Levels of Enzyme Activity and Deoxyribonucleic Acid Synthesis in Mammalian Cells Cultured from the Animal*

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The replication of DNA in the cells of higher forms occurs only during part of the interphase of their division cycle (1-8). As a result, the cycles of plant and animal cells may be divided into four discrete phases: G1, the postmitotic period preceding DNA synthesis; S, the period of DNA synthesis; G2, the premitotic period following DNA synthesis; and M, the relatively brief period of mitotic division (3). The very low rates of DNA synthesis and cell division characteristic of many tissues and organs of the adult mammal imply that control of cell multiplication involves blocks or restrictions in one or more of the phases of the division cycle. In fact, the diploid nature of most of these cells indicates that their control mechanisms are largely confined to the G1, rather than to the G2, period. This is consistent with the observation that the lengths of the S and G2 periods are relatively constant for most mammalian cells, whereas the G1 period generally governs the doubling time (9-11).

Previous observations (12, 13) suggested that kidney cortex cells cultured directly from the rabbit might serve as a useful tool for the study of the chain of events of the G1 period that lead to DNA synthesis and cell division. During this period, marked changes in RNA metabolism were observed in these cells (13). For the first 12 hours of culture, the rate of turnover of nuclear RNA remained at the in vivo level, although the low initial rate of synthesis of stable ribosomal RNA increased progressively. Between 12 and 22 hours, a sharp increase in the turnover rate of nuclear RNA occurred, and this elevated rate was maintained until the onset of DNA synthesis. Addition of p-fluorophenylalanine or low levels of actinomycin D (0.01 μg per ml) prevented the increase in the turnover rate of nuclear RNA, and this elevated rate remained at a-ivo level, although the low initial rate of synthesis of stable ribosomal RNA increased progressively.

Once the high rate of RNA turnover was established in the cultured cells, a progressive appearance with time was observed of cells in which the subsequent synthesis of DNA could no longer be blocked by the addition of previously inhibitory levels of p-fluorophenylalanine or actinomycin D. The possibility was considered that this insensitivity to an amino acid analogue and to an inhibitor of RNA synthesis, acquired late in the G1 period, indicated completion of synthesis of the enzymes needed for DNA replication. To investigate this possibility and to characterize further the final phase of the G1 period, measurements of DNA polymerase and thymidine kinase were made as a function of the age of the cells and after treatment with various chemical agents and ionizing radiation. As controls, the activities of enzymes not directly related to DNA formation (lactic and glucose 6-phosphate dehydrogenases, adenosine deaminase, and hexokinase) were also measured. Increases in the specific activities of each of these enzymes occurred during culture.

The results of these studies are presented in this report. They have led to the recognition of three distinct stages in the period preceding DNA formation. They also suggest that events other than those immediately concerned with the synthesis of the enzymes of the DNA synthetic pathway may have a control function during the G1 period.

EXPERIMENTAL PROCEDURE

Materials—The components of the culture medium and the source of several of the preparations used have been described (12, 13). Actinomycin D was kindly provided by Dr. Elmer Alpert, Merck Sharp and Dohme Research Laboratories, and puromycin was provided by Dr. Stanton M. Hardy, Lederle Laboratories. Glucose 6-phosphate dehydrogenase, used in the estimation of hexokinase, was a commercial preparation (Sigma Chemical Company). DEAE-cellulose was obtained from the Bio-Rad Corporation, Richmond, California.

For the estimation of DNA polymerase, 3H-dCTP was prepared by enzymic phosphorylation of 3H-dCMP (Schwarz BioResearch, Inc.). A crude kinase preparation was obtained by homogenizing 250 mg of an Ehrlich ascites cell acetone powder with 5 ml of 0.025 M potassium phosphate buffer (pH 7.4) followed by centrifugation for 15 minutes at 30,000 × g. The reaction mixture (1.5 ml) contained 1 ml of ascites extract, 100 μmoles of Tris-HCl buffer (pH 7.5), 5 μc of 3H-dCMP (1 μc per μmole), 15 μg of phosphoenolpyruvate (cyclohexylamine salt), 100 μmoles of KCl, 14 μmoles of ADP, 1 μmole of ATP, 50 μmoles of MgCl2, 12.5 μmoles of cysteine, and 1.5 μg of pyruvate kinase (Sigma Chemical Company, Type I). After
incubation for 90 minutes at 37°, the solution was deproteinized with perchloric acid, neutralized, and chromatographed on a column (30 x 1 cm) of Dowex 1-acetate (8% crosslinked). Gradient elution was performed by passing 20 bed volumes of 0.5 M ammonium acetate in 5 M acetic acid through a fixed volume mixer flask containing 500 ml of water. At this point, the solution in the reservoir was changed to 1.0 M ammonium acetate in 5 M acetic acid, and the flow was continued until dCTP was eluted (15 bed volumes). A yield of 60 to 80% was obtained after removal of salt by flash evaporation at 35°.

