DECHLORINATION OF CHLOROETHANE WITH A RECONSTITUTED LIVER MICROSOMAL SYSTEM*

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SUMMARY. A reconstituted microsomal mixed-function oxidase system has demonstrated the capacity to dechlorinate 1,1,2-trichloroethane. The activity of the reconstituted system was stimulated by the presence of 100,000 g supernatant, similar to intact microsomes, and had characteristics of previous reconstituted systems with different substrates.

Previous reports by Van Dyke et al. (1-3) have shown that volatile halogenated anesthetics and chlorinated ethanes can be dehalogenated *in vitro* by enzymes localized in the liver microsomes. Because NADPH and oxygen are necessary for activity, carbon monoxide inhibits the reaction, and the activity is inducible by phenobarbital, the enzymes are believed to be the mixed-function oxidases that metabolize steroids, drugs, and endogenous compounds (4).

It was reported recently that a soluble liver microsomal system containing the mixed function oxidases was separated into three fractions: cytochrome P-450 (the terminal oxidase that activates oxygen), NADPH cytochrome c reductase, and a lipid fraction (5). Recombination of these components produced a system that was capable of hydroxylating drugs, foreign compounds, and endogenous substrates, similar to that of intact liver microsomes (6). To clarify the involvement of the P-450 mixed-function oxidase system in the dehalogenation reaction, the reconstituted microsomal system was examined for this activity.

METHODS

Microsomes.--Male Sprague-Dawley rats (300-350 g) were pretreated with 0.2% sodium phenobarbital in their drinking water (7), fasted

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overnight, and sacrificed the following day. Microsomes were prepared and stored as a suspension in 0.25 M sucrose under nitrogen at -20°C (5). The 100,000 g supernatant (20 mg protein/ml) resulting from the preparation of microsomes was prepared fresh (from similarly treated rats) when this was used in assays.

Preparation of Components.--The reductase and P-450 fractions were prepared, assayed, and stored according to the method of Lu et al. (6). Specific activities of 3 nmoles of cytochrome P-450 and 300-400 units NADPH cytochrome c reductase* per milligram of protein were obtained. Lecithin (dilauryl) was used in place of the lipid fraction (8).

Incubation.--Incubation mixtures contained 2.4 μmol $MgCl_2$, 0.5 μmol NADP, 1 μmol glucose-6-phosphate, 0.5 unit glucose-6-phosphate dehydrogenase, and the P-450, reductase, and lecithin in various amounts in a total volume of 2 ml of 0.05 M Tris-HCl buffer (pH 7.4). The reaction was oxygenated and 1 μliter (10.8 μmol) of uniformly-labeled 1,1,2-trichloroethane-^{36}Cl was added as before to start the reaction (3). The mixture was capped and incubated at 37°C for 0.5 hours. To stop the reaction, 0.2 ml of 15% trichloroacetic acid was added and the flask was allowed to stand uncapped overnight in a hood to allow excess 1,1,2-trichloroethane to volatilize.

Assays.--Total non-volatile, water-soluble ^{36}Cl-labeled metabolites were quantitated before the amount of free inorganic ^{36}Cl was determined.

Total Cl metabolites were determined by centrifuging the precipitated mixture at 1,000 g for 10 minutes, decanting the supernatant, and centrifuging it again at 1,000 g. A 0.2-ml aliquot of the supernatant was dissolved in 10 ml of Diotol (146 g napthalene: 420 ml methanol: 600 ml toluene: 700 ml dioxane: 100 ml Liquifluor) and counted in a liquid scintillation counter.

The amount of inorganic ^{36}Cl was determined as before (2) by precipitating the $^{36}Cl^-$ion with excess silver nitrate. All data were recorded in counts per minute (cpm).

*One unit equals 1 nmol cytochrome c reduced per minute.

Materials.--Lecithin (dilauryl) was purchased from Cal Biochem (San Diego). Uniformly labeled 1,1,2-trichloroethane-^{36}Cl (0.05 mCi/mmol) was obtained from New England Nuclear (Boston) and diluted with carrier before use.

RESULTS AND DISCUSSION

The purified reconstituted microsomal system containing cytochrome P-450, NADPH cytochrome c reductase, and lecithin has the capacity to cleave the carbon-halogen bond (Fig. 1). The system was found to depend primarily on the concentration of cytochrome P-450 and NADPH cytochrome c reductase in the incubation mixture; the system depended less on the concentration of lecithin. The lack of dependency on the lecithin concentration might be explained in two ways: either the lecithin was not the specific lipid required for optimum enzymatic activity or the amount of lipid added completely saturated the small amount of P-450 that was present.

The 1,1,2-trichloroethane is not completely dechlorinated and therefore the higher levels of radioactivity in the total ^{36}Cl than in the inorganic ^{36}Cl indicate the presence of non-volatile water soluble fragments, such as mono- and di-chloroethenol, resulting from the dechlorination of the trichloroethane.

