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METABOLISM OF TRICHLOROETHYLENE

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Abstract—Trichloroethylene was metabolized to chloral hydrate, trichloroethanol and trichloroacetic acid *in vitro*. The three metabolites in the incubation mixture were determined by gas–liquid chromatography using an electron capture detector. The kinetics of the individual steps of the metabolism of trichloroethylene were investigated in rat liver subcellular fractions or recombined fractions. The general features of trichloroethylene metabolism *in vitro* were demonstrated by the conversion of trichloroethyleme to the three metabolites (6 per cent total yield) by the 700 *g* supernatant fraction of rat liver in 2 hr. Oxidation of trichloroethylene to chloral hydrate occurred only in the microsomal fraction of rat liver, as previously reported by Byington and Leibman [5]. (This step was rate-limiting and was stimulated by both phenobarbital and 3-methylcholanthrene pretreatment.) Reduction of chloral hydrate to trichloroethanol occurred in the cytosol of rat liver. This activity was separated into at least three fractions by a DEAE cellulose column—one of them was NADH-dependent and the others were NADPH-dependent.) The formation of trichloroacetic acid from chloral hydrate required cytosol or mitochondria with NAD.

Numerous reports about trichloroethylene (Tri) have been published in the field of industrial health or toxicology [1, 2]. In addition, several biochemical investigations concerning Tri have been carried out. Since Barrett and Johnston [3] in 1939 first detected trichloroacetic acid (TCA) in the urine of dogs that had inhaled Tri vapor, the identification or the quantitative analysis of Tri metabolites in blood or urine has been performed using animals exposed to Tri vapor. In 1949, Butler [4] first reported the excretion of trichloroethanol (TCE) in the urine of dogs. He postulated a mechanism of metabolic conversion of Tri and suggested a pathway from Tri to TCE or TCA via chloral hydrate (CH), although he failed to identify CH experimentally [4]. CH was later isolated by Byington and Leibman [5] as 2,4-dinitrophenylhydrazone in an *in vitro* experiment. Daniel [6] postulated that trichloroethylene oxide is an intermediate from Tri to CH. Characteristics of Tri metabolism *in vitro* were first described by Leibman [7] and Leibman and McAllister [8]. CH is thought to be reduced to TCE by liver alcohol dehydrogenase [9] or oxidized to TCA by the NAD-dependent enzyme from rabbit liver [10].

On reviewing these reports, we felt that the individual steps and general features of the metabolic pathway of Tri *in vitro* had not been experimentally clarified.

Here we describe a simple method for quantitative determination of CH, TCE or TCA in homogenates or subcellular fractions of rat liver using a gas chromatograph equipped with an electron capture detector. Using this method, the kinetics of the individual steps of Tri metabolism were studied in rat liver subcellular fractions or recombined fractions.

EXPERIMENTAL

Reagents

Tri and TCE were obtained from the Kanto Chemical Co. (Tokyo) and were redistilled before use. NAD(H), NADP(H), G-6-P and G-6-P dehydrogenase (yeast) were purchased from the Oriental Yeast Co. Ltd. (Tokyo). Other reagents were obtained from the Wako Pure Chemicals Industries Co. Ltd. (Osaka), and were analytical grade except the organic solvents used for extraction which were fine quality for gas chromatography. DEAE-cellulose (DE-52) was purchased from the Whatman Co. Ltd. (Maidstone, U.K.).

Preparation of enzyme source

Livers of Wistar strain albino rats (male, 200–300 g) were used as the enzyme source. Rats were decapitated and the livers were removed immediately, washed in a medium containing 0.25 M sucrose, 3 mM potassium phosphate buffer (KPB, pH 7.4) and 0.1 mM EDTA, and homogenized in 9 vol. of the medium using a Potter–Elvehjem homogenizer with a Teflon pestle. After centrifugation at 700 *g* for 10 min, the supernatant fraction was strained through cheesecloth. Subcellular fractions were prepared in the usual fashion. Briefly, the 700 *g* supernatant fraction was centrifuged at 5000 *g* for 10 min. The pellet was washed twice with a medium containing 0.25 M sucrose and 3 mM KPB (pH 7.4), and is referred to as the mitochondrial fraction. After

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centrifugation of the 5000 *g* supernatant fraction at 9000 *g* for 10 min, the supernatant fluid was pipetted and is referred to as the 9000 *g* supernatant fraction. The 9000 *g* supernatant fraction was further centrifuged at 105,000 *g* for 60 min. The pellet thus obtained was used for most experiments in the present study and is referred to as the unwashed microsomal fraction. The pellet, washed once in the above medium and once in 1.15% KCl, is referred to as the washed microsomal fraction. The 105,000 *g* supernatant fluid is designated the cytosolic fraction.

Crude aldehyde dehydrogenase from mitochondrial fraction was prepared by sonication according to the method described by Tabakoff *et al.* [11]. All procedures were performed between 0 and 4°.

