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SENSITIVE GAS CHROMATOGRAPHIC METHODS FOR THE DETERMINATION OF VINYL EPOXIDE SYNTHETASE ACTIVITY USING TRICHLOROETHYLENE AS A MODEL SUBSTRATE

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SUMMARY

Two specific and very sensitive methods for the determination of vinyl epoxide synthetase activity in liver microsomes are described. Trichloroethylene, which is used as a substrate, is converted into trichloroethylene oxide by a hepatic epoxide synthetase. Chloral hydrate, the final rearrangement product of trichloroethylene oxide, is determined by electron-capture gas chromatography, either after derivatization with pentafluorophenylhydrazine or after its conversion into chloroform under alkaline conditions. The kinetic parameters of the epoxidation reaction were determined on rat hepatic microsomal suspensions.

INTRODUCTION

It is becoming increasingly evident that many chemically inert exogeneous compounds are converted into biologically reactive intermediates by the microsomal mixed function oxidases[1–3]. Some of these highly reactive metabolites act as the ultimate carcinogens[4,5] and mutagens[6,7] by binding covalently to nucleophilic groups in cellular macromolecules[8]. Electrophilic epoxides belong to this class of electrophilic intermediates and are believed to be responsible for the toxicity, carcinogenicity and mutagenicity of polycyclic hydrocarbons[9–15] as well as of some chlorinated olefins, such as vinyl chloride[16–18], vinylidene chloride[19], 2-chlorobutadiene[19] and trichloroethylene[20,21].

In order to investigate the role of the microsomal enzymatic system in the carcinogenesis and mutagenesis of several chlorinated olefins, we have developed a convenient assay for the specific determination of the vinyl epoxide synthetase activity.

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Trichloroethylene was selected as a model substrate for several reasons:

(1) Trichloroethylene is a carcinogenic compound, which causes liver-cell carcinoma in mice[22]; moreover, it is mutagenic, inducing reverse mutations in *Escherichia coli* strain K12 in the presence of a metabolic activating system[20].

(2) It has been shown[21] to be metabolized into an epoxide intermediate by the mixed function oxidases; this epoxide, which has been shown to bind to cellular macromolecules[22], rearranges spontaneously into trichloroacetaldehyde, which is hydrated to form chloral hydrate; chloral hydrate is further metabolized to either trichloroethanol or trichloroacetic acid, the two major excretion products of trichloroethylene (Fig. 1).

(3) Contrary to the aldehydes resulting from the rearrangement of oxiranes formed during microsomal incubation of most chlorinated olefins, chloral hydrate is very stable in aqueous solution and therefore adequate for a quantitative analysis. Nevertheless, because of the insufficient sensitivity of existing methods for the determination of chloral hydrate in biological media[23–25], it appeared necessary to develop a sensitive method that could be applicable to the determination of the low vinyl epoxide synthetase activities present in some tissues, its kinetic parameters and their modifications under the influence of various pre-treatments of the animals.

