QUANTITATIVE MODELLING APPLIED TO ASPECTS OF NEUROMUSCULAR FUNCTION

A thesis submitted to

The University of Aston in Birmingham

for the degree of

Doctor of Philosophy

by

Anthony John Hancox

April 1981

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SUMMARY

A quantitative mathematical model is proposed which defines synthesis, storage and release of the neuro-transmitter, acetylcholine, at the nerve ending. The approach has been to gather a wide spectrum of information and to formulate a concensus which best describes neuromuscular function. On the basis of this factual evidence (i.e. both quantitative and qualitative), a lumped parameter, quasi time independent model has been conceived, which considers some 50 parameters and variables which appear to define or contribute to aspects of acetylcholine release.

The model seeks to provide an overall picture of neuromuscular function and is used to simulate several aspects of nerve behaviour when evoked in normal, raised calcium, or raised magnesium solutions. An explanation is offered concerning oscillating stores of free acetylcholine (not obvious by electrophysiological experiment) in terms of the general movement of quanta within the nerve ending. The translocation of quanta into three distinct stores is a fundamental feature of the model, and is examined in detail against wider aspects of neuromuscular function. The theoretical results obtained from the model are compared with experimental findings from several sources, and critically discussed. Necessary refinements are suggested and implemented and it is concluded that the model is adequate in describing acetylcholine release. Areas of further development work are detailed so that the model may be extended to more fully simulate general neuromuscular function.

Acetylcholine.
Neurotransmitters.
Neuromuscular junction.
Models, neurological.

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DECLARATION

No part of the work submitted in this thesis has been carried out in collaboration, or has been submitted for any other academic award.

CHAPTER 1

INTRODUCTION

Transmission of electrical signals emanating from the Central Nervous System (i.e. brain and spinal cord) is a complex and continuous process. These signals are used to control a variety of physiological functions, as well as to provide information on environmental conditions of various parts of the body; a fact that each individual takes for granted.

Voluntary movement is one such result of faithfully transmitting these signals. Simple movements often comprise a series of tasks that are very difficult to emulate by machine, and require an extraordinary degree of muscle control. The extent of muscular contraction is governed by the frequency of pulses transmitted down a nerve axon. This transmission process is by no means straightforward as the electrical pathway from nerve to muscle is discontinuous with a small gap or cleft separating the nerve ending from the muscle site. This interface between nerve ending and muscle fibre is often termed the 'neuromuscular' or 'myoneural' junction, and has been the subject of research for many years, although it still constitutes an area of speculation and controversy.

The conduction process at the neuromuscular junction is complex and not well understood, but it is now agreed that the conduction of an electrical signal across the cleft is accomplished by chemical means. The arrival of an electrical pulse or action potential at the nerve

ending, precipitates the effusion of a chemical, acetylcholine, which quickly effuses across the 20 n.m. gap to react with receptor sites on the muscle membrane. The reaction of these sites with this neurotransmitter causes electrical depolarisations to occur and a potential, often referred to as the 'end plate potential' (epp), to become established. The amalgamation of these epps results in a wave of depolarisation over the whole of the muscle membrane and an action potential to arise within the muscle itself; the ultimate reaction being a contraction of the muscle fibre.

Although the above seems to illustrate a series of disjointed processes, the actual effect (i.e. movement) is recognised as a fluid and meaningful action. Any disruption to the processes outlined becomes immediately apparent and in the case of disease (e.g. myasthenia gravis) very distressful to the patient.

A study of the entire conduction process from central nervous system through to a mechanical result in the muscle is an enormous task beyond the scope of the following thesis. This immediate preamble seeks only to set the scene, identify the area of concern (i.e. the neuromuscular junction) and to relate it to the overall conduction system.

There are several reasons why this area has been chosen not least that it is ill defined but also from certain practical considerations that may result. There are many researchers interested in human movement for a

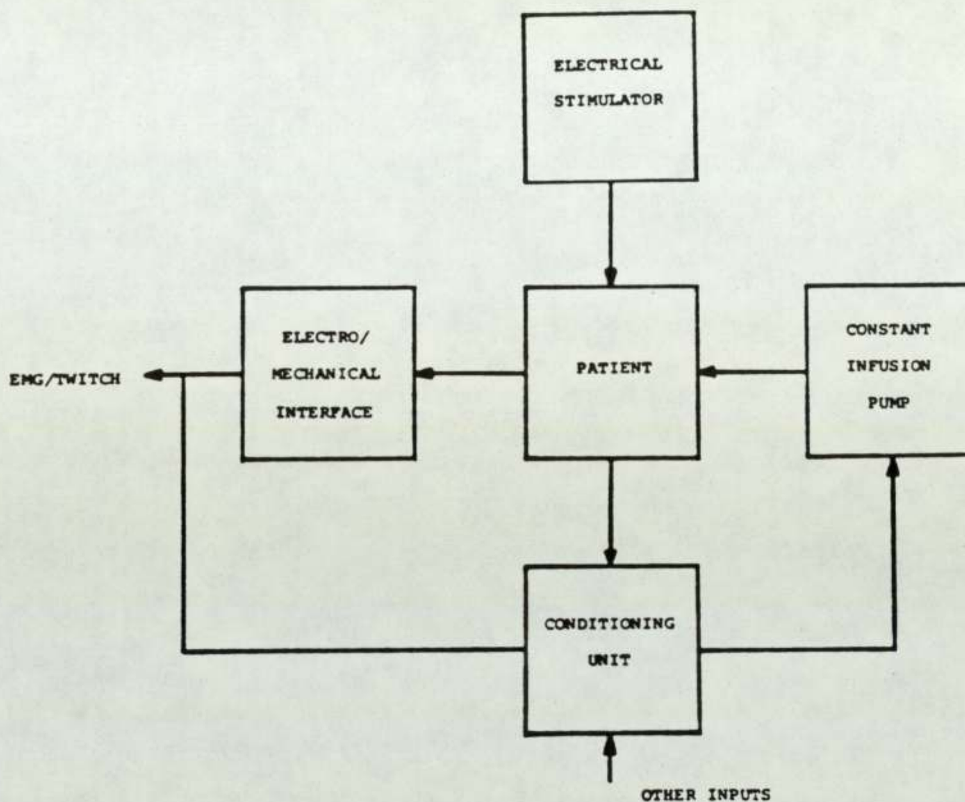
variety of reasons. The scientific disciplines of these researchers is diverse with subjects such as engineering now able to make a significant contribution due to an improvement in technology. However, the full effect of technology is unlikely to be felt until the whole conduction process, in particular the neuromuscular junction, can be explained and quantified in detail.

The present research evolved from a need to stop the conduction process at the neuromuscular junction. There is a need to control muscle tone so as to facilitate particular aspects of patient care. To this end patients are given a muscle relaxant (e.g. Curare) which acts at the neuromuscular junction and effectively blocks neuromuscular transmission. The effect of these relaxants has been known for some years, indeed Curare has been used by some South American tribes as a fatal poison for several centuries before it was pressed into a more respectable service in 1942. Relaxants are used in most anaesthetic procedures to facilitate intubation, and maintain paralysis for the duration of surgery. They are also extensively used on patients who require artificial support for their breathing systems or in the treatment of tetanus and intractable status epilepticus, where the drug acts to prevent destructive spasms. The general philosophy behind the administration of a relaxant is still generally intuitive and scientific methods are rarely used to establish a relationship between patient condition and the quantity of drug to be used. Instead the anaesthetist "gauges" the amount of relaxant needed,

by a consideration of patient height, weight and body surface area, although it is still normal practice to over compensate and cause an 'over kill' situation within the patient. The procedure is carried out with the knowledge that the effect of the relaxant can be reversed by the administration of a further drug, although in several cases this has proved a hazardous process[1].

To ensure control, the patient is carefully monitored by the anaesthetist after the administration of the relaxant and in doing so acts as the control mechanism linking the patient to the life support systems. In consequence the finer points of anaesthesia vary from anaesthetist to anaesthetist and are dependent upon the subjective approach used at the time. The reason, for such a technique arises from difficulties that are presented by biological variations that exist between each patient and the complex physiology involved.

The idea on which this project was founded was to define clearly the basic physiology in a quantitative manner and to subsequently produce a system able to control adequately the degree of neuromuscular blockade at any desired level. The relaxant was to have been continuously infused whilst a suitable nerve was stimulated by electrical impulses so as to provide an electromyograph and isometric muscle twitch. These parameters were then to be used as feedback signals via a suitable conditioning unit to regulate the input of the relaxant, as shown in the block diagram on the following page.



The problem was, however, compounded by inherent delays as the drug reached the site of action, and numerous other stimulii which affect drug action and neuromuscular function. If the output from the nerve ending could be predicted and a known amount of relaxant placed in the extracellular space at a particular time, then competition for the post-junctional receptors could be estimated and hence neuromuscular blockade. However, subsequent work revealed several misconceptions and unknowns regarding the basic physiology at the neuromuscular junction. Attention was therefore turned to this problem in an

attempt to quantify the natural phenomena that exist and to pull together the largest concensus of opinion on the emission of acetylcholine from the nerve ending. Although the ultimate objective of this project remains unaltered, the thesis focuses almost entirely upon synthesis, storage and release of acetylcholine at the nerve ending.

It is still a physical impossibility to measure events taking place in such a tiny space in vivo so most of the information used has come from experiments carried out in vitro. The conception of a mathematical model was necessary as information concerning this aspect of neuromuscular function is limited, and experimental data scarce. The following hypothesis evolves, incorporates and considers, some fifty parameters and variables that perhaps play some part in acetylcholine release and is able to simulate conditions that are impossible to produce or control experimentally. The model seeks to present a method of establishing the viability of these parameters and to quantify their effect on acetylcholine release at the nerve ending. However, before the model is presented it may be as well to consider other models and hypotheses that have evolved from existing knowledge.

CHAPTER 2

AN OVERVIEW OF EXISTING MODELS AND HYPOTHESES

The past thirty years have seen numerous postulations and hypotheses describing various functions of the neuromuscular junction. Some things are now well established fact (e.g. that acetylcholine is the neurotransmitter[2]) but other areas are still speculative (e.g. synthesis, storage and release of acetylcholine).

Techniques have also improved significantly and although _in vivo_ studies are still difficult, _in vitro_ experiments have become a major preoccupation. The discovery that an end plate potential could be generated at the post-synaptic site in the presence of Curare[3,4] has greatly facilitated the study on neuromuscular function. Suppression of the action potential in the muscle fibre (and hence muscle contraction) has greatly improved measurement techniques used to determine the size of the epp[5].

The epp, or in the case of spontaneous release from the presynaptic site, the mini end plate potential (mepp)[6], is thought to be the result of an interaction of quanta or packets of acetylcholine (released from the presynaptic site) with the post-synaptic receptors[7,8]. The problem has always been interpretation between potentials recorded at the post-junctional membrane and the number of acetylcholine molecules or quanta released at the presynaptic site.

Early studies on this end plate activity revealed a

randomness in the occurrence of the epp[9]. For example an epp was not always seen when the nerve was stimulated, and when present was of an unpredictable magnitude. Further statistical studies concluded the output of these epps to follow a Poisson distribution[10,11] and this proposition has been the basis for much of the statistical work carried out to date.

To establish a relationship between the quanta released and epp recorded, there was a need to establish the size of a quantum and to relate this to an expected magnitude of potential at the post-synaptic site. There was also a need to establish quantum content during an evoked response and to determine if changes in epp during tetany were due to a diminution of quantal content or a reduction in quantal size.

As a Poisson distribution for epps had been defined, a derived quotient (equation 1) was said to be equivalent to the expected value of quantum size[12].

$$\text{i.e.} \qquad q = \frac{S_{epps}}{\overline{epp}} \qquad --- \ 1$$

where:-

q = quantum size.

S_{epps} = variance of end plate potential.

$\overline{epp}$ = mean value of separate end plate potentials.

However, the above equation supposes a quantal size to be uniform and to correct for this, should there be a variation, 'q' was said to become a biased estimator and

a more realistic expression evolved as in equation 2.

$$Q = q - \frac{S_{quanta}}{Q} \qquad --- \quad 2$$

where:-

Q = quantum size.

S_{quanta} = variance of quanta produced.

Inaccuracies introduced into the measurement of potentials could also be accommodated as shown in equation 3.

$$Q = q - \frac{S_N}{\overline{epp}} - \frac{S_{quanta}}{Q} \qquad --- \quad 3$$

where:-

S_N = variance due to noise.

Experiment soon revealed quantal size to be uniform[12] and as such $q = Q$.

With quantal size now fixed, attention was directed to establishing a relationship for quantum content in terms of the epp.

Quantum content (m) was defined as in equation 4,

$$m = \frac{\overline{epp}}{Q} = \frac{\overline{epp}^2}{S_{epp}} \qquad --- \quad 4$$

and a direct method established for its measurement. It was noted that reducing the magnitude of the evoked potential to a small fraction of the resting potential caused the epp to become non linearly related to quantum content. Researchers examined this process in detail and

adjusted for this non-linearity by correcting for resting potential (on the muscle fibre concerned) and transmitter equilibrium potential. Quantum content was then redefined as in equation 5.

$$m = \frac{\bar{V}_{epp}}{\bar{V}_{mepp}} \cdot \frac{V_{mt} + \bar{V}_{mepp}}{V_{mt} - \bar{V}_{epp}} \qquad \text{--- } 5$$

where:-

$\bar{V}_{epp}$ = mean amplitude of epp.

V_{mt} = difference between membrane potential and transmitter equilibrium potential.

$\bar{V}_{mepp}$ = mean amplitude of mepp.

This technique has been used to good effect[13], although it assumes a synchronous production of quanta[14].

A method of counting the number of failures in epp has also been used[7,10,15] such that if m was the average quantal content, the expected number of failures would be = e^{-m} [16], assuming a Poisson distribution. The method is limited, however, and unsuitable for measuring quantal contents above 3. It cannot be used to estimate the effect of blocking agents where the amplitude of mepps is severely reduced although this is also true of the direct method. To estimate larger quantal contents necessary when muscle twitch is abolished by the introduction of a non-depolarising muscle relaxant, only the variance method is available. This method is unfortunately imprecise unless extremely long runs of results are produced[17,18]. Using this method the

quantal content may be defined as shown in equation 6.

$$m = \frac{\overline{V}^2_{epp}}{S_v - S_n} \cdot \left(1 + \frac{\sigma^2}{\overline{V}_{mepp}}\right) \qquad \text{--- 6}$$

where:-

σ^2 corresponds to the variance of miniature end plate potentials and provides adjustment for fluctuations in response to a single quanta.

If transmitter equilibrium potential is unknown but it is possible to record both mepps and the evoked response, quantal content may be derived by use of the expression shown in equation 7.

$$m = \sqrt[3]{\frac{\overline{V}^4_{epp}\left(1 + \frac{\sigma^2}{\overline{mepp}^2}\right)}{\overline{V}^2_{mepp}\,(S_v - S_n)}} \qquad \text{--- 7}$$

Although these estimations related measurements of potential to acetylcholine release at the presynaptic site it is difficult to infer more due to a lack of knowledge concerning intermediate events; thus it is difficult to relate these statistical characteristics to underlying physical processes.

A refinement of these statistical models was the suggestion that each junction contained a population of n units, each capable of release in response to a nerve impulse(7). Each unit was assumed to have an average probability $\overline{p}$ and in consequence a mean quantal content

could be derived as indicated in equation 8.

$$m = n\overline{p} \qquad --- 8$$

It was argued that if probability values were small a Poisson distribution would result, whereas large probabilities inferred a binomial distribution. Experimental evidence concerning the output of acetylcholine to the cleft was already available (and shown to follow a Poisson distribution) and consequently unit probabilities were concluded to be small[7].

Around the same time as quantal transmission was postulated[4] the vesicle was discovered in the nerve ending[19,20,21,22]. It soon became fashionable to think of a quantum as a single vesicle although it was not confirmed that acetylcholine resided in this receptical until years later[23], whereupon attempts were made to define the synaptic vesicle as a unit for release. Researchers trying to define n in terms of the number of vesicles available soon realised that the relationship of equation 8 would be destroyed when vesicles were released and in consequence n changed. An assumption was made, however, that vesicles were replenished by synthesis immediately following release, such that the relationship held for a following action potential. Work carried out on the cray fish reinforced this assumption[24,25,26], and with a fixed number of release sites the relationship of equation 8 became a constant.

The probability p may also be regarded as two compounded probabilities; the probability that a release

site is occupied by a quantum between stimuli and the probability that a nerve impulse will activate the occupied site. Consider two probabilities p_1 and p_2 with n_1 the number of sites for release and n_2 the number of sites occupied prior to a stimulus. Immediately following a stimulus the number of sites occupied will be $n_2 (1 - p_2)$ with $n_1 - n_2 (1 - p_2)$ sites free. As demonstrated in the experiments on crayfish the refilling rate must be equal to the rate of release, and occur during the interval between action potentials.

The number of quanta immediately available for release may therefore be defined as in equation 9.

$$m = n_2 p_2 = n_1 \frac{p_1 p_2}{p_1 + p_2 - p_1 p_2} \qquad \text{--- 9}$$

where:-

m is the quantal content.

Although this is an improvement it is still difficult to relate statistics to physiology. However, attempts were made, and it was proposed that there existed a pool of readily releasable acetylcholine and that the amount released by a stimulus was proportional to the size of that pool. This hypothesis was tested(12) by experiment and such a pool identified.

A subsequent model suggested quantal content to be best expressed in terms of the number of quanta available for release at any time as shown in equation 10.

$$m = pC_q V \qquad \text{--- 10}$$

where:-

C_q = concentration of quanta available for release,

and V = constant volume.

The hypothesis proposed a two compartment model where quanta from one store were mobilised into a readily releasable store for subsequent release. The model assumed the rate of transfer between stores to be dependent upon the difference in concentration. For a stimulating frequency f it was proposed that an output would result as expressed in equation 11.

$$fm = K \left[C_p - \frac{2C_q V - m}{2V} \right] \qquad \text{--- 11}$$

where:-

C_p = concentration of quanta available for transfer to the readily releasable store.

Combining 10 and 11 produced an expression for quantal content (to an evoked response) to be as shown in equation 12.

$$m = \frac{KC_p}{f + \frac{K}{V}\left(\frac{1}{p} - \frac{1}{2}\right)} \qquad \text{--- 12}$$

where:-

K is a proportionality constant.

Unfortunately the mathematical model did not fit experimental data at stimulating frequencies below 60 Hz.; obviously the process is not as simple as equation 12

would suggest.

So far only models concerned with quantum content and quantum size have been mentioned. One intriguing factor left out of hypotheses so far described concerns the effects of calcium and magnesium ions. Several workers have shown that changes in external calcium concentration cause corresponding changes in quantal content [27,28,29] although the relationship is non-linear [12]. An increase in the presence of magnesium seems to produce an opposite effect to that of calcium although not quite so pronounced. It seems release of the neurotransmitter is limited by levels of calcium and magnesium with calcium being the primary control. This phenomenon has been reaffirmed by similar experiments carried out on other animals [30,31,32].

One model proposes transmitter release to be related to concentrations of a complex CaX, where X refers to a membrane component. It is also suggested that a further complex MgX is formed but that this has little effect on transmitter release [33]. The amplitude of an epp V thus being described as shown in equation 13.

$$V_{epp} = f \left(\frac{K_1\{Ca\}}{1 + K_1\{Ca\} + K_2\{Mg\}}\right) \qquad \text{--- 13}$$

where:-

K_1 and K_2 are affinity constants,

and f a proportionality constant.

This equation has been further refined [30].

$$V_{epp} = A \left(\frac{K_1\{Ca\}}{1 + K_1\{Ca\} + K_2\{Mg\}}\right)^4 \qquad \text{--- 14}$$

where:-

A is a constant of proportionality.

MgX and CaX are about 0.3 and 1 mM^{-1} respectively.

The main conclusion drawn from the above model is that four ions of calcium react in some way with a membrane component X, and that this complex co-operates with other mechanisms to release one quantum of acetylcholine. In order to establish a single relationship relating effects of both calcium and magnesium to an evoked response (and spontaneous release), a model was constructed which assumed the existence of several complexes. The model proposed the existence of complexes CaX, Ca_2X, Ca_3X, MgX, Mg_2X and Mg_3X and that all of these (together with X itself) but with the exception of Mg_2X contributed in some way to quantal release[31,33]. Upon analysis it was found that a complex Ca_3X provided the most compatible data to that produced by experiment. This model thus predicted the number of calcium ions needed to evoke a quantal release was 3 and not 4 as suggested earlier.

Although a close approximation to the experimental data was obtained there is little direct supporting evidence, and the relationship must still only be regarded as imperical. It has to be stressed that little is known of the influx of calcium ions except that it occurs immediately prior to the release of acetylcholine, and thus appears to trigger in some way transmitter output from the nerve terminal.

Another point worthy of mention during this appraisal

concerns the presence of post-synaptic noise[34]. This noise has been described as the result of a steady depolarisation at the post-synaptic site by acetylcholine, and is made up of a large number of components each the result of a transient opening of an elementary ion channel in the presynaptic membrane. The resultant noise therefore due to fluctuations in the number of channels open from moment to moment[35,36,37,38]. Quantitative analyses of this noise[35,39,40] have predicted the overall response to be made up of events that may be expressed as a function of time (f(t)) and which occur at a frequency of n/sec. The mean value of the ensuing depolarising voltage may thus be expressed as shown by equation 15.

$$V = n \int_{0}^{\tau} f(t)\, dt \qquad \text{--- 15}$$

and its variance $\bar{S}^2$ by:

$$\bar{S}^2 = \overline{(V_t - V)^2} = n \int_{0}^{\tau} (f(t))^2 dt \qquad \text{--- 16}$$

where:-

V_t is the value at time t.

If the function f(t) is taken to be a transient wave rising instantaneously to a value A and then declining exponentially:

$$f(t) = \frac{Ae^{-t}}{\tau} \qquad \text{--- 17}$$

Hence:

$$V = nA\tau \qquad \text{--- 18}$$

and:

$$\overline{s}^2 = \frac{nA^2\tau}{2} \qquad \text{--- 19}$$

from which:

$$\overline{s}^2 = \frac{AV}{2} \qquad \text{--- 20}$$

The importance of studying noise becomes apparent if the characteristically different responses produced by the presence of various drugs at the neuromuscular junction are considered. Life times of drug interaction with post-synaptic receptors may be predicted[36], and isolated from the effects of acetylcholine 'leaking' from the presynaptic site.

All models so far mentioned have arisen from observations of events at the post-synaptic site, and have been produced in an effort to characterise neuromuscular function. It is not the purpose of this chapter to dwell on the interactions of various drugs with post-synaptic receptors, but merely to mention these phenomena in passing. However, as the prediction and estimations of acetylcholine output can only be interpreted by use of post-synaptic events, some detail must be presented regarding the relationship of acetylcholine to these two sites.

Drug interaction (including acetylcholine) at the post-synaptic site has long stimulated research interest and several models have emerged in the literature. A

classical model for receptor action based on the law of mass action, has been applied to the combination of an agonist (e.g. acetylcholine) with the receptor. At equilibrium the proportion of receptors occupied by the agonist can then be related to agonist concentration[41, 42,43], as shown by equation 21.

$$P_A = \frac{K_A\{A\}}{1 + K_A\{A\}} \qquad \text{--- 21}$$

where:-

K_A is the affinity constant for the agonist receptor interaction, and

P_A is the proportion of receptors occupied by the agonist.

With a proportion of the receptors at the post-synaptic site occupied an additional conductance path may be thought to have been introduced such that conductance (G) becomes as in equation 22.

$$G = G_{max} P_A \qquad \text{--- 22}$$

where:

$$\frac{G}{G_{max}} = \frac{K_A\{A\}}{1 + K_A\{A\}} \qquad \text{--- 23}$$

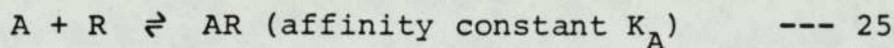
This ultimately produces a voltage which when measured at the post-synaptic site can be used to establish a relationship between agonist concentration and ensuing depolarisations.

i.e.

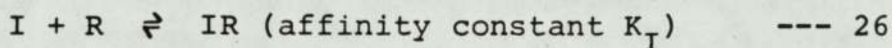
$$\Delta V = \frac{G_{max}K_A\{A\}}{G_o + (G_{max} + G_o)K_A\{A\}} \cdot V_o \qquad \text{--- 24}$$

Unfortunately the value of conductance is not directly proportional to the number of receptors occupied, and thus invalidates the preceding expression for all P_A; at best $G = f(P_A)$.

Many experiments used to determine epp do so by introducing a non-depolarising muscle relaxant so as to depress muscle twitch and hence optimise recording techniques. In consequence the effect of introducing this drug must also be considered. It is assumed that both chemicals (i.e. Acetylcholine and the non depolarising muscle relaxant) compete on a one to one basis for the receptors such that when the law of mass action is applied:



$$A + R \rightleftharpoons AR \text{ (affinity constant } K_A\text{)} \qquad \text{--- 25}$$



$$I + R \rightleftharpoons IR \text{ (affinity constant } K_I\text{)} \qquad \text{--- 26}$$

The proportion of receptors occupied by both agonist and antagonist may therefore be given by the following expressions:

$$P_A = \frac{K_A\{A\}}{1 + K_A\{A\} + K_I\{I\}} \qquad \text{--- 27}$$

$$P_I = \frac{K_I\{I\}}{1 + K_A\{A\} + K_I\{I\}} \qquad \text{--- 28}$$

It is unfortunate that experimental verification of these expressions is difficult. It is impractical, for example, to determine values for P_A, as the use of any labelled agonist will be complicated by the passage of agents into muscle fibres etc. However, a useful approach has been to use the effect of antagonist type drugs[44] such that:

$$P_A = \frac{K_A\{A\}}{1 + K_A\{A\}} = \frac{K_A X\{A\}}{1 + K_A X\{A\} + K_I\{I\}} \qquad \text{--- 29}$$

and

$$X - 1 = K_I\{I\} \qquad \text{--- 30}$$

where:-

X = the dose ratio.

The value of X for a given concentration of antagonist should be constant, irrespective of the magnitude of {A}. Equation 30[44] has been applied successfully to the action of Tubocurarine and several other neuromuscular blocking agents, although significant error is incurred when large concentrations are used[45,46].

It has been necessary to mention these preceding models so as to introduce the two main hypotheses presently used to describe acetylcholine release from the nerve ending, and to appreciate the basis on which these philosophies have been conceived. The main hypothesis, often called the vesicular hypothesis, was first postulated in the early 1950's[8], and further developed into the 1960's[28,29]. The theory supposes the emission of

acetylcholine to be due to a process of exocytosis and has been the basis for most of the work carried out at the neuromuscular junction. This idea of exocytosis has been reinforced over the years but in particular, when presynaptic vesicles were found to contain large quantities of acetylcholine molecules[23].

The vesicular hypothesis states that upon receipt of an action potential at the nerve terminal, vesicles are energised in such a way that some of them are caused to fuse with the outer membrane of the nerve, rupture and spill their charge into the synaptic cleft. Mini end plate potentials are explained in a similar fashion, but here, the fusion of vesicles is deemed to be a product of simple Brownian movement causing spontaneous random events to occur at the post-synaptic site. The collection of numerous data concerning mepps has led researchers to correlate the smallest epp with the release of the contents from one vesicle at the presynaptic site.

There are several pieces of evidence in favour of the release of acetylcholine by exocytosis. Electron micrographs of freeze etched preparations have shown vesicles fused with the presynaptic membrane and their contents apparently discharging into the synaptic cleft[47,48,49,50,51]. It must be pointed out, however, that the number of micrographs available are few and the number of vesicles shown to be in this mode of operation are numerically very small[52,53]. It has been argued that this is to be expected and is explained in terms of the morphology. With a total length of an aborisation

within one junction of the order of 1 mm., a single unit release would occur on average at points roughly 3 μm apart, and in consequence even freezing the specimen at the peak of activity would be unlikely to increase the chances of finding a discharging vesicle.

Vesicle counts have been shown to decrease during stimulations at the mammalian neuromuscular junction[54] as well as in the cat sympathetic ganglion[55,56] and in the electric organ of torpedo[57,58,59]. The presynaptic membrane area has been shown to increase correspondingly during enhanced acetylcholine release at the junction[60,61]; an expected phenomenon if vesicles were indeed fusing with the presynaptic membrane.

One major piece of evidence used to support the vesicular hypothesis is the observation that horse raddish Paroxidase when added to a ringer solution can be found in some synaptic vesicles after rest following a prolonged stimulation[61,62,63]; the inference being that synthesis has recharged the vesicles. Upon restimulating, vesicles which have been charged with horse raddish paroxidase are shown to disappear.

A further piece of useful supporting evidence (be it peripheral) concerns the destruction of mammalian motor nerve terminals by black widow spider venom[64] or botulinum toxin[65]. If as the vesicular hypothesis states, acetylcholine is released directly from synaptic vesicles, then neuromuscular transmission should cease in the absence of these vesicles. Experiments have revealed that an injection of black widow spider venom into the

neuromuscular junction of the frog causes a preliminary increase in mini end plate potential activity followed by a complete cessation of neuromuscular transmission. Subsequent freeze etched specimens examined by electron microscopy have shown a total absence of synaptic vesicles and other organals. When the venom is applied to a cat neuromuscular junction a similar process is shown to occur. An examination about one hour after the injection, revealed a complete absence of synaptic vesicles and the terminal axonal expansion appeared partitioned into several membrane limited vacuoles. The prejunctional membrane had been destroyed and the post-junctional membrane lined with a dense basement membrane along the junctional surfaces. The initial increase in spontaneous discharge of vesicles ceases only when the total store is evacuated[66].

There are, however, serious anomalies within the exocytosis hypothesis. It has been shown in experiments on the ciliary ganglion of the chick that full neuromuscular function is evident even though there are few synaptic vesicles present[67]. It has also been shown that acetylcholine may be released at denervated neuromuscular junctions by the Schwann cells apparently in the total absence of vesicles[68]. In these cases the hypothesis would seem to fail.

Experiments involving stimulating cholinergic tissues have shown that it is acetylcholine in the cytoplasm that is initially depleted and not that of the vesicles[69]. Continued stimulation does, however, deplete the vesicle population and this fact has been interpreted as the

process of exocytosis[58]. Several researchers have shown that by radioactively labelling extracellular choline, newly synthesised acetylcholine is released in preference to that already resident in the vesicular stores[70,71,72,73,74]; the vesicular hypothesis does not account for this release.

A problem is also presented concerning membrane assimilation of discharged vesicles. After fusion, the vesicle membrane would need to become a part of the presynaptic membrane. It has been postulated that should the vesicle membrane become a permanent part of the nerve ending (of approximately 5-10 μm in diameter), then a linear growth of 77-154 cm/hr.[75] could be expected. To prohibit such gross distortions a need for membrane recycling exists, although this technique would be complicated and difficult to envisage. Recent reports have, however, proposed a temporary fusion of the vesicle with the axolemma of the nerve ending from where it may be subsequently recaptured and re-used[56,59,61,76,77,78]. Exocytosis does have a lot of supporting evidence including results of work published recently, concerning the release of acetylcholine with proteins at the neuromuscular junction[79].

It has long been proposed that the vesicle also contains a multitude of other chemicals and proteins[82] which would be released at the same time as acetylcholine, should release be by exocytosis. Ten years ago protein emission from the nerve ending was verified[83,84], and recent work has shown particular proteins known to be

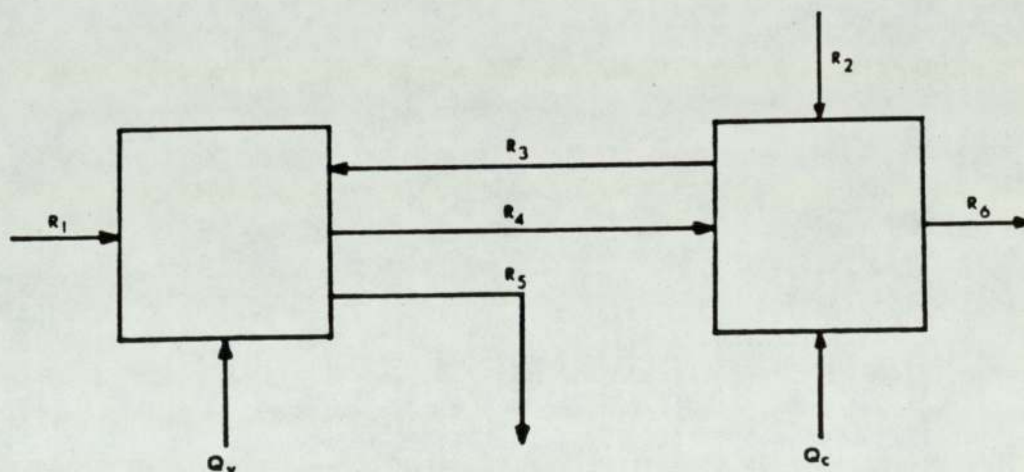
contained in the vesicle, released into the synaptic cleft at the same time as acetylcholine. It must be noted however that the amount of protein released is in excess of that expected to be inside the vesicle.

This recent piece of work does not, however, preclude the main antagonist to the vesicular hypothesis. This considers release of the neurotransmitter to be from a readily accessed pool of acetylcholine resident in the cytoplasm of the nerve terminal and emitted through a gate type mechanism via channels into the synaptic cleft[55,83]; the gates being activated by an influx of calcium ions into the nerve terminal immediately prior to release.

The most serious form of this hypothesis[83] describes two stores of acetylcholine residing within the nerve terminal; a labile store often classed as free acetylcholine, stored in the cytoplasm, and a bound store contained within the membranes of synaptic vesicles seen under electron microscopy. Within the vesicles it is postulated that molecules of acetylcholine are bound on two levels; one fraction loosely bound and able to be released, while the other more tightly bound compartment is not readily released. The hypothesis argues an interaction between two stores of this type is more satisfying than vesicular release into the synaptic cleft. The epps are the result of the release of acetylcholine molecules from the cytoplasm. The hypothesis proposes release in this manner to be a sensible mechanism, similar to that already known to exist with sodium and potassium (i.e. during the propagation of electrical transmission),

and that channels of this type could in fact remove large quantities of acetylcholine molecules into the synaptic cleft in a much more orderly fashion than envisaged by exocytosis.

The philosophy of this model assumes all pools of acetylcholine to be homogeneous, a fact which is itself questionable[84,85,86] although it is suggested that no significant error is evident if this fact is untrue[83]. All compartments are considered to be in a steady state condition and as such adopt a configuration as shown below with R_1 - R_6 corresponding to transfer rates of acetylcholine.



The model assumes no hydrolysis or re-uptake mechanisms at work within the cell[87] although this in part conflicts with other evidence[71]. Compatibility is attempted with various available data concerning synthesis, storage and release of radioactive labelled acetylcholine.

If radioactive labelled choline of specific radioactivity a_p is introduced into the system, then specific radioactivities will be present in both compartments immediately following synthesis. If the specific radioactivity of cytoplasm and vesicular compartments is a_c and a_v respectively then the rate of transfer of radioactive acetylcholine may be given by:

$$\frac{dQ_v}{dt} = a_p R_1 + a_c R_3 - a_v (R_4 + R_5) \qquad \text{--- 31}$$

$$\frac{dQ_c}{dt} = a_p R_2 + a_v R_4 - a_c (R_3 + R_6) \qquad \text{--- 32}$$

Amounts of radioactive acetylcholine in vesicular and cytoplasmic compartments (assuming steady state conditions) correspond to Q_v and Q_c respectively. Equations 31 and 32 may be rewritten in terms of change in specific activities.

$$\frac{da_v}{dt} = \frac{a_p R_1 + a_c R_3 - a_v(R_4 + R_5)}{Q_v} \qquad \text{--- 33}$$

$$\frac{da_c}{dt} = \frac{a_p R_2 + a_v R_4 - a_c(R_3 + R_6)}{Q_c} \qquad \text{--- 34}$$

with $S_v = \frac{a_v}{a_p}$ and $S_c = \frac{a_c}{a_p}$

If S_v and S_c are ratios of specific activity to the choline pool the equations can be further simplified:

$$\frac{dS_v}{dt} = \frac{R_1 + S_c R_3 - S_v F}{Q_v} \quad \text{--- 35}$$

$$\frac{dS_c}{dt} = \frac{R_2 + S_v R_4 - S_c G}{Q_c} \quad \text{--- 36}$$

Most two compartment models produce similar solutions. The solution of the above equations resolves to:-

$$U_v = Ae^{-\lambda 1t} + Be^{-\lambda 2t} \quad \text{--- 37}$$

$$U_c = Ce^{-\lambda 1t} + De^{-\lambda 2t} \quad \text{--- 38}$$

In order to equate transfer rates with measured values the equations are rearranged.

$$R_1 = F(S_v)_o - R_3(S_c)_o \quad \text{--- 39}$$

$$R_2 = G(S_c)_o - R_4(S_v)_o \quad \text{--- 40}$$

$$R_3 = \frac{(\lambda_1 Q_v + F)}{C} \quad \text{--- 41}$$

$$= \frac{(\lambda_2 Q_c + F)}{D}$$

$$R_4 = \frac{FG - \lambda_1 \lambda_2 Q_v Q_c}{R_3} \quad \text{--- 42}$$

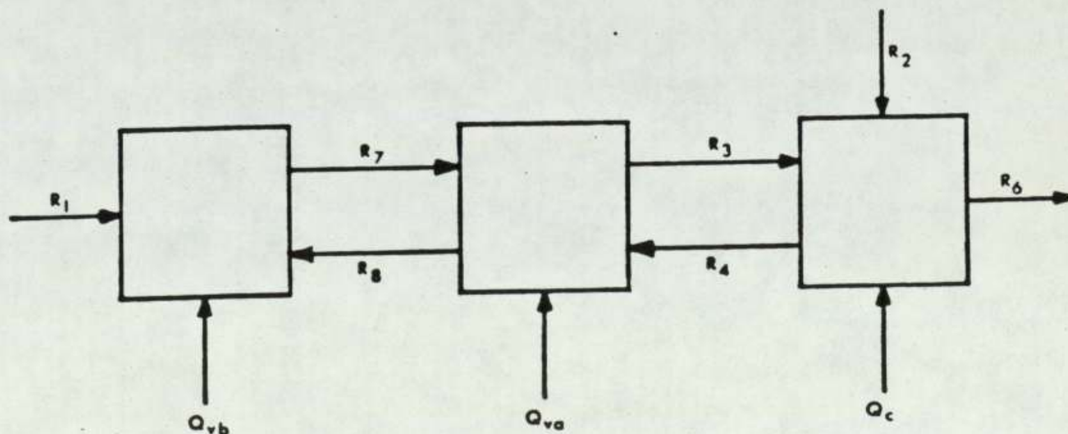
$$R_5 = R_1 + R_3 - R_4 \quad \text{--- 43}$$

$$R_6 = R_2 + R_4 - R_3 \quad \text{--- 44}$$

Application of experimental data to the model[88,89] creates an interesting result. The model gives similar curves to those derived experimentally but the rate constant R_3 was found to be negative indicating unidirectional movement of acetylcholine from vesicular to cytoplasmic store; a result which occurred in both sets of data used. It was proposed that the vesicle population was not homogeneous but heterogeneous[83] and that there were in fact two types of vesicle, some containing acetylcholine which was loosely bound and some tightly bound; a fact that has been corroborated experimentally[90]. In consequence the model resolves to that shown below, and postulates a total rate of synthesis to be as defined by equation 45.

$$R_{TOT} = R_{IL} + R_2 \qquad \text{--- 45}$$

where R_{IL} is synthesis into the loosely bound fraction of the vesicle population.



The model proposes that R_{IL} exactly balances that component of R_3 in reverse mode and ignores that component of synthesis into the tightly bound sections of the vesicle population.

In summary the model contends that acetylcholine release is cytoplasmic and not due to exocytosis. The vesicle population is a reserve supply of acetylcholine and only used in dire need (as synthesis supports release under normal circumstances) but when released it is to the cytoplasm.

There are several advantages to this hypothesis. Over the past ten years it has become increasingly necessary to talk of acetylcholine storage in the nerve ending in terms of at least two compartments; one said to contain free acetylcholine immediately available for release and the other bound acetylcholine which is not so readily released. It has been proposed that the bound acetylcholine corresponds to that resident within the vesicles[91,92] and this store has been shown to be resistant to the hydrolysing effects of acetylcholinesterase [23,90,93]. It is this last fact that provides the main support for the cytosol hypothesis.

It has been shown that a small quantity of acetylcholinesterase when injected into the nerve of an isolated preparation, and using inherent axonal transport for its deliverance to the nerve terminal, (a process taking approximately 2½ hours) causes a sudden cessation in synaptic transmission which had hitherto been normal[94]. Close examination of the preparation revealed the nerve

terminal had suffered no significant decrease in vesicle population. If, as this cytosol hypothesis contends, the major pool of acetylcholine available for release is labile and stored in the cytoplasm of the nerve terminal, it would be accessed quite readily by an injected quantity of acetylcholinesterase; the bound or vesicular pool remaining intact. It has been known for some years that some form of esterase exists within the nerve terminal[45] as its inhibition by use of eserine causes an increase in acetylcholine content to occur.

The hypothesis also explains how newly synthesised acetylcholine can be released more readily than that known to exist in endogenous stores within vesicles, and there is no problem with the formation of a complicated membrane hypothesis.

This hypothesis creates considerable opposition to release by exocytosis, and although it has several attractive ideas, and seems to explain certain phenomena which otherwise evade description, it too has several defects. Release by exocytosis is rejected on the grounds that it would be unable to supply demand unlike the proposed use of select ionic channels. The ionic flux across the membrane may be derived by consideration of the reaction of the membrane to acetylcholine. The derived figure is based on earlier work on similar problems[95] and uses the following relationship[96].

$$\text{Flux} = 2\pi rDnZ \qquad \text{--- 46}$$

where:-

D = diffusion control of acetylcholine (7.6×10^{-6} cm^2/S).

n = concentration in mol/cm^3.

Z = factor depending on the charge interaction.

The basis for supposing that acetylcholine is emitted through channels is based on the fact that this method is proven for the translocation of sodium and potassium ions. The hypothesis highlights evidence that tetraethyl ammonium (TEA) can effectively block a potassium channel[97], and contends that as TEA is a larger molecule than acetylcholine[98] there is no reason to suppose that acetylcholine cannot be effused in a similar fashion, generating a maximum steady flux of around 10,000 molecules/ms/channel. However, there is no direct evidence that TEA actually enters a channel, it could well be that it just blocks the outlet and that the channel diameter is much smaller than a TEA molecule.

It is difficult to imagine channels in the nerve membrane which open upon request selectively to acetylcholine. It would seem probable that if such a channel were opened many more chemicals would follow especially those of smaller molecular size than acetylcholine (e.g. sodium and potassium)[99].

It has also been shown that acetylcholine stores may not be in a steady state condition as the cytosol hypothesis states[99,100]. Recent estimations of store contents every 30 second have shown the amount of

acetylcholine to fluctuate with the amount of free acetylcholine sometimes well in excess of that suggested by electrophysiological evidence. Extensive monitoring of epps in isolated preparations over long periods of stimulation, have established a decline in potential over the first few stimuli followed by a period of relative stability[12], and then a further deterioration. Quantifying stores every 30 seconds during stimulation has revealed an undamped oscillation of store content. If the epp is related to the quantity of acetylcholine released, the cytosol hypothesis must be in conflict with this electrophysiological data. Clearly the situation is not so simply defined.

A further complication to both hypotheses is the claim that vesicles seen by electron microscopy are artefacts produced by the fixation of proteins[53,101]. The argument follows that techniques used to stain and fix sections of the presynaptic site cause proteins to take up shapes similar to the familiar vesicle stores, and that the structures seen by the electron microscope bear no relationship to those in vivo. Standard techniques used to fix biological materials for electron microscopy do denature proteins[102] and alter spacial configurations, and if this is happening at the presynaptic site a powerful argument can be formulated for the cytosol hypothesis. There are, however, certain anomalies with this point of view. For example whether vesicles or artefacts it seems they exist only at the presynaptic site and not post-junctionally. Their arrangement is precise

and always around junctional folds located directly opposite to receptor sites on the post-synaptic membrane[103] and stimulation causes a depletion in their number[104,105]. They have also been shown to be clearly visible when subjected to fixation in glutohaldehyde[83] and embedding in water soluble embedding medium, a process which tends to denature proteins. Although it may be possible that vesicles are artefacts, there seems to be overwhelming evidence to the contrary, and that they are in fact necessary components of the presynaptic site and play an integral part in neuromuscular transmission.

One more model worthy of consideration concerns a quantitative description of end plate currents at the post-synaptic site[106]. A model has been put forward relating the presence of acetylcholine and ionic transport through end plate channels to end plate current measured on the post-synaptic membrane. The model relates the product of the cleft volume (containing the receptors) and the rate of change of concentration with time against, functions for the release (of acetylcholine) at the presynaptic site, diffusion of molecules across the cleft, and subsequent hydrolysis of the chemical by the cholinesterase, as shown by equation 47.

$$\overline{V}\,\frac{d_c(t)}{dt} = f(t) - \int_0^{\tau} G(t-\tau)\ c\ (\tau)\ d - K_E c(t) \quad \text{--- } 47$$

where:-

V = cleft volume.

c = concentration of acetylcholine.

f(t) = function describing release from the nerve terminal.

G(t) = characterises diffusion at the cleft.

K_E = enzymatic hydrolysis.

It is argued that the release function (f(t)) at the presynaptic site is unlikely to make any appreciable contribution to the declining phase of end plate current due to the rapidity of its emission[107], and that the time taken for diffusion of molecules across the synaptic cleft is very small[28]. Time taken in binding acetylcholine molecules to the post-synaptic receptors is also assumed to be very fast, limited only by diffusion. Hydrolysis of the transmitter is said to be extremely rapid, and it has always been assumed that the rate limiting process for events at the post-synaptic site is governed by concentrations of acetylcholine in the cleft.

The model, however, postulates that the concentration of acetylcholine within the cleft could only be present for a short time (due to diffusional and hydrolysis effects) whereas end plate current is known to persist for some time after these events have taken place. The model proposes that the rate limiting process for end plate currents at the post-synaptic site are governed by opening and closing ionic channels in the post-synaptic membrane, and suggests that the action of acetylcholine is only the beginning of an enzymic chain of reactions causing these channels to function. The argument continues that the channels remain open only so long as

the post-synaptic potential is at a particular level, but as the end plate potential falls the channels close and the end plate current is shut off.

The model gives various experimental results as corroborative evidence for its theoretic hypothesis[108, 109,110,111,112] but like most models so far mentioned there are areas of uncertainty. The model postulates that time taken for hydrolysis is small and would have been completed long before the end plate current has ceased to exist. An inherent problem with this assumption is that of the cholinesterases. If a cholinesterase inhibitor is given it has been shown conclusively[113] that the duration of the end plate potential and current is extended to perhaps 50% more than in normal function.

The model assumes a fairly simple function and does not take into consideration any three dimensional effects which must occur at a post-synaptic fold. In consequence although good results occur it would seem that a three dimensional partial differential equation type model should be used, although this technique is dismissed as unnecessary because of the speed at which diffusion occurs in the cleft and the complexity of the resultant mathematics. It has been shown by other research workers that 66% (plus of minus 3%) of acetylcholine molecules released upon stimulation are bound to receptor sites at the post-synaptic interface[114]. This fact is in support of the model as the bound proportion would be hydrolysed fairly quickly and the remainder drift away by diffusion. It must be noted, however, that other

molecules from other synaptic sites could also drift into range of activity by similar mechanisms of diffusion.

