Quantitative Studies of Incorporation of Exogenous Thymidine and 5-Bromodeoxyuridine into Deoxyribonucleic Acid of Mammalian Cells in Vitro*

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SUMMARY

Methods for the synthesis of bromodeoxyuridine-Br\textsuperscript{82} and bromodeoxyuridine-H\textsuperscript{3} (BUdR) are described.

The incorporation of both thymidine and BUdR into the deoxyribonucleic acid (DNA) of rat bone marrow cells in vitro was markedly influenced by concentrations of thymidine and BUdR, respectively, in the incubation media. At equivalent concentrations of respective substrate, the amount of thymidine incorporated always exceeded that of BUdR. Limiting concentrations of both thymidine and BUdR were found, above which little or no further incorporation of the respective compounds occurred. Efficiency of incorporation of each compound, as measured by percentage of the available dose which was incorporated, was highest at low respective substrate concentrations.

The rates of incorporation of thymidine and BUdR into the DNA of Ehrlich ascites tumor cells in vitro were maximal at the beginning of incubation and fell progressively thereafter.

Thymidine and BUdR mutually inhibit the incorporation of the other into DNA. Evidence is presented that this inhibition may be due to competition by the compounds for binding sites on the same enzyme, which is rate-limiting for their entry into the cell and subsequent incorporation into DNA.

The presence of 5-fluorouracil in the medium exerts an enhancing effect on thymidine and BUdR incorporation.

The incorporation of the halogenated analog, 5-bromodeoxyuridine (BUdR), into the deoxyribonucleic acid (DNA) of mammalian cells may confer upon them increased sensitivity to ultraviolet light (18, 19, 23) and x-ray (11, 12, 22–24, 31–33). The possibility of utilizing the radiosensitizing effect of BUdR for selective potentiation of the effect of radiotherapy on localized tumors has been discussed by Djordjevic and Szybalski (12). However, experiments with mice which received BUdR in doses up to 4 mg/day for 8 days failed to indicate either any sensitization of a transplanted tumor to x-ray treatment (21) or any evidence of increased sensitivity of mice to total-body irradiation (25). The failure to elicit radiosensitization by BUdR in vivo may be due, among other reasons, to the fact that an insufficient amount of the administered compound is actually incorporated into DNA under such conditions. The in vitro studies reported here were undertaken to elucidate some of the local tissue factors which might influence the quantitative incorporation of BUdR into DNA. In vivo observations on the distribution, decomposition, and excretion of BUdR in animals will be reported separately.

MATERIALS AND METHODS

The synthesis of both BUdR-Br\textsuperscript{82} and BUdR-H\textsuperscript{3} was undertaken to conduct the study. BUdR-Br\textsuperscript{82} was synthesized by bromination of deoxy-
uridine with Br²2 as follows: Br²2 was prepared from bromide-Br²2 by the distillation method of Weygand et al. (34), starting with about 6 mc. of the isotope and 10 mg. of sodium bromide carrier. While the collecting flask was still immersed in a dry ice-acetone mixture, the bromine crystals were dissolved in a few (1–5) ml. of CCl₄. On the average, 75 per cent of the initial radioactivity was present in the recovered bromine, and the final specific activity was about 750 mc/mg. The measurement of the radiation of the potent source and final distillation product was facilitated by an ionization chamber, which permitted direct measurement of the entire sample. The Br²2-CCl₄ mixture was used to brominate deoxyuridine, dissolved in formamide, by a modification of the Markham procedure described for the bromination of deoxycytidine triphosphate (1). Modification of the procedure followed the step involving the use of aniline. After the addition to the reaction mixture of an equal volume of water and 2 drops of NaOH, the resultant turbid solution was centrifuged. The yellow CCl₄ droplet at the bottom of the tube was removed with a fine Pasteur pipette. The remaining solution was extracted by mixing with a few ml. of ether. The extraction was performed 3 times. The supernatant ether layer was discarded, and the remaining solution was lyophilized. Heating to 60° C. was usually required toward the end of lyophilization to drive off the formamide. The remaining dry powder was taken up in a few ml. of water, and the solution was passed through Dowex 1-CI anion exchange resin. Particulate matter was removed by filtration. The final material and nonradioactive (standard) BUdR⁴ were chromatographed together on paper, with a solvent of butanol:water = 86:14 with 5 per cent ammonia. The radioactivity, as determined by counting with a windowless gas-flow strip counter, was confined to a single area whose position was identical to that of the standard, as localized by ultraviolet light.

