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Review

Aging of Phosphylated Cholinesterases: Mechanistic and Structural Aspects, and New Possibility for Resurrection of Aged Cholinesterases

Patrick Masson [1,*], Andrey V. Nemtarev [2], Tatiana Pashirova [1,2], Samaneh Hajimohammadi [3] and Vladimir Mironov [2]

[1] Institute of Fundamental Medicine and Biology, Kazan Federal University, 18 Kremlyovskaya St., 420008 Kazan, Russia; tatyana_pashirova@mail.ru
[2] Arbuzov Institute of Organic and Physical Chemistry, FRC Kazan Scientific Center of RAS, Arbuzov Str. 8, 420088 Kazan, Russia; a.nemtarev@mail.ru (A.V.N.); mironov@iopc.ru (V.M.)
[3] Department of Pharmacodynamics and Toxicology, School of Pharmacy, Mashhad University of Medical Sciences, Mashhad 91775-1365, Iran; s.hajimohammadii@gmail.com
* Correspondence: masson.pym@gmail.com

Abstract

Cholinesterases (ChEs) are irreversibly inhibited by organophosphorous compounds (OP). Then, OP-inhibited ChEs undergo a reaction that progressively decreases reactivatability. This process called "aging" results from dealkylation of the adduct. Aged ChEs are resistant to antidotal oximes. Structural and conformational changes in the aged phosphylated ChE active site pocket impair enzyme reactivatability. Thus, reactivation of aged ChEs was a challenge for more than 70 years. However, it was postulated that realkylation of aged adducts could lead to reactivation of enzymes. This hypothesis was confirmed in 2018 when a new generation of reactivators, electrophilic quinone methide precursors (QMPs), capable of resuscitating or resurrecting aged ChEs were synthesized. The QMP-mediated resurrection process of ChEs by these first "resurrectors" is still very slow. Thus, substantial optimization in the chemical design of new drugs and drug-targeted delivery are needed before the resurrection approach can be translated into clinically viable therapies. However, despite limitations, the first achievements resolving the non-reactivatability issue of OP-aged cholinesterases and successful administration of these new reactivators can be regarded as a major step forward in improving the therapy of OP poisoning.

Keywords: aging; cholinesterase; quinone methide precursor; organophosphate; reactivator

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1. Introduction

Organophosphates (OPs) are highly toxic thio/oxo phosphoro/no esters that have been widely used for decades. They are pesticides [1], drugs or pro-drugs used in human and veterinary medicine [2], antiwear agents and flame retardants in industrial oils such as tricresyl phosphate (TCP, TOCP) [3], and the most potent chemical warfare agents are OPs [4]. Owing to the high toxicity of these compounds, most OPs are also considered potential terrorist agents. OPs are potent irreversible inhibitors of serine hydrolases [5], including esterases (carboxylesterases, lipases), acylamidases, and proteases. The most important enzymes targeted by OPs are cholinesterases (ChEs). Irreversible inhibition of ChEs leads to accumulation of acetylcholine in synapses, and a blockade of cholinergic transmissions, causing a major cholinergic syndrome. Inhibition of acetylcholinesterase

in peripheral (ganglia and neuromuscular junctions) and central nervous systems is the main cause of the acute toxicity of OPs [4,5]. In addition, irreversible inhibition of other hydrolases in the central nervous system and alkylation of different proteins plays a role in sub-acute toxicity of OPs as well as in non-cholinergic toxicity, in particular long-term post-exposure effects. Although the use of OPs is slowly decaying, OP poisoning is still a major public health concern. Nowadays, accidental and self-poisoning by pesticides is responsible for more than 100,000 deaths a year in the world [5].

Owing to the high risk of accidental and self-poisoning due to the use of OPs in agriculture and in industrial oils, the threat of OP implementation in terrorist acts and in asymmetric conflicts, and environmental consequences of the extensive use of OPs in the world, it is important to improve the medical countermeasures against OP poisoning. However, prophylactic and post-exposure treatments of OP poisoning are imperfect [6,7]. In particular, a post-inhibitory reaction on phosphylated ChEs, called "aging", may dramatically impair the efficacy of antidotes. Fortunately, new molecules synthesized in the past years are aimed at overcoming the aging reaction and lead to resurrection (resuscitation) of aged enzymes. Thus, the present review deals with the molecular bases of the aging process and the quest toward effective medical countermeasures capable of reactivating aged ChEs.

2. Irreversible Inhibition of Cholinesterases

ChEs are progressively inhibited by OPs through phosphylation of their active site serine. Irreversible inhibition of AChE is responsible for the acute toxicity of OPs [5]. There are two ChE sisters: acetylcholinesterase (EC 3.1.1.7; AChE) and butyrylcholinesterase (EC 3.1.1.8; BChE). These enzymes are α/β-hydrolases with a similar 3-D structure [8]. AChE plays a major role in the cholinergic system in terminating the action of the neurotransmitter acetylcholine. BChE has a minor role in the cholinergic system and its other physiological functions are not well known, but it is of importance in pharmacology and toxicology in degradation of drugs and scavenging OP and carbamate toxicants [2]. The overall reaction scheme of ChE inhibition by OPs post-inhibition and reactivation is described in Figure 1.

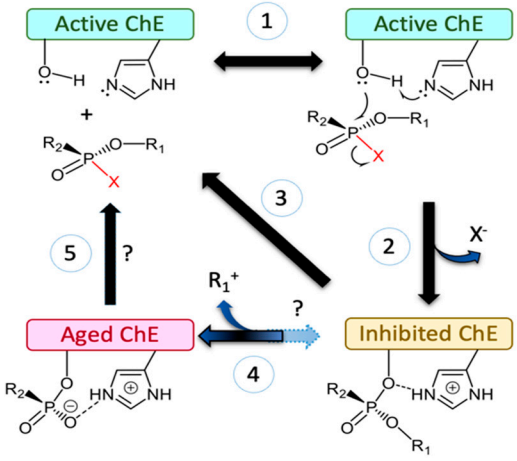

Figure 1. Mechanism of phosphylation, aging and reactivation of cholinesterases by OPs. Two key residues in active center of ChEs are depicted: the catalytic nucleophile (serine, S) (-ÖH) and the catalytic histidine (H). Reaction 1 is formation of reversible complex between enzyme and OP; reaction 2 is phosphylation step of active serine with release of leaving group X^-; reaction 3 is spontaneous water-mediated self-reactivation of enzyme; reaction 4 is dealkylation of the phosphyl adduct, leading to aged enzyme; reactions 4? and 5? are hypothetical direct reverse reactions on aged enzymes.

As seen in Figure 1, the OP molecule first binds reversibly to ChEs' active center (step 1). Then, after formation of reversible complex, the active site (serine, E-ÖH, S198* in human BChE, S203* in human AChE (* the amino acid numbering refers to active center residues in human BChE and human AChE, respectively)) is phosphylated. The phosphylation reaction is accompanied by the release of the OP leaving group X^- (step 2). X^- can be a halide (F-, like in DFP) or an oxo/thio alkyl/aryl ion, like in paraoxon and malathion. Bimolecular rate constants for phosphylation of ChEs range from 106 to 1011 $M^{-1}\cdot min^{-1}$. After phosphylation, water is too weak a nucleophile for fast spontaneous reactivation of phosphylated ChEs. Thus, OPs can be regarded as pseudo-substrates of ChEs [2]. Therefore, the phosphylated enzyme can only be reactivated in a short time by strong nucleophilic agents, like oximates (e.g., pralidoxime, 2-PAM) used as antidotes in emergency treatment of acute OP poisoning [9] (step 3). Then, the post-inhibitory reaction we mentioned complicates the inhibition scheme: the phosphyl–ChE conjugate may undergo a spontaneous dealkylation (step 4) through alkyl–oxygen bond scission. This reaction leads to progressive loss of reactivatability of phosphylated ChE; this process is called "aging" [10]. Thus, aging causes irreversible inactivation (non-reactivatability) of phosphylated enzyme. Aging can be very fast (a few minutes for human AChE phosphonylated by soman), and even ultrafast, less than 15 s for BChE, with cresyl saligenin phosphate (CSP), the active metabolite of tri-ortho-cresylphosphate (TOCP) [11], thus impairing practical reactivation attempts. Conversely, aging after phosphylation by paraoxon and malaoxon is very slow [12]. Aging in the order of hour(s), as for example with DFP (diisopropylfluorophosphate, $t_{1/2}$ = 1 h for BChE) [13], allows us to conveniently study the kinetics of this process and reactivation attempts. The structure of several OP inhibitors of ChEs are presented in Figure 2.

