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ALTERATION OF THE ENZYMOLOGY OF CHLORAL HYDRATE REDUCTION IN THE PRESENCE OF ETHANOL *

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Abstract—The enzymology of chloral hydrate reduction to trichloroethanol was studied in rat liver slices and homogenates. Two enzymes capable of reducing chloral hydrate are present in rat liver and by their properties were found to be aldehyde reductase and alcohol dehydrogenase. The alcohol dehydrogenase catalyzed reaction was very sensitive to the NAD/NADH ratio of the incubation medium and was found to be virtually incapable of performing the reduction with a simulated *in vivo* coenzyme ratio. The aldehyde reductase catalyzed reaction was relatively insensitive to the NADP/NADPH ratio. Hence, only one of the two possible enzyme systems appears to catalyze the reduction *in vivo*. Incubations performed with liver slices in the presence or absence of inhibitors and alternative substrates for the two enzyme systems indicated that in the absence of ethanol only aldehyde reductase catalyzed the reduction of chloral hydrate. About a 1.5-fold increase in the rate of reduction of chloral hydrate was observed when 40 mM ethanol was added to the liver slice incubation. Further, deuterium was incorporated into trichloroethanol when the incubations were performed with deuteroethanol. The increased rate of reduction and the deuterium incorporation were both prevented by the inclusion of alcohol dehydrogenase inhibitors (pyrazole and isobutyramide). Thus, in the presence of ethanol, both alcohol dehydrogenase and aldehyde reductase contribute to the reduction of chloral hydrate. Alcohol dehydrogenase is capable of reducing chloral hydrate in the presence of an oxidizable alcohol because it is converted into an enzyme–NADH complex which can then reduce the compound.

Chloral hydrate is primarily reduced in the body to trichloroethanol, though a small amount is oxidized to trichloroacetic acid [1, 2]. Though never unequivocably proven, the enzyme catalyzing this oxidation is presumably either aldehyde dehydrogenase (aldehyde:NAD oxidoreductase, EC 1.2.1.3) or aldehyde oxidase (aldehyde:O_2 oxidoreductase, EC 1.2.3.1). It was suggested in early work that alcohol dehydrogenase (alcohol:NAD oxidoreductase, EC 1.1.1.1) was responsible for the reduction of chloral hydrate [3], since no other general aldehyde-reducing enzymes were known at the time. More recently, aldehyde reductase (EC 1.1.1.2, alcohol:NADP oxidoreductase) was discovered to exist in many tissues [4–10]. Tabakoff *et al.* [11] demonstrated that aldehyde reductase is the enzyme responsible for chloral hydrate reduction in the brain. Recently, however, deuterium incorporation from deuteroethanol into trichloroethanol was demonstrated [12], again implicating alcohol dehydrogenase in the reduction of chloral hydrate.

Adverse physiological reactions occur when a person simultaneously consumes both ethanol and chloral hydrate [13]. During simultaneous administration of the two drugs, the rate of trichloroethanol formation increases over the rate observed in the absence of ethanol [1, 2]. Since liver contains both alcohol dehydrogenase and aldehyde reductase, two enzymes capable of reducing chloral hydrate to trichloroethanol, two different mechanisms for the enhanced rate of formation of trichloroethanol can be envisioned. The first would require that the aldehyde-reducing enzyme responsible for the formation of trichloroethanol utilize the increased pool of NADH formed during the metabolism of ethanol [14, 15]. The second mechanism requires that alcohol dehydrogenase catalyze the reduction of chloral hydrate utilizing the NADH molecule produced directly from the oxidation of ethanol prior to its dissociation from the enzyme. It has been suggested by Sellers *et al.* [1] that, since alcohol dehydrogenase is converted into an enzyme–NADH complex by the oxidation of ethanol, this complex may be capable of reducing chloral hydrate. Since the dissociation of NADH from the enzyme is the rate-limiting step [16], this enzyme intermediate, which builds up during ethanol metabolism, can reduce the aldehyde. This mechanism implies that, during the reduction of chloral hydrate by alcohol dehydrogenase, a corresponding increase in the rate of oxidation of ethanol would occur.

