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DETERMINATION OF UROCHLORALIC ACID, THE GLUCURONIC ACID CONJUGATE OF TRICHLOROETHANOL, BY GAS CHROMATOGRAPHY WITH ELECTRON-CAPTURE DETECTION AND ITS APPLICATION TO URINE, PLASMA AND LIVER

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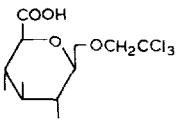
SUMMARY

Gas chromatography with electron-capture detection was used to quantify the glucuronic acid conjugate of trichloroethanol, urochloralic acid. The conjugate was extracted into ethyl acetate—ethanol (19:1, v/v) under acidic conditions, and analyzed as its trifluoroacetylated methyl ester or as its acetylated methyl ester. As little as 0.2 μg of the conjugate was quantified, and the method was applicable to urine, plasma and liver. The synthesis of urochloralic acid from trichloroethanol by rat liver microsomal fraction was demonstrated in the presence of uridine diphosphoglucuronic acid as coenzyme.

INTRODUCTION

We have studied the metabolism of trichloroethylene in vitro using rat liver [1]. It is well known that trichloroethylene is oxidized to its epoxide, which is transformed to chloral hydrate and successively reduced to trichloroethanol (TCE). TCE has been reported to be conjugated with glucuronate in vivo to form urochloralic acid (UCA). Human and animals excrete UCA along with



TCE when they have inhaled trichloroethylene vapour [1—4], or have been administered chloral hydrate [4, 5], triclofos [6], or TCE [4, 7]. However, biochemical studies on the glucuronide formation have not until now been reported. We desired to investigate the formation of UCA in vitro, but found no sensitive method for its determination.

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A number of methods for the determination of UCA have been published [8—12]. In our preliminary experiments, colorimetry of UCA [8, 9] could not be applied to the biochemical studies in vitro because of its low sensitivity and low specificity. With the gas chromatographic methods [10—12] the amount of UCA is calculated as the difference between free TCE and total TCE determined after hydrolysis of UCA by acid or β-glucuronidase. The indirect method for the assay of UCA has been widely applied to serum and urine samples, but this method is more useful if UCA is present in much larger, or about equal, amounts compared to free TCE in the samples. However, when determining a relatively small amount of UCA as, for example, in our in vitro experiment on the conjugation of TCE, the indirect method is inadequate for accurate determination.

Here we describe a direct and sensitive method for the assay of UCA using gas chromatography with electron-capture detection (ECD). The method is applicable to urine, plasma and liver homogenates. Several features of the conjugation of TCE in vitro are also presented here.

MATERIALS AND METHODS

Reagents and instruments

All chemicals were of analytical grade, and the organic solvents for extraction were of especially fine grade; these were purchased from Wako Pure Chemicals (Osaka, Japan). Uridine diphosphoglucuronic acid (UDPGA) was obtained from Yamasa Shoyu (Chiba, Japan), and uridine diphospho-N-acetylglucosamine (UDPAG) and ATP from Sigma (St. Louis, MO, U.S.A.). Authentic methyl ester of acetyl-UCA was synthesized in this laboratory according to the method of Seto and Schultze [13]; authentic UCA was kindly supplied by the Central Institute of Chugai Pharmaceutical Co. (Tokyo, Japan).

Organic solvents were evaporated under reduced pressure by a Speed Vac Concentrator (Savant, U.S.A.).

Extraction of UCA from biological samples

An aliquot (0.5 ml) of plasma, urine, or liver homogenate prepared as described below was added to *n*-hexane—benzene (1:1, v/v, 1.5 ml), stirred vigorously, and centrifuged at 1000 *g* for 10 min. After removal of the organic layer by aspiration, the same procedure was repeated again. After acidification of the aqueous phase with 6 *M* hydrochloric acid (0.1 ml), UCA was extracted twice with ethyl acetate—ethanol (19:1, v/v) (1.5 and 1 ml). The organic solvents were combined, dried over anhydrous sodium sulphate, and evaporated to dryness under reduced pressure.

Rat liver was homogenized in 9 vols. of 1.15% potassium chloride with a Potter-Elvejhem PTFE pestle homogenizer. The homogenate was centrifuged at 700 *g* for 10 min. The supernatant fluid was used for recovery tests. When the formation of UCA in vitro was studied using rat liver microsomal fraction, the incubation mixtures described below were treated in the same manner as described above.

