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Interference with Hepatocellular Substrate Uptake by 1,1,1-Trichloroethane and Tetrachloroethylene[1]

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Interference with Hepatocellular Substrate Uptake by 1,1,1-Trichloroethane and Tetrachloroethylene. KUKONGVIRIYAPAN, V., KUKONGVIRIYAPAN, U., AND STACEY, N. H. (1990). *Toxicol. Appl. Pharmacol.* **102**, 80–90. The effects of chlorinated aliphatic hydrocarbon solvent exposure on hepatocellular transport of some model substrates have been investigated. Exposure of isolated hepatocytes to 1,1,1-trichloroethane or tetrachloroethylene resulted in suppression of uptake of taurocholate, ouabain, and 2-aminoisobutyric acid but not $CdCl_2$ or 3-*O*-methyl-D-glucose. The effect was clearly evident at noncytotoxic concentrations, as indicated by the lack of intracellular enzyme leakage and unaltered intracellular K^+ ion concentration. Moreover, the ultrastructure of solvent-exposed hepatocytes was similar to that of control cells, except for a reduction in membrane microvilli. The suppression of uptake was reversible provided that sufficient time was allowed for the cells to recover. The mechanism of this inhibition may be associated with energy-linked processes, as uptake of taurocholate, ouabain, and 2-aminoisobutyric acid is energy requiring while uptake of $CdCl_2$ and 3-*O*-methyl-D-glucose is not. Cellular ATP was reduced in a dose-dependent manner, but a marked depletion occurred only at cytotoxic concentrations. Na^+–K^+- and Mg^{2+}-ATPase activities in hepatocyte plasma membrane preparations were also inhibited by solvent exposure. The data suggest that 1,1,1-trichloroethane and tetrachloroethylene interfere specifically with energy-dependent hepatic transport functions and that a decrease in ATP levels and/or inhibition of cell membrane ATPases may be the mechanism.

The hepatotoxicity of organic solvents has been extensively studied, especially the mechanisms by which solvents induce cytotoxicity (Reynolds and Moslen, 1974; Berger *et al.*, 1986). Elevations of serum bile acids have been reported in workers who were occupationally exposed to organic solvents (Franco *et al.*, 1986). Interestingly, the increase in the level of bile acids was not always consistent with increases in serum liver enzymes (GPT, GOT, and GGT), which are usually regarded as indices of liver injury (Kaplowitz *et al.*, 1973; Liss *et al.*, 1985). In fact, the elevation of serum liver enzymes would primarily reflect the acute disruption of hepatocellular membranes (Zimmerman and Seeff, 1970) rather than liver dysfunction (Editorial, 1982). Therefore, increased serum bile acid levels may be either a very early sign of hepatocellular toxicity or a sign of liver dysfunction, which is not necessarily associated with cytotoxicity. Consequently, it is of interest to investigate whether exposure to organic solvents leads to the impairment of hepatocyte transport function, especially of bile acids, at a level that does not cause cytotoxicity. This approach is consistent with the views

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expressed by Tamburro and Greenberg (1981), that more appropriate means to monitor liver function to adequately reflect exposure to solvents are required.

The present experiments were therefore designed to investigate whether organic solvents interfere with the processes responsible for hepatocellular transport of bile acids and other model substrates transported by the liver. Appearance of cytotoxicity or necrosis was monitored biochemically and by electron microscopy. Finally, we explored possible mechanisms for observed interferences with hepatic transport. Isolated hepatocyte suspensions were used in these experiments, as this system is well studied, highly reproducible, and relevant to the *in vivo* situation with regard to both the hepatic uptake activities (Christensen, 1975; Schwarz *et al.*, 1975; Eaton and Klaassen, 1978; Stacey and Klaassen, 1980) and the production of cytotoxicity (Klaassen and Stacey, 1982; Guillouzo, 1986). 1,1,1-Trichloroethane (TCE) and tetrachloroethylene (TET) were chosen as they have some associations with liver injury and are both widely used in industry (Commission of the European Communities, 1986a,b).

MATERIALS AND METHODS

Chemicals. Tauro[*carbonyl*-^{14}C]cholic acid, sodium salt, [21,22-^{3}H]ouabain, and carrier-free $^{109}CdCl_2$ were purchased from Amersham (Sydney, NSW); $^{3}H_2O$, dextran-carboxyl[*carboxyl*-^{14}C], 2-amino[1-^{14}C]isobutyric acid (AIB), and 3-*O*-methyl-D-[U-^{14}C]glucose (3-OMG) were from New England Nuclear (Sydney, NSW). The ATP bioluminescent assay kit and all unlabeled compounds were from Sigma Chemical Co. (St. Louis, MO). Highest purity 1,1,1-trichloroethane and tetrachloroethylene were obtained from Aldrich Chemical Co. (Milwaukee, WI).

