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KINETIC CHARACTERIZATION OF THE CATALYSIS OF "ACTIVATED" CYCLOPHOSPHAMIDE (4-HYDROXYCYCLOPHOSPHAMIDE/ ALDOPHOSPHAMIDE) OXIDATION TO CARBOXYPHOSPHAMIDE BY MOUSE HEPATIC ALDEHYDE DEHYDROGENASES*

CARL L. MANTHEY and NORMAN E. SLADEK†
Department of Pharmacology, University of Minnesota, Minneapolis, MN 55455, U.S.A.

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Abstract—A spectrophotometric assay was developed and utilized to directly characterize aldehyde dehydrogenase-catalyzed oxidation of aldophosphamide to carboxyphosphamide by soluble and solubilized particulate fractions prepared from mouse liver homogenates. V_{max} values of 3310 and 1170 nmol/min/g liver were obtained for the soluble and solubilized particulate fractions respectively. K_m values were 22 and 84 μM respectively. Alkaline pH optimums were observed in each case. Aldehyde dehydrogenase-catalyzed oxidation of aldophosphamide by the soluble fraction was markedly more temperature responsive. Catalysis of aldophosphamide and acetaldehyde or benzaldehyde oxidation was apparently by the same isozyme(s) in the soluble fraction. Similarly, low K_m (acetaldehyde/benzaldehyde) and high K_m (acetaldehyde/benzaldehyde) isozymes each apparently catalyzed the oxidation of aldophosphamide in the solubilized particulate fraction. Our findings suggest that (1) oxidation of aldophosphamide to carboxyphosphamide by mouse liver is catalyzed largely by the predominant aldehyde dehydrogenase isozyme present in the soluble fraction (cytosol) of this tissue, and (2) isozymes that catalyze aldophosphamide oxidation are not different from those that catalyze the oxidation of acetaldehyde and benzaldehyde, though the relative contribution of each isozyme within the solubilized particulate fraction to the catalysis of aldophosphamide oxidation remains to be determined.

Cyclophosphamide is the prototype of a group of agents, viz. oxazaphosphorine nitrogen mustards, that are widely used for their antineoplastic and immunosuppressive properties. However, it is itself without pharmacological activity. Thus, its metabolism has been investigated extensively; the results of these investigations have been reviewed [1–3].

Cyclophosphamide is first converted to 4-hydroxycyclophosphamide; this reaction is catalyzed by hepatic mixed-function oxidases. 4-Hydroxycyclophosphamide gives rise to its acyclic aldehyde tautomer, aldophosphamide. 4-Hydroxycyclophosphamide and aldophosphamide are themselves also without pharmacological activity. Aldophosphamide undergoes one of two metabolic fates: β-elimination to phosphoramide mustard, the pharmacologically active species, and oxidation to carboxyphosphamide. The latter reaction is catalyzed by NAD-linked aldehyde dehydrogenase (EC 1.2.1.3, aldehyde:NAD^+ oxidoreductase). Since carboxyphosphamide (1) is pharmacologically inactive, (2) does not give rise to an active metabolite, and (3) is the primary metabolite found in the urine of rodents, dogs, sheep, and humans given cyclophosphamide, its formation represents an important detoxification pathway and has been the subject of several investigations [4–8]. However, kinetic definition of NAD-linked aldehyde dehydrogenase-catalyzed oxidation of aldophosphamide to carboxyphosphamide has, thus far, not been possible even though aldehyde oxidation catalyzed by this enzyme can, ordinarily, readily be quantified by monitoring NADH formation spectrophotometrically. This is because of the unavailability of an aldophosphamide preparation of known concentration and sufficient stability.

Aldehyde dehydrogenase (actually, several isozymes thereof) catalyzes the oxidation of many aldehydes. Thus, various aldehydes, e.g. acetaldehyde and benzaldehyde, have been used as "surrogate substrates" when attempts have been made to gain an understanding of aldehyde dehydrogenase-catalyzed oxidation of aldophosphamide to carboxyphosphamide. In addition to the obvious, the problem, however, is that, while each of the aldehyde dehydrogenase isozymes exhibits a broad substrate specificity, each also exhibits a unique substrate specificity. Thus, for example, it is not known

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† To whom requests for reprints should be addressed, at the University of Minnesota, Department of Pharmacology, 3-260 Millard Hall, 435 Delaware Street S.E., Minneapolis, MN 55455.

whether all isozymes that catalyze the oxidation of acetaldehyde also catalyze the oxidation of aldophosphamide and vice versa.

The present report describes methodology that allows direct spectrophotometric assay of NAD-linked aldehyde dehydrogenase-catalyzed oxidation of aldophosphamide to carboxyphosphamide. Also reported are kinetic constants defining mouse hepatic aldehyde dehydrogenase-catalyzed oxidation of aldophosphamide to carboxyphosphamide, and investigations designed to determined whether oxidation of aldophosphamide, acetaldehyde and/or benzaldehyde is catalyzed by the same isozyme(s).

MATERIALS AND METHODS

Materials. 4-Hydroperoxycyclophosphamide was supplied by Dr. R. F. Borch, Department of Pharmacology, University of Rochester, NY. NAD, *N*-acetyl-cysteine, sodium pyrophosphate, and sodium deoxycholate were purchased from the Sigma Chemical Co., St. Louis, MO. Sodium thiosulfate, sodium cacodylate, and EDTA were purchased from the Fisher Scientific Co., Fairlawn, NJ. Acetaldehyde, benzaldehyde and pyrazole were purchased from the Aldrich Chemical Co., Milwaukee, WI.

Animals. Female, 8- to 12-week-old, DBA/2 mice weighing 18–22 g were bred and raised at the University of Minnesota.

Preparation of 4-hydroxycyclophosphamide/aldophosphamide. 4-Hydroperoxycyclophosphamide (10 μmol) was placed in 0.5 ml of 10 mM cacodylate buffer, pH 6.5, and 50 μmol of sodium thiosulfate in a volume of 0.25 ml was added to initiate the reduction of 4-hydroperoxycyclophosphamide [9, 10]. The temperature was 22°. Reduction was accompanied by a rise in pH necessitating the addition of single drops of 0.3 N HCl to maintain the pH at or below 6.5. It was complete by 30 min at which time the volume and pH were adjusted to 1 ml and 6.5 respectively. The reaction solution was kept at 22° for an additional 90 min to allow *cis*- and *trans*-4-hydroxycyclophosphamide and aldophosphamide to reach equilibrium; it was used in the aldehyde dehydrogenase assay within the next 6 hr. The concentration of aldophosphamide + aldophosphamide hydrate in the solution was 2.23 mM.

Preparation of hepatic soluble and solubilized particulate fractions. Livers were excised from mice immediately after cervical dislocation and were homogenized in ice-cold 1.15% KCl containing 1.0 mM EDTA with the aid of a Dounce homogenizer fitted with a loose pestle. The homogenate was centrifuged at 105,000 *g* and 4° for 60 min, and the resultant supernatant (soluble) fraction was saved for assay. The pellet was resuspended in cold 1.15% KCl containing 1.0 mM EDTA and 0.3% deoxycholate with the aid of a Dounce homogenizer fitted with a tight pestle. This suspension was centrifuged at 105,000 *g* and 4° for 60 min, and the resultant supernatant (solubilized particulate) fraction was saved for assay.

