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DO DNA REPAIR SYSTEMS AFFECT $N^4$-HYDROXYCYTIDINE-INDUCED MUTAGENESIS?**

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It was tested whether mutations induced in *E. coli* by $N^4$-hydroxycytidine (oh$^4$Cyd): (i) undergo mutation frequency decline (MFD) when synthesis of protein is arrested, and (ii) are influenced by polA1, polA107 or xth mutations. It was also investigated whether oh$^4$Cyd may provoke SOS response and prophage $\lambda$ induction. All these processes may involve the action of repair enzymes. It has been shown that none of these processes or repair enzymes affects oh$^4$Cyd-induced mutagenesis.

It is widely believed that base analogues induce the two-way AT$\rightarrow$GC and GC$\rightarrow$AT mutational pathway. This is true for most known base analogues, including 2-aminopurine and 5-bromouracil, and is compatible with the coding ambiguity of base analogues. There is, however, one exception: oh$^4$Cyd, which can imitate both U and C residues in the synthesis in *vitro* of RNA and DNA (Flavell *et al.*, 1974; Budowsky, 1976; Singer & Kuśmierzek, 1982; Topal *et al.*, 1982), in bacteria and bacteriophages induces mutations mainly, if not exclusively, by AT$\rightarrow$GC transitions (Janion & Kajtaniak, 1979; Śledziewska & Janion, 1980; Janion & Glickman, 1980). The reason for this remains unknown. It is reasonable to assume that some cellular processes are responsible for this narrow specificity.

In the preceding paper it was shown that neither the editing function of T4 DNA polymerase nor the mismatch repair enzymes influence the specificity of oh$^4$Cyd-induced mutations (Śledziewska-Gójaska & Janion, 1982). This paper describes an investigation on the possible relationship between cellular processes and oh$^4$Cyd-induced mutagenesis. It was examined whether mutations induced by oh$^4$Cyd may be influenced by MFD conditions, damage in *polA* and *xth* genes, and whether oh$^4$Cyd can cause lysogenic induction. The results presented show that oh$^4$Cyd does not provoke any detectable repair processes.

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1 Abbreviations: oh$^4$Cyd, $N^4$-hydroxycytidine; MFD, mutation frequency decline; AP-sites, apurinic/apyrimidinic sites.
Bacterial strains. Bacterial strains used in this work are listed in Table 1.

Media and plates. LB and M63 were made according to Miller (1972). M was
the E-medium of Vogel & Bonner (1956) enriched with glucose (0.5%). LBE was
the LB-medium enriched with E (20%) and glucose (0.2%). MCSA and M63CSA
were M or M63, respectively, enriched with casamino acids (1%, Difco, vitamin
free). NB contained Difco nutrient broth (15 g/l) and NaCl (5 g/l).

Table 1

E. coli K12 strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>AB1157</td>
<td>supE44 thr1 leu6 proA2 his4 thl argE3 lacY1 galK2 ara14 xyl15 mil1 txx33 strA</td>
<td>Bachmann (1972)</td>
</tr>
<tr>
<td>AB1157 (λ)</td>
<td>λ lysogen of AB1157</td>
<td>Ljunquist et al. (1976)</td>
</tr>
<tr>
<td>AB3027</td>
<td>as AB1157, xthA14 polA20</td>
<td>Glickman et al. (1973)</td>
</tr>
<tr>
<td>NH3016</td>
<td>as AB1157, metE xthA14</td>
<td></td>
</tr>
<tr>
<td>KMBL1788</td>
<td>arg103 bio87 pheA97 thyA301 endA101</td>
<td></td>
</tr>
<tr>
<td>KMBL1789</td>
<td>as KMBL1788 polA107</td>
<td></td>
</tr>
<tr>
<td>KMBL1787</td>
<td>as KMBL1788 polA1</td>
<td></td>
</tr>
<tr>
<td>GY5027 (λ)</td>
<td>strA ampA proB trp his envA uvrB34 λ lysogen</td>
<td>Moreau et al. (1976), Normark et al. (1969)</td>
</tr>
<tr>
<td>GY4015</td>
<td>amp' thr1 leu6 thi1 supE44 lacY tonA21</td>
<td>Moreau et al. (1976), Bachmann (1972)</td>
</tr>
</tbody>
</table>

Soft agar contained Difco trypton (13 g/l), NaCl (5 g/l) and Difco-agar (7 g/l). Plates were solidified with 1.5% of Bacto-agar.

