Bacteriophage inactivation by ascorbic acid and thiol reducing agents: Synergic effect with copper and iron+

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ABSTRACT: When we treated Lambda bateriophage with either ascorbic acid, cystein, glutathione or dithiothreitol, we observed that this reductor agents were able to inactivate the lytic functions of phage. Ascorbic acid, cystein and glutathione presented selfscavenging properties at relatively high concentrations, as well as an optimal concentration at which we observed a maximal bacteriophage inactivation. The former characteristic would explain the antioxidant properties of such chemicals to prevent oxidation in living systems. When we assayed the phage inactivation with mixtures of reductor agents with either copper or iron salts, the inactivating effect was enhanced at least by three orders of magnitude. Such synergic effect was more pronounced in the *in vitro* assays than those performed *in vivo*; this fact led us to postulate the possible existence of a cellular mechanism in bacteria that protects it against oxidative stress.

INTRODUCTION

Oxidative agents have been involved as responsible of some illness as: cancer, heart attacks, apoplexy, arteriosclerosis and arthritis. This oxidants could be endogenous or exogenous. Among the first we have, as an example: those produced by mitochondrial processes of oxidative phosphorylation and the enzymic activity of lipooxygenase, xanthine-

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oxydase/mieloperoxydase system from phagocytes. Among the last have been mentioned some natural components of diets, the ultraviolet radiation, natural radioactive gas (i. e. Radon), cigarette smoke, etc.[4] It has been long suspected that oxidative agents cause damage to living organisms through free radicals production, mainly those from oxygen. Recently, Chevion [2] has reviewed a site-specific mechanism of oxygen free radicals cyclical generation, having reductor agents as electron donors. Some transition metal ions such as iron and copper when in aqueous solution can bind to biological macromolecules (DNA or proteins), catalyzing *in situ* the transformation of superoxide anion ($\dot{O}_2^-$) to hydroxyl radical ($^-\dot{O}H$) by the Haber-Weiss reaction:

$$\dot{O}_2^- + \text{Fe (III)/Cu (II)} \dashrightarrow O_2 + \text{Fe (II)/Cu (I)}$$

$$\text{Fe (II)/Cu (I)} + H_2O_2 \dashrightarrow \text{Fe (III)/Cu (II)} + {}^-\dot{O}H + {}^-OH$$

Besides the superoxide radical, some reductor agents as ascorbic acid, glutathione, etc., are also able to carry on the reduction of the cupric or ferric ions to ferrous or cuprous forms to further generate the hydroxyl radical. In turn, these radicals react with the macromolecules at or near the metal ion binding site for giving adduct products or causing the breakdown of covalent bonds, damaging in this way the macromolecule. It has been described [13,7] that the cupric but not the cuprous ions, have a high affinity for DNA sequences which are rich in guanosine nucleotides and that in presence of an electron donor (i. e. ascorbic acid, hydrogen peroxyde) it is in these sites, where it takes place a double-strand break into DNA [16], leading to irreversible loss of its biological function, since double-strand breaks on DNA are hardly repaired.[2] On the other hand, ascorbate has been reported to be an outstanding antioxidant,[4] due to its capacity to scavenge free radicals, by converting itself in an ascorbate free radical, that is less noxious than the hydroxyl or other radicals, having so a protective role against free radicals injury to cells.[5,8]

Murata *et al.* [11,12] reported the lytic functions inactivation of either DNA (single or double-stranded) or RNA bacteriophages (T1, T2, T3, T4, fd; Ø × 174 and Lambda from *Escherichia coli;* SP01, SP10 and M2 from *Bacillus subtilis*). The phages were inactivated by treating them with either ascorbic acids (L-ascorbic acid, D-araboascorbic acid) or thiol reducing agents (cystein, glutathione, dithiothreitol). The authors described that the phages inactivation was function of exposure time to the reductor agent, as well as some of them shown a inactivation dependent of reductor agent concentration.

