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Flavor quality and ethanol concentration affect ethanol-conditioned flavor preferences

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Abstract

A previous report showed that outbred rats acquired preferences for a sweetened conditioned stimulus (CS) flavor paired with intragastric ethanol. To evaluate the role of sweet taste in ethanol conditioning, this study compared training with sweetened and unsweetened flavors. In Experiment 1, nondeprived rats were trained to drink one flavored solution (CS+, e.g., grape) paired with intragastric infusion of 5% ethanol and another (CS−, e.g., cherry) paired with intragastric water on alternate days. The volume of ethanol solution infused was matched to the volume of flavored solution the rats consumed. The sweet group's flavors initially contained 0.2% saccharin, reduced to 0.1%, 0.05%, and 0% over days; the plain group's flavors were unsweetened. The sweet group drank more and self-infused more ethanol during training and its preference for the CS+ over the CS− (without saccharin) exceeded that of the plain group (75% versus 62%). Experiment 2 equated total ethanol intake in rats trained with two combinations of flavor quality and ethanol concentration. The Sweet5 group drank flavors with 0.2% saccharin throughout training and tests and received 5% ethanol when they drank CS+, while the Plain10 group drank unsweetened flavors and the CS+ was paired with 10% ethanol. Despite equal daily ethanol doses, the Sweet5 group strongly preferred the CS+ (89%) while the Plain10 group avoided it (31%). The two groups continued to show opposite CS+ preference profiles even when both were tested with sweet CS flavors and 10% ethanol infusions. Thus, sweet taste contributes to the development of ethanol-conditioned flavor preferences, and this effect is not explained by a simple enhancement of ethanol intake.

Keywords: Flavor conditioning; Ethanol; Gastric infusions; Saccharin

1. Introduction

Rats do not readily drink plain alcohol solutions but can be induced to do so using a variety of techniques. Once ingested, alcohol can have positive or negative consequences that promote or suppress future consumption, depending in part on the amount of alcohol ingested. This postingestive modulation of alcohol appetite has been investigated using conditioned flavor preference/aversion techniques. While most studies have obtained flavor aversions, there are several reports of alcohol-conditioned flavor preferences. In a study by Sherman et al. (1983), food-deprived rats developed a preference for a flavored sucrose solution that was paired with intragastric intubation of ethanol at a dose of 0.5 g/kg. A dose of 1.0 g/kg failed to condition a flavor preference, and a 2.0-g/kg dose produced a flavor aversion. Several other reports of ethanol-conditioned flavor preferences have been published using food- and/or water-deprived rats (Cunningham and Niehus, 1997; Deems et al., 1986; Fedorchak and Bolles, 1987; Mehiel and Bolles, 1984; Waller et al., 1984). These findings are relevant to human alcohol use since humans typically acquire preferences for the flavors of particular alcoholic beverages.

A potential limitation of most prior ethanol flavor-conditioning studies is that they all involved food- and/or water-deprived rats, whereas alcohol consumption by humans is not dependent upon food or fluid deprivation. We recently reported, however, that intragastric ethanol infusions could condition flavor preferences in nondeprived rats (Ackroff and Sclafani, 2001). The animals were given ad libitum access to food, water, and a flavored noncaloric solution (the conditioned stimulus or CS+) that was paired with a concurrent intragastric infusion of 6% ethanol. On other

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days, a different flavored solution (the CS−) was paired with intragastric water infusions. In subsequent two-bottle tests, the rats significantly preferred the CS+ to the CS−, and this preference was sustained as the ethanol concentration of the infusate was gradually increased to 24%. Since the rats were nether food- nor water-deprived during training, their learned preference for the ethanol-paired flavor was not based on restoration of energy or hydration deficits. This does not necessarily mean, however, that the flavor preference was reinforced by the pharmacological rather than the nutritional effects of ethanol. We have conditioned strong flavor preferences in nondeprived rats using intragastric nutrient infusions (Azzara and Sclafani, 1998; Drucker et al., 1993, 1994; Elizalde and Sclafani, 1990; Lucas et al., 1998; Sclafani and Nissenbaum, 1988; Sclafani et al., 1993), even at what would appear to be calorically insignificant concentrations (Ackroff and Sclafani, 1994).

The present experiments further investigated the ability of ethanol to condition flavor preferences in nondeprived rats. In our initial study, the animals were trained with fruit-flavored CS+ and CS− solutions (Kool-Aid drink mixes) that were initially sweetened with saccharin. Then, following the lead of the sucrose-fading techniques commonly used to initiate oral ethanol consumption (Grant and Samson, 1985; Samson, 1986; Samson et al., 1999), saccharin was faded out before preference testing. Saccharin was initially added to the CS solutions because without it rats drink relatively little of the Kool-Aid flavored solutions, which have a sour taste, when plain water is available (unpublished observations). This problem was avoided in prior nutrient-conditioning studies by using a one-bottle training procedure, i.e., the unsweetened CS solution was the only fluid source during the conditioning phase. Not only do rats learn to prefer the nutrient-paired CS+ flavor to the CS− flavor, they also come to prefer the unsweetened CS+ flavor to plain water. In our initial ethanol study, we did not use this one-bottle training procedure because it would have forced the rats to take ethanol every time they drank, which might have aversive consequences. Therefore, the rats were given ad libitum access to plain water and saccharin-sweetened CS solutions early in training. Most rats drank very little water (< 3 g/day) on CS+ training days, and thus the availability of plain water during training does not appear necessary for ethanol conditioning. Whether initial training with saccharin-sweetened CSs is critical for conditioning is less certain and was investigated in the present experiments.

The role of sweet taste in ethanol conditioning is of interest for several reasons, including the reported correlation between the appetite for sweet drinks and alcoholic beverages in animals and humans (Kampov-Polevoy et al., 1999). Adding saccharin to the both CS flavors during training may facilitate learning because sweet taste increases total CS consumption and thus ethanol intake on CS+ training days. Alternatively, sweet taste may have more subtle effects that enhance flavor conditioning, such as altering drinking patterns, increasing the saliency of the CS+ flavor, and activating cephalic digestive reflexes that alter the post-ingestive disposition of the ethanol. Because the CS+ and CS− are sweetened equivalently, sweetness per se is not differentially associated with the CS+. Another issue concerns the role of sweet taste in the expression of ethanol-conditioned preferences. In our prior study, saccharin was faded out of the CS flavors prior to the critical CS+ versus CS− choice test. This may have affected the outcome of the test, given the results obtained in a recent oral ethanol conditioning study. The rats in that study were trained to consume saccharin-sweetened flavors mixed in ethanol or water, and were tested with both flavors in water. When the flavors were sweet, rats with unrestricted access during the 30-min sessions preferred the CS−, displaying a CS+ preference only when saccharin was removed from the flavors during additional training and testing (Cunningham and Niehus, 1997). Perhaps if we had tested with sweet CSs we would also have found a preference for the CS−.