The model details events and molecular mechanisms at the post-synaptic site to establish equations concerning the action of end plate current, and a forcing function is derived which when applied to the model produces the respective output. It is interesting that the shape of this forcing function (with the respect to time) is remarkably similar to that of diffusion of a spot concentration in a larger medium[115]. It would seem that if post-synaptic activity is directly proportional to acetylcholine concentration within the cleft, a forcing function would result similar to that already explained, although the time scale over which it occurs might be different. It is interesting that there is experimental evidence that the function f(t) dismissed by the model does persist for several milli seconds[116], and in consequence there seems reason for speculation.

All models, expressions, and hypotheses discussed, relate to the problem of defining synthesis, storage and release of acetylcholine at the neuromuscular junction. Although the list has not been exhausted it is hoped this overview has been in a logical sequence and been presented in an unbiased manner. It should now be apparent that although much work has been done to quantify events concerning neuromuscular transmission, there is no overall model capable of reflecting the many facets of neuromuscular function.

It may well be that this solution is still distant,

but it should be possible to try to produce a concensus of opinion from the many reports and conflicting view-points that have been published over the past three decades.

CHAPTER 3

PROPOSED MODEL

A review of the literature has revealed anomalies not fully or adequately explained by either of the two major hypotheses. An alternative approach to the phenomena that comprise neuromuscular function would be to utilise available factual evidence and accommodate some of the most useful evidence into a lumped parameter mathematical model. It is the purpose of this chapter, therefore, to introduce such a model which links known processes together into one overall description of the synthesis, storage and release of acetylcholine into the synaptic cleft.

Although large quantities of acetylcholine are released by evoked response there is evidence to suggest the isolating property of acetylcholinesterase ensures all quanta are used[117]. It is also interesting to note that the muscle responds effectively to smaller numbers of quanta released, showing a high margin of safety[118].

The morphology of the nerve ending is so precisely engineered[119] that it would be no surprise to find a readily releasable store of acetylcholine is set aside for immediate use. A simple system could thus be envisaged linking this readily releasable store (Q_n) to the synaptic cleft via a transfer function describing movement of acetylcholine.

Most learned papers discuss this output in terms of

quanta[6,12,117] and there seems little reason to deviate from this principle here, although doubt still remains as to a quantum corresponding to the charge of acetylcholine held within a vesicle[35,120,121].

It is important at this stage to qualify Q_n as a step input to the system (i.e. $\frac{Q_n}{S}$). Although the store is reduced as its contents are evacuated to the synaptic cleft, it may still be regarded as a step function so long as its magnitude (Q_n) is updated in small discrete time intervals throughout these evacuations.

A transfer function (G10) may be used to define the manner in which the acetylcholine store is depleted. No mechanism has yet been suggested whereby Q_n is replenished but this too will be discussed later in the description.

Defining G10 is difficult and can only be done through speculation. There is still little conclusive evidence to show how molecules of acetylcholine find their way into the synaptic cleft or to quantify the processes involved[6,83]; in consequence there is a grey area which is ill defined but fundamental in describing a quantitative model.

Assume the molecules or vesicles of acetylcholine which comprise the readily releasable store Q_n are free and floating within the cytoplasm of the nerve ending[83,122]. With the advent of an action potential these molecules/ vesicles are caused to migrate through the cytoplasm to the synaptic cleft. Migration implies movement and consequently certain physical principles must be invoked

during the course of this action. A sudden movement of molecules/vesicles indicates some force or prime mover at work which is initiated in some way by the action potential. A force may be created by some form of ionic repulsion which occurs when the action potential upsets the equilibrium, or be the simple result of a concentration gradient across the presynaptic membrane, but like all forces that exist, is balanced by other forces of equal but opposite magnitudes.

To move any mass inherent inertia must be overcome, and as molecules/vesicles are known to migrate quickly at the nerve ending (i.e. the manoeuvre is accomplished in approx. 1 m.s.), acceleration may have a pronounced effect on the manner in which the output occurs.

Molecules/vesicles must also move through a medium much thicker than water, containing many larger particles (in the form of proteins etc.), which requires a force of some magnitude to exist as molecular velocity is high.

To further complicate the situation molecules/ vesicles (of acetylcholine) may be "locked" in position by a complex system of interactive ionic and chemical forces so as to maintain equilibrium in the steady state. Any displacement would require a force capable of offsetting these forces of equilibrium and would be relative to the distance moved. As these molecules/ vesicles reside extremely close to each other, these equilibrium forces may also be significant in defining the characteristic of movement. Movement undoubtedly occurs in a three dimensional plane but to try to define

it in this way would needlessly complicate the proposed model at this time. In consequence a simple equation relating all these forces to a total flow in one direction may be as follows:

$$F = I\frac{d^2x}{dt^2} + U\frac{dx}{dt} + Kx \qquad \text{--- 48}$$

where:-

F = the applied force.

I = inherent inertia of the molecule (vesicle).

U = viscous resistance due to the medium.

K = resistance due to equilibrium forces.

x = distance moved in a direction along the length of the nerve terminal.

Solving the above expression reveals a solution given in equation 49.

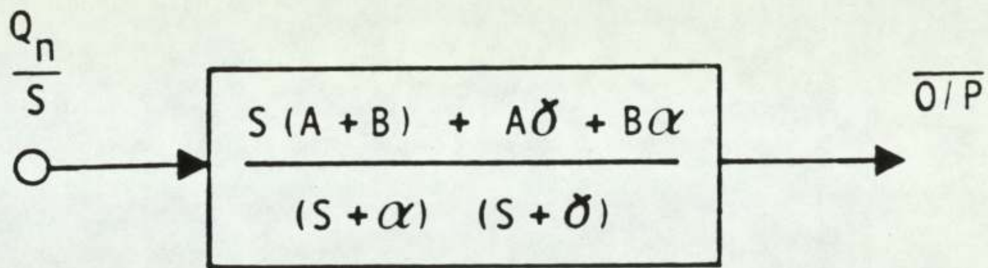
$$x(t) = F\left[\frac{1}{PQ} + \frac{1}{P(P-Q)}e^{-Pt} + \frac{1}{Q(Q-P)}e^{-Qt}\right] \qquad \text{--- 49}$$

P and Q are arbitrary constants.

Although this equation defines distance moved by the molecules/vesicles it could easily lend itself to quantifying output to the synaptic cleft; again a transfer function reflecting a series of exponentials.

For these reasons it seems sensible to choose a transfer function (G10) as defined by equation (50) and represented diagramatically on the next page.

$$G10 = \frac{A}{S + \alpha} + \frac{B}{S + \gamma} \qquad \text{--- 50}$$



The problem now is to decide numerical values for A, B, α and γ.

The movement of molecules/vesicles into the synaptic cleft is swift (i.e. approx. 1 m.s.)[12] but not, so far as it is known, oscillatory. Hence:

$$U^2 \geq 4IK \qquad \text{--- 51}$$

giving real roots for the expression.

It does seem possible that it is a molecule that is being considered and not a vesicle which is very much larger (perhaps 30,000 times) and in consequence would have more inertia.

In the first instance let:

$$\alpha = 100$$

$$\gamma = 10$$

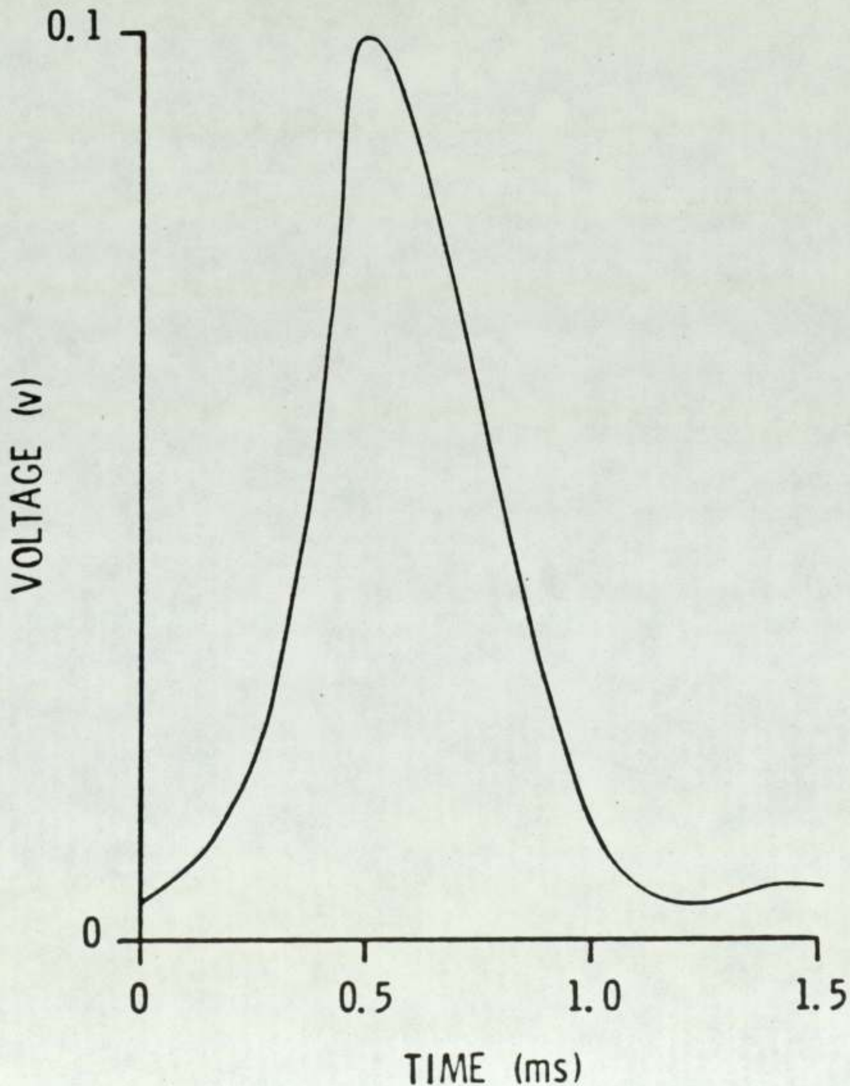
Values for A and B are dependent upon system parameters. If as already stated:

$$I < U \qquad \text{--- 52}$$

substitution reveals that values for A and B will probably be small,

i.e. $$A < 1 \quad\quad B < 1 \qquad \text{--- 53}$$

A problem when using the above system is that of integrating a force component into the model. It is difficult to establish what this force is or how it occurs but the action potential which initiates this phenomenon has been monitored extensively and can be represented as shown below.



Assuming this generated force adopts a similar shape, it may therefore be represented mathematically and inserted into the system.

It has been known for many years that after the arrival of an action potential, but before the emission of acetylcholine from the nerve ending, calcium ions from the extracellular space cross into the nerve ending. That calcium has an influence on acetylcholine release is now undisputed but the mechanism by which it exerts this influence is still unknown, although recent work has shown that the presence or absence of calcium is not the only reason for fluctuations in the release of the transmitter[123,124]. An increase in calcium level causes a corresponding increase in acetylcholine output[125] and when all calcium is expunged from the system (in vitro) complete suppression of acetylcholine output is effected. Changes in spontaneous activity of the mepps has also been attributed to changes in calcium[126].

The presence of magnesium in the extracellular fluid has a similar but opposite effect on acetylcholine release[127]. Increased or decreased magnesium levels, succeed in decreasing or increasing acetylcholine output, but with less effect than corresponding changes in calcium. The effects of these elements has been well documented and much has been made of the statistics of these events[128,129,130].

Both calcium and magnesium ions permanently reside in the extracellular space and various attempts have been made to produce hypotheses that explain how these chemicals

affect acetylcholine release[131,132]. The combined effects of the two elements are complementary although it seems calcium exerts more influence.

The presence of these chemicals must be accommodated into the system. The fact that calcium ions move from the synaptic cleft into the nerve ending indicates some feedback mechanism, and this is reinforced by the manner in which the actual physiological system reacts to changes in concentrations of these ions. It is likely, however, that the biological system responds not only to absolute concentrations but also to deviations from normal, and this should be reflected in the feedback system chosen.

Consider a feedback function of the following form:

$$H4 = AN - \frac{Ca}{Mg} \qquad \text{--- 54}$$

where:-

AN = the normal and expected ratio of calcium and magnesium.

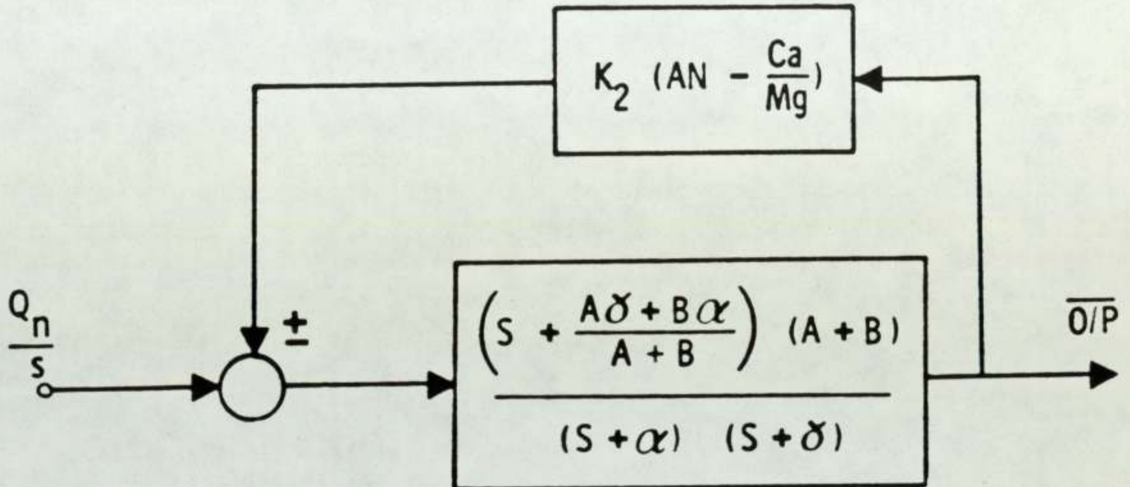
Ca = the actual value of calcium in the system.

Mg = the actual value of magnesium in the system.

Such a function helps to explain the measured outputs from biological preparations during changes in calcium and magnesium concentrations. The biological system seems particularly sensitive to changes in these ions and it does not seem unreasonable to infer some form of amplification if these ions act only as catalysts. With a normal ratio of calcium/magnesium a no feedback mechanism results as shown by equation 55 and represented by the block

diagram below.

$$H4 = K_2 \left(AN - \frac{Ca}{Mg}\right) \qquad \text{--- 55}$$



Although this part of the system is now complete, difficulty will arise when matching calculated outputs to those known to occur in isolated preparations. The value of Q_n can be deduced with some accuracy as can the feedback components, but using values of A, B, α and γ already discussed, the system is unlikely to produce outputs compatible with those known to occur. There are various ways in which the system can be made to output the correct information (e.g. large changes in Q_n, feedback, etc.) but most would be incompatible with existing literature, and present model philosophy.

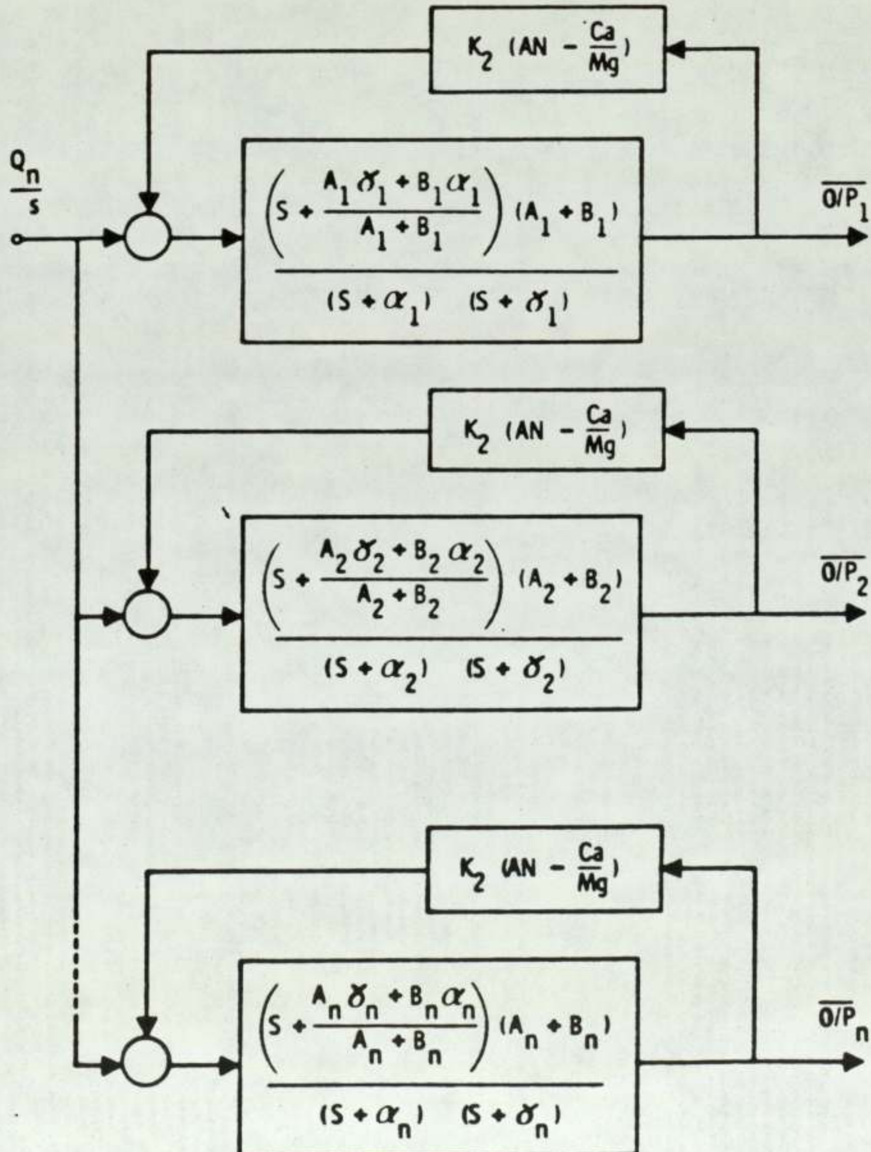
It is possible, however, that the system configuration so far described is correct except that it does not give total release, but the output of only one "channel". It

may be that release of acetylcholine into the synaptic cleft is made through a series of gated channels and not by exocytosis. There are various arguments concerning this aspect of transmitter release, but the configuration of the model so far described tends to indicate a controlled output, possibly through channels. It is equally possible to describe a channel as the point of fusion of a vesicle with the presynaptic membrane as in exocytosis but this would seem a random and uncontrolled event and not in keeping with the model philosophy.

If the channel hypothesis is to be given credance a further transfer function has to be created and accommodated within the system. This transfer function would define the number of channels in the nerve ending and would need to have a numerical value of around 500,000 if the system is to remain unaltered and still provide the correct output. This figure seems in keeping with morphometry of the neuromuscular junction, and the fact that not all channels are operational at the same time, or that those that do function are not fully efficient. The diameter of these channels would be small (approx. 0.3 n.m.) and easily accommodated over the nerve ending.

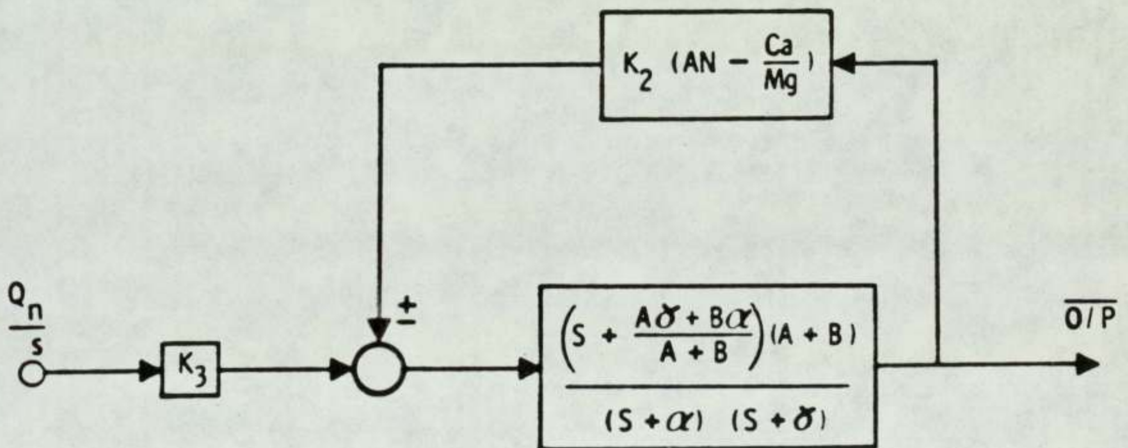
Using this latter hypothesis the model can be further redefined (as shown over the page) yielding a total output as follows:-

$$\overline{O/P} = \sum_{n=1}^{n=n} \frac{Q_n}{S} \cdot \frac{\left\{ S + \frac{(A_n \gamma_n + B_n \alpha_n)}{(A_n + B_n)} \right\} (A_n + B_n)}{(S+\alpha_n)(S+\gamma_n) + \left\{ \left[S + \frac{(A_n \gamma_n + B_n \alpha_n)}{(A_n + B_n)} \right] (A_n + B_n) . K_2 \left(AN - \frac{Ca}{Mg}\right) \right\}} \quad \text{--- 56}$$



If it is assumed that each A, B, α and γ are identical for each channel, a simple approach to the solution of the above model would be to include a transfer function containing a numerical estimate of the number of channels in operation at any time and may be represented as shown in the following block diagram; such that the total output becomes as defined by equation 57.

$$\overline{O/P} = \frac{Q_n}{S} . K_3 . \frac{S + \frac{(A\gamma + B\alpha)}{(A + B)} (A + B)}{(S+\alpha)(S+\gamma) + (S + \frac{(A\gamma + B\alpha)}{(A + B)})(A+B) . K_2 (AN - \frac{Ca}{Mg})} \quad \text{--- 57}$$



One aspect of the model not considered so far concerns resting release of acetylcholine into the synaptic cleft. The activity of mepps has been monitored by several groups[36,121,133,134,135] and has until recently been considered the result of spontaneous discharge of vesicles from the presynaptic site. Reported values for spontaneous resting release of acetylcholine seem to be in broad agreement[71,136,137,138] with a mean value of

around 2.7 p.M/min. Most studies concerning acetylcholine synthesis, storage and release have concentrated on the rat hemidiaphragm[71,138,139] and suggest a mean concentration of around 2.7 x 10^{-16} M/min/end plate, assuming the muscle to have around 10,000 end plates. Recent work has shown the output of acetylcholine necessary to produce mepps to be between 1% and 10%[140, 141,142] of the above figure, so clearly the larger part of acetylcholine emission at rest comes from a source not necessarily responsible for the mepp. It is possible that a steady leakage of acetylcholine occurs[143] through channels that are considered to exist at the end plate and is responsible for the end plate noise experienced at the post-synaptic site[135].

As already discussed, values for A and B are ultimately determined by forces existing in the nerve ending. It is unlikely that these forces will ever be zero, but probably increase in proportion with the concentration of acetylcholine. It is possible therefore that to maintain equilibrium, acetylcholine is leaked into the synaptic cleft causing a steady depolarisation to occur at the post-synaptic site. In consequence it would seem reasonable to assume minimum values for A and B that are not zero but dependent upon concentrations of acetylcholine in the nerve ending. This postulation does not interfere with the basic functions of the model but only refines its operations.

Studies suggest that a readily releasable store has the capacity to store 1,000 quanta[12] but this is perhaps

only 1% of the total available within the nerve ending. This concepts fits in well with the above model[55]. The electrophysiological evidence points to a release of acetylcholine from what might be classed as a mobilisation store into the already defined readily releasable store. The concept of a two compartment system has been around for some time[12,55,144,145,146,147] although single compartment stores have also been proposed[148,149].

The idea is further reinforced by morphological evidence which has revealed dark patches or dense projections which seem to connect the presynaptic membrane to a collection of interconnecting vesicles held together by a series of rodlets[50,52,150,151,152]. These vesicles do not appear to be in Brownian movement but physically attached to the dense projections.

It is possible that these vesicles constitute a mobilisation store (Q_m) and the interconnecting rods form part of a transportation mechanism used to absorb vesicle contents into the dense projections. The electron microscope has revealed a well ordered structure[48,53,104,153] and it seems sensible to assume movement of quanta between stores to be just as well organised.

It is also interesting to note that virtually all vesicles attached to dense projections have electron dense cores. It may be the dense cored vesicles are rich in acetylcholine and ready for release unlike the lighter cored vesicles found in abundance further away from the presynaptic membrane.

Movement of vesicles is likely to produce similar

forces within the nerves (discussed earlier) although the speed of transportation is inherently slower. For similar reasons, a transfer function (G8) defining movement of quanta between stores may be defined as in equation 58; the input to this system being $\frac{Q_m}{S}$.

$$G8 = \frac{C}{S + \delta} + \frac{D}{S + \tau} \qquad \text{--- } 58$$

C, D, δ and τ are lumped parameters defining system elements such as inertia, viscous resistance, etc. For the same reasons explained earlier, these parameters are likely to be numerically small, although larger for vesicles than molecules. From electrophysiological data it appears that the effect of a mobilisation store on the readily releasable store is dependent upon the frequency of stimulation[12,154]. An effect is always apparent at the output (i.e. at the post-junctional membrane) even at frequencies as low as 1 Hz.[154]; higher rates of stimulation cause the effect to become apparent much earlier. As the frequency of stimulation rises, the input from the mobilisation store occurs with less delay. It seems that the urgency of the readily releasable store is transmitted somehow to the mobilisation store.

It is possible that mechanisms governing the rate of acetylcholine release from the mobilisation store are related to changes in concentrations of acetylcholine in the readily releasable store. As the frequency of stimulation is increased the readily releasable store

would be quickly depleted and the change in acetylcholine concentration within the store more apparent. If this change is accommodated in δ and τ the rate of mobilisation will then change with respect to the needs of the readily releasable store. This seems a satisfactory mechanism and may be included in the model by redefining the parameters δ and τ as shown in equations 59 and 60.

Hence:

$$\delta = \frac{Z_1}{K_4 \ (Q_{nz} - Q_n)} \qquad \text{--- 59}$$

$$\tau = \frac{Z_2}{K_5 \ (Q_{nz} - Q_n)} \qquad \text{--- 60}$$

where:-

Z_1 and Z_2 refer to physical properties of the system,

Q_{nz} and Q_n refer to conditions of the readily releasable stores at times t = 0, and t = t respectively.

K_4 and K_5 are constants of proportionality.

Equations 59 and 60 rely on values of the readily releasable store Q_n for a solution. Q_n is a system variable but may be considered to be a constant over a very short time increment. The ultimate solution to this model is to be calculated by digital computer over many increments in time, with model status redefined at each step. In consequence this quasi time independent model cannot be solved in the conventional sense due to complex interactions between inputs and outputs.

It is obvious from the literature that irrespective

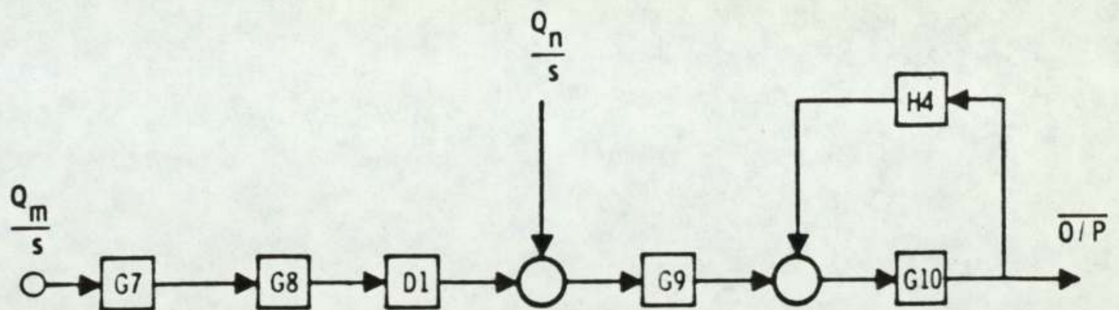
of stimulation frequency a delay is always apparent between the onset of stimulation and an output from the mobilisation store becoming effective[12]. At high frequencies of stimulation the readily releasable store may be almost depleted before an input arrives from the mobilisation store[154]. In consequence a delay due to the physical properties involved must be included in the model which will have a non zero minimum value.

The delay must also be dependent upon the change of concentration of acetylcholine in the readily releasable store and may be defined as follows:

$$D1 = \text{Fixed minimum constant} + \frac{E_K}{Q_{nz} - Q_{nn}} \quad --- 61$$

The fixed constant corresponds to the minimum delay caused by the inherent physical properties of the system, E_K corresponds to a constant of proportionality and Q_{nn} the value of store Q_n that was last transmitted to store Q_m. This technique should ensure a smooth operation and be similar to the physiological situation.

As G9 represents the number of channels of release so must a transfer function (G7) be created to define the number of vesicle pathways used in transferring quanta from the mobilisation store to the readily releasable store. The literature suggests a figure of around 2,200 quanta that may be held in the mobilisation store[12] and this fact may be included in the model as shown on the following page.



The model is now capable of exhibiting many of the phenomena experienced by the electrophysiologist. However, as the literature points out, the nerve ending is capable of storing many more quanta than have been accounted for by mobilisation (Q_m) and readily releasable (Q_n) stores[71,119]. It follows that a further store exists which supplies the mobilisation store, but utilises transportation mechanisms so slow that its effect is not apparent during most electrophysiological experiments.

The literature contains many photographs produced by electron microscopy of vesicles resident within the nerve ending[61,119,155,156,157]; the majority of this population being inhomogeneous[84,85,86]. Many of the less dense vesicles have thicker membranes which, it has been argued, are due to mechanisms involved during membrane recapture after exocytosis[156,158]. This may well be true but it might also suggest a fundamental feature in synthesis of new vesicular acetylcholine[159].

On the basis of present knowledge it is possible to postulate that many of the vesicles in the nerve ending

have incomplete stores of acetylcholine. It follows that the third store now being proposed, must be considered in terms of quanta available for use and not necessarily the number of vesicles that exist. In consequence a minimum of 180,000 quanta may be expected to comprise this latent store (Q_L)[12].

Although the methodology behind transportation of these quanta (perhaps fully charged vesicles) to the mobilisation store is relatively slow[160,161], the vesicles must be subject to similar physical forces as discussed for both mobilisation (Q_m) and readily releasable (Q_n) stores. A transfer function G6 of the following form should therefore be acceptable; with input $\frac{Q_L}{S}$.

$$G6 = \frac{E}{S + \beta} + \frac{F}{S + \theta} \qquad \text{--- 62}$$

Transportation of these vesicles has been a matter for speculation for many years, but recent morphological evidence has related vesicular movement to the presence of microtubules within the nerve ending[162]. Electron micrographs have shown vesicles attached to microtubules[163, 164,165] which appear to terminate in close proximity to the presynaptic membrane (i.e. close to a dense projection) and opposite a post-junctional fold. Initially it was assumed that vesicles rolled down the outside of the microtubules to arrive at release sites, but this hypothesis ran into difficulty when a smooth endoplasmic reticulum was seen to be wrapped round the microtubules. Movement

of vesicles down the outside of the microtubule would thus be impeded by the smooth reticulum although the vesicles always appeared to be well spaced and showed no signs of clustering. Movement in this way is thus speculative and unproven although it is only the translocation of quanta that is considered in this model.

As before when defining G7 and G9 a transfer function must be created (G5) which defines the number of microtubules present and a delay (D2) which will determine when an input to Q_m is made.

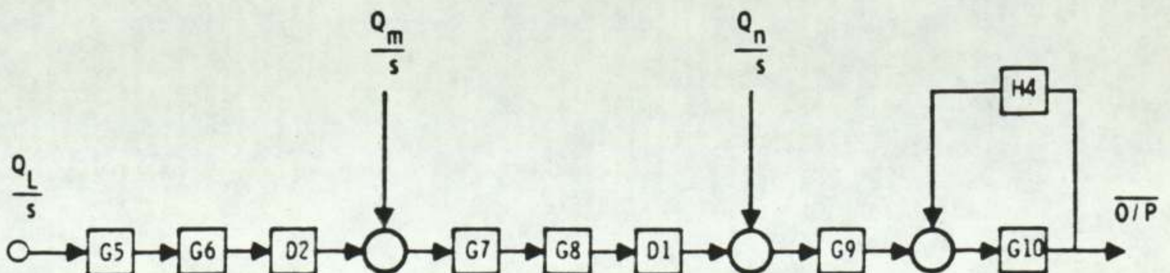
$$D2 = \text{minimum fixed delay} + \frac{E_{KL}}{Q_{mz} - Q_{mm}} \qquad \text{--- } 63$$

where:-

E_{KL} = constant of proportionality, and

Q_{mm} = the value of store Q_m that was last transmitted to Q_L.

The model now proposes three stores. A latent store which contains the majority of quanta available in the nerve ending and a mobilisation store containing sufficient quanta to enable a readily releasable store to function effectively. The model now adopts the following configuration.



Discussion of the mepp has so far been minimal. It has been postulated for some time that vesicles within the nerve ending are in a continuous state of Brownian movement[120], although recent morphological evidence has shown that very precise mechanisms may be at work and that the system in general is well ordered and precisely located. It would seem no accident that microtubules appear to terminate near dense projections located opposite post-junctional folds containing receptors. To argue the emission of acetylcholine is due to an increase in Brownian movement seems unsatisfactory, and the proposed model does not cater for spontaneous random release through 'channels' resident in the dense projections. The mass of vesicles resident in the latent store (Q_L) may well be in a constant state of random agitation and several microscopy studies have revealed vesicles apparently in the process of exocytosis[47,48,49,50,51] although they have not been discovered in sufficient numbers to confirm the exocytosis hypothesis during evoked response.

Although as the model presumes, evoked release of acetylcholine is through well defined channels, it may be that exocytosis is the cause of random mepps and the resulting mini end plate currents[166] which have been the basis of much of the statistical work carried out to explain neuromuscular function[9,35,167,168,169]. Accepting this proposition, other assumptions may be made.

It has already been postulated that many vesicles in this latent store may have incomplete complements of

acetylcholine[170]. If most of the vesicles are in a state of random movement it seems reasonable to expect Brownian movement to cause some of these vesicles to fuse with the presynaptic membrane and release their contents into the synaptic cleft. Research has shown around 10^5 molecules of acetylcholine which, applied to an end plate, cause a depolarisation similar to that of a mepp[142]. This figure is, however, regarded as an upper limit and the later estimates of 1%-10% of resting acetylcholine release seem much more realistic. It is interesting to note that if only 1%-10% of the acetylcholine released at rest can be attributed to spontaneous random events and a mean value for frequency 60-150 events/min. is used[171,172], the spontaneous activity reveals a quantum of release containing 540-5400 molecules of acetylcholine. Several recent estimates of vesicular acetylcholine suggest a figure of 6,000-15,000 molecules per vesicle[120, 141,173] although earlier estimates have varied from 900[174] to around 60,000[175]; the latter figure estimated as being close to the maximum number of molecules that can be packed into a vesicle[61,176].

If random vesicular release from the latent store is responsible for the mepps it would be expected that many of the vesicles released would not contain maximum complements of acetylcholine and again evidence exists in the literature which helps to confirm this hypothesis[121].

If as assumed 10^5 molecules is an over estimate of the number of molecules required to produce a mepp and that a figure of 50,000 molecules is more reasonable,

then if a quantum corresponds to 10,000 molecules, it follows that 1 mepp is caused by an accumulation of 5 quanta released at a rate of 80/sec and corresponding to a mean concentration of 2.48×10^{-19} M/s.

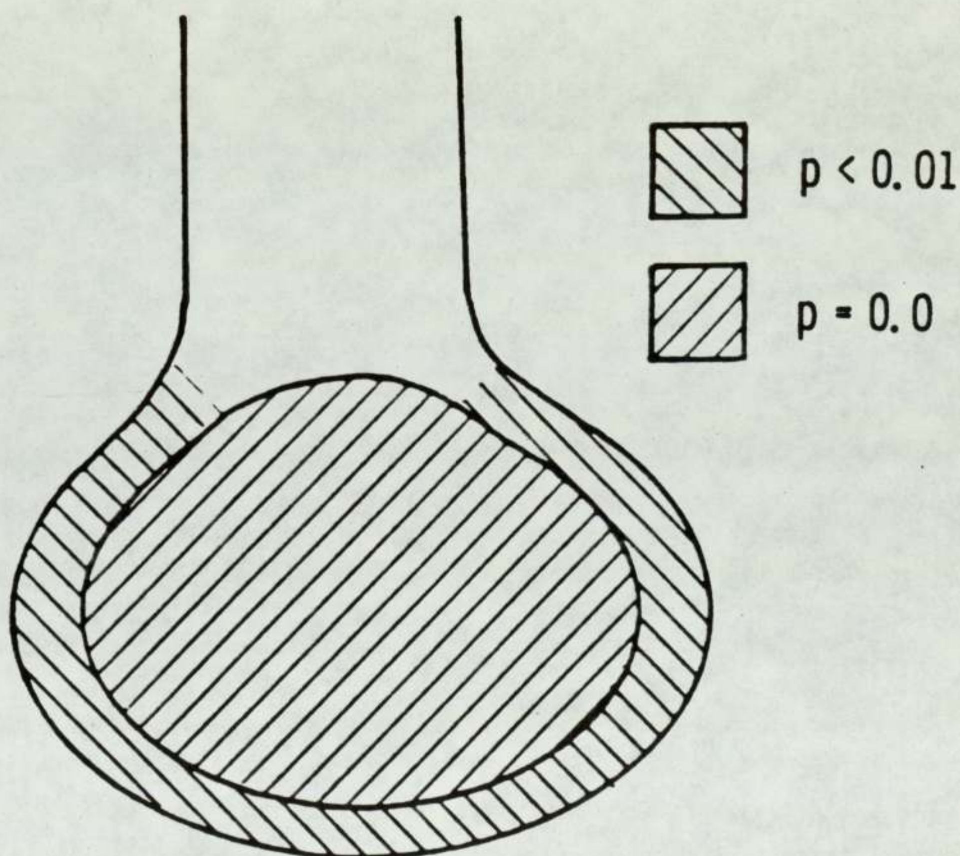
This argument may be further expanded: if this mean concentration corresponds to 5% of the total spontaneous release, then leakage must correspond to around 152 quanta/s. Values for A and B which facilitate this phenomenon are therefore of the order A = 0.0001 and B = 0.0001. These are included in the model as A_o and B_o and are only active during intervals between action potentials.

It is also evident from the many studies on this phenomenon that the release of quanta comprising the mepps is random and follows a Poisson distribution[7]. To incorporate a function within the model capable of producing a random output from the latent store (Q_L) is straight forward but refinements may be included.

It is known that as the stores become severely depleted the mepps decrease in both frequency and size. However, when the stores hold above average quantities of acetylcholine no increase in size or frequency of mepp[71] is noted.

The probability that any particular vesicle will be released by exocytosis is difficult to estimate, but if the distribution of vesicles within the nerve ending is considered to be uniform, it may be that vesicles closer to the membrane have a better chance of release than those situated near the 'core' of the nerve ending. Vesicles

close to the membrane may be assumed to have some probability of release whilst those at the centre, zero probability. The number of vesicles released is small when compared to vesicles available for release and in consequence this probability of release must be small (say $P \leqslant 0.01$). The situation may be more explicitly represented in the following figure.



It would be unrealistic to define a probability of release for individual vesicles such that only a mean probability is incorporated within the model.

Similar arguments may be applied when defining the

number of vesicles available for release. If as already mentioned the total quanta resident within the latent store is 180,000 only a small proportion of this figure (say < 2,000) will be sufficiently close to the presynaptic membrane to have any chance of release and this fact must be included in the model.

A transfer function capable of including all the above criteria is as shown in equation 64.

$$G11 = \overline{BN} \cdot \overline{BP} \left(1 - e^{\frac{1}{K_6(Q_{LZ} - Q_L)}}\right) \quad \text{--- } 64$$

where:-

$\overline{BN}$ = mean fraction of the number of vesicles with a probability of release.

$\overline{BP}$ = mean probability of release.

Q_{LZ} = number of vesicles normal in the latent store.

Q_L = number of vesicles in the latent store at time t.

K_6 = arbitrary constants.

The above transfer function (G11) is not in a form which is normally accepted. However, it may be considered as a constant value with input $\frac{Q_L}{S}$ during the short time the solution is considered to be valid.

The only factors the model does not contain which disable its function are those concerning synthesis and hydrolysis of acetylcholine within the nerve ending. These phenomena have been studied extensively but always in the steady state condition[177,178], as it is still technically impossible to study transient changes of

synthesis or hydrolysis; phenomena that can be swift in function[100].

Synthesis has been studied by various groups on a variety of animals. For example rat brain[179,180,181,182,183,184], ganglion of the cat[185,186,187], rat[188,189] and leech[190], guinea pig ileum[191,192,193], torpedo electric organ[179,194] and rat hemidiaphragm[71] are all organs studied in detail. The application of steady state kinetics has been applied to this data[177,178,194,195,196] and resultant mathematical interpretation carried out. The number of species used may well account for the variety of information available and it is unfortunate that so little information exists on man himself. Some studies, however, are particularly useful[71,186] and these can be used to supply data to the above model.

Treatment of the nerve ending with drugs such as eserine and neostigmine has shown esterases to be present[71,197,198] although the presence of acetylcholinesterase is still uncertain. With the esterase inhibitor eserine resident in a nerve terminal and the total store of acetylcholine is seen to double over a short time, indicating the nerve ending to be capable of holding more acetylcholine than normal and the hydrolysing action of the esterase to be the ultimate limiting factor on synthesis itself[71,197,198]. At rest, the rate of synthesis appears to balance losses of acetylcholine through spontaneous random release, leakage and hydrolysis. It is difficult to determine exact rates of hydrolysis from

the literature, but it would appear that the rate is dependent upon concentrations of acetylcholine held within the particular store. For example, it has been shown that vesicular acetylcholine is unaffected by hydrolysis[100] as the vesicle membrane provides an effective barrier against this action. Both latent and mobilisation stores contain more vesicular acetylcholine than free acetylcholine and consequently the rates of hydrolysis must therefore be smaller than that occurring within the cytoplasm of the nerve ending[71]. It follows that different rates of hydrolysis (for each store) will require varying degrees of definition. In consequence functions describing hydrolysis may be considered to be of the form shown in equations 65, 66 and 67.

$$H1 = \frac{(1 - e^{-(Q_n - Q_{nz})T_1})}{600} \qquad \text{--- 65}$$

$$H2 = \frac{(1 - e^{-(Q_m - Q_{mz})T_2})}{600} \qquad \text{--- 66}$$

$$H3 = \frac{(1 - e^{-(Q_L - Q_{LZ})T_3})}{600} \qquad \text{--- 67}$$

where:-

Q_n, Q_m, Q_L are states of the stores at time t.

Q_{nz}, Q_{mz}, Q_{LZ} are the normal values of the stores.

T_1, T_2, T_3 are arbitrary constants determining rates of hydrolysis.

For reasons similar to those given when justifying equation 64, transfer functions H1, H2 and H3 may be

considered constants with respective inputs of $\frac{Q_n}{S}$, $\frac{Q_m}{S}$ and $\frac{Q_L}{S}$ for the duration that the solution is considered valid. This technique simplifies model simulation, and is well suited to computer analysis. In consequence the model may be considered to adopt a configuration shown on the following page.

Functions representing synthesis are also likely to be similar in form. Differences will exist, however, as will delays on newly synthesised acetylcholine arriving in a particular store. These delays will vary for each store in keeping with the rates of transportation of synthesised acetylcholine, and the model may accommodate these differences by using techniques already described. It has been assumed that quantities of choline and acetyltransferase (the constituent parts of acetylcholine) are adequate and constant, such that total synthesis of acetylcholine is also constant[199] although proportioning and translocation of the transmitter amongst the stores are different and variable[71].

Although choline may be resident in the nerve through inherent hydrolysis there is growing evidence that the majority of choline used in synthesis is derived from the extracellular fluid[84,86,200,201].

Experiments have shown that a high proportion of newly synthesised acetylcholine is swiftly transported to the readily releasable store where it becomes immediately available for release[71]. This process is unlikely to occur in mobilisation and latent stores where acetylcholine molecules are housed in vesicles. In

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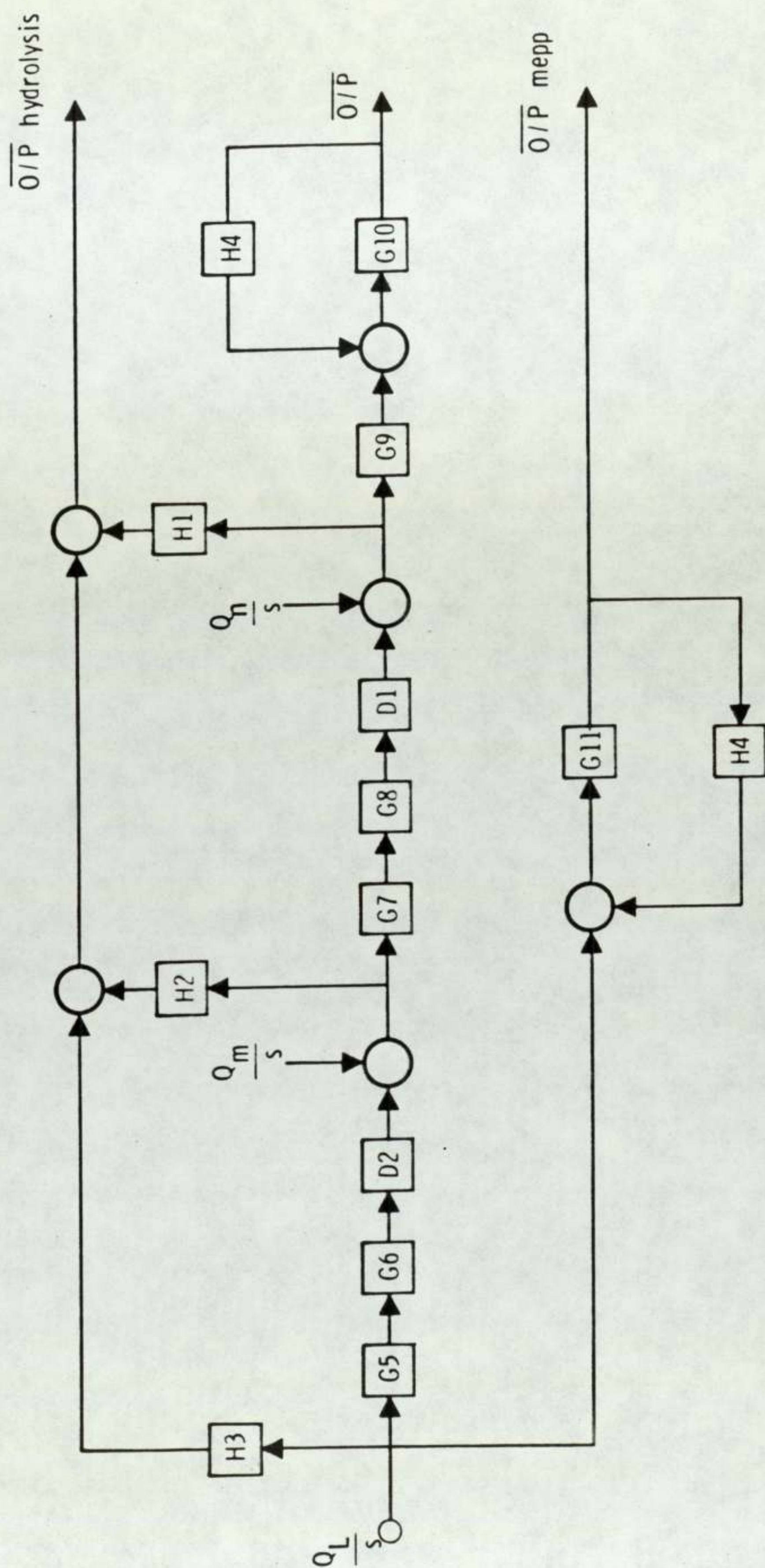


O/P hydrolysis
O/P
O/P mepp
H4
G10
G9
H1
Q_n/s
D1
G8
G7
H2
Q_m/s
D2
G6
G5
H3
Q_L/s
G11
H4

consequence, equations 68, 69 and 70 give functions in keeping with synthesis and equations 71, 72 and 73 create the respective delays in keeping with the appearance of newly synthesised acetylcholine within the stores.

$$G2 = R_L \cdot (1 - e^{-(2Q_{LZ} - Q_L)T_4}) \qquad \text{--- } 68$$

$$G3 = R_m \cdot (1 - e^{-(2Q_{mz} - Q_m)T_5}) \qquad \text{--- } 69$$

$$G4 = R_n \cdot (1 - e^{-(2Q_{nz} - Q_n)T_6}) \qquad \text{--- } 70$$

where:-

R_L, R_m, R_n = arbitrary constants affecting the proportioning of R_{syn}.

