INHIBITIONAL CHOLINESTERASC ENZYME ACTIVITY BY NEW DERIVATIVES OF PHENINDIONE[+]

Falah S.D. AL-Fartusie[*]

Abstract

This study was designed to show the effects of new derivatives of phenindione on the activity of ChE in sera. All compounds studied demonstrated inhibitory effects on the enzyme activity and these effects are increased with increasing the concentrations of the compounds. The compound 2-(3-Chloro propanoyl)-2-phenyl-1,3-indandione has the highest ability to inhibit ChE of other compounds. Kinetic properties of these inhibitors reveal that compounds (3,6,8 and 10) cause mixed type of inhibition, compounds (1,4,7 and 9) act as competitive inhibitors, compound (5) acts as a non-competitive inhibitor and compound (2) behaves as an un-competitive inhibitor. The mechanism of action of these types of compounds acting as inhibitors to the ChE is suggested.

المستخلص

يوضح هذا البحث تأثير مشتقات جديدة للفنيندايون في فعالية أنزيم الكولين أستريز في مصل الدم . أظهرت جميع المركبات المدروسة تأثير تثبيطي في فعالية الأنزيم وان قدرة هذه المركبات على تثبيط الأنزيم تزداد طرديا مع زيادة تركيزها. لقد وجد أن المركب ٢ -(٣ -كلورو بروبانويل)- ٢ -فنيل- ١،٣ -انداندايون يمتلك أعلى قابلية لتثبيط الأنزيم مقارنة ببقية المركبات . أظهرت دراسة الخواص الحركية لهذه المثبطات أن المركبات (١،٤،٧،٩) تسبب تثبيط من النوع التنافسي بينما المركبات (٣،٦،٨،١٠) تظهر تثبيط من النوع المختلط والمركب (٥) يسبب تثبيط غير تنافسي أما المركب (٢) فأنه يسبب تثبيط من النوع اللا تنافسي . أخيرا تم في هذا البحث اقتراح ميكانيكية توضح معقد (الأنزيم- المثبط) الذي يتكون بين أنزيم الكولين أستريز وهذا النوع من المركبات كمثبطات للأنزيم.

Introduction

The term cholinesterase (ChE) was proposed in 1932 by Stedman *et al.* to describe the enzyme which hydrolyzes acetylcholine and other choline esters at a more rapid rate than non-choline esters [1]. There are two main types of cholinesterase: (a) acetylcholinesterase, also called specific cholinesterase, true cholinesterase and acetylhydrolase of acetylcholine, is classified as [EC 3.1.1.7], for being enough but not absolutely specific for acetylcholine.

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* Chemistry Department, College of Science - Al-Mustansiriyah University.

And (b) butyrylcholinesterase, also named pseudocholinesterase and acylcholine acylhydrolase is,classified as [EC 3.1.1.8], this enzyme hydrolyzs not only acetylcholine but also several other esters of choline [2].

Cholinesterase is such an important enzyme especially in transporting the nerve signals from the central and peripheral nervous system. This enzyme has a vital function in the termination of synaptic transmission by hydrolysis of the neurotransmitter acetylcholine (after completing its function) into choline and acetate [3].

Cholinesterase is found in all excitable tissues whether nerve or muscle, central or peripheral, cholinergic or adrenergic, motor or sensory, in most erythrocyte and in placental tissue. Plasma cholinesterase is found much more widely in the central and peripheral nervous system, in most tissues especially the liver and, as its name implies, in the plasma [4].

Plasma cholinesterase is a complex molecule comprising four identical subunits, each subunit consisting of a polypeptide chain of 547 amino acids and nine carbohydrate chains. The molecular weight of each subunit is about 85000 Dalton. The structure of the tetramer, including the complete amino acids sequence and the whereabouts of the disulphide bonds, was determined by Lockridge *et al.* in1987 [5,6].

There are two active sites in cholinesterase known as the anionic site and the esteratic site [7]. It is this esteratic site which actually combines with the carbonyl group of the ester linkage (acetylecholine) and is responsible for the hydrolysis of the ester bond as shown in figure (1).

