

Kinetics For The Inhibition of Serum Acetylthiocholin Esterase Activity by Some New Phenobarbital Derivatives.

Abstract:

This work addresses the kinetic analysis of the interaction of some new prepared Phenobarbital derivatives (A,B,C,D) with human serum acetyl cholinesterase. It was found that these compound (A,B,D) have inhibitory effect at concentrations (10^{-4} , 10^{-6} , 10^{-8} , 10^{-10} M), and observed that elevated inhibition with increasing concentration. From (10^{-10} to 10^{-4} M) for both compounds A,B and elevated inhibition with decrease concentration from 10^{-4} to 10^{-10} M for D. The effect of each A,B,D was reversible in nature. All results about C compound was neglected. Michaelis- Menten constant and maximum velocity for hydrolysis of acetyl thiocholine iodide by Ach E were determined in control and treated systems. Line weaver-Burk plot and their secondary replots indicated that the nature of inhibition was (competitive at concentration 10^{-4} , non competitive at 10^{-10} for A), (non competitive at 10^{-4} , competitive at 10^{-10} for B), (non competitive at 10^{-4} , uncompetitive at 10^{-10} for D) respectively. The value of k_i was estimated also. The mechanism of action of these types of compounds acting as inhibitors to the Ach E is suggested.

Introduction:

Acetylcholinesterase (AChE, E.C. 3.1.1.7) is found in many types of conducting tissue: nerve and muscle, central and peripheral tissues, motor and sensory fibers, and cholinergic and noncholinergic fibers. The activity of Ach E is higher in motor neurons than in sensory neurons [1-3]. AChE is also found in the red blood cell membranes, where it constitutes the Yt blood group antigen. The enzyme exists in multiple molecular forms, which possess similar catalytic properties, but differ in their oligomeric assembly and mode of attachment to the cell surface. In the mammalian brain the majority of AChE occurs as a tetrameric, G4 form (10) with much smaller amounts of a monomeric G1 (4S) form [4].

The catalytic triad (Ser-203, His-447, and Glu-334) (all numbers refer to the amino acid position of human acetylcholinesterase) has been shown to be located at the base of an active site gorge, with the peripheral anionic site in the rim of this gorge, including residues (Tyr-72, Tyr-124, Trp-286, Tyr-841 and Asp-74). Interestingly, these two sites have been described as working in tandem, where occupation of the peripheral anionic site can decrease or increase activity at the catalytic site. For example, at high concentrations both acetylcholine and acetylthiocholine have been shown to bind to peripheral anionic site, leading to substrate inhibition as a result of reduced substrate hydrolysis through steric blockade and/or conformation change in Trp-86 and Tyr-133 residues within the active site gorge⁽⁵⁾.

A cholinesterase inhibitor (or "anticholinesterase") suppresses the action of the enzyme because of essential function chemicals that interfere with the action of AChE are potent neurotoxins, causing excessive salivation and eye-watering in low doses, followed by muscle spasms and ultimately death (examples are : reversible inhibitor compounds which function as reversible competitive or non-competitive inhibitors of cholinesterase are those most likely to have therapeutic uses, these include (organophosphates, carbamates, phenanthrene derivatives, Tacrine). Quasi-irreversible inhibitor compounds which function as quasi-irreversible inhibitors of cholinesterase are those most likely to have use as chemical weapons or pesticides, these include (sarin, soman, phenmedipham) ⁽⁶⁾.

Phenobarbital or phenobarbitone is a barbiturate, first marketed as luminal , it's the most widely used anticonvulsant worldwide and oldest still commonly used ⁽⁷⁾. It also has sedative and hypnotic properties but, as with other barbiturates, has been superseded by the benzodiazepines for these indications. The World Health Organization recommends its use as first-line for partial and generalized tonic-clonic seizures in developing countries. It is a core medicine in the WHO Model List of Essential Medicines, which is a list of minimum medical needs for a basic health care system ⁽⁸⁾. In more affluent countries, it is no longer recommended as a first or second-line choice anticonvulsant for most seizure types though it is still commonly used to treat neonatal seizures ⁽⁹⁾.

