MOLECULAR STUDIES OF SOME EXCITATORY NEUROTRANSMITTERS

AND A PRELIMINARY STUDY OF PROSTAGLANDIN FLEXIBILITY

by

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Abstract

The experimental work detailed here falls into four main areas.

(1) Quantitative structure activity relationship (QSAR) studies

These were used to investigate the "active" conformations of the three neuroexcitant amino acids listed below. A 3–point electrostatic interaction with the receptor was assumed in each case.

(a) **L-Glutamate.** Conformational analysis yielded two possible "active" conformations by careful comparison of the conformational modes of L-ibotenate with those of 1-amino-1, 3-dicarboxy cyclopentane – both L-glutamate agonists.

(b) **L-Aspartate.** Due to the lack of a suitably active conformationally restricted ligand it was unfortunately not possible to predict an "active" conformation.

(c) **Kainate.** The ideal situation arose. A single "active" conformation was defined by comparison of the L-glutamate analogue L-ibotenate with kainate itself – both conformationally restricted. A further hydrophobic binding site might also exist.

(2) Radiolabelled ligand binding studies

These provided the databases for the QSAR studies above. Two binding studies were performed.

(a) *In vitro* **L-[^{3}H] aspartate** studies yielded a hierarchy of potencies for various active displacing ligands for use in the L-aspartate QSAR study above.

(b) **[^{3}H] Kainate** studies also produced a potency data set for use in the kainate QSAR study.

(3) Crystal structure determinations

These were performed to provide detailed information in support of the above areas. Two reported excitatory amino acid antagonists were investigated.

(a) **HA–966**

(b) **α – Methyl – D L – glutamate**

(4) Conformational analysis of the prostaglandins

A computer theoretical study of prostaglandin flexibility was performed. A start was made on correlating potency with a PG molecule's ability to adopt an aligned side chain conformation. During the course of this work a series of papers on the same topic appeared in the literature from Japan.A comparison with that work is included here.

The thrust behind this work came from the desire to understand *how* drugs actually interact with their "receptors" – the first stage in evoking their physiological effect that translates into a tangible change in, say, an illness related symptom.

But first, the concept of the "receptor" must be defined. It is usually a proteo-lipid entity capable of recognising *specific* drugs and binding with them to produce a physico-chemical change.

An understanding of drug interaction events at the molecular or even atomic levels can arguably lead to *rational* drug design. Prior to the early '70s discovery of new drugs was usually by a process of synthesising a vast number of different compounds and screening for activity. This process could be broken down into two levels. Modifications to an existing drug might be made by almost "hunch" and the new structures screened for a *particular* activity – Beecham's series of synthetic penicillin antibiotics was developed in this way. At a more pioneering level completely novel compounds could be synthesised and tested across a broad spectrum of bioassays. ICI found this random approach produced a "hit" rate of only 1 in 400,000! (Wooldridge, 1978 private communication). A hugely higher success rate might be expected from a *rational* approach.

The work detailed here pursues the first leg in the process of the rational design of neurotransmitter drugs – either agonists or antagonists. The method adopted is a variation of *conformational* analysis. Indeed Martin et al (1973) have already successfully designed an anti-Parkinson's disease drug using a conformational approach. Where "viewing" the receptor surface is concerned, conformational analysis is one of many *indirect* techniques where complementarity is assumed between drug and receptor. *Direct* methods – notably x-ray crystallography – may one day visualise a receptor with bound drug – but probably not yet awhile, although this *has* been achieved with substrate bound to an enzyme (Phillips, 1963).

CHAPTER 1

1. Introduction

1.1. The outline

1.1.1. Scope of work

This work describes the pursuit of "active conformations" for three neuroexcitatory molecules – the receptor surfaces were then assumed complementary in both 3-D structure and charge distribution. This is the first step in the process of *rational* drug design introduced in the preamble. The second step of synthesising "designed" drugs is the logical ultimate aim -- not attempted here. Chapter 4 details conformational studies of L-glutamate, L-aspartate and kainate which are all excitatory amino acids in the CNS of both vertebrates and invertebrates. Collection of the data sets using radiolabelled binding assays is described in Appendix A. Supporting crystal structures of two reported amino acid antagonists are given in Appendix B. Chapters 2, 3 and 4 discuss the background required for Chapter 5. Chapter 2 outlines the many theories of drug action at the molecular level. Chapter 3 looks at the physiological events during nervous transmission. The pharmacology of the amino acid neurotransmitters is discussed in Chapter 4.

A theoretical flexibility study was made of the prostaglandins (Chapter 6). These more complex molecules all possess a special structural feature – a pair of hydrophobic side chains. The alignment of these side chains was assessed in relation to potency.

1.1.2. Choice of Approach

A *conformational* approach was employed as detailed in Chapter 5.

Many techniques have been used in the drive toward rational drug design e.g. Hansch and the Free-Wilson or *De Novo* methods (Hansch, 1973; Neely, 1973; Purcell et al, 1973). Hansch's scheme treats potency as a function of a molecule's physico-chemical characteristics whilst the Free-Wilson approach assumes that the substituent groups of a parent compound contribute linearly to biological activity. Hansch's method is not only concerned with a drug's ability to bind the receptor but also its capacity to be absorbed across membranes – an obvious modulator of potency.

The conformational analysis technique used here looks at *only* a drug's ability to bind the receptor – made possible by the choice of data – collection method described in Appendix A (Radiolabelled binding studies). Whilst investigating binding at a receptor surface (as

opposed to investigating the *overall* potency of a drug) it is imperative to collect potency data which relate to a particular receptor exclusively – also no potency modulating factors should be allowed to interfere. This vitally important aspect of the work will be discussed in 5.1.2.

The umbrella term of "conformational analysis" covers two distinct sub-approaches. The first assumes that the "active conformation" (that conformation adopted in binding the receptor) exists amongst the minimum or low energy conformations – often termed "preferred" conformers. Molecular Orbital (MO) calculations, simplistic Classical Energy considerations and X-ray crystallographic studies are all able to furnish information on preferred conformations. The second sub-approach scrutinises all conformations (usually on a rotation grid of each bond) of molecules within a biologically active homologous series. A common conformation (or rather disposition of functional groups) is sought between the molecules of the series to arrive at the assumed "active conformation". The only experimental requirements of this second approach are a reliable set of potency data and a Dryding Model Kit. This was the method of choice used in Chapter 5.

Returning to Hansch, his scheme sought a physico-chemical activity relationship (PAR) whilst the Free-Wilson approach looked for a structure activity relationship (SAR). Since in the conformational analysis used here actual distances between functional groups are measured it can be termed a quantitative structure activity relationship (QSAR) approach.

1.2. Important Considerations in Conformational Analysis

1.2.1. Principle of Complementarity

All the various techniques currently available to "view" receptor morphology are indirect in so much as it is the ligands, active at the receptor, which are studied to yield data which are then assumed to be complementary to those relating to the receptor. Thus, in order to "map-out" the three dimensional morphology of a receptor it is necessary to discover the "active" three dimensional structure of a ligand in terms of:

(i) the corporal shape and steric dimensions

(ii) hydrophilic charges and their disposition within the molecule

(iii) hydrophobic areas and their disposition

1.2.2. Importance of conformation

Since many ligands are flexible, it is not only the atomic configuration of the molecule which is important but also the conformational modes which that configuration allows One or more of these conformations will then correspond to the "active" three-dimensional structure of a ligand mentioned above. Of course a molecule will not normally be completely flexible about all bonds since usually the presence of various chemical groups will restrict relative atomic movement to some extent.

1.2.3. Further thoughts on choice of approach

Inter alia, two techniques which have been extensively applied to the study of molecular conformers, with a view to deducing a possible "active" mode, are X-ray crystallography and molecular orbital (MO) calculations. The crystal structure of a molecule usually reflects one of probably a number of relatively stable conformers of that molecule found in solution commonly with minor distortions due to inter- and intra-molecular hydrogen bonding, etc. on packing. Molecular orbital calculations are commonly used to predict either electronic charge distributions around molecules, or those conformational modes with the highest population densities. In the discussion of "active" conformers, much attention has been focused on these most stable conformers. Indeed Gill (1959, 1964), using Classical Energy considerations was able to demonstrate a relationship between the most highly populated conformers and activity in two series of acetylcholine antagonists. More recent work on the GABA receptor (Clarke, 1976; Steward et al, 1971, 1975; Steward & Clarke, 1975) has demonstrated a further relationship between high probability conformers and activity. However there is no *a priori* reason why a ligand should employ the most stable conformation available to it as its active comformation in binding to its receptor. This is a view which is certainly shared by Nicholson, Suckling and Iverson (1979), Buu, Phil and Van Gelder (1976) and is also mooted by Kier (1975). Indeed Roberts (1974) has cited several examples where a ligand does not bind in its "preferred" conformation.

Many studies (e.g. Borthwick & Steward, 1976a, b) have sought to define accurately the most populated conformation of a ligand in solution on the basis of intramolecular stabilisation of a particular conformation. This conformation is then considered likely to be active. It must be pointed out however that during the process of ligand binding, it is equally likely that a functional group on the receptor will stabilise the ligand in a completely different conformation through intermolecular stabilisation. The distorting influence of intermolecular stabilisation can be observed in most crystal structures.

Against this background, the X-ray crystallographic studies performed here (Appendix B) have only been used to confirm configuration and/or determine charge distributions. Sim-

ilarly the MO calculations referred to here have only been employed to predict charge distributions.

The type of conformational technique described in Chapter 5 does not assume that the active conformer is necessarily a highly populated one -- although this may be so. Where conformationally *restricted* ligands exist in the active homologous series under review, this usually narrows the number of common conformations and, in the most favourable case, a single "active" conformation emerges. This technique has been demonstrated to be successful in the determination of the active conformation of GABA (Nicholson et al, 1979).

1.3. A Word on X-ray crystallography as a direct approach

Of course it would be very instructive if the proteo-lipid receptor could be crystallised and viewed directly by this technique. However, not only are receptors extremely difficult to purify whilst retaining their activity, but proteo-lipids are difficult to crystallise. Isolation and purification of neurotransmitter binding proteins which are thought to represent receptors have been reported for, *inter alia*, acetycholine (De Robertis 1971-73; Klett et al, 1973) and glutamate (shrimp muscle, De Plazas & De Robertis, 1974; locust muscle, Hunt, 1973). Also although the crystal structures of a number of enzymes have been solved -- with and without bound substrate -- the molecular weight of a neurotransmitter receptor proteo-lipid might be many times that of a typical enzyme resulting in very poor resolution of the constituent atoms. The conformation of a bound ligand would then be very difficult to determine -- if the ligand could be distinguished at all! One solution might be to cleave the proteo-lipid receptor into smaller moieties and then to attempt to crystallise the binding site moiety from these. The atomic resolution would then be very much improved but the conformation and integrity of the receptor binding site would probably be dependent on the other moieties. Against this backdrop, direct X-ray crystallographic study of receptors does not appear to be on the short term horizon.

CHAPTER 2

2. Receptor models and the action of neurotransmitters

2.1 Introduction

2.1.1 Two basic theories

The concept of the receptor is inextricably linked to the biological responsiveness of the system under study. Prior to the mid 1960's nearly all the information concerning receptors was deduced from analysis of dose – response data for a variety of agonist and antagonist compounds causing rapid effects in nerve or muscle preparations. With particular reference to the excitant neurotransmitters, the work of Curtis and Watkins (1960, 1963) is particularly illustrative of this type of experiment where compounds were iontophoretically administered to various *in vivo* nerve preparations and the "spike-height" and time scale of the subsequent depolarisations were measured as indicative of compound potency. As a wealth of dose-response data was built up, two basic receptor theories gained credibility; they were the **Occupancy Theory** as developed by Clark (1926, 1927, 1937), Gaddum (1926, 1937), Ariens et al (1954, 1964), Stephenson et al (1956, 1970) and others and the **Rate Theory** developed by Paton et al (1961, 1965, 1964). These two theories have yet to be superceded and all data is still interpreted basically in terms of one of these theories or a combination of the two.

The *occupation theory* assumes that the biological response is proportional to the amount of ligand – receptor complex formed. The *rate theory* assumes that potency is proportional to the rate of receptor occupation. i.e. in the present context, a specific number of neuroactive ligands would need to bind the receptor to "push" a single ion through the channel. We have assumed here, however, that an ion channel is continously "open" whilst the receptor is occupied i.e. equilibrium *occupation* is proportional to potency in the binding studies used in Chapter 5. The studies here do not recognise molecules' differential ability to perturb the receptor (to illicit a response) once bound. This ability was termed "intrinsic activity" by Ariens (1954, 1964) and "efficacy" by Stephenson (1956, 1970).

2.1.2 Two state receptor models

Based on electrophysiological data, it has been proposed that the membrane components of ion channels exist in two states in equilibrium whether the channel is closed and irrespective of the drug responsible for the modulation of conductivity. This construct, based on the model of Monod et al (1965), further assumes that the receptor and ionophore form a strongly interacting membrane – localised complex (the drug-binding protomer) and that

the drug affinity for the receptor in the two states, R (ionophore open) and T (ionophore closed), may be different. If it is assumed that agonists have a greater affinity for the R state and that antagonists a greater affinity for the T state then the resulting perturbations in the equilibrium between the R and T states effectively account for agonism and antagonism. Also this model obviates, to a large extent, the need for consideration of drug "efficacy"

With direct relevance to neurotransmitters, Mohler and Okada (1977) have demonstrated that an agonist and antagonist conformation of the GABA receptor might exist. It has also been suggested (Pert & Snyder, 1974, 1975; Feinberg et al, 1976) that the opiate receptor can assume two different conformations, one favouring antagonists and the other favouring agonists. Young & Snyder (1974) have shown that the glycine receptor also exhibits similar characterisitcs.

Two basic variations of the two-state model have been developed, as outlined extensively by Colquhoun (1973) and in detail by Karlin (1967) and by Changeux et al (1968, 1970). No two-state receptor has yet been identified for L-glutamate, L-aspartate or kainate – the three neuroexcitants under investigation here.

2.2 Receptor binding of flexible ligands

2.2.1 Introduction

Flexible neurotransmitters exist in solution in a number of conformations in equilibrium with each other. On the other hand, when bound to a receptor binding site it is assumed that these flexibile ligands have a single, well defined conformation. The formation of such a ligand – receptor complex must therefore involve a process of conformational selection, the kinetic effects of which will depend on the mechanism of the binding process. Burgen et al (1975) have discussed two contrasting models for this binding process which are described below.

2.2.2 Lock and Key model

This first model is essentially an extension of Fischer's "lock and key" model (Fisher, 1894) in which only those ligand molecules which collide with the binding site in the correct conformation and orientation are assured to form a stable complex. In this single-step binding process all the favourable interactions between each "segment" of the ligand and the corresponding "subsites" on the receptor are formed simultaneously. For a ligand with a large number of conformational degrees of freedom, the proportion of molecules in solution adopting the binding or "active" conformation will usually be quite small which

will, of course, reduce the effective association rate constant by a corresponding amount when compared with say a rigid analogue which is fixed in the active conformation.

2.2.3 The "zipper" model

This alternative model proposes that the flexible ligand and receptor bind with a "zipper" action and is therefore termed by Burgen et al (1975) the "zipper" model. In this model it is supposed that an initial "nucleation" complex is formed by interaction of a single segment of the ligand with its subsite and that this is followed by a series of conformational rearrangements of the partly bound ligand leading to binding of the remaining segments to their appropriate subsites. The association rate constant of the complex, when compared with the "lock and key" model, will be very much greater since not only are the conformational requirements much less stringent but also the orientational requirements. Additionally, Burgen et al (1975) have argued that, following the formation of the initial nucleation complex, conformational rearrangements occur at such a high rate than an adjacent segment could easily be bound to its corresponding subsite within the lifetime of the initial nucleation complex. The "cooperativity" of this sequential binding of molecular segments means that there is a strong probability that nucleation will be followed by propogation leading to complete binding.

It has often been assumed in conformational analysis that the conformation of the bound ligand is more likely to correspond to the predominant conformation of the ligand in solution since if the conformation adopted by the ligand in the complex is one which is poorly populated in free solution then the "lock and key" model predicts that the association rate constant will be low which is not a characteristic expected of a neurotransmitter. However, for the "zipper" model, since the rate of binding is likely to be determined by the nucleation step, the forward rate will be less dependent on the nature of the conformation. The "zipper" model thus provides a mechanism for the rapid binding of ligands even when a conformation of low population in solution is involved.

2.2.4 Kinetic differences between the two models

In terms of kinetics, the difference between these two models for ligand binding can be represented in terms of energy barriers (Fig 2.1). In the "lock and key" model there is a large energy barrier for binding containing translational, orientational and conformational terms. In the zipper model this is replaced by a series of smaller energy barriers corresponding to the sequential molecular segment binding and since the overall rate is related to the height of the largest energy barrier in the reaction sequence the association rate will increase. As the two models only represent alternative pathways for going from the same initial to the same final state the equilibrium K_D must of course be the same for both

models. Therefore the faster association rate constant is accompanied by a faster dissociation rate constant which is entirely consistent with characteristics expected of a neurotransmitter.

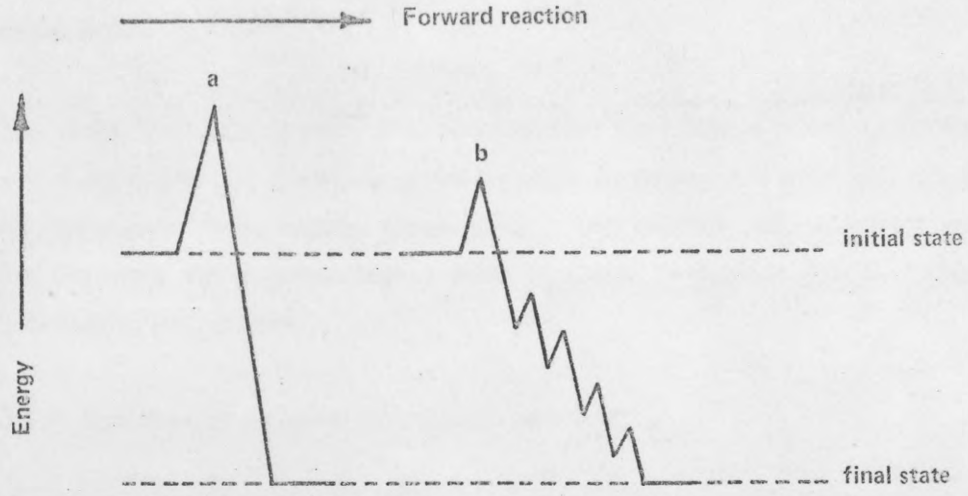


Fig 2.1 Schematic representation of the kinetics of the (a) "lock & key" and (b) "zipper" models

2.2.5 Hand in glove model

This is a basic extension of the zipper model. It assumes there occurs mutual conformational adjustment of both the ligand and receptor during binding. The phrase "hand in glove" was first coined by Williams (1977) although the role of ligand-receptor flexibility was forseen by Schueler (1953) and was embodied in the "induced fit" model of Koshland (1959).

2.2.6 Remote recognition of preferred conformation

The phrase "remote recognition", coined by Kier (1973, & Holtje 1975) embodies the concept that "a molecule makes a preliminary weak bond with a receptor while in a minimum energy state". As the drug molecule approaches the receptor, Kier visualised that "essential features of the drug molecule become oriented in a favourable way with complementary structures on the receptor" before formal binding takes place. That is "the essential phenomenon of the "recognition" of receptor for pharmacophore occurs at a separation distance greater than the distance at which conformational and electronic perturbations ensue".

Due to thermal vibration rotation about single bonds may occur at rates greater than $10^{10}s^{-1}$ (Burgen et al, 1975) and therefore, unless the electrostatic forces between ionisible groups on the ligand and complementary ones on the receptor are very strong, remote recognition does not seem likely except for perhaps very short distances. The favoured "zipper" model does not require "remote recognition" for successful binding of course.

CHAPTER 3

3. Physiology of nervous conduction

3.1 Introduction

3.1.1 Preamble

The major part of this work is concerned with the putative neurotransmitters L-glutamate and L-aspartate. It therefore seems sensible to discuss the essential role that neurotransmitters play in nerve impulse transmission. This chapter also provides a useful background for the early electrophysiological work of Curtis & Watkins (1960, 1963, 1965) on the neuroactive amino acids.

3.1.2 Structure of a typical mammalian nerve cell

Although many different types of nerve cell or neurone exist Fig 3.1 outlines the essential features.

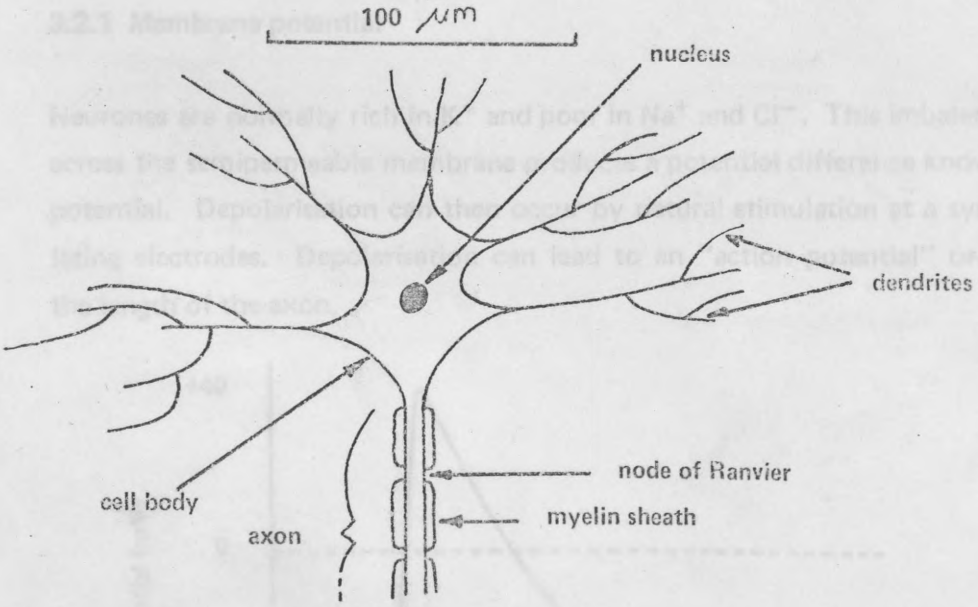


Fig 3.1 Structure of a typical mammalian neurone (After Bowsher 1979)

The dendrites in Fig 3.1 above terminate in boutons also termed synaptic knobs (Fig 3.2) which are normally in contact with the cell body or dendrite of another neurone. It is across the synaptic cleft of Fig 3.2 that neurotransmitter molecules pass to propogate a nervous impulse.

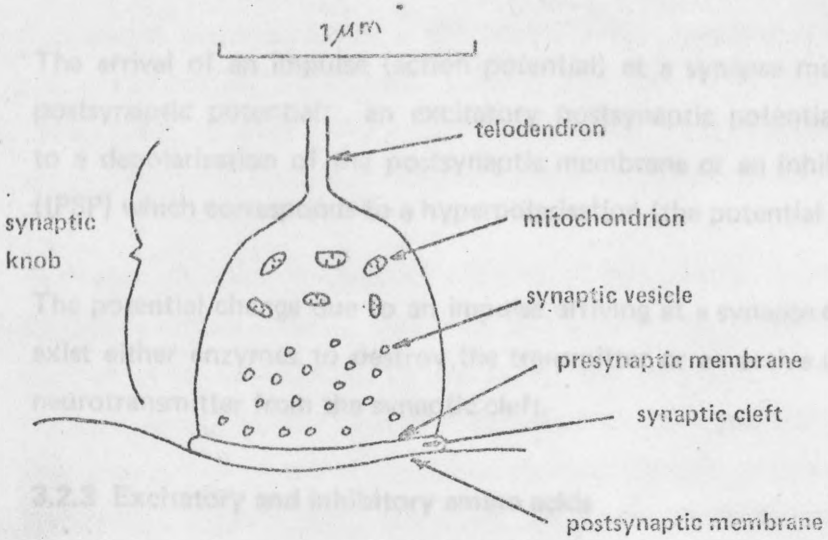


Fig 3.2 Structure of the Synapse (After Bowsher, 1979)

3.2 Nerve impulse conduction

3.2.1 Membrane potential

Neurones are normally rich in K^+ and poor in Na^+ and Cl^-. This imbalance of ionic species across the semipermeable membrane produces a potential difference known as the "resting" potential. Depolarisation can then occur by natural stimulation at a synapse or by stimulating electrodes. Depolarisation can lead to an "action potential" or impulse travelling the length of the axon.

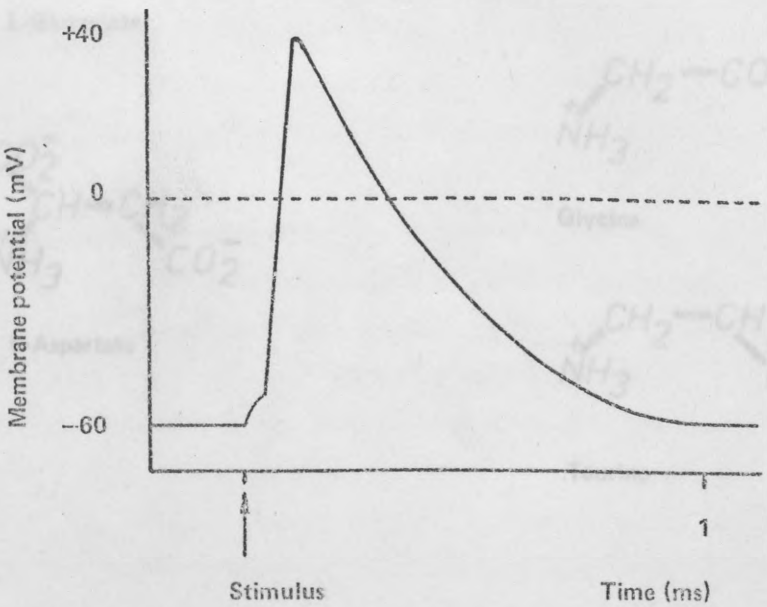


Fig 3.3 Time course of an idealised action potential (after Dowben, 1971)

3.2.2 Postsynaptic potentials

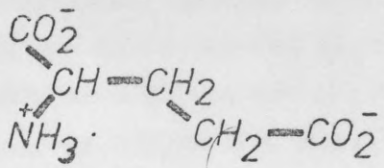
The arrival of an impulse (action potential) at a synapse may cause one of two types of postsynaptic potential: an excitatory postsynaptic potential (EPSP) which corresponds to a depolarisation of the postsynaptic membrane or an inhibitory postsynaptic potential (IPSP) which corresponds to a hyperpolarisation (the potential difference increases).

The potential change due to an impulse arriving at a synapse does not persist because there exist either enzymes to destroy the transmitter or an active uptake system to remove the neurotransmitter from the synaptic cleft.

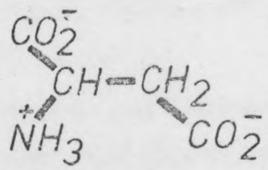
3.2.3 Excitatory and inhibitory amino acids

Many central neurotransmitters have now been identified (Ryall, 1979). They include acetylcholine, 5-hydroxytryptamine, noradrenaline, dopamine, adrenaline, leucine, enkephalin and substance P but the neurotransmitters which are of interest here are the amino acids. The major cerebral amino acids are listed below.

Excitatory

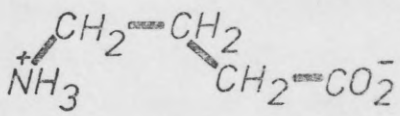


L-Glutamate

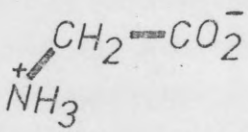


L-Aspartate

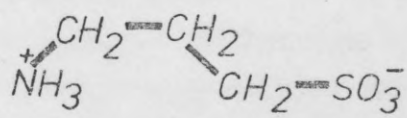
Inhibitory



γ – Aminobutyric acid (GABA)



Glycine



Taurine

The evidence for these amino acids as excitatory or inhibitory neurotransmitters has been discussed in depth by Curtis & Johnston (1974). Although it has been claimed that there are morphological differences between inhibitory and excitatory synapses (Uchizono, 1965, 1967; Bodian 1966; Larramendi et al, 1967) it is the neurotransmitter involved which determines the type of postsynaptic potential produced.

The work discussed throughout the rest of this text is concerned primarily with the two excitatory amino acids L-glutamate and L-aspartate. Both of these amino acids produce depolarisation (excitation) by increasing the postsynaptic membrane's permeability to Na^+ (Krnjevic, 1970). Investigations have also indicated (Ames et al, 1967) that Ca^{2+} may play a role in the conductance change. The inhibitory transmitters produce hyperpolarisation by selectively increasing the postsynaptic membrane's permeability to Cl^- (Krnjevic, 1970). The exact mechanism of permeability change is connected with selective ion pores or channels which the respective transmitters can open by binding to a receptor site associated with the pore. It has been suggested that L-glutamate might initiate a permeability change to Na^+ by displacing Ca^{2+} from the membrane sites which control Na^+ permeability (see Tan, 1975).

4. Pharmacology of the excitatory amino acids

4.1 L-Glutamate and L-aspartate as neurotransmitters

4.1.1 Introduction

Hayashi (1954) was the first to describe the potent excitatory effect of L-glutamate on neurones when applied to the cerebral cortex. The iontophoretic application of L-glutamate to individual neurones has shown that most cells are excited and depolarised by it (Curtis et al, 1960; Curtis 1965; Krnjevic, 1965, 1970; Curtis & Crawford, 1964) and its application closely mimics the effect of synaptic activation (Galindo et al, 1968). The wealth of physiological and biochemical information now gathered, and reviewed relatively recently by Curits & Johnston (1974) and Krnjevic (1974), indicates that L-glutamate and L-aspartate are the main excitatory neurotransmitters in the mammalian brain.

Additionally, there is good evidence that L-glutamate is the excitatory synaptic transmitter in peripheral crayfish muscles (Kravitz et al, 1970; Takeuchi & Takeuchi, 1964; Onodera & Takeuchi, 1976; Dudel, 1974) as well as in synapses of other invertebrates (Usherwood & Cull-Candy, 1975; Takeuchi & Takeuchi, 1972).

Although L-aspartate was initially reported to only act like L-glutamate (Krnjevic, 1974a; Gerschenfeld, 1973), subsequent experiments in lobster muscle (Shank & Freeman, 1975) and the mammalian CNS (Curtis & Johnston, 1974a) have suggested that L-aspartate probably has a distinct role as a neurotransmitter. In addition to its excitatory effects, L-glutamate has also been shown to exhibit inhibitory action in the mammalian brain which does not appear to be mediated by the release of an inhibitory neurotransmitter (Yamamoto et al, 1976, 1977). Inhibitory action of L-glutamate and a number of its analogues has also been demonstrated in the snail (Kerkut et al, 1974). Although recognised to exist, the inhibitory actions of L-glutamate have not been pursued in this work.

Lowagie and Gerschenfeld (1974), working with the crayfish neuromuscular junction, found that χ-methyl-L-glutamate was the most potent blocker of L-glutamate activity of a series of L-glutamate esters tested. However, Haldeman and McLennan (1972) working with spinal and cuneate neurones of the cat found that L-glutamic acid diethyl ester was the most potent blocker of the series, and the methylesters had agonistic effects. This reversed pattern suggests that vertebrate and invertebrate glutamate receptors are probably different. This and other evidence (Constanti & Nistri, 1976) of the discrete identities of vertebrate and invertebrate glutamate receptors compelled us to decide that only vertebrate results should be investigated and discussed in the QSAR studies of Chapter 5.

4.1.2 Discrete receptors

Although L-glutamate and L-aspartate mediate a similar physiological effect they are thought to possess discrete receptors (Buu et al, 1976). In support of this contention, Duggan (1974) reported that spinal interneurones of the rat are slightly more sensitive to L-glutamate than to L-aspartate and the converse was found for Renshaw cells. However, perhaps more importantly, the recent study by Foster and Roberts (1978) has shown that L-glutamate is a far more potent displacer of specifically bound radiolabelled L-glutamate than L-aspartate and conversley, the binding study described here (Appendix A2) has shown that L-aspartate is far more potent at displacing specifically radiolabelled L-aspartate than L-glutamate. Both of these results indicate neurotransmitter selectivity, that is, discrete receptors.

4.2 Agonists of L-glutamate and L-aspartate

4.2.1 Neuroexcitants kainate and NMDA

There have been numerous studies reporting the powerful excitant action of kainate on neurones in rat brain (Shinozaki & Konishi, 1970) rat, frog and rat spinal cord (Johnston et al, 1974; Padjen 1974; Biscoe et al, 1976) in snail ganglia (Walker, 1976) and at the crustacean neuromuscular junction (Shinozaki & Shibuya, 1976; Wheal & Kerkut, 1976). In certain invertebrate systems, however, whilst kainic acid is only a relatively weak direct agonist it potentiates the action of glutamate (Constanti & Nistri, 1975; Daoud & Usherwood, 1975; Walker, 1976). The potent excitatory action of N-methyl-D-aspartate (NMDA) was first reported by Curtis & Watkins (1963).

Shortly after publication of the work of Duggan (1974), mentioned in subsection 4.1.2 above, McCulloch et al (1974) reported that the relative sensitivities found by Duggan were amplified by the administration of the neuroexcitants kainate and NMDA instead of L-glutamate and L-aspartate respectively. From this evidence, coupled with structural considerations it was postulated (Johnston et al, 1974) that kainate may be relatively selective for the glutamate receptor and that NMDA may be selective for the aspartate receptor. However, the recent study of Foster & Roberts (1978) has revealed that kainate displays little ability to displace specifically bound radiolabelled L-glutamate and conversely, it has also been demonstrated (Simon et al, 1976; Johnston et al, 1979) that L-glutamate is only a weak displacer of specifically bound radiolabelled kainate. Similarly, the aspartate binding study described here (Section A2.2) has shown that NMDA exhibits little ability to displace specfically bound radiolabelled L-aspartate. Also Engberg et al (1978) have found that the membrane conductance characteristics produced by NMDA and kainate are very different from those of L-aspartate and L-glutamate. There additionally appears to be a supra-additive effect between iontophoretically administered kainate and L-glutamate

(Shinozaki & Shibuya, 1974). These results, taken together with a wealth of other evidence (e.g. Hall et al, 1978; London & Coyle, 1979) tend to suggest that NMDA and kainate possess their own discrete receptors. Against this background, the high affinity kainate binding work of Simon et al (1976) was enlarged here (see section A2.3) to provide a data base for the structure activity relationship (SAR) study of this putative kainate receptor performed here and described in Section 5.4.

4.2.2 Kainate as a neurotoxin

Although kainate is a potent excitant when applied to mammalian neurones (Shinozaki & Konishi, 1970) the injection of kainate into rat striatum and hippocampus causes lesions whose patterns of damage are similar to those seen in the human afflictions of Huntingdon's chorea and status epilectus respectively (Coyle et al, 1978; Nadler et al, 1978). In fact, kainate is able to selectively destroy neurones in the arcuate nucleus (Olney et al, 1974), striatum (Coyle & Schwarcz, 1976; McGeer & McGeer, 1976; Schwarcz & Coyle, 1977), striatum (Coyle & Schwarcz, 1976) and hippocampus (Nadler et al, 1978) while sparing fibres which pass to or through these regions. Thus, kainate binding sites appear to be physiologically localised to particular brain neurones. It has long been known that administration of high doses of monosodium glutamate to immature animals causes degeneration of neurones in the retina and arcuate nucleus of the hypothalamus (Lucas & Newhouse, 1957; Olney, 1969) and indeed the destructive effects of L-glutamate and kainate appear very similar on striatal (Olney et al, 1974) and arcuate neurones (McGeer & McGeer, 1976) although they are effective at does about two orders of magnitude apart. Taken in isolation, this evidence might suggest that kainate generally exerts its neurotoxic effect at the same sites that L-glutamate exerts its effect. However, in the hippocampus, Nadler et al (1978) discovered that L-glutamate was virtually ineffective in destroying hippocampal pyramidal cells even when it was injected directly into this region at a dose more than three orders of magnitude greater than the minimally effective dose of kainate.

With regard to its neurotoxic mechanism of action, it is considered likely (Nadler et al, 1978) that a relatively high dose of kainate will interact with its excitatory binding site to effect a prolonged depolarisation. This chronic excitation would then lead to an irreversible ionic imbalance resulting in cell dath. If, indeed, the neurotoxic sites are synonymous with the excitation sites then the evidence discussed here again indicates a discrete population of kainate receptors. In support of the hypothesis that kainate exerts its neurotoxic action by overstimulation of the excitatory receptors, it has been found (Olney et al, 1971; Schwarcz et al; 1978) that a correlation exists between the neurotoxic and neuro-excitatory effects of glutamate analogues.

Against the background of the wealth of neurotoxic, electrophysiological and binding evidence in favour of a discrete population of kainate receptors, then in the same way

that endogenous enkephalin was discovered by the action of the exogenous drug morphine at opiate receptors (Hughes, 1975; Terenius & Wahlstrom, 1975), perhaps an endogenous kainate analogue exists which acts as a mediator or modulator of synaptic transmission at sites where kainate receptors exist. The identification of such a compound might help in the mechanisms involved behind Huntingdon's chorea and epilepsy.

4.2.3 Other neuroexcitants

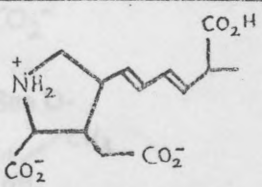
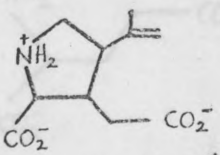
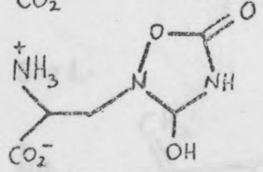
Ibotenic acid, an isoxazole isolated from the mushroom of the genus Amanita, was first reported to possess powerful neuroexcitatory activity by Johnston et al (1968). It is a conformationally restricted anologue of L-glutamate and is discussed in the QSAR study of section 5.2

Quisqualic acid, isolated from seeds of the plant genus Quisqualis, is highly potent as a glutamate agonist both at the crayfish neuromuscular junction (Shinozaki & Shibuya, 1974) and also on frog and rat spinal neurones (Biscoe et al, 1975).

The neuroactivity of the potent excitant, domoic acid, was first discussed by Biscoe et al (1975). It occurs in certain marine algae and is a structural analogue of kainate.

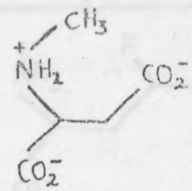
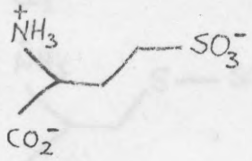
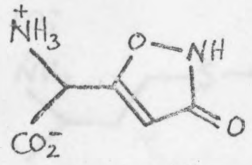
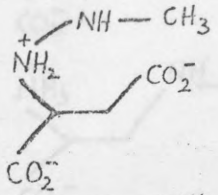
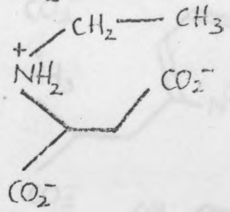
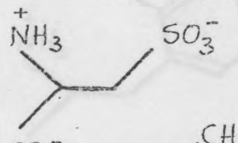
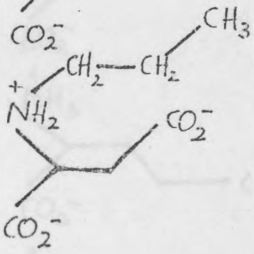
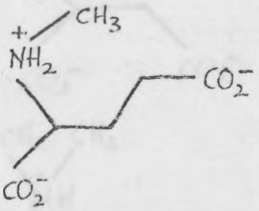
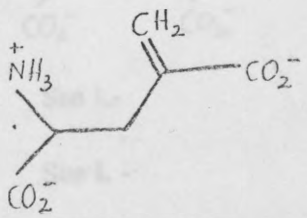
Other neuroexcitants including cycloglutamic acid (Johnston et al, 1974), homocysteic and cysteic acids (Curtis & Watkins, 1963) are discussed in the QSAR studies of chapter 5. Table 4.1 lists the major neuroexcitants in mammalian central neurones.

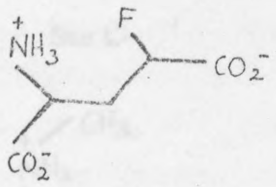
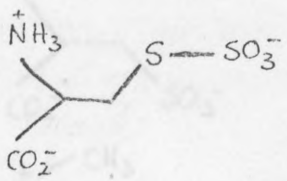
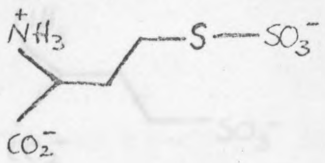
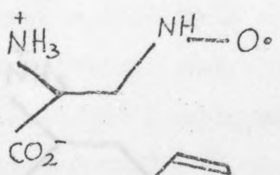
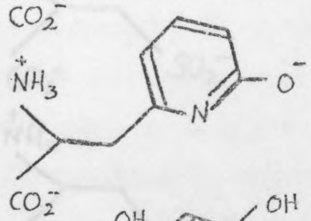
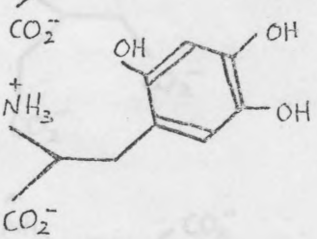
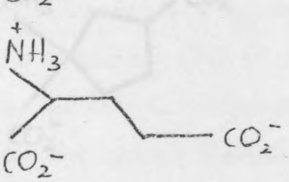
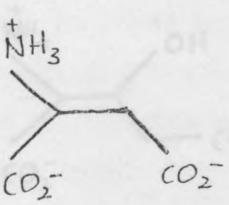
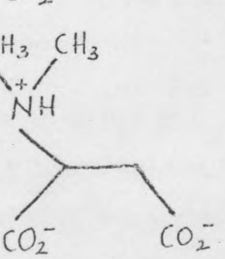
Table 4.1 Compunds reported to be neuroexcitants in mammalian central neurones

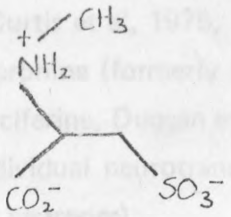
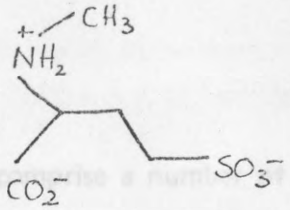
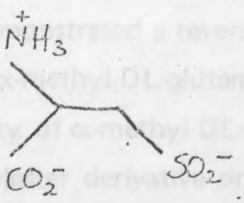
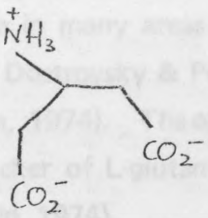
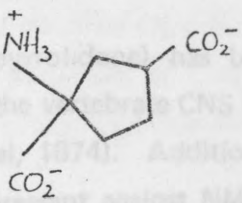
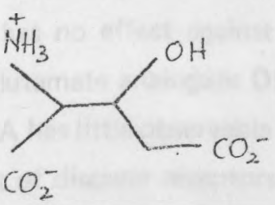
Compound	Structure	Potency*	Reference†
Domoate		50 -> 200	Biscoe et al (1975)
Kainate		40 -> 200	"
Quisqualate		25 -> 200	"

** Potency relative to L-glutamate (x)*

† Usually the first reported activity in mammalian central neurones

Compound	Structure	Potency*	Reference†
N-Methly-D-aspartate (NMDA)		5 — 14	Curtis & Watkins (1963)
D-Homocysteate		3 — 10	"
Ibotenate		2 — 7	Johnston et al (1974)
N-Iminomethyl- D-aspartate		1 — 8	Curtis & Watkins (1963)
N-Ethyl-D-aspartate		1 — 8	"
L-Cysteate		0.5 — 2	"
N-n-Propyl-D-aspartate		0.5 — 2	"
L-Homocysteate	See D-	0.5 — 2	"
N-Methyl-L-glutamate		0.5 — 2	"
N-Methyl-D-glutamate	See L-	0.5 — 2	"
γ - Methylene-L-glutamate		> 1	Curtis et al, (1972)

Compound	Structure	Potency*	Reference†
γ - Fluoro-L-glutamate		>1	Curtis et al (1972)
S-Sulpho cysteine		>1	Bixcoe et al (1975)
2-Amino 4 – thiosulphonylbutyrate		>1	"
β-N-Oxylyl- α, β – diaminoproprionate		>1	"
6-Hydroxy-2 – pyridylalanine		>1	"
6-Hydroxy dopa		>1	"
L-Glutamate		1	Curtis et al (1972)
L-Aspartate		1	"
N,N-Dimethyl- DL-aspartate		0.2 – 1.2	"
D-Glutamate	See L-	0.2 – 1.2	"
D-Aspartate	See L -	0.2 – 1.2	"

Compound	Structure	Potency*	Reference†
D-Cysteate	See L-	0.2 – 1.2	Curtis et al(1972)
N-Methyl-DL-cysteate		0.2 – 1.2	"
N-Methyl-DL-homocysteate		0.2 – 1.2	"
L-Cysteine sulphinate		1	Curtis & Watkins (1960)
β-Aminoglutarate		0.7 – 1	"
Cycloglutamate		0.7 – 0.8	Johnston et al (1974)
β-Hydroxyglutamate		0.7	Curtis & Watkins (1960)

4.3 Putative antagonists

4.3.1 Non-selective blockers

Several compounds have been identified which antagonise the actions of the excitatory amino acids. These include LSD-25 (lysergic acid diethylamide, Boakes et al, 1970), L-methionine-DL-sulphoximine (Curtis et al, 1975; Haldeman & McLennan, 1972; Davies & Watkins, 1973a), 9-methoxyaporphine (formerly numbered "2–", Curtis et al, 1972), and L-5,6-dimethoxy aporphine (nuciferine, Duggan et al, 1973). However all these compounds possess little selectivity for individual neurotransmitters (e.g. McLennan & Wheal, 1976, nuciferine on rat and cat central neurones).

4.3.2 Structural analogues

A second class of antagonists comprise a number of compounds which more closely resemble L-glutamate and have been reported to be more selective in their blocking action. McLennan et al (1971) have demonstrated a reversible blockade of L-glutamate excitation by two related compounds: α-methyl-DL-glutamate and L-glutamic acid diethylester. Although the antagonistic ability of α-methyl-DL-glutamate has been called into question (Curtis et al, 1972), the diethylester derivative on the other hand appears to block excitation produced by L-glutamate in many areas of the vertebrate CNS (Curtis et al, 1972; Haldeman & McLennan, 1972; Dostrovsky & Pomeranz, 1973; Tebecis, 1973; Zieglgansberger & Puil, 1973 and Padjen, 1974). The α-methyl derivative of L-glutamate has also been found to be a potent blocker of L-glutamate activity in the crayfish neuromuscular junction (Lowagie & Gerschenfeld, 1974).

HA-966 (1-hydroxy-3-amino-2-pyrrolidone) has been observed to antagonise glutamate excitation in several regions of the vertebrate CNS (Davies & Watkins, 1972, 1973a, 1973b; Curtis et al, 1973; Clarke et al, 1974). Additionally, Biscoe et al (1977a, 1978) have found that HA-966 is very depressant against NMDA, mildly depressant against both L-aspartate and L-glutamate and has no effect against kainate. A similar pattern was observed for the two long chain glutamate analogues DL-α-aminopimelate and DL-α-amino adipate except that DL –α –AA has little observable depressant effect against L-glutamate. This again is evidence in support of discrete receptors for L-aspartate, L-glutamate, NMDA and kainate.

It is interesting that Dα-AA was predicted to be an antagonist by Hall et al (1977) when they discovered that the DL-form had a depressant effect on spontaneous and amino acid induced excitation of rat thalamic neurones whilst the L-form was weakly excitatory. This prediction was subsequently confirmed by Biscoe et al (1977b).

