The Dynamics of Quantal Neuromuscular Transmission

David Briggs
B.Sc. (Hons)

Thesis submitted to

The University of Aston in Birmingham

for the degree of

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Summary

This thesis describes the development of a new tool to aid the investigation of neuromuscular transmission. The tool is a suite of computer programs which implement a discrete numerical simulation of some of the events occurring during quantal transmission at the vertebrate skeletal neuromuscular junction. It enables the user to investigate the effects of variations in several of the parameters involved, including synaptic morphology, receptor density, and transmitter binding kinetics, some of which are not accessible to the experimenter. Predictions made can then be compared with data obtained from physiological experiments.

This can be used to improve our understanding of the process of neuromuscular transmission, both in normal animals and those suffering from disorders such as myasthenia gravis and other diseases which affect the neuromuscular junction.

Keywords: Acetylcholine Receptor
Computer Simulation
Miniature End-Plate Current
Neuromuscular transmission

To Mum and Dad

Acknowledgements

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Abbreviations

The following abbreviations are used in this thesis:

ACh	Acetylcholine
AChE	Acetylcholinesterase
ACR	Acetylcholine Receptor
ATCh	Acetylthiocholine
DFP	Diisopropylfluorophosphate
E.P.C.	End-plate current
E.P.P.	End-plate potential
Km	Michaelis-Menten Constant
M.E.P.C.	Miniature end-plate current
M.E.P.P.	Miniature end-plate potential
2-D	Two Dimensional
2-PAM	Pyridine-2-aldoxime methiodide
3-D	Three Dimensional

NOTE

In places in this report, real numbers are expressed in the form -1.5 E+5. In this notation, the E replaces "times 10 to the power". As a result of typographical restrictions, Greek letters have been replaced by the name of the letter - alpha, pi etc.

Glossary of Computing Terms

Algorithm - A sequence of instructions used to perform a specific task.

Background - A low priority task performed when the computer has nothing of higher priority to do.

BASIC - an acronym for a popular computer programming language. It stands for Beginner's All-Purpose Symbolic Instruction Code.

Binary - A form of arithmetic using a radix of 2.

Bit - A single BInary digiT. It can have the values 0 or 1.

Block - The unit of physical storage allocation for files on random access media such as magnetic disks. One block is often 512 bytes in size.

Bug - A fault in a computer system, usually an error in a program.

Byte - this is the name given to a type of binary number. It comprises 8 bits and can have 256 different values. It is also the unit of memory storage capacity. One byte can store one character of text.

Compiler - A program that can turn instructions written in a programming language (e.g. FORTRAN) into a sequence of machine code instructions.

Computer - A calculating machine, usually electronic.

Debugging - The fine art of hunting bugs.

Disk - A form of rotating storage medium. Data is normally stored in files comprising a number of blocks. Magnetic disks are random access devices.

File - An organised set of data, normally kept on magnetic tape or disk storage media. Files can be accessed by either sequential or random access methods.

Floating Point - A format in which real numbers are normally stored. It comprises a mantissa or fractional part (e.g. 0.5) and an exponent by which the mantissa should be multiplied.

Floppy disk - A flexible magnetic disk that can be removed from its drive

and read/write mechanism for storage or transportation. They are cheap and can hold up to 1 megabyte.

Foreground - The opposite of background.

FORTRAN - An early computer programming language. It stands for FORmula TRANslation and is used where large quantities of high-speed arithmetic are required. The latest definition of this language is FORTRAN 77, also known as FORTRAN V.

Hard disk - A type of magnetic disk storage. Unlike floppy disks, hard disks are not flexible. The read/write heads on the drive mechanism can be positioned over them more precisely, and this increases their storage capacity. The largest ones can store hundreds of megabytes. They are not cheap.

Head - Data is written to and read from magnetic storage using heads which can be positioned over the disk surface on one of a number of concentric tracks. Each track is further divided up into sectors.

Integer - A number having no fractional part. Integers are normally stored in a different format to real or floating point numbers. An integer would typically require two bytes (or more) of store.

Interpreter - Similar to a compiler, but an interpreter reads the program stored in memory and works out what to do on a line by line basis. This makes it slower than a machine code program to do the same job.

Kilobyte - 1024 bytes. This is normally the unit used for measuring the storage capacity of computers and their storage media.

Machine Code - This is the only language that computers can actually understand, though each machine has a different one...

Mainframe - A type of large computer installation.

Megabyte - One million bytes. This is usually actually 1000 Kilobytes.

Memory - Computers need somewhere to store programs and data. The general name for this is memory. Solid state memory in a computer that can

be both written to and read from is usually give the misnomer Random Access Memory, or RAM, though all solid state memory in a computer is accessed randomly.

Microcomputer - A computer built around a microprocessor.

Operating System - This is the general name given to the set of computer programs that control the operation of the computer itself.

Program - A complete sequence of instructions given to a computer, in order that it may perform some task. It is on a larger scale than algorithm. Programs written in high-level languages have to be translated by a compiler or an interpreter before they can be executed.

RAM - A misnomer given to semiconductor memory devices that can be read from and written to. It stands for Random Access Memory, but it actually means Read / Write Memory. See also ROM.

Random Access - A computing term given to types of memory which can be accessed by a computer specifying an address. As opposed to sequential, random access memories do not have to be read in order. Random access files are stored as numbered blocks. The computer is able to read any one of the blocks in the file by specifying its block number.

Read/Write - A type of memory whose contents can be changed by writing new values into them.

Real - A type of number which has both fractional and integer parts. They are normally stored in floating point format. A real number would normally require four bytes (or more) to store.

Record - A unit of data in a structured file. Records are logical blocks which can be processed as a unit.

ROM - Read Only Memory. The contents of this type of memory cannot be altered by writing new values in. Often used to hold programs.

Sequential - A type of memory which can only be accessed serially - start at the beginning and finish at the end.

Source Code - This is the general name given to programs which are in a human-readable form, as opposed to machine code, which is not. The FORTRAN programs in the Appendix are examples of source code.

Winchester - A type of magnetic disk store in which a hard disk is sealed inside a dust-proof enclosure. They are different from normal hard disks in that the distance between the heads and the disk surface is very small, and the heads actually touch the disk when it is not rotating. They have higher storage capacities than floppy or other hard disks.

Word - This is the basic unit of data inside a computer system. It corresponds to the smallest unit that can be read or written by the computer's processor. It is usually a multiple of 8 bits wide. The Harris' word length is 24 bits, the North Star's (a microcomputer) is only 8.

Section 1 - Introduction

1.1 General Introduction

Motion, produced by the controlled use of specially developed contractile tissues or muscles, is a common behavioural characteristic of animals. Coordination of movement is performed by a nervous system, the complexity of which varies considerably from the lower orders of invertebrates to the higher vertebrates.

Despite the very many species of animals, there are surprisingly few variations in the methods used to communicate information and instructions in the body. Before the development of a recognisable nervous system, tissues used chemical transmitters distributed by diffusion to communicate with other parts of the body. This is only sufficient where the distances concerned are less than a few millimetres. The evolution of the nervous system has improved this delivery system where speed and directionality were advantageous. This applies particularly to the control of locomotion, as improvements give predators and prey evolutionary advantages.

The major differences, in vertebrates and invertebrates, are the degree of development of the nerves (for speed) and in the chemical transmitters used. Practically all vertebrates use the chemical Acetylcholine (ACh) as the transmitter at the junctions between nerves and their fast skeletal muscles - i.e. those used for motion. Other transmitters are used where speed is not so important, though the method is fundamentally the same.

Neuromuscular Transmission: General Principles

This section is a brief introduction to the principles of neuromuscular transmission. The following sections include a more detailed description of

the processes involved as revealed by different research techniques.

Nerves use an essentially electrical method, known as the Action Potential, to convey information. An action potential, however, cannot cross the junction between nerve and muscle cells, and it is here that the chemical transmitter takes over. The nerve terminal itself is swollen where it presses against the muscle cell surface, with a narrow gap - the synaptic cleft - in between. The transmitter is accumulated in the nerve terminals, usually in small spherical bubbles known as vesicles (see Figure 1.1.1). Vesicles in large numbers are a characteristic feature of nerve terminals, which look rather like a sack of marbles as a result.

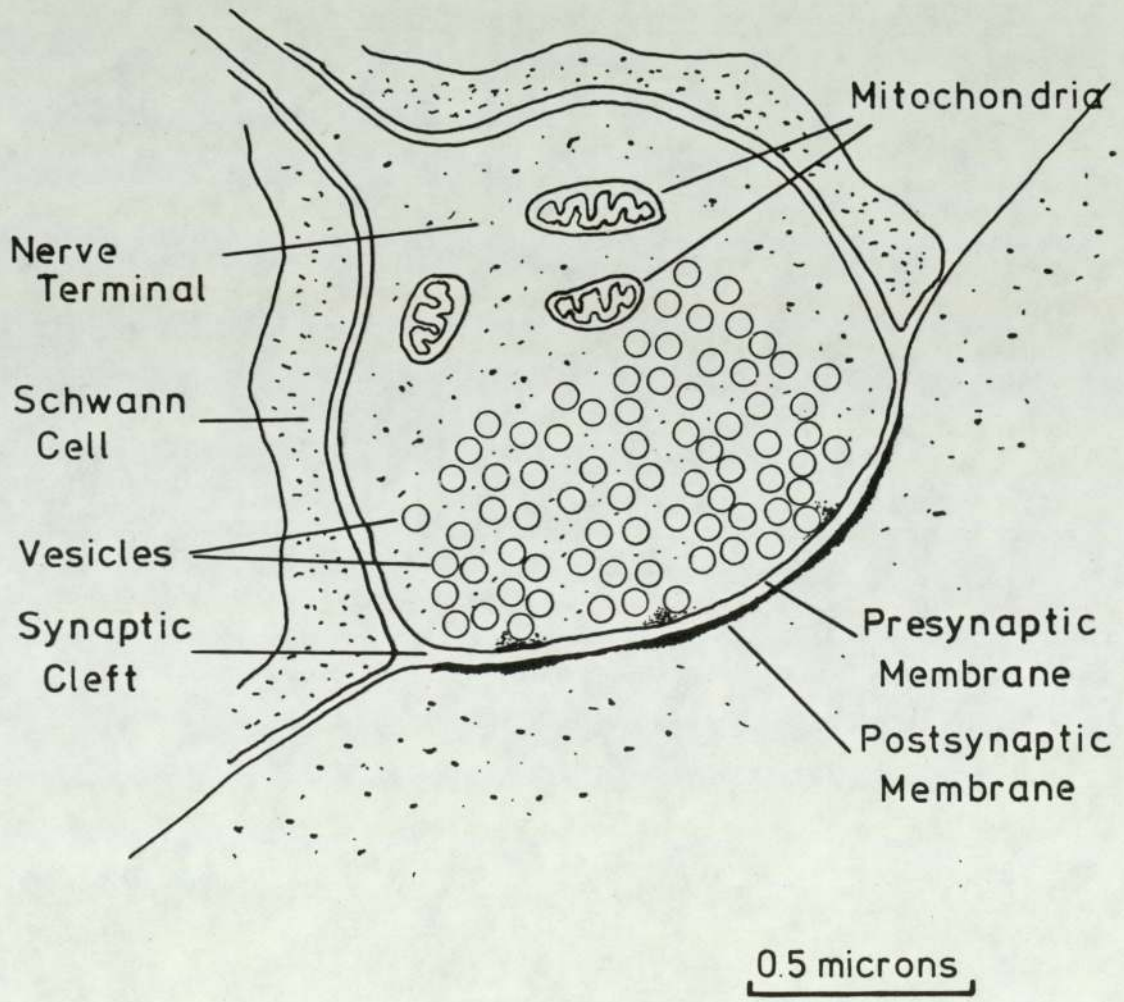

Figure 1.1.1 A Simple Synapse

There is a mechanism in the nerve terminal which results in transmitter being released in relatively constant-sized packets, known as quanta, one of which could correspond to the contents of a vesicle. The release mechanism is controlled by a stochastic process which is partly dependent on the intracellular concentration of some ions, most notably calcium ions. There is a low probability of release taking place under resting conditions and only a small number of quanta are discharged spontaneously each second. When an action potential arrives at the nerve terminal however, the resulting changes in ionic concentration increase the probability to a level where as many as a few hundred quanta can be released more or less simultaneously.

Once released, the transmitter diffuses freely about the synaptic cleft until it comes into contact with receptors on the surface of the muscle cell. The transmitter molecules can bind to these proteins, causing conformational changes in them which open up holes big enough for small ions to pass through the muscle cell membrane. These channels stay open for a short time before the transmitter is released, and they close again.

There is a chemical imbalance across the membrane, the majority of this difference being in the concentration of positively charged sodium ions. This imbalance results in the inside of the muscle cell being at a more negative voltage than the extracellular fluid, typically by about -70 millivolts. Sodium ions flow through the receptor channels, and this reduces the local transmembrane potential towards zero, an effect known as depolarisation. If sufficient channels are opened for the depolarisation at the end-plate to pass through a threshold value, another action potential can be generated in the muscle cell. This can lead to the activation of the muscle's contraction mechanism and, hence, movement.

If nothing was done about it, the transmitter would stay in the synaptic cleft, activating receptors, until it diffused out into the extracellular space. This situation is avoided in most synapses by the incorporation of a faster removal mechanism. In some, the transmitter is taken up into the adjacent cells intact, but in the vertebrate skeletal neuromuscular junction, the acetylcholine is broken down by a special enzyme, Acetylcholinesterase, which rapidly hydrolyses ACh into choline and acetate. Thus the transmitter is turned into an inactive form, allowing the muscle cell to recover.

Normally, each nerve action potential results in more transmitter quanta being released than are actually necessary to generate an action potential in the muscle cell. The excess, sometimes as much as five times too much, gives a safety factor to ensure effective transmission.

Sustained contractions in skeletal muscles are produced by rapid, repetitive stimulation of the muscles, as many as fifty times in a second. The resultant contractions are smoother, stronger, and more controllable than single twitches. It is important that the transmitter is destroyed quickly, so that the muscle cell can respond to the next nerve action potential. The success of neuromuscular transmission relies on the balance between release of transmitter and its prompt destruction.

Disorders of the Neuromuscular Junction:

There are several pathological or pharmacological conditions which affect the effectiveness of neuromuscular transmission. The safety factor could be eroded in many ways:

1 The store of transmitter in the nerve terminal could become depleted.

This would result in a smaller number of quanta being released in response to an action potential.

2 The release mechanism could be affected (particularly by some snake toxins) either increasing or decreasing the probability of release. This could lead to convulsions or to paralysis.

3 The structure of the synaptic cleft could be disrupted in such a way as to increase the distance that the transmitter has to go to reach a receptor, or to reduce the surface area of the postsynaptic membrane, also affecting the number of accessible receptors.

4 Many predators such as snakes use toxins to paralyse their victims. The neuromuscular junction is an obvious target, and many toxins contain compounds that inhibit the binding of transmitter to receptors or prevent the ionic channels from opening and closing properly.

5 Conversely, there are natural inhibitors of the hydrolysing enzyme acetylcholinesterase. These act to potentiate the action of the transmitter with resultant loss of control. Many nerve gases, though not natural substances, have this action.

One condition of particular interest in this respect is Myasthenia gravis. Patients suffering from this disease have difficulty maintaining contractions of their muscles - particularly keeping their eyes open. This was originally thought to be the result of transmitter depletion as short contractions could be made. The treatment of this was, therefore, to use an inhibitor of the Acetylcholinesterase so that the transmitter would last that little bit longer. This treatment is in fact successful, but it is not a lack of transmitter that is primarily responsible. A breakthrough came when research workers attempting to produce antibodies to acetylcholine receptors from electric eels noticed that the rabbits that they were using developed a similar disability. They had produced antibodies, but their own

receptors were so similar to those of the fish that an immune response was directed against them as well. This disease, known as Experimental Auto-Immune Myasthenia Gravis, can kill animals like Guinea pigs.

This led to checks being made with human patients, and a large fraction were found to have anti-ACR antibodies in their blood (Aharonov, Abramsky, Tarrab-Hadzai and Fuchs 1975). These, presumably, are interfering with neuromuscular transmission.

Methods used to Investigate Neuromuscular Transmission:

The electrical signals generated by both nerves and muscles when active are particularly accessible for study purposes. They can often be recorded from the skin of an intact subject without any damage being done. Most of the classic studies, however, have been performed on isolated tissues, some also using electrical recording methods. The electrical methods will be discussed later: the next section is concerned with what the components of the neuromuscular junction look like and how the active components fit together at the molecular level.

1.2 Cytology, Morphology and Biochemistry

Though the neuromuscular junction is a very common structure in evolutionary terms, a wide range of shapes and sizes are found. Some of the features are sufficiently large to be seen under a light microscope, but the finer details require the use of an electron microscope. In combination with radiolabelled, highly specific inhibitors such as alpha-bungarotoxin, it is possible to pin-point the locations of some of the active molecules and even count them. The same inhibitors can also be used to assist in the extraction of these proteins from tissues so that their properties can be studied more readily.

Synaptic Morphology:

The simplest type of synapse is the bouton, where the presynaptic nerve terminal swells into a blob which is pressed against an otherwise flat postsynaptic membrane (Figure 1.1.1). This type of synapse is found in the central nervous system and in the autonomic nervous system. Vesicles varying between 20 and 50 nm in diameter can be detected in the presynaptic nerve terminal. The synaptic cleft itself is usually narrow, some 10 to 20 nm wide, (de Robertis and Bennett 1955, Palay 1956) and contains amorphous material. The postsynaptic membrane is usually flat. The junctions are often surrounded by glial tissue such as Schwann cells which may improve their electrical characteristics.

Vertebrate neuromuscular junctions are more complex. Often, the nerve terminal or motor end-plate is extended over the surface of the muscle cell, sometimes in a trough or gutter. The most noticeable difference is the development of secondary folds which extend the cleft down into the

muscle cell (Figure 1.2.1). These folds are usually perpendicular to the longitudinal axis of the nerve terminal and increase the postsynaptic membrane area considerably. The secondary clefts contain a network of fine fibres shown very clearly by the electron micrographs of Heuser and Salpeter (1979). These fibres form the basal lamella which Zacks and Blumberg (1961a) compare with the mucopolysaccharide or mucoprotein basement membranes found in other tissues.

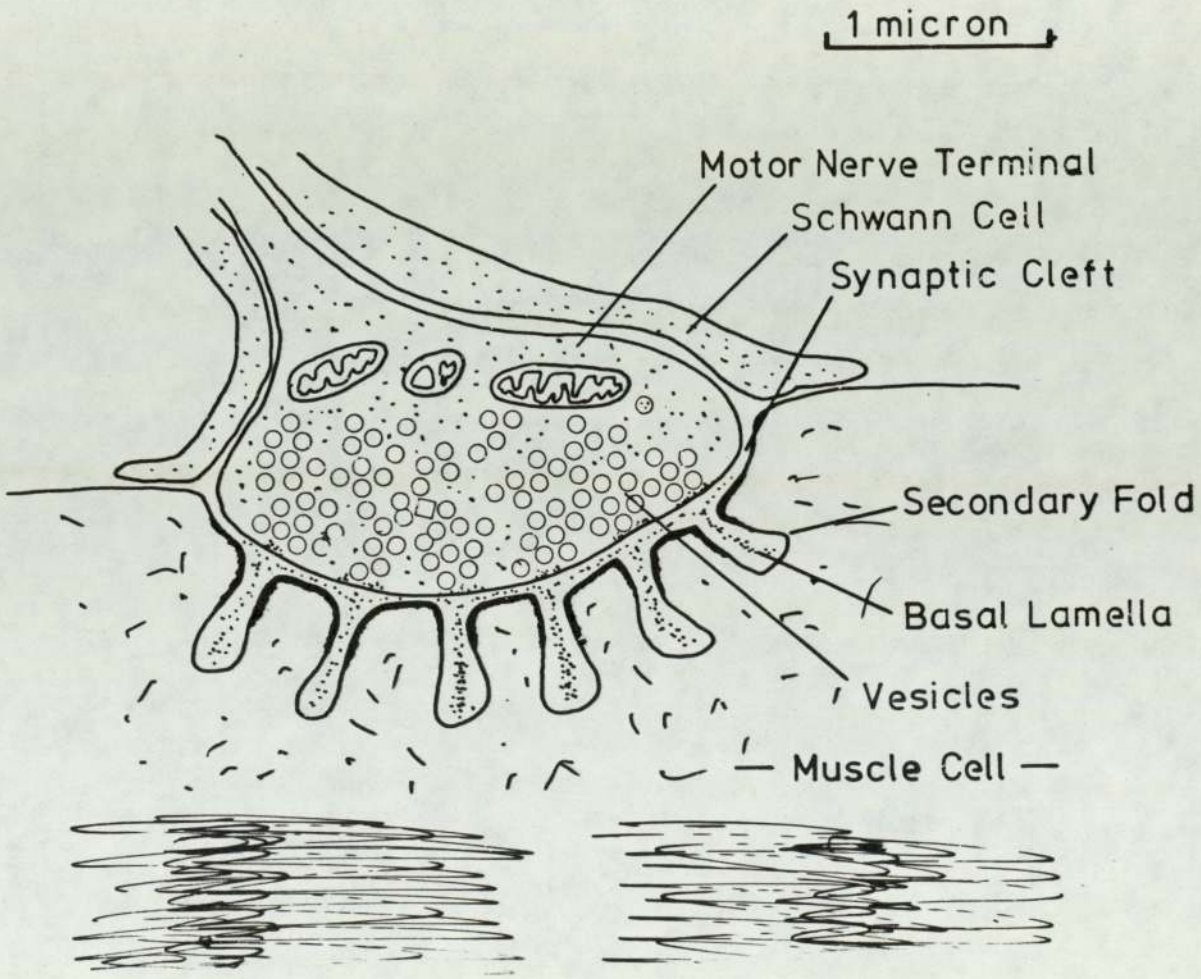

Figure 1.2.1 The Vertebrate Skeletal Neuromuscular Junction

The arrangement of folds varies considerably from muscle to muscle, as well as from one species to another. This type of structure is found in amphibians (Birks, Huxley and Katz 1960), reptiles (Robertson 1956), and

mammals, including man (Zacks and Blumberg 1961a), and is probably essential to their operation as one of the fastest reacting types of synapse found.

Scanning electron microscopy and stereoscopic techniques such as those used by Heuser and Salpeter (1979) have made the three-dimensional structure more apparent, particularly when the nerve terminal and overlying glial cells have been partly removed.

Synaptic Dimensions:

The primary synaptic cleft is generally wider than in the simpler synapses, varying between 20 and 60 nm. The secondary folds are 50 to 150 nm wide and range between 500 and 1000 nm deep. They are separated by gaps up to 2 microns wide. The presynaptic membrane often shows thickenings opposite the secondary clefts. These sometimes have vesicles associated with them and it has been suggested that these are the sites of the release mechanism (Birks et al 1960). The dimensions of motor end-plates in two types of muscle are listed below for the Mouse and the Rat, two mammals commonly used for neuromuscular transmission research.

