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SYNTHESIS AND INTERACTION OF SECONDARY N-NITROSAMINES WITH ACETYLCHOLINESTERASE

by

TSIETSO BERNARD MMUTLE

BSc.(HONS.) (RHODES)

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"I OFTEN SAY THAT WHEN YOU CAN MEASURE WHAT YOU ARE SPEAKING ABOUT, AND EXPRESS IT IN NUMBERS, YOU KNOW SOMETHING ABOUT IT; BUT WHEN YOU CANNOT EXPRESS IT IN NUMBERS, YOUR KNOWLEDGE IS OF A MEAGRE AND UNSATISFACTORY KIND; IT MAY BE THE BEGINNING OF KNOWLEDGE BUT YOU HAVE SCARCELEY, IN YOUR THOUGHTS, ADVANCED TO THE STATE OF SCIENCE, WHATEVER THE MATTER MAY BE".

----LORD KELVIN (1824-1907)

ABSTRACT

Secondary N-nitrosamines: diphenylnitrosamine (DPhNA), dimethylnitrosamine (DMNA), diethylnitrosamine (DENA), dipropylnitrosamine (DPNA), dibutylnitrosamine (DBNA), diethanolnitrosamine (DEtNA), methylnitrosoglycine (MNGly), nitrosopyrrolidine (NPyr), nitrosomorpholine (NMor) and nitrosopiperidine (NPip) were synthesised , and their interaction with acetylcholinesterase (AChE) was investigated.

Analyses of kinetic results show that DMNA (Ki=34.78 μM); DENA (Ki=54.24 μM); DPNA(Ki=60.36 μM); DBNA(Ki=95.54 μM); DEtNA(Ki=43.68 μM) MNGly (Ki=30.18 μM); NPip (Ki=123 μM); NPyr (Ki=66.07 μM), NMor (Ki=73.93 μM) and DPhNA (Ki=20.32 μM) are competitive and reversible inhibitors of acetylcholinesterase, with respect to the substrate, acetylthiocholine chloride, ATChCl . With time they act as irreversible covalent inhibitors with dipropylnitrosamine producing 72% inactivation after 60 minutes.

Scatchard analyses of fluorometric titrations, (K_d=0.75mM-4.09mM); gel chromatography (K_d=0.80mM-4.60mM) and equilibrium dialysis (K_d=0.71mM-4.21mM) for MNGly, DMNA, DEtNA, DENA, DPNA, NPyr, DBNA, NMor and NPip show that these compounds have weaker affinity for the enzyme, as compared to the much tightly binding aromatic DPhNA, K_d values (0.65mM, 0.68mM and 0.68mM) for fluorometric experiments, gel chromatography and equilibrium dialysis respectively. In all cases, the number of binding sites of acetylcholinesterase averaged to four.

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LIST OF ABBREVIATIONS USED

ACh	acetylcholine
AChE	acetylcholinesterase
ATChCl	acetylthiocholine chloride
A_n	asymmetric form of acetylcholinesterase with n catalytic subunits
Bp	boiling point
BSA	Bovine Serum Albumin
Conc.	concentration
^oC	degree Celcius
D	Debye
DBNA	dibutylnitrosamine
DEtNA	diethanolnitrosamine
DENA	diethylnitrosamine
DMNA	dimethylnitrosamine
DPhNA	diphenylnitrosamine
DPNA	dipropylnitrosamine
DTNB	5,5-dithiobis-(2-nitrobenzoic acid)
G_n	globular forms of acetylcholinesterase with n catalytic subunits
IR	Infra Red spectroscopy
Mp	melting point

ABBREVIATIONS USED (Contd.)

MNGly	N-methyl-N-nitrosoglycine
K_m	Michaelis-Menten constant
nAChR	nicotinic acetylcholine receptor
NMor	nitrosomorpholine
NPip	nitrosopiperidine
NPyr	nitrosopyrrolidine
NMR	Nuclear Magnetic Resonance spectroscopy
M_R	relative molecular weight
SDS	Sodium dodecyl sulphate
TLC	Thin layer chromatography
UV	Ultra Violet spectroscopy

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CHAPTER 1 INTRODUCTION

1.1 *HISTORICAL PERSPECTIVES*

The discovery of the first soluble extract of acetylcholinesterase [Acetylcholine hydrolase, EC 3.1.1.7, AChE] from the electric eel Electrophorus electricus and from the electric organ of the ray Torpedo marmorata was obtained in 1938 (1,2,3). An early review on acetylcholinesterase by Nachmanson and Wilson (cited in 1) actually opened further avenues into the study of the enzyme. This was followed by Silman *et al* (4) who further confirmed that large quantities of the enzyme are readily obtainable from the electric organs of the ray and the eel.

1.2 *LOCALITIES OF AChE*

Experimental findings indicate that AChE is a widely distributed enzyme found in a variety of organs: present particularly in excitable tissues such as nerve(5,6), muscle, electrogenic tissue (7), human serum (8), motor cortex (9), foetal human tissues (10), foetal bovine serum (11), basal ganglia (12). Furthermore, it is also found in non-excitable tissues such as the surface of the red blood cell membrane (13,14) and cobra venom (11,15) as well as in Golgi apparatus (16). From the highly specialised organs of Electrophorus electricus and Torpedo marmorata, the enzyme was first extracted, solubilised, purified extensively and then finally obtained in a homogeneous form and ultimately was crystallised (1,3,17). Despite the fact that Electrophorus and Torpedo are the rich sources of AChE, it is realised that the electric organ of Electricus contains exclusively A_n forms, while that of Torpedo contains both A_n and G_n forms alongside each other (1,2,18).

1.3 *STRUCTURE OF AChE*

Studies on the molecular structure of AChE are of great importance, not only for elucidating the mechanism of action of the enzyme, but also clarifying the nature of its association with the excitable membrane, which is a necessary condition for proper understanding in the way the enzyme fulfills its biological role *in situ*. Numerous studies on the molecular structure of the enzyme have been reported in the literature (7,18,19).These indicate that AChE obtained from various sources was found to consist of several components which could be distinguished by their sedimentation coefficients in glucose (7).The distribution of these molecular forms differs from tissue to tissue, even within the same animal (18). These molecular components were further found to differ in their quaternary structure and solubility characteristics. They are conveniently classified according to the nomenclature of Bon *et al.* (20,21) as "asymmetric" (A_n) and "globular" (G_n) formms of n catalytic subunits. [See FIGURES 1(a) and 1(b)].

The A_n forms are elongated structures of AChE and are localized primarily at skeletal neuromuscular junctions, which appears to be mediated by the interaction of the collagen-like tail structure with extracellular basement membrane (13). In these forms, a large head is connected to a fibrous tail of about 500nm in length (4,7,17) . This multisubunit head is found to contain one, two or three catalytic subunit tetramers designated A_4, A_8, and A_{12} respectively. The role of the tail is to anchor the enzyme to the basal lamina within the synaptic cleft (18).

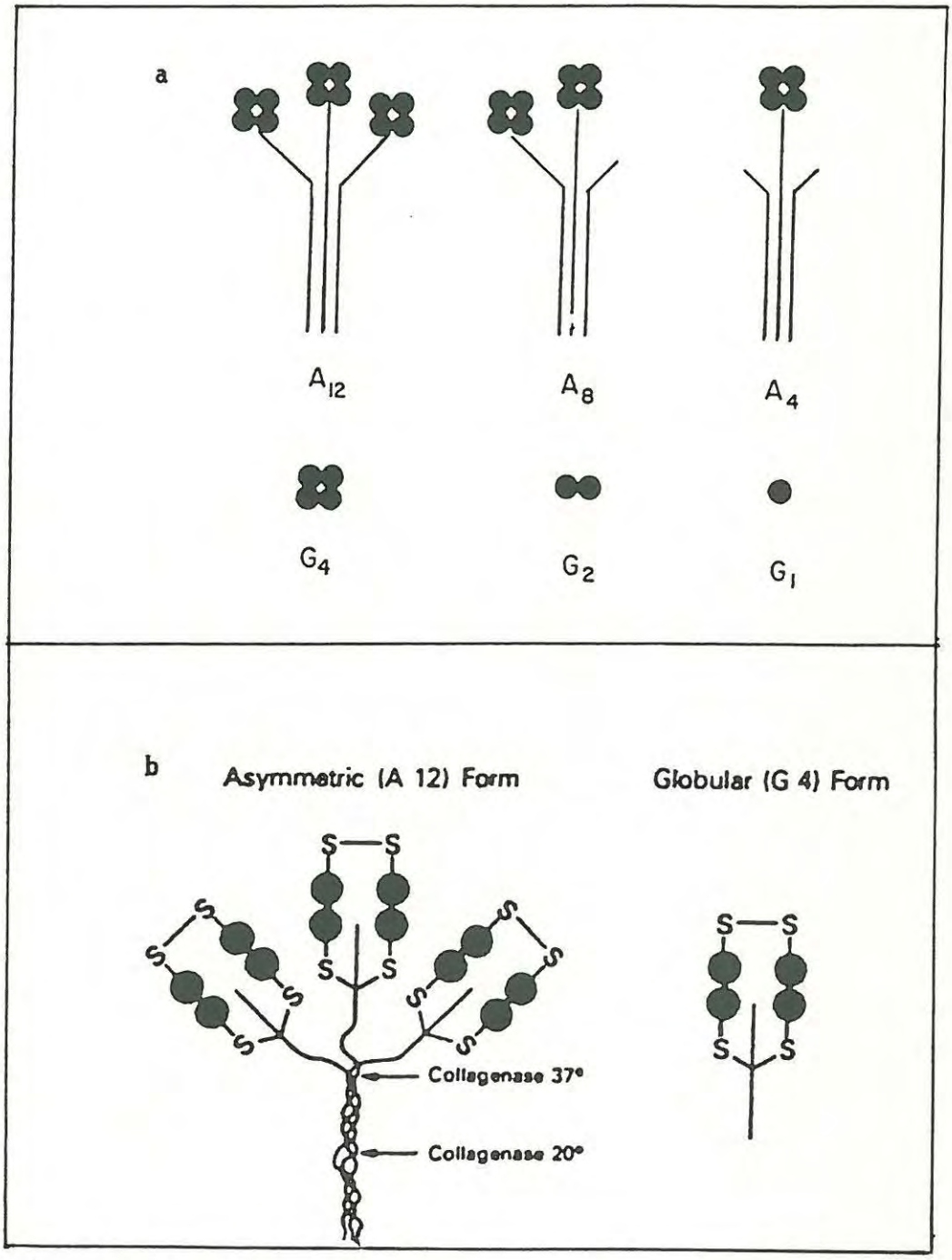


FIGURE 1: *Schematic representation of (a) asymmetric and globular forms of acetylcholinesterase (AChE), and (b) their interconnection by disulphide bonds (18,22).*

The detailed studies substantiated the contention that the fibrous tail is collagenous in nature, and this led to the model shown in FIGURE 2.

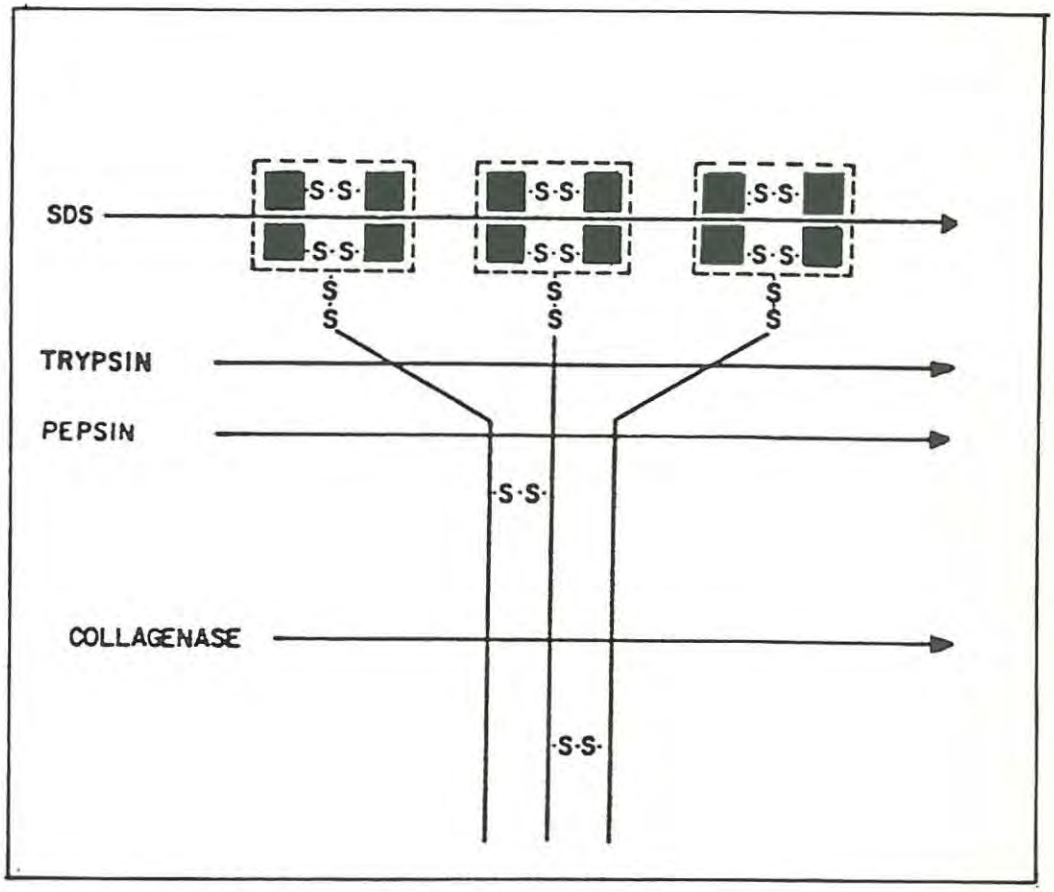


FIGURE 2: *Schematic representation of A_{12} form of AChE from Electrophorus electricus (18).*

In FIGURE 2, the arrows show the points of cleavage by proteases and by sodium dodecyl sulphate (SDS). Three catalytic subunit tetramers are connected via disulphide linkages to a collagen triple helix; the corresponding A_4 and A_8 forms, present in smaller amounts, contain one and two subunit tetramers respectively, similarly connected to the tail structure (7,19).

Properties of the asymmetric forms have been established. Sucrose gradient centrifugation studies show that A_4, A_8 and A_{12} sedimented as 8S, 11S and 18S respectively (1). The three species from eel have been

purified and appear in electron micrographs as clusters of 4, 6-8 and 10 respectively, or more subunits attached to an elongated "tail" (1) [FIGURE 3].

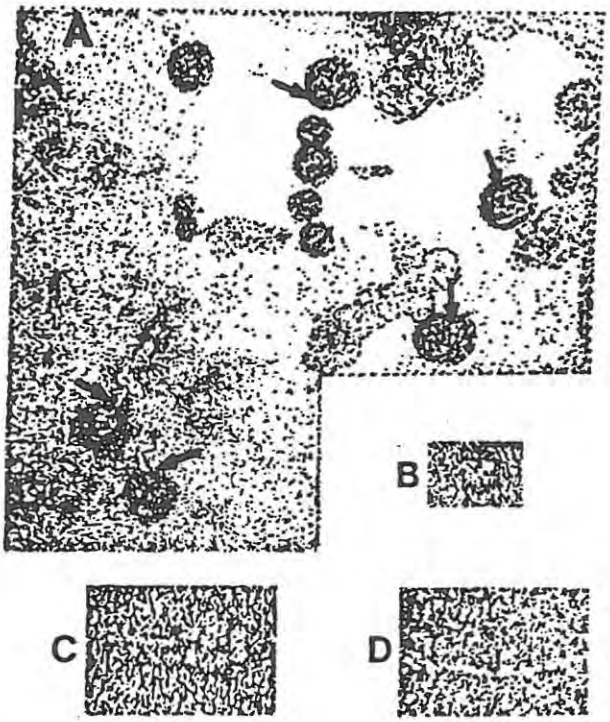


FIGURE 3: *Electron micrographs of various molecular forms of AChE from eel electric organ obtained by negative staining (1).*

In FIGURE 3: (A) shows 14S and 18S species characteristic of highly purified preparation obtained directly from fresh tissue extracts. The field also contains multisubunit "heads" connected to "tails" and separated "tails", magnified 340 000 times, (B) shows a single molecule of 11S species magnified 650 000 times; (C) shows a single molecule of 8S species magnified 600 000 times and (D) is a single molecule of 18S species, magnified 600 000 times.

All three of these asymmetric species may be converted to a globular 11S form by treatment with trypsin , sonication or by allowing ageing (3). The results of electron micrographs of 18S AChE strongly suggest that this molecule contains three sets of subunit tetramers (1), and that the tail of the enzyme is composed of three individual filaments, and each associated with one tetramer, and intertwined in the distal portion of the strand. On the other hand, globular forms of acetylcholinesterase appear to be widely distributed on the outer surface of the membranes (2). These forms consist of monomers, dimers or tetramers of the catalytic subunit and are designated G_1, G_2 and G_4 respectively . Both G_2 and G_4 are recorded to sediment to 8S and 11S respectively (1). These forms can further be subdivided into two groups: (a) those that interact with nonionic detergents following extraction, and presumably correspond to integral membrane proteins *in situ*, and (b) those that do not bind detergent and are presumed to be soluble proteins (13,23,). Thus G_n forms are either hydrophobic or non-hydrophobic. Hydrophobic AChE has also been purified to homogeneity from the brains of a number of vertebrate species such as bovine, rat, human and chicken. In all these cases the purified brain enzyme occurs as a G_4 tetramer(18).

Furthermore, the water-soluble G_n forms in some cases are found to be "lytic" species derived from either the A_n forms or from the hydrophobic G_n forms by endogenous or exogenous removal of the membrane-anchoring domain (8,9). Evidence is available (18,19,24) that hydrophobic AChE is linked through other proteins to the plasma membrane [FIGURE 4]. Phosphatidylinositol is attached via a glycosidic linkage to a glucosamine residue, while the latter is attached to an oligoglycan (23).

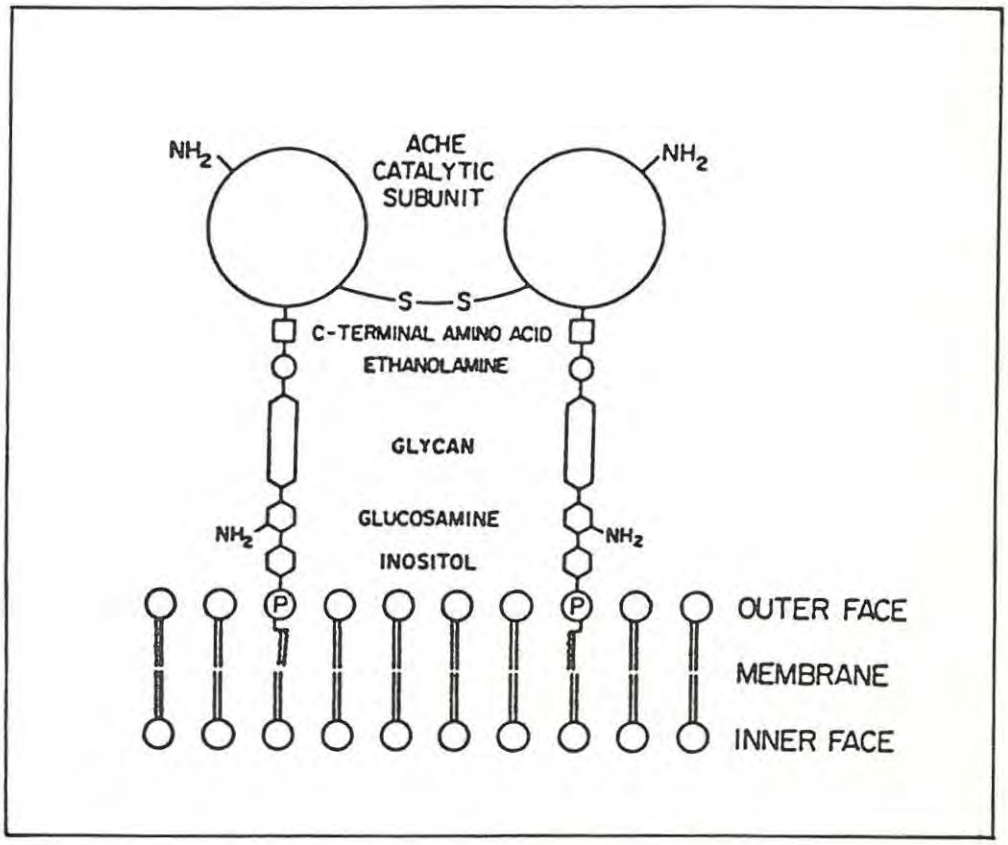


FIGURE 4: *A model of the membrane-anchoring domain of the hydrophobic G_2 form of AChE from Torpedo californica (18).*

1.3.1 Molecular Architecture of the Catalytic Site

The active site of AChE is confirmed to consist of three amino acids viz. serine, histidine and aspartic acid that facilitate enzymatic catalysis (1,18). Two subsites of the active site that interact with cationic substrates and inhibitors have also been identified : a negatively charged subsite and an esteratic subsite. The latter includes the serine hydroxyl, which is the residue presumably acylated or deacylated during a catalytic turnover (1,16) [FIGURE 5].

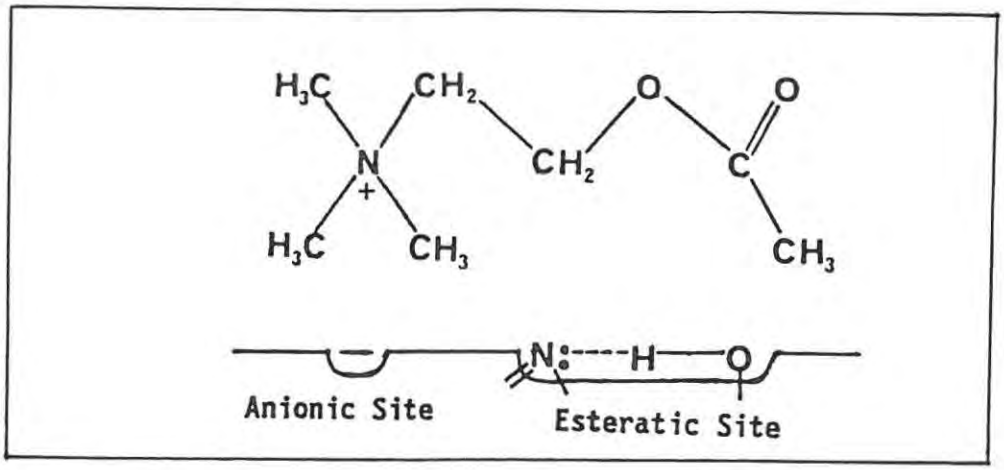


FIGURE 5: *Schematic illustration of the two subsites of acetylcholinesterase and its substrate, acetylcholine*

1.4 *FUNCTIONS OF AChE*

Acetylcholine [ACh] whose molecular structure is illustrated in FIG.5, is found as a metabolite in high concentrations in nervous tissue and in motor nerve tract, as an active neurotransmitter substance. The first discovery of the action of this substance came later in 1914 when Dale (25) tested the actions of many esters and ethers of choline. When a nerve impulse travels down the axon to the synapse, acetylcholine is released from nerve endings (26,27), diffuses across the cleft and interacts with the postsynaptic receptor on the cell membrane (29,30,31).

Acetylcholinesterase is a principal enzyme in impulse coordination in a biological system. Its main biological function is the termination of an impulse at cholinergic synapses by rapidly hydrolysing acetylcholine to acetic acid and choline (16,32,) according to reaction (1).

$$(CH_3)_3N^+CH_2CH_2OCOCH_3 \xrightarrow{AChE} (CH_3)_3N^+CH_2CH_2OH + CH_3COOH \quad ....(1)$$

Acetylcholine — choline — acetic acid

This enzymatic cleavage lowers the concentration of free acetylcholine in the synaptic cleft, and by perturbation of equilibrium, will induce dissociation of the acetylcholine-receptor complex , thus directing the signal to the muscle cell.

