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Conditioning Under Carbolonium Bromide and D-Tubocurarine Chloride Paralysis

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O'SULLIVAN, P. A., T. A. MATYAS AND M. G. KING. *Conditioning under carbolonium bromide and d-tubocurarine chloride paralysis.* PHARMAC. BIOCHEM. BEHAV. 8(4) 357–364, 1978. – The findings of past research using d-tubocurarine chloride as a control procedure in heart rate conditioning have been equivocal. The present experiment compared classical heart rate conditioning under neuromuscular blockade by d-tubocurarine chloride, and a pharmacologically more specific blocker, carbolonium bromide. Both groups were compared to a placebo condition. The results indicated that carbolonium bromide and placebo treated animals acquired deceleratory cardiac conditional responses, but animals treated with d-tubocurarine chloride did not. Unconditional acceleratory responses were found in all groups. The findings were discussed in relation to classical conditioning and the psychopharmacology of neuromuscular blockade.

Carbolonium bromide d-Tubocurarine chloride Classical aversive conditioning Heart rate

PARALYSIS by dimethyl-tubocurarine-chloride (Tubarine) has been used extensively as a control procedure in autonomic conditioning studies investigating both classical conditioning [1, 4, 5, 21, 30] and instrumental conditioning [33, 34, 35]. The main concern of the present study was with classical conditioning under neuromuscular blockade.

The behaviour of the curarized groups in previous classical conditioning studies indicates that the results must be viewed as equivocal. For example, some curarized animals did not show an unconditional response (UCR) to the shock [2]. Others did not show a conditional response (CR) in the presence of a UCR [5]. Still others failed to show either a UCR or a CR [21]. This finding is confirmed by a study in which the UCR and CR disappeared under Tubarine paralysis in animals that had previously acquired a conditioned response in the undrugged state [38]. Further, in one study previously mentioned [28], the authors used an unorthodox method to analyse the HR data. They subtracted the heart rate (HR) during the 10th sec of the conditional stimulus (CS) from that of the 1st sec, instead of the pretrial value or other control observation as is both customary and theoretically desirable. Similarly, the HR immediately following shock was compared to that immediately preceding the shock, instead of a control observation.

Examination of the pharmacological literature indicates that Tubarine produces pronounced autonomic blockade in paralytic doses [9,43]. This pharmacological action interferes with conditioning [2,24]. In addition, Tubarine is known to cross the blood/brain barrier in small quantities [9] and blockade evoked potentials from the cortex of animals under Tubarine paralysis has been reported [37]. Tubarine has been found to produce diminished levels of excitation in a posterior hypothalamus [18].

Probable alternatives to Tubarine [9,24] e.g. a synthetic curare, gallamine triethiodide (Flaxedil), and dimethyl-tubocurarine-iodide, whilst somewhat more satisfactory also produce equivocal findings. For example, conditioned blood pressure (BP) elevations and HR decelerations have been reported using Flaxedil [47], while a later study found both HR and BP UCR's, but debilitated CR's [41]. Dimethyl-tubocurarine iodide treated animals, on the other hand, showed diminished CR's relative to the placebo group, but greater than the Tubarine group, indicating that some autonomic blockade and/or central effect may have occurred [24].

A neuromuscular blocker which has not been used to date in conditioning experiments is Carbolonium Bromide (Imbretil). Imbretil has been extensively reported to have no effect on the autonomic ganglia of man, dog, cat, guinea pig, mouse and rat [8, 9, 10, 11, 12, 15, 17, 22, 23, 28, 31, 40] in paralytic doses. Effects on the cardiovascular system and on intestinal motility do not begin to occur until 20–30 times the relaxing dose is administered [11, 31, 40]. A deep, long lasting paralysis can be achieved by a dose not more than 10 times the relaxing dose even in the rat, which is relatively resistant to Imbretil [7]. In regard to central effects, no studies have reported Imbretil to be centrally active following peripheral administration. The one study comparing Imbretil with a number of drugs known to be centrally active supports this position [29].

The original intention of the Tubarine studies was to examine autonomic conditioning in the absence of skeletal

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muscle activity. Autonomic effects resulting from Tubarine imply interference with at least the performance of the CR and possibly its acquisition. Central effects resulting from Tubarine may also impede acquisition. It is possible that the difficulty in obtaining classical conditioning under neuromuscular blockade with Tubarine is due to the original hypothesis that skeletal muscle involvement is necessary to autonomic CR acquisition [42]. Alternatively the difficulty may be due to prevention of CR acquisition or performance by the nonmuscular effects of Tubarine. The pharmacological evidence indicates that Imbretil is superior in these respects. Consequently the present study compared HR response during classical conditioning under Imbretil with that obtained under the most commonly used paralizing agent, Tubarine, as well as that obtained under placebo conditions.