In view of these considerations, Experiment 1 compared flavor conditioning by intragastric 5% ethanol infusions in rats trained with sweetened and unsweetened CS solutions. As in our prior study, the rats trained with the saccharin-sweetened CS solutions had the saccharin faded out before the final preference test. Because the rats drinking sweet flavors self-administered about twice as much ethanol as those drinking unsweetened flavors, Experiment 2 controlled for total ethanol dose during training by doubling the ethanol concentration in the unsweetened flavor group. New rats were trained with unsweetened flavors paired with infusions of 10% ethanol or sweetened flavors and 5% ethanol infusions. We reasoned that if sweet taste promotes flavor conditioning by increasing total ethanol intake, then a similar effect might be produced by increasing ethanol concentration. Another important feature of Experiment 2 was that saccharin was not faded out of the CS flavors: the critical CS+ versus CS− test was conducted with sweetened flavors.

2. Experiment 1: CS flavor quality

Many techniques for inducing oral ethanol intake and producing ethanol-based conditioned flavor preference have used sucrose- or saccharin-sweetened flavors (Heyman, 1997; Matthews et al., 2001; Roberts et al., 1999; Samson et al., 1996). Other nutrients condition preferences for unsweetened as well as sweetened flavors (e.g., Ackroff and Sclafani, 1994; Azzara and Sclafani, 1998; Drucker et al., 1993, 1994; Elizalde and Sclafani, 1990; Giza et al., 1997; Pérez et al., 1998; Sclafani et al., 1993), although in some situations sweetening the CS flavors with saccharin improves nutrient conditioning (Ackroff and Sclafani, 1994; Lucas and Sclafani, 1989). Using a procedure that conditions ethanol-based preferences for sweet flavors (Ackroff and Sclafani, 2001), this experiment determined if ethanol can also condition preferences for unsweetened flavors. In

addition, bout patterns were analyzed to characterize differences in intake of sweetened and unsweetened flavors.

2.1. Subjects

Adult male Sprague–Dawley rats ($n = 28$; Charles River Laboratories, Wilmington, MA) weighed 390–490 g at the start of the experiment. They were housed in stainless-steel hanging cages with ad lib access to powdered food (No. 5001, PMI Nutrition International, Brentwood, MO; 3.3 kcal/g) and fluid in rooms maintained on a 12:12 light/dark cycle (lights on 1000 h) at 21 °C.

2.2. Surgery

The rats were anesthetized with a mixture of ketamine HCl (63 mg/kg) and xylazine (9.4 mg/kg), and were implanted with a stainless-steel gastric cannula used to attach the infusion catheters as described previously (Elizalde and Sclafani, 1990). Briefly, the cannula was inserted into the fundus of the stomach and secured with a purse-string suture, polypropylene mesh and dental cement. The shaft of the cannula was passed through a small incision in the abdominal wall and skin. When not in use, the cannula was kept closed with a stainless-steel screw.

2.3. Apparatus

The test cages used for intragastric infusion were similar to the ''electronic esophagus'' system previously described (Elizalde and Sclafani, 1990). In brief, the rats were housed in stainless-steel hanging cages ($24 \times 18 \times 18$ cm) with powdered chow available from a food cup accessible through a hole in the back wall of the cage. Drinking fluids were available from stainless-steel sipper tubes located through two small holes (19 mm diameter) at the front of the cage. A slot in the cage floor permitted two catheters attached to the rat's gastric cannula to be connected to a dual-channel infusion swivel located below the cage; the catheters were protected by a flexible stainless-steel spring. Plastic tubing connected the swivel to two peristaltic infusion pumps. The pumps were operated automatically by drinkometer circuits and a microcomputer whenever the rat drank from the sipper tubes. The flow rate of the pumps was 1.6 ml/min and they were controlled by computer software to infuse $\sim$1 ml of fluid for each 1 ml of fluid orally consumed. The microcomputer stored on disk the number of licks emitted during 6-s bins for offline analysis of drinking patterns. The infusion system operated 22 h/day; during the remaining 2 h (1000–1200 h), chow and fluids were not available while the intakes were measured and the infusion system serviced.

2.4. Solutions

The oral test fluids (CSs) were tap water-flavored with 0.05% (w/w) grape and cherry unsweetened Kool-Aid drink mixes (General Foods, White Plains, NY). The Kool-Aid flavors are equally unpreferred to plain water (Elizalde and Sclafani, 1990). The flavor (CS+) paired with intragastric ethanol and the flavor (CS−) paired with intragastric water were counterbalanced across subjects. For one group, the CSs were sweetened during training with sodium saccharin (Sigma, St. Louis, MO) added at 0.2%, 0.1%, and 0.05% (w/w) concentrations, as described in the procedure. Unflavored 0.2% saccharin and tap water were also available to drink during pretraining and preference tests, respectively. Left/right positions of the bottles were counterbalanced across days. The infusates were tap water and 5% ethanol prepared by mixing 95% ethanol and tap water. The energy density of the ethanol solution was 0.287 kcal/g. The amounts of fluid consumed and infused were recorded to the nearest 0.1 g.

2.5. Procedure

After a postsurgery recovery period (10–12 days), the rats were transferred to the test cages where they lived for the remainder of the experiment. They were adapted to the cages for several days with chow and water available ad lib. Then their gastric catheters were attached and they were infused with water whenever they drank water during the next 2 days. The rats were then familiarized with the 0.2% saccharin solution which was available, along with water, for 4 days; water was infused intragastrically whenever they drank either fluid.

The rats were divided into two groups matched for pretraining saccharin preference, food and fluid intake and body weight. They were given 20 days of one-bottle training, in which oral intake of the CS− and CS+ flavored solutions were paired with intragastric infusions of water and 5% ethanol water on odd- and even-numbered days, respectively. The sweet group ($n = 15$) was initially trained with the CS solutions sweetened with 0.2% saccharin (3 days each CS) and then the saccharin concentration was reduced to 0.1% (2 days each), 0.05% (2 days each), and finally, 0% (3 days each CS). The plain group ($n = 13$) was given the same Kool-Aid flavored CS solutions without saccharin during the 20-day training period.