Aldehyde dehydrogenase assay. NAD-linked aldehyde dehydrogenase activity was quantified at 37° by monitoring the appearance of NADH at 340 nm with the aid of a Gilford 2400 recording spectrophotometer. The reaction mixture (1 ml, pH 8.2) contained 4 mM NAD, 32 mM sodium pyrophosphate, 0.1 mM pyrazole, 5.0 mM *N*-acetyl-cysteine, 1.0 mM EDTA, the substrate(s) of interest, and soluble or solubilized particulate fraction prepared from 1–6 mg of liver. The reaction was initiated by the addition of substrate; blank rates were obtained in the absence of substrate. Except where noted, initial rates were used to generate all kinetic values. Preliminary studies (^{31}P-NMR,* TLC) established that aldehyde dehydrogenase-catalyzed oxidation of aldophosphamide was indeed to carboxyphosphamide.

Two-substrate kinetic analysis. Various concentrations of the superior substrate alone, or together with a fixed concentration of the inferior substrate, were included in the incubation medium, and the initial rates of NADH formation were determined; superiority was assigned to the substrate with which, in preliminary experiments, the greater V_{max} was obtained. In each case, reciprocals of initial velocities thus obtained were then plotted against reciprocal concentrations of the superior substrate. Assuming mutual substrate inhibition, i.e. that one isozyme or group of isozymes catalyzes the oxidation of both substrates, the double-reciprocal plot obtained when both substrates are present in the incubation medium will intersect the double-reciprocal plot obtained when the superior substrate alone is present. The concentration of the superior substrate at which this crossover should occur is calculated using the equation $V'K/(V - V')$, where V' and V are the maximal velocities obtained with the inferior and superior substrates respectively, and K is the Michaelis constant for the superior substrate. If mutual substrate inhibition does not occur, i.e. if the oxidation of each substrate is catalyzed by a different enzyme, the two plots will never intersect.

^{31}P-NMR spectrometry. ^{31}P-NMR spectra were obtained with a Nicolet NT-300 WB spectrometer using 12-mm sample tubes, a 121.47 MHz radio frequency, a 2000 Hz spectral width, a 10-μsec pulse width, a 1.0-sec pulse repetition time, and 300 acquisitions. Time points were midpoints of data acquisition. An internal ^{2}H lock and broad band proton decoupling were used. Chemical shifts were measured in parts per million from an external standard of 85% phosphoric acid. Peak identification was based on relative peak positions reported previously [9], and the relative proportions of reaction components were calculated from peak integrals. A precalibrated standard glass electrode was used to measure solution pH; pH values were not corrected for deuterium isotope effect.

RESULTS

Generation of aldophosphamide. 4-Hydroperoxycyclophosphamide was placed into a slightly acidic aqueous solution, and mild reducing conditions were

* Abbreviations: NMR, nuclear magnetic resonance; K_m, Michaelis constant; V_{max}, rate of catalysis when enzyme is saturated with substrate; and Tris, tris(hydroxymethyl)aminomethane.

