

## GENOTOXICITY OF NITROSO PHENOLS IN THE SOS CHROMOTEST: INHIBITORY EFFECT OF ASCORBIC ACID

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### Resumen

*En este trabajo de investigación se han sintetizado los compuestos: 4-nitrosufenol, 2-metil-4-nitrosufenol y 3-metil-4-nitrosufenol. Se ha evaluado la genotoxicidad de los compuestos citados anteriormente en la cepa PQ37 de Escherichia coli mediante el SOS Chromotest. Se ha investigado la influencia de la posición del grupo metilo en el daño genotóxico.*

*Por otra parte, se han determinado las propiedades antígenotóxicas del ácido ascórbico frente a la genotoxicidad causada por 4-nitrosufenol, 2-metil-4-nitrosufenol y 3-metil-4-nitrosufenol en el SOS Chromotest.*

*Se ha investigado la reacción de inhibición de la formación de los nitrosocompuestos anteriormente citados a partir de nitrito de sodio y fenol, 2-metilfenol y 3-metilfenol, con ácido ascórbico. La técnica espectrofotométrica utilizada para el seguimiento de las reacciones ha consistido en la medida de la absorbancia del producto nitrosado a 345 nm con el tiempo.*

*Los nitrosocompuestos estudiados resultaron ser genotóxicos en el SOS Chromotest. El ácido ascórbico disminuye el daño genotóxico de 4-nitrosufenol, 2-metil-4-nitrosufenol y 3-metil-4-nitrosufenol. El ácido ascórbico inhibe la reacción de formación de los nitrosocompuestos citados anteriormente.*

**Palabras clave:** Nitrosufenol; Nitrosación; SOS Chromotest; Ácido ascórbico; Daño oxidativo.

**Summary**

*4-Nitrosophenol and methylated derivatives, 2-methyl-4-nitrosophenol, and 3-methyl-4-nitrosophenol have been synthesised and tested for their genotoxicity towards Escherichia coli strain PQ37 in the SOS Chromotest. Effect of methylation among the various positional isomers, ortho or meta, were investigated. On the other side, antimutagen properties of ascorbic acid were also determined.*

*In order to study the inhibition of nitrosation reaction of phenol, 2-methylphenol and 3-methylphenol with nitrite species, effect of ascorbic acid were investigated, by measuring variation in the absorbance of the nitrosated product at 345 nm vs time.*

*The studied compounds, 4-nitrosophenol and methylated derivatives, 2-methyl-4-nitrosophenol, and 3-methyl-4-nitrosophenol, were found to be genotoxic in the SOS Chromotest. Ascorbic acid dramatically decreased the nitrosophenol synthesis reaction and its genotoxicity in the mutagenic test.*

**Key Words:** Nitrosophenol; Nitrosation; SOS Chromotest; Ascorbic acid; Oxidative damage.

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## 1. Introduction\*

Interest in the carcinogenic and mutagenic nitrosocompounds has grown rapidly in recent years. The chemistry and biochemistry of nitrosocompounds has attracted considerable research owing to their proven toxic, carcinogenic, mutagenic, and teratogenic effects (Sugimura *et al.*, 2002; García-Santos *et al.*, 2002; Martínez *et al.*, 1988; Bartsch *et al.*, 1987).

One group of the most nitrosocompounds studies is the *N*-nitrosocompounds (Sandor *et al.*, 2001). The biological model for the relationship between *N*-nitrosocompounds and the occurrence of the gastric cancer and others cancers has been established several years ago. It has been demonstrated that nitrate itself is a precursor of these carcinogens. The conversion of ingested nitrate to *N*-nitrosocompounds was also verified in human (Mirvish, 1995) and some of these molecules were classified as probable human carcinogens.

However, the mutagenic and carcinogenic activities of nitrosophenols are not well known. The relevance of such types of compounds is that their precursors, phenols and nitrate, are commonly found in foodstuffs. The phenol derivative tyramine, which occurs in cheese, meet extracts, beer, and soybean products, has been identified as one of the precursors largely responsible for the mutagenic activity of certain Japanese soy sauces treated with nitrite (Sugimura *et al.*, 1996; Wakabayashi *et al.*, 1989). Bamethan [1-(4-hydroxyphenyl)-2-butylaminoethanol], a phenolic drug used for a long-term oral treatment of cardiovascular disease, is both nitrosable and a direct-acting mutagen (Sugimura *et al.*, 1996).

