PART - I

EFFECTS OF VARIOUS BENZODIAZEPINES AT THE LEVEL OF
MEMBRANE BOUND ENZYME ACTIVITIES IN DIFFERENT
SUBCELLULAR FRACTIONS OF RAT BRAIN
INTRODUCTION

A variety of drugs influence the central nervous system either as a major part of their therapeutic action or as an unwanted side effect. The study of the drug effects may be hampered by the complexity of the CNS. The adult mammalian CNS is extremely heterogeneous. This heterogeneity is essential for the understanding of ion distribution in the brain. Drugs acting upon the CNS may affect the ion distribution either by altering the downhill movements during excitation or by influencing the subsequent homeostasis (active or, passive) mechanism. Thus it has been suggested that diphenylhydantoin owes its antiepileptic effect to either a reduction of ion movements during excitation (1) or a stimulation of the subsequent active transport of $\mathrm{K}^+$ ion and $\mathrm{Na}^+$ ion (2-5) and a multitude of drugs counteract the $\mathrm{K}^-$-induced stimulation of energy metabolism. These phenomena seem to a large extent to occur in glial cells but ultimately released $\mathrm{K}^+$ ions must of necessity be reaccumulated into neurons and the accumulated $\mathrm{Na}^+$ ion must be extruded again (6). Thus both glial and neuronal cell membrane potentials are affected by drugs particularly by psychopharmacologic drugs.

An understanding of numerous aspects of nervous function including the effects of drugs and toxins requires a detailed knowledge of the molecular architecture of the nervous system. One of the most powerful current techniques for studying the organisation of cells at the molecular level is that of subcellular fractionation. With the introduction of subcellular fractionation
of brain tissue can thus yield preparations of many different kinds of various types of study e.g., (a) Synaptosomes for the study of the mechanisms of synthesis, storage and release of transmitters, the effect of drug thereon, and the identification of new transmitter if any, (b) isolated nuclei, mitochondria and ribosomes for the study of the biochemical reactions characteristic of these organelles, (c) membrane preparations of defined morphological origin for studies of composition, metabolic and permeability properties (7-10).

Synaptosomes are mostly sealed bags of cytoplasm having a continuous external plasma membrane and can be regarded as miniature non-nucleated cells which provide a model system for the study of the permeability of the non-myelinated neuronal membrane and the metabolic properties of the enzymes of neuronal cytoplasm. The synaptosome fraction as normally prepared is also heterogenous being derived from presynaptic nerve terminals of diverse chemical and morphological types—cholinergic, monoaminergic, axosomatic, axodendritic. Synaptosomes may be separated into a number of subpopulations, for example (a) synaptic vesicles occupying about 4% (b) synaptic mitochondria about 24% (c) external synaptic membrane, about 8% and (d) synaptic cytoplasm about 64% of the total synaptosomal volume, making use of discontinuous gradients (11-13) or continuous gradients (14,15).

A considerable number of literatures on synaptosome now exist which showed that intact synaptosomes from variety of vertebrate
species appears to be very similar but regional differences being greater. The synaptosome derived from any given regions of CNS are also heterogenous with respect to chemical composition, i.e., they contain various transmitter substances, enzymes, lipids and small molecules. According to the current concept each neuron possess only one transmitter among ACh, NA, DA, 5HT, GABA and glycine, together with the enzymes responsible for its synthesis, and inactivation sedimented with synaptosomal fraction. Thus the neurons may be classified according to the transmitter they elaborate. Synaptosome have a higher proportion of their total lipid in the form of phospholipid than any other subcellular organelle except mitochondria. All the usual phospholipids are phosphatidyl-choline, ethanolamine, -serine and -inositol, ethanolamine plasmalogens and sphingomyelin and very little cerebroside. Both synaptosome and microsome contain similar molar ratio of cholesterol : phospholipid and ganglioside content (16). The differences in enzyme, proteolipid in nature, composition between vesicle and ESM also suggest the possible difference in lipid composition between these two subsynaptic organelles in addition to the extensive analysis of lipid composition of vesicle and ESM. Synaptosome contain number of enzymes may be classified as follow: enzymes of intraterminal mitochondria; enzymes of soluble cytoplasm which include glycolytic enzymes, transmitter synthesising enzymes and protein synthesising enzymes; enzymes of external synaptic
membrane - these include Na\textsuperscript{+}-K\textsuperscript{+}-ATPase and choline acetyesterase and only one enzyme, Mg\textsuperscript{2+}-activated ATPase of synaptic vesicle (17-19).

Acetylcholine, one of the several naturally occurring esters of choline, is rapidly hydrolysed by two groups of cholinesterase, designated as pseudo and true cholinesterase. (\text{RX} + \text{H}_2\text{O} \xrightarrow{\text{AChe}} \text{ROH} + \text{HX}) where \text{ROA} = \text{carboxylic acid; XH} = \text{choline} and by some aliesterases. The aliesterases are eserine resistant and are not generally concerned in general metabolism. Extensive studies of the differential specificities of cholinester hydrolase in the erythrocyte membrane and serum led to the distinction that AChE (true cholinesterase, E.C. -3.1.1.7) specificity resides primarily on the acyl group 'R' (20) while ChE (pseudocholinesterase E.C. -2.1.1.8) activities are more sensitive to variations in the leaving group 'X' (21). The distribution of AChE, being enriched in grey matter, in subcellular fraction reported that AChE is present in both microsome (lumen of the endoplasmic reticulum) and synaptosome (external synaptic membrane) - such distribution also shown from electron microscopic study (22,23).

The physiological role of AChE in synaptic transmission developed in parallel with the concept of the importance of Ach at the neuromuscular junction. Ionic currents in postsynaptic membrane were shown to be highly sensitive to inhibitors which blocked the access of ACh to the active sites either of AChE or of ACh-receptor.
Such information confirmed the idea that AChE can control the ionic currents produced in post-synaptic membrane at cholinergic synapses by hydrolysing ACh thus destroying its potent against action on the receptor (24). More recent work suggests that the enzymes at the synapse may also help to regulate the level of stored synaptic ACh, perhaps by providing part of the choline used for the resynthesis of ACh (25). In addition to this generally accepted role of AChE at the synapses a direct involvement of the enzyme in the self-propagated excitability of conduction membrane has been suggested by Nachmansohn, 1971 (26). AChE and ACh-receptor are postulated to be involved in a protein assembly which has a general role in controlling ionic currents in both classes of excitable membranes. However, the presence of AChE in tissues other than nervous tissue, e.g., to red blood cells, in which it could not be concerned with ACh metabolism has suggested that even in the nervous system it may have some function additional to its function of destroying ACh.

The effect of various psychoactive drugs and chemical agents on AChE had been studied by various investigators. Imipramine and SKF-525 completely inhibits AChE (27), various organo-phosphates (soman, isopropyl methyl phosphonofluoride) and potent inhibitors of this enzyme. Barbiturate elevate brain ACh levels without inhibiting AChE, giving rise to the possibility that brain ACh levels result from mechanism other than enzyme inhibition (28).
BZP, particularly DZP increases ACh level by decreasing ACh turnover in whole brain and in synaptosomal fraction of brain and BZP had no effect on stria V AChE and cholineacetyl transferase (29,30).

Another membrane bound enzyme that has important role in brain is glutamine synthetase (GS, E.C.6.3.1.2) which causes ATP dependent fixation of ammonia to glutamate. This enzyme is mainly concentrated in microsomal fraction and a definite portion bound to synaptic membrane (31,32). GS has a localization equal to AChE and Na\(^{+}\)-K\(^{+}\)-ATPase, but with higher concentration in the AChE-poor nerve ending membrane. GS may regulate the concentration of this amino acid with neurons. Controlling its transformation into glutamate and GABA. Since GS utilises ATP, the amino acid, glutamine can easily cross the nerve ending membrane. Having such a strategic position in the nerve ending membranes GS could play a synaptic role in those terminals utilizing glutamate and GABA-mechanisms.

Methionine sulfoximine, a drug that produces seizures and inhibits GS activity (33) whereas \(\Delta^9\)-THC stimulates GS activity (34). Thus conditions affecting the energy metabolism of the cell are known to impair GS and to lead to its depletion.

Another important enzyme of neuronal membrane is ATPase, (E.C. 3.6.1.3) adenosine triphosphatase act as energy transducer, liberating energy from high phosphate bonds that is then used in
active transport of ions and other energy-consuming transformations.
The active transport of Na and K is a fundamental and major energy
requiring cellular process, where ATP is the energy source for this transport. In general theree Na\(^+\) are pumped outward and two K\(^+\) inward for every ATP hydrolysed (35) this enzyme system was Na\(^+\)-K\(^+\)-ATPase. The Na\(^+\)-K\(^+\)-ATPase catalyses the hydrolysis of the \(\gamma\)-phosphate of ATP if Mg\(^{2+}\), Na\(^+\) and K\(^+\) are all present and inhibited by ouabain. The presence of Mg\(^{2+}\) ion is essential and for optimal activity of the enzyme the ratio of Na\(^+\)/K\(^+\) lies between 10:1 and 5:1 (36).

It has been postulated that Na\(^+\)-K\(^+\)-ATPase hydrolyzes ATP in a stepwise fashion involving Na\(^+\)-dependent phosphorylation of the enzyme and K\(^+\)-dependent-hydrolysis of the phosphoenzyme (37).

\[
\begin{align*}
E_1 + ADP & \rightarrow E_1^*P + Mg^{2+} + Na^+ & \quad (i) \\
E_1^*P & \rightarrow E_1^*P + Mg^{2+} & \quad (ii) \\
E_2 & \rightarrow E_2^*P + H_2O & \quad (iii) \\
E_2^*P & \rightarrow E_2^*P + K^+ & \quad (iv)
\end{align*}
\]

Skou (38) postulated that phosphorylation of the enzyme and dephosphorylation of the phosphoenzyme might be involved in an important intermediate steps in the overall reaction. Most model for Na and K transport invoke some sequential changes in binding affinities of these ions. Binding of K\(^+\) ion is permitted after the formation of the phosphorylated intermediate in a high Na\(^+\)
environment, but Na binding was absolutely dependent on ATP (39-40). Numerous studies pointed out that lipid is required for Na⁺-K⁺-ATPase reaction as treatment of enzyme with detergents, phospholipases and solvents leads to inactivation. Again addition of lipid, particularly the phospholipids restore the enzyme activity (41-44). Among the various suggestion. The role of phospholipid is to create a negative charge and hydrophobicity near but not at the active site which help cation translocation. An interesting finding is that detergent at low concentration stimulated Na⁺-K⁺-ATPase activities. Detergent not only remove the lipid portion of the membrane fragment but also remove inert protein, reduces the level of Mg⁺²-ATPase and expose the latent enzyme site (45).

There are many reports concerning the activation or inhibition of Na⁺-K⁺-ATPase by various chemicals, drugs and biologically active agents which by any way change the ion-distribution or react with lipids necessary for Na⁺-K⁺-ATPase action. Phenothiazine tranquillisers inhibit Na⁺-K⁺-ATPase also inhibit active transport of K⁺ ion (46). Local anesthetics (procain, atropine) block the influx of Na⁺ that acelerate the activity of membrane ATPase (mainly Na⁺-K⁺-ATPase) (47). It has been known that during DDT poisoning Na⁺ and K⁺ transport in the CNS is disrupted and ultimately inhibit Na⁺-K⁺-ATPase (48). Cardiac glycoside, ouabain inhibit Na⁺-K⁺-ATPase and can be antagonised by increasing K⁺ ion - thus K-dependent dephophorylation may be inhibited by ouabain (49). In general the
antidepressant (ethosuximide and phenytoin) inhibit $\text{Na}^+\text{-K}^+$-ATPase thus the effect of BZP, another antidepressant group of drugs give some insight about their mode of interaction with the neuronal membrane.

