in the following order: gentamicin, kanamycin, amikacin, sisomicin, tobramycin, netilmicin and streptomycin [17]. Neomycin, the most toxic aminoglycoside, is used in local therapy only. The ranking of aminoglycosides with respect to the decrease of their ability to compete with gentamicin for binding sites on renal cortex *in vitro* in our experiments is only partly in accordance with the order of their nephrotoxic potential *in vivo*.

In conclusion it may be said that the uptake of gentamicin into rat renal cortex *in vitro* is saturable and characterized by $K_m = 86.67\ \mu g/ml$ and $B_{max} = 477.47\ \mu g/g$ per 7 min. This uptake could be competitively inhibited by neomycin and kanamycin which are aminoglycoside antibiotics with more free amino groups on their molecule than gentamicin (3). This is in accord with the proposed charge interaction between the cationic polybasic aminoglycosides and the anionic tissue components of the kidney [16].

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Inhibition of acetylcholinesterase and cholinesterase by ellipticine derivatives

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Ellipticine and its derivatives are indole alkaloids with antitumoral properties [1, 2]. The structure of these compounds is depicted in Fig. 1. The cytotoxicity of ellipticines may be related to their affinity for deoxyribonucleic acid to which they combine by intercalation. Elliptinium acetate (**IIb**), a quaternary ammonium derivative, has induced remissions in patients with breast cancer and other malignancies [3, 4]. Some of the side effects observed, such as dyspnea and muscle cramps, suggested an interaction between elliptinium and the cholinergic system [5]. Previous studies on the structure–activity relationship have shown that many reversible inhibitors of acetylcholinesterase (EC 3.1.1.7, AcChE) and butyryl cholinesterase (EC 3.1.1.8, BuChE) contain one or two quaternary ammonium groups [6, 7], or eventually a protonated tertiary amine. The present study reports the interaction of ellipticine derivatives with the catalytic activity of acetylcholinesterase from rat brain and of human sera pseudocholinesterase.

Materials and methods

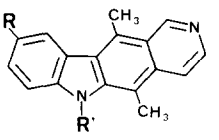
The substrates (i.e. acetylthiocholine ATC and butyrylthiocholine BTC), the chromogen dithionitrobenzoic acid (DTNB) and Triton X-100 were purchased from Sigma Chemical Co. (St. Louis, MO). 2*N*-methyl-9-hydroxyellipticinium* and BD 40 M were obtained from Institut Pasteur Production (Paris, France), and other derivatives were gifts of Dr. Auclair (L.A. 147 C.N.R.S.). Edrophonium chloride was obtained from Hoffmann-La Roche & Co., A.G. (Basel, Switzerland). All other reagents were of analytical grade.

Enzymatic preparations. The two enzymatic preparations used in this study were acetylcholinesterase extracted from the cerebral cortex of male Wistar rats and cholinesterase of human serum.

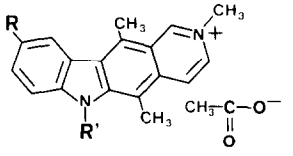
The rat cortices were rapidly removed after decapitation and placed in a cold phosphate buffer, pH 7.4, containing 1% of non-ionic surfactant (Triton X-100). Cortices were then homogenized by Polytron action, and 8 ml of buffer was added to give a final volume of 10 ml. After centrifugation (15 min, 40,000 *g*, 4°), the supernatant was removed and used immediately or frozen at −20°. Enzymatic solutions were always used within 4 days after preparation. Human serum cholinesterase (BuChE) was obtained by centrifugation of whole blood collected on heparin anticoagulant, and diluted to 50% in phosphate buffer.

