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Drug-Drug Heart Rate Conditioning in Rats: Effective USs When Pentobarbital is the CS

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REILLY, S. AND S. REVUSKY. *Drug-drug heart rate conditioning in rats: Effective USs when pentobarbital is the CS.* PHARMACOL BIOCHEM BEHAV 42(4) 633-643, 1992.—Injections of two drugs in sequence may be considered the Pavlovian pairing of one drug as a conditioned stimulus (CS) with a second drug as an unconditioned stimulus (US). If pentobarbital was the CS and *d*-amphetamine or nicotine sulfate the US, then after about four drug-drug pairings the pentobarbital CS produced a higher heart rate (HR) than control conditions. With the same pentobarbital CS, HR conditioning was not obtained with the following USs: atropine, caffeine, lithium chloride, continuous foot-shock, and intermittent foot-shock. Although amphetamine and nicotine are pharmacologically different, a common conditioning mechanism seems indicated because of striking similarities in their parametric effects as USs. There also were strong similarities in these two USs when the conditioned response was a reduced capacity of the CS drug to produce conditioned taste aversions.

Heart rate conditioning Taste aversion learning Pentobarbital Amphetamine Nicotine
Atropine Caffeine Lithium chloride Foot-shock

REVUSKY et al. (8) changed the effect of pentobarbital on heart rate (HR) simply by injecting pentobarbital into rats 30 min prior to an injection of amphetamine. After three to five such pairings, injection of the pentobarbital produced a higher HR than was obtained under control conditions when the drugs were injected 24 h apart. In Pavlovian terminology, the effects of pentobarbital provided the conditioned stimulus (CS), those of amphetamine provided the unconditioned stimulus (US), and the increased HR was the conditioned response (CR).

Other CRs that have repeatedly been shown to be affected by drug-drug pairings are body temperature (13-15) and Avfail [*av*ersion *fail*ure; (5,7)], which is a reduced capacity of the CS drug to produce conditioned taste aversions. The Avfail test depends upon the fact that nearly all drugs, including innocuous doses of recreational drugs, support aversions to a preceding taste. For instance, if a sedative dose of pentobarbital is injected into a rat after it drinks saccharin solution it will later exhibit an aversion to the taste of saccharin. The Avfail effect is an attenuation of this saccharin aversion that occurs if the pentobarbital (or one of certain other drugs) has previously been paired with a second drug. In some cases, the Avfail effect is so marked that the pentobarbital produces no detectable saccharin aversion whatsoever (7).

Because the effects on body temperature and Avfail are obtained with a wide variety of drug-drug combinations, the phenomenon is attributed to conditioning rather than to specific pharmacological interactions, even though some CS-US combinations are apparently ineffective (5,7). One of the present purposes was to find out whether a similar claim concerning CS-US combinations can be made when HR is the CR. We began research into HR conditioning with the pentobarbital-amphetamine combination because these drugs yielded good conditioning in Avfail experiments (5,7). Prior to the present report, pentobarbital and amphetamine was the only pair of drugs reported to have been used in the HR conditioning paradigm except for a report by Wilkin et al. (19) of HR conditioning with ethanol as the CS and lithium as the US. However, as outlined in the General Discussion section, we (8) consider the HR conditioning studied in the present article qualitatively different from the HR conditioning reported by Wilkin et al.

Although drug-drug HR conditioning with the pentobarbital-amphetamine combination has been demonstrated in a number of experiments, the mechanism underlying it remains unclear. If, as would be expected on the basis of our experience with Avfail conditioning (7), only certain US drugs were to produce HR conditioning under our experimental conditions, the pattern of which drugs are effective USs would be a clue to the mechanism(s) underlying this type of conditioning. The first two experiments of the present series were designed

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to detect other USs that would support conditioning to a pentobarbital CS. In Experiment 1, the USs were atropine, lithium chloride (LiCl), continuous shock, and intermittent shock. In Experiment 2, the USs were caffeine and nicotine. Also, amphetamine was used in each experiment to replicate the original effect (8), making any negative results with other USs more convincing.

A different approach to the problem of elucidating the mechanism responsible for drug-drug HR conditioning was taken in Experiments 3 and 4. Using nicotine, the one successful drug identified in the first two experiments, we examined some parametric characteristics of this US when it was paired with the pentobarbital CS. It was our intention to compare these data with the amphetamine dose effect reported by Revusky and Reilly (10) and the forward and backward conditioning determined for the pentobarbital-amphetamine combination by Revusky and Reilly (11). Contrasts and similarities between the effects of these two USs may benefit our understanding of the processes underlying HR conditioning in the drug-drug paradigm.

EXPERIMENT 1

Due to the almost complete absence of substantive information about the mechanism(s) underlying the present HR conditioning effect, our grounds for the selection of US drugs were somewhat intuitive. Atropine was used because, in contrast to amphetamine, it produces a marked elevation of HR. However, following completion of the present experiment we discovered in an unreported ancillary experiment that this tachycardia does not occur if atropine is preceded by an injection of pentobarbital. There was, then, for experimental animals no observable unconditioned effect of atropine in the present experiment. (In the case of every other drug US we used, ancillary work showed that prior injection of pentobarbital did not markedly change the magnitude of the effect of the US on HR under our conditions.)

LiCl was tried since: a) like amphetamine, it produces a marked reduction in HR; b) pairing it as a US with a pentobarbital CS produces pronounced conditioned hyperthermia (13) and Avfail (7); and c) Wilkin et al. (19) reported that LiCl was an effective US in their HR conditioning experiment.

We used shock USs because conditioning with shock can sometimes occur under anesthesia (1) and our standard 32-mg/kg dose of pentobarbital approaches the anesthetic level. Also, both the continuous shock and the intermittent shock are effective stimuli in other preparations. They have been found to attenuate taste aversions produced by amphetamine (9) and to induce analgesia (12). If both types of shocks prove to be effective USs for HR conditioning, it would be conceivable that amphetamine produces HR conditioning by counteracting sedation or something similar.

METHOD

Subjects

One-hundred and thirty-nine naive, male Sprague-Dawley rats obtained from Canadian Breeding Laboratories (St. Constance, Quebec, Canada) served as subjects. They were housed in individual stainless steel, wire mesh cages with unlimited access to dry Purina Rat Chow. All experimentation was conducted in the animal housing room, which was lighted 24 h per day. During the experiment, rats received water on a schedule of 2 days of free access followed by 2 days of deprivation. Prior to the initiation of this schedule, their free-feeding weight range was 165–175 g. At this time, electrodes (stainless steel safety pins) were implanted subcutaneously on the left shoulder and right flank to allow recording of HR.