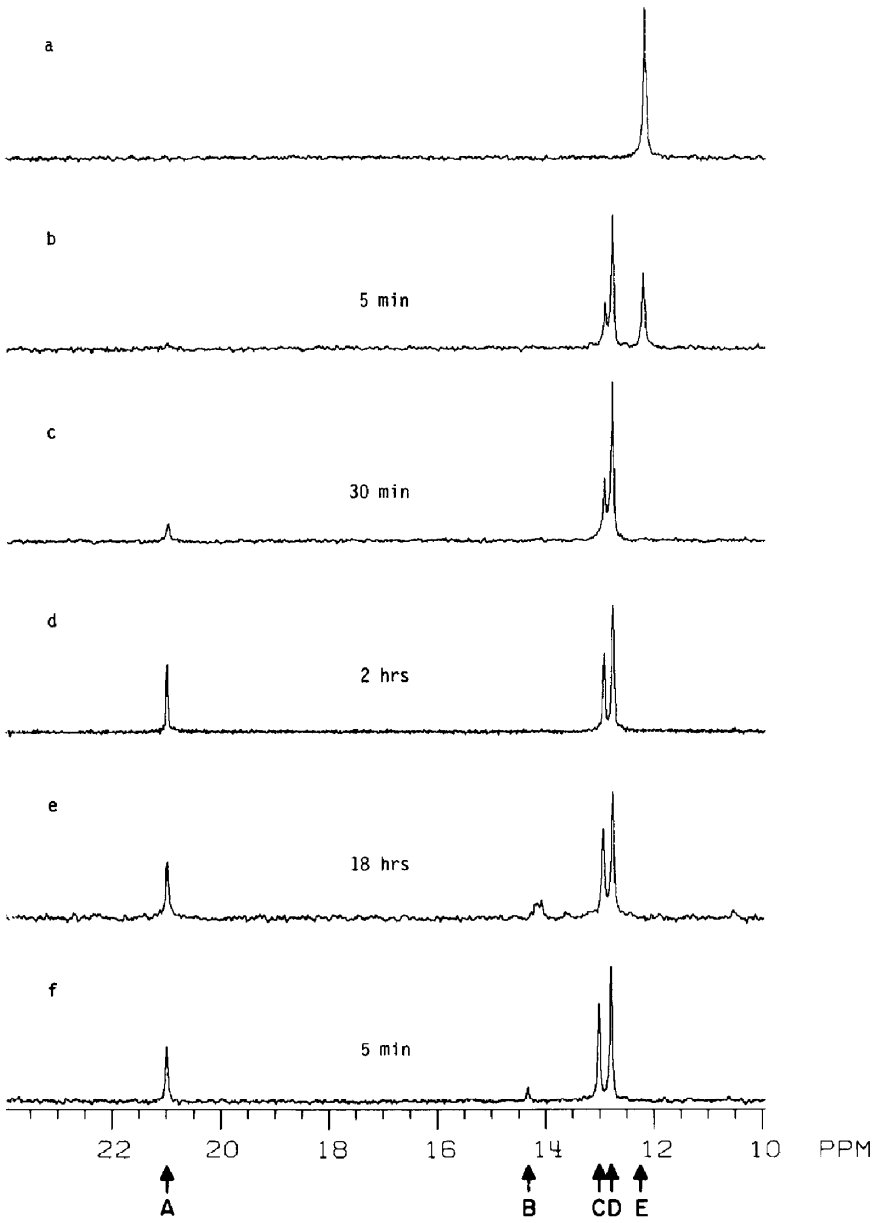

Fig. 1. ^{31}P-NMR spectra showing sodium thiosulfate reduction of 4-hydroperoxycyclophosphamide to 4-hydroxycyclophosphamide/aldophosphamide and the stability of the latter in aqueous solution. ^{31}P-NMR spectra were obtained as described in Materials and Methods. Fig. 1a: 4-Hydroperoxycyclophosphamide (5 mM) was dissolved in 5 mM cacodylate buffer containing 10% ^2H_2O, pH 6.5. Fig. 1b–e. 4-Hydroperoxycyclophosphamide (30 μmol) was dissolved in 2.25 ml of 6.67 mM cacodylate buffer containing 13.3% ^2H_2O, pH 6.5, and aqueous solutions of sodium thiosulfate (0.75 ml, 200 mM) and HCl (10 μl, 0.5 N) were added at t = 0. Spectra were obtained at approximately 15-min intervals. The pH was monitored between data acquisition; microliter volumes of 0.5 N HCl were added when necessary until completion of reduction (<30 min) to maintain the pH at or below 6.5. Upon completion of reduction, the pH was adjusted to exactly 6.5 by adding microliter volumes of 0.1 N NaOH solution; thereafter, the pH remained unchanged for the duration of the experiment. The temperature was maintained at 22° throughout the experiment. Spectra obtained at selected time points are presented. Fig. 1f: Sodium pyrophosphate buffer (1.5 ml, 64 mM, 52°) was added at t = 0 to an aqueous solution of 4-hydroxycyclophosphamide/aldophosphamide (1.5 ml, 20 mM, 22°) prepared as described in Materials and Methods except that ^2H_2O was included, and the pH was quickly adjusted to 8.2 with microliter volumes of 0.1 N NaOH solution. Final temperature was 37°; the solution was kept at this temperature for the duration of the experiment. Spectra were obtained at approximately 20-min intervals; only the first spectrum obtained (t = 5 min) is presented. This and subsequent spectra were used to establish that the conversion of 4-hydroxycyclophosphamide/aldophosphamide to phosphoramide mustard was a first-order process (k = 0.0067 min^{-1}). Key: (A) aldophosphamide + aldophosphamide hydrate (δ21.12); (B) phosphoramide mustard (δ14.35); (C) *trans*-4-hydroxycyclophosphamide (δ13.04); (D) *cis*-4-hydroxycyclophosphamide (δ12.90); and (E) 4-hydroperoxycyclophosphamide (δ12.33).

used to generate a stable aldophosphamide preparation of known concentration. The reaction solution was made slightly acidic and relatively low in buffer concentration to minimize the conversion of aldophosphamide to phosphoramide mustard and acrolein since this conversion is known to be subject to general base catalysis [11]. ^{31}P-NMR spectroscopy established (Fig. 1, a–e) that (1) reduction of 4-hydroperoxycyclophosphamide to a mixture of *cis*- and *trans*-4-hydroxycyclophosphamide and aldophosphamide (hydrate) was completed within 30 min, (2) the reaction products reached full equilibrium within 2 hr, (3) at equilibrium, the relative amounts of *cis*- and *trans*-4-hydroxycyclophosphamide and aldophosphamide (hydrate) were 23:16:11, respectively, and (4) conversion of 4-hydroxycyclophosphamide/aldophosphamide to phosphoramide mustard was minimal (<10%) even at 18 hr; there was no detectable conversion to phosphoramide mustard at 18 hr when the equilibrium mixture was kept at 4° (data not shown). In three such experiments, 22.3 ± 0.5% (mean ± SE) of the 2-hr equilibrium mixture was aldophosphamide (hydrate). This value was used in all subsequent experiments to estimate aldophosphamide (henceforth, used to mean aldophosphamide plus aldophosphamide hydrate) concentrations.

Solutions of aldophosphamide generated in the manner described above were used as the substrate in the enzyme kinetic experiments described below. The latter were conducted at 37° in a 32 mM sodium pyrophosphate buffer, pH 8.2; data acquisition time was <5 min. Conversion of the equilibrium mixture to phosphoramide mustard under these conditions was first-order and slow ($k = 0.0067\ min^{-1}$). At this rate, less than 5% of the equilibrium mixture was converted to phosphoramide mustard during the first 5 min (Fig. 1f). Thus, the aldophosphamide concentration was essentially unchanged (as a consequence of conversion to phosphoramide mustard) over the data acquisition period used in the kinetic experiments, a prerequisite if meaningful constants were to be obtained.

The preceding experiments established that the concentration of aldophosphamide in a solution thus prepared was quantifiable, highly reproducible, and stable. These attributes made such a solution a suitable source of substrate when determining the kinetics of aldehyde dehydrogenase-catalyzed oxidation of aldophosphamide.

Aldehyde dehydrogenase activity: incubation medium. The use of aldophosphamide (generated in the manner described above) as a substrate in the assay for aldehyde dehydrogenase activity in crude fractions placed special requirements on the composition of the reaction mixture. Typically, the substrate is added to a reaction mixture containing enzyme, NAD, buffer, and pyrazole (an alcohol dehydrogenase inhibitor), and the rate of NADH appearance is monitored. However, we noted a rapid inactivation of the enzyme soon after the addition of the aldophosphamide solution to such a reaction mixture (Fig. 2). Inactivation was observed only when the aldophosphamide solution was added, i.e. it was not observed when aldehydes such as acetaldehyde were added. The underlying basis for this

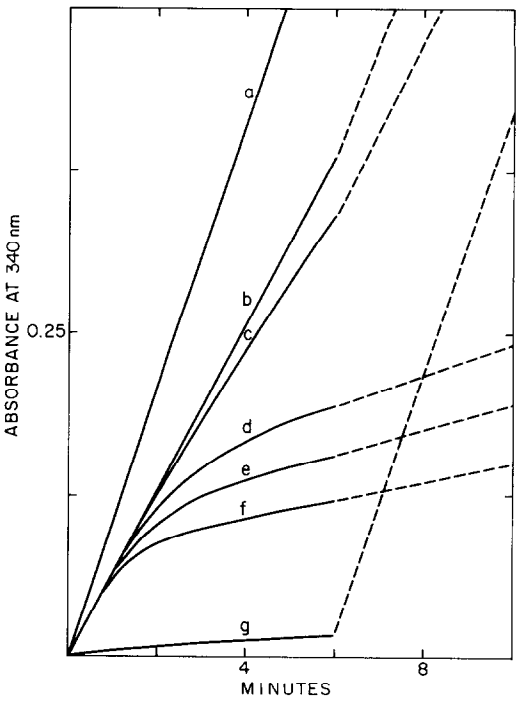

Fig. 2. Prevention with *N*-acetyl-cysteine of 4-hydroxycyclophosphamide/aldophosphamide-induced inactivation of aldehyde dehydrogenase. Aldehyde dehydrogenase activity present in the soluble fraction of 4.5 mg of mouse liver was determined as described in Materials and Methods except that the concentration of *N*-acetyl-cysteine was varied. Key: (a) acetaldehyde (4 mM), *N*-acetyl-cysteine (0 or 5 mM); (b–f) aldophosphamide (110 μM), *N*-acetyl-cysteine (5 or 10, 1, 0.4 0.1 and 0 mM, respectively); (g) *N*-acetyl-cysteine (0 or 5 mM). Acetaldehyde (4 mM) was added to b–g at t = 6 min. Qualitatively similar results were obtained when a solubilized particulate fraction obtained from mouse liver was used as the enzyme source (data not shown).

