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χ-ADH is the sole alcohol dehydrogenase isozyme of mammalian brains: Implications and inferences

(class III alcohol dehydrogenase/tissue-specific enzyme)

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ABSTRACT **Class III (χ) is the only alcohol dehydrogenase (ADH) in human, equine, bovine, simian, canine, and rodent brain and is the first to be identified, purified, and characterized from the brain of humans or other vertebrates. Like the corresponding isozymes from human placenta and liver, the χ-ADH isozymes purified from mammalian brain are neither inhibited by nor do they bind to immobilized pyrazole, and they oxidize ethanol only very poorly (K_m > 2.5 M). Indeed, it would be incorrect to classify them as "ethanol dehydrogenases." They contain 4 g·atom of zinc/mol, bind 2 moles of NAD, and readily oxidize long-chain aliphatic and aromatic primary alcohols. These findings appear to exclude the possibilities that ADH protects the brain of these vertebrates against ethanol or its metabolic products and that the brain can generate energy for cerebral function from ADH-monitored ethanol metabolism. Thus χ-ADH must serve a totally different but as yet unknown role. The failure to detect any ethanol dehydrogenase activity in brain creates an intellectual dilemma only if it is assumed that such an enzyme has evolved and developed as a protective mechanism for ethanol detoxification in that organ, as has been assumed. Tissue and substrate specificities of ADH isozymes are likely to give new insight regarding their physiological roles.**

Oxidation of ethanol has been assumed and generally accepted to be the primary function of human alcohol dehydrogenases (ADH; alcohol:NAD^+ oxidoreductase, EC 1.1.1.1), perhaps because an ADH was actually first isolated and crystallized from yeast (1). The formation of ethanol can hardly be the function of ADH in mammalian liver. Yet it seems to have been taken for granted that ethanol was the primary substrate for the enzymes isolated first from the horse (2) and then from human liver (3). In the ensuing work on these enzymes, differences of rates, specificities, kinetic, structural, and functional details, and the reaction product favored at equilibrium for various substrates of these enzymes were not given specific emphasis in that regard. Hence, the tacit premise that the oxidation of ethanol is the prime target of human ADH has evolved and persisted to the present. Moreover, most of the metabolic activity of the mammalian ADHs has been assumed to be identical with those isolated from liver, where they are most abundant and relatively accessible to isolation and where, in the human, from 75 to 90% of ethanol is apparently metabolized. Long-term human pathology due to ethanol (e.g., alcoholic cirrhosis) primarily affects the liver, further focusing attention on the ADH of this organ. Comparatively speaking, minimal experimental attention has been given to ADH and its behavior in tissues other than the liver.

On administration of ethanol to humans, the effects on brain are the ones that are apparent most rapidly. Hence, the occurrence and function of ADH in that tissue has always been of particular interest, but efforts to uncover facts relevant to ethanol have been singularly disappointing. Most of those studies antedate the awareness of the remarkable number and diversity of structure and specificity of ADH isozymes (4), first noted much earlier (5).

Experimental work failed to reveal any significant ADH activity in brain (6). The level of ethanol oxidation in rat brain is so vanishingly low that only assays of exceptionally high sensitivity could detect it (7). A number of contemporaneous studies employing [^{14}C]ethanol concluded that the perfused rat brain does not metabolize [^{14}C]ethanol at all (8, 9). Immunological work has implied the existence of minuscule amounts of class I† isozymes in human brain (10). However, none of these or other isozymes have been identified, isolated, purified, or characterized from the brains of either humans or other vertebrates.

Among all the isozymes now known, the capacity of class III (χ) ADH to oxidize ethanol is minimal. Whereas all other isozymes are saturated by millimolar or micromolar ethanol, as much as 2.5 M ethanol fails to saturate χ-ADH! In contrast, the K_ms of χ-ADH for pentanol and longer-chain aliphatic alcohols are quite analogous to those of all class I and II isozymes for ethanol (11).

