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ON THE INTERACTION OF DRUGS WITH THE CHOLINERGIC NERVOUS SYSTEM—V.

CHARACTERIZATION OF SOME EFFECTS INDUCED BY PHYSOSTIGMINE IN MICE: *IN VIVO* AND *IN VITRO* STUDIES

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Abstract—Four systemic effects induced by s.c. injection of physostigmine and neostigmine to mice were investigated by the "quadro-test" procedure, a novel design in which salivation, tremor, hypothermia and the rotarod-effects are simultaneously and continuously recorded. The time profiles of these effects were used to construct dose–response curves, which were compared to those of brain acetylcholinesterase (AcChE) inhibition by the drug. A good relationship was found between the dose and time dependency of these pharmacological and biochemical parameters, as well as between the relative potencies of physostigmine and neostigmine and their affinity to the enzyme.

Using scopolamine.Hbr and its quaternary analogue, as well as tertiary cholinergic agonists and other cholinesterase agents, it was found that the physostigmine-induced hypothermia is mostly a central-muscarinic response, while the tremor is most probably a non-muscarinic peripheral effect. The peripheral-muscarinic origin of the salivation was confirmed. The effects measured in the rotarod test were found to be muscarinic and mixed central-peripheral for physostigmine, and non-muscarinic for neostigmine. The lethality caused by physostigmine seems to be partially centrally mediated, while that of neostigmine is peripheral.

Reversible anticholinesterase agents were shown previously to induce changes in brain acetylcholine content, distribution and release [1–4]. Although it is widely accepted that most of the acute effects induced by anticholinesterase agents can be attributed to the accumulation of the neurotransmitter [5], there is some evidence suggestive of direct action of cholinesterase inhibitors at the neuromuscular junction [6–9].

Several investigators described the correlation of cholinesterase inhibition in brain and in the periphery with diverse behavioral effects, both spontaneous and forced [10–17]. This subject was reviewed at length by Brimblecombe [5].

In the present study we undertook to establish the relationship between brain acetylcholinesterase (AcChE) activity and several systemic effects induced by physostigmine in mice. These responses can be differentiated into peripheral versus central and muscarinic versus non-muscarinic. This characterization provides a starting point for the elucidation of the biochemical basis of tolerance to physostigmine.

MATERIAL AND METHODS

Drugs

Physostigmine (eserine) salicylate, neostigmine (prostigmine) bromide, acetylthiocholine (iodide) and 5,5'Dithiobis (2-Nitrobenzoic acid) (DTNB, Ellman reagent) were obtained from Sigma. (−) Scopolamine.HBr ($(\alpha)^{D}_{25} = -13.3°$, C = 2.04 in 1 N HCl) and pilocarpine (HCl) were obtained from Plantex (Israel). The Methiodide salt of scopolamine was prepared from the free base and methyliodide in anhydrous ether. Oxotremorine (free base) and tacrine hydrochloride (1,2,3,4-tetrahydro-9-amino-acridine) were obtained from Aldrich, and (±) 3-acetoxyquinuclidine hydrochloride was prepared according to Robinson *et al.* [18]. The drugs were dissolved in saline and injected s.c. in a constant volume of 0.1 ml/animal. Fresh solutions were prepared every 2–3 days, and stored refrigerated until used.

Animals

The ICR male and female mice used were approximately four weeks old and weighted 19–22 g. Rats of the Wistar strain weighing 200–250 g were used in several experiments. The animals were housed in plastic cages, with food and water available *ad lib.* The temperature and light were kept on constant schedules (ambient temperature 23 ± 0.5°, 12 hr light, 12 hr dark). The animals were allowed a minimum of two days to acclimatise after shipment before any experimental procedure was begun.

Methods

The pharmacological tests will be specified for the mice. Essentially the same methods were applied in the rats experiments.

Immediately prior to each test, groups of animals were removed from the housing cages, placed in new 20 × 30 × 40 cm plastic cages and food and water were withdrawn for the 1–2 hr of the experiments. The

"quadro-test" procedure was employed whereby four effects induced by the drugs were measured continuously in 6-min cycles: hypothermia, salivation, tremor and the effects measured in the rotarod test (vide infra). The experiments were conducted between 14:00 and 23:00.

Hypothermia. Rectal temperature of the animals was recorded by a YSI (model 456 TUC) telethermometer, at a constant ambient temperature (23 ± 0.5°). The rectal temperature of each was read 30 sec after the probe of the telethermometer was inserted 2.5 cm into the rectum. Results are expressed either as the mean temperature reduction, or as the mean rectal temperature, as a function of time. Six mice were tested with every dose.

Salivation and tremor were recorded according to Inch *et al.* [19], and are represented as the percentage of affected animals versus the time. Nine mice were tested with every dose.

Rotarod test. Mice were placed on a rod 32 mm in diameter, rotating at 16.5 rev/min. Sideward movements on the rod were limited by circular discs set 19 cm apart. After 5–10 min of pretraining on the rod all the mice were able to stay on for at least 120 sec. These mice were injected with test drugs and subjected to test trial lasting 120 sec. Mice which fell off the rotarod within the first 30 sec were returned to the rod and the trial was continued for an additional 90 consecutive sec. Those mice unable to stay on the rod within these 90 sec were scored as affected. The mice were tested in this manner every 6 min until a complete recovery from the drug effect was achieved. Results are expressed as the percentage of dropout as a function of time. In each test 9 mice were used per dose.

