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Flavor preferences conditioned by intragastric ethanol with limited access training

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Abstract

In a prior study, ad libitum fed rats learned a strong preference (90%) for a flavored saccharin solution (conditioned stimulus, CS+) paired with concurrent intragastric (IG) infusions of 5% ethanol over another flavor (CS−) paired with water infusions in unlimited access sessions (22 h/day). The present study expanded the investigation of ethanol-conditioned preferences to limited access sessions (30 min/day). Experiment 1 revealed that ad lib or food-restricted rats failed to develop a CS+ preference using the same CS solutions (0.05% Kool-Aid + 0.2% saccharin) and IG infusions that were effective with long-term training. Experiments 2 and 3 mimicked the parameters from a report of successful ethanol conditioning in deprived rats: ethanol (0.5 g/kg) or water was infused intragastrically 5 min before access to sweetened CS solutions flavored with HCl or NaCl. Rats learned to prefer the ethanol-paired CS+ when the flavors were mixed with 5% sucrose but not when mixed with 0.2% saccharin. Experiment 4 revealed that 5% sucrose solutions flavored with 0.25% Kool Aid also supported flavor preference conditioning by IG ethanol (0.5 g/kg). CS+ preferences were obtained in rats trained with ethanol infused 5 min before or concurrent with CS+ intake, but not in rats trained with ethanol infused 30 min before CS+ intake. These data confirm that flavor preferences can be conditioned by IG ethanol using a limited access procedure. However, in contrast to 22 h/day training, 30 min/day training requires more intense CS flavors and a nutritive sweetener. The preference reinforcing actions of ethanol may develop slowly and are thus most effective with long training sessions or when intense CS flavors are used in short training sessions.
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1. Introduction

There are numerous reports of rats developing aversions to flavors that have been paired with the postingestive actions of ethanol (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). This is not surprising, given that ethanol has dysphoric effects at sufficiently high concentrations. However, in some studies, ethanol produced conditioned flavor preferences in rats (Ackroff and Sclafani, 2001a, 2002; Cunningham and Niehus, 1997; Deems et al., 1986; Fedorchak and Bolles, 1987; Mehiel and Bolles, 1984; Sherman et al., 1983; Waller et al., 1984), which is consistent with other evidence showing that ethanol is reinforcing to rats when appropriately administered. Since humans acquire preferences for the distinctive flavors of particular alcoholic beverages, ethanol-conditioned flavor preferences in rats are of considerable interest in modeling the acquisition and persistence of ethanol appetite.

We recently reported that intragastric (IG) infusions of ethanol can produce a significant flavor preference in non-deprived, outbred Sprague–Dawley rats, a relatively low-drinking strain (Ackroff and Sclafani, 2001a, 2002). The rats were trained 22 h/day with one flavored solution (conditioned stimulus, CS+, e.g., grape) paired with IG infusions of 5% v/v ethanol, and on alternate days with a different flavored solution (CS−, e.g., cherry) paired with IG water infusions. In subsequent two-bottle choice tests, the rats strongly preferred (70–90%) the CS+ to the CS− solution when the CS+ remained paired with IG ethanol (reinforced test) as well as when the CS+ was paired with IG water (extinction test). Furthermore, the rats continued to

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prefer the CS+ in reinforced tests as the concentration of the infused ethanol was increased to 24% (Ackroff and Sclafani, 2001a). Yet, rats that were initially trained with 10% ethanol displayed a conditioned aversion rather than a preference (Ackroff and Sclafani, 2002). In addition to ethanol concentration, the quality of the CS flavor was also found to be an important variable. Rats trained with sweetened CS solutions (e.g., grape–saccharin solution) acquired stronger preferences than did rats trained with unsweetened solutions (e.g., grape–water solution) (Ackroff and Sclafani, 2002).

The present study further characterized the flavor preference conditioning effect of IG ethanol by using short limited access (30-min) rather than long unlimited access (22 h) training. Two prior studies by Sherman and coworkers reported preference conditioning by IG ethanol in rats trained 20 min/day (Deems et al., 1986; Sherman et al., 1983), but the interpretation of these results is open to question. That is, the CS solutions were flavored 5% sucrose solutions and the rats consumed more of CS+ sucrose solution on IG ethanol trials than CS− sucrose solution on IG water trials. It is possible, therefore, that the elevated sucrose intake may have contributed to the CS+ flavor preference. Experiment 1 of the present study determined whether IG ethanol infusions would condition a preference for a flavored saccharin solution in rats trained 30 min/day as it does in rats trained 22 h/day (Ackroff and Sclafani, 2001a, 2002). In our long-term studies, the rats had food available ad libitum, whereas in the short-term studies of Sherman et al., the rats were food-restricted. Therefore, the first experiment also investigated the impact of deprivation state on flavor conditioning. The remaining three experiments explored the effect of other procedural variables on preference conditioning by IG ethanol. These included the effects of sweetener (sucrose vs. saccharin), CS flavor, and interval between the IG ethanol infusion and CS consumption.

2. Experiment 1

In a recent study (Ackroff and Sclafani, 2002), ad libitum fed rats trained 22 h/day with flavored saccharin solutions paired with IG infusions of 5% ethanol or water displayed a 90% preference for the ethanol-paired flavor (CS+) in subsequent two-bottle choice tests. Experiment 1 determined if rats would also acquire a CS+ preference using the same CS flavors and IG infusions if training and testing were limited to 30-min/day sessions. The impact of deprivation state (food ad lib vs. deprived) on preference conditioning was also examined. Prior conditioning experiments using IG carbohydrate infusions (glucose, sucrose, Polycose) have obtained robust CS+ preferences in rats trained with flavored saccharin solutions in 22-h and 30-min sessions, and in rats trained nondeprived as well as food-deprived (Ackroff and Sclafani, 1994, 2001b; Azzara et al., 2001; Elizalde and Sclafani, 1990; Lucas et al., 1998; Pérez et al., 1996, 1998). Because deprived rats normally consume more than nondeprived rats, training intake and infusions were limited to equate the CS intakes of the two groups.

