Biochemical Pharmacology, Vol. 29, pp. 3011–3016.
Pergamon Press Ltd. 1980. Printed in Great Britain.

STUDIES ON THE HYPNOTIC EFFECTS OF CHLORAL HYDRATE AND ETHANOL AND THEIR METABOLISM *IN VIVO* AND *IN VITRO*

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(*Received* 26 *April* 1980; *accepted* 21 *July* 1980)

Abstract—The ED_{50} values for chloral hydrate, trichloroethanol and ethanol were determined in mice in terms of loss of righting reflex. Co-administration of chloral hydrate and ethanol had an additive effect, but both chloral hydrate and trichloroethanol at subhypnotic doses significantly prolonged ethanol sleeping time. Chloral hydrate and ethanol, given together, yielded lower blood ethanol levels than when ethanol was given alone. Chloral hydrate was reduced by liver alcohol dehydrogenase (K_m = 7.5 mM). Trichloroethanol was not a substrate for the enzyme but inhibited ethanol oxidation competitively ($K_i = 1.38 \times 10^{-5}$ M). It is proposed that chloral hydrate metabolism to trichloroethanol is increased by ethanol and that this is responsible for the prolonged sleep time.

The interaction between the hypnotic chloral hydrate and ethanol has been the subject of several investigations over a number of years and various theories have been put forward to explain the apparent potentiation of ethanol by chloral hydrate. Friedman and Cooper [1] demonstrated that chloral hydrate could be reduced by the action of an enzyme apparently identical to alcohol dehydrogenase in the presence of NAD^+. On this basis it was proposed that the two drugs competed for the same route of inactivation. However, the reduced metabolite of chloral hydrate, 2,2,2-trichloroethanol, was also found to be a potent hypnotic [2] despite an earlier negative report [3]. Subsequent work has shown, however, that the pretreatment of mice with chloral hydrate results in an increased rate of production of acetaldehyde [4] implying a more rapid rate of ethanol metabolism.

Cabana and Gessner [2] found that the co-administration of chloral hydrate and ethanol shortened the half-life of chloral hydrate in whole mice but did not alter the half-life of trichloroethanol. It would appear, therefore, that chloral hydrate and ethanol, contrary to earlier beliefs, actually hasten their metabolism when given together. Direct evidence of this possibility has recently been provided by Wong and Biemann [5] using ethanol-D_6 and unlabelled chloral hydrate. The co-administration of these two drugs to a rat resulted in the appearance of 1-deutero-2,2,2-trichloroethanol and unlabelled acetaldehyde. This indicates a coupling *in vivo* of the oxidation of ethanol and loss of the α-hydrogen atom to the reduction of chloral hydrate and gain of an α-hydrogen by way of NADH.

The questions remaining to be answered are whether or not chloral hydrate merely prolongs the apparent action of ethanol by the formation of trichloroethanol, which has a longer half-life than either chloral hydrate or ethanol [2], and, if so, whether the initial interaction is only an additive effect due to the central depressant actions of both drugs. The present work has therefore examined the interactions between chloral hydrate, trichloroethanol and ethanol both in terms of their ED_{50} values (loss of righting reflex) and the duration of the loss of righting reflex. Under these circumstances we are able to demonstrate either addition or apparent potentiation depending upon the experimental conditions used.

The *in vitro* interactions between trichloroethanol, chloral hydrate and ethanol have been examined using purified crystalline liver alcohol dehydrogenase under standard assay conditions [6] as well as under conditions which more closely approximate to those occurring in the liver *in vivo*. The results have indicated that the NAD^+/NADH ratio is likely to be an important factor in determining the fate of chloral hydrate and ethanol *in vivo*, and also that the trichloroethanol produced from chloral hydrate is significant in the apparent potentiation of ethanol by chloral hydrate. Some of the present findings have already been presented in a preliminary report [7].

METHODS

Adult LACG mice of either sex from an inbred colony were used throughout. All drugs were obtained from BDH Ltd., Poole, Dorset, U.K., except for pyrazole which was obtained from Koch-Light Ltd., Colnbrook, Bucks, U.K. The purified crystalline enzymes and cofactors used in the kinetic studies were supplied by The Boehringer Corporation Ltd., Lewes, East Sussex, U.K.