On the cell, the free radicals are generated as a defense against exogenous agents, i. e. in blood plasma the free radicals concentration is enhanced by the action of polymorphonuclear phagocites, during the immune response after they have phagocited bacteria or other particles attacking them by producing an excess of free radicals. This radicals are able to difunde into blood plasma where they can cause peroxidation of proteins and membrane's cell lipids. In this case is important the ascorbic acid scavenger role.[4,5]

The scientists interest have been focused into endogenous free radical reactions caused by metabolic processes by themselves as well as in conjuction with the effects of toxical exogenous chemicals. Nevertheless, their attention has been shifted towards inhibition of free radicals generation *in vivo* in order to prevent various diseases in which they may play a contributing role.[15]

In the present work, we describe the Lambda phage inactivation by ascorbic acid and

thiol reducing agents both *in vitro* and *in vivo*. We believe that the phage-bacteria system used may help to the understanding of mechanisms of free radical damage, as well as to prevent or to control their production in the living organisms.

Materials and methods

Microorganism and culture media

The *Escherichia coli* 837 (*arq, met, trp, thy, r— m—*) was used as assay strain as well as indicator bacteria. The phage used was Lambda wild type. The bacteria was grown into L medium: Bactotryptone, 10 g; yeast extract, 5 g; NaCl, 5 g; distilled water to 1 l. For plates, 15 g/l of agar-agar were added. For soft agar, either 6 g/l (lysate phage preparation) or 8 g/l (for lysate phage titering), were added to L medium.

Lysate phage obtention and titering

The methodology described by Miller [9] was followed. Briefly, the 837 strain was grown to 2×10^8 cells/ml, portions of the culture were infected with Lambda phage preparation to give a m.o.i. of 1. The infected cultures were allowed to stand 20 minutes at room temperature; then 4 ml of soft agar were added and the tubes poured into plates containing L-agar medium. The plates were incubated overnight and next day the soft agar layer was scraped, chloroform drops added and the mixture shaked vigorously and centrifuged. The supernatants were stored to 0-4°C until used. For titering, the first part of above technique was used, but infecting with the appropiate phage dilutions, next day the lytic plaques were counted.

Reductor agents and metal salts preparation

The ascorbic acid solution was prepared 100 mM, Glutathione, L-cystein and dithiothreitol were prepared 50 mM, all in distilled water. The solutions were stored in sterilized tubes screw capped at 0-4°C preparing fresh stocks every month.

The metal salts solutions were prepared as follows: ferrous sulfate: 18 mM; cupric sulfate: 20 mM and ammonium ferric citrate: 5 mg/ml, in distilled water.

In vitro phage inactivation

Portions of 0.5 ml bacteriophage Lambda lysates, with titers between 10^8—10^{10} infections centers/ml, were placed into Ependorff tubes, to each portion was added either one reductor agent or its mixture with one transition metal salt, to give the desired concentrations. After the reductor agents or their addition mixtures, the volumes of every tube were adjusted to 0.7 ml with sterilized 0.15 M NaCl. The preparations were stored during 24 hs to 0—4°C. After this time appropiate dilutions were done and 837 strain culture portions infected with 0.1 ml of such dilutions, for titering. Next day the lytic plaques were counted. Having as control a non treated phage preparation, it was taken around 100% and survival percentages were evaluated, from means of six replicates of two different dilutions.

In vivo phage inactivation

The *Escherichia coli* 837 was cultured in L medium added with either ascorbic acid

(neutralized to pH 6.5 at moment of use), L-cysteine, glutathione or dithiothreitol or their mixtures with one transition metal salt, at the optimal inactivation concentrations found in the *in vitro* assays. The bacteria was grown at least four times, diluting it whenever the culture reached the stationary phase, with fresh corresponding medium. The cultures were then centrifuged at 3000 × g and the pellet resuspended into fresh medium in absence of any inactivating agent. Thereafter the cultures were infected with the appropriate Lambda phage dilutions and the process as described for the *in vitro* inactivation followed.

Results

In order to properly asses the Lambda phage inactivation by the reductor agents, we perform the assays at different concentrations of such agents; in the case of ascorbic acid, from 0 to 50 mM and with cysteine from 0 to 10 mM. The obtained data are shown in figure 1, where we can appreciate a minimal percent age of phage survival of 9 when it was treated with ascorbic acid and of 6% in the cysteine treatment case. In both cases the above mentioned maximal degree of phage lytical functions inactivation, suggests the existence of an optimal reductor agent concentration for inactivating the phage.

Unlike the former cases, when we treated the Lambda phage with either glutathione or dithiothreitol, we got the results shown in figure 2, where we can see that by rising the glutathione concentration, there is a corresponding augment in phage inactivation; nevertheless, without being any linear relationship, since there are three different slopes, the first located between 0 and 1 mM, the second between 1 to 5 mM and the third between 5 to 10 mM, suggesting different phage inactivation rates. In concern to the phage treated with dithiothreitol, starting from the 1 mM concentration, we can see from figure 2, a logarithmic inactivation by increasing dithiothreitol concentrations.