The rats then received a series of two-bottle preference tests with the unsweetened CS solutions. In the first test, they were given the choice of the CS+ versus CS− solutions for 4 days. The second test involved CS+ versus water and CS− versus water choices on alternate days for a total of 12 days. Intake of the CS+ remained paired with intragastric infusions of 5% ethanol throughout two-bottle testing, while the intake of the CS− and plain water were paired with intragastric water infusions.

Body weights were estimated as growth of 8.8 g/week, using the weight when the animals were placed in the infusion cages and when they were removed at the end of the experiment as endpoints. The estimated body weights were used to calculate estimated ethanol doses in grams per kilogram.

2.6. Data analysis

The intake data were averaged over the different training and test phases. Drinking patterns were analyzed with a bout defined as a period of drinking containing at least 30 licks and interlick intervals no longer than 5 min. Ethanol intakes per day and per bout were calculated as grams of pure ethanol contained in the infusates. To obtain an estimate of the average ethanol per bout of CS+ intake, the ratio of infused ethanol solution to oral intake was used as a correction factor to account for small variations from the targeted 1:1 ratio. The data were entered in repeated-measures analyses of variance, except for single variable comparisons using *t* tests. For significant main effects, tests of differences between specific means or weighted groups of means (e.g., the three sweetened periods versus the unsweetened period) were performed using least squares weighted means contrasts (and *t* tests). Comparisons of two-bottle preference scores (CS+ intake/total intake $\times 100$) were conducted on arcsine transformed percentage scores. A probability level of .05 was used in all tests.

2.7. Results

2.7.1. Training intakes

The intakes during one-bottle training are shown in Table 1; for the sweet group, means are given for each saccharin period and the final unsweetened period. The plain group, which received the same unsweetened CS flavors throughout training, showed little change in intake of either CS+ or CS− during this period. The sweet group's intakes of both CS flavors were substantially greater than those of the plain group while saccharin was present (46–64 g/day), and intake resembled that of the plain group when the saccharin was removed (32–34 g/day).

Analysis of the training intakes confirmed that the groups consumed the CS solutions differently [Saccharin level × CS type × Group interaction, $F(3,78)=4.02$, $P=.01$]. There were main effects of group [$F(1,26)=43.62$, $P<.0001$] and saccharin level [$F(3,78)=27.83$, $P<.0001$]. Saccharin level interacted with group [$F(3,78)=31.05$, $P<.0001$] and with CS type [$F(3,78)=5.32$, $P<.01$]. Separate analyses confirmed that the sweet group drank more of both CSs than the plain group at 0.2%, 0.1%, and 0.05% ($P<.0001$) but not 0% saccharin. A within-group analysis of the sweet group revealed significant variation of CS intakes with saccharin level and CS type [interaction $F(3,42)=6.12$, $P<.01$]. Intakes of the sweetened CS+ exceeded that of unsweetened CS+ and intakes of sweetened CS− exceeded that of unsweetened CS− ($P<.001$).

Because ethanol infusions were matched to the animals' oral intakes, the sweet group received more ethanol than the plain group during the training period. Analysis of estimated daily ethanol doses during training yielded an interaction of group and saccharin level [$F(3,78)=9.25$, $P<.001$]; in parallel with their unchanging CS intakes, the plain group's ethanol doses remained constant at about 2.5 g/kg/day. During the periods when the CS contained saccharin, the sweet group's ethanol doses were greater than the dose in the unsweetened period (Table 1; $P<.0001$). The groups did not differ significantly in daily dose when both were consuming unsweetened CS.

Energy intakes during the training period differed on CS+ and CS− days; with the exception of ethanol intake during the saccharin period, there were no differences between groups. Total energy intake was calculated as the sum of chow intake and infused ethanol. In a three-way analysis, the only significant effect was the main effect of CS type [$F(1,26)=5.47$, $P<.05$]. Intakes on CS+ days averaged 95.6 kcal, while CS− day intakes were 92.5 kcal. A parallel chow-intake analysis revealed only a main effect of CS type [$F(1,26)=39.49$, $P<.001$]. Chow intake on CS+ days averaged 84.6 kcal. The ethanol calories varied with saccharin level for the sweet group but not during the same periods for the plain group (7.7–8.1 kcal/day) [interaction $F(3,78)=8.99$, $P<.001$]. The sweet group's ethanol calories were greater when the CS+ was sweet (14.9–15.9 kcal) than when the CS+ was unsweetened (8.8 kcal; $P<.0001$).

Table 1
Mean (S.E.M.) oral intake and bout patterns during training

Group		Oral (g/day)[a]		Ethanol (g/kg/day)	Bouts/day		Oral (g/bout)		Ethanol (g/kg/bout)
		CS+	CS−		CS+	CS−	CS+	CS−	
Experiment 1									
Sweet	0.2%	59.0 (4.7)	64.2 (4.2)	5.1 (0.5)	21.2 (1.6)	22.7 (2.0)	2.8 (0.2)	2.8 (0.2)	0.25 (0.02)
	0.1%	51.4 (2.7)	59.4 (4.2)	4.7 (0.3)	20.4 (2.2)	24.0 (2.5)	2.7 (0.2)	2.6 (0.2)	0.25 (0.02)
	0.05%	54.5 (3.2)	45.7 (3.8)	4.6 (0.3)	19.7 (1.6)	22.7 (1.6)	2.9 (0.3)	2.1 (0.2)	0.25 (0.02)
	0%	33.8 (1.7)	31.7 (1.5)	2.7 (0.2)	15.5 (1.1)	15.6 (1.1)	2.3 (0.2)	2.1 (0.1)	0.19 (0.02)
Plain		29.9 (2.0)	30.5 (1.6)	2.5 (0.2)	15.0 (1.3)	15.0 (1.4)	2.1 (0.2)	2.2 (0.2)	0.18 (0.02)
Experiment 2									
Sweet5		48.3 (2.6)	50.4 (3.2)	3.9 (0.2)	21.8 (2.0)	24.8 (2.1)	2.5 (0.3)	2.2 (0.3)	0.20 (0.02)
Plain10		21.8 (0.8)	27.6 (1.4)	3.6 (0.2)	9.1 (0.8)	10.9 (0.8)	2.6 (0.2)	2.7 (0.2)	0.43 (0.04)

[a] Total daily fluid intakes were twice these values, due to the matched intragastric infusions.

