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Aldehyde Dehydrogenase (ALDH) Isozymes in the Gray Short-Tailed Opossum (*Monodelphis domestica*): Tissue and Subcellular Distribution and Biochemical Genetics of ALDH3

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Polyacrylamide gel isoelectric focusing (PAGE-IEF), cellulose acetate electrophoresis, and histochemical techniques were used to examine the tissue and subcellular distribution, genetics and biochemical properties of aldehyde dehydrogenase (ALDH) isozymes in a didelphid marsupial, the gray short-tail opossum (Monodelphis domestica). *At least 14 zones of activity were resolved by PAGE-IEF and divided into five isozyme groups and three ALDH classes, based upon comparisons with properties previously reported for human, baboon, rat, and mouse ALDHs. Opossum liver ALDHs were distributed among cytosol (ALDHs 1 and 5) and large granular (mitochondrial) fractions (ALDHs 2 and 5). Similarly, kidney ALDHs were distributed between the cytosol (ALDH5) and the mitochondrial fractions (ALDHs 2, 4, and 5), whereas a major isozyme (ALDH3), found in high activity in cornea, esophagus, ear pinna, tail, and stomach extracts, was localized predominantly in the cytosol fraction. Phenotypic variants of the latter enzyme were shown to be inherited in a normal Mendelian fashion, with two alleles at a single locus* (ALDH3) *showing codominant expression. The data provided evidence for genetic identity of corneal, ear pinna, tail, and stomach ALDH3 and supported biochemical evidence from other mammalian species that this enzyme has a dimeric subunit structure.*

KEY WORDS: aldehyde dehydrogenase; isozymes; opossum; genetics.

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INTRODUCTION

Aldehyde dehydrogenases (ALDHs; aldehyde:NAD^+ oxidoreductase; EC 1.2.1.3) exhibit extensive multiplicity in mammalian tissues, and the isozymes have been divided into at least three classes of enzyme, based on comparative biochemical properties, tissue and subcellular distributions, and amino acid and DNA sequences. Class 1 ALDHs are localized predominantly in the liver cytosol, exhibit a broad substrate specificity toward a wide range of "natural aldehydes," and apparently function in a detoxifying role in acetaldehyde, biogenic aldehyde, and peroxidic aldehyde metabolism (MacKerrell *et al.,* 1986; Algar and Holmes, 1986, 1989a). Class 2 ALDHs are the major mammalian liver mitochondrial ALDHs, show submicromolar K_m values with acetaldehyde and other short-chain aliphatic aldehydes as substrate, and function in a detoxifying role in aliphatic and biogenic aldehyde metabolism (Algar and Holmes, 1985, 1989a; MacKerrell *et al.,* 1986; Weiner, 1987). Class 3 ALDHs are characterized by their "high K_m" (mM) values with acetaldehyde as substrate and include the major corneal/stomach ALDH, which is found in very high levels in cornea (0.5% wet weight of tissue) and functions in peroxidic aldehyde metabolism (Holmes and VandeBerg, 1986a; Evces and Lindahl, 1989; Algar and Holmes, 1989b; Abedinia *et al.,* 1990). Molecular genetic and protein chemistry studies have also supported the concept of at least three distinct but related classes for this enzyme (Hempel *et al.,* 1984; Hsu *et al.,* 1985; Jones *et al.,* 1988). Semialdehyde dehydrogenases also exhibit high K_m (mM) values with acetaldehyde as substrate, but are localized in liver and kidney mitochondria, and function in semialdehyde metabolism (Forte-McRobbie and Pietruszko, 1986; Algar and Holmes, 1985, 1989a).

Biochemical genetic studies using the mouse model have confirmed the gene multiplicity for mammalian ALDHs, with at least four genes encoding ALDH isozymes being separately localized in the mouse genome: *Ahd-1* (chromosome 4) (Holmes, 1978), *Ahd-2* (chromosome 19) (Timms and Holmes, 1981), *Ahd-4,* and *Ahd-6* (chromosome 11) (Holmes *et al.,* 1988; Rout and Holmes, 1989). In addition, the genes encoding the human ALDH1, ALDH2, and ALDH3 isozymes have been localized on separate chromosomes; on chromosomes 9q, 12q, and 17, respectively (Human Gene Mapping 10, 1989).