We now demonstrate that class III (χ) ADH is the only ADH isozyme in human brain and that of five other mammalian species. We have identified, isolated, purified, and characterized ADH from each of these sources and proven it to have properties virtually identical to those of class III ADH in human liver and placenta.

The demonstration that class III is the sole ADH in cerebral tissue cannot answer any questions as to the presence or absence in brain of any mechanism by which ethanol is detoxified. Indeed the isolation of class III from brain documents the presence of an alcohol dehydrogenase that is *not* an ethanol dehydrogenase; it must have a different function. Minimally, the linguistic inference that "alcohol dehydrogenase" can be considered an "ethanol dehydrogenase" is tantamount to semantic misunderstanding. Maximally, it has proven to be a fallacious premise for critical physiological, biochemical, and pharmacological investigation of ethanol metabolism in man.

The present data would seem to exclude the possibility that χ-ADH could protect cerebral tissues against the effects of ethanol and the metabolic products of its ADH oxidation and generate energy from ADH-monitored ethanol metabolism for cerebral function. Rather, the presence of this sole isozyme in brain (and other tissues) must signal completely

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Abbreviations: ADH, alcohol dehydrogenase; U, unit(s).

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†Class I comprises the α,β,γ family of isozymes (jointly also referred to as "undifferentiated" ADH), class II comprises the π-ADHs, and class III the χ-ADHs, of which at least two forms have been recognized.

different, albeit as yet unknown, physiological and biochemical roles.

The failure to detect any ethanol dehydrogenase activity in brain creates an intellectual dilemma only if it is assumed that the enzyme has evolved and developed as a protective and/or defense mechanism for the detoxification of ethanol in that organ.

It would seem to us that the discovery of the enzymological diversity of the ADH isozymes shifts the emphasis now centering on inquiry regarding the properties of the isozymes of the liver to those of the brain, placenta, testis, and other organs whose ADH isozyme content is much more selective and tissue-specific.

MATERIALS AND METHODS

Human brain was obtained from legal autopsies (with permission) within 12 hr postmortem and was stored at −70°C. Simian frontal cortex of *Macaca nemestrina*, *M. fascicularis*, and *Papio cynocephalus* was obtained frozen from the Regional Primate Research Center (University of Washington, Seattle, WA). Horse brain (frontal cortex) was obtained at slaughter and stored immediately at −70°C, whereas whole cow, dog, and rat brains were purchased from Pel Freez.

ADH was isolated and purified by the procedure of Wagner *et al.* (11). ADH activity assays and metal analyses were as described (12). Retinol (70 μM) oxidation was measured in 100 mM sodium phosphate (pH 7.4) containing 0.02% Tween 80 and 2.4 mM NAD^+ and was monitored by the change of absorption at 382 nm. One unit of enzyme activity was defined as the oxidation or reduction of 1 μmol of coenzyme/min. Protein concentrations were measured by the method of Lowry *et al.* (13), and amino acid analyses were performed by the phenyl isothiocyanate method (14).

Brain tissue homogenates were electrophoresed in starch gels (15) and stained for ADH activity using ethanol (100 mM) and pentanol (35 mM), and in some cases crotyl alcohol (70 mM) or cinnamyl alcohol (1.0 mM), as substrates.‡ $NaDodSO_4$/PAGE was performed by the procedure of Laemmli and Favre (16).

Immunological crossreactivity between the purified brain isozymes and antisera from rabbits immunized with human liver χ-ADH was established by ELISA, using horseradish peroxidase conjugate for detection.

RESULTS

Class III (χ) ADH is the only isozyme found in starch gel electrophoresis patterns of brain homogenates after activity staining with pentanol or crotyl or cinnamyl alcohol (Fig. 1). With ethanol as substrate, ADH bands cannot be detected at all or are only barely visible. Homogenates were obtained from the frontal cortex of four different human brains, from single samples of three simians, *M. nemestrina*, *M. fascicularis*, and *P. cynocephalus*, from the frontal cortex of three horse brains, and from one cow, one dog, and three rat brains. Compared to a human liver homogenate that contains both the cathodic, class I and II isozymes and the anodic, class III (Fig. 2), only class III bands are apparent in the brain homogenates. Their migratory properties vary for each species, as would be expected for species-dependent amino acid replacements. Analogous to the liver and placenta χ-ADH, those from the brains of all but the horse exhibit two closely spaced anodic bands, which may or may not prove to be genetically distinct.

