CHANGES IN SOME ENZYMES INVOLVED IN DNA BIOSYNTHESIS FOLLOWING INDUCTION OF DIVISION IN CULTURED PLANT CELLS

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SUMMARY
The biosynthetic enzymes DNA polymerase, thymidine kinase (TdR kinase) and thymidine monophosphate kinase (dTMP kinase) show characteristic patterns of activity associated with cell division in synchronously dividing tissue cultures of the Jerusalem artichoke. In each case the first major increase in enzyme activity is coincident with and dependent upon DNA replication. In addition the increased activities of all 3 enzymes are not due to the activation of pre-existing enzyme or to the removal of inhibitors. A theory is advanced to account for the close correlation between DNA synthesis and the major rise in activity of the 3 enzymes.

INTRODUCTION
Knowledge of division in cells from higher plants is almost completely restricted to the events of mitosis and the timing of the various phases of division as originally defined by Howard & Pelc (1953). Only in one research school (Stern, 1966) has an attempt been made to discover the nature of the catalytic events which characterize the cell cycle up to and beyond mitosis. This work, performed with synchronously developing microspores from the anthers of species of *Lilium* and *Trillium*, provides the only record of changes in enzyme patterns which precede mitosis and meiosis in higher plants. In the present investigation a synchronously dividing plant tissue culture system has been used (Yeoman, Evans & Naik, 1966; Yeoman & Evans, 1967; Yeoman & Davidson, 1971) to determine the pattern of activity of a group of enzymes involved in the synthesis of deoxyribose nucleic acid (DNA). Particular attention has been accorded to the relationship of these enzymes, thymidine kinase (EC 2.7.1.20), dTMP kinase (EC 2.7.4.4) and DNA polymerase (EC 2.7.7.7) to the onset of DNA replication. The enzymes chosen for this study have all been examined in synchronously dividing systems of microorganisms and animal tissue cultures (Zeuthen, 1964; Mitchison, 1971), but only thymidine kinase (TdR kinase) has previously been studied in higher plant tissue in relation to the cell cycle (Hotta & Stern, 1961, 1963).

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MATERIAL AND METHODS

Plant material

All of the experiments described in this communication were performed with explants isolated from the mature tuber of the Jerusalem artichoke (Yeoman, Dyer & Robertson, 1965). The preparation of the tissue and the culture procedures employed have been described in detail elsewhere (Yeoman & Davidson, 1971). The medium consisted of a simple mineral salts mixture in 4% sucrose with 2,4-dichlorophenoxyacetic acid (2,4-D) at $10^{-4}$ M (Yeoman & Mitchell, 1970). Cell numbers were estimated according to the method described by Brown & Rickless (1949). DNA (Feulgen) per nucleus was measured according to the method described by Mitchell (1969).

Measurement of the rate of incorporation of [\textsuperscript{3}H]\textsuperscript{3}H-thymidine into DNA

The rate of DNA synthesis was estimated by measuring the rate of incorporation of [\textsuperscript{3}H]-thymidine (\textsuperscript{3}H-TdR) into DNA. Thymidine (TdR) labelled with tritium in the ‘6’ position, at a specific activity of 20–25 Ci/mmol was employed. Rate of incorporation of this compound was found to be linear with time for pulses up to 60 min (Evans, 1967). A pulse of 45 min duration was used in all the experiments described in this communication. Sixteen explants were transferred from the culture flask to a 25-ml conical flask containing 5 ml of culture medium, a magnetic stirrer, and \textsuperscript{3}H-TdR at a concentration of 3 \muCi/ml. The contents of the flask were agitated under standard culture conditions for 45 min and then the explants and stirrer were transferred to a 100-ml conical flask containing 8 ml of a $3 \times 10^{-4}$ M solution of unlabelled TdR in distilled water and agitated for a further 15 min. The explants were fixed in cold methanol and stored in the deep freeze at $-20 \, ^\circ C$. The total nucleic acid of groups of 8 explants was extracted in hot perchloric acid according to the method described by Yeoman & Mitchell (1970). The acid hydrolysate was neutralized with 5 N KOH and the tubes cooled in ice to accelerate the precipitation of potassium perchlorate. Aliquots of the clear supernatant were counted using a standard scintillation technique. The results were expressed as counts/min/explant of \textsuperscript{3}H-TdR incorporated into DNA. The rate of uptake of \textsuperscript{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% trichloroacetic acid (TCA), to the average counts incorporated into DNA. This value for uptake does not include the radioactivity removed during the 15-min chase in non-radioactive thymidine. The rate of incorporation of \textsuperscript{3}H-TdR into DNA was not limited by the rate of uptake of \textsuperscript{3}H-TdR into the tissue.

