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Enzyme Activities and Ethanol Preference in Mice

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Alcohol dehydrogenase and aldehyde dehydrogenase, the two principal enzymes of alcohol metabolism, were assayed in the livers of the inbred mouse strains C57BL/6J and DBA/2J. Previous work has shown that animals of various C57BL substrains prefer a 10% ethanol solution to water in a two-bottle preference test, and that animals of various DBA/2 substrains avoid alcohol. In the present study, C57BL/6J mice were found to have 300% more aldehyde dehydrogenase activity than DBA/2J mice and 30% more alcohol dehydrogenase activity. The F_1 generation is intermediate to the parents in preference for the 10% alcohol solution and is also found to possess intermediate levels of alcohol and aldehyde dehydrogenase activity. These experiments suggest a systematic relationship between the behavioral trait of ethanol preference and the activity of aldehyde dehydrogenase and a similar but much less pronounced relationship with alcohol dehydrogenase.

INTRODUCTION

Systematic differences among highly inbred strains of mice in preference for a 10% ethanol solution over tap water have been described by McClearn and Rodgers (1959). Initial attempts to determine the biochemical basis for these differences in preference concentrated on alcohol dehydrogenase (ADH) activity because previous studies had indicated that ADH was the rate-limiting step in alcohol metabolism (Jacobsen, 1952; Westerfeld, 1955). Rodgers *et al.* (1963) showed a significant increase of ADH activity measured in micromoles NAD reduced/min/g liver in high preference C57BL mice as compared to the low preference DBA/2 mice. This study also indicated that moderately severe caloric deprivation did not produce preference for alcohol in the initially low preference mice.

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If ADH were the rate-limiting step, disappearance of blood acetaldehyde should be faster than that of alcohol. However, Schlesinger and co-workers (1966) showed that the level of blood acetaldehyde in DBA/2 mice was significantly greater than that in C57BL mice after ethanol injection. These results suggest that aldehyde dehydrogenase (ALDH) and not ADH may be rate-limiting in ethanol metabolism of mice.

The purpose of this research was to corroborate and extend the previous observations on ADH, and to study directly the ALDH activity of genetically uniform mice which possess a known characteristic alcohol preference.

Studies of nonsegregating populations such as inbred strains and F_1's are capable of generating hypotheses concerning correlations among traits, but more definitive conclusions require research with the appropriate genetically heterogeneous populations (McClearn, 1967).

MATERIALS AND METHODS

Since ALDH and ADH levels have both been shown to increase in mice which have consumed alcohol (McClearn *et al.*, 1964; Dajani, Danielski, and Orten, 1963), all experiments discussed here used mice that had never consumed alcohol.

Female mice of the C57BL/6J and DBA/2J inbred strains and the F_1 generation obtained from matings of C57BL/6J♀ with DBA/2J♂ were used. The animals were from the colony of the Institute for Behavioral Genetics and were derived from breeding stock from the Jackson Laboratory. These inbred substrains are not identical to, but they do not differ significantly in alcohol preference from, the corresponding substrains used in the previously cited research. The C57BL × DBA/2 F_1 animals have been shown to have intermediate preference (McClearn and Rodgers, 1961). The mice were decapitated at 6–7 weeks of age. The livers were immediately removed, weighed to 0.01 g, and homogenized in a Potter-type homogenizer with 9 vol (w/v) of 0.25 M sucrose. The homogenate was centrifuged at $260 \times g$ for 20 min to remove nuclei and debris. The enzyme activities of the liquid were determined and are referred to as whole homogenate activity.

Alcohol Dehydrogenase Assay

Alcohol dehydrogenase (ADH) activity was measured by the method of Theorell and Bonnichsen (1951). A standard incubation mixture routinely contained 2.5 ml of 0.1 M glycine-NaOH buffer, *p*H 9.6, 0.2 ml NAD (10 mg/ml), 0.1 ml 2.0% EtOH, and 0.1 ml liver homogenate. The reaction mixture was incubated for 5–10 min at 30 C, during which time the OD_{340} was continuously recorded. An identical cuvette which contained all reactants except the substrate ethanol was used to measure endogenous activity. This value was subtracted from the increase in optical density found in the presence of the substrate. Theoretical V_{max} was determined by the method of Lineweaver and Burke (1934). The yield of NADH was calculated from the molar extinction of 6.22×10^3 liters-cm/mole.