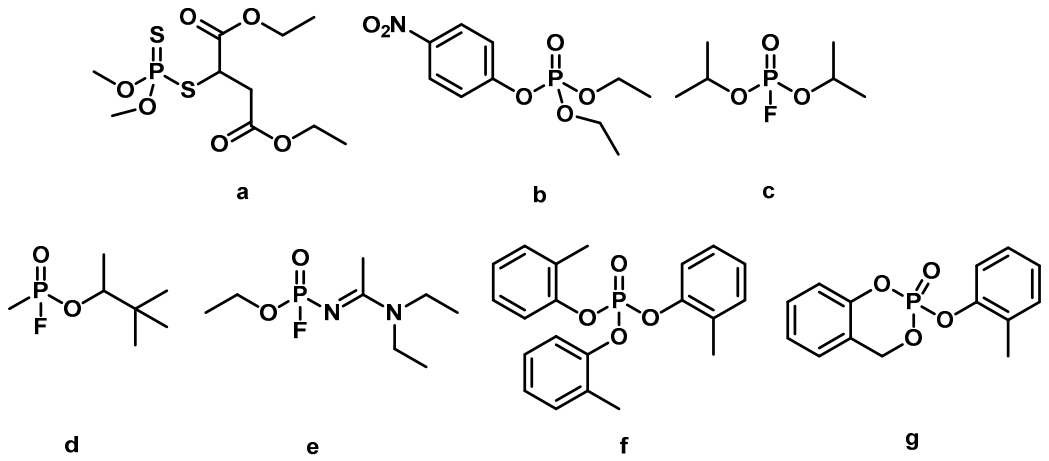

Figure 2. (**a**) Malathion; (**b**) paraoxon; (**c**) DFP (diisopropylfluorophosphate); (**d**) soman; (**e**) Novichok A234; (**f**) TOCP (tri-*ortho*-cresyl phosphate); (**g**) CSP (cresyl saligenin phosphate).

Reactivation of aged ChEs has long been considered a challenge. In particular, direct displacement of the aged adduct (step 5) leading to spontaneous enzyme reactivation is impossible. However, in recent years, drug-mediated reactivation of ChEs through realkylation ($+R_1$) of aged ChEs (reverse reaction, in step 4 and subsequent step 3, called "resurrection" or "resuscitation") was demonstrated to be possible both in vitro [14] and in vivo [15] by using quinone methide precursors (QMPs).

3. Progressive Loss of Reactivatability of Phosphylated Cholinesterases

3.1. Consequence of Dealkylation

The dealkylation reaction of adducts leads to "aged" ChEs that are unable to be reactivated by nucleophiles. The "aging" process depends on the structure of the phos-

phylated enzyme active site pocket. The first-order rate constant of aging (k_a) depends on temperature, pH (aging is favored at low pH), and binding of ligands on the enzyme's peripheral anionic site (PAS) of ChEs. In general, phosphylated BChE ages faster than phosphylated AChE.

However, as a rule, the rate of aging depends on the OP alkyl structure groups. Branched P-O-bound alkyl chains dealkylate much faster than short alkyl chains. Thus, the half-time of aging ($t_{1/2} = Ln2/k_a$) in general ranges from a few minutes to several days. For instance, at physiological pH, human AChE ages with $t_{1/2}$ = 6.3 min after inhibition by soman (dealkylation of pinacolyl chain), $t_{1/2}$ = 8.7 h after inhibition by cyclohexyl–sarin (loss of cyclohexyl chain), and $t_{1/2}$ = 1.5 days after inhibition by VX (loss of ethyl chain) [16] (for a more complete range of typical halt times see [17]). However, as mentioned, for the active metabolite of TOCP, cresyl saligenin phosphate (CSP), aging of ChEs is almost instantaneous and takes place through a double dealkylation process [11,18] (Figure 3). The aging rate also depends on OP enantiomers [19,20]. Therefore, the antidotal treatment of OP poisoning may be dramatically impaired and even impossible when the ChE-OP-adduct undergoes fast aging.

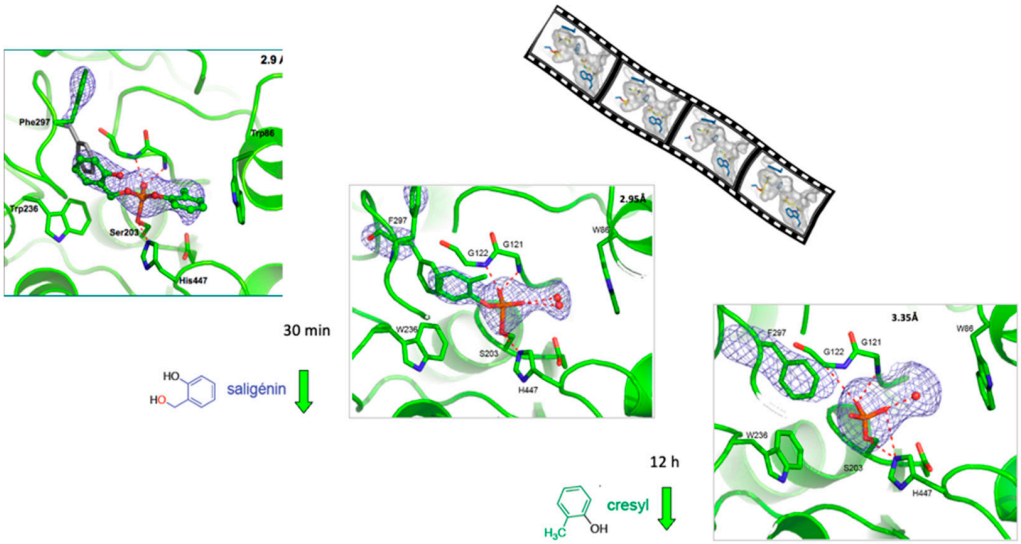

Figure 3. The two consecutive dealkylation steps of CSP-phosphorylated human AChE, leading to phosphate adduct on active site serine as proved by crystallokinetics (adapted from [18]).

3.2. Mechanism of Dealkylation

The aging of ChEs, discovered in the early 50s was thoroughly investigated in the past 30 years, in particular for ChEs and serine proteases inhibited by soman [21]. Dealkylation of phosphylated ChEs is in general a single-step unimolecular SN_1 reaction involving a carbocationic intermediate. However, in the case of CSP-ChE conjugates, X-ray crystallokinetic experiments showed that two consecutive dealkylation steps take place, leading to a phosphate ion bound to the active site serine [11,18] (Figure 3).

The active site residues involved in the molecular mechanism of dealkylation have been identified. First, chemical modification of histidine by diethylpyrocarbonate implicated catalytic histidine in aging. Then, pH dependence and site-directed mutagenesis studies provided conclusive evidence for participation of catalytic histidine (H438/H447) and glutamate (E197/E202) vicinal to the catalytic serine (S198/S203) in the mechanism of dealkylation [22]. Figure 4 provides views of the active center of both human AChE and BChE [23].