Active AChE is very crucial in neuromuscular function (33), and inhibition of the enzyme results in coordination imbalances, muscle paralysis and asphyxiation . Chemical reactions at the junctions between cells involved in the signal transmission are facilitated by close association between the enzyme and its receptor, viz. nicotinic acetylcholine receptor [nAChR] which is located on the postsynaptic membrane (34,36). The presence of acetylcholine on the receptor makes the latter undergo a conformational change which triggers ion permeability by opening a self-contained cation channel (36,37). This smooth functional co-operation between AChE and nAChR is ascribed to their functional similarites. Structurally they both have an "esteratic" and "cationic" subsites in the active sites (38,39,).

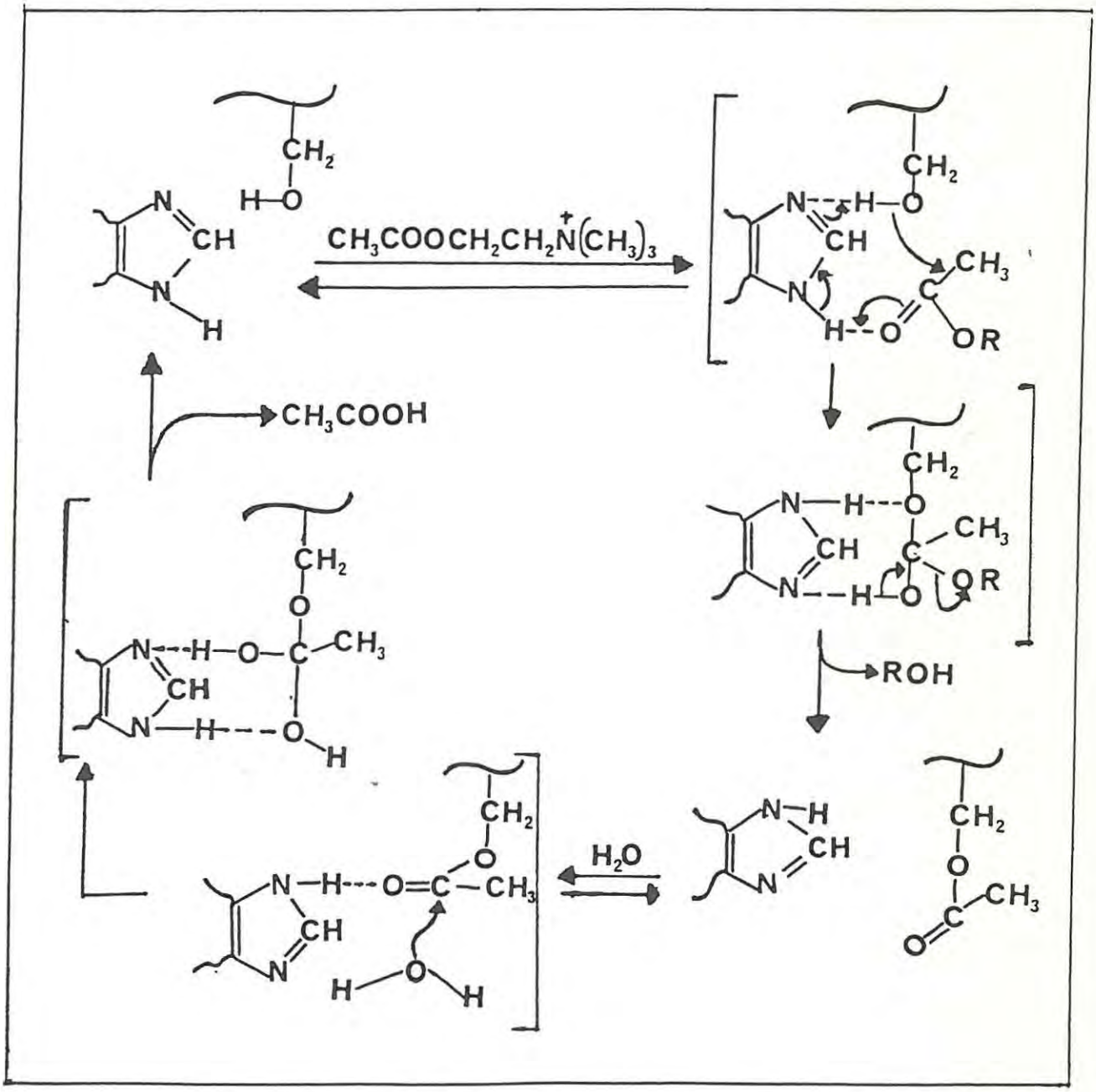
1.5 *PROPOSED CATALYTIC MECHANISM OF AChE*

Kinetic mechanisms for enzyme-catalysed reactions are ordinarily proposed to explain initial-rate data (40,41). It was this very sort of attempted correlation that led Brown (42) in 1902 to suggest that the enzyme and its substrate must combine for a finite period of time before catalysis can occur.

Subsequently, a large number of catalytic mechanisms (43,44,45,) have been proposed to explain the enzyme-substrate interaction at a molecular level. Knowledge of the enzyme's kinetic mechanism is important in order to understand how the enzyme functions as a biological catalyst, as well as how it is regulated in the cell. This information is not only necessary, but also important in order to evaluate inhibition constants for multisubstrate enzymes. The establishment of such a mechanism for an enzyme may further provide information on, among other things, the chemical mechanism, the nature of the transition state (46,47), the geometry of the active site, substrate specificity, inhibition and activation, acidic and basic groups associated with catalysis (47,48), allosteric properties as well as the mode of regulation at the physiological level (49,50,51). The catalytic reaction of AChE involves a double displacement mechanism with a nucleophilic serine at the anionic site with cationic center of the substrates and inhibitors (42,52).

Firstly, an acyl-enzyme is formed. This bears the acetyl group of serine residue of the active site. Secondly, the acyl-enzyme is then hydrolysed off. One such feature is the fact that serine proteases have an evolutionary conserved "catalytic triad" of serine, histidine and aspartate originally called the 'charge relay' system (53,54). The chemically inactive hydroxyl group of serine is activated with the aid of imidazole group of histidine located in its immediate vicinity (32,54,55) [SCHEME 1]. On interaction with the substrate, the activation process begins with the formation of a hydrogen bond between its carboxyl oxygen and the imino-nitrogen of the imidazole, which increases the basicity of the azole nitrogen and leads to the formation of

hydrogen bond between this nitrogen and the serine hydroxyl moiety. There is then a sharp increase in the nucleophilicity of the hydroxyl oxygen and its interaction with the central carbon atom is facilitated.



SCHEME 1 : *The postulated catalytic mechanism of acetylcholinesterase during its hydrolysis of acetylcholine, (ACh) (1).*

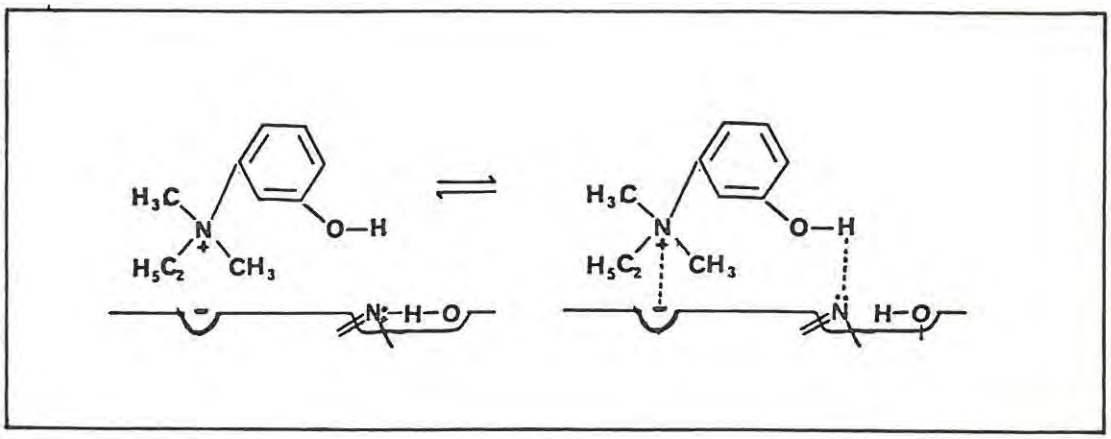
The redistribution of electron density in the eight-membered ring of the enzyme-substrate complex formed results in the formation of a covalent bond between the serine oxygen and the acid group of the substrate and in the elimination of the alcohol group (56,58),the subsequent reaction of the acetylated enzyme with water proceeds analogously, forming an intermediate acyl-enzyme-hydrate complex which then decomposes to the initial enzyme and the acid (1,54,59). In this way, the reactive form of serine hydroxyl is regenerated at the instant of the oriented binding of the substrate molecule, (60,61) [SCHEME 1].

1.6 *INHIBITION STUDIES ON AChE*

With the molecular studies of AChE, and the identification of the amino acids involved at the active site, studies on the inhibition of this enzyme with both natural and synthetic compounds have been well documented (62,63).

Some of the compounds tested include : quaternary derivatives of pyridine and acridine (62), 1-bromo-2 pinacolone (63,64), alkylating agents, N-dansyl aziridine and S-Mercuric-N-dansylcysteine (65), anionic and cationic drugs (66,67), isoquinoline alkaloids (67,68,69), phosphorus-containing molecules such as organophophates (70,71,72), phosphonate esters (73,74,75), sulphur-containing compounds (76,77,78), carbamates (79,80,81), methanesulphonic acid esters (82,83,84), substituted ethylenediamines (85), nitro- and nitroso-containing substances (86,87), aryl esters and anilides (88), ethanol (89), organic acids (90), trimethylammonium bromide (91,92), pyridoxal 5'-phosphate (93,94), phosphorylated alkaloids (95), and a host of others (96-99).

Generally, inhibition on AChE catalysis occurs on the basis of the anionic site binding and orientating substituted ammonium ions, as well as the esteratic site, where the hydrolytic processes occur (100,101). Reversible inhibitors such as substituted ammonium ions may act, not only by competition with the substrate for the active site of the free enzyme, but possibly also by combining with the acetyl-enzyme at the anionic site [see SCHEME 2].



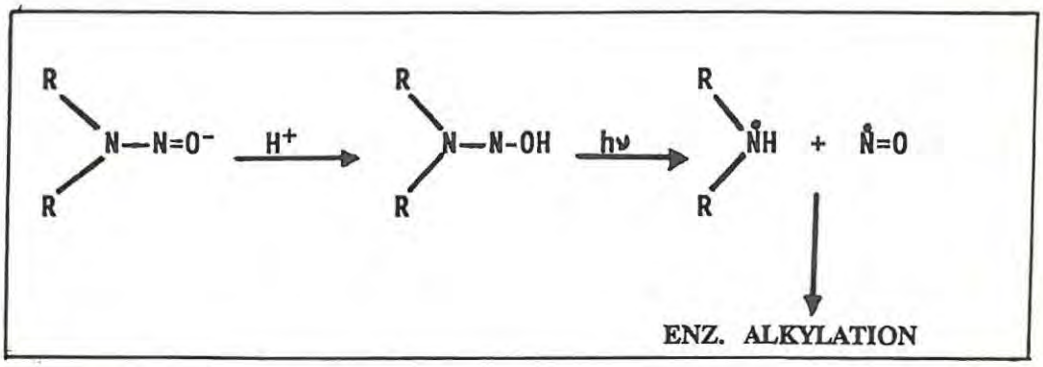
SCHEME 2: *An illustration of an inhibition of AChE by Edrophonium (67).*

Perhaps it should therefore, be easy to imagine that deacylation would be sterically blocked, especially if the water molecule had to approach from the direction of the anionic site . This restriction on the direction of the approaching water molecule is to be anticipated for the reason that acetylcholinesterase catalyses the hydrolysis of acetylcholine. During the regeneration of acetylcholine, a choline molecule attacks the acetyl-enzyme, and this attack comes clearly from the direction of the anionic site (100,101).

1.7 *N-NITROSAMINES AS AChE INHIBITORS*

1.7.1 Properties of N-Nitrosamines

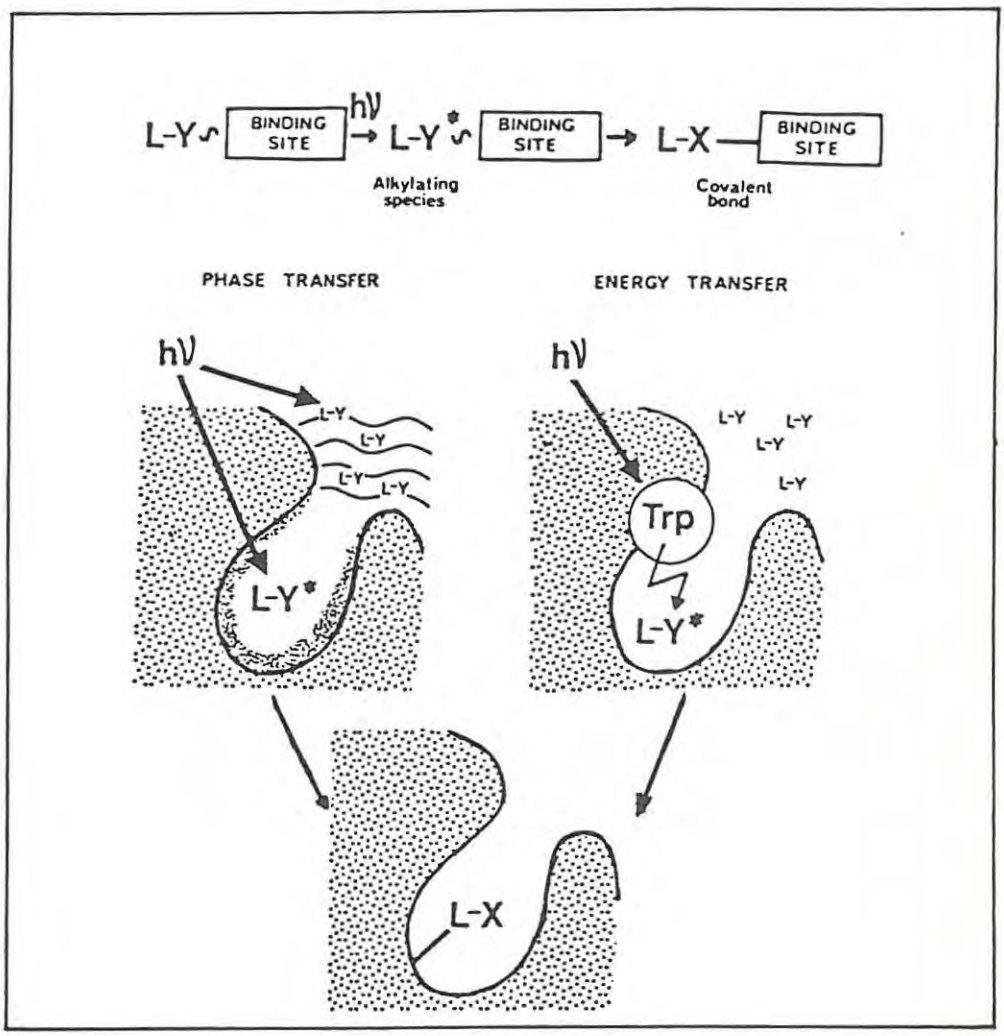
Nitrosamines are good ligands for proteins involved in a cholinergic system. Like other molecules, nitrosamines are able to undergo photolysis, especially in the presence of a dilute acid (107,108,109) [SCHEME 3].



SCHEME 3: *Schematic Representation of Photodecomposition of N-Nitrosamine in the presence of a dilute acidic medium.*

This photolability is also closely associated with the polar character of the nitrosamine (110,111). An interesting additional feature of these molecules is that their [-N=O⁻] moiety is responsible for their affinity and is also the photolabile function (111). Goeldner *et al* (107) have indicated that the nitrosamines were specifically photoactivated after association with a proton at the active site . N-nitrosamines are thus classified as photoaffinity labels or photosuicide inhibitors (87). By definition photoaffinity labels are ligands that have inherent affinity for a binding site, and possess biological activity, but also contain a photosensitive functional group, which when photoactivated is capable of forming a covalent bond at or near the binding site. Their association with the recognition site will ordinarily be reversible till photolysis is initiated (110,111,112).

Furthermore, this specific photoactivation should lead to a highly reactive species which can irreversibly modify the protein in the vicinity of the active site [SCHEME 4].

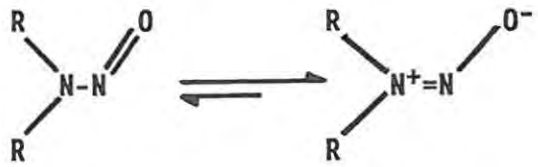


SCHEME 4: *Schematic illustration of photosuicide labelling of the active site of an enzyme by a secondary nitrosamine (107).*

In SCHEME 4, the energy transfer system involves fluorescent amino acid residues, whereby the donor tryptophan (Trp) residues of the protein and the acceptor which is a labelling reagent are in close proximity (107). Furthermore, the photolabile ligand (L-Y) should be stable at the tryptophan excitation wavelength (about 290nm), i.e it must be an

efficient quencher; and it must also be photosensitive at tryptophan emission wavelength (about 340nm). Nitrosamines according to phase transfer induced labelling, will be photoactivated after the association with a proton at the active site (107,110).

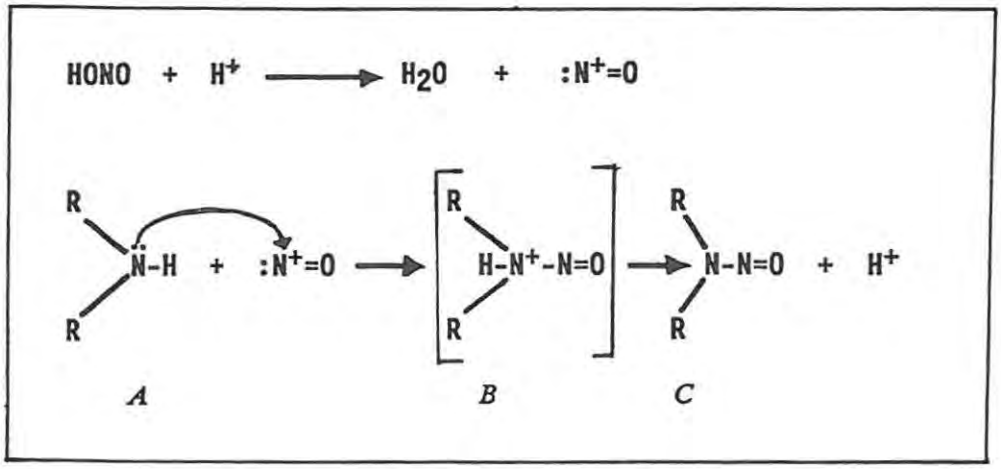
Various experimental physical measurements using UV,IR,(111,112) and NMR (113,114) spectroscopy have been carried out to study the nature and the character of the nitroso functional group (115). Considerable evidence gathered indicates the existence of a partial N=N double bond character,and the location of the negative pole at the nitroso oxygen. It has been established that a proton (or a Lewis acid) would seek the oxygen as the co-ordination site (108,110,112) maintaining the secondary nitrosamines in two resonant forms: [See also APPENDIX 12]



This resonance character of the molecule indicates that the structure of the secondary nitrosamine is partially planar (115).Furthermore, nitrosamines like their respective amines are rich sources of carbocations [See APPENDIX 13]. The latter were found to be capable of alkylating alcoholic residues on the enzyme Chymotrypsin (87). It is thus expected that the generation of such carbonium ions at the active site of an enzyme should alkylate the carboxylate anion of either glutamic or aspartic acid of the subsite and therefore "labelling" it (87,88).

1.7.2 Synthesis

The following nitrosamines were synthesised: DBNA, DPNA, DMNA, DEtNA, NPip, NMor, NPyr, DPhNA and MNGly. Nitrosation of secondary amines occurs in a strongly acidic medium, with sodium nitrite as the nitrosating agent (103,104). During the generation of a nitroso compound, sodium nitrite ($NaNO_2$) reacts with strong acid at 0^oC to generate a nitrosyl ion [$:N^+=O$]. The latter is then transferred readily to the nucleophilic nitrogen of the secondary amine (103,106) [SCHEME 5]



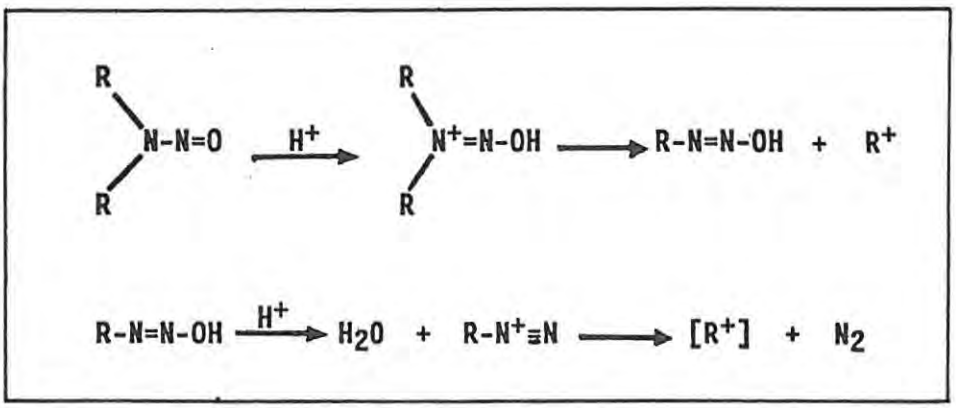
SCHEME 5 :*Reaction Mechanism for generation of N-Nitrosamine from Secondary amine and nitrous acid, where A, B and C are secondary amine, unstable intermediate and secondary nitrosamine respectively.*

1.7.3 Proposed Inhibition Mechanism

It is established that both nitro and nitroso compounds serve as reversible competitive inhibitors to AChE (54,87).The canonical form $R_2N=N-O^-$ is important in representing the structure of nitrosamines. The barrier rotations about the N-N bond are recorded to be 70-160 $KJ.mol^{-1}$, and the dipole moment (u=4.0 D) (117).

It is logical therefore to envisage the blocking of the binding of acetylcholine by the nitrosamine bound to the active site of the enzyme. The binding of the nitrosamine to the active site of the enzyme is considered to be two fold : Firstly, the negatively charged, ionised oxygen of the nitroso group will tend to interact with, at least one protonated residue in the active site of the enzyme within the enzyme-substrate complex [SCHEME 7(a)]. Secondly, the active site of AChE must have a hydrophobic nature, and to support this idea, at least one tryptophan has been shown to be close to the active site (54,63). This implies that the nitrosamine passes from a hydrophilic to a hydrophobic environment, before it can bind to the enzyme. This change of environment is a requisite for effective binding of acetylcholine to the active site, and has already been implicated as an important factor in enzyme catalysis (54).