Preparation of the extract for enzyme assays

The contents of each culture flask were filtered through muslin and the explants retained by the fabric washed 3 times with distilled water. Groups of 100 explants were placed on Whatman No. 1 filter paper to remove the excess surface moisture and then transferred to a mortar at 0 °C for 5 min. The cold grinding medium consisted of 1 ml 0.2 M sodium phosphate buffer at pH 8.0 or 1.5 ml of 0.01 M Tris-maleate buffer at pH 7.2 and contained 0.07 M \beta-mercaptoethanol and 400 \mug/ml of bovine serum albumin (BSA). After preliminary maceration of the tissue, using a pestle and mortar, the tissue was further homogenized using a Kontes hand glass homogenizer. This treatment resulted in the disruption of nuclei, plastids and mitochondria. An aliquot of the resulting crude extract was used for DNA polymerase assays. Finally, when TdR kinase and dTMP kinase activities were to be determined, the homogenate was centrifuged at 2200 \times g for 10 min at 0 °C, and the supernatant fraction retained for use as the enzyme extract. Assays were carried out immediately following the preparation of the extracts.

Enzyme assays

TdR kinase. The assay is a modification of the method described by Hotta & Stern (1963). The enzyme substrate used was thymidine labelled with \textsuperscript{3}H in the ‘6’ position and was supplied by Radiochemicals of Amersham at a specific activity of 20–30 Ci/mol. The total volume of
Enzymes involved in DNA biosynthesis

The reaction mixture was 225 μl and contained 40 μg of bovine serum albumin (BSA); 4.5 μmol \( \beta \)-mercaptoethanol; 0.05 μmol MgCl\(_2\); 0.2 μmol ATP; 0.5 μmol phospho-enol pyruvate; 3.47 nmol thymidine; 0.4 nmol [6-\(^3\)H]thymidine, specific activity 253 μCi/nmol; 0.25 mg phospho-enol pyruvate kinase; 100 μl of enzyme extract; and 40 μmol sodium phosphate buffer. The final pH of the mixture was 8.0.

The reaction mixture was incubated in a shaking water bath at 37 °C for 20 min and the reaction was terminated by the addition of 1000 μl of 95% ethanol. Complete denaturation of the protein was secured by incubation of the mixture in a water bath at 100 °C for 2 min. The precipitated proteins were separated from the mixture by centrifugation at 1000 g for 5 min at 0 °C, the supernatant was decanted into a specimen tube and the pellet re-extracted with 0.2 ml of 70% ethanol, re-centrifuged and the supernatants combined.

The product of the reaction (dTMP) was separated from excess substrate (Tdr) by subjecting a 30-μl aliquot of the ethanolic supernatant to electrophoresis for 4 h on cellulose acetate paper (19 cm long) at 4 °C in 0.5 M ammonium formate buffer pH 3.5 at a potential difference of 200 V. The air-dried papers were cut into a series of 0.5-cm strips down the length of the paper and the activity present in each strip counted using standard scintillation techniques. Blank estimations were carried out by replacing the enzyme extract with buffer. After correction for the blank, enzyme activities were expressed as pmol of TdR converted to dTMP/min/ml extract.

d TMP kinase. This enzyme converts thymidine monophosphate (dTMP) to thymidine diphosphate (dTDP) in the presence of ATP. The radioactive substrate employed was thymidine \([\text{Me-}^3\text{H}]\)-5'-monophosphate (i.e. dTMP labelled with \(^3\)H in the methyl position), supplied at a specific activity of 1 Ci/mmol. The total volume of the reaction mixture was 100 μl and contained 20 μg of BSA; 2.25 μmol \( \beta \)-mercaptoethanol; 0.025 μmol MgCl\(_2\); 0.41 μmol ATP; 0.25 μmol phospho-enol pyruvate; 0.5 μmol \([\text{Me-}^3\text{H}]\)-dTMP (1 μCi/nmol); 20 μmol sodium phosphate buffer; 100 μg phospho-enol pyruvate kinase; and 50 μl of enzyme extract (added last). The final pH of the mixture was 8.0. The assays were performed as described for TdR kinase except that the reaction was terminated by placing the reaction tubes in a boiling water bath for 2 min without the addition of ethanol. The supernatant free from proteins was stored in a deep freeze at -20 °C. The product of the reaction (dTDP) was separated from excess substrate (dTMP) by high-voltage paper electrophoresis of a 30-μl aliquot of the supernatant. Electrophoresis was carried out for 1 h at a potential difference of 1 kV on Whatman No. 3MM chromatography paper (45 cm long) using 0.05 M ammonium formate at pH 3.5. Each electrophoresis path was cut into 24 strips 1 cm wide. The strips were cut in half and the activity present counted using standard scintillation techniques. Blank estimations were carried out by replacing the enzyme extract with buffer. After correction for the blank, enzyme activities were expressed as pmol of dTMP converted/min/ml extract.

