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Ethanol Consumption and Hepatic Enzyme Activity[1]

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ANDERSON, S. M., G. E. McCLEARN AND V. G. ERWIN. *Ethanol consumption and hepatic enzyme activity.* PHARMAC. BIOCHEM. BEHAV. 11(1) 83–88, 1979.—Enzyme activity and ethanol consumption were measured in an F_2 generation derived from the C57BL and C3H inbred mouse strains. A significant correlation (0.25) was found between alcohol dehydrogenase activity and ethanol acceptance in the F_2 generation. Mass selection from a genetically heterogeneous mouse stock, HS/Ibg, has yielded high ethanol acceptance (HEA) and low ethanol acceptance (LEA) lines of mice. The mean ethanol acceptance scores for the fifth generation of these lines are 1.008 and 0.606, respectively. The total liver alcohol dehydrogenase activity was found to be 24% higher in the HEA line than in the LEA line after five generations of selective breeding. No association between cytosolic aldehyde dehydrogenase activity and ethanol acceptance was found in either the F_2 generation or the fifth generation of the selectively bred lines.

Alcohol consumption Alcohol dehydrogenase Aldehyde dehydrogenase Selection

EVIDENCE of genetic influences on voluntary alcohol consumption by experimental animals has been reported by many laboratories [2, 21, 31, 45]. Studies of mice from inbred strains drinking ethanol in a two-bottle choice situation have shown that mice of the C57BL strain consume substantial amounts of alcohol but BALB/c, A and DBA/2 mice choose water almost exclusively [13, 25, 36]. Results from classical Mendelian crosses of inbred strains of mice and selective breeding experiments with rats demonstrate that voluntary alcohol consumption is a polygenic trait with low heritability estimates [9, 26, 44].

Attempts to link variability in rodent alcohol drinking behavior to metabolic differences have focused on the hepatic alcohol catabolizing enzymes alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH). These enzymes eliminate 75–90% of ingested ethanol from the body. Although catalase and the microsomal ethanol oxidizing system (MEOS) can oxidize ethanol to acetaldehyde *in vitro* recent evidence indicates that hepatic ADH is the principle enzyme responsible for the conversion of ethanol to acetaldehyde *in vivo* [4, 16, 43]. The NAD-dependent ALDH's in the liver catalyze the conversion of acetaldehyde to acetate, which is the second step in the catabolism of ethanol [8,19].

Inbred mouse strain differences in liver ADH activity measured *in vitro* have been shown to parallel strain differences in alcohol preference [32]. Studies have found that the alcohol-preferring C57BL mice have higher liver ADH activity than the moderate alcohol consuming C3H and alcohol avoiding DBA/2 and CBA mice [10, 32, 35, 37, 46]. Measurements of hepatic ALDH activity also demonstrate large differences between the C57BL and DBA/2 inbred mouse strains [38]. However, fortuitous associations between traits may be developed within strains during inbreeding and studies in genetically heterogeneous populations are necessary in order to demonstrate relationships between two traits.

In a correlation study using a genetically heterogeneous population which was developed by systematic crossbreeding of inbred strains [28] 10% of the variance in alcohol preference was related to differences in levels of ADH activity [23]. Another experimental approach used to investigate the relationship between ethanol consumption and enzyme activity was selective breeding. After three generations of selection for high and low alcohol preference in mice the high preference line had higher ADH levels than the low line. Unfortunately, low fertility caused a premature termination of that project [23].

A brief test of voluntary alcohol consumption called "alcohol acceptance under thirst motivation" was introduced by McClearn [22]. The similarity in rank ordering of inbred strain means for alcohol preference and for alcohol acceptance under thirst motivation suggests that these are correlated measures. Two divergent lines for ethanol acceptance under thirst motivation are being developed by selective breeding at the University of Colorado. Mass selection has produced two lines of mice (HEA/Ibg and LEA/Ibg)

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which continue to diverge in their ethanol acceptance scores. (A detailed discussion of the selective breeding project can be found in McClearn and Anderson, *Behavior Genetics*, in press.) The main objective of this study was to determine whether differences in alcohol acceptance under thirst motivation, measured in two different genetically segregating populations of mice, are related to variation in levels of hepatic ethanol metabolizing enzyme activities.

EXPERIMENT 1

METHOD

Alcohol acceptance under thirst motivation, ADH, ALDH, ethanol acceptance and alcohol preference were measured in a genetically segregating F_2 generation derived from inbred strains.

