# THE EFFECTS OF REPEATED $^{14}C$ HALOTHANE EXPOSURE IN MICE

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THE ANAESTHETIC ADMINISTRATION of $^{14}C$ labelled halothane has been shown to cause non-volatile halothane metabolites to accumulate in the liver of mice.[1] These labelled halothane metabolites are bound to liver microsomes in proportion to liver blood flow, hepatic arterial oxygen tension and enzyme induction.[2] Cohen[3] showed that repeated weekly injections of labelled halothane caused non-volatile metabolites to accumulate and increase in the liver suggesting stimulation of enzyme induction. In contrast, Topham and Longshaw[4] showed that repeated daily inhalations of labelled halothane produced no accumulation of non-volatile metabolites using whole body auto-radiography techniques. Stevens and co-workers[5] reported that constant subanaesthetic inhalations of halothane for 35 days caused reproducible dose dependent liver toxicity. This could be related to Sawyer's[6] work which showed optimum halothane metabolism at 1/100 MAC. It was hypothesized that increasing halothane concentrations depressed the necessary enzyme systems for halothane metabolism and that optimum metabolism may only be present in the post-anaesthetic period.

In an attempt to clarify the divergent results of other workers, our study measured metabolism of halothane with chronic repeated exposures, determined the specific site of metabolism and assessed possible hepatotoxicity by light and electron microscopy.

## METHODS

Sixty Swiss Webster mice weighing approximately 20 gm were enclosed in an air-tight chamber of known gas volume. An initial chamber concentration of 42 per cent oxygen was required due to the high metabolic rate for mice. Twenty millicuries of $^{14}$carbon halothane were mixed with 6 ml of unlabelled halothane and vapourized by means of a cotton wick and circulating fan. This resulted in a constant chamber concentration of 0.7 per cent to 0.8 per cent halothane measured with a Draeger Narcomed halothane analyzer. Soda lime was placed on the chamber floor to absorb carbon dioxide. Sampling sites for determination of oxygen and carbon dioxide percentage assessed the adequacy of maintenance of normal values. After four hours of exposure the chamber was opened in a fume hood and vented to the atmosphere to remove volatile halothane. The animals were allowed to recover before removal to their usual cages. Figure 1 illustrates the methodology used in this study.

Three mice exposed to halothane and one control mouse were decapitated in five groups on days 1, 5, 8, 11 and 14. The livers were excised rapidly, irrigated to

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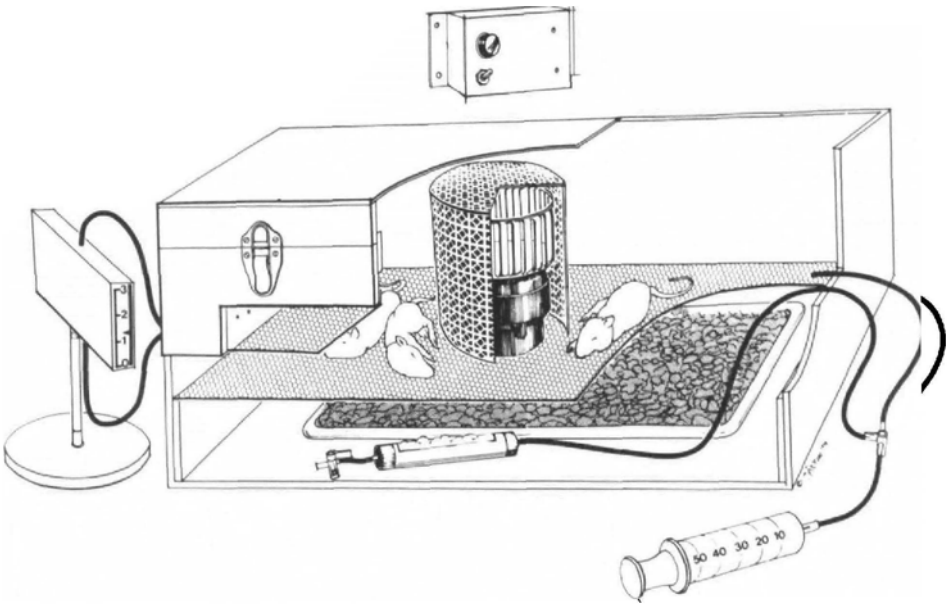