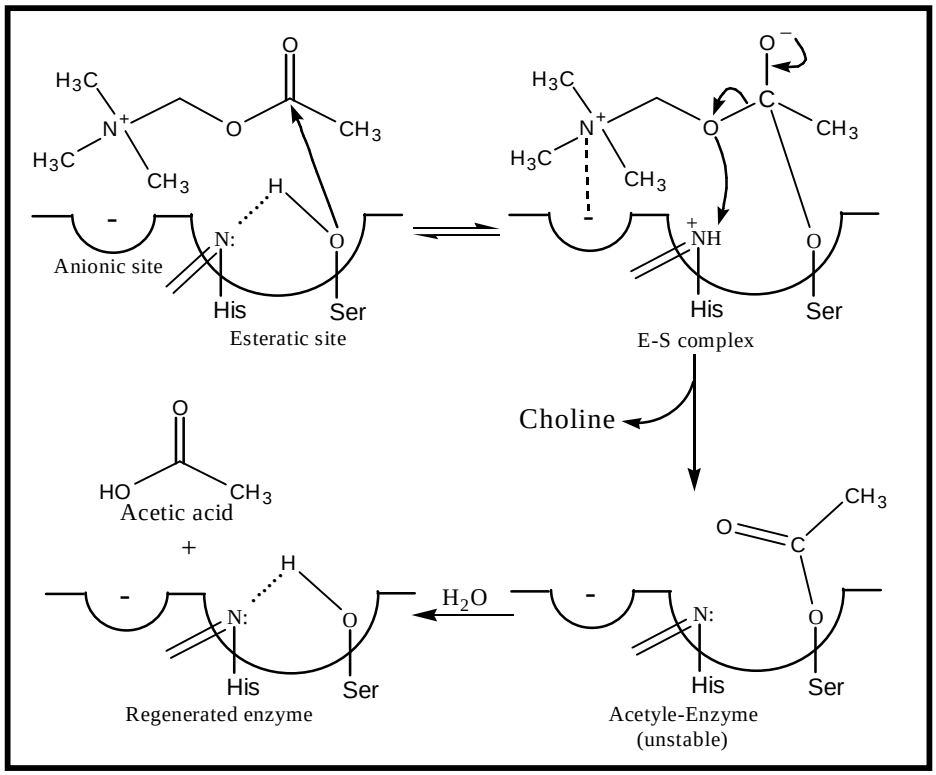


Fig. (1) : The steps involved in ChE-mediated hydrolysis of ACh.

Numerous substances act as ChE inhibitors, they are generally classified into reversible (Carbamate) and irreversible (Organophosphorous) most of these compounds are used as insecticides, pesticides or drugs [8]. The most toxic organophosphates are chemical warfare agents [9]. Some newer chemicals, such as thiosemicarbazone compounds [10], cis-diamminediaquaplatinum (II) [11] and some hetercyclic compounds [12] can also affect the cholinesterase enzyme.

The kinetics of cholinesterase with the inhibitors occurs in two stages as indicated in figure (2) which involves formation of a reversible enzyme-inhibitor complex (EH.IX) followed by formation of the enzyme inhibitor bond with displacement of the leaving group.

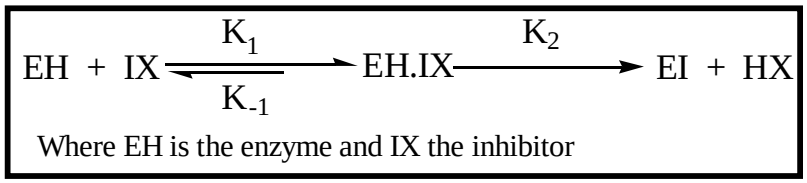


Fig. (2): Scheme shows the general mechanism of Inhibition of ChE.

Materials and Methods

Acetylcholinesterase activity was assayed by Ellman method [13]. The principle of the method is the measurement of the rate of production of thiocholine as acetylthiocholine is hydrolyzed. This was accomplished by the continuous reaction of the thiol with 5,5- Dithio bis-2-nitrobenzoic acid (DTNB) to produce the yellow color of 5- thio-2- nitrobenzoic acid. The rate of color production is measured at 430 nm.

Method

1) ChE activity was measured in human serum using the modified Ellman method [14] as follows:

(50 µl) of DTNB solution (0.001 M) is added to (2.25 ml) of sodium phosphate buffer solution (pH=7.3, 0.2M) , then (10µl) of serum is added , mixed well and (2 ml) of the mixture is transferred to a measuring cell (3 mm), then (34 µl) of acetylthiocholine iodide (ASChI 0.06 M) is added, the change in absorbency is measured before and after adding the substrate at (430 nm) for (3 min). The enzyme activity is calculated as the concentration in µmole of the substrate hydrolyzed to each (ml) of sample in (3 minute) and expressed as (µmole/ 3 min/ml).

2) A stock solution (0.1 M) concentration of each compound, table (1), is prepared and then the following concentrations of ($1X10^{-3}$, $5X10^{-4}$, $1X10^{-4}$, $5X10^{-5}$, $1X10^{-5}$) M are prepared. The different concentrations of the compounds are prepared by diluting with dimethyl sulfoxide (DMSO) solvent using the stock solution. ChE activity is measured in human serum as follows:

(50 µl) of DTNB solution (0.001M) is added to (2.25 ml) of sodium phosphate buffer solution (0.25 ml of inhibitor is mixed with 2 ml of buffer (pH=7.3, 0.2M)), then (10µl) of serum is added, mixed well and (2 ml) of the mixture is transferred to a measuring cell (3 mm), then (34 µl) of ASChI (0.06M) is added, the change in absorbency is measured before and after adding the substrate at (430 nm) for (3 min). The inhibition percentage is calculated by comparing the activity with and without the inhibitor and under the same conditions, according to the equation:

$$\% \text{ Inhibition} = 100 - \left(\frac{\text{The activity in the presence of inhibitor}}{\text{The activity in the absence of inhibitor}} \right) X\ 100$$