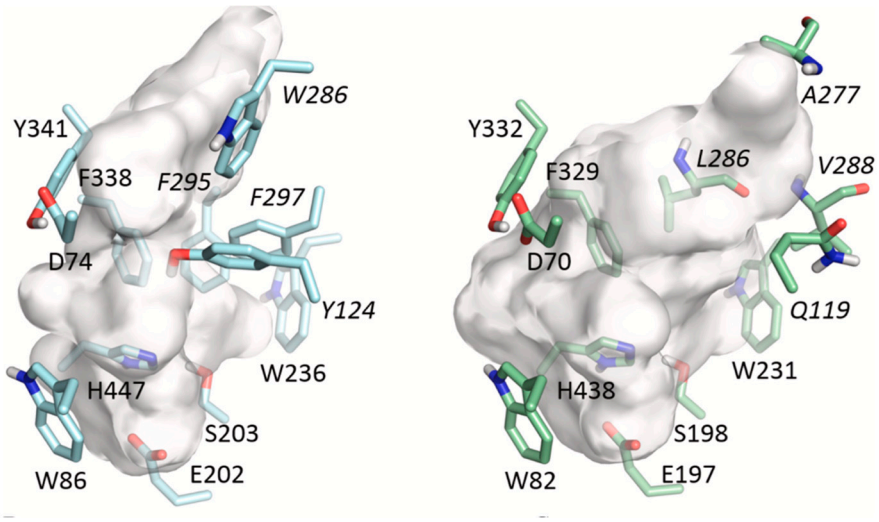

Figure 4. Active site gorge of human AChE (**left**) and human BChE (**right**). The main homologous residues of both enzymes are labeled: two residues of the catalytic triad (S203/S198 and H447/H438), adjacent glutamate (E202/E197), π-cation-binding site (W86/W82), acyl-binding site residues (W236, F295, F297 in AChE; L286, V288 in BChE), main PAS residue (D74/D70) and secondary PAS residues (Y124 in AChE, W286 in AChE, Y341/Y332) [23]. Note that the volume of the active site gorge is 300 Å^3 for AChE and 500 Å^3 for BChE.

The main component of the choline-binding pocket, tryptophan residue (W82/W86), together with E197/E202 stabilize the developing carbocation that will be cleaved [24]. Also, F329/F338 in the acyl-binding pocket was found to play a role in the aging process by stabilizing the imidazolium ring of H447 [25]. The aging rate of phosphylated human AChE is slower than the aging rate of phosphylated human BChE. This difference is related to a difference in mobility of the catalytic histidine: H438 in BChE is always in a conformation favorable to aging [17]. Lastly, D70/D74, the main amino acid residue of the peripheral anionic site (PAS), modulates the rate of aging. This aspartate is part of the Ω loop (C65–C92) that allosterically links the PAS to the choline-binding site of the catalytic center [17,26]. The mutation D70G in human BChE [13] likely induces a change in the position of W82, and disorganizes the water molecule network inside the gorge, which in turn alters stabilization of the carbocationic intermediate of adduct dealkylation. Double-mutant (Y332A/D70G) cycle kinetic analysis of human BChE under high hydrostatic pressure showed that water molecules move more freely in the active gorge of BChE mutated in the PAS than in the gorge of the wild-type enzyme (Figure 5) [27]. Participation of water molecules in the dealkylation process was directly inferred from the dependence of the aging rate on hydrostatic pressure versus osmotic pressure (Figure 5) [28] and from X-ray structure analysis [17,29].

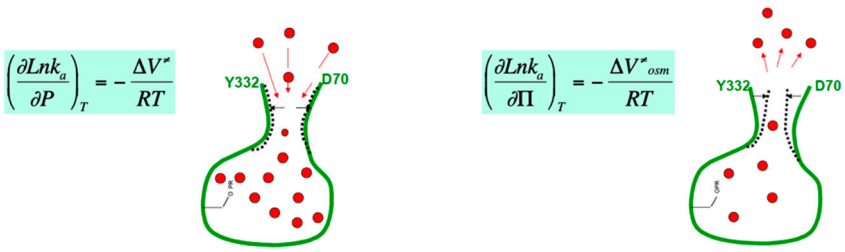

Figure 5. Hydration changes in the active gorge of human BChE, accompanying the dealkylation of OP adducts as probed by kinetics under hydrostatic pressure, P (**left**), and osmotic pressure stress, Π (**right**); k_a is the aging rate and $\Delta V^{\neq}$ is activation volume accompanying the reaction. Water molecules are represented as red balls. The residues Y332 and D70 located at the entrance of the gorge act as a check valve.

Products released from dealkylation of the pinacolyl chain of soman bound to human AChE [30] as well as pH profiles and solvent isotope effects support a "push–pull" mechanism for aging in which tryptophan (W82/W86) and glutamate (E197/E202) exert electrostatic and steric "push", and histidine (H438/H447) and the oxyanion hole act as "pullers" [21,30] (Figure 6).

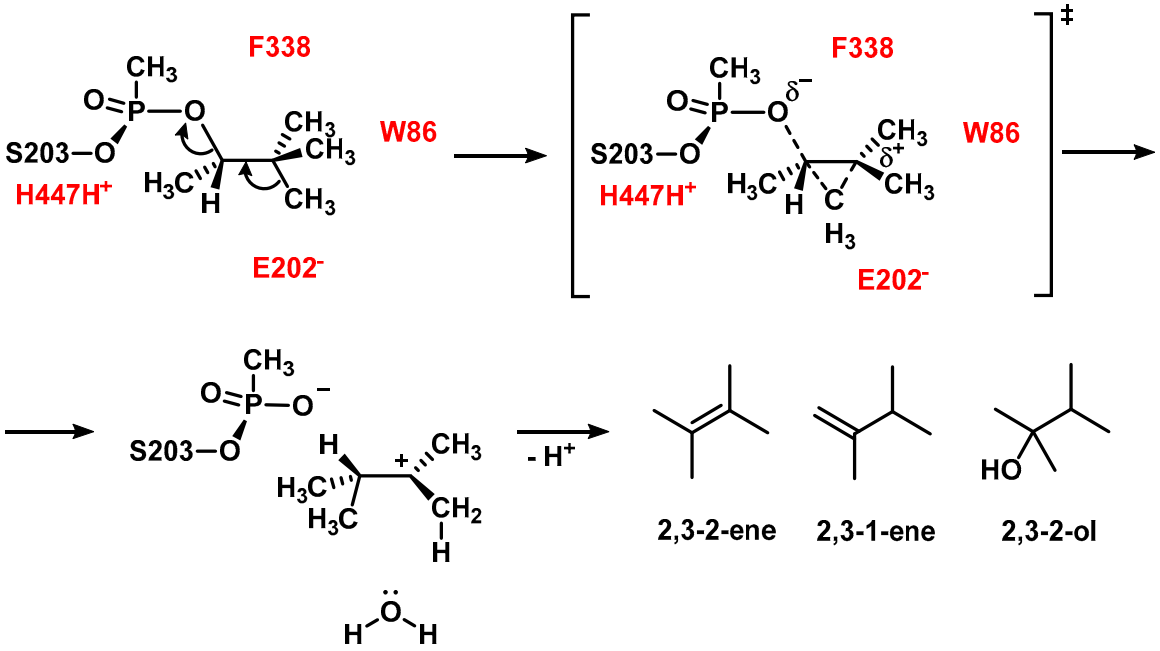

Figure 6. Push–pull mechanism proposed by Kovach for dealkylation of human AChE inhibited by PsCs–soman stereoisomer [21,29,30]. Important active site amino acids involved in dealkylation of the adduct are written in red. Electrostatic and steric "push" involves W86 and E202; concomitant "pull" effect is provided by protonated H447 and the enzyme oxyanion hole. Products of pinacolyl dealkylation are 2,3-dimethyl-2-butene, 2,3-dimethyl-1-butene and 2,3-dimethyl-2-butanol.