FIGURE 1. A representation of the experimental equipment is illustrated.

remove blood and minced with scissors. The liver was homogenized with a teflon pestle in a pre-cooled grinding vessel to which 5 ml of 0.001 M sodium bicarbonate (pH 7.5) was added. The homogenate was transferred to a refrigerated centrifuge pre-cooled to 4°C. It was spun twice at 2000 r.p.m. for ten minutes to remove cellular debris which was discarded after a radioactivity count. The supernatant was centrifuged three times for 30 minutes at 8600 rpm to separate the mitochondrial pellet. After the first 30-minute spin the microsomal supernatant was decanted from the mitochondrial pellet and spun at 36,000 rpm for one hour to obtain the microsomes. The methods used were a modification of two reported techniques.[7,8] After centrifugation, the pellets were placed in scintillation vials with 50 ml of water and 7 ml of the tissue solubilizer, Aquasol. All samples were counted by a liquid scintillation process using a Nuclear Chicago Isocap 300 system. Isotope radiation after amplification was converted to an electronic pulse which was registered as counts per minute. Each sample was counted for one minute, after we were satisfied that longer counting times brought no significant change in counts per minute.

Periodically during our study, liver samples to be centrifuged were taken from halothane and control animals for light and electron microscopy. Tiny samples of microsomal and mitochondrial pellets were also obtained for electron microscopy to verify our technique and determine any possible subcellular liver damage.

At day 14 after the first exposure, the remaining 45 mice were exposed a second time to 15 $\mu$Ci of $^{14}C$ halothane under the same conditions for the same time-period. After recovery, three animals and one control were sacrificed every two or three days in five groups. The livers were removed, homogenized, centrifuged, examined by electron microscopy and the radioactivity counted as previously described. On day 28, the remaining 30 mice were subjected to a third exposure

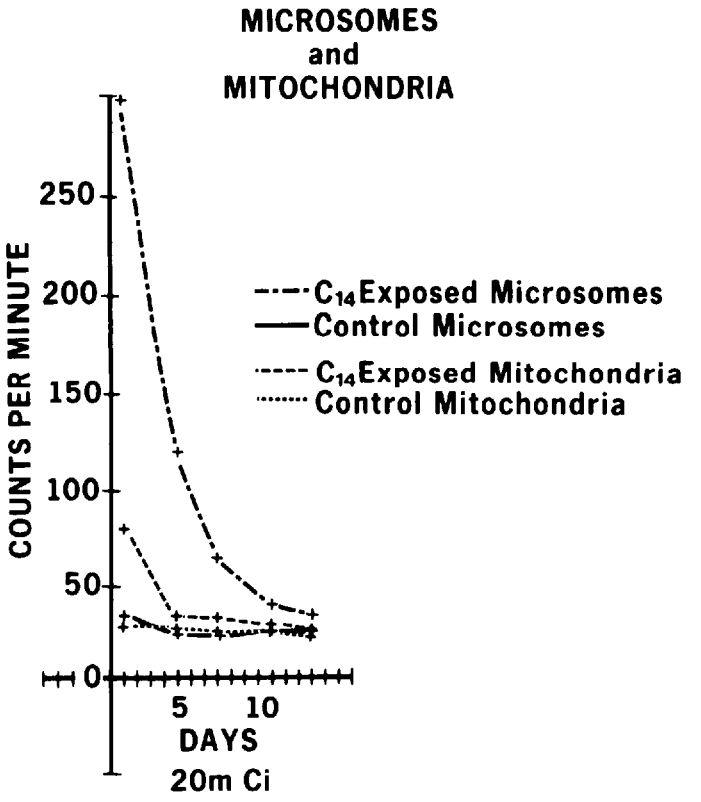


FIGURE 2. The effects of $^{14}C$ halothane exposure on microsomes and mitochondria are documented in exposed and control mice.

of 10 Ci of $^{14}C$ halothane as described before, followed by the sacrifice of 15 animals and controls with liver removal, centrifugation, microscopy and counting. On day 42 the last 15 mice received a fourth exposure to 5 $\mu$Ci of labelled halothane and at the end of two weeks the experiment was completed, by the usual sacrifice, liver removal, centrifugation, examination and counting for both exposure and control mice.