3) A constant concentration of inhibitor ($1X10^{-4}$ M) is used with different substrate concentrations of (0.02, 0.04, 0.06, 0.08, 0.09) M to study the type of inhibition. These different concentrations are prepared from the stock solution of (0.1 M) ASChI. The enzyme activity is determined with and without the inhibitor. Using the Lineweaver- Burk equation by ploting 1/V vs. 1/[S] [15]we calculate the following values: a) Ki . b) Apparent Vmax (Vmapp). c) Apparent Km (Kmapp). d) Type of inhibition.

Results and Discussion

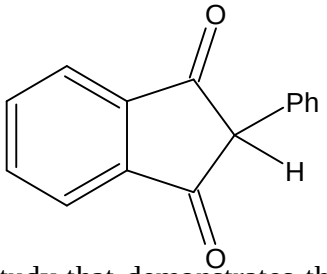
The physiological action and great usefulness of cholinesterase enzyme in the cholinergic transmission (this enzyme catalyzes the hydrolysis of chemical transmitter acetylcholine) make it essential target for a wide range of new enzymatic studies.

This research addresses investigatin of the effects of ten new derivatives of phenindione (2-Phenyl-1,3-indandione) table (1), which have significant biological and chemical effect [16], on human serum cholinesterase activity *in vitro*.

The biochemical tests revealed that all compounds cause (moderate to good) inhibitory effects on the enzyme activity, table (2), the normal value of the enzyme activity ranges between (4.70-6.63) μmole/3 min/ml.

The relationships between compounds concentrations versus the activity of enzyme are shown in figures (3 and 4). From these results it is observe that any increase in compounds concentrations causes increases in percentage of inhibition of enzyme. The greater inhibition of each compound is demonstrated at concentration (0.001 M) as shown in figure (5). From this figure it is observed that compound (8) exhibits higher percentage of inhibition (73.71%) than other compounds. In contrast compound (2) exhibits lower percentage of inhibition (47.42%) than other compounds.

The differences in potency of inhibition from any compound to another are due to the differences in nature of groups substituted instead of hydrogen atom in phenindione compound.



The present work is the first study that demonstrates the effect of these kinds of compounds on the activity of cholinesterase enzyme, but there are other studies that refer to the inhibitory effect of some classes of compounds on the enzyme activity, such as thiosemicarbazone compounds [10], heterocyclic compounds [12], new pyrazole derivatives and Schiff bases [17]. It has also been found that some alkaloids such as cathinone, cathine and ephedrine cause rise in enzyme activity and are classified as reactivators to the cholinesterase enzyme [18].

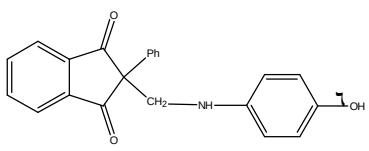
The type of inhibition and kinetic parameters (Kmapp, Vamp and KI) were also determined at different concentrations of substrate and under the same conditions by using Lineweaver-burk equation and one plotted as shown in figures (6 and 7) and table (3).

The results demonstrate are plottedthat compounds (1,4,7 and 9) exhibit the same type of inhibition (competitive inhibition), whilst compounds (3,6,8 and 10) cause mixed type inhibition, the compound (5) acts as a non-competitive inhibitor and finally the compound (2) behaves as an un-competitive inhibitor.

The hydrolysis of acetylcholine by the acetylcholinesterase involves initial attack of hydroxyl group of the amino acid serine residue to form covalent bond with the carbonyl carbon of acetylcholine and attraction of the acetylcholine cationic heads to the enzyme anionic site, which leads to the formation of acetylcholinesterase-acetylcholine complex figure (2), afterwards the acetyl group is catalytically transferred to a serine residue present at the esteratic site while choline molecule is lost and later the hydrolysis takes place to produce acetic acid and regenerated enzyme [19].

In order to understand the action of phenindione derivatives as inhibitors to cholinesterase enzyme, the following proposed mechanism was studied. These inhibitors contain multi electrophilic groups (carbonyl groups) in the essential structure, therefore the hydroxyl group of the amino acid serine residue attacks the carbonyl carbon of inhibitors instead of attacking the carbonyl group of acetylcholine and forms inhibitor-enzyme complex which causes inhibition for the activity of cholinesterase enzyme according to the suggested mechanism in figure (8).

Table (1): The new derivatives of phenindione (2-Phenyl-1,3-indandione) used for inhibited ChE.