Apparatus

Four animals were tested at a time, with one rat in each of four identical test chambers of the type previously described by Revusky et al. (8). Briefly, each chamber consisted of an aluminum shell (12.2 cm high and 19.1 cm diameter) that contained a rigid plastic liner into which the rat was placed. In the center of the steel mesh lid was a swivel that permitted the animal to move freely when its electrodes were attached to the HR monitoring equipment.

Using the method of Revusky et al. (8), HRs were sampled for several seconds at 2-min intervals by the following digitizing system. The amplified signal from each rat was band pass-filtered and then digitized using an 8-bit analog-to-digital converter (ADC). The ADC was interfaced to an 8085 microprocessor running a clock with a 6.14 MHz crystal. A sampling rate of 1 kHz was used. The algorithm measured the time between successive peaks of five R-waves as follows. First, a local maximum was determined. Then, input was locked out for 40 ms to exclude secondary peaks, which might be increased due to noise and thus be read as a second peak. After the lockout period, the system used the previously obtained local maximum as a criterion as to when to start searching for a second local maximum. With each millisecond, the criterion was reduced by $\frac{1}{64}$ of the full scale. When the digital output exceeded the criterion, the system searched for a new maximum. When four determinations of the peak-to-peak time interval were obtained, the two extremes were discarded and the mean of the remaining determinations was used to obtain a duration that was converted to heart beats per minute.

Procedure

There were four conditioning trials and one test trial (Trial 5). Trials were spaced 4 days apart and conducted while rats were 16–20 h water deprived. For all subjects, the CS (32 mg/kg sodium pentobarbital at a concentration of 32 mg/ml in physiological saline) was injected IP after a 20-min acclimation period in the HR chamber. After an additional 30 min, each rat was returned to its home cage upon completion of the trial. Experimental rats received the US as they were being removed from the test chamber, while control subjects received the US 24 h later. The drug USs and their doses were as follows: 20 mg/kg IM *d*-amphetamine sulfate for 22 experimental rats and 11 controls; 10 mg/kg IM *d*-amphetamine sulfate for 14 experimental rats and 9 controls; 50 mg/kg IP atropine sulfate for 14 experimental rats and 6 controls; and 200 mg/kg IP LiCl for 14 experimental rats and 8 controls. Except for LiCl, all US drugs were diluted in saline and injected at 1.0 ml/kg. The LiCl vehicle was distilled water and the injection volume was 10.0 ml/kg. In addition, intermittent shock was the US for 14 experimental rats and 7 controls and continuous shock was the US for 13 experimental rats and 7 controls. For subjects in the shock groups, just after experimental rats were removed from the test chamber they were placed in an operant chamber and shock was delivered through the steel grid floors. The intermittent shock was administered at 2.0 mA for 10 min, 1 s on, 4 s off, from an E1064 Grason-Stadler shock source (scrambled). The continuous shock was a steady 2-mA shock for 2 min. There was no specific measurement of the effect of the shock, but according

to unquantified observations sometimes experimental rats (which were still sedated by the pentobarbital CS injected 30 min earlier) twitched with onset of the shock. However, rats seem never to have been aroused from sedation since there was no overt sign that the shock had been applied just after the shock terminated.

Trial 5, the test trial, was similar to the earlier trials except rats remained in the apparatus for 50 min after the CS injection to increase the time available to observe the CR. The US was omitted since it was superfluous.

Statistical Analyses

Heart rate was determined at 2-min intervals both during the 20-min acclimation period prior to injection of the CS drug and during the subsequent 30–50 min of a trial. Based upon extensive experience with this preparation, we used the mean of the determinations during a criterion period (over 20 min but less than 50 min after the CS injection) to compare the various groups by *t*-tests and *F*-tests, which were two tailed unless otherwise indicated. The use of the mean during a criterion period is valid, while methods based upon the more usual repeated-measures analyses of variance (ANOVAs) are invalid in this type of situation because successive HRs are positively correlated. For this reason, they lead to absurdly high levels of significance (8). Given statistical significance according to this overall criterion, the CR was considered to be apparent during all successive 2-min determinations in which the CR yielded $p < 0.10$, provided at least one of these determinations was in the criterion period. For instance, if the overall criterion was met and all individual *t*-test results from 6 min after CS injection until 50 min after injection yielded $p < 0.10$ the duration of the CR was from 6 min until 50 min after the pentobarbital injection. We also provided for statistical evaluation of unanticipated effects but none emerged.

RESULTS AND DISCUSSION

Within each of the four experiments reported in this article, the different USs (or different doses of the same US) did not significantly affect the results under control conditions and hence for each experiment the data provided by control subjects were pooled. When referring to a US group below, we always mean an experimental group.

In our experiments, a change in HR after the CS injection in experimental rats is not evidence for conditioning because other factors also affect HR. Instead, the conditioned effect must be determined solely by the difference between experimental and control HRs after injection of the CS drug. The changes in HR before and after injection of the CS drug that occur among control animals and that cannot be attributed to conditioning are apparent in the curve for these subjects as shown in Fig. 1 for the test trial, Trial. 5. This control curve indicates that during the first 18 min after the rat is placed in the test chamber, prior to any injection, its HR decreases. This decrease is a recovery from a transient rise in HR that occurred when the rat was moved from its home cage and placed in the test chamber. The handling involved in the injection of pentobarbital also produced a transient rise in HR followed by a recovery to baseline levels. This postinjection elevation of HR is not mainly due to the action of the pentobarbital CS drug since a similar rise in HR is obtained when saline is injected in lieu of pentobarbital (8).

Heart rate conditioning relative to this control performance was demonstrated only by experimental groups for which amphetamine was the US: $p < 0.0001$ and $p < 0.01$, respectively, for the 20- and 10-mg/ks doses of *d*-amphetamine by our criterion measure (a higher mean HR than the pooled controls during the determinations 20–50 min after pentobarbital injection). The individual 2-min determinations also yielded ps < 0.05 (one tailed) throughout this criterion period. (These ps are not statistically independent, but such independence is unnecessary for the conclusion that the CR was present throughout a period during which all ps are significant.) In agreement with a later finding of no marked effect of amphetamine doses on HR (10), there was no significant difference between the 10- and 20-mg/kg amphetamine groups when they were compared directly during the criterion period or for any of the 25 individual 2-min determinations following injection of the CS at $p < 0.05$.

None of the other USs used in this experiment (LiCl, atropine, continuous shock, or intermittent shock) induced changes in HRs of experimental rats significantly different from those of the pooled controls. Furthermore, during the criterion period each of these experimental groups exhibited significantly lower HRs than the 20-mg/kg amphetamine dose ($p < 0.05$, one tailed). When similar comparisons were made with the 10-mg/kg amphetamine group, the differences were significant for the LiCl and atropine US groups but not for the intermittent- and constant-shock groups.