Afterwards we had some knowledge about Lambda phage behavior towards the reductor agents tested, our attention was focussed to the question if mixtures of the described reductor agents were able to present synergic effect towards the phage inactivation. Accordingly to the above objective, we mixed variable quantities of ascorbic acid (from 0.2 to 10 mM) to a constant 1mM cysteine concentration. The results of such assays are shown in figure 3, where we observe that in comparing to the data displayed in figures 1 and 2 (in regard to the inactivation percentages) we did not get significative differences between the data when the reductor agents were alone, to that when the mixtures of them were tested. Besides, we found that the presence of a maximal inactivation point is not longer so notorious, since at a concentration ratio of 1 we got a 10% phage survival value, and at higher ratios, the survival percentages are around 12 to 14.

Once we tested the phage inactivation *in vitro* we were concerned about the question if we were able to have similar results by treating not the phage itself but its host bacteria. To know that, we cultured *E. coli* 837 along several generations into L-broth supplemented with ascorbic acid to give final concentrations of 0.1, 0.2 and 0.25 mM. After culturing four times the bacteria in the corresponding ascorbic acid concentration supplemented to the culture medium, we infected with Lambda phage the differently treated cultures and samples were withdrawn every 10 minutes during 50 minutes, it was diluted and plated into the indicator strain. In an independent fourth assay, the bacteria was cultured as described above, but before infecting it with the phage, the culture was centrifuged and the pellet resuspended into fresh medium without ascorbic acid added. The results of

these assays are shown in the figure 4, where we can see, that alike to the assays performed *in vitro,* there is an optimal inactivating concentration, that in these assays was of 0.2 mM. In the same figure we are also able to see that in all the instances there is a decrease of inactivation percentages at the 20 minutes time after the infection, subsequently, at further times newly increase. Nevertheless, this was not the case that in the assay where ascorbic acid was withdrawn before the phage infection, as you observed into the figure, there was an increase after the signaled 20 minutes, but at 50 minutes there was a decrease of inactivation rate again.

Since we were able to observe that both, *in vitro* and *in vivo* there was phage inactivation, the obvious question to ask was if both treatments had synergic effect, one upon another. So, we performed the inactivation treatments independently as well as together.

The results of these experiments are shown in figure 5 where we watch that a synergic effect did not exist while the maximum *in vivo* phage inactivation was 75%, and *in vitro* was 95%, the combined treatment rendered a 98% inactivation.

Because of Chevion[2] review of the transition metals role in the biological macromolecules inactivation, we decided to test the effects that upon the addition of two such metals, copper and iron, caused on the phage inactivation by ascorbic acid and cysteine, both *in vitro* as well *in vivo.* In the assays we performed the mixtures of ascorbic acid with either ferric, ferrous or cupric ions. These ions were mixed also to either cystein, glutathione

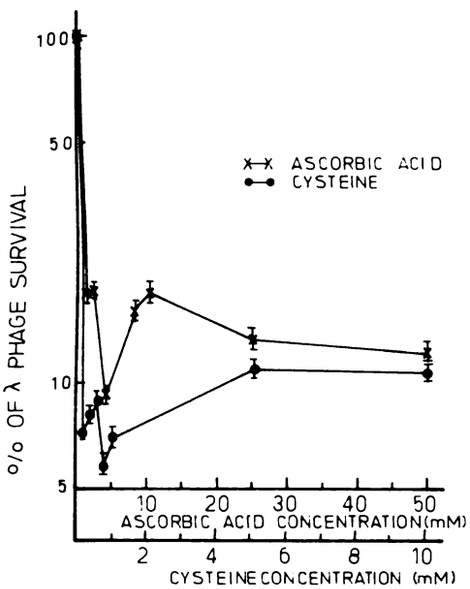

FIG. 1. Lambda phage inactivation by either ascorbic acid or cystein, at different concentrations. The differences between the points of minimal survival (8 mM for ascorbic acid; 0.8 mM for cysteine), and those at any other concentration, were significative as assessed by the analysis of variance after arc sine transformation of percentages[14] ($P<0.05$).