Selective plates. Rif-plates contained the NB-medium and rifampicin (50 µg/ml). SEMhis− and SEMarg− plates contained the E-medium supplemented with glucose (0.5%), Luria broth (1%), thymine (12.5 µg/ml), thiamine (0.17 µg/ml) and the following amino acids (each at 0.2 mM): Pro, Leu, Thr, and either Arg (SEMhis−) or His (SEMarg−). GT-amp plates contained Difco-tryptone (5 g/l), Bacto-peptone (8 g/l), NaCl (5 g/l) and D-ampicillin (15 µg/ml).

Mutation frequency decline. AB1157 cells (10⁸ cells/ml) grown in MCSA were
mutagenized with oh⁴Cyd (500 µg/ml) for 45 min, filtered off, washed and re-
suspended in M-medium without amino acids at half the starting volume. At zero
and at various times of starvation, aliquots were taken for counting Arg⁺ and His⁺
revertants (0.1 ml/plate in duplicate) as well as viable cells.

Mutation to rifampicin resistance. Bacterial cells (about 10⁸ cells/ml) were muta-
genized with oh⁴Cyd (500 µg/ml) for 4 h. Samples of 0.1 ml (in duplicate) were
spread on the rif-plates and, after dilution, on NB-plates, for viable cells count.
A parallel culture growing without mutagen served as control.
Lysogenic induction: (a) In the liquid medium. To AB1157(λ) lysogens (2 - 5×10^7 cells/ml) growing in M63CSA, adenine (0.1 mg/ml) was added and the culture was divided into three parts. To one oh^4Cyd (500 μg/ml) was added, to the second — nalidixic acid (50 μg/ml), and the third served as control. After 90 min of incubation chloroform was added, bacterial debris was centrifuged off, and the titers of phages with AB1157 as a detector strain were estimated.

(b) In the inductest III (Moreau et al., 1976). GY5027(λ) cells growing in LBE were diluted to about 500 lysogens per 0.1 ml. These samples were mixed with various doses of mitomycin C or oh^4Cyd, incubated for 15 min and plated out with the indicator bacteria GY4015 (0.3 ml of overnight grown culture) onto GT-plates. Untreated lysogens served as control. Only ampicillin-resistant detector strain grows on the GT-amp plates and gives plaques. Both growth of GY5027(λ) and lysogenic induction are arrested.

RESULTS

Effect of inhibition of protein synthesis on the frequency of the oh^4Cyd-induced mutations

When mutagenized cells are transferred onto the minimal growth medium under conditions in which protein synthesis is inhibited, frequency of mutation may dramatically decline. This phenomenon called MFD, observed first for u.v. - induced mutagenesis, was subsequently observed for certain chemical mutagens. We exa-

![Graph](image1)

Fig. 1. Effect of starvation of AB1157 cells (MFD) pretreated with oh^4Cyd (500 μg/ml) on frequency of Arg^+ or His^+ reversions. Number of His^+ (O) or Arg^+ (△) revertants; viable cells count (●). The results are average of 3 - 4 independent experiments.
minded the effect of amino acid starvation of oh\textsuperscript{4}Cyd-pretreated AB1157 cells on the yield of Arg\textsuperscript{+} and His\textsuperscript{+} revertants. The results obtained (Fig. 1) show that the number of Arg\textsuperscript{+} and His\textsuperscript{+} revertants as well as the number of viable cells remained at the same level throughout the whole experiment, i.e. up to 90 min of starvation. No decrease in the frequency of oh\textsuperscript{4}Cyd-induced mutations was observed.