DNA polymerase. DNA polymerase was assayed by a method similar to that described by Wever & Takats (1970). The extract was prepared as described for TdR kinase except that the final centrifugation was omitted. The radioactive substrate used was Schwarz [\(2^{-14}\text{C}\)] deoxythymidine triphosphate (dTTP) obtained as a 10 μCi/ml solution in 50% ethanol at a specific activity of 45.5 mCi/mmol. 'Activated' calf thymus DNA was prepared by treatment with crystalline pancreatic DNase as described by Aposhian & Kornberg (1962), and denatured before use by heat treatment followed by rapid cooling in ice. The total volume of the assay mixture was 350 μl and it contained 40 μg of BSA; 1.0 μmol Tris-maleate buffer; 8.46 μmol \( \beta \)-mercaptoethanol; 49.5 μmol Tris-HCl; 429 μmol MgCl\(_2\); 0.43 μmol ATP; 0.19 μmol dATP; 0.19 μmol dGTP; 3.6 mmol [\(2^{-14}\text{C}\)]-dTTP; 125 μg activated denatured calf thymus DNA; and 100 μl extract. The final pH of the mixture was 8.0.

The assay was performed at 30 °C. After 30 min incubation, the reaction was stopped by addition of 0.6 ml of 7% perchloric acid (PCA) following the addition of 0.025 ml of a solution containing 10 mg/ml BSA. The mixture was kept in ice for 10 min and 2 ml of cold water were added. The mixture was centrifuged at 2000 g for 5 min and the supernatant discarded. The pellet was dissolved in 0.3 ml of 0.2 M NaOH and after 1 h 0.6 ml of 7% PCA was added and the procedure repeated until 4 precipitations with PCA had been performed. This ensures that the excess unincorporated labelled dTTP is completely removed. Finally, the pellet was dissolved in 1 ml of 2 M NH\(_4\)OH plated on to a ringed aluminium planchet and counted for 20 min using a Beckman Lowbeta gas flow counter. Control values
were obtained by incubating a sample without enzyme for 30 min. Extract was then added and the reaction stopped at once. After correction for the blank, enzyme activity was expressed as pmol dTTP incorporated/min/ml of extract. DNA polymerase activity was dependent upon the addition of all 4 deoxyribonucleotide triphosphates, and the product of the reaction was shown to be degraded by pancreatic DNase. Addition of primer DNA was essential for activity, activated DNA giving the highest result, while denaturation also increased activity. Poly dAT copolymer was also very active as a primer with these plant extracts. It was shown that DNA prepared from the artichoke tubers was no more active than the calf thymus DNA routinely used.

Previous investigation has shown that all 3 enzymes were being assayed under optimum conditions. The enzyme extract employed exhibited linearity with respect to amount and time (Harland, 1971).

RESULTS

The data presented in Fig. 1 show the periodicity with respect to the increase in DNA in explants induced to divide. This period of DNA replication, which commences at about 12-14 h and is completed by approximately 22 h, is followed by a synchronous division at about 22 h. This result is consistent with the earlier observations of Mitchell (1967) and Yeoman & Evans (1967) and once again confirms the synchronous behaviour of the system with respect to DNA accumulation and cell division. The rate of incorporation of $[3H]$thymidine into DNA in a further experiment is shown in Fig. 2. Two sets of data are presented here: for the rate of uptake and incorporation of $[3H]$thymidine into the tissue; and rate of incorporation into DNA. In this experiment the synchronous division takes place 24 h after excision and the rate of uptake increases about 6-fold during the first 10 h of culture at which time 4% of the added counts have been taken up by the tissue. The rate of uptake decreases markedly after 10 h until 24 h, and then rises again. During the first 12 h of culture the rate of incorporation of $[3H]$thymidine into DNA is extremely low and
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Remains constant. Thereafter the rate increases and reaches a maximum at 22 h (0.7% of added counts in DNA) and then decreases to reach a minimum value at 24 h. Subsequently the rate rises again after 30 h heralding the preparation for the second synchronous division. It is to be noted that the rate of uptake into the tissue was never less than twice the rate of incorporation into DNA and was usually more than five-fold. This would suggest that the rate of incorporation is unlikely to be restricted by the rate of uptake and certainly does not affect the timing of DNA replication, which is the major point of interest in this study.