Mitochondria are the cytoplasmic granules containing the enzymes involved in oxidative metabolism coupled to the production of ATP. Although mitochondria from different tissues are basically similar in their ultrastructure, mitochondria of neural tissue have certain peculiarities the significance of which is unknown. Brain as well as most other mitochondria are frequently located adjacent to intracellular lipid droplets and are the seat of cellular oxidation. The mitochondria are composed of two osmophilic membrane, an inner and an outer membrane and cristae. The cristae is really invaginations of the inner membrane and may have variety of shapes. The membranes strand, which is comprised of a bimolecular array of lipids not only may provide the structural frame work for the mitochondria but also essential to enzymatic organization and function. The mitochondrial membrane comprised of a globular substructure of lipoprotein complexes with no distinct separation of phospholipids and proteins. Rat brain mitochondria contain about 37% lipid, 57% protein and other substances. Of the lipids about 75% phospholipid and some neutral lipids, mainly cholesterol. The major phospholipids are lecithin and serine phosphatides. Cardiolipin is also found in mitochondria of different tissues. Among the other
substances in mitochondria both saturated and unsaturated fatty acid together with nucleic acids both in the form of DNA and RNA are also present in brain tissue. Of the total mitochondrial protein there are some structural protein and some mitochondrial enzyme system consist of the dehydrogenases of TCA cycle, the electron transport scheme, energy coupling enzymes involved in the synthesis of ATP, a protein synthesising system, a fatty acid oxidase and biosynthetic enzymes, several transaminases, a few glycolytic enzymes, such as thiokinase and a variety of miscellaneous enzymes such as MAO (51,52).

Monoamine oxidase (MAO : Oxidoreductase (deaminating) : E.C. 1.4.3.4) is a mitochondrial outer membrane bound oxidoreductase enzyme catalysing the oxidation of a number of variety of alkyl or, arylamines of which the latter group includes the physiological substrate.

\[
R.CH_2.NH_2 + O_2 + H_2O \xrightarrow{MAO} RCHO + NH_3 + H_2O_2
\]

It is necessary to trap the aldehyde with semicarbazide to further oxidation to the corresponding acid. The radical R can be a substituted aryl or alkyl group or even amino alkyl chain upto a certain length which shows maximum velocity. Among all the three classes of amines, tertiary amines are poor substrate, a primary amines with aromatic ring and a hydroxyl substitution at para position (as in tyramine) rather than at ortho and meta position are best substrate for the
enzyme with high rate of degradation (53).

MAO is present more or less in all the organs studied, though highest activity is found in brain and liver (54). There is now a considerable weight of experimental evidences in favour of MAO activity being located in the outer membrane fraction of the mitochondria (55-57) and microsomal MAO activity has been suggested as an artifact of homogenisation and fractionation (57). It was also established that MAO has been exist in multiple forms in all the tissues depending on the type of amine generally present in that tissue. Thus, from the various experimentation it can be concluded that two forms of MAO (A form and B form) has been identified in most of the tissue including brain with varying proportion in different regions (58). These two forms differ in their substrate and inhibitor sensitivity. Preferred substrate for type A are serotonin, norepinephrine and normetaepinephrine, whereas type B preferentially oxidises benzylamine and p-phenylethyl amine. Dopamine, tyramine and tryptamine are acted upon by both the types. It has been suggested that the phospholipids may be responsible for the multiple forms of MAO (59). Recently Manette et al. (60) have proposed that rat liver MAO is a single enzyme, not a group of interrelated enzymes, with two independent catalytic sites. It may be assumed that the reaction at one site alters the conformation, substrate specificity and catalytic behaviour of the second site.
It is well recognised that MAO plays an important role, in the degradation of biologically active monoamines (adrenaline, noradrenaline, dopamine and 5 hydroxy tryptamine) in the both central and peripheral tissue. These amines are stored in the amine storage granules of nervous and endocrine tissue and can be released as quanta either by nerve stimulation or by pharmacological agent and could cause drastic pharmacological effects if the excess is not metabolised. Thus, it is for this reason the enzyme, MAO is provided (61). Perhaps the most important set of observation in this area concerns the property which MAO inhibiting drugs possess of bringing about an improvement in depressed patients (62,63).

MAO inhibiting drugs also prevent amine reuptake (result high intracellular monoamine concentration is expected which is also antidepressive (64). MAO inhibitors appear to have a considerably greater effect on 5HT containing neurons rather than those containing catecholamines since catecholamines and dopamine is mainly metabolised by catecholamine-O methyltransferase, COMT, and that this reaction could proceed deamination (65,66). It has been observed that the level of functional pool of serotonine within the neuron is regulated by the availability of serotonine to MAO and to granular reuptake processes. Deficiency of neuronal serotonine either due to reduced reuptake or due to increased MAO activity or some other factors has been implicated in mental depression and suicide (62,67,68). The function of neuronal MAO may therefore be to metabolise released
neurotransmitter monoamines before and after reuptake into the nerve endings. The reuptake process appears to be governed by the relative concentration of the free intra- and extra-neuronal amines.

MAO activity had already been tested in presence of various drugs and chemical agents. Pargyline inhibits irreversibly MAO activity of rat liver mitochondria (69). It has been observed that iproniazid, amphetamine, pheniprazine, nialamide, and doxapine inhibits MAO activity (70–74). Chiatropic agents depress brain mitochondrial MAO activity under all condition of treatment due to disaggregation of the lipid protein component of the membrane, but Δ⁹-THC, like many other psychoactive agent stimulate brain MAO (75). However, CNS stimulants or, antidepressant has a tendency to show inhibitory effect on MAO activity whereas CNS depressant has the tendency to activate MAO activity. Acute BZPS administration elevate the level of catecholamines and serotonine by reducing the turnover of respective amines in brain (76).

It is somewhat puzzling that mitochondria which generate ATP contain an active ATPase molecule in the innermembrane reponsible for cation transport accross the membrane. In mitochondria the flow of energy between electron transport complex and ATPase is reversible. In these reactions the energy of broken down high energy phosphate bond is utilised in transport phenomenas accross the mitochondrial membrane. Due to the presence a specific protein inhibitor (77,78)
which interacts with the ATPase in such a manner that ATP hydrolysis is blocked but ATP generation takes place the effect of drugs on mitochondrial ATPase is poorly progressed. Among the psychotherapeutic agents phenothiazine derivatives at low concentration inhibit mitochondrial ATPase activity by reacting with the mitochondrial phospholipids responsible for ATPase activity (79). The effect of BZP, another psychoactive substance, on mitochondrial ATPase is of great interest.

Although BZPS are potent anticonvulsant group of drugs and resembles other anticonvulsant dipenylhydantoin and barbiturates in many ways in their action, the mechanism of action of BZPS are still speculative. Here, in the first part of the thesis the action of different BZPS under both in vivo (acute and chronic administration) and in vitro condition of treatment mainly from the standpoint of changes brought about at the level of ion-distribution and microenvironment of some prominent membrane-bound enzymes of nerve cell i.e., Mg$^{2+}$-ATPase, Na$^+$-K$^+$-ATPase, AChE and GS of synaptosomal and microsomal fraction and Mg$^{2+}$-ATPase, Na$^+$$-K^+$-ATPase and MAO of mitochondrial fraction was of particular interest.
**EXPERIMENTAL RESULTS**

The following tables record the effect of three BZPS (viz. DZP, OZP and FZP) on $\text{Mg}^{+2}$-ATPase, $\text{Na}^{+}$-$\text{K}^{+}$-ATPase, AChE and GS activity of microsomal and synaptosomal fractions and $\text{Mg}^{+2}$-ATPase, $\text{Na}^{+}$-$\text{K}^{+}$-ATPase and MAO activity of mitochondrial fraction from rat brain tissue under both *in vivo* (acute with varying times and chronic condition) and *in vitro* condition of treatment.

The effect of acute administration of flurazepam, diazepam and oxazepam on rat brain microsomal $\text{Mg}^{+2}$-ATPase activity is shown in Table 1. It is evident from the result of Table 1 that single administration (i.p.) of all these three benzodiazepines at low dose (10 mg/kg) level do not produce any significant ($P>0.05$) changes of microsomal $\text{Mg}^{+2}$-ATPase activity whereas at higher dose (25 mg/kg) these drugs produce significant ($P<0.05$) stimulation of $\text{Mg}^{+2}$-ATPase activity at different hours of drug treatment e.g., FZP, DZP and OZP causes such change over control 180 minutes, 120 minutes and only 360 minutes respectively after drug administration.
Table 1

Effect of in vivo administration of benzodiazepines on rat brain microsomal Mg$^{2+}$-ATPase* activity

<table>
<thead>
<tr>
<th>Times of treatment (mins)</th>
<th>Low dose (10 mg/kg)</th>
<th>High dose (25 mg/kg)</th>
<th>Low dose (10mg/kg)</th>
<th>High dose (25 mg/kg)</th>
<th>Low dose (10 mg/kg)</th>
<th>High dose (25 mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flurazepam</td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low dose</td>
<td>16.48±1.62</td>
<td>16.68±1.13</td>
<td>16.28±1.01</td>
<td>16.15±1.5</td>
<td>16.33±0.76</td>
<td>16.52±1.18</td>
</tr>
<tr>
<td>High dose</td>
<td>17.92±1.42</td>
<td>16.02±1.26</td>
<td>16.36±1.14</td>
<td>16.71±1.41</td>
<td>16.39±0.93</td>
<td>16.42±2.42</td>
</tr>
<tr>
<td></td>
<td>18.30±1.16</td>
<td>15.36±0.83</td>
<td>17.38±1.43</td>
<td>18.02±0.94</td>
<td>16.54±1.38</td>
<td>17.07±1.85</td>
</tr>
<tr>
<td></td>
<td>18.51±0.93</td>
<td>15.62±1.13</td>
<td>16.87±0.78</td>
<td>20.89±1.38</td>
<td>17.21±0.84</td>
<td>18.24±1.96</td>
</tr>
<tr>
<td></td>
<td>18.77±1.71</td>
<td>22.60±1.42</td>
<td>16.24±2.01</td>
<td>25.86±2.02</td>
<td>15.89±1.64</td>
<td>19.83±1.63</td>
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<tr>
<td></td>
<td>16.82±2.21</td>
<td>25.32±1.83</td>
<td>15.45±1.84</td>
<td>25.52±1.27</td>
<td>16.71±0.77</td>
<td>22.47±0.34</td>
</tr>
</tbody>
</table>

**ATPase activity is expressed as μmoles of Pi liberated/mg protein/hour.

Results are expressed as mean ± SEM of six experiments.

Significantly differ from control : a=P<0.05, b=P<0.01, C=P<0.001

For details/experimental see materials and methods.

Table 2 shows the effect of acute intraperitonial administration of benzodiazepines (viz.FZP,DZP and OZP) on rat brain microsomal Na$^{+}$-K$^{+}$-ATPase activity in varying hours of treatment. It appears from the results of Table 2 that single individual administration of FZP is ineffective to produce any change of Na$^{+}$-K$^{+}$-ATPase activity at any time (within 360 mins) after drug administration. Diazepam at both doses (10 mg/kg and 25 mg/kg) causes a time dependent increase of Na$^{+}$-K$^{+}$-ATPase activity up to 60 mins of drug treatment and then the activity gradually declines to the normal level. Here the low dose produces greater activation of the enzyme, Na$^{+}$-K$^{+}$-ATPase, activity than that observed at higher one.
Oxazepam only at higher dose and at late hours (after 240 mins) produces significant increase of enzyme activity.