On the validity of these assumptions, the reaction mechanism suggests that the starting nitrosamine would decompose, as soon as it reaches the active site, into an alkylating species, $[R^+]$. This generation of a carbocation occurs according to the following process :

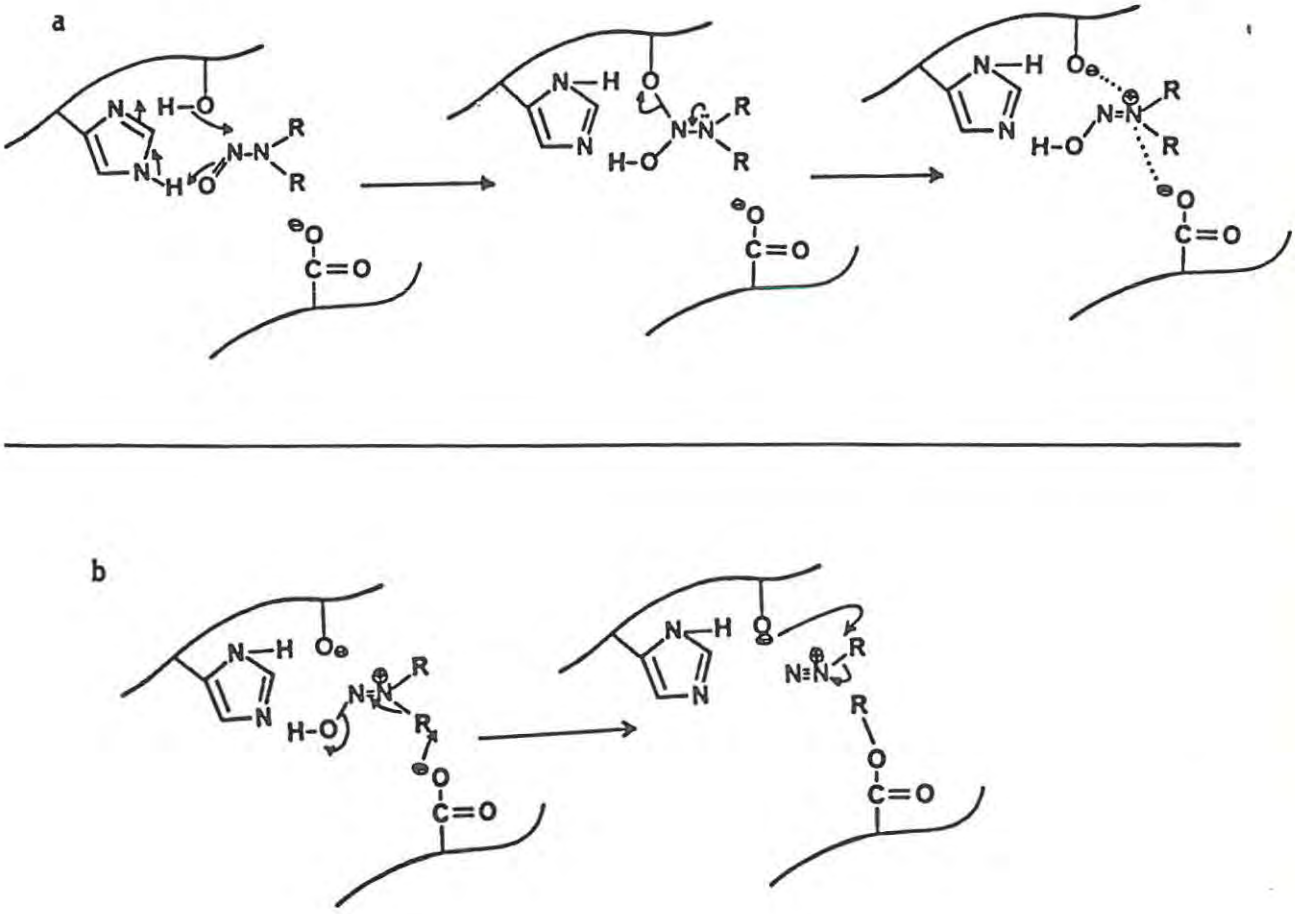


SCHEME 6: *Generation of a carbocation $[R^+]$, during decomposition of a nitrosamine.*

The released carbocation species [R^+] then interacts with serine hydroxyl group in an irreversible way (54).In this way, the substrate molecule is prevented from binding to the enzyme, according to the following simplified reaction :

$$[R^+] + \text{HO-Ser-ENZ} \longrightarrow \text{R-O-Ser-ENZ}$$

The representation of such an irreversible mechanism is depicted in SCHEME 7(b).



SCHEME 7: *Mechanism of interaction of nitrosamine with acetylcholinesterase involving a reversible (a) and irreversible (b) competitive inhibition.*

CHAPTER 2 SYNTHESIS OF SECONDARY N-NITROSAMINES

2.1 MATERIALS and METHODS

2.1.1 Reagents

All the chemical reagents used were of analytical grade. Dibutylamine, diphenylamine, dimethylamine, diethylamine, dipropylamine were all obtained from Hopkins Williams Chemical Co. UK, pyrrolidine, piperidine, morpholine, N-methylglycine and diethanolamine were all obtained from Aldrich Chemical Co. All other chemical and solvents as well as acids were of reagent grade (Holpro).

2.1.2 Methods

General Procedure:

Secondary amine (0.25mol) was added slowly to hydrochloric acid (50ml,5M), initially cooled in ice at 5°C. The solution of the amine-acid was then introduced to a solution of sodium nitrite (20.70g, 0.30mol) in 25ml water contained in a 250ml distilling flask and the mixture azeotropically distilled. The aqueous distillate was extracted several times with ether, and the ether layers combined, dried ($MgSO_4$) and the solvent evaporated. Respective nitrosamines were purified by flash chromatography or recrystallization (118) [TABLE 1].

Diethanolnitrosamine:

Hydrochloric acid (167ml, 2M) was added slowly to a cold solution (10°C) of diethanolamine (35.03g, 0.33mol). The resulting solution was treated dropwise during one hour with cold (5-10°C) solution of sodium nitrite (27.02g, 0.39mol) in water (35ml). The reaction solution was stirred at 10°C for 30 minutes and again at 35°C for one hour then concentrated under reduced pressure at 60°C. The residual mixture was stirred in absolute ethanol (55ml), filtered to remove the insoluble salts and then evaporated again under reduced

pressure. Extraction into ether, filtration and evaporation were repeated several times. The organic layer was dried and evaporated to produce a yellow oil. Distillation of the residue afforded an oil (27.67g, 79%); Bp 110-119°C at 1.0 mmHg (104).

Diphenylnitrosamine:

Diphenylamine (25.39g, 0.15mol) was added in 250ml of warm ethanol and together with sodium nitrite (22ml, 13.80g, 0.20mol) were cooled to below 5°C. The solution of diphenylamine-ethanol was acidified with hydrochloric acid (25ml,5M) under slow and constant stirring. To this mixture, sodium nitrite solution was rapidly added and stirred. The crystals of diphenylnitrosamine were filtered, washed with water and recrystallised (petroleum ether) to afford the product (17.77g, 70% Mp 67-69°C) (102).

N-Methyl-N-nitrosoglycine:

Sodium nitrite (13ml, 7.63g, 0.11mol) was added dropwise to a hot (95°C) solution of N-Methylglycine (8.91g, 0.10mol) dissolved in hydrochloric acid (5M, 5ml). The mixture was heated (1h), cooled, and extracted with ether. The ether layers were combined,dried ($MgSO_4$) and evaporated to afford an oil which crystallised on standing. Recrystallisation (ethylacetate) afforded 90.5% (8.06g, Mp 74-76°C) (105).

TABLE 1: *Properties of N-Nitrosamines*

NITROSAMINE	YIELD(%)	EXPERIMENTAL VALUES		LITERATURE VALUES		REF.
		Mp(°C)	Bp(°C)	Mp(°C)	Bp(°C	
DBNA	78.2	-----	130-134	-----	133	102
DEtNA	79.4	-----	110-119	-----	100-120	104
DENA	80.5	-----	173-175	-----	177	102
DMNA	72.5	-----	146-148	-----	149-150	102
DPhNA	70.5	67-69	------	66.00	-----	102
DPNA	69.3	-----	175-178	-----	176-177	102
MNGly	90.5	74-76	------	75-77	-----	105
NMor	81.1	26-30	96-98/1mmHg	29.00	98/1mmHg	102
NPip	80.1	-----	102-114	-----	100-109	102
NPyr	69.7	-----	110-115	-----	98	102

CHAPTER 3 KINETIC STUDIES

3.1 *ASSAY OF ACETYLCHOLINESTERASE*

3.1.1 MATERIALS and METHODS

3.1.1.1 Materials

All the reagents used were of analytical grade. Acetylthiocholine chloride (ATCh.Cl) and *Ellmans* reagent [5,5-dithiobis-(2-nitrobenzoic acid)] were obtained from Sigma Chemical Co, $NaH_2PO_4.2H_2O$, sodium hydroxide (NaOH), sodium bicarbonate ($NaHCO_3$) were obtained from BDH Chemicals LTD, Poole, England; Acetylcholinesterase (type III, salt free, 100 U/mg lyophilisate from Electrophorus electricus) was purchased from Boehringer Mannheim, West Germany. All other chemicals were of reagent grade.

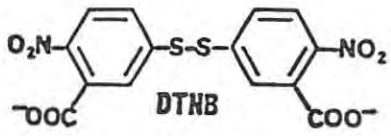
3.1.1.2 Methods

The colourimetric method (119) was employed. The principle involves the hydrolysis of acetylthiocholine chloride by AChE to acetic acid and thiocholine according to Reactions (2) and (3):

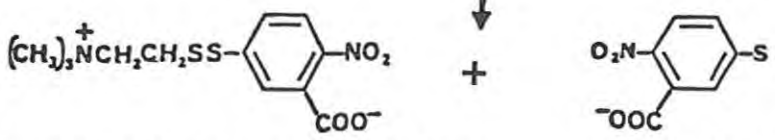
$$(CH_3)_3N^+CH_2CH_2SCOCH_3 + H_2O \xrightarrow{AChE} (CH_3)_3N^+CH_2CH_2SH + CH_3COOH \quad \ldots\ldots (2)$$

Acetylthiocholine thiocholine acetic acid

+



$\ldots\ldots\ldots$ (3)



2-nitrobenzoate-5-mercaptothiocholine 5-thio-2-nitrobenzoate

The catalytic activity is measured by following the increase of the yellow anion, 5-thio-2-nitrobenzoate, produced from thiocholine when it

reacts with DTNB (*Ellmans* reagent) according to reaction (3). The assay mixture (3.14ml) contained phosphate buffer (0.1M, pH 8.0) buffered *Ellmans* reagent (10mM), $NaHCO_3$ (17.85mM, pH 7.0) and acetylthiocholine chloride (478 μM). Aliquots were first made upto 3.12ml with phosphate buffer (pH 8.0) and the reaction initiated by the addition of 0.02ml of acetylcholinesterase [See APPENDIX 9 for Measurements]. The enzyme concentration (4.66 μM) was determined using the absorption coefficient ($A^{1\%}_{1cm}$) of 16.1 at 280nm and M_R of 260 000 .

3.1.2 RESULTS

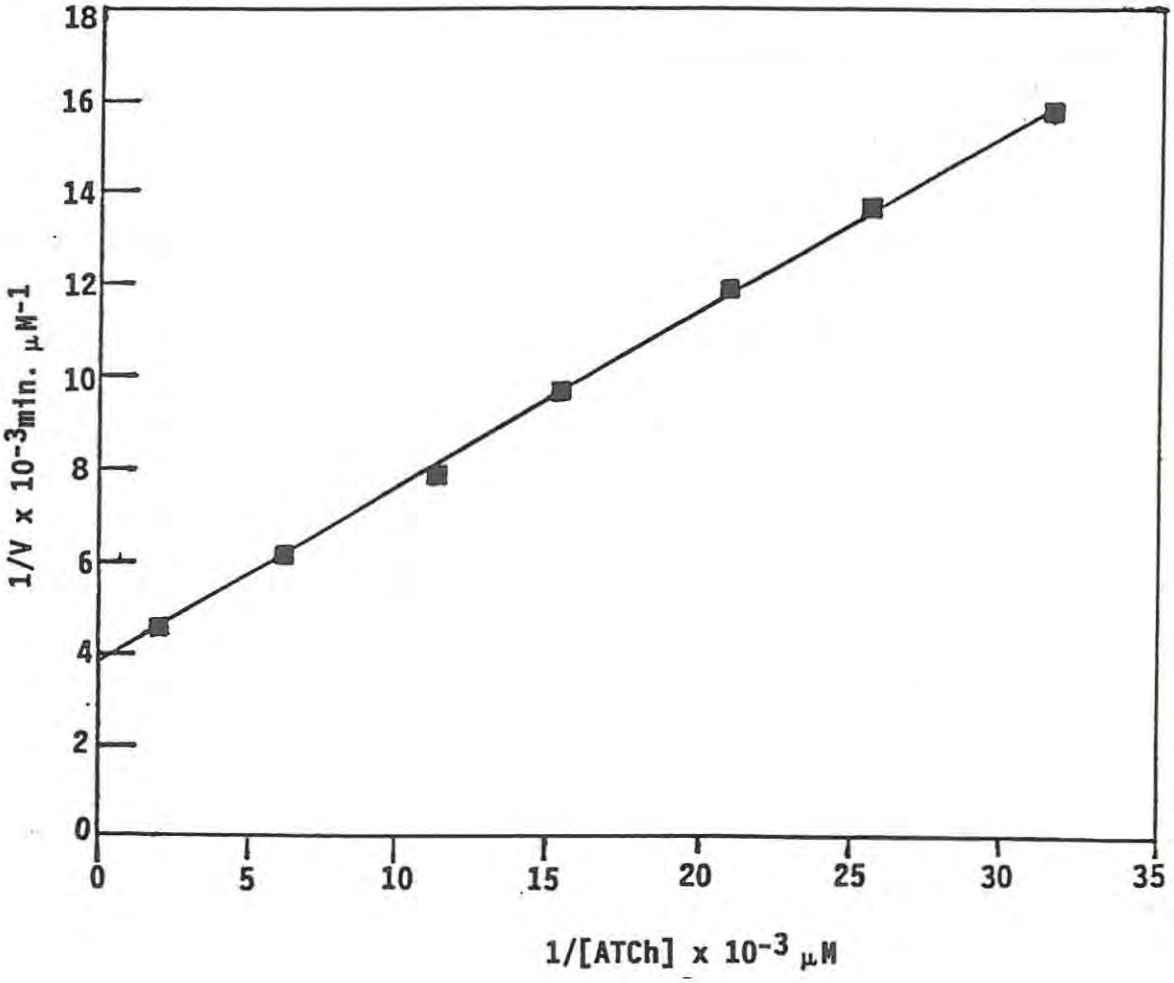


FIGURE 6: *Uninhibited hydrolysis of acetylthiocholine chloride (ATCh.chloride) by acetylcholinesterase (AChE)*

3.1.3 DISCUSSION

The activity of the enzyme was optimised under the stated experimental conditions. The increase in the concentration of the substrate resulted in an overall increase in the velocity of the enzyme-catalysed reaction. However, as the concentration of the substrate was increased, a drop in the enzyme velocity was apparent. This could be interpreted as being due to "substrate inhibition". From these results Vmax and Km were estimated to be 263.16 $\mu M.min^{-1}$ and 102.61 μM respectively.

3.2 *INHIBITION STUDIES*

3.2.1 MATERIALS and METHODS

3.2.1.1 Materials

All the chemicals used were of analytical grade. The nitrosamines were prepared as described in CHAPTER 2.

3.2.1.2 Methods

Inhibition of acetylcholinesterase by interaction with various nitrosamines was studied in the the presence of phosphate buffer (0.1M,pH 7.0), acetylthiocholine chloride (0-478 μM) and a nitrosamine (0-320 μM). Aliquots (2.8ml) were treated with enzyme solution (0.2ml). Polyethyleneglycol (2%) was used as a co-solvent for DPhNA.

3.2.2 RESULTS

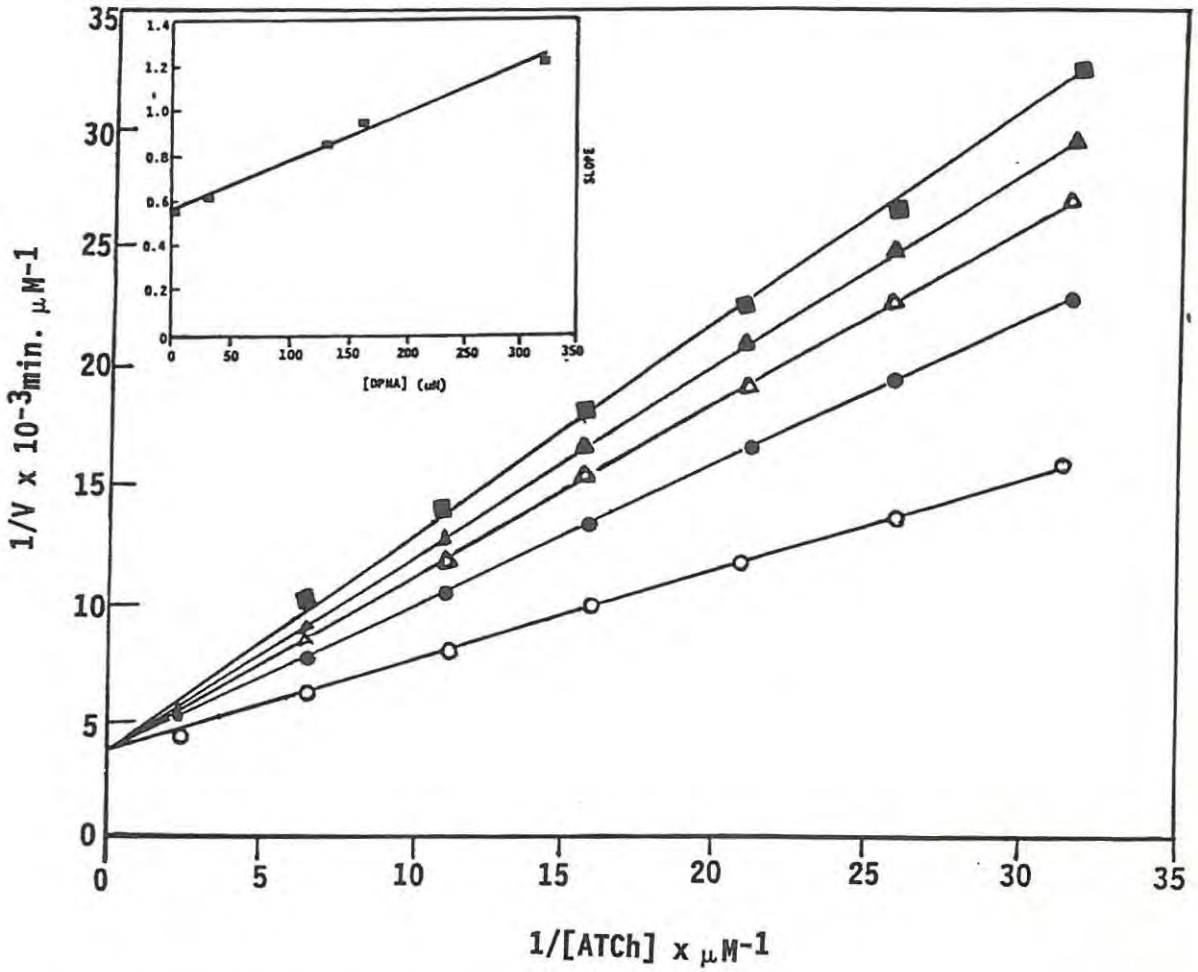


FIGURE 7: *Competitive inhibition of acetylcholinesterase by dipropylnitrosamine (DPNA) at pH 7.0. Concentration of DPNA is (○) 0 μM; (●) 32 μM; (△) 130 μM; (▲) 160 μM; (■) 320 μM. V represents the rate of the enzymatic reaction. The secondary plots represent the slopes of the Lineweaver-Burk lines versus DPNA concentration.*

3.2.2 RESULTS (Contd.)

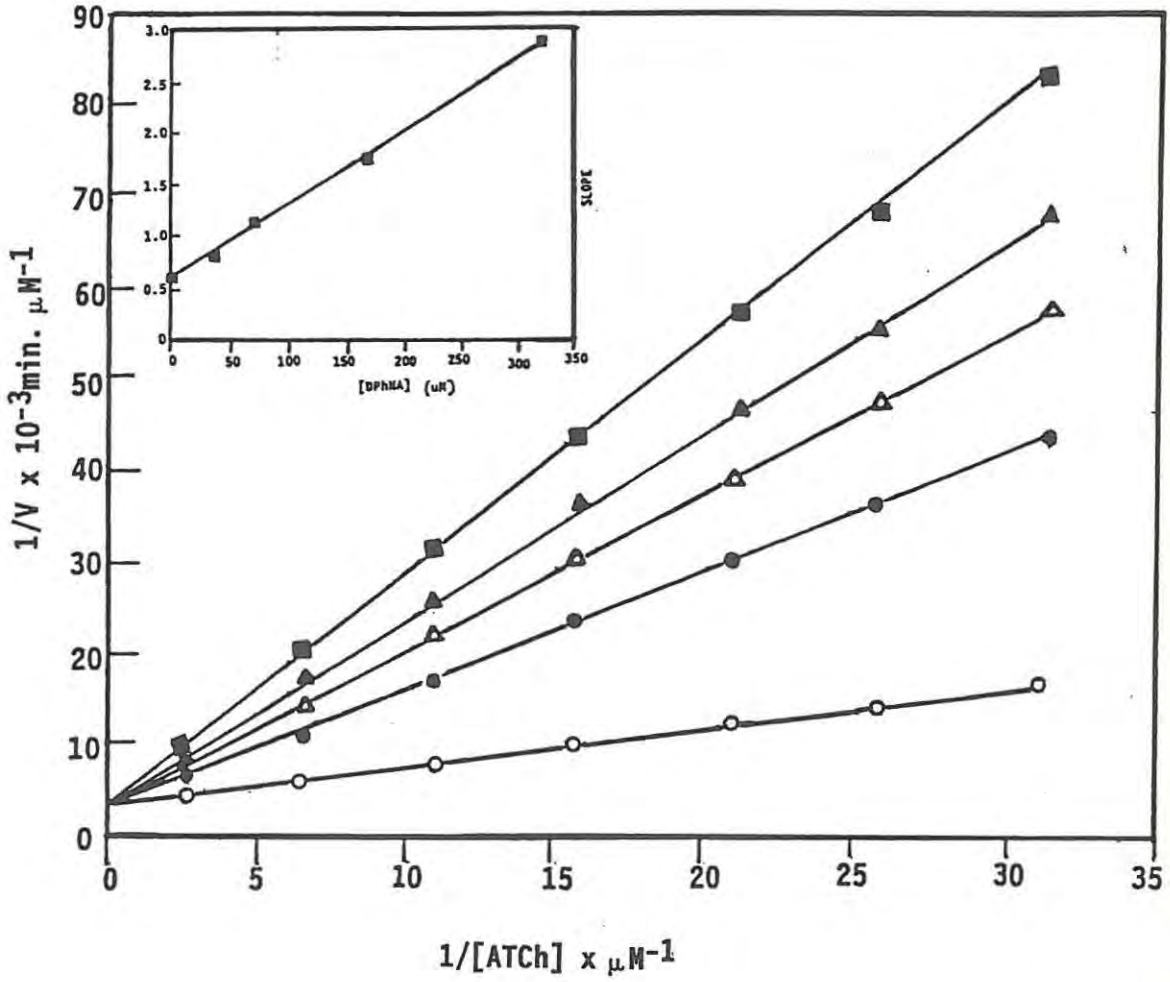


FIGURE 8: *Competitive inhibition of acetylcholinesterase by diphenylnitrosamine (DPhNA) at pH 7.0. Concentration of DPhNA is (○) 0 µM; (●) 32 µM; (◭) 64 µM; (▲) 160 µM; (■) 320 µM The secondary plots represent the slopes of Lineweaver-Burk lines versus DPhNA concentration.*