"*Quadro-test procedure*". During the "6-minute cycle" of this procedure 9 mice were tested: first on the rotarod (2 min), then simultaneously for tremor and salivation (1 min), and finally the rectal temperatures of 6 out of the 9 animals were recorded (3 minutes). Figure 1 is an example of the time profile curves obtained by this procedure for 0.48 μmoles/kg (0.2 mg/kg) physostigmine salicylate. The standard deviation ranged between 8–18% for the peak effect and 10–15% for the duration in all 4 measurements, and for all the doses used.

Acetylcholinesterase (AcChE) activity. The enzymatic hydrolysis of acetylthiocholine was determined in whole brain homogenates spectrophotometrically, according to Ellman *et al.* [20], using a Varian Techtron spectrophotometer model 235. The mice were decapitated and their brains quickly removed and homogenized in a Potter-Elvehjem glass homogenizer fitted with a motor-driven Teflon pestle. From the dependency of AcChE activity on the substrate concentration, it was found that the reaction was linear in the concentration-range of $0.8–6 \times 10^{-4}$ M. This activity was found to depend linearly on the amount of brain homogenate within the range of 1–12 mg of tissue per assay. Thus, a typical reaction mixture contained in a final volume of 3.0 ml, 8 mg tissue, 0.1 ml 0.01 M DTNB and 0.1 M phosphate buffer, pH 8. The tissue was prepared as a 10% homogenate in 0.1 M phosphate buffer, pH 8, containing 0.1 M NaCl + 0.5% Triton X-100, and diluted to a final concentration of 20 mg/ml with 0.1 M phosphate buffer, pH 8.0. All AcChE assays were carried out at 25°, pH 8.0, and were initiated by addition of 0.025 ml of the substrate. No butyrylcholinesterase activity was found under these conditions using butyrylthiocholine as substrate. Determination of the degree of AcChE inhibition by physostigmine and neostigmine was carried out *in vitro* after 10 min preincubation of the diluted brain homogenate (20 mg/ml) with the anticholinesterase drug, at 25°, pH 8.0, using 6×10^{-4} M substrate.

The time profile for the inhibition of brain AcChE by physostigmine *in vivo* and its spontaneous reacti-

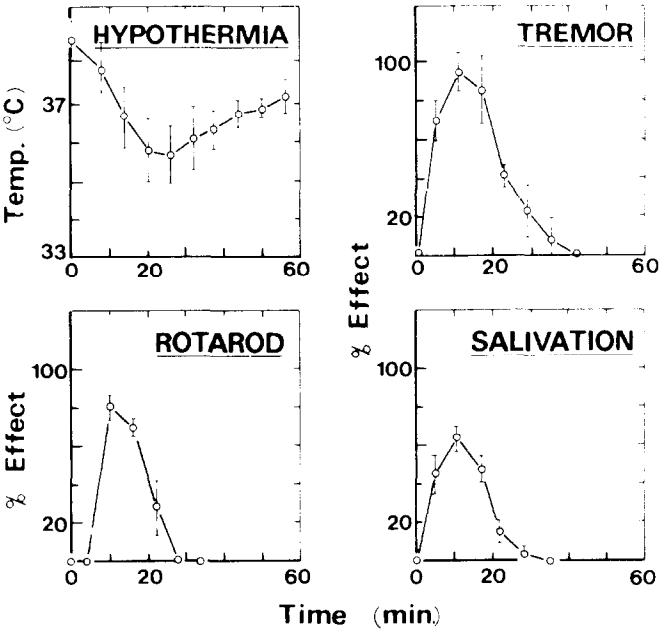

Fig. 1. Time profiles of 4 systemic effects induced by 0.2 mg/kg (0.48 μmoles/kg) physostigmine in mice. Results are the mean ± S.D. of 6 experiments, 9 mice in each, using the "quadro-test" procedure.

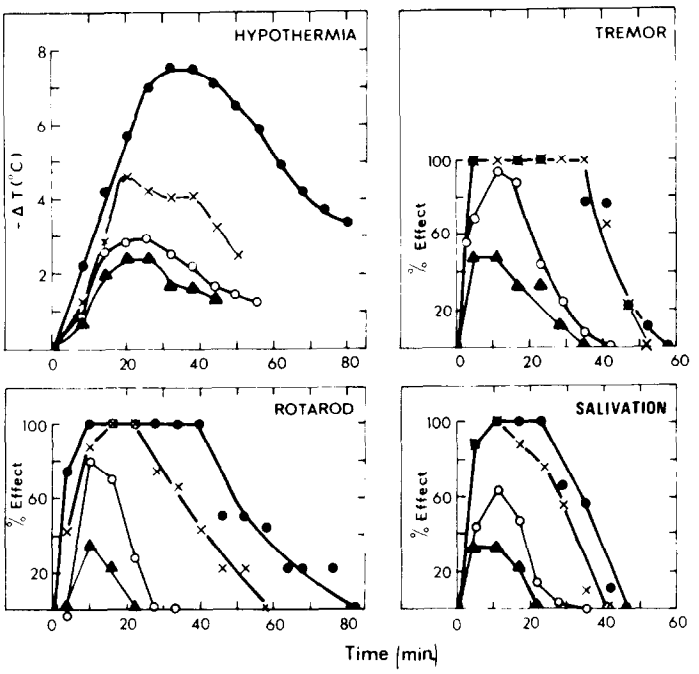

Fig. 2. Dose-dependent time profiles of 4 systemic effects induced by physostigmine in mice. (▲——▲) 0.1 mg/kg (0.24 μmoles/kg); (○——○) 0.2 mg/kg (0.48 μmoles/kg; (×——×) 0.4 mg/kg (0.96 μmoles/kg); (●——●) 0.6 mg/kg (1.44 μmoles/kg). Results are the mean of 3–5 experiments, 9 mice in each. The S.D. is identical to that in Fig. 1.

vation was carried out as follows. At various time intervals after s.c. injection of the drug, 3 animals were decapitated and their whole brains quickly removed. A 10% homogenate was prepared from each brain in an ice-cold 0.1 M phosphate buffer (pH 8.0), as described above. The AcChE activity was determined in each homogenate in triplicate within 4 min from decapitation, using 6×10^{-4} M substrate. This activity was stable at 0° for at least 6 hr and was completely inhibited by addition of 10^{-5} M physostigmine *in vitro*.