Animals

All animals were obtained from the breeding colony at the Institute for Behavioral Genetics, University of Colorado. The animals were maintained under standard laboratory conditions with food and water available *ab lib* and were experimentally naive prior to the studies described here. Twenty-five males and 25 females of an F_2 generation derived from crossbreeding the C57BL/Ibg and C3H/Ibg inbred strains were tested for alcohol preference at 60 ± 5 days of age. At age 120 ± 5 days these same mice were tested for ethanol acceptance under thirst motivation. Between testing sessions for drinking behavior animals were maintained under standard laboratory conditions. Measurements of hepatic ADH and ALDH activity were made on these subjects at 190 ± 9 days of age. All the testing and assays were conducted as described below.

Alcohol Preference Test

Animals were placed in individual stainless steel cages (25×13×10 cm) above which were suspended two 25 ml graduated cylinders with ball bearing sipper tubes extended through the cage tops. One cylinder contained tap water and the other a solution of 10% ethanol in tap water. Daily readings were taken of fluid consumption for a period of fifteen days with the cylinders being interchanged every third day to eliminate position effects. The daily alcohol preference score was recorded as the ratio of alcohol consumed to total daily fluid intake. The alcohol preference ratio for the entire experiment is the mean for the fifteen day period.

Ethanol Acceptance Under Thirst Motivation

For many experimental purposes, a test shorter than the usual two-week preference test would obviously be desirable. A brief version of the standard preference procedure does not serve well because several days are required for the development of preference by C57BL mice. It seemed possible, however, that motivation provided by a brief period of fluid deprivation might yield satisfactory results [22]. In the present study we used a short test of alcohol consumption after a brief period of fluid deprivation (total duration of testing—4 days).

Animals were caged as described above but with only one cylinder of tap water. Water consumption was recorded for two days followed by 24 hr of fluid deprivation. The next day the animals were given 10% ethanol in a graduated cylinder as their only source of fluid. The amount of ethanol solution consumed was recorded 24 hr later. The ethanol acceptance score is defined as the ratio of ethanol consumed to the average daily water intake.

Enzyme Activity Determination

Subjects were removed from their home cages, weighed and immediately sacrificed by cervical dislocation. The liver was quickly removed, washed in 15 ml of ice cold sucrose (0.25 M), weighed and homogenized in 9 volumes (w/v) of fresh ice cold sucrose (0.25 M) in a pyrex tissue grinder with a teflon pestle attached to a power source. One ml of homogenate was considered equivalent to 100 mg of tissue. Samples were then centrifuged at 40,000×G for 1 hr in a Sorvall refrigerated centrifuge, model number RC 2-B. The fresh supernatant fluid was kept in an ice bath prior to the enzyme assays and protein determination.

The alcohol dehydrogenase activity was assayed using a variation of the method of Theorell and Bonnichsen [42]. The reaction mixture contained 2.5 ml 0.06 M glycine/NaOH buffer at pH 9.6 with 0.075 M semicarbazide, 0.2 ml NAD^+ (10 mg/ml), 0.1 ml supernatant fluid, 0.1 ml water and 0.1 ml ethanol (0.3 M). Alcohol dehydrogenase activity was measured as the change in optical density (OD) at 340 nm (NADH production) for 10 min at 20°C in a Gilford spectrophotometer, model number 2400, with a 1 cm light path using 3 ml quartz cuvettes. Endogenous activity as measured by blanks containing 0.1 ml pyrazole (0.03 M) instead of water was subtracted from the change in OD observed in the reaction mixture. Duplicate assays were performed for both the reaction mixture and blank. The initial reaction velocity was used to calculate the enzyme activity.

Supernatant aldehyde dehydrogenase activity was assayed by the method of Racker [30] with the addition of pyrazole to prevent contamination by alcohol dehydrogenase activity [5]. A typical reaction mixture contained 2.5 ml of 0.1 M pyrophosphate buffer at pH 9.6, 0.2 ml NAD^+ (10 mg/ml), 0.1 ml pyrazole (0.03 M), 0.1 ml supernatant fluid, and 0.1 ml propionaldehyde (22 mM). Endogenous activity as measured by blanks containing water in lieu of the substrate, propionaldehyde, was subtracted from the change in OD observed in the reaction mixture. Initial rates of NADH production were measured spectrophotometrically as in the alcohol dehydrogenase assay. The protein content of the supernatant fluids was determined by the Biuret method [14] with the mean value of duplicate measures at OD at 450 nm being used in the data calculations.