Our study involves preparation of many new compounds which derivatives from Phenobarbital which have biochemical important . They are studies and characterization by spectrum methods (U.V, IR, and C.H.N) with determination of values of RF. This derivatives obtain from preparation of ester by reaction 5-ethyl-5-phenylbarbituric acid with ethylbromoacetate, then ester will be react with aqueous hydrazine and utilize it's derivatives to prepare Schiff's bases. Phenobarbital derivatives required a big tension according to biological activities which prove it, for example: these compounds utilize as anti spasmodic, and the other as anti microbial and antifungal^(10,11) ,. Biological activity of Schiff's due to presence of substitutes N-benzylidine derivative which require this type of compounds good biological activity due to some then group which is represent the core of Schiff bases, such as : salicylidene aniline and its derivatives, and the last is very useful as antimicrobial, fungi static agents and exhibit antitubercular activity ⁽¹²⁾. There are many Schiff bases molecule are useful in life such as (Rhodopin) which can combine vitamin A with macroprotein (opsin) ,in addition Schiff's bases have medical property such as diuretic and cordiotonic, and these compound derivative from furfural ⁽¹³⁾.

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 ⁽¹⁴⁾.

Materials and Methods:

Materials:

Acetylcholinesterase activity was assayed by Ellman Method ⁽¹⁵⁾. 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 430nm.

All compounds studied were prepared as following: Byrefluxing mixture of (6.9gm)(5-ethyl-5-phenyl barbituric acid) with (10gm)(bromoethyle acetate) for (6hr.) ^(16,17) the product was vaporized under vacuum pressure until appearance of white crystal (ester), then (4.5gm) from ester was treated with (2.5gm) of aqueous hydrazine and refluxing the mixture for (6hr.) a gain to produce hydrazine derivative, then (0.5gm) of it refluxing with (0.3gm) of aldehydes and ketons in (25ml Ethanol) for (4hr.) the mixture was cooled , dried and recrystallized with suitable solvent.

Blood sampling:

Five ml of blood was drawn from the same subject by vein directly after the clotting, in centrifuge at 3000rpm for 10min., the serum sample was separated and used immediately as a source of enzyme.

Methods:

1) AchE activity was determined in human serum using method of Ellmanetal⁽¹⁵⁾ as follows:

(50μL) of DTNB solution (0.001M) is added to (2.25ml) of sodium phosphate buffer solution (PH=7.3,0.2M), then (10μL) of serum is added, mixed well and (2ml) of the mixture is transferred to a measuring cell (1cm), then (34μL) of acetyl thiocholineiodide (ASChI 0.06M) is added, the change in absorbency is measured before and after adding the substrate at (430nm) for (3min.). The enzyme activity is calculated as the concentration in μ mole of the substrate hydrolyzed to each (ml) of sample in (3min.) and expressed as (μmole/3min./ml).

2) A stock solution (0.01M) concentration of each compounds in table (1) is prepared and then different concentrations (10^{-2} , 10^{-4} , 10^{-6} , 10^{-8} , 10^{-10})M of each compound are prepared by diluting with dimethyl sulfoxide (DMSO) as solvent. ChE activity is measured in human serum as follows:

(50μL) of DTNB solution (0.001M) is added to (2.25ml) of sodium phosphate buffer solution (PH= 7.3,0.2M), 0.25ml of inhibitor is mixed with 2ml of the same buffer, then (10μL) of serum is added, mixed well and (2ml) of the mixture is transferred to a measuring cell (1cm), then (34μl) of (AChI 0.06M) is added, the change in absorbency is measured after adding the substrate at 430nm for 3min.. The inhibition percentage is calculated by comparing the activity with and without inhibitor and under the same conditions according to the equation:

$$\% \text{Inhibition} = 100 - \frac{\text{the activity in the presence of inhibitor}}{\text{100 - The activity in the absence of}} \times 100$$

