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REVERSIBLE INHIBITION OF ACETYLCHOLINESTERASE BY ESEROLINE, AN OPIOID AGONIST STRUCTURALLY RELATED TO PHYSOSTIGMINE (ESERINE) AND MORPHINE

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Abstract—The action of eseroline—(3a*S*,8a*R*)-1,2,3,3a,8,8a-hexahydro-1,3a,8-trimethylpyrrolo[2,3-*b*]indol-5-ol—salicylate was tested on preparations of ChE from different sources and on the longitudinal muscle of guinea-pig ileum. While eseroline is extremely weak-acting on horse serum BuChE (K_i = 208 ± 42 μM), it is a rather strong competitive inhibitor of AChE's, its K_i being 0.15 ± 0.08 μM, 0.22 ± 0.10 μM and 0.61 ± 0.12 μM in electric eel, human RBC and rat brain, respectively. Eseroline inhibitory action on AChE is independent of the duration of pre-incubation and appears fully developed in less than 15 sec. This action is also rapidly reversible; after pre-incubation followed by dilution, maximum enzymic activity is regained within 15 sec. The electrically-evoked contractions of the longitudinal strip were inhibited by concentrations of eseroline in the range 0.2–15 μM, while they were increased by concentrations over 20 μM. In the same preparation, without electrical stimulation, but in the presence of naloxone, eseroline induced contractions at concentrations higher than 5 μM. This effect was antagonized by atropine. The inhibitory activity of eseroline parallels, as regards selectivity, potency and kinetics, that of the phenolic anticurare agent edrophonium, while it differs markedly from that of physostigmine.

Eseroline, (3a*S*,8a*R*)-1,2,3,3a,8,8a-hexahydro-1,3a,8-trimethylpyrrolo[2,3-*b*]indol-5-ol, is derived from physostigmine by hydrolysis of the *N*-methylcarbamyl group [1–5] (Fig. 1). Although, as free base, it is rather unstable and readily oxidizes to the *o*-quinone derivative, rubreserine, on being left to stand in the air [6], the salicylate of eseroline, used in this study, is more stable and can be profitably used in both *in vivo* and *in vitro* experiments [5, 7–9].

Eseroline occupied a prominent position in those early studies which led, first, to the elucidation of the molecular structure of physostigmine by Stedman and Barger [3] and, subsequently, to its synthesis by Julian and Pikl [10].

Stedman [11] observed that eseroline did not possess the miotic activity of physostigmine. As a consequence, he concluded that the methylcarbamyl group of physostigmine was the one mainly responsible for its pharmacological properties. Subsequent studies, carried out directly on serum [6, 12, 13] and on haemolyzed RBC† [13] ChE's, detected only a negligible inhibitory activity in eseroline solutions, thus confirming Stedman's early finding. This, therefore, has until now been a firmly-established point in the structure–activity relationship studies of anti-ChE agents and, as such, it has been repeatedly reviewed [14, 15].

However, since we have recently found [5, 7, 8] that eseroline possesses strong opioid-like antinociceptive activity, which has not been described before, we thought it would be worth re-examining its action on ChE's in greater detail. Moreover, the acquisition of new information on this point appeared interesting also in view of the structural analogy between eseroline and morphine [5, 16].

In this study, while we confirm the inactivity of eseroline on serum BuChE's (pseudo-ChE's), we present experimental evidence that this drug is in fact endowed with remarkable inhibitory activity towards AChE's (true-ChE's) obtained from different sources and that the mechanism of this action differs from that of physostigmine in terms of kinetics.

A preliminary report of this study was presented at the International Symposium on Cholinergic Mechanisms, Florence, March 1980.

MATERIALS AND METHODS

Reagents and drugs. DTNB, ATCh and BuTCh were purchased from Boehringer Mannheim Gmbh (F.R.G.). Physostigmine, physostigmine sulphate and atropine sulphate were purchased from Sigma Chemical Co. (St. Louis, MO). Bacterial protease (29.6 I.U./g) was from Calbiochem-Behring Corp. (Lucerne, Switzerland). Edrophonium chloride was kindly donated by Dr. Merzario (Prodotti Roche,

† Abbreviations used: ACh, acetylcholine; ChE, cholinesterase; AChE, acetylcholinesterase; BuChE, butyrylcholinesterase; ATCh, acetylthiocholine iodide; BuTCh, butyrylthiocholine iodide; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); RBC, red blood cell(s).

