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MUTAGENIC ACTIVITIES OF HYDROPEROXYTHYMINE DERIVATIVES, PRODUCTS OF RADIATION AND OXIDATION REACTIONS

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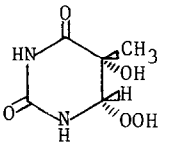
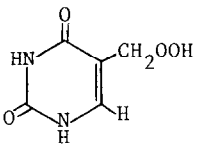
Summary: By using a bacterial test system, it has been shown that hydroperoxy derivatives of thymine and thymidine produced by ionizing radiation, near-UV radiation, and certain oxidation reactions are *highly* mutagenic. Considering that hydroperoxy derivatives of biomolecules have been implicated widely as likely candidates causing mutagenesis, carcinogenesis, and aging, it would be advantageous to screen these compounds when they can be isolated in pure state in order to assess their potential hazards to human health. The findings from these assays would provide information to further our understanding of the mechanism of their mutagenic action.

cis-5,6-Dihydro-6-hydroperoxy-5-hydroxythymine (6-TOOH) produces mutation in *Haemophilus* transforming DNA in the presence of transition metal ions, most effectively Cu^{++}.[1] The mutation frequency was found to be directly proportional to the exposure time and concentration of 6-TOOH and Cu^{++}. Under similar conditions, hydrogen peroxide (HOOH) did not produce mutation. Furthermore,



6-TOOH



α-TOOH

catalase which decomposes HOOH did not lessen the effectiveness of 6-TOOH as a mutagen. On the basis of identification of 6-TOOH as a radiation product of Thy[2] and of 6-TOOH derivative possibly of Thd[3] and DNA,[4] and the recognition of hydroxylated 5,6-dihydrothymines as major products of DNA base damage due to ionizing radiation under aerobic conditions in bacteria[5] and human skin fibroblasts,[6] we propose a molecular mechanism for *in vivo* radiation mutagenesis. *Ionizing radiation produces hydroperoxides of pyrimidines (and purines?) in the DNA or metabolic pool of cells. The hydroperoxides may be stabilized by forming complexes*[7] *with transition metal ions in situ and transport to the*

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targets for chemical action which results in the modification of neighboring or other bases[8] *of the cell genome. Certain of these modified bases could cause base mispairing during replication leading to mutations.*

To determine whether a correlation of chemical reactivity with mutagenicity is possible for pyrimidine hydroperoxides, we compared the efficacies of 6-TOOH and 5-hydroperoxymethyluracil (α-hydroperoxythymine, α-TOOH), in the inactivation of *H. influenzae* transforming DNA. Surprisingly α-TOOH, which is about 10^3-fold less reactive chemically than 6-TOOH,[9] was approximately 10^3-fold more effective in the biological inactivation.[10] *Apparently, the crucial factor in producing radiobiological effects is not the extent of formation of a radiation product but the effectiveness of its action as a mutagen or carcinogen in a particular biological system.* In addition, we also tested the growth inhibitory effects or toxicity of seven ionizing radiation products of Thy derivatives on *Salmonella typhimurium* strain TA100 on agar plates in the presence of 6 mM histidine.[11,12] We found that the approximate I_{50} are 150, 80, 90, 80, and 80 nanomole/plate for the five hydroperoxy derivatives, α-TOOH, 6-TOOH, Thd-2, Thd-3, and Thd-4, respectively. In contrast, the approximate I_{50} is 2×10^5 nmole/plate for Thy glycol and 1.5×10^5 nmole/plate for Thd glycol. The several logs differences between the hydroperoxy derivatives and the glycols clearly indicate the toxicity of the former for *S. typhimurium* is of a high order.

These results indicate that chemical reactivity alone does not account for the observed variances, and it became of interest to assess the possible importance of stereochemical differences between the two types of hydroperoxides, i.e. the planar ring of α-TOOH vs. the half-chair conformation of 6-TOOH and other Thd hydroperoxides. To this end we utilized the *S. typhimurium* mutagenicity test[11,12] to determine whether mutations which could result from different steric interactions with DNA molecules were produced. Two strains of histidine requiring mutants of *S. typhimurium* were used, TA100 and TA98, sensitive to base pair substitutions and to frameshift mutation, respectively.

Table 1. Mutagenic activities of seven radiation products of thymine and thymidine on *S. typhimurium* strains TA100 and TA98 *in vitro* with (+) and without (-) added rat liver microsomal fraction (S_9).

Compound	max tested[a] (nmole)	Number of Revertants per nmole in Excess of Control[b]					
		TA100			TA98		
		$-S_9$	$+S_9$ Ar[c]	$+S_9$ Ph[c]	$-S_9$	$+S_9$ Ar[c]	$+S_9$ Ph[c]
α-TOOH	4	5.2	5.2	4.4	0.8	0.7	1.4
6-TOOH	3	9.3	2.9	0	1.4	0.8	1.4
Thd-2	0.4	15	48	0	0	0	0
Thd-3	7	3.2	8.8	0	0	0	0
Thd-4	0.4	40	0	33	0	0	17.5
Thy glycol	630	0	0.01	0.04	0	0	0
Thd glycol	3,700	0	0.02	0.03	0	0	0.05
Hycanthone[d]	15	65	65	N.D.	30	30	N.D.
2-Aminoanthracene[d]	6	20	80	N.D.	N.D.	80	N.D.
9-Aminoacridine[d]	200	6	10	N.D.	0.2	0.3	N.D.