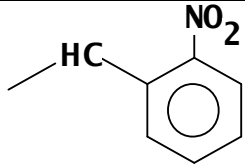
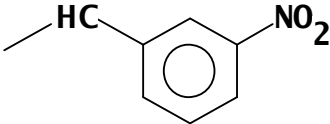
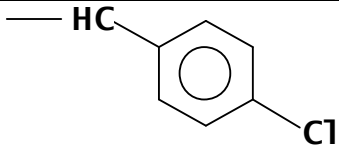
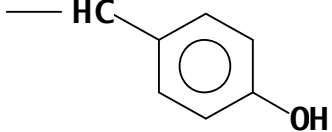
3) A constant concentration of inhibitors (10^{-4} , 10^{-10} μ L) (higher and lower concentration of inhibitors. (10^{-2} M is excluded in the study due to precipitation during experiment) is used with different concentrations of substrate (0.02, 0.04, 0.06, 0.08 M) to study the type of inhibition. These different concentrations are prepared from the stock solution (0.1 M) AChI. The enzyme activity is determined with and without the inhibitions using the lineweaver –Burk equation by plotting $1/V$ vs. $1/[S]$ ⁽¹⁸⁾. The following values were calculated 1) K_i , 2) Apparent V_{max} (V_{mapp}), 3) Apparent K_m (K_{mapp}), 4) type of inhibition.

Results and Discussion:

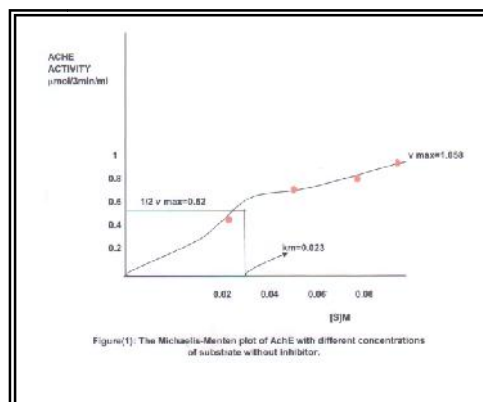
An AChE inhibitor is a chemical that inhibits the cholinesterase enzyme from breaking down acetylcholine, increasing both the level and duration of action of the neurotransmitter acetylcholine ⁽¹⁹⁾.

AChE activity in present study was assayed in the absence and presence of new Phenobarbital derivatives under different substrate concentrations. A series of compounds in table(1) is designed to investigate the effects of these new derivatives of Phenobarbital on AChE activity in vitro.

Table (1): The new derivatives of Phenobarbital used to inhibitedChE.

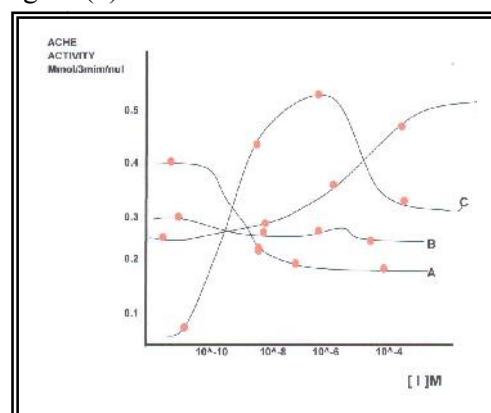
Comp. No.	Comp. name	R	Color	Yield%	m.p.c°	M. Formula	Purification solvents
A	5-ethyl-5-phenyl-1,3-di[N(2-nitrobenzilidin hydrazinyl)]barbituric acid		Yellow	50	(200-203)	C ₃₀ H ₂₇ N ₈ O ₉	Ethanol + Water
B	5-ethyl-5-phenyl-1,3-di[N(3-nitrobenzilidin hydrazinyl)]barbituric acid		Light yellow	40	(185-188)	C ₃₀ H ₂₇ N ₈ O ₉	Ethanol + Water
C	5-ethyl-5-phenyl-1,3-di[N(4-chlorobenzilidin hydrazinyl)]barbituric acid		Yellow crystal	45	(201-203)	C ₃₀ H ₂₅ Cl ₂ N ₆ O ₇	Acetone + Water
D	5-ethyl-5-phenyl-1,3-di[N(4-hydroxybenzilidin hydrazinyl)]barbituric acid		Dark yellow	55	(166-168)	C ₃₀ H ₂₈ N ₆ O ₇	Acetone + Water

Before each set of inhibition experiments, the K_m and V_{max} were determined by using four different concentrations of acetyl thiocholineiodide (0.02, 0.04, 0.06, 0.08)M as in figure (1).



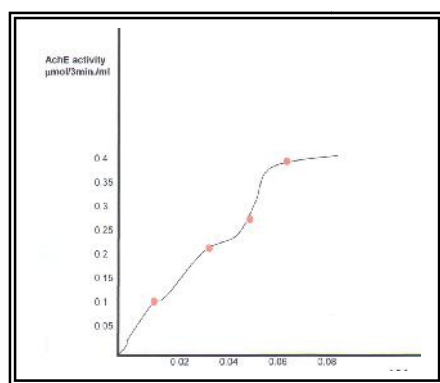
Figure(1): The Michaelis-Menten plot of AChE with different concentrations of substrate without inhibitor

The effect of four concentrations of each inhibitors (A,B,C,D) in AChE activity is illustrated in figure (2).