METHOD

Animals

The animals were 30 male albino Wistar rats between 95 and 130 days old. Animals were housed in pairs in their home cages prior to the experiment.

Apparatus

The CS+ (1000 Hz, dB A) and CS− (4000 Hz, 75 dB a) tones were generated by two audio-oscillators. Rats can easily detect both these frequencies [6]. The tones were delivered through Rola (6MO1) Speakers mounted on a board fixed to two retort standard, 30 cm above the rats' head. The UCS was a 1 mA pulsed DC shock produced by a constant current shock generator. The CS tones and the shock were scheduled by a mini computer and associated logic units. The ECG and EMG signals were amplified and recorded on ultraviolet paper by a Devices polygraph (Model M19). The cardiotachograph was recorded on one channel, the integrated EMG (IEMG) on a second channel and CS presentation on a third.

Procedure

Pilot studies determined that a dose of 10 mg/ml/kg of Imbretil and 3 mg/ml/kg of Tubarine was necessary to maintain paralysis for the duration of the experiment. Drug solutions were kept at 4°C throughout the period of use. Both drugs and the placebo solution (9% NaCl) were administered by intraperitoneal (IP) injection under light ether anaesthesia.

Paralysed animals were artifically respirated by introducing an air tube into the pharyngeal region of the mouth and sealing the mouth opening with surgical tape and modelling clay. The animals were placed supine on the preparation board. A mound of modelling clay maintained the thorax elevated and the respiratory pathways open. The caudal end of the preparation board was elevated 15° to facilitate fluid exit from the pharyngeal area.

Placebo animals were restrained in a calico harness jacket suspended between the 2 retort stands holding the CS speakers. The jacket was laced dorsally and contained openings for the head, the tail and the legs. Restraint by suspension harness ensured not only relative immobility, but also permitted good access to the animal particularly in respect of electrode loci.

Electrocardiograms were obtained from electrodes implanted in the loose dorso–lateral skin folds caudal to the front limbs. Electrodes consisted of 26 ga stainless steel monofilament (Ethicon). Amplification of the ECG signal was constrained by high and low pass filtering. The objective was to produce a signal containing an R wave, imbedded in noise of not more than 50% of the R wave amplitude. This ensured reliable Schmitt triggering in the ratemeter.

The EMG electrodes were Grass stainless steel needle electrodes implanted in the *biceps femoris* muscle of the left hind leg. The EMG signal was calibrated at 50 μV/cm following previous standards [14]. The high frequency cut-off was set at 10 K Hz, and the time constant at 0.03 sec. The EMG pre-amplifier output was fed into a Hewlett-Packard oscilloscope.

Direct EMG was analysed on the oscilloscope to establish that all muscle activity had ceased before the experiment was begun. Direct EMG was monitored throughout the experiment to ensure that the IEMG recorded contained only noise. Both the direct EMG and IEMG observations were used to ascertain that complete paralysis was achieved throughout the experimental period.

Shocks were administered through a Grass stainless steel needle electrode. The electrode was implanted dorsal to the left hind leg in the *gluteus maximus*. Shock return was through the EMG electrodes with the amplifier momentarily blocked.

Each group received 48 acquisition trials. Each of these trials contained a 10 sec CS and a 0.5 sec UCS administered 9.5 sec after CS onset. The 2 CS values and the 3 intertrial interval (ITI) values used were ordered on a fixed quasi-random schedule with these constraints: (1) equiprobable CS+ and CS− occurrence; (b) equiprobable ITI occurrence of 60, 70 and 80 sec; (c) the same CS or ITI values were prohibited from occurring more than twice consecutively; and (d) recorded trials consisted of adjacent CS+ and CS− trials. The recorded trials were 11, 12; 23, 24; 35, 36; and 47, 48.

Data Reduction

Transformation of cardiac period into cardiac frequency introduces errors, which are reflected in the mean, variance and degree of skewness of the basic data [27]. Therefore, ECG data in this experiment were analysed in inter-beat intervals (IBI). Six IBI's were averaged at each of 7 points during the trials. The pretrial baseline was obtained by averaging the 6 beats immediately preceding CS onset. The first CS sample (C_1) was obtained by averaging the 6 beats immediately following CS onset. The second sample (C_2) contained 6 beats in the middle of the CS period and the last sample (C_3) contained 6 beats immediately preceding UCS onset.