BUdR-H³ was synthesized in a manner similar to the above by use of a mixture of stable Br²-CCl₄ to brominate deoxyuridine-H³ (900 mc/m mole). The tritiated product was identified as BUdR-H³ by demonstrating that its mobility on paper was identical to standard BUdR.

In addition to the synthesized compounds described above, thymidine-H³, 900 mc/m mole and thymidine-2-C¹⁴, 1 mc/m mole were also used.

Preparation and analysis of tissues.—Approximately 10⁸ bone marrow cells from the femur and tibia of Long-Evans rats were suspended in 10 ml. of a medium consisting of Gey’s solution plus rat plasma (3:1 v/v). The medium also contained labeled compounds, together with any carrier, in varying molar concentrations. The cell suspensions in 25-ml. Erlenmeyer flasks were incubated for 2 hours at 37° C. by an automatic incubator-shaker. Incubation and subsequent processing of any sample were carried out in duplicate. In one experiment 2.6 × 10⁸ Ehrlich ascites tumor cells, which had been grown intraperitoneally in C57BL mice, were incubated in F4FC solution (28). At the end of incubation an aliquot of the cell suspension was centrifuged, washed twice with 0.9 per cent saline, and homogenized. DNA content was determined according to a modification (16) of the method of Ogur and Rosen (26). The amount of DNA was calculated from the relationship, 1 μmole DNA phosphate (equivalent to 320 μg. DNA) per ml. has an E₀ of 6.9 at 260 μm. Radioactivity of the hydrolyzed DNA extract was determined by counting with a well-type gamma scintillation counter (Br²) or by beta spectrometry with a liquid phosphor counter (C¹⁴, H³, Br²). The activity of any sample was expressed as the per cent available radioactivity in the medium per mg. DNA in the sample. This value, multiplied by the total amount of the labeled compound and its respective carrier, if any, in the medium, expressed as μmolesm, yielded the calculated value of the incorporated compound in μmolesm/mg DNA.

RESULTS

Effect of thymidine or BUdR substrate concentration upon thymidine and BUdR incorporation into DNA.—Thymidine incorporation into the DNA of rat bone marrow cells was studied in the presence of varying concentrations (4 × 10⁻⁷ to 1.6 × 10⁻⁴ M) of thymidine or BUdR substrates, with thymidine-H³ used as the thymidine label (Chart 1). Thymidine incorporation was dependent upon, but not directly proportional to, the thymidine concentration in the medium, and tended toward a maximum at a concentration about 10⁻⁴ M. The effect of increasing substrate thymidine concentration was more marked at substrate concentrations of 1.6 × 10⁻⁴ M or less. Over this lower con-

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² Supplier: Oak Ridge National Lab., Oak Ridge, Tenn.
³ Designed and built by R. Loeveniger, Ph.D.
⁴ California Corporation for Biochemical Research, Los Angeles, Calif.
⁶ The liquid scintillator consisted of 15 ml. of a solution containing 7.0 gm. diphenyloxazole (DPO), 0.15 gm. p-bis-(5-phenyloxazolyl)-benzene (POPOP), and 60 gm. napthalene in 1 liter of toluene-1,4-dioxane (1:4 v/v) containing 30 ml. of ethanol.
centration range, the percentage of the available thymidine-$H^3$ which was incorporated varied relatively little (from 8.6 per cent/mg DNA to 5.7 per cent/mg DNA). Above thymidine concentrations of $4 \times 10^{-6}$ M, the percentage incorporation of thymidine-$H^3$ fell indirectly proportional to the increase in substrate concentration. When BUdR was substituted for thymidine in the medium in equimolar concentration, the amount of incorporated thymidine in DNA was reduced. Inhibition of thymidine incorporation by BUdR was nonlinear and was more noticeable at concentrations of BUdR in excess of $5 \times 10^{-6}$ M.