Isolation of hepatocytes. Hepatocytes from male Sprague–Dawley rats (300–400 g) were prepared by *in situ* perfusion of the liver with 0.03% collagenase (type IV, Sigma). The method followed that of Berry and Friend (1969) with some modifications (Kukongviriyapan and Stacey, 1988). Hepatocytes were resuspended in Hanks' buffer supplemented with 20 mM *N*-2-hydroxyethylpiperazine-*N*'-2-ethane sulfonic acid (Hepes) (medium A) at a concentration of 1.4×10^6 cells/ml. Initial viability of hepatocytes always exceeded 90% as determined by trypan blue exclusion.

Solvent exposure and substrate uptake. Medium A was used as the incubation buffer in all uptake studies, except for the 3-OMG, where glucose was omitted. Hepatocyte suspensions (1.9 ml) were incubated in 25-ml Erlenmeyer flasks at 37°C with continuous shaking. TCE or TET, at various volumes, was applied against the wall of the incubation flask by microsyringe to allow the solvents to evaporate into the gas phase. The flasks were sealed immediately and incubated for 20 min. Uptake of substrates (10 μM taurocholate, 125 μM ouabain, 1 mM AIB, 2 μM $CdCl_2$, and 15 mM 3-OMG) commenced upon the addition of 0.1 ml of substrate containing radiolabeled substrate. In studies with 3-OMG the suspensions were exposed to solvent at 37°C for 20 min and cooled down to 24°C in another water bath for 5 min before the determination of the uptake. Aliquots of cell suspension were removed at appropriate times and the radioactivity in the pellet and supernatant was determined by using the silicone oil centrifugation technique (Eaton and Klaassen, 1978). Samples were also taken at the end of each experiment for determination of protein content (Lowry *et al.*, 1951) and cell membrane integrity, by the measurement of extracellular lactate dehydrogenase (LDH) and alanine aminotransferase (ALT) activities as well as intracellular potassium ion concentration, as previously described (Stacey *et al.*, 1980). Intracellular water space and adherent fluid were estimated using $^{3}H_2O$ and dextran-carboxyl[*carboxyl*-^{14}C] according to Eaton and Klaassen (1978). To investigate the reversibility of the uptake inhibition, cell suspensions exposed to the solvents were washed twice by centrifugation at 50*g* and further incubated for 20 min in the solvent-free medium prior to determination of AIB uptake.

ATP assays. Cell samples were taken after the exposure to solvent and extracted with cold 2.5% trichloroacetic acid for 1 hr. The denatured protein was removed by centrifugation and samples were assayed immediately for ATP content by a bioluminescence method (Leach and Webster, 1986).

Preparation of plasma membranes and exposure to TCE and TET. Plasma membranes from rat livers were prepared according to Loten and Redshaw-Loten (1986) with minor modifications. Briefly, the liver was perfused *in situ* to remove blood cells with ice cold buffer (250 mM sucrose, 10 mM Tris–HCl, pH 7.5), then excised, minced, and homogenized in a loose-fitting Dounce homogenizer with 8 strokes in the same buffer. The homogenate was diluted to 200 ml and centrifuged at 1500*g* for 15 min. The pellet was resuspended and diluted to 75 ml and mixed with 10.1 ml of Percoll (Pharmacia, Uppsala, Sweden) and 1.45 ml of 2 M sucrose. The resulting mixture was centrifuged at 35,000*g* for 20 min. The plasma membrane band was collected, homogenized with 20 strokes

in a Dounce homogenizer, diluted to 75 ml, mixed with 10.1 ml of Percoll, 1.45 ml of 2 M sucrose, and 0.28 ml of 400 mM $CaCl_2$, and centrifuged at 45,000*g* for 10 min. The membrane band was harvested and washed twice by centrifugation at 4500*g* for 10 min.

