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**Substrate specificity of rat liver aldehyde dehydrogenase with
chloroacetaldehydes**

Sharpe, Amy-Joan Lorna, M.S.

The University of Arizona, 1991

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SUBSTRATE SPECIFICITY OF RAT LIVER ALDEHYDE
DEHYDROGENASE WITH CHLOROACETALDEHYDES

by

Amy-Joan Lorna Sharpe

A Thesis Submitted to the Faculty of the
DEPARTMENT OF PHARMACOLOGY AND TOXICOLOGY
In Partial Fulfillment of the Requirements
For the Degree of

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WITH A MAJOR IN TOXICOLOGY

In the Graduate College
THE UNIVERSITY OF ARIZONA

STATEMENT BY AUTHOR

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SIGNED: Amy-Joan L. Sharpe

APPROVAL BY THESIS DIRECTOR

This thesis has been approved on the date shown below:

Dean E. Carter
Dean E. Carter
Professor of Pharmacology and Toxicology

4/26/91
Date

To Basel, whose love and support helped me through and to my
sister, Nancy, who inspired me by example

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ABSTRACT

Chlorinated acetaldehydes have recently been the focus of research interest due to their role as reactive intermediates and their possible occurrence in chlorinated drinking water. The metabolism of these compounds, however, has not been extensively studied. In this study, the in vitro substrate specificity of cytosolic and mitochondrial rat liver aldehyde dehydrogenase toward these compounds was investigated. Both crude and semi-purified preparations of the enzymes were used. Monochloroacetaldehyde was found to be extensively metabolized by this enzyme system. It was metabolized to a greater extent than the standard compound propionaldehyde. Dichloroacetaldehyde was also found to be metabolized by this enzyme, but to a lesser extent than its monochloro-analogue. There was some evidence to suggest, however, that alcohol dehydrogenase and chloral hydrate dehydrogenase may play a significant role in the metabolism of this compound. Chloral hydrate was not metabolized by this enzyme to any appreciable extent.

INTRODUCTION

Chlorinated acetaldehydes have been the focus of recent reasearch due to their occurrence as metabolites of chlorinated ethylenes, ethanes, pesticides and chemotherapeutic drugs. Chlorinated ethylenes and ethanes are extensively used as industrial solvents. These chemicals have been associated with carcinogenicity as well as other toxicologic effects which are thought, in part, due to the formation of chlorinated aldehydes formed as reactive intermediates during metabolism (Bignami et al., 1980; Bolt et al., 1982). In recent years, there has also been some evidence to suggest that these aldehydes are by products of the chlorination of drinking water (Keith et al., 1981). The normal analytical techniques for contaminants in drinking water do not detect these compounds and no EPA limits have been set for their contamination in drinking water.

The metabolism of the chlorinated aldehydes has not been extensively investigated. Although extensive study in the past three decades has been focused on the structural analogue of these compounds, acetaldehyde, these compounds are chemically different and warrant study.

Occurrence of Chloroacetaldehydes

Chloral (trichloroacetaldehyde) is probably the most extensively investigated of these compounds (see figure 1 for structures). This compound, in the hydrated form, has been used therapeutically as a sedative/hypnotic since the late 1800's. Chloral hydrate (CH) has also gained fame from the "knock-out drop" effect that is produced when the drug is taken in combination with ethanol (Sellers et al., 1971a). This effect has been named the "Micky Finn" and is caused by the interaction of the compound with ethanol through mutual inhibition of key deactivating enzymes (figure 2). This compound also occurs as an intermediate in the metabolism of trichloroethylene (TCE) and possibly perchloroethylene (PER). These compounds are extensively used as industrial solvents, especially in the dry cleaning industry, and are common water pollutants. Trichloroethylene and perchloroethylene belong to a class of compounds, known as peroxisome proliferators, thought to be carcinogenic (Williams and Weisburger, 1986). These compounds have been shown to induce liver tumors in mice, and are highly toxic to the kidney of both mice and rats. The metabolism of TCE has been extensively studied and the proposed metabolic pathways are illustrated in figure 3 (Bruckner et al., 1989). There is some evidence that chloroethylene oxide is not an obligate intermediate of chloral. Miller and Guengerich (1982) proposed that chloral

Monochloroacetaldehyde

Dichloroacetaldehyde

Chloral Hydrate

Figure 1 - Structures of the chloroacetaldehydes; monochloroacetaldehyde (MCL), Dichloroacetaldehyde (DCL) and chloral hydrate (CH) - chloral is shown in its hydrated form.

Figure 2 - The interaction of chloral hydrate with ethanol, taken from Sellers et al., 1971.

Figure 3 - The proposed metabolism of trichloroethylene, adapted from Bruckner et al., 1989.

was formed through an oxygenated TCE-P₄₅₀ intermediate, subsequently followed by chlorine migration and the formation of CH. Miller and Guengerich (1983) also found that chloroethylene oxide is not important in the irreversible binding to DNA or protein, this leaves the possibility that chloral could play some part in adduct formation.

