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INHIBITION OF CHOLINESTERASE AND ACETYLCHOLINESTERASE *IN VITRO* BY BUTYROPHENONE NEUROLEPTICS

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Abstract—The inhibitory effect of six butyrophenone neuroleptics: haloperidol, triperidol, moperone, floropipamide, benperidol and droperidol on the activity of partially purified aspecific horse serum ChE and on purified *Electrophorus electricus* specific AChE was studied with AThCh as substrate using Ellman's spectrophotometric method.

All butyrophenones were found to be inhibitors of ChE, the K_i being in the range 10^{-5} M and all followed a fully competitive type of inhibition. There were small differences in K_i-values among various butyrophenones, while there were great differences in K_i-values between the butyrophenones, the two phenothiazines and imipramine, the last three drugs being far more potent competitive ChE inhibitors (K_i 10^{-6}–10^{-7} M) than the butyrophenones.

All the butyrophenones were also found to inhibit AChE, the overall inhibitor constants being in the range 10^{-4} to 10^{-5} M and all showed a mixed type of inhibition. The most potent of the butyrophenones are droperidol and benperidol, the benzimidazolone derivatives. There are no great differences in the K_i-values for the butyrophenones, the two phenothiazines and imipramine for inhibition of AChE.

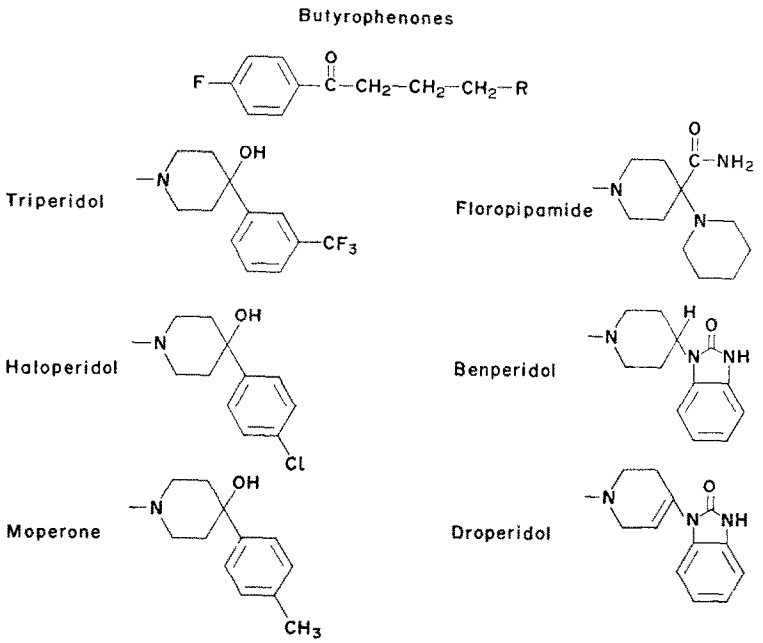
IN A previous study[1] we demonstrated that triperidol, a potent butyrophenone neuroleptic at a concentration 2×10^{-4} M strongly decreased the free ACh* synthesis in fresh brain tissue *in vitro* but was quite without effect on the enzymatic AThCh hydrolysis, causing only a slight inhibition in the thalamus and hypothalamus. The concentration of triperidol which produced a 50 per cent (I_{50}) inhibition in cerebral cortex was about 10^{-3} M.

Poli,[2] on the basis of same histochemical work reported a marked inhibition of ChE and, to a lesser extent, of AChE particularly in the mesencephalon and diencephalon of rabbits chronically treated with haloperidol. It seems therefore that the butyrophenones may have some inhibitory activity against both ChE or AChE.

It is well known that a number of neuroleptics and tertiary amines inhibit these enzymes. Some phenothiazine derivatives have been reported to inhibit ChE of plasma[3–6] and erythrocytes,[3] while brain AChE inhibition was demonstrated by some authors[7] and its absence by others.[6] Imipramine[8,9] has also been demonstrated to be an inhibitor of the ChE found in blood and brain.

The present study will report effects of some butyrophenone neuroleptics on purified preparations of specific AChE from electrical organ of *Electrophorus electricus* and on partially purified preparations of aspecific ChE from horse serum. The results will

*Abbreviations used: ACh, acetylcholine; AThCh, acetylthiocholine; AChE, acetylcholine acetylhydrolase (EC 3.1.1.7); ChE, acylcholine acylhydrolase (EC 3.1.1.8).



be compared with those obtained under the same experimental conditions with some phenothiazine derivatives and imipramine.