The purity of the preparation was analyzed by intracellular and plasma membrane marker enzymes. Succinate–cytochrome *c* reductase and NADPH–cytochrome *c* reductase were measured by the methods of Sottocasa *et al.* (1967), alkaline phosphatase was measured by the procedure of Walter and Schutt (1974), and Na^+–K^+-ATPase and Mg^{2+}-ATPase were measured by the recording spectrophotometric method of Scharschmidt *et al.* (1979). Plasma membrane ATPase activity after exposure to TCE or TET was determined by the following method: Aliquots (40 μl) of plasma membrane suspensions (0.5 mg protein/ml) in 1.89 ml of ATPase assay buffer (to the final concentration of 125 mM Tris, 1 mM EGTA, 120 mM NaCl, 12.5 mM KCl, 5 mM NaN_3, 2.5 mM phosphoenolpyruvate, 0.5 mM NADH, and with or without 2 mM ouabain) were preincubated for 1 min at 37°C. Various amounts of TCE or TET were applied via microsyringe against the wall of the incubation flask, which was then capped tightly and further incubated for 6 min with continuous shaking. Thereafter, 20 μl of an enzyme mixture containing lactate dehydrogenase and pyruvate kinase (20 units of each) and 50 μl of ATP in $MgCl_2$ solution (to final concentrations of 5 mM) were added and mixed well. The assay mixture was immediately transferred to a prewarmed cuvette and sealed with a teflon cap before the rate of NADH oxidation at 340 nm was recorded. In a preliminary study, it was verified that under these experimental conditions solvents did not alter the activities of the coupling enzymes of the assay system. This can be seen from the comparable rates of NADH oxidation between the assay mixtures which were exposed or not exposed to solvents where ATP was substituted with 0.2–1 μmole of ADP in order to initiate the reactions.

Quantitation of TCE and TET by gas chromatography. Cell-free medium or plasma membrane-free medium was obtained by rapid centrifugation of samples in capped microfuge tubes at 10,000*g* for a few seconds or 20 seconds, respectively. The supernatant was extracted with heptane (Hipersolv, BDH, UK) and samples were kept on ice in glass-stoppered tubes without head space until analyzed. Microliter volumes were injected into a gas chromatograph (Varian, Model 3300 with Integrator Model 4290) equipped with a 2 mm × 6 ft column (0.1% SP-1000 on 80/100 Carbopack) and an electron capture detector. Temperature conditions were: injector, 250°C; detector, 250°C; column, 120°C for TCE and 180°C for TET.

Electron microscopy. Hepatocytes exposed to TCE or TET at various concentrations were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer for 1 hr and then postfixed in 1% phosphate-buffered osmium tetroxide for 1 hr. The specimens were dehydrated through graded acetone solutions, embedded in Spurr's resin, sectioned on an Ultracut E ultramicrotome, and stained with uranyl acetate and Reynolds' lead citrate prior to examination with a Phillips 400-HMG electron microscope.

Data analysis. Calculation of intracellular K^+ concentration was based upon the ^3H_2O estimation of an intracellular water space of 3 μl/mg cell protein. Initial rates of uptake were determined from the slopes of the linear portions of the curves which were over 1, 2, 5, and 10 min for taurocholate, $CdCl_2$, ouabain, and AIB, respectively. Uptake of 3-OMG was determined at 15- and 30-sec incubations. Data were evaluated by analysis of variance with Duncan's test. Statistical significance was set at $p < 0.05$.

RESULTS

Cytotoxic effects of TCE and TET. When TCE (2, 5, or 10 μl per flask) and TET (1, 2, and 4 μl per flask) were vaporized, steady state concentrations of TCE and TET in the medium were established within 5 min (Table 1). The application of TCE to the flask did not significantly change the cell viability as there was no increase in LDH and ALT release into the medium, and no decline of intracellular K^+ concentration (Table 1). TET, on the other hand, at 4 μl/flask caused a remarkable cell membrane damage, as there were large increases in LDH and ALT activities in the medium and a marked reduction in intracellular K^+ concentration. The application of 1 and 2 μl/flask did not consistently alter the cell viability.

Ultrastructure of hepatocytes treated with solvents. When control hepatocytes were incubated in medium A for 20 min, numerous microvilli were apparent on the cell surface membrane and the hepatocellular ultrastructure was generally well preserved (Fig. 1A). Exposure of the isolated hepatocytes to 2–10 μl TCE for 20 min did not lead to any significant changes in the ultrastructure, except for a reduction in microvilli on the cell surface, particularly at the highest dose of TCE (Fig. 1B). Similarly, exposure to 1–2 μl TET resulted only in the reduction of cell surface microvilli. Other ultrastructural features were similar to control. However, 4 μl TET (Fig.

