In *Escherichia coli* the *mutT* gene is one of several that acts to minimize mutagenesis by reactive oxygen species. The bacterial MutT protein and its mammalian homolog have been shown to catalyze *in vitro* the hydrolysis of the oxidized deoxyguanosine nucleotide, 8-oxo-dGTP, to its corresponding monophosphate. Thus, the protein is thought to “sanitize” the nucleotide pool by ridding the cell of a nucleotide whose incorporation into DNA would be intensely mutagenic. However, because others have shown *mutT* mutations to be mutagenic under some conditions of anaerobic growth, and have shown 8-oxo-dGTP to be a poor DNA polymerase substrate, there is reason to question this model. We have devised an assay for 8-oxo-dGTP in bacterial extracts. Using this assay, which involves reversed-phase high-performance liquid chromatography and electrochemical detection, we have been unable to detect 8-oxo-dGTP in extracts of three different *mut* mutants of *E. coli*, even after growth of the bacteria in the presence of hydrogen peroxide. Our estimated upper limit for 8-oxo-dGTP content of these bacteria is about 200 molecules/cell, corresponding to a concentration of about 0.34 $\mu$M. When 8-oxo-dGTP was added at 0.34 $\mu$M to an *in vitro* DNA replication system primed with a DNA template that permits scoring of replication errors and with the four normal dNTPs at their estimated intracellular concentrations, there was no detectable effect upon the frequency of replication errors. These findings lead us to question the conclusion that 8-oxo-dGTP is the most significant physiological substrate for the MutT protein.

The action of reactive oxygen species upon cells stimulates mutagenesis in large part by increasing the abundance in DNA of the oxidized guanine derivative, 7,8-dihydro-8-oxoguanine (8-oxoG).\(^1\) This base efficiently pairs with adenine, leading, if unrepaird, to a transversion mutation (1). In *Escherichia coli* the products of three genes play particularly prominent roles in counteracting this genotoxicity, and all three have homologs in mammalian cells. Two of these genes, *mutM* and *mutY*, encode DNA glycosylases, and their actions initiate base excision repair processes at sites occupied by oxoG (1). The third gene, *mutT*, encodes a nucleotidase, which cleaves dGTP to dGMP and pyrophosphate (2) but which Maki and Sekiguchi (3) showed to have much lower $K_m$ for the oxidized dGTP derivative, 8-oxo-dGTP. This finding plus subsequent publications from the same laboratory (4–9) support the concept that the action of the MutT protein is to “sanitize” the nucleotide pool by removing from cells a damaged nucleotide that, if incorporated into DNA, would be strongly mutagenic. In agreement with this model, several investigators (10–12) have shown that addition of 8-oxo-dGTP to an *in vitro* DNA replication system in which replication errors could be scored as mutations stimulated replication errors that were shown by sequence analysis to be transversions.

The human homolog of MutT, hMTH1, has been expressed in *mutT* mutant *E. coli* and shown to suppress the mutator phenotype (5, 8). Thus, both bacterial and mammalian proteins are thought to play the same role in sanitizing the nucleotide pool. The bacterial and human enzymes differ somewhat in substrate specificity; hMTH1 acts upon two oxidized dATP derivatives as well as upon 8-oxo-dGTP (13), and the bacterial enzyme acts upon 8-oxo-GTP, the ribonucleotide analog of 8-oxo-dGTP (14).

Although most published data support the nucleotide pool cleansing role for MutT in ridding the cell of 8-oxo-dGTP, two puzzling observations have been made. First, Fowler et al. (15) showed that under certain growth conditions for *E. coli* the *mutT* mutator phenotype is expressed even when cells are grown anaerobically, and this is under conditions where no appreciable dGTP oxidation should take place. Second, Einolf et al. (16, 17) showed 8-oxo-dGTP to be a poor substrate for several DNA polymerases when compared with dGTP. For four different polymerases (16), the $k_{cat}/K_m$ was $\sim$10$^5$-fold higher for dGTP:C than for either 8-oxo-dGTP:A or 8-oxo-dGTP:C, suggesting that 8-oxo-dGTP levels in cells would have to be quite high in order for it to substitute for dGTP in a significant extent and hence to play a significant role in mutagenesis. Note that in studies with the *in vitro* DNA replication systems (10–12), 8-oxo-dGTP was present at concentrations equal to or greater than those of dGTP, conditions that are unlikely to hold in living cells.

