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RELATIONSHIP BETWEEN ALCOHOL PREFERENCE AND BIOGENIC ALDEHYDE METABOLIZING ENZYMES IN RATS*

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Abstract—Rats were presented water and alcohol solutions [up to 30 per cent (v/v)] in a free choice screening test. High drinkers ingested the equivalent of *ca.* 6 g/kg · day of pure ethanol, at any concentration offered. Low drinkers consumed only *ca.* 2/kg · day, but stopped drinking alcohol solutions at concentrations higher than 7.5%. Males and females exhibited similar drinking habits. As measured by incubations of tissue slices and analysis of the components formed, high as well as low drinkers metabolize serotonin and dopamine at the same rate and produce identical metabolites. However, some small differences in the metabolism of these biogenic amines exist between males and females. The isozyme patterns of both aldehyde reductase (ALRed) and aldehyde dehydrogenase (ALDH) from the brain of high and low drinkers are identical. The levels of activity and isozymes of liver ALRed are the same, as are those of liver ALDH from the matrix of mitochondria which is responsible for the oxidation of acetaldehyde. Though the level of liver ALDH from the cytosol fraction is unaltered, the isozyme pattern is very different. Low drinkers always have three isozymes at pI 5.9, 6.0 and 6.2, while high drinkers have only one isozyme in this range, either at pI 5.9 or 6.2. Both animals have many more isozymes of the enzyme.

Though the effects of ethanol on the organism are well studied, little is known about factors which lead individuals to drink alcoholic beverages. One of the major effects of ethanol is to alter both biogenic amine levels and metabolism [1–4]. The first step in the metabolism of both alcohol and biogenic amines is an oxidation to the corresponding aldehydes, by alcohol dehydrogenase (alcohol:NAD oxidoreductase, EC 1.1.1.1, ADH) or monoamine oxidase (monoamine:O_2 oxidoreductase, EC 1.4.3.2, MAO) respectively. Aldehyde dehydrogenase (aldehyde:NAD oxidoreductase, EC 1.2.1.3, ALDH) further oxidizes these aldehydes to their respective acid derivatives. In the liver, multiple molecular forms of this last enzyme are present in cytosol, mitochondrial and microsomal fractions of the cell [5]; however, most of the oxidation of acetaldehyde takes place in mitochondria [6], where 3,4-dihydroxyphenylacetaldehyde (DOPAL) is also metabolized.† The specificity and the function of the cytosol and microsomal isozymes are not known.

Alternatively, some biogenic aldehydes are reduced to the alcohol form by aldehyde reductase (alcohol: NADP oxidoreductase, EC 1.1.1.2, ALRed). This enzyme is located mainly in the cytosol and also exists in multiple molecular forms [7]. In the presence of alcohol, some biogenic aldehydes which are usually oxidized are found to be reduced [1–4].

Interactions between ethanol and neurotransmitters could be involved in the biochemical mechanisms controlling alcohol abuse and/or addiction. Several authors have found slight variations of ADH and/or ALDH level in some subcellular liver fractions of different strains of rats [8, 9] and mice [10, 11] selected and bred for their alcohol preference. Eriksson [8] has shown that such strains of Wistar rats exhibit differences in the rate of metabolism of ethanol and acetaldehyde. The purpose of this study was to determine if any changes in the isozyme distribution of ALDH and/or ALRed were related to the observed responses of our animals with respect to their drinking habits.

MATERIALS AND METHODS

All chemicals were reagent grade and used, except when stated, without further purification. NAD and NADP were purchased from P-L Biochemicals, Inc. (Milwaukee, WI) and 4-methoxybenzyl alcohol from Aldrich Chemical Co. (Milwaukee, WI). Phenazine methosulfate, nitro blue tetrazolium and *p*-nitrobenzaldehyde were obtained from Sigma Chemical Co. (St. Louis, MO). Acrylamide, *N'*,*N'*-methylene-bis-acrylamide, ammonium persulfate and Photo-flo were products of Eastman Kodak Co. (Rochester, NY). Ampholines were purchased from LKB Instrument, Inc. (Bromma, Sweden), Panheprin was obtained from Abbott Laboratories (Chicago, IL), and all the other chemicals from Mallinckrodt Chemical Works (St. Louis, MO). Double-distilled water was used throughout this work, except for drinking purposes.