The relevance of polyphenols in current biochemistry and chemical technology should be stressed (Siebert *et al.*, 1996). The biosynthetic pathways leading to tannins, anthocyanins, flavonoids, etc, as well as the role of plant polyphenols in flavoring, coloring, leather tanning, and herbal medicines are aspects that support the interest in food science and in knowledge about their potential capacity as nitrosatable substrates. In addition, some polyphenols are able to interact with chlorine or nitrite to yield products with greater mutagenic potential than their original compounds (Lin and Lee, 1992).

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On the other hand, the most important sources of nitrate are the contaminated drinking water and nitrite treated or nitrate containing food products (Sugimura *et al.*, 1996).

In a recent paper, Duarte *et al.* 2000 have investigated the genotoxicity of instant coffee, related to the possible involvement of phenolic compounds (Duarte *et al.*, 1999; Duarte *et al.*, 2000). The genotoxicity of instant coffee seems to be associated with the presence of reactive oxygen species, namely hydrogen peroxide, arising from the auto-oxidation of phenolic compounds.

In order to determinate the genotoxic and carcinogenic effect of C-nitrosocompounds, we have tested the potential genotoxicity of nitrosophenol (NPHE) and methylated derivatives: 3-methyl-4-nitrosophenol (mNPHE) and 2-methyl-4-nitrosophenol (oNPHE), by the SOS Chromotest assay. Moreover ascorbic acid was also investigated as a potencial inhibitor to genotoxicity caused by nitrosophenols action.

## 2. Materials and methods

### 2.1. Chemicals

Phenol (PHE), 2-methylphenol (oPHE), 3-methylphenol (mPHE), 4-nitrosophenol (NPHE) and ascorbic acid were purchased from Sigma-Aldrich (Steinheim, Germany). Sodium nitrite was from Merck (Darmstadt, Germany). 2-Methyl-4-nitrosophenol (oNPHE) and 3-methyl-4-nitrosophenol (mNPHE) were obtained from their precursors in acid medium, sodium nitrite and 2-methylphenol and 3-methylphenol, according to the procedure outlined by Gonzalez-Mancebo *et al.*, 2002. As inhibitor ascorbic acid was obtained from Sigma-Aldrich (Steinheim, Germany).

### 2.2. SOS Chromotest

The genetic features of the *E. coli* PQ37 strain have been described in detail by Quillardet and Hofnung, 1985. It is derived from *E. coli* GC4436 that carries a *sfzA::lacZ* operon fusion. PQ37 also harbors the following critical markers: *uvrA*, which prevents excision repair;  $\text{PHO}^c$ , resulting in constitutive expression of alkaline phosphatase and *rfa* that causes a deficient strain lipopolysaccharide which allows better diffusion of different substances into the cell.

### 2.2.1. SOS spot test

The SOS spot test was performed according to the procedure outline by Quillardet and Hofnung, 1985. In brief, after mixing aliquots of 100 ml containing PQ37 bacteria with 2.5 ml of top agar medium, this volume is added to STA or LB plates. Then, 10 ml from every assayed concentration of analysed nitrosocompounds was added into the plates. Concentrations in the range of 0.12 to 10 mM were used. Acridin and NQO were used as positive controls in a range of 0.1-2 mM. The plates were incubated during 24 hours to allow colorimetric reaction development.