To better understand the role of 8-oxo-dGTP in mutagenesis and the mechanism by which MutT minimizes the effects of oxidative DNA damage, it seemed important for intracellular levels of 8-oxo-dGTP to be measured directly under various conditions. For example, we would expect to see higher levels of 8-oxo-dGTP in *mutT* mutant mutant bacteria than in wild-type cells and lower levels in anaerobic than in aerobic growth conditions. Developing a protocol for extracting and analyzing the 8-oxo-dGTP pool in *E. coli* and carrying out such analyses were the goals of this investigation.
EXPERIMENTAL PROCEDURES

Mutant E. coli Strains and Culture Conditions—E. coli strains used included strain B, the wild-type control from our collection, and three mutT mutants obtained from the E. coli Genetic Stock Center, New Haven, CT. These strains are E. coli DH5α, E. coli BL21, and E. coli DH5α. All bacteria were grown in LB medium, and 50-ml cultures were grown to mid-logarithmic phase for extraction and assays of dNTPs.

Extraction of Nucleotides—Bacterial cultures (50 ml each) were extracted for nucleotide analysis by a modification of the dual extraction procedure using methanol and acid as described in an earlier paper from this laboratory (18). Briefly, bacteria were collected by rapid filtration and extracted with cold 60% methanol, 1% toluene at −20°C followed by drying under vacuum, re-extraction in 5% trichloroacetic acid, centrifugation, extraction of the supernatant with 0.5 or tri-N-octylamine-PF6, drying of the aqueous phase, and dissolution of the solid residue in 100 μl of HPLC buffer (50 μl buffer A, 50 μl buffer B; see below). One modification was that all solutions used for nucleotide extraction contained 0.1 mM desferrioxamine mesylate (Sigma-Aldrich), to prevent oxidation during the extraction procedure (19). The yield of dNTP extracted was determined by subjecting standard dNTPs to identical extraction conditions and then subjecting them to the same HPLC analysis as the extracts. A typical recovery for 8-oxo-dGTP was 64%, and for the standard dNTPs, recoveries ranged from 50 to 80%.

HPLC Nucleotide Analysis—The HPLC method of DiPierro et al. (20) was modified to develop an HPLC assay for simultaneous measurement of 8-oxo-dGTP and the four standard dNTPs. The chromatographic system consisted of a Beckman 126 analytical pump system with a Beckman 166 UV detector. Standard dNTPs and other nucleotides were detected by UV absorbance at a wavelength of 267 nm. Analysis of 8-oxo-dGTP used a Bioanalytical Systems LC-4B amperometric detector with a C4 flow cell, which was connected in series with the UV detector. 8-Oxo-dGTP was detected using a glassy carbon electrode set at a potential of +900 mV versus the Ag/AgCl reference electrode. We obtained 8-oxo-dGTP from Amersham Biosciences, who reported its purity at greater than 99% as determined by HPLC. Our HPLC analysis of the product confirmed this estimate (data not shown).

Chromatographic separation of nucleotides was achieved by using an Alltima C18 column (Alltech Inc., Nicholasville, KY) with a Phenomenex C18 guard cartridge or Discovery C18 (Supelco, Sigma-Aldrich) column with C18 guard column (Supelco). All analytical columns were 250 × 4.6 mm, with a 5-μm particle size. The mobile phase consisted of Buffer A (10 mM tetrabutylammonium hydroxide, 10 mM KH₂PO₄, 0.25% methanol, pH 7.0) and Buffer B (2.8 mM tetrabutylammonium hydroxide, 100 mM KH₂PO₄, 30% methanol, pH 5.5). All mobile phase buffers were filtered and degassed. Separation was achieved with the Alltima C18 column using a 40-min linear gradient of 50% buffer A, 50% buffer B to 30% buffer A, 70% buffer B followed by a wash with 100% buffer B. It was necessary to re-equilibrate the column in 50% buffer A, 50% buffer B for at least 30 min after each run to achieve reproducible retention times for all nucleotides on subsequent runs.