The fact that the rate of incorporation does not decrease further after the completion of the first S-phase is puzzling, particularly when it can be seen from the data presented in Fig. 1 that DNA accumulation is arrested at the time of cell division. Studies on changing mitotic index with time (Yeoman & Aitchison, 1973a) have
shown a clear separation between the peaks of mitotic index which precede divisions one and two and the persistence of a low index of 1–2% between the peaks. This is clear evidence for the presence of a small percentage of dividing cells out of step with the rest of the dividing population but fails to explain the relatively high rate of \(^{3}\text{H}-\text{TdR}\) incorporation observed after the termination of the first \(S\)-period in this and other experiments. It has also been shown, using autoradiography, that RNase fails to remove the label incorporated into the nuclei, suggesting that the added \(^{3}\text{H}-\text{TdR}\) has not been incorporated into RNA. This problem is now under investigation and will be reported in a later communication.

Changes in the activity of TdR kinase during the cell cycle are shown in Fig. 3. From these results it can be seen that at excision the activity of this enzyme is low.
Enzymes involved in DNA biosynthesis

Fig. 4. dTMP kinase activity (●) in relation to the rate of incorporation of [3H]-TdR into DNA (○) during the cell cycle. Activity per cell (■) calculated as in Fig. 3.

and remains so for the first 12 h of the cell cycle. The first rise in activity occurs at about 14 h and thereafter increases sharply reaching a maximum value at 20 h. Subsequently the activity falls reaching a steady value at 22 h, just before division. Activity per cell after division

\[
\frac{\text{Activity per explant}}{\text{Total cell number per explant}}
\]

is approximately the same as the activity per cell at excision. The rate of incorporation of [3H]thymidine into DNA commences just before the rise in enzyme activity and reaches a maximum value at 16 h, thereafter decreasing to a steady value at 22 h.

The change in activity of dTMP kinase during the first cell cycle is shown in Fig. 4. Activity of this enzyme in freshly excised explants is fairly high and decreases markedly after 4 h of culture, reaching a steady value at about 6 h. It is not until 16 h that the activity rises and this increase continues until cell division. Activity per cell after division

\[
\frac{\text{Activity per explant}}{\text{Total cell number per explant}}
\]
Fig. 5. Changes in DNA polymerase activity in explants cultured with (●) and without (○) 2,4-D. Rate of incorporation of $^3$H-TdR into DNA in the culture with 2,4-D is also shown (▲). Explants without 2,4-D fail to synthesize DNA and do not divide.

is approximately the same as at excision. The rate of incorporation of $[^3]$H-thymidine into DNA is extremely low until 10 h, thereafter it increases very sharply, and before the rise in enzyme activity, reaching a maximum value at 18–22 h.

The pattern of change of DNA polymerase activity is shown in Fig. 5. The activity of this enzyme increases very slowly during the early culture period followed by a much more rapid increase commencing at about 20 h. The considerable increase in activity of this enzyme is quite different and in marked contrast to the smaller changes in TdR kinase and dTMP kinase. The rate of incorporation of $[^3]$H-thymidine into DNA is constant at a low level until 16 h after which it rises very rapidly reaching a maximum value at 24 h and then falls sharply reaching a minimum value at 32 h (Fig. 5). Here as with the other 2 enzymes there is a marked coincidence between the onset of DNA replication and the major rise in enzyme activity.

As can be seen in Table 1, the primer requirements of the activity do not show
Enzymes involved in DNA biosynthesis

Table 1. Primer requirements for DNA polymerase activity at different growth phases of the artichoke tuber explants

125 μg DNA were used in each case, except that 60 μg only were used where artichoke DNA acted as the primer. The S-period commenced at 12-13 h, and the first cell division at about 30 h.