It has also been demonstrated (Evans et al, 1977) that low concentrations of Mg^{2+} ions markedly antagonise NMDA-induced depolarisations of frog and rat spinal neurones *in vitro* while responses to kainate were unaffected. Additionally, L-aspartate induced responses were more sensitive to blocking by Mg^{2+} than L-glutamate responses. These results have subsequently been confirmed in cat spinal neurones *in vivo* by Davies & Watkins (1977). Again this is further evidence for discrete receptors for all four neuroexcitants.

The hierarchy of antagonism for Mg^{2+} described above is identical to that of D-α-AA, HA-966 and α,ε-diaminopionelic acid (Evans et al, 1978).

D-α-Aminosuberate, which is a higher homologue of D-α-AA has also been reported to possess blocking action similar to D-α-AA in various rat neurones (Collingridge & Davies 1979).

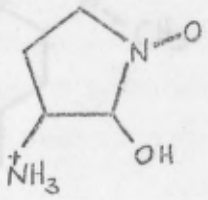
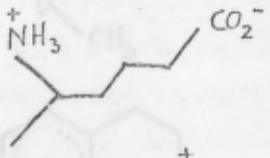
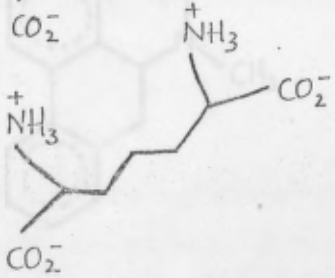
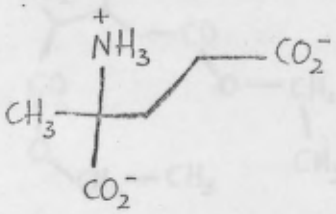
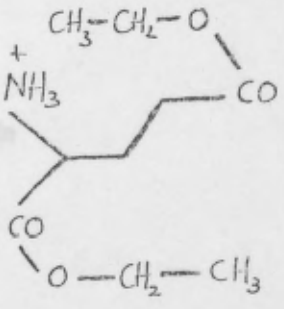
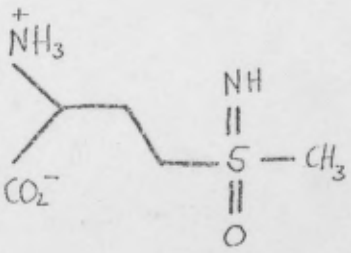
Finally the glutamate analogue DL-2-amino-4-phosphonobutyrate (APB) has been reported to interact with glutamate receptors of locust muscle and to antagonise iontophoretically administered glutamate induced activity in this preparation (Cull Candy et al, 1976; Clements & May, 1974). Similarly Dudel (1977) has found that APB was an effective antagonist of neuroexcitation at the crayfish neuromuscular junction. However, in the vertebrate system the value of the phosphono derivates is still being debated. Watkins et al (1977) have reported that in the dorsal horn interneurones and Renshaw cells of the cat, neither APB or the 3-phosophonoproprionate derivative reduced the excitatory actions of either L-glutamate, L-asparate or DL-homocysteate. On the other hand, White et al (1979)have found that both these phosphono derivatives reduced excitation in various rat neurones. In conclusion, it must be said that not a single antagonist which is exclusively selective against either L-glutamate or L-aspartate has yet been discovered. Table 4.2 list the major neuroexcitant antagonists discussed here.

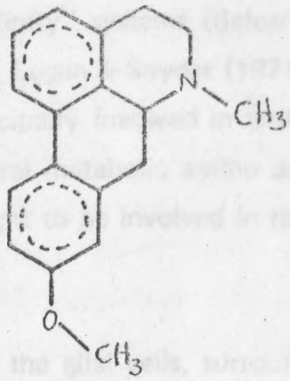
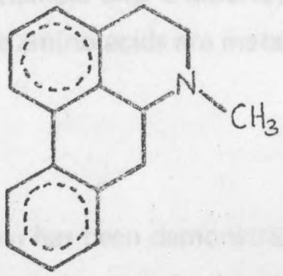
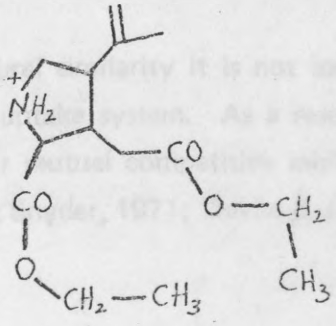
4.4 Active uptake process

4.4.1 Introduction

There is strong evidence that active uptake probably terminates the actions of the excitatory amino acids in the vertebrate (Curtis et al, 1970) and invertebrate CNS (Baker & Potashner, 1971). In contrast to the potentiation of the excitant action of acetylcholine by acetylcholinesterase inhibitors (Curtis & Ryall, 1966) the actions of the excitatory amino acids on Renshaw cells in the cat spinal cord are unaffected by the local administration of a number of inhibitors of the excitatory amino acid metabolising enzymes (Curtis & Watkins, 1975; Curtis, Duggan & Johnston, 1970) suggesting that extracellular enzymatic inactivation does not occur.

Table 4.2 Compounds reported as neuroexcitant antagonists in mammalian central neurones

Compound	Structure	Antagonising	Reference
1-Hydroxy-3-amino-pyrrolid-2-one (HA-966)		NMDA L-Aspartate L-Glutamate	Evans et al (1978) Biscoe et al (1977a,b) Davies & Watkins (1972, 1973a,b) Curtis et al (1973)
D- α-Aminoadipate		NMDA L-Aspartate	Evans et al (1978) Biscoe et al (1977a, b)
α, ε-Diaminopimelate		NMDA L-Aspartate L-Glutamate	"
D- α-Aminosuberate		NMDA L-Glutamate L-Aspartate	Collingridge & Davies (1979) Davies et al (1978)
DL- α-Methylglutamate		L-Glutamate	McLennan et al (1971) Haldeman et al (1972)
L-Glutamic acid diethyl ester		L-Glutamate L-Aspartate	Haldeman et al (1972) Haldeman & McLennan (1972) Curtis et al (1972) Davis & Watkins (1973a) Stone (1973)
L-Methione-DL-sulphoximine		L-Glutamate L-Aspartate	Curtis et al (1972) Haldeman & McLennan (1972) Davies & Watkins (1973)

Compound	Structure	Antagonising	Reference
9-Methoxyaporpine		L-Glutamate	Curtis et al (1972)
L-5,6,-Dimethoxy aporphine (Nuciferine)		L-Glutamate	Duggan et al (1973) Curtis et al (1973) Hind & Kelly (1975) Ben-Ari & Kelly (1975)
Mg^{2+}	Mg^{2+}	NMDA L-Glutamate L-Aspartate	Evans et al (1977,1978) Davies & Watkins (1977)
Kainic acid diethyl ester		L-Glutamate	McDonald et al (1977)
D-Lysergic acid diethylamide (LSD 25)		L-Glutamate	Boakes et al (1970)

4.4.2 High and low affinity uptake

Two kinetically distinct uptake systems have been recognised, these being the so-called "high affinity" and "low affinity" systems (Balcar & Johnston, 1972; Logan & Snyder 1972). Of these two systems, Logan & Snyder (1971) have shown that it is the high affinity uptake system which is principally involved in termination of neurotransmission since, of those tested, all of the general metabolic amino acids exhibit a low affinity system but only those amino acids thought to be involved in neurotransmission exhibit a high affinity system.

It has been shown that it is the glial cells, surrounding the neurones, which exhibit the high affinity uptake of L-glutamate and L-aspartate (Balcar et al, 1977; Schousboe et al, 1977) where, after uptake, the amino acids are metabolised.

4.4.3 Ion dependence

The high affinity uptake system has been demonstrated to be highly Na^+ and K^+ dependent. Active uptake is enhanced by an increase in the external [Na^+] and inhibited by external K^+ (Baker & Potashner, 1971 (invertebrate); Bennett et al, 1973 (vertebrate). This ion dependence would seem to be particularly appropriate in view of the Na^+ and K^+ fluxes known to accompany depolarisation.

In view of their close structural similarity it is not inconceivable that L-aspartate and L-glutamate share a common uptake system. As a result of their kinetically similar high affinity uptake and also their mutual competitive inhibition, many workers have reached the same conclusion (Logan & Snyder, 1971; Davies & Johnston, 1976).

5. Quantitative Structure – Activity Relationship (QSAR) Studies

5.1 Introduction

5.1.1 Outline of QSAR studies

The "active conformations" of ligands active at three receptors were investigated by scrutiny of a ligand's conformational flexibility. This method comprises the selection of a common conformer (or disposition of functional groups) from the conformations of an active homologous series of ligands. Three series of ligands were investigated: those active at the receptors of L-glutamate (5.4), L-aspartate (5.5) and kainate (5.6). These studies were based on data collected by radiolabelled binding assays for L-aspartate and kainate performed here (Appendix A). A number of important points are discussed first, together with a resume of the background work already done (5.3).

5.1.2 Definition of the "active conformation"

As discussed in chapter 1, model-building conformational analysis was chosen as that QSAR technique offering the most merit in the determination of one or more "active conformation(s)" of a ligand. But before "active conformations" can be investigated it is worth emphasising that it is the bound conformation which is under scutiny. Having now defined the "active conformation" for the purposes of the QSAR studies, the following discussion is academic -- but important to bear in mind nonetheless.

Various authors (notably Kier & George, 1973; Kier & Holtje, 1975) have discussed a ligand being attracted to a receptor whilst the ligand is in a "recognition conformation". In most flexible molecules, rotations about single bonds may occur at rates greater than $10^{10} s^{-1}$ (Burgen et al, 1975) as a result of thermal vibration. It therefore seems unlikely that the lifetime of a "recognition conformation" will be sufficiently long for attraction and subsequent binding to take place.

Applying Occam's razor principle of *entia non sunt multiplicanda*, the "zipper" model of binding (Burgen et al, 1975) recommends itself -- discussed in 2.2.3. This model proposes that a "nucleation complex" is initially formed with the receptor by only one segment of the ligand molecule. The remaining parts of the molecule then wriggle, by thermal vitration, to fit the rest of the receptor. (In the case of excitatory amino acid neurotransmitters the most probable "nucleation" site is the rigid $\overset{+}{N}H_3 \circledcirc CHR \circledcirc CO_2^-$ moiety which is common to all the active compounds).

In performing QSAR studies a difficulty arises from Koshland's (1959) theory of "induced fit" termed the "hand in glove" model in 2.2.4. If the receptor site surface is flexible and

able to accomodate ligand analogues which are not *quite* able to assume the "correct" 3-D structure and charge distribution of the endogenous ligand, then the search for a unique or active conformation loses its validity to some extent. In most experimental procedures it is necessary to fix one set of variables whilst investigating another set. Therefore, since the degree of flexibility of the receptor site surface under study was unknown it has been assumed in the work reported here that the receptor site surface was rigid. This assumption is not strictly valid but since the aim of this work was to identify a common "best fit" set of ligands' parameters this must correspond to a complementary set of receptor functional group parameters. Also, to maintain consistency with the receptor rigidity assumption, only those ligands exhibiting high potency were considered in the analysis i.e. only those ligands presumably able to adopt "optimum" conformations when bound to the receptor surface. Those low-potency ligands which *must* necessitate a large degree of receptor distortion when bound were disregarded.

Steward and Clarke (1975) have tentatively deduced the flexibility of the GABA receptor binding site by scrutiny of the intercharge distances of a number of "rigid" GABA agonists. The rigidity of their agonists was based, however, on the most populated conformers.

Also, in the following QSAR studies, the existence or otherwise of lipophilic or "anchoring" sites has not been investigated. Only the disposition of charged groups of active ligands has come under investigation. The fact that a ligand analogue exhibits binding activity demonstrates that it is able to offer up a charge distribution which is acceptable to the receptor. The differences in binding avidity are then presumably due to the ionisibility of the charged groups and also steric hindrance effects produced by non-essential side-chains, rings etc.

Finally, it has been tacitly assumed that, on determination of a unique disposition of charged groups for a ligand, the morphology of the receptor is then complementary both in terms of 3-D spatial characteristics and also charges. Although receptor model building has not been attempted here Smythies has been very active in this area for various other receptors (acetycholine, 1975; convulsants, 1974; prostaglandins, 1979; opiates, 1977; co-carcinogens, et al, 1975).

5.1.3 Potency data collection – why radiolabelled binding assays were used

For these QSAR studies, potency data were collected using radiolabelled binding assays. Details of the potency data collected for the L-aspartate and kainate receptors are given in Appendix A. The potency data of Foster & Roberts (1978) was used to study the L-glutamate receptor. The majority of QSAR studies, however, performed to date on the excitatory neurotransmitters have used potency data gathered neurophysiologically (eg Curtis & Watkins 1960 to Watkins & Curtis 1977) by the iontophoretic application of

compounds to neurones *in vivo*. This type of experiment yields a hierarchy of compounds in terms of their respective abilities to evoke action potentials. However, these data must be treated with caution since they *do not discriminate* between action potentials derived from *inter alia* putative glutamate and aspartate receptors. In addition to this main disadvantage there are other factors which can modulate the magnitude of the response observed. These include.

(i) Differences in diffusion rate of various compounds through surrounding tissue.

(ii) Differential enzyme degredation.

(iii) Differential abilities to act as substrates for the active uptake process.

Thus, the potency of a ligand which binds avidly to the receptor might be considerably reduced by any of the factors listed above. Conversely the response to a ligand which binds less avidly could be potentiated by the above factors working in its favour – particularly factor (iii).

However, the *in vitro* technique of radiolabelled ligand receptor binding offers an important apporach to the collection of a useful data set since it circumvents many of the criticisms levelled at the *in vivo* iontophoretic technique. In most instances, the endogenous ligand (neurotransmitter in this case) is very specific for its receptor. Therefore a relatively unambiguous set of data can be collected in terms of various challenging ligands' ability to displace the "hot" endogenous ligand. Also, by a suitable choice of binding study conditions it is usually possible to eliminate the potency modulating factors relating to the iontophoretic experiments mentioned earlier.

Binding studies of neurotransmitters were pioneered by Sol Snyder in the early '70s. The opiate receptor, though not first thought of as a conventional neurotransmitter receptor, provided the prototype. Using [^{3}H] naloxone (an opiate antagonist) of high specific activity, stereospecific opiate receptor binding was observed in homogenates of mouse brain (Pert & Snyder, 1973, see also Snyder, 1978a). With various modifications the opiate receptor technique allowed demonstration of receptor sites for most neurotransmitters in the brain (Snyder & Bennett, 1976). The field of binding studies has been extensively reviewed by Snyder (1978b) et al (1978). The main disadvantage of this *in vitro* technique is that the receptor purification technique and general experimental conditons might alter the characteristics of the receptor but judicious comparison of the results from both the iontophoretic and the binding studies will usually indicate any anomalies. A less serious criticism of binding studies is that they do not take any account of the "drug efficacy" component of potency as discussed in 2.5. A further criticism is that binding studies do not distinguish between agonists and competitive antagonists although any ambiguity can usually be resolved by reference to the corresponding iontophoretic results.

Thus, on balance, the radiolabelled ligand receptor binding technique was chosen here to provide data on which to perform the subsequent SAR studies.

5.2 Conformational analysis

5.2.1 The concept

It is a fundamental axiom of conformational analysis that the activity of a neurotransmitter analogue is a function of its ability to present charged or polarised groups to the binding site of the receptor in the same relative positions as those in the active conformer of the natural neurotransmitter. Thus a consistent method of intercharge separation measurement is necessary.

5.2.2 Measurement of intercharge separation

In early work on the neurodepressant GABA, Kier et al (1970, 1973, 1974) used measurements based on the nitrogen atom (N^+) and each oxygen atom (O^-) as shown in figure 5.1 below.

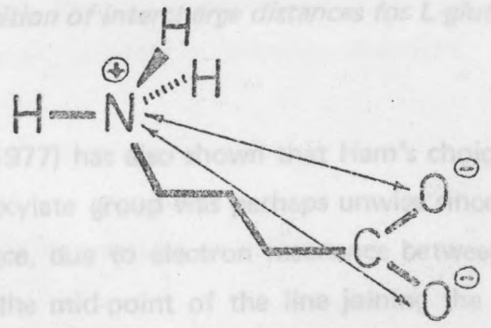


Fig 5.1 Intercharge centre measurements on GABA after Kier (1970, 1973, 1974)

Ham (1974) is an NMR study of GABA again used the nitrogen atom but in the negative region of the molecule used the carboxyl carbon. However, it has been demonstrated by molecular orbital calculations (Borthwick, 1977) that the charge on the ammonium group (N^+H_3) of GABA resides not on the nitrogen atom but is equally distributed amongst the ammonium protons. Thus it is proposed here that any measurements made to this charge

centre be taken to the centroid of the triangle formed by the ammonium protons (Fig 5.2).

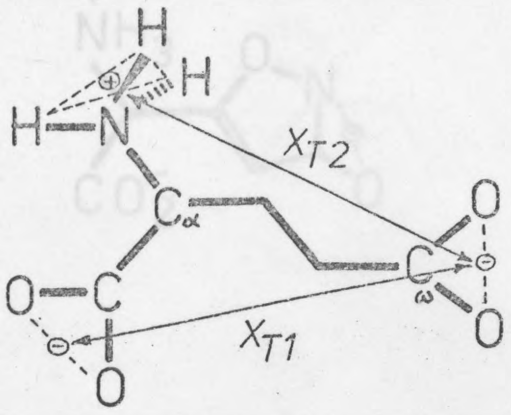


Fig 5.2 Definition of intercharge distances for L-glutamate

Borthwick (1977) has also shown that Ham's choice of the carbon atom for measurements to the carboxylate group was perhaps unwise since this atom carries a substantial positive charge. Hence, due to electron resonance between the two oxygen atoms, it is proposed here to use the mid-point of the line joining the oxygen atoms for measurements to the carboxylate charge centre (Fig 5.2). The assignment of intercharge distances X_{T1} and X_{T2} is also given for L-glutamate in figure 5.2. This basic assignment of distances is employed, with some modifications, for all the analogues of L-glutamate analysed in sections 5.3, 5.4 and 5.5. The intercharge distance between the C_α carboxylate and ammonium groups is fixed and is therefore not normally considered in amino acid conformational analysis. With particular reference to the conformational analysis conducted in section 5.3, Borthwick (1977) proposes that the charge centre of ibotenate, analagous to the C_ω carboxylate group of L-glutamate, be defined as the mid-point of the line joining the oxygen and nitrogen atoms in figure 5.3. Other similar analogues are treated in a similar way.

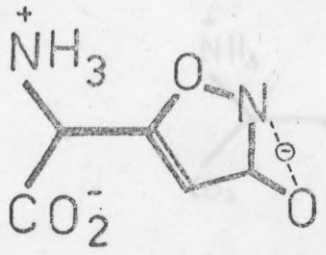


Fig 5.3 Definition of the –CON⁻ group charge centre in ibotenate

Finally, as added support for the above definitions of charge centres, Borthwick (1977) has shown that, to a close approximation, the electron distributions around most charged groups are intra- and inter-molecularly invariant.

5.3 The excitatory amino acids

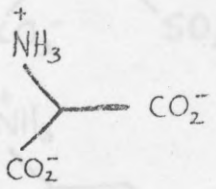
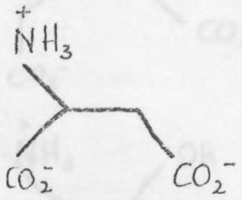
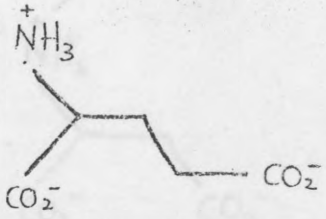
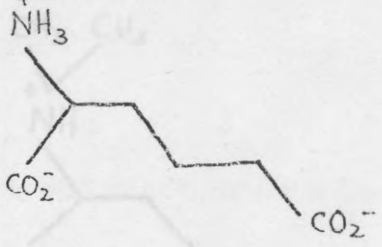
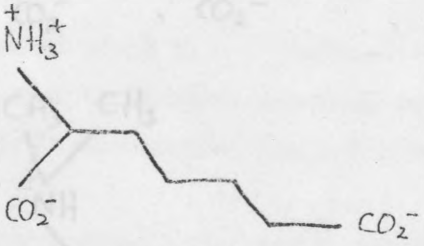
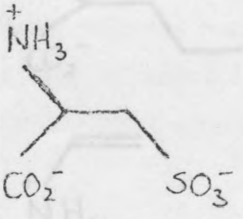
5.3.1 General observations on activity

As a preface to launching into the following SAR studies proper, it is useful to discuss the pioneering work already performed by other workers.

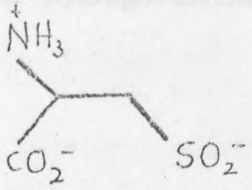
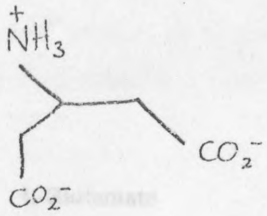
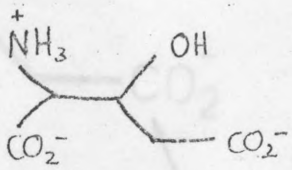
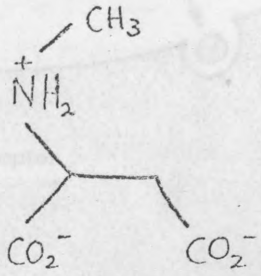
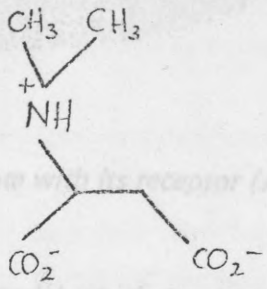
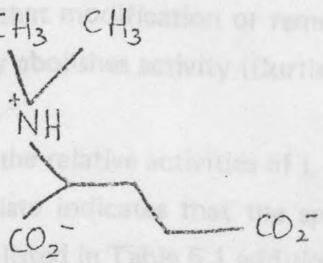
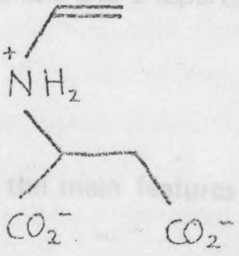
As discussed in Chapter 4, SAR work on the excitatory amino acids was first performed by Curtis & Watkins (1960, 1963, 1965) employing the method of iontophoretic application of compounds to cat neurones. Although this experimental method does not produce a reliable hierarchy of potencies for different receptors, it *does* distinguish between active and non active compounds in the excitatory system as a whole.

Curtis & Watkins systematically tested various analogues of L-glutamate and L-aspartate to determine those molecular characteristics necessary for neuroexcitant activity. Those compounds which they found to be active in their 1960 paper are listed in Table 5.1.

Table 5.1 Amino acids found to possess neuroexcitatory activity by Curtis & Watkins (1960)

Compound	Structure	Activity*
Aminomalonate		+
L-Aspartate		+++
D-Aspartate	See above	++ (+)
L-Glutamate		+++
D-Glutamate	See above	++ (+)
DL -α- Aminoadipate		+
DL-α- Aminopimelate		+
L-Cysteate		+++

* +++ denotes strong response
++ denotes medium response
+ denotes weak response

Compound	Structure	Activity*
L-Cysteine sulphinate		+++
β – Aminoglutarate		++ (+)
β -Hydroxyglutamate		++
N-Methyl-DL-aspartate		++
N : N Dimethyl-DL-aspartate		+
N; N -Dimethyl-DL-glutmate		+
N-Formimino-L-aspartate		++

A superficial inspection of the results indicates that two carboxyl (or quasi-carboxyl) groups and one ammonium group are necessary for activity. These results prompted Curtis & Watkins to postulate a "three-point" hydrogen bonded interaction with the receptor (Fig 5.4).

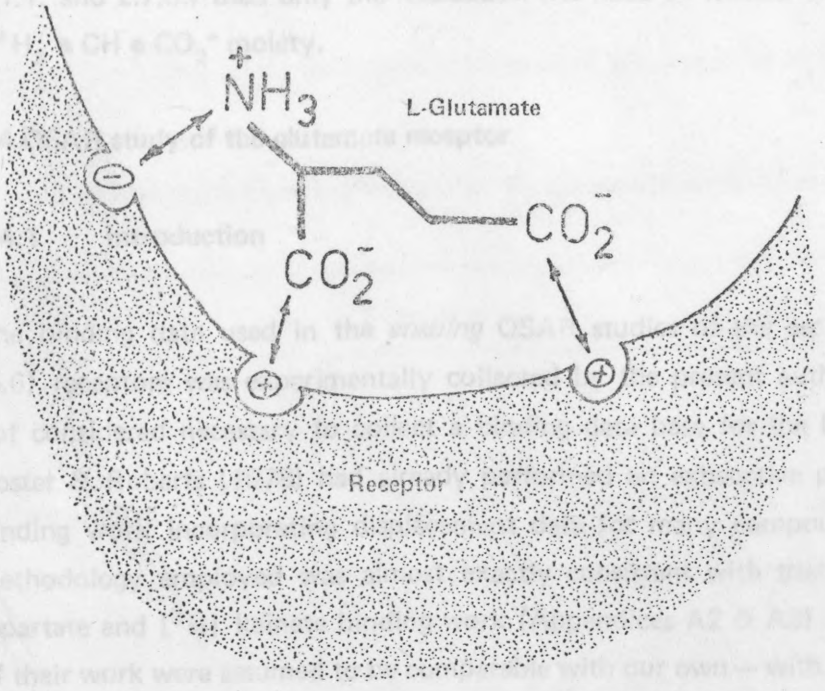


Fig 5.4 Interaction of L-glutamate with its receptor (After Curtis & Watkins, 1960)

This "three-point" interaction model which to date has never been seriously questioned is further supported by the facts that modification or removal of any of the charge centres on the interacting molecule usually abolishes activity (Curtis & Watkins, 1960).

Also the simplistic comparison of the relative activities of L-glutamate with DL - α – amino-adipate and DL - α - aminopimelate indicates that the spatial dispostion of the charged centres is important. (The results listed in Table 5.1 additionally indicate a degree of stereo-specificity in favour of both L-glutamate and L-aspartate).

5.3.2 Features for activity

From the work described above, the main features for excitatory activity may be summarised as follows:

$$^{+}X \bullet CH \bullet CO_2^{-} \bullet (CH_2)_n \bullet Y$$

where X is $N^{+}H_3$ or similar group, Y is CO_2^{-} or similar and n = 0 to 4.

The potency of a compound is also likely to depend on the proportion of molecules ionised at physiological pH since the active groups need to be ionised in order to participate in hydrogen bonding. If the "zipper" model of ligand binding is assumed (as discussed in 5.1.1. and 2.7.3.) then only the nucleation site need be ionised initially – in this case the $N^{+}H_3 \bullet CH \bullet CO_2^{-}$ moiety.

5.4 QSAR study of the glutamate receptor

5.4.1 Introduction

The binding data used in the *ensuing* QSAR studies of the aspartate (5.5) and kainate (5.6) receptors was experimentally collected by the present author. Fortunately it was not considered necessary to collect a binding data base for the L-glutamate system since Foster & Roberts (1978) had already performed an exhaustive piece of [^{3}H] glutamate binding work incorporating displacement data for many compounds. The experimental methodology employed was almost exactly consistent with that used in our own [^{3}H] aspartate and [^{3}H] kainate binding work (Appendices A2 & A3) and therefore the results of their work were assumed to be comparable with our own – with few reservations.

5.4.2 The [^{3}H] glutamate displacement data used

In this study the binding affinity data used were taken from the work of Foster & Roberts (1978) in "High affinity L-[^{3}H] glutamate binding to postsynaptic receptor sites on rat cerebellar membranes"

The abstract reads,

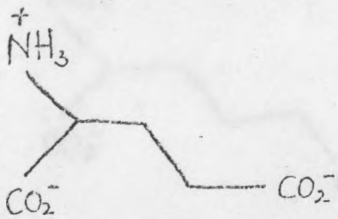
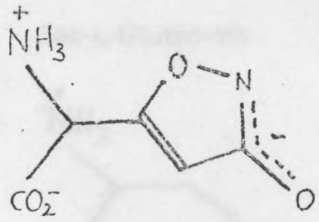
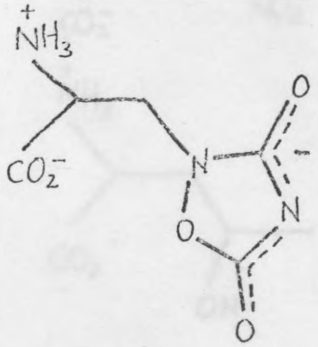
"L-[^{3}H] glutamate binding was investigated in membrane preparations derived from rat cerebellum, an area of the brain where it is likely that a high density of post-synaptic glutamate receptors occurs. Glutamate was bound specifically and, in freshly prepared membranes, was optimal under physiological conditions of pH and temperature and was associated with the synaptic membrane fraction of the cell. Specific binding occurred through a single, high-affinity process with a K_d of 744nM and a capacity of 73 pmol/mg protein. Unlike the findings reported for GABA, the specific binding of glutamate to fresh membranes did not involve an uptake site. Comparison of the potencies of a wide

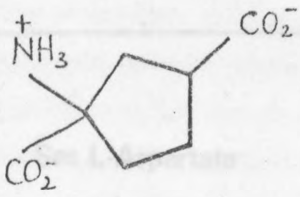
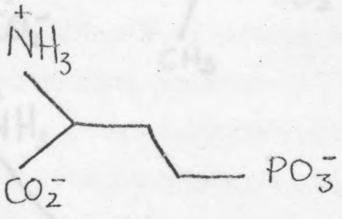
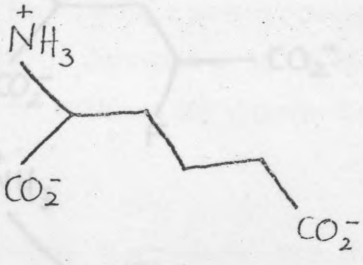
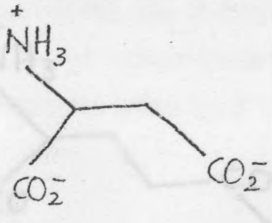
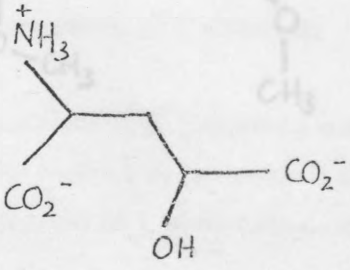
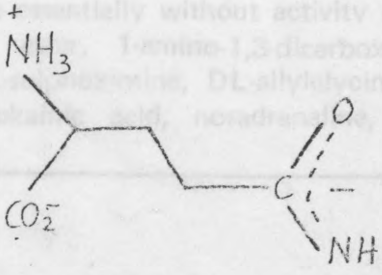
range of compounds with known pharmacological activities demonstrated that their ability to displace specific glutamate binding was consistent with specific interactions with glutamate receptors."

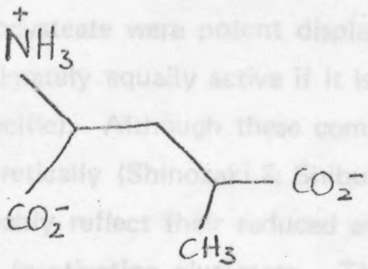
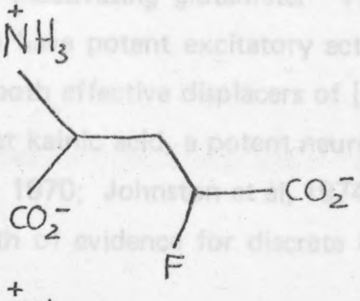
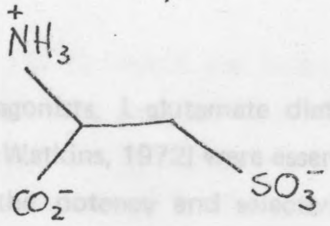
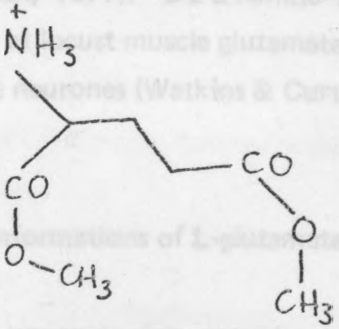
The experimental procedures adopted in the above work were near identical to those employed in the "high affinity L-[^{3}H] aspartate binding to rat cerebellar synaptic membranes" study presented in Appendix A2 except that, in the binding assay, centrifugation was used to separate the membranes from the incubate.

The results of the L-[^{3}H] glutamate binding work are presented in Table 5.2.

Table 5.2 Displacement of L-[^{3}H] glutamate binding in synaptic membranes

Compound	Structure	IC_{50} (μM)	% inhibition at 10^{-3} M
L-Glutamate		4.8	
D,L-Ibotenic acid		8.1	
DL-Quisqualic acid		8.4	
DL-Homocysteic acid		10.8	

Compound	Structure	IC_{50} (μM)	% Inhibition at 10^{-3} M
Trans-1-amino-1,3-dicarboxycyclopentane		17.9	
Cis-1-amino-1,3-dicarboxycyclopentate	See above	18.5	
DL-2-Amino-4-phosphonobutyric acid		25.6	
DL-α-Aminoadipic acid		26.3	
D-Glutamate	See-L-Glutamate	28.8	
L-Aspartate		42.1	
L-Glutamate-γ-hydroxamate		65.9	
L-Glutamine		116.8	

Compound	Structure	IC_{50} (μM)	% Inhibition at 10^{-3}M
D-Aspartate	See L-Aspartate	138.0	
γ-Methyl glutamate			99.0
4-Fluoro-glutamate			99.0
L-Cysteate			92.9
L-Glutamate dimethyl ester			53.9

The following compounds were essentially without activity (% inhibition < 50% at 10^{-3}M): L-glutamate diethyl ester, 1-amino-1,3-dicarboxyhexane, DL-aspartate-hydroxamate, L-methionine-DL-sulphoximine, DL-allylglycine, kainic acid, glycine, amino-oxyacetic acid, dihydrokainic acid, noradrenaline, GABA, N-methyl-DL-aspartate and HA-966.

5.4.3 Observations on the binding data

The binding data is interesting in its own right. L-Glutamate was found to be the most potent displacer of [^{3}H] glutamate specific binding. L-glutamate was about 6x more potent than D-glutamate indicating receptor stereo-specificity. The relative lack of activity of L-aspartate would indicate that glutamate is binding predominantly to glutamate specific sites in the membrane preparation. The three powerful neuroexcitants DL-ibotenate, DL-quisqualate and DL-homocysteate were potent displacers but were slightly less active than L-glutamate (or approximately equally active if it is assumed that these compounds, like glutamate, are stereospecific). Although these compounds are more powerful excitants when applied iontophoretically (Shinozaki & Shibuya, 1974; Johnston et al, 1974) their elevated potencies probably reflect their reduced effectiveness as substrates for the high affinity uptake process inactivating glutamate. The two isomers of 1-amino-1,3-dicarboxycyclopentane which have potent excitatory actions on mammalian central neurones (Hall et al, 1978) were both effective displacers of [^{3}H] glutamate. Other interesting results include the finding that kainic acid, a potent neuroexcitant and proposed glutamate agonist (Shinozaki & Konishi, 1970; Johnston et al, 1974) was found to be almost inactive which ties in with the wealth of evidence for discrete kainate receptors as discussed in Chapter 4.

The proposed glutamate antagonists, L-glutamate diethyl ester and HA-966 (Haldeman & McLennan, 1972; Davies & Watkins, 1972) were essentially without effect on the binding of L-glutamate. However, the potency and selectivity of these compounds has been challenged (Clarke & Straughan, 1977). DL-2-Amino-4-phosphonobutyric acid, which is a potent glutamate antagonist at locust muscle glutamate receptors (Cull-Candy et al, 1976) but is not active on vertebrate neurones (Watkins & Curtis, 1977), was a potent displacer of [^{3}H] glutamate.

5.4.4 Deducing the active conformations of L-glutamate

DL-Ibotenate, having a planar isoxazole ring, provides a useful foundation for a discussion of active conformation. Since the potency of ibotenate is almost equal to that of glutamate it is clear that the active conformation of L-glutamate must involve an extended chain i.e. $\tau = \pi/2$ (Fig 5.5)

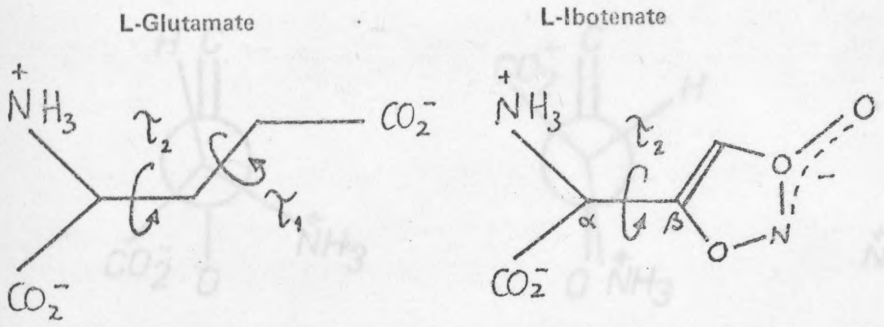
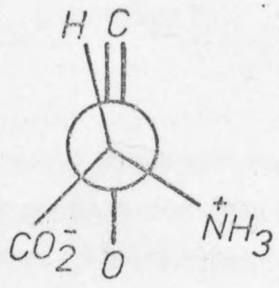


Fig 5.5 Possible bond rotations for L-glutamate and the conformationally restricted analogue L-ibotenate

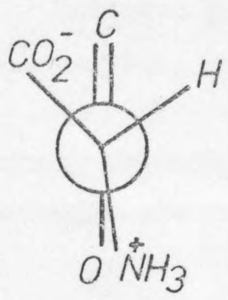
Although Foster & Roberts (1978) tested the racemic mixture DL-ibotenate, only L-ibotenante has been conformationally analysed here. This is because the results of Foster & Roberts above show the receptor to be specific for the L-stereoisomer of glutamate which we have also assumed to be the case for ibotenante – it seems reasonable to presume the N^+H_3 • CHR • CO_2^- moiety binds the receptor surface in the same way for both ligands.

If rotation τ, about Cα-Cβ, of L-ibotenate is now considered (Fig 5.5) then the conformers represented in Fig 5.6 are possible.

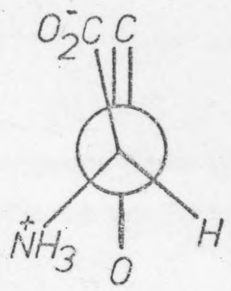
The two isomers of 1 - amino - 1,3 - dicarboxycyclopentane (ADCP) also provide useful information since their conformational flexibility is extremely restricted. Movements of 0.5Å of either C_3 or C_4 out of the plane of the remaining four ring carbons yields the X_T values presented in Fig 5.7.



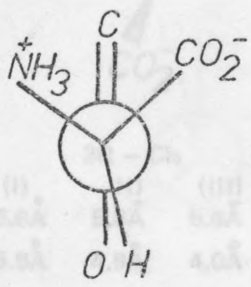
(I) X_{T1} = 5.6 Å*
X_{T1} = 5.6 Å



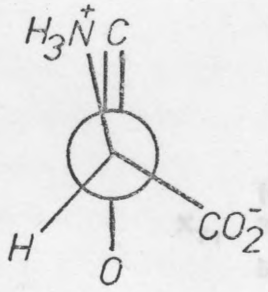
(II) X_{T1} = 5.4 Å
X_{T2} = 5.3 Å



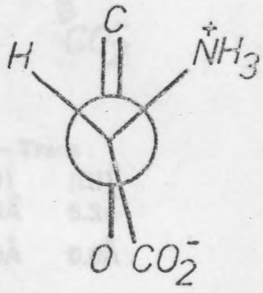
(III) X_{T1} = 5.4 Å
X_{T2} = 5.2 Å



(IV) X_{T1} = 5.0 Å
X_{T2} = 5.4 Å



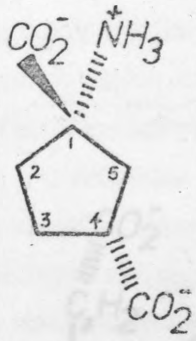
(V) X_{T1} = 4.9 Å
X_{T2} = 5.6 Å



(Vi) X_{T1} = 5.2 Å
X_{T2} = 5.8 Å

Fig 5.6 *L-Ibotenate conformers rotating about $C_\alpha - C_\beta$ (τ_2 Fig 5.5)*

* *For definitions of the intercharge distances X_{T1} and X_{T2} See 5.2*



	3R – Cis		
	(I)	(II)	(III)
X_{T1}	5.6Å	5.8Å	5.6Å
X_{T2}	5.9Å	4.9Å	4.0Å

	3R – Trans		
	(I)	(II)	(III)
X_{T1}	3.6Å	4.6Å	5.3Å
	5.7Å	6.0Å	6.0Å

Where conformer (I) is ideal envelope, C_3 (for 3R-cis) or C_4 (for 3R-trans) 0.5Å down,
(II) is planar,
(III) is ideal envelope, C_3 (for 3R-cis) or C_4 (for 3R-trans) 0.5Å up

NB Other ring conformations exist but the ideal envelope conformers produce the X_T extremes of functional group mobility

Fig 5.7 The X_T distances of the isomers of 1-amino-1,3-dicarboxy-cyclopentance (ADCP)

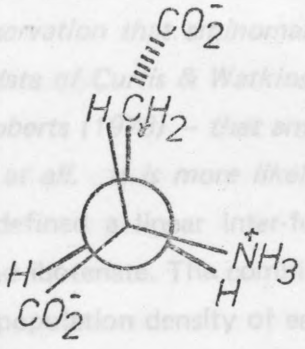
By comparison of the X_T distances with those of L-ibotenate (Fig 5.6) the most closely matched ("matching couplets") conformers are:

L-ibotenate (I) with Cis – ADCP (I)
and Trans – ADCP (III)

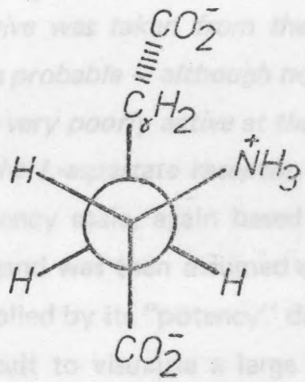
and,

L-ibotenate (VI) with Cis – ADCP (I)
and Trans-ADCP (III)

This would tentatively imply that one of the conformers L-ibontenate (I) or (IV) is the active conformation. Fig 5.8 below visualises the translations of these two conformations into the L-glutamate molecule.



(I) X_{T1} = 5.8Å
X_{T2} = 5.9Å



(II) X_{T1} = 5.3Å
X_{T2} = 6.1Å

Fig 5.8 Possible active conformers of L-glutamate

Both of these conformers can be described as "extended" chain structures.

Molecular orbital calculations using the CNDO/2 method predict L-ibotenate conformer (II) as the most stable (Borthwick & Steward, 1976 a,b) although as we have reiterated *ad nauseam* there is no reason why a conformer's stability should be directly connected with its activity.

Neither homocysteate nor quisqualate can usefully provide further unique conformational information since the former is conformationally analagous to glutamate and the latter

can be likened to ibotenante (see structures in Table 5.2) although the negative charge centre of the quisqualate ring is diffcult to define since, in the crystal structure (Flippen & Gilardi, 1976), the two oxygen substituents of the ring are both doubly bonded and no electron resonance is apparent. Also, molecular orbital calculations have yet to be performed on this molecule to predict charge distribution. Ligands of potency lower than 1-amino-1,3 dicarboxycyclopentane were disregarded for the reason discussed in 5.1.2 – to lend weight to the "receptor rigidity" assumption.

Although in performing an SAR study on the glutamate system Clarke (1975) deduced an extended L-glutamate molecule to be active, Van Gelder (1970) did not have the benefit of the knowledge that L-ibotenate was a rigid potent glutamate analogue. Against the background that Van Gelder assumed a single receptor for both L-glutamate and L-aspartate, he postulated that a folded L-glutamate conformation was the active one.

The work of Clarke (1975) was followed by an interesting study by Borthwick (1977). He assumed that the receptor was so flexible that *every* conformation of various active ligands was capable of interacting with the receptor – based on the observation that molecules as diverse as aminomalonate ($N^+H_3 \bullet CHCO_2^- \bullet CO_2^-$) and ibotenate were active. *(In fact the observation that aminomalonate is weakly active was taken from the electrophysiological data of Curtis & Watkins (1960, 1963). It is probable – although not tested by Foster & Roberts (1978) – that aminomalonate is only very poorly active at the glutamate receptor, if at all. It is more likely to interact with the L-aspartate receptor – see 5.5).* Borthwick defined a linear inter-functional group potency scale, again based on aminomalonate and ibotenate. The combined potency of a ligand was then assumed equal to the sum of the population density of each conformer multiplied by its "potency" derived from the scale. Although an interesting concept, it's difficult to visualise a large number of conformers of the *same* molecule binding the receptor when clearly there must be a very low number – probably one – of conformations which exist in the ligand-receptor complex. A very flexible receptor would also mean that the receptor would lose some of its biologically important selectivity.

It is perhaps interesting that for L-α-methyl glutamate (not tested by Foster & Roberts, 1978) it is the α-methyl substituent which obviously must confer the much reduced neuroexcitant activity (Haldeman et al, 1972; Curtis et al, 1972). This virtual lack of activity might be ascribed to the methyl group sterically hindering or blocking the drug "docking" process. Alternatively, if activity is diminished by an *intra*-molecular effect it is interesting that conformer (I) of the previous scheme is the only one where the α-methyl group has the opportunity to destabilise the conformation by potential interaction with the C_α H-atoms. Indeed, in the crystal structure (Appendix B) the α-methyl group is *trans* to the C_γ atom.

5.4.5 Summary of QSAR study of the glutamate receptor

Two extended chain conformers of L-glutamate emerged from the QSAR conformational analysis as those most likely to represent the "active" conformation of the neurotransmitter when bound to its receptor. Their inter-functional-group distances X_{T1} and X_{T2} lie within the 5–6 Å range deduced by other workers (Clarke, 1975; Borthwick, 1978). This result was obtained by the careful comparison of the conformational modes of L-ibotenate with those of 1-amino-1,3-dicarboxycyclopentane — both L-glutamate agonsits. Confidence can be attached to these findings because the radiolabelled L-glutamate binding work of Foster and Roberts(1978) — the database for the QSAR conformational analysis — probably represents binding to a *single* population of binding sites. We have assumed that these binding sites reflect the postsynaptic neurotransmitter receptors. In support of the L-glutamate binding site as a discrete postsynaptic receptor it is relevant that L-aspartate was only weakly active and kainate inactive in their abilities to displace labelled L-glutamate.

5.5 QSAR study of the asparate receptor

5.5.1 Introduction

The binding data used here was experimentally collected as described in Appendix A2. The electrophysiological work of Curtis & Watkins (1960, 1963, 1965), discussed in section 5.3, looked at excitatory compounds in general and did not distinguish between those compounds active at the glutamate receptor and those active at, *inter alia*, the aspartate receptor. The important aspect of the aspartate binding work used here is that it looks at the aspartate receptor is isolation.

5.5.2 General observations on activity

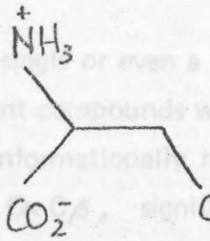
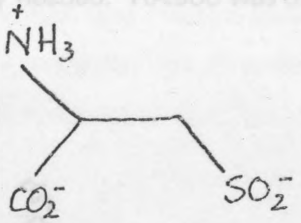
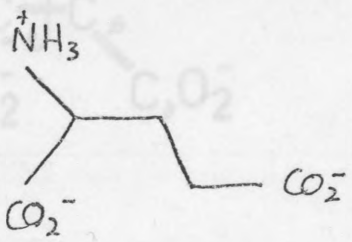
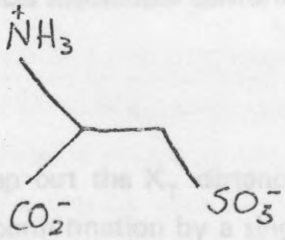
The displacement results of the "high affinity L-[^{3}H] aspartate binding to rat cerebellum synaptic membranes" described in Appendix A2 are listed in Table 5.3. As discussed in subsection A2.4.1 the characteristics of binding fulfil many of the basic criteria necessary for receptor identification.

