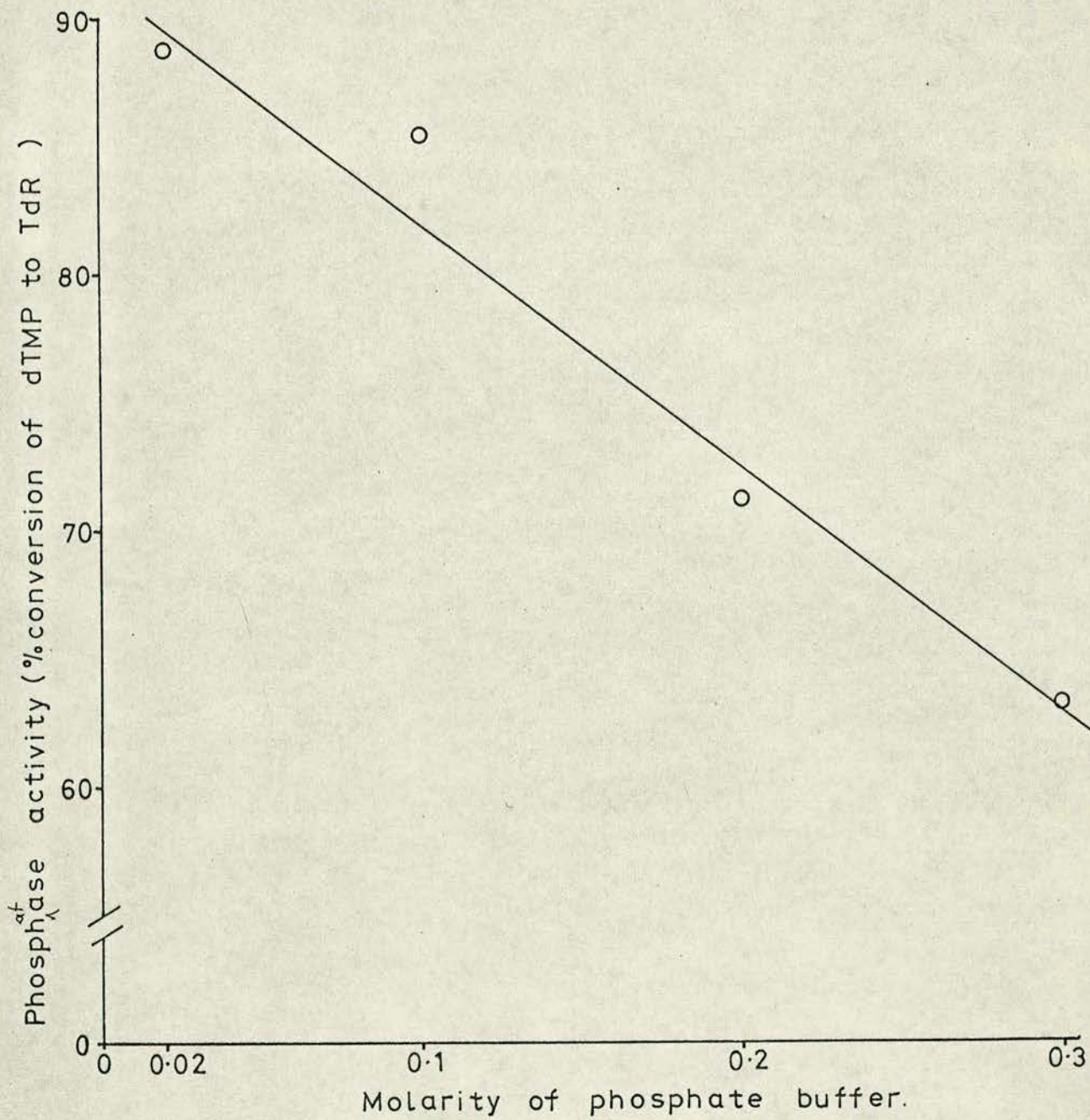
FIG. 3.18 The effect of phosphate buffer molarity on the breakdown of dTMP by phosphatase.

phosphatase activity in the next experiment by manipulation of
the pH.

Experiment 12.

Robertson (1966) working on phosphatase activity in artichoke
tuber tissue found two pH optima, one in the region of pH 5, and a
second in the region of pH 7-8.   Anticipating that further
suppression of phosphatase activity in artichoke shoot extracts
might be achieved by increasing the pH of the phosphate buffer,
the phosphatase test was carried out at a number of different pH
values.   Robertson (1966) had also found that tissue which had
been cultured for 3 or 4 days had a much higher level of
phosphatase activity than freshly excised tissue.   As it was
possible that artichoke shoot tissue was also a richer source of
phosphatase activity than tuber tissue, tests were also carried
out to determine the phosphatase activity of freshly excised
explants in the presence of 0·02M and 0·2M phosphate buffers.

For this experiment, four different extracts were prepared.
a) 345 mg. shoots/0·5 ml. of 0·2M phosphate buffer pH 7·2
b) 707 mg.   "   /1·0 ml. "   "   "   "   pH 8·0
c) 345 mg.   "   /0·5 ml. "   "   "   "   pH 9·1
d) 698 mg. explants/1·0 ml. of 0·02M phosphate buffer pH 7·2.
As in the previous experiment, 6 different tubes each containing
cellulose acetate strips impregnated with ^{3}H-dTMP and 0·5 μl. of
0·1M $MgCl_2$ were prepared.   The following additions were made.

<u>Additions</u> <u>Final molarity and pH</u>

1) 0·1 ml. 0·2M buffer pH 7·2 + 0·1 ml. 0·2M shoot
                    extract pH 7·2   0·2M   7·2

2) 0·1 ml. 0·2M buffer pH 8·0 + 0·1 ml. 0·2M shoot
                    extract pH 8·0   0·2M   8·0

3) 0·1 ml. 0·2M buffer pH 9·1 + 0·1 ml. 0·2M shoot
                    extract pH 9·1   0·2M   9·1

4) 0·1 ml. 0·02M buffer pH 7·2 + 0·1 ml. 0·02M
                explant extract pH 7·2   0·02M   7·2

5) 0·1 ml. 0·4M buffer pH 7·2 + 0·1 ml. 0·02M
                explant extract pH 7·2 approx.0·2M   7·2

6) 0·2 ml. 0·02M buffer pH 7·2 (Blank)

The results were obtained as described for the previous experiment.

As in the previous experiment, the blank showed no conversion of counts into TdR. For the artichoke shoot extracts, the following results were obtained.

pH of reaction medium	Phosphatase activity (% conversion of dTMP to TdR)
7·2	77·1
8·0	48·9
9·1	46·5

A marked suppression of phosphatase activity occurred on increasing the pH from 7·2 to 8·0 but there was little increase in the suppressive effect between pH 8·0 and pH 9·1. As the pH optimum for TdR kinase is in the region of pH 8·0 for most tissues (Cleaver 1967), this pH was adopted in all subsequent experiments.

For freshly excised tuber tissue, the phosphatase test yielded a value of 67·0% conversion to TdR in the presence of 0·02M phosphate buffer pH 7·2, which was reduced to 11·6% in the presence of 0·2M phosphate buffer pH 7·2. Anticipating a further reduction using 0·2M phosphate buffer pH 8·0, it was estimated that phosphatase breakdown of the product of the TdR kinase reaction during a 20 minute incubation period would be negligible for freshly excised tissue.

Experiment 13.

The availability of tubers from the new artichoke crop enabled the investigation to proceed using explant tissue. A time course experiment using asynchronously dividing explants was carried out using the new grinding medium (0·2M phosphate buffer pH 8·0 + 0·5% EtSH + 400 µg./ml. BSA) and the final concentration of buffer in the reaction medium was 0·2M pH 8·0. The extract was prepared using 700 mg. of Day 4 explants (grown in the presence of coconut milk)/ml. of grinding buffer. Incubations of 0, 10, 20 and 30 minutes were carried out in triplicate. The test for phosphatase activity was carried out as previously described, in the presence of 0·2M phosphate buffer pH 8·0.

The time course which was obtained for the TdR kinase reaction is shown in Fig. 3.19 and was linear up to 30 minutes incubation. An incubation period of 20 minutes was chosen for use in all subsequent experiments.

The phosphatase test revealed a conversion to TdR of 43·5%
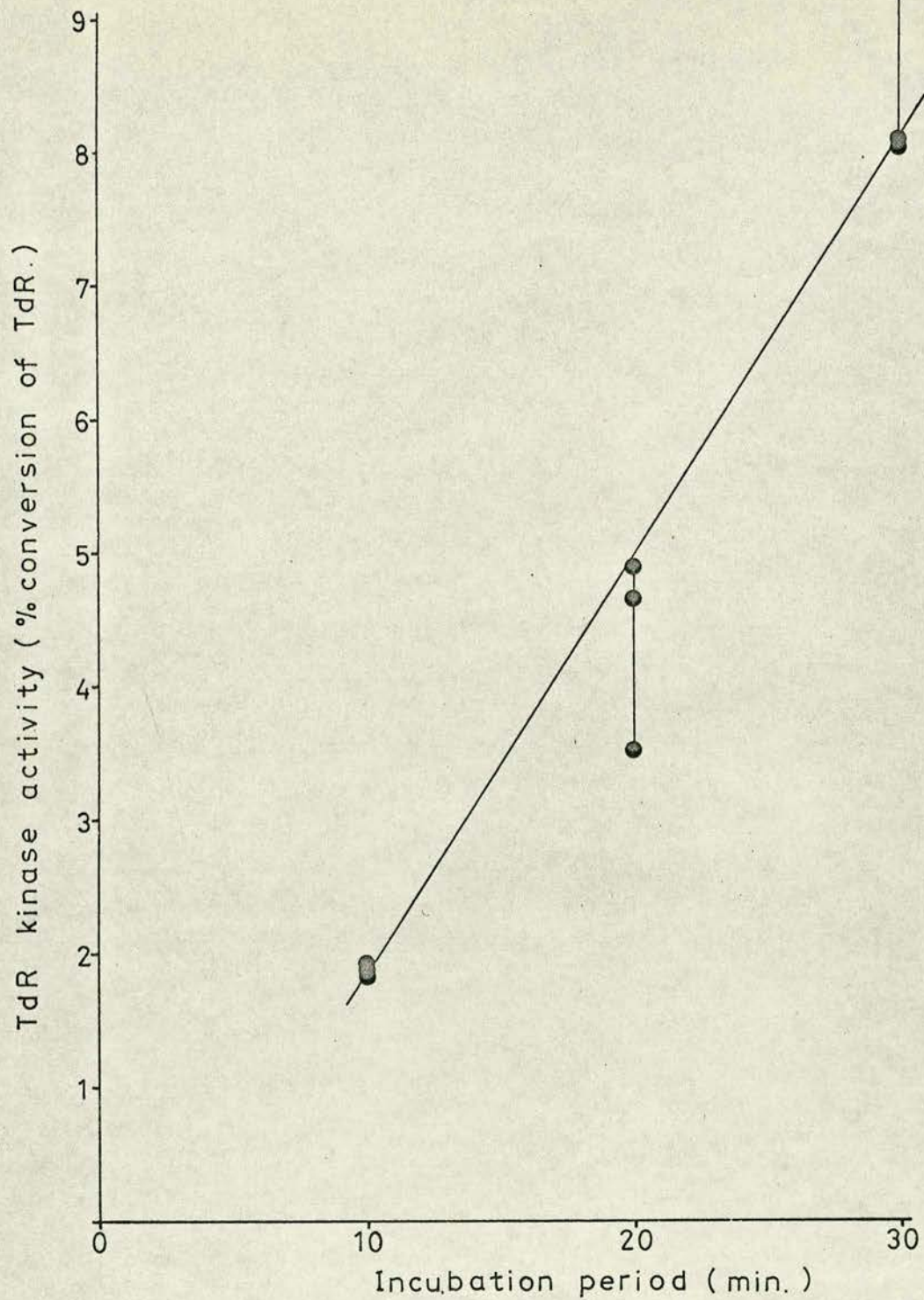
FIG. 3.19   Time course for TdR kinase activity of
day 4 explants assayed in the presence
of 0·2M phosphate buffer pH. 8·0.

in 1 hour.   Assuming that the phosphatase reaction is linear, the value obtained for Day 4 explants is 3-4 times greater than the value for freshly excised explants under these conditions, (see previous experiment) and is in good agreement with the results of Robertson (1966).

This experiment has shown that the TdR kinase reaction for Day 4 artichoke extract is linear over a 30 minute incubation period despite the fact that considerable phosphatase activity is also present.   As all cell cycle experiments were carried out between Days 0 - 2 of culture when the phosphatase activity is relatively low, this method is suitable for the assay of TdR kinase during the cell division cycle.

The method employed in this experiment was used to assay TdR kinase activity in several cell cycle experiments e.g. Experiments 7, 8 and 11 of Chapter 4.   In those experiments which also included assays of dTMP kinase activity such as the following experiment and Experiments 9, 13 and 14 of Chapter 4, the TdR kinase assay was adapted for convenience to resemble the assay of dTMP kinase.

Experiment 14.

An essential prerequisite to the mixed extract experiment (Experiment 13 of Ch. 4) is proof that the enzyme activity is proportional to the volume of extract.   Although this proportionality was indicated in earlier experiments, it had not been shown for the final developed method.

For this experiment, tissue from the 'S' phase of the first division cycle was used (at this time, it had already been shown that TdR kinase activity increased during 'S'). Explants were cultured in the dark in a sucrose + mineral salts + 2,4-D medium and harvested after 22 hours. 200 explants were macerated using pestle and mortar treatment in 1·0 ml. of grinding buffer. Silver sand was not included as an abrasive. Further homogenisation was achieved using a Kontes hand glass homogeniser followed by the standard centrifugation and incubation procedures. The reaction was stopped by treatment at $100^\circ C$ for 2 minutes followed by immersion in an ice bath. After a 1000 g./5 minutes centrifugation at $0^\circ C$, the reaction tubes were stored in a deep freeze at $-20^\circ C$. A 10 µl. aliquot of supernatant only, was required for cellulose acetate electrophoresis and consequently a much better separation was obtained compared with the ethanol extraction method.

In this experiment amounts of extract varying from 0·01 ml. to 0·1 ml. were made up to 0·1 ml. with grinding buffer as required, and the TdR kinase activity of duplicated samples was found.

The results shown in Fig. 3.20, show a linear relationship between volume of extract and enzyme activity.

Experiment 15.

In preparation for Experiment 9 of Chapter 4, a preliminary experiment was carried out to test the effect of a grinding buffer containing 1·0% EtSH, on TdR kinase activity. Because the cell
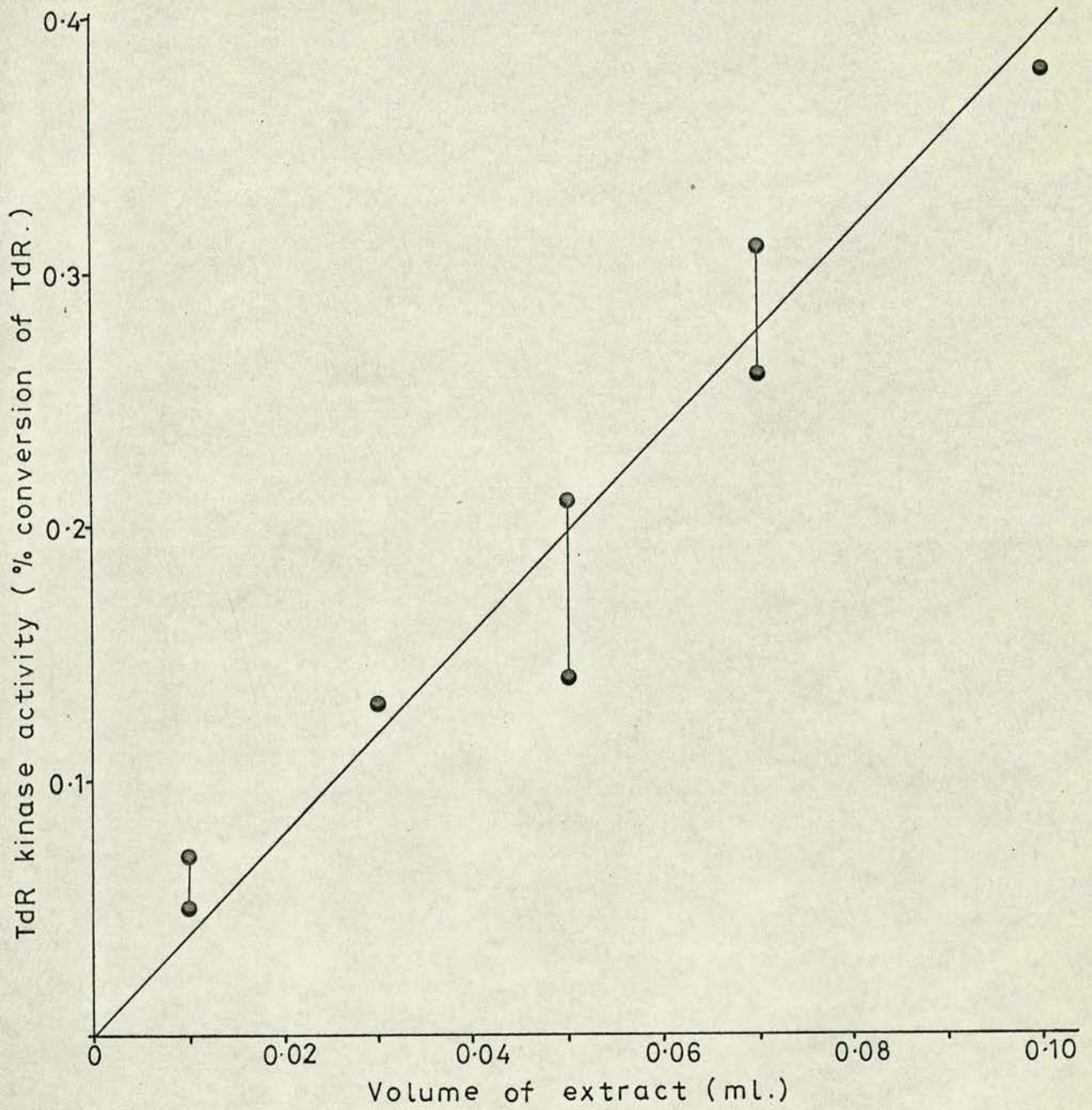
FIG. 3.20 Relationship between volume of extract and
TdR kinase activity of 22 hour explants.

cycle experiment in question was intended to measure activities of DNA polymerase, TdR kinase and dTMP kinase in the same experiment, it was important that tests were carried out in order to check that the presence of 1·0% EtSH in the grinding medium (which was present for reasons associated with the DNA polymerase assay) did not inhibit the activity of either TdR or dTMP kinase.

Two samples of 100 explants were removed from fresh tubers. Extracts were prepared using 1·0 ml. of the following grinding media. 1) 0·2M phosphate buffer pH 8·0 + 400 µg./ml. BSA + 0·5% EtSH. 2) "   "   "   "   "   "   "   "   1·0% "

The assay method was adapted to resemble the dTMP kinase assay and the reaction medium contained

50 µl. radioactive medium

50 µl. extract

10 µl. pyruvate kinase (10 mg. protein/ml.).

Although this reaction medium was of approximately half the volume of that used in previous experiments the ratio of extract:radioactive medium was similar.   The incubation was carried out as in the previous experiment and the reaction was stopped in the same way.

The results of single electrophoresis runs indicated values of 0·21 and 0·33% conversion to dTMP for extracts prepared in the presence of 0·5% and 1·0% EtSH respectively.   These results indicate that the use of 1% EtSH in the grinding medium does not inhibit TdR kinase activity.   There is also some suggestion that increasing the EtSH concentration in the grinding medium from

0·5% to 1·0% may have a stimulatory effect on the activity of TdR kinase.

In this section an assay method has been developed to measure TdR kinase activity in artichoke tissue.   As the level of TdR kinase activity in this tissue is extremely low compared with tissues such as wheat embryo, a successful assay method was developed from the method of Hotta and Stern (1961) by increasing the concentration of tissue in the extract and the specific activity of the substrate, increasing the incubation temperature, by the use of uniform shaking during incubation, by incorporating BSA and β-mercaptoethanol into the grinding medium and finally, by the use of high molarity phosphate buffers of high pH in the reaction medium in order to suppress as far as possible the breakdown of dTMP by phosphatases.   The complete assay method was suitable for measuring the level of TdR kinase activity during the cell division cycle because the activity obtained was proportional to the period of incubation and proportional to the volume of extract.

This assay was designed to measure TdR kinase activity during the first division cycle i.e. during the first day of culture only.   Robertson (1966) has shown that phosphatase activity is relatively low during this period compared with tissue which has been cultured for a number of days.   A linear time course for TdR kinase activity was obtained using Day 4 explants, therefore this method is undoubtedly suitable for the measurement of TdR kinase activity during the cell division cycle.

SECTION C

THYMIDINE MONOPHOSPHATE KINASE AND THYMIDINE MONOPHOSPHATE PHOSPHATASE

The enzyme thymidine monophosphate kinase (dTMP kinase) catalyses the conversion of thymidine monophosphate (dTMP) to thymidine diphosphate (dTDP) according to the following equation (Dixon and Webb 1958)

$$\text{Thymidine monophosphate} + \text{ATP} \longrightarrow \text{Thymidine diphosphate} + \text{ADP}.$$
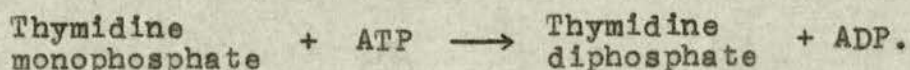
The activity of this enzyme has been measured in a limited number of tissues e.g. Brent et al. (1965) in Hela cells, Johnson and Schmidt (1966) and Wanka and Poels (1969) in Chlorella, and Grav and Smellie (1965) in Landschutz ascites tumour cells. There are no reports on the measurement of this enzyme in higher plants. The basis of the assay system used in all these communications is the conversion of ^{3}H or ^{32}P-labelled dTMP (substrate) to the phosphorylated product, dTDP.

In some organisms the enzyme thymidine diphosphate kinase (dTDP kinase) which catalyses the conversion of dTDP to dTTP by further phosphorylation, has a high affinity for dTDP. For example, in the assay of dTMP kinase activity in Chlorella, Wanka and Poels (1969) noted that more than 60% of the dTDP formed during standard incubation was converted to dTTP, and it was therefore also necessary to measure the counts converted to dTTP.

The accurate measurement of enzyme activity depends firstly

on the successful separation of the substrate and product by
paper chromatography or ion exchange chromatography, and secondly
on the estimation of radioactivity of the product using various
counting techniques.   Separations using paper chromatography are
time consuming and often involve the use of specialized chromatogram
scanning counting equipment whereas elution from ion exchange
columns requires the use of large volumes of solvent.   In view of
this a more rapid separation technique was evolved using high
voltage paper electrophoresis coupled with the direct measurement
of both substrate and product on the paper by scintillation counting.

This section describes experiments leading up to the
successful development of a dTMP kinase assay system for artichoke
tissue.   As the development of the high voltage electrophoresis
procedure is considered to be a critical part of the method, the
development of this technique is also described at this point.

High voltage electrophoresis

Using this technique, preliminary tests were carried out in
order to establish suitable conditions for the adequate separation
of reaction products dTDP and/or dTTP from the substrate dTMP.
Standard solutions of dTMP, dTDP and dTTP were prepared at a
concentration of 10 mg./ml. of 0·001N formic acid.

As an adequate separation of these three standard solutions
could not be obtained by low voltage cellulose acetate electro-
phoresis on 20 cm. cellulose acetate strips, a series of high
voltage paper electrophoresis runs were carried out.   All measure-

ments were made from the baseline to the centre of the spot which was located using UV illumination.

For a 38 minute electrophoresis run of standards,

dTMP moved 8 cm. towards the anode

dTDP   "   12·8 cm.   "   "   "

dTTP   "   13·3 cm.   "   "   "

In a 68 minute electrophoresis run in which the baseline was 7 in. from the cathode end,

dTMP moved 12·0 cm. towards the anode

dTDP   "   19·0 cm.   "   "   "

dTTP   "   21·7 cm.   "   "   "

In a 77 minute electrophoresis run with the baseline only 4 in. from the cathode end,

dTMP moved 14·8 cm. towards the anode

dTDP   "   23·2 cm.   "   "   "

dTTP   "   26·3 cm.   "   "   "

A standard mixture was prepared using 0·1 ml. of each of the three standard 10 mg./ml. solutions.   30 µl. of the standard mixture was streaked onto the baseline.   In an 84 minute electro-phoresis run using a baseline only 4 in. from the cathode end,

dTMP moved 15·4 cm. towards the anode

dTDP   "   24·9 cm.   "   "   "

dTTP   "   27·0 cm.   "   "   "

The combined results of these tests have shown that it is possible to separate dTMP from the di and tri-phosphates using this technique.   It is possible to separate dTDP from dTTP by

increasing the duration of electrophoresis. However, complete separation of the di and tri-phosphates is not essential for the dTMP kinase assay because any counts converted to dTTP are presumably derived from dTDP which is the product of the dTMP kinase reaction.

The dTMP kinase assay.

The dTMP kinase assay was adapted from the successful TdR kinase assay by substituting labelled dTMP in place of TdR in the assay medium. Brent et al. (1965) had also adopted this approach for work on Hela cell cultures.

A radioactive medium of similar composition to that used in the TdR kinase assay was prepared but in this instance the substrate was thymidine-2-^{14}C-5'monophosphate supplied at a specific activity of 57 mCi/mM from Radiochemicals, Amersham.

5 ml. of radioactive medium contained:-

$2 \cdot 5$ ml. $0 \cdot 4$M sodium phosphate buffer pH $8 \cdot 0$

$0 \cdot 025$ ml. $0 \cdot 1$M $MgCl_2$

$6 \cdot 2$ mg. ATP

$8 \cdot 4$ mg. phospho-enol pyruvate (PEP)

1 μCi ^{14}C-dTMP (approx. 1,000,000 counts/min.)

distilled water to a total volume of 5 ml.

The grinding buffer was $0 \cdot 2$M sodium phosphate buffer pH $8 \cdot 0$ containing 400 μg./ml. of BSA and $0 \cdot 5$% EtSH. The tissue was homogenised in a pestle and mortar using silver sand as an abrasive and after a 2200 g. centrifugation at 0°C for 10 minutes the supernatant fraction was retained as the enzyme extract.

For individual assays, the reaction mixture contained

    0·1 ml. of radioactive medium

    0·1 ml. extract (added last)

    0·025 ml. pyruvate kinase (10 mg. protein/ml.)

Incubation was for 20 minutes at $37^{\circ}C$ in a shaking water bath, and the reaction was stopped by placing the tubes in a boiling water bath for 2 minutes (see Johnson and Schmidt 1966). After cooling in an ice bath, coagulated proteins were precipitated by a 1000 g. centrifugation at $0^{\circ}C$ for 5 minutes. The reaction tubes were then stored in a deep freeze at $-20^{\circ}C$ prior to high voltage electro-phoresis.

After high voltage electrophoresis of, for example, a 6 cm. streak of the supernatant, the electrophoresis path was cut into 20 or more 1 x 6 cm. bands. Each individual band was cut into two halves, each half folded and both halves completely immersed in 10 ml. of toluene scintillator inside a counting vial. Each band was counted for 1 minute using a ^{14}C setting. The positions of TdR, dTMP, dTDP and dTTP were identified using standards, observed under UV illumination.

Experiment 1.

A preliminary test for dTMP kinase activity was carried out using 48 hour old wheat embryo tissue (grown as previously described) and freshly excised artichoke explants. Because the wheat embryo tissue exhibited high TdR kinase activity, it was anticipated that it might also provide a rich source of dTMP kinase activity.

Extracts were prepared using 15 wheat embryos/0·5 ml. of grinding buffer and 100 freshly excised explants/ml. of grinding buffer.   Enzyme assays were carried out as described and a blank estimation (no enzyme) was also included.   100 µl. aliquots of supernatant were streaked onto electrophoresis papers and the duration of electrophoresis was 50 minutes.

In all cases there was no apparent radioactive peak observed in the dTDP/dTTP region but a peak was observed in the same position as TdR.   The percentage of the total counts in TdR was estimated as described in Chapter 2.

	% of total counts in TdR
Blank	2·8%
Wheat extract	11·6%
Artichoke extract	3·3%

Although there is no apparent dTMP kinase activity, a marked conversion of dTMP to TdR is observed for the wheat extract due to the action of dTMP phosphatase.   The small TdR peak in the blank sample is attributed to impurities arising from the original enzymic preparation of dTMP from TdR.   A small amount of phosphatase activity is also present in the artichoke extract.

Experiment 2.

In the previous section the effect of high molarity phosphate buffers in suppressing the activity of phosphatase was noted.   In case the failure to detect dTMP kinase activity in the previous experiment was due to a similar inhibition of dTMP kinase activity,

the experiment was repeated in the presence of tris-HCl buffer.
The grinding buffer contained 0·05M tris-HCl buffer pH 8·0 +
400 µg./ml. BSA + 0·5% EtSH and a new radioactive medium was made
up to a final tris-HCl buffer concentration of 0·01M at pH 8·0.

The previous experiment was repeated using the new media
and electrophoresis was for 60 minutes at 1000 volts.   Standard
spots were also included.   The following results were obtained.

	% of the total counts in the	
	TdR region	dTDP/dTTP region
Blank	3·44	no peak
Wheat extract	91·62	"
Artichoke extract	35·1	1·68%
	34·2	no peak

As the apparent dTDP/dTTP peak occurred in only one of the
duplicated artichoke extract estimations it is likely that it
was an artifact of the electrophoretic separation and not a
measurement of enzyme activity.   When these results are compared
with those of the previous experiment it is evident that conversion
to TdR by phosphatase action for both wheat and artichoke is
considerably enhanced in the presence of 0·05M tris-HCl buffer
compared with the use of 0·2M phosphate buffer in the assay medium.

In the next experiment, the ATP concentration was increased
in an attempt to stimulate the formation of dTDP by dTMP kinase
action.

Experiment 3.

As the present concentration of ATP was less than 1mM, the ATP concentration was increased 4 fold by adding ATP to the radioactive medium to give a final concentration of 24·8 mg./5 ml.

A time course experiment was carried out using an artichoke extract prepared using 150 freshly excised explants/1·5 ml. of grinding medium (tris-HCl). The incubation time was varied from 0 to 25 minutes. The electrophoresis was for 60 minutes and standard solutions of TdR and the three nucleotides were also used. The percentage of the total counts in the dTDP/dTTP region of the strip was estimated in addition to the counts converted to TdR. The following results were obtained.

Incubation period (minutes)	% of the total counts in the	
	TdR region	dTDP/dTTP region
0	4·1	1·26
5	28·9	1·00
	28·8	1·30
10	36·5	1·39
	36·1	0·93
15	40·7	2·46
	39·2	1·11
20	45·6	1·22
	44·5	1·28
25	45·5	1·38
	44·7	1·15

From these results it is evident that dTMP phosphatase activity increases during the incubation period but that there is no apparent increase in dTMP kinase activity. The low conversions

Experiment 3.

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ATP concentration was increased 4 fold by adding ATP to the radio-
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grinding medium (tris-HCl). The incubation time was varied from
0 to 25 minutes. The electrophoresis was for 60 minutes and
standard solutions of TdR and the three nucleotides were also used.
The percentage of the total counts in the dTDP/dTTP region of the
strip was estimated in addition to the counts converted to TdR.
The following results were obtained.

Incubation period (minutes)	% of the total counts in the	
	TdR region	dTDP/dTTP region
0	4·1	1·26
5	28·9	1·00
	28·8	1·30
10	36·5	1·39
	36·1	0·93
15	40·7	2·46
	39·2	1·11
20	45·6	1·22
	44·5	1·28
25	45·5	1·38
	44·7	1·15

From these results it is evident that dTMP phosphatase activity
increases during the incubation period but that there is no
apparent increase in dTMP kinase activity. The low conversions

observed in the dTDP/dTTP region are probably due to the presence of background counts in that region as there is no increase during incubation.

In other synchronous systems e.g. <u>Chlorella</u>, there is a high dTMP kinase activity associated with cell division. For this reason it was anticipated that a dividing asynchronous artichoke tissue might provide a richer source of the enzyme.

<u>Experiment 4.</u>

Artichoke explants were grown in liquid culture in the dark for 5 days and extracts were prepared using 700 mg. of tissue/ml. of grinding medium. Reactions were carried out in the presence of 0·05M tris-HCl buffer pH 8·0, and in the presence of 0·2M phosphate buffer pH 8·0. No dTMP kinase activity was detected, and the following conversions to TdR by phosphatase action were noted.

Buffer	% conversion to TdR
Tris-HCl	68·1
	62·5
Phosphate	15·4
	17·9

These observations confirm the results of Experiments 1 and 2 as they show that 0·2M phosphate buffer pH 8·0 markedly suppresses phosphatase activity.

If the dTMP kinase activity of artichoke tissue exists in a particle bound form, it is possible that the present failure to detect this activity is due to the use of a supernatant fraction as the extract.

Experiment 5.

In order to investigate this possibility, the activity of a crude homogenate and of a supernatant fraction was determined in this experiment. Day 7 asynchronously dividing explants grown in liquid culture in the dark, were used to prepare the extract. 700 mg. of tissue was macerated in 1·0 ml. of tris-HCl grinding buffer in a pestle and mortar. Further homogenisation was achieved using a Kontes hand homogeniser (This procedure was included in all subsequent experiments). An aliquot of the crude homogenate was retained and the remainder was subjected to the standard centrifugation procedure to obtain a supernatant fraction. No dTMP kinase activity was detected in either fraction, but the phosphatase activity was possibly slightly higher in the crude homogenate, compared with the supernatant fraction.

	% conversion to TdR
Homogenate	38·9
	40·0
Supernatant	35·2
	34·9

Therefore the inability to detect dTMP kinase activity in this experiment was not due to the mode of preparation of the enzyme extract. As a supernatant fraction is usually used to assay this enzyme, this fraction was used in all subsequent experiments.

In the next experiment, an attempt was made to increase the sensitivity of the dTMP kinase assay, by doubling the amount of radioactive substrate present in the assay medium.

Experiment 6.

In the TdR kinase experiments the specific activity of the substrate was increased by reducing the amount of cold substrate present and by increasing the amount of radioactive substrate. As the radioactive medium for the dTMP kinase assay does not contain any unlabelled substrate, the specific activity of the medium cannot be increased, instead the concentration of ^{14}C-dTMP in the radioactive medium was doubled. For this experiment the radioactive medium contained 2 μCi ^{14}C-dTMP in 5 ml. of medium. An extract was prepared using 700 mg. of Day 12 asynchronously dividing explants (grown in liquid culture) per ml. of a tris-HCl grinding buffer.

No dTMP kinase activity was detected.

In terms of measurable counts the final specific activity of the ^{14}C-dTMP in the reaction medium used in this experiment was approximately $11 \cdot 4 \times 10^7$ cpm./μ mole whereas the specific activity of the ^{3}H-TdR used in the TdR kinase assay was approximately 197×10^7 cpm./μ mole. (These figures and those quoted in the next experiment were derived as shown in Appendix 3). As it was impossible to further increase the specific activity of the ^{14}C-labelled substrate and a tritiated dTMP substrate of higher specific activity was available, a new radioactive medium was prepared using thymidine (methyl-T)-5'-monophosphate supplied at a specific activity of 1,000 mCi/m mole from Radiochemicals, Amersham.

Experiment 7.

The new radioactive medium had the following composition.

0·5 ml. 0·1M tris-HCl buffer pH 8·0

0·025 ml. 0·1M $MgCl_2$

12·4 mg. ATP.

8·4 mg. PEP

0·5 mCi ^{3}H-dTMP

Distilled water to a total volume of 5 ml.

In terms of measurable counts, the final specific activity of this medium was $76·4 \times 10^7$ cpm./μ mole. Using this medium, attempts were made to detect dTMP kinase activity in extracts prepared using 700 mg. of Day 5 asynchronously dividing explants (grown in liquid culture) per ml. of grinding buffer. Extracts were made using the following grinding buffers:-

a) 0·1M tris-HCl buffer pH 8·0 + 400 μg./ml. BSA + 0·5% EtSH.

b) 0·4M phosphate "   "   "   "   "   "

After electrophoresis, a small peak was present in the position of dTDP (identified using standard dTDP). The following conversions were obtained:-

	% of total counts in		dTMP kinase activity	Phosphatase activity
	dTDP	TdR		
Blank	0·372	4·17	-	-
Tris buffer extract	0·824	74·0	0·452%	69·83%
Phosphate " "	1·380	12·5	1·008%	8·33%

It is evident from these results that the use of tris-HCl buffer gives rise to a high phosphatase activity which is suppressed approximately 6 fold by the use of a final concentration of 0·2M phosphate buffer pH 8·0 in the reaction medium. This suppressive effect may be responsible for the elevated dTMP kinase activity observed in the presence of phosphate buffer. As it is possible to measure dTMP kinase activity in the presence of 0·2M phosphate buffer pH 8·0, this buffer was used in reaction media of all subsequent experiments due to its suppressive effect on the breakdown of dTMP by phosphatase action.

Experiment 8.

A time course experiment was carried out using an extract prepared in the presence of phosphate buffer as described in the previous experiment. In order to conserve substrate, the reaction mixture contained:-

50 μl. radioactive medium

50 μl. extract

10 μl. pyruvate kinase (a 10 mg./ml. solution)

This reaction mixture had a similar final composition in terms of molarity to that used previously. Duplicated assays were incubated for periods varying from 0 - 25 minutes. After the electrophoresis strips had been counted, all values were corrected for the blank and enzyme activities were expressed as percentage of dTMP converted to dTDP (dTMP kinase) or to TdR (dTMP phosphatase). The results of this experiment which are shown in Fig. 3.21 indicate
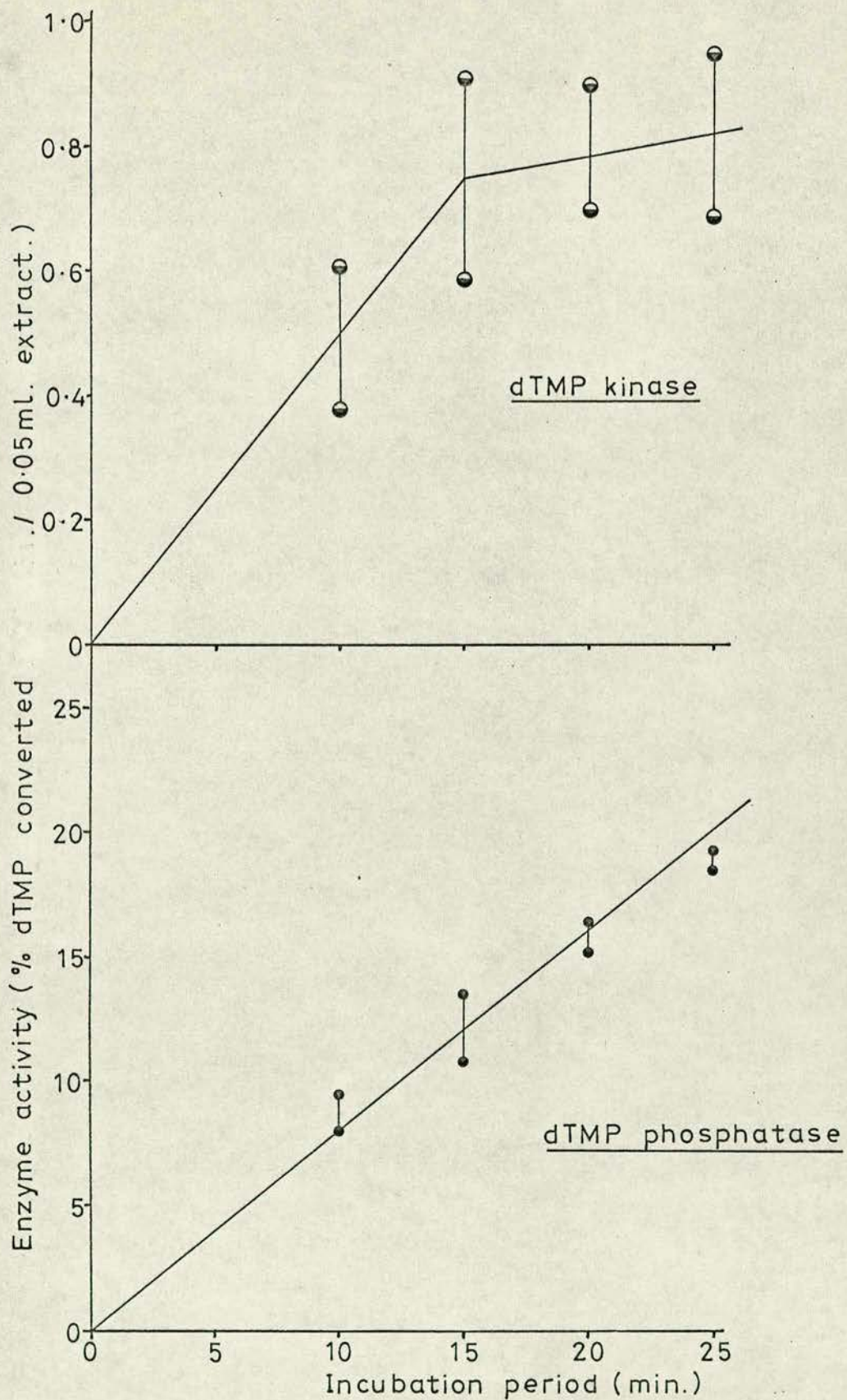
FIG. 3 . 21 Time course for dTMP kinase and dTMP
phosphatase activity of day 5 explants.

that the dTMP kinase reaction was linear up to 15 minutes only.
The non-linearity after 15 minutes may be partially explained by
the linear breakdown of dTMP by phosphatase which was maintained
throughout the 25 minute period.   Despite the suppressive effect
of phosphate buffer, the phosphatase activity of this extract
prepared using Day 5 asynchronous explants, represented almost 20%
breakdown of dTMP during the 25 minute incubation.

Robertson (1966) had shown that phosphatase activity is lower
in freshly excised explants compared with explants which have been
cultured for several days.   Therefore an extract prepared from
freshly excised explants was used in the next experiment.

Experiment 9.

Anticipating the existence of a lower phosphatase activity,
the previous experiment was repeated using an extract prepared from
110 freshly excised explants/ml. of grinding buffer (0·2M phosphate
buffer pH 8·0 + 400 µg./ml. BSA + 0·5% EtSH).   A new radioactive
medium was made up to a final concentration of 0·2M phosphate buffer
pH 8·0 and the ATP concentration was doubled (final concentration
24·8 mg./5 ml.) to ensure that the ATP concentration was adequate
for the dTMP kinase reaction.   The incubation period was varied
from 0 to 30 minutes.

The results in Fig. 3.22 show a linear relationship between
activity and time for both dTMP kinase and dTMP phosphatase,
throughout the 30 minute period.   When these results are compared
with those of the previous experiment it is apparent that non-linearity
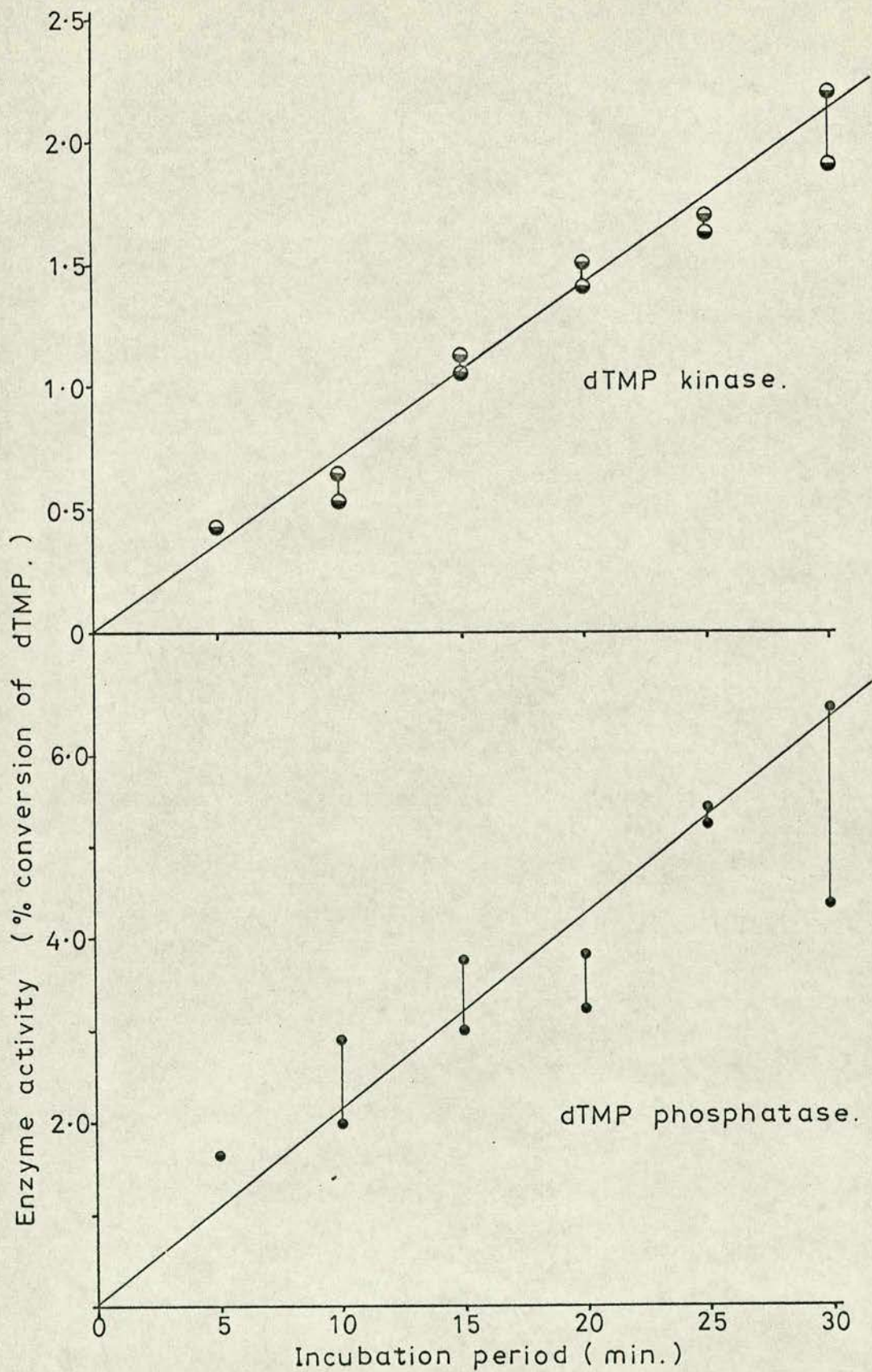
FIG. 3.22 Time course for dTMP kinase and dTMP phosphatase activity of day 0 explants.

of the dTMP kinase reaction occurs when the phosphatase conversion exceeds a value of 10%. Provided that phosphatase activities below this level are maintained during the cell division cycle, then this method is suitable for the measurement of dTMP kinase activity during the cell division cycle.

Experiment 10.

An essential prerequisite to the mixed extract experiment (Experiment 13 of Ch. 4) is proof that the enzyme activity is proportional to the volume of extract. For this experiment, tissue from the 'S' phase of the first division cycle was used (at this time it had already been shown that dTMP kinase activity increased during 'S'). Explants were cultured in the dark in a liquid medium and harvested after 22 hours. An extract of twice the usual concentration was made using 200 explants/ml. of grinding medium.

The total volume of the reaction mixture was 220 μl., made up of 100 μl. of radioactive medium, 20 μl. of pyruvate kinase and varying amounts of extract made up to 100 μl. with grinding buffer.

