effectively blocked by metergoline. A receptor mediated reaction might be expected to show a saturation effect. However, in our experiments, 5-HT at 10^{-8} M does not have such an effect. It is possible that stimulation of (Na^+–K^+)-ATPase by 5-HT is mediated by more than one mechanism, as has been envisaged by us for the activation of (Na^+–K^+)-ATPase by noradrenaline (unpublished observations). Further studies on this receptor-mediated 5-HT stimulation of (Na^+–K^+)-ATPase should provide an insight into the mechanism by which 5-HT generates its depressant action on cerebral cortical neurons.

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Inhibition of [^{3}H]naloxone binding in homogenates of rat brain by eseroline, a drug, with analgesic activity, related to physostigmine

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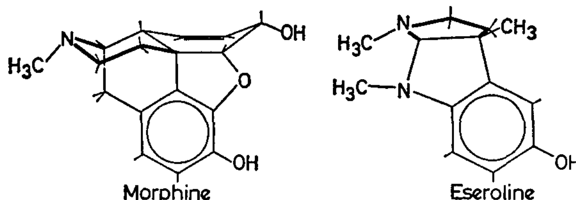
Eseroline, (3aS,8aR)-1,2,3,3a,8,8a-hexahydro-1,3a,8-trimethylpyrrolo [2,3-b] indol-5-ol, is a new analgesic drug derived from physostigmine by hydrolysis of *N*-methyl carbamyl group (Bartolini et al 1978). Eseroline, as free base, is unstable and readily oxidizes to the quinone derivative, rubreserine (Robinson 1965). Its



salts, however, are more stable and can be stored as dry powders or in solution in the presence of an antioxidant, without noticeable loss of activity: in this study we have used the salicylate. Eseroline possesses a remarkable antinociceptive activity comparable in potency to that of morphine: the analgesic doses for eseroline salicylate range from 1 to 5 mg kg^{-1} s.c. in rats and mice depending on the test used (Bartolini et al submitted). In this respect eseroline resembles physostigmine, which also possesses antinociceptive activity in various tests (Flodmark & Wramner 1945; Hendershot & Forsaith 1959; Harris et al 1969; Pleuvry & Tobias 1971). Unlike the latter compound, however, eseroline is devoid of anticholinesterase activity (Ellis et al 1943, confirmed by our experiments on human blood serum) and its analgesic effect is not antagonized by atropine (Bartolini et al 1979). Since the antinociceptive actions of physostigmine are generally considered a consequence of its indirect cholinomimetic activity (Harris et al 1969; Ireson 1970), it appeared of interest to acquire more information on the mechanism of the analgesic action of eseroline.

In the present study we have examined the possibility of a direct interaction between eseroline and opiate receptor sites. To this end, we have evaluated the ability of eseroline in inhibiting stereospecific [^{3}H]-

* Correspondence.

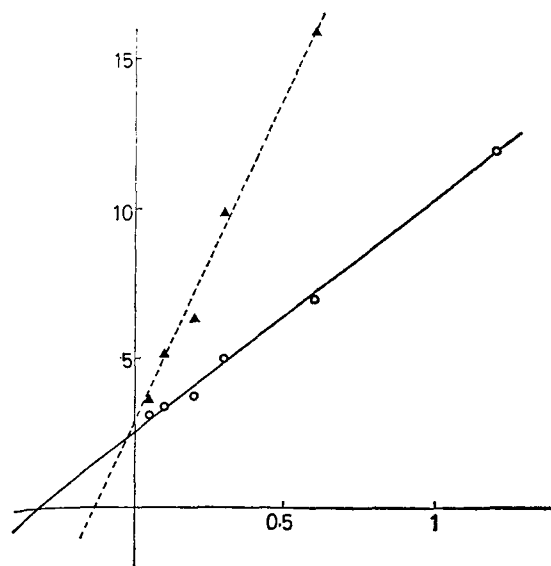


FIG. 1. Effect of eseroline on stereospecific [^{3}H]naloxone binding. Double-reciprocal plot of the [^{3}H]naloxone saturation curves for stereospecific binding in the absence (○) and in the presence of 3 × 10^{-7} M eseroline (▲). Binding assays were carried out using 6 different concentrations of [^{3}H]naloxone, ranging from 0·8 to 20 nM. The points of the graph represent the means of triplicated determinations which varied less than 15%. The experiment was repeated twice. Ordinate: 1/stereospecifically bound [^{3}H]naloxone (pmol)/18 mg original wet tissue. Abscissa: 1/concentration of [^{3}H]naloxone (nM) in the incubation medium.