Species	Tissue	Muscle Fibre Diameter (microns)	End-plate Dimensions (microns)	Source
Mouse	Sternomastoid	40 - 80	40 * 64	Salpeter (1972)
	Diaphragm	18 - 25	23 * 26	Salpeter (1972)
Rat	Sternomastoid	50 - 70	40 * 60	Salpeter (1973)
	Diaphragm	25 - 50	30 * 40	Salpeter (1973)

Histochemistry:

The end-plate is not completely beyond the bounds of light microscopy, and some of the coarser details can be resolved, particularly in amphibian species. For example, Anderson and Cohen (1974) attached a fluorescent dye to alpha-bungarotoxin molecules. These would still bind to acetylcholine receptors and could be visualised using ultra-violet light. The fluorescence showed that the toxin had bound in the region of the postsynaptic membrane, extending into the secondary folds which appeared as bright strips every few microns.

For finer detail, the combination of specific inhibitors, such as alpha-bungarotoxin, radioactive labels, and the electron microscope has been a very powerful tool. For Acetylcholinesterase, the enzyme's function as an esterase was used initially to provide information on its localisation. A thiol-substituted analogue of acetylcholine was the source of sulphur which was released when the false transmitter was hydrolysed by the enzyme. This was then reacted with a lead-containing reagent to precipitate the electron-dense lead sulphide. Some diffusion occurs before the lead sulphide is precipitated, and this spreads the stain over a larger area. Zacks and Blumberg (1961b) found lead sulphide in the synaptic cleft.

Two types of cholinesterase enzyme are found in nerve and muscle tissues. One, AChE, is the acetylcholine-specific Acetylcholinesterase (formally known as Acetylcholine hydrolase, which has the Enzyme Commission classification E.C. 3.1.1.7.). The other, ChE, is known as Pseudo- or non-specific cholinesterase (Acylcholine acylhydrolase E.C. 3.1.1.8.). Using substrates specific to one or other type of cholinesterase, Bergman, Veno, Morizono, Hanker and Seligman (1967) thought that the AChE was in the muscle sarcoplasm, albeit close to the postsynaptic membrane. Teravainen

(1967), using a diazoether - osmium reaction, also found AChE activity associated with the postsynaptic membrane, and ChE close by in the muscle sarcoplasm.

In 1969, Rogers, Darzynkiewicz, Salpeter, Ostrowski and Barnard used the irreversible but relatively non-specific organophosphorus cholinesterase inhibitor Di-isopropyl fluorophosphate (DFP), labelled with radioactive 32-Phosphorus in an attempt to count the numbers of binding sites in mouse muscles. 32-Phosphorus is not very useful for localisation of the enzyme as the powerful beta particles emitted may travel some distance before interacting with the detecting emulsion. However, it is ideal for counting the number of DFP binding sites since a known fraction of the particles produce tracks. Unfortunately, DFP also binds to many other enzymes. A refinement of this method was to use Pyridine 2-aldoxime methiodide or pralidoxime (2-PAM), a specific re-activator of Acetylcholinesterase, to differentiate the AChE from other binding sites. 2-PAM is not particularly effective against DFP and can be useless against other ligands (Silver 1974), but is nonetheless useful as it is specific for AChE in this context.

A better method, used at about the same time by Salpeter (1969), was based on Tritium-labelled DFP to determine the density of binding sites in mouse sterno-mastoid and diaphragm muscles. The beta particles emitted by tritium are much less energetic than those from 32-Phosphorus, so they do not travel as far before being absorbed. This means that this technique can be used for localisation, though absorption of the beta particles by the tissue itself makes it less accurate than 32-Phosphorus for density determination. Indeed, results obtained later gave lower densities (Salpeter, Plattner and Rogers 1972; Barnard, Chiu, Jedrezejcyzk, Porter and Wieckowski 1973). Of the 8,700 DFP-binding sites found per square

micron of postsynaptic membrane, about one third of these could be reactivated by 2-PAM, giving an AChE density of about 2,500 per square micron in the two tissues.

Work in a similar vein on the distribution of receptors has been carried out using Tritiated or 125-Iodine labelled alpha-bungarotoxin. Porter, Barnard and Chiu (1973) found 8,500 bungarotoxin binding sites per square micron in mouse diaphragm, a value almost identical to the DFP-binding sites. Taken as a whole, the numbers for complete end-plates are also very similar, even for different species of vertebrates.

This striking similarity in numbers of binding sites seems to hold for the end-plates studied so far. Barnard et al (1973) take this to imply a functional connection between DFP-binding sites and receptors, even though only a fraction of the DFP-reactive sites are AChE.

At one time, it was thought that the esterase and receptor functions were different parts of the same structure (e.g. Wilson 1967), though more recently it has been possible to separate the two completely without destroying their functionality. They are now believed to be separate proteins, but there are hints of allosteric interactions between them. Stalc and Zupancic (1972) found that alpha-bungarotoxin had an effect on AChE activity. This disappeared when the enzyme preparation was purified further and may have been caused by associated receptors which had not been removed.

Later work (Fertuck and Salpeter 1976) on mouse and on frog (Matthews-Bellinger and Salpeter 1978) has indicated that the receptors are not evenly distributed, being found more densely at the crests of the

secondary folds, that is, adjacent to the nerve terminal, where the density can be greater than 25,000 per square micron, with very few at depths greater than 250 nm into the secondary clefts.

The culmination of the Acetylcholinesterase localisation endeavours came in 1978 when McMahan, Sanes and Marshall managed to remove all of the cellular components of synapses from the frog cutaneous pectoris muscle, leaving behind only the basal lamella - the fibrous network found in the secondary clefts. The remaining tissue was then assayed for cholinesterase activity and it was found in abundance. The use of specific inhibitors demonstrated that most, if not all, of the cholinesterase activity at this neuromuscular junction (basal lamella) was due to Acetylcholinesterase.

Biochemistry - the molecules:

Studies on the molecules themselves are only possible when relatively large amounts can be isolated. Fortunately, there are some rich sources of both AChE and receptors. One such tissue is the electric tissue found in many fish such as electric eels (Electrophorus electricus) which use high voltage electric fields for navigation amongst other things. This tissue, known as an electroplax, is a modified form of muscle which is rather like a battery in structure.

The development of affinity chromatography using specific inhibitors bound to an inert material such as Sepharose gel has also been of considerable benefit. It is possible to extract comparatively small amounts of receptors and cholinesterase molecules from large volumes of unwanted material using this technique (e.g. Rosenberry, Chang and Chen 1972).

Care must be taken when interpreting properties of molecules in vivo from

parameters obtained in vitro. Discrepancies have been observed which may result from disruption of the organisation in the synapse. An example of this has been the study of molecular weights. Different methods of isolation have produced a wide variety of results - for AChE, values as high as 30 million and as low as 70,000 have been obtained (see Silver 1974 and Rosenberry 1975 for extensive reviews). This is probably the result of relatively crude technique which either breaks the molecules up into fragments, or is insufficient to properly separate multi-molecule aggregates.

One reason for this wide range of values was revealed by the work of Bon, Vigny and Massoulie (1979), reviewed in Massoulie and Bon (1982). They identified six different types of AChE which could be extracted. There are three globular forms, each being an aggregate of a number of identical chains. Each chain has a molecular weight estimated at 71,000 daltons, and corresponds to a single enzyme sub-unit with one active site. Bon et al call them G1, G2, and G4, according to the number of sub-units that they

Table 1.2.1 Molecular Forms of Acetylcholinesterase

Form	Chains	Active Sites	Molecular Weight	Stokes' Radius
G1	1	1	71,000	
G2	2	2	191,000	
G4	4	4	341,000	8.1 nm
A4	1*G4 + tail	4	453,000	13 nm
A8	2*G4 + tail	8	747,000	
A12	3*G4 + tail	12	1,062,000	15.5 nm

contain. The other three forms are asymmetric, and have 50 nm long collagen tails. These are called A4, A8, and A12, and they comprise one, two, or three, respectively, of the tetrameric G4 forms, held together by their tails. The molecular weight of the tails is estimated at about 100,000.

The sheer size of the tails implies that they are a part of the structure of the basal lamella itself. Clearly, insufficient 'purification' was leaving numbers of AChE molecules still stuck together by their tails, and excessive fractionation was breaking even the globular parts up into their component parts.

By titrating the AChE active sites with an essentially irreversible ligand (Rosenberry and Bernhard 1971, Rosenberry et al 1972) it has been possible to determine the numbers of active sites per enzyme. These experiments gave 3.3 as the result for an enzyme molecular weight of 260,000. This was thought to be a tetramer at the time.

Similar problems have beset workers trying to determine the molecular weight of the acetylcholine receptor. Changeux, Meunier, Olsen, Weber, Bourgeois, Popot, Cohen, Hazelbauer and Lester (1973) report that a discrepancy between a measured weight of 470,000 and a corrected one of 360,000 was accounted for by large numbers of detergent molecules (Triton X-100) being attracted to the lipophilic part of the receptor molecule which is normally found inside the cell membrane. They thought that the 360,000 species was an aggregate of 6 to 8 sub-units each of about 50,000. A fraction of molecular weight 40,000 was isolated from electroplax by de Robertis (1971). In 1975, Barnard, Dolly, Porter and Albuquerque reported a molecular weight of 370,000 of receptors purified from denervated muscle

(in 0.2% Triton X-100). Adams (1981) suggests 250,000 as the correct value. Unlike the AChE molecule, the receptor's sub-units do not appear to be identical.

Binding studies using 14-Carbon labelled acetylcholine (de Robertis 1971) suggest two binding sites per receptor molecule, one with high, the other with low affinity for the transmitter (Dissociation constants 1 E-7 M. and 1 E-5 M., respectively). Studies by Changeux and Podleski (1968) and Rang (1971) also support the two binding site theory.

Biochemistry - the reactions:

The reaction between Acetylcholine and AChE takes place in three steps. The ACh quaternary nitrogen is attracted to a negatively charged anionic site, with the ester carbonyl group oriented towards an esteratic site. The ester bond is broken, the choline part leaving the acetyl group attached covalently to the enzyme. This acetyl-enzyme is rapidly hydrolysed and the acetate produced departs as well. The values of some of the rate constants (shown in the scheme below as k's) for the reactions have been determined for enzymes from a variety of sources. Some typical values are given in the kinetics section below.

$$\underset{+\ H_2O}{AChE + ACh} \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} \underset{+\ H_2O}{AChE.ACh} \xrightarrow{k_2} \underset{+\ OH}{AChE.A + Ch.H} \xrightarrow{k_3} \underset{+\ Ch.H}{AChE + A.OH}$$

The reaction with receptors is unusual in that the transmitter is really only acting as a moderator of ionic channel conductance. There are no other products. Assuming that two molecules of ACh are required to open the channel, we have the following scheme:

$$ACR + 2ACh \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} ACR.ACh + ACh \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} ACR.2ACh\ (Channel\ Closed) \underset{k_{-3}}{\overset{k_{+3}}{\rightleftharpoons}} ACR.2ACh\ (Channel\ Open)$$

ACh molecules bind to the receptor active sites one at a time. Once two are both bound, there is a delay while the channel changes from the closed to open states. The channel remains open for a time before the transmitter molecules are released and it closes.

Kinetics:

The morphology and histochemistry merely describe the environment in which neuromuscular transmission takes place. Transmission, however, is a dynamic process, the neuromuscular junction having evolved into one of the most rapidly operating types of synapse. Rapid movements and fine control require short response times, and this also demands rapid recovery from each impulse. Not only do the receptors and ion channels have to operate in microseconds, it is essential that the transmitter is eliminated within a few milliseconds.

These properties come under the general heading of kinetics, though enzyme

Table 1.2.2 Acetylcholinesterase Kinetic Constants

Constant	Symbol	Value	Reference
Michaelis-Menten constant	Km	2 - 3 E-3 Molar	(Namba and Grob 1968)
Forward binding rate	k+1	2 E+8 / M / sec	(Rosenberry 1975)
Turnover Number		8.0 E+5 / min	(Rosenberry 1975)

kinetics are usually studied at a more leisurely pace. The parameters typically measured include the substrate concentration which gives half maximal reaction velocity (Km - the Michaelis Menten constant) and the turnover number, which is the number of substrate molecules processed by an active site in one minute under optimum conditions. The reciprocal of this is the turnover time. Conditions for these determinations are important, but there are many complications. AChE, given the right ionic environment in solution, shows a reduction in activity at high substrate concentrations (substrate inhibition). This has not been demonstrated in so-called membrane-bound preparations. Namba and Grob (1968) used a preparation of enzyme-bearing membranes from rat intercostal muscles to give a turnover number of 1.61 E+5 and a Km of 3.1 millimolar at 37°C and pH 8.0. This contrasts with the turnover given by Rosenberry (1975) for solubilized AChE of 8.0 E+5 molecules per active site per minute at 25°C, at a concentration of 2.7 millimolar (equivalent to 13.7 molecules per site per millisecond).

Salpeter and Eldefrawi (1973) corrected Rosenberry's figures for an estimated transmitter concentration in the synaptic cleft of 1.1 E-5 Molar,

Table 1.2.3 Acetylcholine Receptor Kinetic Constants

Constant	Symbol	Value	Reference
First forward binding rate	k+1	3 E+8 / M / sec	(Wathey et al 1979)
First dissociation rate	k-1	1.6 E+4 / sec	(Wathey et al 1979)
Second forward binding rate	k+2	3 E+7 / M / sec	(Sheridan and
Second dissociation rate	k-2	1 E+3 / sec	Lester 1977)

to give a turnover much less than this, 0.18 E+5 per minute, or only 0.3 molecules per active site per millisecond. This concentration may be an underestimate as they assumed that the transmitter was evenly distributed through the synaptic volume, and there is reason to believe that it acts over a more restricted space at a much higher concentration (see later).

Enzyme kinetics relate to the reaction mediated by the enzyme concerned. With the Acetylcholine Receptor, there is no reaction as such. However, the rate constants used in the scheme above can be estimated. Some values are given in Table 1.2.3. Most of these constants can only be estimated using electrical methods, and even then may not be experimentally accessible. Values can be calculated by feeding them into computer-based simulations, which will be described in more detail later. With acetylcholine as the 'enzyme substrate', the rate limiting step (the slowest one) is believed to be the binding of the second transmitter molecule.

1.3 Electrophysiology

Electrical Recording:

A large amount of work on neuromuscular transmission is based on the recording of electrical signals from muscle cells. In amplitude, these signals vary from the Action Potential, usually about 100 millivolts from baseline to peak, to the tiny currents, of the order of 1 pico-amp, which flow through single ionic channels. Their frequency components range from the steady transmembrane potential (0 Hz) to the 'noise' which can be recorded as a result of the opening and closing of individual receptor ionic channels, which extends into the kiloHertz region.

Electrodes are placed in or near to muscle cells, usually in isolated tissue preparations, and these are connected to electronic amplifiers and recording instruments such as oscilloscopes. Some problems arise from the physical sizes of the cells. Electrodes have to be positioned using light microscopes and the cells are very small - mouse diaphragm muscle cells are about 20 microns in diameter. In order to record a reasonably accurate signal, the electrode must be positioned within one or two fiber diameters of the end-plate itself. Also, very fine electrodes are required to penetrate the cells without causing too much damage. A single cell has a high output impedance, so the electrode and amplifier must be chosen to avoid overloading the cell electrically.

The electroplax preparation is also a useful tissue in this respect, as it has very large areas of acetylcholine sensitivity. Changeux and Podleski (1968) studied the effect of some receptor agonists by measuring the depolarisation produced by various doses. They obtained sigmoid dose

response curves. These can by further analysed using a technique known as the Hill Plot which can detect the presence of multiple binding sites and allosteric interactions (Rang 1971). Changeux calculated the Hill coefficient for his dose-response curves as being approximately 2, which was taken to indicate that there are two acetylcholine binding sites per receptor molecule.

A characteristic event that can be recorded from the neuromuscular junction is the miniature end-plate potential or m.e.p.p. This is the effect produced on the muscle cell by the release of a single quantum of transmitter (containing up to ten thousand molecules of acetylcholine) from the motor nerve terminal. The result is a brief depolarisation about one millivolt in amplitude followed by an exponential decay back to the resting membrane potential (usually about -70 millivolts). The recorded amplitude is affected by the position of the electrode, the impedance of the muscle cell as 'seen' by the moving ions, as well as by the dynamic balance between diffusion and binding to the receptors and cholinesterase molecules. There are also complications which result from the movements of ions through the channels.

Voltage Clamping:

It is possible to reduce the effect due to the impedance of the muscle cell by a technique known as voltage clamping. This was originally developed at Seattle by Connor and Stevens (1971). Briefly, two electrodes are required in the muscle cell at the end-plate. One of these is used to measure the voltage, while a feedback amplifier passes current through the other one so as to hold the voltage at a chosen value. The current that is passed can be measured, and it gives a measure of the current that is passing through the cell membrane, hopefully through receptor channels. The signal

resulting from a quantum of transmitter is then known as a miniature end-plate current or m.e.p.c.

The localisation of both electrodes is critical, particularly in narrow muscle fibers such as in mouse diaphragm, as mis-positioning the current passing electrode causes current to be 'wasted', and bad placement of the voltage electrode will give a poor measure of the current passing through the membrane in the target region. However, mouse tissue does have some advantages. The cells' input impedance is high (about 500 kilohms) which means that a large voltage deviation is obtained for an influx of current, and the end-plate dimensions are small, so that only a small volume of cell needs to be clamped.

Electrically, voltage clamping results in much faster signals being obtained, since the exponential relaxation obtained with voltage recording is largely passive recharging of the membrane. This places heavier demands upon the recording equipment and feedback noise is generated in the clamping circuit which detracts from the signal quality. To make things even worse, the normal physiological temperature for the mouse is 37°C. At this temperature, most of the biochemistry works at its fastest. the original Seattle voltage clamp circuit was designed for use in the frog at room temperature and needs some modifications to improve its speed.

Averaging:

The standard way of reducing a high noise component in a repeating signal is to add successive samples together. In this way, the repetitive component adds up, whereas the noise, which is assumed to be random, tends to cancel out. The averaging is usually performed on a digitised sample of the signal. The process of digitisation introduces errors due to the

discrete nature of the output - both in time (sampling) and amplitude (quantisation). The time and amplitude resolution of the digitiser has to be adequate, and the input signal should also be filtered to remove high frequency components which would interact with the sampling rate (aliasing) to produce spurious results.

The release process in the motor nerve terminal is stochastic. This means that it is not possible to predict when a quantum of transmitter will be released, unless an action potential is triggered in the nerve itself. In order to record an m.e.p.c. complete with the baseline signal before the rising phase, it is necessary to use a technique known as pre-trigger recording. Successive samples taken of the input signal are stored in a circular buffer. If the rising part of the signal passes through a threshold value, then samples taken after this point can be stored separately. When sufficient points have been stored, the contents of the circular buffer can be extracted and put on the front, before the trigger point. The only alternative to this method is to record continuously, using a special recorder or moving film from which the signals can be identified and extracted later.

Iontophoresis:

Acetylcholine can be added to preparations by means other than release from the nerve terminal. A common method is to fill a micro-pipette with a solution of agonist. Passing a current through the pipette can force the agonist out of the end, a technique known as iontophoresis. This technique has been used to simulate quantal release, but it is difficult to duplicate the effect of the nerve terminal, which acts as a barrier to diffusion, both into and out of the synapse. Kuffler and Yoshikami (1975) used a development of this method to estimate the number of acetylcholine

molecules in a single quantum, obtaining 10,000 as an upper limit in frog and snake end-plates.

The time that the ionic channel remains open can be estimated using special techniques. In one of these, transmitter is added to the preparation bathing fluid. The acetylcholinesterase would normally be inhibited to permit the acetylcholine concentration in the synaptic cleft to reach a reasonable value. Then, the continuous presence of transmitter results in a more or less continuous opening and closing of receptor ionic channels. The current noise that this produces can be detected using voltage clamping, and statistical analysis can be used to extract the channel open times (see Stevens 1975 for an introduction to the technique).

Patch Clamping:

An alternative technique is known as patch clamping. Rather than placing an electrode inside the muscle cell, this method relies on obtaining a good electrical seal around the tip of an electrode placed against the cell membrane. If the electrode contains an agonist, this will react with receptors in the membrane, causing channels to open. Normally, the motor nerve terminal would have to be removed in order to obtain access to the post-synaptic membrane, though denervated muscles, which have receptors spread over their entire surface, can also be used. The currents which flow through the channels are tiny, of the order of 1 picoamp, but they can be measured and show the rectangular conductance changes as the channels open and close. The channel noise obtained by this method can be analysed statistically to provide a further insight into the events at the molecular level (e.g. see Hoffman and Dionne 1983). These experiments show that the different agonists used do not affect the conductance of individual channels but they do affect the time that each channel stays open.

The initial concentration of transmitter is of the order of 50 - 250 millimolar (2,000 to 10,000 molecules in a 50 nanometre sphere - Kuffler and Yoshikami 1975). This will dilute rapidly as the molecules diffuse, but the concentration will remain high in comparison to the affinity constants for receptor binding for some time. The higher density of receptors immediately adjacent to the release sites would be expected to promote rapid initial binding, resulting in many channels opening in a fairly small area (Matthews-Bellinger and Salpeter 1978).

Land, Salpeter and Salpeter (1980) studied rise-times of m.e.p.c's in lizard intercostal end-plates with the receptors partly inhibited by alpha-bungarotoxin (and with the cholinesterase inhibited also). They found that as the density of available receptors was reduced by further toxin binding, the m.e.p.c. rise time was increased. They concluded that a quantum of transmitter acted on a small area at a saturating concentration. Diffusion, then, was an important factor, though there were others - e.g. binding time and conformational changes - which were not dependent on receptor density. Gage and McBurney (1975) came to a similar conclusion using toad muscles at a range of temperatures between 10 and 30°C. The results of Matthews-Bellinger and Salpeter (1978), obtained from frog cutaneous pectoris, indicated that diffusion is not the predominant factor, particularly at lower temperatures.

Head (1983) has studied m.e.p.c's in rat diaphragm cells and obtained channel open times of 237 microseconds at 37°C from noise analysis. This method also permitted channel conductance to be determined, giving a value of 26 picoSiemens. The decay time constant of the m.e.p.c's was consistently longer than the channel open time which may imply that some transmitter molecules activate more than one receptor before being lost or

hydrolysed.

When many quanta are released simultaneously, Hartzell, Kuffler and Yoshikami (1975) suggest that AChE is able to destroy the transmitter so that the areas acted on by molecules from adjacent release points do not overlap. However, after inhibition of the AChE, this is no longer the case and then the areas do overlap. At the relatively low concentrations of transmitter expected at a late stage of the end-plate current, it is possible that a significant number of receptors which would normally only bind one transmitter molecule (not enough to open the channel) are able to bind a second one as well, thereby opening up the ion channel. This would potentiate the effect - giving a longer e.p.c..

If hydrolysis by acetylcholinesterase is the prime agent effecting the termination of transmitter action, then its inhibition would be expected to extend the m.e.p.c. as transmitter molecules bounce from one receptor to another. However, while bound to receptors, the transmitter is protected from hydrolysis, so how could a significant number of second channel activations be prevented under normal conditions? If the transmitter was released well before the receptor channel closed, or there was a conformational delay before the receptor could bind more transmitter, then this could force the ACh to move further afield, increasing its chance of being hydrolysed.

