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Interrelationships of Alcohol Consumption, Actions of Alcohol, and Biochemical Traits[1]

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ERWIN, V. G., G. E. McCLEARN AND A. R. KUSE. *Interrelationships of alcohol consumption, actions of alcohol, and biochemical traits.* PHARMAC. BIOCHEM. BEHAV. 13: Suppl. 1, 297–302, 1980.—Voluntary alcohol consumption, acute tolerance, and central nervous system (CNS) sensitivity to ethanol are potentially informative measures concerning human alcoholism. Little is understood regarding the associations among these parameters or between these traits and neurochemical processes such as brain protein or brain enzyme activities. A powerful strategy is to assess a large number of characteristics simultaneously on all individuals of a heterogeneous sample. This permits rapid screening of a large number of variables with respect to their interrelationships. Identification can thus be made of those variables that are elements of the causal nexus, and subsequent experimental research can attack the problem of identifying mechanisms. The present study employed mice from the HS/Ibg stock which is maintained by systematic random mating to assure genetic heterogeneity. The results demonstrate that voluntary ethanol consumption and acquisition of acute tolerance to ethanol were positively associated, whereas these measures were not significantly related to CNS sensitivity to ethanol. In addition, ethanol preference was inversely related to soluble brain protein. The activities of the soluble enzymes from brain, aldehyde reductase and glucose-6-phosphate dehydrogenase, were not significantly associated with ethanol preference, acquisition of acute tolerance, or CNS sensitivity to ethanol. Unexpectedly, more than 30 percent of the variance in voluntary alcohol consumption could have been predicted from the measurements of acquisition of acute tolerance, and vice versa.

Ethanol preference Acute tolerance CNS sensitivity to alcohol Brain aldehyde reductase
Brain glucose-6-phosphate dehydrogenase Associations between ethanol preference and actions of alcohol

ETHANOL is known to exert marked depressant actions on motor processes and to lead to motor incoordination and loss of balance [9]. It has also been shown that inbred strains [2] and selected lines [6] of mice differ in brain sensitivity to ethanol, and various inbred strains of mice have been shown to differ markedly in preference for alcohol [17].

It is also well established that administration of alcohol results in the development of acute tolerance [11] and acquisition of acute tolerance to ethanol, utilizing a motor coordination paradigm, was recently demonstrated for inbred strains of mice [7]. The relationship between central nervous system (CNS) sensitivity and acquisition of acute tolerance to alcohol has not been systematically investigated, nor has the relationship been explored between these actions of alcohol and alcohol preference or voluntary alcohol consumption.

A number of neurochemical processes have been postulated to be involved in the effect of ethanol on the CNS and in ethanol preference. The proposed systems include nerve cell membrane composition and order [3] and neurotransmitter amine metabolism [1, 5, 15]. However, the relationships of these neurochemical parameters to the actions of ethanol or to voluntary alcohol consumption have not been elucidated. Therefore, the present research was performed to determine the associations among the above alcohol phenotypes and some specific neurochemical parameters.

The experimental paradigm, involving manipulation of an independent or treatment variable with concomitant or subsequent assessment of a dependent or outcome variable, has been the dominant approach in animal studies of alcohol actions with little attention being given to measures of association. The basic parametric statistics for studying associations are, of course, the regression and correlation coefficients. A contrast is often made between these measures of association and the hypothesis testing t or F statistics in that causation is indicated only by the latter. However, correlations can be of great value in suggesting hypotheses that may be testable by subsequent experiment. Furthermore, there may arise situations in which experimental manipulation of a variable is either technically impossible or "dirty" in the sense that the manipulation is thought to have many diverse confounding effects. In these circumstances advantage may be taken of naturally occurring variation in the putative causal and putative effect variables to determine if they covary. Correlational studies thus permit the study of patterns of interrelationship that might be quite impractical to assess by analysis of variance designs.