Animals

Both male and female Wistar rats (21-days-old, 50–60 g) were obtained from the closed breeding facilities of the Department of Biochemistry of Purdue. The colony contains at least fifty breeding pairs and

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no attempt has been previously made to prevent possible inbreeding. The animals were isolated in individual cages, at least 1 week prior to the first screening test. Anosmic animals were obtained by injection of aqueous 5% $ZnSO_4$ (w/v) in the nasal cavity as described by Alberts and Galef [12].

Screening

The selection of animals with respect to their alcohol drinking habits was performed with a three-bottle choice system, as described by Myers and Holman [13]. The concentration of alcohol was increased by 2.5% (v/v) for 12 consecutive days in a range from 2.5 to 30%. Food was available *ad lib.* The weight of animals was recorded on days 1 and 12 of the screening test.

Preparation of tissues

The blood was drained from the animal by perfusion of 0.9% NaCl solution (w/v) containing 0.025% Panheprin (v/v) which was injected into the blood stream through heart puncture, under ether anesthesia. The edges of the liver lobes were immediately clipped and the perfusion was performed until the perfusate was colorless. The liver was then removed, cooled below 5° on ice and sliced with a Stadie–Riggs microtome for the metabolism studies. Simultaneously, the brain tissues were chopped to *ca.* 1 mm^3 pieces and suspended in 4 vol. (w/v) of Krebs–Ringer buffer [14].

Metabolism studies

The metabolism of dopamine and serotonin were measured as described by Tank *et al.* [4]. The separation of the products from incubations with serotonin was performed by paper electrophoresis in 34.5 mM phosphate buffer, pH 6.5, followed by ascending chromatography in a mixture of isopropanol–NH_4OH–water (40:5:5), at 90° to the direction of electrophoresis.

Isoelectric focusing on polyacrylamide gels

The preparation of stock solutions, gels, samples and the procedures for focusing and staining were performed as modified based upon a procedure originally described by Vesterberg [15].

Preparation of samples. Brain and liver were removed from the animal in the 2 min after decapitation. A small amount (100–500 mg) of either tissue was homogenized with 4 vol. of 10 mM phosphate buffer, pH 7.4, in a Kontes 23 glass–Teflon homogenizer (0.5 min, 1000 rev./min), extracted for 1 hr at 4° and centrifuged in an IEC B-20 centrifuge (30,000 *g*, 1 hr, 4°). The supernatant was dialyzed for 16 hr in 500 vol. of 1 mM phosphate buffer, pH 7.4, and recentrifuged under the same conditions. This supernatant was used for the preparation of isoelectrofocusing samples.

Preparation of the gels. The stock solutions of recrystallized acrylamide and bisacrylamide, 20 and 1% (w/v), respectively, were filtered on a 0.22 μm pore-size membrane filter and stored at 4° in the dark. Ammonium persulfate solution (0.1 per cent) was prepared daily. The gel solution contained 1 ml of each acrylamide and bisacrylamide stock solution, 0.3 ml of 40% Ampholine, pH 5–8, and 3.7 ml of the ammonium persulfate solution, and was degassed under vacuum at 0°. Glass tubes (120 × 2 mm) were soaked overnight in chromic acid, rinsed with distilled water, immersed 15 min in 0.5% Photo-flo and drained briefly. The damp tubes were stopped with rubber caps and filled with 0.25 ml of the gel solution. The top of the gel was overlaid with 10–20 μl water. Polymerization occurred overnight at room temperature. The water from the top of the gels was removed with an unbeveled needle connected to a vacuum line, and replaced with 10 μl of 5% sucrose solution containing 2% Ampholine, pH 5–8. The glass tubes were placed in an electrophoresis cell containing 0.1 M phosphoric acid in the bottom (anodic solution) and overlaid and filled with 4% ethylenediamine, which was used in the top chamber (cathodic solution). A pre-electrofocusing (1–2 hr, 2 mA/12 gels, up to 600 V) established the pH gradient and eliminated strong ions from the gels.

