THE GENETICS OF α-HYDROXYACID OXIDASE AND ALCOHOL DEHYDROGENASE IN THE MOUSE: EVIDENCE FOR MULTIPLE GENE LOCI AND LINKAGE BETWEEN *Hao-2* AND *Adh-3*

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ABSTRACT

Electrophoretic polymorphisms for stomach alcohol dehydrogenase (ADH-C_2) and kidney L-α-hydroxyacid oxidase (HAOX-B_4) have been identified in an Asian subspecies of mouse, *Mus musculus castaneous*. These variants are inherited in a normal Mendelian fashion with two alleles in each case showing codominant expression. The structural gene loci for those enzymes (*Adh-3* and *Hao-2*, respectively) are apparently linked (17.6% recombinants) in this organism, whereas the multiple gene loci for HAOX, *Hao-1* (encoding the A_4 liver isozyme) and *Hao-2*, exhibited independent segregation and are unlinked (50% recombinants). Evidence is presented for 3 ADH loci : *Adh-1*, encoding liver ADH-A_2 which exhibits high activity with ethanol (SELANDER, HUNT and YANG 1969; *Adh-2*, liver and stomach ADH-B_2 using 2-hexene-1-ol as substrate; and *Adh-3*, stomach ADH-C_2 using both benzyl alcohol and 2-hexene-1-ol as substrate.

ALCOHOL dehydrogenase (ADH: E.C.1.1.1.1.) is a zinc metalloenzyme that catalyzes the reversible interconversion of a broad spectrum of alcohols and their corresponding aldehydes and ketones with NAD^+ and NADH, respectively, as coenzymes. The flavin-containing enzyme, L-α-hydroxyacid oxidase (HAOX; E.C.1.1.3.1.), also exhibits a broad substrate specificity and catalyzes the oxidation of a variety of α-hydroxyacids with molecular oxygen:

$$R\text{–}CHOH\text{–}COO^- + 0_2 \rightarrow R\text{–}CO\text{–}COO^- + H_2O_2$$

Previous studies have shown that HAOX exists as two isozymes in mammalian tissues, HAOX-A_4 and B_4 (DULEY and HOLMES 1976), which are encoded by distinct genetic loci in mice (*Hao-1* and *Hao-2*, respectively) (DULEY and HOLMES 1974; HOLMES and DULEY 1975). In contrast, recent reports on mammalian ADH indicate that three separate structural gene loci (*Adh-1*, *Adh-2* and *Adh-3*) encode genetically distinct subunits for this enzyme (A,B and C polypeptides), which hybridize *in vivo* to form genetic (A_2, B_2 and C_2) and hybrid (AB, BC, AC) isozymes in human tissues (SMITH, HOPKINSON and HARRIS 1971, 1972, 1973a,b). Genetic and hybrid ADH isozymes have been also described in other mammalian species (see reviews by PIETRUSZKO 1975a,b).

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Genetic variations for mouse liver ADH (A_2 isozyme genotype *Adh-1*) and HAOX (A_4 isozyme *Hao-1*) have been observed in natural populations of two European subspecies of this organism (SELANDER, HUNT and YANG 1969) and in an inbred strain (NZC) (HOLMES and DULEY 1975) respectively. This paper reports electrophoretic genetic variants for the *Hao-2* and *Adh-3* loci in an Asian subspecies of mouse (*Mus musculus castaneous*) and describes evidence for genetic linkage between these two loci, as well as evidence for nonlinkage between the two peroxisomal enzyme loci, *Hao-1* and *Hao-2*.

MATERIALS AND METHODS

Mouse strains: Two inbred strains of *Mus musculus*, NZC and an Asian subspecies (*Mus musculus castaneous*), were routinely used in these studies. Matings between female *castaneous* mice and male NZC mice were made, and the F_1 offspring were backcrossed with NZC mice to examine segregation and the possible linkage relationships of the *Hao* and *Adh-3* loci. Other mouse inbred strains analysed included CBA/H, C3H, C57BL/6J, C57BL/bMf, Quackenbush (an outbred Swiss-white strain), C_S^b (a non-standard strain from Argonne National Laboratory), SM/J and BALB/c.