naloxone binding to homogenates of rat brain in comparison with physostigmine and morphine.

Eseroline free base, was synthetized from physostigmine using the method of Salway (1912), with minor modifications.

Eseroline free base, was synthesized from physostigof eseroline free base (1·5 g) in ether (100 ml) to salicylic acid (1·3 g) in ether (20 ml), with stirring and bubbling with nitrogen. The white precipitate obtained was collected by filtration, washed with a little ether and dried under vacuum. Eseroline salicylate crystallizes into white needles melting at 178–180 °C, with slight decomposition. Yield 90–95%. [Found: C, 67·4; H, 6·9; N, 8·0. $C_{20}H_{24}N_2O_4$ requires C, 67·4; H, 6·8; N, 7·9%].

Binding measurements were carried out following the basic procedure of Simantov & Snyder (1976), slightly modified. Rat brain homogenates were prepared in ice-cold Tris-HCl 0·05 M buffer (pH 7·4 at 23 °C) with two centrifugation steps (49 000 *g*, 15 min) and preincubated at 37 °C for 30 min. Aliquots of homogenates, corresponding to 18 mg of original wet tissue, were then incubated in triplicate 2 h at 0 °C unless otherwise stated, in the presence of [^{3}H]naloxone (spec. act. 17·1 Ci mmol^{-1}) and unlabelled drugs in a total volume of 2 ml. After incubating, samples were filtered under reduced pressure, through glass fibre disks (Whatman GF–B), washed with 10 ml of ice cold buffer and counted in a Triton X-100 scintillation fluid. Stereospecific binding was calculated by subtracting the binding which occurred in the presence of 1 μM levallorphan from that in the presence of its pharmacologically inactive enantiomer, 1 μM (+)-3-hydroxy-*N*-allylmorphinan (dextrallorphan). The solutions containing eseroline were kept in nitrogen to prevent oxidation.

Table 1. Effect of eseroline, physostigmine and opiates on stereospecific [^{3}H]naloxone binding in homogenates of rat brain. Four to eight concentrations of each drug were incubated with 1·7 nM [^{3}H]naloxone in the presence and in the absence of 100 mM NaCl. Incubations and filtrations were carried out either at 0 °C (incubation time = 2 h) or at 25 °C (incubation time = 20 min). The concentration of drug that produces 50% inhibition of control stereospecific binding (IC50) was determined by log-probit analysis. The data are the means of two independent experiments. In each experiment, the points of the inhibition curves were determined in triplicate. (I) Temperature of binding assay; (II) IC50 in the absence of NaCl (μM); (III) IC50 in the presence of 100 mM NaCl (μM); (IV) sodium response ratio III/II.

	I	II	III	IV
Naloxone	0°	0·0025	0·0036	1·4
Met-enkephalin	0°	0·016	0·350	22
Morphine	0°	0·026	0·650	25
Profadol	0°	0·090	0·900	10
Eseroline	0°	0·270	50	185
Physostigmine	0°	30	90	3
Salicylate	0°	>100	>100	—
Morphine	25°	0·024	0·310	13
Eseroline	25°	0·320	6	19
Physostigmine	25°	11	25	2·2
Salicylate	25°	>100	>100	—

A double reciprocal analysis of the saturation of stereospecific [^{3}H]naloxone binding, in the absence and in the presence of eseroline is shown in Fig. 1. The regression lines of the graph are based on 6 concentrations of [^{3}H]naloxone, ranging from 0·8 to 20 nM. The addition of 3 × 10^{-7} M eseroline produces an increase in the apparent K_d of naloxone binding with no change in the maximum number of binding sites. These results suggest that eseroline interacts with naloxone binding sites in competitive fashion with a K_i apparent of 2·2 × 10^{-7} M.

