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KINETICS OF INHIBITION OF ACETYLCHOLINESTERASE BY SPIN LABELED ACETYLCHOLINE ANALOGS

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A series of spin labeled acetylcholine analogs, in which the number of methylene groups between the quaternary nitrogen and the alcohol oxygen ranged between 1–5, have been examined as inhibitors of electric eel acetylcholinesterase. Evidence is presented suggesting that inhibition of acetylcholinesterase by the spin labeled ACh analogs is due to the high affinity of these compounds for the enzyme, inhibition is competitive and reversible. It has been shown that complex formation is of major importance in the reaction between spin labeled ACh analogs and acetylcholinesterase. The acetylation step has been shown to occur by demonstrating that the leaving group is released as the reaction proceeds. Complex formation has been demonstrated by means of kinetic criteria. Kinetic parameters have been measured for the five compounds, and correlations with alkaline hydrolysis are discussed.

1. Introduction

Two separate active sites can be distinguished on the active surface of acetylcholinesterase: one, an anionic site, is largely concerned with specificity, and the other, the esteratic site, is concerned with hydrolytic processes [1–4]. The principle forces of interaction between acetylcholinesterase and substrates are: (1) Coulombic attraction and Van der Waals' dispersion forces between the negatively charged anionic site and positively charged substrates; and (2) a weak covalent bond between the hydroxyl group of a serine amino acid residue at the esteratic site and the electrophilic carbonyl carbon atom of the ester. In addition Belleau and LaCasse [5] concluded that the environment of the anionic site is hydrophobic and that bulky substituents on the choline nitrogen atom would induce nonspecific molecular perturbations of the enzyme, leading to the compound under examination being an inhibitor. Several workers have shown that, apart from the small hydrophobic portions situated directly in the anionic center (and probably adapted to the nitrogen methyl groups of acetylcholine), there are more extensive hydrophobic regions beyond the anionic site (which adapt to bulky hydrophobic groups) [6–10].

Kitz and Wilson [11], Main [12] and O'Brien [8] have demonstrated the importance of prior complex formation in the reaction of organophosphates, sulfonates and carbamates with acetylcholinesterase.

In order to better understand the nature of the active sites of acetylcholinesterase and acetylcholine receptors, we have been engaged in the synthesis [13], pharmacology [14] and biochemistry [10] of a series of spin labeled acetylcholine analogs. The purpose of this report is to study the mechanism of action of a series of spin labeled probes. This mechanism is of interest in itself, as it bears upon the steric requirements for interaction at the anionic site of acetylcholinesterase and because inhibition may involve functional groups outside the active site.

2. Materials

Acetylcholinesterase (AChE) (*Electrophorus electricus*, EC 3.1.1.7) was a lyophilized salt free powder and contained 340 μmolar units per mg solid

(Sigma type VI); acetylcholine iodide (ACh); acetylthiocholine iodide (ATCh); and Ellman's reagent, [5,5'-dithiobis-(2-nitrobenzoic acid)], (DTNB) were obtained from Sigma Chemical Co., St. Louis, Missouri, USA.

Spin labeled ACh analogs, in which the distance between the nitrogen and the alcohol oxygen was 1–5 carbon atoms, were prepared as previously reported [13]. The compounds are: 2-[N,N-dimethyl-N-(2,2,6,6-tetramethylpiperidine-1-oxylamino)] methyl acetate iodide, (SL-1); 2-[N,N-dimethyl-N-(2,2,6,6-tetramethylpiperidine-1-oxylamino)] ethyl acetate iodide, (SL-2); 2-[N,N-dimethyl-N-(2,2,6,6-tetramethylpiperidine-1-oxylamino)] propyl acetate iodide, (SL-3); 2-[N,N-dimethyl-N-(2,2,6,6-tetramethylpiperidine-1-oxylamino)] butyl acetate iodide, (SL-4); and 2-[N,N-dimethyl-N-(2,2,6,6-tetramethylpiperidine-1-oxylamino)] pentyl acetate iodide, (SL-5).