The pattern of conditioning obtained in Trial 5, the test trial, was first apparent in Trial 4 but was not as marked: For 20 mg/kg amphetamine $p < 0.01$ and for 10 mg/kg $0.05 > p < 0.01$; both p values are based upon a comparison with the pooled controls during the criterion period. No other US yielded any evidence for conditioning on Trial 4, and there was no indication of any conditioning on earlier trials.

The results of the present experiment, then, replicated our earlier studies in that amphetamine was an effective US. Unfortunately, the other stimuli failed to support conditioning and therefore provide no positive information to aid our understanding of the mechanism responsible for HR conditioning.

EXPERIMENT 2

Caffeine and nicotine were used as USs in Experiment 2 because they are potent stimulants that are different pharmacologically from each other and from amphetamine. Neither caffeine nor nicotine unconditionally affected HR in rats under our conditions at the doses we used although, in contrast to amphetamine, they increase HR in humans. Caffeine, like amphetamine, produces vasoconstriction (3), while nicotine produces vasodilation (17). The rationale for trying nicotine and caffeine was based upon a conjecture about how amphetamine might produce HR conditioning. Normally, amphetamine lowers HR indirectly. It produces vasoconstriction and the lowered HR is believed to be a reflex of the vagus nerve resulting from the increased blood pressure (18). On this basis, we supposed that the pentobarbital CS produces a CR to compensate for the lowered HR produced by the amphetamine US. Following the model of physiological conditioning proposed by Eikelboom and Stewart (2), the lowered HR, because it was a reflex to elevated blood pressure rather than directly produced by the CNS, was presumed to function as a US for the CNS. In reaction to this US, the unconditioned response (UR) of the CNS was presumed to elevate HR to compensate for the effect of the US. According to the Eikelboom–Stewart model, the CR would be similar to the UR. This reasoning would be incorrect if nicotine, when substituted for amphetamine, were an effective US because it produces vasodilation

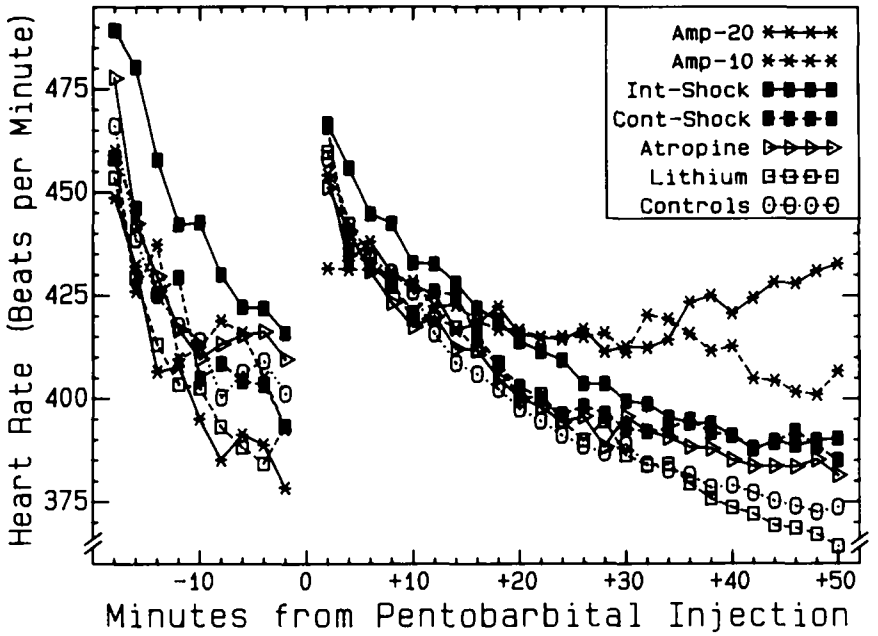

FIG. 1. Mean HRS during Trial 5 of Experiment 1 for groups subjected to different USs and for pooled controls as a function of time from the pentobarbital injection. Negative numbers on the *x*-axis refer to time before pentobarbital injection.

instead of vasoconstriction. On the other hand, we might feel comfortable with such a conclusion if caffeine rather than nicotine were an effective US since it is a vasoconstrictor.

In Experiment 1, conditioning trials were administered in the apparatus used to monitor HR. In Experiments 2, 3, and 4, conditioning was carried out in the home cages since this procedure is easier and, contrary to an earlier report (8) in which an inappropriate control group was used, HR conditioning is not context specific under our experimental conditions (11).

METHOD

Subjects and Apparatus

Ninety-six naive, male Sprague-Dawley rats were used in Experiment 2. Watering schedules, housing, and experimental chambers were as described in Experiment 1.

Heart Rate Monitoring Equipment

Using the more sophisticated method of Revusky and Reilly (11), the amplified output from the electrodes was read at 3-min intervals by means of a Labmaster board and Labpac software (Scientific Solutions, Inc., Solon, OH) for 1.2 s at 1-ms intervals. In each 1.2-s sampling period, data was obtained from 8 rats. Overall control was by a Microsoft QuickBasic program that determined peak amplitudes and translated them into a rate. This rate determination was cross-checked through the program for various conceivable artifacts. It also could be overridden by the operator on the basis of a graphic display of amplified outputs over time that appeared on the video monitor for each rat. The only bias of which we are aware is that a double heart beat (extrasystole), which occurred very rarely, was read as an error and the HR was then redetermined.

Procedure

The USs were 12 mg/kg IM *d*-amphetamine sulfate, 6 mg/kg SC nicotine sulfate, and 150 mg/kg IP caffeine. For each US, there was an experimental and control group with $n = 14$ for each amphetamine group and $n = 17$ for the other groups. The first four trials were administered in the home cages by injecting the CS drug, which was followed by a US injection 30 min later for experimental rats and 1 day later for control rats. All injections were diluted in saline so that the volume injected was 1.0 ml/kg except caffeine, which was injected at 3.0 ml/kg. Experimental rats received control injections of saline by the route of administration of their US injection at the time controls were injected with the US drug. Similarly, controls received saline when experimental rats were injected with the US.

Trials 5 and 6 were conducted in the test chamber. Rats were acclimated in this chamber for 18 min and then injected with the pentobarbital CS. After an additional 33 min, rats were removed from the test chamber. Experimental subjects were immediately injected with the US and returned to the home cage, while control rats were injected with the US on the following day. Saline control injections were administered as in the earlier trials. On the final test trial, Trial 7, rats were not removed from the test chamber until 93 min after the injection of pentobarbital. The US was not injected since it was unnecessary.

