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Interaction of Trichloroethane Isomers with Cytochrome *P*-450 in the Perfused Rat Liver

TAKEHITO TAKANO, YOSHIFUMI MIYAZAKI, AND YUTAKA MOTOHASHI

Department of Hygiene, School of Medicine, Tokyo Medical and Dental University, Tokyo 113, Japan

Interaction of Trichloroethane Isomers with Cytochrome *P*-450 in the Perfused Rat Liver. TAKANO, T., MIYAZAKI, Y., and MOTOHASHI, Y. (1985). *Fundam. Appl. Toxicol.* 5, 353–360. The real-time interactions of 1,1,1-trichloroethane (TCE) and 1,1,2-TCE with cytochrome *P*-450 were observed using *in vivo* optical methods to measure the spectral changes of cytochrome *P*-450 and the reduction–oxidation transition of pyridine nucleotides in the perfused liver of rats treated with phenobarbital. Changes in oxygen consumption and TCE uptake were also measured. The spectral changes of cytochrome *P*-450 indicated that both TCE isomers bound to low spin (substrate free) ferric cytochrome *P*-450 and formed a high spin (substrate complexed) form. However, 1,1,1-TCE bound more tightly to cytochrome *P*-450 and seemed to be only slowly metabolized compared to 1,1,2-TCE. The stoichiometry of the change in oxygen consumption rate to the change in 1,1,1-TCE uptake rate ranged between 5/1 and 9/1, whereas that of 1,1,2-TCE was 1.4 to 2.0. Decreases in reduced pyridine nucleotides associated with TCE administration were significantly larger with 1,1,1-TCE than with 1,1,2-TCE. The inhibitory effect of 1,1,1-TCE on hexobarbital metabolism in the perfused liver was greater than that of 1,1,2-TCE. Considering our previous data indicating that TCE did not stimulate mitochondrial respiration, it is postulated that the far higher amount of oxygen consumption associated with the binding of 1,1,1-TCE to cytochrome *P*-450 than the amount which was necessary to mixed-function oxidation of this compound was due to an uncoupling effect of 1,1,1-TCE on the mixed-function oxidase system. © 1985 Society of Toxicology.

1,1,1-Trichloroethane (1,1,1-TCE) is considered to be a relatively safe solvent on the basis of the LD50 (Goto *et al.*, 1977). Since 1,1,1-TCE is widely used for industrial and domestic purposes, more information about its effects is necessary for an adequate health assessment of the compound.

1,1,1-TCE is known not to be metabolized extensively in the rat and the human (Hake *et al.*, 1960; Monster *et al.*, 1979). However, after exposure of the rat to 1,1,1-TCE, interaction with the cytochrome *P*-450 system is evidenced by induction of cytochrome *P*-450 in the liver (Ando *et al.*, 1981). In addition, incubation of liver microsomes with 1,1,1-TCE produced hydrogen peroxide, although 1,1,1-TCE was not metabolized extensively (Ivanetich and Van Den Honert, 1981).

Therefore, we studied the interaction of the cytochrome *P*-450-dependent system with 1,1,1-TCE in the perfused rat liver by using optical techniques to determine the effect of 1,1,1-TCE on the cytochrome *P*-450 system in the intact liver cell. The perfused liver has been shown to be a good model for studying mixed-function oxidation under conditions similar to those *in vivo* and for the optical measurements of the cytochrome *P*-450 system (Thurman *et al.*, 1979).

For comparative purposes, we also determined the effects of 1,1,2-TCE in the same model, since the 1,1,2-isomer is well known as a compound which has a high metabolic rate in the liver microsomal enzyme system (Van Dyke and Wineman, 1971; Yllner, 1971; Nakajima and Sato, 1979). Furthermore, the dechlorination of 1,1,2-TCE has been demonstrated to be catalyzed by a reconstituted hepatic cytochrome *P*-450 system (Gandolfi and Van Dyke, 1973).