Effect of damage in polA and xth loci on sensitivity to oh\textsuperscript{4}Cyd and its mutagenicity repair. Mutants defective in polA and xth genes are more sensitive to many physical and chemical agents, e.g. u.v.- and \(\gamma\)-radiation, alkylating agents (Glickman \textit{et al.}, 1973; Yajko \& Weiss, 1975), 5-bromouracil (Krych \textit{et al.}, 1979), indicating their deficiency in DNA repair. We examined whether a defect in DNA polymerase I or in endonuclease VI may influence bacterial growth or the ability to induce mutations in the presence of oh\textsuperscript{4}Cyd. The rate of bacterial growth of polA\textsuperscript{+} strain and their two polA mutants: polA1 (defective in 3\textsuperscript{'}→5\textsuperscript{'} polymerase), or polA107 (defective in 5\textsuperscript{'}→3\textsuperscript{'} exonuclease) is shown in Fig. 2. In the presence of oh\textsuperscript{4}Cyd, growth of bacterial cells is slightly decreased but to the extent similar for all bacterial strains tested, polA\textsuperscript{+}, polA1 and polA107.

Likewise, the decrease in growth rate of the NH5016(xth\textsuperscript{-}) oh\textsuperscript{4}Cyd-treated cells was not greater than that of the oh\textsuperscript{4}Cyd-treated control AB1157 strain (data not shown).

![Fig. 2. Effect of oh\textsuperscript{4}Cyd on the growth rate of polA\textsuperscript{+} (A), polA1 (B), and polA107 (C) strains. Bacteria growing in LB medium were divided into two parts, to one oh\textsuperscript{4}Cyd (500 \(\mu\text{g/ml}\)) was added (●), the second served as control (○). At the time indicated the aliquots were removed for viable cells count. The results are average of 3 independent experiments.](image-url)
The effect of damage of polA or xth genes on the mutagenic activity of \textit{oh}^\textit{4}Cyd is shown in Table 2. Induction of the forward mutation to rifampicin resistance was tested after treatment with \textit{oh}^\textit{4}Cyd. The data indicate that neither mutation in DNA polymerase I nor in endonuclease VI exert a significant effect on the frequency of \textit{oh}^\textit{4}Cyd-induced mutations. The frequency of Rif\textsuperscript{r} mutations in all the strains tested was in the range of 1 - 2 per 10\textsuperscript{6} cells, i.e. about 50 - 200-fold higher than that of the spontaneous mutations in polA\textsuperscript{+} strains. PolA mutants exhibit the properties of a mutator strain and show a slightly increased level of spontaneous mutations.

\textbf{Table 2}

\textit{Effect of polA and xthA mutations on the frequency of \textit{oh}^\textit{4}Cyd-induced Rif\textsuperscript{r} mutants}

The results are average of 3 - 4 independent experiments.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>Number of Rif\textsuperscript{r} mutants per 10\textsuperscript{8} cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>spontaneous</td>
</tr>
<tr>
<td>KMBL1788</td>
<td>polA\textsuperscript{+}</td>
<td>4 ± 2</td>
</tr>
<tr>
<td>KMBL1787</td>
<td>polA1</td>
<td>15 ± 5</td>
</tr>
<tr>
<td>KMBL1789</td>
<td>polA107</td>
<td>14 ± 7</td>
</tr>
<tr>
<td>AB1157</td>
<td>polA\textsuperscript{+}xthA\textsuperscript{+}</td>
<td>1 ± 1</td>
</tr>
<tr>
<td>NH5016</td>
<td>polA\textsuperscript{+}xthA14\textsuperscript{-}</td>
<td>1 ± 1</td>
</tr>
<tr>
<td>AB3027</td>
<td>polA20\textsuperscript{-}xthA14\textsuperscript{-}</td>
<td>8 ± 5</td>
</tr>
</tbody>
</table>

