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Reduction of chloral hydrate to trichloroethanol in brain extracts

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THE PRIMARY product of chloral hydrate metabolism is trichloroethanol[1] which is a more potent hypnotic than chloral hydrate *per se*.[2] The conversion of chloral hydrate to trichloroethanol in the liver has been attributed to the action of alcohol dehydrogenase (EC 1.1.1.1.).[3] Although brain tissue has been shown to be capable of reducing chloral hydrate,[4] the enzymes which catalyze this reaction in brain have not been investigated. Although brain tissue has a small amount of alcohol dehydrogenase similar to the well described liver enzyme,[5] both brain and liver have been shown to contain several reductases capable of metabolizing aldehydes to alcohols.[6–9] In addition, reports[10,11] indicating that the free carbonyl form rather than the hydrated gem diol form of aldehydes was the actual substrate for several dehydrogenases prompted us to investigate the metabolism of chloral hydrate in brain tissue and compare it to the metabolism of chloral and two other aldehydes by liver enzymes. Since differential inhibitors of both alcohol dehydrogenase and aldehyde reductase[12,13] (i.e. pyrazole and barbiturates) are available, we attempted to characterize the chloral hydrate metabolizing activity by the use of inhibitor sensitivities as well as by cofactor and substrate specificities.

Male Sprague–Dawley rats, weighing between 250 and 300 g, were decapitated and brains or livers were quickly removed and homogenized in chilled isolation medium, consisting of 0·32 M sucrose containing 0·05 M sodium phosphate (pH 7·4) and 0·05 mM EDTA. All isolation techniques were performed in the cold (0–4°). Brain homogenates were centrifuged at 27,000 *g* for 30 min and the supernatant fluid was subjected to ammonium sulfate fractionation. Protein precipitating between 0 and 40 per cent saturation (24·3 g/100 ml) with ammonium sulfate and between 40 and 60 per cent saturation (an additional 13·2 g/100 ml) was resuspended in 0·025 M sodium phosphate (pH 7·0) containing 0·5 mM mercaptoethanol and 0·05 mM EDTA. The resuspended fractions and the remaining protein solution were dialyzed for 20 hr against phosphate buffer identical to that used for resuspending the protein. Solutions, after dialysis, were centrifuged at 126,000 *g* for 60 min to remove undissolved proteins and the clear supernatant fluid was used for determination of enzyme activity.

Liver homogenates (10%, v/v) were prepared in an isolation medium consisting of 0·32 M sucrose containing 0·05 mM EDTA and 0·05 M sodium phosphate (pH 7·4), and were centrifuged at 27,000 *g* for 30 min. The supernatant fluid was recentrifuged at 126,000 *g* for 90 min. The clear supernatant fluid obtained from the second centrifugation was used for determination of enzyme activity. Protein concentrations were determined by the method of Lowry *et al.*[14]

Chloral hydrate was twice recrystallized from water. Trichloroethanol was quantified by the method of Cabana and Gessner.[15] This method is based on the formation of a specific chromophore of trichloroethanol. Routinely, the reduction of aldehydes was monitored spectrophotometrically by measuring the decrease in absorbance at 340 nm due to the oxidation of NADH or NADPH. (Assay conditions are

described in Tables 2 and 3.) When present, the inhibitors were preincubated with the enzyme for 2 min before the initiation of the reaction by the addition of aldehyde.

All values have been adjusted for optical density changes seen in the absence of aldehyde substrate. Rates of cofactor utilization when aldehyde was absent were never greater than 50 per cent of the experimental rates.* An identical control system, without inhibitor, was monitored simultaneously with all samples containing the various inhibitors, together with appropriate blanks. The reported specific activities (see Tables 2 and 3) are means of 8–12 determinations. The degree of inhibition was calculated from two to four separate determinations.