MATERIALS AND METHODS

Materials. AChE from *Electrophorus electricus*, purified by chromatography and gel filtration and ChE, a lyophilized powder from horse serum were obtained from Worthington Biochemical Corporation, Freehold, N.J. All reagents for the determination of enzymes activity were obtained from Boehringer, Mannheim, Germany. The butyrophenone derivatives were kindly supplied by the Janssen Research Laboratories, Beerse, Belgium and the phenothiazine derivatives by Rhône-Poulenc, France.

Enzyme kinetic measurements. The activity of both enzymes was measured, as previously described[11–12] according to the procedure of Ellman *et al.*[13] with a Beckman DK-2 spectrophotometer and with AThCh iodide as substrate. The final concentrations of the reagents were as follows: phosphate buffer, pH 7·2, 0·039 M, 5,5′-dithio-bis(2-nitrobenzoic) acid 0·21 mM. In the case of AChE, six substrate concentrations ranging from 0·045 to 0·56 mM were used. With ChE, six substrate concentrations ranging from 0·175 to 5·6 mM were used. In the case of AChE each reaction mixture contained 3 ng of enzyme preparation and with ChE reaction mixture contained 4 μg of enzyme preparation. The total reaction volume was 1·4 ml. The reaction was initiated by adding the substrate solution to a cuvette containing all the reagents. The values of extinction at 412 nm were measured once every minute from the second to the sixth min of the reaction. The difference in extinction between the second and the third minute was used to calculate the enzyme activity since after the third minute the velocity of enzymatic reaction slightly decreased. The nonenzymatic hydrolysis

of AThCh was found to be negligible. The butyrophenone bases were dissolved in 2 N hydrochloric acid, subsequently neutralized and added to the reaction mixture at a concentration which did not alter the pH of the enzymatic reaction. The 6 min incubation period was necessary to ascertain if the degree of inhibition by butyrophenones maintained constant in the time.

The K_m-values of AThCh–AChE and AThCh–ChE complexes were determined graphically by plotting 1/v vs 1/[S] according to Lineweaver–Burk.[14] The inhibition kinetics of enzymes with compounds were studied on the Lineweaver–Burk plot as described by Dixon and Webb.[15] Each point of the uninhibited and inhibited reaction was the mean of at least three assays. The slopes and intercepts were determined as recently described by Mahon and Brink[16] from a computer generated linear regression analysis on six points for each line, correlation coefficients for the regression lines being in all cases greater than 0·988. The 95 per cent confidence limits for $1/K_m$, $1/K_p$ and for different values of 1/v and 1/[S] were calculated from the regression analysis data. The inhibitor dissociation constants K_i, with their 95 per cent confidence limits were calculated from the Lineweaver–Burk plots as described by Dixon and Webb.[15]

RESULTS

Inhibition of horse serum ChE by butyrophenone derivatives. It was found in the preliminary experiments with horse serum ChE that when 4×10^{-4} M floropipamide was present in the reaction mixture containing AThCh, the enzyme activity was inhibited to 25 per cent of the control values and all butyrophenones studied inhibited to various degrees the enzyme activity.

The equilibrium conditions were attained rapidly. The degree of inhibition was constant throughout the 6 min incubation period and preincubation for up to 10 min of the inhibitor with enzymes without AThCh did not affect the initial rate measurements.

Lineweaver–Burk plots have demonstrated (Fig. 1) that the plotted lines of 1/v vs 1/[S] in the presence of 4×10^{-5} M concentration of triperidol, floropipamide and droperidol intersect on the vertical axis the line of uninhibited reaction, that is the presence of an inhibitor increased the apparent K_m-value without affecting V_{max}. The graphs for haloperidol, moperone, benperidol and imipramine are not reported in the Fig. 1 because the lines were superimposable on others (for example the graphs for imipramine at 10^{-5} M concentration and for moperone at 6×10^{-5} M concentration were superimposable on that for droperidol at 4×10^{-5} M) and presented the same characteristics of competitive inhibition of the horse serum ChE.

Figure 1 also demonstrates two lines of the enzymatic reaction in the presence of two phenothiazines, chlorpromazine and prochlorperazine of the promazine and piperazine groups respectively. All these compounds produced a competitive type of inhibition.

The K_i values (Table 1) ranged for the butyrophenone compounds from $1{\cdot}9 \times 10^{-5}$ in the case of droperidol to $6{\cdot}1 \times 10^{-5}$ M in the case of haloperidol.

It appears that there is little difference among the various butyrophenones while there is a marked difference in inhibitory activities between butyrophenones and phenothiazines, the last being more active.