Analysis of the drinking patterns revealed constant bout sizes and numbers across training days for the plain group, and changes in these measures as a function of saccharin level in the sweet group (Table 1). In the overall analysis of bout number, there were main effects of group [$F(1,26) = 7.70$, $P < .01$], saccharin level [$F(3,78) = 16.05$, $P < .0001$], and CS type [$F(1,26) = 5.24$, $P < .05$]. Saccharin level interacted with group [$F(3,78) = 12.63$, $P < .0001$] and with CS type [$F(3,78) = 4.78$, $P < .01$]. Separate analyses of the groups showed that the plain group drank the solutions in about 15 bouts/day throughout training, while the sweet group's bout numbers varied with saccharin level [$F(3,42) = 21.85$, $P < .0001$]; the rats drank more bouts of sweetened than unsweetened CS ($P < .0001$). Overall, the sweet group drank fewer bouts of CS+ than CS− [CS type $F(1,14) = 7.81$, $P < .05$]. A separate analysis of bout numbers at the 0% saccharin level showed no effects of group or CS type.

Analyses of bout sizes (and therefore the amounts of fluid self-infused per bout) revealed interactions of group with saccharin level [$F(3,78) = 7.35$, $P < .001$] and CS type [$F(1,26) = 8.93$, $P < .01$] as well as an interaction of CS type and saccharin level [$F(3,78) = 6.91$, $P < .001$]. Separate analyses of the groups showed that the plain group did not change bout size during the training period (2.2 g/bout), but CS type and saccharin level interacted to determine the sweet group's bout sizes [$F(3,42) = 7.51$, $P < .001$]. This was due to two shifts. The sweetened CS+ bout sizes were larger than the unsweetened CS+ bouts (Table 1; $P < .0001$), and CS− bouts were larger at the two higher concentrations than at the 0.05% and 0% saccharin concentrations ($P < .0001$). A separate analysis of bout sizes at the 0% saccharin level showed no effects of group or CS type. Analysis of the estimated ethanol g/kg/bout on CS+ days showed that the sweet group's ethanol bouts were larger than those of the plain group [$F(1,26) = 5.81$, $P < .05$], with an interaction with saccharin level [$F(3,78) = 6.47$, $P < .001$]. Like CS+ bout size, ethanol g/kg/bout did not vary across training periods in the plain group, but the sweet group's dose per bout was greater when the CS+ was sweet than when it was unsweetened (Table 1; $P < .01$).

2.7.2. Preference tests

In the preference test between the two unsweetened CS (Fig. 1), the rats drank more of the CS+ than CS− [$F(1,26) = 47.20$, $P < .0001$] and there was a Group × CS type interaction [$F(1,26) = 8.21$, $P < .01$]. Both groups drank more CS+ than CS− [plain $F(1,12) = 8.71$, $P < .05$, sweet $F(1,14) = 45.54$, $P < .0001$] and the sweet group drank more CS+ and less CS− than the plain group [$F(1,26) = 6.97$ and 4.88, $P < .05$]. The average percent CS+ preference of 75% in the sweet group was greater than the 62% preference of the plain group [$t(26) = 2.38$, $P < .05$].

In the CS versus water tests (Fig. 2), analysis revealed a three-way interaction (CS type × Fluid × Group [$F(1,26) = 9.10$, $P < .01$], so the groups were analyzed separately. For the plain group, there was only an effect of fluid, with CS+ and CS− intakes less than water intake [$F(1,12) = 16.03$, $P < .005$]. In the sweet group, the relative intakes of water and CS depended on the CS type [interaction $F(1,14) = 20.0$, $P < .001$]: the rats drank more CS+ than water ($P < .05$) and less CS− than water ($P < .01$).

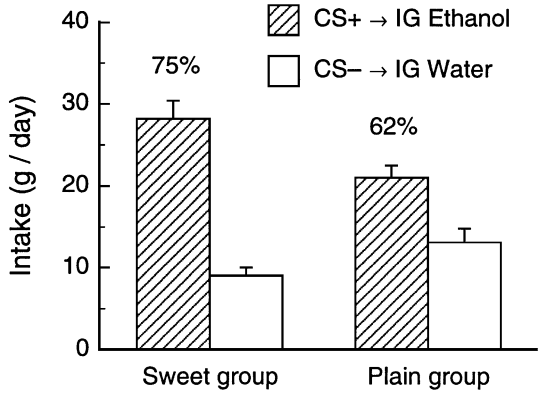

Fig. 1. Mean ± S.E.M. daily intakes of CS solutions by the plain and sweet groups in the CS+ versus CS− two-bottle test of Experiment 1. Both groups were offered unsweetened CS solutions in the test. Mean percentage of total intake consumed as CS+ is shown atop the bars.

2.8. Discussion

The 75% ethanol-conditioned CS+ preference displayed by the sweet group closely replicates the 72% CS+ preference previously obtained with rats trained under similar conditions (Ackroff and Sclafani, 2001). Furthermore, as in the prior study, the sweet rats not only preferred the CS+ to the CS−, both of which were unsweetened during testing, but they also preferred CS+ to plain water. This finding demonstrates that the rats' preference for the CS+ over the CS− was a true preference for the ethanol-paired flavor, rather than simply a learned avoidance of the CS− flavor. The preference for water over CS− in the sweet group in consistent with the preference of untrained rats for water over the sour CS flavors. Taken together, the CS versus water tests show that intragastric ethanol infusions reversed the rats' normal aversion to the unsweetened CS flavors to a preference.

The new finding of the present experiment is that intragastric ethanol infusions conditioned a weaker preference in rats trained with unsweetened CS flavors. Not only did the rats in the plain group display a smaller CS+ preference, relative to the CS−, compared to the sweet group (62% versus 75%); unlike the sweet rats, they failed to prefer the CS+ to plain water. One explanation of these findings is that sweetening the solutions with saccharin during the first 2 weeks of training enhanced total CS intakes and thus increased the amount of ethanol self-infused. The sweetened CSs were consumed in more and larger bouts than the unsweetened CSs, so that sweetness was also associated with

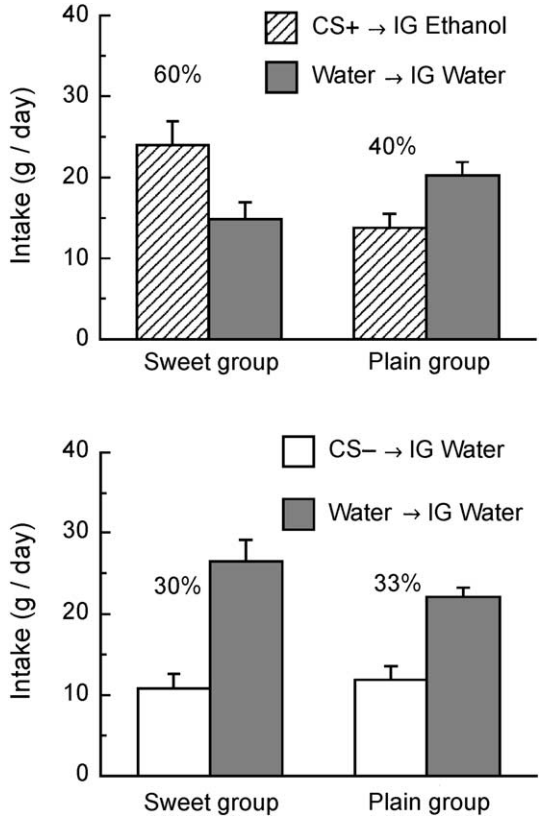

Fig. 2. Mean ± S.E.M. daily intakes of CS solutions and water in the CS+ versus water (top) and CS− versus water (bottom) two-bottle tests of Experiment 1. Both groups were offered unsweetened CS solutions in the tests. Mean percentage of total intake consumed as CS is shown atop the bars.