Homogenate preparation: Livers, kidneys and stomachs were excised from mice freshly killed by exsanguination, rinsed in cold distilled water and homogenized in 50 mm Tris-HCl buffer (pH 7.4) (20% w/v, liver and stomach; 40% w/v, kidney). The homogenates were then centrifuged (50,000 × *g*, 30 minutes) prior to electrophoresis.

Gel electrophoresis and histochemical staining: Homogenate supernatants were subjected to zone electrophoresis on horizontal 10% starch gels at 4° with tris-glycine buffer (20 mM glycine), pH 9.0. A voltage gradient of 20 V/cm was applied for 16 hours. The gels were then sliced, stained for activity and photographed.

HAOX was histochemically stained by the procedure outlined in DULEY and HOLMES (1974) using 8mM glycolate (liver A_4 isozyme) or L-βOH-phenyllactate (kidney B_4 isozyme) as substrate. ADH was stained by the tetrazolium dye procedure (see BREWER 1970), using 10 mM ethanol or 10 mM 2-hexene-1-ol (liver isozymes) or 10 mM benzyl alcohol (stomach C_2 isozyme).

RESULTS

The electrophoretic patterns for variant and hybrid HAOX isozymes are shown in Figure 1. The results for the NZC and hybrid phenotypes in each case are representative of those obtained from F_2 individuals of a NZC (♂) × NZC-*castaneous* (♀) hybrid cross. The wild-type HAOX-A_4 phenotype (genotype *Hao-1*^{a}) was observed for *M.m. castaneous* individuals, as well as for CBA/H, C3H, C57 BL/6J, C57 B1/bMf, Quackenbush, C_S^b, SM/J and BALB/c individuals, whereas the variant phenotype (A_4^1) was found only in NZC mice (genotype *Hao-1*^{b}). The hybrid HAOX-A_4 phenotype (*Hao-1*^{a}*1*^{b}) exhibited five major bands of activity. The wild-type HAOX-B_4 phenotype (genotype *Hao-2*^{a}) was observed in all inbred strains examined with the exception of the *castaneous* mice, which exhibited a faster anodal migrating form (genotype *Hao-2*^{b}). The hybrid HAOX-B_4 phenotype was characterized by a single intermediate broad zone of activity.

Seventy-four progeny from a backcross of (*castaneous* ♀ × NZC ♂) F_1 ♀ × NZC (♂) were examined for segregation at the *Hao-1* and *Hao-2 loci* (Table 1). The loci exhibited a recombination frequency of 50%, and the variation observed

