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STRUCTURAL REQUIREMENTS, SYNTHESIS AND INTERACTION OF SECONDARY NITROSAMINE INHIBITORS WITH ACETYLCHOLINESTERASE FROM *TORPEDO FUSCOMACULATA*

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A series of secondary diaryl and dialkyl nitrosamines have been synthesised and tested as substrates and/or inhibitors of highly purified acetyl- cholinesterase from *Torpedo fuscomaculata*. None were found to act as substrate, but many could selectively inhibit the enzyme. Kinetic analysis has shown that all the nitrosamines act as reversible competitive inhibitors with respect to the substrate, acetylthiocholine chloride; with time they act as irreversible covalent inhibitors. Scatchard analysis indicates that aliphatic nitrosamines have a weaker affinity for the enzyme compared to the aromatic and heterocyclic nitrosamines. In all cases the number of binding sites was four. Pseudo first-order kinetics are observed with the rate constant being proportional to the concentration of the nitrosamine and the order of reaction being equal to one.

KEY WORDS: Acetylcholinesterase, competitive inhibition, nitrosamines

INTRODUCTION

The existence in excitable tissue such as nerve, muscle, and electrogenic tissue of nicotinic acetylcholine receptor (nAChR) and acetylcholinesterase (AChE) both with an affinity for acetylcholine raises the question as to whether, and to what extent, the available neurotransmitter is shared between the two macromolecules. With the exception of polypeptide neurotoxins all ligands of the enzyme are also ligands of the receptor. The ligands of the enzyme can be subdivided into substrates and competitive inhibitors while those of the receptor can be subdivided into agonists and antagonists (competitive blockers). Since it had been found in these laboratories[1] that the powerful carcinogenic nitrosamines acted as agonists to nAChR it was reasonable to suppose that they would interact with the enzyme as well.

Careful selection of the nitrosamines and a study into the kinetics and change of fluorescence observed upon their binding to the enzyme may provide insights into, (i) the molecular mechanism of functional excitation and permeability change, (ii) the principles on which more complex neural functions are based, (iii) intramolecular

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communication and, (iv) functional properties of integral membrane proteins. Binding, kinetic and fluorescent studies critically depend on the specificity of the ligands applied and the properties of the observed changes in fluorescence. Nitrosamines are suitable ligands to use for interaction with the enzyme since they are a rich source of carbocations.[2] The chemical approach to biological problems through investigations of models rests upon the ability of the chosen system to mimic some functions of the biological ensemble. This then implies a full understanding of, amongst other things, the structure, localisation and functions of a particular biological system under study.

MATERIALS AND METHODS

Materials

Sephacryl S-400 was purchased from Pharmacia; bovine serum albumin, acetylcholine, acetylthiocholine chloride and 5,5-dithiobis-(2-nitrobenzoic acid) were obtained from Sigma. Nitrosamines were either prepared as described below or purchased from Aldrich chemical Co. Dialysis membranes were obtained from Spectropor. All other inorganic and buffer materials were of reagent grade. Live electric rays *Torpedo fuscomaculata* were caught in the Bushmans river esturary off the South East coast of South Africa.

Methods

Biochemical Isolation of Acetylcholinesterase from Torpedo fuscomaculata Electric organs were excised from a freshly killed *Torpedo* ray and stored at –80°C until required. Electric tissue (300 g) was homogenised for 14 min in ammonium sulphate solution (5%, 750 ml) and then centrifuged (3000 × g) for 15 min. The supernatant was decanted, the precipitate re-extracted with ammonium sulphate (5%, 250 ml) followed by centrifugation (3000 × g) for 15 min. The precipitate was discarded.

Solid ammonium sulphate was added to the cold (4°C) combined supernatants to a final concentration of 31% and the solution allowed to stand (4°C) for 20 h. The precipitate was collected by centrifugation (40 000 × g) for 15 min and dissolved in cold (4°C) water, the pH adjusted to 7 with 1M NaOH and the total volume adjusted to 90 ml.

Solid ammonium sulphate was added to a final concentration of 20% and the mixture allowed to stand (4°C) for 20 h then centrifuged (54 000 × g) for 10 min. The supernatant was discarded and the precipitate dissolved in cold water (5.0 ml), dialysed against distilled water and finally lyophilised.

Protein determination Protein was measured by the modification of the Lowry[3] method using bovine serum albumin as the standard.

Acetylcholinsterase assay Hydrolysis of acetylthiocholine by AChE was recorded at 410 nm at room temperature following the method according to Ellman.[4] The assay mixture (3.14 ml) contained phosphate buffer (0.1M, pH 8), buffered Ellman's reagent (5,5′-dithiobis-2-nitrobenzoic acid) (10.0 mM), $NaHCO_3$ (17.85 mM, pH 7.0) and acetylthiocholine chloride (0.2 mM). Aliquots were made up to 3.12 ml with phosphate

buffer (pH 8) and the reaction was initiated by the addition of 0.02 ml of the enzyme. The enzyme concentration was determined using an absorption coefficient ($A_{1\ cm}$) of 16.1 at 280 nm and a M_r 260,000.[5]

Equilibrium dialysis These experiments were carried out by the method suggested by McPhie.[6] Acetylcholinesterase (6 $\mu g.ml^{-1}$, 3.0 ml) was dialysed at 4°C against phosphate buffer (20.0 mM, pH 7.6) containing nitrosamine (8 – 20 μM) under constant agitation (16 h). After equilibrium the concentration of nitrosamine outside the dialysis bag was determined. Data were analyst according to Scatchard.[7] Concentrations of nitrosamines were determined spectrophotometrically using published absorption coefficients.[8]

