INDUCTION OF THIOGUANINE- AND OUABAIN-RESISTANT MUTANTS AND SINGLE-STRAND BREAKS IN THE DNA OF CHINESE HAMSTER OVARY CELLS BY $^3$H-THYMIDINE

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ABSTRACT

Cultured Chinese hamster cells were labeled with 6-$^3$H-thymidine or 5-methyl-$^3$H-thymidine and allowed to accumulate damage from $^3$H decays for various periods of time while frozen. The frequencies of cells resistant to 6-thioguanine or ouabain and the amount of DNA damage (i.e., number of single-strand breaks) were determined and compared with the mutation frequencies resulting from X and ultraviolet light irradiation. Whereas $^3$H decays and X rays made only 6-thioguanine-resistant mutants, ultraviolet light made both 6-thioguanine- and ouabain-resistant mutants. $^3$H decays originating at the 6 position were two to three times as effective as decays at the 5-methyl position in making drug-resistant mutants, but decays at both sites were equally effective in making single-strand breaks. Mutants and strand breaks produced by beta irradiation of the nucleus probably are the same irrespective of the site of the decay in thymine; these results indicate that the local trans-mutation effects of $^3$H decay produce more mutations when they occur at the 6 position than at the 5-methyl position.

$^3$H-THYMIDINE is probably the radioactively labeled compound most widely used in cell biology. However, the incorporation of $^3$H-thymidine into DNA, while allowing study of the replication of DNA and the subsequent fate of labeled cells, can cause artifacts due to intracellular irradiation from $^3$H decays. The size of the dose emitted by these decays has been calculated theoretically for $^3$H-thymidine incorporated in cell nuclei of various sizes (STRAUSS 1958; TÄGER and SCHEUERMANN 1970), and the effect of the damage has been determined experimentally in mammalian cells for biological end-points such as cell death (PAINTER, DREW and HUGHES 1958; DREW and PAINTER 1959, 1962; PAINTER and DREW 1959; MARIN and BENDER 1963a,b; BURKI and OKADA 1968, 1970; BURKI et al. 1973; BEDFORD et al. 1975), growth delays (EHMANN et al. 1975), chromosome aberrations (WIMBER 1959; DEWEY, HUMPHREY and JONES 1965; BREWEN and OLIVIERI 1966), and DNA strand breaks (CLEAVER, THOMAS and BURKI 1972; BURKI et al. 1975). The induction of mutations by $^3$H decays has not yet been studied in mammalian cells, but in bacteria it has been demonstrated that the site of $^3$H decays has a large influence on the frequency of mutations produced (PERSON and BOCKRATH 1964, 1965; BOCKRATH, PERSON and

In particular, Krasin et al. (1976) have shown that $^3$H decays are twice as efficient in making arginine reversions in *Escherichia coli* when they occur in the 6 position of thymine as when they occur in the 5-methyl group. In order to determine the relative frequencies of mutations produced by decays originating at the 5-methyl and the 6 positions, I measured the production of both 6-thioguanine-resistant and ouabain-resistant mutants in Chinese hamster cells by ultraviolet (UV) light, X rays, 6-$^3$H-thymidine, and 5-methyl-$^3$H-thymidine. In addition, to ascertain whether mutation frequencies and DNA single-strand breaks were correlated, I measured the number of single-strand breaks induced by $^3$H decays at both the 6 and the 5 positions.