T_4, T_5, T_6 = arbitrary constants affecting the rate of appearance of acetylcholine within the stores.

Q_{Lz}, Q_{mz}, Q_{nz} = normal states of stores.

Q_L, Q_m, Q_n = state of stores at time t.

The above equations (i.e. 68-70) may be considered constants (over a very small time interval) with input R_{syn}/S; R_{syn} representing a mean value of synthesis. A preliminary value may be chosen such that R_{syn} is at least capable of replenishing twice the readily releasable store per second. This value is, however, limited by the ability of the store to accept the newly synthesised acetylcholine and the difficulties that arise in translocating the molecules. In consequence the parameters

R_n and T_6 need to be defined so that the above expression would be capable of supplying spontaneous leakage from channels in the nerve ending (i.e. about 150 quanta/s.). Tentative calculation reveals figures for R_n and T_6 to be 0.08 and 0.001 respectively.

$$D5 = K_7 + \frac{Q_{LEK}}{(2Q_{LZ} - Q_L)} \qquad \text{--- 71}$$

$$D4 = K_8 + \frac{Q_{mek}}{(2Q_{mz} - Q_m)} \qquad \text{--- 72}$$

$$D3 = K_9 + \frac{Q_{nek}}{(2Q_{nz} - Q_n)} \qquad \text{--- 73}$$

where:-

K_7, K_8, K_9 = minimum times required between synthesis and the appearance of newly synthesised acetylcholine in the stores.

$Q_{LEK}, Q_{mek}, Q_{nek}$ = arbitrary constants affecting times of appearance.

Twice normal values of the stores have been used, as experiments have predicted these to be the maximum amounts that may be stored in the nerve ending[71].

A similar procedure has been adopted here for inclusion of equations 68-73 as described earlier when dealing with equations 64-67. Equations 68-73 may be considered as constants over a very small time period and as such incorporated into the model as simple transfer

functions shown by the system configuration on the following page.

These values will, however, change (at each time increment) depending upon the state of the stores. It will be noticed that the system now also contains two functions (i.e. D6 and D7) that have not yet been described. These simply provide delays in the mechanisms responsible for transmitting the state of Q_n to Q_m, or of Q_m to Q_L (i.e. Q_{nn} and Q_{mm}). It seems realistic to suppose that the transmission of this information is not instantaneous but is buffered from small, transient changes; such a delay would provide this necessary function. These delays, although subject to the state of stores as shown in equations 74 and 75, may be regarded as constants during each time increment.

$$\text{Delay D7} = V_1 + \frac{V_2}{Q_{nz} - Q_n} \qquad \text{--- 74}$$

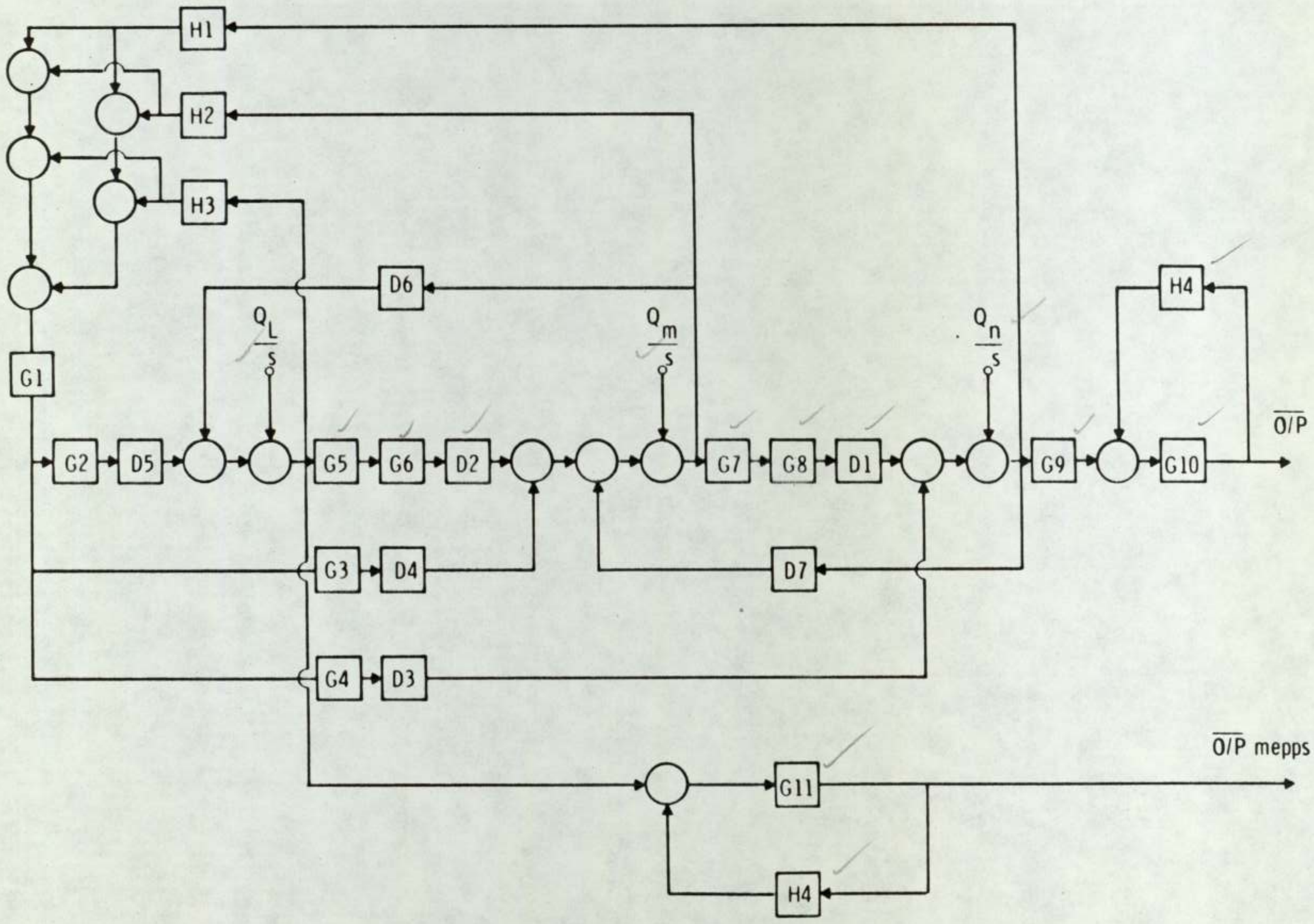
$$\text{Delay D6} = V_3 + \frac{V_4}{Q_{mz} - Q_m} \qquad \text{--- 75}$$

where:-

V_1 and V_3 correspond to minimum delay times due to the physical properties of the system, and

V_2 and V_4 are arbitrary constants.

This proposed model accommodates several of the shortcomings of present hypothesis and being built on data that has gained the largest concensus employs a wide factual basis in its philosophy. Movement of quanta


H1
H2
H3
G1
G2
D5
Q_L/s
D6
G5
G6
D2
G3
D4
G4
D3
Q_m/s
G7
G8
D1
D7
Q_n/s
G9
G10
H4
O/P
G11
H4
O/P mepps

within the model can be broken down for simplicity into a series of transfer functions representing bi-exponentials. Although this type of transfer function was generated by the use of an engineering approach it also seems to stand if considered from other viewpoints.

It seems intuitive that the transfer functions must reflect some form of decaying exponential term, as many natural and biological processes follow this form of mathematical argument (e.g. growth of yeast cultures, decay of radio isotopes, etc.). Assuming the processes involved are more complicated than the study of any one particular phenomenon it is possible that the movement of quanta may be represented by a series of exponentials as shown in equation 76.

$$O/P = \sum_{n=1}^{n=m} A_n e^{-a_n t} \qquad \text{--- } 76$$

where m is an integer defining the number of processes involved.

Rates of uptake and elimination of drugs (or body fluids) have long been a major preoccupation of the pharmacokineticist, who to predict effects, drug levels, etc. has developed various types of mathematical models (202). These models have already proved successful in many areas and when applied to this problem also yield a solution comprising a series of exponential terms.

The proposed model is by no means perfect but it does offer a means of quantifying certain parameters that have hitherto evaded description.

CHAPTER FOUR

RESULTS, VALIDATION AND REFINEMENT OF THE PROPOSED MODEL

Model philosophy was adapted and reproduced in Fortran IV for analysis on a Varian V76 Digital mini computer. The resultant computer programme attempted to balance the smallest discrete time interval that may be used to provide a good approximation to continuous functions generated by the philosophy against practicalities of job time and abilities of the computer. A programme flow chart is contained in Appendix I and provides an explanation of programme construction.

This chapter deals specifically with results provided by the computer after successful analyses of known input data, and provides a comparison to responses measured during physiological experiment. The analysis has taken approximately 1100 computer hours to complete and in consequence only major points requiring a change in model philosophy are considered.

The model was tested essentially for acetylcholine release during an evoked response. Leakage and spontaneous release were also considered, as were transmitter synthesis and hydrolysis. Various parameters and variables were changed to check effect and the model subjected to frequencies of stimulation corresponding to published results.

Of the many papers which have considered acetylcholine storage and release, most were qualitative assessments. Whilst these could not be ignored most of the data needed for an objective assessment was gained by reference to only

a handful of publications[9,12,71,125,127,152,203,204,205,215] and all subsequent references in this chapter to the literature are references to these papers. Response characteristics that the model seeks to mimic are as shown in figures 1, 2 and 3, which are located at the end of this chapter. Figure 1 gives the normal evoked response of human intercostal muscle whereas figures 2 and 3 give muscle responses when subjected to raised calcium and magnesium solutions. The first ten responses are shown by thin lines and represent averages of individual epps, whereas the following six responses are the pooled averages of five sequential epps.

TEST 1 - Initial observations and changes in the basic model

The model was submitted for computer analysis, with all initial parameters and variables corresponding to the numerical values listed in Table I overleaf, and used as shown by the flow chart of Appendix I.

The ultimate intention was to test the model over forty impulses at stimulating frequencies of 1.1, 5.0, 11.0, 20.0, 47.0 and 110.0 Hz. but during initial stages of development only short tests were considered necessary.

Results were encouraging but obvious anomalies were present. A higher output was experienced at a stimulating frequency of 5 Hz. than at 1.1 Hz. although relative stability was achieved. An increase in the frequency of stimulation (i.e. at 11.0 Hz. and 20.0 Hz.) produced a continually falling output as shown in figures 4, 5, 6 and 7 respectively. The solid lines of the graphs represent

the extent of the computer analysis, whereas the dotted lines are merely a probable extrapolation of system response.

TABLE I

BN	BP	α	γ	Q_{nz}
Ca	Mg	AN	T	ANUMB
A	B	FREQ	Q_{mz}	E_K
Z_1	Z_2	Y_1	Y_2	R_{syn}
F	G	Y_{L1}	Y_{L2}	Q_{LZ}
E_{KL}	Q_{nek}	Q_{mek}	Q_{LEK}	P
R_n	R_m	R_L	S_1	P_m
P_L	T_1	T_2	T_3	T_4
T_5	T_6	A_o	B_o	
V_1	V_2	V_3	V_4	

2000	0.01	100	10	1000
1.225	0.42	2.9	0.0015	10
0.5	0.5	1.1	2200	200
100000	200000	0.5	0.5	2000
10000000	20000000	0.5	0.5	180000
20000	30	4000	1000000	500000
0.8	0.09	1.2	800	100
1.0	0.001	0.001	0.0005	0.0001
0.001	0.00001	0.0001	0.0001	
0.5	200	60	100000	

With the exception of the first response all output characteristics were too large and the expected decline in magnitude at the higher frequencies of stimulation was not present. Reasons for these anomalies were found in the mode of operation used in the computer programme.

The action potential when applied to the model was divided into ten discrete steps over a period of 1.5 ms. The output was noted at each step and the effect on the system determined. The interval between action potentials was dealt with in a similar fashion but divided into 100 increments. The model assumed a steady leakage to occur over each time interval, through the channels postulated to exist at the nerve ending, and it was this phenomenon that caused the interchange of curves at low frequencies.

A stimulating frequency of 1.1 Hz. produced a steady output to the cleft with leakage just accommodated by a synthesis input to the model. An increase in frequency (to 5.0 Hz.) caused the interval between action potentials to decrease and in consequence less leakage to occur, enabling synthesis to better satisfy the demands of the readily releasable store (Q_n); the results being a higher output. Plateaus seen during the latter part of stimulation (at low frequencies) were due to an assumption in the computer programme that leakage ceased when the contents of Q_n fell below a predetermined limit. At stimulating frequencies of 11.0 and 20.0 Hz. a sudden cessation in leakage (as store contents were depleted beyond this figure) caused the magnitude of subsequent responses to be abnormally high and thus in conflict with

the literature.

The philosophy of the model was therefore redefined. None of the parameters of the output equation (i.e. equation 57) were considered likely to vary except perhaps K_3 which defined the number of channels available for quantal release at the nerve ending.

K_3 seemed unlikely to be a fixed value but probably dependent upon the concentration of acetylcholine contained in Q_n. Not all channels were likely to be functioning, or those that were, operating at full efficiency. In consequence K_3 was redefined such that:

$$K_3 = P_{max} \frac{Q_n}{Q_{nz}} \qquad \text{--- 77}$$

where:-

P_{max} = maximum number of channels available, and

K_3 = equivalent number of channels operating at full efficiency.

Leakage was now allowed to become a continuous process and was not contained by some limit on store size. It followed that parameters A_o and B_o (i.e. representing forces acting on the channels during the interval between action potentials) although small in value must always be greater than zero.

The programme, rerun with the above amendments, yielded a more satisfactory result as shown in figures 8, 9, 10 and 11. The relationship of the number of quanta released for each action potential was greatly improved although inconsistencies remained. A higher output was

still experienced at 5 Hz. than at a lower frequency. The solution lay in a balance between synthesis input and leakage output during the interval between action potentials. Confirmation was obtained by considering readily releasable store content and total output during stimulation. For example at 1.1 Hz. a total output of around 2,500 quanta had been produced after the third impulse compared with only 300 quanta at 5 Hz. after the same number of stimulations.

The new variable K_3 seemed to be a useful concept and was consolidated within the programme.

TEST 2 - To establish and refine synthesis to the readily releasable store

Equations used to define synthesis in the computer model are as shown below:

$$\text{Delay in synthesis} = 0.025 + \left(\frac{Q_{nek}}{(2Q_{nz} - Q_n)}\right) \quad \text{--- 78}$$

$$\text{Synthesis O/P } Q_n = R_{syn}.R_n.(1 - \exp(-(2Q_{nz} - Q_n).T_6)).TT \quad \text{--- 79}$$

Equation 79 defined the magnitude of synthesis over a discrete time increment (TT) and equation 78 an inherent delay in providing newly synthesised acetylcholine to Q_n. This latter equation shows a minimum system delay of about 25 ms. although essentially it is proportional to the concentration of acetylcholine within the store.

The effect of synthesis on the model was checked in

the first instance by increasing the fixed minimum time to 1 second, thus prohibiting an input from synthesis over the first few stimulii at stimulating frequencies in excess of 1.1 Hz. Results of this simple test indicated the time taken to relocate newly synthesised acetylcholine to have profound effects on the model. The test also showed a swift recovery in output response when the enforced delay had elapsed; leakage was also shown to be too high in these circumstances.

The fixed minimum delay was reduced to 0.25 s. and values for A_o and B_o reduced by a factor of 10, (i.e. to .00001) in an effort to control leakage. Results of this test showed recovery to be swift after the delay had elapsed but with increased effect due to a decrease in leakage. These events still occurred at the wrong time, however, confirming the initial value of 25 ms. to be of the right order.

Original values for A_o and B_o were reinstated (i.e. 0.0001) and the effects of changing the magnitude of synthesis were ascertained. A change in value for R_{syn} or R_n merely changed the magnitude of synthesis in a similar fashion for all stimulating frequencies. The literature, however, showed effects of synthesis to be quite different at various frequencies and this could only be simulated by a change in T_6. An alteration of T_6 from 0.0004 to 0.0005 gave useful results at frequencies above 11 Hz. but a poor comparison with results shown in the literature at lower stimulating frequencies. A further reduction to .0003 improved characteristics above 5 Hz.

but a poor response was still evident at 1.1 Hz.

A_o and B_o were decreased ten times as effects of synthesis had already been shown to deteriorate with high leakage at low frequencies of stimulation. Results of this test showed responses produced at 1.1 Hz. and 5 Hz. to be more representative (of the real situation) reaching similar values after the first few stimulii. A reduction in T_6 produced a further improvement in keeping with the type of characteristic seen in the literature. Leakage was still too high at very low stimulating frequencies and a figure of A_o and B_o was derived (i.e. 0.00005) after several programme runs, which gave a reasonable response.

With T_6 reduced to 0.0002 (to check effect) model response showed a further improvement, although the decline in magnitude of output response was unrepresentative at low frequencies. Synthesis also seemed to occur too quickly but an increase in the minimum delay time to 90 ms. caused a slight improvement with no significant alteration in shape of the characteristics.

A poor response was produced with smaller values of T_6 and further tests showed a figure of 0.00025 to give the best results (at least over the first few impulses) and in some respects similar to responses shown in the literature.

Care was taken throughout this series of tests to contain the influence of the mobilisation store although an input can be seen at a frequency of 1.1 Hz. in figures 12, 13, 14 and 15. It was, however, considered

impractical to continue testing the model under these conditions.

TEST 3 - To establish the effects of synthesis and the mobilisation store on overall model response

The previous test had proved useful in that initial values of parameters used in equations 78 and 79 had been produced. Although further refinement was necessary, this was considered best achieved with the mobilisation store producing an output in a realistic time.

The equations controlling times of transfer from mobilisation (Q_m) to readily releasable store (Q_n) are as shown below:

$$\text{Delay in initiating } Q_m \text{ O/P} = V_1 + \frac{V_2}{(Q_{nz} - Q_n)} \qquad \text{--- 80}$$

Delay in translocating acetylcholine from Q_m to Q_n

$$= 0.05 + \frac{E_K}{(Q_{nz} - Q_n)} \qquad \text{--- 81}$$

Both equations contained an inherent minimum delay (due to physical properties of the system) but the output of both was essentially dependent upon deviations of Q_n from its normal resting state.

Equation 80 determined the delay in transmitting need or requirement of Q_n to Q_m and equation 81 the time required to transfer molecules or quanta of the transmitter from Q_m.

An examination of previous response curves indicated a minimum fixed constant of 50 ms. for equation 81 and

parameter V_1 of equation 80 was also set to the same value. There was no reason to suppose the mechanisms responsible for the delay of equation 81 would be different to those of equation 80 and consequently values for V_2 and E_K were both set to 100, such that a Q_n complement of 900 quanta produced delays of around one second.

These changes caused the computer programme to produce characteristics with several obvious anomalies. An input from Q_m was shown to become effective at a later time to that shown in the literature. The number of quanta input to Q_n was also too high, causing an unrealistic change in output to occur at the cleft. The fall in output for subsequent impulses at all frequencies was too shallow and unrepresentative of data gathered during physiological experiment.

In an effort to produce a representative response the output from Q_n was increased. Firm conclusions had been reached in the literature concerning the quantity of acetylcholine in this store so the value of Q_{nz} was left unaltered although the maximum number of channels was increased slightly. Values for E_K and V_2 were also changed to a figure of 50 to reduce delays in presenting an output from Q_m to Q_n.

Results of these changes were encouraging as the initial value of the first emission was about 98 quanta in keeping a figure of 100 shown in the literature. Changes brought about by parameters E_K and V_2 had also been significant and the time taken to input quanta to Q_n was now seen to be approaching that experienced in the

literature.

The initial slope of the output response was still clearly in error at all frequencies, and attention was focused on the newly formed equation 77 which defined the maximum number of channels available for release to the cleft, at any time.

It was considered improbable that the number of channels could be defined by such a simple expression. The distribution of acetylcholine throughout the cytoplasm of the nerve ending was an unknown quantity, (although an assumption had been made that it was homogeneous) and it was perhaps too optimistic to expect an expression such as that of equation 77 to produce a correct response. Intuition leaned towards an exponential function but there was no valid reason to suppose a solution of this type. The following expression was therefore created as the next simplest approach to be tried in this trial and error situation.

$$K_3 = P_{max} \left(\frac{Q_n}{Q_{nz}}\right)^u \qquad \text{--- 82}$$

An increase in the power u was to cause an increase in slope and this possibility was tested before altering any parameters affecting inputs to Q_n.

With u set to a figure of 1.1, results of the computer simulation were encouraging, with the input from Q_m becoming effective after delays similar to those experienced in the literature. The output responses also showed the initial slope to increase with the frequency of stimulation.

The magnitude of input from the mobilisation store was also unrealistic. This input clearly had to be controlled in such a way as to produce only small amounts as Q_n approached its normal resting state, and larger quantities as the store was depleted.

A consideration of all characteristics suggested a value of 2.0 for u, and that the minimum fixed constant used in equation 81 be reduced to 25 ms. Results produced by rerunning the programme showed the above arguments to have substance with a significant improvement in characteristics at all stimulating frequencies. A comparison with the literature revealed a strong correlation of responses obtained at stimulating frequencies of 110 and 47 Hz. but deviations were present at 11 Hz. and below.

What had also become apparent was the need for a synthesis expression capable of producing a greater input to Q_n as the store content was reduced; in keeping with events expected to occur in reality. Equation 79 therefore had to be redefined.

Any change in R_{syn} or R_n would do little to facilitate this condition and over a fixed time increment the bracketed part of the expression would have to reflect the desired changes in synthesis output.

i.e.

$$(1 - \exp(-(2Q_{nz} - Q_n)T_6)$$

This part of the expression was already capable of producing the right type of effect; a readily releasable store of 900 quanta produced a value of 0.24, whereas a store content of 100 quanta showed a small increase to

0.378. Although synthesis increased as Q_n decreased, it less than doubled whilst Q_n was reduced to very small numbers; clearly this was undesirable in the model and unlikely to occur in reality. An escalation of this difference was needed and in an effort to accomplish this the bracketed term was changed to the following.

$$(1 - \exp(-(2Q_{nz} - Q_n)T_6))^Y$$

Changes in store size from 900 to 100 quanta with a Y value of 4 produced figures of 0.198 and 0.523. The output thus increased by 2½ times with a severe depletion of Q_n. With T_6 increased to 0.0011, the programme was rerun to check this effect.

A very disappointing result was produced which although showing a slight improvement fell far short of that required. Changes in the synthesis expression had now been exhausted and it was clear that the equation would have to be redefined, perhaps by a simpler expression of the form shown below.

$$\text{O/P Synthesis} \propto \left(\frac{Q_{nz}}{Q_n}\right)^x$$

Unfortunately such an expression did not provide any restriction on synthesis output and assumed some mechanism able to supply an ever increasing demand. It was unlikely that such a mechanism could exist and consequently this type of expression was rejected on the grounds that it was unable to reflect the physiological situation.

The exponential term used previously had provided such a restriction, but was unable to provide sufficient

definition between varying requirements of the readily releasable store. It seemed possible that this type of expression was still of use if sensitivity could be increased. This was accomplished by ammending the expression as shown below:

$$\left(1 - \exp\left(- \left(\frac{2Q_{nz} - Q_n}{Q_n}\right)^x\right)\right)$$

A preliminary assessment on the effects of this ammendment are shown in Table II; a value of 4 being assigned to x.

TABLE II

Q_n	$\left(1 - \exp\left(- \left(\frac{2Q_{nz} - Q_n}{Q_n}\right)^x\right)\right)$
900	0.238
800	0.36
700	0.52
600	0.65
500	0.81
400	0.92
300	0.984
200	0.999
100	"
50	"

The inclusion of this change in the computer simulation produced useful results, particularly at higher frequencies of stimulation. At 110 Hz. the response was of the right shape as shown in figures 16, 17, 18 and 19 and indicated by the literature. The lower frequencies of stimulation still produced a slightly higher output than desired but influence from the mobilisation store was still in need of correction.

TEST 4 - To establish a method of output from the mobilisation store and its interaction with a synthesis input at the readily releasable store

A similar argument was applied (as before) to define the number of pathways required to transfer quanta of acetylcholine from Q_m to Q_n. This definition was considered to be more complex than equation 82 as it must reflect the states of both stores. For example, it seemed reasonable to suppose a depletion of the mobilisation store would cause a decrease in the number of translocating pathways, and it seemed equally probable that as the readily releasable store was reduced the number of useful pathways would increase in proportion. In consequence a relationship was postulated relating both conflicting forces, as shown in equation 83.

$$P_m = P_{mmax} \left[\frac{Q_m \cdot (Q_{nz} - Q_{nn})}{Q_{mz} \cdot Q_{nz}} \right]^{\frac{1}{2}} \quad \text{--- 83}$$

It is, however, the prime purpose of the nerve ending to propagate an action potential by the secretion of acetylcholine to the cleft, and consequently the effect

produced by the readily releasable store was considered to be predominant; the square root term (in equation 83) provided this effect.

It was important when defining the manner of transfer pathways to ensure the effect could be adequately quantified. The computer model was altered so that no output was produced by Q_m, thus providing a datum for the comparison of subsequent responses (produced with an output). This procedure was carried out at all stimulating frequencies with results indicating an increase in power of the synthesis expression and the value assigned to R_n (i.e. from 4 to 5, and 0.8 to 1.0 respectively).

The programme was rerun with these changes and produced results similar to those found in the literature. Changes made in the synthesis expression produced a useful contribution but it was apparent that the input from the mobilisation store was still untimely and unavailable in sufficient quantity, although errors between published and model results were becoming smaller. The problem was most evident at a frequency of 47 Hz. where very little mobilisation effect was evident as shown in figures 20, 21, 22 and 23.

Several runs were carried out with changes to the synthesis expression, and eventually with A_o and B_o set to a figure of 0.00006, the index x increased to 8 with a corresponding increase in R_n to 1.5, a series of useful responses was produced. Little change was experienced at low frequencies of stimulation but major changes had

occurred at the higher frequencies, in particular 47 Hz. Leakage was still awry as a cross over of characteristics were evident at 1.1 and 5.0 Hz. as shown in figures 24, 25, 26 and 27. This cross over was due to too large an influx of acetylcholine from the mobilisation store; an effect that seemed to dominate the lower frequency characteristics.

An examination of the values for P_m provided an explanation. Table III shows values of the readily releasable store transmitted to the mobilisation store (i.e. Q_{nn}) and the subsequent number of pathways made available. A power of 0.5 had to be used in equation 83 and Table III shows the effect of increasing this index.

TABLE III

Q_{nn}	$P_m = P_{mmax}(\ )^{\frac{1}{2}}$	$P_m = P_{mmax}(\ )^1$	$P_m = P_{mmax}(\ )^2$
1000	0.0	0.0	0.0
900	$0.316.P_{mmax}$	$0.1.P_{mmax}$	$0.02.P_{mmax}$
800	0.44 . "	0.2. "	0.08. "
700	0.547. "	0.3. "	0.18. "
600	0.63 . "	0.4. "	0.32. "
500	0.707. "	0.5. "	0.5 . "
400	0.77 . "	0.6. "	0.98. "
300	0.84 . "	0.7. "	1.28. "
200	0.87 . "	0.8. "	1.62. "

A power of 0.5 caused the rate of increase in the number of transfer pathways to decrease as the contents of Q_n was depleted; a development in direct conflict to what was required. With the index raised to unity, however, the number of pathways increased significantly, now in direct proportion to the transmitted state of the readily releasable store. The situation was further improved by squaring the expression (i.e. a ratio of approx. 81 between high and low states of the readily releasable store).

The programme was rerun with this change in expression and with A_o and B_o assigned to a figure of 0.00005, and the number of available translocation pathways increased to 2,000, useful results were produced as shown in figures 28, 29, 30 and 31 respectively. The effect of the alterations was as predicted, with the characteristics of the right order of magnitude and the effects of the mobilisation store now in sensible proportion. A simulation with the power increased to 3 further improved the situation, although a problem still existed with the presentation of newly synthesised acetylcholine to Q_n at high frequencies of stimulation.

The form of the synthesis expression had been selected because some sort of limitation had to be imposed on the production of acetylcholine. As the effect of a mobilisation input could now be analysed, it was seen that the power (x) of the synthesis expression had to be of a high order to produce the required response at both ends of the stimulating frequency range. The

range of influence of an increasing x on just two values of Q_n is shown in Table IV.

TABLE IV

Q_n	x=4	$\frac{Q_{n500}}{Q_{n900}}$	x=8	$\frac{Q_{n500}}{Q_{n900}}$	x=10	$\frac{Q_{n500}}{Q_{n900}}$
900	0.248		0.06		0.031	
		3.29		11.0		19.6
500	0.815		0.66		0.6	

Q_n	x=12	$\frac{Q_{n500}}{Q_{n900}}$	x=14	$\frac{Q_{n500}}{Q_{n900}}$	x=16	$\frac{Q_{n500}}{Q_{n900}}$
900	0.015		0.00759		0.0036	
		35.6		64.7		121
500	0.54		0.489		0.436	

Several computer simulations were accomplished with various values of x and corresponding changes in R_n (i.e. to provide adequate compensation as the magnitude of the expression changed). With a power of 16, R_m increased to 20,000, R_n assigned to 8.5 and leakage adjusted (i.e. A_o and B_o set to 0.000025), a reasonable output was at last produced, as shown in figures 32, 33, 34 and 35 respectively.

The output characteristic at a stimulating frequency of 1.1 Hz. was about 11% higher than that shown in the literature and 12.5% at 11.0 Hz. Similar differences were experienced at higher stimulating frequencies with maximum

deviations at 47 Hz. and 110 Hz. being 9% and 11% respectively.

TEST 5 - To establish the effect of a newly synthesised acetylcholine input to the mobilisation store and subsequent changes in output characteristic

The arguments and expressions derived and executed in earlier tests had not considered an input of newly synthesised acetylcholine to the mobilisation store, and it was important at this stage that the model, should seek to simulate this facet of the physiology. The model now required Q_m to be considered not in a state of uninterrupted depletion but in continual receipt of small amounts of acetylcholine.

The basic philosophy had already created equations for such an input, and inherent delays in transferring newly synthesised quanta to this store (i.e. equations 84 and 85).

$$\text{Synthesis O/P}_{Q_m} = R_{syn}.R_m(1-\exp(-(2Q_{mz}-Q_m).T_5).TT \quad \text{--- 84}$$

$$\text{Delay in synthesis} = 0.1 + \frac{Q_{mek}}{(2Q_{mz} - Q_m)} \quad \text{--- 85}$$

For similar reasons outlined earlier equation 84 was refined such that synthesis to the mobilisation store became more sensitive to store content (equation 86).

$$\text{Synthesis O/P}_{Q_m} = R_{syn}.R_m(1-\exp(-(\frac{(2Q_{mz}-Q_m)}{(Q_m)}).T_5)).TT \quad \text{--- 86}$$

These changes were inserted into the computer programme which was rerun with a change of T_5 (to 0.25) over a range

of stimulating frequencies. A rise in output characteristic (quite prominent in the literature) was produced by an input from Q_m and although of too short a duration, the magnitude of oscillation was of the right proportion. Synthesis to the mobilisation store at 110 Hz. was too large resulting in the raised output response shown in figures 36, 37, 38 and 39.

To correct this anomaly several simulations were carried out with various changes to equation 83 with no satisfactory conclusion. Changes in index produced a variety of responses, two of which are considered in Table V overleaf, where the contents of Q_n are compared with the corresponding number of pathways between stores.

A power of 3 exerted little influence on the number of pathways until Q_n was depleted below 900 quanta. A reduction of this index to less than unity caused an opposite effect, with sensitivity decreasing with an increase in reciprocal. The use of a reciprocal caused too great an effect if only small reductions were made in the content of Q_n and therefore seemed unlikely to be of use. With indices greater than unity also providing unrealistic solutions at low levels of store content, it seemed the ideal index must change from a figure greater than unity to a reciprocal, with a depletion of store content below 900 quanta; a procedure that was difficult to justify.

A possibility was considered, however, that acetylcholine flux (along these pathways) was not a continuous process, but was in fact only input to Q_n if the contents were

reduced below a particular value (of say 900), or if sufficient acetylcholine was resident in the pathways to force an entry. This proposition suggested the output from the mobilisation store only became apparent if synthesis failed to meet the demands of Q_n.

TABLE V

Q_n	$P_m = P_{mmax}\ (\ )^3$	$P_m = P_{mmax}\ (\ )^{\frac{1}{4}}$
1000	0	0
900	0.026	19
980	0.212	22.67
970	0.739	25.16
960	1.808	27.1
950	3.64	28.7
940	6.5	30.15
930	10.66	31.4
920	16.43	32.58
910	24.18	33.65
900	34.3	34.6
.	.	.
.	.	.
800	390.6	42.4
.	.	.
.	.	.
500	25000	60

To test this hypothesis a statement was programmed which inhibited an input to Q_n (from Q_m) unless the content of the store decreased beyond a value of 900 quanta,

after which the resulting flux was regulated by the expression shown in equation 87.

$$P_m = P_{mmax} \cdot \frac{Q_m}{Q_{mz}} \cdot \left(\frac{Q_{nz} - Q_{nn}}{Q_{nz}}\right)^Z \qquad \text{--- 87}$$

where $Z = 0.25$.

The result produced similar responses to those seen in the literature. The distinctive 'bumps' seen on earlier output characteristics had disappeared to be replaced by more realistic undulations. The output at low stimulating frequencies was however too high with too little input from the mobilisation store at higher frequencies. The index of equation 87 was changed to 0.5, the number of available pathways increased to around 800 and the programme rerun.

The result of this simulation was to produce a series of responses quite comparable to those known to occur during physiological experiment. A maximum deviation (from the literature) of any point produced by the model during 40 impulses over the whole six stimulating frequencies was about 17% with the majority of points well within this figure as shown by figures 40, 41, 42 and 43.

Although the model was now able to achieve realistic responses it was still possible to continue the refining process but this seemed a waste of resource when errors in the experimental data were considered (i.e. $\geqslant \pm 10\%$). Instead attention was given to broadening the capability of the model.

TEST 6 - To establish the effects of Ca^{++} and Mg^{++} on the computer model

All previous tests had incorporated a feedback expression of the form shown by equation 88.

$$H4 = K_2 \left(AN - \frac{Ca}{Mg}\right) \qquad --- 88$$

The expression produced zero feedback when normal values of calcium and magnesium were considered, and hence had no effect on the overall output characteristics.

As mentioned earlier a multiplier K_2 might exist which was in all probability much greater than unity. Provisional calculations showed a value to be 6500 and rerunning the programme after this adjustment produced encouraging responses for 2 Ca^{++} levels. All responses were of reasonable shape but of too great a magnitude for most of the simulated characteristics. This extra acetylcholine must have been developed somewhere and it seemed that synthesis now provided too large a contribution under these changed circumstances. It also became apparent that leakage was also too large and subsequent runs with decreased synthesis and leakage confirmed this analysis. A permanent incorporation of these changes to the programme although capable of producing reasonable output responses for 2 Ca^{++} would have rendered the model totally ineffective under normal physiological conditions. Clearly refinements were needed to accommodate changes in calcium and magnesium, yet retaining the ability to produce a normal response.

In consequence both leakage and synthesis expressions were radically altered to fulfil these criteria. Several successful normal runs had produced a figure of .000025 for both A_o and B_o which gave a useful contribution to the output response. This figure needed to change with increased calcium in the extracellular fluid, and an expression of the type shown in equation 89, was proposed as a means of providing such a solution.

$$A_o = B_o = J_o \left(\frac{Ca}{Mg}\right)^{K_o} \qquad \text{--- 89}$$

where:-

J_o = 1.724×10^{-6}, and

K_o = 2.5.

Preliminary calculations indicated normal levels of calcium would produce a value of .000025 for A_o and B_o which changed to 0.00014 with 2 Ca^{++} and decreased to small proportions with a similar increase in Mg^{++}. This idea seemed in keeping with electrophysiological evidence which pointed to an increase in spontaneous activity and leakage, with high calcium levels, and a decrease with similar levels of magnesium.

Changes in synthesis due to deviations in levels of these chemicals (from normal) were also accommodated in a similar manner by a change in the index (x) of the synthesis expression of equation 90.

$$\text{Synthesis } O/P_{Q_n} = R_{syn} . R_n . \left(1 - \exp\left(-\left(\frac{2Q_{nz} - Q_n}{Q_n}\right)\right)\right)^{x} . TT \qquad \text{--- 90}$$

Several computer simulations had indicated the index x

was to be of a value approaching 16 under normal conditions but should be reduced significantly (to about 8) with 2 Ca^{++}. x was defined as shown by equation 91, and it was interesting to note parameters that had hitherto been considered constants now becoming variables.

$$x = 47.0 \frac{Mg}{Ca} \qquad \text{--- 91}$$

A change in x also created a requirement to change R_n, and just as the index had become a variable so had this parameter. Changes here were to be more complex than the two previous expressions; a decrease in the value of R_n was needed with an increase in either magnesium or calcium. Normal computer simulations had produced a value for R_n of 5 which had to be significantly reduced with a two fold increase in calcium (or magnesium). To produce this effect an expression (equation 92) was created.

$$R_n = 5.0\, e^{-\left|\frac{Ca}{Mg} - AN\right|} \qquad \text{--- 92}$$

Normal values of calcium and magnesium produced a value of 5 for R_n but a two fold increase in calcium reduced this figure to 0.27 and with a similar increase in magnesium to 1.16.

Rerunning the programme produced encouraging results, but was still unrepresentative of experimental data. A correct response was obtained at 1.1 Hz. but higher frequencies produced too small an output. Values derived for R_n and x were considered excessive and in need of further refinement.

The fall in x (from 16 to 8) following a two fold

increase in calcium had been too dramatic and equation 91 was rearranged (equation 93) so as to produce a figure of around 11.

$$x = 27.3 \cdot \sqrt{\frac{Mg}{Ca}} \qquad \text{--- 93}$$

R_n was also redefined in a manner shown in equation 94 producing values of 1.16 and 2.4 for two fold increases in calcium and magnesium.

$$R_n = 5.0 \cdot e^{-\frac{1}{2}\left|\frac{Ca}{Mg} - AN\right|} \qquad \text{--- 94}$$

The computer programme was rerun with revised expressions and 2 Ca^{++}. Responses produced were in keeping with the literature, the worst deviation at a frequency of 1.1 Hz. being less than 5%, at 11 Hz. less than 9%, and over the whole simulation of six frequencies was less than 20% as shown by figures 44, 45, 46 and 47 respectively.

The programme was again submitted but with normal calcium levels and a four fold increase in magnesium. Results produced at stimulating frequencies up to 11 Hz. were similar to responses produced by physiological experiment, although large discrepancies existed between data at frequencies of 47 and 110 Hz. All characteristics were however of diminished magnitude and this was considered due to the large negative feedback component produced by the expression of equation 88. It was proposed that this expression was more complex, and provisional calculations revealed that a change of the form shown in equation 95 would be of use.

$$H4 = K_2 \left(AN - \frac{Ca}{Mg}\right)^3 \quad --- 95$$

where $K_2 = 764$.

The changed programme was rerun with 4 Mg^{++} and produced comparable results with the experimental evidence. The characteristic produced by a stimulating frequency of 1.1 Hz. deviated by 7% to the experimental results at the worst point. Published results at this frequency show a variety of peaks which also appeared in the computer simulation. The characteristic produced at 11 Hz. deviated by less than 2.5% from the experimental evidence although anomalies still existed in characteristics produced in excess of this frequency as shown in figures 48, 49, 50 and 51.

This change in feedback expression had not significantly affected the systems ability to produce other characteristics as shown in figures 52, 53, 54 and 55 for 2 Ca^{++} and figures 56, 57, 58 and 59 for a normal response; all final values given to parameters and variables being as shown in Table VI overleaf.

Figures 60, 61, 62 and 63 and figures 64, 65, 66 and 67 are included to show model responses with 1.5 Ca^{++} and 2 Mg^{++} respectively, although there is no direct evidence with which to confirm these latter responses. These interpolations do, however, seem relative to known characteristics and add to the general picture of neuromuscular function that the model seeks to provide. The percentage deviations of model response from experimental data in normal, raised calcium, and raised

magnesium solutions was now considered acceptable and is shown by figures 68, 69 and 70.