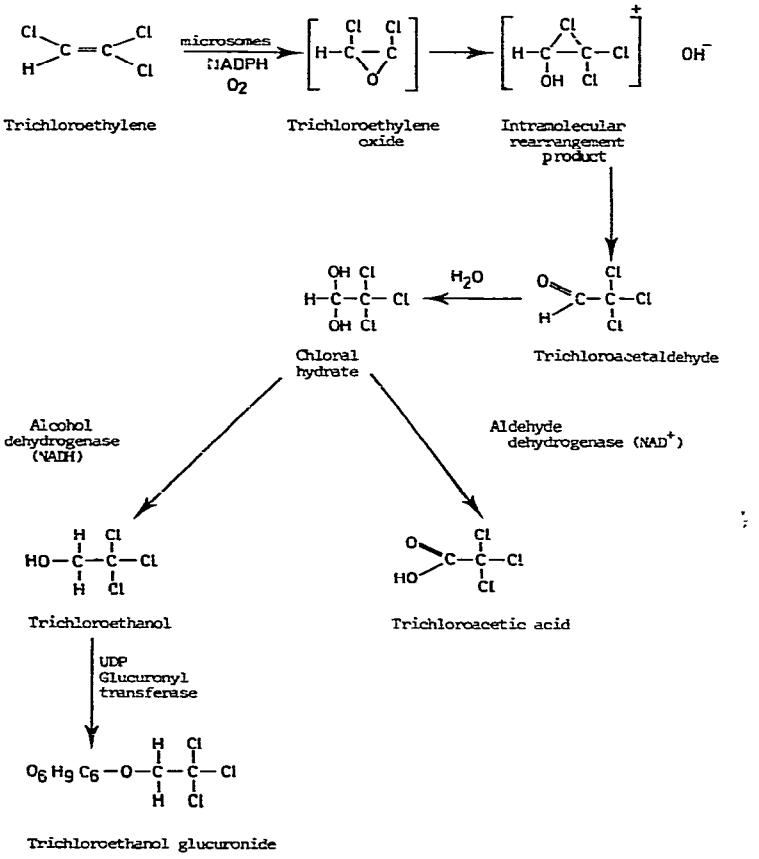

Fig. 1. Proposed metabolic scheme for trichloroethylene.

EXPERIMENTAL

Reagents and chemicals

Trichloroethylene, chloral hydrate, hexanal and pentafluorophenylhydrazine were obtained from Aldrich (Milwaukee, Wisc., U.S.A.).

Hexanal diethylacetal was prepared as described by Vogel[26].

n-Hexane and *n*-heptane (Nanograde quality) were purified by filtration on silica gel activated at 170° and kept on 5 Å molecular sieve, activated at 300°.

Purification of the solvents was necessary in order to remove trace amounts of contaminants, such as chloroform, which could subsequently interfere in the assay.

Apparatus and conditions

A Hewlett-Packard Model 5750 G gas chromatograph equipped with a nickel-63 electron-capture detector (ECD) was used for the measurement of the pentafluorophenylhydrazine derivatives of chloral hydrate. The spiral borosilicate-glass column (2.0 m × 4 mm I.D.) was packed with 3% OV-1 on Supelcoport (80–100 mesh). The conditions for analysis were as follows: injector temperature, 150°; column temperature, 140°; detector temperature, 250°; carrier gas, argon–methane (95:5) at a flow-rate of 50 ml/min.

In the second method, based on the measurement of the chloroform formed, a Perkin-Elmer 3920 B gas chromatograph equipped with a nickel-63 ECD was used. A spiral stainless-steel column (2.0 m × 3 mm I.D.), packed with 10% Apiezon L on Supelcoport (80–100 mesh), was used under the following operating conditions: injector temperature, 200°; column temperature, 80°; detector temperature, 250°; carrier gas, argon–methane (95:5) at a flow-rate of 50 ml/min.

Mass spectrometric determinations were carried out with an LKB 9000 S instrument. Derivatives were injected into the gas chromatograph with a flash heater temperature of 250°, a carrier gas (helium) flow-rate of 30 ml/min and an oven temperature of 180°.

The coiled glass gas chromatographic (GC) column (2.2 m × 3 mm I.D.) was packed with 1% OV-1 on 60–80-mesh Chromosorb W. The Ryhage type of molecular separator was maintained at 270°; mass spectra were recorded at electron energy 70 eV, trap current 60 μA and ion-source temperature 270°.

Animals

Male wistar rats weighing approximately 200–250 g were used routinely. All animals were fed normally and fasted for 24 h before sacrifice by decapitation.

Preparation of microsomes

Livers were immediately removed, weighed and rinsed free of blood. The chilled livers were minced and homogenized at 0° in a Potter–Elvehjem homogenizer in 0.25 *M* sucrose, buffered at pH 7.4 with 0.03 *M* imidazole, to make a 33% (w/v) homogenate.

The microsomes were prepared according to the procedure described by De Duve[27] by subjecting the liver homogenate to fractional centrifugation at 4° in a Heraeus–Chirst ultracentrifuge. The microsomal pellet thus obtained was re-suspended in 0.25 *M* sucrose plus 0.03 *M* imidazole (pH 7.4) with a Dounce homogenizer. Protein concentrations were determined by the method of Lowry *et al.*[28].

