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INTRACELLULAR OXIDATIVE CLEAVAGE OF DNA IN *ESCHERICHIA COLI* BY THE COPPER-1, 10-PHENANTHROLINE COMPLEX

ELEONORE LICKL*, SHIOW-CHYI CHAO†, and WEN-CHANG CHANG†

**Ludwig Boltzmann Institut für Klinische Onkologie, Wolkersbergenstrasse 1, 1130 Wien, Austria; †Institute of Biological Chemistry, Academia Sinica, P.O. Box 23–106, Taipei, Taiwan, R.o.C.*

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Plasmid and chromosomal DNA of *E. coli* during exponential growth are cleaved after treatment with copper(II)-1, 10-phenanthroline complex (1:2) without providing any exogenous reductant. About 1500 copper molecules per cell are present as estimated by atomic absorption analysis. Within the cell the endogenous reducing substances may have participated in the sequential oxidative reactions, which lead to the damage of DNA. A portion of the resultant DNA fragments originates from plasmid DNA as demonstrated by hybridization tests.

KEY WORDS: DNA damage, copper-phenanthroline, *E. coli, in vivo* model.

INTRODUCTION

The copper complex of 1,10-phenanthroline (OP) (1:2), $[Cu^{++}-(OP)_2]$ degrades nucleic acids in the presence of reducing agents and molecular oxygen *in vitro*.[1-12] The reaction involves hydrogen peroxide reacting with a ternary $DNA-Cu^{+}-(OP)_2$ complex in a site-specific mechanism.[13,14] A site-specific cleavage reaction of DNA (single stranded) at lower OP concentrations, and stimulated by ascorbate and H_2O_2, has been reported recently.[42] Experiments with varying conditions — DNA, OP and copper(II) ions were incubated with (1) reducing agents such as thiols, ascorbate or superoxide anion generating systems in the presence of molecular oxygen, (2) NADH and H_2O_2 or (3) reducing agent and H_2O_2 — had not shown any primary sequence specifity previously. Other authors have reported preferred cleavage sequences depending on secondary structural specificities[5,9,15-18] and with oligothymidylated $Cu^{++}-(OP)_2$ complexes.[19]

Models which prove that $Cu^{++}-(OP)_2$ without the addition of other reactants induces the scission of DNA *in vivo* have not been published so far. Aronovitch *et al.*[20] report rapid cell killing of *E. coli* and cleavage of chromosomal DNA in systems consisting of hydrogen peroxide and OP or H_2O_2 and $Cu^{++}-(OP)_2$. In mammalian cells OP prevents the formation of DNA breaks and cell killing after exposure to hydrogen peroxide or a superoxide generating system.[21-23] *E. coli* has been used in studies of the reactivity of the superoxide anion radical and of H_2O_2.[24,32-38]

To prove the *in vivo* DNA scission activity experimental conditions similar to the conditions *in vitro* have to be estabilished inside the cell. Radicals formed homogeneously in bulk solution will have a high probability to react with any near neighbor molecule.[25] As shown *in vitro* the efficiency of the DNA cleavage by the $Cu^{++}-(OP)_2$

system results from the site-specific mechanism where the deleterious radicals are formed in the vicinity of the DNA target and thus react immediately with it.[13] The *in vivo* model must allow the accumulation of the reactants in the cell and must enable the redox reactions and the formation of the ternary biological target $-Cu^{+} -(OP)_2$ complex to occur without hindrance. Phenanthroline is inhibitory to the cell cycle of mammalian cells in culture, however the $Cu^{++} -(OP)_2$ complex at selected concentrations and conditions is not.[26] The mechanism of the negative influence of OP on the growth of *E. coli* is unclear. The enrichment of the $Cu^{++} -(OP)_2$ complex in the cell is the first step. The intracellular concentration of biological reducing agents is known to be rather high, because NADPH, NADH, and thiol compounds such as glutathione and cysteine, are present in every cell.[27] The intracellular H_2O_2 concentration in living bacteria was estimated to be 10^{-8} M under usual growth conditions. [41] So it seems that the environmental conditions in the cell are suitable for the oxidative cleavage reaction cited above.

