Biochemical Pharmacology, Vol. 20, pp. 929–942. Pergamon Press, 1971. Printed in Great Britain

STUDIES ON THE INTERACTION BETWEEN INHIBITORS OF DRUG METABOLISM AND HORSE PLASMA CHOLINESTERASE

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(*Received* 8 *October* 1969; *accepted* 29 *May* 1970)

Abstract—Several inhibitors of drug metabolism were found to inhibit horse plasma cholinesterase activity at concentrations ranging from 0·3 μM to 20 μM. A detailed study of the inhibition was made using 2-diethylaminoethyl 2,2-diphenylvalerate (SKF 525A); 2,4 dichloro-6-phenylphenoxyethyldiethylamine (Lilly 18947) and *N*-(γ-dimethylaminopropyl-iminodibenzyl) (imipramine). The cholinesterase inhibition produced by these three compounds was very fast, competitive and reversible. Only SKF 525A was able to delay the irreversible inhibition of the enzyme by dimethyl-dichlorovinyl-phosphate, indicating that it is probably acting at the esteratic site.

The inhibition by these compounds increases from pH 5·5 to pH 7·5–8·0, then decreases. Since the inhibitors are mainly in the basic form at pH 7·5, the results were interpreted as showing that the active portion of the inhibitor is located at the basic nitrogen atom and that this group interacts on a chemical group of the enzyme having a pK_a about 8·6–9·0.

In agreement with this assumption, changes in basicity of the nitrogen atom of the inhibitors explained very well the differences in inhibitory power between pairs of analog compounds. This hypothesis also explains why bovine erythrocyte acetylcholinesterase exhibited smaller affinity for inhibitors than the horse plasma enzyme, in spite of the similarities in the inhibition at different pH values. Changes in entropy for the inhibition of cholinesterase by Lilly 18947 were also in accordance with this hypothesis.

SEVERAL structurally unrelated compounds share the ability of inhibiting drug metabolizing enzymes.[1, 2] Preliminary experiments from our laboratory showed that they also inhibit horse plasma cholinesterase.

The similar behavior of these compounds acting on two completely different enzyme systems would suggest the possibility that all of them have some portion of their molecules able to interact with chemical groups in both enzymes having similar reactivity. Since the active site of horse plasma cholinesterase has been considerably elucidated, we thought that a study of its blockade by some inhibitors of drug metabolism may give a better understanding of the nature of the interaction shared by these compounds.

In this work we describe our studies on the inhibition of horse plasma cholinesterase by inhibitors of drug metabolism.

MATERIALS AND METHODS

Horse plasma cholinesterase (ChE) was purchased from Nutritional Biochemicals; its specific activity was 5 units per mg (1 unit hydrolyzes 1 μmole of acetylcholine

* The work described was carried out in partial fulfilment of the requirements for the Ph.D. Thesis, University of Buenos Aires, Argentine.

† Recipient of a research grant from the "Consejo Nacional de Investigaciones Científicas y Técnicas", Argentine.

(ACh) per min at pH 8·0 at 37°). Bovine erythrocyte acetylcholinesterase (AChE) was purchased from Sigma Chemical Co.; its specific activity was 1–2 units per mg at 37°.

2-Diethylaminoethyl-2,2-diphenylvalerate hydrochloride (SKF 525A) was a gift from Smith Kline & French Laboratories. 2-Diethylaminoethyl-2-phenyl-2-(2-propene)-4-penten-1-oate hydrochloride (CFT 1201) was supplied by Chemische Fabrik Tempelhof, Preuss and Temmler, Berlin (Germany). Ethyl 2-diethylaminoethyl 2-phenyl-2-ethylmalonate hydrobromide (Sch 5705); ethyl *N*-(2-diethylaminoethyl) 2-phenyl-2-ethylmalonamate hydrobromide (Sch 5706); ethyl 2-diethylaminoethyl 2-ethyl-2-butylmalonate hydrobromide (Sch 5712) were kindly supplied by Societá Italiana Prodotti Schering, Milan (Italy). 2,4 Dichloro-6-phenylphenoxyethyldiethylamine hydrobromide (Lilly 18947) and 2,4 dichloro-6-phenylphenoxyethylamine (DPEA) were gifts from Eli Lilly Co. 10-(3-Dimethylaminopropyl)-phenothiazine hydrochloride (promazine) and 10-(3-dimethylaminoisopropyl)phenothiazine hydrochloride (promethazine) were obtained from Wyeth Laboratories. *N*-(γ-dimethylaminopropyl-iminodibenzyl) hydrochloride (imipramine) was obtained from Geigy Laboratories. Dimethyl-dichlorovinyl-phosphate (DDVP), b.p. 118·5° at 13·5 mm Hg, was prepared by the Organic Chemistry Division from our Institute.