Samples during the CS period were limited to 3 per trial in view of the scoring effort and in view of previous findings which indicate that the CR is a lasting deceleratory trend maximising in proximity to UCS onset time [24]. The first post UCS sample (U_1) was obtained by averaging the 6 beats immediately following the UCS offset. The second sample (U_2) contained 6 beats 5 sec post UCS and the last sample (U_3) contained 6 beats 9 sec post shock.

The procedure of averaging a fixed number of beats, instead of averaging all beats occurring in a nominated time interval such as 1 sec, was adopted for 2 reasons. First, means based on equal sample sizes will have equal statistical stability. The second reason derives from the relative span

of breathing and cardiac cycles in the rat. The mean breathing rate of rats is about 70 cycles/min [19] which gives a mean cycle duration of 0.857 sec. The expected range of HR responding was 350–500 bpm [21]. Six beats within these limits can occupy between 0.72 sec and 1.028 sec. Therefore, the samples should, on the average, distribute evenly over one respiratory cycle.

RESULTS

Analyses for CRs in the CS periods and UCR's in the post CS periods were conducted independently. In addition examination of the raw data indicated that error variances were not homogeneous across the 3 treatment groups. Consequently analyses of variance (ANOVA's) were conducted separately for each drug group. Subsequent examination of the equivalent error mean squares (MS's) for each treatment group confirmed the error variance heterogeneity in both the CS period and the post CS period.

Effects in the CS Period

Placebo group. The mean IBI's observed in placebo rats during the CS period were investigated with a 4 x 2 x 4 ANOVA for repeated measures on all factors [26]. Figure 1 shows that the mean IBI's during the CS tended to increase on most trial blocks. The ANOVA confirmed this typical deceleratory cardiac effect with a significant samples effect, $F(3,29) = 12.902$, $p<0.001$. Figure 1 also suggests that deceleratory cardiac responding improved as trials progressed. By blocks 3 and 4 IBI was increasing steadily during the CS, reaching a minimum mean heart rate at sample C_3 just before the UCS point. This apparent improvement in deceleratory cardiac responding was confirmed by the ANOVA with a significant Blocks x Samples effect, $F(9,81) = 2.058$, $p<0.05$. However placebo treated rats showed some evidence of discrimination between CS+ and CS– occurrence. Figure 1 indicates not only that mean IBI's increased in the samples taken in the later part of the CS period, but that they did so to a greater extent during CS+ trials than during CS– trials. The ANOVA confirmed that cardiac deceleration progressed to a greater extent during CS+ trials than during CS– trials with a significant CS x Samples interaction, $F(3,27) = 10.282$, $p<0.001$. The ANOVA showed no other significant effects in placebo treated animals during the CS period.

Imbretil group. The mean IBI's of Imbretil treated animals were treated with a 4 x 2 x 4 ANOVA for repeated measures over all factors identical to that used for the placebo means. Figure 2 shows the mean IBI's obtained from Imbretil treated rats. Unlike the placebo group means of Fig. 1, cardiac responding appeared more erratic during the first 2 trial blocks in the Imbretil treated rats. However by blocks 3 and 4 particularly Imbretil animals also began to show consistent IBI increases at C_2 and C_3. This acquisition of deceleratory responses during the CS presentation was confirmed by the ANOVA with a significant Blocks x Samples interaction, $F(9,81) = 2.178$, $p<0.05$. No other significant changes were indicated in the Imbretil group means during the CS period.