### 2.2.2. SOS chromotest

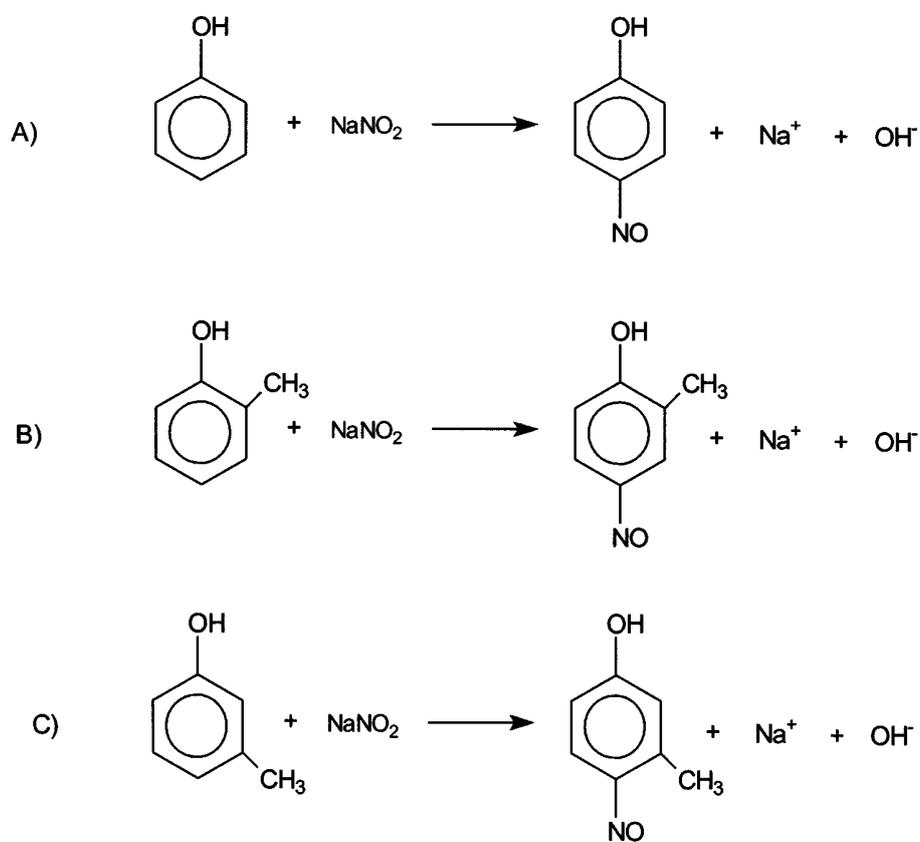
Genotoxicity of nitrosophenols was evaluated in terms of SOS response induction. As a measure of genotoxicity, the SOSIP (SOS-inducing potency) value was calculated from the linear part of the induction factor (IF)-dose response curve. The induction factor (IF) (Quillardet and Hofnung, 1985), was the ratio of the activities of b-galactosidase to alkaline phosphatase at a given concentration divided to the background value (without nitrosocompound). In this way, induction of SOS response can be measured as an increase in b-galactosidase activity, while alkaline phosphatase activity measurement is used for toxicity correction related to proteins biosynthesis inhibition.

In brief, 100ml of 15-hours overnight culture of *E. coli* PQ37 was diluted with 5.0 ml of LB medium containing ampicillin (100mg/ml) and incubated at 37°C (310 K) for two hours approximately (corresponding to  $200 \times 10^6$  CFU/ml). This culture was then diluted 1:10 with fresh medium and fractionated in 600ml portions of the bacterial nutrient dilution. Test substances, dissolved in 20ml sodium phosphate buffer pH 7.0 or DMSO were given. These mixtures were incubated for another two hours at the same temperature and afterwards distributed to two new tubes with equal volumes. b-galactosidase activity was determined using 4-nitrophenyl-b-D-galactopyranoside (ONPG) as substrate. Constitutive alkaline phosphatase activity was determined using 4-nitrophenylphosphate as substrate. All mixtures were incubated at 37°C for 60 minutes in a water bath. Photometrical determinations were performed at 405 nm in a GBC 10e spectrophotometer. Reagents, media and sources are described extensively in (Quillardet and Hofnung, 1985). All results are expressed as mean of at least three experiments.

### 3. Results and discussion

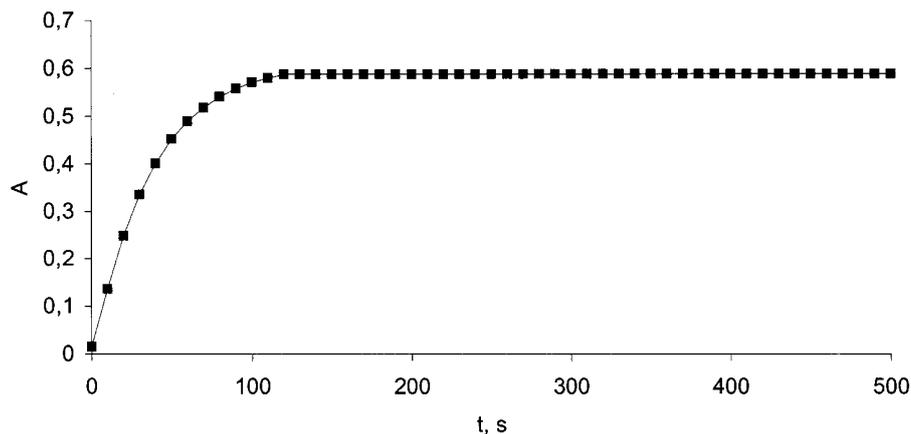
#### 3.1. Formation reaction of *C*-nitrosophenol: Inhibitory effect of ascorbic acid.