ChE and AChE activity were measured by automatic titration with a Radiometer SBR 2/SBU 1 titrigraph according to the procedure of Jørgensen. Unless otherwise stated, all the experiments were conducted at pH 7·2 and 30°, with nitrogen bubbling through the incubation mixture to prevent the absorption of carbon dioxide from atmosphere. ACh concentration is given in each experiment.

In order to correct for nonenzymatic hydrolysis of choline esters and various inhibitors, control determinations were made under the same conditions used for a typical assay except that the ChE was omitted. Appropriate corrections for spontaneous liberation of acidity by the enzyme preparation in the presence or absence of inhibitor were also included.

The ionization constants of SKF 525A (25 μM) Lilly 18947 (1 mM), and imipramine (1 mM) were determined by titration with sodium hydroxide at 30°.

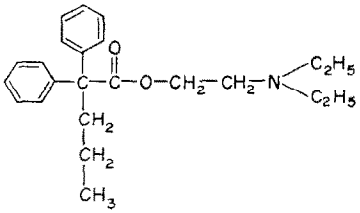
In order to establish the probable existence of precipitation in the aqueous solutions of the inhibitors at different pH and 30°, changes in absorbance at 660 mμ were recorded. Alternatively, decreases in absorbance at 220 mμ of filtered solutions at different pH were employed.

Protein concentrations in enzyme solutions were determined according to the method of Kalckar.[4]

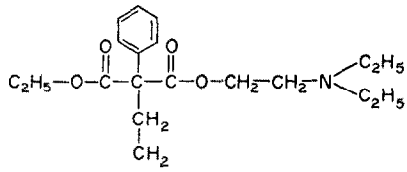
RESULTS

Effect of several compounds on ChE activity. The effect of SKF 525A and the other inhibitors of drug metabolism on ChE was studied. The compounds were incubated for 5 min with ChE before adding ACh (final concentration 1 mM). Their chemical structures, together with the values of their pI_{50}, are presented in Fig. 1. All the compounds tested showed an extremely potent inhibitory action; the concentrations needed for 50 per cent inhibition were all in the range from 0·3 μM to 20 μM.

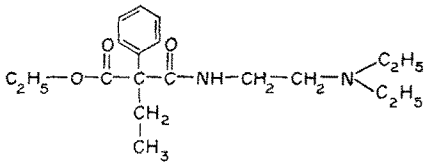
The rate of inhibitory reaction was determined for three representative compounds, SKF 525A (1 μM), Lilly 18947 (2 μM) and imipramine (1 μM). ACh concentration



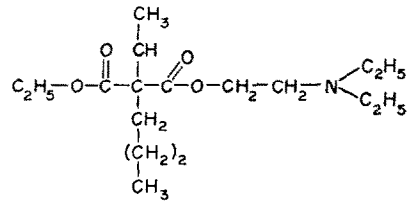
SKF 525A (pI50=6·5)



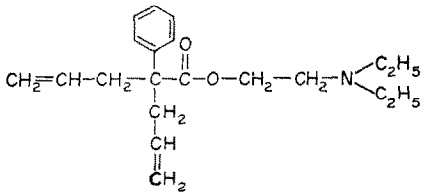
Sch 5705 (pI50=5·0)



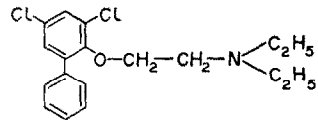
Sch 5706 (pI50=5·3)



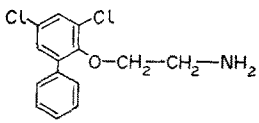
Sch 5712 (pI50=5·0)



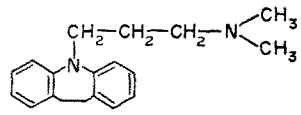
CFT 1201 (pI50=6·0)



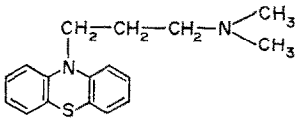
LILLY 18947 (pI50=6·0)



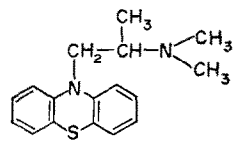
DPEA (pI50=4·7)



IMIPRAMINE (pI50=6·2)



PROMAZINE (pI50=6·3)



PROMETHAZINE (pI50=6·3)

FIG. 1. Structure and anti-ChE activity of some inhibitors of drug metabolism. Different concentrations of the compounds were incubated with ChE for 5 min at pH 7·2 and 30°. Then ACh was added to a final concentration of 1 mM and activity recorded. The inhibitory activity is shown between brackets and is expressed as the negative logarithm of the concentration inhibiting 50 per cent of the enzyme activity (pI_{50}).

was 1 mM, and it was present in the incubation mixture together with the enzyme before the addition of the inhibitor, but percentage of inhibition is independent of the order of adding substrate or inhibitor to the enzyme. The inhibition was very rapid (a period of time smaller than the one needed by the titrigraph to do the initial adjustment of pH) and remained constant for at least 30 min. Results were: SKF 525A, 86 per cent initial inhibition, after 30 min 86 per cent; for Lilly 18947, 67 per cent initial inhibition, after 30 min 67 per cent; for imipramine, 73 per cent initial inhibition, after 30 min 73 per cent.

