Effects on HeLa Cell Division of Physiologic Deoxyribo nucleosides and Deoxyri bonucleotides\textsuperscript{1,2}

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Summary

Mitotic effects of natural deoxyribonucleosides, their 5'-monophosphates, certain 5-halogenated deoxyuridines, and other substances were studied in treated HeLa cells observed in life.

Entry into mitosis was strongly inhibited by 1–2 mM deoxyadenosine, 2 mM deoxyguanosine, 2–4 mM thymidine, or 0.005–1.0 mM 5-fluoro-2'-deoxyuridine. The 5'-monophosphates of the 1st 3 nucleosides had a similar but weaker effect.

Metaphase delay or arrest was caused by deoxyguanosine, deoxyadenosine, and thymidine, in descending order; their 5'-monophosphates were much less effective. The chromosomes were irregularly arranged and 1 or more were often at the polar region long after breakdown of the nuclear membrane, although the spindle appeared unchanged. Metaphase was not inhibited by 2 or 4 mM deoxycytidine or 2 mM deoxyuridine, although 6 mM deoxyuridine did prolong it. Deoxycytidine or its 5'-phosphate partly reversed metaphase prolongation caused by deoxyadenosine or thymidine but not that caused by deoxyguanosine. Although 5-bromo-2'-deoxyuridine at 0.077–1.0 mM did not affect metaphase, 5-fluoro-2'-deoxyuridine prolonged it slightly. Adenosine but not cytidine at 2 mM delayed metaphase somewhat.

Nucleolar reconstruction was retarded in cells after treatment with adenosine, deoxyadenosine, and certain combinations of agents including deoxyribonucleosides.

A nucleolar change resembling that induced by adenosine or 6-thioguanine was produced in interphase cells by a combination of deoxyadenosine, deoxyguanosine, and deoxycytidine-5'-phosphate. A transient and slighter alteration of nucleoli was produced by 5-fluoro-2'-deoxyuridine and by some other deoxyribonucleosides alone or in certain combinations.

Discussion is centered on possible relation of the cytologic effects to interference in DNA metabolism.

Introduction

Certain physiologic purines and pyrimidines, their ribonucleosides, and their ribonucleotides at appropriate concentrations are known to affect various kinds of cells in both interphase and mitosis (3–5, 7, 17, 23, 24, 32). On the other hand, few systematic studies have been made on mitotic effects of natural deoxyribonucleosides and deoxyribonucleotides. Greulich et al. (15) reported an apparent stimulation of mitosis by TdR in the duodenal epithelium of mice, and Barr (1) demonstrated that this effect on barley and HeLa cells actually resulted from a prolongation of metaphase. Reports of the inhibition of both DNA synthesis and cell division by dA led to tests of this agent for its effects on chromosomes in broad bean root tips (25) and in human leucocyte cultures (26); dA produced chromatid gaps, breaks, and interchanges, as well as some excessive chromosomal fragmentation. dG and TdR, as well as dA, are known to be potent inhibitors of DNA synthesis (21, 27, 28, 33, 37, 38, 45).

These and other reasons led to the present study, which deals with mitotic effects on HeLa cells of deoxyribonucleosides, deoxyribonucleotides, certain combinations of these substances, and some related compounds. Information has been gathered with the dual aim of applying it eventually to the elucidation of mitosis and to the chemotherapy of cancer.

Materials and Methods

HeLa cells maintained uncloned in this laboratory were used exclusively. They were cultured on Eagle's basal medium plus 10% horse serum, 1 ml of 200 mm L-glutamine solution and 1 ml of penicillin-streptomycin mixture (5000 units each/ml) being added/100 ml. Routine subcultures were made every 3.5 days in milk dilution bottles (Pyrex, 160 ml). The spent growth medium was discarded and 5 ml of 0.25% trypsin (Difco, 1:250) solution in Gey's balanced salt solution was added to each bottle. After incubation at 37°C for 3 min, within which time all the cells became dislodged from the glass, 5 ml of new growth medium were added to the enzyme solution containing the cells. Then

\textsuperscript{1}This publication is dedicated to Professor Sajiro Makino of Hokkaido University, Sapporo, Japan, on the occasion of his 60th anniversary.

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\textsuperscript{4}Abbreviations used are: dA, deoxyadenosine; dC, deoxycytidine; dG, deoxyguanosine; TdR, thymidine; dU, deoxyuridine; BUdR, 5-bromo-2'-deoxyuridine; FUDR, 5-fluoro-2'-deoxyuridine; CDP, cytidine diphosphate; dCDP, deoxycytidine diphosphate; and AMP, dAMP, dCMP, GMP, dGMP, dTMP, and dUMP denote the 5'-monophosphates of adenosine, deoxyadenosine, deoxycytidine, guanosine, deoxyguanosine, thymidine, and deoxyuridine, respectively. Of these chemicals, dC was used as the hydrochloride and dAMP as the dianion dihydrate.
the mixture was centrifuged for 5 min at 43 × g. The supernatant solution was decanted, and the cells were resuspended in 2 ml of growth medium. After the cells were dispersed by pipetting repeated 15 times, 0.5 ml of this concentrated cell suspension was aspirated with a pipet and distributed to each bottle containing 20 ml of the fresh medium. The bottles were then incubated at 37°C.

**Setting Up of Sykes-Moore Chamber Cultures**

Sykes-Moore tissue culture chambers (51) were used for direct visual observation as well as for time-lapse cinemicrographic study of the behavior of both treated and untreated cells. The chambers were autoclaved completely assembled except for tightening of the screw. The latter was finally tightened just before the chamber was filled with HeLa cell suspension. A sterile No. 25 hypodermic needle was used as the air vent. The needle, attached to a 5-ml syringe, was pushed through a port of the chamber and drilled through the silicone rubber ring. Another 5-ml hypodermic syringe was filled with HeLa cell suspension (about 2.5 × 10⁶ cells/ml, prepared by adding 0.5 ml of the concentrated cell suspension mentioned above to 10 ml of fresh medium) and fitted with a No. 23 needle, which was introduced into the chamber through a port opposite the vent needle. After the chamber was completely filled, the No. 23 needle was drawn out and again inserted in order to introduce several small air bubbles. Then both needles were pulled out. During the first 24 hr of incubation at 37°C, the chamber was kept upper cover glass down to permit the cells to adhere to the glass.

**Medium Change and Treatment with Chemicals**

A medium change was made at 8 hr and at 24 hr after the chamber culture was set up. Thereafter the medium was changed every 12 hr, small air bubbles being introduced unless the chamber was about to be under microscopic study. The chamber was put cover glass down in the incubator. The experiments were begun on the 3rd to 5th day after the setting up, when cells were growing well enough to turn phenol red in the medium slightly yellowish. Adenosine, cytidine, and BUdR, were obtained from Sigma Chemical Co., St. Louis, Mo.; all others (except 6-thioguanine and FUdR) were from Nutritional Biochemicals Corp., Cleveland, Ohio. They were weighed on an electrobalance, Cahn Model M10. If the pH had shifted to the alkaline side after the chemicals were dissolved in the medium, the pH of the latter was corrected by seration with an air mixture containing 5 volume % of CO₂. Each time the culture was perfused with growth medium or with medium containing chemicals under test, the medium was prewarmed to 37°C, No. 25 needles were used, and the volume of fluid perfused was 1 ml. The perfusate was usually not kept at 37°C for more than 10 min before application, in light of possible decomposition or change of the chemicals as a result of reaction with some constituents of the medium or of enzymatic attack. When the period of observation of cells treated with a chemical compound lasted more than 12 hr, the chamber was perfused with medium containing the same substance every 12 hr.