Nitrosation reactions of phenolic compounds is shown schematically in figure 1.



**Figure 1:** Nitrosation reaction of phenolic compounds: A) Phenol; B) 2-Methylphenol; C) 3-Methylphenol

Figure 2 shows a typical kinetic run. All kinetic runs are expressed as mean of at least three experiments.

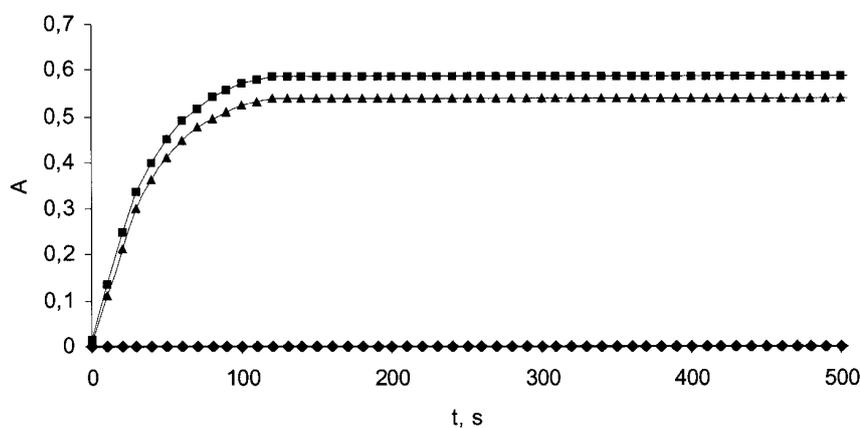


**Figure 2:** Variation in the absorbance of the nitrosated product at 345 nm *versus* time: [PHE] = 0.1  $10^{-3}$  M, [Nitrite] = 0.1  $10^{-3}$  M, pH = 2.70, T = 310 K

In order to block the nitrosation reaction, we have investigated the ascorbic acid effect on *C*-nitrosation, by measuring the variation in the absorbance of the nitrosated product at 345 nm with time. Several concentrations of ascorbic acid were used. Results are shown in figure 3. *C*-Nitrosation reaction of phenol was completely blocked when ascorbate 2mM was used and decreasing of nitrosation occurred from 0.5mM. These results agree with to obtained in other nitrosation reactions studied previously. Thus, *N*-nitrosamines formation is largely inhibited by ascorbic acid (Tannenbaum *et al.*, 1991; Arrigoni and Tullio, 2002). This results show that ascorbic acid blocks nitrosation reaction, leading to formation of *C*-nitrosophenols.

Recently we have described the mechanism of nitrosation of phenolic compounds (Gonzalez-Mancebo *et al.*, 2002, Fernández-Lienres *et al.*, 1997). The reaction mechanism can be explained in terms of aromatic electrophilic substitution by nitrosonium ( $\text{NO}^+$ ) or nitrous acidium ( $\text{NO}_2\text{H}_2^+$ ) ions, kinetically indistinguishable, a mechanism previously suggested in other studies (Challis and Lawson, 1968) and handled by us earlier (Gonzalez-Mancebo *et al.*, 1999).

Inhibitory effect of ascorbic acid can be due to the reduction of



**Figure 3:** Variation in the absorbance of the nitrosated product at 345 nm with time: [PHE] =  $0.1 \cdot 10^{-3}$  M, [Nitrite] =  $10^{-4}$  M, pH = 2.70, T = 310 K; (♦) [Ascorbic] = 2 mM; (▲) [Ascorbic] = 0.5 mM; (■) [Ascorbic] = 0.05 mM.

nitrosating species to NO. This specie, NO, is not a nitrosating agent (Ramirez-Victoria *et al.*, 2001; Chung *et al.*, 2002; Sen *et al.*, 2001; Vermeer *et al.*, 1999).