Dichloroacetaldehyde (DCL) is the least studied of the chlorinated acetaldehydes. Dichloroacetaldehyde is a known metabolite of vinylidene chloride (VDC), a chemical used industrially in the manufacture of Saran-type plastics (Liebler and Guengerich, 1983; Costa and Ivanetich, 1982) and is also presumed to be a metabolite of the pesticides trichlorfon and dichlorvos (Bignami et al., 1980; Löfroth, 1978; see figure 4 for structures). Liebler and Guengerich (1983) showed that the epoxide intermediate of vinylidene chloride, VDC oxide, is not an obligatory intermediate in the formation of dichloroacetaldehyde, similar to the formation of chloral from TCE (figure 5). Vinylidene chloride is also a suspected carcinogen and DCL is thought to possibly play a role in its carcinogenicity (Costa and Ivanetich, 1982). Vinylidene chloride has been shown to be toxic to the liver and kidneys in rodents, dogs and monkeys, and is carcinogenic in mice, producing kidney and liver tumors as well as lung and liver angiosarcoma (Williams and Weisburger, 1986).

Dichlorvos

Trichlorfon

Figure 4 - Structures of the pesticides dichlorvos and trichlorfon.

Figure 5 - Metabolism of vinylidene chloride, adapted from Liebler et al., 1985.

Monochloroacetaldehyde (MCL) is a known metabolite of the industrial chemical vinyl chloride (VC) and a suspected metabolite of the industrial chemical 1,2-dichloroethane (Guengerich et al., 1980) and the antineoplastic drug cyclophosphamide (McCann et al., 1975). Vinyl chloride is used in the production of polyvinyl chloride (PVC). This compound is a known human carcinogen and was discovered as such when persons who cleaned the reaction vessels in polymerization plants developed angiosarcomas of the liver (Williams and Weisburger, 1986). This compound has also been shown to be toxic to the liver and kidney in rodents (Lee et al., 1977). The proposed metabolic pathways for the metabolism of VC and its production of MCL are illustrated in figure 6 (ATSDR/TP-88/25).

Another proposed source of these chlorinated aldehydes is their formation as by products upon the chlorination of drinking water. Keith et al. (1981) reported the presence of chloral in the drinking water of several U.S. cities including Seattle, Philadelphia and New York City. They indicated that chloral was a by product of chlorination rather than an industrial contaminant because of its presence in the drinking water of two cities that obtain their water supplies from uncontaminated upland water (NYC and Seattle). They also proposed that the presence of this compound could be more widespread but is often missed in the analysis of drinking

Figure 6 - Metabolism of vinyl chloride, taken from ATSDR/TP-88/25.

water since the compound is not sufficiently isolated and concentrated by the inert gas stripping technique (volatile organics analysis) prior to GC or GC/MS analysis, a technique most often used in the analysis of drinking water. It is possible that the less substituted chlorinated aldehydes will also exist in water since the degree of substitution depends upon the amount of chlorination used and minimum of chlorination is often used to reduce the concurrent formation of halomethanes. Miller and Uden (1983) have shown the formation of dichloroacetic acid (DCAA), trichloroacetic acid (TCAA), chloroform and chloral hydrate upon the chlorination of aqueous fulvic acid. Trehy and Bieber (1981) also proposed that the formation of these chlorinated aldehydes would be possible upon the chlorination of water containing amino acids (figure 7), but that these compounds have not been detected because they are extremely water soluble. Kringstad et al. (1981) reported the presence of MCL and CH in the spent liquor from kraft pulp chlorination.

Binding of Chloroacetaldehydes to Macromolecules

The carbonyl carbon of these chloroacetaldehydes is an electrophilic center that makes them amenable for attack by nucleophilic compounds. The addition of the chlorines to the adjacent carbon enhances this electrophilicity thereby making it even more susceptible to nucleophilic attack. Tuma and

Figure 7 - The formation of halogenated acetaldehydes from the amino acid aspartic acid, taken from Trehy and Bieber, 1981.

Sorell (1985) demonstrated that acetaldehyde could form stable adducts with proteins through Schiff base formation with an amine (figure 8), or similar adduct formation with a thiol. This binding was shown to decrease with the addition of reduced glutathione, cysteine, or lysine to the reaction mixture. They also showed that metabolically derived acetaldehyde (from ethanol) does form stable and unstable adducts with hepatic proteins in crude liver homogenate. Theoretically, these reactions with proteins to form stable adducts should be more prominent with chlorine substitution of the compound.

It has also been demonstrated by several investigators that monochloroacetaldehyde can covalently bind to nucleic acids. Barrio et al. (1982) showed that MCL reacted with adenosine and cytidine to form 1,N⁶-ethenoadenosine and 3,N⁴-ethenocytidine, respectively, in vitro under acidic conditions (figure 9). Green and Hathway (1978) reacted calf thymus DNA with MCL in an acetate buffer at pH 4.5, to produce etheno-deoxyadenosine, etheno-deoxycytidine and etheno-deoxyguanosine. Sattsangi et al. (1977) reacted guanosine and guanine with MCL under aqueous conditions and physiological pH to form 1,N²-ethenoguanosine and 1,N²-ethenoguanine, respectively. These adducts have also been shown to form upon the reaction of vinyl chloride with DNA and RNA both in vitro, after metabolic activation, and in vivo

Figure 8 - Formation of a Schiff base with an amine and its stabilization or dissociation, taken from Tuma and Sorell, 1985.

1,N⁶-ethenoadenosine 3,N⁴-ethenocytidine 1,N²-ethenoguanine 1,N²-ethenoguanosine

Figure 9 - Structures of adducts formed with nucleic acids and monochloroacetaldehyde.

(Laib and Bolt, 1977, 1978; Green and Hathway, 1978; Bolt et al., 1980).