**Cultural Methods and Determinations**—The preparation of cultures from trypsinized rabbit kidney cortex cells has been described (12). DNA synthesis was measured by the incorporation of 2-[^3H]-thymidine into a fraction insoluble in ethanol and acid; RNA synthesis was estimated with ^[^3H]-cytidine; and protein synthesis, with ^[^3C]-labeled amino acids. The samples were prepared for radioassay in a liquid scintillation spectrometer by a filtration procedure (14) as previously described (13). Homogenates were made in a Dounce homogenizer (tight-fitting pestle) from cells detached from the glass surface with trypsin (0.25%) and suspended in water after three washes with the basal culture medium. Immediately after homogenization, 2 M sucrose, 1 M Tris-HCl (pH 8), and 1 M KCI were added to yield final concentrations of 0.25, 0.1, and 0.005 M, respectively. Protein was measured by the method of Lowry et al. (15).

Radioautography of methyl-[^3H]-thyminde-labeled cultures was carried out as previously described (13) with nuclei prepared from cells homogenized in 0.1 M citric acid. Under the conditions used, a short exposure (24 hours) to Kodak Nuclear Track Emulsion, Type NTB 3, gave heavy labeling of the nuclei, and the background grains were absent.

**Enzyme Assays**—Under the conditions of each assay, the extent of reaction was proportional to the amount of enzyme preparation used. Mixtures of homogenates prepared from cells of different ages yielded the sums of the activities measured separately, indicating an absence of inhibitors in zero time and young cultures. Homogenates of nontrypsinized kidney occasionally exhibited higher specific activities than zero time trypsinized cells, but these differences did not exceed 20%. Rapid freezing and thawing (one to three times) of the homogenates did not significantly alter the levels of any of the enzyme activities. With thymidine kinase, however, the frozen preparations provided better proportionality between the amount of enzyme preparation used and the TMP formed. For each of the enzymes measured, the sum of the activities of the soluble and particulate fractions (separated by centrifugation at 105,000 X g for 1 hour) was equal to the activity of the homogenate.

A unit of enzyme is defined as the amount causing the removal of 1 pmole of a substrate, or yielding 1 pmole of a product, per hour, except in the case of DNA polymerase (1 pmole per hour) and thymidine kinase (1 pmole per hour). Specific activity is expressed as units per mg of protein.

For DNA polymerase, the reaction mixtures (1 ml), prepared as described by Bollum and Potter (16), contained 40 pmoles of Tris-HCl (pH 8), 5 pmoles of MgCl₂, 5 pmoles of ATP, 6 pmoles of sodium phosphoenolpyruvate, 0.05 pmole each of dATP, dGTP, and dTTP, 0.0035 pmole of ^[^3H]-dCTP (6.7 x 10⁶ c.p.m. per pmole), 0.3 mg of thymus DNA, 0.02 mg of crystalline pyruvate kinase (Sigma Chemical Company), and the enzyme preparation. After 60 minutes at 37°, 0.07 ml of 5 M NaOH was added, and the solution was kept at 37° for 16 to 20 hours. At the end of this time, the DNA was precipitated with trichloroacetic acid, and the acid- and ethanol-insoluble fraction was prepared for radioassay as described above.