Comp. number	Structure of compound	Compound name
		

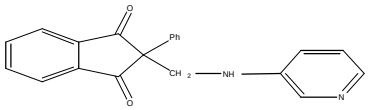
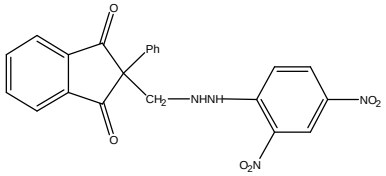
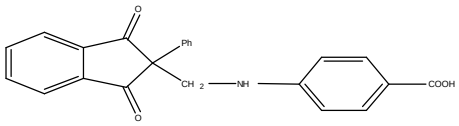
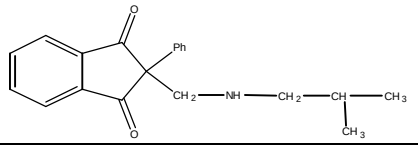
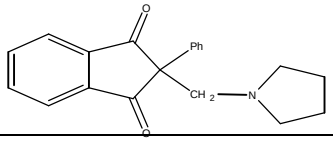
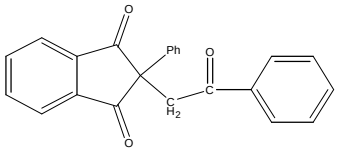
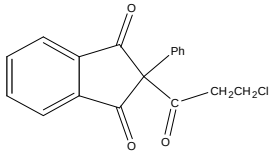
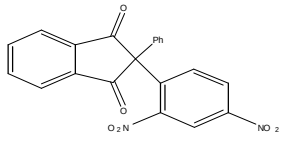
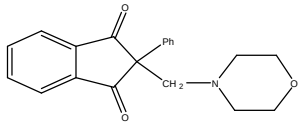
1		2-Methylen-(*N*-*p*-hydroxy anilino)-2-phenyl-1,3-indandione
2		2-Methylen-(*N*-3-amino pyridine)-2-phenyl-1,3-indandione
3		2-Methylen-(*N*-*N*-2,4-dinitro phenyl hydrazino)-2-phenyl-1,3-indandione
4		2-(*N*-*p*-benzoic acid methyl amino)-2-phenyl-1,3-indandione
5		2-Methylen-*N*-(3-methyl propinyl amino)-2-phenyl-1,3-indandione
6		2-Methylen-(*N*-pyrrolidino)-2-phenyl-1,3-indandione
7		2-Phenacyl -2-phenyl-1,3-indandione
8		2-(3-Chloro propanoyl)-2-phenyl-1,3-indandione
9		2-(2,4-dinitro phenyl)-2-phenyl-1,3-indandione
10		2-Methylen-(*N*-morpholino)-2-phenyl-1,3-indandione

Table (2) : The effect of different concentrations of compounds (1-10) on the activity of ChE in human serum.

Inhibitor Conc.[M]	**Enzyme Activity µmole/3 min/ml**	**% Inhibition**	Inhibitor Conc.[M]	**Enzyme Activity µmole/3 min/ml**	**% Inhibition**
Compound 1			**Compound 6**		
NIL	5.03	0.00	NIL	6.63	0.00
0.00001	3.85	23.38	0.00001	5.18	21.89
0.00005	3.23	35.82	0.00005	4.78	27.92
0.0001	2.65	47.26	0.0001	4.33	34.72
0.0005	2.08	58.71	0.0005	3.85	41.89
0.001	1.70	66.17	0.001	3.08	53.58
Compound 2			**Compound 7**		
NIL	5.33	0.00	NIL	5.40	0.00
0.00001	4.70	11.74	0.00001	4.03	25.46
0.00005	4.23	20.66	0.00005	3.63	32.87
0.0001	3.63	31.92	0.0001	3.15	41.67
0.0005	3.15	40.85	0.0005	2.48	54.17
0.001	2.80	47.42	0.001	1.85	65.74
Compound 3			**Compound 8**		
NIL	4.98	0.00	NIL	5.80	0.00
0.00001	4.18	16.08	0.00001	3.53	39.22
0.00005	3.68	26.13	0.00005	3.10	46.55
0.0001	3.20	35.68	0.0001	2.70	53.45
0.0005	2.48	50.25	0.0005	2.08	64.22
0.001	1.78	64.32	0.001	1.53	73.71
Compound 4			**Compound 9**		
NIL	5.53	0.00	NIL	4.70	0.00
0.00001	4.23	23.53	0.00001	3.75	20.21
0.00005	3.63	34.39	0.00005	3.33	29.26
0.0001	3.25	41.18	0.0001	2.85	39.36
0.0005	2.58	53.39	0.0005	2.43	48.40
0.001	2.13	61.54	0.001	1.95	58.51
Compound 5			**Compound 10**		
NIL	5.23	0.00	NIL	6.00	0.00
0.00001	4.40	15.79	0.00001	4.53	24.58
0.00005	3.90	25.36	0.00005	3.83	36.25
0.0001	3.43	34.45	0.0001	3.23	46.25
0.0005	2.73	47.85	0.0005	2.78	53.75
0.001	2.33	55.50	0.001	2.28	62.08