Derivatization of UCA

The residue containing UCA was dissolved in 1 ml of ethyl acetate and 0.1 ml of methanol, into which diazomethane was introduced according to the microscale procedure of Schlenk and Gellerman [14]. After standing at room temperature for about 30 min, the solvent was evaporated to dryness under reduced pressure. To the dried residue, ethyl acetate (175 μl) and trifluoroacetic anhydride (TFAA, 25 μl) were added in turn. For acetylation, pyridine (150 μl) and acetic anhydride (50 μl) were added to the residue. Both acetylating reaction mixtures were allowed to stand at room temperature overnight. In the case of trifluoroacetylation, an aliquot (1 μl) of the reaction mixture was injected into the gas chromatograph after appropriate dilution with ethyl acetate. In the case of the acetylation, excess reagents were evaporated under a nitrogen stream at about 40°C, and the residue was dissolved in ethyl acetate (0.2 ml). An aliquot (1 μl) was subjected to gas chromatography after appropriate dilution with ethyl acetate.

Gas chromatography

The gas chromatographic analysis was carried out on a Shimadzu Model GC-4CM gas chromatograph equipped with an electron-capture detector (^{63}Ni), which was operated at a pulse-rate of 10 kHz and a temperature of 230°C for the methyl ester of trifluoroacetyl-UCA and 270°C for that of acetyl-UCA. The glass column, 2 m × 3 mm I.D., was packed with 1.5% Silicone OV-17 on Shimalite W (AW DMCS), 80—100 mesh. The carrier gas was nitrogen at a flow-rate of 40 ml/min. The electrometer setting was continuously kept at range 10^2, attenuation 8. The injection temperature was 230°C and the column oven temperature 180°C for trifluoroacetyl-UCA, and 270°C and 250°C for acetyl-UCA. The peak height was used for quantification.

Preparation of enzyme source

Livers of male Wistar strain albino rats (200—300 g body weight) were used as enzyme source. Rats were killed by decapitation, and the microsomal fraction was prepared by the method of Schöllhammer et al. [15]. Briefly, liver homogenates were centrifuged at 9000 *g* for 20 min, and the supernatant fluid decanted was centrifuged at 105,000 *g* for 60 min. The pellet thus obtained was suspended in an aliquot of 1.15% potassium chloride, and recentrifuged at 105,000 *g* for 60 min. After repeating this washing procedure, the pellet was suspended in a volume of 1.15% potassium chloride equivalent to the original weight of wet liver; this is referred to as the "washed microsomal fraction".

Incubation

The incubation system for conjugation was constituted according to the method of Schöllhammer et al. [15]. The incubation mixture consisted of 0.1 ml of the washed microsomal fraction, 2.5 m*M* magnesium chloride, 0.5 m*M* TCE, 0.5 m*M* UDPGA and 37.5 m*M* Tris—HCl buffer (pH 7.4) in a total volume of 0.5 ml. The mixture in a 10-ml test tube was incubated at 37°C for 60 min with shaking.

UCA formed in the incubation mixture was extracted, reacted with diazomethane and acetylated as described above.

RESULTS

Reaction conditions for derivative formation

Typical gas chromatograms are shown in Fig. 1a, indicating that symmetrical sharp peaks appeared for both trifluoroacetylation and acetylation.

Trifluoroacetylation. To establish optimum reaction conditions for trifluoroacetylation of methyl urochloralate, the reaction temperature and time, and the amount of TFAA were investigated. UCA (2 mg) was previously reacted with diazomethane as described under Materials and methods, and aliquots corresponding to 5 and 10 μg of UCA were reacted with TFAA under various conditions. As shown in Fig. 1b, the maximum peak height was obtained after reaction at room temperature for about 8 h, and remained constant up to 46 h. However, reaction at 60°C for 6 h resulted in the disappearance of the peak. It required 20—30 μl of TFAA to acetylate the methyl ester quantitatively.

