eral individuals. We collected volatiles by using the smallest number of entrapment filters possible, in order to minimize the quantity of elution solvent and attendant solvent contaminants. A filter was used for up to 7 hours over the course of an evening to collect from whichever individuals happened to initiate hunting (*6*).
14. L. B. Bjostad and W. L. Roelofs, *Science* **220**, 1387 (1983). Four additional *M. cornigera* moth prey species belong to taxonomic groups (Hadeninae in the Noctuidae, and Crambinae in the Pyralidae) for which, for almost all studied species, members of this biosynthetic series are the principal components of the attractant blend [Y. Tamaki, in (*12*), pp. 145–191]. Some moth prey species are known for two other members of the *M. cornigera* species group: *M. dizzydeani* (*1, 2*) and *M. hutchinsoni* (*5*). Attractant blends for all four prey species for which information is available have as their major components members of the set: Z9-14:Ac, Z11-16:Al, and Z11-16:Ac—a subset of the equivalent set for *M. cornigera* prey. Very different sex pheromone compounds are used by some of the moth species captured by other *Mastophora* species (*2, 3*).
15. This study is the first step in our research program of (i) characterizing spider volatiles, (ii) reproducing spider volatile blends with synthetic compounds, and (iii) measuring the attractivity of these blends for various prey species. This is a more practical approach to understanding how spiders attract moths than bioassaying spider material directly. Spider material is very difficult to obtain; volatile collection is labor-intensive, and extraction of these difficult-to-find, hard-to-raise spiders is not practical. Until the factors controlling the variation in spider blends are understood, it will be necessary to perform assays with a variety of moths. Reproducing blends with synthetic compounds alleviates the difficulty of obtaining sufficient assay material.
16. Histological work has identified a candidate gland [A. Lopez, M. K. Stowe, J. C. Bonaric, *Publications Scientifiques Accélérées (Université Rêné Descartes, Clichy)* **8**, 1 (1985); A. Lopez and M. K. Stowe, in preparation].
17. See (*3*) for a review of chemical mimicry systems and the possible effects of mimic-model coevolution on the composition of mimic and model signal blends.
18. W. Steck, E. W. Underhill, M. D. Chisholm, *J. Chem. Ecol.* **8**, 731 (1982); M. S. Mayer and J. R. McLaughlin, *Handbook of Insect Sex Pheromones* (CRC Press, Boca Raton, FL, in press). The effect of a component on the attractiveness of a moth sex pheromone blend is not simply determined by its presence or absence; blend attractiveness usually depends on the ratio of two or more components [W. Steck, E. W. Underhill, M. D. Chisholm, *Environ. Entomol.* **9**, 583 (1980); (*3*)].
19. We thank A. T. Proveau and V. Bauder for technical assistance; P. Greany, J. Coffelt, J. McLaughlin, J. Sivinski, J. Reiskind, S. Johnson, C. Johnson, and W. Icenogle for help and advice; K. V. Yeargan for unpublished information; and H. W. Levi, T. C. Baker, P. E. A. Teal, J. R. Rocca, P. Landolt, M. S. Obin, B. J. Ploger, and L. Fink for critiques of the manuscript. Supported in part by a Harvard Graduate fellowship (M.K.S.) and a National Science Foundation predoctoral fellowship (M.K.S.). Mention of a commercial or proprietary product does not constitute an endorsement by the U.S. Department of Agriculture.

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# Carbon Tetrachloride at Hepatotoxic Levels Blocks Reversibly Gap Junctions Between Rat Hepatocytes

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**Electrical coupling and dye coupling between pairs of rat hepatocytes were reversibly reduced by brief exposure to halogenated methanes ($CBrCl_3$, $CCl_4$, and $CHCl_3$). The potency of different halomethanes in uncoupling hepatocytes was comparable to their hepatotoxicity in vivo, and the rank order was the same as that of their tendency to form free radicals. The effect of carbon tetrachloride ($CCl_4$) on hepatocytes was substantially reduced by prior treatment with SKF 525A, an inhibitor of cytochrome P-450, and by exposure to the reducing reagent β-mercaptoethanol. Halomethane uncoupling occurred with or without extracellular calcium and did not change intracellular concentrations of calcium and hydrogen ions or the phosphorylation state of the main gap-junctional protein. Thus the uncoupling appears to depend on cytochrome P-450 oxidative metabolism in which free radicals are generated and may result from oxidation of the gap-junctional protein or of a regulatory molecule that leads to closure of gap-junctional channels. Decreases in junctional conductance may be a rapid cellular response to injury that protects healthy cells by uncoupling them from unhealthy ones.**

HEPATOCYTES IN SITU OR SOON after dissociation into cell pairs communicate with each other through gap junctions (*1, 2*). One of the functions proposed for gap junctions is mediation of metabolic cooperation among coupled cells under physiological conditions. Liver gap-junctional channels can be closed by various treatments (*2*); uncoupling could serve to disconnect an unhealthy cell from healthy ones (*3*) and thereby protect them from loss of metabolically important substances and from spread of toxic molecules.

