Biochemical Pharmacology, Vol. 55, pp. 1445–1452, 1998.

ISSN 0006-2952/98/$19.00 + 0.00
PII S0006-2952(97)00645-X

Metabolic Activation of Vinyl Chloride by Rat Liver Microsomes: Low-Dose Kinetics and Involvement of Cytochrome P450 2E1

Fatiha El Ghissassi, Alain Barbin*‡ and Helmut Bartsch†*

*International Agency for Research on Cancer, Unit of Gene-Environment Interactions, 150, Cours Albert Thomas, 69372 Lyon Cedex 08, France; and †German Cancer Research Center (DKFZ), Division of Toxicology and Cancer Risk Factors, Im Neuenheimer Feld 280, D-69120 Heidelberg, Germany

Abstract. The metabolism and pharmacokinetics of vinyl chloride (VC) have been extensively studied in rodents and humans, but the maximum velocity (V_{max}) and Michaelis constant (K_m) for the activation of VC by microsomal monooxygenases *in vitro* have not yet been determined. Using a new sensitive assay, the epoxidation of VC by rat liver microsomes (adult Sprague–Dawley) at concentrations from 1 ppm to 10^6 ppm in the gas phase was measured. In the assay, the reactive VC metabolites chloroethylene oxide and 2-chloroacetaldehyde were trapped with excess cAMP, yielding 1,N^6-etheno-cAMP (ϵcAMP) which was quantitated by HPLC fluorimetry. The trapping efficiency of electrophilic VC metabolites by cAMP was close to 10%. The specificity of the method was confirmed by purification of ϵcAMP on an immunogel. The VC concentration in the gas phase was measured by GC/flame ionization detection, while in the aqueous phase it was calculated from the partition coefficient between air and the microsomal suspension. Activation of VC by rat liver microsomes followed Michaelis–Menten kinetics with K_m = 7.42 ± 0.37 (±SD) μM and V_{max} = 4674 ± 46 pmol · mg protein^{-1} · min^{-1}. Inhibitor studies and immunoinhibition assays showed that VC was activated by cytochrome P450 (CYP) 2E1 down to 1 ppm in the air phase. Based on the metabolic parameters determined, the uptake of VC by rats *in vivo* can be accurately predicted. BIOCHEM PHARMACOL **55**;9:1445–1452, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. vinyl chloride; rat liver microsomes; enzyme kinetics; metabolism; CYP 2E1; etheno-cAMP

VC§ induces liver tumors in humans and rodents [1, 2] and is mutagenic in a number of experimental systems [1, 3]. Both adverse effects of VC are mediated through its activation into chloroethylene oxide by microsomal monooxygenases [4, 5]. Four DNA adducts have been identified in rodents exposed to VC (reviewed in [6]), including a major adduct, 7-(2-oxoethyl)guanine, and three minor etheno adducts (1,N^6-ethenoadenine, 3,N^4-ethenocytosine and N^2,3-ethenoguanine) which exhibit promutagenic properties (reviewed in [7, 8]). Chloroethylene oxide and its rearrangement product 2-chloroacetaldehyde can both react *in vitro* with nucleic acid bases to yield etheno derivatives [9, 10].

The pharmacokinetics and metabolism of VC have been well characterized in several mammalian species, including humans [11], where VC is mainly metabolized in the liver [12]. In rats exposed by inhalation, the uptake of VC is perfusion-limited at low concentrations and follows first-order kinetics; above 250 ppm, the metabolic absorption of VC is saturated and the uptake kinetics are of zero-order [13]. Pharmacokinetic studies in humans have shown large interindividual variations in uptake and metabolism of VC [12]. This variability has been confirmed by *in vitro* studies where VC was activated by human liver microsomes [5, 14, 15]. CYP 2E1 is thought to be the major CYP isozyme involved in the bioactivation of VC both in humans [5] and rats [16]. It has been shown that the phenobarbital-inducible CYP 2B1 can also metabolise and activate VC in rats [17, 18]. However, these studies were carried out at high VC concentrations, and the possible involvement of other CYP isoforms at low concentrations was not addressed. In addition, the kinetics of enzymatic activation of VC *in vitro* had not yet been established. For these reasons, we determined the kinetics of VC activation by rat liver microsomes over a large concentration range, from 1–10^6 ppm in the air phase. A sensitive assay, based on the measurement of ϵcAMP formed by the two reactive VC metabolites chloroethylene oxide and 2-chloroacetaldehyde in the presence of cAMP, was used [19, 20]. To

‡ Corresponding author: Dr. A. Barbin, Unit of Gene-Environment Interactions, International Agency for Research on Cancer, 150 cours Albert Thomas, 69372 Lyon Cedex 08, France. Tel. (33) 4-72-73-85-38; FAX (33) 4-72-73-83-29.

§ *Abbreviations:* ϵcAMP, 1,N^6-etheno-adenosine 3′, 5′-cyclic monophosphate; ϵdAdo, 1,N^6-etheno-2′-deoxyadenosine; C_{max}, maximum transhepatic concentration gradient; CYP, liver microsomal cytochrome P450; K_m, Michaelis constant; VC, vinyl chloride; V_{max}, maximum velocity.

Received 5 August 1997; accepted 29 October 1997.

characterise the CYP isozymes involved, the effects of some chemical inhibitors and monoclonal antibodies on VC activation were also examined.

MATERIALS AND METHODS

Chemicals

cAMP, ϵcAMP, ϵdAdo, NADPH, diethyldithiocarbamate, and chlorzoxazone were obtained from Sigma Chemical Co. Formic acid (ultrapure grade) was obtained from Normatom-Prolabo. Sepharose CL 4B and protein A/Sepharose CL 4B were purchased from Pharmacia-LKB. Tetrabutylammonium phosphate (Pic A reagent), obtained from Waters–Millipore, was diluted to a concentration of 5 mM in water before use. Murine monoclonal antibody EM-A-1 (supernatant from hybridoma cell cultures), prepared as described previously [21], was obtained from Professor M. F. Rajewsky (Institute of Cell Biology, University of Essen, Germany). Monoclonal antibodies against rat CYP isozymes (clones 1-91-3, 1-7-1 and 2-66-3, directed against CYP 2E1, CYP 1A1/1A2 and CYP 2B1/2B2, respectively) [22–24] and antibody HyHel (against chicken lysozyme) were obtained from Dr. S. S. Park (Laboratory of Comparative Carcinogenesis, National Cancer Institute, Frederick, MD). Pure (>99.9%) VC was a gift from Elf-Atochem (St Fons, France). Gas mixtures of VC in N_2 (at concentrations of *ca.* 10, 100, 1,000 and 30,000 ppm) were purchased from Airgaz. Before use, VC was transferred to gas sampling bags (Tedlar bags from Chrompack).