Drugs for injection were made up in 0.9% (w/v) saline buffered to pH 7.4 with sodium phosphate buffer (50 mM) and administered i.p.

The 'sleeping time' was defined as the duration of the loss of righting reflex and was recorded to the nearest 0.25 min. The criterion for loss of righting reflex was that the mouse, when placed gently on its

back, made no attempt to right itself within 15 sec. Recovery was checked by replacing the mouse on its back and again observing the criterion. The latency of onset was taken as the time between the injection and the loss of righting reflex. Where two drugs were administered, the time was recorded following the second injection.

The ED_{50} values were defined as the doses which produced loss of the righting reflex in half the animals tested and were calculated using the moving average interpolation method of Weil [8] using at least 4 groups of 6 animals.

Preparation of tissue extracts. The mice were killed by stunning and immediate decapitation. Blood was collected from the trunk onto a heparinized wax block and a 100-μl aliquot taken and mixed with 500 μl 6% (w/v) perchloric acid using a vortex stirrer. The sample was then centrifuged at 1200 *g* for 10 min and the supernatant fraction withdrawn and stored at −20°. Under these conditions the samples could be kept without deterioration for up to four months.

The mouse brain was rapidly dissected out, rinsed briefly in the ice-cold physiological saline to remove surface blood, blotted, weighed, and then homogenized in 2 ml 6% (w/v) perchloric acid using a teflon–glass top-drive homogenizer. The homogenate was centrifuged at 1200 *g* for 10 min and the resultant supernatant fraction withdrawn and stored as before.

Analysis of samples. Ethanol was assayed using a Perkin Elmer F40 Multifract GLC in the F1D mode with a carbopack column at 80° and N_2 as the carrier gas. An internal standard of *n*-propanol was added to the samples prior to the assay. The GLC was calibrated each day using standard ethanol solutions, and the peak areas calculated using a Hewlett Packard 3380a integrator programmed to correct for recovery.

Chloral hydrate and trichloroethanol were assayed using a Pye Unicam 104 GLC in the ECD mode with Carbowax 80 column at 100° and N_2 as the carrier gas. Chloral hydrate was first extracted into *n*-heptane before injection onto the column. The *n*-heptane:water partition coefficient for chloral hydrate was found to be 0.20 at 20°. The overall recovery of chloral hydrate was 19.2 ± 0.19 per cent (mean ± S.E.M. of five estimates) and this correction was applied to all the results. *n*-Heptane itself provided a reference peak which was used as an internal standard. Trichloroethanol was assayed similarly except that water was used as an internal standard, and the overall recovery was 82.0 ± 0.96 per cent.

Assay of alcohol dehydrogenase. The assay method used was that of Bonnichsen and Brink [6] using a Unicam SP1800 spectrophotometer linked to an AR linear recorder. Crystalline horse liver alcohol dehydrogenase was used in all the kinetic studies. Alcohol oxidation was determined by observing the increase in absorbance due to NADH at 340 nm. Assay conditions were as follows: alcohol oxidation, sodium hydroxide–glycine buffer pH 9.6 (50 mM); aldehyde reduction, sodium phosphate buffer pH 6.4 (50 mM). Some assays were also carried out at physiological pH using a sodium phosphate buffer. Initial reaction rates were measured for 3 min following the addition of substrate during which time the rate remained linear. Values for V_{max}, apparent K_m and inhibitor constants (K_i) were determined from Lineweaver-Burk double reciprocal plots of $1/v_i$ against $1/s$ and confirmed by the direct linear plot of V_i against s [9].

Statistical analyses were performed where appropriate using Student's *t*-test for measuring the significance of the difference between the means of independent groups.

RESULTS

Interactions between chloral hydrate and ethanol in vivo. The ED_{50} values for chloral hydrate, trichloroethanol and ethanol are shown in Table 1. On a weight basis, chloral hydrate and trichloroethanol appear to be equipotent and approximately ten times as potent as ethanol in producing loss of the righting reflex. On a molar basis, however, trichloroethanol is seen to be slightly less potent than chloral hydrate. The ED_{50} was then determined for an equipotent mixture of ethanol and chloral hydrate (10:1 by weight). The results shown in Table 1 indicate that the ED_{50} values of the drugs in combination are half the ED_{50} values of either drug given alone, thus indicating an additive effect.

