Biochemical Pharmacology, Vol. 16, pp. 637–646. Pergamon Press Ltd. 1967. Printed in Great Britain

ON THE MODE OF ACTION OF A POTENT CARCINOGEN, 4-HYDROXYLAMINOQUINOLINE 1-OXIDE ON BACTERIOPHAGE T4

MINORU ISHIZAWA and HIDEYA ENDO

Cancer Research Institute, Faculty of Medicine,
Kyushu University, Fukuoka, Japan

(*Received* 30 *December* 1966)

Abstract—The *in vitro* effect of 4-hydroxylaminoquinoline 1-oxide (4-HAQO) on bacteriophage T4 was investigated. Among five derivatives of 4-nitroquinoline 1-oxide (4-NQO), only 4-hydroxylaminoquinoline 1-oxide produced marked inactivation of the phage.

This inactivation effect took place at pH >7·0 and only in the presence of oxygen. Under these conditions, 4-HAQO lost its effect within 10 min.

Spectrophotometric and chemical investigations established that 4-HAQO was oxidized to 4,4′-azoxyquinoline-1,1′-dioxide, indicating the existence of 4-nitrosoquinoline 1-oxide as an intermediate. This led to the conclusion that 4-HAQO itself is not the ultimate compound in the inactivation process of the phage but that an oxidation product is responsible for this event.

The target of the chemical agent in the phage particle is not the tail protein but its DNA.

Evidence for this is presented and the possible connection between these events and the initial step of carcinogenesis by 4-HAQO is discussed.

FOR THE understanding of the initial step of chemical carcinogenesis the recognition of the ultimate derivative of a carcinogenic agent administered, and the elucidation of its target or receptor are essential. The present study of 4-hydroxylaminoquinoline 1-oxide (4-HAQO), a potent carcinogen, was undertaken to attempt a solution of these problems. In 1957 Nakahara and his co-workers[1] discovered the powerful carcinogenic action of 4-nitroquinoline 1-oxide (4-NQO) which had been synthesized previously by Ochiai and his associates[2]. The mode of carcinogenic action of 4-NQO, was thought at first to be due to a non-enzymic substitution reaction between its nitro group and intracellular nucleophilic groups, especially sulfhydryl groups.[3, 4] However, it was shown that 4-NQO was reduced enzymatically to 4-HAQO, 4-aminoquinoline 1-oxide (4-AQO) and 4-aminoquinoline (4-AQ) by microorganism,[5] as well as by mammalian cells,[6, 7] and that a 4-HAQO possessed a high carcinogenic activity,[8, 9] whereas 4-AQO and 4-AQ did not, and that 4-HAQO exhibited also all the biological actions of 4-NQO, such as formation of nuclear inclusion in tissue culture cells,[10] prophage induction in lysogenic bacteria[11] and induction of mutation in microorganism.[12–15] When 4-NQO and 4-HAQO were injected into rats, in mole equivalent of the minimal effective dose of 4-HAQO tumour formation only occurred in the group to which 4-HAQO had been administered,[16] all results suggesting that 4-HAQO might be an active intermediate in 4-NQO carcinogenesis. In order to clarify this point and the

mode of action of 4-HAQO the present studies were undertaken by employing bacteriophage T4 as a model for the target or receptor in the mammalian cell, involved in chemical carcinogenesis. Bacteriophage is a very useful system, having no metabolic apparatus, for testing and analysing the mode of action of compounds that interact *in vitro* with phage particles which consist only of DNA and protein, and can suffer inactivation or induction of mutations.[17] Moreover, the genetic fine structure of the *r*II region of phage T4 has been already analysed in detail.[17]

MATERIALS AND METHODS

Phage and bacteria. The phage strains used were T4B standard type and *r*II mutant *r*287. T4B is designated in this paper as T4r^+ or *r*II$^+$. The bacterial strains used were *Escherichia coli* B, BB and KB. KB is a derivative of *E. coli* K12(λ)[18].