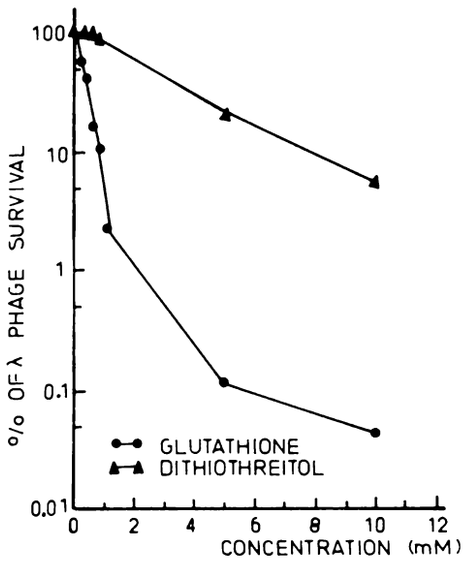

FIG. 2. Lambda phage inactivation by either glutathione or dithiothreitol, at different concentrations.

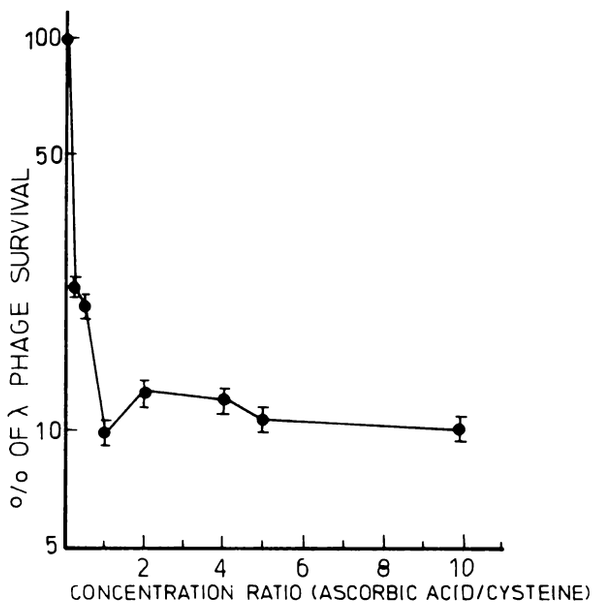

FIG. 3. Lambda phage inactivation by different ascorbic acid cysteine ratios. The cysteine concentration was held constant at 1 mM, while the ascorbic concentration was varied from 0.25 to 10.0 mM.

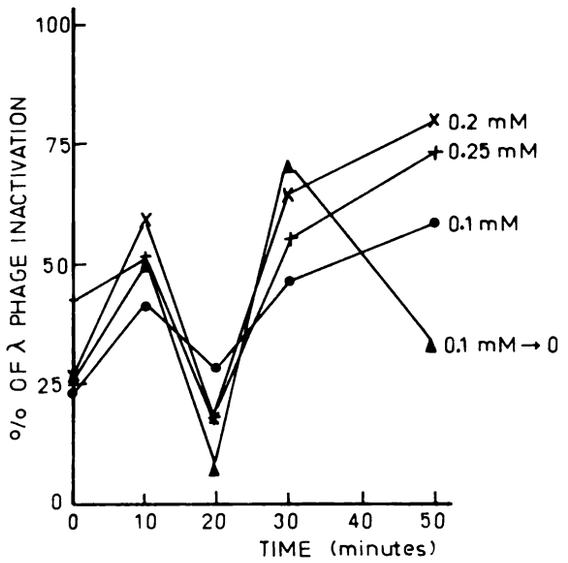

FIG. 4. Inactivation kinetics of Lambda phage with *Escherichia coli* cultures grown in presence of different ascorbic acid concentrations. 0.1 mM ----→ 0 represents a bacterial culture grown in presence of 0.1 mM ascorbic acid and infected in absence of it.

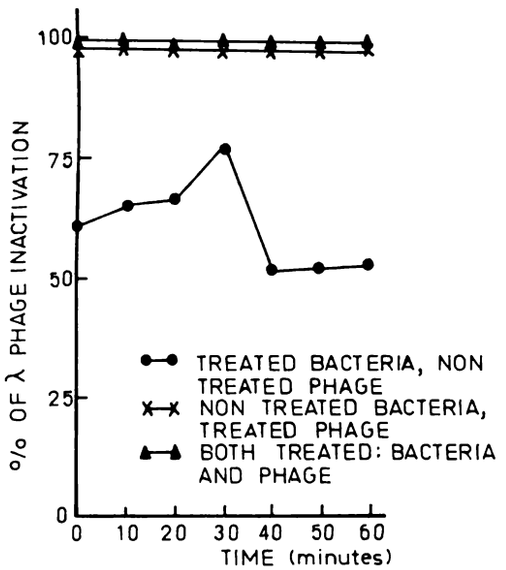

FIG. 5. Kinetics of Lambda phage inactivation *in vivo* and *in vitro*, as well as treated with both procedures.