RESULTS AND DISCUSSION

In each of the test trials, Trials 5 and 6 (Fig. 2) and Trial (Fig. 3), both the amphetamine and nicotine US groups exhibited higher HRs than the pooled controls during the criterion period (24–33 min after the CS injection in Trials 5 and 6 and, in Trial 7, 24–48 min after the CS injection) at $ps < 0.02$. In Trial 7, the results of the *t*-tests for the individual HR

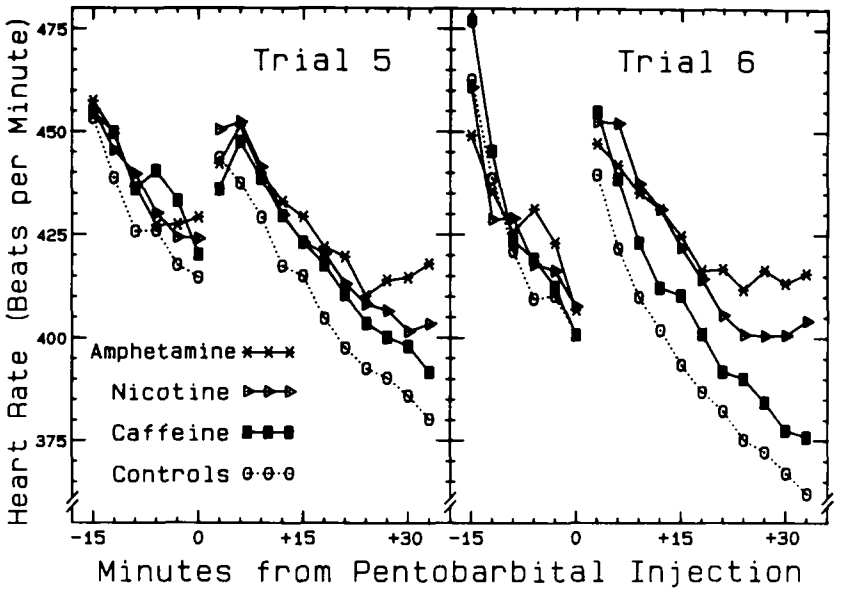

FIG. 2. Mean HRs during Trials 5 and 6 of Experiment 2 for groups subjected to different USs and for pooled controls as a function of time from pentobarbital injection.

determinations spaced 3 min apart indicated significantly higher HRs than the pooled controls during all determinations from 18–87 min after pentobarbital injection for the amphetamine group and from 24–81 min after pentobarbital for the nicotine group. The caffeine group did not exhibit statistically reliable conditioning during the overall criterion period in Trials 5 and 7. However, Trial 6 yielded $p < 0.05$ (one tailed). We did not interpret this as significant HR conditioning due to the negative results in Trials 5 and 7.

The nicotine and amphetamine US effects on HR conditioning were statistically indistinguishable: Over the three test trials, there were 53 HR determinations after injection of the pentobarbital CS. Not one of these yielded a significant difference between the nicotine and amphetamine US groups at

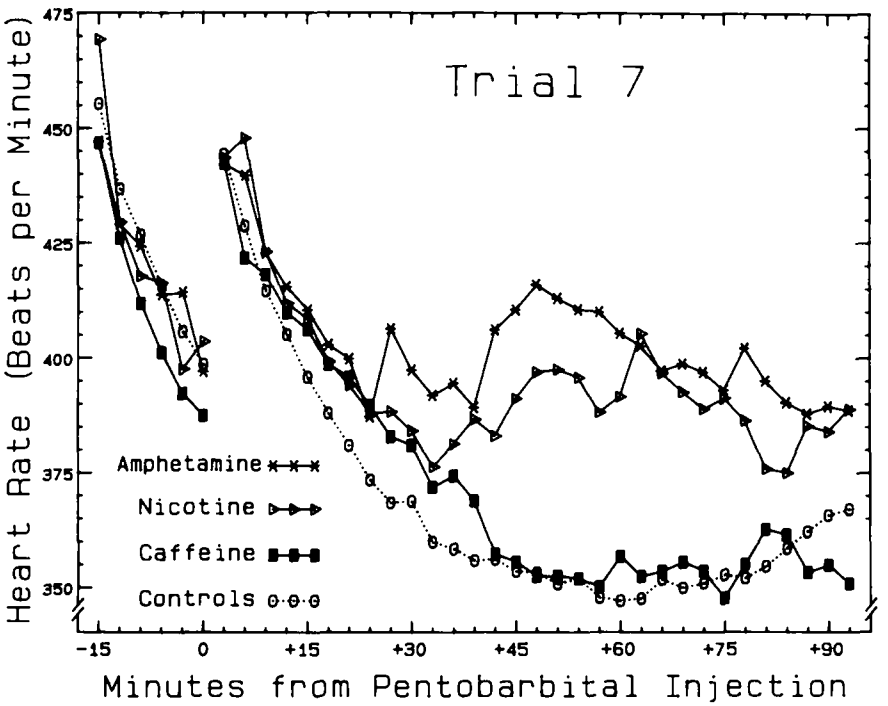

FIG. 3. Mean HRs during Trial 7 of Experiment 2 as a function of time from pentobarbital injection.

$p < 0.05$. So, the tendency in Figs. 2 and 3 toward a larger effect for *d*-amphetamine than for the nicotine sulfate is not evidence for a real difference in the effects of these drugs.

The pattern of results in Figs. 1-3 excludes a number of possible mechanisms of conditioning, as will be explained in the General Discussion section, but we are unable to isolate the specific mechanism on the basis of the results.

EXPERIMENT 3

The similarity in the effects of nicotine and amphetamine in Experiment 2 provides some indication that they may have similar properties as USs for HR conditioning. An alternative possibility, however, is that the similarity was specific to the two particular doses used. Experiment 3 was intended to address this latter interpretation. We were interested in ascertaining the lowest effective dose of nicotine that would support HR conditioning. Our primary intention, however, was to determine how the CR varied with nicotine dose and compare the results to those obtained in our earlier amphetamine dose experiment (10). In that report, we studied HR conditioning with US doses of *d*-amphetamine sulfate ranging from 2-16 mg/kg; the CS was the same 32 mg/kg pentobarbital used in the present experiments. There was a slight trend toward a stronger CR with increases in the US dose, but it was not statistically reliable. The present experiment was of a similar design to the amphetamine dose experiment except nicotine sulfate was the US drug.

METHOD

Subjects and Apparatus

One-hundred and twenty naive, male Sprague-Dawley rats were used in Experiment 3. Watering schedules, housing, and experimental chambers were as described in Experiment 1. The HR monitoring equipment was that used in Experiment 2.

Procedure

The conditions were like those of Experiment 2 with the following exceptions. Each of four experimental groups contained 20 rats and each of the four control groups contained 10 rats. Each pair of experimental and control groups was assigned to a different dose of nicotine sulfate: 0.75, 1.5, 3.0, or 6.0 mg/kg.