LD_{50} *values.* The LD_{50} values of physostigmine and neostigmine with or without preinjection of scopolamine.HBr or scopolamine.CH_3I were determined in 30-day-old animals of both sexes, using 10 mice per dose, according to Behrens [21].

Table 1. LD_{50} values of physostigmine and neostigmine in mice with and without preinjection of scopolamine.HBr or meth-scopolamine

Muscarinic antagonist*	LD_{50} (mg/kg) Physostigmine	Neostigmine
None	1.0 ± 0.1	0.3 ± 0.04
Scopolamine. HBr†	9.3 ± 1.2	1.3 ± 0.22
Scopolamine. CH_3I‡	2.8 ± 0.34	1.2 ± 0.19

* Injected 10 min before administration of the anticholinesterase drug.

† 0.52 μmoles/kg (0.2 mg/kg).

‡ 0.52 μmoles/kg (0.23 mg/kg).

Each dose was tested on 10 mice. Results are the mean of 3 experiments.

RESULTS

Both the peak and the duration of the systemic effects under study were found to be dose-dependent in the range of 0.12–1.45 μmoles/kg (0.05–0.6 mg/kg) physostigmine (Fig. 2) and 0.02–0.6 μmoles/kg (0.006–0.18 mg/kg) neostigmine, which served as a quaternary reference compound. Higher doses could not be used without fatal results and a prior administration of anticholinergic drugs was required (Table 1). The time profiles of the tremor, salivation and rotarod effects are similar for both drugs, while the hypothermia, induced by physostigmine only, has a much longer onset time and duration (Fig. 2). The peak effect obtained at each dose was plotted versus the logarithm of the dose, yielding dose–response curves (Fig. 3) from which ED_{50} values were interpolated (Table 2). It seems that neostigmine is more potent than physostigmine in two of the three common effects—salivation and tremor—while they exhibit approximately the same potencies in the rotarod test (Table 2).

The dose-dependent time profile of brain AcChE inhibition by physostigmine *in vivo* is illustrated in Fig. 4. A maximal obtainable inhibition of 90 per cent was reached starting at 0.4 mg/kg. Increasing the dose to 5 mg/kg (10 min after preinjection with scopolamine.HBr) did not lead to a higher measurable inhibition. This 10 per cent "residual" activity could be completely blocked *in vitro* by 10^{-5} M physostigmine, indicating that it is due to cholinesterase activity. Neostigmine at doses up to 0.2 mg/kg did not inhibit brain AcChE *in vivo* 5, 15, 30 and 60 min after administration, while both drugs inhibited the enzyme *in vitro* (Fig. 5). Plotting the maximal percentage inhibition of brain AcChE by physostigmine *in vivo* vs the logarithm of the dose yielded a dose–response curve comparable to that obtained *in vitro* (Fig. 6): for

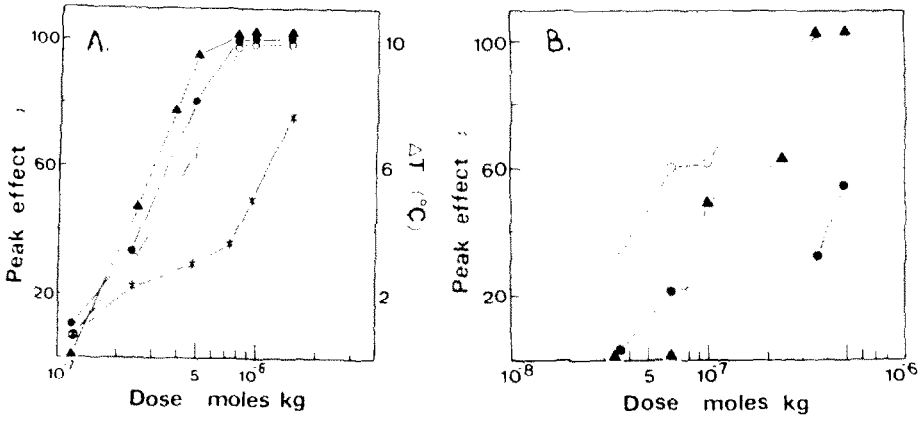

Fig. 3(A). Dose–response curves for the physostigmine-induced systemic effects. The curves were constructed by plotting the peak effects (Fig. 2) versus the appropriate dose. Key: (●——●) rotarod, (▲——▲) tremor, (○——○) salivation and (*——*) hypothermia. (B). Dose–response curves for the neostigmine-induced systemic effects. Key: (●——●) rotarod, (▲——▲) tremor, (○——○) salivation. Results are the mean of 2–4 experiments, 9 mice in each. See legend to Fig. 3(A) for details.

Table 2. ED_{50} values for systemic effects induced by physostigmine and neostigmine in mice

Effect	ED_{50}* physostigmine mg/kg	physostigmine μmoles/kg	neostigmine mg/kg	neostigmine μmoles/kg	EPMR† physostigmine neostigmine
Salivation	0.15	0.36	0.015	0.049	7.3
Tremor	0.10	0.24	0.030	0.10	2.4
Hypothermia‡	0.30	0.72	—	—	—
Rotarod	0.12	0.29	0.13	0.43	0.7

* The data are taken from Fig. 3.
† EPMR: equipotent molar ratio.
‡ Chosen arbitrarily as that dose which induces a maximal hypothermia of 3.5°. Results are the mean of 2–5 experiments, the S.D. of which is 8–18 per cent.