Mutagenicity and Toxicity of Chloroacetaldehydes

The mutagenicity of MCL has been extensively studied. Monochloroacetaldehyde, without metabolic activation, has been shown to be effective in reverting Salmonella typhimurium bacterial tester strains TA 100 and TA 1535 (base-pair substitution strains), E. coli K12 A , Aspergillus nidulans (a point mutation strain) and was weakly active in Streptomyces coelicolor , a base substitution strain (McCann et al., 1975; Rannug et al., 1976; Elmore et al., 1976; Perrard, 1985 and Bignami et al., 1980). Monochloroacetaldehyde was also found to induce mutations in Chinese hamster V79 cells (Huberman et al., 1975). Dichloroacetaldehyde has also been shown to be mutagenic, although much less than MCL, in S. typhimurium TA 100, TA 1535 and TA 98, A. nidulans and S. coelicolor (Bignami et al., 1980; Löfroth, 1978). Both the anhydrous and hydrate forms of chloral were found to be mutagenic in S. typhimurium TA 100 with and without metabolic activation, but only the hydrate form showed some activity with A. nidulans and S. coelicolor (Bignami et al., 1980).

Monochloroacetaldehyde is a very toxic and irritating compound (Lawrence et al., 1972). The LD₅₀ in male Sprague-Dawley rats was 0.00602 ml/kg when given intraperitoneally and 0.07507 ml/kg when given orally. When air was bubbled through

a 30 % solution of MCL at a rate of 2 L/min and groups of five male ICR mice were placed in glass inhalation chambers with a capacity of 8.75 L, the LT_{50} was 2.57 min. The compound produced extensive tissue damage when applied dermally, in a 30 % solution, to rabbits and produced extensive ophthalmic damage in a solution as low as 0.27 %. Hemolysis at a rate of 50 % occurred in rabbit erythrocytes with a 0.3494 M solution (2.75 %). With pretreatment of the compound, there was a dose-related increase in phenobarbital induced sleeping time. In acute intravenous toxicity tests on the effects of MCL in anesthetized rabbits, there was a dose dependent decrease in blood pressure and, with a high dose (0.2 ml/kg), stimulation of the sciatic nerve was blocked. In a 30 day cumulative toxicity study in rats at doses of 0.3 and 0.6 times the LD_{50} there was a mortality rate of 25 and 66.7 % respectively and weight gain was slowed. With the high dose, there was a significant decrease in hemoglobin, hematocrit and erythrocytes, the organ-to-body weight ratios for brain, gonads, heart, kidneys, liver, lungs and spleen were increased and a hematoxylin-eosin stain revealed severe bronchitis, bronchiolitis and bronchopneumonia. With the low dose there was an increase in segmented neutrophils, monocytes and lymphocytes and there was an increase in the organ-to-body weight ratios of lung and brain. Finally in a subacute study, a series of MCL doses ranging from 0.0032-0.00032 ml/kg were

given intraperitoneally 3 times a week for 12 weeks. This produced a subacute LD₅₀ of approximately 0.003 ml/kg. There was a decrease in erythrocytes and lymphocytes and an increase in segmented neutrophils and hemoglobin clotting time and the lungs demonstrated focal, chronic bronchopneumonia and changes in the respiratory epithelium suggestive of a premalignant condition.

Chloral hydrate is not as toxic as MCL. The acute effects of overdose include symptoms of confusion, poor coordination, respiratory distress, apnea and coma. In studies with the mouse following exposure of low doses in drinking water for 14 and 90 days, the effects produced in the male mice included a dose dependent hepatomegaly, an elevation of SGOT and LDH, and increase in aminopyrine N-demethylase and aniline hydroxylase activity, without an increase in P-450 content (Sanders et al., 1982a). Female mice did not experience the same toxic effects as the male mice, but rather with the 90 day exposure of chloral they experienced a significant depression in the humoral immune function (Kauffmann et al., 1982). No data was found on the toxicity of dichloroacetaldehyde.

Aldehyde Dehydrogenase

Aldehyde dehydrogenase is present in several different forms in most organs of the body. The largest amounts of this enzyme are present in the liver (Pietruszko, 1983; Weiner,

1979). This enzyme is responsible for the metabolism of ethanol-derived acetaldehyde to acetic acid and it has been proposed that genetic differences in this enzyme could contribute to alcoholism and alcohol sensitivity (Goedde, 1983; Bosron and Li, 1986). This enzyme also metabolizes endogenous substances such as aldehydes derived from biogenic amines (i.e. 3,4-dihydroxyphenylacetaldehyde derived from dopamine) and corticosteroids (Pietruszko, 1983).

There are three categories of this enzyme, based on their subcellular localization (cytosolic, mitochondrial and microsomal), and two classes based on the K_m value of the enzyme with short chain aliphatic aldehydes, "high K_m " (mM) and "low K_m " (μ M). In the rat there are five different isozymes present, a high K_m and a low K_m isozyme in the cytosol (C I and C II, respectively), a high K_m and a low K_m isozyme in the mitochondria (M II and M I, respectively) and a high K_m isozyme in the microsomal fraction (Tottmar et al., 1973; Koivula and Koivusalo, 1975; Siew et al., 1976; Horton and Barrett, 1975).