The data for \textit{xth} mutants were compared with those for AB1157 as the control strain. We are aware that AB1157, although related, is not the true parental strain of \textit{xth} mutants (Ljungquist \textit{et al.}, 1976). Therefore, small deviations in the frequencies of Rif\textsuperscript{r} mutations induced by \textit{oh}^\textit{4}Cyd (2 \times 10\textsuperscript{-6} for xth\textsuperscript{-} vs 1.2 \times 10\textsuperscript{-6} for xth\textsuperscript{+}), probably reflect the disparities between strains.

\textit{Attempts to provoke prophage induction by \textit{oh}^\textit{4}Cyd treatment}

It seems likely that fragments of single-stranded DNA activate the RecA protein which switches on the SOS system and leads to prophage induction (Bridges & Lehmann, 1982). One of the pleiotropic effects of the SOS response is the increase in mutagenesis (Radman, 1974). It is supposed that this increase is a result of induction of an error-prone DNA polymerase (Radman \textit{et al.}, 1979). Therefore, by examining the ability of \textit{oh}^\textit{4}Cyd to induce prophages, we expect to obtain the following information: (i) whether \textit{oh}^\textit{4}Cyd may provoke DNA degradation and induce the SOS system, and in relevance to it (ii) whether the SOS induced error-prone DNA polymerase may take part in \textit{oh}^\textit{4}Cyd-induced mutagenesis.

The ability of \textit{oh}^\textit{4}Cyd to cause lysogenic induction of the AB1157(\lambda) strain was estimated in the two test-systems: the liquid medium and the inductest III of Moreau \textit{et al.} (1976). In the liquid medium, \textit{oh}^\textit{4}Cyd at 500 \textmu g/ml did not induce any prophages whereas nalidixic acid at a dose of 30 \textmu g/ml caused complete lysis of bacteria. The titer of phages in bacterial lysates was identical for the control
and the oh⁴Cyd-treated cells, namely 8×10⁴ phages/ml. On the other hand, the titer of phages in nalidixic acid-treated bacteria was 10⁹ phages/ml.

The inductest of Moreau et al. (1976) is a very sensitive test for phage induction. It allows for detection of a single induced cell. As can be seen from Fig. 3, oh⁴Cyd-treatment does not cause prophage induction. The number of phages released from bacteria treated with oh⁴Cyd at the doses ranging from 1 µg to 1 mg equalled the spontaneous phage liberation. For bacteria treated with mitomycin C starting at doses of 100 ng, complete phage induction, i.e. 500 induced cells per 500 lysogens, was observed.

![Graph showing lysogenic induction of GY5027(λ) estimated in inductest III (see Methods for details). About 500 lysogens were treated with oh⁴Cyd (●), or with mitomycin C (○) at the doses indicated. The results are average of 4-5 independent experiments.](image)

**DISCUSSION**

All the processes investigated after oh⁴Cyd treatment: mutation frequency decline, sensitivity of polA or xth mutants, prophage induction, might be triggered by repair enzymes.

Mutation frequency decline which occurs under inhibition of protein synthesis may result from excision of pyrimidine dimers at suppressor (tRNA) loci, causing loss of suppressor mutants (“genuine” MFD, Witkin & Wermundsen, 1973) or from excision of other premutational damage throughout the genome (Clarke, 1973). MFD-like processes were observed for mutations induced by 4-nitroquinoline-N-oxide, methoxyamine (Williams & Clarke, 1974), ethyl methanesulphonate (Corran, 1968; Green et al., 1977) and 5-bromouracil (Rydberg, 1977).

oh⁴Cyd due to its specificity (AT→GC transitions) is not able to induce suppressor (tRNA) mutations which occur by GC→AT transition or by any transversion. Therefore the loss of mutants under MFD conditions could be due only to general excision repair. However, no such repair has been observed here.