Preparation of Microsomes

Liver microsomes were prepared from 5-week-old male Sprague–Dawley rats, as described [25]. Pools of microsomes from five animals were used in the experiments. Stock microsomal suspensions (stored at −80°) were prepared in 0.1 M of potassium phosphate buffer (pH 7.4) supplemented with 1 mM of K_2EDTA, 0.1 mM of dithiothreitol and 20% (v/v) glycerol. They contained *ca.* 10 mg of proteins/mL (measured using the Protein Assay from Bio-Rad S.A.) and 0.95 nmol of CYP/mg of proteins (measured according to Omura and Sato, [26]).

Microsomal Assays

Enzymatic reactions were carried out in 39.2 mL serum type vials fitted with screw caps and Teflon-lined septa (Supelco). The microsomal reaction mixture contained 25 mM of cAMP, 1 mM of NADPH and 100 μL of microsomal suspension (~1 mg of proteins) in 1 mL of 50 mM of Tris-HCl buffer, 8 mM of $MgCl_2$, pH 8.0. The mixture was prewarmed in a stirring water bath at 37° for 1 min, and VC was injected at different concentrations into the headspace using gas-tight syringes (Hamilton Co.). The enzymatic reaction was initiated by injecting an NADPH solution and, after a rapid stirring on a vortex, allowed to proceed at 37° for 30 min. It was stopped by addition of 80 μL 0.5 M of formic acid to lower the pH to 4.1. Vials were then transferred at 100° for 30 min to complete the formation of ϵcAMP [19].

Determination of VC Concentration in the Headspace

Concentration of VC in the headspace was measured by GC (118- × 1/8 in. stainless steel column packed with 80/100 mesh Porasil C, Supelco). The GC system was equipped with a hydrogen flame ionization detector. VC was analyzed at a constant oven temperature of 150°. The injector and detector were held at 200°. The helium carrier gas flow was adjusted to *ca.* 20 mL/min.

Determination of Partition Coefficients

The liquid/air partition coefficients for VC were determined with 0.9% (w/v) saline and microsomal suspensions, using a modified version of the headspace vial equilibration technique [27, 28]. One mL of VC at 30,000 ppm was injected into the headspace of a 39.2-mL vial (vial I) containing 1 mL of aqueous phase. The latter consisted of stock microsomal suspension diluted tenfold in 50 mM of Tris-HCl buffer, pH 8.1, and supplemented with 25 mM of cAMP. After 30 min at 37°, 1 mL of headspace from each vial was removed and analyzed by GC to determine its concentration in VC (C_{g1}). One hundred μL of the aqueous phase were transferred (using a gas-tight syringe) to a second vial (vial II) with an internal volume of 1.62 mL. After 30 min at 37°, 100 μL of the gas phase in vial II were collected and analyzed by GC to determine the concentration of VC (C_{g2}). The partition coefficient K was calculated by equation (1):

$$K = \frac{C_{g2}V_{g2}}{(C_{g1}–C_{g2})V_{aq2}} \quad (1)$$

where V_{g2} and V_{aq2} are the volumes of the gaseous and aqueous phases in vial II, respectively.

Quantitation of ϵcAMP

Samples containing ϵcAMP were supplemented with ϵdAdo (40 ng/mL) as an internal standard and analyzed by HPLC fluorimetry using a Waters–Millipore model 510 liquid chromatograph coupled to a Perkin–Elmer LS40 fluorescence detector and to an SP 4290 integrator (Spectra-Physics). Samples were injected onto a Nucleosil C18 column (25 cm × 4.6 mm, 10-μm particle size, Société Française Chromato Colonne) and eluted in acetonitrile/5 mM of Pic A reagent (13.5: 86.5, v/v) at a flow rate of 2 mL/min. The fluorescence of column effluent was monitored at excitation and emission wavelengths of 300 and 415 nm, respectively. The ratio of areas measured for ϵcAMP (retention time ≈ 5.1 min) and ϵdAdo (retention time ≈ 2.8 min) was used to calculate ϵcAMP concentration in the samples. The detection limit was 0.2 pmol.

Purification of ϵcAMP on Immunoaffinity Columns

In some experiments, ϵcAMP formed in the microsomal mixtures was prepurified on immunoaffinity columns before the HPLC analyses. Immunoaffinity columns were prepared with antibody EM-A-1, as described previously [20]. After addition of ϵdAdo as an internal standard, samples were diluted up to 2 mL with 11.5 mM of phosphate-buffered saline (pH 7.4) containing 0.02% (w/v) sodium azide and filtered through 1 mL of a Sepharose CL 4B gel to remove precipitated proteins. They were then loaded at 4° onto immunoaffinity columns and purified [20]. This improved assay permitted the measurement of metabolic activation of VC down to a concentration of 1 ppm in the air.

Determination of the Ratio of VC Metabolised to ϵcAMP Formed

To determine the yield of ϵcAMP formed by reaction of VC metabolites with cAMP, assays of VC activation were carried out in 1.62- and 1.40-mL vials, in the presence of 0.5 mL of microsomal reaction mixture. One hundred or 125 μL of 900 ppm VC were injected into the headspace of the reaction vials and of empty vials (containing no aqueous phase). After incubation for 25 min at 37°, a 100-μL aliquot of the headspace was withdrawn for GC analysis. Forty μL of 0.5 M of formic acid were added to the reaction vials which were further incubated at 100° for 30 min. The amount of VC metabolised Q_M was calculated from equation (2):

$$Q_M = C_{g1}V_T - C_{g2}(V_g + KV_{aq}) \quad (2)$$

where V_T, V_{aq} and V_g are the internal volume of the empty vials and the volumes of the aqueous and air phases in the reaction vials, respectively; C_{g1} and C_{g2} are the headspace concentrations of VC in the empty and reaction vials, respectively, and K is the microsome/air partition coefficient of VC.

