DAMAGES INDUCED IN λ PHAGE DNA BY ENZYME-GENERATED TRIPLET ACETONE

C.F.M. Menck*[1], J.B.Cabral-Neto*, A.Faljoni** and R. Alcantara-Gomes*

* Department of Biophysics, Institute of Biology, Rio de Janeiro State University, CP 46500 - Rio de Janeiro - RJ - 20550 - Brazil

** Department of Biochemistry, Institute of Chemistry, University of São Paulo, CP 20780 - São Paulo - SP - Brazil

SUMMARY. Exposure of λ phage to triplet acetone, generated during the aerobic oxidation of isobutanal by peroxidase, leads to genome lesions. The majority of these lesions are detected as DNA single-strand breaks only in alkaline conditions, so true breaks were not observed. Also, no sites sensitive to UV-endonuclease from Micrococcus luteus were found in DNA from treated phage. The participation of triplet acetone in the generation of such DNA damage is discussed.

INTRODUCTION. DNA alterations can be induced during incubation with enzyme-generated excited carbonyl compounds, as suggested by changes in the DNA circular dichroism spectrum (1,2) and single-strand breaks detected by sedimentation in alkaline sucrose gradients (3). These photochemical like effects could be caused by energy transfer from the excited molecule to DNA and are considered, with many others, "photobiochemical reactions without light", recently reviewed by Cilento (4,5).

The oxidation of isobutanal (IBAL)[2] by horseradish peroxidase (HRP) produces formic acid and acetone, the latter in the electronically excited triplet state (6,7). We have reported that triplet acetone inhibits the λ phage ability to infect Escherichia coli (8). This inactivation was observed even though the phage DNA was injected into the bacteria, suggesting that DNA lesions would be formed and would prevent phage multiplication. In this paper, we confirm the existence of DNA damage in the phage treated with the IBAL oxidation system. Possible involvement of energy transfer in this phenomenon will be considered and discussed.

MATERIAL AND METHODS. The λ phage (cI857tsSam7) were obtained from lysogenic bacteria Escherichia coli by thermal induction and purified to the CsCl gradient (9). DNA labelling and phage or DNA treatment with IBAL oxidation system were performed as described before (8). DNA was extracted with chloroform: isoamyl alcohol (24:1) and dialysed against Tris 10mM, EDTA 1mM, pH 7.0. When necessary, DNA was irradiated with a low pressure germicidal lamp (254nm) at a fluence rate of 0.5 $W.m^{-2}$, dosimetry by Latarjet meter. UV-endonuclease from Micrococcus luteus employed corresponds to fraction III of the purification procedure of Carryer and Setlow (10). DNA incubation with this enzyme was done as already described (11). To test DNase susceptibility to DNase, the enzyme (final concentration 15μg/ml) was added after pretreatment of the phage or DNA with the IBAL oxidation system and N_2 bubling. The incubation was then performed at 37°C for 60 minutes and stopped by ice chilling.

The DNA was sedimented in two different denaturating conditions: (i) in alkaline pH: phage or DNA preparations were lysed in 0.1N NaOH, 3% SDS, 20mM EDTA and aliquots (0.3 ml) were layered on the top of a 4.0 ml linear 5-20% sucrose gradient containing 0.1 M NaCl, 0.1 N NaOH; the centrifugation was at 32000 rpm at 20°C in a SW 50.1 rotor (Beckman) for 2h. (ii) In neutral pH: we used the technique described before (12); basically DNA aliquots (0.3 ml) were layered on the top of a 4.0 ml linear gradients which were 0-15% sucrose (w/v) formamide; centrifugation was at 32000 rpm at 30°C for 4h in the same rotor. In both cases, fractions were collected from the bottom of the tube and processed for DNA analysis as already described (11).