Cell death in a variety of pathological conditions is commonly attributed to a cascade of cytochemical changes that lead to the formation of an excess of oxidants (*4*). Halogenated hydrocarbons—a prime example is carbon tetrachloride ($CCl_4$)—are usually considered to be toxic in the liver by virtue of free-radical generation (*5, 6*), and the toxicity of several hepatotoxins is prevented by prior treatment with α-tocopherol, a free-radical scavenger (*7*). Cytochrome P-450 catalyzes the one-electron reduction of $CCl_4$ to yield as products chloride anion and the trichloromethyl radical (*8*). We report here that halogenated hydrocarbons decrease junctional conductance between hepatocytes. The effect may be due to an oxidant stress caused by free radicals since it is decreased by a blocker of cytochrome P-450 or a reducing agent, and it is not seen in cells in which the halogenated hydrocarbons presumably do not cause oxidant stress.

Dissociated hepatocytes were obtained with a collagenase perfusion technique (*2, 9*). Carbon tetrachloride and the other halomethanes tested were prepared as 5% (by volume) stock solutions in either pure ethanol or dimethyl sulfoxide (DMSO) and then diluted with Dulbecco's phosphate-buffered saline (PBS, Gibco) to the concentration used to perifuse the hepatocytes. At higher concentrations, solutions had to be used within 20 minutes or they lost potency because of separation of the halomethane from the aqueous phase. Electrical coupling was recorded under current clamp with four intracellular microelectrodes (resistance typically 10 to 20 Mohms when filled with 3*M* potassium chloride or 3*M* potassium citrate). Input and transfer resistances were used to calculate the conductance of junctional ($g_j$) and nonjunctional ($g_{nj}$) membranes by means of the pi-T transform (*10*).

When hepatocytes were perifused with $CCl_4$ (650 μ*M*) electrical coupling was blocked almost completely within 1 minute (Fig. 1). This uncoupling was due entirely to closure of gap-junctional channels; $g_j$ decreased to less than 1% of its initial value while $g_{nj}$ decreased slightly, the latter tending to increase coupling. Coupling returned when $CCl_4$ was washed out, although it appeared at a slower rate than that of the development of uncoupling. The incidence of dye coupling, evaluated by injecting Lucifer yellow into one cell of each of a large sample of pairs, went from 100% before treatment to 0% when $CCl_4$ was applied and reversed to 100% when $CCl_4$ was washed out (Fig. 2A).

Three additional halomethanes were tested, and two were found to block hepatocyte coupling (Fig. 2B). The relative potency of the halomethanes in uncoupling was $CBrCl_3 > CCl_4 > CHCl_3 > CH_2Cl_2$; the last was ineffective at the highest dose treated. The order of potency was the same as their

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relative tendency to form free radicals when acted on by cytochrome P-450 and therefore to cause an oxidant stress (*11*). Moreover, the concentrations of the halomethanes required for hepatotoxicity were close to those for uncoupling and again the rank order was the same (*11*). β-Mercaptoethanol, a reducing agent, partially prevented the reduction in dye transfer caused by $CCl_4$. In 430 μ*M* $CCl_4$, the minimal concentration that caused 100% uncoupling, 5 to 10 μ*M* β-mercaptoethanol maintained the incidence of dye coupling at 30 to 35%. Two sulfhydryl reagents, *N*-ethylmaleimide and diamide, have been shown to uncouple other systems (*12*), but the effect of β-mercaptoethanol may be due to a general reduction of oxidative action rather than specific protection of sulfhydryl groups.

Another metabolic product of the potent halomethanes ($CBrCl_3$, $CCl_4$, and $CHCl_3$) is phosgene, which has also been proposed to mediate hepatotoxicity (*13*). Pretreatment with cystamine, an effective phosgene scavenger, at levels up to 20 m*M* had no protective effect against uncoupling (three experiments). Thus phosgene is not likely to be involved.

SKF 525A (5 μ*M*), a nonspecific blocker of cytochrome monooxidases, increased the incidence of dye coupling in the presence of 430 μ*M* $CCl_4$ from 0% to about 40 to 45% (four experiments).

Further evidence for a role of cytochrome P-450 in hepatocyte uncoupling is provided by a comparison to other tissues. Rat ventricular myocytes and frog (*Xenopus laevis*) blastomeres presumably lack cytochrome P-450 and do not metabolize $CCl_4$. At a much higher concentration (1.5 m*M* $CCl_4$) than required to completely block $g_j$ between hepatocytes, $CCl_4$ did not affect coupling in these cells (three experiments on each cell type).