While studying the metabolism of the aldehyde derived from dopamine, 3,4-dihydroxyphenylacetaldehyde, it was shown that both alcohol dehydrogenase and aldehyde reductase were capable of reducing the aldehyde *in vitro* * [17]. It was further shown that *in vivo* aldehyde reductase was responsible for reducing the compound. In the presence of ethanol, however, alcohol dehydrogenase was responsible for an increased rate of reduction of this biogenic aldehyde, presumably by a mechanism similar to that proposed by Sellers *et al.* [1].

The observations of the metabolism of 3,4-dihydroxyphenylacetaldehyde led us to reinvestigate the reduction of chloral hydrate in the presence and

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absence of ethanol. In this study, we report on this investigation performed with rat liver homogenates and slices. A preliminary report of some of these findings has been made [18].

EXPERIMENTAL PROCEDURE

Chemicals. All chemicals were reagent grade and used, except where stated, without further purification. Coenzymes were purchased from P-L Biochemicals, Inc. (Milwaukee, WI). Chloral hydrate was obtained from the Fischer Scientific Co. (Fair Lawn, NJ) and was distilled before use. 2,2,2-Trichloroethanol was obtained from the Eastman Kodak Co. (Rochester, NY). Chlorobutanol was obtained from the Merck Co. (Rahway, NJ) and was sublimed in order to purify the compound. Tris–hydrochloride, dopamine and etiocholan-3β-ol-17-one were obtained from the Sigma Chemical Co. (St. Louis, MO). 4-Hydroxypyrazole was a gift of Dr. David Lester, Rutgers University. Deuteroethanol (CD_3CD_3OD) (98%) was obtained from Stohler Isotope Chemicals (Waltham, MA). Gas chromatographic material (20% carbowax 20 M on Gas-chrom Q) was obtained from Applied Science Lab, Inc. (State College, PA). Distilled water was used in all experiments.

Animals. Both male and female Wistar rats (250–350 g) were obtained from the breeding facilities in the Department of Biochemistry of Purdue University.

Enzyme assays with rat liver homogenates. Rat livers were homogenized in nine parts of 250 mM sucrose-10 mM Tris buffer, pH 7.4, at 4°. The suspension was centrifuged at 39,000 *g* for 1 hr at 4° to remove cellular debris. The supernatant solution was passed through a Sephadex G-25 column (30 × 1 cm) in order to separate the enzymes from the small molecules. The alcohol dehydrogenase and aldehyde reductase activities in the homogenates were measured fluorometrically at 25° with an Aminco filter fluoromicrophotometer. An aliquot of the homogenate was added to a solution of 100 mM sodium phosphate (pH 7.4) containing coenzyme. The spontaneous reaction was allowed to proceed until the endogenous substrates were used up, and then substrate was added. The final volume of the reaction mixture was 1 ml. The concentrations of substrates and coenzyme, as well as of inhibitors, are indicated in Fig. 1.

Incubations with rat liver tissue slices. The liver was rapidly removed from an animal killed by cervical dislocation, cooled to approximately 5°, and then sliced with a Stadie–Riggs tissue slicer. The weight of a slice was between 50 and 70 mg. Incubations were initiated by placing one slice into 10 ml of Krebs–Ringer buffer [19] at 37°. Chloral hydrate (2 mM) and any other effector were added. At 4-min intervals 100 μl of the media were removed, diluted to 1 ml with distilled water, and extracted with 1 ml ether containing 0.8 μg chlorobutanol. The latter compound was used as an internal standard for the gas chromatographic analysis, and all peak heights were related to its peak height. Duplicate incubations were performed with tissue obtained from each animal, and routinely six aliquots were removed from each incubation in order to determine the rate of trichloroethanol formation.