<table>
<thead>
<tr>
<th>DNA primer</th>
<th>Culture period, h</th>
<th>DNA polymerase activity, pmol dTTP incorporated/min/ml extract</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>20</td>
</tr>
<tr>
<td>Native calf thymus</td>
<td>0.4</td>
<td>0.8</td>
</tr>
<tr>
<td>Denatured calf thymus</td>
<td>0.8</td>
<td>1.9</td>
</tr>
<tr>
<td>'Activated' native calf thymus</td>
<td>0.5</td>
<td>1.8</td>
</tr>
<tr>
<td>'Activated' denatured calf thymus</td>
<td>2.0</td>
<td>5.7</td>
</tr>
<tr>
<td>Native artichoke</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>Denatured artichoke</td>
<td>0.8</td>
<td>1.3</td>
</tr>
</tbody>
</table>

any great change over the culture period. With the exception of native artichoke DNA an increase in activity is seen with all the DNA primers tried. It is possible to demonstrate the existence of more than one type of DNA polymerase in a cell by examination of DNA primer requirements. Thus the various DNA polymerase activities in *Escherichia coli* are known to show preferences for different DNA primers. Moses & Richardson (1970) and Chiu & Sung (1972) have shown that primer requirements can vary according to the stage of development in rat brain. The latter authors suggest that in rat brain there are 2 DNA polymerase activities involved, one predominating in proliferating tissue and which prefers denatured template, and another, perhaps involved in repair, which will only utilize native DNA primer. It is of interest therefore that no such clear changes in primer requirements were observed in the present plant system, and so we have no evidence for a change in the type of DNA polymerase through the culture period. Like the maize DNA polymerase (Stout & Arens, 1970) and the rat brain 'proliferating' activity, the present enzyme activity shows marked preference for a denatured DNA primer.

Omission of 2,4-D from the culture medium results in the failure of cell division (Yeoman & Mitchell, 1970) and this has been used to determine whether the changes observed in enzyme activity are related to cell division or to other events taking place during culture. The data presented in Table 2 show that explants cultured without 2,4-D show no marked change in the activity of TdR kinase. Similarly cultures without 2,4-D failed to show any change in the activity of DNA polymerase (Fig. 5). Cultures without 2,4-D show a decrease in dTMP kinase but no subsequent increase was detected (Table 3). Incorporation of [3H]thymidine into DNA is virtually non-existent in cultures without 2,4-D (Table 3).

It seems clear that the increases in activity are related to cell division but it is not possible to be certain whether the changes in activity reflect changes in the amount of enzyme. One possibility is that changes in activity are a result of the presence of activators or inhibitors and that the pattern of activity observed is a reflection of a
Table 2. Comparison of the activity of TdR kinase in cultures with (dividing) and without (non-dividing) 2,4-D

<table>
<thead>
<tr>
<th>Period of culture, h</th>
<th>TdR kinase activity, pmol TdR converted/min/ml extract</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>With† 2,4-D</td>
</tr>
<tr>
<td>0</td>
<td>2·9 (2·7, 3·5, 2·6)</td>
</tr>
<tr>
<td>•18</td>
<td>8·4 (8·8, 8·2, 8·2)</td>
</tr>
<tr>
<td>•20</td>
<td>9·6 (9·0, 8·8, 10·9)</td>
</tr>
<tr>
<td>•22</td>
<td>8·3 (8·6, 8·4, 7·8)</td>
</tr>
</tbody>
</table>

* During the S-period.
† Mean values are stated, with replicate values in parentheses.

Table 3. Comparison of the activity of dTMP kinase in cultures with (dividing) and without (non-dividing) 2,4-D

<table>
<thead>
<tr>
<th>Period of culture, h</th>
<th>dTMP kinase activity, pmol dTMP converted/min/ml extract</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>With 2,4-D Mean values</td>
</tr>
<tr>
<td>0</td>
<td>19·5 (52)</td>
</tr>
<tr>
<td>10</td>
<td>5·5 (49)</td>
</tr>
<tr>
<td>12</td>
<td>7·5 (29)</td>
</tr>
<tr>
<td>24</td>
<td>15·0 (1200)</td>
</tr>
<tr>
<td>26</td>
<td>11·0 (1350)</td>
</tr>
<tr>
<td></td>
<td>14·0</td>
</tr>
</tbody>
</table>

Figures in parentheses are mean counts/min/explant of 
3H-TdR incorporated into DNA, a measure of the rate of DNA synthesis.

change in the pattern of activators or inhibitors. The results of an investigation on the effects of mixing equal volumes of high and low activity extracts are shown in Table 4. (All explants were grown in the presence of 2,4-D.) Before proceeding to a consideration of these data it is important to note that the volume of the extract is proportional to enzyme activity for both TdR kinase and dTMP kinase.