inactivation was not determined, but one possibility, viz. acrolein, was ruled out since 80 nmol of this agent was required to reproduce the first 2 min of the curve designated f in Fig. 2, and, assuming a β-elimination rate constant of $0.0067\ min^{-1}$, *vide ante*, when the reaction mixture contains 493 nmol of 4-hydroxycyclophosphamide/aldophosphamide, only about 6 nmol of acrolein would be generated during this time. Interestingly, when methyl sulfide rather than sodium thiosulfate was used as the reducing agent, enzyme inactivation occurred more slowly, suggesting a role for the reducing agents in aldehyde dehydrogenase inactivation. That role, however, is not a direct one since, by themselves, neither sodium thiosulfate nor methyl sulfide inactivated the enzyme.

Addition of high concentrations of agents that scavenge oxidants and electrophiles, e.g. sulfhydryls, impeded the inactivation. Therefore, *N*-acetyl-cysteine (5 mM) was routinely added to the reaction mixture in all subsequent experiments. Complete protection was achieved (Fig. 2). Sulfhydryls are

Table 1. Catalysis of aldophosphamide, acetaldehyde and benzaldehyde oxidation by mouse liver aldehyde dehydrogenases: Michaelis–Menten constants*

Fraction	Substrate		K_m (μM)	V_{max} (nmol/min/g liver)
Soluble	Aldophosphamide		22 ± 1	3,310 ± 160
	Acetaldehyde		390 ± 46	4,250 ± 90
	Benzaldehyde		0.31 ± 0.02	2,680 ± 170
Solubilized particulate	Aldophosphamide		84 ± 5	1,170 ± 81
	Acetaldehyde	high K_m enzyme	3,940 ± 460	3,490 ± 410
		low K_m enzyme	0.28 ± 0.04	1,460 ± 49
	Benzaldehyde	high K_m enzyme	10,600 ± 990	6,680 ± 140
		low K_m enzyme	0.09 ± 0.01	276 ± 16

* Soluble and solubilized particulate fractions were prepared from the liver of a single mouse and were assayed for aldehyde dehydrogenase activity as described in Materials and Methods. Low substrate concentrations (10 μM acetaldehyde; 10 μM benzaldehyde) and the integrated Michaelis equation [14] were used to determine kinetic constants for catalytic activities exhibiting nanomolar (low) K_m values. Initial rates, relatively high substrate concentrations and double-reciprocal plots of these parameters (see Figs. 4 and 5) analyzed as described by Wilkinson [15] were used to determine kinetic constants for catalytic activities exhibiting micro- and millimolar (high) K_m values. When both high and low K_m (acetaldehyde, benzaldehyde) catalytic activities were present in the subcellular preparation, i.e. solubilized particulate fraction, initial rate values for high K_m activity were calculated by subtracting separately determined V_{max} values for the low K_m catalytic activity from the initial rates obtained when relatively high substrate concentrations were used. Values are the mean ± SE of three to six determinations, each made with fractions obtained from a different mouse.

known to form reversible adducts with aldophosphamide and 4-hydroxycyclophosphamide [9, 12]. However, preliminary ^{31}P-NMR studies in our laboratory (data not shown) as well as a consideration of the concentrations of the reactants that we used and the kinetics of the adduct formation [9] deem it highly unlikely that the concentration of aldophosphamide in the reaction mixture was altered significantly during the first 5 min of the assay by the presence of *N*-acetyl-cysteine.

Aldophosphamide and 4-hydroxycyclophosphamide are also known to form adducts with certain amines, e.g. Tris [9, 13]. Therefore, inclusion of such amines, e.g. as buffers, in the reaction mixture was avoided.

Similarly, ^{31}P-NMR spectroscopy revealed that, at high concentrations of pyrazole, an aldophosphamide-pyrazole adduct characterized by a chemical shift of 20.98 was generated (data not shown). Formation of this adduct presented a potential problem because aldehyde dehydrogenase was inhibited by it. However, at 100 μM pyrazole, formation of the adduct was insignificant (data not shown); this concentration of pyrazole was sufficient to inhibit completely alcohol dehydrogenase (data not shown).

Mouse liver aldehyde dehydrogenase-catalyzed aldophosphamide oxidation. Aldehyde dehydrogenases in the soluble, as well as in the solubilized particulate, fraction of mouse liver catalyzed the oxidation of aldophosphamide. However, enzyme activity in the two subcellular fractions markedly differed with regard to catalytic constants (Table 1) and energy of activation values (Table 2).

K_m and V_{max} values for the soluble fraction-catalyzed reaction were roughly one-fourth and 3-fold, respectively, those for the solubilized particulate fraction-catalyzed reaction. Kinetic constants defining aldehyde dehydrogenase-catalyzed oxidation of acetaldehyde and benzaldehyde by mouse hepatic soluble, and solubilized particulate, fractions were also determined (Table 1). It should be noted that, when either of these aldehydes was

Table 2. Catalysis of aldophosphamide, acetaldehyde and benzaldehyde oxidation by mouse liver aldehyde dehydrogenases: energies of activation*

Fraction	Energy of activation (kcal/mol)		
	Aldophosphamide	Acetaldehyde	Benzaldehyde
Soluble	20.2 (19.2–21.2)	20.4 (19.4–21.4)	23.2 (20.7–25.7)
Solubilized particulate	5.9 (5.3–6.5)	6.2 (5.2–7.2)	6.1 (4.3–7.9)

* Soluble and solubilized particulate fractions of pooled livers obtained from three mice were prepared and assayed for aldehyde dehydrogenase activity at 27, 30, 33, 36, 39 and 42° as described in Materials and Methods. Substrate concentrations were 70 μM aldophosphamide, 4 mM acetaldehyde and 20 μM or 4 mM (soluble and solubilized particulate fractions, respectively) benzaldehyde. Log initial rates were plotted as a function of $1000/K$, and straight-lines were fitted to these plots by the method of least squares. Energies of activation were calculated from the slopes of these lines. Parentheses, 95% confidence intervals.