Effect of dialysis on inhibition by SKF 525A, Lilly 18947 or imipramine. If after incubating the different inhibitors with the enzyme the reaction mixture is subjected to dialysis in the cold (4° for 24 hr) and then ACh is added (1 mM), no inhibition is observed with these three compounds when comparison is made with undialyzed enzyme under the same experimental conditions.

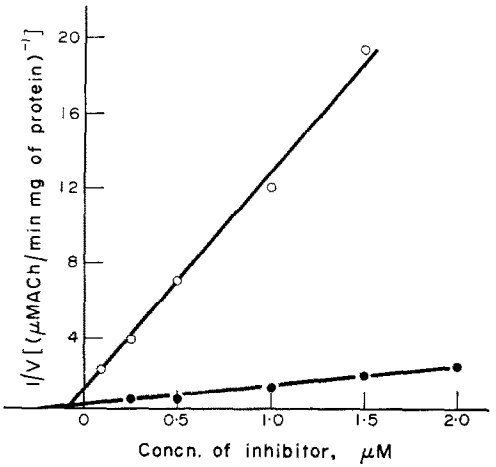


FIG. 2. Inhibition of ChE by different concentrations of SKF 525A. ChE solutions were incubated for 5 min at 30°, pH 7·2, with different concentrations of SKF 525A ranging from 0·1 to 2 μM. Then ACh was added at two different final concentrations, 0·25 mM and 2·5 mM. Activity was recorded and results expressed according to the method of Dixon.[5] —○—, 0·25 mM ACh, —●—, 2·5 mM ACh.

The results were: 1 μM SKF 525A, inhibited 87 per cent before dialysis and 0 per cent after dialysis; 5 μM Lilly 18947, inhibited 100 per cent before dialysis and 2 per cent after dialysis; 5 μM imipramine, inhibited 100 per cent before dialysis and 3 per cent after dialysis.

Effect of SKF 525A concentration on inhibition. SKF 525A concentrations ranging from 0·1 μM to 2 μM were allowed to interact with ChE during 5 min; then ACh was added at two different concentrations, 0·25 mM and 2·5 mM, and activity was recorded. The results plotted according to the method of Dixon[5] are shown in Fig. 2. The inhibition was competitive. The K_i was 0·08 μM.

Effect of substrate concentration on inhibition. Since the method described by Dixon[5] has the disadvantage that quite small experimental errors may lead to large changes in the estimate of K_i,[6] we decided to test the effect of SKF 525A, Lilly 18947 and

imipramine at fixed inhibitor concentrations (1 μM for SKF 525A; 2 μM for Lilly 18947, and 1 μM for imipramine) on enzyme activity at various ACh concentrations ranging from 0·5 mM to 2·5 mM.

Results illustrated in Fig. 3 correspond to the double reciprocal plots according to the method of Lineweaver and Burk[7] of the values found when SKF 525A is the inhibitor. The inhibition was competitive and the K_i (SKF 525A) was 0·11 μM. Lilly 18947 and imipramine behave similarly, their enzyme-inhibitor dissociation constants being: K_i (Lilly 18947) = 0·79 μM and K_i (imipramine) = 0·25 μM.

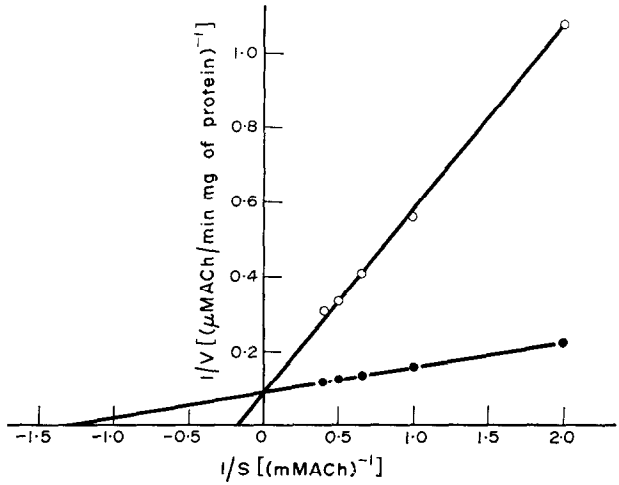


FIG. 3. Competitive inhibition of ChE by SKF 525A. Enzyme and inhibitor (final concentration, 1 μM) were incubated for 5 min at 30° and pH 7·2. Then ACh was added to a final concentration ranging from 0·5 to 2·5 mM and activity recorded. Results are expressed according to the method of Lineweaver and Burk.[7] —●—, Control; —○—, inhibition.