Table 2

<table>
<thead>
<tr>
<th>Times of treatment (mins)</th>
<th>Flurazepam Low dose (10mg/kg)</th>
<th>Flurazepam High dose (25 mg/kg)</th>
<th>Diazepam Low dose (10mg/kg)</th>
<th>Diazepam High dose (25 mg/kg)</th>
<th>Oxazepam Low dose (10mg/kg)</th>
<th>Oxazepam High dose (25 mg/kg)</th>
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</thead>
<tbody>
<tr>
<td>30</td>
<td>6.07±0.38</td>
<td>6.61±0.26</td>
<td>7.43±0.44</td>
<td>6.71±0.82</td>
<td>6.42±0.32</td>
<td>6.48±0.21</td>
</tr>
<tr>
<td>60</td>
<td>5.90±0.47</td>
<td>6.59±0.43</td>
<td>9.46±1.02</td>
<td>7.68±0.43</td>
<td>6.47±0.37</td>
<td>6.56±0.37</td>
</tr>
<tr>
<td>120</td>
<td>6.24±0.43</td>
<td>6.94±0.18</td>
<td>7.66±0.38</td>
<td>5.30±0.63</td>
<td>6.16±0.16</td>
<td>7.03±0.83</td>
</tr>
<tr>
<td>180</td>
<td>5.93±0.18</td>
<td>6.74±0.22</td>
<td>6.15±0.24</td>
<td>5.32±0.46</td>
<td>6.22±0.31</td>
<td>8.42±0.92</td>
</tr>
<tr>
<td>360</td>
<td>6.36±0.09</td>
<td>6.93±0.14</td>
<td>6.26±0.35</td>
<td>6.33±0.49</td>
<td>6.25±0.30</td>
<td>8.64±0.63</td>
</tr>
</tbody>
</table>

*ATPase activity is expressed as &mu;moles of Pi liberated/mg protein/hour.
Results are expressed as mean ± SEM of six experiments.
Significantly differ from control a = P<0.05, b = P<0.01.

It is evident from the result of Table 3, in vitro of FZP, DZP and OZP on rat brain microsomal Mg²⁺-ATPase and Na⁺-K⁺-stimulated ATPase activity, that in vitro treatment of FZP and OZP produces a dose dependent inhibition of both the Mg²⁺-ATPase and Na⁺-K⁺-ATPase activity of rat brain microsomal fraction. Among these changes statistical significance is observed at higher doses but not at lower doses (10⁻⁷/mg protein). A biphasic type of action, i.e., significant stimulation (P<0.01) at low dose (10⁻⁷/mg protein) but significant inhibition at higher doses (20⁻⁷/mg protein, P<0.05; 50⁻⁷/mg protein, P<0.001), of microsomal Mg⁺²-ATPase activity is observed due to in vitro treatment of DZP. The microsomal Na⁺-K⁺-ATPase activity shows dose dependent inhibition when incubated with diazepam.
**Table 3**

Effect of *in vitro* treatment of benzodiazepines on rat brain microsomal Mg$^{2+}$-ATPase* and Na$^{+}$-K$^{+}$ATPase* activity

<table>
<thead>
<tr>
<th>Doses of the drug</th>
<th>Flurazepam</th>
<th>Diazepam</th>
<th>Oxazepam</th>
</tr>
</thead>
<tbody>
<tr>
<td>V/mg protein</td>
<td>Mg$^{2+}$-ATPase</td>
<td>Na$^{+}$-K$^{+}$-ATPase</td>
<td>Mg$^{2+}$-ATPase</td>
</tr>
<tr>
<td>—</td>
<td>15.58±1.31</td>
<td>5.62±0.61</td>
<td>14.81±1.02</td>
</tr>
<tr>
<td>10</td>
<td>13.96±1.11</td>
<td>5.58±0.43</td>
<td>20.87±2.43</td>
</tr>
<tr>
<td>20</td>
<td>12.39±1.43</td>
<td>2.98±0.43</td>
<td>11.54±1.04</td>
</tr>
<tr>
<td>50</td>
<td>11.38±0.78</td>
<td>0.62±0.08</td>
<td>2.07±0.32</td>
</tr>
</tbody>
</table>

* ATPase activity is expressed as µmoles of Pi liberated/mg of protein/hr.

Results are expressed as mean ± SEM of six experiments.

For experimental detail see materials and methods.

Significantly differ from control

a = P<0.05,  b = P<0.01,  C = P<0.001

Results presented in Table 4 indicates that upon single individual administration (i.p.) of either FZP or DZP or OZP produces significant dose dependent and time dependent increase of rat brain microsomal glutamine synthetase activity, with the exception of OZP which at low dose not produce any change in microsomal GS activity. This significancy does not observe within 120 mins of treatment with either of these three drugs. Both DZP and FZP show similar percentage of stimulation of microsomal GS activity and OZP shows less stimulation than FZP and DZP under similar condition of drug treatment.
Table 4
Effect of \textit{in vivo} administration of various Benzodiazepines on rat brain microsomal glutamine synthetase (GS) activity

<table>
<thead>
<tr>
<th>Time of treatment (mins)</th>
<th>Flurazepam</th>
<th>Diazepam</th>
<th>Oxazepam</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Low dose</td>
<td>High dose</td>
<td>Low dose</td>
</tr>
<tr>
<td></td>
<td>(10 mg/kg)</td>
<td>(25 mg/kg)</td>
<td>(10mg/kg)</td>
</tr>
<tr>
<td>6.78±0.5</td>
<td>6.74±0.72</td>
<td>6.25±0.57</td>
<td>6.48±0.62</td>
</tr>
<tr>
<td>30 6.95±0.76</td>
<td>6.78±0.66</td>
<td>5.83±0.34</td>
<td>6.04±0.48</td>
</tr>
<tr>
<td>60 7.05±0.48</td>
<td>7.51±0.43</td>
<td>5.65±0.45</td>
<td>5.74±0.75</td>
</tr>
<tr>
<td>120 8.50±0.92</td>
<td>8.85±1.02</td>
<td>5.24±0.73</td>
<td>4.95±0.31</td>
</tr>
<tr>
<td>180 8.83±1.23</td>
<td>11.4±0.84</td>
<td>10.18±1.21</td>
<td>18.98±1.73</td>
</tr>
<tr>
<td>360 11.63±1.07</td>
<td>25.12±1.25</td>
<td>11.36±1.62</td>
<td>24.29±1.11</td>
</tr>
</tbody>
</table>

*GS activity is expressed as AOD at 540 nm/mg of protein/hour.
Results are expressed as mean ± SEM of six experiments.
For details of the experiments see materials and methods.
Significantly differ from control
a = P<0.05, b = P<0.02, C = P<0.01, d = P<0.001

Table 5 shows the effect of \textit{in vitro} treatment of various benzodiazepines on rat brain microsomal GS activity. Both DZP and OZP causes a dose dependent inhibition of microsomal GS activity being significant from 20 \(\mu\)g protein. The percentage of inhibition is higher in DZP and in OZP, FZP at 10 \(\mu\)g and 20 \(\mu\)g protein produces no change in microsomal GS activity but at 50 \(\mu\)g protein there is significant stimulation of the same enzyme.
Table 5

Effect of *in vitro* treatment of benzodiazepines on rat brain microsomal glutamine synthetase* activity

<table>
<thead>
<tr>
<th>Dose of the drugs (mg/mg protein)</th>
<th>Benzodiazepines</th>
<th>Flurazepam</th>
<th>Diazepam</th>
<th>Oxazepam</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>6.60±0.43</td>
<td>4.48±0.34</td>
<td>5.82±0.33</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>6.53±0.72</td>
<td>2.36±0.07</td>
<td>4.36±0.48a</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>7.82±0.48a</td>
<td>0.92±0.03d</td>
<td>3.81±0.12d</td>
<td></td>
</tr>
</tbody>
</table>

*Glutamine synthetase activity is expressed as ΔO.D.540 nm/mg protein/hour.

Results are expressed as mean ± SEM of six experiments.

For experimental details see materials and methods.

Significantly diff from control: a = P<0.05, d = P<0.001

Results shown in Table 6 indicate that intraperitoneal administration of benzodiazepines e.g. FZP, DZP and OZP (only one at a time) produces a similar type of action on microsomal AChE activity as on microsomal GS activity i.e., all these drugs produce significant dose dependent and time dependent stimulation of microsomal AChE activity between 120 mins to 360 mins after drug treatment but not with OZP at low dose (10 mg/kg) which cannot produce any significant change in microsomal AChE activity.
### Table 6
Effect of in vivo administration of benzodiazepines on rat brain microsomal AChE* activity

<table>
<thead>
<tr>
<th>Time of treatment (mins)</th>
<th>Benzodiazepines</th>
<th>Flurazepam</th>
<th>Diazepam</th>
<th>Oxazepam</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Low dose (10mg/kg)</td>
<td>High dose (25 mg/kg)</td>
<td>Low dose (10 mg/kg)</td>
<td>High dose (25 mg/kg)</td>
</tr>
<tr>
<td>-</td>
<td>8.00±0.56</td>
<td>8.52±0.72</td>
<td>8.15±0.81</td>
<td>8.25±0.54</td>
</tr>
<tr>
<td>30</td>
<td>7.14±0.87</td>
<td>10.07±0.68</td>
<td>8.21±0.56</td>
<td>9.72±1.04</td>
</tr>
<tr>
<td>60</td>
<td>7.23±1.02</td>
<td>10.84±1.43</td>
<td>8.32±0.43</td>
<td>10.11±0.76</td>
</tr>
<tr>
<td>120</td>
<td>9.12±0.83</td>
<td>12.80±1.33</td>
<td>6.88±0.72</td>
<td>14.42±1.43</td>
</tr>
<tr>
<td>180</td>
<td>12.25±1.72</td>
<td>15.81±2.01</td>
<td>9.78±1.07</td>
<td>15.60±1.12</td>
</tr>
<tr>
<td>360</td>
<td>14.00±1.53</td>
<td>28.06±1.82</td>
<td>14.00±1.35</td>
<td>24.06±1.23</td>
</tr>
</tbody>
</table>

* AChE activity is expressed as nmoles of ACh hydrolysed/mg protein/hour.

Results are expressed as mean ± SEM of six experiments

For experimental details see materials and methods

Significantly differ from control

a = P<0.05, b = P<0.02, c = P<0.01, d = P<0.001

It is evident from Table 7 that all these three benzodiazepines cause a dose dependent stimulation of rat brain microsomal AChE activity under in vitro condition of treatment. FZP at 10 and 20 mg protein, DZP and OZP at 10 mg protein increases the enzyme activity but not significantly (P>0.05). The potency is of the order DZP> FZP > OZP.
Table 7
Effect of in vitro treatment of benzodiazepines on rat brain microsomal Acetylcholine esterase* activity

<table>
<thead>
<tr>
<th>Doses of the drugs (mg/mg protein)</th>
<th>Benzodiazepines</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Flurazepam</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>—</td>
<td>8.60±0.74</td>
</tr>
<tr>
<td>10</td>
<td>9.93±1.03</td>
</tr>
<tr>
<td>20</td>
<td>10.59±0.94</td>
</tr>
<tr>
<td>50</td>
<td>15.88±1.31ᵈ</td>
</tr>
</tbody>
</table>

* Acetylcholine esterase activity is expressed as nmoles of ACh dehydrolysed/mg protein/hr.