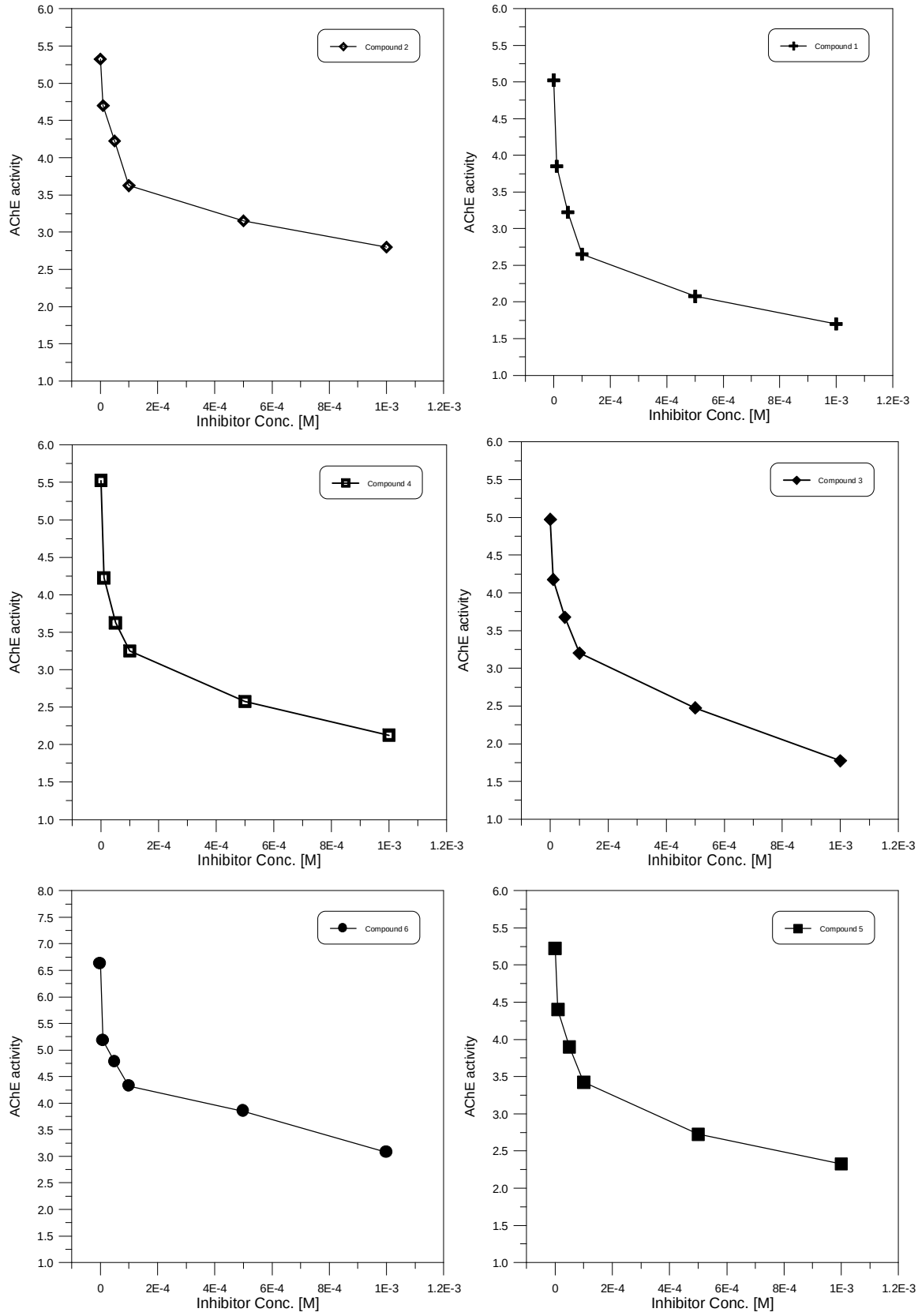


Fig. (3): The relationship between concentrations of compounds (1,2,3,4,5 and 6) and ChE activity.