RESULTS. Phage DNA lesions detected in alkaline sucrose gradients - When λ phage is treated with the IBAL oxidation system, DNA single-strand breaks are observed by sedimenting DNA in alkaline conditions (fig. 1c). No DNA single-strand breaks are detected if the phage is treated with IBAL in the absence of HRP (fig. 1a), or with acetone and formic acid, even in the presence of HRP (fig. 1b). These results suggest the participation of triplet acetone in the process which yields these DNA lesions. However, minor reactions in the IBAL system could produce the highly reactive activated species of oxygen, which are well known DNA damaging agents (13,16). The action of hydroxyl radicals does not seem probable since the reaction mixture contains ethanol, an efficient scavenger of this radical. As shown in table I, superoxide dismutase and catalase, which eliminate superoxide radical and hydrogen peroxide respectively, are not able to protect DNA from damage. Also, the participation of singlet oxygen in the phenomenon described here, which would be itself an interesting result, seems unlikely because: (i) there is no indication of its generation in the IBAL oxidation system, and (ii) deoxyguanosine, an scavenger of singlet oxygen, does not protect DNA when added to the reaction mixture (table I). Nevertheless,

when the incubation of λ phage with the IBAL system is in the presence of tryptophan, an efficient acceptor of triplet acetone emission (17), a significant reduction in the number of DNA lesions is observed (table I). Moreover, production of DNA lesions is dependent on the IBAL concentration (fig. 2), which correlates well with the increase of triplet acetone emission at higher reagent concentrations (data not shown). Thus, triplet acetone seems to be necessary for the appearance of lesions in the phage genome.

Characterization of the DNA damages - In order to distinguish between true single-strand breaks and alkaline-sensitive sites, DNA from treated and untreated phages were extracted and sedimented under denaturating conditions in neutral formamide. The results are presented in fig. 3. Almost not true breaks are detected in the DNA from treated phage (fig. 3b), however if this DNA is submitted to an alkaline pH, before being added to the gradient, it sediments slower (fig. 3c) indicating that most of the lesions induced in the phage genome by triplet acetone are alkaline sensitive sites. No sedimentation changes are observed in DNA from untreated phage when submitted to an alkaline pH (fig. 3a). Also, no UV-endonuclease sensitive sites were detected in the DNA from treated phage (table II). Thus, the DNA lesions induced by triplet acetone in λ phage are mostly alkaline sensitive sites but are not recognized by UV-endonuclease.

Phage genome accessibility to DNase I after treatment with IBAL oxidation system - The deleterious action of triplet acetone in nude DNA has already been described (1,3). However, we recently observed (8) damage in λ phage protein coat after treatment with the IBAL oxidation system. Thus, it was important to verify if the phage DNA is still shielded by the protein coat or is totally exposed for the direct action of triplet acetone. Therefore the nuclease attack on DNA was tested (table III). DNase I is unable to introduce DNA nicks in either untreated or treated phage but yields positive controls for nude DNA. These results show that after phage exposure to triplet acetone the protein coat is still protecting its genome.

DISCUSSION. The induction of DNA damage by triplet acetone, generated either chemically (18) or enzymically (1-3) has already been described. In this paper we present evidence that this effect can also be observed even if DNA is shielded by an envelope of proteins as is the case for λ phage. In fact, the participation of triplet acetone damaging phage genome is strongly supported by our results in which tryptophan is the only scavenger able to protect DNA (table I).

The mechanism that would lead to DNA lesions is unknown. Since triplet acetone is generated inside the enzyme (7) and DNA is not accessible to DNase, a direct action of triplet acetone through molecular collision seems unlikely. Recently, evidence of energy transfer from triplet acetone to aminoacids inside proteins were obtained (19), so participation of the protein coat as an intermediate should be considered.

The analysis of the kind of DNA damage produced can give us some clues on the process involved in its generation. Most of the detected lesions are alkaline sensitive sites and not true ruptures in the DNA phosphodiester chain. This fact excludes all mechanisms that would generate more DNA single-strand breaks than alkaline sensitive sites. Since oxygen radicals yields true breaks in DNA (15,20, our unpublished results) their participation in the process discussed here seems unlikely. Also, although production of thymine dimers by chemically generated triplet acetone has been observed (18), UV-endonuclease from M.luteus is not able to introduce DNA breaks in phage treated with the IBAL oxidation system. This enzyme nicks UV-irradiated DNA at the pyrimidine dimer site (21) and recently the T4 endonuclease V, which has essentially the same activity of the M.luteus enzyme, has been observed to act on apurinic/apyrimidinic sites (22). Therefore, pyrimidine dimers are not produced in phage DNA by triplet acetone treatment, and also, the DNA alkaline sensitive

sites described here do not seem to be apurinic/apyrimidinic sites. There results are in agreement with the possibility that incubation of λ phage with the IBAL oxidation system would promote purine and/or pyrimidine alterations, since these nitrogeneous bases are possible acceptors of the energy from triplet acetone or from an excited intermediated compound in the protein coat.