The critical step in this mechanism involves migration of methyl from C_β to C_α in the pinacolyl chain in the dealkylation transition state. The other proposed mechanism supported by site-directed mutagenesis involves protonation of the pinacoloxy oxygen by protonated catalytic histidine, and subsequent scission of the O–C bond [25]. Resolution of crystal structures of non-aged and aged soman–*Tc*AChE conjugates led to a critical re-examination of both models, and highlighted the role of a water molecule (H-bonded to Y121; Y124 in human AChE) in dealkylation [31]. However, X-ray data are in favor of the push–pull mechanism.

Aging of human BChE inhibited by ethyl-OPs like echothiophate and paraoxon, DFP, and phosphonates (sarin and soman), results from scission of the P–O–C chain. To determine whether there is either breakage of the P–O bond or O–C bond, phosphylation and aging were carried out in ^{18}O-water and the products compared to phosphylation and aging in water. Analysis of aged BChE tryptic peptides by MALDI-TOF/MS provided evidence that the dealkylation was due to O-C breakage assisted by a water molecule and not P-O breakage [32]. For phosphoramidates like tabun, MS and X-ray structure data provided evidence that aging of ChEs proceeds through O-dealkylation, not deamination [33]. Interestingly, it should be noted that ChE adducts of phosphoramidates like novichoki do not age. This may result from electron delocalization along the amidine chain (see Figure 2e). For phosphorodithioates like isomalathion aging is more complex, involving O–C, P–S and S–C cleavages, depending on the enantiomer that reacted with the enzyme [32]. Recently, another mechanism, leading to nucleophile alkylation of the

catalytic histidine, was established for slow aging of ChEs inhibited by pesticides [34]. Therefore, there are multiple pathways for aging of ChEs. Other enzymes, phosphylated by OPs (serine proteases like trypsin, chymotrypsin [21] and cathepsins [35]) are susceptible to aging through these mechanisms.

3.3. Molecular Hypotheses for the Resistance of Aged ChEs to Oxime Reactivators

To explain the non-reactivatability of aged ChEs, earlier studies suggested that a new bond was formed between the aged adduct and an active center residue, thus preventing reactivation. First, it was proposed that the negatively charged oxygen on the phosphorus atom formed an electrostatic shield opposing a nucleophilic attack of oximates on the phosphorus atom [36]. Later, NMR studies provided evidence for formation of a salt bridge between P–O- and protonated catalytic histidine [37]. Moreover, the role of catalytic triad histidine as a base for dephosphylation catalysis is impaired because of its protonation. Then, crystal structures of aged ChEs confirmed the existence of a strong salt bridge in the active center of aged conjugates (Figure 7) that raises the energy barrier for the reactivation reaction [8,31,33].

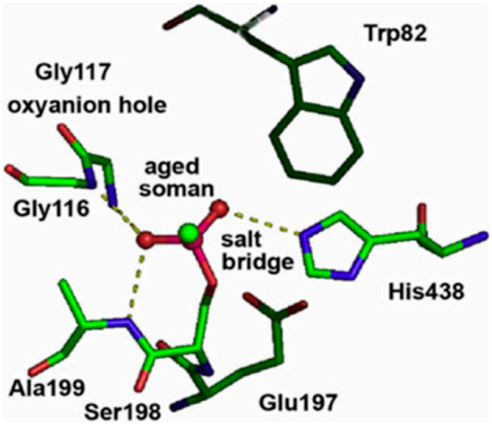

Figure 7. Salt bridge formed between the negatively charged aged soman adduct (methyl phosphonyl) bound to S198 and positively charged H438 of human BChE, PDB code 1p0q [8].

3.4. Structure and Conformational Stability of Aged ChEs

Structural investigations were aimed at understanding whether bonding interaction in the active center was the sole cause of resistance to reactivators of aged ChEs or whether enzyme conformational changes occurred upon dealkylation. First, measurement of fluorescence decay of pyrenebutyl-OPs bound to AChE suggested that the pyrenebutyl group in aged conjugates is more deeply buried than in non-aged conjugates [38]. Then, it was shown that ligand binding was altered in aged AChE compared to non-aged AChE [39]. This was interpreted as a consequence of a topographic change between the active center and the PAS. A binding study of human BChE revealed a significant decrease in the binding affinity of soman-aged BChE for a reversible ligand compared to a non-inhibited enzyme and enzyme inhibited by methyl sulfonyl fluoride, not susceptible to causing aging [40]. In the same work, cross-linking of non-inhibited and soman-aged BChE (tetrameric forms) with dimethylimidates of different chain lengths between enzyme solvent-exposed lysine ε-amino groups showed neither a change in the enzyme quaternary structure nor in the overall conformation of the monomeric subunit between both enzymes. This suggested that only minor conformation changes occur upon dealkylation. We must point out that cross-linking of solvent-exposed lysine residues was recently exploited to allosterically modulate the conformation of AChE, with the purpose of slowing the aging process [41]. To support aging-induced conformational change, enzyme denaturation studies with de-

naturing agents, heat, and hydrostatic pressure showed that the conformational stability of aged ChEs is dramatically increased compared to non-inhibited and non-aged ChEs (Figure 8) [30,42–44].

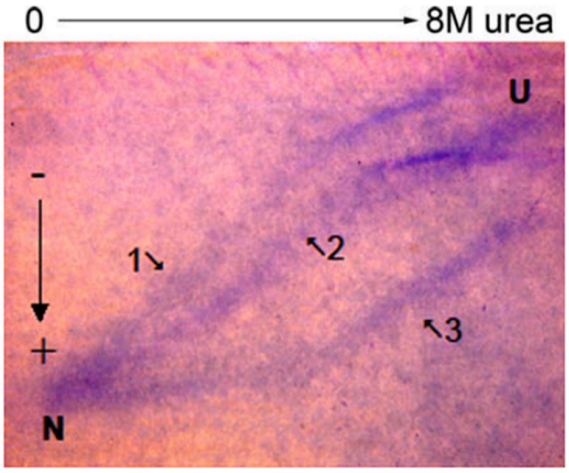

Figure 8. Transverse urea gradient gel electrophoresis (TUGGE) of 1, native BChE tetramer; 2, non-aged-soman phosphonylated BChE; 3, soman-aged BChE. Electrophoretic curves show urea-induced unfolding of enzymes. The urea concentration at mid-point transition of non-inhibited native enzyme is 3.5 M; non-aged enzyme starts to unfold at slightly higher urea concentration. Aged enzyme retains its folded structure up to 4 M urea and the transition mid-point is at 6 M urea. N indicates folded conformation and U is unfolded conformation (for experimental details and theoretical background of TUGGE, see [45,46]).

The pressure-induced molten globule transition of BChE correlating to neutron scattering data indicated a shift in the unfolding onset of aged-BChE [46]. In addition, molecular dynamics of non-inhibited and soman-aged BChE studied by incoherent elastic neutron scattering as a function of temperature showed a significant change in molecular flexibility between both enzyme forms. The increase in the stability of aged BChE correlates with a decrease in molecular flexibility [47]. Thus, formation of the salt bridge and partial dehydration of the active site gorge upon dealkylation explain stability and molecular dynamics changes in aged BChE. Therefore, it can be stated that a decrease in conformational flexibility (dynamics) of aged enzymes contributes to their non-reactivatability by preventing proper adjustment of reactivators in the active site gorge.

