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REACTION OF BROMOTRICHLOROMETHANE DERIVED FREE RADICALS WITH URACIL IN A MODEL SYSTEM. STRUCTURES OF PRODUCTS FORMED

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Free radicals generated by benzoyl peroxide-mediated catalytic decomposition of bromotrichloromethane (eg. trichloromethyl) were allowed to react under nitrogen or under air with uracil. Under nitrogen two reaction products were formed, one was identified as 5-chlorouracil and the other as a 5-bromouracil. Under air, besides the above two products other nine were also formed: 5,6-dihydrouracil; 5-hydroxyuracil; a chlorohydroxy adduct of uracil; a bromohydroxy derivative of uracil having the 5,6 bond in the saturated form; other bromohydroxy derivative of uracil having the double bond intact; 5,6-dihydroxyuracil; two dihalogenated hydroxylated uracil derivatives and one peak we were not able to descipher its structure. No single reaction product formed had carbon centered radicals (eg. trichloromethyl) added from $CBrCl_3$ and consequently would be missed in 'in vivo' covalent binding studies where ^{14}C haloalkane ($CBrCl_3$ or carbon tetrachloride) were employed. If similar reaction products resulted during interaction of $CBrCl_3$ reactive metabolites with uracil in RNAs, significant deleterious effects in their function would be expected. That possibility, however, remains to be established.

KEY WORDS: uracil; trichloromethyl; trichloromethylperoxyl; free radicals; carbon tetrachloride; RNA.

INTRODUCTION

Carbon tetrachloride and bromotrichloromethane are two haloalkanes whose liver toxicity is believed to be related to their biotransformation to trichloromethyl ($\cdot CCl_3$) and trichloromethylperoxyl ($CCl_3O_2\cdot$) free radicals[1–6]. Both free radicals would participate in addition, hydrogen abstraction and other reactions with key cellular constituents to initiate the molecular events ending in liver cell damage[1–6]. Most workers in the field believe, that the covalent binding (CB) of the $\cdot CCl_3$ to cellular constituents and the lipid peroxidation (LP) sparked by the H abstraction from polyunsaturated fatty acids (PUFA), are the critical determinants of these haloalkanes hepatotoxicity[1–6].

There is abundant and detailed information about the reaction products arising during LP and about the interaction of them with cellular components. Many thorough reviews on the subject are available about it[7–12]. No equivalent detailed studies exist about the nature of the reaction products produced when both, $\cdot CCl_3$ and the $CCl_3O_2\cdot$

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interact with protein constituents or DNA components, despite that these target molecules might potentially be at least equally relevant to toxicity than membrane lipids[13]. Recent efforts from others and from our laboratory were directed to fill that gap in the knowledge [14–21].

In the present work, we describe our studies on the chemistry of the interaction between the $\cdot CCl_3$ and the $CCl_3O_2\cdot$ with the RNA base uracil. These studies are needed for future work in biologically relevant systems.

MATERIALS AND METHODS

Chemicals

$CBrCl_3$ was purchased from Aldrich and distilled before use. The original commercial product contained several additives obscuring the GC/MS analysis. Uracil and benzoyl peroxide were purchased from Sigma. The benzoyl peroxide contains about 30% water as stabilizer. The purity of uracil was checked by GC/MS and no detectable amounts of any of the reaction products was originally present in the uracil itself.

Procedures

The study of the 'in vitro' chemical interaction of $CBrCl_3$ with uracil was carried out as follows: 0.2 mg of the base plus 3 ml of $CBrCl_3$ were heated at 100°C for 5 hr with 1 mg of benzoyl peroxide in an ampoule. Reaction mixture and ampoule were purged with either nitrogen or air as indicated in each experiment before sealing. At the end of this period, the solvent was evaporated under nitrogen at 40°C. The residue was then silylated with a mixture of BSTFA: acetonitrile (1:1); 0.2 ml at 100°C for 30 min.

Blanks without benzoyl peroxide or without uracil were run simultaneously. It is not possible to run blanks without $CBrCl_3$ for it is the only solvent of reaction mixture. Further, it is not possible to run the reaction in solvents other than $CBrCl_3$ or CCl_4 (eg. ethanol, glyme, acetone and others) for either they do not dissolve uracil even in the trace amounts necessary for MS detection or they generate themselves free radicals which obscure identification by production of many artifacts. We selected $CBrCl_3$ due to its inherent better reactivity than that of CCl_4.