Media. The composition of the medium is given per liter of deionized water. Broth contained 1% bacto-peptone and 0·5% NaCl. Broth agar: broth with 1·2% bacto-agar. Soft agar: broth with 0·7% bacto-agar. As a synthetic medium, M-9 was employed.[19] M-9 buffer is M-9 minus glucose.

Chemicals. 4-nitroquinoline 1-oxide, 4-hydroxylaminoquinoline 1-oxide, 4-aminoquinoline 1-oxide, 4-aminoquinoline, 4-hydroxylaminoquinoline and 4,4′-azoxyquinoline-1,1′-dioxide were kindly supplied by Professor M. Hamana, Dept. of Pharmaceut. Chemistry in Kyushu University.

Inactivation experiment of phage T4 in vitro. Phage T4r^+ was incubated with the chemical agents by adding one volume of solution of a chemical to nine volumes of the phage suspension preincubated for 5 min at 37°C. The resulting mixture contained approx. 3×10^9 phages/ml and respective concentration of chemicals in 0·1 M-sodium phosphate buffer of pH 7·5 containing 0·1 M-NaCl. Aliquots of the mixture were taken at various time intervals, diluted 100-fold with broth to stop the interaction of the drug, and after appropriate further dilutions, were plated on the indicator strain *E. coli* B. The phage assay was based on agar layer technique.[20] 4-HAQO solution was freshly prepared by dissolving 4-HAQO. HCl in cold 0·02 *N*-HCl and kept at 0°C until the time of the experiment. This procedure is essential for obtaining reproducible results, because of the lability of 4-HAQO in alkaline solution. It is also noteworthy that T4 used in the test was purified by repeated low and high speed centrifugations, because inhibition of the phage inactivation by 4-HAQO had been frequently seen, when crude lysate was used instead of the purified phage. For the inactivation test under anaerobic condition, Thunberg tubes were employed. Before mixing the phage suspension (main compartment) with the drug solution (side arm), the gas phase was replaced three times with pure nitrogen which was passed through Fieser's solution.[21] Since 4-HAQO is extremely sensitive to oxygen at a pH in the alkaline range, even traces of oxygen in nitrogen as well as those dissolved in the reaction mixture have to be completely eliminated.

Marker rescue. All crosses were done with strain BB bacteria as host. Cross reactivation of *r*II$^+$ marker was carried out under conditions similar to those employed by Bautz-Freese and Freese.[22] Bacteria growing exponentially in M-9 were centrifuged, washed and resuspended in M-9 buffer to a density of 2×10^8 cells/ml and starved for 40 min at 37°C. The procedure of the phage inactivation by 4-HAQO was the same as that described in above section, except that M-9 buffer was used instead of broth for 100-fold dilution. For measurement of the survival of the infectivity of T4*r*II$^+$,

free phages were plated on KB. For measurement of the genetic survival of the treated rII^+ phage, the starved bacteria were jointly infected with a multiplicity of 0·1 for rII^+ and of 4 for rII ($r287$) per cell. Adsorption was allowed to proceed for 8 min in M-9 buffer containing 40 μg per ml of L-tryptophan, and anti-T4 serum was added to remove free phages. Five min after addition of anti-serum, the infected bacteria were then diluted and plated on KB.

RESULTS

The active agent. In pursuing the working hypothesis that 4-HAQO is probably the proximate compound of 4-NQO carcinogenesis, and interacts directly with phage particles *in vitro*, affecting the infectivity of the phage, we checked whether 4-NQO and its derivatives (including 4-HAQO) were able to inactivate the phage T4 *in vitro*. As illustrated in Table 1 (the phage was incubated with each drug in buffer, pH 7·5, 30 min at 37°C) only 4-HAQO produced inactivation. In Figs. 1 (a) and 1 (b) the