A further potential complication is suggested by Attwell and Iles (1979). Sodium ions are the main charge carrier through the receptor channels, but the highly localised effect of each transmitter quantum may well produce a depletion of the sodium ions in the synaptic cleft. They used published data for the frog neuromuscular junction and predicted significant

concentration changes both for sodium ions, and also for potassium ions travelling in the opposite direction. These effects would be dynamic, depending on the spread of transmitter and the amount of current flowing, but could also affect the muscle's response.

1.4 Simulations

Limitations:

It is not possible to monitor the binding to and release of transmitter from active sites in the synapse directly using the techniques available at this time. Many other potentially significant parameters, such as synaptic ion concentrations, are also experimentally inaccessible. As the biological experiments were particularly difficult at this stage, it seemed that a completely different approach was required. Therefore, the methods in use for modelling diffusion and simulating neuromuscular transmission were investigated.

Heat Flow:

The earliest attempts to model transmitter action were performed by Eccles and Jaeger (1958), who assumed that diffusion in the cleft was very fast. An even concentration was obtained quickly, and then the effect of the transmitter was limited solely by diffusion from the edges of the synapse. They derived some equations which could be used to determine the remaining transmitter concentration profile in the cleft with time. These, and subsequent work (Jaeger 1960) made use of the earlier work by Carslaw and Jaeger (1941 and 1947) on heat flow in shaped metal blocks.

While the models were fairly simple in shape, linear mathematical models could be used. However, these are insoluble as soon as any complications in shape or conditions are involved, and numerical analysis must be used instead. This is the approach taken by most of the other workers in this field.

One way to skirt this problem was employed by Rosenberry (1979) who developed two kinetic models on an analog computer to predict the amplitudes and time courses of e.p.c's and m.e.p.c's for normal and esterase-inhibited junctions. Sections of the analog computer were used to represent the diffusion, binding and loss of transmitter, with further sections to produce the current flows. Though this sounds relatively simple, it is a form of numerical analysis, and making the model representative of 'real life' is quite a problem. Rosenberry was able to predict the decay phase of e.p.c's and m.e.p.c's accurately, but was less successful with the growth phase.

Numerical Models:

Wathey, Nass, and Lester (1979) constructed a model to test the quantitative data then available on transmission at the neuromuscular junction. Their model was based on partial differential equations representing the radial diffusion of transmitter from a point source in a flat disk-like synapse, with its subsequent fate determined by hydrolysis and binding to receptors. The receptors were given two transmitter binding sites, the channel only opening when both were occupied. The rate-limiting step was the binding and dissociation of the second transmitter molecule. They assumed that receptors were evenly spread over the post-synaptic surface and that transmitter molecules fell off the edge of the synapse irreversibly (down an infinite concentration gradient). The model, which also included transmitter release from receptor sites, was evaluated by a program written in BASIC running on a Nova 2 minicomputer.

They were able to reproduce the growth phase (200 microseconds to peak current amplitude), the peak number of open channels (2000), and decay of

current found in experimental results, predicting also that the decay time constant was 20% greater than the mean channel open time. Another prediction was that the event was highly localised, two thirds of the open channels being within an area of 0.15 square microns, representing two thirds of the channels in this area.

This model was also able to simulate the effects of variations in temperature, transmembrane voltage, cholinesterase and even receptor inactivation.

Adams (1980) constructed a simple two-dimensional geometric model to simulate m.e.p.c's. This model, with some modifications, was later used by Jachter and Sachs (1982) in their work on ionic depletion in the synapse during the course of an m.e.p.c. Though the diffusion model is in a simple shaped synapse, their conclusions about the loss of sodium ions and accumulation of potassium ions through the open ion channels support Attwell and Iles (1979). Their description of the model says that they have used "a method whereby the inhomogeneous diffusion equation with forcing function is integrated by decomposing it into its analytically obtained homogeneous and numerically evaluated inhomogeneous parts". The evaluation was performed on a mini-computer and took some 30 minutes to simulate 10 milliseconds. They are using it to model m.e.p.c's.

Another model, similar to Wathey's has been developed by Land et al (1981). The geometry of the synapse is more complex: they assume one secondary fold which is also liberally coated with receptors. The transmitter is then partitioned into two - one fraction diffusing laterally along the primary

cleft, the other going down the secondary cleft. They make some assumptions relating the density of binding sites to the diffusion, binding and channel opening times, and using data gleaned from their experiments on receptor density (Land et al 1980), are able derive some values for the coefficients concerned. The value predicted for the forward binding coefficient assumes that both first and second molecule binding constants are the same. They also predict that receptor activation is very local, occurring within a radius of 0.3 microns of the release point. The evaluation of the coefficients from their experimental data and the model used a maximum likelihood method and a little more computer time.

Summary:

Given the difficulty of performing 'real' experiments on neuromuscular transmission, the simulation approach offers an alternate method for investigating the fundamental mechanism - the biophysics - of neuromuscular transmission. Its major advantage is that the mechanism of the model is completely accessible to the experimenter. The model can be designed so as to permit the parameters to be altered and the effects on the model determined and compared with experimental results. Variables which need to be readily alterable include three-dimensional geometry, receptor and AChE densities and kinetics.

There is a conceptual problem with the intensively mathematical models. They are normally constructed with several partial differential equations which are not particularly easy to understand unless the user is adequately mathematically educated, and attempts to modify them can produce unexpected interactions. Even the later ones mentioned above are limited in their geometrical scope to relatively simple structures, and most of the end-plates of interest in muscle have very variable structures, being

complicated in three dimensions.

I am of the opinion that true modelling of the binding of transmitter to active sites is best performed at the molecular level. Such a discrete model, operating on a quantum containing between 2,000 and 10,000 molecules, and also a large number of potential binding sites, would give a digital computer a lot of work to do.

Section 2 - Simulation

2.1 System Analysis

The Process under Investigation: a brief recapitulation

Acetylcholine is the transmitter at the neuromuscular junctions of vertebrate fast skeletal muscles. It is synthesised in the presynaptic terminals of motor nerves and stored in vesicles.

Transmitter is released from the motor nerve terminal in quanta, each containing between 2000 and 10000 acetylcholine molecules. The release mechanism appears to be a random process which normally has a low probability. The arrival of an action potential increases the probability considerably, so that many quanta are released simultaneously. Typically, this would be in the range 20 to 200 quanta. It then diffuses freely until it is destroyed by the enzyme acetylcholinesterase or leaves the synaptic cleft. While it is in the synaptic cleft, it can come into contact with acetylcholine receptors which are found on the postsynaptic muscle cell membrane.

Binding of sufficient acetylcholine molecules to the receptor causes a conformational change in the molecule which results in a hole (a non-specific cation channel) being opened up across the membrane. The ionic imbalance between the fluids either results in small ions (Sodium and Potassium) passing through, so as to depolarise the muscle cell from its resting potential of about -70 millivolts.

A single quantum is sufficient to cause a depolarisation of about 1 millivolt, which is not normally sufficient to produce an action potential

in the muscle cell. When a large number of quanta are released as a consequence of the arrival of an action potential the resulting muscle cell depolarisation crosses a threshold value (about -40 millivolts) and an action potential is generated in the muscle cell.

The action potential in the muscle cell sweeps along the cell surface and activates the contractile mechanism and this results in a contraction. The muscle cell requires a few milliseconds to recover from the electrical effects of the action potential before it can respond again. The contraction mechanism is slower to operate, but can integrate the effects of multiple stimulation to produce a greater strength contraction.

Most enzymes are assemblies of sub-units. The protein molecules in question here are no exception. One consequence of this is that they are all believed to bind more than one molecule of transmitter. Sub-units in an enzyme are often functionally connected so that binding at one binding site has an effect on binding at the others. This is normally helpful - a phenomenon known as co-operativity - making it easier for further molecules to bind.

Factors Affecting Quantal Neuromuscular Transmission:

Synaptic geometry is highly variable. The end-plates of fast skeletal muscles normally have very complex structures, with most of the adjacent post-synaptic cell membrane typically being thrown into numerous secondary folds, which increases the surface area available for binding sites and hence the numbers of ionic channels which can be opened at the same time, and also increases the local reservoir of ions to reduce the effects of depletion.

The binding of transmitter to receptors can be a significant factor influencing the time course of the m.e.p.c. Whilst bound, the transmitter is protected from hydrolysis and is also trapped where the chances of hitting another receptor are high. After release from a single point, there may well be local saturation, but unless receptors are actually clustered closely around release points, then binding must thin out further away.

2.2 Simulation Objectives

Scope:

It is not intended that the entire process of neuromuscular transmission be simulated. This would be a rather unmanageable project in the sense that it would be difficult to know where to begin and, more importantly, it would be even harder to decide when it had finished. Instead, the work was restricted to simulating the events occurring as a result of the spontaneous release of one quantum of acetylcholine from a motor nerve terminal at a 'typical' vertebrate skeletal neuromuscular junction.

The target was to predict the numbers of ion channels opened with time by transmitter binding, so that the transmembrane current could be calculated. The model was to run for as long as was feasible, producing results at regular intervals. Correction of the model parameters to make this prediction fit with experimental results was seen as being too complicated and time consuming for the M. Phil. project, although this is the logical next step.

Aims:

The approach taken was to represent the model as simply as possible, with the model's mechanism decoupled as much as possible from its data, enabling either to be altered independently. In this way, alterations (bug fixes and improvements) could be made in a reasonably straightforward way.

The mechanism was to support simple diffusion and binding to active sites. The data included a definition of the three-dimensional structure of the model, the number and starting positions of the transmitter molecules, and

the density and distribution of binding sites. The number of binding sites and the probabilities of transmitter binding for each type of active site could be varied from one run to the next. Different probabilities for successive molecules binding to each active molecule could be used to model cooperativity. It was not envisaged that interactions between adjacent active sites in the model would be required.

The main difference between this model and the others developed previously was in the attempt to aim the simulation at the molecular level - making it a discrete model. Also, by making the data more accessible to the user, the model is easier to understand and modify than conventional numerical models. As a by-product of this approach, the model has been made much more flexible than the alternatives, albeit at a cost in run-time.

Choosing a language:

Program portability is always a problem. Few programming languages are properly standardised, and even these often contain some 'extras' or 'features' specific to a particular machine. While most programs can be transferred between identical systems with little difficulty, porting a program to a different machine from a different manufacturer can be difficult and time-consuming.

As the simulation mechanism is essentially fixed, the major consideration will be speed. To aid portability, a fairly well standardised language should be used, with any machine or operating system specific parts reduced to a minimum. To provide the best possible run-time performance on any machine requires some work to optimise the program and its data structure. A compiled language program will execute faster than an interpreted or semi-compiled one, and may offer better data types. For example, most

computers have a BASIC interpreter to run BASIC programs. This is quite slow in operation, and a substantial speed increase can be obtained by converting the BASIC program to machine code. In contrast, large scientific computers always support a FORTRAN compiler, and often the machines themselves are designed specifically to run FORTRAN programs efficiently (i.e. fast).

Unfortunately, FORTRAN is one of the less well-standardised languages. The approach taken was to use a common subset of FORTRAN wherever possible, with only the input and output operations being machine dependent. The latest versions of the programs are written in FORTRAN 77 and run on a Harris H800 processor with hardware arithmetic and considerable disk storage.

Program Development:

This was seen as falling into two main stages, with the majority of the work involved in development of the fundamental algorithm used by the diffusion mechanism. The data structures were left relatively vague as these are influenced by the programming language implementations.

Early work was carried out on an ICL mainframe using ICL FORTRAN IV, but the poor mainframe programming environment eventually led to the demise of this approach. The early programs were comparatively simple two-dimensional diffusion simulations which ran quite quickly but were unable to model binding and release.

It was obvious that a serious model had to include binding to active sites. This meant that the move from two dimensions to three was essential. However, the two-dimensional programs had little data storage requirements.

The three-dimensional ones, in contrast, needed considerably more store, and a different approach had to be adopted, essentially this meant starting again.

The majority of the 3-D program development was carried out on North Star Horizon microcomputers using WordStar from Micro-Pro as a text-editor. Programs were written in Microsoft FORTRAN 80 and compiled to produce machine code for the 8080 microprocessor, which is very slow. However, from a development environment viewpoint, the facilities offered on the CP/M operating system were far superior to those available on the competitive large mainframe computers (ICL, CDC and Harris). The use of a good editor enabled program development major cycles (edit, compile, run, debug) to be carried out quickly and easily. Also, the Horizon had a large (18 megabyte) Winchester Disk, so data files could be large without fears of running out of space.

The majority of arithmetic operations are carried out on INTEGERS with only the random number generator and binding probability calculations using REAL numbers for added precision. Most computers can perform integer arithmetic operations quickly, but start to slow down where real numbers are concerned.

Having ironed out most of the bugs which were causing incorrect operation, (usually resulting in transmitter mysteriously appearing or disappearing) the program suite was transported (re-typed) onto the Harris H800, where a considerable increase in speed was expected. This meant that some translation from MicroSoft FORTRAN 80 to the Harris FORTRAN 77 was required. The only problems encountered in this process, apart from typing errors, were a result of the different file operations required on the

Harris Vulcan operating system. The main files used to hold the transmitter and active sites data are fairly large FORTRAN binary random access files, and this particular type of file structure is normally highly machine dependent. The great majority of program run-time is spent reading or writing to these files, so optimisation of their structure is important to obtaining good program performance.

There is no way around this problem. Anyone attempting to put the program suite up on another computer, even one having a FORTRAN 77 compiler, will have to solve it reasonably efficiently.

Listings of the main programs can be found in Appendix 2 (Section 5.2).

2.3 Implementation

The Beginning:

The model is based on the Particle in Cell Method (see Potter 1973 for a detailed description). The two- or three-dimensional structure of the synaptic cleft is converted into an array of cells on a rectangular grid or mesh. The cells can be occupied by active sites or numbers of transmitter molecules, and the diffusion algorithm is used to move the transmitter from cell to cell, occasionally bumping into and binding to active sites. A rectangular array had to be used as other types are very difficult to implement on digital computers.

Two Dimensions:

In the 2-D model, the array of cells was only one cell thick, and represented a cross-sectional view of the synaptic cleft (Figure 2.3.1). Later 3-D versions extended the cross-sectional view in thickness, so that differences between the two were minimised.

The initial cell size was chosen to be 10 nanometres square to make the scaling calculations easy. Electron micrographic data from the sources mentioned previously were used to derive a 'typical' structure for a region of the mammalian skeletal neuro-muscular junction. The primary synaptic cleft was made 60 nanometers or six cells deep, with the secondary clefts extending to a total depth of 540 nm downwards. The model was 45 cells wide, with three secondary clefts, a single transmitter 'release point' being above the central cleft. Figure 2.3.1 shows the active sites and some free transmitter molecules in the central cleft after a diffusion run. The distribution of active sites was the same in all three clefts.

Column:	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
Row:															
1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1
2		7	1	8	3		4	14	10	9	1	4		1	2
3	2	1	3	1	11	4	6	14	5	6	4	3	4	3	3
4	1	2	4	2	5	4	5	6	5	5	4	1	1	3	3
5	1	3		6	3	4	11	7	3	4	2	2		2	3
6	2	1	1	7	6	4	3	8	6	5	1	3	5	2	2
7	0	1	2	4	5	6	7	6	9	2	3	4	5	1	1
8	-3	-3	-4	-3	-3	1	9	6	6	10	-3	-3	-4	-3	-3
9	.	.	.	.	-3	6	4	5	2	4	-3	.	.	.	.
10	.	.	.	.	-3	2	3	2	4	2	-3	.	.	.	.
11	.				-3	3	3	5	4	7	-3	.	.		.
12	.				-4	3	1	3	2	1	-4	.			.
13	.				-3	5	2	2	2	2	-3				
14	.				-3	3		2	2		-3				
15	.				-3	2	3	1			-3				
16	.				-3	1		1	1	1	-3				
17	.				-3	2	2				-3				
18	.				-4						-4				
19	.				-3						-3				
20	.	.	.	.	-3	.	.	.	.	1	-3				
21	.				-3						-3				
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23	.				-3						-3				
24	.				-4						-4				
25	.				-3						-3				
26	.				-3						-3				
27	.				-3						-3				
28	.				-3						-3				
29	.				-4						-4				
30	.	.	.	.	-3	.	.	.	.	.	-3				
31	.				-3						-3				
32	.				-3						-3				
33	.				-4						-4				
34	.				-3						-3				
35	.				-2						-2				
36	.				-3						-3				
37	.				-4						-4				
38	.				-2						-2				
39	.				-3						-3				.
40	.	.	.	.	-2	.	.	.	.	.	-2	.	.	.	.
41	.				-4						-4				.
42	.				-2						-2				.
43	.				-3						-3				.
44	.				-4						-4				.
45	.				-2						-2				.
46	.				-2						-2				.
47	.				-4						-4				.
48	.				-2						-2				.
49	.				-2						-2				.
50	.	.	.	.	-4	.	.	.	.	.	-4	.	.	.	.
51	.				-2						-2				.
52	.				-4						-4				.
53	.					-2				-2					.
54	.	.	.	.	.	.	-4	-2	-4	.	.	.	.	.	.

Key:

-1 = Presynaptic Membrane

-2 = Postsynaptic Membrane

-3 = Acetylcholine Receptor

-4 = Acetylcholinesterase

Positive numbers are transmitter molecules.

For clarity, cells which are empty are shown as . or are left blank.

Figure 2.3.1 Part of the Model Array:
Cross-Sectional View of the Central Cleft

The locations of the bounding membranes with their inactive and active sites are defined by filling cells in the appropriate places with values that the program can recognise. All membrane sites are represented by negative numbers, empty cells contain 0, and transmitter molecules are shown by positive numbers. These numbers are all stored as REAL Numbers (see the Glossary) so that that fractional parts of molecules can be stored. (Figure 2.3.1 actually shows transmitter molecules as integers. This is because the results came from the 3-D model - see later.)

Data for the initialisation of the array is contained in a template file which is read in by the main program before it starts the run. Each record in this file specifies the two-dimensional coordinates for a particular site as well as the characteristic negative value. The transmitter's starting coordinates have to be initialised as well, this being done on a complete cell basis. For a typical experiment, the transmitter starting coordinates would be around Column 23, Row 2 of Figure 2.3.1. (A sample of the 3-D model template file is given in Appendix 4.)

The cell resolution is such that the smallest area of membrane that can be defined is a 10 nm square. This would give a theoretical maximum of 10,000 sites per square micron, which is below the maximum values found for receptor density. Similarly, transmitter molecule positions are only known to this resolution.

The 2-D model had a total of about 2500 cells, each containing one real number. The storage required to hold a real number would typically be about four bytes, about 10,000 bytes being needed to store the main array.

There was no restriction on the total number of transmitter molecules that

could be in any one cell. Measurements made by Canepa, Pauling and Sorum (1966), indicate that crystalline molecules of acetylcholine are about 1 nanometre long (ignoring hydration) on their longest dimension, which would give an upper limit of the order of 1000 molecules in any cell. This is only likely to be relevant at the very beginning of the simulation, when the transmitter is packed into a small area, representing a point of release. In fact, the first runs started with all the transmitter in one cell, and 'diffusion' was relied on to bring this down to a more reasonable level within a few cycles.

Assumptions:

The assumptions used were similar to those used to derive the ideal gas laws:

1 Transmitter molecules are independent of each other.

2 They only travel orthogonally to the model's axes.

3 The direction any molecule travels in is independent of the directions it has travelled in previously.

4 Transmitter cannot penetrate active or inactive membrane sites.

5 Active sites are independent of each other.

6 Molecules that travel across the edge of the model are completely lost.

The Diffusion Algorithm - Mark 1:

The 2-D program worked by scanning each cell in the array to see if it contained any transmitter molecules. If so, the total number was divided by 6, one for each possible direction. This normally resulted in fractional molecules being produced as the total would rarely be a multiple of 6. This may seem odd, but what the model is actually calculating is the probability of there being transmitter in a particular cell (cylinder).

Adjacent cells in the model would then be checked to see whether they contained any obstructions. If not, then a portion of the transmitter would be deposited in that cell. If the cell was occupied by an active site, a running total of contacts for that type of site would be increased by the number of transmitter molecules, which would then bounce back to the cell they came from as there was no facility for binding. If the number of contacts for a particular active site was greater than 1.0, then a 'sites active' counter was also incremented.

Molecules travelling out of the plane of the array were deemed not to have changed their two-dimensional coordinates, (i.e. they remained in the same position) and the model grew in thickness with each cycle. Those that reached the edge of the model were allowed to fall off, becoming lost. As it was not possible for molecules to fall back into the model, this represented an infinite concentration gradient at the edge. This was about as close as this model could get to diffusion loss from the edges of a real synapse, losses being controlled by the membrane geometry near the edge.

The array represented the absolute locations of all of the transmitter molecules in two dimensions. After diffusion, the number of transmitter molecules in each of the three secondary clefts was also adjusted so that a coarse track could be kept on their spread.

It was necessary to maintain three arrays so that transmitter molecules entering, already in, and left in a cell could be stored separately. This was because the array had to be processed one cell at a time. If the transmitter leaving one cell for another was put straight into the new cell, that cell's contents would have changed from that at the beginning of the cycle. This can make molecules move very fast in the direction of

scanning, possibly even multiplying them on the way! The use of multiple arrays solved this problem at the cost of increased storage requirements - now some 7500 real numbers, 30,000 bytes.)

One cycle of the model was completed when all cells in the array had been processed. The molecules in the 'entering' and 'left in' arrays were then added together, and the counters (numbers of molecules, active sites contacts etc.) re-set where required, ready for the next cycle.

The program was run for 2000 cycles, with the values for active site contacts and molecule locations being printed every 10 cycles. After each 500 cycles, a complete dump of the main array was produced to allow further checking.

The results from this model indicate the spread of probability of molecules being in each cell (cylinder). As the model is symmetrical about the central secondary cleft, it was possible to reduce the total storage requirements to about half by assuming that diffusion was also symmetrical, with a mirror plane down the centre. It was then only necessary to work through (and store) half of the model, some 4000 real numbers. The central column of cells had to be treated specially as it alone is not duplicated, in order to prevent net movements across the mirror plane.

It was not possible to simulate binding of transmitter to active sites. This was because there was no fixed number of active sites - it increased with the model's thickness as the run continued. The count of molecule to active sites contacts only gave an estimate of the probability of binding. Similarly, there was no way that saturation could be investigated.

The model was able to show how, in the absence of binding, transmitter might spread out from the release site into the central and outer secondary clefts. It was also able to show the relative timing of the wave of transmitter binding probability with respect to receptor and esterase locations, though in the absence of binding, this is not particularly useful. These results are described more fully in the Results section.

