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THE SYNTHESIS AND BIOCHEMICAL AND PHARMACOLOGICAL EVALUATION OF γ-AMINOBUTYRIC ACID ANTAGONISTS.

by

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A thesis submitted to the Council for National Academic Awards in partial fulfilment for the Degree of Doctor of Philosophy

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Memorandum

Except where acknowledgement is made by reference, this thesis and the experiments described were the unaided work of the author. The work was carried out under the supervision of Dr. J. F. Collins in the Department of Chemistry at the City of London Polytechnic in collaboration with Professor T. D. Inch at the Department of Chemistry, Chemical Defence Establishment, Porton Down, Wiltshire, during the period October 1976 - October 1979, and was supported by a studentship from the Science Research Council.

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Publication

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Abbreviations

GABA	Y-Aminobutyric acid
CNS	Central nervous system
$MgSO_4$	Anhydrous magnesium sulphate
bp.	Boiling point
mpt.	Melting point
^{1}Hnmr	Proton nuclear magnetic resonance
DHP	Dihydropicrotoxinin
CPK	Corey-Pauling-Koltum
(^{3}H)-GABA	Y-[$2_1 3_1$ - ^{3}H (N)] - aminobutyric acid
(^{3}H)-Muscimol	[methylene - ^{3}H (N)] - muscimol
(^{3}H)-DHP	α-(8,10 - ^{3}H) - dihydropicrotoxinin
c-GMP	Cyclic guanosine 3' 5' phosphate
c-AMP	Cyclic adenosine phosphate
GAD	Glutamic acid decarboxylase
bicyclic phosphate esters	4-substituted 2,6,7-trioxa-1-phosphabicyclo (2,2,2) octan-1-ones
silatranes	1-(aryl or alkyl) - 2,8,9-trioxa-5-aza-1-silabicyclo (3,3,3) undecanes
TETS	Tetramethylenedisulphotetramine
ACHC	Cis-3-aminocyclohexane carboxylic acid
THIP	4,5,6,7-tetrahydro-isoxazole (5,5,-C) piridin-3-ol
THF	Tetrahydrofuran
TMS	Tetramethylsilicon
J	Coupling constant between hydrogens (cycles per second)
δ	Chemical shift from TMS in ppm

Abstract

The synthesis and biochemical and pharmacological evaluation of γ-aminobutyric acid antagonists. Neil J. McLaughlin.

γ-Aminobutyric acid has been shown to be a major inhibitory neurotransmitter in the central nervous system (CNS). This study is concerned with the examination of a number of GABA antagonists in two *in vitro* assay systems.

A series of cage-structured compounds including bicyclic phosphate esters (2,6,7,-Trioxa-1-phosphabicyclo (2,2,2,) octan-1-oxides with suitable 4-substituents) have been shown to be potent GABA antagonists. These compounds do not act directly on the postsysnaptic GABA receptor site but probably at the same site as another potent GABA antagonist picrotoxinin. It has been shown that the substituent group on the bridge-head on the 4-carbon atom in the molecule plays a very important role in the toxic potency and GABA antagonistic properties of these compounds. A very bulky and branched substituent caused a great increase in potency. This property was utilised in the synthesis of a series of these compounds.

The binding of radiolabelled ligands (both agonists and antagonists) to synaptic membrane fractions can be utilised as a convenient assay for the study of GABA receptors. Such assays using frozen-thawed rat brain synaptosomal membranes has been demonstrated to be stereospecific and saturable using (^{3}H)-GABA, the potent GABA agonist (^{3}H)-muscimol also with GABA antagonists using (^{3}H)-bicuculline methobromide and (^{3}H)-

dihydropicrotoxinin. Utilising these methods a number of agonists and antagonists have been examined to evaluate their ability to displace the stereospecific binding.

The synthesised GABA antagonists have also been used to evaluate their properties on the depolarising effect of GABA on the rat superior cervical ganglion in comparison to results obtained by known GABA antagonists.

The bicyclic phosphate esters were shown to be very toxic when administered to mammals causing seizures and death, this effect being attributed to the antagonism of GABA in the CNS. They were shown to be potent inhibitors of GABA in the superior cervical ganglion. In binding studies with (^{3}H)-muscimol they did not displace any of the bound agonist and also were shown not to displace bound (^{3}H)-dihydropicrotoxinin. The results obtained in this study have been discussed and compared with structure-activity relationships to other compounds of similar structure.

CONTENTS

CHAPTER 1 : Y-AMINOBUTYRIC ACID AS A NEUROTRANSMITTER

1.1 Introduction. γ-Aminobutyric acid (GABA) (Fig.1.1) was first discovered in the mammalian central nervous system (CNS) as one of the amino acids in mouse brain extracts (Roberts and Frankel, 1950a). Later these brain extracts were shown to have a Factor I which had inhibitory effects on the synapses in the crayfish stretch receptor (Bazemore, Elliott and florey, 1957). This inhibitory Factor I was found to be GABA. Further research on GABA eventually established it as a major inhibitory neurotransmitter (Curtis and Watkins, 1960, Krnjević and Phillis 1963, Krnjević and Schwartz 1966, Krnjević 1974, Curtis 1975). Since then a great deal of evidence has been accumulated which supports the proposal that GABA is a naturally occuring inhibitory neurotransmitter.

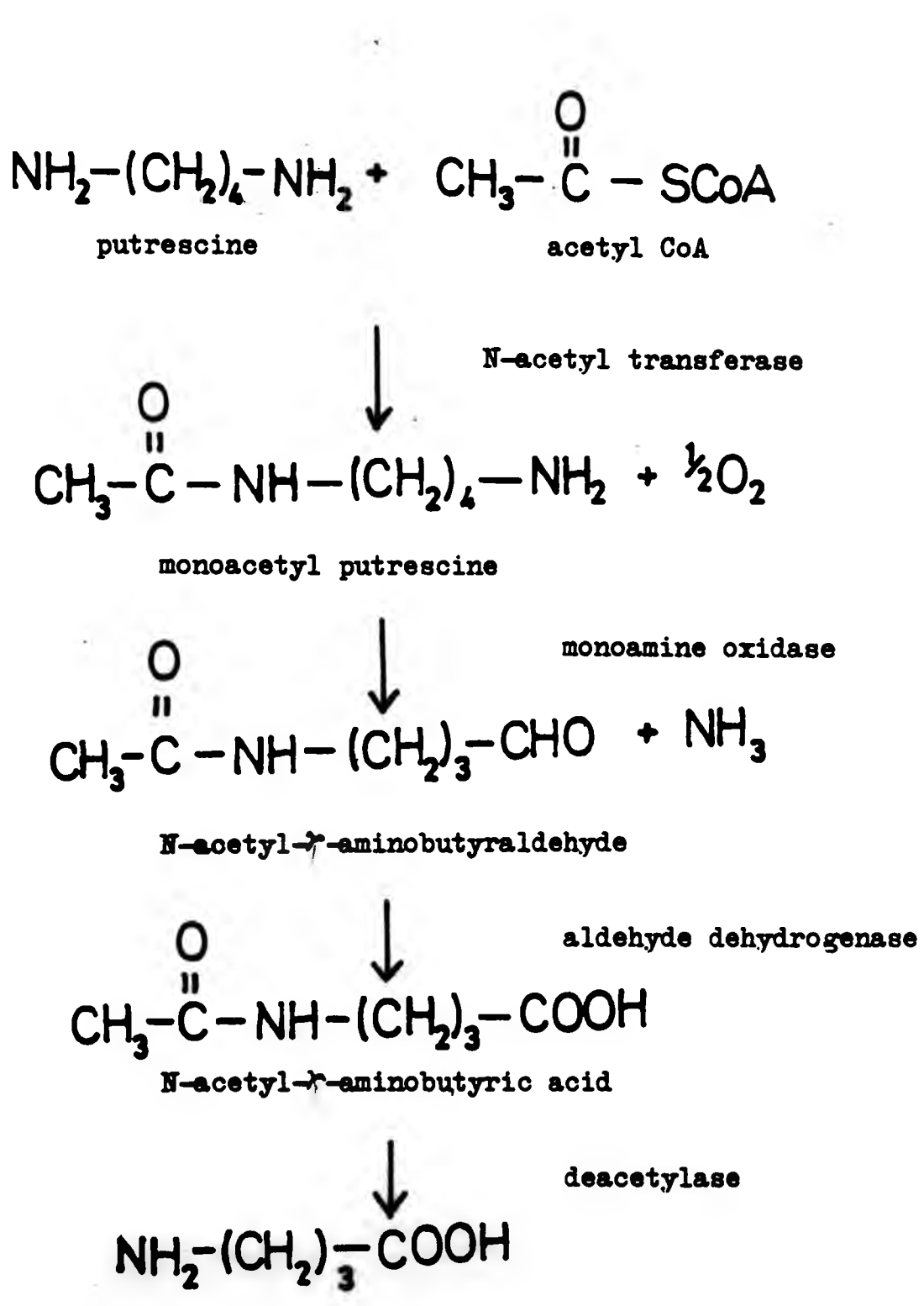
1.2 Evidence to support GABA as a neurotransmitter.

To classify a compound as a neurotransmitter it must be able to fill certain criteria:

1.2.1 Synthesis

GABA is synthesised by the decarboxylation of glutamic acid (Roberts and Frankel 1950b). This is done via an enzymatic process involving L-glutamate-1-carboxylase (EC4. 1.1.15) usually referred to as glutamate decarboxylase (GAD). Evidence for this has been shown with metabolic experiments in which the immediate origin of GABA carbon atoms can be traced through the carbon atoms of glutamate (Bert 1972). The correlation of GAD apoenzyme levels with GABA content in brain areas and cell types has also been shown (Fonnum, Storm-Mathisen and Walberg 1970).

Fig. 1.2. An alternative complete metabolic pathway from putrescine via monoacetyl putrescine and N-acetyl-γ-aminobutyric acid to GABA (Baxter 1976)



An alternate pathway from putrescine via monoacetyl putrescine and N-acetyl-Y-aminobutyric acid to GABA has also been reported to exist in mouse brain (Seliev and Al-Therib, 1974) (Fig. 1.2).

1.2.2 Storage

There must be evidence showing facilities for the storage of the compound where it can easily be transported or made available at the presynaptic nerve terminals.

Fluid left in contact with the brain surface was found to contain only traces of GABA indicating that GABA must be stored inside the nerve cells (Jasper and Koyama 1969, Jasper, Khan and Elliott 1975).

Experiments performed with radioactively labelled GABA showed that half the radioactivity taken up by cortical brain slices was concentrated in the nerve endings which also contained most of the GAD activity (Neal and Iversen 1969). When extracts of these were tested directly on cortical cells they produced a significant inhibition (Krnjević and Whittaker 1965). Immuno-histochemical visualisation of GAD has been used as an indication of the synaptic sites of GABA synthesis (Wood et al 1976).

1.2.3 Release

The transmitter must be released when there is physiological stimulation, this release must be quantal and calcium dependent.

In view of the technical difficulties involved in demonstrating *in vivo* the synaptic release of any transmitter

due to the small amounts released, local calcium ion concentrations and efficient uptake mechanisms, few studies have been performed. However, calcium ion induced release of endogenous GABA has been demonstrated *in vivo* into the fourth ventricle from the surface of the cerebellum and cerebral cortex of the cat (Obata and Takeda 1969, Obata 1976).

Studies on the release of GABA *in vitro* using cortical slices or synaptosomes by electrical or chemical depolarising stimuli have been shown to be calcium dependent (Srinivasen, Neal and Mitchell 1969, De Belleroche and Bradford 1972). This release is difficult to relate to that occuring *in vivo*, except when a degeneration of a pathway does or does not influence the release *in vitro* from slices prepared from the appropriate region of the CNS (Nadler et al 1977).

1.2.4 Physiological action

The physiological action of the compound must be the same when it is applied externally.

The proposed action of GABA is that it is a major inhibitory neurotransmitter in the CNS, this potent depressant effect on central neurones has been demonstrated (Curtis and Watkins 1960). It is therefore necessary to show a direct correlation between the endogenous inhibitory processes and those due to the application of GABA.

The inhibitory action of GABA was shown on cortical neurones (Randić and Straughan 1966, Krnjević and Phillis 1963).

GABA was shown to cause a marked increase in membrane

conductance with a hyperpolarising action with the evoked inhibitory postsynaptic potentials and the effects of GABA being reversed with the depolarisation caused by the injection of chloride ions into the neurones. GABA itself was shown to have no effect when injected intracellularly, indicating that it probably acts on receptors on the surface of the cells.

Most neurones in the CNS are sensitive to the inhibitory effects of GABA (Curtis, Phillis and Watkins 1959, Krnjevic 1970). The mechanism of this action is a pronounced increase in chloride ion permeability (Ten Bruggencate and Engeberg 1971, Curtis et al 1968).

Under <u>in vitro</u> conditions GABA binds in a sodium-independent fashion to synaptic membranes of mammalian brain at sites which can be distinguished with substrate specificity from those associated with the sodium-dependent membrane transport of GABA and related amino acids (De Feudis 1977, Olsen et al. 1978).

Therefore since GABA is found in all parts of the CNS it is likely to be one of the major inhibitory neurotransmitters (Krnjević 1974).

1.2.5. <u>Uptake and Inactivation</u>

Some mechanism or mechanisms must exist in order to inactivate the action of the compound and or remove it from the nerve synapse.

Uptake of GABA was shown in cerebral cortex slices (Elliott and Van Gelder 1958) which could remove GABA rapidly from the medium in which they were soaked. This GABA was held in occluded form <u>and</u> was capable of being released by stimulation.

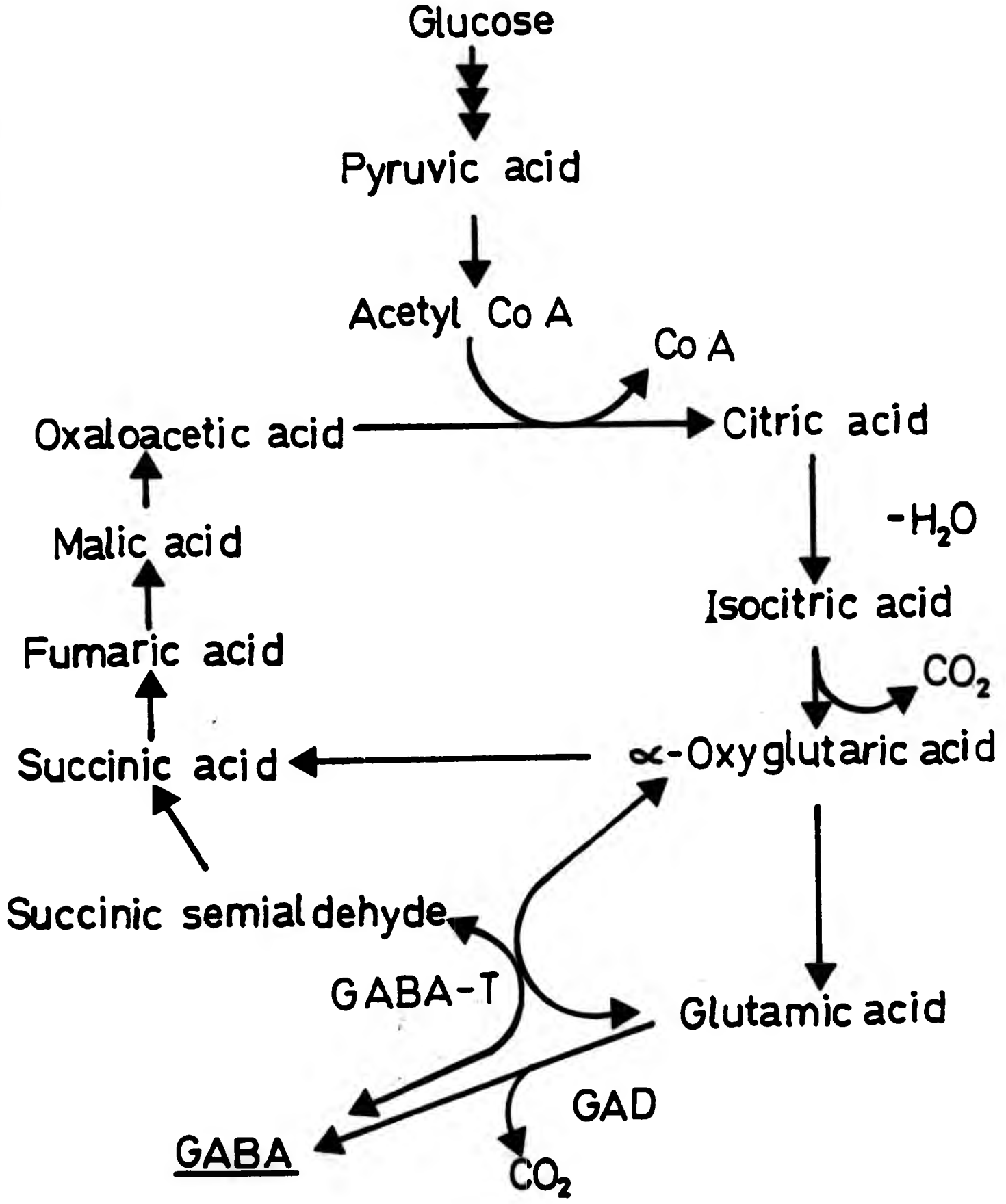
This showed GABA removal was by active uptake into the surrounding cells rather than by metabolic conversion. This was by diffusion and a carrier-mediated cellular uptake into neurones, terminals and glial cells (Martin 1976).

Both low and high-affinity transport mechanisms have been described: the former may be more significant when subsynaptic concentrations of GABA are relatively high immediately after synaptic release, whereas the latter is more important for maintaining very low extracellular concentrations of GABA.

The high-affinity uptake into the cells was shown to be an active process which was sodium dependent (Varon, Weinstein, Baxter and Roberts 1965, Bennett, Logan and Snyder 1972) and did not involve the metabolic conversion of GABA into an inactive substance extracellularly. Uptake into glial cells was shown to be a very important factor in GABA uptake (Henn and Hamberger 1971, Shriefer and Thompson 1974).

The primary degredative pathway of GABA metabolism is through transamination catalysed by a specific enzyme, 4-amino-butyrate : 2-oxyglutarate aminotransferase (EC 2.6.1.19.) commonly known as GABA-T (Roberts and Bregoff 1953). The products of this reaction are succinic semialdehyde and glutamate, these are then rapidly converted to succinate by a reaction catalysed by the enzyme SSA-NAD^+-oxidoreductase (EC 1.2.1.16), commonly known as succinic semialdehyde dehydrogenase (SSA-D) (Albers and Salvador 1958). This pathway is dependent on the availability of

Fig. 1.3. The synthesis and degredation of GABA in relation to the Krebs cycle



tricarboxylic cycle (Kreb's cycle) intermediate α-oxyglutarate. The succinate is returned to the tricarboxylic cycle (Fig.1.3) As with GAD, GABA-T and SSA-D are found in most parts of the brain in conjunction with the presence of GABA (Salvador and Albers 1959).

1.2.6. Antagonism

Pharmacological agents which antagonise the action of the transmitter should have the same effect when the compound is applied directly.

There are several limitations involved with the evaluation of the pharmacological effects of systemically administered antagonists. These are: metabolism of the drug, the blood-brain barrier, selectivity for the site and peripheral effects. Also aqueous solubility is an important factor involved with the study of potential GABA antagonists.

(+)-Bicuculline (Fig.2.4) and picrotoxinin (Fig.2.2) block both the hyperpolarising effect of GABA on neurones and the depolarisation of primary afferent terminals (Curtis and Johnston 1974, Curtis 1978).

Local administration of picrotoxin, a mixture of picrotin and picrotoxinin by electro-osmosis was found to antagonise GABA inhibition of rabbit occulomotor neurones (Obata and Highstein 1970). Picrotoxinin has also been shown to interfere with GABA-activated ionophores (Olsen 1976).

Binding studies suggest that (+)-bicuculline may be a competitive antagonist at the GABA receptors (Zukin, Young and Snyder 1974, Möhler and Okada 1977).

Numerous compounds with a caged structure (Fig. 2.1) such as tetramethylamine disulphotetramine (TETS), silatranes, bicyclophosphate esters ($R-(CH_2O)_3\ P=O$), and bicyclocarboxylate ortho esters ($R-(CH_2O)_3-C-R'$), cause tonic-clonic convulsions and death in higher animals in a manner similar to picrotoxinin and (+)-bicuculline (Casida et al. 1976, Cooper et al. 1978). These substances also inhibit GABA postsynaptic responses (Bowery et al. 1976, Bowery et al. 1977) and have other actions resembling GABA antagonists, such as the elevation of cerebellar cyclic GMP (Mattson et al. 1976). The actions of the cage convulsants can also be reversed by barbiturates (Bowery and Dray 1976, Casida et al. 1976).

The cage convulsants, especially the bicyclophosphate esters will be dealt with and discussed in more detail later.

1.3 Mechanism of GABA Action

1.3.1 Postsynaptic inhibition

The initial effect of an excitatory transmitter is a sub-threshold change in the potential of the cell membrane brought about by a change in the sodium ion permeability, this is an excitatory postsynaptic potential (EPSP). These EPSP's summate to initiate the action potential which is self generating (McLennan 1970).

In the case of inhibitory transmission a transient hyperpolarisation of the membrane is caused, this is the inhibitory postsynaptic potential (IPSP). These IPSP's counteract the EPSP's by lowering the excitability of the membrane and so reducing the change of an action potential. This hyperpolarisation

is brought about by an increase in the membrane permeability causing an inward flux of chloride ions (Obata, Takeda and Shinozaki 1970, Krnjević and Phillis 1963, Ten Bruggencate and Engeberg 1971, Obata, Ito, Ochi and Sato,1967).

1.3.2 Presynaptic inhibition

GABA can act on presynaptic fibres to cause a decrease in the output of excitatory transmitter. This action can be antagonised in a similar fashion in the spinal cord to those actions in postsynaptic inhibition (Curtis et al. 1971, Davidoff 1972).

GABA appears to act presynaptically in the spinal cord and postsynaptically in the brain (Fahn 1976, Otsuka and Konishi 1976).

1.3.3 Excitatory effects of GABA.

On peripheral ganglion cells and their axons GABA has been shown to cause depolarisation.

This has been demonstrated *in vitro* on the rat superior cervical ganglion both intracellularly (Adams and Brown 1973) and extracellularly (Bowery and Brown 1976). This will be described in more detail later.

In vivo observations have also been made on several ganglia in the cat and rabbit, recording potential changes, as a depolarisation, while applying GABA intravenously or intra-arterially (de Groat 1970, de Groat, Lalley and Block 1971, de Groat 1972, de Groat, Lalley and Saum 1972). In both the *in vivo* and the *in vitro* experiments the results of the agonists and antagonist paralleled those found in the CNS.

The probable ionic mechanism of this depolarisation is an increase in the chloride ion permeability of the membrane (Obata 1976). This increase is in the opposite direction to the change brought about in the CNS. Therefore the action of GABA in both the peripheral and central nervous systems is to change the chloride ion permeability of the cell membranes.

1.4. GABA action in invertebrates

GABA has been shown to be a potent inhibitory transmitter in the neuromuscular junctions of some invertebrates. The crayfish stretch receptor neurones were inhibited by GABA causing an increase in chloride ion permeability of the membrane (Tackeuchi and Tackeuchi 1965, Tackeuchi 1976). It was also shown that GABA decreases membrane resistance of crayfish synapses in the ventral cord of the crayfish (Furshpan and Potter 1959).

Particulate fractions of homogenised crustacean muscle were found to bind radioactive GABA and the binding sites having properties consistent with those expected of synaptic receptor sites (Olsen, Lee and Ban 1975).

Iontophoretic methods also showed that GABA stimulated the chloride ion permeability of the membrane in the crayfish muscle (Ticku and Olsen 1977). This would appear to be the same mechanism in mammalian CNS, by increasing the chloride ion permeability of the membrane.

1.5. Regional variation of GABA concentrations in the CNS

1.5.1 Cerebral cortex

Inhibition by GABA in the cerebral cortex was shown

as the stellate cell inhibition of the pyramidal cells (Dreifuss, Kelly and Krnjevic 1979).

Using immunocytochemical techniques to label GAD inhibition by GABA was observed in somata, proximal dendrites and axon terminals of nonpyramidal neurones in the rat visual cortex including the immediate subjacent white matter (Ribak 1978). Terminals formed symmetric synaptic junctions with dendritic shafts and somata of pyramidal and stellate neurones, (Roberts 1978).

1.5.2 Cerebellum

Basket cells lying close to the Purkinje cell layer can inhibit them by clustering their terminals around the axons of the Purkinje cells (Otsuka et al. 1971).

There is also inhibition of granule cells in the Golgi cell regions and it would appear that only granule cells cause inhibition non-GABAergically.

1.5.3 Hippocampus

Pericellular baskets around pyramidal and granule cell somata were shown immunohistochemically to have GAD present and hence these were assumed to be inhibitory with GABAergic terminals (Ribak et al. 1978). There is again GABAergic inhibition of pyramidal cells by basket cells (Ben-Ari, Kanazawa and Sigmond 1976).

1.5.4 Basal Ganglia

Some of the highest levels of GABA are found in the globus pallidus and substantia nigra, therefore GABA pathways are probably associated with extrapyramidal function (Feltz 1971).

Immunohistochemical techniques confirmed the abundance of GAD in both axodendritic and axosomatic synapses in the substantia nigra (Roberts (1978). In the globus pallidus the medium sized neurones appeared also to be GABAergic (Ribak 1978).

1.5.5 Olfactory bulb

The greatest concentration of GABAergic neurones was in the external plexiform layer in the glomeruli of the glomerula layer (Ribak et al. 1977). The granule and periglomerular cells inhibited the mitral cells (Graham 1973).

1.5.6 Retina

There is evidence that GABA is a transmitter in the retina (Neal 1976). GAD-positive terminals were also postsynaptic to bipolar cells and sometimes observed to form - reciprocal synapses with bipolar terminals (Roberts 1978).

1.5.7 Spinal cord

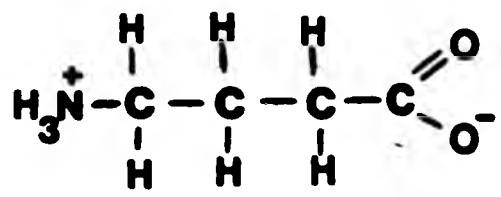
GABAergic synaptic terminals were presynaptic to large and small dendrites and motoneuron somata in the motor nuclei. In addition small GAD terminals were presynaptic to larger axonal terminals which were in turn presynaptic to motoneuron somata. Immunohistochemical techniques to localise the GAD also showed that it was present in terminals presynaptic to dendrites and cell bodies in both dorsal and ventral horns, this evidence is compatible with evidence described earlier that GABA mediates postsynaptic inhibition of spinal interneurones and motorneurones (Roberts 1978).

1.5.8 Other areas

GABA has been found in abundance in all areas of grey matter and is therefore probably the major inhibitory neurotransmitter in the mammalian neuroaxis.

1.6 Stereochemistry of GABA

Fig. 2.1

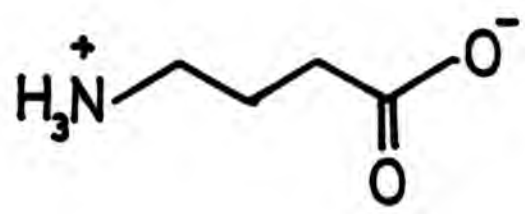
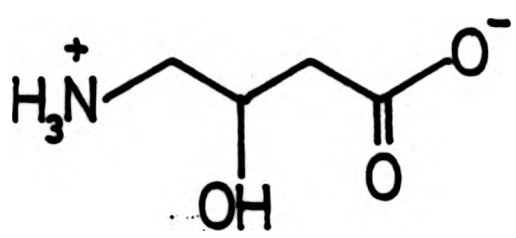
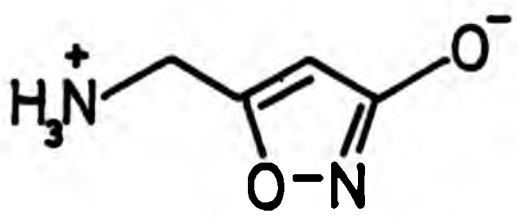
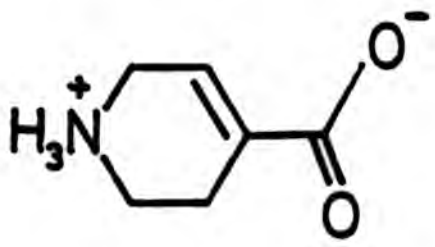
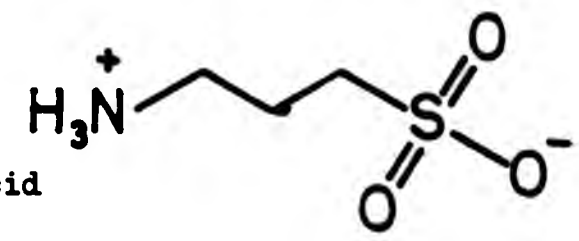


GABA

The above figure shows γ-aminobutyric acid to be a simple achiral molecule which exists as a zwitterion in neutral conditions. The molecule allows free rotation about all the bonds and has three prochiral centres at carbon atoms 2, 3 and 4. These factors can contribute to the shape of the molecule and it would probably be in one specific conformation when it comes into contact with the recognition site on the receptor. Sufficient evidence suggests that a GABA-ionophore complex exists on the surface of excitable membranes and when GABA attaches itself to these sites anion channels are opened. There may be several steps in this process involved with the increase in chloride ion conductance (Enna and Snyder 1975, De Feudis 1977).

The receptor also shows no affinity towards some antagonist suggesting that they do not all react with the receptor site itself but at some place away from the GABA recognition site at the ionophore complex.

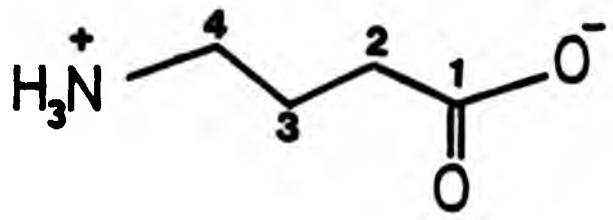
Fig. 1.4. Relative potencies of some amino acids as depolarising agents on rat superior cervical ganglion and as displacing agents of (^{3}H)-GABA binding in CNS tissue.

Compound	Structure	IC_{50} (μM) Binding	Relative Potency Cervical Ganglia
GABA		0.34*	1.0†
γ-amino-β-hydroxy-butyric acid			
muscimol			5.1†
isoguvacine		1.4*	0.23†
3-amino-propane sulphonic acid		**	3.4 X

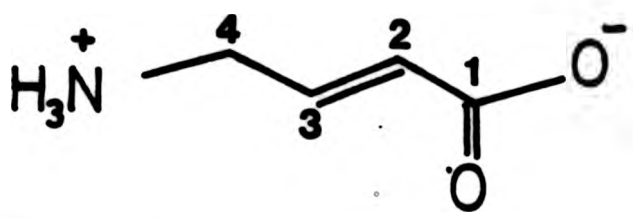
* Krogsgaard-Larsen (1978)
† Bowery et al (1975)
X Bowery and Brown (1974)
†† Olsen et al (1979)
** Enna and Snyder (1977)

Fig. 1.5 Semi-rigid GABA agonists

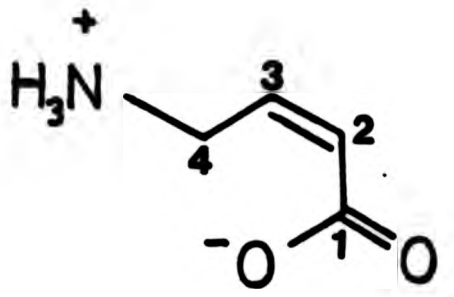
GABA



trans-4-aminocrotonic acid

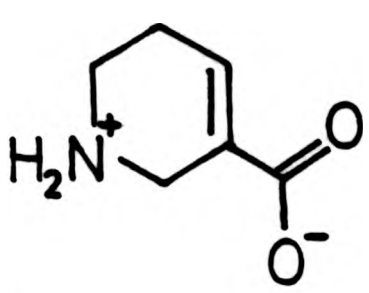


cis-4-aminocrotonic acid

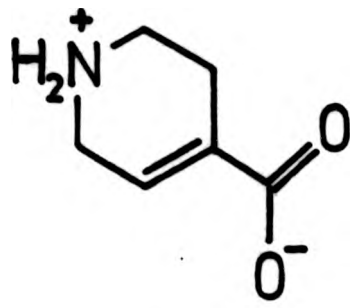


GABA, cis, trans-aminocrotonic acid all reduce the firing rate of spiral interneurones but only GABA and the transisomer are antagonised by bicuculline methochloride.

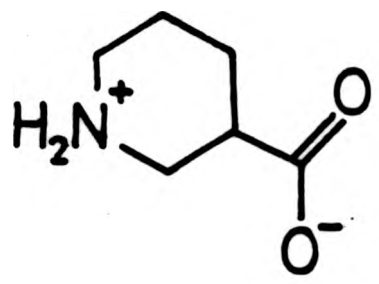
Fig. 1.6. Some structurally-related inhibitors of GABA uptake



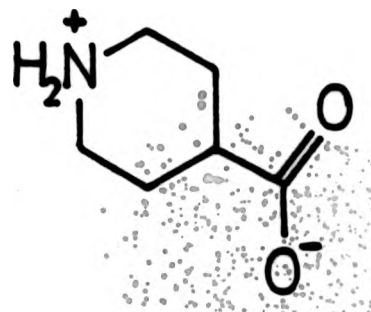
Guvacine



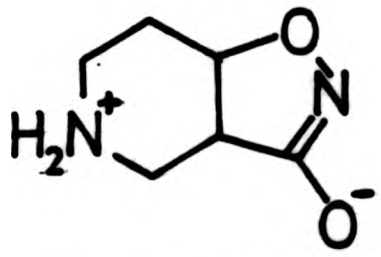
Isoguvacine



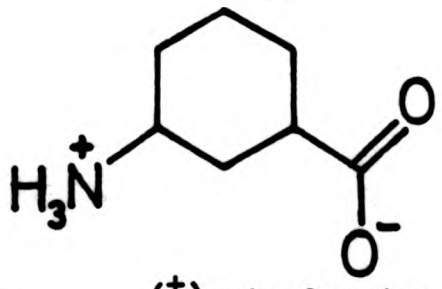
(±)-Nipecotic acid



(±)-Isonipecotic acid



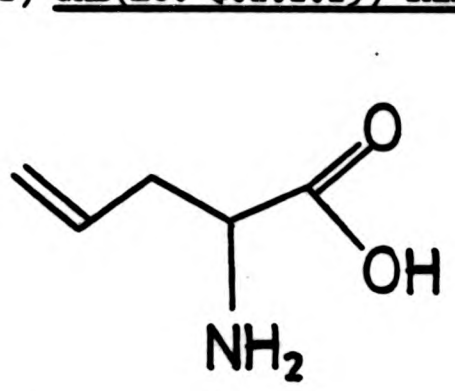
4,5,6,7,-tetrahydro-isoxazolo (4,5-c)-piridin-3-ol (THIP)



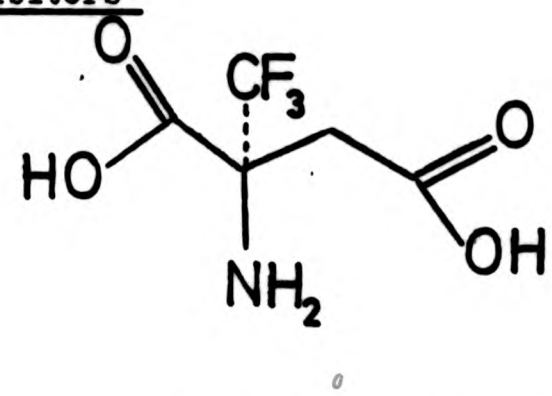
(±)-cis-3-aminocyclohexane carboxylic acid (ACHC)

Fig. 1.7. Compound structures which impair GABA synthesis.

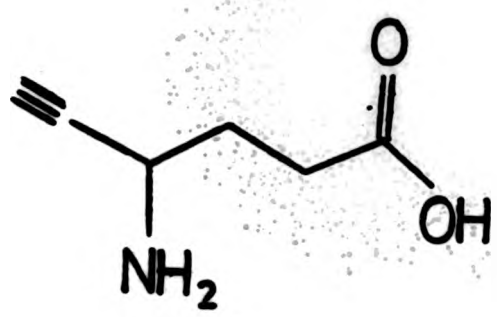
(i) <u>GAD(EC. 4.1.1.15) inhibitors</u>



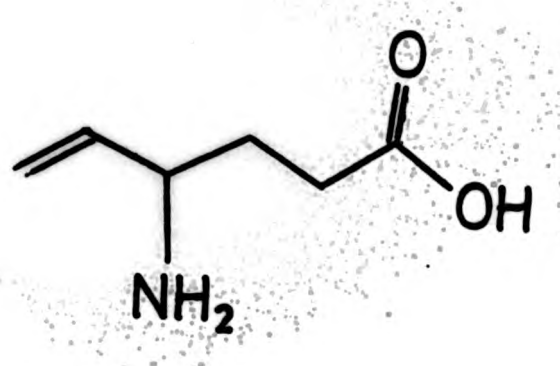
DL-ALLYLCLYCINE



2-TRIFLUORMETHYL GLUTAMIC ACID

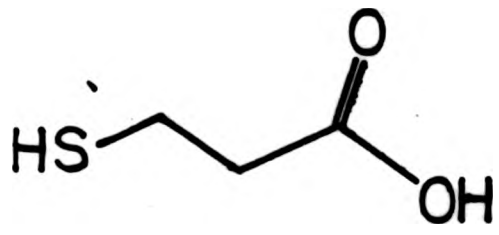


Y-ACETYLENIC GABA



Y-VINYL GABA

(ii) <u>Substrate competitor</u>



3-MERCAPTOPROPIONIC ACID

1.7 Drugs which affect the actions of GABA

1.7.1 GABA Mimetics

The GABA molecule has considerable flexibility as a result of free rotation around the single bonds as shown previously. This conformational mobility can be reduced by analogues of GABA by the incorporation of unsaturation, ring structures or both into the basic transmitter molecule. Such restrictions can result in compounds having more selective actions than that of the transmitter itself, or having little or no action on GABA processes.

It is also possible to investigate the effect of non-rigid, straight chain analogues of GABA but often these molecules with shorter or longer chains were only very weakly active or not active at all - on the depolarisation action on the isolated rat superior cervical ganglion (Bowery and Brown, 1974). Only 3-aminopropane sulphonic acid and β-hydroxy GABA (Fig.1.4) were significantly active. 3-Aminopropane sulphonic acid is approximately three times as active as GABA as it is not taken up by the neuronal transport system, β-hydroxy GABA is taken up by the neuronal transport system and has approximately four times less the potency of GABA.

Trans-, and cis-4-aminocrotonic acid (Fig. 1.5) are both double bond analogues of GABA and depress the firing rate of spinal interneurones induced by GABA, but only the action of the trans-isomer is reduced by bicuculline methochloride (Johnston et al. 1975, Johnston 1976). Cis-4-aminocrotonic acid is an

analogue of GABA in a folded conformation. Various analogues of trans-4-aminocrotonic acid have been synthesised and tested with some success in the selectivity on GABA binding and uptake (Allan and Twichin 1978). These findings supported the concept that extended conformations of GABA were important with respect to the bicuculline sensitive postsynaptic receptors and high-affinity uptake and the proposal that folded conformations of GABA may interact with bicuculline-insensitive receptors (Johnston 1976, Johnston 1978).

Among cyclic amino carboxylic acid the structure of isoguvacine (Fig. 1.4) seems optimal for the activation of GABA receptors (Krogsgaard-Larsen et al. 1977). Experiments for potencies of isoguvacine on the GABA receptor using iontophoretic techniques show that it was 2-4 times more potent an agonist than GABA itself (Curtis et al. 1971b). But these results differ from those obtained using the isolated superior cervical ganglion of the rat which showed it to have a potency of 0.23 times that of GABA (Bowery et al. 1978).

However guvacine (Fig.1.6) is inactive at the GABA receptor but is a potent inhibitor of neuronal uptake (Curtis et al. 1971). The conformations adopted by GABA are folded for neuronal uptake and extended for the GABA receptors.

Cis-3-amino-cyclohexanecarboxylic acid (ACHC) (Fig.1.6) appears to be a selective inhibitor of the uptake of GABA in the nerve terminal but has very little activity on the uptake in glial cells, but little affinity for GABA transaminase (Neal and

Bowery 1977, Bowery et al. 1976b). ACHC has a flexible cyclohexane ring and can therefore exist in numerous conformations, the extremes being where the substituents are diaxial and diequilateral (the two different chair forms of the same ring). The diaxial conformation involves maximum interaction between oppositely charged groups and would therefore hold the molecule in this conformation. In aqueous solution however this does not happen (Hewgill and Jeffries 1955). The diaxial conformation being equivalent to folded GABA and the diequilateral conformation being equivalent to extended GABA. Since it does not act at the GABA receptor as a specific agonist (Bowery et al 1976b) it is assumed that the flexibility of the ring does not permit interaction at the GABA receptor site.

Another relatively potent blocker of GABA uptake is nipecotic acid (piperidine-3-carboxylic acid Fig.1.6)(Johnston et al. 1976). In aqueous solution and in the solid state, nipecotic acid exists in the chair formation with the carboxyl group in an equatorial position. This conformation corresponds to GABA in its folded form (Krogsgaard-Larsen and Johnston 1975).

Muscimol (3-hydroxy-5-aminomethylisoxazole) (Fig.1.4) is a cyclic, conformationally restricted analogue of GABA (Brehm et al. 1972). It is a very potent and selective GABA receptor agonist (Johnston et al. 1968, Curtis et al. 1971a) being also a weak inhibitor of uptake into rat brain slices (Johnston 1971) and cultured astrocyte cells (Schousboe et al. 1978). The

3-isoazolol moiety acts as a masked carboxyl group and restricts conformation (Johnston et al. 1968) and there is relatively free rotation of the aliphatic side chain of muscimol. Muscimol is not a substrate for or an inhibitor of GABA-transaminase, (Johnston et al. 1978). The properties and actions of muscimol will be dealt with in more detail later.

β-(p-chloro)phenyl-GABA (Baclofen, Lioresal) has been reported to have an inhibitory action on the nervous system related to GABA agonism, with GABA-like actions on cat motorneurones (Puil et al. 1976), weak GABA-like activity on the crayfish muscle, it inhibits specific GABA binding to mouse brain receptor sites and shows a weak inhibition of GABA uptake (Olsen et al. 1978c). However other studies show that it does not involve GABA receptors (Curtis et al. 1974c, Naik et al. 1976).

The bicyclic isoxazole, THIP (4,5,6,7-tetrahydro-isoxazole (5,5-c) pyridin-3-ol) an almost rigid muscimol analogue (Fig.1.6) is a less toxic compound than muscimol in vivo in mice and is not susceptible to metabolism (Krogsgaard-Larsen et al. 1978). THIP can penetrate the brain easily after systemic injection and in general the effects of THIP are slightly weaker than, but qualitatively similar to, those observed with muscimol (Scheel-Krüger et al. 1978).

Other methods of affecting GABA actions are possibly by stimulation of the calcium ion induced release of GABA. Although benzodiazepines (e.g. diazepam) stimulate GABA action by inhibiting GABA transport into nerve and glial cells and by

stimulation of release of GABA from cells but this action is not by a calcium ion dependent secretory mechanism but by reversal of the GABA pump (Olsen and Ticku 1977).

Also it would be possible to influence GABA release by stimulation of GABA synthesis or precursors but precursor availability is apparently not critical to GAD activity and there are no examples of these types (Tapia 1975).

1.7.2 Summary of the structure-activity requirements of GABA agonists.

The compound equivalent to GABA in a fully extended conformation tend to be agonists at the GABA recognition site whereas the compounds which are in a folded conformation tend to be blockers of the neuronal and glial uptake of GABA. This suggests that GABA acts at its receptor in the extended conformation.

Binding studies with (^{3}H)-GABA in the presence of a number of substituted GABA analogues would appear to confirm this theory (Iversen and Suckling 1979).

A chiral aspect of the interaction cannot be excluded. Muscimol (Fig.1.4)and 4-aminocrotonic acid (Fig. 1.5) are both very potent agonists and both possess the hydrogen atoms of the prochiral assembly at carbon-4 may be involved. Also in both these compounds the carbon-4 atom has complete free rotation.

Isoguvacine (Fig.1.6)and isonipecotic acid (Fig.1.6) also possess the two hydrogen atoms at the appropriate carbon

atom but since it is part of a ring this carbon atom does not have the same degree of freedom of movement, making these compounds weaker agonists. The ring system of the muscimol however does not appear to interfere with the interaction of the molecule with the GABA-recognition site.

1.7.3 GABA "Inhibitors"

Drugs which act on GABA-mediated inhibition in the mammalian CNS cause convulsions due to a subsequent decrease in GABA levels. The ways in which this inhibition is produced can be categorised into three major different groups:

a) Drugs which impair the synthesis of GABA.

b) Drugs which block synaptic GABA release.

c) Drugs which impair the neurological inhibitory actions of GABA.

1) Drugs which impair the synthesis of GABA.

i. GAD (EC 4.1.1.15) inhibitors

DL-allylglycine (2-amino pent-4-enoic acid) (Fig.1.7)produces irreversible inhibition of cerebral GAD in vivo (Horton and Meldrum 1973). In vitro, both D- and L-allylglycine are only weakly active as GAD inhibitors however (Orlowski et al. 1977).

2-Trifluomethylglutamic acid (Fig.1.7)also inhibits GAD in chick embryo brain in a specific way to cause a decrease in endogenous GABA levels (Rando 1978).

Y-Vinyl GABA (4-aminohex-5-enoic acid) (Fig. 1.7) inhibits GAD *in vitro* irreversibly (Metcalf et al. 1978) and that Y-acetylenic acid (4-aminohex-5-ynoic acid (Fig. 1.7) also causes an irreversible inactivation of GABA *in vitro* (Jung et al. 1978).

ii. Substrate competition

The administration of 3-mercaptopropionic acid (Fig.1.7) to rats causes convulsions preceded by a decrease in GABA levels and GAD activity (Karlsson et al. 1974). This is due to enzymatic conversion to a Keto compound and competition with glutamate. When administered systemically seizures in rodents and baboons are also observed (Horton and Meldrum 1973).

iii. Interference with synthesis or coenzymic function of pyridoxal phosphate

Antivitamin B_6 agents such as pyridoxines (e.g. methoxypyridoxine)and other carbonyl trapping agents decrease GABA levels in the brain and administration of these compounds causes generalised seizures in animals (Killam et al. 1960, Purpura et al. 1960). The cause of the seizures is thought to be due to these compounds being GAD substrate competitors (Meldrum 1975).

2. Drugs which affect the synaptic release of GABA

Tetanus toxin acts presynaptically to reduce the physiologically-induced release of GABA (Curtis et al. 1973, Osborne and Bradford 1973, Davies and Tongroach 1977). Focal

injection of tetanus toxin into the ventral hippocampus induces generalised seizures in the rat comparable to those seen after systemic administration of larger quantities of the toxin (Mellanby et al. 1977).

3. Drugs which antagonise the neuronal inhibitory effects of GABA

The actions of GABA can also be antagonised post-synaptically by several different types of compounds. The modes of action of these compounds will be discussed in greater detail in the next chapter.

i. The phthalide isoquinoline alkaloids

These alkaloids occur naturally in plants of the botanical families Papaveraceae and Fumariaceae. For example (+) -bicuculline is found in Corydalis scoulerii and Aldumia fungosa (Manske 1933).