Aldehyde Dehydrogenase Assay

Assay of whole homogenate for aldehyde dehydrogenase (ALDH) was patterned after the method developed by Erwin and Deitrich (1966). The technique consists of measuring spectrofluorometrically the indoleacetic acid produced by the enzymatic oxidation of indole-3-acetaldehyde bisulfite. A standard assay mixture consisted of 0.2 ml NAD (10 mg/ml), 0.1 ml indoleacetaldehyde bisulfite (varying from 2 to 10 mg/ml), 0.6 ml of 0.1 M pyrophosphate buffer, *p*H 9.6, and 0.1 ml whole liver homogenate. Excess substrate was extracted into 1,2-dichloroethane after raising the *p*H of the solution to approximately 11 by addition of 0.2 ml 1 N NaOH. Three separate control reactions were included in all experiments: no enzyme added, no ethanol added, and a zero incubation-time reaction. Theoretical V_{max} was determined by the method of Lineweaver and Burke. Proteins were determined in all cases by the method of Lowry *et al.* (1951).

A partial purification of ALDH which eliminates all ADH activity was achieved by fractional precipitation with ethanol of a phosphate buffer extract of an acetone-dried powder prepared from the mouse liver. Five grams of mouse liver were homogenized in acetone and subsequent purification steps were patterned after the method of Racker (1949). The resulting powder was extracted at room temperature with 50 ml phosphate buffer (0.1 M, *p*H 7.4) containing 0.25 mM ethylenediaminetetraacetic acid (disodium salt) and 50 mM mercaptoethanol. This solution was centrifuged at 5000 rpm at 0 C for 5 min. Precipitates obtained from the supernatant solution by ethanol fractionation in 5% increments from 20 to 80% were dissolved in the smallest amount possible (about 5 ml) of 0.1 M pyrophosphate buffer, *p*H 9.6, at 0 C. ALDH activity was found in the 35–50% saturated ethanol precipitates. This assay was performed spectrophotometrically in the same manner as the ADH assay. A routine incubation mixture contained 2.5 ml of 0.1 M pyrophosphate buffer, *p*H 9.6, 0.2 ml NAD (10 mg/ml), 0.1 ml 0.1% acetaldehyde, and 0.2 ml enzyme (containing approximately 1 to 2 mg of protein).

Mitochondrial ALDH Assay

Mitochondria were prepared from livers in 0.25 M sucrose by the method of Schneider and Hogeboom (1950). The mitochondria were disrupted by sonification at 0 C with a Measuring and Scientific Equipment Ltd. sonifier at a setting of 3 μ for 1.5 min in 30-sec periods with a 1-min interval for cooling. Mitochondrial-associated ALDH activity was assayed for by the fluorometric method already described.

All biochemicals were reagent grade and were obtained from Sigma Chemical Co., St. Louis, Mo. A Gilford model 2000 spectrophotometer was used to make all visible and ultraviolet measurements. An Aminco Bowman spectrofluorometer was used in the idoleacetic acid assay.

RESULTS

The data presented in this paper were obtained using female mice only. In some related pilot research, male mice were also used and no sex differences were observed.

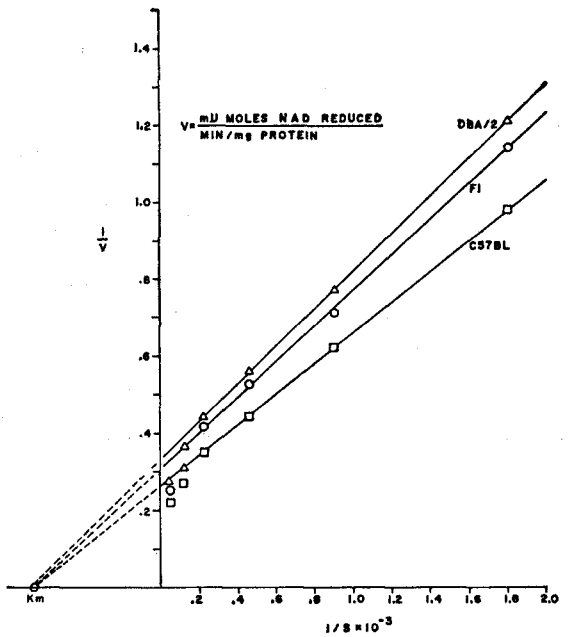

Fig. 1. Double reciprocal plot ($1/v$ vs. $1/s$) of enzyme-catalyzed oxidation of alcohol by NAD using the whole homogenate of mouse liver. Rate of reaction was determined by measuring NADH formation spectrophotometrically as described in text.