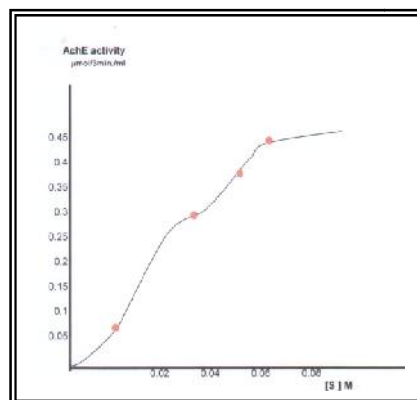


Figure(2): The relationship between concentrations of compound A,B,C,D and AChE activity

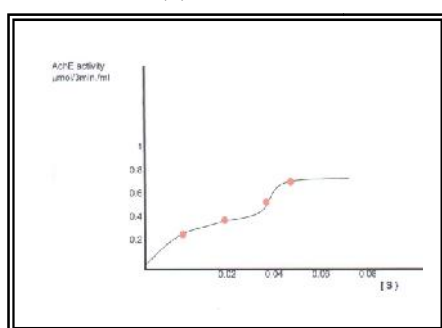
We paid little attention to compound C, which shows unclear inhibition to enzyme activity in figure(2) as a result of uncomplete dissolving of these compound in DMSO. From above figures we notice that the change of AChE activity with $[I]$ is very wide in A & B and it increased from concentration 10^{-4} to 10^{-10} in contrast, shows little inhibition in D but from concentration 10^{-10} to 10^{-4} . The Michaelis – Menten constant (K_m) and maximal Velocity (V_{max}) of AChE with different concentration of substrate with inhibitor A,B,D)) were determined from plots in figure (3), and are summarized in table (2):



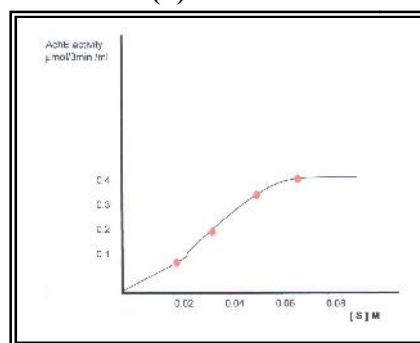
(a) 10^{-4} M of A



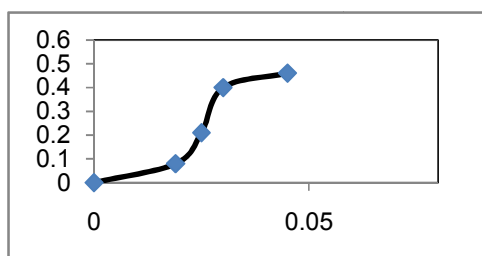
(b) 10^{-10} M of A



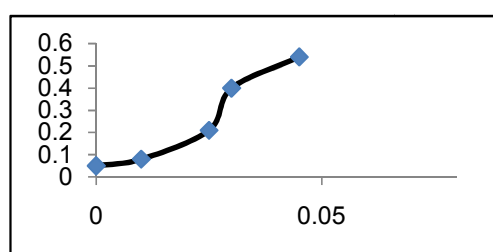
(c) 10^{-4} M of B



(d) 10^{-10} M of B



(e) 10^{-4} M of D



(f) 10^{-10} M of D

Figure (3): The Michaelis-Menten plots of AChE with different concentrations of substrate in presence of inhibitors

Table (2): Michaelis-Menten constant (K_m) and maximal velocity (V_{max}) for human serum AChE in absence and presence of compounds.