larger individual doses of ethanol that occurred more frequently than those obtained by drinking the unsweetened CS+. During the saccharin period, the sweet group's daily ethanol intake was about twice that of the plain group. In addition, their average dose per bout was about 40% larger than that of the plain group, and their number of bouts per day exceeded that of the plain group by 47%, such that the sweet group self-administered more daily "trials" as well as larger doses. The small differences in daily energy intake on CS+ and CS− days occurred in both groups and thus do not explain the greater preference of the sweet group. The reduced chow intake on CS+ days suggests that the animals detected the ethanol energy.

3. Experiment 2: ethanol concentration

Experiment 2 examined the possibility that the stronger ethanol-conditioned CS+ preference displayed by the sweet group in the first experiment was due to their greater self-infused ethanol intake, relative to that of the plain group. Two new groups of rats were trained with CS flavors paired with intragastric ethanol or water. The Sweet5 group was trained with saccharin sweetened CS flavors and intragastric infusions of 5% ethanol, as in the first experiment, except that the saccharin was not faded out of the solutions. The Plain10 group was trained with unsweetened CS flavors, as in Experiment 1, except that CS+ intake was paired with matched intragastric infusions of 10% ethanol instead of 5%. This equated the total ethanol dose of the two groups because the Sweet5 rats consumed approximately twice as much CS+ as did the Plain10 group. (Note that the 5% and 10% ethanol infusions were diluted in half by the orally consumed CS solution, for net concentrations of 2.5% and 5% ethanol in the stomach.) Saccharin was not faded out of the CS flavors for the Sweet5 rats in order to keep their daily ethanol doses stable throughout training and matched to the stable ethanol doses of the Plain10 group. CS+ versus CS− preference tests were first conducted with each group given the same solutions and infusions as used in training. Additional preference tests were then conducted with treatment conditions equated: both groups were tested with sweetened CS flavors and with the CS+ paired with intragastric infusions of 10% ethanol.

3.1. Method

New rats ($n = 24$) of the same sex, strain, age, and supplier were fitted with a gastric cannula and housed as in Experiment 1. The rats were adapted to the test cages, intragastric infusions, and 0.2% saccharin solution as in the first experiment and were divided into two groups ($n = 12$ each) matched for saccharin preference, food and fluid intake, and body weight. The Sweet5 group was trained with grape and cherry CS solutions sweetened with 0.2% saccharin and with the CS+ paired with 5% ethanol infusion. The Plain10 group was given the same Kool-Aid flavors without saccharin and the CS+ was paired with a 10% ethanol infusion. For both groups, the CS− was paired with water infusion. As in the first experiment, the rats were given 20 one-bottle training days with the CS+ and CS− presented on alternate days. However, a 2-day reinforced two-bottle CS+ versus CS− test was interposed midway through the training period to evaluate the course of preference learning.

At the end of one-bottle training, the rats were given two-bottle choice tests with their respective training solutions for 6 days. During the first and last 2 days, intakes of the CS+ and CS− remained paired with intragastric infusion of ethanol and water, respectively (reinforced tests). During Days 3 and 4, intakes of both CS were paired with intragastric water infusions (extinction test).

Next the groups were both shifted to drinking sweet CS solutions paired with intragastric 10% ethanol. The Plain10 group simply had 0.2% saccharin added to their CS flavors for the next 6 days, still paired with 10% ethanol infusions. The sweet group's flavors remained the same but the concentration of the ethanol infusions was increased to 7.5% (2 days) and then 10% (4 days). The rats were weighed periodically throughout the study.

3.2. Results

3.2.1. Training intakes

Table 1 shows the mean intakes for the 10 days of training with each CS. There was a substantial difference in intake of the two groups, with the Sweet5 group drinking about twice as much as the Plain10 group [$F(1,22) = 70.74$, $P < .0001$]. CS+ intakes were lower than CS− intakes [$F(1,22) = 12.49$, $P < .01$]. Ethanol doses on CS+ days and thus energy from ethanol were similar in the two groups [3.6 versus 3.9 g/kg/day, $t(22) = 1.32$, ns]. Chow intakes were greater on CS− days (98.4 kcal) than on CS+ days (86.8 kcal) [$F(1,22) = 129.74$, $P < .001$]. Total energy intakes did not differ between groups and flavors.

Differences in CS intake between the groups were largely due to differences in the number of bouts per day [23.3 for the Sweet5 group, versus 10.0 for the Plain10 group, $F(1,22) = 36.83$, $P < .0001$]. The CS+ was consumed in fewer bouts/day than the CS− [15.5 versus 17.9, $F(1,22) = 26.70$, $P < .001$]. There were no main effects of group or flavor for bout size, but there was an interaction [$F(1,22) = 7.10$, $P < .05$], due to the smaller CS− bout size for the sweet group (Table 1). The resulting bout sizes in g ethanol/kg were twice as large in the Plain10 group than in the Sweet5 group [$t(22) = 5.33$, $P < .0001$]. Mean ethanol bout sizes of the first two and last two CS+ training days were compared to assess changes over the course of the training period. While the Sweet5 group's dose/bout did not change, the Plain10 group increased from 0.29 to 0.47 g/kg/bout [interaction $F(1,22) = 49.33$, $P < .0001$ and simple main effects]. The groups differed marginally at the beginning of training ($P = .06$) and substantially by the end ($P < .0001$).

The comparison of first two and last two days of training was continued for the Plain10 group. Analysis of the oral bout sizes for both CS flavors in the Plain10 group showed that intake per bout rose for both flavors [interaction $F(1,11) = 12.10$, $P < .01$]. Simple main effects indicated that the CS+ bout size was initially less than the CS− size (1.9 versus 2.4 g/bout), but by the end of training they were similar at 2.9 g/bout. Parallel analysis of bout number showed that the Plain10 group altered the number of bouts they drank per day for both flavors [interaction $F(1,11) = 10.51$, $P < .01$]. Simple main effects showed initially similar CS+ and CS− bouts per day (12.5 and 12.0), with a greater decline for CS+ than CS− by the end of training (8.1 versus 10.7 bouts per day). The net effect of these changes in pattern was a constant daily intake during training ($F < 1$).

