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Cholinolytic Antagonism to the Disruptive Effects of Oral Low Doses of Pyridostigmine on Simple Discrimination Performance in Rats[1]

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LIU, W.-F. *Cholinolytic antagonism to the disruptive effects of oral low doses of pyridostigmine on simple discrimination performance in rats.* PHARMACOL BIOCHEM BEHAV **40**(4) 745–749, 1991.—We have previously reported that acute oral administration of low doses (≤12 mg/kg) of pyridostigmine bromide (PYR) to rats resulted in a dose-dependent decrement in reinforcement rate under two different multiple schedules of response-produced water presentation, which involved motivational dysfunction rather than motor impairment and alterations in visual perception. The purpose of the present investigation was to examine further if the anticipated operant behavioral deficits of PYR are mediated by central and/or peripheral cholinergic mechanisms. Lever-press responses of male Sprague-Dawley rats were maintained under a multiple fixed-ratio GO/differential-reinforcement-of-low-rate NO GO, brightness discrimination, schedule of water reinforcement. The effects of the muscarinic antagonists atropine (ATR) and methylatropine (MAT), both at doses of 0.25, 0.5 and 1.0 mg/kg (SC), against a single oral low dose of PYR (12 mg/kg)-induced behavioral disruption were compared. ATR partially antagonized the reinforcement loss of PYR with concomitant dose-related increases in nonreinforced responses, whereas MAT completely antagonized the reinforcement loss without affecting the frequency of nonreinforced responses. These results suggest that in rats, the debilitating effects of oral PYR on operant behavior are primarily due to the stimulation of peripheral muscarinic receptors via its anticholinesterase activity. The increments of nonreinforced responses observed after coadministration of PYR with ATR may reflect a central, excitatory action of ATR which could affect the discrimination performance. The present results have practical implications for the clinical utilization of PYR in combination with the peripherally active muscarinic antagonist in situations where optimal performance is required.

Pyridostigmine Atropine Methylatropine Discrimination Operant behavior Rats

PYRIDOSTIGMINE bromide (PYR), a reversible inhibitor of acetylcholinesterase (AChE), used for the chronic treatment of myasthenia gravis (3,13), has been suggested for use in prophylaxis against intoxication with lethal irreversible AChE inhibitors (4, 6, 7, 11). Although PYR, as a quaternary carbamate, has been alleged to hardly cross the blood-brain barrier (3,15), it has been postulated that PYR may have more central actions than hitherto accepted in view of the following findings: PYR at low doses (≤10% LD_{50}, IP) far below those that cause overt symptoms, interfered with certain behavioral paradigms that involve higher CNS structures in rats (20); PYR has a prophylactic efficacy against intoxication with soman (4, 6, 7, 11), an organophosphate (OP) AChE inhibitor with a predominantly central mode of action (19); and PYR improved memory and attention in patients with senile dementia of the Alzheimer type when given by intravenous infusion (1).

We have recently demonstrated that in rats, oral low doses of PYR (≤15% LD_{50}) resulted in decrements in reinforcement rate under two different multiple schedules, implemented with light-intensity discriminative stimuli, of response-produced water presentation [i.e., fixed-ratio time-out (FR/TO) and fixed-ratio differential-reinforcement-of-low-rate (FR/DRL) schedules (12,14)]. These disruptive effects are associated with motivational dysfunction rather than motor impairment and are not likely to result from alterations in visual perception (12). The present investigation was designed to further examine if the anticipated operant behavioral deficits of oral PYR are mediated by central and/or peripheral cholinergic mechanisms. This was carried out to compare the antagonistic effects of the muscarinic antagonists atropine (ATR), presumed to have both central and peripheral actions, and methylatropine (MAT), presumed to act only peripherally, against a single oral low dose of PYR (12 mg/kg) induced behavioral disruption on operant responding of a simple brightness discrimination task in rats.