Focusing. The tubes were removed from the cell and the upper section of the gels was washed with distilled water. The sample ($5 \leq x \leq 20$ μl) was placed on top of the gels and overlaid with the 2% Ampholine–5% sucrose solution ($10 \leq y \leq 25$ μl, $y = 30 - x$) which was then overlaid by the cathodic solution. The isoelectric focusing was performed up to equilibrium at 4° (2 mA/12 gels, max 900 V, *ca.* 2 hr). The gels were removed from the glass tube by injection of water with a 26-gauge needle.

Enzyme detection. The activity-staining solutions contained 0.5 mg nitro blue tetrazolium, 0.2 mg phenazine methosulfate, 1 mg coenzyme and some substrate, in 2 ml of 0.1 M phosphate buffer, pH 7.4. For aldehyde dehydrogenase detection, NAD and 2.5 mM *p*-nitrobenzaldehyde were used. For aldehyde reductase, NAD or NADP, and 75 mM *p*-methoxybenzylalcohol were chosen [16]. The gels were incubated for 3 hr at 37° in closed tubes in the dark. Stained gels were conserved in 7% acetic acid. Position and intensity of the stain were measured at 546 nm with a Gilford 2520 gel scanner connected to a Honeywell recorder/disc integrator.

Calculations

Except when stated, all values are expressed as mean and standard deviation of duplicate analysis from six animals. Statistical significance was determined by Student's *t*-test for two means with a programmed Hewlett–Packard 25 calculator.

RESULTS

Drinking patterns

The screening tests of our rats did not reveal well-defined groups, but rather a heterogenous distribution with respect to their drinking habits. The daily alcohol intakes of 210 rats averaged over the screening period are presented in Fig. 1. The sex of the animals did not contribute to this distribution. The 30 highest and lowest drinkers were subjected to a second identical screening 2–4 weeks later. Only the animals exhibiting consistent drinking patterns were used for subsequent experiments at least 3 weeks after the last ingestion of alcohol. The results of screening tests of these animals are shown in Fig. 2. At any concentration in the range tested, except 2.5%, the low

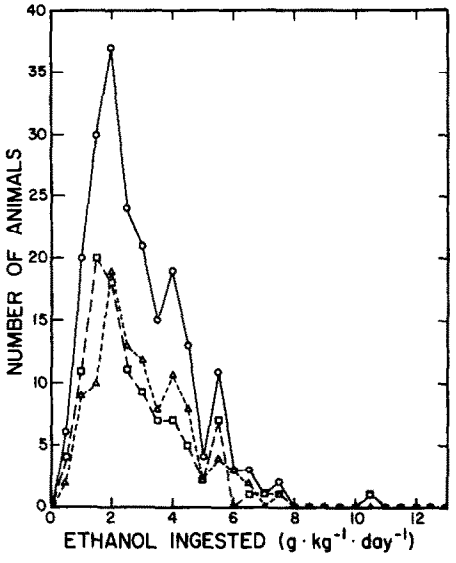

Fig. 1. Distribution profiles of deliberate ethanol ingestion by Wistar rats [male (□), N = 105; female (△), N = 105; both sexes (○), N = 210). Values for ethanol ingested are means of a standard 12 days, twelve concentrations screening test as shown in Fig. 2.

drinkers drank less than half of the volume of alcohol solutions as compared to the high drinkers. At concentrations higher than 7.5%, the low drinkers stopped to drink alcohol solutions. In contrast with these results, the high drinkers never chose to drink only water. As appears from Fig. 2B, the amounts of ethanol ingested voluntarily by either group of animals were fairly constant, at concentrations between 10 and 25%. The destruction of the olfactory system by $ZnSO_4$ [12] did not affect the drinking habits. The measurements were corrected for evaporation but not for spillage by the animals.