TABLE VI

BN	BP	α	γ	Q_{nz}
Ca	Mg	AN	T	A
B	Q_{mz}	E_K	Z_1	Z_2
C	D	R_{syn}	E	F
Q_{LZ}	E_{KL}	Q_{nek}	Q_{mek}	Q_{LEK}
K_{3max}	R_n	R_m	R_L	P_{mmax}
P_L	T_1	T_2	T_3	T_4
T_5	T_6	J_o	K_o	K_2
V_1	V_2	V_3	V_4	K_7
K_8	K_9			

2000	0.01	100	10	1000
1.225	0.42	2.9	0.0015	0.5
0.5	2200	20	100000	200000
0.5	0.5	2000	0.5	0.5
180000	20000	30	2000	1000000
550000	5	0.09	1.2	810
1.0	0.001	0.001	1.0	0.25
0.001	0.00001	0.000001724	2.5	764
0.05	20	60	100000	1.0
0.1	0.01			

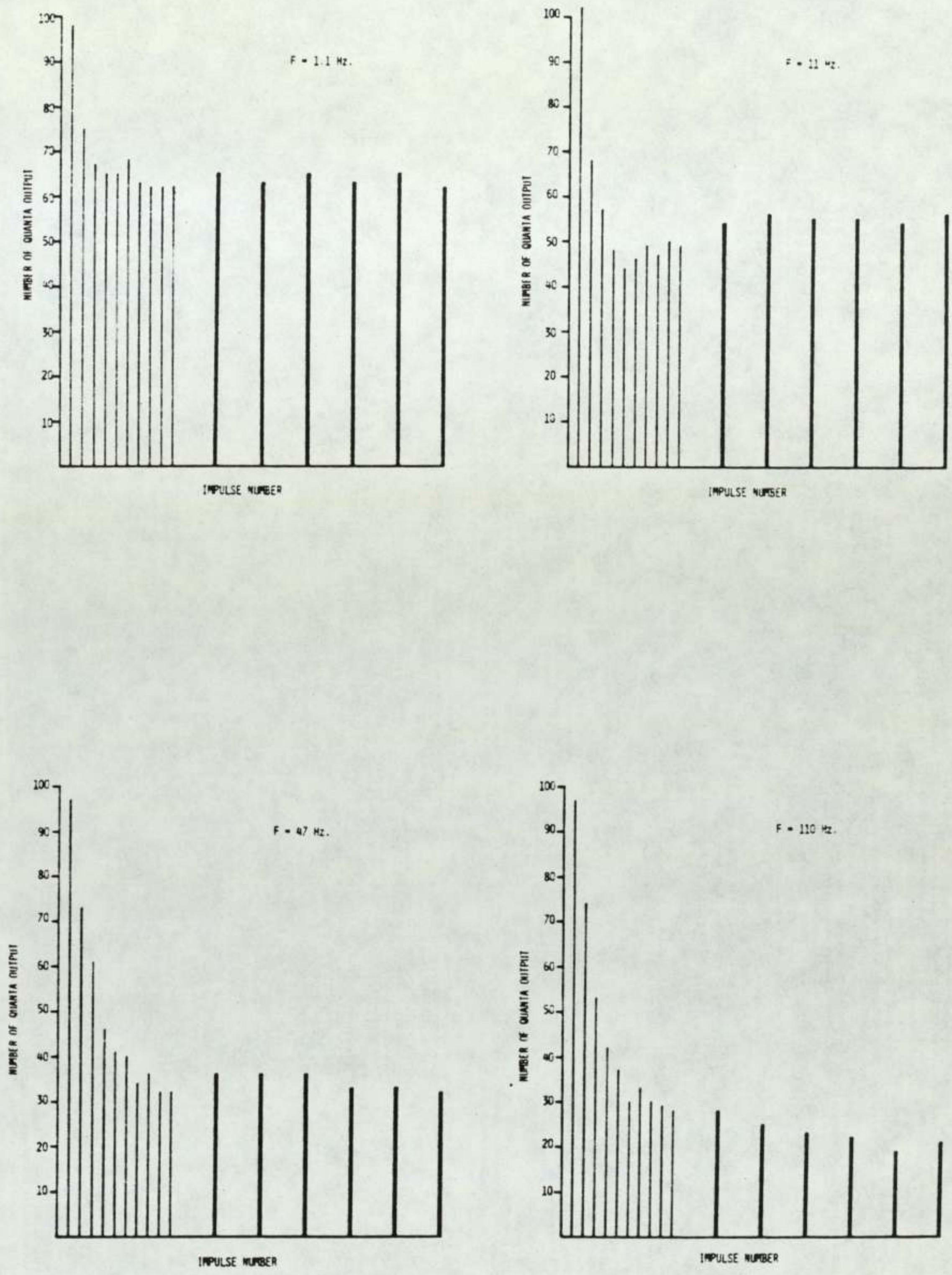
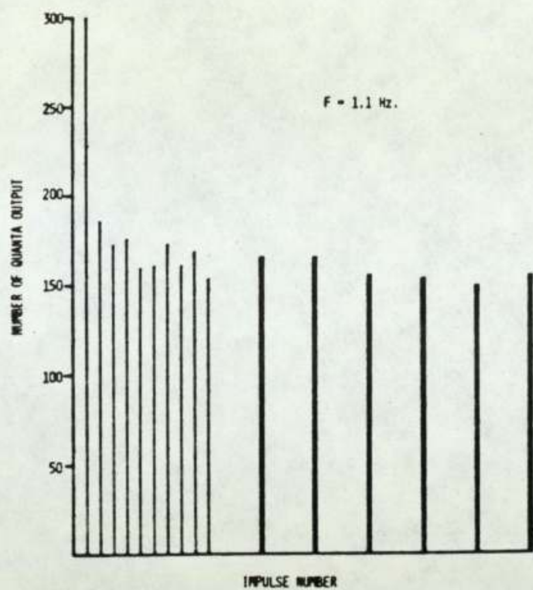
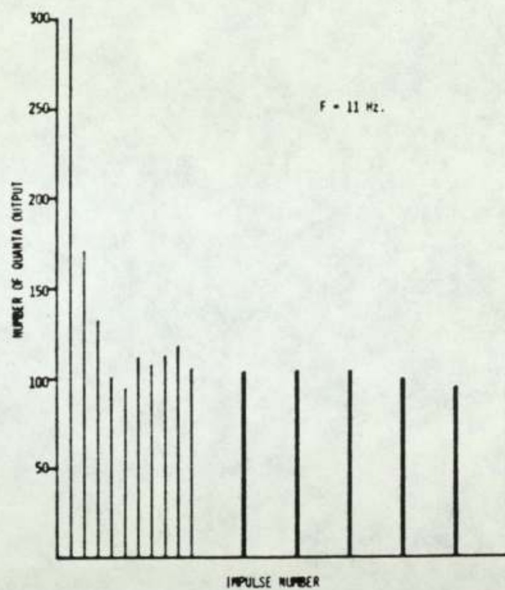
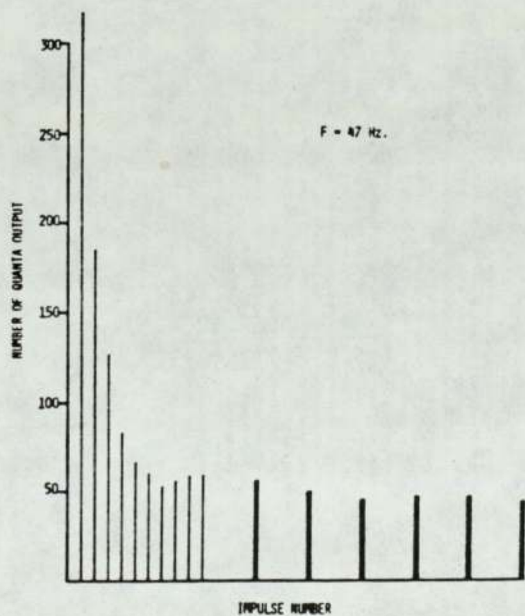


Fig. 1

Reproduced from the work by Elmqvist and Quastel[12]







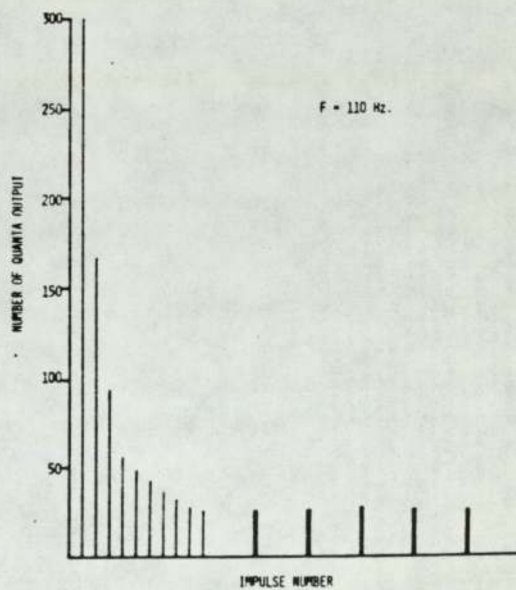


Fig. 2

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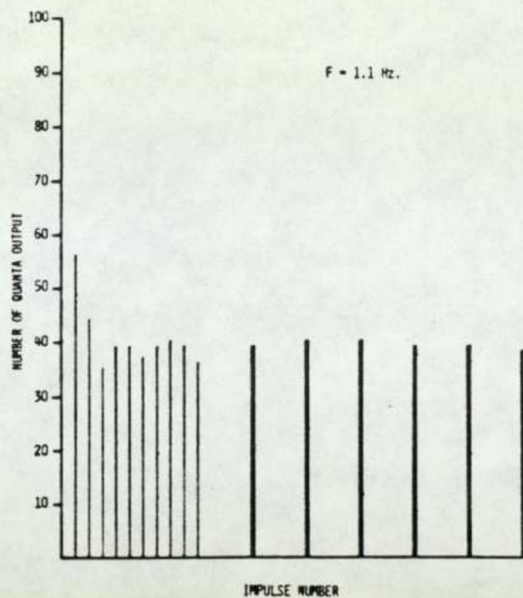
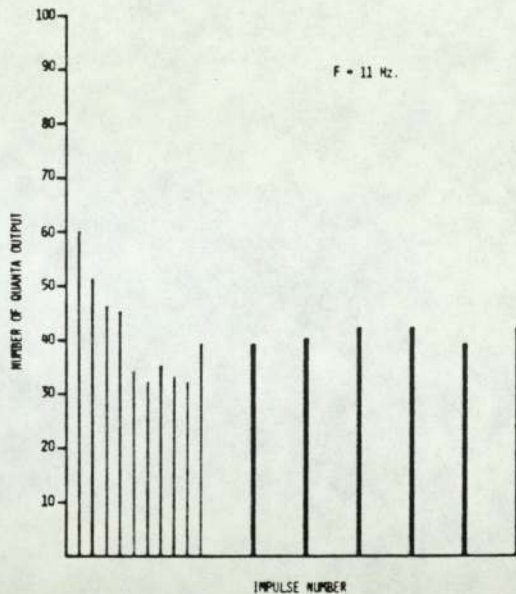
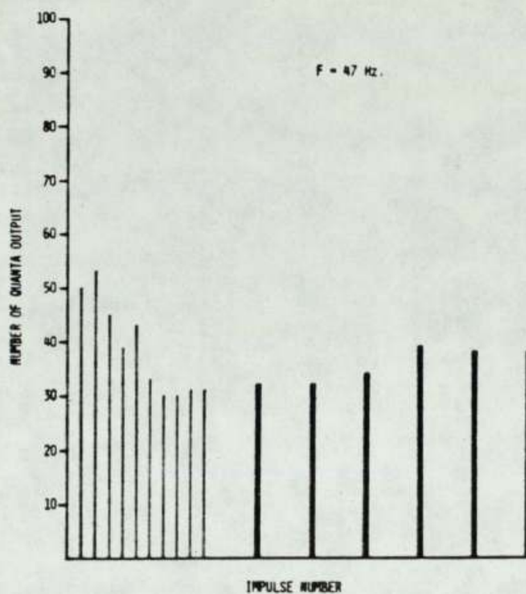






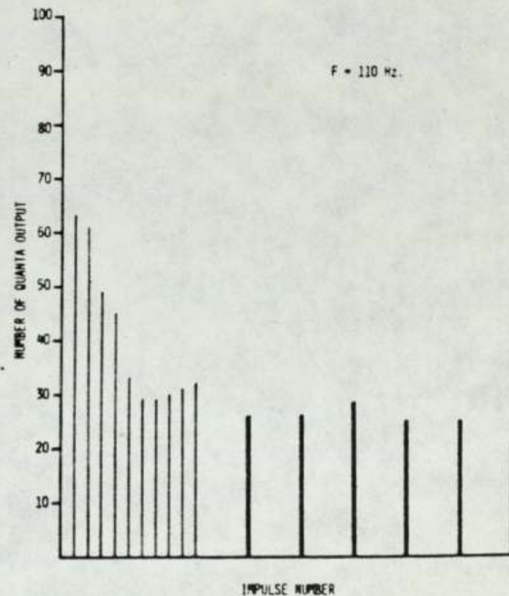


Fig. 3

Reproduced from the work by Elmqvist and Quastel[12]

Fig. 4

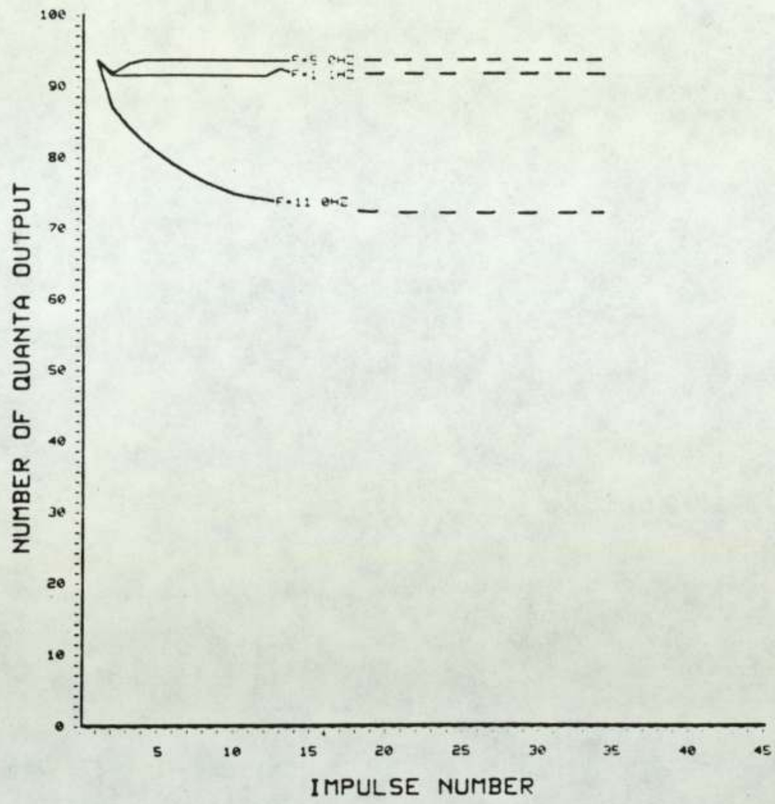


Fig. 5

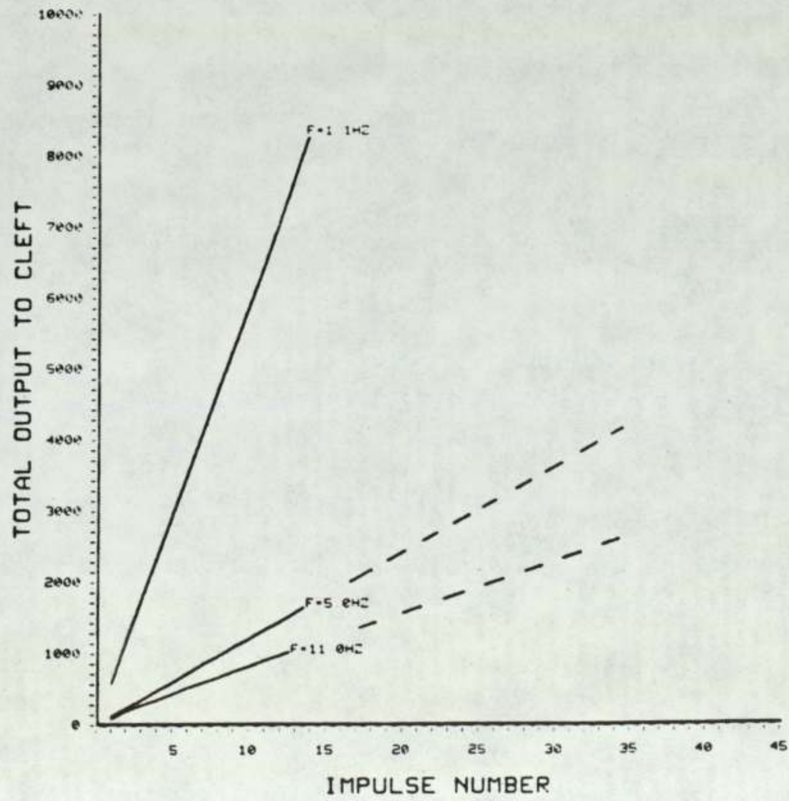
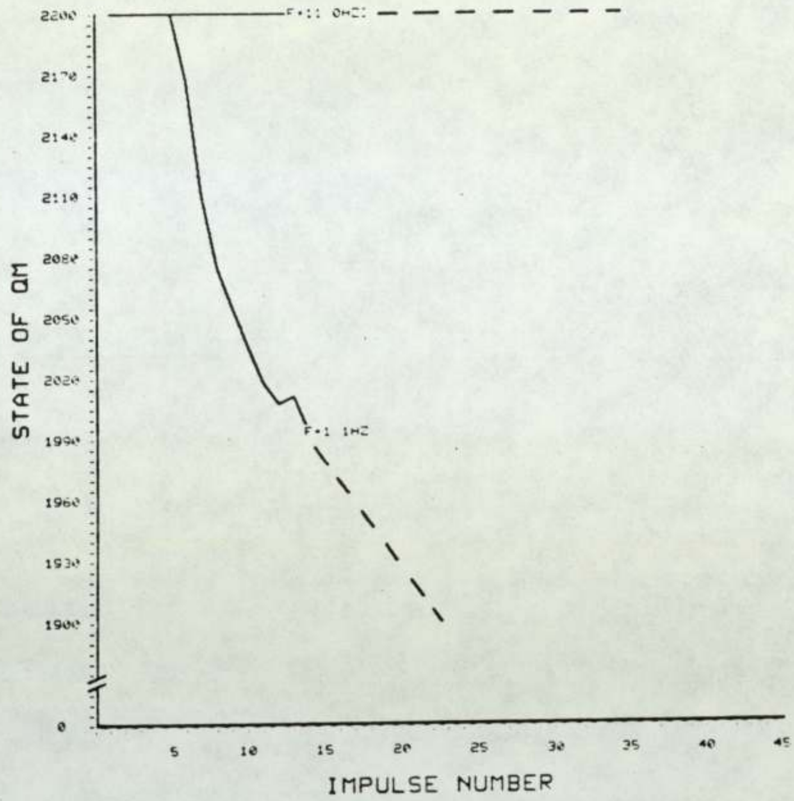


Fig. 6



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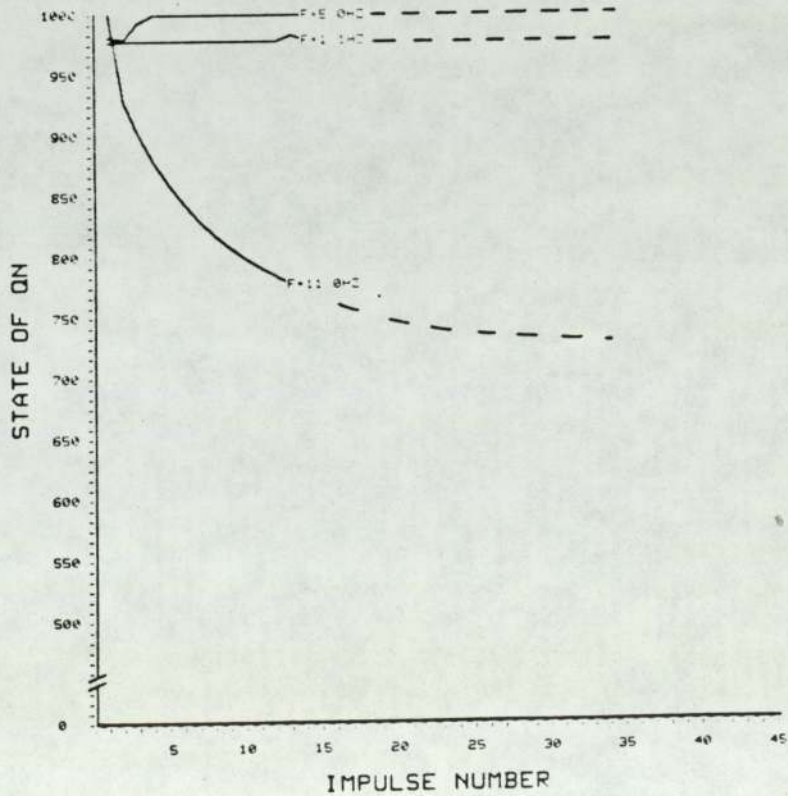

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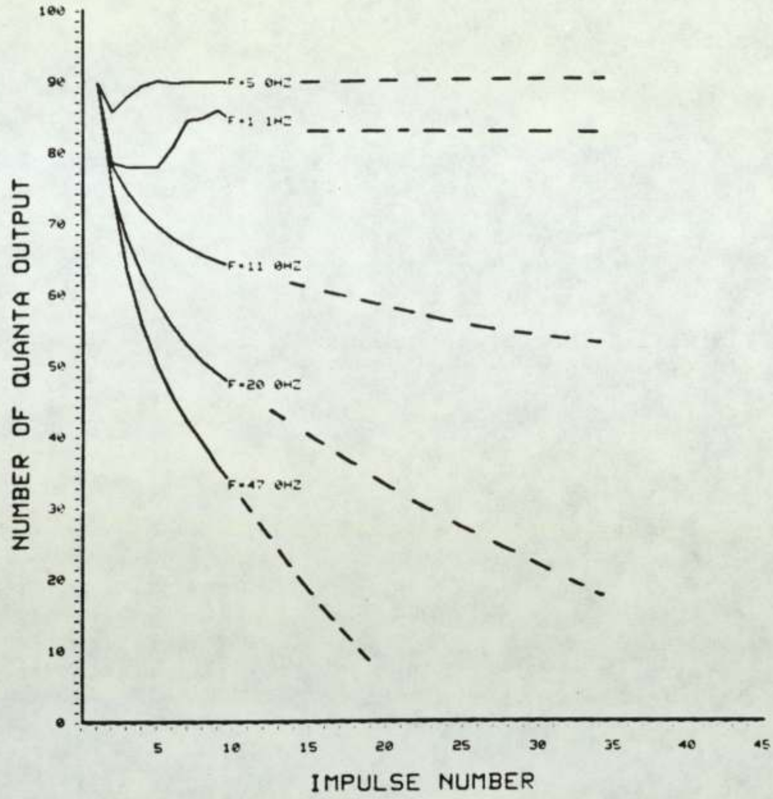

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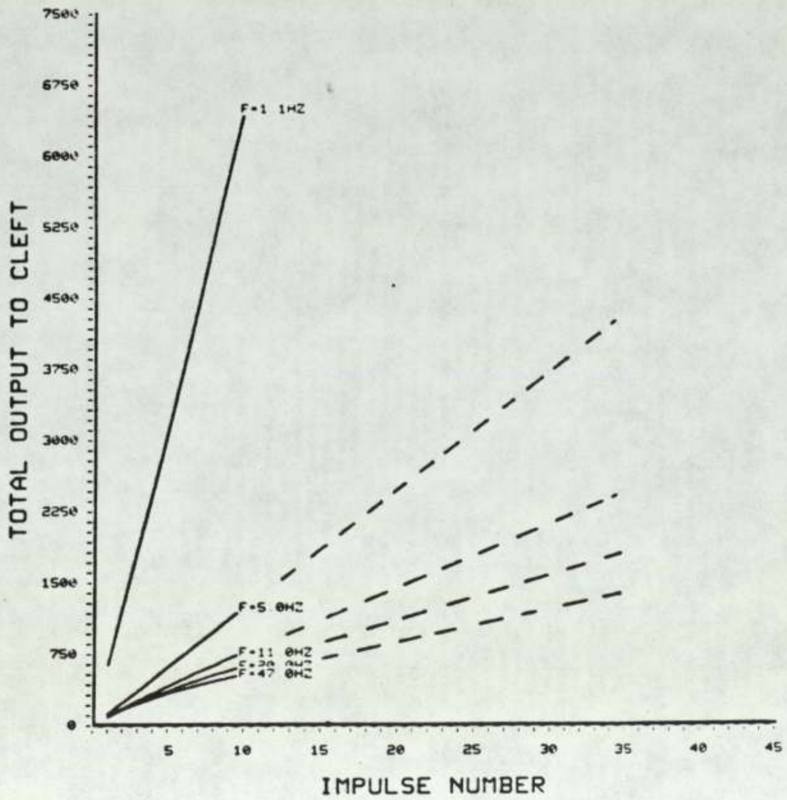

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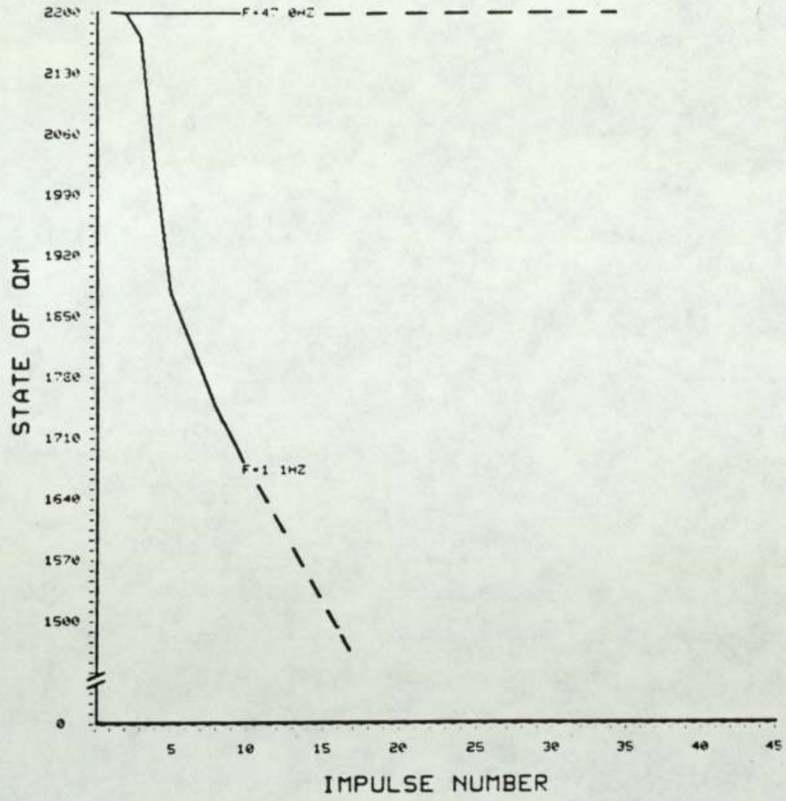

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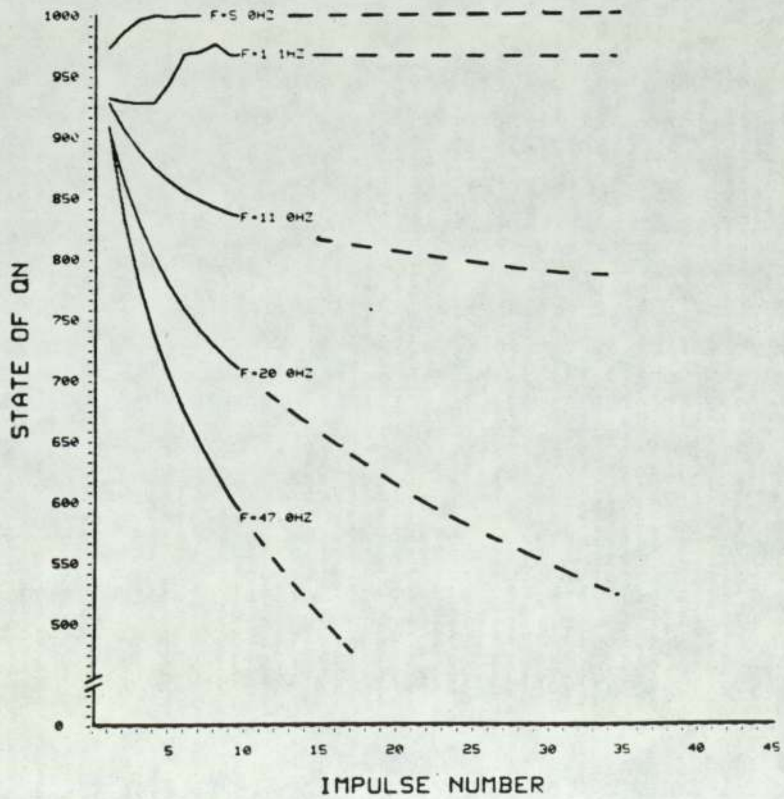

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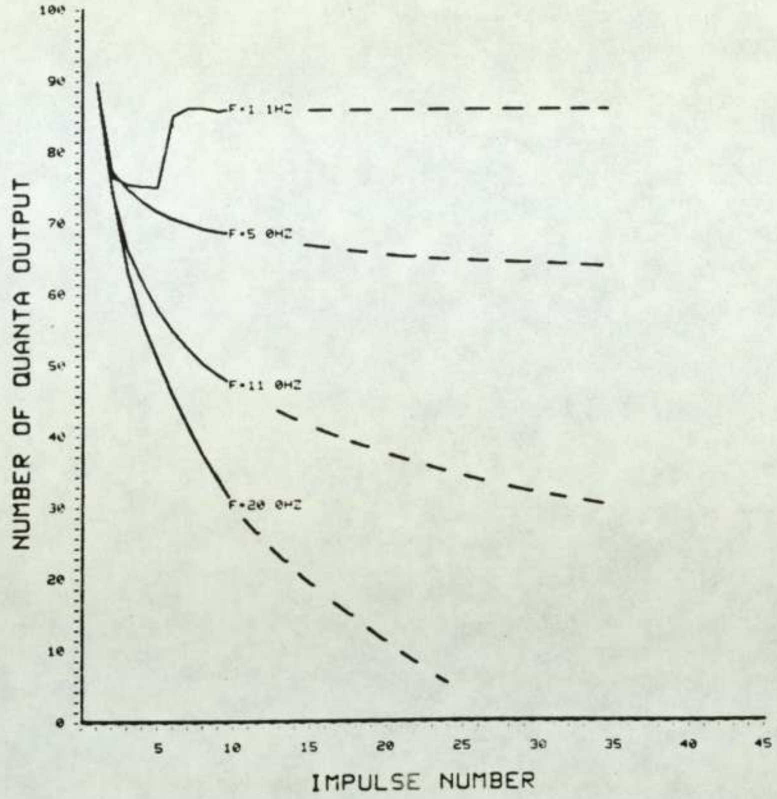

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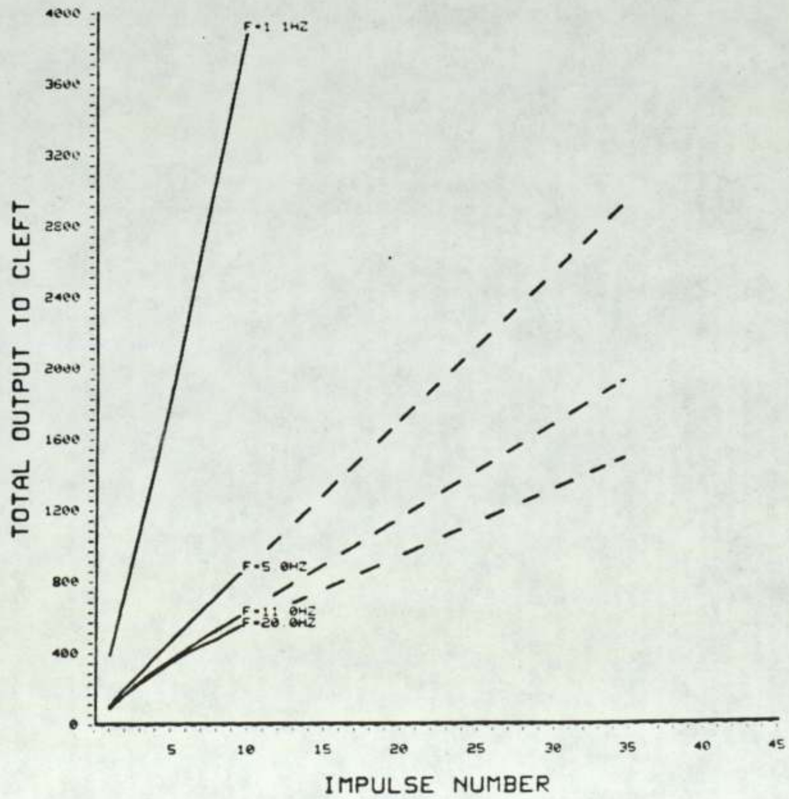

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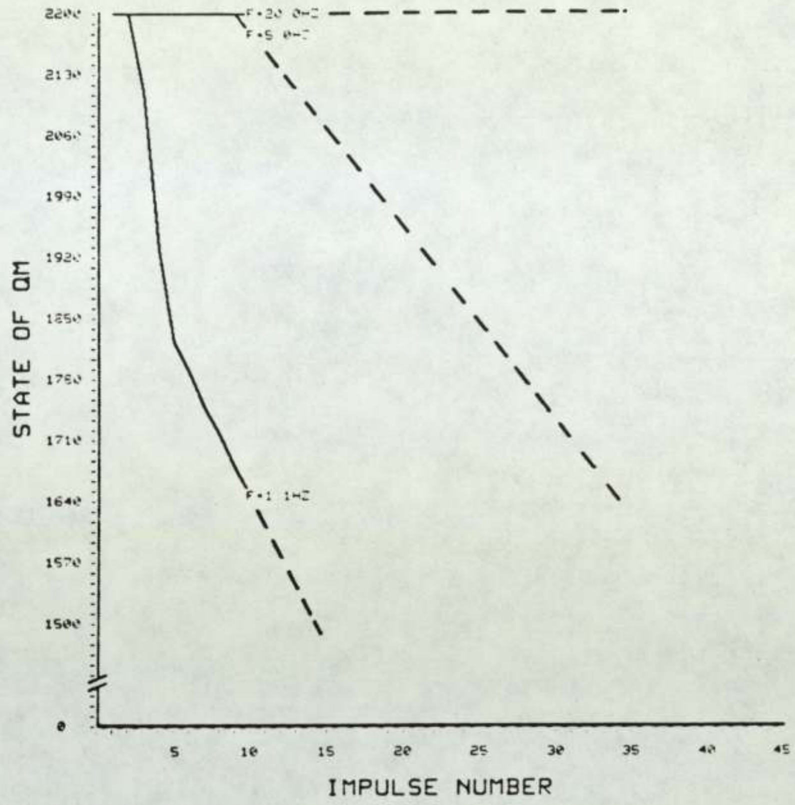

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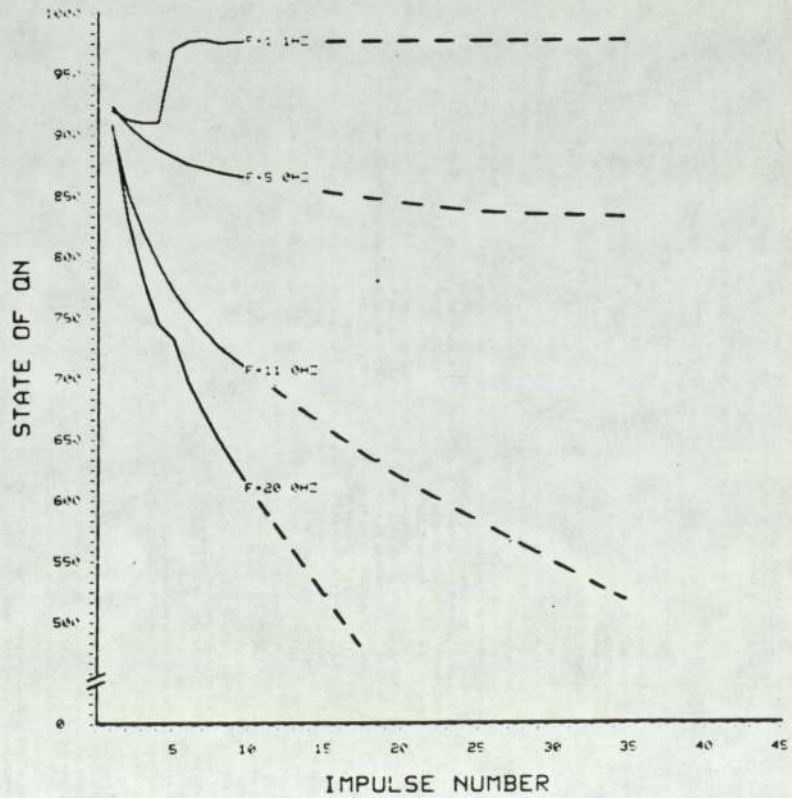

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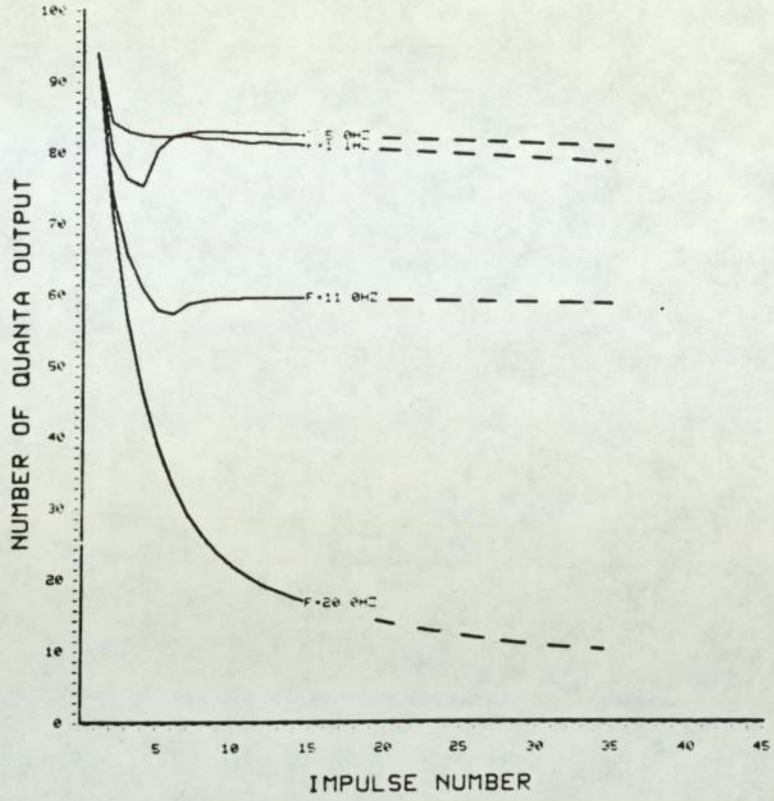

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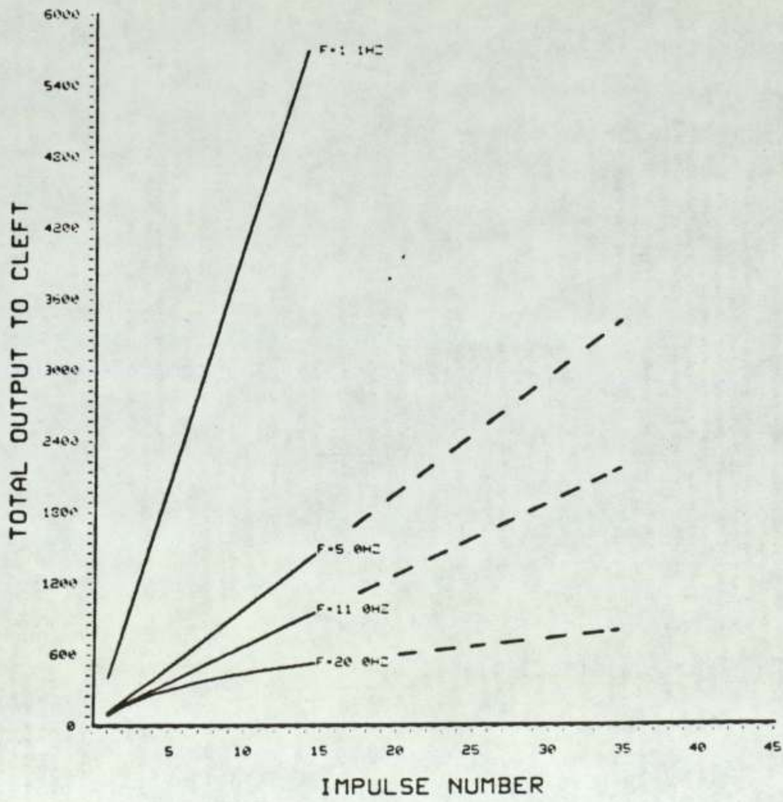

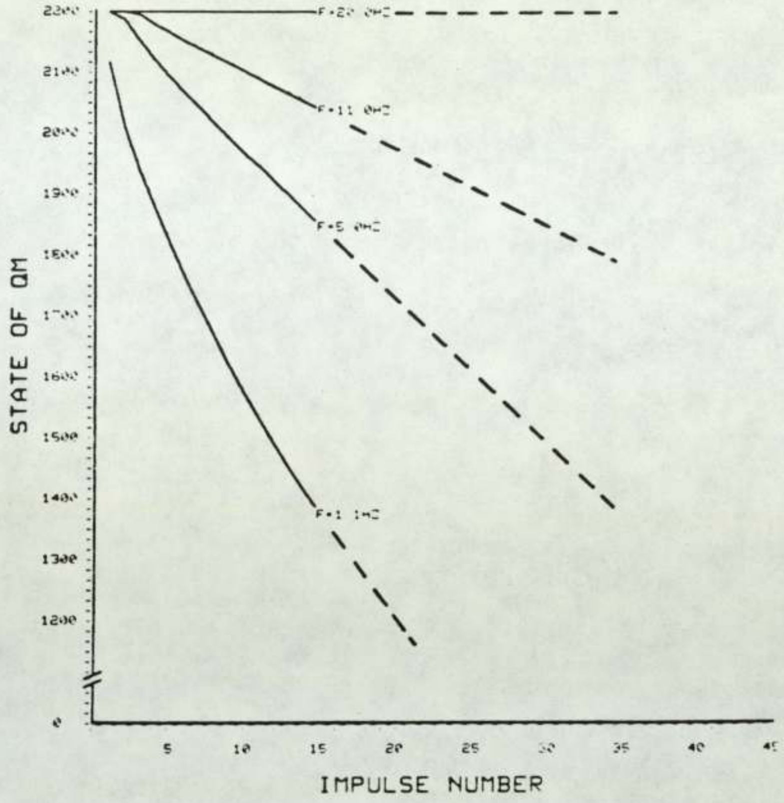

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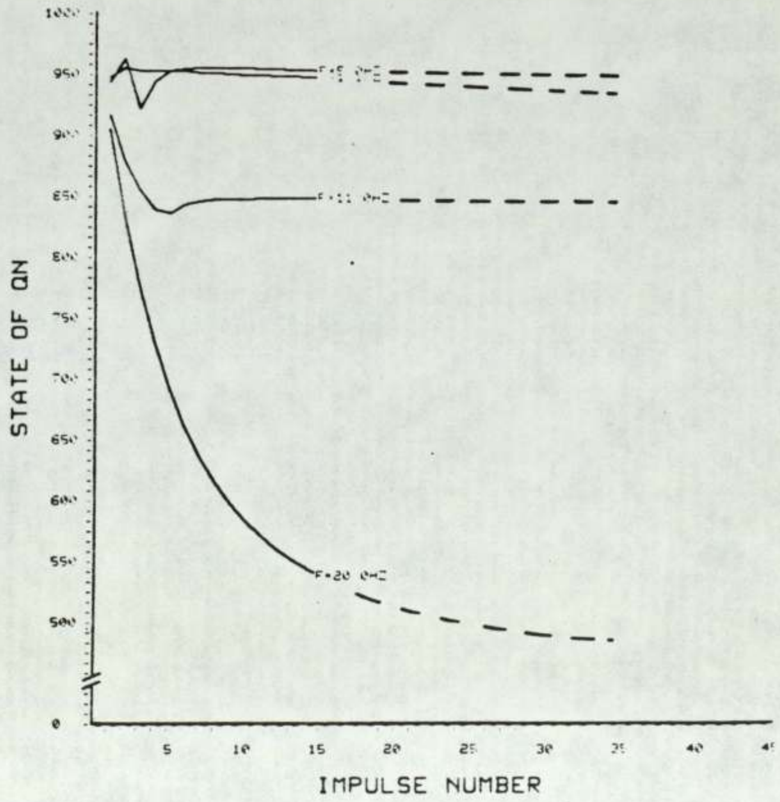
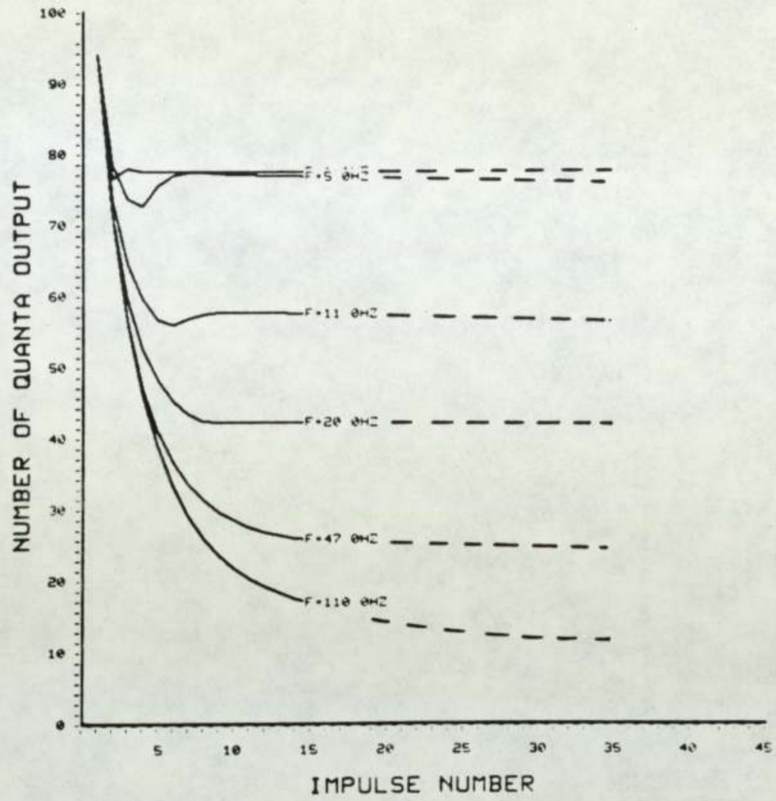


Fig. 19

Fig. 20



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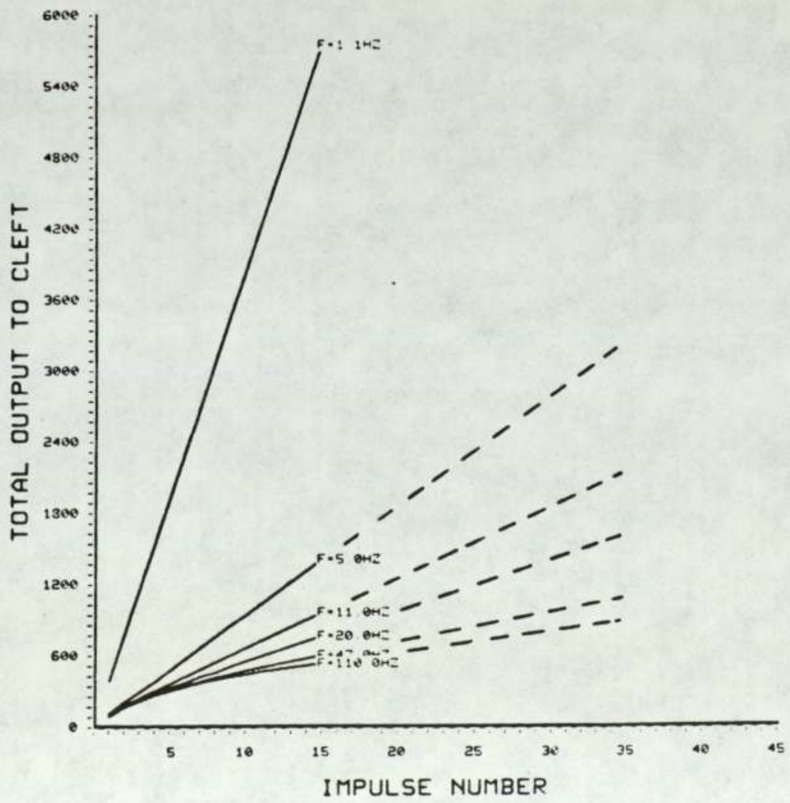

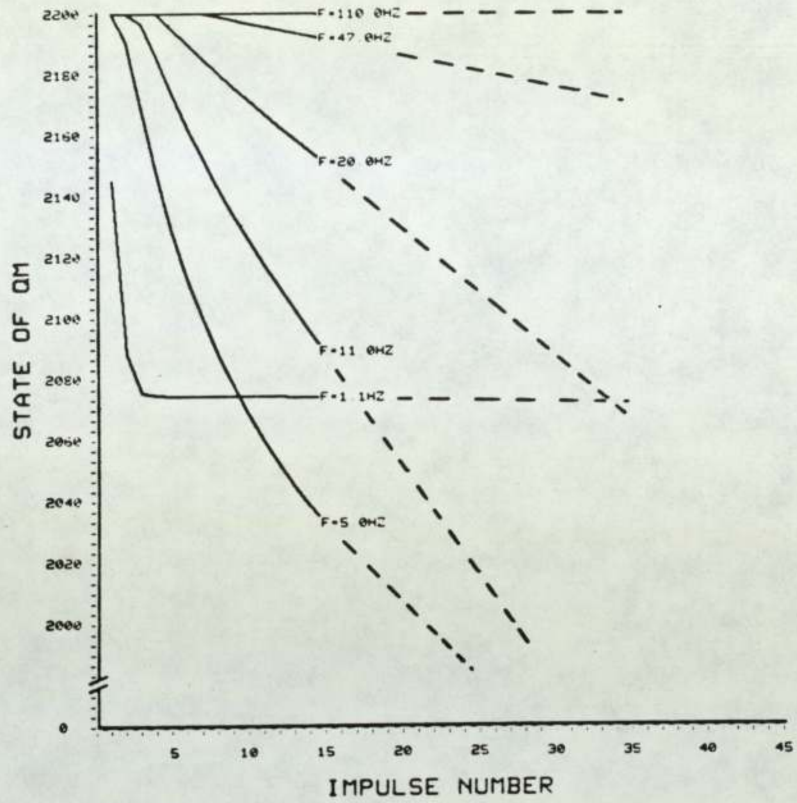

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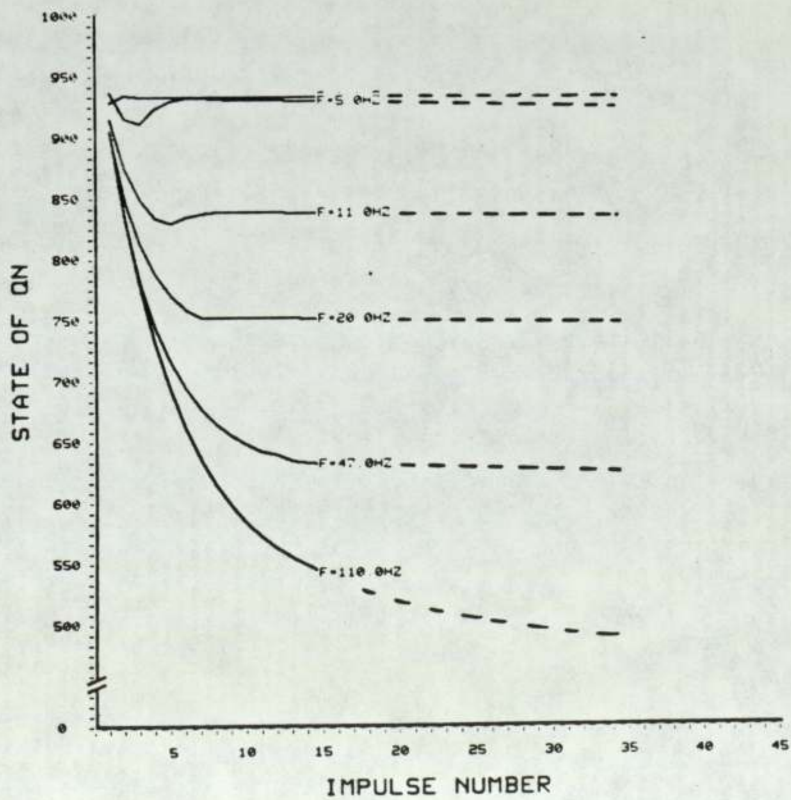
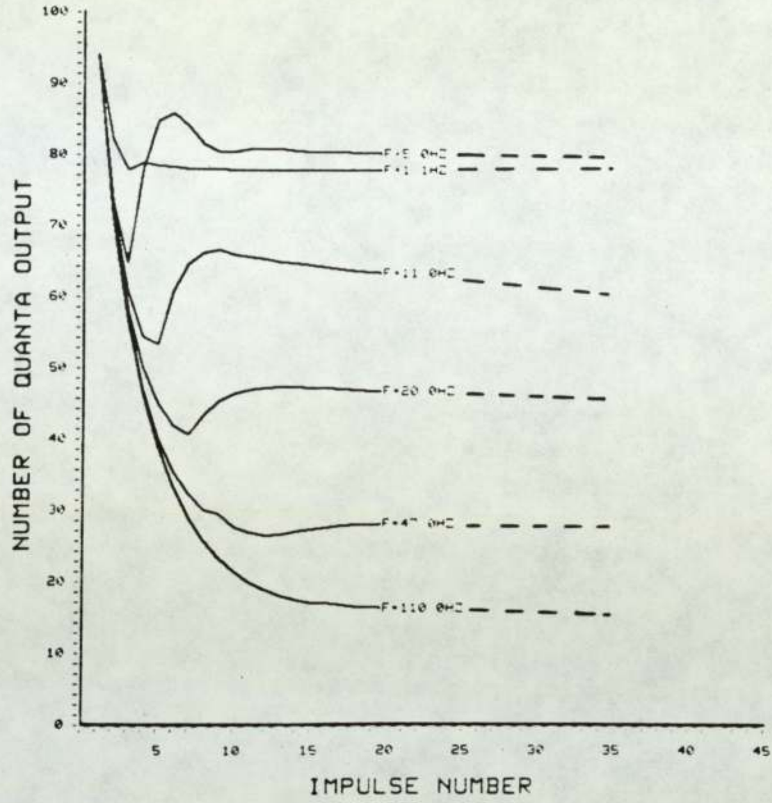


