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Title page

The kinetics of inhibition of human acetylcholinesterase and butyrylcholinesterase by two series of novel carbamates

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Running title page

Running title: Kinetics of inhibition of cholinesterase by novel carbamates

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Abbreviations

ACh, acetylcholine; AD, Alzheimer's disease; AI, aminoindan; ATC, acetylthiocholine; rhAChE, recombinant human acetylcholinesterase; BTC, butyrylthiocholine; hBChE, human butyrylcholinesterase; PE, phenylethylamine; *Tc*AChE, AChE of Torpedo californica; ChE, cholinesterase; MAO-B, monoamine oxidase-B.

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Abstract

Controlled inhibition of brain acetyl- and butyrylcholinesterases (AChE and BChE, respectively) and of monoamine oxidase-B (MAO-B) may slow neurodegeneration in Alzheimer's and Parkinson's diseases. It was postulated that certain carbamate esters would inhibit AChE and BChE with the concomitant release in the brain of the OH-derivatives of rasagiline or selegiline that can serve as inhibitors of MAO-B and as antioxidants. We conducted a detailed *in vitro* kinetic study on two series of novel *N*-methyl, *N*-alkyl carbamates and compared them to rivastigmine, a known anti-Alzheimer drug. The rates of carbamylation (k_i) and decarbamylation (k_r) of recombinant human acetylcholinesterase (rhAChE) were mainly determined by the size of the *N*-alkyl substituent and to a lesser extent by the nature of the leaving group. k_i was highest when the alkyl was methyl, hexyl, cyclohexyl or an aromatic substituent, and lowest when it was ethyl. This suggested that k_i depends on a delicate balance between the length of the residue and its degree of freedom of rotation. By contrast, presumably because of its wider gorge, inhibition of human butyrylcholinesterase (hBChE) was less influenced by the size of the alkyl group and more dependent on the structure of the leaving group. The data show how the degree of enzyme inhibition can be manipulated by structural changes in the *N*-methyl,*N*-alkyl carbamates and the corresponding leaving group to achieve therapeutic levels of brain AChE, BChE, and MAO-B inhibition.

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Introduction

The major role of acetylcholinesterase (AChE) is to catalyze the hydrolysis of acetylcholine (ACh) in cholinergic synapses while the function of butyrylcholinesterase (BChE) is less clearly defined since it can hydrolyze ACh as well as other esters. The elucidation of the three-dimensional structure of AChE of *Torpedo californica* (*Tc*AChE) (Sussman et al., 1991) and of BChE (Nicolet et al., 2003) showed a general similarity between them but indicated differences in the composition of the amino acids lining a deep, narrow gorge at the bottom of which resides a catalytic site. In human BChE, 6 of the 14 aromatic amino acids that line the gorge of AChE are replaced by aliphatic ones (Harel et al., 1992) and consequently it lacks the peripheral site found in AChEs and its acyl pocket is larger (Radic et al., 1993; Saxena et al., 1997).

The observation that progressive deterioration of cholinergic innervation in the cortex contributes to the cognitive deficits in Alzheimer's disease (AD) led to the introduction of the AChE inhibitors for the symptomatic treatment in this disorder (Summers et al., 1986; Ellis, 2005). However, it was found that the membrane bound form of AChE declines in the brains of AD patients as cholinergic nerve terminals are lost, while BChE remains unchanged or is even increased (Lane et al., 2006). AChE levels also decline in rats with lesions of cortical cholinergic neurons but ACh can be maintained by selective BChE inhibitors (Giacobini, 2000). Therefore, it is now accepted that drugs that inhibit both enzymes may be preferable for treating patients with AD to those that are selective inhibitors of AChE like donepezil (Lane et al., 2006).

Although the aetiology of AD is not yet known, increasing evidence indicates that a significant role is played by free radical formation and oxidative stress. Drugs that can either reduce the formation of free radicals and/or protect cells from their damaging effects may slow the progression of neurodegeneration in AD or animal models of the

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condition (Cole et al., 2005; Liu and Ames, 2005). Effective therapy for AD is therefore more likely to be achieved by drugs that incorporate both antioxidant and cholinesterase (ChE) inhibitory activity within the same molecule.

Monoamine oxidase-B (MAO-B) inhibitors, selegiline and rasagiline, slow the progression of Parkinson's disease (Palhagen et al., 2006; Siderowf and Stern, 2006) and prevent the effects of oxidative stress in a variety of models both *in vitro* and *in vivo* (Youdim and Weinstock, 2001). In an attempt to produce compounds having both ChE and MAO-B inhibitory activity we synthesized two series of carbamates based either on selegiline or rasagiline (Sterling et al., 2002) (Fig. 1). It was postulated that the carbamate group would inhibit the enzyme with the concomitant release in the brain of the OH-derivatives of rasagiline or selegiline that are more potent inhibitors of MAO-B than the parent carbamates and retain the antioxidant activity of rasagiline and selegiline (Maruyama et al., 2003; Weinstock et al., 2003).

In designing a carbamate drug for the treatment of AD it is important to optimize and control the rates of carbamylation and decarbamylation both in the brain and peripheral tissues in order to: (a) Obtain the desired rates and levels of inhibition of AChE and BChE; (b) Control the release of the leaving groups of the carbamates that serve as MAO-B inhibitors and antioxidants; (c) Reduce the likelihood of adverse effects like nausea and vomiting because of too rapid inhibition of brain AChE (Jann, 2000). This was attempted by performing a detailed kinetic analysis of the influence of altering the size of the *N*-alkyl moiety of *N*-methyl, *N*-alkyl carbamate and the leaving group on the rate of carbamylation and decarbamylation of recombinant human (rhAChE) and human BChE (hBChE) by two series of drugs. It enabled us to speculate how their predicted interactions with the gorge domains of the enzyme controlled inhibitory activity and its duration.

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Human AChE and *Tc*AChE possess a high degree of structural similarity but the rate of interaction of these enzymes with rivastigmine, a carbamate currently used for the treatment of AD, differs markedly (Bar-On et al., 2002). An additional aim of the current study was therefore to see whether the interaction of some of the novel carbamates with rhAChE differed from that with *Tc*AChE.

Materials and Methods

Enzymes: rhAChE and hBChE, found by sedimentation on sucrose gradient to consist mainly of the tetrameric form, were purchased from Sigma-Aldrich Israel. The enzymes were dissolved in phosphate buffer pH 8 containing 0.01% NaN_3 and 1 mM EDTA to give a final concentration of 20units/ml and stored at -20ºC until use. The purified dimeric tissue-derived *Tc*AChE from *Torpedo Californica* was provided by Prof. Israel Silman Department of Neurobiology Weizmann Institute of Science, Rehovot, Israel.

Reagents: Acetylthiocholine iodide (ATC), butyrylthiocholine iodide (BTC), bovine serum albumin (BSA), and 5,5'-dithiobisnitrobenzoic acid (DTNB) were purchased from Sigma-Aldrich Israel.

Enzymic activity: Enzymic activity was determined by the method of Ellman et al. (Ellman et al., 1961) using ATC as a substrate for rhAChE and *Tc*AChE, and BTC as a substrate for hBChE on a multiscan microplate reader (Labsystems) containing 96 wells. Each well contained rhAChE 0.02 units/ml or BChE 0.025 units/ml, DTNB 0.25 mM, ATC or BTC 1mM, phosphate buffer pH 8.0 (0.1M), containing BSA (0.05%), NaN_3 (0.01%),and 1mM EDTA, in a total volume of 0.2 ml. Measurements with human enzymes were performed at 37°C and with *Tc*AChE at 24°C.