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METHODS

Animals. Thirty-two male albino rats (Wistar strain; Clea Japan, Inc., Japan), weighing 200–230 g, received a chow diet (Clea Japan, Inc., Japan) and water *ad libitum*. The animals were treated with sodium phenobarbital (70 mg/kg, ip) once a day for 4 days to induce hepatic microsomal mixed-function oxidase enzymes before performing perfusion experiments. Rats were fasted for 24 hr prior to surgical preparation under pentobarbital anesthesia (50 mg/kg, ip). Sixteen animals were divided into two groups of eight each for the administration of 1,1,1- and 1,1,2-TCE, respectively, and 16 animals were used in the experiment to study the effect of TCE on hexobarbital metabolism.

Nonrecirculating hemoglobin-free liver perfusion. The perfusion technique used was essentially the same as that reported by Sugano *et al.* (1978). The perfusion fluid was Krebs–Henseleit bicarbonate buffer plus 2 mM xylitol, pH 7.4, saturated with an oxygen and carbon dioxide gas mixture (95:5). During observation of the spectral changes of cytochrome *P*-450, the perfusate was saturated with an oxygen, carbon dioxide, and carbon monoxide gas mixture (92:5:3) (Iyanagi *et al.*, 1981). The perfusion was carried out with a peristaltic pump at a flow rate of 35 ml/min at 31°C. The liver was preperfused for 30 min before administration of TCE.

Scanning reflectance spectrophotometry and surface fluorometry. Optical measurements of the spectral changes in cytochrome *P*-450 and fluorescence of reduced pyridine nucleotides were carried out with a scanning reflectance spectrophotometer and a surface fluorometer (Tateishi Omron, Inc., Japan), respectively (Takano *et al.*, 1980, 1983; Kobayashi *et al.*, 1982). Briefly, the spectrophotometer scans the 420- to 700-nm region in 10 sec, and describes the average spectrum for each of four scans. Results are expressed in terms of the difference in spectrum prior to and after TCE administration by using a memory system. The fluorescence of reduced pyridine nucleotides with a broad peak at 460 nm, emitted when excited by ultraviolet light with a peak at 366 nm, was monitored continuously. Changes in fluorescence were expressed on a percentage scale, with 100% defined as the change of full-reduction under anoxia.

A diagram of the experimental system is shown in Fig. 1.

Oxygen measurement. The oxygen concentration in the perfusion fluid entering the liver corresponded to the oxygen tension of the gas mixture and was monitored with a Clark-type oxygen electrode. The oxygen concentration in the effluent from the liver was also monitored