Thymidine kinase was assayed by a modification of the method described by Kiely (17) for TMP kinase. The reaction mixture (0.25 ml) contained 0.04 μmole of 2-[^3H]-thymidine (2 x 10⁶ c.p.m. per pmole), 2 μmoles of ATP, 4 μmoles of MgCl₂, 6 μmoles of potassium phosphate (pH 7.4), 10 μmoles of Tris-HCl (pH 7.4), and the enzyme preparation. After incubation at 37° for 10 minutes, the mixture was diluted to 1 ml, kept at 100° for 2 minutes, and centrifuged. An aliquot (0.5 ml) was allowed to drain into a pad of DEAE-cellulose (90 mg) prepared by allowing an aqueous suspension to settle under gravity in a Millipore Microanalysis Filter Holder (No. XX 10 025 00). The pad was then washed with suction with 10-ml portions of the following: three times with 0.005 M NH₄HCO₃ twice with acetone, and once with ether. The air-dried pad was suspended in 1 ml of a 1 M methanolic solution of Hyamine hydroxide and assayed by liquid scintillation counting after addition of 10 ml of the toluene-phosphor solution. The incubation conditions employed here were selected for reproducibility and convenience even though a slightly higher activity (20%) was obtainable by halving the MgCl₂ concentration and raising the pH to 8.0.

Lactate dehydrogenase was measured spectrophotometrically by the disappearance of DPNH; glucose 6-phosphate dehydrogenase, by the formation of TPNH; and adenosine deaminase, by the decrease in optical density at 275 mμ in test mixtures containing adenosine (18). Hexokinase was estimated by the increase in optical density at 340 mμ during a 6-minute period in test mixtures (1 ml) containing 100 μmoles of Tris-HCl (pH 8), 5 μmoles of MgCl₂, 200 μmoles of glucose, 1 μmole of ATP, 0.3 μmole of TPN, 10 units of glucose 6-phosphate dehydrogenase, and the enzyme preparation.

**Irradiation Procedure**—All irradiations were performed with a unit operating at 280 kv and 20 ma, and a 0.5 mm Cu filter. The half-value layer was 1.5 mm of Cu. The target distance was maintained at 32 cm where the dose rate of 413 roentgens per minute was constant within 5% over a field 14 cm in diameter. The cells were exposed in groups of four covered 60 mm Petri dishes resting on a heavy Masonite backing. We are indebted to Dr. Elliott C. Laser, Professor of Radiology and Chemotherapy, and to Mr. Richard C. Granke, Assistant Professor of Radiology, University of Pittsburgh School of Medicine, for their kind assistance.

**RESULTS**

**Changes in Enzyme Activity Levels with Age of Cells**—The enzymes studied, with the exception of malic dehydrogenase, fall into two classes. One of the distinctions between the two classes was observed when the increases in activity were studied as a function of the age of the cells. For the members of one of the classes (lactic and glucose 6-phosphate dehydrogenases, adenosine deaminase, and hexokinase), the increases appeared to begin immediately, whereas for the members of the other (DNA polymerase and thymidine kinase), the increases began later, at about the time of DNA synthesis (Table I). For comparison, the specific activity of malic dehydrogenase, which did not change with time, is also shown.

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2 One unit of glucose 6-phosphate dehydrogenase is defined as the amount causing the formation of 1 pmole of TPNH per hour.
Effect of Actinomycin D and p-Fluorophenylalanine on Enzyme Activity Levels—To test their effect on the increase in enzyme activity levels, actinomycin D and p-fluorophenylalanine were used in concentrations just sufficient to prevent DNA replication when they were added at zero time. The results, presented in Tables II and III, show that these inhibitors, added at zero time, drastically suppressed the increases of DNA polymerase and thymidinic kinase but had relatively little effect on the other enzymes tested. A distinct increase in DNA polymerase was observed, however, when the inhibitors were added at 39 or 46 hours, a time when they were unable to prevent DNA synthesis by some of the cells.

### Table I

<table>
<thead>
<tr>
<th>Age of culture hours</th>
<th>Enzyme activities</th>
<th>DNA synthesis c.p.m./culture</th>
<th>units/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DNA polymerase</td>
<td>Thyminde kinase</td>
<td>Lactic dehydrogenase</td>
</tr>
<tr>
<td>0</td>
<td>3.6</td>
<td>1.3</td>
<td>13</td>
</tr>
<tr>
<td>21</td>
<td>3.1</td>
<td>1.4</td>
<td>18</td>
</tr>
<tr>
<td>36</td>
<td>4.4</td>
<td>2.1</td>
<td>21</td>
</tr>
<tr>
<td>46</td>
<td>11</td>
<td>2.1</td>
<td>25</td>
</tr>
<tr>
<td>72</td>
<td>47</td>
<td>7.5</td>
<td>45</td>
</tr>
</tbody>
</table>

The same procedures were used as described in Table I. With cultures incubated for 65 hours, the growth medium was replaced at 0 hours. As indicated, actinomycin D (0.01 µg per ml) was present in one group from 0 to 65 hours, and in another from 39 to 65 hours.