Gel chromatoqraphy The binding of the nitrosamines to the enzyme was studied on a column (1 × 20 cm) of Sephacryl S-400 pre-equilibrated in 0.1M phosphate buffer, pH 7.0. Reaction mixtures (3.0 ml) containing enzyme (1.5 μM) and nitrosamine (2 – 50 μM) were applied to the column, the flow rate being 1.85 $ml.min^{-1}$. The effluent was monitored at 280 nm. The determination of the number of binding sites and the dissociation constants (K_d) of the enzyme-ligand complex was determined according to the method of Scatchard.[7]

Fluorimetric analysis Fluorimetric measurments were made with a Hitachi fluorescence spectrophotometer using an excitation light source from an Xenon 150 lamp; the fluorescence was measured through the cell at an angle of 90° to the incident beam. All fluorimetric titrations were carried out at 20°C in 0.1M phosphate buffer (pH 7.2).

For studies involving binding of the nitrosamines to the enzyme increasing concentrations of ligand (0 – 3 μM) were added to a sample of the enzyme (12.1 $ng.ml^{-1}$). The mixture was excited at 285 nm and emission measured at 330 nm. The fluorescence data were used to determine the dissociation constant (K_d) of the ligands with the complexes formed with the enzyme.

Inhibition studies Inhibition of acetylcholinesterase by the interaction with various nitrosamines was studied in the presence of phosphate buffer (0.1M, pH 8), acetylthiocholine chloride (0 – 500 μM), and ligand (0 – 45 μM). Aliquots (2.8 ml) of such reaction solutions were initiated by the addition of the enzyme (0.2 ml).

Reversibility The test of the reversibility of the interaction was carried out by removal of the nitrosamine by dialysis. A mixture of the nitrosamine (20 μM) and the enzyme preparation (0.2 ml) was incubated at room temperature (1 h). The solution was dialysed against deionised water at 4°C, pH 7.5, 48 h. The enzyme activity was determined at the beginning of dialysis, after 24 h and after 48 h.

Chemical Synthesis of nitrosamines The nitrosamines used in this research were prepared by the classical method of nitrosation in acidic solution with sodium nitrite.[9] The reaction products were isolated and purified by standard techniques. Some of the physical properties of these nitrosamines are given (Table 1).

TABLE 1
Synthesis and Physical Properties of Disubstituted Nitrosamines

$$\begin{array}{c} R^1{-}N - N = O \\ | \\ R \end{array}$$

	Form	R^1	R	Yield(%)	Abbrev.	mp(°C)	bp(°C/mmHg)	Ref.
1.	oil	CH_3	CH_3	73	DMNA	–	146°/750	9
2.	oil	CH_3CH_2	CH_3CH_2	80	DENA	–	173°/750	9
3.	oil	$CH_3(CH_2)_2$	$CH_3(CH_2)_2$	70	DPNA	–	179°/750	9
4.	oil	$CH_3(CH_2)_3$	$CH_3(CH_2)_3$	78	DBNA	–	130°/750	9
5.	crystals	Ph	Ph	70	DPhNA	68°	–	9
6.	oil	CH_3	Ph	61	MPhNA	74°	–	9
7.	oil	CH_3CH_2	Ph	60	EPhNA	81°	–	9
8.	oil	$OH(CH_2)_2$	$OH(CH_2)_2$	80	DEtNA	–	120°/1	10
9.	solid	$-(CH_2)_2O(CH_2)_2-$		81	MorNA	26°	98°/1	9
10.	oil	$-(CH_2)_5-$		80	PipNA	-	110°/750	9
11.	oil	-CH=CH-CH=CH-CH=		69	PyrNA	-	115°/750	9
12.	crystals	CH_3	$-CH_2COOH$	90	MGlyNA	75°	–	11

N-Nitrosodiethanolamine (DEtNA)[10] 2M hydrochloric acid (167 ml, 334 mM) was added slowly to a cold (8°C) stirred diethanolamine (35 g, 334 mM) and the resulting solution treated dropwise over 1 h with a cold (5°C) solution of sodium nitrite (27 g, 391 mM) in water (35 ml). The reaction mixture was stirred at 10°C (30 min) and then at 35°C (1 h). Ethanol (50 mls) was added, the solution filtered and the solvents removed to afford a yellow oil (42 g, 95%) bp = 120°/750mm Hg. ^{1}H n.m.r. δ 3.5 (2H, dt, J = 5Hz, CH_2-N-N(O)); 3.6 – 4 (4H, m, $2CH_2OH$); 4.2 (2H, t, J = 5Hz, CH_2-N-N(O)); 4.7 – 5 (2H, m, 2 × OH).

N-methyl-N-Nitrosoglycine (MGlyNA)[11] Sodium nitrite (7.63 g, 110 mM) in water (15 ml) was added dropwise to a hot (90°C) solution of *N*-methylglycine (8.9 g, 100 mM) dissolved in hydrochloric acid (5M, 5ml). The mixture was heated (1 h), cooled and extracted with ether. The ether layers were combined, dried ($MgSO_4$) and evaporated to afford an oil which crystallised on standing. Recrystallisation (ethyl acetate) afforded yellow crystals 8.06 g; (90%), mp = 74° – 76°C (lit.[11] m.p. = 75°C). ν_{max} 1730, 1440 cm^{-1}. ^{1}H n.m.r. ($CDCl_3$) δ 3.9 (3H, s, CH_3-N); 4.3, 4.7 (2H, 2xs, CH_2).