TABLE 1. EFFECT OF 4-NITROQUINOLINE 1-OXIDE DERIVATIVES *in vitro* ON THE INFECTIVITY OF PHAGE T4

Chemicals tested	Incubation time (min)	Phage titer (per ml)
Non	0	$2{\cdot}7 \times 10^9$
	30	$2{\cdot}45 \times 10^9$
4-nitroquinoline 1-oxide	30	$2{\cdot}2 \times 10^9$
4-hydroxylaminoquinoline 1-oxide	30	$< 5 \times 10^2$
4-hydroxylaminoquinoline	30	$2{\cdot}35 \times 10^9$
4-aminoquinoline 1-oxide	30	$2{\cdot}65 \times 10^9$
4-aminoquinoline	30	$2{\cdot}4 \times 10^9$

Phage T4 r^+ was incubated with each derivative at a final concentration of 0·5 mM in 0·1M-phosphate-0·1M NaCl (pH 7·5) at 37°C. After 30 min, the phage titer was determined as described under Materials and Methods.

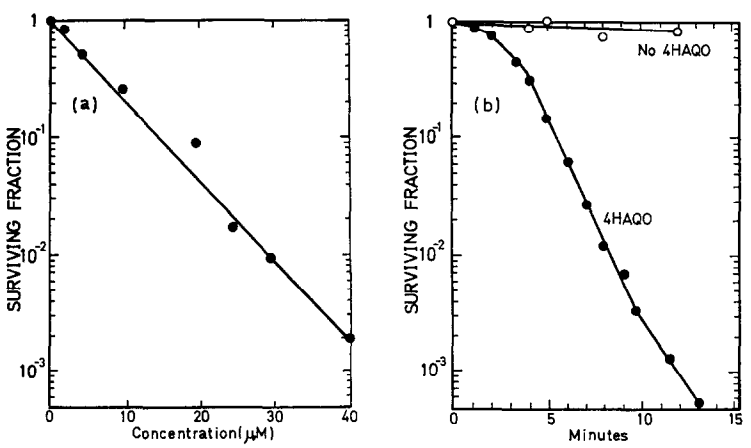


FIG. 1. Effect of 4-HAQO on the infectivity of T4 as a function of concentration and time.
(a) T4 r^+ was incubated for 10 min with various concentrations of 4-HAQO on 0·1 M-phosphate-0·1 M-NaCl (pH 7·5) at 37°C.
(b) T4 r^+ was incubated in the presence (●) or absence (○) of 40 μM-4-HAQO for varying periods in the same buffer, pH 7·5, as in (a) at 37°C. After the incubation phage infectivity was assayed as in Table 1.

phage titer was plotted, respectively, against the concentrations of 4-HAQO and against varying time intervals of interaction; Fig. 2 shows pH dependency of the phage inactivation. These results indicated that the effects on phage were optimally produced at a pH > 7 (alkaline range) and proceeded exponentially in response to the drug dose. Moreover this *in vitro* reaction was temperature dependent and did not proceed at 0°C. During these experiments, it was frequently observed that solutions

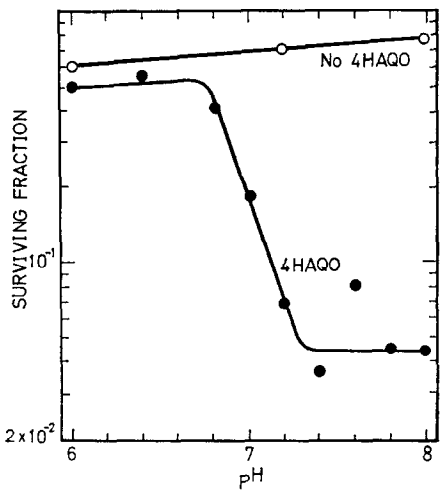


FIG. 2. Effect of 4-HAQO on the infectivity of T4 as a function of pH.
T4 r^+ was incubated for 10 min in the presence (●) or absence (○) of 20 μM-4-HAQO in 0·1 M-phosphate-0·1 M-NaCl having various pH values as indicated at 37°C. Phage assay was the same as in Table 1.