TABLE I

CONCENTRATIONS OF TCE AND TET IN THE MEDIUM AND EFFECTS ON RELEASE OF LIVER ENZYMES AND INTRACELLULAR K^+ CONTENT

Experimental groups	Concn. (μM)[a]	LDH (units × liter^{-1})[b]	ALT (units × liter^{-1})[b]	K^+ (mM)[b]
Control	—	161 ± 26 (A)[c]	43.8 ± 3.8 (A)	126.2 ± 6.2 (A)
TCE 2 μl	234 ± 17	180 ± 40 (A)	54.3 ± 5.0 (A)	129.0 ± 1.3 (A)
TCE 5 μl	526 ± 67	168 ± 32 (A)	51.0 ± 5.0 (A)	127.2 ± 2.0 (A)
TCE 10 μl	1011 ± 91	176 ± 31 (A)	60.0 ± 4.8 (A)	116.3 ± 5.4 (A)
TET 1 μl	21 ± 4	150 ± 34 (A)	48.3 ± 10.0 (A)	121.6 ± 4.1 (A)
TET 2 μl	38 ± 4	154 ± 34 (A)	53.3 ± 8.9 (A)	118.3 ± 9.8 (A)
TET 4 μl	80 ± 16	364 ± 85 (B)	185.0 ± 8.8 (B)	35.5 ± 18.8 (B)

[a] Values are the means ± SE of three separate determinations.
[b] Values are the means ± SE of duplicate determinations each from four experiments.
[c] Values with the same capital letter are not significantly different ($p < 0.05$).

1C) resulted in the total loss of microvilli, marked dilation and whirling of endoplasmic reticulum, degranulation of rough endoplasmic reticulum, aberrant mitochondrial profiles, and the formation of lamellar structures. Moreover, cytoplasmic protrusions were also observed in some cells. The results from the examination of hepatocyte ultrastructures were consistent with the biochemical profiles, and suggested that the condition of exposure to TCE at all concentrations studied, and low and medium concentrations of TET, did not cause appreciable cytotoxicity.

Uptake of taurocholate. The uptake of taurocholate was inhibited by TCE and TET in a dose-dependent manner (Fig. 2). TET at 4 μl caused a marked depression of taurocholate uptake, but this concentration also resulted in marked cytotoxicity.

Uptake of some model substrates. Initial uptake of ouabain and AIB was significantly depressed by TCE and TET (Figs. 3 and 4) at all concentrations employed. It appears from these data that AIB is more sensitive than ouabain to inhibition by TCE but that the degree of inhibition by TET is similar. This is borne out by the subsequent experiments using lower concentrations of TCE and TET. A TET concentration of 0.25 μl/flask did not affect the uptake of AIB and ouabain. TCE at 0.5 μl/flask still significantly inhibited the uptake of AIB but not ouabain and at 0.25 μl/flask had no detectable effects (data not shown). In contrast, the initial uptake of $CdCl_2$ or 3-OMG were not affected significantly by the exposure to solvents (data not shown).

Reversibility of the inhibition by TCE and TET. AIB uptake activity was fully restored in hepatocyte suspensions previously exposed to TCE or TET at subcytotoxic concentrations following the washing and reincubation of cells in open air for 20 min (Table 2). Reincubation times of less than 20 min resulted in only partial recovery of the uptake activity (data not shown).

ATP content. The solvent exposure resulted in a dose-related decline in intracellular ATP content (Table 3). TET at 4 μl/flask produced a marked depletion of ATP which was consistent with the cell damage at this concentration.

Effects on membrane ATPase activities. The profile of marker enzyme activities and enrichments in purified membrane fractions, compared with starting homogenates, indicates that the preparations were derived mainly from hepatocyte plasma membrane. Na^+-K^+-ATPase and Mg^{2+}-ATPase were enriched approximately 8- to 14-fold and alka-