Diphenylnitrosamine (DPhNA)[9] Diphenylamine (25.4 g, 150 mM) dissolved in ethanol (250 ml) was acidified with hydrochloric acid (5M, 25 ml) and the whole cooled below 5°C. Sodium nitrite (13.8 g, 200 mM) in cold (4°C) water (30 ml) was added with stirring and the crystals of diphenylnitrosamine collected by filtration. Recrystallisation (petroleum ether) afforded crystals, 17.8 g (70%), m.p. = 67° – 69°C. (lit.[9] m.p. = 68° – 70°C).

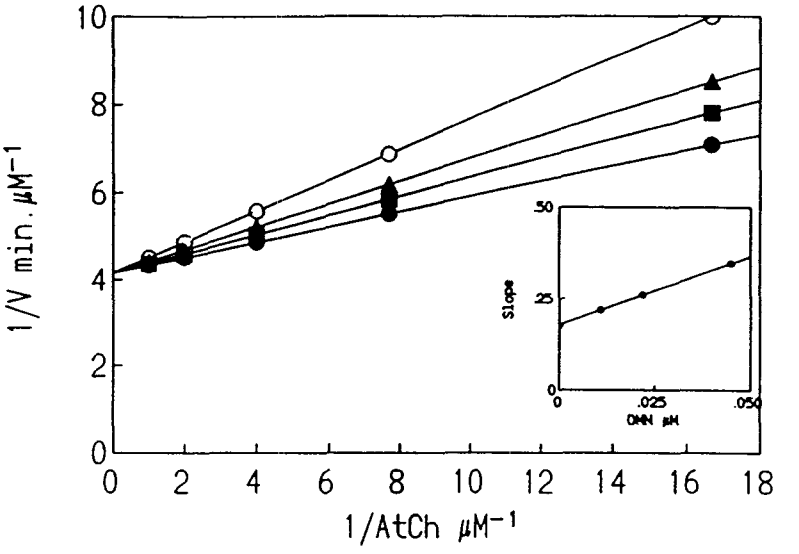


FIGURE 1 Variation in the activity of acetylcholinesterase in the presence of different concentrations of dimethylnitrosamine (DMNA) at pH 7.0; ●, 0 μM; ■, 11 μM; ▲, 22μM; ○, 45 μM. V represents the rate of enzymatic reaction. The secondary plot represents the slopes of the Lineweaver-Burk lines versus DMNA concentration.

RESULTS

A series of nitrosamines were tested as substrates with acetylcholinesterase from *Torpedo fuscomaculata* or as inhibitors with acetylthiocholine chloride as substrate. None were found to be a substrate but many could selectively inhibit the enzyme.

The evidence of a reversible inhibitory mechanism was realised after characteristic Lineweaver-Burk plots (Figure 1) were produced, indicating that all the nitrosamines studied were competitive inhibitors of the enzyme with respect to acetylthiocholine chloride. A replot of the slopes of the double reciprocal plot versus ligand concentration is linear (Figure 1, inset). The relevant kinetic parameters for the nitrosamines are given in Table 2. The K^{app} are the respective Michaelis constants estimated from the intercept on the abscissa; K_i are the inhibitor constants of the enzyme-inhibitor complex calculated either from the extrapolation of the graph of slope versus nitrosamine concentration (Figure 1; inset) or from equation (1),

$$K^{app} = K_m(1 + K_i^{-1}I) \quad (1)$$

where I is the concentration of the particular nitrosamine. Using variable substrate concentrations the values of K_m and V_{max} for acetylcholinesterase were 42 μM and 240 μM.min^{-1} respectively. The aromatic compounds diphenyl-nitrosamine,

TABLE 2
Kinetic and Binding Data of Disubstituted Nitrosamines

		k_1	k_2	K_d (μM)					
	K_i(μM)	(min^{-1})	($M^{-1}.min^{-1}$)	Fluorimetry	p	Equil.Dialysis	p	Gel Chromat.	p
1	25.3	0.44	40.0	5.4	4	5.2	3.9	5.8	3.8
2	27.0	0.41	37.5	6.0	4	6.0	4.1	6.0	3.8
3	29.6	0.38	34.2	6.4	4	6.6	4.2	6.5	3.6
4	32.5	0.34	31.1	7.7	4	8.0	3.8	8.4	3.9
5	8.6	1.29	117.7	2.3	4	1.8	4.1	2.5	4.1
6	10.2	1.09	99.2	3.0	4	2.9	4.1	3.6	4.1
7	12.3	0.91	82.3	3.8	4	3.3	3.9	4.1	3.9
8	21.8	0.51	46.4	4.6	4	4.3	3.7	4.9	4.0
9	73.9	0.15	13.7	2.0	4	2.0	3.9	2.0	3.8
10	123.0	0.09	8.2	4.2	4	4.0	4.0	4.0	3.8
11	66.1	0.17	15.3	1.8	4	2.0	4.0	2.0	4.0
12	15.2	0.73	66.6	4.0	4	3.9	4.1	4.0	4.1

methylphenylnitrosamine and ethylphenylnitrosamine (**5**, **6** and **7**, Table 2) showed a marked inhibition of the enzyme with K_i of 8.6 μM, 10.2 μM and 12.3 μM respectively. The aliphatic nitrosamines (**1 – 4**, Table 2) were less potent as inhibitors with K_i values of 25.3 μM (DMNA), 27.0 μM (DENA), 29.6 μM (DPNA) and 32.5 μM (DBNA). Heterocyclic nitrosamines had K_i values of 66.1 μM (PyrNA), 73.9 μM (MorNA) and 123 μM (PipNA). The problem of solubility with many nitrosamines was overcome by using 2% polyethylene glycol as a cosolvent (in control experiments polyethylene glycol had no effect on the inhibitions of acetylcholinesterase).