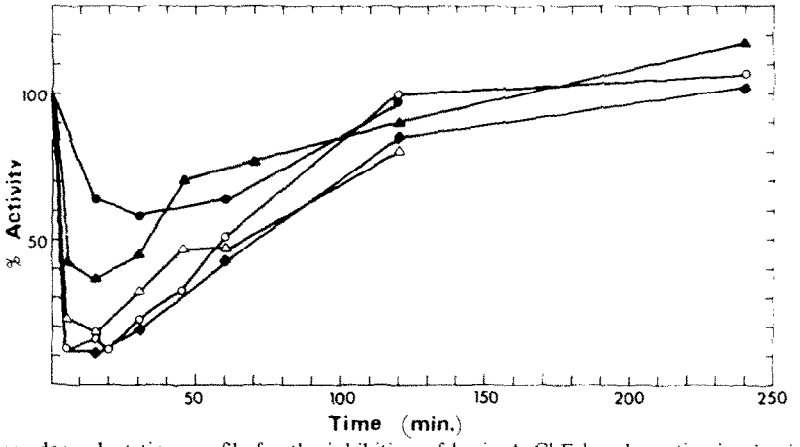

Fig. 4. Dose-dependent time profile for the inhibition of brain AcChE by physostigmine *in vivo*. Mice were injected s.c. with various doses of physostigmine. They were killed in triplicate at different time-intervals and the residual AcChE activity determined 3 times in each brain homogenate. Key: (●——●) 0.05 mg/kg (0.12 μmoles/kg), (▲——▲) 0.1 mg/kg (0.24 μmoles/kg), (△——△) 0.2 mg/kg (0.48 μmoles/kg), (○——○) 0.4 mg/kg (0.96 μmoles/kg) and (◆——◆) 0.6 mg/kg (1.44 μmoles/kg). Results are the mean of 3–6 experiments. The S.D. was found to range between 3–15 per cent. The 100 per cent activity is given as the mean of 180 separate readings.

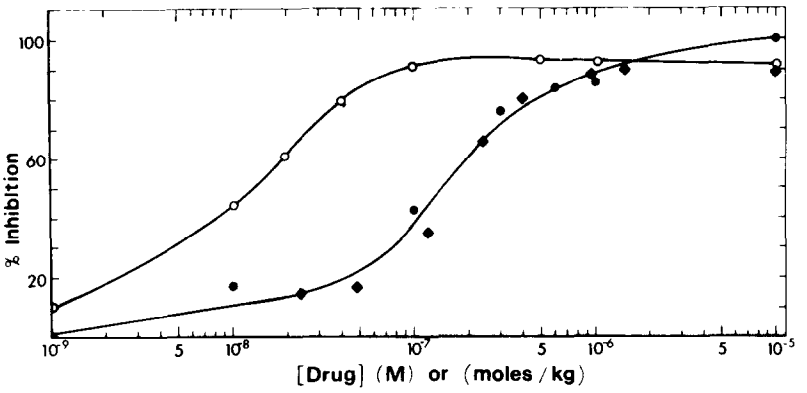

Fig. 5. Dose–response curves for the inhibition of brain AcChE: by physostigmine, (◆——◆) *in vivo* and (●——●) *in vitro*; by neostigmine (○——○) *in vitro*.

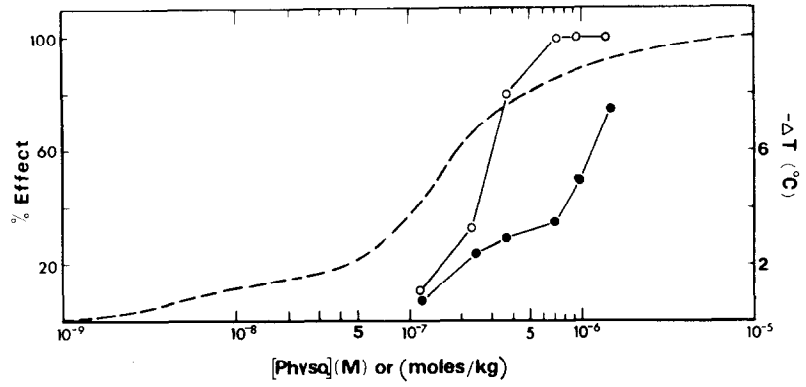

Fig. 6. A comparison between the dose–response curves for the inhibition of brain AcChE by physostigmine *in vivo* (------) and for the physostigmine-induced hypothermia (●——●) and rotarod effects (○——○).