3.2.2 RESULTS (Contd.)

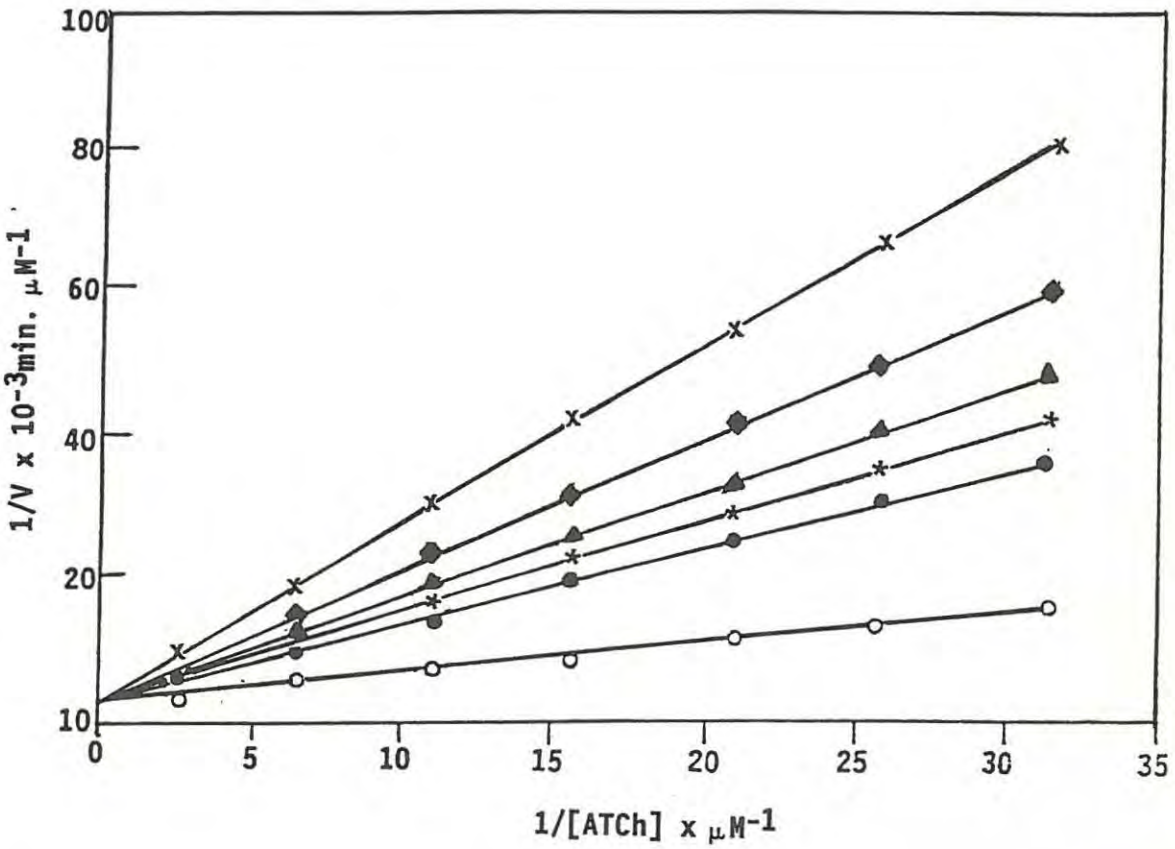


FIGURE 9(a): *Competitive inhibition of acetylcholinesterase (o), at pH 7.0 by diphenylnitrosamine (DPhNA), (×); methylnitrosoglycine (MNGly), (◆); dimethylnitrosamine (DMNA), (▲); diethanolnitrosamine (DEtNA), (✱); diethylnitrosamine (DENA), (●). The concentration of the nitrosamines is 320μM.*

3.2.2 RESULTS (Contd.)

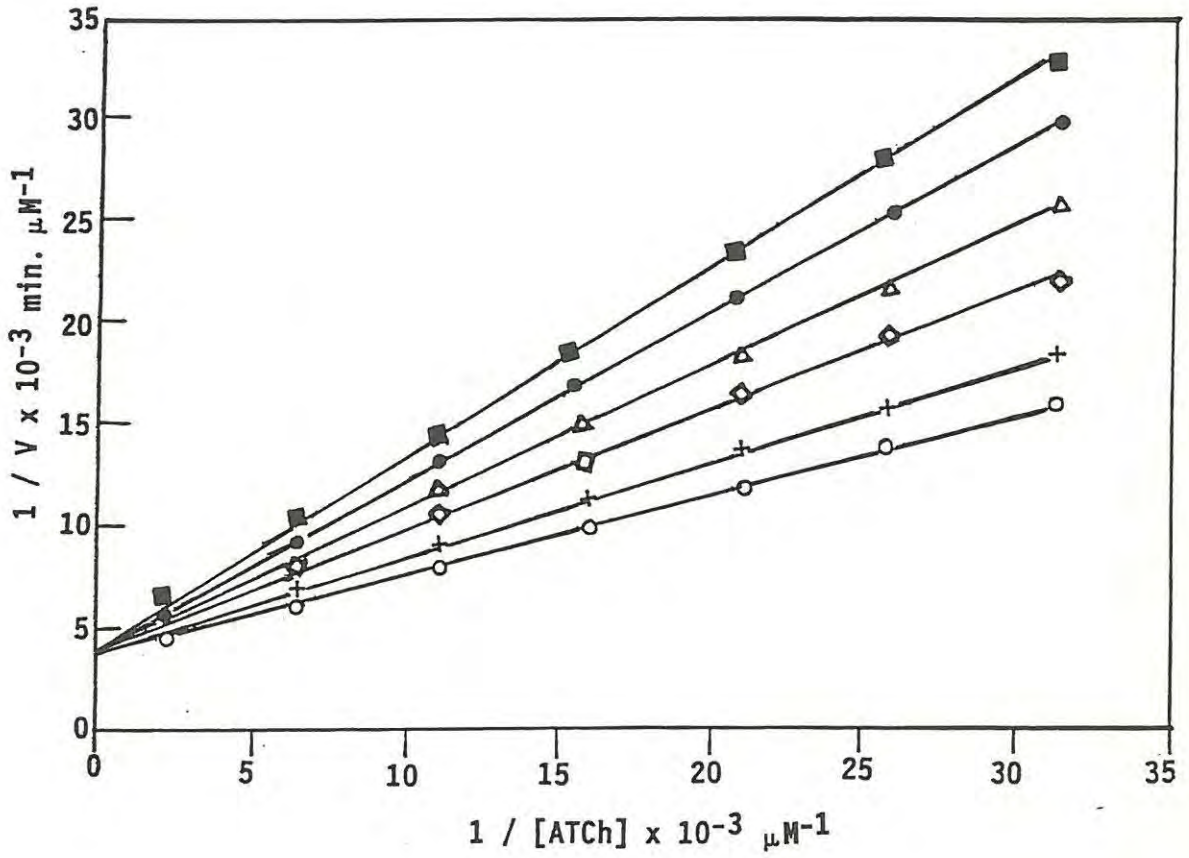


FIGURE 9(b): *Competitive inhibition of acetylcholinesterase (○), at pH 7.0 by dipropylnitrosamine (DPNA), (■); nitrosopyrrolidine (NPyr), (●); nitrosomorpholine (△), ; dibutylnitrosamine (DBNA), (◇); nitrosopiperidine (NPip), (+). The concentration of the nitrosamines is 320μM.*

3.2.2 RESULTS (Contd.)

TABLE 2: *Kinetic Constants of nitrosamines as determined from their respective plots*

INHIBITOR	CONC. (μM)	Km_{App} (μM)	K_i (μM)
DPhNA	32	264.20	20.32
	64	425.79	
	130	759.07	
	160	910.56	
	320	1718.52	
MNGly	32	211.41	30.18
	64	320.20	
	130	544.60	
	160	646.60	
	320	1087.98	
DMNA	32	197.02	34.78
	64	292.43	
	130	486.14	
	160	574.65	
	320	1046.69	
DEtNA	32	177.78	43.68
	64	252.95	
	130	408.00	
	160	478.47	
	320	854.33	

3.2.2 RESULTS (contd.)

TABLE 2 *Kinetic Constants of nitrosamines as determined from their respective plots*

INHIBITOR	CONC. (μM)	Km_{APP} (μM)	K_i (μM)
DENA	32	163.15	54.24
	64	223.68	
	130	348.54	
	160	405.29	
	320	707.98	
DPNA	32	157.01	60.36
	64	211.41	
	130	323.60	
	160	374.60	
	320	646.61	
NPyr	32	152.31	66.07
	64	202.00	
	130	304.51	
	160	351.10	
	320	599.58	
NMor	32	147.02	73.93
	64	191.44	
	130	283.04	
	160	324.68	
	320	546.75	

3.2.2 RESULTS (contd.)

TABLE 2 *Kinetic Constants of nitrosamines as determined from their respective plots*

INHIBITOR	CONC.(μM)	Km_{App}(μM)	K_i (μM)
DBNA	32	136.98	95.54
	64	171.35	
	130	242.23	
	160	274.45	
	320	446.29	
NPip	32	129.30	123.00
	64	156.00	
	130	211.06	
	160	236.09	
	320	369.56	

3.2.3 DISCUSSION

The continual addition of different nitrosamines to the enzyme-catalysed reaction resulted in a decrease of enzyme activity . These results give evidence of a reversible inhibitory mechanism due to the characteristic Lineweaver and Burk (120) plots [FIGURES 7-9]. This further illustrates 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 [FIGS. 7 and 8, inset].

The established kinetic constants show an increase in values from DPhNA(20.32 μM) to NPip(123 μM) [TABLE 2]. Kinetic constants indicate the extent of inhibition and the degree of association imposed on the enzyme by a particular inhibitor.

On the other hand, the values increase with an increase in the length of the aliphatic (R) chain. This means that the potency of inhibition by the nitrosamine decreased with the increase in chain length.

3.3 *TEST OF REVERSIBILITY OF INTERACTION BETWEEN NITROSAMINE AND AChE*

3.3.1 MATERIALS and METHODS

3.3.1.1 Materials

All chemical reagents used were of analytical grade. Acetylthiocholine chloride, *Ellmans* reagent, phosphate buffer (0.1M, pH 8.0), dialysis membrane (obtained from Spectrophor).

3.3.1.2 Methods

The test of reversibility of the interaction of a nitrosamine with acetylcholinesterase was carried out by dialysis (54,69). A mixture of a nitrosamine (32-320 μM) and the enzyme solution (3.0ml) was incubated at room temperature (1hour). The solution was dialysed against deionised water (pH 7.5), at 4^{o}C for 48 hours. The enzyme activity was determined at the beginning of dialysis, after 24 and 48 hours.

3.3.2 RESULTS

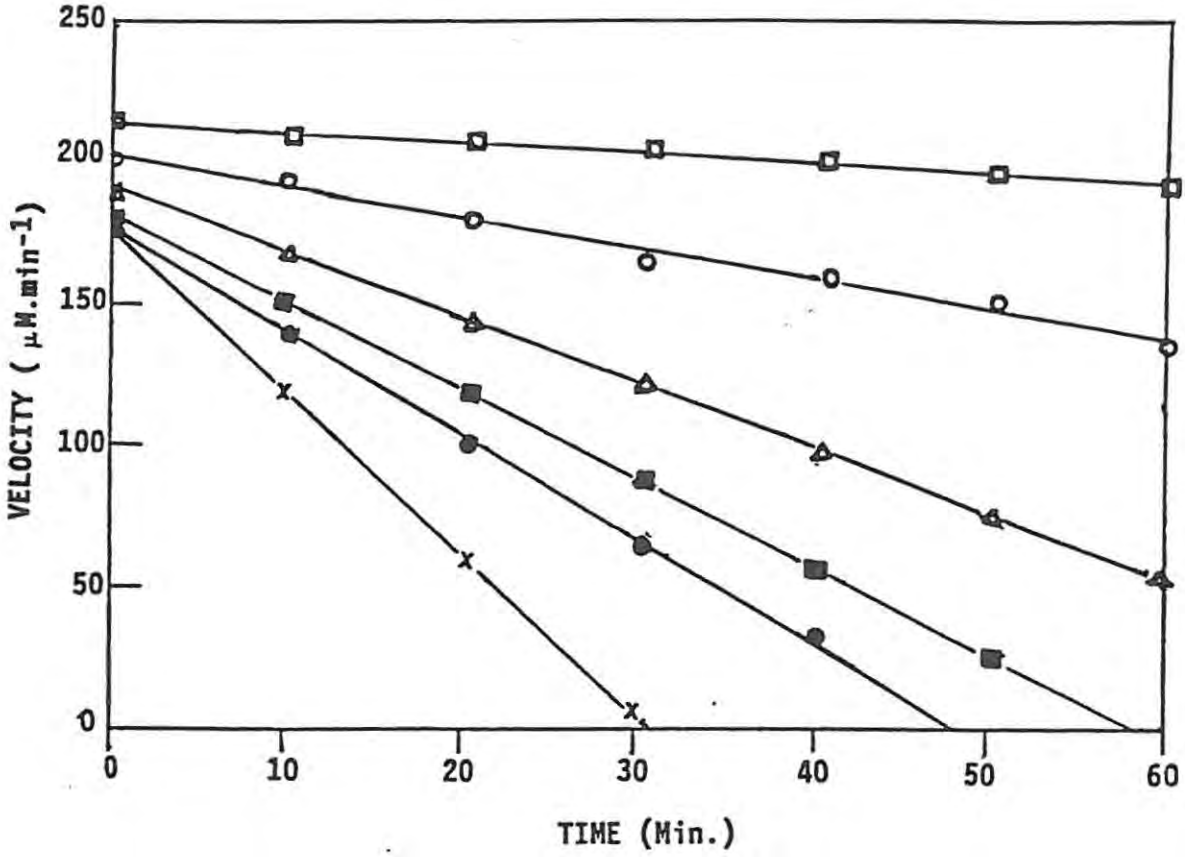


FIGURE 10: *Inactivation of acetylcholinesterase, (AChE) by dipropylnitrosamine, (DPNA) at pH 7.0. Concentration of DPNA is (□), 0μM; (○), 32μM; (△), 64μM; (■), 130μM; (●), 160μM; (x), 320μM.*

3.3.3 DISCUSSION

Incubation of acetylcholinesterase with dipropylnitrosamine resulted in a progressive loss of enzyme activity as depicted in FIG. 10. These results indicate that the inactivation of the enzyme by DPNA was dependent on the concentration of the nitrosamine as well as on the incubation time. When the enzyme was mixed with 32 μM DPNA, 28% of the control activity remained after 60 minutes while it took only 6 minutes in the presence of 320 μM DPNA to reach the same activity.

CHAPTER 4 BINDING STUDIES

A thorough understanding of the biochemical significance of a ligand which is a small molecule that binds to a macromolecule eg. protein or enzyme comes only from a quantitative analysis of the strength of binding. Binding affinity between two molecules is often expressed in terms of an association constant, Ka of the protein-ligand complex (121,122).

In order to make a comprehensive study on the effect of secondary nitrosamines on AChE, it thus became one of the aims of this work to investigate the binding of these molecules to the active side of the enzyme. This objective was accomplished by carrying out the binding studies using three techniques: Equilibrium dialysis, Gel chromatography and Fluorometry.

4.1 *EQUILIBRIUM DIALYSIS*

4.1.1 MATERIALS and METHODS

4.1.1.1 Materials

All chemicals and reagents were of analytical grade. Phosphate buffer, deionised water, dialysis tubes were from Spectrophor.

4.1.1.2 Methods

The experiments were carried out according to the method of McPhie(121). Acetylcholinesterase (15 $\mu g.ml^{-1}$,3.0ml) was dialysed at 4°C against phosphate buffer (20mM, pH 7.6) containing ligand (1.0-7.5mM,1.0ml) under constant agitation for 24 hours. After equilibrium, the concentration of the ligand outside the dialysis bag was determined spectrophotometrically using absorption coefficients (124) [APPENDIX 1].

Data was then analysed according to Scatchard (125), the number of binding sites, p and the dissociation constants, Kd determined according to equations (1) and (2):

$$\nu / [L]_f = p - \nu / K_d \quad ....(1) \qquad \Delta A_x / \Delta A_{xmax} = \nu / p \quad ....(2)$$

where ν are the molecules of bound ligand, p is the number of binding sites, $[L]_f$ is the concentration of free ligand, K_d is the dissociation constant of the ligand-enzyme complex, ΔA_x is the difference in the absorbance of the two solutions at wavelength x and ΔA_{xmax} is the maximal absorbance change.

4.1.2 RESULTS

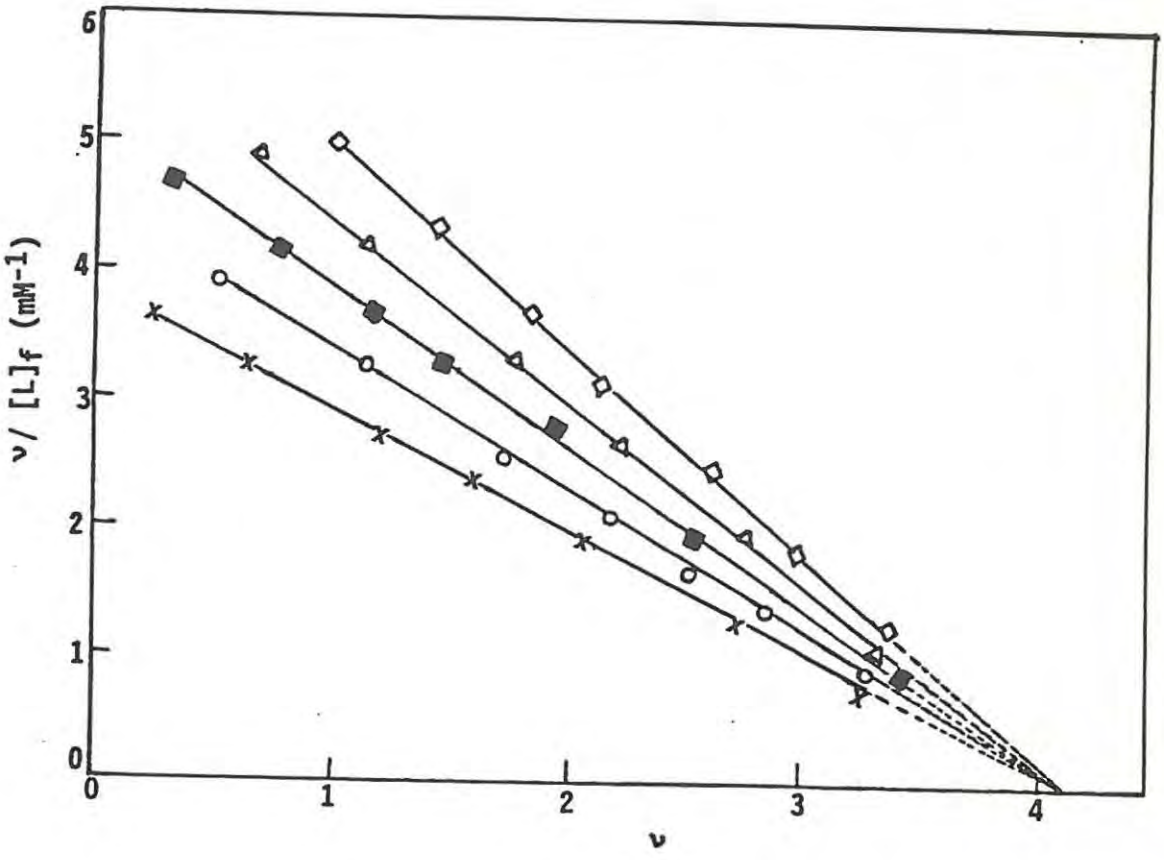


FIGURE 11(a) : *Scatchard plot for binding of DPhNA(◇), MNGly(△), DMNA(□), DEtNA(○) and DENA(×) to AChE as determined by equilibrium dialysis, ν are molecules of bound ligand.*

4.1.2 RESULTS (Contd.)

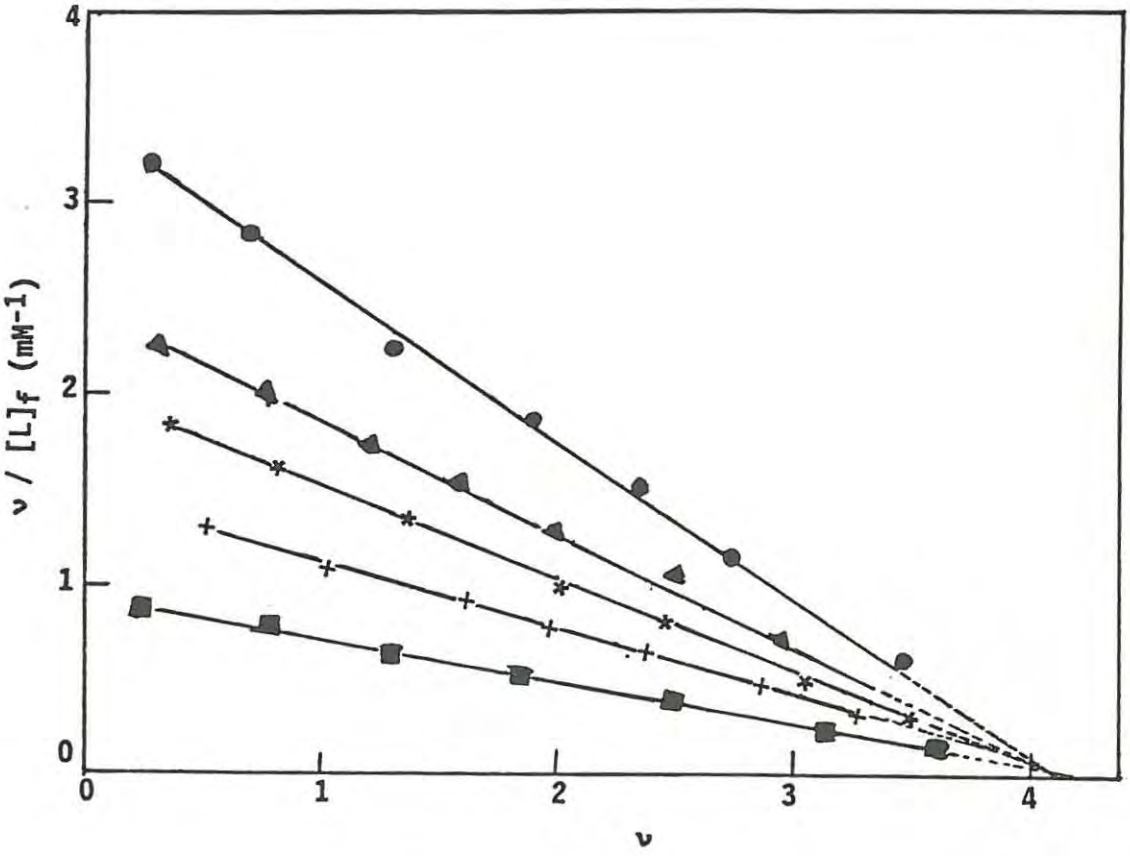


FIGURE 11(b) : *Scatchard plot for the binding of DPNA(●), NPyr(▲), NMor(✱), DBNA(+) and NPip (■) to acetylcholinesterase by equilibrium dialysis*

4.1.2 RESULTS (Contd.)

TABLE 3: *Binding Constants calculated from Scatchard plots of nitrosamines to acetylcholinesterase, as determined by equilibrium dialysis experiments.*

LIGAND	K_d(mM)	p
DPhNA	0.62	4.1
MNGly	0.71	4.1
DMNA	0.80	4.0
DEtNA	0.91	4.1
DENA	1.06	4.1
DPNA	1.22	4.1
NPyr	1.67	4.1
NMor	2.02	4.0
DBNA	2.83	4.0
NPip	4.21	4.0

4.1.3 DISCUSSION

The results show that the extent of binding of the nitrosamine to the enzyme depends on the size and shape of that molecule [APPENDIX 12].The Scatchard analysis of the results [FIGURES 11(a) and 11(b)] show that the enzyme contains a single class of binding sites. TABLE 3 shows the dissociation constants of various nitrosamines, which increase with an increase in the size of the alkyl groups attached to the nitroso group. The heterocyclic morpholine is a weakly polarised molecule due to the lone pair of electrons on the oxygen inside the ring, as such it is a weak nucleophile. Though the molecule is able to solvate the cations within its environment, its high dissociation constant (K_d=2.02mM) shows

that it binds less strongly to the active site of the enzyme. The molecule is relatively bulky compared to the other compounds studied. Upon the generation of an *in situ* quaternary nitrogen, the size of this compound prevents an effective interaction between the molecule and the active site. Another point is that on photodecomposition, NMor is expected to generate a 4-carbon alkylating species, meaning that the longer alkyl group could effect a lower binding to the subsite of the enzyme.