3.2.2. Preference tests

In the first preference test (interposed in the middle of the training period), the groups already exhibited differences in flavor preferences. The Sweet5 group consumed significantly more CS+ than CS− (42.3 versus 10.4 g), while the Plain10 group drank less CS+ than CS− [8.8 versus 18.2 g, Group × Flavor interaction, $F(1,22) = 35.23$, $P < .001$]. The Sweet5 and Plain10 consequently differed in their percent CS+ intakes [80% versus 34%, $t(22) = 5.75$, $P < .001$].

In the post-training preference tests, intakes during the first and second two days of reinforced testing were similar and therefore were combined and compared to the intakes during the 2-day extinction test. As illustrated in the top and center panels of Fig. 3, the groups differed markedly in their intakes and preferences during these two-bottle tests, with the Sweet5 group drinking much more CS+ than CS−

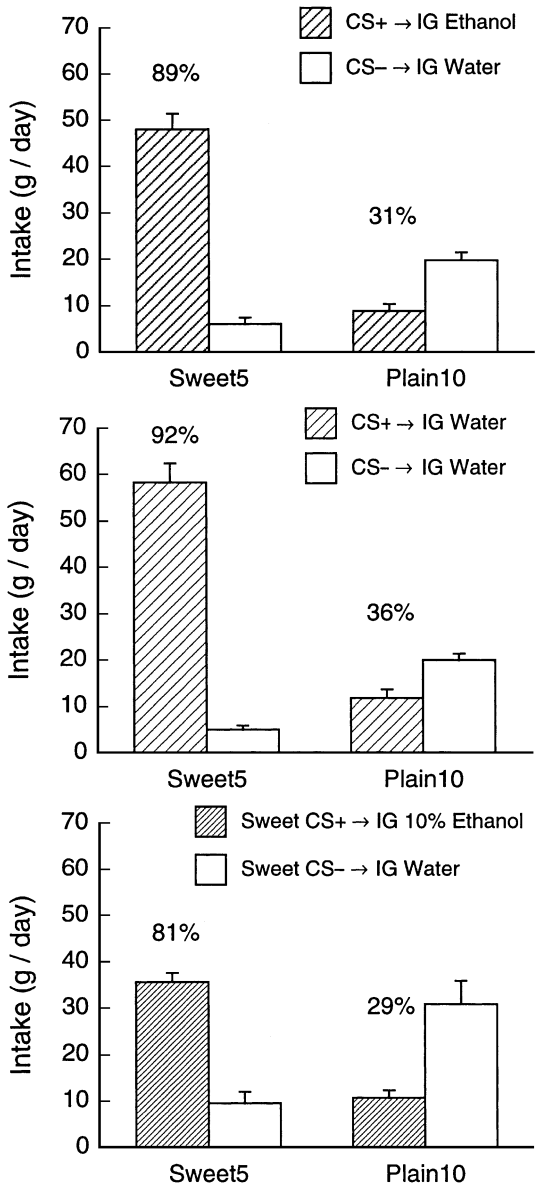

Fig. 3. Mean ± S.E.M. daily intakes of CS solutions in two-bottle tests of Experiment 2. The Sweet5 group and the Plain10 group were given their training CS solutions in the tests. Shown are the initial reinforced preference test with the CS+ paired with intragastric infusion of ethanol (top), the extinction test with both CS solutions paired with water (center), and the preference test with both groups shifted to sweet CS and 10% ethanol infusions. Mean percentage of total intake consumed as CS+ is shown atop the bars.

while the Plain10 group drank less overall and preferred the CS− over the CS+ [Group ×CS interaction, $F(1,22) = 174.6$, $P < .0001$]. There was also a large difference in the group CS+ preference scores [$F(1,22) = 138.05$, $P < .05$] over the 6 days of testing. All of the rats in the Sweet5 group strongly preferred the CS+, with individual preference scores ranging from 74% to 97%. During the reinforced tests, the 3.6-g/kg daily ethanol dose self-administered by the Sweet5 group exceeded the 1.4-g/kg dose of the Plain10 group [$t(11) = 5.52$, $P < .001$].

Within-group comparisons indicated that, overall, the Sweet5 group strongly preferred the CS+ to the CS− [$F(1,11) = 192.40$, $P < .001$]. There was an interaction of CS and test [$F(1,11) = 14.91$, $P < .01$], which reflected greater CS+ intake during extinction than reinforced testing, but similar intakes of CS−. The Plain10 group drank significantly more CS− than CS+ overall [$F(1,11) = 12.59$, $P < .01$] and drank more in the extinction test than in the reinforced test [$F(1,11) = 16.57$, $P < .01$], but there was no CS × Test interaction.

Overall, the group differences in the CS+ preference were sustained when they were tested under identical conditions: sweetened flavors and CS+ paired with 10% ethanol infusions (bottom panel of Fig. 3). The Sweet5 rats continued to consume significantly ($P < .01$) more CS+ than CS−, while the Plain10 group continued to consume significantly ($P < .01$) less CS+ than CS− [CS × Group interaction, $F(1,22) = 104.89$, $P < .0001$]. The percent CS+ intake of the Sweet5 group also remained greater than that of the Plain10 group [81% versus 29%; $t(22) = 7.06$, $P < .001$].

Within-group comparisons indicated that the Sweet5 group's intakes dropped somewhat as the ethanol concentration of the infusion was increased from 5% to 10%. An interaction of concentration and flavor [$F(1,11) = 12.55$, $P < .01$] was due to a significant reduction in CS+ intake at the higher ethanol concentration, with no change in CS− intake. Their CS+ preference score also decreased somewhat from 89% to 81% as ethanol concentration increased [$t(11) = 2.30$, $P < .05$]. Note, however, that the rats self-infused a higher dose of ethanol when the CS+ was paired with 10% ethanol than with 5% ethanol infusions [5.4 versus 3.6 g/kg/day, $t(11) = 6.80$, $P < .0001$].

Overall, the Plain10 group consumed more of the sweetened CS than of the unsweetened CS [$F(1,11) = 10.86$, $P < .01$]. However, they continued to prefer the CS− to the CS+ [$F(1,11) = 14.23$, $P < .01$] and their percentage intakes of CS+ did not change appreciably. Their daily dose of ethanol also did not change much when the CS+ was sweetened (1.4 versus 1.6 g/kg/day).