Acetylation. We examined the acetylation conditions of methyl uro-

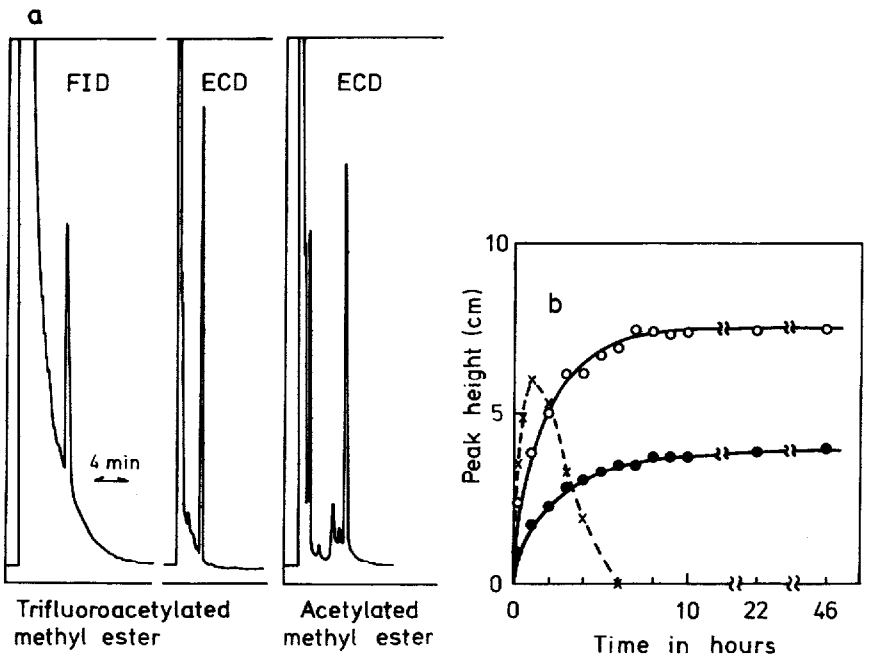

Fig. 1. Typical gas chromatograms obtained from authentic UCA, and reaction conditions for trifluoroacetylation. (a) UCA (10 μg) was reacted with diazomethane and TFAA or acetic anhydride as described under Materials and methods. In the case of the trifluoroacetylation, a 5-μl aliquot of the reaction mixture (0.2 ml) was subjected to gas chromatography with flame-ionization detection (GC—FID), and a 1-μl aliquot after 2000 times dilution with ethyl acetate to GC—ECD. Analytical conditions for GC—FID were as follows: injection and detector temperature 200°C, column temperature 160°C, flow-rate of nitrogen 50 ml/min, attenuation 8, sensitivity 10^3. In the case of acetylation, a 1-μl aliquot of the reaction mixture was injected for GC—ECD after 200 times dilution with ethyl acetate. Analytical conditions were the same as described under Materials and methods. (b) Methyl urochloralate [corresponding to 5 (•) and 10 μg (○) of UCA] was reacted with TFAA (30 μl) in ethyl acetate (0.17 ml) at 25°C (——) and 60°C (- - -) for varius time intervals. In the latter case methyl urochloralate corresponding to 10 μg of UCA was tested. A 5-μl aliquot was subjected to GC—FID.

chloralate with acetic anhydride, by comparison with authentic methyl ester of acetyl-UCA. The best yield was obtained when 25—50 μl of acetic anhydride were added to the reaction mixture (0.2 ml in a total volume with pyridine), followed by standing at room temperature overnight.

Standard and calibration curves

The present assay consists of extraction (Step 1), methylation (Step 2) and acetylation (Step 3). Three experiments were carried out: Step 3 only; Steps 2 and 3; and all the steps. Three curves were prepared as shown in Fig. 2. Accordingly, the difference between standard curves I and II gives the yield of methylation (92%), which proved to fall 40% below 1 μg of UCA in 0.2 ml of the reaction mixture (see inset to Fig. 2). The difference between standard curve II and the calibration curve gives the extraction ratio (89%).

These results were obtained for trifluoroacetylation; the acetylation method gave a similar result (data not shown).

