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Classical Conditioning During Nitrous Oxide Treatment: Influence of Varying the Interstimulus Interval

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GHONEIM, M. M., H. M. EL-ZAHABY AND R. I. BLOCK. *Classical conditioning during nitrous oxide treatment: Influence of varying the interstimulus interval.* PHARMACOL BIOCHEM BEHAV **62**(3) 449–455, 1999.—Classical conditioning of the rabbit nictitating membrane response (NMR) was accomplished by presenting a 75-ms tone conditioned stimulus (CS) at intervals of 0, 100, 200, 400, and 800 ms before the presentation of a 100-ms shock unconditioned stimulus. Following every four paired trials (tone followed by shock), the occurrence of conditioned responses (CRs) was tested on every fifth trial in which only tone was presented (test trials). Three doses of nitrous oxide in oxygen (0, 33, and 67%) were used during conditioning. Nitrous oxide produced dose-dependent decrements of learning. Conditioned responding was related to the interstimulus interval (ISI) by a concave-down function. The higher dose of nitrous oxide caused more decrements of learning at several ISIs compared to the other two doses, changing the shape of the curve. Trace conditioning, which was examined in the present study, was more impaired under the influence of nitrous oxide than conditioning in a previous study, which used the standard delay paradigm. Thus, the drug impairs explicit memory more than implicit memory. © 1999 Elsevier Science Inc.

Anesthetic Nitrous oxide Classical conditioning Interstimulus interval Trace conditioning Learning Explicit memory Implicit memory Nictitating membrane response

NITROUS oxide (N_2O) is the most widely used general anesthetic. It is also commonly used in subanesthetic concentrations in dental, obstetric, and ambulatory surgery settings to produce conscious sedation. It is occasionally abused (20,25, 29). The drug has been proposed as a useful model compound for the study of the sedative effects of drugs (13,37). It is given by inhalation, a steady state is rapidly achieved, and the effects wear off quickly. In addition, apart from the occasional occurrence of nausea and vomiting, it has very few side effects, and is not unpleasant to inhale. The drug produces dose-dependent impairments of memory, cognition, and psychomotor performance (3,5,13,19). It produces a variety of subjective effects that include alterations of time perception (4). Although it functions as a reward in animals (41), it is not a reinforcer in healthy volunteers (43) except in the presence of pain (27). The exact mechanisms by which N_2O exerts its actions on the central nervous system are unknown.

Classical conditioning research with the rabbit nictitating-membrane response (NMR) has provided a substantial and robust set of data on associative learning (14). The NMRs methodological characteristics have led to its achieving the status of a model preparation for the study of associative learning and its neural substrates under different physiological and pathological conditions including the effects of centrally active drugs. The NMR's status is principally attributable to the absence of unconditioned (alpha) NMRs to conditioned stimuli (CSs), a low base rate of NMRs, and the absence of any detectable pseudoconditioned or sensitized NMRs. Basic associative learning is the way organisms learn about causal relationships in the world. In conditioning, one learns an association between a CS and an unconditioned stimulus (US). One of the important conditions for the formation of such an association is temporal contiguity. Conditioned responding (CR) is sensitive to variation in the tempo-

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ral interval between CS and US, being best established when the CS precedes the US by a short (but not too short) interval, and declining rapidly as this interval increases (31).

In a previous study (24), we found that N_2O impaired acquisition of CRs; had no reliable effects upon nonassociative processes; impaired unconditioned response (UR) amplitude; attenuated CS intensity; decremented tone-induced reflex modification of the UR; and demonstrated no evidence of behavioral tolerance. We concluded that N_2O's impairment of CR acquisition was attributable to its attenuation of the intensity of tone CSs and shock USs and/or UR amplitude. These experiments were all done using the basic delay paradigm where the CS and US overlap in time. In the present study, we used trace conditioning where the CS terminates and there is a period of no stimulation between CS offset and US onset (Fig. 1) In trace conditioning, as Pavlov proposed years ago (26), the organism must maintain a "trace" of the CS in the brain in order for the CS and the US to become associated. Several CS-US time intervals were studied. The standard simple delay conditioning represents processing of the CS-US relationship in an automatic reflexive way where the eye blink serves as an adaptive, defensive response to the US; features that are characteristic of nondeclarative or implicit memory. By contrast, trace conditioning represents acquiring conscious knowledge of the CS-US relationship and remembering it across many trials; features that are characteristic of declarative or explicit memory (7). We speculated that nitrous oxide would decrease the acquisition of CRs much more under the trace conditioning paradigm than the standard delay procedure, and that the anesthetic properties of N_2O might modify the standard shape of the concave-down function relating conditioning to the CS-US interval.