As expected, all of the avidly bound compounds ($K_i < 5 \times 10^{-4}$ M) are also electrophysiologically active (Curtis & Watkins, 1960). A cursory inspection of the active compounds also indicates that, like L-glutamate, the L-aspartate receptor requires a "three-point interaction" with the ligand as discussed in section 5.3. In this respect it is interesting that the results show that the separate removal from aspartate of the C_α and C_γ carboxylate groups, to produce β -alanine and glycine respectively, abolishes binding. Both β - alanine

and glycine are strong neurodepressants.

A further striking observation is that the L-aspartate receptor is also very stereospecific. L-aspartate, of all the compounds tested, is the most avidly bound whilst D-aspartate exhibited little displacing activity.

Table 5.3 Potencies of various compounds in displacing specific L- [^{3}H] aspartate binding in rat brain membranes

Compound	Structure	K_i (M)
L-Aspartate		2.1×10^{-6}
L-Cysteine Sulphinate		0.9×10^{-5}
L-Glutamate		4.5×10^{-5}
L-Cysteate		4.5×10^{-4}
DL-Cysteate	See above	4.5×10^{-4}

The following compounds displayed K_i's $> 0.9 \times 10^{-3}$ M : (±) 4-fluoroglutamate, N-methyl-DL-glutamate, ibotenate, DL- α-aminoadipate, N-methyl-D-aspartate (NMDA), DL-pyroglutamate. Additionally the compounds listed next showed little or no displacing activity: D-aspartate, D-glutamate, DL-homocysteate, kainate, L-glutamic acid diethyl ester HCl, quisqualate, L-methionine-DL-sulphoximine, DL-α,ε-diaminopimelate, HA-966, dopamine HCl, noradrenaline HCl, taurine, glycine, acetylcholine Cl, β-alanine, GABA, L-proline, leptazol, naloxone and formamidine sulfinate.

As discussed in subsection 4.2.1, the evidence to date suggests that NMDA possess its own discrete receptor. The results obtained here are not at variance with this contention – neither NMDA or HA966 were significant displacers of L-[^{3}H] aspartate binding. The finding by Curtis & Watkins (1960) that the electrophysiological potency of D-aspartate was almost equal to that of L-aspartate might be explained by the possibility that D-aspartate binds at the NMDA receptor – certainly their stereochemistries are identical. Presumably L-cysteine sulfinate is a more avid binder than L-cysteate because the sulfinate (SO^-_2) group more closely resembles the charge distribution of L-aspartate's equivalent carboxylate group than does L-cysteate's SO^-_3 group.

5.5.3 Deducing the active conformations

It was not possible to arrive at a single or even a number of possible active conformations due to a lack of data – insufficient compounds were active in the binding assay. Although L-aspartate is to some extent conformationally restricted in that the molecule is so short that only the rotation τ_1, about $C\alpha$-$C\beta$, significantly changes its 3-D shape, an analogue with τ_1 restricted was critically needed. HA966 was originally thought to be a candidate – but was inactive.

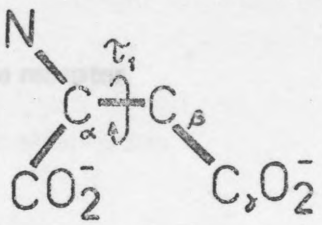


Fig 5.9 Only one rotation produces substantial conformational change in L-aspartate

Occassionally it is possible to map out the X_T distances of two different flexible active analogues and arrive at a unique conformation by a single coincidence of X_T's. The only analogue that *might* have been used with L-aspartate in this way was L-glutamate. However, by Dreiding Model comparison of L-aspartate and folded L-glutamate it was apparent that for "closest fit" the distal carboxylate group of L-glutamate can follow that of L-aspartate through the entire range of L-aspartate conformations rotating through τ_1 in Fig 5.9. A unique active conformation for L-aspartate could not, therefore, be tracked down.

The contention (e.g. Johnston et al, 1974) that L-glutamate must adopt a folded conformation to interact with the L-aspartate receptor is indirectly supported by the obser-

vation that ibotenate, the planar extended chain equivalent of L-glutamate, was almost inactive as an L-[^{3}H] aspartate displacer. A similar argument applies to quisqualate.

5.5.4 Summary of QSAR of the aspartate receptor

Unfortunately, due to a lack of conformationally restricted ligands active in the radiolabelled L-aspartate binding assay (Appendix A2) it was not possible to determine a unique or even small number of conformations representing the "active" mode of the neurotransmitter when bound to its receptor. All the ligands found to be active displacers of L-[^{3}H] aspartate possessed sufficient flexibility to "follow" L-aspartate through its range of conformational modes. The conformationally restricted HA–966 was the main hope – but was found to be inactive. The binding work (Appendix A2), however, was interesting in its own right. It probably represented binding to a single population of binding sites which it was assumed reflected binding to the neurotransmitter postsynaptic receptors. The binding was very stereospecific and was selective. Binding was maximal for L-aspartate itself – NMDA was inactive indicating a separate binding site. Many more conformationally restricted ligands need to be tested for activity before any further progress can be achieved on the "active" conformation aspect.

5.6 QSAR of the kainate receptor

5.6.1 Introduction

Appendix A3 details the binding data used (together with that of Simon et al (1976)) in this conformational study.

As discussed in section 4.2, kainate is a powerful excitant on vertebrate and invertebrate neurones. The overwhelming body of evidence suggests it possesses its own discrete receptor. The compound also exhibits neurotoxic action at higher concentrations. It is considered likely (Nadler et al, 1978) kainate exerts its neurotoxic action at the same site as its excitatory action – by prolonged depolarisation. The binding data referred to below probably only reflects a single population of kainate receptors.

5.6.2 The [^{3}H] kainate displacement data used

This QSAR study was performed using the binding data described in Appendix A3 supplemented by data from the work of Simon et al (1976) in "Binding of [^{3}H] kainate acid, an analogue of L-glutamate, to brain membranes". The procedural details of Simon et al's

work were identical with our own and many identical, overlapping results were obtained (within the limits of experimental error). See Table 5.4.

5.6.3 Observations on the binding data

Firstly, the results are valuable since L-glutamate is found to be only a poor displacer of radiolabelled kainate and L-aspartate is a weaker displacer still. This result supports the contention, discussed in section 4.2, that kainate possesses its own receptor.

Most of the putative neurotransmitters tested so far in other binding work are the most potent displacers of their respective radiolabelled counterparts (L-aspartate, Appendix A2; L-glutamate, Foster & Roberts, 1978; GABA , Zukin et al 1974 etc). It is striking therefore that the most potent displacer of [^{3}H] kainate is kainate itself. It is interesting to speculate that an endogenous ligand might exist which is very similar to kainate — the latter does not occur naturally in either vertebrates or invertebrates.

Both quisqualate and ibotenate possess sufficient flexibility to bind efficiently to both the kainate and the L-glutamate (Section 5.4) receptors. The kainate receptor also seems to be stereospecific. The stereochemistry of the kainate molecule corresponds to L-glutamate (see Fig 5.11) and, in turn, L-glutamate is a more potent displacer of radiolabelled kainate than is D-glutamate.

5.6.4 Deducing the active conformation

In 1974, Johnston et al postulated that kainate and L-glutamate interact with the same receptor on the basis that there is "unique juxtaposition of the equivalent ionisable groups in ..... L-glutamate..... and kainic acids" (Fig 5.10).

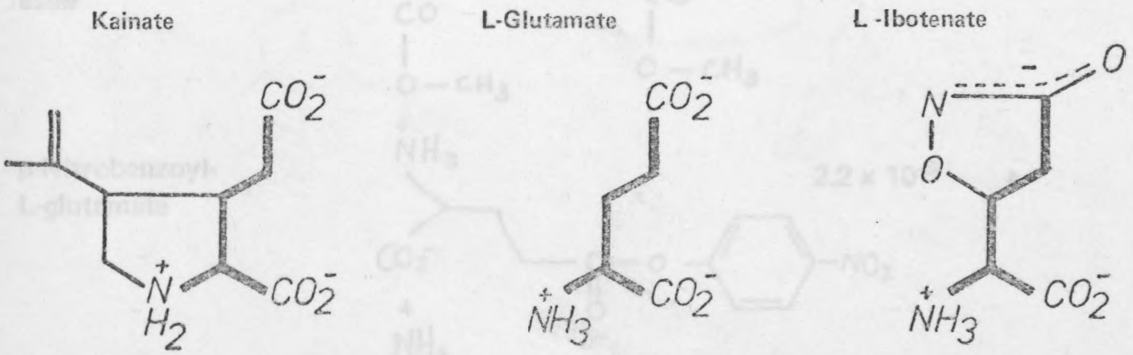
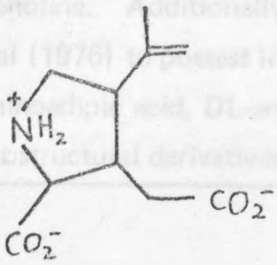
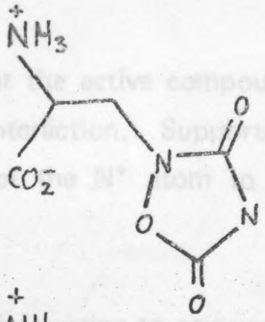
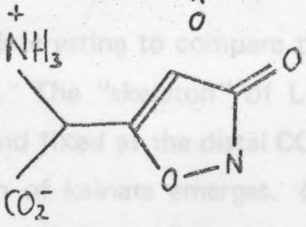
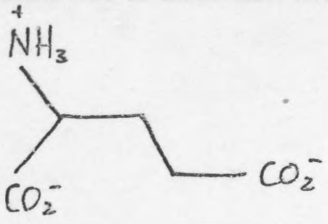
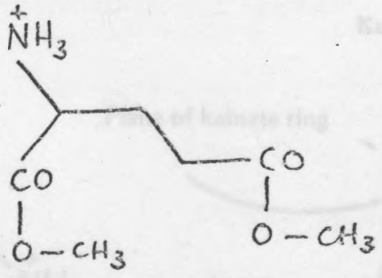
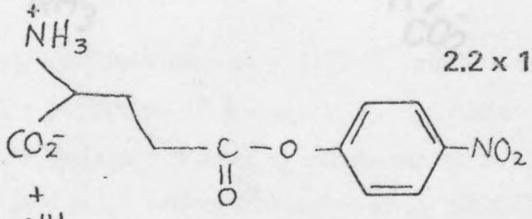
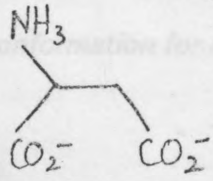


Fig 5.10 Structural congruences with L-glutamate skeleton

Table 5.4 Potencies of various compounds in displacing specific [^{3}H] Kainate binding in rat brain membranes

Compound	Structure	K_i (M)	Reference*
Kainate		1.5×10^{-8} $1.2(\pm 0.6) \times 10^{-8}$	a b
Quisqualate		4.5×10^{-8} $6 (\pm 3) \times 10^{-8}$	a b
Ibotenate		$6 (\pm 3) \times 10^{-8}$	b
L-Glutamate		4.8×10^{-7} $6 (\pm 3) \times 10^{-7}$	a b
L-Glutamic acid dimethyl ester		1.8×10^{-5}	a
p-Nitrobenzoyl-L-glutamate		2.2×10^{-5}	a
L-Aspartate		3.6×10^{-5} $3 (\pm 1) \times 10^{-5}$	a b

* a ..... Simon et al (1976)

b .... Binding study described in Appendix A3

In the binding study performed here (A3), both D-glutamate and HA-966 were found to possess K_i's less than 6×10^{-5}M and similarly Simon et al (1976) found that the following compounds exhibited K_i's $< 6 \times 10^{-5}$M: D-Glutamate, pentobarbital sodium, metrazol, glycine, GABA, serotonin, dl-norepinephrine, acetylcholine. Additionally the following compounds were found by Simon et al (1976) to possess little or no activity: glutaric acid, fumaric acid, DL-aminoadipic acid, DL-amino pimelic acid, proline, dopamine and various other structural derivatives of L-glutamate.

Indeed, a cursory glance at the active compounds listed in Table 5.4 implies a glutamate - like 3-point receptor interaction. Supporting this contention Schwarcz et al (1978) found that esterification of the N^+ atom to produce the N-acetyl derivative abolished neurotoxicity.

Pursuing this theme, it is interesting to compare the structure of kainate with that of the potent displacer ibotenate. The "skeleton" of L-glutamate is conformationally fixed at the N^+H_3 end in kainate and fixed at the distal CO^-_2 end in ibotenante. Therefore a single unique active conformation of kainate emerges. (Fig 5.11). (In fact slight modifications to the conformation are possible due to distortion within the kainate ring structure).

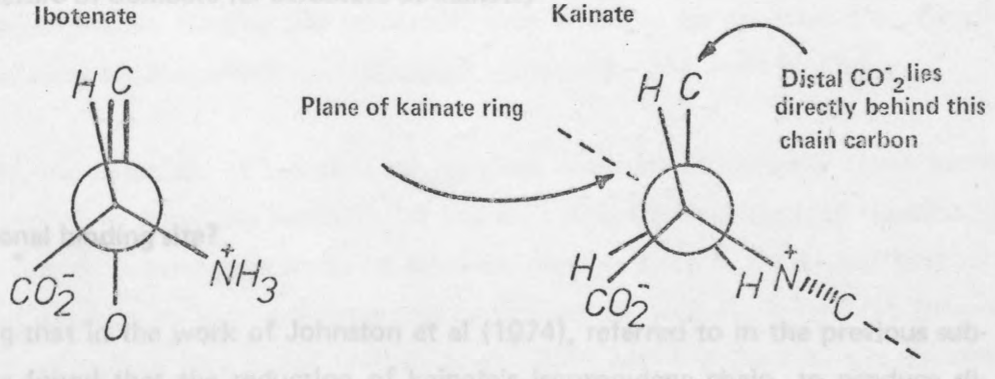


Fig 5.11 Unique conformation for activity

This conformation of ibotenate corresponds to conformer (I) of the glutamate receptor analysis illustrated in Fig. 5.6 and discussed in subsection 5.3.4. This conformer of ibotenate is one of only two thought here to be possibly active at the glutamate receptor. Quisqualate can be easily fitted into this scheme. Though not tested here, the potent neuroexcitant domoic acid (Biscoe et al, 1975) is a structural analogue of kainate (Fig 5.12) and would therefore be expected to interact with the receptor in a manner identical to kainate.

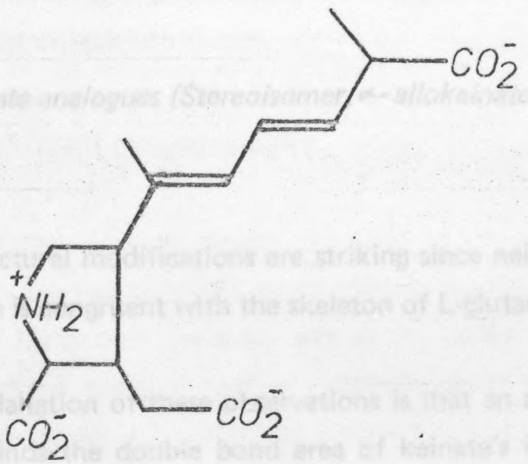


Fig 5.12 Structure of domoate (cf Structure of kainate)

5.6.5 Additional binding site?

It is interesting that in the work of Johnston et al (1974), referred to in the previous subsection, it was found that the reduction of kainate's isopropylene chain, to produce dihydrokainate, abolished neuroexcitatory action in cat neurones and also abolished neurotoxic action in rat striatum and chick retina (Schwarcz et al, 1978). Also a stereo-isomer of kainate, α-allokainate, where the isopropylene side chain is on the other side of the pyrrolidine ring (Fig 5.13) is some ten times less potent than kainate as an excitant of rat spinal neurones (Biscoe et al, 1976).

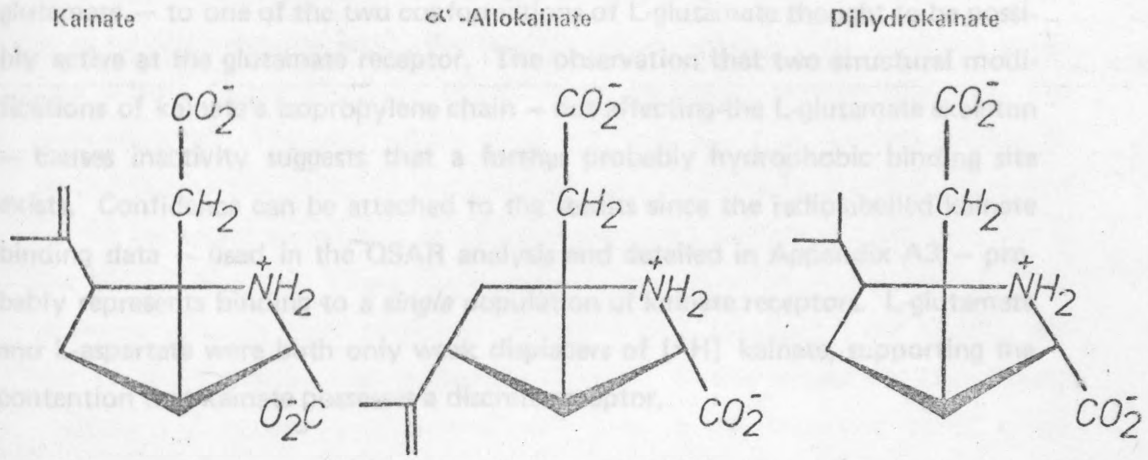


Fig 5.13 Kainate analogues (Stereoisomer, ∝- allokainate after Watase, 1958)

These two structural modifications are striking since neither of them affect that part of the molecule which is congruent with the skeleton of L-glutamate (Fig 5.10).

A possible explanation of these observations is that an additional hydrophobic binding site exists which binds the double bond area of kainate's isopropylene side chain. The facts that ibotenate and L-glutamate do not possess corresponding hydrophobic areas might explain their reduced binding affinities. Table 5.4 lists kainate 4x more potent than ibotenate and 32X more potent than L-glutamate. It would have been interesting to test dihydrokainate and ∝ -allokainate in the binding assay detailed in Appendix A3. If an additional hydrophobic binding site does exist then it would be expected that dihydrokainate show *some* binding affinity – reflecting L-glutamate - like 3-site binding.

Alternatively, the reduction of kainate's isopropylene chain might sterically *hinder* binding – though bearing in mind the flexibility of kainate's ring, this is difficult to visualise (the pyrrolidine ring of kainate appears to be relatively flexible since in the crystal structures, kainate itself exhibits an ideal envelope ring conformation, with C_3 lying 0.6A from the plane of the other four ring atoms (Watase et al, 1958) whilst in the zinc salt (Watase & Nitta, 1957) and ∝-allokainate (Watase, 1958; Cruickshank, 1959) the ring adopts a half-chair conformation).

5.6.6 Summary of QSAR of the kainate receptor

A single "active" conformation of kainate emerges from the conformational analysis by the careful comparison of the conformationally restricted ligand

L-ibotenate with kainate – itself conformationally restricted. This active conformation of kainate corresponds – by comparison with the skeleton of L-glutamate – to one of the two conformations of L-glutamate thought to be possibly active at the glutamate receptor. The observation that two structural modifications of kainate's isopropylene chain – not affecting the L-glutamate skeleton – causes inactivity suggests that a further probably hydrophobic binding site exists. Confidence can be attached to the results since the radiolabelled kainate binding data – used in the QSAR analysis and detailed in Appendix A3 – probably represents binding to a *single* population of kainate receptors. L-glutamate and L-aspartate were both only weak displacers of [^{3}H] kainate, supporting the contention that kainate possesses a discrete receptor.

The result obtained here is gratifying since the ideal situation has arisen – the identification of a unique conformer.

5.7 Summary of QSAR receptor studies

The conformational analysis results obtained in Chapter 5 – based on data collected by *in vitro* radiolabelled ligand binding studies detailed in Appendix A – represent the fruition of the major aim of this thesis: to define ideally a single or small number of possible "active" conformations for the three excitatory amino acids under study – L-glutamate, L-aspartate and kainate.

Back in 1960, Curtis and Watkins proposed a 3-point interaction with the receptor from their electrophysiological results using various glutamate and aspartate analogues. That basic model has been retained here. Electrophysiological potency data, however, possesses the obvious disadvantage that it does not distinguish between responses attributable to different receptors – an analogue might be active at *more* than one receptor. The *in vitro* radiolabelled ligand studies employed here allow *individual* amino acid neurotransmitters to be studied – a major advance on the previous electrophysiological work.

For kainate the ideal situation arose – a single "active" conformer was defined by comparison of the L-glutamate analogue L-ibotenate with kainate itself (5.6.6). In addition to the 3-point electrostatic interaction a further hydrophobic binding site might exist. Conformational analysis of L-glutamate yielded two possible "active" conformers by the careful comparison of the conformational modes of L-ibotenate with those of 1-amino-1,3-dicarboxycyclopentane – both L-glutamate agonists. Analysis of L-aspartate unfortunately did not reveal a finite number of possible "active' conformations due to the lack of a suitably active conformationally restricted ligand.

The radiolabelled binding studies – detailed in Appendix A – are interesting in their own right. Although the kainate work was simply an extension of Simon et al's (1976) binding work, the binding studies using L-[^{3}H] aspartate broke new ground (Appendix A2). At the time, no similar experimental work had been reported. The observations therefore, that DL- α - amino-adipate, N-methyl-D-aspartate (NMDA) and HA-966 were all virtually inactive lent weight to the important contention (4.3.2) that they are all active at a different site – the NMDA receptor. Those few ligands which were active in displacing L-[^{3}H] aspartate were all close structural analogues of L-aspartate; the binding site was far more specific than previously thought. In this respect the L-glutamate analogue L-ibotenate – conformationally fixed in the "extended chain" mode – was almost inactive.

Overall, the results obtained here are very encouraging in that certain "active" conformers have emerged from the analysis making no assumptions about the conformational modes the ligands "prefer" to adopt in solution. Indeed the unique "active" conformation predicted for kainate reflects an "eclipsed" set of atoms (Fig 5.11) which is certainly not a preferred condition of the molecule in solution. This eclipsed conformation, however, is the only one which is consistent with the potencies of the other ligands active at the kainate receptor. Similarly, for the glutamate receptor, one of the two possible active conformations of L-glutamate is an eclipsed form (Fig 5.8).

CHAPTER 6

6. Prostaglandins – Theoretical Approach to Active Conformation

6.1 Introduction to the technique

The prostaglandins (PGs) are the current focus of a massive amount of research effort – mainly by the major drug companies. In fact this Research Group's work on the PGs was at the instigation of May & Baker Ltd., a subsidiary of Rhone-Poulenc May & Baker's prime target was a commercially acceptable "morning after" birth control pill. This family of important naturally occuring drugs, however, has a vast range of potent different actions on different tissues.

The PGs all possess a common chemical structure – a 5-membered ring with two long side-chains from adjacent ring carbons. Certain observations (see 6.3.1.) indicate that these side chains need to be aligned for activity. The work described here was an attempt to correlate potency with a PG molecule's ability to adopt a closely aligned side chain conformation. The method employed here involved computer analysis of conformational modes to identify aligned side chain conformers (Appendix C). The identification process was quantified by means of an index of alignment – the S-index. This index represented the sum of the distances between every backbone atom in one chain and each and every atom in the opposing chain (see Appendix C).

The work was performed in collaboration with P.W. Borthwick and R.H. Lowe in the Department. The involvement of the present author was in helping to formulate, write and develop the computer programme EPAC used in the analysis (see 6.3.3.).

6.2. Prostaglandin Characteristics

6.2.1. Historic Background

In the early 1930's Van Euler (1934) and Goldblatt (1933) independently reported the discovery of a new factor with vasodepressor and smooth muscle stimulating activity. In his 1935 paper, Van Euler provisionally named the factor "prostaglandin" after the fact that it was first discovered in human semen.

6.2.2. Prostaglandin Structure and Activities

Following Van Euler's initial work, it was not until 1960 that Bergstrom and Sjovall reported the isolation of PGE_1 and $PGF_{1\alpha}$ in pure form. In the ensuing years a large family of prostaglandins was found. Their structures are illustrated in Fig. 6.1. below.

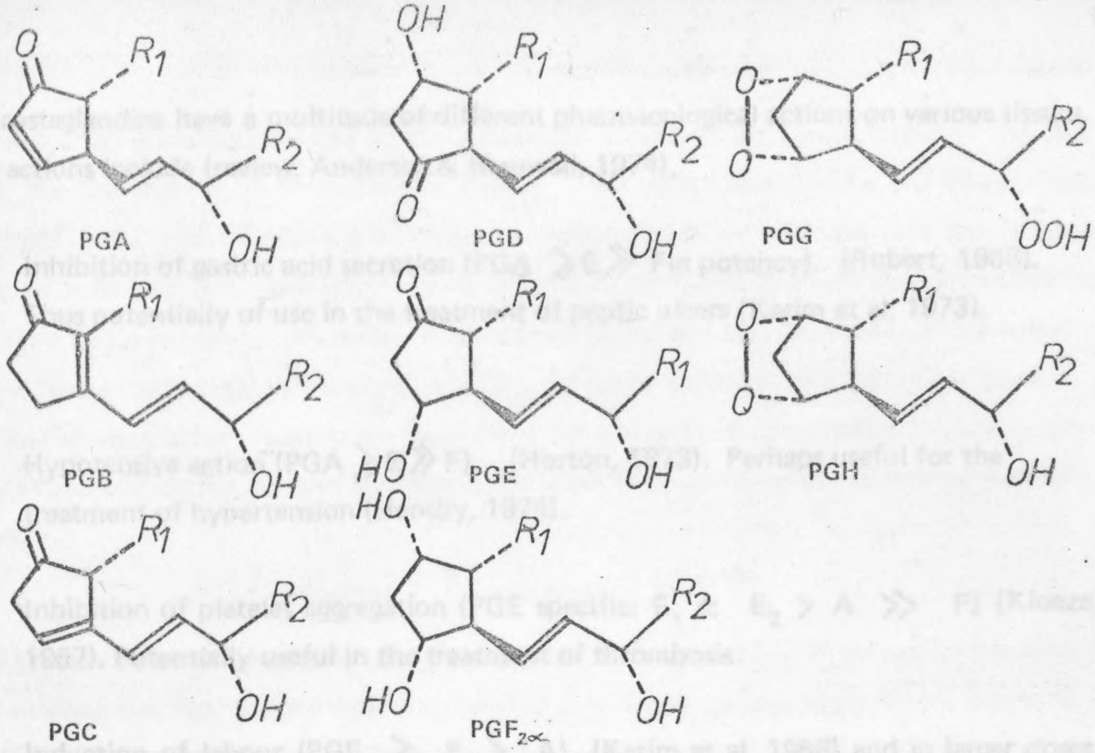


Fig 6.1 Structures of prostaglandins

Prostaglandins have the general classification PGX_n where X defines the form of the ring (above) and n defines the double bonds in the side chains (below) (Fig. 6.2.).

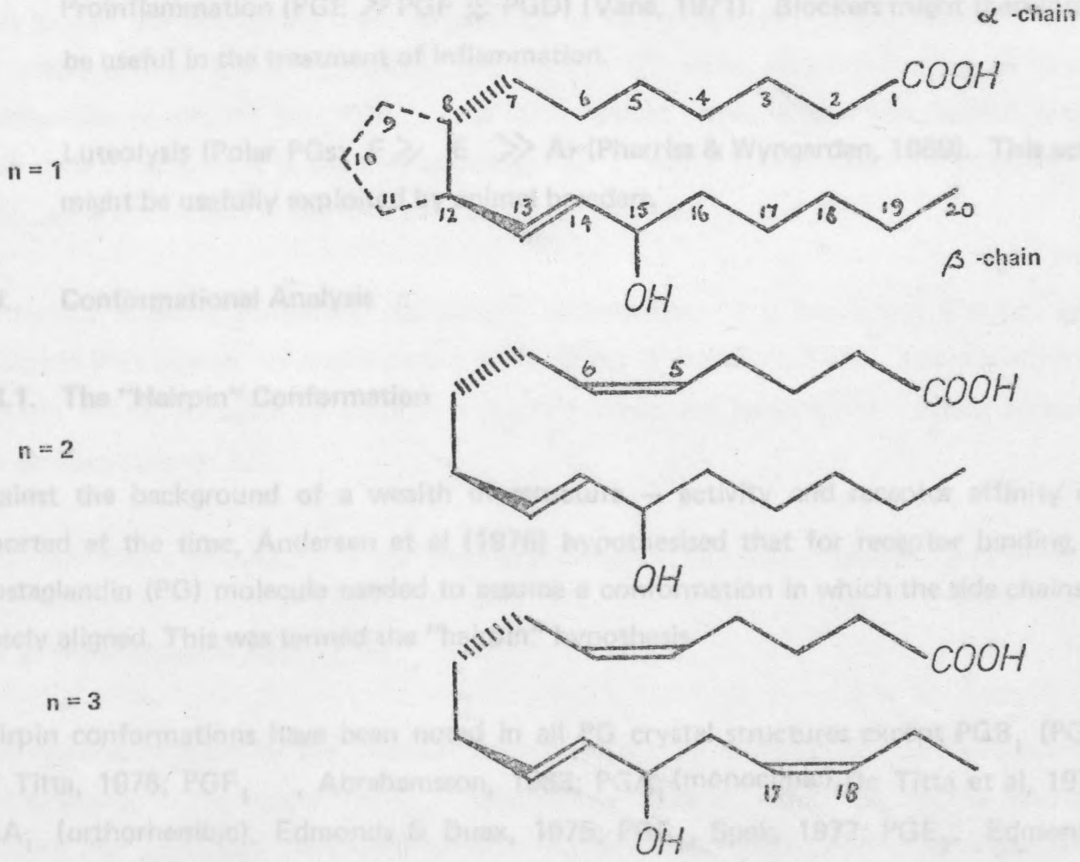


Fig 6.2 Side-chain nomenclature of prostaglandins

The prostaglandins have a multitude of different pharmacological actions on various tissues. These actions include (review, Andersen & Ramwell, 1974),

a) Inhibition of gastric acid secretion (PGA $\gtrsim$ E $\gg$ Fin potency). (Robert, 1968). Thus potentially of use in the treatment of peptic ulcers (Karim et al, 1973).

b) Hypotensive action (PGA $\gtrsim$ E $\gg$ F). (Horton, 1973). Perhaps useful for the treatment of hypertension (Hensby, 1974).

c) Inhibition of platelet aggregation (PGE specific: $E_1 \approx E_2 > A \gg F$) (Kloeze, 1967). Potentially useful in the treatment of thrombosis.

d) Induction of labour (PGE $>$ F $>$ A) (Karim et al, 1968) and in larger doses induction of abortion (Karim & Filshie, 1970).

e) Bronchoconstriction (PGF $\gg$ E $\approx$ A) (Sweatman & Collier, 1968; Mathe et al, 1973; Smith & Cuthbert, 1972) Blockers of PGF might therefore be useful in the treatment of asthma (Horton, 1969).

f) Proinflammation (PGE $\gg$ PGF $\approx$ PGD) (Vane, 1971). Blockers might therefore be useful in the treatment of inflammation.

g) Luteolysis (Polar PGs: F $\gtrsim$ E $\gg$ A) (Pharriss & Wyngarden, 1969). This action might be usefully exploited by animal breeders.

6.3. Conformational Analysis

6.3.1. The "Hairpin" Conformation

Against the background of a wealth of structure – activity and receptor affinity data reported at the time, Andersen et al (1976) hypothesised that for receptor binding, the prostaglandin (PG) molecule needed to assume a conformation in which the side chains are closely aligned. This was termed the "hairpin" hypothesis.

Hairpin conformations have been noted in all PG crystal structures except PGB_1 (PGB_1, De Titta, 1976; PGF_1 , Abrahamsson, 1963; PGA_1 (monoclinic), De Titta et al, 1975a; PGA_1 (orthorhombic), Edmonds & Duax, 1975; PGE_1, Spek, 1977; PGE_2, Edmonds & Duax, 1974; PGF_2 , Langs et al, 1977; PGA_1 & PGB_1, De Titta et al, 1979; review, De Titta et al, 1975b). The circular dichroism (CD) studies of Leovey and Anderson (1975) can also be interpreted as implying the close spatial proximity of the side chains in solution.

The energy of stabilisation, due to the dispersion forces of attraction between the side chains, for the aligned conformation of $PGF_{1\beta}$ has been calculated by Rabinowitz and coworkers (1971) to be about 4.9 kcals mol^{-1} which, together with the ring stabilisation energy of a further 3.8 kcals mol^{-1}, they suggest is considerably more than necessary to maintain a stable conformation state. Interestingly Murakami and Akahori (1977a) found that the "total conformational energy" of $PGF_{1\beta}$ varied by 3.8 kcals mol^{-1} between "sterically allowed" conformers – although these Japanese workers did not identify the minimum energy conformer with a hair-pin conformation. Their total conformational energy was defined by,

$$E = E_{nonbonded} + E_{electrostatic} + constant$$

Where the nonbonded contribution involved the Lennard-Jones "6–12" potential function. These workers also assumed a planar ring for $PGF_{1\beta}$ – which can seriously distort values of the stabilisation energies discussed later in 6.3.5.

Rabinowitz and coworkers (1971) also found that for the PG "1" series the descending order for stabilisation of the aligned conformation is

$$PGE_1 > PGF_{1\alpha} > PGA_1 > PGB_1$$

This is also the descending order of potency of PGs on smooth muscle using the isolated rat uterus (Bergstrom et al, 1968). For the PG "2" series, Rabinowitz and co workers additionally concluded that $PGF_{2\alpha}$ and PGE_2 should exhibit higher stabilisation energies than their counterparts in the corresponding PG "1" series. It is interesting that $PGF_{2\alpha}$ and PGE_2 are more potent in the rat uterus bioassay than $PGF_{1\alpha}$ and PGE_1.

As further evidence supporting the hairpin conformation it is interesting that the 16,16-dimethyl PGs possess enhanced potency (Andersen & Ramwell, 1974). The dimethyl substituent would continue to improve attractive interchain interactions without disturbing the carboxyl terminus.

Thus, the ability to adopt a hairpin confirmation appears probably necessary for PG activity.

6.3.2. Prostaglandin Active Sites

Modification of many of the chemical features of the PG molecule can modulate potency. For example, in the case of $PGF_{2\alpha}$ (Hammarstrom et al, 1976), the specific binding of tritiated $PGF_{2\alpha}$ to a particulate receptor fraction of bovine corpora lutea was modified by the functional group changes illustrated in Fig. 6.3. below.

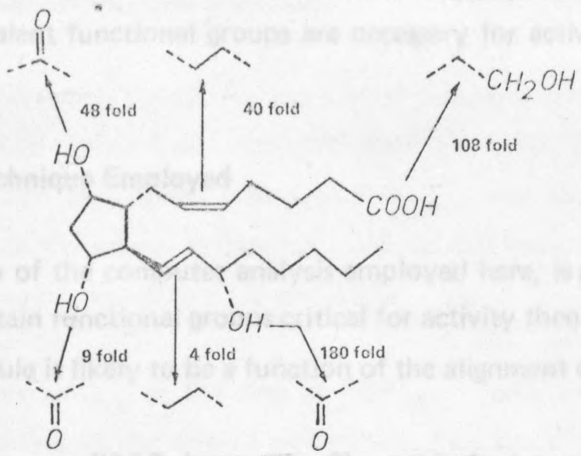


Fig 6.3 Relative importance of functional groups in $PGF_{2\alpha}$ binding to bovine corpus luteum receptor (decreases of Kas following the structural modifications shown), (After Hammarstrom et al, 1976).

Further results for analogues of $PGF_{2\alpha}$ are illustrated in Fig. 6.4. below.

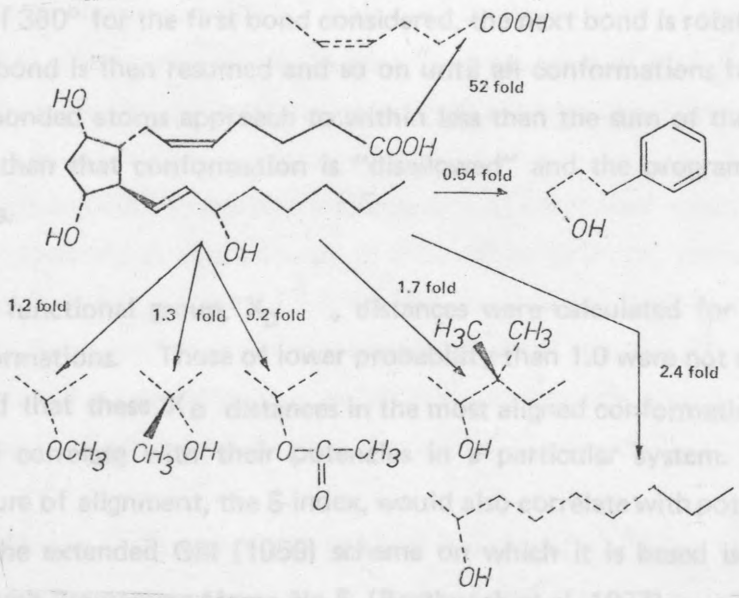


Fig 6.4 Decreases of K s following structural modifications of $PGF_{2\alpha}$ (After Hammarstrom et al, 1976).

From Fig. 6.3. it is quite apparent that for $PGF_{2\alpha}$ activity at the bovine corpus luteum, the terminal carboxyl and 15-hydroxyl groups are very important, closely followed by the 5,6 double bond and the ring 9-hydroxyl. The ring 11-hydroxyl and the 13,14 double bond are of lesser importance.

Fig. 6.4. demonstrates that retention of the hydroxyl character (i.e. lone pair donor) at the 15-hydroxyl group maintains activity. The structural changes at the terminal end of the β-chain do not significantly modulate activity.

These same or equivalent functional groups are necessary for activity in many systems for many PGs.

6.3.3. Computer Technique Employed

The basic assumption of the computer analysis employed here, is provided that a PG molecule possesses the certain functional groups critical for activity then the potency of the molecule is likely to be a function of the alignment of the side-chains.

The computer programme EPAC (appendix C) was devised to assess by systematic and exhaustive bond rotation, the most closely aligned conformation of a PG molecule. The aim of this analysis was to predict whether a newly-designed, "drawing board" PG could adopt an aligned "hairpin" conformation. A PG molecule coming through this screening analysis could then be assayed for activity and investigated further.

The programme considers each bond on a rotation grid of 120°. Following a complete rotation of 360° for the first bond considered, the next bond is rotated by 120°. Rotation at the first bond is then resumed and so on until all conformations have been investigated. If any non-bonded atoms approach to within less than the sum of the appropriate Van der Waal's ratir then that conformation is "disallowed" and the programme prints out the violating atoms.

Inter functional group, X_D , distances were calculated for the allowed probability 1.0 conformations. Those of lower probability than 1.0 were not considered. It was hoped that these X_D distances in the most aligned conformation of each PG molecule could correlate with their potencies in a particular system. It was also hoped that the measure of alignment, the S-index, would also correlate with potency. This entire programme and the extended Gill (1959) scheme on which it is based is discussed in detail in Research Programme Memo No.5. (Borthwick et al, 1977).

6.3.4. The Method Employed – A Critique

As mentioned many times elsewhere in this text, there is no *a priori* reason to believe that the active conformation of a ligand will reflect one of the populated conformations in solution. However, in 1976 when this work was carried out, it was the firmly held belief of our Research Group that a link did exist between biological activity and the most populated conformations. Therefore, it will be noted that in the computer analysis, only those conformers exhibiting a probability of 1.0 were considered. Those conformers whose probabilities were as high as 0.272 or even 0.5 were disregarded. Thus when investigating the bond rotations, in particular those near to the ring structure, an entire "family" of potentially

important conformations could have been "lost".

Using the expression of Salem (1962) for the ($\sigma\sigma$) dispersion attraction between CH_2 -- CH_2 in parallel opposed chains, Rabinowitz and coworkers (1971) calculated that the stabilisation energy between the aligned chains of $PGF_{1\beta}$ was about 4.9 kcal mol^{-1} This value is far more than the energy difference, Δ E, of 0.8 kcals mol^{-1} (Szasz et al, 1948) between conformations of probability 1.0 and 0.272.

Additionally, a pucker at C_{12} in the ring of $PGF_{1\beta}$ adds another 3.8 kcals mol^{-1} of conformation stabilisation to the aligned side chains. Also the binding energy, Δ G, of the prostaglandin receptor interaction is probably in the range 6 - 12 kcal mol^{-1} (Andersen et al, 1976) which again puts the 0.8 kcal mol^{-1} Δ E between conformations in perspective.

6.3.5. Prostaglandin Ring Conformation

In their investigation of the crystal structure conformation of prostaglandins, Rabinowitz and his collaborators (1971) looked at the magnitude of the attractive dispersion forces between aligned side chains of the crystal structure of $PGF_{1\beta}$. They found that the dispersion attraction or "stabilisation energy" between the chains was 4.9 $kcalsmol^{-1}$.

In addition, there is another major source of conformer stabilisation in $PGF_{1\beta}$ and that is the lowering in strain energy of the cyclopentane ring when it is puckered into an ideal envelope with C_{12} some 0.7 Å out of the plane of the other four ring carbons (Abrahamsson, 1963). Rabinowitz and his co workers estimated that this pucker at C_{12} adds another 3.8 kcals mol^{-1} of conformation stabilisation to the side chains in the aligned conformation giving a total gain in stabilisation energy of 8.7 $kcalsmol^{-1}$.

Additionally these workers found that if a model of $PGF_{1\beta}$ is constructed, identical to the crystal structure conformation except that the ring pucker position is not at C_{12} (or C_{ε}) then the inter chain distance is increased by about 1Å and the dispersion forces are reduced to about ¼ of their former value.

Thus the PG ring conformation appears crucial to the molecule's ability to adopt the most closely aligned hairpin conformation. Against this background it was decided to use an "idealised" ring conformation for the computer studies on PGE and PGF where C_{12} was an arbitrary 0.7 Å out of the plane of the other four carbon atoms. Although the PG crystal structures show a variety of ring conformations it is encouraging that both the A and B forms of PGE_1 exhibit C_{12} envelopes (Spek, 1977).

6.3.6 Results and Discussion

The results have been discussed in depth in the Group's Research Memo No 7 (Lowe, 1978). For the bovine corpus luteum (BCL) receptor, binding studies using [^{3}H] PG $F_{2\alpha}$ produced a potency data set for a series of $PGF_{2\alpha}$ analogues. It was then attempted to correlate the affinity data to the "S-index" detailed in Appendix C.1.2. The index was designed as a measure of inter-chain alignment and is equal to the summation of interatomic distances between each α-chain atom and every other atom in the β-chain.

An inverse correlation seemed to exist between the ability of $PGF_{2\alpha}$ anlogues to compete with [^{3}H] $PGF_{2\alpha}$ at its BCL receptor and the predicted index for the most aligned probability = 1, conformer. A better correlation, however, might have been obtained if *all* the "allowed" conformers had been inspected. Only those of probability = 1 were considered here (see 6.3.4).

Lowe (1978) also proposed the interesting idea that the most aligned conformers also possessed the highest stabilisation energies which would be released by the chains splaying – on entering the receptor membrane phase – to ellicit the physiological response.

It was subsequently discovered that Murakami & Akahori (1977b) had performed similar conformational work. In their original work (1974) they tried to correlate conformational energies of sterically "allowed" conformers with potencies of a series of prostaglandins. They concluded that the "total conformational energy" differences between conformations were insufficient to favour particular conformations e.g. for PGE_1 total conformational energy ranged between – 41.26 to -- 38.44 kcals mol^{-1} (difference of 2.82 kcals mol^{-1}). We would interpret the data differently and suggest than an energy difference of almost 3kcalsmol^{-1} could be significant (see 6.3.1). Their second paper published in 1977 (b) is more interesting since it looks at all the allowed conformers – not just the high probability ones. Their study attempted to correlate the populations of "conformational groups" (conformers processing similar X_D distances between functional groups C_1OOH, $C_9 = O$, $C_{15}OH$ and subactive sites C_5 and C_{20}) with potencies of a series of PG's in various bioassays (rat uterus, guinea pig ileum, rabbit jejunum and rat blood pressure). Employing the additional subactive site C_5 tentative correlations were found for guinea pig ileum and rabbit jejunum. Unfortunately the X_D distance C_5 to $C_{15}OH$ – which gives a tentative indication of inter-chain proximity near to the ring (Fig 6.2) - was not minimal for the correlating groups. Using C_{20} as a subactive site, however, the correlating conformational groups for guinea pig ileum did show a tentative propensity for inter-chain align-

ment as measured by the C_1OOH to C_{20} X_D distance. A critique of this work is that if a unique active conformation is assumed then only one conformational group — representing the "active" interfunctional group dispositions -- would be expected to correlate. In fact about half of the groups correlated.

Finally, PG receptor model building has already been attempted (Smythies, 1979; Smythies et al, 1970, 1975) although as pointed out in 6.1, receptor model building was not the main priority here.

APPENDIX A1

Appendix A Radiolabelled – ligand receptor binding studies

A1 Approach to binding studies

A1.1 Historical backdrop

Radiolabelled ligands binding to receptors, as applied to neurotransmitter systems is a relatively new approach to receptor characterisation since it is only within the last decade that the following thee obstacles have been overcome:

(i) Attaining a high ligand selectivity for the receptor.

(ii) Attaining a high ligand affinity.

(iii) Attaining a high specific activity of the radiolabel.

Whereas enzyme assays are facilitated by the amplifying process of enzymological catalysis, neurotransmitter receptor binding assays involve a single labelled molecule per receptor. But if the former three conditions can be achieved then a sufficiently low concentration (probably nanomolar) of the radiolabelled ligand can be used to detect the receptor binding component ("specific binding") of total binding against the background binding to other tissue ("non-specific binding").

It was the successfuly work on the nicotinic cholinergic receptor using ^{125}I-labelled α-bungarotoxin, the snake venom antagonist (Changeux et al, 1970 ; Meunier et al, 1972; Miledi et al, 1971; Raftery et al, 1971) which focused sufficient attention on the technique for it to take off in its application to other neurotransmitter systems.

The elegant characterisation of nicotinic cholinergic receptor of the electric organ of the electric eel, mentioned above, suceeded in part because this receptor constituted as much as 20% of the membrane protein (which eases the stringency for a label of high specific activity) coupled with the fact that the snake venom toxin, used as the radiolabel, combined with the receptor with a very high selectivity and an affinity bordering on the irreversible.

It is perhaps fair to say that the springboard for the present work, using receptors located on synaptic membranes, was the work performed by Young & Snyder (1973) where these investigators demonstrated that strychnine, a potent and selective antagonist of glycine –

induced hyperpolarisations of spinal neurones binds specifically to a component of synaptic membranes which it was assumed was the physiological glycine receptor, since strychnine has negligible affinity for the glycine high affinity uptake system. Also at about the same time, Peck et al (1973) found that the γ -aminobutyric acid (GABA) antagonist bicuculline competitively inhibited GABA binding to synaptosomes (pinched off nerve endings) in which the active uptake site had been inactivated by chlorpromazine.

This pioneering work employing synaptic membranes then led Roberts (1974,1975) and Michaelis (1975) et al (1974) to perform studies on the glutamate receptor itself. This is thus the historical background to the work presented here.