Incubation of acetylcholinesterase with a nitrosamine resulted in a progressive loss of enzyme activity. As shown (Figure 2a) the inactivation of the enzyme by DPhNA was dependent on the concentration of the nitrosamine and on the incubation time. When the enzyme was mixed with 11 μM DPhNA, 22% of the control activity remained after 60 min while in the presence of 45 μM DPhNA it took only 15 min to reach the same activity. After 1 h, 47% activity remained in the presence of 22 μM DMNA and 0% activity if 45 μM DMNA was used. The inactivation followed pseudo first-order kinetics as indicated by typical plots of enzyme activity vs time (Figure 2a). Double log plots of k_1 as a function of nitrosamine concentration (Figure 2b) were also linear yielding a slope of 1 and hence a reaction order (n) of 1 with respect to nitrosamine (equation (2)). The second-order rate constant (k_2) was obtained from the slope of the linear plots of pseudo first-order rate constant (k_1) vs nitrosamine concentration (Figure 2c). The values are also given in Table 2.

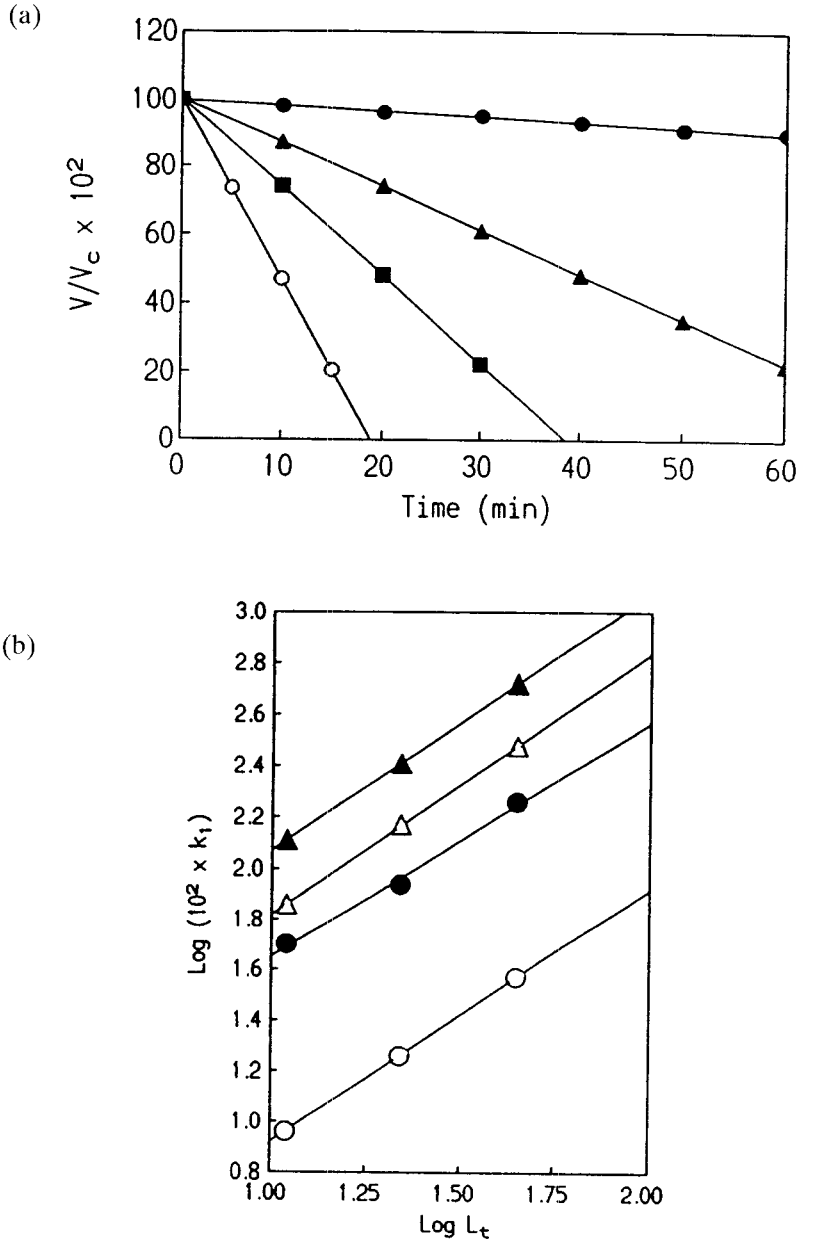
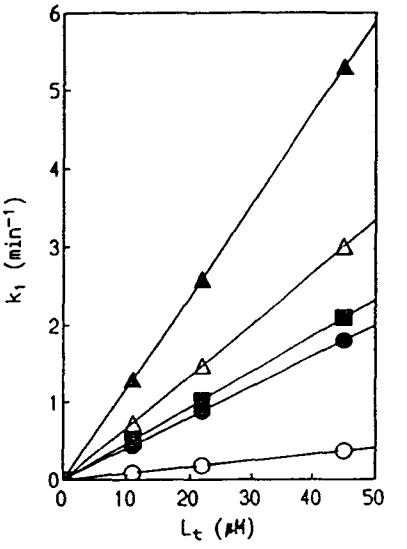