‡Tissues were also stained with lactic acid, yielding the expected lactate dehydrogenase (LDH) isozyme appearance and distribution. Such LDH stains can interfere with the detection of ADH isozymes and must be excluded by suitable controls.

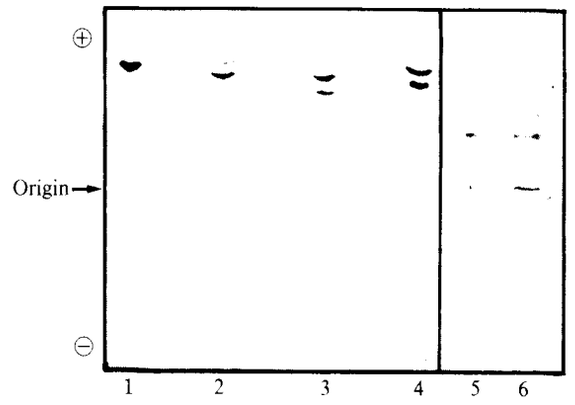

FIG. 1. Activity stained (crotyl alcohol substrate) starch gel electrophoregrams of homogenates of equine (lane 1), bovine (lane 2), canine (lane 3), rabbit (lane 4), and simian (*P. cynocephalus*, lane 5; *M. fascicularis*, lane 6) brain.

Two human brains were dissected, and 2-g samples of anatomically defined sections were homogenized in water. After centrifugation, the homogenates were electrophoresed in starch gel and stained for activity with both ethanol and pentanol. Fig. 2 shows the results for five anatomically distinct samples: right basal ganglia, corpus striatum, vermis, medulla, and right frontal lobe; a liver homogenate is included for comparison. Staining with ethanol as substrate was entirely negative for ADH activity of all brain samples, indicating that class I or II isozymes, present in the liver homogenate, could not be detected. By comparison, stains with pentanol promptly resulted in bands corresponding to class III ADH in all samples. Similarly thalamus, substantia nigra, pons, left parietal and posterior cortex, cerebellum, and the parietal-occipital regions were identical: they contained only class III ADH.

The ADH isozymes were isolated and purified from 20 g of brain of each species by the procedure devised for liver ADH (11, 12). DEAE-cellulose chromatography of bovine brain homogenate resulted in an elution profile that is typical of all species (Fig. 3). Affinity chromatography of the pooled, active fractions from the DEAE chromatography on AMP-hexane-agarose (Fig. 4) yielded virtually homogenous isozymes, as indicated by starch gel electrophoresis (Fig. 5). Close to the major χ-ADH band some preparations contained a second, minor band that also stained only with pentanol.

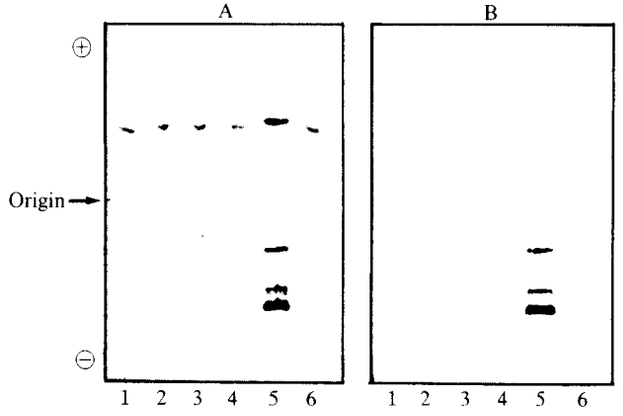

FIG. 2. Starch gel electrophoregrams of homogenates of human right basal ganglia (lanes 1), corpus striatum (lanes 2), vermis (lanes 3), medulla (lanes 4), liver (lanes 5), and right frontal lobe (lanes 6). Samples (≈2 g) were homogenized in an equal volume of water and applied directly to the gel. ADH bands were visualized by activity staining with pentanol (*A*) or ethanol (*B*) as substrate.