Results are expressed as mean ± SEM of six experiments.

For experimental details see materials and methods.

Significantly differ from control

a = P<0.05, b = P<0.03, c = P<0.01, d = P<0.001

Studies on the effect of chronic treatment (treatment was continued for 15 consecutive days with 10 mg/kg/day) of FZP, DZP and OZP on rat brain microsomal Mg2+-ATPase, Na+-K+-ATPase, acetylcholine esterase and glutamine synthetase enzyme activities was presented on Table 8 which showed that all these three benzodiazepines significantly stimulated only GS activity (27% by FZP, 70% by DZP and 48% by OZP) and no noticeable change was observed for microsomal AChE, Na+-K+-ATPase and Mg2+-ATPase by any of the benzodiazepines (FZP, DZP or OZP) tested.
Table 8
Effect of chronic administration of various benzodiazepines on rat brain microsomal, Mg²⁺-ATPase, Na⁺-K⁺-ATPase; acetylcholine esterase (AChE) and glutamine synthetase (GS) enzyme activities

<table>
<thead>
<tr>
<th>Types of drug treatment</th>
<th>Enzymes</th>
<th>ATPase activities*</th>
<th>Studied</th>
<th>AChE activities**</th>
<th>GS activities***</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mg²⁺-ATPase</td>
<td>Na⁺-K⁺-ATPase</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>15.46±1.23</td>
<td>6.05±0.32</td>
<td>7.87±0.5</td>
<td>5.73±0.26</td>
<td></td>
</tr>
<tr>
<td>FZP</td>
<td>18.17±1.45</td>
<td>5.25±0.41b</td>
<td>7.38±0.42</td>
<td>7.23±0.46a</td>
<td></td>
</tr>
<tr>
<td>DZP</td>
<td>11.54±1.31</td>
<td>7.17±0.65</td>
<td>7.98±0.33</td>
<td>9.75±0.72c</td>
<td></td>
</tr>
<tr>
<td>OZP</td>
<td>14.72±0.96</td>
<td>6.39±0.37</td>
<td>7.56±0.75</td>
<td>8.43±0.8c</td>
<td></td>
</tr>
</tbody>
</table>

+ Treatment was continued for 15 consecutive days with 10 mg/kg/day.

* ATPase activities are expressed as µmole of Pi liberated/mg of protein/hr.

** AChE activities are expressed as µmole of ACh hydrolysed/mg of protein/hr.

*** GS activities are expressed Δ OD 540 µ/L/mg of protein/hr. Values are expressed as mean ± SEM of six experiments.

Significantly differ from control is given by,

a = P<0.02, b = P<0.01, c = P<0.001

The effect of acute i.p. administration of FZP, DZP and OZP on rat brain synaptosomal Mg²⁺-ATPase activity is summarised in Table 9. Neither FZP, nor DZP nor OZP at any instant of time (within 360 mins of drug treatment) for both low (10 mg/kg) and high (25 mg/kg) doses can produce any significant changes of rat brain synaptosomal Mg²⁺-ATPase activity. Only FZP at high dose there is a very slight increase of Mg²⁺-ATPase activity upto 120 mins after drug treatment and then gradual normalisation of the synaptosomal Mg²⁺-ATPase activity is observed.
Table 9

Effect of in vivo administration of benzodiazepines on rat brain synaptosomal Mg\textsuperscript{2+}-ATPase activity

<table>
<thead>
<tr>
<th>Time of treatment (mins)</th>
<th>Flurazepam</th>
<th>Benzodiazepines</th>
<th>Diazepam</th>
<th>Oxazepam</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Low dose</td>
<td>High dose</td>
<td>Low dose</td>
<td>High dose</td>
</tr>
<tr>
<td></td>
<td>(10 mg/kg)</td>
<td>(25 mg/kg)</td>
<td>(10 mg/kg)</td>
<td>(25 mg/kg)</td>
</tr>
<tr>
<td>18.60±1.34</td>
<td>18.31±1.46</td>
<td>18.79±1.04</td>
<td>18.54±1.61</td>
<td>18.43±2.12</td>
</tr>
<tr>
<td>18.44±1.51</td>
<td>19.15±1.74</td>
<td>18.62±1.42</td>
<td>18.41±1.75</td>
<td>18.32±1.25</td>
</tr>
<tr>
<td>18.28±1.32</td>
<td>20.48±1.89</td>
<td>18.44±1.18</td>
<td>18.32±1.34</td>
<td>18.41±0.95</td>
</tr>
<tr>
<td>17.27±1.02</td>
<td>21.32±1.32</td>
<td>18.54±1.82</td>
<td>19.51±2.11</td>
<td>18.22±1.46</td>
</tr>
<tr>
<td>18.73±1.24</td>
<td>20.08±2.07</td>
<td>18.33±1.22</td>
<td>18.77±1.86</td>
<td>18.28±1.78</td>
</tr>
<tr>
<td>19.61±1.55</td>
<td>17.87±1.83</td>
<td>16.61±0.95</td>
<td>19.94±1.82</td>
<td>18.05±1.33</td>
</tr>
</tbody>
</table>

* ATPase activity is expressed as µ moles of Pi liberated/mg of protein/hr.

Results are expressed as mean ± SEM of five experiments.

For experimental details see materials and methods.

The effect of three benzodiazepines (viz. FZP, DZP and OZP) on synaptosomal Na\textsuperscript{+}-K\textsuperscript{+} activated ATPase activity of rat brain tissue are depicted in Table 10 which tells that in vivo treatment of DZP and OZP cause significant dose dependent inhibition of Na\textsuperscript{+}-K\textsuperscript{+}-ATPase activity 180 mins after drug administration, but not at short interval of time (e.g. 30 and 60 mins duration of drug treatment) FZP at low dose (10 mg/kg) and only 360 mins after drug treatment significantly stimulated (P<0.05) the enzyme activity and at higher dose (25 mg/kg) synaptosomal Na\textsuperscript{+}-K\textsuperscript{+}-ATPase activity is stimulated significantly 120 mins after drug treatment, i.e., FZP shows both dose dependent (only at 6 hrs) and time dependent (only at higher dose) stimulation of synaptosomal Na\textsuperscript{+}-K\textsuperscript{+}-ATPase activity.
Table 10
Effect of in vivo administration of benzodiazepines on rat brain synaptosomal Na\(^+-\)K\(^+-\)ATPase* activity

<table>
<thead>
<tr>
<th>Time of treatment (mins)</th>
<th>Low dose (10 mg/kg)</th>
<th>High dose (25 mg/kg)</th>
<th>Low dose (10 mg/kg)</th>
<th>High dose (25 mg/kg)</th>
<th>Low dose (10 mg/kg)</th>
<th>High dose (25 mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Flurazepam</td>
<td>Diazepam</td>
<td>Oxazepam</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>30.42±1.62</td>
<td>30.75±1.42</td>
<td>30.81±1.51</td>
<td>30.64±1.46</td>
<td>29.84±1.76</td>
<td>30.26±1.16</td>
</tr>
<tr>
<td>30</td>
<td>29.68±1.73</td>
<td>28.72±1.33</td>
<td>31.72±2.12</td>
<td>30.06±2.08</td>
<td>29.41±2.13</td>
<td>30.07±2.14</td>
</tr>
<tr>
<td>60</td>
<td>28.29±2.02</td>
<td>26.79±2.12</td>
<td>33.48±2.07</td>
<td>29.45±2.01</td>
<td>28.87±1.75</td>
<td>28.36±1.64</td>
</tr>
<tr>
<td>120</td>
<td>27.02±1.48</td>
<td>49.65±3.49</td>
<td>32.68±3.11</td>
<td>27.58±2.24</td>
<td>28.52±2.41</td>
<td>25.12±1.51</td>
</tr>
<tr>
<td>180</td>
<td>32.91±2.42</td>
<td>56.40±4.22</td>
<td>26.25±1.78</td>
<td>22.42±1.78</td>
<td>26.61±1.83</td>
<td>25.04±1.55</td>
</tr>
<tr>
<td>360</td>
<td>36.47±1.58</td>
<td>60.47±2.41</td>
<td>22.74±1.64</td>
<td>19.64±1.82</td>
<td>25.70±1.08</td>
<td>22.34±1.63</td>
</tr>
</tbody>
</table>

* ATPase activity is expressed as μ moles of Pi liberated/mg protein/hr.

Results are expressed as mean ± SEM of six experiments.

For experimental details see materials and methods.

Significantly differ from control a= P<0.05, b= P<0.01, c= P<0.001

The results of in vitro treatment of rat brain synaptosomal Na\(^+-\)K\(^+-\) ATPase and Mg\(^{2+}\)-ATPase are summarized in Table 11. In addition to the vehicle treated control, the effects of varying doses of either FZP or DZP or OZP are shown. There appeared to be a dose related inhibition of both the two enzymes associated with the synaptosomal fraction of rat brain tissue in all these three cases of drug treatment only the changes observed on Na\(^+-\)K\(^+-\) ATPase activity by FZP at low dose (10 μg/mg protein) and Mg\(^{2+}\) ATPase activity by both DZP and OZP at low dose (10 μg/mg protein) are non-significant (P>0.05) with respect to control. Among these drugs, DZP is most active, FZP is least and OZP is in between them so far as in vitro action of these drugs on ATPase activities are concerned.
Table 11
Effect of in vitro treatment of benzodiazepines on rat brain synaptosomal Mg-ATPase* and Na-K-ATPase* activity

<table>
<thead>
<tr>
<th>Doses of the drug (mg protein)</th>
<th>Flurazepam</th>
<th>Benzodiazepines</th>
<th>Oxazepam</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mg-ATPase</td>
<td>Na-K-ATPase</td>
<td>Mg-ATPase</td>
</tr>
<tr>
<td>Vehicle</td>
<td>18.90±1.21</td>
<td>29.76±1.89</td>
<td>18.99±1.42</td>
</tr>
<tr>
<td>10</td>
<td>14.73±1.38</td>
<td>26.38±2.22</td>
<td>16.84±1.63</td>
</tr>
<tr>
<td>20</td>
<td>12.47±1.07</td>
<td>25.57±1.58</td>
<td>10.88±1.07</td>
</tr>
<tr>
<td>50</td>
<td>10.80±0.83</td>
<td>24.57±1.41</td>
<td>2.56±0.54</td>
</tr>
</tbody>
</table>

* ATPase activity is expressed as pmoles of Pi liberated/mg of protein/hr.

Results are expressed as mean ± SEM of six experiments.
For experimental details see materials and methods.
Significantly different from control a=P<0.05, b=P<0.02, c=P<0.01, d=P<0.001.