Fig. 23

Fig. 24



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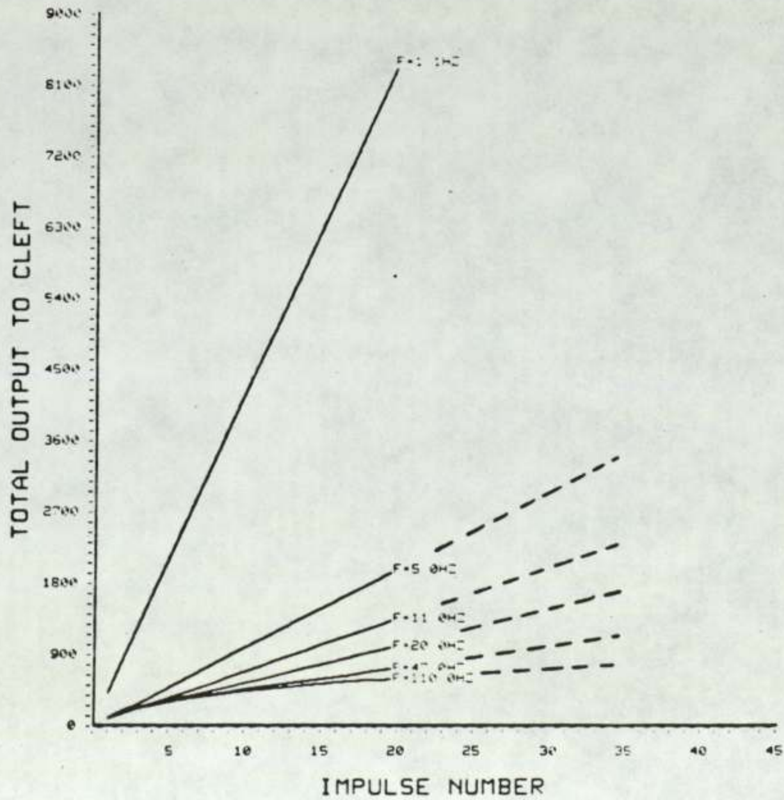

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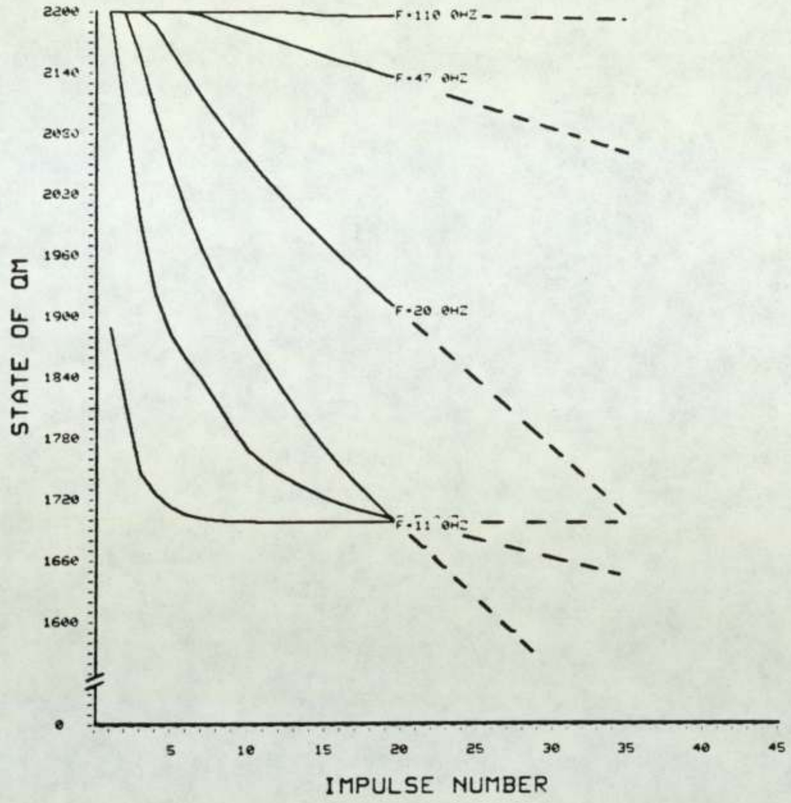

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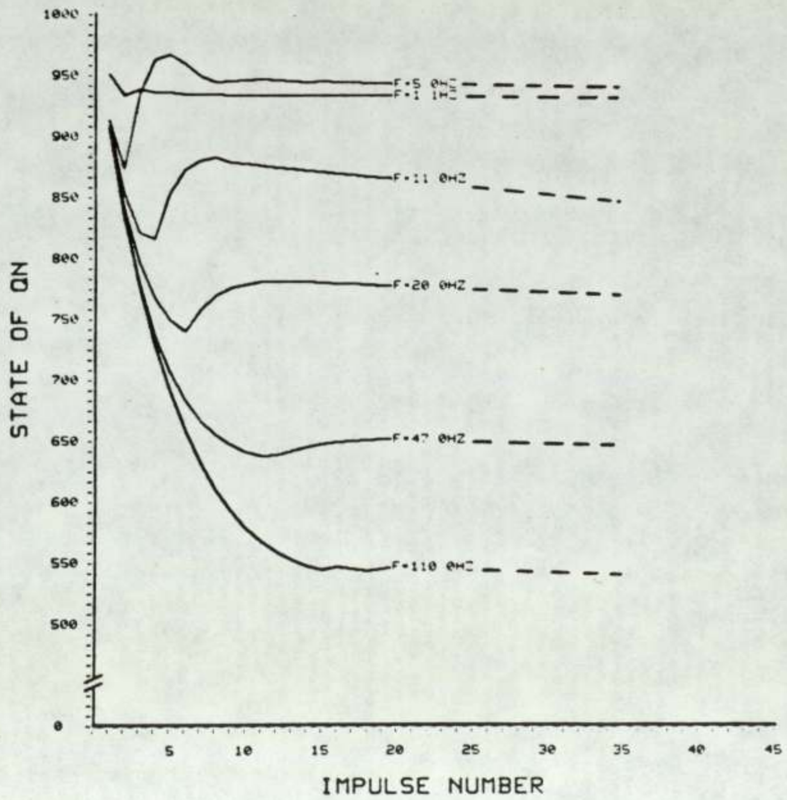

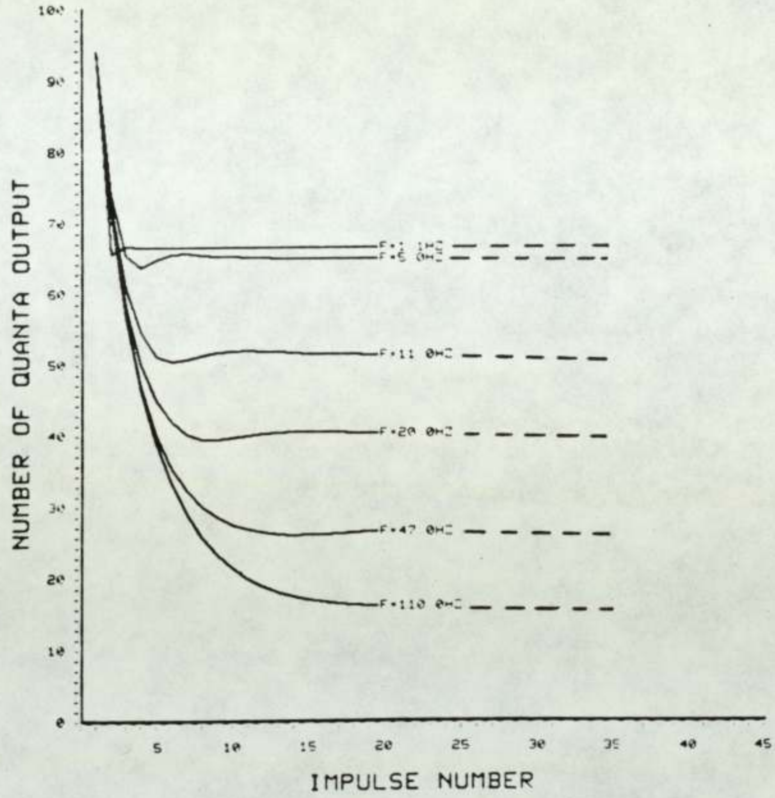

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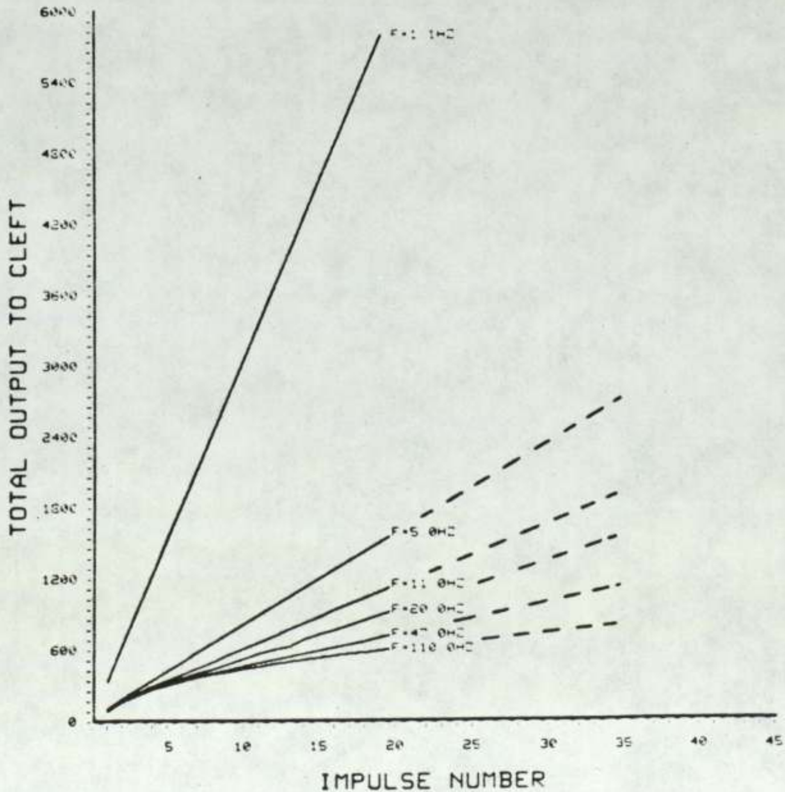
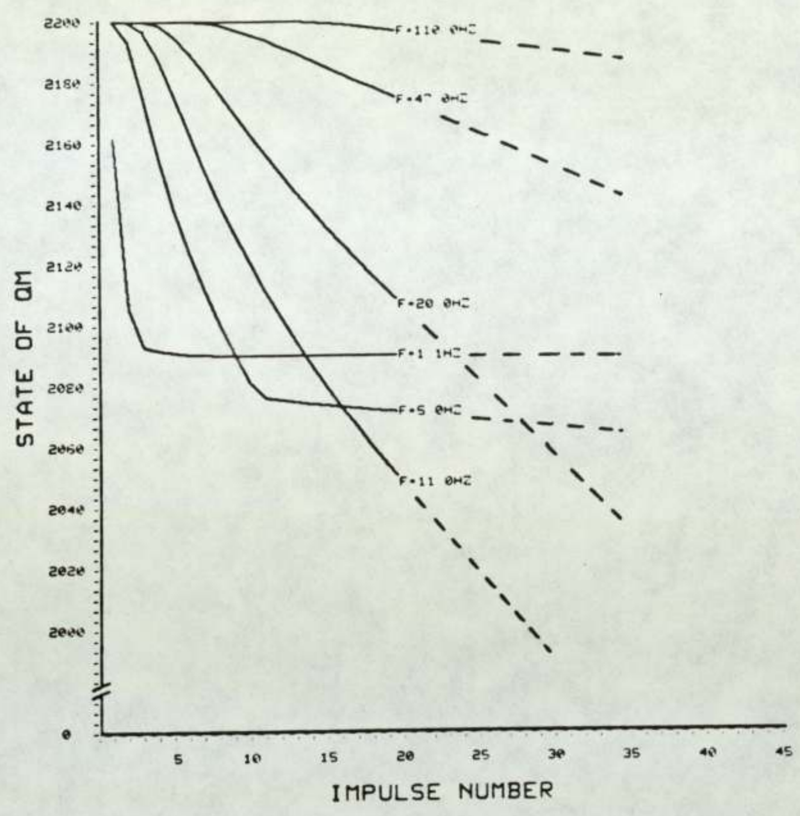


Fig. 29

Fig. 30



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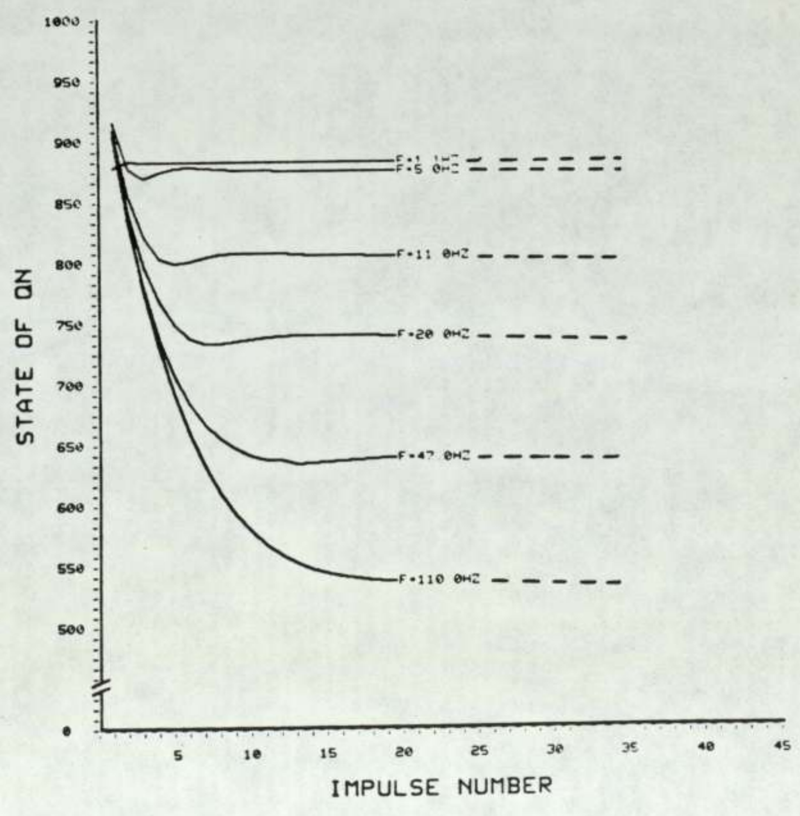

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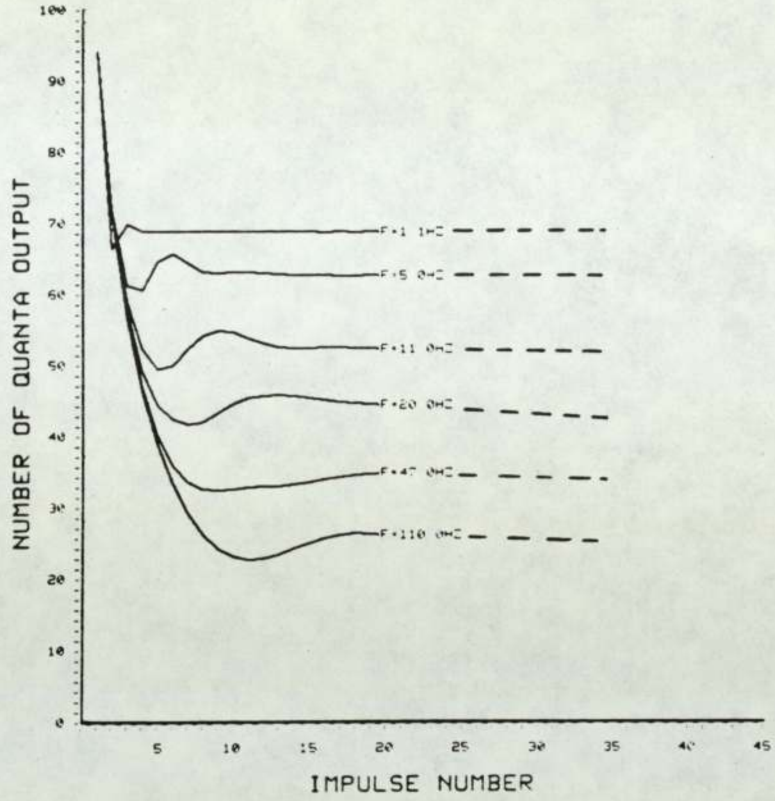

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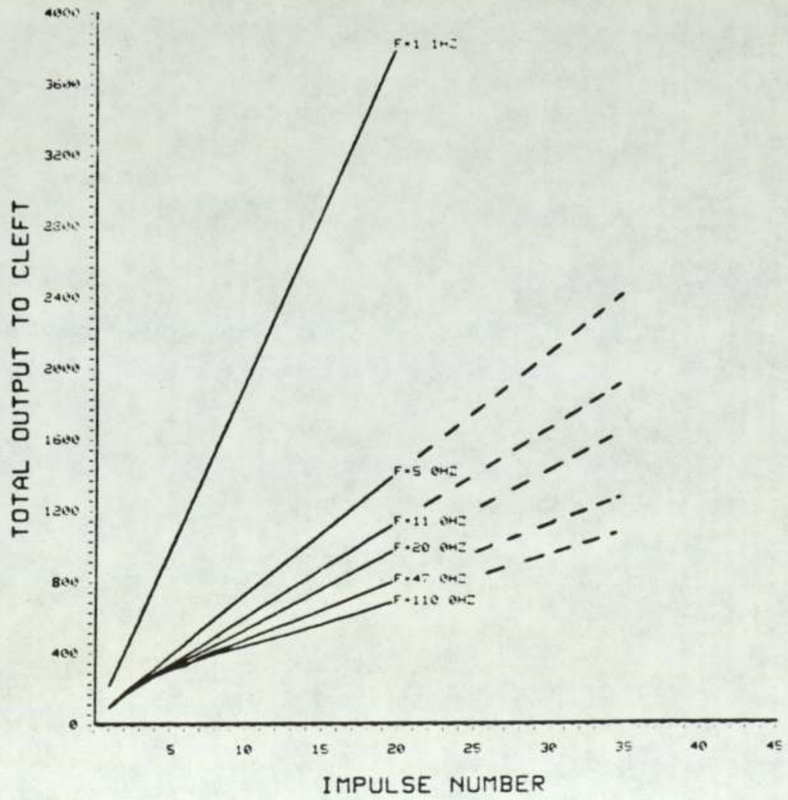

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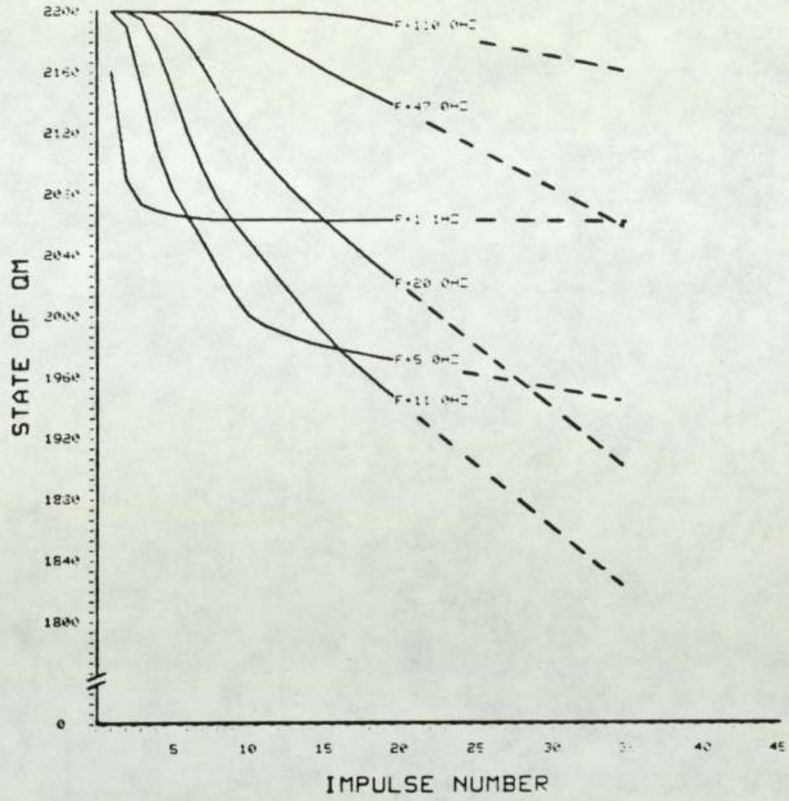

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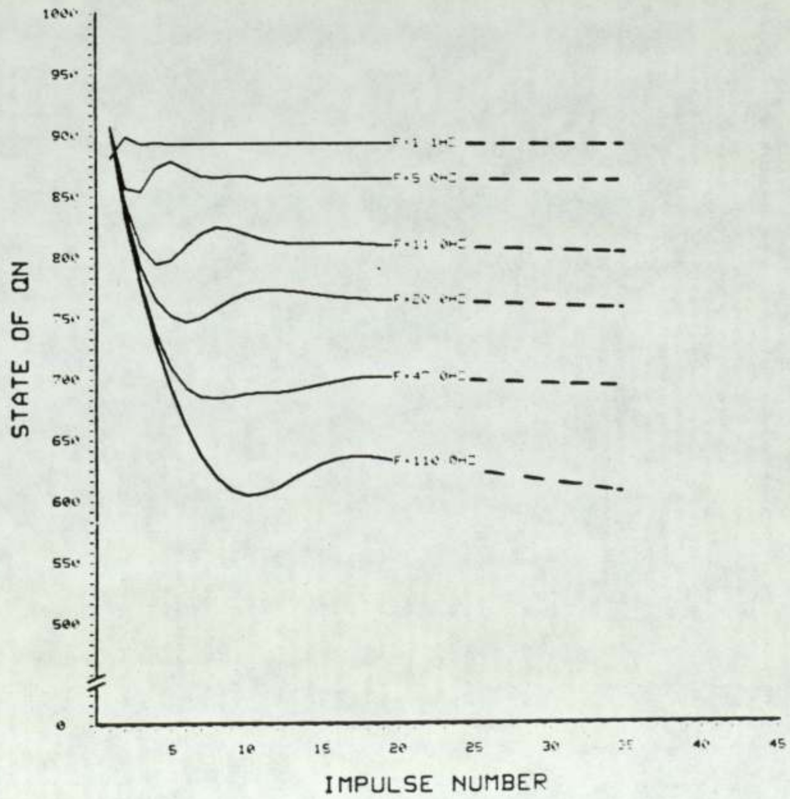

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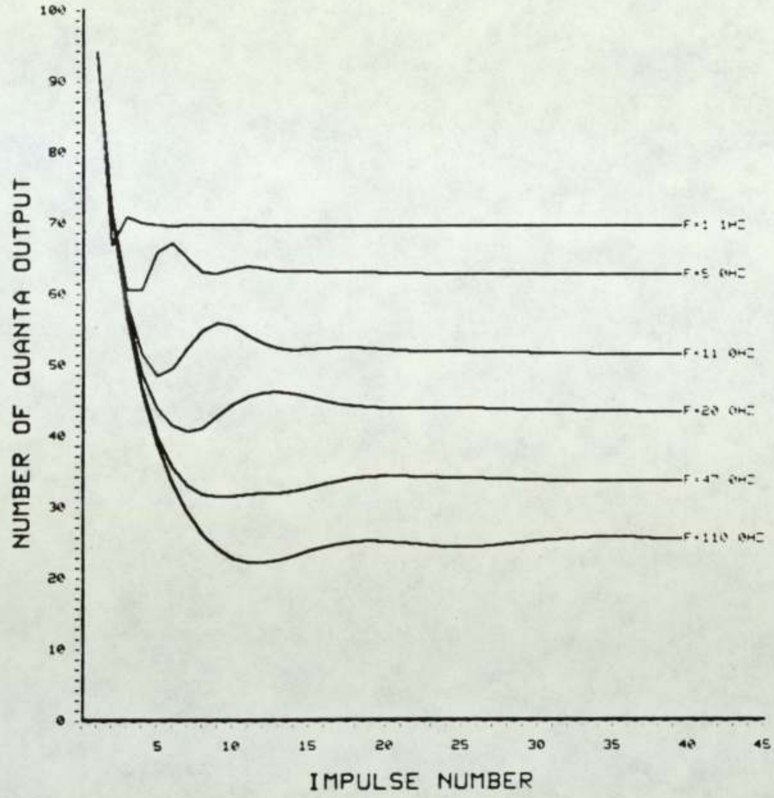

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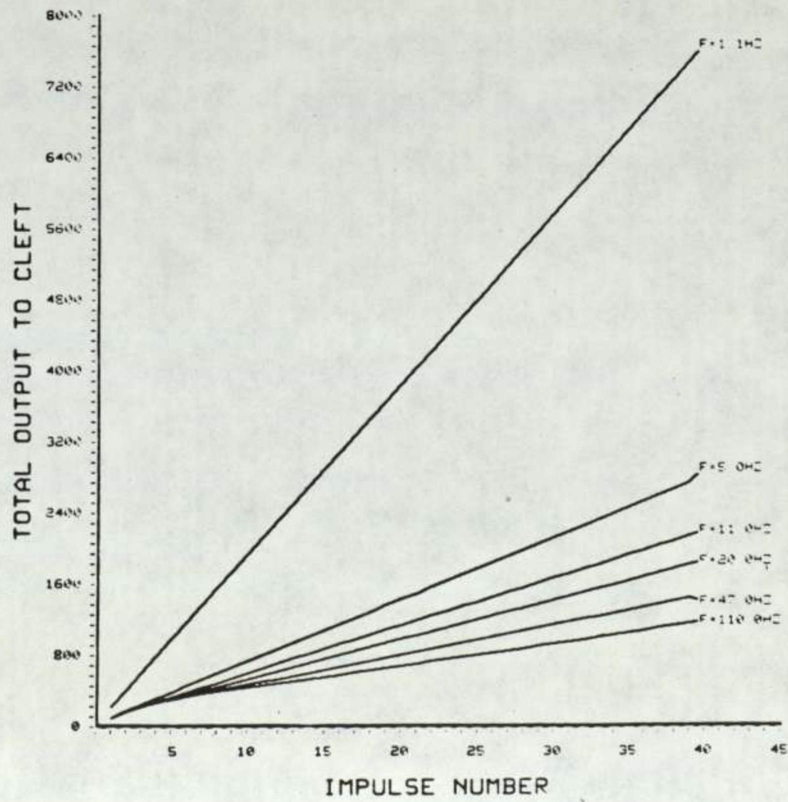

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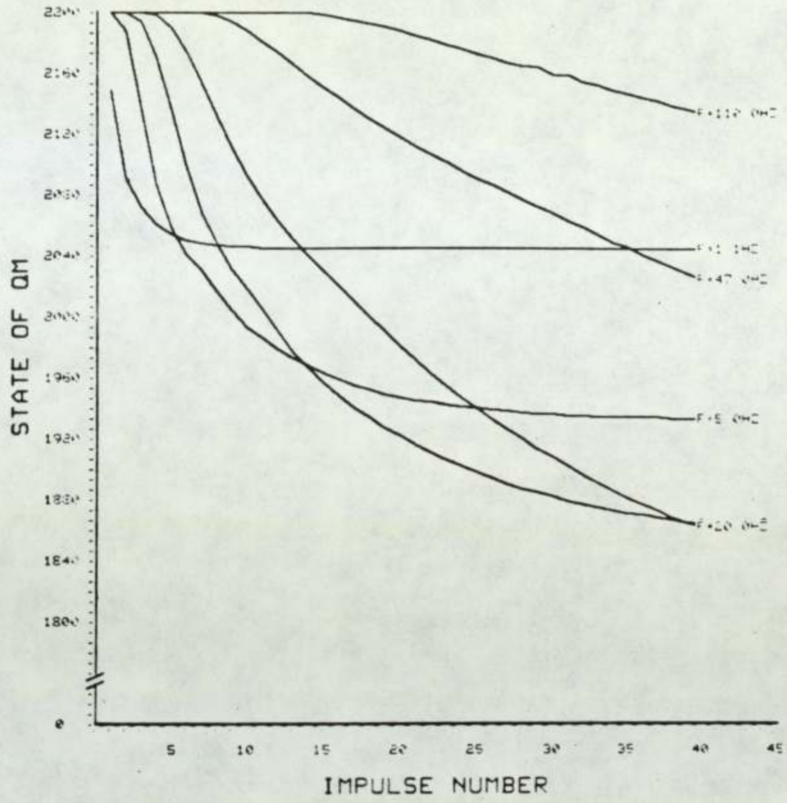

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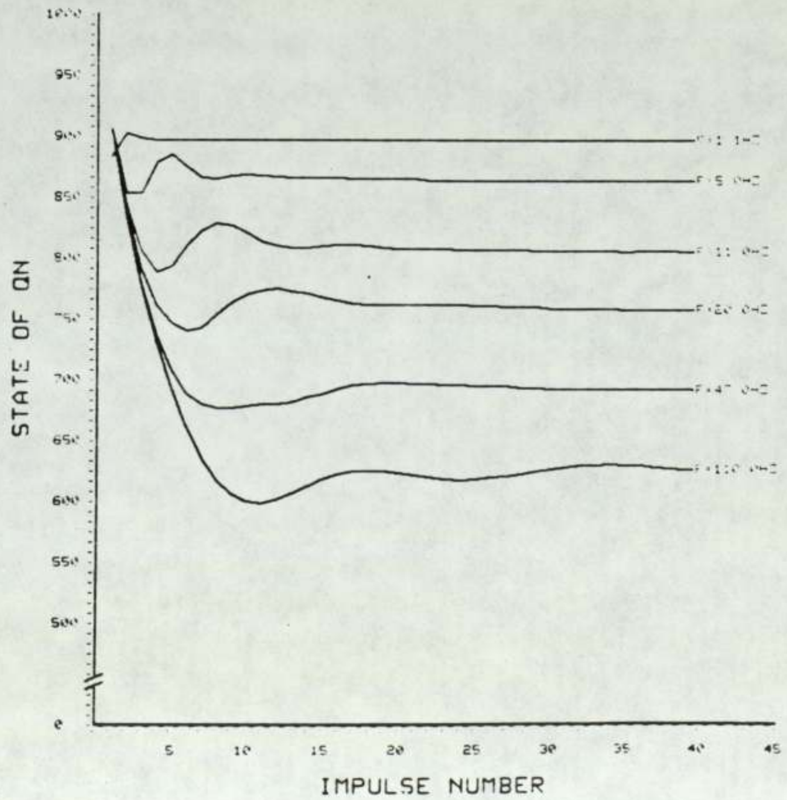

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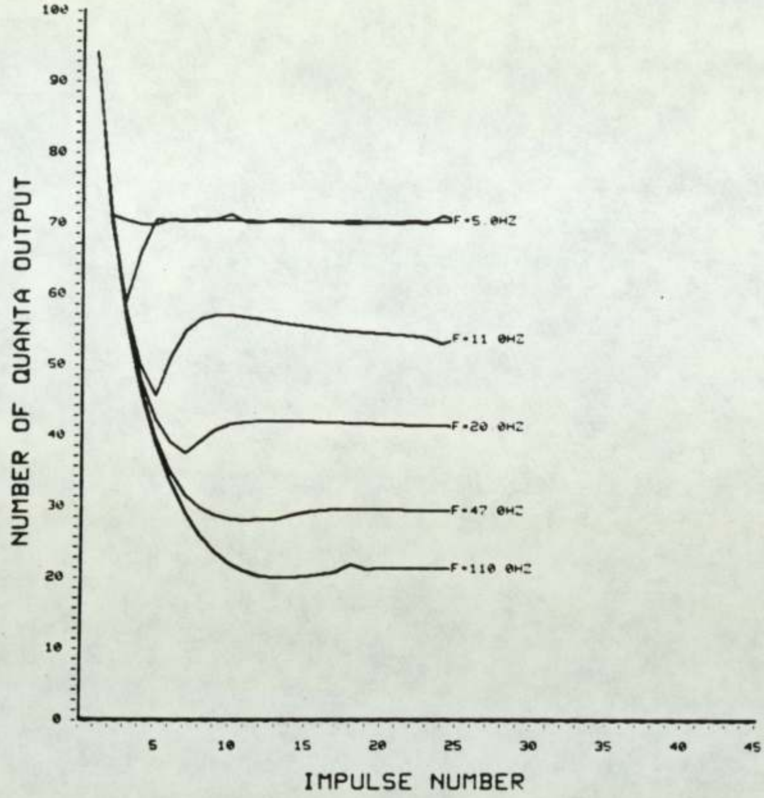

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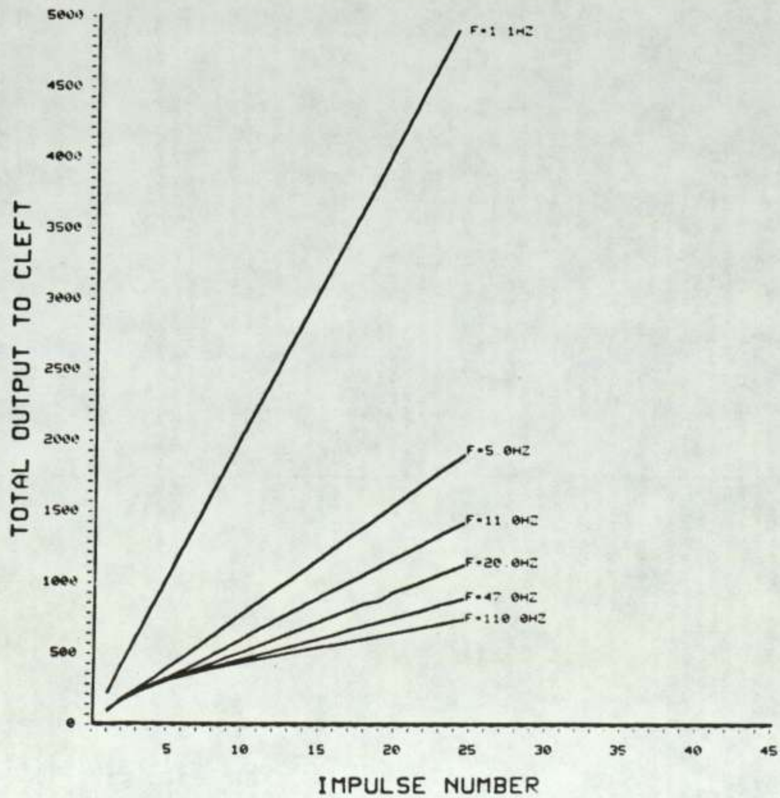

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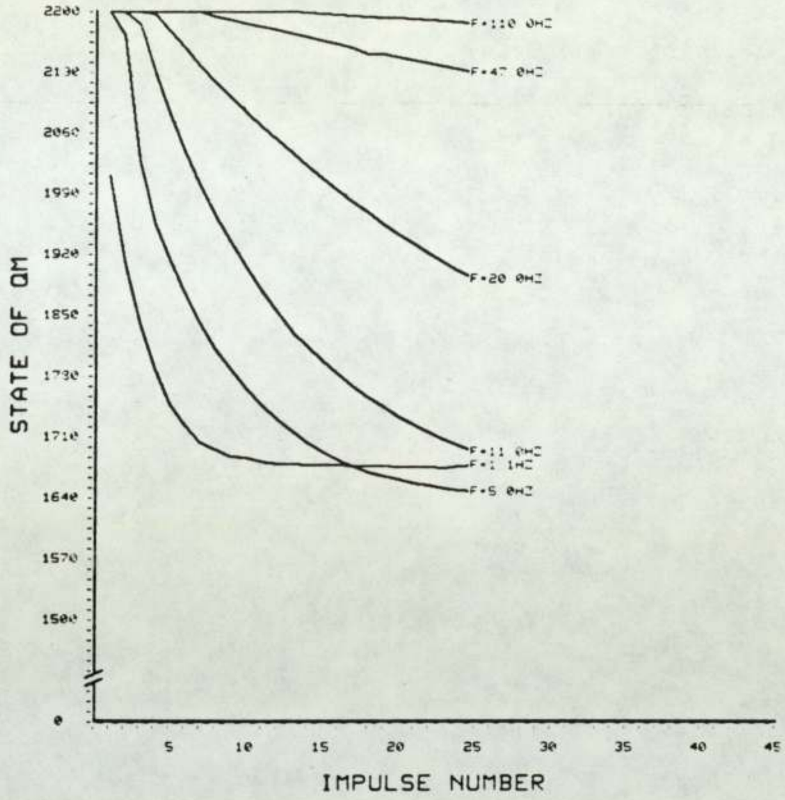

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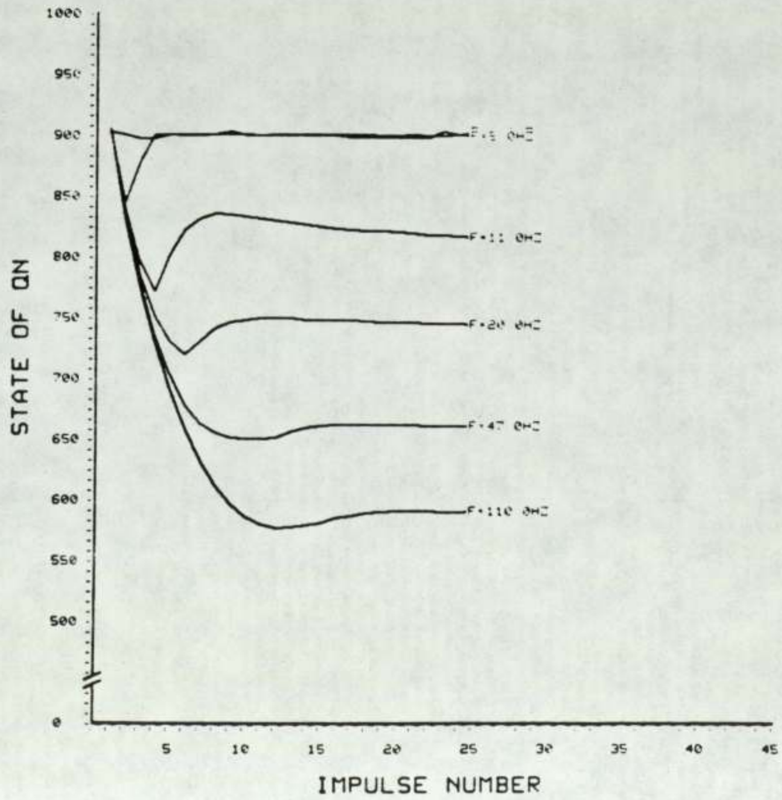

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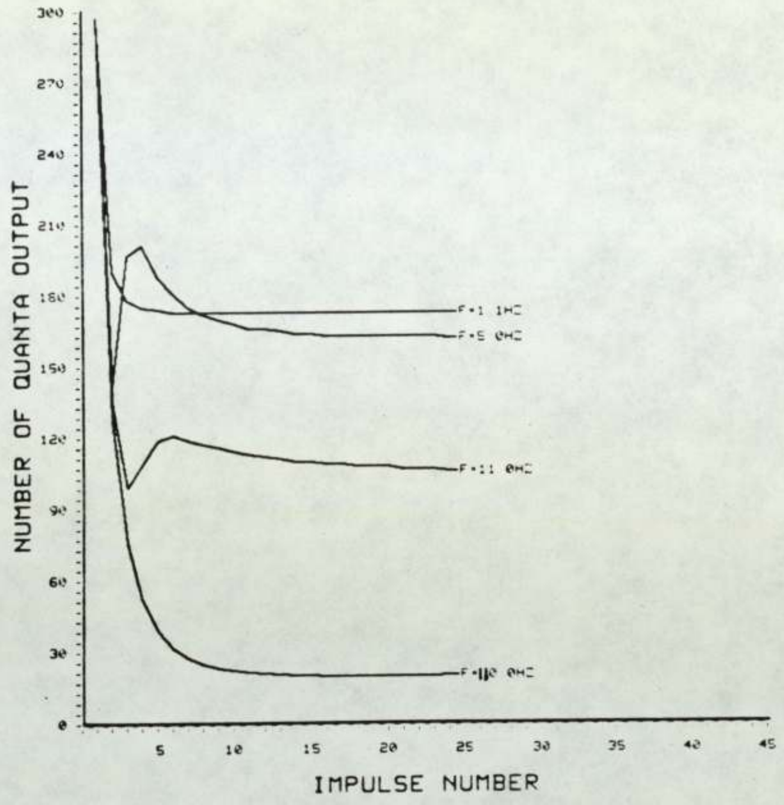

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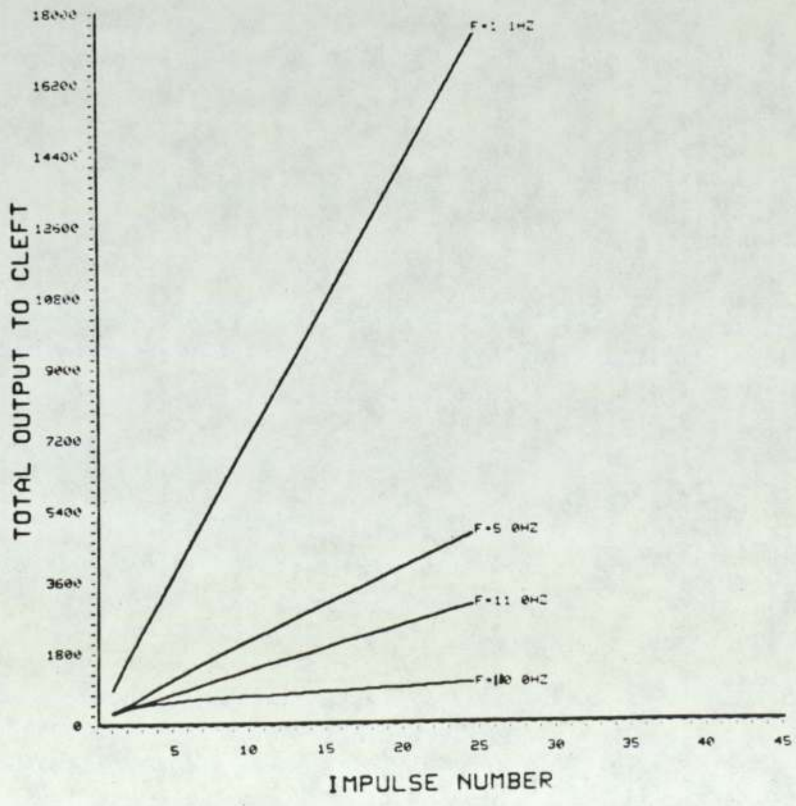

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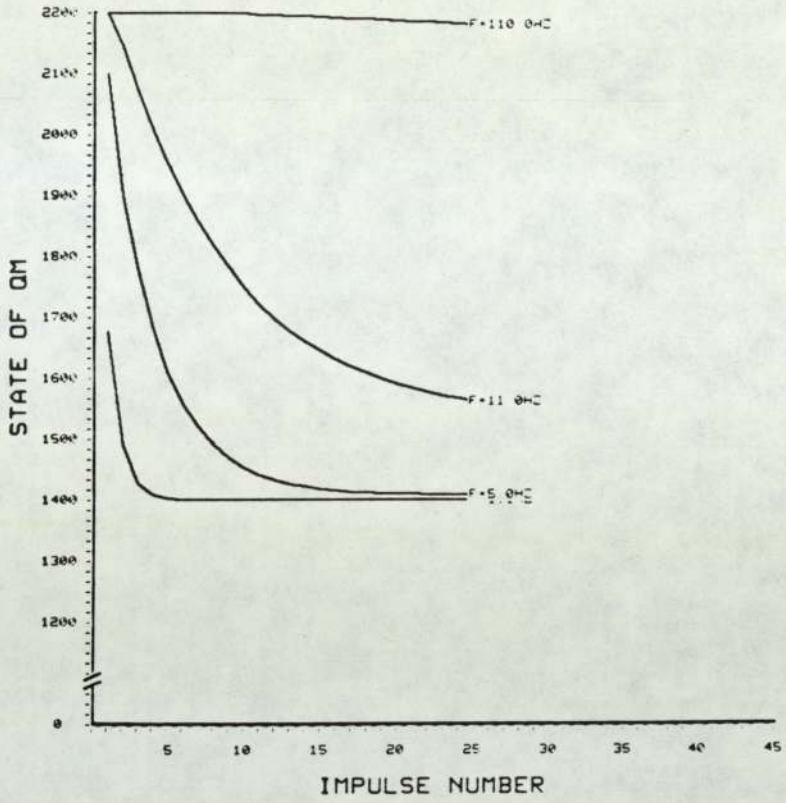

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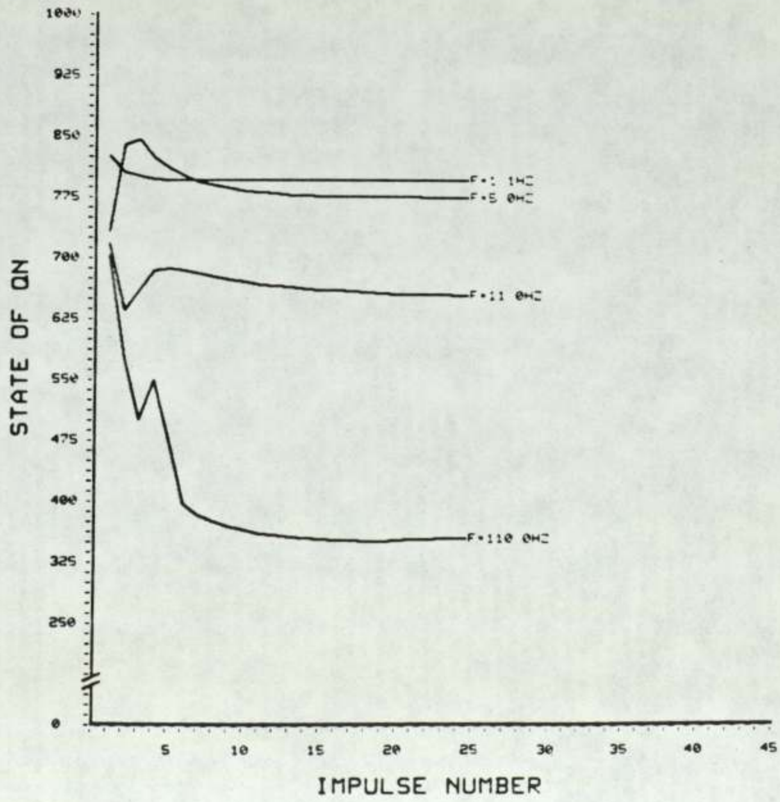