Protein recovered in the 40–60% ammonium sulfate fraction from rat brain cytosol possessed the greatest capacity to metabolize chloral hydrate to trichloroethanol. The utilization of chloral hydrate in the presence of NADPH was five times faster with this fraction from brain than was seen in the presence of NADH. The ammonium sulfate treatment resulted in a 3- to 4-fold increase in specific activity of the protein recovered in the 40–60 per cent fraction compared to the activity in the 27,000 *g* supernatant fluid. Ammonium sulfate fractionation, in addition, eliminated much of the oxidation of NADH and NADPH in control systems to which no aldehyde was added. Approximately 50–70 per cent of the total aldehyde reducing capacity found in the 27,000 *g* supernatant fluid from rat brain was recovered in the 40–60% ammonium sulfate fraction when chloral hydrate was used as substrate. The 40–60% ammonium sulfate fraction also had the greatest capacity to utilize NADPH when *p*-nitrobenzaldehyde was the substrate. Tabakoff and Erwin[6] found similar results for bovine brain aldehyde reductase. Substantially less activity appeared in the protein soluble at 60 per cent saturation with ammonium sulfate; however, the rate of reduction of chloral hydrate in the presence of NADH was nearly equal to that in the presence of NADPH. Although approximately 20 per cent of the total chloral hydrate reducing capacity in the presence of NADPH was found in the 0–40% ammonium sulfate fraction, this fraction exhibited lower specific activity, a greater oxidation of NADPH when no aldehyde was added, and the major portion of aldehyde dehydrogenase activity. The metabolism of chlorat hydrate and *p*-nitrobenzaldehyde by each ammonium sulfate fraction was inhibited by sodium pentobarbital. The 40–60% ammonium sulfate fraction was, therefore, chosen to characterize further the chloral hydrate metabolizing enzymes in brain. Table 1 illustrates a one-to-one stoichiometry between oxidation of NADPH and the production of trichloroethanol. The K_m for

TABLE 1. STOICHIOMETRY OF CHLORAL HYDRATE METABOLISM BY ENZYMES FROM RAT BRAIN*

	NADPH oxidized (nmoles)	Trichloroethanol produced (nmoles)
Expt 1	74	65
Expt 2	73	85
Expt 3	62	53
Mean ± S.D.	69·66 ± 6·65	67·66 ± 14·4

* Incubation mixtures contained: NADPH ($1{\cdot}6 \times 10^{-4}$ M); enzyme (40–60% ammonium sulfate fraction, 4·0 mg protein); sodium phosphate, 0·05 M, pH 7·0. The reaction was initiated by addition of chloral hydrate (1×10^{-3} M) and monitored for approximately 180 min at 340 nm in a Gilford model 2400 spectrophotometer. The reaction was terminated by the addition of sulfosalicylic acid and precipitated protein was removed by centrifugation at 20,000 *g* for 10 min. The presence of trichloroethanol in the supernatant fluid was determined by the method of Cabana and Gessner.[15] Incubation mixtures containing boiled enzyme or missing the chloral hydrate were used as blanks. Trichloroethanol standards, added to incubation mixtures containing boiled enzyme, were carried through the entire procedure for each experiment.

* The only exception was the low activity obtained when chloral hydrate and propionaldehyde were incubated with NADH and the brain enzyme (see Table 2). The decrease in absorbance at 340 nm in incubation mixtures without added substrate corresponded to the oxidation of <0·3 nmole NADPH/min/mg of protein or <0·2 nmole NADH/min/mg of protein in the presence of the 40–60% ammonium sulfate fraction from brain. When no aldehyde was added, less than 1·5 nmoles NADPH or 3·3 nmoles NADH was oxidized in incubation mixture containing the 126,000 *g* supernatant fluid from liver. No significant oxidation of cofactors occurred during the period of observation when no enzyme fractions were added.