Aldehyde dehydrogenase is, apparently, a tetrameric enzyme with a molecular weight in the rat of 320,000 for M II, and approximately 200,000 for the cytosolic enzymes (Seiw et al, 1976; Weiner, 1980). The exception in the rat is the low K_m mitochondrial enzyme, M II, which has a molecular weight of 67,000 and is thought to be a monomer. The microsomal enzyme

primarily metabolizes long chain aldehydes and will not be discussed further (Weiner, 1980). The tetrameric enzyme appears to have four identical subunits, but seems to function with only half-of-the-sites reactivity where only two of the four subunits function at one time (Weiner and Takahashi, 1985). The catalytic activity of the mitochondrial enzyme has been shown to increase two-fold with the addition of Mg^{2+} and a concomitant dissociation of the tetrameric enzyme into a pair of dimers with full-site reactivity (Takahashi and Weiner, 1980). This phenomena seems to also be dependent upon pH and concentration of enzyme (Weiner, 1982a).

The different isozymes present in the cell differ in their subcellular location for different species. In rat, ox and sheep, for instance, the majority of acetaldehyde oxidation occurs in the mitochondria while in the human it occurs in the cytosol (Parrilla et al., 1974; Harrington et al., 1987; Takase et al., 1989). Rats also have aldehyde dehydrogenase enzymes in the cytosol that are inducible by xenobiotics and are associated with chemically induced tumors (Koivula and Rautoma, 1985). These enzymes possess properties that are different than the normal cytosolic enzymes.

Statement of the Problem

The toxicologic significance of these chlorinated

acetaldehydes has been well demonstrated. This significance was first noted because of their role as reactive intermediates of known or suspected carcinogens and has recently become of more concern because of their possible existence in chlorinated drinking water. The metabolism of these compounds, however, has not been examined. Because of the importance of the aldehyde dehydrogenase enzymes in the metabolism of the structural analogue acetaldehyde, it seems likely that this enzyme system could be very important in the metabolism of these compounds. This route of metabolism could also be very important to the resulting toxicity of these compounds, since the chlorinated acids have also been shown to be toxic compounds (Bhat et al., 1991; Kanz et al, 1991).

MATERIALS AND METHODS

Chemicals

Propionaldehyde and monochloroacetaldehyde were purchased from Aldrich Chemical Company, Inc., Milwaukee, WI. Monochloroacetaldehyde was redistilled prior to use. Dichloroacetaldehyde was generously supplied by Dr. Eugene Mash's laboratory at the University of Arizona, Department of Chemistry and was synthesized according to the procedure of Swietoslawski and Silowiecki, 1976. Chloral hydrate was purchased from Fisher Scientific, Fairlawn, NJ. Mercaptoethanol was purchased from Eastman Kodak Co., Rochester, NY. DEAE cellulose was purchased from either Whatman, Hilsboro, OR, or Sigma Chemical Co., St. Louis, MO. All other chemicals were purchased from Sigma Chemical Co., St. Louis, MO.

Animals

Male Sprague-Dawley rats (250-450 g) housed 2-4 per cage were obtained through Sasco Laboratories, Omaha, Ne. All animals were provided food and water ad libitum prior to sacrifice. The light cycle maintained in the animal rooms was 12-hr on and 12-hr off.

Preparation of Subcellular Fractions

Hepatic cytosolic and mitochondrial fractions were prepared by methods described by Koivula and Koivusalo (1975) with some modifications taken from procedures used by Lamé and Segall (1986). For the crude preparation of the enzyme, single rat livers were used. For the purification of the enzymes using column chromatography, the livers were pooled (4-10 livers at a time). The livers were removed immediately after stunning and decapitation of the animal and then placed in ice-cold 0.25 M sucrose, for the crude preparation of the enzymes, and in ice-cold 0.25 M sucrose made up in 10 mM sodium phosphate buffer, pH 7.4, containing 2 mM mercaptoethanol, for the purified preparation of the enzymes (hereby referred to as sucrose solutions). The livers were then minced and homogenized in a Potter-Elvehjem type homogenizer to make a 20 % w/v homogenate. All procedures from this point on were performed at 4°C. This homogenate was then centrifuged for 5 min at 700g in a Beckman J-21C preparative centrifuge. The resultant pellet was discarded and the supernatant was then centrifuged at 4500 g for 10 min. The supernatant from this spin was then centrifuged at 12,000 g for 15 min and 106,000 g for 60 min with the two resulting pellets being discarded (a Beckman L8 preparative ultracentrifuge was used for the 106,000 g spin). The resulting supernatant was used for further cytosolic study

after the removal of the lipoprotein layer.

The pellet resulting from the 4500 g spin was washed twice with the sucrose solution. This pellet contained the mitochondria. The pellet was resuspended in 10 mM sodium phosphate buffer, pH 7.4 (2 ml/g liver). This buffer contained 2 mM mercaptoethanol when preparing the mitochondria for purification using column chromatography. The mitochondrial suspension was then kept at 0°C for 30 min with stirring for approximately 30 sec every 5 min. After the 30 min, sodium deoxycholate (100 mg/ml) was added in 20 μ l aliquots per 1 ml of mitochondrial suspension (or 200 μ l per 10 ml of suspension depending on the total volume of suspension). This solution was then maintained at 0°C for another 30 min with stirring for 30 sec every 5 min. The mitochondrial suspension was then centrifuged at 106,000 g for 60 min to spin down the disrupted membranes. The supernatant from this centrifugation was then use for mitochondrial study.