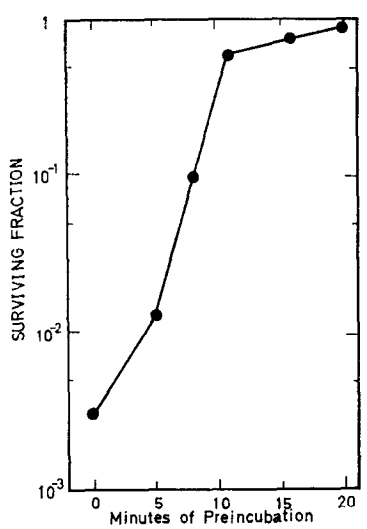


FIG. 3. Stability of T4-inactivating ability of 4-HAQO. 4-HAQO was dissolved in 0·1M-phosphate-0·1M NaCl (pH 7·5) at a concentration of 40 μM and incubated for varying periods at 37°C. At the end of the incubation; a twentieth volume of T4 r^+ suspension was added to each tube and after 10 min incubation at 37°C; phage titer was determined as in Table 1.

of 4-HAQO, prepared by diluting the stock solution in a buffer of pH 7·5, tended to turn yellow as time passed; finally a reddish substance precipitated from the solution that had lost its phage inactivating effect suggesting lack of stability of the starting material. In additional experiments 4-HAQO on its own was incubated in the buffer, pH 7·5, at 37°C and after various intervals aliquots were taken and incubated with the phage for 10 min to establish their inactivating effects (Fig. 3). The result showed clearly that 4-HAQO lost its anti-phage activity completely within 10 min. Simultaneously, the absorption maximum at 360 mμ of 4-HAQO was found to decrease until it disappeared after 10 min (Fig. 4). The questions which arose concerned the

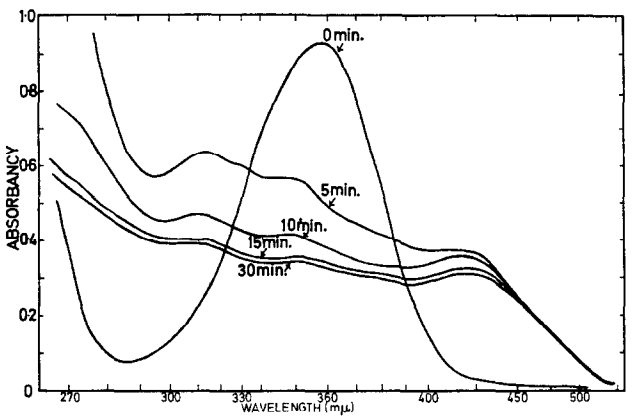
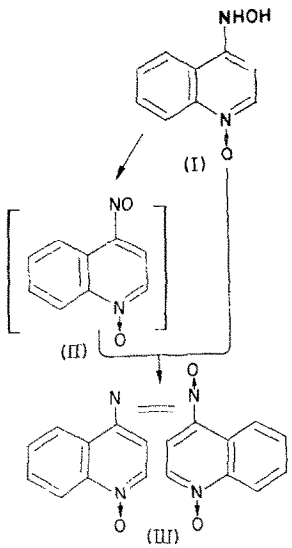


FIG. 4. Change in absorption spectrum of 4-HAQO as a function of time.
Tubes containing 75 μM-4-HAQO in 0·1 M-phosphate-0·1M-NaCl (pH 7·5) were incubated at 37°C. At the indicated times a sample was taken and its spectrum was measured rapidly with a Shimazu (Type SV-50 A) recording photoelectric spectrophotometer at room temperature. Spectra of ultraviolet and visible light regions were measured separately but were combined in the figure.