DNA polymerase I takes part in nearly all repairs of DNA discontinuity (short path repair). It acts on a pathway involving removal of pyrimidine dimers (Cooper & Hanawalt, 1972) and repair of AP-sites in DNA (Verly, 1980). These sites may be
formed spontaneously or as a result of the action of numerous specific DNA-glycosylases, which initiate DNA repair by hydrolysis of modified bases (Lindahl, 1982).

Endonuclease VI, which is a main endonuclease specific for AP-sites in DNA, accounted for 90% of the total AP-endonuclease activity of E. coli (Gossard & Verly, 1978). It nicks DNA at the 5'-side of AP-sites, generating a substrate for DNA polymerase I.

Equal sensitivity of polA or xth mutants and of their parental strain to oh4Cyd and the lack of influence of polA or xth damage on the frequency of oh4Cyd-induced mutations suggest that oh4Cyd does not provoke repair processes involving DNA polymerase and/or endonuclease VI.

Prophage induction is a very sensitive test for the measurement of induction of the SOS response. It occurs after treatment of bacterial cells with many damaging agents, carcinogens and mutagens, including 2-aminopurine and 5-bromouracil (Witkin, 1976; Moreau et al., 1976; Anderson et al., 1980; Mayer et al., 1969; Rydberg, 1977; Krych & Pietrzykowska, 1979).

Prophage induction evoked by 5-bromouracil may be the result of degradation of bromouracil-containing DNA. It was demonstrated that 5-bromouracil residues in DNA undergo conversion to uracil which is subsequently excised from DNA (Krych et al., 1979). Lack of influence of oh4Cyd on prophage induction confirms the resistance of N4-hydroxycytosine residues to excision or its inability to induce DNA degradation. This can explain the high mutational potency of oh4Cyd as compared to 2-aminopurine or 5-bromouracil (Janion, 1978) but cannot explain its mutational specificity. So far only the mismatch repair system seems to excise oh4Cyd-induced mismatches from DNA (Śledziewska-Gójska & Janion, 1982). However, as shown here, this system of repair does not lead to prophage induction.

Another conclusion emerging from this study is that polymerase I does not participate in the mismatch repair system.

In the previous paper (Śledziewska & Janion, 1980) we suggested that N4-hydroxycytosine residues in DNA may undergo secondary reactions, for example reduction to cytosine. If such is the case, the A·oh4C base pairs would lead to mutations (A·oh4C→A·C→G·C) whereas G·oh4C pairs would not (G·oh4C→G·C). This could explain both the mutagenic specificity of oh4Cyd and its resistance to repair enzymes. Only the mismatch repair system can excise natural bases and this system does influence the oh4Cyd-induced mutagenesis.

We are indebted to Dr. I. Pietrzykowska and Dr. M. Trojanowska for the bacterial strains.

REFERENCES


**CZY SYSTEMY REPARACYJNE MOGĄ WPLYWAĆ NA ZMIANĘ MUTACJI INDUKOWANYCH N⁺-HYDROXSYCYTIDYNĄ?**

**Streszczenie**

Badano, czy mutacje indukowane N⁺-hydroksycytidygną u *E. coli*: (a) wykazują zmniejszoną częstotliwość mutacji w warunkach zahamowania syntezy białka, (b) ulegają zmianom pod wpływem mutacji w genach polA1, polA107 lub xth. Badano również, czy N⁺-hydroksycytidygną może wywoływać odpowiedź SOS i indukcję profaga λ. Wszystkie te procesy mogą być ujawniane dzięki działaniom enzymów reparacyjnych. Wykazano, że żaden z badanych procesów lub enzymów reparacyjnych nie zmienia mutagenezy indukowanej przez N⁺-hydroksycytidygną.

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