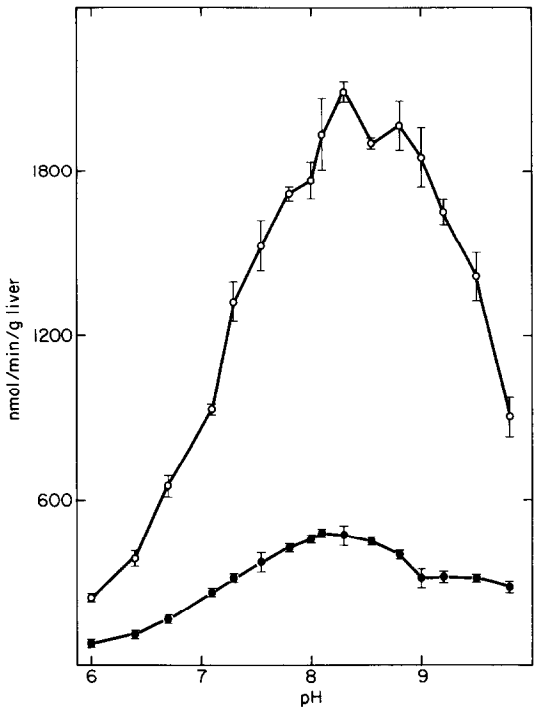

Fig. 3. Effect of pH on the catalysis of aldophosphamide oxidation by mouse aldehyde dehydrogenases. Except for pH, all assay conditions were as described in Materials and Methods. Soluble (○) and solubilized particulate (●) fractions were prepared from the pooled livers of three mice. The substrate was aldophosphamide (70 μM). Data points are the mean and SD of triplicate determinations of initial rates.

used as the substrate, and the solubilized particulate fraction was used as the source of enzyme activity, a single Michaelis constant inadequately defined the observed reaction, i.e. high K_m (millimolar range) and low K_m (nanomolar range) enzymes appeared to be operative in each case.

The energy of activation values for soluble fraction-catalyzed oxidation of aldophosphamide, acetaldehyde, and benzaldehyde were approximately 3-fold greater than those obtained for the solubilized particulate fraction-catalyzed oxidation of these substrates (Table 2).

The pH optimum for aldehyde dehydrogenase-catalyzed oxidation of aldophosphamide was approximately 8.2 for both fractions (Fig. 3). Catalysis of the oxidation of a number of other aldehydes by aldehyde dehydrogenases from several species has been reported to occur optimally at alkaline pH.

Two-substrate kinetic analysis strongly indicated that, in the soluble fraction, the same isozyme or group of isozymes catalyzes the oxidation of aldophosphamide and acetaldehyde or benzaldehyde (Fig. 4 and Table 3). The double-reciprocal plot obtained when both acetaldehyde (superior substrate) and aldophosphamide (inferior substrate) were present in the incubation medium intersected the double-reciprocal plot obtained when acetaldehyde alone was present (Fig. 4A), and the double-reciprocal plot obtained when aldophosphamide (superior substrate) and benzaldehyde (inferior substrate) were present in the incubation medium intersected the double-reciprocal plot obtained when aldophosphamide alone was present (Fig. 4B). Moreover, in both cases, experimentally obtained and calculated crossover values were in close agreement (Table 3).

The high K_m (acetaldehyde, benzaldehyde) isozyme(s) present in the solubilized particulate fraction also apparently catalyzes the oxidation of all three aldehydes. The double-reciprocal plot obtained when acetaldehyde (superior substrate) and aldophosphamide (inferior substrate) were present in the incubation medium intersected the double-reciprocal plot obtained when acetaldehyde alone was present (Fig. 5A), and the double-reciprocal plot obtained when benzaldehyde (superior substrate) and aldophosphamide (inferior substrate) were present in the incubation medium intersected the double-reciprocal plot obtained when benzaldehyde alone was present, (Fig. 5B). However, in both cases, experimentally obtained crossover concentration values were somewhat lower than calculated crossover concentration values (Fig. 5 and Table 3). This is probably because a low K_m (acetaldehyde, benzaldehyde) isozyme was also operative in catalyzing the oxidation of aldophosphamide, *vide infra*. When calculating the expected crossover con-

Table 3. Catalysis of aldophosphamide oxidation by mouse liver soluble fraction, and solubilized particulate fraction high K_m (acetaldehyde, benzaldehyde), aldehyde dehydrogenases: two-substrate kinetic analysis*

Fraction	Substrates (superior/inferior)	Intercept (mM) Calculated	Intercept (mM) Experimental
Soluble	Acetaldehyde/Aldophosphamide	1.42 ± 0.28	1.14 ± 0.10
	Aldophosphamide/Benzaldehyde	0.13 ± 0.02	0.15 ± 0.04
Solubilized particulate	Acetaldehyde/Aldophosphamide	2.20 ± 0.04	1.65 ± 0.03†
	Benzaldehyde/Aldophosphamide	2.14 ± 0.12	1.12 ± 0.07†

* Soluble and solubilized particulate fractions were prepared from the liver of a single mouse as described in Materials and Methods. Experimental and calculated intercept values were obtained as described in the text and in the legends of Figs. 4 and 5. Values are the mean ± SE of three determinations, each made with fractions obtained from a different mouse.

† $P < 0.05$ when calculated and experimental values are compared.

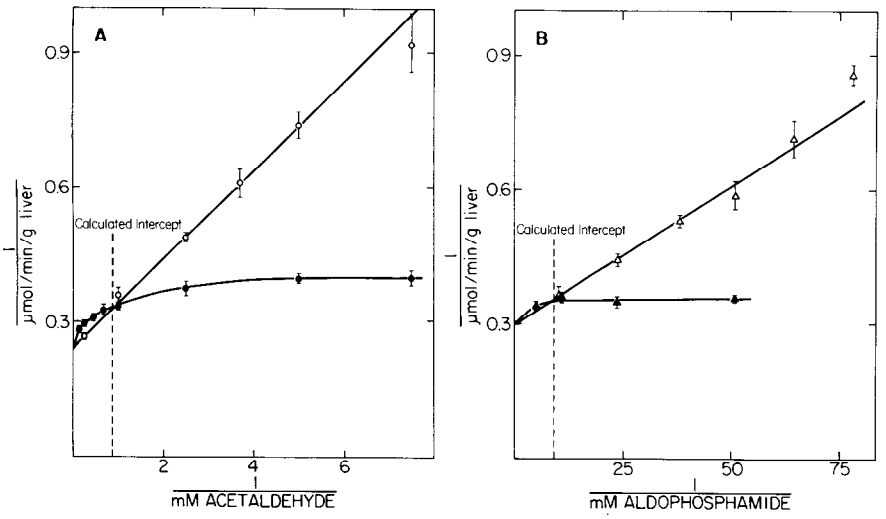

Fig. 4. Two-substrate kinetic analyses of mouse hepatic soluble fraction aldehyde dehydrogenase-catalyzed aldophosphamide/acetaldehyde and aldophosphamide/benzaldehyde oxidation. Assay conditions were as described in Materials and Methods. Data points are the mean and SD of three or more measurements of initial rates. Calculated intercept = $V'K/(V - V')$, where V' and V are the maximal velocities (nmol/min/g liver) for the inferior and superior substrates, respectively, and K is the Michaelis constant (μM) for the superior substrate. (A) A soluble fraction was prepared from the liver of a single mouse and was used to generate all values. Substrates were aldophosphamide alone (data not shown), acetaldehyde alone (○), or acetaldehyde plus 110 μM aldophosphamide (●). V' (aldophosphamide) and V (acetaldehyde) values were 3006 and 4073 respectively; the K (acetaldehyde) value was 404. (B) A soluble fraction was prepared from the liver of a second mouse and was used to generate all values. Substrates were aldophosphamide alone (△), benzaldehyde alone (data not shown), or aldophosphamide plus 20 μM benzaldehyde (▲). V' (benzaldehyde) and V (aldophosphamide) values were 2820 and 3367 respectively; the K (aldophosphamide) value was 20.7.