or dithiothreitol. For the *in vivo* assays the above mentioned mixtures were included into the growth broth at the optimal inactivating concentrations found *in vitro*. It was significative that the host strain did not grow when it was cultured into L-broth added with the mixture of ascorbic acid-copper salt and in order to have grown such culture, we had to grow the bacteria first in L medium plus ascorbic acid, transfering further a portion of this culture to the medium containing the ascorbic acid-copper mixture. The results of these series of experiments showed that (*in vitro* and *in vivo* inactivations): *i*) reductor agents by themselves decrease phage titers in average about three orders of magnitude *in vitro* in comparing to the controls, being the more efficient inactivating agent dithiothreitol. *ii*) *In vivo* the inactivation rates were much less pronunced than *in vitro* being in average around twenty-fold with respect to controls, glutathione presenting the highest inactivation rate. *iii*) By adding Fe^{2+} to ascorbic acid, the inactivating effect was increased 710-fold *in vitro* and 6-fold *in vivo*. Ferric ions both *in vitro* and *in vivo* had less effectiveness than ferrous ones, since the inactivation enhancing actions were of 374-fold and 3-fold, respectively. The copper ions were as effective as Fe^{2+} *in vitro*, while *in vivo* there was not any synergic effect on phage inactivation but a decrease of inactivating action of ascorbic acid by itself by 5-fold. When the mixture of Fe^{2+} plus Cu^{2+} was tested *in vitro*, it was effective as Fe^{2+} alone, but less active for inactivating the phage lytic functions *in vivo* by 4-fold. *iv*) When mixed to cysteine, the ions (Fe^{2+}, Cu^{2+}, Fe^{3+}) shown less effectiveness than their mixtures with ascorbic acid in inactivating the Lambda phage, both *in vivo* as well as *in vitro*; even, as described for ascorbic acid, Cu^{2+} presenting rather a revertion of inactivating action when mixed to this aminoacid. It was the same when cystein was mixed with Fe^{2+}—Cu^{2+}. *v*) *In vitro*, both dithiothreitol and glutathione presented an enhanced inactivating effect upon the addition of Fe^{2+} as well as with the mixture Fe^{2+} plus Cu^{2+}.

Discussion

In the assays done to know if existed an optimal concentration to inactivate bacteriophage Lambda, we found that actually there were concentrations of both ascorbic acid and cysteine in which we detected a maximal inactivation degree of phage lytic functions, which were significatively different from the inactivations obtained from other concentrations of reductor agents ($P < 0.05$). This was not the case when glutathione and dithiothreitol were used as reductor agents, since for the former we found just decreasing inactivation rates as denoted by the different slopes as the concentration was increased, so the higher the concentration, inactivation being less effective; and in the last case, the inactivation rates relationship to concentration being rather logarithmic.

A possible explanation to these results lies into the selfscavening properties of free radicals generated by both ascorbic acid and cysteine, the rationale being that as concentration is raised there is an augment also of two free radicals colliding, provoking the scavening of both of them via an oxido-reduction reaction, being the products ascorbic and dehydroascorbic acids in the case of ascorbate free radicals, and the formation of a disulphide bond for cysteine free radicals.

In concern to the results obtained with glutathione, even though there was not an optimal inactivating concentration, the presence of diminishing slopes suggests that actually exists selfscavening, too, but it might be that the glutathione free radicals were more reac-

tives than those of both ascorbic acid and cysteine and because of that, we didn't find a definite maximal inactivation of phage.

The only exception to the above described behavior was the dithiothreitol case, where there was a logarithmic relationship of inactivation to concentration. It ought be due to the fact that glutathione molecule presents two groups able to form free radicals (the two thiol groups) and when two molecules collide one end of each reacts to form the disulphide bond, but at the other ends, still remain the thiol free radicals which are able to perform the Haber-Weiss reaction to inactivate the phage.