Comparison of the crystal structures of soman-aged AChE to native and non-aged AChE showed only a slight displacement of the catalytic histidine upon formation of the salt bridge at the bottom of the active site gorge [31]. In fact, determination of the crystal structure of the ternary complex between soman-aged AChE and 2-PAM revealed that the oximate group points away from the phosphorus atom, and therefore cannot attack it [33,48]. Then, in the case of ChEs inhibited by phosphoramidates like tabun or novichoki, resistance to oxime reactivators may result from the conjunction of electronic, steric and conformational factors: electron delocalization along the P-N-R chain (amidine chain) and stabilization by the salt bridge. In silico investigations of aging, using QM/MM, shed new light on energetics and molecular events leading to adduct dealkylation [49]. To go further, in particular for investigating new drugs to overcome the aging process, different approaches could be implemented, e.g., transition state modeling with an empirical force-field, simulations and calculation of Gibbs energy profiles using umbrella sampling algorithms. However, these approaches present methodological limitations and drawbacks out of the scope of this review, and show frequent discrepancies between computerized reaction models and experimental reactivity.

4. Attempts to Overcome the Aging Process

Because of the failure of current reactivators to restore the activity of aged ChEs, several strategies have been developed to overcome the aging limitation to antidotal therapy. Some of these efforts were reviewed [50]. In particular, these authors suggested that upregulation of AChE expression can be modulated to increase de novo expression. Unfortunately, this would be too slow to be of practical interest. On the other hand, prophylactic specific miRNA intervention would be more feasible [51]. Let us focus on proposed classical pharmacological modulators. At this point, it is important to note that most of the attempts to overcome the aging process were undertaken either without knowledge of the molecular mechanism of adduct dealkylation or the 3-D structure and dynamics of ChEs. In particular, the implication of a salt bridge formation, leading to active site rigidity and local dehydration, was not taken into account for the rational design of new therapeutic reactivators and aging modulators.

4.1. Modulation of Aging Velocity

Among the different pharmacological approaches that have been implemented, it was attempted to slow the aging process by various effectors administered prior to poisoning. Molecular dynamic simulations indicated that the PAS and the catalytic binding site are coupled via conformational change in the Ω loop, and that mutation of D70/D74 slows the rate of aging [13,52]. Therefore, it is conceivable that occupation of the PAS may affect the rate of aging by altering interactions between the aged adduct and active center residues. Allosteric effectors of the PAS, in particular bis-pyridinium compounds [53] and gallamine [54], showed only a moderate reducing effect on the aging rate. Until recently, none of the PAS effectors were found to be of practical interest for slowing down aging in vivo so far. However, PAS ligands, bifunctional reversible inhibitors and bifunctional cross-linkers that interact with both PAS and the active center are of interest. The very recent work of Katz showed that functionalized polystyrene cross-linkers confer aging resistance due to stabilization of the phosphylated AChE in a conformation preventing aging and favoring reactivation [41]. Despite their limited action, such ligands could serve as scaffolds to make new molecules capable of preventing, slowing or reversing the aging process.

4.2. Attempts to Reactivate Aged ChEs

Reactivation of aged ChEs was considered a challenge until ten years ago [55]. Direct reactivation of aged conjugates using oximes has not been possible. Yet thousands of oximes have been synthesized. However, most oximes have been designed without knowledge of the 3-D structure and molecular dynamics of phosphylated enzymes. Reported structures of complexes between oximes and non-aged and aged phosphylated ChEs provided some clues about the inefficiency of oximes to reactivate aged enzymes [43]. In particular, the 3-D structure of the non-aged tabun-AChE conjugate with the bisquaternary Hagedorn oxime Hlö-7 shows that the oximate function is not oriented toward the P atom [56]. In addition, partial electronic delocalization reinforces interactions of the adduct with the acyl-binding pocket and the oxyanion hole [33]. In the case of inhibition of ChE by compounds such as A234 (cf. Figure 2e), it is obvious that electron delocalization along the amidine chain impairs the nucleophilic attack of oximates on the phosphorus atom. Also, Sanson et al. [43] provided a rationale to understand why 2-PAM cannot reactivate aged ChEs: the X-ray structure of the soman-aged TcAChE-2-PAM complex shows that the pyridinium ring of 2-PAM is stacked against W84 and the oximate function points away from the adduct. Unfortunately, so far, X-ray data and molecular dynamics simulations did not provide

information for optimizing the orientation of the oxime group for effective attack on the phosphorus atom. In silico design of new oximes did not lead to new potent reactivators.

Therefore, the proposed strategy for reactivation of aged ChEs was to realkylate the aged adduct by using an electrophilic molecule, and then to displace the phosphotriester with a nucleophilic compound. Such an approach was difficult because of potential non-specific reactions between the designed electrophilic compounds and multiple biological targets. The first attempts with such molecules, initialized by M. Goeldner (University of Strasbourg, France) in 2005–2008, failed because of the high reactivity of synthesized electrophilic molecules with numerous macromolecules. The non-selectivity of these electrophilic molecules and the high risk of off-target alkylation of numerous biological molecules that could cause a serious iatrogenic effect in vivo led us to cancel this project. In addition, no evaluation of reactivation kinetics versus non-specific alkylation reactions was performed. However, the concept was understood, and a few years later came the appearance of the first molecules capable of re-alkylating, and thus "resuscitating", aged ChEs were synthesized [57,58]. Effective molecules, Mannich phenol quinone methide precursors (QMPs), capable of realkylating aged ChEs and displacing the realkylated phosphyl adduct have been synthesized by Hadad's group [14]. Immediately after publication of the results with the first QMP molecule (Figure 9a) [14], D. Quinn, a prominent scientist who has worked for decades in the field, highlighted the importance and the future of such molecules and labeled the process "resurrection" of the aged enzyme [59]. The resurrection mechanism is not yet completely established. It was postulated that the formation of quinone methide acts as an electrophile with the nucleophilic oxyanion hole [14,60]. Subsequent works with new QMP derivatives confirmed the promising interest of these molecules for resurrection of both aged AChE and aged BChE [14,61]. Hadad's group continues to develop new QMP derivatives displaying dual activities [62] (Figure 9b).

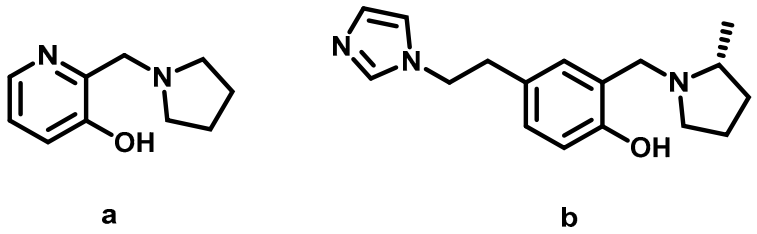

Figure 9. Structure of two QMP derivatives: (**a**) compound C8 [14]; (**b**) compound **5** [62].

Despite the slow resurrection of ChEs (in the order of 10% in 24 h for AChE), QMP derivatives have proved to be effective in vivo [15]. In particular, it was shown that novel dual derivatives, acting both as a nucleophilic reactivator (acting before aging) and an electrophile realkylator (acting on the aged adduct), are very promising. At this point, despite limitations in resurrection kinetics, the in vivo feasibility of this approach was demonstrated. However, the pharmacokinetics and central nervous system accessibility of QMP derivatives and potential new molecules are important issues to consider. The life-time of administered molecules in the blood stream must be long enough to avoid repeated administrations, and molecules must be able to significantly cross the blood–brain barrier to resurrect central ChEs. Also, taking into account the possibility of fast and ultra-fast aging that impairs reactivation of phosphylated ChEs by classical reactivators, the possibility of using these new molecules in prophylaxis must be considered.

5. Conclusions

The structural and physico-chemical bases of the aging process are now well established. The reactivation of aged ChEs remains a challenge after some 70 years of research.