Table 12 shows the effect of acute administration (i.p.) of FZP, DZP and OZP on rat brain synaptosomal glutamine synthetase activity. Both DZP and OZP for both low and high dose levels can not produce any significant change of synaptosomal GS activity, whereas FZP are highly effective in bringing significant dose dependent stimulation of synaptosomal GS activity (170% for 10 mg/kg and 330% for 26 mg/kg dose) at 360 mins (P 0.001). FZP also at higher dose significantly stimulate (100%) GS activity after 180 mins of drug treatment.
Table 12

Effect of *in vivo* administration of benzodiazepines on rat brain synaptosomal glutamine synthetase* activity

<table>
<thead>
<tr>
<th>Time of treatment (mins)</th>
<th>Benzodiazepines</th>
<th>Flurazepam</th>
<th></th>
<th>Diazepam</th>
<th></th>
<th>Oxazepam</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Low dose (10 mg/kg)</td>
<td>High dose (25 mg/kg)</td>
<td>Low dose (10 mg/kg)</td>
<td>High dose (25 mg/kg)</td>
<td>Low dose (10 mg/kg)</td>
<td>High dose (25 mg/kg)</td>
<td></td>
</tr>
<tr>
<td>_</td>
<td>2.69±0.21</td>
<td>2.37±0.18</td>
<td>2.23±0.25</td>
<td>2.41±0.29</td>
<td>2.52±0.2</td>
<td>2.48±0.35</td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>2.61±0.35</td>
<td>2.12±0.14</td>
<td>2.20±0.28</td>
<td>2.46±0.36</td>
<td>2.61±0.46</td>
<td>2.26±0.63</td>
<td></td>
</tr>
<tr>
<td>60</td>
<td>2.41±0.29</td>
<td>1.87±0.42</td>
<td>2.22±0.42</td>
<td>2.58±0.63</td>
<td>2.51±0.72</td>
<td>2.28±0.33</td>
<td></td>
</tr>
<tr>
<td>180</td>
<td>2.26±0.38</td>
<td>2.53±0.75</td>
<td>2.28±0.12</td>
<td>3.03±0.48</td>
<td>2.46±0.5</td>
<td>2.81±0.44</td>
<td></td>
</tr>
<tr>
<td>180</td>
<td>3.00±0.16</td>
<td>4.80±0.63</td>
<td>2.25±0.36</td>
<td>2.93±0.51</td>
<td>2.76±0.52</td>
<td>2.96±0.81</td>
<td></td>
</tr>
<tr>
<td>360</td>
<td>7.28±0.48</td>
<td>10.32±1.73</td>
<td>2.26±0.41</td>
<td>2.60±0.23</td>
<td>2.54±0.51</td>
<td>2.72±0.5</td>
<td></td>
</tr>
</tbody>
</table>

*Glutamine synthetase activity is expressed as ΔO.D.540 mg/mg protein/hr.

Results are expressed as mean ± SEM of six experiments.

For experimental details see materials and methods.

Significantly differ from the control: a = P<0.01, b = P<0.001

Table 13 summarises the effect at of *in vitro* treatment of DZP, FZP and OZP on rat brain synaptosomal GS activity. It is clear from Table 13 that DZP only at high dose (50 µg/mg protein) causes significant inhibition (P<0.01) of rat brain synaptosomal GS activity, but FZP at low dose (10µg/mg protein) produces significant stimulation (P<0.02) of the same enzyme preparation. OZP cannot produce any significant change of synaptosomal GS activity.
Table 13

Effect of in vitro treatment of benzodiazepines on rat brain synaptosomal glutamine synthetase* activity

<table>
<thead>
<tr>
<th>Doses of the drugs (µg/mg protein)</th>
<th>Benzodiazepines</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Flurazepam</td>
</tr>
<tr>
<td>-</td>
<td>2.70±0.23</td>
</tr>
<tr>
<td>10</td>
<td>4.21±0.42</td>
</tr>
<tr>
<td>20</td>
<td>3.36±0.21</td>
</tr>
<tr>
<td>50</td>
<td>2.06±0.12</td>
</tr>
</tbody>
</table>

*Glutamine synthetase activity is expressed as AOD₅₄₀ nm/mg of protein/hour.

Results are expressed as mean ± SEM of six experiments.

For experimental details see materials and methods.

Significantly differ from control: b= P<0.01, c= P<0.001

Both DZP and OZP stimulate the rat brain synaptosomal AChE activity in both dose and time related manner (Table 14). The percentage of stimulation observed in DZP treatment is higher than that observed in OZP treatment, provided the changes are statistically significant over control. It also appears from Table 14 that the effect of FZP administration on rat brain synaptosomal AChE is dose related but time independent i.e., initially an inhibition, significant only at high dose at 60 mins of drug treatment and then stimulation, significant after 360 mins for both the doses of the drug is observed.
Table 14

Effect of in vivo administration of benzodiazepines on rat brain synaptosomal acetylcholine esterase activity

<table>
<thead>
<tr>
<th>Time of treatment (mins)</th>
<th>Flurazepam</th>
<th>Diazepam</th>
<th>Oxazepam</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Low dose (10 mg/kg)</td>
<td>High dose (25 mg/kg)</td>
<td>Low dose (10 mg/kg)</td>
</tr>
<tr>
<td>-</td>
<td>12.04±1.32</td>
<td>12.81±1.13</td>
<td>11.72±0.83</td>
</tr>
<tr>
<td>30</td>
<td>10.26±1.24</td>
<td>9.83±1.05</td>
<td>14.02±1.32</td>
</tr>
<tr>
<td>60</td>
<td>9.66±1.11</td>
<td>8.09±0.98^c</td>
<td>17.02±2.08^a</td>
</tr>
<tr>
<td>120</td>
<td>11.43±1.07</td>
<td>11.72±0.75</td>
<td>18.75±1.26^d</td>
</tr>
<tr>
<td>180</td>
<td>14.62±1.32</td>
<td>14.66±1.34</td>
<td>19.36±1.53^d</td>
</tr>
<tr>
<td>360</td>
<td>20.40±2.11^c</td>
<td>21.07±1.78^c</td>
<td>20.04±1.36^d</td>
</tr>
</tbody>
</table>

*AChe activity is expressed as µ moles of ACh hydrolysed/mg protein/hr.

Results are expressed as mean ± SEM of six experiments.

For experimental details see materials and methods

Significantly differ from control: a=P<0.05, b=P<0.02, c=P<0.01, d=P<0.001

Table 15 showed the effect of in vitro treatment of FZP, DZP and OZP on rat brain synaptosomal AChe activity. The table 15 demonstrate that maximum stimulation (P<0.001) of AChe is observed with low dose of FZP and then gradually falls to basal value and at 50 µg/mg protein dose no change is observed. In contrast to FZP, DZP produces inhibition of synaptosomal AChe activity and such inhibition is maintained for all the doses tested but OZP is ineffective to produce any significant change in synaptosomal AChe activity under similar condition of treatment.
Table 15
Effect of in vitro treatment of benzodiazepines on rat brain synaptosomal acetylcholine esterase* activity

<table>
<thead>
<tr>
<th>Doses of the drug (μg/mg protein)</th>
<th>Benzodiazepines</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Flurazepam</td>
<td>Diazepam</td>
<td>Oxazepam</td>
</tr>
<tr>
<td></td>
<td>10.17±1.22</td>
<td>10.54±0.82</td>
<td>10.27±1.04</td>
</tr>
<tr>
<td>10</td>
<td>22.72±1.31³</td>
<td>6.41±0.88ᵇ</td>
<td>10.04±1.56</td>
</tr>
<tr>
<td>20</td>
<td>20.88±1.04³</td>
<td>6.49±0.66ᵇ</td>
<td>9.73±1.21</td>
</tr>
<tr>
<td>50</td>
<td>12.27±1.27</td>
<td>6.77±0.75ᵇ</td>
<td>9.42±0.98</td>
</tr>
</tbody>
</table>

* AChE activity is expressed as μ moles of ACh hydrolysed/mg protein/hour.
Results are expressed as mean ± SEM of six experiments.
For experimental details see materials and methods.
Significantly differ from control : a=p<0.05, b=p<0.01, c=p<0.001

The effect produced due to the chronic treatment (single i.p. administration of 10 mg/kg per day for 15 consecutive days) of FZP, DZP and OZP on rat brain synaptosomal membrane bound enzyme, particularly Mg²⁺-ATPase, Na⁺-K⁺-ATPase, AChE and GS activity are summarised in Table 16. It appears from Table 16 that FZP produce no significant alteration of any of these enzyme activities. In control, DZP and OZP significantly inhibit AChE and Mg²⁺-ATPase to an equal extent over vehicle treated control, but Na⁺-K⁺-ATPase and GS activities remain unchanged by any of these drug treatment (chronic).
Table 16
Effect of chronic administration of various benzodiazepines on rat brain synaptosomal Mg\(^{2+}\)-ATPase, Na\(^{+}-\)K\(^{+}\)-ATPase, acetylcholine esterase (AChE) and glutamine synthetase (GS) enzyme activities

<table>
<thead>
<tr>
<th>Types of the drug treatment</th>
<th>Enzyme studied</th>
<th>ATPase activities*</th>
<th>AChE activities**</th>
<th>GS activity***</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mg(^{2+})-ATPase</td>
<td>Na(^{+}-)K(^{+})-ATPase</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>17.94±1.14</td>
<td>31.72±1.62</td>
<td>13.1±1.05</td>
<td>2.46±0.13</td>
</tr>
<tr>
<td>FZP</td>
<td>14.99±1.27</td>
<td>33.98±2.06</td>
<td>11.98±1.24</td>
<td>2.97±0.15</td>
</tr>
<tr>
<td>DZP</td>
<td>12.44±0.96</td>
<td>28.69±1.82</td>
<td>8.2±1.11</td>
<td>2.22±0.08</td>
</tr>
<tr>
<td>OZP</td>
<td>13.12±1.31(^a)</td>
<td>30.33±1.61</td>
<td>8.36±0.96</td>
<td>2.31±0.21(^c)</td>
</tr>
</tbody>
</table>

+ Chronic treatment was continued for 15 consecutive days with 10 mg/kg/day for each drug.

* ATPase activities were expressed as μ mole of Pi liberated/hr/mg protein.

** AChE activities were expressed as μ mole of ACh hydrolysed/mg protein/hr.

*** GS activities were expressed as AOD. 540 nm/mg protein/hr.

Results were expressed as mean ± SEM of six experiments.

Significantly differ from vehicle treated control is given by: \(^a\)=P<0.05, \(^b\)=P<0.01, \(^c\)=P<0.001

The effect of acute administration of three benzodiazepines viz., FZP, DZP and OZP on rat brain mitochondrial Mg\(^{2+}\)-activated ATPase activity is shown in table 17 where it is clear that neither FZP nor DZP nor OZP for both low (10 mg/kg) and high (25 mg/kg) dose can produce any significant change of Mg\(^{2+}\)-ATPase activity of rat brain mitochondrial fraction within 6 hours of each drug treatment when compared with that of vehicle treated control groups of rats.
Table 17
Effect of in vivo administration of benzodiazepines on rat brain mitochondrial Mg\(^{2+}\)-ATPase* activity

<table>
<thead>
<tr>
<th>Time of treatment (mins)</th>
<th>Benzodiazepines</th>
<th>Flurazepam</th>
<th>Diazepam</th>
<th>Oxazepam</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Low dose (10 mg/kg)</td>
<td>High dose (25 mg/kg)</td>
<td>Low dose (10 mg/kg)</td>
<td>High dose (25 mg/kg)</td>
</tr>
<tr>
<td>10</td>
<td>10.12(\pm)1.11</td>
<td>10.58(\pm)0.96</td>
<td>10.22(\pm)0.85</td>
<td>10.32(\pm)0.95</td>
</tr>
<tr>
<td>30</td>
<td>10.03(\pm)0.44</td>
<td>10.52(\pm)0.7</td>
<td>10.64(\pm)0.75</td>
<td>10.73(\pm)0.63</td>
</tr>
<tr>
<td>60</td>
<td>9.98(\pm)1.02</td>
<td>11.17(\pm)0.45</td>
<td>12.59(\pm)0.92</td>
<td>11.69(\pm)1.21</td>
</tr>
<tr>
<td>120</td>
<td>9.19(\pm)0.81</td>
<td>8.48(\pm)0.62</td>
<td>12.51(\pm)1.04</td>
<td>11.90(\pm)1.01</td>
</tr>
<tr>
<td>180</td>
<td>8.28(\pm)0.75</td>
<td>8.45(\pm)0.6</td>
<td>12.24(\pm)1.07</td>
<td>12.64(\pm)1.07</td>
</tr>
<tr>
<td>360</td>
<td>10.02(\pm)0.78</td>
<td>9.47(\pm)0.82</td>
<td>10.00(\pm)1.00</td>
<td>8.34(\pm)0.75</td>
</tr>
</tbody>
</table>

* ATPase activity is expressed as \(\mu\) moles of Pi liberated/mg of protein/hour.