centration, V_{max} values for acetaldehyde and benzaldehyde oxidation, reflecting high K_m (acetaldehyde, benzaldehyde) aldehyde dehydrogenase activity only, could be used because the contribution to the total velocity by low K_m (acetaldehyde, benzaldehyde) activity could be subtracted out, *vide ante*. However, the contribution, if any, to the total velocity of aldophosphamide oxidation by low K_m (acetaldehyde, benzaldehyde) activity could not be determined since only a single

Table 4. Catalysis of aldophosphamide oxidation by mouse liver solubilized particulate fraction low K_m (acetaldehyde, benzaldehyde) aldehyde dehydrogenases*

Substrate	Substrate concn. (μM)	v (nmol/min/g liver) Obtained†	Expected‡	Activity ratio (obtained/expected)
Aldophosphamide	100	499 ± 40		
Acetaldehyde	10	1,358 ± 48		
Benzaldehyde	10	257 ± 8		
Aldophosphamide + acetaldehyde	100/10	1,643 ± 81	1,857	0.88§ (0.89 ± 0.02)
Aldophosphamide + benzaldehyde	100/10	607 ± 10	756	0.80§ (0.74 ± 0.03)

* A solubilized particulate fraction was prepared from the liver of a single mouse and was assayed for aldehyde dehydrogenase activity as described in Materials and Methods. Values for "v obtained" are the mean ± SD of triplicate measurements. Altogether, this experiment was performed three times with the solubilized particulate fractions being obtained from three different mice. Mean activity ratios thus determined, and the SEs thereof, are shown in parentheses.

† Velocity obtained at the concentration(s) of substrate(s) indicated. Initial rates were used to calculate these values.

‡ Expected value assuming that catalysis is by two isozymes or groups of isozymes each specific for one of the substrates. This value is the sum of the velocities obtained when aldophosphamide or acetaldehyde, and aldophosphamide or benzaldehyde, alone, were used as substrates at the concentrations indicated.

§ $P < 0.02$ when comparing (one-sided paired t-test) the value obtained with the expected value of 1.0.

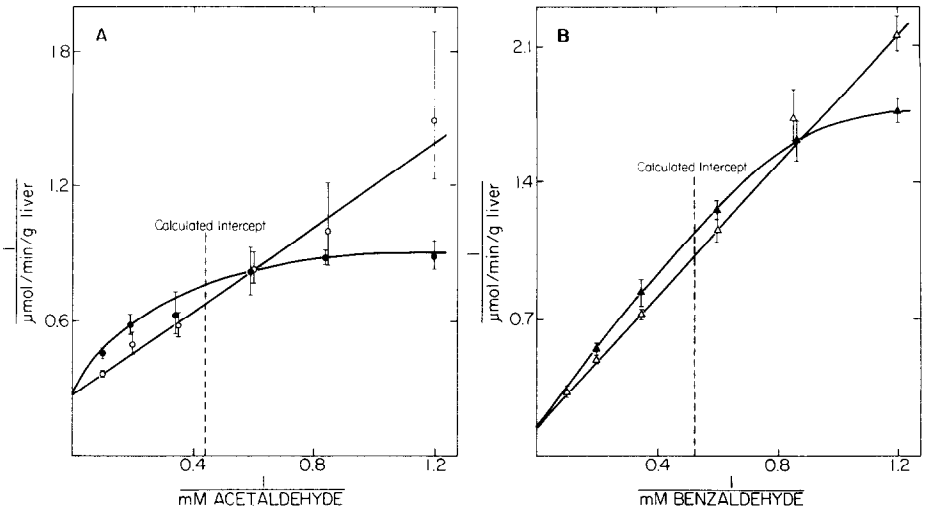

Fig. 5. Two-substrate kinetic analyses of mouse hepatic solubilized particulate fraction high K_m (acetaldehyde/benzaldehyde) aldehyde dehydrogenase-catalyzed aldophosphamide/acetaldehyde and aldophosphamide/benzaldehyde oxidation. Assay conditions were as described in Materials and Methods. Estimates of initial rates resulting from the catalytic action of high K_m (acetaldehyde, benzaldehyde) isozyme(s) were made by subtracting separately determined V_{max} values for low K_m (acetaldehyde. benzaldehyde) activity from the actual initial rate values obtained in these experiments. Data points are the mean ± SD of three or more determinations of net initial rates estimated in this manner. No adjustment in initial rate values was needed for any potential catalysis of aldophosphamide oxidation by the low K_m isozyme(s) because realization of this potential was not possible given the concentrations of acetaldehyde, benzaldehyde and aldophosphamide used in these experiments, the K_m values for low K_m isozyme-catalyzed oxidation of acetaldehyde (0.28 μM) and benzaldehyde (0.09 μM) (Table 1), and, assuming that the low K_m isozyme(s) does(do) in fact catalyze the oxidation of aldophosphamide, the K_m value (70 μM) (Table 1) characterizing this catalysis. Directly put, the, in effect, saturating (for the low K_m isozyme) concentration of acetaldehyde or benzaldehyde used in these experiments would prevent aldophosphamide from ever occupying the catalytic site of this (these) isozyme(s). Calculated intercept = $V'K/(V - V')$, where V' and V are the maximal velocities (nmol/min/g liver) for the inferior and superior substrates, respectively, and K is the Michaelis constant (μM) for the superior substrate. (A) A solubilized particulate fraction was prepared from the liver of a single mouse and was used to generate all values. Substrates were aldophosphamide alone (data not shown), acetaldehyde alone (○), or acetaldehyde plus 200 μM aldophosphamide (●). V' (aldophosphamide) and V (high K_m acetaldehyde) values were 1435 and 3587 respectively; the K (high K_m acetaldehyde) value was 3380. (B) A solubilized particulate fraction was prepared from the liver of a second mouse and was used to generate all values. Substrates were aldophosphamide alone (data not shown), benzaldehyde alone (△), or benzaldehyde plus 200 μM aldophosphamide (▲). V' (aldophosphamide) and V (high K_m benzaldehyde) values were 956 and 6407 respectively; the K (high K_m benzaldehyde) value was 10,900.

K_m value described aldophosphamide oxidation by the solubilized particulate fraction. Since the putative contribution to the total velocity by the low K_m (acetaldehyde, benzaldehyde) isozyme could not be subtracted out, the V_{max} value for aldophosphamide used to calculate the expected crossover point would have been overestimated and its use would have resulted in an overestimation of the crossover concentration. Moreover, if we overestimated the calculated crossover concentration, the conclusion that all high K_m (acetaldehyde, benzaldehyde) isozymes also catalyzed the oxidation of aldophosphamide cannot be made with certainty. This is because an experimental crossover concentration value greater than the calculated crossover concentration value would then be possible; the latter would be consistent with the presence of a high K_m (acetaldehyde, benzaldehyde) isozyme that does not catalyze the oxidation of aldophosphamide.