On the other hand, diethanolnitrosamine bears two hydroxyl groups which also make the molecule polar. These two functional groups are able to form hydrogen bonds with suitable groups in or around the active site of the enzyme. Its dissociation constant (K_d=0.91mM) reflects a stronger binding as compared to NMor. The length of the alkyl groups could disorientate the molecule around the active site, thus resulting in a far less stronger binding as compared to a tighter binding of diphenylnitrosamine (K_d=0.62mM).

4.2 *GEL CHROMATOGRAPHY*

4.2.1 MATERIALS and METHODS

4.2.1.1 Materials

All the chemical reagents used were of analytical grade. Sephacryl-S 400 was obtained from Pharmacia, and Bovine Serum Albumin (BSA) was from Sigma Chemicals Co.

4.2.1.2 Methods

The binding of the nitrosamine to the enzyme was studied on the column (2x20cm) of Sephacryl-S 400, pre-equilibrated in phosphate (0.10mM, pH 7). The reaction mixture (3 ml) which contained the enzyme solution (0.11mM, 1.0ml) and ligand (1.5-7.5mM) were applied to the column after being incubated at room temperature for 10 minutes. The flow rate of elution was 0.85ml.min^{-1} and the effluent was monitored at 280nm. Data was analysed according to Scatchard. The number of the binding sites (p) and the dissociation constant (K_d) of the enzyme-ligand complex were determined according to equations (1) and (2) (PAGE 36).

4.2.2 RESULTS

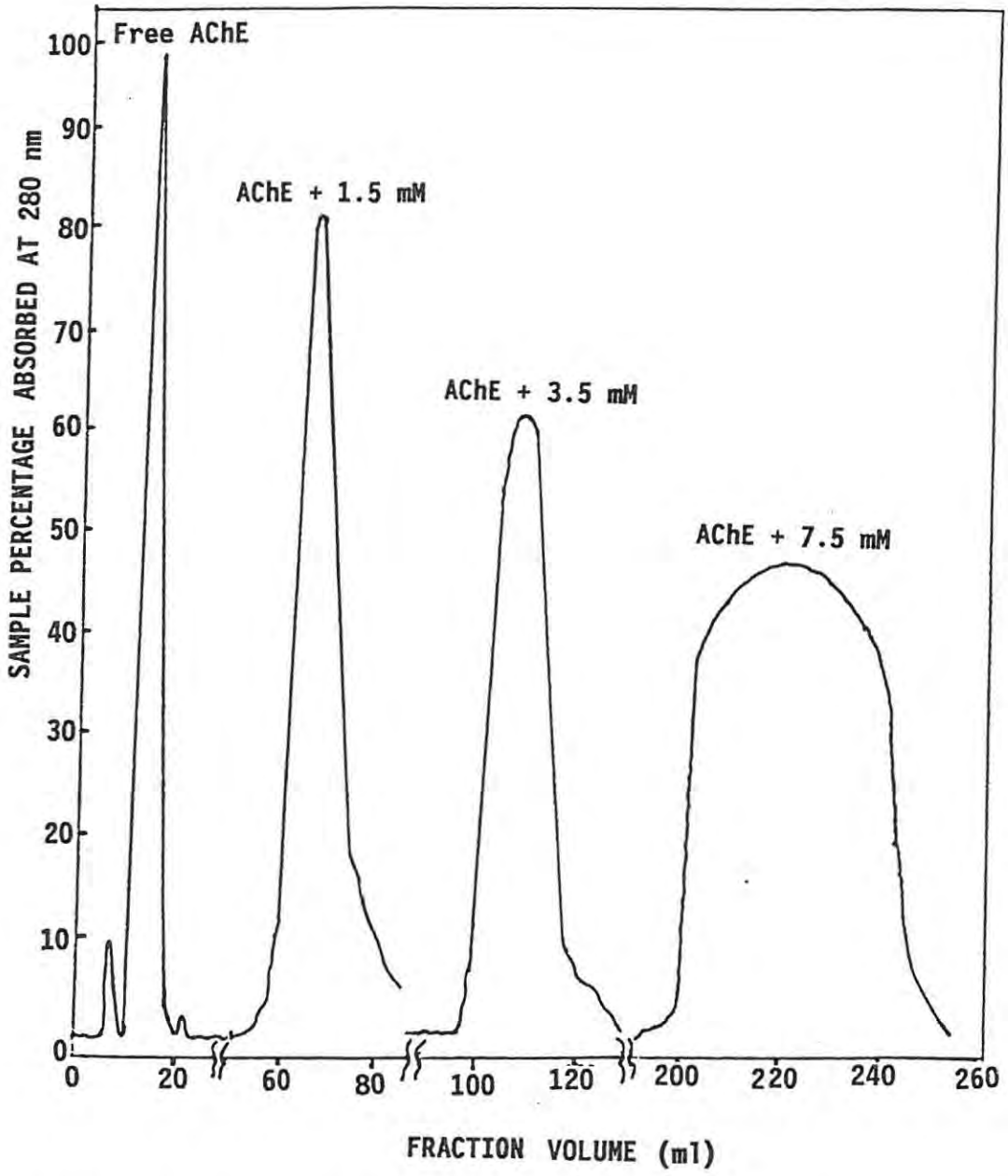


FIGURE 12: *Elution chromatograph of absorbance change on binding of dibutylnitrosamine to acetylcholinesterase (0.11mM) in 0.10mM phosphate buffer (pH 7.0). The eluant was monitored at 280nm*

4.2.2 RESULTS (Contd.)

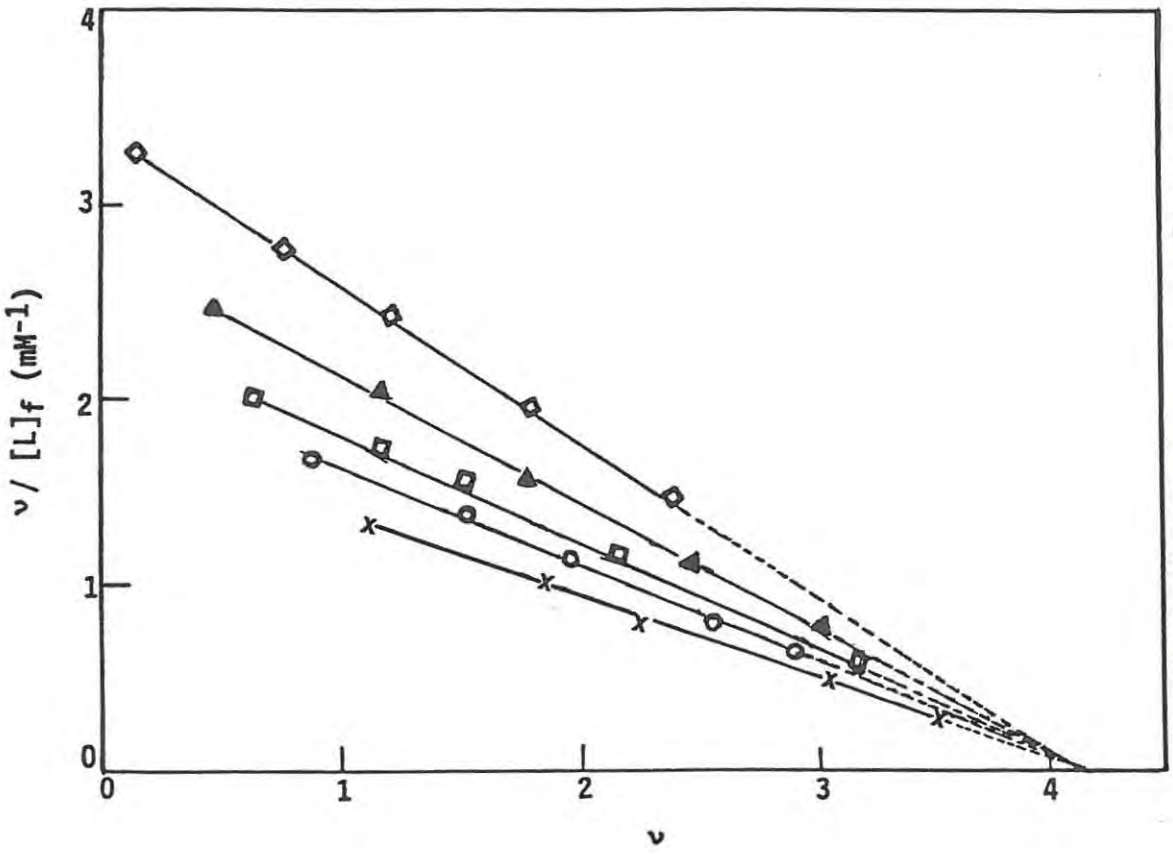


FIGURE 13: *Scatchard plot for binding of DPhNA(◇), MNGly(▲), DMNA(□), DEtNA(○) and DENA(×) to acetylcholinesterase as determined by gel chromatography, ν are molecules of bound ligand.*

4.2.2 RESULTS (Contd.)

TABLE 4: *Binding constants of AChE-Nitrosamine complexes determined from the corresponding Scatchard plot (FIGURE 13), using gel chromatography*

LIGAND	K_d(mM)	p
DPhNA	0.68	3.90
MNGly	0.80	3.93
DMNA	0.87	3.89
DEtNA	0.92	3.89
DENA	1.01	4.00
DPNA	1.25	3.82
NPyr	1.72	3.79
NMor	2.25	3.68
DBNA	2.92	3.76
NPip	4.60	3.83

4.2.3 DISCUSSION

The results obtained from Gel Chromatography indicate the same general pattern as those obtained from Equilibrium dialysis experiments. The dissociation of the enzyme-ligand complex appears to increase with an increase in the alkyl groups. DMNA(0.87mM), DENA(1.01mM), DPNA(1.25mM) and DBNA(2.92mM). This pattern holds true for the cyclic ones: NPyr(1.72mM),

NPip(4.60mM). As before, DPhNA(0.68mM) with its lowest K_d value reflects the strongest binding of the rest of the group.

4.3 *FLUOROMETRIC EXPERIMENTS*

In recent years, Fluorescence Spectroscopy has been developed into a routine experimental procedure for the study of protein-ligand interactions (123,126,127).

4.3.1 MATERIALS and METHODS

4.3.1.1 Materials

All the chemicals used were of analytical grade.

4.3.1.2 Methods

Titration measurements were made with Hitachi Fluoresence Spectrophotometer using an excitation light source from a Xenon-150 lamp; fluorescence was measured through a cell at an angle of 90° to the incident beam. All the experiments were carried out at 20°C in phosphate buffer (0.1M, pH 7.2). The binding studies were carried out with nitrosamines (0-7.5mM), which were added to the enzyme solution ($15.20 mg.ml^{-1}$). The mixture was excited at 285nm and analysed at 330nm. Corrections for inner filter effects were made according to procedure described by Helene *et al.* (128), equation (3):

$$F/F_1 = C = A_T(1-10^{-Ao}) \;/\; Ao(1-10^{-Ao}) \quad .....(3)$$

where F_1 is the observed fluorescence before application of the correction factor for inner filter effects, Ao is the absorbance of the acceptor alone and A_T is the total absorbance of the acceptor-ligand mixture. The corrected fluorescence intensities were used to determine the dissociation constants according to equations (4) and (5):

$$K_d / 1-\theta = [L]_t / \theta - p[A]_t \quad ....(4) \qquad \theta = \Delta F / \Delta F_{max} \quad ....(5)$$

where $[A]_t$ is the total concentration of the acceptor, i.e the enzyme in the system, p is the total number of binding sites, θ is the fractional occupancy of the total acceptor sites by ligand, ΔF_{max} is the increase in fluorescence at full saturation with ligand, ΔF is the increase in fluorescence of a known amount of ligand and $[L]_t$ is the total concentration of ligand.

4.3.2 RESULTS

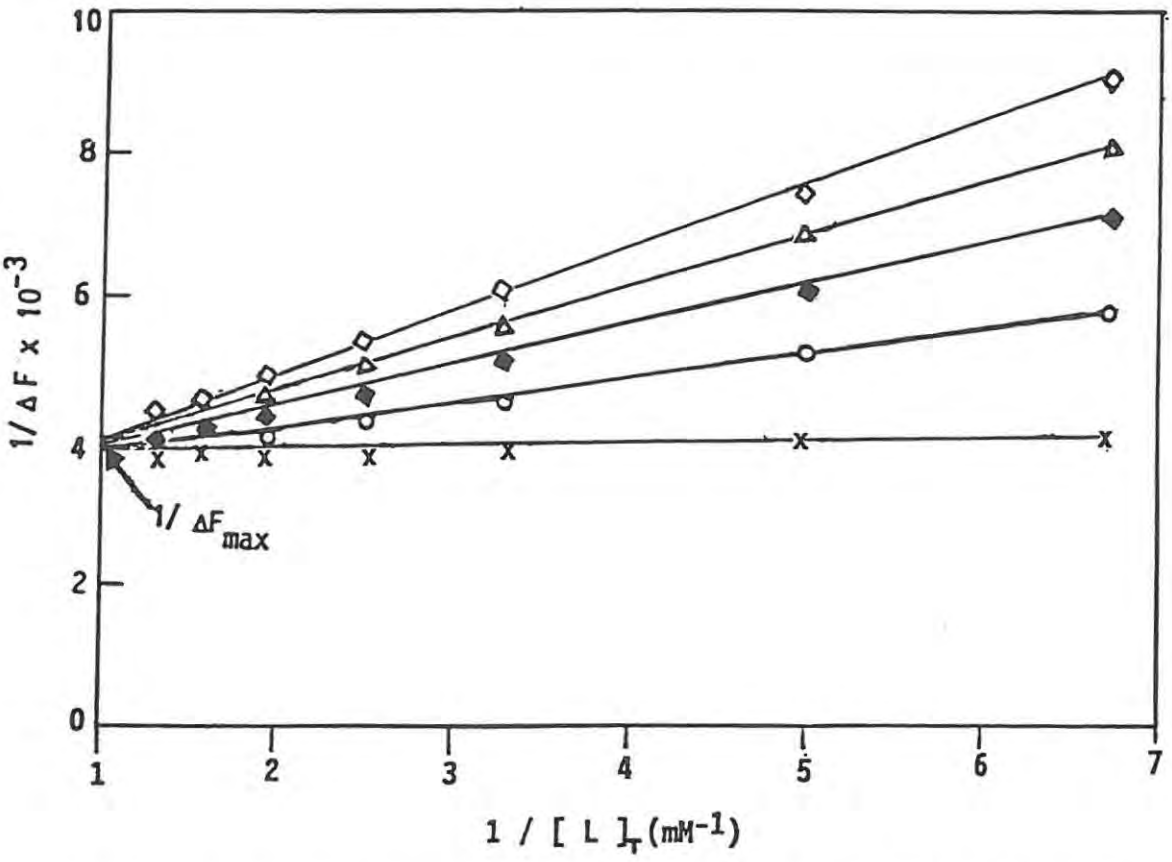


FIGURE 14(a): *Double-reciprocal plots of fluorescence change on binding of DPhNA(◇), MNGly(△), DMNA (◆), DEtNA (○),and DENA (×) to acetylcholinesterase (15.20mg.ml^{-1}) in 0.10M phosphate buffer pH 7.2). The mixture was excited at 285nm and emission measured at 330nm.*

4.3.2 RESULTS (Contd.)

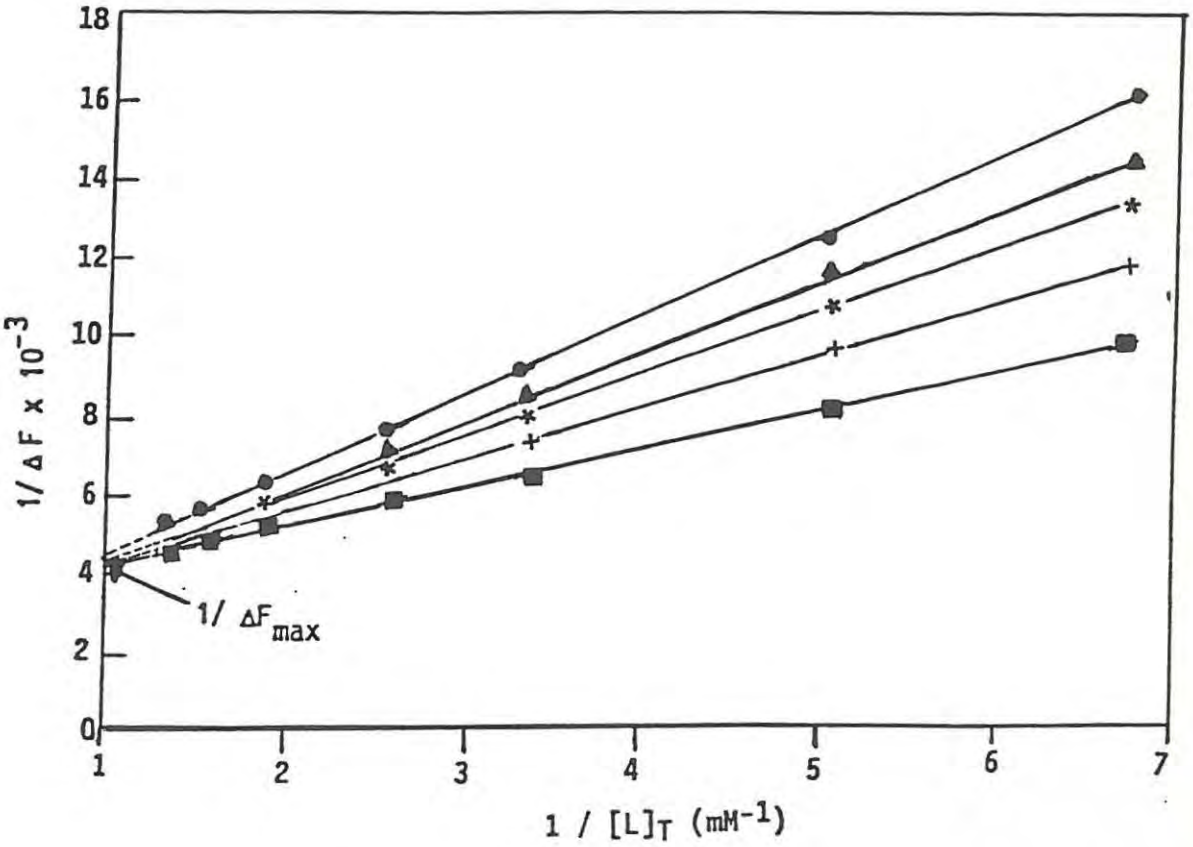


FIGURE 14(b) : *Double-reciprocal plots of fluorescence change on binding of DPNA(●), NPyr(▲), NMor(✱) DBNA(✚) and NPip (■) to acetylcholinesterase (15.20mg.ml^{-1}) in 0.10M phosphate buffer (pH 7.2). The mixture was excited at 285nm and emission measured at 330nm.*

4.3.2 RESULTS (Contd.)

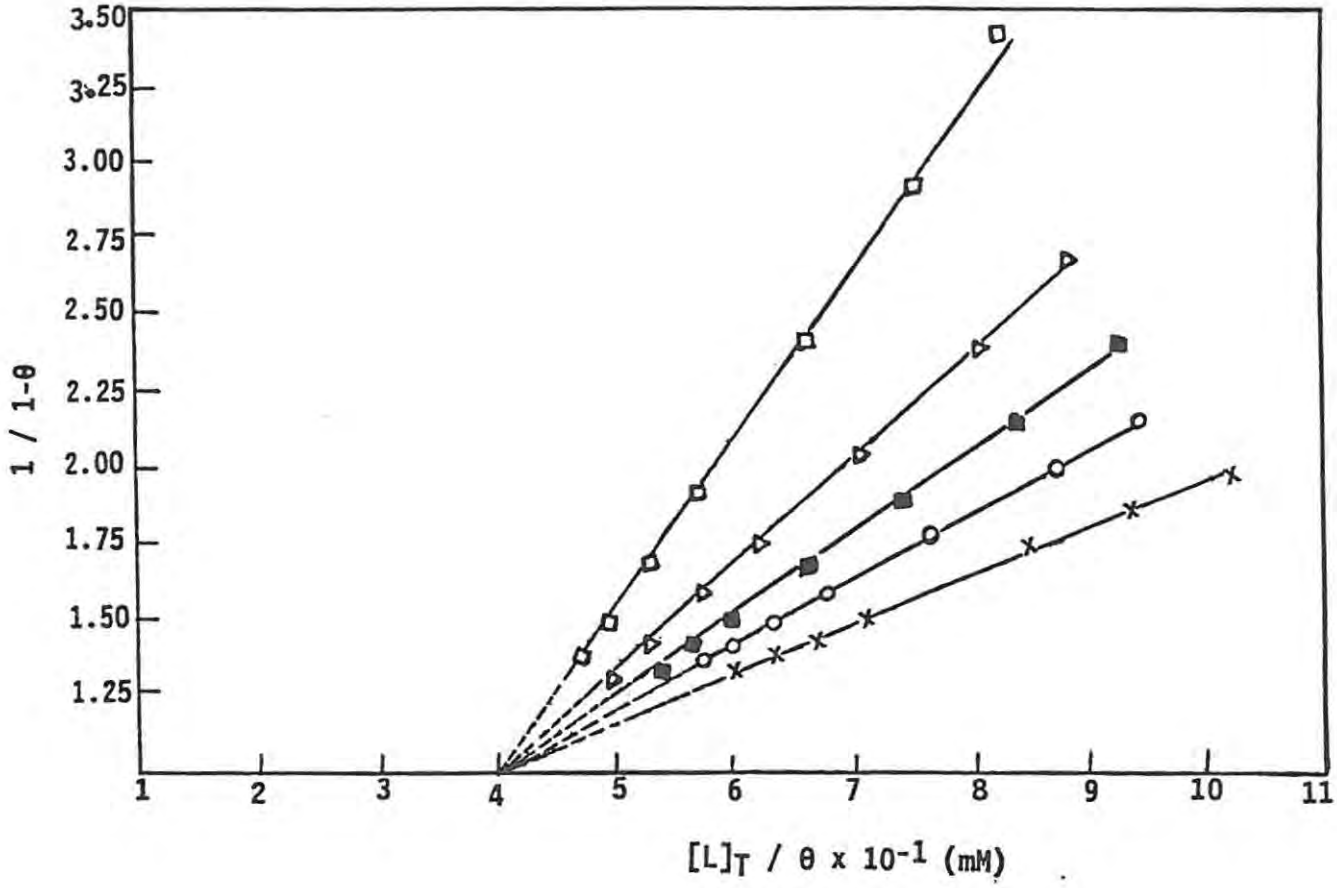


FIGURE 15(a): *Analysis data for the binding of DPhNA(◇), MNGly(△), DMNA(■), DEtNA(○) and DENA(×) to acetylcholinesterase in 0.10M phosphate buffer, pH 7.2.*