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Carbamate inhibitors: The structures of the inhibitors used in this study and of selegiline, rasagiline and rivastigmine are shown in Fig. 1 and Table 1. Since most of the MAO-B inhibitory activity of rasagiline and its derivatives is found in the R enantiomer we also tested some of the compounds in the R-form, and the activity of one of these was compared to that of the S-enantiomer (**2a** and **2b**, respectively). All carbamates were prepared as previously described by Sterling et al. (2002)

Kinetic constants: *Inhibition*. The progressive inhibition of AChE or BChE by carbamates is shown in Scheme 1. Two experimental protocols were used to obtain the constants involved in enzyme carbamylation.

Method A. *Initial rates*: Since the carbamylation phase of the reaction (k_{uni}) is considerably faster than that of decarbamylation (k_r), the two phases can be characterized separately. To avoid interference from regeneration of enzyme activity, the initial velocity was used to determine k_{obs} ($V=k_{obs}$ [E]) at each carbamate concentration. Initial velocities were calculated from the slope of the straight lines obtained from the plots of residual enzymic activity vs. time of incubation prior to addition of substrate. These lines were constructed from measurements made every min for up to 10 min. By using the double-reciprocal plot of $1/k_{obs}$ vs. 1/[inhibitor] (Fig. 2), K_D [μM], k_{uni}, [min^{-1}] and the second-order inhibition rate constant, $k_i = k_{uni}/K_D$ [M^{-1} min^{-1}], were obtained in accordance with eq. 1:

$$(k_{obs})^{-1} = (k_{uni})^{-1} + K_D\,(k_{uni})^{-1}[\text{inhibitor}]^{-1} \qquad (1)$$

Eq. 1 was used under experimental conditions in which [inhibitor]>>[enzyme] and inhibitor concentration was set within 0.3 to 3 times the estimated K_D value, conditions that permitted the use of Eq. 1 for meaningful separation of k_{uni} and K_D.

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***Method B*: *Approach to steady-state*.** Since all the carbamates were found to be stable in aqueous buffered solution at pH 8.0, Scheme 1 indicates that a steady state will be reached when the rates of carbamylation and decarbamylation are equal. For several carbamates the time course of the decrease in enzyme activity was followed for several hours and the rate constants of carbamylation (K_D, k_{uni}) and decarbamylation (k_r) were calculated as previously described (Ashani et al., 1972). Experimental conditions were as described for Method A.

Decarbamylation rate: The rate constants of decarbamylation (k_r) were determined by following directly the recovery of the inhibited enzymes. The enzymes were incubated with each carbamate for at least 2 hrs at a concentration that produced more than 85% inhibition. In order to minimize re-inhibition by excess inhibitor a 1000-fold dilution was made at t = 0 with phosphate buffer pH 8 and the enzyme maintained at a temperature of 37°C. Samples were withdrawn at successive time points and enzyme activity was measured to determine its recovery. Decarbamylation was monitored for 2-4 half-lives of the spontaneous reactivation. Since decarbamylation is a first-order reaction (Scheme 1) and the degree of reactivation monitored for several days exceeded 95% of the expected activity. k_r was computed from the fit of the data points to a single exponential decay curve using the control activity as that of the fully reactivatable enzyme.

Results

(a) Inhibition of rhAChE and hBChE

General: Tables 2 and 3 summarize the kinetic parameters of the inhibition and spontaneous regeneration of rhAChE and hBChE, respectively. It can be seen that the second-order rate constants of the inhibition reaction (k_i) obtained by the initial rate protocol (Method A) are in reasonable agreement with those calculated from the

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equations that describe the rate of approach to steady state and the steady state residual activity (Method B). Furthermore, k_i of rhAChE and hBChE determined for rivastigmine by Method A were in reasonable agreement with those reported previously by the use of different experimental protocols (Bar-On et al., 2002; Darvesh et al., 2003). Thus, Methods A and B are satisfactory for determination of the inhibition rate constants. Since the relative error associated with initial velocity measurements was smaller than that observed with the approach to steady state, and the time required to determine k_{obs} was significantly shorter than that needed to approach a steady state in Method B, comparison of inhibitory potency of the carbamates by Method A appeared to be preferable.

Inhibition of rhAChE and hBChE by the two carbamate series, like that by rivastigmine, was found to be a second-order reaction. According to Scheme 1 the initial velocity of the inhibition follows pseudo first-order kinetics because the concentration of the carbamylated enzyme is insignificant and therefore the initial concentration of the inhibitor is unchanged and the reactivation to restore enzyme activity is negligible. Thus, the double reciprocal plots of $1/k_{obs}$ vs. 1/[carbamate] in which the inhibitor concentration was in near stoichiometry to K_D resulted in reasonably straight lines for all carbamates that are consistent with the inhibition part of Scheme 1.

The second order rate constants of inhibition of hBChE by the majority of the carbamates, including rivastigmine, were greater than those observed for inhibition of rhAChE. Exceptions were **7** that inhibited rhAChE 6-fold more rapidly than hBChE and **6, 9** and **14** that showed no inhibitory activity for hBChE even at 300 μM, the highest concentration tested. The most pronounced increase in k_i of hBChE relative to rhAChE was observed with the compounds of the phenylethylamine series and is attributed to both the decrease in K_D and to the greater k_{uni}.

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Inhibition of rhAChE: The influence of the second *N*-alkyl group in the two series of carbamates on k_i of rhAChE is shown in Fig. 2. In the AI and PE series, substitution of one methyl by an ethyl group resulted in a 14- and 40-fold decrease in k_i of **2a** and **10**, respectively. There were no significant differences in K_D, k_{uni}, or k_i, between the R (**2a**) and S (**2b**) enantiomers (Table 2). The large increase in K_D (decrease in affinity) was the major factor contributing to the reduction in k_i in the AI series when one methyl of the *N*,*N*-dimethyl carbamate was substituted by an ethyl group, with virtually no change in k_{uni}. On the other hand, the reduction in k_i induced by this substitution in the PE series was mainly determined by the large decrease in k_{uni}. Although we do not know why substitution of a methyl by an ethyl group results in a similar k_i values for compounds **2a** and **11** but different k_{uni} and K_D values, it is possible the AI and PE leaving groups cause the ethyl moiety to take up different orientations in the active site. This hypothesis is supported by the observation that rivastigmine, which also has an *N*-methyl,*N*-ethyl carbamyl moiety, was found to be a better inhibitor of rhAChE than its AI and PE analogues, mainly due to an increase in k_{uni}.

Larger alkyl substituents induced a stepwise increase in k_i in both series with increasing length of the alkyl chain until the butyl and hexyl compounds of the AI series reached a similar value to that of the dimethyl analogue. Although these compounds had a lower affinity than the dimethyl analogues their k_i values were similar mainly due to the increase in k_{uni}. By contrast, in the PE series, none of the compounds exhibited a k_i of inhibition of rhAChE greater than that of the dimethyl carbamate, in spite of the fact that k_{uni} of the butyl analogue was 2-fold greater. Introduction of an aromatic group (phenyl, methoxy-phenyl, but not benzyl) resulted in compounds in the AI series that inhibited rhAChE faster than the dimethyl analogue.