The results of the TdR kinase assays are shown in Table 4. The actual activities measured are shown in the left-hand column. The right-hand column shows the arithmetic sum of 8-, 22-, 10- and 24-h activities in all possible combinations. These values are compared with the measured activities of the mixed extracts shown
**Table 4. A comparison of TdR kinase and dTMP kinase activities at different times during the cell cycle in separate and mixed extracts (division at about 30 h)**

<table>
<thead>
<tr>
<th>Period of culture, h</th>
<th>dTMP kinase activity, pmol dTMP converted to dTDP/min/ml extract</th>
<th>TdR kinase activity, pmol TdR converted to dTMP/min/ml extract</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Replicates</td>
<td>Possible additions</td>
</tr>
<tr>
<td>8</td>
<td>8.0</td>
<td>8.0 + 18.0 = 26.0</td>
</tr>
<tr>
<td>*22</td>
<td>5.0</td>
<td>8.0 + 22.0 = 30.0</td>
</tr>
<tr>
<td></td>
<td>18.0</td>
<td>5.0 + 18.0 = 23.0</td>
</tr>
<tr>
<td></td>
<td>22.0</td>
<td>5.0 + 22.0 = 27.0</td>
</tr>
<tr>
<td>Mixture of 8 + 22</td>
<td>20.0</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>27.0</td>
<td>—</td>
</tr>
<tr>
<td>10</td>
<td>5.0</td>
<td>5.0 + 24.0 = 29.0</td>
</tr>
<tr>
<td>*24</td>
<td>13.0</td>
<td>5.0 + 25.0 = 30.0</td>
</tr>
<tr>
<td></td>
<td>24.0</td>
<td>13.0 + 24.0 = 37.0</td>
</tr>
<tr>
<td></td>
<td>25.0</td>
<td>13.0 + 25.0 = 38.0</td>
</tr>
<tr>
<td>Mixture of 10 + 24</td>
<td>28.0</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>30.0</td>
<td>—</td>
</tr>
</tbody>
</table>

* During the S-period.

in the left hand column. It can be seen the results for the mixture agree well with the possible additions.

The results of the dTMP kinase activity assays are also shown in Table 4. Here again there is a fairly high degree of agreement between the 2 sets of values. In a similar way, DNA polymerase activity was found to be additive when samples of high and low activity were mixed. From these results it can be seen that there is no evidence for the presence of activators or inhibitors at the times tested and therefore the changes in activity are probably due to changes in the level of the enzyme.

The results presented demonstrate a marked coincidence between the onset of incorporation of [3H]thymidine into DNA (DNA synthesis) and the major rise in the activity of TdR kinase, dTMP kinase and DNA polymerase. Indeed there is some suggestion that the onset of DNA synthesis precedes the rise in enzyme activity. One method of discovering whether the rise in activity of these enzymes is dependent on DNA synthesis is to prevent DNA synthesis and subsequently investigate the levels of all 3 enzymes. The inhibitor of DNA synthesis used in this study was fluorouridine deoxyriboside (FUdR) which blocks the normal pathway of DNA biosynthesis in this tissue without seriously interfering with the synthesis of RNA or protein (Yeoman & Aitchison, 1973a). The results of this study are shown in Fig. 6 and Tables 5 and 6. DNA polymerase activity increases slowly in the pre-S period and this small rise is unaffected by the presence of FUdR at 22 µg/ml. However, FUdR prevents the sharp rise in activity characteristic of the untreated explants. The Feulgen histograms of explants sampled during the S-period show that DNA synthesis is proceeding only in the absence of FUdR.
Fig. 6. Changes in DNA polymerase activity during a cell cycle in cultures with (●) and without (○) FUdR. The histograms show the spread of DNA (Feulgen) per nucleus in 2 populations of cells, with (lower) and without (upper) FUdR, at 37 h. The vertical line through the histograms indicates the 2C value.

Table 5. The effect of FUdR (22 µg/ml) and FUdR (22 µg/ml) + TdR (333 µg/ml) added at 0 h on the development of DNA polymerase activity in cultured explants

The S-period commenced at 12–13 h.