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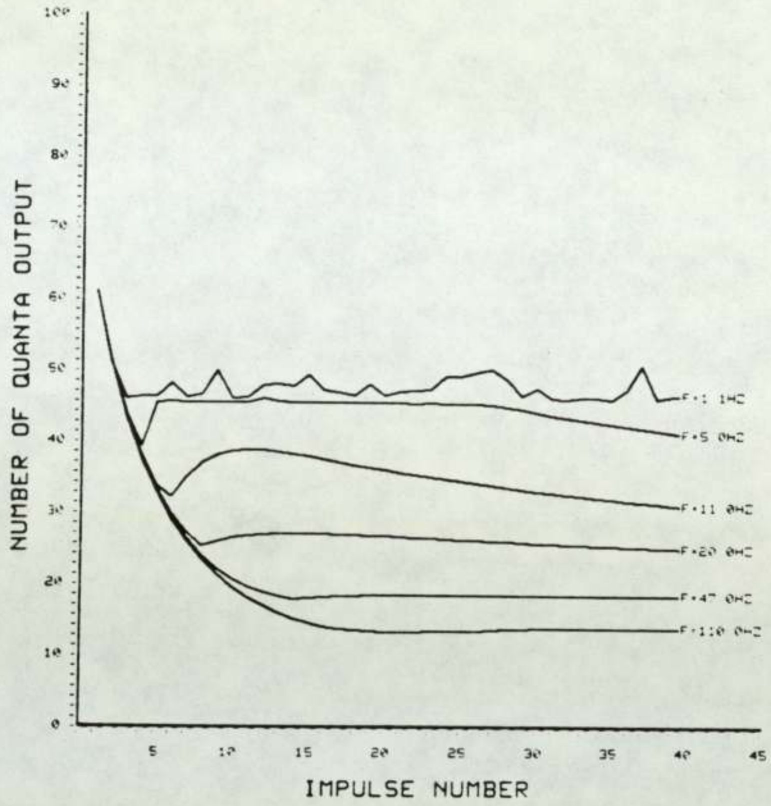

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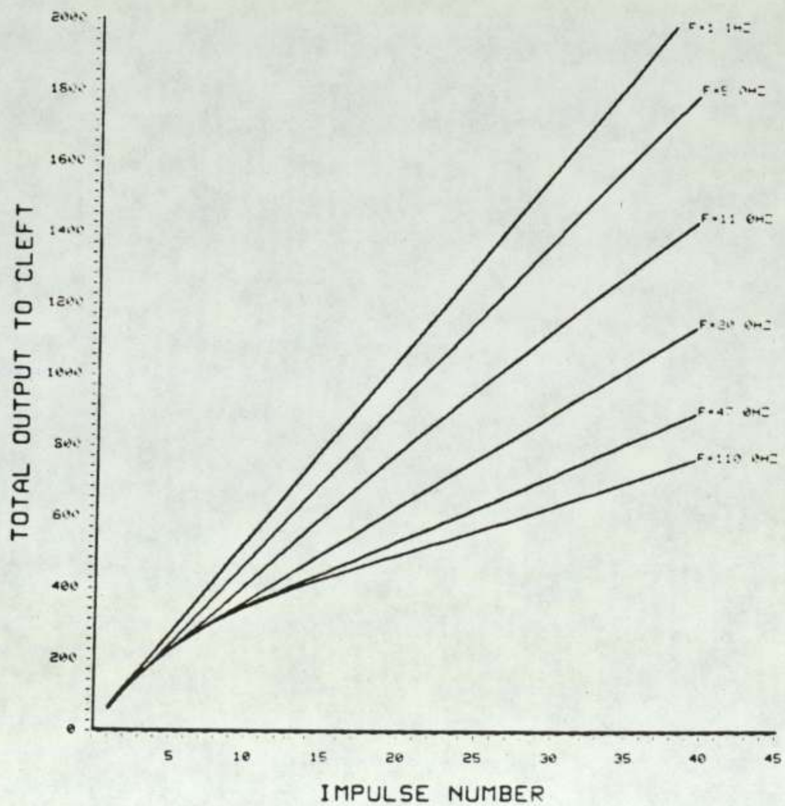

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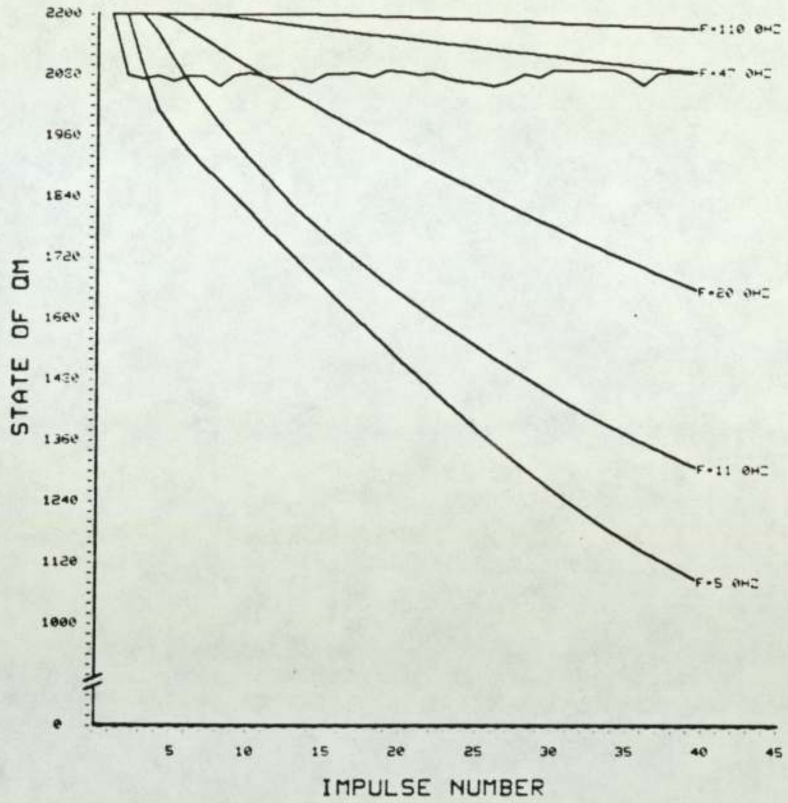

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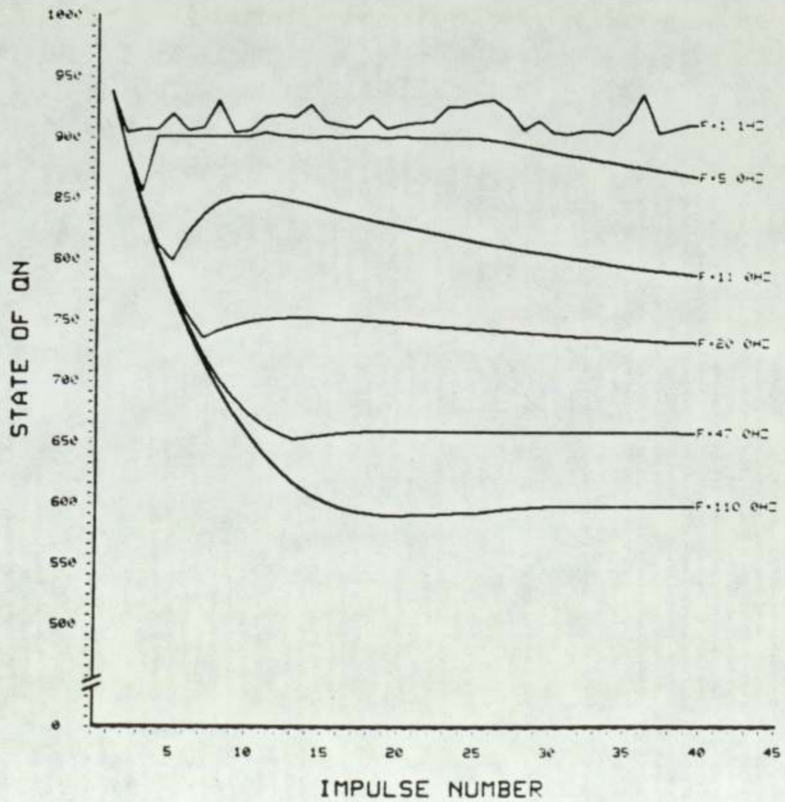

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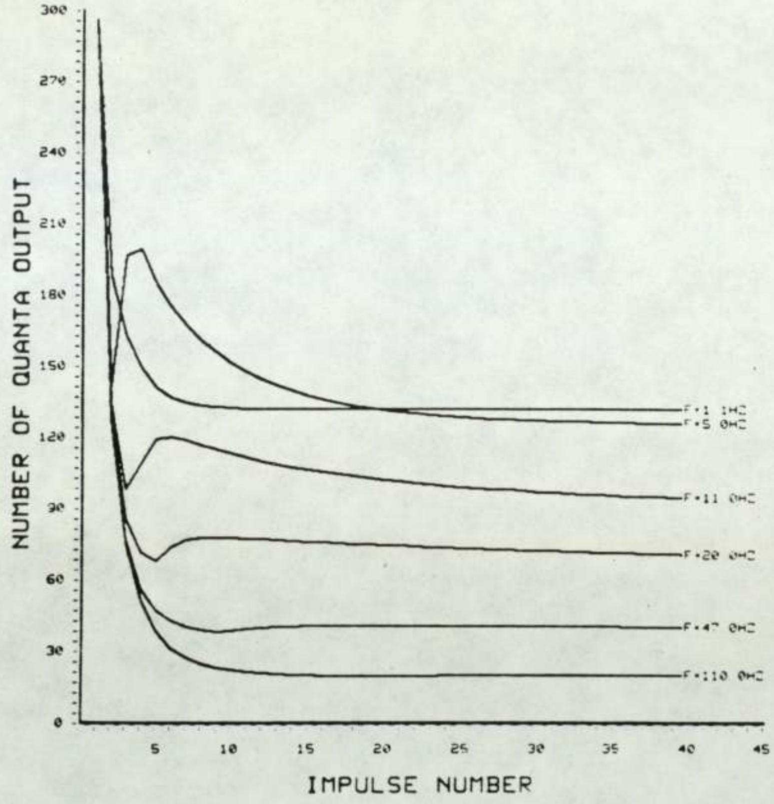

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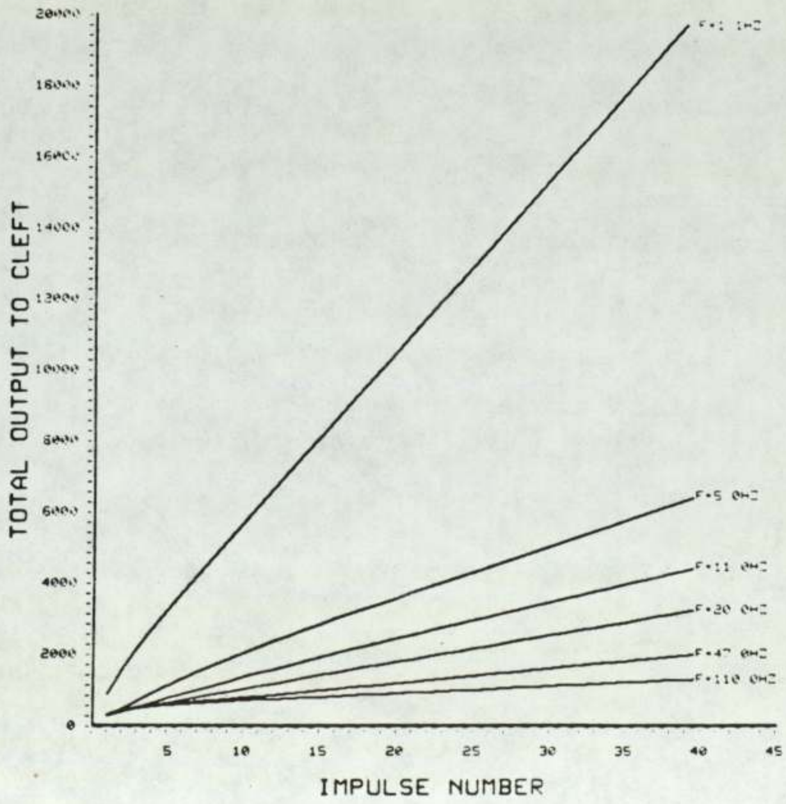

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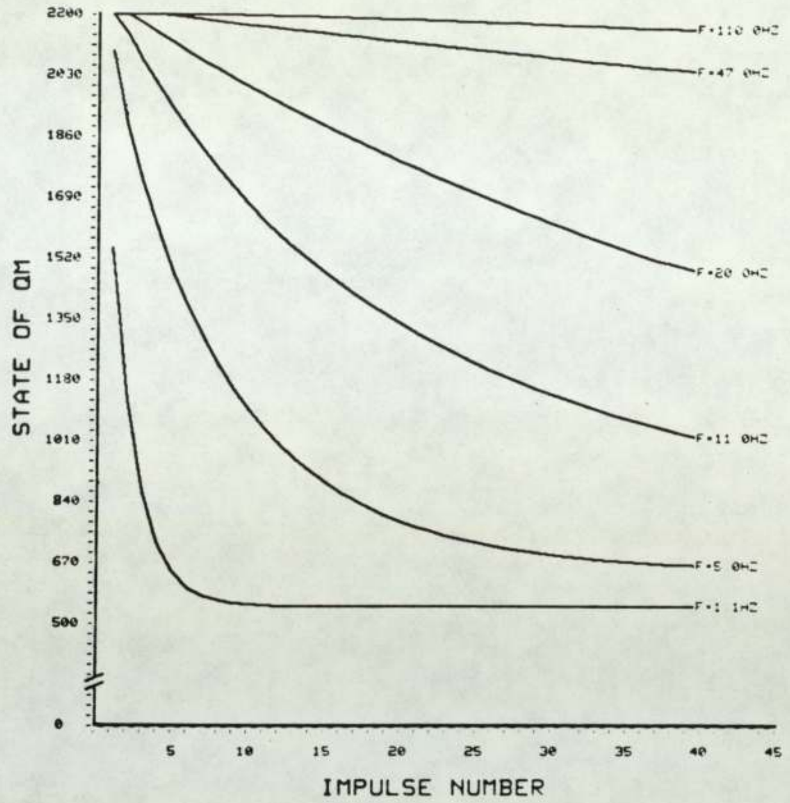
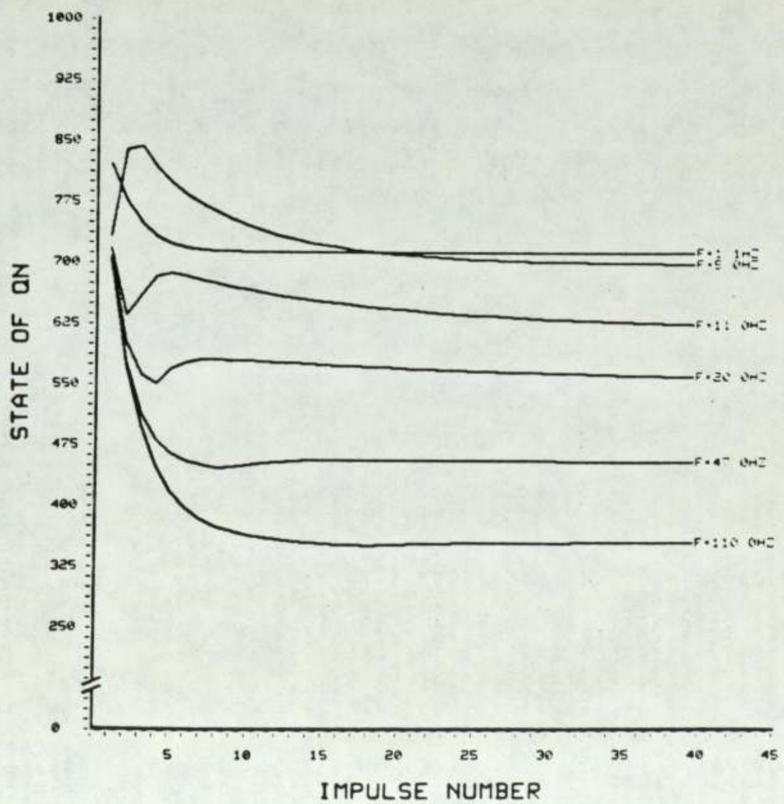


Fig. 55



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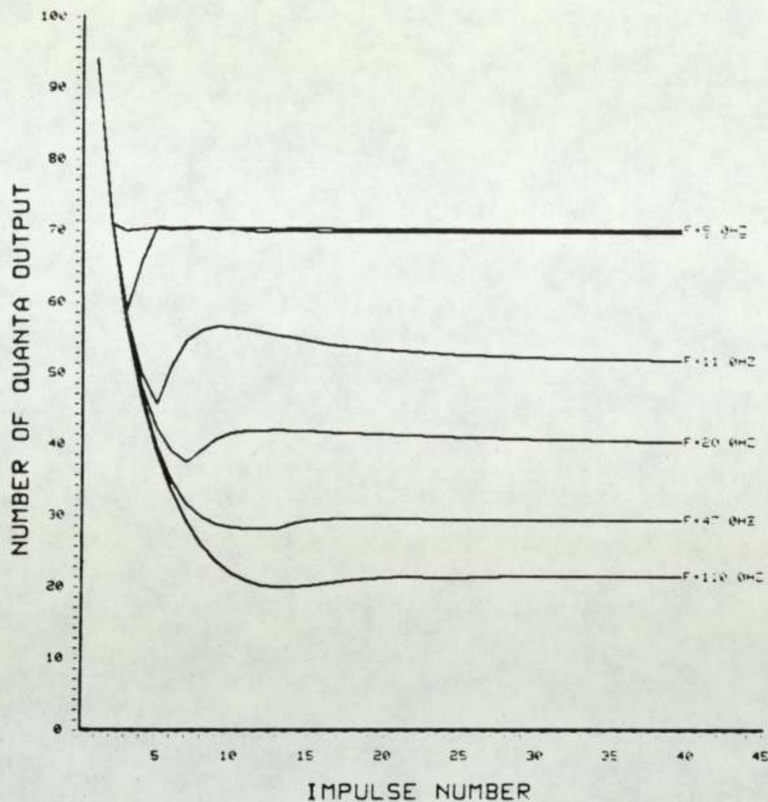

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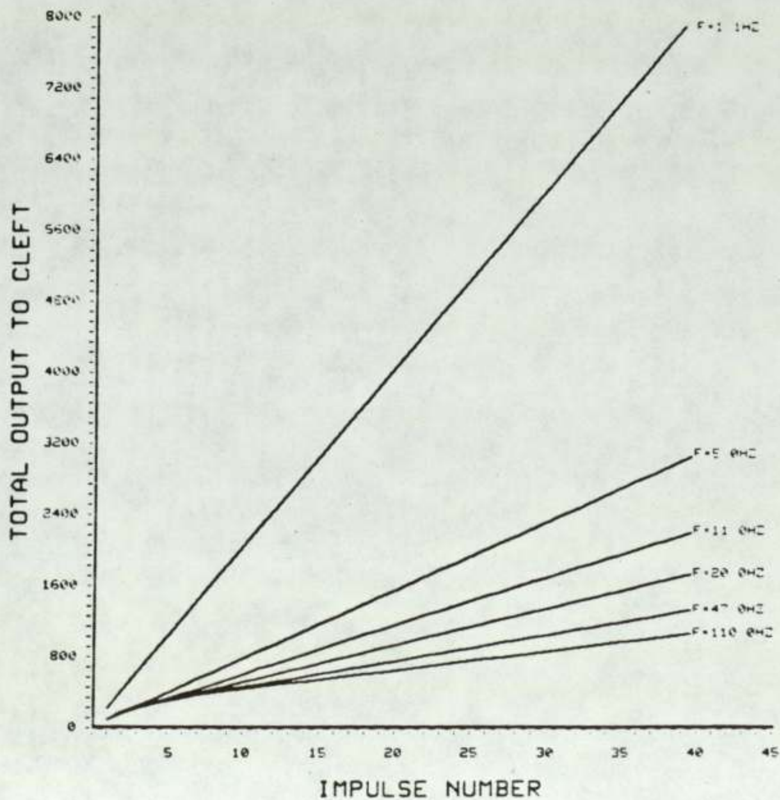

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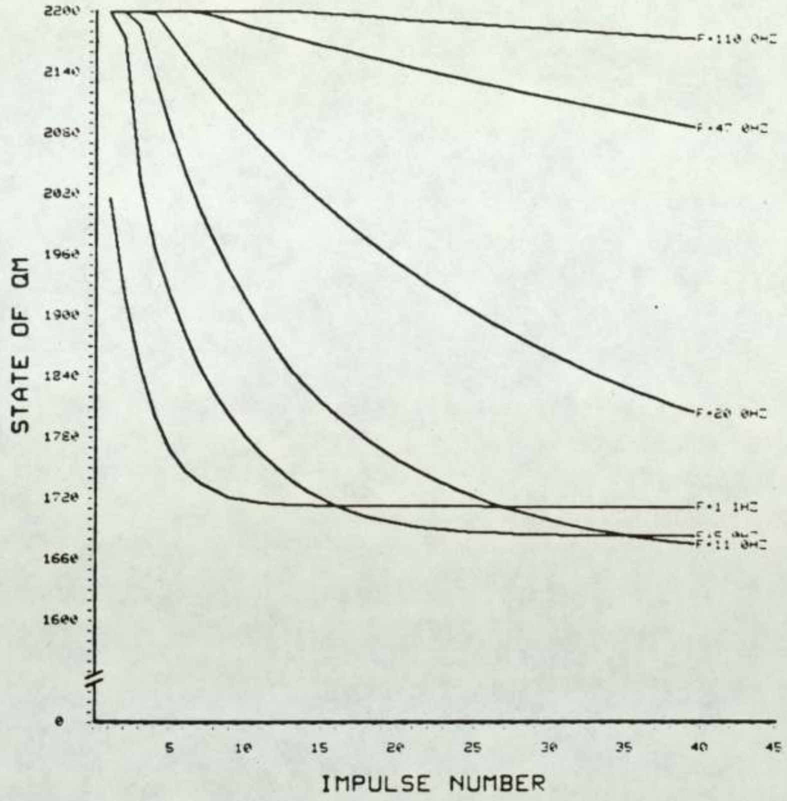

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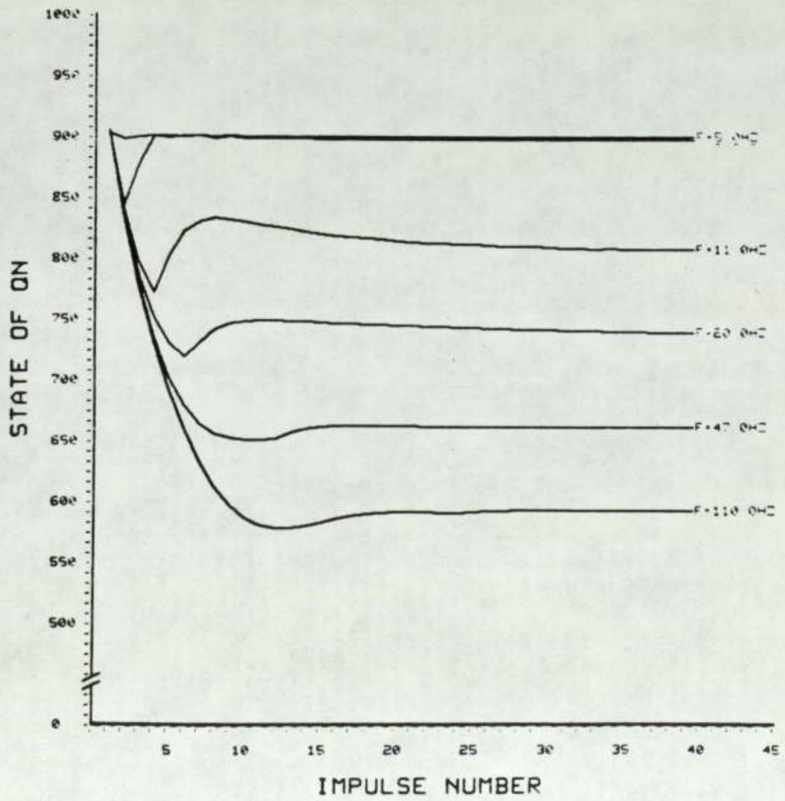
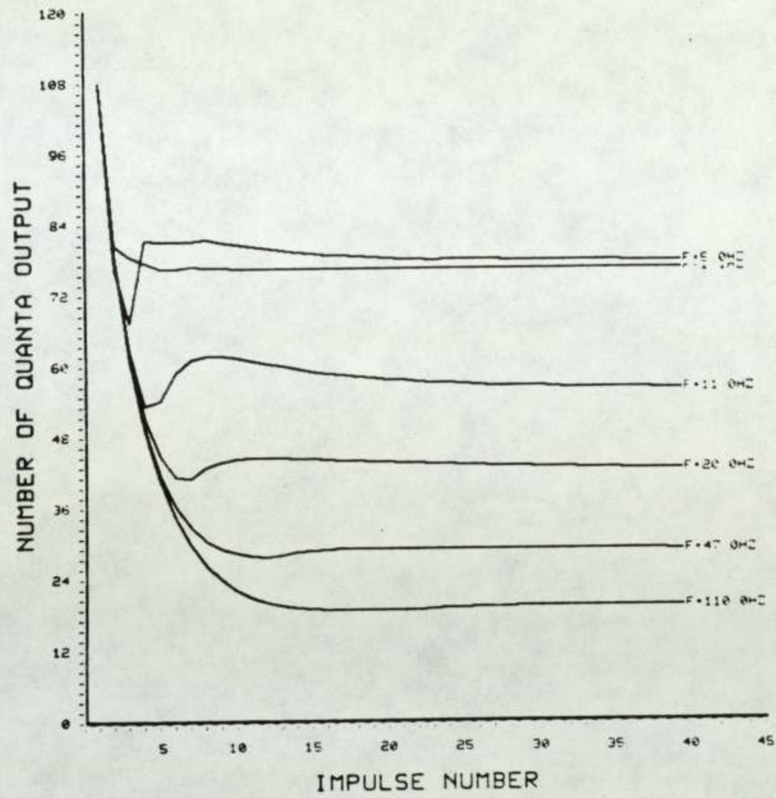


Fig. 60



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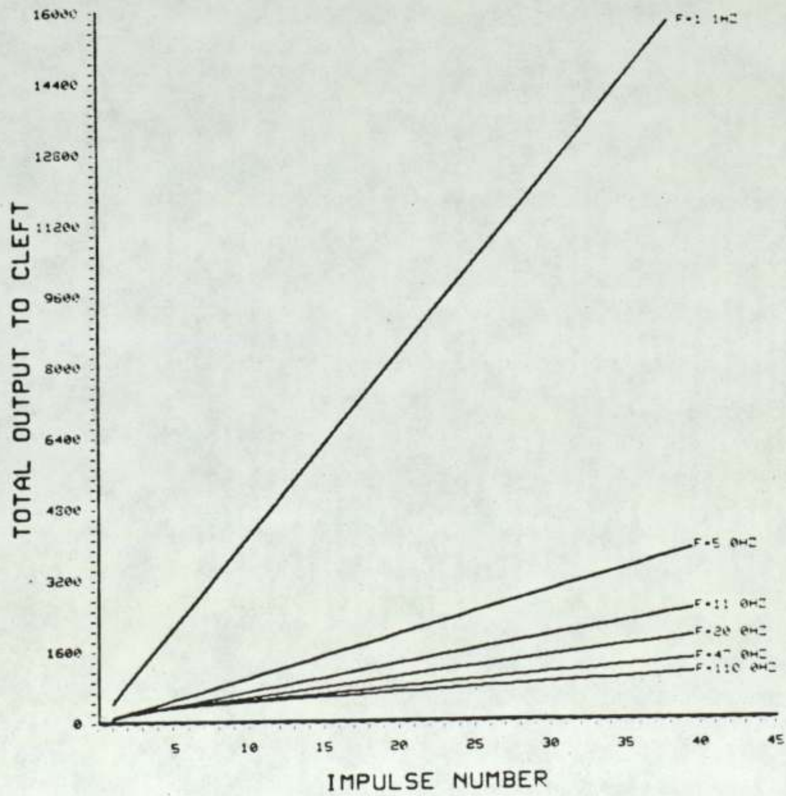

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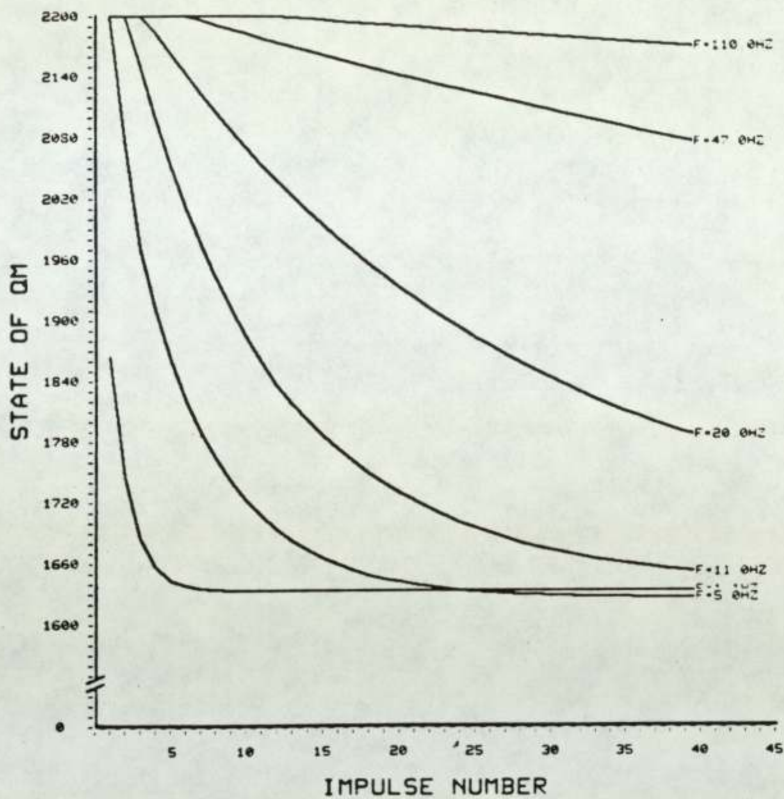

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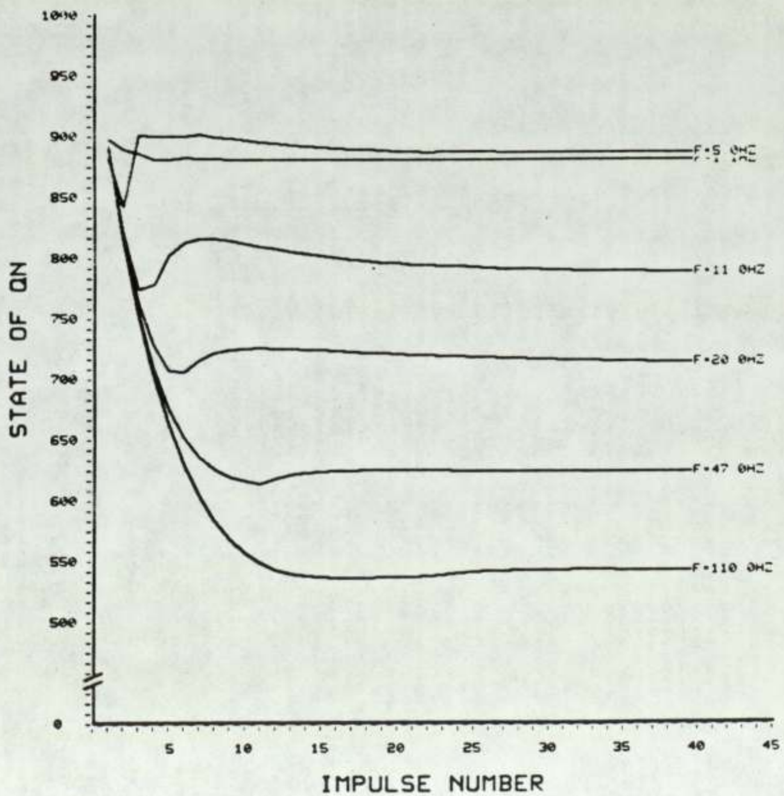

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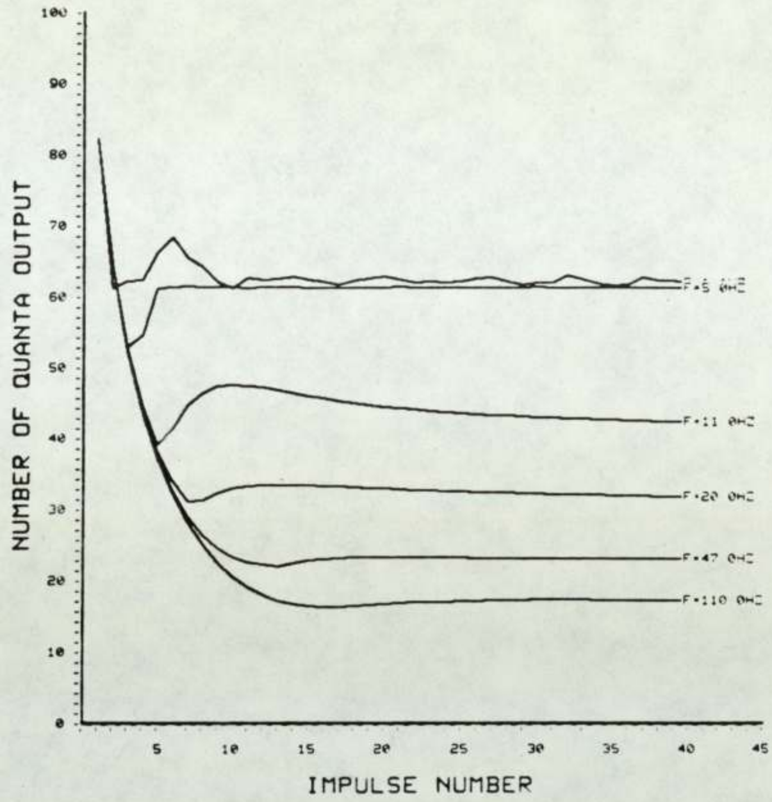

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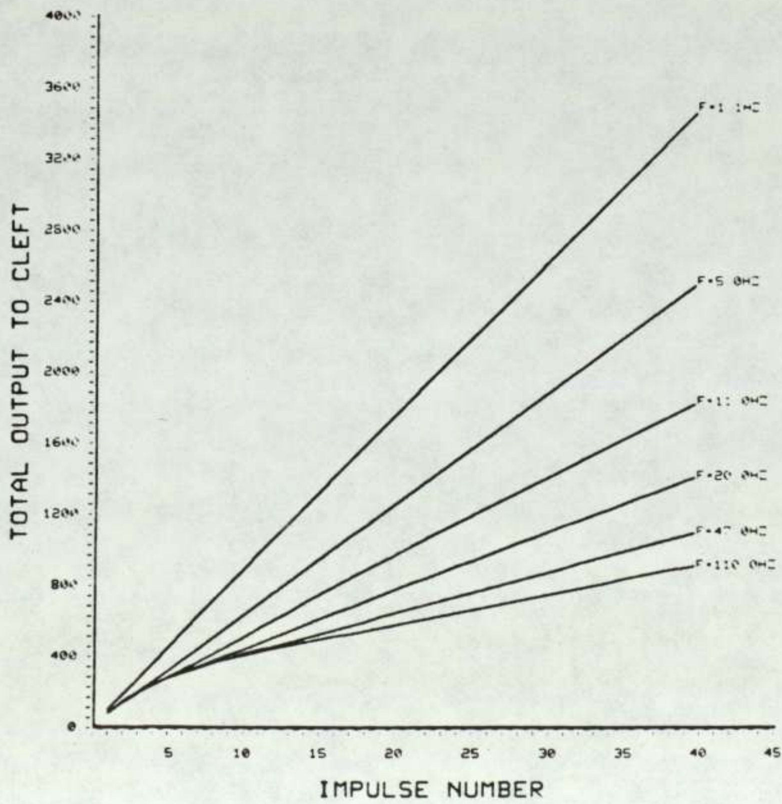


Fig. 66

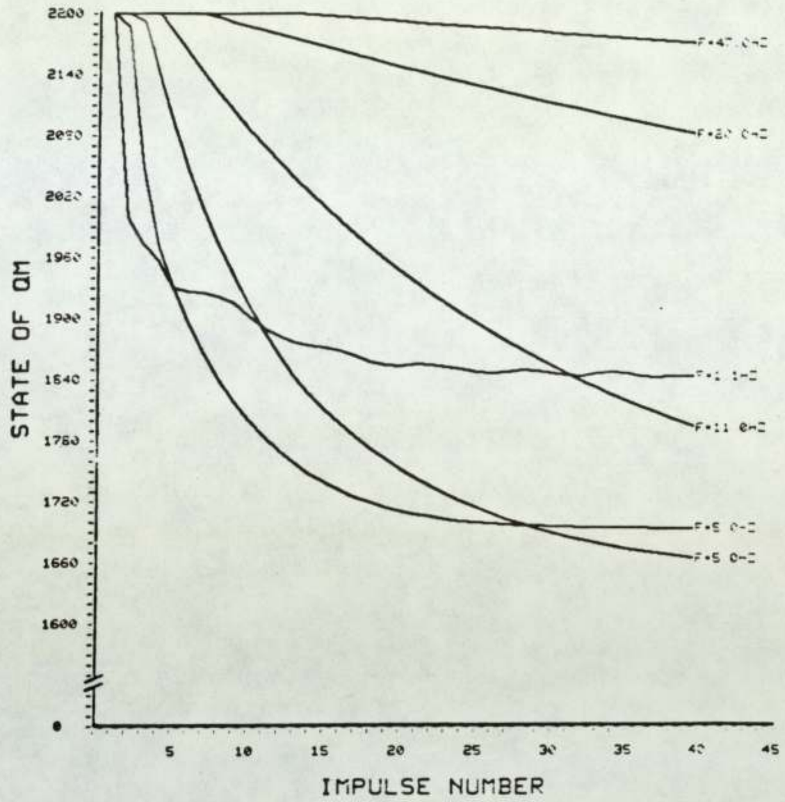
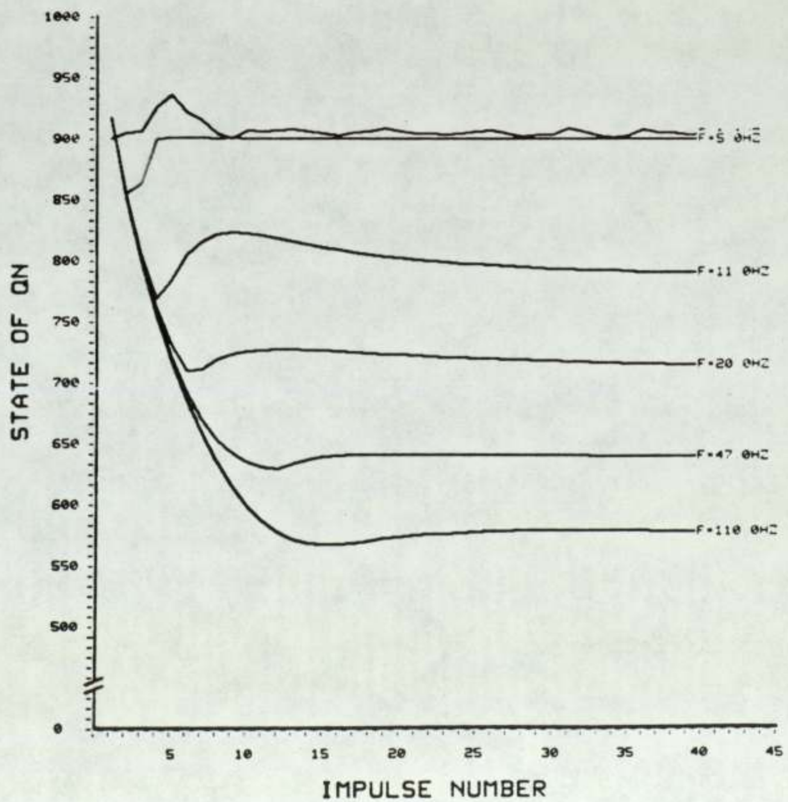


Fig. 67



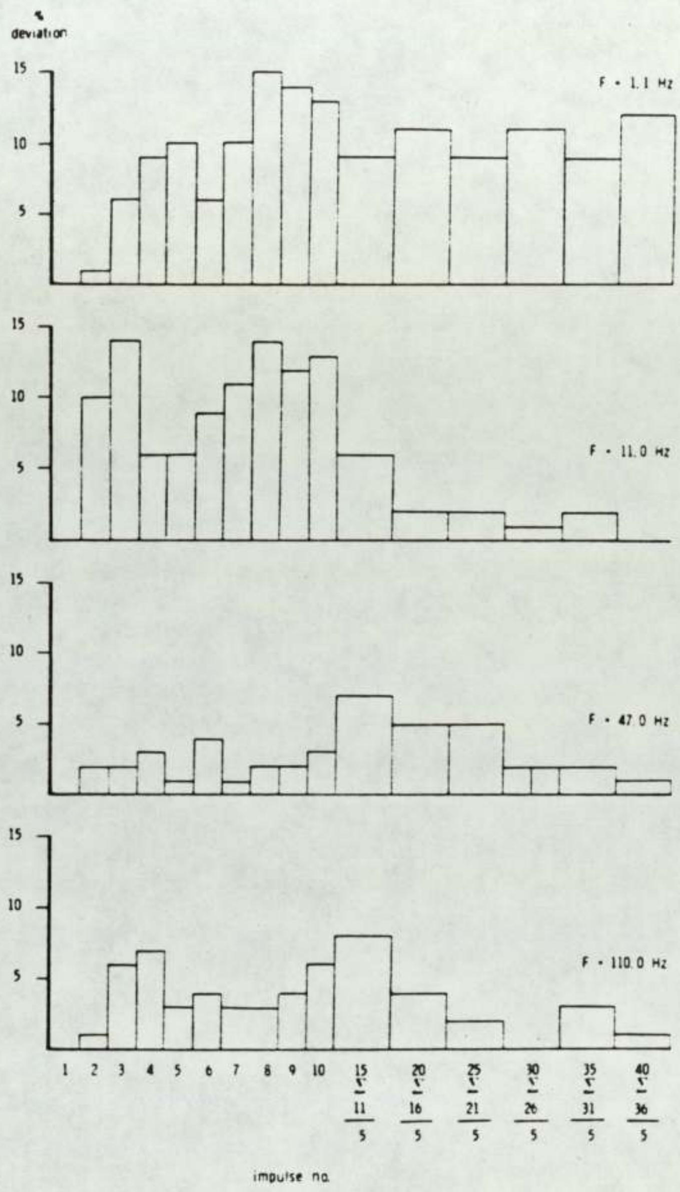


Fig. 68

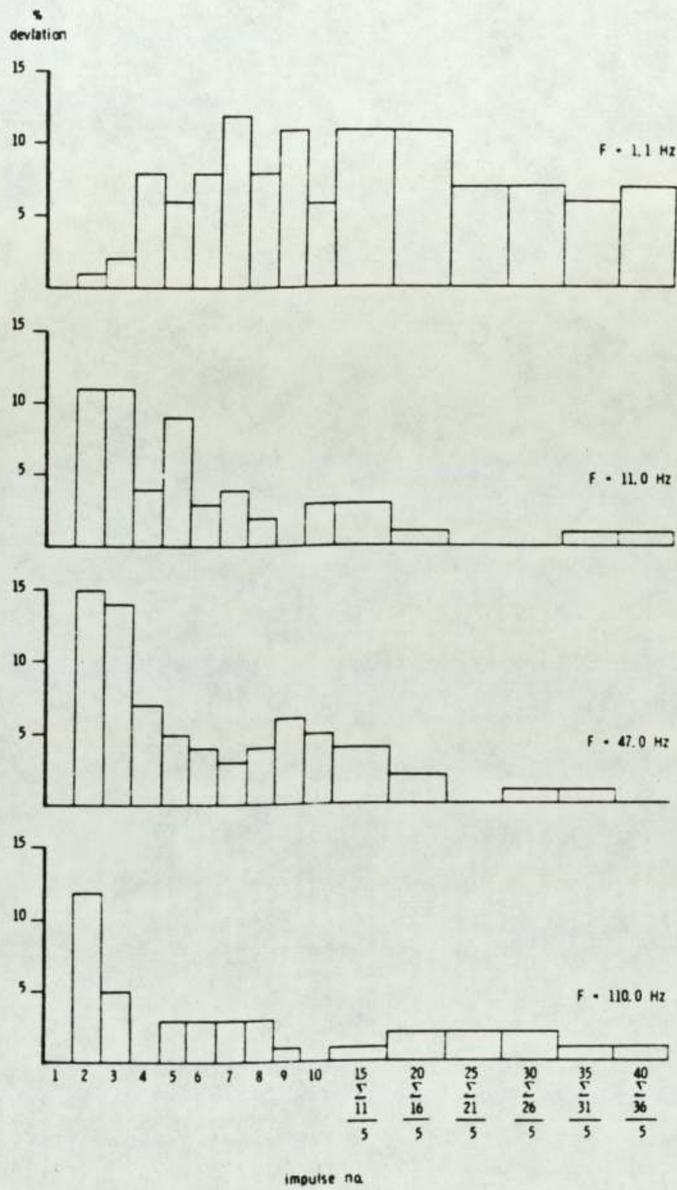


Fig. 69

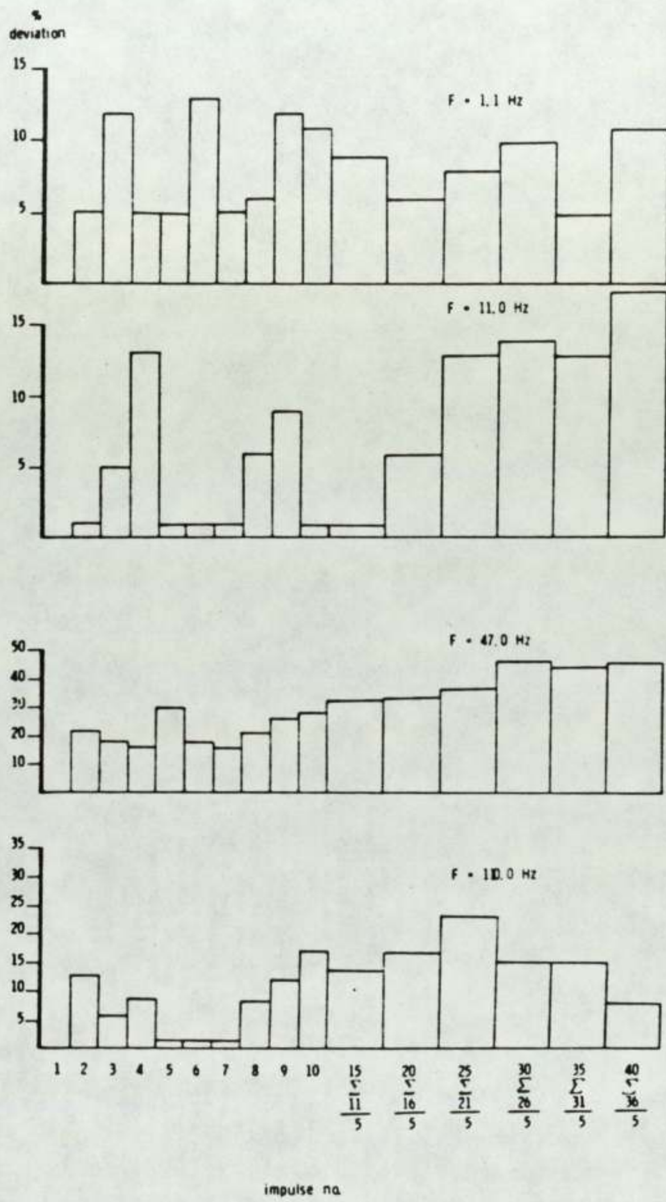


Fig. 70

CHAPTER FIVE

RELEVANCE OF THE MODEL TO WIDER ASPECTS OF NEUROMUSCULAR FUNCTION

The previous two chapters have proposed and refined a mathematical philosophy capable of producing a realistic response to various stimulating frequencies. Model characteristics have so far been compared only with immediate reactions to an evoked response during physiological experiments on isolated preparations, but confirmation of model performance may also be obtained from various other aspects of the literature.