(+)-Bicuculline (Fig.2.4)was shown to have a potent convulsant activity in both vertebrates (Welch and Henderson 1934a) and invertebrates (Olsen 1976). It also inhibits acetylcholinesterase (Svennby and Roberts 1973) and potentiates acetylcholine action (Miller and McLennan 1974).

The demonstration of its antagonism by GABA was first observed by Curtis et al. (1971). (+)-Bicuculline is only sparingly soluble in water and is hydrolysed to a less active derivative, hydroxy acid bicucine (Fig.2,5) (Welch and Henderson 1934b). Quaternary methohalide salts, however, are more aqueous soluble and are also potent selective GABA antagonists (Johnson et al. 1972).

Another active phthalidisoquinaline alkaloid which was active when tested on feline spinal interneurones was corlumine hydrochloride (Fig. 2.1). This has the same configuration is bicuculline and its quaternary methohalide salts, (1S, 9R) about the $C_1 - C_9$ bond. (Fig. 2.4) (Albers and O'Brady 1959, Johnston 1976). In addition bicucine methyl ester (Fig. 2.4) was also active as a GABA antagonist (Jasper and Koyama 1969, Johnston 1976).

ii. Picrotoxin

Picrotoxin is a drug obtained from the seeds of the plants Anamirta paniculata and Anamarita cocculus and consists of a molar ratio of 1:1 of two compounds, picrotin and picrotoxinin. Picrotoxinin (Fig. 2.2) is the active constituent and is about fifty times more potent a convulsant than picrotin (Jarboe et al. 1968). (Fig. 2.2).

Picrotoxinin is not very soluble in water but techniques using electro-osmosis showed that it successfully blocked vestibular inhibition and GABA action of rabbit oculomotor neurones (Obata and Highstein 1970) and the blockade of the actions of GABA and not glycine was also observed in the feline cuneate nucleus (Galindo 1969). In crustacea, picrotoxin also blocks the actions of GABA (Florey 1957).

α-Dihydropicrotoxinin (Fig.2.3), a reduced form of picrotoxinin, is slightly less active as a convulsant than picrotoxinin (Jarboe et al. 1968), but it it possible to label this with (^3H) relatively easily by using tritiated hydrogen to

reduce unlabelled picrotoxinin to form (^{3}H)-α-dihydro-picrotoxinin which could then be used in binding studies to elucidate more about the modes of actions of this drug. This will be discussed fully later.

Other analogues of picrotoxin have been examined for their ability to block the actions of GABA but despite the fact that tutin (Fig.2.2) and shikimin are more aqueous soluble their actions appear to be less specific on GABAergic systems (Curtis et al. 1973b).

iii. Caged convulsants

Several compounds with a caged structure (Fig.2.1), such as bicyclophosphate esters (R-$(CH_2O)_3$ P = O), bicyclocarboxylate ortho esters (R-$(CH_2O)_3$ C - R'), silatranes (N-$(CH_2O)_3$ Si-R), and bicyclophosphite esters (R-$(CH_2O)_3$ -P), tetramethylene disulphotetramine, cause tonic-clonic convulsions and death in higher animals in a manner similar to picrotoxinin and bicuculline (Casida et al. 1976). These substances have been shown to inhibit GABA postsynaptic responses (Bowery et al. 1977) and have other actions resembling GABA antagonists, such as elevation of cyclic GMP in cerebellum (Mattson et al. 1976). These actions are also reversed by barbiturates (Bowery and Dray 1976, Casida et al. 1976).

a) Bicyclic phosphate esters

4-Alkyl-2,6,7 trioxa-1-phosphabicyclo (2,2,2) octan-1-ones were shown to be highly toxic in mice and produced tonic-clonic seizures and consequent death (Bellet and Casida 1973).

They do not act as cholinesterase inhibitors or cause excessive parasympathetic stimulation (Bellet and Casida 1973, Kimmerle et al. 1976). They increase cyclic guanosine 3'5'-phosphate levels in rat cerebellum due to a primary action on the inhibitory GABA mechanisms with no effect on cyclic adenosine monophosphate. (Mattson et al. 1977).

The 4-ethyl homologue is the toxic principle in the smoke produced by burning a fire retardent polyurethane foam based on a trimethylopropane initiated short chain polyol and a phosphate flame retardant (Petajan et al. 1975).

The convulsions were found to be due to antagonism of the actions of synaptically released GABA (Bowery et al. 1976a, Bowery et al. 1977) by examination of the superior cervical ganglion of the rat.

With decerebrated frogs, the injection of a bicyclic phosphate ester directly into the brain cavity produced seizures which could be abrogated by pithing the remainder of the brain (Casida et al. 1976). Cockroaches injected with LD_{50} doses showed an increase in spike frequency of the abdominal nerve cord and the crayfish isolated abdominal nerve cord showed an increase in spike activity although high doses were needed and this preparation was relatively insensitive to these compounds.

The potency of the bicyclic phosphate esters requires a symmetrical cage, high electron density at the 1-position and a suitable hydrophobic 4-substituent (Casida et al. 1976,

Cooper et al. 1978). The most important feature being a suitable hydrophobic 4-substituent (Eto et al. 1976). The mechanism of action and role of these substituents in the bicyclic phosphate esters will be discussed in more detail later.

b) Bicyclic phosphite esters

4-Alkyl 2,6,7-trioxa-1-phosphabicyclo (2,2,2) ocanes ($P-(OCH_2)_3-C-R$) (Fig.2.1)were shown to have essentially the same potencies for the corresponding 4-alkyl substituent as the bicyclic phosphate esters when tested on LD_{50} intraperitoneal injection. In mice, however, they were less stable in aqueous solution (Wadsworth and Emmons 1962) and so the bicyclic phosphate esters have been concentrated on as the bicyclic phosphite esters probably have exactly the same pharmacological properties (Cooper et al. 1978).

c) Bicyclocarboxylate ortho esters

1-substituted-4-substituted-2,6,7- trioxabicyclo (2,2,2) octanes of the type ($R-C(OCH_2)_3-C-R'$) where the phosphorous atom is replaced by carbon in the bicyclic system always yields compounds of greatly reduced toxicity. However, if the optimal substituents are added (i.e. R = H, R' = $t-C_4H_9$) a relatively high toxicity is found showing that the overall configuration in the cage is most important to the toxic and GABA antagonistic properties (Casida et al. 1976). Toxicity was greatly reduced with large alkyl or aromatic substituents on either R' or R ends of the molecule (Milbraith et al. 1979).

d) Silatranes

The 1-(aryl or alkyl)-2,8,9-trioxa-5-aza-1-silabicyclo (3,3,3) undecanes. or silatranes (Fig.2.1)have been shown to have toxic effects producing tonic-clonic convulsions similar to these produced by bicuculline and picrotoxinin when injected intraperitoneally in mice but had little effect on frogs (Voronkov 1966).

In sublethal doses they act as a powerful analeptic and do not cause convulsions in animals with damaged central nervous systems, they also have no effect on the inhibition of acetylcholinesterase and cholinesterase (Voronkov and Lukevičs 1969, Voronkov et al. 1977).

Silatranes elevate the cyclic guanosine 3'5'- phosphate levels in rat cerebellum (cf. other GABA inhibitors) (Milbraith et al. 1979). However the phenobarbital-induced sleeping time in rats is prolonged unlike the other GABA inhibitors (Mattsson et al. 1977b).

The commercially available rodent poison p-chlorophenyl silatrane (1-(—chlorophenyl) -2,8,9-trioxa-5-aza-1-silabicyclo (3,3,3) undecane) Produced by M and T Chemicals Inc. Rothway, N.J.) is equipotent with similar symptoms to those of the other GABA inhibitors after intraperitoneal injection in mice but was shown to be inactive on the isolated mouse diaphragm preparation on which it failed to cause the rapid twitching at low doses noted for ther other GABA inhibitors discussed previously (Casida et al. 1976).

Although the caged structure, similar charge distribution and size of the molecule are similar to the other cage convulsants the silatranes show some difference in pharmacological properties.

e) Tetramethylenedisulphotetramine (TETS)

TETS, (2,6-Dithia-1,3,5,7-tetraza-tricyclo (3.3.1.1. ^{3,7}) decane-2,2,6,6- tetraoxide) (Fig. 2.1) has been shown to antagonise the actions of GABA in the isolated rat superior cervical ganglion (Bowery et al. 1975). It antagonises the actions of GABA on the rat cuneate nucleus, midbrain reticular formation and medulla (Collins et al. 1975, Dray 1975). This is a very toxic compound in mice when injected intraperitoneally and the effects are again reversed by barbiturates (Casida et al. 1976), and when administered systemically it antagonised prolonged spinal inhibition (Curtis and Johnston 1976b).

It has been commercially available as a poison (Produced by the Fish and Wildlife Service, United States Department of the Interior, Denver, Colorado).

When tested by injection into the brain cavity of decrerebrated frogs seizures were produced in the same dose and in the same way as isopropyl bicyclic phosphate ester, these seizures were again abrogated by pithing the remainder of the brain (Casida et al. 1976). TETS antagonises the GABA-mediated conductance in the crab neuromuscular junction (Large 1975).

Therefore it would appear that there is a great similarity in the structure modes of action and effects of the other bicyclic compounds and TETS.

Seizures in baboons after a minimal convulsant dose of TETS has an effect the same as picrotoxinin whereas larger doses of TETS produce generalised seizures resembling the effects of bicuculline (Meldrum 1978).

iv) Other compounds which inhibit the actions of GABA

a) Caprolactams

The cyclized GABA analogue, 2-pyrrolidone, is inactive as a GABA agonist (McGeer et al. 1961) in the crayfish stretch receptor neurone, but higher lactams do show convulsant activity, e.g. substituted caprolactams (e.g. 3,5,5-trimethyl n-butyolactam) and these may be associated with GABA antagonistic properties (Kerr et al. 1976), in rabbit substantia nigra by striopalladial stimulation.. Caprolactam also antagonised GABA-mediated increase in chloride ion permeability in crayfish but had little effect on displacing (^{3}H)-GABA from mouse brain synaptosomal preparations (Olsen et al. 1978).

b) d-Tubocurarine

This is a potent GABA antagonist in the feline cerebral cortex and cuneate nucleus, but it also affects the actions of glycine and is probably a less-specific GABA inhibitor (Hill et al. 1973a, Hill et al. 1973b).

c) Benzyl penicillin

Intravenous injections of benzyl penicillin antagonised GABA-mediated inhibition in the spinal cord of the frog and cat (Davidoff 1972b, Davidoff 1972c). Iontophoretic application of benzyl penicillin also blocked GABA-induced spinal depressions without affecting the glycine-induced spinal depressions (Curtis et al. 1972). The GABA-mediated increase in chloride permeability was antagonised by benzyl penicillin in crayfish muscle, it inhibits GABA binding to receptor sites in mammalian brain synaptosome preparations (Olsen et al. 1978c). However benzyl penicillin was found to show virtually no inhibition of GABA responses in crab muscle (Earl and Large 1973). A structural similarity of benzyl penicillin to GABA has been noticed (Curtis et al. 1972) and the evidence shows that it is a fairly specific GABA antagonist.

However, it has been suggested that the inhibition of GABA produced by penicillin in the rat olfactory cortex and cuneate nucleus in vitro is different to the inhibition produced by bicuculline or picrotoxinin (Pickles and Simmonds 1980). This inhibition may be due to the penicillin blocking the chloride ion channels which are GABA-mediated in this preparation (Pickles 1979). It has been shown also that penicillin decreases chloride ion conductance in crustacean muscle (Hochner et al. 1976). Other evidence shows that benzyl penicillin does block the GABA-mediated opening of the

chloride ion channels so it acts in a similar way to picrotoxinin.

1.8 The role of GABA in neurologic and psychiatric disorders

Research on GABA and its nervous function has helped with an understanding of its malfunction. Defects in the GABA system cause various neurological disorders.

1.8.1 Huntington's Chorea

Huntington's chorea (Huntingdon's disease) is a hereditary extrapyramidal disease which involves a severe reduction of GABA in the corpus striatum and substantia nigra, (Perry et al. 1973). This reduction in GABA appeared to correlate with a reduction in levels of GAD (Bird and Iversen, 1974) but post-mortem effects and the findings that GAD was also reduced in other neurologic disorders casts doubts on the significance of these findings, (Spokes and Koch, 1978, Iversen et al. 1978). In some cases binding of (^{3}H) GABA to this tissue was hardly affected (Enna et al. 1976), indicating that treatment of a patient suffering from Huntington's chorea may be successful by the administration of a GABA-mimetic which proved unsuccessful in some cases (Chase and Tamminga 1978) and in other cases there was some degree of success (Bartholini et al. 1978). These results may have been due to the inability,of the GABA agonists used, to enter the brain.

There is a possibility that two populations of Huntington's patients exist, one with low striatal (^{3}H) GABA

binding and one with normal (^3H) GABA binding (Iversen et al. 1978).

1.8.2 **Parkinson's Disease**

In Parkinson's disease, GAD is reduced in the striatum, globus pallidus and substantia nigra while (^3H) GABA binding is only reduced in the substantia nigra (Marsden 1978). This is probably due to a reduced dopamine input to the striatum and not related to cell loss as the GAD activity of patients treated with L-DOPA appears to be normal suggesting the GABAergic neurones are involved in a feedback loop which mediates the activity of nigrostriatal dopaminergic neurones (Lloyd et al. 1979).

1.8.3 **Schizophrenia**

GABAergic mechanisms associated with schizophrenic symptoms were first suggested by Roberts (1972). A loss of post-mortem GAD activity in several brain regions has been reported (Bird et al. 1977) but these results may have been due to factors before death. A reappraisal of this evidence shows that changes in GAD levels are not associated with schizophrenia (Iversen et al. 1978).

1.8.4 **Vitamin B_6** (Pyridoxine dependency)

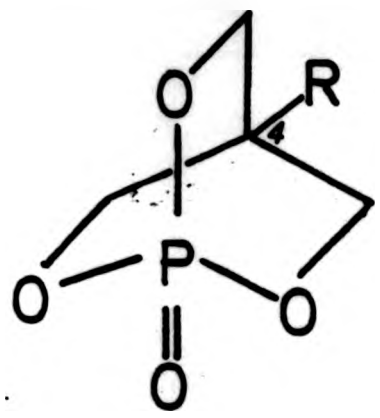
Severe dietary deficiencies of B_6 vitamins results in convulsions which could rapidly be abolished by administration of B_6 (Tower 1956). Then a dependence to B_6 was noted where removal of the vitamin caused a reappearence of the seizures. This is related to its function in the form

of pyridoxal-5-phosphate as a coenzyme for GAD. These seizures could not only therefore be abolished by B_6 but also by intravenous injections of GABA (Tower 1976).

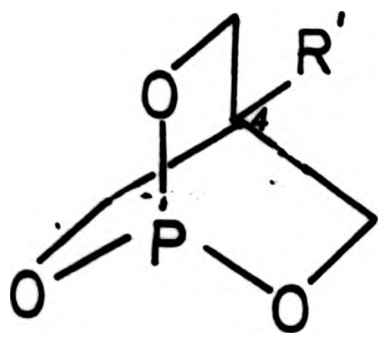
CHAPTER 2 DRUGS WHICH MODIFY THE ACTIONS OF Y-AMINOBUTYRIC ACID

Fig. 2.1. Chemical structures and the names of convulsant "cage" compounds.

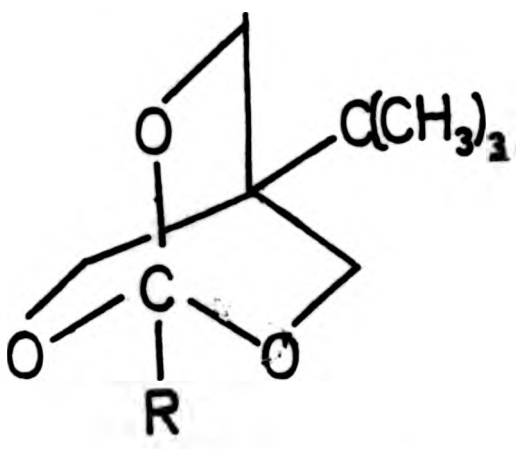
BICYCLOPHOSPHATES
(4-substituted-1-phospha-2,6,7-trioxabicyclo (2,2,2) octane-1-oxides).



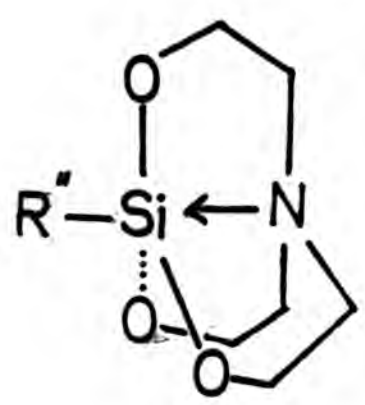
BICYCLOPHOSPHITES
(4-substituted-1-phospha-2,6,7-trioxabicyclo (2,2,2) octanes).



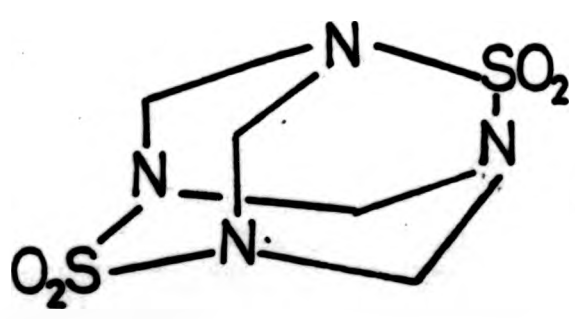
t-BUTYL BICYCLOCARBOXYALTES
(1-substituted-4-t-butyl-2,6,7-trioxabicyclo(2,2,2) octanes).



SILATRANES
(1-substituted-2,8,9,-trioxa-5-aza-1-silabicyclo (2,(3,3,3) undecane).



TETRAMETHYLENEDISULPHOTETRAMINE (TETS)
(2,6-Dithia-1,3,5,7-tetraza-tricyclo (3.3.1.1.^{3,7}) decane-2,2,6,6,-tetraoxide.



2.1 Analogues of Bicyclic Phosphorus Esters

2,6,7-Trioxa-1-phosphabicyclo(2,2,2) octane-1-oxides with suitable 4-substituents (R) (Fig.2.1) were first shown to be highly toxic in mammals causing tonic-clonic convulsions and death within minutes when injected intraperitoneally in mice (Bellet and Casida 1973) by an unknown mechanism.

The toxic signs shown were found to be similar to those produced with picrotoxinin (Casida et al. 1976) and a direct relationship with antagonism of the actions to GABA postsynaptic responses was established (Bowery et al. 1977).

In crayfish isolated abdominal nerve cord the isopropyl derivative (OP $(OCH_2)_3$ C-C_3H_7-i) caused an increase in spike frequencies but relatively large doses were needed and the preparation was fairly insensitive (Casida et al. 1976). The actions of synaptically released GABA were antagonised in the neuromuscular junction of crustaceans by bicyclic phosphates (Korenaga et al. 1977).

In insects and mites various species were found to be insensitive to the toxic effects of bicyclic phosphate esters but in the American cockroach (Periplaneta americana) death was caused after symptoms showing a state of hyperexcitability and motor inco-ordination also the isolated nerve cord preparation showed an increase in the spike frequency (Casida et al. 1976).

In the frog (Rana temporaria) the bicyclic phosphate

esters have been shown to produce seizures in the decerebrated animal which were abrogated by pithing (Casida et al. 1976). These actions on the frog spinal cord were due to potent GABA-inhibition (Bowery et al. 1977).

Subacute toxicity in mice did not affect their susceptibility to subsequent administration and there was no gross effect on major organs in the body after 30 days. Similar findings were also found in chickens and frogs (Casida et al. 1976). There were similar effects on the toxicities in mammals and birds (Kimmerle et al. 1976).

In mammalian isolated tissue preparations there was a rapid twitching caused in the isolated mouse diaphragm after exposure to the isopropyl derivative ($OP(OCH_2)_3C-C_3H_7-i$) and in the isolated superior cervical ganglion of the rat a potent inhibition of the depolarising effect of GABA was produced (Bowery et al. 1977). These actions were due to the antagonism of synaptically released GABA, in cuneate nucleus of the rat (Davidson et al. 1977) and other preparations (Bowery et al. 1976ab, Bowery and Dray 1976) and also the mammalian neuromuscular junction (Korenaga et al. 1977).

Convulsions were produced in photosensitive baboons (Papio papio) which were similar to those produced by bicuculline and picrotoxinin (Meldrum 1978).

Although some bicyclic phosphate esters of modest

activity were reported to inhibit GABA binding to rat brain synaptosomal preparations (Enna et al. 1977) this effect was not seen using high doses of several compounds in GABA binding (Olsen et al. 1978b) or in muscimol binding to rat brain synaptosomal preparations (Bowery et al. 1978b).

In experiments on rat brain synaptosomal preparations using radiolabelled (^{3}H)-α-dihydropicrotoxinin some reports have suggested a displacement which corresponds to the order of toxicity in other preparations (Olsen et al. 1978b, Ticku and Olsen 1979). However the maximum specific binding was very low (approximately 15%) of the total binding. This will be discussed in more detail later.

A similar specific binding using (^{3}H)-dihydropicrotoxinin was also shown in crayfish muscle homogenates and the percentage of specific binding was not greatly improved using a more sophisticated fractionation of the tissue (Olsen et al. 1978d).

The actions of the bicyclic phosphate esters were different from other toxic phosphorus esters which inhibit acetylcholinesterase or cause excessive parasympathetic stimulation (Bellet and Casida 1973, Petajan et al. 1975, Kimmerle et al. 1976, Coult and Wilkinson 1977) and they were shown to have no effect on muscarinic cholinergic transmission (Mattson et al. 1977a).

They had no effect on cyclic-AMP in any parts of the brain (Bellet and Casida 1973, Casida et al. 1976, Coult and

Wilkinson 1977) even in convulsive doses (Mattson et al. 1977a) and no inhibition or stimulation of cyclic-AMP phosphodiesterase in beef heart was demonstrated (Casida et al. 1976).

Cyclic-GMP levels were elevated after intraperitoneal injections of bicyclic phosphates in the rat cerebellum in subconvulsive doses but there was no elevation of cyclic-GMP levels in the cortex (Mattson et al. 1977a). These effects are similar to those agents which interfere with GABA-ergic mechanisms (Mao et al. 1975, Opmeer et al. 1976).

A similar action to bicuculline was observed with bicyclic phosphate esters in the inhibition of the GABA-activated enzyme, adenylate cyclase (EC4.6.1.1.) but they had no effect on the acetylcholine-activated enzyme, guanylate cyclase (EC4.6.1.2.) in the rat cerebellum (Coult and Howells 1979).

There was a decrease in the hexabarbitone sleeping time in mice (Casida 1976) which was attributed to barbiturate reversal of the GABA antagonism produced by these compounds (Bowery and Dray 1976).

There was no involvement with electronic reactions such as phosphorylation and alkylation in the primary actions of these compounds (Eto et al. 1976, Casida 1976). However, it is likely that they are rapidly metabolised or excreted based on their brief actions in several species of animals and lack of cumulative effects (Kimmerle et al. 1976).

Embryos in chicken eggs injected with the ethyl analogue($OP(OCH_2)_3 C-C_2H_5$) developed normally but on hatching there was a very high incidence of fusion of the leg joints (Casida et al. 1976) and the NAD content of the embryo was lowered (Proctor et al. 1976).

A change in the basic shape of the caged structure of the bicyclo compounds caused a loss of high toxicity but substituting the phosphorus for arsenic or carbon showed the equivalent compounds to be equipotent (Cooper et al. 1978).

The importance of the steric role of R substituent of the 4-carbon atom on the bridge head of the molecule for high toxicity has been emphasised. The greater the bulkiness or branching of the bridge-head substituent the higher the toxicity (Eto et al. 1976, Cooper et al. 1978, Milbraith et al. 1979) and the increase in ability to antagonise the actions of GABA has been shown (Bowery et al. 1977, Olsen et al. 1978e). The suggested mode of action of the bicyclic phosphate esters is that symmetrical cage structure blocks a pore-type or anion channel receptor (Bowery et al. 1977, Milbraith et al. 1979, Olsen et al. 1978e) with the two ends of the cage differing in properties, the bridge-head hydrophobic structure fitting in one part of the channel and the small hydrophillic end of the molecule fitting into a separate protein conformation.

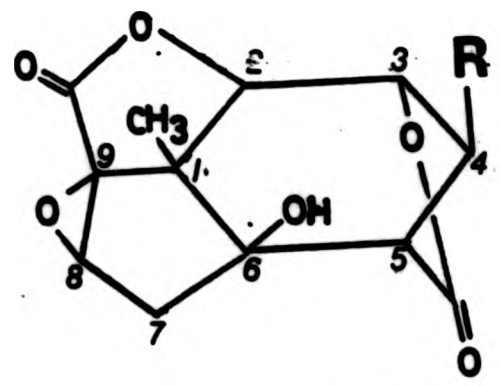
The very bulky and branched bridged-head substituents on the 4-carbon atom as in the tertiary butyl analogue ($OP(OCH_2)_3$

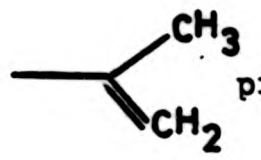
C-C$(CH_3)_3$ and isopropyl analogue (OP$(OCH_2)_3$ C-C_3H_7-i) were the most potent in toxicity (Eto et al. 1976, Cooper et al. 1979) and in their abilities to antagonise the effects of GABA (Bowery et al. 1977) on isolated tissues and in the displacement of (^{3}H)-α-dihydropicrotoxinin binding (Olsen et al. 1978e, Ticku and Olsen 1979).

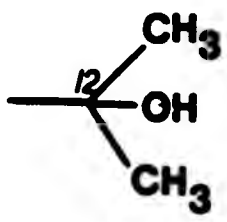
It was therefore decided to synthesise several new bicyclic phosphate esters which had very bulky and branched bridge-head substituents in order to elucidate the optimal size of the hydrophobic end of the molecule.

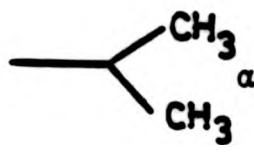
A series of these compounds were then synthesised in order to evaluate their biochemical and pharmacological properties in comparison with the work already described in this chapter. The synthesis and results will be discussed in the next chapter.

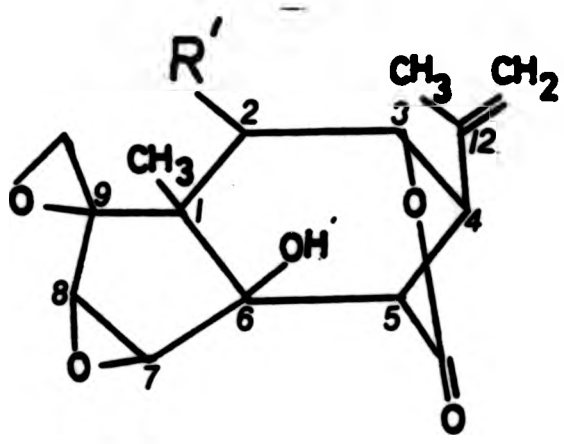
Fig. 2.2. Structures of picrotoxinin and related convulsants which cause GABA antagonism. Also showing the numbering system.



R =  picrotoxinin

R =  picrotin

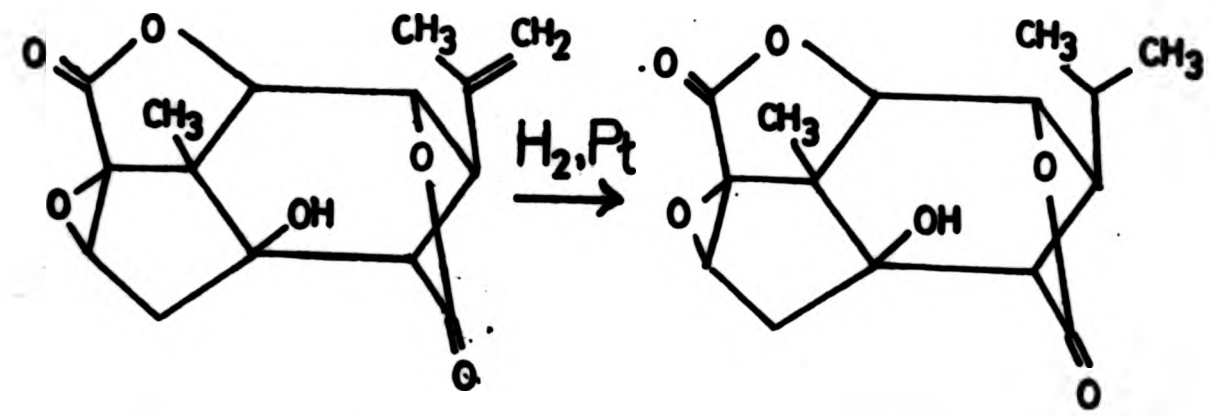
R =  α-dihydro-picrotoxinin



R'=H. Coriamytrin

R'=OH, Tutin

Fig. 2.3. Synthesis of α-dihydropicrotoxinin from picrotoxinin by hydrogenation using a platinum catalyst.



PICROTOXININ

α-DIHYDRO
PICROTOXININ

2. 2 Picrotoxinin

The excitatory effects of picrotoxinin were first associated with the action on GABA in crustacea (Florey, 1951).

At low concentrations of picrotoxinin the synaptic effects of GABA are blocked in several different crustacean preparations. These preparations include the crayfish heart (Florey, 1957); the crayfish stretch receptor (Elliott and Florey 1956, Iwasaki and Florey 1969, and Hori et al. 1978); and the inhibitory neuromuscular junction of the crayfish (Robbins and Van der Kloot 1958, Takeuchi and Takeuchi 1969 and Takeuchi 1976).

In other crustacea, picrotoxinin antagonises the GABA-mediated inhibition of the neuromuscular junction in the lobster (Grundfest et al. 1958 and Shank et al. 1974) and crab (Epstein and Grundfest 1970, Earl and Large 1972).

Picrotoxinin also antagonises the peripheral synaptic inhibition and the inhibitory effects of GABA on insects (Usherwood and Grundfest 1965) and the antagonism of GABA-receptor interaction on the locust muscle (Norman 1978).

In amphibia, picrotoxinin reduces the depolarisation by GABA on the afferent fibres of the spinal cord of the toad in vitro, (Tebécis and Phillis 1969) and in the spinal cord of the frog (Barker and Nicoll 1973), blocking both the dorsal and ventral root responses to GABA, the dorsal root responses being antagonised more potently than the ventral root responses Nicoll et al. 1976).

In mammals picrotoxinin has been shown to antagonise the actions of GABA *in vitro* and *in vivo*.

Picrotoxinin suppresses both vestibular inhibition and the inhibitory actions of GABA on oculomotor neurons in the rabbit (Obata and Highstein 1970).

Picrotoxinin increased the concentration of cyclic-GMP in rat cerebellum in non-convulsant doses which can be antagonised by treatment with benzodiazepines or GABA (Costa et al. 1976). In other work on rat cerebellar cyclic-GMP levels the actions of picrotoxinin are reported to lower these levels and this can be antagonised by the GABA agonist, muscimol (Biggio et al. 1978). In cultured rat cerebellar Purkinje cells, which are spontaneously bioelectrically active, picrotoxinin antagonised the inhibitory actions of GABA. It also increased the firing rate of these cells in low concentrations. This action was reversible but in higher concentrations the firing rate was decreased (Gähwiler 1975). However in the cat cerebellar cortex picrotoxinin did not interfere with the depression of Purkinje cell firing by GABA (Kawamura and Provini 1970). These conflicts in the reported results may be due to the relatively low aque ous solubility of picrotoxinin and so investigations have depended on electro osmotic ejection of picrotoxinin from solutions (Curtis 1964).

As with the frog spinal cord the dorsal and ventral root responses to GABA are antagonised by picrotoxinin in the rat spinal cord in vitro (Bowery and Brown 1972, de Groat 1970, Evans 1978).

When administered systemically picrotoxinin first modifies the spinal dorsal root potentials and the associated excitability changes in afferent fibres (Bell and Anderson 1972, Levy and Anderson 1972). Therefore slow penetration of the blood-brain barrier is an unlikely explanation for this latency in action and it is probably accounted for by the complex inter relationships of the inhibitory systems and neuronal pathways.

When administered intravenously in cats picrotoxinin produces cardiovascular changes which occur in two phases. The first phase is a decrease in arterial pressure and sinus rate due to activation of the central parasympathetic centres and the second phase is an increase in arterial pressure and sinus rate due to activation of sympathetic centres in the brain (DiMicco et al. 1977).

There have been reports of specific antagonism of GABA in cat cuneate neurones (Galindo 1969, Kelly and Renaud 1973).

Picrotoxinin does not displace radioactively-labelled GABA from mammalian synaptosomal brain fractions (Zukin et al. 1974, Enna et al. 1977) or crayfish muscle preparations (Olsen et al. 1977). In invertebrate

preparations picrotoxinin inhibits neurophysiological effects of GABA non-competitively appearing to compete with chloride ions at the ionophore site (Takeuchi and Takeuchi 1979).

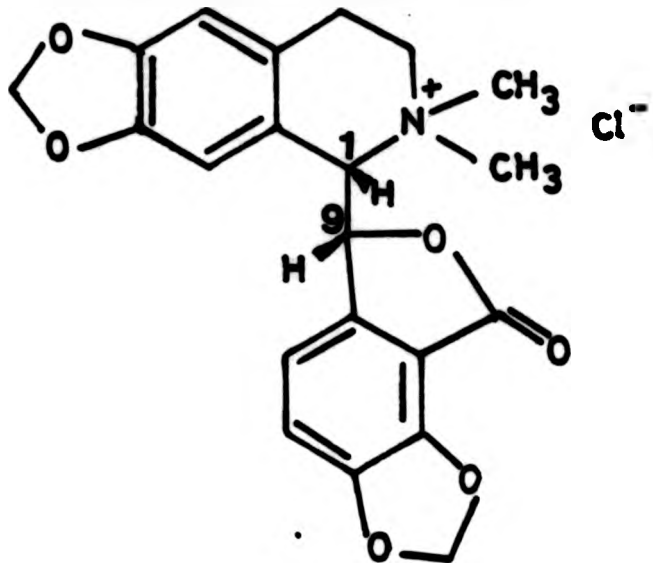
In crayfish muscle picrotoxinin and the less potent but relatively similar analogue α-dihydropicrotoxinin antagonised the GABA-induced, radioactively-labelled chloride ion uptake (Ticku and Olsen 1977, Olsen et al. 1978d). The specific binding was approximately 20% on the microsomal (P_3) fraction of the crayfish tail muscle. Binding was not affected by chloride ion concentration. Bicuculline, another GABA antagonist (Curtis and Johnston 1974), also inhibited the dihydropicrotoxinin binding but only at high concentrations where it causes various non-specific effects (Olsen, 1976, Olsen et al. 1976). This would also suggest the binding sites for GABA and picrotoxinin are different.

Using autoradiography, ^{3}H-dihydropicrotoxinin has been reported to be localised at similar locations to the GABA receptor binding sites in rat brain synaptosomal preparations (Olsen et al. 1978e, Ticku et al. 1978). The GABA binding was found to be enhanced by some detergents (e.g. Triton X100) but treatment with detergents on the dihydropicrotoxinin binding resulted in a destruction of specific binding (Ticku et al. 1978).

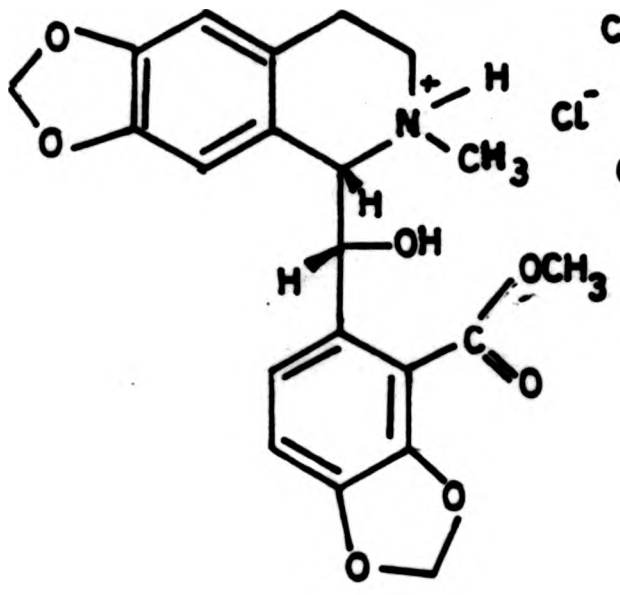
Dihydropicrotoxinin binding in rat brain synaptosomal preparations was also affected by a series of convulsant drugs, the "cage convulsant" drugs (Olsen et al. 1978b) and both depressant and excitant barbiturates (Ticku and Olsen 1978).

The binding studies which are detailed here are reports of the material which has been published to date and will be discussed in greater detail in a later chapter which investigates the specific binding of (^{3}H) - α-dihydropicrotoxinin binding to rat brain synaptosomal fractions.

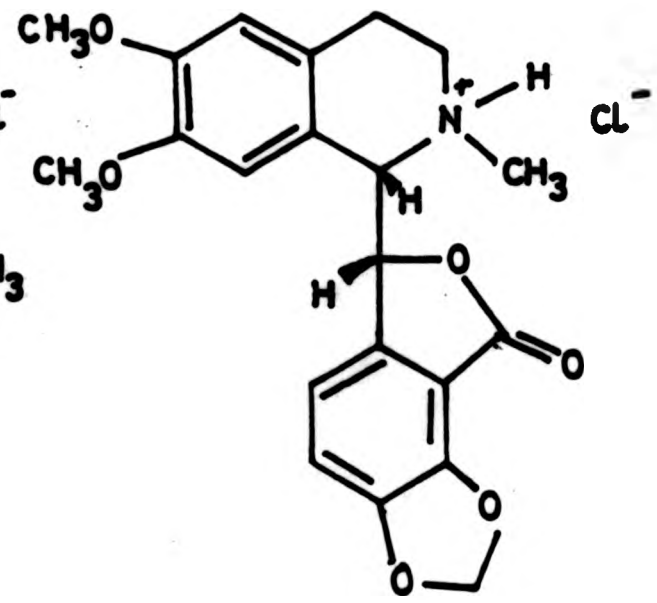
Fig. 2.4. Structures of bicuculline methochloride and those of other GABA antagonists active in mammalian CNS, bicucine ester, hydrochloride and corlumine hydrochloride



BICUCULLINE METHOCHLORIDE

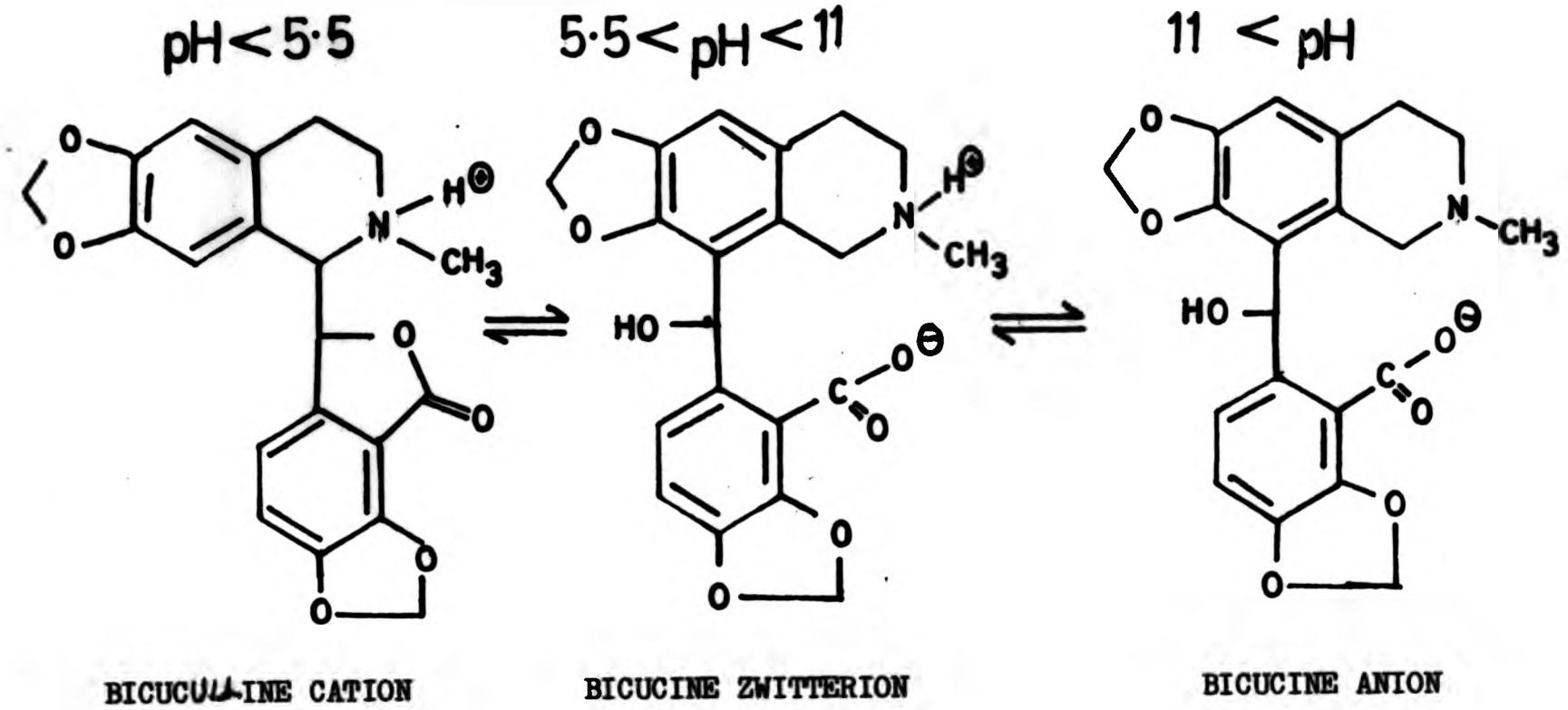


BICUCINE METHYL ESTER HYDROCHLORIDE



CORLUMINE HYDROCHLORIDE

Fig. 2.5. Chemical structures of bicuculline and bicucine in aqueous solutions of various pH values.



2.3 Bicuculline

The phthalideisoquinoline alkaloid bicuculline (Fig.2.4) was shown to be a potent convulsant in rabbits and frogs (Welch and Henderson 1934a), the convulsant activity being shown to be due to antagonism of a GABA-mediated process (Curtis et al. 197a ,b).

In insects bicuculline causes severe convulsions in the house fly (Musca domestica) and also antagonises the GABA-mediated inhibitory postsynaptic potentials (IPSPs) in the cockroach (Periplaneta americana) coxal muscles, (Olsen et al. 1976, Walker et al. 1971).

In crustacea the results obtained with bicuculline have been variable. In the crayfish (Eustacus armatus) stretch receptor the inhibitory action of GABA and impulses in the inhibitory nerve fibres was antagonised (McLennan 1970b, McLennan 1973), and also at the neuromuscular junction of the crayfish (Combarus olarkii) (Takeuchi and Onodera 1972) but this was only a weak antagonistic effect. Conflicting reports on the crayfish stretch receptor suggested bicuculline had little effect (Swagel et al. 1973).

These negative results were supported by bicuculline showing only a weak antagonistic effect on neuromuscular inhibition in the lobster (Homarus americanus) (Shank et al. 1974) and hermit crabs (Earl and Large 1972).

Other work was also indicative of the dubious nature of bicuculline as a GABA antagonist (Godfraind et al. 1970,

Straughan et al. 1971) in invertebrates. However, at physiological pH there is a conversion of bicuculline by hydrolysis to the hydroxyacid bicucine (Fig.2.5) which is a much less active convulsant than bicuculline (Welch and Henderson 1934b). Aqueous solutions of bicuculline at neutral pH can be converted to bicucine by lactone hydrolysis from the lone pair of electrons on the nitrogen atom. This process was slowly reversed by acidification (Olsen 1976, Olsen et al. 1975). This reason may account for the negative results reported above, the solubility at neutral pH of bicuculline in aqueous solution is also much reduced than in acidic conditions. Further, it may precipitate if too high concentrations are used thus giving a negative result in experiments designed for investigation of antagonistic properties.

Quaternisation of bicuculline to produce (+)-bicuculline methiodide ("N-methyl bicuculline"), methochloride and methobromide (Fig.2.4) produced increased water solubility and lactone hydrolysis cannot readily take place in these derivatives. These derivatives have been shown to be more potent convulsants than bicuculline when the blood-brain barrier is bypassed by route of administration in some vertebrates (Pong and Graham 1972, Johnston et al. 1972).

(+)-Bicuculline methiodide was found to be unstable in aqueous solution, however, if left for long periods at room temperature, decomposing to give an inactive, water-

insoluble oil, (Cryer 1979) whereas (+)-bicuculline methochloride was stable (Johnston et al. 1972). Subsequently (+)-bicuculline methobromide was found to be much more easily prepared and just as stable and potent as (+)-bicuculline methochloride (Cryer 1979). Consequently this quaternary salt was used in all experiments.

In the frog (Rana temporaria) spinal cord the actions of bicuculline have been reported to block both dorsal and ventral root responses to GABA specifically (Barker et al. 1975a,b, Nichol et al. 1976), however other reports on this preparation claim that the blockade was not specific to GABA (Pixner 1974).

In the rat spinal cord bicuculline blocked dorsal root responses to GABA (Evans 1978) and specifically antagonised the depolarising effect of GABA on the rat superior cervical ganglion (Bowery et al. 1976a).

In iontophoretic studies doubt has been cast on the effectiveness of bicuculline as a specific GABA antagonist in the rat caudate nucleus (Bernardi et al. 1976), rat cerebellar Purkinje cells (G&whiler 1975) and also in cat (Godfraind et al. 1970).

In rat brain slices bicuculline is insensitive to the high-affinity uptake of radiolabelled GABA (Olsen et al. 1976, Iversen and Johnston 1971) and there was a stimulation

of release of GABA (Johnston and Mitchell 1971). Studies with rat brain synaptosomal fractions have shown that bicuculline acts with the synaptic receptors by displacement of radiolabelled GABA (Curtis et al. 1971a,b, Johnston 1978, Lester and Peck 1978), in a stereospecific manner (Enna et al. 1977). There was a heterogenity of the GABA and bicuculline binding sites (Greenlee et al. 1978) and GABA and bicuculline binding sites may be distinct from each other due to the differential effects of chaotropic salts (e.g. ClO_4^-) in binding with (^3H)-bicuculline methobromide to demonstrate a high and low affinity site (Mohler 1978, Mohler and Okada 1978) and the effects of mild detergents (e.g. Triton X100)(Enna and Snyder 1977).

Bicuculline has a similar effect on the cat cardiovascular system causing a decrease in arterial pressure and sinus rate due to activation of GABA mediated central parasympathetic centres followed by an increase in arterial pressure and sinus rate due to activation of sympathetic centres in the brain (DiMicco and Gillis 1979).

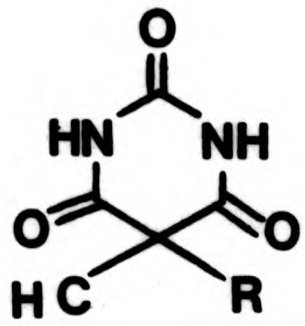
The mode of action of bicuculline is not due to any effect of enzymes involved with GABA synthesis or degradation (Beart and Johnston 1972) nor does it effect the uptake or release of GABA (Johnston and Mitchell 1971).

Other effects of bicuculline not related to its GABA-receptor actions are its inhibition of acetylcholinesterase in mouse brain (Svenneby and Roberts 1973) but no evidence was

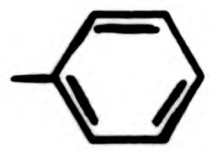
found in cat Renshaw cell firing rates (Curtis et al. 1974).