A recent study of corneal ALDH from a nocturnal South American marsupial species (*Monodelphis domestica*) has described variants for this enzyme, which were used to provide evidence for the genetic identity of corneal ALDH with a major soluble protein (Holmes *et al.,* 1990). In this present investigation, we characterize ALDH isozymes from this animal in terms of multiplicity, tissue and subcellular distribution, and catalytic prop-

erties and describe the phenotypic variability and genetics of a Class 3 enzyme (designated ALDH3) in this organism.

MATERIALS AND METHODS

Animals. The gray short-tailed opossums (*Monodelphis domestica*) were produced at the Southwest Foundation for Biomedical Research. This South American marsupial species is a fecund laboratory animal highly suitable for genetic research (see VandeBerg, 1990; van Oorschot and VandeBerg, 1989). Liver and ear pinna samples were collected from 44 animals selected to be genetically representative of the colony, stored at −80°C, and then surveyed for electrophoretic variation in ALDH1, ALDH2, ALDH3, and ALDH5. Ear pinna or tail samples of 321 offspring and their parents, stored at −80°C, were used to investigate the inheritance of ALDH3 variant phenotypes. Tail samples were used to type for ALDH3 in animals younger than 5 weeks of age because of insufficient size of ear pinnae. Additional animals were killed to obtain tissue samples for a more thorough analysis of the tissue and subcellular distribution and the catalytic properties of *M. domestica* ALDH isozymes.

Chemicals. Acrylamide, *N,N*^{1}-methyene-bisacrylamide and *N,N,N*^{1}*N*^{1}-tetramethylethylenediamine were obtained from Bio-Rad Laboratories (Richmond, Calif.), polyacrylamide gel support medium was from FMC Corporation (Rockland, Maine), isoelectric point calibration kits were from Pharmacia Fine Chemicals (Uppsala, Sweden), and ampholytes (Servalyte 3–10 and 4–7 ranges) were from Serva Fine Chemicals (Garden City, N.Y.). Nicotinamide adenine dinucleotide (NAD), phenazine methosulfate (PMS), methyl thiazolyl blue (MTT), sodium pyruvate, ammonium persulfate, tricine [*N*-tris(hydroxymethyl) methylglycine], acetaldehyde, and heptaldehyde were obtained from Sigma Chemical Co (St Louis, Mo.). All other chemicals were of analytical-grade purity.

Homogenate Preparation. All frozen tissue samples were homogenized in 50 mM tricine–sodium hydroxide, *p*H 8.0, 0.25% sodium deoxycholate buffer using either a Potter homogenizer [liver, heart, lung, kidney (20%, w/v); stomach, intestine (17%, w/v); esophagus (7%, w/v); ear pinna (10%, w/v); tail (25%, w/v)] or a Brinkmann Polytron homogenizer (Model PT 10-35; Westbury, N.Y.) [cornea (7%, w/v)]. The homogenates were then centrifuged (45,000*g*, 40 min, 40°C) prior to isoelectric focusing or cellulose acetate electrophoresis.

Subcellular Fractionation. Samples of freshly dissected liver, kidney, and ear pinna were finely minced and then homogenized gently in a Potter homogenizer with a motor-driven close-fitting Teflon pestle, as a 10% (w/v) suspension in 50 mM tricine–sodium hydroxide buffer (*p*H 7.4), containing

0.25 M sucrose. The homogenates were then subjected to differential centrifugation to obtain a large-granule (mitochondria, peroxisomes, and lysosomes) fraction, in each case (Hogeboom, 1955). These were washed twice in the homogenizing buffer, and then extracted in 2 vol (of original tissue sample) of 50 mM tricine–sodium hydroxide, *p*H 8.0, containing 0.25% sodium deoxycholate, and centrifuged at 45,000*g* for 40 min. Cytosol fractions of opossum liver, kidney, and ear pinna were prepared by extracting finely minced tissue samples in 5 vol of 50 mM tricine–sodium hydroxide, *p*H 8.0, buffer containing 0.25% sucrose, using the Potter homogenizer, and then subjecting the extracts to centrifugation at 45,000*g* for 40 min.