**METHODS**

*Labeling and freezing conditions*: Chinese hamster ovary (CHO) cells were grown in Eagle's minimum essential medium with 10% fetal calf serum, in which they had a population doubling time of 12 hr. A single clone of cells was grown into cultures of approximately $10^8$ cells in each of several roller bottles. The cultures were then labeled with 5-methyl-$^3$H-thymidine or 6-$^3$H-thymidine in a way that would produce a uniform distribution of $^3$H in both strands of DNA throughout the hamster genome without allowing significant amounts of $^3$H-induced radiation damage during the labeling period, so that labeled cells could be frozen to accumulate $^3$H decays at a low dose rate. This was achieved by labeling cultures for about five generations (60 hr) with a large volume (150 ml) of medium containing 5-methyl- or 6-$^3$H-thymidine at high concentration and low specific (0.3 $\mu$Ci/ml, 30 $\mu$m, 0.01 Ci/m mole) and $4\mu$m deoxycytidine. At the end of the 60-hr labeling period, cultures were rinsed and grown for 1 hr in non-radioactive medium to deplete radioactive pools in the cells (Cleaver 1967). The labeled cultures contained 20% fewer cells and the plating efficiencies were 95% of that of unlabeled cultures, indicating that $^3$H irradiation had only a small effect on the cell population during cell growth. Cells were then rinsed with balanced salt solution (Saline A, GIBCO), trypsinized, and resuspended in growth medium containing 10% dimethyl sulfoxide. About $5 \times 10^8$ cells per ml in 1-ml ampules were frozen at $1^\circ$/min and stored in a Revco freezer at $-79^\circ$. Unlabeled cells were frozen at the same time. At the time of freezing, cultures were confluent, and both labeled and unlabeled frozen cultures consisted of more than 90% G1 cells, as determined by flow microfluorimetry. The $^3$H activity of the medium (cpm/ml) was depleted by only 20% from the starting activity. A second pair of 5-methyl-$^3$H-thymidine- and 6-$^3$H-thymidine-labeled populations was prepared on a separate occasion by labeling with a 10-fold higher specific activity of $^3$H-thymidine. After freezing and thawing, unlabeled cells had a plating efficiency of 40-100%, which was not significantly different from that of unfrozen cells.

*Specific activity determinations*: $^3$H activity was determined by fixing known numbers of cells with 4% perchloric acid at $4^\circ$, rinsing them in 4% perchloric acid at $4^\circ$, and then digesting them in 10% perchloric acid at $90^\circ$ for 30 min. The $^3$H activity soluble in hot perchloric acid was measured with a water-miscible scintillation mixture (Aquasol), and counting efficiencies were determined with $^3$H-toluene internal quench standards. The activity was calculated in decays per cell per day. The total number of decays per cell was varied by thawing cells at 2- to 3-week intervals for up to 6 months from the time of freezing.

*Irradiation conditions*: X-ray doses of 300-1000 rads were delivered to some frozen ampules of unlabeled cells at dry ice temperature from a G.E. Maxitron X-ray machine (300 kVp) without added filtration. The dose rate of 250 rads/min was calibrated with lithium fluoride thermoluminescent dosimeters in the same exposure geometry at room temperature (Cleaver, Thomas and Burki 1972; Burki et al. 1975). Cultures were irradiated with 0-20 J/m² of UV light by exposing cell suspensions ($5 \times 10^8$ cells/ml) in 10 ml of phosphate buffered saline in 90-mm plastic petri dishes to an incident dose rate of 1.3 J/m² per sec at 254 nm (calibrated
with a Yellow Springs Instrument Radiometer Number 65). Doses were recorded as J/m² incident on the cell suspension.

**Mutation frequency determinations:** To determine the frequency of mutations among surviving cells induced by ³H decays or X rays, ampules were thawed rapidly to 37° and cells were resuspended in 100-150 ml of growth medium containing 10 µM thymidine and 4 µM deoxycytidine and allowed to grow for 7 days for mutation expression. For experiments with UV light, irradiated cultures were allowed to grow for 7 days for mutation expression. Spontaneous mutation frequencies were measured in control cultures grown under the same conditions as irradiated cultures. The minimum time for mutation frequencies to increase from spontaneous to radiation-induced levels was determined to be 6 days for 6-thioguanine resistance and 2 days for ouabain resistance, in agreement with previous results (Hsie et al. 1975a,b; Van Zeeland and Simons 1976). At the end of 7 days' growth, cultures were trypsinized and the plating efficiencies in both media were calculated. Due to metabolic cooperation in crowded cultures (Cox et al. 1970), 6-thioguanine-resistant mutants could not be accurately recovered at densities above about 10⁶ cells per 90-mm dish and ouabain-resistant mutants above 10⁸ cells per dish. Therefore, only data obtained from dishes with lower densities were used. Mutation frequencies were calculated from the ratio of the plating efficiency in 6-thioguanine or ouabain to that in normal medium.