## RESULTS

Figure 2 illustrates that radioactive metabolites of halothane are present primarily in microsomes and secondly in mitochondria. The microsome counts per minute show a four-fold increase in concentration as compared to the mitochondria. The obtained results added confirmation to the work of Van Dyke[2] and Cohen.[11]

The results of repeated exposures to 20, 15, 10 and 5 millicuries of labelled halothane are shown in Figure 3. The similarity of the exponential washout curves with repeated exposures was verified mathematically by statistical curve analysis and time-constant determination to show good correlation. Statistical curve evaluations indicated that individual microsomal metabolic activity remained constant with repeated exposures. Time-constant analysis suggested that enzyme induction

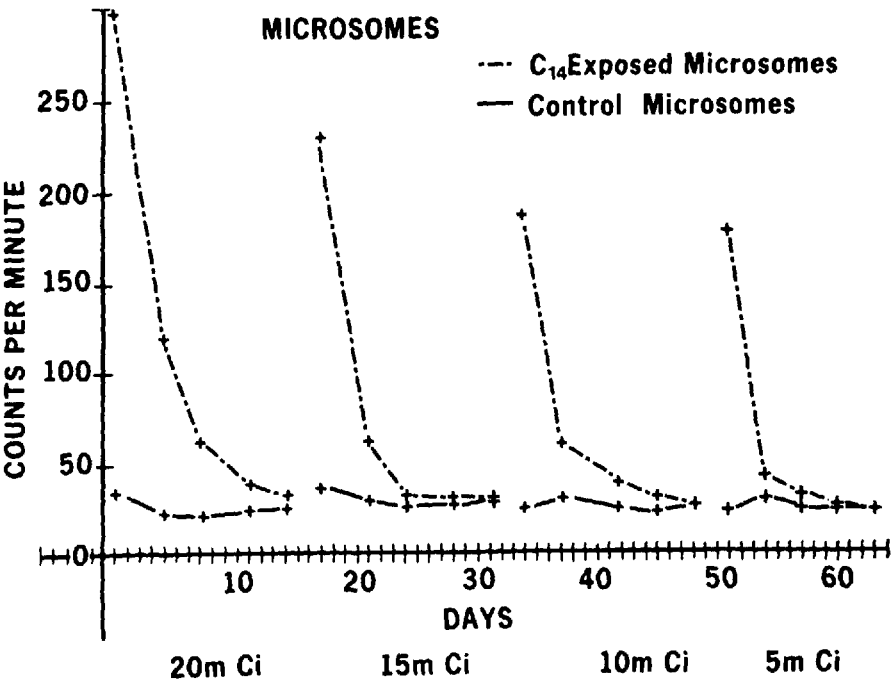


FIGURE 3. The concentrations of metabolites in microsomes are shown for repeated exposures with $^{14}C$.

was occurring. Another feature of this graph is the apparent decrease in metabolite concentration with subsequent exposures. This is artifactual because the dose of labelled halothane was concommitantly reduced. Decreasing halothane exposure doses (20, 15, 10 and 5 $\mu$Ci's) were used in favour of 20 millicuries halothane doses to alleviate possible rising metabolite counts with enzyme induction, possible tissue toxicity with repeated radiation exposure and cost of labelled halothane.

If counts per minute from labelled halothane are converted to 20 millicuries instead of 20, 15, 10 and 5 millicuries, Figure 4 is the result. There is a corrected marked increase in metabolite concentration with repeated exposures. This postulates the presence of either more metabolic sites, becoming available through enzyme induction, or metabolism and excretion beginning earlier with repeated exposures. The variability between metabolite formation and urinary excretion shown by Rehder, *et al.*,[12] Cascorbi, *et al.*,[13] and Cohen, *et al.*[14] tends to oppose the latter theory. Time-constant determinations in our study not only suggest enzyme induction but favour this theory. Our results showed one time-constant was 6.02 days after the first exposure and had fallen to 4.80 days after the fourth exposure. These data suggest a significant increase in functioning microsomes to account for our results.