Isoelectric Focusing and Staining. Homogenate supernatants were subjected to polyacrylamide gel isoelectric focusing (PAGE-IEF) according to the method of Radola (1980). A 2:1 mixture of *p*I ranges 4–7 and 3–10 ampholytes was used to resolve opossum ALDH isozymes. The gels were prefocused at 9°C for 30 min by setting the constant-wattage power supply (E-C Apparatus Corporation, St. Petersburg, Fla.) at 5 W. Aliquots (10–20 μl) of tissue extracts or subcellular preparations were then applied to the gel, and IEF commenced for 30 min at 5 W. After removal of the sample applicator, PAGE-IEF proceeded for a further 30 min at a constant 8 W.

Following IEF, ALDH activity was stained using the following mixture: 100 mM tricine–sodium hydroxide (*p*H 8.0), 0.25 mM NAD, 2.5 mM pyruvate (to inhibit lactate dehydrogenase activity), 0.5 mM PMS, 3 mM MTT, and aldehyde substrate. Various conditions of substrate were used to distinguish different classes of ALDH: acetaldehyde (0.1, 1.0, and 50 mM) and heptaldehyde (1 mM). Appropriate control stains (in the absence of substrate and/or coenzyme) were also used to distinguish oxidase activity on the gels. The gels were stained at 37°C for periods up to 15 min and destained in cold 5% acetic acid for 30 min. Protein stains (for use with protein standards for p*I* determinations) were performed using 0.2% Coomassie blue G in methanol: acetic acid:water (5:5:1) and destained in the same solvent mixture. Following washing, the gels were air-dried at 37°C overnight and photographed.

Cellulose Acetate Electrophoresis and Staining. Tissue extracts (ear pinna, tail, cornea, and stomach) were also subjected to cellulose acetate electrophoresis and stained for ALDH activity as described by Holmes *et al.* (1990).

RESULTS

Tissue Distribution and Substrate Specificities of ALDH Isozymes. Figure 1 illustrates the tissue distribution of ALDH isozymes in *M. domestica,* following PAGE-IEF and staining with either 50 mM acetaldehyde or 1 mM heptaldehyde as substrate. Liver ALDHs were resolved into at least 11 zones of activity, which were designated five different ALDHs, on the basis