As it is shown in Table 1, the concentration of eseroline necessary for 50% inhibition of [^{3}H]naloxone binding (IC50 value), in the absence of Na^+, is 2·7 × 10^{-7} M. In our experiments, eseroline appears in this action appreciably weaker than morphine, met-enkephalin* and profadol, an analgesic drug representing an open model of eseroline (Winder et al 1966). The

*Methionine-enkephalin.

ability of physostigmine in displacing naloxone binding is very low, its IC50 value being 3×10^{-5} M. Analogously, salicylate, present in all incubations with eseroline, does not itself show any affinity for naloxone binding sites. The inhibition of [^{3}H]naloxone binding by eseroline is highly sodium dependent: the IC50 ratio, in the presence and in the absence of 100 mM NaCl, is 185. In our experiments, under the same conditions, morphine, met-enkephalin and profadol presented IC50 ratios for sodium of 25, 22 and 10 respectively. When binding measurements were carried out at 25 °C, the effect of sodium on the inhibition of naloxone binding by eseroline was much less marked (IC50 ratio = 19). Under these conditions, the ratio for sodium of morphine was 13.

The principal finding of this study is that eseroline interacts with naloxone binding sites of rat brain in the presence of sodium in a range of concentrations approximately corresponding to those at which eseroline exerts its pharmacological effects, provided that binding is measured at 25 °C. By contrast, its parent molecule, physostigmine, shows affinity for naloxone binding sites only at concentrations several orders greater than those analgesic for this drug (Harris et al 1969; Pleuvry & Tobias 1971). Moreover, it seems worth pointing out that the 'sodium response ratio' of eseroline is particularly high for binding assays carried out at 0 °C, being the highest value observed in our experiments and among those reported by Simantov & Snyder (1976). Such a sodium-effect is markedly affected by increasing incubation and filtration temperatures, as we have observed also for morphine, in agreement with a previous observation of Creese et al (1975).

These results indicate that inhibition of naloxone binding by eseroline closely parallels that typical of pure opiate agonists, suggesting that interaction of eseroline with brain opiate receptors is a preliminary step in the mechanism of its analgesic activity. This hypothesis appears strengthened also by the observation that analgesia by eseroline is blocked by 1 mg kg^{-1} s.c. of naloxone (Bartolini et al 1979), whereas analgesia by physostigmine is only partially antagonized by naloxone (Howes et al 1969; Pleuvry & Tobias 1971).

These results, however, do not explain why eseroline is a rather weak inhibitor of naloxone binding, when compared with morphine, while the two compounds are approximately equipotent as analgesics. This lack of correlation between binding data and pharmacological effects, reported also for etorphine (Pert & Snyder 1973), meperidine (Pert et al 1973), codeine (Pert & Snyder 1973), Win 35, 197-2 (Kosterlitz & Leslie 1977) and other opiates, might be explained by a marked difference either in the kinetics of the two drugs or in their intrinsic biological activities.

As seen in the structural formulae, eseroline and morphine present precise common chemical features. In particular, when the phenolic moieties of the two molecules are superimposed, the pyrrolidine nitrogen of eseroline is isosteric with the piperidine nitrogen of morphine. Therefore, eseroline, although derived from physostigmine, appears structurally related to morphine also. On the other hand, the carbamyl ester linkage present in physostigmine differentiates this drug from morphine and eseroline and gives it the well known cholinomimetic activity.

To sum up, this finding and the results for displacement of [^{3}H]naloxone, strongly suggest that analgesic activity of eseroline involves a direct interaction with opiate receptors, as happens with morphine-like analgesics. Moreover, our experiments point to the importance of the methyl carbamyl group of physostigmine in determining the biological target of this molecule.

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