**Molecular weight determinations:** To determine the molecular weights of single-stranded DNA in cells irradiated by ³H-thymidine decays while frozen, ampules containing labeled cells were thawed in a water bath at 25° until only a small ice fragment remained. Ampules were then put into an ice bath to minimize strand-break rejoining that might occur during further thawing (Cleaver, Thomas and Burki 1972; Burki et al. 1975). Thawed cell suspensions (0.5 ml) at concentrations of 10⁸ cells/ml were added to 0.5 ml of 0.5 NaOH and 0.1 M EDTA on top of 5-20% alkaline sucrose gradients (0.4 N NaOH, 0.01 M EDTA, 0.1 M NaCl; pH 12.5) in 30-ml cellulose nitrate tubes. After standing for 1 hr at room temperature, gradients were centrifuged in an SW 25.1 rotor at speeds between 10,000 and 25,000 rev/min until the main peaks of DNA moved about halfway down the tubes. Gradients were then collected, radioactivity per fraction was determined, and the weight-average molecular weights were calculated using SV40 form I and II DNA as standards, as described previously (Cleaver, Thomas and Burki 1972; Burki et al. 1975).

**RESULTS**

Mutation frequencies were induced linearly by ³H decays, X rays, and UV light over most of the dose ranges studied (Figure 1). At the highest UV doses, the yield per J/m² decreased (Figure 1A), and at the lowest doses of ³H decays, slight thresholds were apparent (Figures 1C and 1D). The spontaneous mutation frequencies were low (0.2 × 10⁻⁵ for 6-thioguanine resistance and 0.08 × 10⁻⁶ for ouabain resistance) because freshly cloned cells were used throughout; when cultures were subcultured regularly without cloning, higher spontaneous frequencies were obtained.

Linear dose-response curves have also been obtained for mutagenesis at the 6-thioguanine-resistant gene locus in various Chinese hamster cell types using UV light (Hsie et al. 1975b; Van Zeeland and Simons 1976) and ethyl methanesulfonate (Hsie et al. 1975a), although earlier work reported curved dose-response curves using both UV light and X rays (Bridges and Huckle 1970). The precise shape of the dose-response curve may therefore depend on details of procedure such as the population distribution at the time of irradiation,
Figure 1.—Frequencies of drug-resistant CHO cells induced by various radiations. (A) UV light at room temperature; (B) X rays delivered to frozen cells (−79°); (C) ³H decays in frozen cells (−79°) uniformly labeled with 5-methyl-³H-thymidine at a dose rate of 20 decays per cell per day; (D) ³H decays in frozen cells (−79°) uniformly labeled with 6-³H-thymidine at a dose rate of 11 decays per cell per day. ○, 6-thioguanine-resistant mutants; ●, ouabain-resistant mutants.

Indicated errors are estimated assuming that the number of mutant colonies (n) recovered at any particular dose represents a random sample from a larger population, so that the standard error is n½, according to Poisson statistics. The lines are drawn by linear regression analysis using all data points except those at the two highest UV doses and the spontaneous values. Spontaneous values are shown for UV light only. The frequency of ouabain-resistant mutants in A has been amplified by 10 for better display.

the drug concentration used for selection, the expression time, the particular strain of cells used, and the relative viabilities of mutant and wild type cells (Eckardt and Haynes 1976). Because the dose-response curves were linear over most of the dose ranges in these experiments, subtle differences in curve shape at high or low doses will be disregarded for present purposes, although they may be important for later experiments.
TABLE 1
Frequencies of drug-resistant mutant cells induced by UV light, X rays, and *H-thymidine*