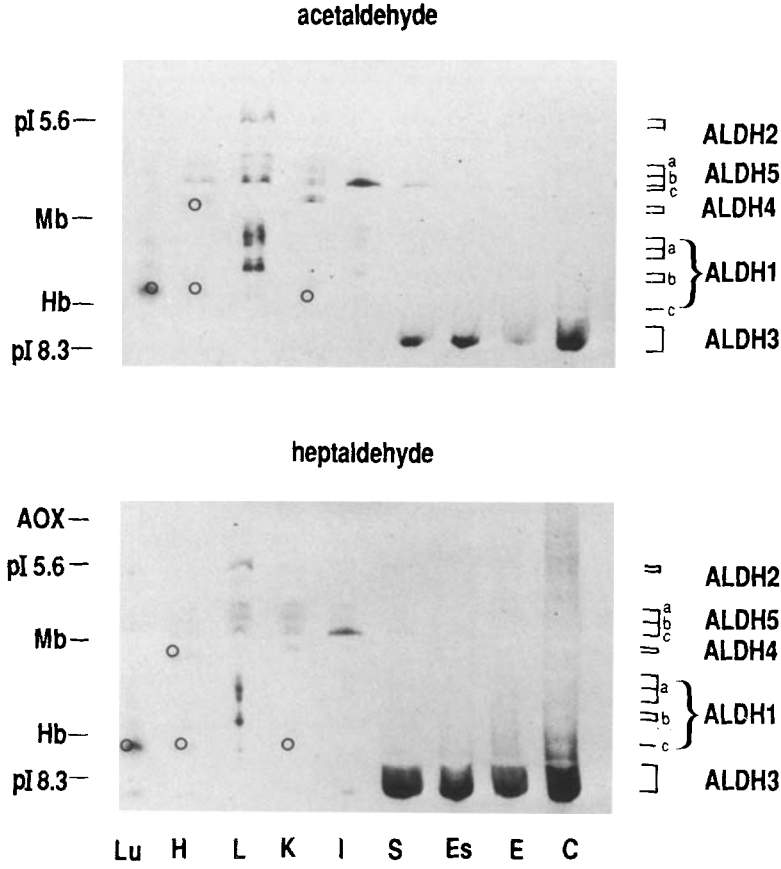

Fig. 1. Agarose-IEF zymograms of *Monodelphis domestica* aldehyde dehydrogenase (ALDH) isozymes, illustrating their differential tissue distribution. ALDH activity zones are indicated at the side of the zymograms. AOX (aldehyde oxidase) zone refers to control activity in the absence of coenzyme. Mb (myoglobin) and Hb (hemoglobin) refer to pigment zones. *p*I (isoelectric point) values are indicated. Tissues examined included lung (Lu), heart (H), liver (L), kidney (K), intestine (I), stomach (S), esophagus (Es), ear pinna (E), and cornea (C). Zymograms stained with 50 mM acetaldehyde (upper) and 1 mM heptaldehyde (lower). (○) Hemoglobin or myoglobin zones.

of their distinct tissue and subcellular distribution and substrate specificity patterns (see Table I). ALDH1 comprised six forms of activity, designated ALDH1a (*p*I values of 6.8, 6.9, and 7.0), ALDH1b (*p*I values of 7.3 and 7.4), and ALDH1c (*p*I of 7.8). Low ALDH1 activity was also observed in intestine and lung extracts. ALDH2 comprised a major (*p*I 5.6) and two minor forms of activity (*p*I values of 5.5 and 5.8) and was more widely distributed, being observed also in lung, intestine, kidney, heart, and stomach extracts. ALDH3

Table I. Properties of Gray Short-Tailed Opossum (*Monodelphis domestica*) Aldehyde Dehydrogenase (ALDH) Isozymes

Property	ALDH1	ALDH2	ALDH3	ALDH4	ALDH5
Tissue distribution[a]	Liver	Liver Kidney	Cornea Esophagus Ear pinna Tail Stomach (Intestine) (Lung) (Liver)	Heart Kidney (Liver)[a]	Wide distribution
Subcellular distribution[b]	Cytosolic	Mito-chondrial	Cytosolic	Mito-chondrial	Cytosolic Mitochondrial
Activity[c]					
Acetaldehyde					
0.1 mM	+	+++	–	–	–
1.0 mM	++	+++	(+)	(+)	(+)
50.0 mM	++	++	+++	++	++
Heptaldehyde					
1.0 mM	++	++	++++	++	++
Isoelectric point	1a (7.0, 6.9, 6.8) 1b (7.4, 7.3) 1c (7.8)	5.5, 5.6, 5.8	7.8, 8.05, 8.3[d]	6.5	5a (6.1) 5b (6.15) 5c (6.3)
Proposed ALDH class	1	2	3	?	?

[a]Parentheses denote trace activity.
[b]Mitochondrial activity based on large-granule preparations.
[c](+), +, ++, +++, and ++++ denote increasing levels of activity.
[d]Different *p*I values refer to variant forms.

(*p*I 8.3) exhibited high levels of activity in cornea, esophagus, ear pinna, tail, and stomach extracts and was also observed in lung, heart, and intestine extracts. The enzyme was characterized by its enhanced activity with 1 mM heptaldehyde as substrate. ALDH4 (*p*I 6.5) was localized predominantly in kidney and heart extracts, with lower levels of activity also observed in liver extracts. ALDH5 exhibited three major forms of activity (designated 5a, 5b, and 5c for the enzymes with *p*I values of 6.1, 6.15, and 6.3, respectively), for which ALDH5c was more widely distributed, and observed in high activity in liver, intestine, kidney, and heart extracts. These results are summarized in Table I.