Metabolism of biogenic amines

The metabolism of neurotransmitters in brain and liver was measured by incubation of tissue samples. The results for the metabolism of dopamine are summarized in Table 1. In incubations of brain tissues, the only statistically significant difference was found in the concentration of 3,4-dihydroxyphenylethanol (DOPET), which was 16% higher in the high than in the low drinkers. No differences were found between high and low drinkers with respect to their liver metabolism. The respective levels of tetrahydropapaveroline (THP) were unaltered in incubations of either liver or brain. Some differences appeared in the levels of metabolites from dopamine when animals of different sex were taken into consideration. As compared to the females, the males showed an 18 per cent lower level of 3,4-dihydroxyphenylacetaldehyde (DOPAL) after incubations of brain tissue. This was related to a higher 3,4-dihydroxyphenylacetic acid (DOPAC) level, even though no statistical significance was reached for this last component, mainly due to broad individual variations. In contrast with brain metabolism, the oxidizing pathway in the liver was more efficient for the males than for the females, as shown by a 22 per cent increase in DOPAC formation. This was accompanied by lower DOPAL accumulation and DOPET level. The results for the metabolism of serotonin are presented in Table 2; the concentrations of every metabolite detected are unaltered for high and low drinkers, as well as for males and females.

Isozymes pattern

The complete isozyme patterns of rat liver and brain aldehyde dehydrogenase, as well as NAD- and NADP-dependent aldehyde reductase activities, are

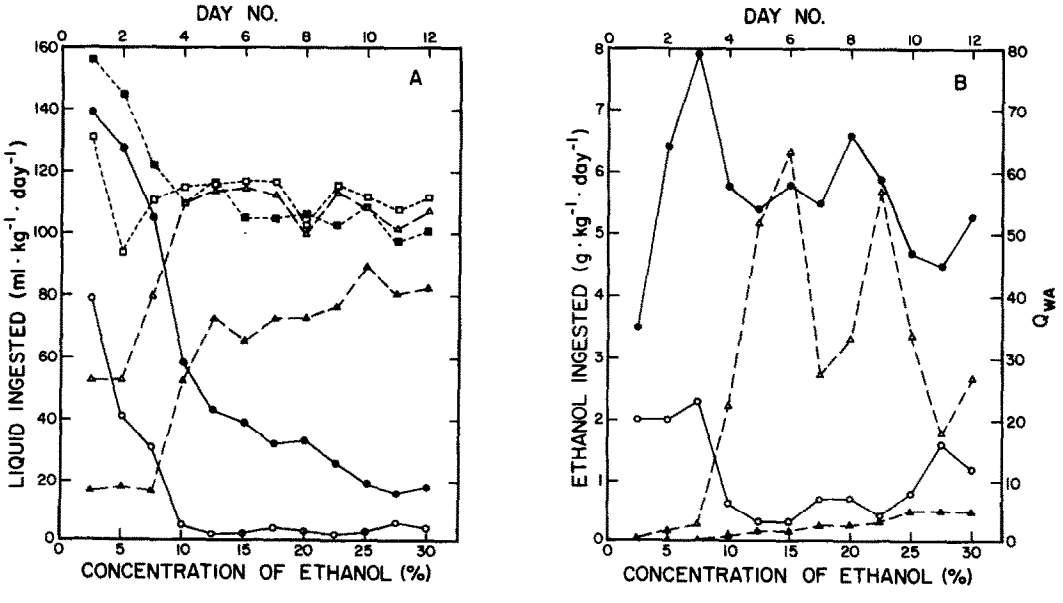

Fig. 2. Drinking pattern of two groups of animals selected with respect to their alcohol preference (high drinkers: ●, ▲, or ■) or rejection (low drinkers: ○, △ or □). (A) Amounts of alcohol (●, ○, ——); water (▲, △, – – – –); and liquid (■, □, · · · · ·) ingested daily. (B) Amounts of pure ethanol (●, ○, ——) ingested daily, and ratio of the ingested volume of water to that of alcohol (Q_{WA}: ▲, △, – – – –). Conditions are as described in Materials and Methods.