In the other hand, we did not find any synergic effect upon inactivation by mixing two of such reductor agents. It could be due to the fact that actually the reductor agents by themselves are not able to form free radicals, but through the Haber-Weiss reaction mediated by transition metal ions, and since we are not able to assert that water used in our assays were completely free of such ions, perhaps the limiting step for getting inactivation of the phage was the quantity of these ions present in the reaction mixture and not the interactions between the reductor agentes by themselves.

It is possible that the above observed facts could explain the outstanding antioxidant role of ascorbic acid [4] in blood.

In concern to our question if the reductor agents were able to carry on inactivation of the phage *in vivo,* we found indeed that it was so. Nevertheless, the inactivation degree was less than *in vitro* perhaps due to the fact that the intracellular concentrations that can be achived are lesser than those added to the culture medium. That the reductor agents are able to cross the cell's envelope is indicated by the evidence that when we incubated the bacteria in the medium additioned with any of the used reductor agents, for further eliminating it at bacteriophage infection time, the inactivation rates were decreased with time.

We found that by adding to reductor agents either copper or iron salts, the phage inactivation was enhanced by three orders of magnitude in average, in comparing to the inactivation caused by these agents by themselves.

We think, again, that maybe this inactivation is not fullfilled *per se* by the reductor agents, but by the trace of the transition metals ions existent naturally on the cells that via the electrons donated by the reductor agents and by means of cyclic redox, generate hydroxil free radicals [2] the most reactive.

In comparing the inactivation rates that we observed *in vitro* to those in vivo, we are able to see some main differences between both: Firstable, since the concentrations used were the same for the two instances, the inactivation *in vitro* was more efficient than *in vivo.* Besides the allready pointed out fact that intracellular concentrations of reductor agents being less than those added to culture medium, if we take in account that bacteria didn't grow in the medium containing ascorbate-copper and that we were able to get growth only when we cultured it into medium containing ascorbate alone, prior to transfer the growth to the mixture ascorbate-copper, and the fact that were not able to found any synergic effect when the phage was treated *in vitro* for further infecting with it treated cultures, it strongly suggests us that it was induced somehow into the cell a mechanism that protects bacterial cell against the injury of free radicals, as described by Kim [6] for *Saccharomyces* or the one described by Moore [10] in which glutathione participates against the damage caused by radiation. Maybe this protection could involve the enhanced production of free radicals scavening proteins as superoxide dismutase, catalase, peroxidase re-

ductase, etc. [3,16] Even though we can't discard that in this protective mechanism there were participation of a DNA damage repair mechanism, we consider that it could participate in a lesser extent.

Another difference was that in both, *in vivo* and *in vitro* ferrous ion was twice effective for phage inactivation than the ferric ion, although we think that it is not significative since iron has a far well known capacity to perform the interconversion of both oxidation states in presence of reductor or oxidative agents.

Also, we are convinced that the phage inactivation is carried out by oxygen free radicals rather than by the reductor agents by themselves; our above statement is futher supported by the reported fact [1] that the ascorbate, cysteine, etc. free radicals are very stable and hence not very reactive in comparing with, let us say, the hydroxil radical. Finally we are in the way of performing experiments trend to elucidate the mechanism by which the free radicals generated by the reductor agents inactivate the phage lytic functions.

Resumen

Al tratarse el bacteriófago Lambda con ácido ascórbico, cisteina, glutatión o ditiotreitol, se observó que estos agentes reductores fueron capaces de inactivar las funciones líticas del fago; con los tres primeros, cuando fueron utilizados a concentraciones relativamente altas, se observaron características que fueron interpretadas como la presencia en los sistemas de ensayo, de un efecto de autoapagamiento de los radicales libres en ellos formados, lo cual podría explicar las propiedades antioxidantes que se han descrito para estas substancias en los seres vivos. Estos tres agentes reductores presentaron concentraciones óptimas a las cuales se observaron los mayores grados de inactivación del bacteriófago. Al ensayarse las mezclas de estos agentes reductores en presencia de sales de fierro o cobre, se observó un aumento en el grado de inactivación del fago, de por lo menos tres órdenes de magnitud, con respecto a la inactivación causada por los agentes reductores solos; siendo este efecto sinérgico más acentuado en los ensayos efectuados *in vitro*, que los llevados a cabo *in vivo*. Este hecho nos condujo a postular que en la bacteria utilizada existe un mecanismo que la protege de los daños causados por acción de los radicales libres de oxígeno.

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