In general, labelled antagonists have provided the most efficient routine receptor labels because of their typically nanomolar affinities being substantially higher than the affinities of the transmitters themselves. However, in the absence of suitable antagonists (see subsection 4.1.4) the binding work described here has either employed the labelled neurotransmitter itself or a high potency agonist.

A1.2 Criteria for Receptor Identification

A1 2.1 Identification

In undertaking a binding study it is essential to make a very careful comparison of the properties of the ligand-binding site with the properties expected of the neurotransmitter receptor to determine if they really are synonymous. The contention that a given binding site represents a receptor may be supported by the ability of the characteristics of the binding to fulfill a basic set of necessary but not sufficient criteria (Curtis, 1961; Birdsall & Hulme, 1976). Failure to meet even onecriterion can call into question the validity of receptor identification.

The five basic criteria are:

(i) Saturability.

(ii) Specificity.

(iii) Kinetics

(iv) Distribution

(v) Pharmacology

These basic criteria will now be discussed followed by other characteristics of binding which may serve as useful secondary criteria.

A1.2.2 Basic criterion (i) Saturability

There are only a finite, commonly small, number of receptors associated with a sample of tissue which implies that a component of these usually high affinity binding sites must saturate with increasing concentrations of the radioligand.

Saturable binding is suggested if a reduction in the radio-ligand binding occurs upon the addition of a large excess of the unlabelled same ligand. If binding increases in proportion to the ligand concentration, which would occur if there are only a large number of very low affinity binding sites, then the radioligand bound will remain the same. However if at least one class of binding sites becomes fully saturated then as a result of the reduced specific activity of the radioligand, in the presence of a large excess of the unlabelled ligand, the amount of bound radioligand will decrease.

The density of binding sites in a sample of tissue can also be important when other ligands competing with radio-ligand binding are under investigation. If all ligands do not yield a similar density of binding sites (as derived by, for example, Scatchard analysis (section A 1.3) then this can imply more than one class of binding site.

A1.2.3 Basic criterion (ii) Specificity

The radioligand employed must be selective or "specific" for the receptor under investigation. Thus concentrations of drugs which are pharmacologically effective at a particular receptor should displace significantly the saturable component of radioligand binding (termed "specific binding") while pharmacoligically effective concentrations of drugs acting at other receptors should be ineffective.

Many biological systems distinguish between stereoisomers of drugs. Thus stereospecificity of the radio ligand itself or unlabelled competing ligands lends support to receptor identification.

A1.2.4 Basic criterion (iii) kinetics

In the normal way for a bimolecular ligand-binding site interaction the dissociation constant (K_D) is equal to the dissociation rate constant (k_{-1}) divided by the association rate constant (k_1). If, after independent determination of these three binding parameters, the above relation is not maintained within the limits of experimental error then this anomalous

behaviour implies a multiplicity of binding sites or some other complicating factor. Also, with respect to the association rate constant, the time course of specific radio-ligand binding should be at least as fast as the onset of the physiological effect. Conversely, the dissociation of specific binding should be at least as fast as the termination of the physiological effect.

A1.2.5 Basic criterion (iv) Distribution

Specific radio-ligand binding should be localised to only those tissues of the brain which are known to exhibit the appropriate physiological response. It should also be highest in those subcellular fractions demonstrated to contain synaptic membranes.

Caution should be exercised when attempting to relate specific binding distribution within regions of the brain with physical distribution of the neurotransmitter (as determined by, for example, autoradiographic studies) since metablic pools of the neurotransmitter may also exist in addition to the neurotransmitter pools.

A1.2.6 Basic criterion (v) Pharmacology

The detailed pharmacology of the specific binding is a very important criterion in identifying a binding wite with a neurotransmitter receptor since a receptor is postulated to exist in the first place as a result of its pharmacology.

Thus drugs which are physiologically effective as agonists or antagonists of the neurotransmitter should significantly displace the specific radioligand binding at relatively low concentrations while physiologically ineffective drugs should compete for specific binding only at relatively high concentrations, if at all.

Ideally both qualitative and quantitative correlation should be obtained between the physiological response to particular drugs and their ability to displace specific binding. However, in practice, both of these are difficult to achieve not as a consequence of the binding studies themselves but as a consequence of the nature of the other pharmacological data with which the binding data are being compared. This aspect has been discussed previously in subsection 5.1.3.

Specific binding affinities also typically differ according to whether an agonist (such as the neurotransmitter itself) or an antagonist has been used as the radioligand which can also influence attempts to correlate specific binding affinities with physiological effectiveness. This differential specific binding phenomenon has been variously explained in terms of preferential specific binding of agonists and antagonists to different receptor conforma-

tional stakes (see section 2.1.2) and in terms of a multiplicity of classes of agonist binding sites (Birdsall & Hulme, 1976).

A1.2.7 Other useful criteria

(i) Tissue concentration linearity

Linearity between tissue concentration and specific radioligand binding must be exhibited to demonstrate the absence of potentially interferring effects, such as receptor or, less commonly, ligand degredation during incubation, or unrecognised endogenous ligands.

(ii) Temperature dependence

All neurotransmitter receptors identified so far have been lipoproteins (Lindstrom, 1978). Thus the tertiary structure of the protein component should begin to be disrupted, resulting in reduced specific binding, at temperatures greater than about 40°C evenutally leading, with increasing temperature, to complete denaturation of the protein component and a concomitant complete loss of specific binding.

(iii) pH dependence

Since neurotransmitter receptors normally operate under conditions of physiological pH ($\sim$ 7.4) it is to be expected that their pH value corresponding to optimal specific binding would be relatively close to pH 7.4.

A 1.3 Useful expressions

A1 3.1 Derivation of the Scatchard model of receptor ligand binding and the evaluation of a competing ligand dissociation constant (K_i)

The simplest case of receptor-ligand binding is that involving a single class of independent noninteracting binding sites.

$$R + L \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} RL$$

Where R = unoccupied receptor, L = unbound ligand, RL = receptor-ligand complex.

At equilibrium,

$$K_D = \frac{k_{-1}}{k} = \frac{[R]\,[L]}{[RL]}$$

Or,

$$K_D = \frac{(N - \bar{\nu})\,PtA}{\bar{\nu}\,Pt} \qquad \text{.....(i)}$$

where K_D = radioligand dissociation constant (M)

N = binding site conc. (moles/mole protein)

$\bar{\nu}$ = bound radioligand conc. (moles/mole protein)

Pt = total protein conc. (M)

A = free radioligand conc. (M)

By rearrangement,

$$\bar{\nu} = \frac{NA}{K_D + A} \qquad \text{.....(ii)}$$

Equation (ii) is a usual formation of the Scatchard (1949) model. This equation has the convenient property that the Scatchard variables $y = \bar{\nu}/A$ and $x = \bar{\nu}$ transform this model to the linear form,

$$y = \frac{N}{K_D} - \frac{x}{K_D} \qquad \text{..... (iii)}$$

In this form the intercept on the y-axis yields N/K_D and the gradient is equal to $-1/K_D$ Equation (iii) is thus the most useful practical formulation of the Scatchard model.

The major advantage of the Scatchard model compared to other methods of plotting saturation data is that all the points on the graph are given equal statistical weight. The experimental error in determining the major variable bound radioligand, is mathematically eliminated from the line fitting procedure because the variable is present on both axes.

(ii) In the presence of a competing unlabelled ligand equation (ii) is modified to yield

$$\bar{\nu}_r = \frac{NA}{K_D 1 + A} \quad \text{..... (iv)}$$

where $\bar{\nu}_r = \bar{\nu}$ in the presence of the competing ligand

K^1_D = apparent K_D

This modification assumes that, for the competing ligand concentration range within which the K^1_D would be measurable practically, the free binding site concentration in the presence of the competing ligand is equal to that in its absence.

But,

$$K^{\cdot}_D = K_D (1 + I/K_i)$$

where I = competing ligand conc. (M)

K_i = competing ligand dissociation constant (M)

By rearrangement

$$K_i = \frac{I}{K^{\cdot}_D/K_D - 1} \quad \text{.....(v)}$$

Equation (v) may be implemented to evaluate K_i from the K_D derived from a Scatchard plot of equation (iv).

A1.3.2 Derivation of the relationship between the competing ligand dissocation constant K_i and the concentration of competing ligand which causes 50% displacement (IC_{50}) of the radio-ligand.

And understanding of the relationship between IC_{50} and K_i under the conditions prevailing in a "typical" binding experiment and the theoretical basis for their determination is critical to appropriate interpretation of the experimental data, as well as for comparison of the literature values of IC_{50}.

Theoretical Analysis

A usual formulation of the Scatchard model (see subsection A1.3.1 equation (ii)) for radioligand binding is given by,

$$\bar{\nu}_r = \frac{NA}{K_D + A} \quad \text{.... (i)}$$

where $\bar{\nu}_r$ = bound radioligand conc. (moles/mole protein)

N = protein binding site conc. (moles/mole protein)

A = free radioligand conc. (M)

K_D = radioligand dissociation constant (M)

In the presence of a competing unlabelled ligand equation (i) is modified to yield,

$$\bar{\nu}_r' = \frac{NA}{K_D' + A} = \frac{NA}{K_D (1 + I/K_i) + A} \quad \text{.....(ii)}$$

where $\bar{\nu}_r'$ = $\bar{\nu}_r$ in the presence of the competing ligand

K_D' = apparent KD

I = competing ligand conc (M)

K_i = competing ligand dissociation constant (M)

When $I = IC_{50}$, $\bar{\nu}_r = 2\bar{\nu}_r'$, then,

$$\frac{2NA}{K_D (1 + IC_{50}/K_i) + A} = \frac{NA}{K_D + A}$$

By rearrangement

$$K_i = \frac{IC_{50}}{1 + A/K_D} \quad \text{..... (iii)}$$

Equation (iii) is parallel to that derived by Cheng & Prusoff (1973) for enzymatic reactions. It is usually valid to assume that the added radioligand concentration is equal to the actual free radioligand concentration at equilibrium. In fact a loss of up to 10% of added readioligand concentration will not significantly affect the estimation of K_i's (Bennett, 1978). Thus equation (iii) may be written,

$$K_i = \frac{IC_{50}}{1 + C/K_D} \quad \text{..... (iv)}$$

where C = added radioligand conc. (M)

A2 High affinity L-[^{3}H] aspartate binding to rat cerebellar synaptic membranes.

A2.1 Introduction

The result of the work described here provide a data base for the structure – activity relationship (SAR) study of the aspartate binding site performed in section 5.5.

The specific L-[^{3}H] aspartate binding demonstrated here displayed many characteristics expected of an interaction with the physiological aspartate synaptic receptor.

A2.2 Materials and methods

A2.2.1 Preparation of cerebellar synaptic membranes

The method of Zukin etal (1974) was employed. The quantities of materials used in the procedure briefly outlined below yield sufficient cerebellar synaptic membrane suspension to, say, investigate the displacement curves of three competing ligands.

Twenty male Sprague-Dawley rats (150 - 250g) were stunned then decapitated and their brains quickly removed. Five cerebella were placed in each of four glass homogeniser tubes containing 18ml ice-cold 0.32M sucrose. The tissue was homogenised using 20 strokes of a Potter-Elvehjem homogeniser fitted with a Teflon pestle. Each homogenate was divided between two ice-cold plastic centrifuge tubes with plastic caps and centrifuged at 1000g for 10 minutes at 4°C. Each S_1 supernatant was decanted into another ice-cold plastic centrifuge tube and the remaining P_1 pellet (crude nuclear fraction) was discarded. The S_1 supernatants were then centrifuged at 17,000g for 20 minutes at 4°C. The resulting P_2 pellets (crude mitochondrial fraction) were transferred by spatula into homogeniser tubes (two pellets per tube) containing 18ml ice-cold distilled water and homogenised using 10 strokes of the homogeniser. The homogenate was sonicated for 60 seconds with an MSE sonicator and the resulting suspension was centrifuged at 8000g for 20 minutes at 4°C. Half of each S_3 supernatant was decanted into another ice-cold plastic centrifuge tube and the other half was used to wash off, with the aid of a gentle jet of supernatant from a Pasteur pipette, the soft buffy upper layer of the pellet (crude mitochondria-myelin (microsomal) fraction) to leave only the lower light-brown layer. The combined supernatant fraction was centrifuged, using metal caps on the centrifuge tubes, at 38000g for 30 minutes at 4°C to obtain the P_4 pellets (crude synaptic membranes). All the P_4 pellets were washed into 100ml ice-cold 0.05M Tris-acetate buffer (pH 7.2 at 25°C) and homogenised with 20 strokes of the homogeniser. The cerebellar synaptic membrane suspension (0.1 - 0.4 mg protein/ml) was kept on ice until used.

A2.2.2. L-[^{3}H] Aspartate binding assay

For the routine measurement of L-[^{3}H] aspartate binding, 0.9ml aliquots of the fresh membrane suspension (0.1 - 0.4mg protein/ml) were dispensed into 5ml plastic incubation tubes using a Cambridge pipette and preincubated at 25°C for 10 min. on a shaking water bath, with 50μl of either 20mM (1mM final concentration) unlabelled L-aspartate, competing ligand under test, or the routine buffer (0.05M Tris-acetate, pH 7.2 at 25°C). The latter 50μl volumes were dispensed using a Cambridge pipette. The incubation proper was initiated by the addition, by Cambridge pipette, of 50μl of 4μM (0.2μM final conc.) L-[^{3}H] aspartate and continued for 15 minutes at 25°C. It was terminated by the addition of 3ml of ice-cold buffer followed by immediate filtration through Whatman GF/C filters under reduced pressure and quickly washed with an additional 6ml of buffer. To reduce inaccuracies associated with droplets of the incubate adhering to the filtration barrel, a short 8ml barrel was used. Also, to facilitate rapid filtration, the scintered glass filter was reverse flushed frequently to remove particles of glass fibre emanating from the GF/C filters. Each filter was vortex mixed in 10ml scintillation cocktail (6.63ml toluene, 3.32ml Triton X-100, 50μl glacial acetic acid and 33mg PPO) and allowed to stand overnight. After thorough vortex mixing, the filter-bound radioactivity was measured using a Packard 3255 liquid scintillation spectrometer. Appropriate modifications to this routine assay were made for the various control studies as described later. Triplicate incubations were performed in parallel for each concentration of displacing ligand. For each batch of synaptic membrane suspension prepared the protein concentration was determined using the method of Lowry et al (1951).

Since the number of counts per sample, N, collected by the scintillation counter was the result of random decay of the tritium label, the inaccuracy associated with N is $\sqrt{N}$. An inaccuracy of about 1% was considered acceptable against the larger inaccuracies inherent in the binding assay and thus about 10,000 counts were required to be collected for each sample. Also 5 min. was considered to be the longest practicible counting period. Therefore, for the routine assay, the stock radioligand was diluted with "cold" ligand (i.e. the specific activity was reduced) to yield, in the binding assay and in the absence of a displacing ligand, 10,000 counts in about 5 min.

The specific activity of the L-[2,3 - ^{3}H] aspartate used was 12 Ci mmol^{-1} and the radioactive concentration was 1m Ci ml^{-1}. Thus the molar concentration of the radioligand was 83 μM. It was found, by trial and error, that 200μl of the 83μM stock radioligand plus 15ml of 2.89 μM "cold" ligand gave 15ml of 4 μM "diluted" radioligand (required in the binding assay) of the appropriate specific activity (4.6 Ci mmol^{-1}) to yield the desired 10,000 counts in about 5 min.

A2.2.3 Analysis of scintillation counter results

For each sample condition of ligand concentration, temperature, pH etc, the basic results sought from the scintillation counter data were the specific binding (in units of pmol/mg^{-1} protein) and associated standard deviation of the triplicate samples, and the nonspecific binding with standard deviation.

The programme SATR , with corresponding flow chart , was written to analyse the scintillation counter data from the saturability study. This programme not only calculates values of specific and nonspecific binding for increasing concentrations of radioligand but also subsequently performs a Scatchard analysis to yield the dissociation constant (K_D) and the maximum specific binding capacity. Modified versions of this basic programme were used to accommodate the analysis of data from the other investigations.

A specimen output from the Packard 3255 liquid scintillation spectrometer is given below:

.5789	175	5.00	3131	2123	626.2	424.6
External Standard Ratio	Sample No.	Counting Time (min)	Red Channel Total Counts	Green Channel Total Counts	Red Channel cpm	Green Channel cpm

The External Standard Ratio (ESR) gives a measure of the optical and chemical quenching of the system and is a function of the percentage efficiency of counting approximated by

$$Y = A + BX + CX^2 + DX^3$$

where Y is the % efficiency, X is the ESR and A,B,C, and D are the polynomial coefficients.

Using a radioactive sample of known activity, an empirical graph of % efficiency v. ESR (Y v X) can be plotted for the PPO/toluene scintillation cocktail used. Computer fitting of the above polynomial to the empirical curve yielded the following coefficients.

$$A = 0.02116$$
$$B = 0.28315$$
$$C = 1.34134$$
$$D = -1.28821$$

Therefore, using the above expression, the cpm (counts per minute) of each sample can be adjusted for quenching to yield dpm (disintegrations per minute). For ESRs above 0.5, the red channel counts were used. Ideally, for each value of cpm, the % efficiency should

be calculated from the corresponding value of ESR. In practice, however the values of ESR between samples varied by so little ($<$2%) that a constant value of ESR was chosen which was the visually estimated average of the range of ESR values for a particular experiment. The inaccuracy involved in this approximation was very small compared with the inaccuracies inherent in the binding assay. As discussed in the "L-[^{3}H] aspartate binding assay" a counting accuracy of circa 1% was aimed for.

A2.3 Binding results

A2.3.1 Saturability of specific L-[^{3}H] aspartate binding

Final L-[^{3}H] aspartate concentrations were varied over the range 0.05 - 2.0 μM. The basic routine assay was followed except that smaller volumes of the membrane suspension and L-[^{3}H] aspartate were used (0.46 and 0.02ml respectively) in order to reduce the amount of radioligand used. (This study consumed a very large proportion of the radioligand available as compared with the other studies).

A 50 μM L-[^{3}H] aspartate stock stock solution (2 μM final concentration) of a suitable specific activity was made up from 100 μl of the 83 μM original stock L-[^{3}H] aspartate plus 350 μl of 41 μM "cold" L-aspartate. For the very low concentrations of radioligand used it was found necessary to count, on the scintillation counter, for greater than 5 minutes in order to bring the statistical counting inaccuracy within reasonable limits. Specific L-[^{3}H] aspartate binding was saturable with increasing concentrations of L-[^{3}H] aspartate in contrast to the nonspecific L-[^{3}H] aspartate binding which increased linearly (Fig. A2.1) Scatchard analysis (Scatchard, 1949) of the specific binding yielded datum points which were approximated to a straight line (Fig. A2.2) using an unweighted least squares best fit, with a dissociation constant (K_D) of 1.2 ($\pm$ 0.4) μM and a specific binding capacity of 27 ($\pm$ 5) pmol/mg protein. A straight line indicates a single population of binding sites (see A1.3.1).

In order to detect the possibility of anomalous binding behaviour the data for saturation of specific binding from Fig. A2.1 were analysed using a Hill plot (Fig. A2.3). The datum points were approximated to a straight line with a Hill coefficient (η) of 1.05 ($\pm$ 0.15).

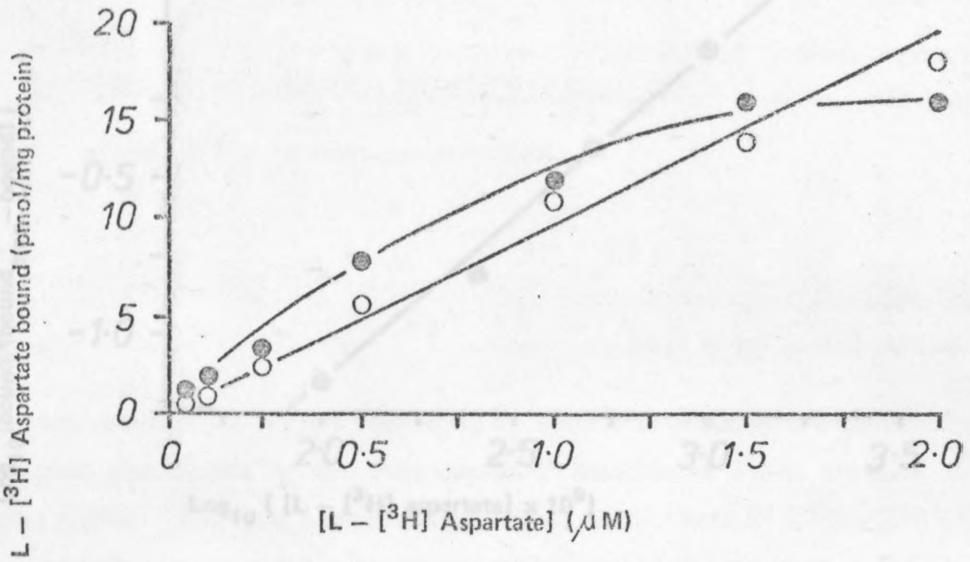


Fig. A2.1 Saturability of specific L – [^{3}H] aspartate binding. The routine assay was followed except that final L – [^{3}H] aspartate concentrations were varied over the range 0.05 – 2.0 μM. Results are means of triplicate determinations which varied by less than 9%. ● Specific; o nonspecific.

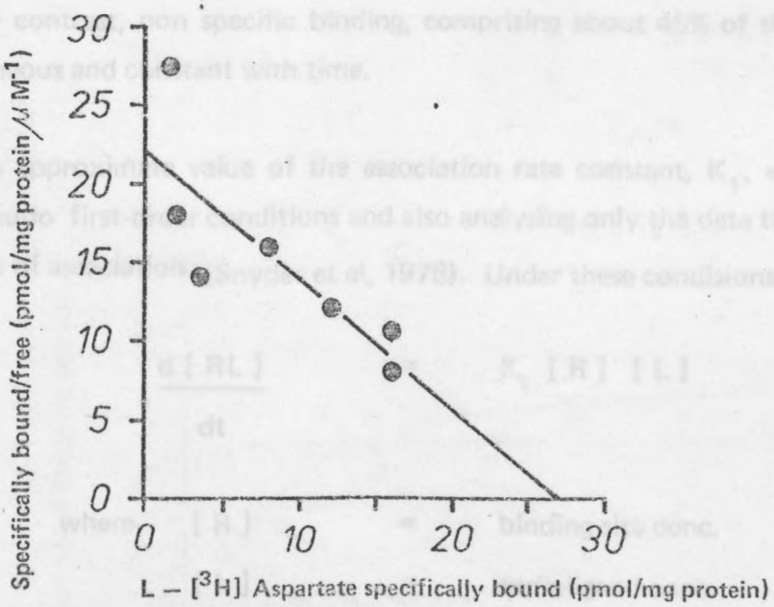


Fig. A2.2 Scatchard analysis of the specific binding data of Fig. A2.1

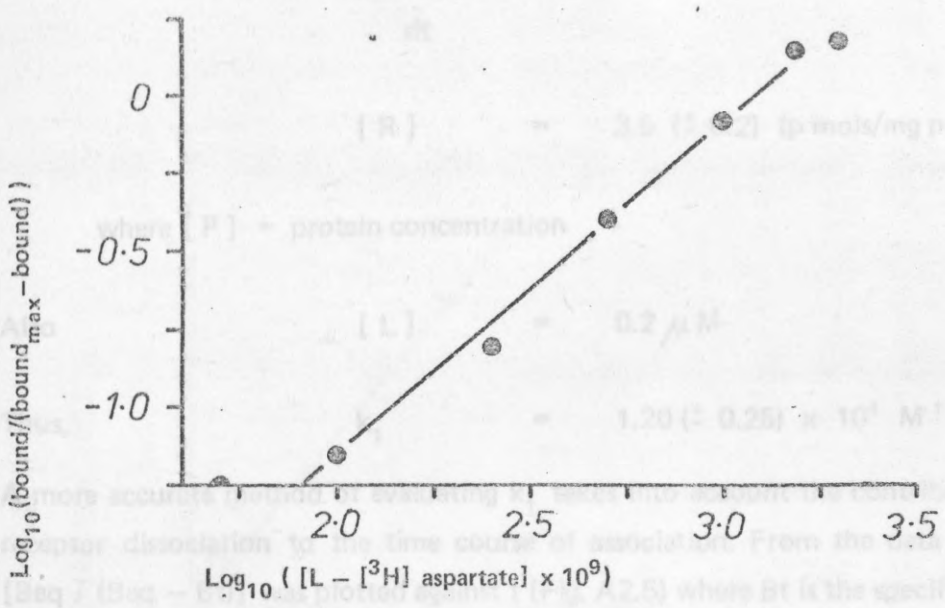


Fig. A2.3 Hill plot of the specific binding data of Fig. A2.1

A2.3.2 Association of specific binding

Samples were assayed during the time interval 0 - 30 minutes at 25° C. The specific binding was half-maximal at about 3.5 minutes and plateaued at about 15 minutes (Fig. A2.4). By contrast, non specific binding, comprising about 45% of the total binding, was instantaneous and constant with time.

An approximate value of the association rate constant, K_1, was determined by assuming pseudo first-order conditions and also analysing only the data through the first half life of association (Snyder et al, 1978). Under these conditions we have,

$$\frac{d[RL]}{dt} = K_1 [R] [L]$$

where [R] = binding site conc.

[L] = radioligand conc.

[RL] = complex conc.

t = time

From Fig. A2.4 the line of best fit through the first half-life was constructed and the gradient was divided by the initial concentrations of free radioligand and binding site to yield k_1 as follows.

Gradient, $\frac{d[RL]}{dt} = 8.3\ (\pm 1.2)$ (f mols/mg protein s^{-1}) x [P]

$[R] = 3.5\ (\pm 0.2)$ (p mols/mg protein) x [P]

where [P] = protein concentration

Also $[L] = 0.2\ \mu M$

Thus, $k_1 = 1.20\ (\pm 0.25) \times 10^4\ M^{-1}\ S^{-1}$

A more accurate method of evaluating k_1 takes into account the contribution of ligand – receptor dissociation to the time course of association. From the data of Fig. A2.4. ln [Beq / (Beq – Bt)] was plotted against t (Fig. A2.5) where Bt is the specific binding at time t. The following expression (Bennett, 1978) related the gradient of this line (k_{obs}) to k_1 and the dissociation rate constant, k_{-1}.

$$k_1 = \frac{k_{obs} - k_{-1}}{[L]}$$

From Fig. A2.5,

$k_{obs} = 3.0\ (\pm 0.5) \times 10^{-3}\ s^{-1}$

Also,

$k_{-1} = 1.4\ (\pm 0.3) \times 10^{-2}\ s^{-1}$ (see subsection A2.3.3)

$[L] = 0.2\ \mu M$

Thus,

$k_1 = 8.5\ (\pm 1.5) \times 10^4\ M^{-1}\ s^{-1}$

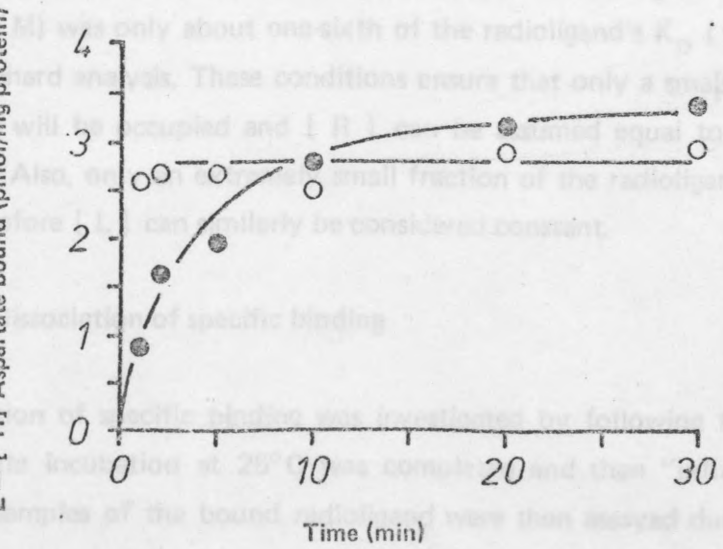


Fig. A2.4 Association of specific binding. The routine assay was followed except that the incubation time was varied over the range 1-30min. Results are means of duplicate determinations which differed by less than 10%. ● Specific; o nonspecific

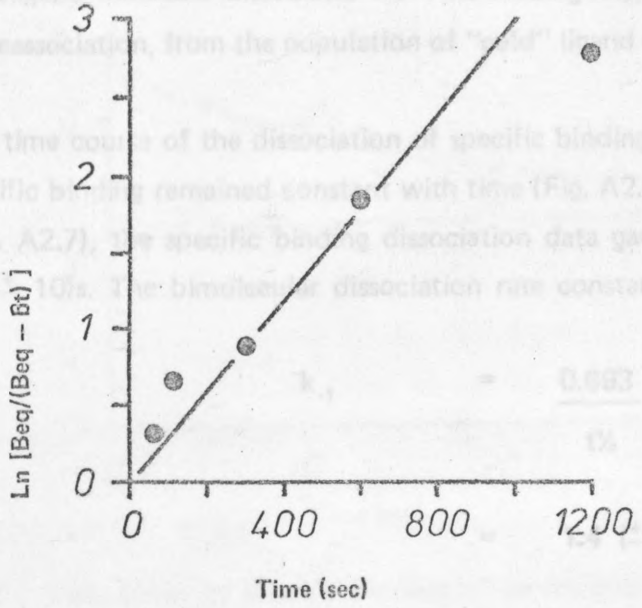


Fig. A2.5 Semilogarithmic plot of the association data from Fig. A2.4

The difference which exists between this value of k_1 and the approximate value derived earlier indicates that the rate of dissociation of the complex contributes significantly to the time taken to attain equilibrium.

Pseudo first-order conditions are, to some extent, justifiable since, in addition to analysing the binding data only through the first half-life, the initial free radioligand concentration (0.2 μ M) was only about one-sixth of the radioligand's K_D ($\sim$ 1.2 μM) as determined by Scatchard analysis. These conditions ensure that only a small fraction of the total binding sites will be occupied and [R] can be assumed equal to B_{max} (maximal binding) x [P]. Also, only an extremely small fraction of the radioligand is bound at equilibrium and therefore [L] can similarly be considered constant.

A2.3.3 Dissociation of specific binding

Dissociation of specific binding was investigated by following the routine assay until the 15 minute incubation at 25°C was completed and then "infinitely" diluting the radioligand. Samples of the bound radioligand were then assayed during a 0 - 30 seconds time interval.

"Infinite" dilution was achieved by the addition of 1mM (final concentration) unlabelled L-aspartate which represents a 5000-fold molar excess of "cold" ligand. Thus, as each radioligand molecule dissociates from its binding site, it will almost certainly be replaced, by reassociation, from the population of "cold" ligand molecules.

The time course of the dissociation of specific binding was relatively fast whereas the non-specific binding remained constant with time (Fig. A2.6). When plotted semilogarithmically (Fig. A2.7), the specific binding dissociation data gave a half-life, $t_{½}$, of the complex of 50 ($\pm$ 10)s. The bimolecular dissociation rate constant, k_{-1}, is given by (Snyder et al, 1978),

$$k_{-1} = \frac{0.693}{t_{½}}$$

$$= 1.4\ (\pm\ 0.3) \times 10^{-2}\ s^{-1}$$

The "time" axis of Fig. A2.6 relates to the interval between the addition of excess "cold" ligand and the initiation of filtration. The datum point at 0 seconds represents no excess of "cold" ligand added. The datum point at 1 second represents the time taken to add an excess of cold ligand and vortex-mix the sample.

The value of K_D calculated from the association and dissociation rate constants ($K_D = k_{-1} / k_1$) was 0.15 ($\pm$ 0.10) μ M which compares with the K_D of 1.2 ($\pm$ 0.4) μ M derived by Scatchard analysis of the saturation data.

The separation time using the filtration technique was 4 ($\pm$ 1) seconds which from the value of k_{-1} corresponds to a loss of specific binding during separation of 6 ($\pm$ 3)%. This loss falls within that value of 10% considered as the maximum acceptable loss (Snyder et al, 1978).

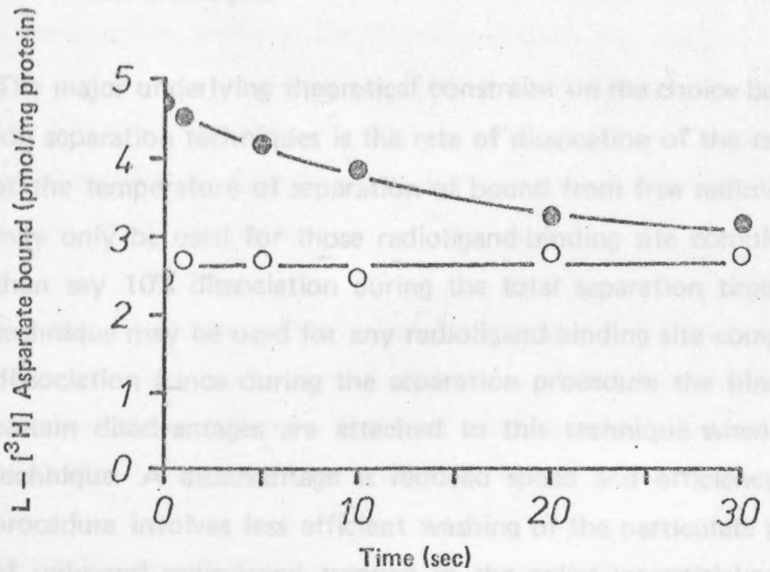


Fig A2.6 Dissociation of specific binding. The routine assay was followed except that after the 15 min incubation, 50 µl 20mM unlabelled L-aspartate, which represents about a 5000-fold molar excess, was added to the incubate. After time intervals over the range 0-30 s the dissociation was terminated according to the routine assay. The values at zero time represent no excess of unlabelled L-aspartate added. Results are means of duplicate determinations which varied by less than 10%.

● *Specific; o nonspecific*

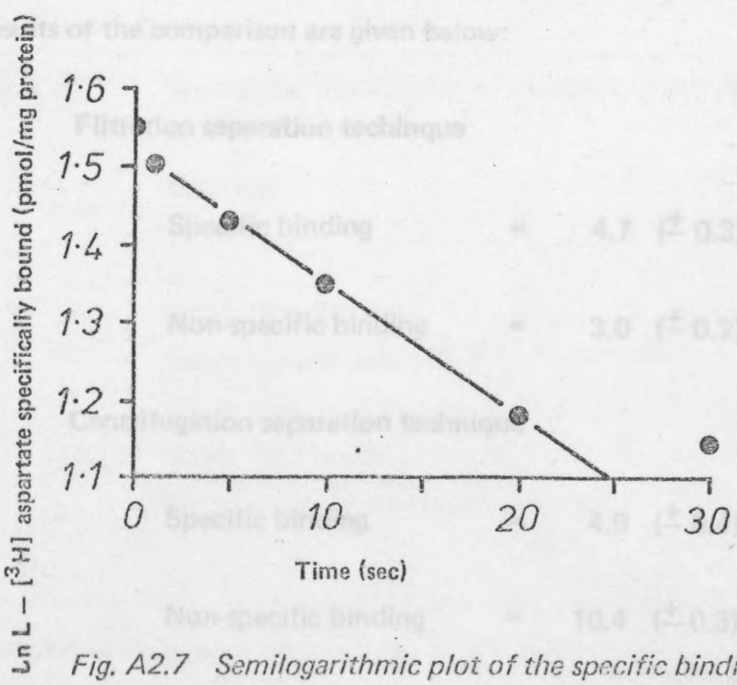


Fig. A2.7 Semilogarithmic plot of the specific binding data of Fig A2.6

A2.3.4 Justification of the use of the filtration separation technique against the centrifugation technique.

The major underlying theoretical constraint on the choice between filtration or centrifugation separation techniques is the rate of dissocation of the radioligand-binding site complex at the temperature of separation of bound from free radioligand. The filtration technique may only be used for those radioligand-binding site complexes which exhibit not greater than say 10% dissociation during the total separation time. Although the centrifugation technique may be used for any radioligand-binding site complex irrespective of the rate of dissociation (since during the separation procedure the binding is always in equilibrium), certain disadvantages are attached to this technique when compared with the filtration technique. A disadvantage is reduced speed and efficiency and also the centrifugation procedure involves less efficient washing of the particulate pellet resulting in higher levels of unbound radioligand trapped in the pellet interstitial space. This effect substantially increases the "nonspecific" binding levels as evidenced below.

A direct comparison was made between the radioligand binding as measured by the two separation techniques. For the centrifugation assay the routine assay was followed until the completion of the 15 minute incubation. Each sample was then centrifuged at 3800g for 10 minutes at 25° C. The supernatant was decanted and the pellets were rinsed superficially with 3ml, then 6ml of ice-cold buffer. The pellets were soluabilised overnight in 1ml of distilled water and after vortex-mixing were added to 1ml of Protosol (New England Nuclear Corp.) and vortex-mixed. Scintillation cocktail was added and after further vortex-mixing the samples were counted by scintillation spectrometry as for the routine binding assay. The results of the comparison are given below:

Filtration separation techinque

Specific binding	=	4.7 ($\pm$ 0.3) pmols/mg protein
Non-specific binding	=	3.0 ($\pm$ 0.2) pmols/mg protein

Centrifugation separation technique

Specific binding	=	4.9 ($\pm$ 0.4) pmols/mg protein
Non-specific binding	=	10.4 ($\pm$ 0.3) pmols/mg protein

The values for specific binding are very similar which may indicate, assuming that a negligible amount of radioligand was dissociated during the superficial rinsing of the pellet for the centrifugation technique, that the glassfibre filters used (Whatman GF/C) collected a

very large proportion of the bound radioligand. Also, the results may indicate that very little dissociation of bound radioligand occurred during the separation time for the filtration technique. The results of the dissociation study also support this contention.

The filtration technique was the preferred choice on two grounds: it was more rapid than the centrifugation technique and also the non-specific binding values were very much lower for the filtration technique.

Non-specific binding was also less for Whatman GF/C than GF/B filters, whilst the specific binding remained very similar. Therefore GF/C filters were used routinely and they also possessed the added advantage that the total separation time was less for GF/C than for GF/B (GF/B were thicker than GF/C).

A2.3.5 Specific binding to subcellular fractions of cerebellum

The various fractions produced during the synaptic membrane preparation procedure were routinely assayed to yield the results given in Table A2.1. If specific L-[^{3}H] aspartate binding represents an interaction with a synaptic receptor then the binding should be mainly associated with the synaptic membrane fraction. In fact the specific binding was maximal in the crude synaptic membrane pellet (P_4) and represented on approximately 5-fold enrichment of specific binding compared to the whole homogenate. Of course the protein concentration of each fraction was determined in order to perform a valid comparison of the amount of specific binding associated with each fraction.

Table A2.1 Subcellular distribution of specific L-[^{3}H] aspartate binding in cerebellum

Fraction	Specific binding (± S.E.M.) (pmol/mg protein)
Whole homogenate	1.1 (± 0.4)
Crude nuclear pellet (P_1)	0.4 (± 0.3)
Crude mitochondrial pellet (P_2)	1.5 (± 0.4)
Crude microsomal pellet (P_3)	4.1 (± 0.2)
Crude synaptic membrane pellet (P_4)	5.2 (± 0.3)

The routine assay, as described in the text, was followed except that suspensions of the various fractions were used instead of the synaptic membrane fraction suspension. Results are means (± S.E.M.) of triplicate determinations.

A2.3.6 Protein concentration-dependence of specific binding

Various dilutions (to X 5) of a synaptic membrane suspension were prepared and routinely assayed. Specific binding increased linearly with protein concentration up to 90 μg protein per 0.9ml aliquot. (Fig. A2.8). The nonspecific binding remained almost constant from zero protein concentration indicating that the nonspecific binding was almost totally attributable to adsorption of the radioligand by the glass-fibre filters.

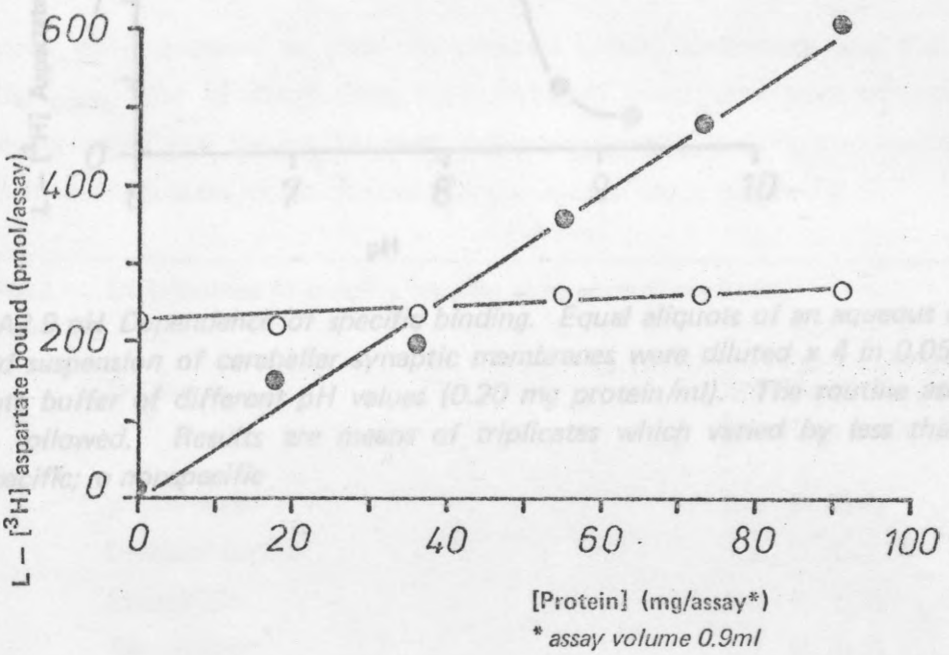


Fig. A2.8 Protein concentration dependence of specific binding. The routine assay, as described in the text, was followed employing various dilutions of a cerebellar synaptic membrane suspension. The method of Lowry et al. (1951) was used to determine protein concentration. Results are means of triplicates which differed by less than 12%. ● Specific; o nonspecific

A2.3.7 pH Dependence of specific binding

In order to obtain samples of varying pH and constant protein concentration, the final P_4 pellet of a synaptic membrane preparation was suspended initially in a small volume ($\sim$ ¼ final volume) of ice-cold distilled water. Aliquots of this suspension were diluted x4 with 0.05M Tris-acetate buffers of various pH values. The pH value of each suspension was directly measured and the routine assay was then followed. Although the buffer system used, 0.05M Tris-acetate, buffers most effectively within the range pH 7.2 - 9.2, a buffer of pH 6.35, outside this range, was used. Some degree of confidence was attached to this latter pH value since it was measured directly.

A pH optimum for specific binding occurred at about pH 7 (Fig. A2.9).

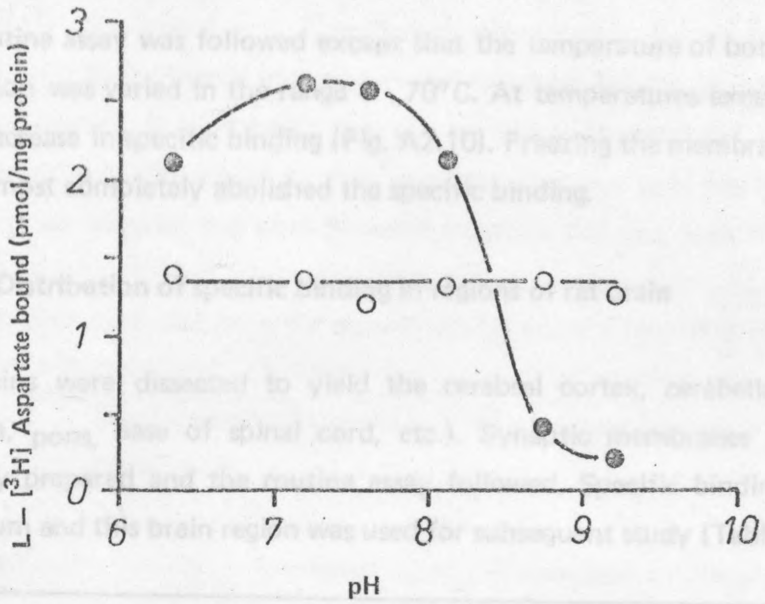


Fig. A2.9 pH Dependence of specific binding. Equal aliquots of an aqueous concentrated suspension of cerebellar synaptic membranes were diluted x 4 in 0.05M Tris-acetate buffer of different pH values (0.20 mg protein/ml). The routine assay was then followed. Results are means of triplicates which varied by less than 10%. ● Specific; o nonspecific

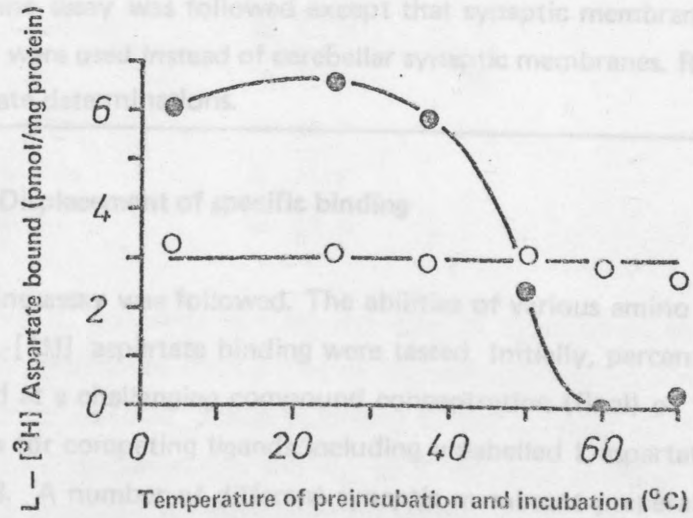


Fig. A2.10 Temperature dependence of specific binding. The routine assay was followed except that the temperature of the preincubation and incubation was varied over the range 4-70° C. Results are means of triplicates which differed by less than 10%. ● Specific; o nonspecific

A2.3.8 Temperature dependence of specific binding

The routine assay was followed except that the temperature of both the preincubation and incubation was varied in the range 4 - 70° C. At temperatures exceeding 37° C there was a steep decrease in specific binding (Fig. A2.10). Freezing the membranes prior to the binding assay almost completely abolished the specific binding.

A2.3.9 Distribution of specific binding in regions of rat brain

Rat brains were dissected to yield the cerebral cortex, cerebellum and the remainder (medulla, pons, base of spinal cord, etc.). Synaptic membranes from each region were routinely prepared and the routine assay followed. Specific binding was maximal for the cerebellum and this brain region was used for subsequent study (Table A2.2).

Table A2.2 Distribution of specific binding in regions of rat brain

Region	Specific binding (± S.E.M.) (pmol/ng protein)
Whole brain	2.3 (± 0.4)
Cerebral cortex	1.3 (± 0.2)
Cerebellum	4.9 (± 0.3)
Remainder	2.7 (± 0.1)

The routine assay was followed except that synaptic membranes of the various regions of the brain were used instead of cerebellar synaptic membranes. Results are means (± S.E.M.) of triplicate determinations.

A2.3.10 Displacement of specific binding

The routine assay was followed. The abilities of various amino acids and drugs to displace specific L-[^{3}H] aspartate binding were tested. Initially, percentage inhibition studies were performed at a challenging compound concentration (final) of 1mM (Table A2.3). Inhibition curves for competing ligands including unlabelled L-aspartate are shown in Figs. A2.11, 12 and 13. A number of different synaptic membrane preparations were used to plot the inhibition curves as evidenced by the different values of specific binding at zero concentration of displacing ligand. (Each preparation produced a slightly different specific binding capacity/mg protein). This did not influence the values of percentage inhibition or IC_{50} as illustrated by the comparison of the inhibition curves for unlabelled L-aspartate in Figs. A2.11 and 13.