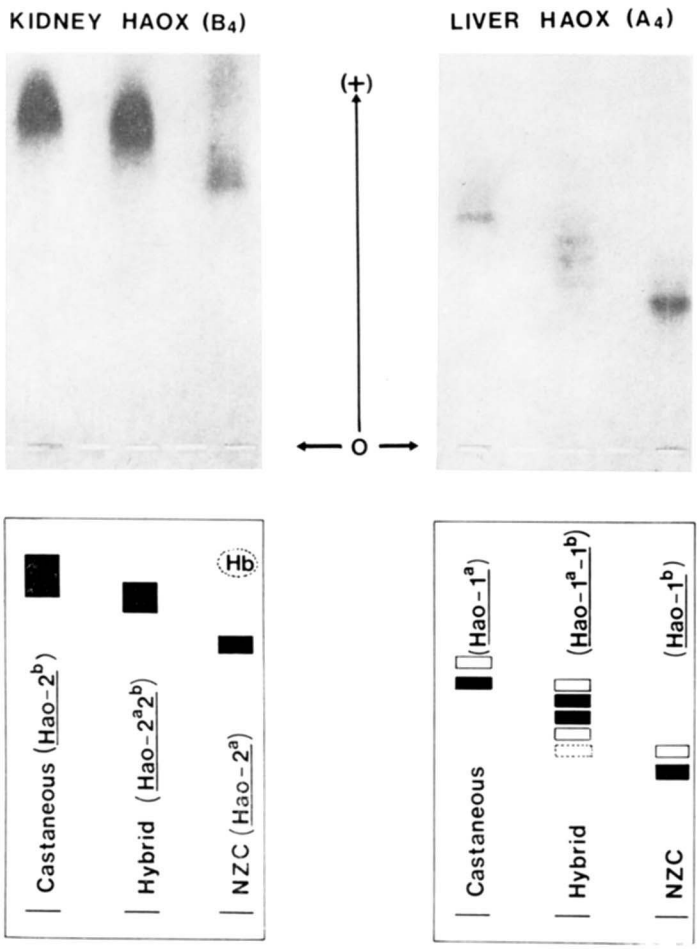

FIGURE 1.—Starch gel zymograms and diagrammatic illustrations of L-α-hydroxyacid oxidase isozymes from mouse inbred strains, *Mus musculus castaneous* and NZC, and their F_1 hybrids. Proposed genotypes are given for the kidney B_4 (*Hao-2* locus) (left diagram) and liver A_4 (*Hao-1*) (right diagram) isozymes.

TABLE 1

Two-point recombination cross between Hao-1 *and* Hao-2*

Parents (♀ × ♂)	Parentals		Recombinants	
	Hao-1^b *Hao-2*^{a} / *Hao-1*^{b} *Hao-2*^{a}	*Hao-1*^{a} *Hao-2*^{b} / *Hao-1*^{b} *Hao-2*^{a}	*Hao-1*^{b} *Hao-2*^{b} / *Hao-1*^{b} *Hao-2*^{a}	*Hao-1*^{a} *Hao-2*^{a} / *Hao-1*^{b} *Hao-2*^{a}
(NZC × *castaneous*) × NZC	18	19	17	20
Total	37		37	

* Recombination frequency = 50%; variation from 1:1:1:1 ratio for unlinked loci was not significant ($\chi^2 = 0.27$; 3 df; $0.975 > P > 0.95$), indicating that the loci are not linked.

from the 1:1:1:1 expected ratio for unlinked loci was not significant ($\chi^2 = 0.27$; 3 df). The loci were therefore segregating independently, which suggests that they are unlinked in these animals.

Figure 2 illustrates the resolution of mouse liver and stomach ADH isozymes by means of starch gel electrophoresis at pH 9.0. The isozymes were histochemically stained with three alcohols to demonstrate their differential specificities. Proposed subunit structures for these isozymes are given. The cathodal migrating liver isozyme (A_2) was most active with ethanol as substrate, whereas the stomach enzyme (C_2) and the anodal migrating liver isozyme (B_2) exhibited high activities with 2-hexene-1-ol. Benzyl alcohol also served as a good histochemical substrate for stomach ADH. The electrophoretic patterns for wild-type, variant and hybrid forms of ADH-C_2 (stomach isozyme) are shown in Figure 2. The results for the NZC and hybrid phenotypes are representative of those obtained from F_2 individuals of a NZC (♂) × NZC-*castaneous* (♀) hybrid cross. The wild-type ADH-C_2 phenotype (proposed genotype *Adh-3*^{a}) was observed for NZC individuals, as well as for SM/J, C3H, BALB/c, CBA and Quackenbush strains, whereas the variant phenotype (C_2^1) was found in *castaneous*, C_s^b and C57B1/6J mice (proposed genotype *Adh-3*^{b}). The hybrid phenotype (*Adh-3*^{a}*3*^{b}) exhibited three zones of activity, of which two were common with the parental forms.