A competitive type of inhibition was also observed with SKF 525A (concentration 1 μM) using benzoylcholine (BzCh) as substrate (concentration range, 0·25 μM to 1 mM). The K_i obtained in this experiment was 0·12 μM, virtually the same value observed when ACh is the substrate. This result and that obtained in the Dixon plot indicate that the inhibition is purely competitive and exclude the possibility of an inhibition like the one called by Dixon and Webb[8] partially competitive, in which there is an EIS complex able to give products.

Inhibition of ChE by DDVP in the presence of inhibitors of drug metabolism. ChE was incubated for 5 min with either 2 μM SKF 525A, 10 μM Lilly 18947, or 5 μM imipramine and then 0·5 μM DDVP was added to the reaction mixture. At 5, 15 and 30 min aliquots were taken and ACh was added to a final concentration of 20 mM (which is thirty times the K_m for ACh). This concentration overcomes the competitive inhibition by the inhibitors of drug metabolism and allowed measurement of the DDVP action.

The results shown in Table 1 indicate that only SKF 525A delays the irreversible inhibitory action of DDVP.

Effect of pH on inhibition. The effect of pH on inhibition by SKF 525A, Lilly 18947 and imipramine was studied by incubating the compounds and ChE at different pH

TABLE 1. EFFECT OF SKF 525A; LILLY 18947 OR IMIPRAMINE ON THE INHIBITION OF ChE BY DDVP*

Compounds in the incubation mixture	Percentage inhibition at different periods of time		
	5 min	15 min	30 min
SKF 525A	47	47	47
DDVP	42	74	91
SKF 525A + DDVP	42	67	66
Lilly 18947	59	59	
DDVP	39	75	
Lilly 18947 + DDVP	85	90	
Imipramine		29	29
DDVP		66	82
Imipramine + DDVP		83	100

* ChE was incubated during 5 min with 2 μM SKF 525A; 10 μM Lilly 18947, or 5 μM imipramine, and then 0·5 μM DDVP was also added to the reaction mixture. At 5, 15 and 30 min, aliquots were taken and ACh was added to a final concentration of 20 mM and activity recorded. During all the process the temperature was held at 30° and the pH at 7·2.

values from 5·5 to 10·0 for 5 min and then assaying the activity at the same pH. The concentrations of the inhibitors were: 1 μM for SKF 525A; 10 μM for Lilly 18947; and 10 μM for imipramine. ACh was used at the final concentration of 1 mM. In Fig. 4 (a) and (b) are shown the graphs of the normal curve showing the pH dependence of activity for ChE as well as the percentage of inhibition by SKF 525A against pH (both determinations were performed employing freshly made ChE solutions). As can be seen, the curve showing percentage of inhibition against pH follows the same pattern as the one of activity against pH, both evidencing a maximum at pH 7·0–8·0.

It is interesting to point out, however, that the pH activity curve shown in Fig. 4 (a) is obtained only with freshly prepared enzyme solutions and that solutions which are several days old but have essentially the same or higher activity do not follow the same pattern in the alkaline pH region. In these preparations, activity not only does not decline but sometimes slightly increases at pH values higher than 7·5 [see Fig. 5 (a)]. These experiments may explain why so many contradictory results are found in literature for the pH activity curve in the alkaline region. The reason may be that during the storage of the enzyme or during its purification some changes in the protein structure would occur which while not affecting significantly the total enzyme activity, alter its response at alkaline pH. However, the curve of percentage of inhibition against pH with these enzyme preparations also declines after pH 7·5. An example of this kind of experiment obtained with 1 μM SKF 525A as inhibitor is shown in Fig. 5 (b).

Since the use of older ChE preparations does not modify the shape of the pH percentage of inhibition curves, we studied the effect of pH on the percentage of