Transformed bacteria carrying a plasmid offer a good model to study the intracellular oxidative cleavage of DNA, the plasmid DNA can be isolated after treatment with a $Cu^{++} -(OP)_2$ complex and its size electrophoretically checked. Hybridization allows the identification of the origin of the nucleic acid fragments.

In this paper we report the intracellular cleavage of DNA after treatment with a $Cu^{++} -(OP)_2$ complex, utilizing endogenous reducing substances for the formation of the $Cu^{+} -(OP)_2$ complex. Within the bacterial cell the ternary DNA $-Cu^{+} -(OP)_2$ complex will be oxidized by hydrogen peroxide and the deleterious hydroxyl radicals formed in a site-specific mechanism cleave the nucleic acids. As the bacteria carry plasmid DNA, cleavage of chromosomal and plasmid DNA can be demonstrated.

EXPERIMENTAL

Materials and methods

E. coli HB 101 were transformed by the calcium chloride procedure with the plasmid pBR 322.[28] Precultures were grown in LB broth at 37°C in the presence of ampicillin (25 μg/ml) and tetracycline (12.5 μg/ml) under shaking at 240 rpm overnight. After dilution 1:100 with fresh broth containing the antibiotics cultures were grown to an OD_{600} of appr. 0.2 under shaking at 240 rpm at 37°C. Samples were taken at indicated times for optical density measurement. Reactants were added during exponential growth (OD_{600} appr. 0.2), and indicated concentrations represent final concentrations.

Plasmid DNA was isolated by the alkaline lysis method.[28] Gel electrophoresis was done in 1.0% agarose (TBE buffer system, 100 V, staining with ethidium bromide).[28] Southern transfer, hybridization and autoradiography were done as described previously.[28,30]

Copper concentrations were estimated in cell pellets harvested after 3 hours of exposure to reagents ($Cu^{++} -(OP)_2$, 1 mM) by centrifugation. Pellets were washed 3 times with water. Samples were prepared by acid digestion according to [29] for measurement of copper concentration in a PERKIN ELMER 372 Atomic Absorption Spectrophotometer. The number of copper ions per cell was estimated based on the copper concentration and cell density in culture before harvest and compared with the copper concentration in parallel cultures without any treatment with $Cu^{++} -(OP)_2$.

All chemicals employed were of analytical grade and were used as received. 1,10-

phenanthroline-monohydrate (OP), $CoCl_2 . 6H_2O$, $ZnCl_2$, $MnSO_4 . H_2O$, diethylenetriaminepentaacetic acid (DETAPAC), 1,4-diazabicyclo-(2,2,2)-octane (DABCO), L-histidine, D-fructose, ethylenediaminetetraacetic acid (EDTA), 8-hydroxyquinoline, peptone, Hefeextrakt were purchased from Merck, $FeSO_4 . 7H_2O$, ethyleneglycol-bis-(βaminoethylether)N, N'-tetraacetic acid (EGTA), 2,2'-dipyridyl, diethyldithiocarbamine acid (Na^+), imidazole were from Sigma, U.S.A.

pBR 322, TRIS, agarose from Bethesda Res. Lab., $CuSO_4 . 5H_2O$ from Wako Chemicals; Nick Translation Kit, Amersham, ^{32}P-dATP (NEG-O12H), Du Pont NEN.

All solutions for the cleavage experiments were prepared with water from a Millipore system which was further double-distilled. Complex solutions were freshly prepared before use.