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Inhibition of hBChE: The influence of increasing the size of the second alkyl group on k_i of hBChE was quite different from that of rhAChE as shown in Fig. 3. Substitution of an ethyl for one methyl group in the AI series produced only a small reduction in k_i of the R-isomer and increased that of the S-isomer 1.8-fold. The latter was due to a decrease in K_D compared to that of **1** (Table 3). The *N*-methyl,*N*-propyl carbamate (**3**) had the lowest k_i value among the aliphatic chain substituents and the *N*-methyl,*N*-benzyl (**8**) the highest of the AI series. Although the dimethyl compound (**1**) had the highest k_{uni}, it had the lowest affinity. It is surprising that compounds **6**, **9** and **14** were inactive as inhibitors of hBChE given the high activity of **8**. More experiments will be required to clarify these observations.

In the PE series, the effect of the leaving group on k_i of hBChE was much greater than any alteration in the size of the alkyl group. The k_{uni} of all the four active compounds was either similar to, or smaller than that of the corresponding AI derivatives, but they all had much lower K_D values, suggesting tighter binding to the enzyme. The k_i value of rivastigmine, which has a [(dimethylamino) ethyl] phenol leaving group was about 2.5-fold higher than that of **11** and about 10- to 20-fold higher than that of **2b**. These findings highlight the significant effect of the size of the leaving group on the rate of carbamylation of hBChE.

(**b) Spontaneous regeneration of carbamylated ChEs**

The rates of spontaneous recovery of enzyme activity (k_r, Scheme 1; Tables 2 and 3) are best determined by the direct method. In all compounds $t_{1/2}$ of the reactivation was >65 min and thus the assay period of 1-2 min did not affect the calculations of k_i, and the data fitted reasonably well to a mono-exponential decay equation (Fig. 4). Decarbamylation of *N*,*N*-dimethyl carbamylated AChE from human erythrocytes produced by pyridostigmine (Ellin and Kaminskis, 1989) was found in reasonable

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agreement with the values reported in Table 2 for the two *N*,*N*-dimethyl carbamylated rhAChE. k_r values that were obtained for the slowly reactivated carbamylated-AChEs ($t_{1/2}$ 300-1000 min) by using the equations that describe the approach to steady state (Method B) were consistently higher than those obtained by the direct method. However, a more reasonable agreement between the two methods was observed whenever the reactivation was faster ($t_{1/2}$ <250 min). This suggests that the duration of incubation at 37^{o}C, the presence of relatively high concentrations of the intact carbamates or the released leaving groups or a combination of all three factors, may modify the rates of recovery of carbamylated enzymes.

The degree of reactivation of the carbamylated enzymes appeared to approach 100%, provided that the enzyme controls were stable during the reactivation period. This was illustrated for rhAChE and hBChE for several compounds (Tables 2 and 3). Thus calculations of k_r were based on 100% recovery of the inhibited ChEs. The rate of decarbamylation was found to be faster for hBChE than for rhAChE for all compounds and was much less affected by the size of the alkyl group. For rhAChE, k_r was greatest when both alkyl groups were methyl in both the AI and PE series, and decreased sharply when one methyl was replaced by ethyl or a larger group (Table 2). A similar trend, though less pronounced, was observed with hBChE (Table 3).

(c) IC_{50} vs inhibition-reactivation constants of carbamates

The majority of studies on structure function relationships for enzyme inhibition by carbamates report their measure of activity in terms of IC_{50}s, the concentration of the drug needed to inhibit the enzyme by 50% after a given time of contact, usually 15 or 30 min. Since the apparent inhibition level is determined by the three constants, K_D, k_{uni}, k_r and not by a single value of IC_{50} at a given time point, the latter gives an erroneous estimate of the effect of changes in structure on inhibitory potency. This is illustrated in Table 4.

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(d) Comparison of inhibition of *Tc*AChE and rhAChE

The dimethyl analogue of the PE series (**10**) showed a similar k_i for inhibition of both *Tc*AChE and rhAChE, while that of the AI series (**1**) was about 20 times higher for rhAChE than *Tc*AChE (Table 5). By contrast, the *N*-methyl,*N*-ethyl carbamates, rivastigmine, **2a** and **11** inhibited rhAChE 150-1000 times faster than *Tc*AChE. Thus the reduction in k_i resulting from substitution of a methyl by an ethyl group was 480-and 8950-fold in *Tc*AChE for the AI and PE derivatives, respectively, compared to 14-and 40-fold in rhAChE. The decarbamylation rates of the *N*,*N*-dimethyl carbamylated *Tc*AChE were about 1/3 of those of rhAChE, while those of the *N*-methyl,*N*-ethyl carbamylated *Tc*AChE were at least 50-75 times lower.

(**e) Comparison of inhibition at steady-state**

The overall differences in the inhibitory potencies of the examined carbamates are illustrated in Table 6 which shows the computation of the %-inhibition at steady-state on an equimolar basis for several carbamates by using the constants listed in Tables 2 and 3. These data show how the levels of enzyme inhibition can be manipulated by structural changes of the *N*,*N*-dialkyl carbamates to achieve ChE inhibition that is relevant for the therapeutic activity of a potential drug for the treatment of AD. In selecting a suitable carbamate for *in vivo* studies, this information should be weighed together with pharmacokinetic and pharmacodynamic behaviour of each inhibitor.

Discussion

The current study focused on the influence of a stepwise increase in chain length and dimensions of the second substituent group in two series of *N*-methyl,*N*-alkyl carbamates on the kinetics of inhibition of rhAChE and hBChE, and recovery of the corresponding carbamylated enzymes. The comparison with rivastigmine, a carbamate

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currently used for the treatment of AD, permitted us to analyze the effect of a third leaving group on the kinetic behaviour of *N*-methyl,*N*-ethyl carbamates.

Most published analyses of structure–activity relationships of carbamates designed as potential drugs for the treatment of AD are based on measurements of IC_{50} (Bartolucci et al., 2006; Bolognesi et al., 2004; Luo et al., 2005, 2006; Sterling et al., 2002; Yu et al., 2001). However, this parameter lacks information on the individual rate constants that govern the approach to, and the steady state level of, enzyme activity and the rate of release of a leaving group designed to exert independent biological activity. The current study demonstrates that the relationship between structure and therapeutic efficacy of carbamates is better defined by the individual rate constants of the inhibitor-enzyme interaction which are more useful in predicting *in vivo* levels of enzyme inhibition.

It was found that the nature and size of the second carbamate alkyl substituent in *N*-methyl,*N*-alkyl derivatives of AI and PE had a similar influence on inhibitory activity (k_i) of rhAChE. The lack of a significant difference in k_i between the enantiomers of the *N*-methyl,*N*-ethyl carbamate derivative of the AI series led us to conclude that the configuration of the chiral carbon in the rigid cyclopentyl moiety of aminoindan is not important for inhibition of rhAChE by the AI derivative, but chirality does influence inhibition by rivastigmine both of rhAChE (Bar-On et al 2002) as well as hBChE, as shown previously by others (Luo et al., 2006; Yu et al., 2001).

Increasing the length of the hydrocarbon chain of the *N*-alkyl substituent from two to six in the AI series resulted in a stepwise increase in k_i until it approached that of the *N*,*N*-dimethyl compound. The introduction of partial rigidity by converting n-hexyl to cyclohexyl decreased k_{uni} but increased its affinity, thereby maintaining a similar rate of carbamylation (k_i). These data suggest that the k_i of rhAChE derived from *N*,*N*-dialkyl substituted carbamate inhibition may be influenced by the degree of freedom of rotation

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of the alkyl residue and its molecular dimensions. This presumably is due to the greater flexibility of the longer alkyl groups like N-hexyl that can exist in more conformations than the shorter N-butyl group and direct the carbamate into a more favorable orientation. The ethyl derivative has the least number of conformations and also the most limited degree of rotation.