3.3. Discussion

Experiment 2 revealed a very strong ethanol-conditioned flavor preference in rats trained and tested with a sweetened CS+ flavor paired with intragastric infusions of 5% ethanol. In contrast, rats trained and tested with an unsweetened CS+ flavor paired with 10% ethanol infusions avoided the CS+ flavor. Yet the daily ethanol doses of the two groups during training were similar. These results demonstrate that the stronger CS+ preference displayed in Experiment 1 by the sweet group relative to the plain group cannot be explained simply by the group differences in daily ethanol dose.

The groups differed in the conditions of the initial reinforced two-bottle tests in terms of CS flavors (sweet versus nonsweet) and ethanol infusions (5% versus 10%). However, when the conditions were equated, with all rats tested with sweet CS flavors and 10% ethanol infusions, the group differences persisted: the Sweet5 rats continued to prefer the CS+ strongly while the Plain10 rats continued to avoid the CS+. These results clearly demonstrate that the opposite preference patterns displayed by the two groups were due to their different training conditions and were not due to differences in the initial preference test conditions.

The 89% CS+ preference displayed by the Sweet5 group is the strongest ethanol-conditioned preference observed to date with outbred rats and it is comparable to the strong preferences produced by intragastric infusions of other nutrients (Sclafani, 1999). The preference persisted during the 2 days of extinction testing and, in fact, absolute CS+ intake increased when it was paired with intragastric water rather than with ethanol, which confirms prior results (Ackroff and Sclafani, 2001). This increased intake presumably represents the release from the satiety actions of the infused ethanol. The extinction data are important because they demonstrate that the Sweet5 rats had acquired a true preference for the CS+ flavor and were not consuming it simply as an instrumental act to obtain ethanol infusions. With other nutrients, conditioned preferences persist for several weeks or more of extinction testing (Elizalde and Sclafani, 1990); the persistence of ethanol-conditioned flavor preferences remains to be established.

4. General discussion

In the present study, intragastric ethanol infusions conditioned a flavor preference in nondeprived outbred rats trained using a saccharin-fading procedure, which replicates our previous results (Ackroff and Sclafani, 2001). The new findings of this study are that ethanol-conditioned preferences can be obtained with unsweetened flavors (Experiment 1) and with sweetened flavors without subsequent saccharin fading (Experiment 2) when the flavors are paired with 5% ethanol infusions. Overall, the conditioned flavor preferences were strongest when the CS+ remained sweetened through training and testing, weakest when they were never sweetened, and intermediate when the sweet taste was faded out. In contrast, pairing an unsweetened flavor with infusions of 10% ethanol infusion resulted in a CS+ avoidance. An important additional finding is that ethanol-con-

ditioned preferences were expressed when the CS+ flavors were unsweetened during the two-bottle test (Experiment 1) as well as when they were sweetened during the test (Experiment 2). Thus, the conditioned preference for the ethanol-paired flavor does not depend on removal of sweetness: the sweet CS+ was preferred despite the potential competition from the sweet CS−. In contrast, rats in a recent oral ethanol conditioning study preferred the CS− when the flavors were sweet and shifted to a CS+ preference only when saccharin was removed (Cunningham and Niehus, 1997). The contrasting results may be due to important differences in procedure, such as the use of food restriction and short daily sessions, in addition to the route of administration.

The negative flavor conditioning response to the 10% ethanol infusions in Experiment 2 is consistent with many prior reports of ethanol-conditioned flavor aversions (Berman and Cannon, 1974; Cannon and Carrell, 1987; Crawford and Baker, 1982; Eckardt et al., 1974; Marfaing-Jallat and Le Magnen, 1979; Miceli et al., 1980; Sinclair, 1984). Nevertheless, this outcome was somewhat surprising, because the conditions of self-administration were benign in the present experiments. That is, the rats controlled the ethanol infusions by their spontaneous drinking behavior, and the total ethanol dose self-administered by the Plain10 rats was no greater than that of the Sweet5 rats. The Plain10 group's relatively low intake of CS+ in two-bottle tests is at best a mild aversion, given that the rats consumed 30% of their intake as CS+ when they need not have consumed any. It is possible that some feature of the higher concentration, such as overly rapid rise in blood ethanol, contributed a negative effect that diminished the net reward of the infusion. Note that the possibility of negative effects that might counteract reward is not limited to ethanol. More concentrated carbohydrates and fats, whether ingested orally or infused, may not be preferred to less concentrated ones (Booth et al., 1972; Lucas and Sclafani, 1989; Lucas et al., 1998).

The failure to obtain flavor preferences with 10% ethanol was a limitation for the acquisition of preferences, but not for their expression. In our previous study, preferences established with 6% ethanol infusions were sustained as the accompanying infusions were increased as high as 24% ethanol (Ackroff and Sclafani, 2001). In the present study, the Sweet5 rats, after having acquired a CS+ preference based on 5% ethanol infusions, maintained a strong CS+ preference when it was paired with 10% ethanol. It is possible that duration of exposure to ethanol accustomed the rats to its effects so that the higher concentrations, introduced gradually, were acceptable. This may reflect the development of tolerance to any aversive effects of the infused ethanol. Studies that have preexposed animals to drugs before flavor–drug pairing have found attenuation of conditioned flavor aversion, suggesting reduced responsiveness to aversive effects with experience (e.g., Gaiardi et al., 1991; Stewart et al., 1991). Other methods of inducing increased ethanol intake in rodents have started with low concentrations that were gradually increased, which may reflect a similar process (e.g., Linseman, 1989; Samson, 1986). In the case of oral ethanol, the salient taste of the ethanol itself, which becomes stronger with concentration, can mediate the learning. This factor is missing in our situation, in which the same flavor must be linked to the effects of more concentrated untasted ethanol.

Together, the results of Experiments 1 and 2 demonstrated that adding a sweet taste to the CS flavors enhanced preference conditioning by intragastric infusions of 5% ethanol. Several possible explanations for this effect can be considered. Adding saccharin to the CS solutions clearly stimulated intake in both experiments. Yet, while sweetened CS intakes, and thus the volume-matched infusions, were greater than unsweetened CS intakes, the conditioning results cannot be attributed simply to greater absolute intake of ethanol: doubling the ethanol concentration to 10% increased the daily dose but did not result in preferences for plain ethanol-paired flavors in Experiment 2. Furthermore, once the Plain10 rats had learned about the 10% ethanol infusions, saccharin did not increase their preference for the CS+ flavor. Total energy intakes (ethanol plus chow) also did not differ between the plain and sweet groups in the two experiments so that overall energy intake does not account for the effect of sweet taste on CS+ preference.