The effect of BUdR substrate concentration on BUdR incorporation was studied, with BUdR-$H^3$ used as the BUdR label (Chart 2). In a manner analogous to thymidine, the amount of BUdR incorporated was dependent on the concentration of BUdR, increasing nearly linearly at low concentrations and tending toward a maximum at a concentration about $2 \times 10^{-5}$ M. However, the amount of BUdR incorporated was less than that of thymidine at identical concentrations of the respective substrate (Charts 1, 2).

In another experiment, with thymidine-$C^{14}$ and BUdR-$H^3$ used as labels, the effect of thymidine substrate concentration upon simultaneous incorporation of thymidine and BUdR was compared (Chart 3). BUdR-$H^3$ was present in the medium in the constant amount of 1 mmole. Some inhibition of BUdR incorporation was noted even at relatively low concentrations of thymidine. This inhibition became progressively greater as the concentration of thymidine substrate was increased. Conversely, the amount of thymidine incorporated increased with increasing substrate concentrations.

The relative competition between thymidine and BUdR, when each was present in the medium in approximately equal amounts, was studied in another experiment. Five media were prepared which differed in their respective content of thymidine and BUdR (A–E, Table 1). In medium A, carrier thymidine and cold BUdR were present in equimolar amounts. Media B, C, D, and E were used as control media. Column 6 shows the mean
amount of incorporated thymidine-H\(^3\) calculated from triplicate determinations and expressed as the percentage of the thymidine-H\(^3\) in the medium per mg. DNA in the sample. The calculated total amount of thymidine incorporated (column 7) with medium A was 1.30 m\(\mu\)mole/mg DNA. In comparing this figure with that obtained with other media, it is apparent that the amount of thymidine incorporated is: (a) increased when the BUdR is replaced with an equal amount of thymidine (compare A to B), (b) unchanged when BUdR is absent from the medium (compare A to D), (c) markedly decreased when thymidine is absent from the medium (compare A to E) or when carrier thymidine is replaced with an equal amount of BUdR (compare A to C).

**Effect of 5-fluorouracil.**—The effect of BUdR substrate concentration on BUdR and deoxyuridine (UdR)\(^7\) incorporation, respectively, was determined with and without added 5-fluorouracil (5-FU). In four series of flasks, the total concentration of labeled and carrier BUdR was varied between \(1.6 \times 10^{-7}\) and \(5 \times 10^{-5}\) M. Two of the series contained BUdR-H\(^3\) in the quantity of 10 \(\mu\)c/2.3 m\(\mu\)mole. The other two series contained an identical amount of UdR-H\(^3\). One series with BUdR-H\(^3\) and UdR-H\(^3\), respectively, also contained 5-FU, \(4 \times 10^{-5}\) M. The results are shown in

<table>
<thead>
<tr>
<th>MEDIUM</th>
<th>QUANTITY OF PYRIMIDINES (m(\mu)moles/10 ml MEDIUM)</th>
<th>H(^3)-THYMIDINE INCORPORATED</th>
<th>TOTAL THYMIDINE INCORPORATED</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Thymidine-H(^3)</td>
<td>Carrier thymidine</td>
<td>BUdR</td>
</tr>
<tr>
<td>(1)</td>
<td>(2)</td>
<td>(3)</td>
<td>(4)</td>
</tr>
<tr>
<td>A</td>
<td>4.55</td>
<td>72.7</td>
<td>72.7</td>
</tr>
<tr>
<td>B</td>
<td>4.55</td>
<td>145.5</td>
<td>145.5</td>
</tr>
<tr>
<td>C</td>
<td>4.55</td>
<td>72.7</td>
<td>72.7</td>
</tr>
<tr>
<td>D</td>
<td>4.55</td>
<td>72.7</td>
<td>72.7</td>
</tr>
<tr>
<td>E</td>
<td>4.55</td>
<td>72.7</td>
<td>72.7</td>
</tr>
</tbody>
</table>