Theoretical V_{max} as determined from a Lineweaver–Burke plot showed C57BL/6J mice to have about 30% more ADH activity ($V_{max} = 3.8$) than the DBA/2J mice ($V_{max} = 3.0$) (Fig. 1) (N = 9 for each group; specific activity defined in millimicromoles product per minute per milligram protein). These results are in agreement with those reported by Rodgers *et al.* (1963) who showed greater ADH activity in inbred mouse strains that preferred to drink alcohol. The ADH activity of the C57BL/6J mice was 20% greater than that of the F_1 animals.

The plot of $1/v$ vs. $1/s$ is linear for substrate concentrations from 0.5 mM to 5 mM. Above 5 mM substrate concentration an increase in reaction velocity occurred.

Extrapolation to the apparent K_m gave identical values (1.6×10^{-3} M) for the three groups tested. This value for mouse liver ADH is similar to that reported by Theorell and Bonnichsen (1951) for horse liver ADH (2.0×10^{-3} M).

ALDH Activity

The data of Fig. 2 summarize the results of individual 0.5-min ALDH assays on 12 subjects from each group. Linearity of reaction velocity was observed up to 5 mM substrate concentration at which point an inhibition of enzyme activity occurred.

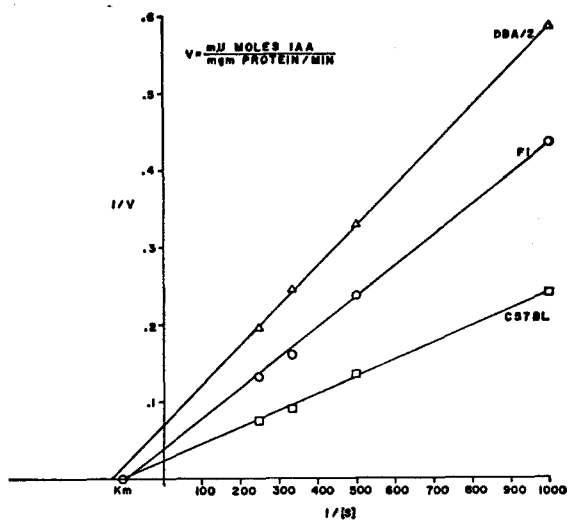

Fig. 2. Double reciprocal plot ($1/v$ vs. $1/s$) of enzyme-catalyzed oxidation of indoleacetaldehyde using the whole homogenate of mouse liver. Rate of reaction was determined by measuring formation of indoleacetic acid spectrophotometrically as described in text.

A similar form of substrate inhibition was also reported by Deitrich (1966). The theoretical V_{max} (millimicromoles indoleacetic acid/mg protein/min) for ALDH in C57BL/6J livers is 52.8, for DBA/2J livers is 14.3, while that for the F_1 livers is 33.3. A common apparent K_m (8.4×10^{-3} M) was observed for all groups tested.

Analysis of variance indicated significant differences between groups at each substrate concentration (F = 308, 170, 159, 82; df = 2, 33). Multiple range tests demonstrated significant differences among all test group means at each concentration.

Production of indoleacetic acid by whole liver homogenate enzyme at a substrate concentration of 2 mM was linear with regard to protein concentration up to 2 mg protein/ml. Production of indoleacetic acid by whole liver homogenate enzyme was linear with time up to 5 min (Fig. 3). Assay conditions of the experiments summarized by Fig. 3 were 2 mg protein/ml with 2 mM substrate concentration and 25 C. Under these conditions the rate of indoleacetic acid production (millimicromoles per minute) by C57BL/6J liver enzyme is 11.2 while that for DBA/2J liver enzyme is 4.8 and that for the F_1 liver enzymes is 6.0.