RESULTS

The addition of a $Cu^{++}-(OP)_2$ complex to transformed bacteria during exponential growth results in growth curves shown in Figure 1. Increasing concentrations of the

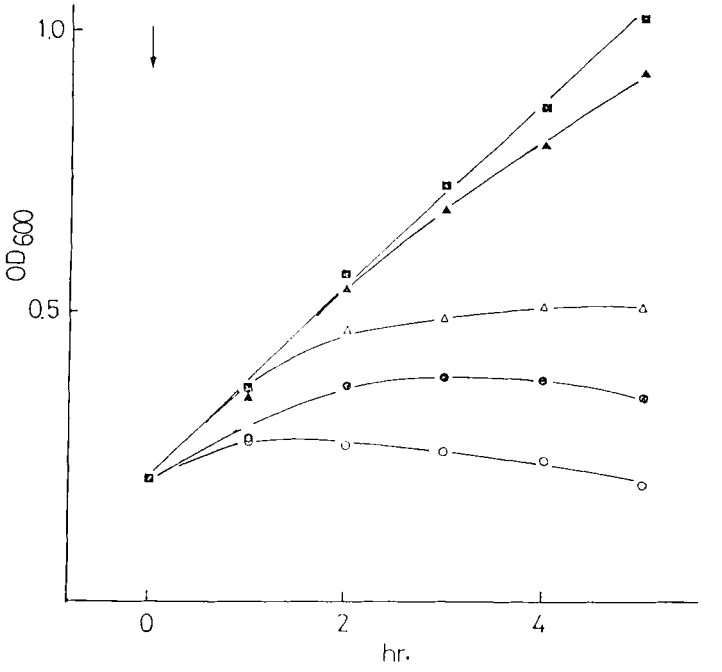

FIGURE 1 Growth curves of *E. coli* treated with $Cu^{++}-(OP)_2$. *E. coli* HB 101, carrying the plasmid pBR 322 were grown overnight at 37°C in LB broth with antibiotics (tetracycline 12.5 μg/ml, ampicillin 25 μl), diluted 1:100 into fresh medium containing the antibiotics. When the OD_{600} reached about 0.2 the following additions were made (indicated with an arrow): (■) no addition, (▲) 0.02 mM $Cu^{++}-(OP)_2$, (△) 0.1 mM, (●) 0.5 mM, (○) 1.10 mM. Incubation was continued at 37°C, aliquots were removed every hour and measured for OD_{600}. Curve (■) also indicates the following additions (0.1 mM and 1.0 mM): DETAPAC, DABCO, L-histidine, diethyldithiocarbamate, D-fructose, EGTA, EDTA, 2,2'-dipyridyl, imidazole, and each chelate with Cu^{++} (1:2), (0.1 mM and 1.0 mM final concentration).

complex reduce the rate of growth as monitored by optical density measurement. OP alone inhibits the growth of *E. coli* when present in concentrations higher than 10^{-5} molar. Other known copper chelators (such as DETAPAC, DABCO, diethyldithiocarbamine acid, histidine, fructose, EGTA, EDTA, 2,2'-dipyridyl, imidazole, 0.1 and 1 mM) do not affect the growth at all, nor when present exclusively, nor together with the copper ion (final concentrations 0.1 and 1 mM). Copper salt does not influence the growth of transformed *E. coli* in LB broth when added at 0.1 or 1 mM, identical uninfluenced growth is monitored after the addition of Fe^{++}, Zn^{++} or Mn^{++} salts (added in tenth millimolar or in millimolar concentration).

Isolated plasmid pBR 322 from *E. coli* grown in broth with added $Cu^{++}-(OP)_2$ complex (1 mM) shows cleavage depending on time (Figure 2). When 1 mM of the $Cu^{++}-(OP)_2$ complex is present, within one generation time the plasmid DNA is cleaved, as shown by the smeared pattern of the electropherogram (Figure 2, lane 9). Isolation of the plasmid DNA after only five minutes of treatment results in an