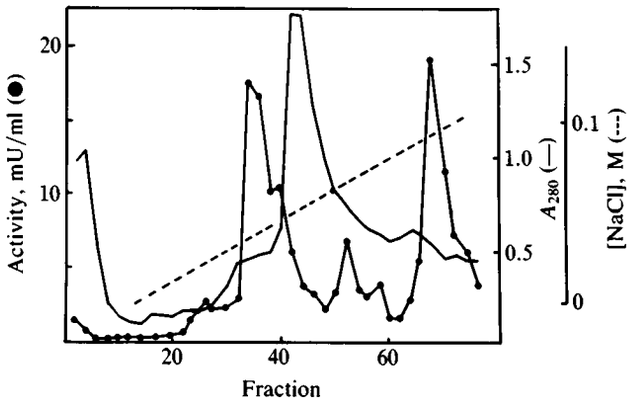

FIG. 3. Chromatography of human brain homogenate on a DEAE-cellulose column (1.5 × 30 cm) equilibrated with 10 mM Tris Cl (pH 7.9). Elution was with a NaCl gradient, as indicated; 4-ml fractions were collected, and activity was measured with 1 mM 1-octanol. mU, milliunits.

This doublet was identified earlier and corresponds to the two χ-ADH isozymes described for placenta (17). Similar doublets have been observed in dog, rat, cow, and monkey brain, but no attempts were made to separate them further. The horse brain sample showed only a single band. $NaDodSO_4$/PAGE of all purified samples resulted in a single band at 37–40 kDa (Fig. 5). Table 1 summarizes the activities for the χ-ADH preparations from each species.

χ-ADH purified from 60 g of human brain cortex was analyzed for metals and amino acid composition, and both were compared to those of χ-ADH from human liver. The zinc content was 3.35 ± 0.04 mol/mol based on an absorptivity A = 0.933, as determined for the liver enzyme (11). The Ni, Cu, Mn, Co, and Fe contents were <0.1 g·atom/mol. The amino acid composition of human brain χ-ADH is virtually the same as that of the liver χ-ADH isozyme (Table 2). The lower serine value for the brain isoenzyme is thought to be due to improved methodology.

The functional properties of χ-ADH that distinguish it from all other ADH isozymes are its anodic migration, complete lack of inhibition by 4-methylpyrazole, and minimal activity towards ethanol and other short-chain alcohols reflecting its failure to exhibit saturation kinetics. Even 2.5 M ethanol does not saturate the enzyme. However, the K_m values of the cerebral (and other) χ-ADHs of each species for long-chain

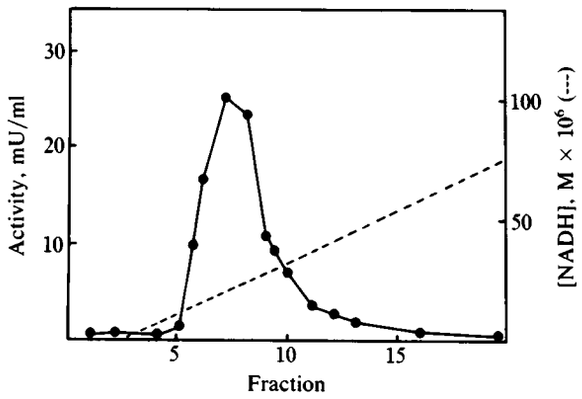

FIG. 4. Agarose-AMP affinity chromatography of active fractions (nos. 32–44) from the DEAE chromatography (see Fig. 1), on a column (1.5 × 5 cm) equilibrated with 5 mM Hepes (pH 7.2). After washing the column with three void volumes of starting buffer, enzyme was eluted with a 0–100 μM gradient (- - -) of NADH. Activity was measured with 1 mM 1-octanol. Active fractions (nos. 7–9) were pooled and used for characterization of the enzyme.