The *N*-methyl,*N*-ethyl congeners of AI and PE, like rivastigmine (Bar-On et al., 2002), were about 150- to 1000-fold more potent as inhibitors of rhAChE than of *Tc*AChE. This finding casts doubt on any mechanistic conclusions drawn from extrapolation of the analysis of the crystal structure of carbamylated *Tc*AChE to rhAChE despite the close similarity of the two enzymes. These observations, and others with phenylmethansulfonyl fluoride (Kraut et al., 2000), highlight the influence of subtle structural differences that can significantly alter the kinetic behaviour of inhibitors towards *T*cAChE and mammalian AChE in aqueous solution. This is further illustrated by the observation that replacement of Tyr337 in rhAChE by Phe did not affect the k_i of rivastigmine even though this mutant enzyme contains the same 14 aromatic residues found in the catalytic gorge of *Tc*AChE (Barak et al., 2005).

Despite these unexplained kinetic discrepancies between the two enzymes, the crystal structures of covalent conjugates, formed between *Tc*AChE and bulky *N*-substituted mono- and dialkyl-carbamates with large leaving groups, provide information on the relative orientation of the covalently bound carbamyl moiety and may assist in the rationalization of results obtained from the kinetics of inhibition of rhAChE by the AI and PE series. While the *N*-methyl,*N*-ethyl carbamyl adduct of rivastigmine disrupted the catalytic triad (Bar-On et al., 2002), the bulky mono-*N*-alkyl carbamates of (*N*-*cis*-2,6-dimethylmorpholino-octyl chain (Bartolucci et al., 1999) and the (*N*-2'-ethyl phenyl) moiety of ganstigmine (Bartolucci et al., 2006) did not change the orientation of His440

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of the catalytic triad of *Tc*AChE. In the latter cases resistance of the carbamylated enzyme to spontaneous reactivation was rationalized by the existence of a strong hydrogen bond between the NH of the mono *N*-carbamyl moiety and the $N^{\varepsilon 2}$ atom of the catalytic His440 that is required for catalysis. With *N*-methyl,*N*-ethyl carbamates such interaction is impossible due to the lack of a hydrogen donor in the carbamyl adduct (Fig. 5). Thus, the increased resistance to reactivation of the conjugates obtained by such compounds may be associated with a substantial change in the orientation of the triad His440 as proposed for rivastigmine (Bar-On et al., 2002).

The 3D structure of *N*-methyl,*N*-ethyl carbamylated *Tc*AChE suggests that the *N*-methyl group faces the entrance and the *N*-ethyl group projects towards the bottom of the gorge (Fig. 5). In rhAChE larger N-alkyl groups may be accommodated in a region that spans the less crowded entrance to the gorge thereby providing tighter binding with less steric constraint. This is consistent with the *Tc*AChE crystal structures for mono N-substituted carbamates with a bulky *N*-substituent (Bartolucci et al., 1999, 2006).

It is noteworthy that both rhAChE and hBChE enzymes reactivate spontaneously at the same rates after inhibition by carbamates bearing the same *N*,*N*-dialkyl moiety. These results substantiate the experimental methodology, show that spontaneous reactivation of the diluted carbamyl enzyme is not affected by the leaving group, and provide strong evidence for the formation of same covalent conjugates of *N*,*N*,-dialkyl carbamylated enzyme regardless of the leaving group. The observation that *N*-ethyl and larger *N*-alkyl substituents form carbamylated enzymes of comparable stability supports the suggestion that the bulkier groups face the entrance to the gorge and therefore do not further affect the mobility of the already perturbed His440.

The rates of carbamylation and decarbamylation of hBChE by most of the compounds were higher than those for rhAChE. The structure of the leaving group played

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a much greater role in inhibition of hBChE than of rhAChE. This was seen in the higher k_i values of the S- relative to the R-isomer and of the PE analogues relative to those of the corresponding AI compounds. The faster inhibition of hBChE by the PE than by the AI carbamates is attributed to their increased affinity rather than to k_{uni} (Table 3). It is likely that the phenylethylamine moiety fits better to the active site of hBChE, leading to increased stabilization of the transition state of carbamylation. This is illustrated in compound **11** in which the transition state energy of binding to hBChE was reduced by about 3.8 kcal/mol relative to rhAChE ($k_{iBChE}/k_{iAChE} = 466$).

The wider gorge and aliphatic residues Leu and Val in hBChE instead of Phe288 and Phe290 in rhAChE (torpedo numbering system) might allow the catalytic histidine enough freedom of mobility to assume an active position enabling decarbamylation of the enzyme via activation of water molecules (see Fig. 5). The presence of Ala in hBChE at the homologous position of Phe330 in *Tc*AChE, or of Tyr337 in rhAChE can further increase the mobility of the catalytic His. Phe331 that is conserved in *Tc*AChE, rhAChE and hBChE, can play an important role in enhancing the affinity (and thus k_i) of the *N*-methyl,*N*-benzyl derivative to hBChE via π-π interactions similar to those proposed for the interaction of Phe331 with Phe330 in *Tc*AChE (Bar-On et al., 2002). This seems to be a rather sensitive parameter in rhAChE as the insertion of a methylene group decreases k_i of the benzyl, relative to those of phenyl and methoxyphenyl derivatives.

Interestingly, the *N*,*N*-dimethyl carbamylated rhAChE and hBChE undergo spontaneous reactivations at similar rates (Tables 2 and 3), as those of mono *N*-methyl carbamylated rhAChE and hBChE (Wetherell and French, 1991). However, the substitution of one *N*-methyl by an *N*-ethyl group stabilizes the inhibited rhAChE relative to hBChE (Tables 2 and 3). These findings probably reflect the steric constraints around

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the acyl pocket of hAChE that are loosened in hBChE. The data may be used to model the *N*,*N*-dialkyl carbamates in the catalytic gorge of both enzymes.

Conclusions

The carbamylation studies with two series of novel compounds and rivastigmine show that a large alkyl substituent attached to the nitrogen of *N*-methyl carbamates provides moderate (rhAChE) to good (hBChE) inhibitors. Since both rhAChE and hBChE are important targets for development of symptomatic treatment of AD, one can design inhibitor molecules that will produce the desired level of inhibition of the two enzymes and also provide a controlled release of an active leaving group such as a MAO-B inhibitor. One of the carbamates (**2a**, ladostigil) produces slowly developing, but sustained AChE and BChE inhibition *in vivo* together with brain selective MAO inhibition (Weinstock et al., 2003) and reduction of oxidative stress in the brain (Shoham et al., 2006).

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Legends for Figures

Scheme 1. Inhibition of cholinesterases by carbamates.

E, enzyme; I, intact carbamate; EI reversible enzyme-inhibitor complex; E' carbamylated enzyme. The carbamate forms a complex analogous to Michaelis-Menten complex (K_D) with cholinesterase prior to carbamylation (k_{uni}), and decarbamylation (k_r) occurs through attack of water in analogous manner to deacetylation. K_{uni} is the unimolecular rate constant and represents the carbamylation phase of the reaction. k_i is the second-order rate constant of the formation of the carbamylated enzyme (inhibition reaction), and it is approximated by k_{uni}/K_D.

Figure 1. Chemical structures of compounds in this study.

Figure 2. Influence of increasing chain length on k_i of inhibition of *rh*AChE

AI = aminoindan derivatives. PE = phenylethylamine derivatives.

Figure 3. Influence of increasing chain length on k_i of inhibition of *h*BuChE

AI = aminoindan derivatives. PE = phenylethylamine derivatives.

Figure 4. Recovery of the activity of rhAChE after inhibition by compound **(11**) and dilution to remove excess inhibitor.