The effects of subhypnotic doses (150 mg/kg) of chloral hydrate and trichloroethanol on the potency of ethanol are illustrated in Fig. 1 where the incidence of loss of righting reflex in groups of 12 mice has been plotted against the log dose of ethanol. Under these conditions, both chloral hydrate and trichloroethanol produced a marked shift of the log dose–response line to the left, implying a synergistic action.

Table 1. Relative potencies of chloral hydrate, trichloroethanol and ethanol

	ED_{50} values (±95% confidence limits) mg/kg	mmoles/kg
Chloral hydrate	199 ± 11.1	1.20 ± 0.067
Trichloroethanol	199 ± 11.2	1.33 ± 0.075
Ethanol	2010 ± 112	43.7 ± 2.4
Ethanol + chloral hydrate (10:1, equipotent mixture): ethanol	1007 ± 113	21.9 ± 2.46
chloral hydrate	99.7 ± 1.18	0.602 ± 0.017
Ethanol (+150 mg/kg chloral hydrate)	503.5 ± 22.5	10.9 ± 0.27
Ethanol (+150 mg trichloroethanol)	374 ± 40	8.13 ± 0.87

* ED_{50} values were calculated by the method of Weil (1952) data from at least 4 groups of 6 animals. The drug combinations were administered together.

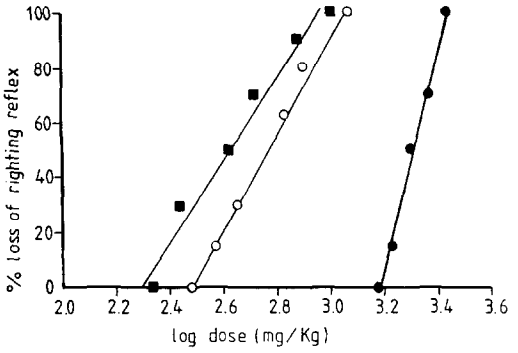

Fig. 1. Effects of chloral hydrate and trichloroethanol on the log dose-response relationship for ethanol. Key: ● Ethanol alone; ○, ethanol + chloral hydrate (150 mg/kg); ■ ethanol + trichloroethanol (150 mg/kg). The ethanol was administered 5 min after the chloral hydrate or trichloroethanol. Each point represents data from a group of 12 animals. Lines were drawn by the method of least squares.

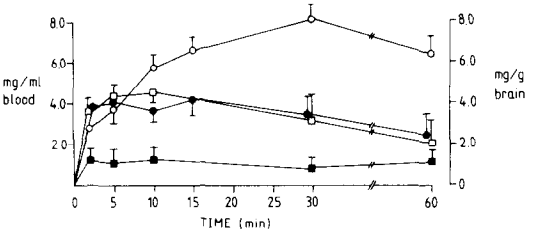

Fig. 2. Brain and blood levels of chloral hydrate and trichloroethanol. Mice were injected at zero time with either 200 mg/kg of chloral hydrate (solid symbols) or trichloroethanol (open symbols). Each point represents the mean of at least six observations. Some of the standard error bars have been omitted for clarity. Key: ● Chloral hydrate in brain; ■, chloral hydrate in blood; ○, trichloroethanol in brain; □, trichloroethanol in blood.

Neither chloral hydrate nor trichloroethanol at 150 mg/kg produced loss of the righting reflex.

The effects of chloral hydrate and trichloroethanol on the latency and duration of the ethanol sleeping time are shown in Table 2. Both drugs, when given in combination with ethanol, produced a significant ($P < 0.02$) shortening of the latency and increase in the duration of the loss of righting reflex. The mean sleeping times following ethanol (2 g/kg), trichloroethanol (200 mg/kg) or chloral hydrate (200 mg/kg) were not significantly different, although chloral hydrate had a significantly ($P < 0.05$) longer latency than either ethanol or trichloroethanol.