Total ion chromatogram (TIC) analysis and mass spectrometric identification of reaction products were performed in a Hewlett Packard Model 5970 B mass selective detector interfaced to HP 5890 gas chromatograph. Chromatographic conditions were as follows: injection port temperature 250°C in the splitless injection mode. Separation was carried out in a fused silica capillary column (12 m×0.2 id) crosslinked with 5% phenyl methyl silicone (0.33 um film thickness) (Hewlett Packard, Palo Alto, Ca.), carrier gas: helium (column head pressure, 100 KPa). Column temperature was maintained at 100°C for 1 min and then increased to 280°C at a ramp velocity of 10°C/min. GC/MS interface temperature was 280°C and ion source was ca. 200°C. Spectra was taken at 70 eV scanning quadrupole from 50 to 750 amu, at 0.61 sec/scan. It is important to emphasize that we had to rely entirely on MS for identification because the very low product yield from the reaction mixture under experimental conditions employed, prevented the use of additional NMR methods to further elucidate the structures.

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RESULTS

Interaction of bromotrichloromethane with uracil under anaerobic conditions

The capillary chromatographic analysis with TIC detection of reaction products when $CBrCl_3$ attacks uracil in the presence of benzoyl peroxide under N_2 atmosphere is showed in Fig. 1. Two peaks corresponding to the reaction products were observed. Peak I (Fig. 2) was identified as 5-chlorouracil. The position of chlorine was elucidated by the mass shift for fragment at m/z 99 reported to be in the spectrum of bis (trimethylsilyl) uracil involving C-4 and C-5 in the pyrimidine ring[22]. Following similar considerations the mass spectrum of peak II, shown in Fig. 3, indicated the presence of bromine on the carbon-5 of the derivatized uracil ring. In the absence of the catalyst, most of the uracil remained unreacted. We observed only peaks I and II in a very low amount.

Interaction of bromotrichloromethane with uracil under aerobic conditions

When the above described reaction was performed under air and in the presence of the catalyst, not only a different pattern of reaction products was obtained, as revealed by the chromatographic analysis (Fig. 4). Yield of reaction products formed under nitrogen (peaks I and II) was also dramatically changed. The lack of adequate non isotopical or deuterated forms of standards did not allow precise quantification of differences in both cases. In addition, nine new products were detected. Peak III was identified as the bis (trimethylsilyl) derivative of 5,6-dihydrouracil. Its presence was confirmed by the injection of a pure sample. Peak IV corresponded to 5-hydroxyuracil,