### 3.2. SOS Spot test: Inhibitory effect of ascorbic acid.

As mentioned before the SOS spot test allows to a simple semi-quantitative procedure and rapid detection of genotoxic agents. Induction of b-galactosidase, related to SOS response activation, is evidenced on indicator plates containing a substrate which releases a blue ring or halo around a zone of inhibition (Quillardet and Hofnung, 1985).

In our studies, after 24 hours of incubation of PQ37 bacteria in STA or LB plates with different concentrations of NPHE and their methylated derivatives, a positive response was obtained (results not show). Staring at NPHE effects, a blue coloration and an inhibition halo were evident when 0.9 mM or higher concentrations were assayed. Although genotoxic activity was also detected from both methylated derivatives, the relative size and intensity of the blue halo were significantly lower. There might be to reasons that justify this fact: a worse diffusion across the indicator plates due to methyl groups, or diminished genotoxicity of these methyl-substituted nitrosophenols assayed. In this sense, differences can be observed analysing the results obtained with oNPHE or mNPHE. A relative order of genotoxicity could be established

among both positional isomers (*ortho*, *meta*) methyl-substituted nitrosophenols referred to nitrosophenol. Thus NPHE seems to be the most genotoxic, followed by oNPHE more than mNPHE. This result suggests the relevant effect of steric, positional or electronic factors in the genotoxicity of nitrosophenols. role of similar effect was seen to oNPHE and mNPHE.

The inhibition effect of the ascorbic acid on the genotoxic response of NPHE and its methylated derivatives was also analysed. We used concentrations in a range of 0.2-2 mM for all of nitrosocompounds assayed or ascorbic acid.

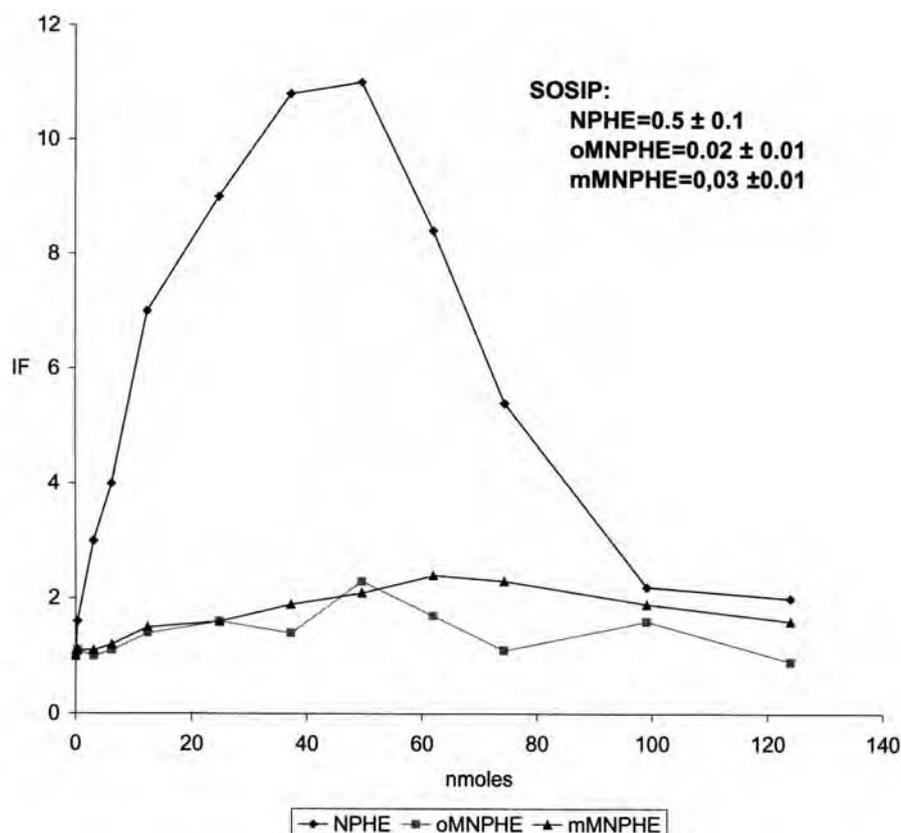
As long as concentrations of ascorbic acid were increased, inhibition halo did not appear in plates incubated with 0.2 mM of nitrosocompounds. However, when 2 mM of NPHE was assayed in presence of distinct concentration of ascorbic acid (0.2 mM, 0.4 mM and 0.8 mM), a genotoxic response was observed.