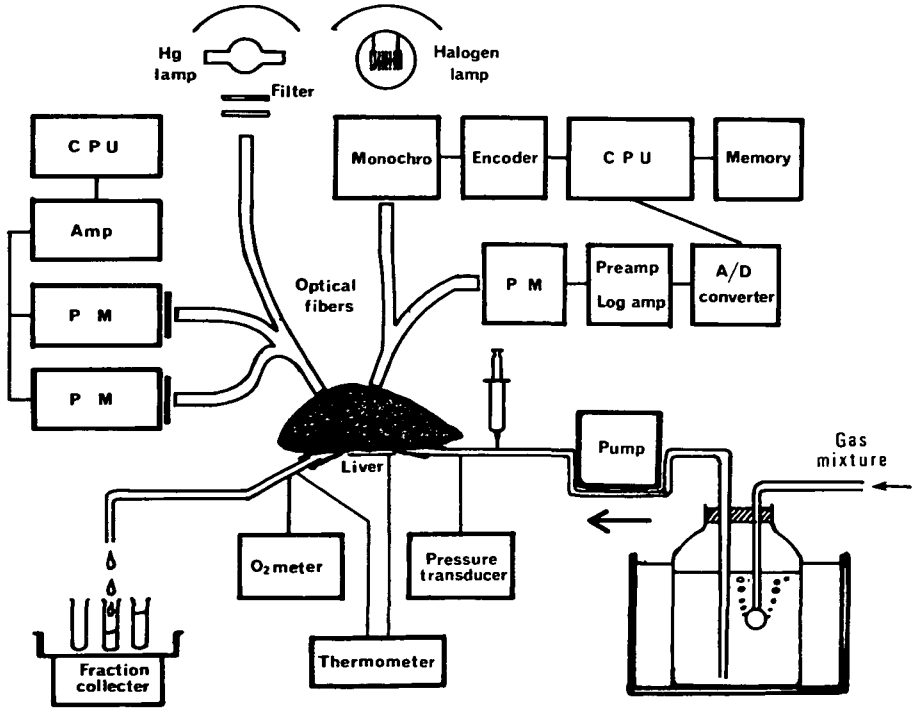

FIG. 1. Schematic diagram of the experimental system. PM: photomultiplier; CPU: central processing unit; Amp: amplifier. (Not drawn to scale.)

continuously through an air-tight connection employing another Clark-type oxygen electrode. The oxygen consumption of the liver was calculated from the difference in oxygen concentration between the influx and efflux perfusates and from the flow rate (Thurman and Scholz, 1969).

Administration and measurement of TCE. 1,1,1- and 1,1,2-TCE were dissolved in the Krebs–Henseleit bicarbonate buffer and introduced into the perfusion circuit with a feeder pump. The duration of infusion of the compound was 4 to 5 min. The volume of the solution administered was sufficiently small as not to influence the oxygen supply to the liver. The concentrations of 1,1,1- and 1,1,2-TCE in the perfusate were measured with a gas chromatograph. Samples of perfusate were extracted just before entering and just after leaving the liver. The uptake of TCE by the liver was calculated from the difference in concentration between the influx and efflux perfusates and from the flow rate.

Assessment of the inhibitory effect of TCE on hexobarbital metabolism. To observe the effect of 1,1,1- and 1,1,2-TCE on hexobarbital metabolism in the perfused liver, the uptake of hexobarbital was measured serially with a high pressure liquid chromatograph with or without TCE infusion. Hexobarbital was administered with another feeder pump at a final concentration of 0.05 mM.

Materials. Specially purified 1,1,1- and 1,1,2-TCE were obtained from Tokyo Industry Co. (Japan). 1,1,2-TCE did not contain any stabilizers. The 1,1,1-isomer contained 2% 1,4-diethylene dioxide as a stabilizer, which was confirmed not to interfere with the experimental observations. All other chemicals were of analytical grade.

Statistical evaluation. Differences between the values were analyzed by Student's t test, and were considered significant when p was less than 0.05.

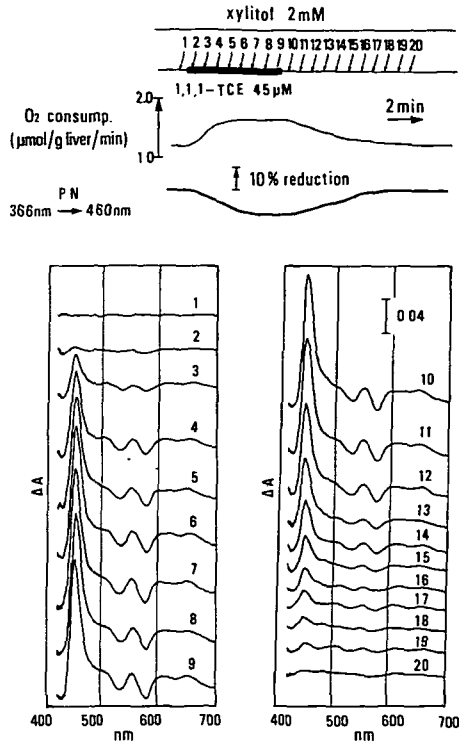

FIG. 2. Typical changes in oxygen consumption, reduced pyridine nucleotides (PN), and spectra following administration of 1,1,1-trichloroethane (TCE) (45 μM as final concentration) in the perfused rat liver. With respect to records of oxygen consumption and fluorescence of PN, the time scale proceeded from left to right; increase in oxygen consumption was recorded as an upward deflection, and decrease in reduced PN was recorded as a downward deflection. From Nos. 2 to 9, 1,1,1-TCE was infused. Each spectrum was observed at the time indicated by the number written in the upper line. Perfusate contained 2 mM xylitol.