Chemical Inhibitions and Immunoinhibitions

Three hundred μM of diethyldithiocarbamate [5], 50 μM chlorzoxazone [29] and 10 mM ethanol [30, 31] were used for chemical inhibition assays. They were added to the microsomal reaction mixtures which were preincubated for 3 min at 23° before the addition of VC and NADPH. The enzymatic reactions were performed as described above.

The immunoinhibition assays were performed with the following monoclonal antibodies against rat liver CYP isozymes: clone 1-91-3 specific for ethanol-inducible CYP 2E1, clone 1-7-1 specific for methylcholanthrene-inducible CYP 1A1/1A2, and clone 2-66-3 specific for phenobarbital-inducible CYP 2B1/2B2 [22–24]. Clone HyHel, an antibody against chicken egg lysozyme, was used as a negative control. The antibodies were incubated with microsomes for 15 to 30 min at 23° before the addition of the other components. Their concentration was selected in order to reach the maximal inhibition of enzymatic activity. The total protein concentration (immunoglobulins plus microsomal proteins) in the reaction mixture was maintained at 1 mg/mL.

Calculation of Data

Unless otherwise specified, results are reported as means ± SD of triplicate assays. Linear and nonlinear regressions were performed using the SigmaPlot software from Jandel Scientific GmbH. Analyses of variance were done with the Stata Statistical Software (Stata Corp.).

Estimation of $K_{m,app}$ Following In Vivo *Inhalation*

$K_{m,app}$ after *in vivo* inhalation was estimated from the Michaelis constant K_m, using a pharmacokinetic model developed by Andersen [32]. Firstly, C_{max}, the maximum transhepatic concentration gradient sustainable at a hepatic blood flow Q_L, was calculated as V_{max}/Q_L, with Q_L = 0.0136 L/min for a 250 g rat (estimated from Fiserova–Bergerova, [33]). Then, the C_{max} and K_m values were used in equation (3) to calculate $K_{m,app}$, the arterial concentration of VC at which the concentration gradient across the liver is $C_{max}/2$:

$$K_{m,app} = \frac{C_{max}}{2} \times \frac{e^d}{(e^d - 1)} \quad (3)$$

where $d = C_{max}/2K_m$.

To estimate K_m after *in vivo* inhalation, the $K_{m,app}$ must be divided by the steady-state blood/gas concentration ratio or effective partition coefficient $(N_{eff})_{1/2}$, given by equation (4):

$$(N_{eff})_{1/2} = \frac{N}{1 + F(C_{max}/2K_{m,app})(Q_T/V_{alv})N} \quad (4)$$

where N is the thermodynamic blood/air partition coefficient of VC (N = 1.68; [34]), F the fraction of cardiac output perfusing the liver (F = 0.22; [33]), Q_T the cardiac output ($Q_T \cong 0.068$ L/min for a 250 g rat; estimated from Fiserova–Bergerova, [33]) and V_{alv} the alveolar ventilation (V_{alv} = 0.084 L/min for a 250 g rat; estimated from Fiserova–Bergerova, [33]).

RESULTS

Optimisation of the Assay

Using closed systems, one limitation with equilibration methods is the depletion of substrate during incubation in presence of the metabolic system. In our study, this was minimised by using vials with an empty volume of 39.2 mL and adding 1 mL of the microsomal system (see below). Control incubations were run without NADPH to demonstrate that VC was not lost unspecifically from the vials. To optimise the assay, the effect of different components on

the activation of VC at 1 ppm in the air phase was investigated. After 30-min incubations, the ϵcAMP formed was purified by immunoaffinity and measured by HPLC fluorimetry. It was found (data not shown) that the yield of ϵcAMP increased linearly with: 1) the concentration of microsomal proteins (with a maximum obtained at 1 mg of protein/mL, followed by a decrease); 2) the concentration of NADPH (up to 0.5 mM of NADPH, followed by a plateau); and 3) the concentration of the trapping agent cAMP (up to 25 mM). Using these optimal conditions (1 mg of protein/mL, 1 mM of NADPH, 25 mM of cAMP), the formation of ϵcAMP as a function of time was then measured in presence of low (1 ppm), intermediate (800 ppm) and high (982,000 ppm) concentrations of VC in the air phase. At all concentrations of VC, the yield of ϵcAMP increased linearly with time during the first 60 min. Our kinetic studies showed that equilibration occurred within 1 min after the addition of NADPH.

Partition Coefficient of VC

The microsomal mixture/air partition coefficient for VC, needed to calculate the substrate concentration in the aqueous phase, was determined to be 0.56 ± 0.13 ($N = 7$). Using the same method, the saline/air partition coefficient was found to be 0.41 ± 0.16 ($N = 4$), which is very close to the value of 0.43 reported by Gargas *et al.* [28].

Ratio of VC Metabolised to ϵcAMP Formed

To determine the efficiency of cAMP in trapping the reactive metabolites of VC, the disappearance of VC from the headspace was measured in parallel to the formation of ϵcAMP in the aqueous phase. In the presence of 25 mM of cAMP, the molar ratio of VC metabolised to ϵcAMP formed was found to be 9.85 ± 0.85 ($N = 6$).