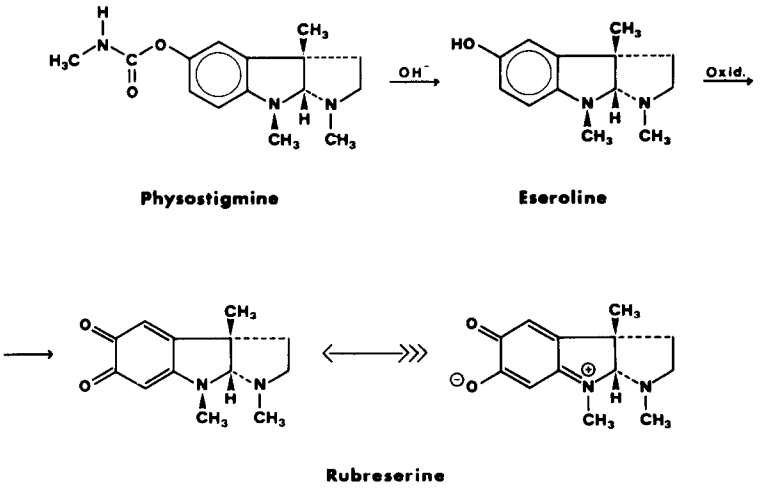

Fig. 1. Reactions leading to the formation of eseroline and rubreserine from physostigmine.

Milan); naloxone hydrochloride was a gift from Dr. M. J. Ferster (Europe Endo Lab. Inc., Brussels, Belgium).

Eseroline free base and eseroline salicylate were synthesized according to the method previously described [5].

Rubreserine was prepared by oxidizing eseroline. Eseroline free base (1 g) was dissolved in 2% aqueous NaOH (100 ml) by gently shaking in a separatory funnel. The alkaline mixture was then extracted 4–5 times with 70 ml aliquots of $CHCl_3$ and the combined extracts evaporated to 4–6 ml. Bright-red needles of rubreserine (m.p. 144–145°) precipitated on addition of petroleum ether (b.p. 50–70°).

Cholinesterases. Purified AChE (714 I.U./mg) from *Electrophorus electricus*, was from Boehringer Mannheim Gmbh (West Germany).

Purified BuChE (15 I.U./mg protein) from horse serum, was purchased from Sigma Chemical Co.

AChE activity from rat brain was prepared according to the method of Ho and Ellman [17], slightly modified. The brain of a Sprague–Dawley rat (200–300 g), killed by decapitation, was homogenized in 5 vol. of ice-cold 0.03 M sodium phosphate buffer, pH = 7.0, with a glass/Teflon homogenizer. The homogenate was centrifuged in the cold at 24,000 *g* for 1 hr. The pellet was then resuspended in the original volume of bacterial protease in buffer (0.16 mg/ml), stirred at 4° for about 20 hr and centrifuged again at 24,000 *g* for 1 hr. The clear supernatant was collected in fractions and, after addition of ammonium sulphate (5% w/v), stored at −50° until used in the activity assays. When tested with ATCh as substrate, the preparation presented approximately 1.3 I.U./ml of AChE activity. Its BuChE activity was negligible.

Human RBC AChE activity. The red cells of 0.1 ml heparinized blood were washed 3 times by centrifugation with 9 ml of 0.9% saline. The washed RBC were resuspended in 50 ml of cold 0.05 M sodium phosphate buffer, pH = 7.2, in saline. AChE assays were performed in glass cuvettes on 1 ml aliquots of the suspension. RBC did not haemolyze during the assay.

ChE assays. ChE activity was determined at 25° and at pH 7.2, by the photometric method of Ellman *et al.* [18], using ATCh, or BuTCh, as substrates. The variations of optical absorbance at 412 nm, were measured at 15 or 30 sec intervals by the aid of a photometer equipped with an automatic data printer (Enzymeter IV, PoliMak S.r.l., Rome, Italy). I_{50} values were calculated graphically from semilogarithmic plots based on 6 scalar concentrations of the inhibitor, in the presence of 0.5 mM ATCh. The solutions containing eseroline were kept under N_2 to prevent oxidation.

Longitudinal strip. The guinea-pig ileum myenteric plexus longitudinal muscle was prepared according to Paton and Vizi [19]. The strip was suspended in a 12 ml organ bath in a Krebs solution having the composition (mM): 118.0 NaCl; 4.7 KCl; 2.5 $CaCl_2$; 1.2 KH_2PO_4; 1.2 $MgSO_4$; 25.0 $NaHCO_3$; 11.0 glucose. The temperature was maintained at 37°. In some experiments, the strip was stimulated with an electrical field at 0.1 Hz, 1 msec of pulse duration and at twice the threshold voltage. Responses were recorded isotonically.