The potency of kainate observed here cannot be reproduced by other workers (Roberts, 1980, private communication) and indeed this result has not been able to be repeated subsequently in our own hands. Therefore it appears that the result for kainate is probably spurious and has not been included in the data base for the aspartate SAR study (Section 5.5). Additionally it is probable that our samples of N-acetyl-D,L-aspartate and N-acetyl-L-aspartate were contaminated by free aspartate since not only has this been found to be the case in other samples but pure N-acetyl-aspartate has not been found to be active in the hands of other workers (Roberts, 1980, private communication). Similarly, these results have also been excluded from the aspartate SAR study. Compounds with % inhibitions $>$ 50% below were recrystallised -- but some time elapsed before their use.

Table A2.3 Displacement of specific L-[^{3}H] aspartate binding in rat cerebellar synaptic membranes

Compound	% Inhibition ($\pm$ S.E.M.) at 10^{-3}M	IC_{50} ($\pm$ S.E.M.) (M)	K_i ($\pm$ S.E.M.) (M)
L-Aspartate	100 ($\pm$ 7)	2.5 ($\pm$ 0.5) x 10^{-6}	2.1 ($\pm$ 0.6) x 10^{-6}
Kainate	102 ($\pm$ 6)	2.5 ($\pm$ 0.5) x "	2.1 ($\pm$ 0.6) "
L-Cysteine Sulfinate	95 ($\pm$ 7)	1.0 ($\pm$ 0.3) x 10^{-5}	0.9 ($\pm$ 0.3) x 10^{-5}
L-Glutamate	89 ($\pm$ 6)	5 ($\pm$ 2) "	4.5 ($\pm$ 2.0) "
N-Acetyl-L-aspartate	79 ($\pm$ 8)	2.0 ($\pm$ 0.5) x 10^{-4}	1.7 ($\pm$ 0.4) x 10^{-4}
N-Acetyl-D, L-aspartate	75 ($\pm$11)	5 ($\pm$ 1) "	4.5 ($\pm$ 1.0) "
L-Cysteate	70 ($\pm$10)	5 ($\pm$ 1) "	4.5 ($\pm$ 1.0) "
D, L-Cysteate	68 ($\pm$ 8)	5 ($\pm$ 2) "	4.5 ($\pm$ 1.5) "
($\pm$) 4-Fluoroglutamate	49 ($\pm$ 6)	10^{-3}	0.9 x 10^{-3}
N-Methyl-D, L-glutamate	39 ($\pm$ 7)	"	"
Ibotenate	38 ($\pm$ 9)	"	"
D, L-α-aminoadipate	25 ($\pm$ 7)	"	"
N-Methyl-D-aspartate	24 ($\pm$10)	"	"
D, L-Pyroglutamate	23 ($\pm$ 7)	"	"

Specific L-[^{3}H] aspartate binding was assayed as described in the text. The IC_{50} values were visually estimated from a plot of log competing ligand concentration vs. specific binding and converted to K_i values according to the equation $K_i = IC_{50}/(1 + L/K_D)$ where L is the radioligand concentration and K_D its dissociation constant (see A1.3.2). The following compounds exhibited no significant displacing activity (% inhibition at 10^{-3} M $\leqslant$ 12%): D-aspartate; D-glutamate; D,L-homocysteate; L-glutamic acid diethyl ester HCl ; quisqualate; L-methionine -- D,L-sulphoximine; D,L-α,ε-diaminopimelate; HA-966; dopamine HCl ; noradrenaline HCl ; taurine; glycine; acetylcholine Cl ; β-alanine; GABA; L-proline, leptazol; naloxone and formamidine sulfinate. Values are the means ($\pm$ S.E.M.) of triplicate determinations.

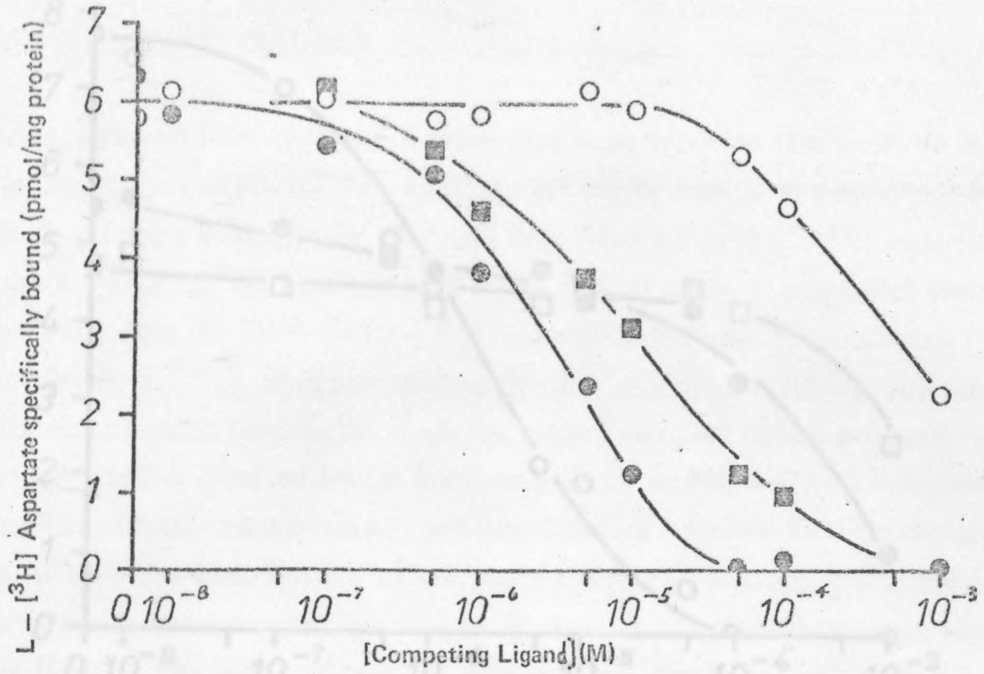


Fig. A2.11 Inhibition curves for various compounds competing with specific L – [^{3}H] aspartate binding. The routine assay was followed employing challenging compound concentrations over the range 10^{-8} – 10^{-3} M. Results are means of triplicate determinations which differed by less than 10%. ● L-Aspartate; o L-cysteate; ■ L-cysteine sulfinate.

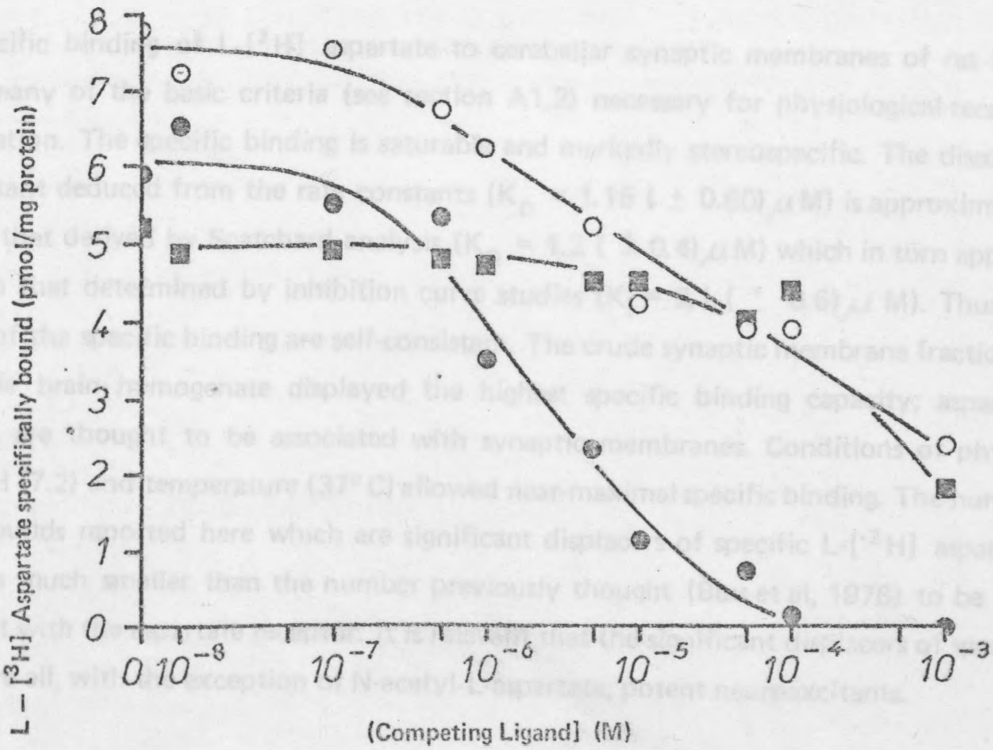


Fig. A2.12 Further inhibition curves. ● Kainate; o L-glutamate; ■ N-acetyl-D,L-aspartate.

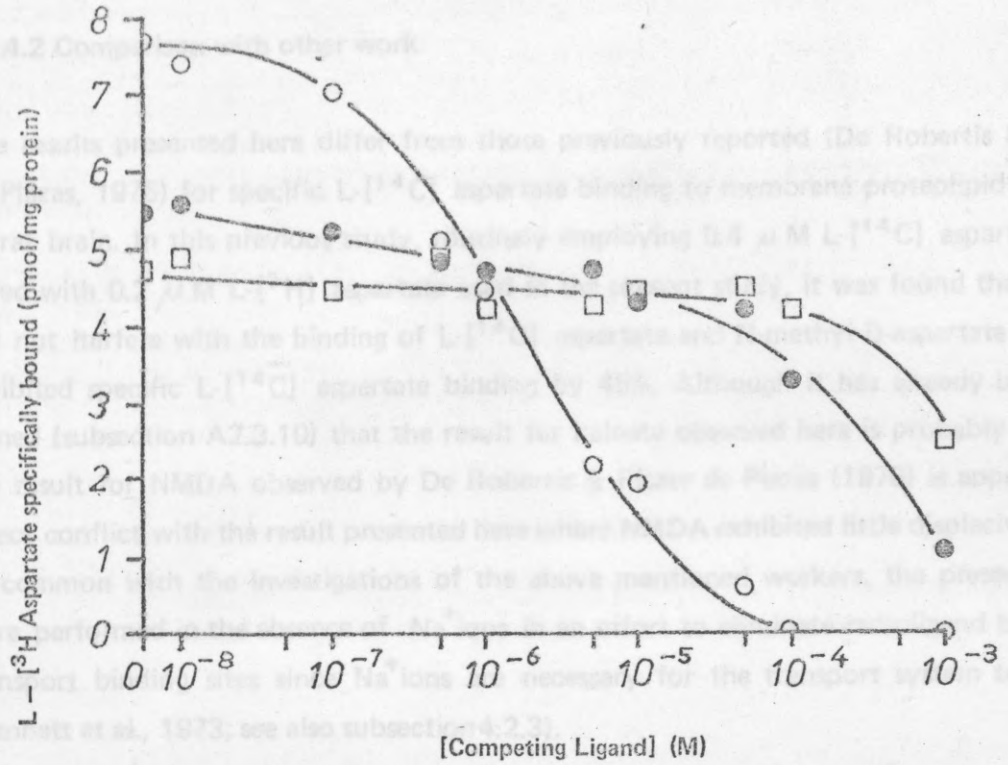


Fig A2.13 Further inhibition curves. ● N-Acetyl-L-aspartate; o L-aspartate; □ D,L-cysteate.

A2.4 Discussion of results

A2.4.1 Characteristics of binding

The specific binding of L-[^{3}H] aspartate to cerebellar synaptic membranes of rat brain fulfills many of the basic criteria (see section A1.2) necessary for physiological-receptor identification. The specific binding is saturable and markedly stereospecific. The dissociation constant deduced from the rate constants (K_D = 1.15 (± 0.60)μM) is approximately equal to that derived by Scatchard analysis (K_D = 1.2 (± 0.4)μM) which in turn approximates to that determined by inhibition curve studies (K_i = 2.1 (± 0.6)μM). Thus the kinetics of the specific binding are self-consistent. The crude synaptic membrane fraction of the whole brain homogenate displayed the highest specific binding capacity; aspartate receptors are thought to be associated with synaptic membranes. Conditions of physiological pH (7.2) and temperature (37° C) allowed near-maximal specific binding. The number of compounds reported here which are significant displacers of specific L-[^{3}H] aspartate binding is much smaller than the number previously thought (Buu et al, 1976) to be able to interact with the aspartate receptor. It is relevant that the significant displacers of specific binding are all, with the exception of N-acetyl-L-aspartate, potent neuroexcitants.

A2.4.2 Comparison with other work

The results presented here differ from those previously reported (De Robertis & Fiszer de Plazas, 1976) for specific L-[^{14}C] aspartate binding to membrane proteolipid fractions of rat brain. In this previous study, routinely employing 0.4 μM L-[^{14}C] aspartate compared with 0.2 μM L-[^{3}H] aspartate used in the present study, it was found that kainate did not iterfere with the binding of L-[^{14}C] aspartate and N-methyl-D-aspartate (NMDA) inhibited specific L-[^{14}C] aspartate binding by 45%. Although it has already been mentioned (subsection A2.3.10) that the result for kainate observed here is probably spurious, the result for NMDA observed by De Robertis & Fiszer de Plazas (1976) is apparently in direct conflict with the result presented here where NMDA exhibited little displacing activity. In common with the investigations of the above mentioned workers, the present studies were performed in the absence of Na^+ ions in an effort to eliminate radioligand binding to transport binding sites since Na^+ ions are necessary for the transport system to operate (Bennett et al., 1973; see also subsection 4.2.3).

A2.5 Computer programme, SATR

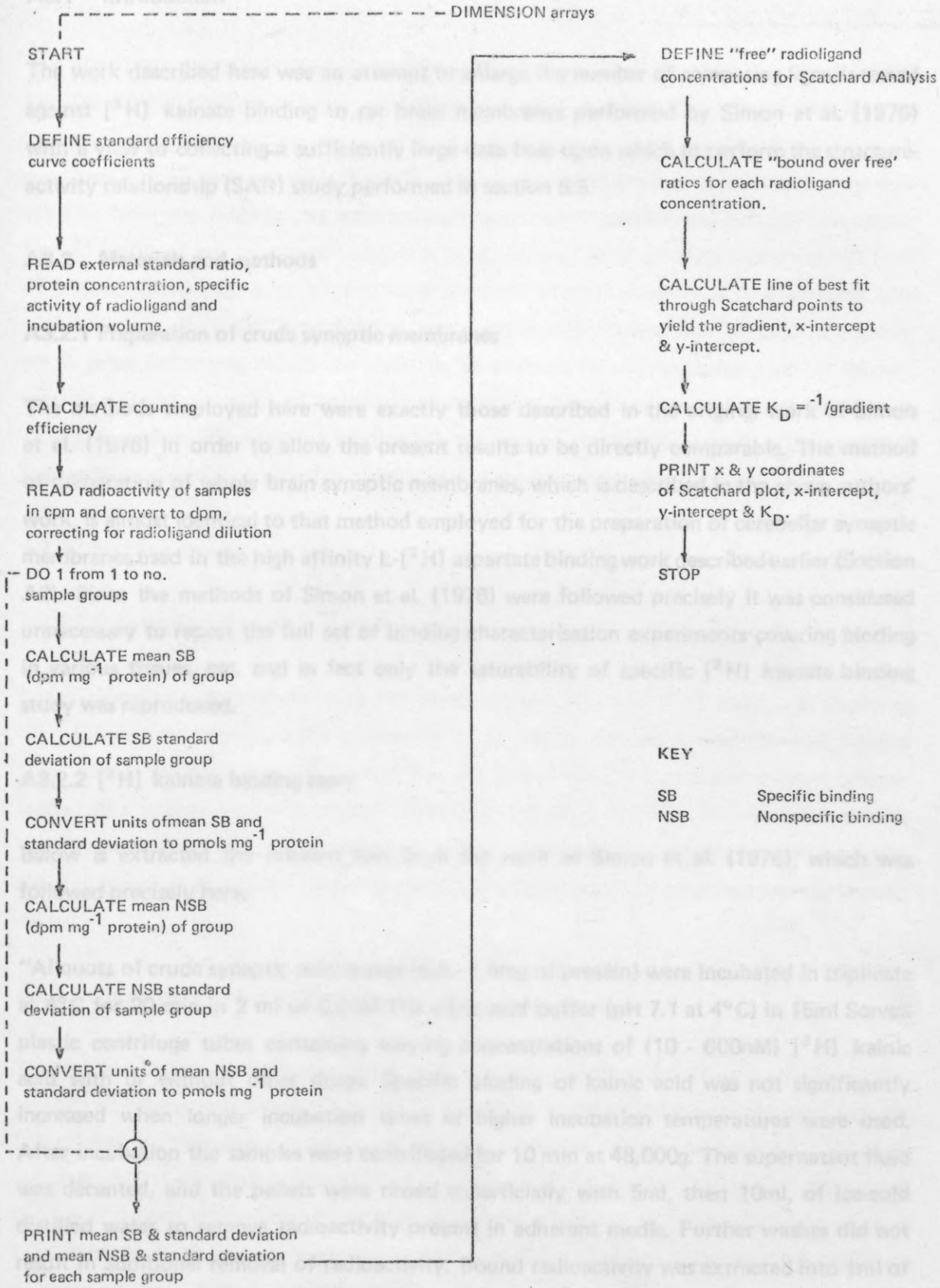
The following programme was written to analyse the scintillation counter data from the saturability study. The programme is discussed in A2.2.3.

Programme SATR Listing

```
      MASTER SATR
      DIMENSION CDPM(38),SDSBDPM(9),SDSBPMOL(9),SDNSBDPM(9),
     XSB(4),APMOLAR(9),X(9),Y(9),BOF(9),SDNSBPMO(9)
      REAL MSBDPM(9),MSBPMOLS(9),MNSBDPM(9),MNSBPMOL(9),NSB(4)
     X,INCVOL,KD
      A=0.02116
      B=0.28315
      C=1.34134
      D=-1.28821
      READ(5,3)ESR,PROTCONC,SA,INCVOL
    3 FORMAT(4F0.0)
      EFF=A+B*ESR+C*ESR*ESR+D*ESR*ESR*ESR
      DO 2 L=1,38
      READ(5,1)CPM
    1 FORMAT(F0.0)
      DPM=CPM/EFF
      CDPM(L)=DPM*22500.0/8300.0
    2 CONTINUE
      IJ=2
      DO 4 M=1,9
      SB(1)=CDPM(IJ)-CDPM(IJ+1)
      SB(2)=CDPM(IJ)-CDPM(IJ+3)
      SB(3)=CDPM(IJ+2)-CDPM(IJ+1)
      SB(4)=CDPM(IJ+2)-CDPM(IJ+3)
      MSBDPM(M)=(SB(1)+SB(2)+SB(3)+SB(4))/(4.0*PROTCONC*INCVOL)
      SUM=0
      DO 5 I=1,4
      SUM=SUM+(SB(I)/(PROTCONC*INCVOL)-MSBDPM(M))**2
    5 CONTINUE
      SDSBDPM(M)=SQRT(SUM/4)
```

```
      MSBPMOLS(M)=(MSBDPM(M)/(2.22*1000))/SA
      SDSBPMOL(M)=(SDSBDPM(M)/(2.22*1000))/SA
      NSB(1)=CDPM(IJ+1)-CDPM(1)
      NSB(2)=CDPM(IJ+1)-CDPM(38)
      NSB(3)=CDPM(IJ+3)-CDPM(1)
      NSB(4)=CDPM(IJ+3)-CDPM(38)
      MNSBDPM(M)=(NSB(1)+NSB(1)+NSB(3)+NSB(4))/(4*PROTCONC*INCVOL)
      SUMM=0
      DO 8 I=1,4
      SUMM=SUMM+(NSB(I)/(PROTCONC*INCVOL)-MNSBDPM(M))**2
    8 CONTINUE
      SDNSBDPM(M)=SQRT(SUMM/4)
      MNSBPMOL(M)=(MNSBDPM(M)/(2.22*1000))/SA
      SDNSBPMO(M)=(SDNSBDPM(M)/(2.22*1000))/SA
      IJ=IJ+4
    4 CONTINUE
      WRITE(6,9)
    9 FORMAT(1X,'SATURATION CURVE RESULTS')
      DO 6 N=1,9
      WRITE(6,7)MSBDPM(N),SDSBDPM(N),MSBPMOLS(N),SDSBPMOL(N)
    7 FORMAT(1X,'SBDPM=',F9.0,' SD=',F9.0,' SBPMOLS=',F6.3,' SD=',
     XF6.3)
    6 CONTINUE
      DO 17 N=1,9
      WRITE(6,11)MNSBDPM(N),SDNSBDPM(N),MNSBPMOL(N),SDNSBPMO(N)
   11 FORMAT(1X,'NSBDPM=',F9.0,' SD=',F9.0,' NSBPMOLS=',F6.3,' SD='
     X,F6.3)
   17 CONTINUE
      APMOLAR(1)=1.0E4
      APMOLAR(2)=2.0E4
      APMOLAR(3)=5.0E4
      APMOLAR(4)=1.0E5
      APMOLAR(5)=2.5E5
      APMOLAR(6)=5.0E5
      APMOLAR(7)=1.0E6
      APMOLAR(8)=1.5E6
      APMOLAR(9)=2.0E6
      DO 10 J=1,9
      BOF(J)=MSBPMOLS(J)/APMOLAR(J)*1.0E6
      X(J)=MSBPMOLS(J)
      Y(J)=BOF(J)
   10 CONTINUE
      SXY,SX,SY,SXS=0
      N=9
      DO 12 J=1,N
      SXY=SXY+X(J)*Y(J)
      SX=SX+X(J)
      SY=SY+Y(J)
      SXS=SXS+X(J)*X(J)
   12 CONTINUE
      GRAD=(N*SXY-SX*SY)/(N*SXS-SX*SX)
      YINT=(SY*SXS-SX*SXY)/(N*SXS-SX*SX)
      XINT=-YINT/GRAD
      KD=-1.0/GRAD
      WRITE(6,13)
   13 FORMAT(1X,'SCATCHARD PLOT X&Y ')
      DO 15 I=1,9
      WRITE(6,14)X(I),Y(I)
   14 FORMAT(1X,F8.3,F10.3)
   15 CONTINUE
      WRITE(6,16)XINT,YINT,KD
   16 FORMAT(1X,'XINT=',F8.3,' YINT=',F10.3,' KD=',F10.3)
      STOP
      END
      FINISH
```

Programme SATR flowchart



A3. [^{3}H] Kainate binding to rat brain synaptic membranes

A3.1 Introduction

The work described here was an attempt to enlarge the number of competing ligands tested against [^{3}H] kainate binding to rat brain membranes performed by Simon et al. (1976) with a view to collecting a sufficiently large data base upon which to perform the structure-activity relationship (SAR) study performed in section 5.5.

A3.2 Materials and methods

A3.2.1 Preparation of crude synaptic membranes

The methods employed here were exactly those described in the original work of Simon et al. (1976) in order to allow the present results to be directly comparable. The method of preparation of whole brain synaptic membranes, which is described in the above authors' work, is almost identical to that method employed for the preparation of cerebellar synaptic membranes used in the high affinity L-[^{3}H] aspartate binding work described earlier (Section A2). Since the methods of Simon et al. (1976) were followed precisely it was considered unnecessary to repeat the full set of binding characterisation experiments covering binding in various tissues, etc. and in fact only the saturability of specific [^{3}H] kainate binding study was reproduced.

A3.2.2 [^{3}H] kainate binding assay

Below is extracted the relevant text from the work of Simon et al. (1976), which was followed precisely here.

"Aliquots of crude synaptic membranes (0.5 - 1.5mg of protein) were incubated in triplicate at 4°C for 20 min in 2 ml of 0.05M-Tris citric acid buffer (pH 7.1 at 4°C) in 15ml Sorvall plastic centrifuge tubes containing varying concentrations of (10 - 600nM) [^{3}H] kainic acid with or without other drugs. Specific binding of kainic acid was not significantly increased when longer incubation times or higher incubation temperatures were used. After incubation the samples were centrifuged for 10 min at 48,000g. The supernatant fluid was decanted, and the pellets were rinsed superficially with 5ml, then 10ml, of ice-cold distilled water to remove radioactivity present in adherent media. Further washes did not result in additional removal of radioactivity. Bound radioactivity was extracted into 1ml of Protosol (New England Nuclear Corp.); 10ml of toluene phosphor was added and the radioactivity was assayed by liquid scintillation spectrometry in a Packard Tricarb Model 3375

Counting efficiency was 32%". (A Packard Tricarb Model 3255 was used in the work described here. Counting efficiency was 34%) . Under these conditions, binding activity was linear with tissue concentration used (0.5 - 2.0mg protein).

"Routine assays were performed in triplicate utilizing 40nM [^{3}H] kainic acid with parallel samples containing 0.1mM kainic acid to obtain "blank" values. The thawed, resuspended synaptic membrane fractions from whole rat brain were used. About 1.0mg of protein was included in each sample. 'Specific [^{3}H] kainic acid binding' was obtained by subtracting from the total bound radioactivity the amount not displaced by high concentrations (0.1mM) of kainic acid or L-glutamic acid. At the higher concentrations used (0.1mM or greater), blank values for binding were the same whether kainic acid or L-glutamic acid was used as a displacer. Total binding was approximately twice that of specific binding. When tubes containing membrane fractions were placed in a boiling water bath for 10 min, binding activity was reduced to that of blank values. There was no measurable metabolism of kainic acid in the pellet or medium at the end of the binding assays as the extracted radioactivity chromatographed as a single peak coicident with authentic kainic acid".

A3.3 Binding results

A3.3.1 Saturability of specific [^{3}H] kainate binding

Specific binding was saturable with increasing concentrations of [^{3}H] kainate in the range 10 - 250nM, in contrast to the nonspecific [^{3}H] kainate binding which increased linearly (Fig. A3.1). Scatchard analysis yielded Fig. A3.2 in which the datum points were approximated to a straight line with a dissociation constant (K_D) of 90 ($\pm$ 20)nM and a specific binding capacity of 0.9 ($\pm$ 0.1) pmol/mg protein. These results are broadly consistent with those of Simon et al. (1976) where the values were 59nM and 1.03 pmol/mg protein respectively.

A3.3.3 Displacement of specific binding

The routine assay was followed. Since the main objective of this work was to expand the number of compounds tested by Simon et al. (1976) for their abilities to displace specific [^{3}H] kainate binding, four additional compounds were introduced: HA-966, α-methyl-D, L-glutamate, N-methyl-D, L-aspartate and N-methyl-D-aspartate. Of course the final number of compounds tested here covered a number of those which Simon et al (1976) found to be of high displacing potency, in order to compare the displacing abilities of these common ligands between the two sets of experiments. It will be seen that these "common" results are nearly identical between the work of Simon et al (1976) and the work presented here. Table A3.1 includes those compounds tested. Corresponding inhibition curves for competing ligands including unlabelled kainate are shown in Figs. A3.3 and 4.

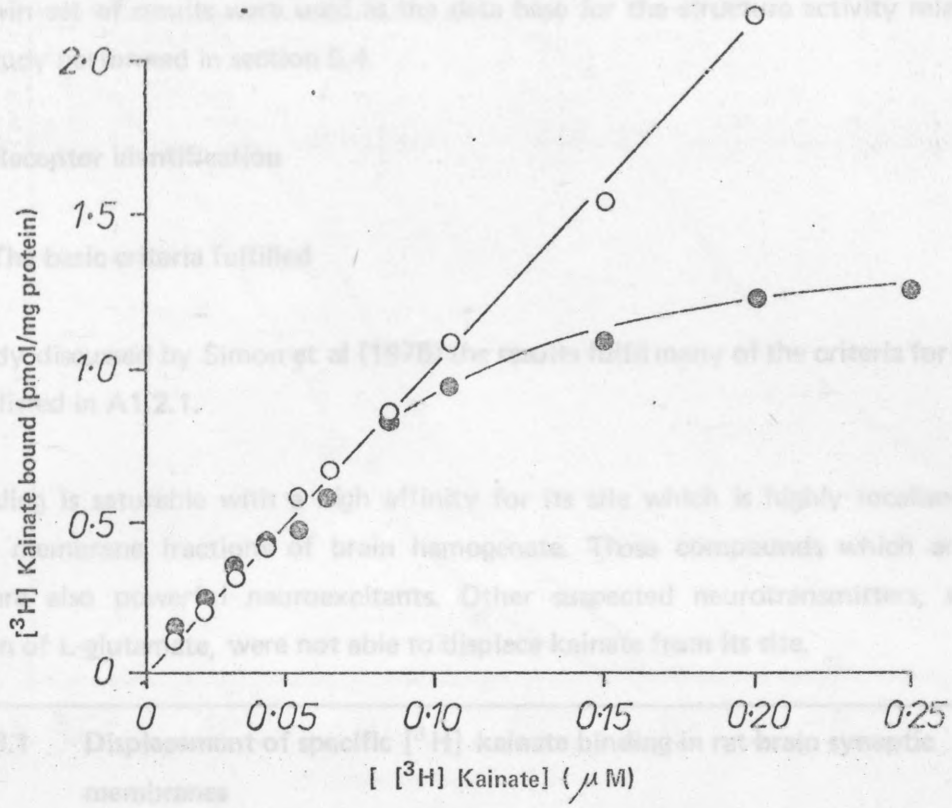


Fig A3.1 *Saturation of specific [³H] kainate binding*
● Specific; o nonspecific

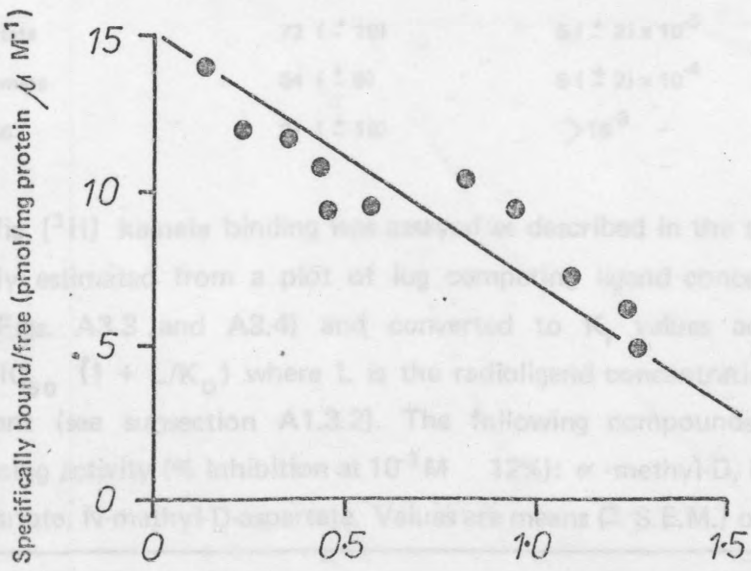


Fig A3.2 *Scatchard analysis of the specific binding data of Fig A3.1*

These twin set of results were used as the data base for the structure activity relationship (SAR) study performed in section 5.4.

A3.4 Receptor identification

A3.4.1 The basic criteria fulfilled

As already discussed by Simon et al (1976) the results fulfil many of the criteria for receptor identity listed in A1.2.1.

The binding is saturable with a high affinity for its site which is highly localised to the synaptic membrane fractions of brain hamogenate. Those compounds which are avidly bound are also powerful neuroexcitants. Other suspected neurotransmitters, with the exception of L-glutamate, were not able to displace kainate from its site.

Table A3.1 Displacement of specific [^{3}H] kainate binding in rat brain synaptic membranes

Compound	% inhibition (± S.E.M.) at 10^{-3}M	IC_{50} (± S.E.M.) (M)	K_i (± S.E.M.) (M)
Kainate	101 (± 7)	$2 (\pm 1) \times 10^{-8}$	$1.2 (\pm 0.6) \times 10^{-8}$
Quisqualate	98 (± 6)	$1 (\pm 0.5) \times 10^{-7}$	$0.6 (\pm 0.3) \times 10^{-7}$
Ibotenate	99 (± 7)	1 (± 0.5) "	0.6 (± 0.3) "
L-Glutamate	95 (± 8)	$1 (\pm 0.5) \times 10^{-6}$	$0.6 (\pm 0.3) \times 10^{-6}$
L-Aspartate	72 (± 10)	$5 (\pm 2) \times 10^{-5}$	$3 (\pm 1) \times 10^{-5}$
D-Glutamate	54 (± 9)	$5 (\pm 2) \times 10^{-4}$	$3 (\pm 1) \times 10^{-4}$
HA - 966	21 (± 10)	$> 10^{-3}$	$> 0.6 \times 10^{-3}$

Specific [^{3}H] kainate binding was assayed as described in the text. The IC_{50} values were visually estimated from a plot of log competing ligand concentration vs. specific binding (Figs. A3.3 and A3.4) and converted to K_i values according to the equation $K_i = IC_{50} (1 + L/K_D)$ where L is the radioligand concentration and K_D its dissociation constant (see subsection A1.3.2). The following compounds exhibited no significant displacing activity (% inhibition at 10^{-3}M 12%): α -methyl-D, L-glutammate; N-methyl-D, L-aspartate; N-methyl-D-aspartate. Values are means (± S.E.M.) of triplicate determinations.

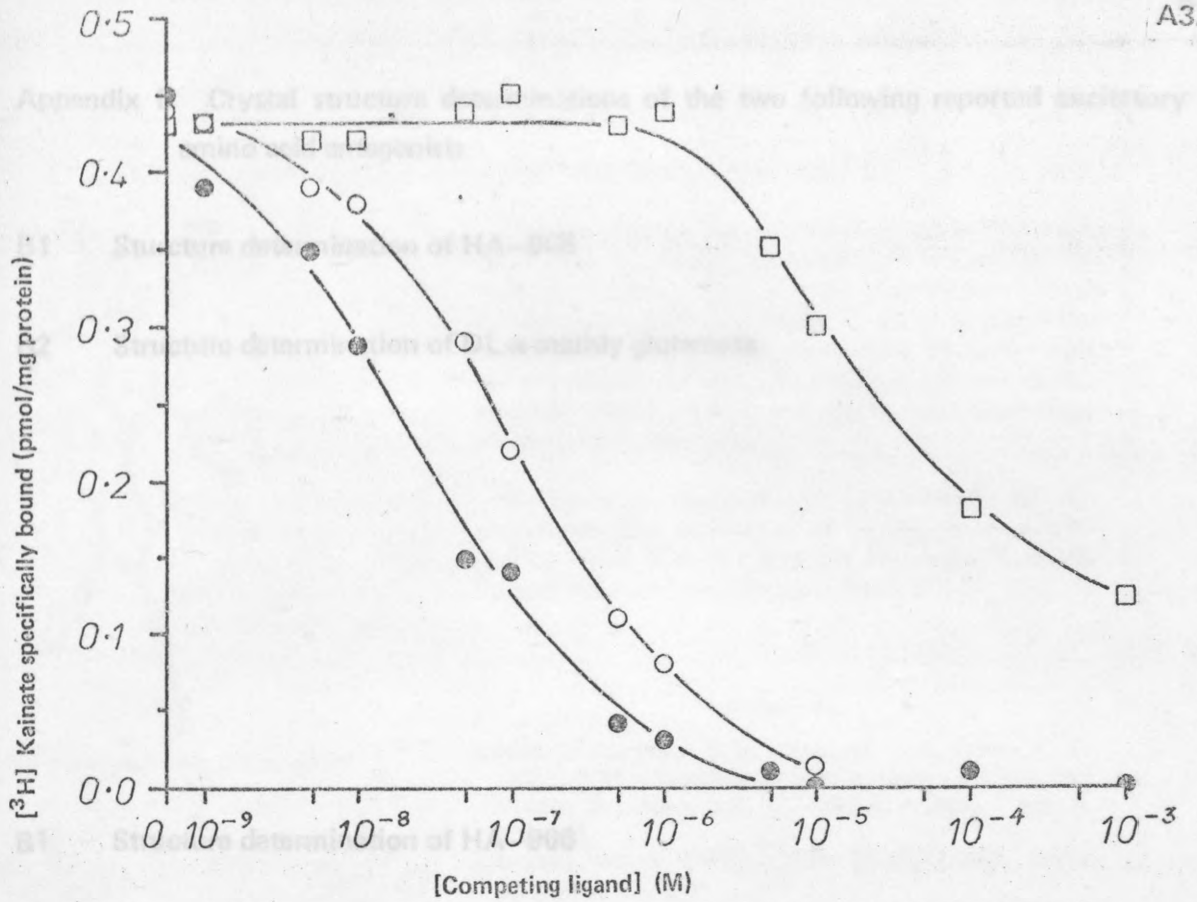


Fig A3.3 *Inhibition curves for various compounds competing with specific [^{3}H] kainate binding. The routine assay was followed employing challenging compound concentrations over the range 10^{-9} – 10^{-3} M. Results are means of triplicate determinations. ● Kainate; o quisqualate; □ L-aspartate.*

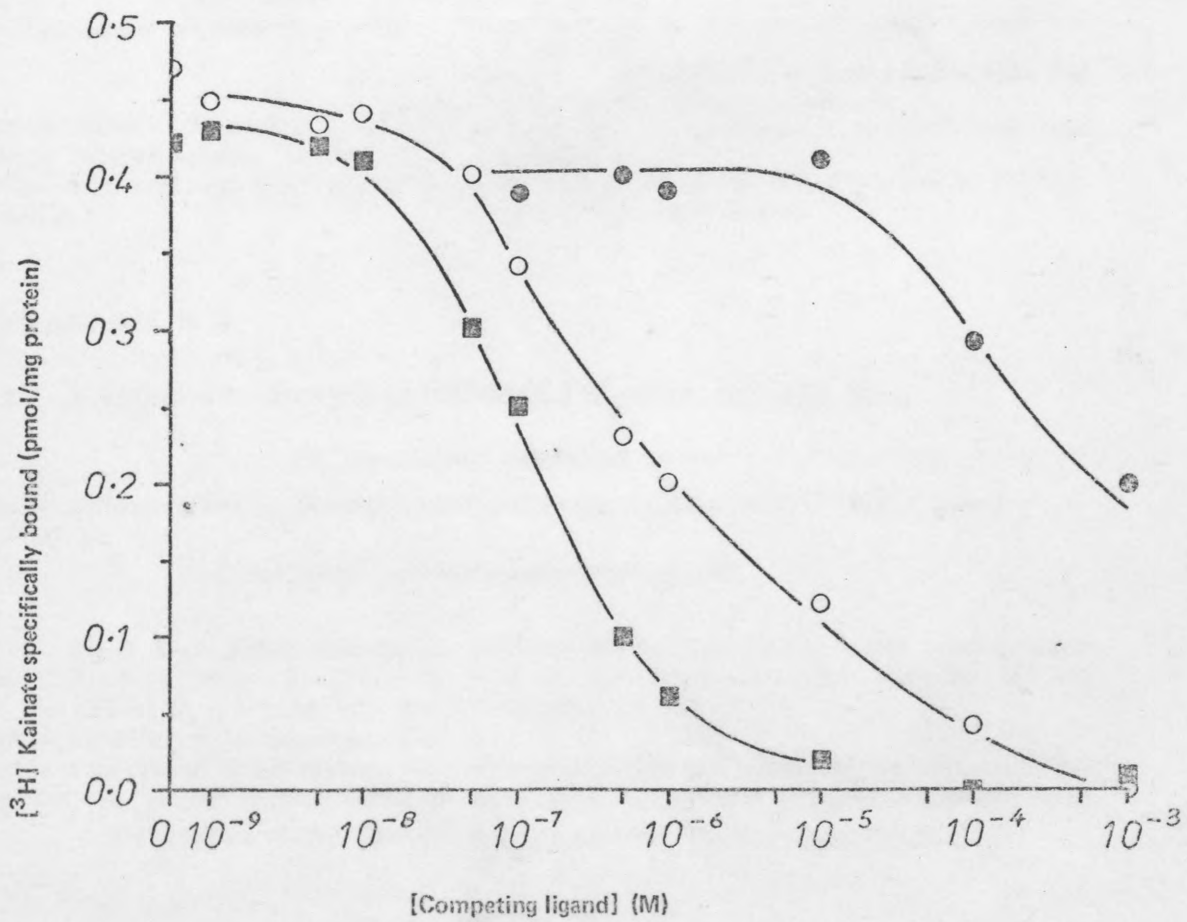


Fig A3.4 *Further inhibition curves. ■ Ibotenate; o L-glutamate; ● D-glutamate*

Appendix B Crystal structure determinations of the two following reported excitatory amino acid antagonists

B1 Sturcture determination of HA–966

B2 Structure determination of DL-α-methly glutamate

B1 Structure determination of HA–966

B1.1 The published structure

The following reprint was taken from Acta Crystallographica (1980) B36, 1969-1972.

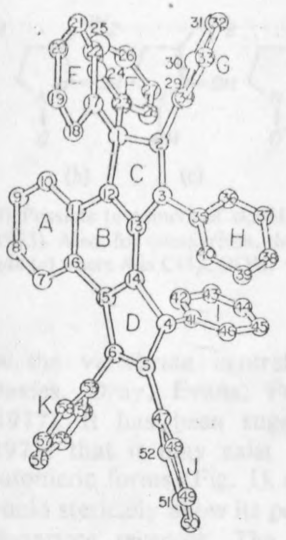


Fig. 1. The atomic numbering scheme used.

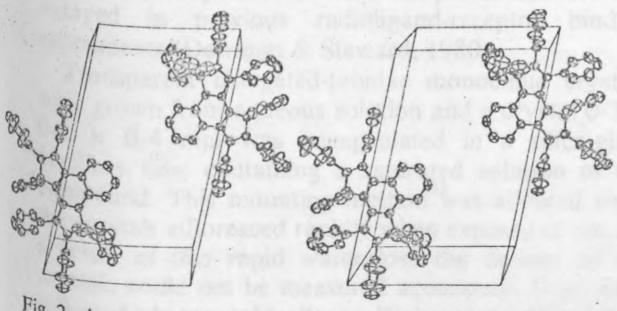


Fig. 2. A stereoscopic diagram of the contents of one unit cell.

and *K*, which are connected to the nucleus by sp^2–sp^2 bonds, make large dihedral angles, in the range 59–86°, with the five-membered rings to which they are bonded (Figs. 1 and 2).

In the naphthalene system the distances in ring *A* are very close to those measured and calculated by molecular-orbital methods in naphthalene itself (Cruickshank & Sparks, 1960). However, in ring *B* the bond character appears to be modified by fusion with the five-membered rings. Throughout the rest of the molecule bond distances and angles, other than those noted above, appear normal.

We thank M. Anschel and P. D. Readio for the preparation and purification of $C_{58}H_{40}$, supported in part by Grant 2563-A1 from the Petroleum Research Fund of the American Chemical Society.

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Acta Cryst. (1980). B**36**, 1969–1972

D,L-3-Amino-1-hydroxy-2-pyrrolidone Trihydrate (D,L-HA-966)

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Abstract. $C_4H_8N_2O_2.3H_2O$, $M_r = 170{\cdot}2$, monoclinic, $P2_1/c$, $a = 11{\cdot}86$ (8), $b = 6{\cdot}84$ (14), $c = 11{\cdot}73$ (14) Å, $\beta = 114{\cdot}94$ (2)°, $U = 863$ Å^3, $D_x = 1{\cdot}31$ Mg m^{-3}, $Z = 4$; final $R = 0{\cdot}056$ for 977 unique reflections. The compound exists as a zwitterion in the crystal. The pyrrolidine ring has an almost perfect envelope conformation and very little hydrogen bonding occurs between the water molecules and the HA-966 molecules.

Introduction. The title compound has been reported as a selective antagonist at the physiological aspartate

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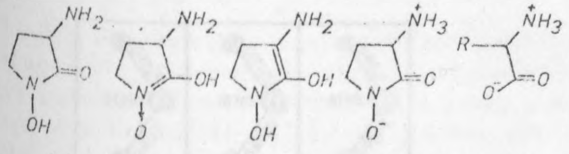

(*a*) (*b*) (*c*) (*d*) (*e*)

Fig. 1. (*a*)–(*d*) Possible tautomers of D,L-HA-966 after Davies & Watkins (1973). Also, for comparison, the α-amino acid moiety of L-aspartate (*e*) where *R* is CH_2COOH.

receptor in the vertebrate central nervous system (Biscoe, Davies, Dray, Evans, Francis, Martin & Watkins, 1977). It has been suggested (Davies & Watkins, 1973) that it may exist in one of several different tautomeric forms (Fig. 1), only one of which (Fig. 1*d*) would sterically allow its potential interaction with the aspartate receptor. The present structure determination was undertaken to determine which tautomer was present in that sample of D,L-HA-966 assayed in previous radioligand-receptor binding experiments (Derricott & Steward, 1980).

Transparent elongated-tabular monoclinic crystals were grown from aqueous solution and a crystal 0·3 × 0·2 × 0·4 mm was encapsulated in a silica-glass capillary tube containing a saturated solution of the compound. This mounting method[i] was adopted since the crystals effloresced rapidly when exposed to air. As a result of this rapid water loss the density of the crystals could not be measured accurately. Data were collected photographically on Weissenberg film packs, each containing four films, with Ni-filtered Cu *Kα* radiation (λ = 1·5418 Å).[ii,iii] Levels *h*0*l* to *h*6*l* were collected and also the *hk*0 level in order to obtain inter-level scale factors. The films were processed by the SRC Microdensitometer Service (Daresbury Laboratory, Daresbury, Warrington WA4 4AD, Cheshire) to yield structure factor data and a non-linear least-squares refinement of the cell dimensions. Lp corrections were applied but no absorption corrections [μ(Cu *Kα*) = 0·936 mm^{-1}] were made; 977 unique reflexions were used in the refinement.

The systematic absences *h*0*l*, $l = 2n + 1$ and 0*k*0, $k = 2n + 1$ uniquely defined the space group $P2_1/c$.[ii]

The structure was solved by direct methods (*SHELX* 76, Sheldrick, 1976).[iv] All the non-H atoms in the asymmetric unit, except for the water O atoms, were revealed by an *E* map calculated with 168 reflexions ($E \geq 1{\cdot}2$). Isotropic full-matrix least-squares refinement (R = 0·40) followed by a difference synthesis revealed two of the three water O atoms and also two H atoms. Further isotropic refinement (R = 0·25) revealed the third water O atom and the remaining H atoms were located after further anisotropic refinement.[v] The H atoms bonded to the waters O(4) and O(5) were difficult to locate as a consequence of three or four peaks appearing, in a difference synthesis, proximal to each O atom. Those peaks which formed a dimensionally reasonable hydrogen-bond network were chosen as the most likely candidates and this was justified by their subsequent satisfactory refinement; the other peaks did not refine. The appearance of these spurious peaks may indicate a degree of disorder, although a disordered model, with fractional site-occupation factors, was unsuccessfully tried. H atoms bonded to C were included in calculated positions (C–H 1·08 Å, H–C–H 109·5°) and refined with a riding model in which C–H vectors were constant in magnitude and direction but not position. N–H and O–H lengths were constrained

Table 1. *Final fractional atomic coordinates* (×10^4)

	x	*y*	*z*
N(1)	1532 (3)	5391 (4)	1239 (2)
C(2)	1046 (3)	7075 (5)	786 (3)
C(3)	1479 (3)	7646 (6)	−204 (3)
C(4)	2089 (4)	5811 (7)	−412 (4)
C(5)	2322 (4)	4532 (6)	710 (4)
N(2)	408 (3)	8307 (4)	−1361 (3)
O(1)	1374 (2)	4525 (4)	2212 (2)
O(2)	337 (3)	8024 (4)	1110 (2)
H(1)*	2123 (3)	8858 (6)	72 (3)
H(2)	−242 (36)	7218 (56)	−1682 (46)
H(3)	721 (42)	8716 (73)	−2006 (35)
H(4)	83 (45)	9588 (45)	−1179 (47)
H(5)*	2954 (4)	6166 (7)	−461 (4)
H(6)*	1478 (4)	5087 (7)	−1268 (4)
H(7)*	3287 (4)	4581 (6)	1373 (4)
H(8)*	2058 (4)	3037 (6)	426 (4)
O(3)	3531 (3)	3135 (5)	3834 (3)
H(9)	3679 (47)	1815 (39)	3666 (49)
H(10)	2737 (27)	3597 (74)	3287 (40)
O(4)	4964 (3)	6271 (5)	3688 (3)
H(11)	4519 (52)	5097 (57)	3708 (59)
H(12)	5482 (39)	6454 (80)	4557 (21)
O(5)	3756 (4)	−200 (6)	2633 (4)
H(13)	4016 (56)	−1530 (40)	2832 (59)
H(14)	4319 (47)	404 (91)	2364 (56)

* Atoms in calculated positions.