Results are expressed as mean \(\pm\) SEM of six experiments.

For details of the experiments see materials and methods.

Table 18 shows the effect of in vivo treatment of FZP, DZP and OZP on rat brain mitochondrial Na\(^+\)-K\(^+\)-ATPase activity at different hours of treatment. It appears that both DZP and OZP significantly inhibit rat brain mitochondrial Na\(^+\)-K\(^+\)-ATPase activity only at 6 hours after drug treatment in a dose dependent manner and the inhibition is greater in DZP than in OZP. FZP also inhibit the same enzyme activity a bit earlier (60 mins after for high dose and 180 mins after low dose) than the two other benzodiazepines, in both dose and time treated manner.
Table 18

Effect of in vivo administration Benzodiazepines on rat brain mitochondrial Na\(^+\)-K\(^+\)-ATPase* activity

<table>
<thead>
<tr>
<th>Time of treatment (mins)</th>
<th>Flurazepam</th>
<th>Benzodiazepines</th>
<th>Diazepam</th>
<th>Oxazepam</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low dose (10 mg/kg)</td>
<td></td>
<td>Low dose (10 mg/kg)</td>
<td>High dose (25 mg/kg)</td>
<td>Low dose (10 mg/kg)</td>
</tr>
<tr>
<td>5.25±0.34</td>
<td>5.25±0.26</td>
<td>4.61±0.35</td>
<td>4.62±0.4</td>
<td>4.78±0.33</td>
</tr>
<tr>
<td>30</td>
<td>5.15±0.65</td>
<td>4.78±0.72</td>
<td>5.21±0.5</td>
<td>4.76±0.43</td>
</tr>
<tr>
<td>60</td>
<td>5.45±0.43</td>
<td>4.28±0.78(a)</td>
<td>5.36±0.44</td>
<td>5.17±0.62</td>
</tr>
<tr>
<td>120</td>
<td>4.59±0.56</td>
<td>3.84±0.17(b)</td>
<td>5.06±0.31</td>
<td>4.72±0.35</td>
</tr>
<tr>
<td>180</td>
<td>4.02±0.27(a)</td>
<td>3.69±0.2(b)</td>
<td>5.57±0.21</td>
<td>5.05±0.41</td>
</tr>
<tr>
<td>360</td>
<td>3.61±0.05(b)</td>
<td>3.45±0.14(b)</td>
<td>2.58±0.12(c)</td>
<td>1.62±0.1(c)</td>
</tr>
</tbody>
</table>

* ATPase activity is expressed as µ mole of Pi/mg of protein/hr
Results are expressed as mean ± SEM of six experiments.
For experimental detail see materials and methods
Significantly differ from control : \(a=P<0.05, b=P<0.001, c=P<0.01\)

Table 19 shows the effect of in vitro treatment of FZP, DZP and OZP separately on rat brain mitochondrial Mg\(^{2+}\)-ATPase and Na\(^+\)-K\(^+\)-ATPase activities. It is evident from Table 19 that FZP does not produce any significant changes in mitochondrial Mg\(^{2+}\)-ATPase and Na\(^+\)-K\(^+\)-ATPase activity. The Mg\(^{2+}\)-ATPase and Na\(^+\)-K\(^+\)-ATPase activities are inhibited by both DZP and OZP in a dose dependent manner, but DZP shows greater percentage of inhibition than OZP, DZP at 20 µ and 50 µ/mg protein doses significantly inhibits (\(P<0.001\)) both Mg\(^{2+}\)-ATPase and Na\(^+\)-K\(^+\)-ATPase where as OZP significantly inhibits Na\(^+\)-K\(^+\)-ATPase at both 20 µ and 50 µ/mg protein (\(P<0.001\)) and Mg\(^{2+}\)-ATPase at 50 µ/mg protein (\(P<0.01\)). The lowest dose (10 µ/mg protein) for both DZP and OZP are ineffective to bring about any change in the above mentioned enzyme activities.
Table 19
Effect of in vitro treatment of benzodiazepines on rat brain mitochondrial Mg²⁺-ATPase and Na⁺-K⁺-ATPase activity

<table>
<thead>
<tr>
<th>Doses of the drug (mg/mg protein)</th>
<th>Flurazepam</th>
<th>Benzodiazepines</th>
<th>Diazepam</th>
<th>Oxazepam</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mg²⁺-ATPase</td>
<td>Na⁺-K⁺-ATPase</td>
<td>Mg²⁺-ATPase</td>
<td>Na⁺-K⁺-ATPase</td>
</tr>
<tr>
<td>Vehicle</td>
<td>11.06±2.01</td>
<td>4.87±0.34</td>
<td>10.59±1.21</td>
<td>5.01±0.33</td>
</tr>
<tr>
<td>10</td>
<td>10.94±1.33</td>
<td>3.78±0.32</td>
<td>10.49±0.88</td>
<td>3.73±0.61</td>
</tr>
<tr>
<td>20</td>
<td>10.73±0.74</td>
<td>4.14±0.48</td>
<td>2.49±0.12 b</td>
<td>1.70±0.07 b</td>
</tr>
<tr>
<td>50</td>
<td>10.64±0.86</td>
<td>5.47±0.75</td>
<td>0.64±0.05 b</td>
<td>0.93±0.13 b</td>
</tr>
</tbody>
</table>

*ATPase activity is expressed as μ moles of Pi liberated/mg protein/hour.

Results are expressed as mean ± SEM of six experiments.

For experimental detail see materials and methods

Significantly differ from control : a=P<0.01, b=P<0.001

Table 20 shows the effect of acute single intraperitoneal administration of FZP, DZP and OZP on rat brain mitochondrial monoamine oxidase activity at different hours of treatment. It is evident from the table that MAO activity remains unaffected by FZP treatment and DZP for both the doses and OZP at higher dose (25 mg/kg) only 6 hours after drug treatment produce significant inhibition of the same enzyme activity. DZP shows greater percentage of inhibition (≈40% for high dose and 30% for low dose) of MAO activity than that observed for OZP (≈32% for high dose).
Table 20

Effect of *in vivo* administration of benzodiazepines on rat brain mitochondrial MAO* activities

<table>
<thead>
<tr>
<th>Time of treatment (mins)</th>
<th>Flurazepam Low dose (10 mg/kg)</th>
<th>High dose (25 mg/kg)</th>
<th>Diazepam Low dose (10 mg/kg)</th>
<th>High dose (25 mg/kg)</th>
<th>Oxazepam Low dose (10 mg/kg)</th>
<th>High dose (25 mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>29.85±1.29</td>
<td>29.18±1.61</td>
<td>31.65±1.33</td>
<td>31.59±1.05</td>
<td>30.25±1.45</td>
<td>29.93±1.5</td>
</tr>
<tr>
<td>30</td>
<td>29.62±0.84</td>
<td>30.23±1.33</td>
<td>31.46±1.72</td>
<td>31.5±0.56</td>
<td>30.42±1.13</td>
<td>30.5±1.21</td>
</tr>
<tr>
<td>60</td>
<td>27.35±1.27</td>
<td>34.03±1.04</td>
<td>31.79±0.86</td>
<td>31.33±1.11</td>
<td>30.15±1.25</td>
<td>23.85±1.46</td>
</tr>
<tr>
<td>120</td>
<td>28.8±1.44</td>
<td>25.7±0.95</td>
<td>29.78±1.21</td>
<td>33.03±1.32</td>
<td>29.86±0.88</td>
<td>28.75±0.73</td>
</tr>
<tr>
<td>180</td>
<td>27.91±1.14</td>
<td>27.0±1.23</td>
<td>31.21±1.61</td>
<td>30.55±1.24</td>
<td>28.64±1.06</td>
<td>26.16±0.92</td>
</tr>
<tr>
<td>360</td>
<td>30.45±1.26</td>
<td>28.38±0.92</td>
<td>21.03±0.75&lt;sup&gt;a&lt;/sup&gt;</td>
<td>18.25±1.21&lt;sup&gt;a&lt;/sup&gt;</td>
<td>27.62±1.00</td>
<td>20.72±0.67&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

* MAO activity is expressed as Δ OD 420 μm/100 mg of protein/hr.
Results are expressed as mean ± SEM of six experiments
For experimental details see materials and methods
Significantly differ from control a = P<0.001

Table 21 shows the effect of *in vitro* treatment of FZP, DZP and OZP on rat brain mitochondrial MAO activity, where all these drugs show dose dependent inhibition of MAO activity but the degree of inhibition is of the order of DZP>OZP>FZP. DZP significantly inhibits MAO activity for all the three doses tested; OZP significantly inhibits MAO activity at 20 μg/mg protein dose and FZP only at 50 μg/mg protein dose inhibits the mitochondrial MAO activity.
Table 21
Effect of in vitro treatment of benzodiazepines on rat brain mitochondrial MAO* activity

<table>
<thead>
<tr>
<th>Doses of the drug (\mu g/mg protein)</th>
<th>Benzodiazepines</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Flurazepam</td>
</tr>
<tr>
<td>10</td>
<td>28.57±1.62</td>
</tr>
<tr>
<td>20</td>
<td>27.20±1.11</td>
</tr>
<tr>
<td>50</td>
<td>18.13±1.43^b</td>
</tr>
</tbody>
</table>

* MAO activity is expressed as A 0.420 mm/100 mg protein/hour.

Results are expressed as mean ± SEM of six experiments. For experimental details see materials and methods. Significantly differ from control, a = P<0.05, b = P<0.01, c = P<0.001.

Results presented in Table 22 shows the effect of chronic (10 mg/kg/day for 15 consecutive days) administration of FZP, DZP and OZP (one at a time) on rat brain mitochondrial membrane bound (e.g. Mg^2+-ATPase, Na^+-K^+-ATPase and monoamine oxidase) enzyme activities. It is evident from the Table 22 that these drugs do not produce any significant (P>0.05) change of either of these above mentioned three enzyme activities.
<table>
<thead>
<tr>
<th>Types of drug treatment</th>
<th>Enzymes studied</th>
<th>ATPase activity*</th>
<th>MAO activity **</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mg$^{+2}$-ATPase</td>
<td>Na$^{+}$-K$^{+}$-ATPase</td>
</tr>
<tr>
<td>Vehicle</td>
<td></td>
<td>8.95±0.65</td>
<td>4.93±0.35</td>
</tr>
<tr>
<td>PZP</td>
<td></td>
<td>8.81±0.72</td>
<td>6.01±0.45</td>
</tr>
<tr>
<td>DZP</td>
<td></td>
<td>10.02±0.55</td>
<td>6.02±0.28</td>
</tr>
<tr>
<td>OZP</td>
<td></td>
<td>9.24±0.82</td>
<td>5.93±0.31</td>
</tr>
</tbody>
</table>

* Chronic treatment was continued for 15 consecutive days with 10 mg/kg/day for each drug.