The NADPH-generating system contained the following constituents in a final volume of 2 ml: 0.05 *M* Tris–hydrochloric acid, pH 7.8; 10 m*M* $NADP^+$; 40 m*M* glucose 6-phosphate; 6.25 m*M* magnesium chloride; 0.05 m*M* manganese (II) chloride and 4 μl of glucose 6-phosphate dehydrogenase (350 U/mg).

Methods

The NADPH-generating system (2.0 ml) was pre-incubated at 37° for 15 min, in glass-stoppered tubes and in the presence of 25 μl of a 7.5% methanolic solution of trichloroethylene. The reaction was initiated by the addition of 0.30 ml of the microsomal suspension, corresponding to 0.50 mg of protein. Incubation was carried out at 37° in a metabolic shaker for 8 min. The reaction was stopped by adding 0.2 ml 60% perchloric acid to the incubation mixture. The tubes were centrifuged and the supernatant was removed and washed twice with *n*-hexane in order to eliminate the unreacted trichloroethylene.

Two alternative GC methods were developed for the determination of the chloral hydrate formed: the first, the "derivatization method", is based on the measurement of pentafluorophenylhydrazine derivative of chloral hydrate, and the second, the "chloroform method", on the determination of the amount of chloroform formed after its conversion under alkaline conditions.

Derivatization method. A fixed amount (15 μl) of an acetonitrile solution of hexanal diethylacetal (20 μl dissolved in 1 l), used as the internal standard, was added to an aliquot (1.0 ml) of the supernatant. A 0.3-ml volume of a 0.095 *M* solution of pentafluorophenylhydrazine in 3 *N* hydrochloric acid was introduced and the mixture was left for 6 h at room temperature.

The derivatives of both chloral hydrate and the internal standard were extracted by shaking for 2 min with 1.0 ml of *n*-hexane. The organic phase was washed with 1.0 ml 3 *N* hydrochloric acid and aliquots (1–2 μl) were injected on to the gas chromatograph.

Chloroform method. This method takes advantage of the easy conversion of chloral hydrate into chloroform under alkaline conditions (the haloform reaction).

An aliquot (1.0 ml) of the previously neutralized supernatant was treated with 100 μl of 5 *N* sodium hydroxide solution for 15 min and simultaneously shaken with 2.0 ml of *n*-heptane containing an adequate amount of carbon tetrachloride used as an internal standard.

Aliquots of the organic phase (1–2 μl) containing both chloroform and carbon tetrachloride were directly injected into the gas chromatograph.

RESULTS AND DISCUSSION

Derivatization method

Typical GC results are shown in Fig. 2. The pentafluorophenylhydrazine derivatives of the two aldehydes were clearly separated. No chloral hydrate peak was observed in gas chromatograms with samples similarly obtained from extracts of control reaction mixtures using boiled microsomes, indicating that non-enzymatic oxidation of trichloroethylene is negligible.

The identities of the pentafluorophenylhydrazine derivatives were established by mass spectrometry, as shown in Figs. 3 and 4.

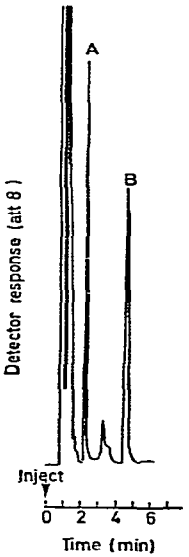

Fig. 2. Typical gas chromatogram of the derivatives of participants in the vinyl epoxide synthetase assay, as extracted from the incubation mixture. Separation of the pentafluorophenylhydrazine derivatives of chloral (A) and hexanal (B).

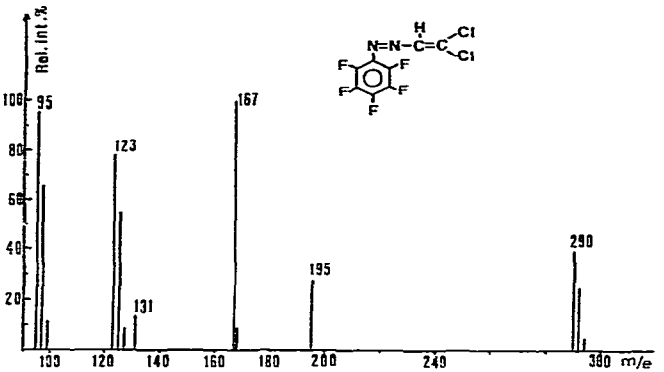

Fig. 3. Mass spectrum of the pentafluorophenylhydrazine derivative of chloral hydrate.