Incubation conditions

Warburg flasks (15 ml) were used as incubation vessels. The incubation mixture was introduced into the main chamber of the flask. The complete incubation mixture consisted of 3.0 ml of an enzyme source corresponding to 0.3 g wet weight of liver, 0.25 μmoles each of NAD and NADP, 60 mmoles G-6-P, 7.5 mmoles $MgCl_2$, 50 mmoles nicotinamide and 0.1 ml of 1 M KPB (pH 7.4) with a total volume of 3.6 ml. Tri (0.1 ml) was placed in the center well of the flask as substrate. After enclosing O_2 gas, the mixture was incubated at 37° for 1 hr with shaking. When G-6-P dehydrogenase was used in the experiment, 12.5 units were added to the incubation mixture; when CH or TCE was used as substrate, 0.1 ml of aqueous solution of CH (30 mM) or TCE (30 mM) was added. The mixtures were incubated under the same conditions as described above, but using 25-ml Erlenmeyer flasks as open incubation vessels. Immediately after incubation, the flasks were cooled in an ice bath. The reaction mixtures were transferred to 10-ml test tubes and stored frozen until analysis.

The oxidation of CH to TCA by mitochondrial crude aldehyde dehydrogenase was also assayed spectrophotometrically in the presence of NAD or NADP. The incubation mixture was constituted according to Tabakoff *et al.* [11] with a total volume of 1.0 ml. The increase of absorbance at 340 nm was followed on a Hitachi 124 spectrophotometer equipped with a recorder.

Determination of CH, TCE, and TCA

TCE analysis. The reaction mixture (1–100 μl) and 0.1 ml of 0.1 M KPB (pH 5.8) were placed in a 10-ml test tube. *n*-Hexane (1–4 ml) was added to the mixture, and the test tube was stirred vigorously in a Vortex mixer. An aliquot (2 μl) of the *n*-hexane layer was injected into the gas chromatograph, as described below.

A calibration curve (concentration versus peak height) was prepared using TCE aqueous solution in the same manner as described above.

CH and TCA analyses. CH was assayed using a modified method of Wells and Cimbura [12]; TCA was assayed using the method of Helbolsheimer and Funk [13]. The reaction mixture (0.1–1.0 ml) was evaporated to dryness under reduced pressure to remove Tri and TCE completely. The residue was suspended in the original volume of 0.1 M KPB (pH 5.8), and half the volume (0.05–0.5 ml) of the suspension was introduced into a 5-ml ready-made ampule. CH in the ampule was extracted with ethyl ether (2 ml), which was diluted twice with benzene, and an aliquot (2 μl) was injected into the gas chromatograph after appropriate dilution with benzene–ether (1 : 1). The organic layer containing CH in the ampule was removed by aspiration, and the aqueous layer was washed with ethyl ether (2 ml × 3) in order to remove CH thoroughly. Ethyl ether was then thoroughly removed from the aqueous layer in the ampule under a gentle stream of N_2 gas. Benzene (1.0 ml) was added to the washed aqueous solution, and the ampule was sealed and heated at 95–98° for 2 hr. After cooling, the ampule was opened, and an aliquot (2 μl) of the benzene layer was injected into the gas chromatograph.

A calibration curve (concentration v. peak height) was prepared using a CH or a TCA aqueous standard solution in the same manner as described above, except that the evaporation procedure was omitted.

Gas chromatography

A Hitachi gas chromatograph, model 163, with a nickel 63 electron capture detector was used. The glass column (2 m × 3 mm) was packed with 5% Silicone GE SE-30 on Chromosorb W (AWDMCS), 80–100 mesh. The electron capture detector was maintained at 200° for the analysis of TCE, at 170° for CH, and at 140° for chloroform formed from TCA through heating, and the column at 140°, 80° and 60° respectively. The injector block was adjusted to the detector temperature. The flow rate of carrier gas (nitrogen) was 20–40 ml/min. The electron capture detector was operated at pulse intervals of 100 μsec, and the electrometer setting was kept continuously at range 10^2, attenuation 32.

Protein determination

An aliquot (0.05 to 0.2 ml) of liver homogenate was analyzed by the biuret method [14].

RESULTS

Determination of CH, TCE, and TCA

The recovery of CH added to liver homogenate was 96.7 ± 4.0 per cent (N = 3).

On TCE analysis, a coefficient of variation of 2.6 per cent was obtained from triplicate determinations of the same sample.

On TCA analysis, CH must be thoroughly removed, since CH is also converted to chloroform under the same reaction conditions. The recovery of TCA, added to liver homogenate, was 98.7 ± 4.8 per cent (N = 3). The yield of the conversion of TCA into chloroform was calculated to be 81.9 ± 4.2 per cent (N = 4), from the standard curve of chloroform.

General features of Tri metabolism

Effects of incubation time and amount of enzyme. To clarify the overall profile of Tri metabolism, experiments using the 700 *g* supernatant fraction as the enzyme source were performed. Figure 1a shows the effect of incubation time on the formation of CH,