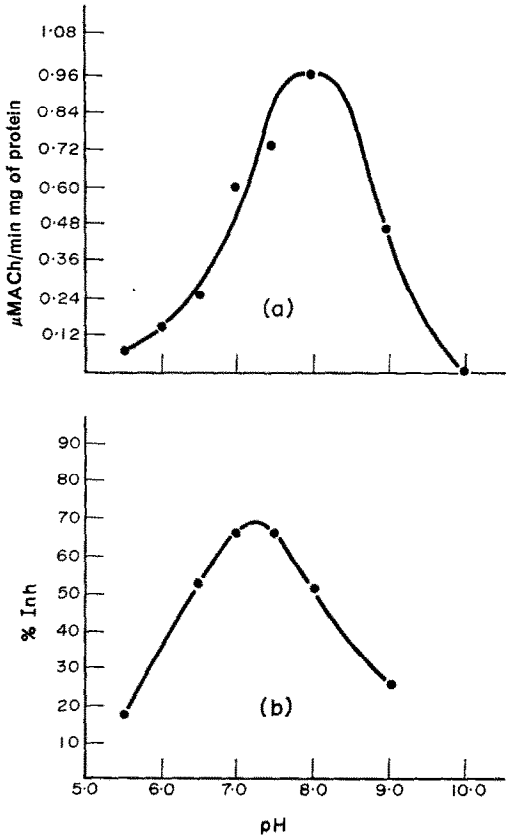


FIG. 4. pH-Dependence of ChE activity and ChE inhibition by SKF 525A. SKF 525A, 1 μM, was incubated with a fresh preparation of ChE for 5 min at the given pH, then ACh was added (final concentration, 1 mM). (a) Variation of ChE activity with pH; (b) variation of the percentage of inhibition of ChE by SKF 525A with pH. All the determinations were performed at 30°. Similar results were obtained with Lilly 18947 (10 μM) and imipramine (10 μM).

inhibition by Lilly 18947 and imipramine, Fig. 6 (a) and (b), with them. The possibility of working at pH 10 allowed us to have better estimations of the pK of the group being ionized in the enzyme at alkaline pH.

Since the decrease in the percentage of inhibition observed at the alkaline pH could be interpreted as a reduction in the amount of inhibitor caused by destruction or precipitation of the inhibitor at alkaline pH, we evaluated the significance of these factors in the results. In one experiment, the percentage of inhibition obtained when the pH is held at 7·5 during incubation with 1 μM SKF 525A and also after ACh addition (final concentration, 1 mM) was compared with that resulting if incubation with inhibitor is made at pH 9·0, and then the system is brought to pH 7·5 and activity recorded. The results were: for the process carried out totally at pH 7·5, 57 per cent inhibition; for the process with previous incubation at pH 9·0, 52 per cent inhibition.

As can be seen from the above-mentioned experiments, no important destruction occurred during the short periods of time required for incubation and recording of

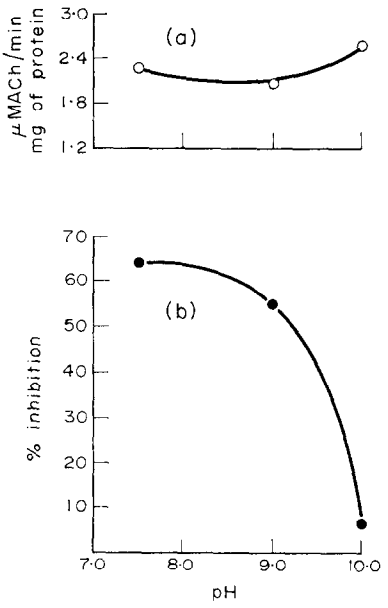


FIG. 5. pH-Dependence of ChE activity and ChE inhibition by SKF 525A using "aged" ChE solutions. Conditions were the same as in Fig. 4, but using "aged" ChE solutions and recording activity only in the alkaline zone. (a) Variation of ChE activity with pH; (b) variation of the percentage of inhibition by SKF 525A with pH.

activity. In support of this result, spontaneous liberation of acidity by SKF 525A at alkaline pH, whether it is in presence or in absence of the enzyme preparation, is not significant in short periods of time. They are important, however, with some of the compounds of the Sch 5705 type.

In order to know if precipitation occurs at the alkaline region, we have measured the absorbance at 660 mμ of the incubation mixtures immediately after activity recording at the given pH. We could not observe precipitation with 10 μM SKF 525A; 10 μM Lilly 18947; or 10 μM imipramine in the range of pH from 5 to 10. However, higher concentrations of SKF 525A and Lilly 18947 (e.g. 100 μM) precipitate at alkaline pH. Supporting these experiments, we also found that the same type of percentage of inhibition pH curves is obtained if the medium contains methylcellosolve in amounts as high as 20 per cent, conditions during which the basic form of SKF 525A does not precipitate. All these above-mentioned observations demonstrate that none of the pH percentage of inhibition curves obtained for ChE is affected by any of these factors.

Since the correct interpretation of pH percentage of inhibition curves requires the knowledge of the pK of the three compounds, we have measured them according to the directions given in methods. The values were: SKF 525A, 6·7; Lilly 18947, 6·7; and imipramine, 7·5.