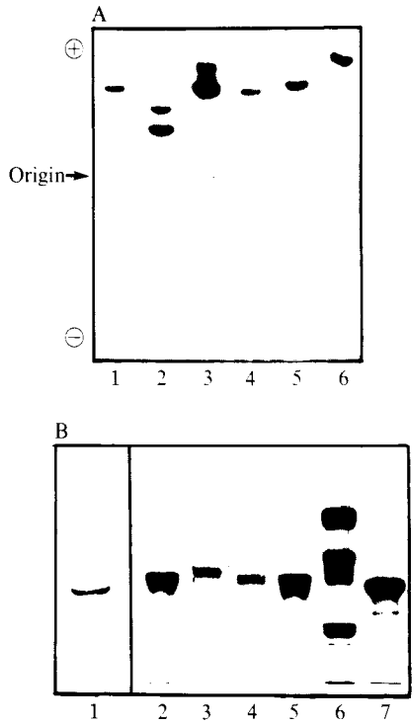

FIG. 5. Electrophoresis of purified class III ADH preparations from vertebrate brain tissues. (*A*) Starch gel electrophoresis at pH 8.2 (activity stain with crotyl alcohol as the substrate). Lanes: 1, human; 2, simian; 3, bovine; 4, rat; 5, canine; 6, equine. (*B*) $NaDodSO_4$/PAGE (Coomassie blue R-250 stain). Lanes: 1, human; 2, equine; 3, canine; 4, rodent; 5, bovine; 7, simian. Lane 6: molecular weight standards (bovine serum albumin, M_r 66,000; ovalbumin, M_r 46,000; carbonic anhydrase, M_r 29,000).

alcohols are similar to those of the human liver χ-ADH (Table 3). The bovine isozyme is the sole exception, with slightly lower K_m values for all substrates. Methanol is not a substrate for any of the χ-ADHs. Thus far, judging by K_m values, 12-hydroxydodecanoic acid is the best substrate for all of the χ-ADHs isolated in this work.

The kinetic details and substrate specificity of the horse brain isozyme were studied in greater detail (Table 4). Long-chain primary aliphatic alcohols are the best substrates, and their K_m values decrease as a function of side-chain length. The brain and human liver class III ADH isozymes do not oxidize secondary alcohols (2-octanol and cyclohexanol), primary alcohols with adjacent polar groups (ethylene glycol, 2-aminoethanol, and sphingosine), and several unsaturated alcohols (retinol, 2-butyn-1-ol, 3-hexyn-1-ol), nor do these compounds inhibit the enzyme. 4-Methylpyrazole (5 mM) does not inhibit any brain ADH, as

Table 1. Class III ADH from brain tissue of various mammals (final purification step)

Source[a]	Volume, ml	Protein, μg/ml	Activity, mU/ml	Specific activity,[b] U/mg	Yield,[c] %
Equine	3.5	23	77	3.3	15
Canine	7.3	11	17	1.6	10
Rat	5.6	27	14	0.52	21
Bovine	7.2	11	30	2.7	13
Simian	7.2	190	39	0.20	20
Human	6.6	13	10	0.75	6

[a]Twenty grams of tissue.
[b]With l-octanol (1 mM) as substrate.
[c]Based on the original activity in the dialyzed homogenate.

Table 2. Amino acid composition of human brain χ-ADH

Amino acid	Residues per subunit: Liver χ-ADH[a]	Brain χ-ADH[b]
Asx	25	24
Glx	36	35
Ser	29	22
Gly	45	43
His	8	8
Arg	9	10
Thr	24	23
Ala	34	35
Pro	18	18
Tyr	7	8
Val	37	37
Met	6	7
Ile	24	26
Leu	21	24
Phe	12	14
Lys	31	32
Total	366	366

[a]Ref. 11.
[b]Average of duplicate determinations after 24-hr hydrolysis with 6 M HCl.

would be expected for class III ADH. $NADP^+$ is not a cofactor. For all substrates the K_m values of χ-ADH from human liver and brain are virtually the same.