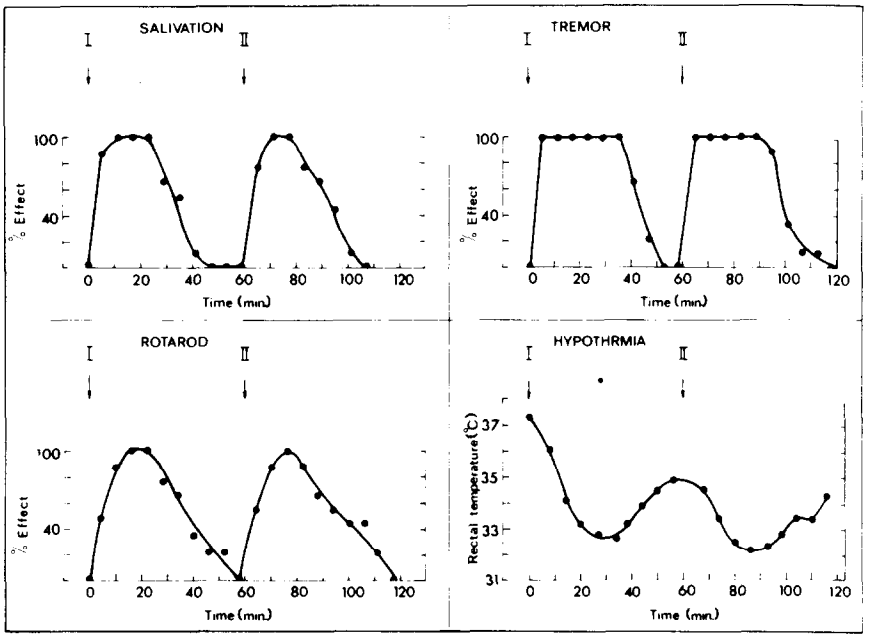

Fig. 7. A 2-cycle experiment of the physostigmine-induced systemic effects. 0.4 mg/kg (0.96 μmoles/kg) physostigmine were injected to the same group of animals at a 1-hr interval and the time profile of systemic effects followed continuously after the 1st (I) and 2nd (II) injection.

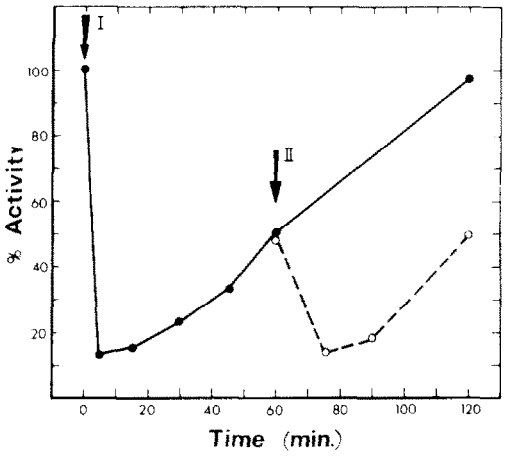

Fig. 8. A 2-cycle experiment of the physostigmine-induced brain AcChE inhibition *in vivo*. Mice were injected with 0.4 mg/kg (0.96 μmoles/kg) at 1-hr interval and decapitated at various time intervals after the 1st (I) and 2nd (II) injection. The time profile of brain AcChE inhibition is represented (broken line). In addition, the spontaneous reactivation of the enzyme after one injection of this dose (taken from Fig. 5) is represented too (solid line).

example, a 60 per cent inhibition occurred at 0.1×10^{-6} M or at 0.1 μmoles/kg. Interestingly, this dose was found to be the minimum required for the induction of measurable systemic effects.

This point was further investigated in a "two-cycle" experiment, in which both the systemic effects and brain AcChE activity were measured after two consecutive injections of 0.4 mg/kg physostigmine given one hour apart to the same group of mice (Figs 7 and 8). The termination of the systemic effects was accompanied by recovery of the enzyme to 40 per cent of its pre-injection activity, and the time of peak effects coincided with that of maximal inhibition (90 per cent) in both cycles.

Characterization of the systemic effects induced by physostigmine was attempted using scopolamine.HBr and its quaternary analogue, scopolamine.CH_3I. It was found that these antagonists do not modify the pattern of brain AcChE-inhibition by physostigmine *in vivo*, when given 10 min before the latter, in the dose range of 0.2–0.4 mg/kg. The kinetics of the antagonism of physostigmine-induced systemic effects by scopolamine HBr is depicted in Fig. 9. Administration of scopolamine.HBr 10 min before physostigmine resulted in a complete blockade of the salivation and hypothermia, and about 80 per cent antagonism of the rotarod effects. Scopolamine.CH_3I, on the other hand, antagonized the salivation only. A similar pattern was observed when neostigmine was used (Table 3). It should be noted that 10 min was found to be the time of peak presence of labeled scopolamine.HBr in mouse brain and the time of maximal mydriatic activity of both antagonists [22]. The tremor, unexpectedly, was not affected by either antagonist and therefore it was necessary to establish whether a similar pattern exists for other cholinergic drugs and in other strains of animals. It was found that the tremor induced in mice by three anticholinesterase agents—physostigmine, neostigmine and tacrine (Table 3)—and in rats by physostigmine (Table 4) was not blocked either by scopolamine.HBr or by scopolamine.CH_3I while the tremor caused by acetylcholine-like muscarinic tertiary drugs—oxotremorine, (±)-3-acetoxyquinuclidine and pilocarpine—could be completely antagonized, as demonstrated for mice (Table 3).