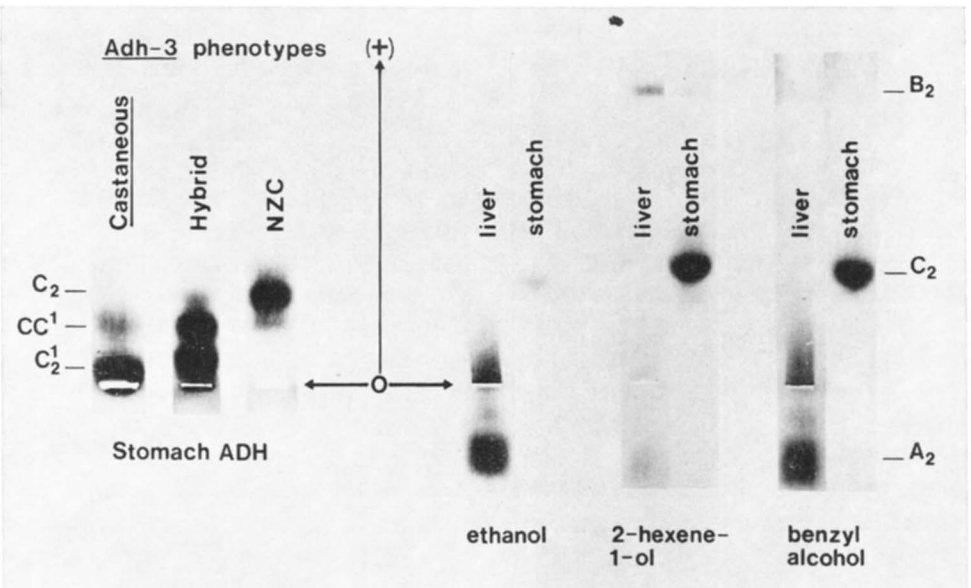

FIGURE 2.—Left diagram—Starch gel zymograms of *Adh-3* phenotypes from mouse inbred strains, *Mus musculus castaneous* and NZC, and their F_1 hybrids. Proposed subunit structures are given. Right diagram—Starch gel zymograms of mouse (NZC strain) liver and stomach alcohol dehydrogenase isozymes stained using ethanol, 2-hexene-1-ol and benzyl alcohol as substrates. Proposed subunit structures are given.

TABLE 2

Two-point recombination cross between Hao-2 *and* Adh-3*

Parents (♀ × ♂)	Parentals		Recombinants	
	Hao-2^a *Adh-3*^{a} / *Hao-2*^{b} *Adh-3*^{b}	*Hao-2*^{a} *Adh-3*^{a} / *Hao-2*^{a} *Adh-3*^{a}	*Hao-2*^{a} *Adh-3*^{b} / *Hao-2*^{a} *Adh-3*^{a}	*Hao-2*^{a} *Adh-3*^{a} / *Hao-2*^{b} *Adh-3*^{a}
(NZC × *castaneous*) × NZC	32	29	6	7
Total	61		13	

* Recombination frequency = 17.6%; variation from 1:1:1:1 ratio for unlinked loci was significant ($\chi^2 = 31.4$; 3 df; $P < 0.005$), indicating that the two loci are linked.

The F_2 backcross progeny previously mentioned were also examined for segregation between the *Adh-3* locus and the *Hao* loci. The *Adh-3* and the *Hao-2* loci exhibited a recombination frequency of 17.6%, and the variation observed from the expected 1:1:1:1 ratio for unlinked loci was significant ($\chi^2 = 31.4$; 3 df), thus indicating that these loci are localized on the same chromosome in mice. (Table 2).

DISCUSSION

Prior to this study, there have been a number of reports indicating that mammalian liver and kidney HAOX are isozymes and are encoded by separate genetic loci. Substrate specificity studies, using purified preparations of HAOX, gave the first indication of biochemical differences between these enzymes. Rat and pig liver HAOX were shown to preferentially oxidize short-chain aliphatic hydroxyacids and exhibited maximum activity with glycolate (KUN, DECHARY and PITOT 1954; ROBINSON *et al.* 1962; NAKANO *et al.* 1968; SCHUMAN and MASSEY 1971), whereas rat and pig kidney contained a form of HAOX exhibiting maximum activity with long-chain aliphatic and aromatic α-hydroxyacids (ROBINSON *et al.* 1962; NAKANO, TSUTUMI and DANOWSKI 1967). Subsequently, the first genetic variant for HAOX was observed in NZC strain mice and used in genetic studies of this enzyme (DULEY and HOLMES 1974; HOLMES and DULEY 1975). The locus for the liver isozyme (*Hao-1*) was localized on chromosome *2* of the mouse, between *a* (agouti) and *Cs* (structural gene for catalase). In addition, it was observed that genetic variation at the *Hao-1* locus did not have any effect on the electrophoretic properties of the kidney isozyme, and that purified rat liver and kidney HAOX differed in their amino acid compositions (DULEY and HOLMES 1976).