The results of this experiment which are shown in Fig. 3.23 indicate a linear relationship up to 0·1 ml. of extract for both dTMP kinase and dTMP phosphatase activities. In almost all subsequent experiments, extract equivalent to 0·05 ml. of the above extract was used, to ensure that all activities coincided with the linear part of the curve.
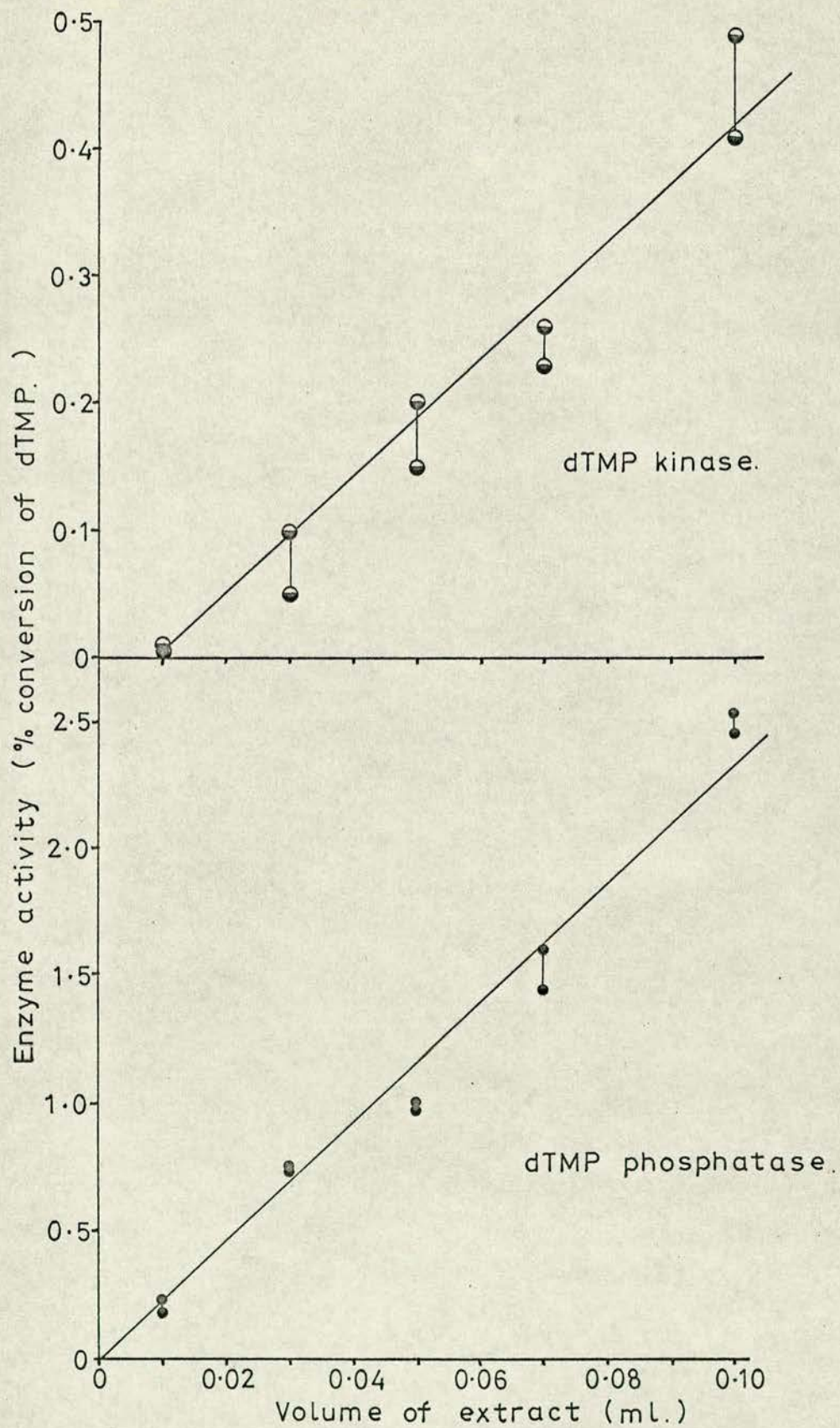
FIG.3.23 Relationship between volume of 22 hour extract and enzyme activity, for dTMP kinase and dTMP phosphatase.

Experiment 11.

In preparation for Experiment 9 of Chapter 4, a preliminary
experiment was carried out to test the effect of a grinding buffer
containing 1·0% EtSH on dTMP kinase activity.   The reason for
including 1·0% EtSH and the practical details of this experiment
have already been described for TdR kinase in Experiment 15 of the
previous section.

The results of single electrophoresis runs indicated values
of 0·66% and 0·68% conversion to dTDP for extracts prepared in the
presence of 0·5% and 1·0% EtSH respectively.   These results
indicate that the use of 1% EtSH does not inhibit dTMP kinase
activity.   There is no evidence of a stimulatory effect as was
obtained for TdR kinase.

With respect to the phosphatase activity, values of 3·03%
conversion to TdR and 1·53% conversion were obtained in the presence
of 0·5% and 1·0% EtSH respectively.   In view of the large variation
often associated with phosphatase results these values can only
suggest an inhibition by higher concentrations of EtSH.

In this section an assay method has been developed to measure
dTMP kinase activity in artichoke tissue.   The method was
originally adapted from the TdR kinase assay described in the
previous section by substituting dTMP in place of TdR as the
substrate for the reaction.

The assay was unsuccessful using a low specific activity [14]C-

labelled substrate but when a high specific activity ^{3}H-labelled substrate was used, a successful assay method was developed. In order to suppress phosphatase activity as far as possible, phosphate buffer was included in the assay medium at a concentration of 0·2M.

The final assay method was shown to be suitable for measuring dTMP kinase activity of artichoke extracts because the activity obtained was proportional to the period of incubation and proportional to the volume of extract. However, this method was only quantitatively valid provided that the accompanying phosphatase activity of the extract represented less than 10% conversion of the common substrate dTMP. The purpose of this assay was to measure the level of dTMP kinase activity during the first division cycle which occupies only the initial period of culture. Robertson (1966) had shown that phosphatase activity is relatively low during this period compared with the activity of tissue which had been cultured for a number of days.

Although phosphatase activity was suppressed as far as possible by the use of 0·2M phosphate buffer pH 8·0, the suppressed activity of this enzyme was also proportional to the incubation period and proportional to the volume of extract. Therefore this method is also suitable for estimating the suppressed dTMP phosphatase activity of artichoke extracts although it must be emphasized that this enzyme is not being assayed under optimal conditions.

<u>CHAPTER 4</u>

<u>EXPERIMENTAL RESULTS II</u>

CELL CYCLE EXPERIMENTS

The previous chapter has described the way in which
satisfactory assay methods for a number of different enzymes were
developed for use with the artichoke system.   These assay methods
have been used to investigate patterns of enzyme activity during
the cell division cycle.

The experiments described in this chapter are confined to the
first synchronous cell division in artichoke tissue.   All the
cultures were prepared in low intensity green light and grown in
the dark at 25°C, in order to obtain a high percentage cell
division, in the region of 60%.

All experiments were performed during the 'artichoke season'
(which extends from November to April, when the mean division time
of the first division cycle is less than 30 hours (Evans 1967)).
Experiments carried out early in the 'season' have a comparatively
short division time (22 - 24 hours), whereas for experiments carried
out later in the season, the mean division time is extended to 28
or 30 hours.   Because of this variation, it is often impossible
to make direct comparisons between the results of individual
experiments unless a large amount of relevant data is collected
for each experiment.

The enzymes chosen for study are all associated with DNA
metabolism and include enzymes of degradation in addition to
enzymes of the biosynthetic pathways.   In all cell cycle experiments

particular attention was paid to the accurate measurement of the
timing and extent of DNA synthesis.   In addition to examining
the pattern of enzyme activities during the cell cycle, the aim
of these experiments was to try and explain the mechanism whereby
enzyme levels are regulated in an attempt to answer the question:-
"Is DNA synthesis the cause or effect of the varying enzyme
patterns?"

SECTION A

THE ENZYMES 'NATIVE'-DNAase, 'DENATURED'-DNAase and
DNA POLYMERASE

Although there are few reports in the literature, of DNAase
activity during the cell division cycle, a marked periodicity in
the activity of this enzyme was observed by Stern (1961) prior to
the second meiotic division in Lilium longiflorum anther tissue.
In view of this, it was anticipated that a study of 'native'-DNAase
activity during the cell division cycle in artichoke tissue might
produce similar results.

DNA polymerase is, of all the enzymes, reputed to be the
most closely associated with DNA biosynthesis and it is easily seen
how changes in the availability of this enzyme could provide a
direct control mechanism for DNA synthesis.

The mechanism of action of bacterial DNA polymerase involves
the close association of this enzyme with a DNAase enzyme which
has substrate preference for denatured-DNA (Kornberg 1969).
Anticipating a possible correlation during the cell division cycle,
between the patterns of activity of DNA polymerase and the
'denatured'-DNAase enzyme from artichoke tissue, these two enzymes
were often assayed within the same experiment.   However, it
should be emphasized that the DNA polymerase assays were carried
out by Dr. Jackson using an assay method which was shown to yield
quantitatively valid results in artichoke tissue by Jackson (1970).
Since the assays carried out in joint experiments made use of the
same extracts, it is relevant to include the DNA polymerase results
in this section.

Experiment 1.

The aim of this experiment was to establish patterns of activity during the first division cycle for the enzymes 'native'-DNAase, 'denatured'-DNAase and DNA polymerase and to compare any variations in activity with the timing and extent of DNA synthesis. In order to establish continuity with the results of previous workers for this system, a number of standard parameters were also measured including cell number and levels of total nucleic acid, DNA and acid resistant protein.

For this experiment, 18 flask cultures were prepared, each containing about 160 explants. All cultures were prepared in low intensity green light and grown in the dark at $25^{\circ}C$. By setting up 10 of the cultures 18 hours before sampling began and 8 cultures immediately prior to sampling, the culture periods from 0 to 16 hours and from 18 to 36 hours were sampled in parallel at 2 hourly intervals. Thus the 0 hour and 18 hour cultures were sampled together, then two hours later the cultures which had been growing for 2 hours and 20 hours were sampled and so on.

At each sampling time the procedure was as follows:-

a) 0·5 ml. of the growth medium was removed aseptically from a random sample of flasks and used to test for possible contamination by micro-organisms. Results of these tests are recorded in Appendix 2a.

b) 16 explants were removed for estimation of

(i) rate of uptake of ^{3}H-thymidine into the tissue and

(ii) rate of DNA synthesis as indicated by the rate of

incorporation of ^{3}H-thymidine into DNA.

The remaining explants were washed 3 times in distilled water and
retained on muslin.

c) 100 explants were removed for preparation of the enzyme

extracts used to assay all 3 enzymes. Assays for both of

the DNAase enzymes were carried out in triplicate and

assays for DNA polymerase were carried out in duplicate.

d) 5 explants were removed for the estimation of cell

number (only in certain samples).

e) 2 or 3 samples of 15 explants were removed for the

standard estimations of DNA, total nucleic acid and acid

resistant protein.

This order of sampling was adopted as a standard procedure used in
all cell cycle experiments and has been described in full for this
experiment only.

Results

Changes in cell number (Fig. 4.1)

Division commenced after about 24 hours and at this time only
a few cell divisions had occurred. Division was complete by
28 hours. It was estimated that 62% of the cells had divided, a
figure which is comparable to the results of Davidson (1971) for
a dark grown system.

In this, and in all subsequent experiments the period of cell
division will be represented on all graphs as follows :—
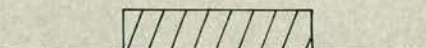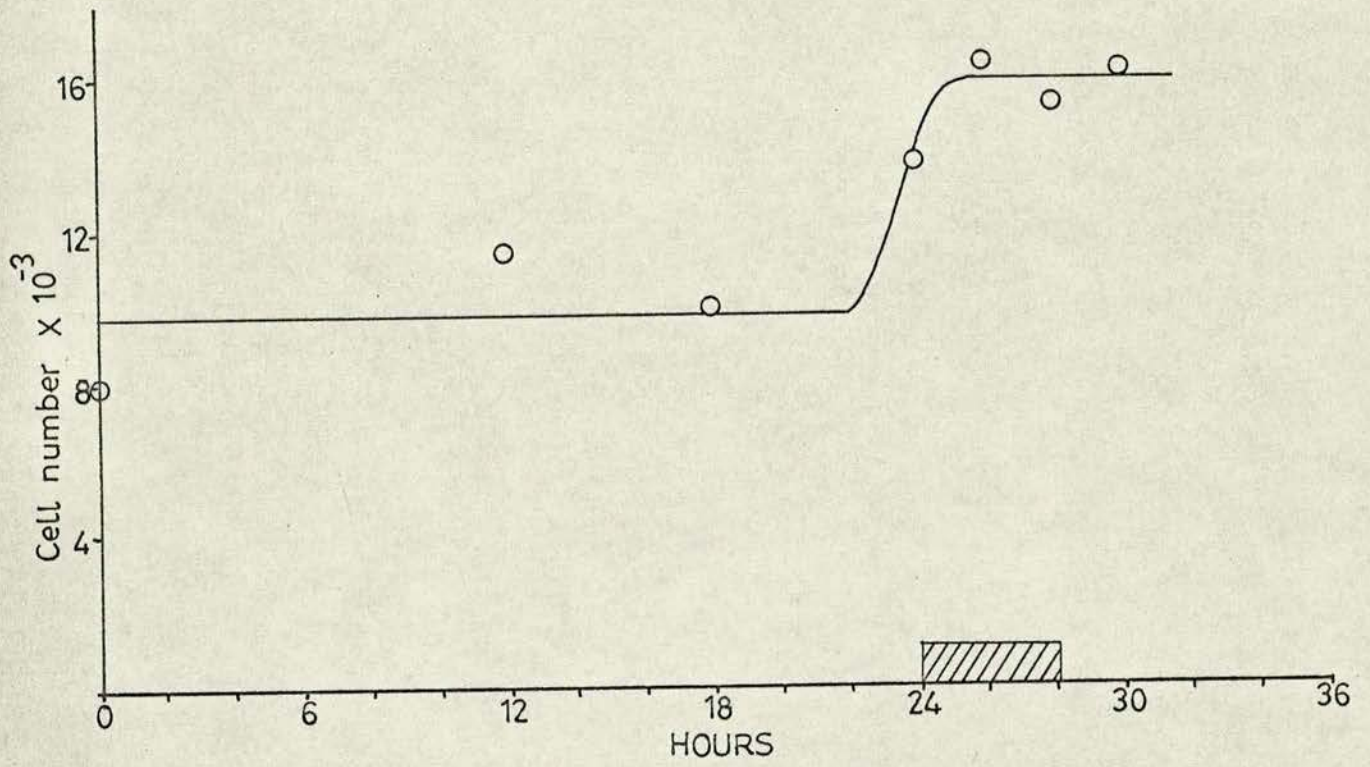
FIG. 4.1 Cell number changes in a 'dividing' system.

Chemical estimation of DNA levels using Burton's method

      The values shown in Fig. 4.2 stay constant for the first part of the cycle but by 20 hours it is apparent that there has been a definite increase in the level of DNA, amounting to an increase over the basal value of approximately 62%. The fact that this figure is the same as that obtained for percentage cell division agrees well with the results of Mitchell (1967) who has shown in this system that DNA synthesis occurs only in those cells which divide in the first wave of cell division.

      The pattern of increase in the level of DNA is similar to that observed by Evans (1967) for a system prepared in the light and in the presence of coconut milk and grown in the dark.

Rate of uptake of ^{3}H-thymidine into the tissue and rate of incorporation of ^{3}H-thymidine into DNA (Fig. 4.3).

      A sharp increase in the rate of uptake of ^{3}H-thymidine into the tissue begins after 4 hours of culture and reaches a maximum value at 10 hours when it is estimated that, of the total counts available, 4% have been taken up into the tissue. The rate of uptake then falls sharply until about 14 hours when a less rapid decline is noticed which reaches a minimum value at the beginning of division. A further increase which begins after 24 hours and continues until a plateau value is reached between 26 and 32 hours is followed by a possible decline which begins at 34 hours.

      Values for the rate of incorporation of ^{3}H-thymidine into DNA appear to remain constant for the first 10 hours and the sharp
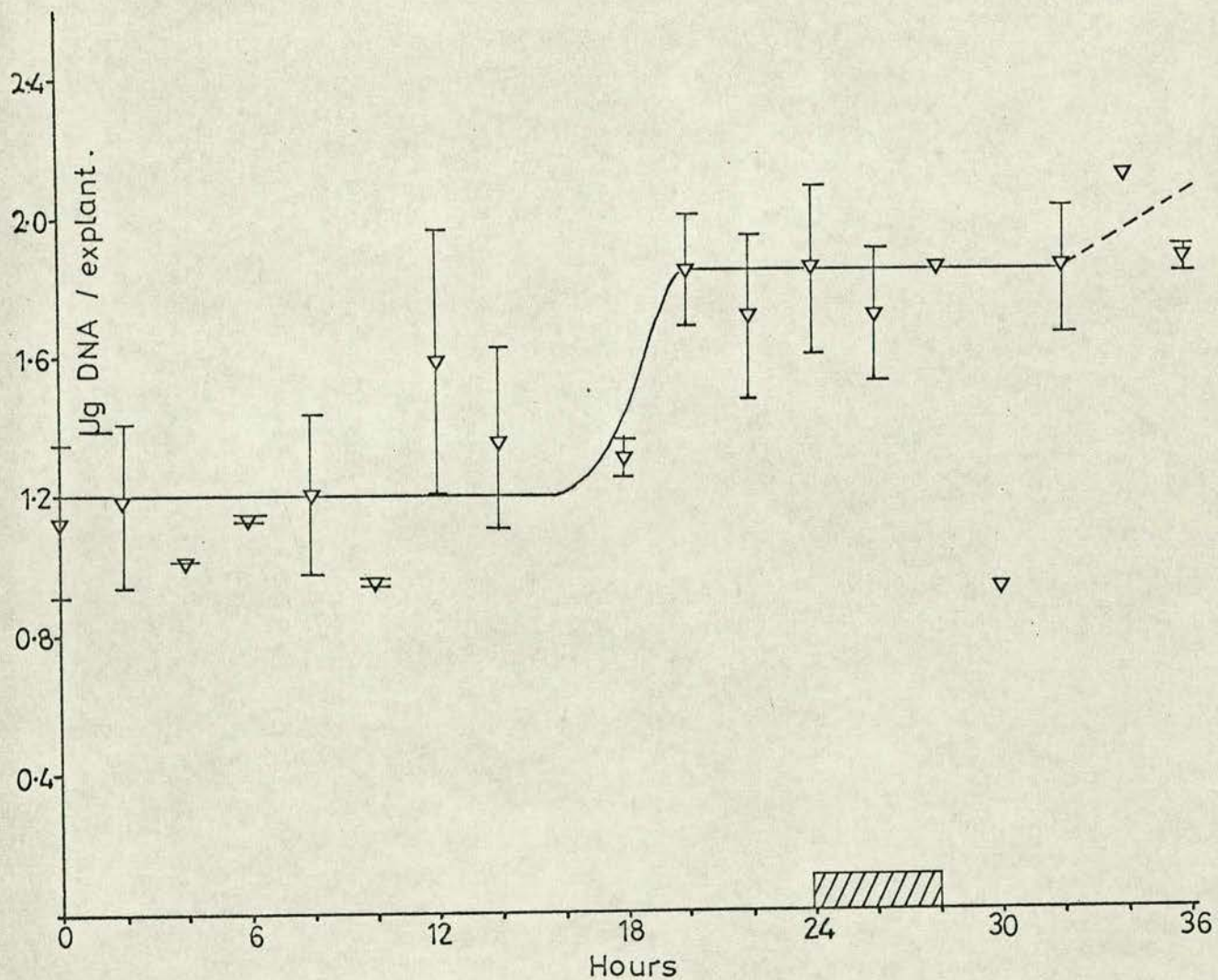
FIG. 4·2   Changes in the level of DNA in a 'dividing' system.
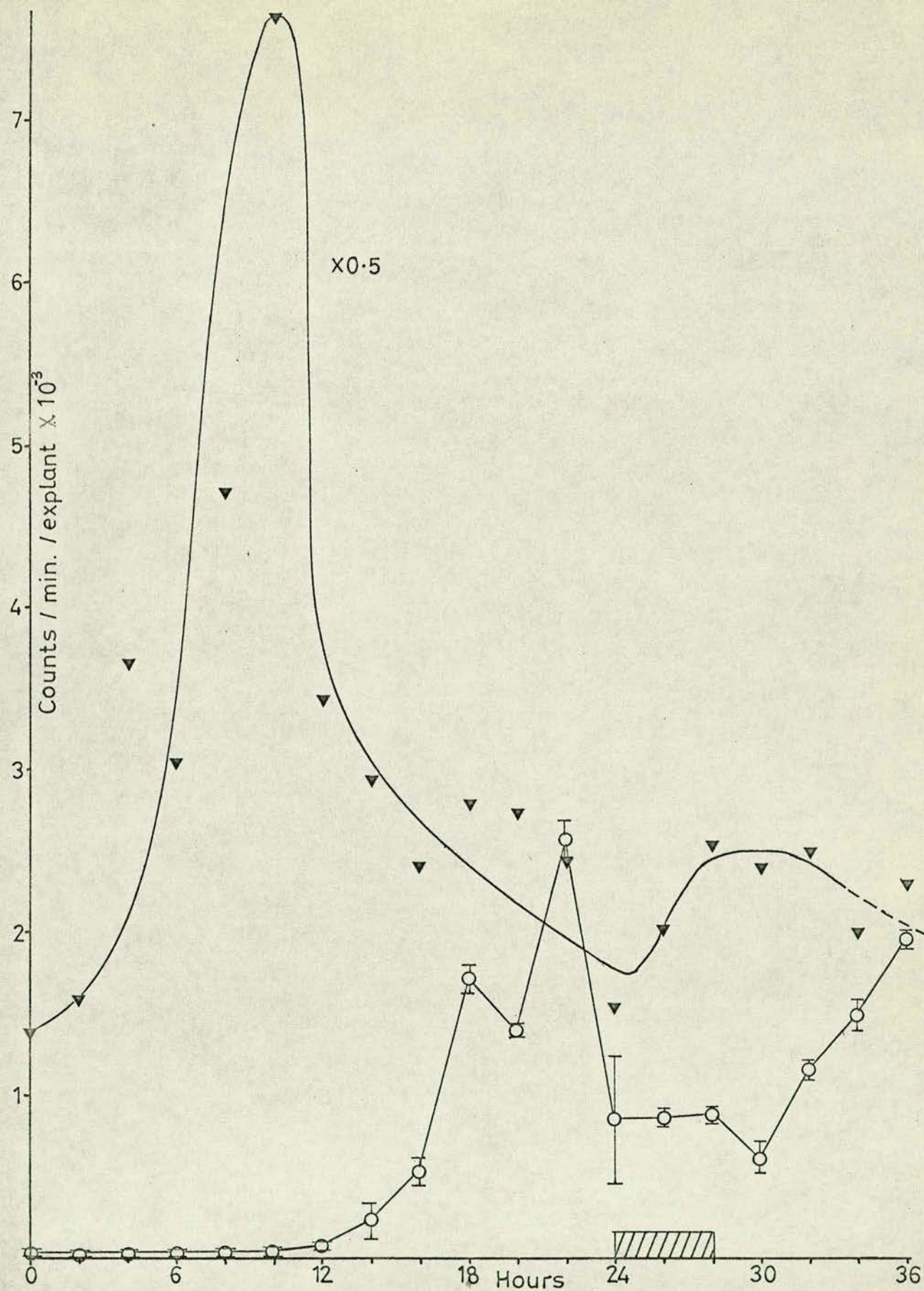
FIG. 4·3   Changes in the rate of uptake of ^{3}H-thymidine into the tissue (▼) and in the rate of incorporation of ^{3}H-thymidine into DNA (O) in a 'dividing' system.

increase which begins between 12 and 14 hours marks the beginning
of the 'S' phase.   The increase continues until 18 hours after
which a slight fall is noted at 20 hours.   The maximum incorporation
occurs at 22 hours when it is estimated that 0·7% of the total
counts added have been incorporated into DNA.   The 'S' phase
appears to end at 24 hours when the rate of incorporation falls to
a plateau value maintained until a further increase occurs from
32 hours onwards.   This second increase is indicative of the
beginning of the 'S' phase of the second division cycle.

The results displayed in Fig. 4.3. largely verify those
obtained by Evans (1967) who measured these same parameters for a
system excised in the light and grown in the dark in the presence
of coconut milk.   However, the pattern of the rate of uptake of
^{3}H-thymidine into the tissue need not be associated with cell
division and may be a property of freshly excised tissue.   The
latter possibility is suggested by the fact that there is no marked
increase in the rate of uptake associated with the second division
cycle.

Data obtained for the incorporation of ^{3}H-thymidine into DNA
is also comparable with the results of Evans (1967), except for
the plateau in the curve at 20 hours.   This difference could be
a reflection of the larger variation inherent in experiments where
samples are removed from different flasks.

In ability to define the accurate timing of the 'S' phase,
measurement of the rate of DNA synthesis by incorporation of

^{3}H-thymidine into DNA is far superior to Burton's chemical method
for DNA.   Whereas the radioactive method marks the beginning of
'S' at about 14 hours, the first increase in the level of DNA
measured using Burton's method occurs between 18 and 20 hours.
This discrepancy of 4 or 6 hours which is due to the insensitivity
of the technique could seriously affect the interpretation of the
results of enzyme experiments.

Estimation of the rate of DNA synthesis by incorporation of
^{3}H-thymidine into DNA is thus a useful indicator of the commencement
and progress of DNA synthesis.   In this experiment it is clear that
the 'S' phase of the first division cycle begins at 14 hours and
ends at 24 hours and also that this experiment covers part of the
second division cycle in which the 'S' phase begins at about
32 hours.

Total nucleic acid.

Total nucleic acid is a term which includes both RNA and DNA,
but as the percentage of DNA is very small, this is largely a
reflection of RNA levels.   There have been several estimations of
the DNA content of total nucleic acid in the artichoke system.
Using both chemical and polyacrylamide gel techniques, Fraser (1967)
obtained a value approximating to 12% and Mitchell (1967) using a
histochemical technique estimated a value of less than 10%,
whereas Evans (1967) achieved a value of 1·7% using DNA phosphorus
analysis.

Changes in the level of total nucleic acid are shown in Fig.4.4.
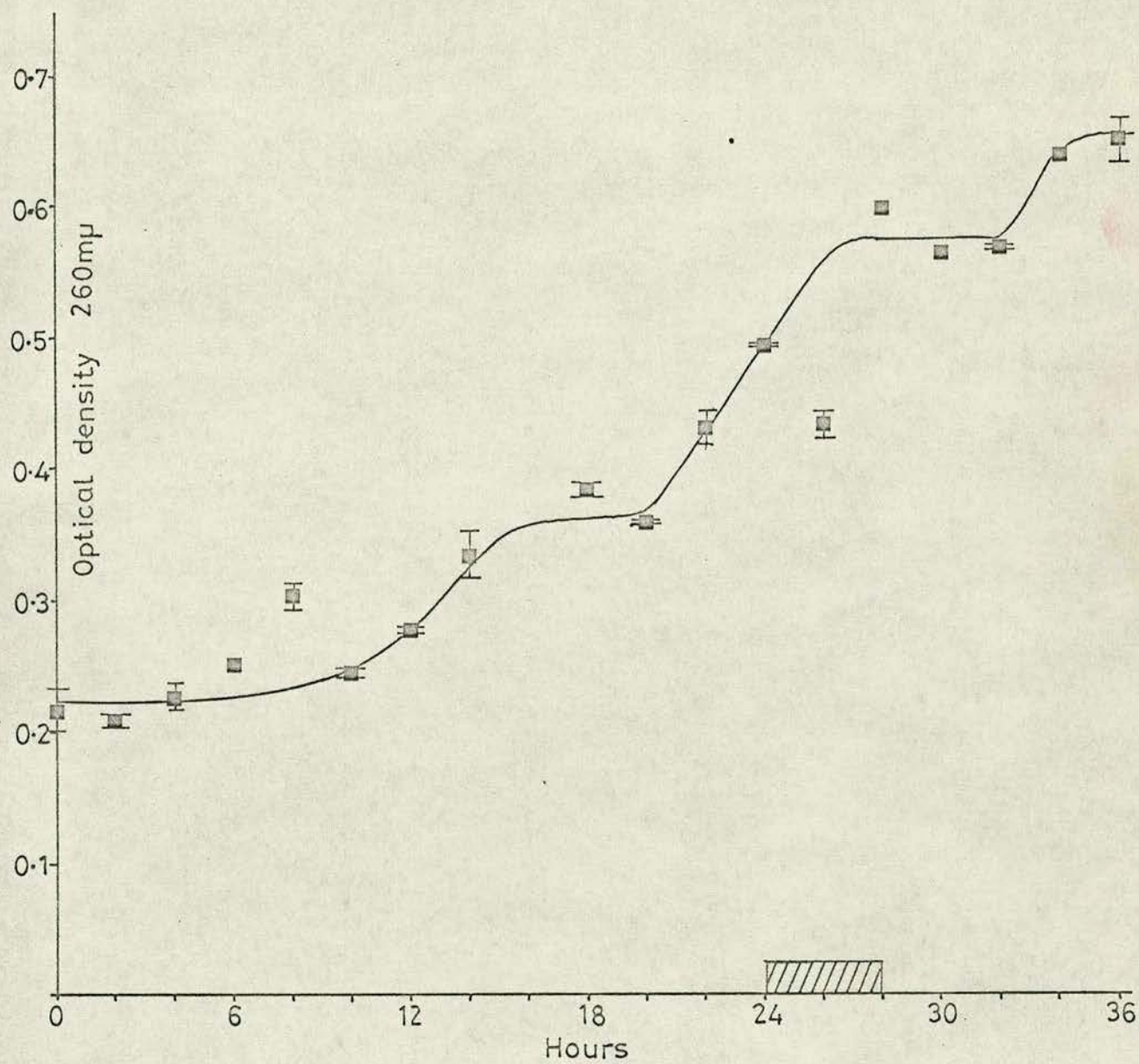
FIG. 4.4 Changes in the level of total nucleic acid in a 'dividing' system.

The first increase occurs early in the culture period and a plateau value is reached about half way through the 'S' phase representing an increase of about 77%. The second much larger increase takes place towards the end of 'S' and is completed at about the time of division. The total increase amounts to 177%. A third small increase occurs during the early part of the second cycle. The overall pattern agrees with the results of Evans (1967) for a system excised in the light and grown in the dark in the presence of coconut milk. The overall increase, however, is considerably larger in this experiment, a fact which is almost certainly due to the larger proportion of dividing cells characteristic of explants excised in low intensity green light and grown in the dark.

Protein content due to acid resistant protein (Fig. 4.5)

The acid resistant protein is contained in the residue from the perchloric acid treatment used to obtain a hydrolysate of total nucleic acid. Over the first 12 hours of the experiment there appears to be a gradual increase. By 14 hours there has been a definite increase and a plateau value is reached at about 18 hours which represents an 88% increase over the zero time value. The second increase which begins towards the end of 'S' and reaches a plateau value at the end of division amounts to a final increase over the zero time value of 162%. Subsequently there are no further increases.

This pattern of changes is in close agreement with the results of
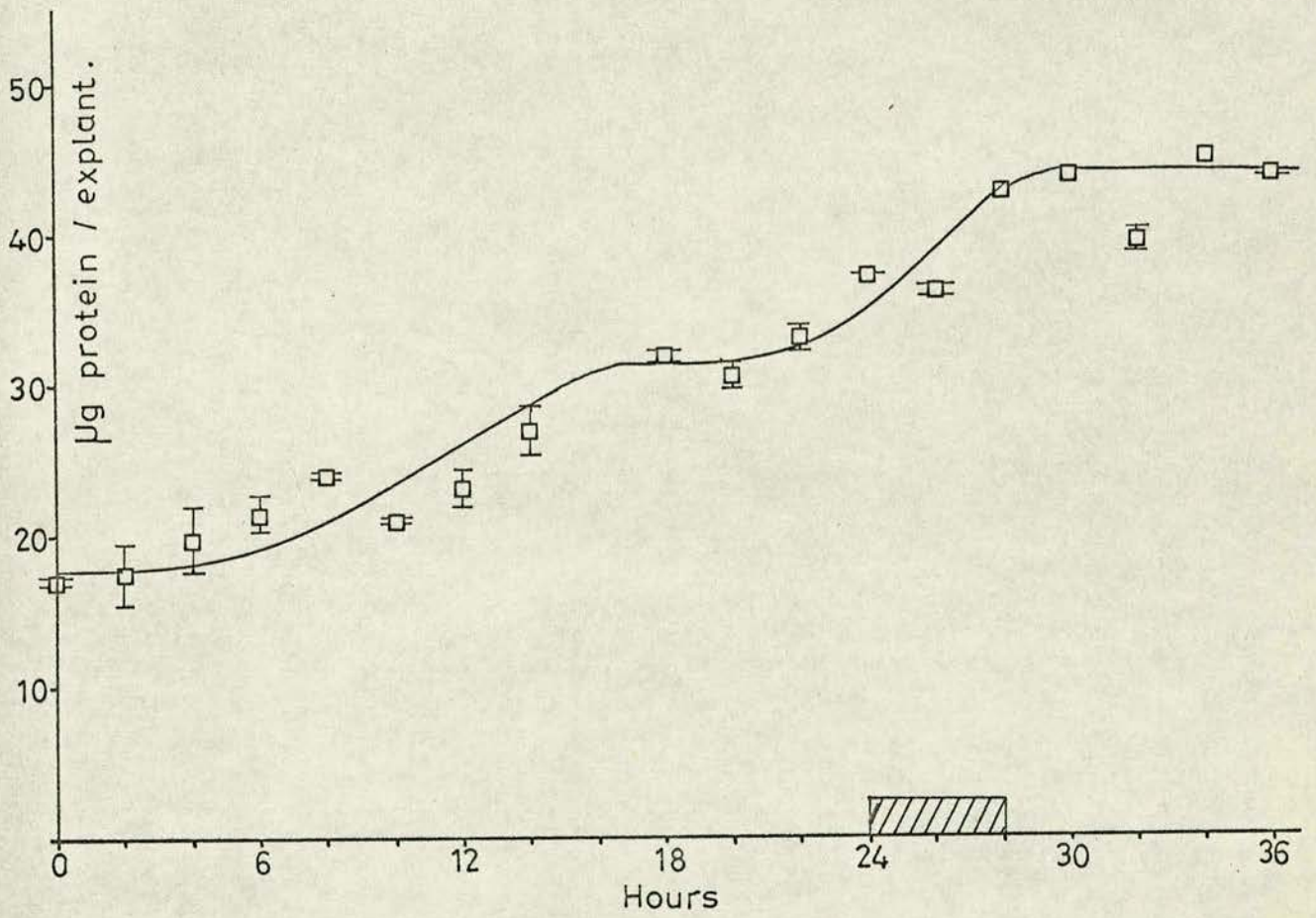
FIG. 4.5 Changes in the level of acid resistant protein in a 'dividing' system.

Evans (1967) for a system excised in the light and grown in the dark in the presence of coconut milk, but as was previously mentioned for total nucleic acid, the percentage increases are somewhat larger due to differences in the preparation of the cultures.

Activity of 'native'-DNAase

Variations in the activity of 'native'-DNAase are compared with the rate of DNA synthesis in Fig. 4.6. A considerable increase in activity occurs during the first part of the cycle to reach a maximum value around 14 hours. There is a decline in activity which takes place after 14 hours and a plateau value is reached by 24 hours which remains more or less constant for the rest of the experiment. When this pattern is compared with the timing of DNA synthesis it is apparent that the rise in enzyme activity ceases rather abruptly after the beginning of 'S'. A possible explanation of this relationship could be that during the 'S' phase, the newly synthesized DNA present in the extracts considerably dilutes the specific activity of the labelled substrate used in the assay, causing an apparent fall in enzyme activity during 'S'. On the other hand, the fact that there is no marked increase in the activity of this enzyme during the pre-'S' phase of the second cycle may indicate that the pattern of activity observed in this experiment is not exclusively associated with cell division.
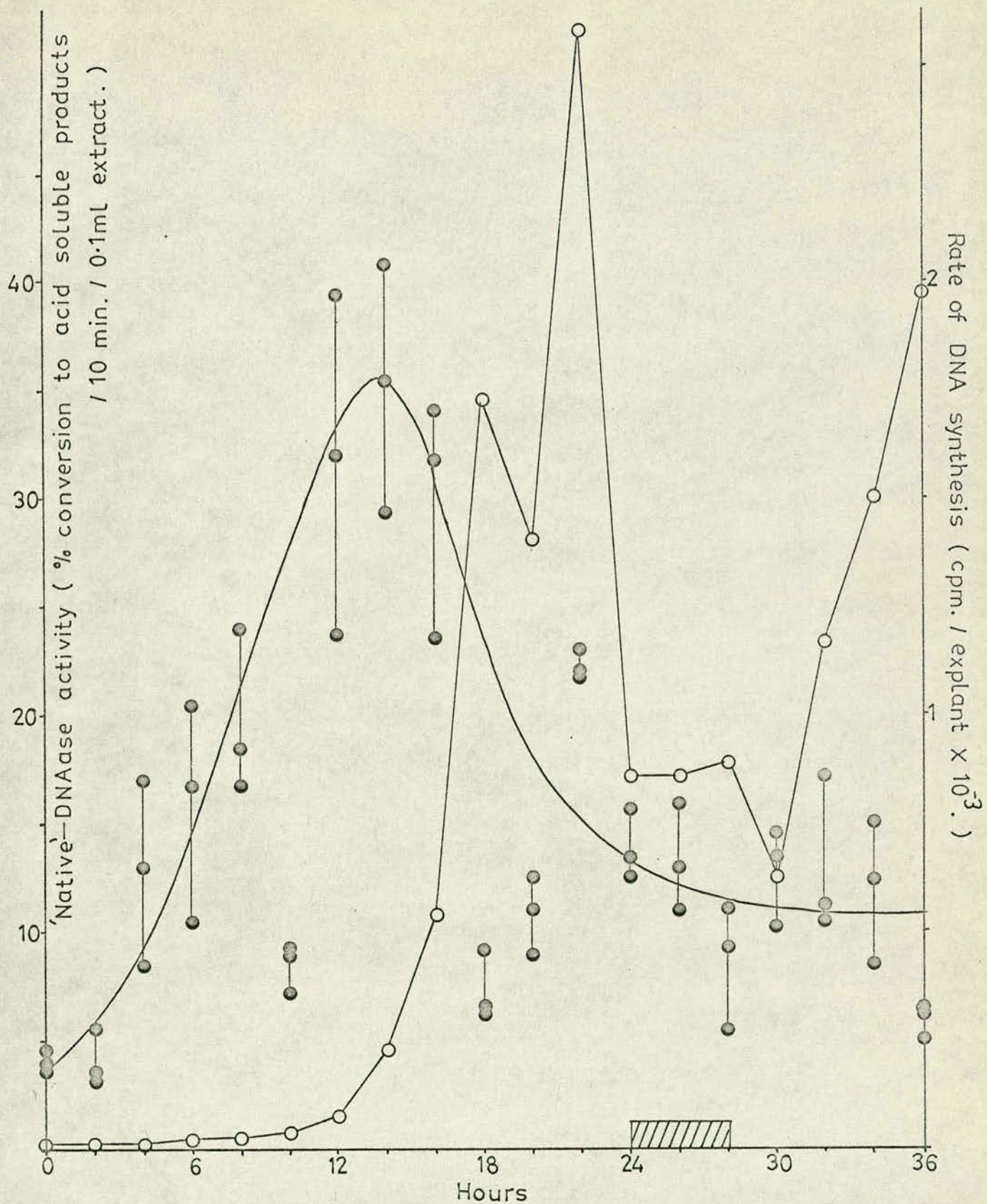
FIG. 4.6   Activity of 'native'-DNAase (⬤) in relation to the rate
of DNA synthesis (○) in a 'dividing' system.

Activity of 'denatured'-DNAase

Results obtained for the activity of the DNAase which
hydrolyses denatured artichoke DNA are compared with the rate of
DNA synthesis in Fig. 4.7.   The average activity from 22 to
36 hours is considerably higher than the average activity from
0 to 18 hours.   It can in fact be proved that these two sets of
values are statistically different using a simple d test (See
Appendix 4 for statistical proof of this).   The fact that there
is a larger variation within triplicated activities recorded after
22 hours also suggests that an increase has occurred.   The pattern
of increase shown in Fig. 4.7. is an abrupt increase occurring at
20 hours (approximately 6 hours after the beginning of 'S') and
amounts to an increase of 47 per cent.   This analysis does not
exclude the possibility that there is a gradual rise in activity
taking place which begins before 20 hours.   No increase in
activity is shown in association with the second division cycle
but perhaps this experiment does not cover enough of the second cycle
for an increase to be detected.

Final conclusions regarding a pattern for this enzyme cannot
be made until further experiments similar to this one have been
carried out.

Activity of DNA polymerase (Fig. 4.8)

There may be a gradual increase in the activity of DNA
polymerase from 0 to 18 hours followed by a sharp increase at about
22 hours.   At 24 hours a maximum activity is observed constituting
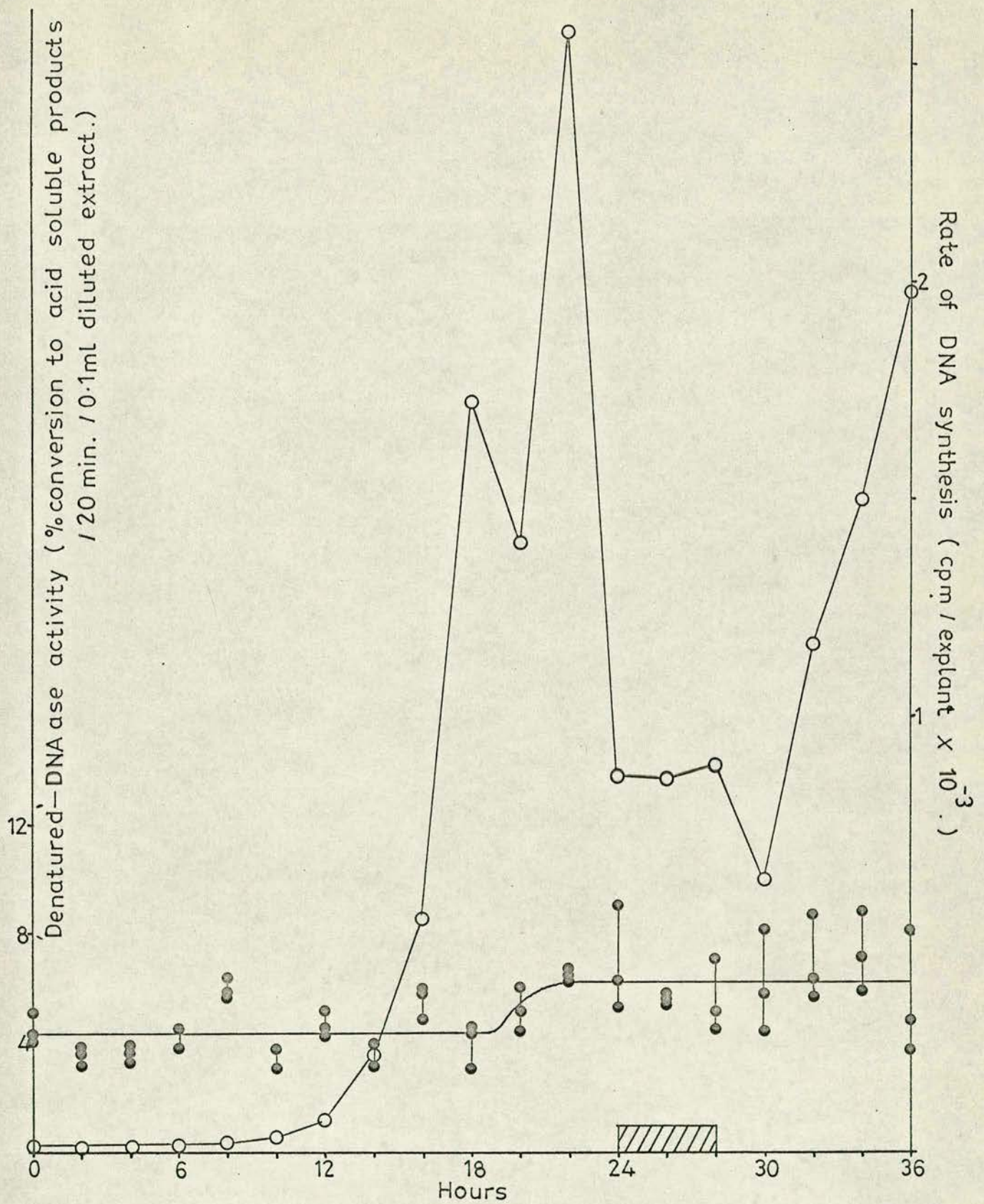
FIG.4.7 Activity of 'denatured'-DNAase (●) in relation to the rate of DNA synthesis(O) in a 'dividing' system.
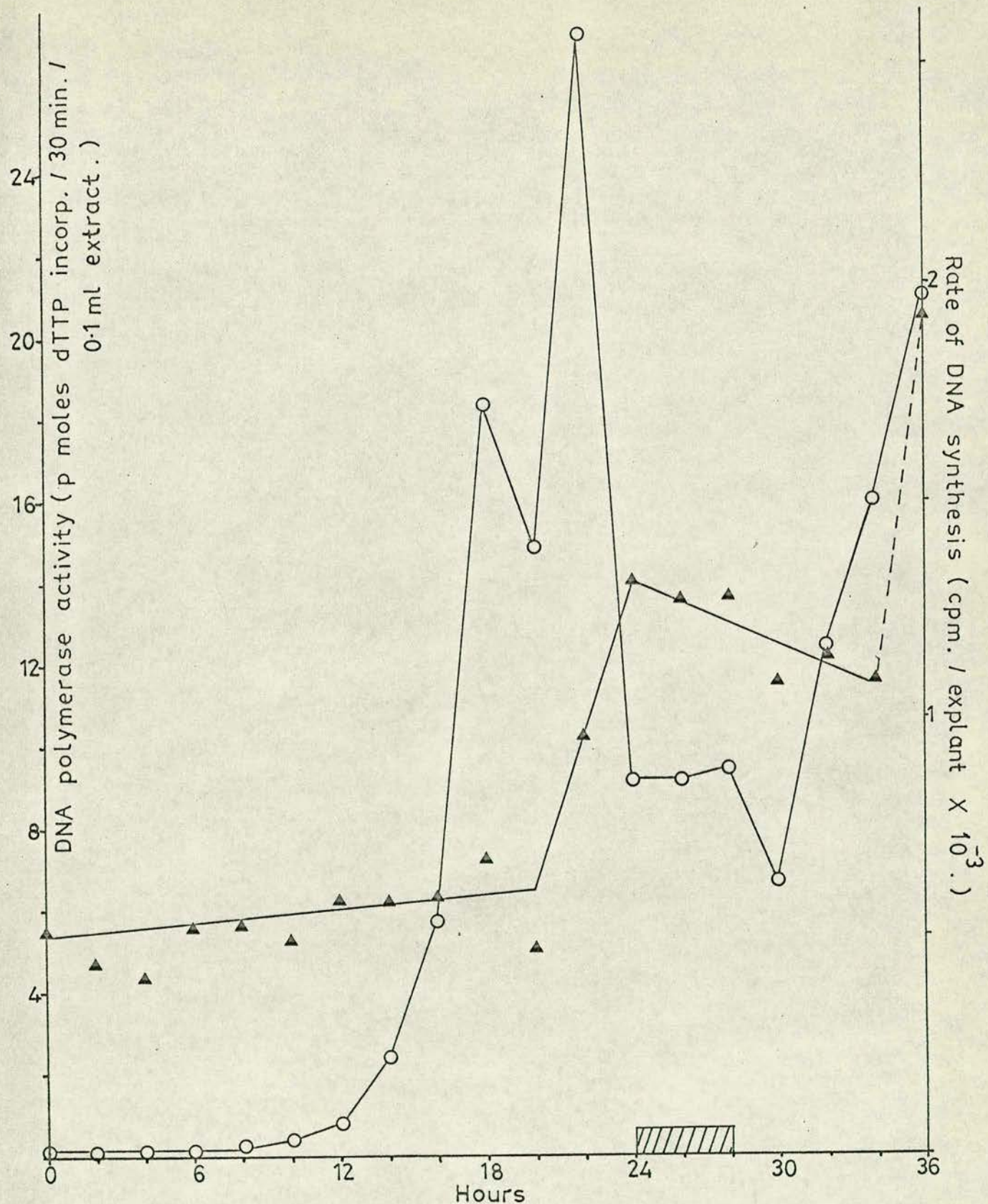
FIG. 4·8   DNA polymerase activity (▲) in relation to the rate
of DNA synthesis (O) in a 'dividing' system.

a 160% increase compared with the zero value.   A gradual decrease in activity is then observed until 34 hours, followed by a second sharp increase at 36 hours.   Thus when the pattern of activity is compared with the rate of DNA synthesis in Fig. 4.8, it is apparent that during the first division cycle a sharp increase in DNA polymerase activity occurred 6 - 8 hours after the beginning of 'S', whilst for the second division cycle this difference is only 2 - 4 hours.

By measuring such standard parameters as cell number and levels of total nucleic acid, DNA and acid resistant protein, it has been established that the experimental system is behaving in the way described by previous workers.   So far in this investigation the results tend to be more variable than previous results but this is easily explained by the fact that in this experiment each individual sample is represented by a different culture flask due to the large amount of material required for enzyme assays. Patterns of activity for the enzymes 'native'-DNAase, 'denatured'-DNAase and DNA polymerase have been described for a 'dividing' system.   There may be some significance in the fact that activities of the enzymes 'denatured'-DNAase and DNA polymerase were observed to increase at approximately the same time during the first division cycle.   However, this association is not supported by the fact that during the second division cycle the increase in DNA polymerase activity at 36 hours was not accompanied by an increase in 'denatured'-DNAase activity.

Final conclusions regarding patterns of enzyme activity cannot be made until repeat experiments have been described.

Experiment 2.

This experiment which is almost an exact replica of Experiment 1, was designed to confirm the patterns of enzyme activities previously described. As in Experiment 1, such standard parameters as cell number, and levels of total nucleic acid, DNA and acid resistant protein were measured in order to establish continuity with the results of previous workers.

The conditions used for setting up this experiment were as described for Experiment 1. This time however, the experimental period was 32 hours and two series of cultures were used, 9 prepared 16 hours before sampling began and 8 prepared immediately prior to sampling. In this way the times from 16 to 32 hours and from 0 to 16 hours were sampled in parallel at 2 hourly intervals. This time an overlap point was included at 16 hours which gave two 16 hour samples, one from the first and one from the second series of cultures. Sampling was carried out as for Experiment 1, the only difference being that in the DNA polymerase assay, the activity of the supernatant fraction of the extract was estimated in addition to the activity of the total extract (see Ch. 2. D. 3).

For this experiment estimations of the degree of contamination of culture media by micro-organisms are presented in Appendix 2b.

Results

Changes in cell number (Fig. 4.9)

The first division which commenced at about 26 hours was completed by 30 hours when it was estimated that approximately 50% of the cells had divided.

Chemical estimation of DNA levels using Burton's method (Fig. 4.10)

The level of DNA appears to remain constant during the first part of the cycle. The first increase is detected at 26 hours and continues until 30 hours when a plateau value is reached amounting to an increase of approximately 77% over the basal value. This value is not in complete agreement with the figure obtained for percentage cell division as it was in Experiment 1, a discrepancy which is possibly due to the fact that there were an insufficient number of values on the final plateau of the DNA curve.