Product analyses. Trichloroethanol concentrations

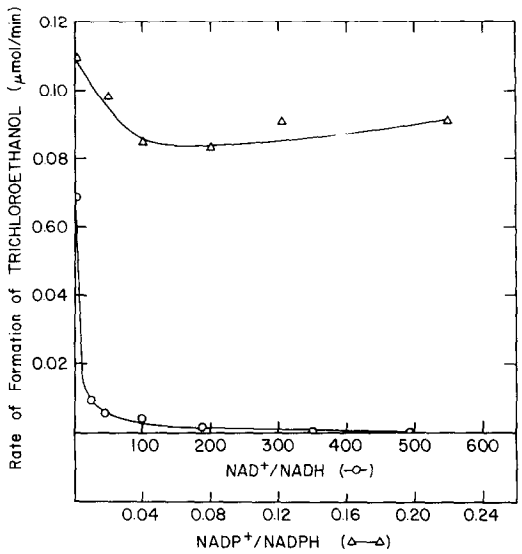

Fig. 1. Effect of oxidized coenzyme on the reduction of chloral hydrate. The effect of the redox potential on the reduction of chloral hydrate was studied by monitoring the velocity of the reaction in the presence of different amounts of oxidized coenzyme. The reaction velocity was measured by monitoring the rate of fluorescence change with time. A 25-μl sample of homogenate was used throughout. When NADH (13.2 μM) (○) was used, the NAD concentration was varied between 0 and 7.8 mM: when NADPH (5.3 μM) (△) was used, the NADP concentration was varied between 0 and 1.2 μM. All the incubations contained 2 mM chloral hydrate. Pyrazole (10 mM) was added when NADPH was used as the cofactor in order to inhibit any alcohol dehydrogenase catalyzed reaction.

were determined by a gas chromatographic procedure based upon the original report of Garrett and Lambert [20]. The analyses were performed with a Varian model 1200 gas chromatograph fitted with a 6 ft stainless steel column packed with 20% carbowax 20 M on Gas-chrom Q. An electron capture detector was employed. The operating temperature of the column was 150° and the carrier flow (N_2) was 120 ml/min. The ether layer (1 μl) was injected into the instrument and the concentrations of components were determined by their peak heights. Each analysis was run in duplicate, and many separate incubations were performed with liver slices obtained from any one animal. Control extractions revealed that the recovery of product was greater than 95 per cent.

Treatment of the data. The rates of trichloroethanol production measured in the duplicate control incubations were averaged and this new rate was normalized to be 100 per cent. The rates of trichloroethanol production for the duplicate runs with effectors added were then averaged, and this rate was normalized to its percentage of the average control rate from the same animal. These calculations were performed to minimize variations in the data arising from differences in the rates of trichloroethanol production between animals. There was an average difference of 9 per cent between the mean and either duplicate control run performed on any individual animal. A Student's *t*-test was used to determine statistical significance of the data.

Mass spectrographic analyses of deuterated

products. Incubations were performed as indicated above except that deuterated ethanol was used. At the end of the incubations the sample was extracted twice with equal volumes of ether, and the ether was concentrated to a volume of 1 ml. A Finnigan model 3700 chromatograph-mass spectrometer equipped with a 4 ft column of 20% carbowax 20 on Gas-chrom Q was employed. Mass fragments were produced upon electron impact. The column temperature and inlet temperature were 40 and 300°, respectively; the carrier gas flow (He) was 20 ml/min and the accelerating voltage was 70 eV. The ratio of the peak heights at mass 113 and 114 which correspond to $(CCl_2–CH_2OH)^+$ and $(CCl_2–CHDOH)^+$ was measured manually and the ratio was used to estimate the incorporation of deuterium [12].

RESULTS

Incubations with rat liver homogenates were performed in order to determine if both alcohol dehydrogenase and aldehyde reductase were capable of reducing chloral hydrate to trichloroethanol. Double reciprocal plots of the data, obtained when the enzyme activities were separately measured, showed that the K_m values for chloral hydrate with alcohol dehydrogenase and aldehyde reductase are 1.8 mM and 6 mM respectively. There was *ca.* 1.5 times more aldehyde reductase activity than alcohol dehydrogenase activity under V_{max} conditions. In order to substantiate further the fact that the NADH-dependent reaction measured was due to alcohol dehydrogenase and not to aldehyde reductase, the reactions were run in the presence of 10 mM pyrazole, an alcohol dehydrogenase inhibitor [21]. This concentration was found to inhibit completely the NADH-mediated reduction of chloral hydrate. Pyrazole had no effect on the NADPH-dependent reduction of chloral hydrate to trichloroethanol.