With the Discovery C18 column, separation of all nucleotides could be achieved in 25-min linear gradient of 50% buffer A, 50% buffer B to 40% buffer A, 60% buffer B, with buffer B containing 6% acetonitrile in addition to the substances listed above. All separations were performed at a flow rate of 1.5 ml/min. Authentic nucleotide standards were used to identify nucleotides in the elution profile and develop calibration curves for all nucleotides. Injections (50 μl each) of bacterial extract were performed in duplicate with material in the second injection containing internal standards of dATP, dCTP, dGTP, dTTP, and 8-oxo-dGTP. Data from these second runs were used to verify identifications made from elution times, as well as to calculate recoveries of each nucleotide and apply these figures to correct nucleotide data from the extract for variable losses of material during nucleotide extraction and separation.

8-Oxo-dGTPase Assay—The assay for 8-oxo-dGTPase activity was performed as described by Bialkowski and Kasprzak (21). To prepare cells for extraction of enzyme activity, 50-ml bacterial cultures were grown to mid-log phase and then centrifuged to pellet the cells. After washing the cell pellet in 20 mM Tris-buffered saline, the cells were once again recovered by centrifugation and resuspended in 500 μl of 20 mM Tris-HCl, pH 7.4. Cells were lysed by sonication followed by centrifugation to pellet cell debris. A sample of the resulting supernatant was used to determine protein concentration, and the rest was transferred to chilled polycarbonate Ultracentrifuge tubes (Beckman No. 343778) and centrifuged for 1 h at 49,000 rpm, 4°C in a Beckman TL-100 Ultracentrifuge. Three fractions of the supernatant (100 μl each) with protein concentrations of more than 4 mg/ml were filtered through individual 30 K Nanosep filters until passage was complete. A typical 8-oxo-dGTPase activity of the resulting ultrafiltrates was determined by incubating the ultrafiltrate (5 μl) in a reaction mix (total volume = 60 μl) containing 40 μM 8-oxo-dGTP, 5 mM MgCl₂, and 100 mM Tris-HCl, pH 8.5, at 37°C for 4 h; this was followed by the addition of 20 μl of 50 mM Na₂EDTA to terminate the enzymatic activity. The reaction mixture was analyzed by HPLC using an Alltima C18 column (5 μm, 250 × 4.6 mm, Alltech Inc.) at a flow rate of 1.0 ml/min, with nucleotides detected by UV absorbance at 293 nm. Aliquots (50 μl) of the reaction mixture were chromatographed isocratically with 100 mM NaH₂PO₄, pH 5.5, and 100 μM Na₂EDTA at a flow rate of 1.0 ml/min. 8-Oxo-dGTP (Amersham Biosciences) and 8-oxo-dGMP (prepared as described by Bialkowski and Kasprzak (21)) were used for calibration. The enzymatic activity in each extract is described as pmol of 8-oxo-dGMP formed/min/mg of protein.

Analysis of Replication Errors by in Vitro DNA Synthesis—We analyzed replication errors during DNA synthesis in vitro by the method of Roberts and Kunkel (22), in which a cytosolic extract of HeLa cells is supplemented with a modified M13 phage replicative form DNA containing an SV40 replication origin and part of the E. coli lacZ gene capable of β-complementation. The lacZ gene contains an opal mutation in codon 7 of the gene for the α-complementing peptide. Missed substitution mutations at this site generate a peptide in which α-complementation is readily detected by plating in the presence of X-gal, a chromogenic β-galactosidase substrate. The specific DNA construct used in these experiments is identified as M13mp2SV/opal-7 in another paper from this laboratory (23), and the specific methods we used are as described in that reference. DNA synthesis reactions were carried out using HeLa S3 cell cytosolic extract (75 μg protein), 1 μg of SV40 T antigen (Molecular Biology Resources, Milwaukee, WI), 80 ng of DNA template, and nucleotides at the following concentrations: dATP, 60 μM; dCTP, 30 μM; dGTP, 10 μM; dTTP, 60 μM; and 8-oxo-dGTP (Amersham Biosciences); concentrations varied as indicated. Additional reaction components included 30 mM HEPES buffer, pH 7.8, 7 mM MgCl₂, 200 μM each of CTP, GTP, and UTP, 4 mM ATP, 0.5 mM dithiothreitol, and 25

![Graph](http://www.jbc.org/Downloaded from http://www.jbc.org by guest on July 23, 2018)
μg of bovine serum albumin. Reaction mixtures were incubated for 4 h at 37°C.