### Table II

<table>
<thead>
<tr>
<th>Additions to growth medium</th>
<th>Incubation period</th>
<th>Enzyme activities</th>
<th>DNA synthesis c.p.m./culture</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>hours</td>
<td>DNA polymerase</td>
<td>Thymidine kinase</td>
</tr>
<tr>
<td>None</td>
<td>0</td>
<td>4.2</td>
<td>1.7</td>
</tr>
<tr>
<td>None</td>
<td>29</td>
<td>4.8</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>65</td>
<td>5.3</td>
<td>15</td>
</tr>
<tr>
<td>Actinomycin (at 0 hour)</td>
<td>65</td>
<td>5.6</td>
<td>3.0</td>
</tr>
<tr>
<td>Actinomycin (at 39 hours)</td>
<td>65</td>
<td>14</td>
<td></td>
</tr>
</tbody>
</table>
Table III
Effect of p-fluorophenylalanine on enzyme activity levels

The same procedures were used as described in Table I. With cultures incubated for 71 hours, the growth medium was replaced at 46 hours. As indicated, p-fluorophenylalanine (0.75 mM) was present in one group from 0 to 71 hours, and in another from 46 to 71 hours.

<table>
<thead>
<tr>
<th>Additions to growth medium</th>
<th>Incubation period</th>
<th>Enzyme activities</th>
<th>DNA synthesis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>hours</td>
<td>units/mg protein</td>
<td>c.p.m./culture</td>
</tr>
<tr>
<td>None</td>
<td>0</td>
<td>4.3 1.4 10 0.6</td>
<td>1.0 1.5</td>
</tr>
<tr>
<td>None</td>
<td>46</td>
<td>5.5</td>
<td>800</td>
</tr>
<tr>
<td>None</td>
<td>71</td>
<td>19 10 22 2.0</td>
<td>2.7 3.6</td>
</tr>
<tr>
<td>DL-p-Fluorophenylalanine (at 0 hours)</td>
<td>71</td>
<td>4.6 2.2 28 1.7</td>
<td>1.9 2.9</td>
</tr>
<tr>
<td>DL-p-Fluorophenylalanine (at 46 hours)</td>
<td>71</td>
<td>14</td>
<td>2620</td>
</tr>
</tbody>
</table>

Table IV
Effect of EDTA on enzyme activity levels

The same procedures were used as described in Table I. With cultures incubated for 76 hours, the growth medium was replaced at 51 hours. As indicated, EDTA (0.4 mM) was present in one group, and EDTA (0.4 mM) plus ZnCl₂ (0.04 mM) in another, from 0 to 76 hours. A third group contained EDTA only from 51 to 76 hours.

<table>
<thead>
<tr>
<th>Additions to growth medium</th>
<th>Incubation period</th>
<th>Enzyme activities</th>
<th>DNA synthesis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>hours</td>
<td>units/mg protein</td>
<td>c.p.m./culture</td>
</tr>
<tr>
<td>None</td>
<td>0</td>
<td>5.3 1.7 13 0.7</td>
<td>0.8 1.9</td>
</tr>
<tr>
<td>None</td>
<td>51</td>
<td>10 3.0</td>
<td>340</td>
</tr>
<tr>
<td>None</td>
<td>76</td>
<td>33 6.0 43 2.1</td>
<td>1.7 4.7</td>
</tr>
<tr>
<td>EDTA (at 0 hours)</td>
<td>76</td>
<td>10 1.7 45 3.0</td>
<td>2.2 5.8</td>
</tr>
<tr>
<td>EDTA (at 51 hours)</td>
<td>76</td>
<td>37 5.6</td>
<td>2400</td>
</tr>
<tr>
<td>EDTA + Zn²⁺ (at 0 hours)</td>
<td>76</td>
<td>27 6.8 46 3.0</td>
<td>2.7 4.9</td>
</tr>
</tbody>
</table>

Table V
Effect of x-radiation on enzyme activity levels

The same procedures were used as described in Table I. The cultures (48 per group) were irradiated with a dose of 3000 r at 3 hours, the medium was replaced at 40 hours, and the cells were harvested at 70 hours.