There were four conditioning trials in the home cage prior to two test trials in the test chamber. With rare exceptions, animals were run in squads of eight. Each squad contained 0-2 animals from each of the eight groups and groups were balanced, as in earlier experiments, for assignment to each of the eight experimental chambers. Heart rates were sampled at 3-min intervals during Trials 5 and 6.

Trials were administered 4 days apart while rats were 16-20 h water deprived. During the home cage conditioning trials, all rats were injected with the CS drug (32 mg/kg IP sodium pentobarbital at a concentration of 32 mg/ml). Then, 30 min later, experimental animals were injected SC with the appropriate nicotine dose (diluted in saline for an injection of 1.0 ml/kg) while controls were injected with an equal volume of saline. Control animals received the US injection a day later, when experimental rats received an injection of saline.

Trials 5 and 6 were conducted in the test chambers. The CS drug was injected following an 18-min acclimation period and animals remained in their chambers for an additional 48 min. For experimental animals, the US drug was administered when rats were removed from the chambers upon completion of the trial; control animals received the US on the following day. Saline injections were administered as in earlier trials. Trial 6 was identical to Trial 5 except the nicotine US was not injected.

RESULTS AND DISCUSSION

On each of Trials 5 and 6 (Fig. 4), all experimental groups exhibited the HR CR relative to the pooled controls ($ps < 0.05$ during the criterion period). There was, however, no statistically significant effect of the nicotine US doses on the magnitude of HR conditioning, although there was a very weak and insignificant tendency for conditioning to be more pronounced at the highest nicotine dose, 6 mg/kg. In Trial 6, each experimental group exhibited significantly higher HRs ($ps < 0.05$) than pooled controls on each determination from 24 min after pentobarbital injection until the end of the session.

This finding is similar to our earlier experiment in which amphetamine also failed to uncover a noticeable effect of US dose (10). In Experiment 1 (Fig. 1), 20 mg/kg amphetamine produced HRs higher than the 10-mg/kg dose, but this difference also was not statistically significant. Hence, the results are similar for both US drugs over a range of reasonable doses, suggesting that the similarities between effects produced by the amphetamine and nicotine USs in Figs. 2-4 did not occur because of the particular doses selected.

One of our original intentions, both in the present experiment and in our corresponding work with amphetamine (10), was to find a threshold dose for the US that might produce the CR. In both cases, we overestimated how low this threshold might be and have no idea how much lower the threshold dose is relative to our lowest effective dose. However, the practical implications are clear. Our two lowest effective doses, 2 mg/kg *d*-amphetamine sulfate and 0.75 mg/kg nicotine sulfate, are small fractions of the lethal dose and only a sixteenth part of doses that we had been able to use routinely with rodents. Many humans consume these drugs for recreational purposes in doses that are much higher relative to a lethal dose than those used here. It is possible, then, that both amphetamine and nicotine in doses routinely taken by many humans can change the effect of pentobarbital on HR in humans.

EXPERIMENT 4

Revusky and Reilly (11) injected rats with 32 mg/kg sodium pentobarbital as the CS and 16 mg/kg *d*-amphetamine sulfate as the US. There were three forward conditioning groups with 30-, 90-, and 270-min delays between CS and US injections. There also were three backward conditioning (US injected prior to the CS) groups with the same delays. The control data were pooled from forward and backward groups with a 1-day interinjection interval since the results of the groups were statistically identical. Heart rate conditioning occurred at the 30-min forward delay and did not occur at the 270-min forward delay. Although a small effect seemed apparent with the 90-min forward delay, it was not statistically significant. Unexpectedly, conditioning occurred at all three backward delays: 30, 90, and 270 min.

The question of what underlies backward conditioning will not be considered until the General Discussion section, because the rationale for Experiment 4 does not depend upon it. Suffice it to say for the present that conditioning with back-

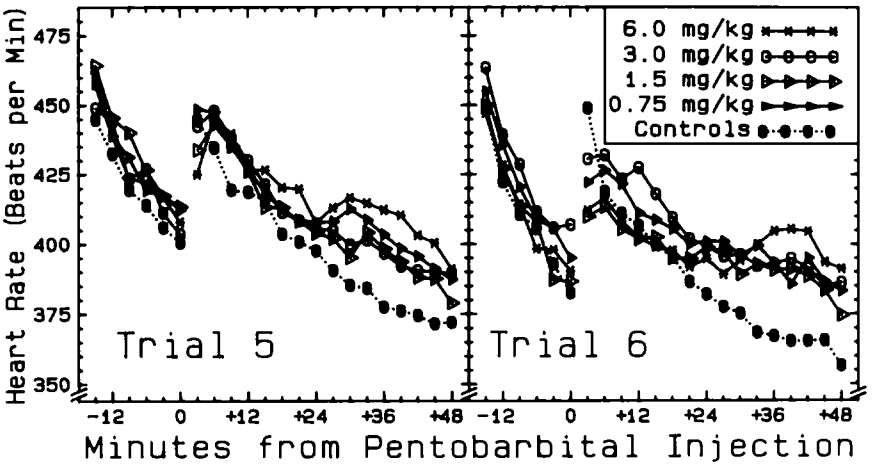

FIG. 4. Mean HRs in Experiment 3 for groups subjected to different nicotine US doses and for pooled controls as a function of time from pentobarbital injection.

ward delays of up to 270 min is unprecedented in the learning literature and, if similar backward conditioning were to occur with nicotine as the US, it would suggest that nicotine and amphetamine produce HR conditioning through a common mechanism. The present experiment explored this possibility and used the same design as our earlier experiment with amphetamine (11) and hence the groups were PN30, PN90, PN270, PND, NP30, NP90, NP270, and NPD, where PN and NP refer to forward and backward conditioning, respectively, the numbers refer to the delay (min) between the two injections, and D means the delay was 1 day. As in the earlier experiment, the test period lasted until 270 min after pentobarbital injection.

After Revusky and Reilly (11) tested rats subjected to pairings of pentobarbital and amphetamine by the HR measure, they administered an Avfail test to the same rats and obtained a different pattern of results than that obtained with HR conditioning. They interpreted these results as evidence for Avfail conditioning with forward delays of up to 270 min. There was no evidence for backward conditioning of Avfail. In the present experiment, we repeated this follow-up Avfail procedure with our rats, which differed only in that they had been subjected to a nicotine US. Because this overall procedure is complex, it has been outlined in Table 1, to which reference will be made in the following Method section.

METHOD

Subjects and Apparatus

One hundred and ninety-two naive, male Sprague-Dawley rats were used in Experiment 4. Watering schedules, housing, and experimental chambers were as described in Experiment 1. The HR monitoring equipment was that used in Experiment 2.