Replication reaction mixtures were treated with DpnI endonuclease to eliminate unreplicated DNA molecules (22), and DNA in the mixtures was transferred by electroporation into E. coli NR9162 (mutS). This mixture was plated on E. coli CSH50, an X-gal-complementing host (22), in the presence of X-gal at a density sufficient to give about 2000 plaques/plate. Mutant plaques were identified by their blue color.

**RESULTS**

**Electrochemical Detection of 8-Oxo-dGTP**—Because electrochemical detection is routinely used to assay 8-oxo-dGMP levels in DNA, and because it is more sensitive than UV absorbance-based methods, we established an electrochemical detection and quantitation protocol for 8-oxo-dGTP. Fig. 1 shows a voltammogram for a solution of authentic 8-oxo-dGTP. A strong signal with a midpoint potential of about +700 mV was seen. No such signal was seen with dGTP. For detection of 8-oxo-dGTP, thereafter in HPLC streams we applied +900 mV. Fig. 2 shows separation of a mixture of standard nucleotides, detected by ultraviolet absorbance, plus an 8-oxo-dGTP standard detected electrochemically at + 900 mV when subjected to identical chromatographic conditions. With this elution protocol, 8-oxo-dGTP, with a retention time of about 37 min, was clearly separated from the eight common ribo- and deoxyribonucleoside triphosphates. In separate experiments (not shown) we found that none of the standard ribo- or deoxyribonucleoside triphosphates showed a discernible peak when analyzed by HPLC and electrochemical detection at +900 mV. We conclude, therefore, that 8-oxo-dGTP can be detected and quantitated in the presence of an excess of each of the standard nucleoside triphosphates.

**8-Oxo-dGTP in Bacterial Extracts**—Next we attempted to detect and quantitate 8-oxo-dGTP in E. coli extracts. Fig. 3 shows the elution profile analyzed by electrochemical detection for extracts of E. coli B and a mutT mutant, T-198. No signal was seen in either extract at 37 min. The mutT mutant showed a peak at about 39 min, but that peak did not represent 8-oxo-dGTP, as revealed by mixing 50 pmol of authentic 8-oxo-dGTP standard with the extract prior to analysis and seeing a peak corresponding to that added material at 37 min.

So far we have not identified the material in the T-198 extract that elutes at 39 min, but we note that a similar peak was seen in the analysis of extracts of two other mutT mutants (data not shown). In preliminary attempts to identify this material, we treated standard nucleotides with H2O2 and ascorbate and analyzed these reaction mixtures by HPLC and electrochemical detection. dCTP and GTP were the only nucleotides to yield electrochemically active material under these conditions. The oxidized dCTP eluted shortly after authentic dCTP, and the GTP oxidation product eluted immediately before authentic GTP. These preliminary experiments suggest that the unknown material, which may provide a clue...
to the MutT function, is not an oxidized derivative of a standard nucleoside triphosphate.

In our analyses of the other two mutT mutants we were similarly unable to detect material with an elution time of 37 min, corresponding to 8-oxo-dGTP (data not shown). As expected for mutT mutants, all three strains that we studied lacked detectable 8-oxo-dGTPase activity (Fig. 4). This was true whether the cells were grown aerobically, anaerobically, or aerobically in the presence of 2.5 mM hydrogen peroxide.

Next we asked whether 8-oxo-dGTP could be detected in bacteria, either wild-type or mutT-negative, that were oxygen-stressed by growth in the presence of 2.5 mM hydrogen peroxide. For this experiment we analyzed extracts from larger numbers of cells than depicted in Fig. 3. This allowed the visualization of a number of peaks in the elution profiles (Fig. 5), but none of them increased significantly in peroxide-stressed as compared with normal bacterial cultures. Although one of the peaks was seen at an elution time of 37 min in this experiment, it did not represent 8-oxo-dGTP, because addition of authentic 8-oxo-dGTP to one of the extracts generated a distinct peak at 36 min (elution times for 8-oxo-dGTP varied slightly depending upon the age of the column). We conclude that 8-oxo-dGTP levels are below the limits of detection by our method, whether the bacteria are mutT-plus or -minus and whether or not the cells are stressed by growth in hydrogen peroxide.