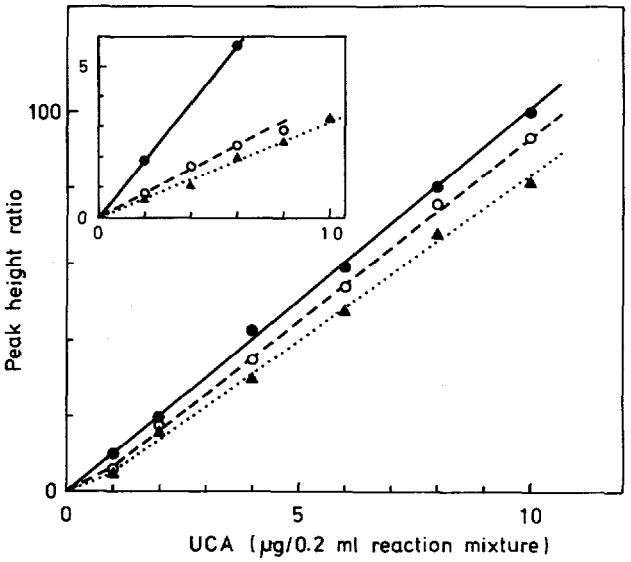

Fig. 2. Standard and calibration curves of the methyl ester of trifluoroacetyl-UCA. Standard curve I (•): UCA (1 mg) was dissolved in ethyl acetate (about 1 ml), reacted with diazomethane at room temperature for 30 min, and excess reagent and the solvent were evaporated. The residue was dissolved in ethyl acetate (1.0 ml), from which standard solutions (corresponding to 100 μg and 10 μg UCA per ml ethyl acetate) were prepared. An aliquot (corresponding to 0.2—10 μg of UCA) of the standard solutions was subjected to trifluoroacetylation, and to gas chromatography as described under Materials and methods. Standard curve II (○): The UCA standard solutions in ethyl acetate (100 and 10 μg/ml) were prepared, and an aliquot (corresponding to 0.2—10 μg of UCA) of the standard solutions was subjected to methylation and trifluoroacetylation, and to gas chromatography as described under Materials and methods. Calibration curve (▲): An aqueous solution (0.5 ml) containing 0.2—10 μg of UCA was prepared, extracted, and derivatized as described under Materials and methods. A 1-μl aliquot of the reaction mixture was injected into the gas chromatograph after appropriate dilution with ethyl acetate. The peak height obtained from 10 μg of UCA in standard curve I was multiplied by a dilution factor (2000), and is represented as 100 in the figure.

Recovery tests

In order to apply the present procedure to the assay of UCA in urine, plasma and liver homogenates, typical gas chromatograms were taken after trifluoroacetylation or acetylation (see Fig. 3), and then recovery tests were carried out. As shown in Fig. 3a, the trifluoroacetylation method was applicable to plasma and liver. However, trifluoroacetylation was found to be unsuitable for UCA determination in urine, since a large peak behind the methyl ester of trifluoroacetyl-UCA interfered (data not shown). Therefore, the acetylation method had to be applied to urine, and the separation of the peaks was satisfactory (Fig. 3b). For the assay of UCA in liver homogenates the acetylation method was also applicable, and was time-saving (compare the chromatograms in Fig. 3a and b). Acetylation could be applied to plasma, but a chromatogram is not shown.