Action of SKF 525A on AChE. AChE is also inhibited by SKF 525A. For example, a 10 μM concentration of this compound inhibits ChE 100 per cent and AChE only 30 per cent (ACh final concentration, 0·5 mM). This difference may arise in part from

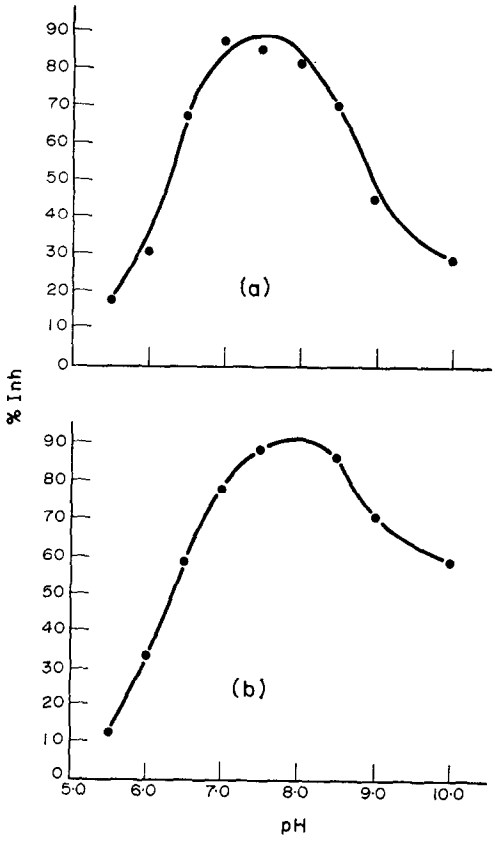


FIG. 6. pH-Dependence of ChE inhibition by Lilly 18947 and imipramine. Experimental conditions are the same as in Fig. 4, except that "aged" ChE solutions were employed. (a) Variation of the percentage of inhibition of ChE by 10 μM Lilly 18947 with pH; (b) variation of the percentage of inhibition of ChE by 10 μM imipramine with pH.

the greater affinity for ACh exhibited by AChE compared with that shown by ChE but also from the smaller affinity for the inhibitor shown by AChE.

Figure 7 presents Lineweaver–Burk plots of the results of studies of inhibition at different ACh concentrations. SKF 525A concentration was 100 μM and ACh concentration ranged from 0·06 mM to 1 mM.

It is clear that the inhibition is competitive. The K_i was 11·7 μM. The dependence of AChE activity and percentage of inhibition on the pH is shown in Fig. 8 (a) and (b); (SKF 525A concentration, 100 μM; ACh concentration, 0·12 mM). It can be observed that there is a maximum of inhibition at pH 7·0 to pH 8·0 as for ChE, but in contrast to the results obtained with that enzyme, inhibition at pH 5·5 does not decline; moreover, there seems to occur a slight increase in inhibition.

Since as it was mentioned above that SKF 525A at higher concentrations precipitates at alkaline pH, we evaluated the incidence of precipitation in our results. A 100 μM solution of SKF 525A was adjusted at different pH values from 7·0 to 10 and then filtered through a double layer of Whatman 3 MM filter paper. This operation was made under a nitrogen atmosphere to prevent changes in pH because of the carbon

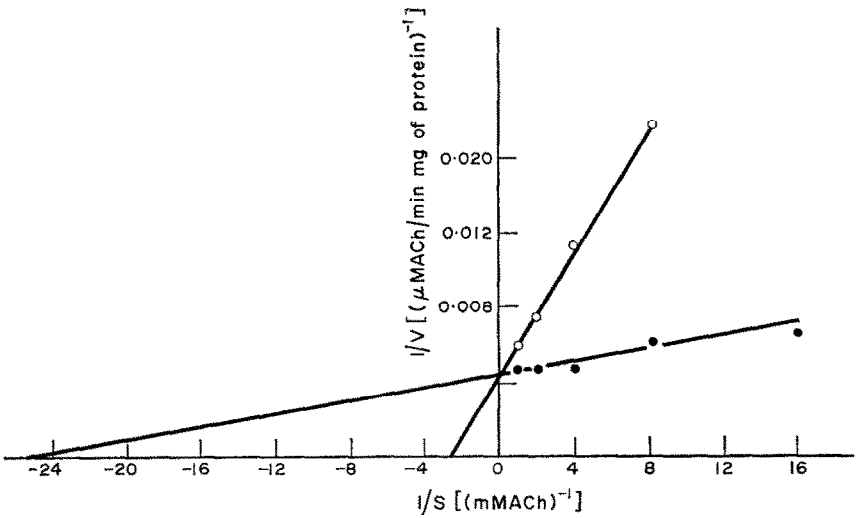


FIG. 7. Lineweaver–Burk plot for the inhibition of AChE by SKF 525A. Enzyme and inhibitor, 100 μM, were incubated for 5 min at 30° and pH 7·2. Then ACh was added at final concentrations ranging from 0·06 to 1 mM and activity recorded. Results were plotted according to Lineweaver and Burk.[7] —●—, Control; —○—, inhibition.

dioxide from air. After adjusting the acidity to pH 2, absorbance at 220 mμ was measured. A 100 μM standard solution of SKF 525A was run simultaneously. Precipitation was negligible at pH 7·2 and at pH 10 reached a value of 15·3 per cent of the total amount of SKF 525A. This small decrease excludes the possibility that precipitation was responsible for the significant changes observed at the alkaline region.