<table>
<thead>
<tr>
<th>DNA polimerase</th>
<th>Thymidine kinase</th>
<th>Lactic dehydrogenase</th>
<th>Glucose 6-phosphate dehydrogenase</th>
<th>Adenosine deaminase</th>
<th>Hexokinase</th>
<th>Malic dehydrogenase</th>
<th>DNA synthesis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (0 hours)*</td>
<td>3.7</td>
<td>1.4</td>
<td>12</td>
<td>0.6</td>
<td>1.2</td>
<td>1.5</td>
<td>38</td>
</tr>
<tr>
<td>Control (70 hours)</td>
<td>21</td>
<td>18</td>
<td>32</td>
<td>1.4</td>
<td>3.6</td>
<td>3.6</td>
<td>36</td>
</tr>
<tr>
<td>X-ray (70 hours)</td>
<td>6.1</td>
<td>3.6</td>
<td>43</td>
<td>1.6</td>
<td>3.4</td>
<td>3.4</td>
<td>40</td>
</tr>
</tbody>
</table>

* Refers to zero time trypsinized cells.

During the period preceding DNA synthesis (31 hours), the small fraction of insensitive cells remained essentially constant, but it increased markedly at 55 hours when some cells were actively synthesizing DNA.

In contrast to the constancy of the x-ray-sensitive fraction of the population up to 31 hours, a part of it had become resistant to actinomycin D (0.01 ag per ml) as previously observed (13). Thus, with the same cells used in the x-ray experiments, actinomycin D added at 6, 31, and 55 hours resulted in DNA formation (expressed as % of the control value) of 0.6, 11, and 50%, respectively. Similar results were obtained with 0.75 mM p-fluorophenylalanine and 0.4 mM EDTA.

These data suggested that cells in the terminal phase of the G₁ period had become resistant to the chemical agents but were still sensitive to inhibition by x-rays and that the x-ray resistant population represented cells that were already forming DNA at the time of irradiation. To test for a correlation between x-ray resistance and DNA synthesis, ³H-thymidine was used to estimate the number of nuclei replicating DNA before and after exposure to x-rays (Table VII). As can be seen from the table.
Efforts directed towards an understanding of the cellular changes in the table, largely but not completely blocked DNA replication, as described in "Cultural Methods and Determinations." Periods as indicated, and the cells were harvested and assayed as described in Table I, received EDTA (0.5 mM) and others were exposed to X-ray.

Rate of Amino Acid Incorporation as Function of Age of Cells—Efforts directed towards an understanding of the cellular changes.

No new nuclei entered the period of DNA synthesis following exposure of 49½-hour cultures to 3000 r. Essentially all the nuclei that had been synthesizing DNA before irradiation, however, continued to do so afterwards. In contrast, when p-fluorophenylalanine was added at 494 hours, the number of nuclei synthesizing DNA increased even in the presence of a concentration of the amino acid analogue that completely suppressed DNA replication when added at zero time.

Effect of Inhibitors Added During Period of DNA Synthesis—It was of interest to determine whether concomitant protein and RNA synthesis were obligatory for DNA replication. To test this point, puromycin and high levels of actinomycin D (1.7 μg per ml) and p-fluorophenylalanine (5 mM) were added to cultures which were forming DNA (Table VIII). Puromycin, as shown in the table, largely but not completely blocked DNA replication, whereas actinomycin D and p-fluorophenylalanine had much less effect on DNA formation.

TABLE VI
Lack of effect of EDTA and x-rays on RNA synthesis

<table>
<thead>
<tr>
<th>Treatment of cells</th>
<th>Pulse period with H-lysine</th>
<th>0-1 hour</th>
<th>4-5 hours</th>
<th>23-24 hours</th>
<th>total c. p.m. in RNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>1260</td>
<td>970</td>
<td>2880</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EDTA</td>
<td>1060</td>
<td>1330</td>
<td>3200</td>
<td></td>
<td></td>
</tr>
<tr>
<td>X-ray</td>
<td>1160</td>
<td>1040</td>
<td>2440</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Effect of inhibitors added during period of DNA synthesis