HR Conditioning and Testing

In unspecified details, the procedures of Experiment 3 were used. The eight groups in the HR experiment and the number of animals that provided test trial data are indicated in the two leftmost columns of Table 1. Due to the large number of subjects and the very long test of HR conditioning, the start of the experiment was staggered over 12 consecutive days with 16 animals, 1-3 from each group, receiving injections each day. The five conditioning trials were administered at 4-day intervals in the home cages by the procedure outlined in the third column of Table 1. The sodium pentobarbital dose was 32 mg/kg IP and the nicotine sulfate dose was 6.0 mg/kg SC. After completion of the conditioning stage of the experiment, all animals received an identical test in the HR monitoring apparatus as indicated in Table 1. Pentobarbital was injected 21 min after placement in the test apparatus and nicotine was injected after another 270 min. The rationale for subjecting all rats to the US on this test was related to the logic of the later Avfail procedure as was explained elsewhere for the corresponding experiment with an amphetamine US (11). The first 17 HR readings were spaced 3 min apart, the next 5 readings were at 6-min intervals, and thereafter readings were at 15-min intervals.

Avfail Procedure

Rats were maintained on the water deprivation schedule for two more 4-day cycles. During the second cycle, all animals received, as appropriate to their group, a final pairing of pentobarbital and nicotine identical to the first 5 HR conditioning trials. (This is not shown in Table 1.) On the day following these injections, animals were allowed 3-h access to unflavored tapwater. For the next 5 days, drinking time was restricted to 15 min each day. Rats retained their previous group designations except for the formation of a control group by assignment of two to four rats from each earlier group. This is why there were fewer rats in each Avfail group than in the corresponding HR conditioning group.

The single Avfail conditioning trial and the Avfail test trial are outlined in the final two columns of Table 1. In the Avfail conditioning trial, all animals were allowed 15-min access to a 3.0% (v/v) solution of Heinz cider vinegar and, as bottles were removed, given an injection of the pentobarbital CS except for controls, which were injected with saline. Administered 2 days later, the test trial was identical to the conditioning trial except the pentobarbital injection was omitted. The data are reported as preference ratios $V/(V + W)$, where V is the weight of the vinegar solution consumed on a trial and W is the weight of the unflavored water consumed on the preceding day. Statistical analysis of differences in vinegar prefer-

TABLE 1

PROCEDURES FOR THE HEART RATE EXPERIMENTATION AND THE FOLLOWING AVFAIL EXPERIMENT

	HR Experimentation			Avfail Experimentation		
Group	*n*	Conditioning	Test	*n*	Conditioning	Test
PN30	22	P-30 min-N	P-270 min-N	20	Vin-P	Vin
PN90	25	P-90 min-N	P-270 min-N	21	Vin-P	Vin
PN270	26	P-270 min-N	P-270 min-N	22	Vin-P	Vin
PND	21	P-1,440 min-N	P-270 min-N	19	Vin-P	Vin
NP30	22	N-30 min-P	P-270 min-N	20	Vin-P	Vin
NP90	25	N-90 min-P	P-270 min-N	22	Vin-P	Vin
NP270	26	N-270 min-P	P-270 min-N	23	Vin-P	Vin
NPD	21	N-1,440 min-P	P-270 min-N	18	Vin-P	Vin
Control				23	Vin-S	Vin

P refers to injection of pentobarbital. N refers to injection of nicotine. The arabic numeral immediately preceding min refers to the number of minutes between the two injections; 1,440 is the number of minutes in 1 day. Vin refers to 15 min of free access to vinegar solution. If followed by P, it means that pentobarbital was injected as the vinegar was removed. S indicates that saline was injected in lieu of pentobarbital.

ence on the test day was by means of analysis of covariance (ANCOVA), with preference on the conditioning day as the covariate.

RESULTS AND DISCUSSION

HR Conditioning

The overall criterion period for HR conditioning was the same as that used in the corresponding experiment reported by Revusky and Reilly (11). The determinations 24, 27, 30, 36, 42, and 48 min after pentobarbital injection were averaged with the 36-, 42-, and 48-min determinations weighted twice as heavily as the others because they represented twice as much time. Later determinations were not used to define the basic CR because the precedent from our work with amphetamine (11) was that the CR would disappear 60–90 min after pentobarbital injection. Figure 5 shows mean HRs during the criterion period as a function of the delay between the two drug injections for PN groups (forward conditioning) and NP groups (backward conditioning). Groups PND and NPD, the groups subjected to a 1-day delay, exhibited statistically identical HRs and their results were pooled and shown as the control level. Conditioning (higher HRs than among the pooled controls) was significant at a forward delay of 30 min and at backward delays of 30 and 90 min (both $p < 0.002$). Conditioning was not demonstrated for any other experimental group ($p > 0.05$). The mean HRs during the criterion period were statistically similar among all groups that exhibited conditioning and also were statistically similar among all experimental groups that failed to exhibit conditioning ($p > 0.05$ for all such pairwise comparisons by the Newman-Keuls test). In addition, there was a significant difference for each possible comparison between a group that exhibited conditioning and one that did not ($p < 0.05$, Newman-Keuls). These results are almost identical to earlier results in which the US was amphetamine (11) except conditioning was obtained with a 270-min backward delay with the amphetamine US.

Figure 6 shows the difference in HR between experimental groups and pooled controls. The PN groups and NP groups are shown separately for graphic clarity.

Noteworthy in Fig. 6 is the similar time course of the CR among each of the three groups that exhibited statistically significant conditioning: PN30, NP30, and NP90. The CR reached a peak 36 or 42 min after pentobarbital injection and nearly disappeared 75 min after that injection. When the US was amphetamine (11), the same similarity in the time course of the CR was apparent among all groups that exhibited conditioning. In passing, the reader is cautioned against inferring anything into the occurrence, on the curve shown for Group PN90 in Fig. 6, of a peak 75 min after pentobarbital injection. Although the HR at this single point is significantly higher ($p < 0.05$) than the control level, this is not true at any other such time, either during the criterion period or afterward. Due to the large number of statistical determinations, the signifi-

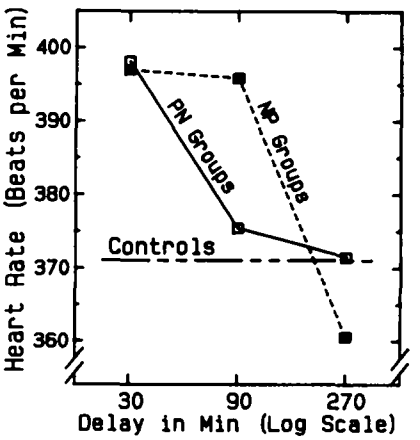

FIG. 5. Mean HRs 21-48 min after pentobarbital injection during the test trial (Trial 6) of Experiment 4 as a function of the delay between the two injections during conditioning shown separately for the PN and NP groups. The control level is pooled for the PND and NPD groups, which did not differ significantly.