[a]Maximum amount per plate tested.

[b]Number of spontaneous revertants averaged 165 and 24 for TA100 and TA98, respectively.

[c]Assays were carried out in the presence of S_9 fractions from either aroclor (Ar) or phenobarbital (Ph) treated rats.

[d]These standard mutagens were run concurrently with the radiation products. Hycanthone was a gift of Dr. S. Archer. 2-Aminoanthracene and 9-aminoacridine were kindly supplied by Dr. V. Dunkel.

Mutagenicity studies were conducted at concentrations which produced less than 5% growth inhibition. The results are summarized in Table 1. The five hydroperoxy derivatives had extremely high mutagenic activities ranging from 3.2 to 40 revertants per nmole detected with strain TA98. In general, the hydroperoxy derivatives required no metabolic activation (Table 1, columns 2 and 5) by the use of a microsomal/cytosal fraction, S_9, from the liver of rats

induced either with a polychlorinated biphenyl mixture (Aroclor 1254, columns 3 and 6) or phenobarbital (columns 4 and 7). It should be pointed out that the highest mutagenic activity of 48 revertants per nmole was exhibited by Thd-2 in the presence of Aroclor induced microsomes, a level approaching those of potent mutagens and carcinogens as hycanthone, 2-aminoanthracene, and 9-aminoacridine (Table 1). Under similar conditions, Thd-3 could also be metabolized to a somewhat more potent mutagen by S_9. Thd-4 had high mutagenic activities with strain TA100 in the absence of microsomes and with TA98 in the presence of microsomes induced by phenobarbital. Clearly, these hydroperoxy derivatives are mutagenic to *Salmonella* especially when compared with the extremely low (barely significant) activities of Thy and Thd glycols.

The above findings clearly show that hydroperoxy derivatives of Thy and Thd produced by γ-radiation are highly toxic and mutagenic. These compounds are also products of oxidation and photo-oxidation reactions of biological relevance. These reactions were used in the present study to prepare the desired products in sufficient amounts for biological testing and for their identification and characterization. As can be seen, we have taken advantage of the Milas reaction condition[13] to generate HO· and HOO· from HOOH under the influence of near-UV light to prepare sufficient quantities of 6-TOOH and α-TOOH, which have been identified as the major radiation products of Thy[2] and possibly DNA.[4] *cis*- and *trans*-Pyrimidine glycols were also prepared by the same procedure.[14] However, irradiation of Thd and HOOH with near UV light gave glycols and hydroperoxides of Thd used in these experiments were obtained by peroxidation of the corresponding Thy glycol.[15] A solution of 3 ml of 50% HOOH containing 20 mg of the glycol and 50 µl of conc. HCl was stirred for 3 hr at room temperature. After concentration the mixture was separated by preparative tlc [Merck 60F-254 silica gel, ethyl acetate—2-propanol—H_2O(75:16:9)]. The hydroperoxides were located by KI-saturated MeOH spray, extracted from the chromatograms with abs. MeOH and purified by rechromatography. *trans*-Thd Glycol yielded two isomers tentatively identified as *trans*-5,6-dihydro-6-

hydroperoxy-5-hydroxythymidine (*trans*-ho$_2^6$ho^{5}hThd) with R_f 0.48 (Thd-1) and R_f 0.69 (Thd-2) and the *cis*-glycol gave two isomers tentatively identified as *cis*-ho$_2^6$ho^{6}hThd with R_f 0.53 (Thd-3) and R_f 0.64 (Thd-4), respectively. These products correspond to those formed by γ-radiation of Thd in aerated aqueous solution.[3]

In conclusion, using a bacterial test system we have shown that hydroperoxy derivatives of Thy and Thd produced by ionizing radiation, photoreactions, or oxidation reactions are *highly* mutagenic. If this is a reflection of the general toxicity of these compounds in man, radiation products as well as certain oxidation products should be considered potential human health hazards and should definitely be screened when they can be isolated, purified, and synthesized. Such information would be of value in elucidating the molecular basis of mutations induced by oxidation products.

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7. *Cu^{++} was found not to facilitate radical formation but rather to form a stable complex with 6-TOOH. Thus, increasing the lifetime of the reactive 6-TOOH relative to its decomposition in a biological system renders it more effective as a mutagen.* This suggestion was based on two observations. First, electron-spin-resonance spectra were not detected with 6-TOOH and 6-TOOH and Cu^{++} complexes in the absence or presence of t-nitrosobutane, a spin-trap. Second, stability of the complexes was clearly indicated when the synthetic [2-^{14}C]-6-TOOH and Cu^{++} complexes were applied on paper or thin-layer plates for chromatography.
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15. Thd glycols were prepared according to the method of S. Iida and H. Hayatsu (1971) Biochim. Biophys. Acta 228, 1-8.