The actions of acetylcholine have been shown to be potentiated (Miller and McLennan 1974) and bicuculline also has a direct effect on axonal membrane (Freeman 1973) and lobster muscle membrane conductances (Shank et al. 1974).

Fig. 2.6. Structure of barbiturates with different substitutions which give rise to convulsant or depressant properties.



R = $-CH(CH_3)-CH_2-CH(CH_3)-CH_3$ DMBB (Convulsant)

R =  Phenobarbital (Depressant)

R = $-CH(CH_3)-CH_2-CH_2-CH$ Pentobarbital (Depressant)

2.4 The actions of barbiturates

The actions of barbiturates may be due to their effects on the GABA-mediated inhibitory transmission (Nicholl et al 1975). Pentobarbital has been reported to prolong GABA-mediated responses in frog motorneurones(Nicholl 1975a), cultured spinal cord cells (Ransom and Barker 1976), hippocampus (Nicholl et al. 1975) and the superior cervical ganglion (Bowery and Dray 1976, Dray and Bowery 1979). This action can be reversibly antagonised by picrotoxinin (Nicholl 1975a, McDonalds and Barker 1978).

Pentobarbital, at therapeutic concentrations does not alter GABA uptake or release (Peck et al. 1976, Olsen et al. 1977) by acting presynaptically nor does it inhibit binding on the postsynaptic GABA receptor (Enna and Snyder 1977, Peck et al. 1976, Collins and Cryer 1978, Olsen et al. 1978a) but it can prevent (+)-bicuculline from displacing GABA from its binding site (Collins and Cryer 1978).

Barbiturates also have an effect on cation conductance mechanisms (Blaustein 1976, Sato et al. 1976). Pentobarbital also inhibited α-DHP binding in rat brain membranes suggesting the site of action of it being at the picrotoxinin binding site in the GABA recepto.- ionophore influencing the chloride ion channels (Ticku and Olsen 1978). However the convulsant barbiturate DMBB (Fig. 2.6) inhibited ^{3}H-α-DHP binding much more potently, suggesting a similar inhibition of GABA-mediated effects in closing the chloride ion channel and depressant and anticonvulsant barbiturates could prolong GABA-mediated

inhibition by increasing the lifetime of the activated ion channel.

2.5 Action of benzodiazepines.

Benzodiazepines have been shown to facilitate GABA-ergic transmission in the mammalian CNS (Costa et al. 1976, Guidotti et al. 1979, Haefely et al. 1975, 1979).

A highly specific, high affinity binding of radiolabelled benzodiazepines to proteins in synaptic membranes in the CNS has been demonstrated *in vitro* (Möhler and Okada 1977b, c, Braestrup and Squires 1977) and *in vivo* (Chang and Snyder 1978). Although there is an excellent correlation between the affinities of various benzodiazepines for binding sites in accordance with their muscle relaxant actions (Möhler and Okada 1977b) no other psychotropic drugs or neurotransmitters inhibit benzodiazepine binding including GABA, barbiturates, glycine and strychnine (Haefely et al. 1979, Creese 1978) but GABA and muscimol increase benzodiazepine binding (Regan et al. 1980, Tallman et al. 1978).

Distribution of benzodiazepine binding sites in the CNS is uneven but it may be located presynaptically on GABA neurons or post-synaptically on GABA-ceptive neurons. A marked decrease in their number in the striatum combined with a fall of GAD activity in Huntington's Chorea patients suggests a presynaptic location (Okada et al. 1978).

Benzodiazepines reversed the bicuculline induced GABA blockade in rat isolated superior cervical ganglion and medullary

neurons (Bowery and Dray 1978, Dray and Straughan 1976) but did not inhibit (^{3}H)-bicuculline binding in rat synaptic membranes (Möhler and Okada 1977a).

(^{3}H)-DHP binding was inhibited by benzodiazepines in rat synaptic membranes (Olsen et al. 1980, Ticku et al. 1981) and picrotoxinin antagonised the actions of benzodiazepines *in vivo* (Stein et al. 1975).

There was no inhibition of (^{3}H)-GABA binding (Snyder and Enna 1975, Olsen et al. 1978c) or (^{3}H)-muscimol binding (Beaumont et al. 1978)) in mammalian brain synaptic membranes. However, studies on the sodium independent (^{3}H)-GABA binding on fresh synaptic membranes of the rat cerebral cortex cause in increase in the affinity of the membranes for GABA suggesting that the benzodiazepines bind to an endogenous modulator which is located on the GABA receptor. The modulator has been isolated and benzodiazepines bind specifically and with high affinity with the same rank order as their anxiolytic actions (Guidotti et al. 1979, Costa and Guidotti, 1979).

Other observations *in vivo* have suggested the existence of two types of benzodiazepine receptor, one of which is not associated with the GABA receptor and could mediate the anxiolytic actions and the second type of receptor coupled to the modulator of the GABA receptor is responsible for the other pharmacological effects of the benzodiazepines and not their anxiolytic actions (Klepner et al. 1979, Huot et al. 1981).

CHAPTER 3 : CHEMICAL SYNTHESIS AND ACUTE TOXICITIES OF BICYCLIC PHOSPHATE GABA ANTAGONISTS

3.1 Introduction

3.2 Preparation of 4-(2,2,dimethyl propyl) 2,6,7, trioxa-1-phosphabicyclo (2,2,2) octan-1-one.

3.3 Preparation of 4-(1,2, dimethyl propyl) 2,6,7, trioxa-1-phosphabicyclo (2,2,2) octan-1-one.

3.4 Preparation of 4-cyclopentyl 2,6,7,-trioxa-1-phosphabicyclo (2,2,2) octan-1-one.

3.5 Attempted preparation of 4-(1,1,2, trimethyl propyl (THEXYL) 2,6,7, trioxa-1-phosphabicyclo (2,2,2) octan-1-one.

3.6 Attempted preparation of 3,4, dimethyl pentanal.

3.6.1 By metal assisted borohydride reduction of an acid chloride

3.6.2 By the use of Bis (triphenyl-phosphine) Copper (I) tetraborohydrate

3.6.3 By the use of chlorosuccindimide oxidation of the alcohol.

3.7 Attempted preparation of 3,4,4 trimethyl pentanal

3.7.1 By reduction of the corresponding acid chloride with lithium tri-t-butoxy-aluminohydride.

3.7.2 By acid hydrolysation from 1-methoxyl, 3,3,4 trimethyl 1,2, pentene

3.8 Attempted preparation of 1,1, 2 trimethyl/ ethoxypropane by increase in the carbon chain using trimethyl oxosulphonium iodine/sodium hydride complex.

3.9 Acute toxicity of bicyclic phosphate esters

3.9.1 Method

3.9.2 Results

3.10 Discussion of synthetic routes

3.11 Conclusions

3.1 Introduction

The actions of the bicyclic phosphates have been documented and described earlier. An optimum size for the hydrophobic bridge-head on the 4-carbon atom has been shown to be essential for high toxicity. It was therefore decided that efforts would be concentrated on synthesising compounds which had extremely bulky and hydrophobic bridge-head substituents. These would be predictably very toxic in mammals and hence possibly very potent GABA antagonists which could be useful as tools in order to elucidate more information about the GABA system.

Experimental

The following general techniques were used throughout the text:

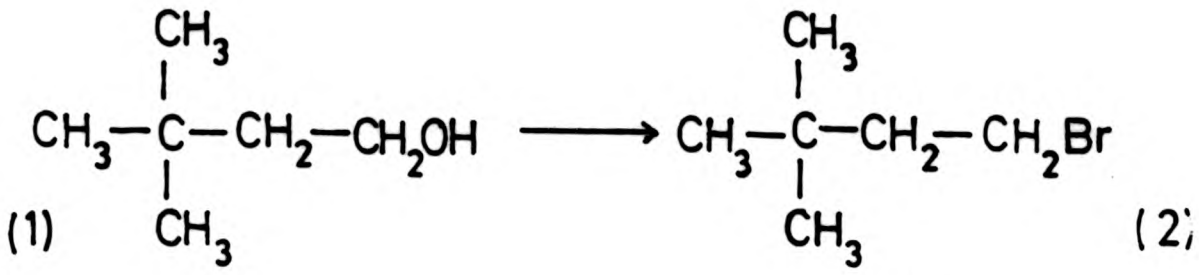
Melting points were measured on an electro-thermal melting point apparatus (sealed tubes were used for toxic compounds).

Infra-red spectra were recorded on a Perkin-Elmer 237 grating spectrophotometer.

Nmr spectra were recorded on a high frequency JOEL C-60H or C90Q spectrometer, performed by Mr. C. Pottage.

Carbon, hydrogen and nitrogen analyses were performed by Mrs. E. Chittenden. LD_{50} values were performed by Mr. P. Williams, C.D.E., Porton Down, Wiltshire.

CHART I



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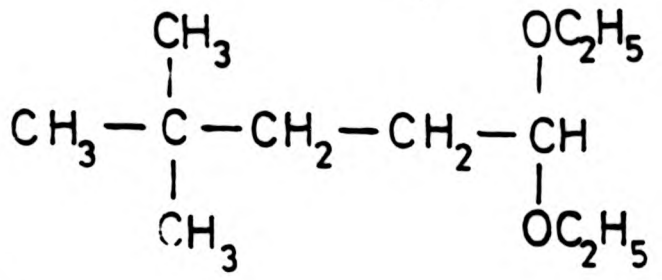

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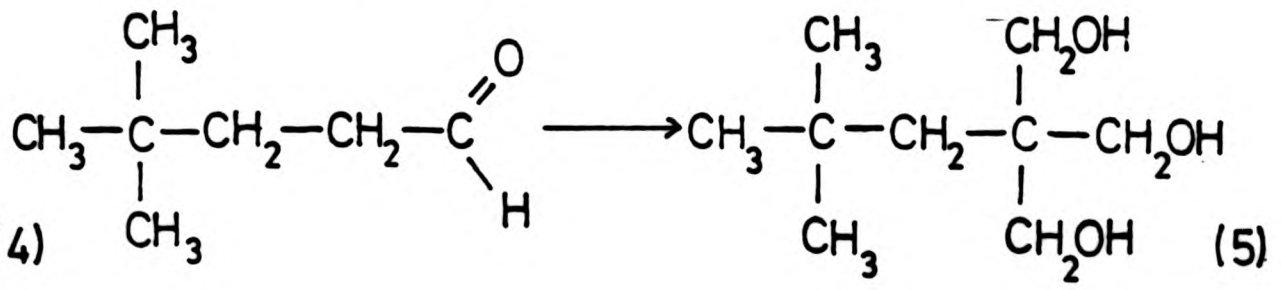

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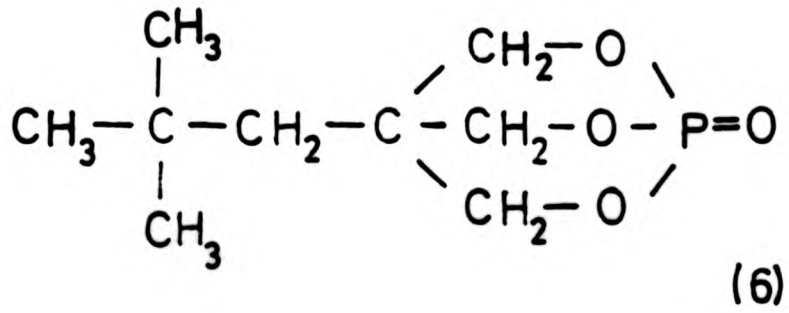

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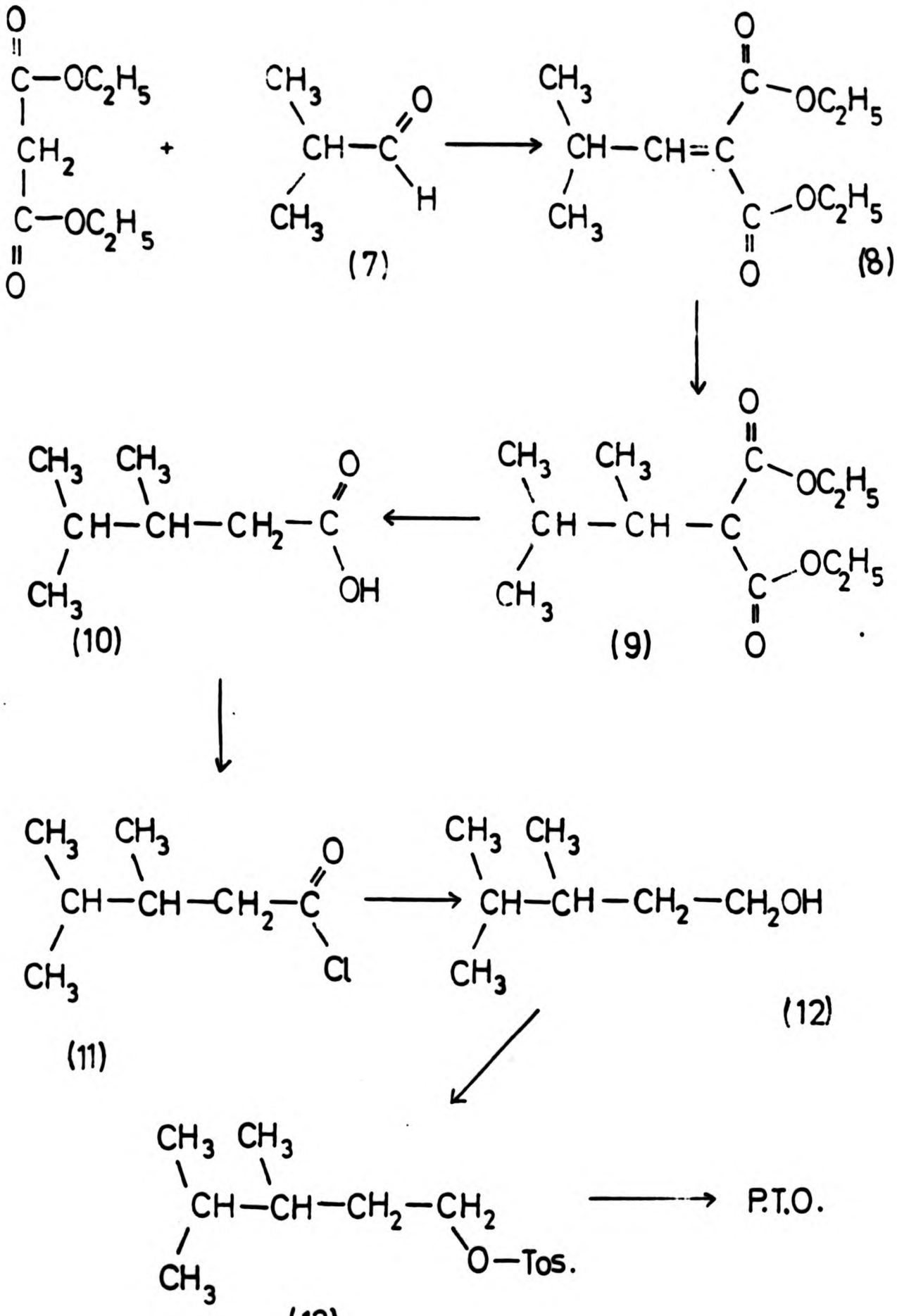

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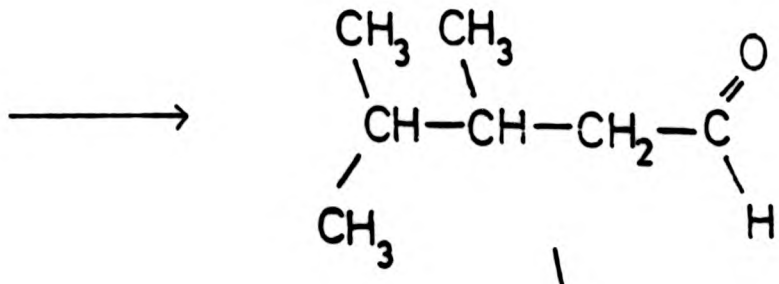

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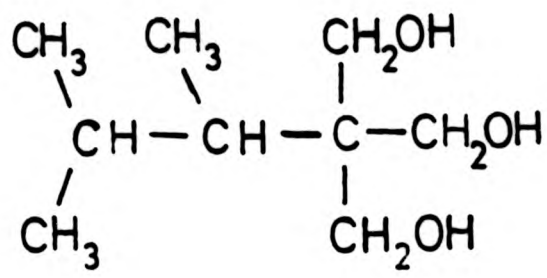

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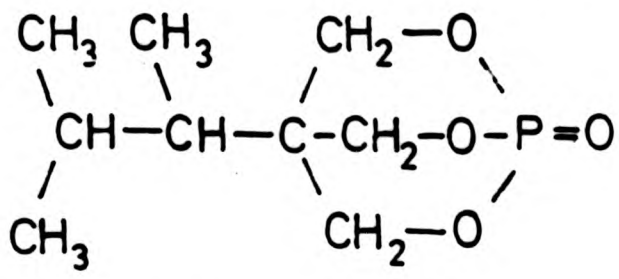

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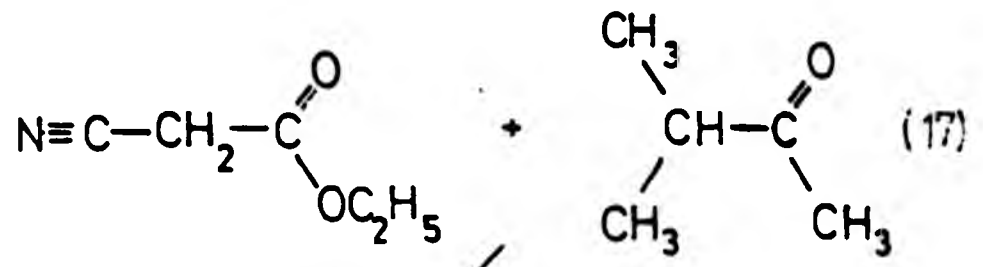

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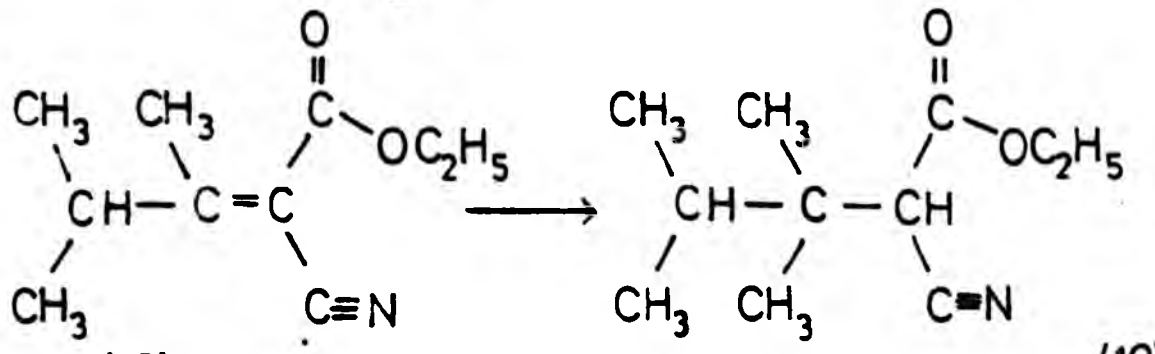

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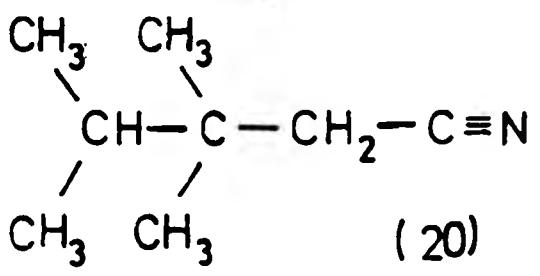

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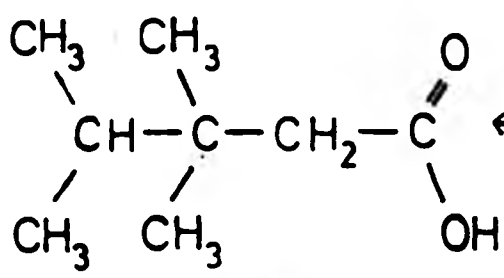

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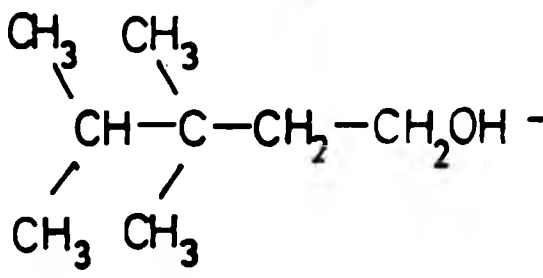

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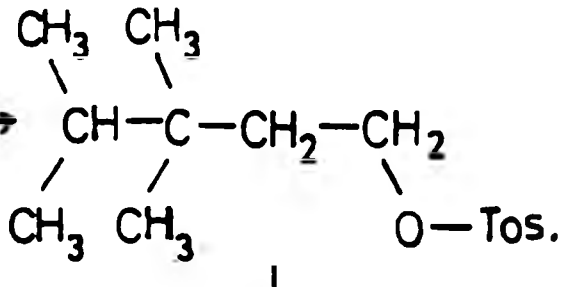

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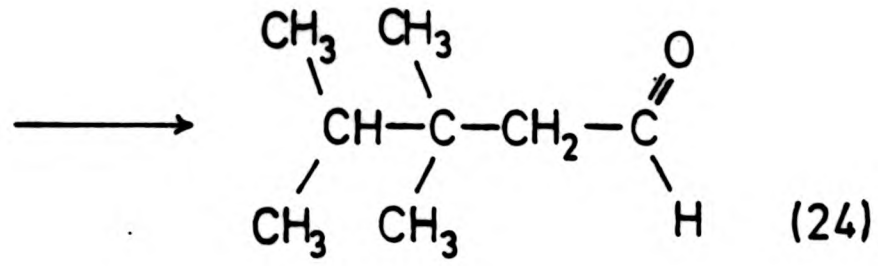

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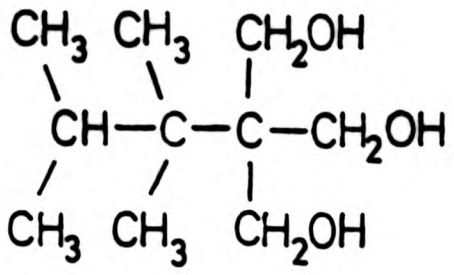

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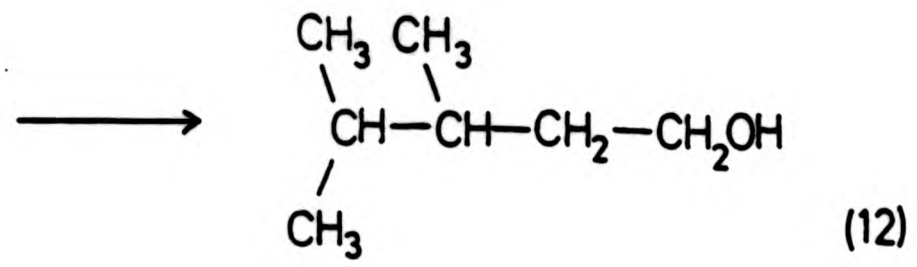

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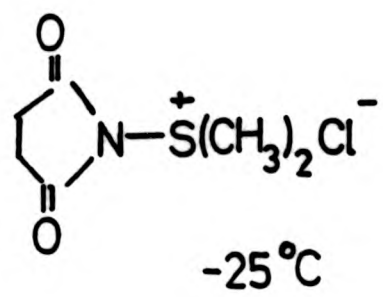

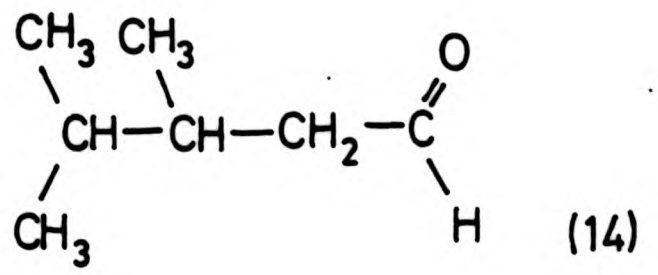

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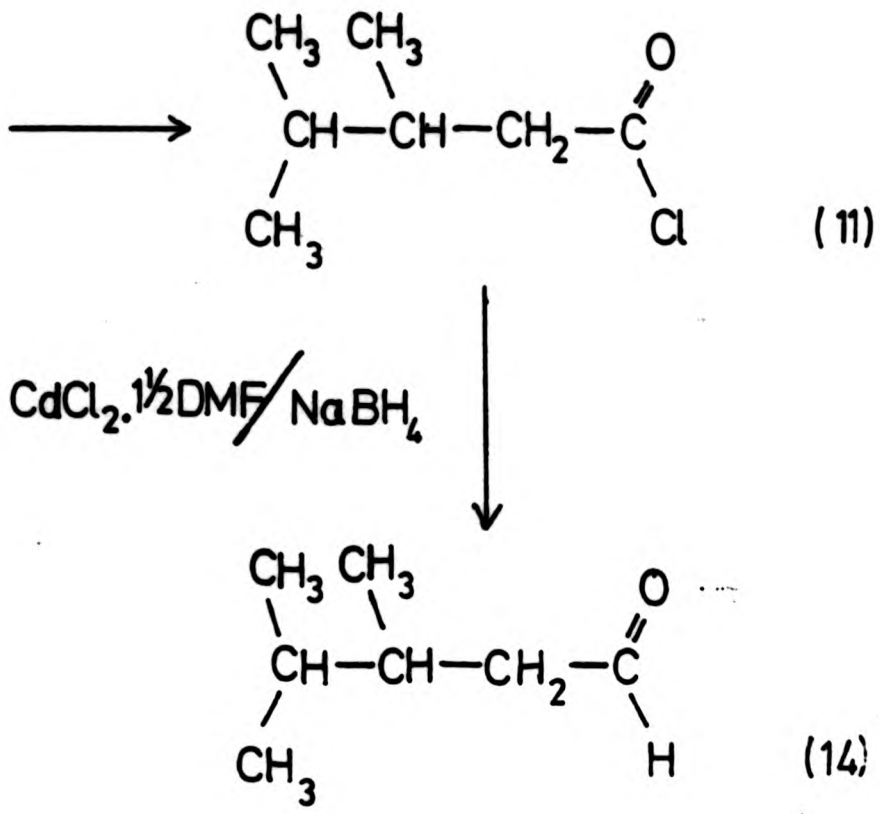
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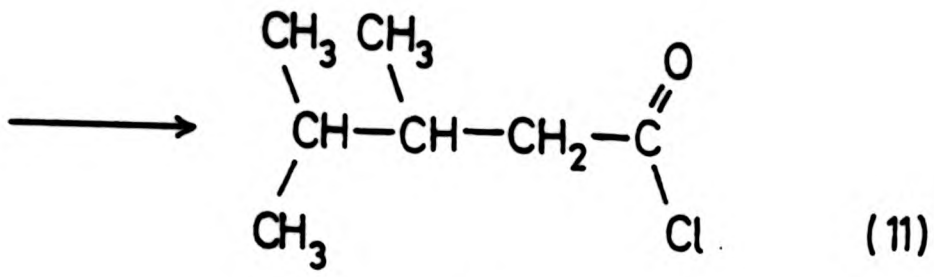
O
CH—CH—CH2—C
CH3
Cl
(11)
CdCl2.1½DMF/NaBH4
CH3 CH3
O
CH—CH—CH2—C
CH3
H
(14)

CHART VI



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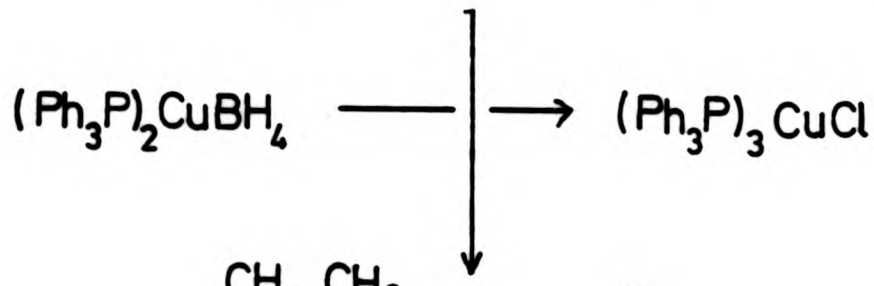

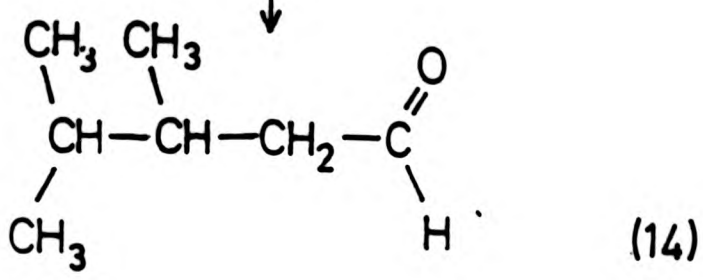

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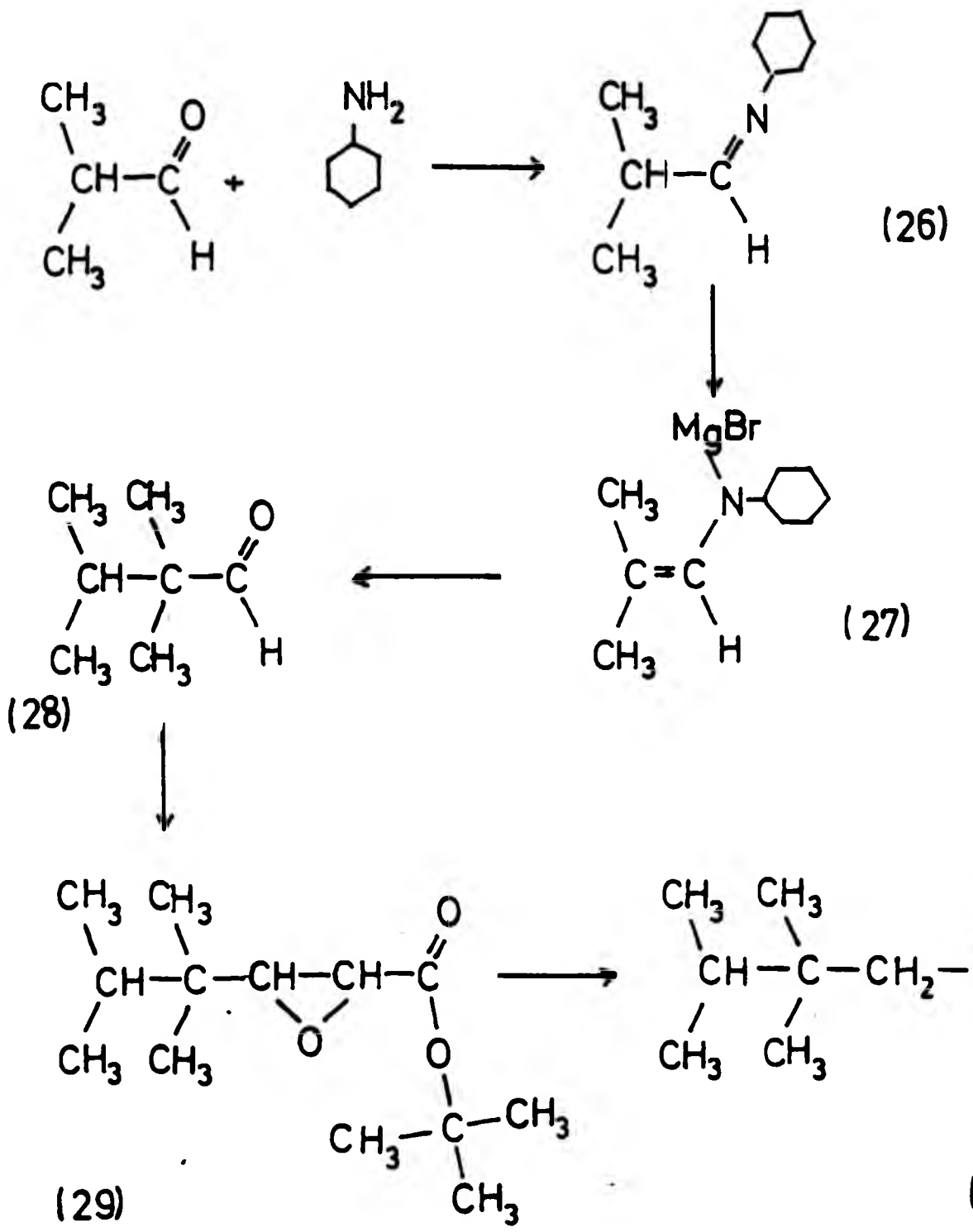
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O
CH
C
H
CH3
+
NH2
CH3
N
CH
C
H
CH3
(26)
MgBr
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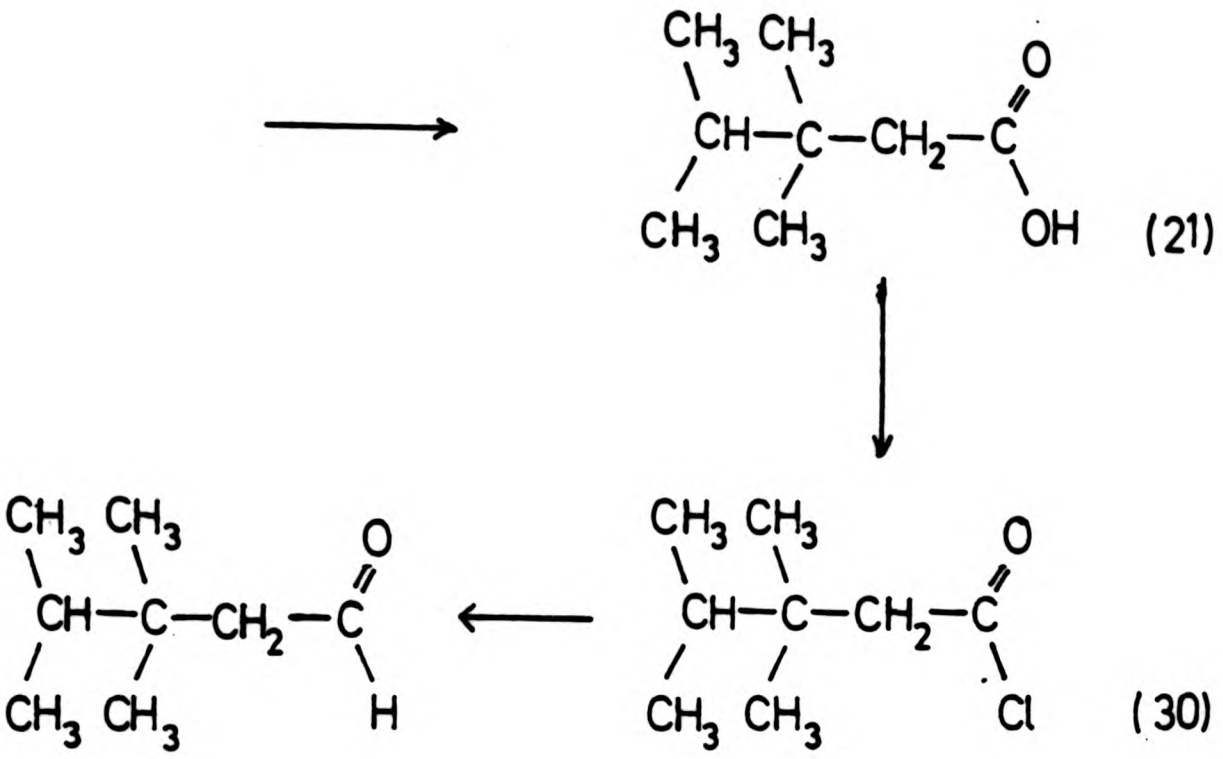
N
C=C
H
CH3
(27)
CH3 CH3
O
CH
C
C
H
CH3 CH3
(28)
CH3 CH3
O
CH
C
CH
CH
C
O
O
CH3
C
CH3
CH3
CH3 CH3
(29)
CH3 CH3
O
CH
C
CH2
C
H
CH3 CH3
(24)

CHART VIII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O
CH3 CH3
OH
(21)
CH3 CH3
CH—C—CH2—C
O
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Cl
(30)
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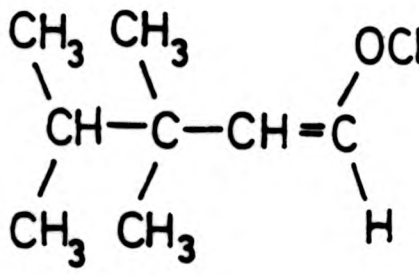
O
CH3 CH3
H
(24)

CHART IX



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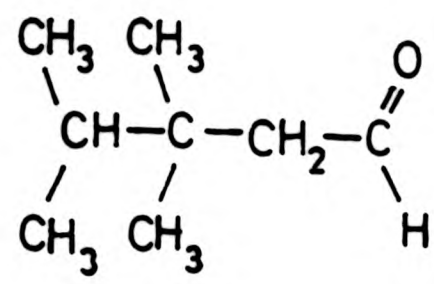

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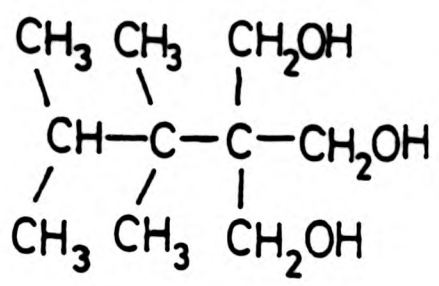

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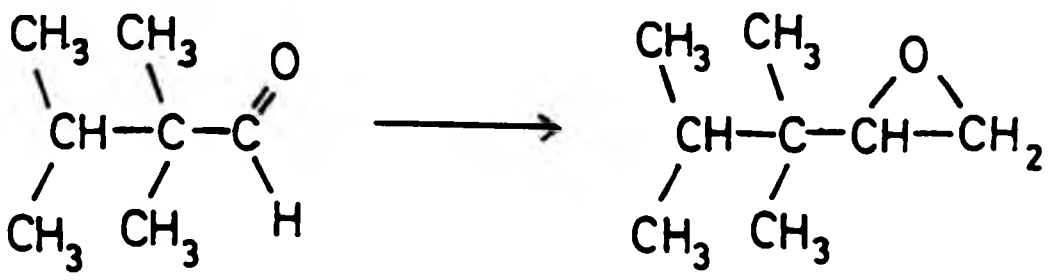

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(32)

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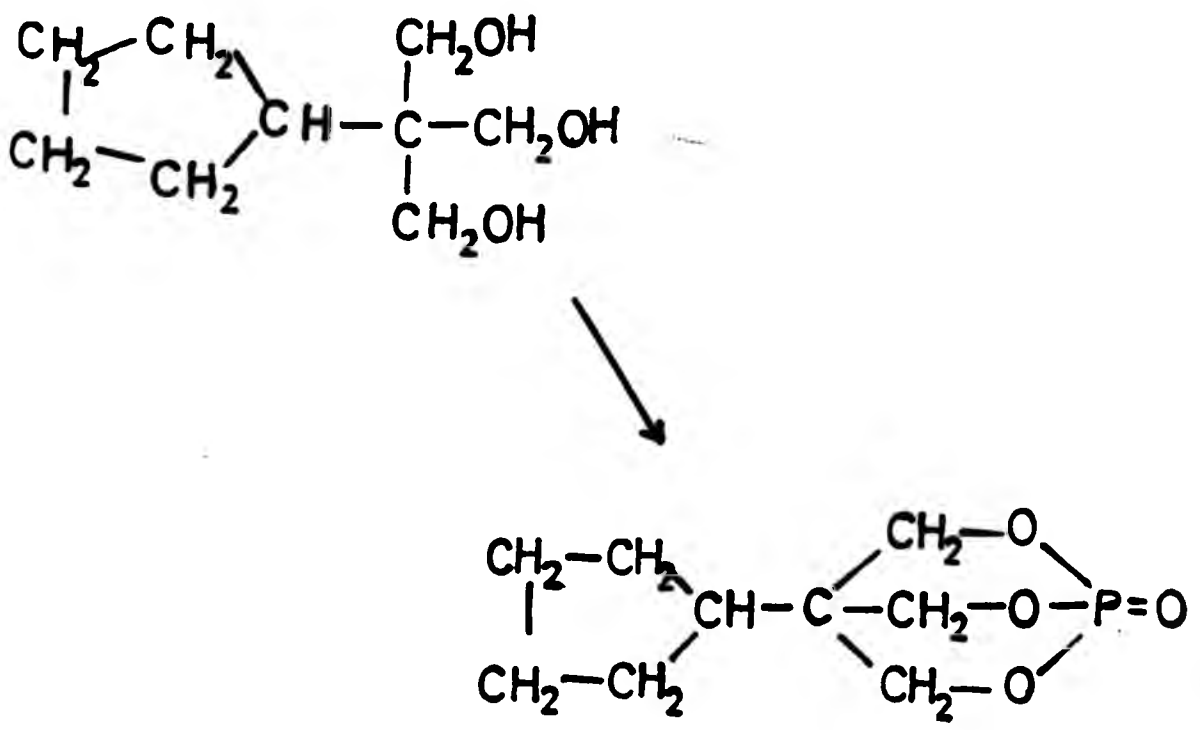

3.2 Preparation of 4-(2,2.dimethyl propyl (NEOPENTYL) 2,6,7, trioxa-1-phosphabicyclo (2,2,2) octan-1-one. (CHART I)

3,3 dimethyl butan-1-ol (64.0g, 0.63 moles) (1) and 48% hydrobromic acid (157 cm^3, 0.92 moles) were refluxed together for 3 hours. The organic layer was extracted with 5% sodium hydrogen carbonate, washed with water, dried with magnesium sulphate and distilled to give Bromo-3,3,dimethyl butane (2) (BP = 35-37°C at 12 mmHg. Yield = 74%)

The Bromo-3,3,dimethyl butane (2) (76.5g, 0.46 moles) was prepared into a grignard reagent in dry ether (137 cm^3) by reaction with magnesium (11.4g, 0.47 moles) and to this was added triethylorthoformate (68.5g, 0.46 moles) and stirred on a boiling water bath for 1 hour. The reaction mixture was then poured into water and saturated with ammonium chloride solution (1000cm^3). The ether layer was dried with magnesium sulphate and concentrated before distillation of 1,1, diethoxy 4,4, dimethylpentane (B.P. = 64 - 74°C at 12 mmHg, Yield = 52%) (3)

The 1,1, diethoxy, -4,4,dimethylpentane (22.7g, 0.12 moles) was refluxed for 10 minutes with 10% hydrochloric acid (150 cm^3), washed with saturated sodium hydrogen carbonate solution (50 cm^3), washed with water, the organic layer separated and dried with magnesium sulphate. This was distilled to give 4,4, dimethylpentanal (B.P. 55°C at 12mmHg, Yield = 25%). (4)

The 4,4, dimethyl pentanal (4.85g, 0.04 moles)(4) was added to 10% sodium hydroxide solution 31cm^3, 0.06 and 40% methanol solution (20cm^3, 0.2 moles) and stirred at room temperature for 2 hours. The mixture was then heated to 50°C for 1 hour. This was then continuously extracted with chloroform for 5-6 hours and after concentrating was run on a silica gel column using chloroform:methanol (9:1) as the elutant. The fraction obtained which produced an Rf value of 0.36 when chromatographed using the same elutant and located with 50% sulphuric acid spray, appeared on the silica plate as a black spot when the plate was heated. This was concentrated and the product recrystallised from dichloromethane; appeared as white needle-shaped crystals (0.597g, Yield = 12%, m.pt 137-139°C uncorrected, Empirical formula $C_9H_{20}O_3$, H = 11.44%, O = 27.23%, results for microanalysis : C = 56.60%, H = 10.60%, values differing due to one molecule of water present).

Analysis repeated:

$C_9 H_{20} O_3$ Mwt = 176

Calculated	C 61.33	H 11.44
Found	C 61.72	H 11.17

The 2,2, bishydroxymethyl-4,4,dimethyl pentan-1-ol(0.597g, 3.4 mmole) (5) was stirred for 5-6 hours with phosphorous oxychloride (0.52g, 3.4 mmole). The products went liquid/solid after 30 minutes, the flask was then heated to 70°C for 1 hour and water ($20cm^3$) added. The reaction mixture was then boiled until the product had dissolved in the water, this was then allowed to cool and white needle-shaped crystals of 4-(neopentyl) 2,6,7, trioxa-1-phosphabicyclo (2,2,2) octan-1-one (6) formed. These were filtered and allowed to dry over calcium chloride in a dessicator for 3 days. (0.350g. Yield = 47%, mpt. (sealed tube) 198-200°C (uncorrected). Empirical formula $C_9 H_{17} O_3$, theoretical values for micro-analysis : C = 49.09%, H = 7.78%, results for micro-analysis C = 49.19%, H = 7.65%. These figures are well within the 0.3% limits and can therefore be assumed to be the correct compound. MW = 220.21. NMR spectrum ($90MH_z$) with respect to TMS δ values.

δ : 0.89 (S) 9H ($3 \times CH_3$); 1.24 (S) 2H (CH_2); 4.57 (d) 6H ($3 \times CH_2$ cyclo, J = $6.8H_z$ P - coupling).

$C_9H_{17}O_3P$

Calculated	C	49.09	H	7.78
Found	C	49.19	H	7.65

The 2,2, bishydroxymethyl-4,4,dimethyl pentan-1-ol(0.597g, 3.4 mmole) (5) was stirred for 5-6 hours with phosphorous oxychloride (0.52g, 3.4 mmole). The products went liquid/solid after 30 minutes, the flask was then heated to 70°C for 1 hour and water ($20cm^3$) added. The reaction mixture was then boiled until the product had dissolved in the water, this was then allowed to cool and white needle-shaped crystals of 4-(neopentyl) 2,6,7, trioxa-1-phosphabicyclo (2,2,2) octan-1-one (6) formed. These were filtered and allowed to dry over calcium chloride in a dessicator for 3 days. (0.350g. Yield = 47%, mpt. (sealed tube) 198-200°C (uncorrected). Empirical formula $C_9 H_{17} O_3$, theoretical values for micro-analysis : C = 49.09%, H = 7.78%, results for micro-analysis C = 49.19%, H = 7.65%. These figures are well within the 0.3% limits and can therefore be assumed to be the correct compound. MW = 220.21. NMR spectrum ($90MH_z$) with respect to TMS δ values.

δ : 0.89 (S) 9H ($3 \times CH_3$); 1.24 (S) 2H (CH_2); 4.57 (d) 6H ($3 \times CH_2$ cyclo, J = $6.8H_z$ P - coupling).

$C_9H_{17}O_3P$

Calculated	C	49.09	H	7.78
Found	C	49.19	H	7.65

3.3 <u>Preparation of 4-(1,2, dimethyl propyl) 2.6.7. trioxa-1-phosphabicyclo (2,2,2,) octan-1-one</u>. (CHART II)

2-methyl propanal (72g, 1 mole) (7) was condensed with diethyl malonate (160g, 1 mole) in the presence of benzene (100cm^3), piperidine (4.5cm^3) and glacial acetic acid (12cm^3) until 1 mole (18g) of water was collected. The product was washed with water (3 x 100cm^3), dried with magnesium sulphate and distilled to give diethyl isobutylidene malonate.(8)(b.pt.118-120°C, 12 mmHg. Yield = 78%) (Cope et al. 1941).