TABLE 2. METABOLISM OF CHLORAL HYDRATE AND OTHER ALDEHYDES BY RAT BRAIN ENZYMES*

Substrate	Cofactor	Sp. act.†	Inhibitor (M)	Inhibition (%)
Chloral hydrate	NADPH	0·51 ± 0·09	0	
			Pyrazole	
			1×10^{-2}	0
			Pentobarbital	
			1×10^{-3}	81·5
			5×10^{-4}	79·0
p-Nitrobenzaldehyde	NADPH	5·05 ± 0·15	0	
			Pyrazole	
			1×10^{-2}	0
			Pentobarbital	
			1×10^{-3}	83·2
			5×10^{-4}	71·6
Propionaldehyde	NADPH	0·24 ± 0·12	0	
			Pyrazole	
			1×10^{-2}	0
			Pentobarbital	
			1×10^{-3}	42·5
			5×10^{-4}	23·7
Chloral hydrate	NADH	0·12 ± 0·03	0	
			Pyrazole	
			1×10^{-2}	0
			Pentobarbital	
			1×10^{-3}	
			5×10^{-4}	
p-Nitrobenzaldehyde	NADH	0·51 ± 0·09	0	
			Pyrazole	
			1×10^{-2}	
			Pentobarbital	
			1×10^{-3}	23·0
			5×10^{-4}	0
Propionaldehyde	NADH	0·05 ± 0·02	0	
			Pyrazole	
			1×10^{-2}	0
			Pentobarbital	
			1×10^{-3}	0
			5×10^{-4}	0

* Incubation mixtures (1 ml total volume) contained: NADH or NADPH ($1{\cdot}6 \times 10^{-4}$ M); enzyme (rat brain 40–60% ammonium sulfate fraction, 0·6 to 1·8 mg protein); and sodium phosphate, 0·05 M (pH 7·0). When present, the inhibitors were added approximately 2 min before the initiation of the reaction by addition of the substrate. The substrate concentrations were: chloral hydrate, 1×10^{-3} M; propionaldehyde, 1×10^{-3} M; *p*-nitrobenzaldehyde, $6{\cdot}6 \times 10^{-4}$ M. The reaction was monitored by the decrease in optical density at 340 nm using either a Gilford model 2400 or Beckman Acta III spectrophotometer.

† Specific activity is expressed as nmoles cofactor oxidized/min/mg of protein in the presence of substrate ± standard deviation.

chloral hydrate was determined to be $1{\cdot}9 \times 10^{-4}$ M. The K_m for NADPH has been previously shown to be $1{\cdot}2 \times 10^{-6}$ M.[16] It was of interest to find, however, that high concentrations of NADPH inhibited the metabolism of both chloral hydrate and *p*-nitrobenzaldehyde. Under standard assay conditions (Table 2), no oxidation of NADPH was evident at $1{\cdot}0 \times 10^{-3}$ M concentration of this cofactor. All further studies were, therefore, performed in assay mixtures containing $1{\cdot}6 \times 10^{-4}$ M NADPH or NADH, a concentration which produced maximal brain enzyme activity.

Table 2 demonstrates that the metabolism of chloral hydrate in brain tissue was unaffected by pyrazole in concentrations up to $1{\cdot}0 \times 10^{-2}$ M. However, when NADPH was used as a cofactor for the reaction, the metabolism of both chloral hydrate and *p*-nitrobenzaldehyde was strongly inhibited by sodium pentobarbital. Pentobarbital inhibition of NADPH oxidation was less marked when propionaldehyde served as the substrate. Pentobarbital had little or no effect on the metabolism of aldehydes (Table 2) when

TABLE 3. METABOLISM OF CHLORAL HYDRATE AND OTHER ALDEHYDES BY RAT LIVER ENZYMES*

Substrate	Cofactor	Sp. act.†	Inhibitor (M)	Inhibition (%)
Chloral hydrate	NADPH	1·3 ± 0·3	0	
			Pyrazole	
			1×10^{-2}	39·5
			1×10^{-3}	35·7
			Pentobarbital	
			1×10^{-3}	6·4
			5×10^{-4}	14·0
p-Nitrobenzaldehyde	NADPH	13·1 ± 3·4	0	
			Pyrazole	
			1×10^{-2}	66·5
			1×10^{-3}	43·5
			Pentobarbital	
			1×10^{-3}	51·8
			5×10^{-4}	45·7
Propionaldehyde	NADPH	4·9 ± 1·4	0	
			Pyrazole	
			1×10^{-2}	92·8
			1×10^{-3}	77·5
			Pentobarbital	
			1×10^{-3}	13·0
			5×10^{-4}	0
Chloral hydrate	NADH	3·5 ± 1·0	0	
			Pyrazole	
			1×10^{-2}	100
			1×10^{-3}	86·5
			Pentobarbital	
			1×10^{-3}	0
			5×10^{-4}	0
p-Nitrobenzaldehyde	NADH	69·5 ± 6·2	0	
			Pyrazole	
			1×10^{-2}	91·2
			1×10^{-3}	51·5
			Pentobarbital	
			1×10^{-3}	21·5
			5×10^{-4}	47·3
			1×10^{-4}	9·5
Propionaldehyde	NADH	93·4 ± 16·2	0	
			Pyrazole	
			1×10^{-2}	94·3
			1×10^{-3}	88·3
			Pentobarbital	
			1×10^{-3}	0
			5×10^{-4}	0