fate of 4-HAQO and the identity of the reddish precipitate. 4-HAQO (1mM) was incubated in buffer, pH 7·5, at 37°C for 3 hr and the resulting reddish precipitate was collected and purified by silica gel chromatography. Details of this procedure will be reported elsewhere. When the visible, u.v. and i.r. spectra of this product were compared with those of 4,4′-azoxyquinoline-1,1′-dioxide(III) (supplied by Professor M. Hamana) the physical properties of these two materials were indistinguishable, and thus the two compounds are identical. It is not unlikely that 4-HAQO(I) was first in part oxidized (air) in alkaline solution to the nitroso compound (II) from which by interaction of the nitroso group with hydroxylamino group of (I), the reddish azoxy compound was produced. The question whether this oxidative condensation process is actually linked causally with the phage inactivation by 4-HAQO could be answered by allowing the drug to act on the phage under anaerobic conditions. Figure 5 shows clearly that under anaerobic conditions inactivation by 4-HAQO of the phage could be observed only to a slight extent whereas under aerobic conditions, as before, a marked decrease of the phage titer was demonstrated. The slight inactivation of the phage under anaerobic conditions may be due to traces of oxygen remaining in the reaction system in spite of the repeated replacement of air by nitrogen gas;



however some intermolecular oxido-reduction reaction (disproportionation reaction), cannot be excluded.

Receptor. The next problem to be solved was the identity of the target attacked by 4-HAQO in the phage particle. There are two constituents of the phage that may act as receptors for the drug: the tail protein of the phage which recognizes the host

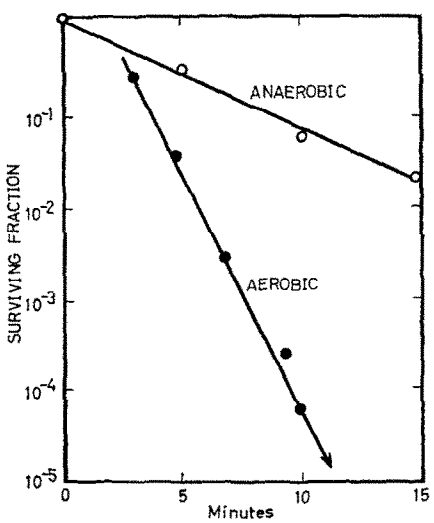


FIG. 5. Effect of oxygen on the T4-inactivating capacity of 4-HAQO.
T4 r^+ was incubated for different times with 60 μM-4-HAQO in 0·1 M-phosphate-0·1 M NaCl (pH 7·5) under aerobic (●) and anaerobic (○) condition; phage titer was determined as in Table 1.

in a specific manner, and attaches the phage to the bacterial surface so that the transfer of the genetic material of the phage into host cell is secured; the other is the genetic material itself. The study of the mechanism of the action of 4-HAQO must be correlated therefore with the functions of these two main parts and the phage-inactivating

capacity of the drug: T4 phages were treated for various periods with 4-HAQO. The plaque forming ability (curve (a), Fig. 6) and the adsorbing capacity (curve (b), Fig. 6) of these phages were then determined. As shown, the phage treated with 4-HAQO preserved completely its adsorbing capacity to the host cell, in spite of the loss of

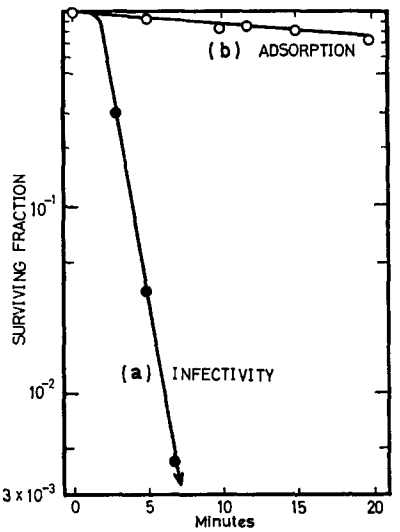


FIG. 6. The absorbing capacity of 4-HAQO-treated T4. Log-phase *E. coli* B ($2{\cdot}9 \times 10^8$ cells/ml) were infected with an average of 3 phage particles/cell which had been treated with 60 μM 4-HAQO for different periods in 0·1 M-phosphate-0·1 M NaCl (pH 7·5) at 37°C. After 10 min adsorption at 37°C in broth containing 0·005 M-KCN, anti-T4 serum was added for 5 min, then the infected cells were diluted and plated on agar plate for measuring the number of survival cells. Curve (a), preservation of T4-infectivity (plaque former); Curve (b), preservation of the bacterial killing ability of T4.