** MAO activities were expressed as A.O.D.420 miJ/mg protein/hour.

Results are expressed as mean ± SEM of six experiments.
DISCUSSION

Because central nervous system is very rich in membranous structure and that the major site of action of many psychopharmacologic drugs is on the CNS, the neuronal membranes have been supposed to play an important role in BZP action. Another important aspects of neuronal membrane, in contrast to other cell membranes, is its unusually high lipid and protein content together with carbohydrate and nucleic acids, which allow interaction under both in vivo and in vitro condition with various neurotropic agents to produce characteristic biochemical and biophysical changes.

Microsomal Membrane Bound Enzymes

It is evident from the present investigation that all the three BZPS under (in vivo) condition increase the activities of rat brain microsomal ATPase, AChE and GS (Tables 1, 2, 4, 6). These changes due to BZPS may indicate that psychopharmacological activity of BZPS is related to the changes in cation transport and mostly dose and time dependent except for Na⁺-K⁺-ATPase activity of DZP treated rats which shows higher stimulatory effect at low dose than that observed at higher dose i.e., biphasic action of the enzyme Na⁺-K⁺-ATPase, by DZP treatment, due to the difference in membrane permeability is observed (80).
In contrast to in vivo treatments in vitro treatment with BZPS (Tables 3, 5, 7) significantly inhibit above mentioned microsomal enzymes except for GS which is stimulated by FZP even in in vitro condition. These differences between in vivo and in vitro effects of the same drug on the same neuronal membrane bound enzyme activity may reflect differences in local concentration of the each drug in the two systems which may not have identical condition of ionic environment. The present changes of ATPase activity due to BZPS treatment may be explained on the basis of the altered sensitivity of the enzymes towards ions. It had been shown that the inhibition of microsomal Na⁺-K⁺-ATPase activities by alcohol and several general depressants is due to the inhibition of cation transport with the production of CNS depression (81). Such inhibition of cation transport by BZPS at the microsomal membrane may possible also be the reason of in vitro ATPase inhibition by BZPS. The decreased microsomal Mg²⁺-ATPase activity (in vitro) by BZPS may be due to depletion of Mg²⁺-ion resulting from the release of divalent cations (particularly Ca²⁺-ion) from the membrane (82). Such a release of Ca²⁺-ion by BZPS may also be expected from the observed increased activity of AChE by these drugs.

ATPase activity by BZPS under in vivo condition may produce a change in the conformation of the microsomal ATPase molecule,
cationic activators. The results of these experiments, may give reasonable grounds to believe that the differential sensitivity of brain microsomal ATPase towards cation and may have some relationship to changes induced by BZP administration. It has been observed that the increased GS activity in both microsome and synaptosome in presence of FZP (in vivo, Table 4,12) is probably functionally involved in the increased utilisation of NH₃ appeared from the decreased level of biogenic amine may be responsible for the increased turnover by FZP (83). It has been reported that a decreased level of NH₃, due to increased activity of GS is associated with sedation (84,85). In this context FZP may be regarded as a more potent sedative than DZP or OZP as DZP and OZP stimulate only microsomal GS activity in vivo whereas FZP stimulate both microsomal and synaptosomal GS activity in vivo. In contrast to the action of FZP, DZP and OZP inhibit GS activity (in vitro, Table 5). From the results of in vitro effect of these three BZPS on GS activity possible a different mechanism of action of FZP and DZP/OZP may be expected so far as their action on glutamic acid-GABA system is considered.

In the present investigation microsomal AChE activity is also increased in both in vivo (Table 6) and in vitro (Table 7) treatment with these drugs. This suggest that BZPS act directly on the ACh-cholinesterase system of microsomal fraction. It is
well known that AChE of brain microsomal fraction serves as reserve AChE (96) and stimulation of brain microsomal AChE by BZPS give rise to less possibility of sparing of microsomal Ach (87). Such as increased activity of AChE by BZPS put further support to the antagonising action of this group of drugs on several anticholinesterases as observed by other investigators (88-90). In view of the lipophilic nature of BZPS and in addition to the idea that phospholipids are required for maintaining the function of many membrane bound enzymes (91) in CNS, it is possible that BZPS some how affect the hydrophobic-hydrophilic balance at the membrane surface that regulates the storage, release and secretion processes in the neurons.

Synaptosomal Membrane Bound Enzymes

It was well established that a variety of psychoactive drugs like chlorpromazine, amitryptiline, cannabis, diphenyl hydantoin and chlodiazepoxide hydrochloride changes the activity of both Na\(^{+}\)-K\(^{+}\)-ATPase, and Mg\(^{2+}\)-ATPase depending on the condition of the experiments(592-95). From the present study, it is evident that BZPS being psychoactive drugs, show no alteration of the synaptosomal Mg\(^{2+}\)-ATPase under \textit{in vivo} condition of treatment (Table 9) but all these drugs (DZP, OZP and FZP) under \textit{in vitro} condition (Table 11) inhibit the same enzyme in a dose dependent manner and the inhibition are in the order of DZP>OZP>FZP.
Although benzodiazepines are potent anticonvulsant and possess some structural similarities with other anticonvulsant, diphenylhydantoin, BZPS under in vivo condition of treatment do not obey Gellbert's hypothesis (93) the inhibition of vesicular Mg$^{2+}$-ATPase activity is a characteristic property of all the anticonvulsant drugs. This unchanged total synaptosomal Mg$^{2+}$-ATPase activity due to BZPS treatment in the present experiments may be explained on the basis of degree of heterogeneity of the synaptosomal fraction (obtained by the method of Gray and Whittekar) as a result the effect of these drugs on synaptosomal Mg$^{2+}$-ATPase activity may not be detected because the vesicle which contain most of the synaptosomal Mg$^{2+}$-ATPase activity contribute only some 5% to synaptosomal protein (50). Again the inhibition of synaptosomal Mg$^{2+}$-ATPase activity under in vitro condition by all these three benzodiazepines (Table 12) reflect that the drug concentration is higher in in vitro condition than in vivo condition which is sufficient to produce significant inhibition of vesicular Mg$^{2+}$-ATPase activity in crude synaptosomal preparation and is in accord with previous observation (93).

Clearly, the significance of Mg$^{2+}$-ATPase has to be established. It has been observed that the inhibition/synaptosomal Mg$^{2+}$-ATPase activity is not due to the depletion of Mg$^{2+}$-ion but may be due to decrease in the K$^+$/Na$^+$ ratio (95) resulting from
increase synaptosomal Na level. Such an inhibition of Mg^{2+}-ATPase activity may in some way be related to the degree of inhibition of amine uptake into the vesicle resulting an increased concentration of amines by these drugs, as already observed by various investigators (71,96-99).

Again the inhibition of synaptosomal Na^{+}-K^{+}-ATPase activity both in vivo and in vitro condition by DZP and OZP treatment is in agreement with the work of Geilbert et al. (1976) (50), Ueda et al. (1970) (100) and Maisov et al. (1976) (101) and the pharmacological activity of BZP was expected to mediate through the drug induced changes in membrane permeability. However, this view is not generally accepted but it may help to explain many of the observed biochemical changes of BZPs; clinically important anticonvulsant and antianxiety drug. In view of the known lipid solubility of BZP (102), like other depressant drugs it appears likely that in usual therapeutic condition (dose) DZP like DPH may stabilise membrane which have been rendered unstable in some way (103). This membrane action implies an effect of the drug on the membrane constituents and binding of both protein and phospholipid represents a mechanism of this effect, as proteolipids appears to be involved in specific drug receptors (104,105).
In contrast to DZP/OZP, FZP stimulated Na\(^+\)-K\(^+\)-ATPase activity of rat brain synaptosomal fraction under in vivo condition of treatment, which in turn maintain an apparent low level of Na due to Na efflux (or with a concurrent gain of K\(^+\)-ion)\(^{(106)}\). Such drug induced changes in the membrane permeability may be a common mechanism for the inhibitory action of both depressant and antidepressant group of drugs on synaptosomal Na\(^+\)-K\(^+\)-ATPase, located near the internal surface of the neuronal membrane and is exposed to axoplasmic ionic environment and represent the enzymatic basis of Na\(^+\)-K\(^+\)-transport across the cell membrane \(^{(107)}\).

Such change in membrane permeability due to the inhibition of synaptosomal Na\(^+\)-K\(^+\)-ATPase by DZP/OZP may inactivate the Na conductance (decreased Na efflux) or, increase K conductance (increase K-influx) or both as a result a transient increase of Na\(^+\)-ion near the internal membrane and decreased of K\(^+\)-ion there may be observed\(^{(106)}\). But Ueda et al. \(^{(1970)}\)\(^{(106)}\) has observed a competitive inhibition of Na\(^+\)-K\(^+\)-ATPase against K\(^+\)-ion and DZP which supports the interaction between these ions and the drug, DZP at the catalytic site of the allosteric enzyme ATPase \(^{(108)}\). Thus, it may be hypothesised that benzodiazepines (particularly DZP or OZP) like diazonium salts \(^{(106)}\), may form covalent bonds with many of the functional groups of the protein ATPase located out side permeability barrier of the
plasma membrane and may inhibit the binding of the ATP at the active side due to conformational change caused by drug-action (K⁺-ion) interaction, as a result inhibition of Na⁺-K⁺-ATPase activity is observed.

It is clear from the results of present experiments that P2P, D2P and O2P produce very little or, no change of synaptosomal Na⁺-K⁺-ATPase activity (which is also observed for other neuronal membrane bound enzyme activity) within 60 minutes of drug administration, but these drugs (P2P and D2P) change GABA concentration within 60 minutes (Part-II). Thus, it may be expected that the changes in GABA concentration in brain by these drugs may be the primary effect produced by B2P group of compounds. Such changes in GABA concentration by B2P may also change membrane permeability of the synaptic membrane.

This inhibition of synaptosomal Na⁺-K⁺-ATPase by B2PS under acute treatment is possible associated with the following:

(a) Increased accumulation of ATP which may stabilise the binding and compartmentation of putative neurotransmitter in the synaptic junction (110).

(b) Decrease release and reuptake process of putative neurotransmitter (111). In favour of this interpretation also is the suggestion made from present work that acute treatment of B2P exerts an inhibitory effect on the
reuptake process of inhibitory neurotransmitter, GABA in the rat brain.

(c) Increased polarisation of the neuronal membrane with concomitant release of ACh.

The increased synaptosomal AChE activity as observed with in vivo action of BZP may possible be linked with the metabolism of increased ACh level in brain (95,113) but not in agreement with Consolo et al. (1975) (113a) who observed that DZP is not able to alter the activity of both cholineacetyltransferase activity and acetylcholineesterase activity in vitro condition but increased the brain ACh level. It has been observed that barbiturate exert their effect on brain ACh levels by a mechanism other than enzyme inhibition (114). From this study and the similarity of BZP with barbiturate may indicate that the increased level of ACh in rat brain is not due to the result of changes at the level of metabolising enzyme activities rather due to the reduced turnover of ACh by the drug, DZP (115,116). Recently it has been observed that GABA-receptor antagonist, bicuculline inhibits AChE competitively (117). Hence, the increased activity of AChE by another GABA-receptor agonist, BZP, can not be ignored as an artifact. As benzodiazepines contain a basic proton donor group (N-atom) in its nucleus may play an important role in AChE activity, and may bind at the
esteric site or, the anionic site of the enzyme molecule like other psychiatric drugs. This action of the drug at the allosteric site, distinct from active centre (122,123) of the molecule where cations, particularly the increased level of Mg$^{+2}$-ion due to DZP action, produced their effect and this in turn changes the conformation of the enzyme molecule in such a way that an increased action with ACh which produces an increase level due to BZP action, is favoured.