The different substrate specificities for *M. domestica* ALDH isozymes were further analyzed using a sequence of PAGE-IEF analyses of ALDHs from liver, kidney, and ear pinna extracts with increasing concentrations of acetaldehyde (0.1, 1.0, and 50 mM) or with 1 mM heptaldehyde as substrate (Fig. 2, Table I). ALDH1, ALDH2, and ALDH5 groups of isozymes were active at all concentrations of acetaldehyde examined, with ALDH2 exhibit-

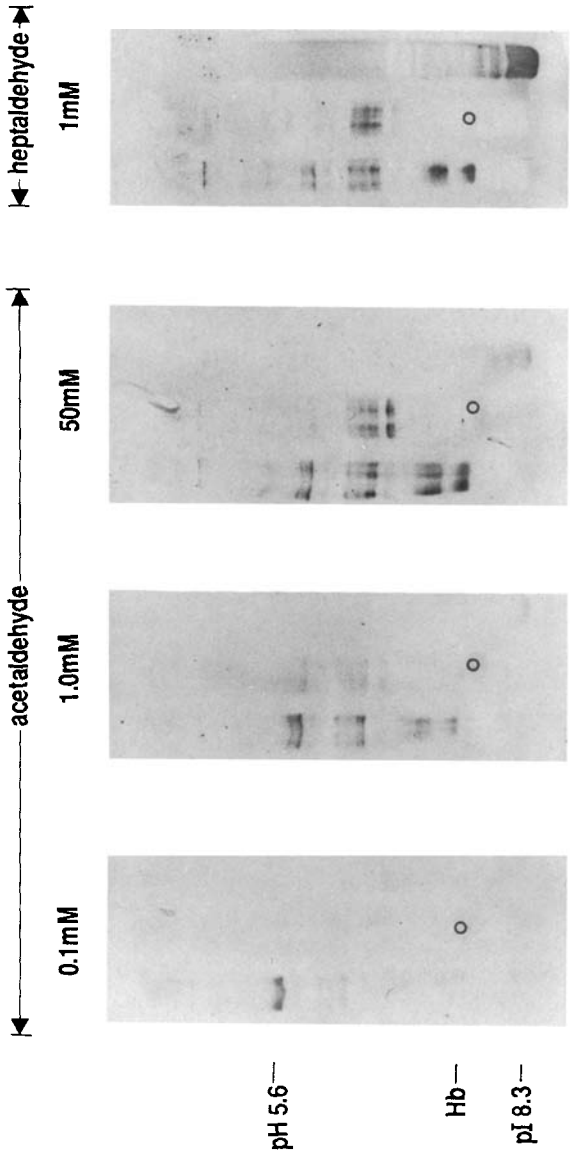

Fig. 2. Agarose-IEF zymograms of *Monodelphis domestica* aldehyde dehydrogenase (ALDH) isozymes, illustrating their differential substrate specificities. ALDH activity zones are indicated at the side of the zymograms. Hb (hemoglobin) refers to a pigment zone. *pI* (isoelectric point) values are indicated. Tissue extracts examined included liver (L), kidney (K), and ear pinna (E). Zymograms stained with 0.1, 1.0, and 50 mM acetaldehyde and 1 mM heptaldehyde as substrates. (○) Hemoglobin zone.

ing properties consistent with this enzyme having a "low K_m" (μM) for this substrate. ALDH1 and ALDH5 isozymes increased in activity across the range of acetaldehyde concentrations used, consistent with an "intermediate K_m" value, whereas ALDH3 and ALDH4 increased dramatically in activity in the 1–50 mM acetaldehyde range and showed properties observed for "high-K_m" ALDHs. In addition, as observed in both Fig. 1 and Fig. 2, ALDH3 exhibited a major preference for heptaldehyde as substrate.