2.1. Subjects

Male Sprague–Dawley rats ($n = 29$) were born in our lab from randomly paired outbred parents purchased from Charles River Laboratories (Wilmington, MA); they weighed 358–501 g at surgery. The rats were housed in stainless steel hanging cages with ad lib access to water in rooms maintained on a 12:12 light/dark cycle (lights on 1000 h) at 21 °C. Access to powdered chow (No. 5001, PMI Nutrition International, Brentwood, MO; 3.3 kcal/g) was either ad libitum or restricted, as described in the procedure.

2.2. Surgery

The rats were anesthetized and fitted with a gastric catheter according to a technique adapted from Davis and Campbell (1975). Briefly, silastic tubing (id: 1.02 mm; od: 2.16 mm) was inserted into the fundus of the stomach and secured with sutures and polypropylene mesh. The tubing was routed under the skin to the head where it was connected to a luer-lock assembly fixed onto the skull with stainless steel screws and dental cement. Intramuscular penicillin (30,000 U, 0.1 ml) was given following surgery.

2.3. Apparatus

Training and testing took place in plastic cages ($23 \times 24 \times 31.5$ cm) with a stainless steel grid floor and slotted plastic top. The slot permitted plastic tubing protected by a flexible stainless steel spring to connect the output port of a swivel on a counterbalanced lever to the rat's luer-lock assembly. The swivel's input port was connected by plastic tubing to a 30-ml syringe positioned in a variable-speed pump. The rats had access to one or two stainless steel drinking spouts through holes in the front wall of the cage, centered 32 mm apart. The spouts were attached to drinking bottles mounted on motorized holders that positioned the spouts at the front of the cage at the start of the sessions and retracted them at the end of the sessions. Spill trays were located below the bottles. Licking behavior was monitored by an electronic drinkometer interfaced to a microcomputer that activated the syringe pump within 3 s of the rat's initiation of drinking. The infusion rate was 1.3 ml/min and the oral intake-to-infusion was maintained at approximately 1:1 by the computer software.

2.4. Solutions

The oral test fluids (conditioned stimuli, CSs) were 0.2% (w/w) sodium saccharin solutions (Sigma, St. Louis, MO) flavored with 0.05% (w/w) grape or cherry unsweetened Kool-Aid drink mixes prepared with tap water (General

Foods, White Plains, NY). The flavor (CS+) paired with IG ethanol and the flavor (CS −) paired with IG water were counterbalanced across subjects. Unflavored 0.2% saccharin was also available to drink during pretraining. Left/right positions of the flavored solutions were counterbalanced across days. The infusates were tap water and 5% (v/v) 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

To insure that the rats would readily drink the CS solutions during the 30-min training and test sessions, they were adapted to drink unflavored saccharin in the test cages under food-deprived and nondeprived conditions. They were first housed in the cages for 20 h with access to 0.2% saccharin solution, water, and food; the saccharin and water drinking spouts were automatically positioned to the front of the cages for 30 min every hour. All rats were then food-restricted (to 90% ad lib body weight) and adapted to drink the saccharin solution during six 30-min/day sessions in test cages. Their daily chow ration and ad lib water were provided in the home cage 1 h after the session. Ad lib chow was then restored, and the rats were given an additional five 30-min sessions; chow was available in the home cage, except for 1 h before to 1 h after the 30-min sessions. Gastric catheter surgery was performed and, after several recovery days, 30-min/day pretraining sessions with saccharin were resumed. For the first 3 days, the rats were not infused and then for the next four sessions they were co-infused with water intragastrically when they drank the saccharin solution. The rats were then divided into two groups matched for saccharin intake and body weight. The ad lib group ($n = 14$) continued to receive unlimited chow, and the deprived group ($n = 15$) was placed on food restriction for the remainder of the experiment. They were reduced to the target weight (90% of ad lib weight) during the next 5 days, while both groups continued with daily 30-min saccharin sessions.

Flavor conditioning involved giving the rats eight one-bottle training sessions (30 min/day) with the CS+ solution paired with IG 5% ethanol on even-numbered days and the CS− solution paired with IG water on odd-numbered days. Both the oral and IG amounts were limited to a maximum of 6 ml. The left–right positions of the CS solutions varied following an ABBA sequence. Following one-bottle training sessions, a two-bottle preference test was conducted with unlimited access to the CS+ and CS− solutions for two 30-min sessions. The left–right positions of the bottles were reversed on the second day. During this test, the rats were not infused. A second cycle of eight training sessions and two test sessions was then conducted.

Training and test sessions were conducted between 0900 and 1230 h. Food was returned to all animals in their home cages 1 h after the end of the session. The CS intakes were measured to the nearest 0.1 g (corrected for spillage) and IG infusions were measured to the nearest 0.5 ml. The training conditions for this and subsequent experiments are summarized in Table 1. The experimental protocol was approved by the Institutional Animal Care and Use Committee of Brooklyn College.

2.6. Data analysis

Intake data during one-bottle training and the two-bottle test sessions were averaged over days within training/test cycles and analyzed using repeated-measures analyses of variance. Individual comparisons were evaluated using simple main effects, Newman–Keuls or *t* tests as appropriate. A significant difference between the two-bottle intakes of the CS+ and CS− was taken as primary evidence for a preference. The two-bottle intakes of the individual rats were also expressed as percent CS+ intakes (CS+ intake/total intake × 100) and analyzed following an inverse sine transformation (Kirk, 1995).

2.7. Results

The groups did not differ in training intakes and consumed similar amounts of CS+ and CS− solutions (Table 2).