Synaptosomal GS activity is stimulated only by FZP and not by DZP and OZP. This again shows that FZP and DZP, OZP may not have the similar mechanism of action on GABA-glutamate-glutamine system which is partially responsible for the anticonvulsant action of these drugs. Results of acute in vivo study indicate that GS activity is stimulated by FZP may be responsible for the higher glutamine level or, lower glutamic acid level in brain beside the role of GAD. The anticonvulsant action of FZP may be due to the change in GABA/glutamate ratio or higher glutamine level in brain rather than the increased GABA level, as convulsive agent methionine sulfoximine reduced the glutamine level and GS activity (124,126) in brain.

Another point worth noting is that during chronic (10 mg/kg/day for 15 days) treatment with FZP and DZP/OZP show different action on synaptosomal membrane bound enzyme activities. The
The unchanged activity of the enzymes by the chronic use of FZP may be due to the fact that FZP, during chronic treatment may entirely and quickly be converted to polar metabolites (121,128) than DZP/OZP which may interact less strongly than DZP/OZP with the membrane lipids. In the acute condition, BZPS remain mostly in lipid soluble form even after metabolism having little chance to form hydroxylated polar metabolite and hence producing more interaction with the membrane components. Both DZP and OZP show slight inhibition of both AChE and Mg\(^{+2}\)-ATPase activity giving rise to the possibility that ACh level may be increased by chronic treatment not due to their increased release of ACh but due to the retarded destruction of release ACh.

Here, while discussing the mode of action of BZP on neuronal membrane another point is worth noting by both DZP and FZP (Tables 3, 13A) exhibit a biphasic action, like many other psychotropic agent (129,130) meaning that two types of action at two drug concentrations; mainly stimulatory at low dose and inhibitory at higher one on the membrane bound enzymes. In order to explain such action of the drug on enzyme activity recently Dutta et al. (1977) (131) have proposed a model which vested critically on the hypothesis of the formation of an unstable triple complex, i.e., (enzyme-sub-drug) which under certain physiological condition might produce biphasic action.
This model endeavours to prove that the differential action of the drugs on the enzymes due to the possible alteration in the membrane permeability (i.e., reduced permeability at lower concentration and increased permeability at higher one) rather than the conformational difference of the enzyme E₁ and E₂ at these two drug concentration due to the differences in polarity of the surrounding medium (132,133). This possible change in membrane permeability due to the formation of triple complex may result in the shift in the transition temperature of the membrane considerable to a lower value which allow the membrane lipid to pass to a less orientated structure i.e., "melting of the lipid chains" occur at the higher drug concentration than the lower one, leading to an effective increase of the membrane fluidity and permeability to water and several other organic molecule including the drug molecule (134,136).

Mitochondrial Membrane Bound Enzymes

It is clear from the present investigation that benzodiazepines do not produce any noticeable change in mitochondrial Mg⁺²-ATPase (Table 17) but inhibit mitochondrial Na⁺-K⁺-ATPase activity (Table 18) under in vivo condition of treatment which
is in agreement with Spector's (1972) (137) observation that all anticonvulsant or antiepileptic drugs inhibit mitochondrial \( O_2 \) uptake or \( O_2 \) consumption and this may subsequently attenuated the subcellular function of the CNS. According to recent finding of Roosdrop et al. (1977) (138). BZPS can bind with the positively charge arginine residue of rat brain mitochondrial membrane and such binding of BZPS like other arginyl binding reagent can inhibit the complex V of mitochondrial oxidative phosphorylation system which contains both the enzymes responsible for ATP-synthesis and hydrolysis (139) but devoid of respiratory chain components. Such inhibition of mitochondrial \( Na^+ - K^+ \)-ATPase by BZPS will affect the cation pump operating at the mitochondrial membrane in such a fashion that the \( Na \) concentration will be higher there and such an increased \( Na \) concentration may given rise to the possibility of increased release of biogenic amines by these drugs as observed by various investigators. Again under \textit{in vitro} condition DZP and OZP inhibit mitochondrial ATPase in a dose dependent manner but not by FZP (Table 19) which may be due to the similar type of binding of at least DZP and OZP in rat brain mitochondrial membrane.

Again it has been reported in the present investigation that FZP and OZP do not produce any perceptable change of MAO activity under \textit{in vivo} condition, only DZP inhibits MAO activity 6 hours after drug treatment (Table 20). In contrast to \textit{in vivo} treatment,
all these three drugs inhibit MAO activity under \textit{in vitro} condition and the potency of inhibition are in the order of DZP>OZP>FZP which can not explain the differential action of FZP on dopamine level when compared with DZP or OZP (140), but can put additional support to the anticonvulsant potencies of these drugs which are in the order of DZP>OZP>FZP as also observed by others (141). It may be explained from the present survey that the increased level of NE, DA and 5HT in different regions of rat brain 2 hours after DZP treatment (98) did not necessarily be due to the decrease catabolism of these amines by DZP as upto two hours DZP produce no change in MAO activity. The function role of MAO activity is to detoxicate the released biogenic amine responsible for the different condition of mood and behaviour of an individual. Since MAO activity is not affected by BZPS the increased monoamine level in brain may be controlled by these drugs due to reduced turnover of these amines which was also proposed by many workers. The \textit{in vitro} inhibition of MAO by these drugs may represent an inhibition of reuptake process involved in amine turnover, as a consequence an increased level of free intraneuronal amine (98,140) due to reduced turnover is expected. It is evident from the present study that unlike acute treatment chronic treatment of BZPS show no significant change of mitochondrial membrane bound ATPase as well as MAO activity (Table 22).
Such an unchanged activity of the enzymes may be explained on the basis of the stabilisation of membrane due to chronic BZPS treatment as chronic DZP treatment increased phospholipid concentration by inhibiting phosphodiesterase activity (142). Phospholipids are the essential component of the mitochondrial membrane and are also responsible for maintaining the enzyme conformation as a result no change in enzyme activity is observed due to chronic BZPS treatment.
SUMMARY

The results obtained in the study of FZP, DZP and OZP induced changes in different membrane bound enzymes at different subcellular fractions of rat brain tissue may be summarised as follows:

(A) ATPase activity in different subcellular fractions:

1. A single i.p. administration of FZP, DZP and OZP at low dose (10 mg/kg) did not produce any significant change of Mg$^{2+}$-ATPase activity within six hours after drug treatment, but FZP, DZP and OZP at higher dose (25 mg/kg) stimulated microsomal Mg$^{2+}$-ATPase activity in the order of DZP$>$FZP$>$OZP.

2. Acute administration (i.p.) of FZP were not effective to bring any change in microsomal Na$^+$-K$^+$-ATPase activity; OZP under similar condition of treatment stimulated the same enzyme preparation at higher dose and at late hours. A single i.p. administration of DZP showed a greater stimulation at lower dose at an early interval of drug treatment and then gradually normalised.

3. FZP, and OZP under in vitro condition of treatment inhibited microsomal Na$^+$-K$^+$-ATPase and Mg$^{2+}$-ATPase activity but DZP under similar condition of treatment inhibited microsomal Na$^+$-K$^+$-ATPase and showed biphasic action (i.e., stimulation at low dose and inhibition at higher dose) on microsomal Mg$^{2+}$-ATPase activity.
4. Chronic treatment (10 mg/kg/day for 15 days) with FZP, DZP or OZP did not cause any change in microsomal or synaptosomal or mitochondrial Na\(^{+}\)-K\(^{+}\)-ATPase activity. Only microsomal Mg\(^{2+}\)-ATPase was stimulated and synaptosomal Mg\(^{2+}\)-ATPase was inhibited by chronic treatment with FZP and both DZP and OZP.

5. Synaptosomal Mg\(^{2+}\)-ATPase activity was not affected by the acute in vivo treatment with FZP, DZP and OZP, whereas in vitro treatment with these drugs produced dose dependent inhibition of the same enzyme preparation and was of the order of DZP > OZP > FZP.

6. Acute i.p. administration of FZP showed stimulation of synaptosomal Na\(^{+}\)-K\(^{+}\)-ATPase activity but the other benzodiazepines viz., DZP and OZP inhibited the same enzyme under similar condition of drug treatment. In in vitro condition of treatment, both DZP and OZP showed very high inhibitory effect on synaptosomal Na\(^{+}\)-K\(^{+}\)-ATPase activity but FZP also inhibited the same enzyme very slightly.

7. Chronic treatment with FZP, DZP and OZP showed no change in synaptosomal Na\(^{+}\)-K\(^{+}\)-ATPase activity, but synaptosomal Mg\(^{2+}\)-ATPase activity was inhibited by DZP and OZP but not by FZP under same condition of treatment.
(B) Acetylcholinesterase activity in different subcellular fraction:

1. Acute i.p. administration of BZPS caused significant stimulation of microsomal AChE activity in a dose dependent and time dependent manner (not pronounced upto 60 mins) which was in the order of FZP > DZP > OZP.

2. Both DZP and OZP showed time and dose dependent stimulation of synaptosomal AChE activity, but the potency of DZP was greater than that of OZP. Acute FZP treatment showed biphasic type of action on the same enzyme i.e., inhibition at initial phase and stimulation at latter phase (after 6 hours).

3. Microsomal AChE activity was somewhat resistant to produce any change due to chronic treatments with BZPS. Synaptosomal AChE activity was depressed by the chronic treatment with DZP and OZP but not with FZP.

4. In in vitro treatment with BZPS (viz. DZP, OZP and FZP) stimulated microsomal AChE activity in a dose dependent manner, and synaptosomal AChE activity remained unaffected by OZP, inhibited by DZP (more or less similar for all the doses).

(C) Glutamine synthetase activity in different subcellular fractions:

1. Acute administration of BZPS, generally, resulted in a dose-dependent and time-dependent (prominant after 180 mins of treatment) stimulation in the activities of microsomal GS. The potencies were of the order of FZP > DZP > OZP.
2. DZP and OZP had no effect on synaptosomal GS activity but single i.p. injection of FZP produced dose dependent stimulation of the same enzyme (synaptosomal GS) activity.

3. Chronic treatment of FZP or DZP or OZP had no effect on synaptosomal GS activity but stimulated microsomal GS activity.

4. In **in vitro** treatment of DZP and OZP caused dose dependent inhibition of microsomal GS activity and very little stimulation of the same enzyme preparation was observed with FZP only at higher dose (50 μg/mg protein) under similar condition of treatment.

5. **In vitro** treatment of FZP produced stimulation of synaptosomal GS activity only at low dose (10 μg/mg protein); OZP showed no change of the same enzyme and DZP under similar condition of treatment produced significant inhibition of GS activity only at higher dose (50 μg/mg protein) of the drug.

(D) Monoamine oxidase activity in mitochondrial fraction:

1. Single i.p. administration of FZP or, OZP did not produce any change in MAO activity (within 6 hours) but DZP only 6 hours after drug treatment produced dose-dependent inhibition of the same enzyme.
2. Chronic treatment with FZP, DZP and OZP showed no change in Monoamine oxidase activity.

3. In vitro treatment of DZP and OZP resulted in dose related decrease of MAO activity and FZP also inhibited the same enzyme only at higher dose (50 μg/mg protein) under similar condition of treatment.
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