METHOD

Subjects

Eight male Sprague-Dawley rats weighing 350 to 400 g were used. The subjects were water-deprived for 22 h but received ad

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lib food. Animals had received PYR (3–12 mg/kg, PO) previously (12), but had been drug-free for four weeks before the start of this study. They were housed individually in a room with a 12-h day-night light cycle (light on from 6:00 a.m. to 6:00 p.m.).

Apparatus and Procedure

The rats were trained to press a lever for water presentation (0.01 ml tap water) on a multiple FR-10 GO/DRL-10″ NO GO light-intensity discrimination schedule, i.e., this schedule was implemented with a 10-s limited hold of bright and dim houselights, respectively. The eight rats were separated into two groups of 4 rats each; one for ATR + PYR and the other for MAT + PYR testings. Testing was carried out between 9:00 a.m. and 2:00 p.m. in four identical rat operant chambers located in sound-attenuating boxes. Events were scheduled by an Omron C-20 controller (Japan) and recorded by an Acer 1100 computer (Taiwan, R.O.C.). Each animal was placed in the same box 5 days a week (Monday–Friday) at approximately the same time each day for 120-min sessions. The number of reinforcers received and nonreinforced DRL-respondings emitted were recorded. The rats were normally dosed with drugs on Fridays, and the performance on Thursday served as control data. The extensive details of apparatus and training procedure have been presented in a previous paper (12).

Drug Preparation and Administration

Both atropine sulfate (ATR) and methylatropine bromide (MAT) (Sigma Chemical Company, Chicago, IL) were dissolved in sterile isotonic saline (0.9%), and pyridostigmine bromide (synthesized by the Organic Chemistry Unit of our division) was dissolved in distilled water (dH_2O). Both ATR and MAT (0.25, 0.5 and 1.0 mg/kg, SC), and PYR (12 mg/kg, PO) doses are expressed in terms of total salts. Both the subcutaneous injections with ATR or MAT, and the oral gavages with PYR were given immediately prior to the 2-h experimental sessions. Injection and gavage volumes were kept constant at 1.0 ml/kg and 5 ml/kg, respectively. The doses of ATR and MAT selected were behaviorally inactive in our preliminary experiments, with the exception of 0.5 and 1.0 mg/kg ATR which decreased reinforcement rate to about 85% and 80% of baseline control, respectively, but both were without significant effects on nonreinforced response. A single oral dose of PYR (12 mg/kg) was chosen from prior experimental studies of acute PYR effects on lever pressing behavior in rats (12,14) that it produced reinforcement loss by about 50–60% of baseline levels, an effect ideal for studying the antagonists with PYR interactions.

Statistical Analysis

Data on the number of reinforcements obtained as a percent of the baseline control levels in the drug interaction studies were subjected to a repeated measures analysis of variance (ANOVA). Mean contrasts comparison using Tukey's HSD test were subsequently determined. Since the group variances among the treatments on nonreinforced respondings were heterogenous, these data were subjected to a nonparametric ANOVA (Kruskal-Wallis test) followed by a Tukey-like multiple comparison test for treatment effects. p-Values <0.05 were considered statistically significant for treatments effects.

RESULTS

Baseline Control Performance

The rate of operant responding in the multiple FR/DRL schedule was very stable across these experiments. During the 120-min control sessions, the animals in the ATR plus PYR group (N = 4) received 389 ± 83 reinforcers and emitted 100 ± 42 nonreinforced DRL respondings (mean ± SEM); while the animals in the MAT plus PYR group (N = 4) received 218 ± 33 reinforcers and emitted 54 ± 19 nonreinforced DRL respondings. Although the ATR + PYR group had higher rates of responding than those of the MAT + PYR group, their variability ratios (i.e., SE/mean) for reinforcers received and nonreinforced respondings emitted were about the same (i.e., 0.2 and 0.4, respectively). The finding that the average number of reinforcers obtained by the former group of animals was greater than 360, the theoretical maximum number of reinforcers during the 120-min session, was due to the fact that one of the four rats could sometimes receive two reinforcements within a 10-s period of the FR-10 responding.