Rate of uptake of ^{3}H-thymidine into the tissue and rate of incorporation of ^{3}H-thymidine into DNA (Fig. 4.11)

The rate of uptake of ^{3}H-thymidine into the tissue begins to increase between 0 and 2 hours and continues to increase until a maximum is reached around 10 hours when it is estimated that 2·7% of the total counts added have been taken up into the tissue. The rate of uptake begins to fall between 12 and 14 hours and continues to do so until 24 hours. A second smaller rise in the rate of uptake appears to take place between 26 and 32 hours.

A low rate of incorporation of ^{3}H-thymidine into DNA is
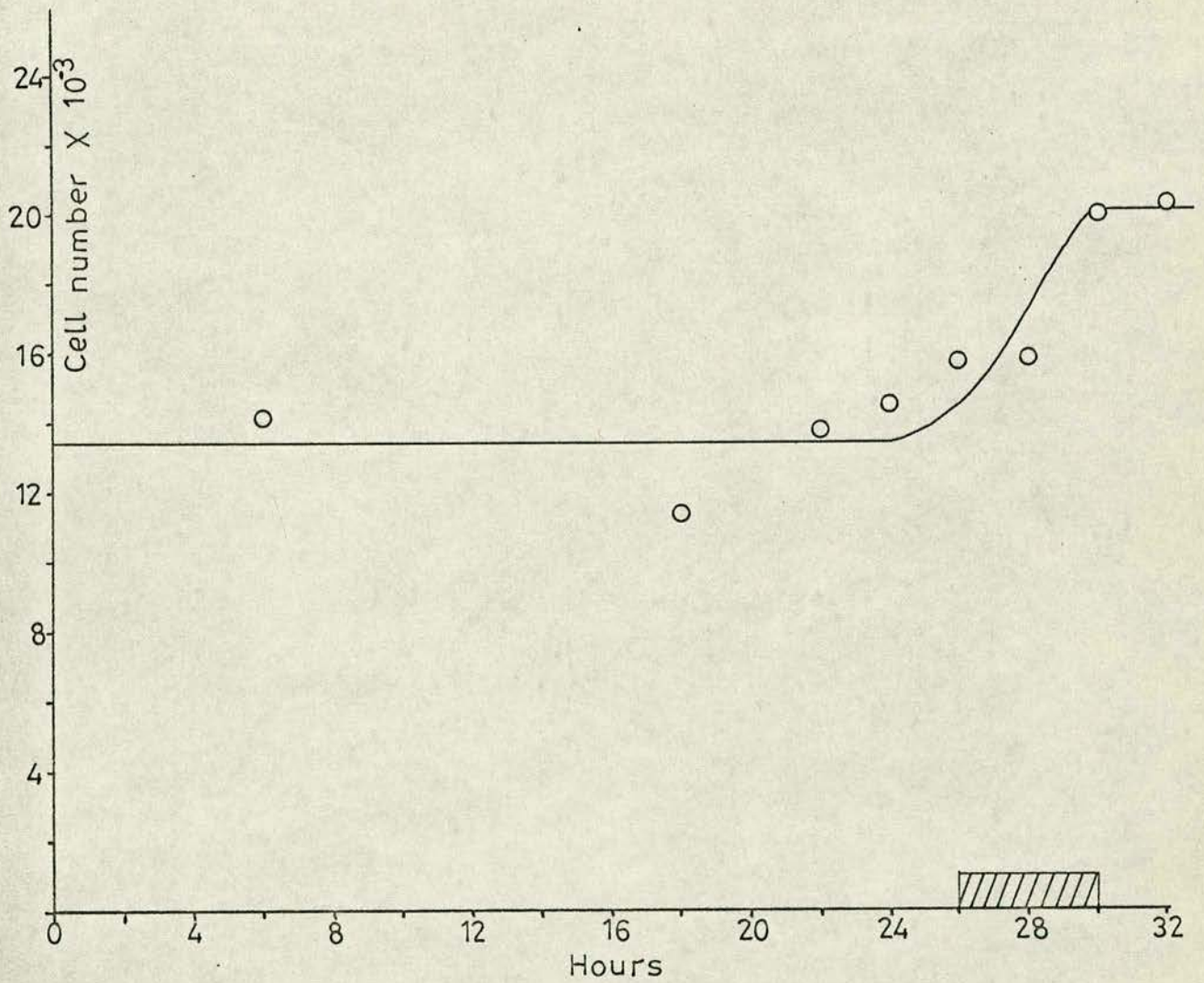
FIG. 4.9   Cell number changes in a 'dividing' system.
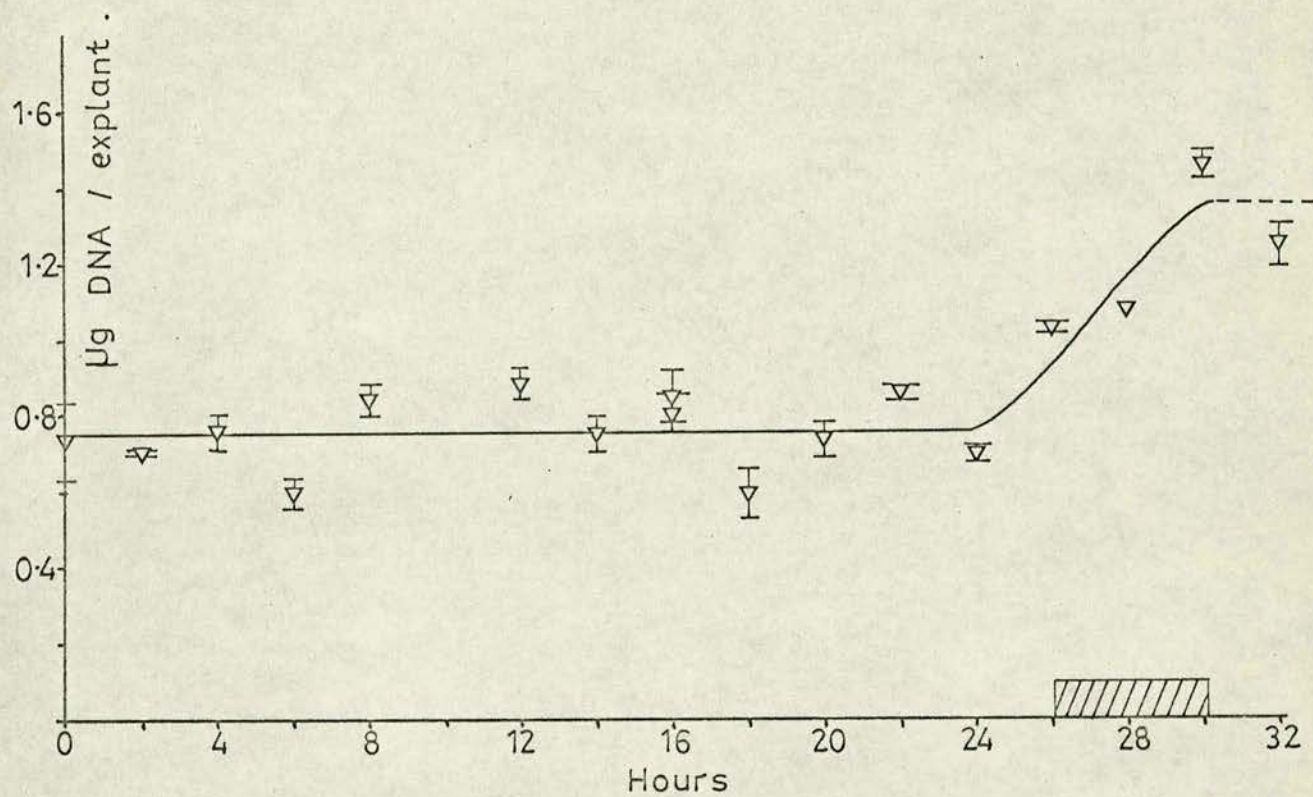
FIG. 4·10   Changes in the level of DNA in a 'dividing' system.
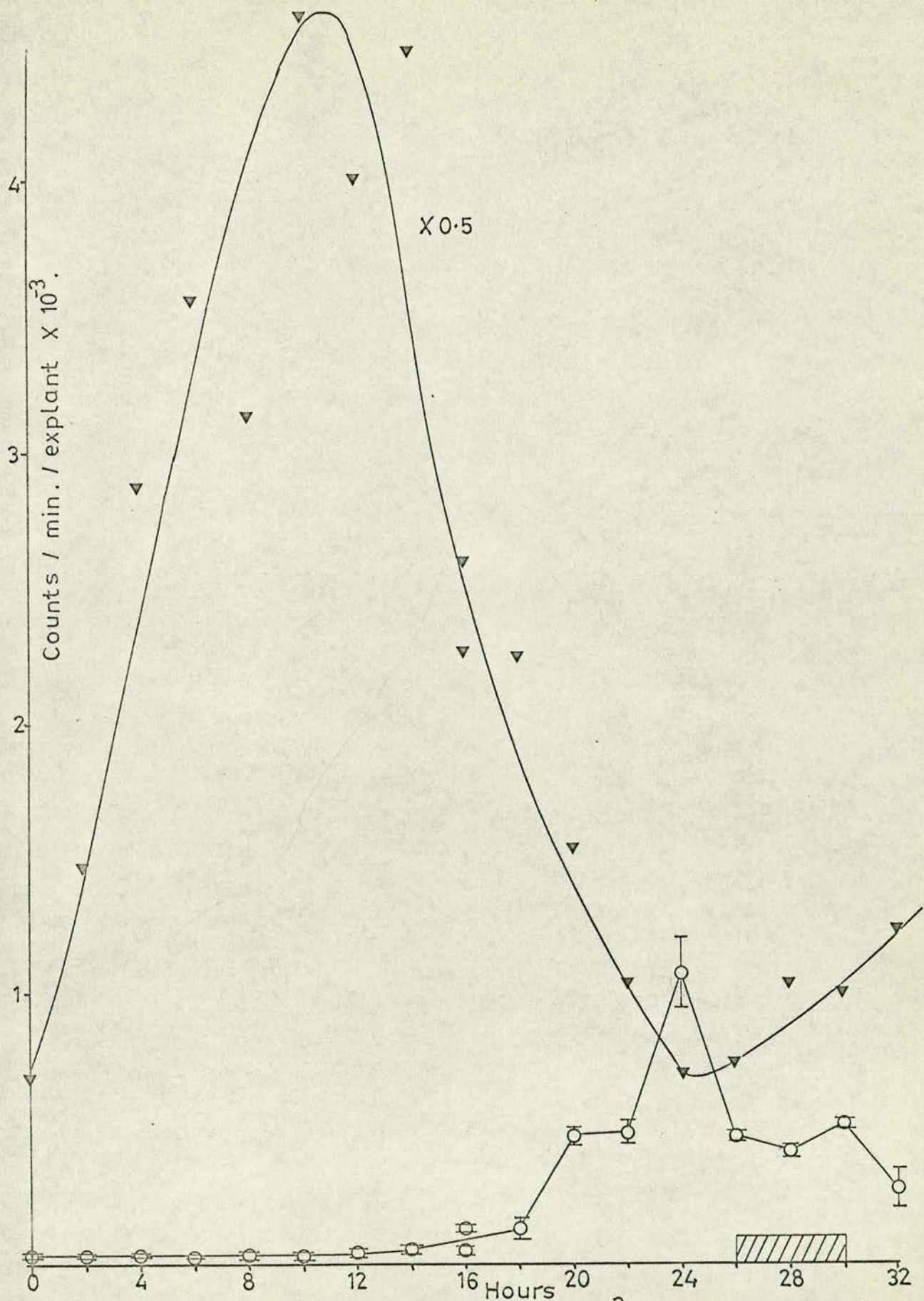
FIG. 4.11 Changes in the rate of uptake of ³H-thymidine into the tissue (▽) and in the rate of incorporation of ³H-thymidine into DNA (O) in a 'dividing' system.

maintained during the first part of the experiment.   The first substantial increase in rate which marks the beginning of 'S' is shown in the 16 hour sample belonging to the 16 - 32 hour series of cultures.   The rate of incorporation at 18 hours is similar to that recorded at 16 hours rising to a higher value at 20 and 22 hours and reaching a maximum value at 24 hours when it is estimated that 0.3% of the total counts added have been incorporated into DNA. A fall in the rate of incorporation at 26 hours marks the end of 'S' and what appears to be a plateau value is maintained until the experiment ends at 32 hours.

Changes in the level of total nucleic acid

The data presented in Fig. 4.12 show an initial fall in the value from 0 to 2 hours.   A basal value is maintained until 6 hours after which there is a small increase and a plateau value is reached from 10 to 18 hours which amounts to a 22% increase over the basal value.   A further, much larger rise, which begins at about the beginning of 'S' and ends at about the time of division may amount to a final increase of 100% over the basal value.   No further increases were recorded.

Acid resistant protein content (Fig. 4.13)

After an initial fall in the value from 0 to 2 hours a basal value is maintained followed by an increase which begins at about 6 hours.   A plateau value which is maintained from about 10 to 18 hours amounts to an increase of 29% over the basal value.   A further increase occurs during 'S' and a plateau value is reached at about the beginning of division which is maintained until the

FIG.4.12.

Changes in the level of total nucleic acid in a 'dividing' system.
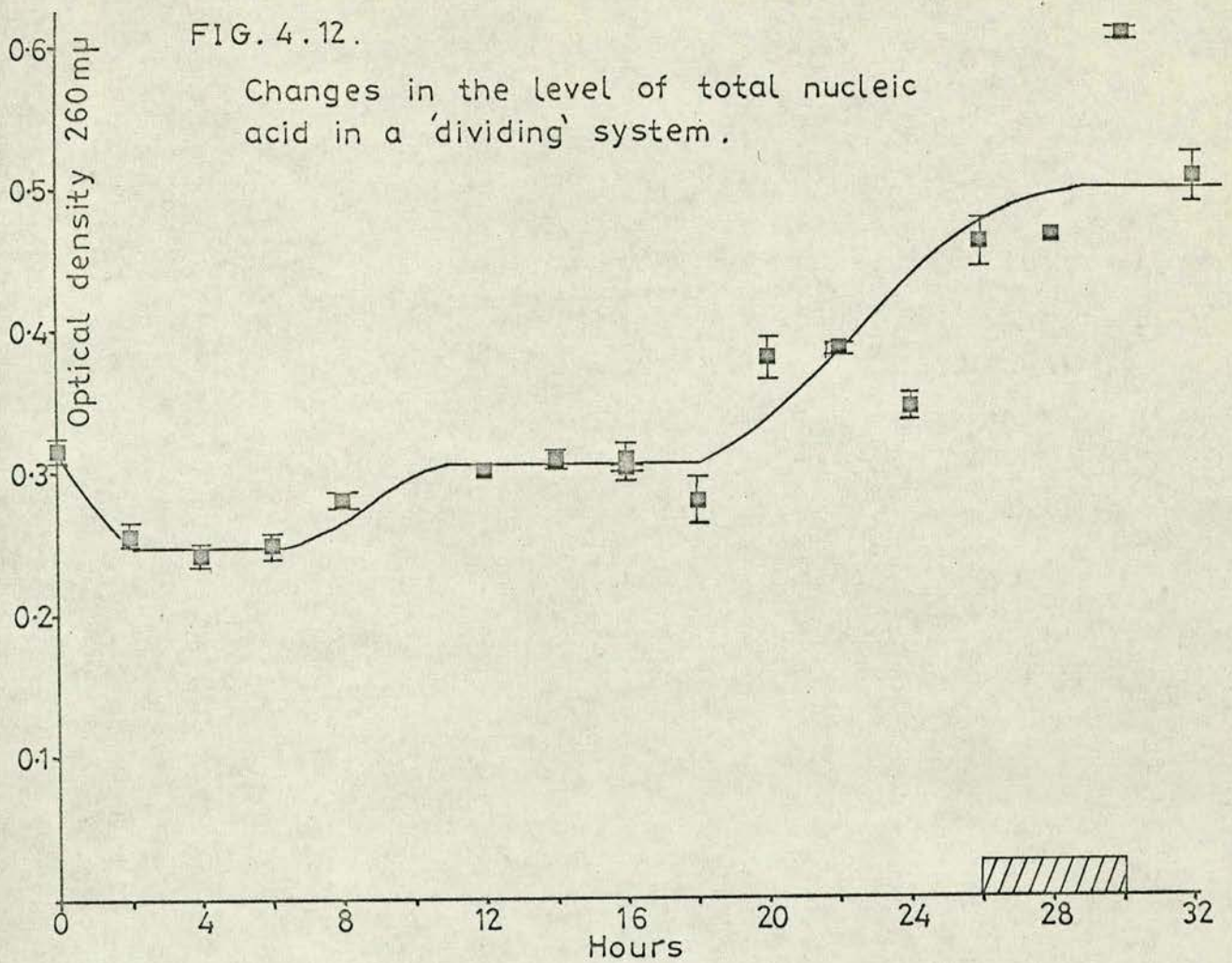
FIG.4.13.

Changes in the level of acid resistant protein in a 'dividing' system.
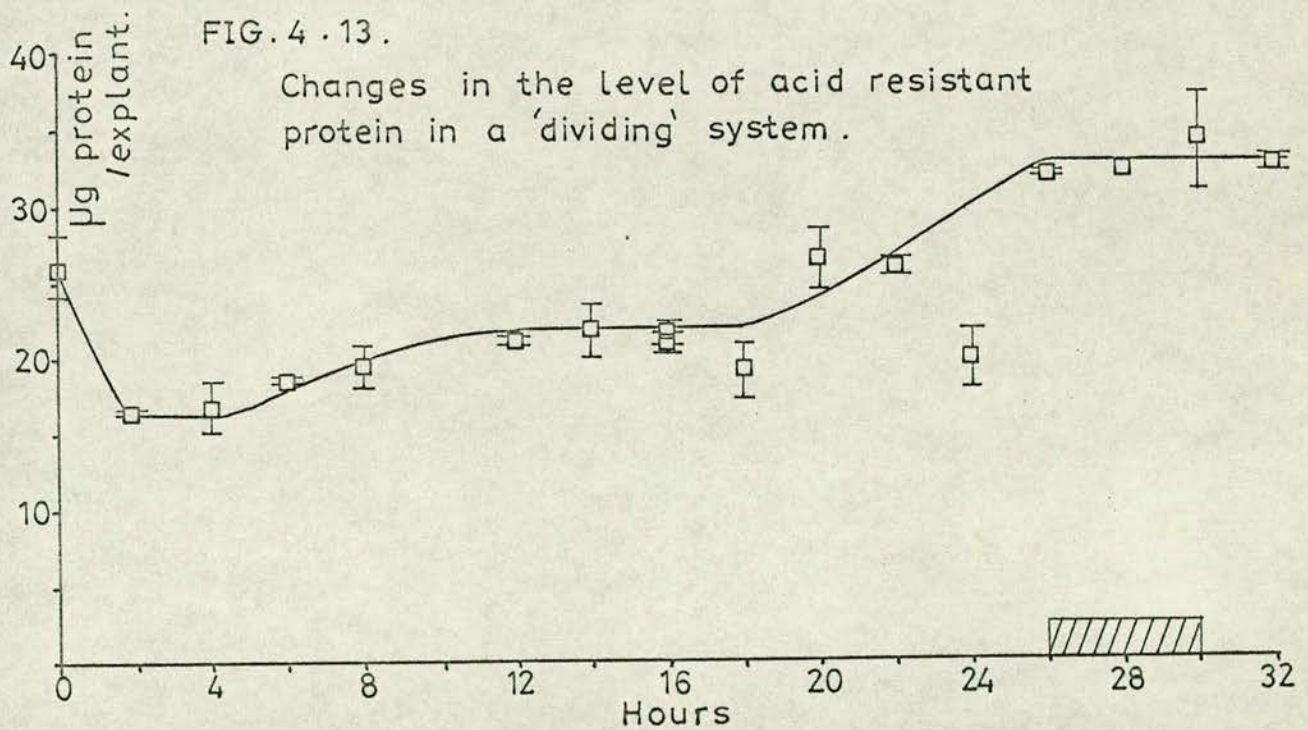
experiment ends at 32 hours.   The final plateau value amounts to an increase of 94% over the basal value.

In this experiment the pattern of acid resistant protein levels closely resembles the pattern obtained for levels of total nucleic acid, a similarity which was first observed by Evans (1967) for a system excised in the light and grown in the dark in the presence of coconut milk.

'Native'-DNAase activity

Variations in the activity of 'native'-DNAase are compared with the rate of DNA synthesis in Fig. 4.14.   A considerable increase in activity occurs during the first part of the cycle reaching a maximum value at 16 hours.   The decline in activity, which begins after 16 hours, continues until the experiment ends at 32 hours.

When the pattern of activity is compared with the rate of DNA synthesis, it is apparent that the decline from maximal activity begins at approximately the same time as the beginning of 'S'.   There may be some significance in the fact that in the duplicated 16 hour samples, the highest activity was observed in the sample in which 'S' had not started.

'Denatured'-DNAase activity

The activity of 'denatured'-DNAase is compared with the rate of DNA synthesis in Fig. 4.15.   During the first part of the cycle there appears to be a decline in 'denatured'-DNAase activity and a fairly constant level of activity is maintained until the experiment ends at 32 hours.
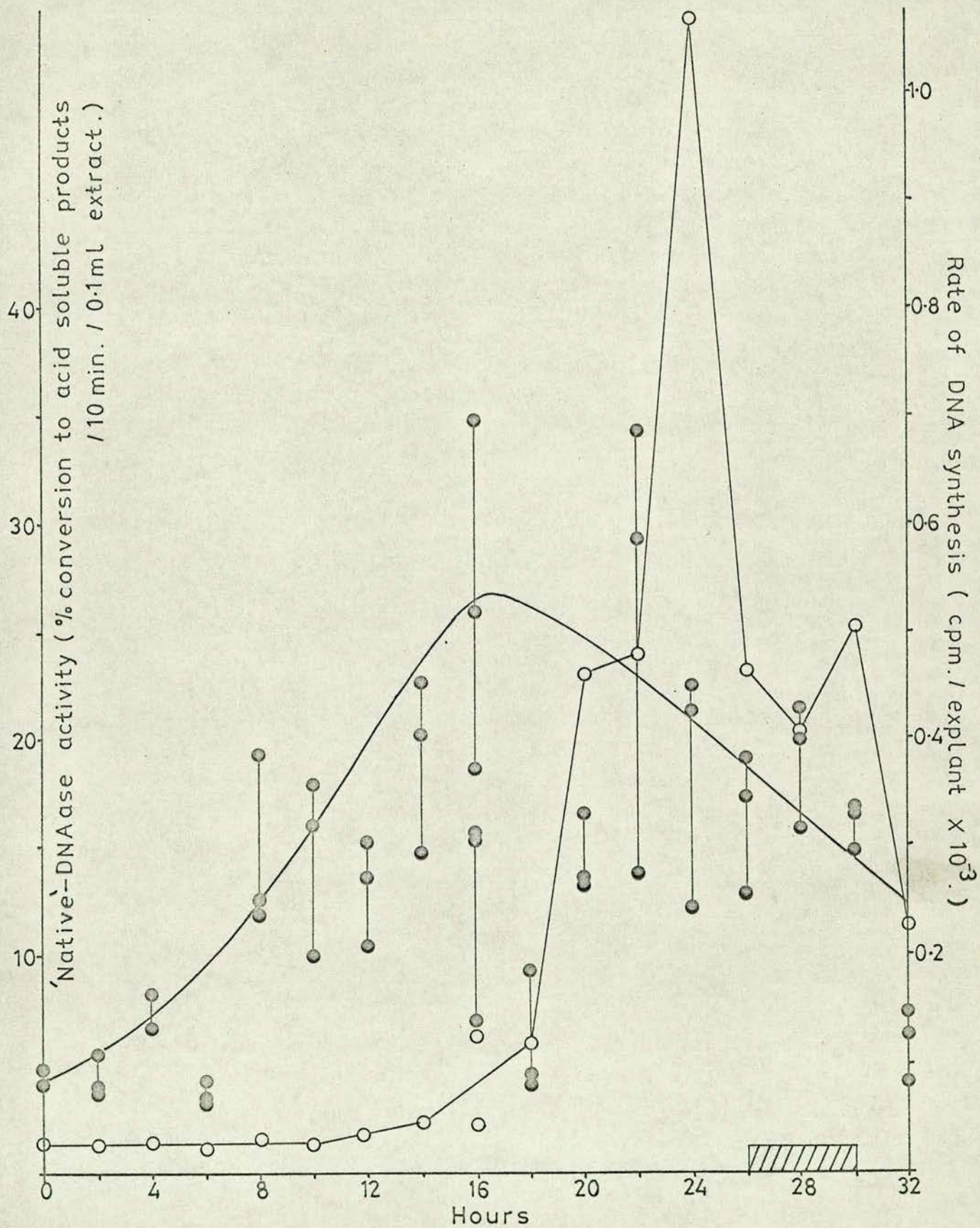
FIG. 4.14   Activity of 'native'-D.NAase (◉) in relation to the rate of DNA synthesis (O) in a 'dividing' system.
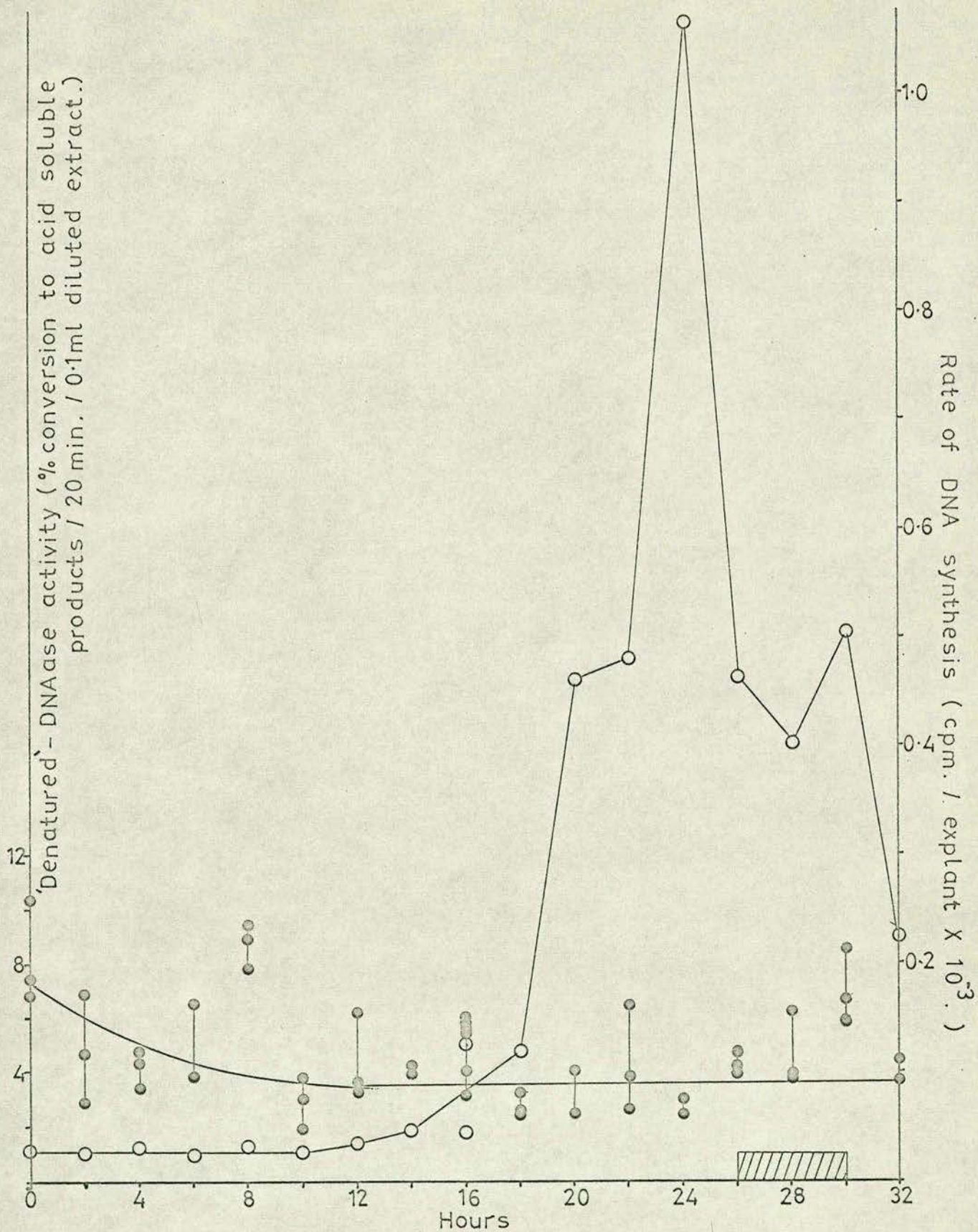
FIG. 4.15   Activity of 'denatured'-DNAase (●) in relation to the
rate of DNA synthesis (O) in a 'dividing' system.

<u>DNA polymerase activity</u>

Data for the DNA polymerase activity of both particulate and supernatant fractions of the extract are compared with the rate of DNA synthesis in Fig. 4.16. One feature which is immediately apparent is that the total extract shows a much higher activity when compared with the supernatant fraction. This suggests that some of the activity of the total extract is present as a bound enzyme.

The DNA polymerase activity of the total extract may show an initial fall from 0 to 2 hours which is followed by a small gradual increase during the first part of the cycle. The first sharp increase in activity is observed in one of the duplicated 16 hour samples, in fact in the one in which 'S' has started. The activity continues to increase steadily throughout the 'S' phase, rises even more sharply at the end of division and continues to increase until the experiment ends at 32 hours. At the beginning of division, the increase in activity compared with the 2 hour value is 380% and when the experiment ended at 32 hours this percentage has increased again to a value of 780%.

The DNA polymerase activity of the supernatant fraction also shows a gradual increase during the first part of the cycle. However, the first major rise in activity, which is recorded at 22 hours occurs about 6 hours after the beginning of 'S'. The value remains constant until 26 hours followed by a second much larger increase which takes place from 28 hours onwards. This pattern is not identical to that observed for the total extract.

The total activity of the extract consists of the activity of
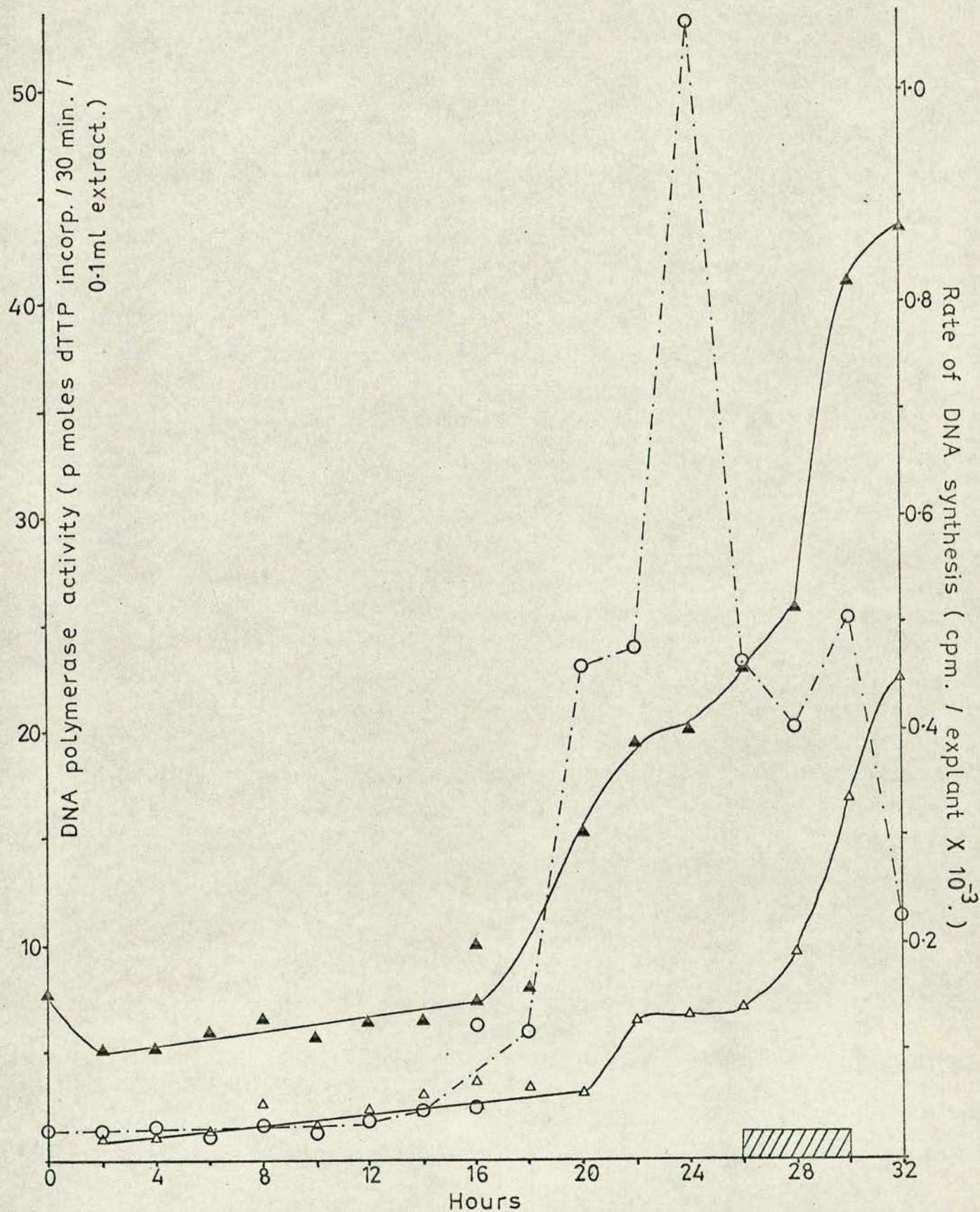
FIG. 4.16 DNA polymerase activity of the total extract (▲) and the supernatant fraction (△) in relation to the rate of DNA synthesis (O) in a 'dividing' system.

a soluble enzyme together with the activity of a bound enzyme.
The soluble enzyme activity is equivalent to the activity of the
supernatant fraction whilst the activity of the bound enzyme can be
calculated by subtracting the activity of the supernatant fraction
from the total activity of the homogenate. Using this simple
calculation, it can be estimated what percentage of the total
activity is represented by the bound and soluble enzyme components
at any one time.   These data are presented in histogram form in
Fig. 4.17 from which it is apparent that the bound enzyme is nearly
always present in a greater proportion than the soluble enzyme.
Various trends can be seen during the cell cycle.   The percentage
activity due to the bound enzyme tends to fall during the first
part of the cycle but shows an increase during 'S' after which it
declines again to a value lower than that at the beginning of the
experiment.   The percentage activity due to the soluble enzyme
increases during the first part of the cycle and tends to decrease
during 'S'.   A further increase is recorded during division and
when the experiment ends at 32 hours the percentage of soluble
enzyme activity slightly exceeds the percentage of bound enzyme
activity.

The results of this experiment have largely confirmed those
reported in Experiment 1.

In this experiment the period of time before division (the
lag phase) was greater than that recorded in Experiment 1.   This
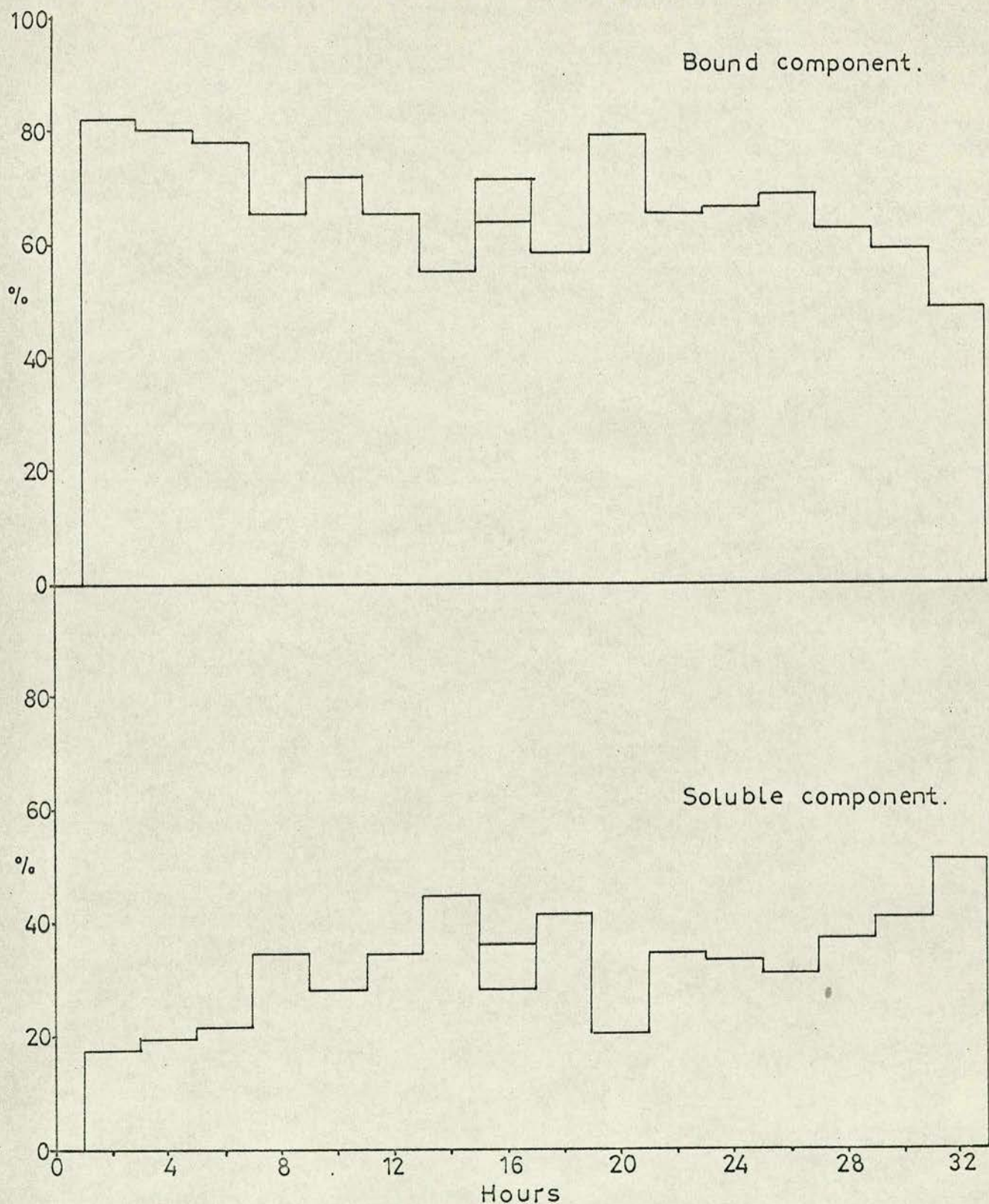
FIG. 4·17. Percentage of the total DNA polymerase activity which is represented by bound and soluble components during the cell division cycle.

discrepancy can be explained by the results of Evans (1967) on the basis that experiments carried out later in the season exhibit longer lag phases.   The dates on which all experiments were carried out are listed in Appendix 5.

A possible explanation of why the percentage cell division in this experiment was lower than that recorded in Experiment 1 is that cultures set up towards the end of the season may have a reduced capacity for growth (Robertson 1966).

Patterns for uptake of ^{3}H-thymidine into the tissue agree remarkably well in the two experiments.   The peak in the rate of uptake into the tissue, which occurs during the first part of the cycle, may be somewhat broader and lower in this experiment but attains a maximum value at about the same time in both experiments (10 hours).   In both experiments the rate of uptake falls during 'S' to reach a minimum value towards the end of 'S'.

Data for incorporation of ^{3}H-thymidine into DNA are similar to those presented in Experiment 1, except that in this experiment it is apparent that the 'S' phase of the second division cycle has not been reached.   It may be significant that a shoulder similar to that observed in Experiment 1, exists in the rising part of the curve, this time at 20 to 22 hours.

These data also demonstrate by the overlap values of different cultures at 16 hours, the variation which can exist between two cultures which have been growing for the same length of time and under the same conditions.   In one of the cultures, the 'S' phase has started whilst in the other, it has not.

It is clear that the accurate timing of the 'S' phase can only be obtained from the method employing incorporation of ^{3}H-thymidine into DNA, which is far superior to Burton's chemical method in this respect. Whereas the radioactive method defines the beginning of 'S' as 16 hours in this experiment, the first definite increase in the level of DNA measured by Burton's method occurs between 24 and 26 hours. This discrepancy of 8 - 10 hours is even larger than the discrepancy of 4 - 6 hours observed in Experiment 1.

Patterns of changes in the levels of DNA, total nucleic acid and acid resistant protein are all similar to those observed in Experiment 1. In this experiment, however, the final percentage increases are all comparatively lower, a property which may be associated with the reduced percentage cell division.

The fall in the levels of total nucleic acid and acid resistant protein which occurred between 0 and 2 hours in this experiment was not observed in Experiment 1. This phenomenon, which is often observed in cell cycle experiments may be a consequence of the initial loss of certain substances from damaged cells on the surface of the explants, into the culture medium.

The pattern of 'native'-DNAase activity described in Experiment 1 has been confirmed in this experiment.

The apparent slow decline in 'denatured'-DNAase activity during the first part of the cycle was not observed in Experiment 1. The increase in activity which began during 'S' in Experiment 1, did not occur in this experiment.

The pattern of DNA polymerase activity due to the total extract which was observed in this experiment, does not resemble that obtained in Experiment 1. The percentage increase in activity obtained at the end of division in this experiment is far greater than that obtained in Experiment 1. The marked increase in DNA polymerase activity which coincides with the beginning of 'S' in this experiment, occurs 6 - 8 hours after the beginning of 'S' in Experiment 1. These differences are inexplicable since similar reaction conditions were observed in both experiments.

In view of certain discrepancies between the results of the first two experiments, further experiments are required to establish patterns of activity for 'denatured'-DNAase and DNA polymerase, during the first division cycle.

Experiment 3.

In this experiment the activity of 'denatured'-DNAase was measured during the first cell division cycle in relation to the rate of DNA synthesis. The assays were carried out using a higher level of enzyme activity than that used in the two previous experiments and for this reason it was anticipated that any increases which might occur during the cell cycle would be larger in absolute terms and therefore more well defined.

The conditions used for setting up this experiment were as described for Experiment 1. This experiment covered a 28 hour period and involved setting up three series of cultures. The first 5 cultures were prepared 20 hours before sampling began and

the second 5 cultures were prepared 10 hours prior to sampling.
In this way, the period of time from 20 to 28 hours and from 10 to
18 hours were sampled in parallel at intervals of 2 hours.   The
third series of cultures which was prepared immediately prior to
sampling contained only 3 flasks for sampling at 0 hours, 4 hours
and 8 hours.   No overlap point was included in this experiment
because of the technical difficulty of sampling 3 cultures at the
same time.

At each sampling time 5 explants were removed for cell counting,
16 explants removed for estimation of the rate of DNA synthesis
and 100 explants taken for preparation of the enzyme extract.

Because this experiment also included assays for dTMP kinase
and dTMP phosphatase, the preparation of the extract was modified
(Ch. 2. D. 2.)   The final composition of the assay mixture was
similar to that used in the two previous experiments except that
the extract was not diluted quite so much in order to obtain an
initial percentage conversion of approximately 10%.   Assays for
'denatured'-DNAase activity were carried out in duplicate and the
results were based on both 1 minute and 10 minute counts for each
sample.

Results

Changes in cell number (Fig. 4.18)

The first division commenced at about 24 hours and was
completed between 26 and 28 hours when it was estimated that
approximately 65% of the cells had divided.
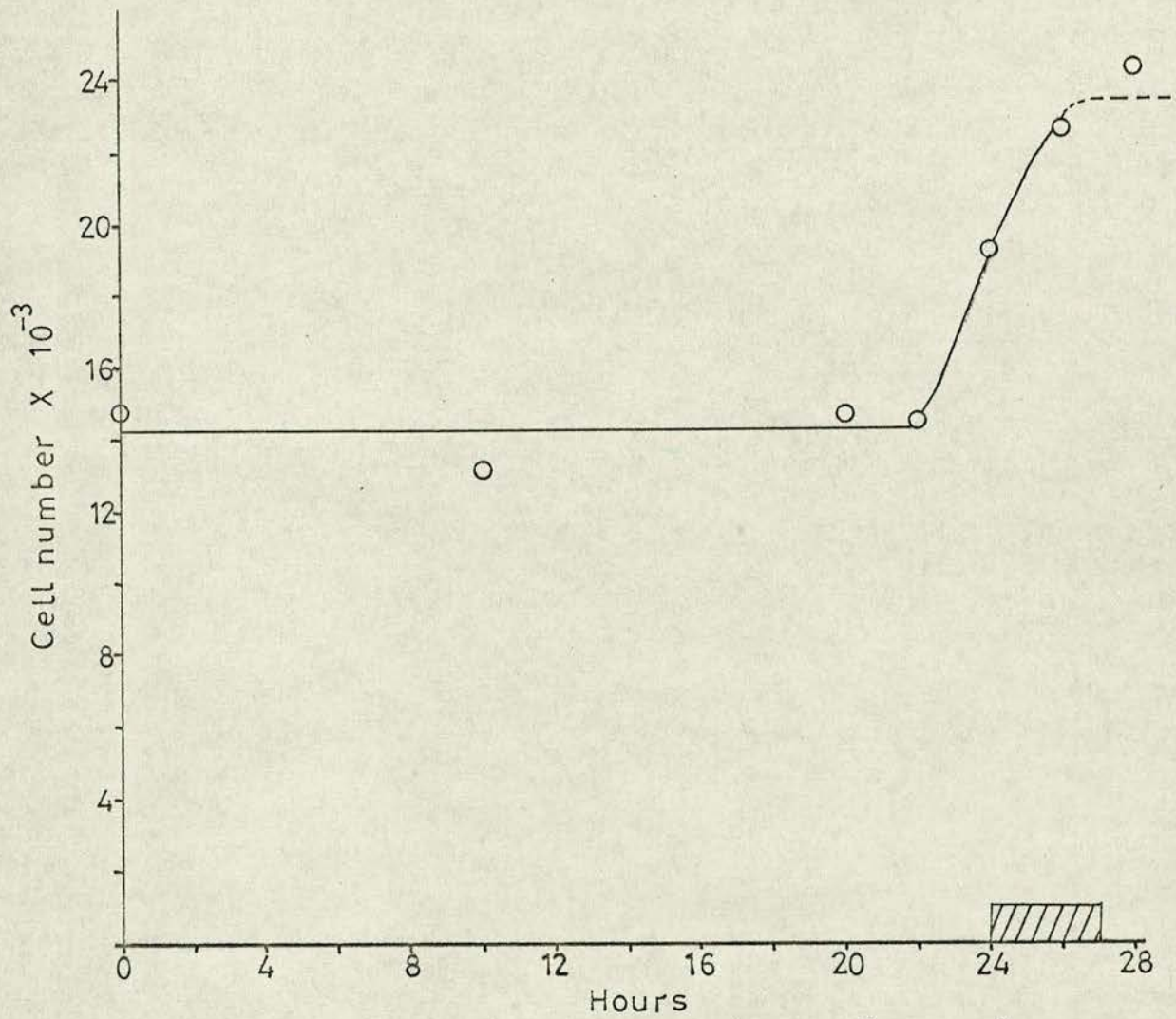
FIG. 4.18   Cell number changes in a dividing system.

The rate of DNA synthesis

Data for the rate of DNA synthesis measured by rate of incorporation of ^{3}H-thymidine into DNA are presented in Fig. 4.19. A low value is maintained during the first 16 hours of the cycle and the first sharp increase which marks the beginning of 'S' is detected at 18 hours.   The rate of incorporation continues to rise sharply until 20 hours and remains approximately constant until 22 hours.   Another sharp increase is observed at 24 hours followed by the suggestion of a fall in rate at 26 hours, and at 28 hours the highest value is recorded.

'Denatured'-DNAase activity

The activity of 'denatured'-DNAase (based on both 1 minute and 10 minute counts) during the first division cycle is shown in Fig. 4.20.   When results are expressed on a 1 minute count basis the activity appears to remain constant throughout the experiment. When the results are expressed on a 10 minute count basis, there is less variation between duplicated values, but the overall pattern of activity is the same.

The 65% cell division obtained in this experiment is in good agreement with the results of Experiment 1 and with the results of Davidson (1971).