The brain and blood levels of chloral hydrate and trichloroethanol following the administration of 200 mg/kg i.p. of either drug are shown in Fig. 2. Both chloral hydrate and trichloroethanol attained higher concentrations in the brain than in the blood between 10 and 60 min of injection. Both brain and blood levels of trichloroethanol were significantly greater ($P < 0.05$) than those of chloral hydrate during the same time period. The peak brain level of

Table 2. Effects of chloral hydrate, trichloroethanol and ethanol on sleeping times

Drug treatment	Latency (min)	Duration (min)
Ethanol (2 g/kg)	3.75 ± 1.2	13.8 ± 4.3
Chloral hydrate (200 mg/kg)	7.10 ± 1.8	5.70 ± 2.9
Trichloroethanol (200 mg/kg)	3.81 ± 0.67	6.71 ± 3.1
Ethanol + chloral hydrate (2 g/kg) (200 mg/kg)	1.54 ± 0.07†	78.5 ± 7.2‡
Ethanol + trichloroethanol (2 g/kg) (200 mg/kg)	2.13 ± 0.20†	73.6 ± 6.4‡

* Each value represents the mean ± S.E.M. from at least eight observations. Sleeping times were defined as described in Methods.
† Latency < latency for either drug alone, $P < 0.05$.
‡ Duration > duration for either drug alone, $P < 0.01$.

Table 3. Effects of pyrazole on chloral hydrate and trichloroethanol sleeping times

	Chloral hydrate (200 mg/kg)		Trichloroethanol (200 mg/kg)	
Sleeping time	Control	Pyrazole	Control	Pyrazole
Latency (min)	7.8 ± 1.8	2.3 ± 0.16	3.8 ± 0.7	6.2 ± 0.5
Duration (min)	5.7 ± 2.9	36.7 ± 6.4 $P < 0.01$	6.7 ± 3.0	41.3 ± 8.6 $P < 0.01$†

* Mice were injected i.p. with 200 mg/kg pyrazole or saline as control 10 min prior to injection of chloral hydrate or trichloroethanol by the same route. Each value represents the mean ± S.E.M. of at least six observations.
† P values refer to the appropriate control value and were estimated from one-tailed *t*-test.

trichloroethanol also appeared to occur later than that for chloral hydrate.

The significance of the possible conversion of chloral hydrate to trichloroethanol by alcohol dehydrogenase *in vivo* was examined by observing the effects of the alcohol dehydrogenase inhibitor pyrazole on the chloral hydrate and trichloroethanol-induced sleep times. The results are shown in Table 3. Pyrazole itself, at the dose used (200 mg/kg), had no detectable depressant action but, given 10 min prior to either chloral hydrate or trichloroethanol, produced a highly significant ($P < 0.01$) prolongation of the sleeping time. The latency of onset of loss of the righting reflex following chloral hydrate was significantly decreased, although in the case of trichloroethanol it was increased. In a few mice, the brain concentrations of chloral hydrate and trichloroethanol were determined at the same time they regained their righting reflex. The mean values obtained were 3.8 ± 0.6 mg/g and these were not altered by the pyrazole pretreatment.

The interaction *in vivo* between chloral hydrate and ethanol in terms of their distribution between the brain and blood was examined by administering ethanol (2 g/kg) or chloral hydrate (200 mg/kg) either alone or in combination and assaying brain and blood levels of the two drugs over 1 hr. The peak brain concentration of ethanol (9.8 mg/g) and chloral hydrate (8.0 mg/g) occurred 5 min post-injection. After 1 hr, the brain ethanol concentration was half the peak value and that of chloral hydrate 80 per cent of the peak value. The blood levels followed a similar pattern. The coadministration of ethanol and chloral hydrate resulted in lower blood and brain levels of ethanol compared to those observed following ethanol alone, although the differences were not statistically significant ($P < 0.1$). The blood and brain levels of chloral hydrate were not significantly altered by the co-administration of ethanol at any time between 5 and 60 min post-injection.