FIGURE 2 Inactivation of acetylcholinesterase by diphenylnitrosamine (DPhNA) at pH 7.0 (a) Concentration of DPhNA is •, 0 μM; ▲, 11 μM; ■, 22 μM, ○, 45 μM. V and V_c are enzyme activities of experimental and control. (b) Apparent order with respect to DPhNA concentrations (c) Plot of k_1 obtained at various concentrations of DPhNA against ligand concentration.

(c)



(cont'd Fig. 2)

$$\log k_1 = n \log (\text{Ligand}) + \text{Log}k_2 \quad (2)$$

Typical fluorescence binding curves and the analysis as a plot of $1/(1-\theta)$ versus (ligand)/ θ are shown for the effect of the nitrosamines on acetylcholinesterase (Figures 3 and 4). From the plots the dissociation constant (K_d) for a single binding site for the nitrosamines can be estimated (equations (3) and (4)) and are given in Table 2

$$K_d/(1-\theta) = (L)_t/\theta - \text{P}(A)_t \quad (3)$$

$$\theta = \Delta F/\Delta F_{\max} \quad (4)$$

where $(A)_t$ is the total concentration of acceptor in the system, p is the number of binding sites per mole, θ is the fractional occupancy of total acceptor sites by ligand, ΔF is the change in fluorescence in the presence of a known amount of ligand, $\Delta F_{\max}$ is the change in fluorescence at full saturation with ligand and $(L)_t$ is the total concentration of ligand.

In each case the number of binding sites was four (Figure 4). The Kd for the titration of the nitrosamines showed for aliphatic nitrosamines an increase as the number of carbon atoms increased. DMNA had a K_d value of 5.4 μM. DPNA with a 3-carbon chain had a K_d value of 6.4 μM and the 4-carbon DBNA was represented by a K_d value of 7.7 μM.

The aromatic nitrosamines bind relatively tightly with K_d values of 2.3 μM (DPhNA) 3.0 μM (MPhNA) and 3.8 μM (EPhNA). Even the heterocyclic nitrosamines show marked binding in 2.0 μM for MorNA and 4.2 μM for PipNA.

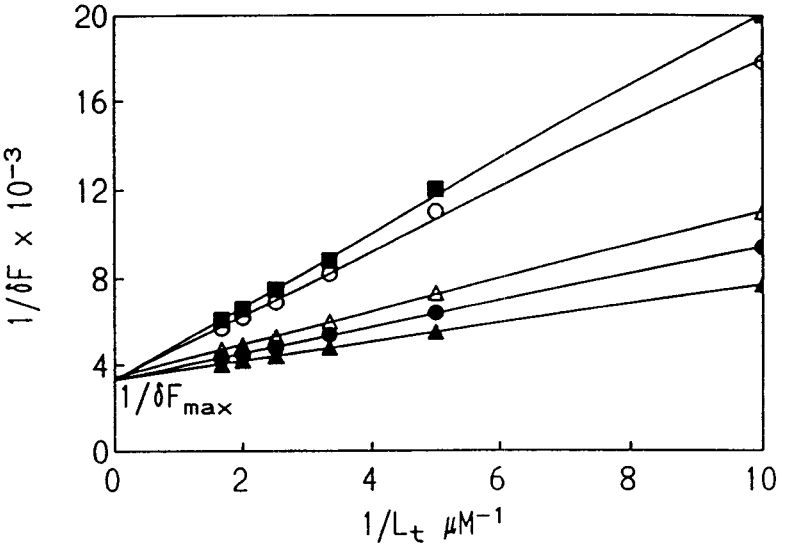


FIGURE 3 Double reciprocal plots of fluorescence change on binding of dibutyl-nitrosamine (▲) (DBNA), dipropylnitrosamine (●) (DPNA), diethanol-nitrosamine (△) (DEtNA), diphenylnitrosamine (○) (DPhNA) and morpholinenitrosamine (■) (MORNA) to acetylcholinesterase.

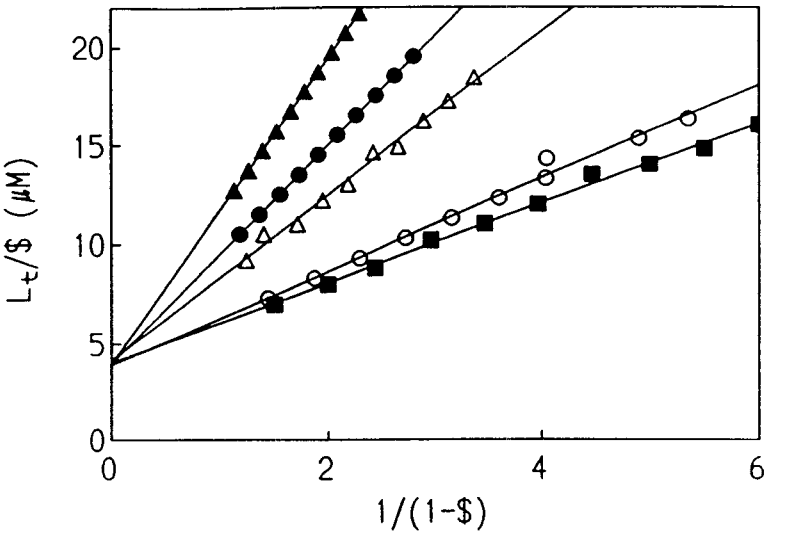


FIGURE 4 Analysis data for the binding of dibutylnitrosamine (▲) (DBNA), dipropylnitrosamine (●) (DPNA), diethanolnitrosamine (△) (DEtNA), diphenylnitrosamine (○) (DPhNA) and morpholinenitrosamine (■) (MORNA) to acetylcholinesterase.