**TABLE 1**

**THYMIDINE INCORPORATION INTO DNA OF CELLS INCUBATED IN PRESENCE OF NEARLY EQUIMOLAR AMOUNTS OF BOTH THYMIDINE AND BUdR**

**CHART 4.** Increasing BUdR concentration had an enhancing effect on BUdR incorporation (cf. Chart 9) and, conversely, an inhibiting effect on UdR incorporation. The inhibition of UdR incorporation was slight at a BUdR concentration of \(2 \times 10^{-6}\) M and marked at \(5 \times 10^{-5}\) M. Under the influence of 5-FU, the amount of BUdR incorporated increased from 0.02 to 0.3 \(\mu\)mole when the BUdR concentration was increased from 2.3 to 263 \(\mu\)M.

The effect of 5-FU on the incorporation of thymidine was studied in a separate experiment, with the use of thymidine-H\(^3\). In each of four series of flasks the concentration of thymidine was the same \((4.76 \times 10^{-5}\) M), whereas that of 5-FU was varied from \(4 \times 10^{-5}\) to \(4 \times 10^{-3}\) M (Table 2). Thymidine incorporation in the absence of 5-FU was 1.17 \(\mu\)mole/mg DNA. The incorporation increased by 0.1 \(\mu\)mole in the presence of \(4 \times 10^{-3}\) M 5-FU. This increase was in the same range as that calculated for BUdR incorporation at the

\(^7\) Incorporation of UdR via the pathway UdR \(\rightarrow\) UdR-3'-monophosphate \(\rightarrow\) thymidine-5'-monophosphate is assumed.
same 5-FU concentration (cf. Chart 4). A similar increment in thymidine incorporation occurred at successive tenfold concentrations of 5-FU. An increase in exogenous thymidine incorporation in the presence of 5-FU is in agreement with previous observations in this and other laboratories (9, 13, 16). The effects of 5-FU can be attributed to its conversion \textit{in vitro} to 5-fluorodeoxyuridine monophosphate (5-FUdRMP) (6), which inhibits the enzymatic conversion of deoxyuridine-5'-phosphate to thymidine-5'-phosphate (4).

Rate of incorporation of \textit{BUdR} and thymidine.---A series of nine flasks, each containing \(2.6 \times 10^8\) Ehrlich ascites tumor cells suspended in 10 ml. of F4FC solution (31) containing \(0.3\) mmoles of thymidine-H\(^2\) and 51 m\(\mu\)M of \textit{BUdR}-Br\(^{38}\), were placed in a water bath at 37° C. Five minutes later, and at 15-minute intervals thereafter, a flask was removed, and the cells were centrifuged and processed for measurement of DNA content and H\(^2\) and Br\(^{38}\) radioactivity in DNA. The cumulative incorporation of thymidine and \textit{BUdR} in DNA with time is shown in Chart 5. After comparable time intervals a greater percentage of the available thymidine was incorporated than \textit{BUdR},

even though the concentration of the latter in the medium was about 22 times that of thymidine. The incorporation rate of each compound was relatively high initially and fell progressively during the 2-hour period of study. The total quantity of \textit{BUdR} incorporated into the DNA of ascites cells by the end of 2 hours was 0.2 mmoles/mg DNA. This value is in close agreement with that derived graphically at a concentration of 51 m\(\mu\)M (5.1 \(\times\) 10\(^{-5}\) M) of \textit{BUdR} substrate (Chart 2) in the experiment in which incorporation of \textit{BUdR} into DNA of bone marrow cells was studied.

### TABLE 2

<table>
<thead>
<tr>
<th>Series</th>
<th>5-FU (molarity)</th>
<th>Thymidine incorporated (mmoles/μg DNA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4(\times)10(^{-5})</td>
<td>1.17*</td>
</tr>
<tr>
<td>2</td>
<td>4(\times)10(^{-4})</td>
<td>1.27</td>
</tr>
<tr>
<td>3</td>
<td>4(\times)10(^{-4})</td>
<td>1.42</td>
</tr>
<tr>
<td>4</td>
<td>4(\times)10(^{-3})</td>
<td>1.51</td>
</tr>
</tbody>
</table>

* Duplicate determinations in each series agreed within 2 per cent.