Cultures were prepared as described in Table I. The medium in each culture was replaced by fresh growth medium at 49½ hours. Where indicated, cultures were irradiated at 49½ hours with a dose of 3000 r and p-fluorophenylalanine (0.75 mM), when used, was present only during the indicated pulse periods. For the estimation of DNA synthesis, cultures were pulsed with 2-14C-thymidine (as in Table I), while other cultures, used for the estimation of the number of labeled nuclei (see "Cultural Methods and Determinations"), were pulsed with H-thymidine (5 × 10⁴ c.p.m. per culture, 1.2 × 10⁴ c.p.m. per μmole). Cells were harvested and assayed at the end of the pulse periods as described in "Cultural Methods and Determinations." Nuclei were scored as labeled or unlabeled.

**TABLE VII**
Effect of x-radiation on DNA synthesis as a function of dose and age of cells

Cultures were prepared as described in Table I. The medium in each culture was replaced by fresh growth medium at 48 hours, and the radioactive substrates and the various inhibitors were added as indicated at 63 hours. The cultures were harvested 1 to 4 hours later and assayed as described in "Cultural Methods and Determinations" except that the thymidine-labeled cells were kept for 20 hours in 0.3 M NaOH at 37°C prior to acid precipitation. Inhibitor concentrations were: puromycin, 0.4 mg per ml; actinomycin D, 1.7 μg per ml; and p-fluorophenylalanine, 5 mM. The labeled substrates were: 1-14C-leucine (4 × 10⁴ c.p.m. per culture, 2.5 × 10⁴ c.p.m. per μmole), H-lysine (6 × 10⁴ c.p.m. per culture, 3.2 × 10⁴ c.p.m. per μmole), and 2-14C-thymidine (3 × 10⁴ c.p.m. per culture, 5 × 10⁴ c.p.m. per μmole).

**TABLE VIII**
Effect of inhibitors added during period of DNA synthesis

Cultures were prepared as described in Table I. The medium in each culture was replaced by fresh growth medium at 48 hours, and the radioactive substrates and the various inhibitors were added as indicated at 63 hours. The cultures were harvested 1 to 4 hours later and assayed as described in "Cultural Methods and Determinations" except that the thymidine-labeled cells were kept for 20 hours in 0.3 M NaOH at 37°C prior to acid precipitation. Inhibitor concentrations were: puromycin, 0.4 mg per ml; actinomycin D, 1.7 μg per ml; and p-fluorophenylalanine, 5 mM. The labeled substrates were: 1-14C-leucine (4 × 10⁴ c.p.m. per culture, 2.5 × 10⁴ c.p.m. per μmole), H-lysine (6 × 10⁴ c.p.m. per culture, 3.2 × 10⁴ c.p.m. per μmole), and 2-14C-thymidine (3 × 10⁴ c.p.m. per culture, 5 × 10⁴ c.p.m. per μmole).

**Fig. 1.** Effect of x-radiation on DNA synthesis as a function of dose and age of cells. Cultures of trypsinized kidney cells were prepared in Petri dishes as described in Table I, and some of them were exposed to the indicated doses of ionizing radiation at 6, 31, or 55 hours. With the cultures irradiated at the two earlier times, and with unirradiated controls, the medium was replaced at 48 hours with fresh growth medium containing methyl-[H]-thymidine (5 × 10⁴ c.p.m. per culture, 1.2 × 10⁴ c.p.m. per μmole). The cultures irradiated at 55 hours and a separate group of unirradiated controls were similarly treated at 60 hours. All the cultures were harvested at 72 hours, and after treatment with 0.3 M NaOH for 18 hours at 37°C, the radioactivity incorporated into DNA was estimated as described in "Cultural Methods and Determinations." DNA synthesis is shown as the % of the radioactivity incorporated by the comparable, unirradiated control cultures. The symbols indicating age of culture when irradiated are: O, 6 hours; ●, 31 hours; and □, 55 hours.

* Similar results were obtained with 14C-leucine as substrate.
kidney cortex cell can be subdivided into three phases (I, II, and III). During the first phase, lasting for 12 hours, the turnover of amino acid incorporation rate began to increase immediately on cultivation and by 4 hours had attained a level about 5 times the initial one. For comparison, the rate of incorporation of \( ^3H \)-cytidine into RNA by the same cells is also shown in Fig. 2. No early increase occurred.