METHODS

Subjects

Experimentally naive New Zealand white albino rabbits of either sex weighing approximately 2 kg upon arrival were obtained from local suppliers. Animals were housed individually with free access to tap water and given 60 g of Teklad rabbit chow daily. Consistent with their rearing conditions, animals were kept in constant light. On the day following receipt, the rabbits were prepared for experiment by placement of a suture loop (6-0 Ethilon Monofilament; Ethicon) in the posterior margin of the nictitating membrane (NM). Fur surrounding the right eye was removed, and two wound clips (Autoclip) attached to the skin over the paraorbital region at a distance 10 mm posterior to the canthus and 15 mm apart in the vertical direction.

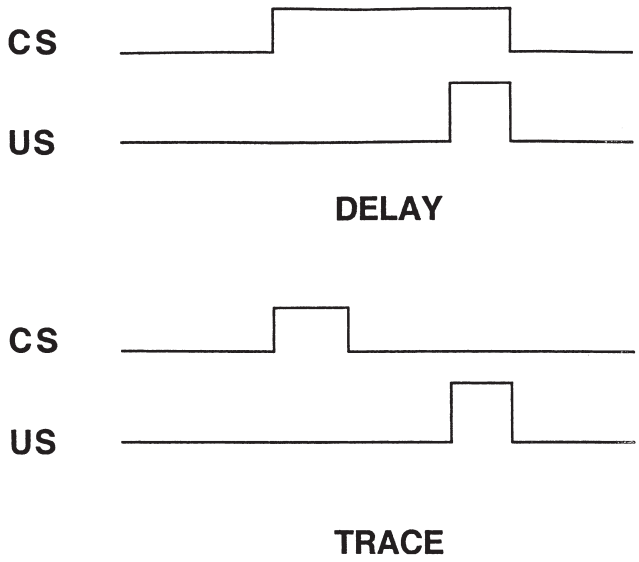

FIG. 1. The temporal relationship between the CS and US for the delay and trace conditioning procedures.

Apparatus and General Procedure

On each of the experimental days, rabbits were positioned in Plexiglas restrainers and placed in individual sound-attenuated chambers. A muzzle headmount containing a photosensitive Polaroid transducer was positioned and secured on the animal's head. The rotary armature of the transducer was attached to the NM with a horizontal bar (22-G needle) with one end hooked into the suture loop on the NM and the other end fixed with a set screw to the end of the rotary armature. A nictitating-membrane response (NMR) was defined as an extension of the NM of at least 0.5 mm. Resolution of the phototransducer was determined to be 0.06 mm movement (extension). Electrodes for delivery of the unconditioned stimulus (US) were attached to the wound clips. The US consisted of an electrical shock (60 Hz) of fixed intensity (3 mA) and duration (100 ms). An audio-oscillator with 11.4 cm diameter speaker for delivery of the conditioned stimulus (CS) was positioned approximately 20 cm above and 8 cm in front of the rabbit's head. The CS consisted of a 1-kHz tone of fixed duration (75 ms) and intensity (84 dB). The time lapse between the onset of the CS and the onset of the US, which was defined as the interstimulus interval, was varied between groups (0, 100, 200, 400, and 800 ms). Analog-to-digital conversion, response analysis, and experimental control were done using an Apple II/FIRST computer system (14).

Anesthetic Gas Delivery

N_2O was mixed with oxygen (O_2) at concentrations of either 0, 33, or 67%, using separate flowmeters and delivered to individual animals at flow rates of 3 liters/min. The composition of inspiratory gas delivered was initially adjusted and periodically confirmed by a gas analyzer that was calibrated daily before use. Each animal was fitted with a specially designed anesthesia mask attached to a Jackson-Rees modified Ayre's T-piece (39). Expired gases were scavenged and exhausted outside the building.