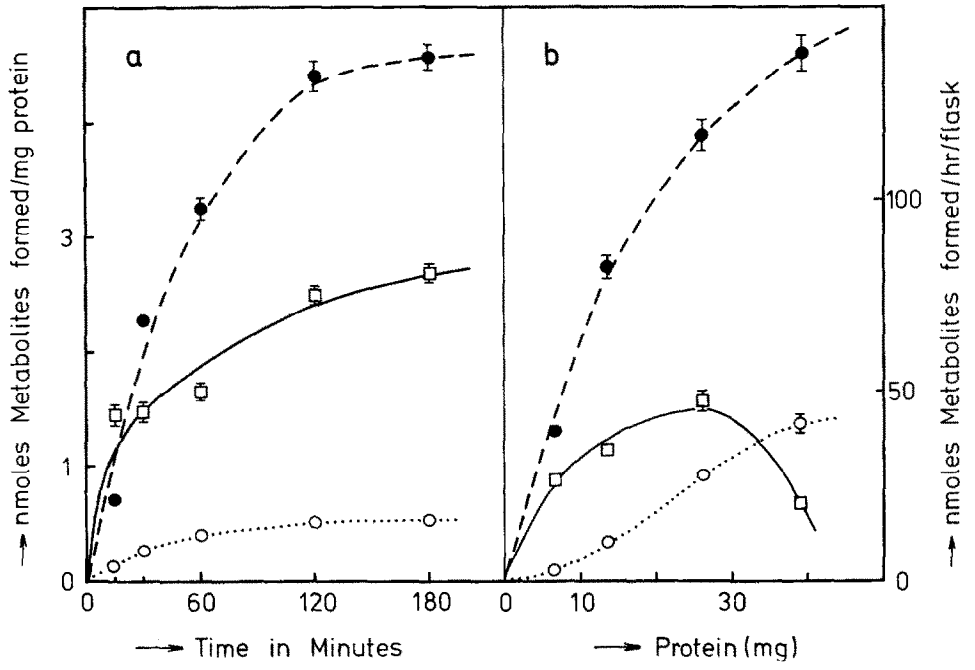

Fig. 1. Effect of incubation time and amount of enzyme source on overall Tri metabolism. In panel a, Tri (0.1 ml) was incubated with 3.0 ml of the 700 *g* supernatant fraction of the rat liver in the complete system described under Incubation Conditions at 37° for 15, 30, 60, 120 or 180 min. In panel b, Tri (0.1 ml) was incubated with various amounts of the 700 *g* supernatant fraction from 0.5 ml (about 6 mg protein) to 3.0 ml in the complete system at 37° for 60 min. In both panels, □——□ represents the formation of CH, ●– – –● that of TCE, and ○···○ that of TCA.

TCE, and TCA, and on the rates of their formation, revealing that TCA reaches a plateau after 120 min, and CH and TCE after 180 min. In this experiment, about 6 per cent of the amount of Tri added had been metabolized to the three metabolites after 120 min of incubation. Figure 1b shows the dependency of the amount of the 700 *g* supernatant fraction on the formation of the three metabolites. If the total activity is expressed as the sum of the amounts of the three metabolites formed in the flask, the sum leveled off at 200 nmoles (corresponding to about 4 per cent yield based on Tri remaining in the reaction mixture) in a flask that contained 2.0 ml of the enzyme source.

Requirements of cofactors. Cofactor requirements for overall Tri metabolism were examined using dialyzed 9000 *g* supernatant fraction [against the homogenizing medium (2 l. × 3) for 5 hrs] as the enzyme source. The results are shown in Table 1. Omission of any one of the five cofactors from the complete system caused a unique decrease in the sum of the three metabolites. Nicotinamide, NAD, and NADP were indispensable cofactors for Tri metabolism. The cofactors required for the formation of TCA from Tri cannot be clearly assessed from the data in Table 1, but the data suggest that NAD rather than NADP may be required for the formation of TCA.

Individual steps of Tri metabolism

Intracellular localization and role of subcellular fractions. Tri metabolism was studied in the subcellular fractions of rat liver using Tri and CH as substrates. The results are summarized in Tables 2 and

Table 1. Requirements of cofactors for Tri metabolism by the dialyzed 9000 *g* supernatant fraction of rat liver*

Additions					Amounts of metabolites (nmoles/mg protein)			
G-6-P	NADP	NAD	Nicotinamide	Mg^{2+}	CH	TCE	TCA	Sum
+	+	+	+	+	0.498 ± 0.044	6.87 ± 0.12	0.338 ± 0.034	7.70 ± 0.12
–	+	+	+	+	1.03 ± 0.05	5.02 ± 0.17	0.302 ± 0.035	6.35 ± 0.27
+	–	+	+	+	1.86 ± 0.20	3.21 ± 0.14	0.291 ± 0.018	5.36 ± 0.57
+	+	–	+	+	0.985 ± 0.054	3.65 ± 0.23	0.151 + 0.015	4.79 ± 0.28
+	+	+	–	+	0.995 ± 0.002	2.61 ± 0.16	0.232 ± 0.028	3.84 ± 0.14
+	+	+	+	–	0.591 ± 0.044	6.11 ± 0.25	0.237 ± 0.008	6.94 ± 0.25
–	–	–	–	–	0.104†	ND‡	0.170	0.274

* Tri (0.1 ml) was incubated with the 9000 *g* supernatant fraction in each incubation system at 37° for 60 min.
† N = 2.
‡ Not detectable.

Table 2. Formation of CH, TCE, and TCA from Tri in subcellular fractions of rat liver*