Table 1. Relative concentrations of the metabolites isolated from incubations of brain and liver tissues with dopamine*

Organs	Animals	DOPAL (%) (Mean ± S. D.)	DOPET (%) (Mean ± S. D.)	DOPAC (%) (Mean ± S.D.)	THP (%) (Mean ± S. D.)
Brain	Low drinkers	21.2 ± 4.1	30.2 ± 4.5	38.2 ± 9.3	10.4 ± 4.4
	High drinkers	19.2 ± 3.6	34.9 ± 5.9	36.7 ± 7.0	9.2 ± 2.5
	P	NS†	<0.05	NS	NS
	Females	22.3 ± 3.2	33.6 ± 5.2	34.7 ± 6.5	9.4 ± 3.6
	Males	18.2 ± 3.6	31.4 ± 6.0	40.2 ± 8.9	10.2 ± 3.6
	P	<0.01	NS	NS	NS
Liver	Low drinkers	18.6 ± 5.9	23.6 ± 6.6	49.3 ± 14.2	8.5 ± 3.2
	High drinkers	15.6 ± 3.1	20.9 ± 6.0	55.7 ± 8.8	7.9 ± 2.7
	P	NS	NS	NS	NS
	Females	19.1 ± 5.0	24.7 ± 7.0	47.2 ± 13.2	9.0 ± 3.3
	Males	15.0 ± 3.8	19.8 ± 4.6	57.8 ± 8.0	7.4 ± 2.3
	P	<0.05	<0.05	<0.05	NS

* The metabolites are: DOPAL, 3,4-dihydroxyphenylacetaldehyde; DOPET, 3,4-dihydroxyphenylethanol; DOPAC, 3,4-dihydroxyphenylacetic acid; and THP, tetrahydropapaveroline.
† NS = not significant.

Table 2. Relative concentrations of the metabolites isolated from incubation of brain and liver tissue with serotonin*

Organ	Aldehyde (Mean ± S. D.)	Alcohol (Mean ± S. D.)	Acid (Mean ± S. D.)
Brain	26.5 ± 3.7	49.7 ± 6.2	23.9 ± 5.9
Liver	17.5 ± 4.3	39.7 ± 13.0	43.6 ± 15.1

* Aldehyde: 5-hydroxyindolacctaldehyde; alcohol: 5-hydroxyindolethanol; and acid: 5-hydroxyindolacetic acid. No significant differences were found between either high and low drinkers or males and females. Values are means ± standard deviations of duplicate analysis from twelve animals.

to be published elsewhere;* a preliminary isoelectric focusing result has been presented [17] which showed that at least six isozymes of liver aldehyde dehydrogenase can be identified from the cytosolic fraction. The isozyme patterns of brain ALDH as well as liver and brain aldehyde reductase are the same in the high and the low drinking animals. However, in the cytosol fraction of the liver only, a major difference is found between high and low drinkers. The low drinkers exhibit a triple peak at pI 5.9, 6.0 and 6.2, although only one peak is found in high drinkers, either at pI 5.9 or at pI 6.2 (Fig. 3). Even though the number of isozymes from cytosol differ between high and low drinkers, the total ALDH activity remains constant in this fraction.

DISCUSSION

The first step to generate a highly inbred strain of animals selected for their alcohol preference is a screening test which allows discrimination between different drinking habits. Though no definite polymodal distribution is observed, rats from our colony clearly exhibit a heterogenous behavioral pattern in this respect (see Fig. 1). The general shape of the curves is not Gaussian, suggesting a genetically controlled drinking behavior not linked to the sexual chromosomes, since distribution profiles are similar for males and females. Groups of high and low drinkers are not apparent from the profiles in Fig. 1. However, a subsequent rescreening of animals distributed on both extremes of the curves is sufficient to allow the selection of rats with consistent drinking habits and consistent isozyme patterns. An individual's isozyme patterns were not determined as a function of age; however, within a group, animals between 100 g (*ca.* 4-weeks-old) and 700 g (males, *ca.* 8-months-old) exhibit identical isozyme distributions and drinking habits.