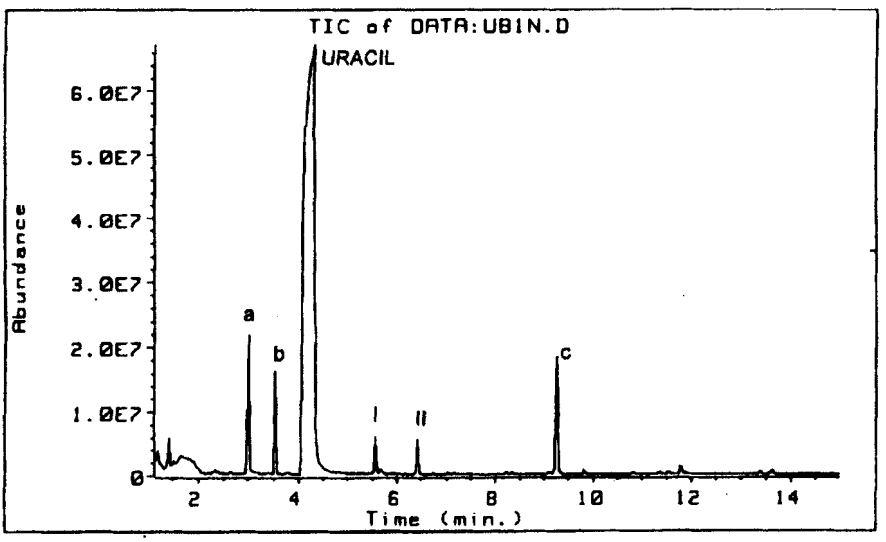

FIGURE 1 Gas chromatogram obtained from a sample of the reaction mixture between uracil and bromotrichloromethane in the presence of benzoyl peroxide and under nitrogen atmosphere, after trimethylsilylation. Column, fused silica capillary crosslinked with 5% phenylmethyl silicone, programmed at 10°C/min, from 100°C to 280°C. Unreacted base peak is denoted as uracil and peaks a to c represent reaction products derived from benzoyl peroxide: a, benzoic acid TMS ester; b, diphenyl ether and c, a phtalic acid bis-TMS ester. Only two peaks arising from the base were observed, I and II. See methods for other details.

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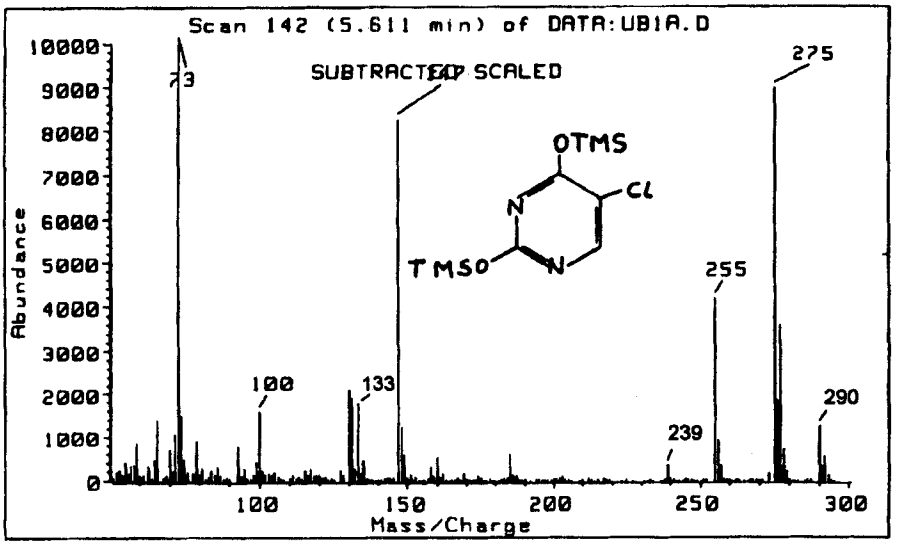

FIGURE 2 Mass spectrum taken from peak I in Fig. 1. $M^{+\cdot}$ appeared at m/z 290/292 and $(M\text{-}15)^+$ at m/z 275/277. Other masses were m/z 255 $((M\text{-}Cl)^+)$, m/z 239 $((M\text{-}15\text{-}HCl)^+)$, m/z 133/135 ($Me_2SiO^+C{\equiv}CCl$) and m/z 100 ($Me_2SiO^+C{\equiv}N$).