Expt.	Fraction	Amounts of metabolites (nmoles/mg protein, N = 3) CH	TCE	TCA	Sum
1	700 *g* Supernatant	0.810 ± 0.062	5.52 ± 0.25	0.653 ± 0.059	6.98 ± 0.27
2	9000 *g* Supernatant	0.657 ± 0.050	7.53 ± 0.15	0.646 ± 0.025	8.77 ± 0.08
3	2 + 4	0.652 ± 0.031	5.80 ± 0.24	0.476 ± 0.026	6.87 ± 0.27
4	Mitochondrial	0.324 ± 0.078	ND†	0.453 ± 0.051	0.78 ± 0.04
5a	Microsomal (unwashed)	20.3 ± 0.4	0.60 ± 0.03	ND	20.9 ± 0.5
5b	Microsomal (washed)	1.88 ± 0.02	ND	ND	1.88 ± 0.02
5c	Microsomal (washed)‡	12.3 ± 0.5	0.253 ± 0.009	ND	12.6 ± 0.3
6	Cytosolic	ND	0.053 ± 0.003	0.186 ± 0.033	0.24 ± 0.04
7	4 + 5b	2.06 ± 0.29	0.097 ± 0.005	0.623 ± 0.114	2.78 ± 0.34
8	4 + 6	ND	0.333 ± 0.008	0.413 ± 0.023	0.75 ± 0.03
9	5b + 6	0.782 ± 0.007	7.21 ± 0.10	0.396 ± 0.011	8.39 ± 0.10
10	5b + 4 + 6	0.641 ± 0.057	5.39 ± 0.39	0.445 ± 0.055	6.48 ± 0.30

* Tri (0.1 ml) was incubated with each subcellular fraction in the complete system at 37° for 60 min.
† Not detectable.
‡ G-6-P dehydrogenase (12.5 units) was added to the incubation mixture.

3. Table 2 shows that CH is the major compound produced from Tri by the unwashed microsomal fraction or the washed microsomal fraction in the presence of G-6-P dehydrogenase (Expts. 5a and 5c). The washed microsomal fraction, however, showed 9.3 per cent of the activity level of the unwashed fraction, indicating that G-6-P dehydrogenase might be adsorbed on the unwashed fraction. TCE was the major compound formed from CH in the cytosolic fraction (compare Expts. 6 and 9 in Table 2, and Expt. 6 in Table 3). As for the formation of TCA from CH, the mitochondrial fraction had the highest specific activity (Expt. 4 in Table 3) and the cytosolic fraction catalyzed the oxidation of CH to TCA (Expt. 6 in Table 3) but, the microsomal fraction contributed little to the formation of TCA. Tri-and CH-metabolizing activities were completely reconstituted from each subcellular fraction respectively (compare Expt. 1 with 10 and 2 with 9 in both tables).

Properties of the activity in the individual steps of Tri metabolism. Since intracellular localization of each activity and the role of the subcellular fractions were demonstrated, the details of the individual steps of Tri metabolism were examined.

(a) Formation of CH by the rat liver microsomal fraction. Effects of incubation time and amount of enzyme on the first step oxygenation of Tri were examined using the unwashed microsomal fraction. The results, shown in Fig. 2, indicate that the level of CH reached a plateau after 180 min, and that the formation of CH was linear with microsomal protein

Table 3. Formation of TCE and TCA from CH in subcellular fractions of rat liver*

Enzyme sources		Amounts of metabolites (nmoles/flask or mg protein) TCE			TCA			
Expt.	Fractions	Total activity	Specific activity	Yield† (%)	Total activity	Specific activity	Yield (%)	Total yield
1	700 *g* Supernatant	1260 ± 40	28.1 ± 0.9	42.0	119 ± 1	2.65 ± 0.03	4.0	46.0
2	9000 *g* Supernatant	1473 ± 31	45.8 ± 0.3	49.0	117 ± 16	3.64 ± 0.50	3.9	52.9
3	2 + 4	1320 ± 10	35.8 ± 0.8	44.0	121 ± 14	3.28 ± 0.38	4.0	48.0
4	Mitochondrial	10.6 ± 2.3	2.2 ± 0.5	0.4	161 ± 18	33.9 ± 3.8	5.4	5.8
5	Microsomal (washed)	21.8 ± 0.6	4.9 ± 0.1	0.7	6.2 ± 0.1	1.38 ± 0.02	0.2	0.9
6	Cytosolic	1413 ± 64	71.0 ± 3.2	47.1	82 ± 11	4.12 ± 0.55	2.7	49.8
7	4 + 5	24.2 ± 2.3	2.6 ± 0.3	0.8	109 ± 22	11.8 ± 2.4	3.6	4.4
8	4 + 6	1250 ± 20	50.8 ± 0.8	41.7	76 ± 5	3.09 ± 0.20	2.5	44.2
9	5 + 6	1517 ± 49	62.2 ± 2.0	50.6	84 ± 2	3.45 ± 0.08	2.8	53.4
10	4 + 5 + 6	1316 ± 41	45.2 ± 1.4	43.9	86 ± 6	2.95 ± 0.21	2.9	46.8

* CH (3 μmoles) was incubated with each subcellular fraction in the complete system at 37° for 60 min.
† Total activity of each metabolite/CH added (3 μmoles/flask) × 100.