The diethyl isobutylidene malonate (167g, 0.78 moles) was added to an iodomethane grignard reagent (0.78 moles, in dry ether (300cm^3). The mixture was cooled to 0°C and copper (I) chloride (1.6g) was added. The mixture was stirred for a further 30 minutes and then poured onto cold 10% sulphuric acid (650cm^3), washed with sodium hydrogen carbonate solution, water and dried with magnesium sulphate. Then it was concentrated and distilled to give diethyl (1,2,dimethyl propyl) malonate.(9)(b.pt118-120°C, 12mmHg. Yield = 92%). (Eliel et al. (1956)

The diethyl (1,2,dimethyl propyl) malonate (9) (164g, 0.76 moles) was refluxed with potassium hydroxide (164g, 0.76 moles) for 10 hours and allowed to cool. The product was then washed with ethanol (0.78 litres) and dissolved in water. The solution was then acidified with

concentrated hydrochloric acid, washed with water and extracted with ether. This was dried with magnesium sulphate and concentrated. The residues then heated to 160°C for 5 hours and allowed to cool. This was distilled to give dl, 3,4, dimethyl pentanoic acid. (10) (b.pt. 109°C, 12mmHg, Yield = 58%).

The dl, 3,4, dimethyl pentanoic acid (10) (58g, 0.45 moles) was refluxed with thionyl chloride (66.6g, 0.55 moles) for 1 hour. This was cooled and distilled to give 3,4, dimethyl pentoyl chloride (b.pt. 45-55°C, 12mmHg. Yield = 87%). (11)

The 3,4, dimethyl pentoyl chloride (11) (22.5g, 0.15 moles) in dry diethyl ether ($100cm^3$) was added to Lithium aluminium hydride (7.2g, 0.19 moles) in dry diethyl ether, ($250\ cm^3$). The products were concentrated and distilled to give 3,4, dimethyl pentan-1-ol. (b.pt. 71°C, 12mmHg. Yield 84%). (12)

The 3,4, dimethyl pentan-1-ol (8.1g, 0.33 moles) was dissolved in pyridine ($20cm^3$) and tosyl chloride (13.5g, 0.07 moles) was added at 0°C. This was then poured into water after 1 hour and extracted with ether. The product was dried with magnesium sulphate, concentrated to give a yellow oil, 3,4, dimethyl pentan-1-yl tosylate, (13) with some pyridine present and this was used directly in the next stage, by addition to a mixture of sodium hydrogen carbonate

(15g. 0.18 moles) in dimethyl sulphoxide ($20cm^3$). This was heated to 150°C in an inert atmosphere of nitrogen, allowed to cool, thrown into water and extracted with ether. This was washed with water (3X $50cm^3$), dried with magnesium sulphate, concentrated and distilled in an inert atmosphere of nitrogen to give 3,4, dimethyl pentanal. (b.pt. 40-42°C. Yield = 20%). (Kornbleum et al. 1959). (14)

The 3,4, dimethyl pentanal (4.91g, 43 mmole) (14) was added to 10% sodium hydroxide solution ($26cm^3$, 65 mmoles) and stirred at room temperature for 2 hours. The mixture was then heated to 50°C for 1 hour and then continuously extracted with chloroform for 5-6 hours. After concentrating it was run down a silica gel column using chloroform : methanol (9:1) as the elutant. The fraction obtained which produced an Rf value of 0.32 when chromatographed using the same elutant and located on the silica gel plate appeared as a black spot after spraying with 50% sulphuric acid and heating the plate. This was concentrated and the product recrystallised from dichloromethane as a white solid. (1.76g, m.pt. 79-81°C uncorrected. Yield = 23%).

The 2,2, bishydroxymethyl 3,4, dimethyl pentan-1-ol (1.76g, 1.0 mmole)(15) was stirred for 5-6 hours with phosphorous oxychloride (1.5g, 1 mmole). The products went liquid/solid after 30 minutes, this was then heated up to 70°C for 1 hour

and water ($50cm^3$) added. This was then boiled until the product had dissolved in the water, allowed to cool and white needle-shaped crystals of 4-(1,2, dimethyl propyl) 2,6,7, trioxa-1-phosphabicyclo (2,2,2,) octan-1-one (16) formed; these were filtered and allowed to dry over calcium chloride in a dessicator for a few days. (281.2mg, Yield = 13%, m.pt, (sealed tube) 190°C uncorrected).

NMR spectrum ($90MH_z$) with respect to TMS δ values

δ: 0.81 (d) 6H (2 x CH_3, J = $6.8H_z$); 0.91 (d) 3H (1 x CH_3, J = $6.8H_z$); 1.4 (m) 1H (2-CH); 1.8 (m) 1H (3-CH); 4.6 (d) 6H (3 x CH_2 cyclo, J = $6.8H_z$ P-coupling).

$C_9H_{17}O_3P$ mwt = 220

Calculated	C	49.09	H 7.78
Found	C	48.82	H 7.76

3.4 <u>Preparation of 4-cyclopentyl 2,6,7,-trioxa-1-phosphabicyclo (2,2,2) octan-1-one</u>.(CHART XI)

2-cyclopentyl-2-hydroxymethyl propane 1,3, diol (0.6g, 3.4 mmole) was stirred for 5-6 hours with phosphorous oxychloride (0.52g 3.4 mmole). The products were liquid/solid after 30 minutes, this was then heated to 60°C for 1 hour, and water ($20cm^3$) added. The water was boiled until the product completely dissolved in it, then allowed to cool and white needle-shaped crystals of 4-cyclopentyl 2,6,7-trioxa-1-phosphabicyclo (2,2,2) octan-1-one formed. These were filtered and dried over calcium chloride in a dessicator for three days (300 mg, Yield = 50%, m.pt. (sealed tube) 214-216°C uncorrected).

NMR spectrum ($90MH_z$) with respect to TMS δ values

δ: 1.58 (m) 9H (cyclopentyl H's); 4.5 (d) 6H (3 x CH_2 cyclo, J = $6.8H_z$ P - coupling).

$C_9H_{15}O_3P$ mwt = 218

Calculated	C 49.99	H 7.10
Found	C 49.54	H 6.93

3.5 Attempted preparation of 4-(1,1,2, Trimethyl propyl (THEXYL)) 2,6,7,-Trioxa-1-Phosphabicyclo (2,2,2,) Octan-1-one.

(See Chart III)

A grignard reagent was prepared using Iodomethane (96.1g 0.68 mole) and magnesium (16.2g, 0.68 mole) in dry diethyl ether (280cm^3). This was then added to Ethyl 2-cyano 3 isopropyl butanoate (122.5g, 0.68 moles) in ether (280cm^3) with copper (I) chloride as a catalyst. The temperature was kept to -15^{o}C and when the grignard was added the mixture was refluxed for 1 hour, allowed to cool and poured onto cold 10% sulphuric acid (300cm^3). It was separated and extracted with ether (3X 100cm^3) before adding aqueous 10% sodium thiosulphate (100cm^3). Then it was dried with magnesium sulphate and concentrated before distillation of ethyl 2-cyano-, 3,3,4 trimethyl , pentanoate. (85.5g, b.pt. 126^{o}C. 12mmHg. Yield = 64%).

The thexyl cyanoate was prepared (85.5g, 0.43 moles), added to boiling potassium hydroxide (81.9g) in water (102cm^3) and refluxed for 6 hours, before cooling and acidifying with 50% concentrated hydrochloric acid (250cm^3). A solid was obtained which was filtered and the solution extracted with diethyl ether then dried with magnesium sulphate and concentrated before distillation of 3,3,4 Trimethylpentanonitrile(48.0g, b.pt. 61^{o}C. 12mmHg. Yield = 89%).

3,3,4, Trimethyl pentanonitrile (48.0g, 0.38 moles) was heated under reflux with 50% concentrated sulphuric acid ($350cm^3$) for 10 hours and allowed to cool. This was then extracted into diethyl ether ($2 \times 20cm^3$) and extracted from the ether into 1M sodium hydroxide solution ($200cm^3$) then acidified with concentrated hydrochloric acid before extracting back into ether ($100cm^3$). The product was dried with magnesium sulphate and concentrated before distillation of 3,3,4 trimethyl pentanoic acid. (19.3g, b.pt. 118°C. 12mmHg. Yield = 35%).

3,3,4 trimethyl pentanoic acid (6.2g, 43 mmole) in dry diethyl ether ($70 cm^3$) was added to Lithium Aluminium hydride (2.0g, 52 mmole) in dry diethyl ether ($70cm^3$) to reduce it to 3,3,4 trimethyl pentan-1-ol. (5.6g, b.pt. 84°C, 12mmHg. Yield = 75%).

3,3,4 trimethyl pentanal (4.2g, 32 mmole) was dissolved in pyridine ($12cm^3$) and to this was added tosyl chloride (6.35g, 32 mmole) dissolved in pyridine ($17cm^3$) and stirred for 1 hour. The reaction mixture was poured into water and separated with ether before drying with magnesium sulphate and concentrating. The mixture was then added to a solution of sodium hydrogen carbonate (8.0g) in dimethyl sulphoxide ($60cm^3$) and heated to 150°C for 5 minutes in an inert atmosphere of

nitrogen before distillation of 3,3,4, trimethyl pentanal under nitrogen as a colourless liquid. (1.7g, b.pt. 42-46°C, 12mmHg under nitrogen. Yield = 41%).

NMR spectrum ($90MH_z$) with respect to TMS δ values.

δ: 0.84 (d) 6H (2 x CH_3 isopropyl, J = $6.8H_z$); 1.02 (s) 6H (2 x CH_3, 3-carbon); 1.52 (m) 1H (CH isopropyl J = $6.8H_z$); 2.24 (d) 2H (CH_2, J = $3.0H_z$); 9.88 (t) 1H (aldehyde carbon, J = $3.0H_z$) $C_7H_{14}O$ mwt = 114.

The 3,3,4 trimethyl pentanal (1.7g, 13 mmoles) was added to 10% sodium hydroxide solution ($8cm^3$, 20 mmoles)- and 40% methanol solution $5cm^3$,65 mmoles), and stirred at room temperature for 2 hours. The mixture was then heated to 50°C and stirred for a further hour at this temperature before continuously extracting with chloroform for 5 to 6 hours.

After concentrating it was run on a silica gel column using chloroform : methanol (9:1) as the elutant. Fractions obtained with the lowest Rf value of 0.40 after locating on a silica gel plate with 50% sulphuric acid spray and heating to give a black spot were collected and concentrated to form a white liquid/solid which when sampled for presence of the triol by means of NMR/IR analysis showed that the fraction collected was the corresponding diol. Therefore apparently no triol was formed in this reaction by the method which under similar conditions consistently forms the triol. This may have been due to unknown variations in

the conditions used and it may in fact be possible to produce the triol but as such a great deal of effort was put into making the aldehyde by several unsuccessful methods previously, time and material were not available to pursue this line unfortunately.

3.6 Attempted preparation of 3.4. dimethyl pentanal

3.6.1 by metal assisted borohydride reduction of an acid chloride (II) (CHART V)

The cadmium chloride and dimethyl formamide were prepared from anhydrous $CdCl_2$ and dry DMF to give $CdCl_2$. $1\frac{1}{2}$DMF (7.5 mmoles).

Sodium borohydride (0.46g, 12 mmole) was dissolved in acetonitrile ($60cm^3$) and hexamethyl phosphoramide ($3.0cm^3$) and then stirred for 5 mins with $CdCl_2$. $1\frac{1}{2}$DMF (2.2g 7.5 mmoles) at -5 to 0^oC (ice-salt bath) (II) 3,4, dimethylpentoyl chloride (1.8g, 12 mmole) in acetonitryl ($12cm^3$) was added with rapid stirring for 5 min. This was then washed with dilute hydrochloric acid, saturated sodium hydrogen carbonate and water before extracting with ether and distilling over nitrogen. No useful product was obtained from this reaction. (Johnstone and Telford 1978).

3.6.2 <u>Attempted preparation of 3,4 dimethyl pentanal (14) by the use of Bis (triphenylphosphine) Copper(I) tetrahydroborate in the reduction of acid chloride</u> (CHART VI)

(i) <u>Bis (triphenylphosphine) copper(I) tetrahydroborate</u>

Hydrated copper sulphate (16g. 64 mmole) was stirred in a solution of triphenylphosphine (54g, 200 mmole) in ethanol (1400 cm^3) to give, after 1 hour, a pale green solution. Finely powdered sodium borohydride (12g, 320 mmole) was added whilst stirring, until a pink precipitate formed, hydrogen was given off. After 1 hour, the crude product was collected by filtration and dissolved in chloroform (500 cm^3). The reaction mixture was filtered as ethanol (800 cm^3) was added to the filtrate. The precipitate was collected by filtration, and washed with ethanol and ether to give bis (triphenylphosphine) copper (I) tetrahydroborate (30g, 50 mmole, 78% based on copper sulphate) as white needles. m.pt. 169-174°C (uncorrected.)

(ii) Reduction of 3,4 dimethylpentoyl chloride (II)

(CHART VI)

3,4, dimethyl pentoyl chloride (7.5g, 49 mmole) in acetone (860 cm^2) was treated with triphenyl phosphine (26g, 99 mmole). To this solution, at room temperature, solid bis (triphenylphosphine) copper (I) tetrahydroborate (297g, 49.. mmoles) was added and the reaction mixture stirred for 45 min. The white precipitate, tris (triphenylphosphine) copper chloride (43.5g, 48.8 mmole) was removed by filtration and the acetone filtrated and concentrated to dryness. The residue was extracted with ether (860 cm^3) and concentrated before redissolving in chloroform (550 cm^3) and the resulting solution stirred over copper (I) chloride (8.6g). The reaction mixture was filtered, the chloroform evaporated and the residue extracted with methanol, dried with $MgSO_4$ and concentrated before distillation. Very little aldehyde was found mixed with the products but the complex was retrieved as Tris (phenylphosphine) copper (I) chloride. (43.5g, 48.8 mmole, 99.7%) (Fleet, Fuller and Harding, 1978).

3.6.3 **Attempted preparation of 3,3,4 trimethyl pentanal (24) and 3,4 dimethyl pentanal (14) by the use of a chlorosuccinamide oxidation of the alcohol (12)**

(CHART IV)

N-chlorosuccinamide (11.4g, 85 mmole) was dissolved in dichloromethane (260 cm^3) and cooled to 0°C with nitrogen passing over. To this dimethyl sulphide (7.5 cm^3) was added and cooled further to -25°C (CO_2-CCl_4 bath). Then 3,4, dimethyl pental-1-ol (6.63g, 57 mmole) dissolved in dichloromethane (65 cm^3) was added and the reaction mixture stirred for 2 hours at -25°C. Then Triethylamine (5.3g, 90 mmole) in dichloromethane was added dropwise and stirred for 5 min before allowing the mixture to return to room temperature. The reaction mixture was extracted with ether, washed with 1% hydrochloric acid and water before drying over magnesium sulphate. The product could then be distilled over nitrogen at reduced pressure for collection. Only a small sample, b.pt. 42-60°C, 12 mmHg, had about 50% aldehyde with most of the product being unreacted alcohol. This method was therefore unsatisfactory for the synthesis of either the thexyl (24) or dimethyl propyl (14) aldehydes. (Corey and Kim, 1972).

3.7 Attempted preparation of 3,3,4 Trimethyl pentanal

(CHART VII)

(i) Isobutyraldehyde, 2 methyl propanal (180 cm^3) was added dropwise to cyclohexylamine (99g, 1 mole) and refluxed on a Dean-Stark condenser until 18 cm^3 of water had been collected. The residue was distilled under reduced pressure over nitrogen to give isobutyraldehyde cyclohexylimine (26) (13g b.pt. 12mmHg 84-85°C, 90%).

(ii) Ethyl magnesium bromide was prepared by reaction of magnesium (21.4g, 0.89 mole) with ethyl bromide (98g, 0.89 mole) in dry tetrahydrofuran (200 cm^3) and refluxed for 30 min. Then isobutyraldehyde (136g, 0.89 mole) in tetrahydrofuran (150 cm^3) was added dropwise and refluxed for 6 hours before cooling. Isopropyl bromide (110.4g, 0.89 mole) was added and the reaction mixture was refluxed for a further 6 hours before pouring onto 10% (w/v) hydrochloric acid (1,000 cm^3) and refluxed for a further 3 hours. The reaction mixture was extracted with ether, washed with sodium hydrogen carbonate, washed with water, dried over $MgSO_4$ and concentrated before distillation at reduced pressure over nitrogen to give 2,2,3 trimethyl butanal (28) (20g, b.pt. 12 mmHg, 38-43°C, 20%). (Stork Reaction)

(iii) Sodium (1.2g, 52 mmole) was added to xylene ($15\ cm^3$) and heated until the sodium melted. This was then stirred vigorously and allowed to cool to form sodium sand, which was further cooled to $5^{\circ}C$ (acetone-CO_2 bath). T-butyl chloroacetate (7.9g, 52 mmole) mixed with (28) 22,3 trimethyl butanal (6.0, 52 mmole) was added dropwise keeping the temperature at $5^{\circ}C$. The product was then poured onto water, separated, wahsed with salt water, dried with magnesium sulphate and distilled at reduced pressure. The lower boiling xylene ($32-50^{\circ}C$, 12mmHg) was discarded and only the product boiling at $100^{\circ}C$, 12mmHg collected, as t-butyl 4,4,5 trimethyl 2,3 epoxy-hexanoate (29) (2.34g, b.pt. 12 mmHg $120-130^{\circ}C$. 20%).

(iv.a) (29) (2.34g, 10 mmole) was refluxed with 10% hydrochloric acid ($50\ cm^3$) for 1 hour before cooling and separating with ether. This was washed with sat. sodium hydrogen carbonate, washed with water, dried over $MgSO_4$ and distilled over reduced pressure. The product obtained was only starting material (29) so more drastic conditions were used.

(iv.b) The (29) was pyrolised by heating up to $200^{\circ}C$, unfortunately this did not produce the

hoped for reaction either, so this method to synthesise the thexyl aldehyde (24) was also rejected.

3.7.2 Attempted preparation of 3,3,4trimethyl pentanal by reduction of the corresponding acid via preparation of the acid chloride with lithium tri-t-butoxy-aluminohydride (CHART VIII)

(i) 3,3,4 trimethyl pentanoic acid (21) (19.3g, 134 mmole) was refluxed with thionyl chloride (19.4g, 168 mmole) for 1 hour and the product distilled at reduced pressure to give 3,3,4 trimethyl pentoyl chloride (30) (18.5g, b.pt. 12mmHg, 89%)

(ii) 3,3,4 trimethyl pentoylchloride (8.5g, 52 mmole) was dissolved in dry tetrahydrofuran (50 cm^3) and cooled to -25°C (acetone-CO_2 bath). Then lithium tri-t-butoxy aluminohydride (25.4g, 100 mmole) in dry tetrahydrofuran (150 cm^3) was added dropwise, and stirred for 1-2 hours until the reaction mixture had reached room temperature. This was then thrown onto ice (800g) and 10% HCl added (200 cm^3), extracted with ether, washed with sat. sodium hydrogen carbonate, washed with water and dried over $MgSO_4$ before distillation at reduced pressure. b.pt. 12mmHg 88-142% produced an impure product with only 10-20% of the correct aldehyde present so the synthesis was rejected. (Brown and McFarlin, 1956; Ho, et al. 1970).

was added dropwise with stirring, and the mixture heated on a water bath to 50°C for 1 hour. This was allowed to cool and water (20 cm^3) was added. Then it was extracted with ether, washed with water, dried with $MgSO_4$ and distilled over nitrogen at reduced pressure. b.pt. 12mmHg 36-40% but this was an impure mixture of starting materials and the method was again rejected as being unsuitable.

3.7.3 Attempted preparation of 3,3,4 trimethyl pentanal by acid hydrolysation from 1 methoxyl 3,3,4 trimethyl 1,2, pentene (CHART IX)

1 methoxyl 3,3,4 trimethyl 1,2, pentene (5.9g, 45 mmole) was refluxed with 5% HCl (30 cm^3) for 1 hour. It was separated and the product washed with sat. sodium hydrogen carbonate, washed with water, dried with $MgSO_4$ before distillation, at reduced pressure to give the aldehyde. (1.0g, b.pt. 12mmHg 48-52°C, 17%). This product was not pure and contained about 25% of 2,2,3 trimethyl butanal which could not be separated, and interfered with the next stage, triolisation, so much that this method was also rejected.

3.8 Attempted preparation of 1,2,2, trimethyl ethoxy propane by increase in the carbon chain using trimethyl oxosulphonium iodine, sodium hydride complex from 3,3, 4 trimethyl pentanal. (CHART X)

Sodium hydride (80% dispersion) was washed with dry ether to get rid of the mineral oils and dried over nitrogen to drive off any excess ether. Then trimethyl oxosulphonium iodide (13.2g, 60 mmole) in dimethyl sulphoxide (60 cm^3) was added. 2,2,3 trimethyl butanal (28) (6.0g, 52 mmole) in dimethyl-sulphoxide (20 cm^3)

TABLE 1

Results for intravenous injection into female albino mice showing the mortality onset times and times to death.

1 (a) For Neopentyl PBTO

Dose (mg/Kg)	Mortality Rate	Onset Times	Times to Death
0.13	0/6	30 sec - 1 min	-
0.16	1/6	15 sec - 1 min	1 min 30 secs
0.20	4/6	30 sec - 1 min	2 - 9 min
0.25	4/6	Immediate - 1 min	1 - 2 min
0.31	10/10	Immediate - 30 sec	30 secs

TABLE 1 (b) of various Lethal Dose values for i.v. in mice on Neopentyl PBTO showing the limits and probit slope.

	Doses with (limits) Mg Kg^{-1}	Probit Slope	(± S.E. of slope)
LD10	0.150 (0.102 - 0.175)		
LD30	0.178 (0.140 - 0.203)		
LD50	0.200 (0.170 - 0.231)	10.25	± 2.75
LD70	0.225 (0.197 - 0.276)		
LD90	0.267 (0.231 - 0.379)		

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0.20	4/6	30 sec - 1 min	2 - 9 min
0.25	4/6	Immediate - 1 min	1 - 2 min
0.31	10/10	Immediate - 30 sec	30 secs

TABLE 1 (b) of various Lethal Dose values for i.v. in mice on Neopentyl PBTO showing the limits and probit slope.

	Doses with (limits) Mg Kg^{-1}	Probit Slope	($\pm$ S.E. of slope)
LD10	0.150 (0.102 - 0.175)		
LD30	0.178 (0.140 - 0.203)		
LD50	0.200 (0.170 - 0.231)	10.25	$\pm$ 2.75
LD70	0.225 (0.197 - 0.276)		
LD90	0.267 (0.231 - 0.379)		

TABLE 2

Results for intravenous injection into female albino mice showing mortality, onset times and times to death.

2.(a) For Dimethyl propyl PBTO

Dose (mg/Kg)	Mortality Rate	Onset Times	Times to Death
0.5	3/3	immediate-15 sec	1-2 min
0.3	2/3	30 sec-60 sec	1-2 min
0.25	2/3	10 sec-30 sec	1-2 min
0.15	1/3	30 sec	4 min
0.10	0/3	30 sec-2min	recovered by 2 min

Table 2 (b)

Table of various Lethal Dose values for i.v. in mice on Dimethyl Propyl bicyclic phosphate showing the limits and probit slope.

	Doses with (limits) Mg Kg^{-1}	Probit Slope	($\pm$ S.E. of slope)
LD10	0.991 (0.005-0.178)		
LD30	0.167 (0.036-0.242)	5.17	
LD50	0.212 (0.103-0.372)	$\pm$ 2.18	
LD70	0.267 (0.181-0.919)		
LD90	0.375 (0.258-5.381)		

Mrs. C. Barcley carried out this analysis.

3.9 Acute toxicity of bicyclic phosphate esters

The cage compounds, including the bicyclic phosphate esters, were found to be highly toxic in mice, producing seizures and consequent death (Bellet and Casida, 1973). This was found to be due to the antagonism of the actions of synaptically released GABA (Bowery et al. 1976 and 1977) by examination of the superior cervical ganglion of the rat.

To establish the acute toxicity in mice, intravenous injections were performed with various doses to give an LD_{50} which may then be compared to other LD_{50} experiments performed by other bicyclic phosphates of known toxicity in mice and their subsequent potencies of antagonism on the actions of GABA.

3.9.1 Method

The compounds were dissolved in dimethylsulphoxide for intravenous administration to female albino mice in the tail. The mice were randomised into groups of six.

3.9.2 Results:

Severe toxic-signs were seen at all doses and onset times and times to death appeared to be dose-related.

Initial signs were tremor and panting respiration with occasional vociferation. This was followed by a brief phase of violent convulsions and terminated by prostration and marked tonic extensor spasms. At this point some of the animals were salivating and haematuria was observed. Many of the mice became cyanosed. Death appeared to be due to respiratory failure. Surviving animals often had bouts of convulsions when prostrate. Most surviving animals recovered within 10 minutes, recovering their upright posture although remaining tremorous and subdued for some time. At the maximum dose used of 0.31 mg/Kg there was 100% mortality with 10 mice used, death being in less than 30 seconds in all cases.

TABLE 1 shows the effect of 4-(2,2,Dimethyl propyl)-2,6,7,-trioxa-1-phosphabicyclo (2,2,2) octan-1-one. Statistical analysis using probit analysis maximum likelihood showed the compound to have an LD_{50} of 0.20 $\pm$ 0.03 mg/Kg with complete mortality at 0.31 mg/Kg.

These results for the Neopentyl PBTO show that it is a very potent convulsant in mice having about half the potency of t-butyl PBTO (t-butyl PBTO toxicity i.v. in mice LD_{50} is 0.12 with limits (0.10 - 0.14) $mg.Kg^{-1}$), (Cooper et al. 1978) and has a similar potency to isopropyl PBTO (toxicity

i.p. in mice LD_{50} is 0.18 (0.15 - 0.21 with limits) $mg.Kg^{-1}$) (Bellet and Casida 1973). All other cage convulsants which have been tested have not been as potent as the Neopentyl PBTO in their convulsant LD_{50} effect in mice, (Cooper et al. 1978, Eto et al. 1976, Milbraith et al. 1979, Casida et al. 1977).

3.10 Discussion of synthetic routes

All the syntheses of the bicyclophosphates involved the cyclisation of the corresponding triol into the bicyclophosphate using phosphorus oxychloride. (Cooper et al. 1978). The major synthetic problems arose with the preparation of the parent aldehydes which were used to synthesise the triols by reaction with 5% aqueous sodium hydroxide (0.15 mole) and 40 % formaldehyde (0.4 mole) (Dermer and Solomon, 1954).

(i) The parent aldehyde (4) was synthesised by the acid hydrolysis of diethoxy 44 dimethyl pentane (3) giving a 25% yield of the product.

The starting material used was the primary alcohol (1) which had one carbon atom less than the aldehyde necessitating the lengthening of the basic carbon chain.

This was done by bromination of the alcohol in order to make a grignard reagent which could then be added to triethyl orthoformate to give (3) which could be hydrolysed.

Preparation of dimethyl propyl PBTO

This method of lengthening the carbon alkyl chain was tried several times by making the grignard agent and reacting with triethyl orthoformate for the dimethyl propyl compound but all of these resulted in

failure even when dimethyl phenyl orthoformate was used instead of the trimethyl orthoformate.

Therefore some different synthetic route had to be established to synthesise the parent aldehyde needed to make the triol and hence the corresponding bicyclophosphate ester.

It was assumed that some method of either oxidising the corresponding alcohol (12) in Chart II to the aldehyde or reducing the corresponding acid (10) in Chart II would be a suitable method since the original simple synthetic route successfully used for the Neopentyl compound would not work for this compound.

CHART IV. The alcohol (12) was prepared and then oxidised using a complex prepared from dimethyl sulphide and N-chlorosuccinamide under carefuuly controlled conditions (Vilsmaier and Sprügel 1972; Corey and Kim, 1972). Even though excellent yields were reported for this process, in practice little success was established with a little aldehyde present in the bulk of unreacted alcohol. After several attempts to obtain good yields by changing solvent from toluene to dichloromethene, this method was rejected in order to concentrate on other methods to synthesise this aldehyde. The same method also proved unsuccessful for the thexyl aldehyde (24).

CHART V. No direct reductions of the corresponding acid (10) and (21) were practical as the conditions needed to reduce an acid are often too severe for the reduction to stay at the aldehyde stage without reducing to an alcohol so the reduction methods used were those with the corresponding acid chloride (11) and (30).

A metal-assisted borohydride reduction of the acid chloride which has been reported to give good yields (60-80%) (Johnstone and Telford, 1978), was attempted using cadmium chloride/dimethylformamide but again this failed to work. (See Methods and Chart V).

A second method of reducing the acid chloride (11) and (30) to the corresponding aldehyde attempted, was by the use of Bis(triphenylphoxphine) Copper (1) tetraborate. This has been reported as simple and effective, giving yields of 86%, (Fleet, Fuller and Harding 1978). It did not work in these experiments with the acid chloride and aldehyde being lost and the copper complex being obtained unchanged at the end of the reaction. (See Methods and Chart VI).

In Chart VII a simple three stage synthesis enabled the preparation of an aldehyde with a carbon atom less than the aldehyde needed (28). This was done by the condensation of 2 methyl formaldehyde and

cyclohexylamine to give good yields of isobutyraldehydecyclohexylimine (26). To this, using a grignard reagent, the Stork Reaction was performed with the addition of the alkyl group to give the basic thexyl shape which was important to the final product. This aldehyde (23) however, could not be reacted directly with the sodium hydroxide and methanol solutions to obtain the desired triol because the first two substituted methyl groups were attached directly to the carbon atom which would form the bridge head in the bicyclophosphate ester. It was therefore essential to increase the basic carbon chain by one carbon atom. This was first attempted by the addition of t-butyl chloroacetate, using sodium sand and proved fairly successful, isolating t-butyl 4,4,5 trimethyl 2,3 epoxyhexanoate (29) in a yield of 20%. However, when an attempt was made to hydrolise this into the desired aldehyde using 10% HCl it was not successful, having no effect on the epoxyhexanoate (29). When more drastic conditions were used and (29) was pyrolised the molecule turned into a polymer which was of no use, therefore this method of increasing the carbon chain was rejected and a different method then attempted (Chart X).

This method involved increasing the carbon chain from the aldehyde (28) in an epoxy propane compound (32) similar to that in the previous attempt, described above. This was done using an oxosulphonium iodine/ sodium hydride complex.

The products, after distillation and analysis proved to be an impure mixture of starting materials, so again the method of increasing the carbon chain from the aldehyde (28) was dropped in favour of other methods of preparation of the desired aldehyde.

Some success was obtained by the acid hydrolysis of (1 methoxyl) 3,3,4 tri-methyl 1,2 pentene (31) in Chart IX), but a mixture of two aldehydes (31) and (28) in a ratio of about 3 : 1 respectively was obtained. This mixture was difficult to separate by distillation, and when used as a mixture in the triolisation only a slight trace of the correct triol (25) was obtained, which was not enough to separate and ring close with the phosphoryl chloride. Most of the product of this reaction was the diol inferring the possibility of the close proximity of the multibranched alkyl chain to the carbon atom which is to form the bridge-head has some effect in preventing the formation of the bulky triol.

3.11 Conclusions

As mentioned earlier the difficulties in the synthesis of the bicyclic organophosphorus esters were encountered in the synthesis of the aliphatic aldehyde which could be triolised fairly simply and then ring closed with phosphorus oxychloride for the final products.

Synthesis of these aldehydes was fairly simple in the case of the cyclopentyl and neopentyl compounds because of the success of acid hydrolysis of the diethoxyl compounds. When this was attempted for the dimethyl propyl and thexyl compounds it failed, therefore other methods of making the corresponding aldehydes had to be attempted. Several methods outlined previously were tried from the literature available, with varying degrees of success, often giving very low and unsatisfactory yields and by products, which were difficult to separate from the aldehyde which was needed for the triolisation.

Eventually the tosylation method proved successful in both cases of dimethyl propyl and thexyl compounds. This method gave the comparatively good yield of over 40%, and the products formed were relatively pure which was essential for the next stage involving triolisation of the aldehyde.

When the dimethyl pentanal was successfully triolised a comparatively good yield of 23% was obtained for the triol. This was successfully ring closed to give the dimethyl propyl bicyclophosphate, but due to the surprisingly high water solubility of this compound a low yield of 13% was obtained because some was lost in the excess of water in the crystalisation.

When triolisation of the thexyl compound was attempted from the aldehyde, suprisingly it did not work as expected and shown in all the other comparable reactions. After purification by four runs on the silica column it was found analytically that there was no corresponding triol present and that the reaction had caused the aldehyde to form into the diol. Unfortunately the reaction conditions could not be changed for other reactions because all the aldehyde was used in the one reaction, and time did not permit for the resynthesis of the thexyl aldehyde. This was a pity because it was this bridge head substituent (thexyl) which most resembled that of the t-butyl substituted bicyclo phosphate, the most potent compound. The lipophillicity, size and shape of the thexyl group would probably cause the corresponding bicyclophosphate to be an extremely potent compound and so help as a tool in the understanding of how these antagonist have their actions.

CHAPTER 4 : PHARMACOLOGICAL EVALUATION OF GABA ANTAGONISTS AND SYNTHESISED BICYCLIC PHOSPHATES ON THE ACTIONS OF GABA AND CARBACHOL ON THE ISOLATED SUPERIOR CERVICAL GANGLION OF THE RAT

4.3.7 The effect of the depolarisation caused by carbachol using a set concentration of several GABA antagonists and varying the concentration of carbachol to obtain a dose-response relationship.

4.3.8 Conclusions

4.1 Introduction

GABA was demonstrated to be involved in the inhibitory mechanisms of sympathetic ganglia (De Groat, 1966, 1969). When GABA was applied to the cat superior cervical ganglion *in vivo* by intra-arterial injection it caused a depolarisation which was different from the effects shown by stimulating cholinoreceptors in the ganglion and appeared to resemble the central inhibitory effects of GABA (De Groat 1970). This depolarisation was different from the hyperpolarisation caused by GABA in mammalian centralneurones (Krnjević and Schwartz 1967, Dreifuss et al. 1969). However there was a similarity with the central actions of GABA with respect to uptake and transport mechanisms for GABA in the ganglion (Bowery and Brown 1972).

GABA appeared to cause a depolarisation of the neurones by a membrane permeability change to chloride ions (Adams and Brown 1974) which was comparable to permeability changes in central neurones measured in a similar way with intracellular electrodes (Krnjević and Schwartz 1967).

The difficulties encountered with iontophoretic application of drugs and the possibilities of the effects of endogenous GABA (Curtis et al. 1971c) could more easily be assessed under *in vitro* conditions using an extracellular recording technique with surface electrodes (Pascoe 1956, Brown 1966) in which drugs could be applied in known

concentrations.

The depolarisation can be detected on the ganglion surface when measured with respect to the postganglionic trunk, this depolarisation was transient and peaked after 10-15 seconds before diminishing with GABA still present. It was a low amplitude depolarisation with a maximum peak of approximately 2 millivolts (Bowery and Brown 1975).

The cholinomimetic, carbachol, also depolarised the ganglion but this was a sustained depolarisation lasting several minutes with a transient hyperpolarisation on washing out the drug. Bicuculline and picrotoxin selectively antagonised the depolarising actions of GABA with much less effect on those of carbachol. (Bowery and Brown 1974).

This depolarisation was not mediated by acetylcholine receptors as it was not blocked by concentrations of the acetylcholine antagonists, hexamethonium or hyoscine, in sufficient concentrations to antagonise the effects of carbachol.

4.2 Methods and Materials.

The method used was that essentially described by Bowery and Brown (1974) with a modification for superfusion (Brown and Marsh 1975).

Male Wistar rats, about 250g in weight were anaesthetised with urethane (1.4g/Kg, intraperitoneally). The superior cervical ganglion was removed with suitable lengths of pre- and post-ganglionic trunks attached. The connective tissue sheath was

stripped off and the ganglion mounted vertically with two silver/silver chloride electrodes placed in contact with the ganglion and its postganglionic trunk. The preparation was superfused with Krebs-Henseleit solution, aerated with 95% oxygen : 5% carbon dioxide, at a constant rate of 1cm^3/ minute.

All the drugs used were added to the superfusion solution. The ganglion surface potential, measured with respect to the postganglionic trunk, was continuously monitored with the electrodes connected to a high impedence ($<$1MΩ) X-T recorder (Smiths Servoscribe 1S) to produce a continuous voltage-time record. A full scale deflection of 2mV or 5mV was routinely used.

The depolarising agonist drugs (GABA and carbachol) were applied for one minute at 15 minute intervals. Antagonist drugs were added to the superfusion fluid and applied continuously 10 minutes before addition of agonist doses. The antagonists were then washed out for 15 minutes with Krebs-Henseleit solution before the addition of more agonist doses. Hyoscine (2.6μM), a muscarinic antagonist, was present in the superfusion fluid to limit the action of carbachol to the nicotinic receptors (Bowery and Brown 1974).

The following compounds were used:

λ-amino-n-butyric acid (4 amino-n-butyric acid, GABA; BDH); carbachol (carbaminoyl choline chloride, BDH); hyoscine hydrobromide (Martindale Samoore); picrotoxinin (Sigma); bicuculline methobromide; Isopropyl bicyclic phosphate

(4-(1 methyl ethyl) 2,6,7 trioxo-1-phosphabicyclo (2.2.2) octan-1-one); dimethyl propyl bicyclic phosphate (4-(1,2, dimethyl propyl) 2,6,7 trioxa-1-phosphabicyclo (2.2.2) octan-1-one); neopentyl bicyclic phosphate (4-(2,2, dimethyl propyl) 2,6,7 trioxa-1-phosphabicyclo (2.2.2) octan-1-one); cyclopentyl bicyclic phosphate (4-(cyclopentyl) 2,6,7 trioxa-1-phosphabicyclo (2.2.2) octan-1-one); tertiary butyl bicyclic phosphate (4-(1,1, dimethyl ethyl) trioxa-1-phosphabicyclo (2.2.2) octan-1-one.

The superfusion solution was Krebs-Henseleit solution with the following composition:

Sodium chloride, NaCl, 118.0mM; Potassium chloride, KCl, 4.8mM; Magnesium sulphate hydrated, $MgSO_4 . 7H_2O$, 1.19mM; Potassium dihydrogen phosphate, KH_2PO_4, 1.18mM; Calcium chloride, $CaCl_2$, 2.52mM; sodium hydrogen carbonate, $NaHCO_3$, 25.0mM; D-glucose, $C_6H_{12}O_6$, 11.0mM, Plus hyoscine hydrobromide, 2.6μM, added to suppress any muscarinic actions of carbachol. The solution was aerated with a 95% oxygen : 5% carbon dioxide mixture to bring the solution to pH 7.4.

4.3 RESULTS: AND CONCLUSIONS

4.3.1 The effect of the depolarisation caused by GABA using a set, submaximal dose and varying the concentration of the antagonists.

A dose of GABA of 30μM was chosen as the set dose because it gave approximately 70-80% of the maximal response. This was applied for 1 minute each time.

(4-(1 methyl ethyl) 2,6,7 trioxo-1-phosphabicyclo (2.2.2) octan-1-one); dimethyl propyl bicyclic phosphate (4-(1,2, dimethyl propyl) 2,6,7 trioxa-1-phosphabicyclo (2.2.2) octan-1-one); neopentyl bicyclic phosphate (4-(2,2, dimethyl propyl) 2,6,7 trioxa-1-phosphabicyclo (2.2.2) octan-1-one); cyclopentyl bicyclic phosphate (4-(cyclopentyl) 2,6,7 trioxa-1-phosphabicyclo (2.2.2) octan-1-one); tertiary butyl bicyclic phosphate (4-(1,1, dimethyl ethyl) trioxa-1-phosphabicyclo (2.2.2) octan-1-one.

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4.3 RESULTS: AND CONCLUSIONS

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Fig.4.1. Effect of increasing concentrations of t-butyl bicyclophosphate (■), picrotoxinin (○) and (+)-bicuculline methobromide (▽) on ganglion depolarisation produced by γ-aminobutyric acid (GABA) at a submaximal concentration of 3μM. Depolarisation is expressed as % reduction in response obtained in the absence of antagonist. Each point and vertical bar gives the mean and standard error for at least six measurements. Lines are drawn by eye.

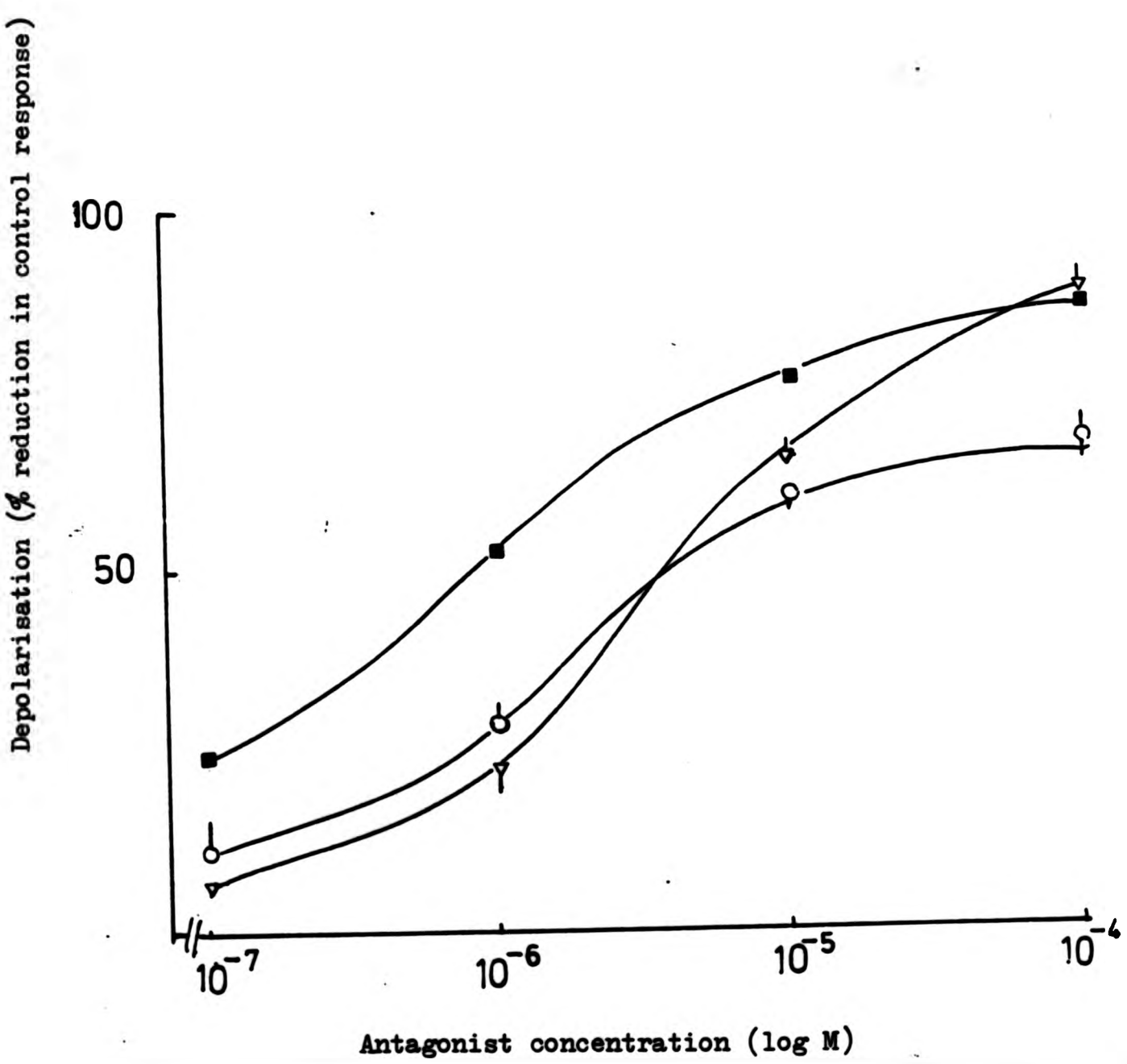


Fig.4.2. Effect of increasing concentrations of isopropyl bicyclophosphate (▽) and cyclopentyl bicyclophosphate (▼) on ganglion depolarisation produced by γ-aminobutyric acid (GABA) at a submaximal concentration of 3μM. Depolarisation is expressed as % reduction in response obtained in the absence of antagonist. Each point and vertical bar gives the mean and standard error for at least seven measurements. Lines are drawn by eye.