Effect of temperature on the inhibition by Lilly 18947. The kinetic constants for the inhibition of ChE by Lilly 18947 were measured at two different temperatures, 20° and 30°. The other experimental conditions were like those described in Fig. 3, except that the inhibitor concentration was 1·5 μM. The pK_i at 20° was 5·64 and at 30° was 5·74. By application of the equations described by Webb,[10] the value for the change in enthalpy and entropy was calculated. The change in enthalpy for the inhibition was −4·21 kcal and the entropy change was 40·34 e.u. (entropy units).

DISCUSSION

Several compounds having well-established properties as drug metabolizing enzyme inhibitors were tested for depression of ChE activity. All were found to inhibit the enzyme at rather low concentrations, suggesting that some specific interaction might be involved.

Our experiments on the inhibitory action by SKF 525A, Lilly 18947 and imipramine show that these compounds act very rapidly, reversibly and competitively, and that the inhibition is constant for at least 30 min. The degree of inhibition is independent of the order of addition of substrate and inhibitor to the enzyme.

Of particular interest was the finding that the inhibition of ChE by SKF 525A, Lilly 18947 and imipramine was competitive. This indicates that the inhibitor competes for the same site on the enzyme as does the substrate. This is really not surprising

since SKF 525A is an ester structurally very similar to ACh. However, the same argument cannot be used to explain the behavior of Lilly 18947 (and presumably DPEA) and even less the one of imipramine (and presumably promethazine and promazine) since the former lacks an ester bond and the latter lacks even the oxygen linked to the dialkylaminoethyl group.

Inspection of the structures of all the compounds tested (see Fig. 1) suggests that the R_2-aminoethyl portion of the molecules may have an important role in their inhibitory power since it is present in all of them. Consideration of the effect of pH on enzyme activity and on the percentage of inhibition of enzyme activity by SKF 525A, Lilly 18947 and imipramine lends support to this concept. Several authors have previously observed changes in pH during the enzymatic hydrolysis of ACh by ChE.[9] From those and other studies[11–13] arises the possibility of the existence in the enzyme active site of an esteratic site having a hydroxyl group from serine and an imidazol group from histidine, as well as an acidic group presumably being a hydroxyl group from tyrosine.

The increase in the percentage of inhibition produced by SKF 525A, Lilly 18947 or imipramine in the range of pH from 5·5 to 7·5 clearly indicates that the cationic groups present in inhibitors do not play an important role since the percentage of cationic form of SKF 525A present at pH 5·5 is 94 per cent and only 13·7 per cent at pH 7·5. Similar conclusions can be attained for Lilly 18947 and imipramine since their

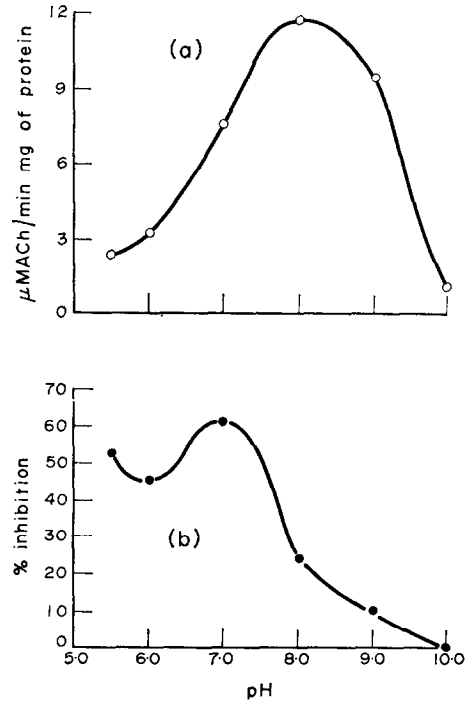


FIG. 8. pH-Dependence of AChE activity and AChE inhibition by SKF 525A. The inhibitor at a final concentration of 100 μM was incubated with AChE for 5 min at pH 7·2 and 30°; then ACh was added (final concentration, 0·12 mM) and activity recorded. (a) Variation of AChE activity with pH; (b) variation of the percentage of AChE inhibition by SKF 525A with pH.

pK values are not too far from that value (6·7, 6·7 and 7·5 respectively). This supposition is reinforced by our results with AChE. If the cationic form of the inhibitor were really an important factor, AChE should be far more sensitive to these inhibitors (and this showed not to be the case) because it is well known that the plasma enzyme has an affinity for cations which is only about 7 per cent of the one exhibited by the AChE.[9] On the other hand, our results with the AChE enzyme at different pH values (Fig. 8) show that the cationic group in SKF 525A might have an important action at the very acid portion of the curve.