The reversible equilibrium characterised in Figure 3 and 4 by the interaction of nitrosamine with acetylcholinesterase has been characterised by gel chromatography and equilibrium dialysis. In either case the number of binding sites and the dissociation constant (K_d) of the enzyme ligand complex was determined according to Scatchard[7] (equation (5) and (6)).

$$L_b = \text{P}(\Delta A_x/\Delta Ax_{\max} \tag{5}$$

$$L_b/L_f = (p - L_b)/K_d \tag{6}$$

where ΔA_x is the difference in the absorbance of the two solutions at wavelength x, $\Delta Ax_{\max}$ is the maximal absorbance change, L_b is the concentration of bound ligand, p is the number of binding sites per mole, $(L)_f$ is the concentration of free ligand, K_d is the dissociation constant of the ligand-enzyme complex. Results indicate that the enzyme contains a single class of binding sites (Figure 5). The calculated dissociation constants of the various nitrosamine-AChE complexes are presented in Table 2. From equilibrium dialysis the K_d values for the titration of the nitrosamines with the enzyme showed an increase from 5.2 μM (DMNA) to 6.6 μM (DPNA) and 8.0 μM (DBNA). The aromatic nitrosamines bind relatively strongly with a dissociation constant of 1.8 μM (DPhNA), 2.9 μM (MPhNA) and 3.3 μM (EPhNA).

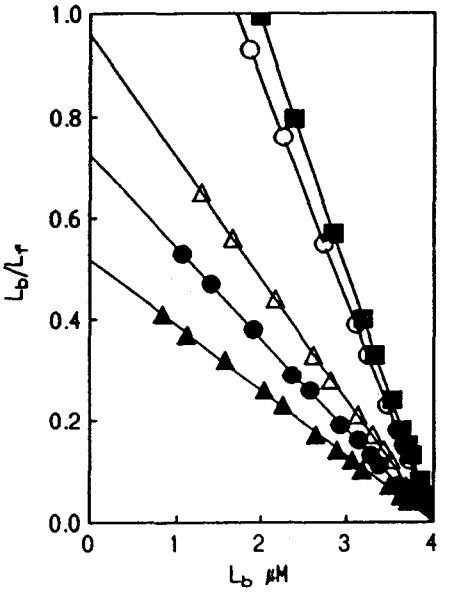


FIGURE 5 Scatchard plot for the binding of dibutylnitrosamine (▲) (DBNA), dipropylnitrosamine (•) (DPNA), diethanolnitrosamine (△) (DEtNA), diphenylnitrosamine (○) (DPhNA) and morpholinenitrosamine (■) (MORNA) to acetylcholinesterase.

Results from gel chromatography showed a similar trend. The dissociation constants for the titration of the nitrosamines with the enzyme increased with the number of carbon atoms: DMNA (5.8 μM), DPNA (6.5 μM) and DBNA (8.4 μM). Diphenylnitrosamine exhibited a strong binding affect (K_d = 2.5 μM), the other two slightly different at 3.6 μM (MPhNA) and 4.1 μM (EPhNA). Interestingly the heterocyclic nitrosamines (**9** and **10**) showed a reasonable binding of 2 μM (PIPNA) and 4 μM (MORNA). The number of binding sites on the enzyme averaged at 3.8 from both gel chromatography and equilibrium dialysis.

DISCUSSION

The potency of a particular nitrosamine in the interaction with AChE is determined by the dissociation constant for the enzyme-inhibitor complex. This fit into the active site of the enzyme, reflected by the values of K_d (or K_i) is largely determined by the size, structure, and configuration of the inhibiting molecule. The capability of the inhibitor to bind non covalently at, or close to, the active site could also influence these values.

As a result of extensive studies of the enzyme[12] it is well documented that the active site of AChE has two subsites, 4–5 Å apart, termed the anionic and esteratic sites. Brestkin *et al.*[13] suggest that the anionic site consists of a carboxylate ion derived from the side chain of a glutamic or aspartic acid residue. The more complex esteratic site has a serine hydroxyl which becomes acylated on interaction with the substrate, acetylcholine. Th chemically inert hydroxyl group is activated by the imidazole of a histidine residue in the immediate vicinity. The nitroso group (N = 0) of the nitrosamine resembles the carbonyl group (C = 0) of acetylcholine and it is suggested that this interacts with the activated serine hydroxyl to form an enzyme-inhibited complex (Figure 6a). This allows an *in situ* quaternary nitrogen to develop which simultaneously interacts with the nucleophilic serine hydroxyl or the anionic subsite that is in close proximity.