3. Methods

3.1. Assay of acetylcholinesterase activity

Inhibition of acetylcholinesterase was examined by the method of Ellman et al, [15] by using a Varian Techtron Model 635 UV-Vis Spectrophotometer. Measurements were made at 37°C by observing ATCh hydrolysis at 412 nm using DTNB. In a typical experiment, the reaction mixture contained 10^{-4} M ATCh, 10^{-4} M DTNB, 0.1 M NaCl, 0.02 M $MgCl_2$, 0.02 M sodium phosphate buffer at pH 7.0 and 0.1 μg enzyme (0.033 μmolar units) in a final volume of 4.0 ml.

3.2. Enzymatic hydrolysis of SL-2

Enzymatic hydrolysis of SL-2 by electric eel AChE was carried out by incubating 68 units of the enzyme (800 times the enzyme concentration used in regular assay for AChE activity), with 1 μmol of SL-2 in a volume of 1 ml of pH 7.4 phosphate buffer containing 0.1 μmol of NaCl and 0.02 μmol of $MgCl_2$ at 38°C. At various times, the reaction was stopped and the concentration of unreacted SL-2 was determined as the ferric-acethydroxamic acid complex [16].

3.3. Alkaline hydrolysis of ACh and analogs

The choline ester was made up in distilled water at 10 mM and 0.1 ml was added to 0.1 ml of 20 mM NaOH and diluted to a final volume of 1 ml at 38°C. At various time intervals, the reaction was stopped and the concentration of unreacted starting material was determined as the ferric-acethydroxamic acid complex [16].

4. Results

The spin labeled ACh analogs (SL-1 through SL-5) inhibited electric eel AChE. Preliminary results indicated that the residual enzymatic activity was a function of the concentration of the spin labeled probe [10].

The spin labeled ACh analogs were examined by conventional methods to test for competitive inhibition. Lineweaver–Burke plots for the inhibition of AChE by the spin labeled ACh analogs were obtained (fig. 1). The results clearly demonstrate that the inhibition is reversible and competitive. From the double reciprocal plots, the values of K_m and K_i were determined. The reversible inhibition constant, K_i, for the inhibition of AChE by spin labeled ACh analogs were also determined by eq. (1), which is based on an equilibrium assumption as well as the Briggs–Haldane modification of the Michaelis–Menten law [17].

$$v_0/v = 1 + [I]/K_i(1 + [S]/K_m)\,. \qquad (1)$$

In this expression, v_0/v is the ratio of the uninhibited to inhibited rate of hydrolysis, and K_m (2.5×10^{-4}M) was obtained from the hydrolysis of ATCh by AChE [18]. Since the equation does not apply to inhibition in the presence of excess substrate, less than optimal substrate concentrations were used. When v_0/v is plotted against [I], a linear relationship is obtained in which the x-axis intercept gives:

$$-K_i(1 + [s]/K_m)\,.$$

The effect of varying the inhibitor concentration was determined at two concentrations. The results for SL-2 are shown in fig. 2. Since the inhibition varies with the substrate concentration, the equilibrium is largely competitive. The K_i values at the two substrate concentrations agreed to within 3%. Similar results

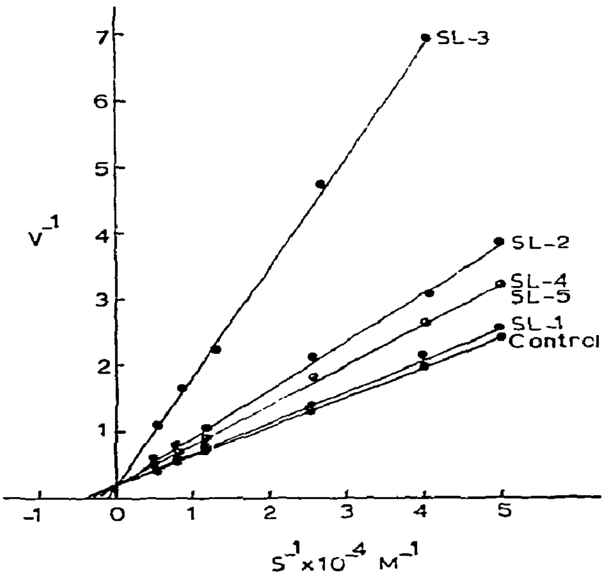


Fig. 1. Lineweaver–Burke plots for the inhibition of electric eel AChE (38°C) by spin labeled ACh analogs (2.5×10^{-4} M); ATCh concentration 10^{-4} M.