RESULTS

Spectral Changes of Cytochrome P-450 in the Perfused Rat Liver

Typical records are shown in Figs. 2 and 3. The difference spectra were observed promptly after TCE administration and the shape of each spectrum was the same for 1,1,1- or 1,1,2-TCE administration. The spectral changes were reversible after cessation of TCE infusion in both cases, but the time for disappearance of the difference spectrum was different. 1,1,1-TCE did not release from cytochrome *P*-450 as fast as 1,1,2-TCE. The spectral change of cytochrome *P*-450 following TCE administration represented a conversion from low spin (substrate free) ferric cytochrome *P*-450 to the high spin (substrate complexed) form and the formation of a triplex complex of substrate, reduced cytochrome *P*-450, and carbon monoxide (Iyanagi *et al.*; 1981).

These spectral changes were observed at concentrations larger than 11.3 μM 1,1,1-TCE in the perfusate, whereas for 1,1,2-TCE

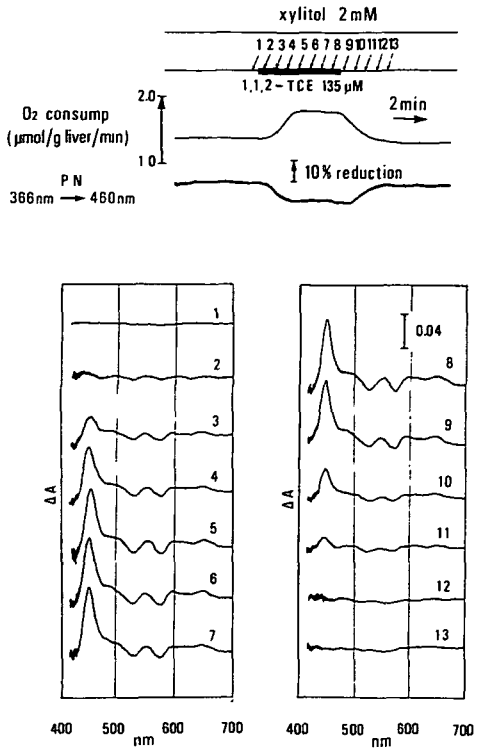

FIG. 3. Typical changes in oxygen consumption, reduced pyridine nucleotides (PN), and spectra following administration of 1,1,2-trichloroethane (TCE) (135 μM as final concentration) in the perfused rat liver. The numbers on the spectra mean the same as in Fig. 2.

spectral changes were detected at concentrations larger than 45 μM. 1,1,1-TCE induced larger spectral changes than the 1,1,2-isomer at the same concentration. The height of the absorbance difference between 450 and 490 nm ($\Delta A_{450-490}$), representing the amount of triplex complex, increased in proportion to the increase of TCE concentration in the perfusate (Fig. 4). Similarly, $\Delta A_{600-576}$, which is mainly the result of substrate–cytochrome *P*-450 complex formation (Iyanagi *et al.*, 1981), also increased with TCE concentration (Fig. 5). Both ΔA's of 1,1,1-TCE were significantly larger than those of the 1,1,2-isomer at the same concentration.

However, with regard to TCE uptake, the uptake of 1,1,1-TCE was not larger than that of 1,1,2-TCE (Tables 1 and 2).

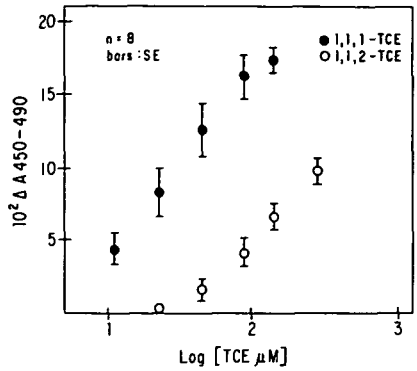

FIG. 4. Changes in absorbance difference ($\Delta A_{450-490}$) associated with trichloroethane (TCE) administration into perfused liver.