Purification of Isozymes by Column Chromatography

Prior to purification by column chromatography, the cytosolic and mitochondrial fractions were subjected to ammonium sulfate precipitation (Koivula and Koivusalo, 1975; and Seiw et al, 1976). The cytosolic proteins were precipitated by the addition of ammonium sulfate to make a 30

to 80% saturated solution. The mitochondrial preparation was subjected to both a 30 to 55% and a 55 to 80% saturation. The 30 to 55% precipitate contained enriched mitochondrial ALDH I (low K_m) and was purified further by column chromatography. The 55 to 80% precipitate contained enriched mitochondrial ALDH II (high K_m) and was not purified further. The precipitates were resuspended in 10 mM sodium phosphate buffer, pH 7.4, containing 2 mM mercaptoethanol (10 mM buffer) and were dialyzed (Spectrapor #1 cellulose dialysis tubing) overnight against 40-100 volumes of the same buffer with one buffer change after approximately 6 hr.

The dialyzed solutions (mitochondrial 30-55 % ammonium sulfate dialysate and the cytosolic dialysate) were applied to a DEAE cellulose column treated with the 10 mM buffer (2.5 x 40 cm for the cytosolic solution and 2.5 x 8 cm for the mitochondrial solution). The columns were initially eluted with the 10 mM buffer, approximately 500 ml for the cytosolic column and 45 ml for the mitochondrial column. The columns were then eluted with a 50 mM sodium phosphate buffer, pH 7.4, containing 2 mM mercaptoethanol, approximately 500 ml for the cytosolic column and 80 ml for the mitochondrial column. The high K_m and low K_m cytosolic enzymes (C I and C II, respectively) were eluted with the 10 mM and 50 mM buffers, respectively. The low K_m mitochondrial enzyme (M I) was eluted with the 50 mM buffer. Each fraction collected (10 ml

each) was assayed for ALDH activity. The fractions with the greatest amount of activity were combined and were analyzed for protein content using a modified Lowry method (Hartree, 1972).

Enzyme Activity Assays

The activity of aldehyde dehydrogenase was measured according to the procedures of Tottmar et al. (1973) with some modifications from Hjelle and Petersen (1983). The reaction was monitored by following the reduction of NAD^+ to NADH spectrophotometrically at 340 nm. The reaction mixture was made up in 50 mM sodium phosphate buffer, pH 7.4 and contained NAD^+ , pyrazole (an alcohol dehydrogenase inhibitor) and rotenone (an NADH oxidase inhibitor) in concentrations such that the final concentration in the cuvette was 1.6 mM, 0.8 mM and 1.6 μM , respectively. The substrates were made up in aqueous solution and the reactions were started by the addition of enzyme.

RESULTS

Crude Subcellular Enzyme Preparations

As a first step toward the elucidation of the metabolism of chloroacetaldehydes by aldehyde dehydrogenase, crude subcellular preparations of the mitochondrial and cytosolic enzymes were used. Propionaldehyde was used as a standard substrate so that relative activity could be established. Propionaldehyde is metabolized in much the same way that acetaldehyde is metabolized and is a more stable compound.

With the crude cytosolic preparation of the enzymes, it was found that monochloroacetaldehyde had a greater specificity for the enzyme than does propionaldehyde (figure 10A). At a concentration of 5 mM MCL, the specific activity was approximately twice as great as the specific activity with propionaldehyde as the substrate at approximately the same concentration. The difference in the specific activity was not quite as great at lower concentrations of the substrates, but MCL was always greater than propionaldehyde.

Dichloroacetaldehyde behaved very differently in this preparation when compared with monochloroacetaldehyde. At low concentrations of this substrate, the specific activity was lower than propionaldehyde, but at a concentration of 5 mM, the specific activity was about equal to that of

propionaldehyde at approximately the same concentration (figure 10A). It is also interesting to note that no maximum in the reaction velocity with DCL was observed with the concentrations of substrate used, but a maximum appeared to occur for both MCL and propionaldehyde. The activity with chloral hydrate (CH) was negligible.

With the crude mitochondrial preparation of the enzymes, monochloroacetaldehyde again had greater activity with the enzyme than propionaldehyde (figure 10B). With this enzyme, however, the difference in activity was greater at lower concentrations of the substrate than at higher concentrations. The curve shows some signs of substrate inhibition with a maximal reaction velocity at 0.5 mM, although this evidence is not conclusive since the velocity at 5 mM increased rather than decreased with respect to the activity at 0.5 mM. The activity of the enzyme with dichloroacetaldehyde and chloral hydrate was negligible compared to MCL and propionaldehyde.

The Lineweaver-Burk plots for these substrates and enzymes were all nonlinear with the exception of monochloroacetaldehyde in the crude cytosolic preparation (figures 11A and 11B, the curvature of the MCL plot with the mitochondria is difficult to see, but this plot is also curved). Since there is known to be two isozymes in each of these subcellular fractions, this was not a surprising result.

Figure 11 - Lineweaver-Burk plots for the crude ALDH enzymes. (A) Cytosolic, (B) mitochondrial - this graph does not show the DCL data since the activity was very low.

However, the linearity of the MCL plot in the cytosol indicated that perhaps only one of the isozymes present in this subcellular fraction acted on this substrate. The K_m and V_{max} for MCL with the crude cytosolic enzyme were 0.13 mM and 13.57 nmol NADH formed/min per mg protein, respectively. No other K_m and V_{max} values were calculated for these plots.