Table 1
Summary of groups and training conditions in the three experiments

Experiment	Groups	CS solutions	Infusion	Sequence
1	Deprived, ad lib	0.05% cherry and grape Kool-Aid in 0.2% saccharin (6 ml limit)	5% ethanol (6 ml limit)	Simultaneous co-infusion, amount matched to CS intake
2	Sucrose	0.1% HCl and 3% NaCl in 5% sucrose (no limit)	5% ethanol (0.5 g/kg)	Infusion followed 5 min later by CS access
3	Sucrose	0.1% HCl and 3% NaCl in 5% sucrose (no limit)	5% ethanol (0.5 g/kg)	Infusion followed 5 min later by CS access
	Saccharin	0.1% HCl and 3% NaCl in 0.2% saccharin (no limit)	5% ethanol (0.5 g/kg)	Infusion followed 5 min later by CS access
4	CS0	0.25% cherry and grape Kool-Aid in 5% sucrose (no limit)	5% ethanol (0.5 g/kg)	Infusion started when rat begins drinking
	CS5, CS30	0.25% cherry and grape Kool-Aid in 5% sucrose (no limit)	5% ethanol (0.5 g/kg)	Infusion followed 5 or 30 min later by CS access

There was a small increase in average intake from the first to the second training cycle [4.5–4.9 g; Cycle: $F(1,27)=4.40$, $P<.05$]. Body weight differed as a function of group [Deprived 389 g, Ad lib 492 g; $F(1,27)=49.20$, $P<.0001$] and cycle [$F(1,27)=15.15$, $P<.001$] because the ad lib group gained weight over time. Because the food-restricted rats were smaller than the ad lib rats, but drank similar amounts, the average self-administered ethanol doses per session were greater for the deprived group (0.47 g/kg) than the ad lib group (0.36 g/kg) [$F(1,27)=4.54$, $P<.05$].

Intakes in the preference tests are shown in Fig. 1. Overall, the rats drank significantly less of the CS+ flavor than the CS− flavor [4.4 vs. 6.5 g/30 min, $F(1,27)=6.30$, $P<.05$]. The groups did not differ; the only significant effects were a main effect of cycle [greater intakes in the first test, $F(1,27)=4.75$, $P<.05$] and the interaction of group and cycle [the ad lib group drank less overall in the second test; $F(1,27)=23.98$, $P<.001$]. The percent CS+ intakes (Fig. 1) of the groups did not differ and the effect of cycle and the interaction was not significant. Averaged over groups and cycles, the rats consumed 46% (±4%) of their total intake as CS+. Seven of 15 deprived rats preferred the CS+ by 60% or more (range 62–77%) in the first test, but only two rats preferred it in the second test (70% and 79%). Only 1 of the 14 ad lib rats expressed a preference (93%) in the first test, but this increased to six rats in the second test (range 62–78%).

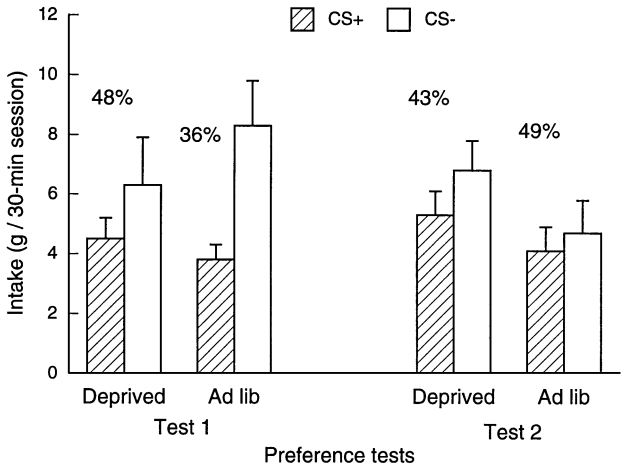

Fig. 1. Mean ± S.E.M. CS solution intakes in the two-bottle preference tests of Experiment 1. The deprived group was maintained on restricted chow rations; the ad lib group had unrestricted home cage chow access. The CS solutions were 0.05% grape and cherry Kool-Aid in 0.2% saccharin. Numbers atop the bars are mean percentage intakes of the CS+.

2.8. Discussion

This experiment revealed a weak aversion to the CS+ flavor that was paired with IG infusions of 5% ethanol. This is in marked contrast to the significant preference displayed by rats trained and tested 22 h/day with the same CS solutions and IG infusions (Ackroff and Sclafani, 2001a). Deprivation state does not account for these discrepant results, since the ad lib and deprived groups displayed a similar lack of preference for the CS+. This occurred despite a probable difference in ethanol's effects: the deprived rats presumably had empty stomachs and therefore more rapid absorption and higher blood ethanol levels than the ad lib rats. The rats in the present study had limited access to the CS solutions (6 ml) during training and their self-administered doses of ethanol could be construed as ineffectively small. Note, however, that most rats did not consume all of the available CS+, particularly in the first cycle. Furthermore, the 0.47-g/kg dose for the deprived group was very close to the 0.5-g/kg dose that conditioned an ethanol-based preference in the early studies of Sherman et al. (Deems et al., 1986; Sherman et al., 1983).

Table 2
Mean training intakes (g) in one-bottle sessions

Experiment	Group		CS+	CS−
1	Deprived	Train 1	4.6	4.6
		Train 2	4.8	5.1
	Ad lib	Train 1	4.4	4.5
		Train 2	4.7	4.9
2	Sucrose		7.2	4.7
3	Sucrose		8.0	7.1
	Saccharin		3.4	4.2
4	CS0		12.9	13.4
	CS5		16.5	17.0
	CS30		15.7	15.3

3. Experiment 2

The mild CS+ aversion displayed by the food-restricted rats in Experiment 1 conflicts with the significant ethanol-conditioned flavor preference reported in the earlier study of Sherman et al. (1983). Their training methods differed in several respects from the one used in the first experiment. Most notably, Sherman et al. intubated the rats with a fixed dose (0.5 g/kg) of 5% ethanol or an equivalent volume of water several minutes prior (exact interval not specified) to presenting the CS solutions. In addition, their CS solutions were sweetened with 5% sucrose rather than saccharin. To determine if the "backward" conditioning protocol and the use of sucrose were important variables, we conducted an experiment with rats trained with 5% sucrose solutions flavored with 0.05% Kool-Aid flavors. In training, the CS+ solution was presented 5 min after the rats were given an IG infusion of 0.5 g/kg ethanol. This conditioning procedure produced only a weak CS+ preference (61%) after the first training cycle which disappeared after a second training cycle (Ackroff and Sclafani, unpublished findings). The Sherman et al. study used 0.1% HCl and 3% NaCl as CS flavors added to 5% sucrose, which may be more intense or distinctive than the Kool-Aid flavors used in our experiments. (The latter share a citric acid taste and

differ primarily in odor.) Experiment 2, therefore, trained rats with the same CS solutions as used by Sherman et al. (1983). Also, to facilitate comparison with the Sherman et al. study, this and subsequent experiments used food-restricted rats.