This genetic distinction has been confirmed by the current study, since *Hao-1* and *Hao-2* segregated independently among backcross F_2 individuals (Table 1). The data suggest that these loci are not linked in this organism. Similar results have recently been reported for *Hao-1* and a genetic locus (*Dao*) encoding another peroxisomal enzyme, D-amino acid oxidase (DAOX) (HOLMES 1976). Consequently, it can be concluded that the structural gene loci for peroxisomal enzymes are not coordinately localized on mouse chromosomes in the form of a higher organism "operon."

Recent studies have demonstrated that rat liver and kidney HAOX are tetramers at infinite dilution (A_4 and B_4 isozymes, respectively), containing subunits of approximately 40,000 in molecular weight (DULEY and HOLMES 1976; PHILLIPS *et al.* 1976). Both isozymes exhibited association-dissociation behavior at higher concentrations of enzymes, forming octameric and higher molecular weight aggregated species (PHILLIPS *et al.* 1976). The results of Figure 1 for liver HAOX are consistent with a tetrameric subunit structure with five allelic isozymes being observed in the hybrid phenotype (A_4, A_3 A′, $A_2A'_2$, AA'_3, A'_4). In contrast, kidney HAOX exhibited a single broad zone of activity in the hybrid organism, which resembled more closely the *castaneous* phenotype in electrophoretic mobility. This probably reflects the higher activity of the *Hao-2*^{b} isozyme in these mice, as well as association-dissociation behavior that would disrupt normal isozyme patterns for tetrameric allelic isozymes.

ADH multiplicity was first reported by DALZIEL (1958), and later studies showed that ADH isozymes are differentially distributed in mammalian tissues (see PIETRUSZKO 1975a,b). ADH is a dimeric protein (DRUM *et al.* 1967), capable of forming homo and heterodimeric isozymes, as well as multiple forms resulting from epigenetic modification. Three genetic isozymes have been reported in human and horse tissues (SMITH, HOPKINSON and HARRIS 1971, 1972, 1973a,b; PIETRUSZKO, 1975a,b) and are designated as A_2, B_2, C_2. Some species (*e.g.*, rat) exhibit only the A_2 isozyme in liver (JORNVALL 1973). In contrast, horse liver has three major forms, A_2 (also called EE), AB (ES) and B_2 (SS) (THEORELL *et al.* 1966; PIETRUSZKO and THEORELL 1969) and human liver has all three polypeptides present to form a complex mixture of homo- and heterodimeric enzymes, A_2 (also called α_2), AB($\alpha\beta$), B_2(β_2), AC($\alpha\gamma$), C_2(γ_2), and BC($\beta\gamma$) (SMITH, HOPKINSON and HARRIS 1971, 1972, 1973a,b). Other human tissues exhibited simple patterns of activity: adult lung and kidney had ADH-B_2 predominating, whereas adult stomach contained almost exclusively ADH-C_2.

The results of this communication provide evidence for three distinct isozymes for mouse liver and stomach ADH, which are distinguished on the basis of substrate specificity, genetic variation and tissue distribution (Figure 2). Mouse liver has 2 forms, one exhibiting high activity with ethanol (presumably A_2) and another which is active with 2-hexene-1-ol (designated B_2). Stomach ADH (C_2) is similar in substrate specificity to the human (SMITH, HOPKINSON and HARRIS 1972) and rat stomach enzymes (CEDERBAUM *et al.* 1975) in exhibiting high activity with benzyl alcohol and 2-hexene-1-ol.

The genetic distinctness of mouse *Adh-3* is confirmed by the results of this study, since genetic variations of ADH-C_2 did not affect the electrophoretic properties of the mouse liver isozymes. In addition, segregation analyses provided evidence for linkage (17.6% recombination frequency) between the *Hao-2* and *Adh-3* loci. The chromosomal location for these loci is at present not known.

In summary, the present results provide evidence that multiple gene loci for the peroxisomal enzyme, HAOX, (*Hao-1* and *Hoa-2*) are localized on separate chromosomes in the mouse. Evidence is also presented that at least two and possibly three gene loci encode ADH isozymes in mouse tissues. Genetic variation of

the stomach ADH isozyme (C_2) was used to demonstrate genetic distinctness from the liver isozymes and linkage between the locus encoding this isozyme (*Adh-3*) and *Hao-2*.

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