Having established the fact that both liver alcohol dehydrogenase and aldehyde reductase can reduce chloral hydrate, additional *in vitro* experiments were run to ascertain which enzyme could be performing the reaction *in vivo*, where a physiological ratio of coenzymes occurs. The ability of the two enzyme systems to reduce chloral hydrate in the presence of various coenzyme ratios was measured; the data are presented in Fig. 1. A 2 mM concentration of chloral hydrate was employed because this level is below the LD^{50} for rats (500 mg/kg) [22]. It can be noted from the data that, as the ratio of NAD/NADH increases to near physiological levels between 100 and 1000 [23], the ability of alcohol dehydrogenase to act as a reducing enzyme decreases dramatically. This fact was reported previously for the reduction of *p*-nitrobenzaldehyde* [17]. Theoretical calculations by Purich and Fromm [24] showed that alcohol dehydrogenase would not function effectively as a reducing enzyme when the ratio of NAD/NADH approached physiological levels. Very little alteration in the velocity of the aldehyde reductase catalyzed reaction occurred as the ratio of NADP/NADPH changed. At a ratio of 0.02, approximately that reported to exist in the liver [23], no large loss in reducing activity of aldehyde reductase was observed.

In order to show that aldehyde reductase was truly the enzyme catalyzing the reduction of chloral hydrate *in vivo*, additional experiments were performed utilizing rat liver slices, rather than homogenates. These incubations do not require the addition of coenzymes, since the slices are capable of regulating the endogenous coenzyme levels. The incubations were performed in the presence and absence of known inhibitors of alcohol dehydrogenase and aldehyde reductase. The data are graphically presented in Fig. 2. The average rate of trichloroethanol formation was 0.176 ± 0.024 μmole/min/liver slice. This rate was normalized to be equal to 100 ± 13.6 per cent for statistical analysis. Pyrazole and isobutyramide, inhibitors of alcohol dehydrogenase [25], did not cause a large inhibition of trichloroethanol formation, though pyrazole at either 2 mM or 10 mM did cause some inhibition (*ca.* 20 per cent $P < 0.01$). The inhibition observed with pyrazole could be due to 4-hydroxypyrazole, a metabolite of pyrazole [26], which was found to be an aldehyde reductase inhibitor (K_i + 0.39 mM) (data not shown). Liver slice incubations with 50 μM 4-hydroxypyrazole also caused a 20 per cent inhibition of trichloroethanol formation. Phenobarbital, a partial inhibitor of aldehyde reductase [5], caused a 30 per cent inhibition in the rate of reduction of chloral hydrate. Lastly, 3,4-dihydroxyphenyacetaldehyde, derived from dopa-

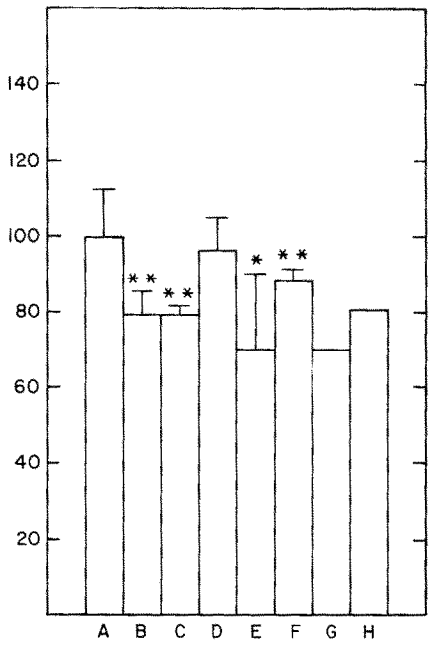

Fig. 2. Effects of inhibitors and enzyme substrates on the reduction of chloral hydrate by rat liver slices. The effects of the added compounds were measured by comparing the rate with the effector to the rate in its absence. All incubations contained 2 mM chloral hydrate, and the rate in the absence of any effector was normalized to 100 per cent. The various effectors added, and the number of experiments performed with the effectors were as follows: control, 13 (A); 10 mM pyrazole, 3 (B); 2 mM pyrazole, 3 (C); 10 mM isobutyramide, 3 (D); 5 mM phenobarbital, 3 (E); 2 mM dopamine, 3 (F); 10 mM *p*-nitrobenzaldehyde, 2 (G); and 50 μM 4-hydroxypyrazole, 1 (H). Error bars indicate standard deviations when n is greater than 2. The single asterisk (*) indicates $P < 0.05$; the double asterisk (**) indicates $P < 0.01$.