TABLE 1

ANTAGONISM OF ATROPINE AGAINST REINFORCEMENT LOSS INDUCED BY PYRIDOSTIGMINE UNDER A MULTIPLE FR/DRL SCHEDULE OF WATER PRESENTATION IN RATS (N = 4)*

Treatments	Mean (± SEM)% of Baseline Rate of Reinforcement	p Values Versus Vehicle	p Values Versus Pyridostigmine
dH_2O + Saline (PO + SC)	92.6 ± 2.3	—	<0.01
Pyridostigmine (12 mg/kg, PO) + Saline (SC)	53.7 ± 7.2	<0.01	—
Pyridostigmine (12 mg/kg, PO) + Atropine (0.25 mg/kg, SC)	74.1 ± 10.2	NS	NS
Pyridostigmine (12 mg/kg, PO) + Atropine (0.5 mg/kg, SC)	82.2 ± 5.5	<0.05	<0.05
Pyridostigmine (12 mg/kg, PO) + Atropine (1.0 mg/kg, SC)	58.9 ± 4.8	<0.05	NS

*Pyridostigmine (PO) and atropine (SC) were both/either given immediately prior to the 2-h experimental sessions. There were significant differences among the five treatments, $F(4,12) = 5.69$, $p<0.01$ (a repeated measures ANOVA followed by Tukey's HSD test).

Atropine Antagonism

The effects of a single dose of PYR (12 mg/kg, PO) alone and in combination with the centrally active muscarinic antagonist ATR (0.25, 0.5 and 1.0 mg/kg, SC) on reinforcements, expressed as percent of baseline control levels, are shown in Table 1. The overall effect of the PYR + ATR combination on reinforcements was moderately significant, $F(4,12) = 5.96$, $p<0.01$. PYR alone produced a significant ($p<0.01$) decrease in the number of reinforcers received by 53.7 ± 7.2% of baseline control. ATR significantly attenuated the reinforcement loss of PYR in a dose of 0.5 mg/kg only ($p<0.05$).

The effects of PYR + ATR in combination on nonreinforced responding are shown in Fig. 1. The left half (Panel A) of Fig. 1 shows data for the individual subjects: the 5 baseline days, and the effects of drug combination, PYR and vehicle. The right half (Panel B) of this figure shows the average data (mean ± SEM) expressed as the percent of baseline control. A nonparametric ANOVA (i.e., Kruskal-Wallis test) yielded a significant effect for nonreinforced responding ($H = 10.98$, $p<0.05$). The nonpa-