The first effective molecules capable of reactivating aged ChEs have been synthesized, and at this point support the proof of concept of realkylation. This achievement shows the direction for design and synthesis of more potent molecules capable of providing therapeutic solutions. However, despite the actual slow QMP-mediated kinetics of aged ChE resurrection, a significant step forward was accomplished in the past decade with the discovery and development of QMP derivatives. The medical applicability of these first molecules remains a concern. The pharmacokinetics, bioavailability, in vivo stability (metabolism) and potential toxicity (selectivity) of these molecules have to be determined before translation into medical use.

The first issue to be solved is the in vitro reactivity and kinetics of resurrection of aged ChEs. Starting from the recent achievements in synthesis and functional evaluation of active QMPs, molecular docking of new molecules and QM/MM simulations shed light on the requirements for improving the efficiency of realkylators aimed at resuscitating aged phosphylated ChEs [57,60,63]. In silico works are important to optimize future molecules: determination of factors that determine the proper binding of new molecules, the structure of reaction intermediates and reaction transition states, evaluation of energy barriers and stabilization energies of individual reactions and overall resurrection process from Gibbs energy profiles. Then, in silico data are expected to provide directions for designing new, more potent molecules for fast resurrection of aged ChEs. Furthermore, although artificial intelligence was not yet applied to the field, it is obvious that soon it will be implemented for the design of new reactivators and in silico approaches.

After creation of new molecules, in vivo studies will determine the conditions of use of these molecules in prophylaxis and/or post-exposure treatments. Then, special attention must be paid to the development of nano-formulations of new reactivators and their implementation in non-invasive routes, e.g., the nose-to-brain route, for CNS-specific delivery. In particular, this method has proven to be easy and effective for the protection/treatment of mice poisoned by a model OP [64] and drug delivery to the brain [65,66]. These will certainly be major steps forward in improving the prophylaxis and emergency post-exposure therapy of acute OP poisoning, even in the case of the most feared nerve agents. At the same time, conditions of sustained post-exposure therapy will be determined by toxicological and pharmacodynamic studies.

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Abbreviations

The following abbreviations are used in this manuscript:

AChE	Acetylcholinesterase
BChE	Butyrylcholinesterase
ChE	Cholinesterase
DFP	Diisopropylfluorophosphate
OP	Organophosphorous compound
PAS	Peripheral anionic site
QMP	Quinone methide precursor

References

1. Singh, A.; Singh, A.; Singh, A.; Singh, P.; Singh, V.; Singh, Y.; Tuli, H.S.; Abdulabbas, H.S.; Chauhan, A. Chemistry, Metabolism and Neurotoxicity of Organophosphorus Insecticides: A Review. *Nat. Environ. Pollut. Technol.* **2023**, *22*, 1867–1880. [CrossRef]
2. Masson, P.; Shaihutdinova, Z.; Lockridge, O. Drug and pro-drug substrates and pseudo-substrates of human butyrylcholinesterase. *Biochem. Pharmacol.* **2023**, *218*, 115910. [CrossRef]
3. Hageman, G.; Mackenzie Ross, S.J.; Nihom, J.; van der Laan, G. Aerotoxic syndrome: A new occupational disease caused by contaminated cabin air? In *Advances in Neurotoxicology*; Elsevier: Amsterdam, The Netherlands, 2022; pp. 77–132.
4. Gupta, R.C. (Ed.) *Handbook of Toxicology of Chemical Warfare Agents*; Elsevier: Amsterdam, The Netherlands, 2020.
5. Eddleston, M. Novel Clinical Toxicology and Pharmacology of Organophosphorus Insecticide Self-Poisoning. *Annu. Rev. Pharmacol. Toxicol.* **2019**, *59*, 341–360. [CrossRef]
6. Masson, P. Novel approaches in prophylaxis/pretreatment and treatment of organophosphorus poisoning. *Phosphorus. Sulfur. Silicon Relat. Elem.* **2016**, *191*, 1433–1443. [CrossRef]
7. Gorecki, L.; Soukup, O.; Korabecny, J. Countermeasures in organophosphorus intoxication: Pitfalls and prospects. *Trends Pharmacol. Sci.* **2022**, *43*, 593–606. [CrossRef]
8. Nicolet, Y.; Lockridge, O.; Masson, P.; Fontecilla-Camps, J.C.; Nachon, F. Crystal Structure of Human Butyrylcholinesterase and of Its Complexes with Substrate and Products. *J. Biol. Chem.* **2003**, *278*, 41141–41147. [CrossRef] [PubMed]
9. Ratandeep; Ayushi; Garima; Devi, L.S.; Pooja. Role of Acetylcholinesterase (AChE) reactivators in the treatment of Organophosphorus poisoning: In vivo, in vitro, and in silico studies. *Chem. Biol. Lett.* **2023**, *10*, 538.
10. Masson, P.; Nachon, F.; Lockridge, O. Structural approach to the aging of phosphylated cholinesterases. *Chem.-Biol. Interact.* **2010**, *187*, 157–162. [CrossRef] [PubMed]
11. Carletti, E.; Schopfer, L.M.; Colletier, J.-P.; Froment, M.-T.; Nachon, F.; Weik, M.; Lockridge, O.; Masson, P. Reaction of Cresyl Saligenin Phosphate, the Organophosphorus Agent Implicated in Aerotoxic Syndrome, with Human Cholinesterases: Mechanistic Studies Employing Kinetics, Mass Spectrometry, and X-ray Structure Analysis. *Chem. Res. Toxicol.* **2011**, *24*, 797–808. [CrossRef] [PubMed]
12. Gao, M.; Ni, Z.; Li, G.; Wu, G.; Huang, B. Study on Spontaneous Reactivation and Aging of Acetylcholinesterase Inhibited by Paraoxon and Malaoxon in Ten Species. *Int. J. Mol. Sci.* **2023**, *24*, 14213. [CrossRef]
13. Masson, P.; Froment, M.-T.; Bartels, C.F.; Lockridge, O. Importance of aspartate-70 in organophosphate inhibition, oxime re-activation and aging of human butyrylcholinesterase. *Biochem. J.* **1997**, *325*, 53–61. [CrossRef]
14. Zhuang, Q.; Franjesevic, A.J.; Corrigan, T.S.; Coldren, W.H.; Dicken, R.; Sillart, S.; DeYong, A.; Yoshino, N.; Smith, J.; Fabry, S.; et al. Demonstration of In Vitro Resurrection of Aged Acetylcholinesterase after Exposure to Organophosphorus Chemical Nerve Agents. *J. Med. Chem.* **2018**, *61*, 7034–7042. [CrossRef]
15. Lovins, A.R.; Miller, K.A.; Buck, A.K.; Ensey, D.S.; Homoelle, R.K.; Murtha, M.C.; Ward, N.A.; Shanahan, L.A.; Gutti, G.; Shriwas, P.; et al. 4-Amidophenol Quinone Methide Precursors: Effective and Broad-Scope Nonoxime Reactivators of Organophosphorus-Inhibited Cholinesterases and Resurrectors of Organophosphorus-Aged Acetylcholinesterase. *ACS Chem. Neurosci.* **2024**, *15*, 1813–1827. [CrossRef]
16. Jun, D.; Kuca, K.; Bajgar, J.; Hruby, M.; Kucka, J.; Renault, F.; Masson, P. Phosphotriesterase modified by poly[N-(2-hydroxypropyl)methacrylamide]. *Toxicology* **2007**, *233*, 235. [CrossRef]
17. Nachon, F.; Asojo, O.A.; Borgstahl, G.E.O.; Masson, P.; Lockridge, O. Role of Water in Aging of Human Butyrylcholinesterase Inhibited by Echothiophate: The Crystal Structure Suggests Two Alternative Mechanisms of Aging. *Biochemistry* **2005**, *44*, 1154–1162. [CrossRef] [PubMed]
18. Carletti, E.; Colletier, J.-P.; Schopfer, L.M.; Santoni, G.; Masson, P.; Lockridge, O.; Nachon, F.; Weik, M. Inhibition Pathways of the Potent Organophosphate CBDP with Cholinesterases Revealed by X-ray Crystallographic Snapshots and Mass Spectrometry. *Chem. Res. Toxicol.* **2013**, *26*, 280–289. [CrossRef] [PubMed]
19. Keijer, J.H.; Wolring, G.Z. Stereospecific aging of phosphonylated cholinesterases. *Biochim. Biophys. Acta-Enzymol.* **1969**, *185*, 465–468. [CrossRef] [PubMed]
20. Wandhammer, M.; Carletti, E.; Van der Schans, M.; Gillon, E.; Nicolet, Y.; Masson, P.; Goeldner, M.; Noort, D.; Nachon, F. Structural Study of the Complex Stereoselectivity of Human Butyrylcholinesterase for the Neurotoxic V-agents. *J. Biol. Chem.* **2011**, *286*, 16783–16789. [CrossRef]
21. Kovach, I.M. Stereochemistry and secondary reactions in the irreversible inhibition of serine hydrolases by organophosphorus compounds. *J. Phys. Org. Chem.* **2004**, *17*, 602–614. [CrossRef]
22. Saxena, A.; Doctor, B.P.; Maxwell, D.M.; Lenz, D.E.; Radic, Z.; Taylor, P. The Role of Glutamate-199 in the Aging of Cholinesterase. *Biochem. Biophys. Res. Commun.* **1993**, *197*, 343–349. [CrossRef]
23. Mukhametgalieva, A.R.; Lushchekina, S.V.; Aglyamova, A.R.; Masson, P. Steady-state kinetic analysis of human cholinesterases over wide concentration ranges of competing substrates. *Biochim. Biophys. Acta-Proteins Proteom.* **2022**, *1870*, 140733. [CrossRef]