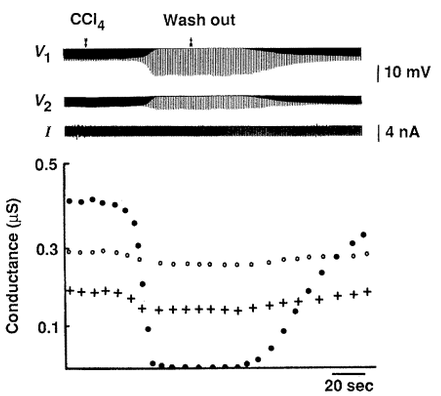


**Fig. 1.** Carbon tetrachloride reversibly blocks junctional conductance ($g_j$) in pairs of hepatocytes. In this experiment current pulses ($I$) were applied alternately through an electrode in each cell. The resulting voltages ($V_1$, $V_2$) were measured by separate electrodes. In each cell the larger vertical deflections measure the input resistance of that cell, and the smaller deflections measure the transfer resistance from the other cell. Cells were perifused with 650 μ*M* $CCl_4$ in PBS. The flow rate was approximately 2 ml per minute (dish volume, 1 ml). In all experiments ($n$ = 10) $g_j$ (●) was reduced by more than 99% and $g_{nj}$ [(○) cell 1; (+) cell 2] were reduced by less than 20% during exposure to $CCl_4$ at the concentration tested.

It has been proposed that halomethane hepatotoxicity depends primarily on entrance of extracellular calcium (*14*) and, in a number of tissues, increase in cytoplasmic $Ca^{2+}$, $[Ca^{2+}]_i$, to relatively high levels does decrease $g_j$ (*3, 15*). However, the toxicity of several hepatotoxins was recently found to persist in $Ca^{2+}$-free medium (*7*).

In dissociated rat hepatocytes $CCl_4$ (1 m*M*) caused a slight increase in $[Ca^{2+}]_i$ and $CBrCl_3$ caused a decrease (*6*), although both uncoupled (Fig. 2B). For a further assessment of the role of $Ca^{2+}$, the effects of $CBrCl_3$, $CCl_4$, $CHCl_3$, and $CH_2Cl_2$ on hepatocyte coupling were studied in saline containing submicromolar $Ca^{2+}$ obtained by adding EGTA to nominally $Ca^{2+}$-free saline. Uncoupling in low $Ca^{2+}$ required at most a slightly higher concentration of $CCl_4$ and $CHCl_3$ compared to 1 m*M* $Ca^{2+}$ (Fig. 2B). In two experiments $[Ca^{2+}]_i$ was measured by means of a $Ca^{2+}$-sensitive microelectrode (*16*) during $CCl_4$ application in low $Ca^{2+}$. There was no change in $[Ca^{2+}]_i$ during total block of $g_j$.

In hepatocytes as in many other tissues, increase in cytoplasmic acidity causes uncoupling (*2*). In five experiments intracellular $pH$ ($pH_i$) of hepatocyte pairs was monitored by means of an $H^+$-selective microelectrode (*2*) during $CCl_4$ application. In three experiments $pH_i$ decreased negligibly. In two experiments, $pH_i$ decreased from 7.15 before $CCl_4$ to 7.00 during $CCl_4$ and increased to the initial value after $CCl_4$ was washed out. In two further experiments cells were first perifused with saline solution containing 10 m*M* $NH_4Cl$, which raised $pH_i$ to 7.8. When 650 μ*M* $CCl_4$ was added to the ammonium solutions, reversible uncoupling was still seen, and the minimal $pH_i$ obtained was 7.65. Since $pH_i$ must decrease to between 6.4 and 6.5 to block $g_j$ in hepatocytes (*2*), cytoplasmic acidification was not responsible for uncoupling by $CCl_4$.

We recently showed that elevation of intracellular adenosine 3′,-5′-monophosphate (cAMP) concentration increased $g_j$ and the amount of inorganic orthophosphate incorporated into the main gap-junctional polypeptide, MP27 (*17*). Enzyme systems involved in activating or deactivating the channel, for example, cAMP cascades, could be affected by $CCl_4$. The possibility that uncoupling by $CCl_4$ is due to a change in the phosphorylation state of MP27 was tested by immunoprecipitation, SDS–polyacrylamide gel electrophoresis, and autoradiography as described (*17*). Neither the amount of [$^{32}$P]orthophosphate incorporation into MP27 nor the specific site of phosphorylation, determined from tryptic fingerprints, was affected by 650 μ*M* $CCl_4$.