Kinetics of VC Metabolism

The kinetic parameters for VC oxidation by rat liver microsomes were determined by measuring the formation of ϵcAMP at concentrations of VC in the headspace ranging from 1 to 982,000 ppm. ϵcAMP was separated by HPLC together with a known amount of ϵdAdo, used as an internal standard, and quantitated by fluorimetry. The specificity of the assay was confirmed by performing a prepurification of ϵcAMP formed in the microsomal mixture on an immunoaffinity gel (specific for $1,N^6$-ethenoadenine nucleotides or nucleosides) before analysis by HPLC fluorimetry: similar levels of ϵcAMP were found with and without the immunopurification step. This immunopurification step was necessary for accurate measurement of ϵcAMP at low concentrations (e.g., ϵcAMP formed in the presence of 1 or 2 ppm of VC). Since at these low concentrations, the presence of contaminating ϵcAMP in the commercial cAMP preparation interfered with the analysis, cAMP was prepurified on the immunogels before use.

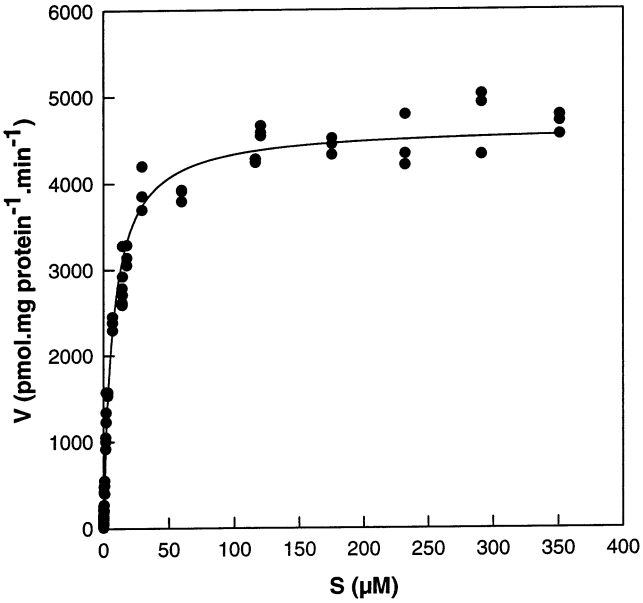

FIG. 1. Kinetic of metabolic activation of VC by rat liver microsomes *in vitro*. The solid line is the computer fit of the Michaelis-Menten equation to the data. The following constants were deduced: V_{max} = 4674 ± 46 pmol/mg protein/min and K_m = 7.42 ± 0.37 μM.

The curve representing the metabolic rate V (pmol VC metabolised/mg protein/min) as a function of the substrate concentration S (μM) was characteristic of Michaelis–Menten kinetics (Fig. 1). In Fig. 1, V was obtained from the quantity of ϵcAMP measured multiplied by 9.85, and S was calculated from the initial concentration of VC in the gas phase, using a partition coefficient of 0.56. A correction was made as follows to take into account depletion of the substrate due to metabolic degradation (this depletion was not negligible, i.e. >10%, below 500 ppm VC). A numeric simulation showed that the substrate concentration in the aqueous phase and the metabolic rate both decreased in a virtually linear fashion with time under our experimental conditions. Therefore, the average substrate concentration S during the 30-min incubation was calculated and used to determine the kinetic parameters for VC activation (Fig. 1). In absence of VC, a background of 21.3 ± 5.1 pmoles of ϵcAMP ($N = 6$) was measured in the microsomal system, following a 30-min incubation at 30°. This was probably due to the concurrent, NADPH-dependent formation of ϵcAMP by lipid peroxidation products in the microsomal suspension [20]. The metabolic rate V was corrected for this background (Fig. 1).

Nonlinear regression of the data shown in Fig. 1 (obtained between 1 and 15,000 ppm VC) yielded the following Michaelis–Menten parameters: $K_{m,app}$ = 7.42 ± 0.37 (±SD) μM and V_{max} = 4674 ± 46 pmol/mg protein/min. The Lineweaver–Burk plot representation showed a linear relationship between 1/V and 1/S for atmospheric concentrations of VC ranging from 40 to 15,000 ppm (not shown).

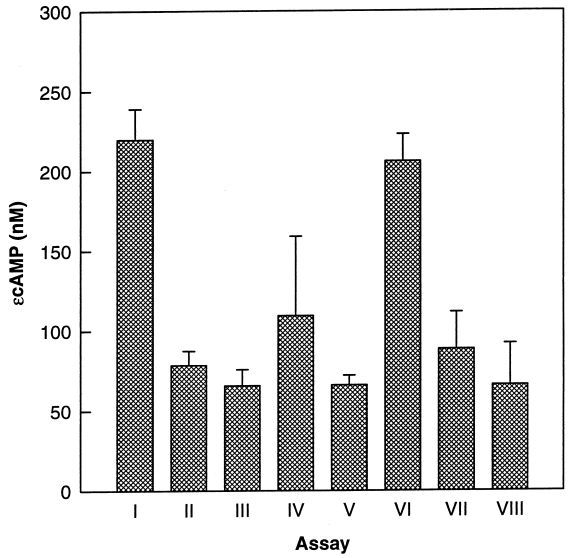

FIG. 2. Formation of ϵcAMP from cAMP in presence of rat liver microsomes and 2 ppm VC: effects of different substrates or inhibitors of CYP 2E1. Assays (1 mL) contained 25 mM of cAMP and 1 mg of microsomal proteins. Additions (+) or omissions (−) in the different assays were as follows: I, + VC; II, + VC, + diethyldithiocarbamate (300 μM); III, − VC, + diethyldithiocarbamate; IV, + VC, + chlorzoxazone (50 μM, dissolved in 20 μL of methanol); V, − VC, + chlorzoxazone; VI, + VC, + 20 μL of methanol; VII, + VC, + ethanol (10 mM); VIII, − VC, + ethanol. Values represent means ± SD of 3 to 5 determinations. With the exception of assay VI, all other assays were significantly different from assay I ($P < 0.001$, Bonferroni multiple comparison test).