Time Calibration:

In order to compare the results with experimental data, it is necessary to establish how much time one cycle represents. This is a function of the cell size and the 'temperature'. Diffusion of solvent molecules in a solvent is a complicated process at the molecular level (Stein 1962). To avoid getting bogged down with lattice energy and Maxwell-Boltzmann distribution calculations, not to mention electrical charge-charge interactions, the model used was aimed at a higher level. Negrete, del Castillo, Escobar and Yankelevich (1972) describe a model which considers the synapse as a thin, flat space of infinite size which is bounded by two planes. At time zero, a finite amount of solute M suddenly appears in the model on a line perpendicular to the planes. Diffusion takes place from this line with radial symmetry, so only the distance from the release point needs to be known. The differential equation of the system is solved to give:

$$C = \frac{M}{4 \pi t h D} \exp(-r^2 / 4 D t)$$

where C is the concentration at radius r, time t, for a model height h and diffusion coefficient D. From this can be derived two values - the time tmax at which maximal concentration occurs at a particular radius r, and

Cmax, the maximal concentration itself.

$$t_{max} = r^2 / 4 D$$

$$C_{max} = \frac{M}{pi\ r^2\ h} \exp(-1)$$

The model is quite similar to the two dimensional simulation array, given a thickness of 10 nm. The only major difference was that the model given above is continuous, and the simulations are performed on the rectangular

Table 2.3.1 Concentration of transmitter during diffusion simulation

Program DIFTEST5 (ICL 1904S)

Cell :	1	2	3	4
Cycle	Molecules contained in each cell			
0	0.000	0.000	0.000	0.000
10	0.042	13.400	23.234	0.427
20	5.073	37.919	53.631	6.573
30	12.814	46.172	58.718	14.198
40	18.215	46.999	56.430	19.328
50	21.279	45.219	52.398	22.171
60	22.792	42.638	48.233	23.519
70	23.362	39.929	44.392	23.965
80	23.372	37.342	40.976	23.884
90	23.057	34.961	37.972	23.502
100	22.557	32.804	35.336	22.956

lattice. A program was constructed that would simulate three dimensional diffusion in such a model, using a 30 * 30 cell array as the 'infinite space'. The standard number of transmitter molecules (10,000) were placed at coordinates 15, 15 and the program started off. The contents of each of the cells in the array was printed every 10 cycles. After the run had finished, four cells were selected for the next stage of the calculation. They had to be far enough away from the release point that the concentration maximum was not immediate, and yet sufficiently close that the maximum was reached in less than 100 cycles. The concentration values for each of these cells are shown in Table 2.3.1 above.

From this table, the maximum concentration obtained in each of the cells and the time in cycles at which it occurred can be estimated - bearing in mind that the sampling, one cycle every ten, is rather coarse. The theoretical times at which the maxima occur can be calculated using the

Table 2.3.2 Theoretical Times to Maximum Concentration

Cell	1	2	3	4
Coordinates	10, 20	10, 15	11, 13	8, 15
Distance from Release Point	70.7 nm	50 nm	44.72 nm	70 nm
Time (microsecs)				
Eccles	1.644	0.8224	0.6579	1.612
Land	3.125	1.5625	1.25	3.06

equation given above. The coordinates for each cell and the theoretical times to maximum concentration are shown in Table 2.3.2. This was then divided by the number of cycles to give the time calibration. Unfortunately, there is some disagreement in the literature as to the correct value for acetylcholine's diffusion coefficient. Eccles and Jaeger (1958) give it as 7.6 E-6 $cm^2.sec^{-1}$, but Land et al (1980) correct this to a minimum of 4 E-6 using their simulation to generate the maximum likelihood value. Wathey et al (1979) use a value of 3 E-6. Values of tmax for both Eccles' and Land's diffusion coefficients are shown. Times calculated using Eccles' value are shorter as the molecules, moving faster, arrive in the cell sooner.

The cycle in which the maximum occurs is only estimated to the nearest 10, and in most cases lies between two. This produces a range of possible time

Table 2.3.3 Time Calibration Estimates

Cell	1	2	3	4
Minimum (cycles)	70	30	30	70
Maximum	80	40	40	70
Eccles:				
Minimum (ns)	20	21	16	20
Maximum (ns)	23	27	22	23
Land:				
Minimum (ns)	39	39	31	38
Maximum (ns)	44	52	42	44

calibrations in nanoseconds per cycle, which are given in Table 2.3.3. The range in the diffusion coefficients makes a more accurate calibration impossible.

To cross-check the above figures, the theoretical concentration maxima were also calculated. Fortunately, these are not dependent upon the diffusion coefficient. The values obtained for the four cells are given in Table 2.3.4.

Table 2.3.4 Theoretical Concentration Maxima (Molecules per cell)

Cell	1	2	3	4
Theoretical	23.41	46.83	58.55	23.90
Estimated	23.37	47.00	58.72	23.97

The values predicted by both methods are pretty close, even allowing for the discrete simulation integrating the concentration over a finite volume.

Implications:

The time calibration determines the time during which molecules of transmitter remain bound to active sites. Using a mid-range compromise calibration of 30 nanoseconds per cycle indicated that for a channel open time of about 250 microseconds, some 7500 cycles would be required. Similarly, the fastest reported turnover time of acetylcholinesterase is only about 30 microseconds, (Silver 1974). This would still take 1000 cycles to complete.

Restrictions of the 2-D Model:

The most significant restriction of the 2-D model is its inability to model binding of transmitter to active sites. This prevents the buffering effects of binding on diffusion from being investigated. It also prevents any measure of receptor saturation, which would be expected to occur in the immediate vicinity of the release 'point'. The figures indicating the spread of transmitter will also be overestimated as binding would be expected to slow everything down somewhat. Also, as there is no binding, there is also no useful timing information relating to channel opening times.

Moving to Three Dimensions:

Modelling in three dimensions enabled binding to individual sites to be monitored. It also meant that the model had to be completely re-designed as the strategy used in the 2-D approach was not readily upgradeable without needing a much larger computer to hold the cell array.

The target was then to duplicate the 2-D experiment in 3-D so that the results could be compared. Some new features were included - specifically, multiple binding of transmitter to each receptor and cholinesterase location - and this led on to the inclusion of a simple form of cooperativity between the binding at the active sites of each molecule.

The most important change to the program design was in the way the data was stored. As there was no simple way of storing the three dimensional structure in any of the accessible computers (all at the same time), the three-dimensional coordinates for each transmitter molecule were stored in a random access file. Together with a flag value which was used to indicate whether the molecule was free, bound, or lost. These were all integers,

requiring one word of store each, giving a total of 40,000 words, at least 80,000 bytes. Also, as individual molecules are modelled, the symmetry which enabled the 2-D models to be halved in size was no longer possible.

The diffusion mechanism was changed so that molecules were not split - each molecule being examined in turn, and if it was free (i.e. not lost or bound), a random number generator was used to decide in which of the six possible directions it was to move. The cell in that direction could then be checked to see if it contained an active site or an inactive membrane site. In the case of an active site with unused binding capacity, another random number was generated to represent the chance that molecule had of binding. Tables of required probabilities were used to determine if the random number was high enough, and if so, binding occurred. This changed the state of the active site of course, and if it had become fully bound, it was deemed to be active - either hydrolysing the transmitter, or having an open ion channel.

Early versions (North Star CP/M + Microsoft FORTRAN 80) held a small active sites array in memory while the new diffusion algorithm was under test, but this eventually had to go the same way as the transmitter - into a random access file - to minimise the amount of storage required.

The two-dimensional initialisation template was modified to include a third coordinate field, and the initialisation of the transmitter and active sites files was separated from the main diffusion program, the files being set up prior to the main run. The active site geometry was unchanged.

The record length of the active sites file is determined by the number of active sites in a cylinder - or - how thick the model is. The test target

was a thickness of 64 cells. With the same depth and width measurements as before, the total model volume is 155,520 cells. There are some 22,000 membrane cells in the active sites file, 15,744 of these being really active, which represents a considerable saving in space. Two integers are stored for each active site, one of these gives the state of binding, the other being used to hold the time, in cycles, when the most recent molecule became bound.

The Random Number Generator:

In many simulation programs, random numbers are used to minimise the effects of number patterns on the results. Most digital computers are unable to generate truly random numbers. Instead, they use a variety of methods to generate numbers that are nearly random - pseudo-random. These almost always use the previous result as the means for calculating the next one, so producing a sequence of pseudo-random numbers. This sequence normally repeats itself after a certain length, the trick being to make the repeat distance of the sequence very long, whilst producing 'good' randomness. The random number generator used here for diffusion direction and binding probability is based on the multiplicative congruence method using values from Thomson (1982). In this method, the previously generated number r(i), is multiplied by a fixed number (u). The result of this is then divided by a large, usually prime, number (m), and the remainder from this division gives the next pseudo-random number r(i+1) in the sequence.

$$r(i+1) = (u * r(i)) \bmod m$$

Mod is the name given to the 'divide and give the remainder' operator. The values used were 254 for u, and 65537 for m. Calculations were all performed using Real numbers. The sequence was seeded with a starting value

of 1.0, so that simulation runs would be repeatable, and it repeated after 65536 numbers, when all possible values between 1 and 65536 had been produced. For diffusion direction, the pseudo-random number was divided by six, and the remainder, plus 1, gave a value in the range 1 to 6 inclusive. The probability number range was reduced to between 0 and 1.0 by dividing the random number by 65536.0.

All this real arithmetic took some time, so efforts were taken to make more efficient use of the numbers generated. One method used was to fill a short array with direction numbers, and then to step through this array. However, even with the length of the array being chosen carefully, unacceptable patterning was produced - a bit like all the molecules going in the same direction each cycle, only a bit more subtle - and this approach was dropped. Pseudo-random numbers were then generated from the sequence whenever required, which increased the run-time on the North Star, using 8080 software arithmetic, by some 30%. Later version running on the Harris H800 were able to take advantage of the hardware arithmetic unit available, so that the calculation overhead became insignificant.

The main consequence of changing the data storage from in-memory to random access (disk) files was an increase in run-time. This is largely the result of accessing the active sites file, as the program works through the transmitter file in order, reading the coordinates for several transmitter molecules into memory at the same time. However, there is no correlation (apart from the random number sequence) between the locations of successive molecules, so each one could involve a read and write operation on the active sites file. The program is optimised to some extent to minimise file operations, but excessive optimisation can result in poor portability.

With the increase in run-time, it became impractical to run the program all in one go, as the early 2-D programs had run. This also made it necessary to split the functions up into a suite of programs, one for file initialisation, one to run the simulation for a fixed duration, and several others to look through the data files for auditing purposes. Descriptions of the programs that make up the suite are given in Appendix 1.

Further Assumptions:

Some new assumptions were made to facilitate implementation. These are as follows:

7 Transmitter binding to active sites can be determined by probability - they do not need to have energy levels. Binding can then be determined by a random number.

8 Active site activity is not dependent upon any electrical changes which might result from transmitter binding.

9 Apart from co-operativity effects, binding of successive molecules to an active site is independent.

10 The cross-sectional structure of the synaptic cleft can be considered as being constant when viewed along a secondary cleft or fold.

Assumption 8 is a fundamental restriction of the model. This information is not available until the results from the simulation run have been processed. Assumption 10, which also applies to the positions of active sites, is to minimise the size of the initialisation template file.

Current Restrictions:

1 Spontaneous release from binding is not implemented. Indeed, nor

is any kind of release mechanism.

2 Time delays due to conformational change are not implemented, though the data to implement them is available.

2.4 How to use the Simulation

Introduction:

This section is intended as a User's Guide to the Simulation program suite. It indicates the sequence of actions needed both to define the parameters for a run and also how to get them into the computer. It does assume that the programs are already available on the computer and that the user understands how to access them. It also assumes that the user has a working knowledge of computer programming, preferably using FORTRAN.

What is Defined Where:

Several of the simulation's parameters can be modified. These include:

1. The synaptic geometry - size and layout.
2. The densities of Receptor and Esterase active sites.
3. The number of molecules that can bind to each active molecule.
4. The relative probabilities for binding of successive molecules.
5. The number of transmitter molecules released.

Changes in the layout of the model, the densities of active sites, and the initial number of transmitter molecules can all be made fairly easily. A new initialisation template file must be produced containing the required data. If this is sufficiently similar to previous versions, then editing may be all that is required. A sample template file is included in Appendix 4 to show the format required for the three-dimensional simulation programs, and further details are given later in this section. This file should not be changed during a simulation run as it is used by several of the programs.

The binding probability arrays are contained in the main program. They can be modified, either in values, or in size, and though the main program will then have to be re-compiled, no other programs are affected. This is not the case when alterations are made to the size of the model array. Any alterations in the dimensions affect all of the programs in the suite so they must all be made to match.

In practice, it is unlikely that any increase in the number of transmitter molecules will be required, though reductions will improve the run-time performance.

Redefining the Geometry:

The most obvious first step is to define the three-dimensional geometry of the target model. Bearing in mind the restriction that the cross-sectional structure must be constant, this stage is simply a matter of defining that structure, and setting up a suitable data file. The same file contains the starting locations for the transmitter molecules, normally placed in a small number of cells to minimise the size of the file.

The format expected in this file is fixed. Each line in the file contains three non-negative integers in the range 1 to the maximum allowed for each dimension. These integers are entered in fields of three (numeric) characters, for the K (1 to KMAX), J (1 to JMAX) and I (1 to IMAX) coordinates, followed by a six character field. This last field is where the characteristic value for an active site (negative) or the number of transmitter molecules to go in that cell is specified. For active sites, only the first two coordinates are significant as the whole I cylinder is defined in one go. Transmitter molecules must have their full 3-D coordinates set. Values out of range (e.g. I < 1 or I > IMAX) may not be

detected by the compiled FORTRAN program and can produce strange results. Take care. The line or record number in this file is used to calculate the pointers to be used to access the data in the active sites file.

Active Sites Characteristic Values:

-1 Inactive site - normally reserved for presynaptic cells

-2 Inactive site - normally reserved for postsynaptic cells

-3 Receptor site - with no transmitter bound

-4 Esterase site - with no transmitter bound

Having decided on the geometry, this file can be generated. It should be checked very carefully to make sure that there are no gaps in the model membranes. These can let transmitter through to the 'inside' of cells. There is no check included in DIFFUSE6 for membrane sides, so this could result in binding on both sides of the membrane! The data file used for the demonstration run actually contained two 'holes'. Fortunately, these were both in the pre-synaptic surface, and were sufficiently close to the edge of the model as to make no significant difference.

Altering the Binding Probabilities:

There are two arrays, one for receptor binding (RBIND), the other for esterase binding (EBIND) and they contain values between 0.0 and 1.0 for comparison against a random number generated in the same range during the program run. If the random number is greater than the binding value, binding occurs. The arrays contain the values required for one, two, three and so on molecules to bind respectively. There is no restriction on the number of molecules that can bind other than the storage required, the lengths of the arrays being defined appropriately using EMAX and RMAX - see

below. The values are initialised at the start of a run from DATA statements in the program. Current values are given below. The value drops for successive molecules, making it easier for them to bind.

Binding Probabilities Used Currently:

Active Site	Maximum	Transmitter Molecule			
		1st	2nd	3rd	4th
Esterase	EMAX = 4	0.875	0.75	0.5	0.375
Receptor	RMAX = 2	0.75	0.5		

Reconfiguring the Programs:

The sizes of the arrays used by the programs are set using manifest constants, that is symbolic names which have a constant value associated with them. Their values are defined in FORTRAN 77 by the use of the PARAMETER statement. Changes in the configuration can be made by simply changing the values given. However, some have important repercussions on the structure of the programs and the random access files, so changes should be made with care. The constants' names and current values are detailed below:

Name	Meaning	Value
EMAX	- Maximum number of transmitter molecules that can bind to a single esterase active site. Binding probabilities are defined in the main program.	(4)
IMAX	- Maximum thickness of the model array. This is also used to define the number of active sites in a record	(64)

in the random-access file ACTIVE1.

JMAX - Maximum height of the model array. This is also one of the dimensions of the pointers array. (54)

KMAX - Maximum width of the model array. This is another dimension of the pointers array. (45)

LMAX - Number of cycles that the main program will run for before stopping. 500 cycles take about 10 hours on the Harris H800 as a control point job. (500)

RMAX - Maximum number of transmitter molecules that can bind to a single receptor active site. The probabilities are defined within the main program. (2)

SLICE - Same as TMAX (32)

TMAX - The number of transmitter molecules in each record in the random-access file TRNSMT1. (32)

Changes to the dimensioning constants will affect the amount of memory required to store the pointers array and the random access file buffers. Increases in the number of active sites in the template file will increase the size of the active sites file. Small modifications are not likely to have much effect on program run-time.

When changes are made, they must be made to all programs in the suite or the resulting mismatch will produce odd results or program termination.

The number of cycles that the program runs for before stopping is defined by LMAX. With a value of 500, the run takes about 10 hours (overnight) on the Harris H800. The upper limit for the program as it stands should be considered to be 1000 cycles, but this may take longer than 24 hours to complete. To continue from where the previous run left off, it is only

necessary to re-start the job without initialising the files.

Having successfully made any changes to the program source file, this should then be recompiled using the FORTRAN 77 compiler. This operation will produce an object file which has to be linked with the FORTRAN run-time support library to form an executable job. On the Harris, this is done using the Vulcaniser.

Running a Simulation:

A further file has to be produced. This is the job control or command file, which the computer uses to sequence the programs in the job. A sample job control file is included in Appendix 3.

The sequence of running the auditing programs is not important. It is only necessary that all the information that is required for later analysis is obtained at this stage, or it will be lost when the main program is next run.

The simulation run proper can then be set up. The first program to run is the random access file initialiser LOADAT (Job name LOAD64). Having set these files up, the run can be started by activating the job. Successive runs will carry on from where the last one left off, in lumps of LMAX cycles.

2.5 Results

This thesis is a report on the work involved in the development of the simulation as an investigative tool. As such, the majority of results obtained so far have been from tests which were mainly intended to detect bugs. The development programme is not completed yet as there are still further enhancements to be made, though the operation of the diffusion and binding algorithms have been checked out. In particular, no attempt has been made to include release of bound transmitter. This may be a tricky problem to surmount without incurring a considerable increase in run-time, particularly if a random component is to be included in the binding times.

The Results so far:

The time represented by one cycle is identical for both the two and three-dimensional models. The size of the model cell is also the same, which, given identical set-up data files, should make both simulations directly comparable. This comparison cannot be complete, as the difference in the method of storage makes some of the data inaccessible during runs of the 3-D model. Also, of course, the models' capabilities are not identical, so there should be some differences.

The Two-Dimensional Model:

Table 2.5.1 shows the results from the 2-D model. 10,000 molecules of transmitter were 'released' from a point above the central of the three secondary clefts, (at Column 23, Row 2 on Figure 2.3.1, page 44). The location breakdown is based on the K (horizontal) coordinate. Transmitter in the cells between K = 16 and K = 30 (i.e. actually in the part of the array shown in Figure 2.3.1) is deemed to be in the central cleft, outside

Table 2.5.1 2-D Model Transmitter distribution and 'binding'

Program CLEFTEST4, Data DATA7 (ICL 1904S, 19-Jul-78)

Cycle Number	Total Mol's	Central Cleft Mol's	Outer Clefts Mol's	Central Receptor Contacts	Outer Receptor Contacts	Central Esterase Contacts	Outer Esterase Contacts	Lost Mol's
0	9999.9	9999.9	0.0	0.0	0.0	0.0	0.0	0.0
100	9998.6	8170.8	1827.8	253.9	68.7	53.3	15.7	1.4
200	9921.7	6864.5	3057.2	248.1	108.0	51.7	24.3	78.2
300	9684.8	6147.9	3536.9	234.8	125.8	49.0	27.5	315.1
400	9356.2	5661.2	3695.0	223.3	134.8	47.0	28.9	643.7
500	8999.4	5291.3	3708.1	213.0	138.8	45.5	29.6	1000.6
600	8644.7	4990.8	3653.9	203.5	139.9	44.4	29.9	1355.3
700	8305.1	4736.5	3568.6	194.5	139.2	43.6	29.9	1694.9
800	7985.2	4515.4	3469.8	186.1	137.2	43.1	29.9	2014.8
900	7686.1	4319.6	3366.5	178.1	134.6	42.7	29.9	2313.9
1000	7407.2	4143.9	3263.3	170.6	131.4	42.4	29.8	2592.8
1100	7147.5	3984.8	3162.7	163.4	127.9	42.3	29.8	2852.5
1200	6905.6	3839.6	3066.0	156.6	124.3	42.2	29.8	3094.5
1300	6679.9	3706.4	2973.5	150.3	120.6	42.1	29.8	3320.1
1400	6469.1	3583.5	2885.6	144.3	116.8	42.1	29.8	3530.8
1500	6272.2	3469.9	2802.3	138.6	113.2	42.0	29.9	3727.8
1600	6087.7	3364.3	2723.4	133.3	109.6	42.0	29.9	3912.3
1700	5914.6	3265.9	2648.7	128.3	106.1	41.9	30.0	4085.3
1800	5752.2	3174.1	2578.1	123.7	102.7	41.9	30.0	4247.9
1900	5599.2	3088.0	2511.2	119.3	99.4	41.8	30.1	4400.8
2000	5455.2	3007.3	2447.9	115.2	96.3	41.7	30.1	4544.8

this region, the molecules will either be in the outer clefts, or lost off the edge. Figure 2.5.1 shows how the total population of 10,000 transmitter molecules is split into these three categories during the simulation run. The difference between the top of the vertical axis and the stars shows the transmitter that has been lost, with the round dots dividing the molecules still in the model into the central and outer clefts. The time, in cycles, is shown on the horizontal axis. The time calibration is taken as 30 ns per cycle, so the total run length represents 60 microseconds.

In a similar fashion to the transmitter location, the 'binding' to receptors and cholinesterase (Esterase) sites is divided into central and outer cleft contributions. These are shown graphically in Figures 2.5.2 and 2.5.3 for receptors and cholinesterases respectively. The solid dots show the central cleft total, and the open stars that in the outer clefts.

In both cases, the central cleft value rises very rapidly from zero to a peak value after about 100 cycles (actually 120, equivalent to only 3.6 microseconds from the moment of release) and then starts to decline. The proportion in the outer clefts rises more slowly to a peak at about 600 cycles (18 microseconds) before declining. The values for 'esterases' are much smaller than those for receptors because the distribution of receptors is more favourable - there being far more receptors near the top of the secondary clefts. A similar pattern is seen, though the later decline is balanced by more even spreading into the deeper parts of the clefts where the higher density of cholinesterase sites offsets the effect of the steadily reducing number of molecules.