Ethanol Fractionation

Using an ethanol-fractionated extract of liver acetone powder as a source of ALDH (see Methods), a 2.4 times increase in the initial velocity of this enzyme in C57BL/6J liver was found as compared to the DBA/2J mice (Table I). The F_1 generation is again intermediate to the parent strains.

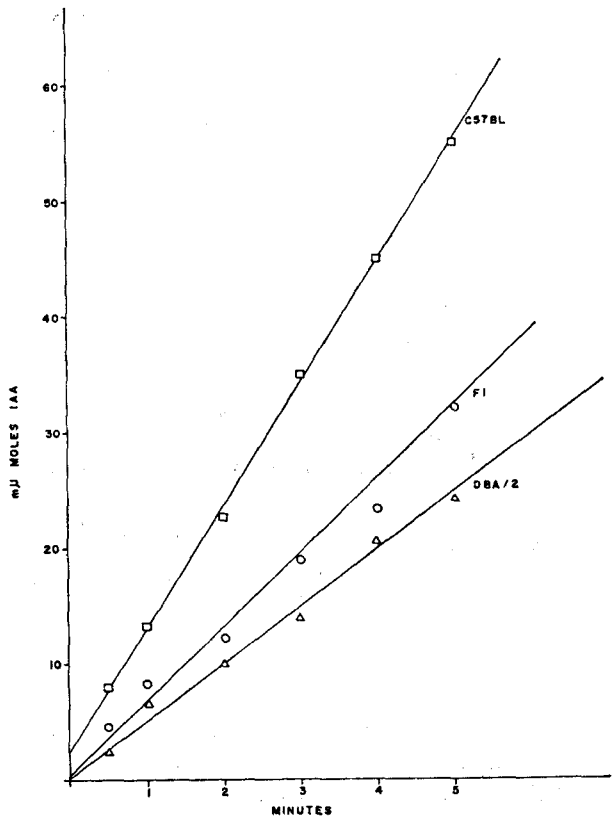

Fig. 3. Plot of rate of formation of indoleacetic acid as a function of time. Reaction conditions are described in the text.

Table I. ALDH Activity in the Partially Purified Precipitate from Ethanol Fractionation[a]

Test group	mμmoles NAD/mg protein/min	Animals assayed
C57BL/6J	8.1 (7.3–8.9)[b]	15
F_1	5.0 (4.5–5.4)	15
DBA/2J	3.2 (2.6–3.8)	15

[a] Conditions: 0.2 ml NAD (10 mg/ml), 0.1 ml substrate (0.1% acetaldehyde), 0.1 ml solubilized protein, 0.6 ml pyrophosphate buffer. Reaction run at 30 C. Activity measured in initial velocity. Precipitate prepared as described in text.

[b] Range.

Table II. Distribution of Aldehyde Oxidizing Capacity Between the Mitochondria and Supernatant Fractions in the Livers of C57BL/6J, DBA/2J, and F_1 Generation[a]

Test group	Aldehyde oxidizing capacity (% of total activity)	
	Supernatant	Mitochondria
C57BL/6J (3)[b]	82.1	14.3
F_1 (3)	75.3	20.0
DBA/2J (3)	80.4	16.7

[a] Conditions: 2 mM NAD, 2 mM substrate, 0.1 M pyrophosphate buffer (*p*H 9.6) used to bring volume to 1 ml; 25 C, 0.5-min reaction time. Mitochondria and supernatant prepared as described in text.
[b] Number of individuals (tested separately).

Mitochondrial ALDH

Rat liver mitochondria have been reported to possess ALDH activity (Deitrich, 1966). A liver mitochondrial-associated ALDH was also found in all three mouse groups tested in these experiments. This enzyme activity accounted for 10–20% of the total aldehyde oxidizing capacity in the mouse liver homogenate. The mitochondrial ALDH activity in the mouse groups was in approximately the same ratios as the soluble ALDH activity. Table II lists the percent of total liver homogenate of aldehyde oxidizing ability recovered in the mitochondrial and supernatant fractions.

DISCUSSION

Although both ADH and ALDH have greater activity in the inbred mouse strain that prefers 10% ethanol solution to water, differences in activity at the second metabolic enzyme, ALDH, may be more critical in determining preference. ADH found in the livers of C57BL/6J had approximately 30% more activity than that in DBA/2J livers, while at the same time ALDH activity was 300% greater. The large difference in ALDH activity was verified by two different and separate assays. One assay used the whole homogenate enzyme with a fluorometric determination of product formation, and another used a partially purified ethanol-fractionated acetone powder extract with a spectrophotometric determination of reaction velocity.