Cholinesterase assay. The determination of enzyme

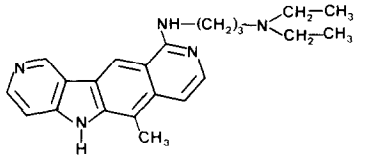
* Elliptinium acetate (DCI) : Celiptium.®



Ia : Ellipticine (R = R' = H)
Ib : 9-hydroxy-Ellipticine (R = OH ; R' = H)
Ic : 9-methoxy-Ellipticine (R = OCH_3 ; R' = H)
Id : 9-fluoro-Ellipticine (R : F ; R' = H)
Ie : 6 N-methyl 9-hydroxy-Ellipticine (R = OH ; R' = CH_3)



IIa : 2N-methyl-Ellipticinium (NME) (R = R' = H)
IIb : 2 N-methyl 9-hydroxy-Ellipticinium (NMHE), (R = OH ; R' = H)
IIc : 2,6 NN'-dimethyl 9-hydroxy-Ellipticinium (R = OH ; R' = CH_3)



III : 10-diethylamino-propylamino 6-methyl (5H)-pyrido-[3',4']-4,5-pyrrolo-(2-3 isoquinoline) (BD 40).

Fig. 1. Structural formulae of ellipticine derivatives and analogues.

activities was performed according to the method of Ellman *et al.* [8] with minor modifications. ATC and BTC solutions were prepared fresh daily by dissolving 0.15 mmole in 5 ml of phosphate buffer at pH 7.4. The same buffer was used for the preparation of Ellman's reagent (23.8 mg DTNB in 50 ml phosphate buffer), and the final solutions of tested drugs. The enzymatic reaction was carried out on a parallel fast analyser (Rotochem®, Aminco), and the increase of absorbance at wavelength 405 nm was measured every minute. Two preincubations were performed at 37°. Twenty μl of the enzymatic preparation was added to 430 μl of DTNB solution, and, on the other hand, 130 μl of substrate dilution (ATC : 0.062–1 mM ; BTC : 0.062–0.5 mM) was mixed with 20 μl of the tested drug or the phosphate buffer. The enzymatic reaction was started by automatic rapid centrifugation, and the enzyme kinetics were followed for 10 min at 37°. The increase of absorbance per min was linear and represented the rate of the reaction, calculated as the mean of nine determinations, according to the fact that during this time interval the reaction was of first order in relation to the substrate concentration.

Results and discussion

All compounds were incubated with both enzymes at different final concentrations (1–100 μM). Kinetic data analysed by the method of Lineweaver–Burk [9] indicated that enzymatic inhibition was non-competitive (Fig. 2a for AcChE inhibition, and Fig. 2b for BuChE inhibition). Inhibition constants (K_I)were calculated as follows:

$$K_I = \frac{[I]\,S}{S'-S}$$

where $[I]$ = inhibitor concentration (mole/l.), S = Lineweaver–Burk plot slope without inhibitor, and S' = Lineweaver–Burk plot slope with inhibitor, and are reported for every ellipticine and for edrophonium chloride in Table 1. K_{I_1} was the value of K_I for AcChE, K_{I_2} for BuChE, and the ratio indicated the preferential action. Representation of these data using the method of Dixon [10] gave similar results to those described in Table 1 (data not shown).

Ellipticines (**I**) and ellipticinium salts (**II**) were respectively weak and strong inhibitors of cholinesterases. This difference may be related to their chemical structure, and to the biochemical properties of the enzymes [11, 12]. Tertiary amines **I** were not good inhibitors as the nitrogen of the carbazol ring presented a pK_a in the range 6.0–6.3. So at pH 7.4 amines were not, in their great majority, protonated and could not be attracted by the anionic site of the enzyme [13]. Quaternary ammonium (ellipticinium) compounds were potent inhibitors of the cholinesterases and could be compared with edrophonium. This reaction was immediate, reversible, and data plots indicated that the mechanism was non-competitive. Derivatives **II** were positively charged at all values of pH, and could be attracted by the anionic site of the enzymes. Furthermore, they presented an important hydrophobic zone capable of reacting upon the enzyme hydrophobic areas [14]. The greater significance of these areas for BuChE [15] could explain the fact that the K_{I_1}/K_{I_2}ratio was superior than that for the quaternary ammonium compounds. Derivative **IIb**, less hydrophobic, presented a ratio of 3, whereas derivatives **IIa** and **IIc** showed a ratio greater than 50.

The analogue **III** exhibited a different type of reactivity. It was a good inhibitor of BuChE, and yet a better inhibitor of AcChE. At pH 7.4, the nitrogen in position 9 of the isoquinoline ring was not charged for the reasons explained above, to the opposite of the α-nitrogen of the aliphatic group ($pK_a > 10$). This compound could react upon the anionic site with its aliphatic nitrogen, the interatomic distance between the quaternary ammonium and the hydrophobic structure being greater in this case then with ellipticinium **II**.