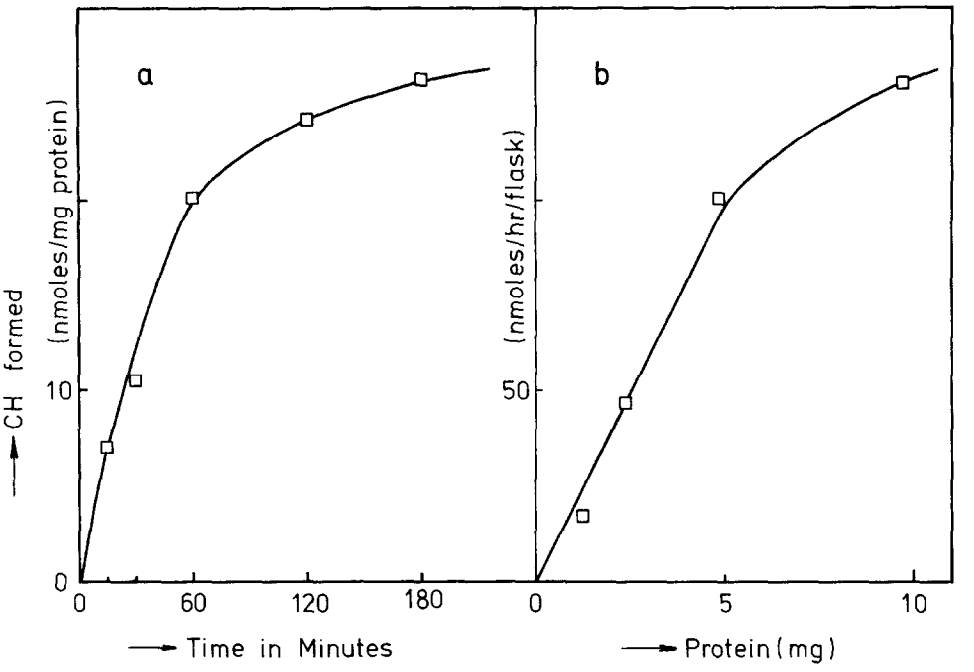

Fig. 2. Properties of the formation of CH from Tri in the unwashed microsomal fraction. Panel a shows the time course of the CH formation by the unwashed microsomal fraction from 15 to 180 min in the complete system as described under "Incubation Conditions". In panel b, Tri (0.1 ml) was incubated with various concentrations of the microsomal protein at 37° for 60 min.

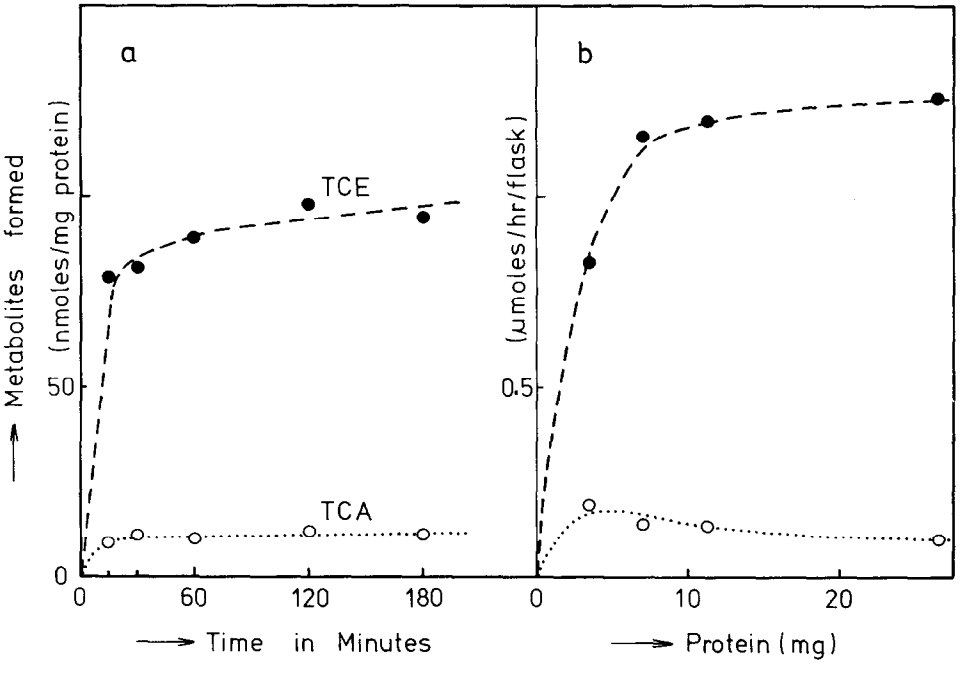

Fig. 3. Properties of the formation of TCE and TCA from CH in the cytosolic fraction. Panel a shows the time course of the formation of TCE and TCA from CH by the cytosolic fraction from 15 to 180 min in the complete system as described under "Incubation Conditions". In panel b, CH (3 μmoles) was incubated with various concentrations of the cytosolic fraction at 37° for 60 min. Key: ●– – –● represents the formation of TCE, and ○···○ that of TCA.

Table 4. Requirements of cofactors for the formation of CH from Tri by the microsomal fraction of rat liver*

Fraction	Additions								CH formed (nmolcs/mg protein)
	G-6-P	NADP	NAD	Nicotinamide	Mg^{2+}	NADPH	NADH	G-6-P dehydrogenase	
Unwashed microsomes	+	+	+	+	+	−	−	−	21.9 ± 2.0†
	+	+	−	+	+	−	−	−	15.1‡
	−	+	+	+	+	−	−	−	8.9 ± 0.5
	−	−	−	+	+	−	−	−	0.35
	−	−	−	+	+	+	−	−	4.8 ± 0.6
	−	−	−	+	+	−	+	−	3.2 ± 0.5
Washed microsomes	+	+	+	+	+	−	−	+	14.0 ± 0.7
	+	−	+	+	+	−	−	+	4.9 ± 0.3
	+	+	−	+	+	−	−	+	10.2 ± 0.6
	+	+	+	+	+	−	−	−	0.82
	−	−	−	−	−	−	−	−	0.68

* Tri (0.1 ml) was incubated with the unwashed or washed microsomal fraction in each incubation system at 37° for 60 min.
† Mean ± S.D. (N = 3).
‡ Mean (N = 2).