Table 2. *Torsion angles* (°) *involving non-hydrogen atoms*

The sign convention is as defined by Klyne & Prelog (1960).

C(5)–N(1)–C(2)–C(3)	0·0 (4)
C(5)–N(1)–C(2)–O(2)	179·2 (3)
O(1)–N(1)–C(2)–C(3)	176·1 (3)
O(1)–N(1)–C(2)–O(2)	−4·7 (5)
C(2)–N(1)–C(5)–C(4)	−10·8 (4)
O(1)–N(1)–C(5)–C(4)	173·1 (3)
N(1)–C(2)–C(3)–C(4)	70·8 (4)
N(1)–C(2)–C(3)–N(2)	132·2 (3)
O(2)–C(2)–C(3)–C(4)	−168·4 (4)
O(2)–C(2)–C(3)–N(2)	−47·1 (5)
C(2)–C(3)–C(4)–C(5)	−16·8 (4)
N(2)–C(3)–C(4)–C(5)	−136·4 (3)
C(3)–C(4)–C(5)–N(1)	16·4 (4)

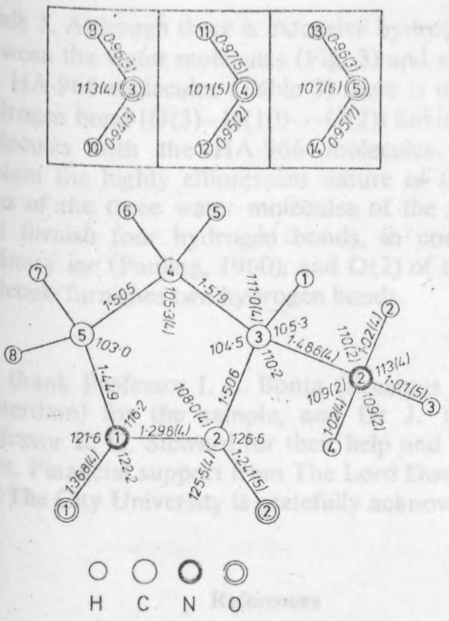

Fig. 2. Atomic numbering scheme, bond lengths (Å) and angles (°) for D,L-HA-966. E.s.d.'s are 0·006 Å for bonds and 0·3° for angles except where indicated otherwise. Bond angles not included on the figure are: N(2)–C(3)–C(4) 112·7, C(3)–N(2)–H(3) 109 (2)°.

(±0·02 Å) at 1·02 and 0·96 Å respectively. A common isotropic temperature factor [0·086 (5) Å²] was refined for all H atoms except for those bonded to the waters O(4) and O(5). A larger common isotropic temperature factor [0·12 (1) Å²] was refined for the latter, not inconsistent with the possible disorder mentioned earlier. Two strong reflexions exhibiting extinction were omitted from the final refinement which then converged to $R = 0·056$ for 130 parameters.

Final atomic coordinates are given in Table 1 and the torsion angles involving non-H atoms in Table 2. The molecular geometry is illustrated in Fig. 2.*(vi)

* Lists of structure factors and anisotropic thermal parameters have been deposited with the British Library Lending Division as Supplementary Publication No. SUP 35223 (7 pp.). Copies may be obtained through The Executive Secretary, International Union of Crystallography, 5 Abbey Square, Chester CH1 2HU, England.

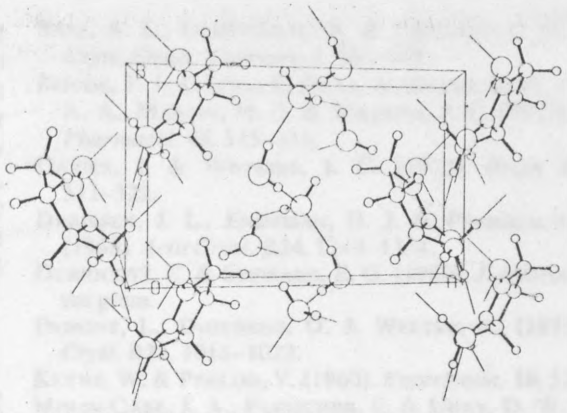

Fig. 3. Crystal packing diagram projected along c.

Discussion. HA-966 exists, in the crystal, as that tautomer (Fig. 1*d*) which is the most similar to the α-amino acid region of L-aspartate (Fig. 1*e*). This may support the contention (Biscoe *et al.*, 1977) that HA-966 may act as a direct antagonist at the physiological aspartate receptor.

In common with L-aspartate (Thyagaraja Rao, Srinivasan & Valambal, 1968; Derissen, Endeman & Peerdeman, 1968), HA-966 exists as a zwitterion in the crystal where the N(2) amino group is protonated and the O(1) hydroxyl group deprotonated. A degree of electron resonance around the N(1), C(2), O(2) portion of the molecule is reflected in the bond lengths: C(2)–O(2) is slightly longer than a normal double bond (Pople & Beveridge, 1970) whilst N(1)–C(2) is shorter than expected. The H atoms of the ammonium group adopt a staggered conformation [C(4)–C(3)–N(2)–H(2) 58·4°].

The pyrrolidine ring of HA-966 has an almost perfect C(4) envelope conformation where C(4) lies 0·31 Å from the C(2), O(2), N(1) plane; C(3) and C(5) both deviate from this by only 0·02 Å. Many other substituted 2-pyrrolidones adopt similar ring conformations (Dupont, Dideberg & Welter, 1975; Aubry, Marraud, Protas & Néel, 1972; Ball, Desiderato & Freeman, 1973; Molin-Case, Fleischer & Urry, 1970).

The packing is illustrated in Fig. 3 and the geometry of the intermolecular hydrogen bonding is given in

Table 3. *Hydrogen-bond lengths* (Å) *and angles* (°)

A–H···*B*	Symmetry of acceptor	*A*–H	H···*B*	*A*···*B*	∠*A*–H···*B*
N(2)–H(2)···O(1)	$-x, 1-y, -z$	1·02 (4)	1·70 (4)	2·727 (4)	180 (24)
N(2)–H(3)···O(1)	$x, \frac{1}{2}-y, z-\frac{1}{2}$	1·01 (5)	1·87 (5)	2·815 (4)	154 (14)
N(2)–H(4)···O(2)	$-x, 2-y, -z$	1·02 (4)	1·72 (4)	2·718 (4)	166 (18)
O(3)–H(9)···O(5)	x,y,z	0·96 (3)	1·86 (3)	2·813 (6)	170 (16)
O(3)–H(10)···O(2)	x,y,z	0·94 (3)	1·70 (3)	2·639 (4)	180 (25)
O(4)–H(11)···O(3)	x,y,z	0·97 (5)	1·83 (5)	2·787 (5)	170 (24)
O(4)–H(12)···O(3)	$1-x, 1-y, 1-z$	0·95 (2)	1·77 (2)	2·724 (5)	180 (19)
O(5)–H(13)···O(4)	$x, y-1, z$	0·96 (3)	1·89 (3)	2·813 (6)	160 (10)
O(5)–H(14)···O(4)	$1-x, y-\frac{1}{2}, \frac{1}{2}-z$	0·95 (7)	1·87 (7)	2·775 (6)	160 (23)

Table 3. Although there is extensive hydrogen bonding between the water molecules (Fig. 3) and also between the HA-966 molecules (Table 3) there is only a single hydrogen bond [O(3)–H(10)···O(2)] linking the water molecules with the HA-966 molecules. This may explain the highly efflorescent nature of the crystals. Two of the three water molecules of the asymmetric unit furnish four hydrogen bonds, in common with ordinary ice (Pauling, 1960), and O(2) of the HA-966 molecule furnishes two hydrogen bonds.

I thank Professor I. L. Bonta (Erasmus University, Rotterdam) for the sample, and Dr J. Trotter and Professor E. G. Steward for their help and encouragement. Financial support from The Lord Dowding Fund and The City University is gratefully acknowledged.

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Acta Cryst. (1980). B**36**, 1972–1975

Structure of Dimethyl 4,4′-Methylenebis(phenylcarbamate): a Model for MDI Units in Polyurethane Hard Segments

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(*Received* 5 *July* 1978; *accepted* 19 *March* 1980)

Abstract. $C_{17}H_{18}N_2O_4$, monoclinic, $P2_1/b$, a = 5·157 (3), b = 9·800 (3), c = 31·472 (11) Å, γ = 93·90 (3)°, Z = 4, D_m = 1·30, D_c = 1·316 Mg m^{-3}. The structure was refined to R = 0·06 based on diffractometer data collected at 258 K. The molecule is V-shaped with a C–CH_2–C angle of 114·5 (4)°. The angle between the phenyl-ring planes is 90·0° and the planes of the urethane groups are at angles of 10·2 and 39·4° to their adjacent phenyl rings. The molecules are linked into sheets by C=O···H–N hydrogen bonds between the urethane groups. The title compound furnishes a conformational model for the diol-linked 4,4′-methylenediphenyl diisocyanate (MDI) units in the hard segments of certain polyurethane elastomers.

Introduction. Polyurethane elastomers are block copolymers composed of alternating hard (urethane) and soft (polyester or polyether) segments. The elastomeric properties of these materials are attributed to microphase separation: the urethane segments segregate to form crystalline domains which serve as crosslinks between the soft-segment chains. One commercially important polyurethane system has hard segments composed of diol-linked 4,4′-methylenediphenyl diisocyanate (MDI). The structure of the hard domains has not been determined by direct study of the polymer, due to the low quality of the X-ray diffraction data, but it is believed that hydrogen bonding between the urethane groups is an important factor in the virtual (non-covalent) crosslinking of the chains (Bonart, Morbitzer & Muller, 1974). The title compound is methanol-capped MDI, and its structure has been determined as a model for the structure of the polymer system.

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B1.2 Notes to the structure determination

The following notes usefully support and expand those sections of the preceding "short communication" which, though routine, illustrate the background work. The notes enumerated below are annotated in the published text.

(i) *Crystal mounting* See Fig B1.1 below

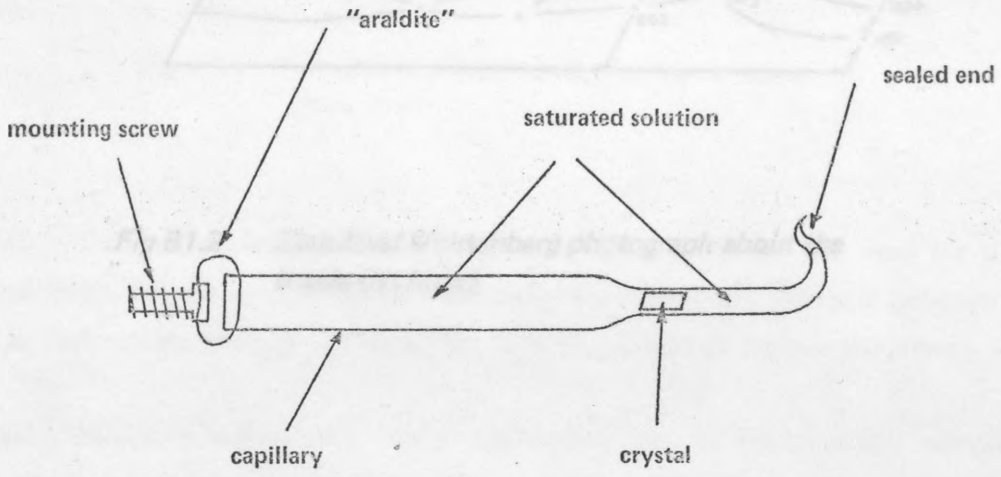


Fig B1.1 Crystal mounting method

(ii) *Space group determination.* Preliminary oscillation photographs, displaying m_x symmetry, indicated a possible crystal-axis parallel with the capillary tube axis and, after crystal alignment (Stout & Jensen, 1968), a zero-level Weissenberg photograph showed the unit cell to be monoclinic, rotating about the b-axis (Fig B1.2). A different crystal aligned along the c-axis produced the zero-level Weissenberg photograph shown in Fig B1.4. The systematic absences hOl, l = 2n + 1 (Figs B 1.2 & 3) and OkO, k = 2n + 1 (Fig B 1.4) uniquely defined the space-group $P2_1/c$.

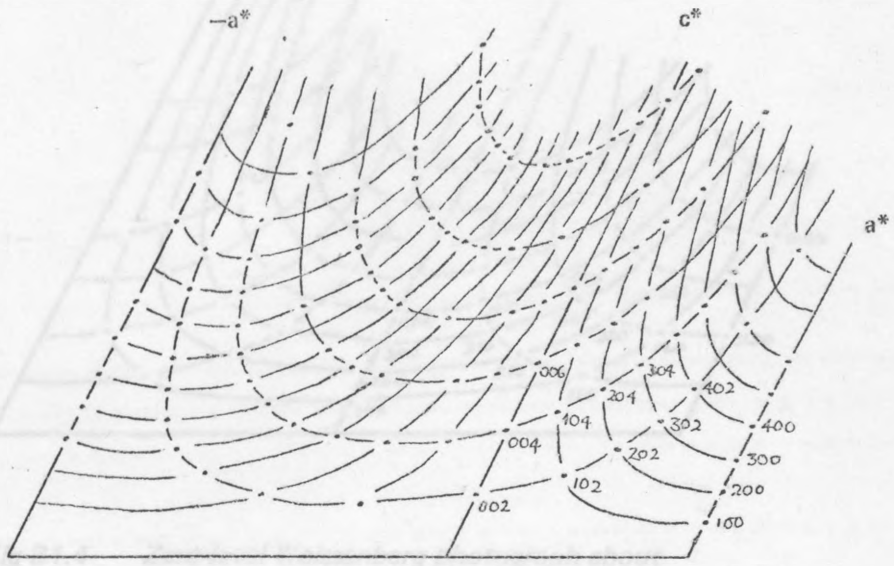


Fig B1.2 Zero-level Weissenberg photograph about the b-axis (Ni filter)

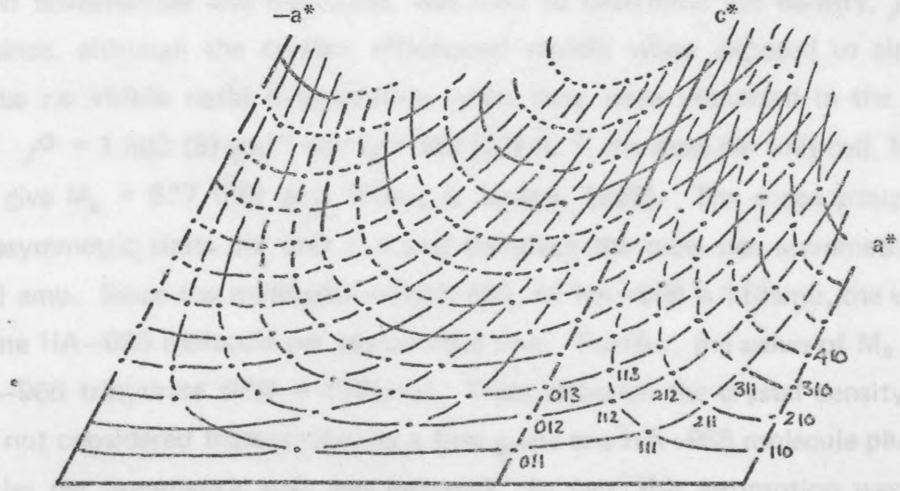


Fig B1.3 First-level Weissenberg photograph about the b-axis (cf B1.2)

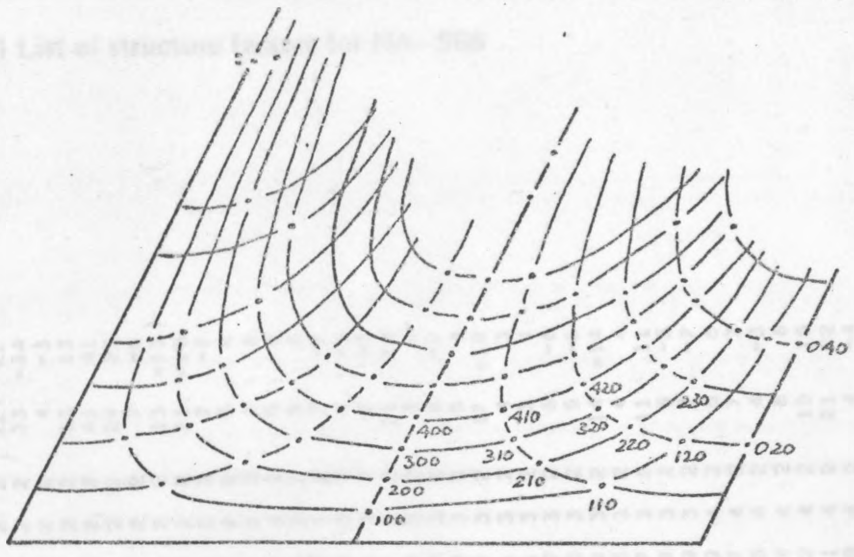


Fig B1.4 Zero-level Weissenberg photograph about the c-axis

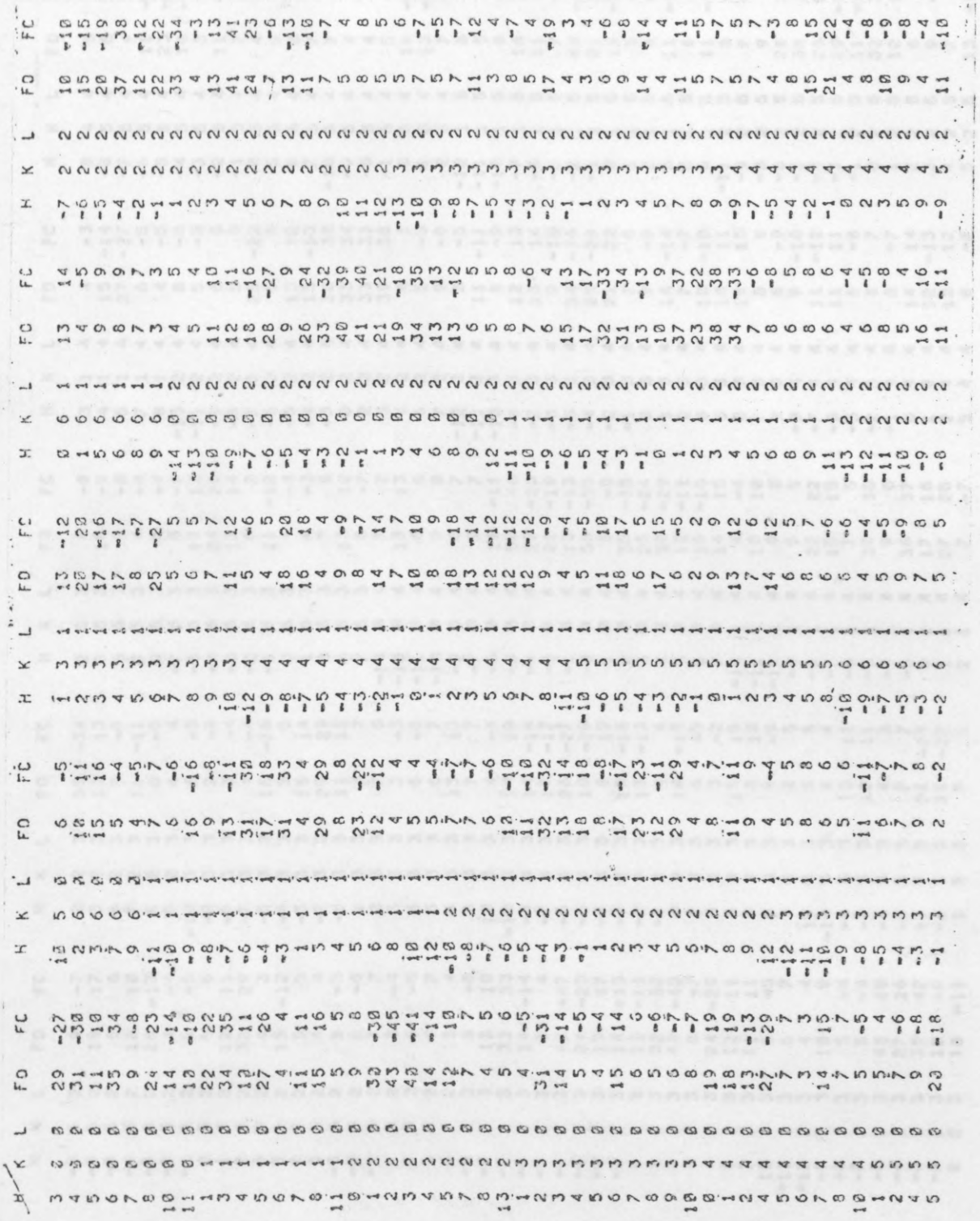
(iii) *Data collection.* Although Ni-filtered CuKα radiation was used for the zero and first levels, for the remaining levels sufficient separation was observed between the Kα and Kβ reflections to allow the use of the layer line screen to exclude the latter.

(iv) *Structure refinement.* As a preliminary step to the structure refinement it was desirable to determine both the number of HA–966 molecules per asymmetric unit and also the number of water molecules per HA–966 molecule. The flotation method, employing carbon tetrachloride and n-pentane, was used to determine the density, ρ, of the crystals since, although the crystals effloresced rapidly when exposed to air, there appeared to be no visible rapid deterioration when they were immersed in the liquids above. From ρ = 1.303 (8) gml^{-1} and U = 863 (32) Å^3, the mass per unit cell, M_u, was calculated to give M_u = 677 (30) amu (Stout & Jensen, 1968). The space group $P2_1/c$ implies four asymmetric units per unit cell and therefore the mass per asymmetric unit, M_a, is 169 (8) amu. Since the molecular weight, MW, of HA–966 is 116amu, the value of M_a suggests one HA–966 molecule per asymmetric unit. Further, the value of M_a corresponds to HA–966 trihydrate (MW = 170amu). Thus, although the crystal density determination was not considered trustworthy, as a first guess one HA–966 molecule plus three water molecules per asymmetric unit was assumed. In fact, this assumption was subsequently, during the structure refinement, found to be accurate.

(v) *Inclusion of "unobserved" reflections.* During the latter stages of the refinement the unobserved reflections were generated (programme UNOBX, B1.3) and included in the structure factor (IFI) data in order to aid the refinement and reduce R.

(vi) *Lists of structure factors and anisotropic thermal parameters.* These are given in Tables B1.1 and B1.2 respectively.

Table B 1.1 List of structure factors for HA–966



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-6	5	2	6	-6
-5	5	2	16	-13
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5	5	2	6	7
6	5	2	5	-4
7	5	2	8	-8
8	5	2	10	10
9	5	2	33	33
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11	5	2	43	-47
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14	5	2	14	-13
15	5	2	11	-11
16	5	2	32	33
17	5	2	43	-42
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20	5	2	12	11
21	5	2	11	11
22	5	2	5	-5
23	5	2	6	7
24	5	2	18	-18
25	5	2	4	-4
26	5	2	45	45
27	5	2	27	26
28	5	2	39	42
29	5	2	10	-9
30	5	2	13	-11

H	K	L	FO	FC
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-7	4	3	13	13
-6	4	3	8	-8
-5	4	3	11	-11
-4	4	3	6	6
-3	4	3	6	6
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5	4	3	7	6
6	4	3	13	13
7	4	3	7	7
8	4	3	6	6
9	4	3	6	7
10	4	3	13	13
11	4	3	7	7
12	4	3	6	6
13	4	3	7	7
14	4	3	11	-11
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17	4	3	21	-19
18	4	3	13	-13
19	4	3	55	55
20	4	3	8	-9
21	4	3	35	-35
22	4	3	25	-24
23	4	3	30	-29
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25	4	3	16	-16
26	4	3	14	15
27	4	3	4	-4
28	4	3	10	10
29	4	3	9	8
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32	4	3	15	15
33	4	3	7	5
34	4	3	30	30
35	4	3	9	8
36	4	3	36	37
37	4	3	17	16
38	4	3	27	26
39	4	3	7	-7

H	K	L	FO	FC
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-7	3	4	15	-14
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-5	3	4	6	-5
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7	3	4	5	-5
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10	3	4	11	11
11	3	4	8	-9
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13	3	4	13	14
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32	3	4	8	-7
33	3	4	14	14
34	3	4	12	-13
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36	3	4	8	-7

H	K	L	FO	FC
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10	2	5	38	37
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14	2	5	32	31
15	2	5	12	11
16	2	5	6	5
17	2	5	9	-8
18	2	5	7	7
19	2	5	13	-14

H	K	L	FO	FC	H	K	L	FO	FC	H	K	L	FO	FC	H	K	L	FO	FC	H	K	L	FO	FC
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H	K	L	FO	FC
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1	1	8	9	-9
5	1	8	6	6
7	1	8	5	5
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0	5	8	5	4

H	K	L	FO	FC
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H	K	L	FO	FC
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2	2	10	9	-9
3	2	10	9	-8
5	2	10	7	8
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H	K	L	FO	FC
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2	3	10	6	-6
3	3	10	4	-4
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2	1	11	5	-4
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-9	2	11	8	8
-6	2	11	4	-3
-4	2	11	4	4
-3	2	11	14	14
-2	2	11	5	5
-11	3	11	8	-9
-10	3	11	6	-5
-9	3	11	7	-8
-6	3	11	5	6
-4	3	11	6	-6
-3	3	11	9	-10
-1	3	11	4	4
0	3	11	11	12
1	3	11	5	5
2	3	11	8	9
-6	4	11	6	-6
-5	4	11	8	-9
-3	4	11	6	-6
-1	4	11	6	-6
0	4	11	8	-8
-7	5	11	3	3
-6	5	11	6	6
-5	5	11	7	6
-4	5	11	6	5
-2	5	11	6	4

H	K	L	FO	FC
0	5	11	2	-2
-12	0	12	13	12
-11	0	12	18	18
-10	0	12	7	7
-9	0	12	10	9
-8	0	12	9	9
-7	0	12	11	11
-5	0	12	10	11
-4	0	12	8	7
-3	0	12	7	7
-2	0	12	10	-8
-1	0	12	10	-10
0	0	12	12	-12
1	0	12	6	5
-12	1	12	9	-9
-8	1	12	8	7
-7	1	12	5	5
-6	1	12	11	11
-5	1	12	13	13
-4	1	12	7	7
-3	1	12	6	6
-2	1	12	7	8
0	1	12	4	4
-11	2	12	4	-4
-10	2	12	12	-11
-9	2	12	7	-7
-8	2	12	6	-5
-7	2	12	12	-12
-6	2	12	7	-7
-5	2	12	5	-5
-4	2	12	5	5
-3	2	12	12	12
-1	2	12	5	-4
0	2	12	4	-4
1	2	12	5	-5
-9	3	12	4	-3
-6	3	12	4	4
-5	3	12	4	-4
-4	3	12	9	-9
-3	3	12	11	-11
-1	3	12	6	7
0	3	12	5	6
-7	4	12	7	-6
-4	4	12	6	6
-4	5	12	6	-5
-10	1	13	6	6
-9	1	13	6	6
-1	1	13	4	4

H	K	L	FO	FC
-10	2	13	3	-3
-9	2	13	3	-3
-7	2	13	3	-2
-5	2	13	4	-4

H	K	L	FO	FC
-4	2	13	11	-11
-3	2	13	6	-8
-2	2	13	6	-3
-1	2	13	4	-4

H	K	L	FO	FC
-7	3	13	7	-7
-6	3	13	5	-5
-4	3	13	7	-7

H	K	L	FO	FC
-8	0	14	4	-3
-4	0	14	4	4
-9	1	14	5	5

H	K	L	FO	FC
-7	1	14	4	-4
-6	1	14	3	-3
-7	2	14	5	4

Table B 1.2 Anisotropic Thermal Parameters ($Å^2 \times 10^4$)

	U_{11}	U_{22}	U_{33}	U_{23}	U_{13}	U_{12}
N(1)	358 (16)	223 (17)	243 (13)	24 (12)	180 (12)	52 (13)
C(2)	431 (20)	186 (19)	267 (16)	– 15 (15)	217 (15)	13 (17)
C(3)	434 (21)	316 (23)	265 (17)	28 (15)	201 (16)	16 (17)
C(4)	623 (28)	581 (31)	405 (22)	125 (20)	341 (21)	295 (24)
C(5)	427 (23)	483 (26)	429 (22)	70 (20)	314 (19)	177 (20)
N(2)	509 (20)	207 (18)	269 (15)	57 (12)	265 (14)	59 (14)
O(1)	712 (18)	263 (15)	454 (15)	82 (13)	437 (14)	180 (14)
O(2)	455 (15)	273 (14)	303 (12)	86 (11)	237 (11)	63 (12)
O(3)	465 (17)	490 (20)	447 (17)	60 (15)	84 (14)	23 (16)
O(4)	804 (24)	537 (21)	440 (16)	39 (16)	261 (17)	–129 (18)
O(5)	993 (31)	607 (27)	1053 (32)	148 (23)	665 (26)	155 (22)

B 1.3 Programme UNOBS to generate unobserved reflections.

The following programme generated on cards, suitable for SHELX76 input, those unobserved reflections which fell within a unique quadrant of the limiting sphere but which were not systematically absent. The unique quadrant was chosen to be consistent with that yielded by the data-reduction programme of the SRC Microdensitometer service and was defined by h: +ve & -ve, k +ve and l : +ve (cf structure factor indices Table B1.1). Only a single quadrant of the limiting sphere is unique for a monoclinic space group since all other quadrants are symmetry related. The programme is not general and was written for the $P2_1/c$ space group in particular since section A screens for the systematic absences OkO, k = 2n + 1 and hOl, l = 2n + 1. Section B screens for existing observed reflections. The remainder of the programme is self-explanatory.

```
      MASTER UNOBS
      INTEGER OHKL(3,2000),CHKL(3)
      DIMENSION T(12)
      READ(5,1)(T(J),J=1,12)
    1 FORMAT(12A6)
      WRITE(6,2)(T(J),J=1,12)
    2 FORMAT(1H1,12A6)
      READ(5,3)A,B,C,BETA
    3 FORMAT(4F10.5)
      WRITE(6,4)A,B,C,BETA
    4 FORMAT(1H1,'CELL CONSTANTS'//1H1,'A=',F8.4,4X,'B=',F8.4,
     X4X,'C=',F8.4,'BETA=',F8.2)
      READ(5,5)LAMBDA
    5 FORMAT(F10.5)
      WRITE(6,6)LAMBDA
    6 FORMAT(1H1,'WAVELENGHT=',F8.4)
      NOBR=0
      DO 12 I=1,2000
      READ(5,11)(OHKL(J,I),J=1,3)
   11 FORMAT(3I4)
      IF(OHKL(1,I).EQ.0.AND.OHKL(2,I).EQ.0.AND.OHKL(3,I)
     X.EQ.0)GOTO 13
      NOBR=NOBR+1
   12 CONTINUE
   13 AS=1/(A*SIN(BETA))
      BS=1/B
      CS=1/(C*SIN(BETA))
      ROLS=2/LAMBDA
      JMAX=ROLS/AS
      KMAX=ROLS/BS
      LMAX=ROLS/CS
      NUR=0
      NCR=0
      II=1
   23 DO 7 I=1,JMAX+1
      NJ=I-1
      DO 8 J=1,KMAX+1
      NK=J-1
      DO 9 K=1,LMAX+1
      NL=K-1
      D=SQRT((NJ*AS)**2+(NK*BS)**2+(NL*CS)**2)
      IF(D.LE.ROLS)GOTO 10
      GOTO 9
   10 CHKL(1)=NJ*II
      CHKL(2)=NK
      CHKL(3)=NL
      IF(CHKL(1).EQ.0.AND.CHKL(3).EQ.0)GOTO 19
      GOTO 20
   19 NN=INT(CHKL(2)/2)
      AA=CHKL(2)/2
      IF(NN.LT.AA)GOTO 9
   20 IF(CHKL(2).EQ.0)GOTO 21
      GOTO 24
   21 MM=INT(CHKL(3)/2)
      BB=CHKL(3)/2
      IF(MM.LT.BB)GOTO 9
   24 NCR=NCR+1
      DO 16 J=1,NOBR
      IF(CHKL(1).EQ.OHKL(1,J).AND.CHKL(2).EQ.OHKL(2,J)
     X.AND.CHKL(3).EQ.OHKL(3,J)GOTO 9
   16 CONTINUE
      NUR=NUR+1
```

A

B

```
      WRITE(7,17)(CHKL(I),I=1,3)
   17 FORMAT(3I4,'     1.00     1.00')
    9 CONTINUE
    8 CONTINUE
    7 CONTINUE
      IF(II.LT.0)GOTO 22
      II=-1
      GOTO 23
   22 WRITE(6,18)NOBR,NCR,NUR
   18 FORMAT(1H1,'NO.OBS.REFLNS=',I7/
     X1H1,'NO.CALC.REFLNS=',I7/
     X1H1,'NO.UNOBS.REFLNS=',I7)
      STOP
      END
      FINISH
```

Glossary of variables

CHKL	Calculated hkl reflections
OHKL	Observed hkl reflections
D	Distance($Å^{-1}$) from reflection hkl to centre of limiting sphere
ROLS	Radius($Å^{-1}$) of limiting sphere
NOBR	Number of observed reflections
NCR	Number of calculated reflections
NUR	Number of unobserved reflections

B2 Structure determination of DL-α-methyl glutamate

B2.1 The published structure

The following reprint was taken from Acta Crystallographica (1979) B35, 2230–2232.

Acta Cryst. (1979). B35, 2230–2232

D,L-α-Methylglutamic Acid Hemihydrate

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Abstract. $C_6H_{11}NO_4.\frac{1}{2}H_2O$, $M_r = 170{\cdot}2$, monoclinic, $C2/c$, $a = 21{\cdot}466$ (5), $b = 8{\cdot}233$ (2), $c = 10{\cdot}573$ (3) Å, $\beta = 119{\cdot}81$ (1)°, $U = 1621$ Å^3, D_m (flotation) = 1·39 (1), $D_x = 1{\cdot}39$ Mg m^{-3}, $Z = 8$, melting point 429 (2) K. Final $R = 0{\cdot}046$ for 1870 unique reflexions. The compound exists as a zwitterion in the crystal. The side chain adopts a *trans* conformation in contrast to the *gauche* conformation of the parent L-glutamic acid. The water molecule is located in a special position on a twofold axis and forms four hydrogen bonds within the structure.

Introduction. The structure determination of the title compound was undertaken to investigate a possible conformational explanation for its activity at glutamate receptors in the vertebrate central nervous system (Haldeman, Huffman, Marshall & McLennan, 1972; Curtis, Duggan, Felix, Johnston, Tebécis & Watkins, 1972).

Transparent, tapered-acicular crystals were grown from water and a crystal *ca* 0·4 × 0·45 × 0·45 mm was selected for intensity measurements.[i] Data were collected on a Philips PW 1100 four-circle diffractometer with graphite-monochromated Mo $K\alpha$ radiation ($\lambda = 0{\cdot}71069$ Å). All unique reflexions were measured in the range $6{\cdot}0 \le 2\theta \le 60{\cdot}0°$ with an ω–2θ scan, a constant scan speed of 0·05° s^{-1} and an ω scan range of $(1{\cdot}0 + 0{\cdot}05 \tan\theta)°$. Cell dimensions were calculated from the diffractometer settings of 22 high-angle reflexions. Lp corrections but no absorption correction [μ(Mo $K\alpha$) = 0·072 mm^{-1}] were applied and 1870 unique reflexions with $I \ge 3\sigma(I)$ were used in the refinement.

The systematic absences *hkl*, $h + k = 2n + 1$ and *h*0*l*, $l = 2n + 1$ indicated possible space groups $C2/c$ and Cc; the centrosymmetric space group was suggested by the E statistics ($\langle|E^2 - 1|\rangle = 0{\cdot}95$) and confirmed by the structure solution and satisfactory refinement.

The structure was solved by direct methods (*SHELX* 76).[ii] An E map calculated from 253 reflexions with $E \ge 1{\cdot}5$ showed all the non-hydrogen atoms; the O atom of the water molecule [O(5)] is located on a twofold axis. Isotropic full-matrix least-squares refinement ($R = 0{\cdot}27$) followed by a difference synthesis revealed five H atoms: the remaining H atoms were located after further anisotropic refinement. H atoms bonded to C were included in calculated positions (C–H 1·08 Å, H–C–H 109·5°) and refined with a riding model in which C–H vectors were constant in magnitude and direction but not position. The N–H and O–H lengths were tied (±0·02 Å) to two free variables which refined to 0·92 and 0·89 Å respectively. A common isotropic temperature factor [0·047 (2) Å^2] was refined for all H atoms. The thermal parameters of O(4) are high suggesting that there may be some disorder in this portion of the molecule. Thirteen strong reflexions exhibiting extinction effects were omitted from the final refinement which converged

Table 1. *Fractional atomic coordinates* (×10^4)

	x	*y*	*z*
C(1)	3747 (1)	3815 (2)	541 (1)
C(2)	3600 (1)	4340 (2)	1765 (1)
C(3)	3483 (1)	2851 (2)	2502 (2)
C(4)	4090 (1)	1585 (2)	3035 (2)
C(5)	3960 (1)	255 (2)	3848 (2)
C(6)	2940 (1)	5459 (2)	1131 (2)
N	4236 (1)	5277 (2)	2880 (1)
O(1)	3270 (1)	2986 (1)	−453 (1)
O(2)	4321 (1)	4267 (2)	606 (1)
O(3)	3542 (1)	−909 (2)	3036 (1)
O(4)	4210 (1)	248 (2)	5143 (2)
H(1)*	2473 (1)	4869 (2)	266 (2)
H(2)*	3064 (1)	6531 (2)	709 (2)
H(3)*	2833 (1)	5803 (2)	1990 (2)
H(4)	4317 (10)	6283 (22)	2578 (21)
H(5)	4201 (10)	5463 (25)	3696 (19)
H(6)	4655 (9)	4720 (23)	3231 (23)
H(7)*	3441 (1)	3262 (2)	3427 (2)
H(8)*	2988 (1)	2270 (2)	1725 (2)
H(9)*	4596 (1)	2165 (2)	3754 (2)
H(10)*	4107 (1)	1076 (2)	2111 (2)
H(11)	3439 (11)	−1643 (24)	3513 (22)
O(5)	5000	2142 (2)	7500
H(12)	4775 (10)	1477 (24)	6737 (20)

* Atoms in calculated positions.

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Table 2. *Torsion angles* (°)

Standard deviations are 0·4°.

C(5)–N(1)–C(2)–C(3)	0·0	C(7)–C(2)–C(3)–C(4)	−177·5	C(3)–C(4)–C(5)–N(1)	0·0
C(5)–N(1)–C(2)–C(7)	177·9	C(7)–C(2)–C(3)–C(8)	0·0	C(13)–C(4)–C(5)–N(1)	178·5
C(6)–N(1)–C(2)–C(3)	−178·6	C(2)–C(3)–C(4)–C(5)	0·0	C(3)–C(4)–C(13)–O(14)	−173·8
C(6)–N(1)–C(2)–C(7)	0·0	C(2)–C(3)–C(4)–C(13)	−178·4	C(5)–C(4)–C(13)–O(14)	8·2
C(2)–N(1)–C(5)–C(4)	0·0	C(8)–C(3)–C(4)–C(5)	−177·2	C(3)–C(8)–O(10)–C(11)	180·0
C(6)–N(1)–C(5)–C(4)	178·5	C(8)–C(3)–C(4)–C(13)	4·5	O(9)–C(8)–O(10)–C(11)	0·0
N(1)–C(2)–C(3)–C(4)	0·0	C(2)–C(3)–C(8)–O(9)	6·9	C(8)–O(10)–C(11)–C(12)	175·8
N(1)–C(2)–C(3)–C(8)	177·0	C(4)–C(3)–C(8)–O(10)	−172·8		

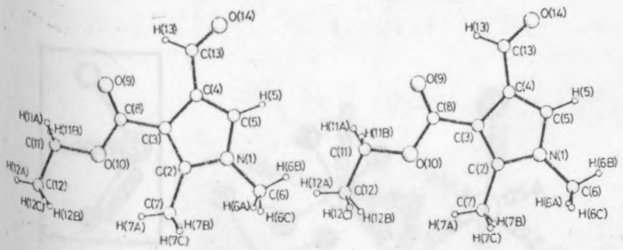

Fig. 1. A stereodrawing of the molecule.

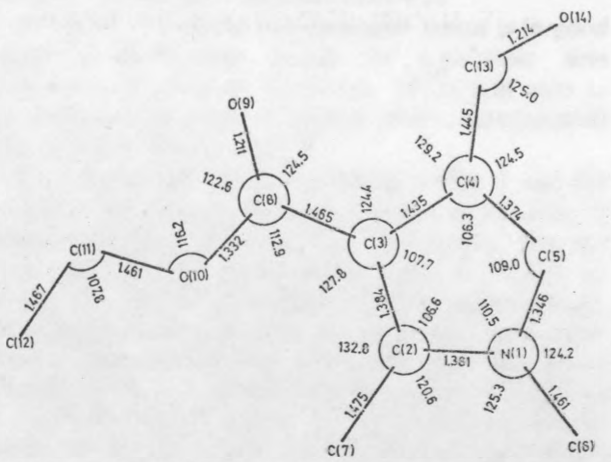

Fig. 2. Bond lengths (Å) and angles (°) involving the non-hydrogen atoms (standard deviations are 0·004 Å and 0·2° respectively).

on a difference Fourier map. The positional and thermal parameters were refined by a full-matrix least-squares method. The programs *SHELX* 76 (Sheldrick, 1976) and *PLUTO* 78 (Motherwell & Clegg, 1978) were used. Refinement with anisotropic temperature factors for the non-hydrogen atoms and isotropic temperature factors (not refined) for H atoms gave a discrepancy index $R = 0{\cdot}049$. Final positional parameters are given in Table 1.*(iii)

Discussion. A stereoscopic drawing of the molecule, showing the atom-numbering scheme, is in Fig. 1. Bond lengths and angles (involving non-hydrogen atoms only) are presented in Fig. 2. The internal dimensions of the molecule are, in general, normal but present some points of interest. The lengthening of the formal double bonds C(2)–C(3) and C(4)–C(5) and the shortening of the other three bonds of the pyrrole ring indicate considerable delocalization of the double-bond π electrons. N(1)–C(5) is much shorter than N(1)–C(2) and this difference may be explained in terms of contributions from mesomeric forms (Sheldrick & Becker, 1978).

As expected, the pyrrole ring is planar. No deviations from the calculated plane exist for the five ring atoms. The near planarity of the molecule is illustrated by the torsion angles between the bond segments listed in Table 2.

The packing of the molecules in the crystal is determined by van der Waals interactions only. No evidence for hydrogen bonds exists and there are no intermolecular contacts shorter than the respective van der Waals diameters.

*Lists of structure factors and thermal parameters have been deposited with the British Library Lending Division as Supplementary Publication No. SUP 34390 (9 pp.). Copies may be obtained through The Executive Secretary, International Union of Crystallography, 5 Abbey Square, Chester CH1 2HU, England.

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Table 2. *Torsion angles* (°) *involving non-hydrogen atoms*

Sign convention as defined by Klyne & Prelog (1960).

C(1)–C(2)–C(3)–C(4)	52·9 (2)
C(2)–C(3)–C(4)–C(5)	175·2 (1)
C(4)–C(3)–C(2)–C(6)	174·4 (1)
C(4)–C(3)–C(2)–N	−66·5 (2)
C(3)–C(2)–C(1)–O(1)	58·6 (2)
C(3)–C(2)–C(1)–O(2)	−122·5 (2)
C(6)–C(2)–C(1)–O(1)	−63·6 (2)
C(6)–C(2)–C(1)–O(2)	115·3 (2)
N–C(2)–C(1)–O(1)	178·3 (1)
N–C(2)–C(1)–O(2)	−2·8 (2)
C(3)–C(4)–C(5)–O(3)	85·6 (2)
C(3)–C(4)–C(5)–O(4)	−94·0 (2)

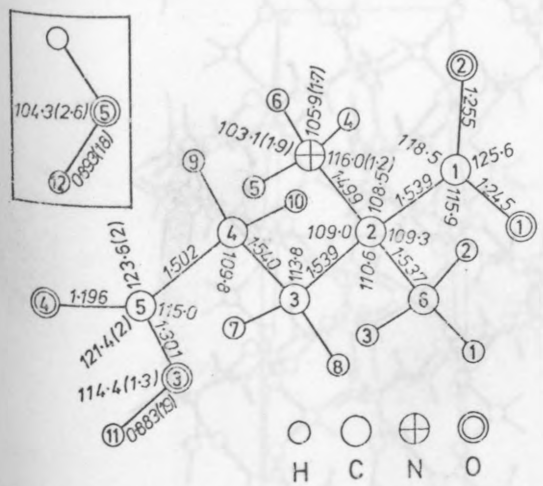

Fig. 1. Atomic numbering scheme, bond lengths (Å) and angles (°) for D,L-α-methylglutamic acid. E.s.d.'s are 0·002 Å for bonds and 0·1° for angles except where indicated otherwise. Bond lengths and angles not included on the figure are given below. Bond lengths (Å): N–H(4) 0·935 (16), N–H(5) 0·915 (16), N–H(6) 0·907 (16). Bond angles (°): C(2)–N–H(5) 111·0 (1·2), C(2)–N–H(6) 113·0 (1·3), H(4)–N–H(5) 106·8 (1·8), C(1)–C(2)–C(3) 110·8 (1), C(6)–C(2)–N 108·4 (1).

to $R = 0{\cdot}046$, $R' = 0{\cdot}053$ ($R' = \sum w^{1/2}\Delta/\sum w^{1/2}|F_o|$) for 126 parameters: reflexions were weighted as $w = 1/\sigma^2(F_o)$.

Final atomic coordinates are given in Table 1 and the torsion angles involving non-hydrogen atoms in Table 2. The molecular geometry is illustrated in Fig. 1.*

Discussion. In common with L-glutamic acid (Hirokawa, 1955; Lehmann, Koetzle & Hamilton, 1972), D,L-α-methylglutamic acid exists as a zwitterion in the crystal. The carboxyl group at C(1) is deprotonated [C(1)–O(1) 1·245 (2), C(1)–O(2) 1·255 (2) Å] whereas that at C(5) is protonated [C(5)–O(3) 1·301 (2), C(5)–O(4) 1·196 (2) Å]. The H atoms of the protonated amino group adopt a staggered conformation [H(6)–N–C(2)–C(1) −55·2°].

The chain C(2), C(3), C(4), C(5) adopts a *trans* conformation in contrast to L-glutamic acid (Lehmann, Koetzle & Hamilton, 1972) and L-glutamic acid in a mixed crystal with L-pyroglutamic acid (Taira & Watson, 1977) in which the chains adopt a *gauche* conformation (Table 3). However, comparison with similar diacidic α-amino acids indicates that the two latter structures are atypical, the *trans* conformation being adopted in all other cases (Table 3).

The geometry of the non-hydrogen atoms is in good agreement with that found for L-glutamic acid (Lehmann, Koetzle & Hamilton, 1972) and also L-glutamic acid in a mixed crystal with L-pyroglutamic acid (Taira & Watson, 1977).