Changes in Oxygen Consumption

In the steady state before TCE administration, the average oxygen consumption rate of the perfused liver as 1.45 ± 0.33 (mean ± SD; μmol/g liver/min). The oxygen consumption increased following the administration of TCE and returned to the original rate after cessation of TCE administration, as shown in Figs. 2 and 3. The increment in oxygen consumption rate (ΔO_2 consumption) during 1,1,2-TCE administration was ap-

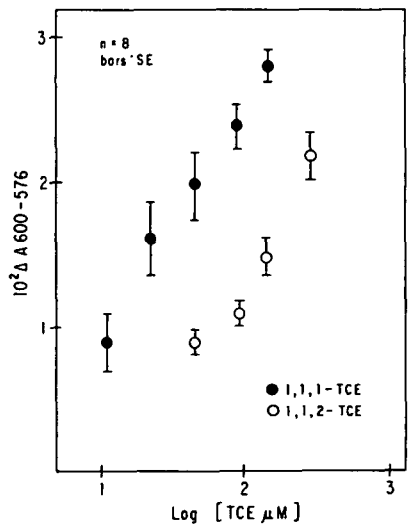

FIG. 5. Changes in absorbance difference ($\Delta A_{600-576}$) associated with trichloroethane (TCE) administration into perfused liver.

TABLE 1

UPTAKE OF 1,1,1-TRICHLOROETHANE (TCE) AND THE RATIO OF THE CHANGE IN OXYGEN CONSUMPTION RATE TO THE CHANGE IN 1,1,1-TCE UPTAKE RATE (ΔO_2/TCE UPTAKE)[a]

	1,1,1-TCE concentration in the perfusate (μM)				
	11.3	22.5	45.0	90.0	135
Uptake of TCE (nmol/g liver/min)	30 ± 9	39 ± 10	84 ± 30	113 ± 63	125 ± 69
ΔO_2/TCE uptake	8.3 ± 3.1	8.7 ± 1.7	5.8 ± 1.7	6.9 ± 5.2	5.0 ± 4.4

[a] Mean ± SD; n = 8.

proximately proportional to the increase in 1,1,2-TCE concentration in the perfusate and to the uptake of 1,1,2-TCE. However, ΔO_2 consumption during 1,1,1-TCE administration was not linearly correlated with the uptake of 1,1,1-TCE (Fig. 6). The ratio of ΔO_2 consumption to TCE uptake is shown in Tables 1 and 2. In the perfused liver, 1,1,1-TCE caused a much larger amount of oxygen consumption than 1,1,2-TCE or hexobarbital. Five to nine moles of oxygen were consumed by the uptake of one mole of 1,1,1-TCE, whereas 1.4–2 moles of oxygen were used for one mole of 1,1,2-TCE. The stoichiometry of oxygen to hexobarbital in the perfused liver during its oxidative metabolism was reported to be 2.2–2.4 (Sies and Brauser, 1970). The stoichiometry of oxygen to hexobarbital in the present study was 2.1 ± 0.3 (mean ± SD, n = 16).

Changes in Reduced Pyridine Nucleotides

Decreases in 460-nm fluorescence were observed following TCE administration, corresponding to net oxidation of pyridine nucleotides (Figs. 2 and 3). Although the supply of reducing equivalent may be rate-limiting for mixed-function oxidation in the perfused liver (Thurman *et al.*, 1979), in the present experiments, the reduced pyridine nucleotide supply was regarded as adequate due to the addition of xylitol to the perfusate (Reinke *et al.*, 1980; Iyanagi *et al.*, 1981; Belinsky *et al.*, 1983). In part, the interfering absorbance from the cytochrome *P*-450 triplex which has an absorption peak at 450 nm could reduce the fluorescence signal reaching the photomultiplier; however, the contribution from such interference to the values obtained in the present condition was thought to be