METHOD

Reagents and chemicals utilized in these studies were of the highest purity obtainable from commercial sources;

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p-nitrobenzaldehyde was purchased from Aldrich Chemical Company; NADP, NADPH, and glucose-6-phosphate were supplied by Sigma Chemical Company. Ethanol utilized in these studies was 95% USP grade obtained from Mallinckrodt, Inc.

Enzyme and Ethanol Assays

Two weeks after the final ethanol administration, animals were sacrificed by decapitation and within 30 to 60 sec brains were removed and placed in ice-cold 0.01 M sodium phosphate buffer, adjusted to pH 6.8 with 0.1 M sodium hydroxide. Homogenization of brains and centrifugation of the homogenates were conducted at 0° to 4°C. Brains were blotted on Whatman filter paper, weighed, and homogenized in 4 volumes of 0.01 M sodium phosphate buffer, pH 6.8. The brain homogenates were centrifuged at 100,000 g for 60 min in a Beckman LS50 ultracentrifuge; the resulting supernatant fluid was removed and aliquots were taken for enzyme assays and protein determinations. The resulting membrane and insoluble protein pellets were resuspended in the original volume of 0.01 M sodium phosphate buffer, pH 6.8, and aliquots were taken for protein determinations. Protein concentrations were determined by the Biuret method using bovine serum albumin as a standard [8].

Aldehyde reductase activity was determined as previously described [18] utilizing p-nitrobenzaldehyde (0.5 mM) and NADPH (0.05 mM) in a 2-ml reaction mixture containing 0.01 M sodium phosphate buffer, pH 6.8, at 30°C. Reaction velocities were estimated from the rates of NADPH disappearance and were linear up to 5 min with protein concentrations used in these experiments (0.2 to 0.4 mg protein). Assays were performed with a Beckman model 25 recording spectrophotometer at 340 nm. Glucose-6-phosphate (G-6-P) dehydrogenase activity was determined by standard spectrophotometric methods [4]. Glucose-6-phosphate (1 mM) and NADP (1 mM) were added with enzyme (0.05 to 0.1 mg protein) to a 2.0-ml reaction mixture containing 0.01 M sodium phosphate buffer, pH 6.8. Rates of NADPH formation at 30°C were determined as described above.

Blood samples were taken by retro-orbital sinus technique [16] utilizing 40 μl microcapillary tubes (MICROCAPS). The blood samples were immediately placed in 1 ml of 3 percent perchloric acid and aliquots were taken for estimation of ethanol concentrations utilizing a spectrophotometric procedure [10]. Blood ethanol levels were then calculated based upon a standard curve obtained from a similar procedure with varying concentrations of ethanol added to blood samples.

Voluntary Ethanol Consumption

The voluntary ethanol consumption was described by two indices, one being the mean ethanol preference ratio over a 15-day preference test [17], the other being the quantity of ethanol consumed in g per kg per day. Each animal was individually caged and was given free choice of a bottle containing tap water or a bottle containing 10% v/v ethanol in tap water. The volume of water and ethanol consumed was recorded daily and the position of the bottles was alternated every third day to balance out position effect. The preference ratio is defined as the total volume of 10 percent ethanol solution intake divided by the total intake (total 10 percent ethanol solution plus total water).

CNS Sensitivity

The initial CNS sensitivity of each animal to ethanol was determined as previously described [7]. In these studies ethanol was administered at 2 g per kg, intraperitoneally, and after approximately 15 min the animals were unable to balance themselves on a fixed 1/2-inch-diameter horizontal wooden rod. Immediately prior to ethanol administration each mouse had been trained through several trials to remain on the horizontal rod for a minimum of 3 min. Animals were tested for their ability to regain balance on the horizontal rod with the criterion of remaining on the rod for 1 min. At this time, defined as T_1, a 40-μl blood sample was taken by the retro-orbital sinus bleeding technique and blood ethanol determined as described above. The blood ethanol level at this initial regaining of balance endpoint was defined as the initial CNS sensitivity, and a value was obtained for each animal.