The apparent binding constant of ADH is the same for all mice (Fig. 1); the ALDH binding constants were also similar (Fig. 2). However, because crude liver homogenates were used in these assays, further tests will be needed in order to establish the true binding constants of these enzymes. Of course, it would be valuable to know whether the different enzyme activities observed in C57BL and DBA/2 mice result from different amounts of the same enzymes or the presence of chemically distinct proteins.

Acetaldehyde, the product of the first enzymatic step in ethanol metabolism and substrate for the ALDH catalyzed reaction, is an extremely toxic substance. Its accumulation leads to poisoning of the circulatory and respiratory systems (Jacobsen, 1952). There is evidence that consumption of alcohol by mice deficient in ALDH activity will lead to acetaldehyde accumulation (Schlesinger, Kakihana, and Bennett, 1966). After ethanol injection, DBA/2 mice do possess a higher level of blood acetaldehyde than C57BL mice.

A possible cause of alcohol preference can be argued from these experiments. Alcohol preference might be related to ability to metabolize alcohol. Avoidance of alcohol could be caused by an enzymatic deficiency which leads to a buildup of highly toxic acetaldehyde. Although, at this time, the ultimate molecular basis for alcohol preference is not understood and a causal relationship between enzymatic activities and alcohol preference has not been shown, our results strongly suggest a relationship between this behavioral trait and the activity of liver enzymes.

REFERENCES

Dajani, R. M., Danielski, J., and Orten, J. M. (1963). The utilization of ethanol. *J. Nutrition* **80:** 196.

Deitrich, R. A. (1966). Tissue and subcellular distribution of mammalian aldehyde oxidizing capacity. *Biochem. Pharmacol.* **15:** 1911.

Erwin, G., and Deitrich, R. A. (1966). Brain aldehyde dehydrogenase. *J. Biol. Chem.* **241:** 3533.

Jacobsen, E. (1952). The metabolism of ethyl alcohol. *Pharmacol. Rev.* **4:** 107.

Lineweaver, H., and Burke, D. (1934). Determination of enzyme dissociation constants. *J. Am. Chem. Soc.* **56:** 458.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951). Protein measurement with the folin phenol reagent. *J. Biol. Chem.* **188:** 265.

McClearn, G. E., and Rodgers, D. A. (1959). Differences in alcohol preference among inbred strains of mice. *Quart. J. Studies Alc.* **20:** 691.

McClearn, G. E., and Rodgers, D. A. (1961). Genetic factors in alcohol preference of laboratory mice. *J. Comp. Physiol. Psychol.* **54:** 116.

McClearn, G. E., Bennett, E. L., Hebert, M. J., Kakihana, R., and Schlesinger, K. (1964). ADH activity and previous ethanol consumption in mice. *Nature* **303:** 793.

McClearn, G. E. (1967). Genes, generality, and behavioral research. In *Behavioral Genetic Analysis.* Edited by Jerry Hirsch. McGraw-Hill, New York, Chap. 15, p. 307.

Racker, E. (1949). Aldehyde dehydrogenase, a diphosphopyridine nucleotide-linked enzyme. *J. Biol. Chem.* **177:** 883.

Rodgers, D. A., McClearn, G. E., Bennett, E. L., and Hebert, M. J. (1963). Alcohol preference as a function of its caloric utility in mice. *J. Comp. Physiol. Psychol.* **56:** 666.

Schlesinger, K., Kakihana, R., and Bennett, E. L. (1966). Effects of tetraethylthiuramidisulfide (antabuse) on metabolism and consumption of ethanol in mice. *Psychosomat. Med.* **28:** 514.

Schneider, W. C., and Hogeboom, G. H. (1950). Intracellular distribution of enzymes. *J. Biol. Chem.* **183:** 123.

Theorell, H., and Bonnichsen, R. K. (1951). Studies on liver alcohol dehydrogenase I. *Acta Chem. Scand.* **5:** 1105.

Westerfeld, W. W. (1955). Metabolism of alcohol. *Texas Rept. Biol. Med.* **13:** 559.