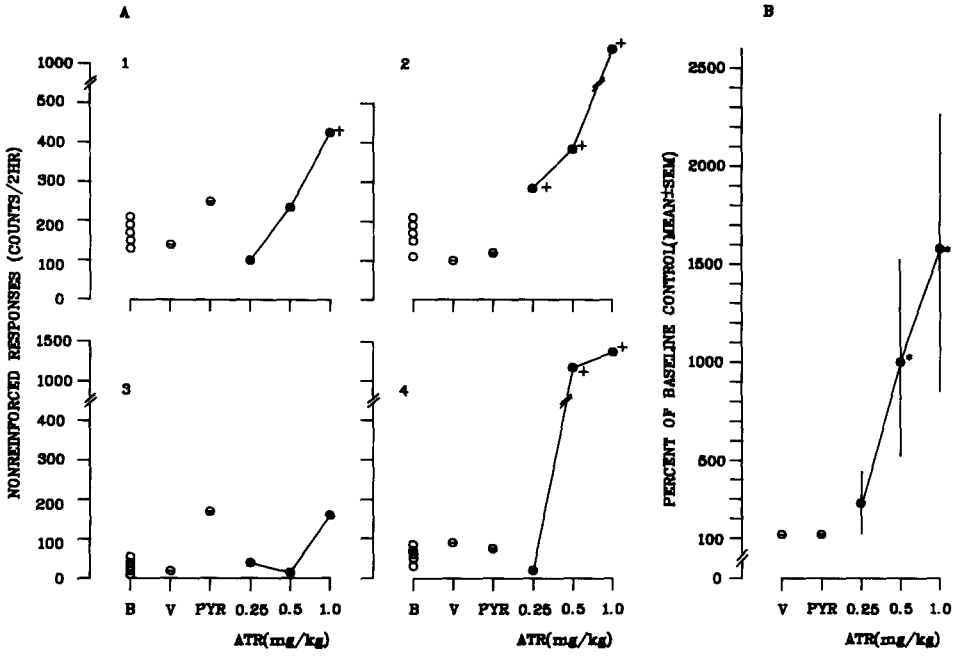

FIG. 1. Effects of PYR administered alone or in combination with atropine (ATR) on nonreinforced responses under the multiple FR/DRL schedule of water reinforcement in rats. PYR (12 mg/kg, PO) and ATR (0.25, 0.5 and 1.0 mg/kg, SC) were both/either given immediately prior to the 2-h experimental sessions. Left (panel A): Individual dose-effect curves of 4 subjects. Points above B are for 5 respective baseline sessions; points above V are for 1 vehicle session. + indicates response increments at least two times greater than both the respective baseline control and PYR-treated levels. Right (panel B): Group data expressed as percentage of baseline control (N = 4). *$p \leq 0.05$, vs. PYR alone, nonparametric ANOVA (Kruskal-Wallis test) followed by Tukey-like multiple comparison procedure.

rametric Tukey-type multiple comparison test yielded significant effects for ATR doses of 0.5 mg/kg ($p = 0.05$) and 1.0 mg/kg ($p < 0.05$) in combination with PYR. This increasing trend of ATR effect with increasing dose for nonreinforced responding can also be seen from the data for the individual subjects, on the left half of this figure. As the dose of atropine increased, the number of rats which exhibited a striking increase in responding, as defined arbitrarily by at least a two times greater than both the respective baseline control and PYR treated levels, increased proportionately. The incidence of behavioral activation was 1/4, 2/4 and 3/4 for 0.25, 0.5 and 1.0 mg/kg doses of ATR, respectively.

Methylatropine Antagonism

The effects of a single dose of PYR (12 mg/kg, PO) alone and in combination with the peripherally active muscarinic antagonist MAT (0.25, 0.5 and 1.0 mg/kg, SC) for reinforcers expressed as percent of baseline control are shown in Table 2. A repeated measures ANOVA revealed a highly significant overall effect of the PYR + ATR combination on reinforcement, $F(4,12) = 14.1$, $p < 0.001$. PYR alone produced a significant ($p < 0.001$) decrease in the number of reinforcements received by $54.1 \pm 1.6\%$ of baseline control. MAT caused a statistically significant antagonism of the reinforcement rate decreasing effect of PYR, in a dose-related manner, with significant effects for MAT at doses of 0.5 mg/kg ($p < 0.05$) and 1.0 mg/kg ($p < 0.001$).

The effects of the PYR + MAT combination on nonreinforced responding are shown in Fig. 2. The Kruskal-Wallis test revealed an insignificant overall effect of the MAT + PYR

TABLE 2

ANTAGONISM OF METHYLATROPINE AGAINST REINFORCEMENT LOSS INDUCED BY PYRIDOSTIGMINE UNDER A MULTIPLE FR/DRL SCHEDULE OF WATER PRESENTATION IN RATS (N = 4)*

Treatments	Mean (± SEM)% of Baseline Rate of Reinforcement	*p* Values Versus Vehicle	*p* Values Versus Pyridostigmine
dH_2O + Saline (PO + SC)	94.5 ± 4.0	—	<0.001
Pyridostigmine (12 mg/kg, PO) + Saline (SC)	54.1 ± 1.6	<0.001	—
Pyridostigmine (12 mg/kg, PO) + Methylatropine (0.25 mg/kg, SC)	65.3 ± 3.7	<0.01	NS
Pyridostigmine (12 mg/kg, PO) + Methylatropine (0.5 mg/kg, SC)	73.8 ± 5.4	<0.05	<0.05
Pyridostigmine (12 mg/kg, PO) + Methylatropine (1.0 mg/kg, SC)	89.8 ± 6.6	NS	<0.001

*Pyridostigmine (PO) and methylatropine (SC) were both/either given immediately prior to the 2-h experimental sessions. There were significant differences among the five treatments, $F(4,12) = 14.1$, $p < 0.001$ (a repeated measures ANOVA followed by Tukey's HSD test).