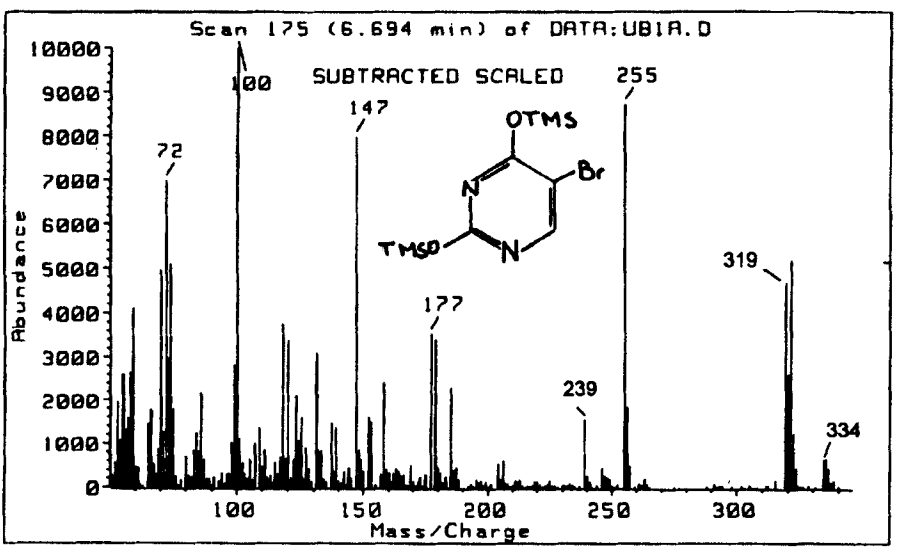

FIGURE 3 Mass spectrum taken from peak II in Fig. 1 and $M^{+\cdot}$ and $(M\text{-}15)^+$ were found at m/z 334/336 and m/z 319/321 respectively. Other important peaks were m/z 255 $((M\text{-}Br)^+)$, m/z 239 $((M\text{-}15\text{-}HBr)^+)$ and m/z 177/179 ($Me_2SiO^+C{\equiv}CBr$).

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which was also shown by others to be a radiation induced hydroxyl adduct of cytosine[23]. It was not possible to identify peak V whose mass spectrum is shown in Fig. 5. In effect, on one hand molecular mass would be at m/z 346 as it would correspond to the TMS derivative of 5-hydroxy-5,6-dihydrouracil. However, fragmentation pattern did not agree with that reported for that compound by Dizdaroglu[24]. On the other hand, the corresponding 6-hydroxy isomer was reported to be unstable in hot acid solutions[24], and probably it could not resist the silylation step. Peak VI, whose spectrum is shown in Fig. 6, corresponded to a chloro hydroxy adduct of uracil. It was not possible from the spectrum to determine the absolute position of substituents, though it was almost identical to the one reported in a previous work from our laboratory for the case of cytosine[19]. Next peak was identified as a bromohydroxy derivative of uracil. Molecular mass indicated that 5,6-double bond would be saturated (peak VII, Fig. 7). When this double bond was conserved through the reaction, a significantly different spectrum was obtained. In effect, peak VIII (spectrum in Fig. 8) corresponded to an bromo hydroxyuracil adduct, whose new hydroxyl substituent was underivatized. We inferred that in this case, planar configuration of the molecule probably avoided silylation by the presence of a bulky bromine close to the hydroxyl group. Peak IX resulted to be a dihydroxylated adduct of the base. We observed it previously as a hydroxyl radical-induced product of cytosine in deaminated form[19]. In addition, this compound was reported to be a radiation-induced product of cytosine[23]. Two last peaks corresponded to dihalogenated hydroxylated derivatives of uracil, both of them showing to have 5,6-bond as a saturated one. Peak X (Fig. 9) was identified as a bromo chloro adduct. Peak XI (Fig. 10) corresponded to the dibrominated analog. In both cases the hydroxyl group was silylated. This peak was overlapped with at least two other major peaks not possible to separate under the different chromatographic conditions tested. In a diluted sample we were able to observe three major components having closely related spectra, so we concluded that they might be isomeric structures of dibromo hydroxyuracil.

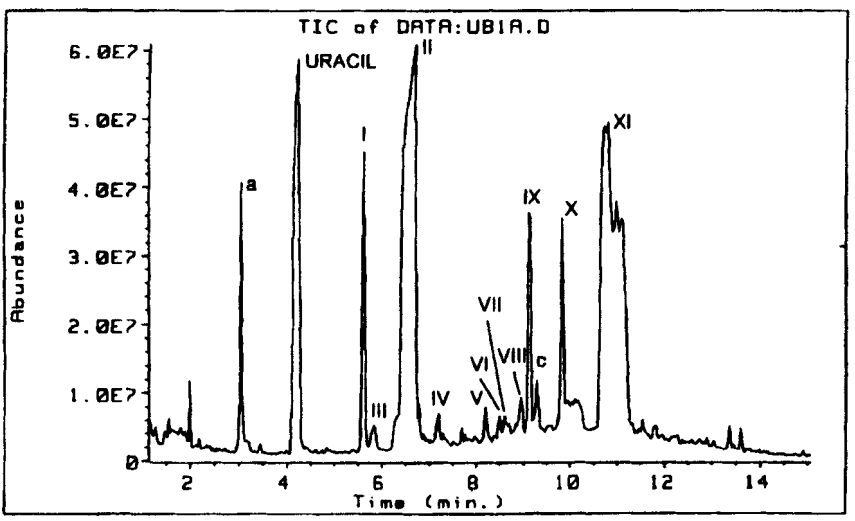

FIGURE 4 Gas chromatogram obtained from a sample of the reaction mixture between uracil and bromotrichlorometane under air atmosphere and in the presence of benzoyl peroxide. See Methods and Fig. 1 for other details.