4.3.2 RESULTS (Contd.)

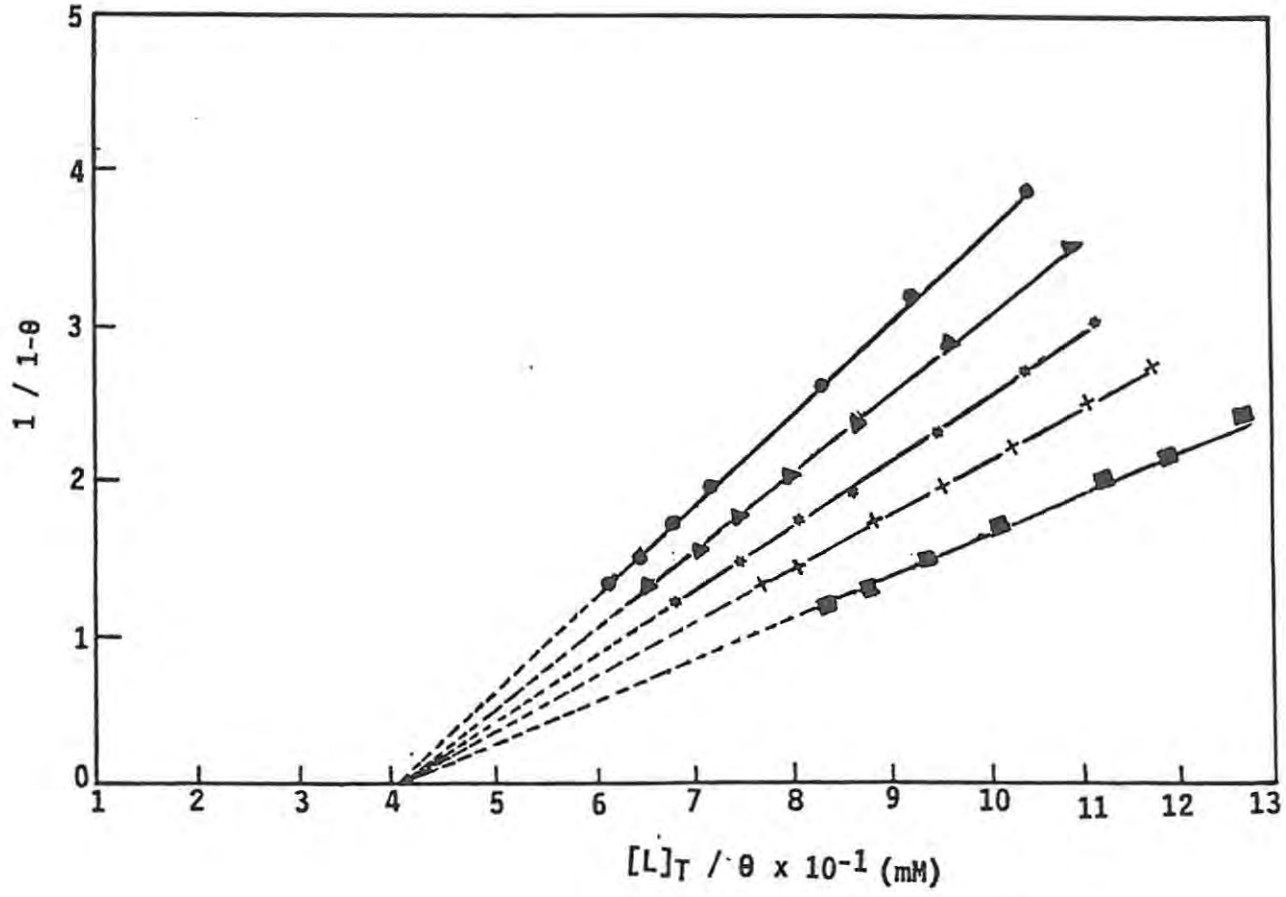


FIGURE 15(b): *Analysis data for the binding of DPNA(●), NPyr(▲), NMor(✱), DBNA(+) and NPip(■) to acetylcholinesterase in 0.10M phosphate buffer, pH 7.2*

4.3.2 RESULTS (Contd.)

TABLE 5: *Binding Constants calculated from analysis data plots [FIGURES 15(a) and (b)]*

LIGAND	K_d(mM)	p
DPhNA	0.65	4.0
MNGly	0.75	3.9
DMNA	0.88	4.1
DEtNA	0.94	4.0
DENA	1.59	3.8
DPNA	2.00	4.0
NPyr	2.43	3.9
NMor	2.86	4.2
DBNA	3.44	4.0
NPip	4.09	4.1

4.3.3 DISCUSSION

The analysis of these results show characteristic plots of 1/1-θ versus [LIG.]/θ to be linear, thus showing effect of these molecules on the enzyme [FIGS.14(a)-15(b)]. From these plots, the extent of dissociation of the AChE-ligand complex can be estimated. TABLE 5 shows the dissociation constants determined from the above figures. From these results, the number of binding sites averages to 4, which was in good agreement to the other results from gel chromatography and equilibrium dialysis. There is an increase in the dissociation constants from DPhNA(0.65mM) to NPip(4.09mM). Again the same argument holds, that the aromatic nitrosamine DPhNA depicts a much tighter binding to the enzyme.

CHAPTER 5 DISCUSSION

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, localization and functions of a particular biological system under study.

It was therefore the purpose of this project to synthesise a series of secondary N-nitrosamines from their respective secondary amines; and to interact them with acetylcholinesterase, in order to study their effect on catalysis, their type of inhibition and their extent of binding to the active site of the enzyme. The choice of nitrosamines for this study is based on their established properties . The successful synthesis of these compounds by classical methods of nitrosation were carried out, for which good yields were recorded in their purified forms [TABLE 1]. The obtained melting and boiling points are comparable to the literature values. It could be established in this project that nitrosamines actually do affect catalysis of the enzyme as inhibitors in a reversible and competitive way with respect to the substrate acetylthiocholine chloride (FIGURES 7-9(a)). Kinetics results were analysed using standard graphical technique of Lineweaver and Burk (120). The lines were drawn by computer calculation for linear competitive inhibition (EQUATION 6):

$$1/V = 1/V_{max}\ [K_m(1+I/K_i)].1/S + 1/V_{max} \quad ....(6)$$

A replot of the slopes of double reciprocal plot versus ligand concentration are linear (FIGS.7 and 8,inset). The relevant kinetic parameters for the nitrosamines are presented [TABLE 2], where K_m and

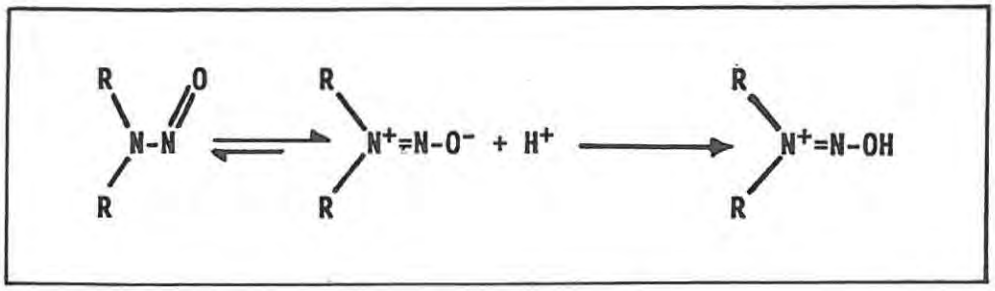
Km_{App} are the respective Michaelis contants, K_i is the inhibitor constant of the enzyme-inhibitor complex calculated from equation (7) and from the extrapolation of the graph-slope versus nitrosamine concentration. According to this graph, the K_i should be 260 μM, while Table 2 shows K_i is 60 μM per subunit.

$$Km_{APP} = K_m(1+I/K_i) \quad .....(7)$$

where I is the concentration of the particular nitrosamine. With variable substrate concentrations values for K_m and V_{max} for acetylcholinesterase were established as 102.61 μM and 263.16 $\mu M.min^{-1}$ respectively.

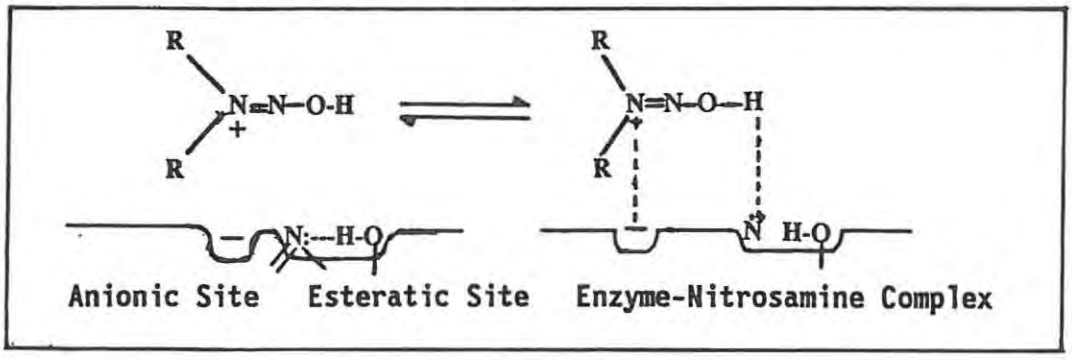
The overall potency of a particular nitrosamine in the interaction of AChE could be established considering the dissociation and inhibitor constants. These values reflect the 'fit' of the nitrosamines into the active site of the enzyme. Such values depend on the size, configuration and the structure of such nitrosamines . Furthermore, the potency of the nitrosamine to bind non-covalently at or near to the active site plays a significant role. The more lipophilic or hydrophobic the nitrosamine, the less the ability to bind and inhibit the enzyme (54). As the length of the alkyl chain attached to the nitroso group increases so there is a decrease in activity of the enzyme. The kinetic data indicates an increase in the K_i values from DPhNA(20.32 μM), MNGly(30.18 μM), DMNA (34.78 μM), DEtNA(43.68 μM), DENA(54.24 μM), DPNA(60.36 μM), NPyr (66.07 μM), NMor(73.93 μM), DBNA(95.54 μM) and NPip(123.00 μM). These values show that the aromatic DPhNA is a far more potent inhibitor than the other nitrosamines.

From the well established properties of nitrosamines, one can appreciate that the nitroso group [-N=O] of the nitrosamine resembles the carbonyl group [-C=O] of acetylcholine. By the same latitude of reasoning, the nitroso group interacts with the activated serine hydroxyl to form an eight-membered enzyme-inhibitor complex. Furthermore, perhaps a more acceptable reasoning is that an *in situ* quaternary nitrogen is developed [SCHEME 8 and APPENDIX 12]:



SCHEME 8: *The illustration of quaternary nitrogen anticipated to be generated in, or in the vicinity of the active site of acetylcholinesterase, in situ.*

This quaternary nitrogen may then interact with the nucleophilic serine hydroxyl or the anionic subsite that is in close proximity (54),[SCHEME 8]. The canonical form of the nitrosamine once bound could be expected to prevent the access of acetylthiocholine to the active site thereby competitively inhibiting the enzyme [SCHEMES 7 and 9].



SCHEME 9: *Illustration of competitive inhibition of AChE by a quaternary form of a nitrosamine*

Fundamentally, two properties for the binding of the nitrosamines to the active site of the enzyme could be implied (54): First, one protonated residue in the active site of the enzyme allows the formation of the enzyme-substrate complex. Secondly, the AChE active site must be hydrophobic in nature, and have at least one tryptophan residue (63). This means that when a nitrosamine binds to the enzyme, the former ought to pass from a hydrophilic to a hydrophobic environment, which has already been implicated as an important factor in enzyme catalysis. Nitrosamines as rich sources of carbocations are able to alkylate the active site. The alkylating species [R^+], [APPENDIX 13] label the carboxylate anion of the anionic subsite, consequently enhancing the competitive nature of the inhibition. The inhibition of the enzyme by these alkylating species is postulated to occur according to mechanism shown in [SCHEME 6] . The calculated inhibitor constants reflect the ease of transition between the hydrophilic and the hydrophobic environment due to the overall stability, energy as well as size of the carbocations and the intermediates.

A general discussion of the K_i values for each nitrosamine is relevant at this stage. This will help the reader to understand the degree to which individual nitrosamine binds to the active site of the enzyme. This diversity in values should be due to the different forms of structures and sizes that each nitrosamine assumes.

The lowest K_i value is obtained for DPhNA(20.32 μM). This indicates that the nitrosamine has high potency as well as strong inhibitory ability on the enzyme. This can be explained by the fact that the two benzyl rings found in the aromatic diphenylnitrosamine at least do

appear to be highly stable in generating the corresponding intermediates [Ph-$N^{+}\equiv N$] and [Ph^{+}]. The generation of a [Ph^{+}] appears very unlikely . This leaves one with the conclusion that this high inhibition could be due to the *in situ* quaternary nitrogen developed within the nitrosamine molecule, after binding to the active site. The excessive electron density in the aromatic rings stabilise this positively charged nitrogen, thus influencing stronger interaction with anionic subsite of the enzyme causing a potent competitive inhibition. This is analogous to the situation of acetylcholine and for the quaternary ammonium salts binding to the enzyme.

Other phenyl-derivative inhibitors however such as phenyltrialkyl ammonium salts were found to be potent inhibitors of the enzyme. They have been reported to bind much tigher to the enzyme than the natural substrate (54). Therefore, if DPhNA decomposes into a diazonium intermediate [Ph-$N^{+}\equiv N$] and/or [Ph^{+}], then these intermediates would still effect ionic interaction at the anionic subsite.

The K_i values increase from MNGly (30.18 μM) to NPip (123.0 μM). From SCHEME 6 showing the generation of carbocations, a short description concerning the potency of individual nitrosamines could be made. MNGly with its carboxylic acid group as a polar functional group is able to form hydrogen bond with other carboxylic groups in the active site or in the vicinity of the latter. This bond could result in the nitrosamine being less potent than DPhNA and generating much stronger inhibition than the rest of the other nitrosamines studied. On the other hand, DEtNA (K_i=43.68 μM) has a hydrophilic hydroxyl group which is also polar, as well as the lipophilic or hydrophobic ethyl group.

Though the hydroxyl group is able to enter into hydrogen bond formation with suitable groups on the enzyme e.g serinyl hydroxyl, the effectiveness of such a bond is counteracted by the overall effect of the lipophilic ethyl groups. DENA(K_i=54.24 μM), DPNA(K_i=60.36 μM) and NPyr(K_i=66.07μM) upon decomposing into carbocations are expected to generate a two, three and four carbon alkylating species respectively.

The results advance a logical explanation that the inhibition in these studies cannot only be intepreted in terms of the shape and conformation of the molecules, but the size of the alkyl groups as well. NMor (K_i=73.93 μM) shows a very weak polarity and hence a weak nucleophilicity. Though the oxygen with its unshared electrons is able to solvate cations within its environment, it remains a less potent inhibitor of the enzyme. This could be attributed to the length of the alkyl groups it possesses . DBNA (K_i=95.54 μM) and NPip (K_i=123.0 μM) show even a lower degree of inhibition. This, according to Whiteley *et al* (54) points out that the more lipophilic the nitrosamine, the less the ability of binding and inhibition of the enzyme.

Though nitrosamine carbocations can label the carboxylate moiety of the enzyme, this labelling may not necessarily and exclusively be limited to this subsite. Upon decomposition of the nitrosamine, the alkylating species could either alkylate other amino acids in a non-specific manner. Mechanism of inhibition [SCHEME 7], shows that the alkylating species inhibit acetylcholinesterase in a competitive and irreversible way. The extent of inhibition is attributed to the number as well as the length of the carbocation generated . According to APPENDIX 13, each

dialkylnitrosamine will generate a pair of carbocation species: dimethylnitrosamine [CH_3^+], diethylnitrosamine [$CH_3CH_2^+$], dipropylnitrosamine [$CH_3CH_2CH_2^+$] and dibutylnitrosamine [$CH_3CH_2CH_2CH_2^+$]. Logically, one of these species will interact covalently with the serine hydroxyl, imparting an irreversible inhibition on the enzyme. Furthermore, the remaining species may be trapped by reaction medium to afford the corresponding alcohol according to reaction (4):

$$R^+ + OH^- \longrightarrow R\text{-}OH \quad \ldots\ldots\ldots\ldots (4)$$

This reaction depends on the rate of decomposition of the nitrosamine, the proximity of such species to the active site as well as the actual conformation of the enzyme at that point in time. In the process of decomposition to yield the carbocation species, the rate-limiting step of mechanism should be the dissociation of the C-N bond in the nitrosamine. It must further be stated that the binding of nitrosamine to the enzyme forming a complex is influenced by the solvating properties of the catalytic site (54). The rate at which this process proceeds can be attributed to a change of solvating conditions in the active site, resulting from the binding of the nitrosamine with the enzyme esteratic site.

On the other hand, diethanolnitrosamine generates two [$HOCH_2CH_2^+$]. One of these species is anticipated to interact with the active site. In this case, the other species is not anticipated to be trapped by the medium to afford an alcohol. Instead, it can bind elsewhere on the enzyme either within the active site, or in its proximity.

Methylnitrosoglycine will generate two carbocations [CH_3^+] and [$HOOCCH_2^+$] according to their order of formation. During interaction with the enzyme, [CH_3^+] is able to interact covalently with the active site and also be trapped by the medium whereby it will form methanol. On The other hand, [$HOOCH_2^+$] is able to covalently bind to the active site but cannot afford an alcohol.

The cyclic nitrosopyrrolidine, nitrosopiperidine and nitrosomorpholine, generate species that are positively charged on both ends: NPyr [$CH_2^+CH_2CH_2CH_2^+$], NPip [$CH_2^+CH_2CH_2CH_2CH_2^+$] and NMor [$CH_2^+CH_2OCH_2CH_2^+$]. Electrostatic repulsion might results from the interaction of the two ends of an individual nitrosamine, rendering a weaker binding and a less stonger inhibition. While it is possible to envisage the binding to the enzyme by one end, the other might trapped by the basic medium to form alcohol moiety.

It is clear that, relative to the environment of the binding site, the nitrosamines cannot increase the basicity of this environment during the generation of an alkylating species. An assumption is that the rearrangement could involve a conformational change in the enzyme molecule, leading to the exposure of some new basic groups inside the active site.

It was also the aim of this project to study the extent of binding of these molecules to the active site of the enzyme. This was accomplished by means of Equilibrium dialysis, Gel Chromatography and Fluorimetric titrations. The results of these three techniques show a similar trend [APPENDIX 11]. The analyses of such results indicate that the enzyme

contained a single class of binding sites [FIGURES 11(a)-15(b)]. The dissociation constants of various nitrosamine-AChE complexes are presented [APPENDIX 11]. As observed in binding studies, an increase in the dissociation constants was shown from these three techniques.

The kinetic and binding studies results show a similar trend. The values of the dissociation constants obtained from the three techniques [APPENDIX 11] are averaged:DPhNA(K_i=20.32 μM, K_d=0.65mM); MNGly (K_i=30.18 μM,K_d=0.75mM);DMNA(K_i=34.78μM,K_d=0.85mM);DEtNA(K_i=43.68 μM, K_d=0.92mM); DENA(K_i=54.24 μM, K_d=1.22mM); DPNA(K_i=60.36 μM, K_d=1.49mM); NPyr(K_i=66.07 μM, K_d=1.94mM); NMor(K_i=73.93 μM, K_d=2.38mM); DBNA (K_i=95.54 μM, K_d=3.06mM) and NPip(K_i=123 μM, K_d=4.30mM). The smaller values in both the K_i and K_d values signify the extent of inhibition as well as binding of the nitrosamine at the active site of the enzyme.

The observed low values indicate stronger inhibition potency as well as a tighter binding of the corresponding ligand to the active site, while the higher values reflect the opposite.

These results tend to support the idea that two discrete sites exist on the enzyme, and also provide evidence indicating that the binding sites could be independent and indistinguishable. The linearity of the Scatchard plots was used to determine the number of binding sites which averaged to four.

CHAPTER 6 *SUMMARY*

Acetylcholinesterase is confirmed to be found in a number of organs. It has been shown to be associated primarily with excitable membranes, cholinergic synapses, and endplates of neuromuscular junctions. It is also found in a few non-neuronal cells like erythrocytes in which its function is unclear.

Its physiological role is to ensure that the nerve impulse is of a finite duration, by lowering the concentration of acetylcholine. Furthermore, subcellular fractionation and cytochemical studies show that the enzyme is closely associated with the surface membrane of the excitable cell. However, the precise relationship of acetylcholinesterase to the excitable membrane is not clearly understood, and various studies indicate that a significant portion of the enzyme is not tightly associated with the plasma membrane (54).

A substantial amount of evidence concerning the structure, locations and function of the enzyme have been reported. Mechanisms by which AChE catalyses its reactions, and by the way such reactions are inhibited have been postulated. Kinetic studies in the presence of nitrosamines indicate their capacity as competitive and reversible inhibitors of the enzyme. It was further shown that with time secondary N-nitrosamines act as competitive irreversible covalent inhibitors. The *in situ* quaternary nitrogen developed in the vicinity of the active site facilitates a competitive and reversible inhibition, while the alkylating carbocation facilitates a competitive covalent and irreversible inhibition.

A study of the kinetics as well as changes in fluorescence observed upon their binding to the enzyme, may provide information on the basic principles of intracellular communication, and on the functional properties of integral membrane proteins.

The facts that the reversible inhibition by the nitrosamines is competitive, and the subsequent irreversible inactivation is essentially the same toward hydrolysis of acetylthiocholine, support the proposal that the active site may be more specifically characterised by these nitrosamines.

Inhibition constants show that the size, the structure and the conformation of the molecule have influence on the extent of binding to the active site, consequently bearing an overall influence on inhibition. Binding studies also reveal to a certain extent the nature of binding ,the number of binding sites and the degree of binding to the active site of enzyme. It becomes apparent from these results that the smaller methyl groups belonging to dimethylnitrosamine and methylnitrosoglycine would not be expected to interfere with the process of binding, while it is probable that the larger and bulky ligands induce a decrease in binding capacity. It can be pointed out that the generation of carbocations by the nitrosamines within or around the microenvironment of the enzyme, in the neighbourhood of the active site can be assumed to be a reality. Fluorometric titrations, steady state kinetics, equilibrium perturbations and ligand binding studies, all support the proposed models for nitrosamine binding to acetylcholinesterase as favourable candidates to mimic acetylcholine, the natural substrate of the enzyme.

CONCLUSION

It is clear that the objectives of this project have been attained. The following points constitute the findings in this project :

(a) nitrosamines could be synthesised in large, purified quantities following classical method of nitrosation.
(b) inhibition of acetylcholinesterase by secondary N-nitrosamines is twofold: competitive and reversible as well as covalent competitive and irreversible.
(c) the size, shape and conformation of the inhibitor do influence the extent of inhibition and binding to the active site of the enzyme.
(d) acetylcholinesterase has independent binding sites.
(e) the number of binding sites averaged to four .

In the final analysis, as a point of departure, it is well documented that the dialkyl nitrosamines are carcinogenic. This, therefore precludes them for use as medical drugs to mimic acetylcholine or to inhibit acetylcholinesterase. Hence on this score they cannot be of much value to the human society especially in pathological diseases like erythroblastosis, thallasaemia major and also haemolytic disease. However the case may be, LIFE NOT BEING A BED OF ROSES, MUST GO ON !