The similarity between the pattern of the rate of DNA synthesis observed in this experiment and those obtained in Experiments 1 and 2, is confined to the first part of the cycle.   In this experiment
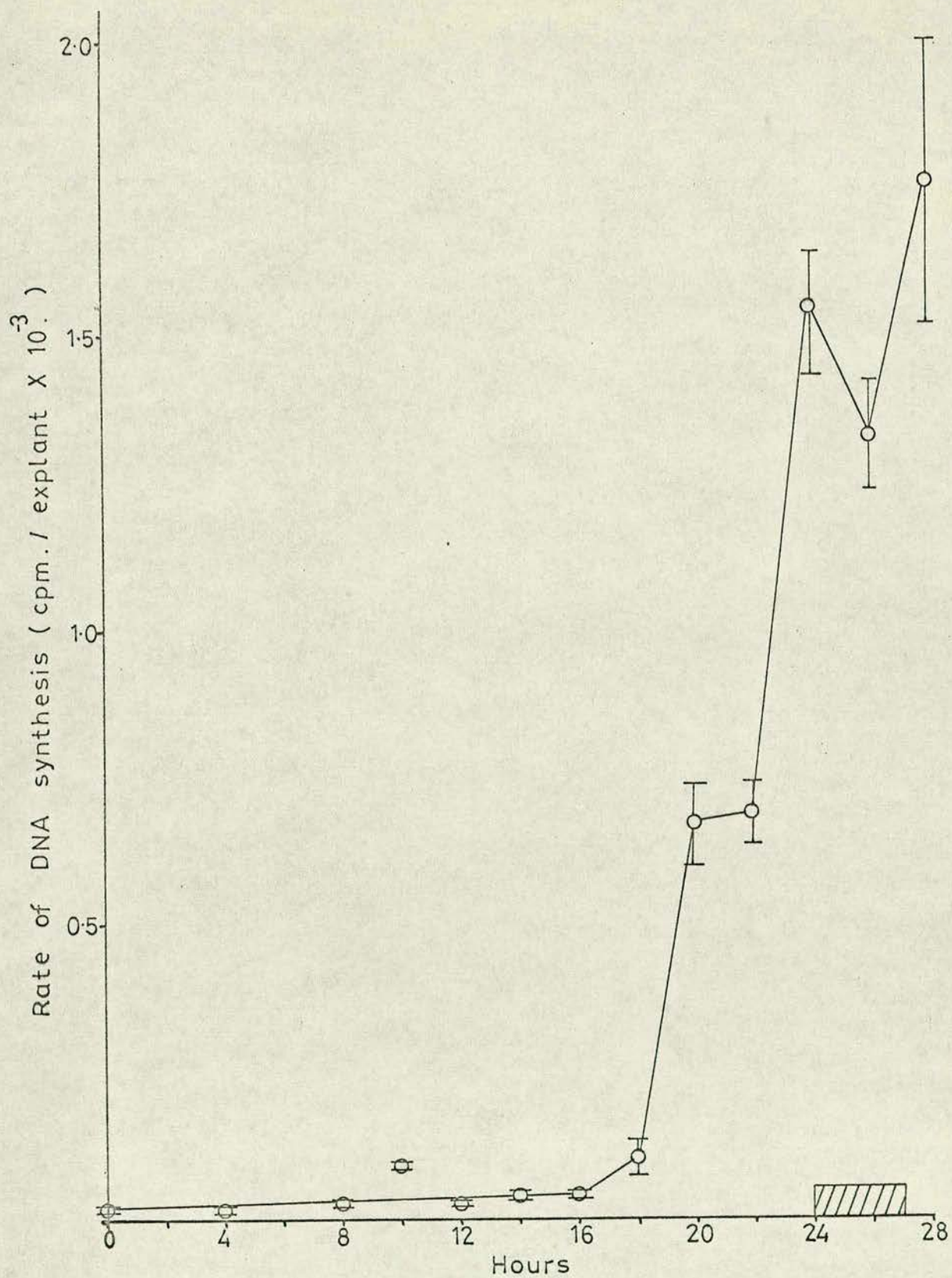
FIG . 4 . 19   Changes in the rate of DNA synthesis in a ´dividing´ system.

based on 1 minute counts.

based on 10 minute counts.
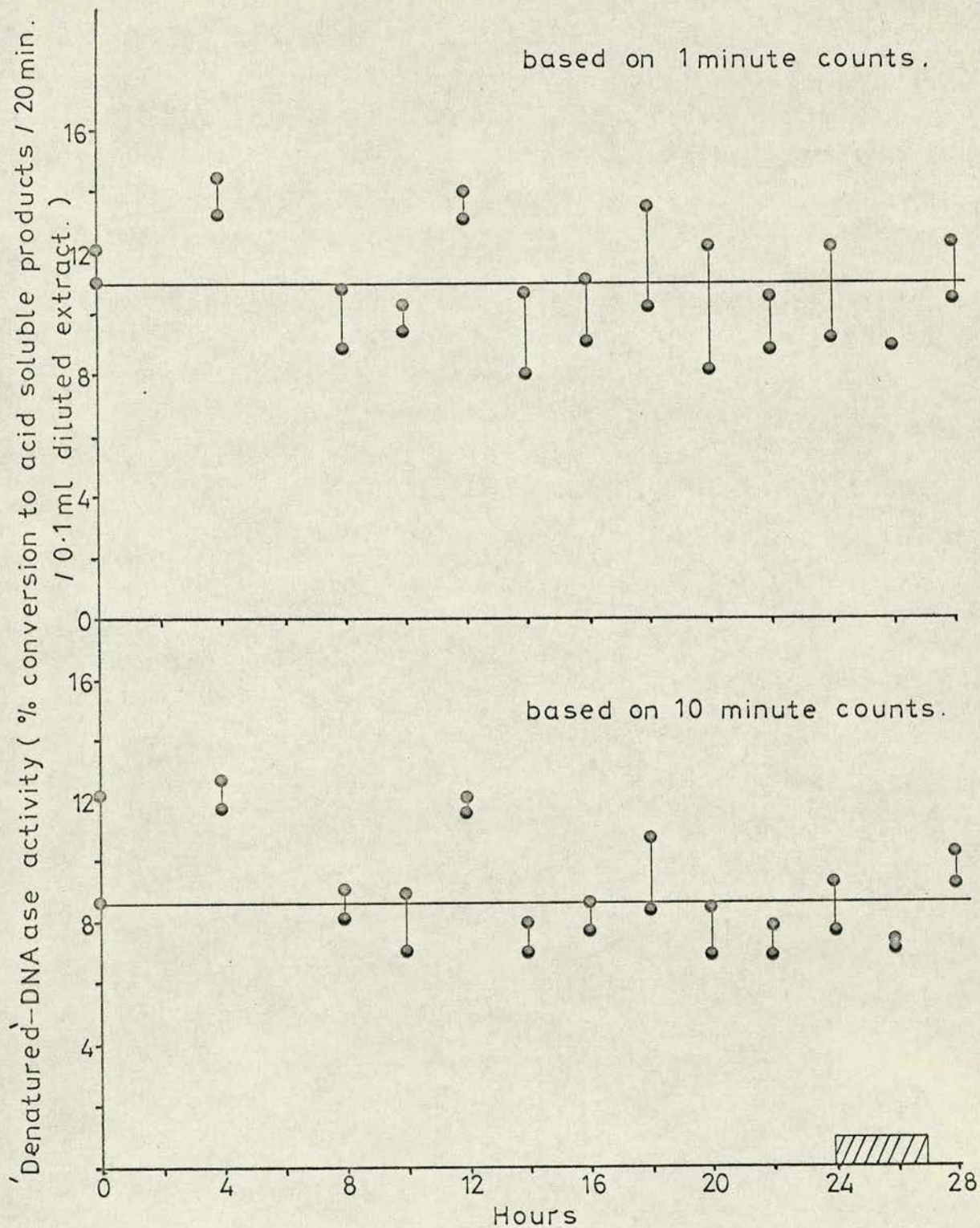
FIG. 4 . 20 Activity of 'denatured'-DNAase in a 'dividing' system based on 10 minute and 1 minute counts.

the pattern differs from previous experiments in that there is no significant fall in the rate marking the end of 'S', and the value continues to increase during division.   This type of pattern, however, is consistent with the results obtained from experiments which were also carried out early in the season. (See Appendix 5 for dates of all experiments)

The pattern of 'denatured'-DNAase activity during the cell cycle observed in this experiment is similar to the pattern observed in Experiment 2.   In Experiment 2, and in this experiment, the activity of 'denatured'-DNAase appeared to remain constant through-out the cell division cycle whereas in Experiment 1, it was possible to prove that a definite increase in 'denatured'-DNAase activity occurred during the experiment.

From a consideration of the results of all 3 experiments, it is probable that the activity of 'denatured'-DNAase remains constant during the first division cycle but that a small gradual increase may be detected when the activity is measured over a prolonged period such as the 36 hour period used in Experiment 1.

Experiment 4.

The aim of this experiment was to measure DNA polymerase activity during the cell division cycle in relation to the rate of DNA synthesis, in order to provide further confirmation of the results obtained in Experiments 1 and 2.

Jackson (1970), had previously shown that when enzyme extracts were prepared using a grinding buffer which contained 1·0%

β-mercaptoethanol (EtSH), the DNA polymerase activity of the total extract became soluble and resided in the supernatant fraction of the extract. Subsequently Jackson had shown that the total DNA polymerase activity of an extract prepared in the presence of 0·5% EtSH is the same as the activity obtained using the supernatant fraction of an extract prepared in the presence of 1·0% EtSH.

In this experiment, all extracts were prepared in the presence of 1·0% EtSH and the activities measured were those of the supernatant fraction.

This experiment covered a 28 hour period and involved preparation of two series of cultures. The first 8 cultures were prepared 14 hours before sampling began and the second 8 cultures were prepared immediately prior to sampling. In this way, the culture periods from 14 to 28 hours and from 0 to 14 hours were sampled in parallel at intervals of 2 hours. As in Experiment 2, an overlap point was included, this time at 14 hours, which gave two 14 hour samples, one from the first and one from the second series of cultures.

At each sampling time, 0·5 ml. of culture medium was removed aseptically for estimation of microbial contamination (see Appendix 2c). In addition, 5 explants were removed for cell counting, and a further 5 for Feulgen estimation of DNA levels. Sixteen explants were removed for estimation of the rate of DNA synthesis, and 100 explants were used to prepare the enzyme extract.

Because this experiment included assays of TdR kinase and

dTMP kinase activities (the results of which are described in a later section), the preparation of the extract was modified by substituting 0·2M sodium phosphate buffer pH 8·0 instead of tris-maleate buffer in the grinding medium (see Ch. 2. D.3)

Results

Cell number changes (Fig. 4.21)

The first division commenced at about 22 hours and was completed by 26 hours, when it was estimated that approximately 63% of the cells had divided.

Feulgen DNA levels (Table 4a)

Data are available only for the period 10 to 24 hours, and the level of DNA appears to increase throughout this period from a basal value of 100% at 10 hours to a value of 138% by 24 hours.

Rate of DNA synthesis (Fig. 4.22)

During the first part of the cycle a low rate of DNA synthesis is maintained and the first major increase which marks the beginning of 'S' is recorded at 12 hours. The values obtained from the two 14 hour samples are markedly disparate, that of the first series of cultures (14 to 28 hours) being much higher than that of the second series of cultures (0 to 14 hours), although 'S' has undoubtedly started in both. The rate recorded at 16 hours is lower than the highest 14 hour value giving rise to the possibility that a shoulder might exist on the curve. A further increase occurs at 18 hours and the rate remains approximately constant
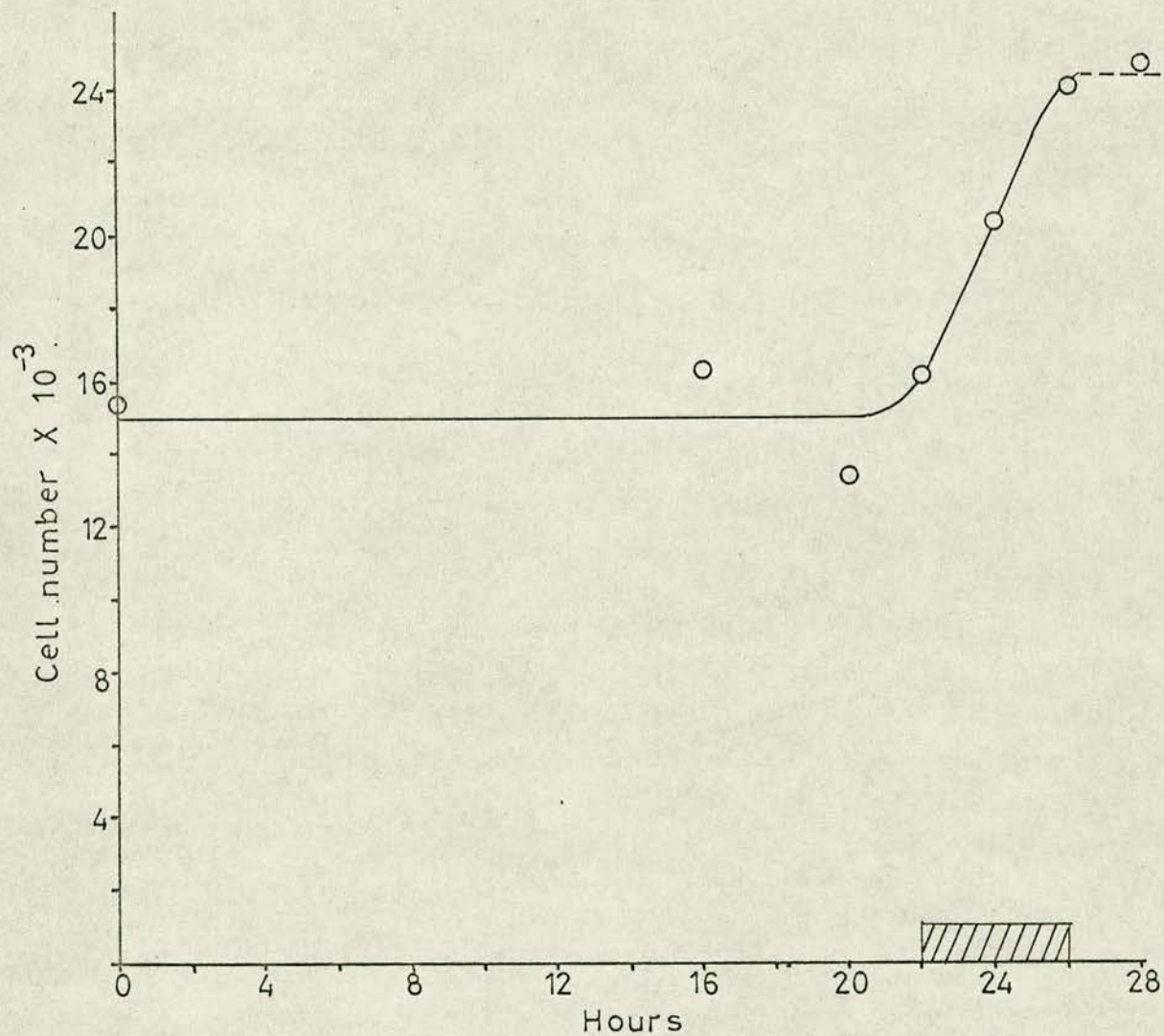
FIG. 4.21 Cell number changes in a 'dividing' system.

Period of culture (hours)	Feulgen DNA levels (% base value)
10	100·0
14	103·9
16	105·2
18	120·5
20	119·5
22	125·0
24	137·8

Table 4.a.   DNA measured using the Feulgen
            method in a 'dividing' system.
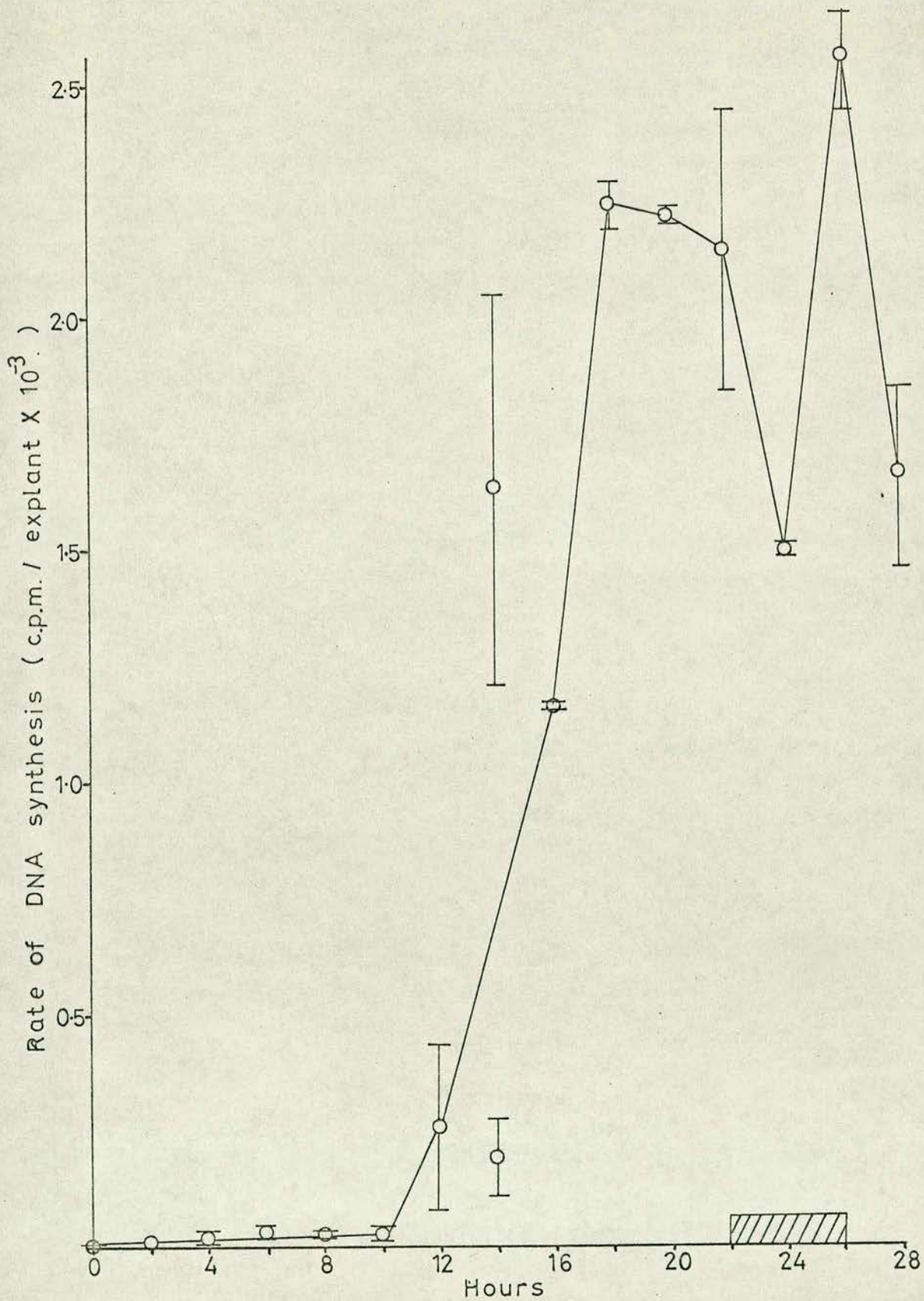
FIG. 4 · 22 Changes in the rate of DNA synthesis in a 'dividing' system.

until 22 hours.   A slight fall in the rate at 24 hours is followed by a further rise at 26 hours and a further fall at 28 hours.

DNA polymerase activity

The DNA polymerase activity during the first division cycle is compared with the rate of DNA synthesis in Fig. 4.23.   A low DNA polymerase activity is maintained during the first part of the cycle and the first major increase in activity occurs at about the beginning of 'S'.   The activities recorded in both 14 hour samples are similar.   The activity continues to increase until 18 hours when it may reach a plateau value which is maintained until the beginning of division and amounts to a 1600% increase over the basal value.   A further sharp increase in activity takes place during division and when the experiment ends at 28 hours the DNA polymerase activity shows a total increase of 3500% over the basal value.   A correlation coefficient of 0·988 between the activity of DNA polymerase and Feulgen DNA levels has been calculated for the period 10 to 24 hours (See Appendix 6).

The 63% cell division obtained in this experiment is in good agreement with the results of Experiments 1 and 3 and with the results of Davidson (1971).

The Feulgen DNA data for this experiment are similar to the results of Mitchell (1967) as a fairly steady increase in the level of DNA occurs during 'S'.

The pattern shown by the rate of DNA synthesis in this
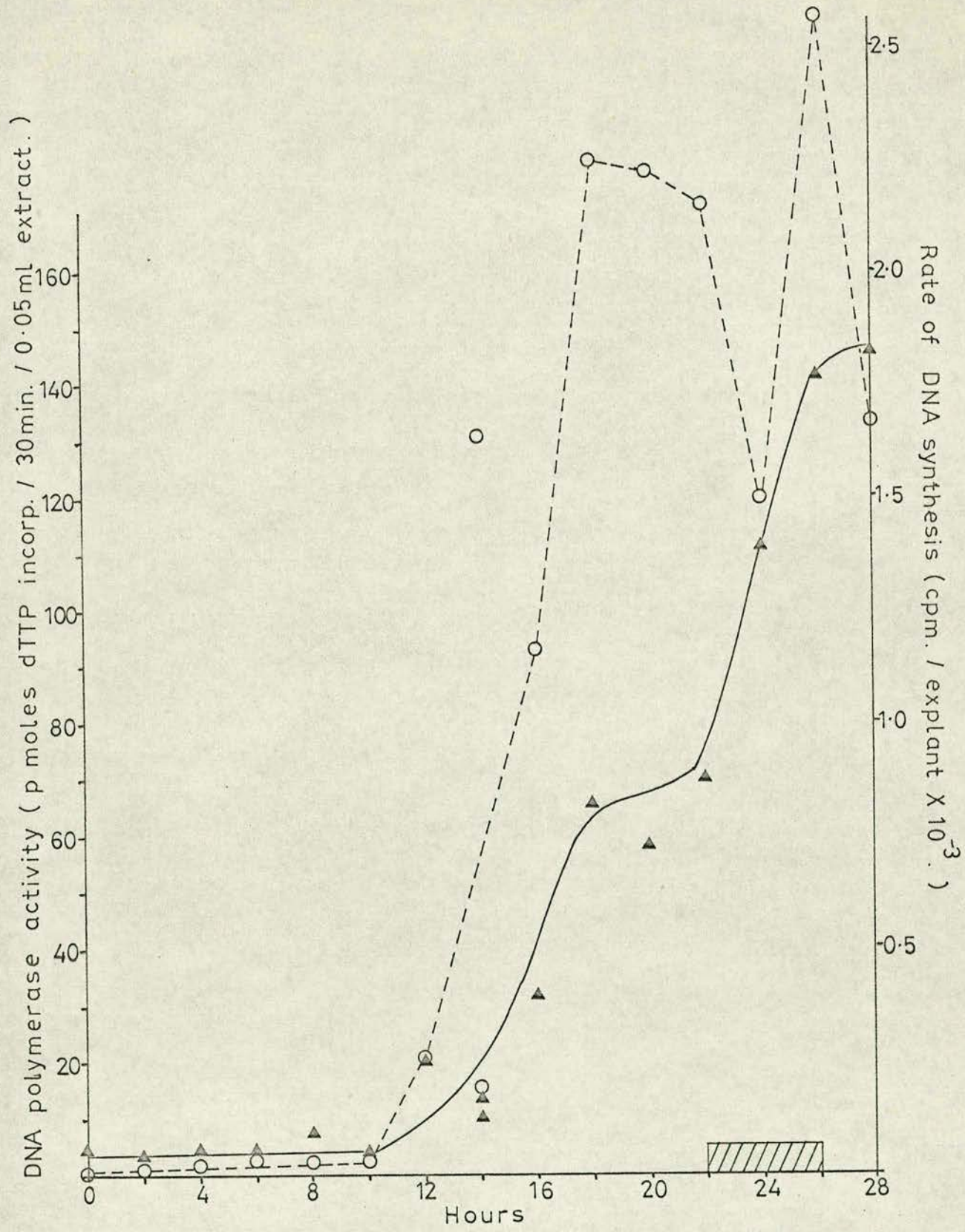
FIG. 4·23 Changes in DNA polymerase activity(▲) in relation to the rate of DNA synthesis (O) in a 'dividing' system.

experiment is similar to that obtained in Experiment 3, in that
it shows no fixed definition of the end of 'S', and the value
also continues to fluctuate during division.   As mentioned in
Experiment 3, this type of pattern may be characteristic of
experiments carried out early in the season (see Appendix 5 for
dates of all experiments).

If the higher rate of DNA synthesis recorded at 14 hours is
closer to the true value than the lower 14 hour rate then there may
be a shoulder in the rising part of the curve in this experiment,
similar to those observed in all previous experiments.

As previously observed in Experiment 2, the first marked rise
in DNA polymerase activity cannot be separated from the beginning of
'S'.   However, the gradual rise in DNA polymerase activity which
was observed during the first part of the cycle in Experiments 1
and 2 is absent in this experiment.

There may be some significance in the fact that in this
experiment, the DNA polymerase activities of both 14 hour samples
were only slightly increased over the basal value, despite the
enormous difference in rate of DNA synthesis values between the two
samples.

The pattern of DNA polymerase activity observed in this
experiment is basically similar to that obtained in Experiment 2.
However, the percentage increase in activity at the end of division
is enormous when compared with the percentages recorded in both
Experiments 1 and 2.

The correlation coefficient between Feulgen DNA levels and

DNA polymerase activity is 0·988.   With 5 degress of freedom this means that the correlation between the two variables is highly significant and there is a probability of less than 1·0% that this correlation is due to error.

In order to establish whether the patterns of enzyme activities so far described are associated with a 'dividing system', it is important that these activities are measured in a 'non-dividing' system.

Experiment 5.

In this experiment the activities of 'native'-DNAase, 'denatured'-DNAase and DNA polymerase were measured in a 'non-dividing' system during a 30 hour period, to determine whether or not the patterns of enzyme activity so far established were associated with the cell division cycle.

For this experiment, 'non-dividing' cultures were prepared by omitting the synthetic auxin 2,4-D from the culture medium (Ch.2.B.) Conditions used for preparing and growing the cultures were as usual, except that the culture medium was a solution containing only sucrose and mineral salts.   A single series of 10 cultures was prepared immediately prior to sampling.   At each sampling time throughout the 30 hour period 5 explants were removed for cell counting and 100 explants were used to prepare the enzyme extract. Assays for both 'native'-DNAase and 'denatured'-DNAase activity were carried out at least in duplicate, and in triplicate where volumes of extract would allow.

The DNA polymerase activity of the total extract was
assayed as described for Experiments 1 and 2.   In addition to the
activities measured during the 30 hour period, several DNA polymerase
activities were measured much later, after 43·5 hours and after
65 hours of culture.   A single 'dividing' culture was sampled at
42 hours and the DNA polymerase activity measured.   A DNAase 1
control was carried out as described in Chapter 2. D. 3. on the
product of DNA polymerase action at 65 hours.

Results

Cell Number

Cell number estimations during the 30 hour period are shown
in the following table from which it is evident that no marked
increase in cell number has taken place.

Growth Period (hours)	Average cell number per explant
0	13750
15	15750
20	14000
24	13000
27	13875
30	13000

The absence of division figures in the cell macerates also
confirmed that cell division had not taken place.

'Native'-DNAase activity

The activity of 'native'-DNAase during the 30 hour period is shown in Fig. 4.24 from which it is apparent that a substantial increase in activity occurs during the first 18 hours of culture followed by a gradual decline in activity which continues until the experiment ends at 30 hours.

'Denatured'-DNAase activity (Fig. 4.25)

The activity of 'denatured'-DNAase remains approximately constant throughout the culture period and the straight line in Fig. 4.25 accommodates almost all the points (Denatured-DNAase activity is expressed as % conversion to acid soluble products/20 min./0·1 ml. diluted extract).

DNA polymerase activity (Fig. 4.26)

The activity of DNA polymerase remains constant throughout the 30 hour period at an average value of 5·05 p moles dTTP incorporated/ 30 minutes/0·1 ml. extract.

At 43·5 hours the value was maintained at 4·7. By 65 hours however, two separate estimations of DNA polymerase activity yielded values of 2·0 and 2·8 respectively which may indicate a gradual fall in activity over a prolonged period. When the products of DNA polymerase action at 65 hours were treated with a preparation of DNAase 1, a value of 0·6 was obtained, indicating that the product of DNA polymerase action was DNA. The single 'dividing' culture which was sampled after 42 hours of growth, yielded a DNA polymerase activity of 36·8, a figure which is comparable to values obtained at the end of division in Experiment 2.
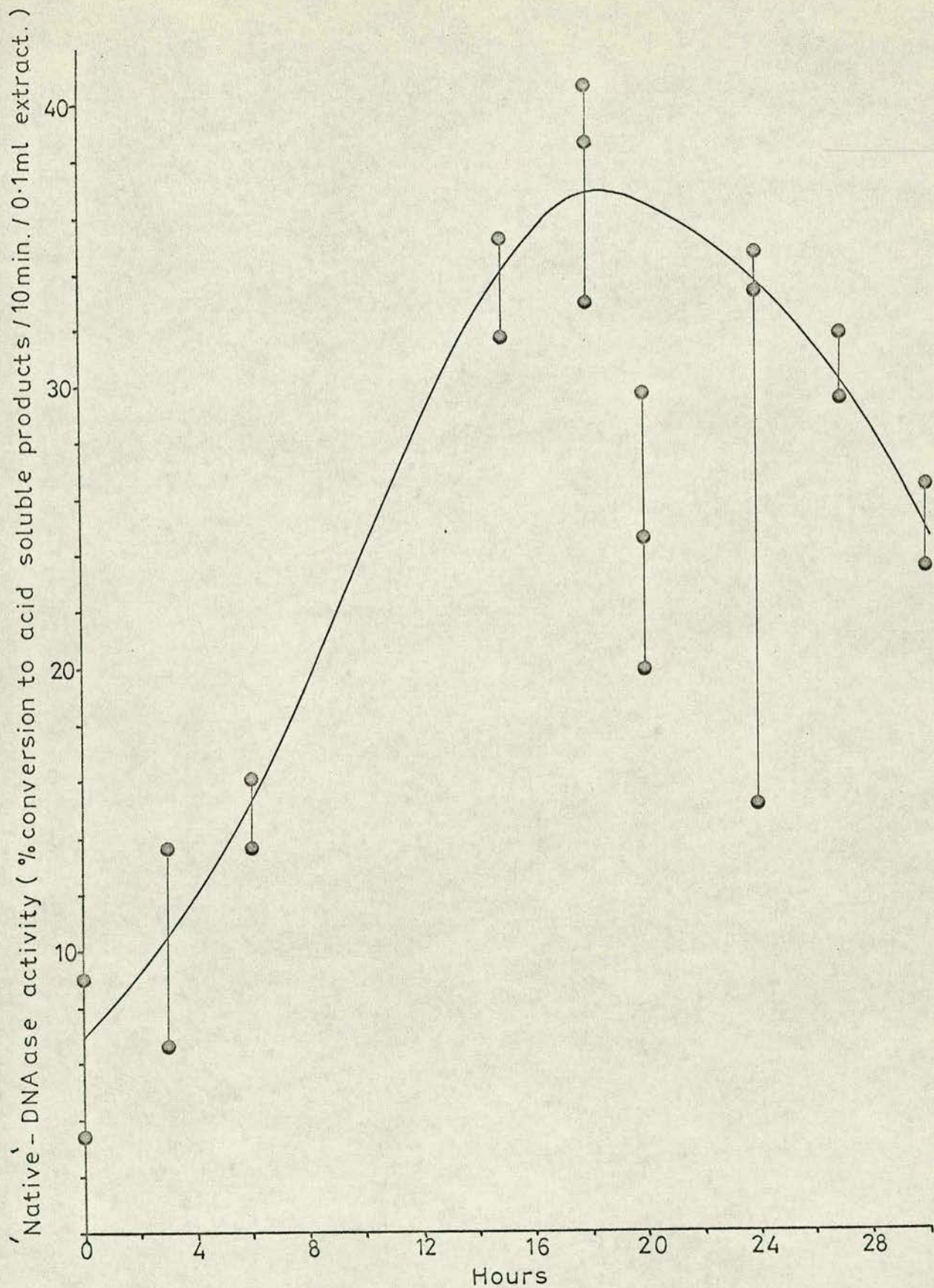
FIG. 4.24 'Native'- DNAase activity during a 30 hour period in a 'non-dividing' system.
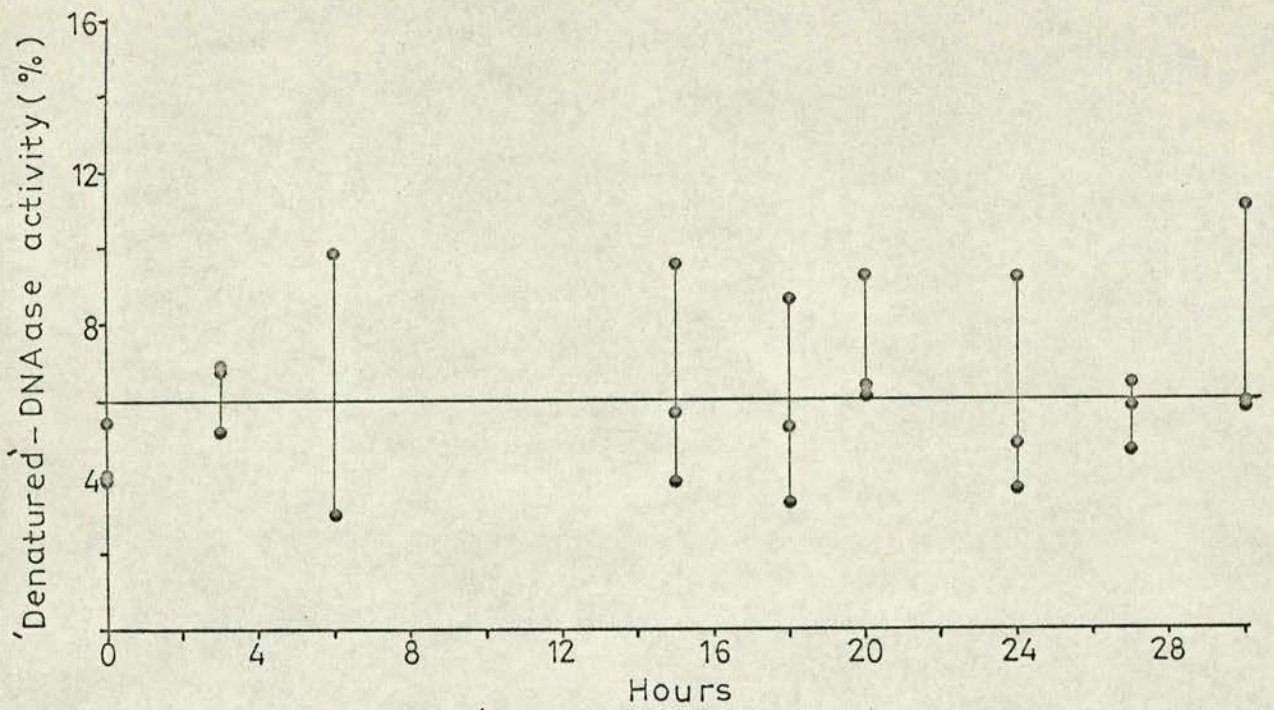
FIG.4.25 'Denatured' - DNAase activity during a 30 hour
period in a 'non—dividing' system.
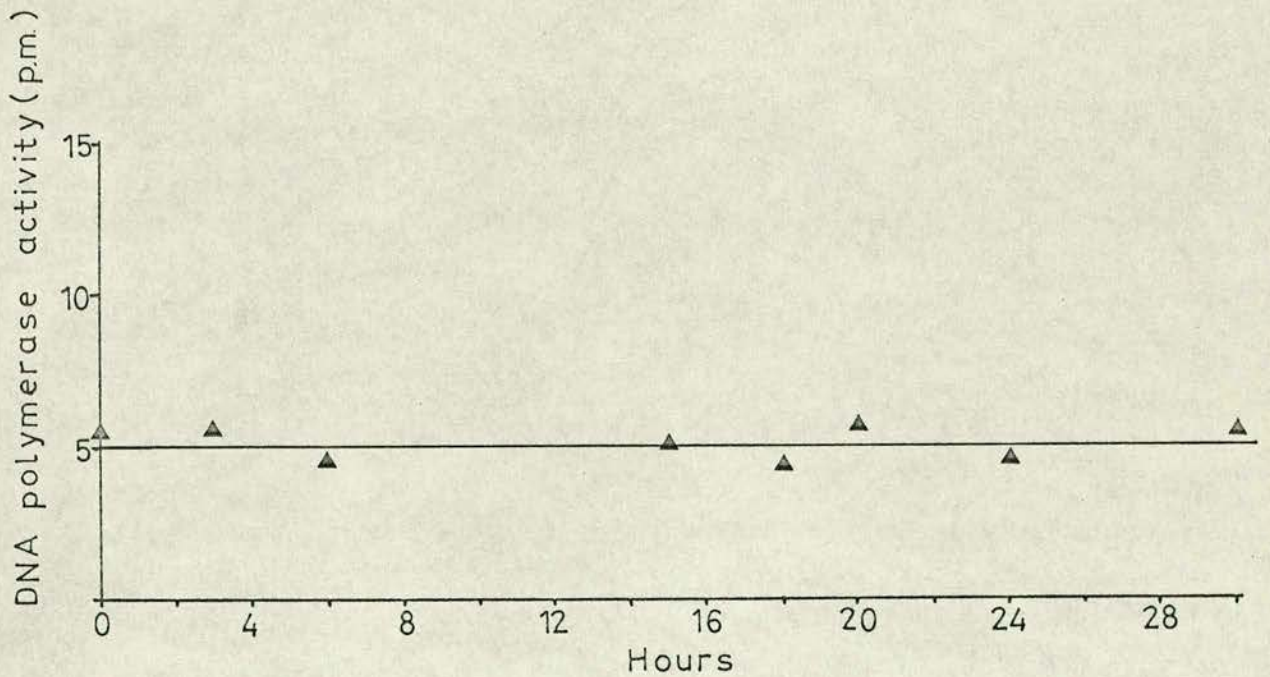
FIG.4.26 DNA polymerase activity during a 30 hour
period in a 'non-dividing' system.

In this experiment the cell number values have confirmed
the absence of cell division. This experiment was carried out
only 6 days after Experiment 2, in which cell division had started
by 26 hours (See Appendix 5 for dates of experiments). The 30 hour
period used in this experiment has adequately covered the period
of time in which division would have occurred if this had been a
'dividing' system, therefore, it is valid to compare the results
obtained in this experiment with those of previous experiments.

The pattern of 'native'-DNAase activity in a 'non-dividing'
system is similar to that observed in the 'dividing' systems
examined in Experiments 1 and 2. This characteristic pattern of
'native'-DNAase activity is therefore a property of freshly
excised artichoke tissue in culture. Although the patterns of
activity are similar in all 3 experiments there are differences in
the time taken to reach maximum 'native'-DNAase activity, which is
18 hours for this experiment, 16 hours in Experiment 2 and 14 hours
in Experiment 1. To determine whether the maximum activity really
occurs later in a 'non-dividing' system or whether this discrepancy
is merely due to variation between samples, an experiment must be
carried out in which several 'dividing' and 'non-dividing' cultures
prepared at the same time are grown in parallel and sampled at the
same time.

The fact that the constant 'denatured'-DNAase activity
maintained throughout this experiment is similar to the patterns
observed in Experiments 2 and 3 for 'dividing' systems, suggests
that the pattern of activity for this enzyme is not associated with

the cell division cycle but is merely a property of freshly excised artichoke tissue in culture.

In this experiment the activity of DNA polymerase remained constant throughout the 30 hour period. Therefore, the gradual rise in DNA polymerase activity during the first part of the cycle which was observed in Experiments 1 and 2, and the sharp rise in activity during 'S' which was observed in Experiments 1, 2 and 4, are probably both exclusively associated with a 'dividing' system.

Experiment 6.

In this experiment several 'dividing' and 'non-dividing' cultures were prepared simultaneously and sampled at the same time, to determine whether there was any difference between the activities of 'native'-DNAase in the two situations.

A series of 3 'dividing' cultures were prepared in the presence of 2,4-D and 2 'non-dividing' cultures were prepared by omitting 2,4-D from the culture medium.

The sampling time chosen was 18 hours which incidentally had given low activity values in both experiments using 'dividing' systems (Experiments 1 and 2) and a high value in a 'non-dividing' system (Experiment 5). Extracts were prepared as described in Experiment 1. Assays for 'native'-DNAase activity were carried out in triplicate on the extract from each culture.

Results

'Native'-DNAase activities are shown in Table 4b from which it is apparent that despite considerable variation, there is no significant difference between activities in 'dividing' and 'non-dividing' systems at 18 hours.

The average 'native'-DNAase activity at 0 hours is only slightly lower than the lowest activity observed at 18 hours hence it is probable that in this experiment, the peak of 'native'-DNAase activity occurred prior to 18 hours, and that the 18 hour point is on the falling part of the curve.

This experiment has confirmed that the activities of 'native'-DNAase after 18 hours of culture, are similar in 'dividing' and 'non-dividing' systems.

Period of culture (hours)	24D presence	Activity of 'native'-DNAase (% conversion to acid soluble products / 10 min./0·1 ml extract)	
		Actual values	Average values
0	-ve	5·8 6·4 7·5	6·6
18A	+ve	6·9 7·3 7·1	7·1
18B	+ve	8·6 9·2 6·3	8·0
18C	+ve	14·0 8·3 11·6	11·3
18D	-ve	10·9 8·3 9·2	9·5
18E	-ve	6·9 7·0 6·8	6·9

Table 4b.   The activity of 'native'-DNAase after 18 hours of

culture in both 'dividing' and 'non-dividing' systems.

Summary of Section A

The results from this series of experiments can be summarised as follows:-

1.   By measuring such standard parameters as cell number, levels of DNA, total nucleic acid and acid resistant protein, it has been established that the experimental system is behaving in the way described by previous workers.   Although the patterns of increase are similar to those observed by Evans (1967) for a system excised in the light and cultured in the dark in the presence of coconut milk, the absolute increases are far larger, a fact which is almost certainly related to the fact that the system used in the present series of experiments was excised in low intensity green light and grown in the dark.

2.   The patterns of rate of uptake of ^{3}H-thymidine into the tissue and of the rate of DNA synthesis are similar to those described by Evans (1967).

In Experiments 1 and 2, measurement of the rate of DNA synthesis was shown to be a more accurate indicator of the onset and progress of DNA synthesis than measurement of DNA levels using Burton's chemical method.

In Experiments 1 - 4, the rates of DNA synthesis are similar during the first part of the cycle and indicate without exception the possibility of a shoulder in the rising part of the curve.

In Experiments 3 and 4 however, this method fails to define the timing of the end of 'S'.   As experiments 3 and 4 were carried

out much earlier in the 'artichoke season' than Experiments 1 and 2 it is possible that the inability to define the timing of the end of 'S' has seasonal implications.

3.   A characteristic pattern of 'native'-DNAase activity during the cell division cycle was described in Experiment 1 and verified in Experiment 2.   That this pattern is not exclusively associated with cell division but is merely a property of freshly excised artichoke tissue in culture, has been shown in Experiments 5 and 6.

4.   The activity of 'denatured'-DNAase during the cell division cycle remained constant in Experiments 2 and 3.   As a similar pattern was observed for the 'non-dividing' system examined in Experiment 5, it is probable that this pattern of activity is not associated with the cell division cycle but is merely a property of freshly cultured artichoke tissue.   The small increase in activity which occurred during a 36 hour period in the 'dividing' system examined in Experiment 1, suggests that instead of being constant, the pattern of activity described above may take the form of a gradual rise over a prolonged period but this rise may be too small to be detected in experiments of 32 hours or less duration.

5.   The total DNA polymerase activity of the extract was measured for a 'dividing' system in Experiments 1 and 2, and for a 'non-dividing' system in Experiment 5.   By using a higher concentration of β-mercaptoethanol, the total DNA polymerase activity of the extract was made soluble and measured for a 'dividing' system in Experiment 4 using a supernatant fraction of the extract.

The slow rise in DNA polymerase activity which was observed during the first part of the cycle in Experiments 1 and 2 was absent in Experiment 4.

The major rise in DNA polymerase activity occurred 4 to 6 hours after the beginning of 'S' in Experiment 1, whilst in Experiments 2 and 4 the rise occurred at approximately the same time as the beginning of 'S'. The overall rise in activity was similar in pattern in Experiments 2 and 4 and in both experiments amounted to an enormous percentage increase over the original value.

Both the slow rise in DNA polymerase activity during the first part of the cycle (Experiments 1 and 2), and the major rise which occurs during 'S' (Experiments 1, 2 and 4) are almost certainly associated with cell division as neither were shown in the 'non-dividing' system which was examined in Experiment 5.

SECTION B

ENZYMES OF THYMIDINE METABOLISM

Periodicity in thymidine kinase (TdR kinase) activity
during the cell division cycle has been described in a large number
of synchronous systems (See Appendix 1) including one higher plant
system (Hotta and Stern, 1965).   In view of the successful
incorporation of ^{3}H-thymidine into DNA which was described in
Section A, it was anticipated that TdR kinase would be active in
artichoke tissue.

In bacteria, the enzyme TdR kinase has been assigned the
role of 'scavenger' (Okazaki and Kornberg 1964a and b), since the
main biosynthetic route of DNA precursors is via phosphorylated
derivatives.   As this argument might also apply to higher plants
an additional investigation was carried out to measure the activity
of thymidine monophosphate kinase (dTMP kinase), an enzyme which is
almost certainly on the direct pathway of DNA biosynthesis.   There
are no other reports from higher plant systems concerning the
activity of dTMP kinase during the cell cycle although data is
available from a number of other synchronous systems (see Appendix 1).

As a result of the dTMP kinase assay in artichoke tissue, it
was possible to obtain a quantitative measurement of suppressed
activity of thymidine monophosphate phosphatase (dTMP phosphatase),
the enzyme which degrades dTMP to TdR.   The activity of this
particular phosphatase has not previously been measured during the
cell division cycle in any synchronous system, although there are

several reports concerning phosphatase activities assayed using conventional methods (See Appendix 1).

The primary aim of experiments described in this section was to investigate the activities of TdR kinase, dTMP kinase and dTMP phosphatase during the cell division cycle in relation to the rate of DNA synthesis. When patterns of activity had been established, it was then possible to determine which of these patterns were associated with the cell division cycle and what the possible control mechanisms might be.

Experiment 7.

In this experiment, the activity of TdR kinase was measured in relation to cell number and levels of total nucleic acid and DNA, during the first division cycle. An estimation of the rate of DNA synthesis was not included as this was actually the first cell cycle experiment carried out, and at that time the superiority of the isotopic method over Burton's chemical method in defining the onset of the 'S' period was not realised.

In this experiment a series of 13 cultures was prepared. The conditions of preparation and growth of the cultures were as described in Experiment 1. The experiment covered a 26 hour period during which one culture was removed every 2 hours. At each sampling time, 5 explants were removed for cell counting, 3 samples of 15 explants were removed for estimation of total nucleic acid and DNA levels, and 100 explants were used to prepare the enzyme

extract.   TdR kinase assays were carried out in triplicate for
each extract.

Results

Cell number changes (Fig. 4.27)

Division commenced after about 22 hours and the cell number
continued to increase until the experiment ended at 26 hours, when
it was estimated that approximately 36% of the cells had divided.
This percentage cell division is rather low compared with the
results of Experiments 1, 3 and 4 and with the results of Davidson
(1971) for dark grown cultures.   As a plateau value had not been
reached by the end of the experiment it is probable that division
was not complete by 26 hours.

Changes in the level of DNA (Fig. 4.28)

A low level of DNA is maintained during the first part of
the cycle and the first detectable increase occurs at 14 hours.
The level continued to increase until about the beginning of
division when a plateau value is reached which amounts to an increase
over the basal value of about 90%.   From these data it is
apparent that 'S' has started by 14 hours.