The speed of diffusion in the absence of binding is shown by the count of lost molecules. Though there are always more molecules in the central cleft

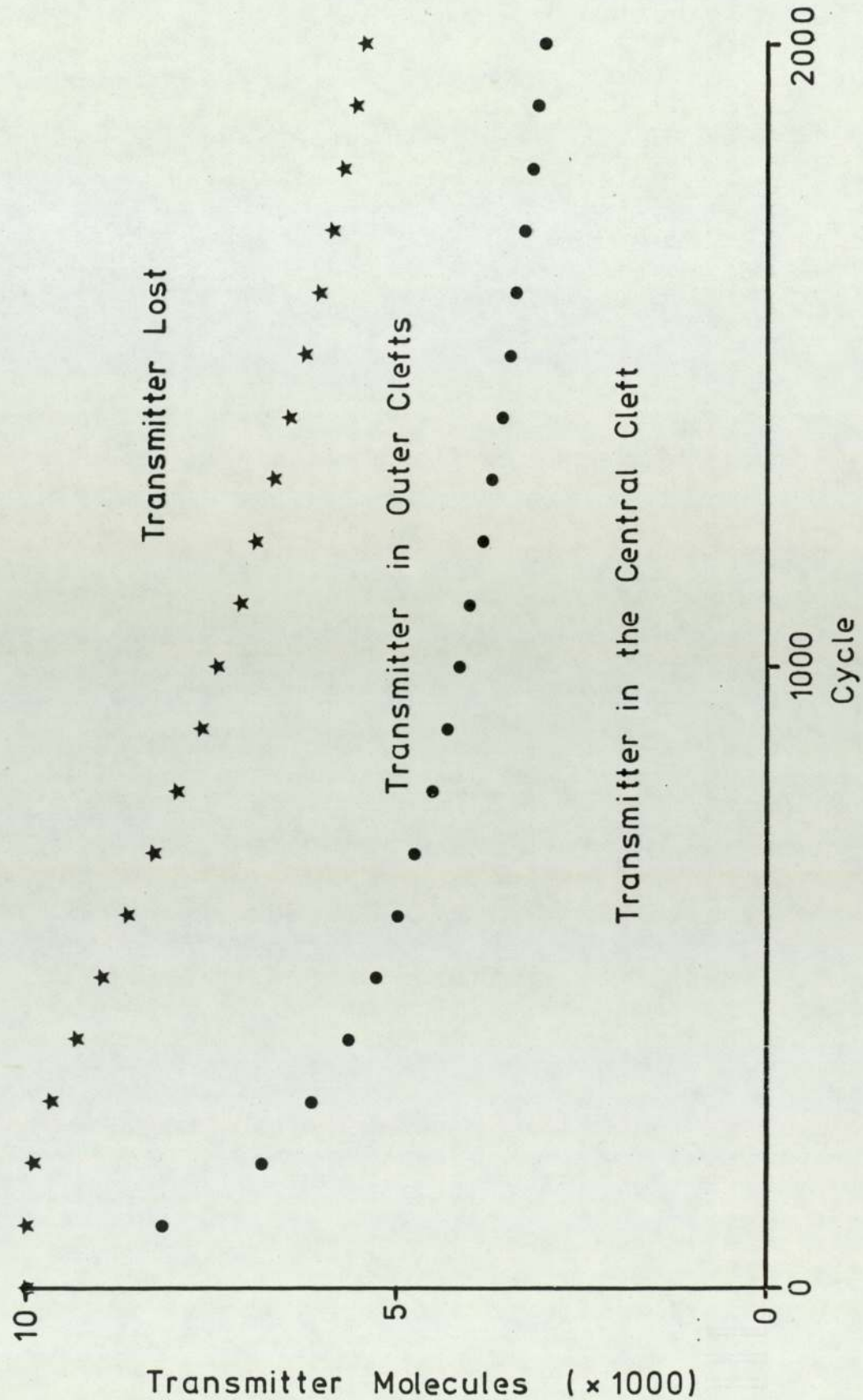

Figure 2.5.1 The Distribution of Transmitter in the 2-D Model.
At the start, all of the transmitter is over the central secondary cleft. As the model progresses, shown by the time in cycles, this distribution changes with some molecules diffusing into the outer clefts, some even becoming lost by falling off the edge.

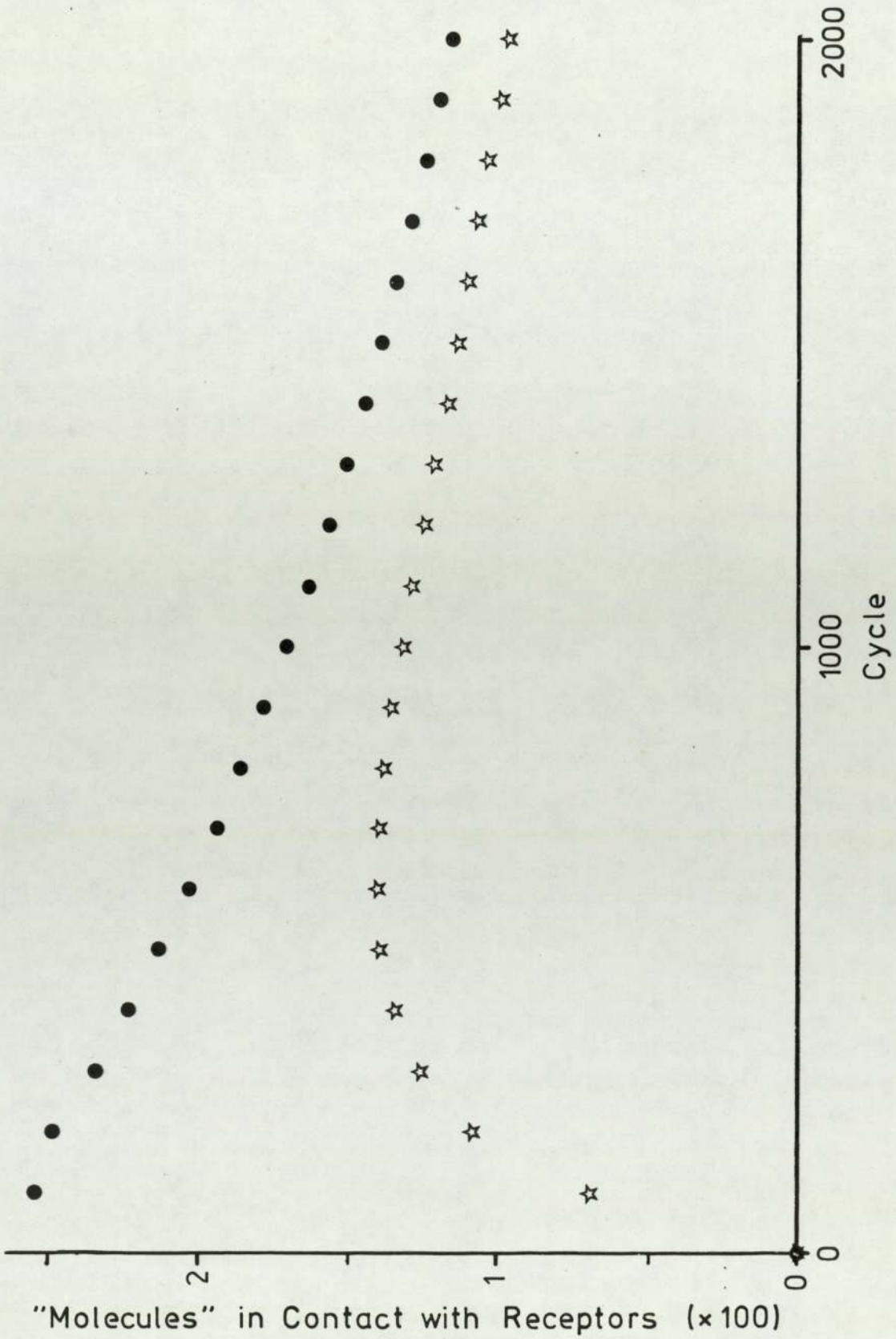

Figure 2.5.2 Transmitter in Contact with Receptor Active Sites
The number of molecules in contact with receptors in the central cleft (shown ●) rises rapidly from zero at the start to a peak, before declining. The value for the outer clefts (☆) rises more slowly to a lower peak before declining. Model progress is shown in cycles.

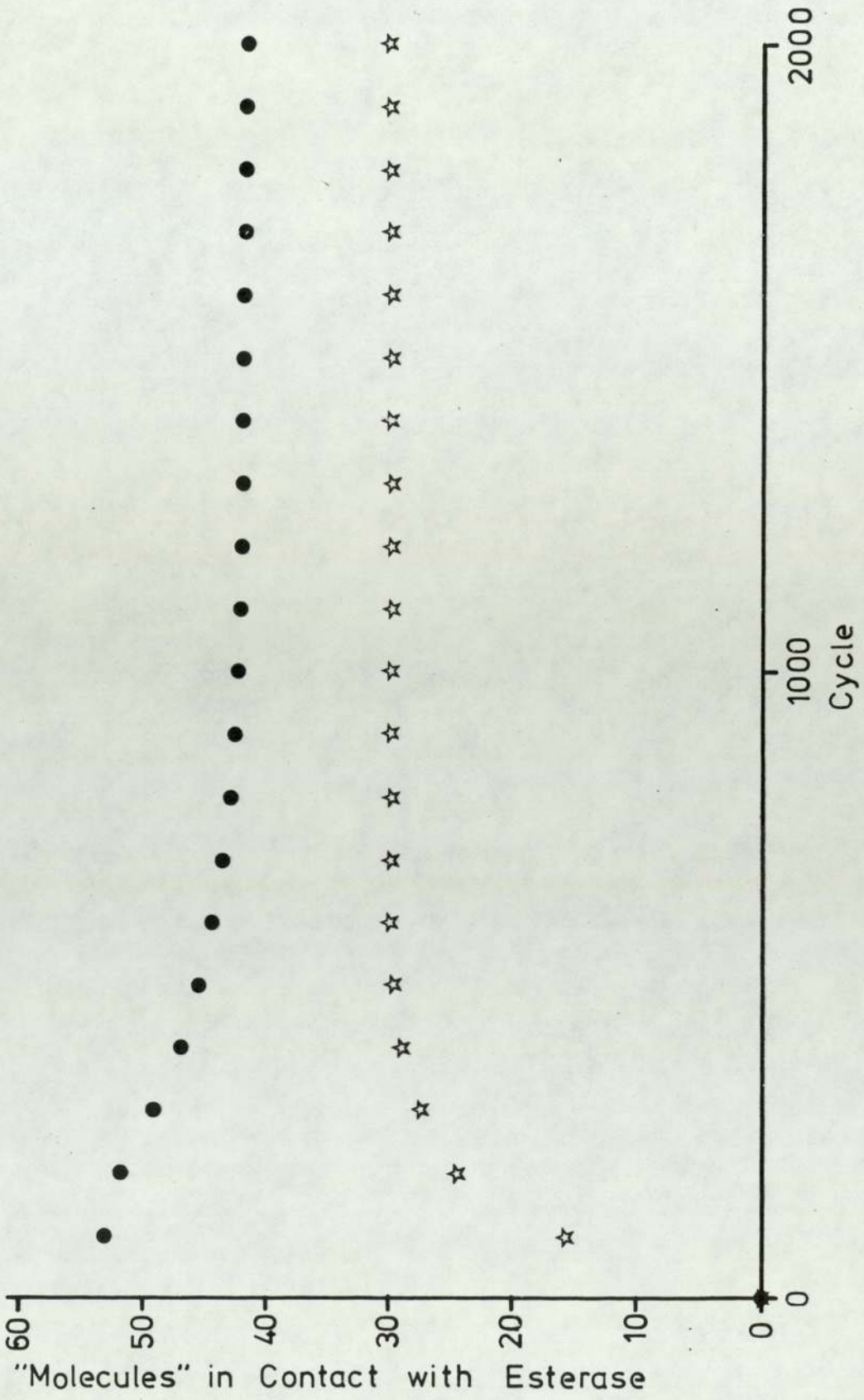

Figure 2.5.3 Transmitter in Contact with Cholinesterase
The number of molecules in contact with cholinesterase in the central cleft (shown as ●) rises rapidly from zero to a peak, before falling slowly. The value for the outer clefts (☆) rises more slowly to a steady value. Model progress is shown in cycles.

than the outer two, half of the initial 9999.9 have left the central cleft by cycle 600, (18 microseconds), and by the end of the run at cycle 2000, nearly half have left the model completely by falling off the edges. The model is not true to reality in this respect, as most end-plates are considerably wider than the 450 nm modelled here, and most if not all of the lost molecules would have been spreading into further adjacent secondary folds.

After 2000 cycles, the model has grown to 40 microns in 'virtual' thickness (2000 cells in each direction from the initial 1). This is still a reasonable value, though it is larger than the typical mouse diaphragm end-plate, and indicates that diffusion loss is not likely to be a major cause of transmitter removal in the short term.

The Three-Dimensional Model:

The starting conditions for the 3-D model were almost identical to those of the 2-D model described above. The starting coordinates for the transmitter included a third dimension, the I coordinate being 32. The major difference between the 2-D and 3-D models is the effect of binding to active sites.

Table 2.5.2 below gives the abridged results from this run. Note that, as whole molecules only are permitted in this model, there are no decimal places in the table. As the results below are only those from the first full run of the program, there are gaps which are partly the result of the reduced accessibility of the data while the program is actually running.

The distribution of transmitter molecules, both free and bound, between the central of the three secondary clefts and the outer two, is not known for

Table 2.5.2 3-D Transmitter distribution and binding

Program DIFFUSE6, Data DATA64, (Harris H800, 18-Aug-83)

Cycle Number	Total Mol's	Central Free Mol's	Outer Free Mol's	Central Receptor Binding	Outer Receptor Binding	Central Esterase Binding	Outer Esterase Binding	Lost Mol's
0	9999	9999		0		0		0
100	9997	8976		827		194		2
200	9921	7725		1714		482		78
300	9814	6628		2500		686		185
400	9648	5539		3240		869		351
500	9464	2681	1916	3854		1013		535
600	9228	3706		4382		1140		771
700	9011	3043		4739		1229		988
800	8840	2563		4988		1289		1159
900	8701	2126		5234		1341		1298
1000	8607	1039	713	2850	2602	846	557	1392
1100	8561	1534		5585		1442		1438
1200	8495	1348		5677		1470		1504
1300	8462	1199		5748		1515		1537
1400	8412	1035		5835		1542		1587
1500	8380	632	290	3012	2887	919	640	1619
1600	8351	829		5949		1573		1648
1700	8340	764		5995		1581		1659
1800	8323	697		6035		1591		1676
1900	8304	642		6060		1602		1695
2000	8286	481	106	3103	2983	947	666	1713

the majority of the run above. It is not currently dumped on a cycle-by-cycle basis, and the program to extract it from the transmitter random access file was written during this run. Where the distribution is known, the values are shown in the appropriate column in the table. Where only the total is known, this is shown as a single column inbetween the two others.

Figure 2.5.4 shows the distribution of transmitter between the model components as for Figure 2.5.1, but now there are two categories of bound molecules. These are, of course, those bound to receptors and those bound to cholinesterase active sites. On the other hand, the distribution of molecules between the clefts is not known. Free transmitter is shown by the solid circles, with the portion bound to receptors between this line and the line of open stars. The molecules bound to cholinesterase ('Esterase') active sites are shown between the receptor binding and the solid stars, which mark the lost fraction.

The most obvious difference is that the numbers of molecules bound are much higher. This is also reflected in the lower number falling off the edge. As binding is currently irreversible, there is no decline in the numbers of bound molecules. Where available, the data for the distribution between the central and outer clefts indicates that about half of the total transmitter becomes bound in the outer two. Figures 2.5.5 and 2.5.6 show the total numbers of transmitter molecules bound to receptor and cholinesterase active sites, respectively, as the solid circles. Below the asterisks are the contributions made by the central cleft binding sites alone. Note that neither of these values have reached a peak in the 2000 cycles, though both have levelled off after a very rapid initial rise.

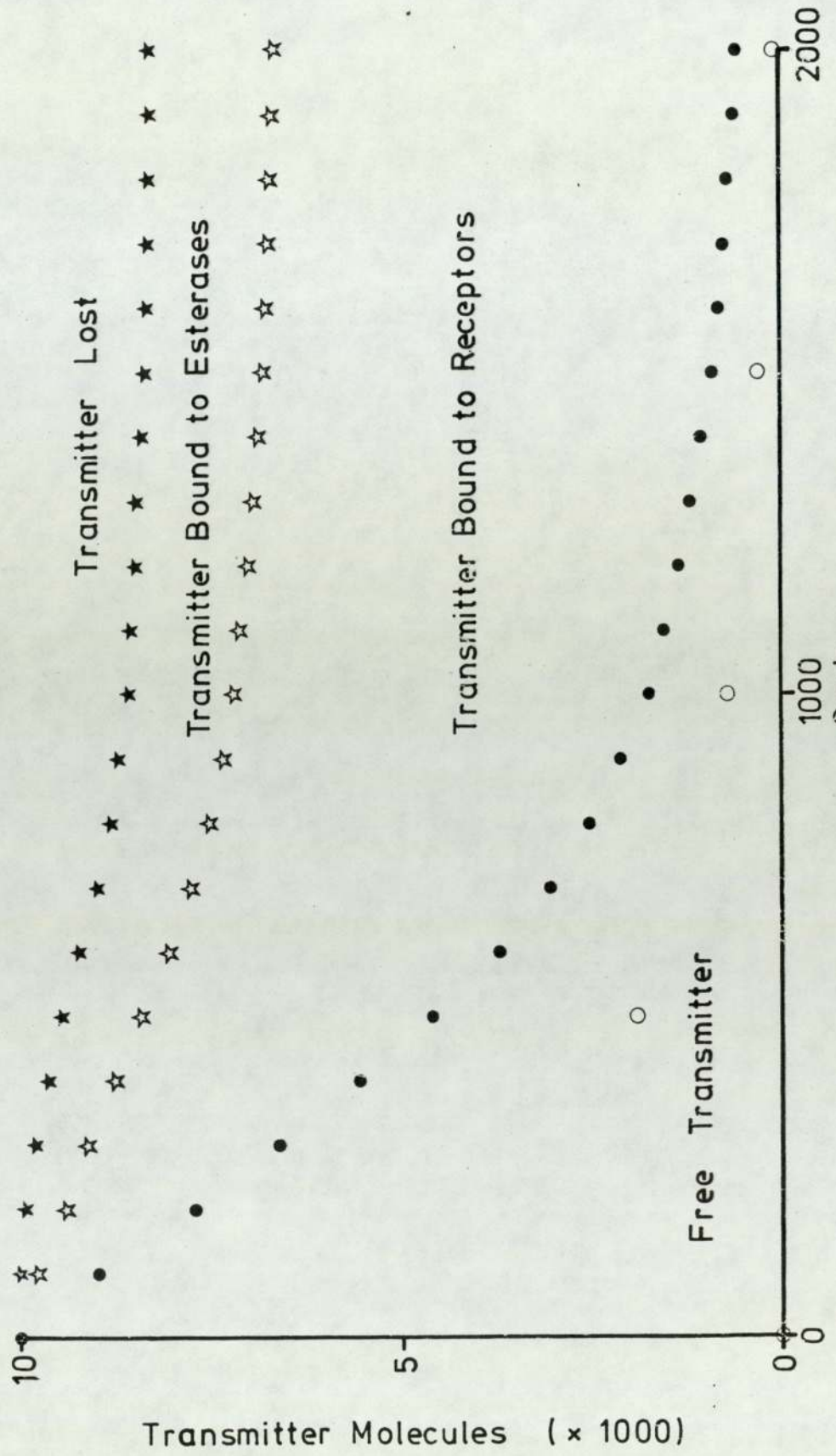

Figure 2.5.4 Distribution of Transmitter in the 3-D Model. Initially, all 10,000 transmitter molecules are free. As time progresses, many become bound to receptors and cholinesterase active sites. A small proportion become lost in the 2,000 cycles of this run. The figure above shows the contributions each compartment makes towards the total number.

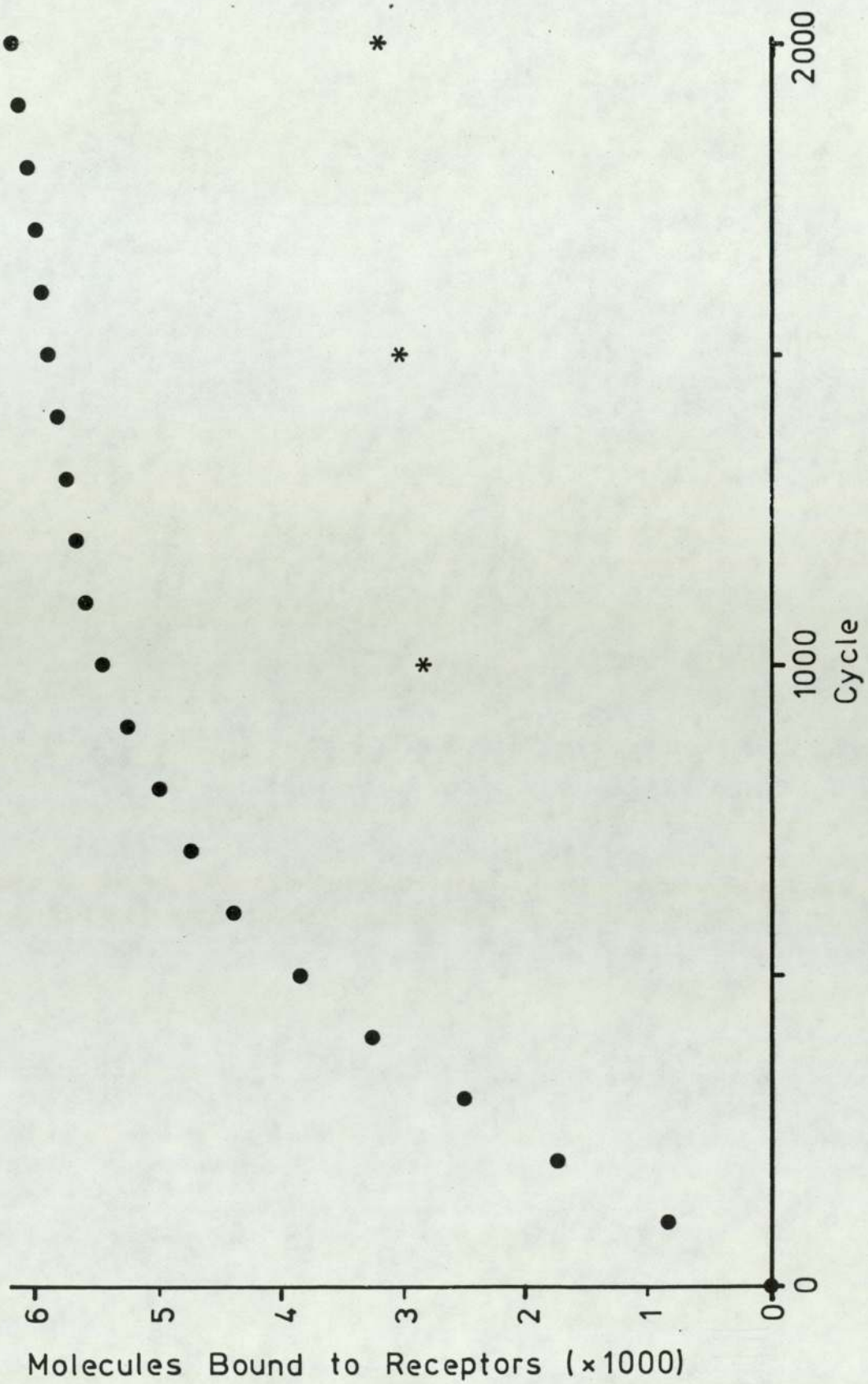

Figure 2.5.5 Transmitter Molecules Bound to Receptor Active Sites The number of transmitter molecules bound to receptors rises monotonically with time. After 1000 cycles, the rate of rise falls to a low value. Peak amplitude is not reached in this model. The proportion of the total made up by transmitter in the central cleft is shown by the asterisks.