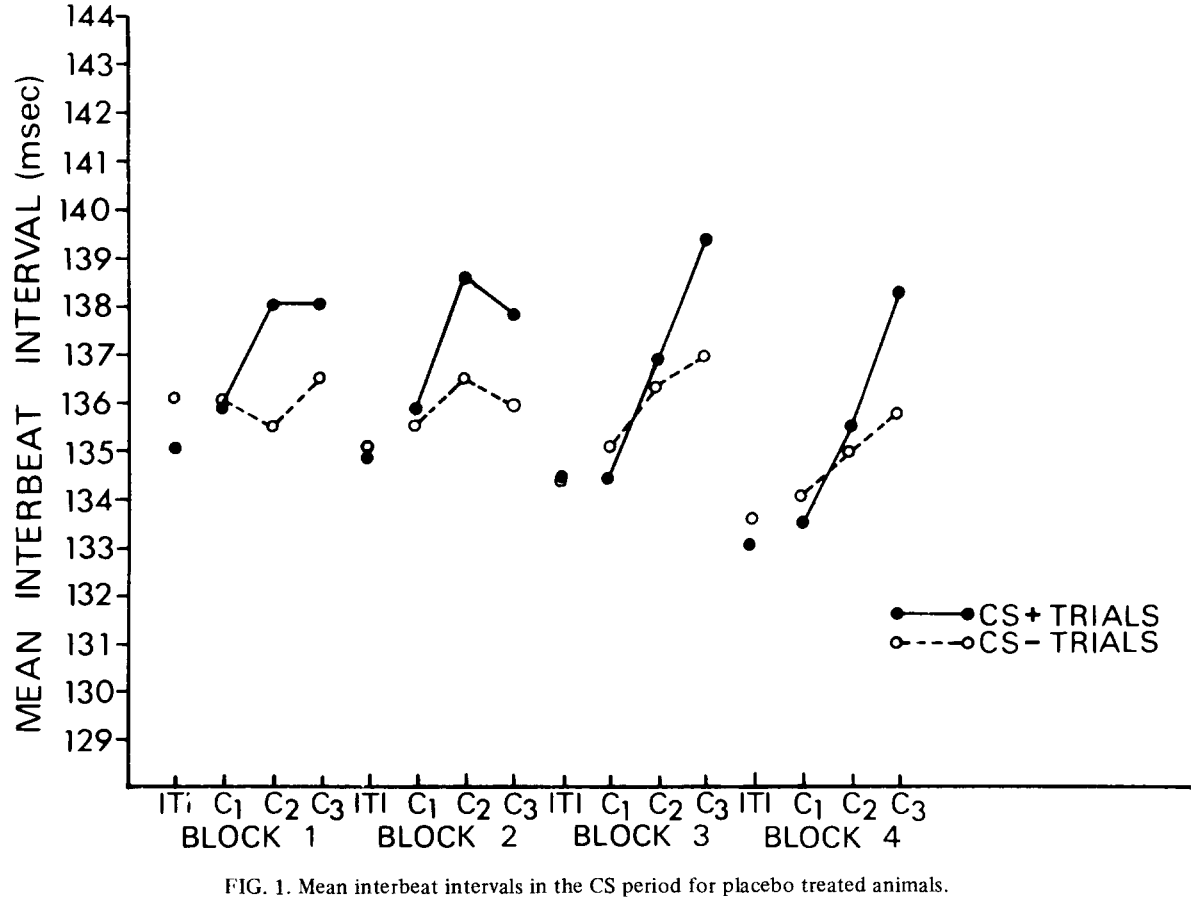

FIG. 1. Mean interbeat intervals in the CS period for placebo treated animals.