### DISCUSSION

The results indicate that the quantitative incorporation of exogenous thymidine into the DNA of mammalian cells \textit{in vitro} is markedly affected by the substrate thymidine concentration. Such a dependence of incorporation rate upon substrate concentration is reminiscent of the product-substrate dependence observed in many enzymatic reactions (5). It is not possible to determine from our data whether the increased incorporation of exogenous thymidine is associated with a propor-
ilar results were observed by Cheong et al. (7) in their experiments with H.Ep. #1 cells derived from a human cervical carcinoma. If one assumes that net DNA synthesis is not increased in the presence of BUdR, then an increasing BUdR incorporation with increasing substrate concentration implies either a competition of BUdR with thymidine for one or more enzymes involved in their phosphorylation and subsequent incorporation into DNA, or a feedback inhibition of thymidine synthesis, possibly by interfering with the reductive conversion of cytidine diphosphate to deoxycytidine diphosphate, as has been suggested by Opara-Kubinska et al. (27). Although it is appreciated that several enzymatic steps are involved in the incorporation of thymidine into DNA (2, 3), a quantitative assessment of a competition between thymidine and BUdR was attempted by analyzing the data as though their introduction into the cell and subsequent incorporation into DNA involved a single rate-limiting step. According to the data presented in Charts 1 and 2, the reciprocal of incorporation rate (mamoles/mg DNA/2 hr) was plotted as the ordinate against the reciprocal of substrate concentration (5) for thymidine and BUdR, respectively. A linear relationship was obtained for each compound. The intersection of the curves, the Michaelis constant (K_m) was calculated (Table 3). The enzyme inhibitor constants (K_i) of BUdR for thymidine incorporation, and of thymidine for BUdR incorporation, were calculated according to the method of Dixon (10). The results, shown in Table 3, indicate that the K_m of BUdR is approximately equal to the K_i of BUdR for thymidine incorporation. Conversely, the K_m of thymidine is nearly equal to the K_i of thymidine for BUdR incorporation. These results may be interpreted as indicating that BUdR and thymidine, or their respective derivatives, are acted upon by the same rate-limiting enzyme and may compete with each other for binding sites on this enzyme.

Using a much higher concentration (10^{-4} m) of BUdR than was employed in our experiments, Eidinoff et al. (14, 15) observed inhibition of thymidine incorporation into DNA of human tissue in vitro. At such a concentration Cheong et al. (7) observed inhibition of cell growth. In the present experiments some inhibition of thymidine incorporation occurred at concentrations of BUdR as low as 10^{-4} m, at which concentration growth was not inhibited (7).

An initial high rate of uptake of thymidine by Ehrlich ascites cells was reported by Crathorn and Shooter (8). A similar time pattern of incorporation of thymidine into DNA has been reported by Friedkin and Wood (17), using chick bone marrow cells, and by Smellie et al. (30), who studied the effects of Ehrlich tumor cell extracts upon DNA synthesis in a cell-free system in vitro. Our data indicate that the initial rate of incorporation of BUdR into DNA, like that of thymidine, is relatively high and that the greatest percentage of the administered BUdR is incorporated with low concentrations. To accomplish radiosensitization of cells, the incorporation of BUdR into both strands of DNA in large amounts is presumably desirable (19). By extrapolating the in vitro data, it is proposed that this could be accomplished by often repeated administration of small doses of BUdR, or considerably less efficiently, by administering larger amounts less frequently. No increment in BUdR incorporation can be expected, however, with doses resulting in local concentrations of the compound in excess of 4 X 10^{-5} m. BUdR incorporation would be facilitated also by simultaneous administration of a compound which inhibits endogenous thymidylate synthesis, e.g., 5-FU or, better, 5-FUdR (20).

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