It was experimentally determined that our practice of perfusing exactly 1 ml of fluid resulted in a concentration of the test substance within the chamber 0.65 times its concentration in the perfusion medium. In this communication, all the concentrations indicated are those of the fluid used for perfusion. The final concentration in the chamber of each of the chemicals applied should therefore be obtained by multiplying the concentration shown in this paper by the factor, 0.65.

**Direct Observation and Time-Lapse Motion Picture Study**

The tissue culture chamber, completely filled with fluid and containing no air bubble, was transferred onto the stage of a Zeiss phase-contrast microscope in an electric incubator kept at 37°C. The illuminator was a low voltage lamp (6 v, 15 w) installed in the microscope base. Before entering the substage condenser, the light passed through a green interference filter and a heat-reflecting filter, which together removed most of the heat rays emitted by the lamp. A 100× oil-immersion phase objective was chiefly used. An 8× Zeiss ocular was employed for direct observation and a 2× Zeiss ocular for cinemicrographic work. The time-lapse cinemicrographic equipment consisted of a Cine-Kodak Special II camera, a prism viewfinder (Emdeco), a time-lapse drive control (Emdeco), and a camera film driving mechanism (Emdeco). Usually prophasic cells whose mean nuclear diameter did not exceed 20 μ as measured with an ocular micrometer were chosen, and their processes of division were followed with direct visual observation or by means of photographs taken with the time-lapse equipment at intervals of about 3 sec, with 1 sec of exposure time. The film used was exclusively 16-mm Kodak Plus-X reversal film. The processed films were analyzed for duration of mitotic phases and for possible mitotic abnormalities with the aid of a Craig Projecto-Editor.

**Results**

**Division in Untreated HeLa Cells**

At very early prophase no change could be seen in the cells except that a slight retraction of the cytoplasmic extensions had occurred, the nuclear contents were slightly more refractile, and the nucleoli, whose surface had become rough, were somewhat less dense than those of interphase cells. As the cells entered early prophase, very fine chromosomal threads came into view adjacent to the inner surface of the nuclear membrane. At mid-prophase the chromosomes appeared thicker, and they grew much thicker and shorter at late prophase, still being close to the inner nuclear surface. At the end of this stage the nuclear membrane disappeared. The chromosomes moved in the spindle then being formed until they aligned on the equator at the end of metaphase. In most cells, simultaneously with disappearance of the nuclear membrane, the cytoplasm began to contract rapidly and the cells took on more or less spherical form during metaphase. In anaphase, chromosomes separated into daughter chromatids, which moved to opposite poles. As they did so, the cell elongated in the direction of the spindle axis. Then a cleavage furrow appeared across the middle of the cell. It deepened with time, until the cell was constricted into its 2 daughters. Almost simultaneously with completion of the cleavage furrow, a mid-body developed across the narrow cytoplasmic bridge still connecting the daughter cells. Concurrently the nuclear membrane appeared around the chromosomes, which fused together at the
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TREATMENTS

Control
1-2 mM dA
2 mM dG
2-4 mM TdR
2 mM dC
4 mM dC
2 mM dU
6 mM dU
2 mM adenosine
2 mM cytidine
1 mM dAMP
2 mM dCMP
2 mM dGMP
2 mM dTMP
2 mM dA + 2 mM dCMP
2 mM dA + 2 mM dG + 4 mM dCMP
2 mM dG + 1 mM NaH₂PO₄ + 1 mM Na₂HPO₄
2 mM dG + 2 mM dCMP
2 mM dG + 2 mM dC
2 mM dT + 2 mM dCMP
4 mM TdR + 2 mM dC
0.077 mM BUDR
1 mM BUDR
0.005-0.077 mM FUDR
1 mM FUDR

Metaphase
Anaphase

End of cleavage
Midbody appears
Nuclear membrane appears
Nucleoli appear

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Metaphase (min)</th>
<th>Anaphase (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>36.1 ± 16.9 (43)</td>
<td>4.1 ± 0.7 (42)</td>
</tr>
<tr>
<td>1-2 mM dA</td>
<td>77.6 ± 43.8 (20)</td>
<td>5.4 ± 1.0 (17)</td>
</tr>
<tr>
<td>2 mM dG</td>
<td>130.5 ± 85.1 (12)</td>
<td>3.4 ± 0.4 (5)</td>
</tr>
<tr>
<td>2-4 mM TdR</td>
<td>56.0 ± 39.9 (19)</td>
<td>4.2 ± 0.8 (13)</td>
</tr>
<tr>
<td>2 mM dC</td>
<td>38.6 ± 24.6 (12)</td>
<td>3.7 ± 0.9 (11)</td>
</tr>
<tr>
<td>4 mM dC</td>
<td>26.7 ± 5.9 (13)</td>
<td>5.2 ± 1.7 (10)</td>
</tr>
<tr>
<td>2 mM dU</td>
<td>30.2 ± 5.1 (9)</td>
<td>4.1 ± 1.1 (6)</td>
</tr>
<tr>
<td>6 mM dU</td>
<td>71.8 (3)</td>
<td>3.0 (1)</td>
</tr>
<tr>
<td>2 mM adenosine</td>
<td>50.1 ± 33.7 (16)</td>
<td>3.3 ± 0.6 (10)</td>
</tr>
<tr>
<td>2 mM cytidine</td>
<td>30.5 ± 13.7 (11)</td>
<td>3.7 ± 0.7 (7)</td>
</tr>
<tr>
<td>1 mM dAMP</td>
<td>40.4 ± 14.8 (10)</td>
<td>4.2 ± 0.8 (9)</td>
</tr>
<tr>
<td>2 mM dCMP</td>
<td>53.6 ± 16.7 (15)</td>
<td>4.0 ± 0.8 (15)</td>
</tr>
<tr>
<td>2 mM dGMP</td>
<td>63.0 ± 78.5 (11)</td>
<td>4.6 ± 0.9 (7)</td>
</tr>
<tr>
<td>2 mM dTMP</td>
<td>45.6 ± 15.3 (11)</td>
<td>4.7 ± 0.7 (11)</td>
</tr>
<tr>
<td>2 mM dA + 2 mM dCMP</td>
<td>45.9 ± 23.5 (10)</td>
<td>4.2 ± 0.9 (10)</td>
</tr>
<tr>
<td>2 mM dA + 2 mM dG + 4 mM dCMP</td>
<td>156.4 ± 76.5 (9)</td>
<td>4.7 (3)</td>
</tr>
<tr>
<td>2 mM dG + 1 mM NaH₂PO₄ + 1 mM Na₂HPO₄</td>
<td>84.8 ± 42.7 (12)</td>
<td>3.4 ± 0.7 (13)</td>
</tr>
<tr>
<td>2 mM dG + 2 mM dCMP</td>
<td>69.2 ± 45.6 (20)</td>
<td>4.8 ± 1.8 (13)</td>
</tr>
<tr>
<td>2 mM dG + 2 mM dC</td>
<td>63.1 ± 45.2 (24)</td>
<td>4.2 ± 1.0 (17)</td>
</tr>
<tr>
<td>2 mM dT + 2 mM dCMP</td>
<td>38.6 ± 14.1 (21)</td>
<td>4.2 ± 1.1 (18)</td>
</tr>
<tr>
<td>4 mM TdR + 2 mM dC</td>
<td>34.2 ± 10.4 (13)</td>
<td>3.6 ± 0.7 (10)</td>
</tr>
<tr>
<td>0.077 mM BUDR</td>
<td>33.9 ± 17.4 (24)</td>
<td>4.3 ± 0.7 (22)</td>
</tr>
<tr>
<td>1 mM BUDR</td>
<td>40.9 ± 16.5 (20)</td>
<td>4.6 ± 1.5 (20)</td>
</tr>
<tr>
<td>0.005-0.077 mM FUDR</td>
<td>40.6 ± 34.3 (14)</td>
<td>4.4 ± 0.6 (12)</td>
</tr>
<tr>
<td>1 mM FUDR</td>
<td>63.0 ± 80.4 (8)</td>
<td>5.3 ± 1.0 (7)</td>
</tr>
</tbody>
</table>

 Results are expressed as the mean ± S.D. Figures in parentheses indicate the number of observations.