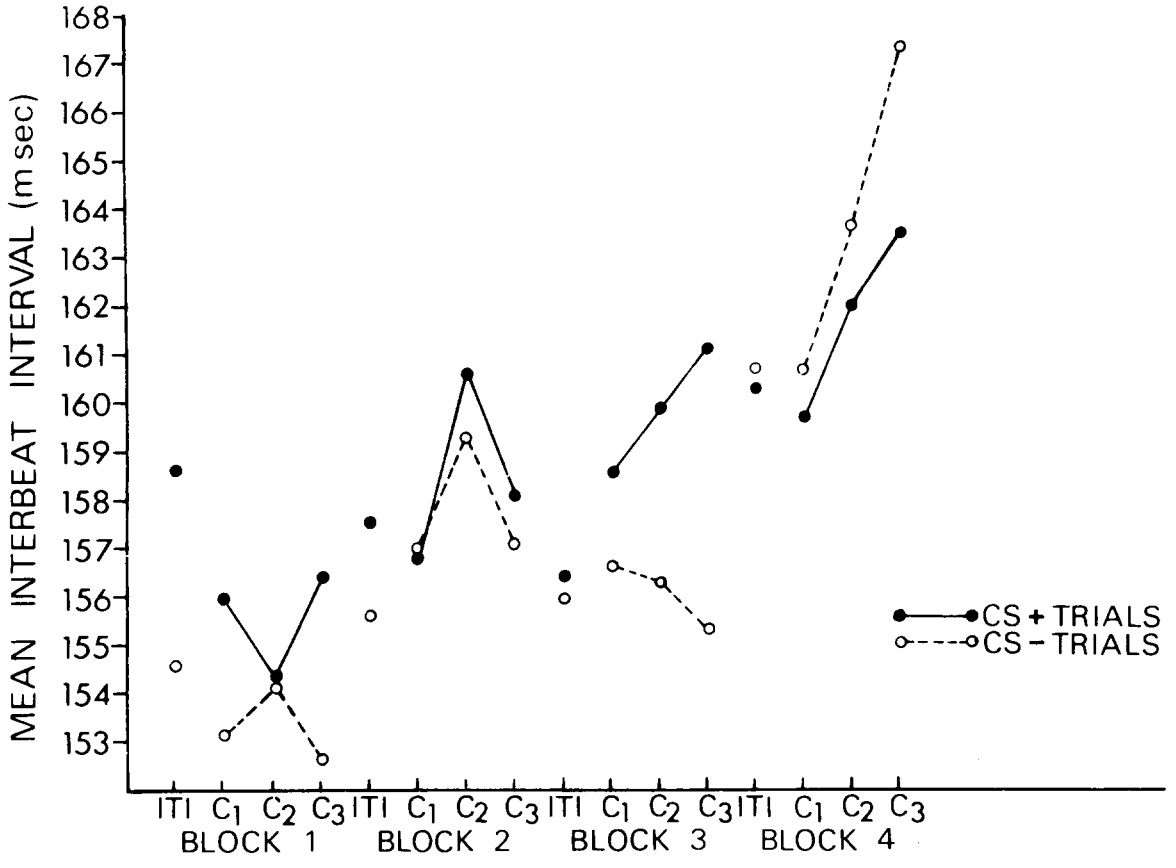

FIG. 2. Mean interbeat intervals in the CS period for the Imbretil treated animals.

Tubarine group. The ANOVA for Tubarine data was also a 4 x 2 x 4 factorial design with repeated measures over all factors, identical to those used for Placebo and Imbretil data. Figure 3 shows the IBI means of Tubarine treated rats observed in the CS period. Apart from a general increase in basal IBI, a previously obtained finding [21], no marked or consistent changes were apparent. The ANOVA indicated that the upward drift in baseline occurred reliably since the Blocks main effect was significant, $F(2,37) = 4.074$, $p<0.02$. Furthermore, the ANOVA failed to detect any other reliable changes, as Fig. 3 suggests. The failure of the Tubarine group to show the deceleratory response to the CS obtained in the placebo and Imbretil groups cannot be attributed to lesser sensitivity for the effect in the Tubarine ANOVA. Examination of the equivalent error MS's in general, but of the Blocks x Samples effects in particular, showed that the error MS was smallest in the Tubarine ANOVA. This indicates that the Tubarine ANOVA was the most sensitive of the three since the factorial arrays were identical and the frequencies of observations were identical. It was concluded therefore that whilst Imbretil and placebo treated rats acquired deceleratory cardiac responses to the CS, Tubarine treated rats failed to do so.

Effects in the Post-CS Period

Placebo group. The mean IBI's observed in placebo treated rats following CS termination were analysed with a 4 x 2 x 4 ANOVA for repeated measures over all factors [26]. The ITI values were included in the samples factor to provide a baseline reference. Figure 4 shows the mean IBI's obtained following signaled shock and signaled no-shock. Placebo animals showed the typical IBI reduction immediately following shocks (U_1). This returned to base in the subsequent samples (U_2 and U_3) with a suggestion of a deceleratory overshoot. Conversely following no-shock trials the mean IBI remained relatively steady and if anything slightly elevated. The ANOVA indicated that the averaged samples differed significantly, $F(3,27) = 4.560$, $p<0.02$, but more importantly it showed that the HR increase and recovery was particularly a feature of shock trials since the CS x Samples interaction was very close to the 5% significance level, $F(3,27) = 2.861$, $p<0.056$. The ANOVA obtained no other significant effects.

Imbretil group. The ANOVA was identical to that used for placebo observations. Figure 4 shows that following shocks, Imbretil treated rats, like placebo treated animals, also showed a rapid IBI reduction followed by baseline recovery with a suggestion of overshoot. In contrast following no-shock trials, mean IBI's were relatively steady. By comparison to the placebo UCR the Imbretil UCR appears much larger, but this must be regarded in the context of much larger error variances. The ANOVA indicated significant effects among the averaged samples, $F(3,27) = 9.763$, $p<0.001$, but also indicated that this was not equally so following CS+ and CS− trials, $F(3,27) = 7.177$, $p<0.005$, thus confirming the suggestion in Fig. 4 that IBI