Data presented in Table 4 indicate that the low K_m (acetaldehyde, benzaldehyde) isozyme(s) found in the solubilized particulate fraction also apparently catalyzes the oxidation of aldophosphamide. Rates of NADH formation were determined in reaction mixtures containing aldophosphamide, acetaldehyde, benzaldehyde, aldophosphamide + acetaldehyde or aldophosphamide + benzaldehyde. The rate obtained when two substrates are present should equal the sum of the rates obtained when each of the substrates is present alone if oxidation of each substrate is catalyzed by a different isozyme or group of isozymes. Very low concentrations of acetaldehyde and benzaldehyde were used in these experiments. Therefore, catalysis of acetaldehyde or benzaldehyde oxidation was essentially all by low K_m isozymes, i.e. the high K_m isozyme(s) was(were) not operative with regard to catalyzing the oxidation of these substrates. Based on the data presented in Fig. 5, it can be assumed, however, that the latter was(were) operative in the catalysis of aldophos-

phamide oxidation, since suitably higher concentrations of this substrate were used. Thus, if catalysis of aldophosphamide oxidation was exclusively by the high K_m isozyme(s), and given that catalysis of acetaldehyde or benzaldehyde oxidation was exclusively by the low K_m isozyme(s), the rate obtained when aldophosphamide and acetaldehyde or benzaldehyde were present should have equalled the sum of the rates obtained when aldophosphamide alone and acetaldehyde or benzaldehyde alone were present. On the other hand, if aldophosphamide oxidation was catalyzed by both the high and the low K_m isozymes, and again given that acetaldehyde or benzaldehyde oxidation was catalyzed exclusively by the low K_m isozyme(s), the obtained rates should have been less than the sum of those obtained when aldophosphamide alone and acetaldehyde or benzaldehyde alone were present. In fact, when aldophosphamide and acetaldehyde or benzaldehyde were both present, the obtained rates were significantly less than the sum of those obtained when they were present alone.

DISCUSSION

The Michaelis–Menten kinetic constants reported herein strongly support a significant *in vivo* role for hepatic aldehyde dehydrogenase activity in clearing aldophosphamide from mice. Thus, using these constants and the relationship (see appendix for derivation),

$$\text{ADMHC} = \{[(V_{\max,s} \times A)/(A + K_{m,s})] + [(V_{\max,p} \times A)/(A + K_{m,p})]\} \times (1/A),$$

where ADMHC is the theoretical aldehyde dehydrogenase-mediated hepatic clearance, $V_{\max,s}$ and $V_{\max,p}$ are maximum velocities for mouse hepatic soluble and solubilized particulate, respectively, fraction aldehyde dehydrogenase-catalyzed oxidation of aldophosphamide, $K_{m,s}$ and $K_{m,p}$ are the Michaelis constants for mouse hepatic soluble and solubilized particulate, respectively, fraction aldehyde dehydrogenase-catalyzed oxidation of aldophosphamide, and A is the concentration of aldophosphamide in plasma, aldehyde dehydrogenase-mediated hepatic clearance would be, in theory, $\geq$116 ml/min when the plasma concentration of aldophosphamide is $\leq 10\ \mu$M. Since hepatic blood flow is less than 2 ml/min in mice [16], it seems likely that actual mouse hepatic aldehyde dehydrogenase-mediated aldophosphamide clearance is flow limited.

Aldehyde dehydrogenases residing in the soluble, as well as in the solubilized particulate, fraction were found to be capable of catalyzing aldophosphamide oxidation, but the soluble fraction exhibited a nearly 3-fold greater $V_{\max}$ and 4-fold lower K_m than did the solubilized particulate fraction. Thus, we would argue that, at least in mice, the aldehyde dehydrogenase isozyme(s) found in the soluble fraction is(are) of primary importance with regard to hepatic clearance of aldophosphamide. Using the Michaelis–Menten kinetic constants obtained in the present investigation and the relationship (see appendix for derivation),

$$F_s = [(V_{\max,s} \times A)/(A + K_{m,s})]/\{[(V_{\max,s} \times A)/(A + K_{m,s})] + [(V_{\max,p} \times A)/(A + K_{m,p})]\},$$

where F_s is the fraction of the theoretical aldehyde dehydrogenase-mediated hepatic aldophosphamide clearance effected by soluble fraction aldehyde dehydrogenase, we estimate that, at aldophosphamide concentrations of $\leq 10\ \mu$M, more than 90% of hepatic aldophosphamide oxidation is catalyzed by aldehyde dehydrogenase activity present in the soluble fraction.

It is uncertain whether each of the several mouse hepatic aldehyde dehydrogenase isozymes that are known to catalyze the oxidation of acetaldehyde and/or benzaldehyde, etc., also catalyzes the oxidation of aldophosphamide to carboxyphosphamide. Possible, too, are isozymes that catalyze the oxidation of aldophosphamide but not that of other aldehydes.

Results of the two-substrate kinetic experiments reported herein are consistent with the notion that the isozyme(s) that catalyzes the oxidation of aldophosphamide also catalyzes the oxidation of acetaldehyde and/or benzaldehyde and, most probably, other aldehydes, and vice versa. This view was further supported by the energy of activation experiments. The energy of activation values describing the catalytic activity of a given substrate-nonspecific isozyme are often very similar regardless of the substrate used to make these determinations [17]. Energy of activation values were nearly identical when oxidation of aldophosphamide, acetaldehyde and benzaldehyde by the soluble fraction was examined. Likewise, little difference among energy of activation values was found when oxidation of these three aldehydes by the solubilized particulate fraction was studied.

A single isozyme is probably responsible for the bulk of the relevant activity found in the soluble fraction [18, 19]. However, at least three different isozymes are known to partition with the solubilized particulate fraction: a high and low K_m (acetaldehyde) mitochondrial isozyme and a high K_m (acetaldehyde) microsomal isozyme [20, 21]. In the present investigation, solubilized particulate fraction aldehyde dehydrogenase-catalyzed oxidation of aldophosphamide was fully defined by a single (intermediate) Michaelis constant. However, at least two isozymes, viz. a high K_m (acetaldehyde, benzaldehyde) isozyme and a low K_m (acetaldehyde, benzaldehyde) isozyme, catalyzed the oxidation of aldophosphamide in this fraction.