All brain χ-ADH isozymes crossreact with rabbit antibodies to human liver χ-ADH (data not shown). The slopes of all ELISA activity vs. dilution profiles are similar, underlining the similarity of their affinities and crossreactivities. Further, human brain χ-ADH does not crossreact with antibodies against human liver class I and II ADH.

DISCUSSION

The liver has been the primary source for the isolation and characterization of mammalian ADHs, owing to the abundance of the enzyme in that organ. Until about 1978, conclusions regarding the function of the human enzyme derived from studies that had to be performed with an "undifferentiated" liver enzyme. Since human liver ADH was first shown to be a mixture of isozymes (5) the systematic exploration of their existence, number, enzymatic specificity, and tissue distribution has completely altered perspectives on the role(s) of human ADH. Activities measured with the undifferentiated mixture of isozymes yield erroneous conclusions regarding potential functions—if any—of the individual components of the mixture. In fact, π- and χ-ADH (classes II and III) were not even components of the mixture, since they do not bind to the affinity column employed for its isolation. They are more anodic on starch gel electrophoresis and were discovered later (15, 17). All of the isozymes now known are recognized to comprise three classes, based on their physical, enzymatic, and immunological properties (4, 20) as well as their remarkable differences in substrate specificities and kinetics (11, 12, 21, 22). However, information regarding their distribution in different organs is still quite sketchy.

χ-ADH, detected and isolated only recently (11), is apparently present in all organs examined so far. It oxidizes ethanol very poorly and therefore is detected in starch gel electrophoresis only when pentanol or higher aliphatic alcohols are used for activity staining. There was no reason, of course, to perform such stains in studies of tissue distribution prior to the recognition of the substrate specificity of this ADH class. The present results further demonstrate that only class III ADH is detected in human, bovine, simian, equine, rat, and canine brain. This contrasts with the isozyme distribution in

Table 4. Specificity of class III ADH from horse brain

Substrate[a]	K_m, M × 10^6	k_{cat}, min^{-1}	k_{cat}/K_m, $mM^{-1}·min^{-1}$
NADH[b]	<1	—	—
NADPH[b]	NR	—	—
NAD^+[c]	100	—	—
$NADP^+$[c]	NR	—	—
12-Hydroxydodecanoic acid	34	420	12,300
Cinnamyl alcohol	110	290	2,700
Cinnamyl alcohol[b]	2,400	180	77
1-Octanol	360	430	1,200
Octanal[d]	660	740	1,100
3-Phenylpropanol	3,800	480	130
3-Methylcrotyl alcohol	6,200	550	89
Crotyl alcohol	17,000	810	47
1-Pentanol	24,000	410	17
Ethanol	>2,400,000	—	—
Methanol[e]	—	NR	—

NR, no reaction.
[a]Unless otherwise indicated, assay was done in 100 mM glycine/2.4 mM NAD^+ at pH 10.0.
[b]100 mM sodium phosphate, 1 mM octanol, pH 7.4.
[c]100 mM sodium phosphate, 10 mM cinnamyl alcohol, pH 7.4.
[d]100 mM sodium phosphate, 58 μM NADH, pH 7.4.
[e]No detectable reaction with 1 M methanol.

Table 3. K_ms of class III ADHs from brain and liver

Substrate[a]	K_m, M × 10^6: Brain Equine	Canine	Rat	Bovine	Simian	Human	Liver Equine[b]	Simian[c]	Human[d]
NAD^+[e]	100	240	170	60	140	130	ND	59	25
12-Hydroxydodecanoic acid	34	70	69	18	58	54	60	240	100
Cinnamyl alcohol	110	370	500	34	200	300	ND	ND	ND
1-Octanol	360	590	380	160	410	450	400	500	1200
Crotyl alcohol	17,000	32,000	48,000	3300	22,000	21,000	ND	ND	ND
Ethanol	NS	NS	NS	NS	NS	NS	NS	NS	NS
Methanol	NA	NA	NA	NA	NA	NA	NA	NA	NA

ND, not done; NS, not saturated up to 2.5 M ethanol; NA, no observable oxidation up to 1.0 M methanol.
[a]100 mM glycine/2.4 mM NAD^+, pH 10.0.
[b]Ref. 18.
[c]Ref. 19.
[d]Ref. 11.
[e]100 mM sodium phosphate/10 mM cinnamyl alcohol, pH 7.4.

other tissues. The liver can contain all isozymes now known, whereas the class I β subunit is the only one in lung, skin, hair roots, and adult kidney (23, 24), and class III is the only one in placenta (17, 25).