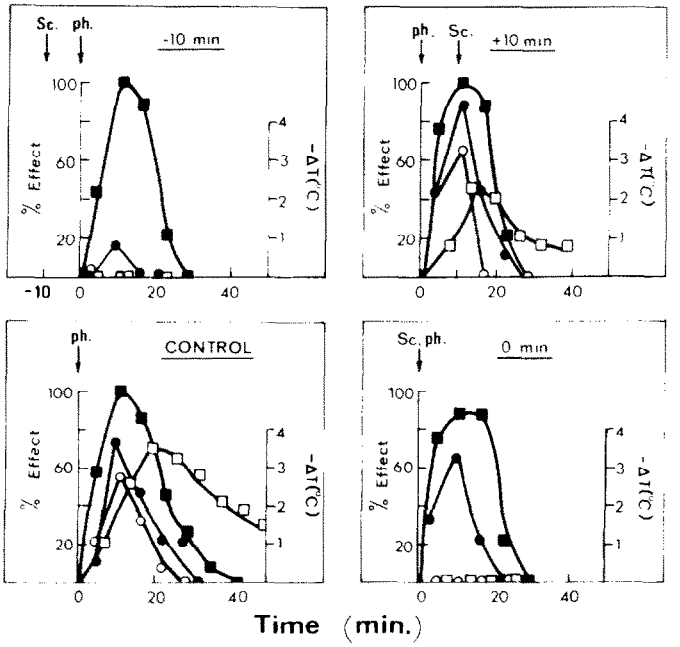

Fig. 9. Antagonism by scopolamine. HBr of the physostigmine-induced systemic effects. Scopolamine.HBr (0.2 mg/kg; 0.52 μmoles/kg) was administered at the indicated time-intervals relative to physostigmine (0.2 mg/kg; 0.48 μmoles/kg). Key (■——■) tremor, (●——●) rotarod, (○——○) salivation and (□——□) hypothermia.

Table 3. Antagonism by scopolamine.HBr and scopolamine.CH_3I of systemic effects induced by cholinergic drugs in mice.

Drug	Effect*	Antagonist† None peak (%)	None dur.‡ (min)	Scopolamine.HBr peak (%)	Scopolamine.HBr dur. (min)	Scopolamine.CH_3I peak (%)	Scopolamine.CH_3I dur. (min)
			Anticholinesterases				
Physostigmine (0.2 mg/kg)	T	100	43	100	30	100	35
	S	60	32	0	0	0	0
	H§	2.9	35	0	0	2.5	40
	R	80	29	17	16	66	34
Neostigmine (0.1 mg/kg)	T	100	60	100	53	100	53
	S	100	65	0	0	0	0
	R	33	20	44	22	55	22
Tacrine (10 mg/kg)	T	100	64	100	59	88	35
	S	100	50	0	0	0	0
	H	2.3	62	0	0	2.1	60
	R	62	62	22	34	77	40
			Acetylecholine-like				
Oxotremorine (0.08 mg/kg)	T	100	84	0	0		
	S	100	94	0	0		
	H	5.8	51	0	0		
	R	77	88	0	0		
(±)3-acetoxy-quinuclidine (6 mg/kg)	T	88	46	22	23		
	S	100	46	0	0		
	H	5.6	48	0	0		
	R	44	62	0	0		
Pilocarpine (10 mg/kg)	T	88	53	0	0		
	S	100	120	0	0		
	H	3.0	56	0	0		
	R	22	28	0	0		

* T = tremor; S = salivation; H = hypothermia; R = rotarod.

† 0.2 mg/kg scopolamine.HBr and 0.23 mg/kg scopolamine.CH_3I (0.52 μmoles/kg) given 10 min before the cholinergic drugs.

‡ Dur. = duration, from injection to complete recovery (tremor, salivation, rotarod) or to 25 per cent recovery (hypothermia).

§ The peak hypothermic effect is expressed as the maximal temperature reduction.

Results are the mean of 3–5 experiments. The S.D. is as specified in Methods.

DISCUSSION

The systemic effects chosen for evaluation of tolerance to a certain drug must meet a few basic criteria: (1) they should be "nonlearned", (2) easily recorded in large groups of animals and (3) the response should be dose- and time-dependent, since both reduction of the peak effect and shortening of its duration are extensively used to define tolerance states [23]. For these reasons, we chose to characterize four physo-

Table 4. Antagonism by scopolamine.HBr of some systemic effects induced by physostigmine in rats

Drugs		Systemic effects: Tremor	Salivation	Hypothermia*	Rotarod
Physostigmine (0.4 mg/kg)	peak (%)	100 ± 8	100 ± 10	1.8 ± 0.2	100 ± 6
	duration† (min.)	53 ± 6	47 ± 5	44 ± 5	50 ± 6
Physostigmine (0·4 mg/kg) + Scopolamine.HBr‡ (0.4 mg/kg)	peak (%)	100 ± 10	0	0	0
	duration (min.)	53 ± 7	0	0	0

* The peak hypothermic effect is expressed as the maximal temperature reduction.

† Time from injection to complete recovery (salivation, tremor, rotarod) or to 25 per cent recovery (hypothermia).

‡ Given 10 min before physostigmine.

Results are the mean of 3–5 experiments.

Table 5. The relationship between the duration of brain AcChE inhibition and the systemic effects induced by physostigmine *in vivo*

Dose mg/kg	Duration of brain AcChE inhibition* (min)	Duration of systemic effects† (min)			
		Rotarod	Salivation	Tremor	Hypothermia
0.05	0	0	0	0	0
0.10	20 ± 3	22 ± 3	22 ± 5	34 ± 4	30 ± 5
0.20	38 ± 7	28 ± 3	34 ± 5	42 ± 6	38 ± 7
0.40	50 ± 5	58 ± 2	42 ± 4	54 ± 5	44 ± 4
0.60	56 ± 3	82 ± 6	46 ± 7	58 ± 5	55 ± 6

* Data are taken from Fig. 4, from injection to recovery to 40 per cent of initial activity.

† Data are taken from Fig. 2, from injection to complete recovery (rotarod, salivation, tremor) or to 25 per cent recovery (hypothermia).

Results are the mean of 2–5 experiments. $P < 0.0025\%$ for all 4 measurements, as determined in student's *t*-test for significance of correlation coefficients.

stigmine-induced effects—tremor, salivation, hypothermia and those measured in the rotarod test. While the first three are natural and spontaneous, the rotarod test represents a behavioral model of a forced response. Neostigmine, which does not readily enter the brain, severed as a quarternary reference compound.