RESULTS AND DISCUSSION

Significant Pearson Product Moment correlations calculated among all experimental measures are presented in Table 1. The correlation between ADH activity and ethanol acceptance scores indicates that approximately 7% of the variance in ethanol acceptance scores in this population is associated with variation in ADH activity. Although the correlation between ADH and ethanol consumption in the F_2 generation, derived from C57BL and C3H strains is lower than would be expected from the high correspondence between the means of these two measures among inbred strains, these data confirm results of a previous study on alcohol preference using another genetically segregating mouse population, the HS/Ibg strain [23], and are consistent

TABLE 1

PEARSON PRODUCT MOMENT CORRELATION COEFFICIENTS BETWEEN EXPERIMENTAL MEASUREMENTS OF AN F2 GENERATION DERIVED FROM A CROSS OF THE C57BL AND C3H INBRED MOUSE STRAINS (MALES AND FEMALES COMBINED, N=50)

	1.	2.	3.	4.	5.	6.	7.	8.	9.
1. Body weight	—	0.65	0.04	−0.29	0.33	0.27	0.41	−0.27	−0.47
2. Liver weight		—	0.06	−0.11	0.59	0.63	0.80	0.01	−0.33
3. mg protein/g liver			—	−0.26	−0.06	−0.18	−0.03	0.20	0.06
4. ADH/g liver				—	0.24	0.69	0.14	0.25	0.19
5. Cystosolic ALDH/g liver					—	0.64	0.94	−0.08	−0.14
6. Total liver ADH						—	0.71	0.16	−0.11
7. Total liver cystosolic ALDH							—	−0.04	−0.20
8. Ethanol acceptance								—	0.27
9. Alcohol preference									—

Correlation coefficients > 0.23 are significant at $p<0.05$

with evidence for the polygenic nature of measurements of voluntary alcohol consumption [9,26].

This correlational analysis involves measurement of alcohol consumption and enzyme activities in the same individual. Procedures such as this are sometimes complicated by possible effects of experimental experience, in that measurement of the first trait may influence the second trait. Increases in ethanol oxidation reported in rats after chronic ethanol consumption have been demonstrated to be related to metabolic factors exclusive of ADH activity [4]. The conflicting evidence concerning increases in liver ADH activity and liver ALDH activity in rodents ingesting low levels of ethanol [2, 6, 15, 27, 40] may be attributable to differences in: (1) duration of ethanol exposure, (2) animal maturity, and (3) amount of time elapsed between ethanol exposure and sacrifice of the animal. Although an increase in ADH activity was demonstrated in mice following a two-week period of forced alcohol consumption, the enzyme activity returned to base line level after a three-week period of water consumption [27]. Alteration of enzyme activity due to previous ethanol ingestion seems unlikely in this report because sacrifice of the subjects for enzyme assay was delayed until two months after termination of ethanol ingestion.

The low correlation (r=0.27) found between ethanol acceptance and alcohol preference indicates that these tests are not equivalent. Although they have some parameters in common, the difference between them is substantial. This small correlation between the ethanol acceptance score and the alcohol preference score within animals is surprising in view of the similarity of inbred strain mean rank ordering for these tests [22].

This discrepancy may be explained by the daily variation in alcohol consumption and the low reliability of these measures. Alcohol consumption within individual animals is subject to daily fluctuations and graphs of such data present an erratic picture [24]. When measuring consumption in groups of animals this daily variation in individual scores will not be apparent from the group means, but when calculating a statistic based on deviations of individual scores from a group mean these fluctuations will be important. Similarly, when recording a mean score for two weeks of testing daily fluctuations will balance out, but the ethanol acceptance test is too brief for that compensatory effect. For example, in this sample of mice only about 40% of the variance in 15 day alcohol preference scores can be accounted for by variation in first day preference scores (r=0.63). The population means for the first day preference score and the 15-day preference score, however, are equivalent, 26.5% and 26.0%, respectively.

EXPERIMENT 2

METHOD

The purpose of this experiment was to determine if five generations of selective breeding for alcohol acceptance under thirst motivation produced significant differences in hepatic enzyme activities between the two divergent lines of mice. Although the distribution of the ethanol acceptance scores of the fifth generation of the HEA/Ibg and LEA/Ibg lines overlap, the population means are significantly different ($t=9.2$: see Fig. 1). After five generations of selective breeding the estimate of the realized heritability, h^2, is 0.18 ± 0.04, $p<0.025$ [39]. This estimate of the realized heritability from the divergent selection experiment is calculated from the regression of cumulative response on cumulative selection differential [11]. The estimation of the error of h^2 is obtained from the variance of the regression coefficient which is calculated from the experimental data [17].