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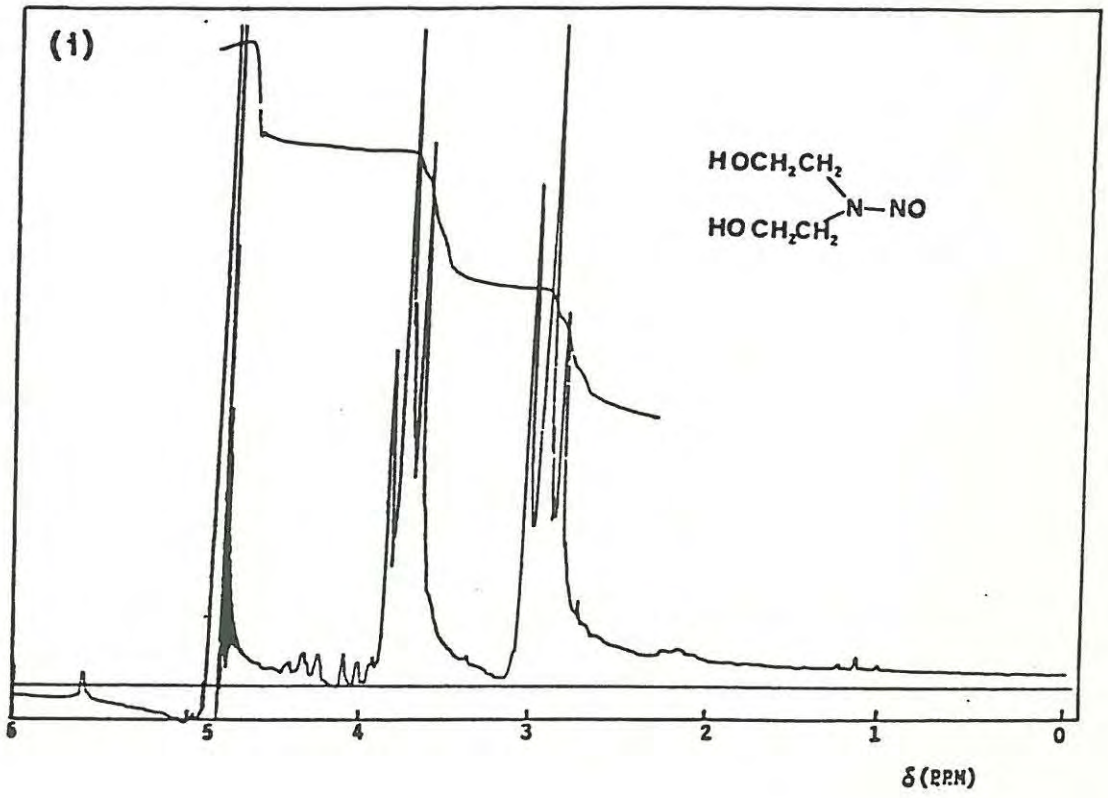
APPENDIX 1

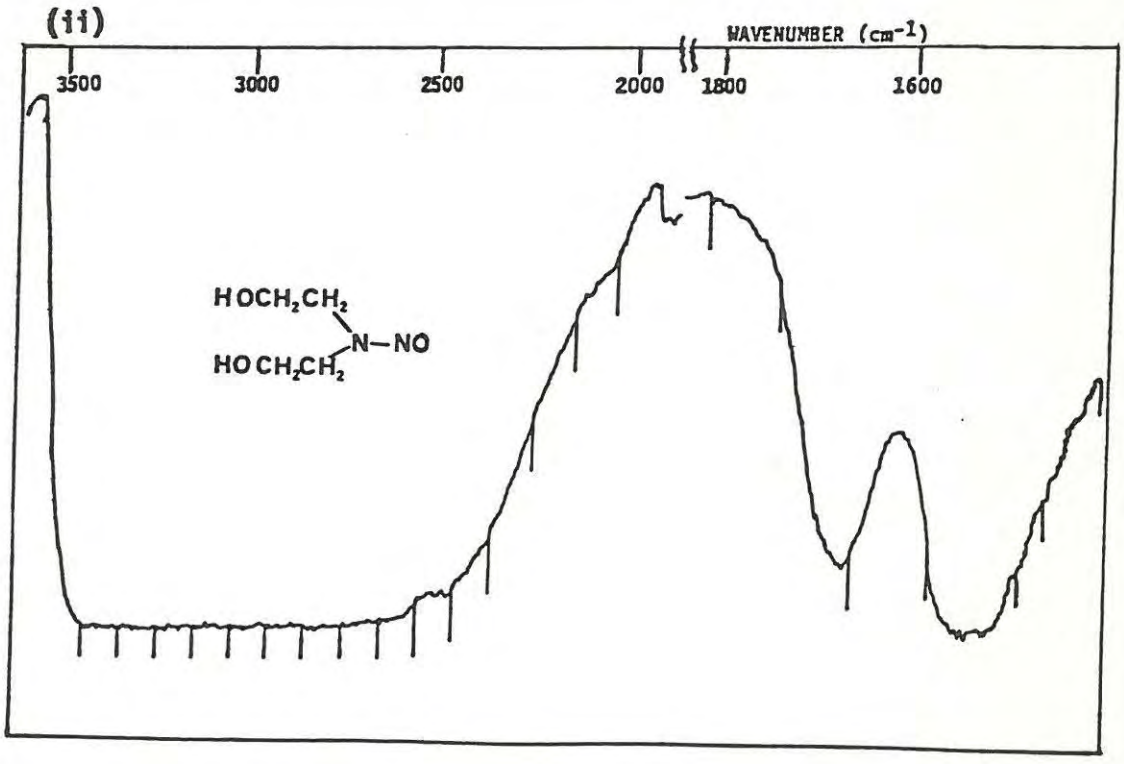
MOLAR ABSORPTION COEFFICIENTS OF N-NITROSAMINES USED IN THE TEXT

NITROSAMINE	log ε	λ_{max} (nm)
DBNA	1.95	350
DENA	1.95	350
DEtNA	1.95	350
DMNA	1.95	350
DPNA	1.95	350
DPhNA	3.88	290
MNGly	2.00	346
NPip	1.95	350
NPyr	1.95	350
NMor	1.95	350

APPENDIX 2

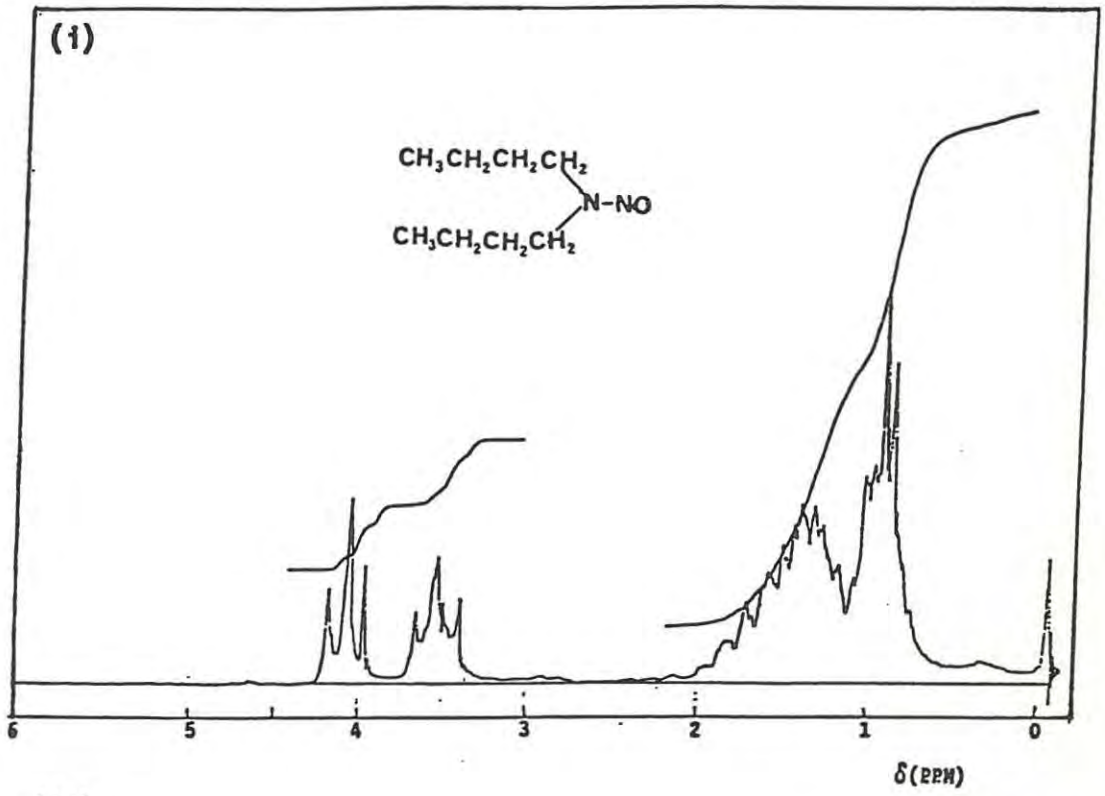
^{1}H-NMR(i) and IR(ii) SPECTRA OF DIETHANOLNITROSAMINE

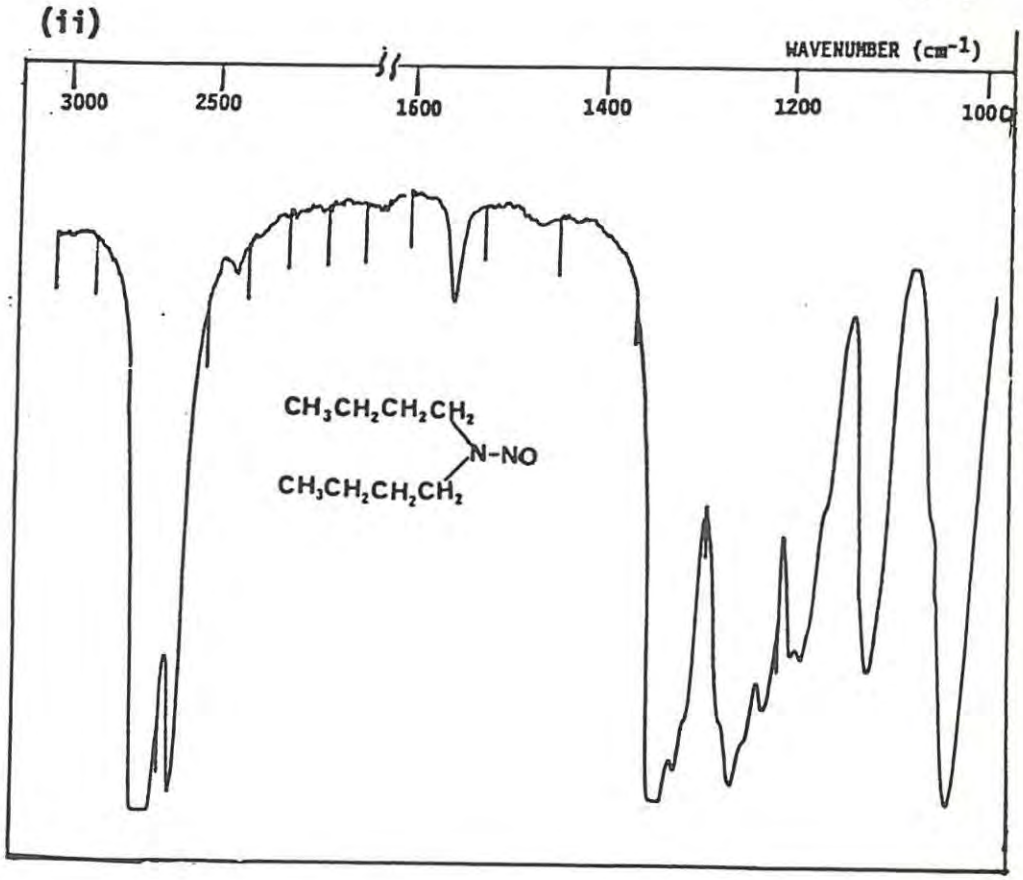




APPENDIX 3

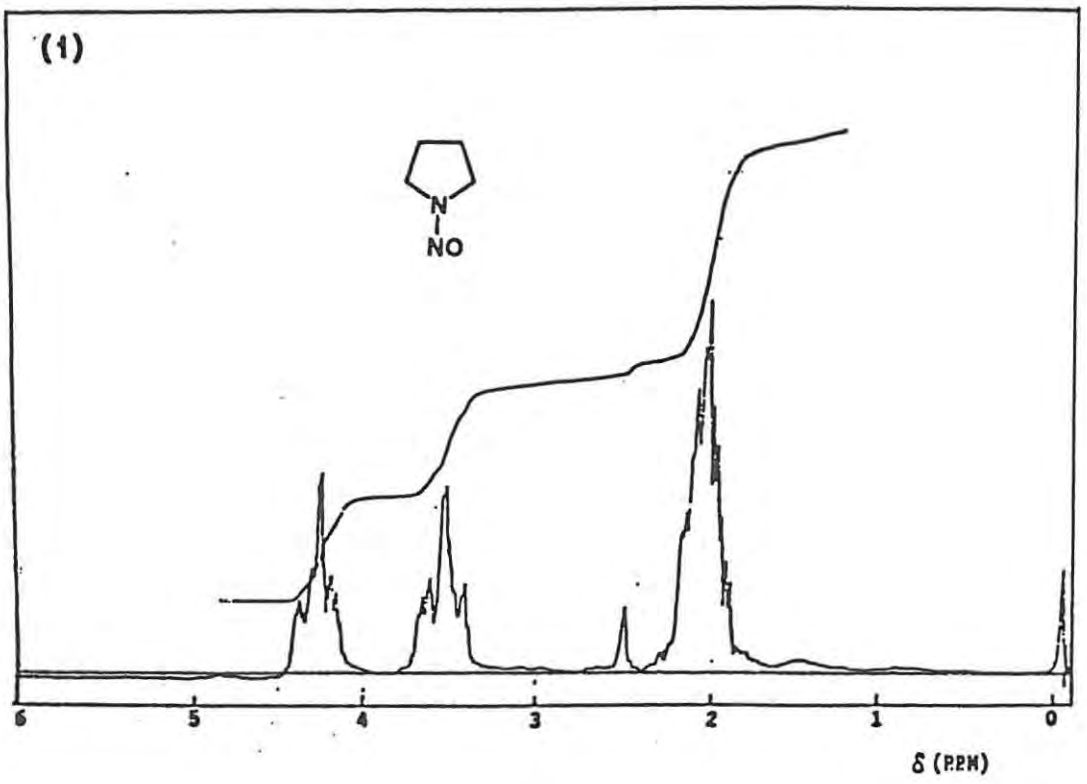
^{1}H-NMR(i) and IR(ii) SPECTRA OF DIBUTYLNITROSAMINE

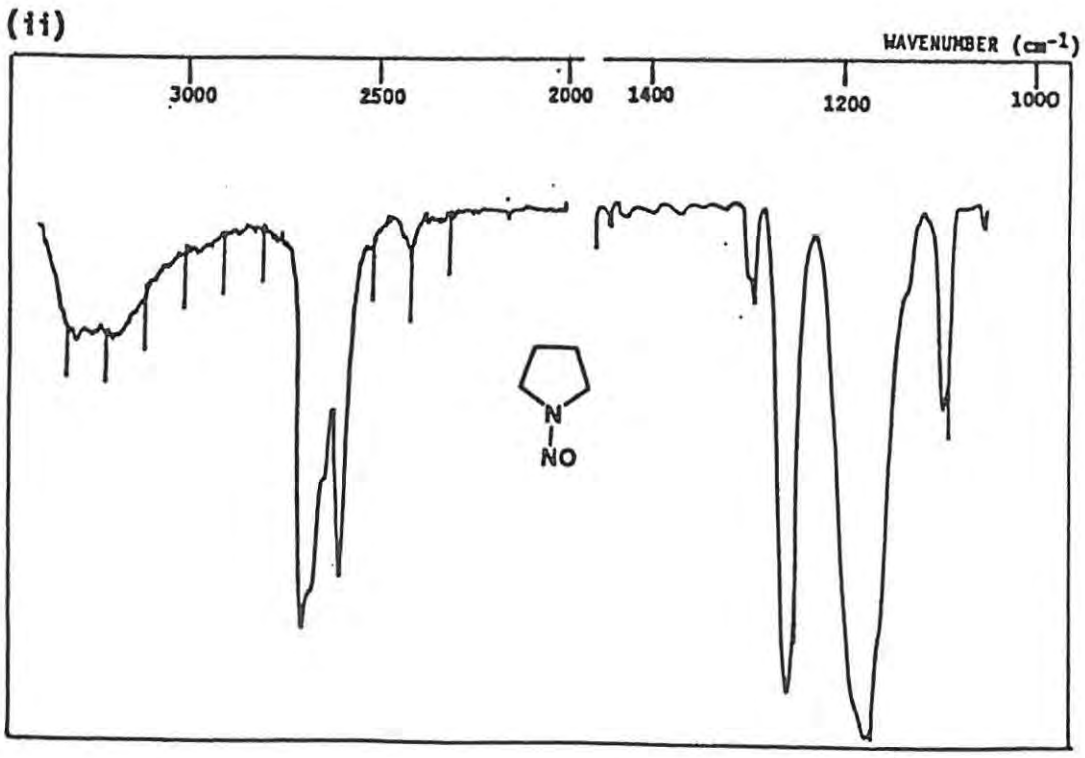




APPENDIX 4

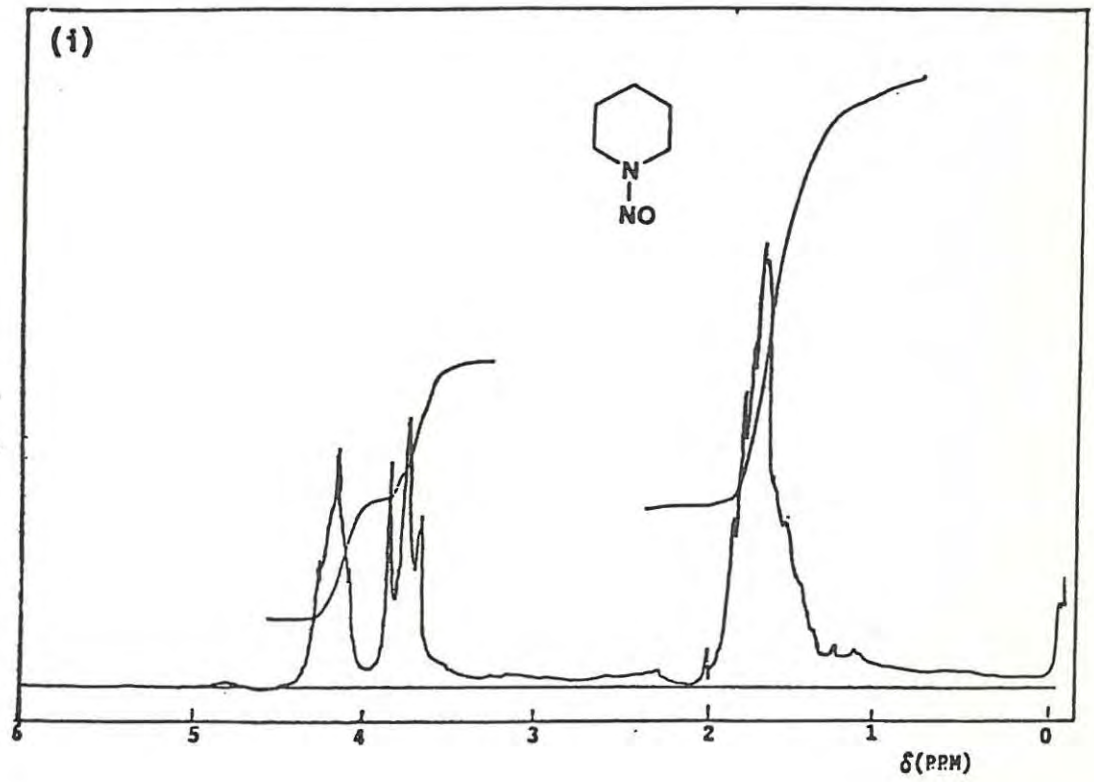
^{1}H-NMR(i) and IR(ii) SPECTRA OF N-NITROSOPYRROLIDINE

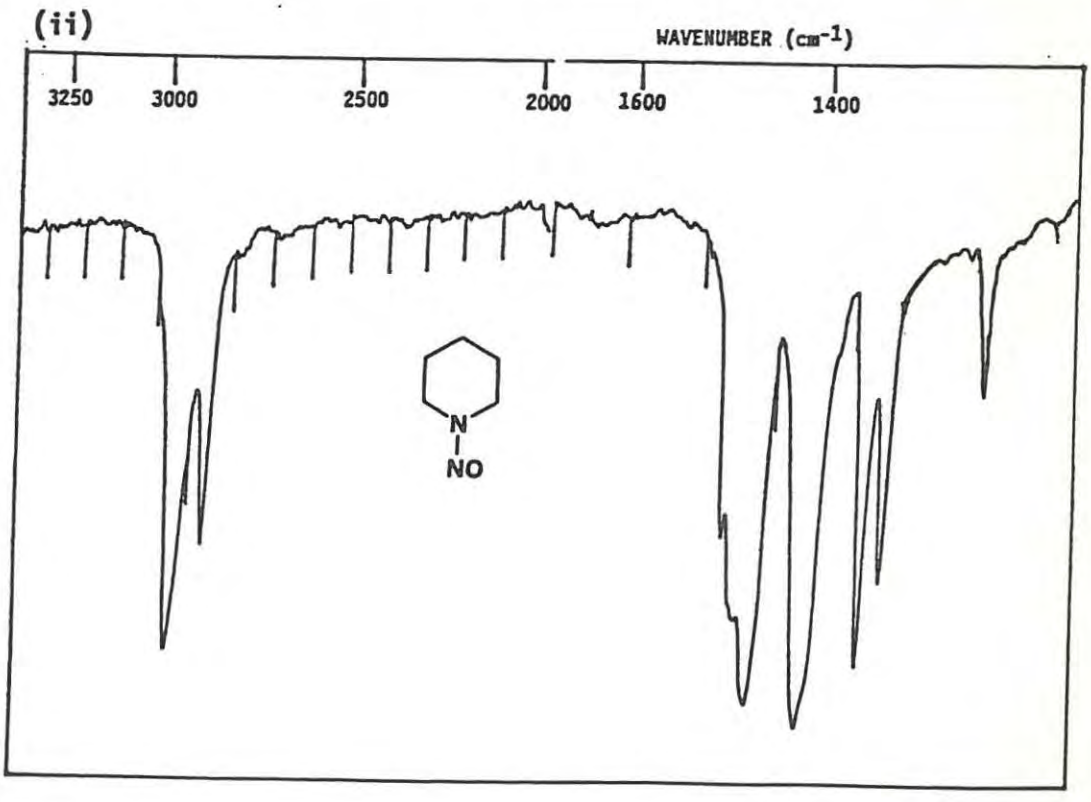




APPENDIX 5

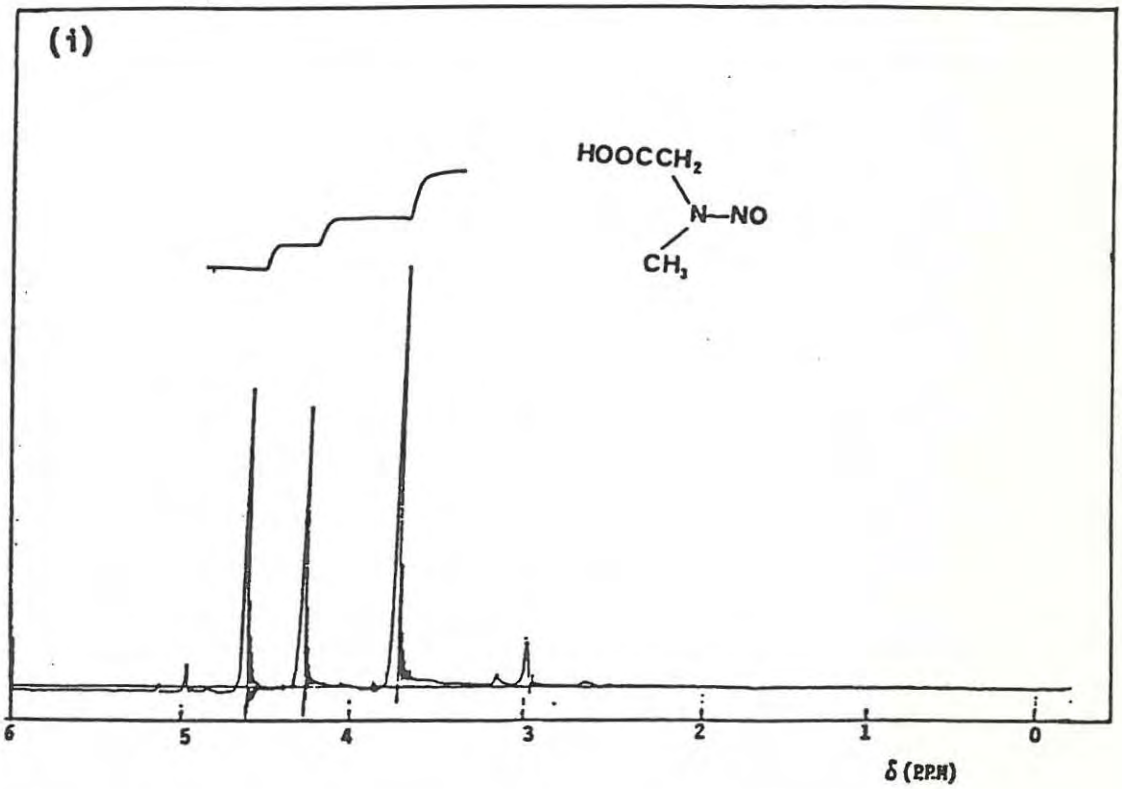
^{1}H-NMR(i) and IR(ii) SPECTRA OF N-NITROSOPIPERIDINE