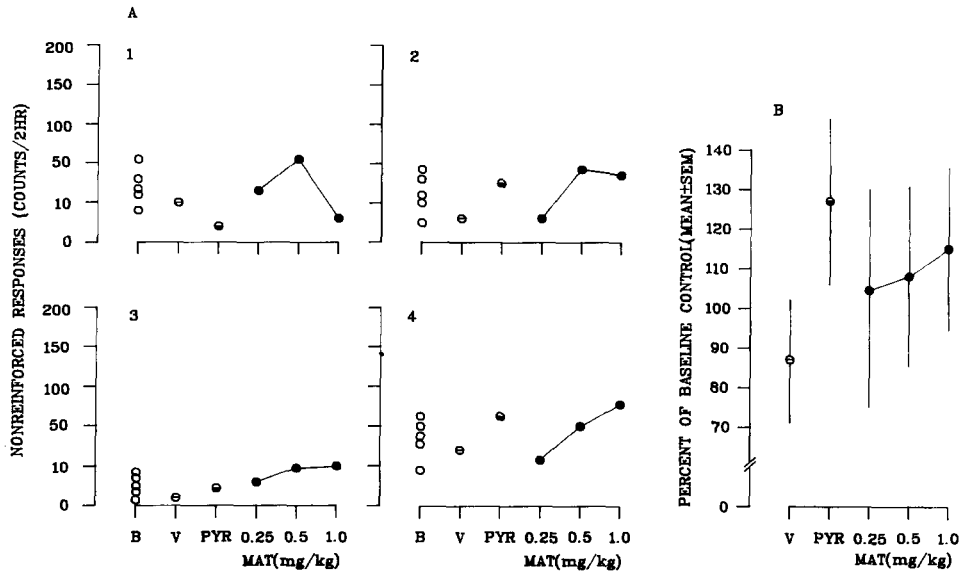

FIG. 2. Effects of PYR administered alone or in combination with methylatropine (MAT) on nonreinforced responses under the multiple FR/DRL schedule of water reinforcement in rats. PYR (12 mg/kg, PO) and MAT (0.25, 0.5 and 1.0 mg/kg, SC) were both/either given immediately prior to the 2-h experimental sessions. Left (panel A): Individual dose-effect curves of 4 subjects. Points B are for 5 respective baseline sessions; points above V are for 1 vehicle session. Right (panel B): Group data expressed as percentage of baseline control (N=4). Other details as in Fig. 1.

combination on nonreinforced responding ($H=5.94$, $p>0.05$). Obviously, PYR alone or in combination with MAT did not affect performance in nonreinforced responding.

DISCUSSION

PYR, orally administered in rats at a low dose of 12 mg/kg alone, produced a moderate decrease in reinforcements obtained (by 50–60% of baseline levels), without significantly affecting the frequency of nonreinforced responses above control levels, a finding that replicated previous study from our laboratory (12). The interaction of ATR (0.25, 0.5 and 1.0 mg/kg, SC) and PYR (12 mg/kg, PO) upon the simple brightness discrimination task indicated that only a single dose of ATR (0.5 mg/kg) caused a significant antagonism of PYR-induced reinforcement loss; however, coadministration of ATR with PYR produced an ATR dose-related increase in nonreinforced responses. Administration of the behaviorally inactive doses of MAT (0.25, 0.5 and 1.0 mg/kg, SC) significantly antagonized the reinforcement loss of PYR in a completely dose-related manner without affecting the frequency of nonreinforced responses.