As shown in Figure 1, the P-450 and reductase components age rapidly since the activity is decreased after storage at -85°C, under nitrogen for 1 week. Possibly, specific labile forms of the cytochrome P-450 and reductase are necessary for maximum dechlorination activity, since Lu et al. (6) have isolated two forms of the cytochrome with different substrate specificity.

Lu et al. (6) reported that NADH could be used as the source of reducing equivalents with the reconstituted system, but that it was inferior to NADPH in this respect. In the dechlorination reaction, NADPH was also much more effective than NADH as a source of reducing equivalents (Table 1). Table 1 shows that the addition of both NADPH and NADH results in an additive effect. Whether the NADH is active through NADPH cytochrome c reductase or through a contaminating NADH reductase-cytochrome b_5 system is undetermined.

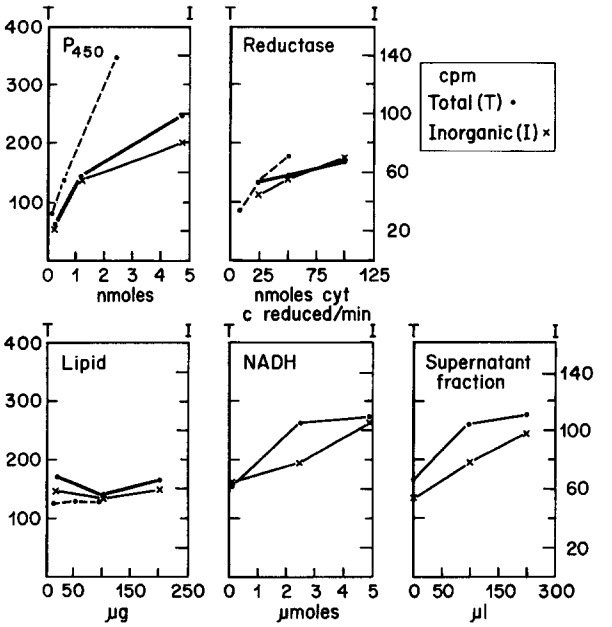

Fig. 1. Factors influencing dechlorination with reconstituted microsomal system. Except for the component listed on each particular graph, all other components were held constant. NADH and supernatant were held constant or varied only in the two graphs where they are indicated. With fresh components (----), the constant concentrations were 0.5 nmol cytochrome P-450, 25 units NADPH cytochrome c reductase, and 50 µg lecithin. Constant concentrations with 1-week-old components (——) were 1 nmol cytochrome P-450, 50 units NADPH cytochrome c reductase, and 100 µg lecithin. NADPH generating system was present in all assays. Results are average of duplicates from replicate samples. (T = total ^{36}Cl metabolite; I = inorganic $^{36}Cl^-$ portion.)

Table 1 also indicates the sensitivity of the reconstituted system to carbon monoxide, supporting the involvement of cytochrome P-450.

The addition of 100,000 g supernatant from the preparation of liver microsomes from phenobarbital-treated rats stimulated dechlorination activity in the presence of NADPH. Stimulation is even greater when supernatant is added in the presence of both NADPH and NADH. Van Dyke and Wineman (3) also

Table 1.--Requirements for Dechlorination

Additions	Relative activity*
None	17 ± 1
NADPH	100
NADPH + CO	17 ± 5
NADH	34 ± 11
NADPH + supernatant	118 ± 15
NADPH + NADH (2.5 μmol)	109 ± 3
NADPH + NADH + supernatant	149 ± 17

Assayed as described in Methods. All contained 1 nmol cytochrome P-450, 50 units NADPH cytochrome c reductase, and 100 μg lecithin. NADPH = 0.5 μmol NADP, 1 μmol glucose-6-phosphate, 0.5 units glucose-6-phosphate dehydrogenase, and 2.4 mol $MgCl_2$. NADH = 2.5 μmol. Supernatant = 100 μl (2 mg protein).

*Data expressed as relative activity to microsomes + NADPH, and are shown as average of duplicate experiments analyzed for both total ^{36}Cl and inorganic ^{36}Cl ± SD.

observed this stimulation by the supernatant when intact liver microsomes were used. Preliminary evidence indicates that the supernatant factor is associated with a soluble protein and is very labile.

When the amounts of cytochrome P-450, NADPH cytochrome c reductase, lecithin, and NADPH are constant, an increase in the concentration of both NADH and supernatant causes an increase in the dechlorination activity to a point of saturation in the presence of each other (Fig. 1). The additional stimulation of activity caused by increasing the amounts of NADH and supernatant in the presence of each other indicates different mechanisms of stimulation.

Thus it appears that the reconstituted microsomal enzyme system containing cytochrome P-450, NADPH cytochrome c reductase, and lecithin is capable of dechlorinating 1,1,2-trichloroethane. NADPH is required for activity while NADH is approximately 30% as effective. The reconstituted system has characteristics that are similar to those of intact microsomes, and 100,000 g liver supernatant stimulates the activity of both.

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