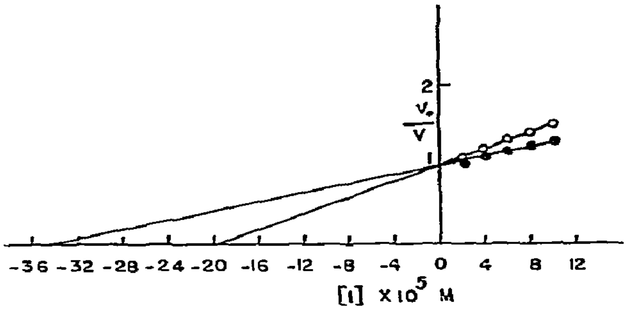
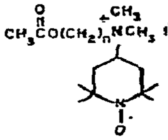


Fig. 2. Inhibition of electric eel AChE (38°C) by spin labeled ACh analog SL-2; ATCh concentrations 10^{-4} M (●) and 2.5×10^{-4} (○).

were obtained for the other spin labeled ACh analogs and their inhibition constants are listed in table 1. The results show that lengthening of the carbon chain separating the alcohol oxygen and the quaternary nitrogen was accompanied by an increase in anticholinesterase activity. The increase in inhibitory potency was observed only up to the propyl radical (SL-3). Once this cut-off point was reached, further lengthening of the alkyl chain (SL-4) decreased anticholinesterase activity. Further increasing the alkyl chain to five carbons (SL-5), did not change the K_i for AChE.

If inhibition of AChE by this series of compounds is due, in part to the electrophilicity of the carbonyl carbon atom, it might be expected that their inhibitory potency would parallel their stability towards alkaline hydrolysis. Table 2 lists the pseudo first and second order rate constants for the base catalyzed hydrolysis of spin labeled ACh analogs. The results indicate that there is no correlation between the rate of hydrolysis of these compounds and their inhibition of AChE. Thus SL-1 which caused the least inhibition of AChE was least hydrolyzed by the base. Also SL-3, which was the most potent inhibitor of this series, had the fastest rate of hydrolysis, followed by SL-5, SL-4 and SL-2. It is noteworthy that the alkaline hydrolysis of SL-3 is very close to that of the substrate, ATCh.

Table 1
Inhibition constants (K_i), dissociation constants (K_a), acetylation constants (k_2), and bimolecular rate constants (k_i) for inhibition of electric eel acetylcholinesterase with a series of spin labeled acetylcholine analogs [a)]



Analog	n	K_i (M)	K_a (M)	k_2 (min^{-1})	k_i (M^{-1} min^{-1})
SL-1	1	$(2.56 \pm 0.1) \times 10^{-3}$	$(1.25 \pm 0.05) \times 10^{-3}$	0.10 ± 0.01	$(8.00 \pm 0.4) \times 10$
SL-2	2	$(3.34 \pm 0.2) \times 10^{-4}$	$(1.93 \pm 0.07) \times 10^{-4}$	0.13 ± 0.02	$(9.26 \pm 0.4) \times 10^2$
SL-3	3	$(3.70 \pm 0.2) \times 10^{-5}$	$(3.85 \pm 0.2) \times 10^{-5}$	0.33 ± 0.03	$(8.57 \pm 0.4) \times 10^3$
SL-4	4	$(5.93 \pm 0.25) \times 10^{-4}$	$(6.06 \pm 0.3) \times 10^{-4}$	0.25 ± 0.02	$(4.13 \pm 0.25) \times 10^2$
SL-5	5	$(6.00 \pm 0.3) \times 10^{-4}$	$(5.88 \pm 0.3) \times 10^{-4}$	0.25 ± 0.03	$(4.25 \pm 0.3) \times 10^2$

[a)] Results are given as average ± S.D.