High or low drinkers differ not only in their liver ALDH isozyme pattern, but in many aspects of their drinking behavior. Both groups consistently drink between 100 and 120 ml/kg · day. However, the amounts of water and ethanol solution they ingest differ as well as their total intake of pure ethanol. The average high drinkers drink 6 g/kg · day independently of the concentration of ethanol presented to them. The low drinkers do not drink significant amounts of ethanol solutions at any concentration higher than 7.5%. At lower concentrations these animals drink up to 2 g/kg · day.

It has been shown in animal models that strains which drink alcohol can have higher (mice [11]) or lower liver ADH (rats [9]) than strains which reject it. Eriksson [8] suggests that the levels of liver ALDH may also differ. Recent work in our laboratory as well as that of others [6] shows that the enzyme from the matrix of the mitochondria is the enzyme-oxidizing acetaldehyde. By measuring the concentration of the matrix isozyme on the isoelectric focusing gels it was found that within experimental error this enzyme is at the same level in the liver of high and low drinkers. Though total ALDH levels were not

* A. W. Tank, D. Berger, J. A. Thurman, W. R. Bensch and H. Weiner, manuscript submitted for publication.

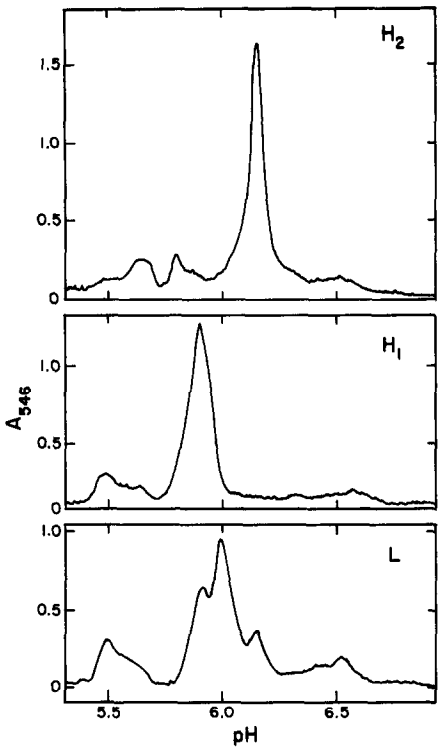

Fig. 3. Partial isozyme patterns of rat liver aldehyde dehydrogenase from cytosol fraction. Isoelectric focusing (0.16 mA/gel, up to 900 V, *ca.* 2 hr) on 5% polyacrylamide gels (2 × 80 mm) containing 2% Ampholine, pH 5–8. Curves are representative patterns of low (L) and high (H_1, H_2) drinkers. A complete isozyme pattern was presented in Ref. 17 (and will also be published in A. W. Tank, D. Berger, J. A. Thurman, W. R. Bensch and H. Weiner, manuscript submitted for publication).

measured by assays *in vitro*, a relative quantitation of this enzyme was calculated from isoelectric focusing gels scanned after activity staining. No significant difference is observed between our two groups of animals. This is not in total agreement with previous results by Koivula *et al.* [9]. The different substrates used and the exclusion of particulate isozymes from the gels (microsomal and some bound mitochondrial isozymes [5]) could account for this small discrepancy. Furthermore, the individual variations in a heterogenous strain of animals are greater than in a highly inbred strain. Thus, small group variations within our colony could be masked by broader individual variations. It appears that no definitive conclusion can as yet be made as to the roles played by ADH and ALDH levels in the determination of alcohol preference.