TABLE 2

UPTAKE OF 1,1,2-TRICHLOROETHANE (TCE) AND THE RATIO OF THE CHANGE IN OXYGEN CONSUMPTION RATE TO THE CHANGE IN 1,1,2-TCE UPTAKE RATE (ΔO_2/TCE UPTAKE)[a]

	1,1,2-TCE concentration in the perfusate (μM)			
	45.0	90.0	135	270
Uptake of TCE (nmol/g liver/min)	97 ± 17	180 ± 27	230 ± 59	367 ± 113
ΔO_2/TCE uptake	1.9 ± 0.6	1.6 ± 0.5	1.6 ± 0.5	1.5 ± 0.5

[a] Mean ± SD; n = 8.

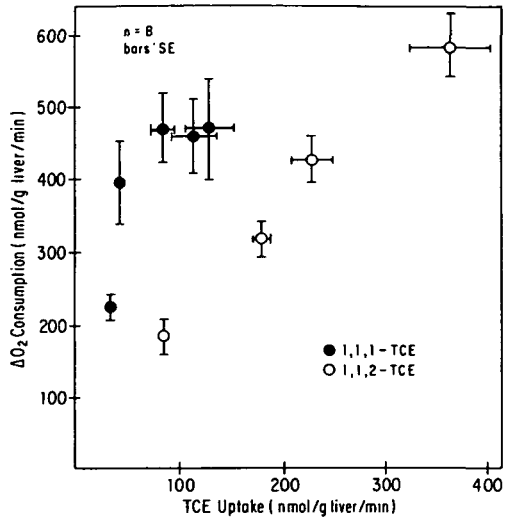

FIG. 6. Correlation between increment of oxygen consumption associated with trichloroethane (TCE) administration and TCE uptake into perfused liver.

small (Takano *et al.*, 1983). The percentage decrease in the reduced level of pyridine nucleotides increased with TCE concentration in the perfusate and, as shown in Fig. 7, this decrease was significantly greater with 1,1,1-TCE than that obtained with 1,1,2-TCE at the same concentration.

Inhibitory Effect of TCE on Hexobarbital Uptake by Liver

The hexobarbital uptake by the perfused rat liver was suppressed during TCE infusion as shown in Table 3. The inhibitory effect of 1,1,1-TCE on hexobarbital uptake was significantly greater than that of 1,1,2-TCE under the present conditions.

DISCUSSION

The liver perfused with hemoglobin-free medium can be free from hypoxia at the appropriate flow rates (Goodman *et al.*, 1973; Lemasters *et al.*, 1983; Matsumura and Thurman, 1983). We have previously demonstrated that in the present experimental system, oxygen was supplied adequately to the tissue at a flow rate of 35 ml/min at 31°C in the perfused liver of rats of similar body weight. Furthermore, mixed-function oxidation was not influenced by the oxygen concentration when the latter exceeded 0.88 mM (Takano *et al.*, 1982). The spectra of reduced cytochromes in the respiratory chain did not increase under enhanced oxygen utilization due to the TCE or hexobarbital administration in the present study, which also confirmed the adequacy of the oxygen supply.

Evidence was obtained that both 1,1,1- and 1,1,2-TCE bound to cytochrome *P*-450 and showed type I spectral changes in the intact liver cell. However, the interaction between 1,1,1-TCE and mixed-function oxidase displayed some differences from those of 1,1,2-TCE which is well known to be metabolized by the cytochrome *P*-450 system *in vitro* (Van Dyke and Wineman, 1971; Yllner, 1971; Nakajima and Sato, 1979; Gandolfi and Van Dyke, 1973).