RESULTS

Inhibition of cholinesterases. Table 1 shows the inhibition constants, K_i, for eseroline, edrophonium and rubreserine towards ChE from different sources. Eseroline inhibited all tested ChE preparations in a competitive, or prevalently competitive, way. Its inhibitory activity, however, was much more potent on AChE from electric eel, human RBC and rat brain than on BuChE from horse serum. K_i values for edrophonium were, on the whole, similar to those for eseroline, while rubreserine was much weaker than eseroline on AChE preparations, but somewhat stronger on horse serum BuChE. Salicylate, present in all solutions of eseroline, did not itself show anti-ChE activity.*

* Data not shown.

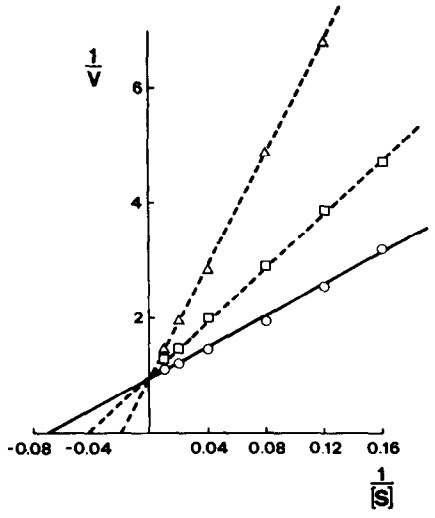

Fig. 2. Inhibition of electric eel AChE by eseroline: Lineweaver–Burk plots. The assay conditions were as described in the footnotes to Table 1. (○), buffer; (□), 0.1 μM and (△), 0.4 μM eseroline salicylate. S, ATCh in 10^{-5} M; *V*, Δ absorbance/min. The points on the graph represent the mean values of 3 separate experiments.

Figure 2 shows the Lineweaver–Burk plots for electric eel AChE inhibition by two different concentrations of eseroline.

To measure the effect of the contact time between inhibitor and enzyme on eseroline action, aliquots of eel AChE were preincubated with a fixed concentration of eseroline salicylate for varying times prior to the addition of ATCh. The change in optical absorbance was measured within 15 sec of the start of the reaction. The results of 3 replicated experiments indicated that the inhibitory action of eseroline was already fully developed in those samples where eseroline and substrate were added simultaneously, that is at zero pre-incubation time.

The mean I_{50} value of eseroline towards eel AChE was 0.6 μM. In contrast to eseroline, the I_{50} value of physostigmine markedly depended on the duration of pre-incubation, averaging 2.6 μM, 0.18 μM and 0.02 μM at zero, 1 and 15 min pre-incubation times, respectively.

The reversibility of the anti-AChE activity of eseroline was assessed by dilution. Aliquots of a solution containing AChE from electric eel, pre-incubated with 0.8 μM eseroline salicylate, were diluted 60 times with DTNB in buffer. The samples were then assayed for enzymic activity at various times after dilution. The assays were completed within 15 sec of the addition of the substrate. The results of 3 replicated experiments indicated that the mean percent enzyme inhibition already dropped from 61% to 0% in those samples in which dilution and addition of ATCh were carried out simultaneously.

Actions on the longitudinal strip. As it is shown in Fig. 3, which represents a typical experiment, eseroline dose-dependently inhibited the electrically-induced contractions of the longitudinal muscle strip of guinea-pig ileum, in concentrations up to 1 μM. This effect was antagonized by naloxone. When eseroline concentrations were in the range 1–15 μM, the electrically-evoked contractions were still inhibited, but inhibition seemed to decrease with increasing doses. The contractions, however, were actually increased by concentrations of eseroline over 20 μM (see graph in Fig. 3). Naloxone–HCl (0.22 μM), administered in the presence of eseroline concentrations over 1 μM, induced the contracture of the strip (see recording in Fig. 3). Thus, naloxone, by blocking the inhibitory effect of eseroline, revealed the stimulating action of the latter compound on this preparation. The washing of the muscle, previously exposed to concentrations of eseroline higher than 5 μM, did not immediately restore the basal contraction height, but, on the contrary, induced a transitory inhibition of the evoked contractions (see recording in Fig. 3). This washing-dependent inhibition was proportional to the doses of eseroline used before washing.