Mitochondrial studies with repeated exposures show two significant features when corrected metabolite counts are compared. Figure 4 illustrates that statistical curve analysis was similar with four exposures. Mitochondria differ from microsomes in their markedly reduced metabolite count. The significant feature is their

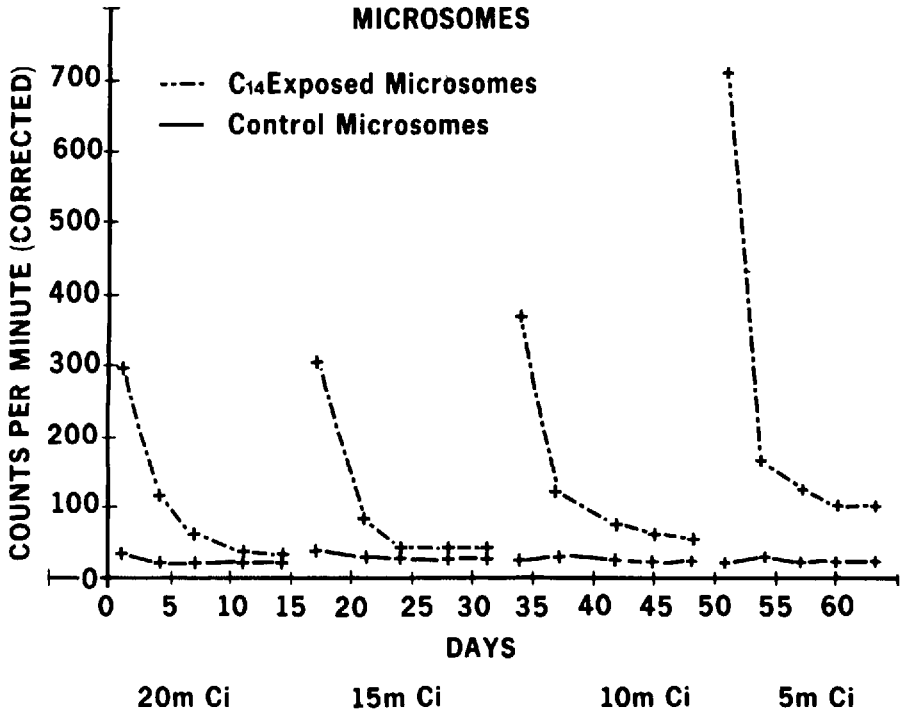


FIGURE 4. Assuming all mice had been subjected to 20 mCi halothane, the corrected concentration of microsomal metabolites is shown with repeated exposures.

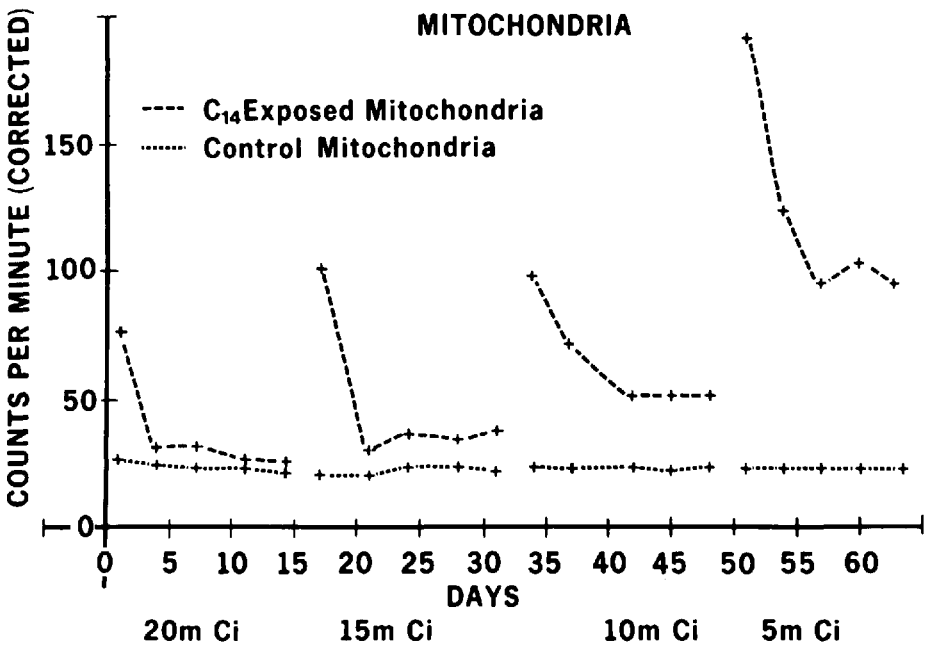


FIGURE 5. The corrected concentrations of mitochondrial metabolites reveal reduced metabolite levels and delayed washouts when compared to Figure 4.