Interactions between chloral hydrate and ethanol in vitro. The metabolism of chloral hydrate and ethanol by alcohol dehydrogenase was investigated *in vitro* using a purified enzyme extract under conditions favouring either oxidation or reduction of the substrate. Ethanol was readily oxidized by alcohol dehydrogenase in the presence of NAD^+ ($K_m = 1.11$ mM), whereas chloral hydrate was not oxidizable, but was reduced in the presence of NADH ($K_m = 7.5$ mM). The addition of chloral hydrate to assay mixtures of ethanol and NAD^+ produced apparent inhibition of ethanol oxidation, possibly due to the coupled reduction of chloral hydrate by NADH produced from the forward reaction. Trichloroethanol was not a substrate for alcohol dehydrogenase, but inhibited the oxidation of ethanol *in vitro*. A Lineweaver-Burk double reciprocal plot (Fig. 3) indicated that the inhibition was of the competitive type with respect to ethanol and that the inhibitor constant (K_i) was 1.38×10^{-5} M.

The influence of the ratio of NAD^+ to NADH on the rate of oxidation of ethanol and reduction of chloral hydrate was determined at pH 7.4 in order to provide a better approximation of the conditions existing *in vivo*. The results are shown in Fig. 4. It is apparent that ethanol oxidation is less favoured

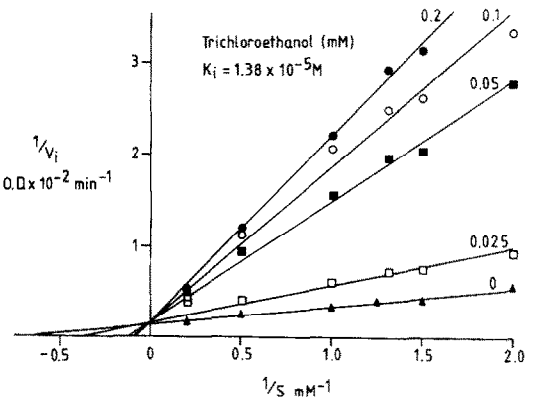

Fig. 3. Lineweaver-Burk double reciprocal plot showing inhibition of alcohol dehydrogenase by trichloroethanol. Assays were performed in triplicate with varying concetrations of ethanol and excess NAD^+. The value of the inhibitor constant for trichloroethanol was calculated from the change in slope of the lines which were fitted by means of least squares.

at increasing NAD^+/NADH ratios, whereas chloral hydrate reduction increases. Thus, at NAD^+/NADH ratios of >30, chloral hydrate reduction will occur at a faster rate than ethanol oxidation in spite of chloral hydrate having a lower affinity for the enzyme.

DISCUSSION

The interaction between the hypnotic chloral hydrate and ethanol have provoked considerable interest, partly as a result of the popular belief in the efficacy of the 'Micky Finn' (a mixture of chloral hydrate and ethanol) [10]. It was originally held that chloral hydrate and ethanol reacted to form chloral alcoholate in solution and that this complex was

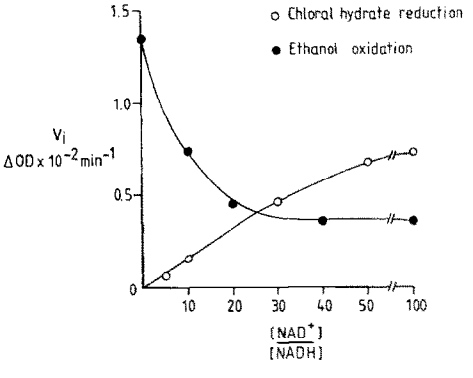

Fig. 4. Effect of NAD^+/NADH ratios on the metabolism of chloral hydrate and ethanol by alcohol dehydrogenase *in vitro*. The reaction mixture consisted of either chloral hydrate or ethanol (5 mM) and 0.05 u of liver alcohol dehydrogenase in sodium phosphate (pH 7.4) buffer. Each point represents the mean of quadruplicate determinations of the initial velocity over 2 min periods at 22°. Standard errors lie within the points on the graph.

responsible for the increased hypnotic action of the mixture [11]. However, it has since been shown that chloral alcoholate dissociates fairly rapidly in aqueous solution to yield free ethanol and chloral hydrate [12]. It is now apparent that the reduction of chloral hydrate to trichlorocthanol can bc coupled to the oxidation of ethanol to acetaldehyde [5], so the relative hypnotic potency of trichloroethanol is an important factor in considering thc hypnotic effects of chloral hydrate.