Total nucleic acid (Fig. 4.29)

The total nucleic acid level decreases from 0 to 2 hours and
a minimum value is reached at 4 hours.   The first increase occurs
at 6 hours and the plateau value which is maintained until 12 hours
represents a 23% increase over the average 2 - 4 hour value.   A
further increase takes place from 14 hours onwards and the plateau
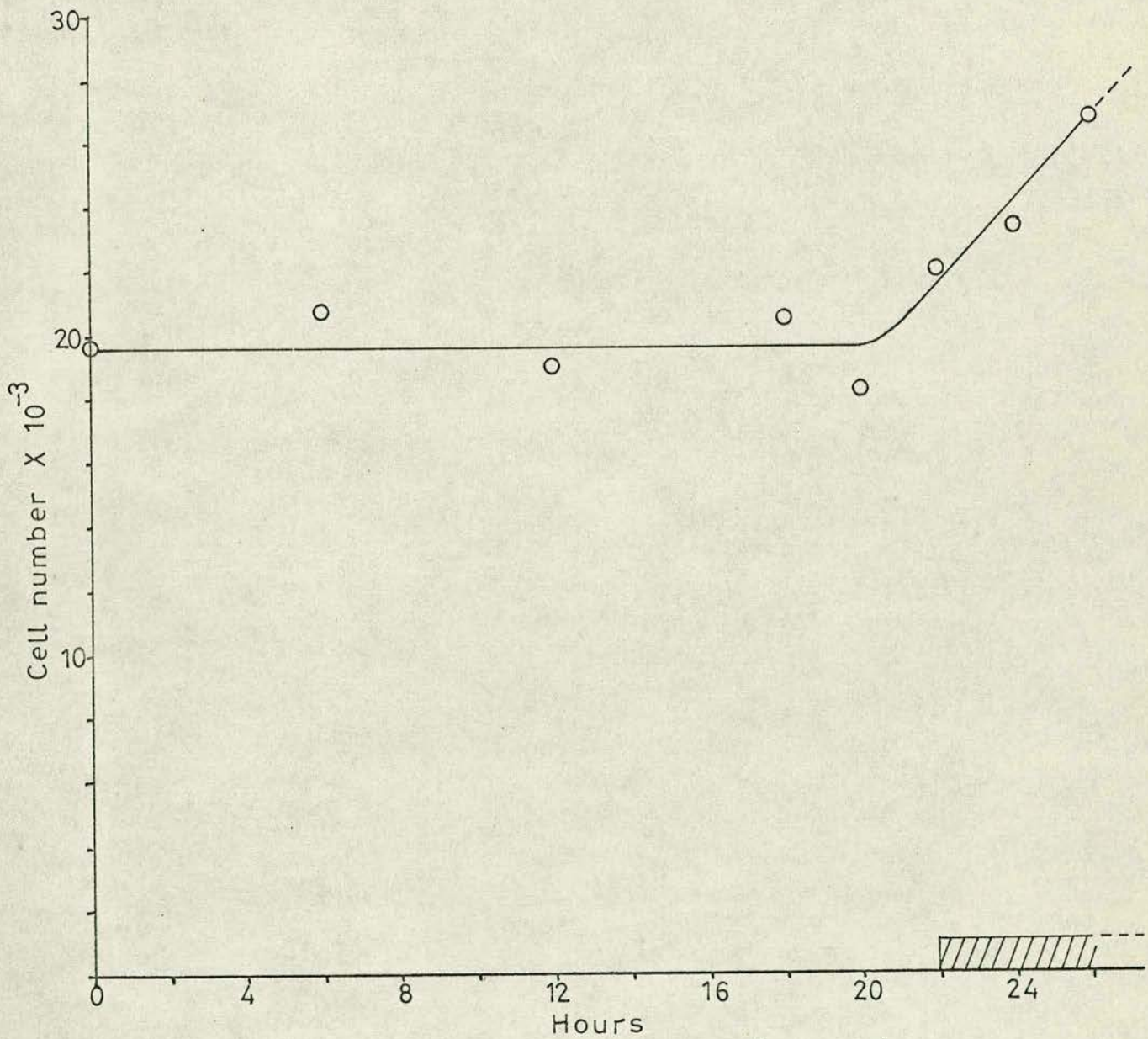
FIG. 4.27 Cell number changes in a 'dividing' system.
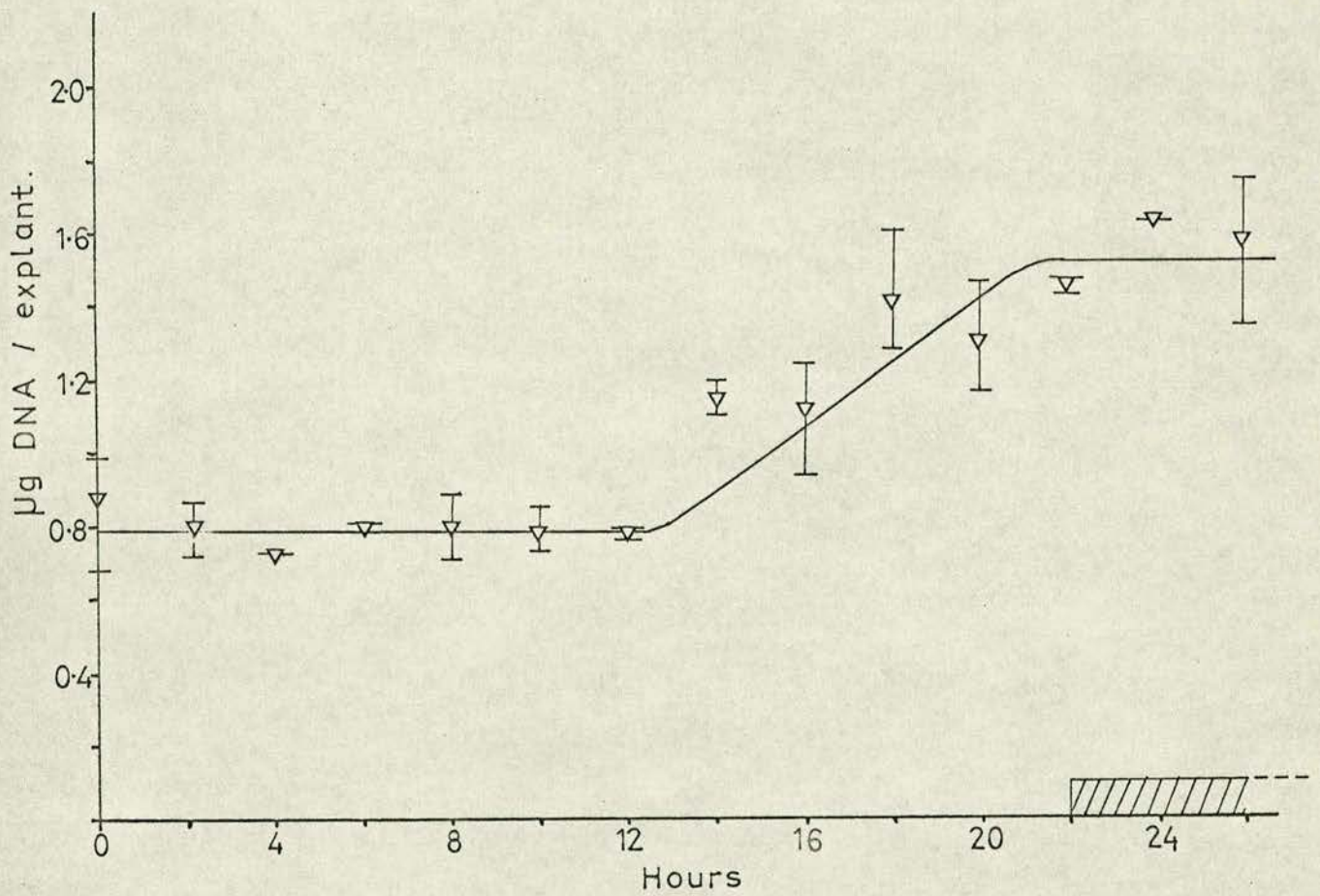
FIG. 4.28   Changes in the level of DNA in a 'dividing'
system.
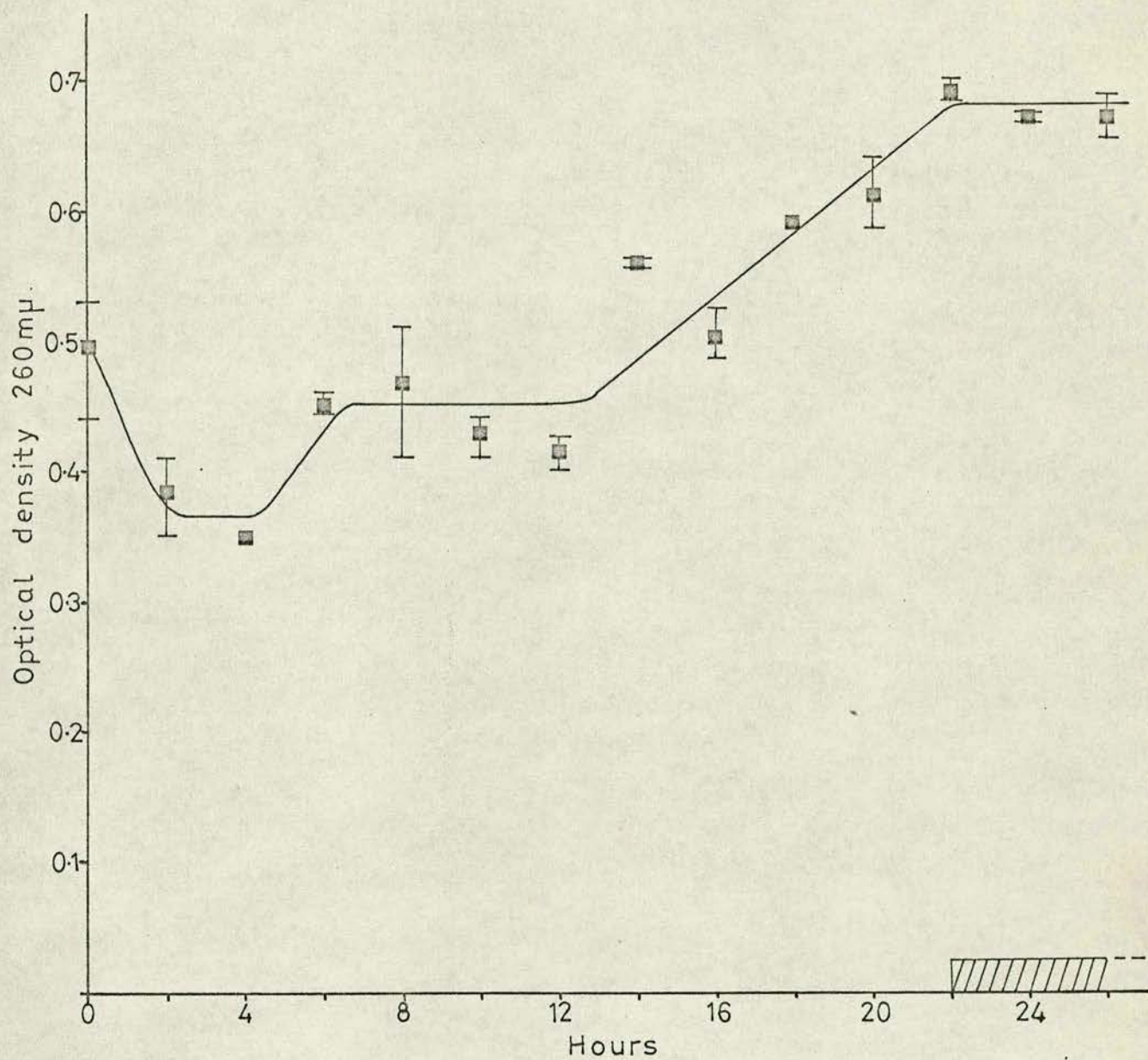
FIG. 4.29 Changes in the level of total nucleic acid in a 'dividing' system.

value which is reached at the beginning of division is maintained until the end of the experiment representing a total increase of 87% over the 2 - 4 hour value.

TdR kinase activity.

Data for TdR kinase activity during the first division cycle are presented in Fig. 4.30.   After an initial decrease in activity from 0 to 2 hours, a low value is maintained until 12 hours.   The activity begins to increase at 14 hours and continues to increase until a maximum value is reached at 20 hours which represents an increase of 871% over the average 2 - 12 hour value or 166% over the average 0 hour value.   A fall in activity occurs at 22 hours and a fairly constant value is maintained until the experiment ends at 26 hours.   The average plateau value from 22 to 26 hours represents a 647% increase over the average 2 - 12 hour value, and a 105% increase over the average 0 hour value.

The activities described above are expressed on a per explant basis.   If activities are expressed on a per cell basis the pattern deviates from the above only during the period of cell division (see broken line in Fig. 4.30), and the apparent fall in activity after 20 hours is emphasized.   This decrease in activity continues and when the experiment ends at 26 hours the activity expressed on a per cell basis is only slightly higher than the average 0 hour value.
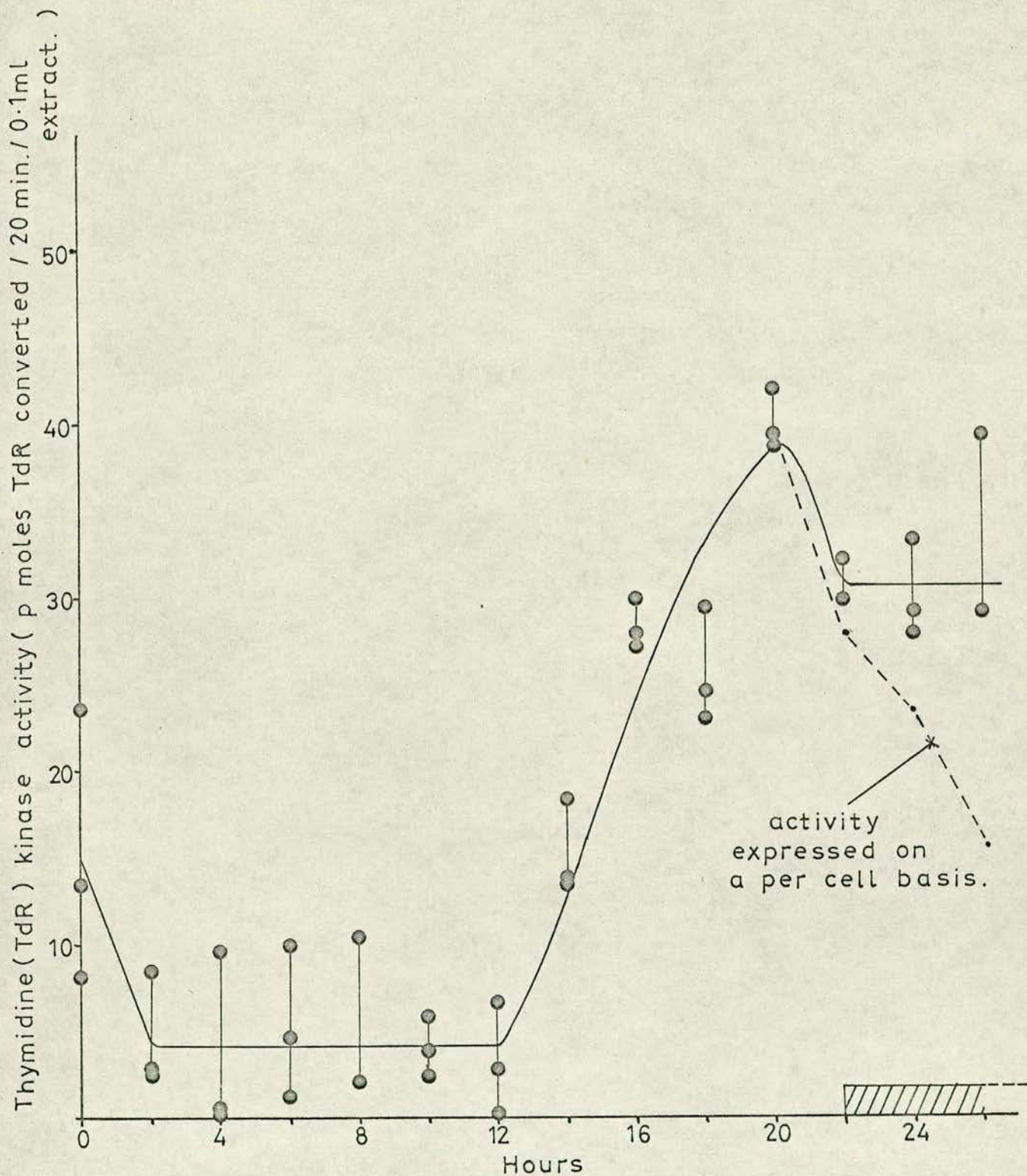
FIG. 4.30   TdR kinase activity during the first division cycle.

The cell number values before division in this experiment are higher than those recorded in all previous experiments. This may be a reflection of the fact that this experiment was carried out very early in the season, before the artichoke tubers had expanded to their maximum size, hence the cells were smaller and consequently each standard explant contained more cells than usual (see Appendix 5 for the dates of experiments).

In this experiment the patterns of increase in DNA and total nucleic acid levels are similar to those observed in previous experiments.

A pattern of TdR kinase activity during the cell division cycle has been established in this experiment and values have been expressed both on a per explant basis and on a per cell basis. Final conclusions regarding a pattern of TdR kinase activity during the cell division cycle cannot be made however, until these observations have been repeated by further experiment.

Experiment 8.

This experiment was designed to confirm the pattern of TdR kinase activity already described, in relation to cell number and levels of total nucleic acid and DNA. In addition the rate of DNA synthesis was measured by incorporation of ^{3}H-thymidine into DNA

The conditions used for preparation and growth of the cultures were as described in Experiment 1. In this experiment which covered a 28 hour period a single series of 12 cultures was prepared. At each sampling time, 5 explants were withdrawn for

cell counting, 2 samples of 15 explants were removed for estimation of total nucleic acid and DNA levels, 16 explants were used to measure the rate of DNA synthesis and 100 explants were used to prepare the enzyme extract.   At 30 hours, a culture was sampled only for the estimation of cell number and levels of total nucleic acid and DNA.

In this experiment the method used for measuring the rate of DNA synthesis differed slightly from that used in other experiments, because a higher concentration of ^{3}H-thymidine (5 μCi/ml. culture medium) was used and also the 'chase' in cold thymidine solution occupied a period of 30 minutes instead of 15 minutes.   Otherwise the method was as described in Ch. 2.C.4a. TdR kinase assays were carried out in triplicate for each extract.

Results.

Cell number changes (Fig. 4.31)

Division commenced at about 22 hours and was completed by 26 hours when it was estimated that approximately 61% of the cells had divided.

Changes in the level of DNA (Fig. 4.32)

A low DNA level is maintained during the first part of the cycle and the first marked increase occurs at 20 hours.   The level continues to increase until about 24 hours when a plateau value may be reached which amounts to an increase of about 66% over the basal value.   A further increase may occur at 30 hours.
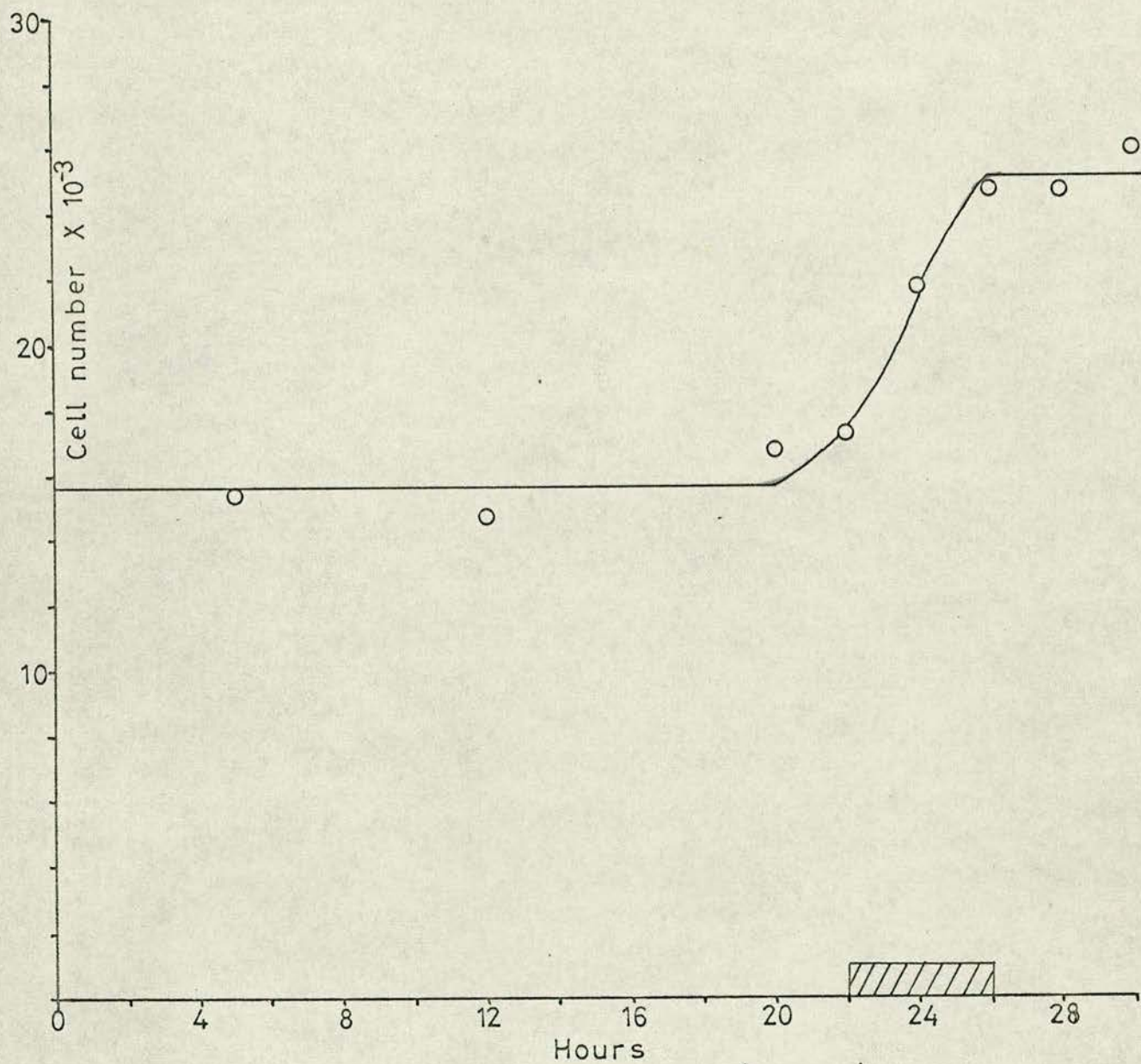
FIG. 4·31 Cell number changes in a dividing system.
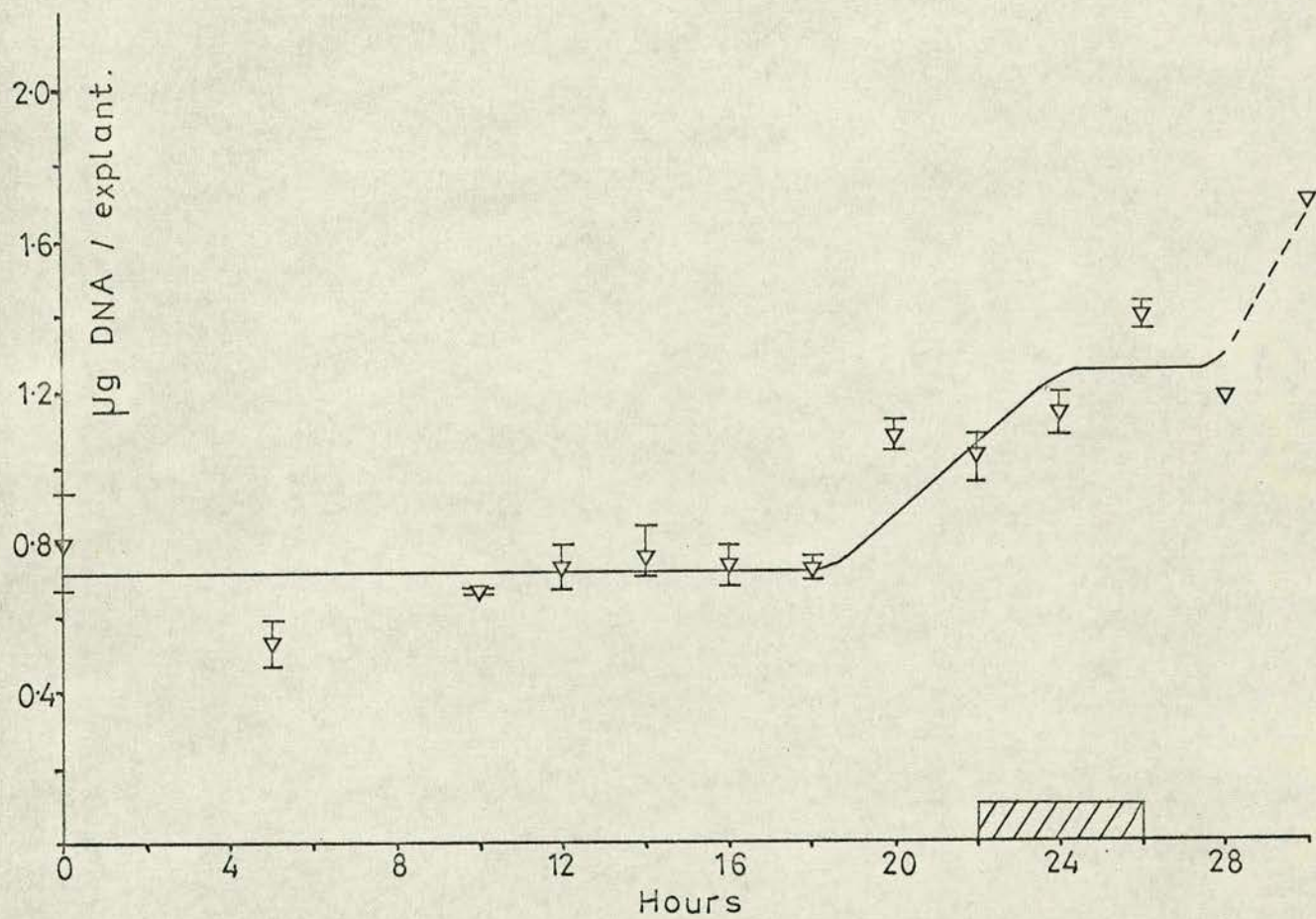
FIG. 4.32   Changes in the level of DNA in a 'dividing' system.

Total nucleic acid (Fig. 4.33)

Due to the absence of sufficient data during the period 0 - 10 hours, a pattern of total nucleic acid levels during this period can only be inferred. After an initial fall in level during the first few hours to reach a basal value, the first increase begins between 5 and 10 hours. The plateau value which is maintained between 12 and 18 hours may amount to an increase of 33% over the basal value. A further increase which begins at 20 hours and continues until the end of division may reach a plateau value at about 26 hours which amounts to a final increase of 110% over the basal value. A further increase may begin at 30 hours.

Rate of DNA synthesis

Data for the rate of DNA synthesis measured by incorporation of ^{3}H-thymidine into DNA are presented in Fig. 4.34. A low rate of DNA synthesis is maintained during the first part of the cycle. The first major increase which marks the beginning of 'S' is detected at 12 hours. The rate continues to rise steadily until 16 hours when a maximum value is reached. At 18 hours the rate declines to a lower value which is maintained until 20 hours. At 22 hours the rate falls to an even lower level marking the end of 'S'. An approximately constant rate of DNA synthesis is maintained between 22 and 28 hours.

TdR kinase activity

The activity of TdR kinase during the first division cycle is compared with the rate of DNA synthesis in Fig. 4.35.

During the first 12 hours of the cycle a relatively low level
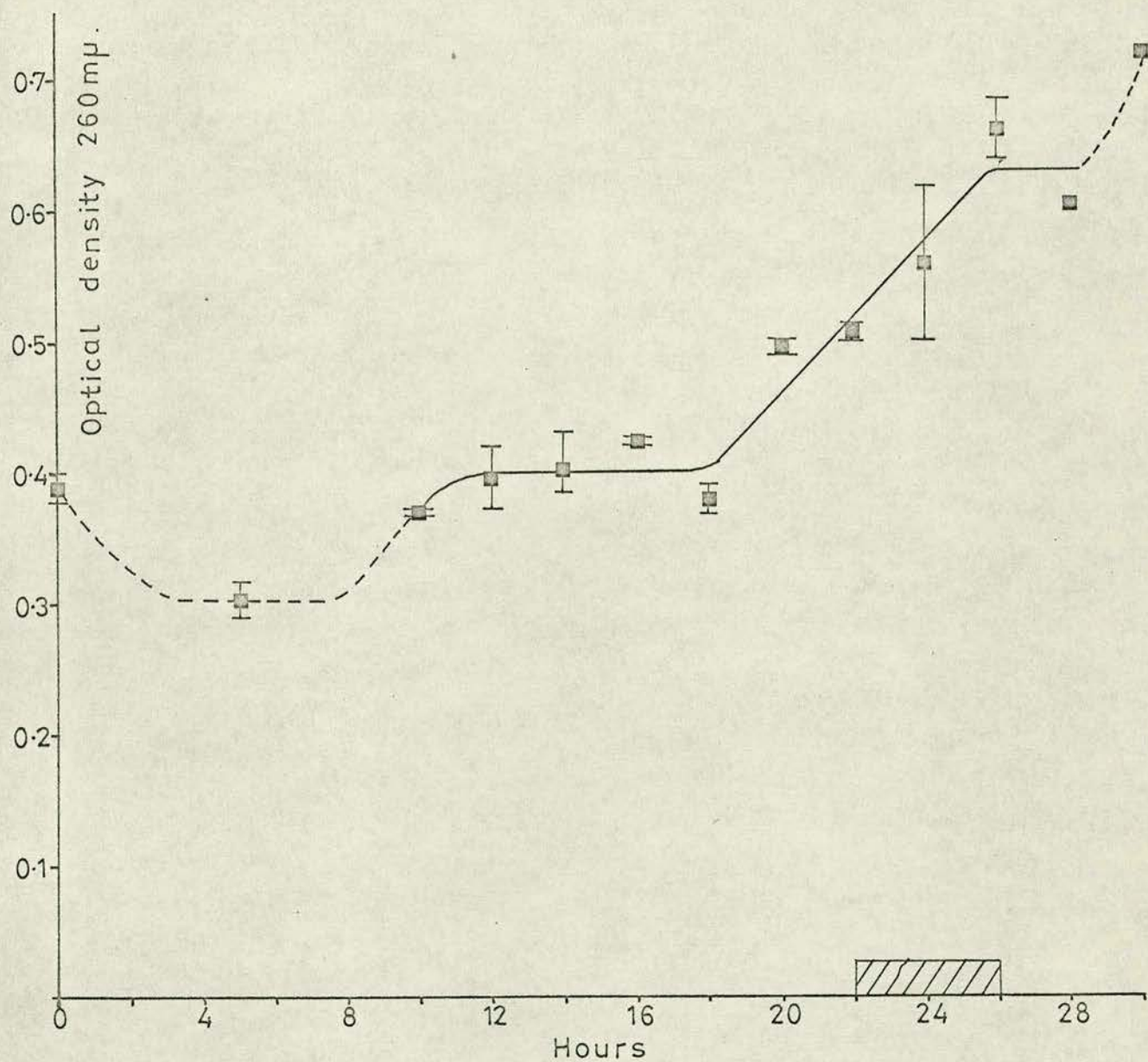
FIG. 4.33   Changes in the level of total nucleic acid in a 'dividing' system.
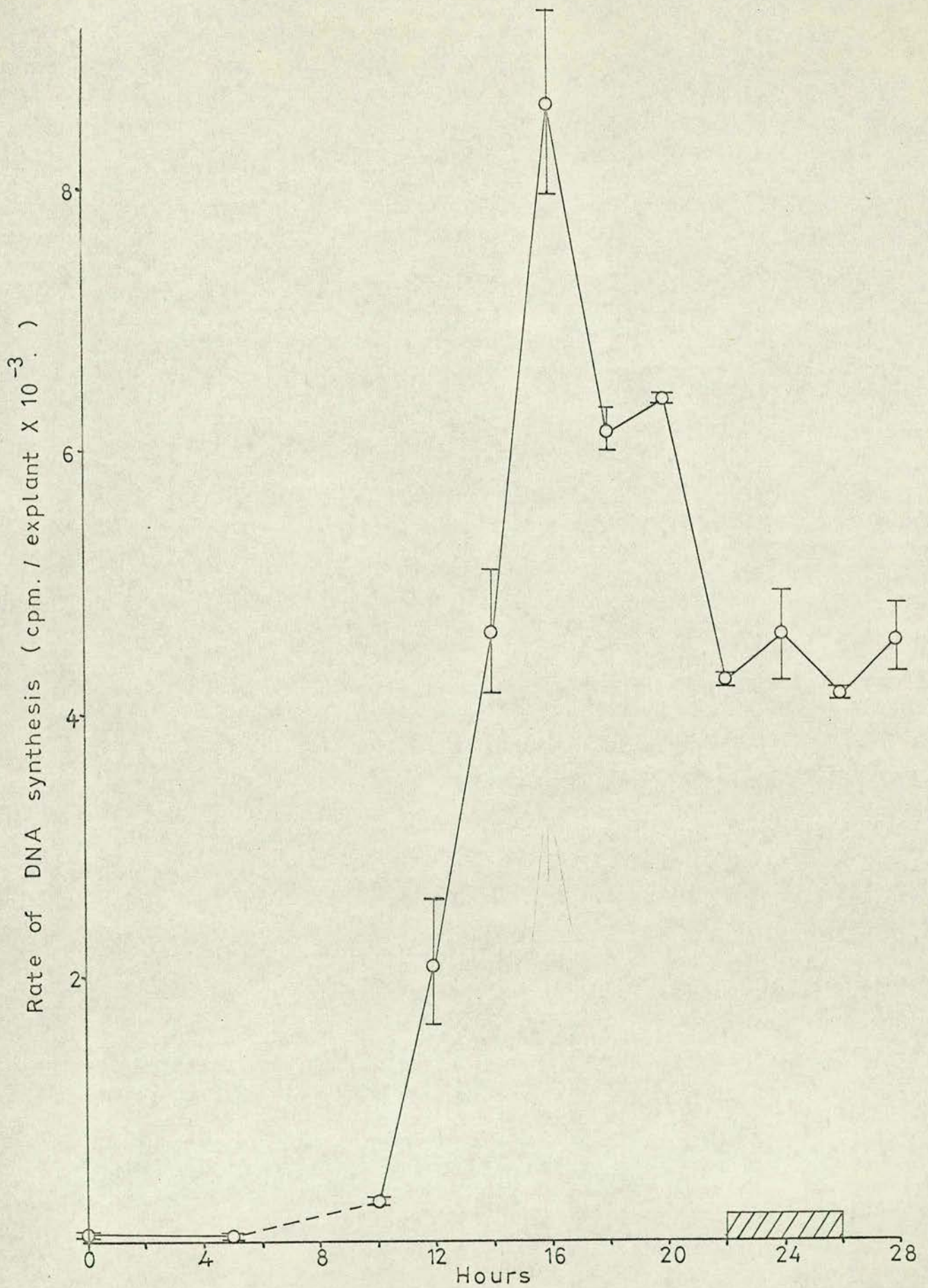
FIG. 4.34 Changes in the rate of DNA synthesis in a
'dividing' system.
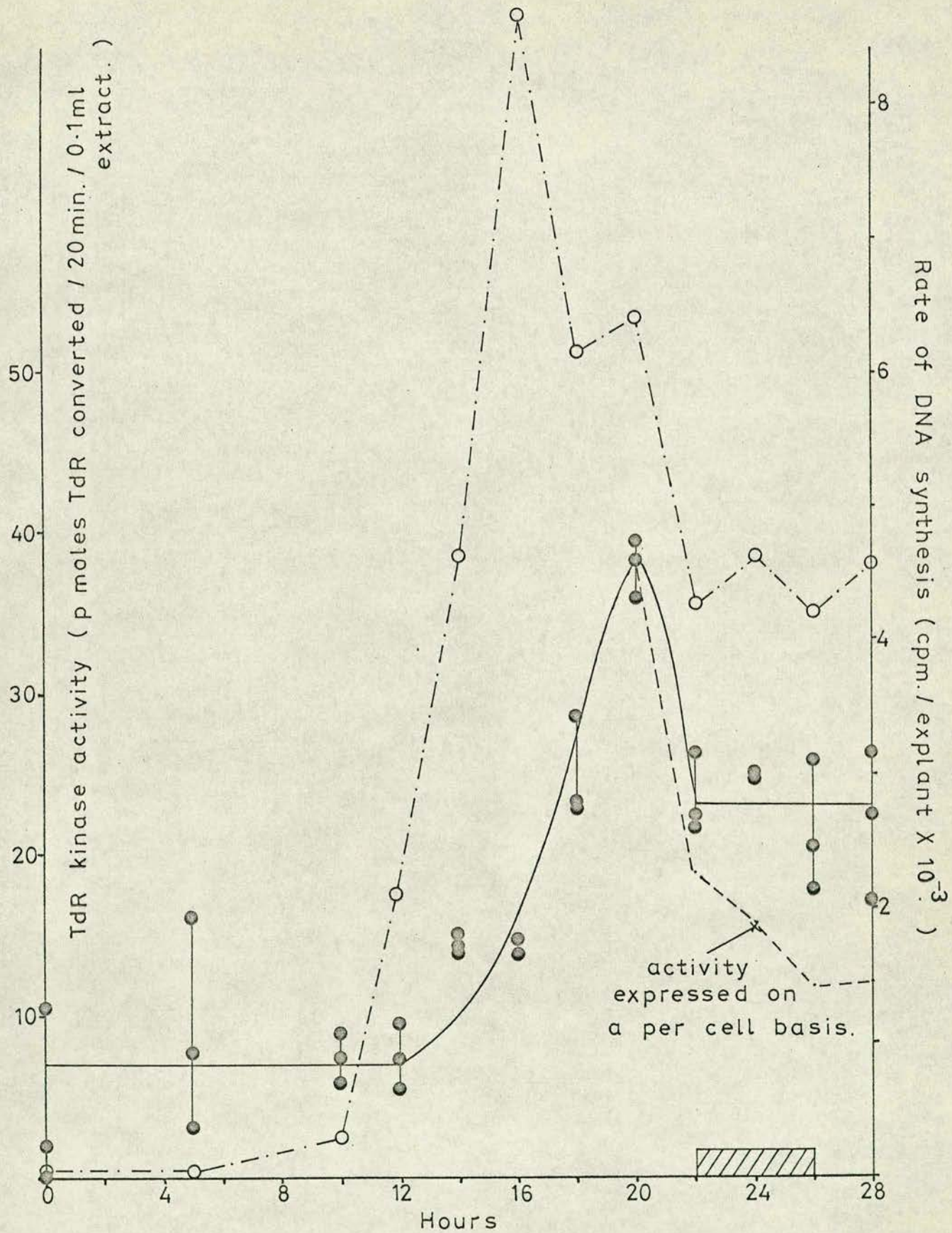
FIG. 4.35 TdR kinase activity (◉) in relation to the rate of DNA synthesis (○) during the cell cycle.

of activity is maintained which rises to a higher value at 14 hours, approximately 2 hours after the beginning of 'S'.   The activity continues to increase until a maximum value is reached at 20 hours which represents a 433% increase over the average 0 - 12 hour value. A marked fall in activity occurs at 22 hours and a fairly constant activity is maintained from 22 to 28 hours, which represents an increase of about 228% over the average 0 - 12 hour value.

When average activities are expressed on a per cell basis, (see broken line in Fig. 4.35) the peak in activity at 20 hours is emphasized and the value falls during division to reach a low value at the end of division which is only slightly higher than the original pre-'S' value.

In this experiment, the figure of 61% obtained for percentage cell division agrees fairly well with the figure of 66% obtained for the increase in DNA levels.   This is consistent with the results of Experiment 1.

The 61% cell division obtained in this experiment is similar to results of previous experiments and to the results of Davidson (1971) for a dark grown system.

The pattern of increase in DNA levels shown in this experiment is similar to that observed in Experiments 1, 2 and 7.   In Experiment 7, the first increase in DNA level was observed at 14 hours whereas in this experiment which was carried out less than 4 weeks later (see Appendix 5) the first increase in DNA level was observed at

20 hours.   This discrepancy may be partially explained by the fact

that

^ in Experiment 7 data for DNA levels were based on 3 estimations,

whereas in this experiment results were based on 2 estimations.

As observed in Experiments 1 and 2, results of this experiment

have confirmed that measurement of the rate of DNA synthesis gives a

much more accurate estimation of the onset of the 'S' period than

does the chemical method of measuring DNA.   In this experiment there

is a discrepancy of 8 hours between the two methods which is in line

with the discrepancies noted for Experiments 1 and 2 of the

previous section.

The pattern of increase in total nucleic acid levels is similar

to that observed in Experiments 1, 2 and 7 and the percentages

increases recorded in this experiment are similar to those described

in Experiments 2 and 7.

The pattern of TdR kinase activity shown in this experiment

largely confirms the results of Experiment 7.   The initial decrease

in activity observed between 0 and 2 hours in Experiment 7 was not

apparent in this experiment.   The peak in activity observed at

20 hours in Experiment 7 was even more marked in this experiment.

As observed in the previous experiment, when TdR kinase

activities are expressed on a per cell basis, the activity at the

end of division in this experiment is similar to that observed

before 'S'.

Before final conclusions are made regarding a pattern of TdR

kinase activity during the cell cycle, the activity of this enzyme

will be measured in one more experiment.

Experiment 9

This experiment was designed to confirm the patterns of TdR kinase activity already described and in addition, to investigate the activity during the division cycle of an enzyme which is on the main pathway of DNA biosynthesis, namely dTMP kinase. Other parameters which were measured in this experiment included cell number, and the rate of DNA synthesis. Anticipating that a more accurate estimation of DNA levels might be given by the Feulgen method, this method was used during the 10 - 24 hour period only. As a result of the dTMP kinase assay it was also possible to estimate the suppressed activity of dTMP phosphatase during the cell division cycle.

In view of the observations in several micro-organisms, that sugars containing TdR derivatives may function as intermediates in cell wall synthesis (this topic has been reviewed by Cleaver 1967), the possible association of TdR kinase with cell wall formation in the artichoke system was investigated.

In addition to estimating the rate of DNA synthesis by incorporation of ^{3}H-TdR into DNA, an extra procedure was included to see if any ^{3}H-TdR remained in the residue, which included the cell wall fraction, after the nucleic acids had been removed by perchloric acid (PCA) extraction.

This experiment also included the assays for DNA polymerase activity described in Experiment 4.

The conditions used for the preparation and growth of the cultures were as described in Experiment 1.

This experiment covered a 28 hour period and involved preparation of 2 series of cultures. The first 8 cultures were prepared 14 hours before sampling began, and the second 8 cultures were prepared immediately prior to sampling. In this way the culture periods from 14 to 28 hours and from 0 to 14 hours were sampled in parallel at intervals of 2 hours. As in previous experiments an overlap point was included which gave two 14 hour samples, one from the first and one from the second series of cultures.

At each sampling time 0·5 ml. of culture medium was removed aseptically for estimation of microbial contamination (the results of this estimation are tabulated in Appendix 2c). In addition, 5 explants were removed for cell counting and a further 5 taken for estimation of DNA levels by the Feulgen method. 16 explants were removed for estimation of the rate of DNA synthesis and 100 explants were used to prepare the enzyme extract.

In this experiment the extract was prepared as described in Ch. 2.D.5 for dTMP kinase except that for reasons already given in Experiment 4 in connection with the DNA polymerase assay, the grinding medium contained β-mercaptoethanol (EtSH) at a concentration of 1%.

In this experiment, assays of TdR kinase activity, and dTMP kinase activity were carried out in duplicate for each extract. For convenience the TdR kinase assay was modified to resemble the dTMP kinase assay as described in Ch.2.D.4.

Results

Cell number changes (Fig. 4.21 - opposite Experiment 4)

The first division commenced at about 22 hours and was completed by 26 hours when it was estimated that 63% of the cells had divided.

Feulgen DNA levels (Table 4a - opposite Experiment 4)

Data are available only for the period 10 to 24 hours, and the level of DNA increases throughout this period from a basal value of 100% at 10 hours to 138% by 24 hours.

Rate of DNA synthesis

The rate of DNA synthesis during this cell cycle experiment is shown in Fig. 4.22 (opposite Experiment 4) and in Fig. 4.36 (opposite this experiment) and the pattern has been described in detail in Experiment 4. At this point it is sufficient to mention only the more important details. The 'S' period begins at about 12 hours and there is considerable variation between the rates of DNA synthesis recorded in the two 14 hour samples. The rate of DNA synthesis in this experiment shows no fixed definition of the end of 'S' and the value continues to fluctuate during division. In Fig. 4.35 the rate of DNA synthesis is shown in relation to the ^{3}H-thymidine counts left in the residue after removal of the nucleic acids by PCA hydrolysis. The counts left in the residue have been plotted on a relatively large scale (x 25) compared with the rate of DNA synthesis, in order to show the two trends on the same graph.

The general pattern shown by both parameters is similar. The efficiency of extracting all the counts using 3 consecutive
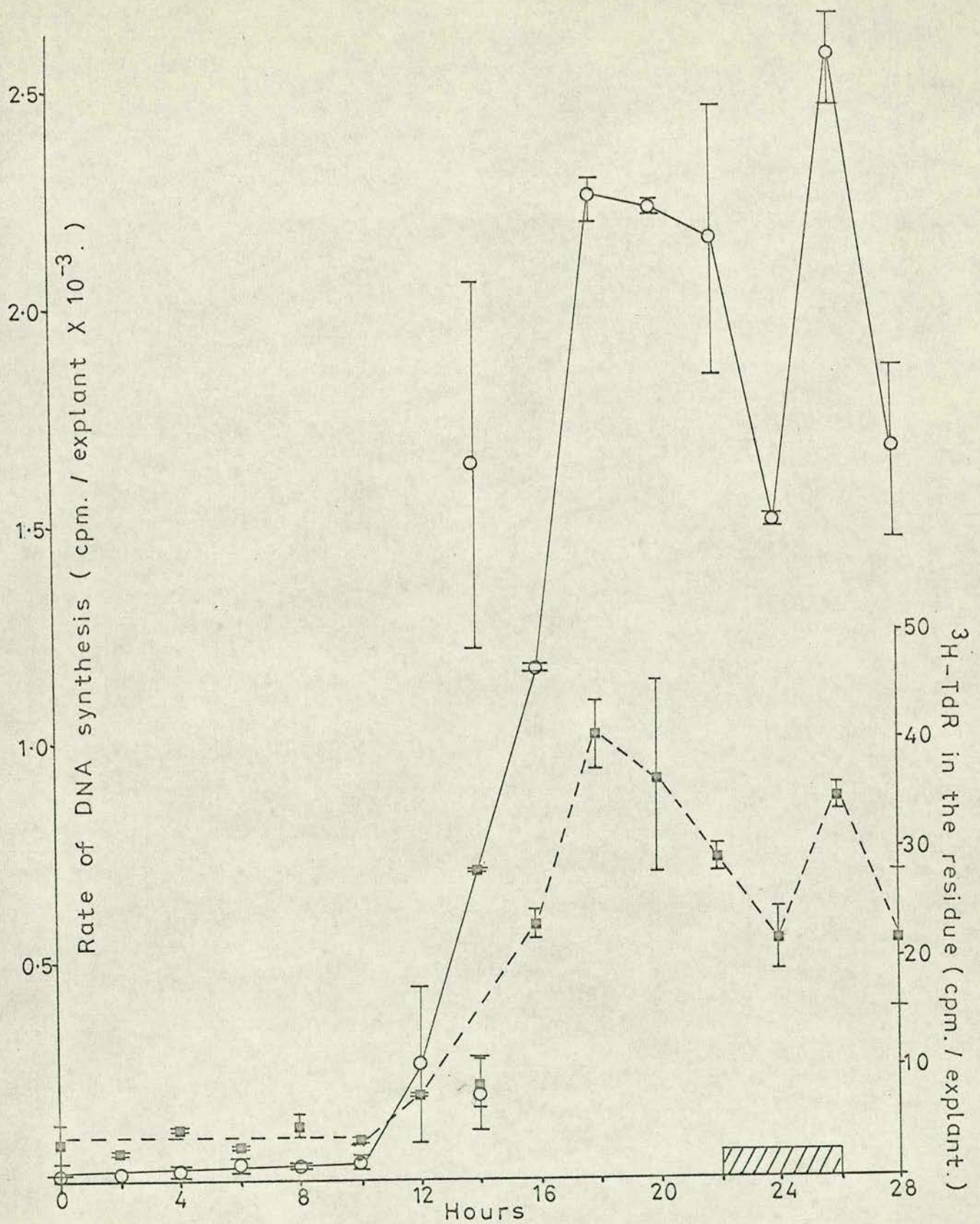
FIG. 4·36   Changes in the rate of DNA synthesis (O) in relation to ^{3}H-TdR in the residue (■) during the cell cycle.

extractions with 0·5N PCA and the percentage of counts left in
the residue, were calculated from the combined data and are
shown in Table 4c.   The efficiency of the PCA extraction increases
during 'S' when more ^{3}H-thymidine counts are incorporated into DNA.
During the period 12 - 28 hours in which high rates of DNA synthesis
are recorded the efficiency of the PCA extraction exceeds 90%
increasing to over 98% after 18 hours.

TdR kinase activity

TdR kinase activity during the cell division cycle is
compared with the rate of DNA synthesis in Fig. 4.37.   A low TdR
kinase activity is maintained during the first part of the cycle
and begins to increase after about 12 hours, at approximately the
same time as the beginning of 'S'.   A steady increase in activity
occurs between 12 and 22 hours, and from 22 to 28 hours the activity
appears to remain constant.   The average 22 - 28 hour value shows
an increase of 150% over the average 0 - 10 hour value.

When average TdR kinase activities are expressed on a per cell
basis (see broken line in Fig. 4.37) a decrease in activity occurs
from 22 hours onwards, and when the experiment ends at 28 hours the
value has declined to a level which is only slightly higher than the
original pre-'S' level.