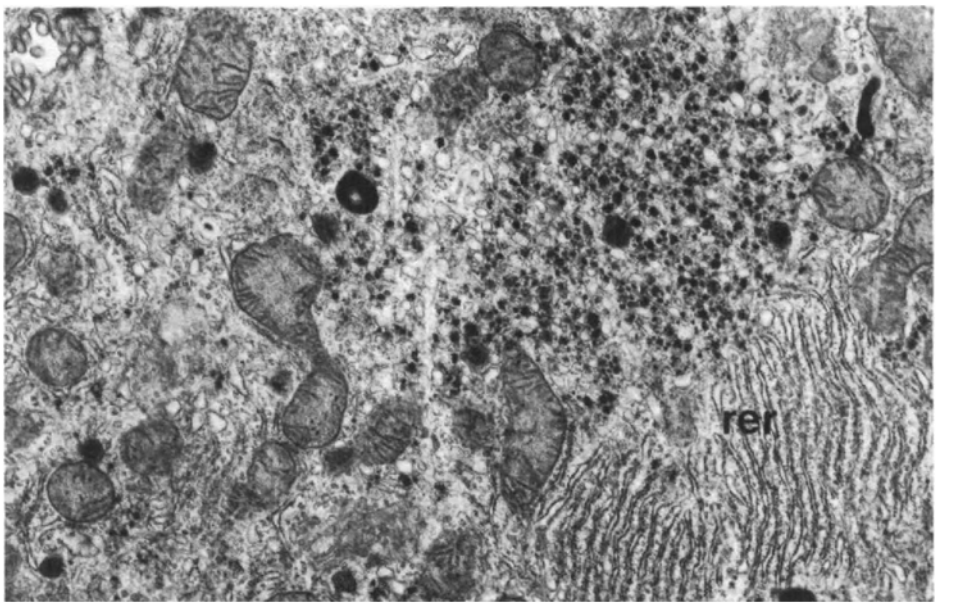


FIGURE 6. An electron micrograph from a control mouse magnified 13,000 times is illustrated as a norm. rer = rough endoplasmic reticulum.

prolonged rate of clearance, as manifested by time constants of 14.83 days after four exposures. This value is approximately 2.5 times the microsome value and explains the apparent increase in mitochondrial metabolites with repeated exposures.

Since metabolite concentrations do not appear to increase in microsomes with repeated exposures, but may increase in the mitochondria, it was necessary to examine liver tissue to determine a possible association of metabolites and hepatotoxicity. Liver tissue from exposed and control animals was examined by light and electron microscopy. Light microscopy revealed that control animals had mixed neutrophilic and lymphocytic infiltration present focally around some hepatic veins and portal tracts. In mice subjected to repeated exposures, occasional focal necrosis, less than one per lobule, was centrolobular in distribution with mixed neutrophilic and lymphocytic infiltrates. No degenerative changes such as increased eosinophilia, vacuolation of cytoplasm or swelling or balling of liver cells were observed. Therefore, light microscopy revealed no significant evidence of hepatotoxicity in exposed or control animals.

Figure 6 shows an electron micrograph of a portion of a hepatocyte from a control mouse, magnified 13,000 times. The rough endoplasmic reticulum with granules of ribosomes attached is labelled and appears in parallel arrays. The smooth endoplasmic reticulum appears as vesicles with interstitial glycogen. Mitochondria with lamellar cristae are shown. This sample photomicrograph is compared with Figure 7 which documents portions of three hepatocytes from a mouse subjected to four halothane exposures. At a magnification of 11,000 times, the rough and smooth endoplasmic reticulum are unchanged. A normal bile canaliculus is shown. The mitochondria show no abnormalities. From these elec-