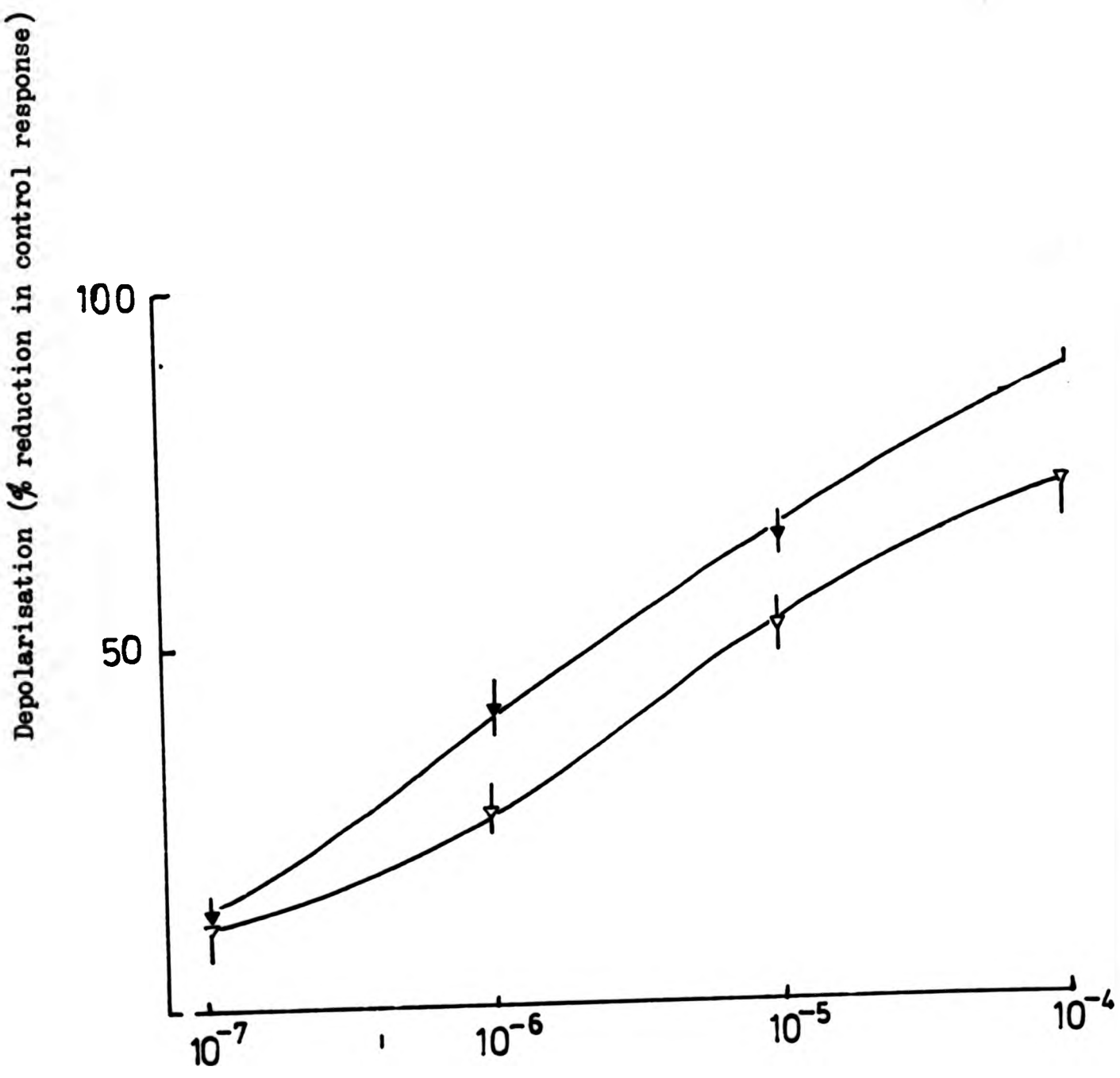
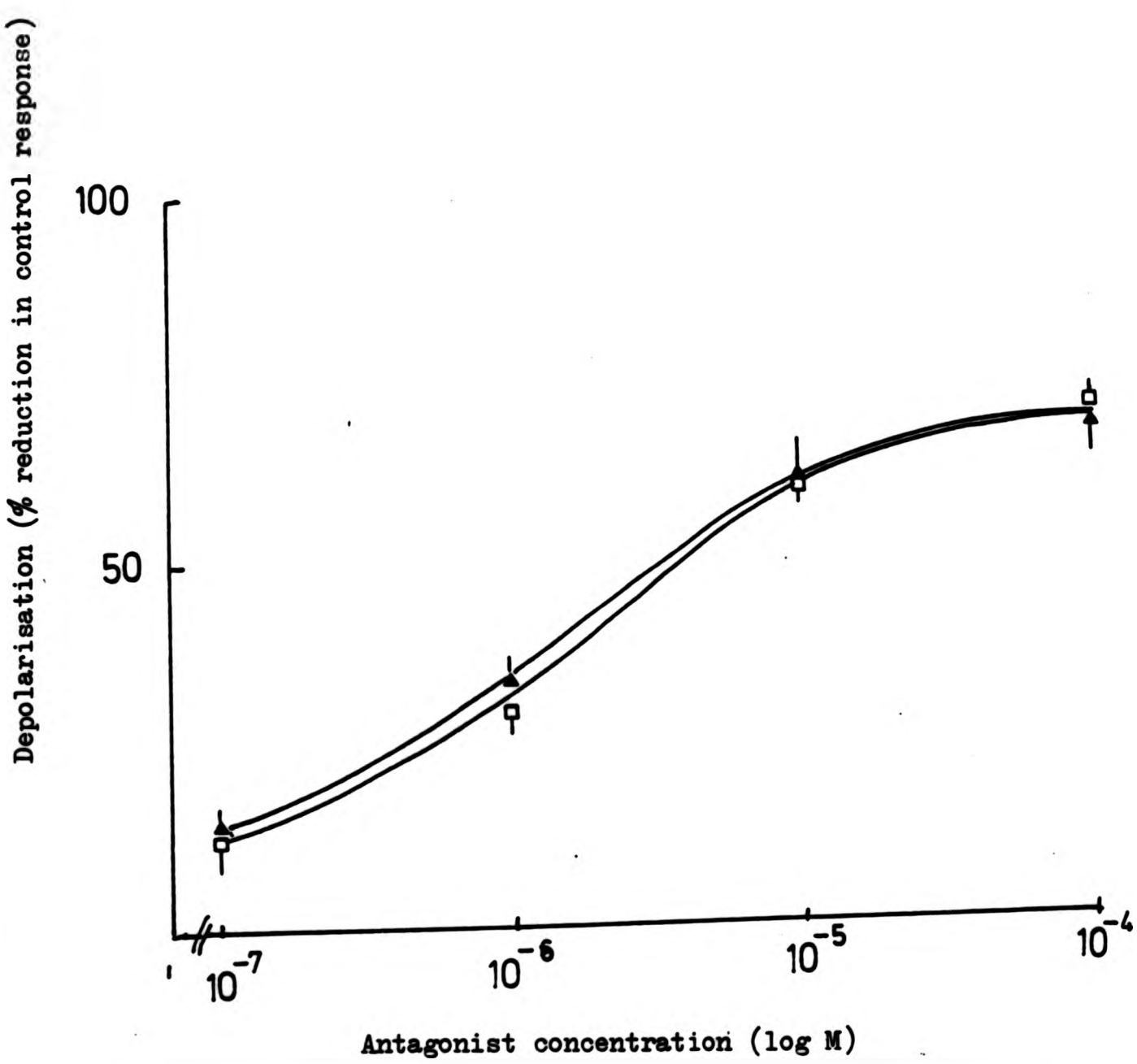


Fig.4.3. Effect of increasing concentrations of Neopentyl bicyclophosphate (□) and Dimethyl propyl bicyclophosphate (▲) on ganglion depolarisation produced by γ-aminobutyric acid (GABA) at a submaximal concentration of 3μM. Depolarisation is expressed as % reduction in response obtained in the absence of antagonist. Each point and vertical bar gives the mean and standard error for at least 7 measurements.



Four concentrations for each antagonist were tested these being 10^{-7}M, 10^{-6}M, 10^{-5}M and 10^{-4}M.

The antagonist was applied 10 minutes previous to the dose of GABA and a reduction in the depolarisation due to GABA was seen in all antagonists tested, even at the lower concentration of 10^{-7}M antagonist present in the superfusing fluid. The antagonist was then washed out completely (30–60 minutes) before another concentration of antagonist could be applied and the effect on the depolarisation to GABA (30μM) could be measured on the polygraph.

Approximately seven determinations for each concentration of antagonist were performed in order to determine the percentage inhibition of the GABA response. The results of these determinations were collated and analysed showing the mean per centage inhibition of GABA with standard errors of the mean for 6–9 determinations.

These results were then represented graphically (Figs. 4.1 - 4.3). From these graphs it could be seen that all seven of the antagonists tested were very effective in their ability to inhibit the depolarisation caused by GABA in this preparation.

The IC_{50} values (the concentration which caused a 50% reduction in response to GABA) were calculated from the graphs and tabulated in Table 3.

TABLE 3

The IC_{50} values (the concentration of antagonist which caused a 50% reduction in the response to GABA), calculated from the values obtained in Figs. 4.1-4.3 for each antagonist.

Antagonist	IC_{50} Value
(+)-Bicuculline methobromide	7.4×10^{-6}M
Picrotoxinin	3.1×10^{-6}M
Cyclopentyl bicyclic phosphate.	1.8×10^{-6}M
Neopentyl bicyclic phosphate	3.0×10^{-6}M
Dimethyl propyl bicyclic phosphate	1.0×10^{-6}M
Isopropyl bicyclic phosphate	5.1×10^{-6}M
Tertiary butyl bicyclic phosphate	8.6×10^{-7}M

i. <u>(+) - Bicuculline methobromide</u>

An IC_{50} value of 7.4×10^{-6}M was obtained with bicuculline which proved to the the least potent in antagonism of the responses to GABA (30μM).

ii. <u>Picrotoxinin</u>.

An IC_{50} value of 3.1×10^{-6}M was obtained for picrotoxinin showing it to be approximately twice as potent as (+) - bicuculline methobromide in this preparation.

iii. <u>Cyclopentyl bicyclic phosphate.</u>

An IC_{50} value of 1.8×10^{-6}M was obtained showing it to be a more potent GABA antagonist than picrotoxinin and (+) - bicuculline methobromide but not as potent an antagonist as the tertiary butyl bicyclic phosphate.

iv. <u>Neopentyl bicyclic phosphate</u>.

An IC_{50} value of 3.0×10^{-6}M was obtained for this; again being more potent than (+) - bicuculline methobromide, about equipotent to picrotoxinin and not as potent as the tertiary butyl bicyclic phosphate.

v. <u>Dimethyl propyl bicyclic phosphate</u>.

An IC_{50} value of 1.0×10^{-6}M was obtained for this compound and was more potent than all the other compounds in inhibiting GABA but again not as potent as the tertiary butyl derivative.

vi. <u>Isopropyl bicyclic phosphate</u>.

An IC_{50} value of 5.1×10^{-6}M showed it to be the least potent of the bicyclic phosphate esters which

were tested here but it was more potent than (+) - bicuculline methobromide.

viii. Tertiary butyl bicyclic phosphate

This was the most potent antagonist tested with an IC_{50} of 8.6×10^{-7}M which was 8.6 times the potency of bicuculline methobromide.

4.3.2 Conclusions:

The most potent antagonist of the effects of GABA on depolarisation of the surface of the superior cervical ganglion of the rat was the tertiary-butyl analogue. This finding would agree with other structure-toxicity studies on the bicyclic phosphates (Eto et al. 1976, Bowery et al. 1977, Cooper et al. 1978, Olsen et al. 1979). In accordance with other structure-toxicity studies the other bicyclic phosphates were in the order of dimethyl propyl, cyclopentyl, neopentyl and the isopropyl derivative being the least active, this was however of the same order as (+) - bicuculline methobromide which was found to be the least active of compounds tested.

Picrotoxinin was found to be equipotent as the neopentyl bicyclic phosphate and of the same order of potency as the other bicyclic phosphates in agreement with Davidson et al. (1977). In this study, however, it was found to be more than twice as potent as (+) - bicuculline methobromide which is in contradiction with some of the studies on mammalian GABA-ergic systems (Gahwiler 1975, Olsen et al. 1975). When the higher

concentrations of picrotoxinin were applied they were found to be difficult to wash out of the preparation and therefore possibly acting as a partially-reversible antagonist.

TABLE 4

Table of % inhibition (or potentiation) of the depolarising effect of carbachol (33μM) on the superior cervical ganglion of the rat in the presence of a high concentration (10^{-4}M) of several GABA antagonists.

Antagonist	No. of determinations	% Inhibition of response to carbachol (33μM) by antagonist (10^{-4})	Inhibition or Potentiation caused
(+)-Bicuculline methobromide	7	31.9 ± 2.4%	Inhibition
Picrotoxinin	6	21.5 ± 4.0%	Inhibition
Cyclopentyl bicyclic phosphate	7	- 4.9 ± 1.3%	Potentiation
Neopentyl bicyclic phosphate	7	-16.0 ± 4.8%	Potentiation
Dimethyl propyl bicyclic phosphate	7	-20.6 ± 7.0%	Potentiation
Isopropyl bicyclic phosphate	6	-14.2 ± 2.5%	Potentiation
Tertiary butyl bicyclic phosphate	5	-19.5 ± 3.9%	Potentiation

4.3.3 <u>The effect of the depolarisation caused by carbachol using a set, submaximal dose and a single high concentration of antagonist</u>.

A dose of carbachol of 33μM was chosen as the set dose because it gave approximately 80% maximal response. This was applied for 1 minute each time.

The highest concentration of antagonist (10^{-4}M) was used to test the effects of carbachol after previously obtaining a depolarising response in the presence of no antagonist.

Approximately six determinations for each antagonist dose of 10^{-4}M were performed in order to determine the effects of the antagonists on carbachol. The results of these experiments are tabulated in Table 4 showing the mean percentage inhibition of carbachol with standard errors of the mean for 5-7 determinations.

i. <u>(+) - bicuculline methobromide</u>.

At the concentration used (10^{-4}M) there was an inhibition of approximately 30% of the normal control response to carbachol.

ii. <u>Picrotoxinin</u>.

This also caused an inhibition to the effects of carbachol but not as potently as (+) - bicuculline methobromide giving a 20% reduction from the control value.

iii. <u>The bicyclic phosphates</u>.

All five compounds tested caused a potentiation in

the depolarising effect of carbachol (33μM) on the ganglion ranging from a 5% potentiation for the cyclopentyl derivative to a 20% potentiation with the tertiary butyl derivative and the dimethyl propyl derivative.

Conclusions:

All determinations were done only at the highest concentration of antagonist (10^{-4}M). The inhibition of carbachol responses caused by (+) - bicuculline methobromide was found to be in agreement with reports on the cervical ganglion (Bowery et al. 1978b, Cryer 1979). It is not known whether the actions of (+) - bicuculline methobromide are specific for carbachol antagonism.

Picrotoxinin also inhibited the depolarising actions of carbachol in a similar fashion to (+) - bicuculline methobromide but would appear not to be as potent an antagonist. The mechanisms of these actions have not been investigated thoroughly and could be non-specific actions due to the high concentrations of antagonists in the preparation as there was very little antagonism for concentrations which dramatically affected the actions of GABA on the ganglion.

In all cases with the bicyclic phosphates there was a potentiation of the depolarisation of the ganglion surface on

application of carbachol; this was followed by a transient hyperpolarisation when the carbachol was washed out. The mechanism of action of this effect is not known but may be non-specific due to the high concentration of antagonist applied. When lower concentrations of bicyclic phosphates, sufficient to antagonise the depolarising actions of GABA were used very little effect on depolarisation caused by carbachol was observed.

Fig.4.5.Log concentration-depolarisation relationship for γ-aminobutyric acid (GABA) on rat superior cervical ganglion in the absence (▽) and in the presence of 23μM dimethylpropyl bicyclophosphate (■) and 24μM picrotoxinin (▲). GABA doses were applied for 1 min periods at 15 min intervals. Antagonists were added 30 min before testing to allow equilibration. Each point and vertical bar gives the mean and standard errors for at least 5 observations.

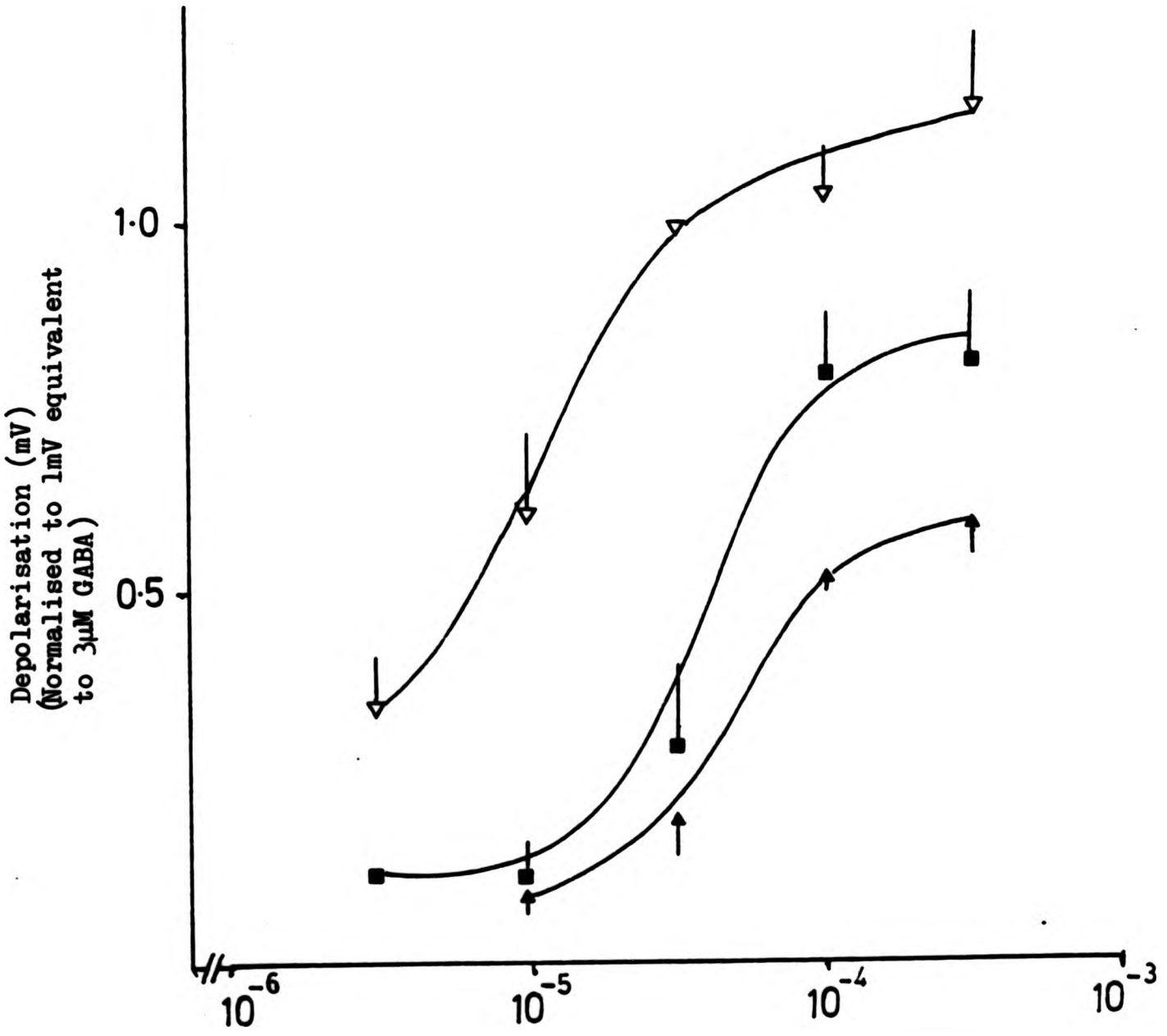


Fig.4.6. Log concentration-depolarisation relationship for γ-aminobutyric acid (GABA) on rat superior cervical ganglion in the absence (▽) and in the presence of 6.5μM (+)-bicuculline methobromide (▲) and 23μM cyclopentyl bicyclophosphate (□). GABA doses were applied for 1 min periods at 15 min intervals. Antagonists were added 30 min before testing to allow equilibration. Each point and vertical bar gives the mean and standard errors for at least 5 observations.

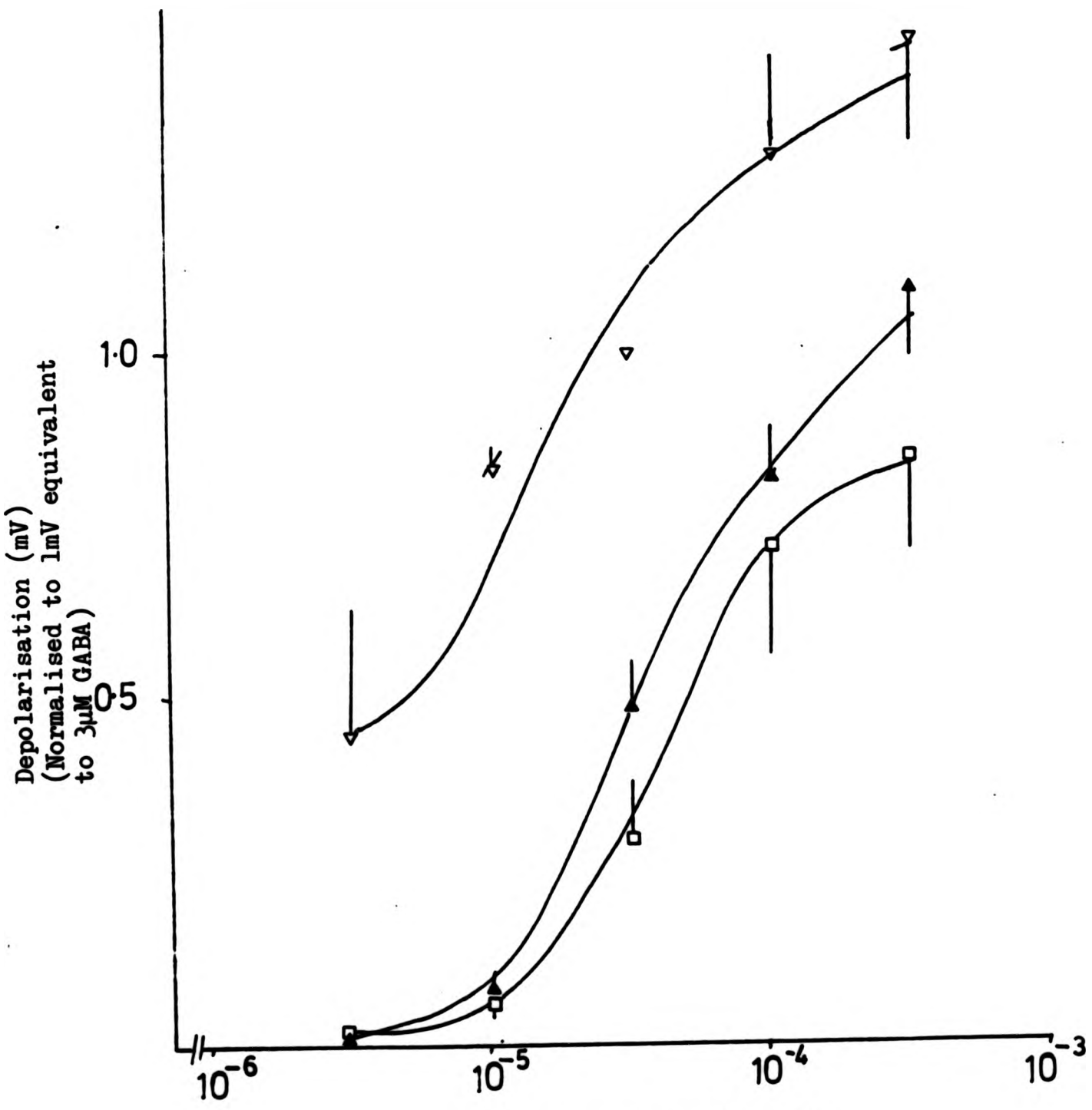
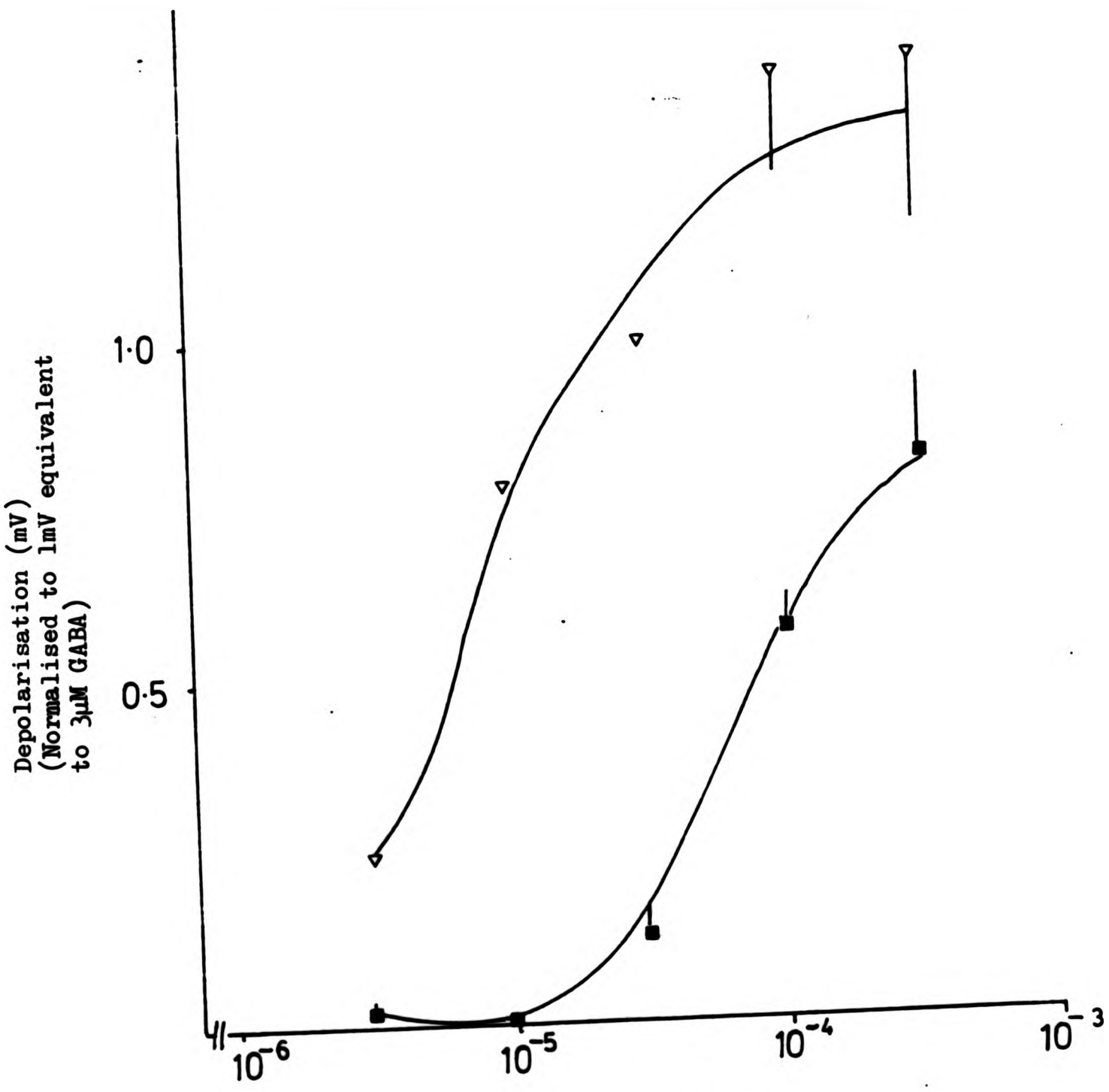


Fig.4.7. Log concentration-depolarisation relationship for γ-aminobutyric acid (GABA) on rat superior cervical ganglion in the absence (▽) and in the presence of 23μM Neopentyl bicyclophosphate (■). GABA doses were applied for 1 min periods at 15 min intervals. Antagonists were added 30 min before testing to allow equilibration. Each point and vertical bar gives mean and standard errors for at least 5 observations.



4.3.5 <u>The effect of the depolarisation caused by GABA using a set concentration of several antagonists and varying the concentrations of GABA in order to obtain a dose-response relationship</u>.

The concentration of antagonist applied in each case was that which gave a 50-60% inhibition of the submaximal concentration of GABA determined in the previous set of experiments.

A range of 5 increasing concentrations of GABA was used in order to obtain a range of results which would provide a good dose-response curve. The concentrations used were 3×10^{-6}M for the minimum concentration up to 3×10^{-4}M to obtain a <u>maximum</u> effect.

The results from 5-8 experiments were used in the control (without the presence of any antagonist) and repeated in the presence of a predetermined concentration of antagonist.

The results of these experiments are represented graphically in Figs. 4.4 - 4.7.

The graphs were plotted semi-logarithmically where depolarisation in mV (normalised to 1mV for a concentration of 3×10^{-5}M GABA) on the ordinate against the log of the concentration of GABA as a control experiment and also in the presence of antagonist on the abscissa.

i. <u>(+) - Bicuculline methobromide</u> (6.5×10^{-6}M)

A shift in the dose-response curve to the right indicating competitive antagonism to GABA as the control and experiment in the presence of antagonist

curves were parallel to each other.

ii. Picrotoxinin (2.4×10^{-5}M)

This also caused a significant inhibition of the responses to GABA but the maximum height of the dose response curve with picrotoxinin present was reduced to about 50% of that of the control dose-response curve indicating a non-competitive type of antagonism.

iii. The bicyclic phosphates. ($2.3-2.5 \times 10^{-5}$M)

Again the antagonism in these experiments seemed to be of a non-competitive nature with all the dose-response curves being moved to the right and also reduced in height indicating that a maximal response would not be obtained with a greater increase in the concentration of GABA applied. The results from the graphs show that there was little difference in the effects of all the different derivatives used.

4.3.6 Conclusions:

There was some difference between the effects of (+) - bicuculline methobromide, the bicyclic phosphates and picrotoxinin indicating they may not have the same mode of action in the antagonism of the effects of GABA in this preparation., suggesting different modes of action.

The least effective in shifting the dose-response curves to GABA depolarisation of the cervical ganglion from the graphs would appear to be (+) - bicuculline methobromide but it must be noted that the concentration of this drug in the experiments was about one fourth that of the other drugs used

so this would account for the unexpected apparent weakness of (+) - bicuculline methobromide thus it was not possible to make any direct comparisons with the effect of the other compounds.

Picrotoxinin was also a potent inhibitor of the depolarising effects of GABA and the inhibition produced appeared to be non-competitive suggesting its actions are at a different site to that of the GABA receptor. These findings are in agreement that picrotoxinin exerts its actions at the ion channel (Ticku and Olsen 1977). Picrotoxinin was more potent than some of the bicyclic phosphates but the results showed that potencies of these compounds were similar to that of picrotoxinin.

All the analogues of the bicycylic phosphates had similar potencies and were potent inhibitors of the depolarising actions of GABA. Similar results in agreement with those found for the isopropyl analogue by Bowery et al. (1976a, 1978a) were obtained in these experiments. The tertiary butyl compound was also shown to have a similar antagonistic activity as found by Bowery et al. (1977). The other analogues of bicyclic phosphates all appeared to exert a similar antagonistic activity and this method would appear to be a useful one to elucidate the potency of these compounds but not a good method for distinguishing between the different analogues.

Fig.4.8. Log concentration-depolarisation relationship for carbachol on rat superior cervical ganglion in the absence (○) and in the presence of 6.5μM (+)-bicuculline methobromide (■). Carbachol doses were applied for 1 min periods at 15 min intervals. Antagonists were added 30 min before testing to allow equilibration. Each point and vertical bar gives the mean and standard error for 6 observations.

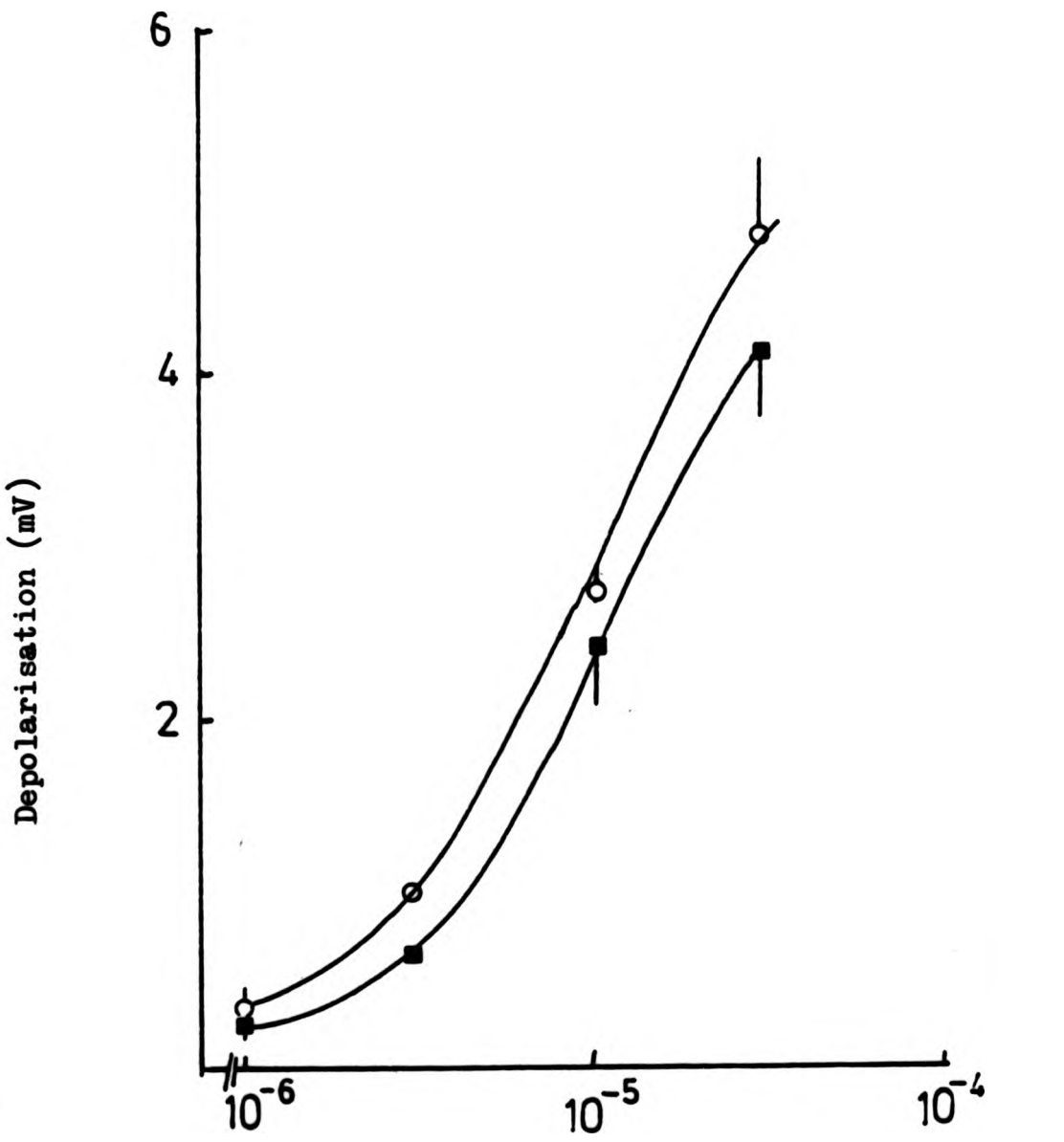


Fig.4.9. Log concentration-depolarisation relationship for carbachol on rat superior cervical ganglion in the absence (O) and in the presence of 25μM picrotoxinin (▲). Carbachol doses were applied for 1 min periods at 15 min intervals. Antagonists were added 30 min before testing to allow equilibration. Each point and vertical bar is the mean and standard error for at least 4 observations.

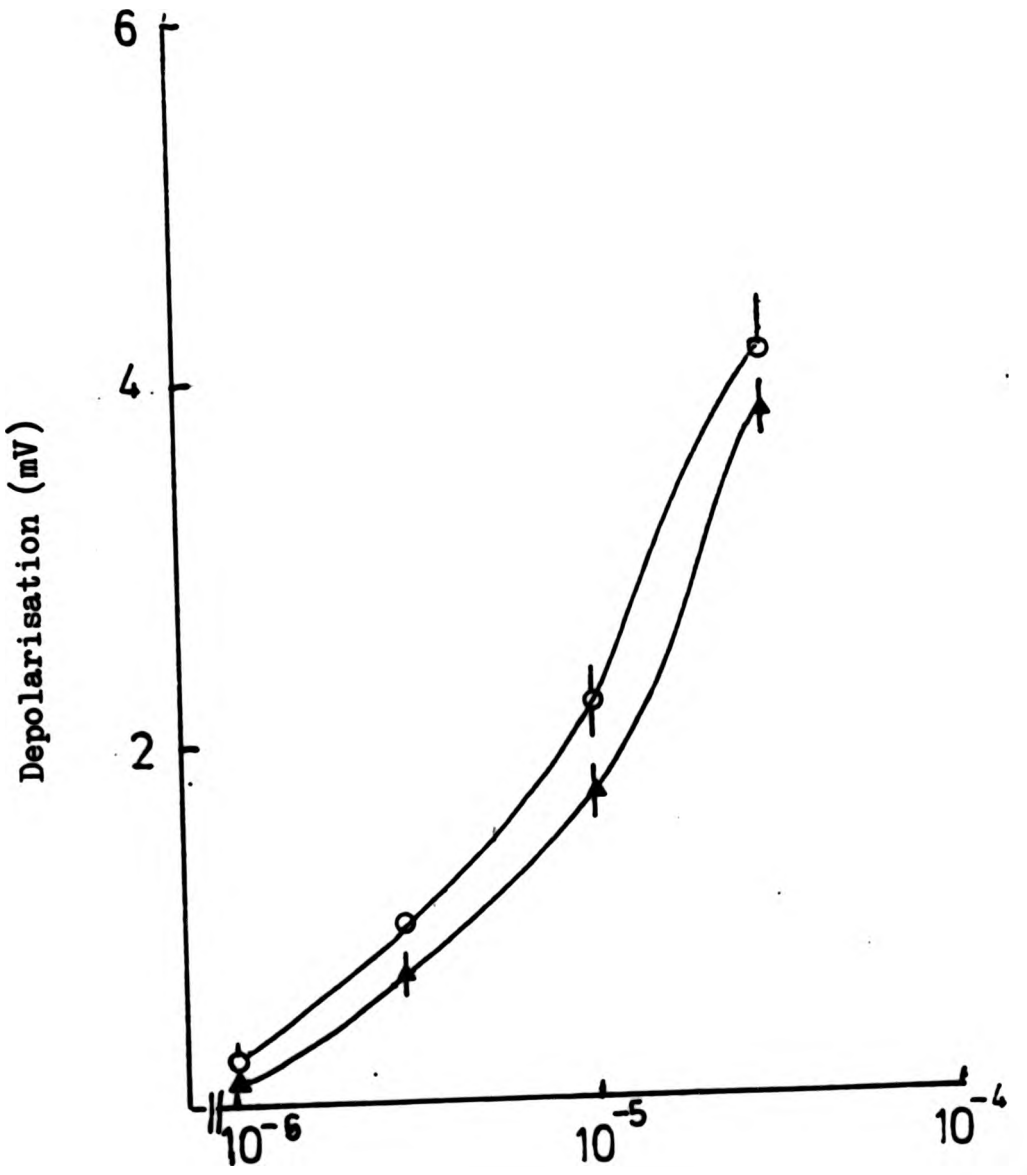


Fig.4.4. Log concentration-depolarisation relationship on rat superior cervical ganglion for γ-aminobutyric acid (GABA) in the absence (▽) and in the presence of 23μM t-butyl bicyclophosphate (□) and 25μM isopropyl bicyclophosphate (○). GABA doses were applied for 1 min periods at 15 min intervals. Antagonists were added 30 min before testing to allow equilibration. Each point and vertical bar gives the mean and standard errors for at least 5 observations. Lines are drawn by hand.

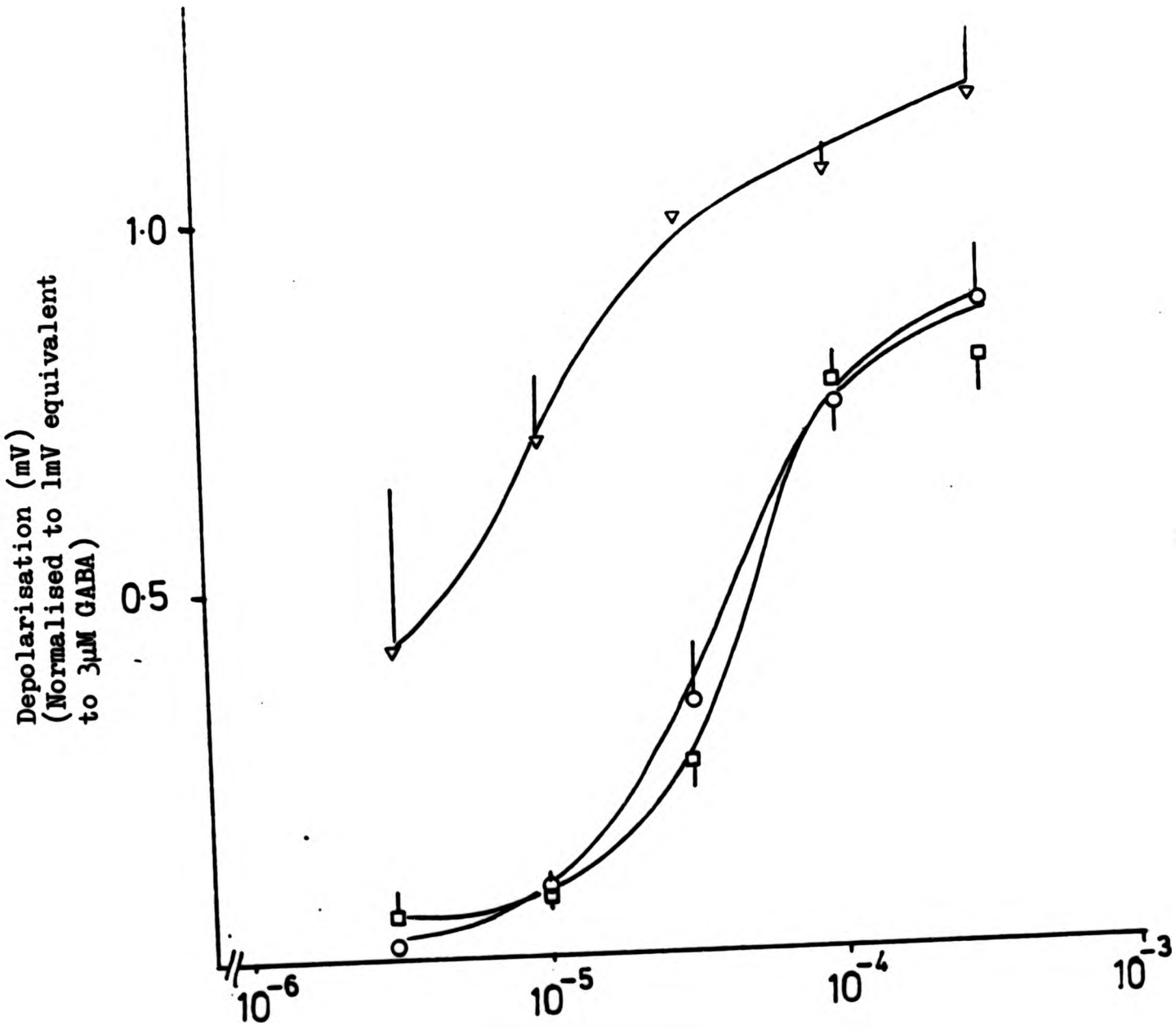
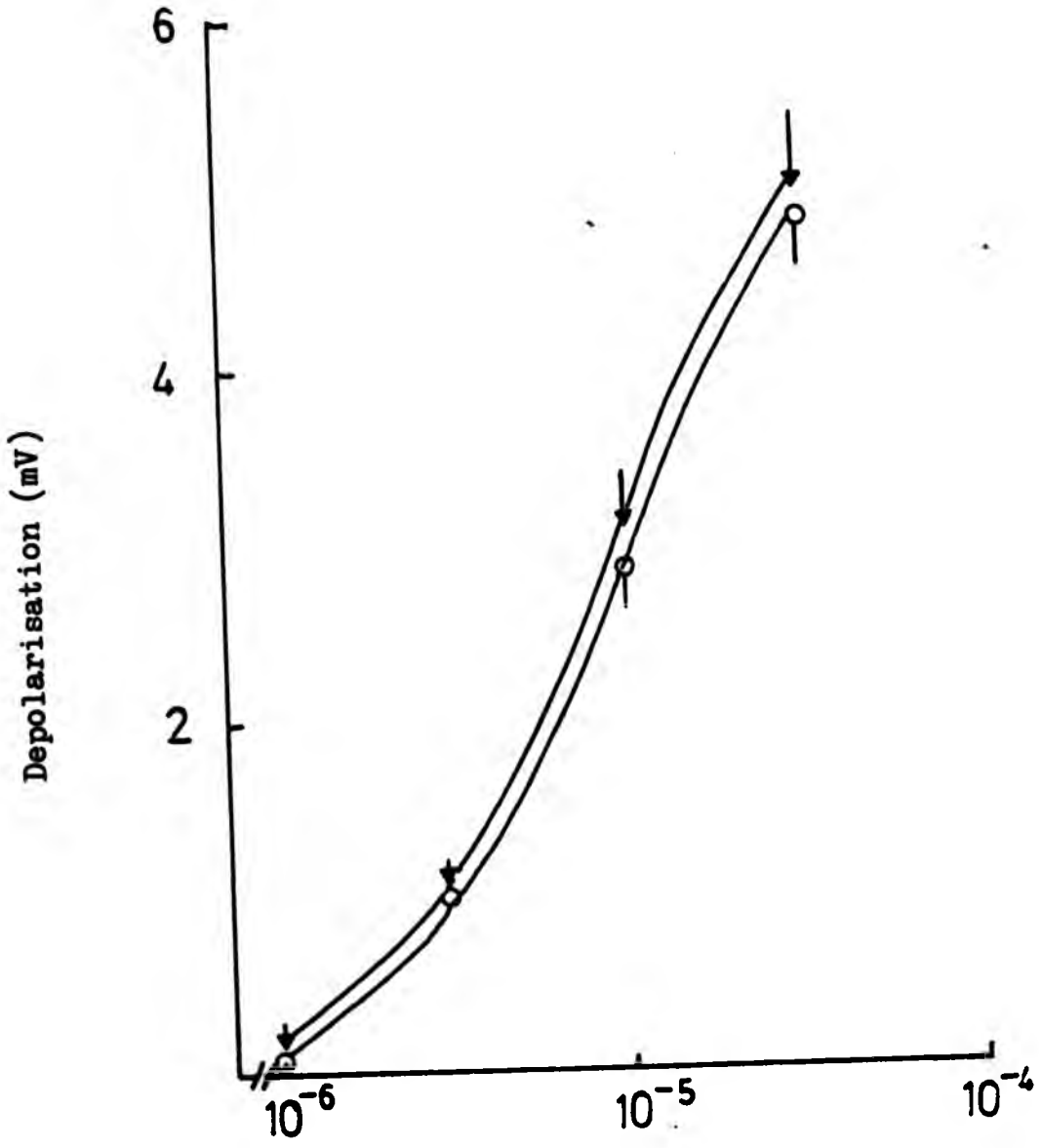


Fig.4.10. Log-concentration relationship for carbachol on rat superior cervical ganglion in the absence (o) and in the presence of 23μM Neopentyl bicyclophosphate (▼). Carbachol doses were applied for 1 min periods at 15 min intervals. Antagonists were added 30 min before testing to allow equilibration. Each point and vertical bar is the mean and standard error for at least five observations.



4.3.7 <u>The effect of the depolarisation caused by carbachol using a set concentration of several GABA antagonists and varying the concentration of carbachol to obtain a dose-response relationship</u>.

As in the previous experiment the concentration of antagonist used was that which gave a 50-60% inhibition of the submaximal concentration of GABA.

A range of four increasing concentrations of carbachol was used in order to obtain a range of results to give a dose-response curve. The concentrations used were 1 x 10^{-6}M for the minimum concentration up to 3 x 10^{-5}M for the maximum concentration.

The results from 4 - 6 experiments were used in the controls (without antagonist present) and then repeated in the presence of antagonist.

The graphs were again plotted semi-logarithmically where depolarization in mV(normalised to 1mV for a concentration of carbachol of 3 x 10^{-5}M)
on the ordinate against the log of the concentration of carbachol as a control and also in the presence of antagonist on the abscissa. (Figs. 4.8 - 4.10)

i. <u>(+) - Bicuculline methobromide</u> (6 x 10^{-6}M)

A slight shift of the dose-response curve to the right shows that (+) - bicuculline methobromide does have an antagonistic effect on carbachol. This is not

a great antagonism, and as reported previously there was about a 30% reduction in the response to carbachol with a mugh higher concentration of the antagonist.

ii. Picrotoxinin (2.4×10^{-5}M)

Again there was a slight shift of the dose-response curve to the right indicating some antagonism but as reported previously in high concentrations of picrotoxinin (10^{-4}M) there was only a 20% reduction in the response to carbachol.

iii. Neopentyl bicyclic phosphate (2.4×10^{-5}M)

The dose-response curve was shifted slightly to the left but the effect was insignificant. This would appear however to have a slight potentiating effect on the responses to carbachol as shown in higher concentrations in the previous experiments.

4.3.8 Conclusions:

The results using GABA antagonists, with carbachol, in lower concentrations (i.e. those which would effectively block the responses to GABA significantly), are inconclusive as the results indicated in the graphs but it could be seen that there was a trend for (+) - bicuculline methobromide and picrotoxinin to shift the dose-response curves to the right and this would suggest some antagonism as borne out with the higher concentrations used previously. However the trend to

shift the dose-response curve to the left with neopentyl bicyclic phosphate also bears out that these compounds have a slight potentiating effect on the actions of carbachol even in large concentrations are needed.

CHAPTER 5 : A STUDY OF THE GABA RECEPTOR USING BINDING STUDIES ON A SYNAPTOSOMAL FRACTION OF RAT BRAIN

5.8.4 Binding of (^{3}H)-muscimol to rat brain synaptosomal preparation in the presence of GABA agonists and antagonists

5.8.5 Conclusions

5.9 Binding studies using (^{3}H)-α-dihydropicrotoxinin on rat brain synaptosomal membranes

5.9.1 Specific and non-specific binding of (^{3}H)-α-dihydropicrotoxinin

5.9.2 Calculation of the number of binding sites from stereospecific binding

5.9.3 Binding of (^{3}H)-DHP to rat brain synaptosomal preparation in the presence of GABA agonists and antagonists

5.9.4 Conclusions

A Study of the GABA receptor using binding studies.