Table 2
Pseudo-first order and second order rate constants for the alkaline hydrolysis of acetylcholine and analogs [a]

Compound	k_1 (min^{-1})	Relative k_1	k_2 ($M^{-1}\ \text{min}^{-1}$)	Relative k_2
ACh	0.40 ± 0.05	100	226 ± 15.0	100
ATCh	0.21 ± 0.01	53	108 ± 9.0	48
SL-1	0.08 ± 0.00	20	4 ± 0.4	2
SL-2	0.04 ± 0.00	10	19 ± 1.0	8
SL-3	0.18 ± 0.01	45	92 ± 6.0	41
SL-4	0.06 ± 0.00	15	30 ± 4.6	13
SL-5	0.10 ± 0.01	25	48 ± 4.6	21

[a] Results are given as average ± S.D.

In the subsequent examination of the kinetics of inhibition of AChE by spin labeled ACh analogs, SL-2 was chosen as a model compound, because it has the same number of carbon atoms between the active centers as does ACh. Fig. 3 shows that inhibition lines intercept the axis at points below 100% activity, i.e., the enzyme is inhibited at zero time. This must be taken to demonstrate the presence of complexed enzyme and inhibitor. At sufficiently high concentrations of inhibitor, the progressive inhibition lines become parallel (fig. 3) indicating saturation of the enzyme. This phenomenon is predicted by the kinetic scheme normally assumed to represent phosphorylation and carbamylation of AChE by phosphates and carbamates:

$$\begin{tikzcd} E + I \arrow[r, shift left, "k_1"] \arrow[rr, bend right, "k_i"'] & EI \arrow[l, shift left, "k_{-1}"] \arrow[r, "k_2"] & EI' \arrow[r, "k_3"] & E + \text{acid}, \quad (2) \end{tikzcd}$$

where E is the free enzyme, I is the inhibitor, EI is the intermediate reversible complex whose formation is controlled by the equilibrium constant, K_a ($= k_{-1}/k_1$), EI' is the carbamylated or phosphorylated enzyme whose rate is governed by k_2 and k_i ($= k_2/K_a$) is the rate constant governing the overall rate of inhibition (the bimolecular rate constant). To calculate acetylation constant, k_2, and the dissociation constant for enzyme inhibition complex, (K_a), one may employ eq. (3) relating progressive inhibition to time and inhibition concentration, as derived by Main [12] for the kinetic scheme given in eq. (2) assuming that $[I] \gg [E]$ and $k_{-1} \gg k_2$,

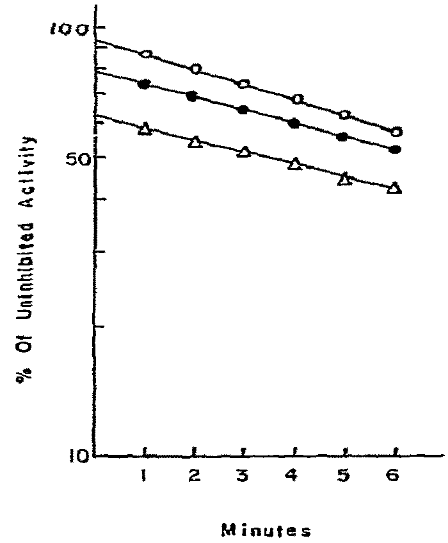


Fig. 3. Inhibition of electric eel AChE (38°C) by SL-2 at concentrations of 10^{-5} M (○), 10^{-4} M (●) and 10^{-3} M (△). Measurements were made at 37°C by observing ATCh hydrolysis at 412 nm in a reaction mixture containing: 10^{-4} M ATCh, 10^{-4} M DTNB, 0.1 N NaCl, 0.02 M $MgCl_2$, 0.02 M sodium phosphate buffer at pH 7.0 and 0.1 mg enzyme (0.033 μ molar units) in a final volume of 4.0 mL. Ordinate is on log scale.