* Incubation mixtures (1 ml total volume) contained: NADH or NADPH ($1{\cdot}6 \times 10^{-4}$ M); enzyme (rat liver cytosol 0·1 to 0·3 mg protein); and sodium phosphate, 0·05 M (pH 7·0). When present, the inhibitors were added approximately 2 min before the initiation of the reaction by addition of the substrate. The substrate concentrations were: chloral hydrate, 1×10^{-3} M; propionaldehyde, 1×10^{-3} M; *p*-nitrobenzaldehyde, $6{\cdot}6 \times 10^{-4}$ M. The reaction was monitored by the decrease in optical density at 340 nm using either a Gilford model 2400 or Beckman Acta III spectrophotometer. The liver preparation was shown to reduce NAD^+ (1×10^{-3} M) in the presence of ethanol (5×10^{-3} M) with a specific activity of 20 nmoles NAD^+ reduced/min/mg of protein. This activity was inhibited 97 per cent by the inclusion of 1×10^{-3} M pyrazole in the reaction mixture.

† Specific activity is expressed as nmoles cofactor oxidized/min/mg of protein in the presence of substrate ± standard deviation.

NADH was the cofactor. Propionaldehyde proved to be the least actively metabolized substrate of the three aldehydes included in Table 2. Oxidation of NADPH (0·14 nmole NADPH oxidized/min/mg of protein) was also noted when acetaldehyde ($1·0 \times 10^{-3}$ M) was incubated with the 40–60% ammonium sulfate fraction from the brain cytosol. However, no reduction of NAD^+ or $NADP^+$ was noted spectrophotometrically when ethanol ($5·0 \times 10^{-3}$ M) and the brain preparation were incubated in sodium pyrophosphate–glycine buffer (pH 8·7) containing semicarbazide.*

Liver metabolism of the aldehydes differed significantly from aldehyde metabolism by brain (Table 3). With liver cytosol, the rates of aldehyde reduction were greater with NADH as the cofactor than with NADPH. With NADH as the cofactor for the liver enzymes, propionaldehyde showed the highest specific activity and pyrazole strongly inhibited the reaction. However, pyrazole ($1·0 \times 10^{-2}$ M) was less effective as an inhibitor when NADPH was used as a cofactor for the reduction of *p*-nitrobenzaldehyde or chloral hydrate (Table 3). Pentobarbital did not inhibit the reduction of chloral hydrate or propionaldehyde when NADH was the cofactor (Table 3). Pentobarbital also had little effect on the reduction of these two substrates in the presence of NADPH. However, pentobarbital did inhibit the reduction of *p*-nitrobenzaldehyde when either NADH or NADPH served as cofactors for the liver cytosol enzymes. The pentobarbital inhibition (Table 3) of *p*-nitrobenzaldehyde reduction in the presence of NADH was, however, erratic. The higher concentration ($1·0 \times 10^{-3}$ M) of pentobarbital was found to produce consistently less inhibition than a concentration of $5·0 \times 10^{-4}$ M. We are currently examining the effect of pentobarbital on more purified preparations of liver aldehydes metabolizing enzymes.