A correlation coefficient of 0·923 between TdR kinase activity
and Feulgen DNA levels has been calculated for the period 10 - 24
(Statistical Data are shown in Appendix 6).   For 5 degrees of freedom
this means that the correlation between the two variables is highly

Period of culture (hours)	Residual counts (% of total)	Efficiency of DNA extraction (%)
0	13·7	86·3
	49·5	50·5
2	18·1	81·9
4	20·9	79·1
	16·9	83·1
6	8·6	91·4
	7·4	92·6
8	16·7	83·3
	12·4	87·6
10	4·9	95·1
	13·6	86·4
12	9·5	90·5
	1·7	98·3
14_1	2·3	97·7
	1·3	98·7
14_2	3·8	96·2
	5·7	94·3
16	1·9	98·1
	2·1	97·9
18	2·0	98·0
	1·6	98·4
20	1·3	98·7
	2·0	98·0
22	1·2	98·8
	1·5	98·5
24	1·3	98·7
	1·6	98·4
26	1·4	98·6
	1·3	98·7
28	1·5	98·5
	1·0	99·0

Table 4c.   The efficiency of DNA extraction by the standard

PCA procedure and the percentage of residual counts.
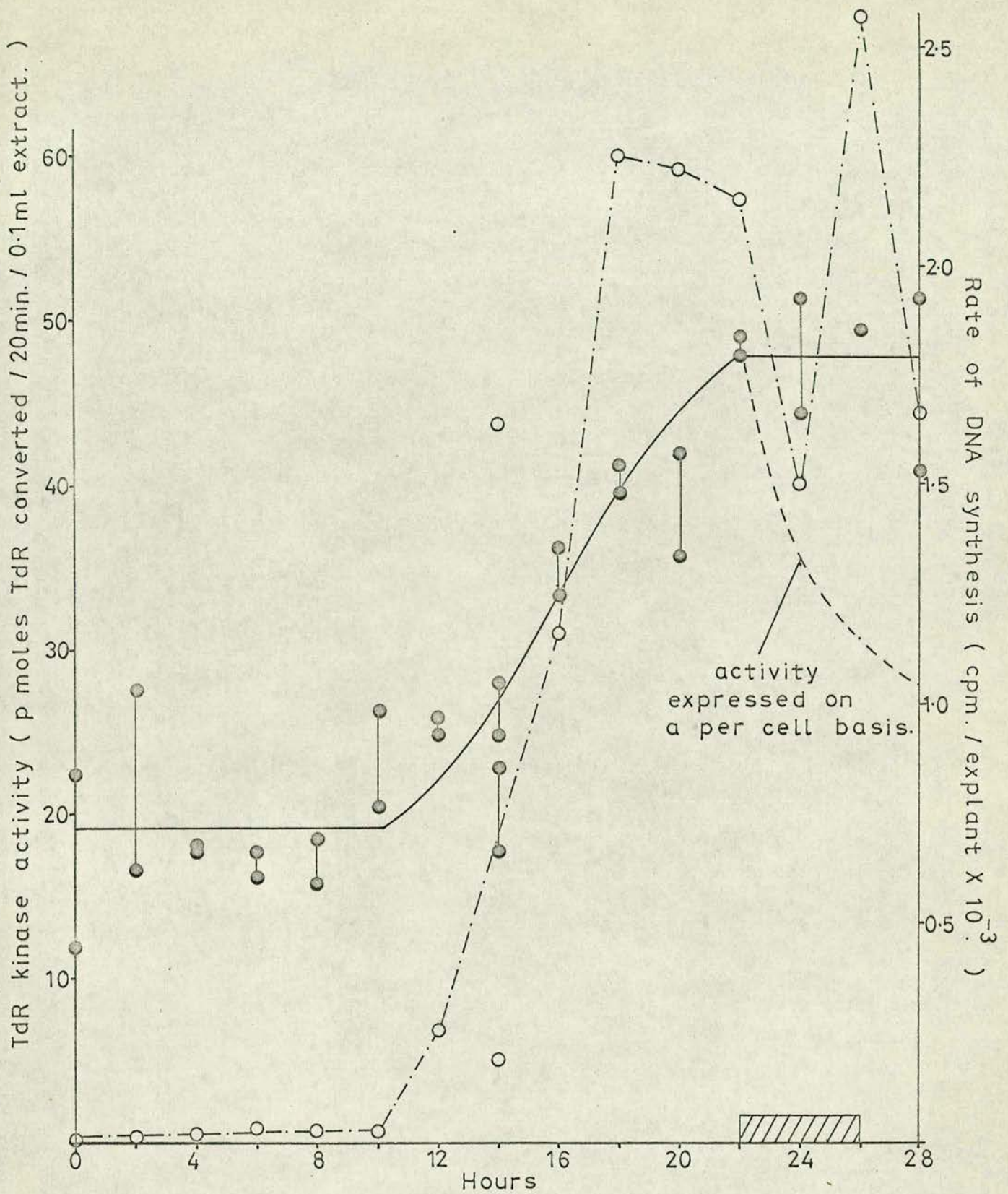
FIG. 4.37. TdR kinase activity (●) in relation to the rate of DNA synthesis (O) during the cell cycle.

significant and there is a probability of less than 1% that this correlation is due to error.

dTMP kinase activity

The dTMP kinase activity during the division cycle is compared with the rate of DNA synthesis in (Fig. 4.38). During the first few hours of the cycle a high dTMP kinase activity is maintained which appears to decrease to reach a basal value from 6 hours to 14 hours and represents a decrease of 45% with respect to the 0 hour value. The first increase in activity occurs at 16 hours, approximately 4 hours after the beginning of 'S'. The activity continues to increase from 16 hours to 26 hours when a plateau value may be reached which amounts to an increase of 219% over the average 6 - 14 hour value. When the dTMP kinase activity is expressed on a per cell basis the pattern becomes modified during the division period (see broken line in Fig. 4.38). The activity expressed on a per cell basis reaches a maximum value at 22 hours followed by a steady decline in activity during division. At 28 hours the dTMP kinase activity expressed on a per cell basis is only slightly higher than the basal activity recorded before the beginning of 'S'.

A correlation coefficient of 0·933 (see Appendix 6) between average dTMP kinase activities and Feulgen DNA levels has been calculated for the period 10 - 24 hours. With 5 degrees of freedom, this means that the correlation between the two variables is highly significant and there is a probability of less than 1% that this correlation is due to error.
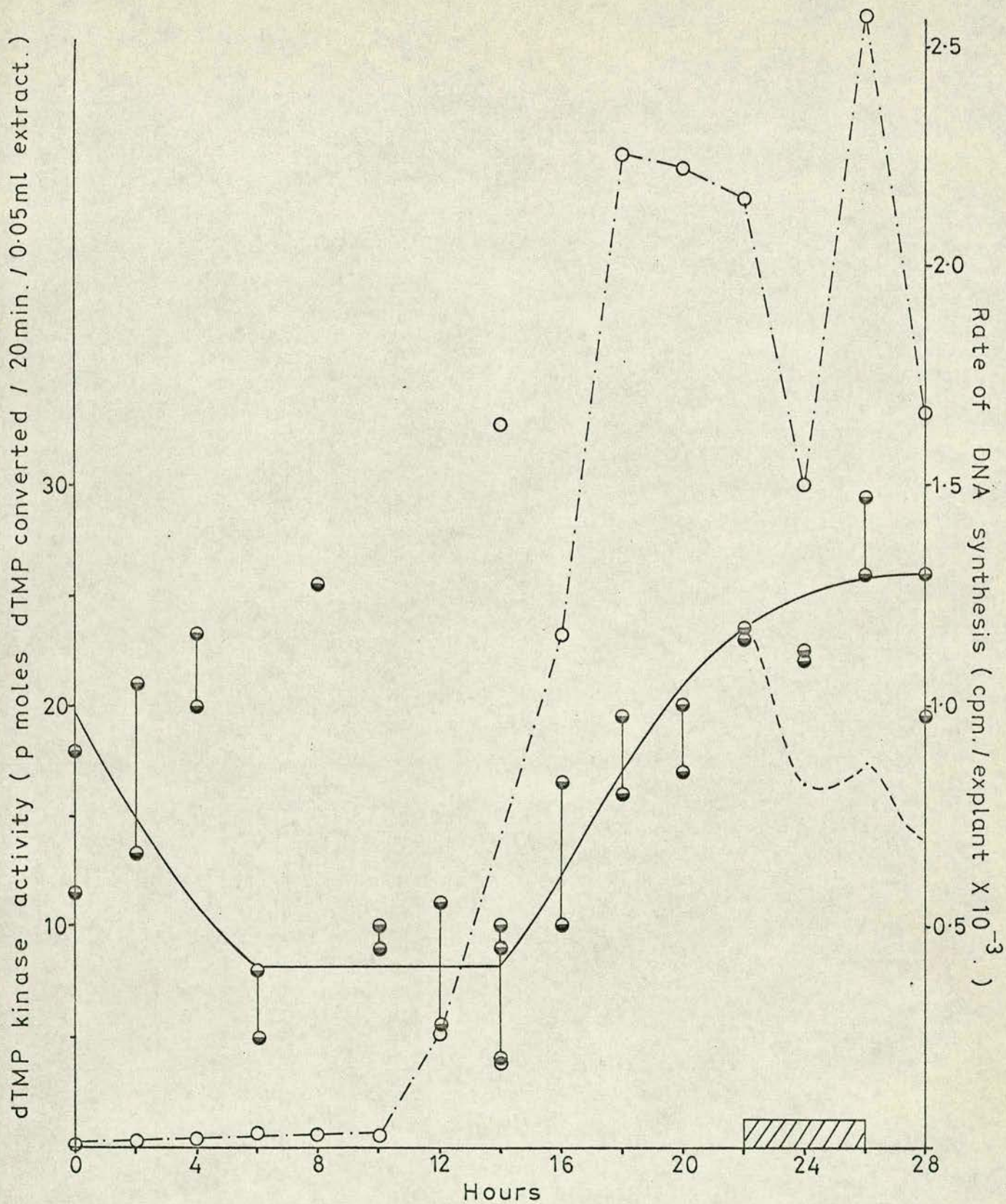
FIG. 4.38. dTMP kinase activity (◓) in relation to the rate
of DNA synthesis (○) during the cell cycle.

(− − − − activity expressed on a per cell basis.)

<u>dTMP phosphatase activity</u> (Fig. 4.39)

Due to considerable variation between samples the data for this enzyme are difficult to interpret and for some samples extra points have been added to the graph representing repeated electrophoresis runs.   In the absence of any marked increases during the experiment and ignoring the 2 and 4 hour values, it is possible that a gradual increase in activity may take place over the 28 hour period.

The 63% cell division obtained in this experiment is consistent with the results of previous experiments and with the results of Davidson (1971).

The Feulgen DNA data which are available for the period 10 - 24 hours of this experiment have been used to calculate correlation coefficients between the average activities of DNA polymerase (Experiment 4), TdR kinase and dTMP kinase, and the level of DNA.   There may be some significance in the fact that the correlation coefficient of 0·988 shown for DNA polymerase is higher than the coefficients of 0·923 and 0·933 calculated for TdR kinase and dTMP kinase respectively.

The pattern shown by the rate of DNA synthesis in this experiment is similar to that obtained in Experiment 3, in that it shows no fixed definition of the end of 'S', and the value also continues to fluctuate during division.   As mentioned in Experiment 3, this type of pattern may be characteristic of experiments carried
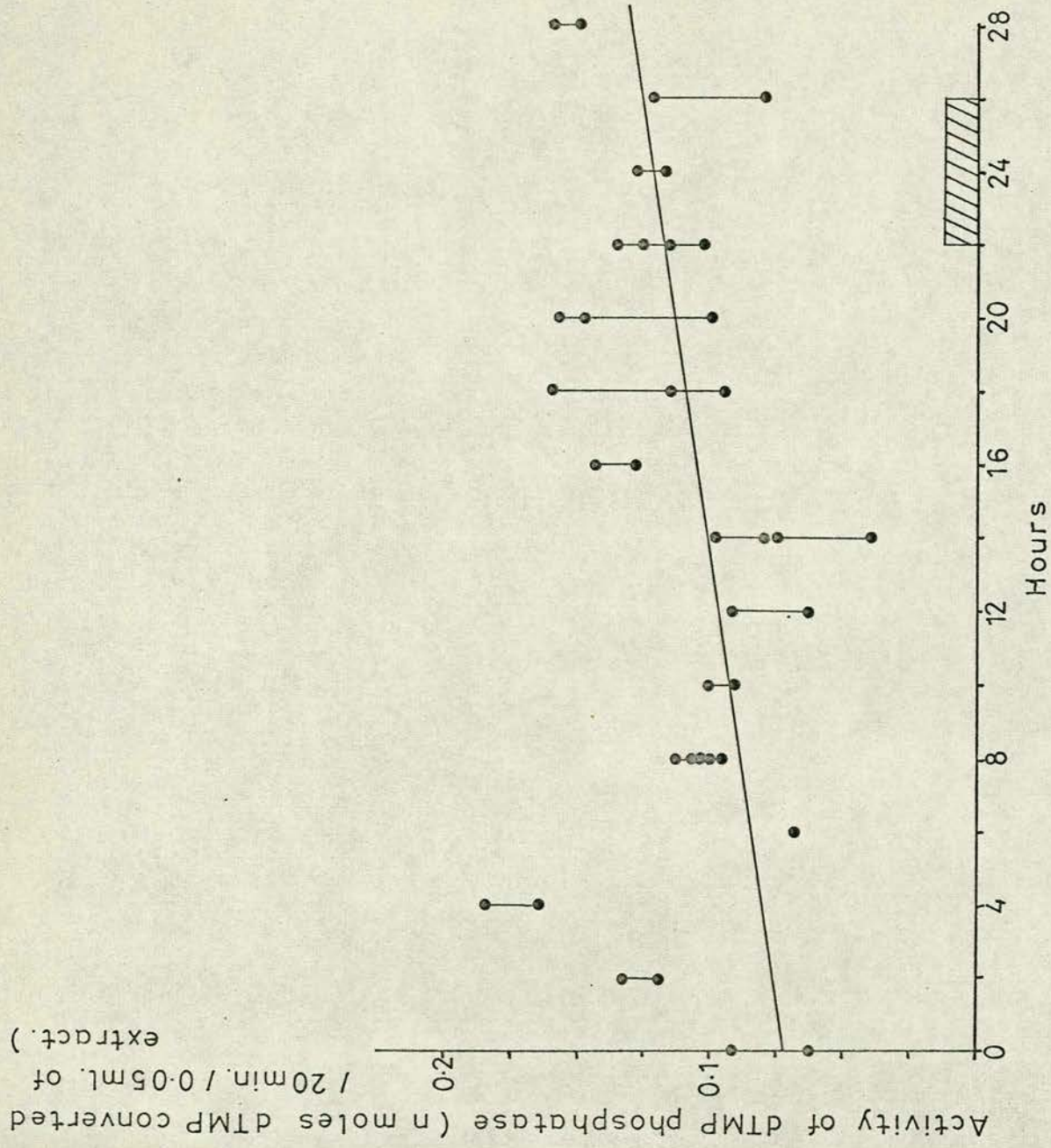
FIG. 4.39 Activity of dTMP phosphatase in a 'dividing' system.

out early in the season (see Appendix 5 for dates of all experiments).
As mentioned in Experiment 4, there may also be a slight shoulder on
the rising part of the curve similar to those observed in almost
all previous experiments.

In this experiment the extraction of ^{3}H-labelled DNA by PCA
hydrolysis between 12 and 28 hours is over 90% efficient, and the
few counts which remain in the residue show a similar pattern to
the rate of DNA synthesis curve.   In order to determine whether these
few remaining counts are contained in DNA residues which are removable
by further PCA hydrolysis, or whether they are present in some other
residue which is not DNA, it is important that more extensive
hydrolysis is carried out in the next experiment.

The pattern of TdR kinase activity during the cell cycle in
this experiment is fairly similar to those already described.   As
in Experiment 8, the first major rise in activity occurs at the
same time as the beginning of 'S'.   In this experiment, however, a
plateau level of activity is maintained from the beginning of
division onwards and no maximum activity is observed prior to
division as in Experiments 7 and 8.

At this point it is relevant to emphasize that in this
experiment a modified assay method was used to assay TdR kinase and
also that a higher concentration of EtSH was present in the
grinding medium compared to the concentration used in Experiments 7
and 8.   A preliminary experiment which is described in Ch. 3 has
indicated that increasing the EtSH concentration from 0·5% to 1% in

the grinding medium, may effectively increase the activity of TdR kinase whilst having a negligible effect on the activity of dTMP kinase.   Although a higher EtSH concentration was used in this experiment, the percentage increase in the level of TdR kinase activity after division compared with the 0 hour value, was inter-mediate to the percentage increases recorded in Experiments 7 and 8.

When TdR kinase activities were expressed on a per cell basis the data obtained in this experiment are similar to those of Experiments 7 and 8 in that the activity declined during division to reach a value at the end of the experiment which was only slightly higher than the pre-'S' value.

A pattern of dTMP kinase activity during the cell division cycle has been established in this experiment.   A fall in the level of dTMP kinase activity may occur during the first few hours of the cycle to reach a stationary basal value.   The first marked increase in activity begins several hours after the beginning of 'S' and continues until the end of division when a plateau value may be reached.

The pattern of dTMP phosphatase activity established in this experiment may take the form of a gradual increase in activity over the 28 hour period.

Final conclusions regarding the patterns of dTMP kinase activity and dTMP phosphatase activity during the cell division cycle cannot be made until further experiments have been carried out.

Experiment 10

This experiment was designed to confirm and extend the results of the previous experiment regarding patterns of dTMP kinase and dTMP phosphatase activity during the cell division cycle. Particular attention has been paid to the pre-'S' period, because of the sharp fall in dTMP kinase activity observed in the previous experiment.

From the previous experiment there was some suggestion that the small percentage of residual ^{3}H-thymidine counts from the rate of DNA synthesis procedure was due to incomplete PCA extraction. Therefore an attempt was made in this experiment to remove the residual counts by more stringent PCA hydrolysis (see Ch. 2.C.4.)

This experiment covered a 28 hour period and involved preparation of 3 series of cultures. The first 5 cultures were prepared 20 hours before sampling began and the second 5 cultures were prepared 10 hours prior to sampling. In this way the culture periods from 20 to 28 hours and from 10 to 18 hours were sampled in parallel at intervals of 2 hours. The third series of cultures which was prepared immediately prior to sampling consisted of only 3 flasks for sampling at 0 hours, 4 hours and 8 hours. No overlap point was included in this experiment because of the technical difficulty of sampling 3 cultures at the same time.

At each sampling time, 5 explants were removed for cell counting, 16 explants were removed for estimation of the rate of DNA synthesis and counts in the residue, and 100 explants were used to prepare the enzyme extract.

In contrast to Experiment 9, the extract was prepared in the

presence of 0.5% EtSH.   Assays for dTMP kinase were carried out in duplicate for each enzyme extract.

This experiment also included the assays of 'denatured'-DNAase activity described in Experiment 3 of the previous section.

Results

Cell number changes (Fig. 4.18 - opposite Experiment 3)

The first division commenced at about 24 hours and was completed between 26 and 28 hours when it was estimated that approximately 65% of the cells had divided.

Rate of DNA synthesis

The rate of DNA synthesis during this cell cycle experiment is shown in Fig. 4.19 (opposite Experiment 3) and in Fig. 4.40 (opposite this experiment).   The pattern has already been described in Experiment 3 and at this point it is sufficient to mention only the more important details.   The 'S' period begins at about 18 hours and there may be a shoulder on the rising part of the curve from 20 to 22 hours.   In this experiment, the rate of DNA synthesis shows no fixed definition of the end of 'S' and a high rate is maintained during the period when the cell number is increasing.

In Fig. 4.40 the rate of DNA synthesis is shown in relation to the ^{3}H-thymidine counts left in the residue after the removal of nucleic acids by the standard PCA hydrolysis procedure, and after a more stringent PCA hydrolysis procedure.   The counts left in the residue have been plotted on a relatively large scale (x 40) compared
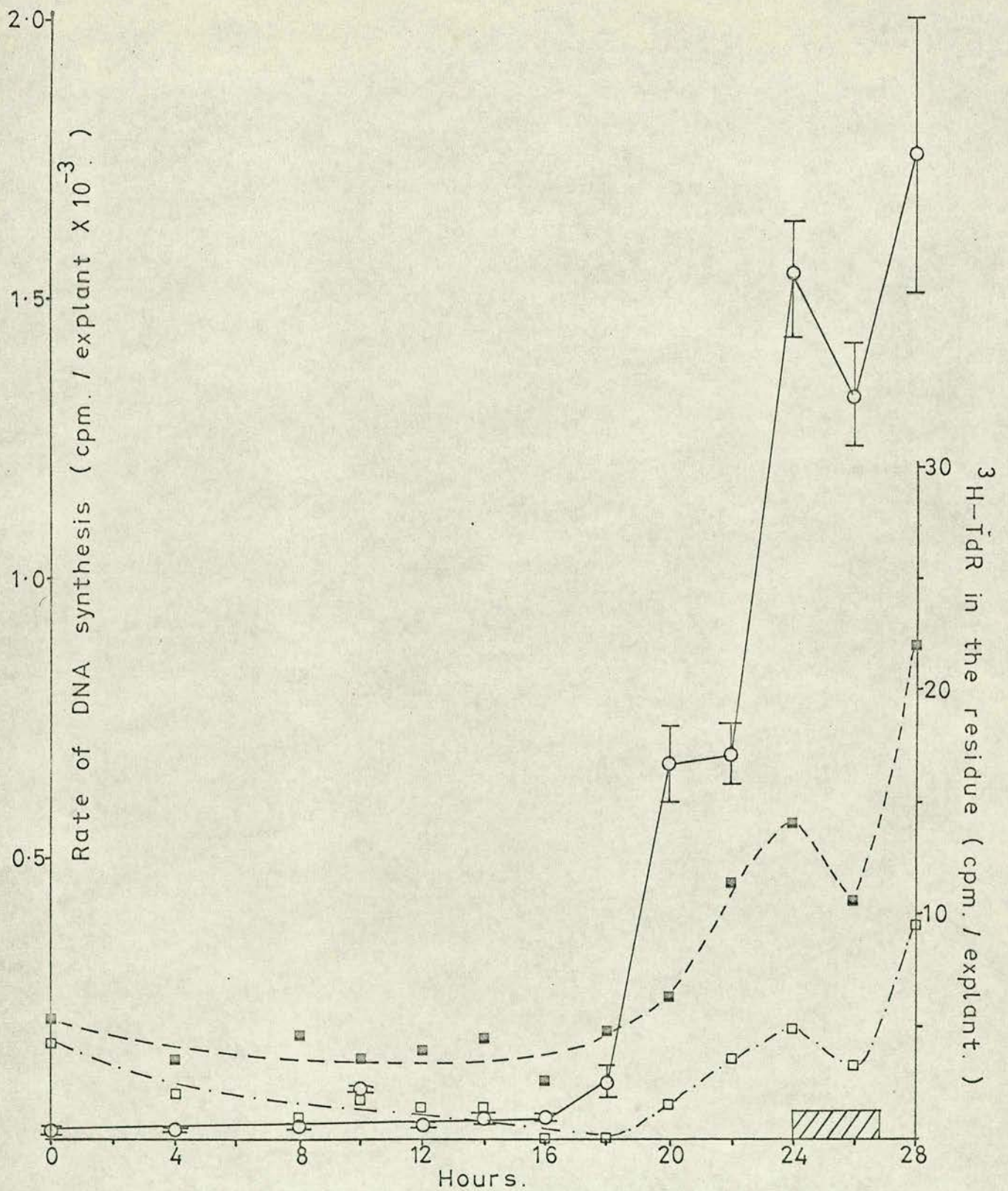
FIG. 4.40 Changes in the rate of DNA synthesis (O) during
the cell cycle, in relation to ^{3}H—TdR in the
residue a after the standard PCA extraction (■)
and b " " second " " (□)

with the rate of DNA synthesis in order to show all 3 trends on the same graph. The three different measurements in Fig. 4.40 show a similar pattern during the cell cycle.

The percentage of the total counts remaining in the residue and hence the efficiency of extraction of DNA has been calculated for all values obtained both before and after the second PCA hydrolysis and these data are shown in Table 4d. The efficiency of extraction of ^{3}H-labelled DNA using the standard PCA hydrolysis procedure is greater than 95% during the 'S' period and can be increased to over 99% using the second PCA treatment. At excision, and in the very early pre-'S' period, exhaustive extraction failed to remove all of the counts.

dTMP kinase activity

The dTMP kinase activity during the cell division cycle is compared with the rate of DNA synthesis in Fig. 4.41. An initial fall in dTMP kinase activity occurs during the first few hours of the cycle and by 8 hours a basal value is reached which is maintained until 18 hours. The initial fall in activity constitutes a decrease of about 55% from the average 0 hour value.

The first marked increase in activity is observed at 18 hours, approximately 2 hours after the beginning of 'S' and the activity continues to increase to reach a maximum value at the beginning of division which constitutes a 213% increase over the basal value. The activity may decrease during division to a level which represents an increase of 133% over the basal value.

Period of culture (hours)	First PCA extraction		Second PCA extraction	
	Residual counts (% total)	Efficiency of DNA extraction (%)	Residual counts (% total)	Efficiency of DNA extraction (%)
0	26·4	73·6	21·5	78·5
4	19·4	80·6	10·8	89·2
8	14·7	85·3	2·5	97·5
10	3·8	96·2	2·1	97·9
12	13·0	87·0	5·0	95·0
14	10·8	89·2	3·3	96·7
16	6·2	93·8	0·0	100·0
18	4·5	95·5	0·0	100·0
20	0·9	99·1	0·2	99·8
22	1·7	98·3	0·5	99·5
24	0·9	99·1	0·3	99·7
26	0·8	99·2	0·2	99·8
28	1·2	98·8	0·5	99·5

Table 4d.   The efficiency of DNA extraction by the first and second PCA procedures and the percentage of residual counts.
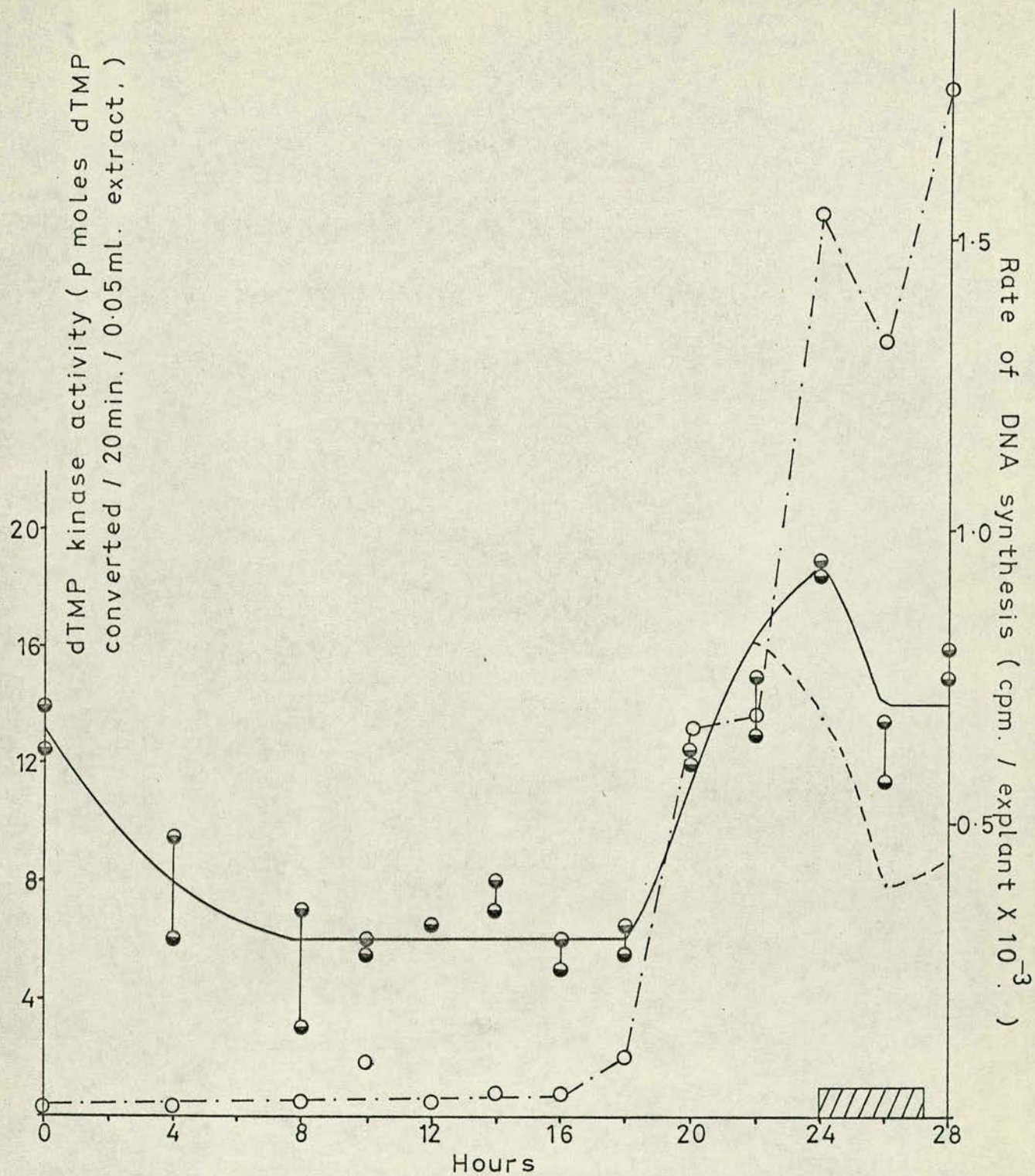
FIG. 4.41 dTMP kinase activity (◑) in relation to the rate
of DNA synthesis (O) during the cell cycle.

(— — — — activity expressed on a per cell basis.)

When average dTMP kinase activities are expressed on a per cell basis (see broken line in Fig. 4.41), the peak in activity occurs between 22 and 24 hours and the value tends to decrease during division. Between 26 and 28 hours the average dTMP kinase activity expressed on a per cell basis is only slightly higher than the original pre-'S' value.

dTMP phosphatase activity (Fig. 4.42)

The enormous variation between consecutive values makes it difficult to assign any fixed pattern to the data. However, it is possible that a gradual increase in dTMP phosphatase activity occurs during the 28 hour period.

The 65% cell division obtained in this experiment is in good agreement with the results of previous experiments and with the results of Davidson (1971).

The pattern shown by the rate of DNA synthesis in this experiment is similar to that described in Experiment 9 in that it shows no fixed definition of the end of 'S' and a relatively high value is maintained throughout division. This type of pattern may be characteristic of experiments carried out early in the season (see Appendix 5 for dates of experiments). As mentioned in Experiment 3, the shoulder on the rising part of this DNA synthesis curve is similar to that observed in almost all previous experiments.

In this experiment the extraction of ^{3}H-labelled DNA by the standard PCA hydrolysis procedure is over 95% efficient during the
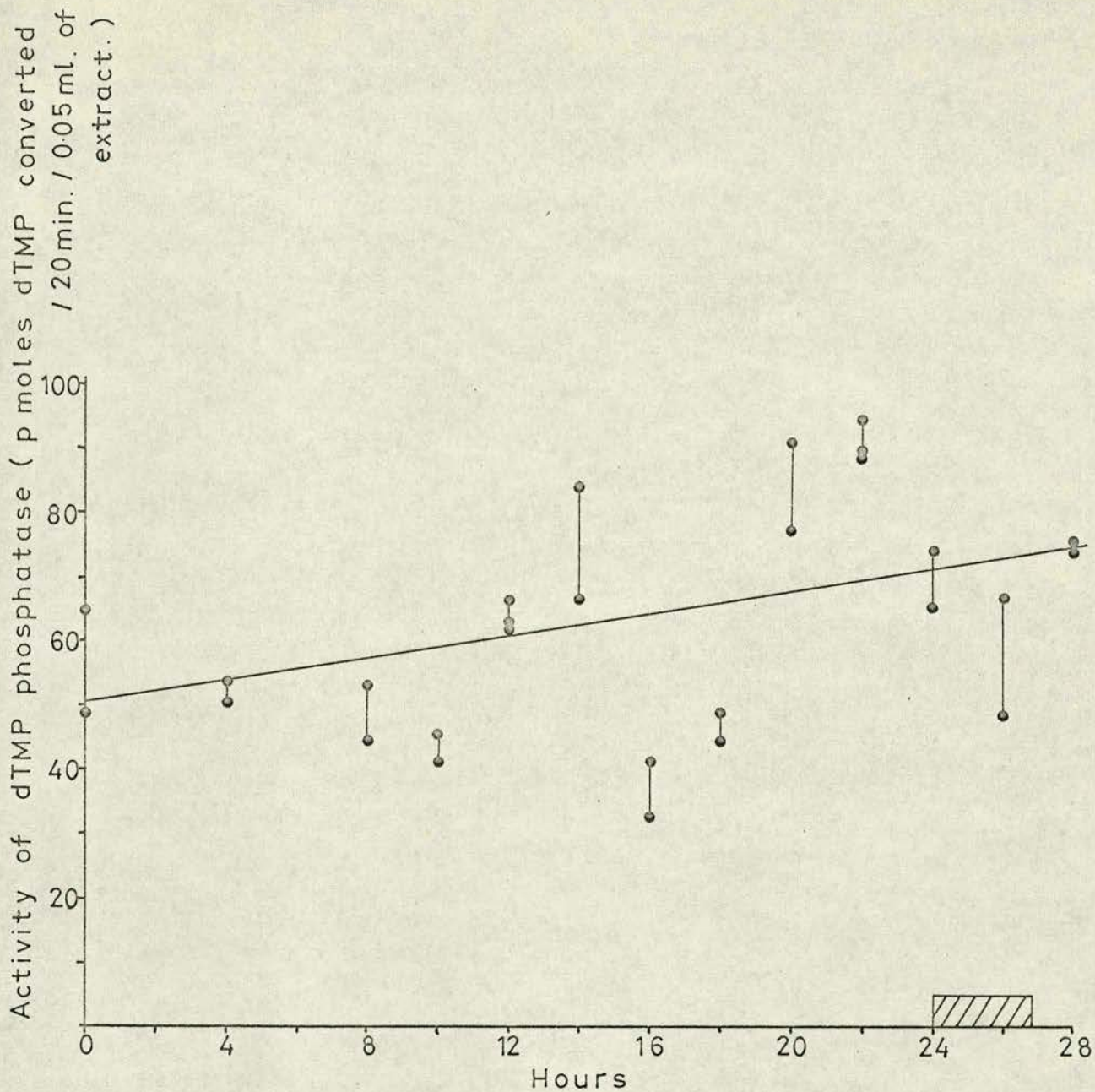
FIG. 4.42   Activity of dTMP phosphatase in a 'dividing' system.

'S' period, and the few counts which remain in the residue show a similar pattern to the rate of DNA synthesis.   The fact that a certain proportion of these counts are removable by further PCA hydrolysis suggests that they are contained mainly in DNA residues which are difficult to remove and it is unlikely that any of the ^{3}H-TdR has been incorporated into other cell constituents, e.g. cell wall components.   Retention of counts in freshly excised material, and material sampled early in the pre-'S' period suggests that DNA may exist in a different 'state' at that time.

The pattern of dTMP kinase activity during the cell division cycle is similar to that described in Experiment 9, except that in this experiment it is clear that the activity tends to fall during the first few hours of the cycle.   The increase in activity during 'S' which occurred 4 hours after the beginning of 'S' in Experiment 9 occurs 2 hours after the beginning of 'S' in this experiment.   The increase in activity of 213% over the basal value obtained at 24 hours in this experiment is similar to the increase of 219% obtained between 26 and 28 hours in Experiment 9.   In this experiment however, the dTMP kinase activity may tend to fall during division whereas in Experiment 9, the activity may have increased slightly during division.

When dTMP kinase activities are expressed on a per cell basis, the pattern of decline during division to reach a value at the end of division which is only slightly higher than the pre-'S' value is similar in Experiment 9 and in this experiment.

The gradual increase in the activity of dTMP phosphatase during

the 28 hour period which was observed in this experiment has confirmed the pattern obtained in Experiment 9.

Since patterns of activity during the cell division cycle have now been established for the enzymes TdR kinase, dTMP kinase and dTMP phosphatase, it remains to determine whether these patterns are associated with the cell division cycle or whether they are merely a property of freshly excised artichoke tissue in culture.

Experiment 11

This experiment was designed to determine whether the increase in TdR kinase activity, which occurs during 'S' of the first division cycle, also occurs in a 'non-dividing' system.   As in Experiments 5 and 6, 'non-dividing' cultures were obtained by omitting the synthetic auxin 2,4-D from the culture medium.

In this experiment a series of 4 'dividing' and 4 'non-dividing' cultures each containing 120 explants, were prepared simultaneously under the usual conditions.   After culture periods of 18, 20 and 22 hours, one of each type of culture was removed and assayed for TdR kinase activity using the method employed in Experiments 7 and 8. The activity at 0 hours was also determined.   TdR kinase assays were carried out in triplicate for each extract.

At 24 hours one of each type of culture was sampled for cell number determination only.

Results

For the culture grown in the presence of 2,4-D, the average cell number per explant at 24 hours was approximately 16,250 and the presence of mitotic figures indicated that cell division had started. The culture grown in the absence of 2,4-D gave an average cell number per explant of 14,750 and no division figures were observed in the cell macerate.

TdR kinase activities for the various extracts are shown in Table 4e, from which it is apparent that the average activities at 18, 20 and 22 hours when 2,4-D is absent, are similar to the 0 hour value. In 'dividing' cultures the average TdR kinase activity at 20 hours represents a 227% increase compared with the 0 hour value whilst the activity at 22 hours represents an increase of 173% compared with the 0 hour value.

In this experiment, cell division had commenced in the 'dividing' cultures by 24 hours. As the 'S' period usually begins approximately 10 hours before the beginning of division (see Experiments 1, 2, 4, 8 and 9) it is safe to assume that the period 18 to 22 hours in this experiment coincides with the 'S' period. In the 'dividing' cultures, the increases in TdR kinase activity of 227% at 20 hours and 173% at 22 hours compared with the 0 hour value, are in the same order of magnitude as the corresponding increases observed in Experiments 7, 8 and 9.

This experiment has clearly shown that the increase in TdR kinase activity which occurs during 'S' in a 'dividing' system and is absent in a 'non-dividing' system, is directly associated with cell division.

Period of culture (hours)	TdR kinase activity (p moles TdR converted/ 20 min./0·1 ml. extract)			
	+ 2,4-D		-2,4-D	
	Actual values	Average values	Actual values	Average values
0	5·4 5·1 7·0	5·8	5·4 5·1 7·0	5·8
18	17·5 16·3 16·3	16·7	4·7 6·6 7·0	6·1
20	17·9 17·5 21·8	19·1	7·0 7·8 6·6	7·1
22	17·1 16·7 15·6	16·5	7·0 5·8 7·0	6·6

Table 4e.   TdR kinase activities in 'dividing' and 'non-dividing' systems.

Experiment 12

This experiment was designed to determine whether the increase in dTMP kinase activity, which occurs during 'S' of the first division cycle and the gradual increase in dTMP phosphatase activity which occurs throughout the first division cycle are confined to a 'dividing' system.

As the pattern of dTMP kinase activity established in Experiments 9 and 10 involved a preliminary decrease in activity during the first few hours of the cycle, it was also necessary to determine whether this fall in activity is confined to a 'dividing' system.

As in Experiments 5,6 and 11, 'non-dividing' cultures were prepared by omitting the synthetic auxin 2,4-D from the culture medium.

This experiment involved the preparation of 4 'dividing' cultures and 4 'non-dividing' cultures each containing about 130 explants. The cultures were sampled after periods of 10, 12, 24 and 26 hours. At each sampling time one 'dividing' culture and one 'non-dividing' culture were removed. Sixteen explants from each flask were used to estimate the rate of DNA synthesis and 100 explants were used to prepare an extract for the assay of dTMP kinase and dTMP phosphatase. The extract was prepared using the method employed in Experiment 10. Enzyme activities and rate of DNA synthesis at 0 hours were also determined.

Results

Rate of DNA synthesis

Data for the rate of DNA synthesis are shown in Table 4f. from which it is evident that 'S' has started in the 'dividing' cultures by 24 and 26 hours. In the 'non-dividing' cultures, the rate of DNA synthesis which is initially low at 10 and 12 hours has reached a slightly higher value by 24 hours which is approximately 8% of the 24 hour value in the 'dividing' culture.

dTMP kinase activity (Table 4g)

In both 'dividing' and 'non-dividing' systems the initially high activity at 0 hours has shown a 73% decrease by 10 to 12 hours.

In the 'dividing' system the dTMP kinase activity at 24 hours has increased 181% over the 10 - 12 hour value and at 26 hours this increase is approximately 110%. No increases in dTMP kinase activity are recorded in the corresponding 'non-dividing' cultures.

The results from the dTMP kinase and rate of DNA synthesis data are compared in semi-diagrammatic form in Fig. 4.43. As the accurate timing of the beginning of 'S' is not known for this experiment, the value indicated on the diagram is only an estimate.

dTMP phosphatase activity (Table 4h)

The activity of dTMP phosphatase appears to show a gradual increase during the 26 hour period in both 'dividing' and 'non-dividing' systems.

This experiment has shown that the increase in dTMP kinase activity during 'S' of the first division cycle is exclusively a

| Period of culture (hours) | Rate of DNA synthesis (counts/min./explant) | | | |
| | +2,4-D | | -2,4-D | |
	Replicate values	Average value	Replicate values	Average value
0			45·9 58·2	52·0
10	49·5 48·7	49·1	49·6 53·0	51·3
12	27·3 31·3	29·3	22·5 16·9	19·7
24	1452·0 948·2	1200·1	73·0 111·5	92·2
26	1844·0 926·0	1385·0	- -	-

Table 4f. Rate of DNA synthesis in 'dividing' and 'non-dividing' systems.

Period of culture (hours)	dTMP kinase activity (p moles dTMP converted to dTDP/20 min./0·05 ml. extract)			
	+2,4-D		-2,4-D	
	Replicate values	Average value	Replicate values	Average value
0	19·5 22·5	21·0	19·5 22·5	21·0
10	5·5 3·0	4·2	7·5 6·0	6·7
12	7·5 7·0	7·2	5·5 1·0	3·2
24	15·0 17·0	16·0	4·0 6·5	5·2
26	11·0 14·0	12·5	6·5 5·5	6·0

Table 4g.   dTMP kinase activity in 'dividing' and
'non-dividing' systems.
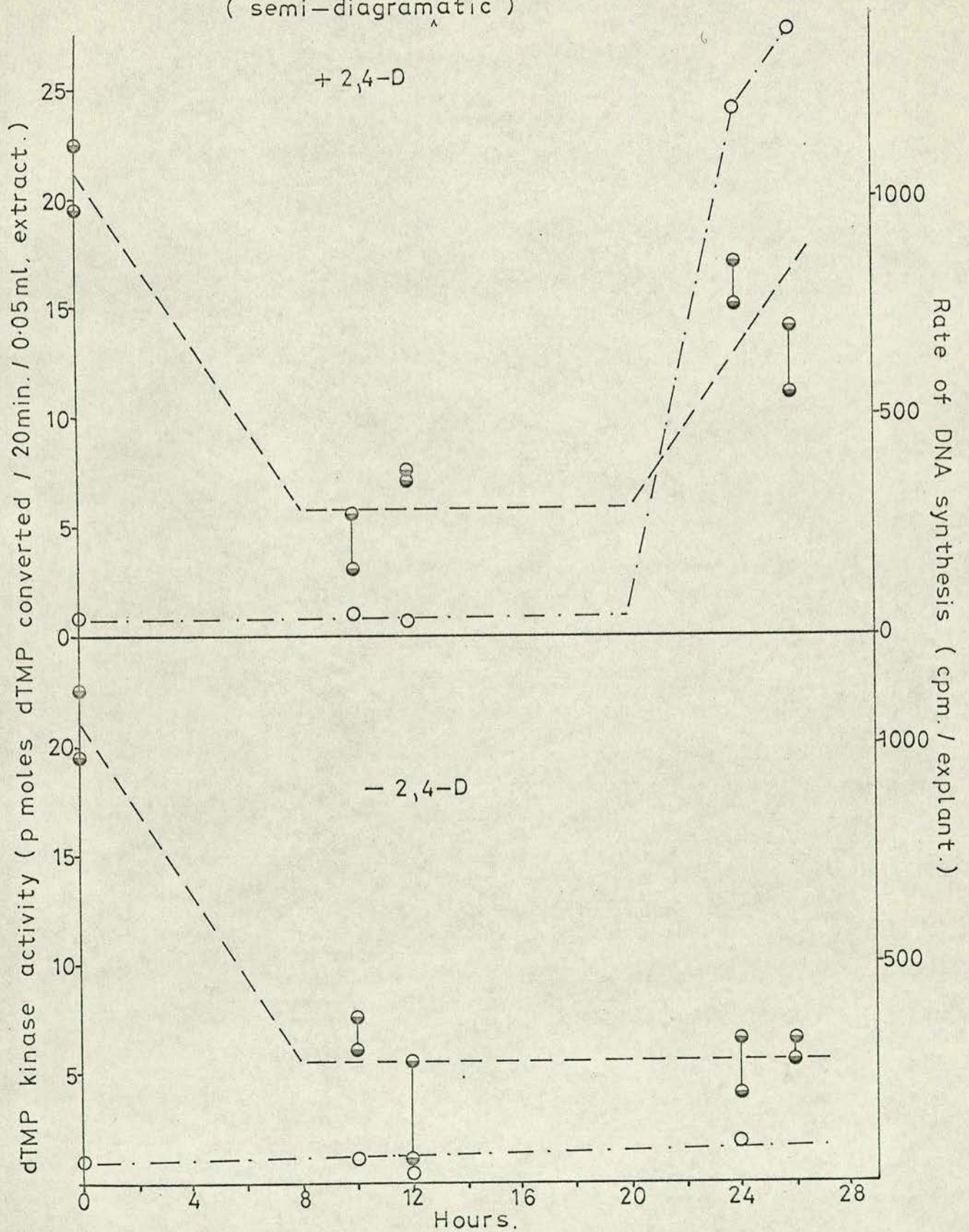
FIG. 4.43   dTMP kinase activity (◓) in relation to the rate of DNA synthesis (O) in 'dividing' and 'non-dividing' systems.

| Period of culture (hours) | dTMP phosphatase activity (p moles dTMP converted to TdR/20 min./0·05 ml. extract) | | | |
| | +2,4-D | | −2,4-D | |
	Replicate values	Average value	Replicate values	Average value
0	42·0 41·5	41·7	42·0 41·5	41·7
10	45·0 44·5	44·7	52·5 44·5	48·5
12	57·0 51·5	54·2	61·5 55·0	58·2
24	70·0 76·0	73·0	88·0 67·5	77·7
26	94·5 85·5	90·0	93·0 74·8	83·9

Table 4h. dTMP phosphatase activity in 'dividing' and
'non-dividing' systems.

property of this 'dividing' system.

The increases in dTMP kinase activity of 181% and 110% recorded in the 24 and 26 hour 'dividing' cultures are in the same order of magnitude as the increase of 219% observed in Experiment 9 and the increases of 213% and 133% observed in Experiment 10.

The initial fall in dTMP kinase activity which occurred between 0 and 10 hours in this experiment was similar in both 'dividing' and 'non-dividing' systems and is therefore a property of freshly excised artichoke tissue in culture.   In this experiment the initial fall in activity of 73% compared with the 0 hour value was slightly higher than the figures of 45% and 55% obtained in Experiments 9 and 10 respectively.

In this experiment the gradual increase in dTMP phosphatase activity which was observed over a 28 hour period in Experiments 9 and 10 occurs in both 'dividing' and 'non-dividing' systems, hence this particular enzyme pattern is not associated with the cell division cycle but is merely a property of freshly excised artichoke tissue in culture.

Now that it has been established that the increase in TdR kinase and dTMP kinase activity during 'S' of the cell division cycle are confined to a 'dividing' system, it remains to determine whether these increases are a result of enzyme activation or whether they are a result of de novo enzyme synthesis.

Experiment 13

This experiment was designed to determine whether the increases in TdR kinase and dTMP kinase activities during 'S' of the first division cycle, are caused either by activation of pre-existing enzyme or by the removal of enzyme inhibitors during this period. If either inhibitors or activators were present, then the effect of mixing equal volumes of extract of high and low activities would not be additive.   It has already been shown that volume of extract is proportional to enzyme activity for both TdR kinase and dTMP kinase (Ch. 3), and it is therefore valid to carry out this experiment.

This experiment involved the preparation of 4 'dividing' cultures for sampling at 8, 10, 22 and 24 hours.   The cultures were set up so that the 8 hour and 22 hour cultures were sampled together and the 10 hour and 24 hour cultures were also sampled together.   At each sampling time the two extracts were prepared as described for dTMP kinase except that in this experiment 150 explants were macerated in 0·75 ml. grinding medium in order to produce an extract of approximately twice the normal concentration.

After the extracts had been prepared, 4 different reaction tubes were set up in duplicate for each enzyme.

For the 8 hour and 22 hour extracts:-

	^{3}H*-medium	Extract (μl)	Grinding buffer (μl)	pyruvate kinase (10 mg. protein/ ml.) (μl)
Blank	100	-	100	25
8 hour control	100	8 hour extract - 50	50	25
22 hour control	100	22 hour extract - 50	50	25
8 hr. + 22 hr. mixture	100	8 hour extract - 50 22 hour extract - 50	-	25

This procedure was repeated for the 10 hour and 24 hour extracts.

Results.

TdR kinase activity

The results of all TdR kinase assays are shown in Table 41. The actual activities obtained are shown in the left hand column. The right hand column shows the sum of 8 hour + 22 hour activities in all possible combinations for comparison with the activities of the 8 hour + 22 hour mixture shown in the left hand column. The 10 hour and 24 hour activities have been treated in a similar fashion.

---

* ^{3}H-medium - the medium containing substrates and cofactors necessary for the TdR kinase or dTMP kinase assays.

Reaction	TdR kinase activity (p moles TdR converted to dTMP/20 min.)	
	Replicate values	Possible additions
8 hour control	2·75	10·22
	3·93	11·00
22 hour control	7·47	11·20
	7·07	9·82
8 hr. + 22 hr. mixture	8·65	
	10·61	
10 hour control	5·11	13·76
	3·14	10·61
24 hour control	8·65	11·79
	7·47	12·58
10 hr. + 24 hr. mixture	13·75	
	12·97	

Table 41.   TdR kinase mixed extract data.

The results for the 8 + 22 hour mixture agree well with the possible additions.   Good agreement is also shown for the 10 + 24 hour mixture.

dTMP kinase activities (Table 4j)

The dTMP kinase results are also tabulated as described above.

The value of 27·0 for the 8 + 22 hour mixture agrees well with the possible additions.   The duplicate value of 20·0 may be rather low due to error variation.

Results for the 10 + 24 hour mixture are similar to some of the possible additions.   However the value of 13·0 obtained for one of the duplicated 10 hour activities is probably higher than it should be.

dTMP phosphatase activities (Table 4k)

The dTMP phosphatase results are also tabulated as described above.

The activity of the 8 + 22 hour mixture is in good agreement with the additive values.

The activity of the 10 + 24 hour mixture is higher than two of the additive values and lower than the remaining two.   The value of 90, obtained in one of the 24 hour controls may be rather low compared with the duplicate value of 177 and may be in part responsible for the above discrepancy.

It is clear from the results of this experiment that the increase in TdR and dTMP kinase activities during 'S' of the cell division cycle, and the gradual increase in dTMP phosphatase activity

Reaction	dTMP kinase activity (p moles dTMP converted to dTDP/20 min.)	
	Replicate values	Possible additions
8 hour control	8·0	26·0
	5·0	27·0
22 hour control	18·0	23·0
	22·0	30·0
8 hr. + 22 hr. mixture	20·0	
	27·0	
10 hour control	5·0	29·0
	13·0	38·0
24 hour control	24·0	37·0
	25·0	30·0
10 hr. + 24 hr. mixture	28·0	
	30·0	

Table 4j.   dTMP kinase mixed extract data.