* The 99% confidence interval on the mean does not overlap that on the corresponding mean of control cells.

† The 95% confidence interval on the mean does not overlap that on the corresponding mean of nontreated control cells, except in the experiment with 2-4 mM TdR. In the latter, the 90% confidence interval on the mean duration of the metaphase overlaps slightly that on the corresponding mean of control cells. The "t" test as recommended by Snedecor (50) for the case in which the "t" test as recommended by Snedecor (50) for the case in which the end of anaphase, and the chromosomes gradually lost their optical density. The nucleoli first appeared about 17-21 min after anaphase began. The cells spread out and began to assume their interphase appearance, although the young interphase nucleus could usually be distinguished by its content of heteropyknotic granules.

The following criteria for the mitotic stages were adopted in this study: prophase, from the very early prophase described above to breakdown of the nuclear membrane; metaphase, from membrane breakdown to the onset of chromosome separation; anaphase, from the onset of this separation to the start of cleavage; telophase, from the start of cleavage to the first appearance of the nucleoli.

The average length of mitosis in untreated cells was slightly less than 1.5 hr. The whole prophase lasted less than 30 min. The mean duration of the metaphase ± its standard deviation was 36.1 ± 16.9 min (Table 1). This stage was of considerable length in a few cells (Chart 1). The duration of metaphase was not conspicuously affected by the diameter of the nucleus at prophase, the time after setting up of the culture, or the time after the last feeding (Chart 1), within the limits of the experimental conditions. Chart 1 gives the impression that metaphase duration remained almost constant within the 1st 6 hr after feeding but was somewhat prolonged after 15 hr had passed. A comparison by "t" test of the duration of metaphase in these 2 different periods after the last feeding showed, however, that the difference from the control value is significant at the 5% level.

end of anaphase until: End of cleavage
Midbody appears
Nuclear membrane appears
Nucleoli appear

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A summary of data on mitotic abnormalities seen in the untreated, control HeLa cells is presented in Tables 2A and 2B.

TABLE 1

THE MEAN DURATION AND STANDARD DEVIATION OF DELIMITED SEGMENTS OF MITOSIS IN TREATED AND CONTROL HeLa CELLS

Metaphase (in min)

<table>
<thead>
<tr>
<th>Treatments</th>
<th>From start of anaphase until:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>End of cleavage</td>
</tr>
<tr>
<td>1-2 mM dA</td>
<td>7.5 ± 1.3 (31)</td>
</tr>
<tr>
<td>2 mM dG</td>
<td>8.4 ± 1.2 (17)</td>
</tr>
<tr>
<td>2-4 mM TdR</td>
<td>8.3 ± 2.9 (11)</td>
</tr>
<tr>
<td>2 mM dC</td>
<td>9.8 ± 1.1* (15)</td>
</tr>
<tr>
<td>4 mM dC</td>
<td>7.5 ± 1.2 (5)</td>
</tr>
<tr>
<td>2 mM dU</td>
<td>8.4 ± 1.5</td>
</tr>
<tr>
<td>6 mM dU</td>
<td>9.2 ± 1.4 (5)</td>
</tr>
<tr>
<td>2 mM adenosine</td>
<td>7.8 ± 0.9 (7)</td>
</tr>
<tr>
<td>2 mM cytidine</td>
<td>7.1 (2)</td>
</tr>
<tr>
<td>1 mM dAMP</td>
<td>9.0 ± 0.7 (5)</td>
</tr>
<tr>
<td>2 mM dCMP</td>
<td>8.8 ± 1.4 (15)</td>
</tr>
<tr>
<td>2 mM dGMP</td>
<td>8.1 ± 1.8 (10)</td>
</tr>
<tr>
<td>2 mM dTMP</td>
<td>8.8 ± 0.3 (3)</td>
</tr>
<tr>
<td>2 mM dA + 2 mM dCMP</td>
<td>9.4 ± 1.2 (11)</td>
</tr>
<tr>
<td>2 mM dA + 2 mM dG + 4 mM dCMP</td>
<td>10.2 ± 2.0* (10)</td>
</tr>
<tr>
<td>2 mM dG + 1 mM NaH₂PO₄ + 1 mM Na₂HPO₄</td>
<td>7.9 ± 1.2 (7)</td>
</tr>
<tr>
<td>2 mM dG + 2 mM dCMP</td>
<td>8.2 ± 1.2 (4)</td>
</tr>
<tr>
<td>2 mM dG + 2 mM dC</td>
<td>8.6 ± 0.7 (5)</td>
</tr>
<tr>
<td>2 mM dT + 2 mM dCMP</td>
<td>8.6 ± 1.1 (10)</td>
</tr>
<tr>
<td>4 mM TdR + 2 mM dC</td>
<td>9.7 ± 1.6 (5)</td>
</tr>
<tr>
<td>0.077 mM BUDR</td>
<td>8.3 ± 1.3 (20)</td>
</tr>
<tr>
<td>1 mM BUDR</td>
<td>8.1 ± 2.1 (13)</td>
</tr>
<tr>
<td>0.005-0.077 mM FUDR</td>
<td>8.4 ± 0.9 (23)</td>
</tr>
<tr>
<td>1 mM FUDR</td>
<td>8.6 ± 1.7 (14)</td>
</tr>
</tbody>
</table>

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3. Metaphasic abnormalities.

2. Prophasic abnormalities.

This should be consulted in connection with Table 2.

present study in both treated and untreated dividing cells are
considered, all the kinds of abnormalities encountered in the
Division in Treated HeLa Cells

Before the experimental results with the tested substances are
considered, all the kinds of abnormalities encountered in the
present study in both treated and untreated dividing cells are
itemized with some explanations in the following tabulation.
This should be consulted in connection with Table 2.

1. Preprophasic inhibition, or inhibition of interphasic cells from
entering prophase.

2. Prophasic abnormalities.
   a. Arrest at early prophase. Sometimes, arrested prophasic
cells reverted to the interphasic appearance.
   b. Prolongation of early prophase. This category included only
those cells in which the whole prophase lasted more than 35
min. No prophase exceeded 74 min in this study. Once cells
entered midprophase, they proceeded to prometaphase
without being inhibited.
   c. Conspicuous change in nuclear shape in middle or late
prophase.
   d. Premature rounding up of cell body. Normally, contraction
of the cell began with nuclear membrane breakdown.