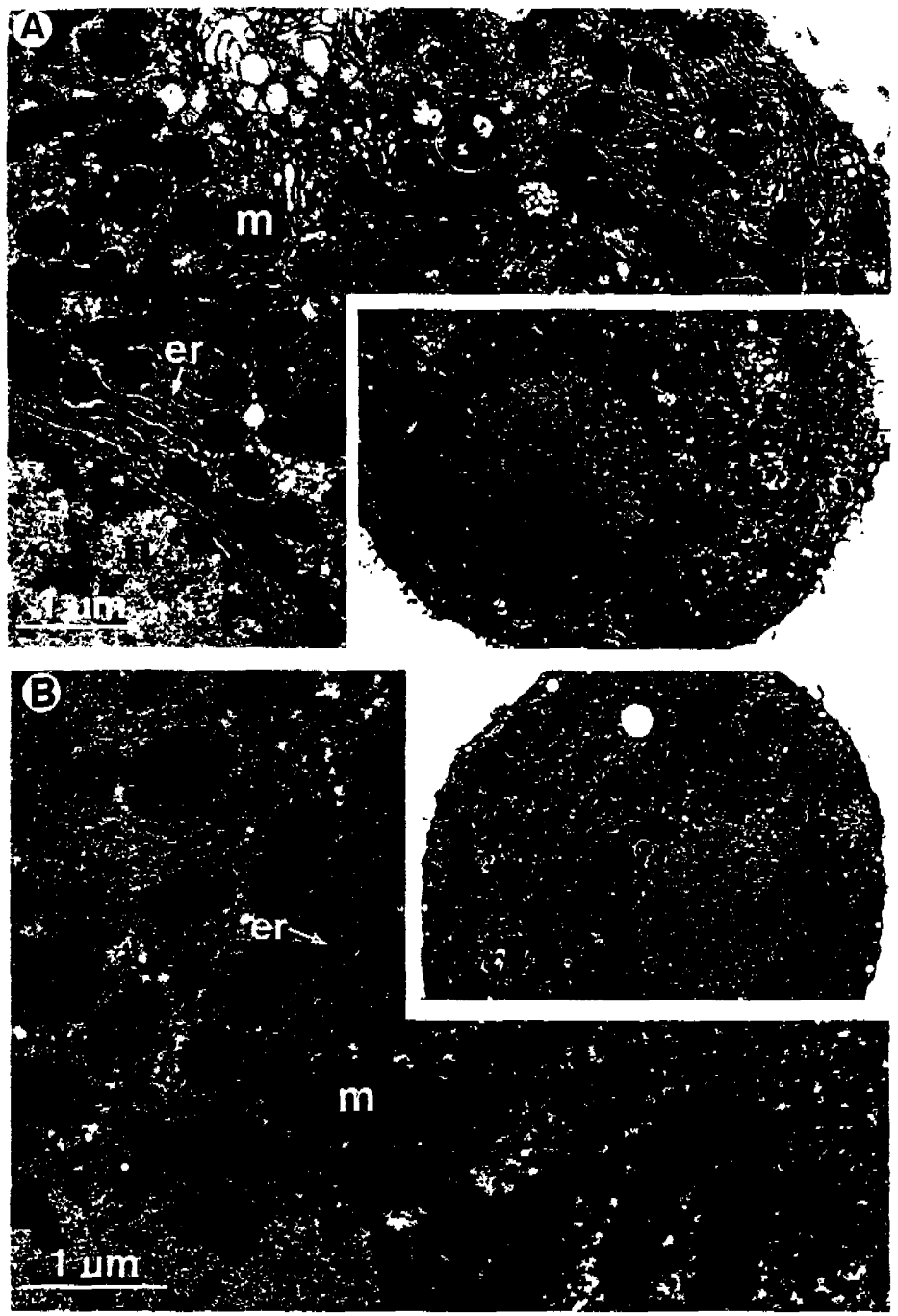

FIG. 1. Transmission electron micrographs from control and solvent-exposed isolated rat hepatocytes. (A) Control cell incubated for 20 min in medium without solvent. Note microvilli on cell surface (inset ×3450), ER, and mitochondria (×17,000). (B) Exposed to 10 µl/flask of TCE for 20 min. Surface microvilli are greatly reduced (inset ×3450) although mitochondria retain the characteristic coupled configuration and rough ER remains intact (×22,000). (C) Exposed to 4 µl/flask of TET for 20 min. Loss of surface microvilli, cytoplasmic protrusion (inset ×3450). Aberrant mitochondrial profiles, degranulation, and dilatation of rough ER (×17,000).