Experimental Design

A total of 118 rabbits was divided into 15 squads (there were three doses of the drug and five interstimulus intervals) of eight animals each, except for two squads that contained only seven animals. Within each squad, an ISI was assigned and subjects were randomized to receive either 0, 33, or 67% N_2O. On the day prior to experiments (adaptation day) rabbits received a 105-minute adaptation session, during which no stimuli were presented or N_2O delivered (animals breathed 100% O_2). NMRs occurring during this time were recorded and used to obtain a measure of baseline responding. Following adaptation day, animals were conditioned over an 8-day period using an interstimulus interval (ISI) of either 0, 100, 200, 400, or 800 ms. Each day of conditioning consisted of an initial 30-min of equilibration at the selected N_2O level

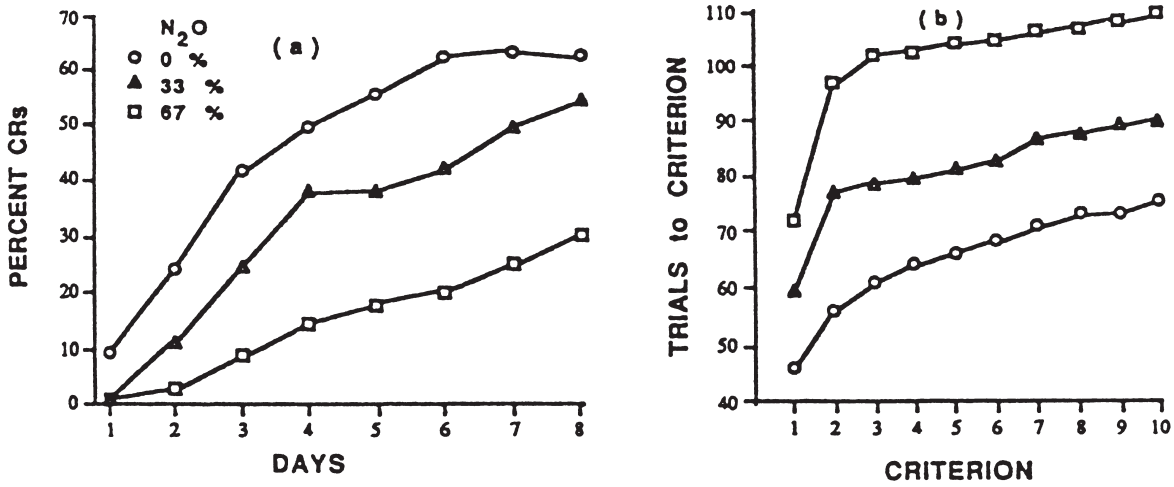

FIG. 2. Percentage CRs, pooled across all five intrerstimulus intervals (ISIs), as a function of the 8 days of paired CS-US conditioning (a) and number of trials to criteria of 1–10 consecutive CRs (b) for the three groups of animals with different doses of nitrous oxide. The number of animals in the control and low- and high-dose nitrous oxide groups were 39, 39, and 40, respectively. The data were based on test trials only.

followed by 75 trials. Trials were presented in a train of four paired CS-US presentations followed by 1 CS (tone) alone presentation (test trial). Amplitude (mm of extension) and onset latency (ms) of responses (NMR) to the US and CS were recorded. A response was recorded as a CR during the tone alone (test trial) if the onset latency occurred within a window of 800 ms duration after the onset of the CS.

Statistical Analysis

A repeated-measures analysis of variance was performed on the data with follow-up analyses to localize significant sources of variation carried out by the method of Tukey's (40) honest significant difference (*hsd*). The level of significance was set at $p < .05$.

RESULTS

Acquisition of CRs was attenuated by N_2O. Figure 2 presents the percentage of CRs on the tone-alone (test) trials across the 8 days of the paired CS-US procedure as a function of N_2O dose. Nitrous oxide caused a dose-dependent retardation of percentage CRs. This was reflected as a main effect of dose, $F(2, 103) = 31.286$, $p < 0.001$. Tukey follow-up tests indicated that the control group had higher percentage CRs than both the low- and high-dose of N_2O groups ($p < 0.01$ for both). The interaction of dose × days was also significant, $F(14, 721) = 4.366$, $p < 0.001$, reflecting the increasing relative impairment from the high dose of N_2O on successive days.