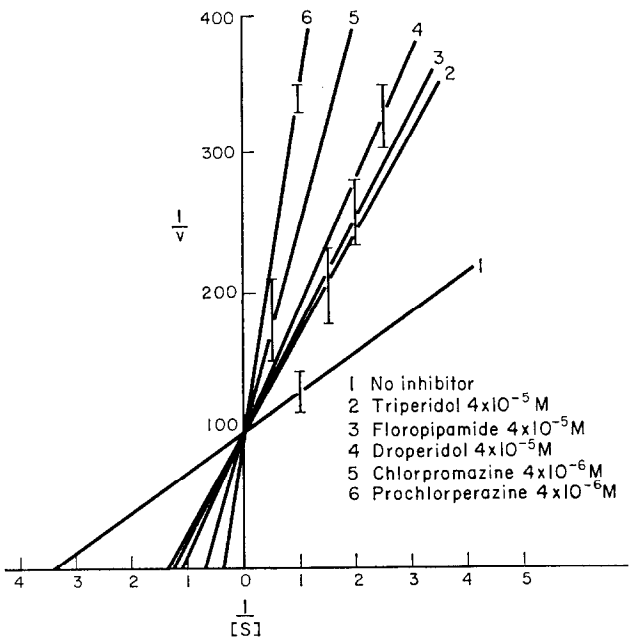


FIG. 1. Lineweaver–Burk plot of the inhibition *in vitro* of horse serum ChE by some butyrophenones and phenothiazine neuroleptics indicating a competitive type of inhibition. Activity of ChE was measured by Ellman's spectrophotometric method. 1/v is the reciprocal of the velocity of enzymatic reaction expressed as mmoles of AThCh hydrolyzed per minute in the reaction mixture. 1/[S] is the reciprocal of the substrate AThCh concentration expressed as mM. The slopes and intercepts were determined according to Mahon and Brink[18] by a computer-generated linear regression analysis on six points for each line (each point is the mean of at least three separate assays) correlation coefficients being in all cases greater than 0·987 with $P < 0{\cdot}01$. The 95 per cent confidence limits for slopes of each line were calculated from the regression analysis data for 1/v values corresponding to 1/[S] values from 0·5 to 3, and since small differences among them were found for the same line, only one value of confidence limits for each line is presented. The K_m-value was found to be $2{\cdot}9 \times 10^{-4}$ M ($2{\cdot}5 \times 10^{-4}$ M to $3{\cdot}5 \times 10^{-4}$ M) and V_{max} 10·3 (9·1–11·9) μmoles of AThCh hydrolysed per minute.

Inhibition of Electrophorus electricus *AChE by butyrophenone derivatives.* It was found in the preliminary experiments with *Electrophorus electricus* AChE that when 4×10^{-4} M floropipamide was present in the reaction mixture containing AThCh, the enzyme activity was inhibited to 66 per cent of the control values, i.e. to a lesser extent than ChE. The other butyrophenones inhibited to various degrees this enzyme activity.

Lineweaver–Burk plots (Fig. 2) have demonstrated that the plotted lines of 1/v vs 1/[S] in the presence of 5×10^{-5} M floropipamide and 4×10^{-5} M moperone intersect the line of the uninhibited enzymatic reaction in the area on the left between the vertical and horizontal axes, thus the inhibition for these compounds is apparently of mixed type, while for 4×10^{-5} M benperidol the point of intersection is on the horizontal axis hence the inhibition appears to be apparently of a noncompetitive type. The lines for other butyrophenones: haloperidol, triperidol, droperidol and for imipramine, not shown in Fig. 2 present the same characteristics of apparently mixed type of inhibition. For the two phenothiazines studied apparently the same characteristics of mixed type inhibition were also demonstrated (Fig. 2).

TABLE 1. INHIBITION OF HORSE SERUM ChE AND *Electrophorus electricus* AChE BY BUTYROPHENONE DERIVATIVES, PHENOTHIAZINES AND IMIPRAMINE*

Compounds	Horse serum ChE		*Electrophorus electricus* AChE†		$K_{i\ AChE}$ / $K_{i\ ChE}$
	$K_i \times 10^5$ M	95% Confidence limits	$K_i \times 10^5$ M	95% Confidence limits	
Triperidol	2·6	1·6–3·4	7·0	6·1–9·1	2·6
Haloperidol	6·1	5·3–6·6	14·0	12·0–17·0	2·3
Moperone	2·7	2·3–3·1	12·0	9·0–20·0	4·4
Floropipamide	2·4	1·9–2·9	25·0	8·0–40·0	10·4
Benperidol	2·0	1·5–2·4	5·0	4·8–5·4	2·5
Droperidol	1·9	1·6–2·1	2·8	2·2–4·2	1·5
Chlorpromazine	0·11	0·08–0·12	11·0	8·0–16·0	100·0
Prochlorperazine	0·046	0·043–0·05	11·0	9·0–12·0	239·0
Imipramine	0·46	0·38–0·55	83·0	82·0–84·0	180·0

* Experimental conditions and elaboration of the data as described in the Fig. 1. K_i were calculated from Lineweaver–Burk plots.[15]

† In the case of *Electrophorus electricus* AChE the overall K_i values are presented.