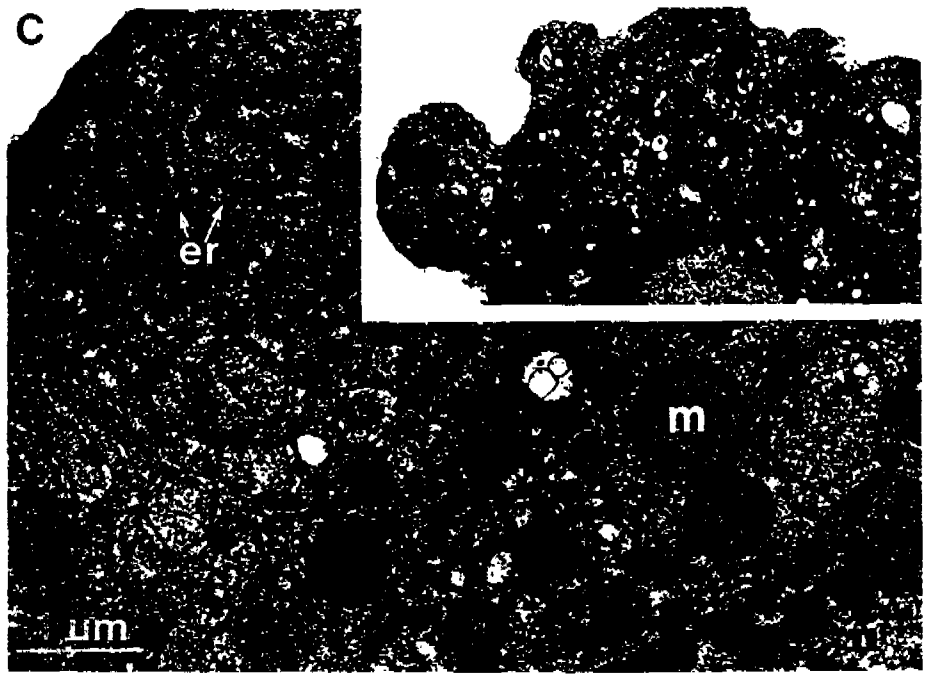

FIG. 1—*Continued*

line phosphatase 10-fold. Marker enzyme activities for endoplasmic reticulum (NADPH–cytochrome *c* reductase) and mitochondria (succinate–cytochrome *c* reductase) were approximately 0.5- and 0.05-fold that of homogenate.

Concentrations of TCE and TET in the assay media after incubation for 6 min were

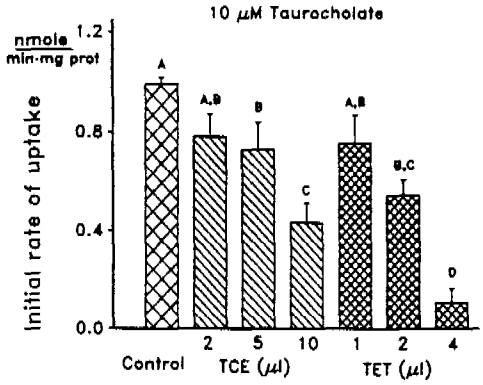

FIG. 2. Effect of TCE and TET on initial rate of uptake of taurocholate. Hepatocytes were incubated with various amounts (μl) of TCE or TET which were applied via the vapor phase for 20 min and initial velocity of uptake of 10 μM taurocholate was measured. Bars with the same capital letters are not significantly different ($p < 0.05$, $n = 4$ or 5).

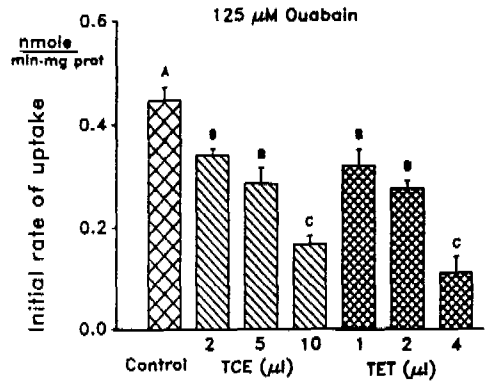

FIG. 3. Effect of TCE and TET on initial rate of uptake of ouabain. Hepatocytes were incubated with various amounts of TCE or TET which were applied via the vapor phase for 20 min and initial velocity of uptake of 125 μM ouabain was measured. Bars with the same capital letters are not significantly different ($p < 0.05$, $n = 3$).

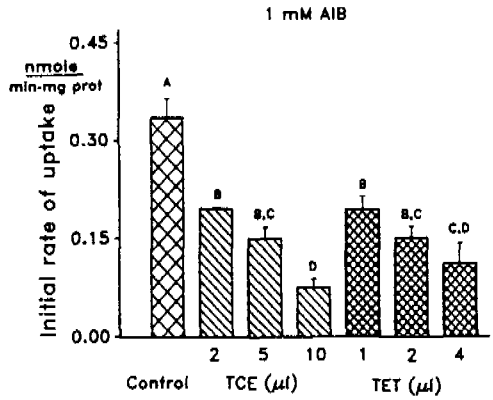

FIG. 4. Effect of solvents on initial rate of AIB uptake. Initial rate of 1 mM AIB uptake was determined after hepatocytes had been exposed to TCE or TET for 20 min. Bars with the same capital letters are not significantly different ($p < 0.05$, $n = 3$).

approximately the same as those found in the cell suspension experiments (data not shown). Exposure of hepatocyte plasma membranes to TCE and TET resulted in a decrease in ATPase activities (Table 4). The inhibition was dose dependent and occurred to about the same degree with both Mg^{2+}- and Na^+–K^+-ATPases.