3.1. Method

Male Sprague–Dawley rats ($n=15$; 349–408 g) purchased from Charles River Laboratories were used. They were fitted with gastric catheters as in Experiment 1. Two weeks after surgery, the rats were familiarized with saccharin by giving them ad lib access to 0.2% saccharin, water, and chow for 3 days. Several rats drank less saccharin than water and to enhance the saccharin preference, sucrose was added to the saccharin solution (1 day each with 2% and 1% sucrose). A final day of plain saccharin ended this exposure phase. The rats were then given a 20-h session in the test cages with 0.2% saccharin presented 30 min every hour to adapt them to the bottle retractors as in Experiment 1. For the next 10 days, they were adapted to food restriction (to 90% of ad lib body weight) and to 30-min sessions with 0.2% saccharin; on the last 6 days they were infused with water (1% of body weight; 3–4 ml) 5 min before the saccharin was presented. Food rations were given to the animals in their home cages 1 h after the end of the daily session.

Flavor conditioning consisted of 12 daily sessions with an infusion (1% of body weight; water or 0.5 g/kg dose of 5% ethanol) ending 5 min prior to 30-min access to the CS solutions; solution intakes were unlimited. The CSs were 5% sucrose solutions flavored with 0.1% HCl or 3% NaCl (w/v); the flavor–infusate pairs were counterbalanced across subjects. Water infusions preceded CS− access on odd-numbered days and ethanol infusions preceded CS+ access on even-numbered days. Following one-bottle training sessions, a two-bottle preference test was conducted with the CS+ vs. CS− solutions for two 30-min sessions; the rats were not infused on these test days. They were then given four additional one-bottle training sessions with the CS+ and CS− paired with infusions followed by a second two-bottle preference test which lasted for six sessions.

3.2. Results

During the initial one-bottle training, the rats drank more of the CS+ than the CS− [mean intakes 7.2 and 4.7 g, $F(1,14)=21.39$, $P<.001$] and their intakes increased over days [$F(5,70)=21.02$, $P<.0001$]. Intake of the CS+ increased more than that of the CS− [interaction, $F(5,70)=7.81$, $P<.0001$]. The rats drank similar amounts of CS+ and CS− in the first two training sessions (3.3–3.4 g/30 min), but more CS+ than CS− in subsequent training sessions ($P<.05$).The mean energy yields per session (from sucrose and ethanol) were 2.5 kcal on CS+ days and 0.9 kcal on CS− days. In the second one-bottle training period, the mean intakes were very similar to the end of the first training period, with CS+ intake greater than CS− intake [9.6 vs. 6.1 g, $t(14)=3.18$, $P<.01$].

The results of the two-bottle test sessions are shown in Fig. 2. Overall, the rats drank more CS+ than CS− [mean 8.7 and 3.3 g/30 min, $F(1,14)=16.00$, $P<.01$], and intakes did not change from Test 1 to Test 2. The percent CS+ preferences in the two tests were 73% (±5%) and 71% (±5%), respectively; 12 of the 15 rats preferred the CS+ by at least 60% in the first test (range 67–95%) and 11 in the second test (range 62–96%). Analysis across sessions within tests showed only a significant flavor effect [$F(1,14)=15.39$, $P<.01$] in the first test. In the second, 6-day test, intake increased across days [$F(5,70)=5.63$, $P<.001$] with no interaction.

3.3. Discussion

This experiment revealed a significant preference for the ethanol-paired CS+ flavor which replicates the results previously reported by Sherman et al. (1983). The present data further demonstrate that the CS+ preference was persistent in that it remained stable over the course of six two-bottle sessions (Test 2) in the absence of IG ethanol infusions. These data contrast with the weak CS+ avoidance obtained in Experiment 1, which used a different pair of flavors presented in noncaloric saccharin solution and paired with concurrent IG infusions. Thus, the difference in results could be due to flavor set, sweetener type, timing of infusion relative to CS intake, or some combination of these factors.

The present data also replicate the findings of Sherman et al. (1983) in that the rats consumed significantly more of the CS+ solution than of the CS− solution during one-bottle

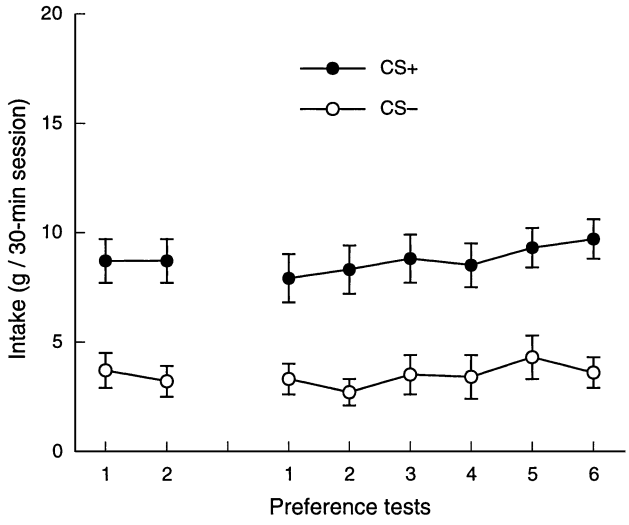

Fig. 2. Mean ± S.E.M. CS solution intakes during the two-bottle preference tests of Experiment 2. The individual days of testing are represented: the two days of Test 1 followed the first 12 days of training, and the 6 days of Test 2 followed an additional 4 days of one-bottle training. The CS solutions were 0.1% HCl and 3% NaCl in 5% sucrose.

training. The ethanol-induced increase in CS+ acceptance developed over sessions, suggesting that it was a conditioned rather than an unconditioned response to the IG ethanol infusion. (CS+ acceptance refers to increased one-bottle intake of CS+ relative to CS−, and is distinguished from CS+ preference which refers to differential intake in two-bottle choice tests.) The increased CS+ acceptance may reflect an ethanol-conditioned enhancement in the rats' evaluation of the CS+ flavor as also expressed by their CS+ preference in the two-bottle test (see Sclafani, 2001). Consistent with this view, there was a positive correlation between one-bottle CS+ acceptance (as measured by CS+ minus CS− difference scores) and two-bottle CS+ preference (percent CS+ intake; r^2=.31, $P<.05$). However, other factors may have contributed to the increased CS+ acceptance produced by the ethanol infusions. Some human studies report that alcoholic beverages have an "appetizer" effect that increases subsequent food intake (Westerterp-Plantenga and Verwegen, 1999; Yeomans et al., 1999). One interpretation offered for this effect is that subjects may increase their food intake to reduce the pharmacological consequences (e.g., "lightheadedness") of the ethanol (Westerterp-Plantenga and Verwegen, 1999). Whatever the cause of the increased CS+ acceptance, it is possible that rats' increased energy intake from sucrose on CS+ training days may have contributed to their subsequent preference for the CS+ flavor over the CS− flavor. This possibility was explored in Experiment 3.