Reaction	dTMP phosphatase activity (p moles dTMP converted to TdR/20 min.)	
	Replicate values	Additive value
8 hour control	84	271
	44	229
22 hour control	187	231
	185	269
8 hr. + 22 hr. mixture	237	
	269	
10 hour control	88	178
	81	258
24 hour control	90	171
	177	265
10 hr. + 24 hr. mixture	225	
	229	

Table 4k.   dTMP phosphatase mixed extract data.

which is a property of freshly excised tissue, are not controlled by the presence of activators or by the removal of inhibitors.

The increase in TdR and dTMP kinase activities are closely associated with DNA synthesis and from the experiments so far described it is difficult to establish which comes first, DNA synthesis or the rise in enzyme activity.   An indication of the answer to this question can be obtained by further experiment involving the use of a specific inhibitor of DNA synthesis.

Experiment 14

By using a specific inhibitor of DNA synthesis, this experiment was designed to determine whether or not the increase in TdR and dTMP kinase activities during 'S' is dependent on DNA synthesis.

The specific inhibitor of DNA synthesis used in this experiment was Fluoro-uridine deoxyriboside (FUdR), a substance which, because of its resemblance to deoxyuridine (UdR) becomes converted to the monophosphate (dFUMP) by the tissue and blocks the conversion of dUMP $\longrightarrow$ dTMP, a reaction which is essential to the normal pathway of DNA biosynthesis.   In organisms which possess the enzyme TdR kinase, this inhibition can be overcome if a high concentration of TdR is also present in the culture medium.

The above relationships are summarized in the following diagram:-

$$- - \rightarrow \text{dUMP} \xrightarrow{\quad//\quad} \text{dTMP} \xrightarrow{\text{dTMP kinase}} \text{dTDP} \longrightarrow \text{dTTP} \rightarrow \rightarrow \boxed{\text{DNA}}$$

```
                        dTMP
- - → dUMP  ─//─→  dTMP  kinase, dTDP ──→ dTTP →  →    DNA

            dFUMP
uridine       ↑          thymidine
kinase        |           kinase
            FUdR    ↑
                   TdR
```
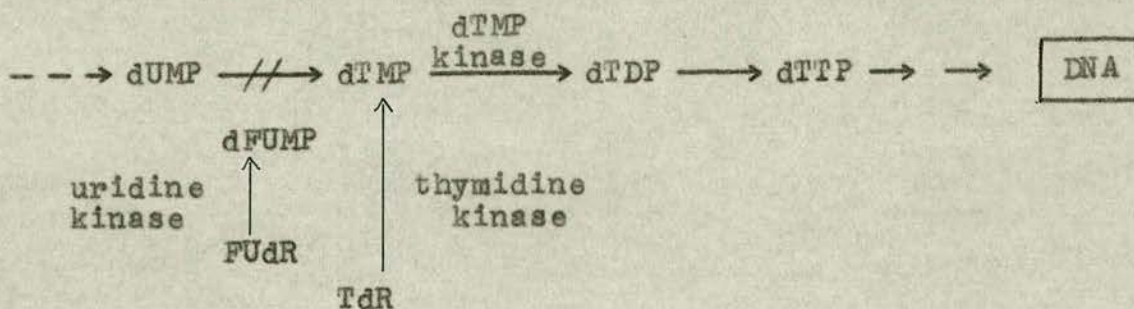
Jackson (1970) has shown that the increase in DNA polymerase activity during 'S' in the artichoke system could be prevented if a high concentration of FUdR (22 µg./ml.) was present throughout the culture period. At this concentration of FUdR the inhibition of DNA synthesis reflected by Feulgen DNA levels was more or less complete. The inhibition of DNA polymerase activity due to the presence of FUdR was almost completely reversible if 333 µg./ml. TdR was also present in the culture medium.

In this experiment the effect of the above concentration of FUdR was examined with respect to the increase in TdR kinase and dTMP kinase activities during 'S'. DNA levels were measured using Burton's chemical method, as in this experiment the rate of DNA synthesis could not be measured because of the possible reversal effect by TdR. An attempt was also made to reverse the proposed inhibition of TdR and dTMP kinase activities using 333 µg./ml. TdR.

This experiment involved the preparation of 5 cultures, each containing approximately 160 explants. Two 'dividing' cultures and two cultures containing 22 µg./ml. FUdR in the culture medium were prepared. One of each type of culture was sampled after periods of 24 and 26 hours. One culture containing 22 µg./ml. FUdR and 333 µg./ml. TdR in the culture medium was prepared for sampling at 26 hours.

At each sampling time, 3 samples of 15 explants were taken from each culture for determination of DNA levels by Burton's method whilst 100 explants were used to prepare the enzyme extract.

Extracts were prepared as described for dTMP kinase (Ch. 2.D.5.).
Assays for TdR kinase and dTMP kinase activities were carried out
in duplicate for each extract.   The activities of a 0 hour extract
were also measured.

Results

DNA levels

DNA levels estimated using Burton's method are shown in
Table 41.

Although no marked increase in the level of DNA had occurred
by 24 hours in the 'dividing' culture the level at 26 hours was
about 25% increased over the 0 hour value.   At 26 hours, the
'dividing' culture also showed a 25% increase in DNA level compared
with the 26 hour culture grown in the presence of FUdR.   The
culture grown in the presence of both FUdR and TdR may show partial
reversal with respect to DNA levels.

Activity of TdR kinase, dTMP kinase and dTMP phosphatase

Enzyme activity data are shown in Table 4m.

In the 'dividing' cultures at 24 and 26 hours the activity of
TdR kinase shows a substantial increase compared with the value at
0 hours.   When FUdR is present however, the activities at 24 and 26
hours are only slightly higher than the activity at 0 hours.   For
the culture grown in the presence of FUdR and TdR, the TdR kinase
activity was similar to the activities obtained for cultures grown
in the presence of FUdR only, and no reversal was detected.

The dTMP kinase activities of the 0 hour extract and the

Period of culture (hours)	FUdR presence	TdR presence	µg. DNA/explant	
			Actual values	Average value
0	-	-	0·79 0·80 1·05	0·88
24	-	-	0·91 0·80 1·05	0·92
24	+	-	1·03 0·98 1·04	1·01
26	-	-	1·03 1·15 1·15	1·11
26	+	-	0·92 0·89 0·87	0·89
26	+	+	0·97 0·95 0·99	0·97

Table 41. The effect of FUdR, or FUdR + TdR presence on DNA

measured using Burton's method.

Period of culture (hours)	Presence of		TdR kinase activity (p moles TdR converted/20 min./0·1 ml. extract)	dTMP kinase activity (p moles dTMP converted to dTDP/20 min./0·05 ml. extract)	dTMP phosphatase activity (p moles dTMP converted to TdR/20 min./0·05 ml. extract)
	FUdR	TdR			
0	-	-	5·1 4·7	11·0 10·5	52·5 55·0
24	-	-	14·9 16·1	11·5 11·0	103·0 97·5
24	+	-	5·5 7·9	4·5 7·0	97·0 88·5
26	-	-	21·6 19·3	13·0 11·0	111·0 71·5
26	+	-	11·8 7·5	6·5 2·5	85·5 103·5
26	+	+	6·3 8·3	7·5 5·5	87·0 85·5

Table 4m.   The effect of FUdR, or FUdR and TdR presence on the
activities of TdR kinase, dTMP kinase and dTMP
phosphatase.

'dividing' 24 hour and 26 hour cultures are high.   When FUdR is
present however, the dTMP kinase activities at 24 hours and 26 hours
are much reduced compared with the 'dividing' cultures.   For the
culture grown in the presence of FUdR and TdR, the dTMP kinase
activity was similar to activities of cultures grown in the presence
of FUdR only, and no reversal effect was observed.

The activities of dTMP phosphatase show a different trend in
that the slow rise in activity has taken place in the 'dividing'
cultures over the 26 hour period but this is not significantly
affected by the presence of FUdR, or FUdR and TdR.

This experiment has confirmed the results of Jackson (1970)
in that it has shown for the 26 hour samples, that 22 µg./ml. FUdR
inhibits DNA synthesis during 'S'.   The extent of the FUdR inhibition
of DNA synthesis was approximately 100% at 26 hours.

The DNA data for this experiment do not confirm that 'S' has
started by 24 hours.   It is in fact probable that 'S' has started
in the 'dividing' 24 hour culture for a number of other reasons.
Firstly, Experiments 1, 2 and 8 have shown that use of Burton's
method is not the best way to estimate the accurate timing of 'S',
as this method usually gives a value which is at least 4 hours behind
the value indicated by rate of DNA synthesis data.   Secondly, this
experiment was carried out only 1 week later than Experiment 12 (see
Appendix 5 for dates of Experiments), in which it was shown from
rate of DNA synthesis data, that 'S' had started by 24 hours, and it

is most unlikely that the length of the pre-'S' phase would increase in 7 days.

Further evidence to support the supposition that 'S' has in fact begun in the 24 hour culture is the enzyme data. In previous experiments the increases in TdR kinase and dTMP kinase activities have apparently never preceded the beginning of 'S', and in this experiment the activities of both enzymes have increased by 24 hours.

The increase in TdR kinase activity during 'S' is prevented if cultures are grown in the presence of 22 μg./ml. FUdR. Although the DNA data may indicate at least a partial reversal of the FUdR inhibition by TdR, no reversal effect was observed for TdR kinase activity.

In this experiment, the dTMP kinase activity at 0 hours is similar to the activities observed for 'dividing' cultures at 24 and 26 hours. In Experiments 9, 10 and 12 it has been established that the dTMP kinase activity at 0 hours always decreases during the first few hours of culture to reach a basal value. The increase in dTMP kinase activity which occurs during 'S' often restores the activity to a level similar to its original 0 hour value and the similarity of the 0, 24 and 26 hour values in this experiment suggests that this increase has occurred by 24 hours.

In the presence of FUdR, the dTMP kinase activities at 24 and 26 hours were much reduced, to a level comparable to the basal pre-'S' values observed in previous experiments, hence it is probable that the increase in dTMP kinase activity which occurs

during 'S' has been prevented due to the presence of FUdR. With respect to dTMP kinase activity, the presence of 333 µg./ml. TdR in the culture medium did not reverse the inhibitory effect of FUdR.

It has already been proved that the gradual increase in dTMP phosphatase activity during the first 30 hours or so of culture, is not associated with cell division. This observation is confirmed by the fact that in this experiment the increase occurs whether FUdR is present or not showing that the increase is independent of the DNA synthesis which is associated with the cell division cycle.

Assuming that FUdR is a specific inhibitor of DNA synthesis, this experiment has indicated that the rise in activities of TdR kinase and dTMP kinase during 'S' is dependent on DNA synthesis.

Summary of Section B

The results from the series of experiments in this section can be summarized as follows:-

1. By measuring such standard parameters as cell number, and levels of total nucleic acid and DNA, it has been established that the experimental system is behaving in the way described by previous workers. Although the patterns of increase observed in this and the previous section are similar to those described by Evans (1967) for a system excised in the light and cultured in the dark in the presence of coconut milk, the absolute increases are far larger, and this is almost certainly associated with the fact that in this investigation both the preparation and culture of explants was in the dark.

2.   Patterns of the rate of DNA synthesis observed in this and the previous section, with the exception of Experiment 8, suggest the presence of a shoulder in the rising part of the curve during 'S'.

In Experiments 9 and 10 (alias Experiments 4 and 3 respectively of Section A) there is no fixed definition of the end of 'S'. As these experiments were carried out much earlier in the 'artichoke Season' than Experiments 1 and 2, it is possible that the inability to define the end of 'S' is related to the physiological state of the tubers.

In Experiment 8, measurement of the rate of DNA synthesis by incorporation of ^{3}H-TdR into DNA was shown to be a more accurate indicator of the onset of 'S' than measurement of DNA by Burton's

chemical method, thus confirming similar observations made in Experiments 1 and 2 of the previous section.

3.   The standard extraction of ^{3}H-labelled DNA from explants during the estimation of rate of DNA synthesis was shown in Experiments 9 and 10, to be over 90% efficient during the 'S' period.   The small percentage of counts remaining in the residue could be partially removed by further rigorous extraction (Experiment 10), which suggested that they were present in DNA residues which were difficult to remove thus excluding the possibility that ^{3}H-TdR was being incorporated into other insoluble products such as cell wall materials.

4.   A characteristic pattern of increase in TdR kinase activity during the cell division cycle, was described in Experiment 7 and verified in Experiments 8 and 9.   The fact that no increase in activity was observed in a 'non-dividing' system (Experiment 11) shows that the pattern observed in Experiments 7, 8 and 9 is primarily associated with cell division.

5.   A characteristic pattern of dTMP kinase activity during the cell division cycle was described in Experiment 9 and verified in Experiment 10.   The results of Experiment 12, in which a 'non-dividing' system was examined, revealed that the increase in dTMP kinase activity during the 'S' period was associated with a 'dividing' system, whilst the initial decrease in activity during the first few hours of culture was merely a property of freshly excised tissue in culture.

6.   The gradual increase in dTMP phosphatase activity during 28

hours of culture in a 'dividing' system was established in Experiment 9 and verified in Experiment 10. The results of Experiment 12 in which a 'non-dividing' system was examined revealed that this increase was not associated with the cell division cycle but was merely a property of freshly excised artichoke tissue in culture.

7.   The results of Experiment 13 have shown that the increase in TdR kinase and dTMP kinase activities during the cell division cycle, and the gradual increase in dTMP phosphatase activity which is a property of freshly excised artichoke tissue in culture, are not controlled either by the presence of activators or by the removal of inhibitors.

8.   The results of Experiment 14, in which FUdR was used as a specific inhibitor of DNA synthesis have indicated that the increase in TdR kinase and dTMP kinase activity during 'S' of the cell division cycle is dependent on DNA synthesis.

CHAPTER 5

DISCUSSION

Knowledge of the events of the cell division cycle in higher plants is largely restricted to the visible parts of cell division i.e. mitosis and cytokinesis, or the timing of the events of the cell cycle as described by Howard and Pelc (1953).   The events of interphase however, are less well known and the information available has largely been derived from studies with synchronously dividing systems.

Cell synchrony during the initial stages of growth of the artichoke callus system was first described by Yeoman, Evans and Naik (1966), and since then this system has been characterized with respect to the many cytological and physiological changes which take place during interphase of the first synchronous division [e.g. Yeoman and Evans (1967); Mitchell (1967) and (1968); Yeoman, Tulett and Bagshaw (1970); and Yeoman (1970)].

In a higher plant, the process of cell division is largely confined to certain defined areas of meristematic cells such as the apex of the root or shoot.   In this situation, the daughter cells which are the products of division grow back to the original size of the parent cell before dividing again, thus a definite cyclic pattern is established.   In the developing artichoke callus however, cells which are initially quiescent are induced to divide, and the events which follow excision and culture of explants in a medium with 2,4-D, are those events which accompany the preparation of a cell for division.

Initial growth of the cultured explant involves two fairly synchronous divisions followed by divisions in which synchrony decreases and eventually a state of asynchrony is reached. The experiments described in this thesis are mainly confined to the first division cycle which is certainly more synchronous than the second division cycle, although the events of induction certainly complicate the interpretation of the results with respect to division.

Since the early studies in this laboratory, manipulation of certain growth conditions such as preparation of cultures in low intensity green light and subsequent growth in complete darkness have produced cultures in which a higher percentage of the constituent cells divide synchronously, than if cultures were prepared in light and grown in darkness with occasional exposure to light [Fraser, Loening and Yeoman (1967); Yeoman and Davidson (1971)]. In this study, it was desirable to achieve a high percentage cell division in order to obtain the maximum expression of biochemical changes associated with this process, such as the possible changes in enzyme activities, therefore all cultures were prepared in low intensity green light and grown in darkness.

In this investigation, all enzyme activities and the rate of DNA synthesis were measured using radiochemical techniques which are generally more sensitive than other analytical methods. In addition, the quantitative validity of each enzyme assay method was demonstrated for artichoke tissue. This degree of confidence in the experimental techniques increases the validity of the established patterns of enzyme activity with respect to the timing and extent of

DNA synthesis during the first division cycle.

When the results of all enzyme experiments are considered, the patterns of changes during the cell division cycle can be divided into two broad categories depending on whether the pattern is associated with cell division or not.   Whether or not each individual enzyme pattern was associated with cell division was determined by the use of 'dividing' and 'non-dividing' systems, cultured in the presence and absence of 2,4-D respectively.

The enzymes which exhibited patterns of increase associated with cell division were the biosynthetic enzymes thymidine (TdR) kinase, thymidine monophosphate (dTMP) kinase and DNA polymerase. In contrast to the above, the activities of certain degradative enzymes such as deoxyribonucleases specific for the breakdown of native and denatured artichoke DNA, and the suppressed activity of thymidine monophosphate (dTMP) phosphatase were similar in both 'dividing' and 'non-dividing' systems and these patterns of activity were attributed to a property of freshly excised artichoke tissue in culture.

During the initial culture period the activity of 'native'-DNAase exhibited a marked increase, whilst the activity of 'denatured'-DNAase and the suppressed activity of dTMP phosphatase appeared to show a very gradual increase.   It is probable that the patterns of activity of degradative enzymes are associated with autolysis, which takes place in the cells on the surface of the explant which are in contact with the culture medium.   These cells eventually form a rind surrounding the explant and are immediately

above the layer of dividing cells (Yeoman, Naik and Robertson 1968).
Using histochemical techniques, Robertson (1966) has shown the
development of other catabolic enzymes, acid and alkaline phosphatases,
in this layer of cells during development of the artichoke callus.

In the developing microspore system of <u>Lilium longiflorum</u>,
the anther tissue which surrounds the microspores and eventually
breaks down may resemble the outer rind of artichoke callus cells.
In the lily anther system, Stern (1961) described a periodic
deoxyribonuclease breakdown of native DNA, during interphase of the
second division of meiosis.   The function of the appearance of
DNAase at a specific time during interphase was attributed to the
breakdown of DNA in the anther tissue in order to provide a pool of
deoxyribosides which would induce the synthesis of nucleoside
kinases in the microspores, resulting in DNA synthesis and cell
division.

There is some evidence from other synchronous systems that the
activity of DNAase with a substrate specificity for native DNA does
not vary greatly during the cell cycle. [Walther and Edmunds (1970)
for Euglena and Gold and Helleiner (1963) for L cells].   In the
rat liver system however, the activity of alkaline deoxyribonuclease
increased 2 - 3 fold prior to the initial peak of DNA synthesis and
remained elevated for at least 70 hours (O'Connor 1971).

In the artichoke system the pattern of activity for 'denatured'-
DNAase did not resemble the pattern for 'native'-DNAase which
strongly suggests that these activities are due to two completely
different enzymes.   Added evidence is the existence of two different

pH optima and the dissimilar patterns of the pH dependence curves.
Furthermore, the activity of 'denatured'-DNAase is considerably
greater than that of 'native'-DNAase in artichoke tissue.

In the artichoke system, the activity of 'denatured'-DNAase
optimally active at pH 6·4 remained approximately constant during
the culture period, although it is possible that this pattern may
take the form of a gradual increase over a prolonged period of culture.
Although this pattern of activity was not associated with cell
division, it might be reasonable to suppose that the enzyme activity
was at such a high level during the initial culture period that an
increase in activity during the cell division cycle was not required
for the growth of this system.

In other synchronous systems there have been several reports of
increased activity associated with the cell cycle for DNAase
enzymes with a substrate specificity for denatured DNA. In
synchronously growing cultures of Chlorella, the activity of a
deoxyribonuclease highly specific for denatured DNA and optimally
active at pH 8·5, increased during the period of DNA synthesis,
(Schonherr et al. 1970). A similar observation was made for
synchronous cultures of Euglena by Walther and Edmunds (1970), for
an enzyme optimally active at pH 7·5. In both of these reports,
the substrate for the enzyme assay was labelled DNA isolated from
Escherichia coli, whereas the substrate used to assay the 'pH 6·4
enzyme' of artichoke extracts was labelled artichoke DNA. It is
possible, though unlikely that the action of 'denatured'-DNAase
on a substrate DNA isolated from the same organism might exhibit

a different pattern of activity during the cell cycle than the action on a foreign DNA substrate.

It is now accepted that DNA synthesis in bacteria involves a close association between an endonuclease highly specific for single stranded DNA and DNA polymerase. Kornberg (1969) has attributed both activities to a single enzyme complex. Although this association has not been demonstrated for higher plants, it was anticipated that there may be a correlation during the cell division cycle between the patterns of activity of 'denatured'-DNAase optimally active at pH 6·4 and DNA polymerase. For this reason, the activities of both enzymes were often measured in the same experiment, but no such correlation was found. From this evidence it would appear that the 'denatured'-DNAase optimally active at pH 6·4 is not associated with DNA biosynthesis. However, this finding does not exclude the possibility that a low DNAase activity associated with DNA biosynthesis might remain undetected in artichoke extracts, masked by the high activity of the pH 6·4 enzyme.

The pattern of activity of another hydrolytic enzyme; dTMP phosphatase, was also measured in the artichoke system. Although the activity of dTMP phosphatase was suppressed as far as possible by the use of high molarity phosphate buffer of high pH in the assay medium, the assay of the suppressed activity was shown to be quantitatively valid (Ch. 3), and was considered adequate to reflect any fluctuations in activity which might occur during the culture period. The pattern of dTMP phosphatase activity during the culture period was similar to the pattern described for 'denatured'-DNAase.

There are no other reports in the literature of dTMP phosphatase activity during the cell cycle, however, a few reports exist of phosphatase activity measured using more conventional assay methods. In the developing microspore of Lilium, Stern (1961) reported that both phosphomonoesterase and phosphodiesterase activities showed a steady increase both prior to and after the second meiotic division. In L cells synchronized with FUdR and TdR, Gold and Helleiner (1963), reported no marked rise or fall in triphosphatase activity during the cell cycle.

In the majority of micro-organisms, the synthesis of phosphatase enzymes can be derepressed by lowering the phosphate concentration of the culture medium. During synchronous growth of Chlorella pyrenoidosa, Knutsen (1968) has described the repressed syntheses of acid and alkaline phosphatases. A stepwise increase in repressed phosphatase activities occurred during the cell cycle and maximum derepression coincided with the period of DNA synthesis.

In bacteria, alkaline phosphatase activity increased continuously during the cell cycle when assayed under repressed conditions. Under induced or derepressed conditions however, the pattern of increase was stepwise with a doubling point once every cell cycle [Kuempel et al. (1965) in Escherichia coli, and Donachie (1965) in Bacillus subtilis].

In synchronous cultures of yeast, Gorman et al. (1964) reported a two step increase of derepressed alkaline phosphatase activity during the cell cycle of a diploid strain of Saccharomyces cerevisiae. In all the above reports for micro-

organisms, derepressed phosphatase activity doubled during DNA synthesis. In synchronous cultures of <u>Schizosaccharomyces pombe</u> however, Mitchison and Creanor (1969) observed that the doubling point of derepressed alkaline phosphatase activity occurred approximately one third of a cell cycle later than DNA synthesis.

From these reports, it is apparent that phosphatase activity increases during the cell cycle in micro-organisms. Any changes in phosphatase activity which occur in association with the cell cycle in artichoke tissue, would almost certainly be masked by the high acid and alkaline phosphatase activities associated with the autolytic effect in the rind cells of the cultured explant (Robertson 1966).

In contrast to the enzymes already discussed, certain bio-synthetic enzymes present at low levels in artichoke tissue, showed patterns of activity associated with cell division. Before considering the possible significance of these increases it is important to compare the different modes of expression of enzyme activity used for this and for other synchronous systems.

Several different methods have been used to express the results of enzyme experiments for other synchronous systems described in the literature. For suspension cultures of cells, activities are often expressed on a per cell basis, and therefore take account of cell number changes during the cell cycle. In other instances, enzyme activities have been expressed as a specific

activity, i.e. with respect to the protein content of the extract, and therefore take account of protein changes during the cell cycle.

For the artichoke system, enzyme activities were always expressed on a per explant basis and therefore did not take account of changes in any other parameter during the cell division cycle. Certain enzyme results however were also expressed on a per cell basis, to permit further interpretation of the results and to enable comparisons to be made with the results of other workers.

In the artichoke system, the activities of TdR kinase, dTMP kinase and DNA polymerase increased during the first division cycle and a different pattern of activity was established for each of these enzymes. Each pattern was established from the results of at least two separate experiments. However, due to the large amounts of tissue required for preparation of enzyme extracts, it was necessary to sample from different culture flasks throughout these experiments, which inevitably must lead to some degree of sampling variation.

A consideration of the variation inherent in cell cycle experiments of this particular design may help to explain why the results of replicated enzyme experiments were not always identical. Although all the cultures for one experiment were prepared and incubated using identical conditions, the results of experiments where an overlap point was included, often demonstrate how two cultures, incubated for the same length of time, can exhibit different 'stages' with respect to the cell cycle.

The pattern of TdR kinase activity during the cell cycle was investigated in three different experiments. The results of

two of these experiments revealed a possible peak in activity at the end of 'S' followed by a decline in activity during the period of cell division, whilst the results of the third experiment revealed a 'step-pattern' of activity. In the first two experiments, the peak in activity relied upon the activity of a single extract. As the third experiment was carried out using a more refined assay technique, it was concluded that the 'step-pattern' is probably the true pattern, although it is possible that due to sampling variation, the existence of a true peak was not detected in the third experiment.

The pattern of dTMP kinase activity during the first division cycle was investigated in two separate experiments. Although the pattern for this enzyme involved a decline in activity during the initial culture period which was not associated with cell division, the pattern of increase during 'S' was similar to that described for TdR kinase. In the first experiment, a stepwise increase in dTMP kinase activity was observed, whilst the results of the second experiment revealed a possible peak in activity prior to division based on the activity of a single extract. Further experiments may be needed to establish which of these patterns is the true pattern, but certain results from other synchronous systems may also help to clarify this point.

In synchronous cultures of Chlorella, Johnson and Schmidt (1966) also observed a decline in dTMP kinase activity during the initial culture period. In the same organism, Wanka and Poels (1969) observed that the activity of dTMP kinase also decreased during the period of cell division. Both these observations were

attributed to the unstable nature of this particular enzyme, so that an immediate decline in activity occurs when the enzyme is not being synthesized.

Another source of variation was particularly noticeable in the experiments which involved assays for dTMP kinase activity. In the first experiment, high dTMP kinase activities were observed in several cultures during the initial culture period whilst in the second experiment, a high activity was confined to the 0 hour culture. Owing to the fact that the first experiment was carried out very early in the 'artichoke season', this high activity was attributed to a seasonal effect, associated with the use of small tubers not yet expanded to their full size, in which a small percentage of cells was still in a dividing condition. Evidence for this explanation was provided by the observation that several weeks before the first cell cycle experiment, the dTMP kinase activity of freshly excised tissue was sufficiently high to enable a time course experiment to be carried out. Moreover, less than one week before the first experiment, the activity of freshly excised tissue was approximately 3 times greater than it was at the time of the first experiment. For assays carried out using identical conditions, with tissue from freshly harvested tubers, the following activities were obtained:-

Date of experiment.	dTMP kinase activity (p moles dTMP converted/20 min./0·05 ml. extract)
4.11.70	70
11.11.70	33
18.11.70 (Ex. 9)	11·5
	8·0

Although all the tubers used in the first cell cycle experiment (Ex.9) were from the same plant, each culture was prepared from an individual tuber, and it is suggested that the use of different tubers in different stages of development, was responsible for the high dTMP kinase activities observed in several cultures during the initial culture period.

It has now been established that the activities of TdR kinase and dTMP kinase exhibit patterns of increase during the cell cycle. Criteria which may be used to indicate whether or not such increases are due to a _de novo_ synthesis of enzyme protein, include the use of protein synthesis inhibitors and mixed extract experiments. Conclusive proof of a _de novo_ synthesis of enzyme protein would involve complex isolation and purification procedures for individual enzyme proteins. The approach which was used to tackle this problem in the artichoke system with respect to the observed increases in TdR kinase and dTMP kinase activities, was the use of mixed extract experiments. For both of these enzymes, mixed enzyme extracts prepared from cultures sampled before the increase began and after the increase had started, gave additive activity values. Therefore the increased activities were not due to activation of pre-existing enzyme, the removal of inhibitors or to the removal of the influence of competing enzyme systems. It is therefore probable that the increased activities of these enzymes were due to a net synthesis of enzyme protein.

When expressed on a per cell basis, the levels of TdR and dTMP kinases after division were fairly similar to the levels recorded

immediately prior to the increase.   This suggests that the function of the increased activity of these enzymes is to provide the daughter cell products of cell division with an amount of enzyme equivalent to that present in the parent cell.

The pattern of DNA polymerase activity during the first division cycle did not resemble the patterns described for TdR and dTMP kinase.   Firstly, a very gradual increase in DNA polymerase activity during the initial period of culture, was observed in two separate experiments.   Although the major increase in DNA polymerase activity occurred at approximately the same time as the other enzyme increases, this increase was enormous by comparison, and in two individual experiments the activity continued to increase during the period of cell division.

A possible explanation of the mammoth increase in DNA polymerase activity during the first division cycle in artichoke tissue, is that an extremely high level of DNA polymerase activity is required for cell division.   The low level of activity associated with freshly excised tuber tissue may be inadequate for this purpose.

The interpretation of the DNA polymerase results is somewhat complicated by the use of different concentrations of β-mercapto-ethanol (EtSH) in the grinding medium, for different cell cycle experiments.   For enzyme homogenates prepared in the presence of 0·5% EtSH, the total DNA polymerase activity consisted of a soluble and a bound enzyme component.   However, for homogenates prepared in the presence of 1·0% EtSH, Jackson (1970) observed that the DNA polymerase activity became completely soluble under these conditions

and resided in the supernatant fraction of the homogenate.

The fact that this enzyme activity is affected by EtSH concentration, suggests that the DNA polymerase enzyme of artichoke extracts contains an abundance of -SH groups. The DNA polymerase enzyme of E. coli (Kornberg's enzyme) is also an '-SH enzyme'.

In this investigation, DNA polymerase assays were carried out using homogenates prepared in the presence of 0·5% EtSH in two experiments. A third experiment employed a supernatant fraction of a homogenate prepared in the presence of 1·0% EtSH. In the third experiment the pattern of activity was fairly similar to that observed in the second experiment although the extent of the increase was far greater. In the first experiment however, the pattern of increase was stepwise and fairly similar to the patterns for TdR and dTMP kinase.

Some of the discrepancies between the DNA polymerase results of the three cell cycle experiments may be explained on the basis that in the first two experiments the enzyme was possibly being assayed under far from optimal conditions. Firstly, once the importance of the EtSH effect had been recognised, more care was taken to ensure that this volatile substance was added to the grinding medium at the beginning of the experiment. A particularly low concentration of EtSH may have been responsible for the low DNA polymerase activities observed in the first experiment. Secondly, all the incubations for the DNA polymerase assay were carried out using an ordinary water bath, not a shaking water bath,

and hence the tendency of cellular particles in crude homogenates to settle out under these conditions, may have resulted in less effective mixing between the substrate and partially bound enzyme in the first two experiments, compared with the third experiment in which a supernatant fraction was used.

A mixed extract experiment similar to that already discussed for TdR and dTMP kinases, was performed by Jackson (1970) for DNA polymerase activity of extracts prepared in the presence of 0·5% EtSH.   As the results of this experiment were also additive, it was concluded that the increased activity of DNA polymerase during the cell cycle assayed under these conditions, was not due to activation of pre-existing enzyme or the removal of inhibitors, but was probably also due to a net synthesis of enzyme protein. No mixed extract data is available for extracts prepared in the presence of 1·0% EtSH, but it is unlikely that this factor could markedly affect the results.

Other reports of DNA polymerase activity during the cell cycle are confined to algal and animal systems.   During synchronous growth of Chlorella, Schönherr and Wanka (1971) observed that DNA polymerase activity increased almost continuously during the cell cycle and did not correlate with DNA synthesis. During the burst of cell division following partial hepatectomy in the rat liver system, DNA polymerase activity increased at the same time as the increase in TdR incorporation into DNA, and remained elevated for at least 60 hours, despite waves of DNA synthesis (Ove et al. 1969).

In mouse fibroblast cultures synchronized with FUdR and TdR, the DNA polymerase activities of particulate and supernatant fractions of the extract were measured (Littlefield et al. 1963). In a number of replicate experiments there was a slight increase in the DNA polymerase activity of the particulate fraction during 'S', which was equivalent to $9 \cdot 0\%$ of the decrease in activity observed for the supernatant fraction during this period. In view of the fact that bound enzymes are more difficult to assay, these workers suggested that at the time of DNA synthesis, part of the supernatant polymerase activity may become particulate and therefore less active in terms of the usual assay conditions. Similar results were obtained from cultures of L cells synchronized with FUdR and TdR (Gold and Helleiner 1963).

Friedman and Mueller (1968), identified two types of DNA polymerase in synchronized Hela cells, which had different requirements in terms of -SH concentration, pH, salt concentration and ATP. The cytoplasmic polymerase was not associated with the cell cycle. In cultures synchronized with amethopterin and TdR, lysates prepared from cells which had been synthesising DNA for 3 hours were more active with respect to DNA polymerase activity than after 1 hour, whilst at 15 hours (after cell division) a very low activity was found.

Some of these results are similar to those obtained in the second cell cycle experiment with artichoke tissue, in that there was a suggestion of an increased contribution towards the total enzyme activity by a bound DNA polymerase activity during 'S'. However, the

capacity to solubilize the DNA polymerase activity of artichoke tissue using 1·0% EtSH is probably an artificial situation, in that a concentration of-SH groups of this magnitude is unlikely to occur in the _in vivo_ cell. Furthermore the -SH concentration within the cell may determine the relative proportion of soluble and bound DNA polymerase activity. It is therefore possible that the results obtained in the first cell cycle experiment in which the EtSH concentration may have been extremely low, were more indicative of the _in vivo_ situation.

Stern (1960) has shown that the pattern of available -SH groups in cell extracts varies during interphase of the second meiotic division in the developing microspore of _Lilium longiflorum_. Therefore it is possible to envisage an _in vivo_ control of DNA polymerase activity, where the -SH concentration within the cell regulates the relative proportion of bound and soluble enzyme during the cell cycle.

An important feature which relates to the interpretation of the enzyme results of this investigation in artichoke tissue, is the timing of enzyme changes with respect to the onset of DNA synthesis. In this series of experiments, two different methods were used to estimate the timing and extent of DNA synthesis. In several instances, measurement of the rate of DNA synthesis by incorporation of labelled thymidine into DNA was far more accurate in this respect, than Burton's chemical estimation of DNA. This indicates the superiority of the radiochemical method over at least

one commonly employed chemical method and supports the view that radiochemical techniques are generally more sensitive than other analytical methods.   This comparison has shown that the use of an insensitive DNA technique could conceivably lead to the misinter- pretation of the timing of enzyme changes with respect to the onset of DNA synthesis.   Hence it is important when comparing these results with those of other workers for other systems that their mode of DNA measurement should also be considered.

Measurement of the rate of DNA synthesis during the cell division cycle in the artichoke system provided much useful information about the timing of the events of interphase.   In all experiments it clearly defined the onset of 'S' and in experiments which were carried out towards the end of the 'artichoke season', it also clearly defined the end of 'S'.   However, at the beginning of the artichoke season when the lag phase of the first division cycle was shortest, there was often no fixed definition of the end of 'S' by this method.   This anomaly is thought to be a consequence of less synchrony in the system at the beginning of the 'season', which is emphasized by the fact that each sample was withdrawn from a different culture flask.

Evans (1967) has also measured the rate of DNA synthesis during the first division cycle in the artichoke system, for explants excised in the light and cultured in darkness, in the presence of coconut milk.   In this instance, all samples were withdrawn from the same culture flask and the curve obtained was similar in pattern to the data of Experiments 1, 2 and 8 (Ch. 4),

which were carried out at the middle or the end of the season.
From this comparison, it is suggested that the results of experiments
in which samples are withdrawn from different culture flasks, are
less susceptible to sampling variation later in the 'artichoke
season'.   (Between January and April).

This investigation includes five separate estimations of the
rate of DNA synthesis during the cell division cycle and in four of
these there is the suggestion of a shoulder in the rising part of
the curve.   In the case where this shoulder is absent, there is
evidence of a shoulder on the descending part of the curve.
Although these deviations from a normal peak pattern may be the
result of sampling from different cultures, the data of Evans (1967)
based on determinations carried out on explants sampled from the
same culture flask do not completely disagree with this hypothesis
as there may be a shoulder on the descending part of this curve.

In the literature there are several reports from other systems
that DNA synthesis during 'S' is discontinuous.   Using auto-
radiography, Hamilton (1969) identified a pause in mid-'S' due to
reduced isotope incorporation after short pulse labelling with
^{3}H-TdR in hard palate epithelial cells of the rat.   Stubblefield
and Gay (1970) examined DNA synthesis by quantitative tritium auto-
radiography during the cell cycle, for each chromosome type of the
Don C chinese hamster cell line.   Many chromosomes exhibited 3
separate steps of synthesis with reduced TdR incorporation between
3 and 4 hours and again between 5 and 6 hours of the 8 hour 'S'
period.   In the same report, these workers describe a 3 step pattern

of incorporation of ^{14}C-TdR into the acid soluble fraction of
the cell measured using conventional scintillation counting
techniques.

These observations provide support for the theory that in
the artichoke system, the rate of DNA synthesis curve need not take
the form of a normal curve.   In order to confirm the true shape of
the curve, more cell cycle experiments are needed in which all the
samples of explants are withdrawn from the same flask in order to
minimise sampling error.

Cleaver (1967) has suggested that in rate of DNA synthesis
studies, TdR labelled with tritium in the methyl position may be
more specifically incorporated into DNA than TdR labelled in the
6 position, due to the fact that some organisms, including many
plant tissues, are a rich source of the enzyme, thymidine oxidase.
(See Appendix 7 for labelling positions of TdR).   Thymidine
oxidase catalyses the conversion of thymidine (TdR) to thymine.
By loss of the methyl group, thymine can be converted to derivatives
of uracil which eventually become incorporated into RNA.
Alternatively, by conversion to thymidine and subsequent phosphoryl-
ation, thymine can be incorporated into DNA.   Cleaver has suggested
that, by use of methyl labelled ^{3}H-TdR, any conversion to uracil
derivatives would involve the loss of label, whereas use of TdR
labelled in any other position (including the -6 position) could
result in labelling of RNA.   This is a valid criticism, since the

estimation of the rate of DNA synthesis depends on measurement of
label in a total nucleic acid hydrolysate which contains both
RNA and DNA.

However, in the current investigation, the use of TdR-6-T
appeared to label DNA specifically for the 45 minute pulse periods
used.   If any incorporation into RNA had taken place, then it is
likely that the beginning of 'S' would have coincided with the
first increase in total nucleic acid levels.   In 3 different cell
cycle experiments for which rate of DNA synthesis and total nucleic
acid levels were measured, the first increase in total nucleic acid
occurred many hours before the beginning of 'S'.

Grivell and Jackson (1968) have observed that the inability
to incorporate added TdR specifically into DNA in Neurospora crassa
and other micro-organisms, may be associated with the absence in
these organisms of the enzyme TdR kinase.   In these organisms the
only pathway of TdR conversion available is degradation to thymine.

As artichoke tissue has been shown to possess TdR kinase
activity, a direct incorporation of labelled TdR into DNA is
possible mediated by phosphorylation, and it is unlikely that the
pathway of degradation to thymine is important in this tissue.

It is clear that the activities of certain biosynthetic
enzymes increase during the cell division cycle in the artichoke
system, and that these increases are in each case probably a result
of net protein synthesis.   There have been several suggestions

arising from this investigation that the observed enzyme increases occur after the onset of DNA synthesis and are dependent on DNA synthesis.

Firstly, in all cell cycle experiments the enzyme increases always occurred consequent with or subsequent to the onset of 'S'. Secondly, patterns of enzyme increase were not associated with a 'non-dividing' system, in which DNA synthesis had not occurred. Thirdly, there was a definite correlation between the levels of enzyme activity during 'S' and the level of DNA estimated using the Feulgen method.

The Feulgen method of DNA estimation is a refined histochemical technique and has been used by Mitchell (1967) to examine the pattern of DNA synthesis during the first division cycle in the artichoke system.   Although this method was not employed extensively in this investigation, it was used to measure the level of DNA during 'S' in a single cell cycle experiment (Ex. 4 alias 9) which included assays for TdR kinase, dTMP kinase and DNA polymerase activities.

The approximately linear relationships between average activities of each of these enzymes and Feulgen DNA levels during 'S' (Figs. 5.1 and 5.2) enabled correlation coefficients to be calculated (see Appendix 6) for each of these enzymes in the same experiment.   In each case the relationship was highly significant below the 1% level, therefore there is a real relationship between the activities of these enzymes and DNA synthesis during 'S'.
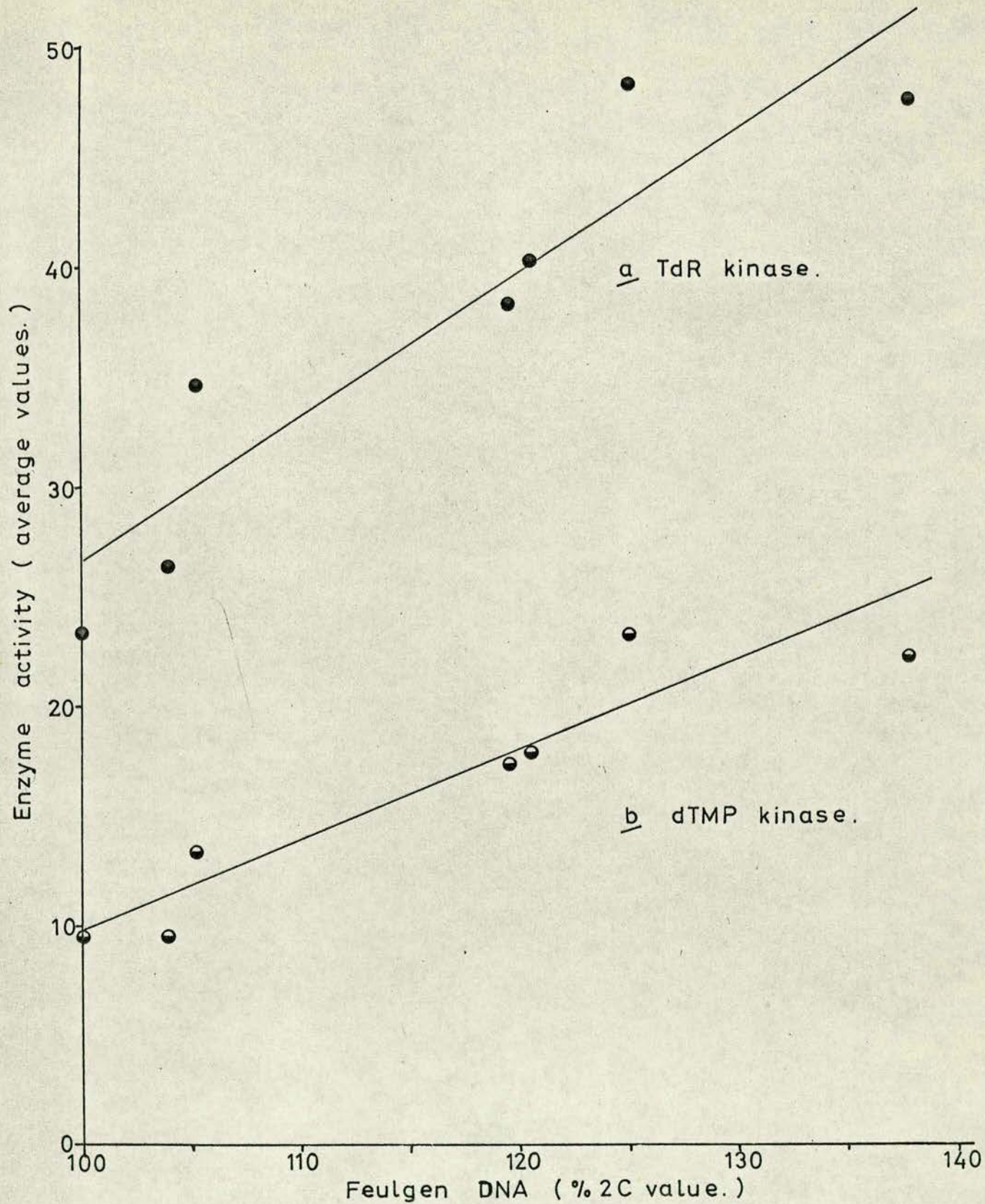
FIG. 5.1 The relationship between Feulgen DNA level and enzyme activity for a TdR kinase and b dTMP kinase.
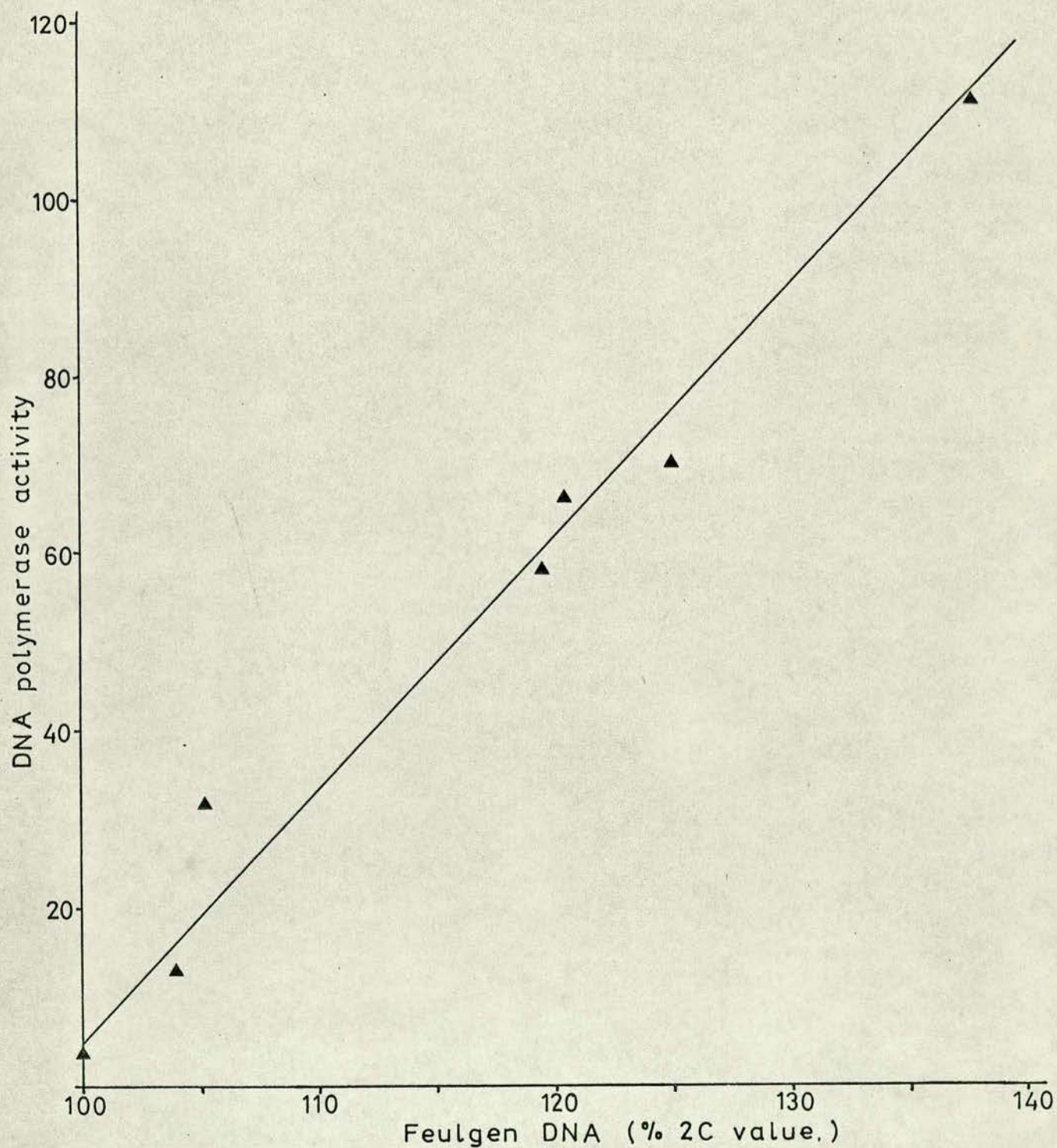
FIG. 5·2 The relationship between Feulgen DNA level and DNA polymerase activity during 'S'.

The correlation coefficient of 0·988 obtained for DNA polymerase was higher than the coefficients of 0·923 and 0·933 obtained for TdR kinase and dTMP kinase respectively. This may reflect the closer association of DNA polymerase with DNA synthesis. Is it a coincidence that the enzyme TdR kinase which is the most remote from DNA of these three enzymes on the pathway of DNA biosynthesis, should give the lowest correlation value?

The correlation data alone does not indicate which comes first, DNA synthesis or increased levels of enzyme, but when the answer to this question is provided from the results of inhibitor experiments it is possible that these data may reflect the order of the different enzyme syntheses.

When FUdR was used as a specific inhibitor of DNA synthesis (Experiment 14), no increase in TdR kinase or dTMP kinase activity was observed, suggesting that the increased enzyme levels during 'S' are dependent on DNA synthesis. It was therefore concluded that in the artichoke system, the increase in TdR kinase and dTMP kinase levels during 'S', were the result rather than the cause of DNA synthesis.