Salivation is a well-known muscarinic peripheral response to cholinergic drugs [19, 24]. The five-fold higher potency of neostigmine relative to physostigmine (Table 2) may reflect a higher affinity of the former towards the salivary gland cholinesterase. Indeed, using brain AcChE as a model, it was found that neostigmine is 10 times more potent an inhibitor *in vitro* than physostigmine, the ID_{50} values being 1.25×10^{-8} and 1.4×10^{-7} M, respectively (Fig. 5). Furthermore, it is tempting to suggest that the two-fold higher lethality of neostigmine (Table 1) is also a reflection of its higher affinity towards the enzyme. This lethality seems to be peripheral in the case of neostigmine, since both scopolamine.HBr and its methiodide salt increased the LD_{50} value by the same factor (Table 1). On the other hand, a central involvement in the lethality of physostigmine is probable, since scopolamine.HBr proved to be about 3 times more potent than scopolamine.CH_3I in increasing the physostigmine LD_{50}. Indeed, antagonism to physostigmine lethality was used as a screening procedure for the anti-parkinsonian activity of drugs, all of which were believed to act centrally [25]. It is noteworthy that the LD_{50} values of both drugs are 3–10 times higher than their respective ED_{50} values. A similar ratio was reported by Chermat *et al.* [26], for i.p. injections.

It was suggested previously that temperature regulation involves a cholinergic mediation [27–28]. However, the reports in the literature vary as to the effects of cholinergic drugs on body temperature, depending on the route of administration of the drugs [26, 29–33]. In our study all the tertiary muscarinic agonists and cholinesterase inhibitors used caused a marked hypothermia in mice when injected s.c. (Table 3). This response could be completely blocked by prior administration of scopolamine.HBr (Fig. 9, Tables 3 and 4), implying a muscarinic mediation. The central origin of the hypothermia was established by two means: (1) no detectable effects were induced by neostigmine, which does not affect the brain AcChE *in vivo* in doses up to its LD_{50} and (2) no significant antagonism of the physostigmine-induced response was caused by the quaternary analogue scopolamine.CH_3I which does not readily enter the brain (Table 3). Similar results were reported by Varagić *et al.* [33]. The complete antagonism of the hypothermia by scopolamine.HBr could not be attributed to decreased uptake of the cholinesterase inhibitor by the brains of the scopolamine-treated mice, since no change was found in the pattern of brain AcChE inhibition *in vivo* in the latter, relative to control. Chermat *et al.* [26] also presented dose–response curves for the hypothermic effect of physostigmine in mice, but this response was much less pronounced than what we measured. This discrepancy can be attributed to strain differences and to i.p. versus s.c. injections.

The circadian fluctuations observed in the rectal temperatures of the mice during the day (07:00–24:00) ranged from 37 ± 0.3–38.8 ± 0.3, with a minimum at about 12:00 and a maximum around 24:00. However, the maximal hypothermic effect induced by physostigmine could not be directly correlated either with the circadian rhythm or with the initial temperature, as suggested previously for the pilocarpine-induced hypothermia [34–35].

The tremor induced by anticholinesterase agents could not be blocked by prior administration of scopolamine.HBr or scopolamine.CH_3I, in contrast to the results with tertiary muscarinic agonists (Table 3). These results imply a non-muscarinic mediation of the tremorogenic effect of the former. This is in contradiction to other reports in which atropine [36] and scopolamine [37] were found to antagonize the physostigmine-induced tremor in rats. The reasons for these discrepancies are not clear but may be the result of differences in the antagonists' doses and/or in the experimental design: for example, we repeated the procedure reported by Dandiya and Bhargave [37] and found that, although the tremor was not blocked by scopolamine.HBr (5 mg/kg), its duration was remarkably shorter. It is most probable that the long intervals used by these investigators to evaluate physostigmine's activity led them to overlook the tremor. On the other hand, Sethy and Van Woert [36] found that neither benztropine nor trihexylphenidyl blocked the physostigmine-induced tremor in rats,

while the oxotremorine-induced tremor was completely antagonized.

The rotarod test could be considered as measuring a tremorogenic side-effect. We found, however, that in physostigmine-tolerant animals there is a complete dissociation of these responses: while a full tremor could be induced, no measurable effects were observed in the rotarod test [38]. Furthermore, while 80 per cent of the rotarod effects were blocked by scopolamine.HBr, the tremor was totally unaffected (Table 3). It seems, therefore, that the rotarod effects are completely independent of the tremor. Our results seem to imply further that physostigmine and neostigmine induce the rotarod effects through different mechanisms: while those produced by the former could be almost completely blocked by preadministration of scopolamine.HBr, indicating their muscarinic nature, no such antagonism was observed for the latter (Table 3). It may be suggested that this effect of neostigmine is due to its direct nicotinic action at the neuromuscular junction [6–9]. The central involvement in the physostigmine-induced rotarod effects is reflected in the lack of antagonistic activity by scopolamine.CH_3I (Table 3).

In an attempt to correlate the pharmacological activity of physostigmine with biochemical events, we compared the dose-dependent time profile of the former (Fig. 2) with the kinetics of brain AcChE inhibition (Fig. 4), using 3 different approaches. (1) A comparison of peak effects revealed that a minimum of 60 per cent inhibition of this enzyme is sufficient to produce measurable effects, while 90 per cent inhibition is required to elicit the maximal systemic responses (Fig. 6). These results are in good agreement with those reported by others, as reviewed by Brimblecombe [5]. (2) The duration of the systemic effects changes concomitantly with the duration of the inhibition of brain AcChE *in vivo* to less than 40 per cent of its initial activity (Table 5). (3) The same pattern was found after a second injection of physostigmine to the same group of animals (Figs 7 and 8), indicating an intrinsic correlation between the pharmacological and biochemical activities of this drug.