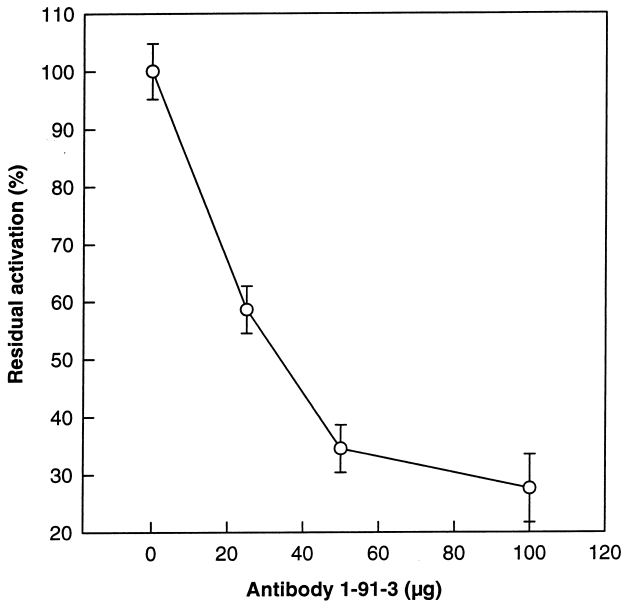

FIG. 3. Activation of VC (4000 ppm) by rat liver microsomes: inhibitory effect of antibody 1-91-3 directed against CYP 2E1. Two hundred-microliter microsomal suspensions containing 100 μg of proteins were preincubated at room temperature with varying amounts of antibody 1-91-3 (completed with a nonspecific antibody to keep the total protein concentration constant). After 15 min, microsomal reactions were started by the addition of cAMP (25 mM), NADPH (1 mM) and VC. Values (expressed as % residual activation) represent means ± SD of 3 determinations.

The parameter values obtained using this linear transformation were: $K_{m,app} = 6.44 \pm 0.58$ μM; $V_{max} = 4589 \pm 296$ pmol/mg of protein/min. No significant deviation from this linear relationship was observed at low concentrations of VC, down to 1 ppm: the slope of the reciprocal plot was $(1.417 \pm 0.044) \times 10^{-3}$ pmol/mg of protein/min · μM between 40 and 15,000 ppm, and $(1.402 \pm 0.054) \times 10^{-3}$ pmol/mg of protein/min · μM between 1 and 24 ppm. This indicates that a single isozyme was involved in the bioactivation of VC. The metabolic rate measured at 982,000 ppm VC was also consistent with these kinetics.

Chemical Inhibitors of VC Oxidation

To verify whether CYP 2E1 was the only isozyme involved in the activation of VC at low concentrations, microsomal assays were carried out at 2 ppm VC in the presence of chemical inhibitors of CYP 2E1 (Fig. 2). In the presence of 300 μM of diethyldithiocarbamate, the formation of ϵcAMP was inhibited by 64% (assay II) as compared to the assay without inhibitor (assay I). Addition of 50 μM of chlorzoxazone (dissolved in 20 μL of methanol) to the reaction mixture led to a similar reduction (50%) in the formation of ϵcAMP (assay IV). Methanol alone (at 2% final concentration) had no significant effect on the activation of VC (assay VI). In contrast, the formation of ϵcAMP was inhibited by 60% in the presence of 10 mM of ethanol (assay VII). The residual levels of ϵcAMP formed in the presence of VC and either of the three inhibitors (assays II, IV, VII) were similar to the levels measured in the absence of VC (assays III, V, VIII), thus indicating that a complete inhibition of VC oxidation was achieved under these conditions.

Immunoinhibition of VC Oxidation

Microsomal reactions were also performed in the presence of several monoclonal antibodies that inhibit specific CYPs. Clone 1-91-3, directed against CYP 2E1, was an efficient inhibitor of the oxidation of VC by rat liver microsomes, at high (Fig. 3; 4,000 ppm VC) as well as low VC concentrations (Fig. 4; 1 ppm VC). The other two antibodies tested, 1-7-1 and 2-66-3, did not inhibit the formation of ϵcAMP (data not shown). The level of ϵcAMP measured after microsomal incubations in presence of 1 ppm of VC and of antibody 1-91-3 (Fig. 4, assay II) was similar to the background level detected in microsomal mixtures without VC (Fig. 4, assay III).

Prediction of Metabolic Constants In Vivo

Under the assumption that all the metabolic absorption of VC *in vivo* is accounted for by the hepatic tissue [12], we can use the metabolic constants determined in this study to estimate the rate constants for the metabolism of VC in

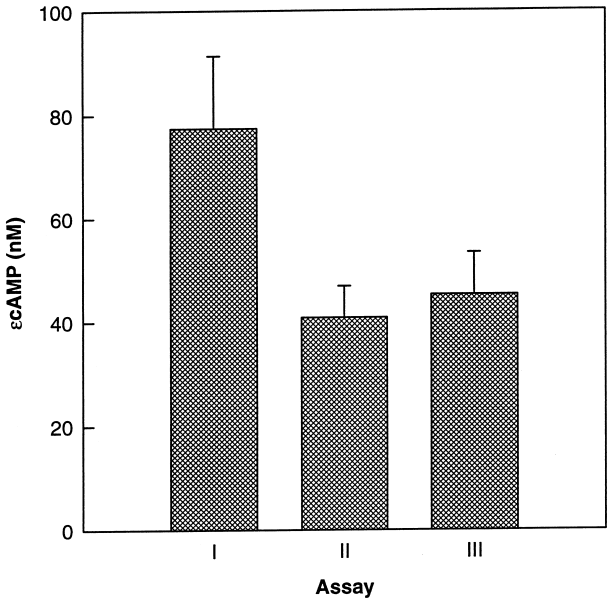

FIG. 4. Formation of ϵcAMP from cAMP in presence of rat liver microsomes and 1 ppm of VC: inhibition by antibody 1-91-3 directed against CYP 2E1. Assays (1 mL) contained 25 mM of cAMP and 0.5 mg (assays I and II) or 1 mg (assay III) of microsomal proteins. I: with 0.5 mg of antibody HyHel (against chicken lysozyme); II: with 0.5 mg of antibody 1-91-3; III, control without VC. Values represent means ± SD of 6 experiments. Data in assay II were significantly different from those in assay I ($P < 0.001$, Bonferroni multiple comparison test).

rats. The *in vivo* V_{max} can be extrapolated directly from the *in vitro* V_{max}. Considering that the liver represents 4% of rat body weight and contains approximately 1% by weight of microsomal proteins, we can calculate a maximum rate of metabolic uptake of 112 μmol of VC/kg bw/hr in rats. Because the metabolic absorption of VC is perfusion-limited [13], the *in vitro* K_m value cannot be directly converted into an inhalational K_m in rats. Applying the pharmacokinetic model developped by Andersen [32] to describe the metabolism of inhaled gases and vapours (see Materials and Methods), the following constants were calculated: C_{max} = 34.31 μmol/L of blood; $K_{m,app}$ = 19.04 μmol/L of blood; $N_{(eff)1/2}$ = 1.28; inhalational $K_{m,app}$ = 14.88 μmol/L of air (equivalent to 360 ppm).