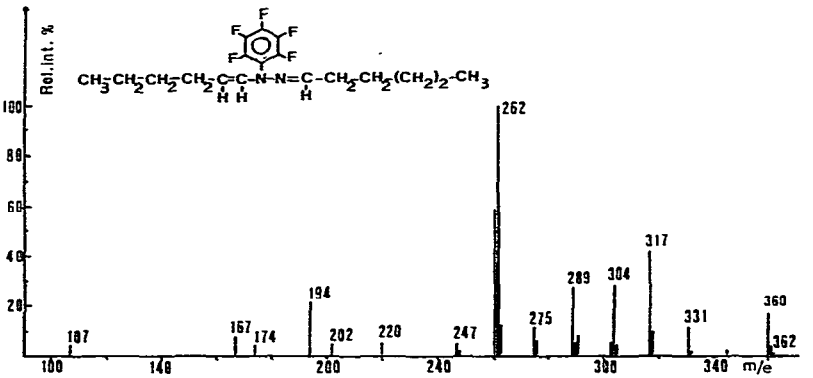

Fig. 4. Mass spectrum of the pentafluorophenylhydrazine derivative of hexanal diethylacetal.

The mass spectrum of 2,2-chloro-1-pentafluorophenylazoethylene (Fig. 3) shows a molecular ion at *m/e* 290, accompanied by isotopic peaks at *m/e* 292 and 294. The relationship between the intensities of these ions is in accordance with the presence of two chlorine atoms in the molecule. Moreover, the presence on the one hand of fragmentation peaks at *m/e* 195 ($C_6F_5N \equiv N$) and 167 ($C_6F_5^+$), belonging to the aromatic moiety of the molecule, and on the other hand at

m/e 123 $\left(N \equiv \dot{\overset{+}{N}} - CH = \underset{\displaystyle Cl}{\overset{\displaystyle Cl}{\overset{|}{\underset{|}{C}}}} \right)$ and 95 $\left({}^{+}CH = \underset{\displaystyle Cl}{\overset{\displaystyle Cl}{\overset{|}{\underset{|}{C}}}} \right)$

belonging to the aliphatic moiety of the molecule and accompanied by the same isotopic peaks (*m/e* 125, 127 and 97, 99) as those mentioned for the molecular ion, indicates clearly the nature of the product that was formed.

The mass spectrum of the pentafluorophenylhydrazine derivative resulting from the condensation of two molecules of hexanal dehydrodiethylacetal in acidic medium is illustrated in Fig. 4. It shows a small molecular ion at *m/e* 362 and main fragmentation peaks at *m/e* 360$^+$ (—H_2), 331$^+$ (360—C_2H_5), 317$^+$ (360—C_3H_7), 304$^{+\cdot}$ (360—CH_2 = CH–CH_2–CH_3), 289$^+$ (304$^{+\cdot}$—CH_3), 275$^+$ (304$^{+\cdot}$—C_2H_5), 262$^{+\cdot}$ (304$^{+\cdot}$—CH_3–CH= CH_2 as evidenced by the presence of a metastable peak at *m/e* 225.2; calculated value 225.8), which are in good agreement with the previously mentioned condensation reaction and with the structure of the pentafluorophenylhydrazine derivative that was formed.

The concentrations of chloral hydrate formed after incubation of trichloroethylene with the microsomal preparations were calculated from a calibration graph (Fig. 5) constructed from chromatograms for biological samples containing various amounts of chloral hydrate and a fixed amount (1500 ng/ml) of the internal standard.