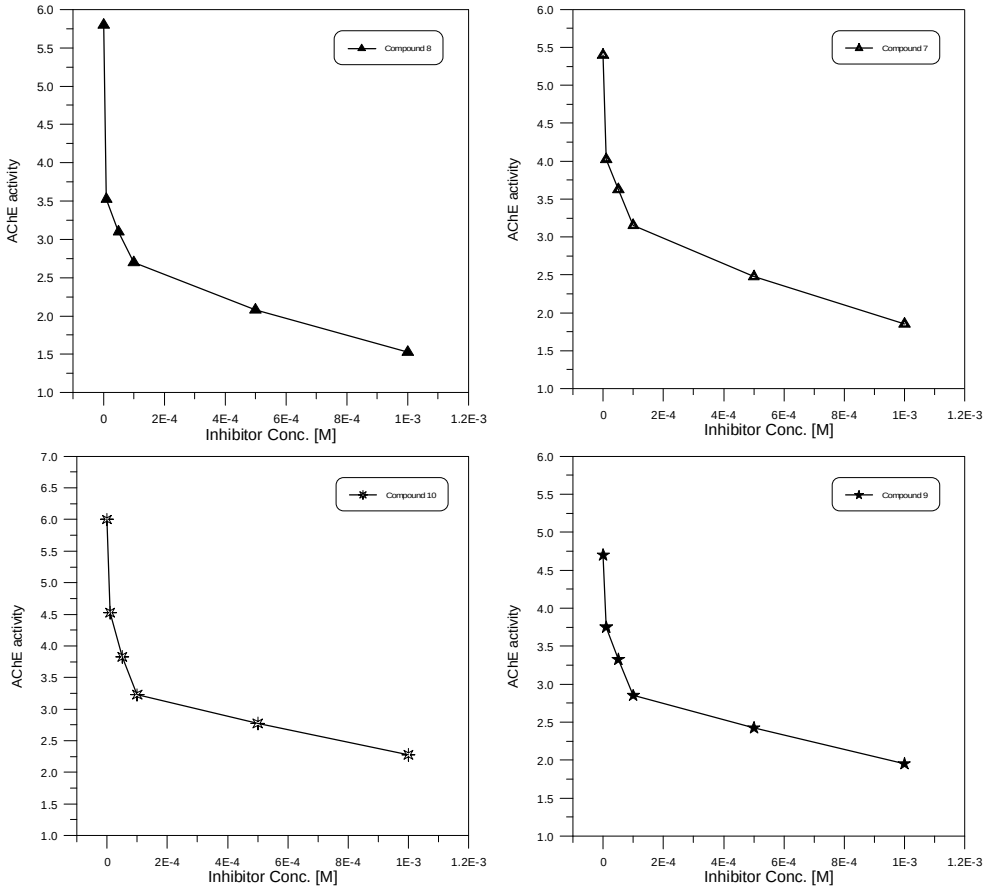


Fig. (4): The relationship between concentrations of compounds (7,8,9 and 10) and ChE activity.

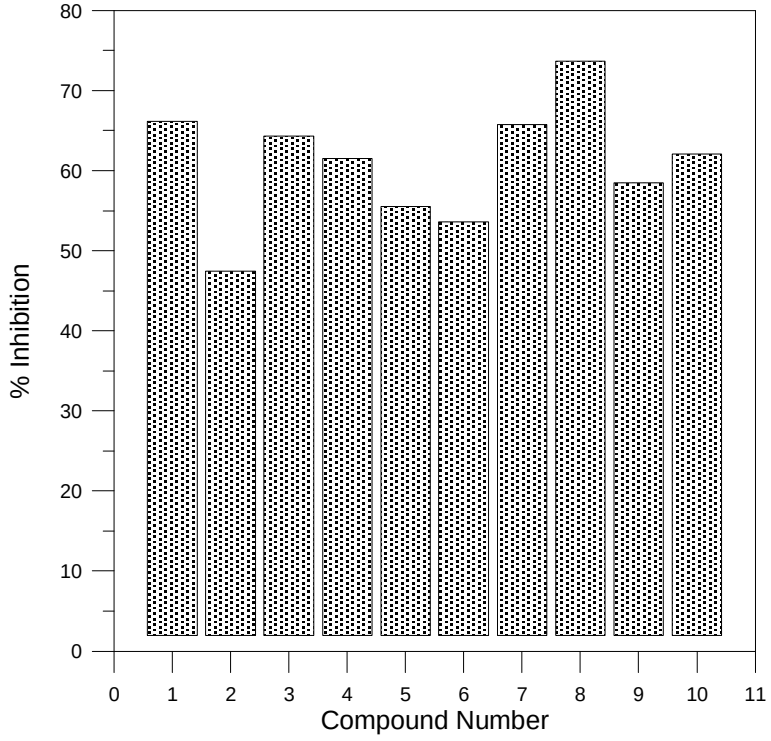


Fig. (5) : The percentage of inhibition of ChE by (0.001M) of compounds (1-10).

