Metabolism of Trichloroethylene in Liver Microsomes

I. Characteristics of the Reaction[1]

KENNETH C. LEIBMAN

Department of Pharmacology and Therapeutics, University of Florida College of Medicine, Gainesville, Florida

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SUMMARY

The conversion of trichloroethylene to chloral hydrate was shown to take place in liver microsomes of rats, rabbits, and dogs, in a reaction requiring NADPH and oxygen. A stable, lyophilized preparation of microsomal and soluble fractions of liver homogenates in Tris buffer was used, from which a fraction could be obtained which resembled fresh microsomes in behavior. The effects of a number of activators and inhibitors of the enzyme system are described.

INTRODUCTION

It has long been recognized that trichloroethylene is largely converted, in man and in other mammals, to trichloroacetic acid, trichloroethanol, and the glucuronide of the latter [see Defalque (2) for a review of this and other aspects of the pharmacology of trichloroethylene]. Because these final products of metabolism are the same as those found after the administration of chloral hydrate, Butler (3) postulated that chloral hydrate is an intermediate in the metabolism of trichloroethylene. The present paper describes a reaction occurring in liver microsomes, requiring NADPH[2] and oxygen, in which trichloroethylene is converted to a nonvolatile polyhalogenated substance. In the accompanying paper (4), the identification of this metabolite as chloral hydrate, and its conversion to trichloroethanol and trichloroacetic acid, are reported.

[1] A preliminary report of part of this work has appeared (1).

[2] The following abbreviations are employed: EDTA, disodium ethylenediamine tetraacetate; Tris, tris(hydroxymethyl)aminomethane; NAD and NADH, oxidized and reduced forms, respectively, of nicotinamide-adenine dinucleotide; NADP and NADPH, oxidized and reduced forms, respectively, of nicotinamide-adenine dinucleotide phosphate; G6P, glucose-6-phosphate.

MATERIAL AND METHODS

Tissue preparations. Male Holtzman rats, about 150 g, or New Zealand White rabbits, 1–2 kg, were used. Animals were killed by decapitation after stunning. Livers were immediately excised, washed in cold 0.9% NaCl solution, and homogenized with Potter-Elvehjem homogenizers in four volumes of medium, usually 0.1 M Tris-HCl buffer, pH. 7.5. Homogenate was centrifuged for 15 min at 9000 *g*. If it was desired to collect the fraction containing nuclei and mitochondria, the sediment was resuspended in buffer and centrifuged again. The original supernatant fraction after centrifugation at 9000 *g* was frozen and stored at −15°, or else it was lyophilized, stored at −15°, and reconstituted as needed by suspending in water.

When microsomal fraction, free of soluble material, was desired, the 9000 *g* supernatant fraction was centrifuged for 1 hour at 105,000 *g*, the sediment was rehomoge-

nized in 0.1 M Tris buffer, pH 7.5, and again centrifuged at 105,000 *g*. The supernatant fraction was discarded; the pellets were frozen until required and were then homogenized in 0.1 M Tris buffer, pH 7.5.

Incubation conditions. The standard system contained 100 μmoles nicotinamide, 50 μmoles $MgSO_4$, 6 μmoles EDTA, 0.06 μmole NADP, 1 mmole Tris-HCl buffer, pH 7.5, enzyme preparation, and water to make 3 ml. In early experiments, 3 ml aqueous solution containing 3 μl trichloroethylene was added, the mixture was equilibrated for 2 min at 0° with a stream of air saturated with trichloroethylene vapors, and the flasks were stoppered and incubated. In later experiments, 3 ml of an aqueous emulsion containing 12 mg Tween 80® polysorbate, 0.9 ml cottonseed oil, and 120 μl trichloroethylene (prepared in a Virtis homogenizer) was added, and the mixture was incubated open to the air. Incubations were carried out for 2 hr at 37° with gentle shaking. Deviations from this standard procedure are indicated where appropriate. Reactions were terminated by the addition of 0.6 ml of 10% Na_2WO_4 and 1.2 ml of 1 N H_2SO_4; after centrifugation, the aqueous layer was drawn off from between the sedimented denatured protein and the supernatant oil layer. After neutralization of the aqueous portion, a current of air was bubbled through it for 30 min; preliminary experiments showed that this removed all trichloroethylene. The solution was then assayed for chloral hydrate by the method recently described (5). Under these conditions, accumulation of 0.01–0.02 μmole of chloral hydrate was detectable. The activity of different enzyme preparations varied, and day-to-day variation of assay results occurred with the same preparations, probably because of the difficulty of preparing identical emulsions. Comparisons were therefore always made in simultaneous experiments, in which replicates agreed to within an average of about 5%.