The observed effects of increasing nonreinforced responding after the coadministration of ATR with PYR are in a manner similar to previous reports from other laboratories which demonstrated that coadministration of ATR and the centrally acting muscarinic agonists physostigmine or oxotremorine in monkeys increased response rates on temporal controlled responding for food reinforcement and shock avoidance, despite the rate-decreasing effects of either drug alone (16,18). This response rate-increasing effect has been suggested as ATR has nonmuscarinic behavioral excitatory effects that were unmasked in the presence of cholinomimetics (17).

Elsmore et al. (5) applied signal detection theory to a delayed brightness discrimination performance in rats following treatment with ATR and MAT. They found that both drugs reduced rates of responding in a dose-related fashion; however, effects of the drugs on sensitivity (response accuracy) were quite different, with atropine producing greater performance decrements than methylatropine on the four-s-delayed trials, which support the conclusion that ATR effects on rate of responding is primarily due to peripheral factors, while effects on qualitative features of discriminative performance are central in origin. Accordingly, the observed antagonistic effects of both drugs on PYR-induced reinforcement loss (i.e., decrements in reinforced responding) in the present brightness discrimination task, a paradigm qualitatively similar to the delayed conditional discrimination used by above authors, suggest that the debilitating effects of oral PYR on operant performance are primarily due to peripheral cholinergic mechanisms, while the striking increases in nonreinforced responding (inhibited response) after coadministration of ATR and PYR are likely resulted from the nonmuscarinic behavioral excitatory actions of ATR (17).

In a preclinical safety assessment, Kluwe et al. (10) administered PYR orally (capsule gavage) to dogs at doses as low as 0.05 mg/kg every 8 hours (daily dose of 0.15 mg/kg), which inhibited red blood cell (RBC) AChE by approximately 10%, produced some evidence of gastrointestinal (GI) side effects, but had no systemic toxicity. This suggests that orally administered PYR at a dose normally used as a prophylactic against OPs or as a therapeutic in other clinical conditions such as myasthenia gravis (3,13) and Alzheimer's dementia (1) may have muscarinic side effects on GI tract to normal human subjects, since PYR is relatively poorly absorbed from the GI tract (3,15). In fact, the existing data of clinical safety evaluation of oral PYR in therapeutic levels did consistently report that GI disturbance is the most common adverse effect of PYR (3,8). However, studies, including preclinical and clinical evaluations, on a direct correlation between GI disorders and sensory-motor performance have not yet been conducted. Whatever the causal relationship, previous studies from this laboratory have found that the detrimental effects of oral PYR on rat brightness dis-

crimination performance resulted from motivational dysfunction rather than motor impairment, which is unlikely to be related to alterations of visual perception (12). The oral coadministration of MAT with PYR completely antagonized the reduction of the corresponding unconditioned water intake of PYR in a dose-related manner, while ATR did so with narrow dose range and without dose-dependency (unpublished observations). Moreover, the degree of AChE inhibition after oral administration with sign-free and/or toxic doses of PYR in rodents was found to be much greater and longer in the ileum than in RBC, diaphragm and brain regions (unpublished observations). Taken together, the observed detrimental effects of oral PYR on brightness discrimination in the present findings and our previous studies on operant behavior in rats may be due to the stimulation of peripheral muscarinic receptors in the GI tract via its anticholinesterase activity.

Based on estimates of the range of effective antagonism and the adverse behavioral effects of muscarinic antagonists, MAT was a better antagonist of PYR-induced behavioral suppression than ATR; and ATR had behavioral excitatory effects when coadministered with PYR, while MAT did not have such. These results have practical implications for the clinical utilization of the combination of PYR with the peripherally acting muscarinic antagonists in situations in which optimal performance is required, such as in military settings.