DISCUSSION

Using an assay based on the trapping of the reactive metabolites chloroethylene oxide and 2-chloroacetaldehyde by cAMP to yield the fluorescent product ϵcAMP [19], we investigated the kinetics of VC oxidation by rat liver microsomes. These kinetics were established over a large range of VC concentrations, including low concentrations to which humans have been and even may be exposed today. The data show that VC oxidation follows Michaelis–Menten kinetics, with a V_{max} of 4674 ± 46 pmol · mg protein^{-1} · min^{-1} and a K_m of 7.42 ± 0.37 μM. As confirmed by inhibition and immunoinhibition assays, a single isozyme, CYP 2E1, is involved in the activation of VC by hepatic microsomes from adult, noninduced Sprague–Dawley rats, at concentrations ranging from 1 to 982,000 ppm.

Despite numerous reports on the metabolism of VC, its kinetic constants had not yet been reported, reflecting the general difficulty in studying enzyme kinetics of volatile organic chemicals (see e.g. [16, 35]). Most often, the kinetics of these volatile compounds have been established by measuring their disappearance from the air phase in the presence of an aqueous microsomal suspension (gas uptake methods; e.g. [27, 36, 37]). The problems associated with these assays have been discussed by Hwang *et al.* [35]. They include large variations in kinetic results and insignificant depletion rate of substrate in the gas phase. In this work, we applied a variation of the vial-equilibration method [27] and measured the reactive metabolites formed in the aqueous suspension, following their trapping with cAMP [19, 20]; thereby, we were able to measure activation of VC down to 1 ppm. One disadvantage of our method is that analysis of multiple vials is required. A similar system, but with adenosine as the trapping agent, was previously used by Guengerich *et al.* [5] to study the activation of VC in the presence of human hepatic microsomes; however, it was not designed for studies at low substrate concentrations.

Lineweaver–Burk plots have been used to determine how many isozymes could contribute to the microsomal metabolism of dialkylnitrosamines [38, 39]: polyphasic kinetics were considered as indicating the involvement of several isozymes with different K_m and V_{max}. The reciprocal plot for VC activation by rat liver microsomes shows a single regression line between 982,000 and 1 ppm, thus pointing to a single isozyme.

The involvement of CYP 2E1 in the metabolic activation of VC by human liver microsomes has been demonstrated previously by Guengerich *et al.* [5]. Earlier studies with isolated rat hepatocytes had indicated that the metabolic activation of VC (at 100 ppm in the gas phase) was inhibited by ethanol, thus pointing to an ethanol-sensitive CYP [40]. More recently, Barton *et al.* [16] provided evidence for the metabolism of VC in Sprague–Dawley rats being associated with the activity of CYP 2E1. The phenobarbital-inducible CYP 2B1 is also able to oxidize VC [17, 18], but is present at very low levels in the liver from untreated rats [41]. To confirm that CYP 2E1 was involved in the activation of VC at low concentrations (1 and 2 ppm), we carried out chemical inhibition and immunoinhibition assays. The formation of ϵcAMP was efficiently inhibited by diethyldithiocarbamate, chlorzoxazone and ethanol (Fig. 2). Diethyldithiocarbamate has been established as a mechanism-based, selective inhibitor of human P450 2E1 [5]. Chlorzoxazone, which is oxidized by CYP 2E1, inhibits the metabolism of other CYP 2E1 substrates in a competitive manner [29]. Ethanol is known to be an inhibitor of CYP2E1-related activities *in vitro* [30, 31].

Gas uptake studies in rats have shown that, at low concentrations, the metabolic absorption of VC is perfusion-limited, following first-order kinetics; above 250 ppm,

the kinetics become zero-order [13]. A Michaelis–Menten model has also been applied to describe with a good approximation the metabolism of VC in rats [42]. Assuming that the liver is the major organ involved in the metabolic activation of VC [12], the *in vitro* V_{max} obtained in this study can be converted directly into an *in vivo* V_{max}, yielding a value of 112 μmol/kg bw/hr. In male Wistar rats, Filser and Bolt [13] measured a V_{max} of 110 μmol/kg bw/hr. Gehring *et al.* [42] calculated the metabolic constants for inhalation of [^{14}C]-VC in male Sprague–Dawley rats from the total radioactivity bound to the carcasses and found a V_{max} of 91.3 μmol/kg bw/hr. Omitting an outlying data point from these data, Chen and Blancato [43] recalculated a V_{max} of 107.5 μmol/kg bw/hr. The concordance of the V_{max} extrapolated from *in vitro* measurements with the values determined in experimental animals shows that virtually all the metabolic absorption of VC *in vivo* occurs in the liver.

To convert the *in vitro* K_m into an inhalational $K_{m,app}$, we used the equations established by Andersen [32] and calculated a value of 14.88 μmol/L of air. Filser and Bolt [13] reported data compatible with an inhalational $K_{m,app}$ of 10 μmol/L of air and Gehring *et al.* [42] reported a value of 13.76 μmol/L of air. After recalculation, the latter studies yielded a K_m of 14.93 μmol/L of air [43]. Therefore, our predicted metabolic parameters *in vivo* are very close to those determined experimentally. This further supports the theoretical model developped by Andersen [32] to describe the metabolic absorption of inhaled genotoxicants under perfusion-limited conditions and suggests that the pharmacokinetics of VC in humans could be predicted with an acceptable accuracy from the kinetic constants measured with human liver microsomes.