Figure 5. *N*-methyl,*N*-ethyl carbamyl moiety in the active site of rivastigmine-*Tc*AChE covalent conjugate.

The thin lines show the shortest nonbonding contacts (3.2-3.9Å) with neighbouring side chain atoms. $N^{\varepsilon 2}$ is the imidazole nitrogen atom of His440 that is expected to catalyze

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decarbamylation. The dotted lines show the shortest distances of $N^{\varepsilon 2}$ and the acyl carbon from water molecules (about 4.5Å away from both atoms). The arrow indicates the direction of the gorge entrance. (Adapted from Bar-On et al., 2002; 1GQR.pdb).

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Tables

TABLE 1

Carbamates tested for rhAChE and huBChE inhibition (see Fig 1 for structures)

Series	Compound	R	Steric form
Aminoindans (AI)			
	1	CH_3	R
	2a	C_2H_5	R
	2b	C_2H_5	S
	3	n-C_3H_7	R
	4	n-C_4H_9	S
	5	n-C_6H_{13}	Racemate
	6	cyc-C_6H_{11}	Racemate
	7	C_6H_5	Racemate
	8	$CH_2C_6H_5$	Racemate
	9	*p*-OCH_3-C_6H_4	Racemate
Phenylethylamines (PE)			
	10	CH_3	Racemate
	11	C_2H_5	Racemate
	12	n-C_3H_7	Racemate
	13	n-C_4H_9	Racemate
	14	Cyc-C_6H_{11}	Racemate

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TABLE 2

Kinetic parameters for inhibition and reactivation of rhAChE a 37°C

Compound	*Method* [a]	K_D (μM) [b]	k_{uni} (min^{-1}) [b]	k_i ($M^{-1}min^{-1}$) $x10^3$ [c]	k_r (min^{-1}) $x10^{-3}$ [d]
Aminoindans					
1	A	9 ± 2	0.09 ± 0.01	10.1 ± 1.0	8.4 ± 0.4 (82%)
2a	A	110 ± 50	0.08 ± 0.02	0.73 ± 0.17	0.30 ± 0.01 (94%)
	B	176 ± 37	0.14 ± 0.008	0.79 ± 0.54	1.6 ± 0.1
2b	A	90 ± 40	0.07 ± 0.02	0.81 ± 0.15	0.40 ± 0.01 (79%)
	B	90 ± 57	0.06 ± 0.04	0.66 ± 0.04	1.6 ± 0.05
3	A	42 ± 3	0.06 ± 0.01	1.43 ± 0.04	0.38 ± 0.02 (90%)
	B	15 ± 3	0.05 ± 0.01	0.71 ± 0.03	0.70 ± 0.06
4	A	190 ± 30	1.05 ± 0.08	5.38 ± 0.38	0.40 ± 0.08 (100%)
5	A	140 ± 20	1.21 ± 0.12	8.33 ± 0.28	0.31 ± 0.01 (99%)
6	A	31 ± 4	0.28 ± 0.03	8.93 ± 0.24	(<5% at 92h) [e]
7	A	14 ± 3	0.35 ± 0.07	25.6 ± 1.3	1.60 ± 0.04 (88%)
8	A	39 ± 6	0.15 ± 0.02	3.85 ± 0.10	0.20 ± 0.01 (53%)
9	A	17 ± 8	0.24 ± 0.11	14.1 ± 0.6	0.60 ± 0.01 (81%)
Phenylethylamines					
10	A	17 ± 3	0.23 ± 0.04	13.3 ± 0.3	9.6 ± 0.3 (93%)
	B	42 ± 21	0.46 ± 0.21	11.0 ± 0.48	19.4 ± 7.5
11	A	54 ± 9	0.02 ± 0.002	0.33 ± 0.02	0.40 ± 0.04 (99%)
	B	119 ± 15	0.03 ± 0.01	0.21 ± 0.01	1.0 ± 0.1
12	A	88 ± 12	0.05 ± 0.004	0.56 ± 0.03	0.20 ± 0.03 (63%)
13	A	127 ± 5	0.57 ± 0.01	4.52 ± 0.08	0.40 ± 0.02 (88%)
14	A	55 ± 13	0.08 ± 0.02	1.46 ± 0.03	(<17% at 165h) [e]
	B	34 ± 13	0.05 ± 0.01	1,36 ± 0.11	1,4 ± 0.2
Rivastig-mine	A	55 ± 11	0.25 ± 0.04	4.54 ± 0.14	0.3 ± 0.01 (94%)

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[a] A = Initial rates; B = Steady-state. [b] Calculated from the double-reciprocal plot of eq.1. Values represent mean ± SEM

[c] Calculated from $k_i = k_{uni}/K_D$. [d] Calculated from the direct measurements as described in the Experimental Section. Figures in parenthesis are % reactivation observed for last data point. The longest monitoring of reactivation was 336 h for **5** and the shortest 24 h for **1**. [e] Not determined.

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TABLE 3

Kinetic parameters for inhibition and reactivation of hBChE at 37°C

Compound	*Method* [a]	K_D (μM) [b]	k_{uni} (min^{-1}) [b]	k_i (M^{-1} min^{-1}) $x10^3$ [c]	k_r (min^{-1}) $x10^{-3}$ [d]
Aminoindans					
1	A	120 ± 12	2.6 ± 0.2	21.7 ± 0.4	6.3 ± 0.2 (88%)
2a	A	60 ± 8	0.99 ± 0.13	16.5 ± 0.1	2.4 ± 0.1 (98%)
	B	27 ± 10	0.32 ± 0.11	11.8 ± 0.14	2.3 ± 0.2
2b	A	10 ± 1	0.39 ± 0.05	39.2 ± 1.0	2.4 ± 0.1 (96%)
	B	9 ± 3	0.30 ± 0.09	31.3 ± 0.97	4.5 ± 0.7
3	A	80 ± 4	0.66 ± 0.03	8.3 ± 0.1	1.2 ± 0.2 (91%)
	B	80 ± 13	0.57 ± 0.08	7.1 ± 0.2	3.5 ± 0.6
4	A	60 ± 5	1.84 ± 0.12	30.7 ± 0.6	0.8 ± 0.2 (85%)
5	A	10 ± 5	0.41 ± 0.17	41.0 ± 1.1	1.2 ± 0.1 (72%)
	B	17.8 ± 8	0.60 ± 0.24	34.4 ± 1.1	2.3 ± 0.4
6	A	Inactive [e]			
7	A	34 ± 14	0.14 ± 0.04	4.1 ± 0.5	3.4 ± 0.5 (86%)
	B	44 ± 22	0.14 ± 0.04	3.3 ± 0.3	2.7 ± 0.2
8	A	3 ± 2	0.13 ± 0.08	50 ± 5	0.90 ± 0.02 (68%)
9	A	Inactive [e]			
Phenylethylamines					
10	A	1 ± 1	0.15 ± 0.12	148± 20	6.5 ± 0.1 (90%)
11	A	7 ± 2	0.99 ± 0.31	142 ± 2	2.3 ± 0.2 (98%)
12	A	3 ± 1	0.30 ± 0.09	111 ± 2	1.3 ± 0.1 (72%)
13	A	3 ± 2	0.28 ± 0.15	100 ± 20	0.8 ± 0.1 (80%)
14	A	Inactive [e]			
Rivastig-mine	A	1.0 ± 0.1	0.33 ± 0.06	333 ± 22	2.3 (99%)

[a-d] As for Table 2. The longest monitoring of reactivation was 41h for **4** and **13** and the shortest, 6 h for **1**. [e] No inhibition observed up to 300 μM. Values represent mean ± SEM

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TABLE 4

Comparison of inhibition of rhAChE expressed as k_i and IC_{50}

Compound	$k_i (M^{-1} min^{-1}) x10^3$ [a]	$IC_{50} (\mu M)$ [b]		
		15'	*30'*	*120'*
Aminoindans				
1	10.1	27.1	16.6	11.7
2a	0.73	144.4	92.0	29.1
3	1.43	48.4	26.9	6.5
4	5.38	49.2	21.2	5.8
8	3.85	18.6	7.9	1.5
Phenylethylamines				
10	13.3	6.1	4.3	2.5
11	0.33	220	125	20.5
12	0.56	114.5	56.8	15
13	4.52	56.6	29.6	7.0
14	1.46	30.5	15.1	4.5

[a] Taken from Table 2. [b] IC_{50} = concentration of compound required to inhibit enzyme by 50% at specified time of incubation.