The effect of eseroline on guinea-pig ileum myenteric plexus longitudinal muscle which is not electrically stimulated, but perfused with naloxone–HCl (0.27 μM), was investigated in 5 experiments. Under these conditions, eseroline exerted no action on the muscle tone in concentrations up to 1 μM (Fig. 4). At higher doses, however, it induced contractions in the strip. This contractile response had a very slow development (6 min) in comparison with the response obtained by ACh administration. Atropine sulphate (0.7 μM) completely eliminated the contractions evoked by eseroline salicylate (20 μM) in 2 experiments, while it caused an 80% mean reduction of the contraction in the other 3 experiments.

DISCUSSION

The principal finding of this study is that eseroline, which represents the basic polycyclic moiety of physostigmine, is endowed with remarkable, rapidly reversible, inhibitory activity on preparations of AChE from different sources. This finding was indirectly confirmed also by our experiments on the longitudinal muscle of guinea-pig ileum (Figs. 3 and 4), although in this case the anti-ChE response of eseroline was complicated, and partially masked, by the opioid-like action of the drug. The anti-ChE activity of eseroline appears to be selectively directed towards AChE, since the K_i constant of this drug on serum BuChE was about 400–1300 times higher than those observed in the case of AChE (Table 1).

Stedman [11] observed that eseroline did not possess the miotic action of physostigmine. Subsequently, Schweitzer *et al.* [12] found that eseroline possessed negligible inhibitory activity on horse serum ChE. This evidence convinced Stedman and his associates that the urethane (methylcarbamyl) group of physostigmine was essential for its anti-ChE activity and, probably, for most of its pharmacological actions. The results of this study, however, indicate that the above conclusions probably apply only to serum BuChE's. Yet it appears surprising that the anti-ChE activity of eseroline has not been detected by previous *in vivo* [11, 12] and *in vitro* [13] tests. This may be explained by the fact that eseroline is rather rapidly inactivated in biological media. In fact, it readily oxidizes to rubreserine (Fig.

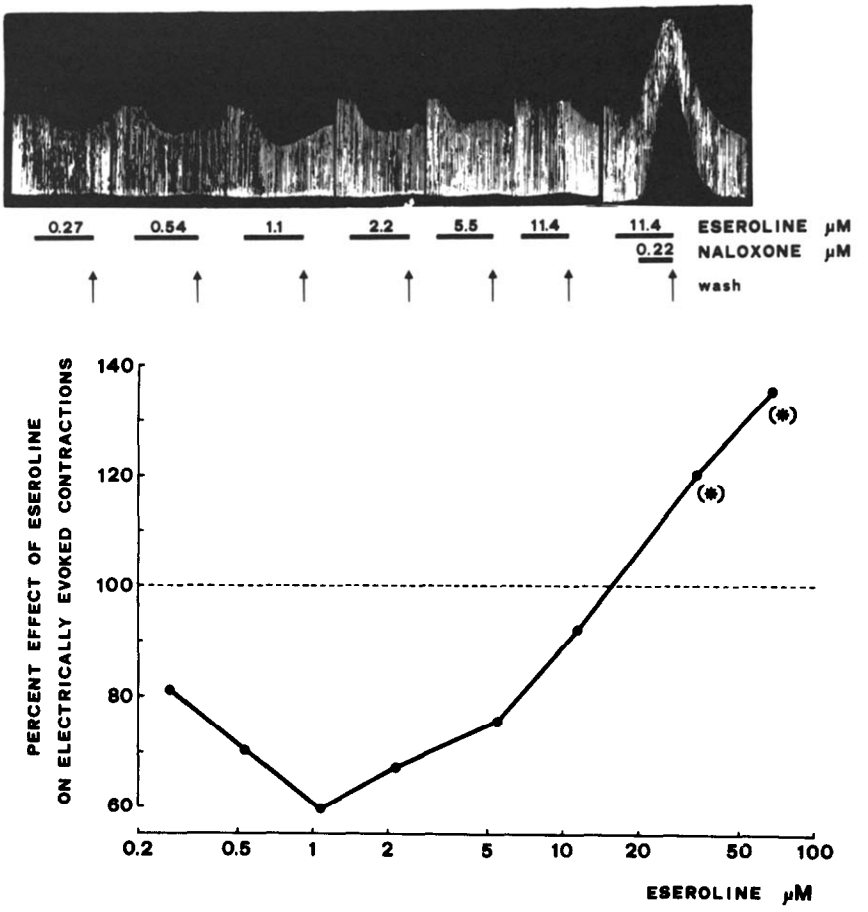

Fig. 3. Effect of graded doses of eseroline salicylate on electrically-evoked contractions of guinea-pig ileum myenteric plexus longitudinal muscle strip. Field stimulation: 1 msec; 0.1 Hz; at twice the threshold voltage. Upper section: recording of a typical experiment. Lower section: dose–response curve from the same experiment. The dotted line represents the normalized contraction height before adding the drugs. * Responses not shown in the recording. The experiment was repeated 7 times with closely similar results.