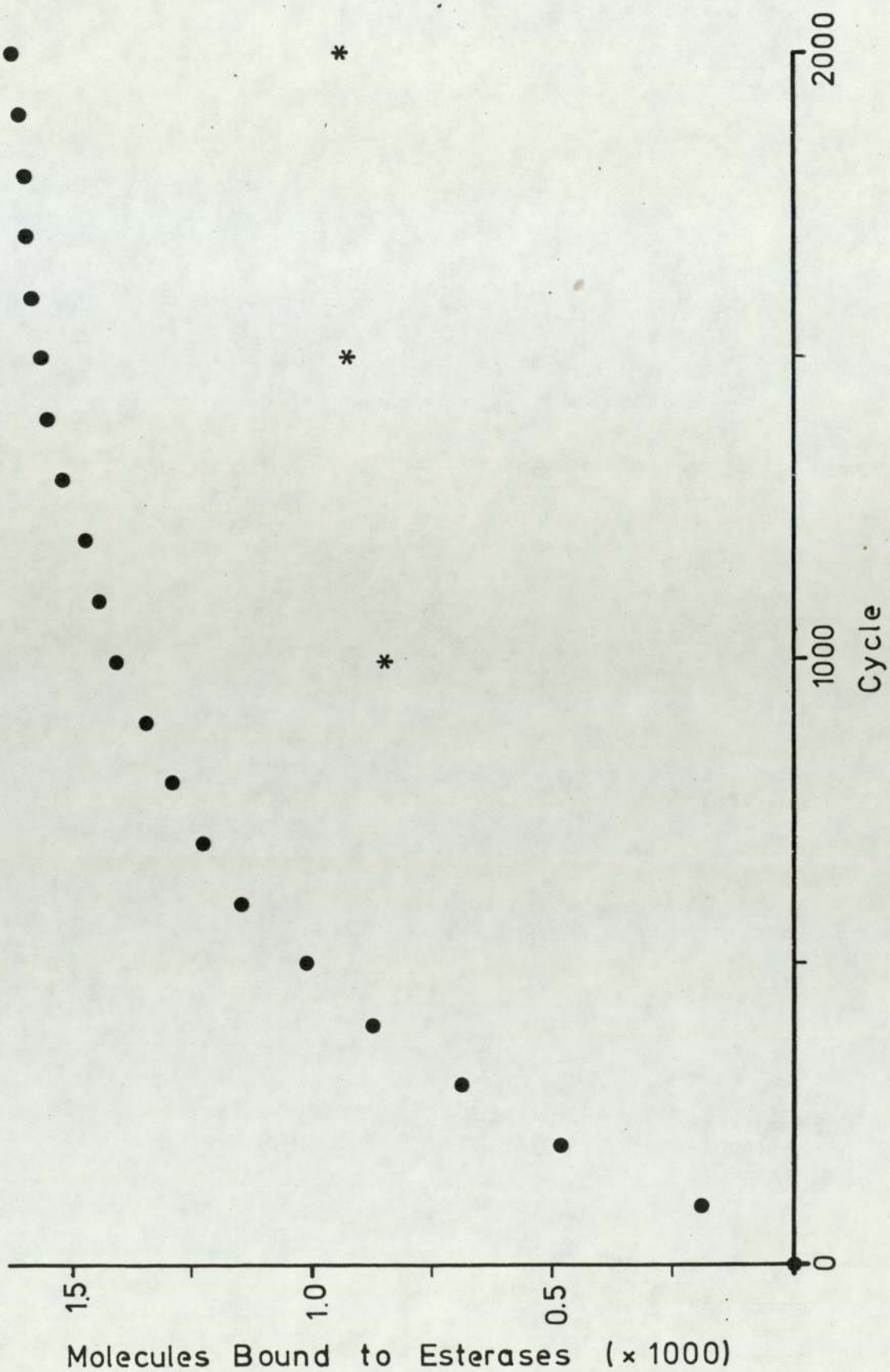

Figure 2.5.6 Transmitter Molecules bound to Cholinesterase Active Sites
The number of transmitter molecules bound to cholinesterase rises rapidly from zero. The rate of rise decreases steadily, but even after 2000 cycles, a peak value has not been reached. Of these, the number of molecules bound in the central cleft is shown by the asterisks.

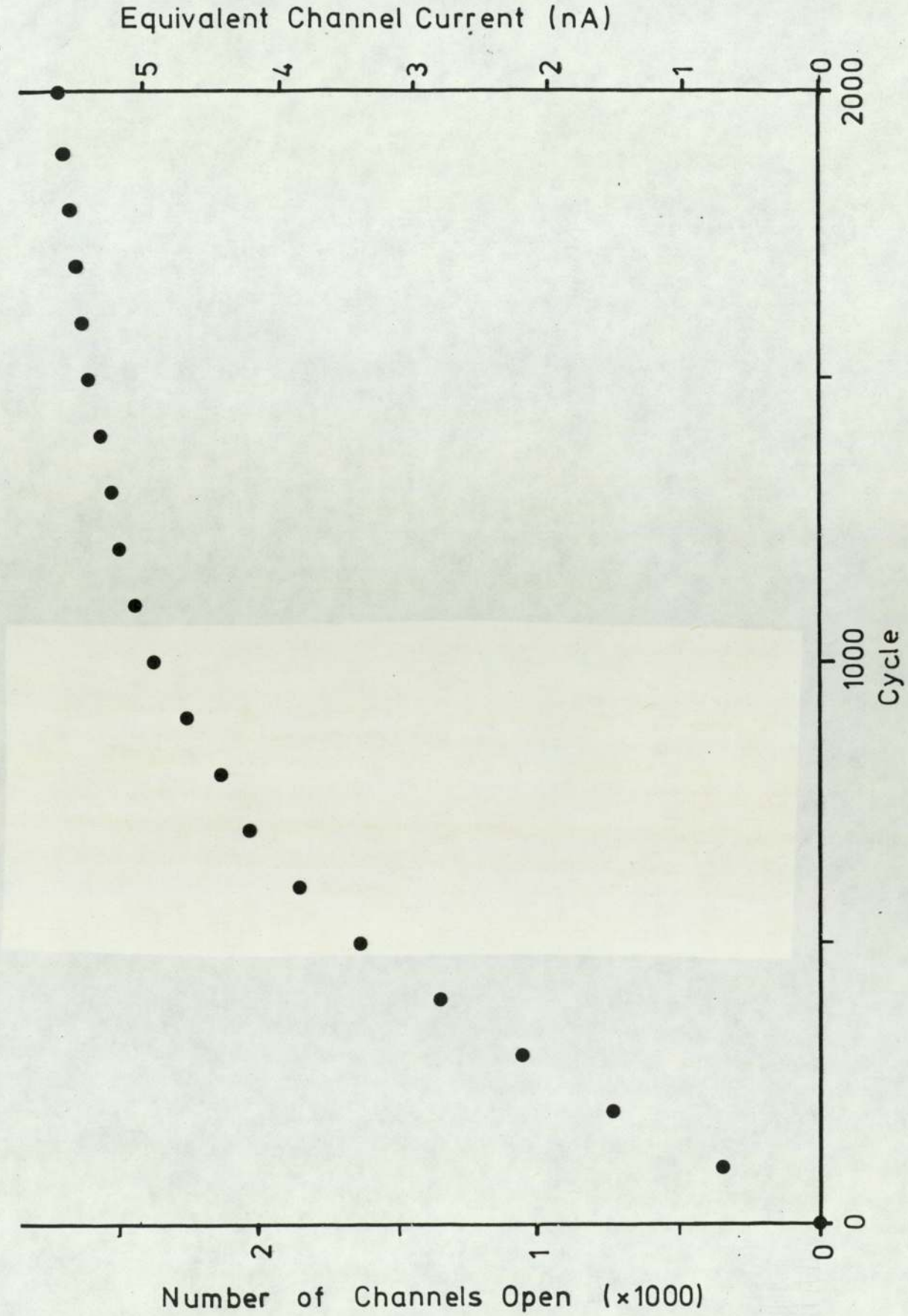

Figure 2.5.7 The Number of Open Receptor Channels
Not all receptors are able to bind two transmitter molecules. Those that do open their ionic channels. Using 2.08 picoamps as the current flowing through each channel, the total equivalent current is also shown above.

Initial Transmitter in model = T

Transmitter bound singly to Receptors = T1

Transmitter bound doubly to Receptors = T2

Transmitter Efficiency = (T2 / T) * 100 %

Receptor Occupancy = T2 / (T1 + T2) * 100 %

Of the 10,000 transmitter molecules released at the beginning of the model, an increasing number become bound to receptors (see Figure 2.5.5). A large proportion of the receptors are able to bind two molecules and open their ionic channels. The progress of this is shown in Figure 2.5.7 where the equivalent current which flows through the membrane is calculated on the basis of a channel conductance of 26 picoSiemens (Head 1983) and a resting membrane potential of -80 millivolts. These values are tabulated in Table 2.5.3 below, together with the instantaneous current flow. The very short channel current rise time predicted by this model is smaller than electrophysiological measurements indicate, but there are no delays in this model for conformational changes.

Table 2.5.3 also includes the percentage of the initial 10,000 transmitter molecules that become doubly bound to receptors and produce open channels. This value, which starts from zero, rises to a surprisingly high value of nearly 54%. This value is in the column titled Transmitter Efficiency.

Of the total number of transmitter molecules bound to receptors, a very high proportion, approaching 90%, are effective and open the ionic channel. This value, which is tabulated as Receptor Occupancy, implies that there is an area of almost complete receptor saturation which is 'filled in' as the model progresses. This is strong support for the theory that the transmitter acts on a small area of receptors at a saturating concentration as very few of the receptors within range have not become fully bound.

Table 2.5.4 shows the equivalent results for cholinesterase binding, though as transmitter molecules can be hydrolysed one at a time, the concept of Occupancy is not relevant.

Table 2.5.3 Active Site Data - Receptor Binding

Program DIFFUSE6, Data DATA64, (Harris H800, 18-Aug-83)

Cycle Number	Receptor Binding	Channels Open	Current (nA)	Receptor Occupancy	Transmitter Efficiency
0	0	0	0.00	0.0 %	0.0 %
100	827	350	0.73	84.6	7.0
200	1714	731	1.52	85.2	14.6
300	2500	1064	2.21	85.1	21.2
400	3240	1371	2.85	84.6	27.4
500	3854	1639	3.41	85.0	32.7
600	4382	1861	3.87	84.9	37.2
700	4739	2030	4.22	85.6	40.6
800	4988	2142	4.46	85.8	42.8
900	5234	2250	4.68	85.9	45.0
1000	5452	2373	4.94	87.0	47.4
1100	5585	2430	5.05	87.0	48.6
1200	5677	2484	5.17	87.5	49.6
1300	5748	2517	5.24	87.5	50.3
1400	5835	2557	5.32	87.6	51.1
1500	5899	2596	5.40	88.0	51.9
1600	5949	2622	5.45	88.1	52.4
1700	5995	2643	5.50	88.1	52.8
1800	6035	2664	5.54	88.2	53.2
1900	6060	2680	5.57	88.4	53.6
2000	6086	2693	5.60	88.4	53.8

Table 2.5.4 Active Site Data - Cholinesterase Binding

Program DIFFUSE6, Data DATA64, (Harris H800, 18-Aug-83)

Cycle Number	Esterase Binding	Fully Bound	Transmitter Disposal (%)
0	0	0	0.0
100	194	31	1.9
200	482	100	4.8
300	686	140	6.8
400	869	170	8.6
500	1013	197	10.1
600	1140	214	11.4
700	1229	233	12.2
800	1289	243	12.8
900	1341	253	13.4
1000	1403	266	14.0
1100	1442	273	14.4
1200	1470	279	14.7
1300	1515	290	15.1
1400	1542	294	15.4
1500	1559	298	15.5
1600	1573	300	15.7
1700	1581	304	15.8
1800	1591	306	15.9
1900	1602	309	16.0
2000	1613	313	16.1

Active Site Saturation:

To ensure that the high receptor occupancy figures were not produced by insufficient receptors being included in the model, a further program, READA3, was written after the run had finished. The results of this - binding figures for each of the 22,528 sites, are even more bulky than the logs from which the above tables were abstracted.

The saturation results are not very easy to display in two dimensions - Figure 2.5.8 is an attempt at such a diagram.

There is a clear region of saturation at the top of the model, in the primary synaptic cleft. This extends laterally, forwards and back, just to the edges of the model (i.e. 220 nm sideways, 300 nm along the cleft axis). It also covers part of the sides of the central secondary fold, reaching about 200 nm from the release point at its lowest. The region wraps around into the outer two secondary folds as well, though it only extends downwards by a few cells. The total area can be obtained from the binding data in the tables above as about 3000 cells, 0.3 square microns, which is greater than the value of 0.15 square microns predicted by Wathey et al (1979), but is considerably less than that given in Land et al (1981), where the region has a radius of 0.3 microns, extending in three planes.

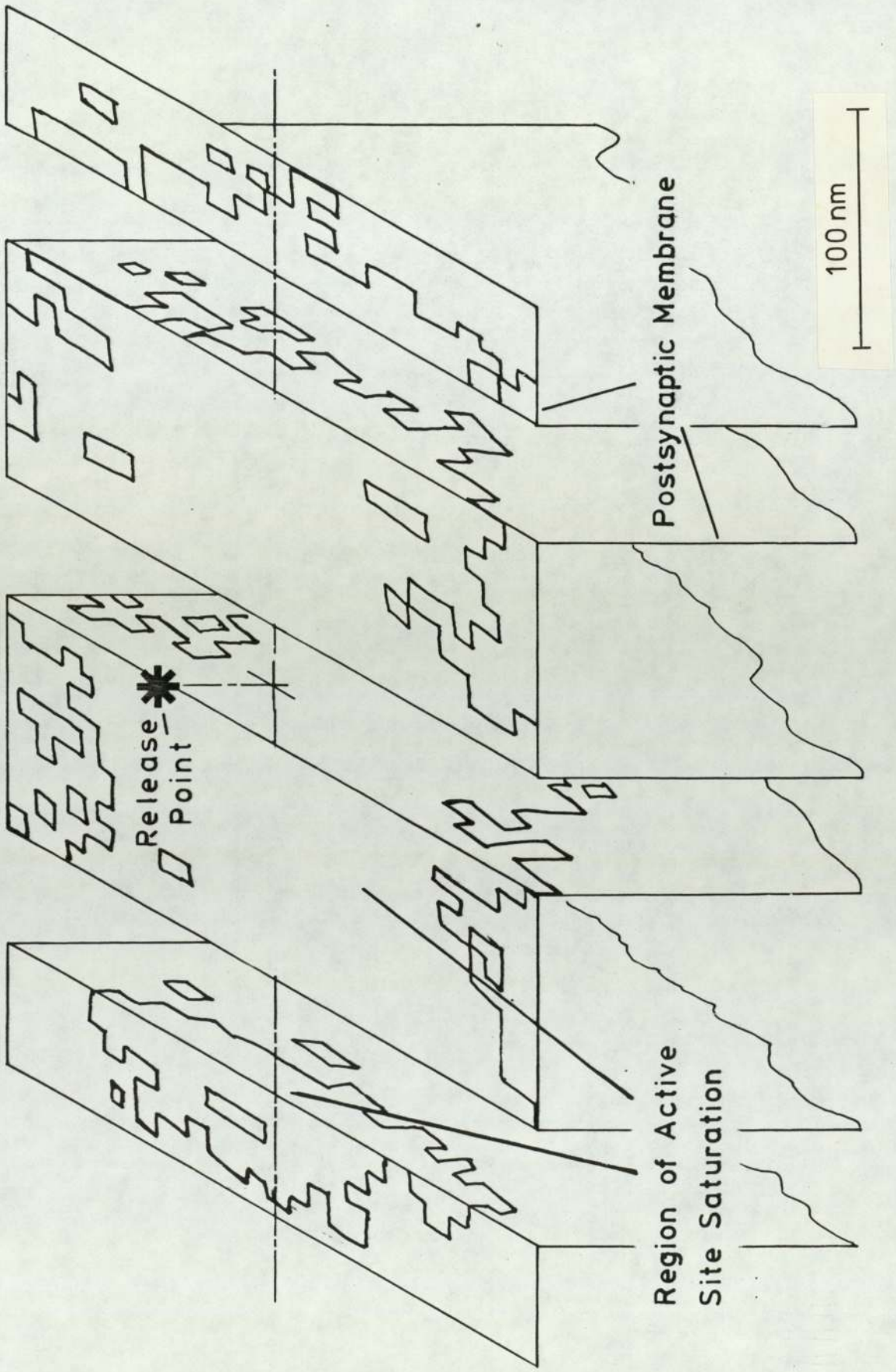

Figure 2.5.8 The Region of Active Site Saturation
The transmitter is released at the point marked by the star over the central secondary cleft. The presynaptic membrane is not shown. The zone of active site saturation is outlined.

Section 3 - Discussion

3.1 Discussion

Introduction:

This method would not have been possible without the modern digital computer. The same comment also applies to all the other simulation methods described previously. Even Rosenberry's early analog computer model almost certainly required some calculation to extract the differential equations. The discrete model described here is particularly greedy for storage and although it can be run on a small floppy disk based microcomputer, it would benefit considerably from being run on a large computer with something like a megabyte of fast RAM. Unlike the numerical methods, it does not involve any complicated arithmetic, the most involved process being the generation of random numbers.

The Model:

As in any computer program, and perhaps more than most, the model described here is dependent upon its data. Bad data will produce bad results, meaningful results only being produced by a combination of a good program with good data. By being adaptable, this simulation includes many opportunities for bad values to slip in, so how reliable are the results produced so far?

The main areas for error include the time calibration, the synaptic geometry, the diffusion and binding algorithms, not forgetting the binding coefficients. It also relies on there being adequate resolution with a cell size of 10 nanometres and a time resolution of one cycle.

Time Calibration:

The method used for time calibration relies heavily on an accurate value being obtained for the diffusion coefficient of acetylcholine. The value of 7.6 E-6 $cm^2.sec^{-1}$ (7.6 E-10 $m^2.sec^{-1}$ in S.I. units) given by Eccles and Jaeger (1958) was taken from diffusion in a 'normal' solution. How closely the conditions in the synaptic cleft match the ideal experimental ones is difficult to judge. This value gives a time calibration of 20 - 25 nanoseconds per cycle. The value given by Land et al (1980, 1981), 4 E-6 $cm^2.sec^{-1}$ (4 E-10 $m^2.sec^{-1}$), is influenced by their experiments on modelling diffusion in synapses with reduced receptor active sites. While this is clearly based on diffusion in the synaptic cleft itself, the assumptions made in the model are likely to infuence the accuracy of this estimate, particularly if there is any significant difference between the organisation of binding sites on the postsynaptic membrane and their assumed even spread. This value indicates that the time calibration is in the range 35 - 45 nanoseconds. Both of these estimates can be improved by re-running the calibration experiment with a finer sampling resolution to pinpoint the concentration maximum predictions in time.

The binding results presented here do not rule out the simple two-plane geometry used, as only a small amount of transmitter actually became bound in the outer secondary folds, most of the 'Outer' binding was actually on the postsynaptic surface of the primary cleft. However, the binding in both primary and secondary clefts does seem to indicate that the two-dimensional disk geometry used by Wathey et al (1979) is over-simplified.

Synaptic Geometry:

Apart from the two minor errors which occurred in the transportation of the initialisation template file, the starting data for the two and three

dimensional simulations were the same. However, there are two problems with this particular geometry. These are:

1 There are insufficient Acetylcholine Receptors at the peaks of the secondary synaptic folds, the maximum possible density on a 10 nm square cell size being 10,000 molecules per square micron. The work of Matthews-Bellinger and Salpeter (1978) on frog estimated that there were far more than this - about 26,000 per square micron. On a cubic lattice, this would indicate a repeat distance of about 6 nm. Heuser and Salpeter (1979), however, were able to measure the repeat distance in quick-frozen electroplax. Their electron micrographs indicate that the receptors are arranged in relatively straight lines, with a receptor to receptor distance of about 8.5 nm. This would give a density in the region of 14,000 per square micron.

The model receptor density could be increased by altering the cell size, though the model size would need a corresponding increase as well. An increase in the numbers of receptors would be expected to reduce the size of the area of saturation, possibly also reducing the time taken to reach maximum transmembrane current. It would be necessary to repeat the time calibration calculations for a new cell thickness.

2 The distribution of acetylcholinesterase sites was also incorrect. As revealed by McMahan et al (1978) these should have been positioned in the basal lamina - in the synaptic cleft itself. Removing them from the postsynaptic membrane would also increase the density of receptors that could be obtained. The synaptic cleft in the current model is entirely free of obstructions. Placing active sites in the cleft might obstruct the diffusion of transmitter - perhaps this accounts for Land et al's low value for the ACh diffusion coefficient. This modification should

increase the proportion of transmitter which becomes bound to cholinesterase in the early part of the run as the enzyme would then be 'on the way' to the receptor sites.

The Diffusion Algorithm:

The only problem noticed with the diffusion algorithm is a result of the minimum step size. Molecules which diffuse do so in steps of 10 nanometres. This movement, which produces a sort of checkerboard pattern in the three dimensional model as molecules enter and leave alternate sets of cells, is known as dispersion (Potter 1973) and is common to particle-in-cell models. The normal way to eliminate it is to introduce a diffusion coefficient which varies inversely with distance - decreasing as the distance travelled increases. This solution was considered incompatible with the simulation's objectives, which were to model binding and channel opening, not necessarily producing an even concentration gradient. This problem is largely a cosmetic one, though it can produce some very pretty results!

Nevertheless, the results given by the time calibration experiment do indicate that the algorithm is fairly accurate, despite not being continuous.

The Binding Algorithm:

There are two problems with this. The first has an important influence upon the results obtained. The values used for the binding probabilities for both receptor and esterase active sites are almost certainly too low - making it too easy for transmitter molecules to become bound. The positive cooperativity used will make matters worse, but this was included as a test and will need to be corrected. There is little evidence of cooperativity in acetylcholinesterase kinetics, but this could be a result of the

preparation methods used. It is not likely to be a major effect, as Wilson (1967) calculated the energies required for ACh to bind to the enzyme's active site. He concluded that little or no stress was set up in the acetylcholine molecule, which implies little conformational change in the enzyme sub-unit.

The rate coefficients quoted for Acetylcholine binding to receptors give the binding of the second molecule as the rate-limiting (i.e. slowest) step. To model this correctly, the binding probability for the second molecule should be reduced (that is, the value in the RBIND array should be increased) to a value below that for the first. Making binding to either receptors or cholinesterase less likely is bound to influence the spread of transmitter - probably allowing it to spread through a larger volume. The results obtained from the 3-D simulation show that with maximum binding, most of the transmitter becomes bound to the receptors on the crests of the postsynaptic folds. If Attwell and Iles (1979) and Jachter and Sachs (1982) are correct in their predictions of ionic depletion during the course of a single m.e.p.c., then a greater current would be obtained if the transmitter binding was spread over a larger membrane area, particularly if this made use of the potential ionic reservoir deep in the secondary folds.