Compounds	K_m (M)	V_{max} ($\mu\text{mol}/\text{ml}/\text{min.}$)
<i>Control</i>	0.023	1.058
<i>A</i>		
10^{-4}	0.062	0.391
10^{-10}	0.031	0.441
<i>B</i>		

10^{-4}	0.038	0.5
10^{-10}	0.039	0.366
D		
10^{-4}	0.04	0.59
10^{-10}	0.071	1.09

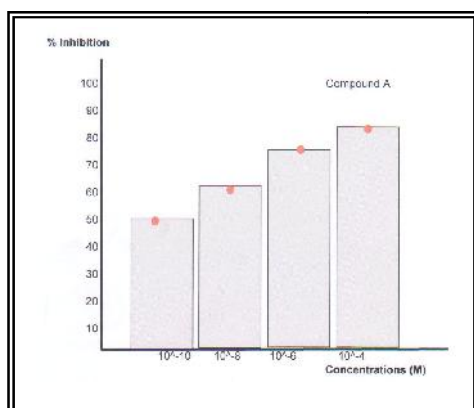
It is very clear from this presentation that K_m was higher in the presence of Phenobarbital derivatives. This indicates that the affinity of ASCh (substrate) decreases in presence of each inhibitors for both concentrations (10^{-4} , 10^{-10})M.

The biochemical tests revealed that all compound cause good inhibitory effects on enzyme activity table (3).The normal value of the enzyme activity range between $(1.5 - 2.7)\mu\text{mol}/3\text{min.}/\text{ml}$.

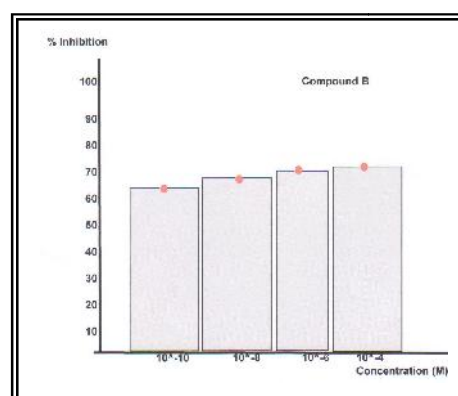
Table (3): The effect of different concentrations of compounds on the AChE activity in human serum

	Inhibition con. (M)	Enzyme activity $\mu\text{mol}/3\text{min.}/\text{ml}$	%Inhibition
A	10^{-4}	0.166	80.08
	10^{-6}	0.180	78.4
	10^{-8}	0.266	68.07
	10^{-10}	0.4	51.99
B	10^{-4}	0.25	69.99
	10^{-6}	0.275	66.99
	10^{-8}	0.291	65.07
	10^{-10}	0.3	63.99
D	10^{-4}	0.5166	37.99
	10^{-6}	0.375	54.99
	10^{-8}	0.3	63.99
	10^{-10}	0.266	68.07

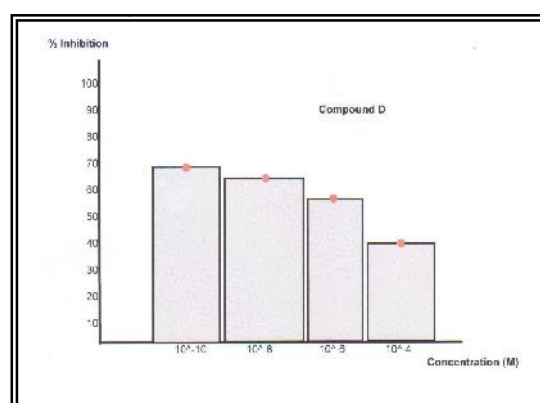
That mean the greater inhibition of A & B is demonstrated at concentration (10^{-4} M) and in compound D at (10^{-10} M) as shown in figure (4):



Compound A



Compound B



Compound D

Figure (4): The percentage of inhibition of AChE by different concentrations (10^{-4} , 10^{-6} , 10^{-8} , 10^{-10})M of compounds A,B,D

It's observed that the substituted with 2-nitrobenzil (A) and 4-hydroxy benzyl (B) and 3-nitro benzyl (D) derivatives of Phenobarbital compound exhibits the main cause of inhibition and different position of NO_2 group in A & D also show that the inhibitory effect of D inversely to A. In same line there are other studies that refer to the inhibitory effect of some classes of compounds on enzyme activity such as phenindione⁽²⁰⁾, organophosphates and carbamates⁽²¹⁾, tacrine⁽²²⁾, prophin⁽²³⁾.