In a preliminary experiment, trichloroethylene remaining in the aqueous portion after tungstic acid treatment, measured by difference in determinations before and after bubbling, was found to be 0.3 mM; chloral hydrate found after bubbling amounted to 15% of this value.

RESULTS

In preliminary experiments, no accumulation of nonvolatile polyhalogenated substance from trichloroethylene could be demonstrated in rat liver homogenates, unless NAD or NADP was present. The system with homogenate of 600 mg (wet weight) of liver, was saturated by approximately 3 mM NAD. Addition of nicotinamide or $MgSO_4$ accelerated the reaction. The activity at pH 7.4–7.5 was greater than those at pH 6.4, 7.1, 7.9, or 8.4.

The 9000 *g* supernatant fraction of rat or rabbit liver homogenates is capable of the formation of chloral hydrate from trichloroethylene. Table 1 shows that the system is more responsive to NADP than

TABLE 1

Trichloroethylene metabolism in liver 9000 g supernatant fractions

Fresh fractions from 400 mg liver were incubated for 2 hr with saturated aqueous trichloroethylene in the presence of nicotinamide, 16.7 mM, and $MgSO_4$, 8.3 mM.

Species	Nucleotide	Chloral hydrate formed (μmoles)
Rat	NAD, 10^{-3} M	0.14
Rat	NADP, 10^{-4} M	0.21
Rabbit	NAD, 10^{-3} M	0.10
Rabbit	NADP, 10^{-4} M	0.30

TABLE 2

Metabolism of trichloroethylene in 9000 g supernatant fractions of various rat organs

Fresh fractions from 200 mg of tissue were assayed in the standard system with saturated aqueous trichloroethylene.

Organ	Chloral hydrate formed (μmoles)
Liver	0.20
Lung	0.01
Spleen	<0.01
Heart	<0.01
Kidney	<0.01
Brain	<0.01

to NAD. In Table 2, assays for this activity in the 9000 *g* supernatant fractions from several rat tissues are shown. Significant activity was found only in liver.

TABLE 3
Methods of presenting substrate

Fresh 9000 *g* supernatant fraction from 200 mg of rat liver was incubated under the standard conditions. Final concentration in the incubation mixtures of the substrate and dispersants are indicated below.

Trichloroethylene dispersion	Chloral hydrate formed (μmoles)
Saturated in water	0.10
Half-saturated in 0.2% Triton X-100	0.03
Half-saturated in 1% methyl Cellosolve	0.03
Half-saturated in 0.2% Tween 80	0.08
2% in pluronic F_{68}–lecithin–oil emulsion	0.33
2% in emulsion with 10% cottonseed oil and 0.2% Tween 80	0.66

In Table 3 are described various methods of presenting the substrate to the microsomal system. The concentration of trichloroethylene in the reaction mixture could be increased by adding it in solution in various wetting agents. These, however, appear to inhibit the microsomal enzyme system. Presenting the substrate in the form of an emulsion proved to be successful, and a simple emulsion of trichloroethylene, cottonseed oil, and water, stabilized with Tween 80 polysorbate, was adopted.

The stability of frozen 9000 *g* supernatant fractions of rat liver was found to be quite variable. Although the loss of activity of some preparations was negligible even after 2 weeks, others lost 25–50% of their activity per day at −15°. When 9000 *g* supernatant fractions from 20% homogenates of rat liver in 0.1 M Tris buffer, pH 7.5, were lyophilized, the resulting dry preparation could routinely be kept for 2 months or longer at −15° with negligible loss of activity. The lyophilized preparation had approximately 75% of the activity of an equivalent amount of fresh 9000 *g* supernatant fraction. This lyophilized preparation catalyzes many other reactions which have been demonstrated by others to be microsomal NADPH-requiring reactions, such as the hydroxylation of acetanilide, the dealkylation of aminopyrine, the side-chain oxidation of pentobarbital, the sulfoxidation of the thioether linkage of chlorpromazine, and the metabolism of phenylbutazone. We have not, however, been able to demonstrate the glucuronide conjugation of phenols with this preparation, under conditions where the phenol is readily conjugated in fresh liver preparations.