The crystal packing is illustrated in Fig. 2 and the geometry of the intermolecular hydrogen-bonding is given in Table 4. O(3)–H(11)···O(1) bonds link the molecules to form zigzag chains which are linked by N–H(5)···O(2) and N–H(6)···O(2) hydrogen bonds. Interstitial water molecules act as further hydrogen-bond bridges across the zigzag structure. The water molecule is unusual in that it furnishes four H bonds as in ordinary ice (Pauling, 1960); hydrogen-bonded arrays involving a maximum of three hydrogen bonds to water are more commonly found in hydrated crystal structures.

It has been suggested that *trans*-L-glutamic acid is the active conformer at the glutamate receptor (Johnston, Curtis, Davies & McCulloch, 1974). D,L-α-Methylglutamic acid, which exhibits weak antagonistic activity (Haldeman, Huffman, Marshall & McLennan,

Table 3. *Torsion angles* (°) *corresponding to* C(2)–C(3)–C(4)–C(5) *of* D,L-α-*methylglutamic acid*

	Reference	
D,L-α-Methylglutamic acid	This work	175·2 (1)
L-Glutamic acid	I	−73·1 (2)
L-Glutamic acid (in a mixed crystal with pyroglutamic acid)	II	−65·2 (8)
Ca L-glutamate.$3H_2O$	III	174·6
Ca L-glutamate chloride.H_2O	IV	170·2 (2)
Cu glutamate.$2H_2O$	V	182·9 (6)
Zn glutamate.$2H_2O$	VI	183·4 (6)
L-Glutamic acid.HCl	VII	−173·1 (5)
DL-*O*-Serine phosphate.H_2O	VIII	−168·1
DL-Homocysteic acid	IX	165

I, Lehmann *et al.* (1972). II, Taira & Watson (1977). III, Einspahr & Bugg (1974). IV, Einspahr, Gartland & Bugg (1977). V, Gramaccioli & Marsh (1966). VI, Gramaccioli (1966). VII, Sequeira, Rajagopal & Chidambaram (1972). VIII, Putkey & Sundaralingam (1970). IX, Clarke & Steward (1977).

*Lists of structure factors and anisotropic thermal parameters have been deposited with the British Library Lending Division as Supplementary Publication No. SUP 34393 (10 pp.). Copies may be obtained through The Executive Secretary, International Union of Crystallography, 5 Abbey Square, Chester CH1 2HU, England.

Table 4. *Geometry of intermolecular hydrogen-bonding*

A–H···*B*	Symmetry of acceptor	*A*–H (Å)	H···*B* (Å)	*A*···*B* (Å)	∠*A*–H···*B* (°)
O(5)–H(12)···O(4)	x, y, z	0·89 (2)	1·82 (2)	2·705 (2)	173 (2)
O(3)–H(11)···O(1)	$x, -y, \frac{1}{2}+z$	0·88 (2)	1·72 (2)	2·597 (2)	174 (2)
N–H(4)···O(5)	$1-x, 1-y, 1-z$	0·94 (2)	1·99 (2)	2·832 (2)	148 (2)
N–H(5)···O(2)	$x, 1-y, \frac{1}{2}+z$	0·94 (2)	1·92 (2)	2·821 (2)	160 (2)
N–H(6)···O(2)	$1-x, y, \frac{1}{2}-z$	0·91 (2)	1·95 (2)	2·814 (2)	158 (2)

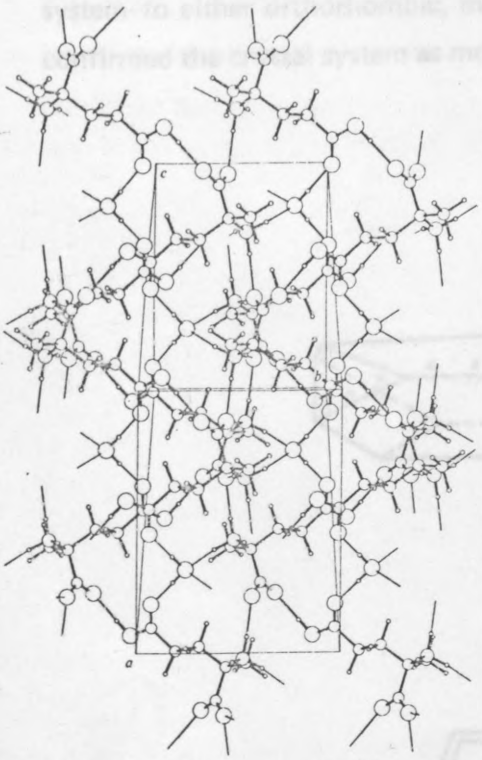

Fig. 2. Crystal packing diagram projected along a*.

1972) or inactivity (Curtis *et al.*, 1972), has been found in this study also to exist in the *trans* conformation. The implications of this will be discussed elsewhere.

We thank Drs P. G. Owston and E. M. McPartlin for use of the diffractometer at The Polytechnic of North London. Financial support from The Lord Dowding Fund and The City University is gratefully acknowledged.

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B2.2 Notes to the structure determination

The following notes describe the background work, which, for the sake of brevity, were not included in the preceding short communication. The notes enumerated below are annotated in the text.

(i) *Crystal morphology.* Habit diagrams of a typical crystal are shown in Fig 2.1. The morphology was not helpful in selecting a likely crystal system but conoscopic (convergent light) observations revealed the crystal to be biaxial which restricted the crystal system to either orthorhombic, monoclinic or triclinic. X-Ray photographic analysis later confirmed the crystal system as monoclinic.

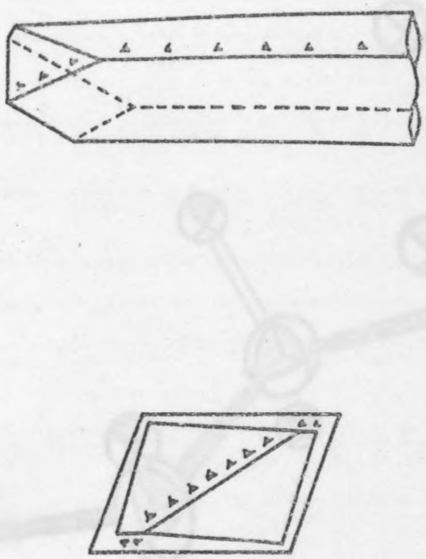


Fig B2.1 Crystal habit diagrams

(ii) *Structure refinement.* As a preliminary step to the structure refinement the number of α-methyl glutamic acid (α-MG) molecules per unit cell and the number of water molecules per α-MG molecule were determined. From the crystal density (ρ = 1.39 (1) Mgm^{-3}) derived by the flotation method, and the unit cell volume (U = 1621 (1) $\AA^3$) the mass per unit cell, M_u, was calculated as 1357 (10) amu. Since the molecular weight of α-MG is 161 amu, M_u corresponds to eight α-MG molecules and four water molecules (MW α–MG hemihydrate = 170 amu).

(iii) *Lists of structure factors and anisotropic thermal parameters.* These are given in Tables B2.1 and B2.2 respectively. Fig B2.2, drawn using the programme ORTEP, illustrates the thermal parameters listed in Table B2.2 and should be compared with Fig 1 of the published text.

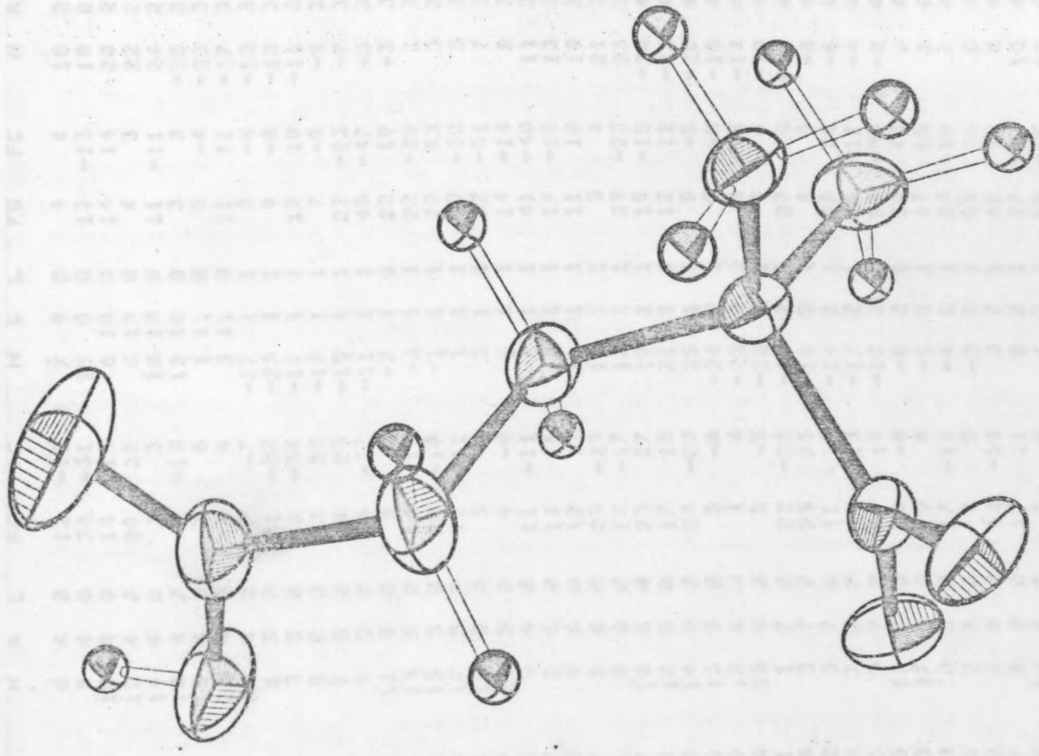
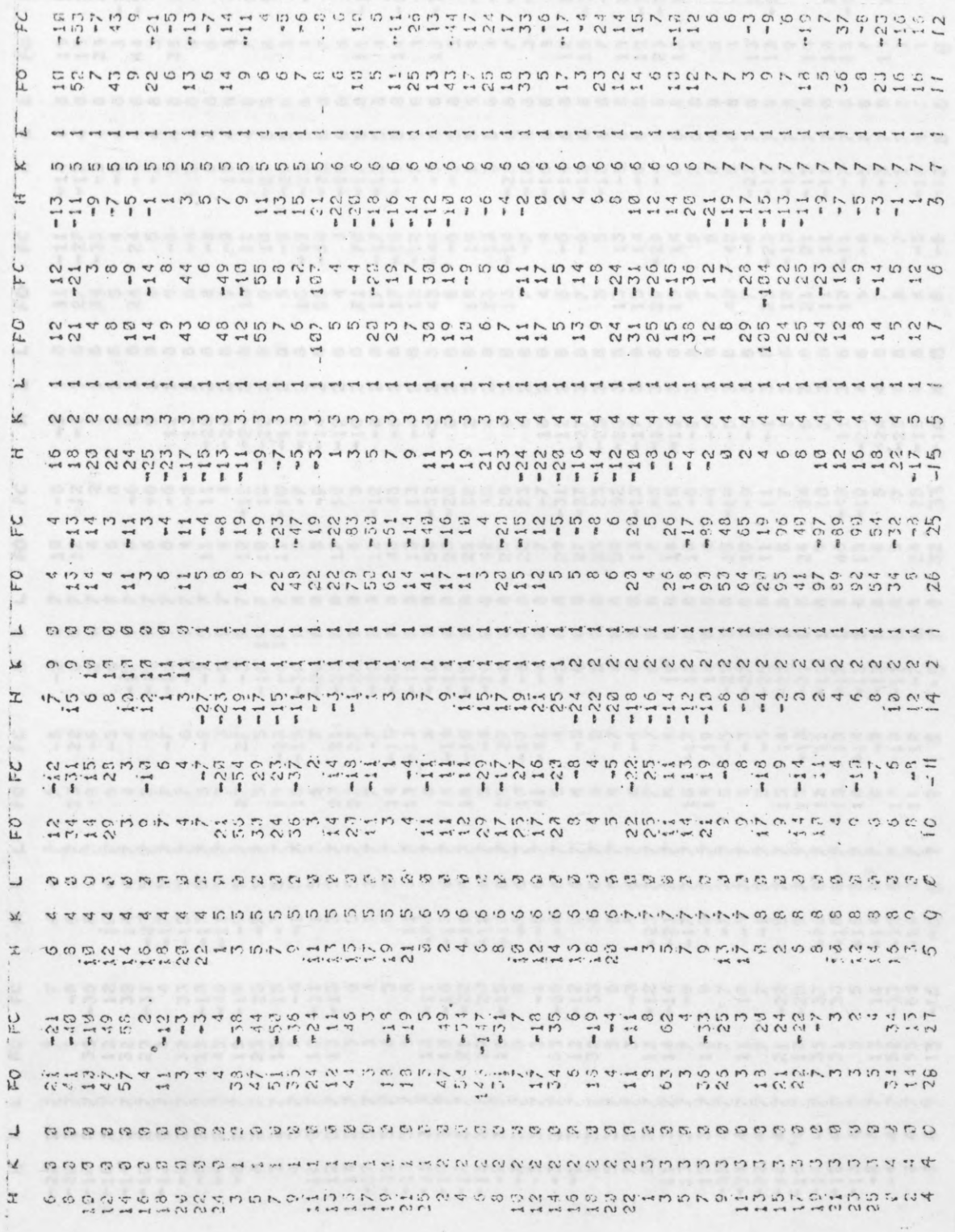


Fig B2.2 *ORTEP illustration of the atomic thermal parameters listed in Table B2.2*

Table B2.1 List of Structure factors for DL-α-methyl glutamate



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-10	10	1	4	5
-4	10	1	8	8
-2	10	1	7	7
0	10	1	7	-8
2	10	1	8	-8
4	10	1	4	5
6	10	1	13	15
8	10	1	7	7
12	10	1	4	-5
-7	11	1	5	6
-5	11	1	5	-5
-1	11	1	7	-6
1	11	1	11	-11

H	K	L	FO	FC
5	11	1	8	9
-26	0	2	13	-13
-24	0	2	10	10
-22	0	2	10	-9
-20	0	2	35	-35
-18	0	2	16	16
-16	0	2	5	4
-12	0	2	15	13
-10	0	2	24	-24
-8	0	2	60	-55
-6	0	2	116	-115
-4	0	2	49	54
-2	0	2	9	9
0	0	2	43	-46
2	0	2	47	-50
4	0	2	3	3
6	0	2	6	-4
8	0	2	61	-61
10	0	2	36	37
12	0	2	35	-34
14	0	2	63	-65
16	0	2	45	44
18	0	2	23	23
20	0	2	15	-16
22	0	2	6	-6
-21	1	2	14	-15
-19	1	2	15	15
-17	1	2	11	11
-15	1	2	8	-7
-13	1	2	22	22
-11	1	2	32	-30
-9	1	2	11	-10
-7	1	2	81	85
-5	1	2	82	85
-3	1	2	79	-81
3	1	2	10	7
5	1	2	3	-5
7	1	2	10	11
9	1	2	14	11
11	1	2	9	9
13	1	2	32	-33
15	1	2	5	6
17	1	2	11	12
19	1	2	4	-3
21	1	2	5	4
23	1	2	5	4
-24	2	2	10	-10
-20	2	2	17	18

H	K	L	FO	FC
-18	2	2	3	3
-16	2	2	7	-10
-14	2	2	29	29
-12	2	2	21	21
-10	2	2	27	26
-8	2	2	6	5
-6	2	2	73	-71
-4	2	2	60	62
-2	2	2	27	28
0	2	2	38	40
4	2	2	14	-15
8	2	2	22	23
10	2	2	5	4
12	2	2	10	-9
14	2	2	4	4
16	2	2	8	7
18	2	2	6	5
20	2	2	14	14
22	2	2	7	-7
-27	3	2	11	11
-23	3	2	16	-16
-21	3	2	6	-7
-19	3	2	13	13
-17	3	2	4	5
-13	3	2	20	-19
-11	3	2	50	-51
-7	3	2	12	12
-5	3	2	61	57
-3	3	2	42	43
3	3	2	14	-18
5	3	2	7	8
7	3	2	27	30
9	3	2	2	-3
11	3	2	15	-15
15	3	2	8	-7
17	3	2	15	-14
19	3	2	11	12
21	3	2	9	9
23	3	2	8	-8
-26	4	2	10	-9
-24	4	2	5	-5
-20	4	2	8	8
-18	4	2	10	10
-16	4	2	22	-23
-14	4	2	18	-19
-12	4	2	16	17
-10	4	2	3	-2
-8	4	2	22	-22

H	K	L	FO	FC
-6	4	2	24	-22
-4	4	2	14	16
-2	4	2	11	10
0	4	2	4	6
8	4	2	7	-8
14	4	2	4	-6
16	4	2	3	-4
18	4	2	9	-9
22	4	2	7	6
-25	5	2	5	-5
-23	5	2	11	11
-19	5	2	10	11
-17	5	2	28	28
-15	5	2	17	-17
-13	5	2	32	-32
-11	5	2	6	6
-9	5	2	28	27
-7	5	2	31	31
-5	5	2	29	29
-3	5	2	35	33
3	5	2	5	-4
5	5	2	57	56
7	5	2	4	-5
11	5	2	37	37
15	5	2	13	-12
17	5	2	4	-4
19	5	2	4	-3
21	5	2	9	8
-22	6	2	6	6
-20	6	2	5	6
-14	6	2	6	5
-10	6	2	26	-27
-6	6	2	31	31
-4	6	2	10	11
-2	6	2	12	12
0	6	2	7	8
2	6	2	40	-42
4	6	2	19	-19
6	6	2	27	28
8	6	2	23	24
10	6	2	7	-6
12	6	2	5	-5
14	6	2	5	4
16	6	2	5	-6
18	6	2	4	-5
20	6	2	7	7
-21	7	2	14	15
-10	7	2	4	-4

H	K	L	FO	FC
-17	7	2	6	-6
-13	7	2	27	-27
-11	7	2	26	-26
-9	7	2	21	21
-7	7	2	23	24
-5	7	2	12	-12
-3	7	2	11	-11
-1	7	2	8	-6
1	7	2	18	-18
3	7	2	19	-19
5	7	2	15	-15
7	7	2	7	7
9	7	2	7	6
11	7	2	5	-5
13	7	2	10	10
15	7	2	15	-13
17	7	2	21	-20
-18	8	2	8	-8
-16	8	2	12	12
-12	8	2	14	-15
-10	8	2	5	-5
-8	8	2	11	11
-6	8	2	8	9
-4	8	2	4	-4
-2	8	2	4	4
0	8	2	5	5
4	8	2	10	-10
8	8	2	17	19
10	8	2	7	-7
14	8	2	7	7
16	8	2	9	-9
-17	9	2	9	8
-15	9	2	12	13
-13	9	2	8	-8
-11	9	2	6	-6
-9	9	2	4	3
-7	9	2	20	19
-5	9	2	5	5
-3	9	2	4	-3
-1	9	2	10	10
1	9	2	4	-4
3	9	2	4	-3
7	9	2	10	9
9	9	2	15	15
13	9	2	4	-4
-14	10	2	8	-8
-6	10	2	4	-3
-4	10	2	14	11

H	K	L	FO	FC	H	K	L	FO	FC	H	K	L	FO	FC	H	K	L	FO	FC	H	K	L	FO	FC
-10	2	11	4	-4	-7	5	11	11	11	-21	1	12	18	13	-10	4	12	8	-7	-15	3	13	5	-5
-6	2	11	11	11	-5	5	11	11	-11	-19	1	12	39	30	-8	4	12	14	14	-13	3	13	9	8
-4	2	11	7	7	-3	5	11	6	7	-15	1	12	21	-21	-6	4	12	10	13	-9	3	13	10	-10
-2	2	11	15	-15	-1	5	11	7	-8	-13	1	12	5	-7	-4	4	12	9	-9	-7	3	13	11	10
2	2	11	16	-17	1	5	11	14	-14	-11	1	12	18	-18	-2	4	12	10	-10	-5	3	13	9	10
4	2	11	4	3	-22	6	11	11	11	-9	1	12	5	-4	-13	5	12	13	-13	-22	4	13	7	6
-27	3	11	8	-8	-20	6	11	14	-14	-7	1	12	18	19	-11	5	12	11	-11	-18	4	13	15	-15
-23	3	11	11	12	-16	6	11	10	10	-5	1	12	13	13	-9	5	12	14	14	-14	4	13	6	6
-21	3	11	4	6	-14	6	11	22	-22	-1	1	12	10	-10	-7	5	12	8	8	-12	4	13	6	-6
-17	3	11	7	-7	-12	6	11	7	-7	3	1	12	9	-8	-5	5	12	9	-8	-10	4	13	5	-5
-15	3	11	21	-22	-10	6	11	19	18	-25	2	12	21	-21	-3	5	12	4	-4	-8	4	13	4	5
-13	3	11	8	-8	-6	6	11	16	-17	-24	2	12	5	-3	-18	6	12	8	-7	-17	5	13	9	-8
-9	3	11	13	13	0	6	11	14	-14	-22	2	12	15	16	-12	6	12	5	5	-22	0	14	14	-14
-7	3	11	16	16	-17	7	11	7	7	-18	2	12	6	7	-10	6	12	4	4	-20	0	14	8	-8
-5	3	11	12	-12	-15	7	11	13	-13	-16	2	12	7	-6	-8	6	12	5	-5	-18	0	14	12	11
-3	3	11	6	-5	-13	7	11	15	-15	-14	2	12	17	-16	-25	1	13	4	-4	-16	0	14	11	11
-1	3	11	9	-10	-11	7	11	5	5	-10	2	12	5	-4	-23	1	13	6	-5	-14	0	14	16	-15
1	3	11	15	-16	-9	7	11	13	13	-8	2	12	4	4	-21	1	13	10	10	-10	0	14	13	12
3	3	11	7	6	-7	7	11	10	9	-2	2	12	7	-7	-17	1	13	12	-12	-8	0	14	14	-15
5	3	11	11	12	-5	7	11	8	-8	0	2	12	9	-9	-15	1	13	4	-5	-6	0	14	17	-17
-24	4	11	18	18	-24	0	12	17	-17	-25	3	12	6	-7	-13	1	13	8	7	-21	1	14	6	-6
-22	4	11	13	10	-22	0	12	7	7	-23	3	12	9	9	-9	1	13	19	-18	-17	1	14	14	13
-20	4	11	8	8	-20	0	12	25	25	-17	3	12	10	16	-5	1	13	14	14	-15	1	14	4	3
-16	4	11	21	-21	-18	0	12	8	7	-15	3	12	4	-4	-3	1	13	8	-8	-11	1	14	9	9
-12	4	11	14	14	-16	0	12	9	-8	-13	3	12	9	-8	-1	1	13	5	-5	-7	1	14	18	-18
-8	4	11	23	23	-14	0	12	6	6	-11	3	12	11	-11	-24	2	13	7	-7	-20	2	14	5	-6
-6	4	11	9	10	-12	0	12	7	-7	-7	3	12	19	19	-22	2	13	15	14	-18	2	14	6	6
-4	4	11	15	-15	-10	0	12	9	-9	-5	3	12	11	11	-20	2	13	13	11	-14	2	14	4	4
0	4	11	5	-6	-8	0	12	13	13	-1	3	12	4	-5	-18	2	13	9	-8	-12	2	14	18	19
4	4	11	10	9	-4	0	12	18	17	-22	4	12	11	11	-16	2	13	6	-5	-10	2	14	6	-5
-23	5	11	13	10	-2	0	12	8	8	-20	4	12	8	8	-6	2	13	9	9	-3	2	14	7	-8
-19	5	11	4	-3	0	0	12	15	-14	-18	4	12	10	-9	-2	2	13	4	-3	-17	3	14	4	-5
-17	5	11	15	15	2	0	12	5	-4	-16	4	12	11	-10	-21	3	13	14	14	-15	3	14	7	-6
-15	5	11	7	-8	-27	1	12	4	3	-14	4	12	12	11	-19	3	13	5	5	-13	3	14	11	10
-13	5	11	23	-23	-25	1	12	18	-18	-12	4	12	4	4	-17	3	13	10	-11	-9	3	14	12	-12
-9	5	11	26	26	-23	1	12	14	-14															

Table B2.2 Anisotropic Thermal Parameters ($\mathring{A}^2 \times 10^4$)

	U_{11}	U_{22}	U_{33}	U_{23}	U_{13}	U_{12}
C(1)	264 (6)	217 (6)	174 (6)	21 (5)	111 (5)	37 (5)
C(2)	221 (6)	218 (6)	186 (6)	−21 (5)	101 (5)	−10 (5)
C(3)	284 (6)	279 (7)	271 (7)	1 (6)	166 (6)	−39 (6)
C(4)	403 (8)	283 (7)	413 (9)	104 (7)	237 (7)	15 (7)
C(5)	455 (9)	289 (8)	304 (8)	40 (7)	178 (7)	−27 (7)
C(6)	309 (7)	353 (8)	316 (8)	−27 (7)	135 (6)	87 (6)
N	260 (5)	255 (6)	191 (5)	−34 (5)	125 (4)	−38 (5)
O(1)	289 (5)	384 (6)	264 (5)	−127 (5)	120 (4)	−32 (4)
O(2)	320 (5)	476 (7)	245 (5)	−71 (5)	182 (4)	−104 (5)
O(3)	650 (8)	386 (7)	305 (6)	4 (5)	237 (6)	−179 (6)
O(4)	1438 (17)	682 (11)	303 (7)	−58 (8)	290 (9)	−584 (12)
O (5)	469 (10)	272 (80)	396 (9)	0	197 (8)	0

Appendix C E-PACE computer programme

C.1 Development of the programme

C.1.1 Introduction

The programme was developed principally for application to prostaglandin (PG) molecules although other compounds may be studied using this method.

As discussed in subsections 6.3.3 and 6.3.4, the programme was only required to generate all those molecular conformers which exhibit a probability assignment of 1.0. Thus, subsequent to a 120° rotation about a bond, the probability assignment of the newly generated conformer must be deduced in order to determine whether that conformation is allowable. The programme was also required to detect superposition of atoms within a molecular conformation in order to disallow those conformations exhibiting this condition.

In order to accurately measure the inter-functional group, X_D, distances it was necessary to include "dummy" atoms in the structure. Those functional groups thought to be likely reactive sites are discussed in subsection 6.3.2. See also subsection 5.2.2. for the general principles of X_D measurement.

C.1.2. The S-index

It was also considered useful to obtain some indication of the conformational proximity of the side chains of the PG molecule and thus the "S index" was introduced. The S index, which is a summation of interatomic distances between defined atoms of opposing chains of the PG molecule for a given conformation, may be expressed as –

$$S = \Sigma_i \left(\Sigma_j r_{ij} \right)$$

where i labels atoms in chain α

j labels atoms in chain β

r_{ij} is the distance between atom i and j

As discussed in subsection 6.3.4 the stabilisation energy of an aligned conformation can be large. This methylene chain-chain attractive dispersion force can be equated to hydrophobic force. A provision for externally defining an S-index limit was made so that conformers not adopting an aligned conformation could be excluded from the analysis.

C.2 Explanation of the programme

C.2.1 Preface

Prior to the output of atom labels the numeric input form of the labels was converted to an alphanumeric output form in which the atomic number is replaced by the element symbol.

The following text sections are designed to be read in conjunction with their corresponding flowcharts (C.2.10) which are usually self-explanatory. If any programme variable or array name discussed in the text is not simultaneously defined then definitions may be found in the glossary (subsection C.2.9).

C.2.2 Input data

In order to generate the required molecular conformers, an initial conformer of probability assignment 1.0 must be set up. From the structural formula, the molecule was constructed using standard bond lengths and angles (Pople & Beveridge, 1970). The construction process was performed by the computer programme SPIN, which was a reduced and modified version of the programme MOJO (Borthwick, 1972). A three atom structure was initially defined and then, using the appropriate bond lengths, bond angles and torsion angles as input, the remaining atoms were added sequentially. In order to facilitate the calculation of X_D distances "dummy" atoms were also added to the molecular structure to represent charge centres where no "real" atoms exist e.g. double bonds. However, a restriction is that the dummy atoms must always be added at the end of the construction sequence to enable them to be recognised by the E-PACE programme as such. The output of the SPIN programme comprised a list of numeric atom-labels* with their corresponding x, y and z atomic coordinates, which provided the E-PACE input of the first column of the array ATINF and the array ATCO respectively.

The array ATINF (atomic information) of dimensions 99 by 17 contained the atomic data required by the programme, each row of the array corresponding to a different atom. As mentioned earlier, column 1 contained the numeric atom-labels; colume 2 contained the atomic number of each atom and column 3 contained either of the integers 0,1 or 2 which define those interatomic distances to be included in the S index. An interatomic distance was included only if one atom contained the integer 1 in colume 3 and the other atom an integer 2, or *vice versa*. For example, in the case of the PG molecule, the "backbone" atoms

** The first digit of each numeric atom-label denotes the atomic number which implies the element concerned, whilst the final two digits denote the sequence number within that periodic table element. The atomic number of a dummy atom is zero.*

of chain α may containe the integer 1 and those of chain β contain the integer 2; all other atoms then contained the integer 0. Column 4 contained either the integer 0 or 1; th integer 1 defined that atom to be required for the calculation of X_D distances. Columns 5 to 8 contained the numeric atom-labels of those atoms which are directly covalently bonded to the atom concerned. In addition, columns 9 to 17 contained the labels of those atoms which are not directly covalently bonded but which are within a two-bond link from the atom considered. This information was required since all these atoms were neglected from the Van der Waals' steric analysis with respect to the atom under consideration (see C.2.7). It was a requirement of the programme that the sequence of atoms considered must be consistent between the arrays ATCO and ATINF.

The input data also comprised the title (TITLE) of the molecule; the number of real atoms in the molecule (NOAT); the number of dummy atoms (NODU); the number of atoms (NOTA) located at the termini of the complete set of rotors considered; the number of rotors considered (NRO) and a value of the S index limit (SLIM). Data was also required for input into the atomic connectivity array, CONNECT, which contained the connectivity of each atom at the rotors' termini, the purpose of which is discussed in section C.2.4. Additionally, the subroutine ORBIT (C.2.8), which performed the appropriate rotations about the rotors, required for each rotor the number of atoms in the rotated group (NGP) and also the numeric atom-labels of those atoms.

C.2.3 Main programme (Refer Flowchart 1, Programme 2)

The structure of the main programme is adequately outlined in flowchart 1 (C.2.10) and listed in programme 2 (C.2.11). The various sections of the programme are discussed in the following text sections; however, the method of generation of the conformers requires explanation here.

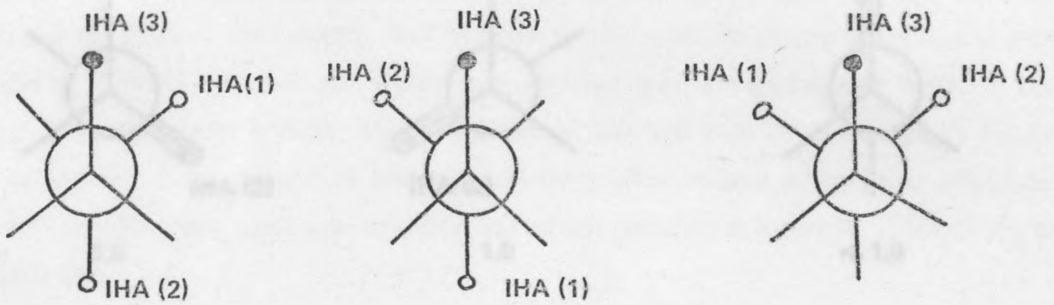
The rotors were considered sequentially beginning at rotor number 1 (NOB = 1). Generation of the two primary conformers involved only rotation about this rotor. Subsequent to reassumption of the initial conformation, the 3rd conformation was generated by a 120° rotation about rotor number 2. Rotation was then transferred back to rotor number 1 to generate two further conformers and then rotation about rotor number 2 resumed. Thus, by successive sequential rotations about all the rotors the complete set of conformers was generated. The actual sequence of the rotors is irrelevant except that, once arbitrarily chosen, that sequence must be maintained.

C.2.4 Section to deduce the species of rotor; whether $C_1 - C_2$ or $C_1 - \tilde{C}_1$

As discussed by Borthwick and coworkers (1977), the $C_1 - C_2$† and $C_1 - \tilde{C}_1$† rotors need only be considered during this preliminary investigation. The species of rotor may be distinguished by inspection of the connectivities of the atoms at the termini of the rotors, which are contained in the array CONNECT. However the connectivity inspection must be referred to the appropriate element since heteroatoms* may comprise rotor termini which may exhibit valencies different from that of carbon. In this respect, however, provision was only made for the heteroatoms nitrogen and oxygen since none of the PG molecules studied contained any other heteroatoms at rotor termini.

C.2.5 Section to deduce the local conformation about a $C_1 - C_2$ rotor (refer Flowchart 2)

Subsequent to rotation about a $C_1 - C_2$ rotor the local conformation of that rotamer was required in order to assign a probability to the corresponding molecular conformer. It was assumed that there were only three possible rotamer conformations which are illustrated below (Fig. C.1) with their corresponding probability assignments.



rotamer:	I	II	III
probability:	1.0	1.0	≤ 1.0

Fig C.1 Rotation about a $C_1 - C_2$ rotor

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† C_2 denotes methylene — CH_2

C_1 " methine — C_2H

$\tilde{C}_1$ " methene — $C{=}H_2$

* *A "heteroatom" is defined as any atom, other than carbon, with an atomic number greater than six.*

In order to distinguish between the rotamers (I, II and III) above, the distances of heavy atom (1) (IHA(1)) to IHA(3) and IHA(2) to IHA(3) were measured. After a rotation of 120° the measurements were repeated and the procedure repeated again after an additional rotation of 120°. (A further rotation of 120° then returned the rotamer to its initial conformation). Then, if either of the maximum distances of the sets IHA(1) to IHA(3) and IHA(3) corresponded to the initial rotamer then, by inspection of the Newman diagrams above, the rotomer exhibited a probability assignment of 1.0; if not then the rotomer was assigned a probability assignment of < 1.0. Provision was also made for viewing the rotor in the opposite direction, since each rotomer exhibited only a 1-fold rotation axis about its rotor axis.

C.2.6 Section to deduce the local conformation about a $C_1 - \tilde{C}_1$ rotor (Refer Flowchart 3)

In common with C.2.5 the local conformation about a rotor was required but here a $C_1 - \tilde{C}_1$ rotor was under investigation. The three classically possible rotomers are illustrated below (Fig. C.2) with their corresponding probability assignments.

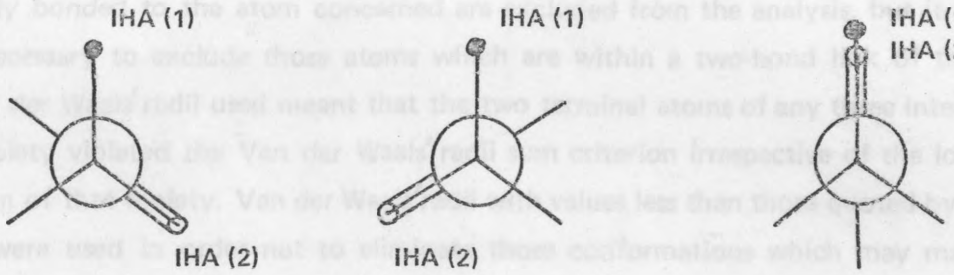


Fig C.2 Rotation about a $C_1 - \tilde{C}_1$ rotor

In order to deduce the conformation of the rotomer the distance of heavy atom (1) (IHA(1)) to IHA(2) was measured. After a subsequent bond rotation of 120° the distance IHA(1) to IHA(2) was again measured and the measuring procedure repeated again after a further rotation of 120°. (An additional rotation of 120° then returned the rotomer to its initial conformation). The minimum of the set of distances was then found. By inspection of the Newman diagrams above it is clear that if the initial rotomer corresponded to the minimum

then it had a probability assignment of < 1.0; if not then it had a probability assignment of 1.0.

C.2.7 Subroutine SIADVDW (Refer to Flowchart 4)

Incorporated into this subroutine was a section which tested for Van der Waals' steric exclusion of a conformer, a section which calculated the required X_D distances and also a section which calculated a value of the S index.

Since dummy atoms were required only for the calculation of X_D distances they were excluded from the Van der Waal's steric exclusion analysis and also from theS index summation.

The "Van der Waals' steric exclusion analysis" refers to the detection of superposition of atoms within a molecular conformer in order to disallow those conformations. For each conformation the analysis detected if any two atoms approached to within less than the sum of their respective Van der Waals' radii. Of course, those atoms which are directly covalently bonded to the atom concerned are excluded from the analysis, but it was also found necessary to exclude those atoms which are within a two-bond link of the atom. The Van der Waals' radii used meant that the two terminal atoms of any three interbonded atom moiety violated the Van der Waals' radii sum criterion irrespective of the local conformation of that moiety. Van der Waals' radii with values less than those quoted by Pauling (1960) were used in order not to eliminate those conformations which may marginally violate the Van der Waals' radii sum criterion yet which could exist by small, local distortion of torsion angles.

It is interesting to note that those conformations exhibiting this condition are probably those which would be the most stabilised by hydrophobic forces. An arbitrary value of half the standard Van der Waals' radius (Pauling, 1960) was adopted for each element.

The purpose and design of the S index has already been outlined in C.1.2 and mode of calculation outlined C.2.2. It only remains to add that the "defined" atoms, between which the interatomic distances are calculated for inclusion in the S index, refer to the 'backbone' atoms of each chain of the PG molecule.

Similarly, X_D distances have already been discussed in C.1.1 and their mode of calculation outlined in C.2.2.

C.2.8 Subroutine ORBIT incorporating the subroutine SUBORB (Refer Flowchart 5)

Essentially the subroutine ORBIT, in conjunction with the subroutine SUBORB, performed bond rotations by the predefined incremental angle of 120°. The end product of the rotation procedure was a new set of atomic coordinates in the array ATCO corresponding to a new molecular conformer. For each rotor only those atoms which were defined in the array ORBAT were rotated. Thus, in commuting from one conformer to the next, only a proportion of the atomic coordinates were replaced.

The subroutine SUBORB simply set up the rotation matrix:

$$\begin{bmatrix} \cos\theta & \sin\theta & 0 \\ -\sin\theta & \cos\theta & 0 \\ 0 & 0 & 1 \end{bmatrix}$$

in order to perform the rotation by the predefined angle θ.

C 2.9 Glossary of programme variable and array names

(i) Contained within the main programme

AMAX	(R* array)	maximum value contained in DIST.
AMIN	(R variable)	minimum value contained in DIST.
ARSN	(I* variable)	array sequence number of element in ATINF.
ATCØ	(R array)	atomic coordinate array
ATINF	(I array)	atomic information array
ATM	(I array)	atomic symbol array
ATMAT	(I array)	atomic matrix array
ATNØ	(I array)	atomic symbol array
DIST	(R array)	interatomic distance array
DX	(R variable)	interatomic change in x-coordinate
DY	(R variable)	interatomic change in y-coordinate
DZ	(R variable)	interatomic change in z-coordinate
IAN1	(I variable)	atomic number of atom in ORBAT (NOB, 1)
IAN2	(I variable)	atomic number of atom in ORBAT (NOB, 2)
IARS	(I variable)	ATINF sequence number
IATØ	(I array)	atomic symbol array
IHA	(I array)	ATINF sequence number corresponding to a "heavy atom"
ISEQ	(I array)	atomic sequence numbers within a periodic table element.

** R denotes 'real' and I denotes 'integer'.*

IXDA	(I array)	array containing ATINF sequence numbers corresponding to atoms defining the X_D distances.
IZNO	(I array)	atomic number array
LBR	(I variable)	last rotor rotated
MATSUM	(I array)	atomic connectivity array
MAXR	(I array)	maximum interatomic distance array
NGP	(I array)	array containing the number of atoms in the rotated group for each rotor.
NOAT	(I variable)	number of real atoms in the molecule
NOB	(I variable)	number of the rotor.
NODU	(I variable)	number of dummy atoms introduced
NOGP	(I variable)	number of atoms in the rotated group
NOSA	(I variable)	number of atoms located at the termini of the set of rotors considered
NRB	(I variable)	number of rotors.
NROT	(I variable)	incremental angle of rotation
NTOR	(I variable)	array containing angles to which each rotor has been rotated.
ORAS	(I array)	array containing ATINF sequence numbers of atom labels contained in ORBAT
ORBAT	(I array)	array containing atom labels of atoms contained in the rotated group
SENO	(I array)	atomic sequence numbers within a periodic table element.

SEQN	(I array)	atomic sequence numbers within a periodic table element
SIADIND	(R variable)	S index
SIADLIM	(R variable)	S index limit
TITLE	(R array)	title of the molecule under consideration
VIOLI	(I variable)	atom violating the Van der Waals' radii sum analysis
VIOLJ	(I variable)	atom violating the Van der Waals' radii sum analysis
XD	(R array)	array containing X_D distances
ZNO	(I array)	atomic number array

(ii) Contained within the subroutine SIADVDW but not within the main programme

SUMVDW	(R variable)	sum of the atomic Van der Waal's radii.
VDWC	(R variable)	Van der Waals' radius of carbon
VDWH	(R variable)	Van der Waals' radius of hydrogen
VDWN	(R variable)	Van der Waals' radius of nitrogen
VDWO	(R variable)	Van der Waals' radius of oxygen

(iii) Contained within the subroutine ORBIT but not within the main programme

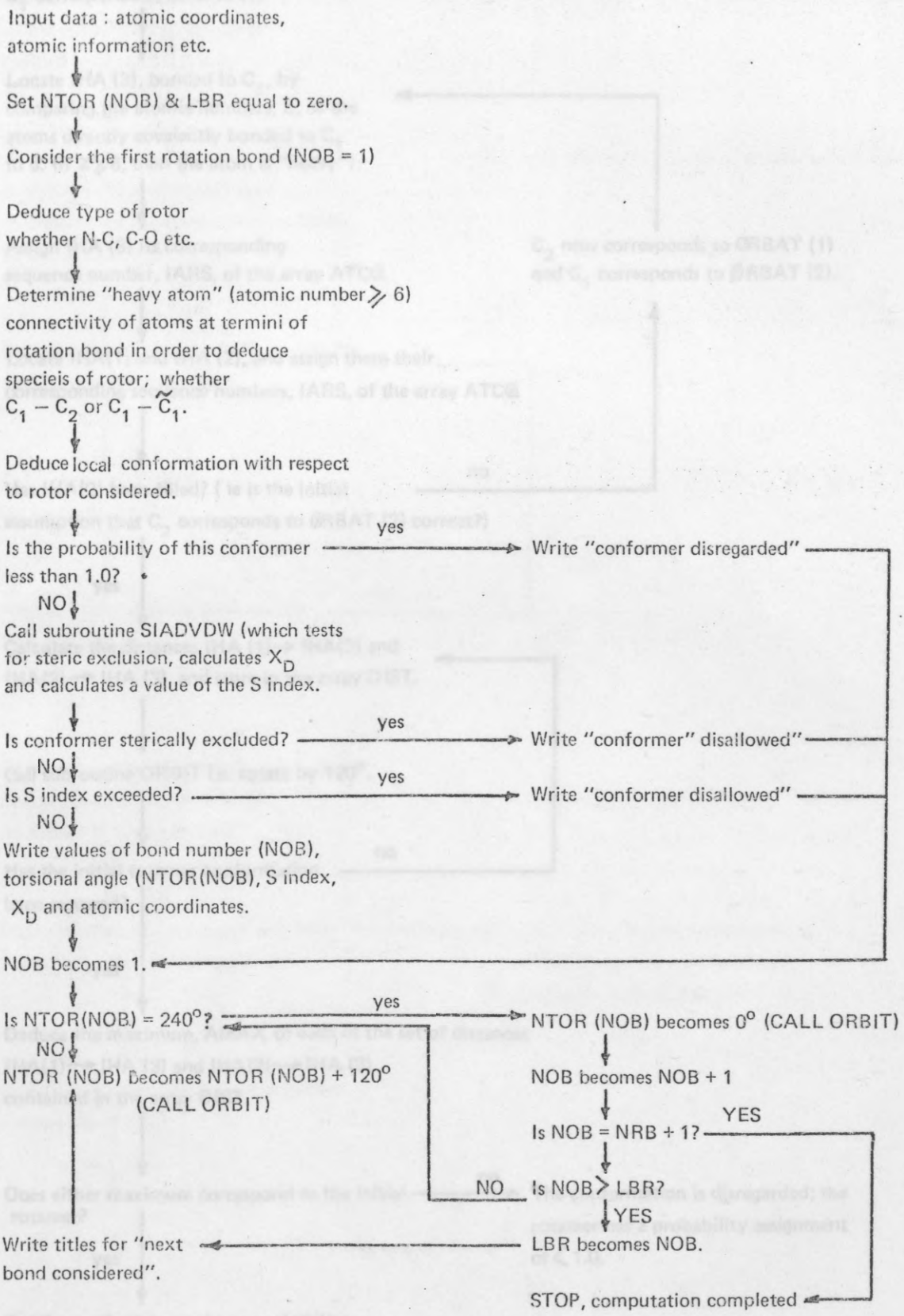
ANGROT	(R variable)	angle rotated
ANGY	(R variable)	angle y
ANGZ	(R variable)	angle z
TRAN	(R array)	translation

(iv) Contained within the subroutine SUBORB but not within the main programme

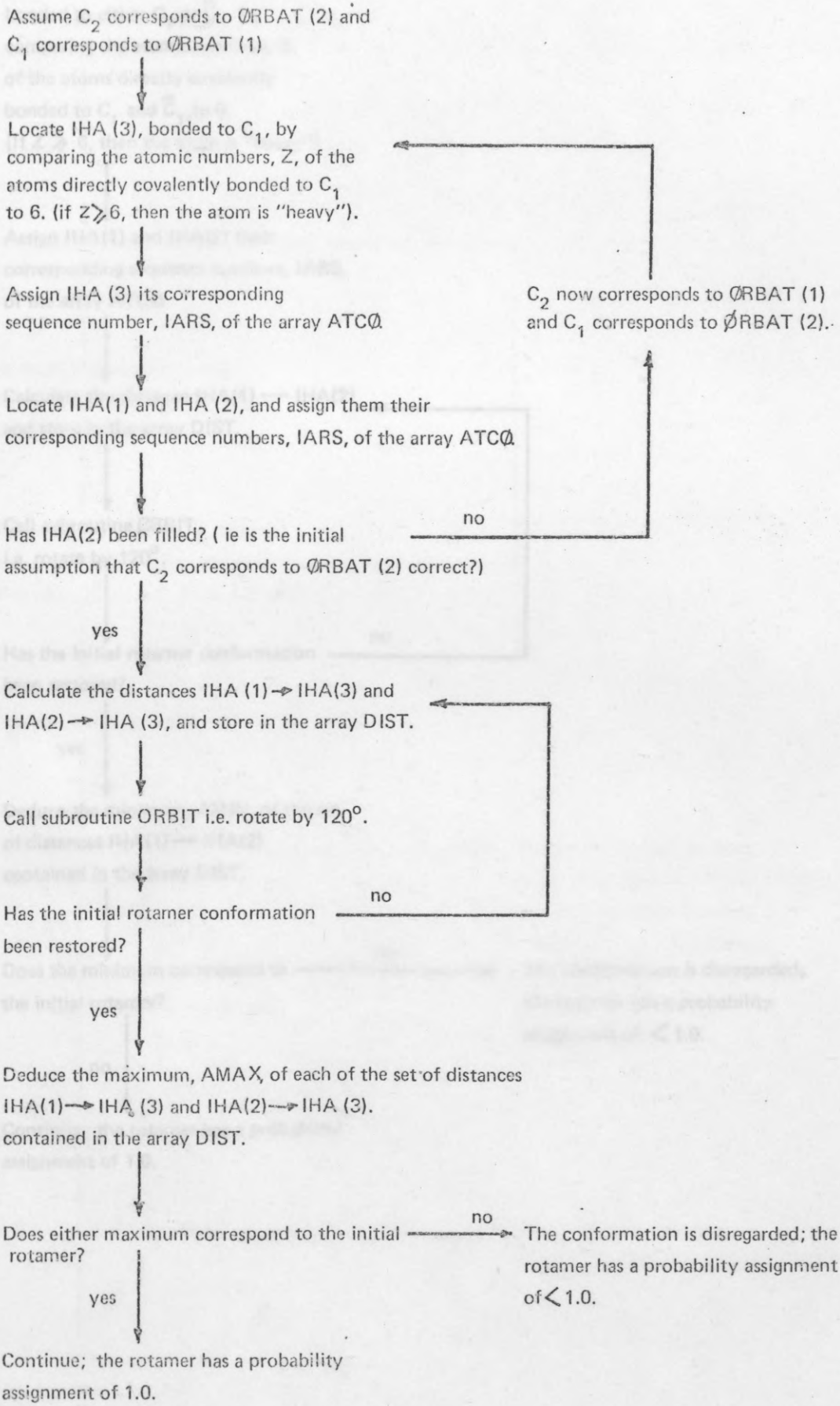
XA	(R array)	x coordinate
YA	(R array)	y coordinate
ZA	(R array)	z coordinate