4. Experiment 3

As noted above, the interpretation of the ethanol-conditioned flavor preference obtained in Experiment 2 and in the Sherman et al. studies is problematic because the greater sucrose intake on CS+ training days may have contributed to the rats' CS+ preference. The present experiment determined if IG ethanol conditioning is dependent upon the use of a caloric sweetener by comparing preference conditioning in rats trained with flavored sucrose and flavored saccharin as CS solutions. Except for the difference in sweeteners, the training flavors and procedure were similar to the first part of Experiment 2.

4.1. Method

Male Sprague–Dawley rats ($n=32$; Charles River Laboratories) weighing 362–486 g were fitted with gastric catheters as in Experiment 1. The rats were adapted to drink an unflavored saccharin solution as in the prior experiment. They were then divided into two groups equated for body weight and saccharin intake. The sucrose group was trained with 0.1% HCl and 3% NaCl flavored 5% sucrose solutions paired with IG infusions of ethanol (0.5 g/kg) or water for 12 one-bottle sessions (30 min/day). The saccharin group was similarly trained except that the flavors were presented in 0.2% saccharin solutions. Following training, the rats were given a two-bottle test with the CS+ vs. CS− for six sessions.

4.2. Results

Overall, the sucrose group consumed more of the CS solutions during one-bottle training than did the saccharin group [$F(1,30)=28.64$, $P<.001$]. Intakes increased over days [$F(5,150)=32.30$, $P<.001$], and the sucrose group's intake continued to increase after the saccharin group's had stabilized [interaction, $F(5,150)=5.64$, $P<.001$]. In contrast to the differential intakes in Experiment 2, the one-bottle intakes of the CS+ and CS− did not differ for either group. Table 2 shows that the sucrose group drank about twice as much as the saccharin group. Corresponding energy intakes on CS+ and CS− sessions for the sucrose group were 2.7 and 1.4 kcal, respectively; the saccharin group received 1.2 kcal ethanol energy on CS+ days and no energy on CS− days.

In the two-bottle choice test, overall CS+ intake exceeded CS− intake [$F(1,30)=21.73$, $P<.0001$], and the sucrose rats drank more than the saccharin rats [$F(1,30)=47.71$, $P<.0001$]. There was a Group × CS interaction and individual tests indicated that the sucrose group, but not the saccharin group, drank more CS+ than CS− [Fig. 3; $F(1,30)=6.16$, $P<.05$]. The percent CS+ intake of the sucrose group also exceeded that of the saccharin group but this difference was not significant [64% (±4%) vs. 59% (±5%)]. Ten of 16 sucrose rats preferred the CS+ by at least 60% (range 60–85%) and 9 of 16 saccharin rats preferred the CS+. CS+ intakes were stable across the six sessions of the two-bottle test (data not shown).

4.3. Discussion

In confirmation of Experiment 2, the rats in the sucrose group displayed a significant preference for the ethanol-

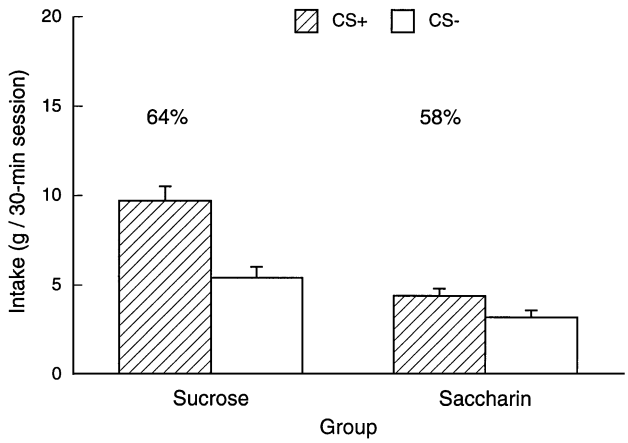

Fig. 3. Mean ± S.E.M. CS solution intakes in the two-bottle preference test of Experiment 3. The CS solutions were 0.1% HCl and 3% NaCl in 5% sucrose (sucrose group) or 0.2% saccharin (saccharin group). Numbers atop the bars are mean percentage intakes of the CS+.

paired CS+ flavor. In contrast, the saccharin rats did not consume more CS+ than CS − in the two-bottle test. These data indicate that a caloric sweetener is more effective than a noncaloric sweetener in supporting ethanol-conditioned flavor preferences. The interpretation of this result in not straightforward, however, because the sucrose rats consumed almost twice as much of the CS solutions during training and testing than did the saccharin rats. Their elevated CS intakes may have resulted because 5% sucrose has a more preferred taste than does 0.2% saccharin (Smith and Sclafani, 2002) and/or because of the postingestive reinforcing action of sucrose (Sclafani, 2001).

The CS+ preference displayed by the sucrose rats in the present experiment was somewhat weaker than that obtained in Experiment 2 (64% vs. 72%, $P>.05$). This may have occurred because the sucrose rats, unlike those in the previous experiment, did not consume more CS+ than CS− during training. Since similar training procedures were used in the two experiments, it is unclear why increased CS+ acceptance was observed in Experiment 2 but not in the present experiment. Note that the one-bottle CS+ intakes were similar in the two experiments, but that CS− intake was higher in the present experiment than in Experiment 2. These differences aside, the present data indicate that IG ethanol can condition a significant CS+ preference even if training intakes of the CS+ and CS− solutions do not differ.