TABLE 2

REVERSIBILITY OF TCE AND TET SUPPRESSION ON AIB UPTAKE

Experimental groups	Initial rate of uptake (nmole/min/mg protein)[a]
Control[b]	30.61 ± 2.72 (A)[c]
TCE 5 μl[d]	12.90 ± 4.27 (B)
TCE 5 μl and wash out[b]	28.71 ± 4.05 (A)
TET 2 μl[d]	12.79 ± 3.04 (B)
TET 2 μl and wash out[b]	25.80 ± 3.69 (A)

[a] Values are the means ± SE from four experiments.
[b] Hepatocytes were incubated with or without solvent for 20 min, washed twice by centrifugation, and further incubated in solvent-free buffer for 20 min before determination of AIB uptake.
[c] Values with the same capital letter are not significantly different ($p < 0.05$).
[d] Determination of the uptake was performed immediately after incubation with the solvent.

TABLE 3

TCE AND TET EFFECT ON INTRACELLULAR ATP CONTENT

Experimental groups	ATP (nmole/mg protein)[a]
Control	19.92 ± 0.49 (A)[b]
TCE 2 μl	17.48 ± 0.89 (B)
TCE 5 μl	16.51 ± 0.86 (B)
TCE 10 μl	13.09 ± 1.10 (C)
TET 1 μl	17.42 ± 0.94 (B)
TET 2 μl	16.53 ± 1.14 (B)
TET 4 μl	6.69 ± 1.54 (D)

[a] Values are the means ± SE from four experiments.
[b] Values with the same capital letter are not significantly different ($p < 0.05$).

DISCUSSION

Correlations between *in vitro* and *in vivo* hepatotoxicity have been observed with several chemicals tested (Tyson *et al.*, 1983a; Tyson, 1987; Tyson and Stacey, 1989). Furthermore, some chemicals which cause impairment of hepatic transport function *in vivo* have been reported to suppress the uptake or efflux of bile acids in isolated hepatocytes (Stacey, 1986, 1988; Van Dyke and Scharschmidt, 1987; Stacey and Kotecka, 1988). Consistent *in vivo* and *in vitro* results may allow the use of this *in vitro* system to study the possible mechanism which occurs in the intact organism. The inhibition of taurocholate transport is of particular interest as it may account for shifts in serum bile acids observed in tetrachloroethylene-treated rats in the absence of other signs of hepatotoxicity (unpublished observations).

Isolated hepatocytes exposed to TCE or TET at or below 10 μl/flask or 2 μl/flask, respectively, gave no evidence of cell death by biochemical assays of cell membrane integrity. Ultrastructural examination is consistent with the biochemical assays, as there was no appreciable alteration compared to controls, except for a reduction and shortening of microvilli. In contrast, cells exposed to TET at 4 μl/flask showed evidence of cell death by

TABLE 4

INHIBITION OF ATPase ACTIVITIES BY TCE AND TET

Experimental group[a]	Amount of solvent (μl/flask)	Specific activity (μmole Pi/mg protein/hr)	
		Mg^{2+}-ATPase	Na^+-K^+-ATPase
Control	—	30.16 ± 2.12[b] (A)[c]	10.74 ± 1.48 (A)
TCE	2	26.51 ± 2.12 (A, B)	9.26 ± 1.81 (A, B)
TCE	5	22.55 ± 2.94 (B, C)	5.78 ± 1.25 (B)
TCE	10	16.86 ± 1.18 (C, D)	5.72 ± 0.99 (B)
TET	1	27.67 ± 2.18 (A)	7.67 ± 1.23 (A, B)
TET	2	20.55 ± 1.21 (B, D)	8.40 ± 1.21 (A, B)
TET	4	15.12 ± 1.67 (D)	6.80 ± 0.51 (B)

[a] Hepatocyte plasma membrane was incubated with assay mixture as described under Materials and Methods. ATPase assay reaction was initiated after mixture containing plasma membrane had been exposed to solvents for 6 min.

[b] Values are means ± SE of triplicate determinations from each of three separate membrane preparations.

[c] Values with the same capital letters are not significantly different ($p < 0.05$).

marked release of cytoplasmic enzymes, loss of K^+, and subcellular changes. Dysfunction in transport by hepatocytes is clearly demonstrated at solvent concentrations which are not associated with cell death or irreversible cell damage, for which we have used the term cytotoxicity elsewhere in this paper. The loss of cell membrane microvilli has been previously reported (Perrissoud *et al.*, 1981; Stacey and Fanning, 1981; Tyson *et al.*, 1983b) in isolated hepatocytes exposed to CCl_4 and was suggested to be a consequence of the effects which lead to the cell death, because the concentrations of CCl_4 used also caused cell membrane damage, as indicated by the leakage of intracellular enzymes. In contrast to this previous interpretation, our data suggest that the reduction of microvilli may well reflect a dysfunction of the hepatocytes but is not necessarily associated with cell death.