3. Metaphasic abnormalities.
   a. Occurrence of polar (centrophilic) chromosomes, or a 3-
group metaphase. A polar chromosome is one that stays
near a spindle pole after the others have aligned on the
equator. If there are groups of such centrophilic chromo-
somes at both poles, in addition to the gathering at the
equatorial plate, the figure is termed a 3-group metaphase
(44). This abnormality has been regarded as an expression
of a spindle disorder (43, 49), possibly resulting from partial
inhibition of spindle fibers (49). Kinetochores or portions
of chromosomes contributing to kinetochore activity may
be involved in the induction of this abnormality (4), since
there is much evidence for the importance of the kineto-
chore in mitotic chromosomal movements (e.g., 9, 18, 19,
48, 52, 54). Three-group metaphases can be produced by
various substances and physical agents, and sometimes
they appear "spontaneously" in untreated normal as well as
malignant cells (e.g., 4, 6, 49). They have been suggested to
arise from effects on sulfhydryl groups (43) or nucleic acid
metabolism (6). All the polar chromosomes observed in the
present study moved to the equator before anaphase and di-
vided equally at this stage. Thus no persistent polar chro-
mosome that could have contributed to unequal distribu-
tion of chromosomes was observed.
   b. Irregularity of chromosome arrangement on the equator.
Disorders in the kinetochores or spindle are suspected.
   c. Fragmentation of chromosomes. The akinetic fragments
moved abnormally. This was observed in only 1 cell, which
had been treated with 1 mM BUdR for 50.5 hr.
   d. Lowering of optical density of chromosomes. This change
was usually seen in prolonged metaphase and was often ac-
companied by indistinctness of the spindle border.
   e. Partial suppression of spindle development, or formation of
a small spindle.
   f. Conspicuous oscillation of spindle, or active movement of
mitotic apparatus, easily observed directly.
   g. Eccentric location of spindle. In some cases of prolonged
metaphase, the spindle was off-center in the cell.
   h. Indistinctness of spindle border, often noted in prolonged
metaphase.
   i. Formation of cytoplasmic processes or blebs (Figs. 17, 18).
   j. Unusual contraction or rounding up of cell body (Fig. 12).
Cells with this abnormality were very refractile. Moorhead
and Hsu (36) observed that high refractility was common
among cells with aberrations.
   k. Prolongation of metaphase.

4. Anaphasic abnormalities.
   a. Asynchronous separation of chromosomes.
   b. Laggard chromosomes accompanied or unaccompanied by
formation of micronuclei at telophase. In most cases the
leggards were seen to catch up with the other chromosomes
eventually and fuse with them. Only rarely did they remain
separate from the major chromosome group and form micro-
nuclei.
   c. Precession of 1 or more chromosomes.
   d. Formation of chromosome bridge.
   e. Invisible (perhaps interzonal) connection between ends of
sister chromatids. For several min after the separation of
sister chromatids, their distal ends behaved as though con-
ected by an invisible threadlike element. Similar observa-
tions were reported by Nakanihchi et al. (39) between the
homologues of bivalents that had received localized β-ir-
radiation and had formed a sticky bridge.
   f. Nondivision of 1 or more chromosomes.
   g. Conspicuous oscillation of mitotic apparatus.
   h. Insufficient elongation of spindle. This was observed in 1 cell
treated with 4 mM dC for 1 hr and 40 min.
   i. Moving back and reunion of once-separated sister chromo-
some groups.

CHART 1.—Metaphase duration in untreated HeLa cells with
respect to time since last feeding.

Division in Treated HeLa Cells

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without being inhibited.
   c. Conspicuous change in nuclear shape in middle or late
prophase.
   d. Premature rounding up of cell body. Normally, contraction
of the cell began with nuclear membrane breakdown.
TABLE 2A
MEAN DURATION OF METAPHASE IN CELLS SHOWING EACH TYPE OF MITOTIC ABNORMALITY WITH RESPECT TO DIFFERENT TREATMENTS

<table>
<thead>
<tr>
<th>Treatment</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
<th>G</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>71.1 (2)</td>
<td>37.6 (1)</td>
<td>95.5 (5)</td>
<td>75.0 (1)</td>
<td>169.5 (1)</td>
<td>40.9 (1)</td>
<td>90.8 (1)</td>
</tr>
<tr>
<td>1-2 mM dA</td>
<td>44.5 (2)</td>
<td>37.4 (1)</td>
<td>112.3 (4)</td>
<td>92.3 (4)</td>
<td>36.2 (1)</td>
<td>94.0 (1)</td>
<td>39.0 (2)</td>
</tr>
<tr>
<td>2 mM dG</td>
<td>70.5 (2)</td>
<td>12.8 (1)</td>
<td>108.8 (3)</td>
<td>207.7 (5)</td>
<td>165.9 (2)</td>
<td>114.0 (1)</td>
<td></td>
</tr>
<tr>
<td>2-4 mM TdR</td>
<td>148.0 (5)</td>
<td>193.1 (6)</td>
<td>247.0 (2)</td>
<td>139.0 (1)</td>
<td>91.0 (8)</td>
<td>116.8 (3)</td>
<td></td>
</tr>
<tr>
<td>2 mM dTMP</td>
<td>30.8 (1)</td>
<td>68.8 (2)</td>
<td>44.2 (2)</td>
<td>139.0 (1)</td>
<td>118.5 (3)</td>
<td>122.7 (4)</td>
<td>45.2 (2)</td>
</tr>
<tr>
<td>2 mM dCMP</td>
<td>57.0 (1)</td>
<td>148.0 (5)</td>
<td>193.1 (6)</td>
<td>247.0 (2)</td>
<td>139.0 (1)</td>
<td>118.5 (3)</td>
<td>122.7 (4)</td>
</tr>
<tr>
<td>0.007 mM BUDr</td>
<td>24.9 (2)</td>
<td>88.2 (1)</td>
<td>32.7 (2)</td>
<td>56.6 (1)</td>
<td>44.9 (3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 mM BUDr</td>
<td>37.6 (1)</td>
<td>44.7 (1)</td>
<td>37.6 (1)</td>
<td>153.5 (1)</td>
<td>37.6 (1)</td>
<td>274.7 (1)</td>
<td>274.7 (1)</td>
</tr>
<tr>
<td>0.005-0.077 mM FUDr</td>
<td>153.5 (1)</td>
<td>37.6 (1)</td>
<td>274.7 (1)</td>
<td>274.7 (1)</td>
<td>274.7 (1)</td>
<td>59.3 (1)</td>
<td></td>
</tr>
</tbody>
</table>

Key to column headings: A, Prolongation of early prophase; B, premature rounding-up of cell body; C, centrophilic chromosome or 3-group metaphase; D, irregular chromosome arrangement during metaphase; E, lower chromosomal optical density or indistinct spindle border; F, partial suppression of spindle development; G, oscillation or conspicuous movement of mitotic apparatus; H, occurrence of cytoplasmic processes in metaphase.

Figures without parentheses indicate mean duration of metaphase in min; those in parentheses are the number of cells that showed the abnormalities concerned.

j. Tripolar division.
k. Premature appearance of precursor of midbody. The midbody usually appeared at the same time as the cleavage furrow was completed and the nuclear membrane was formed. In some cases the first evidence of midbody formation was a row of dense granules on the equatorial plane at later anaphase or early telophase, before completion of the cleavage furrow. The midbody developed by the aggregation of such granules, which must be identical with those dense bodies seen, by electron microscopy, being associated on the continuous spindle fibers in rat erythroblasts (10). In the present report only those cases were included in this category in which the presumptive midbody was noted within 4 min of the start of anaphase.
l. Abnormal cytoplasmic processes.
m. Strong bubbling.
5. Telophase abnormalities.
a. Formation of irregularly shaped nuclei. This was seen in but 1 cell, which had been treated with 1 mM BUDR for 50.5 hr.
b. Failure of nuclear membrane formation.
c. Formation of micronuclei.
d. Retardation of nucleolar formation, or formation of small nucleoli. If the nucleoli first appeared later than 25 min after the start of anaphase or if they were unusually small, the case was included in this category.
e. Cytokinetic irregularities: distortion or suppression of cleavage, regression of once-started cleavage furrow, and cleavage on 1 side alone. Cytokinetic irregularities were sometimes followed by formation of 1 daughter cell with 2 nuclei and another with none.
f. Abnormal shrinkage or refractility of cytoplasm during cleavage.
g. Bubbling all over the cell surface.
h. Premature appearance of midbody precursor.
i. Protrusions of cytoplasmic blebs or irregular outline of daughter cells after cleavage, sometimes accompanied by high refractility of the cytoplasm.
6. Abnormalities in early interphase.
a. Unusual contraction of cell body.