its plaque forming ability. Next, attention was paid to the tail protein function of T4 to inject its DNA into the host cell and the function of that DNA itself. If the tail protein function had been left intact and the target of 4-HAQO, involved in the loss of plaque forming ability of the phage was DNA, the host cell infected with the 4-HAQO treated phage would contain the damaged phage-DNA, the function of which is capable of being rescued by a suitable recombination system. Cross reactivation tests were carried out by infecting *E. coli* BB with the 4-HAQO treated T4*r*II$^+$ and T4*r*II (*r*287) simultaneously and by plating the infected cells on *E. coli* K 12(λ), which allows only the growth of *r*II$^+$. As shown in Fig. 7, considerable recovery of *r*II$^+$ gene function was obtained in the cell jointly infected with *r*II$^+$ and *r*II (7a), but not in the cell infected with *r*II$^+$ alone (7b). These results showed clearly that the 4-HAQO-damaged DNA was present in the host cells.

DISCUSSION

The present study revealed that 4-HAQO (I) itself which is only fully active in presence of oxygen is not a proximate compound but that one of its oxidation products is responsible for the phage inactivation *in vitro*. Thus, 4-nitrosoquinoline 1-oxide (II), or the free radical, the presence of which as an intermediate between 4-HAQO and 4-nitrosoquinoline 1-oxide was confirmed by Nagata *et al.*,[23] seems to be the most likely candidate, particularly in view of the fact that because of their unstable nature

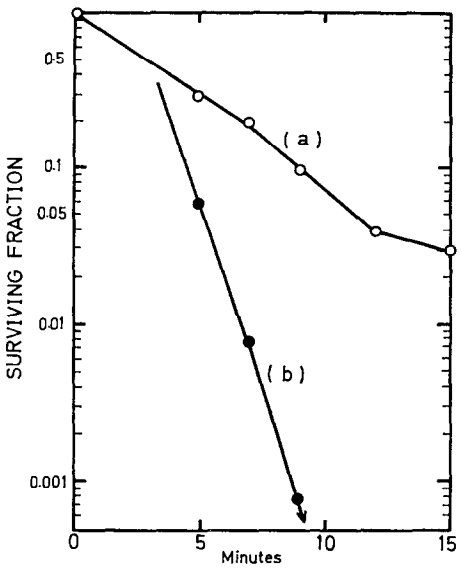


FIG. 7. Rescue of *r*II$^+$ marker of 4-HAQO-treated T4*r*II$^+$ by cross-reactivation with non-treated T4*r*II. Phage T4 *r*II$^+$ was treated with 50 μM-4-HAQO under the same condition as in Fig. 5. *E. coli* BB ($2{\cdot}2 \times 10^8$ cells/ml) were infected jointly with T4*r*II$^+$ and *r*II mutant *r*287 at multiples of 0·1 and 4/cell respectively. After 8 min adsorption in M-9 buffer containing 40 μg/ml at L-tryptophan, anti-T4 serum was added for 5 min, the infected cells were immediately diluted and plated on *E. coli* KB. Curve (a), preservation of T4*r*II$^+$-infectivity on *E. coli* KB; Curve (b), preservation of T4 *r*II$^+$-infectivity reactivated by joint infection with *r*287 on KB. (Survival of *r*287 alone on KB as a control of joint infection was 4×10^{-4}.)