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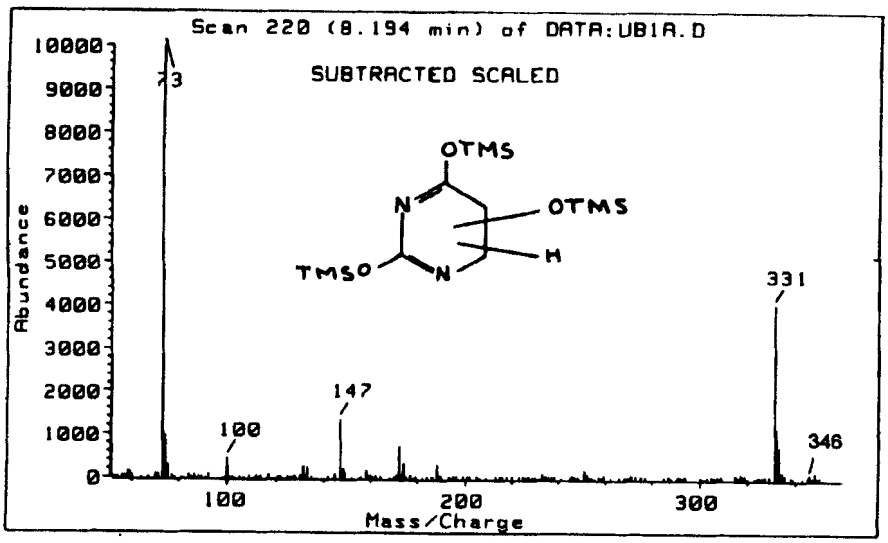

FIGURE 5 Mass spectrum of unknown peak V in Fig. 4.

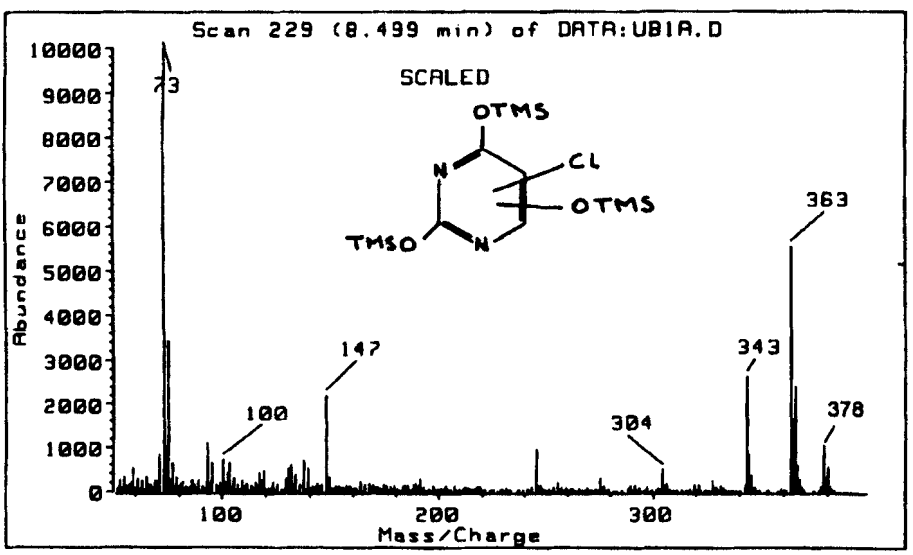

FIGURE 6 Mass spectrum taken from peak VI in Fig. 4. $M^{+\cdot}$ appeared at m/z 378/380 and $(M\text{-}15)^{+}$ at m/z 363/365 was the base peak. Other relevant fragments were m/z 343 ($(M\text{-}Cl)^{+}$) and m/z 304/306 ($(M\text{-}TMSH)^{+\cdot}$).