General Remarks on Time Relations of Mitotic Phases in Treated Cells

Table 1 gives the mean duration and standard deviation of delimited segments of mitosis after treatment with different...
### TABLE 2B

**Mean Duration of Metaphase in Cells Showing Each Type of Mitotic Abnormality with Respect to Different Treatments**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mitotic abnormality&lt;sup&gt;a, b&lt;/sup&gt;</th>
<th>I</th>
<th>J</th>
<th>K</th>
<th>L</th>
<th>M</th>
<th>N</th>
<th>O</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>113.8 (1)</td>
<td>35.8 (1)</td>
<td>41.2 (2)</td>
<td>47.4 (7)</td>
<td>73.5 (2)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1–2 mm dA</td>
<td></td>
<td>138.1 (3)</td>
<td>36.0 (1)</td>
<td>99.0 (1)</td>
<td>114.0 (1)</td>
<td>27.3 (1)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 mm dG</td>
<td></td>
<td>238.5 (2)</td>
<td>99.0 (1)</td>
<td>44.8 (1)</td>
<td>76.9 (7)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2–4 mm TdR</td>
<td></td>
<td>73.6 (6)</td>
<td>30.5 (2)</td>
<td>60.8 (1)</td>
<td>33.0 (1)</td>
<td>21.0 (1)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 mm dC</td>
<td></td>
<td>92.6 (2)</td>
<td>30.1 (1)</td>
<td>66.0 (1)</td>
<td>41.8 (1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 mm dC</td>
<td></td>
<td>23.7 (1)</td>
<td>33.2 (1)</td>
<td>56.0 (1)</td>
<td>20.8 (1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 mm dU</td>
<td></td>
<td>59.7 (1)</td>
<td>30.3 (1)</td>
<td>33.0 (1)</td>
<td>30.8 (1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6 mm dU</td>
<td></td>
<td>193.7 (1)</td>
<td>40.1 (1)</td>
<td>56.0 (1)</td>
<td>125.0 (1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 mm adenosine</td>
<td></td>
<td>122.0 (1)</td>
<td>122.0 (1)</td>
<td>120.2 (2)</td>
<td>118.4 (1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 mm cytidine</td>
<td></td>
<td>109.9 (6)</td>
<td>95.5 (1)</td>
<td>58.0 (1)</td>
<td>36.7 (1)</td>
<td>72.0 (2)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 mm dAMP</td>
<td></td>
<td>104.1 (5)</td>
<td>27.1 (1)</td>
<td>53.8 (1)</td>
<td>45.2 (2)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 mm dGMP</td>
<td></td>
<td>125.0 (5)</td>
<td>41.8 (1)</td>
<td>41.2 (1)</td>
<td>48.3 (2)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 mm dTMP</td>
<td></td>
<td>34.3 (2)</td>
<td>41.8 (1)</td>
<td>25.2 (1)</td>
<td>43.2 (1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 mm dCMP</td>
<td></td>
<td>0.77 mm BuDR</td>
<td>31.9 (2)</td>
<td>26.4 (2)</td>
<td>59.5 (2)</td>
<td>30.1 (3)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 mm TdR + 2 mm dCMP</td>
<td></td>
<td>25.0 (1)</td>
<td>38.9 (4)</td>
<td>26.4 (2)</td>
<td>59.9 (2)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.005–0.077 mm FUdR</td>
<td></td>
<td>65.2 (2)</td>
<td>46.3 (3)</td>
<td>26.4 (2)</td>
<td>26.3 (1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 mm FUdR</td>
<td></td>
<td>59.3 (1)</td>
<td>22.4 (1)</td>
<td>37.6 (1)</td>
<td>123.7 (3)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Figures without parentheses indicate mean duration of metaphase in min; those in parentheses are the number of cells that showed the abnormalities concerned.

<sup>b</sup> Key to column headings: I, Conspicuous contraction of cell in metaphase; J, asynchronous separation of chromosomes in anaphase; K, laggard chromosome in anaphase; L, formation of chromosome bridge; M, tripolar division; N, premature appearance of midbody precursor; O, retardation of nucleolar formation; P, cytokinetic irregularity or abnormal cytoplasmic contraction during cleavage.

Chemicals. It can be noted that (a) no significant acceleration took place in any mitotic phase or segment as the result of treatment, (b) some cells showed delay or inhibition of division at metaphase (delay sometimes occurred in early prophase or in telophase), and (c) anaphase was least susceptible to changes in chemical environment. The 95% confidence intervals were calculated from Table 1 on the mean duration of anaphase and on the durations of various periods from the start of anaphase to completion of the cleavage furrow, to appearance of the midbody, to 1st appearance of the nuclear membrane, or to the appearance of nucleoli in each treated group. Where the interval did not overlap that of the corresponding mean of the control, this fact was indicated by a footnote in the table. Taking into account the errors in measurement of these periods, however, we hesitate to conclude that the chemicals used caused any deceleration or acceleration in these postmetaphasic stages of division. It is of interest that Oishi (41) reported that duration of anaphase and telophase in HeLa and HL-C cells showed no consistent difference between untreated cells and those treated with Carzinophilin or thio-TEPA, whereas metaphase was delayed in the treated cells.

In our study, prolongation or arrest of metaphase was especially noticeable among cells treated with certain compounds. Efforts were therefore made to correlate metaphase prolongation with specific mitotic abnormalities. Table 2 indicates the mean duration of metaphase in cells with each mitotic abnormality with respect to treatment with different chemicals. It is apparent that metaphase generally lasted longer in cells with such abnormalities as occurrence of 1 or more polar chromosomes (3-group metaphases are included in this category), irregularity of chromosome arrangement on the equator, unusually strong contraction of cytoplasm at metaphase, lowering of optical density of chromosomes, and/or indistinctness of the spindle border. On the other hand, metaphase was usually not prolonged in cells with the other mitotic abnormalities listed in the table, as is clear if we compare each entry in Table 2 with the mean duration of metaphase of the same treatment group in Table 1.

**Effects of Deoxyribonucleosides**

Severity of effects generally decreased in the order dG, dA, TdR, dC, and dU. The cytologic effects of the 1st 2 especially were much the same.

dA produced morphologic changes in both interkinetic and mitotic cells. Some interphase cells showed a transient change in...
nucleoli within the 1st hr of exposure to 1 or 2 mM dA. This change was very similar to, but slighter than, the adenosine-induced nucleolar damage mentioned below. Among 21 observed cells, 2 exhibited a prolongation of early prophase, and 1 was arrested at this stage. The most conspicuous effects of dA, however, were (a) inhibition of cells from entering mitosis, very few mitotic figures being seen within the 1st 5 or 6 hr of treatment (this condition lasted at least more than 24 hr), and (b) inhibition of division at metaphase. Prolongation of metaphase was noticed already in cells that were under observation as prophase cells within 30 min after exposure to the compound began (Chart 2). Other abnormalities encountered are listed in Table 2.

dG was also very active. Cells treated with 2 mM dG were markedly inhibited from entering mitosis, the number of cells in prophase being greatly reduced in cultures treated for more than 5 or 6 hr. A transient change in nucleoli, just as in cells treated with dA, was also noted in many cells about an hr after treatment with dG. Out of 14 cells followed, 2 were arrested at early prophase. Metaphase prolongation or arrest was also characteristic of dG, and this effect was found in all cells whose observation from prophase was begun after the treatment was started (Chart 3). dG was the most potent in its metaphase-delaying action among the deoxyribonucleosides tested. In prolonged metaphases, chromosome arrangement on the equator was usually irregular and polar chromosomes were often seen, although the size and shape of the spindle seemed not to be altered. This suggests that dG affected the function of the kinetochore. Other abnormalities observed are given in Table 2.