5.1 Introduction

Investigation of the biochemical nature of receptors is important in the understanding of the basic physiology of the actions of transmitters and also to investigate the mechanism of action of drugs which exert their actions at the receptor site or those which are related to the receptor site. These methods can therefore be useful in the designing of new drugs which may exert their effects at these sites (Young et al. 1976).

The technique of binding studies with a suitable radioactive ligand allows convenient investigation in an *in vitro* system. It is important that the binding site really represents a physiological or pharmacological receptor.

Correlations between binding studies and other *in vitro* preparations include opiate receptor binding affinities with the effectiveness in inhibiting or blocking inhibition of electrically induced contractions of guinea-pig ileum (Creese and Snyder, 1975).

Clearly in the measurement of *in vivo* responses to a drug many more variables are involved and not just the affinity of the drug for its receptor; these may be reduced by application of drugs by microiontophoresis and measuring the response of individual neurons but this method, although giving good comparative data among drugs, is often difficult to quantify. However, dopamine receptor binding has been used to predict *in vivo* behavioural responses to some antischizophrenic drugs (Creese et al. 1976).

This technique with binding studies with radiolabelled ligands does allow convenient investigation of an *in vitro* system. In these binding studies however, it is important that the actual

binding observed must be actual binding to the receptor sites and not those involved with uptake mechanisms, or non-specific sites such as enzymes or charged moeties in the tissues being used. For example some radiolabelled ligands can bind to inert materials, radiolabelled opiates bind to glass fibre filters with affinities in the nanomolar range to exhibit an apparent pharmacological specificity but obviously not matching any biological effects (Snyder et al. 1975a).

To ensure that the binding is to the actual receptor the following basic criteria must be fulfilled: (Snyder, 1975b, Birdsall and Hulme, 1976).

1. Specificity.
2. Saturability.
3. Comparability.
4. Localisation.

5.1.1 Specificity.

Concentrations of drugs which are pharmacologically active at a particular receptor site should displace the saturable component of the binding and pharmacologically active concentrations of drugs acting at different receptors should be ineffective. Known structure-activity relationships must be complied with and the receptor must have a high affinity for the radiolabelled ligand. For example in the mammalian muscarinic cholinergic system only those drugs such as acetylcholine will displace the radioligand in pharmacologically active concentrations and the effects of other types of drugs such as noradrenaline do not displace the radioligand (Snyder et al. 1975b).

5.1.2 Saturability.

A requirement for binding is that there are only a finite number of sites in the tissue and these are of high affinity. This refers to reversible ligands used with dissociation constants in the nanomolar range. Therefore the higher the concentration of radiolabelled drug added the binding will increase to the point where all sites are occupied, but no further, so it is saturated.

Too large a number of sites, accompanied by a low affinity suggests that the binding is not to a receptor. Maximal den.sities of neurotransmitter receptor-binding sites are usually about 10-100 pmoles ligand bound per gram of tissue. If all ligands do not yield a similar number of binding sites then it is probable they cannot be identified with the same receptor. These criteria have been shown in the binding of (^{3}H) quinudidinyl benzylate to muscarinic cholinergic receptor binding in guinea-pig ileum muscle (Yamamura and Snyder, 1975).

5.1. 3 Comparability.

The pharmacology of the binding should correlate quantitatively with the pharmacology of receptor-mediated effects and physiological responses. Excellent correlations have been reported between measurements of stimulation and blockade of β-receptor adenylate cyclase activity and measurements of competition for (^{3}H)-dihydroalprenololbinding (Mukherjee et al. 1976).

5.1. 4. Localisation.

Binding should be localised to tissues which are known to

show the pharmacological response and should not be found where the transmitter is known to be absent.

Autoradiographic studies using (^{3}H) muscimol on rat cerebellar tissues have shown that is is only associated with those areas which have the characteristics of the GABA-receptor, being saturable with kinetic constant similar to those in homogenate preparations (Palacios et al. 1980, DeFeudis et al. 1980).

5.2. Binding studies with brain tissues on the opiate receptor.

The method of using binding studies with radiolabelled ligands was originally developed to study the opiate receptor by Goldstein et al, (1971).

The analgesic potency of the opiates is highly stereospecific and almost all the pharmacological activity of these stereoisomers is found in those with a configuration of (-) - morphine for example (+) and (-) - levorphanol. The (+) - isomer dextrorphan, has been shown to be pharmacologically inert and has neither agonist nor antagonist properties (Kosterlitz et al. 1974, Pert and Snyder 1973).

Studies with the opiate narcotic (^{3}H) - (-) - levorphanol as the radioligand on crude P_1 fractions of mouse brain tissue showed that the total stereospecific binding was only about 2%. However, using a modification of Goldstein's procedure, reports of stereospecific binding of opiates to rat brain homogenates represented a major part of the total binding (Simon et al. 1973, Terenius 1973, Pert and Snyder 1973). The modifications involved using very low concentrations of labelled opiate with high specific activity and washing membranes after incubation to remove unbound

and loosely bound radioactivity.

These stereospecific binding sites were found to be closely associated with membrane fractions of tissue homogenates most concentrated in the synaptosomal cell fraction of the homogenate suggesting location of the sites is in the vicinity of synapses (Pert et al. 1974, Hitzman et al. 1974).

However, despite the small amount of stereospecific binding produced by Goldstein, he was able to demonstrate the existence of three different types of interaction between the opiate and the receptor tissues; thus enabling him to differentiate between stereospecific interactions and non-specific interactions of the opiate and receptors:

a) Non-saturable interaction which consisted of physical solution of lipophillic opiate molecules in the lipid membranes of the tissue.

b) Non-specific saturable binding consisting of interactions between the protonated nitrogen atom in the opiate and anionic groups in the membrane proteins and macromolecules.

c) Stereospecific interaction of the (-) - opiate with the actual opiate receptor.

Therefore, in order to calculate the stereospecific binding to the opiate receptor several experiments were carried out:

i) The tissue was incubated with (-) - opiate which was the radiolabelled ligand to give the sum of all three types of binding listed above.

ii) The tissue was incubated with excess (+) - opiate and radiolabelled (-) - opiate. The bound radioactivity could

then be measured and subtracted from the total bound radioactivity above to give the non-specific saturable binding.

iii) The tissue was then incubated with excess non-radiolabelled (-) - opiate and radiolabelled (-) - opiate and radioactivity measured. This procedure caused the radiolabelled (-) - opiate to be excluded from the non-specific and the stereospecific sites. Therefore the stereospecific binding could be obtained by subtraction of this from that calculated for the non-specific sites.

The studies discussed so far did not distinguish between a potent agonist or a potent antagonist in the binding assay. This problem was resolved when it was shown there was an inhibition of binding of all agonists and an enhancement of binding of antagonist was produced in the presence of sodium ions (Pert and Snyder 1974) demonstrated by increase in the number of high affinity binding sites for naloxone and a decrease in the high affinity binding sites for dihydromorphine. These results were consistent with a model involving the conformational change of the opiate receptor in the presence of sodium ions. When a pure antagonist (e.g. naloxone) was allowed to compete with a labelled antagonist, there was little or no change in the IC_{50} of the competitor when sodium ions were added. But when an unlabelled agonist (e.g. dihydromorphine) was allowed to compete with a labelled antagonist, the IC_{50} for the agonist increased dramatically in the presence of sodium ions (Simon et al. 1975) suggesting a change in affinity for the receptor but no change in the number of binding sites.

then be measured and subtracted from the total bound radioactivity above to give the non-specific saturable binding.

iii) The tissue was then incubated with excess non-radiolabelled (-) - opiate and radiolabelled (-) - opiate and radioactivity measured. This procedure caused the radiolabelled (-) - opiate to be excluded from the non-specific and the stereospecific sites. Therefore the stereospecific binding could be obtained by subtraction of this from that calculated for the non-specific sites.

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Fig. 5.1. A schematic model of the opiate receptor (after Snyder 1975a).

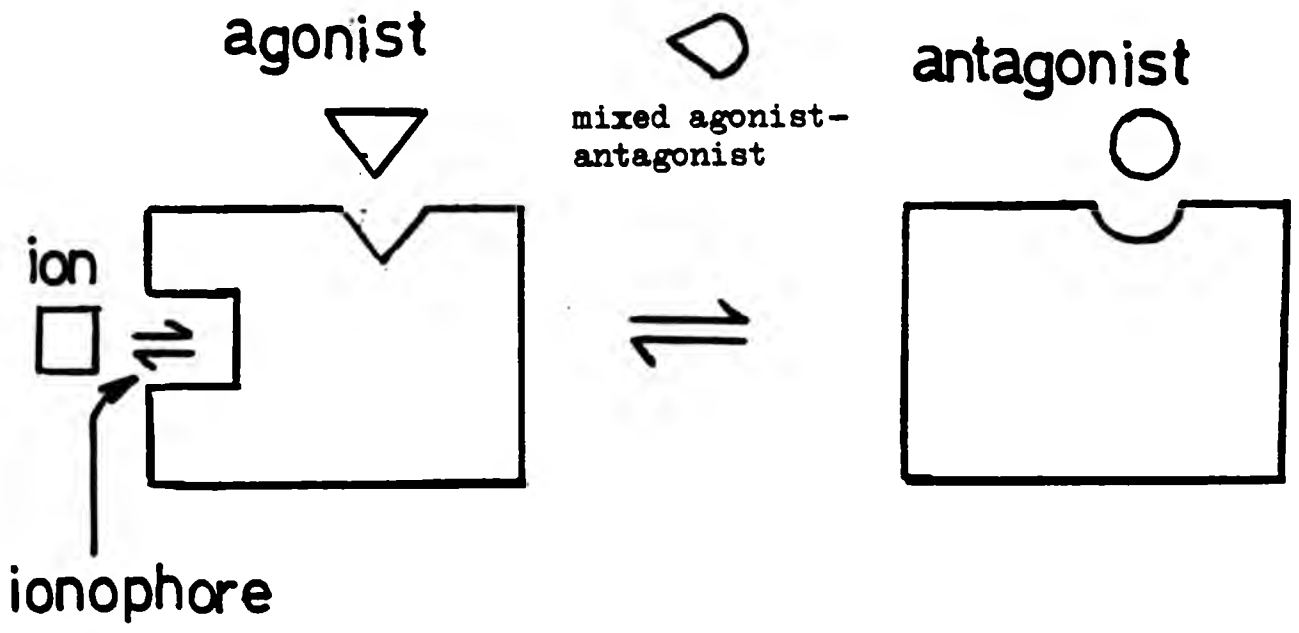
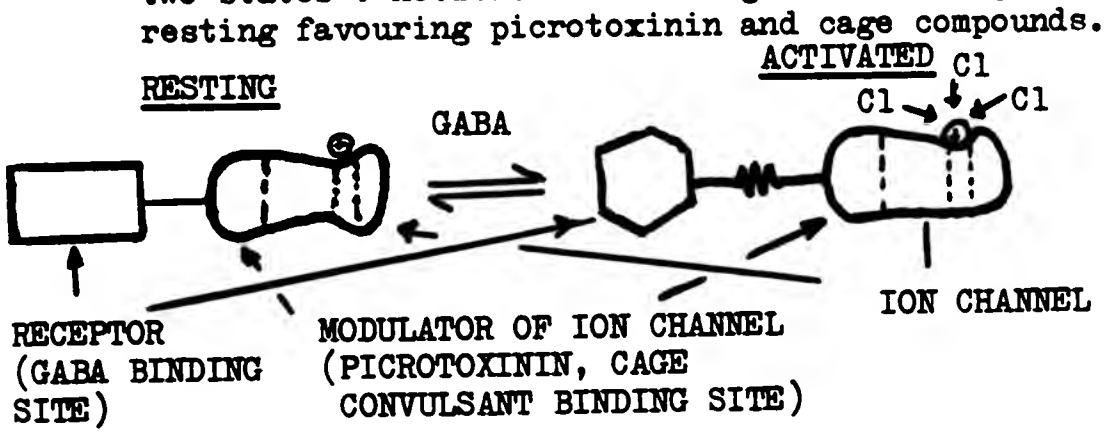


Fig. 6.2. Model of GABA receptor-ionophore indicating action of GABA, picrotoxinin and related cage convulsants. The receptor (GABA binding site) and chloride ion channel (picrotoxinin, cage convulsant site) exists in two states : Activated favouring GABA binding and resting favouring picrotoxinin and cage compounds.



A model for the allosteric effect of sodium ions on opiate receptor is shown in Fig.5.1. (Snyder 1975b, Simon 1975). When sodium ions are bound to the allosteric site there is a change in the shape of the receptor molecule. This causes an alteration of the opiate binding site which can now bind antagonists with greater affinity, and agonists with reduced affinity.

These results show that the opiate receptor has a certain plasticity permitting it to exist in various conformational states. Obviously in vivo the cells would be bathed in physiological fluids containing sodium ions and would normally exist in the state which would be receptive to opiate antagonists.

But if the receptor is mobile and was able to shuttle between the inside and outside of cell membranes there would be a ificant difference in sodium ion concentration which would the conformation of the receptor which may explain the of tolerance to opiates (Simon 1979).

if an agonist opiate was applied it would provoke of the antagonist conformation of receptor to an and reduce the sodium binding to the receptor alteration of membrane permeability.

developed for the opiate receptor has been the glycine receptor. Studies with the antagonist (^{3}H) - strychnine showed that elicited by glycine involved an increase in the conductance (Young and Snyder 1973). Chloride ions the binding of (^{3}H) - strychnine and also decreased the

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But if the receptor is mobile and was able to shuttle between the inside and outside of cell membranes there would be a significant difference in sodium ion concentration which would affect the conformation of the receptor which may explain the phenomenon of tolerance to opiates (Simon 1979).

Also if an agonist opiate was applied it would provoke a transformation of the antagonist conformation of receptor to an agonist conformation and reduce the sodium binding to the receptor complex causing an alteration of membrane permeability.

5.3. The Glycine receptor.

The model developed for the opiate receptor has been extended to include the glycine receptor. Studies with the radiolabelled glycine antagonist (^3H) - strychnine showed that hyperpolarisation elicited by glycine involved an increase in the chloride ion conductance (Young and Snyder 1973). Chloride ions reduced the binding of (^3H) - strychnine and also decreased the

affinity of glycine for the binding sites (Young and Snyder 1974). However it has not been established whether these interactions distinguish between glycine agonists and antagonists.

Whether or not strychnine binds directly to the chloride ion channel is unclear but work done on (^{3}H) - strychnine binding with chloride ion displacement suggested that chloride interacts with strychnine binding in a co-operative fashion and that glycine and strychnine bind to two different sites (Snyder and Young 1975).

Strychnine binding associated with the ionic conductance mechanism for chloride with glycine interacting with the recognition site also has implications for elucidating synaptic features of other neurotransmitters including GABA.

Picrotoxin blocks GABA effects non-competitively in analogy to the strychnine glycine interactions. Studies in neuromuscular junctions of invertebrates (Takeuchi and Takeuchi 1969, Brookes and Werman 1973) showed that chloride ions reduce the ability of picrotoxin to block GABA effects and that conceivably the GABA interactions take place between the GABA recognition site and the chloride ionophore where picrotoxin may act.

5.4. The GABA receptor

The first studies of the GABA receptor utilising the binding assay technique were carried out by Snyder and his co-workers. A similar synaptosomal tissue technique was used that was developed in the study of the opiate receptor described previously.

(^{3}H)-GABA was used as the radioligand (Zukin et al. 1974, Enna and Snyder, 1977). A sodium-independent specific binding of GABA to synaptic membrane fractions of rat brain would appear to be associated with the GABA receptor. This binding was found to differ from sodium-dependent binding to subcellular particles of brain tissue which was involved with the high-affinity transport of GABA into the nerve-terminals and glia (DeFeudis 1973, Kuriyama et al. 1968). The sodium-dependent binding was probably due to the attachment of GABA to these uptake sites (Roberts and Kuriyama 1968).

The sodium-independent binding of GABA to these synaptic membranes was facilitated by a freezing-thawing process which appeared to destroy the uptake sites and a washing process to get rid of excess endogenous GABA already present (Zukin et al. 1974).

This study also employed a similar synaptosomal preparation to study the sodium-independent binding of a potent GABA agonist which was labelled with tritium ((^{3}H)- muscimol) and also a further study with the tritium labelled GABA antagonist (^{3}H) - α - dihydropicrotoxinin. The findings related to the binding of these ligands will be described later in the chapter.

5.4.1 Definition of the specific binding of (^{3}H) GABA.

In their studies Snyder and his co-workers defined the specific binding of GABA as the binding which was displaced by a high concentration of GABA (10^{-3}M) or (+) - bicuculline (10^{-4}M), the remainder being the non-specific binding.

However, this definition did not differentiate between specific and non-specific saturable binding as described in the binding studies with the opiates. In order to demonstrate the stereospecific binding sites which was analogous to the opiate receptor, incubations were performed using (+) - bicuculline methohalide and the pharmacologically inert (-) - bicuculline methohalide. The difference being the stereospecific binding (Enna and Snyder 1977).

The above definition assumed that all the drugs were acting at the same receptor but this has been shown not to be the case with the GABA antagonists picrotoxinin (Enna and Snyder 1977, Olsen et al.1977) and bicyclic phosphates (Ticku and Olsen 1978, Enna et al. 1977). The actions of these antagonists will be discussed later.

5.4.2 Distribution of specific (^{3}H) - GABA binding.

In a whole brain homogenate the specific binding of (^{3}H) - GABA (bicuculline displacable) was about 10% of the total binding, but during subsequent fractionation of the tissue the specific ratio increased progressively. In the crude mitochondrial pellet (P_2 fraction) this increase was about 50% and in a crude

synaptic fraction increased to 70% specific binding (Zukin et al. 1974).

The regional distribution of sodium-independent GABA binding did not always correlate closely with endogenous GABA (Zukin et al. 1974, Enna and Snyder 1975) in rat brain, but this may have been due to the surface area of the postsynaptic membranes varying independently of the transmitter content of the nerve terminal. In monkey brain regions however there was a close correlation between endogenous GABA and sodium - independent GABA binding (Enna et al. 1975). More recently immunocytochemical studies in brains have shown a much greater correlation between sodium - independent GABA binding and postsynaptic GABA receptors (Roberts 1979).

5.4.3 Saturabilitv of (^{3}H) - GABA binding

Specific (^{3}H) - GABA binding to synaptosomal membranes was found to be saturable with increasing concentration, half maximal binding occuring at about 10nM. The non-specific binding was not saturable and increased linearly with increasing concentrations of (^{3}H) - GABA (Enna and Snyder 1975).

5.4.4 Sodium - dependent and sodium - independent binding.

Two completely different types of (^{3}H) - GABA binding have been identified:

i) Sodium - dependent binding.

In the presence of sodium ions (^{3}H) - GABA binding to synaptosomal membranes appears to involve the uptake site and is hardly affected by (+) - bicuculline (Peck et al. 1973,

Martin 1976). A high-affinity transport of GABA into the nerve terminals was involved which was inhibited by cis-3-amino-cyclohexane carboxylic acid (ACHC) (Collins et al. 1975, Bowery et al 1976b) and also into glial cells which could be inhibited by β-alanine (Meldrum 1979). There is also a low-affinity uptake of (^{3}H) - GABA involved in rat brain synaptosomes which is also sodium dependent (Levi and Raitery 1973).

ii) Sodium - independent binding.

In the absence of sodium ions it was shown that the specific (^{3}H) - GABA binding was with the postsynaptic GABA receptor. This binding was inhibited by (+) - bicuculline but not by (-) bicuculline (Zukin et al. 1974, Enna and Snyder 1975). If the brain synaptosomal tissue was frozen at -20°C and stored this facilitated the specific binding of (^{3}H) - GABA causing some destruction of the sodium-dependent uptake sites in nerve endings and glia. Antagonists of the sodium - dependent uptake sites such as nipecotic acid and ACHC had very little effect on the sodium dependent binding (Krogsgaard-Larsen and Johnston 1978, Johnston et al. 1979).

5.4.5 Treatment of tissues with ions.

i) Cations.

Lithium, potassium and magnesium ions were found to have little effect on the specific sodium dependent binding of (^{3}H) - GABA to freshly prepared membranes. Cupric and mercuric ions however were found to be potent inhibitors of specific (^{3}H) - GABA binding but none of the cations had any effect on

the non-specific binding (Enna and Snyder 1977).

ii) Anions.

Specific sodium - independent binding to (^{3}H) - GABA was reduced by thiocyanate, nitrate and iodide while they enhanced the potency of (+) - bicuculline (Enna and Snyder 1977).

In all experiments with the sodium - independent binding of (^{3}H) - muscimol to rat brain synaptosomal membranes no ions were added to the Tris-citrate buffer used as the incubating medium.

5.4.6 Treatment with detergents.

The detergent, Triton X-100 in concentrations of 0.05% increased the sodium - independent binding of (^{3}H) - GABA about five times and also produced from Scatchard analysis two binding sites for (^{3}H) - GABA, a high affinity site and a low affinity site (Enna and Snyder 1977).

A similar increase in (^{3}H) - muscimol sodium - independent binding was produced with Triton X-100 in concentrations of 0.05% to yield only one binding site on Scatchard analysis (Beaumont et al. 1978) in mouse brain synaptosomal tissues the addition of Triton X-100 and several other detergents to the preparation had little effect on the sodium - independent (^{3}H) - muscimol binding (Wang et al. 1979). This difference may be attributed to freeze-thaw cycle and washing which removed an endogenous inhibitor of GABA (Greenlee et al. 1978, Guidotti et al. 1978, 1979).

In all experiments the tissue was freeze-thawed and washed before using and no treatment with any detergent was employed. It was assumed that any endogenous GABA or inhibitor of GABA had been removed by this process (Greenlee et al. 1978).

5.4.7 Treatment with enzymes.

Sodium - independent (^{3}H) - GABA receptor binding was resistant to treatment by several enzymes (e.g. neuraminidase, trypsin or phospholipidase). However, with sodium - dependent specific (^{3}H) - GABA binding in fresh synaptosomal preparation was reduced by 50% with trypsin and 80% with phospholipase.

In all experiments with (^{3}H) - muscimol sodium - independent binding to rat synaptosomal preparations no enzymes were employed.

5.4.8 Endogenous modulator of GABA

There is a substantial difference in the kinetic properties of (^{3}H) - GABA binding sodium - independent to brain synaptosomal preparations when used fresh or whether the preparation has been through a freeze-thaw and washing sequence (Enna and Snyder 1977, Olsen 1978a) suggesting that there may be an endogenous agent which modulated the affinity of GABA for its receptor which was washed out during the freeze-thaw and washing process. This modulator was purified and when added to a freeze-thawed and washed preparation the binding of GABA was decreased and the high affinity site masked as in GABA binding to fresh tissue (Toffano et al. 1978).

It has been proposed that this modulator is a protein kinase which is involved with the interaction of benzodiazepines as their facilitatory function of GABA (Guidotti et al. 1979).

5.5. The use of (^{3}H) - muscimol in GABA binding.

5.5.1 Introduction:

Muscimol (5-aminomethyl-3-isoxazolol) is one of the alkaloid compounds occuring naturally in the fly agaric mushroom, amanita muscaria (Wasser 1967, Theobald et al, 1968, Eugster 1969). It is a potent agonist of the (+) - bicuculline - sensitive GABA postsynaptic receptor, being six or seven times more potent than GABA itself (Krogsgaard-Larsen et al. 1975, Johnston 1976, Beaumont et al. 1978, Johnston et al. 1979), and has been used extensively for studies on the central GABA system.

It is a relatively toxic compound, having an LD_{50} after intraperitoneal injection in mice of 2.5mg/kg (Lloyd et al. 1969) but this may be due to its toxic metabolites after rapid conversion in vivo. (Ott et al. 1975, Naik et al. 1976, Biggio et al. 1977).

The 3-isoxazolol moiety of muscimol can be regarded as a masked carboxyl group of fixed conformation with relatively free rotation of the aliphatic side chain (Krogsgaard-Larsen et al. 1979). X-ray crystallographic structure and molecular orbital calculations with studies of protolytic properties (pK_A values) show that low value of acidic function is an indication of high degree of delocalisation of the negative charge of the corresponding anion, the lower the pK_A value the higher the affinity for the GABA

receptor, pK_A values for GABA zwitterion are 4.0; 10.7, and pK_A values for muscimol are 4.8; 8.4 (Krogsgaard-Larson et al. 1975, 1979).

Muscimol is not a substrate or inhibitor of GABA-transaminase (Johnston et al, 1979) and has a low affinity for the high affinity GABA uptake system in rat brain slices (Johnston 1971).

When injected into the substantia nigra an increase in dopamine metabolites was observed (Cheramy et al. 1978, Scheel-Kruger et al. 1979) and if pretreated with kainic acid there was an increase in cerebellar GMP levels (Biggio et al. 1979).

It has some convulsant and some anticonvulsant properties, causing audiogenic seizures in rodents (Anlezark et al. 1978) and acting as an anticonvulsant (Lloyd et al. 1979, Meldrum 1979, Delini-Stula 1979).

Clearly muscimol is a useful tool in the identification of the postsynaptic GABA receptor and this study utilises a radiolabelled ligand in the study of (^3H) - muscimol binding to the GABA receptor in rat brain synaptosomal preparations.

5.5.2 Specific binding of (^3H) - muscimol.

The specific binding of sodium-independent (^3H) - muscimol can be defined as the difference of tissue incubated with (^3H) - muscimol and that of a similar experiment but with an added high concentration of GABA ($10^{-3}M$). This was found to be about 80% specific with 20% non-specific binding in several synaptosomal preparations of different species (Beaumont et al.

1978, Snodgrass 1978, Leach and Wilson 1978, Williams and Risely 1979). This correlated with the 85% specific binding for (^{3}H) - GABA under similar conditions (Enna and Snyder 1977).

5.5.3 Distribution of specific, sodium - independent (^{3}H) - muscimol binding.

Specific (^{3}H) - muscimol binding was found to be most enriched in the crude synaptosomal fraction (P_2) consisting of about 75% of the total observed in all the subcellular fractions of rat brain (Beaumont et al. 1978).

The regional distribution of (^{3}H) - muscimol binding was shown to have its highest density in the cerebellum with varying concentrations in other regions of the brain and less than 10% of that in the cerebellum was found in the spinal cord by filtration assay or centrifugation (Beaumont et al. 1978, Williams & Risley 1979). These results were consistent with those for (^{3}H) - GABA sodium - independent binding in rat brain regions (Enna and Snyder 1975).

Further studies on rat brain slices using an autoradiographic study for (^{3}H) - muscimol binding also observed highest (^{3}H) - muscimol binding in the cerebellum and similar binding in other regions of the brain consistent with (^{3}H) - muscimol binding in synaptosomal preparations and also show a pharmacological specificity comparable with that of the GABA receptors (Palacios et al. 1980).

5.5.4 The effect of sodium ions on the specific binding of (^{3}H) - muscimol.

In both fresh and previously frozen rat brain synaptosomal membranes the addition of sodium ions to the incubating medium caused a decrease in specific (^{3}H) - muscimol binding (Beaumont

et al. 1978).

At a concentration of 100mM sodium chloride there was a decrease of specifically bound (^{3}H) - muscimol of about 15% in fresh tissue and a decrease of about a third in previously frozen tissue.

In similar preparations using specifically bound (^{3}H) - GABA and the same concentration of sodium chloride a 10-fold increase in specifically bound (^{3}H) - GABA was observed (Enna and Snyder 1977).

These results indicate that muscimol has little effect on the sodium dependent high-affinity uptake of GABA in neuronal and glial cells and the specific binding is sodium - independent exerting its effects on the postsynaptic GABA receptor hence making it a useful tool in the evaluation of GABA agonists and antagonists on the post-synaptic GABA receptor.

5.5.5 Effects of freezing and washing on specific (^{3}H) - muscimol binding.

The specific binding of (^{3}H) - muscimol to fresh tissue is very low (Leach and Wilson 1978, Beaumont et al. 1978, Maurer 1979). The specific binding can be almost doubled if the tissue is frozen and thawed (Beaumont et al. 1978)or dialised (Maurer 1979).

These processes have the effect of removing endogenous compound like GABA or sodium ions which would interfere with the assay.

This compared favourably to results obtained with specific sodium - independent (^{3}H) - GABA binding (Enna and Snyder

1975, 1976, Wong and Horng 1977). It has also been suggested that there is an endogenous modulator of GABA of higher molecular weight which is also washed out during the freeze-thaw and washing processes which decreased the GABA binding because it masked the high-affinity binding site (Toffano et al. 1978) and this modulator was involved with benzodiazepines function as facilitators of the effects of GABA (Guidotti et al. 1979). All experiments were therefore carried out after the freeze-thaw and washing process so these agents could not interfere with the assay.

5.5.6 The effects of detergents on specific (^{3}M) - muscimol binding.

Treatment with a concentration of 0.25% of the detergent Triton X-100 has been reported to increase specific sodium-independent (^{3}H) - muscimol binding by 2-fold in rat brain synaptosomal tissues by both filtration assays and centrifugation assays (Beaumont et al. 1978, Williams and Risley 1979).

However no increase in specific binding of (^{3}H) - muscimol was reported using Tritonised mouse brain synaptosomal tissues (Wang et al. 1979).

Treatment with another detergent, Tween 20, at a concentration of 0.1% enhanced specific (^{3}H) - muscimol binding 40%, (Wang et al. 1979).

Treatment with 0.1% Lubrol PX did not decrease the specific binding of (^{3}H) muscimol but as with Triton X-100 the ability of GABA and β-alanine to compete with (^{3}H) - muscimol was increased. The potency of the antagonist (+) - bicuculline to displace (^{3}H) - muscimol binding however was decreased (Wang et al. 1979).

In specific sodium - independent binding of (^{3}H) - GABA to rat brain synaptosomal preparations treatment with 0.05% Triton X-100 increased 5-fold and the potency of 3-aminopropane sulphonic acid to displace (^{3}H) - GABA was increased whereas the potency of (+) - bicuculline in the displacement of (^{3}H) - GABA was decreased.

In view of the conflicting evidence above all experiments were carried out in the absence of detergents in order to perhaps resemble physiological conditions in the CNS more closely.

5.6. <u>The use of (^{3}H) - α - dihydropicrotoxinin((^{3}H) - DHP) binding in rat synaptosomal preparations</u>.

5.6.1 <u>Introduction</u>:

Picrotoxin, a natural product of plants from the Minispermaceae family, consists of a molar ratio of 1:1 of picrotoxinin and the less active picrotin (Jarboe et al. 1968).

Picrotoxinin is a potent inhibitor of the inhibitory synaptic transmission mediated by GABA (Johnston 1978) and its action is not in direct competition with the GABA recognition site but by inhibition of the GABA regulated chloride permeability on the postsynaptic membrane (Takeuchi and Takeuchi 1969, Ticku and Olsen 1977).

This effect on chloride ion permeability being possibly due to its action at a GABA receptor-ionophore complex (Ticku and Olsen 1978, Olsen et al. 1978a, d, Olsen et al. 1979).

α-Dihydropicrotoxinin (DHP) has similar actions to picrotoxinin but has one seventh the activity as a convulsant

(Jarboe et al. 1968).

α-Dihydropicrotoxinin can be obtained by reduction of the double-bond in picrotoxinin. This is done by catalytic hydrogenation at room temperature under 1 atmosphere of hydrogen using platinum on charcoal as the catalyst (Mercer and Robertson 1936). (Fig.2.3).

This method could be used to radioactively label picrotoxinin by using tritiated hydrogen as the gas under the above conditions to give (^{3}H) - α - dihydropicrotoxinin ((^{3}H) - DHP) (Ticku et al. 1978b) as a useful tool to study binding of (^{3}H) - DHP to the proposed ionophore site on the postsynaptic tissues.

5.6.2 Specific binding of (^{3}H) - α - dihydropicrotoxinin.

The specific binding of (^{3}H) - DHP has been defined by the Riverside Group as the difference of tissue incubated with (^{3}H) - DHP and that of a similar experiment with an added high concentration of non-radiolabelled α - dihydropicrotoxinin (0.1mM). This was found to be about 15% specific and 85% non-specific in rat synaptosomal fractions (P_2) Ticku et al. 1978b, Ticku and Olsen, 1979, Ticku et al. 1979). A similar report for specific binding of (^{3}H) - DHP was found in a microsomal (P_3) fraction of crayfish muscle which was not enhanced with a more sophisticated fractionation of the tissue (Olsen et al. 1978d).

5.6.3 Distribution of (^{3}H) - DHP binding in brain tissue.

Binding of (^{3}H) - DHP varied with brain region being higher in the cerebellum and cerebral cortex than the brain stem (Ticku et al. 1978b). This distribution was of a similar ratio to

that of (^{3}H) - GABA binding although about 2.3 times lower (Enna and Snyder 1975).

The location of (^{3}H) - DHP binding sites in rat brain parallels closely, but not exactly, that of GABA receptor sites with greatest amounts in the synaptosomal (P_2) and light microsomal (P_3) fractions (Ticku et al. 1978a, b).

The quantity of binding sites (Ticku et al. 1978b) has been found to be about twice that of (^{3}H) - GABA binding sites (Olsen and Greenlee 1976, Olsen et al. 1978a.).

These reports suggest that the majority of GABA and DHP binding sites may be coupled.

5.6.4 Effect of sodium and chloride ions on specific binding of (^{3}H) - DHP.

(^{3}H) - DHP binding was not significantly affected by variation of the chloride ion concentration from 0-400mM in either crayfish muscle preparations or rat brain synaptosomal preparations (Olsen et al. 1978d, Ticku et al. 1978b). It is unlikely that (^{3}H) - DHP would complete with chloride ions as it is not a charged molecule.

There was no effect on (^{3}H) - DHP binding on rat brain synaptosomal membranes when a complete replacement of sodium ions with potassium ions was undertaken (Ticku et al. 1978b).

5.6.5 The effects of detergents and protein reagent treatment on specific binding of (^{3}H) - DHP.

In contrast with its effect on (^{3}H) - GABA binding and (^{3}H) - muscimol binding (Enna and Snyder 1977, Beaumont et al. 1978), treatment with the detergent Triton X-100 inhibited (^{3}H) - DHP binding to brain synaptosomes in the rat. At a concentration of

0.1% Triton X-100 there was a 90% inhibition of the specific binding (Ticku et al. 1978a).

Diethylpropylcarbonate in concentrations of 1mM had little effect on specific (^{3}H) - DHP binding in contrast to a reduction by half with specific (^{3}H) - GABA binding (Ticku et al. 1978a).

N-ethylmaleimide in concentrations of 1mM reduced specific (^{3}H) - DHP binding by about 90% but specific (^{3}H) - GABA binding was only reduced by about 15% (Ticku et al. 1978a).

These differential effects of the detergent and the two protein reagents suggest that sites for binding of GABA and DHP are distinct.

5.6.6 Equilibrium dissociation constants.

The equilibrium dissociation constant (K_D) reported for (^{3}H) - DHP binding was found to be 1-2μM using 63nM concentration of (^{3}H) - DHP (Ticku et al. 1978b, Olsen et al. 1978d), and this value is much higher than that usually found for transmitter antagonists and would therefore raise the question whether this binding site is relevant to meaningful drug effects (Snodgrass 1979).

5.6.7 Summary of the actions of GABA agonists and antagonists on the specific binding of (^{3}H) - DHP.

i) Picrotoxin analogues

Tutin(Fig.2.2)was found to be the most potent in the displacement of (^{3}H) - DHP binding in rat membranes and crayfish muscle membranes having IC_{50} values of 0.35μM and 0.5μM respectively (Ticku et al. 1978a, Olsen et al. 1978d), being slightly more potent than picrotoxinin (IC_{50} 0.4μM and 0.6μM), and dihydropicrotoxinin (IC_{50} 1.1μM and 2.1μM) whereas picrotin (Fig.2.2.)

was a weak displacer of (^{3}H) - DHP binding (IC_{50} 70μM).

ii) <u>(+) Bicuculline</u>

An IC_{50} value for displacement of (^{3}H) - DHP in rat brain membranes of 100μM (Olsen et al. 1979), was a much higher value for that reported in specific (^{3}H) - GABA binding in the same tissue (Enna and Snyder 1975, Johnston et al. 1979).

iii) <u>Bicyclic phosphates</u>.

As previously reported the bicyclic phosphates have little effect on displacement of specific (^{3}H) - GABA binding (Bowery et al. 1977) but on the several analogues tested, having R-substituents; t-butyl, isopropyl, n-propyl, ethyl and methyl bicyclic phosphate the IC_{50} values for displacement of (^{3}H) - DHP in rat brain membranes appeared to follow their respective biological activities. The t-butyl bicyclic phosphate being the most potent and had about half the potency in (^{3}H) - DHP displacement as α-dihydropicrotoxinin (Olsen et al. 1979, Ticku and Olsen 1979). However α-dihydropicrotoxinin was shown to be less active than t-butyl bicyclic phosphate as a convulsant (Eto et al. 1976, Jarboe et al. 1968).

iv) <u>GABA and muscimol</u>.

As expected GABA did not displace specific (^{3}H) - dihydropicrotoxinin binding in concentrations of up to 1mM (Olsen et al. 1979).

Muscimol was also ineffective in displacement of (^{3}H) - DHP binding in rat brain membranes in concentrations up to 100μM (Ticku et al. 1978a).

v) Barbiturates.

The convulsant barbiturate (±) - 5 - (1,3-dimethyl butyl) - 5 - ethyl barbituric acid (DMBB) was the most potent displacer of specific (^{3}H) - DHP binding in rat brain membranes with an IC_{50} value of 0.05μM which was nearly ten times that of picrotoxinin. Other convulsant barbiturates were also found to be potent displacers of (^{3}H) - DHP binding (Ticku and Olsen 1978a, Olsen et al. 1979). However, the depressant barbiturates were much less active in displacement of (^{3}H) - DHP binding. Pentobarbital was 10,000 times less potent than DMBB and phenobarbital was eighty times less potent than the pentobarbital (Ticku and Olsen 1978a, Olsen et al. 1979).

vi) Others.

A range of t-butyl-bicyclocarboxylates with suitable R-substituents on the bridge head of the molecule were found to have similar potencies as the bicyclic phosphates in displacement of (^{3}H) - DHP binding in rat brain membranes (Olsen et al. 1979).

The potent GABA antagonist tetramethylenedisulphotetramine (TETS) (Fig.2.1) (Collins et al. 1975, Bowery et al. 1975) displaced specific (^{3}H) - DHP binding in rat brain

membranes with a similar potency to t-butyl bicyclic phosphate (Olsen et al. 1979, Ticku and Olsen 1979). p-Chlorophenylsilatrane, a caged compound with potent convulsant activity (Fig. 2.1) (Eto et al. 1976) was about half as potent as TETS in displacing specific (^3H) - DHP binding (Olsen et al. 1979, Ticku and Olsen 1979).

5.7. The Binding Assay.

a) Preparation of the tissue.

The preparation of the synaptosomal (P_2) fraction of the rat brain tissue was basically that used by Zukin et al. (1974). This will be described in full later.

b) Incubation of the tissue.

The rates of association and dissociation of GABA to its receptor are very rapid (Young et al. 1976, Zukin et al. 1974). This may not be the case with all drugs tested and because of saturable, non-specific actions this may not necessarily be reversible (Enna and Snyder 1975).

In the case of a readily reversible non-labelled ligand, at the end of the incubation period, the labelled ligand and non-labelled ligand should be in equilibrium with the receptor, but because the non-labelled ligand is usually in excess it is more likely to occupy the receptor sites.

c) Termination of incubation.

The incubation can be stopped in two ways, either by filtration under reduced pressure and washing or by centrifugation.

Although the filtration method is a quick and simple process as described for (^3H) - muscimol binding (Williams and Risely 1978, Leach and Wilson 1978, Snodgrass 1978) there may be binding to the filter by the radiolabelled ligand which would cause an increase in non-specific binding. The washing process may also cause some specifically bound (^3H) - muscimol to be washed off the filter to cause more inaccuracies. Due to the rapid dissociation of agonist from the GABA receptors the filtration

assays are difficult (Snodgrass 1979) and this method is not an equilibrium process.

The other method is by centrifugation of the tissue as described by Zukin et al. 1974. The great advantage of this method is that, at all times, there is an equilibrium between the bound and unbound drugs. The termination of incubation can be rapidly achieved by pelleting the tissue, rapidly removing the supernatant by suction and carefully removing any excess supernatant by drying the disposable centrifuge tube. This method also greatly reduces any non-specific binding. Due to all the obvious advantages of the centrifugation method all binding experiments were carried out this way.

5.7.1 **Method:**

Preparation of the synaptosomal (P_2)fractions.

Ten rats (Sprague-Dawley, 250-350 g, male or female) were killed by decapitation, their brains rapidly removed and homogenised in 15 volumes of ice cold 0.32M sucrose solution using a Potter-Elvehjem glass homogeniser fitted with a Teflon pestle. The homogenate was then centrifuged at 1000xg for 10 minutes on a bench centrifuge. The Pellet (P_1) was discarded and the supernatant centrifuged at 20,000xg (13K on an MSE High-Speed 18 Centrifuge). The supernatant osmotically shocked by suspending it in cold, distilled water and dispersed using a high-speed stirrer (Kinematica PCV-2, setting No. 6 for 20 seconds). The suspension was then centrifuged at 8,000xg (8K on an MSE High-Speed 18 centrifuge). The

supernatant was collected and the pellet, a bilayer with a soft buffy upper layer which was rinsed carefully with the supernatant to collect it. The mitochondrial pellet was discarded. The combined supernatant was then centrifuged at 40,000xg (18K on an MSE Hi-Spin 21 centrifuge) for 20 minutes. The supernatant was discarded and the pellet resuspended in ice-cold distilled water with a high-speed stirrer (Kinematica PCV-2, setting No. 3 for 10 seconds) and centrifuged again at 40,000xg (18K on an MSE Hi-Spin 21 centrifuge) for 20 minutes. This final crude synaptosomal membrane (fraction P_2) had the supernatant discarded and the pellet was frozen at $-30^{\circ}C$ for at least 24 hours before use.

5.7.2 <u>The Assay</u>.

The frozen pellet was allowed to thaw and was then suspended in $50cm^3$ of distilled water and left for 30 minutes at room temperature. A sample was removed for protein determination using a modified method of Lowry et al. (1951). The protein sample was dissolved in sodium hydroxide (0.2M) for 30 minutes. This revised method being necessary for a more accurate determination of the protein content of the tissue because under normal conditions the protein from the synaptosomal fraction did not always dissolve and inaccuracies occured when the concentration was worked out from the standard curve which was plotted using readily soluble bovine albumen as the protein standard.

The suspension was then adjusted in volume with distilled water to give a final concentratinn of 1mg of protein/cm^3. Then $1cm^3$ aliquots of the suspension were transferred into the appropriate number of $1.5cm^3$ Eppendorf tubes and centrifuged at 8000xg for

2 minutes using a Microfuge B, bench centrifuge.

The supernatant was removed by suction and discarded. Each pellet was resuspended in 0.05M Tris-citrate buffer (adjusted to pH 7.1) and incubated with the appropriate unlabelled drug (dissolved in the buffer whenever possible) for 10 minutes followed by a further 10 minute incubation time after the addition of the radiolabelled drug (either (^{3}H) - muscimol, 5nM or (^{3}H) - α - dihydropicrotoxinin, 5nM).

Incubation was terminated by centrifugation at 9,000xg for 5 minutes on the Microfuge B. The supernatant was rapidly removed by suction and the insides of all the tubes carefully wiped with cotton buds to remove any excess supernatant.

The pellets were dissolved overnight in Soluene tissue solubiliser (0.1cm^3). After neutralisation of the Soluene with 1.5M hydrochloric acid (0.1cm^3) the solubilised pellets were transferred in the opened Eppendorf tubes to glass scintillation vials and to each sample was added 11cm^3 of scintillation cocktail. The vials were shaken thoroughly to ensure all the solubilised tissue had dissolved in the cocktail.

Samples were allowed to stabilise for 1 hour before counting on an ICN 2700 liquid scintillation counter using a medium quench counting efficiency of 25%. The efficiency being previously determined from a standard solution of (^{3}H) - muscimol of known activity.

Between 6 and 12 determinations in triplicate for each

concentration of unlabelled drug were used against (^{3}H) - muscimol, 5nM and (^{3}H) - α - dihydropicrotoxinin ((^{3}H) - DHP), 5nM. Any determination differing by more than 10% of the mean was discarded.

5.7.3 **Materials:**

The scintillation cocktail consisted of:

toluene : Triton X-100 (2:1); 2,5 - diphenyloxazole (PPO), 0.5%; and 1,4 - bis (5-phenyloxazol-2-yl) - benzene (POPOP), 0.05%.

Radiolabelled drugs:

Methylamine - (^{3}H) - muscimol (specific activity, 12.5 Ci/mmole) was obtained from the Radiochemicas Centre, Amersham. It's radiochemical purity was checked by thin-layer chromatography on silica gel in an isopropanol : methyl ethyl ketone : ammonia (65 : 15 : 25) system and found to be 97% radiochemically pure. The Rf. value corresponded to that of unlabelled muscimol. The l ability of the label was tested by boiling (^{3}H) - muscimol (1mCi) in water for 48 hours. Less than 5% of the radioactivity associated with (^{3}H) - muscimol was lost (D. C. Warrell, the Radiochemical Centre : private communication).

(^{3}H) - α - dihydropicrotoxinin (specific activity 30Ci/mmole) was obtained from New England Nuclear, U.S.A.

All bicyclic phosphates were synthesised as described in Chapter 3 in this thesis (+) and (-)-bicuculline methobromide, α-dihydropicrotoxinin and isoguvacine hydrochloride were prepared by Dr. J. F. Collins. All the other compounds were obtained from commercial sources.

Results:

5.8. Binding studies using (^{3}H) - muscimol on rat brain synaptosomal preparations.

5.8.1 Calculation of the number of receptor sites from the stereospecific binding.

The specific (^{3}H) - muscimol binding was obtained by subtracting from the total bound radioactivity the amount not displaced by a high concentration of GABA (10^{-3}M) for 1mg of protein used. A mean value of 6 experiments was worked out for one set with 5nM of (^{3}H) - muscimol and the value for the results of the other set with 5nM of (^{3}H) - muscimol plus 10^{-3}M GABA present. These were converted to disintegrations per minute/mg of protein and subtracted to give the stereospecific (^{3}H) - muscimol binding.

The calculated stereospecific (^{3}H) - muscimol

binding = 3644 disintegrations per minute/mg of protein.

Specific activity of muscimol = 19 Curies/mmole.

1 Curie = 2.22×10^{12} disintegrations per minute.

= 1.9×10^{-4} moles of (^{3}H) - muscimol.

Therefore 3466 dpm stereospecific binding = $\dfrac{3466}{(2.22 \times 10^{12}) \times (1.9 \times 10^{4})}$

= 8.217×10^{-14} moles of (^{3}H) - muscimol/mg of protein.

= 0.08 pmoles of (^{3}H) - muscimol/mg of protein

Therefore there are 0.08 pmoles of stereospecific binding of (^{3}H) - muscimol for each mg of synaptosomal tissue.

Fig. 5.1. Total (▲) and non-specific (△) binding of ^{3}H muscimol with increasing concentrations of(^{3}H) muscimol synaptic membrane suspensions. (P_2 fractions) which had previously been frozen were incubated (1.0mg protein/tube) in Tris-citrate buffer (pH 7.1). Non-specific binding was determined from assays conducted in the presence of 1mM GABA. Values are the means of triplicate determinations, each experiment being replicated twice.