In conclusion, the kinetic constants measured in this study should be useful in physiologically based pharmacokinetic modelling and cross-species extrapolation (see, e.g. [36, 43, 44]). The assay can also be applied to investigate the metabolism of VC, and of other carcinogens that form etheno adducts [7], by human liver microsomes and at low substrate concentrations.

This work was supported in part by contracts with INSERM (Lyon) and Elf Atochem (Paris) and by the Weisbrem-Benenson Foundation (Paris). We thank Dr. S. S. Park for providing the antibodies against CYP isozymes and Dr. M. F. Rajewsky for the anti-εdAdo antibody.

References

1. IARC, Some monomers, plastics and synthetic elastomers, and acrolein. In: *IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans*, Vol. 19, pp. 377–438. IARC, Lyon, 1979.
2. Simonato L, L'Abbé KA, Andersen A, Belli S, Comba P, Engholm G, Ferro G, Hagmar L, Langard S, Lundberg I, Pirastu R, Thomas P, Winkelmann R and Saracci R, A collaborative study of cancer incidence and mortality among vinyl chloride workers. *Scand J Work Environ Health* **17:** 159–169, 1991.
3. Giri AK, Genetic toxicology of vinyl chloride—a review. *Mutation Res* **339:** 1–14, 1995.
4. Barbin A, Brésil H, Croisy A, Jacquignon P, Malaveille C, Montesano R and Bartsch H, Liver-microsome-mediated formation of alkylating agents from vinyl bromide and vinyl chloride. *Biochem Biophys Res Commun* **67:** 596–603, 1975.
5. Guengerich FP, Kim DH and Iwasaki M, Role of human cytochrome P-450 IIE1 in the oxidation of many low molecular weight cancer suspects. *Chem Res Toxicol* **4:** 168–179, 1991.
6. Bolt HM, Vinyl halides, haloaldehydes and monohaloalkanes. In: *DNA Adducts: Identification and Biological Significance* (Eds. Hemminki K, Dipple A, Shuker DEG, Kadlubar FF, Segerbäck D and Bartsch H), pp. 141–150. IARC Scientific Publications No. 125, IARC, Lyon, 1994.
7. Bartsch H, Barbin A, Marion MJ, Nair J and Guichard Y, Formation, detection, and role in carcinogenesis of ethenobases in DNA. *Drug Metab Rev* **26:** 349–371, 1994.
8. Grollman AP and Shibutani S, Mutagenic specificity of chemical carcinogens as determined by studies of single DNA adducts. In: *DNA Adducts: Identification and Biological Significance* (Eds. Hemminki K, Dipple A, Shuker DEG, Kadlubar FF, Segerbäck D and Bartsch H), pp. 385–397. IARC Scientific Publications No. 125, IARC, Lyon, 1994.
9. O'Neill I, Barbin A, Friesen M and Bartsch H, Reaction kinetics and cytosine adducts of chloroethylene oxide and chloroacetaldehyde: direct observation of intermediates by FTNMR and GC-MS. In: *The Role of Cyclic Nucleic Acid Adducts in Carcinogenesis and Mutagenesis* (Eds. Singer B and Bartsch H), pp. 57–73. IARC Scientific Publications No. 70, IARC, Lyon, 1986.
10. Guengerich FP, Roles of the vinyl chloride oxidation products 1-chlorooxirane and 2-chloroacetaldehyde in the *in vitro* formation of etheno adducts of nucleic acid bases. *Chem Res Toxicol* **5:** 2–5, 1992.
11. ECETOC. *The Mutagenicity and Carcinogenicity of Vinyl Chloride: A Historical Review and Assessment*. ECETOC Technical Report No. 31, ISSN 0773-8072-31, Brussels, Belgium, 1988.
12. Bolt HM, Filser JG, Laib RJ and Ottenwälder H, Binding kinetics of vinyl chloride and vinyl bromide at very low doses. *Arch Toxicol Suppl* **3:** 129–142, 1980.
13. Filser JG and Bolt HM, Pharmacokinetics of halogenated ethylenes in rats. *Arch Toxicol* **42:** 123–136, 1979.
14. Bartsch H, Sabadie N, Malaveille C, Camus AM and Richter-Reichhelm HB, Carcinogen metabolism with human and experimental animal tissues: interindividual and species differences. In: *Advances in Medical Oncology, Research and Education* (Ed. Birch JM), pp. 179–187. Pergamon Press, Oxford and New York, 1979.
15. Sabadie N, Malaveille C, Camus AM and Bartsch H, Comparison of the hydroxylation of benzo(a)pyrene with the metabolism of vinyl chloride, *N*-nitroso-morpholine, and *N*-nitroso-*N'*-methylpiperazine to mutagens by human and rat liver microsomal fractions. *Cancer Res* **40:** 119–126, 1980.
16. Barton HA, Creech JR, Godin CS, Randall GM and Seckel CS, Chloroethylene mixtures: pharmacokinetic modeling and *in vitro* metabolism of vinyl chloride, trichloroethylene, and *trans*-1,2-dichloroethylene in rat. *Toxicol Appl Pharmacol* **130:** 237–247, 1995.
17. Guengerich FP and Watanabe PG, Metabolism of [^{14}C]- and [^{36}Cl]-labeled vinyl chloride *in vivo* and *in vitro*. *Biochem Pharmacol* **28:** 589–596, 1979.
18. Baker MT and Ronnenberg WC, Contrasting effects of 1,1,1-trichloroethane on [^{14}C]vinyl chloride metabolism and activation in hepatic microsomes from phenobarbital- and isoniazid-treated rats. *Toxicol Appl Pharmacol* **119:** 17–22, 1993.
19. Rinkus SJ and Legator MS, Fluorometric assay using high-