<table>
<thead>
<tr>
<th>Radiation</th>
<th>Dose rate (decays per cell per day)</th>
<th>Dose range</th>
<th>Frequencies of drug-resistant mutants†</th>
<th>6-3H-thymidine</th>
<th>X-ray equivalence (rads/decay)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>6-thioguanine resistant</td>
<td>5-3H-thymidine</td>
<td></td>
</tr>
<tr>
<td>UV light</td>
<td>0–10 J/m²‡</td>
<td>0–10 J/m²</td>
<td>3.6 ± 0.2 × 10⁻⁵</td>
<td>1.0 ± 0.2 × 10⁻⁶</td>
<td>—</td>
</tr>
<tr>
<td>X rays</td>
<td>0–1 krad</td>
<td>0–1 krad</td>
<td>1.3 ± 0.4 × 10⁻⁷</td>
<td>0</td>
<td>—</td>
</tr>
<tr>
<td>5-3H-thymidine</td>
<td>20 0–4000 decays per cell</td>
<td>20 0–4000</td>
<td>3.7 ± 0.4 × 10⁻⁸</td>
<td>0</td>
<td>2.2 ± 0.3</td>
</tr>
<tr>
<td>6-3H-thymidine</td>
<td>11 0–2000 decays per cell</td>
<td>11 0–2000</td>
<td>8.2 ± 0.9 × 10⁻⁸</td>
<td>0</td>
<td>0.63</td>
</tr>
<tr>
<td>5-3H-thymidine</td>
<td>253 1500–6000 decays per cell</td>
<td>253 1500–6000</td>
<td>1.4 ± 0.1 × 10⁻⁸</td>
<td>0</td>
<td>0.11</td>
</tr>
<tr>
<td>6-3H-thymidine</td>
<td>260 1500–4000 decays per cell</td>
<td>260 1500–4000</td>
<td>5.2 ± 1.3 × 10⁻⁸</td>
<td>0</td>
<td>0.41</td>
</tr>
</tbody>
</table>

* At room temperature, —79°, and —79°, respectively.
† Mutant frequencies were calculated from the ratio of the plating efficiency in 6-thioguanine or ouabain to that in normal medium.
‡ 6-thioguanine.
§ Ouabain.
Comparisons between the yields of the two kinds of mutations over the linear portions of the curves (Figure 1, Table 1) show that \(^3\text{H}\) decays resembled X rays in producing only 6-thioguanine-resistant mutants, whereas UV light produced both 6-thioguanine- and ouabain-resistant mutants. More striking is the observation that in each pair of experiments with \(^3\text{H}\) in the 5-methyl and 6 positions of DNA thymine, \(^3\text{H}\) in the 6 position produced two to three times more 6-thioguanine mutations than \(^3\text{H}\) in the 5-methyl position.

When strand breaks were measured, however, there was no detectable difference in the frequency of breaks from decays originating in either position of DNA thymine (Figure 2). These measurements were made on the same samples as were used for the high-dose-rate experiment on mutagenesis (Table 1). Thus, the number of single-strand breaks did not correlate with mutation frequency.

**DISCUSSION**

6-thioguanine probably kills cells by being incorporated into DNA, and perhaps also into RNA, via an enzyme pathway involving hypoxanthine-guanine phosphoribosyl transferase. Cells resistant to 6-thioguanine are likely to be produced by any of a variety of mechanisms that alter the specificity or cause loss

![Figure 2](image-url)
of the activity of this enzyme, including deletions, frameshifts, and point mutations. Ouabain, on the other hand, kills cells by binding to a membrane-bound Na⁺-K⁺ ATPase that is encoded by a pair of autosomal alleles in a diploid cell (Baker et al. 1974). Loss of the binding sites produced by one allele due to deletion or frameshift mutation will either leave ouabain binding sites on the membrane and not confer ouabain resistance to the cell, or will totally inactivate cellular ATPase function due to the codominance of this allele (Baker et al. 1974). Probably only point mutations that produce altered sites in the membrane ATPase which do not bind ouabain will confer ouabain resistance to the cell. Thus, it is possible that ionizing radiations (X rays and ³H decays), which cause 6-thioguanine-resistant but not ouabain-resistant mutants (Arlett et al. 1975) (Figure 1), induce point mutations much less efficiently than they induce frameshifts and deletions; whereas, UV light, which caused both 6-thioguanine-resistant and ouabain-resistant mutants (Figure 1), induces all three kinds of mutations efficiently. This hypothesis is partially supported by data for E. coli, in which ionizing radiations cause predominantly deletions, whereas UV light causes similar frequencies of both point mutations and deletions (Ishii and Kondo 1975).

Theoretical calculations based on the distribution of energy from ³H decays within spheres the size of mammalian nuclei estimate the beta dose from ³H decays to be 0.20–0.38 rad per decay (Täger and Scheuermann 1970; Cleaver, Thomas and Burki 1972; Bedford et al. 1975; Burki et al. 1975). Comparison of these theoretical values with the experimental values obtained for 5-methyl-³H-thymidine for mutagenesis [0.11–0.28 rad per decay (Table 1)], DNA strand breakage [0.48 rad/decay (Cleaver, Thomas and Burki 1972) and 0.53 rad per decay (Burki et al. 1975)], and repair replication [0.34 rad/decay (Painter and Young 1974)] suggests that mutations at the 5-methyl position and the other biological effects are due largely to the beta particle emitted during ³H decay.