$$1/[I] = (1/2.303)(dt/d\log V)(k_2/K_a) - 1/K_a, \quad (3)$$

where [I] is the inhibitor concentration; t is the time of incubation; $d\log V$ is the change, caused by inhibition, in velocity of the reaction. When $1/[I]$ is expressed as a function of $dt/d\log V$, a straight line is obtained in which the slope will be k_i; the y-axis intercept becomes $-1/K_a$ and the x-axis intercept is $1/k_2$. Intersection of the axis by the extrapolated line would support the assumption of a reversible intermediate, as well as providing a means of evaluating k_2, K_a and k_i. K_a in the case of reversible inhibitors is identical to K_i, the conventional expression for the reversible inhibition constant of E·I, the enzyme inhibitor complex.

The Main [12] equation is only applicable when the inhibition time is short enough so that the k_3 step can be ignored. Fig. 4 shows that in the present investigation, the reaction of SL-2 which AChE was judged by inhibition of the enzyme is linear over a short period of time (six minutes) and in every case the inhibition approaches an apparent equilibrium. Similar results were obtained for the other spin labeled ACh analogs. Fig. 5 shows representative data which demonstrate that the inhibition of AChE by SL-2 is first order. For all compounds tested, short time intervals were used, so that the subsequent leveling off does not interfere with the kinetic studies. Fig. 6 shows that the kinetic finding were indeed fully compatible with the Main equation. Thus one can evaluate K_a and k_2. Similar plots were obtained for all probes. Table 1 lists K_a (dissociation constant), k_2 (acetylation constant) and k_i (bimolecular rate constant) for each of the spin labeled ACh analogs, along with the conventional expression for reversible inhibition constant, K_i. These data show that SL-3 is the most effective inhibitor.

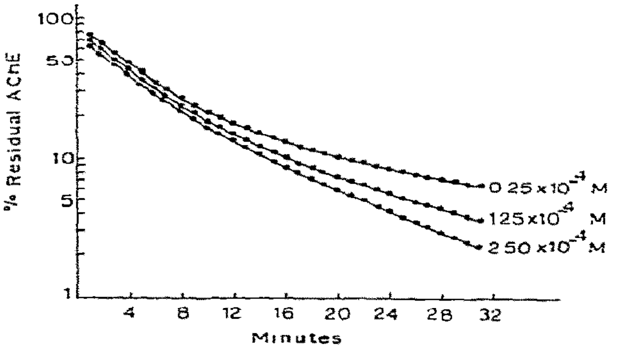


Fig. 4. Progress on inhibition of electric eel AChE (38°C) by SL-2 at various concentrations, to show achievement of steady state, ATCh concentrations 10^{-4} M. Assay conditions as described in fig. 3. Ordinate is on log scale.

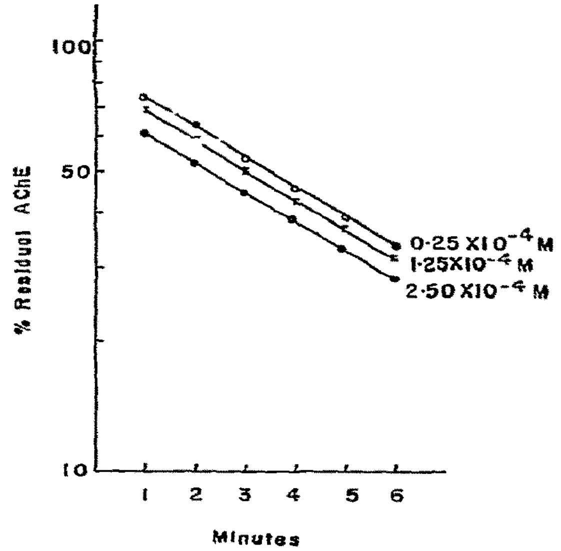


Fig. 5. Progress of inhibition of electric eel AChE (38°C) by SL-2, ATCh concentration 10^{-4} M.