Acute Tolerance

Immediately after the blood sample was taken at T_1, animals were injected with a second dose of ethanol (1 g per kg). Following this administration, the animals again lost their ability to balance on the horizontal rod, and each animal was then repeatedly monitored until it was able to maintain its balance for 1 min. Following this second regaining of balance end-point (T_2), a second blood sample was drawn and the blood ethanol level determined. The difference in blood ethanol levels between T_2 and T_1 was taken as an index of the acquisition of acute tolerance [7] (in the case of a positive number) and as an acquisition of increased CNS sensitivity (in the case of a negative number). For convenience, the index will simply be described as "acute tolerance." The statistical reliability of this measure was determined with 18 male and female DBA/Ibg mice and was found to be quite satisfactory ($r=0.87$, $p \leq 0.001$). The reliability value for the CNS sensitivity index was $r=0.63$, $p \leq 0.006$.

Genetically Heterogeneous Mice

In a correlational study inbred strains of mice can provide only limited information because their variance is exclusively environmental in origin. Systematically maintained genetically heterogeneous stocks are the material of choice in correlational research [12]. Thus, mice of the genetically heterogeneous population, HS/Ibg, from the Institute for Behavioral Genetics at the University of Colorado, were employed. The development of this HS/Ibg stock from inter-crossing 8 inbred strains of mice and its subsequent maintenance have been reported previously [14]. In the present study, each mouse was separately caged and maintained prior to and throughout the experiments in animal quarters with constant temperature (70 ± 2°F), humidity (50 ± 5%), and air flow. Both male and female mice in equal numbers were used in this study, and the preference experiments were initiated at an age of 49 to 50 days. The age of the mice at the time of initial CNS sensitivity and acute tolerance tests ranged from 80 to 85 days, and the age at the time of sacrifice ranged from 143 to 150 days.

Partial Correlations

The method of partial correlations [19] was chosen to analyse the data. This procedure provides a description of an association between two variables, while adjusting for concomitant linear effects of one or more additional variables.

TABLE 1

CORRELATIONS OBTAINED IN A PRELIMINARY STUDY WITH 20 HS/IBG MICE

	1.	2.	3.	4.	5.
1. Preference ratio	—	0.47*		−0.42*	−0.67*
2. Acute tolerance†	0.47*	—		−0.51*	−0.63*
3. CNS sensitivity‡	−0.22	−0.35	—		
4. Brain aldehyde reductase§	−0.42*	−0.51*	0.35	—	
5. Soluble brain protein¶	−0.67*	−0.63*	−0.12	0.30	—

Values are partial correlation coefficients with the effects of sex, age, and weight removed. All values are shown below the diagonal, but to assist the reader significant values (*$p \leq 0.05$) are repeated above the diagonal.

†Acute tolerance was defined as the difference in g ethanol per dl of blood between initial and second regaining of balance (see Method).

‡CNS sensitivity was the blood ethanol observed in g per dl at initial regaining of balance (T_1).

§Enzyme activities were expressed as absorbance (A) change at 340 nm per min per mg protein.

¶Soluble brain protein was expressed as mg protein per ml.

TABLE 2

MEAN VALUES OF VARIABLES USED IN CORRELATIONS OF ETHANOL ACTIONS

Variable	Mean	Standard deviation	n
Preference ratio	0.1170	0.0393	49
Ethanol consumption*	2.4915	0.9504	49
CNS sensitivity†	0.1645	0.0492	48
Acute tolerance‡	0.0719	0.0575	48
Soluble brain protein§	6.8812	0.6709	48
Brain aldehyde reductase¶	0.0177	0.0019	48
Brain G-6-P dehydrogenase¶	0.1480	0.0213	48
Insoluble brain protein§	28.6958	2.2739	48

Values represent means and standard deviations for the indicated number of HS/Ibg mice. Methods of measurement of each variable in each mouse are described in the Method and Procedure section.