the rapid loss of the phage-inactivating ability of 4-HAQO can be explained. Moreover, azoxyquinoline dioxide (III) being insoluble in water has no effect on the phage *in vitro*. However, there is a faint possibility that 4-HAQO is inserted into phage-DNA, and is converted *in situ* into the azoxy compound which changes gradually the normal function of DNA. This is based on the assumption that the target (receptor) involved in the loss of infectivity of the phage by 4-HAQO is DNA; our studies have provided proof for this and demonstrated that the tail protein function is not impaired. The results also suggest the possibility of mutants appearing in the surviving fraction of the treated phage. Okabayashi *et al.* have reported the mutagenic action of 4-HAQO on *Aspergillus niger*[14] and *E. coli*.[15] The mechanism of these mutations, however, still remains unknown. As already mentioned, one of the main reasons why we employed T4 in the present study was to analyse the character of the 4-HAQO induced mutation by using *r*II system. We observed that the treatment of T4*r*$^+$ (wild-type) with 4-HAQO *in vitro* augmented the frequency of the appearance of *r*- (rapid-lyzing type) plaques for about 20 times. This value, however, can only be noted in the surviving population of T4*r*$^+$, highly inactivated by 4-HAQO. The identification and characterization of T4*r*II mutation, induced by this agent, will be reported elsewhere.

The indication that the damaged site of the 4-HAQO-treated phage is DNA, led us also to examine a direct interaction between 4-HAQO and nucleic acid or its components. So far as melting range of DNA, and u.v. absorption characteristics of

nucleic acid bases, nucleosides and nucleotides are concerned, their changes which are usually observed in their reaction with hydroxylamine, nitrous acid, and alkylating agents, could not be detected after interaction with 4-HAQO. The effect of 4-HAQO *in vitro* described in this paper has also been studied by Ono *et al.*[24] by using transforming DNA of *Bacillus substilis*. They obtained very similar results to ours concerning the inactivation by 4-HAQO of the DNA function but they did not succeed in demonstrating any chemically or physicochemically changes through the direct interaction between the drug and DNA. Quite recently, Nagata *et al.*[25] have demonstrated an intercalation of not only 4-HAQO but also of 4-NQO with DNA by using the flow dichroism method. As the latter does not inactivate phage *in vitro* it seems unlikely that this inactivation has any connection with the intercalation of 4-HAQO. Finally, allowing for the fact that the phage interaction in the present study is a model system, what light can it shed on the initial events of carcinogenesis by 4-HAQO, particularly with regard to the question, whether the mechanism of such carcinogenesis represents a somatic mutation or the deletion of a specific protein from the cytoplasm. As pointed out in the introductory part, our present investigation was directed towards a solution of these problems.

While phage inactivation itself has nothing to do with chemical carcinogenesis as a whole, it is conceivable that the initial step of carcinogenesis is, like that of phage inactivation, an interaction between the proximate carcinogen, produced in the cell, and a cellular target or receptor. Making this assumption it seems not unlikely that a cell is destined to become a cancer cell, when such an interaction between the proximate compound, presumably formed by an oxidation process from 4-HAQO, and DNA of the cell takes place.

How does the instability of 4-HAQO under the conditions described in this paper fit in with its behaviour in carcinogenesis? On the one hand Endo and Kume[16] found that cancer could be produced in rats by a single injection of 4-HAQO; on the other hand it was clearly demonstrated that this drug changes *in vitro* to an insoluble azoxy compound and looses its activity *vis-à-vis* phage within 10 min. Taking these two facts together, but bearing in mind one's ignorance as to the degree of stability or lability of 4-HAQO in combination with the phage particle, one could speculate that the time interval necessary for the "determination of the fate" of a somatic cell, whether to become cancerous or to remain normal, may be very short indeed.

Acknowledgement—The authors are indebted to Dr. M. Hamana for his generous gift of 4-HAQO derivatives; to Drs. T. Kamiya and N. Kinoshita for their critical comments on the manuscript.

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