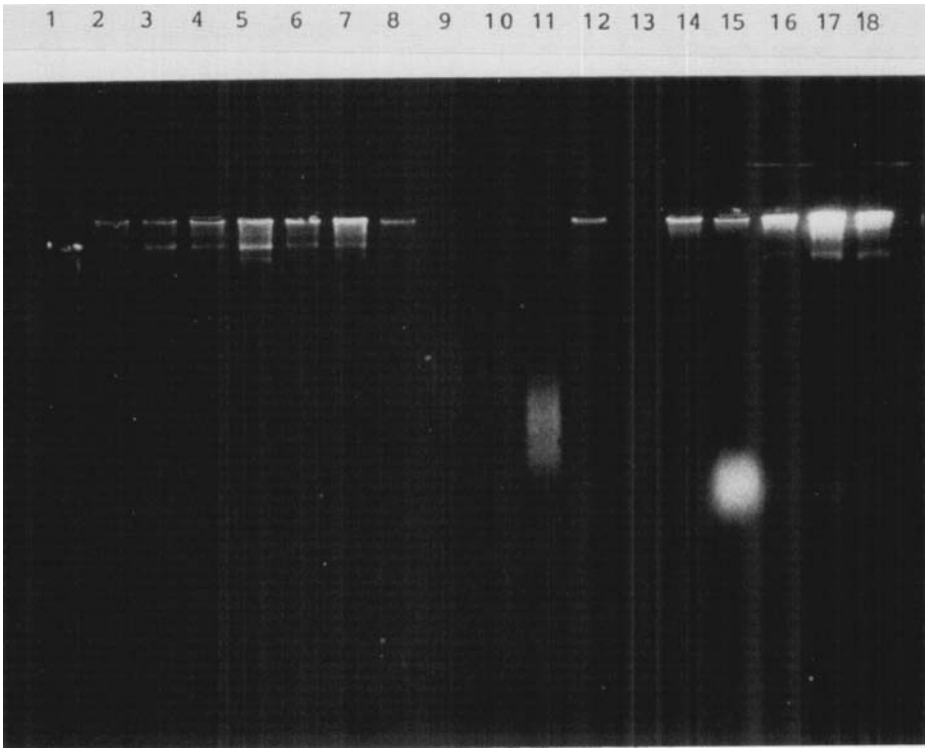

FIGURE 2 Agarose gel electrophoresis of isolated plasmid DNA after treatment with $Cu^{++}-(OP)_2$. Exponentially growing bacteria were treated with complexes at indicated concentrations and times. Lane (1) DNA marker, (2), (3), (18) no addition, (4) 0.1 mM $Cu^{++}-(OP)_2$ for 5 min, (5) 0.1 mM $Cu^{++}-(OP)_2$ for 20 min, (6) 0.1 mM $Cu^{++}-(OP)_2$ for 40 min, (7) 0.1 mM $Cu^{++}-(OP)_2$ for 60 min, (8) 1 mM $Cu^{++}-(OP)_2$ for 5 min, (9) 1 mM $Cu^{++}-(OP)_2$ for 20 min, (10) 1 mM $Cu^{++}-(OP)_2$ for 40 min, (11), (13) 1 mM $Cu^{++}-(OP)_2$ for 60 min, (12) 1 mM OP for 60 min, (14) 1 mM $Zn^{++}-(OP)_2$ for 60 min, (15) 1 mM $Mn^{++}-(OP)_2$ for 60 min, (16) 1 mM $Co^{++}-(OP)_2$ for 60 min and (17) 1 mM $Fe^{++}-(OP)_2$ for 60 min.

uncleaved DNA pattern (Figure 2, lane 8). Lane 12 of Figure 2 shows the plasmid DNA pattern when the bacteria were incubated with 1 mM of OP without the metal ion for one hour. Identical uncleaved pattern of plasmid DNA was obtained, when other metal ion-OP complexes (1:2) were added for one hour during exponential growth in millimolar concentration (Figure 2, lanes 14–17). $Fe^{++}-(OP)_2$ (lane 17) shows weak tendency to a cleavage reaction, although the optical density measurements show no difference with mentioning. The metal ions are quoted according to their increasing stability constants with 1,10-phenanthroline: $Mn^{++} < Fe^{++} < Co^{++}$.[31]