Related to the methylated derivatives, mNPHE and oNPHE, the inhibition halo is clearly reduced when 0.4 mM or higher concentrations of ascorbic acid were added. So, it seems that an equimolar relation between ascorbic acid and the analysed nitrosocompounds allows the blocking of the genotoxic/mutagenic effect of the later compounds. Such relation should be lower for oNPHE and mNPHE. Paying attention to NPHE, it is possible that the ascorbic acid blocks the genotoxic activity of the nitroso group, since there are not impediments of junction to that molecule. On the other side, ascorbic acid exhibits large difficulties to join to the methylated derivatives, due to the methyl groups presence, which might block genotoxicity associated to -NO group by steric effects. Moreover, it is possible that such methylations could produce mutations in DNA, but without inducing an activation of SOS response, because such mutations do not imply the formation of single strand DNA zones (Quillardet and Hofnung, 1993).

### 3.3. SOS Chromotest: Inhibitory effect of ascorbic acid.

Genotoxicity associated to nitrosophenols (NPHE and its derivatives: mNPHE and oNPHE), aim of this study, was also quantified by the SOS chromotest assay. Concentrations assayed were in a range of 0 to 40.7 mM. Results are shown in figure 4.

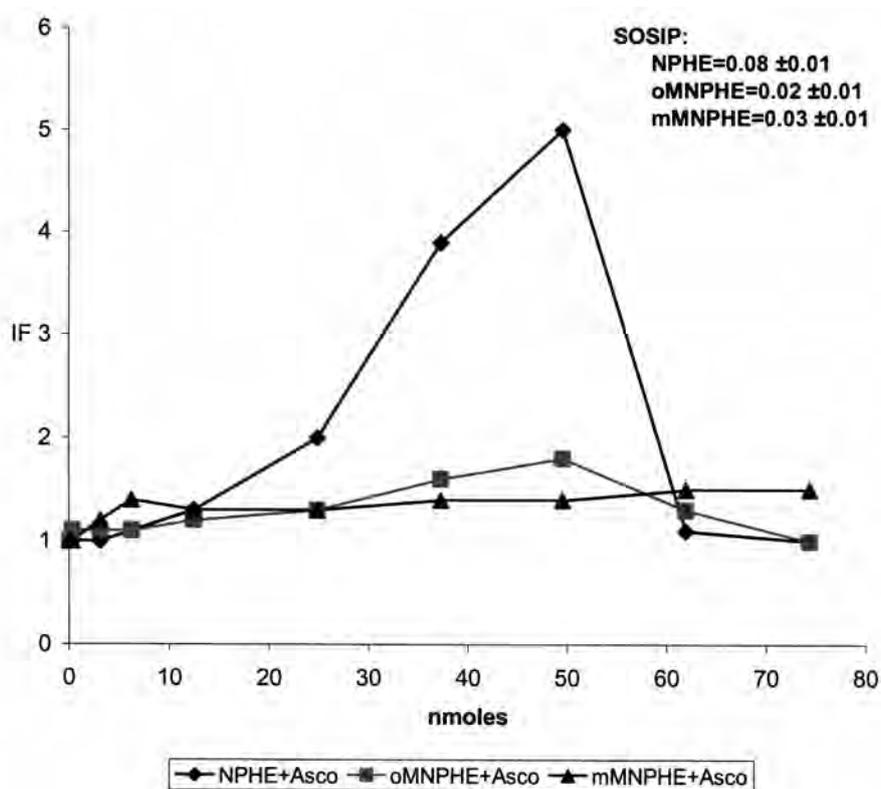
The IF values are represented *versus* nanomols of nitrosocompounds (figure 4) showing a clear induction of the SOS response by NPHE (SOSIP =



**Figure 4:** SOS Chromotest assays (PQ37) in presence of NPHE, mNPHE and oNPHE. IF: Induction Factor.

0.5 ± 0.1). Such compound shows an interval between 1.5-5.0 nanomols, in which it has a genotoxic/mutagenic effect. Higher concentrations should be considered as bacteriotoxic due to dramatic inhibition of protein biosynthesis inferred by alkaline phosphatase activity measured. Related to oNPHE and mNPHE, we obtained SOSIP values of 0.02 ± 0.01 and 0.03 ± 0.01, respectively. Thus, all compounds tested in our studies should be considered as genotoxics, as described by Quillardet and Hofnung (Quillardet and Hofnung, 1985, Quillardet and Hofnung, 1993).