From these considerations we assume that two possibilities exist: either the main place of interaction between enzymes and inhibitors is the esteratic site, or the interaction is located near or at an anionic site. Our experiments in which we looked for a delaying effect by SKF 525A, Lilly 18947 or imipramine on the inhibition of ChE by DDVP were very useful in answering these questions. It has been well established that DDVP, as well as all the other organophosphorus esters with anticholinesterase activity, irreversibly inhibit the enzyme by interaction with the esteratic site.[11] Consequently, any other reversibly bound inhibitor with greater affinity for the esteratic site will delay the inhibition by DDVP.

In our experiments with SKF 525A, Lilly 18947 and imipramine, only the first compound delayed the inhibition. This may indicate that only SKF 525A (and probably also the compounds having an ester bond in the molecule) would be attached to the esteratic site through the acyl group to the alkyl phosphate-binding site. The other compounds should have to be entirely bound to the enzyme anionic site or to a place close to it through the tertiary amine portion of their molecules.

Our experiments on the inhibition of AChE are in agreement with this possibility. Firstly, the groups involved in the interaction on both enzymes seem to be the same; this conclusion is reached from the similarity in the pH of maximum inhibition and the decline in inhibition in the alkaline zone.

On the other hand, the affinity of SKF 525A for AChE is far smaller than the one it has for ChE (the K_i for AChE is 11·7 μM and the K_i for ChE is 0·11 μM); this implies that the same groups have more difficulty to interact in AChE than in ChE. This striking difference might arise from the presence at the AChE active site of two anionic sites;[14] these groups are responsible for the higher affinity for cations shown by AChE when compared with that exhibited by ChE, which has only one of these groups at the active site.[14] The presence of this additional negatively charged group at the anionic site of AChE might very well reduce the affinity of a neighbour chemical group in interacting with an electron rich portion of SKF 525A. That is, since there are only quantitative differences between the competitive action of SKF 525A on both enzymes and there are only marked differences in one portion of their enzyme active site, the place of interaction should have to be the one where the difference is localized, the anionic site. The same arguments are valid if one assumes that AChE has one anionic site and ChE none.[14]

In the ester type of inhibitor, the interaction on the anionic site is also the most important, but the extra binding to the esteratic site would provide further affinity for the enzyme, as can be derived from the smaller K_i obtained with SKF 525A than with Lilly 18947 or imipramine.

Supporting the proposed mechanism, it can be seen that a replacement of an oxygen in Sch 5705 for a less electron attracting nitrogen atom to give Sch 5706 is

accompanied by an increase in inhibitory power, probably because it allows more negative charge remaining at the level of the dialkylamino nitrogen group.

Further evidence can be obtained by comparing DPEA with Lilly 18947 since the two inhibitors are reasonably alike in properties and size. In this case the difference in binding energy may be related to the different interactions of the basic portions of their molecules on the enzyme (see structures in Fig. 1).

The application of the equation described by Webb[15] to our values gave a difference in their interaction energy of −1·79 kcal/mole. The increased binding of Lilly 18947 over DPEA could be accounted for on the basis of greater dispersion energy, but it is more likely because of the increase in basicity of the nitrogen atom produced by the positive inductive effect of ethyl groups.

The differences in structure between promazine, promethazine and imipramine do not lead to changes in affinity.

In considering the nature of the chemical group of the enzyme active site interacting with the R_2-aminoethyl portion of the inhibitors, we do not know very precisely its identity. Notwithstanding, it is possible to anticipate some of its characteristics; one is that it should be at the anionic site or very close to it. Another derives from the curves of inhibition at different pH. The decrease in the percentage of inhibition in the alkaline region, when all these inhibitors are in the basic form, indicates that some group in the enzyme active site undergoes ionization to a form unable to interact with the inhibitors. From the curves of percentage of inhibition against pH for SKF 525A, Lilly 18947 and imipramine, an estimative value for the pK of that group can be obtained (about 8·6–9·0).

The supposition that the interaction between enzymes and inhibitors involves a proton donor group from the enzyme and a basic nitrogen atom from the inhibitor is supported by our experiments on the changes in the pK_i for Lilly 18947 with temperature. The entropy change accompanying the process of inhibition was 40·34 e.u. As pointed out by Laidler,[16] markedly positive values like the one we obtained are evidence that oppositely charged groups are interacting and displacing water of hydration. However, since our entropy value exceeds the magnitude of the results expected when only an ion neutralization occurs,[17] we assume that in addition there is a considerable structural change occurring during the process of formation of the enzyme–inhibitor complex.

Acknowledgements—We thank Dr. David Jollow from the Laboratory of Chemical Pharmacology (N.I.H., U.S.A.) for improving the English of our original manuscript, and to the Organic Chemistry Division of our Institute for their gift of a sample of purified DDVP.

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