To show the efficiency of the DNA cleavage bacterial cultures were incubated with the $Cu^{++}-(OP)_2$ complex for 3 hours, then the broth changed to fresh one — the complex concentration in the broth therefore very diluted — and the optical density measured every hour. Depending on the complex concentrations used in the first 3 hours continuous growth or stagnation can be found (Figure 3). When the concentration of the copper complex added is higher than 0.5 mM, the exchange of broth does not result in any further growth (curve O–·–·–·–O in Figure 3), the amount of surviving bacteria is negligible. With a complex concentration of 0.1 mM appr. 50%

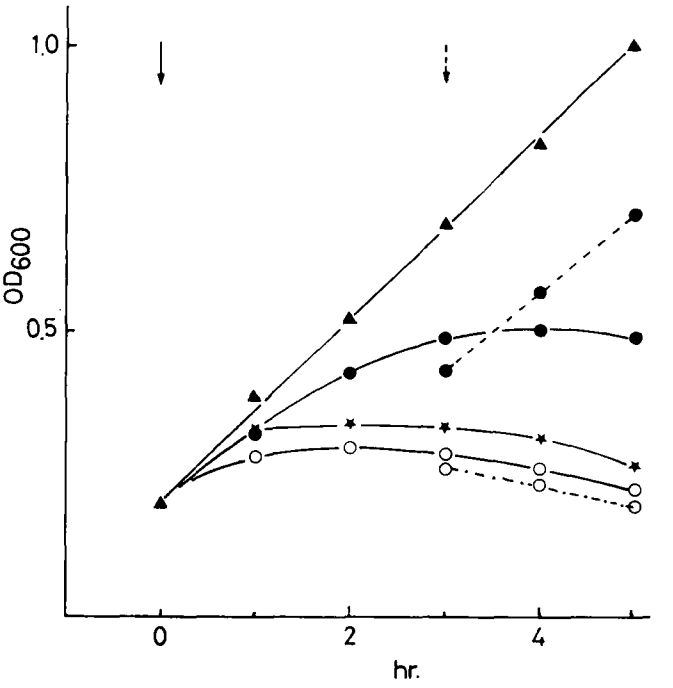

FIGURE 3 Growth curves of *E. coli* after treatment with and removal of $Cu^{++}-(OP)_2$. Cultures of *E. coli* HB 101, with plasmid pBR 322, were grown overnight at 37°C, shaken at 240 rpm, in LB broth with antibiotics, diluted 1:100 in fresh medium containing antibiotics. When OD_{600} reached about 0.2 (indicated with a full line arrow) the following additions were made: (▲)——(▲) no addition, (●)——(●) 0.1 mM $Cu^{++}-(OP)_2$, (*)——(*) 0.5 mM, (○)——(○) 1 mM. Incubation was continued at 37°C and shaking for 3 hours. Then the bacteria were spun down, washed once with LB broth, replaced in fresh medium and incubated as usually. (●)– – –(●) (0.1 mM $Cu^{++}-(OP)_2$ the first 3 hours) with and without antibiotics, (○)– · – –(○) (0.5 mM $Cu^{++}-(OP)_2$ the first 3 hours) with and without antibiotics. Aliquots were removed every hour and measured for OD_{600} (broken lines).

of bacteria are intact after 3 hours of incubation and after removing or at least extensively diluting of the complex from the broth and providing fresh one growth will continue exponentially (curve o----o in Figure 3). Complex concentrations of 0.5 mM or higher in the broth for 3 hours damage plasmid and chromosomal DNA of *E. coli* on such a large scale, that after exchange of broth no growth resp. no surviving bacteria can be found. To control the transformation of all bacteria the growth curves are monitored in the presence and the absence of antibiotics (Figure 3, broken lines). Bacteria without plasmid pBR 322 DNA would not show any resistance to the presence of tetracycline and ampicillin and therefore not continue to grow. If only plasmid DNA would have been destroyed by $Cu^{++}-(OP)_2$ mediated reactions, in a medium free of antibiotics such bacteria would continue to grow after the complex has been removed from the broth.