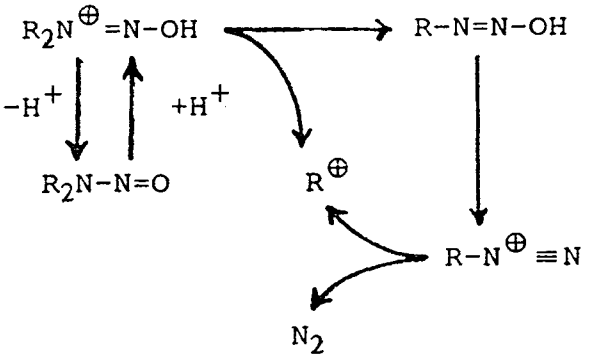
This article has shown that initially nitrosamines inhibit AChE reversibly and competitively with respect to the substrate acetylthiocholine chloride. A kinetic analysis of the inhibition of the enzyme by dimethylnitrosamine is presented (Figure 1) using the standard graphical technique of Lineweaver and Burk.[14] The lines are drawn by computer calculation for linear competitive inhibition (equation (7)) and least square analysis.[15]

$$V^{-1} = V_{\max}^{-1}(K_m(1 + K_i^{-1} \cdot I))S^{-1} + V_{\max}^{-1} \tag{7}$$

The canonical form $R_2N^{\oplus} = N{-}O^{-}$ is of importance in representations of the structure of these compounds and the dipole moment (μ = 4.0D) and barrier rotations about the N-N bond support this idea.[16] In either case the inhibitor prevents the access of acetylcholine to the active site thereby competitively inhibiting the enzyme.

The more lipophilic the nitrosamine the less the ability to bind and inhibit the enzyme. As the degree of alkylation of the nitroso group increased so the overall binding and inhibitory power decreased DENA (K_i = 27 μM), DPNA (K_i = 29.6 μM) and DBNA (K_i = 3.5 μM) (Table 2). The small methyl groups of DMN would not be expected to interfere with the process of inhibition while it is probable that

the larger bulky ligands induce a decrease in binding capacity. The least lipophilic diphenylnitrosamine was the most potent competitive inhibitor studied. This can be explained by the stability and reactivity of the nitrosamine itself and the difficulty in forming the corresponding intermediates ($AR-N^{\oplus} \equiv N$) and $AR^{\oplus}$. It has previously been established[2] that nitrosamines are a rich source of carbocations (Scheme 1).



[Scheme 1] Pathway for nitrosamine degradation

With diphenylnitrosamine this decomposition does not take place. Instead the *in situ* quaternary nitrogen compound (Scheme 1) developed within the nitrosamine molecule after binding to the active site interacts strongly with the anionic subsite of the enzyme causing a potent competitive inhibition. The reaction mechanism suggests that a protonated amino acid residue in the enzyme active site combines with the nitrosamine within the enzyme-substrate complex,[12] followed by a decomposition into the alkylating species (R^{+}). A change in environment from hydrophilic to hydrophobic is a prerequisite for good binding and has already been invoked as an important factor in enzyme catalysis.[17] Acetylcholinesterase active site is hydrophobic thus when the nitrosamines bind to the enzyme they pass from a hydrophilic to a hydrophobic environment. Obviously the ease of transition between the hydrophilic and hydrophobic environment coupled with the overall stability, energy, and size of the carbocation and intermediates formed will be reflected in the value of K_i for the nitrosamine. It is suggested that the nitrosamines act as suicide inhibitor and label the glutamic or aspartic carboxylate ion of the anionic subsite, thereby causing a loss of enzymatic activity through an irreversible mechanism (Figure 6b).

It is quite likely that the unstable carbocation generated may alkylate other amino acid such as the esteratic serine subsite in a non-specific manner or be reacted upon by the medium to afford the corresponding alcohol. It is obvious that the binding of the nitrosamines with the enzyme is influenced by the solvating properties of the catalytic site at which the reaction occurs. The acceleration of this process can be attributed to a change of the solvating conditions in the active centre resulting from the binding of the nitrosamine with the enzyme esteratic site. The nitrosamines themselves cannot increase the basicity of the microenvironment of the binding locus for the generated carbocation. We anticipate that the carbocation formed by the intrinsic decomposition

of the nitrosamine would be released within the hydrophobic environment of the active site and so would not be scavenged by the medium. In order to define a competitive inhibitor it must bind more tightly to the enzyme than does the substrate. Secondly the conformation of the inhibitor must be consistent with the structure of the transition state postulated for the enzyme. An initial event in the normal catalytic reaction involves formation of a covalent bond between a nucleophilic group of the enzyme and the substrate. A most important aspect of mechanism based inhibitors is that they first interact with the enzyme to form a reversible E — I complex and then form a covalent bond with a nucleophilic catalyst of the enzyme. There must be accumulation of a covalently bound complex which may be considered analogous to a steady state intermediate of the normal reaction (Figure 6). The simple expedient of placing a nitroso group in proper juxtaposition to the requisite substrate functionality supports this argument. Chemical modification of a COOH (at R^1) such as reduction to an alcohol (**8**, Table 2) or a conversion to an alkyl chain (**10**, Table 2) have definitely established that the acid group is essential for binding. These observations may be rationalised by assuming that a salt bridge between the inhibitor and a cationic amino acid residue on the enzyme is required. The K_i values of MGlyNA, DiEtNA and PipNA are 15.2 μM, 21.8 μM and 123 μM respectively (Table 2). If the K_m for the actual substrate is 42 μM then MGlyNA binds nearly three times tighter, DiEtNA twice as tight and PipNA three times less to the enzyme than does the substrate.