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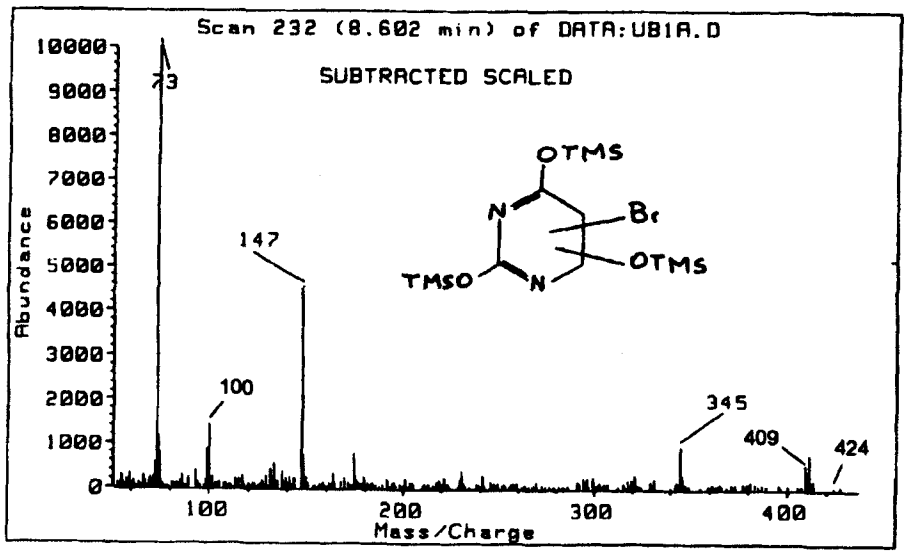

FIGURE 7 Mass spectrum taken from peak VII in Fig. 4. $M^{+\cdot}$ was found at m/z 424/426 and confirmed by m/z 409/411 $((M-15)^{+})$. The loss of bromine leads to fragment at m/z 345.

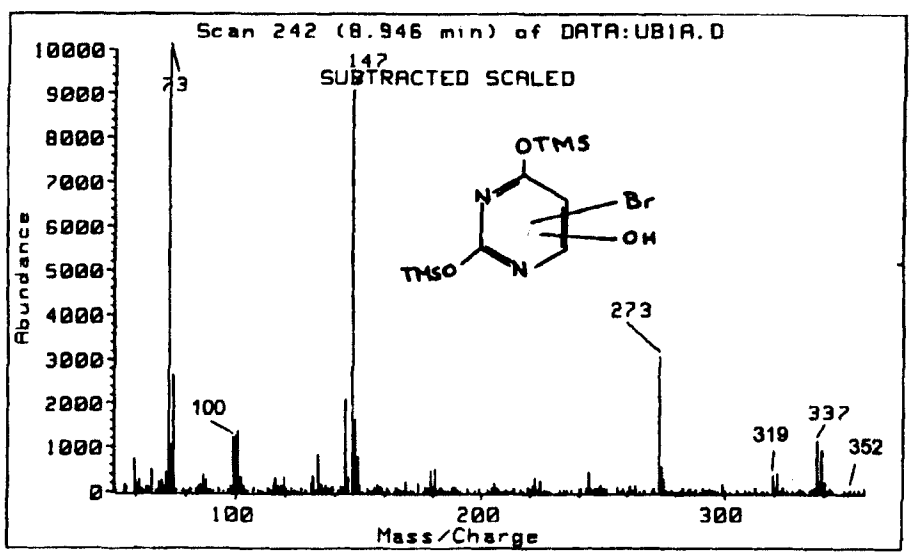

FIGURE 8 Mass spectrum taken from peak VIII in Fig. 4. $M^{+\cdot}$ and $(M-15)^{+}$ were found at m/z 352/354 and m/z 337/339 respectively. Other fragments were m/z 319/321 (loss of water from $(M-15)^{+}$), and m/z 273 $((M-Br)^{+})$.