Observers have shown quite conclusively that little change in magnitude of end plate potential is produced when using stimulating frequencies up to and including 5 Hz.[9,12,71,203]. The initial depression in the 5 Hz. characteristic of the model (see figure 56 of chapter 4) is due to a depletion of the readily releasable store in the absence of synthesis and an influx from the mobilisation store. Once these mechanisms are invoked, however, a typical characteristic of the type observed during physiological experiment is produced.

In an effort to confirm this type of response to stimulating frequencies of 5 Hz. and below the model was presented with two further frequencies of 6.0 Hz., and 0.5 Hz. At 6 Hz. the output characteristic was quite different to that produced at lower frequencies confirming no sudden change in model function at 11.0 Hz. The characteristic produced at 0.5 Hz. was, however, very

similar to those already produced at 1.1 and 5.0 Hz. as shown overleaf in figures 71, 72, 73 and 74 respectively. The oscillatory response was expected as the time increment (TT) chosen for this simulation was large.

The philosophy assumed decreases in end plate potential, seen during physiological experiments, to be due to a fall in quantal content brought about by a depletion of the readily releasable store. This assumption has been confirmed recently[206] where acetylcholine output (per stimulus) has been measured and compared with resultant end plate potentials at the post-synaptic site.

The initial store contents are as estimated by experiment on human muscle preparations, although various other values could have been used (e.g. cat, rat). This seemed inappropriate however when the long term ambitions of the model were considered, and consequently the simulated output response is lower than in some estimations provided by the literature[141]. Several experiments seeking a value for quantal output have made use of a preparation of the phrenic nerve in the hemi-diaphragm of the rat. The model has used some of this information to reinforce certain arguments although quantitative evidence is diffuse and difficult to assess in many cases. For example, one experiment gives quantal output in the rat hemi-diaphragm preparation to be approximately 400 quanta[141] but a more recent investigation has shown the age of the preparation to be a salient feature of this estimation; the maximum output varying between 20-360

Fig. 71

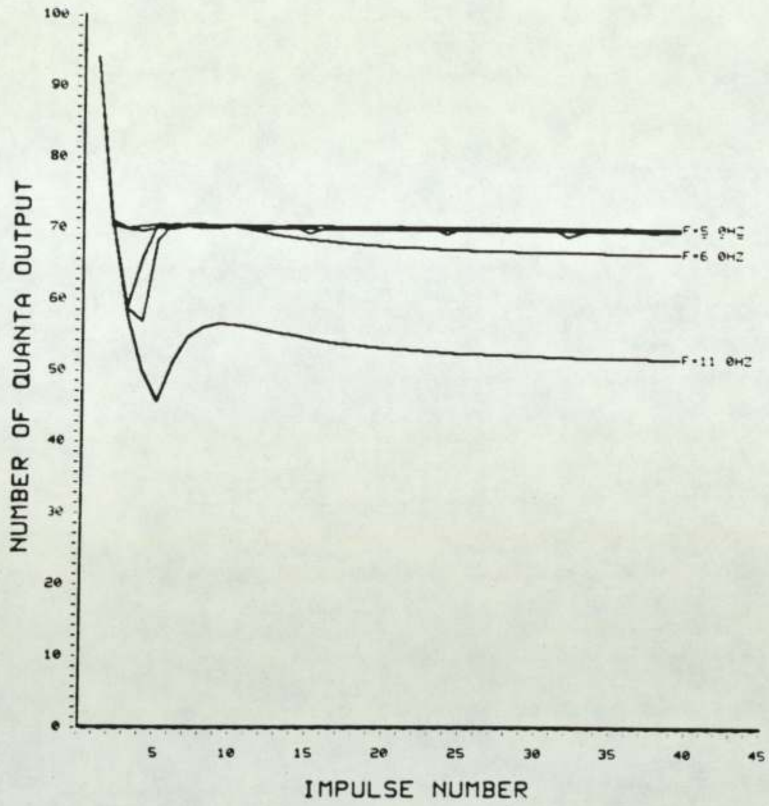


Fig. 72

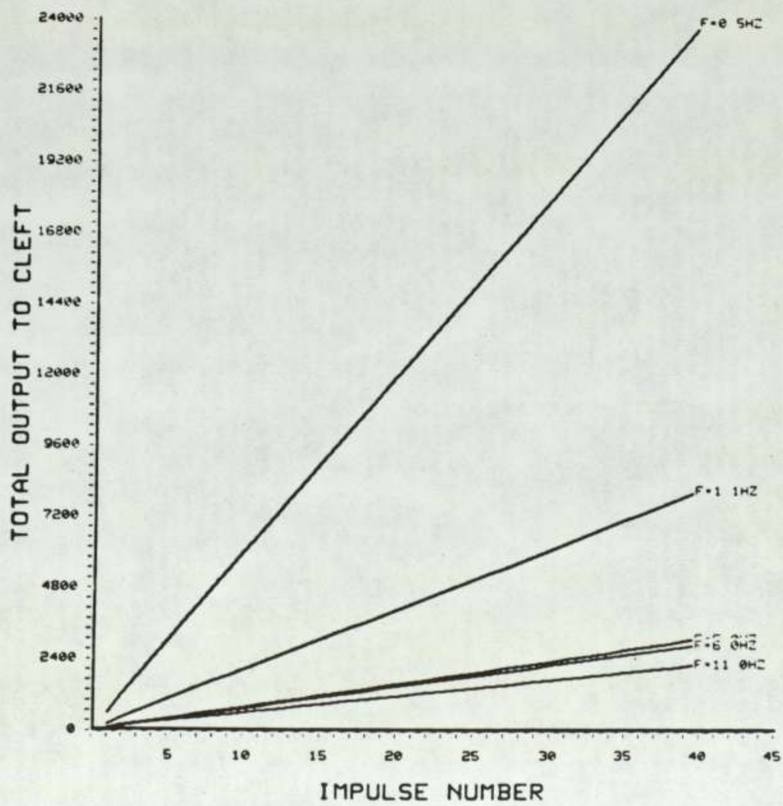


Fig. 73

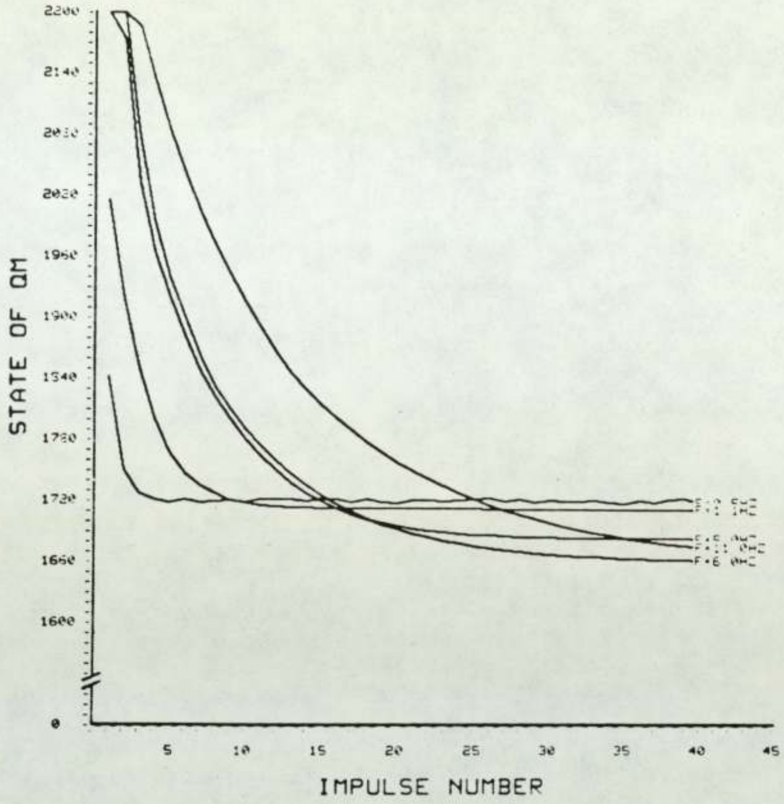
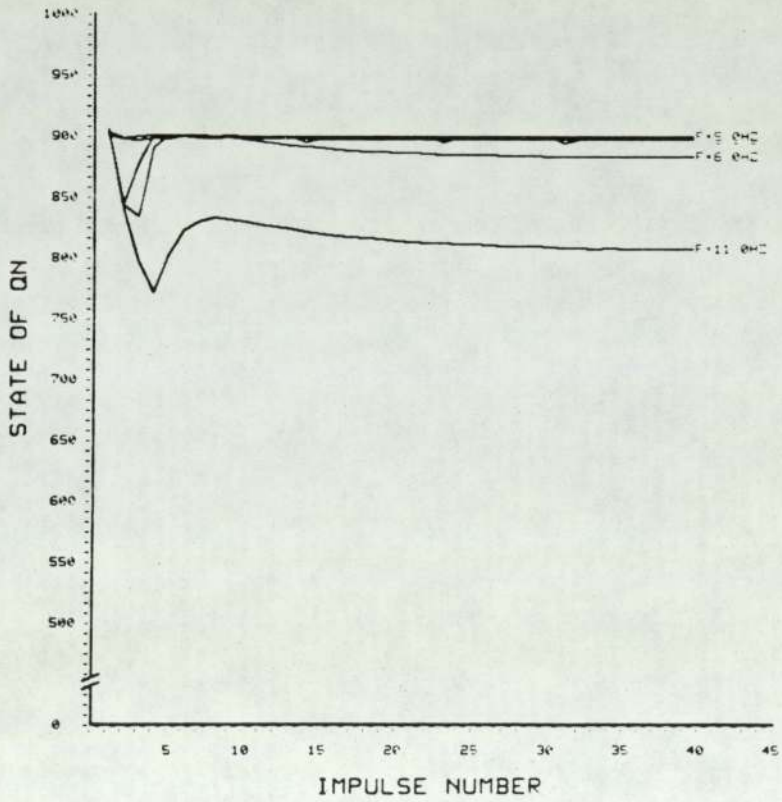


Fig. 74



quanta over the first 375 days of life. It is possible of course that a similar phenomenon exists in humans but no data is available. In consequence there is little direct quantitative evidence with which to validate this model, although various inferences and opinions may be used to complement what data does exist and substantiate some of the assumptions and findings[12,71].

Overall model philosophy although complex, may be considered to comprise three distinctly different processes used to define storage and movement of acetylcholine within the nerve terminal. Although independent in operation, an interrelationship exists which connects all parts of the model through stimulii, defined by a series of equations, to give both magnitude and timing. It seems no accident therefore, that the final result of these processes is to produce an output to the cleft which appears to mimic end plate potentials seen at the post-synaptic site during physiological experiment.

Experimental evidence shows a falling magnitude (of end plate potential) during the first few impulses of stimulation, followed by an increase in output and then a further slow deterioration in size. This increase in end plate potential following the initial decline has been attributed to a potentiation of fractional release (of acetylcholine) from a mobilisation store, and this fact is reflected in the model response. A rise in end plate potential seems to occur (to a greater or lesser extent), at all frequencies of stimulation, and is potentiated when levels of extracellular Ca^{++} are raised;

facts which are again reproduced in the model.

Further evidence is provided by the response of isolated preparations immersed in a physiological solution containing a high concentration of Mg^{++}. End plate potentials were found to vary in a manner not experienced with preparations in normal or raised Ca^{++} solutions. The model produces a similar type of output, inferring the philosophy outlined earlier to bear some resemblance to reality. Model philosophy assumes extracellular Mg^{++} to affect only the immediate output mechanisms to the cleft, and not to alter the manner in which acetylcholine is transferred internally. In consequence although output from the mobilisation store may be normal, leakage, synthesis and evoked output become inhibited in such an environment.

Experiments conducted on isolated human muscle preparations in the presence of high concentrations of Mg^{++} exhibit phenomena not easily explained. The literature shows preparation responses to stimulating frequencies of 11 and 47 Hz. to be similar, indeed, plotting these two characteristics as one graph shows no distinction between them. The results of a statistical analysis suggests this composite characteristic to comprise data produced from the same statistical population.

This raises several interesting questions. Published data, if correct, indicates a constant output for stimulating frequencies well in excess of 11 Hz. and continuing at least to 47 Hz. A significant decrease in end plate magnitude is seen when a stimulating frequency

of 110 Hz. is used suggesting a fall in characteristic to occur at a frequency greater than 47 Hz. The model does not and cannot mimic such a response although outputs produced at a stimulating frequency of 47 Hz. are similar to those published in the literature at 110 Hz.

Consideration was given to the possibility that a mistake had been made during publication (of the experimental data) but a personal communication with the author[207] revealed that although the original data was no longer available, this conclusion could not be substantiated. The experimental data must therefore be assumed correct and in consequence the model incapable of mimicking physiological events when preparations are exposed to extracellular mediums with a high concentration of Mg^{++}. The confusion and general lack of understanding of the relationship of calcium and magnesium ions to physiological response, makes it impossible to convert model function at this time.

It will be noticed, however, that the range of calcium and magnesium solutions that are likely to exist in vivo (even in the very ill patient) are well short of levels created during in vitro experiments. Amounts of these chemicals in the proportions used in the literature do not exist in reality and in consequence distortions may have been produced during experiment.

Quantitative experiments against which evoked responses of the model are compared, were the product of an averaging technique produced by only five muscle fibres

in solutions of raised Ca^{++} and Mg^{++}. Although normal responses were produced following averaged estimations on 26 similar fibres, it is unfortunate that so much emphasis must be placed on these results; there being little comparable evidence on human muscle. If a system to regulate neuromuscular blockade in humans in to evolve, it seems only sensible to base this model on relative data. Quantitative and qualitative information produced over the past 30 years on other animal preparations such as frog and rat[9,203,208,209,210] has, however, been carefully considered and used wherever possible.

Incorporating effects of calcium and magnesium into the model has been simplistic in approach. The action of calcium in the model is considered to be direct and not influenced by the manner in which nerve impulses are propagated at the nerve ending[125]. This may not however be the case. Although it has been suggested that common conductance pathways are used by calcium ions for both spontaneous and evoked responses[211] it is possible that very complicated chemical mechanisms are activated by an influx of Ca^{++} and it is these that are responsible for changes in acetylcholine output. For example a possible role of sialic acid containing substrates has recently been postulated[212], where the substrates act as Ca^{++} receptors. Further recent work on the ability of calcium to influence release has also indicated these mechanisms to be indirect, with some other factor present (e.g. protein) which reacts with the calcium ion to form the necessary complex capable of

invoking release[68,131,213].

Although an increase in Ca^{++} tends to increase both evoked and spontaneous release[214] it is not the only ion capable of doing so. The presence of raised potassium and lowered sodium has also been shown to cause changes in spontaneous release irrespective of amounts of calcium present[215]. The parameter K_2 which forms part of the model feedback expression may well be a variable which incorporates levels of potassium and sodium although model response under these circumstances could not be corroborated by experimental evidence. The model does not cater for changes in sodium and potassium but assumes normal conditions throughout all simulations. Under these circumstances, an increase in Ca^{++} is the only reason for an increase in the frequency of the mepp[214,216]. This effect is reflected implicitly in the model, by providing an overall increase in the amount of acetylcholine released by spontaneous activity; an opposite effect taking place with increased Mg^{++}, with leakage changing in proportion to the amounts of these chemicals in extracellular solution.

What the model cannot mimic, however, are structural changes that take place if levels of calcium are increased to high proportions[217] for long periods of time. The model does, however, produce results that are obviously unrealistic if high calcium values are used. Magnesium does not appear to have this ability to irreversibly change the structure of the neuromuscular junction but its effects are not well documented and in consequence

not readily modelled.

There are other indications besides comparisons to evoked response output which reinforce model philosophy. An exhaustive series of experiments carried out in the early 1970's[71] and recently confirmed in part[218] on synthesis, storage and release of acetylcholine in isolated rat diaphragm muscle, lends weight to arguments produced by the model. Although exact and absolute values were unpublished, results were given as a percentage of total available store population measured at the time. Hemicholinium-3 introduced into the physiological salt solution, inhibited synthesis of extracellular choline and thus emission of acetylcholine became totally dependent on the content of endogenous stores resident within the nerve terminal. The experiments provided an estimation (over 360 nerve impulses at a stimulating frequency between 1-20 Hz.) of approximately 0.022% of total store size, released with each stimulating impulse. If model stores (predicted earlier) are combined, the total available acetylcholine may be considered to be 183,200 quanta, and an output of 0.022% of this total would produce an average around 40 quanta/stimulus to the cleft. It follows that such an output, in the absence of synthesis, would emanate from a store with capacity to provide 4,580 impulses. The experimental evidence suggests that if all measured acetylcholine were resident in the nerve terminal, impulses containing 0.022% of total store size (in the absence of synthesis) would be produced in response to around 4,600 stimulations. It must be noted, however,

that a significant proportion of measured acetylcholine would have come from the surrounding muscle. Other observations have shown similar preparations to release about 0.033% of total store size per impulse[219] when stimulated at a frequency of 1 Hz.

In the presence of eserine (i.e. a cholinesterase inhibitor) store sizes are known to double in content over a period of approximately one hour[71]. This facet of the physiology has been accommodated in synthesis expressions used by the model, and these in turn have contributed significantly in producing the response curves shown in chapter 4. The inference that some form of esterase or hydrolysing component is present within the nerve terminal seems a reasonable proposition, as under normal conditions store sizes are unable to attain those levels achieved during the eserine experiments.

It seems logical that store sizes should increase in excess of normal resting levels immediately following tetany. There are numerous observations of isolated preparations producing significant increases in epp when restimulated after a short rest (approx. 8 sec.) following tetany. The shape of the output characteristic remains unaltered but the magnitude is enhanced. This phenomenon, termed post-tetanic potentiation, is probably due to a large influx of quanta from various sources following a severe depletion of the readily releasable store.

It has not been possible to use available computing facilities to carry out such a simulation. The computer model normally requires an uninterrupted period of 26 hours

to produce responses to 40 impulses over six frequencies of stimulation. Calculations reveal the use of the model to simulate post-tetanic potentiation in the manner just described to be impractical under present circumstances, although an estimation of probable model function may be considered.

Tetany would quickly deplete the readily releasable store to a low value which would not improve with an increase in synthesis. Stimulii would be presented to the mobilisation store indicating the severity of this depletion and in response large numbers of quanta would be released and made available to the readily releasable store within a short period of time. The output characteristic would soon reflect the presence of this new input in a manner previously described, but would be unable to provide a lasting effect. The needs of the readily releasable store would soon cause the mobilisation store to become depleted and signals would be created invoking release mechanisms of the latent store which holds most of the available acetylcholine in vesicles. This part of model function would not be evident during tetany and is not apparent in any of the results shown in chapter 4, but would nevertheless occur.

The literature shows that restimulating the preparation only a short time following tetany does not produce this phenomenon of post-tetanic potentiation, indicating the event to be time dependent. The model would require a significant time (several seconds) to move vesicular quanta between latent and mobilisation stores, although the

number involved would be large. In consequence, once this mechanism has been initiated it should be possible for the mobilisation store to be refilled well in excess of its normal steady state capacity. This in turn would generate a large influx of quanta to the readily releasable store again causing an inflation of normal store content. In these circumstances it can be predicted with some certainty, that subsequent stimulation would cause an output response in excess of that produced during normal working conditions.

Although the above theory of operation is speculative, testing the model under certain conditions does produce an output which tends to reinforce the argument of a large quantal movement of acetylcholine between latent and mobilisation stores. If the model is presented with a stimulating frequency of 0.0167 Hz. (i.e. 1/minute) it seems reasonable to expect the magnitude of the second impulse to be very similar to that of the first; the readily releasable store being replenished during the long interval between stimuli. The number of time increments used to define this interval was increased to 10,000 in an effort to retain model accuracy, although job time increased dramatically (i.e. about 100 fold). In practical terms, a 24 hr. period was now required to produce results from only three stimulations and these are as shown in Appendix II.

These results have not been included in chapter 4 as the simulation is incomplete. The magnitude of response for all three stimulations is similar, as

expected, with readily releasable store replenished within the interval. It will also be noticed that the content of the mobilisation store is in a state of oscillation and this raises several interesting points.

The initial content of the store (i.e. of some 2,200 quanta) is shown to increase significantly during the stimulation, although this effect is not obvious in the state of the readily releasable store. The constance of Q_n is due to the predominance of leakage and hydrolysis which remove quanta at a rate appropriate to the store inputs at the time; similar conditions do not prevail in the mobilisation store. This remarkable condition of undamped oscillation within the model seems at face value to be unrealistic, as the total physiological system shows remarkable control (e.g. voluntary movement). There is, however, evidence within the literature concerning an oscillation in store content of free acetylcholine[100].

A series of experiments performed on the electric organ of torpedo measured amounts of free acetylcholine with a time resolution of one second, during the course of stimulation. Stimulating frequencies of about 5 Hz. were provided for varying periods of time up to 6 minutes and electrophysiological data was produced prior to the removal of the specimen for estimation of remaining transmitter. Results showed a decreasing electrophysiological output as expected (and confirmed by many workers) but of more significance a store of free acetylcholine resident within the biological preparation that was in a state of oscillation. At least two types

of oscillation were evident; a fast wave occurring every two seconds superimposed on a slower wave with a period of approximately 4 minutes. These oscillations were said to be caused almost entirely by levels of free acetylcholine existing within the preparation; earlier work having shown the amount of bound acetylcholine to stay at a constant value.

These observations are particularly relevant to the model and are not accounted for in current hypotheses. The model reflects to some extent this phenomenon in the results of Appendix II, which show it is possible for the mobilisation store of the model to be in a state of undamped oscillation. This condition is not reflected in the state of the readily releasable store, or output to the cleft, and therefore would remain undetected during electrophysiological experiment. It is possible that the contributing factor to the slow oscillation may be from synthesis. The suggestion that full effects from synthesis may not be present for some minutes after stimulation[220] has been around for some time and the model caters for this event by linking synthesis to store content.

One interesting feature present in the model but not appearing in the literature, concerns the influence exerted by the mobilisation store on the readily releasable store. The literature, when considering two stores of this type, often suggests a mobilisation store is held only in a state of readiness and rarely used, as synthesis is more than capable of supplying most needs of

a readily releasable store. Model function, however, causes the mobilisation store to play a fundamental role in quantal output. Even in a resting state this store supplies a steady input to the readily releasable store, even though synthesis occurs simultaneously. In consequence this mobilisation store is not used merely in cases of dire need, but is an integral part of the release mechanism of the model.

The model is however, imperfect, and certain phenomena are still omitted due to lack of experimental evidence. The phenomenon of synaptic delay is probably the most significant observable omission in the model. This delay has been shown to be random, ranging from 0.29 m.s. to 2.3 m.s. and is not due to a failure of an action potential to reach the nerve ending[221]. Neither does it represent diffusion time of acetylcholine molecules across the synaptic cleft. A similar spot concentration of the chemical when put into a beaker of suitable solution produces an estimated diffusion time to be around 1 μs for a distance equivalent to that of the synaptic cleft[107].

The interval therefore originates from a delay in releasing the transmitter following the arrival of an action potential at the nerve ending. It would be a simple matter to put the output of the model through a probability function generator to enable the output, to produce a Poisson or binomial distribution as suggested by earlier workers, but this technique would serve no useful purpose. The philosophy of the model has been

painstakingly presented to show all mathematical features are in some way representative of known physiological events, and to accommodate synaptic delay in the manner just described could only debase model function.

There is a growing view that quantal release of acetylcholine does not follow a Poisson but a binomial distribution[222,223]. If this is true, the probability of release becomes much higher than has hitherto been considered, and in consequence synaptic delay may well be related to physical and chemical conditions of the system and eventually accommodated by the model. Indeed, statistical analyses have shown that as store contents become constant (i.e. during stimulation) any decrease in the number of quanta released per stimulus becomes almost entirely due to the condition of store size; the probability of release remaining constant. It is possible that the delay arises from complex movements of calcium ions which enter the nerve terminal prior to acetylcholine release, and it is unfortunate that so little is known of this ionic movement.

Facilitation is also omitted by the model: that is the ability of the nerve ending to produce (sometimes) an increase in output on subsequent impulses[224]. Work has been produced linking this effect with residual calcium remaining fixed to release sites[225] but how this occurs (or even if it is true) is still unknown. A recent paper has shown a decrease in the permeability of the membrane to Ca^{++} in the presence of cholinergic agents[226]. However, these experiments carried out on the

frog neuromuscular junction provide information in conflict with events known to occur at mammalian sites and further clouding the issue.

Other parameters are also omitted that are known to affect neuromuscular function. Variations in sodium and potassium[124,140] levels have already been mentioned as fundamental in release of the transmitter. Changes in age[227], temperature[228], pH, pO_2 and pCO_2 are also neglected, although evidence exists which relates the condition of these parameters to the stability of neuromuscular function. It is probable that these variables can ultimately become a part of what are at present system constants.

The model is complex and difficult to validate fully, making the introduction of further unknowns an added complication that at present serves no useful purpose. However, the model is also flexible and with an increase in computer power, would be able to accommodate some of these omitted variables and parameters in its present state, and thus may ultimately be of some use in describing acetylcholine release at the neuromuscular junction.

CHAPTER SIX

CONCLUSIONS

The model detailed in chapters 3 and 4 appears able to mimic acetylcholine release at a nerve ending stimulated at various frequencies. Although most of the validation concerns model evoked response,various other considerations have been discussed (e.g. post-tetanic potentiation and facilitation) and tested in part.

The effects of calcium, and magnesium ions in the extracellular fluid have been considered, and the model caused to produce outputs similar to those seen during physiological experiment. Model response is limited in this area and although unable to mimic high concentrations of either calcium or magnesium ions produced during *in vitro* experiments, it does produce useful results over a wide range of conditions that may be expected *in vivo*.

Oscillations in the contents of free acetylcholine detected during experiment is also seen in model output. The model suggests these oscillations to be due to the transfer of large numbers of quanta from latent to mobilisation store, which would also explain why these oscillations remain undetected during electrophysiological experiment.

The presence and use of a mobilisation store is considered to be fundamental in producing an evoked response in the model. Most of the literature suggests that the presence of a mobilisation store would not be

apparent during normal release at the neuromuscular junction, but would be available only in times of "dire need". The model makes use of a mobilisation store even at low stimulating frequencies to produce comparable responses to those known to occur in reality. Model philosophy concludes this to be a sensible mechanism, and the result of a well organised, and precise morphometry.

Vigorous validation of the model is impossible at present as there is little available quantitative evidence on human muscle preparations. Model philosophy has, however, been constructed from a wide spectrum of information, gathered from a variety of animals, and it seems no accident that simulated responses concur with available human data.

The model has evolved by considering overall neuromuscular function, and not just a single facet in isolation. Approximately 50 parameters and variables that comprise neurotransmitter release have been related to three distinct processes at work within the nerve ending. Steady state conditions such as synthesis and hydrolysis have been accommodated, as has the transient event of evoked response.

Finally the model has been specifically structured for maximum flexibility so that various parameters omitted at present may be included at a later date, for example levels of potassium and sodium. The proposed approach would therefore appear to offer a systematic means of testing hypotheses in a quantitative manner, and perhaps more importantly to guide the formulation of future experimental protocols.

CHAPTER SEVEN

FUTURE WORK

The model has been produced as a preliminary effort in establishing a means of ultimately controlling neuromuscular blockade in the anaesthetised patient. In consequence any future work must examine the possibility of model function under simulated conditions of neuromuscular block brought about by the presence of a non-depolarising muscle relaxant. To this end there are three distinct courses of action that might be persued in the continuance of this work.

(i) Model expansion

The latter part of chapter 5 has pointed to areas of the present model in need of further attention. Before attempting to include extra variables, or parameters, the computer programme should be transferred to a more powerful machine so that job time can be reduced, and effects such as post-tetanic potentiation or facilitation examined more closely.

Assuming this procedure can be accomplished there are several factors that may then be considered. Body temperature for example may prove useful, and its' effect can be readily seen when examining facilitation and post-tetanic potentiation[229] (i.e. the evoked response increases but there is a decrease in spontaneous activity).

Levels of electrolytes are also important and should be included in model function. Calcium and magnesium have already been considered, but simulations of sodium and potassium must be produced. Body pH also affects neuromuscular function and this might be included at the same time.

(ii) <u>A model defining the pharmacokinetics of a muscle relaxant</u>

A model might be constructed which can be used to simulate the passage of a muscle relaxant from its injection as a bolus into the blood stream, to its presence at the neuromuscular junction. This is not an easy task however as no two relaxants behave in an identical manner. Both drug distribution, and elimination are difficult to model accurately, and there are many factors that affect drug action which are difficult to simulate (e.g. blood flow, respiratory acidosis/alkalosis, antibiotics, presence of anaesthetic vapour, etc.).

The situation is further complicated if different relaxants are considered to be present simultaneously. For example in the real situation a patient may be given an amount of succinylcholine (a depolarising relaxant) to facilitate intubation prior to surgery. To ensure adequate blockade during an operation, a dose of a non-depolarising relaxant (e.g. Pancuronium Bromide) will also be administered. The time interval between the administration of these drugs, and the basic physiology of the patient (e.g. level of pseudo cholinesterases)

will then determine the level and type of blockade produced.

To model such a situation is an enormous task and should not be undertaken lightly.

(iii) <u>To measure the amount of block present and relate this to the present model</u>

This is perhaps an alternative to the singularly difficult task just mentioned. Methods have already been devised, which attempt to quantify neuromuscular blockade[230,231,232,233] with varying degrees of success. In most operating theatres little effort is made to establish the state of neuromuscular block, although there seems little agreement on a standard test that could be used to do so.

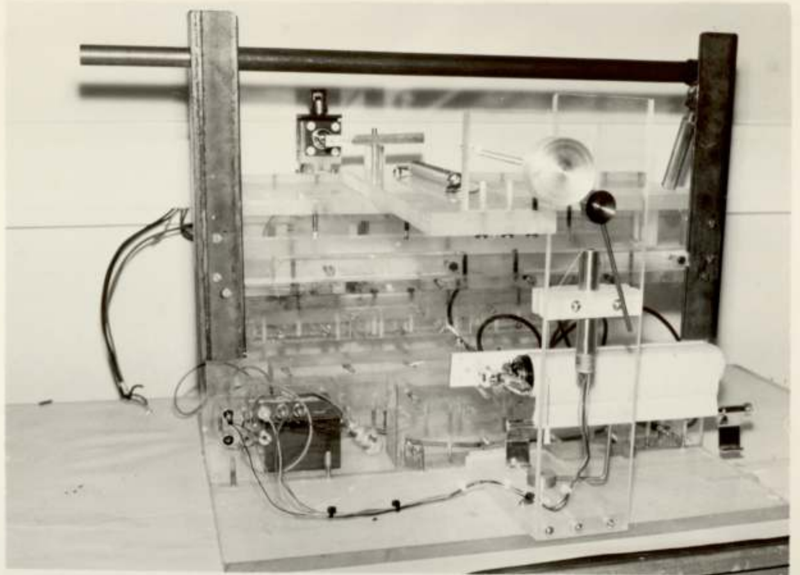
It is possible that further refinement of the present model backed up by experimental evidence concerning neuromuscular blockade, may prove useful in defining a practical and safe control system. To this end a jig has been constructed which measures isometric force produced by the adductor pollicis muscle of the thumb. The hand is held in its physiological resting position, while stimulation is provided through suitable apparatus to the ulnar nerve at the elbow. Apparatus has also been constructed to record either isometric twitch, or isotonic displacement of the thumb, and programmes have been constructed which carry out an analogue to digital conversion on the recorded data, and present it in a manner suitable for manipulation.

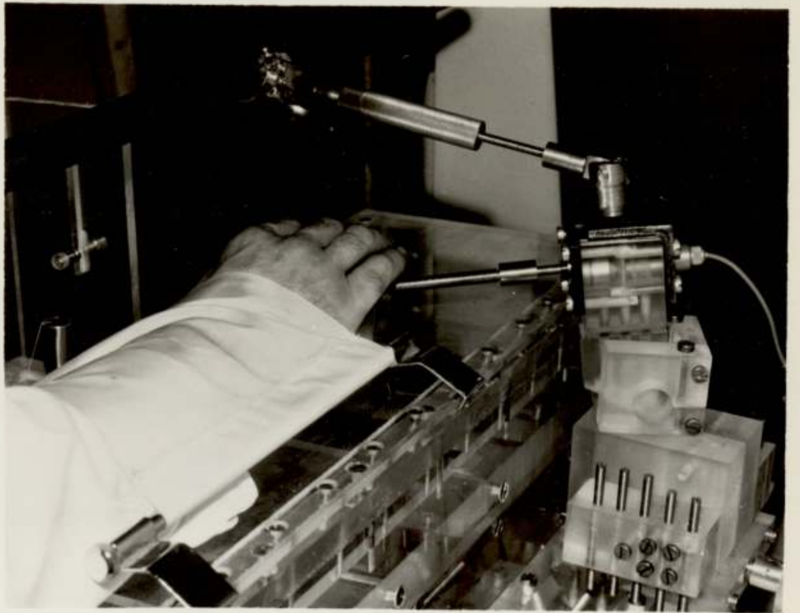
Photographs of the jig and recording system are shown overleaf.

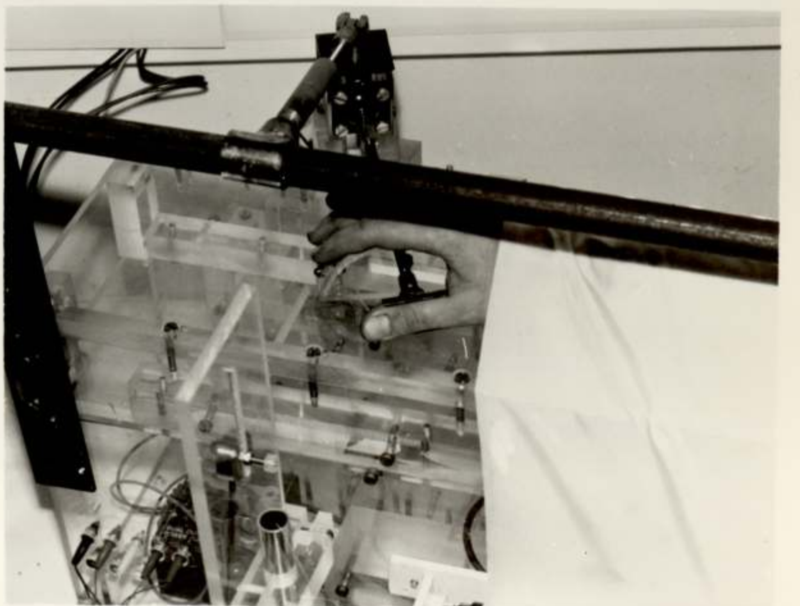
A series of useful experiments may be to occlude the venous return of an arm, and inject a sufficient quantity of muscle relaxant so as to cause paralysis of the limb. With the ulnar nerve continually stimulated a picture of the onset of blockade may be established. With the occlusion (to the venous return) removed it should be possible to gain useful information concerning the dissociation of the relaxant from the neuromuscular junction.

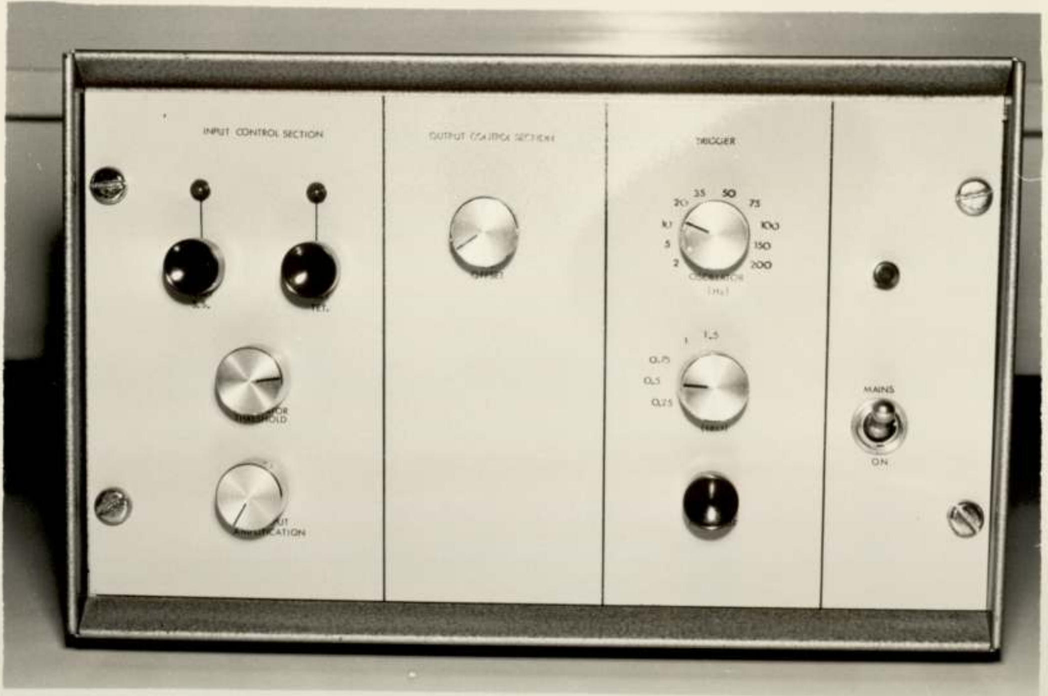
Using this technique, and measuring several other parameters (e.g. blood flow, heart rate, temperature, blood gases, etc.) it may be possible to simulate the event and link this to the model presented in this thesis.

Whichever method is used the process is difficult and will require many years of work to adequately describe in a quantitative fashion this phenomenon of neuromuscular function.








INPUT CONTROL SECTION
OUTPUT CONTROL SECTION
TRIGGER
MAINS
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CEC
DataTape
VR-3300

APPENDIX I

The computer used to test the hypothesis was a Varian V76 mini computer, used as a system orientated general purpose machine for on line data processing. The system utilized a semiconductor core with 16 Bit word lengths, and approximately 25 K words of usable memory.

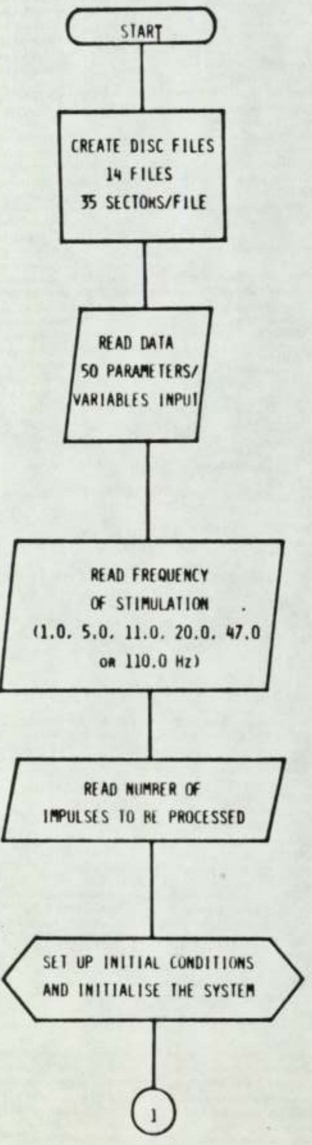
When translated into its binary version, the programme with two real arrays of 2000 elements each could just be loaded and operated by the machine. The programme creates a large quantity of data that is accessed and updated constantly throughout the analysis. As the machine was unable to contain this data in core, 14 scratch files were created on hard disc. These files may be considered to be equivalent to 14 real arrays each of 2000 elements. The programme was constructed in such a way that only two arrays needed simultaneous manipulation at any time. Information was transferred from disc to the arrays held in core, manipulated, and an updated version returned to the scratch files.

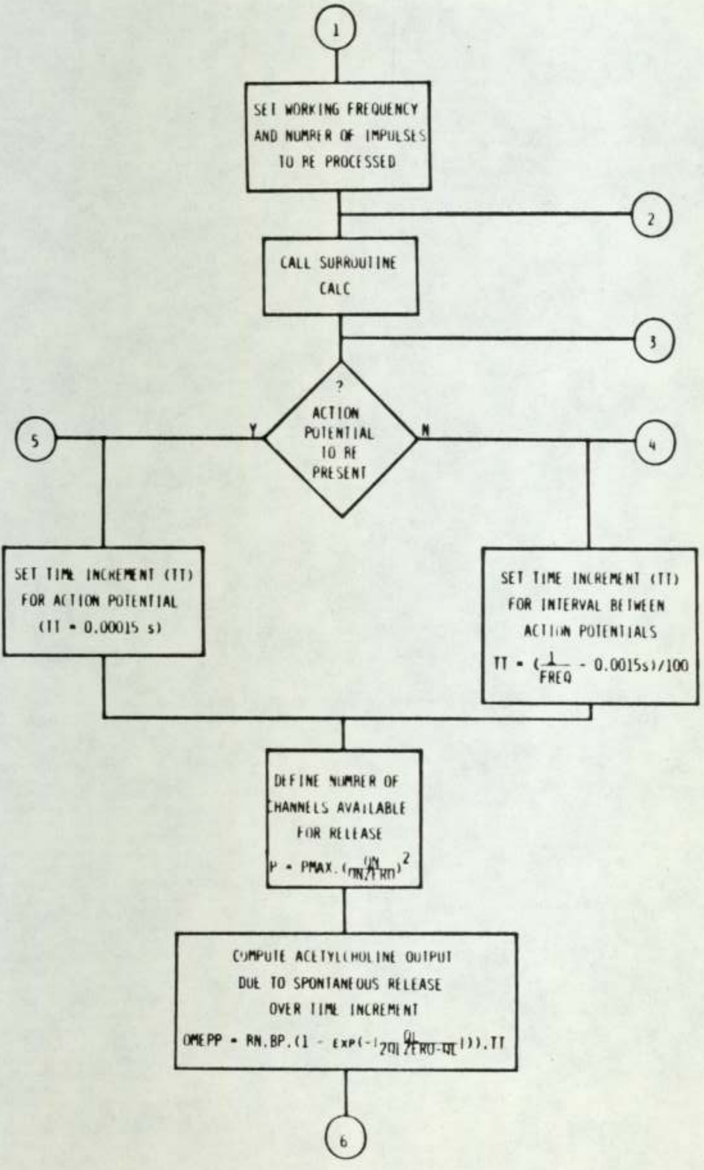
This procedure was inherently slow and caused a full six frequency simulation, each of 40 impulses, to take approximately 26 hours to complete. The computer was heavily used during the working week and these simulations could only be carried out at weekends, or a part simulation overnight.

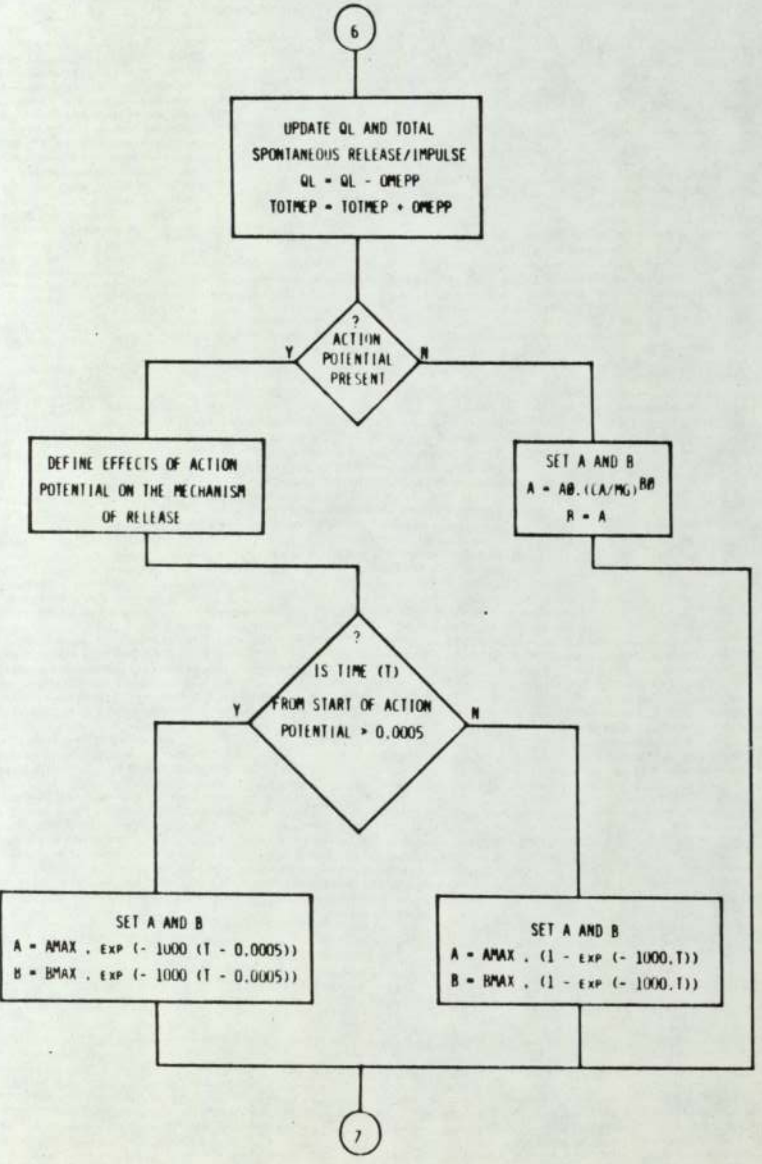
The analysis has taken some 65 useful simulations requiring approximately 1100 computer hours. System

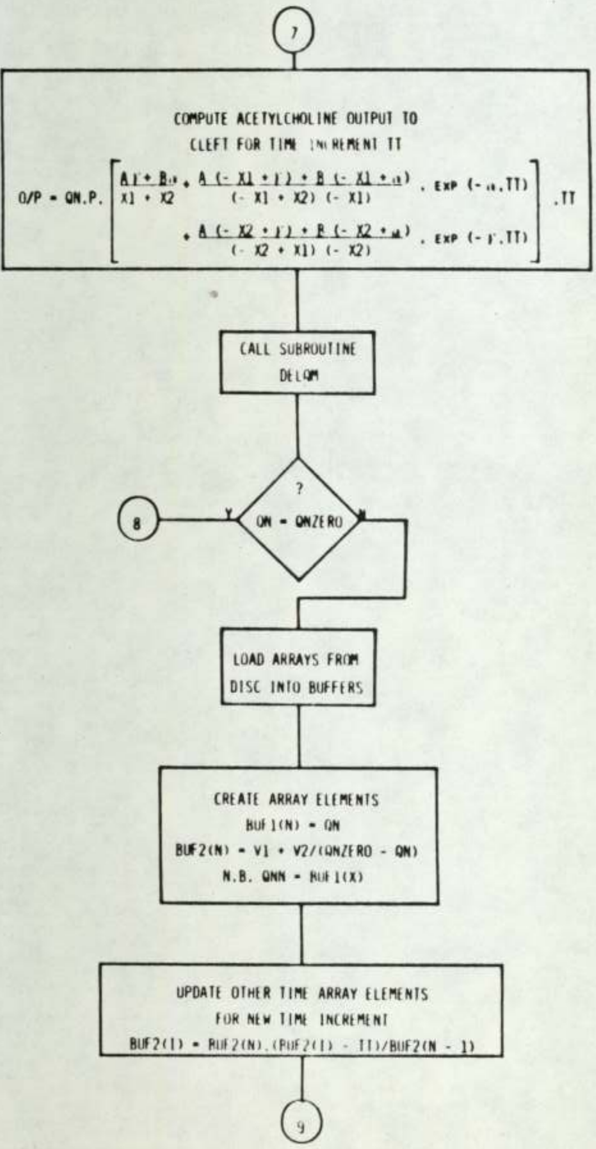
parameters and variables were input to the machine by paper tape, and data produced during the simulation was stored on disc prior to the production of a hard copy. All graphs shown in chapter 4 were produced separately by another programme (but using the data stored on disc) and output directly to a electrostatic printer of resolution 0.01".