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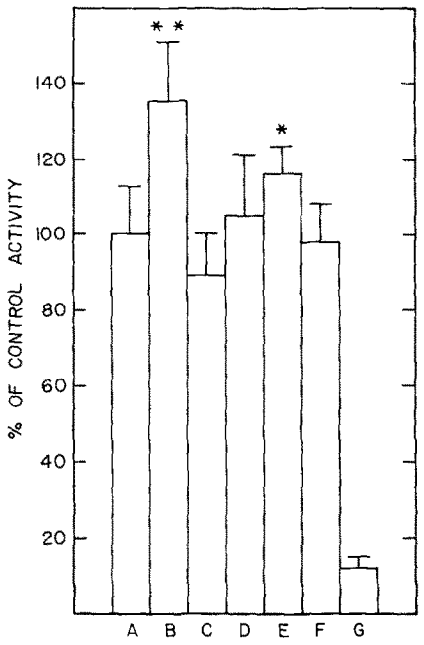

Fig. 3. Effects of compounds on the reduction of chloral hydrate by rat liver slices. The effects of various substances on the reduction of chloral hydrate were monitored by comparing the rate of reduction with the effector to the rate in its absence in slices obtained from the same animal. The rate of chloral hydrate reduction in incubations containing only 2 mM chloral hydrate was normalized to 100 per cent. The various effectors added to incubations and the number of experiments performed with these effectors were as follows: control, 13 (A); 40 mM ethanol, 4 (B); 40 mM ethanol and 10 mM pyrazole, 3 (C); 40 mM ethanol and 10 mM isobutyramide, 3 (D); 1 mM eticholan-3β-ol-17-one, 3 (E); 10 mM lactate, 3 (F); and reduction by 100 μl of blood in the absence of the rat liver slices, 3 (G). Error bars indicate standard deviations. The single asterisk (*) indicates $P < 0.05$; the double asterisk (**) indicates $P < 0.01$.

mine, and *p*-nitrobenzaldehyde compounds, shown previously to be reduced *in vivo* by aldehyde reductase* [17], slowed the rate of reduction of chloral hydrate as would be expected if an alternative substrate for the enzyme were present. These results, coupled with those obtained from the *in vitro* assays, lead us to conclude that *in vivo* aldehyde reductase is the enzyme primarily responsible for reducing chloral hydrate in liver, as it is in brain.

Ethanol was found to increase the rate of production of trichloroethanol by 34 per cent; these data are presented in Fig. 3. Pyrazole and isobutyramide prevented this increased rate in the reduction of chloral hydrate in the presence of ethanol. This fact shows that the enhanced rate is not due to the mere presence of ethanol, but to its metabolism. To verify that a substrate for alcohol dehydrogenase must be metabolized in order to observe the increased rate of reduction of chloral hydrate, incubations were carried out in the presence of a steroid, eticholan-3β-ol-17-one, which is a substrate for rat liver alcohol dehydrogenase [17]. The

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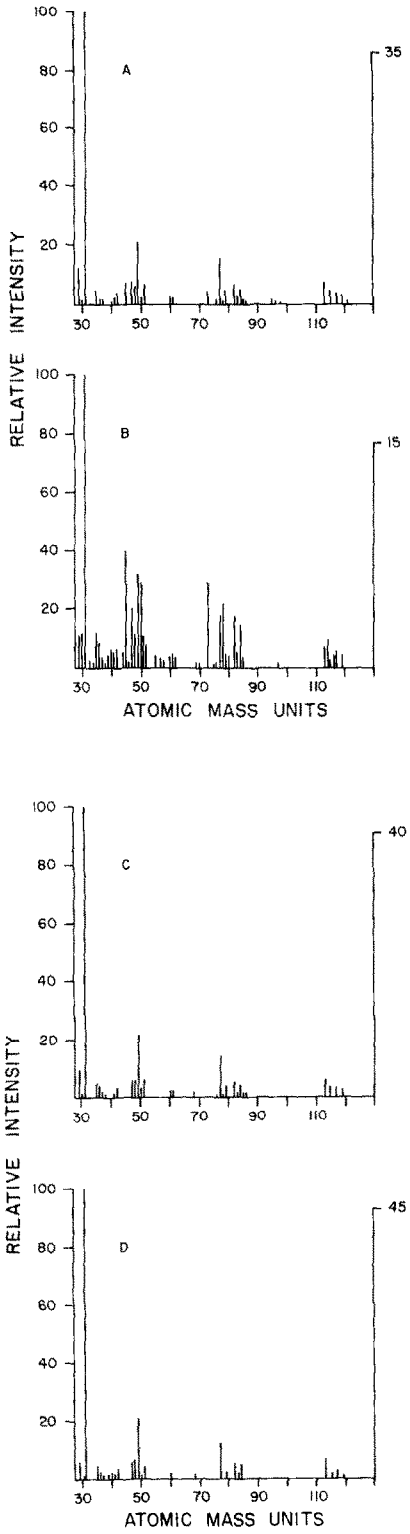