The K_i-values (Table 1) ranged for the butyrophenone compounds from $2·8 \times 10^{-5}$ M for benperidol to $2·5 \times 10^{-4}$ M for floropipamide. In fact the 4-anilinopiperidine butyrophenones, which have the benzimidazolone ring, appear relatively the most active. In the case of AChE inhibition, other drugs tested had a similar inhibitory activity, unlike the results for ChE, where the phenothiazines were more potent inhibitors than the butyrophenones.

DISCUSSION

The first observations on the inhibition of enzymatic hydrolysis of ACh by butyrophenones were made on mammalian brain.[1,2] In the present kinetic studies, however, the purified preparations of *Electrophorus electricus* AChE and horse serum ChE were used.

It is well known that AChE isolated from the electrical organ and AChE from mammalian brain are quite similar, both in substrate specificity and also in relative rates of utilization of most of the typical substrates used in the kinetic studies.[17] Mahon and Brink[16] have reported for the rat brain AChE a K_m-value of $1·2 \times 10^{-4}$ M and a K_m $1·7 \times 10^{-4}$ M for *Electrophorus electricus* AChE using AThCh as substrate.

In these studies AThCh was used as substrate for both enzymes since Patočka and Bajgar[18] have recently shown that the rate of hydrolysis of AThCh by brain AChE is identical to that of ACh in equivalent concentration. Ecobichon and Israel[19] have also reported similar K_m- and V_{max}-values for both substrates using *Electrophorus electricus* AChE. AThCh is also an optimal substrate for horse serum ChE, the rate of hydrolysis being about 70 per cent of that for butyrylthiocholine, the best substrate known.[17]

In the present study it was demonstrated that all the butyrophenones studied are inhibitors of horse serum ChE and *Electrophorus electricus* AChE.

A fully competitive type of inhibition (Fig. 1) was found for butyrophenones with horse serum ChE and there are not great differences among various butyrophenones

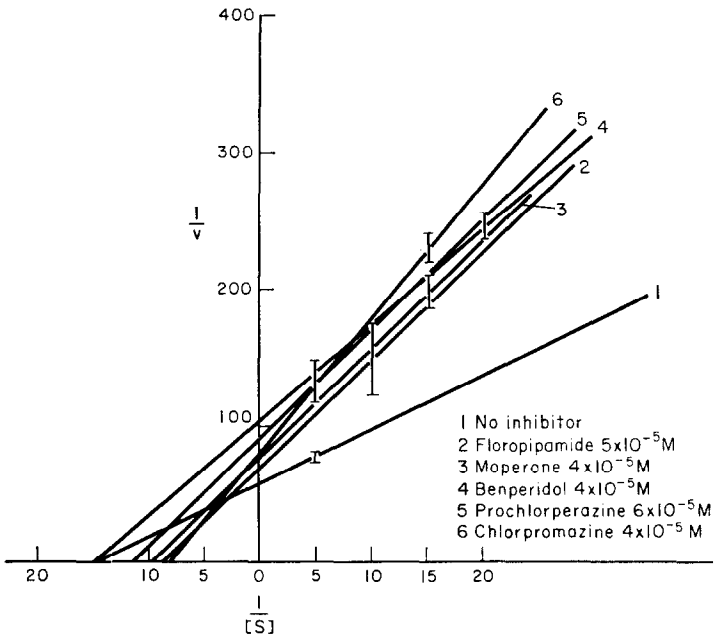


FIG. 2. Lineweaver–Burk plot of the inhibition *in vitro* of *Electrophorus electricus* AChE by some butyrophenone and phenothiazine neuroleptics indicating an apparently mixed type of inhibition. Activity of AChE with AThCh as substrate, 1/v, 1/[S], and the statistical elaboration of the data are as described in Fig. 1. Correlation coefficients for the regression lines on six points for each line (each point is the mean of at least three separate assays) were greater than 0·988 with $P<0{\cdot}01$. The 95 per cent confidence limits were calculated as described in Fig. 1. The K_m-value was found to be $6{\cdot}7 \times 10^{-5}$ M ($6{\cdot}3 \times 10^{-5}$ to $7{\cdot}3 \times 10^{-5}$ M) and V_{max} 16·9 (15·9–18·2) μmoles of AThCh hydrolysed per minute.