The high drinkers were found to drink amounts of alcohol as high as 6 g/kg · day at all concentrations presented. The low drinkers truly reject alcohol solutions for, even at concentrations where they do consume some ethanol, they always drink more water. This can be seen in Fig. 2b, where, except at 2.5%, the low drinkers always have a Q_{WA} greater than 1.0; this indicates that they choose to drink water rather than ethanol solutions. Opposite results are found with the high drinkers. At concentrations below 7.5% the high drinkers choose to drink the ethanol solution ($Q_{WA} \simeq 0.1$), but at concentrations higher than 10%, the animals take most of their liquid as water. Thus, the high drinkers seem to stop drinking when they have consumed *ca.* 6 g/kg, even though Wistar rats are able to metabolize more ethanol in a single day (*ca.* 10 g/kg · day) [8]. Some authors attribute this control of alcohol ingestion to acetaldehyde accumulated in the organism [8, 10]. The factors which discourage the low drinkers to drink ethanol at concentrations above 7.5% are probably related to taste [18].

Eriksson [8] has shown that small differences in the rate of disappearance of ethanol and acetaldehyde may occur in his highly inbred strains from Wistar rats. Preliminary work with some of our male high and low drinkers is in agreement with his observations.

Since ethanol affects both metabolism and levels [1–4] of biogenic amines, the isozyme patterns of the enzymes involved in the degradation of these components were investigated. No differences are detected when the isozyme patterns from the brain of high and low drinkers were compared. However, though the isozymes of liver ALRed are identical in both groups (data not shown), the liver ALDH isozyme patterns of high and low drinkers differ greatly in the region pI 5.9 to 6.2. The low drinkers always possess three isozymes in the cytosol fraction, while the high drinkers always have only one. Each type of drinker possesses many other isozymes of ALDH [17].

Two types of isozyme patterns are identified from the liver of all the high drinkers tested: either one isozyme at pI 5.9, or another at pI 6.2 (see Fig. 3). In the pattern of all the low drinkers tested, these two isozymes are present, along with a third isozyme of intermediate pI (6.0). The latter is never formed alone, or with just one of the others, suggesting that this pI 6.0 isozyme is a hybridization product from the pI 5.9 and 6.2 isozymes. These isozyme patterns (Fig. 3) are consistent with a genetic model where high drinkers would be homozygous, either genes aa or bb, and low drinkers heterozygous ab. However, an alternative, where each isozyme could be the product of separate genes, two of them being absent or totally repressed in the high drinkers, cannot be excluded at this time.

The specificities of the cytosol ALDH isozymes are not known. From the metabolic studies presented, it can safely be concluded that these isozymes are not primarily involved in biogenic aldehyde oxidation, for the high and low drinkers, though possessing different isozyme patterns, metabolize dopamine and serotonin at the same rate. Liver ALDH can oxidize some steroids [19, 20] and, conceivably, these isozymes could be involved in the degradation of such compounds. In any case, there appears to be a good correlation between the drinking behavior of the individual animal and the liver ALDH isozyme patterns. Whether this correlation is at the origin of the individual drinking habits or is more coincidence is not yet known. It can be concluded from the results presented that it will be important to look for isozyme patterns and not total enzyme activity when comparing high and low drinkers. Breeding experiments are underway to determine if the offspring will possess the same correlation of isozyme pattern and drinking behavior.

An animal was classified as a high or low drinker prior to the determination of the isozyme pattern. It could be argued that the ingestion of ethanol for 12 days caused the disappearance of the two isozymes in the high drinker. It has been shown by Deitrich *et al.* [21] that cytosol ALDH isozymes can be induced by drugs. However, chronic treatment of animals with ethanol reduces the activity of ALDH in the mitochondrial fraction of the liver, and the cytosol isozymes are only slightly affected [22]. Thus, it appears unlikely that the ethanol ingested during the screening tests caused such dramatic alteration of the isozyme distributions which were measured at least 2 weeks after the screening tests were completed.

The relationship between biogenic amines and alcohol consumption is not well understood. Dopamine and serotonin are deaminated at the same rate by high and low drinkers. The high drinkers, however, produce slightly higher DOPET levels as a result of dopamine metabolism in the brain. It is interesting to note that this increase in DOPET formation is detected even in the absence of ethanol in the organism. Such a small difference in the overall balance of dopamine metabolites, as well as the more dramatic changes in the isozyme patterns of liver ALDH, could be related to the biochemical mechanisms which control alcohol preference in the rats.

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