When NADPH served as a cofactor, the conversion of chloral hydrate to trichloroethanol by enzymes found in the cytosol from brain tissue represented 7–8 per cent of liver cytosolic metabolism of chloral hydrate per g of tissue (wet weight). The activity of the brain cytosol was found to be approximately 25 nmoles chloral hydrate converted/min/g of original brain tissue. The metabolism of chloral hydrate by enzymes derived from brain differed considerably from the metabolism by liver enzymes. The cofactor specificity of the brain fraction, the relative substrate specificity (*p*-nitrobenzaldehyde being the preferred substrate) and the efficacy of pyrazole and pentobarbital as inhibitors indicate that the enzymes metabolizing chloral hydrate in brain are similar, if not identical to the aldehyde reductase(s) (EC 1.1.1.2.) described by Tabakoff and Erwin,[6] and Erwin *et al.*,[7] and differ from the liver alcohol dehydrogenase. Our results, however, do support the determinant role of alcohol dehydrogenase (EC 1.1.1.1.) for the liver metabolism of chloral hydrate, but also indicate the presence of other enzymes capable of reducing aldehydes. Erwin and Deitrich[8] have recently resolved three peaks of aldehyde reducing activity after subjecting liver cytosol to calcium phosphate gel chromatography.

Cabana and Gessner[2] have reported a significant potentiation of conversion of chloral hydrate to trichloroethanol *in vivo* in mice upon co-administration of ethanol. Similar results have been obtained by Kaplan *et al.*[17] In man, Sellers *et al.*[18] demonstrated not only an increased production of trichloroethanol after co-administration of ethanol and chloral hydrate, but also demonstrated an increase in plasma ethanol concentration in the presence of the endogenously formed trichloroethanol. Ethanol potentiation of chloral metabolism was hypothesized[2,18] to be a result of linking between ethanol oxidation and chloral hydrate reduction via the increased formation of an NADH alcohol dehydrogenase complex[19] which could accept the chloral as substrate. Our results, however, suggest that such an interaction might not be of consequence in brain tissue, since the reduction of chloral hydrate was noted in an enzyme fraction which could not be shown to metabolize ethanol. On the other hand, our results demonstrate a significant effect of barbiturates on chloral hydrate metabolism by enzymes from brain.

The oxidized and reduced products of the biogenic amines in brain tissue are produced by aldehyde dehydrogenase[20] and aldehyde reductase[16] respectively. The deaminated products of the biogenic amines (i.e. aldehydes, alcohols, etc.) have been implicated in the physiology of sleep[20–22] and their involvement in states of addiction to hypnotics has been hypothesized.[23] Barbiturates,[13] phenothiazines[6,24] and acetaldehyde[25] have all been demonstrated to interfere with brain metabolism of biogenic aldehydes. Similarly, chloral hydrate has been demonstrated to be an inhibitor of aldehyde dehydrogenase derived from brain.[26] A shunting of the metabolite pattern from the oxidized products of the amines to the reduced products in the presence of either ethanol[27] or chloral hydrate[28] was demonstrated with peripheral tissue, but has not been replicated with central nervous tissue.[27,28] Our present results indicate that chloral hydrate, which in itself has hypnotic activity,[29] might also compete for the reductive pathway of "biogenic" aldehydes metabolism in brain. Thus, certain pharmacologic effects of chloral hydrate might be mediated through increased steady state levels of biogenic amine metabolites.

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* Assays for alcohol dehydrogenase activity were performed in assay mixtures containing 0·03 M sodium pyrophosphate, 0·01 M glycine and 0·03 M semicarbazide HCl (twice recrystallized from water), pH 8·7.

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Can allosteric effectors of acetylcholinesterase control the rate of ageing of the phosphonylated enzyme?

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IT IS now reasonably well established that compounds related to (+)-tubocurarine bind to electric eel acetylcholinesterase (EC 3.1.1.7; AChE) in low ionic strength media at a point distinct from the catalytic site, and in so doing alter the hydrolytic activity of the enzyme.[1,2] Similar allosteric effects have been noted for AChE from mammalian sources.[3–5] The inhibition of AChE by organophosphorus compounds can be relieved by the administration of pyridinium aldoximes such as 2-PAM (2-hydroxyiminomethyl-*N*-methylpyridinium iodide), but the reactivation by the oximes becomes diminished with time after poisoning, an effect known as "ageing".[6] This is due to the loss of an O-bonded alkyl group from the