Replication Errors Analyzed by in Vitro DNA Replication—Using standard 8-oxo-dGTP and extracts representing as many as $1.5 \times 10^9$ bacterial cells, we determined that we could have detected as little as 6 pmol in a bacterial extract using our electrochemical detection method (data not shown). Given the number of bacterial cells represented by each extract we assayed, this lower limit of detection would correspond to about 240 molecules/cell. Based upon previous dNTP pool size measurements from this laboratory (24), 240 molecules/E. coli cell would correspond to an approximate intracellular concentration of 0.34 mM. We then asked whether 8-oxo-dGTP at this concentration is significantly mutagenic in an in vitro replication system containing the four standard dNTPs at their approximate intracellular concentrations. For this experiment we used the system of Roberts and Kunkel (22), in which a cytosolic extract of HeLa cells is programmed with a modified M13 phage replicative form DNA containing part of the E. coli lacZ gene capable of α-complementation and an SV40 replication origin. The lacZ gene contains an opal mutation in codon 7 of the gene for the α-complementing peptide. Most substitution mutations at this site generate a peptide in which α-complementation is readily detected by plating in the presence of a chromogenic β-galactosidase substrate. In the presence of supercoiled RF DNA from this phagemid and SV40 T-antigen, replication in this system initiates at the SV40 origin and is semiconservative (22). For this experiment we provided the standard dNTPs at concentrations estimated to exist inside a HeLa cell nucleus: 60 μM dATP, 60 μM dTTP, 30 μM dCTP, and 10 μM dGTP (25). As shown in Table I, the addition of 8-oxo-dGTP to this system at 0.34 μM yielded, after replication and analysis, a mutant fraction indistinguishable from that seen with an otherwise identical replication mixture containing no 8-oxo-dGTP. The mutant fraction increased slightly as the 8-oxo-dGTP concentration was increased to 10 μM, but as the numbers of mutant plaques counted were quite small, the calculated increases in mutant fraction are probably not statistically significant.

Thus, 8-oxo-dGTP appears not to be significantly mutagenic when exposed to a replication system at its highest possible intracellular concentration in the presence of dGTP at 10 μM, the estimated concentration in a HeLa cell nucleus. E. coli contains dGTP at an estimated concentration of 100 μM (24), which is 10-fold higher. If 0.34 μM 8-oxo-dGTP is not detectably mutagenic in the presence of dGTP at 10 μM, it seems unlikely that the oxidized nucleotide would be mutagenic in the presence of dGTP at a 10-fold higher concentration.

DISCUSSION

We have developed a separation and analytical system that allows the detection of as little as 6 pmol of 8-oxo-dGTP in a bacterial extract containing the normal nucleotides at their ordinary concentrations. However, when extracts of wild-type

FIG. 4. Lack of detectable 8-oxo-dGTPase activity in extracts of wild-type and mutT mutant E. coli strains. Anaerobic cultures were grown to mid-log phase in a Bactron anaerobic chamber (Sheldon Mfg.). Error bars represent the standard error for three independent cultures treated identically. To each H2O2-treated culture (50 ml each) was added H2O2 to 5 mM every 20 min for 2 h until the cultures were in mid-log phase. Measurements of H2O2 levels in the cultures established that frequent additions were necessary to maintain levels close to 5 mM.

Lack of detectable 8-oxo-dGTPase activity (Fig. 4). This was true whether the cells were grown aerobically, anaerobically, or aerobically in the presence of 2.5 mM hydrogen peroxide.