The fact that a preinjection of scopolamine.HBr did not modify the pattern of AcChE inhibition, while 3 out of 4 systemic effects were blocked (Fig. 9, Tables 3 and 4), points to the muscarinic receptor as a mediator in these responses. The possible involvement of brain AcChE and the muscarinic receptor in the tolerance to physostigmine is reported elsewhere [38, 39].

REFERENCES

1. A. Bartolini, R. Bartolini and E. F. Domino, *Neuropharmacology* **12,** 15 (1973).
2. V. H. Sethy and M. H. Van-Woert, *J. Neurochem.* **23,** 105 (1974).
3. A. T. Modak, W. B. Stavinoha and S. T. Weintraub, *Archs Int. Pharmacodyn. Thér.* **217,** 293 (1975).
4. H. D. Fischer, I. V. Schwarzenfeld and W. Oelszner, *Acta biol. med. germ.* **34,** 435 (1975).
5. W. R. Brimblecombe, in *Pharmacology Monographs* (Ed. P. B. Bradely) p. 63. University Park Press, Baltimore (1974).
6. W. F. Riker and W. C. Wescoe, *J. Pharmac. exp. Ther.* **88,** 58 (1946).
7. W. F. Riker, *Pharmac. Rev.* **5,** 1 (1953).
8. S. Thesleff, *J. Physiol., Lond.* **151,** 598 (1960).
9. G. Werner and A. S. Kuperman, in *Handbuch der Experimentellen Pharmakologie. Cholinesterases and Anticholinesterases Agents* (Ed. G. B. Koelle), p. 570. Springer-Verlag, Berlin (1963).
10. J. M. Barnes and F. A. Denz, *Br. J. indust. Med.* **11,** 11 (1954).
11. A. Banks, Ph.D. Thesis. University of London (1957).
12. N. K. Stacek, *Pharmac. Toxic.* **6,** 559 (1959).
13. R. W. Russel, R. H. J. Watson and M. Frankenhauser, *Scand. J. Psychol.* **2,** 21 (1961).
14. I. S. Kagan, *J. High Nerv. Act.* **12,** 530 (1962).
15. M. E. Goldberg, H. E. Johnson, J. B. Knaak and H. F. Smyth, Jr., *J. Pharmac. exp. Ther.* **141,** 244 (1963).
16. S. N. Pradhan and R. M. Mhatre, *Res. Commun. Chem. Path. Pharmac.* **1,** 682 (1970).
17. J. A. Rosencrans and E. F. Domino, *Pharmac. Biochem. Behav.* **2,** 67 (1974).
18. J. B. Robinson, B. Belleau and B. Cox, *J. med. Chem.* **12,** 848 (1969).
19. T. D. Inch, D. M. Green and P. B. J. Thompson, *J. Pharm. Pharmac.* **25,** 359 (1973).
20. G. L. Ellman, K. D. Courtney, V. Andres, Jr and R. W. Featherstone, *Biochem. Pharmac.* **7,** 88 (1961).
21. B. Behrens, *Arch. exp. Path. Pharmak.* **140,** 237 (1929).
22. M. Rehavi, S. Maayani and M. Sokolovsky, *Biochem. Pharmac.* **26,** 1559 (1977).
23. C. C. Hug, in *Chemical and Biological Aspects of Drug Dependence* (Eds S. J. Mulé and H. Brill), p. 307. CRS Press, Cleveland (1972).
24. G. B. Koelle, in *The Pharmacological Basis of Therapeutics* (Eds L. S. Goodman and A. Gilman), 5th edn., p. 445. MacMillan Publishing Company, NY (1975).
25. T. Nose and M. Kojima *Eur. J. Pharmac.* **10,** 83 (1970).
26. R. Chermat, P. Simon and J. R. Boissier, *J. Pharmac., Paris* **7,** 227 (1976).
27. P. Lomax, *Int. Rev. Neurobiol.* **12,** 1 (1969).
28. P. Lomax, R. S. Foster and W. E. Kirkpatrick, *Brain Res.* **15,** 431 (1969).
29. J. Baird and W. J. Lang, *Eur. J. Pharmac.* **21,** 203 (1973).
30. G. Metcalf, R. D. Myers and P. C. Redgrave, *Br. J. Pharmac.* **55,** 9 (1975).
31. K. G. Johnson, *Br. J. Pharmac.* **53,** 489 (1975).
32. R. D. Myers and T. L. Yaksh *Physiol. Behav.* **3,** 917 (1968).
33. V. M. Varagić, M. Žugić and T. Kožić, *Naunyn-Schmiedebergs Arch. Pharmak* **270,** 407 (1971).
34. M. J. Friedman and J. H. Jaffe, *J. Pharmac. exp. Ther.* **167,** 34 (1969).
35. M. J. Friedman, J. H. Jaffe and S. K. Sharpless, *J. Pharmac. exp. Ther.* **167,** 45 (1969).
36. V. H. Sethy and M. H. Van-Woert, *Biochem. Pharmac.* **22,** 2685 (1973).
37. P. C. Dandiya and L. P. Bhargava, *Archs Int. Pharmacodyn Thér* **176,** 157 (1968).
38. S. Maayani, Y. Egozi, I. Pinchasi and M. Sokolovsky, *Psychopharmacology* in press (1977).
39. I. Pinchasi, S. Maayani and M. Sokolovsky, *Biochem. Pharmac.* **26,** 1071 (1977).