*Ethanol consumption is defined as g ethanol per kg per 24 hr.

†CNS sensitivity is the blood ethanol observed in g per dl at initial regaining of balance (T_1).

‡Acute tolerance is defined as the difference in g ethanol per dl of blood between initial and second regaining of balance (see Method).

§Soluble and insoluble brain proteins are expressed as mg protein per ml.

¶Enzyme activities are expressed as absorbance (A) change at 340 nm per min per mg protein.

The use of partial correlations minimizes the possibility of "spurious" associations. That is, two variables may appear to be associated only because they are both correlated with a third, more basic, variable. Thus, although causality cannot be directly ascertained with correlational techniques, the methods allow the investigation of conditional associations between variables of interest.

In this study, it was quite possible that some or all of the observed association among the variables of interest might have been due to effects of age, sex, or body weight. To assure that these factors would not artificially enhance levels of association, correlations were calculated controlling for the effects of age, sex, and body weight at the time of testing.

Procedures

Two independent correlational studies were conducted and are referred to hereafter as Study 1 and Study 2. Study 1 was performed with 20 HS/Ibg mice (10 males and 10 females) and was considered a pilot study since the sample size was not large enough to provide satisfactory statistical analyses. Consequently, a replicate experiment was conducted with 48 HS/Ibg mice (24 males and 24 females). In addition to replicating all of the measures of ethanol actions and biochemical parameters in Study 1, Study 2 included determinations of alcohol consumption as well as ethanol preference and G-6-P dehydrogenase activity as well as aldehyde reductase activity. Obviously all enzyme and neurochemical systems relevant to the actions of ethanol could not be measured in this study. However, concentrations of soluble and insoluble brain proteins and activities of the enzymes, aldehyde reductase and G-6-P dehydrogenase, were determined. Aldehyde reductase was chosen because of its role in the degradation pathway for norepinephrine [18], and G-6-P dehydrogenase was measured as an important, well-known cytosolic enzyme associated with brain energy metabolism.

RESULTS

Table 1 presents partial correlations from Study 1 among ethanol preference, acquisition of acute tolerance, CNS sensitivity to ethanol, soluble brain protein, and brain aldehyde reductase activity. It is of interest that preference and acute tolerance were significantly positively associated ($r=0.47$, $p \leq 0.05$), whereas neither of these measures was significantly related to CNS sensitivity. Both ethanol preference and tolerance measures were significantly inversely related to brain aldehyde reductase ($r= -0.42$ and -0.51, respectively) and to soluble brain protein ($r= -0.67$ and -0.63, respectively).

The mean values and standard deviations for the variables used in Study 2 are presented in Table 2. Because they are derived from a systematically maintained heterogeneous stock, as distinct from inbred strains or haphazardly maintained stocks, these values may have some merit as norms.

TABLE 3

CORRELATIONS AMONG ETHANOL CONSUMPTION, CNS SENSITIVITY, TOLERANCE, AND OTHER VARIABLES (n=48)

	1.	2.	3.	4.	5.	6.	7.	8.
1. Preference ratio	—	*0.95*		*0.51*	*−0.46*		0.27*	
2. Ethanol consumption†	*0.95*	—		*0.61*	*−0.41*			
3. CNS sensitivity‡	−0.02	−0.07	—	−0.34*				
4. Acute tolerance§	*0.51*	*0.61*	−0.34*	—				
5. Soluble brain protein¶	*−0.46*	*−0.41*	0.01	−0.06	—	*−0.45*	*−0.68*	
6. Brain aldehyde reductase#	0.16	0.14	0.01	−0.02	*−0.45*	—	*0.47*	
7. Brain G-6-P dehydrogenase#	0.27*	0.11	0.01	−0.11	*−0.68*	*0.47*	-	
8. Insoluble brain protein¶	−0.08	−0.16	0.16	−0.15	0.01	−0.19	−0.15	—

Values are partial correlation coefficients with the effects of sex, age, and weight removed. All values are shown below the diagonal, but to assist the reader significant values ($p \leq 0.001$) in italics are repeated above the diagonal, and values with an asterisk are significant at $*p \leq 0.05$.