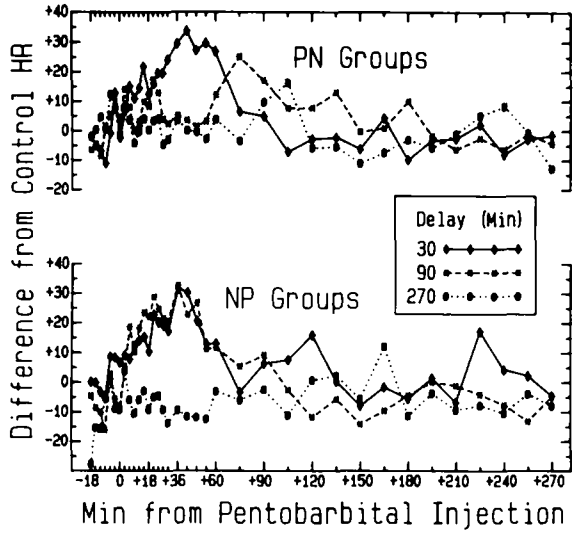

FIG. 6. Number of heart beats per minute by which various groups exceeded the HR of the pooled PND and NPD controls for each determination of Trial 6 in Experiment 4. The significant conditioning exhibited by Groups PN30, NP30, and NP90 is evident as a positive value peaking 36 or 42 min after injection of the pentobarbital CS drug.

cant effect at 75 min may well be due to chance. There was no parallel effect in our earlier experiment when amphetamine was the US (11) despite the similarity of the curves in all other respects.

Avfail Results

The Avfail results yielded the same statistical decisions as earlier results (11) in which amphetamine was the US instead of nicotine. Both amphetamine and nicotine counteract the pentobarbital CS drug and, as a result, the Avfail effect becomes confounded with a second effect, drug habituation, which, like Avfail, reduces the capacity of pentobarbital to yield conditioned taste aversions. As explained in more detail elsewhere (11), while the time between the two injections decreases the total effect of drug habituation diminishes because the two drugs counteract each other more completely. As drug habituation diminishes, the capacity of the CS drug to produce a conditioned taste aversion increases. Because Avfail does not occur with US–CS (backward) pairings (6), habituation is the only determinant of the strength of the vinegar aversions among the NP groups. The net result is that the vinegar aversion produced in the NP groups diminishes ($p < 0.001$, ANCOVA) as the interinjection delay increases (Fig. 7) because the effective habituation increases with this delay. In the case of the PN groups, there is an Avfail factor in addition to the habituation factor. Like the habituation factor, Avfail diminishes the capacity of pentobarbital to produce a vinegar aversion, but, in contrast to habituation, Avfail becomes more potent as the interinjection interval diminishes. Thus, for the PN groups in Fig. 7 the vinegar aversions are not markedly affected by the interinjection interval because the strengths of Avfail and prior drug habituation counteract each other (although there is an overall difference among the PN groups that reflects no obvious pattern in Fig. 7 and barely meets the criterion for significance at $p < 0.05$).

Since the NP curves are affected only by the effectiveness of the earlier habituation, while the PN curves are affected both by this factor and by Avfail, Avfail is demonstrated by factoring out the habituation effect, that is, by comparing PN and NP groups at the same delay. Since both such groups are equally affected by habituation, if the preference for the PN group is higher it must be due to Avfail as explained in more detail elsewhere (11). By this criterion, Avfail was exhibited at $p < 0.001$ at the 30- and 90-min delays and at $p < 0.05$ at the 270-min delay. There was no Avfail effect at the 1-day delay (1,440 min). When amphetamine was the US (11), the Avfail effect was too weak to completely prevent pentobarbital from producing taste aversions and all PA and AP groups had vinegar aversions relative to the controls at $p < 0.01$ except for a marginal aversion at the 90-min forward delay ($p < 0.05$, one tailed).

Although statistical inference cannot be validly used to compare groups in different experiments, the pattern of Avfail results in Fig. 7 seems nearly identical to that obtained when amphetamine was the US except the result at the 270-min forward delay with nicotine was not as marked as it was with amphetamine. If this difference is real, it is probably due to weaker conditioned vinegar aversions in the NP270 group than in the corresponding group of the earlier amphetamine experiment (11). This means that any weaker effect with nicotine was likely due to a difference between the backward conditioning groups that served as control groups in the Avfail experiment rather than due to a difference in the forward conditioning (experimental) groups. These relatively weak vinegar aversions in Group NP270 made it difficult to detect any further weakening of the vinegar aversion, and hence an Avfail effect, in Group PN270. Because of this methodological shortcoming, we believe that no firm conclusion can be reached about the relative strengths of the underlying Avfail effects at the 270-min delay with nicotine and vinegar even

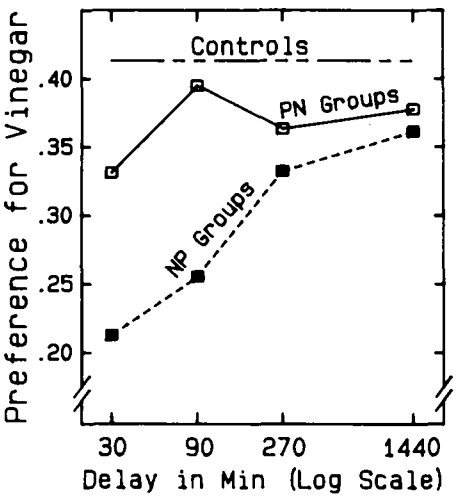

FIG. 7. Preference for vinegar solution during the Avfail test trial of Experiment 4. The curves designated *PN Groups* and *NP Groups* include only rats subjected not only to the indicated pairings but to an injection of pentobarbital just after drinking vinegar solution. The value of 1,440 on the *x*-axis is the number of minutes in 1 day and refers to Groups PND and NPD. The horizontal line labeled *controls* indicates the mean vinegar preference of rats that were not injected with pentobarbital after drinking saccharin solution. These controls had been pooled from subgroups subjected to different forward or backward delays between pentobarbital and nicotine injections.

though such an effect was prominent at this delay in the case of amphetamine in our earlier experiment (11) and was marginal for nicotine in the present experiment.