The cyclic nitrosopyridine, (PyrNA, **11**) nitrosopiperidine (PipNA, **10**) and nitrosomorpholine (MorNA, **9**) generate species that would be positively charged at both ends (scheme 1). Electrostatic repulsion results affording a weaker binding and less inhibitory power of the nitrosamine.

Detection of the intrinsic fluorescence change in a protein upon binding with ligands is one of the simplest and most direct methods to study ligand-induced conformational change. In the present investigation the quenching of fluorescence of the enzyme upon binding with the nitrosamines was used to study the enzyme-ligand interactions. The decrease in relative fluorescence on titration with the ligand supports a change in environment of the nitrosamine moiety when the binding to the protein takes place. This may arise due to the presence of non polar regions in or around the tryptophan-containing binding site of the enzyme.

Our findings from fluorescence titrations on the bindings of the nitrosamines tend to support that two discrete sites exist on AChE. The dissociation constants estimated agree closely with those obtained from equilibrium dialysis and gel chromatography. Furthermore our investigations provide a substantial amount of evidence indicating that the binding sites are independent and indistinguishable. The linearity of the Scatchard plots (Figure 5) from the binding of the nitrosamines to the enzyme further supports the absence of subunit interactions.

The facts that the reversible inhibition by the nitrosamine is competitive and the subsequent irreversible inactivation is essentially the same toward hydrolysis of acetylcholine support the proposal that the active site may be more specifically characterised by these nitrosamines. Fluorometric titrations, steady-state kinetics, equilibrium pertubations and ligand binding experiments all support the proposed models for nitrosamine binding to acetyl-cholinesterase (Figure 6a and 6b).

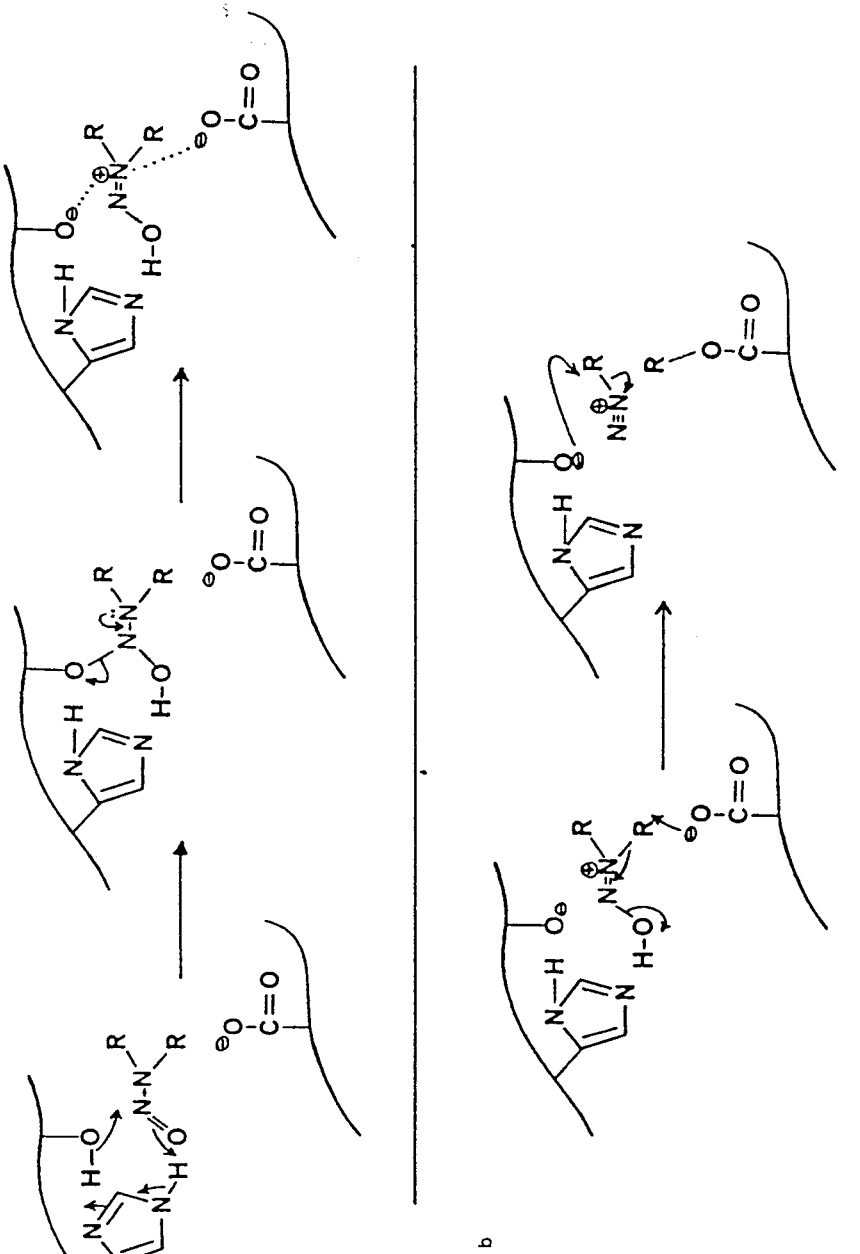


FIGURE 6 Suggested mechanism of interaction of nitrosamines with acetylcholinesterase involving (a) reversible and (b) irreversible inhibitory mechanisms.

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