Whatever these lesions are, they may be responsible for at least part of the λ phage inactivation already described (8). Since phage inactivation is similar in the DNA repair proficient E.coli or in the uvrA recA double mutant, the DNA lesions observed here are apparently not repaired in E.coli through the pathways controlled by the products of those genes.

The biological implications of the results shown in this paper are unknown, however the production of excited carbonyl compounds in the vicinity of cell genome could generate DNA damage. In fact, photon emission close to DNA have already been observed. UV and visible ultra-weak radiation are emitted from cell culture, a phenomenon probably connected with cellular mitoses (23). Popp et al. (24) proposed that the different topological configurations of DNA may act as photon storage within the cells. Thus, the phage DNA lesion induced by an excited carbonyl compound may correspond to a natural phenomenon, and this could be correlated to "spontaneous" deleterious processes in the genome, such as mutagenesis.

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FOOTNOTES

Page 1

1. To whom correspondence should be addressed.
2. Abbreviations: IBAL, isobutanol; HRP, horseradish peroxidase; mol. wt., molecular weight.

TABLE I

Effect of various agents in DNA damage induction by IBAL oxidation system

Treatment	% DNA lesions [b]
IBAL + HRP [a]	100
IBAL + HRP + Catalase (50 µg/ml)	97
IBAL + HRP + Superoxide dismutase (50 µg/ml)	88
IBAL + HRP + deoxyguanosine (1 mM)	92
IBAL + HRP + tryptophan (1 mM)	3

(a) For phage treatment the concentrations were: IBAL 37 mM and HRP 3 µM.

(b) DNA lesions were calculated from alkaline sucrose gradient analysis.

TABLE II

Test for UV-endonuclease sensitive sites

Treatment	Number of UV-endonuclease sensitive sites/10^7 daltons (c)
untreated	- 0,04
IBAL + HRP (a)	- 0,03
UV-irradiated (b)	0,27

(a) For phage treatment the concentrations were IBAL 88 mM and HRP 3 µM.

(b) λ DNA was irradiated with UV at $10 J/m^2$.

(c) UV-endonuclease sensitive sites were determinated by formamide sucrose gradient analysis.

TABLE III

Phage DNA accessibility to DNase

Treatment	Number of nicks introduced by DNase/10^7 daltons[b]
phage	0,01
phage + IBAL + HRP[a]	- 0,03
DNA	0,53
DNA + IBAL + HRP[a]	0,24

(a) For phage or DNA treatment the concentration were IBAL 88 mM and HRP 3 µM.