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REFERENCES

1. Agnoli, A.; Martucci, N.; Manna, V.; Conti, L.; Fiorananti, M. Effect of cholinergic and anticholinergic drugs on short term memory in Alzheimer's dementia. A neuropsychological and computerized electroencephalographic study. Clin. Neuropharmacol. 6:311–323; 1983.
2. Borland, R. G.; Brennan, D. H.; Nicholson, A. N.; Smith, P. A. Studies on the possible central and peripheral effects in man of a cholinesterase inhibitor (pyridostigmine). Hum. Toxicol. 4:293–300; 1985.
3. Breyer-Pfaff, U.; Mairer, V.; Brinkmann, A. M.; Schumm, F. Pyridostigmine kinetics in healthy subjects and patients with myasthenia gravis. Clin. Pharmacol. Ther. 37:495–504; 1985.
4. Dirnhuber, P.; French, M. C.; Green, D. M.; Leadbeater, L.; Stratton, J. A. The protection of primates against soman poisoning by pretreatment with pyridostigmine. J. Pharm. Pharmacol. 31:295–299; 1979.
5. Elsmore, T. F.; Parkison, J. K.; Leu, J. R.; Witkin, J. M. Atropine effects on delayed discrimination performance in rats. Pharmacol. Biochem. Behav. 32:971–975; 1989.
6. Gall, D. The use of therapeutic mixtures in the treatment of cholinesterase inhibition. Fundam. Appl. Toxicol. 1:214–216; 1981.
7. Gordon, J. J.; Leadbeater, J.; Maidment, M. P. The protection of animals against organophosphate poisoning by pretreatment with a carbamate. Toxicol. Appl. Pharmacol. 43:207–216; 1978.
8. Johnston, A.; Hedges, A.; Turner, P. A study of the interaction between oxytetracycline and pyridostigmine. Hum. Toxicol. 7:263–266; 1988.
9. Kay, L. D.; Morrison, J. D. The effects of ingestion of 60 mg pyridostigmine bromide on contrast sensitivity in man. Hum. Toxicol. 7:347–352; 1988.
10. Kluwe, W. M.; Page, J. G.; Toft, J. D.; Ridder, W. E.; Chung, H. Pharmacological and toxicological evaluation of orally administered pyridostigmine in dogs. Fundam. Appl. Toxicol. 14:40–53; 1990.
11. Leadbeater, L.; Inns, R. H.; Ryland, J. M. Treatment of poisoning by soman. Fundam. Appl. Toxicol. 5:S225–S231; 1985.
12. Liu, W. F. Acute effects of oral low doses of pyridostigmine on simple visual discrimination and unconditioned consummatory acts in rats. Pharmacol. Biochem. Behav. 41:251–254; 1992.
13. Rubinstein, E.; Federman, D. D. Scientific American Medicine—Neurology: Disease of muscle and the neuromuscular function. New York: Scientific American; 1983.
14. Shih, J. H.; Liu, W. F.; Lee, S. F.; Lee, J. D.; Ma, C.; Lin, C. H. Acute effects of oral pyridostigmine bromide on conditioned operant performance in rats. Pharmacol. Biochem. Behav. 38:549–553; 1991.
15. Somani, S. M.; Roberts, J. B.; Wilson, A. Pyridostigmine metabolism in man. Clin. Pharmacol. Ther. 13:393–399; 1972.
16. Witkin, J. M. Central and peripheral muscarinic actions of physostigmine and oxotremorine on avoidance behavior of squirrel monkeys. Psychopharmacology (Berlin) 97:376–382; 1989.
17. Witkin, J. M. Behavioral pharmacology of compounds affecting muscarinic cholinergic receptors. In: Barrett, J. E.; Thompson, T.; Dews, P. B., eds. Advances in behavioral pharmacology, vol. 7. Hillsdale, NJ: Lawrence Erlbaum; 1990: 79–118.
18. Witkin, J. M.; Markowitz, R. A.; Barrett, J. E. Physostigmine-insensitive behavioral excitatory effects of atropine in squirrel monkeys. Pharmacol. Biochem. Behav. 32:309–315; 1989.
19. Wolthuis, O. L.; Berends, F.; Meeter, E. Problems in the therapy of soman poisoning. Fundam. Appl. Toxicol. 1:183–192; 1981.
20. Wolthuis, O. L.; Vanwersch, R. A. P. Behavioral changes in the rat after low doses of cholinesterase inhibitors. Fundam. Appl. Toxicol. 4:S195–S208; 1984.