up to about 5 mg. The formation of CH from Tri by the unwashed microsomal fraction was studied at various pH values; a pH optimum of 7.4 ± 1.5 (KPB), was observed which corresponds to that reported by Leibman [7]. In a separate experiment, an apparent K_m value for Tri was determined in the unwashed microsomal fraction; it was 1.75 mM. In this experiment, various concentrations of Tri acetone solution (0.1 ml) were added directly to the incubation mixtures. In all other experiments, Tri was catabolized as vapor, as described in "Incubation Conditions". A difference was found in the yield of CH between the two incubation systems, namely one-third of the CH in the latter system was formed in the former. In this experiment, the addition of acetone (0.1 ml) did not interfere at all with Tri metabolism. Leibman and McAllister [8] reported an apparent K_m value of 9×10^{-2} M for Tri in the incubation system containing Tri–oil–polysorbate emulsion.

Requirements of cofactors for the formation of CH are summarized in Table 4. All the data support the findings reported by Leibman [7]. This step requires the NADPH-generating system or NADPH, and NAD(H) appears to play a supplementary role in the oxygenation.

(b) Formation of TCE and TCA from CH by the rat liver cytosolic fraction. Properties of the reaction involving the formation of TCE and TCA from CH were also studied, using the cytosolic fraction as enzyme source. The results are shown in Fig. 3. The reaction rates of TCE and TCA formation markedly increased within the first 15 min and reached plateaus after 120 min and 30 min respectively. In a separate experiment, an apparent K_m value of 6.0 mM was obtained for the formation of TCE from CH, but the value for the TCA formation could not be calculated.

Table 5 summarizes the cofactor requirements for the formation of TCE and TCA by the cytosolic fraction. It shows that both NADH and NADPH or the NADPH-generating system were required for the reduction of CH to TCE, and NAD, but not NADP, was required for the oxidation of CH to TCA. The latter finding was reported by Cooper and Friedman [10]; the NADPH-dependent reduction of CH to TCE was also postulated by Tabakoff *et al.* [15]. Characterization of the CH-reducing activity will be discussed later.

(c) Formation of TCA from CH by the mitochondrial fraction. The mitochondrial fraction showed the highest specific activity for the oxidation of CH to TCA. The oxidative activity of the intact mitochondrial fraction was 33.9 ± 3.8 nmoles·hr^{-1}·(mg protein)$^{-1}$ in the complete system as shown in Table 3, whereas the activity was 92.3 nmoles·hr^{-1}·(mg protein)$^{-1}$ (N = 2) in the system containing no cofactors. These data suggest that the endogenous cofactors present in the intact mitochondrial fraction were sufficient for the oxidation of CH. For these reasons, we prepared crude aldehyde dehydrogenase from the mitochondrial fraction by sonication, according to the method of Tabakoff *et al.* [11], and examined cofactor requirements and affinity of CH to the enzyme. NAD, but not NADP, was required for the oxidation of CH, and an apparent K_m value of

Table 5. Requirements of cofactors for the formation of TCE and TCA from CH by the cytosolic fraction of rat liver*

Expt.	Additions							Amounts of metabolites† (nmoles/mg protein)		
	G-6-P	NADP	NAD	Nicotinamide	Mg^{2+}	NADPH	NADH	TCE	TCA	Sum
1	+	+	+	+	+	−	−	90.0 ± 1.4	5.1 ± 0.2	95.1 ± 1.5
2	−	+	+	+	+	−	−	86.3 ± 9.4	11.9 ± 1.4	98.2 ± 9.2
3	+	−	+	+	+	−	−	84.2 ± 4.5	8.4 ± 0.4	92.6 ± 4.3
4	+	+	−	+	+	−	−	96.6 ± 6.9	2.1 ± 0.1	98.7 ± 3.7
5	−	−	+	+	+	−	−	73.6 ± 3.7	15.8 ± 2.0	89.4 ± 3.4
6	−	+	−	+	+	−	−	91.8 ± 3.6	2.1 ± 0.4	93.9 ± 3.3
7	−	−	−	+	+	−	−	42.9 ± 0.8	4.2 ± 1.1	47.1 ± 1.0
8	−	−	−	−	−	−	−	33.4 ± 1.1	4.2 ± 0.8	37.6 ± 0.9
9	−	−	−	+	+	+	−	93.0 ± 2.3	2.5 ± 0.7	95.5 ± 2.3
10	−	−	−	+	+	−	+	88.7 ± 3.3	15.5 ± 0.8	104.2 ± 4.0

* CH (3 μmoles) was incubated with the cytosolic fraction in each incubation system at 37° for 60 min.
† Each value is the mean ± S.D. (N = 3).

62.5 mM was obtained for CH at 25°. Details will be presented in a subsequent paper.

Effect of pretreatment of rats with phenobarbital or 3-methylcholanthrene on the formation of CH from Tri

The influence of PB pretreatment of the rats on Tri metabolism has been reported by several investigators *in vitro* and *in vivo* [8, 16]. Here, the effect of 3-MC pretreatment on the metabolism of Tri was investigated using the unwashed microsomal fraction, and the activity was compared with that of the PB-pretreated microsomes. As shown in Table 6, both PB and 3-MC caused about a 3-fold increase in the oxidation of Tri to CH in the complete system.