1,1,2-TCE was taken up by the perfused liver with proportional increases in oxygen consumption and decreases in reduction level of pyridine nucleotides. The relationship between ΔO_2 consumption and 1,1,2-TCE uptake was linear ($r = 0.85$). The stoichiometry

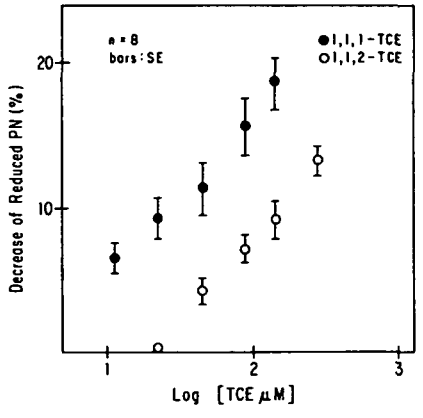

FIG. 7. Decrease of reduced pyridine nucleotides associated with trichloroethane (TCE) administration into perfused liver.

TABLE 3

SUPPRESSION OF HEXOBARBITAL (HB) UPTAKE DURING TRICHLOROETHANE (TCE) INFUSION IN THE PERFUSED RAT LIVER

Addition	n^a	HB uptake (nmol/g liver/min)	$\frac{\text{Decrement of HB uptake}}{\text{TCE uptake}} \times 100$
None (control)	16	101.3 ± 10.3[b]	—
45 μM 1,1,1-TCE	16	77.6 ± 9.0[c]	28.2
45 μM 1,1,2-TCE	8	88.8 ± 12.8[d]	12.9

[a] The number of the liver.
[b] Mean ± SD.
[c] Significant difference from the value for control, $p < 0.01$.
[d] Significant difference from the value for 45 μM 1,1,1-TCE, $p < 0.05$.

of ΔO_2 consumption to 1,1,2-TCE uptake was 1.4–2.0, which was close to that of hexobarbital in the perfused liver. Therefore, it appears that 1,1,2-TCE was metabolized by the cytochrome *P*-450 system in the intact liver cell.

On the other hand, it seems that 1,1,1-TCE was not metabolized as well as the 1,1,2-isomer in the perfused liver. In contrast to the linear uptake of 1,1,2-TCE, the uptake of 1,1,1-TCE saturated when the perfusate concentration was larger than 45 μM. In addition, the spectral changes representing the increase of amount of 1,1,1-TCE binding to cytochrome *P*-450, the ΔO_2 consumption, and the decrease of reduced pyridine nucleotides during 1,1,1-TCE administration were each significantly larger than comparable values obtained for the 1,1,2-isomer at the same concentration. These observations are compatible with *in vitro* data showing that the metabolic rate of 1,1,1-TCE is very slow compared to the 1,1,2-isomer in a solution of microsomal enzymes (Nakajima and Sato, 1979; Ivanetich and Van Den Honert, 1981). Furthermore, it has been found that incubation of liver microsomes with 1,1,1-TCE produces hydrogen peroxide, whereas incubation with 1,1,2-TCE does not (Ivanetich and Van Den Honert, 1981). The present results suggest that in the intact liver cell 1,1,1-TCE tightly bound to cytochrome *P*-450 and was only slowly metabolized, exhibiting a larger amount of complex formation and consequently a larger spectral change as compared to 1,1,2-TCE. The spectral changes lasted longer even after cessation of perfusion with 1,1,1-TCE. Considering our previous observations that 1,1,1-TCE did not stimulate mitochondrial respiration (Takano and Miyazaki, 1982), it is likely that 1,1,1-TCE acted as an uncoupler of the mixed-function oxidase system. The larger increase in oxygen consumption and oxidation of pyridine nucleotides elicited by 1,1,1-TCE are compatible with this explanation. Similarly, the stronger inhibitory effect of 1,1,1-isomer on hexobarbital metabolism could also be explained in this manner.

In conclusion, 1,1,1-TCE was not an inert agent to the mixed-function oxidase system in the liver. This compound bound to cytochrome *P*-450 and caused futile oxygen consumption. This phenomenon should be taken into consideration in the health assessment of 1,1,1-TCE.

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