The actual concentrations of solvents in the buffer determined by gas chromatography were about 10 times higher for TCE than TET (Table 1) despite administration of comparable quantities (TCE, 20, 50, and 100 μmoles/flask; TET, 10, 20, and 40 μmoles/flask). The lower concentration of TET in the medium is likely to be due to lower water/air partition coefficients (TCE and TET are 0.93 and 0.43, respectively) and to high lipid solubility (oil/water partition coefficients for TCE and TET are 383 and 4458, respectively) (Sato and Nakajima, 1979). Thus, more TET would be expected to readily partition into the cells, and this may be the reason why TET is apparently more cytotoxic than TCE in spite of the lower concentrations found in the medium. It is noted that the degree of inhibition of taurocholate, ouabain, and AIB transport by TCE and TET is not the same. A possible explanation is that the three substrates are transported by distinct systems (Eaton and Richards, 1986; Shotwell *et al.*, 1983), although there is some contention that ouabain may be taken up into the liver cells by the bile acid transport system (Frimmer and Ziegler, 1988).

The interaction of solvent with transport mechanisms is not specific for a particular system as we observed interference with at least three probable transport systems. However, general perturbation of plasma membrane lipid resulting in the alteration of fluidity or mass density of lipid bilayer is rather unlikely (Franks and Lieb, 1978), as the uptakes of 3-OMG and $CdCl_2$ are not affected. Nevertheless, we cannot entirely rule out that possibility in the present experiments. Direct

measurement of membrane fluidity by fluorescence polarization may provide more conclusive evidence. 3-OMG, a hexose sugar, is known to be transported by facilitated diffusion (Craik and Elliott, 1979). On the other hand, $CdCl_2$ uptake is largely by simple diffusion (Stacey and Klaassen, 1980). However, at low concentrations of $CdCl_2$, a saturable component of uptake is evident (unpublished observation) but this has not yet been proven to be carrier mediated. Neither of these transport systems require energy. In contrast, the transport systems for taurocholate, ouabain, and AIB are all energy dependent (Schwarz *et al.*, 1975; Le Cam and Freychet, 1977; Eaton and Klaassen, 1978).

Berger *et al.* (1986) have previously reported that CCl_4 at concentrations that did not cause intracellular enzyme leakage strongly inhibited hepatocellular oxygen consumption. The effect was immediate and probably mediated by direct solvent action. This suggests that subcytotoxic concentrations of solvent may ultimately cause depletion of cellular ATP, which is consistent with our findings as there is a dose–response relationship between concentration of solvent and cellular ATP level. This effect may be distinct from the cytotoxic effect, since there was a large decrease in ATP at the clearly cytotoxic concentration of TET (Table 3). However, a linear relationship between the cellular ATP level and inhibition of uptake does not necessarily indicate a cause and effect relationship. The inhibition of uptake could result from other independent effects or the depletion of ATP may play a partial or indirect role, as it was observed that the small decrease in cellular ATP occurred concurrently with the marked reduction in uptake, particularly that of AIB. Joseph *et al.* (1978) have shown that uptake of alanine and serine decreased in direct proportion to the declining level of cellular ATP. Since AIB is an alanine analog and transported largely by the same mechanism as alanine (Kilberg, 1986), the disparity between the degree of inhibition of AIB uptake and ATP depletion suggests that other mechanisms may also contribute to the suppression of transport.

It has been recognized that some halogenated compounds can inhibit Na^+–K^+-ATPase in several membrane preparations (Koch *et al.*, 1971; Sharom and Mellors, 1980) including hepatocyte plasma membranes (Rufeger and Frimmer, 1976). Indeed, the Na^+–K^+-ATPase pump is thought to be the ultimate driving force for most of the secondary active transport systems (Christensen, 1975). Our data show that TCE and TET, in the concentration range studied, inhibited membrane Na^+–K^+-ATPase activity. Our data are also consistent with the report that inhibition by low concentrations of halogenated compound is reversible (Rufeger and Frimmer, 1976). Therefore, interaction between TCE or TET at low level concentrations with membrane components does not result in denaturation of, or covalent interaction with, membrane protein. Moreover, the dissipation of transmembrane Na^+ gradient by inhibition of the Na^+–K^+-ATPase may not be directly responsible for the observed inhibition, since uptake of ouabain is sodium independent (Eaton and Klaasson, 1978). It is possible that dissipation of the Na^+ gradient may result in diminution of other secondary or tertiary active transports which ultimately drive the transport of ouabain.

In conclusion, it has been shown that TCE and TET inhibit the uptake of three actively transported substances but are without effect on uptake of those not requiring metabolic energy. The presence of these chlorinated hydrocarbons caused a small depletion of ATP and an inhibition of cell membrane ATPases, suggesting that this could be the mechanism of the observed inhibition of the uptake of the actively transported substrates.

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