Concentrations of inhibitors that prevented both the increase in the rate of RNA formation and the subsequent formation of DNA (13) had no effect upon the early rise in the rate of amino acid incorporation. Thus, with no inhibitor, with actinomycin D (0.01 \( \mu \)g per ml), and with \( p \)-fluorophenylalanine (0.75 \( \mu \)m), added at zero time, the rates of incorporation during exposure to \( ^3C \)-lysine between 3 and 0 hours were 4.1, 5.1, and 3.2 times greater, respectively, than during the 0- to 1-hour exposure period. With \( ^3C \)-leucine as substrate, the comparable values were 5.1, 3.8, and 4.8. EDTA, similarly, had no effect upon the increased rate of incorporation of the amino acids.

**DISCUSSION**

The synthesis of DNA by rabbit kidney cortex cells is a strongly repressed function in vivo. Transfer of the cells to an environment in vitro, however, results in the removal, for some of the cells, of the controls that previously prevented entry into the period of DNA replication. Although the nature of the controls that have been abolished is presently unknown, some information is now available about the metabolic events that begin once control has been relaxed and culminate in DNA synthesis and cell division.

On the basis of the results presented in this report and in the previous publication (13), the G1 period (see Footnote 1) of the kidney cortex cell can be subdivided into three phases (I, II, and III). During the first phase, lasting for 12 hours, the turnover of nuclear RNA is maintained at a constant rate, identical with the rate observed in the intact organ in vivo. Several changes, however, have been observed during Phase I: progressive increases in the rates of ribosomal RNA synthesis (13) and amino acid incorporation into protein; gradual increases in the specific activities of several enzymes; a decrease in RNase activity (12); and a sharp rise in the pool size of acid-soluble lysine and histidine and presumably of other amino acids as well.\(^4\) The increase in amino acid pool size suggests that a change in permeability of the cell membrane may be an important event in this phase. Whether these changes are on the pathway leading to DNA replication or represent parallel processes is not known.

The roughly parallel response of ribosomal RNA synthesis and incorporation of amino acids into protein suggests that these two processes are to some extent associated, i.e. the cells react to the medium in vitro by increasing their ribosome population. This phenomenon is reminiscent of a similar one observed in bacteria where the cells respond to a transfer from a simple medium to a more complex one with an immediate increase in the rate of RNA synthesis (19).

Between 12 and 20 hours, the kidney cells pass from Phase I to Phase II, characterized by an increased rate of turnover of nuclear RNA. This transition is blocked by the same low doses of \( p \)-fluorophenylalanine and actinomycin D that prevent subsequent synthesis of DNA. It is probable that individual cells make the transition from Phase I to Phase II relatively abruptly, the interval from 12 to 20 hours resulting from the somewhat limited state of synchrony in this population. During Phase II, the cells remain sensitive to low levels of \( p \)-fluorophenylalanine and actinomycin D and, in addition, exhibit a requirement for 

\[ \text{Zn}^{++} \]. The data suggest specific protein synthesis late in Phase I as a requirement for entering Phase II and synthesis of specific proteins, one or more of which may be a 

\[ \text{Zn}^{++} \] protein, during Phase II. Protein synthesis during this period, however, does not lead to increased levels of DNA polymerase or thymidine kinase, enzymes associated with DNA synthesis. On the other hand, increases in the specific activities of four enzymes not directly related to DNA formation (lactic and glucose 6-phosphate dehydrogenases, adenosine deaminase, and hexokinase) do occur during this time.

In the final recognizable phase of the G1 period, Phase III, the sensitive processes of Phases I and II have been completed. \( p \)-Fluorophenylalanine and actinomycin D are no longer effective in preventing subsequent DNA formation and a \( \text{Zn}^{++} \) requirement can no longer be demonstrated. Nevertheless, a period of several hours must elapse before the onset of DNA synthesis and before the appearance of increased levels of DNA polymerase and thymidine kinase. During this time, the cells remain sensitive to x-irradiation, and this sensitivity does not diminish until the cells are actively synthesizing DNA. The fact that the individual events requiring new protein synthesis, such as the transition from Phase I to Phase II, are not x-ray-sensitive suggests that a much larger target volume is associated with the process or processes that go on during the third phase. If replication of chromosomal DNA requires an intact chromosomal structure (see, for example, Krause and Plaut (20)), the large x-ray target may be the chromosome itself.