5. Experiment 4

The results of Experiments 2 and 3 along with those of Sherman et al. (1983) indicate that ethanol-conditioned preferences can be obtained using limited access training sessions if rats are trained with 5% sucrose solutions flavored with 0.1% HCl and 3% NaCl. In contrast, 5% sucrose flavored with 0.05% Kool-Aid flavors produced only a weak and transitory CS+ preference in rats trained in daily 30-min sessions (Ackroff and Sclafani, unpublished data). It may be that HCl and NaCl are more effective as CS flavors than Kool-Aids because they are distinctive stimuli or because they are more intense stimuli, at the concentrations used, and thus are more readily associated with differential postingestive reinforcing effects. A second study from Sherman's laboratory (Deems et al., 1986) reported a significant CS+ preference with 5% sucrose solutions containing 3% flavor extracts (banana and almond/lemon). Thus, short-term ethanol conditioning is possible using intense odor-based as well as taste-based CS flavors. Although 0.05% Kool-Aid flavor mixes were effective in our long-term ethanol-conditioning studies as well as in many short-session studies involving IG carbohydrate or fat infusions, it may be that more concentrated flavors are needed for short-term ethanol conditioning.

To test the possibility that flavor salience is an important feature for limited access preference conditioning with ethanol, the present experiment determined the efficacy of concentrated Kool-Aid flavors to support conditioning when added to 5% sucrose solutions. Several conditioning studies involving orally consumed ethanol used Kool-Aid flavors at concentrations averaging 0.25% (Fedorchak and Bolles, 1987; Mehiel, 1991; Mehiel and Bolles, 1984, 1988), and therefore this concentration was used in the present experiment. It was of interest to identify effective CS flavors other than HCl and NaCl because of the limited number of taste modalities available for use as CSs and because 3% NaCl is hypertonic and has postingestive consequences (Rabe and Corbit, 1973).

A second aim of Experiment 4 was to determine the importance of the temporal interval between IG ethanol infusion and presentation of the CS+ flavor on preference conditioning. Experiments 2 and 3 used a 5-min unconditioned stimulus (US) to CS interval based on the prior work (Sherman et al., 1983). Note that Sherman et al. used a backward conditioning design for practical, not theoretical reasons; i.e., it was necessitated by the manual intubation procedure used to deliver the ethanol. Nevertheless, recent place conditioning findings in mice indicate that IG infusion of ethanol 5 min prior to exposure to the CS+ side is more effective than a 0-delay procedure in conditioning a place preference (Cunningham et al., 2002). Although comparable results were not obtained with rats, ethanol-conditioned place preferences are more difficult to obtain in rats compared to mice (Bormann and Cunningham, 1998). The present experiment therefore compared flavor preference conditioning in rats by IG ethanol infusions presented 0, 5, and 30 min prior to presentation of the CS+ flavor. The 30-min interval was used because this interval was effective in obtaining an ethanol-conditioned place preference in mice (Cunningham et al., 1997), and also supports flavor preference conditioning by IG glucose infusions in rats (Sclafani et al., 1998).

5.1. Method

Male Sprague–Dawley rats (Charles River Laboratories) weighing 343–403 g were fitted with gastric catheters. The rats were pretrained to drink an unflavored saccharin solution in the test cages as in Experiments 2 and 3. They were then given 12 one-bottle training sessions (30 min/day) with the CS+ and CS − solutions followed by four two-bottle test sessions. The CS solutions consisted of 5% sucrose solutions flavored with 0.25% grape or cherry Kool-Aid. The CS5 group ($n=15$), which was run first, had the CS+ and CS− solutions presented 5 min after the end of the IG infusions of 5% ethanol (0.5 g/kg) and water, respectively. The CS30 group ($n=13$) had the CS solutions presented 30 min after the end of the IG infusions. The CS0 group ($n=15$), which was run concurrently with the CS30 group, was given access to the CS+ or CS− solution and each rat was infused with ethanol or water, respectively, after it had made 10 licks. Although this made the infusion contingent

on licking, it ensured simultaneous exposure to the CS and US.

5.2. Results

The one-bottle intakes (Table 2) were somewhat smaller in the CS0 group, but overall the average CS intakes of the CS0, CS5, and CS30 groups did not differ significantly (13.1, 16.8, and 16.6 g/30 min, respectively). Overall, CS+ and CS− intakes did not differ, but there was a significant interaction between CS and training session [$F(5,200) = 28.00$, $P < .0001$]. Intakes of both CSs increased over training sessions, but the increase was greater for the CS− than CS+. By the end of training, CS+ intake was significantly less than CS− intake [mean of last 2 days, 17.9 vs. 20.8 g, $F(1,40) = 41.84$, $P < .0001$]. The groups did not differ in their energy intakes during one-bottle training, and overall they consumed more energy on CS+ sessions (sucrose + IG ethanol) than on CS− sessions (sucrose only) [4.0 vs. 2.9 kcal, $F(1,40) = 216.63$, $P < .0001$].

In the two-bottle preference test, the CS0 and CS5 groups, but not the CS30 group, drank more CS+ than CS− (Fig. 4). This was confirmed by a Group × CS interaction [$F(2,40) = 9.97$, $P < .001$] and simple mean effects showed that CS+ intake exceeded CS− intake ($P < .01$) in groups CS0 and CS5. Averaged over the four test sessions, percent intakes of the CS+ were 61% (±4%) and 64% (±4%) for the CS0 and CS5 groups, which did not differ but exceeded the 44% (±3%) of the CS30 group [$F(2,40) = 7.73$, $P = .001$]. The percent CS+ intakes of the CS0 and CS5 groups were stable over the four test sessions except for an unexplained loss in preference in Session 2 for the CS0 rats. The percent CS+ intakes during the last two test sessions were 68% (±5%) and 64% (±5%) for the CS0 and CS5 groups, respectively. In these sessions, 8 of 15 CS0 rats and 10 of 15 CS5 rats preferred the CS+ by at least 60% (range 63–95% CS0; 65–96% CS5); only 1 of the 13 CS30 rats expressed a CS+ preference (83%).