24. Barak, D.; Ordentlich, A.; Segall, Y.; Velan, B.; Benschop, H.P.; De Jong, L.P.; Shafferman, A. Carbocation-Mediated Processes in Biocatalysts. Contribution of Aromatic Moieties. *J. Am. Chem. Soc.* **1997**, *119*, 3157–3158. [CrossRef]
25. Shafferman, A.; Ordentlich, A.; Barak, D.; Stein, D.; Ariel, N.; Velan, B. Aging of somanyl-acetylcholinesterase adducts: Facts and models. *Biochem. J.* **1997**, *324*, 996–998.
26. Mukhametgalieva, A.R.; Nemtarev, A.V.; Sykaev, V.V.; Pashirova, T.N.; Masson, P. Activation/Inhibition of Cholinesterases by Excess Substrate: Interpretation of the Phenomenological b Factor in Steady-State Rate Equation. *Int. J. Mol. Sci.* **2023**, *24*, 10472. [CrossRef]
27. Masson, P. Double-mutant Thermodynamic Cycles under High Hydrostatic Pressure. In *Advances in High Pressure Bioscience and Biotechnology II*; Springer: Berlin/Heidelberg, Germany, 2003; pp. 61–68.
28. Masson, P.; Clery, C.; Guerra, P.; Redslob, A.; Albaret, C.; Fortier, P.-L. Hydration change during the aging of phosphorylated human butyrylcholinesterase: Importance of residues aspartate-70 and glutamate-197 in the water network as probed by hydrostatic and osmotic pressures. *Biochem. J.* **1999**, *343*, 361–369. [CrossRef] [PubMed]
29. Viragh, C.; Kovach, I.M.; Pannell, L. Small molecular products of dealkylation in soman-inhibited electric eel acetylcholinesterase. *Biochemistry* **1999**, *38*, 9557–9561. [CrossRef]
30. Viragh, C.; Akhmetshin, R.; Kovach, I.M.; Broomfield, C. Unique Push−Pull Mechanism of Dealkylation in Soman-Inhibited Cholinesterases. *Biochemistry* **1997**, *36*, 8243–8252. [CrossRef]
31. Sanson, B.; Nachon, F.; Colletier, J.-P.; Froment, M.-T.; Toker, L.; Greenblatt, H.M.; Sussman, J.L.; Ashani, Y.; Masson, P.; Silman, I.; et al. Crystallographic snapshots of nonaged and aged conjugates of soman with acetylcholinesterase, and of a ternary complex of the aged conjugate with pralidoxime. *J. Med. Chem.* **2009**, *52*, 7593–7603. [CrossRef]
32. Li, H.; Schopfer, L.M.; Nachon, F.; Froment, M.-T.; Masson, P.; Lockridge, O. Aging Pathways for Organophosphate-Inhibited Human Butyrylcholinesterase, Including Novel Pathways for Isomalathion, Resolved by Mass Spectrometry. *Toxicol. Sci.* **2007**, *100*, 136–145. [CrossRef] [PubMed]
33. Carletti, E.; Li, H.; Li, B.; Ekström, F.; Nicolet, Y.; Loiodice, M.; Gillon, E.; Froment, M.T.; Lockridge, O.; Schopfer, L.M.; et al. Aging of Cholinesterases Phosphylated by Tabun Proceeds through O-Dealkylation. *J. Am. Chem. Soc.* **2008**, *130*, 16011–16020. [CrossRef] [PubMed]
34. Miller, K.A.; He, Y.; Allen, S.K.; McElroy, C.A.; Callam, C.S.; Hadad, C.M. Unprecedented Alkylation of the Catalytic Histidine in the Aging of Cholinesterases after Inhibition by Organophosphorus Pesticides. *Chem. Res. Toxicol.* **2025**, *38*, 503–518. [CrossRef]
35. Bouknight, K.D.; Jurkouich, K.M.; Compton, J.R.; Khavrutskii, I.V.; Guelta, M.A.; Harvey, S.P.; Legler, P.M. Structural and kinetic evidence of aging after organophosphate inhibition of human Cathepsin A. *Biochem. Pharmacol.* **2020**, *177*, 113980. [CrossRef]
36. Harris, L.W.; Fleisher, J.H.; Clark, J.; Cliff, W.J. Dealkylation and Loss of Capacity for Reactivation of Cholinesterase Inhibited by Sarin. *Science* **1966**, *154*, 404–407. [CrossRef] [PubMed]
37. Segall, Y.; Waysbort, D.; Barak, D.; Ariel, N.; Doctor, B.P.; Grunwald, J.; Ashani, Y. Direct observation and elucidation of the structures of aged and nonaged phosphorylated cholinesterases by phosphorus-31 NMR spectroscopy. *Biochemistry* **1993**, *32*, 13441–13450. [CrossRef]
38. Amitai, G.; Ashani, Y.; Gafni, A.; Silman, I. Novel pyrene-containing organophosphates as fluorescent probes for studying aging-induced conformational changes in organophosphate-inhibited acetylcholinesterase. *Biochemistry* **1982**, *21*, 2060–2069. [CrossRef]
39. Berman, H.A.; Decker, M.M. Kinetic, equilibrium, and spectroscopic studies on dealkylation ("aging") of alkyl organophosphonyl acetylcholinesterase. Electrostatic control of enzyme topography. *J. Biol. Chem.* **1986**, *261*, 10646–10652. [CrossRef] [PubMed]
40. Masson, P.; Marnot, B.; Lombard, J.-Y.; Morelis, P. Etude électrophorétique de la butyrylcholinestérase agée après inhibition par le soman. *Biochimie* **1984**, *66*, 235–249. [CrossRef]
41. Katz, F.S.; Trakht, I.; Pecic, S.; Rinderspacher, A.; Schneider, L.; Landry, D.W.; Stojanovic, M.N. Conformational Constraint for Prevention of Aging of Organophosphate-Inhibited Acetylcholinesterase. *ChemBioChem* **2025**, *26*, e202500186. [CrossRef]
42. Masson, P.; Goasdoue, J.-L. Evidence that the conformational stability of 'aged' organophosphate-inhibited cholinesterase is altered. *Biochim. Biophys. Acta-Protein Struct. Mol. Enzymol.* **1986**, *869*, 304–313. [CrossRef]
43. Ashani, Y.; Gentry, M.K.; Doctor, B.P. Differences in conformational stability between native and phosphorylated acetylcholinesterase as evidenced by a monoclonal antibody. *Biochemistry* **1990**, *29*, 2456–2463. [CrossRef] [PubMed]
44. Masson, P.; Gouet, P.; Clery, C. Pressure and Propylene Carbonate Denaturation of Native and "Aged" Phosphorylated Cholinesterase. *J. Mol. Biol.* **1994**, *238*, 466–478. [CrossRef]
45. Masson, P.; Lockridge, O. Butyrylcholinesterase for protection from organophosphorus poisons: Catalytic complexities and hysteretic behavior. *Arch. Biochem. Biophys.* **2010**, *494*, 107–120. [CrossRef]
46. Masson, P.; Lushchekina, S. Conformational Stability and Denaturation Processes of Proteins Investigated by Electrophoresis under Extreme Conditions. *Molecules* **2022**, *27*, 6861. [CrossRef]