The present results indicate that trichloroethanol is somewhat less potent than chloral hydratc in terms of ED_{50}, particularly when taking into account that higher brain levels of trichloroethanol can be achieved after i.p. administration of a given dose compared to chloral hydrate (Fig. 2). In terms of duration of the loss of righting reflex, trichloroethanol appears to be more potent than chloral hydrate, which probably reflects the higher maintained brain concentration (Fig. 2) rather than an inherent difference in sensitivity of the brain to the two drugs.

The ability of either chloral hydrate or trichloroethanol to potentiate the effects of ethanol has been tested both in terms of ED_{50} values (Table 1) as well as duration of sleeping time (Table 2). The observation that a mixture of half the equipotent doses of chloral hydrate and ethanol has the same ED_{50} as either drug at the full dose (Table 1) is generally regarded as an indication of a purely additive effect [13]. This design of experiment eliminates the problems associated with interactions at the metabolic inactivation stages which is not the case when duration of loss of righting reflex is considered.

Apparent potentiation of ethanol could be demonstrated by the co-administration of subhypnotic doses of either chloral hydrate or trichloroethanol when the ED_{50} for ethanol was significantly decreased (Table 1 and Fig. 1). The prolongation of the sleeping time by pyrazole administered prior to chloral hydrate or trichloroethanol (Table 3) could also be described as potentiation since it is thought to act specifically by the inhibition of alcohol dehydrogenase and blockade of the metabolism of the alcohol [14, 15].

Measurement of blood and brain levels of chloral hydrate and ethanol following their administration either alone or in combination showed that chloral hydrate reduced the blood ethanol level (although not significantly) rather than increasing it, indicating that the potentiation of ethanol sleeping time was not due to inhibition of ethanol metabolism. This observation is consistent with the rise in blood acetaldehyde level observed by Greaven and Roach [4] and implies an increased rate of ethanol oxidation in the presence of chloral hydrate. Earlier workers have found that trichloroethanol appears quite rapidly in the brain following the peripheral administration of chloral hydrate [3], but a comparative study on the interactions between trichloroethanol and ethanol indicated no potentiation but merely additive effects [16]. Trichloroethanol is, however, a potent hypnotic which is retained in the brain much longer than chloral hydrate (see Fig. 2 and Ref. 3). Thus the apparent potentiation of ethanol sleeping time by chloral hydrate can be explained in terms of the increased rate of metabolism of chloral hydrate to trichloroethanol in the presence of ethanol.

The significance of ethanol metabolism in the termination of its hypnotic action *in vivo* is still unclear since ethanol is unusual in that it is metabolized at a constant rate *in vivo* which is independent of the blood level [17]. This may be due to the low affinity of ethanol for alcohol dehydrogenase (K_m = 2.5 mM), or the rate limiting availability of NAD as co-factor for the enzyme [18]. The demonstration of the dependence on the NAD^+/NADH ratio of the oxidation of ethanol and reduction of chloral hydrate (Fig. 4) lends further support to this view. The NAD^+/NADH ratio in whole liver *in vivo* has been found to be ~4 [19], although much higher ratios can be found in specific cell fractions [20], which should favour the oxidation of an exogenous substrate such as ethanol. The importance of the levels of the pyridine nucleotides in the liver during the metabolism of ethanol is well illustrated by the fact that, during the metabolism of ethanol in the rat, a significant fall in the NAD^+ and rise in the NADH levels can be detected [19].

Shultz and Weiner [21] have recently shown that aldehyde reductase can reduce chloral hydrate in rat liver *in vitro* in a reaction which is not sensitive to the NAD^+/NADH ratio. This enzyme may well be important in the metabolism of chloral hydrate *in vivo*.

In conclusion. it would appear that, under appropriate conditions, chloral hydrate can appear to potentiate ethanol, if prolongation of sleeping time is considered, but that, in terms of ED_{50}, the drugs are merely additive. Potentiation, in the strict sense, can only be demonstrated conclusively by use of isobolograms which require a large number of ED_{50} values to be determined for a series of drug combinations [21].

Acknowledgements—We are indebted to the South West Regional Forensic Science Laboratory for their assistance in the GLC assay of ethanol. The highly competent assistance of Mr. Jon Lynas is also gratefully acknowledged.

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