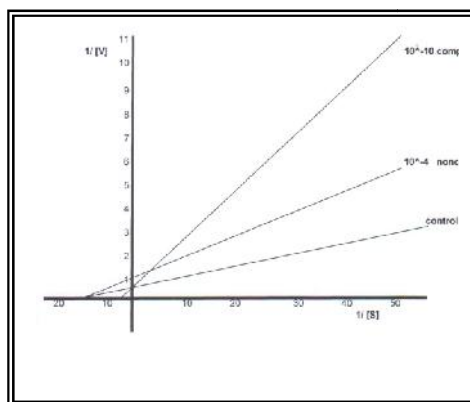
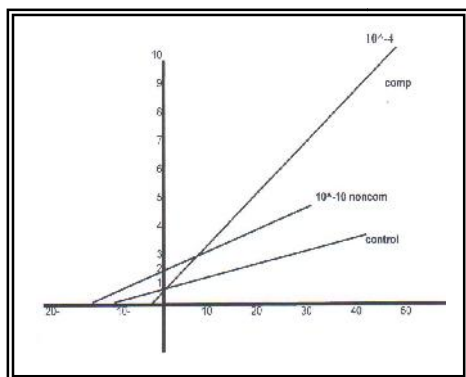
Study Type of Inhibition:

The second part of this study include determine the type of inhibition and kinetic parameters (K_{mapp} , V_{mapp} , and K_i) at different concentrations of substrate and under the same conditions by using Linweaver-Burk equation and one plotted as shown in figures (5), table (4):.

Table (4): the kinetic properties of AChE with A,B and D compounds.

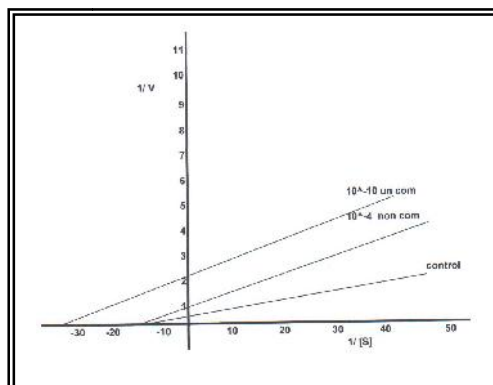
Compounds No.	$K_{\text{mapp}}(\text{M})$	$V_{\text{mapp}}(\mu\text{mol/ml/min.})$	$K_i (\text{M})$	Inhibition
10^{-4}	0.066	2	2.5×10^{-5}	Competitive

A	10^{-10}	0.066	2	1.42×10^{-11}	Non comp.
B	10^{-4}	0.066	2	1×10^{-4}	Non comp.
	10^{-10}	0.066	2	2.5×10^{-5}	Competitive
D	10^{-4}	0.066	1.25	1×10^{-4}	Non comp.
	10^{-10}	0.066	1.25	3.4×10^{-11}	Un comp.



(a)

(b)



(c)

Figure (5): Lineweaver-Burk Plots of AchE with each Concentrations (10^{-4} , 10^{-10})M of compounds

a – compound A

b – compound B

c – compound D

The results demonstrated that (A,B,D) exhibit different type of inhibition at concentrations (10^{-4} , 10^{-10}). Unlike the situation of irreversible inhibition, however the binding is weak and the enzyme activity is restored when the inhibitor dissociates from the enzyme inhibitor complex. 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 attractive of the acetylcholine cationic heads to the enzyme anionic site, which leads to the formation of acetylcholinesterase-acetyl choline complex(ES), 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⁽²⁴⁾.

In order to understand the action of A,B,D as inhibitors to cholinesterase enzyme, the following proposed mechanism was studied :

- a) Molecular interactions between the atoms N,O of the Phenobarbital moieties with the active site.
- b) The Phenobarbital compounds containing moieties of substitution N-benzylidinederivatives.Froed et al⁽²⁵⁾., suggest theory of non competitive inhibition based on the binding of inhibitor to the acetyl enzyme and the free enzyme was proven correct by demonstrating that inhibition ion increase the steady-state concentration of acetyl enzyme, as predicted by the theory by contrast, the traditional theory that the inhibitor bind to the enzyme-substrate complex and free enzyme predicts that the amount of acetyl enzyme will be drastically reduced when the inhibition is highA third theory involving all three types of binding remains possible.

Conclusion:The presentstudy concludes that aromatic Phenobarbital derivatives showed an inhibitory effect on AChE with different types of inhibition.

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