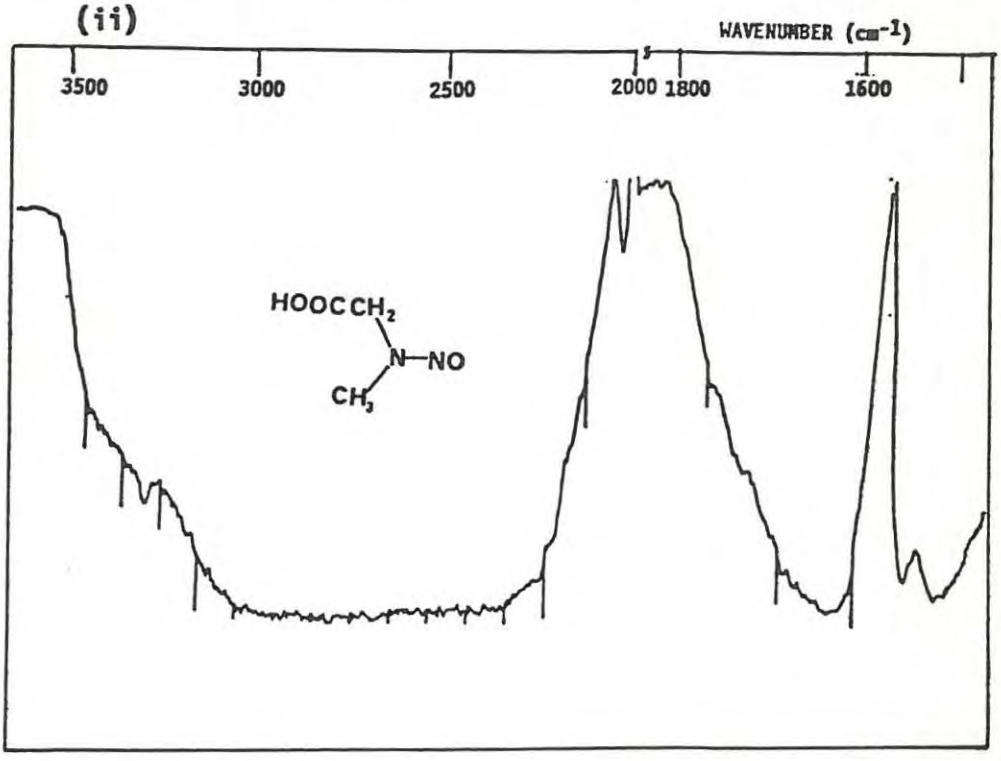




APPENDIX 6

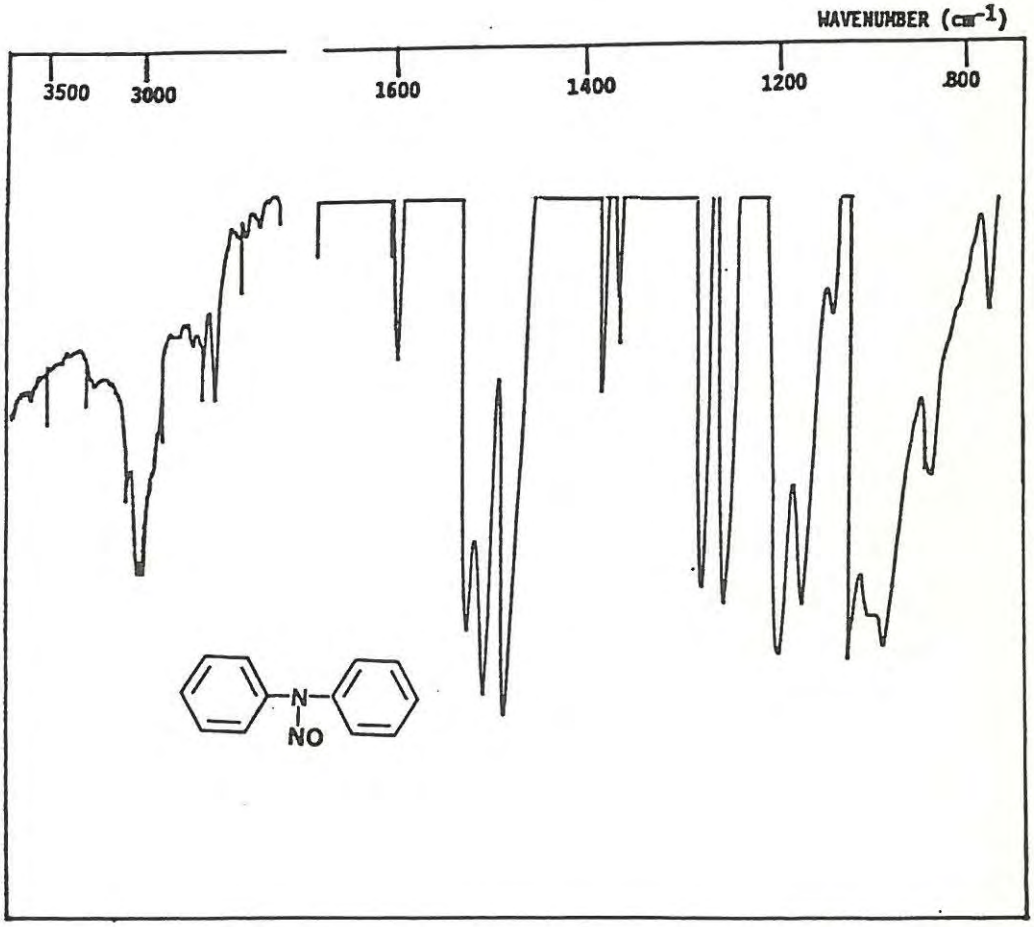
^{1}H-NMR(i) and IR(ii) SPECTRA OF N-METHYL-N-NITROSOGLYCINE





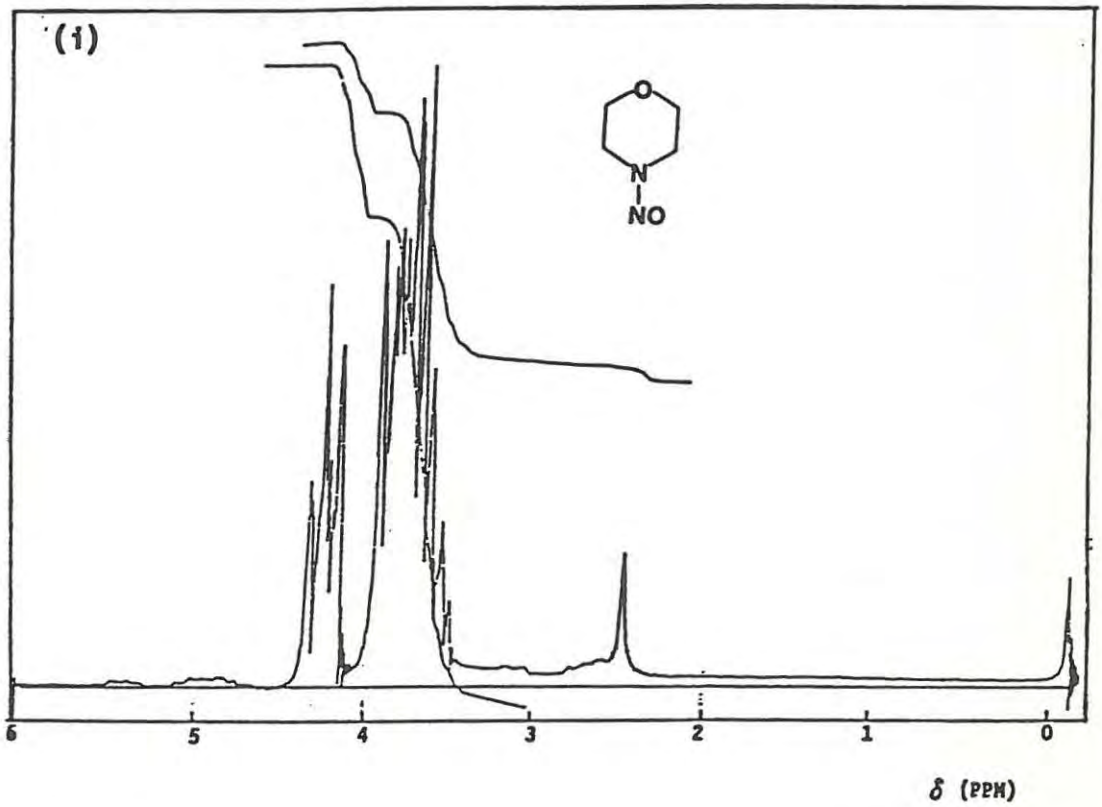
APPENDIX 7

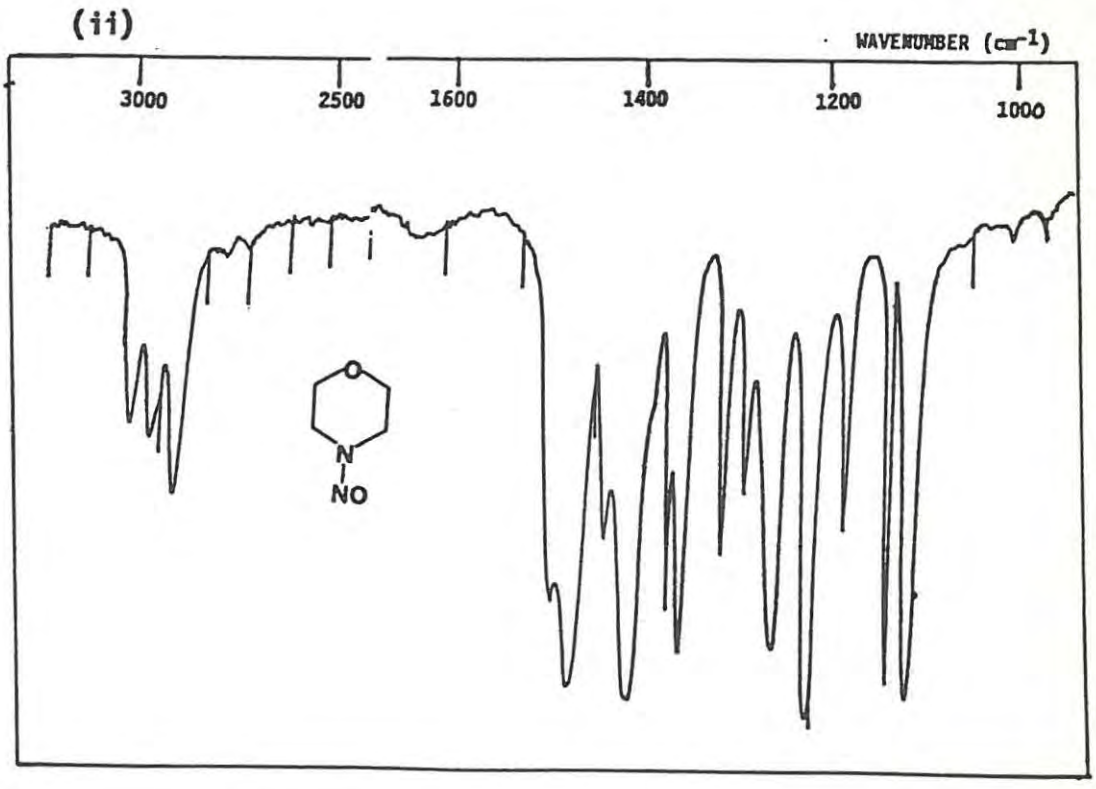
IR SPECTRUM OF DIPHENYLNITROSAMINE



APPENDIX 8

^{1}H-NMR(i) and IR(ii) SPECTRA OF N-NITROSOMORPHOLINE





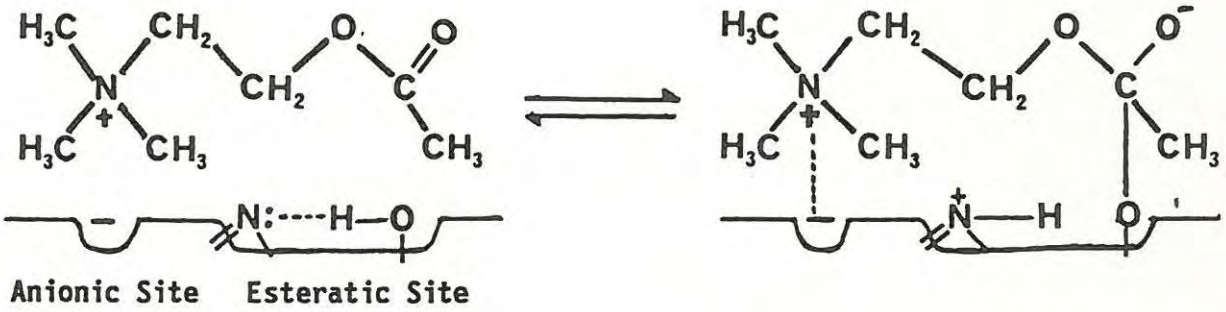
APPENDIX 9

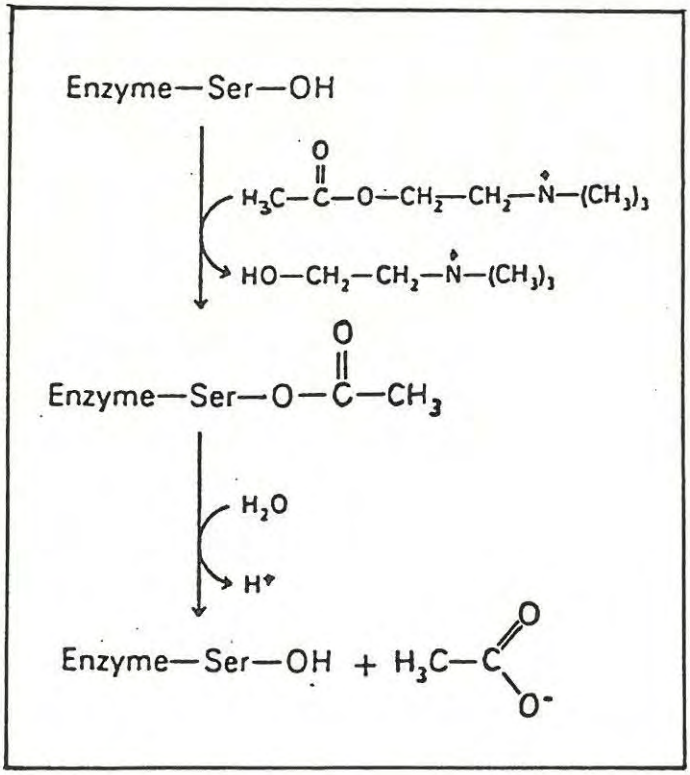
MEASUREMENTS FOLLOWED IN THE ASSAY AND KINETIC STUDIES OF ACETYLCHOLINESTERASE

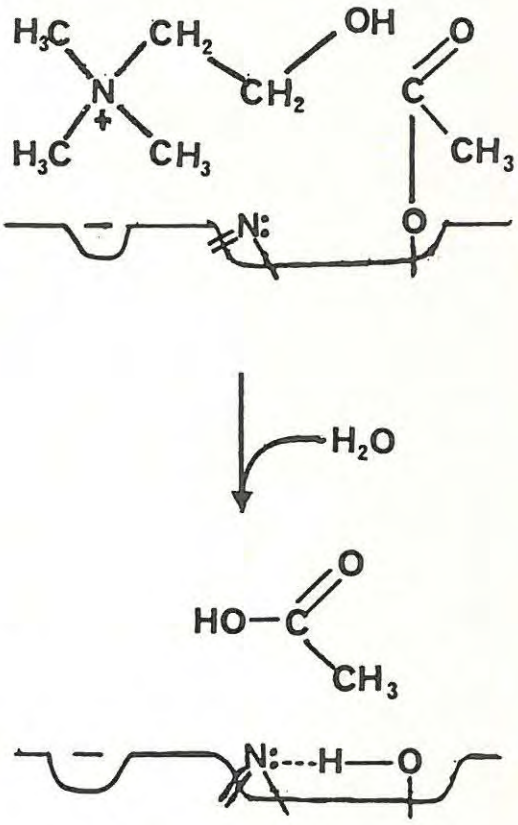
PIPETTE SUCCESSIVELY INTO CUVETTE:		CONC.IN ASSAY MIX.
Buffer (pH 8.0) 0.1M	3.0ml	Phosphate 100mM
ATCh Solution	0.02ml	Acetylthiocholine 0.0 μM,32 μM,39 μM, 48 μM,64 μM,92 μM, 159 μM,478 μM
DTNB Solution	0.10ml	DTNB 315 μM Buffer(pH7.2) 100mM $NaHCO_3$ 560 μM
Inhibitor	0.01ml	Nitroso compounds 0.0 μM,32 μM,64 μM, 130 μM,160 μM,320 μM
Incubate at 30°C for 10 minutes		
Enzyme	0.01ml	Vol. fraction 6.4 μL

APPENDIX 10

GENERAL AND SIMPLIFIED ILLUSTRATIONS OF CATALYTIC REACTION MECHANISM OF ACETYLCHOLINESTERASE







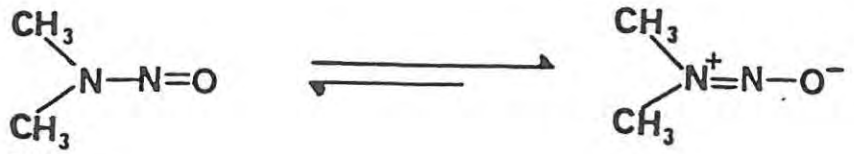
APPENDIX 11

BINDING CONSTANTS DETERMINED FROM STUDIES USING FLUOROMETRIC TITRATIONS, GEL CHROMATOGRAPHY AND EQUILIBRIUM DIALYSIS

LIGAND	EQUIL. DIALYS.		GEL CHROM.		FLUOR. TITR.	
	K_d(mM)	p	K_d(mM)	p	K_d(mM)	p
DPhNA	0.62	4.1	0.68	3.90	0.65	4.0
MNGly	0.71	4.1	0.80	3.93	0.75	3.9
DMNA	0.80	4.0	0.87	3.89	0.88	4.1
DEtNA	0.91	4.1	0.92	3.89	0.94	4.0
DENA	1.06	4.1	1.01	4.00	1.59	3.8
DPNA	1.22	4.1	1.25	3.82	2.00	4.0
NPyr	1.67	4.1	1.72	3.79	2.43	3.9
NMor	2.02	4.0	2.25	3.68	2.86	4.2
DBNA	2.83	4.0	2.92	3.76	3.44	4.0
NPip	4.21	4.0	4.60	3.83	4.09	4.1

APPENDIX 12

MOLECULAR AND RESONANCE STRUCTURES OF SECONDARY NITROSAMINES SYNTHESISED AND USED AS INHIBITORS/LIGANDS OF ACETYLCHOLINESTERASE

1. 

DMNA

2.

DENA

3.

DPNA

4.

DBNA

5.

DEtNA

APPENDIX 12 (Contd.)

6.

HNGly

7.

NPyr

8.

NPip

9.

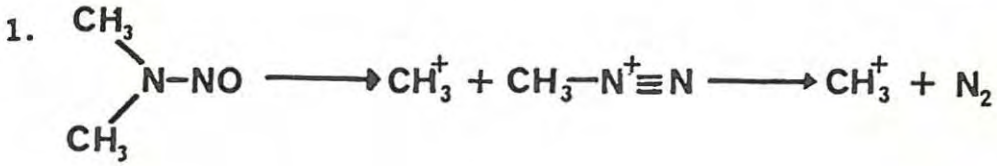
NMor

10.

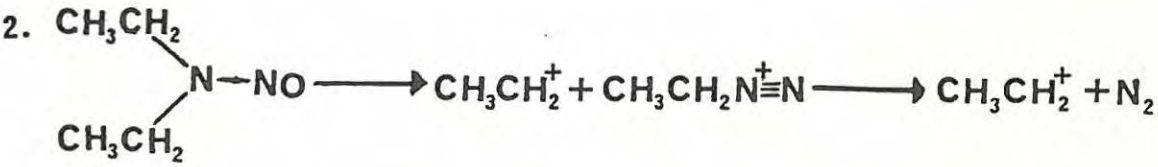
DPhNA

APPENDIX 13

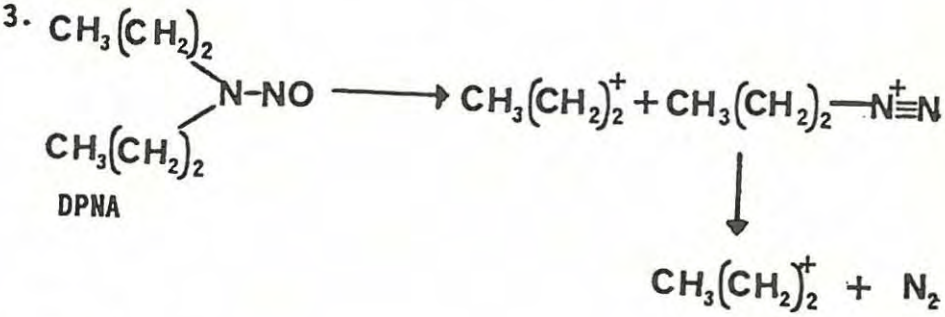
SIMPLIFIED REACTIONS SHOWING GENERATION OF CARBOCATION ALKYLATING SPECIES FROM DIFFERENT N-NITROSAMINES

1. 

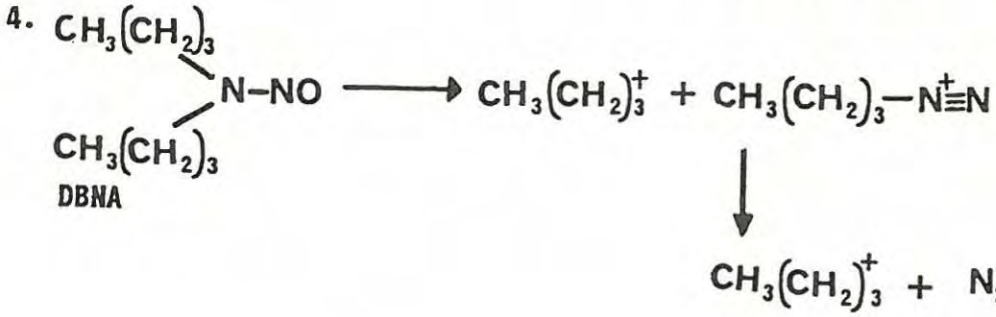
DMNA

2. 

DENA

3. 

DPNA

4. 

DBNA

APPENDIX 13 (Contd.)