Subcellular Distribution in Liver, Kidney, and Ear Pinna. Figure 3 illustrates the differential subcellular distribution of liver and kidney ALDH isozymes and the major localization of ear ALDH3 in the cytosol fraction. The liver cytosolic fraction (Channel 1) exhibited extensive multiplicity for this enzyme, with two forms of ALDH1 and one form of ALDH5 exhibiting high levels of activity. The liver large-granule (mitochondrial) fraction (Channel 3) showed both ALDH2 and ALDH5 (particularly ALDH5c) activities, whereas the detergent extract of opossum liver (Channel 2) exhibited all of these forms of activity. Kidney ALDH5 also showed properties consistent with a dual localization in cytosolic and large-granular (mitochondrial) preparations, whereas ALDH2 and ALDH4 were predominantly observed in kidney large-granular extracts (Channels 4–6).

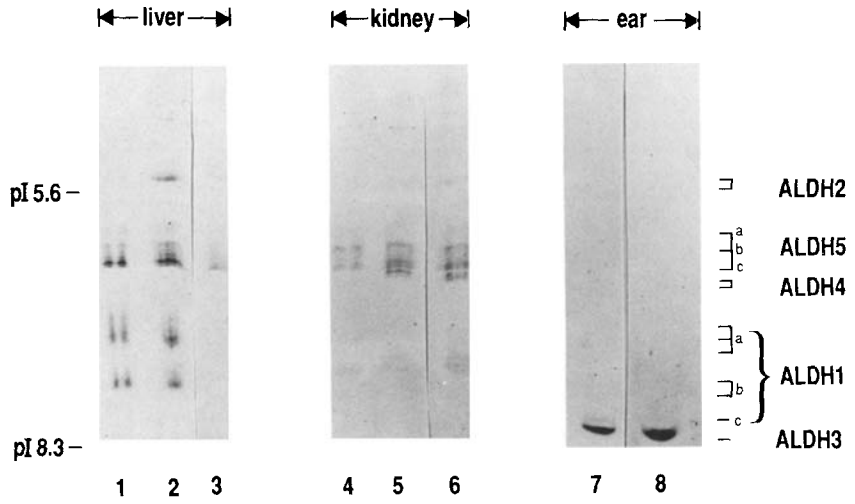

Fig. 3. Agarose-IEF zymograms of *Monodelphis domestica* aldehyde dehydrogenase (ALDH) isozymes, illustrating their differential subcellular distribution between cytosolic and mitochondrial (large granular) preparations. ALDH activity zones are shown at the side of the zymograms. *p*I (isoelectric point) values are also indicated. Subcellular fractions and tissue extracts examined included liver cytosol (1), liver detergent extract (2), liver mitochondria (3), kidney cytosol (4), kidney detergent extract (5), kidney mitochondria (6), ear pinna cytosol (7), and ear pinna detergent extract (8). Zymograms stained with 50 mM acetaldehyde as substrate.

Survey for Variation in Liver ALDHs. Analyses were undertaken of the PAGE-IEF patterns of liver ALDH isozymes, ALDH1, ALDH2, and ALDH5, among the 44 selected panel members. No variant patterns were observed.

Genetics of ALDH3. Figure 4 shows electrophoretic variant phenotypes observed for ALDH3 in stomach, ear pinna, and corneal extracts. Tail extracts (not shown) displayed the same phenotypes. The normal pattern observed for most animals was designated B (Channel 1, in each case) and exhibited a single zone of activity, whereas the variant patterns showed either three forms of activity (Channel 2; designated AB phenotype) or a single zone of activity (Channel 3; designated A phenotype) migrating more slowly toward the cathodal edge of the plate. The family data on the inheritance of these variant phenotypes are shown in Table II. The data are in accordance with codominant allelic inheritance of two alleles, *ALDH3*A* and *ALDH3*B,* at a single autosomal locus (designated *ALDH3*). The variant patterns indicate that the quaternary structure for *ALDH3* is dimeric.