GENERAL DISCUSSION

We attempted to produce HR conditioning by pairing a wide variety of USs with a pentobarbital CS. Only amphetamine and nicotine were found to be effective USs in this paradigm. Both these drugs are stimulants, but caffeine, also a stimulant, was ineffective. It is not immediately obvious how to account for conditioning in terms of a characteristic common to nicotine and amphetamine that is not shared by caffeine, but we can exclude certain theories. Similarly, there is no reasonable sense in which stress per se can be considered an adequate US for HR conditioning with the pentobarbital CS since atropine, caffeine, lithium, and two types of electrical shocks did not produce noticeable CRs and these events could be viewed as just as stressful (if not more) as our lowest nicotine and amphetamine US doses.

As explained in the introductory section of Experiment 2, the only specific mechanism for HR conditioning by an amphetamine US that we considered supposed that it was indirectly due to vasoconstriction produced by amphetamine. This possibility can be excluded because nicotine, which is an effective US, is a vasodilator, while caffeine, which was not noticeably effective as a US, is a vasoconstrictor.

In considering other substances used in drug-drug conditioning as a means of determining its mechanism, the only other reported instance of HR conditioning through drug-drug associations involved an ethanol CS and a lithium US (19). However, the HR effect reported by Wilkin et al. (19) does not resemble the present conditioning and is almost certainly due to a different underlying mechanism. In the ethanol-lithium case, the CR is greatest 2 min after the ethanol CS injection and disappears after 10 min. This is quite different from the pattern shown in Fig. 7. Other differences have been discussed in detail elsewhere (8).

Same Mechanism for Conditioning Produced by Nicotine and Amphetamine

It would be tempting to suppose that nicotine and amphetamine each produce conditioning by different mechanisms because they have little in common except for being stimulants, which has already been excluded as the causative factor. However, the HR conditioning results when nicotine was the US (Experiments 3 and 4) are so similar to those for amphetamine (10,11) that they clearly indicate a common mechanism as follows.

1. Both the nicotine and amphetamine USs produce conditioning within four trials (Experiment 2).
2. The conditioning produced by both nicotine (Experiment 3) and amphetamine (10) are remarkably unaffected by the US dose.
3. The change in the CR over time is similar for nicotine (Experiment 4) and amphetamine (11).
4. For both nicotine (Experiment 4) and amphetamine (11), the effects of the forward CS-US delay on HR conditioning are extremely similar. In each case, there is unequivocal conditioning at a 30-min forward delay and no trace of conditioning at a 270-min forward delay. Also, in each case there is some indication of conditioning with the 90-min forward delay but it is nowhere near statistical reliability.
5. Both US drugs produce HR conditioning with backward delays of 30 and 90 min, but only amphetamine produces conditioning at the 270-min backward delay. We believe the difference at the 270-min delay must be due to some factor that we cannot identify because we do not know the mechanism underlying the conditioning. (It conceivably may involve the fact that nicotine is faster acting than amphetamine in most respects.) Consideration of what may be responsible for this present backward conditioning will be deferred until the next section, but the important factor here is that both US drugs produce backward conditioning over delays far longer than ever reported in the conditioning literature.
6. There is not as much specific detail by which Avfail conditioning can be compared with HR conditioning, but in all pertinent details the effects of nicotine are similar to those of amphetamine, even though Avfail is not affected by paradigmatic variations in the same way as HR conditioning. Backward pairings did not produce Avfail neither with a nicotine US nor, in earlier experiments, with an amphetamine US (6,7,11). Forward pairings of up to 90 min produced an Avfail effect both with nicotine and with amphetamine (11). The only difference between Avfail effects with the two drugs was that amphetamine also produced strong conditioning at the 270-min delay (11), while nicotine produced weak conditioning (Experiment 4). As indicated in our discussion of Fig. 7, this may well be due to a difference among the control groups subjected to the 270-min delay and not specifically due to a difference in the underlying Avfail effect.

Backward Conditioning

Backward conditioning, particularly at long delays, is so widely reputed to be impossible that its present occurrence must be discussed even though we have no explanation for it. It is tempting to explain it in terms of indirect forward conditioning. That is, although the interstimulus interval has been defined here operationally in terms of the temporal delay between injections the CSs and USs are drug effects whose onset and offset are less easy to define. For instance, if the effective US was to occur 300 min after the US drug was injected and the CS effect was to occur very soon after injection the "true" CS-US delay would be a forward delay of under 30 min with a backward interinjection interval of 270 min. However, we are unenthusiastic about any such explanation of the backward conditioning obtained in Experiment 4 because it solves one mystery by postulating a second mystery and would be considered absurd in the absence of a strong theoretically based disbelief in backward conditioning. All drugs used here have overt effects within 15 min of injection and hence such a theory must imply that the effective CS and/or the effective US is not an overt effect but some later aftereffect. We consider this implausible and would wonder why the overt drug effects would not produce associative interference to prevent conditioning (4). In our analysis of the similar backward conditioning obtained with amphetamine, which occurred with delays up to 270 min, we suggested that it was more reasonable to suppose that this backward HR conditioning is a special adaptation for homeostatic regulation (11).

Clinical Implications

Taukulis and Brake (16) demonstrated that the effects of diazepam for which it is prescribed clinically can be changed if it is paired with chlorpromazine. Furthermore, specific clini-

cal effects are changed differently by the very same pairings: Diazepam's efficacy as a muscle relaxant is reduced while its capacity to reduce anxiety is increased. Since diazepam and chlorpromazine are sometimes prescribed in combination, this finding clearly has important medical implications.

The present CRs are not of such direct medical relevance since pentobarbital is not used clinically either to affect cardiac function or produce taste aversions. But, there is no reason to believe that pentobarbital is the only drug that can be changed in its effects by amphetamine or nicotine or that cardiac CRs are the only potential CRs. If the targeted therapeutic effects of certain other drugs (particularly sedatives) can be changed by nicotine or amphetamine in the same way the effects of pentobarbital on HR were changed in the present study, then drug-drug associations are of great clinical relevance. Drug-drug associations that occur with the low doses, few CS-US pairings, and over the same wide range of forward and backward intervals as the effects demonstrated here are almost certain to develop in the normal course of medical drug administration, which often involves prescription of a number of drugs in addition to those the patient may ingest on his or her own. Thus, it is likely that the effects of drugs used to manipulate cardiac function may change as a result of other drugs consumed by the patient (i.e., as a result of drug-drug conditioning). Nor need such effects be limited to cardiac conditioning. For instance, suppose a heavy cigarette smoker is prescribed a sedative that acts like pentobarbital and the sedation response is affected much like HR. After the sedative has been taken on four occasions or so, its effectiveness might well be changed because the patient is effectively pairing the sedative with nicotine.

With one notable exception (16), the influences of inter-drug associations on therapeutically relevant drugs have yet to be documented. If the results reported in the present article have generality, then the ramifications of drug-drug conditioning on medical practice could prove substantial.

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