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TABLE 5
Comparison of carbamylation and decarbamylation of *Tc*AChE and rhAChE by some carbamates

Carbamate	*Carbamylation, k_i ($M^{-1}min^{-1}$ x10^{3})*		*Decarbamylation, k_r (min^{-1} x 10^{-3})*	
	TcAChE	*rhAChE*	*TcAChE*	*rhAChE*
1	0.48 ± 0.02	10.1 ± 1.0	2.6 (78% at 21 h) [a]	8.4
2a	0.0010 ± 0.0001	0.73 ± 0.17	(3% at 17 h) [b]	0.3
Rivastigmine	0.004 ± 0.002	4.54 ± 0.14	(4% at 20 h) [b]	0.3
10	17.9 ± 0.6	13.3 ± 0.3	2.5 (70% at 21 h)	9.6
11	0.0020 ± 0.0002	0.33 ± 0.02	(<2% at 15 h) [b]	0.4

[a] % reactivation at specified time. [b] Not determined

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TABLE 6

Theoretical calculations of inhibition (%) at steady state by 1 μM *N*-CH_3, *N*-substituted carbamates

Carbamate	*N-substituent*	*rhAChE*	*hBChE*
1	methyl	52 [a]	77 [a]
10	methyl	57	92
2a	ethyl	71	87
11	ethyl	56	98
Rivastigmine	ethyl	94	99
4	butyl	93	97
13	butyl	92	99
7	phenyl	99	54

[a] % inhibition was calculated by using the steady state equation: $E_{free}/E_{inhib} = k_r k_{uni}^{-1}(1+ K_I/I)$. Constants were taken from Tables 2 and 3

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Scheme 1

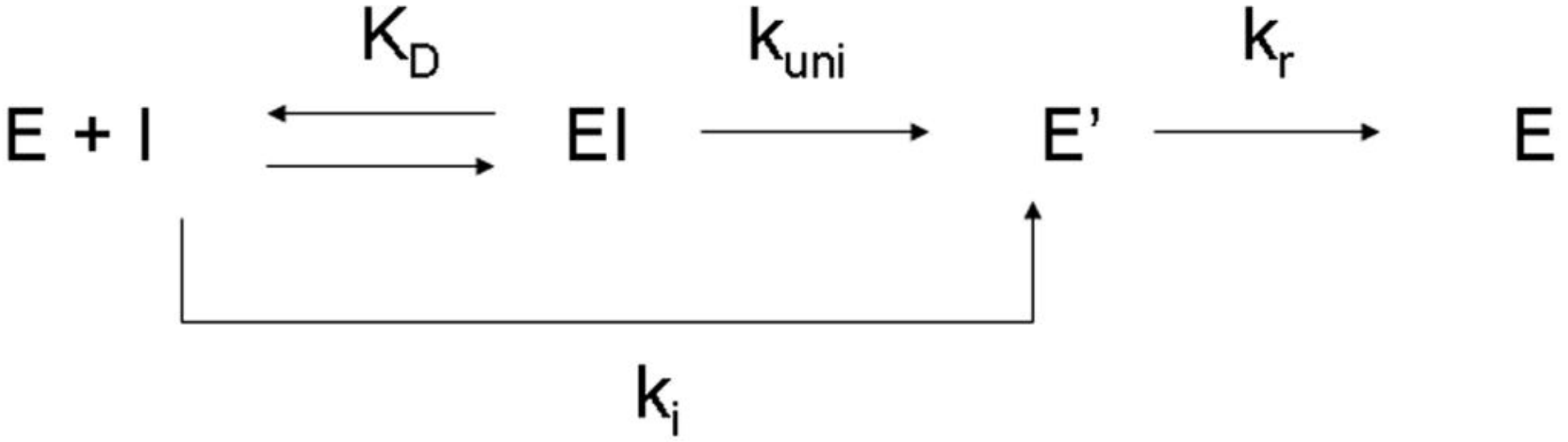
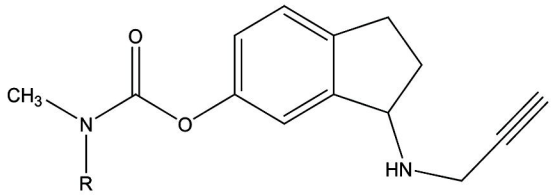
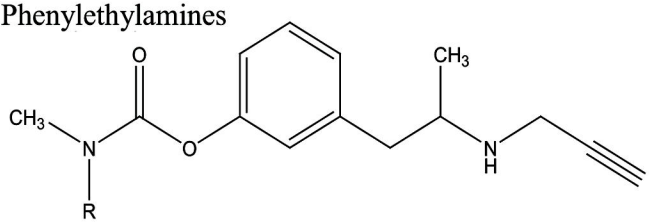