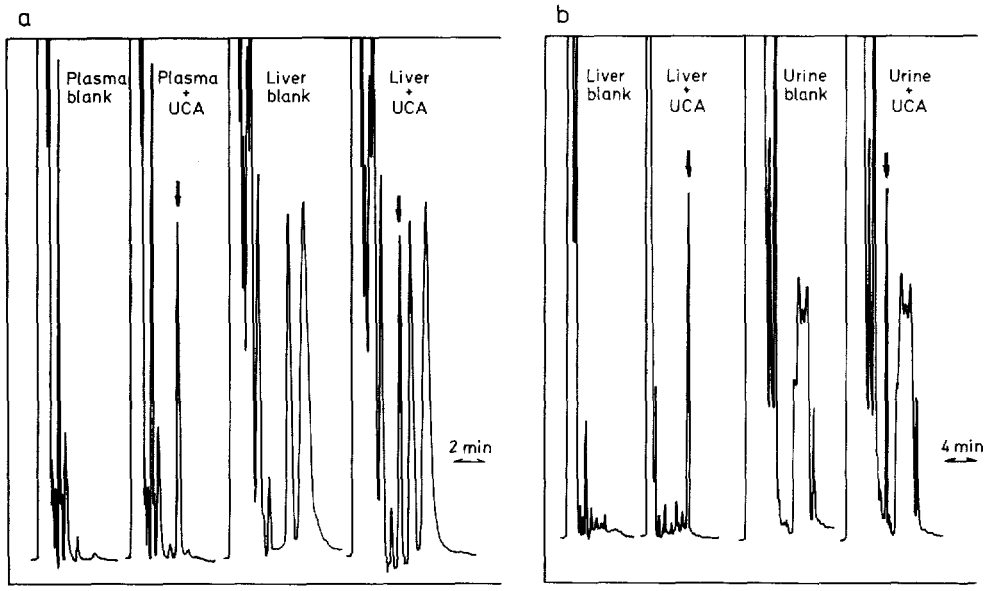

Fig. 3. Typical gas chromatograms obtained from biological samples with and without addition of UCA. After addition of UCA (1 μg) or no addition to biological samples (plasma, liver and urine), they were treated as described under Materials and methods. In the case of trifluoroacetylation (a), a 1-μl aliquot of the reaction mixture was injected into the gas chromatograph after 200 times dilution with ethyl acetate. In the case of acetylation (b), a 1-μl aliquot was subjected to gas chromatography after 20 times dilution. The arrows in the figure indicate the peaks of the derivatives.

Varying amounts of UCA were added to each sample, and the amount of UCA was measured as the methyl ester of trifluoroacetyl-UCA for plasma and liver homogenates, and that of acetyl-UCA for urine. The results are summarized in Table I, indicating that good recoveries were obtained in all cases.

UCA formation by rat liver microsomal fraction

In order to clarify the overall profile of the in vitro formation of UCA, experiments using rat liver microsomal fraction as the enzyme source were per-

TABLE I

RECOVERY TESTS

Biological samples (0.5 ml) with and without addition of UCA were treated in the same manner as described in Materials and methods. Each value represents the mean of duplicate determinations.

Specimen	UCA added (μg)	UCA determined (μg)	Recovery	
			%	Mean ± S.D.
Urine	0	0		97.3 ± 6.9
	1	0.870	87.0	
	2	1.96	98.0	
	3	2.99	99.7	
	5	5.30	106.0	
	10	9.60	96.0	
Plasma	0	0		84.1 ± 4.3
	1	0.876	87.6	
	2	1.73	86.5	
	3	2.42	80.7	
	5	3.91	78.2	
	10	8.74	87.4	
Liver homogenate	0	0		97.4 ± 4.5
	1	0.994	99.4	
	2	1.91	95.5	
	3	2.87	95.7	
	5	4.61	92.2	
	10	10.4	104.0	

formed, and the amount of UCA was determined by the present procedure (acetylation method). The results were as follows.

Effect of incubation time, pH and amount of enzyme. Fig. 4a shows the effect of incubation time on the formation of UCA, revealing that the conjugation proceeds even after 120 min. In this experiment, about 4.5% of the amount of TCE added had been conjugated to UCA after 120 min of incubation. Fig. 4b depicts the effect of pH on the formation of UCA, indicating that the pH optimum is around 7. The dependency of the amount of microsomal fraction on the formation of UCA is shown in Fig. 4c. About 12 nmol of UCA were formed in a test tube that contained about 5 mg of microsomal protein.

Requirements for cofactors. Cofactor requirements for the conjugation were examined. The omission of UDPGA from the complete system described under Materials and methods caused no formation of UCA, while omission of magnesium chloride decreased the formation of UCA from 5.06 nmol/mg protein in the complete system to 2.69 nmol/mg protein. The addition of ATP and UDPAG to the complete system increased the formation of UCA to 17.5 nmol/mg protein; this finding will be discussed later.