The acquisition of CRs was a significant function of the CS-US interval, as indicated by a significant ISI effect, $F(4, 103 = 51.896$, $p < 0.001$. Tukey tests indicated that the 200-ms ISI group had the maximum percent CRs, which was higher than for the 400-ms group ($p < 0.05$), the 800-, 100-, and 0-ms groups ($p < 0.01$). The effects of N_2O on percent CRs were also a function of ISI, as indicated by significant interactions of dose × ISI, $F(8, 103) = 3.685$, $p < 0.001$, and dose × ISI × days, $F(56, 721) = 3.017$, $p < 0.001$. Figure 3 presents the mean percentage of CRs for different ISIs as a function of N_2O doses. Nitrous oxide depressed the levels of CR percents in a dose-related manner, while keeping the dose–response curves for both doses in a pattern similar to that obtained for controls ISI, i.e., with the 200 ms ISI showing the highest level of CR percent, followed by the 400 ms ISI and then by the 800, 100, and then the 0-ms ISIs.

Figure 4 presents the percentage CRs across the 8 days of conditioning as a function of N_2O doses for individual ISIs. For the O-ms ISI, all the animals failed to demonstrate any evidence of CR acquisition. Control animals demonstrated significant acquisition of CRs at the remaining ISIs ($p < 0.001$), with maximum acquisition occurring at the 200-ms interval ($p < 0.01$). The high dose of N_2O blocked acquisition of CRs at both the 100 and 800 ms ISIs; CR percentages were not different from baseline responding rate at those ISIs. Animals treated with both doses of N_2O demonstrated significant acquisition of CRs at both the 200 and 400 ms ISIs ($p < 0.001$), but performance was significantly lower than that demonstrated by controls ($p < 0.01$). The onset latencies

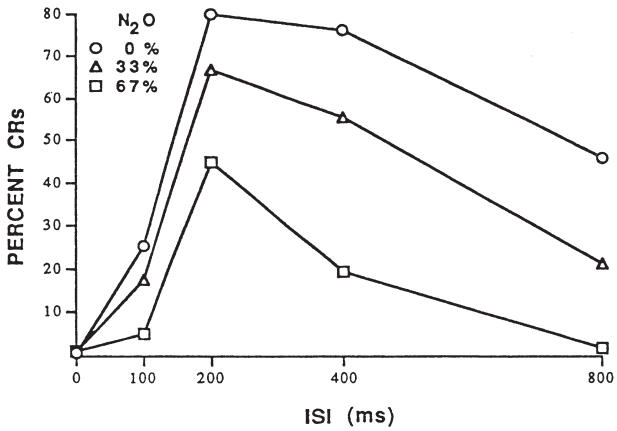

FIG. 3. Percentage CRs, pooled across the 8 days of paired CS-US conditioning, as a function of interstimulus intervals (ISIs) for the three groups of animals with different doses of nitrous oxide. The data were based on test trials only.