In order to determine the time of the injury that induces metaphase delay in cells treated with this compound, cultures were irrigated with medium containing 2 mM dG while cells were being observed under the microscope. When the agent was added shortly after the nuclear membrane had disappeared, no metaphasic delay or chromosomal abnormality occurred in 2 cells observed, although a moderate delay occurred in another (Chart 3). This result can be interpreted in 2 ways: (a) in order for the cells to exhibit the effect they must be treated just before or during prophase, or (b) it takes a little more than 0.5 hr for the concentration of the substance within the cells to become high enough to cause the metaphasic inhibition. In view of the rapidity with which dG enters cells (see below), the first possibility seems to be more likely.

TdR was tested for effects on cell division at 2 and 4 mM. Cells in prophase became less frequent in cultures treated with it for more than several hr. No morphologic effect on late interphase cells was noted. The salient effect of this compound was also an inhibition of metaphase, although there were marked individual differences among cells in their reaction to TdR: some cells were delayed in metaphase, while others were not. The metaphasic delay was already seen in some cells that entered prophase within 30 min of treatment. The effects of TdR on
Deoxyribonucleosides and Cell Division

HeLa cell division are summarized in Tables 1 and 2 and in Chart 4.

dC was tested at 2 and 4 mM for its effects on division. In marked contrast to the 3 nucleosides mentioned above, dC caused little abnormality within at least the 1st several hr of exposure. Nor was metaphase delayed, except in 1 cell that underwent a tripolar division. In cultures treated with 2 mM dC, 1 mitosis out of 13 observed was arrested in early prophase. Two cells showed prolongation of early prophase. In cultures treated with 4 mM dC, chromosomal bridges were formed in 2 cells. In another cell, spindle elongation in anaphase was inadequate (Tables 1, 2, and Chart 5).

The mean duration of metaphase in cells treated with 4 mM dC (Table 1) was significantly shorter than the value given for the untreated controls. The former value, however, was for cells within the 1st 6 hr of treatment, while the latter was for control cells during the entire 1st 24 hr after feeding. The value for treated cells did not differ significantly from the mean duration of control metaphases studied within the 1st 6 hr after feeding.

dU at 2 mM had no deleterious effect on mitotic or interphasic cells. Metaphase duration was within a normal range. At 6 mM, however, dU did appear to delay metaphase (Tables 1, 2, and Chart 6).

Effects of Ribonucleosides

Extensive studies with adenosine have been carried out on both dividing cells and interphasic cells. Hughes (17) and Lettré (32) reported on the morphologic alteration of interphasic nucleoli by adenosine, and Siebs (49) studied its effect of inducing metaphasic abnormalities with polar chromosomes in cultured fibroblasts and tumor cells. It is a preprophasic inhibitor (17).

In the present study, the effects of this physiologic ribonucleoside were compared with those of the deoxyribonucleosides. Cells treated with 2 mM adenosine began to undergo a characteristic nucleolar alteration within 20 min or so: either the middle portion lost optical density and became marginated with the apparently unaltered, peripheral dense portion (Fig. 20), or the nucleolus broke up into separate granules (Fig. 21), as Hughes (17) and Lettré (32) described. Sometimes filamentous structures became visible in the nucleolus, as Lettré (32) reported, and as Dénués and Mottram (13) observed in human cancer cells in hypotonic medium. This change was transient in our material, nucleoli being restored to normal structure within 4-5 hr. It is noteworthy that an essentially similar change in nucleoli followed treatment with 0.01-1.0 mM 6-thioguanine or a combination of dA, dG, and dCMP. A similar but slighter alteration of nucleoli was noted in cells treated with dA, dG, FUdR, dA plus dCMP, or dG plus dCMP. The nucleolar change produced by these compounds seemed to differ morphologically from that produced by 4-nitroquinoline-N-oxide (47) or actinomycin D (46).

Nucleoli at prophase were similarly affected by adenosine. Some prophasic cells with altered nucleoli went through mitosis.

Charts 5, 6, and 7 illustrate the effects of these compounds on metaphase duration in HeLa cells.
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with no other abnormality. Some other treated cells had prolonged prophases. Among 17 mitoses studied, 1 was arrested at early prophase. One cell exhibited irregularity of chromosomal arrangement and delay of metaphase, and another showed some irregularity in cleavage.

Other minor abnormalities were also seen in cells exposed to adenosine. After several hr of treatment, mitotic cells were considerably decreased. Mean duration of metaphase in cells treated with 2 mM adenosine was somewhat prolonged, although the 90% confidence interval on the mean did overlap to some extent that on the mean of the untreated metaphases (Table 1, Chart 7). In 16 cells observed, no polar chromosome was found more than 20 min after the nuclear envelope disappeared. Hughes (17) described the action of adenosine in retarding nucleolar recon-

Effects of Deoxyribonucleotides

Effects of the 4 kinds of deoxyribonucleotides are shown in Table 1 and 2 and in Charts 9–12. Cell division was inhibited to
Deoxyribonucleosides and Cell Division

Reversal of Metaphasic Inhibition Caused by Deoxyribonucleosides

As mentioned above, dG, dA, and TdR, in descending order, strongly inhibited HeLa cells in metaphase. One common biochemical activity of these 3 is a potent inhibition of the conversion of CDP to dCDP and hence of the biosynthesis of DNA. Therefore, attempts were made to release the cells from metaphasic inhibition by simultaneous treatment with dC or dCMP, on the assumption that this inhibition was caused by the inhibitory effect of the deoxyribonucleosides on the synthesis of deoxycytidylate and DNA. As Tables 1 and 2 and Charts 13 and 14 indicate, 2 mM dC and 2 mM dCMP tended to reverse the adverse effects of 4 mM and 2 mM TdR, respectively. Similarly, 2 mM dCMP antagonized metaphase prolongation by 2 mM dA.

Chart 12.—Metaphase duration in HeLa cells treated with deoxycytidine-5'-monophosphate.

Chart 13.—Metaphase duration in HeLa cells treated with thymidine plus deoxycytidine.

a degree after several to 24 hr of exposure to 1 mM dAMP, 2 mM dGMP, or 2 mM dTMP. This effect was much weaker than that of their corresponding nucleosides. A transient nucleolar change caused by 2 mM dGMP was similar to but much slighter than that induced by adenosine. Except for 2 mM dGMP, which markedly prolonged metaphase of a minority of treated cells, the nucleotides caused only a slight or no prolongation of metaphase. The 95% confidence interval on the mean duration of metaphase in cells treated with 2 mM dCMP did not overlap that on the mean of the control metaphases, whereas those on the mean durations of metaphase in cells treated with the other nucleotides did overlap. The incidence of mitotic abnormalities induced by these compounds was correspondingly low. Cells treated with 2 mM dCMP often exhibited oscillation of the mitotic apparatus during metaphase readily detectable by means of direct visual observation.