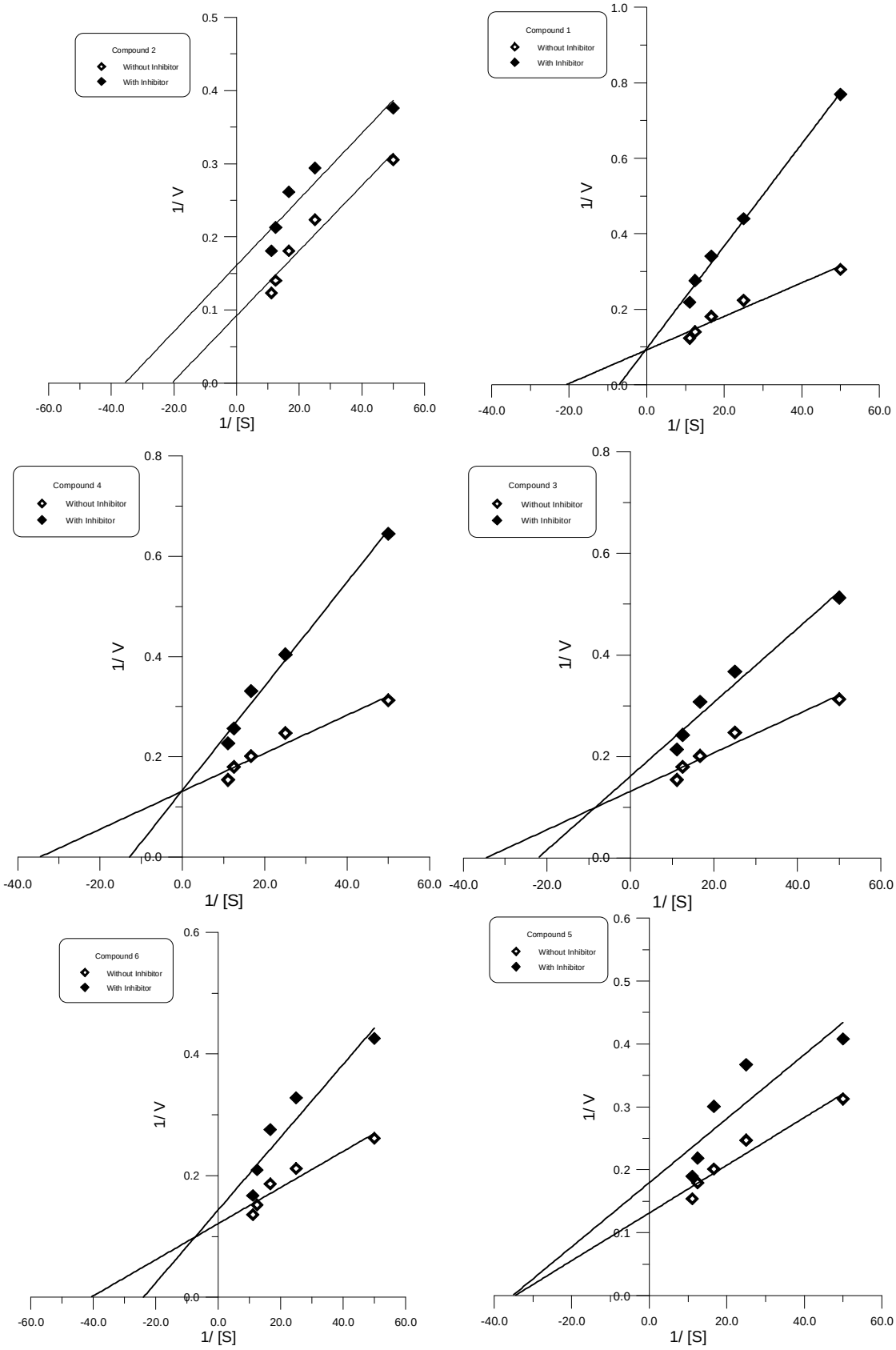


Fig. (6): Lineweaver-Burk plots of ChE with compounds (1,2,3,4,5 and 6).