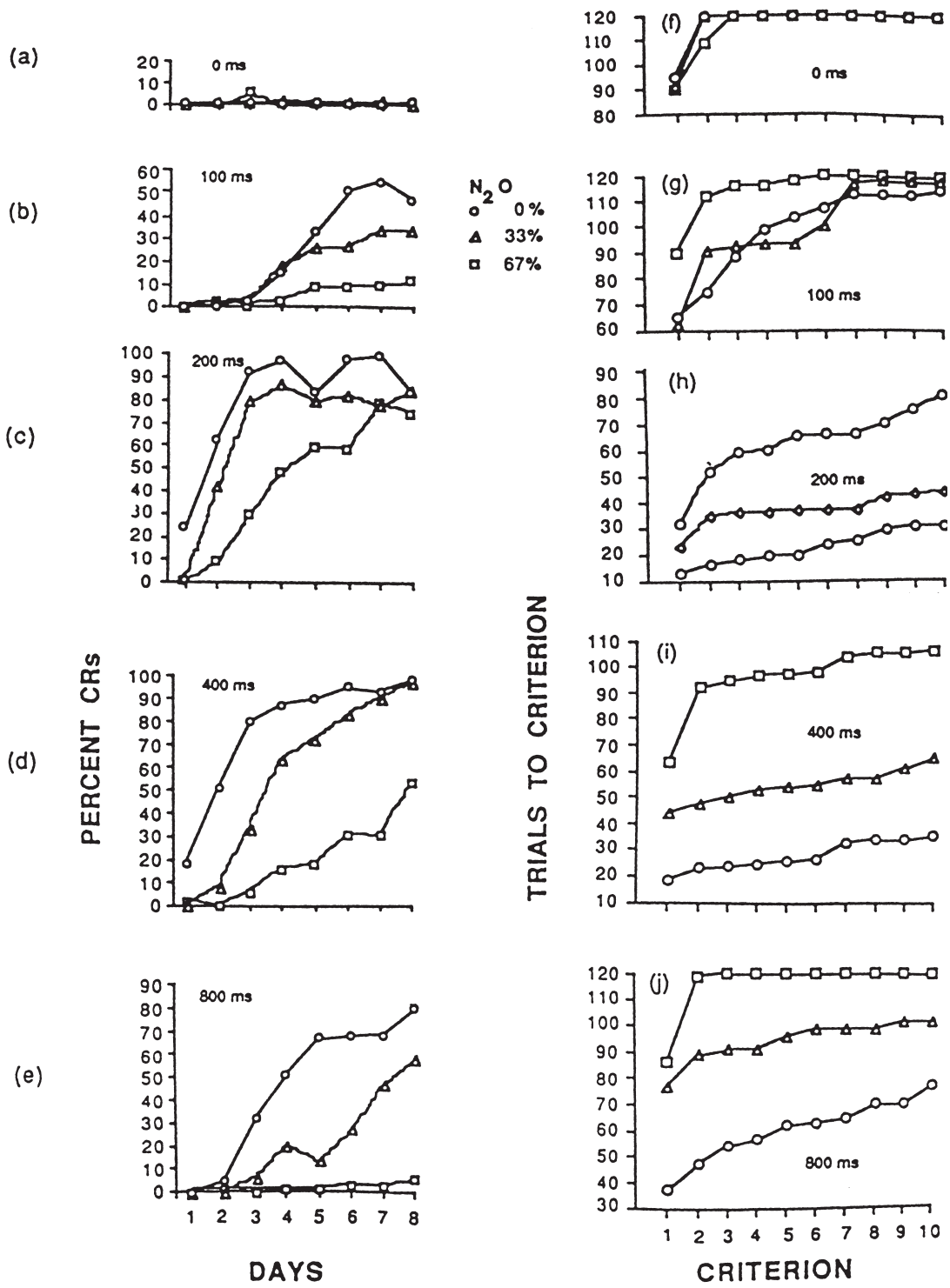

FIG. 4. Percentage CRs as a function of days, interstimulus intervals (ISIs), and nitrous oxide doses (a–e, left side) and number of trials to each criterion of 1–10 consecutive CRs (f–j, right side) on CS-alone test trials. The numbers at the upper left of each plot indicate the ISI in milliseconds.

(measured from the onset of CS until the onset of CR) were greater (mean of 482 ms) for the 0-ms group, which did not show conditioning, than for all other groups ($p < 0.01$ for all). The mean onset latencies for the 100, 200, 400, and 800 ms ISI groups were 128, 154, 221, and 418 ms, respectively. There were no significant differences between onset latencies for 100, 200, and 400 ms groups. The interaction of ISI $\times$ dose was significant for onset latency, $F(8, 103) = 3.132$, $p < 0.005$.

The amplitudes of CRs were a function of N_2O dose and ISI, as indicated by significant main effects of dose, $F(2, 103) = 16.646$, $p < 0.001$, and ISI, $F(4, 103) = 6.672$, $p < 0.001$. The interaction of dose and ISI was also significant, $F(8, 103) = 2.042$, $p < 0.05$. Tukey tests indicated that both the control group (100% oxygen) and the low dose N_2O group had significantly higher amplitudes of CRs than the high dose N_2O group ($p < 0.01$). There were no significant differences between CR amplitudes of the control and the low-dose N_2O groups.

DISCUSSION

The rabbits used in the present studies were sedated, rather than anesthetized. The potency of anesthetic agents is commonly assessed by their minimum alveolar concentrations that prevent 50% of subjects from moving in response to a supramaximal painful stimulus (MAC) (11). MAC for nitrous oxide in the rabbit has not been measured; based on findings for other anesthetics, MAC for nitrous oxide would be expected to be higher than in humans. MAC has been determined for halothane, enflurane, and isoflurane in rabbits. The values are higher than those in humans, i.e., the rabbit needs more anesthetic to prevent its movement in response to a noxious stimulus (Surgical skin incision) (10,30). Clinically, administration of 1.3 MAC prevents movement in nearly all patients during surgery (34). MAC for nitrous oxide in humans is 105–110% (17). It cannot be used alone at 1 atmosphere and still provide a minimum of 21% oxygen. Therefore, it is commonly administered with other anesthetics.