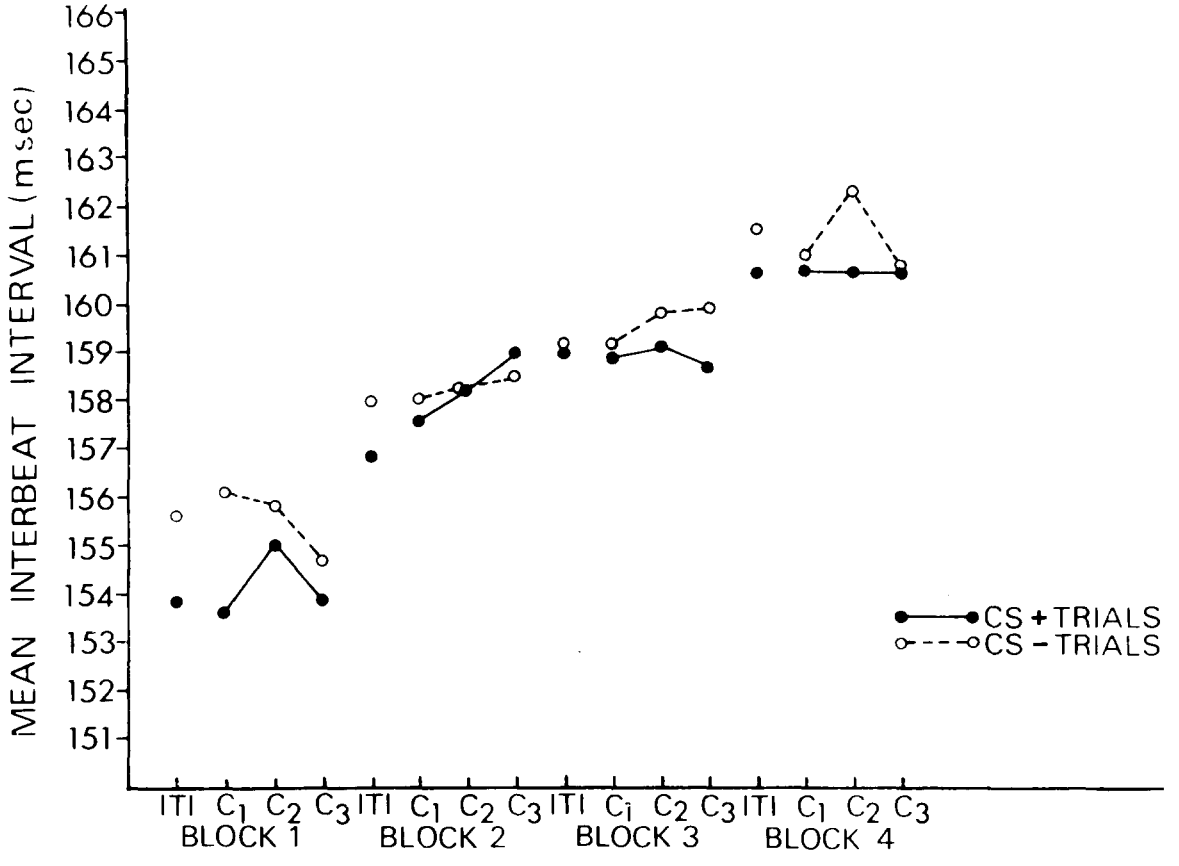

FIG. 3. Mean interbeat intervals in the CS period for the Tubarine treated animals.