Animals

All animals were obtained from the breeding colony at the Institute for Behavioral Genetics, University of Colorado. The animals were maintained under standard laboratory conditions. Twelve males and 12 females (155 ± 5 days old), who were untested siblings of animals tested in the fifth selected generation, from both the HEA/Ibg and LEA/Ibg lines, were weighed, sacrificed and their livers assayed for hepatic ADH and ALDH activities. The assay procedures are identical to those described for Experiment 1.

RESULTS AND DISCUSSION

A comparison of the means of the experimental measurements for HEA/Ibg and LEA/Ibg animals is presented in Table 2. Significant differences between the two lines were found for liver weight and total ADH activity. If a proportion of the variance in ethanol acceptance under thirst motivation

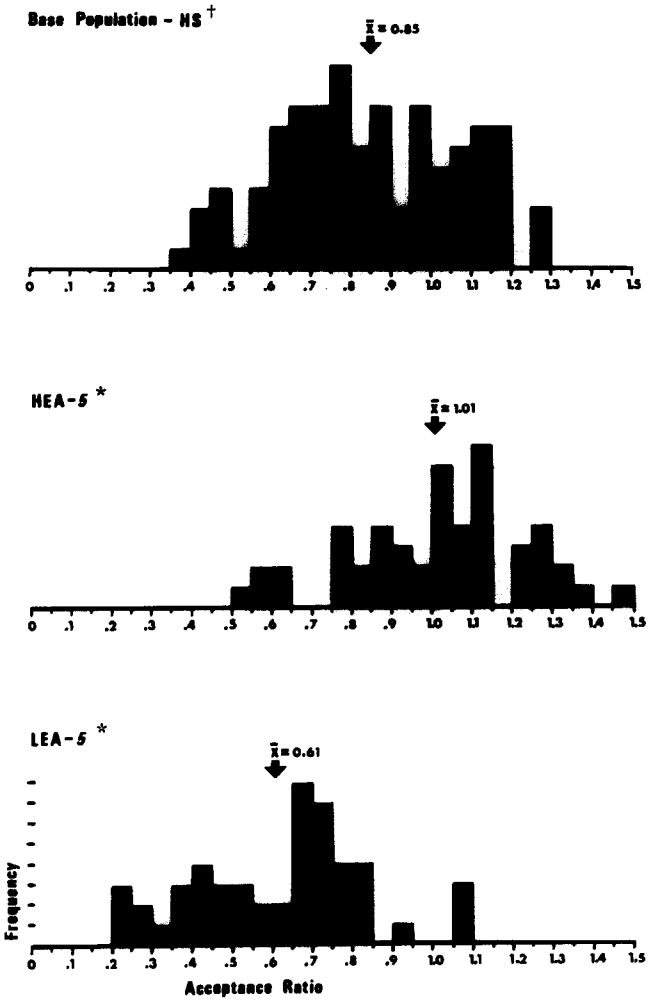

FIG. 1. Alcohol acceptance scores from the HS base population and the fifth selected generation of high and low ethanol acceptance lines. †n=51 males and 49 females: *n=25 males and 25 females.

TABLE 2

MEANS AND STANDARD ERRORS OF EXPERIMENTAL MEASUREMENTS FOR THE FIFTH SELECTED GENERATIONS OF THE HIGH AND LOW ETHANOL ACCEPTANCE LINES

Measure	HEA/Ibg-5 (N = 24)	LEA/Ibg-5 (N = 24)
Body weight - (g)	33.03 ± 1.33	30.32 ± 0.65
Liver weight - (g)	1.88 ± 0.07	1.63 ± 0.04‡
mg protein/g liver	1.29 ± 0.04	1.28 ± 0.05
ADH/g liver*†	1.58 ± 0.05	1.48 ± 0.05
Cytosolic ALDH/g liver*†	1.01 ± 0.04	1.00 ± 0.03
Total liver ADH*	2.96 ± 0.14	2.40 ± 0.09§
Total liver cytosolic ALDH*	1.96 ± 0.16	1.64 ± 0.07

*Enzyme activity is expressed as μmoles/min.

†Because there is no difference in the liver protein concentration of the two lines of mice, enzyme activities expressed per g liver, parallel specific activities.

‡$p<0.01$

§$p<0.005$

can be accounted for by differences in the activity of ethanol metabolizing enzymes, we expect to see some difference in the ADH and/or ALDH activities in divergent lines selected for ethanol acceptance. The mean liver cytosolic ALDH activity of the first generation low ethanol acceptance line (LEA-1) is 14% higher ($p<0.05$) than the mean for the first generation of the high ethanol acceptance line (HEA-1) [3]. If this difference is correlated with the selection criterion, ethanol acceptance under thirst motivation, we would expect liver cytosolic ALDH activity to parallel further divergence of the mean ethanol acceptance scores in later generations of the two selectively bred lines. Data from the fifth generation suggest that the first generation line mean difference in cytosolic ALDH activity was due to random variation between the two lines and is not related to ethanol acceptance.