The effects of the various components of the standard assay system on the conversion of trichloroethylene to chloral hydrate are shown in Table 4. Nicotinamide, which protects pyridine nucleotides against enzymic destruction (6), and EDTA both activate the system. Mg^{++}, which stimulates many, but not all, microsomal drug-metabolizing enzymes (7), has only a slight effect on this lyophilized preparation. The activity of fresh 9000 *g* supernatant fraction of rat liver was much more responsive to Mg^{++}; 8.3 mM $MgSO_4$ caused as much as 70% increment in chloral hydrate production. Such a fraction which had been frozen for 1 day, however, while losing 25–50% of its activity, also became completely unresponsive to Mg^{++}. $MgCl_2$ gave the same stimulation as did $MgSO_4$. As shown in

TABLE 4
Effects of various substances

The complete system contained 50 mg of lyophilized preparation, 6 μmoles G6P, and the components of the standard assay system with substrate in emulsion.

Omissions	Chloral hydrate formed (μmoles)
None	0.36
Nicotinamide	0.21
$MgSO_4$	0.32
EDTA	0.21
G6P	0.35

Table 4, G6P exhibits no influence on this system; it also has no effect in fresh preparations. One fresh dog liver supernatant fraction, which had only about one-tenth the activity of rat liver fractions, was activated by Mg^{++} and nicotinamide, and slightly inhibited by G6P. In different lyophilized preparations from rat liver, optimum NADP concentration varied from 10^{-5} to 10^{-4} M.

In an experiment performed in sealed flasks, production of chloral hydrate in an atmosphere of nitrogen was 30% of that in oxygen. Because of the volatility of the substrate, however, it was not possible to conduct a rigorously anaerobic experiment.

Conversion of trichloroethylene to chloral hydrate by various lyophilized rat liver fractions in the presence of NADP is shown in Table 5. The activity was entirely in the supernatant fraction after centrifugation at 9000 *g*, which contains the microsomes and soluble enzymes. The fraction sedimenting at 9000 *g*, which consists of nuclei and mitochondria, possessed no activity, but strongly inhibited that of the 9000 *g* supernatant fraction. This is consistent with preliminary observations that fresh 9000 *g* supernatant fractions showed much greater activity than did the whole homogenates from which they had been prepared. These results are similar to those of Axelrod (8, 9), who showed that factors present in liver nuclei and mitochondria of rats, mice, dogs, and guinea pigs, but not in those of rabbits, inhibit microsomal *O*- and *N*-dealkylases. In a preliminary experiment, the fraction of rabbit liver containing nuclei and mitochondria was found to accelerate markedly the microsomal oxidation of trichloroethylene.

Centrifugation of a reconstituted lyophilized 9000 *g* supernatant preparation at 105,000 *g* for 1 hour yielded a pellet and a supernatant fraction, neither of which showed any significant activity toward trichloroethylene (Table 5). However, on combination of these two fractions, much of the activity was restored. There was thus apparently no solubilization of the enzyme system during the process of lyophilization, storage, and reconstitution.

Further experiments with the microsomal fraction prepared from the lyophilized preparation are presented in Table 6. No significant conversion of trichloroethylene to chloral hydrate occurred in this fraction in the presence of NADP unless a NADPH-generating system was present. Thus, in the presence of NADP and either the solu-

TABLE 5

Trichloroethylene metabolism in lyophilized rat liver fractions

Lyophilized preparations derived from 250 mg fresh liver were assayed with substrate in emulsion in a system similar to the standard, but with 10^{-4} M NADP. Microsomal and soluble fractions were prepared from lyophilized 9000 *g* supernatant fraction by centrifugation after reconstitution with water.

Fraction	Chloral hydrate formed (μmoles)
1. 9000 *g* supernatant fraction	0.48
2. 9000 *g* sediment fraction	0.01
3. Fraction 1 + fraction 2	0.04
4. Microsomal fraction	0.05
5. Soluble fraction	0.01
6. Fraction 4 + fraction 5	0.37

TABLE 6

Trichloroethylene metabolism in lyophilized rat liver microsomes

Microsomes, and soluble fraction when used, derived from 370 mg of rat liver, were incubated with substrate in emulsion in a system similar to the standard assay system, except that 3 μmoles of NADP were used as indicated. Other additions as indicated were G6P, 6 μmoles; yeast G6P dehydrogenase, 12.5 units; reduced pyridine nucleotides, 0.75 μmole every 15 min for 2 hr.