We can say that NPHE, oNPHE, and mNPHE are a mutagenic substances in the SOS Chromotest. This test does not detect mutations, but is an indirect means of detecting agents that invoke the SOS-repair pathway.



**Figure 5:** SOS Chromotest assays (PQ37) in presence of NPHE, mNPHE and oNPHE and ascorbic acid. [Ascorbic]= 0.4 mM. IF: Induction Factor.

Possibly, these compounds induced damages would activate the *sfi:lacZ* genic fusion expression, probably by -NO (Ning and Xiaobai, 1997). Then, it could produce the induction of SOS response, given that the nitroso group could join nitrogenated bases, generating single sequences of DNA, which is the trigger of the *Rec A* gene activation, that is finally the responsible for the activation of the *sfi:lacZ* genic fusion expression (Quillardet and Hofnung, 1993). This capacity to produce mutagenicity due to oxidative damages generated by the nitroso group is similar to that obtained before with other aromatic nitrosocompounds by other authors (Ning and Xiaobai, 1997).

It is possible that methyl group in aromatic nitrosocompounds inhibit the oxidative action of nitroso group due to steric effects. On this way, we could explain such low SOSIP values obtained, the lower inhibition zones

observed in STA plates, on the methylated derivatives compared to NPHE. In this sense, we can say that when the compound has a substituent near the functional group, such as those included in our experiment, this substituent may have an influence on the enzymatic reduction of nitroso group. The results obtained in our study, are comparable to those obtained with other aromatic nitroso compounds (Haack *et al.*, 2001). As it is known, the molecule reactivity depends, fundamentally, of two factors: an electronic one and a steric one. On mechanisms in which aromatic molecules participate, the electronic effects are much less important than steric ones. On this way, our results are also comparable to those obtained by other authors working with nitrosamines, as the nitrosopiperidine (Wilcox *et al.*, 1991). In that compound, the methyl group insertion makes a steric impediment unfavourable for the attack by the NO specie, what might be transcribed in a low toxicity (Wilcox *et al.*, 1991). Also, it is possible that as long as the substituent group is being included to the nitrosophenol, the interaction between compounds and the cell can be reduced, probably during the compound penetration into the bacteria (Haack *et al.*, 2001).

In order to study the effect of ascorbic acid on mutagenic capacity of NPHE and derivatives, different assays of SOS chromotest were performed.

This way, different concentrations of studied nitroso compounds were analysed in presence of 0.04 mM of ascorbic acid.

Figure 5 shows the IF for NPHE, oNPHE, and mNPHE with and ascorbic acid. The IF in presence of ascorbic acid is notably lower than the IF obtained without it. This fact demonstrates an effective protection of ascorbic acid from the NPHE genotoxic effect.

We can say that it blocks the nitroso group action that would implicate less oxidative damage and, consequently, less induction of the SOS response. Thus, it can observe that the genotoxic/mutagenic values of NPHE (for 50 nanomoles, IF= 11), were reduced to half when ascorbic acid was added to the experiment (IF= 5). Clearly, the inhibitory effect on genotoxic activity of NPHE is higher than 50 %. On the other hand, the ascorbic acid did not produce any effect on the DNA damage response on the oNPHE and mNPHE.

As we have commented before, the presence of methyl group in oNPHE and mNPHE favours the steric effects. This fact could explain that the ascorbic acid had no inhibitory effect on SOS response induction.

In summary, taken into account all obtained results, we could say that

NPHE has a mutagenic effect, acting in a range of concentration as 0.1-16.4 mM, and when it is present in higher concentrations (from 20.5 mM) this compound is bactericide/toxic. Such mutagenic effect would be produced by oxidative damages on the DNA generated by nitroso group (NO) of the NPHE. Then, after DNA damage, single strand zones into the DNA will be released, which would produce SOS response activation.

Ascorbic acid not only inhibits the nitroso compound formation reaction, but also blocks the NO group, responsible of the genotoxic potency of nitrosophenol, as it may be inferred from our results. *o*NPHE and *m*NPHE exert their genotoxicity due to their methyl-substituent instead of oxidative pathways related to  $-NO$  group reactivity. This assertion is supported with no detected genotoxicity inhibition due to ascorbic acid action.

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