Column Purified Enzyme Preparations

The purification of the individual isozymes in the cytosol and mitochondria was carried out using DEAE cellulose column chromatography to yield what has been defined in the literature as a low K_m and a high K_m isozyme, with respect to acetaldehyde, in each subcellular fraction. All four of these isozymes had a different activity profile with respect to the chlorinated aldehydes.

The cytosolic high K_m enzyme (CI) showed no significant activity toward any of the chlorinated aldehydes (figure 12A). The chloroaldehyde with the greatest amount of activity was MCL followed by DCL and CH, respectively. The only significant activity was with MCL at low concentrations (0.025 and 0.05 mM) where the activity approached that of propionaldehyde.

The low K_m cytosolic enzyme (CII) behaved very differently toward MCL and DCL than the high K_m enzyme (Figure 12B). The activity of this enzyme with MCL was much

Figure 12 - Plots of reaction velocity (V) as a function of the substrate concentration for the purified cytosolic enzymes. (A) C I (high K_m), (B) C II (low K_m).

greater than the activity with propionaldehyde. A maximal velocity with this substrate was reached at approximately 0.25 mM, at which point the reaction velocity was 3-4 times greater than that of propionaldehyde. This substrate also clearly inhibited the enzyme at concentrations greater than 0.25 mM.

Dichloroacetaldehyde was much more active with the low K_m cytosolic enzyme than with the high K_m enzyme. The activity of this substrate at a concentration of 5 mM was approximately 65 % of the specific activity of propion-aldehyde at about the same concentration. This compares to the 10 % of propionaldehyde activity with the other cytosolic enzyme. The activity of this enzyme with chloral hydrate was again negligible.

Monochloroacetaldehyde was metabolized by the low K_m mitochondrial enzyme (MI) in much the same way that it was metabolized by the low K_m enzyme in the cytosol (Figure 13A). The specific activity was much greater than that with propionaldehyde (approximately 4 times greater), a maximal reaction velocity was reached at approximately 0.25 mM and substrate inhibition occurred at concentrations greater than 0.25 mM. Dichloroacetaldehyde, however, presented a much different activity profile. The specific activity with DCL was only about 10-15 % of the activity with propionaldehyde at 5 mM. Chloral hydrate metabolism was again negligible.

Figure 13 - Plots of reaction velocity (V) as a function of the substrate concentration for the purified mitochondrial enzymes. (A) M I (low K_m) (B) M II (high K_m).

The activity of the high K_m mitochondrial enzyme with mono-chloroacetaldehyde was different than any of the other isozymes. At low concentrations of the substrate the activity was greater than with propionaldehyde and at high concentrations the activity was less than with propionaldehyde, with the activities being approximately equal at a concentration of 0.5 mM. The activity of this enzyme with dichloroacetaldehyde and chloral hydrate was negligible.

In figures 14-16 the data presented above for the purified enzymes is shown by plotting the data for one substrate on each graph to show enzyme specificity for the substrates. The B portions of the figures have expanded scales to show the activities of certain enzymes. The low K_m mitochondrial enzyme (M I) clearly had the greatest amount of activity with all of the substrates and the high K_m cytosolic enzyme had the lowest activity. For propionaldehyde, the high K_m mitochondrial enzyme (M II) was greater in activity than the cytosolic enzyme at high concentrations. At lower concentrations of propionaldehyde, however, the cytosolic enzyme dominate metabolism. The activities of both mono- and dichloroacetaldehyde with the low K_m cytosolic enzyme (C II) were greater than both of the high K_m enzymes.

One difficulty encountered in measuring the activity

Figure 14 - Plots of the reaction velocity (V) as a function of propionaldehyde concentration for the purified ALDH enzymes. Plot (B) shows C I, C II and M II alone to better define these plots.

Figure 15 - Plots of the reaction velocity (V) as a function of monochloroacetaldehyde concentration for the purified ALDH enzymes. Plot (B) shows C I, C II and M II alone to better define these plots.

Figure 16 - Plot of reaction velocity (V) as a function of dichloroacetaldehyde concentration for the purified ALDH enzymes.

and determining kinetic parameters was the nonlinearity of the Lineweaver-Burk plots obtained from these data (see figure 17 for examples). This nonlinearity was decreased by increasing the amount of enzyme in the reaction mixture.

Figure 18 shows this relationship between protein concentration and linearity of the Lineweaver-Burk plots. The K_m values calculated for the different substrates were calculated from the most linear Lineweaver-Burk plots and are presented in table 1. The V_{max} values calculated (also shown in table 1) were calculated from data collected for the different substrates from the same preparation of enzyme, since there was some variability in the specific activities among the different preparations of the enzyme.

An interesting effect was observed with the low K_m cytosolic enzyme and the chlorinated aldehydes, especially with DCL and CH. During the individual assays, there was an initial decrease in the absorbance before there was an increase (see figure 19). With dichloroacetaldehyde, this "dip" in absorbance was shown to decrease with increasing pyrazole in the reaction mixture, thereby indicating that reaction with some alcohol dehydrogenase contaminant could be responsible for this effect. The occurrence of this absorbance "dip" was a consistent reaction of the enzyme preparation with DCL and CH, and a transient occurrence with MCL.

Figure 17 - Lineweaver-Burk plots for the purified ALDH enzymes. Propionaldehyde is used as the substrate.