Our results indicate that N_2O produces dose-dependent decrements of learning of the classically conditioned NMR. Learning in the control group followed a concave-down function relating the frequency of conditioned responding to the length of the ISI; learning was absent at O ms ISI, increased at the 100 ms ISI, reached a maximum at the 200 ms ISI, and then progressively declined at the 400- and 800-ms intervals. Learning under the influence of N_2O showed a similar concave-down function with respect to ISI. Classical conditioning accounts (1,18,26) have described the associative memory mechanism by which organisms bridge time gaps between CS and US onset in terms of a "neural stimulus trace." These accounts share the key proposition that the central representation of a stimulus trace persists for an appreciable time after its termination. At too short or too long ISI, the CS trace is presumed to be too weak to produce appreciable increments in associative strength. CR-ISI functions are presumed to reflect the intensity variation of the CS trace over time, and thereby reflect the momentary strength of the associative memory bridging the gap between the onsets of the CS and US. Nitrous oxide changed the shape of the concave-down function, mainly through the high dose affecting acquisition at several ISI intervals more than the other two doses. The effects of scopolamine (15) and morphine (23) are similar to that of nitrous oxide. D-Lysergic acid diethylamide, in contrast to CNS depressants, enhances the rate of CR acquisition across a similar range of ISIs (16).

Nitrous oxide reduces the ability of the tone stimulus to increase the amplitude of the UR, i.e., it reduces the ability of tone to produce facilitation of the nictitating membrane reflex at different CS-US intervals (24). Impairment of time perception, which is characteristic of the actions of nitrous oxide (3,4) and other anesthetics may also impair classical conditioning. Assessment of the time that elapses between successive presentations of stimuli may be important for learning (2). There are a lot of similarities between time discrimination and classical conditioning. The similarities suggest that factors that affect one may similarly affect the other (28). It is, therefore, possible that impairment of timing may be another mechanism by which N_2O decreases the acquisition of CRs.

Use of classical conditioning procedures in behavioral pharmacology to study associative learning necessitates control of nonassociative or performance factors. We believe that the effects of nitrous oxide on CR acquisition are due to impairment of associative learning for the following reasons: nitrous oxide, in the dosages used, has no reliable effects upon nonassociative performance processes such as NMR baseline responding, and NMRs to the CS attributable to sensitization and pseudoconditioning effects that may occur when the CS and US are presented in an explicitly unpaired fashion (24). Also, the similarity of the dose-dependent effects of N_2O on trials to criterion and the overall levels of CRs (Fig. 2) indicate that the pattern of the drug's effects were essentially the same before and after CR occurrence. This similarity suggests that the principal effect of N_2O was not on the performance/execution of CRs but on the entry of conditioning components (CS, US, and/or UR) into the associative processes governing acquisition.

Finally, in another experiment (12) we used isoflurane, a more potent anesthetic with potentially more liability to produce a performance deficit. We tested the rabbits for 6 days of acquisition training while receiving the anesthetic. Then, after 1 day of rest, the animals were given 3 days of extinction consisting of presentations of CS- alone while breathing 100% oxygen. The information that was learned during administration of isoflurane was not retained during extinction. The overall frequency of CRs during extinction and/or rate of decline are taken as measures of the strength of acquisition without the possible confounding of performance factors that may oper-

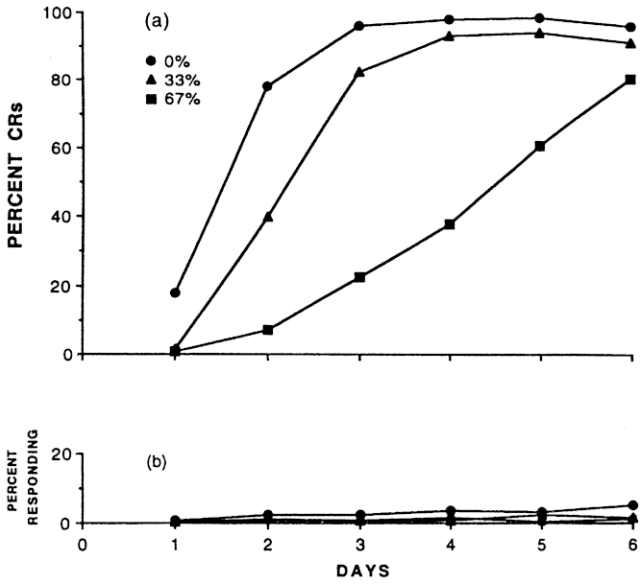

FIG. 5. Effects of nitrous oxide on acquisition of CRs during the paired CS-US training using the delay paradigm. (a) The mean percentage of CRs for animals receiving 100% oxygen ($n = 10$), 33% nitrous oxide ($n = 11$), and 67% nitrous oxide ($n = 11$). (b) The mean percentage of baseline responses during the 400 ms occurring immediately prior to the tones (24).

ate in acquisition. The results did not suggest that isoflurane effects on acquisition were due to performance factors.