47. Gabel, F.; Masson, P.; Froment, M.-T.; Doctor, B.P.; Saxena, A.; Silman, I.; Zaccai, G.; Weik, M. Direct Correlation between Molecular Dynamics and Enzymatic Stability: A Comparative Neutron Scattering Study of Native Human Butyrylcholinesterase and its "Aged" Soman Conjugate. *Biophys. J.* **2009**, *96*, 1489–1494. [CrossRef]
48. Carletti, E.; Aurbek, N.; Gillon, E.; Loiodice, M.; Nicolet, Y.; Fontecilla-Camps, J.-C.; Masson, P.; Thiermann, H.; Nachon, F.; Worek, F. Structure–activity analysis of aging and reactivation of human butyrylcholinesterase inhibited by analogues of tabun. *Biochem. J.* **2009**, *421*, 97–106. [CrossRef]
49. Tang, X.; Zhu, L.; Chen, J.; Hu, J.; Zhang, Q.; Wang, W. Reaction pathway for reactivation and aging of paraoxon-inhibited-acetylcholinesterase: A QM/MM study. *Comput. Theor. Chem.* **2014**, *1035*, 44–50. [CrossRef]
50. Zhuang, Q.; Young, A.; Callam, C.S.; McElroy, C.A.; Ekici, Ö.D.; Yoder, R.J.; Hadad, C.M. Efforts toward treatments against aging of organophosphorus-inhibited acetylcholinesterase. *Ann. N. Y. Acad. Sci.* **2016**, *1374*, 94–104. [CrossRef] [PubMed]
51. Hajimohammadi, S.; Lockridge, O.; Masson, P. New views on physiological functions and regulation of butyrylcholinesterase and potential therapeutic interventions. *Front. Mol. Biosci.* **2025**, *12*, 1625318. [CrossRef] [PubMed]
52. Shafferman, A.; Ordentlich, A.; Barak, D.; Stein, D.; Ariel, N.; Velan, B. Aging of phosphylated human acetylcholinesterase: Catalytic processes mediated by aromatic and polar residues of the active centre. *Biochem. J.* **1996**, *318*, 833–840. [CrossRef]
53. Štalc, A.; Šentjurc, M. A contribution to the mechanism of action of SAD-128. *Biochem. Pharmacol.* **1990**, *40*, 2511–2517. [CrossRef]
54. Dawson, R.; Dowling, M.; Poretski, M. Assessment of the competition between tacrine and gallamine for binding sites on acetylcholinesterase. *Neurochem. Int.* **1991**, *19*, 125–133. [CrossRef]
55. Quinn, D.M.; Topczewski, J.; Yasapala, N.; Lodge, A. Why is aged acetylcholinesterase so difficult to reactivate? *Molecules* **2017**, *22*, 1464. [CrossRef] [PubMed]
56. Ekström, F.J.; Åstot, C.; Pang, Y.-P. Novel Nerve-Agent Antidote Design Based on Crystallographic and Mass Spectrometric Analyses of Tabun-Conjugated Acetylcholinesterase in Complex with Antidotes. *Clin. Pharmacol. Ther.* **2007**, *82*, 282–293. [CrossRef]
57. An, Y.; Zhu, Y.; Yao, Y.; Liu, J. Is it possible to reverse aged acetylcholinesterase inhibited by organophosphorus compounds? Insight from the theoretical study. *Phys. Chem. Chem. Phys.* **2016**, *18*, 9838–9846. [CrossRef]
58. Khavrutskii, I.V.; Wallqvist, A. β-Aminoalcohols as Potential Reactivators of Aged Sarin-/Soman-Inhibited Acetylcholinesterase. *ChemistrySelect* **2017**, *2*, 1885–1890. [CrossRef]
59. Quinn, D.M. Resurrection Biology: Aged Acetylcholinesterase Brought Back to Life. *J. Med. Chem.* **2018**, *61*, 7032–7033. [CrossRef]
60. Ferreira, L.V.F.; Santos, T.M.R.; Tavares, C.A.; Rasouli, H.; Ramalho, T.C. Atomistic Origins of Resurrection of Aged Acetylcholinesterase by Quinone Methide Precursors. *Molecules* **2024**, *29*, 3684. [CrossRef]
61. Franjesevic, A.J.; Sillart, S.B.; Beck, J.M.; Vyas, S.; Callam, C.S.; Hadad, C.M. Resurrection and Reactivation of Acetylcholinesterase and Butyrylcholinesterase. *Chem.—Eur. J.* **2019**, *25*, 5337–5371. [CrossRef] [PubMed]
62. Lovins, A.R.; Miller, K.A.; Homoelle, R.K.; Hoover, H.J.; McElroy, C.A.; Callam, C.S.; Hadad, C.M. Imidazole as a Pendant Reactivation Ligand Increases Efficacy Scope for Reactivation and Resurrection of Organophosphorus-Inhibited/Aged Cholinesterases by Quinone Methide Precursors. *ACS Chem. Neurosci.* **2026**, *17*, 962–975. [CrossRef]
63. Yoder, R.J.; Zhuang, Q.; Beck, J.M.; Franjesevic, A.; Blanton, T.G.; Sillart, S.; Secor, T.; Guerra, L.; Brown, J.D.; Reid, C.; et al. Study of para-Quinone Methide Precursors toward the Realkylation of Aged Acetylcholinesterase. *ACS Med. Chem. Lett.* **2017**, *8*, 622–627. [CrossRef] [PubMed]
64. Pashirova, T.N.; Zueva, I.V.; Petrov, K.A.; Lukashenko, S.S.; Nizameev, I.R.; Kulik, N.V.; Voloshina, A.D.; Almasy, L.; Kadirov, M.K.; Masson, P.; et al. Mixed cationic liposomes for brain delivery of drugs by the intranasal route: The acetylcholinesterase reactivator 2-PAM as encapsulated drug model. *Colloids Surf. B Biointerfaces* **2018**, *171*, 358–367. [CrossRef] [PubMed]
65. Gandhi, S.; Shastri, D.H.; Shah, J.; Nair, A.B.; Jacob, S. Nasal Delivery to the Brain: Harnessing Nanoparticles for Effective Drug Transport. *Pharmaceutics* **2024**, *16*, 481. [CrossRef] [PubMed]
66. Qiu, Y.; Huang, S.; Peng, L.; Yang, L.; Zhang, G.; Liu, T.; Yan, F.; Peng, X. The Nasal–Brain Drug Delivery Route: Mechanisms and Applications to Central Nervous System Diseases. *MedComm* **2025**, *6*, e70213. [CrossRef] [PubMed]