Oxazaphosphorines, e.g. cyclophosphamide, that give rise to aldophosphamide often exhibit a margin of safety more favorable than that exhibited by other nitrogen mustards; differentially greater aldehyde dehydrogenase-catalyzed inactivation of these agents in critical normal tissues, e.g. pluripotent hematopoietic progenitor cells, accounts for some, if not all, of the "oxazaphosphorine-specific" selectivity [22, 23]. However, acquired resistance to the oxazaphosphorines can have, as its basis, increased aldehyde dehydrogenase activity [24–26]. The multiplicity of aldehyde dehydrogenase isozymes capable of catalyzing the oxidation of aldophosphamide may be of considerable significance in that regard. Thus, the isozyme found in oxazaphosphorine-resistant L1210 leukemia cells appears to be identical to the major isozyme found in mouse hepatic soluble fraction [8], while preliminary studies suggest that mouse

pluripotent hematopoeitic cells are protected by a different isozyme. Aldehyde dehydrogenase isozymes differ markedly in their sensitivity to inhibitors. For example, murine isozymes differ by more than 100-fold in their sensitivity to either disulfiram or chloral hydrate [20, 27]. Such differences offer the possibility of inhibiting tumor isozymes without inhibiting isozymes protective of critical normal cells, thereby selectively sensitizing tumor cells to the oxazaphosphorines.

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APPENDIX

Derivation of the equation used to calculate ADMHC

The Michaelis–Menten equations defining the rates of *in vivo* mouse hepatic soluble and solubilized particulate fraction aldehyde dehydrogenase-catalyzed aldophosphamide oxidation are

$$(V_{\max,s} \times A)/(A + K_{m,s}) \quad (1)$$

and

$$(V_{\max,p} \times A)/(A + K_{m,p}), \quad (2)$$

respectively, where $V_{\max,s}$ and $V_{\max,p}$ are maximum velocities for mouse hepatic soluble and solubilized particulate, respectively, fraction aldehyde dehydrogenase-catalyzed oxidation of aldophosphamide, $K_{m,s}$ and $K_{m,p}$ are the Michaelis constants for mouse hepatic soluble and solubilized particulate, respectively, fraction aldehyde dehydrogenase-catalyzed oxidation of aldophosphamide, and A is the concentration of aldophosphamide; the latter is assumed to be roughly equal to the concentration of aldophosphamide in plasma.

The rate of total liver aldehyde dehydrogenase catalyzed-aldophosphamide oxidation is the sum of Equations 1 and 2:

$$[(V_{\max,s} \times A)/(A + K_{m,s})] + [(V_{\max,p} \times A)/(A + K_{m,p})]. \quad (3)$$

The volume of plasma cleared of aldophosphamide by the liver per unit time (hepatic clearance) is obtained by dividing Equation 3 by the concentration of aldophosphamide present in plasma:

$$\{[(V_{\max,s} \times A)/(A + K_{m,s})] + [(V_{\max,p} \times A)/(A + K_{m,p})]\} \times (1/A). \quad (4)$$

When the K_m and $V_{\max}$ values given in Table 1 and a pharmacologically relevant plasma concentration of aldophosphamide are substituted into Equation 4, the theoretical aldehyde dehydrogenase-mediated hepatic clearance of aldophosphamide (ADMHC) is obtained for a mouse with a 1 g liver.

Derivation of the equation used to calculate F_s

Dividing Equation 1 by the concentration of aldophosphamide in plasma yields

$$[(V_{\max,s} \times A)/(A + K_{m,s})](1/A), \quad (5)$$

the soluble fraction-mediated hepatic clearance of aldophosphamide. Dividing Equation 5 by Equation 4, viz. the total aldehyde dehydrogenase-mediated hepatic clearance of aldophosphamide, yields

$$[(V_{\max,s} \times A)/(A + K_{m,s})]/\{[(V_{\max,s} \times A)/(A + K_{m,s})] + [(V_{\max,p} \times A)/(A + K_{m,p})]\}, \quad (6)$$

the fraction of the total aldehyde dehydrogenase-mediated hepatic clearance of aldophosphamide that is effected by the soluble fraction aldehyde dehydrogenase (F_s).

REFERENCES

1. N. E. Sladek, in *Metabolism and Action of Anti-cancer Drugs* (Eds. G. Powis and R. A. Prough), p. 48. Taylor & Francis, London (1987).
2. O. M. Friedman, A. Myles and M. Colvin, *Adv. Cancer Chemother.* **1**, 143 (1979).
3. N. Brock and H-J. Hohorst, *Z. Krebsforsch.* **88**, 185 (1977).
4. D. L. Hill, W. R. Laster, Jr. and R. F. Struck, *Cancer Res.* **32**, 658 (1972).
5. P. J. Cox, B. J. Phillips and P. Thomas, *Cancer Res.* **35**, 3755 (1975).
6. B. E. Domeyer and N. E. Sladek, *Biochem. Pharmac.* **29**, 2903 (1980).
7. B. E. Domeyer and N. E. Sladek, *Biochem. Pharmac.* **30**, 2065 (1981).
8. C. L. Manthey and N. E. Sladek, *Proc. Am. Ass. Cancer Res.* **27**, 1672 (1986).
9. G. Zon, S. M. Ludeman, J. A. Brandt, V. L. Boyd, G. Ozkan, W. Egan and K. Shao, *J. med. Chem.* **27**, 446 (1984).
10. R. F. Borch, T. R. Hoye and T. A. Swanson, *J. med. Chem.* **27**, 490 (1984).
11. J. E. Low, R. F. Borch and N. E. Sladek, *Cancer Res.* **43**, 5815 (1983).
12. U. Draeger, G. Peter and H-J. Hohorst, *Cancer Treat. Rep.* **60**, 355 (1976).
13. R. F. Borch and K. M. Getman, *J. med. Chem.* **27**, 485 (1984).
14. M. Dixon and E. C. Webb, *Enzymes*, 3rd Edn., p. 65. Academic Press, New York (1979).
15. G. N. Wilkinson, *Biochem. J.* **80**, 324 (1961).
16. G. B. Dannielson, K-H. Eriksson, B. Karlmark and B. Schildt, *Acta chir. scand.* **137**, 621 (1971).
17. M. Dixon and E. C. Webb, *Enzymes*, 3rd Edn., p. 175. Academic Press, New York (1979).
18. E. M. Algar and R. S. Holmes, *Int. J. Biochem.* **18**, 49 (1986).
19. G. P. Timms and R. S. Holmes, *Genetics* **97**, 327 (1981).
20. E. M. Algar and R. S. Holmes, *Int. J. Biochem.* **17**, 51 (1985).
21. R. G. Little, II and D. R. Petersen, *Comp. Biochem. Physiol.* **74C**, 271 (1983).
22. F. R. Kohn and N. E. Sladek, *Biochem. Pharmac.* **34**, 3465 (1985).
23. F. R. Kohn, G. J. Landkamer, C. L. Manthey, N. K. C. Ramsay and N. E. Sladek, *Cancer Res.* **47**, 3180 (1987).
24. J. Hilton, *Biochem. Pharmac.* **33**, 1867 (1984).
25. J. Hilton, *Cancer Res.* **44**, 5156 (1984).
26. N. E. Sladek and G. J. Landkamer, *Cancer Res.* **45**, 1549 (1985).
27. T. Koivula and M. Koivusalo, *Biochim. biophys. Acta* **397**, 9 (1975).