Figure1

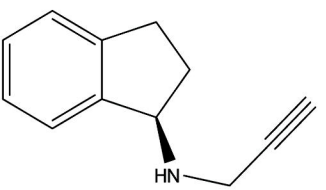
Aminoindans



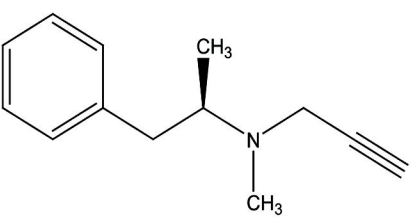
Phenylethylamines



Rasagiline



Selegiline



Rivastigmine

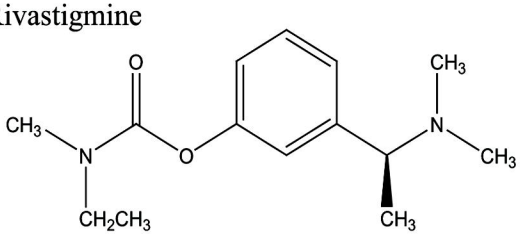


Figure 2

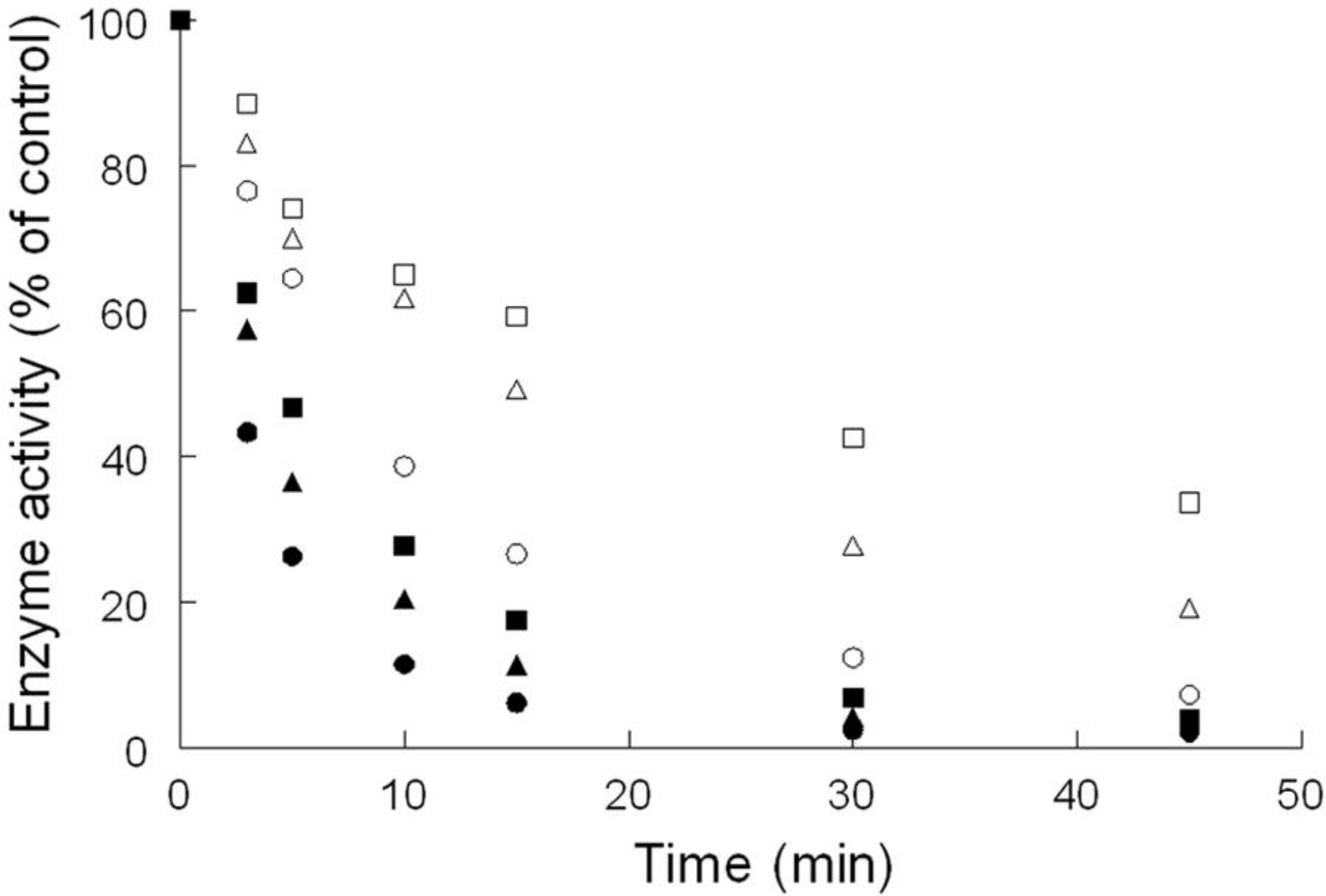

Enzyme activity (% of control)
100
80
60
40
20
0
0
10
20
30
40
50
Time (min)

Figure 3

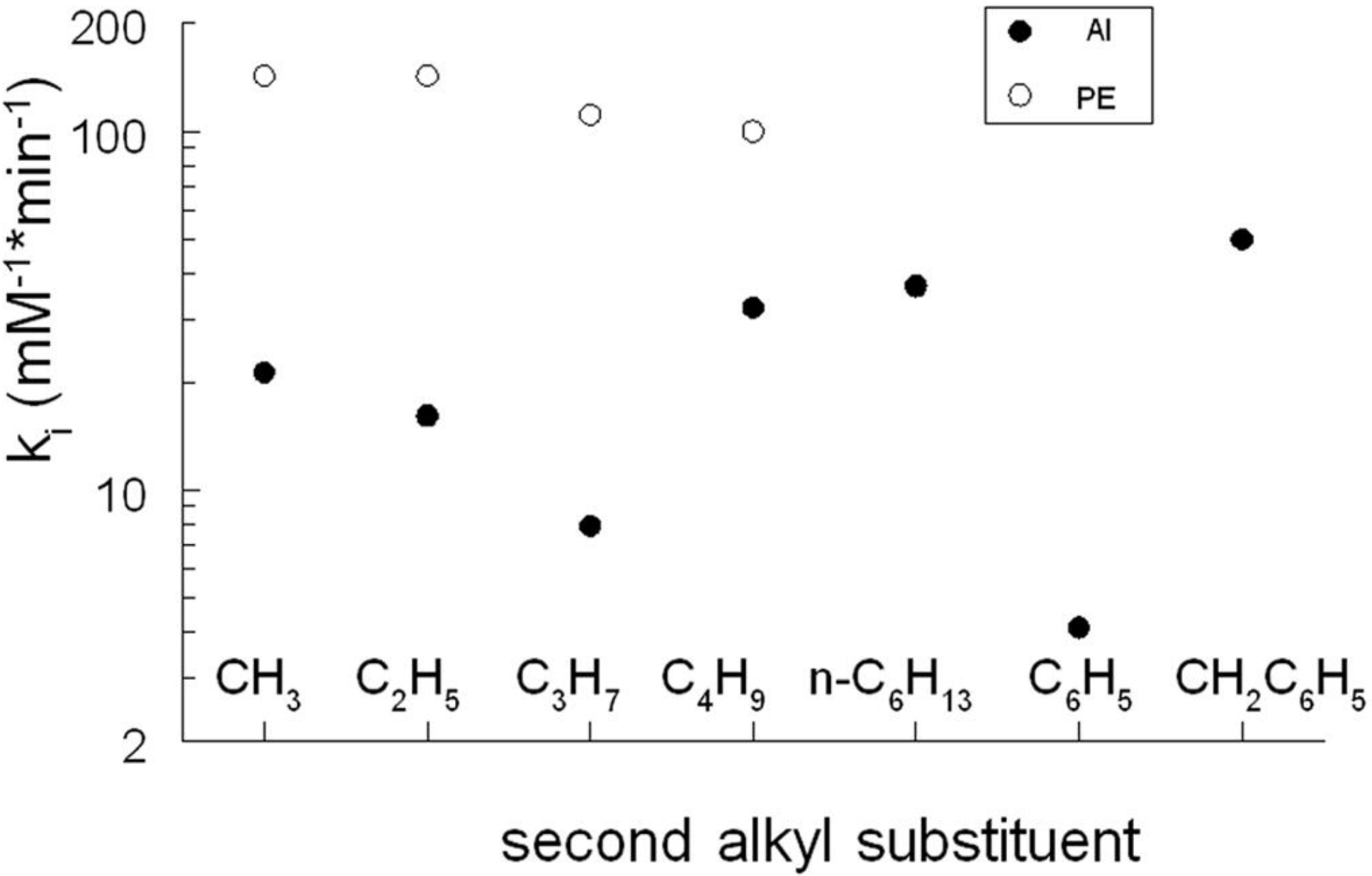

200
100
10
2
k_i ($mM^{-1}*min^{-1}$)
Al
PE
CH_3
C_2H_5
C_3H_7
C_4H_9
n-C_6H_{13}
C_6H_5
$CH_2C_6H_5$
second alkyl substituent