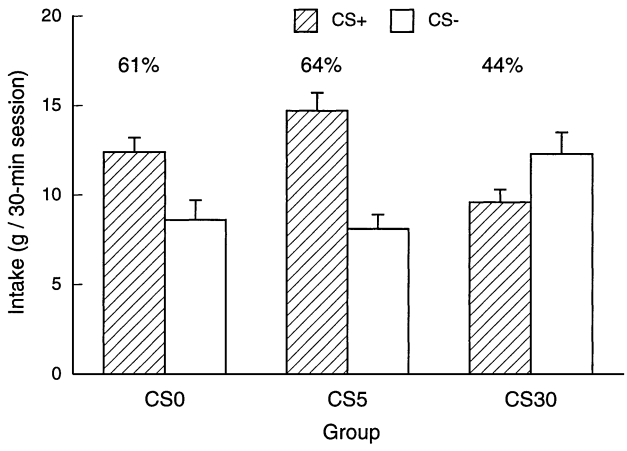

Fig. 4. Mean ± S.E.M. CS solution intakes in the two-bottle preference test of Experiment 4. The CS solutions were 0.25% grape and cherry Kool-Aid in 0.2% saccharin. The groups are named for the US–CS delay during training: the CS0 group's infusion began when they began licking the CS, whereas the CS5 and CS30 groups' access to the CS solutions began 5 and 30 min after the end of the infusion. Numbers atop the bars are mean percentage intakes of the CS+.

5.3. Discussion

The CS5 group's CS+ preference was comparable to that observed with the sucrose groups of Experiments 2 and 3. This suggests that the 0.25% Kool-Aid flavor mixes are as effective as HCl and NaCl in supporting ethanol-conditioned preferences when presented in 5% sucrose solutions. However, CS intakes were about twice as great as those obtained in the two preceding experiments. Thus, 0.25% Kool-Aid + 5% sucrose solutions are much more acceptable to rats than are HCl + sucrose and NaCl + sucrose solutions. The ability of Kool-Aid flavors, which to humans are primarily distinguishable by odor, to support flavor conditioning by IG ethanol is consistent with the earlier report of an ethanol-conditioned preference for sucrose solutions flavored with 3% flavor extracts (banana vs. almond/lemon) (Deems et al., 1986). Thus, pure tastes (salty and sour) and odor–taste mixtures (Kool-Aid mixes, flavor extracts) can serve as CSs in ethanol conditioning studies if they are sufficiently intense.

The present data also revealed that IG ethanol infusions 5 min prior to or simultaneous with presentation of the CS+ were equally effective in conditioning a flavor preference. In contrast, IG ethanol infusions 30 min prior to CS+ presentation failed to condition a flavor preference. Thus, it is not necessary to infuse ethanol several minutes prior to consumption of the CS+ to condition a preference in brief daily sessions. This is consistent with the results of long-term training sessions in which ethanol was infused intragastrically as the animals consumed the CS+ solution (Ackroff and Sclafani, 2001a).

6. General discussion

The present study confirms and extends prior reports (Deems et al., 1986; Sherman et al., 1983) that IG ethanol infusions can condition flavor preferences in food-deprived rats trained in limited access sessions. However, ethanol infusions were not effective with all CS flavors including some that supported ethanol-conditioned preferences in animals trained 22 h/day (Ackroff and Sclafani, 2001a, 2002). These effective and ineffective training procedures provide insight into the limitations of ethanol reward and are discussed in turn.

In Experiment 2, rats displayed a significant preference for a NaCl–sucrose or HCl–sucrose solution that, during training, was consumed 5 min after an IG infusion of 0.5 g/kg ethanol. This closely replicates the results obtained by Sherman et al. (1983) with the same salty–sucrose and

sour–sucrose mixtures. In their experiment, the rats were intubated with 0.5 g/kg ethanol or water a few minutes before 20-min access to the CS+ or CS− solutions. Other rats that were intubated with 1.0 g/kg ethanol failed to acquire a significant CS+ preference, whereas rats intubated with 2.0 g/kg ethanol during training displayed an aversion to the CS+ flavor. Sherman et al. assessed flavor preferences in a single two-bottle test following a series of one-bottle training sessions. Experiment 2 of the present study revealed that the CS+ preference remained stable over six consecutive two-bottle test sessions in the absence of reinforcement with IG ethanol infusions. Thus, ethanol-conditioned preferences, such as those obtained with other nutrients, appear to represent a long-lasting change in the animal's evaluation of the CS+ flavor (Elizalde and Sclafani, 1990).

As in the earlier Sherman et al. study, the rats in Experiment 2 consumed more CS+ than CS− during one-bottle training. This complicates the interpretation of the CS+ preference because the CS+ flavor was associated with greater sucrose intake, relative to the CS−, as well as IG ethanol during training. The sucrose rats in Experiment 3, however, did not drink reliably more CS+ than CS− during training although they also displayed a CS+ preference in the choice tests. Furthermore, the CS5 and CS0 rats trained with Kool-Aid flavored sucrose solutions in Experiment 4 also preferred the CS+ in choice tests although by the end of training they drank more CS− than CS+. Taken together, these results demonstrate that elevated CS+ intake, and therefore elevated sucrose energy intake, is not required for conditioning flavor preferences with IG ethanol infusions.

Experiment 4 also revealed that flavor preferences are produced by IG ethanol infusions that are concurrent with the intake of the CS+ as well as by infusions that end 5 min before CS+ presentation. The concurrent infusion procedure mimics the normal course of events when animals drink a flavored ethanol solution, i.e., the onset of the drug's postingestive actions follow (assuming some processing delay) rather than precede or coincide with the oral stimulation provided by the CS+ solution. Thus, the IG infusion technique represents a realistic model of normal ethanol drinking, and provides for precise control of dose while eliminating ethanol's flavor as a confounding factor.

While IG ethanol infusions paired close in time with the intake of flavored sucrose solutions reliably conditioned flavor preferences in the present and prior limited access studies (Deems et al., 1986; Sherman et al., 1983), they were ineffective in conditioning preferences for flavored saccharin solutions in Experiments 1 and 3. Yet, the same flavored saccharin solutions used in Experiment 1 (0.05% grape or cherry in 0.2% saccharin) supported robust ethanol conditioning in rats trained 22 h/day (Ackroff and Sclafani, 2001a). The flavored saccharin solutions were also effective CSs in many other short-term conditioning studies using various nutrient infusions (glucose, sucrose, maltodextrin, corn oil, casein: Sclafani, 1999) and thus their ineffectiveness in the present study does not reflect a general limitation of these solutions.