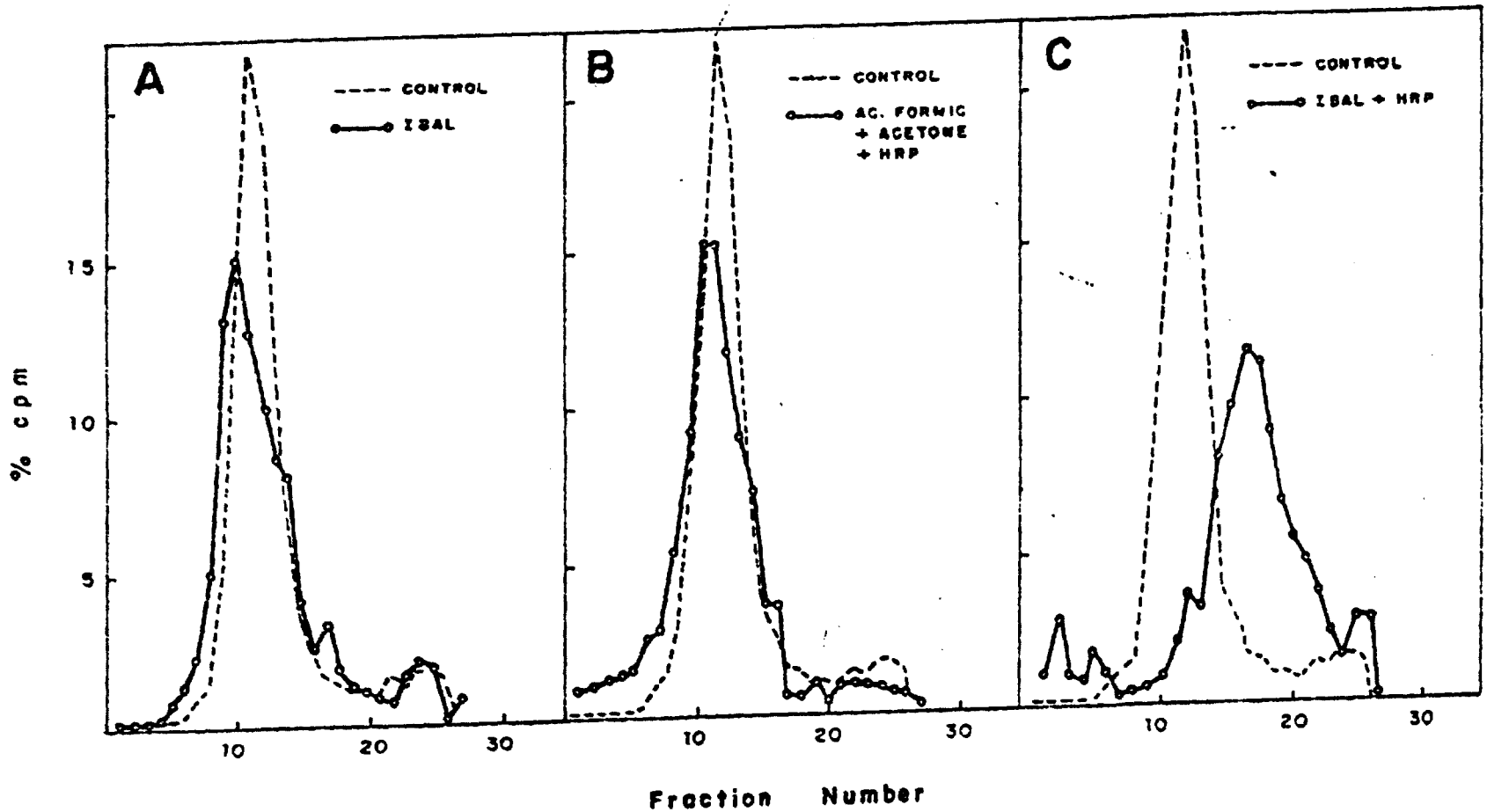
(b) Sensitivity to DNase were calculated from alkaline sucrose gradient analysis.

FIGURE LEGENDS

FIG. 1 - Phage DNA damages by IBAL oxidation system - λ phage containing ^{3}H-DNA were treated (-o-o-) with IBAL 88 mM (a); formic acid 1 mM; acetone 1 mM and HRP 3 μM (b) or IBAL 88 mM and HRP 3 μM (c). Then, phage were lysed and the DNA sedimented in alkaline sucrose gradients. The broken lines represent DNA from untreated phage. Sedimentation is from right to left.

FIG. 2 - Phage DNA damage X IBAL concentrations - λ phage were treated with IBAL at the indicated concentrations and HRP 3 μM, and DNA analysed in alkaline sucrose gradients.