The intake pattern was clearly influenced by flavor quality, with sweet flavors consumed in more frequent bouts than unsweetened flavors, leading to a greater number of more potent "trials" that could facilitate the CS–US association. The oral bouts were larger for sweetened than for unsweetened flavors in Experiment 1. When unsweetened bout sizes were small, perhaps 5% ethanol's reward was too weak to support the development of a strong preference. However, a problem for the bout size/dose explanation is that doubling the ethanol concentration in Experiment 2 had only minimal impact on oral bout size and thus sharply increased the ethanol dose per bout, but in the absence of sweet taste the larger dose was associated with CS+ avoidance.

The increase in bout size over the course of training for the Plain10 group does not seem compatible with the development of an aversion. Rather, the increased bout size may reflect the development of tolerance. Initially, bout size was smaller for the CS+ than for the CS−, which might have been due to attempts to minimize an aversive effect, or a response to a satiating effect of the ethanol. If tolerance to an aversive effect of CS+ drinking occurred, it could manifest as an increase in CS+ bout size with experience. This did occur, but bout size increased for the CS− as well, suggesting little differentiation of response to ethanol- and water-paired flavors, which is not consistent with stimulus-controlled tolerance. Furthermore, the animals did not increase their daily intake of the CS+ during training, as might be expected if they were

becoming more tolerant. If the animals did become more tolerant of a mild negative effect, this may not have been sufficient to overcome the initially learned association with the CS+ flavor, When offered the choice, they drank more of the CS− than the CS+, and they self-infused less than half their "tolerated" daily training dose during the preference tests. The group's response thus looks like an aversion based on ethanol, yet the increasing size of CS+ bouts is not typical of aversive responses. A recent study (Ford et al., 2002) found that rats with a continuous choice of 10% ethanol and water shifted their bout patterns over time, consuming ethanol in fewer but larger bouts, just as the Plain10 rats did. This altered pattern was interpreted as initiation of ethanol drinking in the absence of specific techniques such as sucrose fading.

Bout numbers also varied with flavor quality and ethanol concentration, so that the number of daily trials might explain some of the differences in resulting preferences. The sweet group in Experiment 1 consumed the CSs in more daily bouts than the plain group, and the Sweet5 group drank the CS+ in more bouts than the Plain10 group in Experiment 2. These differences are likely to be driven by the sweet flavor, given the sweet group's shift to intake patterns like those of the plain group when their saccharin was removed. If the number of bouts was an important determinant of the resulting strength of preference, then training a plain group twice as long as a sweet group might equate them. However, the Sweet5 group in Experiment 2 displayed a CS+ preference after 10 training days that was greater than that displayed by the plain group in Experiment 1 after 20 days of training (80% versus 62%), so it seems unlikely that the total number of bouts over the course of training is a critical factor.

Bout number may serve a largely regulatory role in this situation. Behavioral work with many drugs supports the idea that animals work to maintain a preferred level of drug, and intake patterns are the rats' primary means of controlling ethanol levels. Rats drinking ethanol maintain a constant bout size when ethanol concentration is constant (e.g., Gill et al., 1986) and reduce bout size when concentration is increased (Samson et al., 1992). However, there are hints that this behavioral tactic is under partial control of flavor quality: alcohol-preferring P rats drinking ethanol adjust bout size to ethanol concentration to attain a constant blood ethanol (Murphy et al., 1986), but P rats drinking flavored solutions with concurrent intragastric ethanol, as in the present study, attained higher blood levels with higher infused ethanol concentrations (Waller et al., 1984). In the present studies, the quality of the flavor appears to determine bout size fairly strongly when ethanol concentration is the same (Experiment 1), given the increased sweet bout size relative to unsweetened flavors. However, in Experiment 2, Plain10 bouts were unexpectedly as large as Sweet5 bouts. If sweet taste and ethanol concentration produce changes in bout size or dose that are poorly controlled by immediate postingestive feedback, then the time until the next bout is the primary means of regulating the ethanol dose. Increasing the dose per bout with sweetness or concentration of the ethanol would then require prolonging the interbout interval, which translates to a limit on the number of bouts per day.

Sweet taste may enhance flavor preference conditioning by altering ethanol absorption or metabolism in a way that increases ethanol's rewarding effects. For example, modulation of blood ethanol levels might improve the net rewarding action by eliminating an aversively high peak ethanol level. A number of studies have evaluated the effects of sweetening ethanol on its postingestive handling. When such effects are found, they appear to be limited to sugars, rather than sugar substitutes like saccharin. For example, consumption of an ethanol–sugar mixture was greater than consumption of ethanol–saccharin or plain ethanol, but sampled blood ethanol levels for the two sweetened solutions were similar (Matthews et al., 2001; Roberts et al., 1999). Thus, it appears unlikely that saccharin's improvement of flavor preferences is due to a direct alteration of ethanol metabolism.

In studies of animal learning, saccharin has been regarded as a source of reward and of memory facilitation (Messier and White, 1984; Stefurak and van der Kooy, 1992). However, the general paradigm for the production of conditioned preferences or enhancement of memory for learned tasks has involved a comparison of saccharin and no-saccharin conditions. While this is also true in the present studies, within the sweet conditions both of the stimuli to be distinguished are saccharin-sweetened. Thus, saccharin must exert its enhancing effect in spite of its action on both the ethanol-paired and water-paired flavors. This might occur because saccharin increases the salience or intensity of the CS flavors and thereby enhances the animal's attention to the flavors, rendering them more associable with their postingestive effects. Sweet taste is reported to activate dopamine and opioid "reward" systems in the brain, which are also implicated in alcohol appetite (Koob et al., 1998; Mark et al., 1994; Slawecki et al., 1997; Yamamoto and Sawa, 2000). Therefore, it may be the interactive effects of sweet taste and infused ethanol on central reward systems that facilitate flavor preference conditioning. The robust conditioned preferences produced by the intragastric training procedures used here provide a model system to explore these and other alternative explanations of the sweetness effect.

The present data suggest that at least part of the success of oral sucrose-fading procedure for inducing ethanol intake is to increase the animals' exposure to ethanol in a context (i.e., sweet taste) that enhances its reward. In addition, oral sucrose and saccharin fading procedures can confer the effects of their attractive flavors on ethanol by flavor–flavor conditioning (Fanselow and Birk, 1982; Holman, 1975), and sucrose can additionally provide powerful postingestive reward to supplement these actions. By the time the sucrose-trained rat is drinking ethanol in water, it has a rich history of association

with sweet taste and carbohydrate calories in addition to ethanol's pharmacological and caloric effects.

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