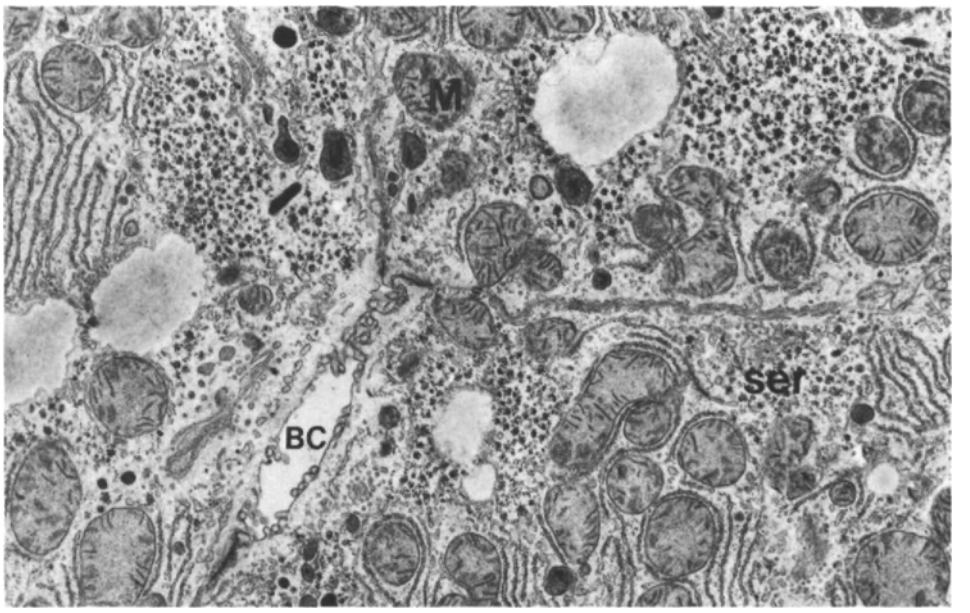


FIGURE 7. A sample micrograph from 1 of 15 mice subjected to 4 halothane exposures shows no abnormalities and is comparable to Figure 6. BC = bile canaliculus, SER = smooth endoplasmic reticulum.

tron microscopy studies, it was concluded that evidence for liver toxicity was not apparent. In regards to enzyme induction, no marked evidence for hypertrophy or alteration of the endoplasmic reticulum was shown. However, this diagnosis would require morphometric analysis and multiple electron micrographs to declare accurately that enzyme induction was present. Although others claim this assessment is relatively easy,[15] our pathology department was unable to substantiate this claim.

## CONCLUSIONS

Several conclusions were drawn from our study. First, the primary site for accumulation of non-volatile halothane metabolites is in the microsomes. A smaller less significant radioactivity is present in the mitochondria. The repeated exposures in our study did not allow complete clearance of metabolites from mitochondria because of the longer time-constants. Secondly, repeated chronic exposures to labelled halothane produced the significant finding of increased concentrations of metabolites which are contrary to the findings of Topham and Longshaw.[4] The basis of these results is postulated to be increased metabolism by enzyme induction. Supportive evidence was obtained from exponential curve analysis and time-constant determinations. Thirdly, the results of light and electron microscopy showed no significant evidence of hepatotoxicity in any liver section submitted to pathology assessment. Our final conclusion was drawn on the basis of all these results. Despite the accumulation of metabolites, primarily in the microsomes, there is no evidence of liver toxicity in mice subjected to repeated chronic anaesthetic exposures to halothane.

The entity of so-called "halothane hepatitis" in humans has been postulated to occur after repeated exposures,[16] enzyme induction with higher metabolic rates, abnormalities in enzyme metabolism, failure to excrete toxic metabolites or hypersensitivity reactions. This study now provides clues to the rational use of halothane anaesthesia. In our study repeated exposures, with probable enzyme induction and normal removal of halothane metabolites from the liver, showed no incidence of halothane hepatotoxicity in mice. This suggests that a hypersensitivity phenomenon or a secondary inducing agent is the culprit in liver toxicity. In the case of a hypersensitivity or immune response, the low incidence of toxicity does not justify the abolition of halothane as an extremely useful inhalation agent. In regards to inducing agents, polychlorobiphenyl has been shown to be a potent agent in the presence of halothane to cause lipid peroxidation and liver disease in mice.[15] The actual association in humans has not been shown but the link may have been forged prior to our present state of knowledge. On the basis of all this knowledge it can be said that the enigma of "halothane hepatitis" continues to enslave the enlightened and confound the foolish.

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