FIG. 3 - Alkali-sensitive sites in DNA from treated phages - λ phage were untreated (A) or treated (B,C) with IBAL oxidation system (IBAL 88 mM, HRP 3 μM). The DNA's were extracted and sedimented in dormamide sucrose gradients. In A and C, before sedimentation the DNA's were submitted to alkaline pH (NaOH 0,1N) for 10 minutes, then pH was neutralized (to pH=8.0) by adding HCL, and Tris-HCL (50 mM). The broken lines represent DNA from untreated phage. Sedimentation is from right to left.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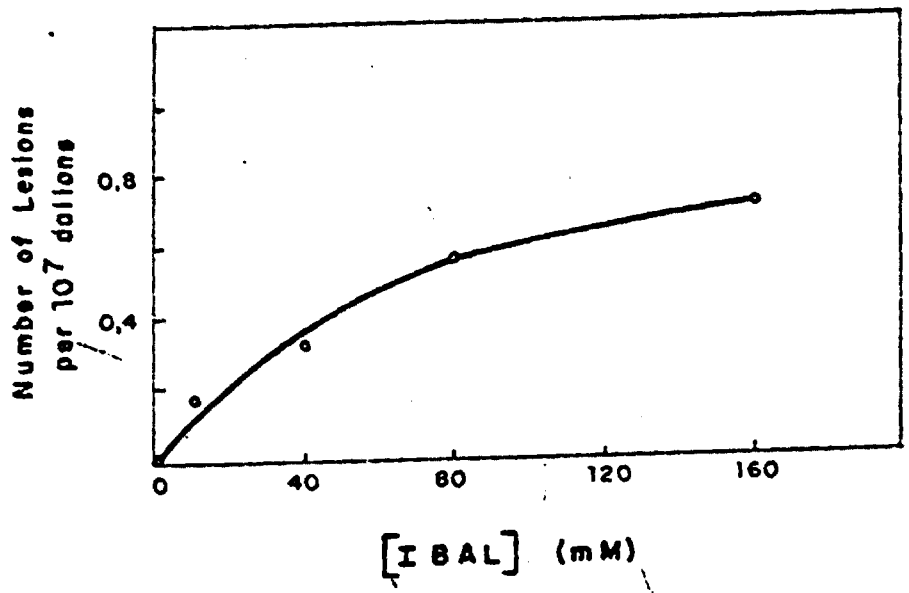

Fig 2

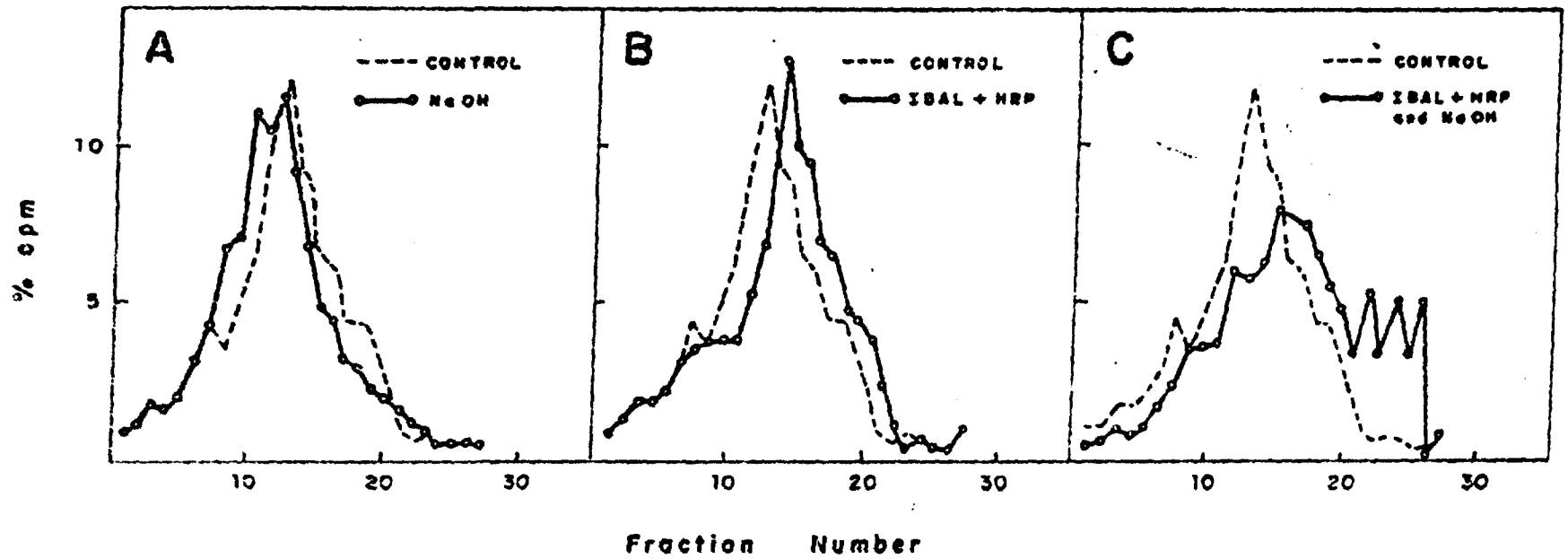
A
CONTROL
NaOH
B
CONTROL
IBAL + HRP
C
CONTROL
IBAL + HRP
and NaOH
% cpm
10
5
10
20
30
Fraction Number

Fig 3