pressure liquid chromatography for the microsomal metabolism of certain substituted aliphatics to 1,N^6-ethenoadenine-forming metabolites. *Anal Biochem* **150:** 379–393, 1985.
20. El-Ghissassi F, Barbin A, Nair J and Bartsch H, Formation of 1,N^6-ethenoadenine and 3,N^4-ethenocytosine by lipid peroxidation products and nucleic acid bases. *Chem Res Toxicol* **8:** 278–283, 1995.
21. Eberle G, Barbin A, Laib RJ, Ciroussel F, Thomale J, Bartsch H and Rajewsky MF, 1,N^6-etheno-2′-deoxyadenosine and 3,N^4-etheno-2′-deoxycytidine detected by monoclonal antibodies in lung and liver of rats exposed to vinyl chloride. *Carcinogenesis* **10:** 209–212, 1989.
22. Park SS, Fujino T, West D, Guengerich FP and Gelboin HV, Monoclonal antibodies that inhibit enzyme activity of 3-methylcholanthrene-induced cytochrome P-450. *Cancer Res* **42:** 1798–1808, 1982.
23. Park SS, Fujino T, Miller H, Guengerich FP and Gelboin HV, Monoclonal antibodies to phenobarbital-induced rat liver cytochrome P-450. *Biochem Pharmacol* **33:** 2071–2081, 1984.
24. Park SS, Ko IY, Patten C, Yang CS and Gelboin HV, Monoclonal antibodies to ethanol induced cytochrome P-450 that inhibit aniline and nitrosamine metabolism. *Biochem Pharmacol* **35:** 2855–2858, 1986.
25. Camus AM, Geneste O, Honkakoski P, Béržiat JC, Henderson CJ, Wolf CR, Bartsch H and Lang MA, High variability of nitrosamine metabolism among individuals: role of cytochromes P450 2A6 and 2E1 in the dealkylation of *N*-nitrosodimethylamine and *N*-nitrosodiethylamine in mice and humans. *Mol Carcinog* **7:** 268–275, 1993.
26. Omura T and Sato R, The carbon monoxide binding pigment of liver microsomes. II. solubilization, purification and properties. *J Biol Chem* **239:** 2379–2385, 1964.
27. Sato A and Nakajima T, A vial-equilibration method to evaluate the drug-metabolizing enzyme activity for volatile hydrocarbons. *Toxicol Appl Pharmacol* **47:** 41–46, 1979.
28. Gargas ML, Burgess RJ, Voisard DE, Cason GH and Andersen ME, Partition coefficients of low-molecular-weight volatile chemicals in various liquids and tissues. *Toxicol Appl Pharmacol* **98:** 87–99, 1989.
29. Peter R, Bocker R, Beaune PH, Iwasaki M, Guengerich FP and Yang CS, Hydroxylation of chlorzoxazone as a specific probe for human liver cytochrome P-450IIE1. *Chem Res Toxicol* **3:** 566–573, 1990.
30. Peng R, Tu YY and Yang CS, The induction and competitive inhibition of a high affinity microsomal nitrosodimethylamine demethylase by ethanol. *Carcinogenesis* **3:** 1457–1461, 1982.
31. Subramanian U and Ahmed AE, Intestinal toxicity of acrylonitrile: *in vitro* metabolism by intestinal cytochrome P450 2E1. *Toxicol Appl Pharmacol* **135:** 1–8, 1995.
32. Andersen ME, A physiologically based toxicokinetic description of the metabolism of inhaled gases and vapors: analysis at steady state. *Toxicol Appl Pharmacol* **60:** 509–526, 1981.
33. Fiserova Bergerova V, Extrapolation of physiological parameters for physiologically based simulation models. *Toxicol Lett* **79:** 77–86, 1995.
34. Gargas ML, Seybold PG and Andersen ME, Modeling the tissue solubilities and metabolic rate constant (V_{max}) of halogenated methanes, ethanes, and ethylenes. *Toxicol Lett* **43:** 235–256, 1988.
35. Hwang IY, Reardon KF, Tessari JD and Yang RS, A gas-liquid system for enzyme kinetic studies of volatile organic chemicals. Determination of enzyme kinetic constants and partition coefficients of trichloroethylene. *Drug Metab Dispos* **24:** 377–382, 1996.
36. Hilderbrand RL, Andersen ME and Jenkins LJ, Prediction of *in vivo* kinetic constants for metabolism of inhaled vapors from kinetic constants measured *in vitro*. *Fundam Appl Toxicol* **1:** 403–409, 1981.
37. Cantoreggi S and Keller DA, Pharmacokinetics and metabolism of vinyl fluoride *in vivo* and *in vitro*. *Toxicol Appl Pharmacol* **143:** 130–139, 1997.
38. Tu YY and Yang CS, High-affinity nitrosamine dealkylase system in rat liver microsomes and its induction by fasting. *Cancer Res* **43:** 623–629, 1983.
39. Yoo JS, Guengerich FP and Yang CS, Metabolism of *N*-nitrosodialkylamines by human liver microsomes. *Cancer Res* **48:** 1499–1504, 1988.
40. Hultmark D, Sundh K, Johansson L and Arrhenius E, Ethanol inhibition of vinyl chloride metabolism in isolated rat hepatocytes. *Chem Biol Interact* **25:** 1–6, 1979.
41. Guengerich FP, Wang P and Davidson NK, Estimation of isozymes of microsomal cytochrome P-450 in rats, rabbits, and humans using immunochemical staining coupled with sodium dodecyl sulfate-polyacrylamide gel electrophoresis. *Biochemistry* **21:** 1698–1706, 1982.
42. Gehring PJ, Watanabe PG and Park CN, Resolution of dose-response toxicity data for chemicals requiring metabolic activation: example—vinyl chloride. *Toxicol Appl Pharmacol* **44:** 581–591, 1978.
43. Chen CW and Blancato JN, Incorporation of biological information in cancer risk assessment: example—vinyl chloride. *Cell Biol Toxicol* **5:** 417–444, 1989.
44. Reitz RH, Gargas ML, Andersen ME, Provan WM and Green TL, Predicting cancer risk from vinyl chloride exposure with a physiologically based pharmacokinetic model. *Toxicol Appl Pharmacol* **137:** 253–267, 1996.