Expected for

Similar peaks were observed in dNTP pool size measurements from this laboratory (24), 240 molecules/E. coli cell would correspond to an approximate intracellular concentration of 0.34 μM. We then asked whether 8-oxo-dGTP at this concentration is significantly mutagenic in an in vitro replication system containing the four standard dNTPs at their approximate intracellular concentrations. For this experiment we used the system of Roberts and Kunkel (22), in which a cytosolic extract of HeLa cells is programmed with a modified M13 phage replicative form DNA containing part of the E. coli lacZ gene capable of α-complementation and an SV40 replication origin. The lacZ gene contains an opal mutation in codon 7 of the gene for the α-complementing peptide. Most substitution mutations at this site generate a peptide in which α-complementation is readily detected by plating in the presence of a chromogenic β-galactosidase substrate. In the presence of supercoiled RF DNA from this phagemid and SV40 T-antigen, replication in this system initiates at the SV40 origin and is semiconservative (22). For this experiment we provided the standard dNTPs at concentrations estimated to exist inside a HeLa cell nucleus: 60 μM dATP, 60 μM dTTP, 30 μM dCTP, and 10 μM dGTP (25). As shown in Table I, the addition of 8-oxo-dGTP to this system at 0.34 μM yielded, after replication and analysis, a mutant fraction indistinguishable from that seen with an otherwise identical replication mixture containing no 8-oxo-dGTP. The mutant fraction increased slightly as the 8-oxo-dGTP concentration was increased to 10 μM, but as the numbers of mutant plaques counted were quite small, the calculated increases in mutant fraction are probably not statistically significant.

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DISCUSSION

We have developed a separation and analytical system that allows the detection of as little as 6 pmol of 8-oxo-dGTP in a bacterial extract containing the normal nucleotides at their ordinary concentrations. However, when extracts of wild-type
and mutT bacteria were compared, we were not able to detect the oxidized nucleotide even at this level in either wild-type or mutant cell extracts, whether or not the bacteria had been subjected to aerobic growth in the presence of X-gal. Mutant plaques were identified by their blue color. Background refers to pre-existing mutants present in the phagemid preparations that were not subjected to in vitro replication.

Our observations with an in vitro replication error detection system are consistent with the results of Einolf et al. (16), who reported that 8-oxo-dGTP is a very poor substrate, as compared with dGTP, for four different DNA polymerases: E. coli polymerases I and II, T7 polymerase, and HIV reverse transcriptase. Three of those polymerases do not play their primary roles in chromosomal DNA replication. However, more recently Einolf and Guengerich (17) observed the same result when they analyzed 8-oxo-dGTP as a substrate for a mammalian replicative polymerase, DNA polymerase δ, in the presence of proliferating cell nuclear antigen. Our in vitro experiments were done with a human cell extract system (22) that carries out semiconservative replication initiating at normal replication origins. Using a mutational target in which most or all substitution mutations can be detected, we saw no significant increase over the control mutant fraction when replication was carried out in the presence of standard 8-oxo-dGTP at the experimentally determined upper limit of its intracellular concentration. These observations, coupled with expression of the mutT mutator phenotype under anaerobic growth conditions (15), suggest that the mechanism by which the MutT protein counteracts oxidative mutagenesis needs to be re-evaluated.

Table I

| 8-oxo-dGTP | Total plaques counted | Mutant plaques counted | Mutant fraction |
|------------|-----------------------|-----------------------|----------------|
| µM         |                       |                       |                |
| 0          | 160,000               | 6                     | 3.75           |
| 0.34       | 190,200               | 7                     | 3.68           |
| 1.0        | 239,400               | 10                    | 4.18           |
| 3.4        | 313,000               | 15                    | 4.79           |
| 10         | 198,600               | 9                     | 4.53           |
| Background | 726,200               | 23                    | 3.17           |

8-Oxo-dGTP Pool Size Measurements in E. coli

Replication reactions in a HeLa cell extract were carried out as described by Martomo and Mathews (23), with the phagemid identified in that reference as M13mp62/Svogal-7 and dNTP concentrations as follows: dATP, 60 µM; dCTP, 30 µM; dGTP, 10 µM; dTTP, 60 µM. After incubation, unreplicated DNA was cleaved by digestion with DpnI endonuclease, and DNA was transformed into recipient bacteria by electroporation and allowed to replicate. The resultant phages were plated on a co-complementing host in the presence of X-gal. Mutant plaques were identified by their blue color. Background refers to pre-existing mutants present in the phagemid preparations that were not subjected to in vitro replication.

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Assessing the Metabolic Function of the MutT 8-Oxodeoxyguanosine Triphosphatase in *Escherichia coli* by Nucleotide Pool Analysis

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