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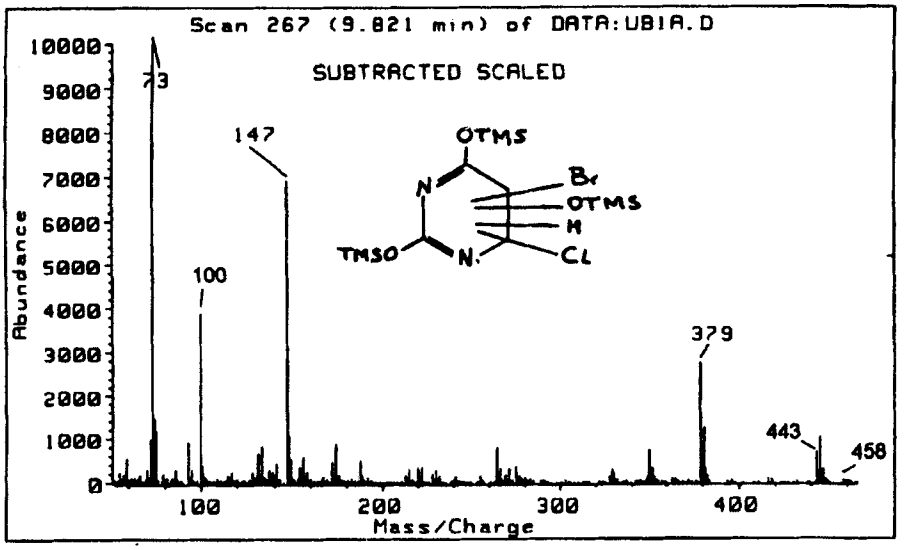

FIGURE 9 Mass spectrum taken from peak X in Fig. 4. $M^{+\cdot}$ appeared at m/z 458/460/462 and $(M-15)^{+}$ at m/z 443/445/447. Other fragment corresponded to the loss of bromine from $M^{+\cdot}$ (m/z 379/381).

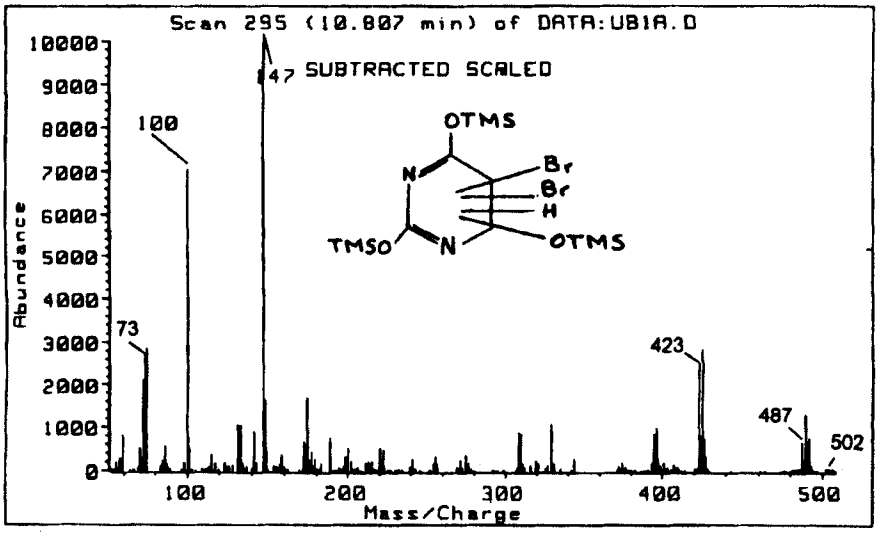

FIGURE 10 Mass spectrum taken from peak XI in Fig. 4. $M^{+\cdot}$ appeared at m/z 502/504/506 and $(M-15)^{+}$ at m/z 487/489/491. Other fragment corresponded to the loss of bromine from $M^{+\cdot}$ (m/z 423/425).

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When benzoyl peroxide was omitted from the reaction mixture, most of the base remained unreacted and only six of the above reported adducts were observed (peaks I, II, IV, VII, VIII and IX).

DISCUSSION

Our here described results indicate that in a chemical model system free radicals generated from $CBrCl_3$ were able to interact with uracil to give at least eleven reaction products. Reaction under oxygen was far more intense than under anaerobic conditions. No single reaction product formed contained C from $CBrCl_3$. This is important, for if similar reaction products to those here reported were formed in uracils during CCl_4 or $CBrCl_3$ poisoning, they would be missed during the usual experiments we and other toxicologists performed in the past, to determine the so called covalent binding and using the ^{14}C-labeled haloalkane[1–4]. The formation of hydroxylated uracil derivatives might be of some relevance under aqueous in vivo conditions. Production of monohalogenated uracil adducts in contrast, might not be likely to occur under biological conditions, where activation of $CBrCl_3$ to $\cdot CCl_3$ would be non homolytic and lead to the halide anion rather than to the halogen radical[1–4].