Jackson (1970) also used FUdR as a specific inhibitor of DNA synthesis in the artichoke system and found that a similar situation existed with respect to DNA polymerase activity. In this series of experiments however, the Feulgen method was used as an alternate way of measuring DNA levels. In contrast to the TdR kinase results, the inhibition of the DNA polymerase increase during 'S' and the inhibition of DNA synthesis (Feulgen) by FUdR, was completely

reversible if the initial culture medium contained a TdR concentration of 333 µg./ml., or partially reversible if this concentration of TdR was added at least 4 hours before the beginning of 'S'.

Although it proved impossible to reverse the FUdR inhibition of TdR kinase synthesis in this way, partial reversal may have occurred with respect to DNA levels. Moreover, it is maintained that a possible explanation of the failure to detect reversal may be associated with the position of TdR kinase with respect to TdR, on the pathway of DNA biosynthesis. The specificity of the FUdR inhibition of DNA synthesis relies on the ability of its mono-phosphate derivative (dFUMP) to inhibit the enzymic formation of dTMP as previously described (Experiment 14). However, in the reversal situation high concentrations of FUdR and TdR are present in the culture medium and in the explant tissues. The uptake of ^{3}H-TdR into the tissue during the cell cycle was described in Experiments 1 and 2. It is proposed that, although reversal of TdR kinase activity may have taken place, it was not possible to measure this. The extracts for the enzyme assays inevitably contained large amounts of unlabelled TdR, which could considerably reduce the specific activity of the ^{3}H-TdR in the reaction medium and therefore produce an activity lower than expected. The successful reversal of DNA polymerase activity described by Jackson (1970) may be a reflection of the remoteness of this enzyme from TdR, on the pathway of DNA biosynthesis.

A similar explanation can be tendered to explain the observed failure to reverse the FUdR inhibition of dTMP kinase synthesis during 'S', by a high concentration of TdR.   Dilution of the specific activity of the enzyme substrate by an excess of unlabelled dTMP, derived from unlabelled TdR via the TdR kinase reaction, is probably responsible for this failure.   It is maintained that it might be possible to detect reversal for both TdR and dTMP kinases, if the explants were subjected to an exhaustive dialysis procedure, designed to remove unlabelled substrate from the cells, prior to the enzyme assay.

Further indications of the temporal relationship between DNA synthesis and increased enzyme activity during the cell cycle are contained in the literature of other synchronous systems.   Hotta and Stern (1961) described a marked periodicity in TdR kinase activity during interphase of microsporogenesis in Lilium longiflorum and in Trillium erectum.

The brief appearance of TdR kinase activity prior to DNA synthesis in the microspores of Lilium was inhibited by Actinomycin D (an inhibitor of nucleic acid synthesis) and chloramphenicol (an inhibitor of protein synthesis), which suggested that enzyme synthesis and not merely enzyme activation was occurring (Hotta and Stern, 1963a).   The removal of TdR kinase some 6 - 12 hours after the onset of synthesis in lily microspores, was inhibited by subjecting the cells to anaerobic conditions, suggesting that the breakdown of TdR kinase is an energy-requiring process.   In addition,

the regulation of enzyme formation and removal was drastically up-
set by the presence of agents causing chromosome breakage (Hotta
and Stern 1963b).

In _Lilium longiflorum_, the appearance of TdR kinase activity
coincided with the appearance of a large pool of deoxyribosides.
As TdR kinase activity was shown to be inducible by its substrate
(TdR) in a heterogeneous population of wheat cells, experiments were
carried out to test for inducibility during the cell cycle.
Potentially inducible cells were found to be susceptible to the
inductive effect of TdR immediately prior to the interval when TdR
kinase activity normally appears (Hotta and Stern 1965).

Although it is possible that a cell cycle system of such long
duration as the lily system provides better resolution of the events
of the cell cycle than most systems, it is peculiar in that it
represents the interphase between the first and second meiotic
divisions rather than a true mitosis.   The DNA data for this system
may be subject to some doubt since the beginning of 'S' was defined
as the time when an increase occurred in the amount of DNA
(measured using inorganic phosphorus analysis) which was soluble
in 0·25M NaCl (Stern 1960).

In synchronous cultures of the slime mould, _Physarum polycephalum_
Sachsenmaier and Ives (1965) observed that the activity of TdR
kinase increased during mitosis to reach a peak during 'S'.   However,
this cell cycle system is unusual as there is no well defined G_1
(or pre-'S') phase so that 'S' commences immediately after mitosis

ends. It is therefore possible that the increase in TdR kinase activity is really confined to the 'S' phase and that the observed increase during mitosis is a result of asynchrony in the Physarum cell population. Such a consideration brings these results in line with results from the artichoke system.

Further support for an increase in TdR kinase activity before the beginning of 'S' is provided by the observations of Littlefield (1966) for mouse fibroblasts synchronized with FUdR and TdR. In this system, TdR kinase activity began to increase prior to 'S' and the pattern of increase was stepwise. When enzyme activities were expressed as specific activities i.e. with respect to the protein content of the extract, there was a decrease in activity towards the end of 'S' due to continued protein synthesis after the cessation of TdR kinase synthesis. The increase in TdR kinase activity could be prevented by the inhibitors Actinomycin D and puromycin and this was interpreted as an inhibition of the protein synthesis machinery prior to the onset of DNA synthesis. However, no DNA synthesis data is provided in this report and it is difficult to see how TdR kinase activity could have started to increase before the onset of 'S', since in systems synchronized in this way, DNA synthesis should begin immediately after TdR reversal.

Results from all the other systems which have been described support the view that TdR kinase activity increases at the same time as the onset of 'S'. In the regenerating rat liver system, Bollum and Potter (1959) found that TdR kinase activity increased

at the same time as the onset of DNA synthesis.   A similar result
was described by Lieberman et al. (1963) for cultured kidney cells
from the rabbit.

For Hela cells synchronized using amethopterin and TdR,
Stubblefield and Mueller (1965) observed that TdR kinase activity
expressed on a per cell basis, increased during 'S' and decreased
during the period of cell division.   Brent et al. (1965) obtained
a similar result for Hela cells synchronized by selective detachment
of mitotic cells in a calcium deficient medium.   In this situation,
TdR kinase activity expressed on a per cell basis, increased during
'S' to reach a maximum value approximately 5 hours after the peak
of DNA synthesis.   The activity then fell sharply during
subsequent division.

In colcemid synchronized chinese hamster fibroblasts,
Stubblefield and Murphree (1967) described an increase in TdR kinase
activity during 'S' which could not be inhibited by DNA synthesis
inhibitors added at the beginning of 'S'.   However, inhibition
occurred when FUdR and amethopterin were used to regulate the size
of the dTMP pool.   At this point it is relevant to note that both
these compounds are also inhibitors of DNA synthesis and it is
therefore possible to interpret these results as an inhibition of
enzyme activity as a result of inhibition of DNA synthesis during 'S'.

It is evident that the majority of reports in the literature
of TdR kinase activity during the cell cycle, agree with the results
obtained from the artichoke system.   It may be a coincidence that
many of the reports of increased TdR kinase activity during 'S' rely

on more refined DNA measurement e.g. Brent et al. (1965) used
incorporation of labelled TdR into DNA, whilst many reports of
increased TdR kinase activity before 'S' rely on less accurate DNA
techniques e.g. Hotta and Stern (1961) used inorganic DNA
phosphorus analysis.

There are very few reports in the literature of dTMP kinase
activity during the cell cycle, and those which do exist are
confined to algal and animal systems.   In synchronous cultures
of Chlorella pyrenoidosa, Johnson and Schmidt (1966) described an
increase in dTMP kinase activity which began prior to the onset
of DNA synthesis.   In this report, the level of DNA was measured
by inorganic phosphorus analysis and the enzyme activity was
expressed as units per µg. of cellular phosphorus.

Wanka and Poels (1969) attempted to relate the increase in
dTMP kinase activity to the control of DNA synthesis regulation in
Chlorella.   In this report, dTMP kinase activity began to
increase approximately 2 hours before the onset of DNA synthesis
which was determined using Burton's method.   The enzyme increase
was prevented by the presence of 15 µM actidione, which is a potent
inhibitor of protein synthesis in Chlorella.   The results of a mixed
extract experiment using extracts from three different parts of
the life cycle ruled out the possibility that enzyme changes were
due to activators or inhibitors.

In Euglena, Cook (1968) described an initial transient
appearance of dTMP kinase activity early in the light period of

the cell cycle.   However, these results are presented in the form
of a diagram and no experimental data is given.

In contrast to the above, the results of Brent et al. (1965)
for Hela cells synchronized by selective detachment of mitotic cells
in a calcium deficient medium, are in good agreement with results
from the artichoke system.   These workers used the incorporation
of ^{3}H-TdR into DNA to determine the rate of DNA synthesis.   The
dTMP kinase activity expressed on a per cell basis increased during
'S', reaching a maximum activity about 5 hours after the peak of
DNA synthesis, followed by a gradual fall during subsequent division.

It is suggested that discrepancies between the dTMP kinase
results from the artichoke and Hela cell systems and the algal
systems may be due to the use of less sophisticated DNA techniques
in the algal systems.

The respective roles of TdR kinase, dTMP kinase and DNA
polymerase on the pathway of DNA biosynthesis can be demonstrated
as follows, (Cleaver 1967).
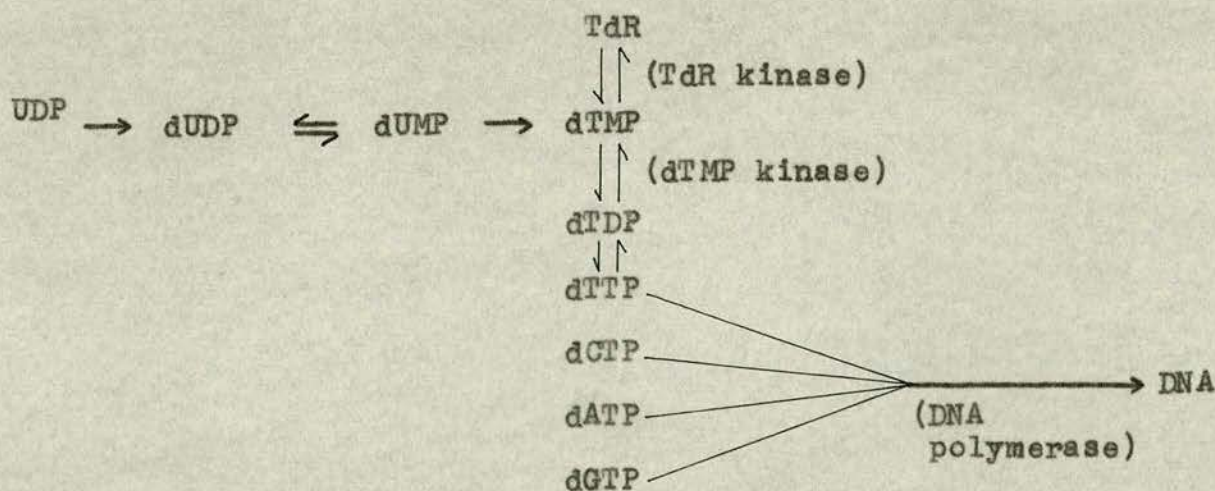
$$
\begin{array}{l}
\text{TdR} \\
\quad \downarrow\uparrow \text{ (TdR kinase)} \\
\text{UDP} \rightarrow \text{dUDP} \rightleftharpoons \text{dUMP} \rightarrow \text{dTMP} \\
\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad \downarrow\uparrow \text{ (dTMP kinase)} \\
\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad \text{dTDP} \\
\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad \downarrow\uparrow \\
\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad \text{dTTP} \\
\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad \text{dCTP} \\
\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad \text{dATP} \rightarrow \text{DNA} \\
\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad \text{dGTP} \quad \text{(DNA polymerase)}
\end{array}
$$

In bacteria, the enzyme TdR kinase has been assigned the role of 'scavenger' (Okazaki and Kornberg 1964a and b) since this enzyme is not normally on the direct pathway of DNA biosynthesis and its major function is probably the utilization of products from dead cells.   The main biosynthetic route of TdR nucleotide precursors of DNA is via the enzyme dTMP synthetase which catalyses the conversion of deoxyuridine monophosphate (dUMP) to dTMP.

Observations that sugar compounds containing thymidine derivatives have been identified in a number of organisms, have been reviewed by Cleaver (1967) and alternative roles for thymidine compounds are suggested.   For instance, Counts and Flamm (1966) identified a compound in mouse liver, which contained glycogen together with a phosphorylated derivative of TdR.   In cell free extracts of Pseudomonas aeruginosa, Kornfeld and Glaser (1960) described the enzymic formation of thymidine diphosphate (TDP)-glucose and its conversion to TDP-rhamnose.   This organism has been shown to secrete a rhamnose-containing lipid into the culture medium.

Although there have been no observations of this kind for higher plant tissues, it might be reasonable to envisage a possible role for thymidine nucleosides as intermediates in carbohydrate synthesis, in a similar way to the known role of UDP-glucose in this process.   In this situation, the incorporation of labelled TdR into substances other than DNA would be expected and if such compounds were associated with cell wall synthesis then labelling might be associated with the insoluble fraction of the cell.

In order to test the possibility that TdR might be incorporated into insoluble cell wall materials during the cell cycle in the artichoke system, in addition to estimation of the rate of DNA synthesis by incorporation of ^{3}H-TdR into DNA, an extra procedure was included to see if any ^{3}H-TdR remained in the residue (which included the cell wall fraction) after the nucleic acids had been extracted by standard perchloric acid (PCA) hydrolysis.   In Experiment 9, it was observed that during the 'S' period less than 10% of the ^{3}H-TdR counts remained in the residue after standard PCA extraction.   As the pattern of residual counts during the cell cycle resembled the pattern of the rate of DNA synthesis, it was suggested that the residual counts merely represented DNA residues which were difficult to remove from the tissue by the standard PCA procedure.   This procedure was repeated in the next experiment (Experiment 10) and similar results were obtained.   In this experiment however, an attempt was made to extract a proportion of the residual counts by a more stringent PCA hydrolysis procedure involving 3 consecutive extractions, each of 1 hour duration, with a total of 3·0 ml. of 1·0N PCA.   As the combined procedures were successful in extracting over 99% of the total counts during 'S', it was concluded that a large scale incorporation of ^{3}H-TdR into insoluble residues, was not occurring in the artichoke system.

However, during the pre-'S' period in both experiments a larger proportion of TdR counts remained in the residue than was observed for the 'S' period, and in the second experiment these

counts were also more difficult to remove by further PCA hydrolysis. This observation is attributed firstly to the fact that very little ^{3}H-TdR incorporation into DNA occurred during the pre-'S' period and as the counts were low this may have led to more error in the percentage values, and secondly that the DNA probably exists in a different physical state during the pre-'S' period and may therefore be more difficult to extract than the newly synthesized DNA produced during 'S'.

The results of the tests which involved measurement of residual counts in the artichoke system merely exclude the possibility of a large scale incorporation of ^{3}H-TdR into insoluble cell wall material. These results may not completely exclude the possible association of soluble TDP-sugar compounds with the biosynthesis of carbohydrate materials in this tissue.

Several theories of enzyme regulation have arisen from the results of work with a wide range of synchronous systems. In the only other higher plant system, for which enzyme data is available, the developing microspore system of Lilium, Hotta and Stern (1961) have advanced a theory to explain all the enzyme changes which were observed prior to the onset of DNA synthesis. It is proposed that the initial increase in deoxyribonuclease activity which may be associated with the surrounding anther tissue, provides a large pool of deoxyribosides which induces the synthesis of nucleoside kinases (e.g. TdR kinase). Because of the possible role of such enzymes in DNA replication, this leads to the onset of DNA synthesis

and subsequent cell division.

An explanation of this sort is not suited to the artichoke system as all the enzyme changes which were associated with the cell division cycle occurred either at the same time as, or after the onset of DNA synthesis.   Furthermore, the results of mixed extract and inhibitor experiments in the artichoke system suggested that the enzyme changes were a result rather than a cause of the onset of DNA synthesis.

There have been several theories advanced by individual workers to explain other observed enzyme increases before the beginning of 'S'.   Littlefield (1966) explained the periodic synthesis of TdR kinase in mouse fibroblasts, by constitutive synthesis of messenger-RNA for this enzyme immediately prior to DNA replication. Stubblefield and Murphree (1967) explained the observed increase in TdR kinase activity during 'S' in the chinese hamster cell system, by feedback control of enzyme synthesis regulated by the size of the thymidylate (dTMP) pool.

In Chlorella, Johnson and Schmidt (1966) and Shen and Schmidt (1966) have explained the observed increase in dTMP kinase and dCMP deaminase activities before the beginning of 'S', on the basis that all the enzymes on the dTTP pathway may be simultaneously limiting the rate of DNA synthesis, and that the synthesis of these enzymes may be co-ordinated.   Sequential induction of enzyme synthesis by the substrates of enzymes on the same pathway is suggested, and it is proposed that genes controlling the enzymes of

the dTTP pathway might exist within the same operon.

However, Wanka and Poels (1969) state that the above suggestion is only valid if a reasonably constant ratio exists between enzyme activity and the rate of DNA synthesis, throughout the cell cycle. These workers found that although DNA synthesis in Chlorella ceased after 18 hours, high activities of the enzymes dTMP kinase and uridine kinase were maintained. This observation therefore contradicts the proposal of Johnson and Schmidt (1966) that DNA synthesis in Chlorella is regulated by the level of enzymes such as dTMP kinase.

In Chlorella, Knutsen (1965) and (1968) has shown that the maximum inducibility of the enzymes nitrate reductase and acid and alkaline phosphatase, coincides with the period of DNA synthesis. This shows a connection between the induction of enzyme activity and DNA synthesis. Schmidt (1969) has recently reviewed the control of enzyme synthesis in Chlorella with reference to observations in yeast and bacterial systems.

Some suggestions that DNA polymerase activity in synchronous systems of animal cells might be regulated by the change from a soluble to a bound form of the enzyme during 'S' of the cell cycle, have already been considered.

The most comprehensive theory which has been advanced to explain observed enzyme increases during 'S', is that the enzyme increase coincides with replication of the structural gene for that enzyme, during the period of DNA synthesis. This theory has arisen mainly from observations with synchronous cultures of yeasts and bacteria.

In _Escherichia coli_, both synthesis of DNA and replication of the genome is non-random and sequential (Cairns 1963) (Nagata 1962). In bacteria, DNA synthesis occupies 70% of the cell cycle and enzymes can be induced (or derepressed) at any period during the cell cycle, but in yeasts and higher organisms, this is only possible during the period of DNA synthesis. Donachie (1964), observed that the rate of enzyme synthesis in _Neurospora crassa_ is directly proportional to the number of genes present if the basal, fully repressed enzyme level is measured.

In a diploid strain of the yeast _Saccharomyces cerevisiae_ which contained 2 non-allelic genes for each of the enzymes invertase and alkaline phosphatase, Gorman et al. (1964) described a 2 step increase in activity of both of these enzymes during the cell cycle. In addition, a hybrid yeast derived from two different strains of _Saccharomyces_, each of which contained an immunologically distinct species of β-glucosidase, produced two marked periods of β-glucosidase synthesis per life cycle.

Donachie (1965), described stepwise patterns during the cell cycle in _Bacillus subtilis_ for the enzymes ornithine transcarbamylase (OTCase) and argenine transcarbamylase (ATCase), whilst alkaline phosphatase (APase) was synthesized continuously. This worker suggested that each of the enzyme steps was regulated by a combination of the doubling of the structural gene for the enzyme and the instability of the enzyme.

Masters and Pardee (1965) related synthesis of several enzymes

including histidase, OTCase and sucrase in <u>B. subtilis</u>, to genetic
maps prepared using mutants unable to produce these enzymes.   The
order of enzyme synthesis corresponded fairly well with the order
of the genetic markers for the respective enzymes, on the
<u>B. subtilis</u> genome.   Tauro and Halvorson (1966) found a correlation
between the number of non-allelic genes for α-glucosidase activity
and the number of periods of enzyme synthesis during the cell
cycle in <u>S. cerevisiae</u>.

Mitchison (1969) has recently reviewed the subject of enzyme
synthesis during the cell cycle in microorganisms, and has
classified enzyme patterns into the four basic categories of
1. 'step', 2. 'peak', 3. 'exponential' and 4. 'linear'.   The first
two categories include enzymes which are synthesized at a specific
time during the life cycle (discontinuous).   Whether the enzyme
pattern takes the form of a 'step' or 'peak' may depend on the
stability of the enzyme.   The second two categories include
enzymes which are synthesized continuously throughout the lifetime
of the cell and are largely representative of the situation in
yeasts and bacteria.

The theories of enzyme regulation in micro-organisms can be
adapted fairly easily to the higher plant situation.   Although it
is not yet possible to carry out exhaustive genetical tests such
as those described for bacterial and yeast systems, it is widely
accepted that the replication of the chromosomes in higher organisms,
including higher plants, occurs in a genetically controlled sequence.

The first indication that there was a regular sequence in the duplication of chromosomes arose from observations in the root cells of Crepis by Taylor (1950).   The DNA subunits at the ends of the chromosomes began replication early, and the direction of replication proceeded towards the centromeres.   In the root cells of Bellevalia however, simultaneously replicating DNA appeared to be rather uniformly distributed among the chromosomes, for after a short period of contact with ^{3}H, all the chromosomes were usually labelled from end to end (Taylor 1958).

There have been many reports of asynchronous duplication of chromosomes in animal cells and recently, Stubblefield and Gay (1970) have described patterns of tritium labelling for each individual chromosome type of the Don C chinese hamster cell line. The species of artichoke used in this investigation however, may not be suitable for this type of study, since each individual nucleus has a large chromosome complement (2n = 102).

This investigation on the varying pattern of enzyme activities during the cell division cycle in the artichoke system, have revealed that the activity of certain enzymes associated with the DNA synthesis pathway e.g. TdR kinase, dTMP kinase and DNA polymerase, increase during the period of DNA synthesis.   For the enzymes TdR and dTMP kinase and for DNA polymerase (Jackson 1970) the increase is probably due to a de novo synthesis of enzyme protein.

The pattern of increase in TdR and dTMP kinase activities was in the form of a single 'step', similar to the 'step-patterns' of enzyme increase reported from bacterial and yeast systems. Although bacteria are procaryotic cells with a simple cellular organisation, yeasts are eucaryotic cells and are therefore of the same type as higher plant cells. Hence a similar explanation of enzyme changes with respect to replication of the genome is possible.

There are a large number of suggestions for future research which arise from the results of this investigation. Due to the sampling variation encountered in enzyme experiments where samples are withdrawn from different culture flasks, it would be advantageous to devise an efficient technique for the mass culture of explants. However, such a technique would inevitably be prone to serious microbial contamination, so that success would rely on a supply of sterile material and extremely effective aseptic techniques.

A valuable extension of the current investigation might also be the measurement of TdR kinase, dTMP kinase and DNA polymerase activities during the second division cycle in the artichoke system. In this system the second division cycle has a shorter lag phase than the first and may be more typical of a higher plant mitotic cycle as it is well separated from the induction of division processes associated with the lag phase of the first division cycle. However, a major disadvantage of the second division cycle is the fact that it is less synchronous than the first.

Investigation of the properties, mode of action and site of

action of such enzymes as TdR kinase, dTMP kinase, and DNA polymerase in artichoke tissue, might lead to a better understanding of the pathway of DNA biosynthesis in a higher plant tissue. For instance, Wanka et al. (1964) separated the partially purified TdR kinase enzyme from wheat seedlings into two separate protein bands P and T by starch paste electrophoresis, and suggested a 2 subunit structure for the enzyme. As all other plant tissues examined contained component P, it was suggested that enzyme activity is controlled by formation and degradation of component T. If component T could serve as part of several different kinases, then several enzyme activities could be controlled by the synthesis of a single protein. This hypothesis is rendered as a possible explanation of the appearance of several kinase activities at the onset of DNA synthesis in several synchronous systems. Similar experiments in artichoke tissue could prove or disprove this theory.

The identification of an endonuclease enzyme with a substrate specificity for denatured DNA which is associated with DNA polymerase action in artichoke tissue, might be possible if partial purification procedures were used in order to remove contaminants such as the pH 6·4 enzyme. The method which was used to assay 'denatured'-DNAase activity in this study, relied upon the release of acid soluble products and was therefore directed towards the measurement of exonuclease activity. Although some workers have employed a similar technique to measure endonuclease activity (e.g. Linn and Lehman (1965) in Neurospora crassa), better assay methods more specific for endonuclease are available; for example, the

method of Geidushek and Daniels (1965) involves retention of the oligonucleotide products of the reaction on cellulose nitrate membrane filters.

Another suggestion for future experiment is an investigation designed to find out whether the TdR kinase activity of artichoke tissue, is inducible by its substrate (TdR) and if so, to measure the inducibility of this enzyme during the cell cycle in relation to the changes in uninduced enzyme activity. As previously mentioned, experiments of this type have been carried out by Hotta and Stern (1965) in the developing microspore system of Lilium. Further evidence from other higher plant tissues that TdR kinase is an inducible enzyme includes a report by Hotta and Stern (1965) for wheat embryo tissue and a more recent report by Macleod (1971), of induction in the primary root of Vicia faba. This approach could then be extended to cover other higher plant enzymes which might be inducible e.g. nitrite reductase and nitrate reductase.

In this investigation, a DNA synthesis inhibitor was used in one experiment. Although the inhibitor FUdR is widely accepted as a specific inhibitor of DNA synthesis in bacteria, its effects on higher plant tissue are less well known. The results of the FUdR experiment were interpreted on the assumption that FUdR was specifically inhibiting DNA synthesis and no other process, in the artichoke system. A valuable extension to this work would therefore be an examination during the cell division cycle in artichoke tissue, of the effect of this inhibitor on the levels of total nucleic acid, protein and other parameters which increase

prior to DNA synthesis.   The artichoke system is ideal for testing the effects of inhibitors in this way.

Experiments designed to inhibit enzyme increases during 'S' by the use of inhibitors of RNA and protein synthesis are complicated by the fact that increases in RNA and protein precede the onset of 'S' in this system (Evans 1967).   Therefore, it would be necessary to add these inhibitors to the cultures immediately prior to the onset of 'S'.   Whether or not these substances could penetrate the cells fast enough to produce an inhibition of the enzyme increase is a matter of conjecture.

Finally, a study of the patterns of activity during the cell division cycle for enzymes which are associated with other processes besides DNA synthesis, e.g. enzymes of respiration, RNA synthesis etc., in relation to the rate of DNA synthesis, might aid the interpretation of the control of enzyme synthesis in a higher plant tissue.

In the future, knowledge of individual enzyme patterns, coupled with studies on the protein synthesis machinery of the cell, could lead to an understanding of the synthesis of individual enzyme proteins, during the cell division cycle, in a higher plant system.

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APPENDIX 1

List of enzyme activities which have been measured in other synchronous systems.

BACTERIA

1. ### Escherichia coli

        alkaline phosphatase (APase)
        dehydroquinase (DHQase)
        argenine transcarbamylase (ATCase)
        histidase   (Kuempel et al. 1965)

        leucine amino peptidase
        protease   (Kogama and Nishi 1965)

        glycyl glycine dipeptidase   (Nishi and Hirose 1966)

        ATCase
        β-galactosidase   (Masters et al. 1965)

2. ### Bacillus subtilis

        sucrase
        histidase   ("   "   "   ")

        ornithine transcarbamylase (OTCase)
        ATCase
        APase   (Donachie 1965)

        histidase
        ATCase
        OTCase
        DHQase
        sucrase   (Masters and Pardee 1965)

ANIMALS

1. ### Regenerating rat liver

        TdR kinase   (Bollum and Potter 1959)

        dCMP deaminase
        dTMP synthetase   (Maley and Maley 1960)

'denatured'-DNAase
DNA polymerase   (Ove et al. 1969)

alkaline DNAase   (O'Connor 1971)

2. Rabbit kidney cells

    DNA polymerase
    lactic dehydrogenase
    G-6-P dehydrogenase
    malic dehydrogenase
    adenosine deaminase
    hexokinase   (Lieberman et al. 1963)

3. Mouse fibroblasts

a. FUdR synchronized

    DNA polymerase   (Littlefield et al. 1963)
    DNA polymerase
    triphosphatase
    'native'-DNAase   (Gold and Helleiner 1963)

b. Amethopterin synchronized

    ribonucleotide reductase
    DNA polymerase   (Turner et al. 1968)

4. DON C chinese hamster fibroblasts

    TdR kinase   (Stubblefield and
                                Murphree 1967)

    lactate dehydrogenase
    G-6-P dehydrogenase   (Klevecz and Ruddle 1968)

    ribonucleotide reductase   (Murphree et al. 1969)

5. Hela cells

a. amethopterin synchronized

    TdR kinase   (Stubblefield and
                                Mueller 1965)

    DNA polymerase   (Friedman and Mueller 1968)

b. TdR synchronized

        polyadenosine diphosphoribose polymerase
        RNA polymerase   (Smulson et al. 1971)

c. synchronized by mitotic selection in a calcium deficient medium

        TdR kinase
        dTMP kinase   (Brent et al. 1965)

        dCMP deaminase   (Gelbard et al. 1969)

PLANTS

1. slime moulds

    <u>Physarum polycephalum</u>
        TdR kinase
        G-6-P dehydrogenase   (Sachsenmaier and Ives
                                        1965)

2. Yeasts

    <u>Saccharomyces cerevisiae</u>
        protease
        dipeptidase   (Sylvén et al. 1959)

        α-glucosidase   (Tauro and Halvorson 1966)

        DNA polymerase   (Eckstein et al. 1967)

        α-glucosidase
        alkaline phosphatase
        invertase   (Gorman et al. 1964)

    <u>S. fragilis/S. dobzhanskii</u> hybrid
        β-glucosidase   ("   " "   ")

    <u>Schizosaccharomyces pombe</u>
        ATCase
        OTCase   (Bostock et al. 1966)

        maltase
        sucrase
        acid phosphatase
        alkaline phosphatase   (Mitchison and Creanor 1969)

        tryptophan synthetase
        alcohol dehydrogenase
        homoserine dehydrogenase   (Robinson 1969)

3. Photosynthetic algae

 Chlorella pyrenoidosa

 nitrate reductase   (Knutsen 1965)

 dTMP kinase   (Johnson and Schmidt 1966)

 dCMP deaminase   (Shen and Schmidt 1966)

 acid phosphatase
 alkaline phosphatase   (Knutsen 1968)

 uridine kinase
 dTMP kinase   (Wanka and Poels 1969)

 ribulose-1,5-diphosphate
     carboxylase   (Molloy and Schmidt 1970)

 'denatured'-DNAase   (Schönherr et al. 1970)

 DNA polymerase   (Schönherr and Wanka 1971)

 Euglena gracilis

 dGMP kinase
 dTMP kinase   (Cook 1968)

 'native'-DNAase
 'denatured'-DNAase   (Walther and Edmunds 1970)

4. Higher plants

 Lilium longiflorum

 DNAase
 phosphatase
 phosphodiesterase   (Stern 1961)

 TdR kinase   (Hotta and Stern 1961)

 TdR kinase (induced)   (Hotta and Stern 1965)

 Trillium erectum

 TdR kinase   (Hotta and Stern 1961)

 Helianthus tuberosus

 'native'-DNAase
 'denatured'-DNAase
 DNA polymerase
 TdR kinase
 dTMP kinase
 dTMP phosphatase   (See Experimental Results
                                       Chapter 4)

APPENDIX 2

Period of culture (hours)	colonies formed/0·5 ml. medium		Maximum number of organisms/ ml. culture medium
	after 2 days at room temperature	after 3 days at room temperature	
4	0	0	0
8	0	0	0
12	48	50	100
16	5	5	10
18	31	34	68
22	78	94	198
26	0	0	0
30	12	17	34
34	0	9	18

a. Estimation of the number of micro-organisms per ml. of culture medium for Experiment 1 (Ch. 4).

Period of culture (hours)	colonies formed/0·5 ml. medium after 3 days at room temperature	Maximum No. organisms/ml. culture medium
2	0	0
4	0	0
6	2	4
8	0	0
10	0	0
12	0	0
14	0	0
16A	0	0
16B	0	0
18	0	0
20	1	2
22	0	0
24	0	0
26	0	0
28	0	0
30	0	0
32	0	0

b. Estimation of the number of micro-organisms present in the culture medium for Experiment 2 (Ch. 4).

Period of culture (hours)	colonies formed/0·5 ml. medium after 3 days at room temperature	Maximum No. of organisms/ ml. culture medium
2	0	0
4	0	0
6	0	0
8	0	0
10	0	0
12	1	2
14(1)	0	0
14(2)	0	0
16	1	2
18	0	0
20	1	2
22	2	4
24	1	2
26	2	4
28	0	0

c. Estimation of the number of micro-organisms present in the culture medium for Experiment 4 (alias Experiment 9) of Ch. 4.

APPENDIX 3

Estimation of the specific activities (in terms of measurable counts) of the radioactive media quoted in Ch. 3 section C.

1. For the ^{3}H-TdR medium used in Experiment 8 (Ch.4).

Specific activity of original preparation was 23·5 Curies/m mole

or 104 mCi/mg.

0·5 mCi of this was used to prepare 5 ml. of radioactive medium.

If 104 mCi of radioactivity is contained in 1 mg.

then 0·5 mCi "   "   "   "   "   " $\frac{0·5}{104}$ x 1 = 0·0048 mg.

or 4·8 µg.

The amount of unlabelled TdR added to 5 ml. of radioactive medium was 42 µg.

∴ Total TdR = 42 + 4·8 = 46·8 µg. ≃ 47 µg.

Molecular weight of TdR = 242

∴ 47 µg. = $\frac{47}{242}$ µ moles = 0·194 µ moles.

1 mCi = 3·7 x 10^7 disintegrations/sec.

= 3·7 x 10^7 x 60 dis./min.

= 3·7 x 10^7 x 60 x $\frac{34·5}{100}$ counts/min. (where the counting efficiency of ^{3}H is 34·5% (see Ch. 2))

∴ 0·5 mCi = 3·7 x 10^7 x 60 x $\frac{34·5}{100}$ x ½ cpm

= 3·82 x 10^8 cpm

In terms of measurable counts

specific activity of radioactive medium = 38·2 x 10^7 cpm/0·194 µ moles

or 197 x 10^7 cpm./µ mole

2. <u>For the ^{14}C labelled dTMP medium</u> used in Experiment 6 of

   Chapter 3 section C.

Specific activity of the original preparation was 57 mCi/m mole.

1 µCi of this preparation was contained in 2·5 ml. of radioactive

medium.　No unlabelled dTMP was added.　Therefore the specific

activity of the medium remains the same.

The specific activity in terms of measurable counts was calculated

as follows:-

57 µCi of radioactivity are contained in 1 µ mole

1 mCi = 3·7 x 10^7 dis./sec.

∴ 1 µCi = 3·7 x 10^4 " "

   or 3·7 x 10^4 x 60 dis./min.

   or 3·7 x 10^4 x 60 x $\frac{80·8}{100}$ counts/min. (where the efficiency
   of counting ^{14}C is
   80·8% - see Ch. 2)

   = 19·55 x 10^5 cpm.

∴ 57 µCi = 57 x 19·55 x 10^5 cpm

∴ specific activity in terms of measurable counts

   = <u>11·4 x 10^7 cpm./µ mole.</u>

3. <u>For the ^{3}H labelled dTMP medium</u> used in all the cell cycle
   experiments (Ch.4)

Specific activity of original preparation was 1,000 mCi/m mole

   or 3·1 mCi/mg.

0·5 mCi of this preparation was contained in 5 ml. of radioactive

medium.

No unlabelled dTMP was added.   Therefore the specific activity
of the preparation remains the same.

specific activity of the original preparation

$$= 1000 \text{ mCi/m mole or } 1000 \text{ μCi/μ mole.}$$

For an ^{3}H medium, from the labelled TdR calculation

$$0 \cdot 5 \text{ mCi} = 3 \cdot 82 \times 10^8 \text{ cpm}$$

$$\therefore \quad 1 \cdot 0 \text{ mCi} = 7 \cdot 64 \times 10^8 \text{ cpm.}$$

In terms of measurable counts

$$\text{specific activity} = \underline{76 \cdot 4 \times 10^7 \text{ cpm/μ mole.}}$$

APPENDIX 4

Statistical proof for Experiment 1 (Ch. 4) concerning the enzyme 'denatured'-DNAase.

In Experiment 1, two populations of values exist:-

      1. from 0 hours to 18 hours
      2. from 22 hours to 36 hours.

Series 1		Series 2	
Hours	'denatured'-DNAase activity (% conversion to acid soluble products)	Hours	'denatured'-DNAase activity (% conversion to acid soluble products)
0	4·3	22	6·4
	5·0		6·2
	4·1		6·7
2	3·2	24	9·0
	3·8		5·3
	3·7		6·3
4	3·5	26	5·7
	3·7		5·6
	4·0		5·4
6	4·6	28	7·1
	3·8		4·5
8	6·4		5·1
	5·7	30	5·9
	5·9		8·1
10	3·8		4·4
	3·1	32	8·7
	3·1		5·7
12	4·2		6·3
	4·5	34	6·8
	5·2		7·1
14	4·0		5·9
	3·2	36	8·1
	3·2		4·8
16	6·0		3·7
	5·8		
	4·9		
18	4·3		
	3·0		
	4·6		

Consider 2 large samples.   In the first there are n_1 observations, the mean is $\bar{x}_1$ and the standard deviation is S_1. The corresponding quantities in the second sample are n_2, $\bar{x}_2$ and S_2.   In order to compare the two samples, i.e. to test whether the true means μ_1 and μ_2 are equal:-

$$d = \frac{\bar{x}_1 - \bar{x}_2}{\sqrt{\dfrac{S_1^{\,2}}{n_1} + \dfrac{S_2^{\,2}}{n_2}}}$$

(where d refers to a table of d values)

Calculation of Variance (Vx)

Series 1	Series 2
n = 29	n = 24
$\Sigma x_1 = 124 \cdot 6$	$\Sigma x_2 = 150 \cdot 8$
$\Sigma x_1^2 = 561 \cdot 84$	$\Sigma x_2^2 = 995 \cdot 4$
$\bar{x}_1 = 4 \cdot 30$	$\bar{x}_2 = 6 \cdot 28$

$$SSx_1 = \Sigma x_1^2 - \frac{\Sigma^2 x_1}{n_1}$$

$$= 561 \cdot 84 - \frac{124 \cdot 6^2}{29}$$

$$= 26 \cdot 5$$

$$Vx_1 = \frac{SSx_1}{n_1 - 1} = \frac{26 \cdot 5}{28}$$

$$= 0 \cdot 946$$

but $S_1^2 = Vx_1$

$$SSx_2 = \Sigma x_2^2 - \frac{\Sigma^2 x_2}{n_2}$$

$$= 995 \cdot 4 - \frac{150 \cdot 8^2}{4}$$

$$= 47 \cdot 9$$

$$Vx_2 = \frac{SSx_2}{n_2 - 1} = \frac{47 \cdot 9}{23}$$

$$= 2 \cdot 08$$

$S_2^2 = Vx_2$

$$d = \frac{\bar{x}_1 - \bar{x}_2}{\sqrt{\dfrac{S_1^{\,2}}{n_1} + \dfrac{S_2^{\,2}}{n_2}}}$$

$$= \frac{4 \cdot 30 - 6 \cdot 28}{\sqrt{\dfrac{0 \cdot 946}{29} + \dfrac{2 \cdot 08}{24}}}$$

$$= 5 \cdot 74$$

On the d table P is less than $0 \cdot 01$ or 1%.

Therefore there is a low probability that the difference in means
is due to error, and therefore there must be a real difference
between the two populations of values.

APPENDIX 5

The dates upon which all experiments in Chapter 4 were carried out.

Experiment	Date
1	17/3/70
2	9/4/70
3	12/1/71
4	19/11/70
5	15/4/70
6	6/5/70
7	5/11/69
8	2/12/69
9	as Ex. 4.
10	as Ex. 3.
11	early January/70
12	3/3/71
13	15/2/71
14	10/3/71

APPENDIX 6

Calculation of correlation coefficients between enzyme activities and Feulgen DNA levels during 'S', for Experiment 4 (alias 9) of Ch. 4.

1. DNA polymerase

Culture period (hours)	Feulgen DNA (% 2C value) (x)	DNA polymerase activity (p moles) (y)
10	100·0	3·6
14	103·9	13·2
16	105·2	31·8
18	120·5	66·0
20	119·5	58·2
22	125·0	70·0
24	137·8	111·0

$$\text{correlation coefficient } (r) = \frac{S.P.xy}{\sqrt{SSx.SSy.}}$$

Calculation of sum of squares (SS).

$\Sigma x^2 = 95276\cdot59$ $\Sigma y^2 = 26162\cdot68$

$\Sigma x = 811\cdot9$ $\Sigma y = 353\cdot8$

$\Sigma^2 x = 659181\cdot6$ $\Sigma^2 y = 125174\cdot4$

$\dfrac{\Sigma^2 x}{n} = 94168\cdot8$ $\dfrac{\Sigma^2 y}{n} = 17882\cdot0$

$SSx = \Sigma x^2 - \dfrac{\Sigma^2 x}{n}$ $SSy = \Sigma y^2 - \dfrac{\Sigma^2 y}{n}$

$= 1107\cdot8$ $= 8280\cdot7$

Calculation of sum of products (S.P.)

$S.P.xy = \Sigma xy - \dfrac{\Sigma x \Sigma y}{n}$

$= 2994\cdot8$

Correlation coefficient (r) = $\dfrac{S.P.xy}{\sqrt{SSx.SSy.}}$ = $\dfrac{2994 \cdot 8}{3028 \cdot 7}$

$$= \underline{0 \cdot 988}$$

Significance of r

Standard deviation of r = Sr = $\sqrt{\dfrac{1 - r^2}{n - 2}}$

$$= \sqrt{\dfrac{1 - (0 \cdot 988)^2}{5}}$$

$$= 0 \cdot 06708$$

$d = \dfrac{r}{Sr}$ = $\dfrac{0 \cdot 988}{0 \cdot 0671}$

$$= 14 \cdot 72 \text{ at 5 degrees of freedom.}$$

On the d table, for d = 14·72 at 5 degrees of freedom there is a probability of less than 0·01 (or 1·0%) that the deviation of r is due to sampling error, therefore there is a real relationship between x and y.

2. TdR kinase.

Culture period (hours)	Feulgen DNA (% 2C value) (x)	TdR kinase activity (Av.) (p moles) (y)
10	100·0	23·3
14	103·9	26·4
16	105·2	34·7
18	120·5	40·3
20	119·5	38·3
22	125·0	48·4
24	137·8	47·8

$SSx = 1107 \cdot 8$ as previously

$$\Sigma y^2 = 10162 \cdot 3$$

$$\Sigma y = 259 \cdot 2$$

$$\Sigma^2 y = 67184 \cdot 6$$

$$\frac{\Sigma^2 y}{n} = 9597 \cdot 8$$

$$SSy = \Sigma y^2 - \frac{\Sigma^2 y}{n} = 564 \cdot 5$$

$$S.P.xy = \Sigma xy - \frac{\Sigma x \Sigma y}{n} = 30793 \cdot 2 - 30063 \cdot 5$$

$$= 729 \cdot 7.$$

$$\text{Correlation coefficient } (r) = \frac{S.P.xy}{\sqrt{SSx.SSy.}} = \frac{729 \cdot 7}{790 \cdot 8}$$

$$= \underline{0 \cdot 923}$$

$$Sr = \sqrt{\frac{1 - r^2}{n - 2}} = \sqrt{\frac{1 - (0 \cdot 923)^2}{5}}$$

$$= 0 \cdot 170$$

$$d = \frac{r}{Sr} = \frac{0 \cdot 923}{0 \cdot 170}$$

$$= 5 \cdot 43$$

On the d table, for $d = 5 \cdot 43$ at 5 degrees of freedom there is a probability of less than $1 \cdot 0\%$ that the deviation of r is due to sampling error, therefore there is a real relationship between x and y.

3. dTMP kinase

Culture period (hours)	Feulgen DNA (% 2C value) (x)	dTMP kinase activity (Av.) (p moles)
10	100·0	9·5
14	103·9	9·5
16	105·2	13·3
18	120·5	17·8
20	119·5	17·3
22	125·0	23·3
24	137·8	22·3

$SSx = 1107·8$ as previously

$$\Sigma y^2 = 2013·7$$

$$\Sigma y = 113·0$$

$$\Sigma^2 y = 12769·0$$

$$\frac{\Sigma^2 y}{n} = 1824·1$$

$$SS.y = \Sigma y^2 - \frac{\Sigma^2 y}{n}$$

$$= 189·6$$

$$S.P.xy = \Sigma xy - \frac{\Sigma x \Sigma y}{n} = 13533·9 - 13106·4$$
$$= 427·5$$

Correlation coefficient $(r) = \dfrac{S.P.xy}{\sqrt{SSx.SSy.}} = \dfrac{427·5}{458·3}$

$$= 0·933$$

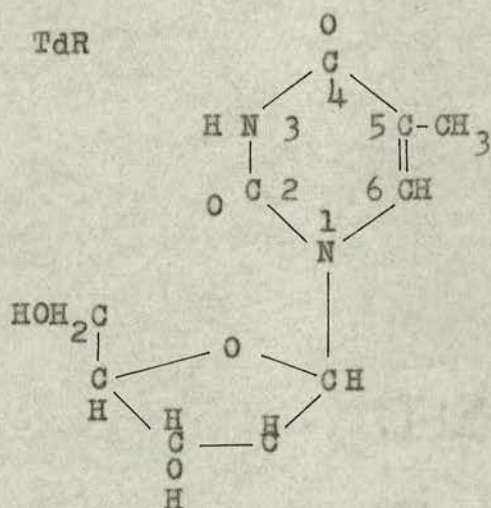$$Sr = \sqrt{\frac{1 - r^2}{n - 2}} = \sqrt{\frac{1 - (0·933)^2}{5}}$$

$$= 0·161$$

$$d = \frac{r}{Sr} = \frac{0·933}{0·161} = 5·79.$$

On the d table, for $d = 5·79$ at 5 degrees of freedom, there is a probability of less than 1·0% that the deviation of r is due to sampling error, therefore there is a real relationship between x and y.

APPENDIX 7

Miscellaneous Data

1. Labelling of thymidine and related compounds.

TdR

Substrates used in
this investigation
were:-

$6-^3H-TdR$

$2-^{14}C-dTMP$

$5(methyl)-^3H-dTMP$.

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2. Total p moles of TdR per 0·1 ml. of radioactive medium for each individual experiment of Ch.4.

Ex. 7.	3814
Ex. 8.	3867
Ex. 9.	3916
Ex.11.	3888
Ex.13.	3930
Ex.14.	3930

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3. Calculation of centrifugal force (g)

$$g = 1118 \times R \times rpm^2 \times 10^{-8}$$

(where R = radius of centrifuge rotor + buckets

rpm = revolutions/minute)

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4. Molecular weights.

^{3}H-TdR   242

^{3}H-dTMP   322

β-mercaptoethanol   78   (density 0·7 gm./cc.)

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5. Units.   m mole = 10^{-3} mole

μ mole = 10^{-6} mole

n mole = 10^{-9} mole

p mole = 10^{-12} mole

ABSTRACT OF THESIS

Name of Candidate   Jennifer Harland.

Address   4 Parkfield Avenue, Tollesby, Middlesbrough, Teesside.

Degree   Ph.D.   *Date*   September 1971

Title of Thesis   Changes in the pattern of enzyme activities during the
cell division cycle.

The initial aim of this investigation was to examine the patterns
of activity for a number of different enzymes during the cell division
cycle in a higher plant tissue.   The system employed in this study
was cultured tissue isolated from the tubers of the Jerusalem
artichoke (<u>Helianthus tuberosus</u>. L.).   Explants cultured in the
presence of a mineral salts medium containing sucrose and the synthetic
auxin 2,4-dichlorophenoxyacetic acid (2,4-D) exhibit a cell population
which divides synchronously for two or three divisions.   For cell cycle
experiments, all cultures were prepared in low intensity green light
and grown in liquid culture in darkness, in order to obtain the maximum
percentage cell division.

The enzymes chosen for this study were all associated with
deoxyribonucleic acid (DNA) metabolism and included enzymes of
biosynthesis and degradation.   Prior to investigations of enzyme
patterns during the cell division cycle, quantitatively valid assay
methods were established for each individual enzyme.

Cell cycle experiments were confined to the first synchronous cell
division of the artichoke system and the activity of each enzyme was
measured with particular reference to the onset and extent of DNA
synthesis as indicated by the rate of incorporation of labelled
thymidine into DNA.   Characteristic increases in the levels of DNA,
total nucleic acid, acid resistant protein and cell number during the
cell cycle, confirmed that the system was behaving as reported by
previous workers.

The activities of two deoxyribonuclease enzymes with substrate
affinities for native and denatured DNA respectively, and the suppressed
activity of thymidine monophosphate phosphatase, showed characteristic
patterns during the culture period which were not associated with cell
division but were a property of freshly excised tissue in culture.   The
biosynthetic enzymes DNA polymerase, thymidine (TdR) kinase and
thymidine monophosphate (dTMP) kinase however, showed characteristic
patterns of activity associated with cell division and in each case
the first major increase in enzyme activity was coincident with, or
subsequent to the onset of DNA synthesis.
The/

Use other side if necessary.

The increased activities of TdR and dTMP kinases during 'S'
were neither due to activation of pre-existing enzyme nor to the
removal of inhibitors.   FUdR, an inhibitor of DNA synthesis prevented
these enzyme increases during 'S', which suggests that the increases
occur as a result of DNA synthesis.

The results of these investigations have shown that the artichoke
system is suitable for an extensive examination of enzyme patterns
during the cell cycle.