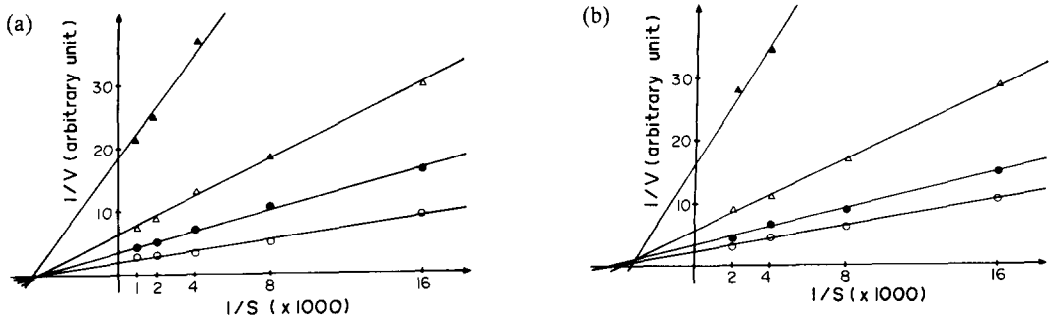


Fig. 2. Cholinesterase inhibition by elliptinium acetate **IIb** as analysed by the graphical method of Lineweaver–Burk. (a) AcChE inhibition. Control (○), elliptinium acetate 5 μM (●), 20 μM(△) and 100 μM(▲). (b) BuChE inhibition. Control (○), elliptinium acetate 1 μM(●), 5 μM(△) and 25 μM(▲).

Table 1. Ellipticine inhibition constants (K_I)* of cholinesterases†

	K_{I_1} : AcChE inhibition	K_{I_2} : BuChE inhibition	$\frac{K_{I_1}}{K_{I_2}}$
Ia	10^{-2}	$5.1\,10^{-3}$	1.9
Ib	10^{-2}	$9.8\,10^{-3}$	1.1
Ic	$1.3\,10^{-2}$	n.c.	—
Id	$1.3\,10^{-2}$	$8.1\,10^{-3}$	1.6
Ie	10^{-2}	$7.4\,10^{-3}$	1.4
IIa	$3.4 \pm 1.3\,10^{-4}$‡	$4.3 \pm 1.6\,10^{-6}$	79.1
IIb	$9.0 \pm 2.1\,10^{-6}$	$2.7 \pm 0.6\,10^{-6}$	3.3
IIc	$1.6 \pm 0.7\,10^{-4}$	$2.4 \pm 1.1\,10^{-6}$	66.7
III	$1.3 \pm 0.5\,10^{-6}$	$1.1 \pm 0.5\,10^{-5}$	0.1
Edrophonium	$8.3 \pm 0.9\,10^{-6}$	$3.8 \pm 1.0\,10^{-6}$	2.1

* Expressed in mole/l.

† Each value represents the mean of three measurements conducted with three concentrations of inhibitor and four concentrations of substrate (except for **I**, one measurement).

‡ Mean ± standard error of the mean.

In summary, tertiary amines were found to have a weaker activity than quaternary ammonium salts on acetylcholinesterase and pseudocholinesterase. The inhibition by ellipticinium was reversible and non-competitive. Inhibition constants were in the range 10^{-4}–10^{-6}M. The K_I for acetylcholinesterase, usually greater than that for butyrylcholinesterase, might by explained by differences between respective enzyme hydrophobic areas. The data indicate that drugs at therapeutic doses might decrease the activity of cholinesterases, as serum levels 1 hr after infusion were neighbouring 10^{-6}M. These observations are important for the understanding of clinical side effects, following human therapy with elliptinium, and for the selection of future ellipticine derivatives in clinical drug screening. Indeed, about 20–30% of treated patients presented side effects suggestive of a cholinergic mechanism. Studies are in progress to determine whether other components of the cholinergic system (i.e. muscarinic and nicotinic receptors) are involved in this interaction.

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