†Ethanol consumption is defined as g ethanol per kg per 24 hr.

‡CNS sensitivity is the blood ethanol observed in g per dl at initial regaining of balance (T_1).

§Acute tolerance is defined as the difference in gm ethanol per dl of blood between initial and second regaining of balance (see Method).

¶Soluble and insoluble brain proteins are expressed as mg protein per ml.

#Enzyme activities are expressed as absorbance (A) change at 340 nm per min per mg protein.

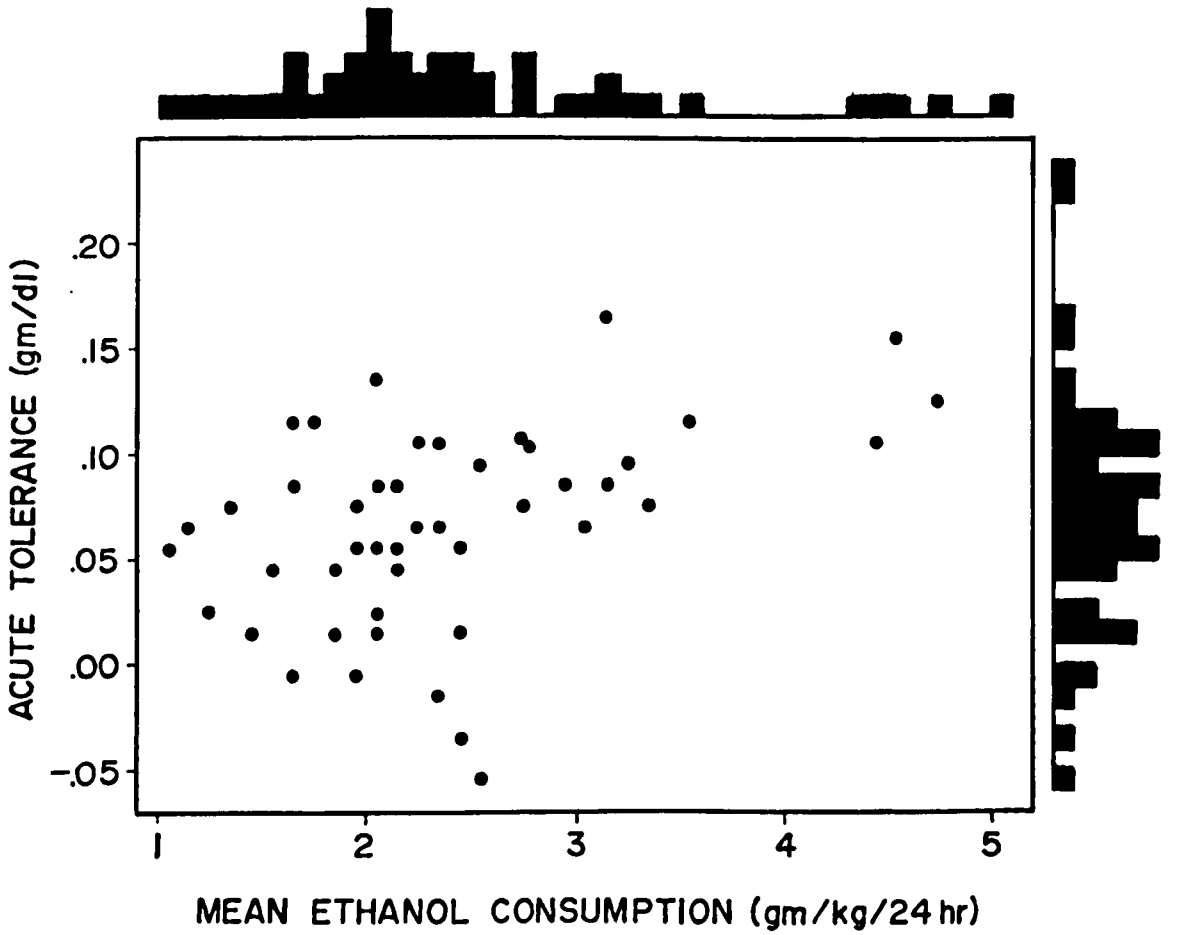

FIG. 1. Scattergram and Frequency Distributions for Acute Tolerance and Voluntary Ethanol Consumption. Methods for determining acute tolerance (g ethanol per dl blood) and voluntary ethanol consumption (g ethanol per kg per 24 hr) are described in the text. The frequency distributions represent the numbers of animals observed at the given values for acute tolerance and voluntary ethanol consumption.

The mean acute tolerance value (0.072 g per dl or 72 mg per dl) is of particular interest in that values ranged from −65, describing increased sensitivity, to 230 mg per dl, indicating acute tolerance.

Table 3 presents results of the correlational analysis of Study 2. The significant correlations include a partial correlation coefficient of 0.95 between the preference ratio and ethanol consumption expressed in g per kg per 24-hr period. This high correlation demonstrates the essential equivalence of the two measures. The significant ($p \leq 0.001$) correlations between preference and acute tolerance (r=0.51), between ethanol consumption and acute tolerance (r=0.61), and between preference (or consumption) and soluble brain protein (r=−0.46 and −0.41, respectively) replicate well the associations observed in Study 1. However, Study 2 results failed to replicate the associations between brain aldehyde reductase activity and acute tolerance or preference obtained in Study 1. These results demonstrate the value of replicate experiments in eliminating spurious associations.

The data in Table 3 also indicate that brain aldehyde reductase and brain G-6-P dehydrogenase, both of which require NADP or NADPH as cofactors, are positively associated (r=0.47) and that these enzymes are negatively associated with soluble brain protein (r=−0.45 and −0.68, respectively). The insoluble membrane protein obtained from brain was not significantly associated with any of the other parameters measured.