Additions	Chloral hydrate formed (μmoles)
NADP	0.04
NADP + G6P + G6P dehydrogenase	0.45
NADP + soluble fraction	0.26
NADPH	0.29
NADH	0.09

ble fraction of rat liver or G6P and G6P dehydrogenase, chloral hydrate formation could be observed. Alternatively, activity was demonstrated when NADPH was added frequently in small amounts during the incubation. NADH substituted only very poorly for NADPH.

Because EDTA causes an acceleration of the conversion of trichloroethylene, the inhibitory effect of a number of metallic cations was tested. As shown in Table 7, iron, cadmium, and zinc salts were the most potent of those tested. It is interesting to compare these results with the case of the microsomal demethylation of isoflavones (10), which is greatly inhibited by cadmium ion, unaffected by ferric salts, and stimulated by high concentrations of ferrous salts. Cupric, mercuric, and silver salts were moderately inhibitory of trichloroethylene oxidation; these cations have been shown to be inhibitors of a number of microsomal drug-metabolizing enzymes, as well as of NADPH oxidation in liver microsomes (10–12). The other cations tested were rather weak inhibitors. The anions accompanying these cations were shown not to influence the assay at the concentrations employed.

TABLE 7
Effect of various cations

The standard system with 75 mg lyophilized enzyme and substrate in emulsion was used, except that EDTA was omitted. Molar concentrations of cations are shown in parentheses.

Cation	Per cent inhibition
Fe^{++}	45 (1×10^{-5}); 89 (1×10^{-4})
Cd^{++}	56 (5×10^{-5}); 91 (3×10^{-4})
Zn^{++}	36 (5×10^{-5}); 55 (6×10^{-5}); 97 (5×10^{-4})
Fe^{+++}	9 (1×10^{-5}); 61 (7×10^{-5}); 90 (1×10^{-3})
Cu^{++}	58 (1×10^{-4}); 100 (1×10^{-3})
Hg^{++}	9 (1×10^{-5}); 50 (1×10^{-4})
Ag^{+}	41 (2×10^{-4}); 93 (5×10^{-3})
Co^{++}	37 (1×10^{-3}); 65 (2×10^{-3})
Pb^{++}	8 (1×10^{-4}); 33 (1×10^{-3})
Ca^{++}	9 (1×10^{-3})
Al^{+++}	36 (2×10^{-3})

Gillette and co-workers (13) have shown that certain demethylating and hydroxylating enzymes of liver microsomes are stimulated by pyrophosphate buffer to a greater extent than by phosphate or Tris buffers. They ascribed this effect to blockage of pyrophosphatases which destroy pyridine nucleotides. Figure 1 shows, however, that

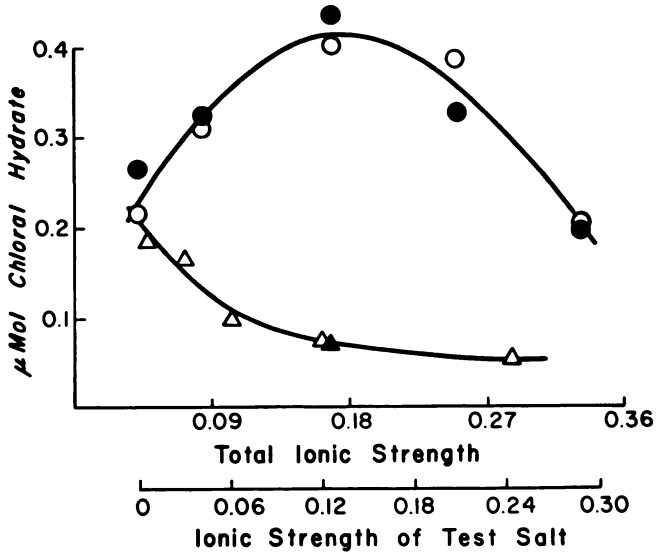

FIG. 1. *Effect of various salts on microsomal oxidation of trichloroethylene*

The standard system was used with 75 mg lyophilized enzyme preparation and substrate in emulsion. ○, sodium chloride; ●, potassium chloride; △, sodium pyrophosphate buffer, pH 7.5; ▲, sodium-potassium phosphate buffer, pH 7.5.

the microsomal oxidation of trichloroethylene was inhibited by sodium pyrophosphate and that this inhibition increased with increasing concentration. Phosphate buffer caused the identical inhibition. That this phenomenon was not an effect of the ionic strength or of the specific cation of the phosphate and pyrophosphate salts is shown by the stimulatory effect of sodium and potassium chlorides at ionic strengths up to about 0.2. At higher concentrations of neutral salt, activity of the microsomal enzyme system again fell. Furthermore, pyrophosphate did not affect the inhibition of trichloroethylene metabolism by the large-particle fraction of rat liver, although such inhibition of the microsomal metabolism of aminopyrine, aniline, and *p*-nitroanisole have been reported to be relieved by pyrophosphate (13).