These results confirm the observations of others (21) that x-irradiation during the G1 period blocks entry into the period of

\(^4\) Unpublished observations.
DNA synthesis. Irradiation damage incurred at any time during the G1 period appears to be expressed just prior to the onset of DNA synthesis. The possibility that nuclear RNA synthesis during the G1 period represents the x-ray-sensitive process (see, for example, Abrams (22)) is eliminated by the observed resistance of RNA turnover to inhibition by x-irradiation. This result is in apparent disagreement with several reported observations that whole body irradiation interferes with nuclear RNA synthesis (23–26). It is possible that these observations in vivo represent secondary effects resulting from disturbed homeostasis.

The data presented here do not permit a detailed analysis of the events leading to competence for DNA replication. They do suggest, however, that two successive waves of new or increased RNA synthesis occur resulting in the formation of proteins for the transition from Phase I to II and from Phase II to III. Interestingly, processes of this type, by far the most sensitive ones of the division cycle, appear to be limited to the G1 period. Thus, it was difficult to suppress DNA formation by cells already in the S period. Relatively large doses of actinomycin D and p-fluorophenylalanine had little effect on the initial rate of DNA synthesis. Even the inhibition by puromycin was incomplete and may simply reflect a requirement for concomitant histone synthesis. Bollum (29) has shown that DNA must be subjected to a change of state (denaturation) before it can subject the replicative state. The observation of Bollum and Potter (35) occur late in both systems, generally at the same time of DNA synthesis. The observation of Bollum et al. (36), however, that x-irradiation at 16 hours blocked subsequent DNA synthesis but not the increased levels of DNA polymerase and thymidine kinase does not agree with the findings in the kidney system where it has not been possible to obtain increased enzyme levels without DNA synthesis. Several reports (37–39) more compatible with the kidney observations suggest the importance of further investigation of the regenerating liver system.

SUMMARY

Six enzymes have been studied that have specific activities which increase when kidney cortex cells are cultured directly from the rabbit. These enzymes can be divided into two classes on the basis of two differences. First, for the members of one of the classes (lactic and glucose-6-phosphate dehydrogenases, adenosine deaminase, and hexokinase), the increases in specific activity appear to begin immediately, whereas for the members of the other (DNA polymerase and thymidine kinase), they begin later, at about the time of DNA formation. Second, agents that prevent DNA synthesis (ethylendiaminetetraacetate, which removes the Zn++ from the growth medium, and low concentrations of actinomycin D and p-fluorophenylalanine) also suppress the increases of DNA polymerase and thymidine kinase but have little or no effect upon the members of the other group. Added later, when DNA replication by some of the cells can no longer be prevented by them, these inhibitors are similarly less effective in suppressing the rise in DNA polymerase. Ionizing radiation, like the chemical inhibitors, affects only the enzymes related to DNA synthesis.

Temporal differences in the action of the inhibitory agents have led to the recognition of three phases in the period preceding DNA formation. Little is known about the first phase (0 to 12 hours), although it is assumed that the events culminating in DNA synthesis are initiated at this time. During this phase, progressive increases occur in the rates of ribosomal RNA synthesis and amino acid incorporation into protein.

Passage (12 to 22 hours) to the next phase involves an increase in the rate of nuclear RNA turnover. This transition is blocked by low doses of actinomycin D and p-fluorophenylalanine but not by ionizing radiation, and Zn++ is not required. The second phase is characterized by the continued susceptibility of the cells to the chemical inhibitors and by the appearance of a requirement for Zn++.

Ionizing radiation affects one of the final cellular changes in the period preceding DNA replication. Thus, cells in the third

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* S. Kishimoto and I. Lieberman, unpublished observations.
phase can pass into the period of DNA synthesis despite the presence of previously inhibitory levels of actinomycin D, p-fluorophenylalanine, and ethylenediaminetetraacetate. These cells, however, can still be prevented by x-rays from entering the period of DNA formation.

Once cells have entered the synthetic period, ionizing radiation and high levels of actinomycin D and p-fluorophenylalanine have relatively little effect on the initial rate of DNA formation. Even inhibition of protein synthesis by puromycin is not capable of completely suppressing DNA synthesis in such cells, although the rate of synthesis is markedly reduced.

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