The correlation between acute tolerance and voluntary ethanol consumption is particularly interesting because the non-destructive nature of the measurements opens avenues for further research not only with animal models but also directly with human subjects. This correlation is displayed in the scatterplot of Fig. 1, together with frequency distributions of the two variables.

DISCUSSION

The data presented in this study demonstrate the extent to which voluntary consumption of ethanol, acquisition of acute tolerance to and CNS sensitivity to ethanol are associated in a genetically heterogeneous population of mice. Although it might be assumed *a priori* that such diverse actions of alcohol and the voluntary consumption of alcohol are controlled by separate mechanisms, these studies clearly demonstrate that, in mice, ethanol preference and voluntary alcohol consumption are not associated with initial CNS sensitivity to ethanol. This observation is important in that it demonstrates that these two parameters are mechanistically distinct. Another principal finding is the significant positive association between voluntary ethanol consumption and the acquisition of acute tolerance. It appears that as much as 30 to 35 percent of the variance in voluntary ethanol consumption may be predicted by the acquisition of tolerance, or vice versa. While these data do not suggest any causal relationship, they show that ethanol preference or voluntary ethanol consumption and the acquisition of tolerance may be influenced in part by the same causal nexus. Previous research on the genetics of alcohol preference [13] has demonstrated that voluntary ethanol consumption is influenced by a polygenic system. The data presented in this paper suggest that the mechanisms underlying preference and acute tolerance share a subset of the total polygenic system.

There is no immediate, obvious explanation for the replicable negative association between soluble brain protein concentrations and voluntary ethanol consumption. Whether this relationship is due to differences in concentrations of specific soluble proteins or to variations in levels of all soluble proteins remains to be determined.

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