The greater number of mutations observed with 6-³H-thymidine-labeled cells [corresponding to an effective dose of 0.41–0.63 rad per decay (Table 1)] indicates that a local effect associated with transmutations at the 6 position in DNA produces mutations more efficiently than the emitted beta particle does. The transmutation processes (³H to ³He and associated recoil, excitation, and charge transfer) are probably more damaging for decays at the 6 position than at the methyl group, because a radioactive decay on the pyrimidine ring is more disruptive. A study in E. coli has reported a greater mutagenic effect for decays at the 6 position than at the methyl position (Person, Snipes and Krasin 1976). In the experiments reported here the greater mutagenic effect of the 6 than the methyl position only increased the frequencies of 6-thioguanine-resistant mutants, but did not result in the induction of any ouabain-resistant mutants (Figure 1, Table 1), indicating that the local damage produced by transmutation is qualitatively similar to that produced by ionizing and beta-particle radiation.

No difference between ³H decays at the 6 position and the 5-methyl position was observed when DNA damage in the form of single-strand breaks was
TABLE 2

*Estimated dose rates to mammalian cells in the $G_I$ phase resulting from the incorporation of 5-methyl-$^3$H-thymidine of various specific activities for one and several generations*

<table>
<thead>
<tr>
<th>Specific activity of $^3$H-thymidine (Ci/mmole)</th>
<th>One cell cycle</th>
<th>Several cell cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(one strand labeled)</td>
<td>(both strands labeled)</td>
</tr>
<tr>
<td></td>
<td>Dose rate (rads/min)</td>
<td>Total dose in 24 hr (krads)</td>
</tr>
<tr>
<td>0.36</td>
<td>0.5</td>
<td>0.72</td>
</tr>
<tr>
<td>1.9</td>
<td>3</td>
<td>4.3</td>
</tr>
<tr>
<td>3</td>
<td>4.8</td>
<td>6.9</td>
</tr>
<tr>
<td>6</td>
<td>9.5</td>
<td>13.7</td>
</tr>
<tr>
<td>20</td>
<td>31.8</td>
<td>45.8</td>
</tr>
</tbody>
</table>

*These estimates are based on the assumptions that mammalian cells contain $6 \times 10^{-12}$ g of DNA, or $2.4 \times 10^6$ thymines per $G_I$ nucleus; that continuous growth in high concentrations of thymidine results in the DNA acquiring the specific activity of exogenously supplied $^3$H-thymidine [approximately true for molarities in excess of $1 \mu$M (CLEAVER 1967)]; that a specific activity of 30 Ci/mmole is equivalent to 1 $^3$H atom per thymidine molecule; that the $^3$H decay rate is $10^{-7}$ per atom per min; and that one $^3$H decay is equivalent to 0.4 rad of X rays. The estimates are therefore the maximum to be expected from continuous growth in $^3$H-thymidine, and different conditions from those assumed here will result in somewhat different dose rates.

measured (Figure 2). The reason for this may be that the emitted beta particle from $^3$H decays is predominant in single-strand break induction and that the transmutation processes produce mutations, but few breaks.

Calculation of the expected dose rate to cells that are grown for one or more cell cycles in 5-methyl-$^3$H-thymidine of commercially available specific activities (Table 2) indicates that labeling of cells with $^3$H of even the lowest specific activity results in a dose rate that will undoubtedly have measurable biological effects. Such potential artifacts should therefore be evaluated in any labeling experiments.

Finally, in consideration of biological hazards that may result from accidental exposure to $^3$H-thymidine or environmental contamination with $^3$H in other forms, the possible influence of the sites at which $^3$H is located should be taken into account, although such position effects may be important for only a small number of sites in DNA (Table 1) (KRASIN et al. 1976; PERSON, SNIPES and KRASIN 1976) and may be no more than two to three times the radiation dose from the $^3$H beta particle.

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3H-THYMIDINE-INDUCED MUTATIONS


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