TABLE 8
Tests of possible inhibitors of trichloroethylene oxidation

The standard assay system was used with 75 mg of lyophilized enzyme preparation and substrate in emulsion. Negative numbers indicate that the rate in the presence of the test compound was greater than control.

Test compound	Concentration (M)	Inhibition (%)
Cytochrome c	5×10^{-6}	95
Sodium cyanide	1×10^{-3}	6
Cytochrome c + NaCN	As above	9
Methylene blue	1×10^{-5}	21
Methylene blue	3×10^{-5}	40
Methylene blue	5×10^{-5}	99
Menadione	5×10^{-5}	62
Menadione	5×10^{-4}	95
Flavin mononucleotide	5×10^{-5}	66
Flavin mononucleotide	5×10^{-4}	98
Sodium fluoride	5×10^{-3}	−2
Sodium azide	1×10^{-3}	16
Sodium iodoacetate	5×10^{-3}	36
Sodium *p*-chloromercuribenzoate	5×10^{-5}	15
Sodium *p*-chloromercuribenzoate	1×10^{-4}	44
Sodium *p*-chloromercuribenzoate	2×10^{-4}	76
2,4-Dichlorophenol	1×10^{-3}	54
α,α'-Dipyridyl	1×10^{-3}	−7
o-Phenanthroline	1×10^{-3}	11
Sodium diethyldithiocarbamate	1×10^{-3}	31
N-Ethylmaleimide	1×10^{-3}	67
Sodium phenobarbital	4×10^{-3}	42
Sodium pentobarbital	4×10^{-3}	46
Acetazolamide	2×10^{-3}	10
Acetaldehyde	1×10^{-3}	−4
Benzaldehyde	1×10^{-3}	0
SKF 525A	8×10^{-3}	39

The inhibition of the system by a number of other agents is shown in Table 8. Cytochrome c, methylene blue, menadione, and flavin mononucleotide were all potent inhibitors of the conversion of trichloroethylene. Such electron-accepting compounds, which may shift the oxidation of NADPH to alternate pathways and thus lower the concentration of reduced coenzyme available for the formation of the active oxygen donor system, inhibit a large number of microsomal drug-metabolizing enzymes (14). In such cases, as well as in the present study, the inhibition by cytochrome c has been shown to be reversed by cyanide ion, presumably because of inhibition by the latter of cytochrome oxidase in the preparation. Cyanide itself, or other metabolic inhibitors such as fluoride, azide, or iodoacetate, inhibited insignificantly or slightly at fairly high concentrations. These compounds have little or no effect on many microsomal drug-metabolizing enzymes (12, 15–18); the latter two compounds, however, which caused slight inhibition of trichloroethylene metabolism, have been shown to inhibit the microsomal oxidation of hexobarbital at high concentrations (19). Trichloroethylene oxidation was inhibited by *p*-chloromercuribenzoate and by the catalase inhibitor 2,4-dichlorophenol to extents comparable to other microsomal drug-metabolic reactions (12, 16, 17). α,α'-Dipyridyl and *o*-phenanthroline did not significantly affect trichloroethylene conversion, in common with several other microsomal systems (12, 18, 19); α,α'-dipyridyl, however, inhibits O-dealkylation (10) and acetanilide hydroxylation (16) at comparable concentrations. Diethyldithiocarbamate weakly inhibited trichloroethylene metabolism, despite the fact that the system is also inhibited by copper ion. Inhibition of the

trichloroethylene reaction by *N*-ethylmaleimide was somewhat greater than in the case of hydroxylation and dealkylation of aniline derivatives (7). Phenobarbital and pentobarbital were weak inhibitors; these barbiturates inhibit a number of microsomal enzymes *in vitro,* but greatly stimulate the activity of the same enzymes 48 hr after they had been administered *in vivo* (20). It has recently been found that the microsomal metabolism of trichloroethylene is greatly enhanced after pretreatment of rats with phenobarbital (21). Acetazolamide, which is a weak inhibitor of the NADH-monodehydroascorbate transhydrogenase of retinal and ciliary microsomes (22), and acetaldehyde and benzaldehyde, inhibitors of the "chloral hydrate dehydrogenase" described by Cooper and Friedman (23), had little or no effect upon trichloroethylene oxidation. Finally, SKF 525A (β-diethylaminoethyl diphenylpropylacetate) inhibited the microsomal metabolism of trichloroethylene only at quite high concentration. This agent, which is a quite potent inhibitor of many microsomal drug-metabolic systems, has little or no effect upon a number of others (24). Both competitive and noncompetitive behavior have been reported for the action of SKF 525A on various microsomal enzymes (14). Kinetic analysis in the present case (Fig. 2) indicated that SKF 525A behaves as a noncompetitive inhibitor in this assay, with $K_I = 1.1 \times 10^{-2}$ M. This experiment was performed by measuring the extent of reaction within the linear period of chloral hydrate formation, which lasts for approximately 30 min.