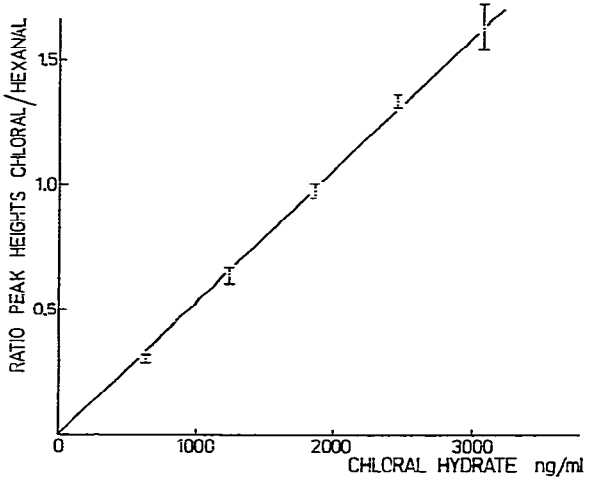

Fig. 5. Calibration graph for the determination of chloral hydrate in microsomal suspension (1500 ng/ml of internal standard added) according to the derivatization method.

(ratio of the peak height of derivatized chloral hydrate to that of derivatized internal standard plotted against concentration).

The detection limits, under the conditions applied, permit the determination of 30 ng of chloral hydrate per millilitre of incubation mixture. Lower detection limits can easily be obtained by concentrating the final organic phase. The precision was determined by measuring the peak-height ratio after adding known concentrations of chloral hydrate to a blank microsomal preparation. Relative standard deviations did not exceed 4% (five determinations) for concentrations ranging from 150 to 1000 ng/ml.

Chloroform method

The conversion of chloral hydrate into chloroform is dependent on time, temperature and sodium hydroxide concentration.

Working at room temperature (20–24°), the reaction was complete after 10 min and maximal for sodium hydroxide concentrations ranging from 0.01 to 1.00 *N* (Fig. 6). At sodium hydroxide concentrations lower than 0.01 *N*, the conversion into chloroform was incomplete; on the other hand, chloroform was progressively degraded into dichlorocarbene when the sodium hydroxide concentration was higher than 1.00 *N*.

Fig. 7 represents a typical electron-capture gas chromatogram obtained after extraction of chloroform (A) and carbon tetrachloride (B) from a microsomal incubation mixture.

The concentrations of chloral hydrate were determined from a calibration graph (Fig. 8), obtained by adding various amounts (10–200 ng/ml) of chloral hydrate and a fixed amount of carbon tetrachloride (10 ng/ml) to the microsomal preparation, carrying out the described procedure and plotting the ratio of the peak height of chloroform to that of carbon tetrachloride against concentration of chloral hydrate.

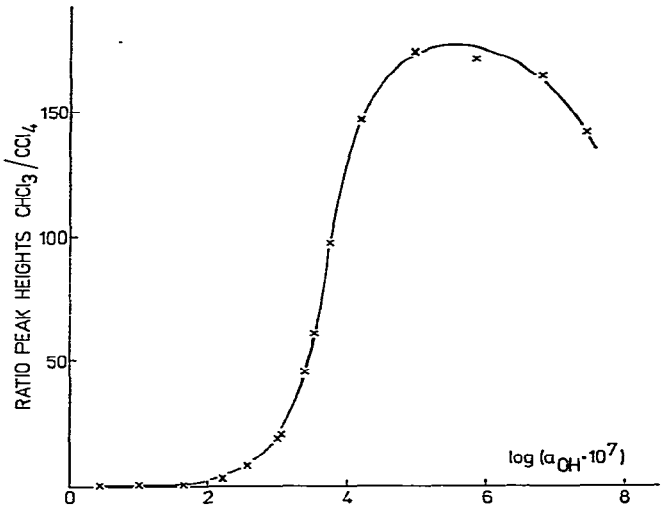

Fig. 6. Variation of the concentration of chloroform produced as a function of OH^- activity (a_{OH}) (temperature, 24°; reaction time, 10 min). The OH^- activities at high NaOH concentrations were calculated from mean activity coefficients given by Robinson and Stokes[29].