DISCUSSION

The results of this study have provided evidence for extensive multiplicity of aldehyde dehydrogenase (ALDH) in gray short-tailed opossum tissues. These ALDHs were resolved using polyacrylamide gel isoelectric focusing

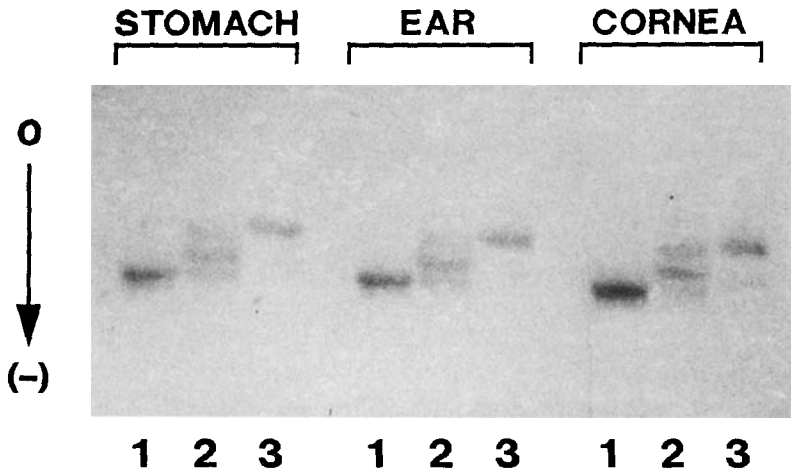

Fig. 4. Cellulose acetate zymograms of stomach, ear pinna, and cornea aldehyde dehydrogenase (ALDH) from *Monodelphis domestica,* illustrating variant phenotypes for ALDH3 among individuals. Animals 1 and 3 represent the proposed homozygous variant phenotypes (designated B and A, respectively); animal 2 shows a three-banded ALDH3 phenotype, consistent with a heterozygous pattern (designated AB).

Table II. Family Data on the Inheritance of ALDH3 in *Monodelphis domestica*

Parental phenotypes[a]	Phenotype of offspring[b]		
	A	AB	B
A × B	0	8	0
B × B	0	0	19
B × AB	0	128	131
AB × AB	9	15	11

[a]The data for the reciprocal crosses are combined, because there were no significant differences between the phenotypic ratios produced.
[b]Chi-square values were calculated for each mating type in which segregation occurred, comparing the observed phenotypic ratio among the offspring with Mendelian expectations. There were no significant differences from Mendelian expectations at the 5% level.

(PAGE-IEF) methods and distinguished on the basis of their differential subcellular and tissue distributions and preferential staining properties with aliphatic aldehyde substrates. The enzymes were conditionally classified into one of three classes (see Table I) previously reported for mammalian ALDHs (Greenfield and Pietruszko, 1977; Algar and Holmes, 1985, 1989a,b; Hsu *et al.,* 1985; MacKerrell *et al.,* 1986; Jones *et al.,* 1988).

A complex group of six ALDH isozymes, provisionally designated Class 1 isozymes, exhibited high activity in the liver cytosol and only low activity in two other tissue extracts examined. These enzymes showed "intermediate K_m" properties with acetaldehyde as substrate and exhibited high activity with 1 mM heptaldehyde (Fig. 2, Table I). The restricted distribution of opossum Class 1 ALDHs is comparable to that of the mouse enzyme (Rout and Holmes, 1985) but is in contrast to human (Harada *et al.,* 1980; Duley *et al.,* 1985) and baboon (Holmes and VandeBerg, 1986b) liver cytosolic ALDH, where a wide tissue distribution of Class 1 ALDH has been reported. The multiplicity observed for opossum liver cytosolic ALDH1 is also comparable to the corresponding mouse liver enzyme, for which four or five forms have been shown to be encoded by a single locus (Timms and Holmes, 1981; Rout and Holmes, 1985). It may be noted that the major liver cytosolic ALDH in human and mouse functions in acetaldehyde, biogenic aldehyde, and peroxidic aldehyde metabolism (MacKerrell *et al.,* 1986; Algar and Holmes, 1989a).