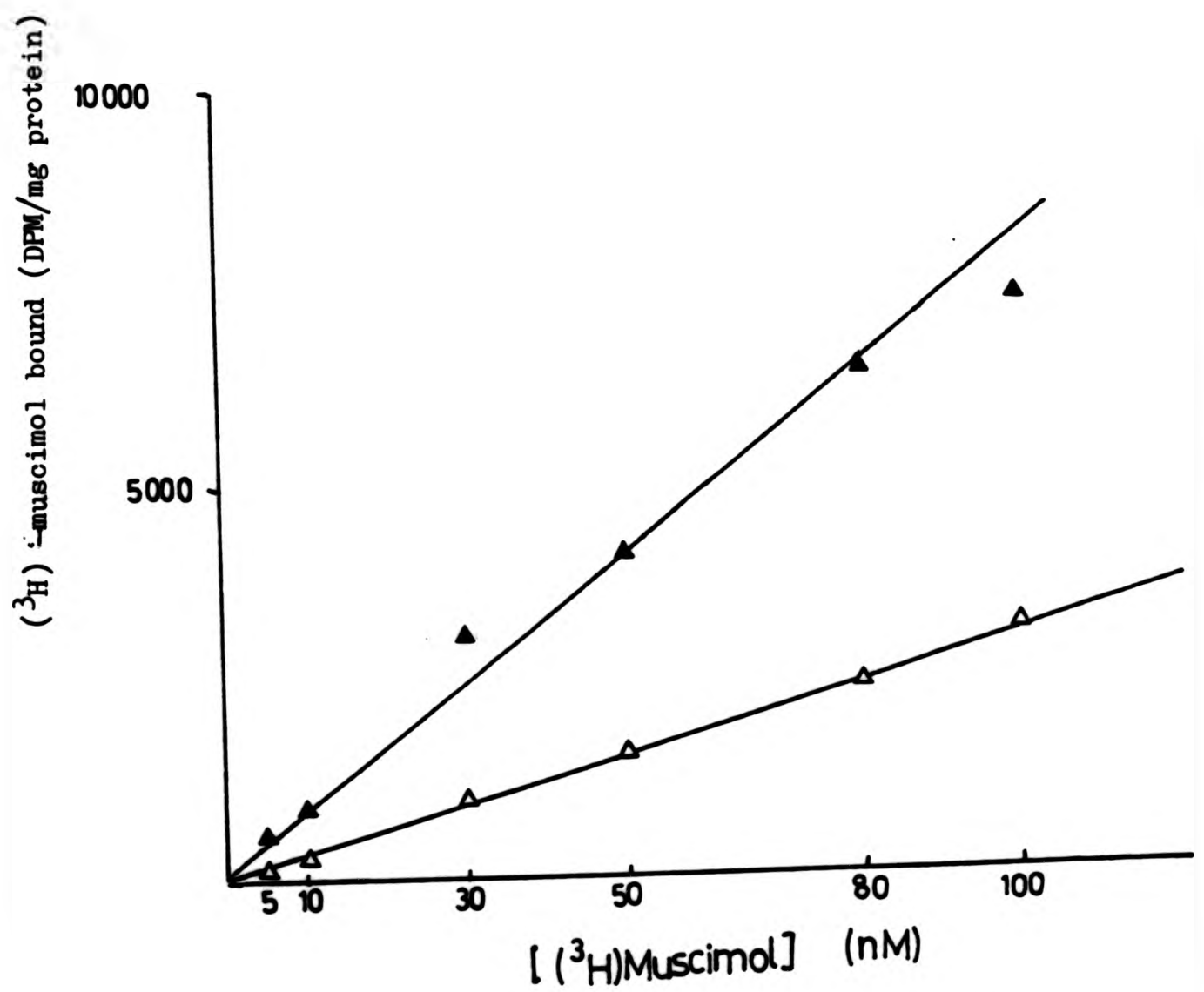


Fig. 5.2. Saturation of specific (^{3}H) muscimol binding with increasing concentrations of (^{3}H) muscimol. Values for specific binding being obtained after subtracting the non-specific binding from total binding in Fig. 5.1.

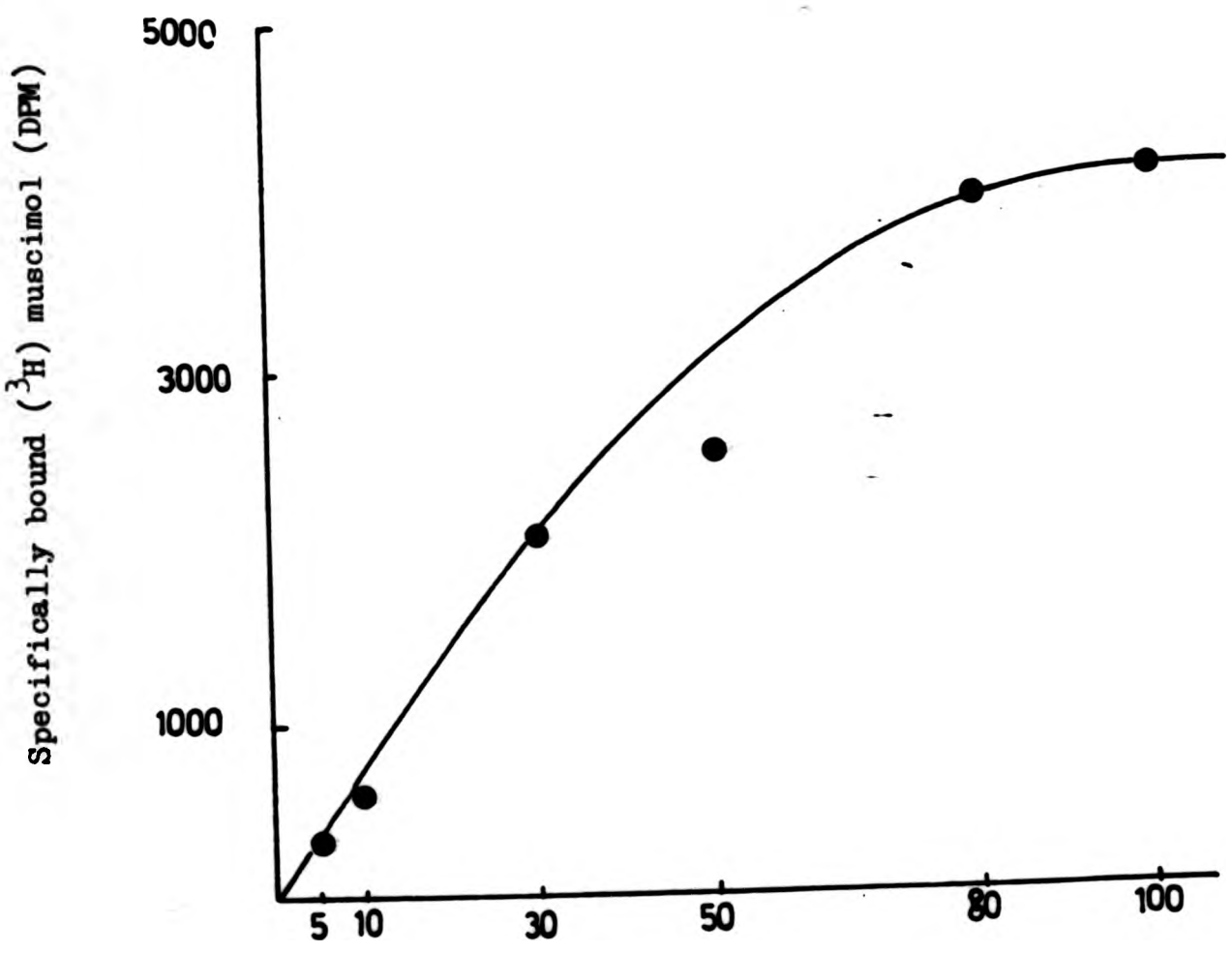
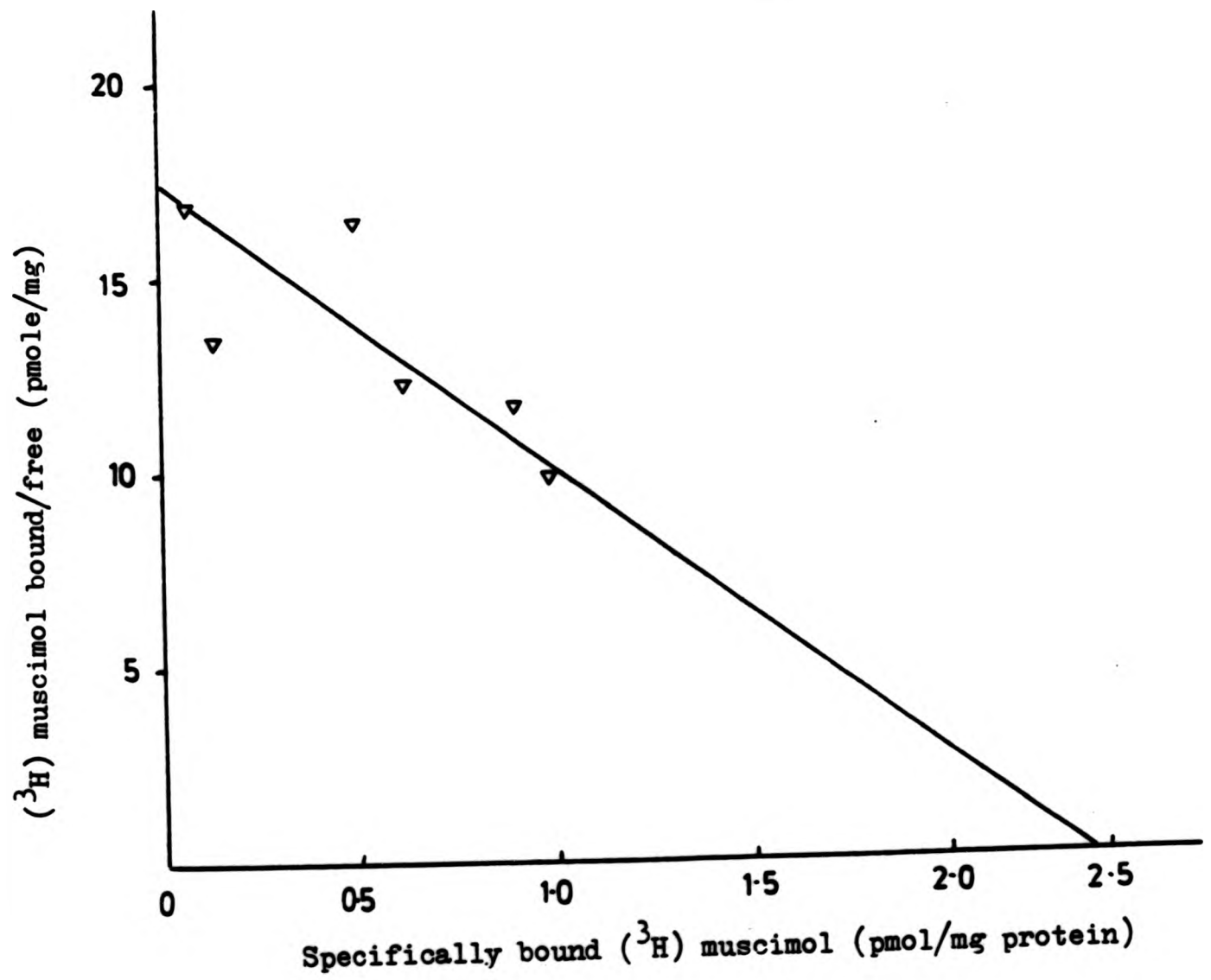


Fig. 5.3. Scatchard plot of saturation of specific (^{3}H) muscimol binding using increasing concentrations of (^{3}H) muscimol. Data obtained from Fig.5.1. showing K_D = 7.4nM and Bmax = 2.37 pmole/mg.



5.8.2 Specific and non-specific binding of (^{3}H) - muscimol.

Two sets of experiments were done in triplicate with increasing concentrations of (^{3}H) - muscimol (5nM - 100nM) on 1mg of synaptosomal fractions. The second set of experiments had a high concentration of GABA (10^{-3}M) to displace all specifically bound (^{3}H) - muscimol.

The results of these experiments were represented graphically (Fig.5.1.) with binding of (^{3}H) - muscimol in dpm/mg protein plotted against the concentration of muscimol.

The values for each concentration of (^{3}H) - muscimol were then subtracted to give the specific binding of muscimol in dpm/mg protein and this plotted against the concentration of (^{3}H) - muscimol. This was represented graphically in (Fig.5.2.)

From the graph it showed that an increase in concentration of (^{3}H) - muscimol after 80-90nM did not increase the specific binding capacity of the tissue, showing that the specific binding was saturable and indicating there were a finite number of receptors present in the tissue.

5.8.3 Scatchard analysis of specific (^{3}H) - muscimol binding in rat brain synaptosomal tissue.

Scatchard analysis of GABA displacement of (^{3}H) - muscimol using rat brain synaptic membranes plotting bound muscimol/free (pmol/mg protein) against specifically bound

(^{3}H) - muscimol (Fig.5.3.) showed that there was only one possible slope for the line of best fit indicating the presence of one binding site of high affinity.

This result was in agreement with some results previously documented with specific (^{3}H) - muscimol binding (Maurer 1978, Snodgrass 1978, Wang et al. 1978, DeFeudis et al. 1980, Kingsbury et al. 1980, Palachios et al. 1980), but reports of another high affinity binding site have also been produced with Scatchard analyses indicating two separate slopes on the graph (Williams and Risely 1978) and a possibility of two sites in the report by Wang et al. (1978)and Bernasconi et al. (1980).

The slope of the graph was fitted by least squares linear regression. The equilibrium dissociation constant (K_D) was estimated as the negative reciprocal of the slope of the line of best fit and was 7.4nM.

This homogenous binding site having a Bmax, the maximum number of binding sites, calculated by the abscissa intercept of the line was 2.37 pmoles/mg of protein.

5.8.4 Binding of (^{3}H) - muscimol to rat brain synaptosomal preparation in the presence of GABA agonists and antagonists.

Assuming that the unlabelled ligand behaves at the receptor site in a similar way to the labelled ligand (which is not the case when antagonists are used) and that binding

phenomena follows classic mass action, then the concentration of unlabelled ligand of which maximum binding of labelled ligand is displaced by 50% (IC_{50}) this will be a reasonable approximation of the apparent equilibrium dissociation binding constant.

The IC_{50} values for all unlabelled GABA agonists and antagonists were calculated by measurement from the graphs of log concentration of unlabelled compound against (^3H) - muscimol binding. This was done by measuring from the graph the concentration of compound which caused a 50% reduction in specific binding of (^3H) - muscimol.

(^3H) - muscimol binding in the absence of unlabelled ligand was approximately 6,000 dpm and the non-specific binding was approximately 1,500-2,000 dpm.

The graphs plotted (Figs. 5.4-5.9) showed displacement of (^3H) - muscimol binding at a concentration of 5nM to 1mg of synaptosomal (P_2) fraction of rat brain by unlabelled agonists and antagonists against the log of the concentration of the unlabelled drugs.

All the results were the means with standard errors for 3-5 determinations done in triplicate. The IC_{50} values are shown in Table 5.

The relative potencies of the GABA agonists on (^3H) - muscimol binding were muscimol > 3-aminopropane sulphonic acid > isoguvacine > GABA > β - hydroxy GABA.

Fig. 5.4. Displacement of specific (^{3}H)-muscimol binding to rat brain synaptic membrane suspensions (P_2 fractions) (1mg protein/tube) assayed with 5nM (19Ci/mmole) radioactive ligand. Specific binding was measured in the presence of various concentrations of GABA (▲), B-hydroxy GABA (▼) and 3-Aminopropane sulphonic acid (□). Each point and vertical line on the graph being the mean and standard error for 3-5 separate determinations done in triplicate.

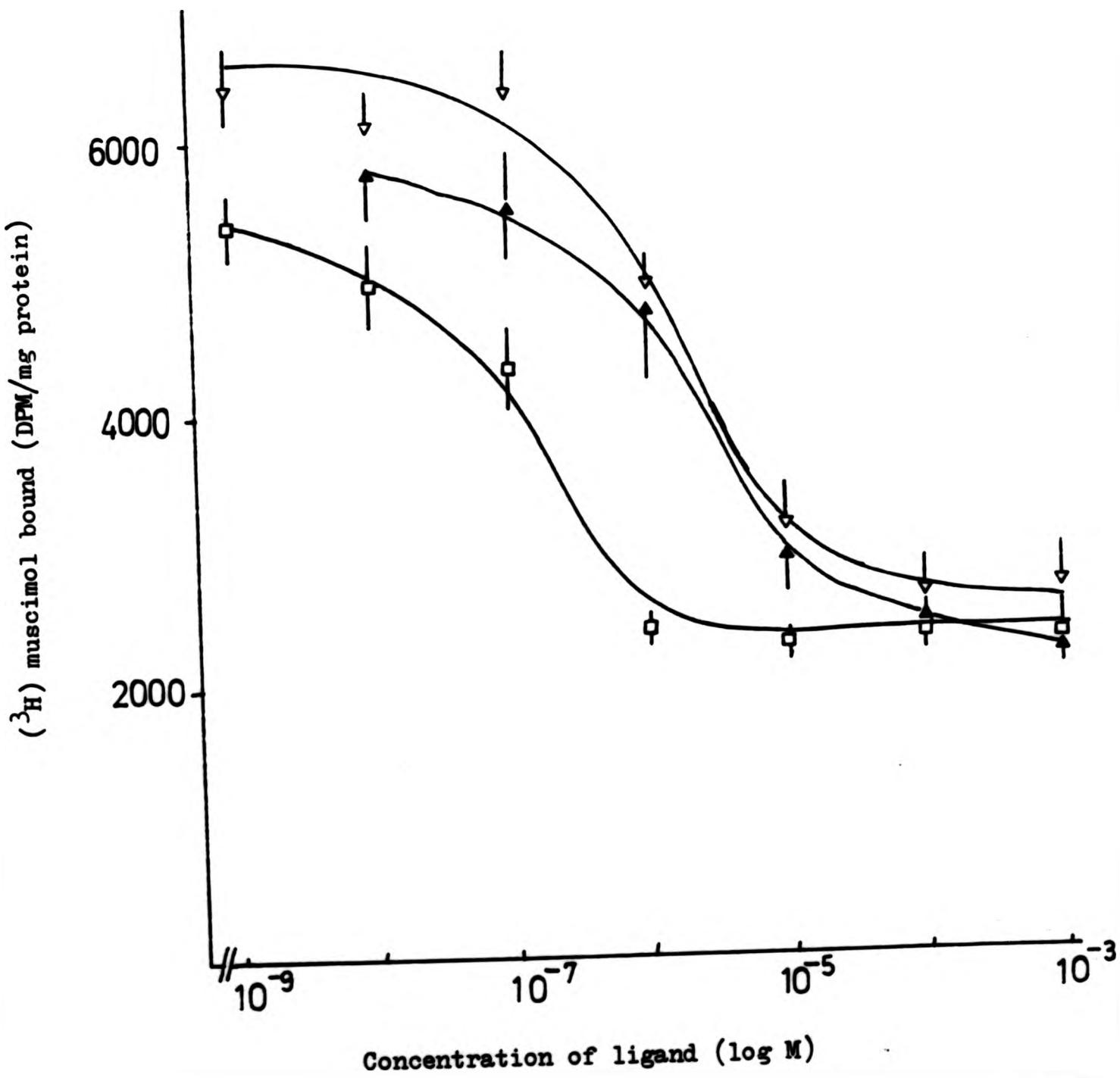


Fig.5.5. Displacement of specific (^{3}H)-muscimol binding to rat brain synaptic membrane suspensions (P_2 fractions) 1mg protein/tube) assayed with 5nM (19Ci/mmole) radioactive ligand. Specific binding was measured in the presence of various concentrations of isoguvacine (■) and muscimol (O). Each point and vertical line on the graph being the mean and standard error for 3-5 separate determinations done in triplicate.

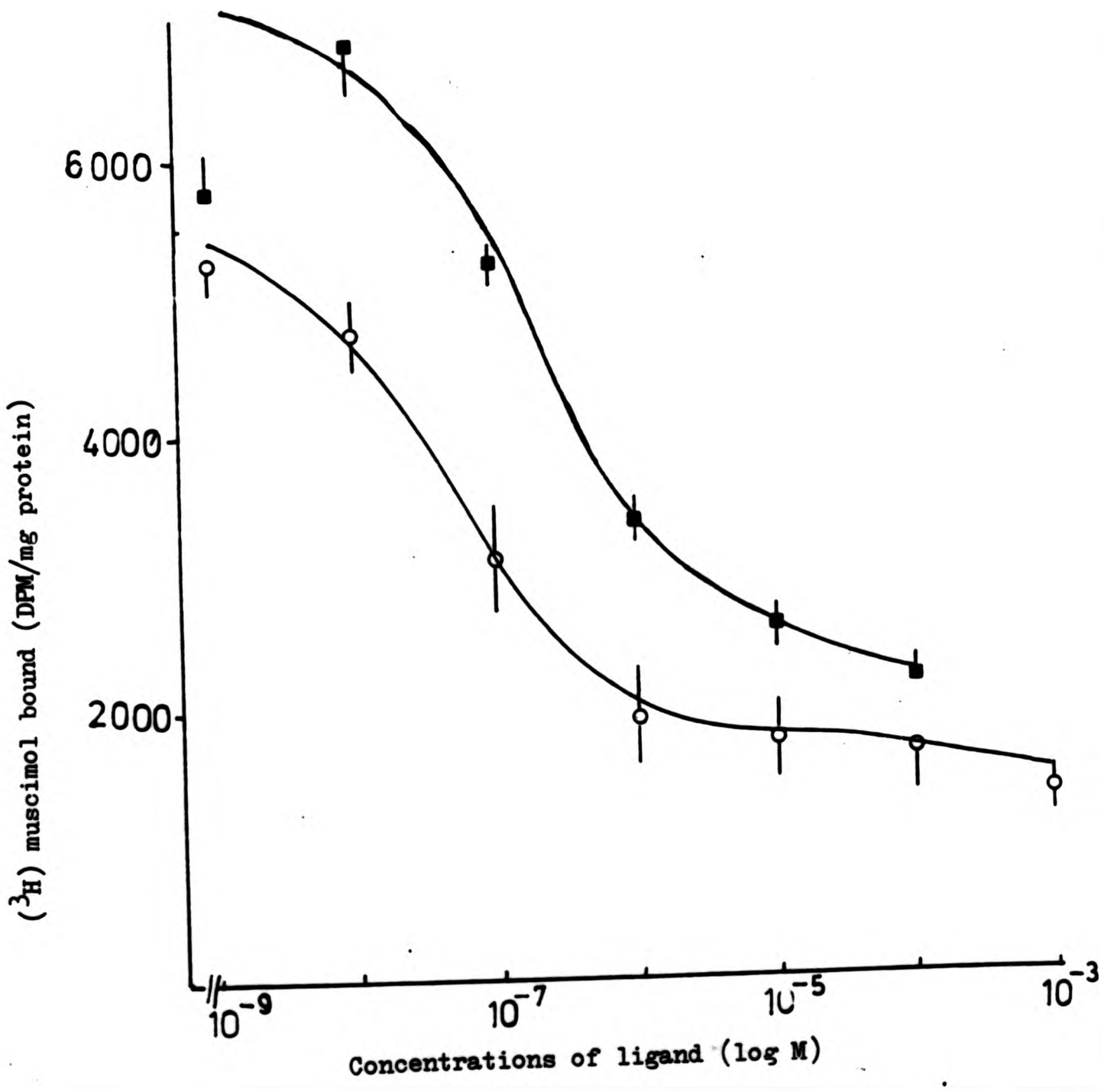


Fig. 5.6. Displacement of specific (^{3}H)-muscimol binding to rat brain synaptic membrane suspensions (P_2 fractions) 1mg protein/tube) assayed with 5nM (19Ci/mmole) radioactive ligand. Specific binding was measured in the presence of various concentrations of (+)-bicuculline methobromide (■) and (-)-bicuculline methobromide (□). Each point and vertical line on the graph being the mean and standard error for 3-5 separate determinations done in triplicate.

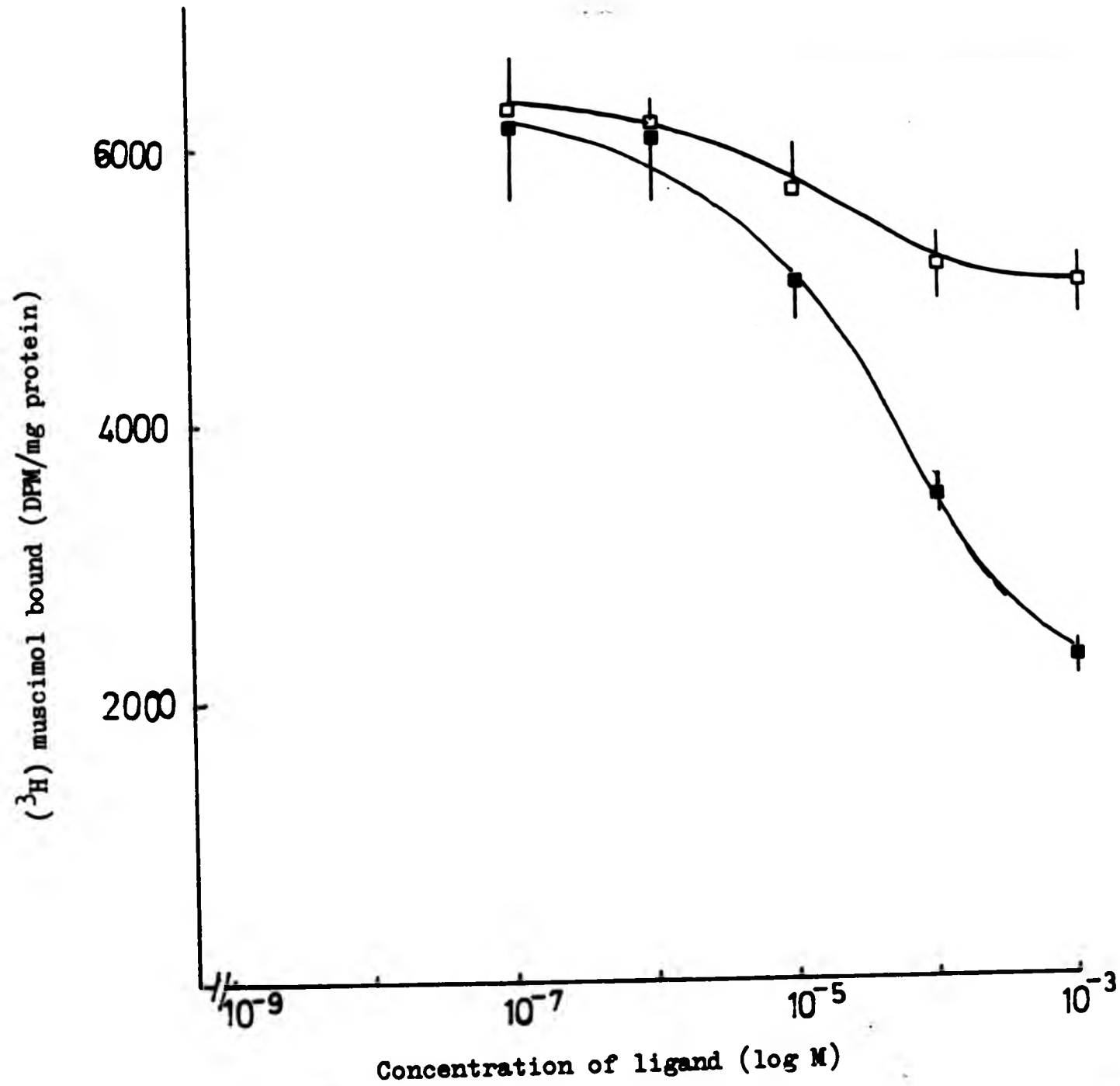


Fig. 5.7. Displacement of specific (^{3}H)-muscimol binding to rat brain synaptic membrane suspensions (P_2 fractions) 1mg protein/tube) assayed with 5nM (19Ci/mmole) radioactive ligand. Specific binding was measured in the presence of various concentrations of picrotoxinin (▲) and α-dihydropicrotoxinin (△). Each point and vertical line on the graph being the mean and standard error for 3-5 separate determinations done in triplicate.

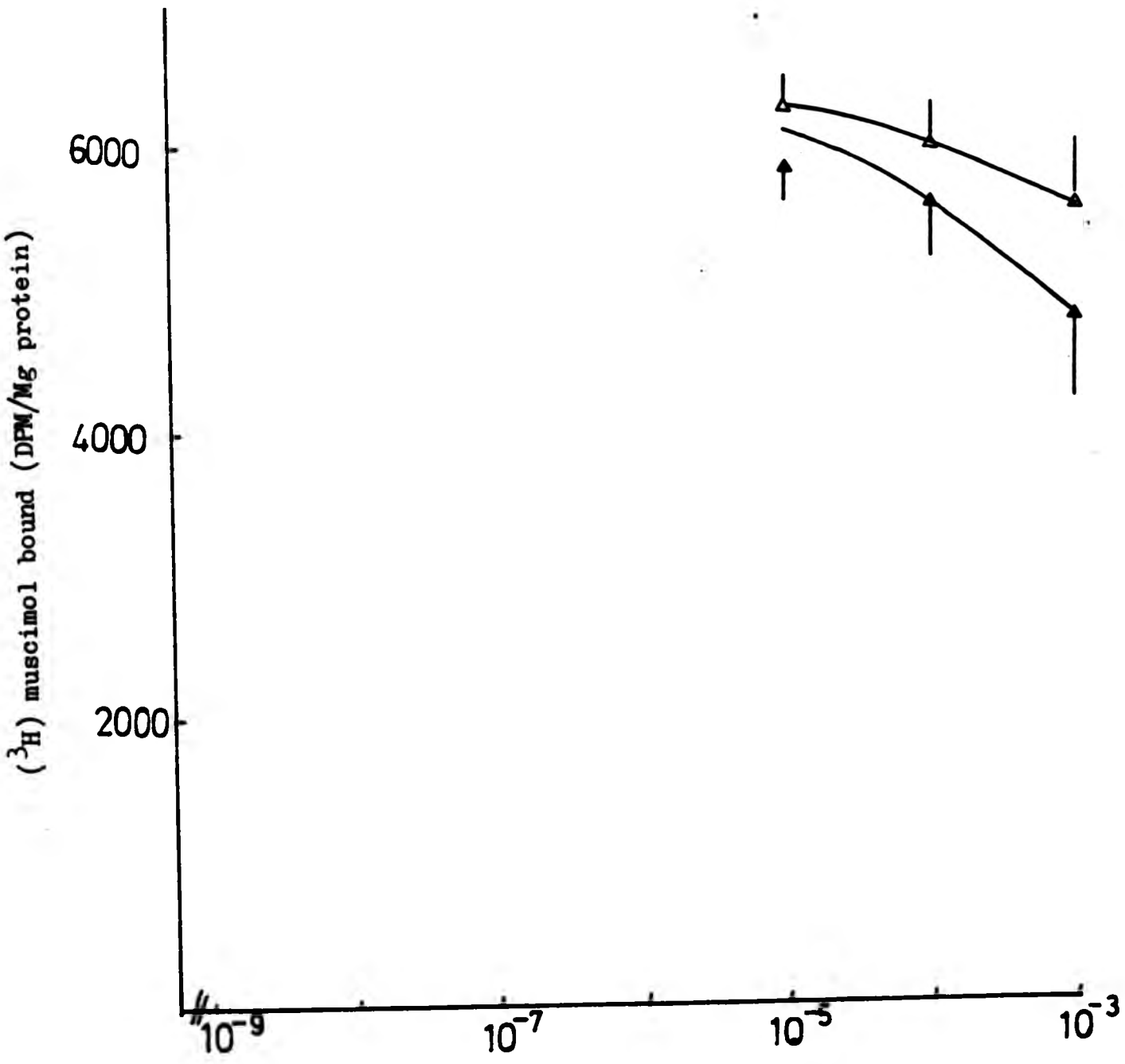


Fig. 5.8. Displacement of specific (^{3}H)-muscimol binding to rat brain synaptic membrane suspensions (P_2 fractions) (1mg protein/tube) assayed with 5nM (19Ci/mmole) radioactive ligand. Specific binding was measured in the presence of various concentrations of dimethyl propyl bicyclophosphate (▼) and neopentyl bicyclophosphate (O). Each point and vertical line on the graph being the mean and standard error for 3-5 separate determinations done in triplicate.

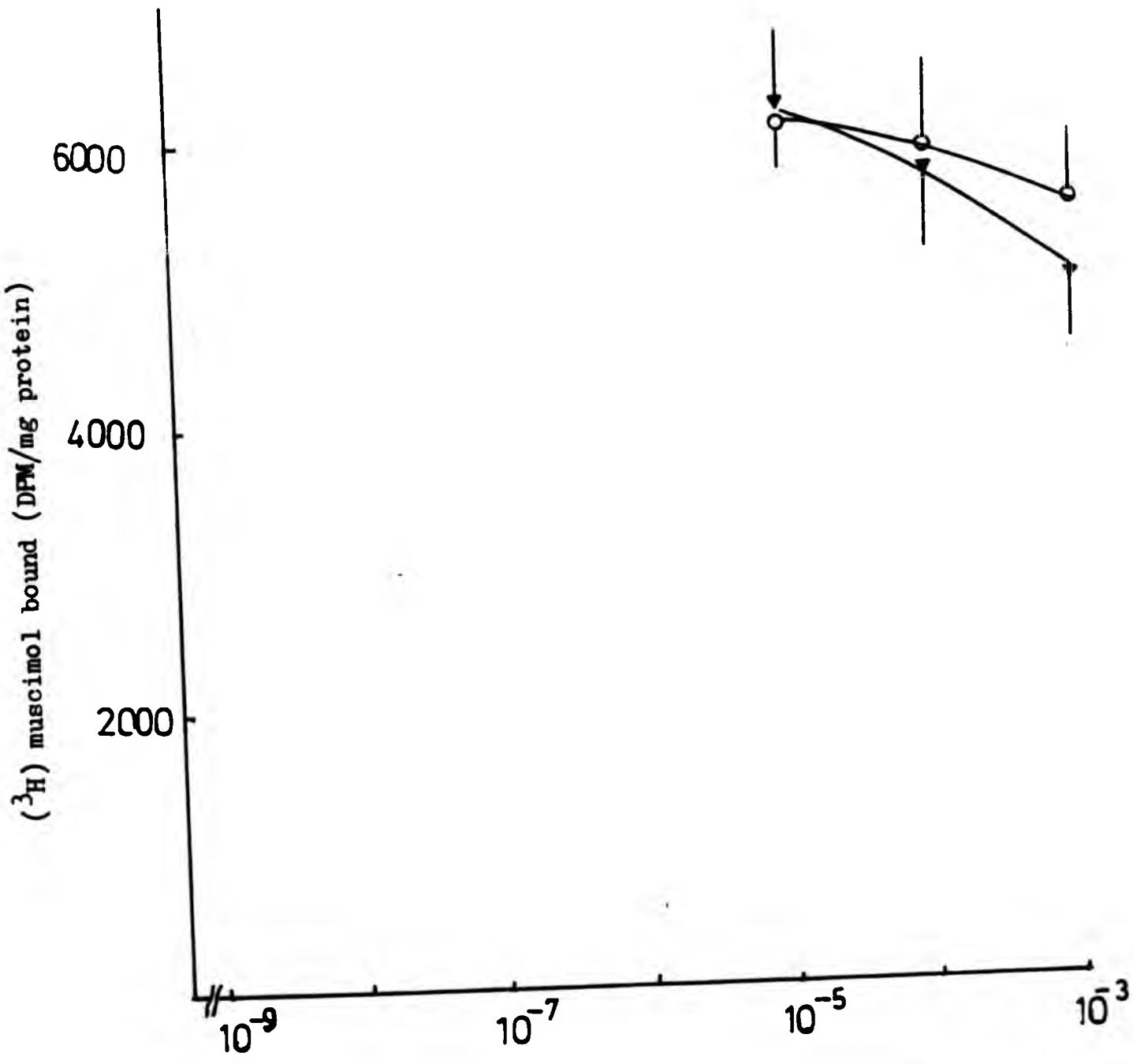


Fig. 5.9. Displacement of specific (^{3}H)-muscimol binding to rat brain synaptic membrane suspensions (P_2 fractions) 1mg protein/tube) assayed with 5nM (19Ci/mmole) radioactive ligand. Specific binding was measured in the presence of various concentrations of cyclopentyl bicyclophosphate (▽) and phenyl bicyclophosphate (▼). Each point and vertical line on the graph being the mean and standard error for 3-5 separate determinations done in triplicate.

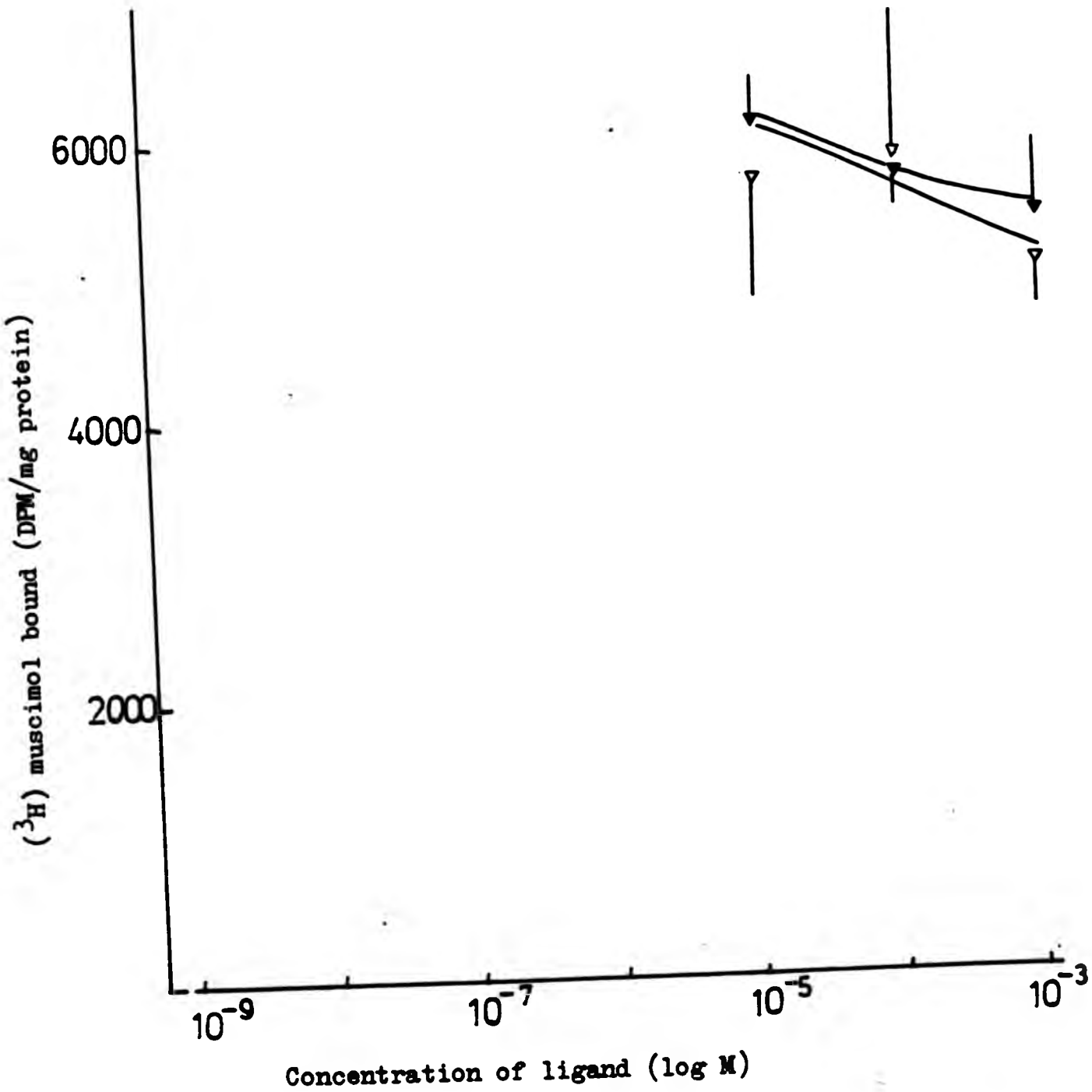


TABLE 5

Inhibitors of (^{3}H)-muscimol binding to rat brain synaptosomes

Compound	IC_{50} (μM)
Muscimol	0.012
3-Aminopropane sulphonic acid	0.087
Isoguvacine hydrochloride	0.40
GABA	1.08
β-hydroxy GABA	1.80
(+)-Bicuculline methobromide	180
(-)-Bicuculline methobromide)	
Picrotoxinin)	
α-Dihydropicrotoxinin)	>1000
Neopentyl bicyclic phosphate)	
Dimethyl propyl bicyclic phosphate)	
Cyclopentyl bicyclic phosphate)	
Phenyl bicyclic phosphate)	

The following substances had no effect up to 1000μM t-butyl bicyclic phosphate, isopropyl bicyclic phosphate, tetramethylenedisulphotetramine (TETS), Cis-3-amino-cyclohexane carboxylic acid (ACHC), glycine, (±)-nipecotic acid. None of these substances have been represented graphically.

All results for the binding were determined in sodium-free buffer using frozen, thawed and thoroughly washed membranes as described previously. All results were made from 3-5 separate determinations done in triplicate.

i) Muscimol

An IC_{50} value of 12nM was obtained from the graph analysis. This result was of the same order as reported on (^3H) - muscimol binding being about half that reported in mouse brain and calf cerebellum tissue (Leach and Wilson 1978, Wang et al. 1978), about a quarter of that in neuron-enriched culture of rat (DeFeudis et al. 1980) and double that reported for rat brain binding (Beaumont et al. 1978). These results also compared favourably to the results obtained for muscimol in (^3H) - GABA binding (Krogsgaard-Larsen and Johnston 1978, Johnston et al. 1979, Olsen et al. 1979) in the same tissue preparation.

ii) 3-Aminopropane sulphonic acid

An IC_{50} value of 87nM, approximately one seventh the potency of muscimol in its ability to displace (^3H) - muscimol was obtained. A result of 100nM in mouse brain fractions for this was obtained and would be in good agreement with the above result, (Wang et al. 1978), and 160nM in cultured rat cerebellum (DeFeudis et al. 1980). However, 3 - amino-propane sulphonic acid was found to be equipotent with muscimol in rat brain fractions in other studies (Beaumont et al. 1978).

In (^3H) - GABA binding studies 3 - aminopropane

sulphonic acid was found to be one sixth as active as muscimol which would agree with the results obtained above, (Enna and Snyder 1977).

iii) Isoguvacine hydrochloride.

An IC_{50} value of 0.4μM was obtained which was of the same order as the result in mouse brain tissue (Wang et al. 1978). However, the result for Isoguvacine was found to be a more effective displacer of (^3H) - muscimol than GABA itself, in agreement with Lodge et al. (1978). In studies with (^3H) - GABA binding there was some conflicting evidence,with Olsen et al. (1978b) in agreement with this and Krogsgaard-Larsen and Johnston (1978) in disagreement on the relative potencies of GABA and isoguvacine.

iv) GABA

An IC_{50} value for GABA at 1μM would appear to be high in comparison to results obtained in the mouse and rat brain (^3H) - muscimol binding experiments (Leach and Wilson 1978, DeFeudis et al. 1980, Wang et al. 1978), and also with (^3H) - GABA binding in similar preparations (Enna and Snyder 1977, Johnston et al. 1978), but it was found to be of the same order and this result may account for the discrepancy and disagreement over the results obtained in comparison to isoguvacine.

v) <u>β-hydroxy GABA</u>

An IC_{50} value of 1.8μM was obtained showing it to be about half as potent as GABA in (^3H) - muscimol displacement. Although no direct comparison can be made with other work on (^3H) - muscimol binding the result agrees with all other work on (^3H) - GABA binding (Young et al. 1976, Olsen et al. 1979).

All the agonists tested were shown to be effective in displacing (^3H) - muscimol from the membranes and it was assumed that their actions were at the GABA recognition site. However when the antagonists were tested only (+) - bicuculline methobromide was able to displace the (^3H) - muscimol binding to a significant extent. All the other compounds were tested up to a concentration of 1mM but even at these very high concentrations a maximum of 10-15% of the specific binding was displaced.

i) <u>(+) - Bicuculline methobromide</u>.

A high IC_{50} value of 180μM was obtained for (+) - bicuculline methobromide which was nearly twice that obtained for the methochloride salt in the calf cerebellum (Leach and Wilson 1978). This was approximately 200 times less potent than GABA in displacing (^3H) - muscimol binding. However, the relative ratio of the potencies of GABA and (+) - bicuculline in both (^3H) - muscimol binding and (^3H) -

GABA binding to those membranes is similar (Enna et al. 1977, Enna and Snyder 1977, Beaumont et al. 1978, Wang et al. 1978).

ii) <u>(-) - Bicuculline methobromide</u>.

Although at very high concentrations (1mM) (-) - bicuculline methobromide failed to displace (^{3}H) - muscimol binding to the rat brain membranes there was a 15-20% reduction in the binding at the highest concentration, but this was probably a non-specific effect. This agrees with evidence in (^{3}H) - muscimol binding (Beaumont et al. 1978, Straughan 1978), although an IC_{50} value was obtained in (^{3}H) - GABA binding studies in brain membranes a very high value was obtained for this and the (-) - isomer was found to be about 100 x less potent than the (+) - isomer.

iii) <u>Picrotoxinin and α - dihydropicrotoxinin</u>.

Again, even at very high concentrations (1mM), these compounds only produced 15-20% displacement of (^{3}H) - muscimol. This agrees with picrotoxin used to displace (^{3}H) - muscimol where concentrations from 10μM - 1mM failed to produce an IC_{50} value (Beaumont et al. 1978, Leach and Wilson 1978, Straughn 1978, Wang et al. 1978, DeFeudis et al. 1980).

Picrotoxin used to displace (^{3}H) - GABA binding from brain membranes in concentrations of up to 1mM also failed to produce an IC_{50} value (Enna et al. 1977, Olsen et al. 1979).

iv) Bicyclic phosphate esters.

Six different analogues of the bicyclic phosphates were tested up to concentrations of 1mM. None of them produced any displacement more than 10-15% of (^{3}H) - muscimol binding. However isopropyl bicyclophosphate was shown to produce an IC_{50} of 135μM in (^{3}H) - muscimol binding (Straughn 1978) and isopropyl bicyclic phosphate also has been shown to give an IC_{50} value of 200μM in (^{3}H) - GABA binding (Enna et al. 1977). The results for dimethyl propyl, neopentyl, phenyl and cyclopentyl derivatives have been represented graphically (Figs. 5.8 and 5.9).

v) Others

(±) - Nipecotic acid, a potent inhibitor of GABA uptake into neuronal and glial cells (Brehm et al. 1978) had little effect of (^{3}H) - muscimol binding in concentrations of 1000μM in agreement with Beaumont et al. (1978) and Wang et al. (1979).

The inhibitory amino acid, glycine (Curtis and Johnston 1974b, McGeer et al. 1978) was also ineffective in displacing (^{3}H) - muscimol in concentrations of 1000μM which again agreed with results obtained in binding to mouse brain fractions (Wang et al. 1978).

Tetramethylenedisulphotetramine (TETS), an

inhibitor of GABA (Bowery et al. 1975, Collins et al. 1975) also had no effect on displacement of (^{3}H) - muscimol in this preparation, in concentrations of 1000μM.

Cis-3-amino-cyclohexane carboxylic acid (ACHC), a potent blocker of neuronal GABA uptake (Collins et al. 1975, Bowery et al. 1976b, Johnston et al. 1978), also had little effect on (^{3}H) - muscimol binding in concentrations of 1000μM.

5.8.5 Conclusions

Results from the Scatchard analysis showed there was only one high affinity binding site for (^{3}H) - muscimol which had an equilibrium dissociation constant (K_D) of 7.4nM.