Current views recognize a number of different types of learning and memory involving different neural systems in the brain. A major distinction is between the capacity for conscious recollection of facts and events ("declarative memory" or "explicit memory") and a heterogeneous collection of nonconscious learning capacities ("nondeclarative memory" or "implicit memory") that are expressed through performance and that do not require access to any conscious memory content (33). It is difficult to dissociate the latter two memory systems in animals and determine whether a particular task taps into one of those systems or the other. No one knows any method that would allow one to identify "conscious recollection" in nonverbal animals. An example of the difficulty is offered by classical conditioning using the standard delay procedure. On the face of it, this would seem to involve acquisition of a "conscious" association of one event with another, and be a form of explicit memory (21). However, the demonstration that amnesic patients show relatively normal classical conditioning accompanied by complete failure to recollect the conditioning episode (8,9,38,42) forces one to conclude the opposite, i.e., that classical conditioning involves procedural or implicit memory. Evidence from eye-blink conditioning of healthy human subjects also suggests that even if they were aware of the CS-US contingency during learning they blink to the tone CS after associative learning has taken place reflexively, rather than by explicitly recollecting the training episodes (42).

Other evidence that supports the classification of associative learning using the standard delay procedure as involving the implicit memory system comes from studies of animals, in which it has been shown that the cerebellum and its associated brain stem tracts contain the necessary circuitry for the acquisition, storage, and expression of the CR (35). Transection of the brain stem just rostral to the pons spares both the acquisition and retention of the CR (22). If the entire forebrain is not needed for conditioning, then conscious knowledge of the CS-US associations seems less likely to play an essential role in learning (32).

On the other hand, trace classical conditioning seems to be more dependent on the declarative or explicit memory system. This is supported by studies in animals in which large bilateral lesions of the hippocampus made before training markedly impaired this type of conditioning, while the same lesions had no effect of the delay CRs (36). The hippocampus and related structures of the medial temporal lobe support declarative memory (33). Amnesic patients acquired delay conditioning at a normal rate, but failed to acquire trace conditioning. For normal volunteers, awareness was unrelated to successful delay conditioning, but was a prerequisite for successful trace conditioning (7).

Our previous results with delay conditioning (24) showed that the placebo group reached an asymptote of CR acquisition of over 95% by the third day of training, while the group that was treated with 67% N_2O reached an acquisition of CRs of 75% on the sixth day (Fig. 5). In the present study, using an 800-ms ISI, the control group reached an acquisition level of 68% on day 6, while the group that was treated with 67% N_2O demonstrated no learning. Thus, as was expected, the trace CR was more difficult to learn than the CR based on the standard delay procedure, and was more impaired by N_2O. This is consistent with our previous finding in humans that a subanesthetic concentration of N_2O impaired performance on a test of declarative memory, i.e., word recall, more than performance on tests of nondeclarative memory, i.e., word completion and category generation (5). There has been some inconsistency in the literature, with some findings [e.g., (6)] suggesting that subanesthetic concentrations of volatile anesthetics suppress both declarative and nondeclarative memory at the same concentrations. Methodological problems may have contributed to these inconsistent results. The tight control of experimental procedures afforded by animal experiments should provide convincing results. It seems that studies of both explicit and implicit memories are needed for assessments of effects of anesthetics on learning and memory.

In summary, nitrous oxide produced dose-dependent decrements of learning and changed the shape, but not the fundamental pattern, of the concave-down function relating conditioning to the ISI. The decrease in learning may be due to decrease in the intensity or durational properties of the tone-CS, thus blocking the increments of associative strength at each ISI. It may be due to attenuation of the shock-US and/ or UR amplitude. It may also be due to impairment of time discrimination. Trace conditioning in the present study was more impaired than conditioning under the standard delay paradigm in our previous study.

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