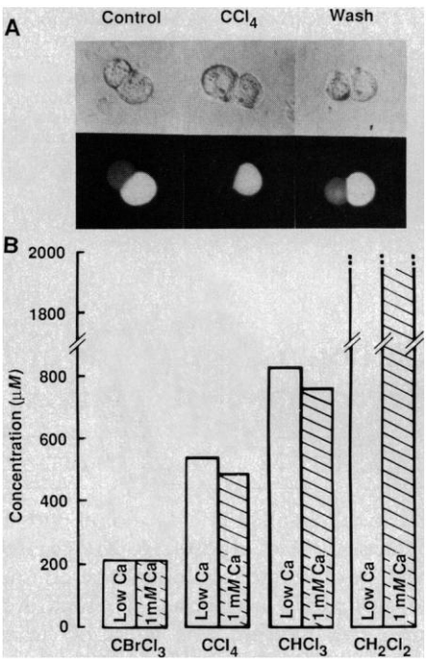


**Fig. 2.** Halomethanes reversibly block dye transfer between hepatocyte pairs. To determine the incidence of dye coupling after various treatments Lucifer yellow (5% w/v in 150 m*M* LiCl) was injected iontophoretically into one cell of each of 15 pairs under each condition. (**A**) Untreated pair in a culture in which the incidence of dye coupling was 100% (left). A pair after $CCl_4$ (540 μ*M*) had blocked dye transfer (middle); the incidence of coupling was 0%. Uncoupling was reversed by washing; 100% of the cells were dye-coupled (right). The cells were approximately 20 μm in diameter. (**B**) Histogram showing the minimal concentrations of halomethanes that reduced incidence of dye coupling to 0% in primary cultures of hepatocytes bathed in saline containing 1 m*M* $Ca^{2+}$ (shaded bars) or submicromolar $Ca^{2+}$ (unshaded bars). The low $Ca^{2+}$ concentration was obtained by adding 0.1 m*M* EGTA to $Ca^{2+}$-free PBS prepared with deionized water. The halomethane concentration necessary for 100% uncoupling was determined by injection of dye into at least 15 cell pairs in each of five different experiments. Near the critical region halomethane concentration was increased in 30- to 45-μ*M* increments. The measurements were carried out within 10 minutes after the halomethane addition. The highest dose of $CH_2Cl_2$ tested was 1.94 m*M*, which did not decrease the incidence of dye coupling; higher concentrations were not tested because at 1.94 m*M* cells became granular and many died within a few minutes, perhaps as a result of a solvent effect. Uncoupling by $CBrCl_3$, $CCl_4$, and $CHCl_3$ was reversed by washing, and the minimal concentration required to cause 100% uncoupling was independent of whether dimethyl sulfoxide or ethanol was the solvent used.

We conclude that the action of halomethanes on gap junctions is not mediated by an increase in intracellular free $Ca^{2+}$ or acidity or by changing the phosphorylation state of the main gap-junctional protein. The pharmacological data support the suggestion that uncoupling is mediated by free radicals.

Free-radical generation in situ after $CCl_4$ administration occurs very rapidly [within 0.5 to 2 minutes (*18*)], and this time course is consistent with the rapid onset of uncoupling in our experiments. Since the effect of the halomethanes is rapidly reversed by perifusion with control saline, the presumed oxidative reaction should also be reversed. Two reaction schemes that have been proposed as the mechanism of $CCl_4$ hepatotoxicity are covalent binding of reactive metabolic products and lipid peroxidation, mainly of membranous structures (*6*). Covalent binding of free radicals to the channels could cause a conformational change that closed the channels, but the rapid reversal after washing makes this mechanism unlikely. Peroxidation of surrounding lipids could also affect the channels, but the membrane would have to be rapidly repaired. Alternatively, free radicals could oxidize sulfhydryl groups to disulfide bonds (*19*). This oxidation reaction affects the activity of many biologically important macromolecules and might mediate uncoupling, which could then be reversed by any of several enzymatic processes (*20*).

Although the detailed mechanism of halomethane uncoupling is not known, the role of cytochrome P-450 and free-radical formation is indicated by the specificity to hepatocytes, the protective action of SKF 525A, and the identity of the order of halomethane potency in uncoupling and in generating free radicals when acted on by cytochrome P-450.

Other chemicals, such as 12-*O*-tetradecanoylphorbol-13-acetate (TPA), benzoyl peroxide, 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane (DDT), and cigarette smoke, which induce free-radical generation (*21*) and cause uncoupling (*22*), may act through a similar oxidative mechanism.

The biological meaning of uncoupling is unclear. It would prevent loss of necessary metobolites by surviving cells due to flow to dying ones and prevent the spread of toxic substances such as $Ca^{2+}$ and oxidized glutathione in the opposite direction. However, uncoupling would also prevent healthy cells from rescuing unhealthy ones by a similar exchange. Free-radical generation occurs in a variety of pathological and toxicological conditions that can lead to cell death (*4*). It is as yet undetermined whether uncoupling is protective, whether it is part of the causal chain leading to cell death, or whether it is a side effect accompanying a more critical lesion.

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