Figure 18 - Plots of the correlation coefficient (r^2) from Lineweaver-Burk plots as a function of protein concentration using propionaldehyde as the substrate. (A) Purified cytosolic enzymes, (B) purified mitochondrial enzymes.

<u>Enzyme</u>	<u>Prop</u>		<u>MCL</u>		<u>DCL</u>	
	<u>K_m</u> [*]	<u>V_{max}</u> ^{**}	<u>K_m</u> [*]	<u>V_{max}</u> ^{**}	<u>K_m</u> [*]	<u>V_{max}</u> ^{**}
CI	0.27	100	---	---	0.35	23.4
CII	0.032	100	0.046	582	0.13	54.9
MI	0.044	100	0.057	462	0.038	12.9
MII	0.20	100	0.024	55.5	0.286	3.44

* expressed in mM

** expressed as nmol NADH formed/min per mg protein

Table 1 - Table of K_m and V_{max} values. V_{max} of propionaldehyde is set equal to 100 and the rest of the values are in relation to propionaldehyde

Figure 19 - Effect of increasing pyrazole concentration on dichloroacetaldehyde metabolism with cytosolic ALDH I. - (●) 1.6 mM pyrazole and 5 mM DCL, (▲) 8 mM pyrazole and 5 mM DCL.

DISCUSSION

This work demonstrated the striking difference in the metabolism of the chlorinated acetaldehydes by aldehyde dehydrogenase. Monochloroacetaldehyde was metabolized by these enzymes to a greater extent than either dichloroacetaldehyde or chloral hydrate. This metabolism occurred primarily in the mitochondria and also exceeded the metabolism of propionaldehyde by the two low K_m enzymes that are located in the liver (one in the cytosol and one in the mitochondria). The only significant metabolism of dichloroacetaldehyde occurred with the low K_m enzymes in the cytosol and mitochondria. The greatest extent of metabolism of this compound relative to propionaldehyde, however, was with the low K_m cytosolic enzyme. Chloral hydrate was not metabolized to any appreciable extent with any of the isolated isozymes of aldehyde dehydrogenase, as would be expected since this compound is a known competitive inhibitor of this enzyme (Koivula and Koivusalo, 1975). Using K_m values as an index, the affinity of the ALDH enzymes for MCL was approximately the same as propionaldehyde with the low K_m enzymes, and was approximately ten-fold greater than propionaldehyde with the high K_m mitochondrial enzyme. The affinity of these enzymes for DCL was consistently lower than the affinity for propionaldehyde with the exception of the low K_m mitochondrial

enzyme, where it was slightly higher. The greatest difference in the affinity for DCL when compared to propionaldehyde was with the low K_m cytosolic enzyme where the affinity of the enzyme for DCL was approximately ten-fold lower than for propionaldehyde. The K_m values determined for propionaldehyde were slightly higher than those reported in the literature (Koivula and Koivusalo, 1975; Siew et al., 1976). These values in the literature, however, appear to be variable. The relative K_m values determined in these experiments for the individual enzymes, however, were consistent with that reported in the literature.

The variability in the metabolism of these structurally similar aldehydes may be related to the degree of hydration and electron withdrawing characteristics of these compounds. Weiner et al. (1982b) investigated the capability of fluoral hydrate to act as an inhibitor of this enzyme. Fluoral hydrate is a structural analogue of chloral hydrate with similar hydrating properties (approximately 99 % hydrated in aqueous solution). The CF_3 -group of fluoral hydrate, however, is a greater electron withdrawing group than the CCl_3 -group of chloral hydrate thereby making the water of hydration held more tightly to the compound. Fluoral hydrate was found not to inhibit this enzyme, thereby deducing that the simple hydration of the compound was not the cause of its competitive inhibition. Weiner et al. (1982b) proposed that the

inhibition of aldehyde dehydrogenase by chloral hydrate was due to the formation of a covalent complex between chloral hydrate and the enzyme (see figure 20, compound II), the proposed mechanism of inhibition by cyclopropanone. In order for this intermediate to form, an exchange of an -SH of hydration (from a cysteine in the active site) for a water of hydration would have to occur. In the case of fluoral hydrate k_{-1} (see figure 20) is probably much greater than k_2 since the CF_3 -group of fluoral hydrate is so electron withdrawing. This mechanism could also explain the differences in metabolism of MCL, DCL, and CH. The electron withdrawing capabilities of these compounds and their degree of hydration differ. These characteristics are the greatest for CH and the least for MCL. Therefore DCL and MCL will form this covalent complex intermediate to a lesser extent than CH allowing metabolism to occur more rapidly.

The role of aldehyde dehydrogenase in the metabolism and detoxification of monochloroacetaldehyde is clearly an important one. The activity of this enzyme, particularly the low K_m isozymes, was very high and exceeded that of propionaldehyde. In the metabolism of vinyl chloride, monochloroacetic acid (MCA) has been shown to be a urinary

Figure 20 - Interaction of chloral hydrate with the active site of aldehyde dehydrogenase, taken from Weiner et al., 1982b.

metabolite with both inhalation and oral exposures in rats (Hefner et al., 1975; ATSDR/TP88/25, 1989). This metabolism appears to be dose dependent, with MCA being formed at high doses and conjugation with sulfhydryl groups predominating at low doses. Therefore, this metabolic pathway could be very important in the detoxification of MCL.