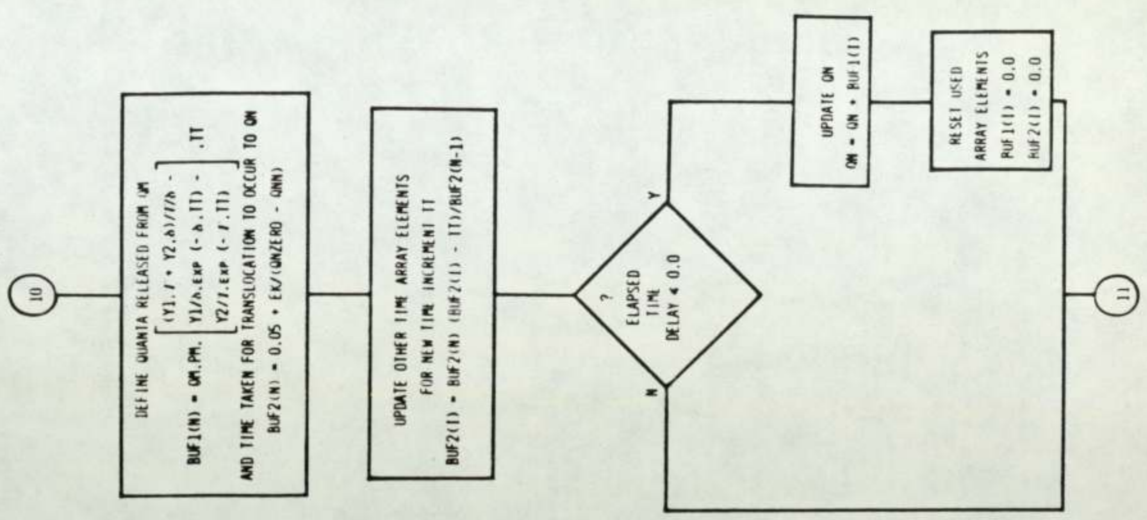
The following flow chart is intended to explain programme function, and to provide the reader with an insight into how the model philosophy (detailed in chapter 3) has been adapted for implementation by a digital computer, although the actual computer programme is not contained in this appendix. Techniques used to solve equations and manipulate data are also omitted, but it is hoped that sufficient information is provided to successfully describe programme function.


START
CREATE DISC FILES
14 FILES
35 SECTORS/FILE
READ DATA
50 PARAMETERS/
VARIABLES INPUT
READ FREQUENCY
OF STIMULATION
(1.0, 5.0, 11.0, 20.0, 47.0
OR 110.0 Hz)
READ NUMBER OF
IMPULSES TO BE PROCESSED
SET UP INITIAL CONDITIONS
AND INITIALISE THE SYSTEM
1

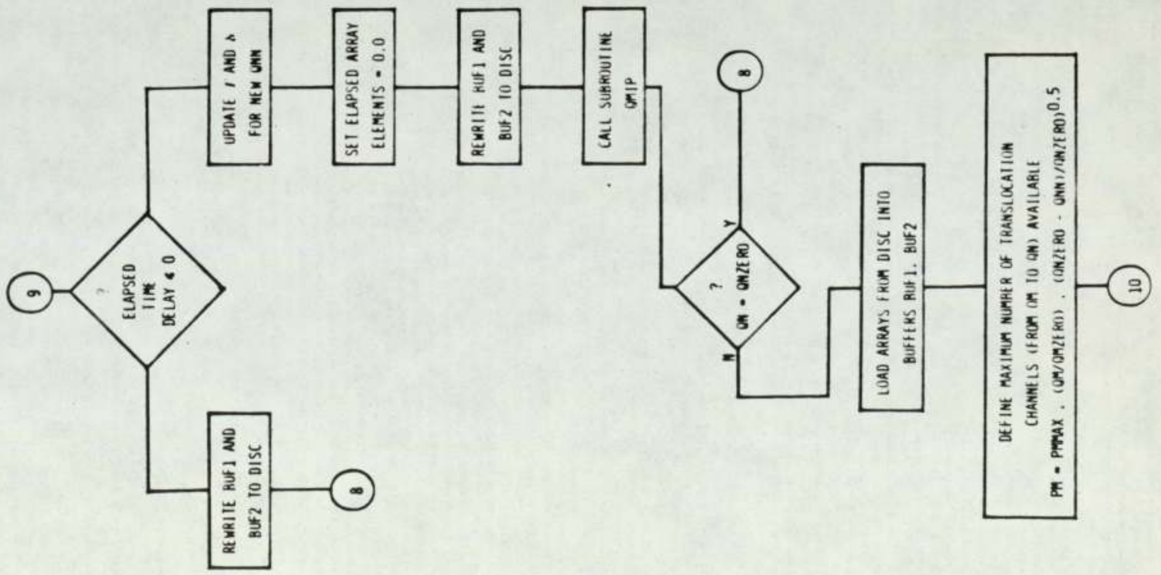

1
SET WORKING FREQUENCY
AND NUMBER OF IMPULSES
TO BE PROCESSED
2
CALL SUBROUTINE
CALC
3
?
ACTION
POTENTIAL
TO BE
PRESENT
Y
N
5
4
SET TIME INCREMENT (TT)
FOR ACTION POTENTIAL
(TT = 0.00015 s)
SET TIME INCREMENT (TT)
FOR INTERVAL BETWEEN
ACTION POTENTIALS
DEFINE NUMBER OF
CHANNELS AVAILABLE
FOR RELEASE
COMPUTE ACETYLCHOLINE OUTPUT
DUE TO SPONTANEOUS RELEASE
OVER TIME INCREMENT
6


6
UPDATE QL AND TOTAL
SPONTANEOUS RELEASE/IMPULSE
QL = QL - OMEPP
TOTMEP = TOTMEP + OMEPP
?
ACTION
POTENTIAL
PRESENT
Y
N
DEFINE EFFECTS OF ACTION
POTENTIAL ON THE MECHANISM
OF RELEASE
SET A AND B
A = AØ.((A/MG)BØ
B = A
?
IS TIME (T)
FROM START OF ACTION
POTENTIAL > 0.0005
Y
N
SET A AND B
A = AMAX . EXP (- 1000 (T - 0.0005))
B = BMAX . EXP (- 1000 (T - 0.0005))
SET A AND B
A = AMAX . (1 - EXP (- 1000.T))
B = BMAX . (1 - EXP (- 1000.T))
7

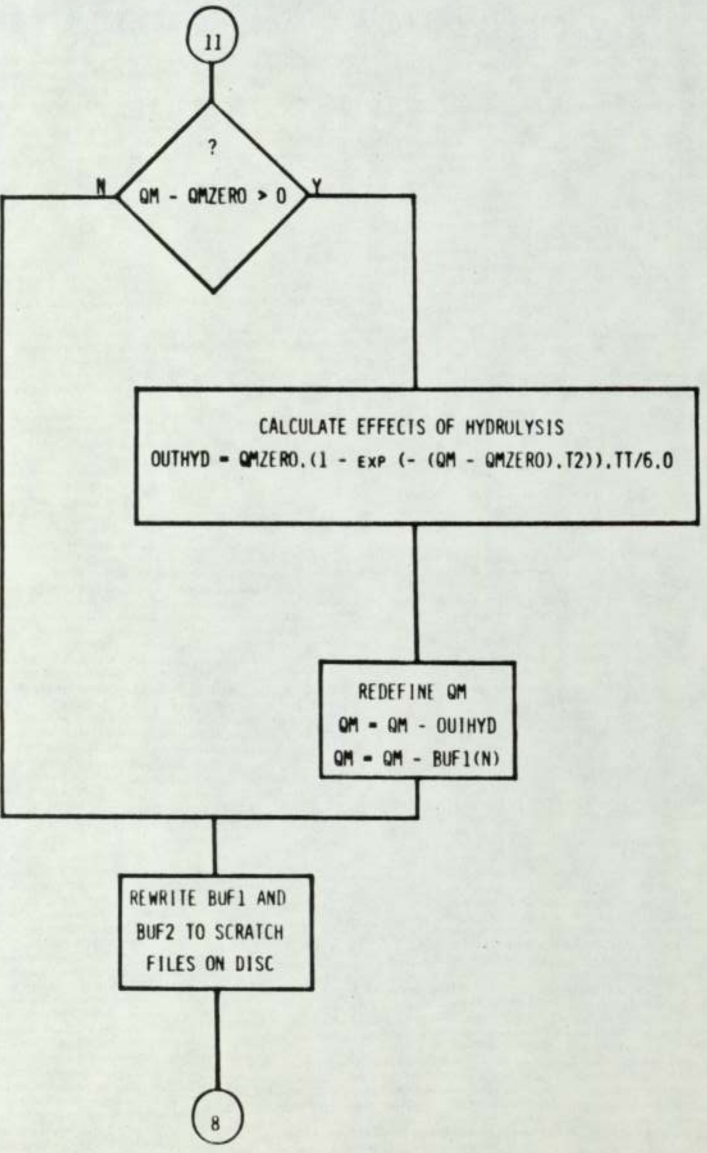

7
COMPUTE ACETYLCHOLINE OUTPUT TO
CLEFT FOR TIME INCREMENT TT
O/P = QN.P.
X1 + X2
(- X1 + X2) (- X1)
(- X2 + X1) (- X2)
.TT
CALL SUBROUTINE
DELQM
?
QN = QNZERO
Y
N
8
LOAD ARRAYS FROM
DISC INTO BUFFERS
CREATE ARRAY ELEMENTS
BUF1(N) = QN
BUF2(N) = V1 + V2/(QNZERO - QN)
N.B. QNN = BUF1(X)
UPDATE OTHER TIME ARRAY ELEMENTS
FOR NEW TIME INCREMENT
BUF2(I) = BUF2(N).(BUF2(I) - TT)/BUF2(N - 1)
9

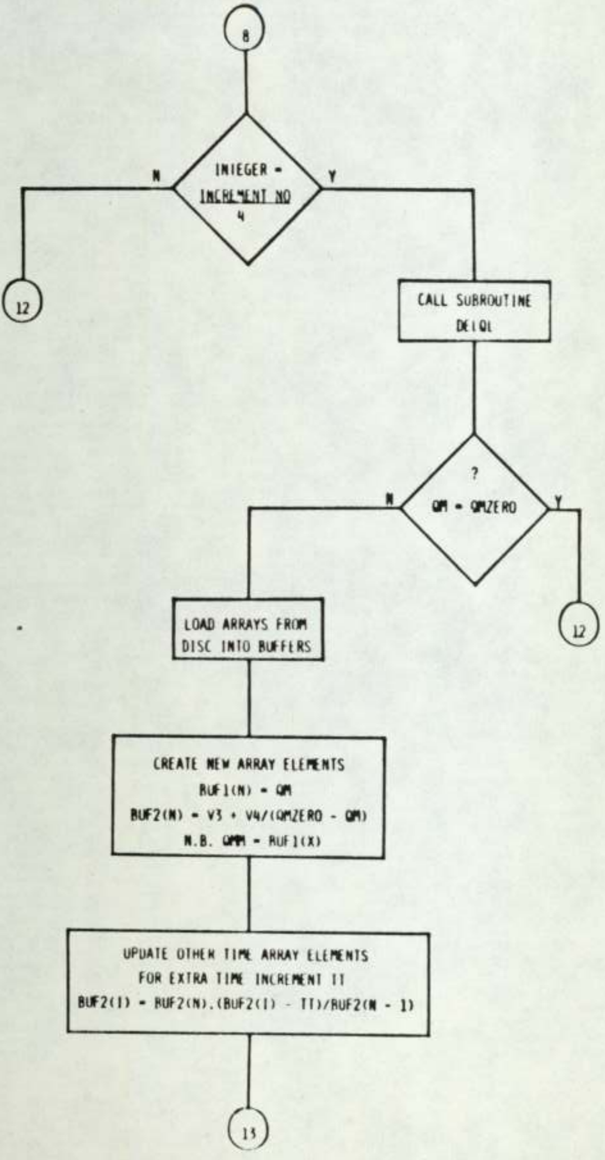


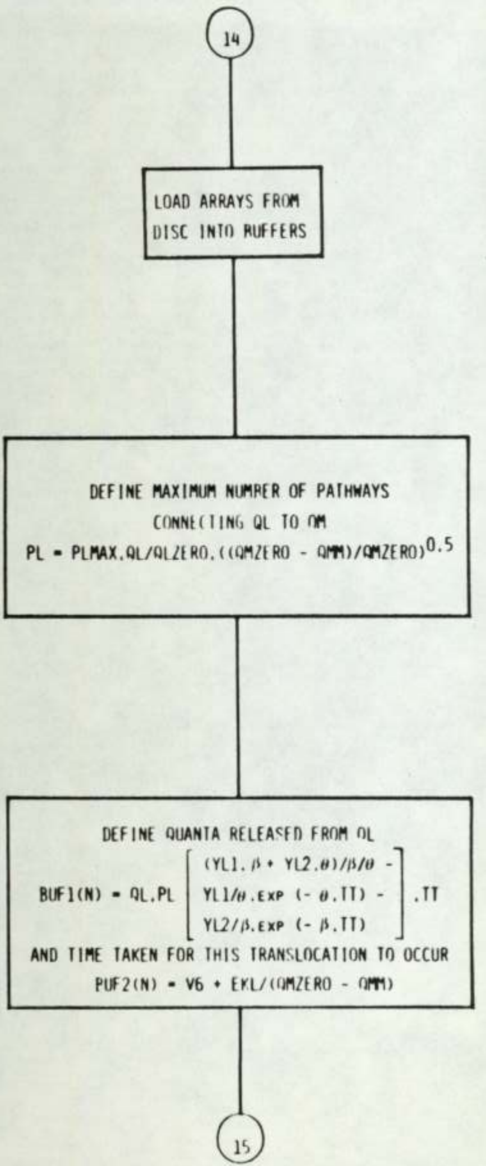
10
DEFINE QUANTA RELEASED FROM QM
BUF1(N) = QM.PM. (Y1.γ + Y2.λ)/γλ - Y1/λ.EXP (-λ.TT) - Y2/γ.EXP (-γ.TT) .TT
AND TIME TAKEN FOR TRANSLOCATION TO OCCUR TO QN
BUF2(N) = 0.05 + EK/(QNZERO - QNN)
UPDATE OTHER TIME ARRAY ELEMENTS
FOR NEW TIME INCREMENT TT
BUF2(I) = BUF2(N) (BUF2(I) - TT)/BUF2(N-1)
?
ELAPSED
TIME
DELAY ≤ 0.0
Y
N
UPDATE QN
QN = QN + BUF1(I)
RESET USED
ARRAY ELEMENTS
BUF1(I) = 0.0
BUF2(I) = 0.0
11

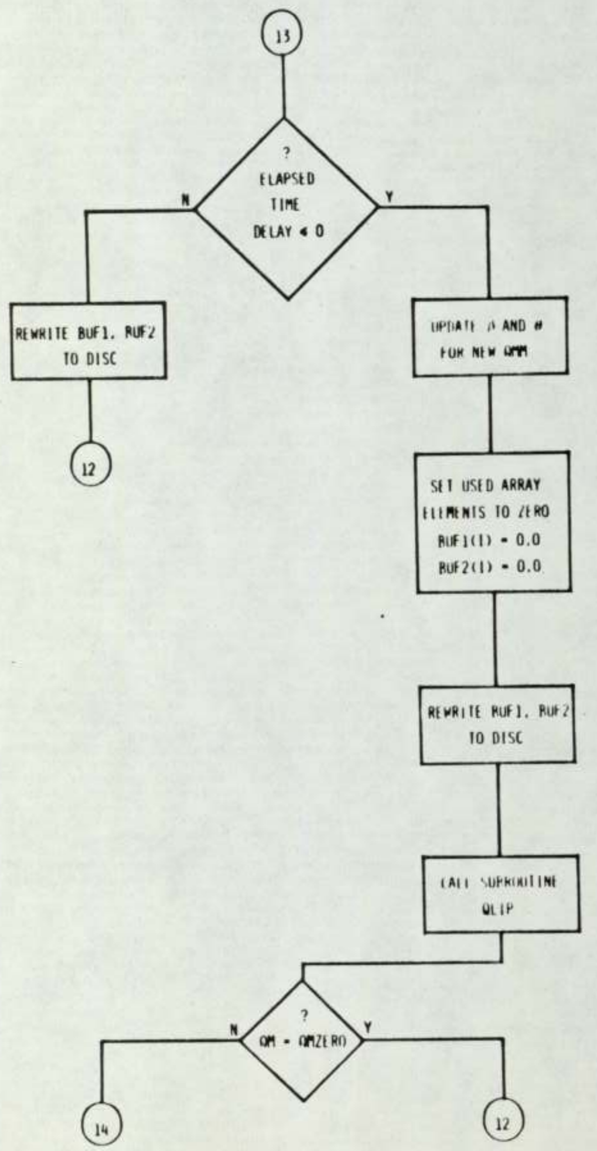


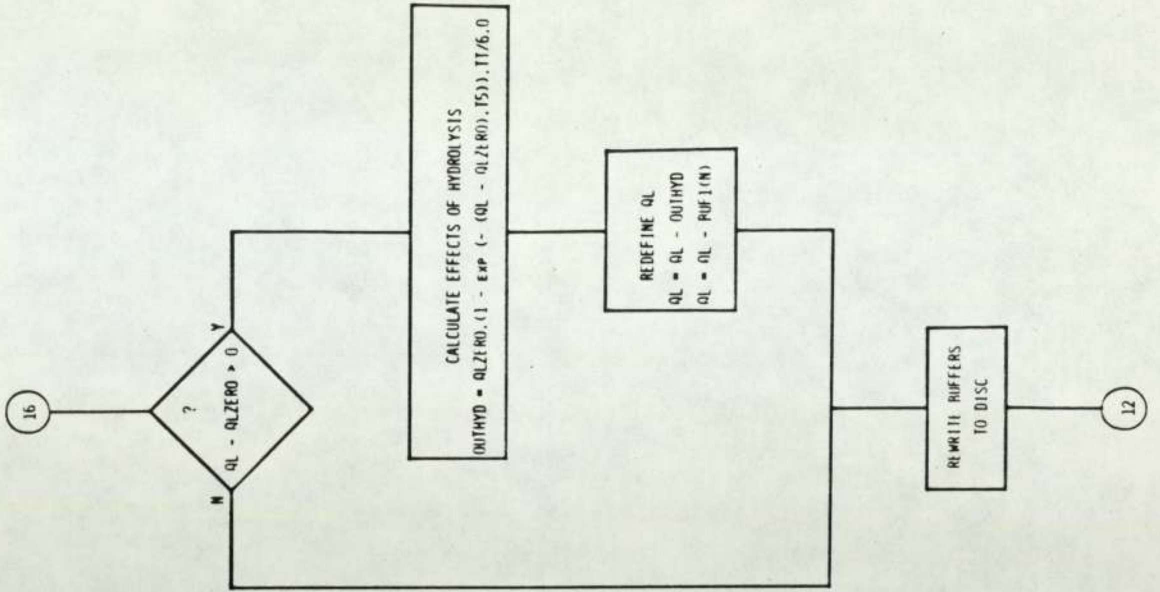
9
?
ELAPSED
TIME
DELAY ≤ 0
REWRITE BUF1 AND
BUF2 TO DISC
8
UPDATE γ AND λ
FOR NEW QNN
SET ELAPSED ARRAY
ELEMENTS = 0.0
REWRITE BUF1 AND
BUF2 TO DISC
CALL SUBROUTINE
QMIP
?
QM = QMZERO
Y
N
8
LOAD ARRAYS FROM DISC INTO
BUFFERS BUF1, BUF2
DEFINE MAXIMUM NUMBER OF TRANSLOCATION
CHANNELS (FROM QM TO QN) AVAILABLE
PM = PMMAX . (QM/QMZERO) . (QNZERO - QNN)/QNZERO)0.5
10

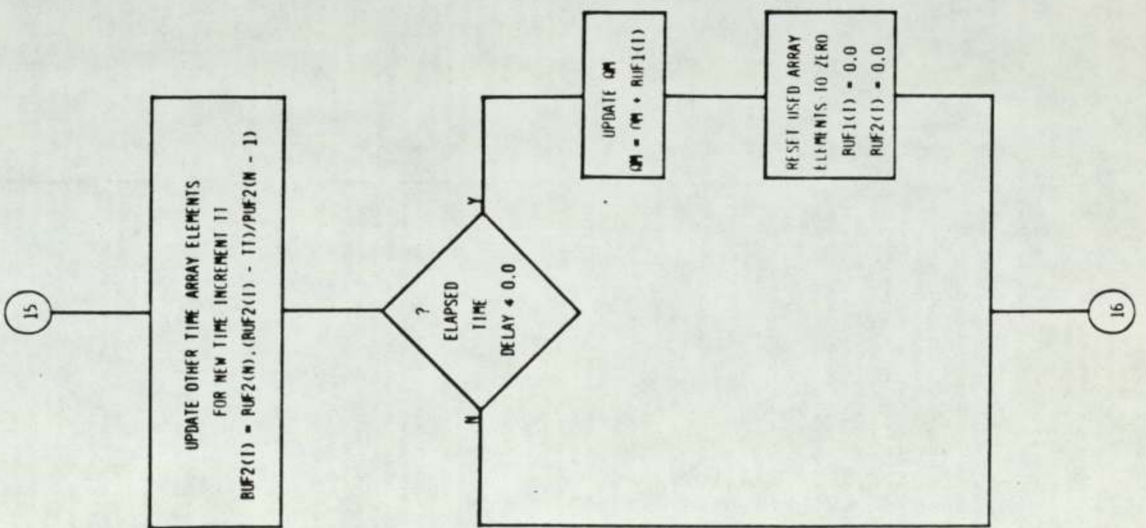

11
?
QM - QMZERO > 0
N
Y
CALCULATE EFFECIS OF HYDRULYSIS
OUTHYD = QMZERO.(1 - EXP (- (QM - QMZERO).T2)).TT/6.0
REDEFINE QM
QM = QM - OUTHYD
QM = QM - BUF1(N)
REWRITE BUF1 AND
BUF2 TO SCRATCH
FILES ON DISC
8

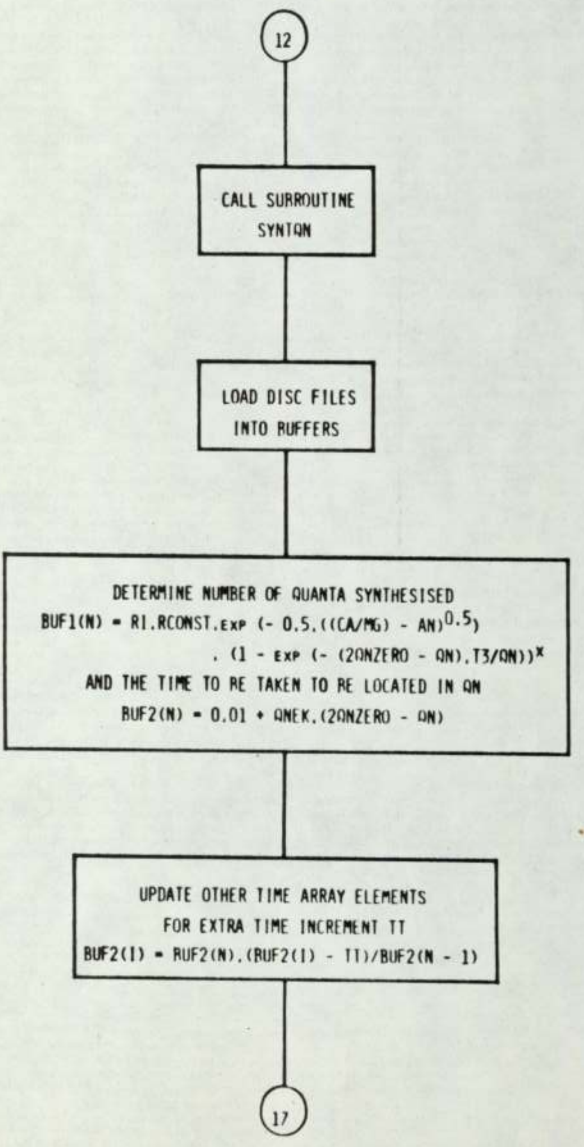

8
INIEGER =
INCREMENT NO
4
N
Y
12
CALL SUBROUTINE
DELQL
?
QM = QMZERO
N
Y
12
LOAD ARRAYS FROM
DISC INTO BUFFERS
CREATE NEW ARRAY ELEMENTS
BUF1(N) = QM
BUF2(N) = V3 + V4/(QMZERO - QM)
N.B. QMM = BUF1(X)
UPDATE OTHER TIME ARRAY ELEMENTS
FOR EXTRA TIME INCREMENT TT
BUF2(I) = BUF2(N).(BUF2(I) - TT)/BUF2(N - 1)
13


14
LOAD ARRAYS FROM DISC INTO RUFFERS
DEFINE MAXIMUM NUMRER OF PATHWAYS CONNECTING QL TO QM
PL = PLMAX.QL/QLZERO.((QMZERO - QMM)/QMZERO)^0.5
DEFINE QUANTA RELEASED FROM QL
BUF1(N) = QL.PL [(YL1.β + YL2.θ)/β/θ - YL1/θ.EXP (- θ.TT) - YL2/β.EXP (- β.TT)] .TT
AND TIME TAKEN FOR THIS TRANSLOCATION TO OCCUR
PUF2(N) = V6 + EKL/(QMZERO - QMM)
15

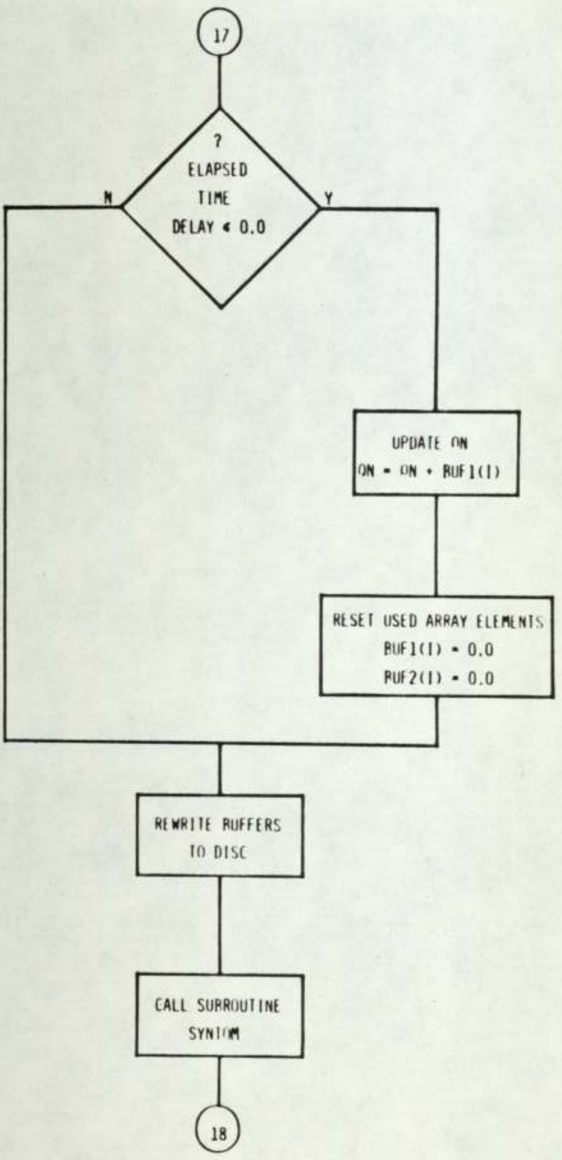

13
?
ELAPSED TIME DELAY ≤ 0
N
Y
REWRITE BUF1, RUF2 TO DISC
12
UPDATE β AND θ FOR NEW QMM
SET USED ARRAY ELEMENTS TO ZERO
BUF1(1) = 0.0
BUF2(1) = 0.0
REWRITE RUF1, RUF2 TO DISC
CALL SUBROUTINE QLIP
?
QM = QMZERO
N
Y
14
12


16
?
QL - QLZERO > 0
Y
N
CALCULATE EFFECTS OF HYDROLYSIS
OUTHYD = QLZERO.(1 - EXP (- (QL - QLZERO).T5)).TT/6.0
REDEFINE QL
QL = QL - OUTHYD
QL = QL - BUF1(N)
REWRITE BUFFERS
TO DISC
12

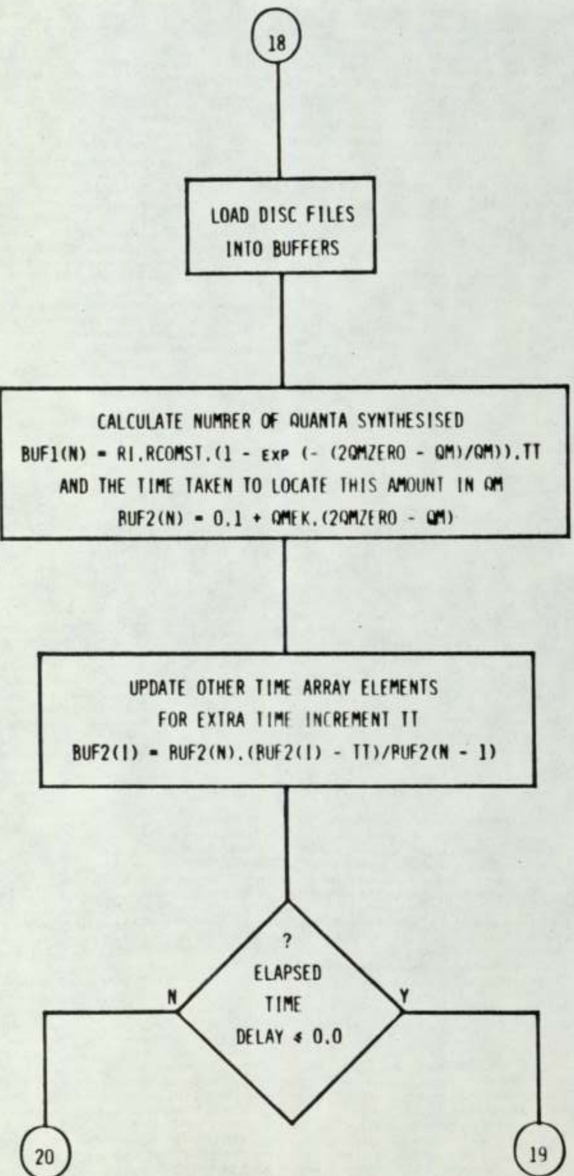

15
UPDATE OTHER TIME ARRAY ELEMENTS
FOR NEW TIME INCREMENT TT
BUF2(I) = BUF2(N).(BUF2(I) - TT)/BUF2(N - 1)
?
ELAPSED
TIME
DELAY ≤ 0.0
Y
N
UPDATE QM
QM = QM + BUF1(I)
RESET USED ARRAY
ELEMENTS TO ZERO
BUF1(I) = 0.0
BUF2(I) = 0.0
16

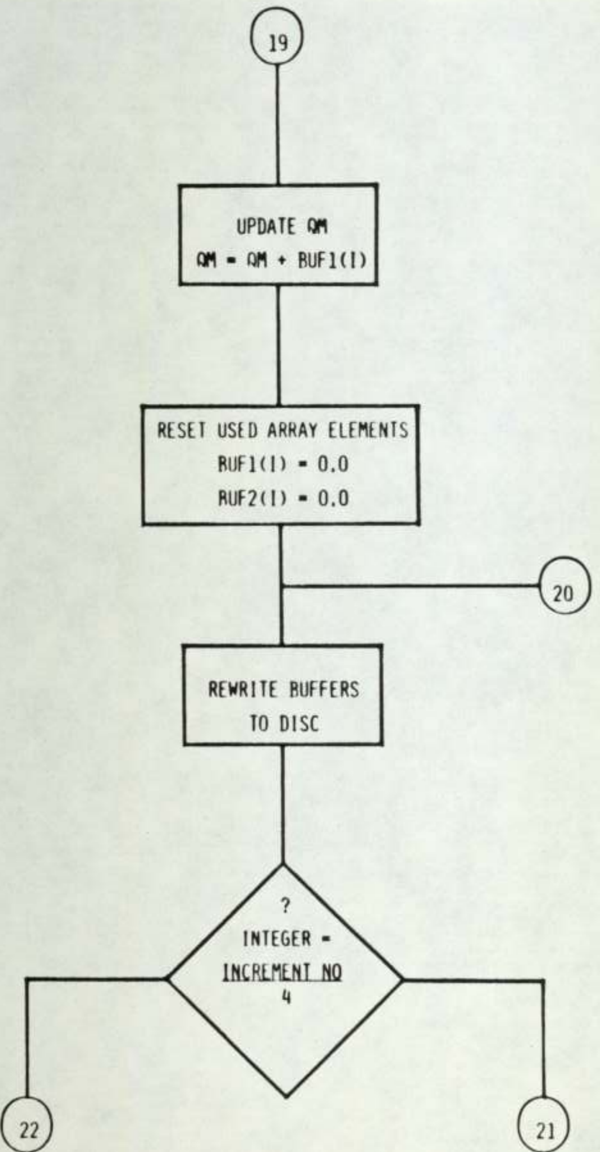


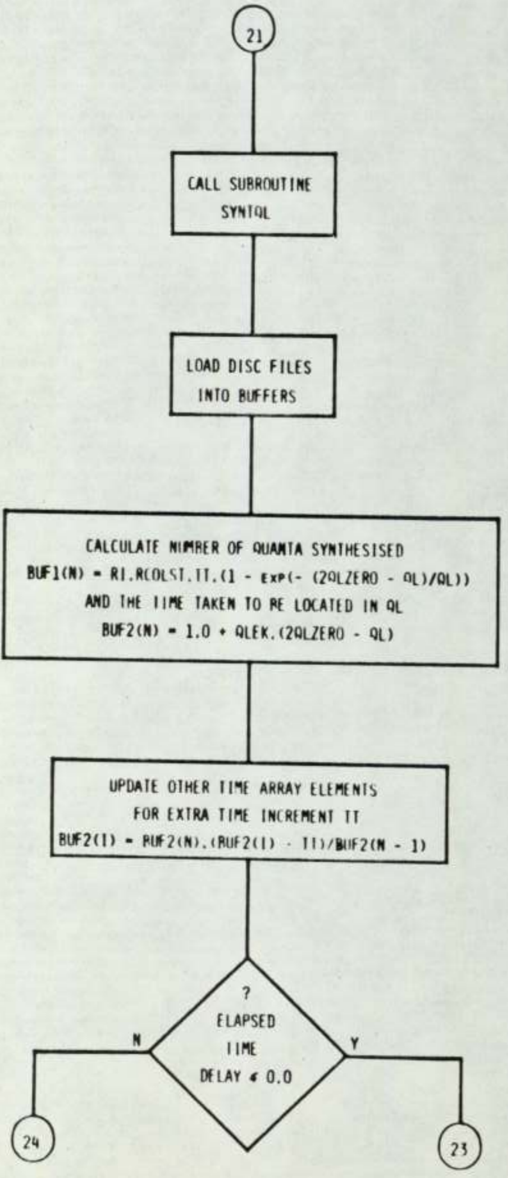
12
CALL SURROUTINE SYNTQN
LOAD DISC FILES INTO BUFFERS
DETERMINE NUMBER OF QUANTA SYNTHESISED
BUF1(N) = RI.RCONST.EXP (- 0.5.((CA/MG) - AN)^0.5)
. (1 - EXP (- (2QNZERO - QN).T3/QN))^x
AND THE TIME TO BE TAKEN TO BE LOCATED IN QN
BUF2(N) = 0.01 + QNEK.(2QNZERO - QN)
UPDATE OTHER TIME ARRAY ELEMENTS
FOR EXTRA TIME INCREMENT TT
BUF2(I) = BUF2(N).(BUF2(I) - TT)/BUF2(N - 1)
17



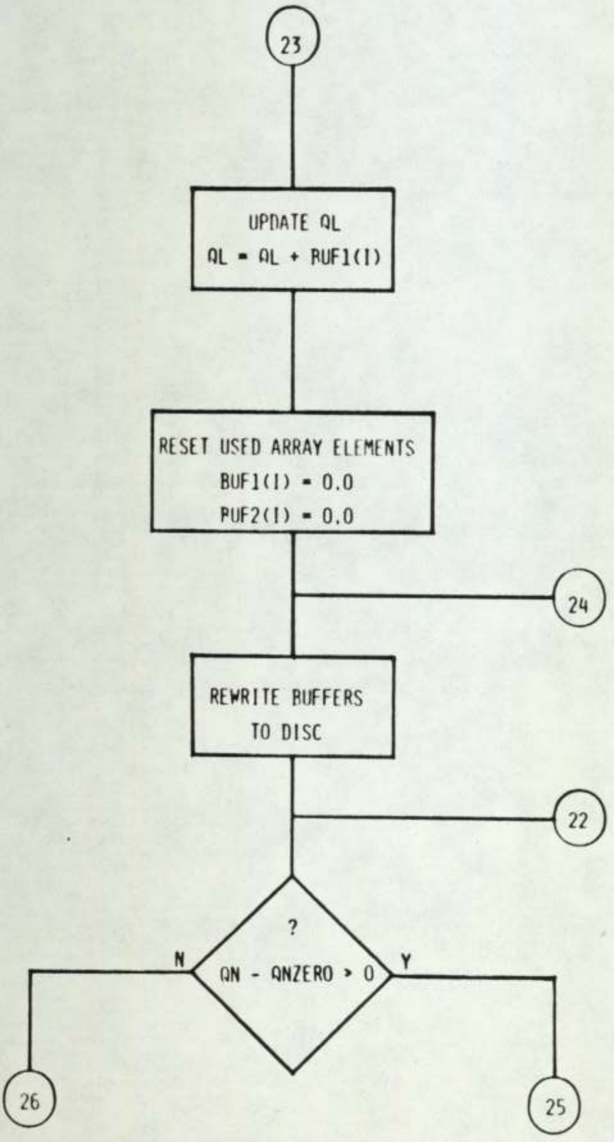
17
?
ELAPSED
TIME
DELAY ≤ 0.0
N
Y
UPDATE QN
QN = QN + BUF1(I)
RESET USED ARRAY ELEMENTS
BUF1(I) = 0.0
BUF2(I) = 0.0
REWRITE BUFFERS TO DISC
CALL SURROUTINE SYNTQM
18


18
LOAD DISC FILES
INTO BUFFERS
CALCULATE NUMBER OF QUANTA SYNTHESISED
BUF1(N) = RI.RCOMST.(1 - EXP (- (2QMZERO - QM)/QM)).TT
AND THE TIME TAKEN TO LOCATE THIS AMOUNT IN QM
BUF2(N) = 0.1 + QMEK.(2QMZERO - QM)
UPDATE OTHER TIME ARRAY ELEMENTS
FOR EXTRA TIME INCREMENT TT
BUF2(I) = BUF2(N).(BUF2(I) - TT)/BUF2(N - 1)
?
ELAPSED
TIME
DELAY ≰ 0.0
N
Y
20
19

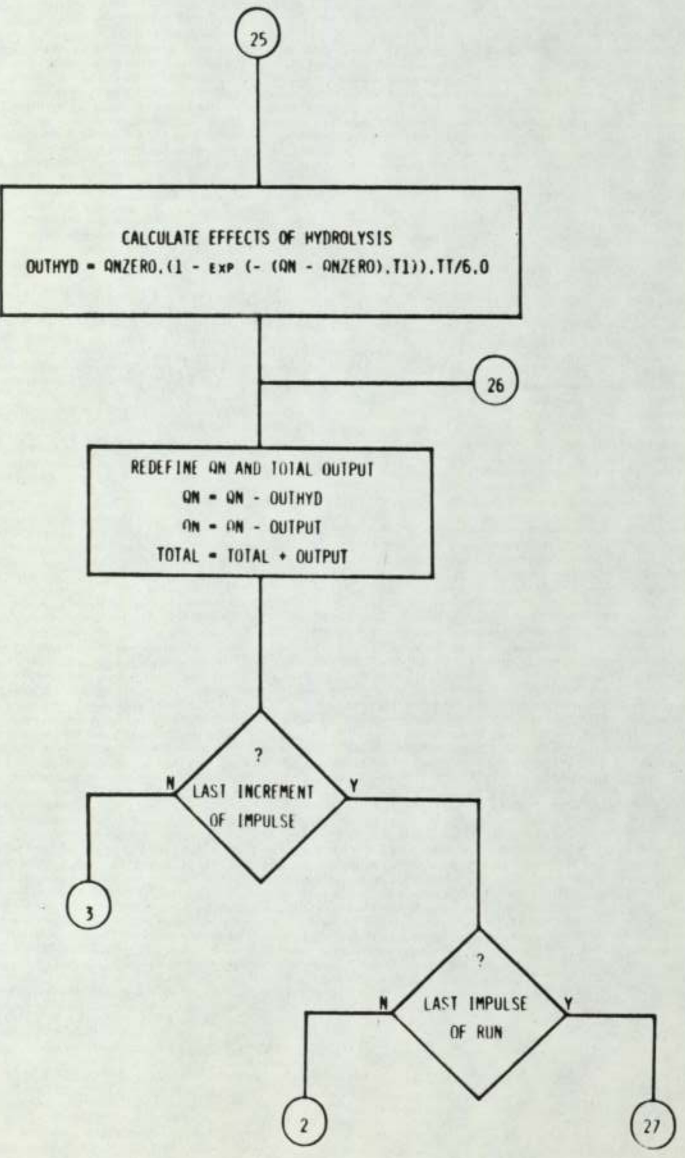

19
UPDATE QM
QM = QM + BUF1(I)
RESET USED ARRAY ELEMENTS
BUF1(I) = 0.0
BUF2(I) = 0.0
20
REWRITE BUFFERS
TO DISC
?
INTEGER -
INCREMENT NO
4
22
21

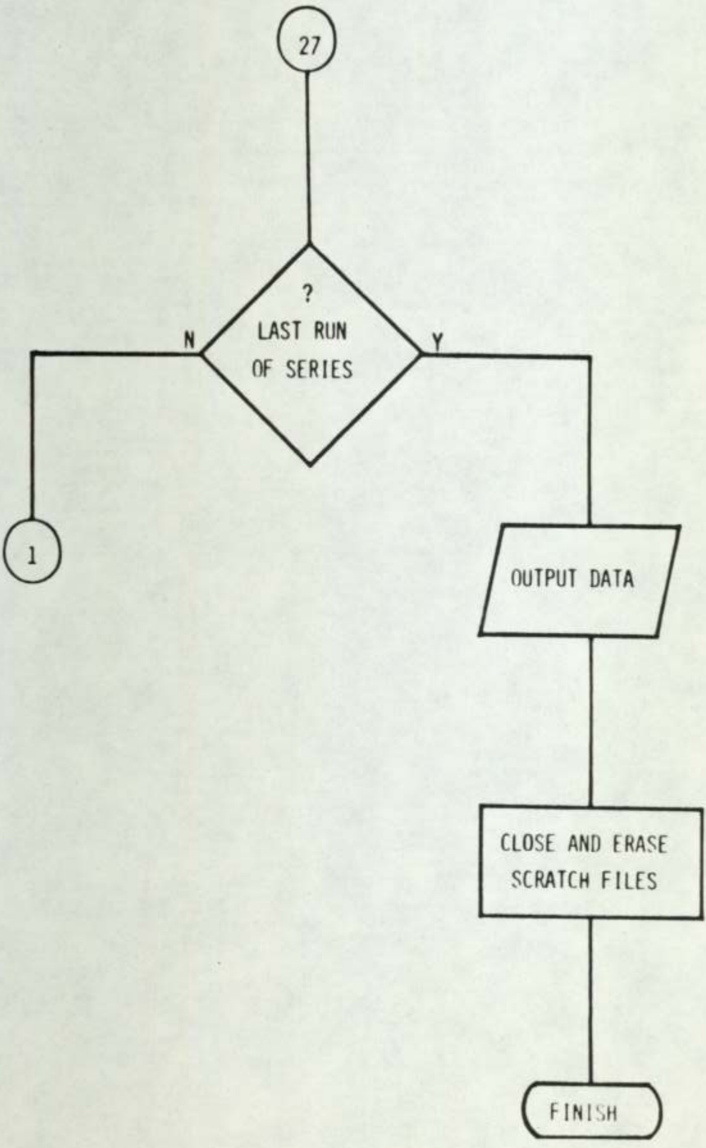


21
CALL SUBROUTINE
SYNTQL
LOAD DISC FILES
INTO BUFFERS
CALCULATE NUMBER OF QUANTA SYNTHESISED
BUF1(N) = RI.RCOLST.TT.(1 - EXP(- (2QLZERO - QL)/QL))
AND THE TIME TAKEN TO BE LOCATED IN QL
BUF2(N) = 1.0 + QLEK.(2QLZERO - QL)
UPDATE OTHER TIME ARRAY ELEMENTS
FOR EXTRA TIME INCREMENT TT
BUF2(I) = BUF2(N).(BUF2(I) - TT)/BUF2(N - 1)
?
ELAPSED
TIME
DELAY ≤ 0.0
N
Y
24
23



23
UPDATE QL
QL = QL + BUF1(I)
RESET USED ARRAY ELEMENTS
BUF1(I) = 0.0
BUF2(I) = 0.0
24
REWRITE BUFFERS
TO DISC
22
?
QN - QNZERO > 0
N
Y
26
25


25
CALCULATE EFFECTS OF HYDROLYSIS
OUTHYD = QNZERO.(1 - EXP (- (QN - QNZERO).T1)).TT/6.0
26
REDEFINE QN AND TOTAL OUTPUT
QN = QN - OUTHYD
QN = QN - OUTPUT
TOTAL = TOTAL + OUTPUT
?
LAST INCREMENT
OF IMPULSE
N
Y
3
?
LAST IMPULSE
OF RUN
N
Y
2
27


27
?
LAST RUN
OF SERIES
N
Y
1
OUTPUT DATA
CLOSE AND ERASE
SCRATCH FILES
FINISH

APPENDIX II

Results produced by the model when stimulated at a frequency of 1/min.

Impulse Number	Quanta Output	State of readily releasable store Q_n	State of mobilisation store Q_m	Total O/P to cleft
1	94.16	1000.0	2200	0.0
2	91.74	991.0	5025	7683
3	83.26	956.0	2047	15345
4	94.2	1001.0	6869	23897

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