<table>
<thead>
<tr>
<th>Period of culture, h</th>
<th>Presence of FUdR</th>
<th>Presence of TdR</th>
<th>DNA polymerase activity, pmol dTTP incorporated/min/ml extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>−</td>
<td>−</td>
<td>4.1</td>
</tr>
<tr>
<td>24</td>
<td>+</td>
<td>−</td>
<td>10.4</td>
</tr>
<tr>
<td>24</td>
<td>−</td>
<td>−</td>
<td>38.3</td>
</tr>
<tr>
<td>24</td>
<td>+</td>
<td>+</td>
<td>36.1</td>
</tr>
</tbody>
</table>

The inhibitory effect of FUdR can be overcome if TdR at a concentration of 333 µg/ml is added at the same time as the inhibitor (Table 5). It has also been shown that FUdR is not effective as an inhibitor of the rise in enzyme activity, if added at the beginning of the S-period. The data presented for TdR kinase and dTMP kinase (Table 6) show that FUdR added at the outset of the experiment prevents the sharp rise in activity characteristic of the untreated series. DNA levels estimated using Burton's method are also shown in Table 6. Although no marked increase in the level of DNA has occurred by 24 h in the culture without FUdR the level at 26 h is about 25% greater than the 0-h value. At 26 h the culture without FUdR shows a greater DNA level than the culture of the same age grown in the presence of
Table 6. The effect of FUdR (22 μg/ml) on the levels of activity of TdR kinase and dTMP kinase and the amount of DNA per explant (FUdR added to the explants at the beginning of the culture period)

<table>
<thead>
<tr>
<th>Period of culture, h</th>
<th>Presence of FUdR</th>
<th>TdR kinase activity, pmol TdR converted/min/ml extract</th>
<th>dTMP kinase activity, pmol dTMP converted/min/ml extract</th>
<th>μg DNA/explant</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>-</td>
<td>2·6</td>
<td>11·0</td>
<td>0·79</td>
</tr>
<tr>
<td></td>
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FUdR. The high value at 24 h in the culture growth with FUdR suggests incomplete inhibition in this sample. Even at 26 h DNA synthesis is only partially completed and division takes place between 30 and 35 h.

DISCUSSION

A striking characteristic of the constituent cells of Jerusalem artichoke explants is their close similarity to one another and the ease with which they can be induced to divide by the addition of a simple nutrient medium with 2,4-D. A further point is that the cells which are induced to divide do so with a high degree of synchrony and it is this latter property which has made this and previous investigations so fruitful in the elucidation of events which take place during the induction of division and the cell cycles which follow. It has already been shown that the accumulation of RNA and protein take place in a series of steps (Evans, 1967; Mitchell, 1968, 1969) and that the onset and completion of DNA replication is a well defined event occupying a large proportion of the cell cycle (Mitchell, 1967). Against such a background it is not unreasonable to expect that enzymes concerned with the synthesis of such macromolecules will behave in a similar fashion. It would appear from the results presented here that this expectation has been realized, for all the enzymes studied show a marked periodicity with respect to their activities.

It is clear from the data presented in this communication that the activities of TdR kinase, dTMP kinase and DNA polymerase exhibit patterns of increase during
the cell cycle. Approaches which may be used to discover 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. However, conclusive proof of *de novo* synthesis of an enzyme necessitates the isolation of individual enzyme proteins which has rarely been accomplished in this context. The approach in this investigation has been the use of mixed extracts. For TdR kinase, dTMP kinase and DNA polymerase, mixed enzyme extracts prepared from cultures sampled before the increase had begun, and after the increase had started, gave values for the activity of the enzyme equivalent to the sum of the activities of the separate extracts. This proves that the increased activities were not due to the activation of pre-existing enzymes, the removal of inhibitors or to the removal of the influence of competing enzyme systems. In addition Aitchison (unpublished observations) has shown that cycloheximide, a potent inhibitor of protein synthesis in this tissue, stops DNA replication as well as preventing the rise in activity of glucose-6-phosphate dehydrogenase and hexokinase, 2 other enzymes associated with the preparation for cell division in this tissue (Yeoman & Aitchison, 1973b). This evidence taken together with the mixed extract data is consistent with the view that the increase in activity of these enzymes is due to a net synthesis of enzyme protein.

All of the enzymes studied here are present in small amounts in the quiescent cells of the original explant and increase only in response to conditions which induce cell division, suggesting that they are closely linked with preparation of the cell for division. Each enzyme shows a characteristic pattern of change. However, in all 3 cases the major increase accompanies the onset of DNA replication. More convincing proof of the existence of this close coincidence emerges when the increase in the amount of DNA is plotted against enzyme activity (Fig. 7), where it can be seen that the activities of all 3 enzymes show a high correlation with DNA synthesis. Further, the correlation coefficient of 0.988 obtained for DNA polymerase is higher than the coefficients of 0.923 and 0.933 calculated for TdR kinase and dTMP kinase respectively and this may reflect the closer association of DNA polymerase with DNA synthesis. However, the correlation data above are only suggestive of an interdependence between these 2 events and do not show which takes precedence. Evidence in favour of the increased rate of enzyme synthesis (or decreased rate of enzyme degradation: Trewavas, 1972) being dependent on DNA synthesis is provided by the experimental series with FUdR. Here it can be seen that the addition of an inhibitor which stops DNA synthesis also prevents the increase in the level of all three enzymes. Presumably then the increased enzyme levels observed during S must constitute a preparation for the second synchronous division as these increases occur too late to contribute significantly to the round of DNA replication which precedes the first synchronous division. This means that the quiescent cells of the explant may at least initiate DNA synthesis without a substantial increase in any of these 3 DNA-related enzymes. It must of course be remembered that a small increase in the level of DNA polymerase precedes the onset of DNA replication.