The provisional designation of opossum ALDH2 as a mammalian Class 2 ALDH was based on the following observations: localization in liver mitochondria (Fig. 3), "low-K_m" properties with acetaldehyde as substrate (Fig. 2, Table I), and its low *p*I value (5.6), which is comparable to that of the corresponding isozyme in human, baboon, and mouse liver (Harada *et al.,* 1980; Duley *et al.,* 1985; Rout and Holmes, 1985; Holmes and VandeBerg,

1986b). This enzyme is widely distributed in opossum tissues and, if similar in properties to the Class 2 human and mouse ALDHs, may function in the detoxification of aliphatic aldehydes (including acetaldehyde) and in biogenic aldehyde metabolism (MacKerrell *et al.*, 1986; Algar and Holmes, 1989a).

The major opossum corneal and stomach ALDH (designated ALDH3) showed low activity and "high-K_m" properties with acetaldehyde as substrate (Fig. 1, Table I) and preferred heptaldehyde under the staining conditions used. The enzyme also exhibited variant phenotypes, which were readily resolved using cellulose acetate electrophoresis (Fig. 4), and showed patterns consistent with ALDH3 being a dimeric enzyme encoded by a single locus (designated *ALDH3*), with two codominant alleles (designated *ALDH3*A* and *ALDH3*B*). This interpretation was supported by the results of family studies of ALDH3 (Table II). The observed biochemical and genetic properties for opossum ALDH3 were comparable to those reported for baboon (Holmes and VandeBerg, 1986a; Algar *et al.*, 1990), mouse (Holmes *et al.*, 1988), rat (Evces and Lindahl, 1989), and bovine (Abedinia *et al.*, 1990) corneal ALDH, which has been proposed by the latter workers as performing a dual function in the cornea in the detoxification of ultraviolet-induced peroxidic aldehydes and in the direct absorption of ultraviolet light. A recent genetic study of opossum corneal ALDH has provided evidence for the genetic identity of corneal ALDH with a major soluble protein in this anterior eye tissue (Holmes *et al.*, 1990). ALDH3 has been provisionally designated a Class 3 enzyme, based on its comparable biochemical and genetic properties to the corneal and stomach ALDH from other mammalian species.

Several other forms of opossum ALDH were resolved by PAGE-IEF, which also exhibited high-K_m properties with acetaldehyde as substrate. ALDH4 was localized predominantly in kidney large granules (mitochondria) and was comparable in isoelectric point value to those human and mouse mitochondrial ALDHs, which use acetaldehyde poorly as substrate, but function in semialdehyde metabolism. Human liver ALDH4 (Forte-McRobbie and Pietruszko, 1986) and mouse liver and kidney AHD-1 (Algar and Holmes, 1989a) have been purified and characterized as semialdehyde dehydrogenases functioning in succinic semialdehyde (γ-amino butyric acid metabolism) and glutamic γ-semialdehyde (derived from amino acid metabolism) metabolism and are localized in kidney mitochondria. Thus, it is possible that opossum ALDH4 may perform a similar function. ALDH5 exists as three major forms of activity, being widely distributed in opossum tissues and apparently localized in both the cytosolic and the large granular fractions of isotonic liver and kidney extracts. This enzyme exhibits high-K_m

(mM) properties with acetaldehyde as substrate, and also uses heptaldehyde, under the conditions used.

In conclusion, we have presented evidence for the subcellular and tissue distribution of aldehyde dehydrogenase isozymes in a laboratory marsupial, *Monodelphis domestica,* and have described the genetics of variant phenotypes for the major corneal, ear pinna, tail, and stomach ALDH (designated ALDH3). The results revealed extensive multiplicity and distinct biochemical and tissue/subcellular distribution characteristics for these isozymes. A provisional classification scheme was proposed for opossum ALDHs, which separated these enzymes into at least three classes, based upon comparable properties previously reported for human, baboon, and mouse ALDHs.

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