Fig. 4. Mass spectra of tricholoroethanol formed from incubations of rat liver slices. The incubation conditions were identical to those described in Experimental Procedure. Panel A: 2 mM chloral hydrate; panel B: 2 mM chloral hydrate and 40 mM deuteroethanol; panel C: 2 mM chloral hydrate, 40 mM deuteroethanol and 10 mM isobutyramide; and panel D: 2 mM chloral hydrate, 40 mM deuteroethanol and 10 mM pyrazole.

rate of reduction was increased in the presence of this alternative substrate for alcohol dehydrogenase, but not as dramatically as it was by ethanol (15 per cent compared to 34 per cent).

NADH levels increase during ethanol metabolism [14, 15]. If the only reason for the increased rate of trichloroethanol formation were that more NADH was being produced during ethanol metabolism, any other substrate which is rapidly oxidized by an NAD-dependent reaction should produce a similar effect. In order to investigate the effects of changing the NADH level on the reduction of chloral hydrate, incubations were performed in the presence of lactate, a compound whose oxidation would also change NADH levels. Previous studies have shown that addition of lactate increases pyruvate formation under nearly identical conditions [17]. Thus, the NADH/NAD ratio will be altered in the presence of lactate since lactate–pyruvate are in thermodynamic equilibrium [27]. As can be seen from the data presented in Fig. 3, lactate had no effect on the rate of chloral hydrate reduction. Thus, a simple increase in NADH levels is not the cause of the increased rate of metabolism of chloral hydrate. Blood itself was not effective in catalyzing the reduction of chloral hydrate.

The increased rate of reduction of chloral hydrate in the presence of ethanol may be due to a direct involvement of alcohol dehydrogenase in the reduction of chloral hydrate. In order to provide independent support for this, the reduction of chloral hydrate was performed in the presence of deuteroethanol. If only alcohol dehydrogenase were involved in the reduction, all of the trichloroethanol produced would be labeled with deuterium. If, however, the metabolism is normally due to aldehyde reductase, and only in the presence of ethanol would alcohol dehydrogenase be partially involved, less than 100 per cent deuterium incorporation would be obtained. The data presented in Figs. 1 and 2 suggest that up to 35 per cent of the metabolism may be caused by alcohol dehydrogenase when ethanol is present. Analysis of the mass spectrum (Fig. 4) indicates that the product is *ca.* 60 per cent labeled with deuterium. The alcohol dehydrogenase inhibitors, pyrazole and isobutyramide, prevented the deuterium incorporation into trichloroethanol.

DISCUSSION

Though alcohol dehydrogenase can reduce chloral hydrate very effectively in an *in vitro* assay, it does not seem to be capable of reducing the compound *in vivo* in the absence of ethanol. This is due to the fact that the NAD/NADH ratio in the cell is so large that the enzyme is primarily in an enzyme–NAD complex, which is primed to oxidize compounds rather than reduce them. Thus, our data indicate that aldehyde reductase is the enzyme reducing the compound in liver. Therefore, the conclusions reached by Tabakoff *et al.* [11] with brain should hold true for every organ in the body, since aldehyde reductase is ubiquitous and alcohol dehydrogenase is found primarily in liver. Aldehyde reductase, which requires NADPH as a cofactor, is in its normal enzyme–NADPH complex as a result of the NADPH/NADP ratio of the cell, and is primed to reduce aldehydes. The metabolic role of the two enzymes then seems to be that alcohol dehydrogenase is present primarily to oxidize alcohols to aldehydes, while aldehyde reductase is responsible for reducing aldehydes to alcohols.