Delays which result from conformational changes occuring in the receptor molecule after the first transmitter molecule has bound could affect the accessibility of the second binding site. Though the data structure to include a delay between the binding of the two transmitter molecules is built in to the program, it is not currently used. Any delay that reduces binding to receptors is likely to permit the transmitter to diffuse further from the initial release point, either sideways into the adjacent clefts or down into the depths of the secondary folds. This would also be expected to

reduce the rate of rise of transmembrane current, and would probably similarly affect the peak current reached. Land et al (1980) predict a time delay due to channel opening of 55 microseconds. This occurs after a diffusion time of 50 microseconds and a binding time of 40 microseconds. If delays of this order were included in the model, few, if any, channels would have opened by the time the run reached cycle 2000.

The second problem is that, although the four sub-units of the Acetylcholinesterase molecule are functionally independent, the molecules in the model do not start to 'hydrolyse' their substrate until all four active sites are occupied. This enables the same algorithm to be used for binding to both cholinesterase and receptor molecules. However, this means that if the last transmitter molecule is very late in binding, a long delay will occur while hydrolysis occurs before further ACh can be bound. Any effect is not likely to be noticeable in the saturation region, where complete binding is likely to be fast, or well away from it, where the cholinesterase is unlikely to become saturated. In this case, the transmitter is not hydrolysed, but is still trapped and unable to react with any further receptors. There may be a region in between where the effect becomes significant. This could reduce the rate of removal of the transmitter.

It was not practical in this method to include any kind of inter-molecular interactions other than binding to active sites. There are far too many molecules to take into account individually. Interactions such as electrostatic attraction are possible with a polar molecule like acetylcholine, the most likely effect being the accumulation of a shell of water molecules which might get in the way of binding. It is unlikely that there are any significant long-range effects. An effect of postsynaptic

membrane surface charge on transmitter binding has been suggested, with a possible mechanism being described by Schengrund and Nelson (1975). This involves an enzyme, sialidase, which is normally found in membranes. The effect was produced by decreased Sodium or increased Potassium ion concentrations, just as you might expect to occur when receptor channels are open. To avoid this potential hazard, any effects are assumed to be much slower than a single m.e.p.c. though this is for convenience.

The Results:

Given that there are some flaws in the data used for both two- and three-dimensional simulations which will need further work to correct, there are still some useful comparisons that can be made. The 2-D model shows how the transmitter would diffuse in the given geometry in the total absence of binding. It is, perhaps, surprising that so much transmitter stays inside the synaptic model: even after as long as 2000 cycles, 60 microseconds of diffusion, only half of the transmitter has fallen off the edge.

As the release site is immediately over the central secondary cleft, it is not surprising that the number of transmitter molecules in contact with central cleft receptors rises very rapidly to a maximum after only 120 cycles (3.6 microseconds). Then, as there is no facility for binding these molecules, they start to 'leak' into the outer clefts.

Under these conditions, the outer clefts also seem to play a significant part, potentially binding as much as one third of the total transmitter in a comparatively short time (peak reached at about cycle 500, 15 microseconds). The number of receptor contacts is, perhaps, a better indicator of likely efficiency, but this too reaches a peak after only 600

cycles (18 microseconds).

The 3-D model corrects the chief failing of the 2-D version, namely the lack of binding. As the binding constants are probably lower than they should be, the results from this simulation represent the opposite extreme - maximum binding. This is demonstrated by the area of saturated active sites around the release point, and the large number of receptors which are fully bound - nearly 80%. It is also probably responsible for the high proportion of transmitter (53%) which has become doubly bound to receptors. The number of channels which open as a result (2600+) is somewhat higher than the more typical value of 2000 obtained by Wathey et al (1979).

The comparatively low proportion of transmitter molecules which bind to esterase active sites (16%) is, in contrast, likely to be an underestimate resulting from the incorrect location of these enzymes in the postsynaptic membrane. Moving them into the basal lamella should increase the amount of transmitter which is hydrolysed early in the run. They are likely to cause some obstruction to free diffusion and may appear to slow diffusion down slightly.

It would be expected that reducing the probabilities for acetylcholine binding to receptors would enable the transmitter to move further from the release site before becoming bound. This would also be expected to increase its chances of being hydrolysed, but the increased mobility might well increase the amount of binding that takes place in the adjacent secondary cletts. If this reaches a significant fraction (say, greater than one quarter) of the total binding that produces open channels, then even the more complex geometry model used by Land et al could prove to be over-simplified. No doubt they could use the work of Jaeger (1960) to

include further secondary folds, but it may not be possible to evaluate the model that results.

Conclusions:

The process of validating a model is lengthy, and is not really complete until the model can cope with every imaginable test. This one has been designed to be flexible enough to permit ready modification and has produced some results which show it to be a potential competitor to the numerical models used by other workers in the field.

Unlike the numerical simulations, this discrete model was designed to be more readily modifiable, particularly in the area of synaptic geometry. This flexibility should enable it to be useful in the further study of quantal and even multi-quantal neuromuscular transmission.

As one of the products of the simulation is essentially a list of open channels, complete with their co-ordinates, it may be possible to combine it with another, similar, model to investigate whether the ionic concentration changes in the synaptic cleft are significant. This would be duplicating the work of Jachter and Sachs (1982) with a different model.

4.1 Further Work

There is clearly more work to do on this computer model. The main failing at this stage is the lack of either spontaneous or 'normal' release from binding to receptor or esterase active sites. While there would not be any 'normal' release of active transmitter from Acetylcholinesterase, the enzyme does not yet become available for further hydrolysis.

The model does lend itself to the investigation of the effects of synaptic geometry on neuromuscular transmission. An interesting test would be to examine the effect of secondary clefts on transmitter diffusion and binding. It is also possible to alter the distribution, density, and binding parameters for the active sites and the effects of these modifications can also be predicted. For example, introducing a conformational delay between the binding of successive transmitter molecules to receptors would be likely to force the transmitter to move further afield. The lower concentration produced would reduce the number of receptors that achieve double binding, and hence the number of channels opened. A delay after both molecules are bound, which is more likely as some physical movement of protein chains is probably needed in channel opening, would merely shift the onset of opening back from time zero.

It would be interesting to test whether the effective areas of transmitter released from adjacent release sites overlap (c.f. Hartzell et al 1975). With this model it is simply a matter of including the extra transmitter at the beginning, providing the geometry is suitable - Hartzell (1975) estimated that the normal distance between release sites was of the order of 2 microns - some 200 cells - apart.

It is not possible to continue this line of research indefinitely without some comparison with 'real life'. As many of the model's parameters are not experimentally accessible, it may not be possible to check them physically. However, experiments such as the Land group have performed, making alterations to the effective receptor density and examining the effects of this on the m.e.p.c. are feasible. Similarly, alterations in the proportion of functional cholinesterase can be made. Land et al, of course, inhibited as much of the cholinesterase as they could for their experiments in 1980.

Of the alternative simulations, Wathey et al (1979) and Land et al (1981) have both started with results of biological experiments. They have fitted the data into their models and turned the handle to see what came out, altering their models until they produced a 'reasonable' m.e.p.c..

Section 5 - Appendices

5.1 The Program Suite

The simulation can be split up into several independent functional units. These have been developed as separate programs which comprise the program suite. This has the advantage ot reducing the amount of memory required to hold the simulation while it is running, and simplifies development, but has the disadvantage of increased complexity - it is slightly more difficult to run. On computers with a batch job facility, this is a minor problem as job control files or command files can be produced to control the sequencing. The greatest difficulty is the limited access to intermediate data. As each program runs, particularly the main diffusion program, intermediate data is used and over-written. The programs themselves have to be written to produce adequate auditing information (results) as they go. The random access files are also quite large and backing up can be a problem (it is on the Harris).

Porting the programs to another computer will require changes to all of the programs as they use machine-dependent file formats and accessing mechanisms. Further details of programs, including listings, are included in Appendix 2.

The Programs:

On the Harris, files containing source code (i.e. FORTRAN 77) and runnable jobs must have different names, unlike many file systems where only the file type needs to be different. In the table below, the Program Name is the name of the source file, and the Job Name is the name of the executable job file. The suffix -64 is used to indicate that these are compatible with the dimensions of data file DATA64.

Program Name	Function	Job Name
LOADAT	Initialises the transmitter and active sites random access files from the template file. Also initialises the program run link data file LINK for the start of the run.	LOAD64
DIFFUSE6	The current main diffusion simulation program. It uses the link file to initialise its internal data structure. Assuming that the transmitter and active sites random-access files are set up correctly, it then runs for a fixed number of cycles and writes the end-of-run dump file and a new link file. Cycle-by-cycle results are written into a log file for later processing. Binding probability values are defined statically in this program.	DIFF64
READA1	Low-level audit program used to check the structure and contents of the active sites random access file.	RDA164
READA2	Quick program to count up active sites in their random access file and tabulate according to levels of binding. This program has to be altered if any radical changes to the binding mechanism are made.	RDA264
READA3	Reads through the active sites file and dumps the number of molecules bound to each site in the array. Also prints a binding table as READA2.	RDA364

READD1	Low-level audit program used to check the structure and contents of the end-of-run dump file.	RDD164
READD2	Audit program to read and print the distribution of free transmitter molecules as held in the dump file. Also prints the contents of the active sites pointer array so that the template file can be checked.	RDD264
READT1	Low-level audit program used to check the structure and contents of the transmitter random access file.	RDT164
READT2	Quick transmitter random access file check program - adds up the total numbers of free, bound, and lost molecules.	RDT264
READT3	Program to find and print the 2-D distribution of transmitter molecules which are bound to active sites.	RDT364

All of the above programs include statically-defined file names which they use for input and output data. These names can be changed by editing and re-compiling the source programs, but are currently set to the names of the following files. Dynamic (i.e. user input) filenames are not suited to jobs run in batch mode - without a user at a terminal.

The Files Used:

Several files are required during each simulation run. The names of the files and their structures must match those used in the programs above. As the names are defined statically in the programs, care must be taken to read or print output files before the are over-written by the next part of

the run. The file types are either F-A for FORTRAN formatted ASCII (i.e. text) or R-A for random-access binary files. The latter are highly machine dependent and changes would almost certainly be required if the programs are to be ported to another computer.

Filename	Contents	Type
ACTIVE1	The active sites random access file. Contains status and timing information for the active sites defined in the template file, stored in groups of one cylinder per file record. The contents are in binary to avoid format conversions on each read or write operation. It is initialised by LOADAT, and is read and written to by DIFFUSE6. Being unformatted binary, it cannot be printed, but its structure can be checked by READA1.	R-A
D6.LOG	The cycle-by-cycle output from DIFFUSE6. Includes the cycle number, numbers of free and lost transmitter molecules, transmitter bound to receptors and esterases, numbers of fully bound sites and sites which are not fully bound. It should be printed after each run.	F-A
DATA64	Initialisation template file. It can be produced using a text editor once the geometry has been determined. It contains the starting locations for all active sites and transmitter molecules. It is also used to initialise the random-access files before a run, and also to initialise the active sites pointer array	F-A

during a run. This file should not be altered during a run or the pointers will not match the active site records in ACTIVE1. The current file is for a model that is 64 cells thick, hence the name.

DUMP — The end-of-run dump of the active sites pointer array. It also contains the 2-D distribution of free transmitter molecules and active sites. The file is printable and its contents can be checked using READD1. F-A

LINK — Run to run link file - initialises the current cycle number and free active sites counts. It is in a compatible format to that used in D6.LOG. F-A

TRNSMT1 — The transmitter random access file. Contains 3-D coordinates and status information for the transmitter molecules defined in the template file. The coordinates are stored as integers, and the status indicates whether each molecule is bound, free to diffuse, or has become lost through falling off the edge or hydrolysis. It's structure can be checked by READT1. R-A

5.2 Program Listings

LOADAT is the program used to initialise the random access files TRNSMT1 and ACTIVE1 from the initialisation template. It also writes LINK to define the starting conditions.

```
      PROGRAM LOADAT
C Last Update 18-Aug-83 DB
C Trailer values added to Random Access Files for EOF Detection
C LUNs increased by 10 to avoid conflicts
C Entered on H800 26-Jul-83 DB
C 2-D Template File Load
C 3-D Transmitter File Load
C 3-D Active Site File Load
C Declare and Type Variables
      INTEGER IMAX, JMAX, KMAX, SLICE
      INTEGER A, I, J, K, L, M, N, O, P, R, S, T
      INTEGER TRANSM, ACTIVE, AIPTR, AOPTR, ASPTR
      INTEGER FREEE, FREER, TLOST, TOTALE
      INTEGER TOTALH, TOTALM, TOTALO, TOTALR, TOTALS, TOTALT
C Define the configuration-dependent constants
      PARAMETER (IMAX = 64, JMAX = 54, KMAX = 45, SLICE = 32)
C Dimension the temporary transmitter storage array
      DIMENSION TRANSM (4, SLICE), ACTIVE (2, IMAX)
      DIMENSION ASPTR (2, JMAX, KMAX)
      WRITE (3, 110)
110   FORMAT (' Diffusion File Setup Program LOADAT 18-Aug-83')
C Initialise all constants and counters
C AIPTR counts up active site input records
      AIPTR =  0
C AOPTR is used to calculate the ACTIVE1 output file record number
      AOPTR =  0
      FREEE =  0
      FREER =  0
      TLOST =  0
      TOTALE = 0
      TOTALH = 0
      TOTALM = 0
      TOTALO = 0
      TOTALR = 0
      TOTALS = 0
      TOTALT = 0
C Clear the active sites pointer array ASPTR
      FOR K = 1, KMAX
        FOR J = 1, JMAX
          ASPTR (1, J, K) = 0
          ASPTR (2, J, K) = 0
        END FOR
      END FOR
C R counts input records, M, AIPTR, and AOPTR output file records.
C N is a temporary array pointer for TRNSMT1, P for ACTIVE1
      L = 0
      R = 0
      M = 0
      N = 0
      P = 0
```

```
      WRITE (3, 120)
C Open the input data file and read in non-zero values
C LUN 16, File DATA64
      OPEN (UNIT=16, FILE='        DATA64')
120   FORMAT (1H , 'Input File Opened')
C Also open the transmitter array file for write
C LUN 17, File TRNSMT2
      OPEN (UNIT=17, FILE='        TRNSMT2', ACCESS='DIRECT', RECL=500)
C and the active sites file
C LUN 18, File ACTIVE2
      OPEN (UNIT=18, FILE='        ACTIVE2', ACCESS='DIRECT', RECL=500)
      WRITE (3, 125)
125   FORMAT (1H , 'Random Access Files Opened')
C Now loop, reading values in from the template file
C A format error is FATAL
130   READ (16, 140, END=160) K, J, I, T
140   FORMAT (I3, I3, I3, I6)
C If I is zero or negative, it means a whole row
C Count Input file records in R
      R = R + 1
C Dispatch to appropriate file write routine
      IF (T) 144, 130, 141

C Now stuff the transmitter into the random file
141   TOTALT = TOTALT + T
      DO 142 S = 1, T
C First fold into TRANSM dimensions
C N is counting around the temporary output array
        N = MOD (N, SLICE) + 1
        TRANSM (1, N) = I
        TRANSM (2, N) = J
        TRANSM (3, N) = K
        TRANSM (4, N) = 0
C If the temporary array is not full, read some more
        IF (N .LT. SLICE) GO TO 142
C M is counting output file records
        M = M + 1
C If there is a write error, it is FATAL
        WRITE (17, REC=M) ((TRANSM (0, P), 0 = 1, 4), P = 1, SLICE)
142   CONTINUE
C Now see if there is any more in the input file
      GO TO 130

C Active Sites are processed here - they can only be handled in complete
C rows (records). Set up the record pointers first
144   AIPTR = AIPTR + 1
      AOPTR = AIPTR
C Pointer Array Store Order is (((VALUE, RECNO) JMAX) KMAX)
      ASPTR (1, J, K) = T
      ASPTR (2, J, K) = AOPTR
C The pointer gives the actual absolute record number for the write
C Set up the values and also count free active sites
      FOR P = 1, IMAX
        IF (T .EQ. -3) FREER = FREER + 1
        IF (T .EQ. -4) FREEE = FREEE + 1
        ACTIVE (1, P) = T
        ACTIVE (2, P) = 0
```

```
      END FOR
      WRITE (18, REC=AOPTR) ((ACTIVE (O, P), O = 1, 2), P = 1, IMAX)
C Now see if there is any more in the input file
      GO TO 130

C Hit the end-of-file and bounce to here
160   CLOSE 16
      WRITE (3, 170) R
170   FORMAT (1H , I4, ' Records Loaded')
C Flush the last set out of the transmitter temporary array
C There is a bug here which loses the last transmitter molecule
      N = MOD (N, SLICE)
      WRITE (3, 171)
171   FORMAT (' Adding trailer values')
C Fill the unused elements with zero's
      FOR I = N, SLICE
        FOR J = 1, 4
          TRANSM (J, I) = -1
        END FOR
      END FOR
      M = M + 1
      WRITE (17, REC=M) ((TRANSM (O, P), O = 1, 4), P = 1, SLICE)
C Now do the same for the Active sites file
      AIPTR = AIPTR + 1
      AOPTR = AIPTR
      FOR P = 1, IMAX
        ACTIVE (1, P) = -1
        ACTIVE (2, P) = -1
      END FOR
      WRITE (18, REC=AOPTR) ((ACTIVE (O, P), O = 1, 2), P = 1, IMAX)
C Now we can safely close the transmitter and active sites files
180   CLOSE 17
      CLOSE 18
C Now open the link data file LINK on LUN 19
      OPEN (UNIT=19, FILE='        LINK')
      WRITE (19, 185) L, TOTALT, TOTALM, TLOST, TOTALR, TOTALE,
     1 TOTALO, TOTALH, FREER, FREEE
185   FORMAT (10I7)
      ENDFILE 19
C Add the active sites totals together
      FREER = FREER + FREEE
      WRITE (3, 190) M, TOTALT, FREER
190   FORMAT (1H , I4, ' Records written', I6,
     1 ' transmitter molecules and', I5,
     2 ' active sites found.')
2010  WRITE (3, 2020)
2020  FORMAT (1H ,'Load Completed.')
      END
```