There are several important differences between unlimited and limited access ethanol training that may account for the different results obtained with flavored saccharin solutions. Most notably, with long-term training the rats consumed 10–20 CS bouts/day, in contrast to the single bout/day taken with short-term training. The multiple bouts and higher total ethanol dose/day would be expected to facilitate training. Bout size may also be a factor: rats in the long-term studies drank the CS+ in bouts that resulted in typical ethanol doses of 0.2–0.3 g/kg/bout (Ackroff and Sclafani, 2001a, 2002), whereas the ethanol doses/session of the ad lib and deprived groups in Experiment 1 were 0.36 and 0.47 g/kg. Yet, the 0.5-g/kg dose used in Experiments 2 to 4 conditioned preferences for flavored sucrose solutions. It may be that the postingestive reinforcing action of ethanol is weaker than other nutrients and therefore requires more salient CS flavors to obtain preference conditioning in limited access sessions.

Sucrose may potentiate ethanol conditioning in the limited access procedure for several reasons. Five percent sucrose has a more preferred taste than does 0.2% saccharin. Prior work indicates that saccharin, at its most preferred concentrations (0.2–0.4%), is "isopreferred" to only 2–3% sucrose (Smith and Sclafani, 2002). One consequence of this taste difference is that rats drank considerably more of the sucrose–CS solutions in Experiment 3 than of the saccharin–CS solutions. This greater exposure to the CS flavors may have facilitated ethanol conditioning. It is also possible that the postingestive actions of sucrose interact with ethanol to render a CS+ session more rewarding. There is some evidence for a positive effect of concurrent food in ethanol conditioning of place preferences: rats given food on both sides of the box learned to prefer the 0.5-g/kg ethanol-paired side (Stewart and Grupp, 1981, 1985). A related possibility is that the concurrent food need only be present at the beginning of ethanol exposure and then can be removed or faded out (Cunningham and Niehus, 1997). In our 22-h/day experiments in which IG ethanol conditioned preferences for flavored saccharin solutions, the rats had continuous access to chow, so that ethanol may have usually been taken in the context of chow nutrients (Ackroff and Sclafani, 2001b, 2002). The mechanism by which concurrent food enhances ethanol reward is not clear; one possibility is that it favorably alters ethanol absorption, perhaps by altering gastric emptying and changing the time course of the rise in blood ethanol (e.g., Matthews et al., 2001; Roberts et al., 1999, but see Czachowski et al., 1999; Gauvin, 1999). Alternatively, the independent rewarding effects of ethanol and nutrients may summate to enhance learning.

In addition to sucrose, intense cue flavors appear to be necessary for ethanol conditioning in the limited access situation. That is, 0.05% Kool Aid flavors added to sucrose were minimally effective (Ackroff and Sclafani, unpub-

lished findings), whereas 0.25% Kool Aid flavors, 3% flavor extracts (Deems et al., 1986), and 0.1% HCl and 3% NaCl (Experiments 2 and 3, Sherman et al., 1983) all supported ethanol conditioning. Strong cue flavors may facilitate ethanol conditioning in limited access training because they produce a more persistent memory trace. This would facilitate conditioning if the rewarding effect of the IG ethanol infusions were delayed. However, if delayed reward onset was the primary cause of ethanol's weak conditioning effect, then infusing the ethanol 30 min prior to CS+ access should improve conditioning, whereas just the opposite effect was observed in Experiment 4. Further work is needed to elucidate the reasons why limited access ethanol conditioning requires intense CS flavors.

Ethanol is not unique in being a more effective reinforcer of flavor preferences in long-term as opposed to short-term sessions. Several studies demonstrate that IG fructose infusions, unlike glucose infusions, fail to condition flavor preferences in animals trained 30 min/day, although fructose conditioning is possible with longer sessions (20–22 h/day). Also, like ethanol conditioning, long-term fructose conditioning is facilitated when sweetened rather than unsweetened CS are used (Ackroff and Sclafani, 2002; Sclafani and Ackroff, 2002). This apparent similarity between the conditioning requirements of ethanol and fructose may be because they are both selectively metabolized in the liver, which may be the source of their postingestive reinforcement actions. Prior workers have proposed that flavor conditioning by ethanol is mediated by its nutritive effects, based on the similar flavor preferences produced by ethanol and various isocaloric nutrient solutions analyzed with between-group oral (Mehiel and Bolles, 1988) and IG (Sherman et al., 1983) procedures. However, in a direct within-group comparison, rats trained with IG ethanol and isocaloric glucose significantly preferred the glucose-paired flavor (Sherman et al., 1983). However, since even isocaloric solutions of different carbohydrates (glucose, fructose, sucrose, maltose) differ in their preference conditioning actions (Ackroff and Sclafani, 1991; Ackroff et al., 2001; Azzara and Sclafani, 1998; Sclafani et al., 1993, 1999), we cannot infer that differences between carbohydrate and ethanol actions would provide an unambiguous criterion for nutrient versus pharmacological reinforcement. Distinguishing between ethanol's pharmacological and nutritive reward effects in oral administration and conditioning studies remains a persistent challenge (Sherman et al., 1988).

The experiments in this study describe some of the necessary conditions for ethanol-based flavor preference learning with limited access training. Although it is possible to get strong preferences for ethanol-paired flavors using long-term sessions, there are advantages to a limited access conditioning procedure. These include a more direct control over the amount and timing of the animals' daily ethanol dose. This would be important in studies of the effects of drugs on ethanol conditioning. Finally, despite the requirements for particular CS flavors, the ability of ethanol infusions to reliably condition flavor preferences using the techniques of the present and previous studies (Deems et al., 1986; Sherman et al., 1983) contrasts with the flavor and preference aversions obtained with many other conditioning procedures. Ethanol-conditioned flavor preferences in rats may serve as a useful model for how humans acquire preferences for alcoholic beverages.

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