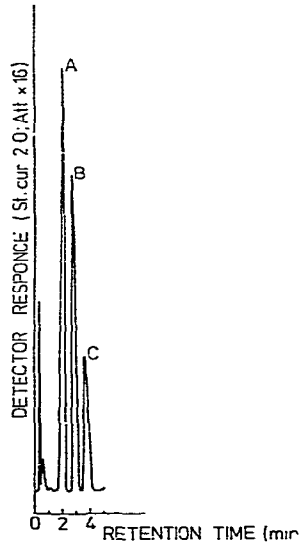

Fig. 7. Typical gas chromatogram of the participants in the vinyl epoxide synthetase assay (chloroform method) as extracted from the incubation mixture. Separation of chloroform (A), carbon tetrachloride (B) and *n*-heptane (C).

Concentrations as low as 3 ng per millilitre of incubation mixture can be determined with this procedure. Relative standard deviations ranged from 5 to 8% (six determinations).

The chloroform method is simpler and quicker than the derivatization method but suffers the disadvantage of being specific for chloral hydrate and therefore restricted to the determination of the vinyl epoxide synthetase activity using trichloroethylene

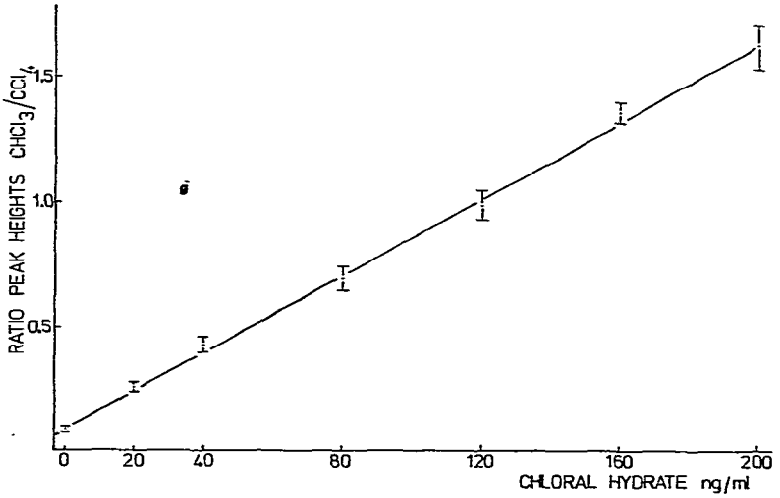

Fig. 8. Calibration graph for the determination of chloral hydrate in microsomal preparation (10 ng/ml of carbon tetrachloride added) according to the chloroform method; six determinations were performed at each concentration.

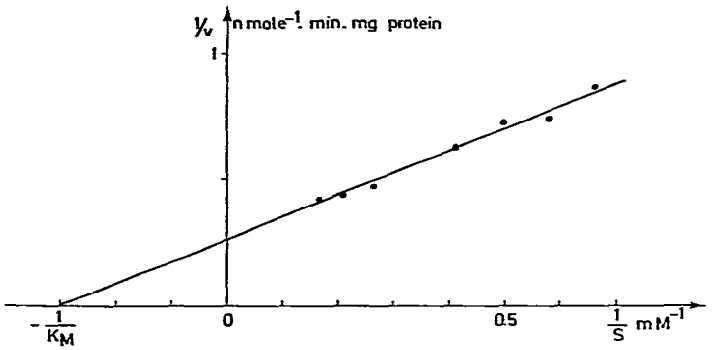

Fig. 9. Lineweaver–Burk double reciprocal plot of rat liver microsomal oxidation of trichloroethylene.

as the only possible model substrate. The derivatization method, on the other hand, can be extended to every aldehyde, such as those formed from vinylic compounds other than trichloroethylene.

The vinyl epoxide synthetase activity of rat liver microsomes was shown to be maximal at pH 7.8 and linear with time up to 8–9 min. Kinetic measurements demonstrated that the reaction rate increases linearly with protein concentration up to 0.25 mg per millilitre of incubation mixture. Lineweaver–Burk plots (Fig. 9) obtained under Michaelis–Menten experimental conditions permitted the determination of the kinetic parameters of the enzymatic activity: $V_{max} = 3.8$ nmol/min · mg protein; $K_m = 3.3$ mM.

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