The best direct evidence for the acetylation theory would be to prove that in the course of inhibition, there is indeed a leaving group, that is, the spin labeled choline analog. This is demonstrated by fig. 7 for

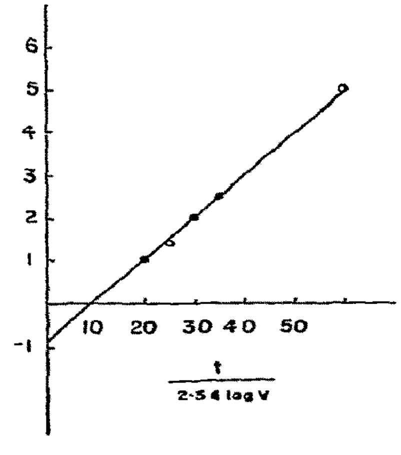


Fig. 6. Main plot for inhibition of electric eel AChE (38°C) by SL-2. ATCh concentration of 10^{-4} M.

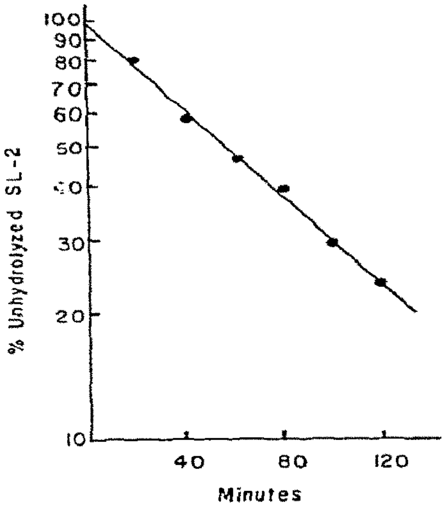


Fig. 7. Hydrolysis of SL-2 by large excess of electric eel AChE (38°C). Half-life from graph is 58 minutes, k_3 = 0.012 $\min^{-1}$. See methods for hydrolysis conditions.

SL-2, in which the concentration of the probe is in large excess of AChE. From fig. 7, one can calculate a k_3 of 0.0112 $\min^{-1}$. This value shows that k_3 is the rate determining step, since it is about a factor of ten smaller than k_2. Thus the acetylation of the enzyme takes place at a rate determined by k_2, and further reaction must await regeneration of the enzyme, which is determined by the slow k_3.

5. Discussion

Spin labeled ACh analogs inhibit AChE in a reversible manner as indicated by the characteristic time-independent and concentration dependent behavior of AChE inhibition. Lineweaver–Burke plots demonstrated the competitive nature of the inhibition [10].

The results of progressive inhibition studies with spin labeled ACh analogs at high concentrations demonstrate a saturation phenomenon, which may only be explained by the assumption that the acetylation step is proceeded by an inhibitor–enzyme complex. In order that the rate of progressive inhibition may reach the observed maximum, it must be assumed that a stage is reached in the incubation vessel at which time all available enzyme is complexed with inhibitor.

These observations make it reasonably certain then that inhibition of AChE by spin labeled ACh analogs follows a pathway similar to that normally assumed for inhibition by sulfonates [11], organophosphates [12] and carbamates [19], i.e., complexing is followed by acetylation. The third step, regeneration, takes place very slowly. Measurements of kinetic parameters showed progressive and large increases in affinity with lengthening of the carbon chain up to SL-3. The inhibition of AChE by these compounds is not due to the inhibition of the deacetylation step (k_3) by either the spin labeled ACh analog or the spin labeled choline analog. This conclusion was drawn from the observation that the ester would be hydrolyzed at low concentrations, which was not the situation. Spin labeled ACh analogs must, therefore be unable to acetylate the enzyme because of an unfavorable interaction in the first stage. Since there is no correlation between the acetylating ability of these compounds and the degree of inhibition of AChE, and since their hydrocarbon radicals are not capable of any specific interactions, the increase in the inhibitory potency with the lengthening of the alkyl chain can be attributed to the improvement of the conditions of absorption of the spin labeled ACh analogs onto the anionic portion of the active center. These facts are in accord with the hypothesis that there are on the surface of AChE other hydrophobic portions outside of the anionic center, or beyond it [6–9]. The inhibitory effect of these compounds can be explained if we assume the existence of some hydrophobic fold or pocket on the enzyme surface onto which the bulky piperidinoxyl group can be absorbed in a complimentary manner. The cut-off at SL-3, implies that the corresponding hydrophobic portion on the enzyme is of limited size. The piperidinoxyl group induces, non-specific molecular perturbations which involve hydrophobic bond formation [20]. EPR data have shown that this "hydrophobic anionic site" is on the surface of the enzyme and is either planar or a curvature of large radius [21].