The functional importance of subcellularly localized forms of ALDH in the mouse liver has not been elucidated. However, recent demonstrations of numerous forms of ALDH in rat liver suggest that the acetaldehyde generated during ethanol metabolism is oxidized to acetate predominantly in mitochondria [19,29].

After five generations of selection the HEA mice were found to have livers that weighed 15% more than livers of LEA mice ($p<0.01$). Although not statistically significant for a sample of this size, ADH activity per gram liver was found to be 7% higher in the HEA-5 line than in the LEA-5 line. These two differences combined to produce a 24% higher level of total ADH activity in the HEA-5 than in LEA-5 ($p<0.005$).

GENERAL DISCUSSION

Alcohol preference, assessed in a two-bottle choice test, is frequently used as a measure of voluntary alcohol consumption in laboratory animals. Inbred strains of mice studied in other tests of alcohol consumption have been found to have their means rank ordered similarly to their rank ordering for alcohol preference [22]. In the present study we found a low correlation between alcohol preference and ethanol acceptance under thirst motivation when both traits were measured in a genetically hetereogeneous population. A test of voluntary consumption after a brief period of fluid deprivation is obviously not equivalent to an ad lib choice between water and alcohol. Scattergrams of data points of the F_2 generation used in this study comparing these two measures, however, demonstrate that animals which have high preference scores also have high acceptance scores and subjects with low acceptance scores are extreme alcohol nonpreferrers. Voluntary alcohol consumption can be thought of as a complex behavior involving many different parameters. Only some of these variables are commonly shared between the measurements of alcohol preference and ethanol acceptance under thirst motivation.

In rat strains selectively bred for divergent alcohol consumption by Eriksson [9] the ANA strain which has low voluntary ethanol consumption was found to have higher ADH activity per gram liver: lower mitochondrial ALDH activity per gram liver [18]: slower reduction in blood ethanol concentration and higher levels of acetaldehyde in the blood and liver after IP injections of ethanol [7, 8, 12]. Although data on total liver ADH activity, which may be more relevant to *in vivo* metabolism, are not published these data suggest that the difference in alcohol consumption between the ANA and AA rat strains is related to variability between the strains in alcohol metabolic rate. The corre-

spondence between the accumulation of acetaldehyde and low alcohol consumption suggested by the metabolic differences between the selectively bred ANA and AA rat lines is consistent with: (1) lower liver ALDH activity and higher blood and brain acetaldehyde levels after ethanol injections in the alcohol avoiding DBA/2 inbred mouse strain [36, 38, 41]; (2) the correspondence between ethanol consumption and ALDH activity in the livers and brains of Wistar rats [1,2]; and (3) a reduction in voluntary ethanol consumption by inbred mice after the administration of tetraethylthiuramdisulfide (Antabuse), an inhibitor of ALDH activity [34].

Although no differences in specific activity of either ADH or ALDH were found between the P (alcohol-preferring) and NP (alcohol-nonpreferring) selectively outbred and inbred rats a higher rate of ethanol oxidation measured in isolated hepatocytes; an apparently more rapid decrease in blood ethanol concentration after ethanol injections and the higher body weight of females from the P strain suggest a greater metabolic capacity for alcohol catabolism in the alcohol-preferring P strain [20]. Higher levels of total liver ADH activity (which may accompany the body weight differences between the NP and P females) would be consistent with the data on the selectively bred HEA and LEA mouse lines presented in this study.

The possible importance of metabolic capacity in determining alcohol consumption suggested by the larger liver size and higher liver to body weight ratio in the high ethanol acceptance line is consistent with previous evidence of a parallel between increased liver weight and greater ethanol consumption in lactating C57BL mice [33]. Measurements of hepatic ADH and ALDH activity in future selected generations of these divergent lines will provide further evidence for the role of these enzymes in the physiological mechanisms involved in voluntary alcohol consumption. A comparison of the three selectively bred divergent lines for alcohol consumption in rodents presented in this discussion is complicated by species differences, diversity in the alcohol-related phenotypes studied and variability in the method of expressing enzyme data. In spite of these problems, the combined results from these studies suggest that voluntary consumption of ethanol by rats and mice is related to differences in the metabolism of alcohol. The diversity of mechanisms revealed through the phenotypes of the three different selection projects illustrates the numerous parameters in the physiology of alcohol.

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