Calculations of the number of copper ions uptaken into a single bacterial cell were done by measuring the copper content of extensively washed *E.coli* after 3 hours of growth in LB broth with antibiotics and 1 mM of $Cu^{++}-(OP)_2$. About 1500 copper molecules per cell were found, when calculated from the optical density of the culture and the amount of copper found in the bacterial pellet; in same amounts of reference cultures without addition of $Cu^{++}-(OP)_2$ the content of copper molecules was below detection limits.

By hybridization the positions of any bands complementary to the radiation probe (^{32}P-pBR 322) can be localized by autoradiography. The amount of hybridized DNA fragments (Figure 4, part B, lane 3) is less than the fragments stained by ethidium bromide. This can be seen easily by comparison of the intensities of the staining with ethidium bromide, 100 ng, 200 ng pBR 322, respectively, and the smeared pattern obtained from the treated sample (Figure 4, part A, lane 2, 4 and 3) and the intensities of the autoradiogram. The less intensive autoradiogram in Figure 4, part B, lane 3 (corresponding to the smeared pattern in part A) can be explained in this way, that not only fragments from the plasmid are present in the smeared pattern but also fragments originated from the chromosome. The hybridization locates only the positions of the ^{32}P-labeled pBR 322.

DISCUSSION

The cleavage of plasmid and chromosomal DNA in *E. coli* is a result of the nuclease activity of $Cu^{++}-(OP)_2$ complex in the presence of reducing agent and molecular oxygen. In the experiments reported here only the copper chelate is externally supplied, the reducing agent and oxygen are endogenous and will respond immediately to penetrating substances. The kinetic mechanisms of the reaction of $Cu^{++}-(OP)_2$ complexes in the presence or the absence of DNA, but in the presence of superoxide anion radicals or any reductant, and H_2O_2 lead to the production of hydroxyl radicals.[12] If any specific reaction is expected, radical-forming reactions have to occur near the target. Illplaced radical reactions can have no result, but if the harmful radicals are formed in the vicinity of bacteria in a broth, the membrane or nucleic acids may be damaged.[20]

The metabolism of each cell is maintained by a highly efficient environmental system, which includes buffer systems and redox active substances. The *in vivo* model of DNA cleavage takes advantage of the reductants present in every cell, which direct the cleavage reaction via the amount of hydrogen peroxide present. The ingestion of