Neither isobutyramide nor pyrazole, which are alcohol dehydrogenase inhibitors, was capable of dramatically inhibiting the formation of trichloroethanol in incubations with rat liver slices. The small amount of inhibition seen when pyrazole is added to rat liver slices is probably not a direct effect of the compound, since the amount of inhibition is not related to the concentration of pyrazole added. The concentrations of pyrazole used have been shown to essentially eliminate ethanol metabolism, but have also been shown to have slight effects on other enzymes [28]. If alcohol dehydrogenase were the major enzyme involved in chloral hydrate reduction, a dramatic inhibition in the rate of reduction should have been observed. Pyrazole, as well as isobutyramide, was effective though in preventing the enhanced rate of reduction when ethanol was included in the incubation media. Thus, the enhanced rate in the presence of ethanol is due to the metabolism of ethanol.

The fact that the changes in NADH pool size alone do not change the rate of reduction of trichloroethanol suggests that the mechanism of the ethanol enhancement of trichloroethanol production must not be due to the simple formation of NADH during ethanol oxidation. Acetaldehyde, which is produced during ethanol metabolism, is not responsible for the increased rate of trichloroethanol formation, since the steroid substrate for alcohol dehydrogenase mimics the effect of ethanol.

We have shown previously that, in the presence of ethanol, alcohol dehydrogenase is converted from an oxidizing enzyme (E-NAD) into a reducing enzyme (E-NADH), which, prior to dissociation of NADH, can reduce aldehydes to alcohols* [17]. This type of observation was reported previously by others utilizing many different substrates [29–31]. Only in the presence of ethanol, then, would both alcohol dehydrogenase and aldehyde reductase be capable of reducing chloral hydrate. It appears, at least under the conditions of the incubations reported here, that approximately one-third of the trichloroethanol formed in the presence of ethanol is produced from the alcohol dehydrogenase catalyzed reaction.

A possible objection to the explanation for partial deuterium incorporation into trichloroethanol when incubations were performed with deuterated ethanol is that alcohol dehydrogenase could use the existing NADH pool in the cell to reduce chloral hydrate. If this were the case, the D-NADH formed would dissociate from the enzyme and be exchanged for an H-NADH in the existing NADH pool. Experimental evidence reported here refute the importance of this possibility. Alcohol dehydrogenase does not seem to operate as a reducing enzyme even when the NADH level is high, as noted by the lack of a lactate effect on the rate of trichloroethanol reduction. For the horse liver enzyme, the rate of dissociation of NADH is 7 sec^{-1}, while the rate of hydride transfer is >250 sec^{-1} [32]; hence, it is highly improbable that D-NADH will dissociate prior to being transferred to trichloroethanol. We conclude

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that the incomplete deuteration of the trichloroethanol indicates that two different enzymes are involved in its reduction in the presence of ethanol. A similar pattern of deuterium incorporation with whole animals has been reported recently by Wong and Biemann [12]. This observation indicates that the metabolism of chloral hydrate must be similar, if not identical, in whole animals and in tissue slice incubations. Even during the metabolism of ethanol, which allows alcohol dehydrogenase to become a reducing enzyme, aldehyde reductase is always operating as a reducing enzyme.

Since the involvement of alcohol dehydrogenase implies clearly that the rates of both ethanol oxidation and chloral hydrate reduction would be enhanced, it can be speculated that the adverse physiological effects of the two drugs are due to an increased rate of formation of trichloroethanol, as well as the increased formation of acetaldehyde. The combination of these two alterations may somehow lead to the hypnotic "knockout" effect observed when the drugs are consumed simultaneously.

Ethanol is known to alter the metabolism of many drugs in the body, possibly by the induction of microsomal P-450 activity [33]. The enhancement of this enzyme system may be responsible for the alteration of metabolism of some compounds in the presence of ethanol. The mechanism proposed in this paper, which is similar to that reported by others, may be of a general nature. If the metabolism of some xenobiotic compound produces an aldehyde intermediate, the presence of ethanol converting alcohol dehydrogenase from an oxidizing enzyme to a reducing enzyme may then cause an increased rate of reduction of this foreign compound. What the total physiological effect of this alteration in metabolism is, is certainly not known. Depending upon the compound, though, the newly formed alcohol may produce an adverse physiological response in the animal.

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