Chart 14.—Metaphase duration in HeLa cells treated with thymidine plus deoxycytidine-5'-monophosphate.

Chart 15.—Metaphase duration in HeLa cells treated with deoxyadenosine plus deoxycytidine-5'-monophosphate.
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Chart 16.—Metaphase duration in HeLa cells treated with deoxyguanosine plus deoxycytidine.

Chart 17.—Metaphase duration in HeLa cells treated with deoxyguanosine plus deoxycytidine-5'-monophosphate.

Chart 18.—Metaphase duration in HeLa cells treated with deoxyadenosine plus deoxyguanosine plus deoxycytidine-5'-monophosphate.

Chart 19.—Metaphase duration in HeLa cells treated with deoxyguanosine plus monobasic and dibasic sodium phosphates.

The inhibitory action of dG was only partly blocked, however, by dC or dCMP at 2 mM (Tables 1, 2, Charts 16, 17).

It is also known that dA or its triphosphate inhibits the reduction of guanosine and cytidine phosphates to the corresponding deoxyribonucleotides in Ehrlich ascites tumor cells (27, 28). Hence, in order to overcome the inhibition of DNA synthesis by dA, one might have to add both dG and dC. The experiment shown in Chart 18 was carried out to see whether or not HeLa cells were subject to metaphase delay when treated with a combination of dA, dG, and dCMP. There resulted a marked delay of metaphase in addition to a higher incidence of mitotic abnormalities (Tables 1, 2). The nucleoli in interphase cells became less dense and revealed fibrous structure, much as after adenosine, within the 1st 35 min of treatment. The nucleolar change was very pronounced by 4.5 hr. Nucleolar reappearance in telophase was retarded in some cells. This combination of the compounds was rather toxic to the cells, most of which became detached from the coverslip within the 1st 24 hr of application.

Because deoxyribonucleosides inhibited metaphase more strongly than the corresponding nucleotides, the experiment shown in Chart 19 was undertaken in order to test the effect of adding phosphate radicals with dG. In this experiment, 2 mM dG was accompanied by 1 mM NaH2PO4 plus 1 mM Na2HPO4. As comparison of Chart 19 with Charts 3 and 10 reveals, this experiment resulted in failure of phosphate radicals alone, as inorganic phosphate, to change the high effectiveness of 2 mM dG to the low effectiveness of 2 mM dGMP.
Deoxyribonucleosides and Cell Division

Effects of 5-Halogenated Deoxyuridines

Two concentrations of BUdR were tested for their effects on cell division (Tables 1, 2, Charts 20, 21). At the 0.077-mM level, mitosis proceeded almost as in untreated cells within several hr after the treatment, although slight abnormalities were seen. In cells treated for more than 24 hr, abnormalities increased in both spindle and chromosomes. In cells treated for more than 40 hr, cleavage was often abnormal. Mean duration of metaphase was within the normal range. At the 1.0-mM level, morphologic abnormalities were more frequent than at the lower concentration within the 1st several hr of treatment, but metaphase was not prolonged. In cells of H.Ep.2 treated with the same substance at 1.0 mM for 24 hr, no evidence of metaphasic delay had been observed (6). In HeLa cells treated for more than 20 hr, cytoplasmic as well as chromosomal abnormalities were frequently seen. In 1 cell treated for 50.5 hr, there was multiple chromosomal fragmentation, and many akinetic fragments were seen just outside the metaphase spindle. Many polar chromosomes were also observed. Subsequent chromosome separation was irregular, and many bridges formed; cytokinesis was also not regular. On the whole, mitotic abnormalities induced by BUdR increased progressively with time of exposure. This might be expected if the total amount of BUdR incorporated into DNA in place of TdR increased with time after treatment.

FUdR is known as a strong and specific inhibitor of thymidylate synthetase and consequently of DNA synthesis as well (12). Accordingly, cultures treated with 0.005, 0.077, or 1.0 mM FUdR for more than several hr had markedly reduced numbers of dividing cells. Interphase cells did not show noticeable change until some 20 hr after the exposure, when some shrunken, granular cells appeared. These degenerated cells increased in number with time, and many came loose from the coverslip. By the 4th day, few viable cells were seen. They looked barely normal, and each had a larger nucleus and cytoplasm, although whether this had resulted from selective damage to smaller cells, from swelling by imbibition, or from excessive synthesis of cellular material in small cells that survived is not known. Eidinoff and Rich (14) had observed to the contrary that the nuclear size of H.Ep.1 cells remained approximately constant throughout 72 hr treatment with $4 \times 10^{-7}$ mM FUdR.

Cultures treated with 1 mM FUdR for 24 hr included some interphase cells in which nucleoli had undergone a slight change (Fig. 22) similar to that induced by adenosine. Some cells had nuclei with many chromatin particles evenly distributed within them. After treatment for 48 hr, many cells showed granular mitochondria intermingled with normal filamentous ones. Metaphase was prolonged in a few of the cells treated with FUdR, although mean duration of metaphase in cells treated with either of these concentrations did not differ significantly from normal (Table 1, Charts 22, 23). Various mitotic abnormalities increased in incidence, as early as within several hr of exposure, in proportion to the concentration of FUdR. Cultures treated with each of these concentrations for more than 20 hr had a con-
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CHART 23.—Metaphase duration in HeLa cells treated with 1.0 mm 5-fluoro-2'-deoxyuridine.

![Graph showing metaphase duration in HeLa cells treated with 1.0 mm 5-FUdR.](chart.png)

A considerable proportion of abnormal metaphases, which were prolonged, displayed an irregular arrangement of chromosomes, some being polar, and had, in some cases, a small spindle and/or irregular cytoplasmic processes. Because of a higher frequency of 3-group and scattered metaphases in the cells treated with 0.008–1.0 mm 5-fluoro-2'-deoxyuridine, it had been concluded (6) that this substance had a metaphase-delaying effect, although it did not completely arrest the metaphase.

**Discussion**

Permeability of the plasma membrane should be considered in any encompassing study of the mechanism of mitotic inhibition by a chemical compound; the agent must enter the cell through the plasma membrane, and its concentration in the intracellular phase must attain some level. According to Jacques (20), all 7 normal and synthetic nucleosides studied by him entered the intracellular phase of Ehrlich ascites cells rapidly and were then enzymatically split into their component pyrimidine base and pentose, except for azauridine. His research included dU, dTdR, and Fu'dR. There is much evidence in the literature for a rapid rate of penetration of other ribo- and deoxyribonucleosides. According to Hughes (17), adenosine, not being ionized, presumably enters the cell more rapidly than the ionizing nucleotides. Perhaps for this reason adenosine has a greater effect on nucleoli than does the nucleotide. Mills (35) considered dC to be readily available to erythrocytes by diffusion from plasma. Therefore, it should be appropriate to assume that all the nucleosides used here readily penetrate the plasma membrane.

This may not be true for nucleotides. Leibman and Heidelberger (31) presented evidence for a very small penetration of intact ribonucleoside-5'-phosphates into the cell. Hampton et al. (16) have shown that 5-ido-2'-deoxyuridine-5'-phosphate is less effective than the parent nucleoside as a source of 5-ido-uracil within the cell, as a result of the difficulty with which the phosphorylated compound passes through natural membranes. Wilson and Wilson (55) did not obtain evidence for permeability of the intestinal epithelium to GMP or AMP. From these facts we might assume also that the deoxyribonucleotides we used penetrated less readily than the corresponding nucleosides. Although Nemer (40) has shown that very young sea urchin embryos appear able to use exogenous dTMP and dUMP, he did not comment on the ability of these nucleotides to cross the cell membrane of the fertilized eggs. There is evidence that nucleotides are dephosphorylated at the cell surface by phosphatases, giving rise to the corresponding nucleosides and phosphate ions. These are able to enter the intracellular phase, where they can be incorporated into nucleic acids and nucleotides of acid-soluble fractions (16, 31). These facts may be important in the interpretation of the effect on mitosis of any nucleoside or nucleotide.