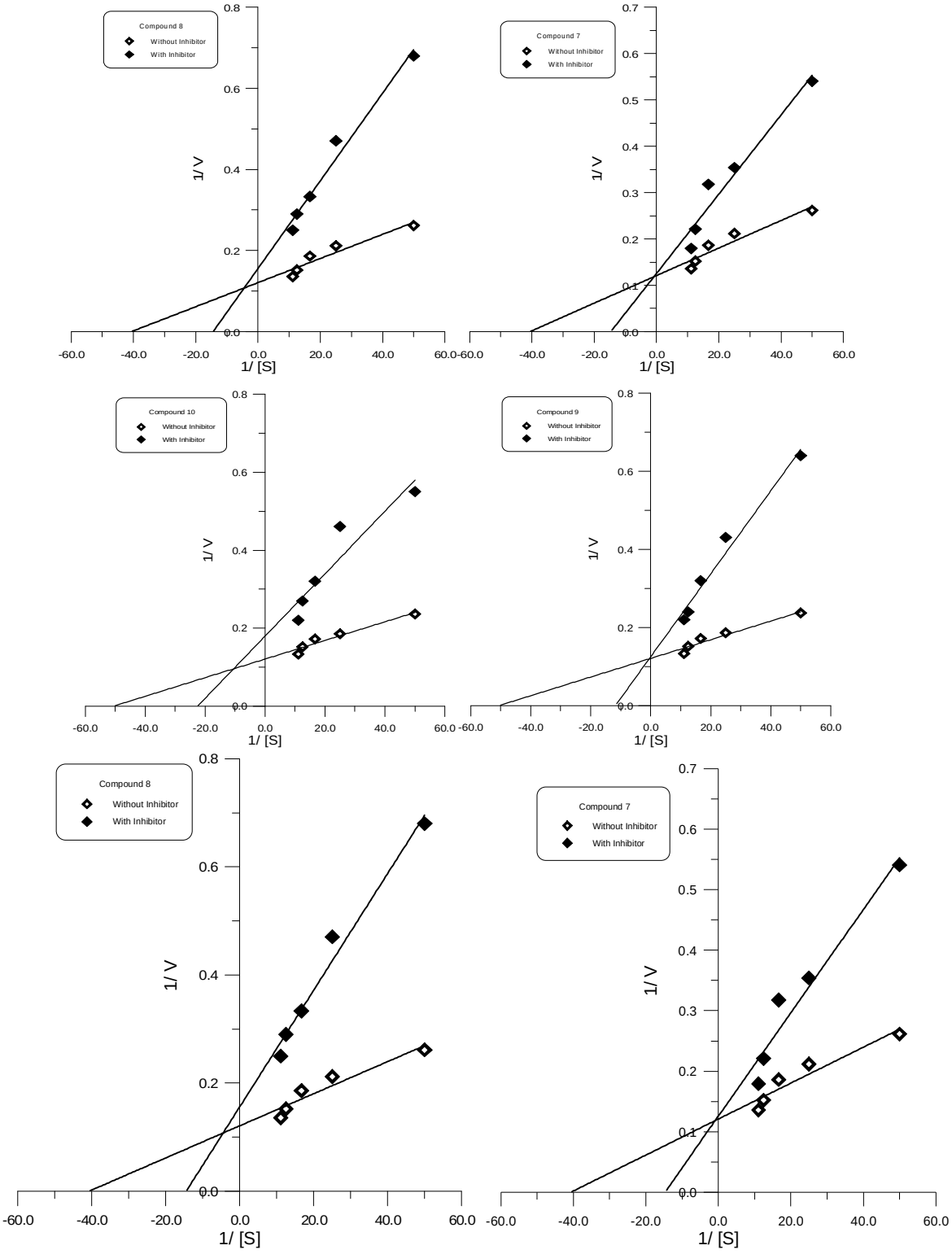


Fig. (7): Lineweaver-Burk plots of ChE with compounds (7,8,9 and 10).

Table (3) : The kinetic properties of ChE with compounds (1-10).

Comp. number ($5*10^{-4}$ M)	**Kmapp (M)**	**Vmapp (µmole/ml/min)**	**Ki (M)**	**Type of inhibition**
1	0.143		$4.5*10^{-5}$	Competitive
2	0.028	6.25	$1.28*10^{-4}$	Un-Comp.
3	0.045	6.06	$2.9*10^{-5}$	Mixed
4	0.077		$6.2*10^{-5}$	Competitive
5		5.7	$2.9*10^{-4}$	Non-Comp.
6	0.042	7.2	$4.2*10^{-5}$	Mixed
7	0.067		$5.7*10^{-5}$	Competitive
8	0.071	6.67	$2.8*10^{-5}$	Mixed
9	0.083		$3.17*10^{-5}$	Competitive
10	0.044	5.7	$1.7*10^{-5}$	Mixed

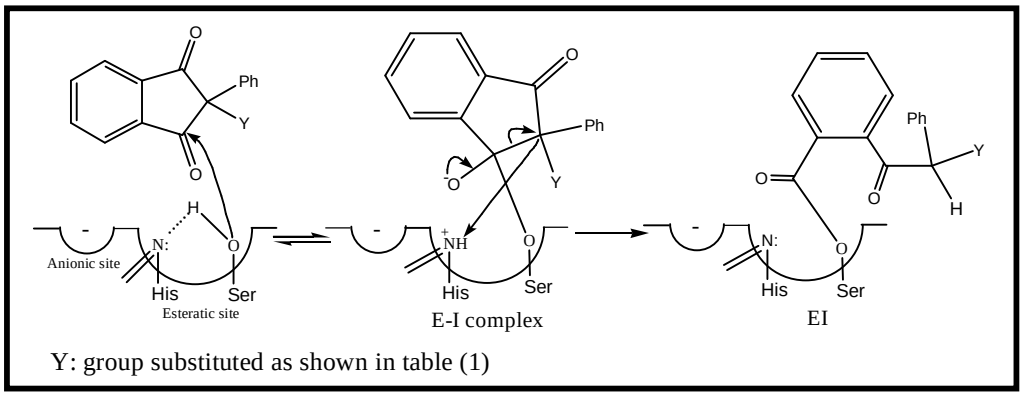


Fig. (8) : Supposed mechanism for inhibition ChE by derivatives of phenindione.

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