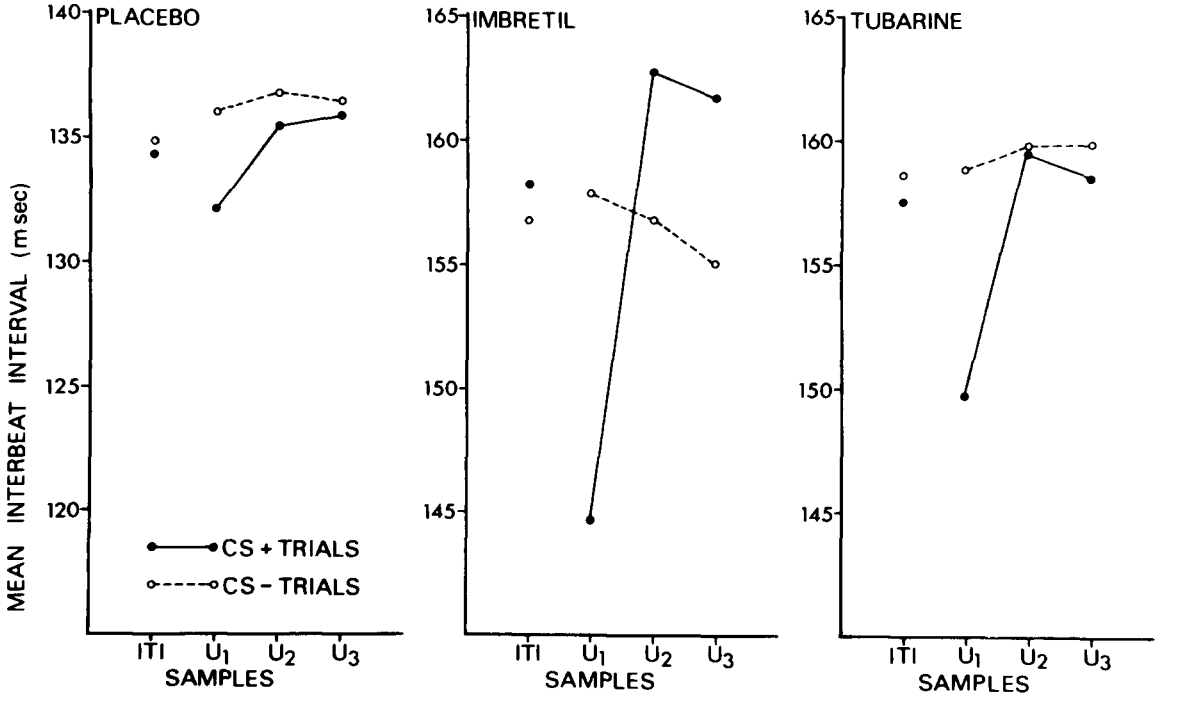

FIG. 4. Mean interbeat intervals in the post CS period for the placebo, Imbretil and Tubarine treated animals.

reduction and recovery was specifically a feature of shock trials.

Tubarine group. The ANOVA was identical to that performed for placebo and Imbretil data. Figure 4 shows that as in the placebo and Imbretil groups shocks also elicited the IBI reduction and recovery with slight overshoot in Tubarine treated rats. Also, in parallel to the other 2 groups, the no-shock trials produced no effects in the post-CS period. The UCR appears smaller than that obtained in the Imbretil group but larger than that obtained in the placebo group. The ANOVA showed that the averaged IBI means for CS+ were significantly shorter than the CS− means, $F(1,9) = 15.763$, $p<0.005$, and that significant differences existed between the averaged samples, $F(3,27) = 12.245$, $p<0.001$. However both these main effects were qualified by the significant CS × Samples interaction, $F(3,27) = 10.035$, $p<0.001$. This confirmed the suggestion from Fig. 4 that the HR acceleration and subsequent recovery was specifically a feature of the post-CS period of CS+ rather than CS− trials. It was concluded therefore that all 3 groups showed significant UCR's.

In order to determine whether the UCS's of the 3 drug conditions were as disparate as Fig. 4 suggests the F ratio was calculated for the Drugs × CS × Samples effect that would have obtained if the 3 drug groups had been analysed together. This indicated a near significant Drugs × CS × Samples interaction, $F(6,81) = 3.343$, $p<0.06$. Allowing that under violation of the homogeneity of variance assumption, α values may be in error by as much as 0.03 [26], the current data still suggests a trend with a Type I error rate of less than 9%. It seems probable therefore that UCR's under the 2 neuromuscular blockers, and particularly under Imbretil, were larger than the UCR's obtained from the placebo treated animals.

DISCUSSION

The main aim of the present experiment was to determine if rats can acquire autonomic CR's when they are paralysed by a neuromuscular blocker known to be of more specific pharmacological action than Tubarine. The reliable decelerative responses acquired over trials in the CS period by the Imbretil group indicate that rats can acquire autonomic responses even when the skeletal musculature is paralysed.

However the conditioning effects under Imbretil were not as clear as those of placebo-treated rats. As a comparison of Figs. 1 and 2 suggests, the deceleratory response in the Imbretil group, although apparently larger by the final trial block, was not consistently evident as early as in the placebo group. The placebo group also showed reliable differentiation of CS+ and CS−, with CS+ trials producing larger deceleration. The failure of the Imbretil group to show such a discrimination does not seem to be attributable to the larger error variance obtained in the Imbretil condition. Even using the correspondingly smaller placebo group error variance the effect would not have reached significance. This suggests that the CR generalized to the CS− in the Imbretil animals. As an hypothesis, it is possible that animals whose CNS and autonomic nervous system remain functional are sensitized to fear effects by the additional stress induced with paralysis.