Our chief interest in the present study is in metaphase prolongation. Some of the agents decreased the frequency of cells entering mitosis, and we may tentatively ascribe this effect of preprophase inhibition to their known interference in DNA synthesis. Let us consider whether it may be reasonable to attribute the delay or arrest of metaphase to the same interference.

Among the 4 naturally occurring deoxyribonucleosides, dG, dA, and TdR have now been shown to have the striking effect of delaying or arresting metaphase in dividing HeLa cells at the concentrations tested. This confirms the finding of Barr (1), who reported that exogenous TdR at 2 mm brought about prolongation of metaphase in HeLa cells after both 26 and 45 hr of treatment, according to the ratio of metaphasic to prophasic cells counted in fixed and stained preparations. It is noteworthy that dC alone did not have a detrimental effect on division and that this compound or its 5'-phosphate could reverse the metaphase-delaying effect of TdR and of dA, although not that of dG. These 3 active deoxyribonucleosides are seen to be more effective as metaphase poisons than dU or its halogenated derivatives, BUdR and Fu'dR.

The relevant information with respect to DNA synthesis is as follows. The triphosphates of dA, dG, and TdR inhibit the conversion of CDP to dCDP and hence ultimately inhibit the synthesis of DNA (37, 45) dA also inhibits the reduction of guanosine nucleotide to deoxyguanosine nucleotide (38). Fu'dR is also a potent inhibitor of DNA synthesis (12). According to Painter and Drew (42), the time from the end of the DNA synthetic phase to the beginning of the next prophase ranged from 3 to 10 hr in HeLa cells grown in 10% horse serum. As metaphasic inhibition by the 3 deoxyribonucleosides in the present study was already seen in cells that were in prophase at the time of treatment with dG or in cells that entered prophase within 30 min of treatment with dA or TdR, it is obvious that these affected cells were, when treated, in prophase or toward the end of G2, a period of the cell cycle in which DNA synthesis is generally considered to have been completed. It need not be assumed that treated cells pass through the G2 phase at the same speed as untreated ones; rather, treated cells may slow down at this stage. Thus, Vicia faba root cells treated with 0.01 m Fu'dR were delayed in G2 (2). It may therefore be that the metaphasic inhibition found in the present study is not related to interference in DNA synthesis by these nucleosides. If so, the delay of TdR or dA inhibition of metaphase was almost reversed by dC or dCMP and is therefore parallel to the reversal of inhibition of DNA synthesis by dC or dCMP. Moreover, both inhibition of metaphase was only partially reversed by dC or dCMP, and dA inhibition was not reversed but was enhanced by...
the addition of dG plus dCMP. Further evidence in favor of this view is given by the fact that FUdR did not strongly inhibit the metaphase. Yet this substance at 0.001 HIM, which is lower than the lowest concentration we employed, reduced the rate of DNA synthesis in HeLa cells essentially to zero (53).

There is no definite proof, however, that a very small amount of DNA is not synthesized during G₂ or visible mitosis. Indeed, Kihlman et al. (26) have suggested that some synthesis of DNA may continue late into the G₂ period, despite failure of autoradiography to indicate it, because cytosine arabinoside caused chromosomal breakage in human leukocyte cultures, even when added as late as 1 hr before the hypotonic processing began; this agent is known to block the reduction of CDP to dCDP (11). Biesele (6) has suggested that interference in a crucial synthesis of a final bit of DNA might be a possible cause of metaphase delay by certain antinucleic acid antimetabolites.

In view of the conflicting considerations given above, we do not consider the problem of the relationship between metaphasic inhibition and suppression of DNA synthesis to have been settled by the present study.

The present discussion has been centered on possible adverse effects of physiologic but antimitotic deoxyribonucleosides on DNA metabolism. There are other possible mechanisms of mitotic inhibition or interference by these agents. The arrest of metaphase by means of amino acid analogs (8) suggests the complexity of the problem, and our further studies (unpublished results) with a number of other inhibitors are devoted to a wider exploration.

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References


Deoxyribonucleosides and Cell Division

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Fig. 1-6.—Regular course of division in the HeLa cell.

Fig. 1.—Very early prophase. Time interval from the moment when this photograph was taken is given in min at the upper left corner of each figure of this set. × 1900.

Fig. 2.—Middle to late prophase, 14 min later than the preceding stage. × 1900.

Fig. 3.—Prometaphase, 3.5 min after breakdown of the nuclear membrane. × 1900.

Fig. 4.—Metaphase. × 1900.

Fig. 5.—Late anaphase. × 1900.

Fig. 6.—Early interphase. The nuclear membrane and nucleoli have already appeared, although the nucleus has not yet become spherical. The midbody is evident. × 1900.
FIGS. 7-12.—Metaphase arrest in a HeLa cell produced by 2 mM dGMP. This cell had been treated for 91 min before the photograph of Fig. 7 was taken. Time interval in min from this 1st photograph is given at the upper left corner of each figure.

Fig. 7.—Very late prophase. Nucleoli and nuclear membrane are almost gone. × 1900.

Fig. 8.—Prometaphase. × 1900.

Fig. 9.—Prometaphase, with a few polar chromosomes, of which several are seen in this photograph, at both poles of the spindle. × 1900.

Fig. 10.—Chromosome arrangement on the equator is somewhat irregular. × 1900.

Fig. 11.—Protracted metaphase, with chromosome arrangement still somewhat irregular. × 1900.

Fig. 12.—The cell is still strongly refractile and has not entered anaphase at 247 min after Fig. 7 was photographed. × 1900.
FIGS. 13-18.—Process of division arrest in a HeLa cell that had been treated with 0.077 mM FUDR for 3 hr 29 min before the photograph in Fig. 13 was taken. Time interval in min from the moment when Fig. 13 was photographed is given in the upper left corner of each figure.

FIG. 13.—Early prophase, which was somewhat prolonged. X 1900.

FIG. 14.—Midprophase. X 1900.

FIG. 15.—Prometaphase, 5.5 min after breakdown of the nuclear membrane. X 1900.

FIG. 16.—Metaphase. X 1900.

FIG. 17.—Protracted metaphase. There are abnormal cytoplasmic processes. The border between spindle and cytoplasm is indistinct, although chromosomes are almost regularly arranged on the equator. X 1900.

FIG. 18.—Protracted metaphase, more than 2 hr after this cell was photographed in metaphase in Fig. 16. X 1900. 
Figs. 19–22.—Miscellaneous effects of certain nucleosides.

Fig. 19.—A 3-group metaphase in a HeLa cell treated for about 6 hr with a combination of 2 mM deoxyadenosine, 2 mM deoxyguanosine, and 4 mM deoxycytidine monophosphate. × 1900.

Figs. 20–21.—Nucleolar change in HeLa cells treated for 50 min with 2 mM adenosine. × 1900.

Fig. 22.—Nucleolar change similar to that induced by adenosine in a HeLa cell treated with 1 mM FUdR for 30 hr. Unlike the preceding 2 figures, however, many chromatin granules were evident in the nucleus. × 1900.
Effects on HeLa Cell Division of Physiologic Deoxyribonucleosides and Deoxyribonucleotides

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