in the inhibitory activity (Table 1). In fact only the butyrophenone chain with the distance between the piperidine nitrogen and carbonyl carbon of approx. 5·7 Å[20] appears to be essential for the binding with the active sites of the enzyme, the intersite distance of 4·0–5·5 Å found by different authors[21] being consistent with this hypothesis. The binding of the substrate at the esteratic site is, however, to a negative ester group rather than to the carbonyl carbon,[21] the ester group being absent in the butyrophenones.

The same competitive type of inhibition was found with two phenothiazines studied, in agreement with the findings of Usdin *et al.*[3] for human plasma with ACh as substrate. The competitive type of inhibition found for imipramine with horse serum ChE is in agreement with the results of Perkinson *et al.*[9] obtained on canine plasma and ACh, for desipramine.

The interaction of butyrophenones, two phenothiazines and imipramine with *Electrophorus electricus* AChE appears to be of a more complex nature than their interaction with horse serum ChE. On the basis of the present results it is not possible to further characterize the apparently mixed inhibition of AChE. One must remember that the reaction scheme for AChE involves two enzyme–substrate intermediates: the active enzyme–substrate complex and the acetyl–enzyme.[22,23] Compounds giving apparently mixed inhibition may be expected to compete with the substrate for

the free enzyme (competitive element) as well as to interfere with further reactions, i.e. the breakdown of the enzyme–substrate complex, the deacetylation of the acetyl–enzyme or both. In the case of AThCh as substrate, the deacetylation is a rate-limiting step in the overall reaction of hydrolysis.[23]

To clarify whether the inhibitor combines with the enzyme–substrate complex or with the acetyl–enzyme, blocking deacetylation, it would be necessary to do additional experiments with a number of substrates carefully chosen for their different rates of the single steps in the overall reaction of hydrolysis.[22]

In conclusion the apparently mixed *Electrophorus electricus* AChE inhibition by butyrophenones found in the present experiments may be a mixture of competitive and noncompetitive inhibition or a mixture of competitive and uncompetitive inhibition. If the first possibility were true, there would not be a plausible explanation for the qualitatively different result of noncompetitive inhibition for benperidol, since its structure is similar to others, especially to that of droperidol. A mixture of competitive and uncompetitive inhibition for all compounds would explain the apparently different result for benperidol by supposing that the K_i-values for competitive and uncompetitive inhibition were roughly equal.

An apparently mixed type of inhibition by some phenothiazines was found by Usdin *et al.*[3] for human erythrocyte AChE with acetyl-β-methylcholine as substrate and the K_i for chlorpromazine was very similar ($0{\cdot}7 \times 10^{-4}$ M found by Usdin *et al.*[3] towards $1{\cdot}1 \times 10^{-4}$ M in the present work).

The results on imipramine agree with those of Ho *et al.*[8] for rat brain AChE and AThCh, the K_i-values are of the same order of magnitude, but are in contrast to those reported by Osborne and Sigg[24] who state that anticholinesterase activity of this compound is about a thousand times more potent.

In conclusion these results demonstrate that none of the butyrophenone compounds may be considered as potent an anticholinesterase agent. All the butyrophenones studied are more potent inhibitors of a specific horse serum ChE than the *Electrophorus electricus* AChE. The $K_{i\ \mathrm{AChE}}$–$K_{i\ \mathrm{ChE}}$ ratio varies from 1·5 to 10·4 (Table 1) for butyrophenones while values of 100 to about 240 were found for two phenothiazines and imipramine. Phenothiazines and imipramine appear to be weak inhibitors of AChE, but relatively potent inhibitors of ChE whereas the butyrophenones are weak inhibitors of both enzymes.

The phenomenon reported in our previous study[1] that is the presence of a slight inhibitory effect of triperidol *in vitro* on the enzymatic ACh hydrolysis in some areas of brain such as thalamus, hypothalamus and cerebellum, and the absence of inhibitory effect in cerebral cortex in all probability is due to the fact that triperidol is quite three-times more potent an inhibitor of aspecific ChE than AChE. It is well known[25] that in the areas as thalamus, hypothalamus and cerebellum about 40 per cent of the enzymatic hydrolysis is due to aspecific ChE while in the cerebral cortex nearly all ACh hydrolysis is due to AChE.

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