Table 6. Effect of PB or 3-MC pretreatment on the formation of CH from Tri by the microsomal fraction of rat liver*

Pretreatment	CH [nmoles·hr^{-1}·(mg protein)$^{-1}$]	Ratio
Control	18.6 ± 2.9†	1.0
PB	58.3 ± 4.4	3.1
3-MC	54.0 ± 6.1	2.9

* Tri was incubated with the unwashed microsomal fraction with or without pretreatment in the complete system at 37° for 60 min.
† Mean ± S.D. (N = 3).

Oxidation of TCE to CH

To understand how TCE is oxidized to CH, the reverse of the reaction mentioned above, TCE was incubated with the 700 *g* supernatant fraction of the rat liver as described under "Incubation Conditions". The formation of CH and of TCA from TCE (60.6 nmoles/mg protein) was 0.90 ± 0.07 (1.6 per cent yield) and 0.27 ± 0.05 nmole/mg protein (0.44 per cent). The overall metabolism of TCE in the 700 *g* supernatant fraction was much smaller than that of CH to TCE (42.0 per cent yield) and to TCA (4.0 per cent) as shown in Table 3. Furthermore, we incubated TCE with the unwashed microsomal fraction in the complete system to obtain information on the oxidation of TCE to CH because methanol and ethanol are known to be oxidized to the corresponding aldehydes not only in the cytosol but also in microsomes using NADPH and oxygen[17–19]. In the experiment on the oxidation of TCE to CH, 7.7 nmoles of CH·hr^{-1}·(mg microsomal protein)$^{-1}$ were formed from TCE, whereas 18.6 nmoles of CH·hr^{-1}·(mg microsomal protein)$^{-1}$ were formed from Tri under the same conditions.

Separation of enzymes involving the reduction of CH to TCE

As shown in Table 5, enzymes reducing CH to TCE with NADH or NADPH existed in the cytosolic fraction of the rat liver. As for the enzyme that required NADH as a cofactor, alcohol dehydrogenase was reported by Friedman and Cooper [9]. Table 5 shows that NADPH-dependent reduction occurred to a degree equal to that of NADH-dependent reduction, although Friedman and Cooper [9] found the former to be smaller than the latter. Separation of the CH-reducing enzymes was thus attempted by DE-52 column chromatography, as a preliminary experiment. The precipitate from 40–70% ammonium sulfate saturation of the cytosol, corresponding to 8 g liver, was applied to a DE-52 column (1.6 × 45 cm). CH reducing activity separated into at least three fractions, peaks I, II and III (see Fig. 4). Peak I showed NADH dependency and sensitivity to pyrazole, a typical inhibitor of alcohol dehydrogenase. Peaks II and III showed NADPH dependency; the latter peak also utilized NADH to a lesser extent.

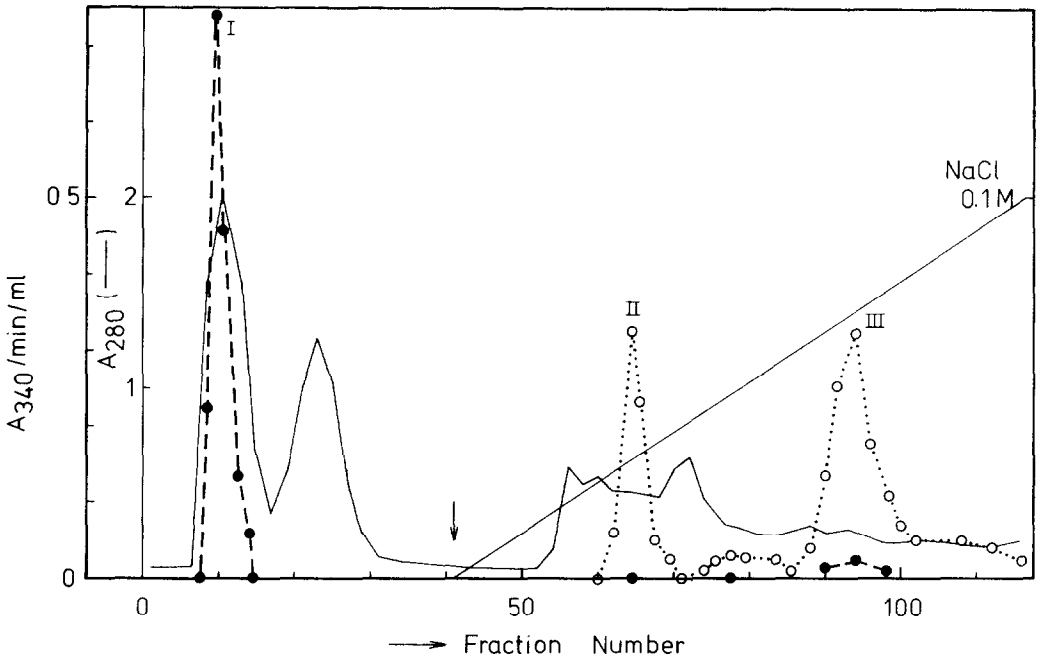

Fig. 4. Elution profile of CH reducing enzymes from a DE-52 column. The gradient was started at Fraction 41 and consisted of 5 mM Tris–phosphate buffer (pH 8.0) containing 5 mM 2-mercaptoethanol (150 ml) and the buffer containing 0.1 M NaCl (150 ml). Protein was approximated by absorption at 280 nm (———) and enzyme activity was determined by measuring the decrease in absorbance at 340 nm. The assay system consisted of 50 mM KPB (pH 7.0),0.16 mM NADH or NADPH, 5 mM CH, and enzyme source with a total volume of 1.0 ml. Key: ●– – –● represents an NADH-dependent activity of CH reduction, and ○···○ NADPH-dependent activities. Each fraction consisted of 4.0 ml.