Concerning the possible mechanisms of formation of the obtained products we envisaged as a likely possibility the following: a) Under anaerobic conditions the process would be initiated by a hydrogen abstraction at the C-5 position of the uracil moiety, mediated by eg. phenyl radicals (formed by thermal decomposition of benzoyl peroxide) or by free radicals arisen from the solvent (in fact, reaction is still observed in the absence of the peroxide). The resulting free radical in the target molecule would then abstract a halogen atom from the $CBrCl_3$ solvent to give I and II products. b) Under aerobic conditions, in contrast, initiation would possibly proceed by a $CCl_3O_2\cdot$. mediated H abstraction from uracil to give uracil radicals. Target molecule radicals would preferentially react with O_2 to produce hydroperoxy intermediates. These intermediate forms would decompose to produce the hydroxy groups reported in the products. The halogenation of those uracils would result from the interaction between the isomeric free radical form of the hydroxy uracils with the solvent. The proposed mechanism is depicted in Fig. 11.

Considering the potential biological significance of the here reported reactions between the $\cdot CCl_3$ and the $CCl_3O_2\cdot$ and uracil moieties, only speculations can be made at present. However, if both radicals were formed nearby uracil-containing components it would not be unexpected that at least part of the alterations here described took place. It is known that uracil is present in the different RNAs instead of the thymine in DNA[25].

Three kinds of RNA were described: messenger RNA (m-RNA); transfer RNA (t-RNA) and ribosomal RNA (r-RNA)[25].

RNA synthesis in a non biologically active form occurs in the nucleus and the protein synthesis process using the active m-RNA; t-RNA and r-RNA is located in the rough endoplasmic reticulum (RER)[25].

Previous studies from our laboratory evidenced that CCl_4 is activated to reactive metabolites, presumably $\cdot CCl_3$ in the liver nuclei[26–28] and also in the liver RER and SER components of the endoplasmic reticulum[29] and consequently both, exposure of uridine moieties from precursor inactive RNAs in the nucleus or of active RNAs in the RER is quite possible. Whether some of these alterations are involved in the very well known deleterious effects of CCl_4 on the liver protein synthesis process[29–36] remains to be established.

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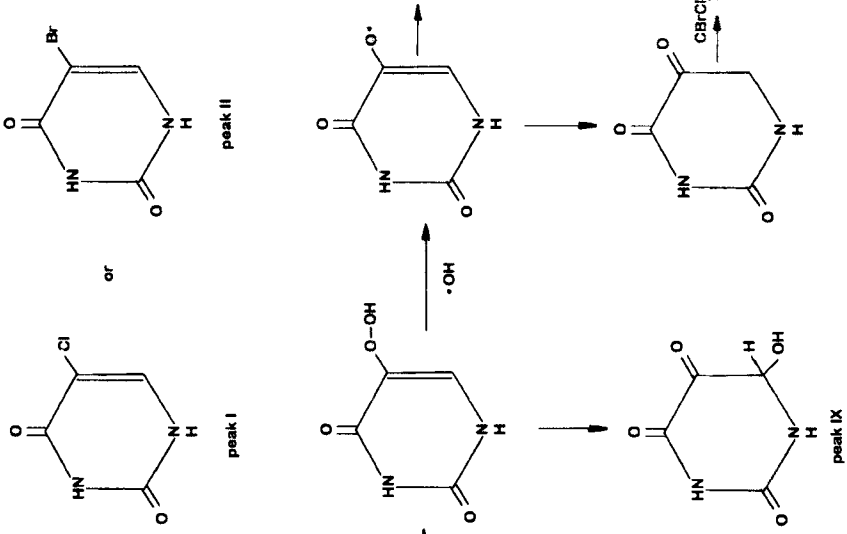

FIGURE 11 A proposed mechanism for the formation of most of the reaction products observed when bromotrichloromethane derived free radicals react with uracil in the model system. X· represents a free radical initiating moiety present in reaction mixture, in the absence (eg. phenyl or solvent derived radicals) or presence (eg. CCl_3O_2·) of oxygen.

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