The brain χ-ADH from all species here examined and the χ-ADH of human liver and placenta are 80-kDa cathodic homodimers. Like the liver χ-ADH isozymes, those from brain are unstable and must be stored at −195°C if activity is to be preserved for longer than 1 month. The exclusive interaction of these isozymes with antisera from rabbits immunized with human liver χ-ADH and their failure to crossreact with rabbit antibodies produced against classes I and II further confirms their identity as class III ADH.

χ-ADH is the first mammalian ADH for which ethanol is so poor a substrate that no activity can be detected with it in gels. The failure of as much as 2.5 M ethanol to saturate χ-ADH is unprecendented for an enzyme that readily forms a Michaelis complex with pentanol, octanol, and 12-hydroxydodecanoic acid, substrates that are oxidized readily by χ-ADH with micro- or millimolar K_m values, typical of those for other ADH isozymes. The brain χ-ADH from each species exhibits both these enzymatic characteristics and the lack of inhibition by 4-methylpyrazole.

The failure of the brain χ-ADHs to oxidize methanol and secondary alcohols is identical to the properties of liver and placenta χ-ADH (11, 18, 25). Thus all of the χ-ADH isozymes now known preferentially oxidize long-chain aliphatic alcohols. Preliminary information has been obtained on additional alcohols and aldehydes of physiological importance, and present indications are that the substrate specificity of each class of ADH is distinct and metabolically significant. Whatever the primary function of χ-ADH, it cannot be ethanol oxidation.

The virtual absence from brain, neural tissue in general, placenta, and testis of any ADH isozymes that significantly oxidize ethanol and reduce acetaldehyde constitutes important information. The specificity and selectivity of tissue distribution are remarkable. The functional consequences cannot be accidental and the presence of but one isozyme, χ-ADH, is hardly fortuitous.

The failure of χ-ADH to oxidize ethanol to any significant extent shows that the organs that contain solely this ADH variant cannot generate energy from this source, nor can the isozyme protect these tissues from possible toxic effects of ethanol or its metabolic products. Since cerebral effects of ethanol account for the major immediate clinical manifestations of its ingestion, it would seem incongruous that of all tissues the brain should have been left enzymatically unprotected from its effects, if the evolutionary intent had been to protect it by making ADH isozymes the guard. Similar rueful conjectures could be advanced regarding the position in which the presence of only χ-ADH places the placenta and testes, their functions, and their products.

The data suggest that χ-ADH likely plays a different and hitherto unrecognized physiological role that is not easily identified inductively. One clue may be that χ-ADH readily oxidizes or reduces higher aliphatic alcohols or aldehydes, and the substrates here examined do narrow the presumable substrate specificity of χ-ADH (Table 4). Aromatic alcohols such as benzyl alcohol and 2-phenylethanol are unlikely substrates of χ-ADH, as are secondary alcohols (including sterols and retinol) and alcohols containing polar groups adjacent to the hydroxyl.

The functional similarity of χ-ADH in all species examined thus far is striking (Table 3), as is their high degree of crossreactivity in immunoassays using human liver class III ADH antibodies. All of these findings suggest that all class III ADH isozymes are highly conserved and exercise a critical metabolic function, though sequence studies are needed to confirm these conclusions.

Within a single species, man, χ-ADH of brain and liver are identical. The apparently ubiquitous distribution of class III ADH in other tissues as well as its uniqueness in brain, placenta, and testis (unpublished observations) suggests important but yet undiscovered role(s) for this group of isozymes. This raises the broader question whether the presumable function and organ distribution of ADH correlate with physiological roles of this and other isozymes in different tissues.

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