As expected, in contrast to both placebo and Imbretil treated rats, Tubarine treated rats failed to show evidence of HR CR's. Given the pharmacological specificity of Imbretil [8, 9, 10, 11, 12, 15, 17, 22, 23, 28, 29, 31, 40] and the non-specificity of Tubarine [9, 18, 37, 43], these results support the conclusion that previous failures to obtain classically conditioned autonomic responses under neuromuscular blockade [5, 21, 24, 38, 41] were probably due to nonspecific actions of the blockers rather than to an inability to acquire autonomic CR's without skeletal muscle involvement [42].

In addition to the demonstration of reliable CR's in the placebo group the conclusion that adequate conditioning parameters were obtained in all groups is strengthened by the demonstration of significant HR UCR's in all 3 treatment conditions. The finding that Tubarine treated rats can show reliable, topographically characteristic, HR UCR's is in contrast with some previous findings [1, 21, 24, 38], but in agreement with another [5]. Because of the procedural variations which include dosage, premedication, and UCS administration, it is difficult to account for the similarities and disagreements. However, within the present parameters, there is the interesting finding that Tubarine treated rats though failing to produce a HR CR did produce a UCR. This suggests hypotheses conceived in terms of either the peripheral anticholinergic effects of Tubarine [9,43], or alternatively its central effects [18,37]. One possible hypothesis is that the UCR occurs and the CR does not because the UCR is primarily an accelerative response under noradrenergic sympathetic mediation, while the CR is primarily a decelerative response, under cholinergic parasympathetic mediation, which is inhibited by the anticholinergic action of Tubarine. Another possible hypothesis is that the UCR, because it is an unconditioned reflex, may be less open to the central effects of Tubarine than is the CR, which has to be acquired and is presumably dependent on more complex perceptual and associative processes. Recent evidence that parasympathetic withdrawal is also implicated in the HR UCR mediation [16, 25, 32], as well as the suggestion of a decelerative overshoot on the recovery arm of UCR's in the present study, seems to support the position that vagal performance is sufficiently competent to at least contribute to the HR UCR under Tubarine blockade. This in turn accords more importance to the explanation based on central effects. If this is so the conclusion suggested is that failure to observe CR's under Tubarine is not simply due to an inability to perform the acquired CR, but due to an impedance of learning. A recent failure to demonstrate CR's using transfer designs in both directions and neuromuscular blockade with dimethyltubocurarine-chloride supports this suggestion [39].

Another finding which emerged from the UCR results was that placebo group UCR's tended to be smaller than UCR's under neuromuscular blockade, particularly when Imbretil blockade was used. However this may be due simply to a law of initial value effect [46]. The pretrial baseline of placebo injected rats was a mean IBI of 134.6 msec; that is the baseline HR was consistently above that of the 2 chemically restrained group, which showed a mean pretrial IBI of 157.5 msec for Tubarine and 158.1 msec for Imbretil. A comparison of Fig. 1 with Figs. 2 and 3 supports this view. The elevated baseline of the mechanically restrained placebo group may have been due to the suspension restraint technique used in the present study since restraint of rats is known to be a potent stressor [20,45].

A final effect which emerged from the data was the heterogeneity of error variances across the treatment groups. As Tables 1 and 2 indicate, this was primarily due to large variances in the Imbretil treated animals. These imply wider differences between Imbretil treated rats in respect to the several effects tested by the ANOVA. These individual differences may be due to variations in absorption of the drug by rats: absorption of Imbretil by other species is superior to that of the rat [7]. Together with the difficulties associated with rat restraint, the absorption problems constitute a recommendation against the rat preparation for studies concerned with learning under muscular paralysis.

In conclusion, the present study has shown that classical conditioning can elicit acquired autonomic responses under muscular paralysis. Carbolonium bromide is recommended as a preferred paralytic agent and dimethyl-tubocurarine-chloride is not recommended. Preparations using phylogenetically advanced species to the rat seem desirable both on behavioral and psychopharmacological grounds. The success of carbolonium bromide in the demonstration of classical conditioning of autonomic responses suggests that a parallel approach may be useful in the controversy regarding skeletal muscle involvement in instrumental learning of autonomic responses. Since recent failures to replicate instrumental autonomic learning under neuromuscular blockade [36] may be due either to the nonspecific action of the blocker used, or a genuine inability to learn without skeletal muscle involvement, studies with the more specific neuromuscular blocker carbolonium bromide seem indicated, preferably with a species other than the rat.

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