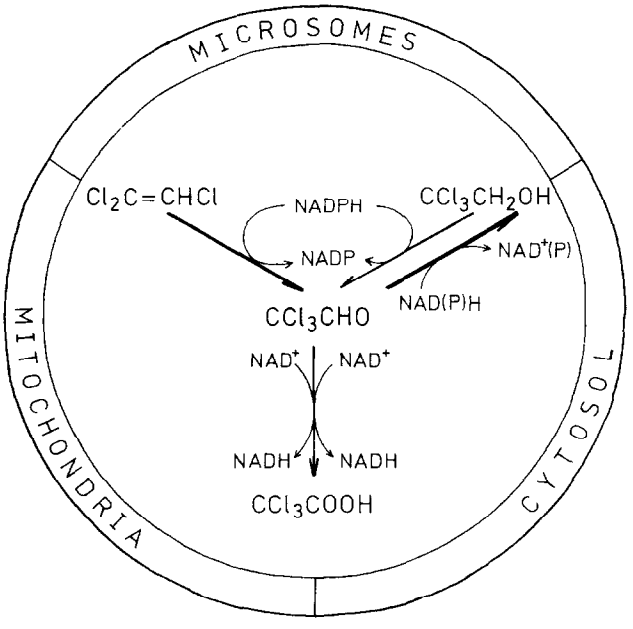

Fig. 5. Metabolic scheme of Tri in the rat liver.

DISCUSSION

Determination of the metabolites of Tri has been carried out using a colorimetric method based on the Fujiwara reaction [20–22], by which studies of Tri metabolism *in vitro* have been performed [5, 7, 8, 16]. The colorimetric method was much less sensitive and specific for each metabolite than the method presented here. We have established a determination for small amounts of the metabolites of Tri applicable to *in vitro* experiments.

The data presented here support a metabolic scheme for Tri in the rat liver, as shown in Fig. 5. In this scheme, the formation of urochloralic acid (TCE glucuronide) is not given because little urochloralic acid was formed from Tri or CH in the complete system using the 700 *g* supernatant fraction.

The first step, the oxidation of Tri to CH, is known to be catalyzed by the microsomal mixed function monooxidases. The finding that both PB and 3-MC pretreatment stimulated this step indicates that both cytochrome P-450 and P-448 take part in this step. Pelkonen and Vainio [23] reported spectral interactions of chlorinated ethylenes with cytochrome P-450 of liver microsomes from variously treated rats, and showed that the more chlorine atoms in the molecule the larger the type I spectral change and the smaller the spectral dissociation constant. They also demonstrated that treatment of rats with 3-MC decreased the magnitude of the type I spectral change, whereas PB increased it. However, they did not examine metabolite (CH) formation. In our own experiment, dichloroethylenes (1,1-, cis-1,2- and *trans*-1,2-), Tri or tetrachloroethylene was incubated with the unwashed microsomal fraction in the complete system as described under "Incubation Conditions", and the respective stable metabolites, dichloroacetaldehyde, CH and TCA, were assayed gas chromatographically. First step oxygenation activity increased in ascending order from tetrachloroethylene, *trans*-dichloroethylene, 1,1-dichloroethylene cis-dichloroethylene to Tri.* The data suggests that stereoselectivity affects the actual interaction of a compound with microsomal cytochrome P-450 and is contradictory to the findings reported by Pelkonen and Vainio [23]. Recently, Moslen *et al.* [24] reported the enhancement of the metabolism of Tri and tetrachloroethylene by variously treated rats *in vivo*, and also showed that 3-MC did not cause as much enhancement as PB, judged by trichlorinated urinary metabolites (μmoles/24 hrs per animal). The effect of 3-MC pretreatment on the metabolism of Tri should be studied in more detail; such studies are now in progress.

The next reductive step from CH to TCE was reported to be catalyzed by liver alcohol dehydrogenase (EC 1.1.1.1) [5, 9]. Tabakoff *et al.* [15] studied the reduction of CH to TCE in brain extracts or liver cytosol and postulated the presence of enzymes other than alcohol dehydrogenase that are capable of reducing CH. NADPH-dependent aldehyde and ketone reductases have recently been reported in the literature [25–29]. In the present paper, at least two NADPH-dependent enzymes capable of reducing CH were demonstrated to be present in rat liver cytosol. Purification and characterization of these enzymes are in progress.

In studies of the oxidative step of CH to TCA, an NAD-dependent enzyme was partially purified from rabbit liver cytosol by Cooper and Friedman [10]. They also reported that the activity oxidizing CH was completely recoverable in the soluble fraction of rabbit liver; the activity in other subcellular levels was not studied. As shown in Table 3, the mitochondrial fraction alone, as well as the cytosolic fraction alone, catalyzes the oxidation of CH. The recombination of mitochondria with cytosol, however, decreased the oxidative activity, because reduction of CH to TCE by cytosol predominates over oxidation. In addition, the apparent oxidative activity was recoverable in the recombined fraction of microsomes and cytosol (compare Expt. 2 with 9). The role of mitochondrial aldehyde dehydrogenase (EC 1.2.1.3) [30] in the oxidation of CH remains to be clarified.

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