This dose related importance of the aldehyde dehydrogenase pathway also appears to be important for the metabolism of dichloroacetaldehyde. Although this compound is not metabolized as extensively as MCL by this system, dichloroacetic acid has been found to be a urinary metabolite of trichlorfon and dichlorvos (Casida et al., 1962; Matsumura, 1985). Although dichloroacetic acid is a postulated metabolite of vinylidene chloride, it has not yet been identified. This is probably due to the detoxification of DCL by the sulfhydryl conjugation pathway predominating. When the glutathione pool is depleted, the role of aldehyde dehydrogenase could become important. This is demonstrated by the metabolism of dichlorvos and trichlorfon in which glutathione conjugation occurs at one end of the molecule while dichloroacetic acid is formed from the opposite end of the molecule (see figure 2). A possible explanation for the absence of DCA as a metabolite of vinylidene chloride is that the formation of DCL is not the only intermediary pathway that occurs with this compound.

Figure 21 - Metabolism of dichlorvos and trichlorfon,
adapted from Matsumura, 1985.

This compound also forms 2-chloroacetyl chloride which is further metabolized to monochloroacetic acid and glutathione metabolites. The relatively small amount of dichloroacetaldehyde formed from this compound is apparently conjugated with glutathione. Upon high dose exposures and depletion of the glutathione pool, however, this route of metabolism could also become important.

The results obtained from the metabolism of DCL by aldehyde dehydrogenase was unexpected as compared to MCL. Dichloroacetaldehyde metabolism with the crude cytosolic preparation of the enzyme was approximately equal to that of propionaldehyde at a concentration of 5 mM. At this concentration, DCL apparently had not reached its maximal velocity, but propionaldehyde had. However, with both of the purified cytosolic enzymes DCL did reach a maximal velocity at this concentration, as did propionaldehyde, but the combined activities of DCL were only about 50 % of the combined activities of propionaldehyde. Therefore, there must be another NADH-producing reaction with DCL occurring in the cytosol. The enzyme most likely responsible for this extra activity in the crude cytosolic preparation is chloral hydrate dehydrogenase. This enzyme is cytosolic and is thought to be responsible for the generation of most of the trichloroacetic acid from chloral (Cooper and Friedman, 1958).

Dichloroacetaldehyde also displayed a very interesting

phenomena with the purified high K_m cytosolic enzyme. A "dip" in the absorbance was seen upon the initiation of the reaction and before any increase in absorbance was observed. This phenomena is probably due to the presence of alcohol dehydrogenase in the enzyme preparation, since this "dip" was decreased in the presence of increased pyrazole (an alcohol dehydrogenase inhibitor). This apparent metabolism of DCL by alcohol dehydrogenase (ADH) in the presence of an inhibitor of this enzyme and the absence of any exogenous NADH (the cofactor for ADH) indicates that perhaps this enzyme could play a significant role in the metabolism of DCL.

The nonlinearity of the Lineweaver-Burk plots was an unexpected finding. The increase in linearity observed with an increase in the enzyme concentration would suggest that this effect is somewhat concentration dependent. Takahashi and Weiner (1980) reported that magnesium stimulated the dissociation of the tetrameric structure of horse liver ALDH into dimers with a concurrent increase (two-fold) in the catalytic activity of the enzyme. This dissociation also changed the reactivity of the enzyme from half-of-the-sites to full-site reactivity. They therefore concluded that the dissociation of the enzyme uncovered two active sites that were previously inactive. Weiner (1982) later reported that this enzyme dissociation also appeared to be pH and concentration dependent. This might explain the nonlinearity

of the Lineweaver-Burk plots if a mixture of the dimers and the tetramer were present in the assay conditions. This dissociation of the enzyme, however, does not explain the nonlinearity of the Lineweaver-Burk plots with the high K_m mitochondrial enzyme, which is thought to be a monomer. MacGibbon et al. (1977) reported nonlinear Lineweaver-Burk plots using sheep liver cytoplasmic ALDH with propionaldehyde and acetaldehyde as substrates. They explained this result as activation of the enzyme at high substrate concentrations. Dickinson and Hart (1982) proposed that this effect was the result of the formation of an abortive complex such as E-NADH that would liberate the product of the reaction more rapidly than the normal product complex. This effect would also be decreased with increasing enzyme concentration.

An interesting observation noted in the metabolism of monochloroacetaldehyde with both of the low K_m enzymes was substrate inhibition. In general, this type of inhibition is regarded as uncompetitive inhibition by the substrate at a site other than the active site (Palmer, 1985). Dickinson and Hart (1982) reported such an effect for sheep liver ALDH with propionaldehyde as the substrate, but only at low concentrations of NAD^+ ($<0.33 \text{ mM}$). No other report of such an effect was found.

Conclusions

The role of aldehyde dehydrogenase in the metabolism of monochloroacetaldehyde is important. This metabolic capacity was not as predominant in the metabolism of dichloroacetaldehyde and was insignificant for chloral hydrate. Alcohol dehydrogenase and chloral hydrate dehydrogenase may play a more important role than aldehyde dehydrogenase in the metabolism of DCL, as is the case with chloral hydrate.

It would appear that the degree of hydration and the electrophilicity of the substituent group adjacent to the carbonyl carbon plays a role in the affinity and catalytic activity of these enzymes with the substrates.

The activity of aldehyde dehydrogenase would appear to be dependent upon the concentration of both the substrate and the enzyme. Substrate inhibition was demonstrated as well as the possibility of substrate activation and enzyme dissociation.

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