Figure 4

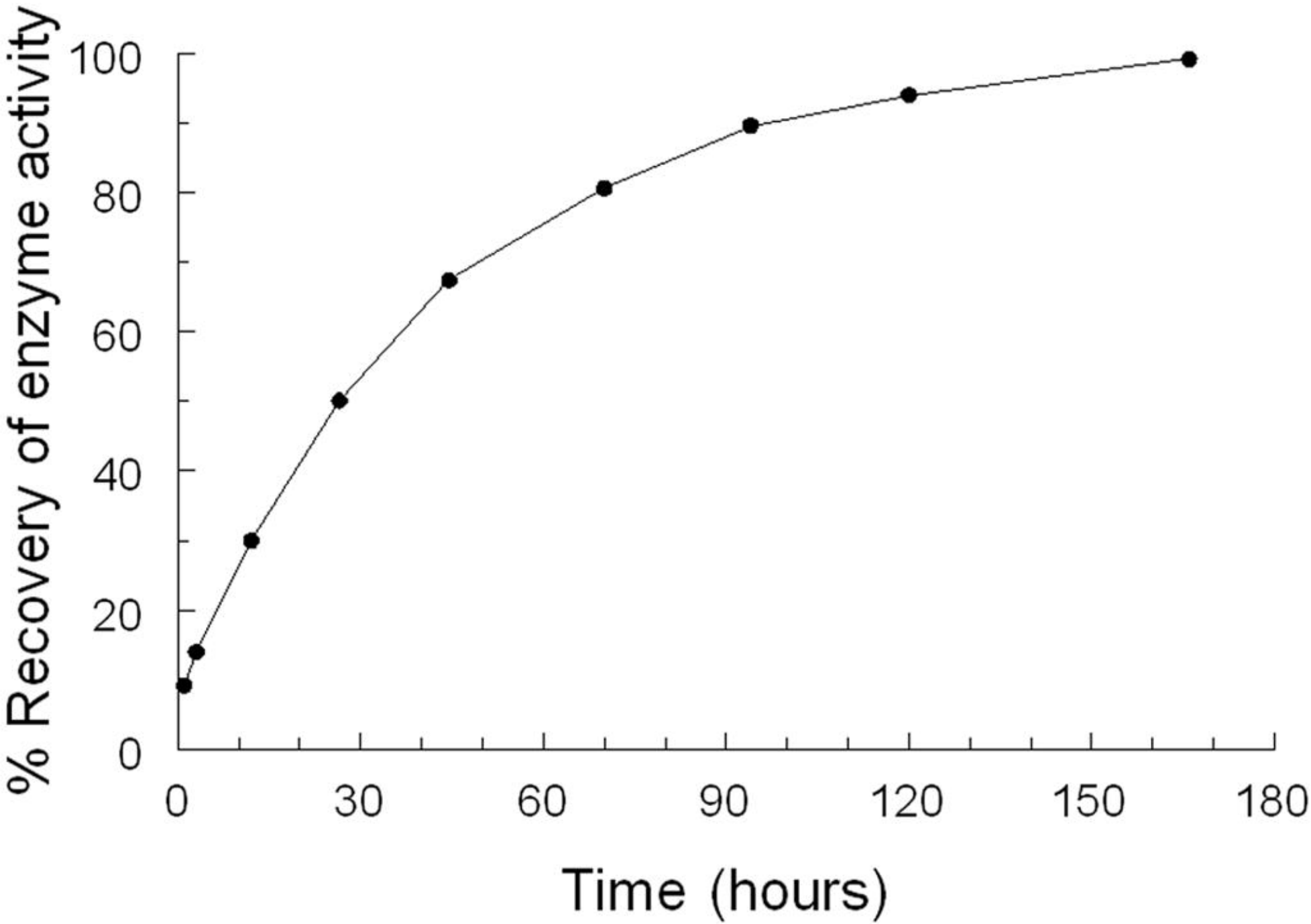
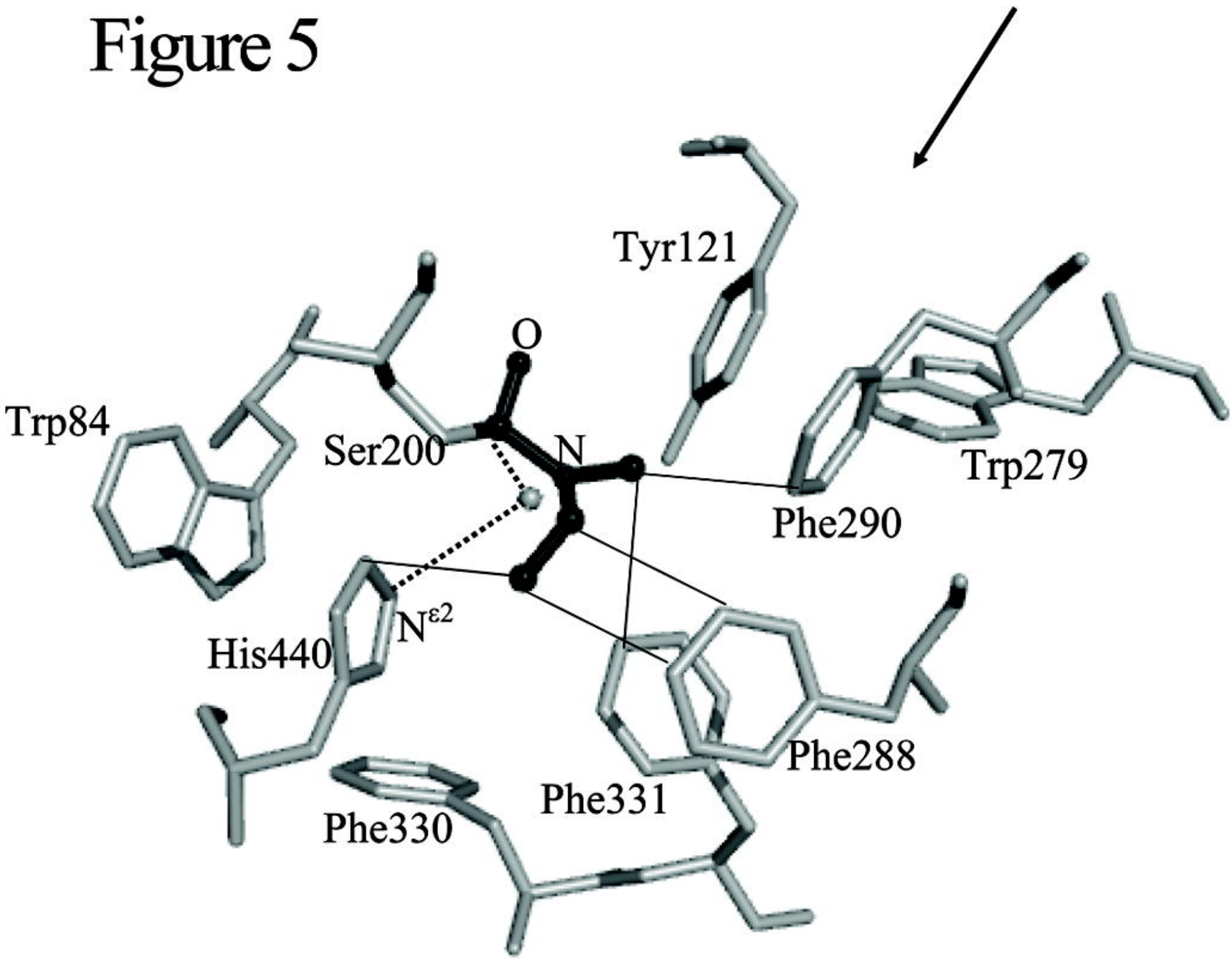

% Recovery of enzyme activity
100
80
60
40
20
0
0
30
60
90
120
150
180
Time (hours)

Figure 5


Tyr121
O
Trp84
Ser200
N
Trp279
Phe290
$N^{\varepsilon 2}$
His440
Phe288
Phe331
Phe330