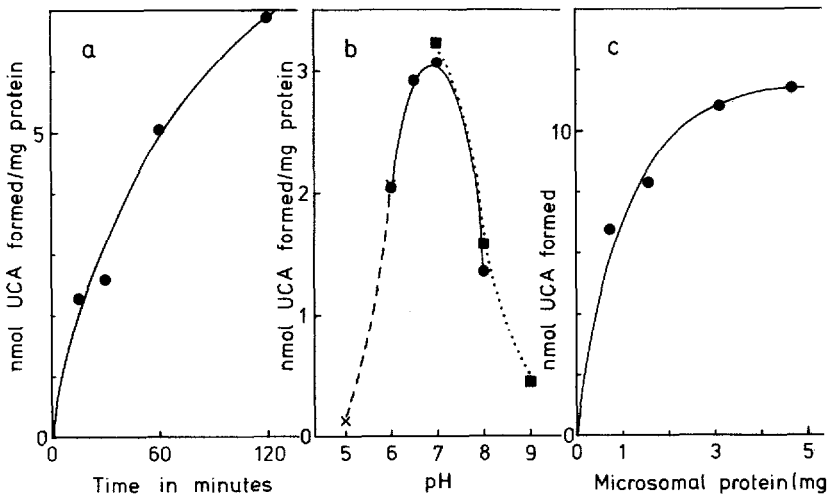

Fig. 4. Effect of incubation time, pH and amount of enzyme source on the formation of UCA. (a) TCE (0.5 m*M*) was incubated with microsomal protein corresponding to 100 mg of wet weight liver in the system described under Incubation at 37°C for 15, 30, 60 or 120 min. (b) Incubation was conducted at 37°C for 60 min as described above, except that acetate (×), potassium phosphate buffer (•) or Tris—HCl (▪) was used to examine the effect of pH on the formation of UCA. (c) TCE (0.5 m*M*) was incubated with various amounts of microsomal fraction from 0.05 to 0.3 ml (about 5 mg of protein) at 37°C for 60 min as described above.

DISCUSSION

As described in Introduction, UCA has been determined as TCE after hydrolysis. Attempts to apply the methods so far published to an in vitro study of the conjugation of TCE with glucuronate were unsuccessful. When UCA was synthesized from TCE by rat liver microsomal fraction, a much smaller amount of UCA than TCE was formed. In this case we had first to remove a large excess of free TCE through repeated extraction with *n*-hexane, but the TCE could not be removed completely. Therefore the exact amount of UCA could not be determined from the difference between total TCE after hydrolysis and remaining TCE by gas chromatography. However, evaporation of the incubation mixture under reduced pressure resulted in complete removal of free TCE, and UCA could be determined as TCE after hydrolysis. By this method, it is not proved whether only glucuronide was determined or other conjugates. For this reason we desired to determine UCA itself.

At first we tried to determine UCA as its trimethylsilylated methyl ester as reported by Verweij and Kientz [16]. That is, UCA was reacted with diazomethane, followed by treatment of the dried residue containing methyl urochloralate with pyridine (0.15 ml), trimethylsilane (50 μl) and hexamethyldisilazane (50 μl) at room temperature for 30 min. The derivative was subjected to gas chromatography with flame-ionization detection. The reproducibility of derivative formation was found to be unsatisfactory.

We thus established two derivatization procedures applicable to biological samples, i.e. trifluoroacetylation and acetylation of the hydroxyl groups of the glucuronic acid moiety. When both methods were compared, the ECD response

of the methyl ester of acetyl-UCA was about one-tenth that of trifluoroacetyl-UCA. However, in practice, the sensitivity of the two methods was almost the same, since at least 100 times dilution of the reaction mixture was necessary to avoid tailing due to TFAA. On the other hand, the acetylated derivative could be analyzed without such interference even after five times dilution of the reaction mixture. In the case of liver homogenates, several peaks appeared behind the acetylated derivative (see Fig. 3); and the trifluoroacetylation method was not applicable to urine as described in Results. As a whole, the acetylation method seems to be more advantageous than the trifluoroacetylation method for analysis of UCA.

UDP-glucuronyltransferase (EC 2.4.1.17), which is responsible for the formation of UCA from TCE, is predominantly located in the liver endoplasmic reticulum [17]. The latency of the enzyme activity in the microsomes has been reported by several investigators [18—24] who studied the conjugation of *p*-nitrophenol, phenolphthalein or bilirubin with glucuronate in vitro. They previously treated the microsomes with perturbants or several reagents to activate the enzyme activity. Similar induction phenomena were observed for TCE as described in the present paper. The addition of ATP and UDPAG, which are supposed to be allosteric effectors for the liver enzyme, resulted in about a four-fold increase in the conjugation of TCE. The concentration of UDPAG in this experiment was 1.24 m*M*, while that in liver was reported to be 0.32 m*M* [25]. Magnesium ion was also shown to stimulate the conjugation of TCE.

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