1), even at mildly alkaline pH, if simple precautions are not taken [5, 6]. Furthermore, oxidation of eseroline to rubreserine may also proceed enzymically, being catalyzed by indophenoloxidases [20] and by peroxidases.* Rubreserine is much weaker than eseroline against AChE's, but slightly stronger against horse serum BuChE (Table 1). As a consequence, concentrated solutions of eseroline may slowly acquire anti-BuChE activity on being left to stand in the air. Under our experimental conditions (see Materials and Methods), the anti-AChE activity of eseroline remained constant and reproducible for at least 45 min after the beginning of the enzymic reaction. However, this apparent instability does not prevent eseroline from showing remarkable pharmacological actions (such as analgesia, decreased spontaneous locomotor activity, hypothermia, etc. [7–9, 12]) when injected systemically. Other factors, therefore, such as the rapid reversibility of AChE inhibition by eseroline and a consequent very brief action duration *in vivo* could provide an alternative explanation.

The inhibition of AChE by eseroline differs from that by physostigmine in many respects. Eseroline inhibits AChE in a competitive way (Fig. 2), regardless of the duration of pre-incubation, while the

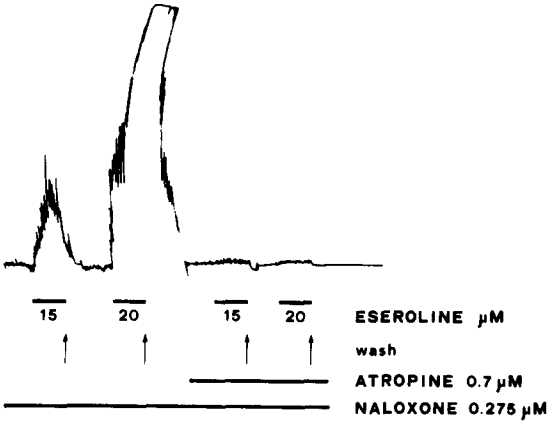

Fig. 4. Effect of eseroline salicylate, and antagonism by atropine sulphate, on the isolated unstimulated guinea-pig ileum myenteric plexus longitudinal muscle strip, perfused with Krebs solution containing naloxone–HCl.

* G. P. Sgaragli and A. Galli: unpublished observation.

Table 1. K_i constants for eseroline and related drugs towards ChE's from different sources

	Electric eel* (μM)	Human RBC* (μM)	Rat brain* (μM)	Horse serum† (μM)
Eseroline	0.15 ± 0.08	0.22 ± 0.10	0.61 ± 0.12	208 ± 42
Edrophonium	0.11 ± 0.04	0.16 ± 0.07	0.20 ± 0.09	75 ± 15
Rubreserine	54 ± 18	24 ± 6	43 ± 12	89 ± 27

Enzymes were pre-incubated with inhibitor at 25° for 15 min before adding the substrate.
Values (mean ± S.E.M. of 3 separate determinations) were calculated from Lineweaver–Burk plots for 2 different concentrations of inhibitor and 6 different concentrations of substrate, in the range 3.12–100 × 10^{-5} M.
* ATCh as substrate.
† BuTCh as substrate.

inhibition by physostigmine appears competitive only for very brief pre-incubations [21, 22]. The K_i constant of eseroline varies considerably in relation to the different ChE preparations tested. By contrast, physostigmine is about equally potent against all ChE's [23], including serum BuChE, where eseroline is very weak. Also the time course of AChE inhibition by eseroline differs markedly from that by physostigmine. The action of eseroline appears already fully developed during the 15 sec necessary to measure enzymic activity by our method. AChE inhibition by physostigmine, on the contrary, gradually increases with the incubation time reaching a plateau in approximately 30 min [22, 24, 25]. As a consequence, the ratio of the relative potencies of eseroline and physostigmine as AChE inhibitors is greatly affected by the extension of the pre-incubation time. In our experiments on eel AChE, the mean I_{50} value for eseroline was 4.3 times lower than that for physostigmine at zero pre-incubation time, but it was 3.3 and 30 times higher at 1 and 15 min pre-incubation times, respectively. The results of the dilution experiments stress the reversible character of AChE inhibition by eseroline. After pre-incubation, then dilution, maximum enzymic activity was regained within the 15 sec necessary to measure the reaction speed. By contrast, AChE pre-incubated with physostigmine regains half maximum activity in approximately 38 min [26]. Thus, dissociation of eseroline from AChE, as well as association, appeared practically instantaneous.