The main diffusion program DIFFUSE6 is listed next. It reads through the initialisation template file DATA64 to initialise the pointers into the active sites file, then reads LINK to set up the start cycle number. Then it carries out the simulation for LMAX cycles before writing the internal data to file DUMP, re-writing LINK ready for the next time, and exiting.

```
      PROGRAM DIFFUSE6
C Last Update 18-Aug-83 DB
C Copied from DIFFUSE5, 11-Aug-83
C Trailer values on TRNSMT1 detected as EOF
C All file LUNs increased by 10 to avoid conflicts
C Active Sites and Transmitter Random Access Files
C 2-D Template to pointer array load
C Listfile output on LUN 8
C Declare and Type Variables
      INTEGER EMAX, IMAX, JMAX, KMAX, LMAX, RMAX, TMAX
      INTEGER G, H, I, J, K, L, M, N, O, P, Q, R, S, T
      INTEGER OLDI, OLDJ, OLDK, AIPTR, AOPTR, ACTIVE, ASPTR
      INTEGER DIRECT, FREER, FREEE, RECORD, TRPTR
      INTEGER LEAVE, RANDOM, MOVING, COMMN, REMAIN
      INTEGER TOTALE, TOTALH, TOTALM, TOTALO, TOTALR, TOTALS
      INTEGER TOTALT, TLOST, TEMP1, TEMP2, TRANSM
      INTEGER RLEN
      LOGICAL ACHNGE, TCHNGE, L1, L3, L5
      REAL    FIRST, LASTNO, NEWNO, EBIND, RBIND
      REAL    PROB1, PROB2, PROB3
C Define dimension constants - EMAX, IMAX, JMAX, KMAX, RMAX, TMAX
      PARAMETER (EMAX=4, RMAX=2)
      PARAMETER (IMAX = 64, JMAX = 54, KMAX = 45, TMAX = 32)
C Set the number of cycles per run
      PARAMETER (RLEN = 500)
C Dimension all arrays
C The Leaving Direction Array
      DIMENSION DIRECT (3, 6)
C The Transmitter Buffer, Active Sites Buffer, and Pointer arrays
      DIMENSION TRANSM (4,TMAX), ACTIVE (2,IMAX), ASPTR (2,JMAX,KMAX)
C The Binding Probability Arrays
      DIMENSION EBIND (EMAX), RBIND (RMAX)
C Load up constant arrays
C ***** WARNING - TRANSM is in I, J, K, order, DIRECT is K, J, I
      DATA DIRECT /-1, 2*0, 1, 3*0, -1, 2*0, 1, 3*0, -1, 2*0, 1/
C Directions 1=Left, 2=Right, 3=Up, 4=Down, 5=Forward, 6=Back
C              K-      K+      J-      J+       I-       I+
C            - 0 0   + 0 0   0 - 0   0 + 0    0 0 -    0 0 +
C            K J I   K J I   K J I   K J I    K J I    K J I
C Binding constant constants -give cooperativity
      DATA EBIND /0.875, 0.75, 0.5,0.375/
      DATA RBIND /0.75, 0.5/
C Seed the random number generators
      NEWNO = 1.0
      PROB2 = 1.0
C Open the log file - will destroy any previous contents
      OPEN (UNIT=18, FILE='        D6.RUN')
C Print Headings
      WRITE (3, 110)
110   FORMAT (' 3-D Diffusion Program DIFFUSE6 18-Aug-83')
C Initialise the main arrays
C The transmitter buffer array - limit TMAX
      DO 115 I = 1, TMAX
```

```
        DO 115 J = 1, 4
          TRANSM (J, I) = 0
115   CONTINUE
      ACHNGE = .FALSE.
C The Active Sites buffer array - limit IMAX
      DO 118 I = 1, IMAX
        ACTIVE (1, I) = 0
        ACTIVE (2, I) = 0
118   CONTINUE
C and the active sites pointer array - limits 2, JMAX, KMAX
      DO 120 K = 1, KMAX
        DO 120 J = 1, JMAX
          ASPTR (1, J, K) = 0
          ASPTR (2, J, K) = 0
120   CONTINUE
      RECORD = 0
C Open the input data file and read in non-zero values
C LUN 16, file DATA64
      OPEN (UNIT=16, FILE='        DATA64')
      WRITE (3, 125)
125   FORMAT (' DATA64 opened - READING')
C Now loop, reading in values until EOF
C A format error is fatal
130   READ (16, 140, END=160) K, J, I, T
140   FORMAT (I3, I3, I3, I6)
C Counting records up in RECORD
      RECORD = RECORD + 1
C We are only interested in active sites - ignore the rest
      IF (T) 150, 130, 130
C Calculate the record number in the active sites file
150   AIPTR = AIPTR + 1
      AOPTR = AIPTR
      ASPTR (1, J, K) = T
      ASPTR (2, J, K) = AOPTR
      GO TO 130

C Hit the end-of-file and bounce to here
C The Active Sites pointer array is now set up to match the random
C access files.
C Now close the input file
160   CLOSE 16
      WRITE (3, 162) RECORD
162   FORMAT (1H , I4, ' records loaded')

C Now open the run link file LINK on LUN 16
170   OPEN (UNIT=16, FILE='        LINK')
      READ (16, 172) L, TOTALS, TOTALM, TLOST, TOTALR, TOTALE,
     1 TOTALO, TOTALH, FREER, FREEE
172   FORMAT (10I7)
      CLOSE 16
C Set the end-of-run cycle count
      LMAX = L + RLEN
C Now open the transmitter random access file on LUN 7
      OPEN (UNIT=17, FILE='        TRNSMT2', ACCESS='DIRECT', RECL=500)
C and the active sites file on LUN 9
      OPEN (UNIT=19, FILE='        ACTIVE2', ACCESS='DIRECT', RECL=500)
      WRITE (3, 180)
180   FORMAT (1H ,'Random Access files opened - STARTING')
C These are used to keep a track of what is in ACTIVE ( )
```

```
      AIPTR = 0
      AOPTR = 0
      WRITE (18, 190)
190   FORMAT (1H ,'Cycle  Static  Moving  Lost  BoundR',
     1 ' BoundE  Open  Hydro.  Free R  Free E')
      WRITE (18, 1530) L, TOTALS, TOTALM, TLOST, TOTALR, TOTALE,
     1 TOTALO, TOTALH, FREER, FREEE
C Outer loop - go through entire array until LMAX
C First adjust L for end-of-last-run value
      L = L + 1
      DO 2000 L = L, LMAX
C Re-initialise the transmitter file record pointer
        TRPTR = 0
C If all non-negative ASPTR (1, *, *) are zeroed here, then
C it can be used to keep a 2-D track on the transmitter molecules
        DO 200 K = 1, KMAX
          DO 200 J = 1, JMAX
            IF (ASPTR (1, J, K) .GT. 0 ) ASPTR (1, J, K) = 0
200     CONTINUE
C Reset the counters
C TOTALM and TOTALS count the numbers of moving and static tmolecules
C TLOST counts the number of molecules lost this cycle
        TOTALM = 0
        TOTALS = 0
        TLOST  = 0
C This line marks the beginning of the transmitter file WHILE loop
300     TRPTR = TRPTR + 1
C Reset the transmitter record altered flag
        TCHNGE = .FALSE.
        READ (17, REC=TRPTR)
     1    ((TRANSM (I, J), I = 1, 4), J = 1, TMAX)
C Now loop around the transmitter buffer array
        DO 1000 M = 1, TMAX
          IF (TRANSM (1, M) .LT. 0) GO TO 1200
C Calculate a new direction using RANDFN
          LASTNO = NEWNO
          NEWNO  = RANDFN (LASTNO)
          RANDOM = IFIX (AMOD (NEWNO, 6.0)) + 1
C Negative status = lost, zero = ok, positive = bound
          IF (TRANSM (4, M)) 1000, 310, 900
C OK, so get next K, J, I coordinates
C ***** WARNING - TRANSM is in I, J, K, order, DIRECT is K, J, I *****
310       OLDI = TRANSM (1, M)
          OLDJ = TRANSM (2, M)
          OLDK = TRANSM (3, M)
          I = OLDI + DIRECT (3, RANDOM)
          J = OLDJ + DIRECT (2, RANDOM)
          K = OLDK + DIRECT (1, RANDOM)
C Logical variables still used for ASPTR array bound checking
          L1 = K .GT. 0 .AND. K .LE. KMAX
          L3 = J .GT. 0 .AND. J .LE. JMAX
          L5 = I .GT. 0 .AND. I .LE. IMAX
C This assumes that edge losses can occur in any direction
C It outside, the flag as a lost molecule
          IF (L1 .AND. L3 .AND. L5) GO TO 320
C It falling off in I+ or I- directions (L5 .FALSE.), bounce back
          IF (.NOT. L5) GO TO 402
          TRANSM (4, M) = -1
          TLOST = TLOST + 1
```

```
          TCHNGE = .TRUE.
C Nothing more to do, so
          GO TO 1000

C If inside the array, then is the destination occupied
320       IF (ASPTR (1, J, K) .LT. 0) GO TO 400
C Got here, so the molecule can move into I, J, K
C Update the position and dump array values
          ASPTR (1, J, K) = ASPTR (1, J, K) + 1
          TOTALM = TOTALM + 1
          TRANSM (1, M) = I
          TRANSM (2, M) = J
          TRANSM (3, M) = K
          TCHNGE = .TRUE.
C Nothing more to do in this case, so
          GO TO 1000

C Active Site hits are processed here. Check for really active
400       IF (ASPTR (1, J, K) .LT. -2) GO TO 410
C Inactive, occupied cells or edge losses in I+ and I- directions.
C Just leave the molecule where it was, update ASPTR (2,,). No need
C to alter TCHNGE or update the file.
402       ASPTR (1, OLDJ, OLDK) = ASPTR (1, OLDJ, OLDK) + 1
          TOTALS = TOTALS + 1
C Nothing more to do in this case so
          GO TO 1000

C Check the active sites pointers, flush the buffer if necessary and
C read in the next one.
410       AOPTR = ASPTR (2, J, K)
C If the record is the same one, don't bother to read it in again
          IF (AOPTR .EQ. AIPTR) GO TO 420
C If the old one has not been altered, just read in the next
          IF (.NOT. ACHNGE) GO TO 415
C Write the record back to file and fetch the replacement
C All errors cause the program to abort after saving the current status
          WRITE (19, REC=AIPTR, ERR=2016)
     1      ((ACTIVE (O, N), O = 1, 2), N = 1, IMAX)
415        READ (19, REC=AOPTR, ERR=2012)
     1      ((ACTIVE (O, N), O = 1, 2), N = 1, IMAX)
          AIPTR = AOPTR
          ACHNGE = .FALSE.
C Binding to active sites is dealt with next
C Esterase and receptors- E's are even, R's are odd. Split them up
420       TEMP2 = IABS (ACTIVE (1, I))
          IF (MOD (TEMP2, 2) .EQ. 0) GO TO 500
C Receptors only here: RMAX gives the maximum no. of binding sites
C Reduce the range of TEMP2 to 1 to RMAX + 1
C NOTE - this range reduction is different as it is to get an array index
          TEMP2 = (TEMP2 - 1) / 2
C It already maximum no. bound, then just bounce
          IF (TEMP2 .LT. 1 .OR. TEMP2 .GT. RMAX) GO TO 402
C No, so generate a random probability of binding
          PROB1 = PROB2
          PROB2 = RANDFN (PROB1)
          PROB3 = PROB2 / 65536.0
C It the energy is big enough
          IF (PROB3 .LT. RBIND (TEMP2)) GO TO 402
C then bind the molecule, adjust the status and flag values
```

```
C The transmitter molecule does not actually move though.
C First set the new binding level
          ACTIVE (1, I) = ACTIVE (1, I) - 2
C and record the last binding time as a record of channel opening time
          ACTIVE (2, I) = L
          ACHNGE = .TRUE.
          TRANSM (4, M) = 1
          TOTALR = TOTALR + 1
          TCHNGE = .TRUE.
C If we have maximum occupancy, open the channel
          IF (TEMP2 .LT. RMAX) GO TO 1000
            FREER = FREER - 1
            TOTALO = TOTALO + 1
          GO TO 1000

C Binding to Esterases: Reduce range as before
500       TEMP2 = (TEMP2 - 2) / 2
C If already maximum bound, bounce
          IF (TEMP2 .LT. 1 .OR. TEMP2 .GT. EMAX) GO TO 402
C No, so generate a new binding probability
          PROB1 = PROB2
          PROB2 = RANDFN (PROB1)
          PROB3 = PROB2 / 65536.0
C If the binding energy is big enough, let it stick
          IF (PROB3 .LT. EBIND (TEMP2)) GO TO 402
C Enough energy, so bind it etc
          ACTIVE (1, I) = ACTIVE (1, I) - 2
C and record the binding time
          ACTIVE (2, I) = L
          ACHNGE = .TRUE.
          TRANSM (4, M) = 1
          TOTALE = TOTALE + 1
          TCHNGE = .TRUE.
C If all sites are occupied, off it goes
          IF (TEMP2 .LT. EMAX) GO TO 1000
            FREEE = FREEE - 1
            TOTALH = TOTALH + 1
          GO TO 1000

C All the diffusion and binding has been processed for this molecule.
C Check bound transmitter for spontaneous release from binding -
C Currently do nothing
900       CONTINUE

C Now we have finished all TMAX molecules in this record. If the molecule
C data has changed, write it back to disk, then movde on to the next record.
1000    CONTINUE
        IF (.NOT. TCHNGE) GO TO 300
        WRITE (17, REC=TRPTR, END=2000)
     1     ((TRANSM (I, J), I = 1, 4), J = 1, TMAX)
        GO TO 300

C Now we have arrived at the end of the file, hopefully safely as there
C is no other way to get here.
1200    CONTINUE

C Just dump the totals - no headings
1520    WRITE (18, 1530) L, TOTALS, TOTALM, TLOST, TOTALR, TOTALE,
     1   TOTALO, TOTALH, FREER, FREEE
```

```
1530    FORMAT (1H , 10I7)

C The Outer Loop ends here - check if there is anything left
        IF ((TOTALS + TOTALM) .EQ. 0) GO TO 2010
2000  CONTINUE

      WRITE (3, 2002)
2002  FORMAT (' End of run - tidying up')
      IF (L .GT. LMAX) L = LMAX

C Now we have got to LMAX cycles or the array is empty.
C First write the active sites buffer back to disk if it has changed
C Assumes that AOPTR has not been corrupted
2010  IF (.NOT. ACHNGE) GO TO 2020
        WRITE (19, REC=AOPTR, ERR=2016, END=2016)
     1    ((ACTIVE (0, N), 0 = 1, 2), N = 1, IMAX)
      GO TO 2020

2012  WRITE (3, 2014) AOPTR
2014  FORMAT (1H ,'Error reading ACTIVE1 record ',I7,' exiting.')
      GO TO 2020

2016  WRITE (3, 2018) AOPTR
2018  FORMAT (1H ,'Error writing ACTIVE1 record ',I7,' exiting.')

2020  WRITE (3, 2030)
2030  FORMAT (1H ,'Test Completed')
C Now open the run link file for write
      OPEN (UNIT=16, FILE='        LINK')
      WRITE (3, 2100)
2100  FORMAT (1H ,'Writing to LINK')
      WRITE (16, 2110) L, TOTALS, TOTALM, TLOST, TOTALR, TOTALE,
     1   TOTALO, TOTALH, FREER, FREEE
2110  FORMAT (10I7)
C Close the link file
9000  ENDFILE 16
C Now dump the contents of ASPTR to DUMP
      OPEN (UNIT=16, FILE='        DUMP')
      WRITE (3, 9010)
9010  FORMAT (1H ,'Writing DUMP')
      DO 9100 K = 1, KMAX
        DO 9100 J = 1, JMAX
          WRITE (16, 9020) J, K, ASPTR (1, J, K), ASPTR (2, J, K)
9020      FORMAT (4I7)
9100  CONTINUE
C Close the dump file
      ENDFILE 16
C Close the random access files
      CLOSE 17
      CLOSE 19
C and close the listing file as well

      ENDFILE 18
      END

C Random Number Function from RANDOM.FOR
C Generates pseudo-random real numbers using r(i+1)=u*r(i)mod M
C where M is the Fermat prime 65537 and u is the primitive root 254
C Translated from W.E. Thomson, PCW Vol 5 No 6, p133
```

```
C      NEWNO = RANDFN (LASTNO) Returns a value from 0 to 65536
C       INDEX = AMOD (NEWNO, 6.0) + 1 gives 1 to 6
C       PROB3 = PROB2 / 65536.0 gives 0 to 1.0

       FUNCTION RANDFN (A)
       RANDFN = AMOD (254.0 * A, 65537.0)
       RETURN
       END
```

The last two programs are examples to demonstrate how data can be extracted from the data files TRNSMT1, ACTIVE1, and DUMP. READA3 is the program used to display the number of transmitter molecules bound to each active site.

```
      PROGRAM READA3
C Last update 6-Oct-83 DB
C Copied from READA2 6-Oct-83 DB
C Entered on Harris 9-Aug-83 DB
C Program to scan the active sites file ACTIVE2
C Declare and type variables
      INTEGER IMAX, JMAX, KMAX
      INTEGER I, J, K, M, N, O, P, Q, R, S, T
      INTEGER AIPTR, AOPTR, ACTIVE, ERROR
      INTEGER FREEE, FREER, RECORD
      INTEGER TOTALS, TOTAL, TEMP
C Define dimensioning constants
      PARAMETER (IMAX = 64, JMAX = 54, KMAX = 45)
C Dimension all arrays
C The active sites sorting array and the file buffer array
      DIMENSION TOTALS (12), ACTIVE (2, IMAX)
C Print headings
      WRITE (3, 110)
110   FORMAT (' Binding Distribution Program READA3',
     1        ' 6-Oct-83')
C Initialise constants and run counters
      ERROR = 0
      TOTAL = 0
C Initialise the arrays -
      FOR I = 1, 12
        TOTALS (I) = 0
      END FOR
      FOR I = 1, IMAX
        ACTIVE (1, I) = 0
        ACTIVE (2, I) = 0
      END FOR
C Now open the active sites file on LUN 19
      OPEN (UNIT=19, FILE='        ACTIVE2', ACCESS='DIRECT', RECL=500)
      WRITE (3, 120)
120   FORMAT (' Active sites file opened - Reading')
      RECORD = 0
      AIPTR  = 0
C Now loop, reading in records until EOF ?????
130   RECORD = RECORD + 1
C Calculate the file record number - MACHINE DEPENDENT 1 to 1 for Harris
      AIPTR = AIPTR + 1
      AOPTR = AIPTR
      READ (19, REC=AOPTR)
     1     ((ACTIVE (J, I), J = 1, 2), I = 1, IMAX)
C Trailer record is full of -1s - just check ACTIVE (2, 1)
      IF (ACTIVE (2, 1) .LT. 0) GO TO 2000
C Now loop through the record and partition the contents
      DO 500 I = 1, IMAX
        TEMP = IABS (ACTIVE (1, I))
C Now zero the output value as default
        ACTIVE (1, I) = 0
C We are only interested in active sites - ignore the rest
        IF (TEMP .GT. 12 .OR. TEMP .LT. 1) GO TO 400
          TOTALS (TEMP) = TOTALS (TEMP) + 1
C Reduce the range to the sites we are interested in
```

```
          TEMP = TEMP - 3
C and lop off to indicate bound molecules only
          TEMP = (TEMP - MOD (TEMP, 2)) / 2
          IF (TEMP .GT. 0) ACTIVE (1, I) = TEMP
          GO TO 500

400       ERROR = ERROR + 1
          ACTIVE (1, I) = 0

500       CONTINUE
C Now the whole record has been processed, so print the binding data
          WRITE (3, 510) RECORD, (ACTIVE (1, I), I = 1, IMAX)
510       FORMAT (1H , I3, 64I2)

C Now go and get the next file record
600       GO TO 130

C End-of-file error branch should come here
2000      CLOSE 19
        RECORD = RECORD - 1
        FOR I = 1, 12
          TOTAL = TOTAL + TOTALS (I)
        END FOR
        WRITE (3, 2020) RECORD, TOTAL, ERROR
2020    FORMAT (' ',I4,' Records, ',I5,' active sites, ',I5,' errors')
        WRITE (3, 2030)
2030    FORMAT (' Active Site Breakdown -1 to -12')
        WRITE (3, 2040) (TOTALS (I), I = 1, 12)
2040    FORMAT (1H , 12I6)
        WRITE (3, 2050)
2050    FORMAT (' End of Program')
        END
```

READT3 is used to check the transmitter file for bound molecules, printing the number bound to each cylinder of active sites in the cross-sectional view of the model.

```
      PROGRAM READT3
C Last Update 25-Aug-83 DB
C Copied from READT2 25-Aug-83
C 3-D Transmitter binding check
C Each record contains 32 * 4 integers, stored * 4 first
C where these are I, J, K, and STATUS values
C Declare and Type Variables
      INTEGER IMAX, JMAX, KMAX, SLICE
      INTEGER I, J, K, L, M, N, O, P, R, S, T
      INTEGER BOUND
      INTEGER TRANSM
      INTEGER TBOUND, TFREE, TLOST, TOTALT
C Define the dimension constants
      PARAMETER (SLICE = 32)
      PARAMETER (IMAX = 64, JMAX = 54, KMAX = 45)
C Dimension the temporary transmitter storage buffer array
      DIMENSION TRANSM (4, SLICE)
      DIMENSION BOUND (JMAX, KMAX)
      WRITE (3, 110)
110   FORMAT (' Transmitter Binding Check Program READT3 ',
     1  '25-Aug-83')
C Initialise all counters
      R = 0
      M = 0
      N = 0
      TBOUND = 0
      TFREE  = 0
      TLOST = 0
      TOTALT = 0
C Initialise the binding record array
      FOR K = 1, KMAX
        FOR J = 1, JMAX
          BOUND (J, K) = 0
        END FOR
      END FOR
C Now scan through the Dump file to initialise the active site locations
C LUN 16, File DUMP
      OPEN (UNIT=16, FILE='        DUMP')
      WRITE (3, 120)
120   FORMAT (' Initialising active site locations')
C Now read in records from DUMP. Each contains J, K, Type and Pointer
121   READ (16, 122, END = 128) J, K, T, P
122   FORMAT (4I7)
      IF (T .LT. 0) BOUND (J, K) = T
      GO TO 121

C Close the Dump file
128   CLOSE 16
C Now open the random access transmitter file
C LUN 16, TRNSMT2
      OPEN (UNIT=16, FILE='        TRNSMT2',ACCESS='DIRECT', RECL=500)
      WRITE (3, 129)
129   FORMAT (' Transmitter file opened - reading')
C Now loop, reading in values from file until we hit the end
C Records are counted in R
```

```
130   R = R + 1
      READ (16, REC=R)
     1  ((TRANSM (O, P), O = 1, 4), P = 1, SLICE)
      DO 150 N = 1, SLICE
        IF (TRANSM (1, N)) 160, 150, 140
140       TOTALT = TOTALT + 1
          IF (TRANSM (4, N) .LT. 0) TLOST  = TLOST  + 1
          IF (TRANSM (4, N) .EQ. 0) TFREE  = TFREE  + 1
          IF (TRANSM (4, N) .LE. 0) GO TO 150
            TBOUND = TBOUND + 1
            J = TRANSM (2, N)
            K = TRANSM (3, N)
            BOUND (J, K) = BOUND (J, K) + 1
150   CONTINUE
C Now see if there are any more records
      GO TO 130

C Hit the end-of-file and bounce to here
160   CLOSE 16
C Adjust R
      R = R - 1
      WRITE (3, 170) R
170   FORMAT (1H , I4, ' Records Loaded')
      WRITE (3, 190) TOTALT
190   FORMAT (1H , I6, ' Transmitter molecules found.')
      WRITE (3, 200) TBOUND, TFREE, TLOST
200   FORMAT (1H ,I5, ' bound,', I5, ' free,', I5, ' lost.')

      WRITE (3, 210)
210   FORMAT (' Binding Breakdown : ')
      FOR I = 1, 3
        WRITE (3, 220) I
220     FORMAT ('1Cleft ',I3)
        FOR J = 1, JMAX
          K = (I - 1) * 15 + 1
          L = I * 15
          WRITE (3, 230) J, (BOUND (J, M), M = K, L)
230       FORMAT (' ', I3, 15I7)
        END FOR
      END FOR

      END
```

5.3 Job Control File

The following example shows the format of the job control file for DIFFUSE6 program runs. The *USERNAME* and *QUALIFIER* fields in the JOB command have been altered to protect my account on the Harris H800. They should be replaced with values appropriate to the user.

The first line is the job definition, similar to the job control cards found on many mainframe installations. It specifies the job's processing requirements, allowing the operating system to schedule it appropriately. On the Harris, a job length of 5000 c.p.u. seconds rates Priority 0, the lowest possible. It takes about 10 hours to complete this job.

The following lines define the sequence of programs to activate. The diffusion program DIFF64, naturally, is the first. This is then followed by several of the audit programs which search through the transmitter and active sites files to see just what has happened.

The $EOJ line tells the system that this is the End of the Job.

```
$JOB D64,*USERNAME*,*QUALIFIER*,OUT=D64LOG,SIZE=95,TIME=5000,LINES=1000
DIFF64
RDA264
RDD264
RDT364
$EOJ
```

5.4 Template File Format

The template file contains initialisation records for both the active sites and the transmitter random access files. The format is fixed. The K, J, and I coordinates for each cell value are defined by three, three character integer fields, with the value itself following as a six character integer.

These coordinates represent the position on the horizontal (K), vertical (J) and 'thickness' (I) axes, respectively, of the cell required.

Active site records, i.e. those whose value is negative, refer to complete cylinders in the model, so the I co-ordinate is set to zero. Transmitter molecules have a positive value, and all three co-ordinates must be set. This file is not complete, being a representative section of file DATA64 which has over three hundred lines.

```
  1  1  0    -1
  2  1  0    -1
  4  1  0    -1
  .  .  .     .
  .  .  .     .
 43  1  0    -1
 44  1  0    -1
  1  8  0    -3
  2  8  0    -3
  3  8  0    -4
  4  8  0    -3
  5  8  0    -3
  .  .  .     .
  .  .  .     .
 23  2 32  2500
 23  3 32  1500
 23  2 31  1500
 23  2 33  1500
```

5.5 Sample Program Output

The following is a short section of the run output put into file D6.LOG by DIFFUSE6 (job DIFF64). It includes the cycle by cycle totals that are calculated as the diffusion continues. As there is one line of output for every cycle, 2000 lines is a bit excessive for an example.

The column headings are largely self-explanatory, BoundR and BoundE are the numbers of transmitter molecules bound to Receptor and Esterase sites respectively. Of these, the fully bound active sites are in the next two columns - channels open and enzymes hydrolysing. The number of free active sites - i.e. those not fully bound - are in the last two columns.

Cycle	Static	Moving	Lost	BoundR	BoundE	Open	Hydro.	Free R	Free E
1500	47	875	0	5899	1559	2596	298	8156	4694
1501	48	873	0	5900	1559	2596	298	8156	4694
1502	45	876	0	5900	1559	2596	298	8156	4694
1503	38	880	1	5901	1560	2597	298	8155	4694
1504	34	882	1	5902	1560	2598	298	8154	4694
1505	40	874	1	5903	1560	2599	298	8153	4694
1506	50	864	0	5903	1560	2599	298	8153	4694
1507	44	867	1	5905	1560	2599	298	8153	4694
.	.	.	.	.	.	.	.	.	.
.	.	.	.	.	.	.	.	.	.
.	.	.	.	.	.	.	.	.	.
.	.	.	.	.	.	.	.	.	.
1737	36	711	0	6006	1585	2649	305	8103	4687
1738	47	700	0	6006	1585	2649	305	8103	4687
1739	37	709	0	6007	1585	2650	305	8102	4687
1740	41	705	0	6007	1585	2650	305	8102	4687
1741	30	714	0	6008	1586	2651	305	8101	4687
1742	35	708	1	6008	1586	2651	305	8101	4687
1743	37	703	0	6011	1586	2653	305	8099	4687
1744	47	692	0	6012	1586	2653	305	8099	4687
1745	44	694	1	6012	1586	2653	305	8099	4687
1746	37	701	0	6012	1586	2653	305	8099	4687

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