In other preparations using rat brain reports of a single high affinity binding site were of the same order as results for K_D ranging from 1.88nM to 13nM (Snodgrass 1978, Bernasconi et al. 1980, Kingsbury et al. 1980). In mouse brain fractions a high affinity binding site with a K_D of 10nM was obtained (Wang et al. 1979).

However, reports of two sites, one of low affinity and one of high affinity show a different dissociation constant value for the high affinity site with values ranging from 9nM - 60nM (Beaumont et al. 1978, Wang et al. 1979, Williams and Risley 1979).

The maximum number of binding sites for (^{3}H) -

muscimol (Bmax) found in the preparation was found to be 2.37 pmoles/mg of protein. Other results showed very similar observations in rat brain tissues : 3.3 pmoles/mg protein (Snodgrass 1978), 2.8 pmoles/mg protein (Beaumont et al. 1979), 2.34 pmoles/mg protein (Kingsbury et al. 1980), and using a radioreceptor assay Bmax of 2.08 pmoles/mg protein (Bernasconi et al. 1980). The high affinity binding Bmax in mouse brain also correlated well with the results found here being 2.1 pmoles/mg protein (Wang et al. 1979).

However two reports showed a much lower Bmax than those reported above. On rat synaptosomal binding a high affinity site Bmax of 0.51 pmoles/mg of protein was found (Williams and Risley 1979) and also using a radioreceptor assay a Bmax of 0.28 pmoles/mg of protein was found (Palacios et al. 1980).

Overall the results from Scatchard analysis obtained here would agree with most of the work undertaken for the specific binding of (^{3}H) - muscimol.

The (^{3}H) - muscimol appeared to bind in a specific fashion to the synaptic GABA receptor in rat brain membranes. The substrate specificity of the (^{3}H) - muscimol binding sites provides strong evidence that muscimol is highly specific for the GABA receptor because of the (^{3}H) - muscimol receptor showing appreciable affinity and stereospecificity only for those drugs which are known to act

neurophysiologically with the GABA receptor. In addition the results with the GABA agonist drugs on (^{3}H) - muscimol compare very favourably with similar experiments using (^{3}H) - GABA.

As expected only (+) - bicuculline methobromide had any significant effect on the displacement of (^{3}H) - muscimol from its receptor with (-) - bicuculline methobromide having little effect. These results again confirmed reports using (^{3}H) - GABA on the same tissues.

Similar results and reports using auto-radiographic techniques also support the evidence that (^{3}H) - muscimol is acting at the GABA receptor (DeFeudis et al. 1980).

The lack of effect of picrotoxinin and α-dihydropicrotoxinin on (^{3}H) - muscimol binding is in accord with other results on brain synaptosomes (Beaumont et al. 1978, Williams and Risley 1979) and with (^{3}H) - muscimol binding using autoradiographic techniques on cultured brain cells (DeFeudis et al. 1980, Palacios et al. 1980), and supported with their lack of effect of (^{3}H) - GABA binding (Enna et al. 1977, Olsen et al. 1979) would support the contention that they act on a GABA regulated chloride ionophore (e.g. Olsen et al. 1978a,b).

The lack of effect of the bicyclic phosphates in

(^{3}H) - muscimol binding (Straughn 1978) and in (^{3}H) - GABA binding (Enna et al. 1977) except in very high concentrations would also support the contention that they also act at a similar site to picrotoxinin (Olsen et al. 1978b, Ticku and Olsen 1979).

(±) - Nipecotic acid, a potent inhibitor of GABA uptake into neuronal and glial cells (Brehm et al. 1978) and cis - 3 - amino - cyclohexane carboxylic acid (ACHC), a potent inhibitor of GABA uptake into neuronal cells (Collins et al. 1975, Bowery et al. 1976b, Johnston et al. 1978) both had little effect of (^{3}H) - muscimol binding again correlating with sodium independent (^{3}H) - GABA binding (Krogsgaard-Larsen and Johnston 1978, Johnston et al. 1979) and that muscimol has little or no affinity for the sites related to GABA uptake (Krogsgaard-Larsen and Johnston 1978).

The inhibitory amino acid, glycine (Johnston 1974b, Snyder 1975), also had little effect on (^{3}H) - muscimol binding corroborating evidence by Wang et al. (1979) and Beaumont et al. (1978). Again this compares favourably with results in (^{3}H) - GABA binding (Olsen et al. 1978b).

Tetramethylenedisulphotetramine (TETS) a potent inhibitor of GABA in the rat superior cervical ganglion (Bowery et al. 1975) and rat cuneate nucleus (Collins et al. 1975) had little effect on (^{3}H) - muscimol displacement as it

did in (^{3}H) - GABA displacement (Enna et al. 1977), suggesting an action at some site away from the GABA receptor, possibly at a similar site of action as picrotoxinin (Ticku and Olsen 1979).

Some of the values for the IC_{50} for some of the agonists which were tested seemed higher than reported elsewhere but this may have been due to the treatment of tissue with detergents which increased both (^{3}H) - GABA binding (Enna and Snyder 1977) and (^{3}H) - muscimol binding (Beaumont et al. 1978, Williams and Risley 1979), whereas no such treatment was used in any of the tissue preparations in these experiments.

Fig. 5.10. Total (●) and non-specific (○) binding of (^{3}H)-α-dihydropicrotoxinin (DHP) with increasing concentrations of (^{3}H)-DHP. Synaptic membrane suspensions which had previously been frozen were incubated (1.0 mg protein/tube) in Tris citrate buffer (pH 7.1). Non-specific binding was determined from assays conducted in the presence of 1mM picrotoxinin. Values are the means of triplicate determinations, each experiment being replicated 2-3 times.

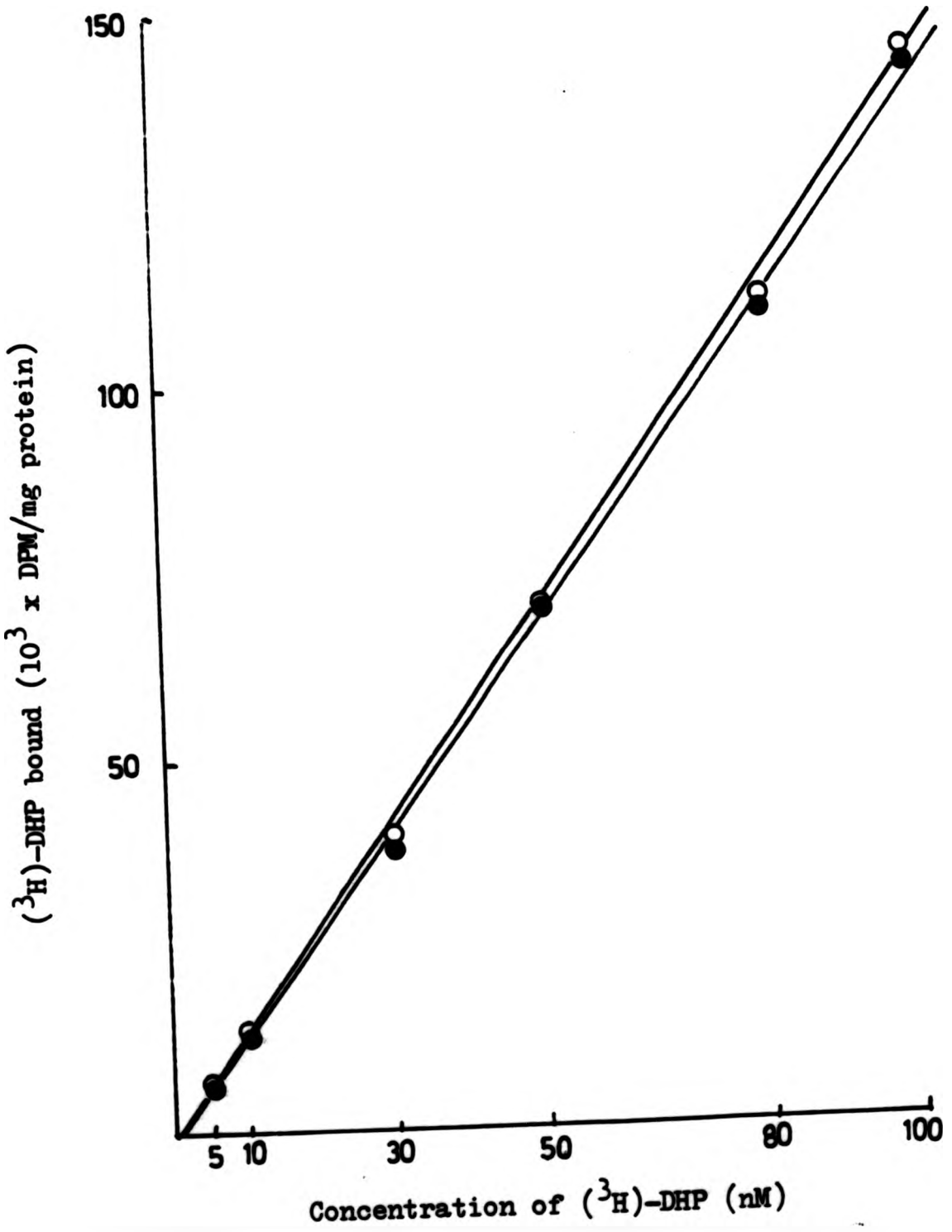


Fig. 5.11. Displacement of (^{3}H)-DHP binding to rat brain synaptic membrane suspensions (P_2 fractions) 1mg protein/tube) assayed with 5nM (19.0 Ci/mmole) radioactive ligand. Binding was measured in various concentrations of picrotoxinin (■) and α-dihydropicrotoxinin (□). Each point and vertical line on the graph being the mean and standard error for 4-11 separate determinations done in triplicate.

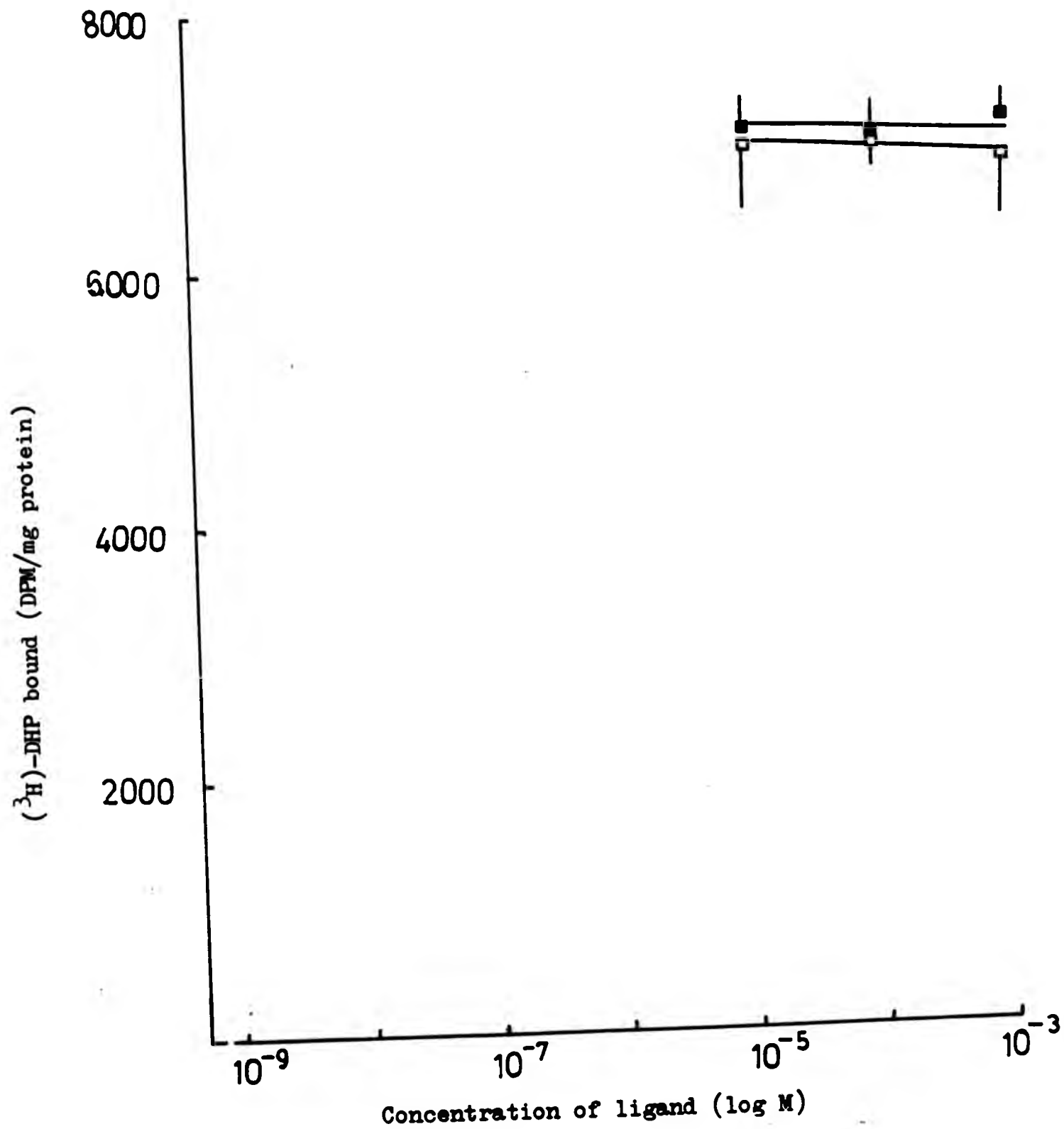
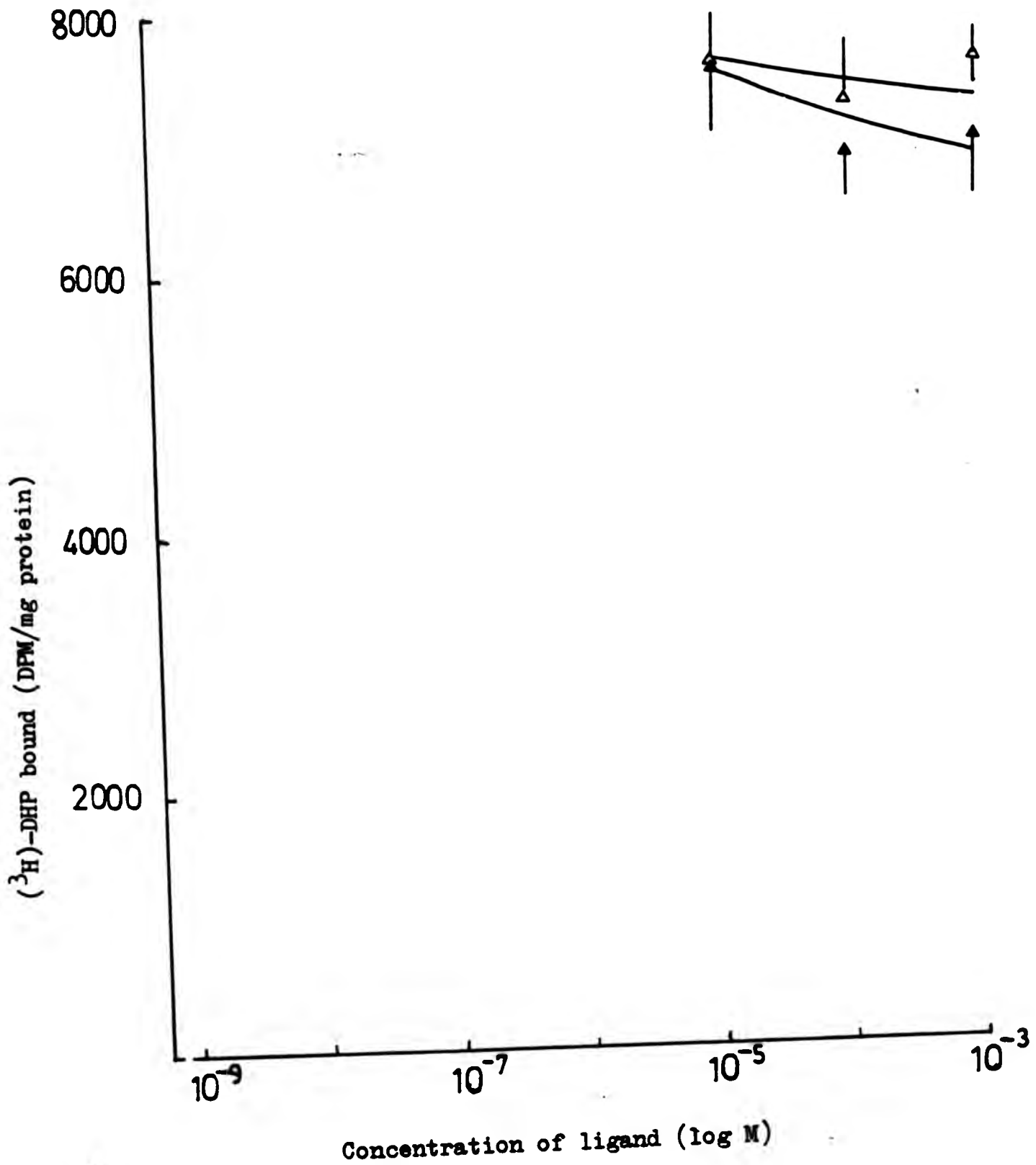


Fig. 5.12. Displacement of (^{3}H)-DHP binding to rat brain synaptic membrane suspensions (P_2 fractions) 1mg protein/tube) assayed with 5nM (19.0 Ci/mmole) radioactive ligand. Binding was measured in various concentrations of neopentyl bicyclophosphate (△) and (+)-bicuculline methobromide (▲). Each point and vertical line on the graph being the mean and standard error for 4-11 separate determinations done in triplicate.



5.9. **Binding Studies using (^{3}H) - α - dihydropicrotoxinin on rat brain synaptosomal membranes.**

5.9.1 **Specific and non-specific binding of (^{3}H) - α - dihydropicrotoxinin.**

Two sets of experiments done in triplicate with increasing concentrations of (^{3}H) - α - dihydropicrotoxinin (5nM - 100nM) on 1mg of synaptosomal fractions. The second set of experiments had a high concentration of picrotoxinin (10^{-3}M) to displace any specifically bound (^{3}H) - α - dihydropicrotoxinin.

The results of these experiments were represented graphically (Fig.5.10) with binding of (^{3}H) - α - dihydropicrotoxinin in dpm/mg of protein plotted against concentration of (^{3}H) - α - dihydro-picrotoxinin.

The results from the graph showed that there was no displacement at any concentration of (^{3}H) - α - dihydropicrotoxinin by a high concentration of picrotoxinin. It therefore must be concluded that for these experiments there was no specific binding.

5.9.2 **Calculation of the number of receptor sites from stereospecific binding.**

Since no stereospecific binding was found in any of 11 experiments, each done in triplicate using 5nM (^{3}H) - α - dihydropicrotoxinin and ones with a high concentration

of picrotoxinin (10^{-3}M) the number of receptor sites could not be calculated.

5.9.3 **Binding of (^{3}H) - α - dihydropicrotoxinin to rat brain synaptosomal preparation in the presence of GABA antagonists and agonists.**

Assuming that the unlabelled ligand behaves at the receptor site in a similar way to the labelled ligand (which is not the case when agonists are used) and that binding phenomena follows classic mass action, then the concentration of unlabelled ligand of which maximum binding of labelled ligand is displaced by 50% (IC_{50}) this will be a reasonable approximation of the apparent equilibrium dissociation binding constant.

(^{3}H) - α - dihydropicrotoxinin binding in the absence of unlabelled ligand was approximately 7,000 dpm and the non-specific binding was approximately 7,000 dpm.

Graphs plotted (Figs.5.11-5.12) showed displacement of (^{3}H) - α - dihydropicrotoxinin binding a a concentration of 5nM to 1mg of synaptosomal (P_2) fraction of rat brain by unlabelled antagonists against the log of the concentration of unlabelled antagonists.

All results were the means with standard errors for 4-11 determinations done in triplicate. The results are shown in Table 6 .

TABLE 6

Inhibitors of (^{3}H)-α-dihydropicrotoxinin binding to rat brain synaptosomes

Compound	IC_{50} (μM)
Picrotoxinin	)
α-Dihydropicrotoxinin	) > 1000
(+)-Bicuculline methobromide	)
Neopentyl bicyclic phosphate	)

The following compounds had no effect up to 1000μM t-butyl bicyclic phosphate, dimethyl propyl bicyclic phosphate, cyclopentyl bicyclic phosphate, GABA, 3-aminopropane sulphonic acid, (-)-bicuculline methobromide. None of these compounds have been represented graphically.

All results for the binding were determined in sodium-free buffer using frozen, thawed and thoroughly washed membranes as described previously. All results were made from 4-11 separate determinations done in triplicate.

TABLE 7.

Results showing the action of various agonists and antagonists on the synaptosomal fraction of the rat brain which was incubated with (^{3}H)-dihydropicrotoxinin at a concentration of 5nM).

	Cold drug incubated	Radioactivity residual in P_2 synaptosomal fraction after incubation with cold drug and (^{3}H)-DHP (5nM) in disintegrations per minute as means with standard errors. (Number of experiments for each concentration is shown in brackets.		
		Concentration of drug added		
		10^{-3}M	10^{-4}M	10^{-5}M
1.	Picrotoxinin	7084 ± 257 (33)	6972 ± 184 (24)	7137 ± 168 (24)
2.	α-Dihydro-picrotoxinin	6963 ± 652 (27)	7006 ± 244 (15)	6862 ± 250 (15)
3.	(+)-Bicuculline Methobromide	7573 ± 642 (12)	6898 ± 354 (15)	7031 ± 469 (12)
4.	Neopentyl PBTO	7537 ± 490 (9)	7311 ± 574 (6)	7593 ± 202 (12)
5.	Cyclopentyl PBTO	6857 ± 380 (24)		
6.	t-Butyl PBTO	7351 ± 307 (12)		
7.	GABA	7110 ± 560 (24)		

N.B. All experiments were carried out with internal blank controls for each batch of binding studies undertaken and these showed that in all the cases there appeared to be no displacement of the (^{3}H)-DHP.

i) <u>Picrotoxinin and α - dihydropicrotoxinin.</u>

Neither compound showed any significant displacement of (^{3}H) - α - dihydropicrotoxinin and in all experiments performed there appeared to be no significant difference between the results with high concentrations of the unlabelled compound and the results obtained for the controls, with no displacement of unlabelled compound. The results are shown in Table 7 No IC_{50} could therefore be obtained.

ii) <u>(+) - Bicuculline methobromide and (-) - bicuculline methobromide</u>.

Again there was no significant difference between the control experiments with 5nM (^{3}H) - α - dihydro-picrotoxinin and the results obtained with concentrations of (+) - bicuculline methobromide in concentrations of 10^{-5} - 10^{-3}M. No IC_{50} value could therefore be established from this. (-) Bicuculline methobromide also had no effect in concentrations of 10^{-3}M.

iii) <u>The bicyclic phosphates</u>.

Four different bicyclic phosphates were tested up to concentrations of 10^{-3}M. The neopantyl bicyclic phosphate represented in Fig. 5.12 appeared to have no effect at all on (^{3}H) - α - dihydropicrotoxinin binding. Similar results were obtained for dimethyl propyl, t-butyl and cyclopentyl analogues in concentrations of 10^{-3}M.

iv) Others

The GABA agonist 3-aminopropane sulphonic acid and GABA itself had no effect on (^{3}H) - α - dihydropicrotoxinin binding in concentrations of 10^{-3}M.

5.9.4 Conclusions

None of the GABA antagonists or agonists caused any displacement of (^{3}H) - α - dihydropicrotoxinin in concentrations of up to 10^{-3}M in any experiment compared to the control. Even the use of a separate, different batch of (^{3}H) - α - dihydropicrotoxinin obtained from a different source produced no displacement at all. The results here are in complete contrast to those obtained by the Riverside group using a similar rat brain synaptosomal method of binding but only obtaining a specific binding of less than 12% (Ticku et al. 1978b). Other work on this preparation has produced displacement of specific binding to rat brain tissues with unlabelled picrotoxinin, bicyclic phosphates, (+) - bicuculline, barbiturates, bicyclocarboxylates, benzyl penicillin, TETS and silatrane,(Ticku and Olsen 1978a, b, Ticku et al. 1978a,b and Olsen et al. 1979b).

The Riverside group have also obtained a specific binding of (^{3}H) - dihydropicrotoxinin of about 16% in crayfish muscle preparation. Displacement by picrotoxinin, tutin and α - dihydropicrotoxinin were obtained (Olsen et al. 1978d).

Although all the results have been obtained by the Riverside group, no other group of workers has published any results confirming a specific binding of (^{3}H) - α - dihydropicrotoxinin and all the results obtained in the experiments here are in complete disagreement, and that no specific binding of (^{3}H) - α - dihydropicrotoxinin can be obtained in sodium free buffer, using frozen, thawed and thoroughly washed membranes of P_2 fractions of rat brain in tris-citrate buffer at pH 7.4, but the results obtained by the Riverside group for rat brain synaptosomal membrane preparations and crayfish muscle microsomal fractions were performed using fresh tissue without the frozen, thawing and washing process, and all binding assays were performed in 0.2M sodium chloride - 5mM sodium phosphate buffer at pH 7.0 which probably accounts for the discrepancies in results obtained here, compared to their results.

Two different batches of (^{3}H) - DHP were used for the experiments, one from the Radiochemical Centre, Amersham, specific activity 30.0 Ci/mmole and the other from New England Nuclear, specific activity 19.0 Ci/mmole. Neither were displaced by any of the agonists or antagonists used in these experiments.

CHAPTER 6 : DISCUSSION AND CONCLUSIONS

6.1 Introduction

The actions of a drug, to be classed as such, must qualify in three major criteria:

i. It must have a high effective potency (often acting in concentrations of the nanomolar range).

ii. It must have chemical specificity, showing a great difference in potency between optical isomers (e.g. effects of (+)-and (-)-bicuculline binding to GABA receptors, the (+)- isomer being 2 to 3 orders of magnitude more potent than the (-)-isomer, (Enna et al. 1977) or a slight change in a chemical side chain of compounds (e.g. in the bicyclic phosphates a change in the side chain from isopropyl to n-C_6H_{13} reduced the potency by more than three orders of magnitude), (Casida et al. 1976).

iii. Biological specificity must be demonstrated (e.g. adrenaline has marked effect on cardiac muscle but very little effect on striated muscle).

These criteria have led to the generally accepted theory that some cells possess specific regions which interact in some way with the drug to cause an effect; these regions on the cells are the drug receptors.

Several different types of receptors have been characterised and various theories have been proposed as drug-receptor models (Rang, 1971, Hollenberg 1978). Some

of these theories have been applied to this study where they are applicable to the GABA receptor.

6.2 The receptor model

A fundamental assumption that if a drug is to have any action it must be bound. The site of this initial binding is termed the receptor and on first approximation in simple chemical terms as a bimolecular drug-receptor interaction (Equation [1]).

$$D + R \underset{K_{-1}}{\overset{K}{\rightleftharpoons}} DR \qquad \text{Response} \qquad [1]$$

Where D = drug or neurotransmitter, R = receptor, DR = the drug-receptor complex, and the equilibrium dissociation constant K_D is given by the quotient of the rate constants, K_{-1} / K_1 . This simple equilibrium forms the basis of the theories of drug action (Clark 1937, Paton 1961, Paton and Waud 1964).

If the magnitude of the biological response is proportional to the amount of drug-receptor complex formed and a bimolecular action is assumed in equation [1] then at equilibrium the law of mass action can be applied to give the equilibrium dissociation constant (K_D) in equation [2]

$$K_D = \frac{[D]\ [R]}{[DR]} \qquad [2]$$

where: [D] , [R] and [DR] represent the concentrations of free drug receptor and drug-receptor complex respectively.

However there must be a finite amount of receptors and a maximal response would be obtained if all these receptors were occupied by the drug to give the equation [3].

$$\text{Response} = \frac{\text{Maximal Response} \times [D]}{K_D + [D]} \qquad [3]$$

From this the concentration which produces half-maximal response $[D]_{0.5}$ (usually termed ED_{50}) can be equated with the equilibrium dissociation constant for the drug receptor interaction.

6.3 The two-state receptor model

The observation of sigmoid concentration - effect curves for GABA (and carbachol) could not be attributed to artifacts of measuring the ionic conductance as the response.

Therefore an allosteric model based on the sigmoid velocity-substate relationship observed with enzymes (Monod et al.1965) was adapted to models of drug action (Karlin 1967, Changeaux et al. 1967).

The observations, based on electrophysiological data, suggested that the membrane components involved ion transport (ion "channels") existed in two states, either open or shut, irrespective of the drug responsible for the modulation of conductivity.

In these receptor models it is assumed that the receptor and ionophore form a closely acting membrane-localised complex which only exists in two conformations for which the affinity for the drug for these sites may be different. In the absence of any drug an equilibrium will be adopted between the T-state (closed) and R-state (open).

In the case of potent agonists the affinity for the R-state (open) is much more than that of the T-state (closed). Therefore agonists will shift the equilibrium to the R-state and open the ion channel. In the case of potent antagonists the affinity for the T-state (closed) is far greater than that of the R-state (open) shifting the equilibrium to close the ion channels. The affinity for an agonist or antagonist drugs for either conformations site would determine its efficacy in opening or closing the ion channels.

The observation of high and low affinity binding sites for the binding of muscarinic agonists (Birdsall and Hulme 1976) and GABA agonists (Enna and Snyder 1976, Beaumont et al. 1978) may be rationalized in terms of this two state model.

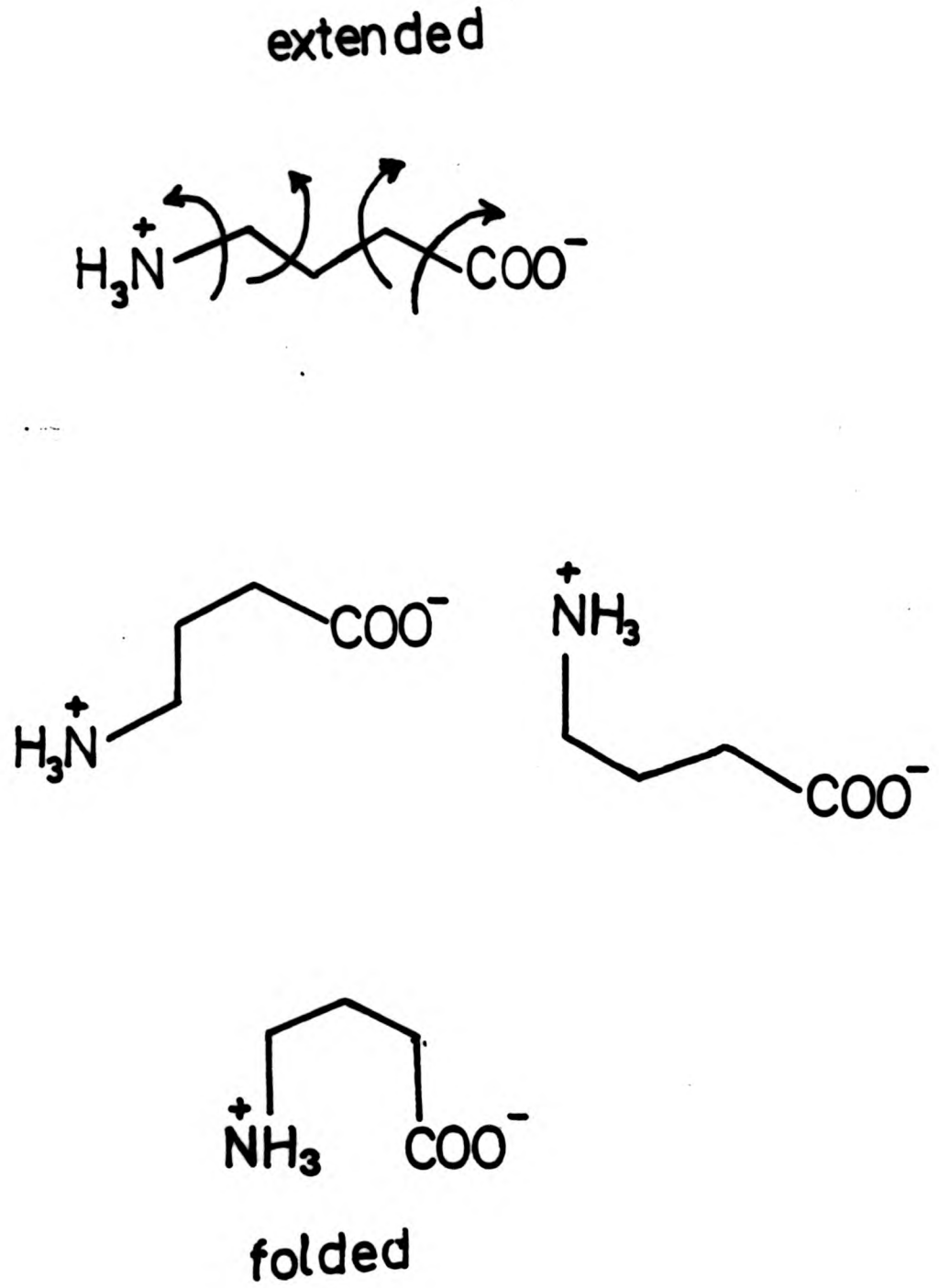
It has been suggested that receptors for neurotransmitters are able to exist in more than one conformation (Rang 1971, Snyder 1978) and that they are

multi-subunit proteins (Cuatrecases 1974, Changeux et al 1967). These subunits may be a group of oligomers (Karlin 1967) or a lattice-like network (Changeux et al 1967) with the constraint that units in the same oligomer are forced to adopt the same conformation or neighbours in the same lattice interact in such a way that they all adopt the same conformation.

Clearly these models predict that a single membrane-localised receptor recognition macromolecule can display more than one affinity for a given ligand so the Hill coefficient can assume any value and this would explain fractional values obtained for various drugs (Rang 1971, Colquhoun 1973).

Highly co-operative binding of transmitter molecules to successive receptor subunits could account for the complex sigmoid dose-response curves (Rang 1971, Colquhoun 1973). Alternatively, binding of more than one transmitter molecule to receptors could be needed to produce an ionophore response (Olsen et al. 1975, Brookes and Werman 1973), the latter model being supported by binding studies in synaptic membranes does not appear to be co-operative (Olsen 1976).

Fig. 6.1. Bond rotation in the GABA molecule producing different conformations.



Probably a combination of co-operativity and high reaction order seems to be plausible for the neurotransmitter to the receptor or receptor/ionophore coupling events. Ionophores may be co-operatively activated by allosteric interactions through either direct receptor-ionophore interactions or membrane lattice effects (Levy et al. 1975). Another model proposed involves changes in membrane mobility of the receptor and ionophore macromolecules. When transmitter is bound to the receptor these ionophore aggregates are stabilised and an opening of the chloride ion channel formed (Olsen 1976).

6.4 The active conformation of GABA

The GABA molecule has considerable flexibility as a result of free rotation around the single bonds (Fig. 6.1). This conformational mobility can be reduced in analogues of GABA by incorporation of unsaturation and/or ring structures into the basic molecular structure. The first studies using GABA analogues of restricted conformation to probe the molecular aspects of GABA interaction were performed on the crayfish stretch receptor (McGeer et al. 1961). The application of some of these analogues was done in mammalian CNS in the cat cerebral cortex (Curtis et al 1971a). New GABA analogues of restricted conformation were tested for in

other GABA-mediated processes such as high-affinity uptake (Beart et al 1972a) and GABA transamination (Beart et al. 1972b). Ligand binding with synaptosomal membrane preparations has also been used to elucidate GABA processes with these analogues (Enna and Snyder 1975). The studies were accompanied with structure-activity speculations with respect to charge separation on the molecule or whether it was in the extended form (Beart et al 1971, Johnson et al 1975) and/or the folded form (Steward et al 1971, Van Gelder 1974).

One of these restricted isomers is muscimol (Fig. 1.4) in which the restriction of conformaton of the molecule by linking C3 and C1 atoms where the 3-isoxazol moiety acts as a masked carboxyl group to restrict its conformation (Johnston et al. 1968). Muscimol is a potent selective bicuculline-sensitive GABA agonist and binds to rat brain membranes with a higher affinity than GABA also muscimol and GABA bind to common sites (Beaumont et al 1978, Palacios et al 1980). The effects of muscimol would appear to be exerted at the GABA recognition receptor site because muscimol has very little effect on the transport-related uptake recognition site, being more than 1,000 times less potent than GABA itself for this site (Krogsgaard-Larsen and Johnston 1975) and it is not a

substrate for nor an inhibitor of GABA transaminase (Johnston et al 1979).

Restriction of rotation around the N-C4 bond provided the GABA antagonist (+)-bicuculline Fig. 2.4). Structural similarities between GABA and (+)-bicuculline have been noted (Curtis et al. 1970a, Johnston 1976). Also rotation around the single bond joining the two ring structures (C1-C9) is restricted (Andrews and Johnston 1973). Ligand binding studies with GABA, (+)-bicuculline and muscimol, indicate that these compounds share some common binding sites (Enna et al 1977, MÖhler and Okada 1977, Beaumont et al 1978). Bicuculline also has a stereoselective action, the (-)-isomer being much less active than the (+)-isomer (Enna and Snyder 1977). (+)-Bicuculline has other actions but appears to have no effect on GABA high affinity uptake or GABA transamination (Johnston 1976, 1978).

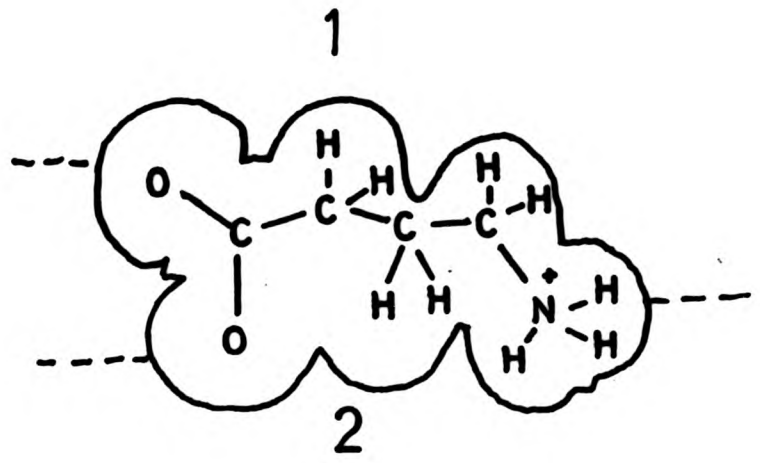
Trans-4-aminocrotonic acid (Fig. 1.5) has restriction of rotation around C3-C2 bond and has higher activity than GABA with respect to the GABA binding site (Beart et al 1972a) also it acts as a substrate for GABA transaminase and has activity similar to GABA for the high affinity GABA uptake. However, the cis-isomer (Fig. 1.5) has little activity in GABA

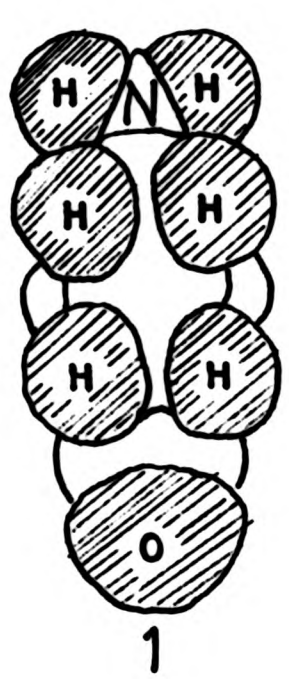
binding and is inactive against GABA high affinity uptake or transamination (Johnston et al 1975, 1979).

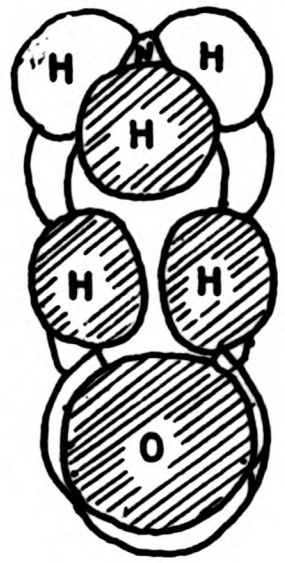
There are many other ways to restrict the conformation of GABA analogues and clearly some of these have selective actions on particular GABA processes, often acting with a higher potency than GABA itself. These actions provide evidence for a multiplicity of GABA receptors such as the postsynaptic GABA receptor, GABA neuronal uptake, GABA transaminase and GABA glial uptake.

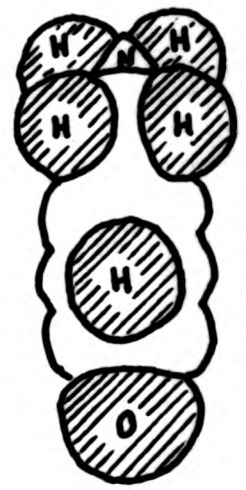
It has been suggested that the extended conformation of GABA binds to the receptor-recognition site and that the folded conformation of GABA binds to the transport-recognition site (De Feudis 1977). It is also possible that the GABA molecule attaches itself to the receptor-recognition site with one side of the extended conformation and to the transport-recognition site with the opposite side of the extended molecule (Roberts 1979). This suggests that if the two sites are in close proximity, attachment of a GABA molecule to the transport site may automatically remove a molecule from the receptor-recognition site and cause an opening of the anion channel.

Fig. 6.3. CPK (Corey-Pauling-Koltum) models of the projections of the faces of the fully extended GABA molecule showing side, top (1), and bottom (2) views. Also shown CPK model of top view of muscimol which is similar to that of the top view of the GABA model (from: Roberts et al. 1979).









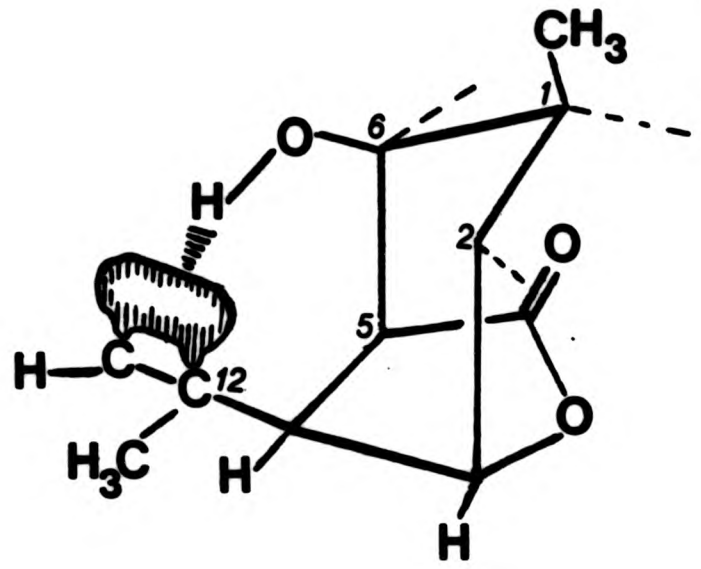
The similarities of the conformations of GABA (top view) and muscimol space filling CPK models (Fig. 6.3) show that this side is the most probable, which will facilitate binding to the receptor recognition site (Roberts 1979). Also similarities were noticed between the bottom view of the extended GABA model and some of the compounds known to have their actions at the GABA transport site. This suggestion eliminates the process of a passive equilibrium dissociation of the transmitter and anion channel opening.

6.5 The anion channel

The GABA molecule itself does not have any direct effect on the channel opening site and it is likely that picrotoxinin and several cage compounds, including the bicyclic phosphates, have their inhibitory actions on or around this site (Fig. 6.2).

In the case of picrotoxinin binding this prevents the opening of the chloride ion channel with these actions being reversible with depressant and anticonvulsant barbiturates and potentiated by convulsant barbiturates (Ticku and Olsen 1978a).

Fig. 6.4. The structure of picrotoxinin showing the features necessary for GABA antagonism (the dotted lines represent bonds to other parts of the molecule not involved in antagonism).



Several compounds similar in structure to picrotoxinin have been examined for their convulsant activity, of which picrotoxin, α-dihydro picrotoxinin, tutin and coryamytrin (Fig. 2.2) have been shown to be potent GABA antagonists(Kelly and Beart 1975a). For the most potent activity as GABA antagonist several conclusions were drawn from the shape of the molecules (Fig. 2.2). The compound must have a lactone function containing C-5 and C-3 of the skeleton. The carbonyl function must be cis to the fused ring structure and in combination with the bridgehead hydroxyl group at C-6. The alkyl side chain features already described appear to comprise an absolute structural requirement for activity.

Acetylation of the C-6 hydroxyl or cleaving the lactone ring leads to a great reduction in potency. The oxitane ring and the lactone ring joining the C-2 and C-9 (absent in tutin and coryamytrin) do not appear to be necessary for activity (Fig. 6.4).

The π (pi) Cloud of the isopropyl side chain and the hydrogen of the C-6 hydroxyl interact to form a hydrogen bond which holds the side chain approximately planar to the lactone ring (joining C-3 and C-5).

The postulated site of action of these compounds has a lipophillic cleft which would easily incorporate the isoprenyl side chain indicating the reason for the decrease in activity of α-dihydro-picrotoxinin, which has a saturated side-chain and is therefore less lipophillic.

The action of picrotoxinin could be to shorten the lifetime of the GABA-activated opening/formation of this channel or possibly to prevent the formation/opening of the channel. It is unlikely that picrotoxinin blocks the opening physically because of its size since pentobarbitone reverses both picrotoxin and bicyclic phosphate GABA antagonism (Bowery and Dray 1976) and that it also competes with picrotoxinin for the same receptor or one in a closely related position.

6.6 Prospects

Malfunction of the GABA system in the brain may cause various neurological and psychiatric disorders, such as Huntington's chorea, involving reduction in levels of GABA in certain brain areas. Administration of a GABA-mimetic has been shown to be effective in the treatment of some patients (Bartholini et al. 1978) but unsuccessful in others (Chase and Tanminga 1978). Parkinson's Disease also involves a reduction in GABA function in the substantia nigra (Lloyd et al. 1979). Also malfunction of GABAergic neurones is thought to be an important factor in epilepsy (Meldrum 1975) and schizophrenia (Iversen et al. 1978, Meldrum 1979).

Since the GABA antagonists do not exert their actions at the same site as agonists they can be of no use in evaluation or the clinical treatment of these dysfunctions. They may however help in elucidation of the overall effects of the inhibitory GABA system especially as the distribution of GABA in the CNS varies and there may be different developments of the system with age.

The use of evaluation clinically is limited because of their potency in producing seizures and death in a wide range of species (Casida et al. 1976, Eto et al. 1976) does not detract from their usefulness as a pharmacological tool in the elucidation of the GABA receptor complex helping to characterize the specificity and efficacy between the different

antagonistic sites of action; the bicyclic phosphates acting at the picrotoxin sensitive anion channel rather than the bicuculline sensitive site of action.

6.7 Conclusions

Although the syntheses involving production of a highly-branched, lipophillic substituent on the bridge-head of the bicyclic phosphate esters produced the predicted potencies in GABA antagonism, other compounds possessing such groups need to be synthesised in order to give some more insight into the nature of the receptor of channel involved in the process. The thexyl compound which was ultimately not possible to synthesise at this time should have produced a very potent antagonist due to its shape and the lipophillicity of this group. This work in collaboration with more research into the synthesis and evaluation of analogues of picrotoxinin and the silatranes would be very useful in order to compare the effects, site of action and potencies of these compounds.

Development of binding studies utilising the GABA system on different tissues would also prove invaluable in the study of antagonists. This done in conjunction with studies involving the radiolabelling of other antagonists such as the silatranes, bicyclocarboxylates and the bicyclic phosphate esters themselves would bring about major advances in the understanding of the interactions between the different types of antagonists

and their sites of action involving the GABA receptor complex and its indirect actions. I see development of the study of GABA antagonists in these directions the most useful in the study for further understanding of the inhibitory actions of GABA.

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