It is now generally assumed that the inhibition of AChE by physostigmine and in general by carbamates, proceeds according to the following reaction scheme [26, 27]:

$$E + I \rightleftarrows E\text{–}I \rightarrow E' \rightarrow E + \text{products}$$

where E–I represents a reversible complex, in which the inhibitor, I, is bound to the enzyme, E, by non-covalent bonds; E′ represents an inactive carbamylated derivative of the enzyme. E′ may slowly break down to regenerate free enzyme thus giving the hydrolysis products of the inhibitor [28], i.e., methylcarbamic acid and eseroline itself when physostigmine acts as inhibitor. Eseroline, lacking the reactive carbamyl group, cannot firmly block AChE. Its inhibitory activity, therefore, unlike that of carbamates, is expressed solely by the equilibrium leading to the formation of the E–I complex and can be interpreted completely in terms of affinity.

Besides eseroline, other well known anti-ChE agents such as 3-hydroxyphenyltrimethylammonium and edrophonium (3-hydroxyphenyldimethylethylammonium) [29, 30], carry a free phenolic group. In our experiments, this chemical similarity between eseroline and edrophonium was closely reflected in the similar anti-ChE activities of the two drugs towards all the enzyme preparations tested. Moreover, edrophonium, like eseroline, reaches equilibrium with AChE very rapidly [31]. Eseroline hydrochloride, however, injected into the cat, is reported [12] to induce only a very feeble potentiation of neuromuscular responses, while edrophonium causes a marked potentiation of contractile responses and possesses strong anticurare activity [32, 33]. The difference in the actions of the two drugs on the neuromuscular junction is probably due to the presence in edrophonium of a quaternary nitrogen which is lacking in eseroline [29, 34].

On the other hand, eseroline as a tertiary amine is expected readily to cross the blood–brain barrier and to exert actions on the CNS. This was confirmed by the recent finding that eseroline possesses a strong analgesic activity [8, 9], along with other central effects [7]. However, the analgesia induced by eseroline does not appear to be directly related to AChE inhibition, since it is not antagonized by atropine, while it is by the opioid antagonist naloxone [8]. A direct involvement of opioid receptor(s) in this action of eseroline seems now well established [5, 8, 9]. Nevertheless, since there is good evidence [35, 36–39] that cholinomimetic drugs can potentiate the analgesic effects of opioids, the possibility that AChE inhibition might play a role in the eseroline analgesic effect cannot be ruled out, especially when high doses are injected.

This possibility of a dual mechanism for eseroline actions is strengthened by the results obtained on guinea-pig ileum longitudinal muscle. Using this preparation, in fact, we observed (Fig. 3) that, while at low concentration eseroline acted as a pure opioid agonist, at high concentration it presented both opioid and cholinomimetic activities, the latter being the more readily reversible of the two on washing.

This anti-AChE activity of eseroline appears interesting also because it may help to shed light on the mechanisms of opioid tolerance and physical dependence. There is, in fact, evidence that opioid agonists cause a reduction of cholinergic activity [40–42], which is considered to be mainly responsible for the development of tolerance of and physical dependence on these drugs [43, 44–47]. Assuming

the validity of such a mechanism, therefore, eseroline, being able by means of AChE inhibition to counterbalance the depression of ACh release which follows opioid receptor(s) stimulation [9], should in itself not induce tolerance and physical dependence. This point is at present being investigated.

To sum up, this study has presented evidence that eseroline, while practically devoid of anti-BuChE activity, is nonetheless endowed with remarkable anti-ChE activity *in vitro*, which is selectively directed towards AChE's of nervous tissue and erythrocytes. This inhibitory activity of eseroline, unlike that of physostigmine, develops instantaneously in the presence of AChE and is rapidly reversible. This property of eseroline is particularly interesting in view of its central actions and its close structural resemblance to morphine.

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