The data described above suggest that spin labeled ACh analogs have poor (low) k_2 values and compensated by low K_a constants. Consequently, one would expect the inhibitor concentration to be close to the K_a, and therefore expect that the observed inhibition would depend markedly, on substrate concentration. The results support the application of the Main equation, and show that spin labeled ACh analogs are substrates for AChE, with high affinity, low acetylation rates and even lower deacetylation rates. One cannot neglect the complex formation step; on the contrary, virtually all the differences in anticholinesterase activity among the spin labeled probes were due to differences in complexing ability. It is concluded that the inhibition of AChE by the spin labeled analogs is due to rapid formation of the complex, EI, and slower conversion of EI to EI′ (with concomitant replenishment of EI from E and I). Spin labeled ACh analogs may, therefore, prove to be useful new tools for the study of the topography of the active center of the enzyme and receptor. The utility of these spin labeled compounds as probes may be difficult, however, because of their low dissociation constants. Bound labeled might be difficult to detect without large amounts of enzymes.

Acknowledgement

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References

[1] I.B. Wilson and F. Bergmann, J. Biol. Chem. 185 (1950) 479.
[2] I.B. Wilson and F. Bergmann, J. Biol. Chem. 185 (1950) 683.
[3] I.B. Wilson, J. Biol. Chem. 197 (1952) 215.
[4] F. Bergmann, I.B. Wilson and D. Nachmansohn, J. Biol. Chem. 186 (1950) 693.
[5] B. Belleau and G.J. LaCasse, J. Med. Chem. 7 (1964) 768.
[6] M.I. Kabachnik, Vest. Acad. Nauk SSSR 5 (1968) 86.
[7] P. Bracha and R.D. O'Brien, Biochem. 7 (1968) 1545.
[8] P. Bracha and R.D. O'Brien, Biochem. 7 (1968) 1555.
[9] P. Bracha and R.D. O'Brien, Biochem. 9 (1970) 741.
[10] M.B. Abou-Donia and G.M. Rosen, Int. J. Biochem. 6 (1975) 399.
[11] R. Kitz and I.B. Wilson, J. Biol. Chem. 237 (1962) 3245.
[12] A.R. Main, Science 144 (1964) 992.
[13] G.M. Rosen and M.B. Abou-Donia, Synth. Comm. 5 (1975) 415.
[14] G.M. Rosen, M.B. Abou-Donia, J.Z. Yeh and D.B. Menzel, Res. Comm. Chem. Path. Pharm. 12 (1975) 317.
[15] G.C. Ellman, K.D. Courtney, V. Andres and R. Featherstone, Biochem. Pharmacol. 7 (1961) 88.
[16] S. Hestrin, J. Biol. Chem. 180 (1949) 249.
[17] K.J. Laidler, The Chemical Kinetics of Enzyme Action (Oxford University Press, New York, 1958).
[18] H. Lineweaver and D. Burke, J. Amer. Chem. Soc. 56 (1934) 658.
[19] R.D. O'Brien, Mol. Pharmacol. 4 (1968) 121.
[20] B. Belleau, J. Med. Chem. 7 (1964) 451.
[21] M.B. Abou-Donia and G.M. Rosen, Int. J. Biochem. 7 (1976), in press.