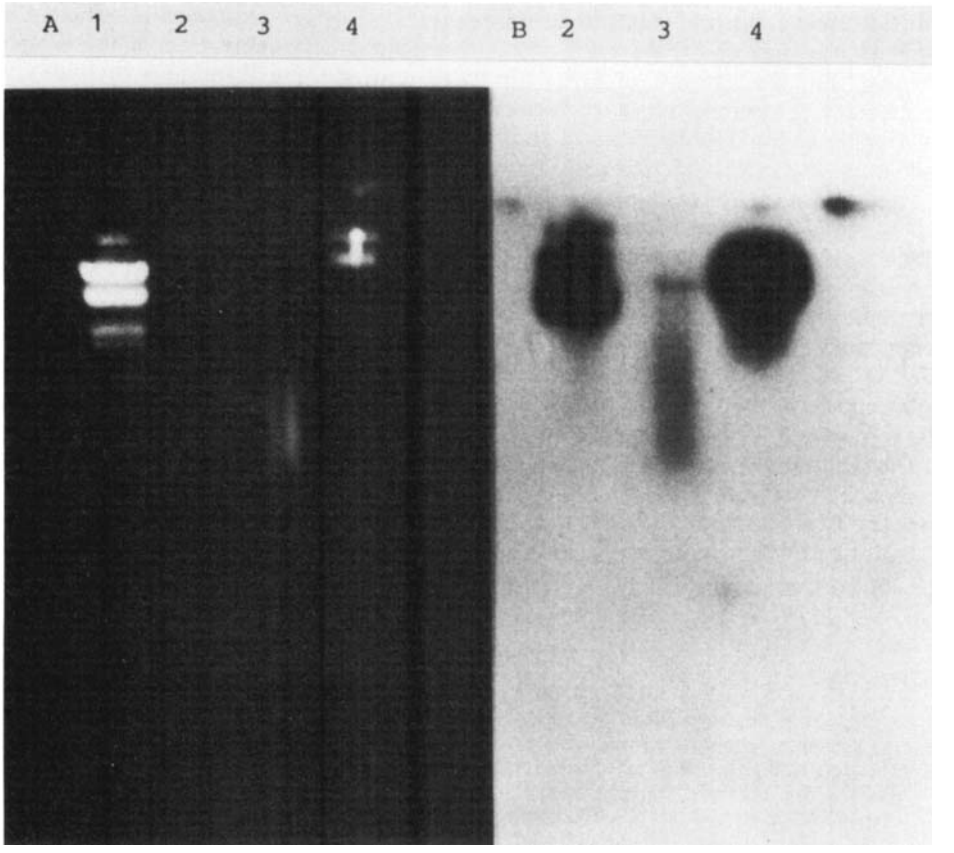

FIGURE 4 Agarose gel electropherogram of DNA from *E. coli* stained with ethidium bromide (A) and hybridized with ^{32}P-pBR 322 (B). (1) marker DNA, (2) 100 ng pBR 322, (3) isolated fragments of DNA from cultures with plasmid pBR 322, treated for 1 hr with 1.0 mM $Cu^{++}-(OP)_2$, (4) 200 ng pBR 322.

the $Cu^{++}-(OP)_2$ into the bacterial cell starts the reaction mechanisms as investigated by Goldstein and Czapski.[11-13] Each cell is packed with DNA, a typical *E. coli* cell accommodates a genome of 1.36×10^{-3} m of DNA,[40] so that the so-called site-specific mechanism[13] is most likely to occur; in addition the reductant is available all the time. Subsequently a low percentage of surviving cells and a smeared pattern of isolated plasmid DNA prove the oxidative scission reaction on nucleic acids. The strong correlation between the complex concentrations and the survival of bacteria after removing the copper complex from the medium demonstrates that DNA is cleaved to different extent. The isolation of the plasmid DNA, which is used as an indicator of DNA cleavage, shows damage, when the copper complex concentration is 1.0 millimolar. At 0.1 mM for 1 hr. the complex does not cleave DNA into small fragments, as does 1 mM (Figure 2). The hybridization experiments also prove that only some portions of the DNA fragments were originated from pBR 322. All nucleic acids in the bacterial cell, which are within reach of the entering copper complex, will be cleaved.

Inside the cell the presence of a certain amount of copper complexes is sufficient to result in cleavage of DNA, since not only the actual concentration of the copper complex, but the amount of H_2O_2 produced manages the formation of hydroxyl radicals and the number of strand breaks. Regarding the rapid metabolism of bacteria — 20 minutes for doubling its size and forming two cells — the bacterial cell should have quite high capacity to supply necessary reductant for the reaction to occur.

This *in vivo* model of oxidative DNA cleavage uses simple and carefully chosen experimental conditions. It is important not to have free phenanthroline in the broth, which would affect the growth of *E. coli*, tenth millimolar of OP added to the broth decrease the percentage of surviving cells. The $Cu^{++}-(OP)_2$ complex is most suitable for the formation of the ternary complex with the biological target, when ingested. When used in millimolar concentration no other renowned copper chelate (DETAPAC, diethyldithiocarbamate, imidazole, cysteine, EDTA) can also cleave DNA in this model at the same extent, when added with the cupric ion, nor does any other metal-phenanthroline complex under comparable conditions.

The defense mechanisms against oxidative stress are biochemical antioxidant systems, enzymatic and non-enzymatic ones.[39] They will act on the appearance of the copper complex, which will change the intracellular equilibrium. Hydrogen peroxide is ubiquitous but may also be produced at a higher rate during the detoxication of the the copper complex.

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