A close coincidence between a rise in TdR kinase and DNA synthesis has also been observed in synchronously dividing rat liver cells (Bollum & Potter, 1959),
Enzymes involved in DNA biosynthesis

Fig. 7. The relationship between Feulgen DNA level and enzyme activity. A, TdR kinase (●, correlation coefficient (c.c.) = 0.923) and dTMP kinase (■, c.c. = 0.933). B, DNA polymerase (c.c. = 0.988). Calculated regression lines drawn through the values.

cultured cells from rabbit kidney (Lieberman, Abrams, Hunt & Ove, 1963), and synchronously dividing HeLa cells (Brent, Butler & Crathorn, 1965; Stubblefield & Mueller, 1965). There are however very few published reports of the pattern of dTMP kinase activity during the cell cycle in eukaryotes and those which do exist are confined to algae and animal systems. Here again there is a close coincidence of DNA synthesis with increase in enzyme activity. In synchronous cultures of Chlorella pyrenoidosa, Johnson & Schmidt (1966) have described an increase in dTMP kinase activity which began prior to the onset of DNA synthesis. Wanka & Poels (1969) report a similar result also with Chlorella. In contrast Brent, Butler & Crathorn (1965) showed that in synchronously dividing HeLa cells the rise in dTMP kinase activity occurred during S and reached maximum activity about 5 h after the peak of DNA synthesis. Patterns of DNA polymerase activity during the cell cycle have been reported by Littlefield, McGovern & Margeson (1963) and Gold & Helleiner (1964) in synchronized mouse fibroblast cells. They have shown that no large change in the total activity of DNA polymerase occurs during the cell cycle although some reciprocal changes were observed between the particulate and soluble enzyme fractions. In contrast Friedman & Mueller (1968) and Friedman (1970) have demonstrated that the nuclei of cultured HeLa cells accumulate DNA polymerase activity prior to DNA replication. The nuclear polymerase activity increased 3 to 5-fold as cells approached S, remained high until the completion of DNA replication and then declined while the soluble polymerase activity remained relatively constant throughout the cell cycle. Also in cultured rabbit kidney cells (Lieberman et al., 1963) the activity of DNA polymerase begins to increase at about the time that DNA synthesis is initiated. A similar coincidence between DNA synthesis and rise in activity
of the enzyme has been reported by Loeb & Agarwal (1971) during the transformation of human lymphocytes and by Hecht (1972) during the meiotic cycle in Lilium.

It seems clear from published work and the results presented here that there is a coincidence between the increases in the levels of TdR kinase, dTMP kinase and DNA replication but reports vary as to which event takes precedence. Evidence from previous studies with eukaryotic cells is conflicting but favours a temporal relationship between DNA synthesis and rise in DNA polymerase activity. One hypothesis can be advanced which is consistent with the facts and does account for the close relationship that exists between DNA replication and increased levels of all 3 enzymes in this investigation. This is that during the cell cycle transcription occurs at a constant rate depending on the number of appropriate gene copies and as these copies are replicated they immediately become active in transcription and the extra mRNA produced is immediately available for translation. This is similar to the gene dosage effect in bacteria where the expression of activity is limited by the number of gene copies specifying the appropriate mRNA. Observed levels of enzyme are then due to the balance between synthesis and degradation of mRNA and protein which changes only at S. As the increase in level of the DNA-related enzymes does not present a strict doubling during S it must be assumed that for the rest of the cell cycle some other factor controls the level of enzyme (Varner, 1971). It is also possible that some other factor or condition is limiting the level of the enzyme and for this group relaxation of the control shortly after the initiation of DNA synthesis is perhaps coincidental. For instance it might be due to a change in the structure of the nuclear envelope, increasing the flux of mRNA into the cytoplasm.

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REFERENCES


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