DISCUSSION

The results described here show the conversion of trichloroethylene to chloral hydrate to take place in rat liver microsomes in a reaction requiring NADPH and oxygen. The characteristics of this process are quite typical of those of a large group of drug-metabolizing enzymes present in liver microsomes. Thus, the system is stimulated by nicotinamide and Mg^{++} and is inhibited by factors present in the nuclei and/or mitochondria of rat liver. The reaction is also inhibited by agents which cause the removal of NADPH via an altered electron-transport system, and by other com-

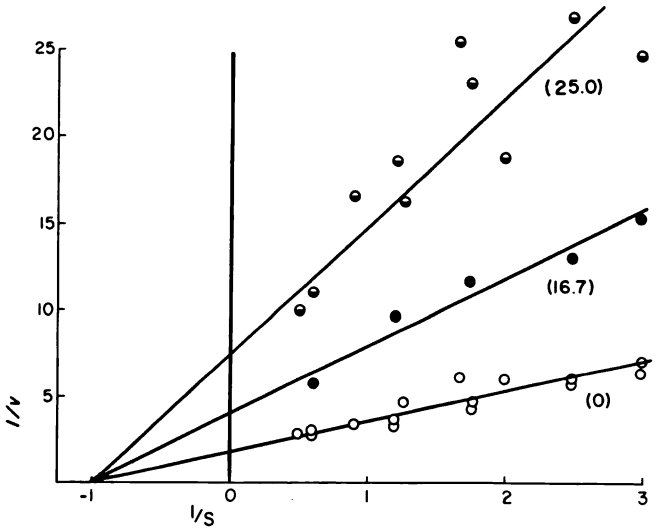

FIG. 2. *Inhibition of microsomal trichloroethylene metabolism by SKF 525A*

Standard system as in Fig. 1, except that concentration of substrate in emulsion was varied, and incubation time was 15 min. Numbers in parentheses represent micromolar concentration of inhibitor. S = per cent trichloroethylene in the system; v = micromoles chloral hydrate formed during the assay.

pounds which typically inhibit microsomal drug-metabolizing enzymes, including, to a certain extent, the "prolonging agent" SKF 525A. Like other microsomal transformations, that of trichloroethylene is but little affected by respiratory and glycolytic inhibitors such as cyanide, azide, fluoride, and iodoacetate.

Lyophilized powders derived from 9000 *g* supernatant fractions of liver homogenates in Tris buffer have proved to be very useful preparations in this work. The stability of the enzyme system responsible for trichloroethylene oxidation in fresh frozen preparations was quite erratic, while that of the lyophilized preparation was in general very dependable. Leadbeater and Davies (25) have recently described a similar lyophilized preparation in which demethylating and hydroxylating enzymes were well preserved. Their lyophilized preparations, however, offered no advantage over frozen preparations stored at —40°. Whether the differences in relative stability of frozen and lyophilized preparations between these authors' report and the current work are due to the use of a lower storage temperature by Leadbeater and Davies than that used here, or to a greater lability of the trichloroethylene-oxidizing enzyme system compared to other microsomal enzymes, is not known. However, the stability at —15° of the trichloroethylene-metabolizing enzyme in fresh frozen preparations appears to be lower and more erratic than that reported for some other microsomal systems (8, 12, 19).

It is interesting to note that reconstituted lyophilized 9000 *g* supernatant fractions of rat liver homogenates not only are active with respect to a number of enzyme activities, but may be centrifugally fractionated into microsomal and soluble fractions in the same manner as fresh supernatant fractions. The structural integrity of the particulate enzyme complex in this preparation is thus evident.

Much of the work reported here was accomplished before the final elucidation of the reaction product as chloral hydrate. The experiments are described, however, in the light of this identification (4).

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