C 2.10 The flowcharts

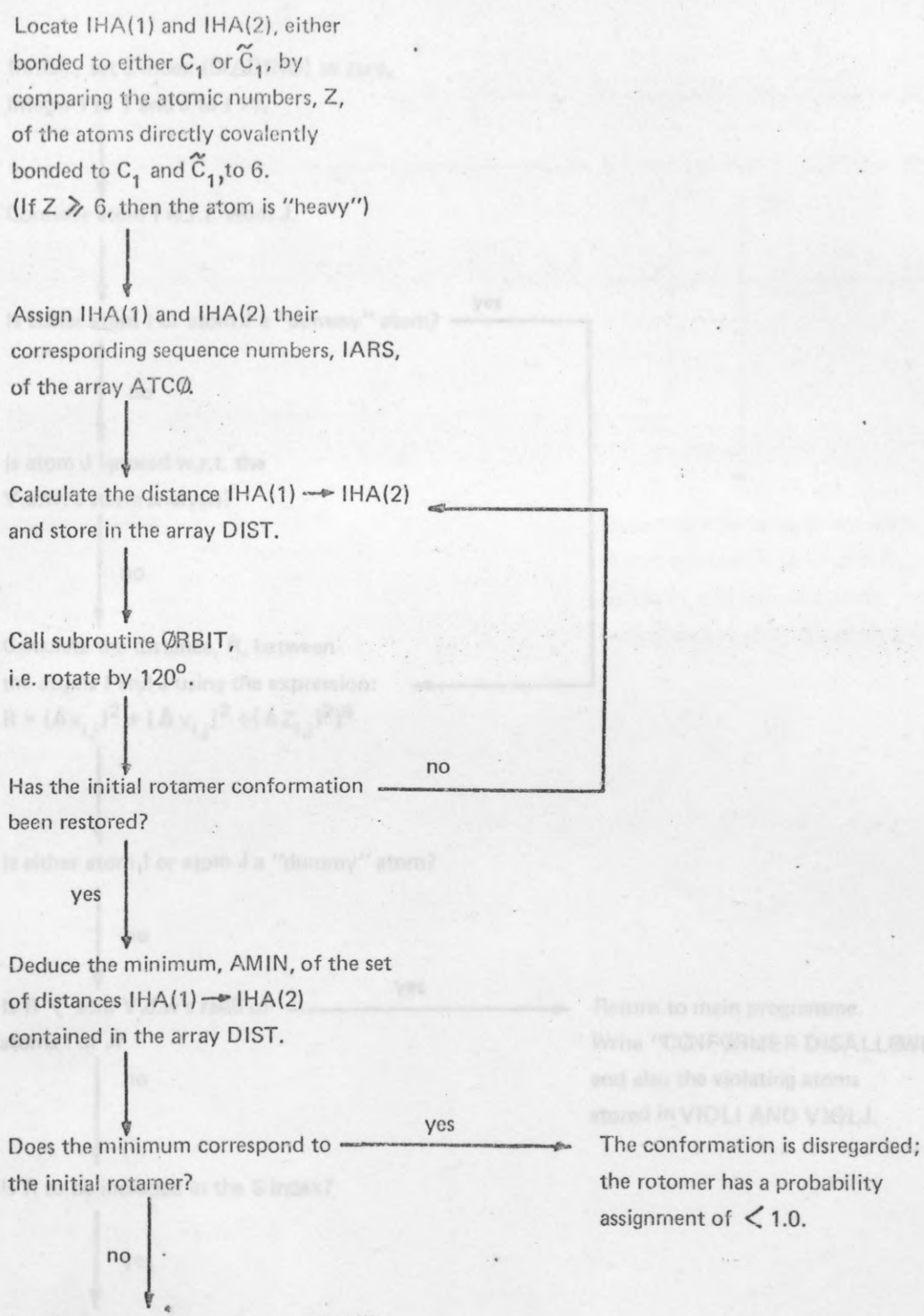
Flowchart 1 The main programme



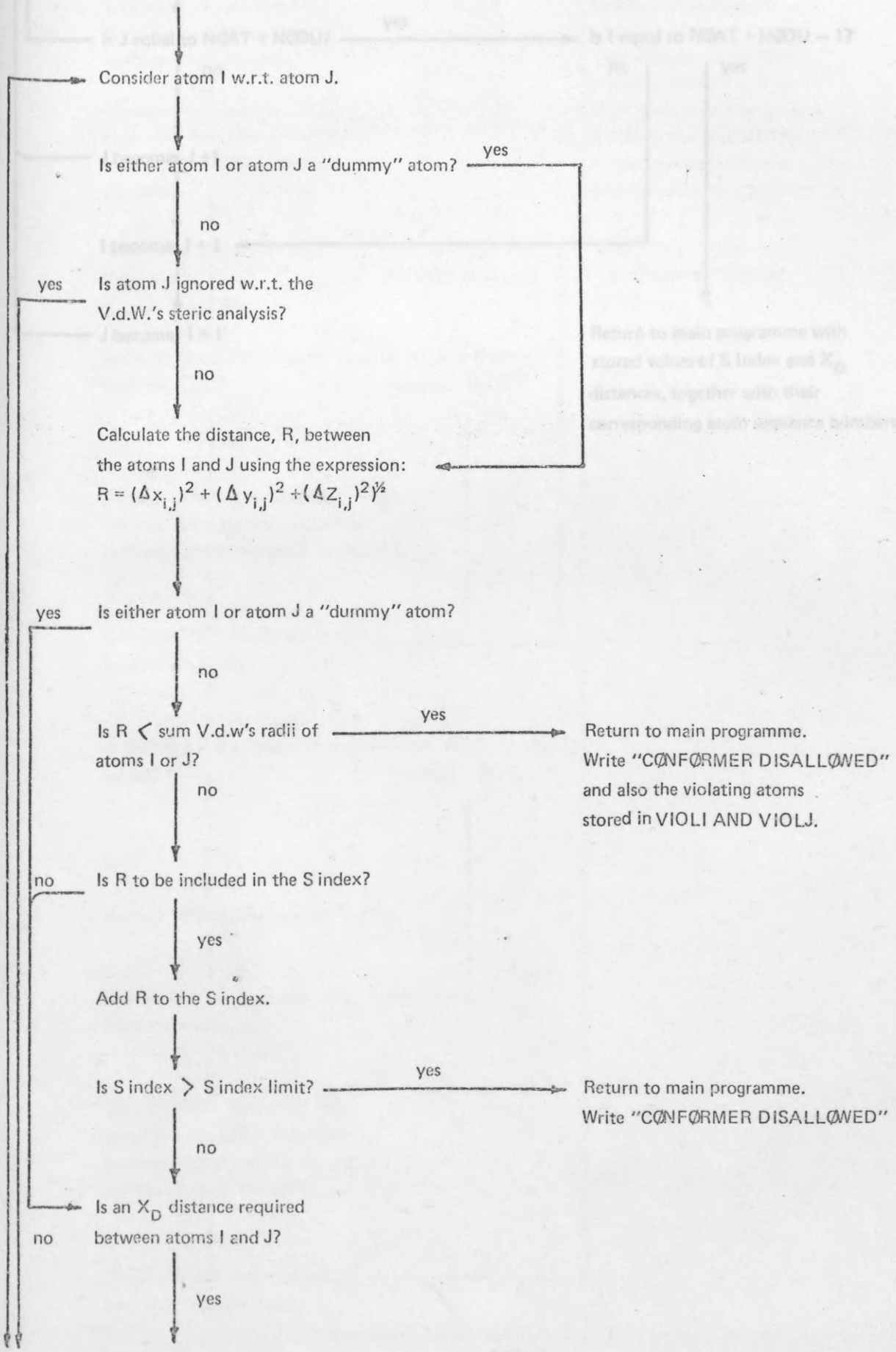
Flowchart 2. Section to deduce the local conformation about a C_1-C_2 rotor

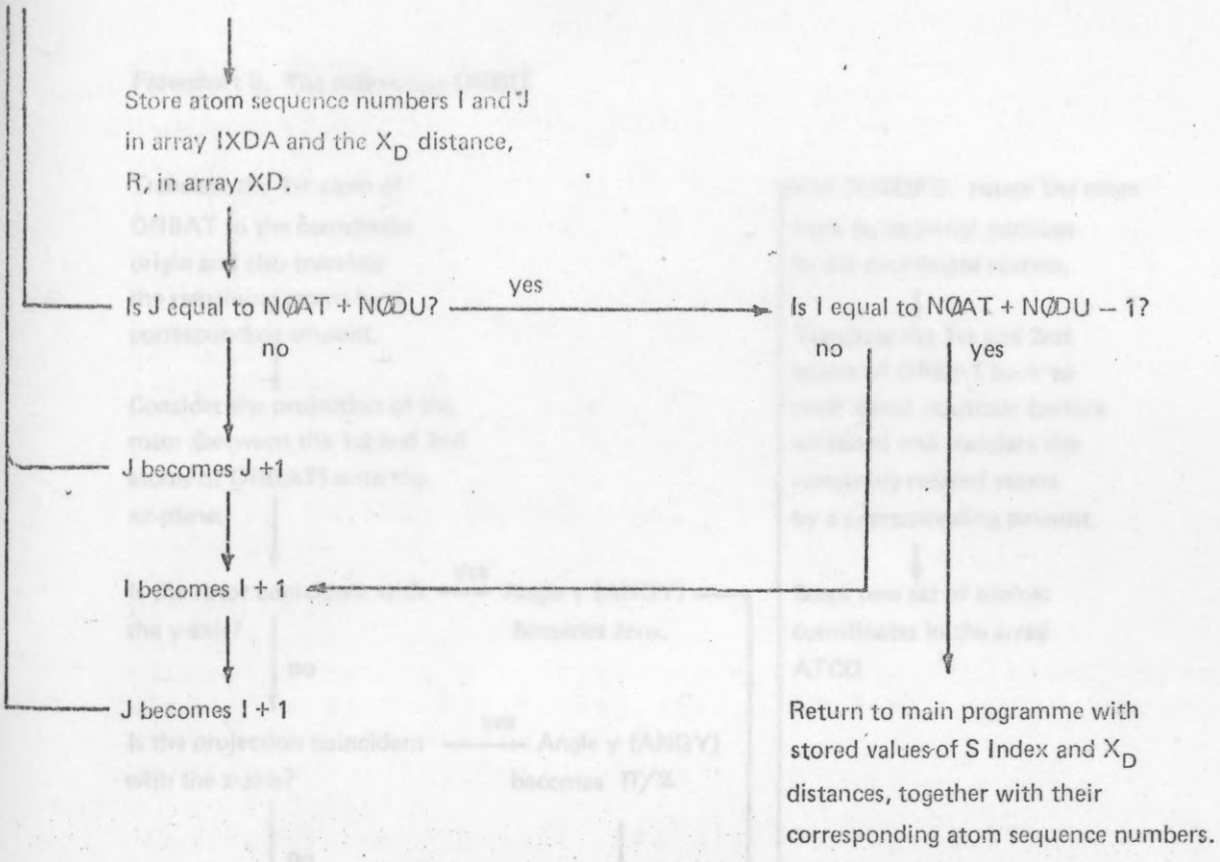


Flowchart 3. Section to deduce the local conformation about a $C_1 - \tilde{C}_1$ rotor

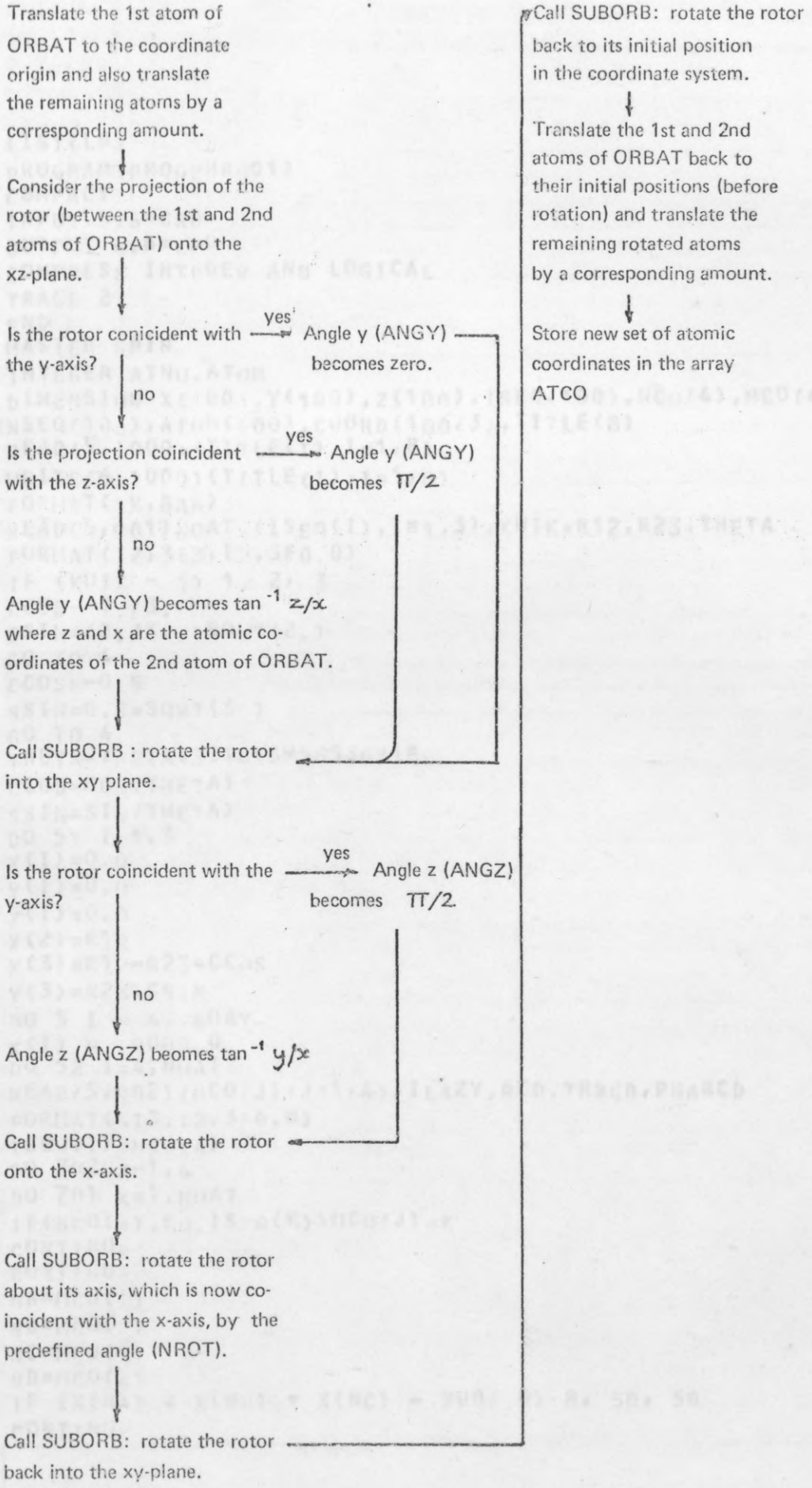


Flowchart 4. Subroutine SIADVDW




Store atom sequence numbers I and J in array IXDA and the X_D distance, R, in array XD.
Is J equal to NØAT + NØDU?
yes
Is I equal to NØAT + NØDU – 1?
no
yes
J becomes J +1
I becomes I + 1
J becomes I + 1
Return to main programme with stored values of S Index and X_D distances, together with their corresponding atom sequence numbers.

Flowchart 5. The subroutine ORBIT



C 2.11 Programme Listings

Programme 1.Spin

```
      LIST(LP)
      PROGRAM(PROGPHR091)
      COMPACT
      INPUT 5,5=CRD
      OUTPUT 6,6=LPO
      COMPRESS INTEGER AND LOGICAL
      TRACE 2
      END
      MASTER SPIN
      INTEGER ATNO,ATOM
      DIMENSION X(100),Y(100),Z(100),ISEQ(100),NCO(4),MCO(4),ATNO(100),
     XNSEQ(100),ATOM(100),COORD(100,3),TITLE(8)
      READ(5,1000)(TITLE(I),I=1,8)
      WRITE(6,1000)(TITLE(I),I=1,8)
 1000 FORMAT(1X,8A4)
      READ(5,901)NOAT,(ISEQ(I),I=1,3),KWIK,R12,R23,THETA
  901 FORMAT(I2,3I3,I2,3F0.0)
      IF (KWIK - 1) 1, 2, 3
    1 CCOS=-1./3.
      SSIN=(2./3.)*SQRT(2.)
      GO TO 4
    2 CCOS=-0.5
      SSIN=0.5*SQRT(3.)
      GO TO 4
    3 THETA=THETA*3.14159265E/180.
      CCOS=COS(THETA)
      SSIN=SIN(THETA)
    4 DO 51 I=1,3
      X(I)=0.0
      Y(I)=0.0
   51 Z(I)=0.0
      X(2)=R12
      X(3)=R12-R23*CCOS
      Y(3)=R23*SSIN
      DO 5 I = 4, NOAT
    5 X(I) = 10000.0
      DO 52 I=4,NOAT
      READ(5,902)(NCO(J),J=1,4),ILAZY,RCD,THBCD,PHABCD
  902 FORMAT(4I3,I2,3F0.0)
      ISEQ(I)=NCO(4)
      DO 702 J=1,4
      DO 701 K=1,NOAT
      IF(NCO(J).EQ.ISEQ(K))MCO(J)=K
  701 CONTINUE
  702 CONTINUE
      NA=MCO(1)
      NB=MCO(2)
      NC=MCO(3)
      ND=MCO(4)
    7 IF (X(NA) + X(NB) + X(NC) - 7000.0) 8, 50, 50
    8 CONTINUE
```

```
      IF (ILAZY - 8) 79, 78, 79
   78 RBC=SQRT((X(NC)-X(NB))**2+(Y(NC)-Y(NB))**2+(Z(NC)-Z(NB))**2)
      X(ND) = X(NC) + (X(NC) - X(NB))*RCD/RBC
      Y(ND) = Y(NC) + (Y(NC) - Y(NB))*RCD/RBC
      Z(ND) = Z(NC) + (Z(NC) - Z(NB))*RCD/RBC
      GO TO 52
   79 XA = X(NA) - X(NC)
      YA = Y(NA) - Y(NC)
      ZA = Z(NA) - Z(NC)
      XB = X(NB) - X(NC)
      YB = Y(NB) - Y(NC)
      ZB = Z(NB) - Z(NC)
      XYB=SQRT(XB**2+YB**2)
      K = 1
      IF (XYB - 0.1) 9, 10, 10
    9 K = 0
      XPA = ZA
      ZPA = -XA
      XA = XPA
      ZA = ZPA
      XPB = ZB
      ZPB = -XB
      XB = XPB
      ZB = ZPB
      XYB=SQRT(XB**2+YB**2)
   10 COSTH = XB/XYB
      SINTH = YB/XYB
      XPA = XA*COSTH + YA*SINTH
      YPA = YA*COSTH - XA*SINTH
   11 RBC=SQRT(XB**2+YB**2+ZB**2)
      SINPH = ZB/RBC
      COSPH=SQRT(1.-SINPH**2)
      XQA = XPA*COSPH + ZA*SINPH
      ZQA = ZA*COSPH - XPA*SINPH
   12 YZA=SQRT(YPA**2+ZQA**2)
      COSKH = YPA/YZA
      SINKH = ZQA/YZA
      IF (ILAZY - 1) 13, 14, 15
   13 COSD = 1.0
      SIND = 0
      GO TO 21
   14 COSD = 0.5
      SIND=0.5*SQRT(3.)
      GO TO 21
   15 IF (ILAZY - 3) 16, 17, 18
   16 COSD = -0.5
      SIND=0.5*SQRT(3.)
      GO TO 21
   17 COSD = -1.0
      SIND = 0
      GO TO 21
   18 IF (ILAZY - 5) 19, 20, 22
   19 COSD = -0.5
      SIND=-0.5*SQRT(3.)
      GO TO 21
   20 COSD = 0.5
      SIND=-0.5*SQRT(3.)
   21 COSA = -1.0/3.0
```

```
      SINA=(2./3.)*SQRT(2.)
      GO TO 29
   22 IF (ILAZY - 7) 23, 24, 26
   23 COSD = 1.0
      SIND = 0
      GO TO 25
   24 COSD = -1.0
      SIND = 0
   25 COSA = -0.5
      SINA=0.5*SQRT(3 )
      GO TO 29
   26 IF (ILAZY - 9) 27, 28, 28
   27 CONTINUE
      GO TO 29
   28 THBCD=THBCD*3.1415926536/180.
      PHABCD=PHABCD*3 1415926536/180.
      SINA=SIN(THBCD)
      COSA=COS(THBCD)
      SIND=SIN(PHABCD)
      COSD=COS(PHABCD)
   29 CONTINUE
      XD = RCD*COSA
      YD = RCD*SINA*COSD
      ZD = RCD*SINA*SIND
   30 YPD = YD*COSKH - ZD*SINKH
      ZPD = ZD*COSKH + YD*SINKH
      XPD = XD*COSPH - ZPD*SINPH
      ZQD = ZPD*COSPH + XD*SINPH
      XQD = XPD*COSTH - YPD*SINTH
      YQD = YPD*COSTH + XPD*SINTH
      IF (K - 1) 31, 32, 31
   31 XRD = -ZQD
      ZRD = XQD
      XQD = XRD
      ZQD = ZRD
   32 X(ND) = XQD + X(NC)
      Y(ND) = YQD + Y(NC)
      Z(ND) = ZQD + Z(NC)
   52 CONTINUE
      DO 41 I=1,NOAT
       COORD(I,1)=X(I)
      COORD(I,2)=Y(I)
      COORD(I,3)=Z(I)
41    CONTINUE
      DO 600 I=1,NOAT
      WRITE(6,700)ISEQ(I),(COORD(I,K),K=1,3)
  700 FORMAT(1X,I5,1X,3(F10.3))
      GOTO 600
  601 WRITE(6,602)ATOM(I),NSEQ(I),(COORD(I,K),K=1,3)
  602 FORMAT(1X,A1,I1,2X,3(F10.3))
  600 CONTINUE
      GOTO 650
   50 WRITE(6,958)NA,NB,NC
  958 FORMAT(1H0,38HCOORDS OF 1 REFERENCE ATOM UNAVAILABLE,3I6)
  650 STOP
      END
      FINISH
```

```
      LIST(LP)
      PROGRAM EPAC
      INPUT 5=CRD
      OUTPUT 6=LPD
      COMPACT
      COMPRESS INTEGER AND LOGICAL
      TRACE 2
      END
      MASTER EPAC
      DATA BYD/1HH/,CAR/1HC/,NIT/1HN/,OXY/1HO/,DUM/1HD/
      DIMENSION ATCO(99,3),NGP(20),ORBAT(20,50),ATINF(99,17),
     XCONNECT(50,2),NTOR(20),DIST(3,2),AMAX(2),MAXR(2),
     XIHA(3),TITLE(8),ATNO(2),SENO(2),IATO(2),ISEQ(2)
     X,ATOM(2),IZNO(2),IXDA(99,2),XD(99),ORAS(20,50)
     X,ZNO(2),SENO(2),ATM(2)
      INTEGER ATINF,ORBAT,CONNECT,ARSH,ATOM,SEQN,ATNO,ORAS
     X,ZNO,SENO,ATM,VIOLI,VIOLJ
      COMMON/COMA/ATINF,NOAT,NODU,SLIM,STND,IXDA,XD
     X,VIOLI,VIOLJ
      COMMON/COMB/ORAS,NGP,NRDT
      COMMON/COMC/ATCO,NDB
C
C
C     THE E-PACE INPUT DATA
C
C
      READ(5,1)(TITLE(I),I=1,8),NOAT,NODU,NOTA,NRO,SLIM
    1 FORMAT(8A4,/(I2,1X),F0.0)
      DO 4 N=1,NOAT+NODU
      READ(5,3)ATINF(N,1),(ATCO(N,M),M=1,3)
    3 FORMAT(I3,1X,3F9.0)
    4 CONTINUE
      DO 15 N=1,NOAT+NODU
      READ(5,2)(ATINF(N,M),M=2,17)
    2 FORMAT(3X,3I3,1X,4I3,1X,9I3)
   15 CONTINUE
      DO 6 I=1,NOTA
      READ(5,5)(CONNECT(I,M),M=1,2)
    5 FORMAT(2I3)
    6 CONTINUE
      DO 8 I=1,NRO
      READ(5,7)NGP(I)
    7 FORMAT(I2)
      NOGP=NGP(I)*
      READ(5,1001)(ORBAT(I,M),M=1,NOGP)
 1001 FORMAT(50I3)
    8 CONTINUE
      DO 11 N=1,NRO
      NTOR(N)=0
   11 CONTINUE
      DO 19 K=1,NRO
      DO 18 J=1,NGP(K
      DO 1000 L=1,NOAT+NODU
      IF(ORBAT(K,J).EQ.ATINF(L,1))ORAS(K,J)=L
 1000 CONTINUE
```

```
18 CONTINUE
19 CONTINUE
C
C
C     THE MAIN PROGRAMME
C
C
      LBR=0
      NROT=12
      NOB=1
      WRITE(6,12)SLIM
12    FORMAT(1X////1X,'THIS PROGRAM GENERATES CONFORMATIONS OF PROBABIL
     :ITY 1.000 WITCH */
     :1X,'----------------------------------------------------------
     :-'/
     :1X,'ARE CONTAINED WITHIN AN S INDEX LIMIT OF ',F10.3/
     :1X,'----------------------------------------------------'////)
      WRITE(6,16)(TITLE(I),I=1,8)
16    FORMAT(1X,8A4/1X,'----------------------------------------'////)
      WRITE(6,13)
13    FORMAT(1X,'BOND NUMBER NEXT CONSIDERED FOR ROTATION: 1'/1X,
     :'-----------------------------------')
      DO 17 I=1,2
      ATNO(I)=ORBAT(NOB,I)/100
      SERN(I)=ORBAT(NOB,I)-ATNO(I)*100
      IF(ATNO(I).EQ.1)CALL COPY8(ATOM(I),HYD)
      IF(ATNO(I).EQ.6)CALL COPY8(ATOM(I),CAR)
      IF(ATNO(I).EQ.7)CALL COPY8(ATOM(I),NIT)
      IF(ATNO(I).EQ.8)CALL COPY8(ATOM(I),OXY)
      IF(ATNO(I).EQ.0)CALL COPY8(ATOM(I),DUM)
17    CONTINUE
      WRITE(6,14)ATOM(1),SERN(1),ATOM(2),SERN(2)
14    FORMAT(1X,'BETWEEN ATOMS:',1X,A4,I2,'=',A4,I2/1X,
     :'-----------------------')
C
C
C     SECTION TO DEDUCE THE SPECIES OF ROTOR
C
C
20    DO 21 I=1,2
      ATNO(I)=ORBAT(NOB,I)/100
      SERN(I)=ORBAT(NOB,I)-ATNO(I)*100
      IF(ATNO(I).EQ.1)CALL COPY8(ATOM(I),HYD)
      IF(ATNO(I).EQ.6)CALL COPY8(ATOM(I),CAR)
      IF(ATNO(I).EQ.7)CALL COPY8(ATOM(I),NIT)
      IF(ATNO(I).EQ.8)CALL COPY8(ATOM(I),OXY)
      IF(ATNO(I).EQ.0)CALL COPY8(ATOM(I),DUM)
21    CONTINUE
      DO 22 I=1,NOTA
      IF(CONNECT(I,1).EQ.ORBAT(NOB,1))M=CONNECT(I,2)
      IF(CONNECT(I,1).EQ.ORBAT(NOB,2))M=CONNECT(I,2)
22    CONTINUE
      IAN1=ATNO(ABS(NOB),1)+2
      IAN2=ATNO(ABS(NOB),2)+2
      GOTO(37,37,37,37,37,23,24,25)IAN1
23    GOTO(37,37,37,37,37,26,27,28)IAN2
24    GOTO(37,37,37,37,37,29,30,31)IAN2
25    GOTO(37,37,37,37,37,32,33,34)IAN2
```

```
   26 IF(II.EQ.3.0.AND.N..0.3)GOTO 70
      GOTO 30
   27 IF(II.EQ.3.0.AND.N..0.2)GOTO 70
      GOTO 30
   28 IF(II.EQ.3.0.AND.N..0.1)GOTO 70
      GOTO 30
   29 IF(II.EQ.2.0.AND.N..0.3)GOTO 70
      GOTO 30
   30 WRITE(6,35)
   31 IF(II.EQ.2.0.AND.N..0.1)GOTO 70
      GOTO 30
   32 IF(II.EQ.1.0.AND.N..0.3)GOTO 70
      GOTO 30
   33 IF(II.EQ.1.0.AND.N..EQ.2)GOTO 70
      GOTO 30
   34 WRITE(6,36)
   35 FORMAT(1X,'IS AN H-H BOND CORRECT?')
      STOP
   36 FORMAT(1X,'IS A C-C BOND CORRECT?')
      STOP
   37 WRITE(6,38)
   38 FORMAT(1X,'AN ERROR IN THE DATA INPUT EXISTS')
      STOP
C
C
C     SECTION TO REDUCE THE LOCAL CONFORMATION ABOUT A C1-C2 ROTOR
C
C
   70 DO 73 I=1,2
      ARSN=0
      DO 77 L=1,NOAT
      IF(ORBAT(NO,I).EQ.ATINF(L,1))GOTO 78
      GOTO 77
   78 ARSN=L
      GOTO 69
   77 CONTINUE
   69 DO 72 J=5,8
      IARS=0
      DO 66 L=1,NOAT
      IF(ATINF(ARSN,J).EQ.ATINF(L,1))GOTO 67
      GOTO 66
   67 IARS=L
      GOTO 68
   66 CONTINUE
      GOTO 72
   68 IF(ATIN(IARS,2).GE.6)GOTO 71
      GOTO 72
   71 DO 79 K=1,2
      IF(ATINF(ARSN,J).EQ.ORBAT(NOB,K))GOTO 72
   79 CONTINUE
      IHA(I)=IARS
   72 CONTINUE
   73 CONTINUE
      DO 74 I=1,3
      DX=ATCO(IHA(2),I)-ATCO(IHA(1),1)
      DY=ATCO(IHA(2),I)-ATCO(IHA(1),2)
      DZ=ATCO(IHA(2),I)-ATCO(IHA(1),3)
      DIST(I,1)=SQRT(DX*DX+DY*DY+DZ*DZ)
```

```
      CALL ORBTT
   74 CONTINUE
      AMIN=AMIN1(DIST(1,1),DIST(2,1),DIST(3,1))
      IF((DIST(1,1)-AMIN).LT.1.0E-09)GOTO 75
      GOTO 40
   75 WRITE(6,76)NOB,ATOM(1),SEQN(1),ATOM(2),SEQN(2),NTOR(NOB)
      GOTO 42
   76 FORMAT(1X,'BOND NUMBER =',I2,',BETWEEN ATOMS ',A1,I2,'&',A1,I2,
     X' ,TORSIONAL ANGLE =',I3,',PROB.<1.000,CONF. DISREGARDED')
C
C
C     SECTION TO DEDUCE THE LOCAL CONFORMATION ABOUT A C1-C1(TILDE)
C     . ROTOR
C
C
   39 DO 81 I=1,3
      IHA(I)=0
   81 CONTINUE
      M=1
      N=2
   82 L=1
      ARSN=0
      DO 87 I=1,NOAT
      IF(ORBAT(NOB,M).EQ.ATINF(I,1))GOTO 88
      GOTO 87
   88 ARSN=I
      GOTO 10
   87 CONTINUE
  101 DO 84 J=5,8
      IARS=0
      DO 103 I=1,NOAT
      IF(ATINF(ARSN,J).EQ.ATINF(I,1))GOTO 104
      GOTO 103
  104 IARS=I
      GOTO 105
  103 CONTINUE
      GOTO 84
  105 IF(ATINF(IARS,2).GE.6)GOTO 83
      GOTO 84
   83 DO 95 K=1,2
      IF(ATINF(ARSN,J).EQ.ORBAT(NOB,K))GOTO 84
   95 CONTINUE
      IHA(3)=IARS
   84 CONTINUE
      ARSN=0
      DO 97 I=1,NOAT
      IF(ORBAT(NOB,N).EQ.ATINF(I,1))GOTO 96
      GOTO 97
   96 ARSN=I
      GOTO 98
   97 CONTINUE
   98 DO 86 J=5,8
      IARS=0
      DO 106 I=1,NOAT
      IF(ATINF(ARSN,J).EQ.ATINF(I,1))GOTO 108
      GOTO 106
  108 IARS=I
      GOTO 107
```

```
106 CONTINUE
    GOTO 86
107 IF(ATIN(IARS,2).GE.6)GOTO 85
    GOTO 86
 85 DO 99 K=1,2
    IF(ATIN(IARS,J).EQ.ORBAT(NOB,K))GOTO 86
 99 CONTINUE
    IHA(L)=IARS
    IF(L.EQ.2)GOTO 100
    L=2
 86 CONTINUE
100 IF(IHA(2).EQ.0)GOTO 89
    GOTO 90
 89 M=2
    N=1
    GOTO 82
 90 DO 92 J=1,3
    DO 91 I=1,2
    DX=ATCO(IHA(3),1)-ATCO(IHA(1),1)
    DY=ATCO(IHA(3),2)-ATCO(IHA(1),2)
    DZ=ATCO(IHA(3),3)-ATCO(IHA(1),3)
    DIST(J,I)=SQRT(DX*DX+DY*DY+DZ*DZ)
 91 CONTINUE
    CALL ORDIT
 92 CONTINUE
    DO 94 I=1,2
    AMAX(I)=AMAX1(DIST(1,I),DIST(2,I),DIST(3,I))
    DO 93 K=1,3
    IF((AMAX(I)-DIST(K,I)).LT.1.0E-09)MAXR(I)=K
 93 CONTINUE
 94 CONTINUE
    IF(MAXR(1).EQ.3.AND.MAXR(2).EQ.2)GOTO 75
    IF(MAXR(1).EQ.2.AND.MAXR(2).EQ.3)GOTO 75
C
C
C
C
C
 40 CALL SIADVDU(&40,&50)
    WRITE(6,41)NOB,ATOM(1),SEQN(1),ATOM(2),SEQN(2),NTOR(NOB)
   X,SIND
 41 FORMAT(' BOND NUMBER ',I2,' BETWEEN ATOMS ',A1,I2,'&',A1,I2,
   X' ',I2,' TORS.ANGLE =',I3,' "S" INDEX =',F8.3)
    DO 60 I=1,99
    IF(IXDA(I,1).GT.0)GOTO 62
    GOTO 60
 62 N=IXDA(I,1)
    M=1
 63 IZNO(M)=ATIN(N,1)/100
    ISEQ(M)=ATIN(N,1)-IZNO(M)*100
    IF(IZNO(M).EQ.1)CALL COPYS(IATO(M),IVD)
    IF(IZNO(M).EQ.6)CALL COPYS(IATO(M),CAR)
    IF(IZNO(M).EQ.7)CALL COPYS(IATO(M),NIT)
    IF(IZNO(M).EQ.8)CALL COPYS(IATO(M),OXY)
    IF(IZNO(M).EQ.9)CALL COPYS(IATO(M),SUN)
    IF(I.EQ.IXDA(I,2))GOTO 64
    N=IXDA(I,2)
    M=2
```

```
      GOTO 63
64    WRITE(6,65)(ATO(1),ISEQ(1),ATO(2),ISEQ(2),XD(I)
65    FORMAT( X,8,'XD BETWEEN ATOMS ',A1,I2,'&',A1,I2,'=',F7.3)
60    CONTINUE
      WRITE(6,55)
55    FORMAT(1X,'ATOMIC COORDINATES :')
      DO 54 N=1,NUMAT
      IZNO(1)=ATNO(N,1)/100
      ISEQ(1)=ATNO(N,1)-IZNO(1)*100
      IF(IZNO(1).EQ.1)CALL COPY8(ATOM(1),HYD)
      IF(IZNO(1).EQ.6)CALL COPY8(ATOM(1),CAR)
      IF(IZNO(1).EQ.7)CALL COPY8(ATOM(1),NIT)
      IF(IZNO(1).EQ.8)CALL COPY8(ATOM(1),OXY)
      IF(IZNO(1).EQ.0)CALL COPY8(ATOM(1),DUM)
      WRITE(6,53)(ATO(1),ISEQ(1),(ATCO(N,M),M=1,3)
53    FORMAT(1X,A1,I2,3F8.3)
54    CONTINUE
48    NOB=1
42    IF(NTOR(NOB).EQ.240)GOTO 44
43    NTOR(NOB)=NTOR(NOB)+NROT
      CALL ORBIT
      GOTO 20
44    NTOR(NOB)=0
      CALL ORBIT
      NOB=NOB-1
      IF(NOB.LE.NROT1)STOP
      IF(NOB.GT.LBR)GOTO 45
      GOTO 42
45    LBR=NOB
      DO 59 I=1,2
      ATNO(I)=ORBAT(NOB,I)/100
      SEQN(I)=ORBAT(NOB,I)-ATNO(I)*100
      IF(ATNO(I).EQ.1)CALL COPY8(ATOM(I),HYD)
      IF(ATNO(I).EQ.6)CALL COPY8(ATOM(I),CAR)
      IF(ATNO(I).EQ.7)CALL COPY8(ATOM(I),NIT)
      IF(ATNO(I).EQ.8)CALL COPY8(ATOM(I),OXY)
      IF(ATNO(I).EQ.0)CALL COPY8(ATOM(I),DUM)
59    CONTINUE
      WRITE(6,46)LBR
46    FORMAT(1X//1X,'BOND NUMBER NEXT CONSIDERED FOR ROTATION:',I3/1X,
     X'=========================================')
      WRITE(6,47)(ATOM(1),SEQN(1),ATOM(2),SEQN(2)
47    FORMAT( X,'BETWEEN ATOMS:',1X,A1,I2,'-',A1,I2/
     X1X,'---------------------')
      GOTO 43
49    K=1
      L=VIOLI
56    ZNO(K)=ATINF(L,1)/100
      SEQ(K)=ATINF(L,1)-ZNO(K)*100
      IF(ZNO(K).EQ.1)CALL COPY8(ATM(K),HYD)
      IF(ZNO(K).EQ.6)CALL COPY8(ATM(K),CA )
      IF(ZNO(K).EQ.7)CALL COPY8(ATM(K),NIT)
      IF(ZNO(K).EQ.8)CALL COPY8(ATM(K),OXY)
      IF(ZNO(K).EQ.0)CALL COPY8(ATM(K),DUM)
      IF(L.EQ.VIOLJ)GOTO 57
      K=2
      L=VIOLJ
      GOTO 56
```

```
      WRITE(6,51)NOB,ATOM(1),SEQN(1),ATOM(2),SEQN(2),NTOR(NOB)
     X,ATM(1),SENO(1),ATM(2),SENO(2)
      GOTO 48
   50 WRITE(6,52)NOB,ATOM(1),SEQN(1),ATOM(2),SEQN(2),NTOR(NOB)
      GOTO 48
   51 FORMAT(1X,'BOND NUMBER ',I2,',BETWEEN ATOMS ',A1,I2,'&',A1,I2,
     X',TORSIONAL ANGLE =',I3,',STERIC VIOLATION BETWEEN',
     X' ATOMS ',A1,I2,'&',A1,I2)
   52 FORMAT(1X,'BOND NUMBER ',I2,',BETWEEN ATOMS ',A1,I2,'&',A1,I2,
     X',TORSIONAL ANGLE =',I3,',"S" INDEX EXCEEDED, CONFORMER DISALLOW
     XED')
      STOP
      END
C
C
      SUBROUTINE STADBU(*,*)
C
C
      INTEGER ATINF(99,17),VIOLI,VIOLJ
      COMMON/COMA/ATINF,NOAT,NODU,SLIM,SIND,IXDA(99,2),XD(99)
     X,VIOLI,VIOLJ
      COMMON /COMC/ATCO(99,3),NOB
      M=0
      SIND=0.0
      DO 1 L=1,99
      IXDA(L,1)=0
      IXDA(L,2)=0
      XD(L)=0.0
    1 CONTINUE
      DO 6 I=1,NOAT*NODU-1
      DO 5 J=I+1,NOAT*NODU
      IF(ATINF(I,2).EQ.0)GOTO 401
      IF(ATINF(J,2).EQ.0)GOTO 401
      DO 2 K=3,17
      IF(ATINF(J,1).EQ.ATINF(I,K))GOTO 5
    2 CONTINUE
  401 DX=ATCO(I,1)-ATCO(J,1)
      DY=ATCO(I,2)-ATCO(J,2)
      DZ=ATCO(I,3)-ATCO(J,3)
      R=SQRT(DX*DX+DY*DY+DZ*DZ)
      IF(ATINF(I,2).EQ.0)GOTO 400
      IF(ATINF(J,2).EQ.0)GOTO 400
      VDWH,VDWC,VDWN,VDWO=0.0
      L=I
    3 IF(ATINF(L,2).EQ.1)VDWH=VDWH+1.0
      IF(ATINF(L,2).EQ.6)VDWC=VDWC+1.2
      IF(ATINF(L,2).EQ.7)VDWN=VDWN+1.4
      IF(ATINF(L,2).EQ.8)VDWO=VDWO+1.6
      IF(L.EQ.J)GOTO 4
      L=J
      GOTO 3
    4 SUMVDW=VDWH+VDWC+VDWN+VDWO
      VIOLI=I
      VIOLJ=J
      IF(R.LT.SUMVDW)RETURN 1
      IF(ATINF(I,3).EQ.0)GOTO 400
      IF(ATINF(J,3).EQ.0)GOTO 400
      IF(ATINF(I,3).EQ.1.AND.ATINF(J,3).EQ.1)GOTO 400
```

```
      IF(ATIN(I,3).EQ.2.AND.ATIN(J,3).EQ.2)GOTO 400
      SIND=SIND*R
      IF(SIND.GT.SLIM)RETURN 2
  400 IF(ATIN(I,4).EQ.1.AND.ATIN(J,4).EQ.1)GOTO 7
      GOTO 5
    7 M=M+1
      IXDA(M,1)=I
      IXDA(M,2)=J
      XD(M)=R
    5 CONTINUE
    6 CONTINUE
      RETURN
      END
C
C
      SUBROUTINE ORB
C
C
      INTEGER ORAS(20,50)
      COMMON/COMB/ORAS,NGP(20),NROT
      COMMON/COMC/ATCO(99,3),NOB
      DIMENSION TRAN(3)
      ANGROT=FLOAT(NROT)/57.2958
      DO 15 I=1,3
   15 TRAN(I)=ATCO(ORAS(NOB,1),I)
      DO 4 J=1,3
      DO 3 I=1,NGP(NOB)
    3 ATCO(ORAS(NOB,I),J)=ATCO(ORAS(NOB,I),J)-TRAN(J)
    4 CONTINUE
      IF(ATCO(ORAS(NOB,2),3).EQ.0..AND.ATCO(ORAS(NOB,2),1).EQ.0)GOTO 7
      IF(ATCO(ORAS(NOB,2),1).EQ.0.)GOTO 5
      Y=ATCO(ORAS(NOB,2),3)/ATCO(ORAS(NOB,2),1)
      Y=ABS(Y)
      ANGY=ATAN(Y)
      GOTO 6
    5 ANGY=3.14159/2.0
    6 CONTINUE
      IF(ATCO(ORAS(NOB,2),1).LE.0..AND.ATCO(ORAS(NOB,2),3).LT.0.)
     XANGY=ANGY-3.14159
      IF(ATCO(ORAS(NOB,2),1).GT.0..AND.ATCO(ORAS(NOB,2),3).LT.0.)
     XANGY=-ANGY
      IF(ATCO(ORAS(NOB,2),1).LE.0..AND.ATCO(ORAS(NOB,2),3).GE.0.)
     XANGY=-ANGY+3.14159
      GOTO 8
    7 ANGY=0.
    8 CONTINUE
      CALL SUBORB(ANGY,3,1,2,2,3,1,NGP,ORAS)
      IF(ATCO(ORAS(NOB,2),2).EQ.0..AND.ATCO(ORAS(NOB,2),1).EQ.0.)
     XGOTO 50
      IF(ATCO(ORAS(NOB,2),1).EQ.0.)GOTO 9
      Z=ATCO(ORAS(NOB,2),2)/ATCO(ORAS(NOB,2),1)
      Z=ABS(Z)
      ANGZ=ATAN(Z)
      GOTO 10
    9 ANGZ=3.4159/2.0
   10 CONTINUE
      IF(ATCO(ORAS(NOB,2),1).GT.0..AND.ATCO(ORAS(NOB,2),2).GT.0.)
     XANGZ=-ANGZ
```

```
      IF(ATCO(ORAS(NOB,2),1).LE.0..AND.ATCO(ORAS(NOB,2),2).LE.0.)
     XANGZ=-ANGZ+3.14159
      IF(ATCO(ORAS(NOB,2),1).LE.0..AND.ATCO(ORAS(NOB,2),2).GT.0.)
     XANGZ=ANGZ-3.14159
      GOTO 50
  500 ANGZ=0.0
  501 CALL SUBORB(ANGZ,1,2,3,1,2,3,NGP,ORAS)
      CALL SUBORB(ANGROT,2,3,1,3,1,2,NGP,ORAS)
      CALL SUBORB(-ANGZ,1,2,3,1,2,3,NGP,ORAS)
      CALL SUBORB(-ANGY,3,1,2,2,3,1,NGP,ORAS)
      DO 12 J=1,3
      DO 11 I=1,NGP(NOB)
   11 ATCO(ORAS(NOB,I),J)=ATCO(ORAS(NOB,I),J)+TRAN(J)
   12 CONTINUE
      RETURN
      END
C
C
      SUBROUTINE SUBORB(ANG,I1,I2,I3,I4,I5,I6,NGP,ORAS)
C
C
      COMMON/CONC/ATCO(99,3),NOB
      DIMENSION ORAS(20,50),XA(3,3),YA(3,3),ZA(3,3),NGP(20)
      INTEGER ORAS
      XA(1,1)=COS(ANG)
      XA(1,2)=-SIN(ANG)
      XA(2,1)=SIN(ANG)
      XA(2,2)=COS(ANG)
      XA(1,3),XA(2,3),XA(3,1),XA(3,2)=0.
      XA(3,3)=1.0
      DO 2 I=1,NGP(NOB)
      J=ORAS(NOB,I)
      YA(1,1)=ATCO(J,1)
      YA(1,2)=ATCO(J,2)
      YA(1,3)=ATCO(J,3)
      DO 1 K=1,3
      ZA(1,K)=0.
      DO 1 M=1,3
    1 ZA(1,K)=ZA(1,K)+XA(K,M)*YA(1,M)
      ATCO(J,1)=ZA(1,4)
      ATCO(J,2)=ZA(1,5)
    2 ATCO(J,3)=ZA(1,6)
      RETURN
      END
      FINISH
```

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