A Dissertation in Two Parts: The development of an in vivo nerve-muscle model in the rat; and an experiment “The interaction between rocuronium and thiopentone” to test the validity of the model.

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INTRODUCTION

Experimentation involving small animals has, over the years, been of major importance in the development of our current understanding of nerve-muscle physiology. This dissertation describes the development of an *in vivo* model of the rat nerve-muscle interface at the Faculty of Health Sciences at the University of Cape Town. Such a model was not previously available to the faculty. The development was the result of collaboration between the Departments of Anaesthesia and Human Biology.

Research and teaching are the reasons for the introduction of the model. The object of this dissertation is to show that the model developed is suitable for these activities.

The first chapter gives a short history of the development of our current understanding of nerve-muscle physiology and illustrates the importance of animal experimentation in the accumulation of that knowledge. The various types of animal models which have been used over the years are described and compared. The reasons for our choice of model are explained.

The second chapter gives a detailed description of the model and its development. We describe the anaesthetic technique employed, the preparatory dissection and the workings of the model.
The third and fourth chapters describe an experiment performed using the model. The experiment investigates the effect of thiopentone on the neuromuscular blocking effects of rocuronium. In Chapter Three the pharmacology of thiopentone and rocuronium is discussed, and the available literature relating to the interaction is reviewed.

In Chapter Four, the experiment is described and its findings discussed.

This is followed by a short concluding Chapter and References.
CHAPTER ONE

In this chapter a short history of the discoveries leading to our current knowledge of the nerve - muscle interface will be given. This will show that to a large extent, the basis of our understanding rests on information obtained from animal experimentation. Thereafter the available nerve - muscle models will be described and compared, and the reasons for our choice of model given.

The history of nerve - muscle physiology begins with the writings of the ancient Greeks. The oldest writings are those of the Hippocratic school dating to three centuries BC, although some contain ideas which are considerably older. In these writings the function of the muscles, or flesh, is simply to hold the body together. Movement is brought about by the tendons or nerves. The same name, neuron, is used for both. Blood vessels transport blood and pneuma or breath by which movement is initiated (1).

Over the next five hundred years or so these ideas changed somewhat. Herophilus of the Alexandrian school was the first to distinguish nerves from tendons and ligaments. Erasistratus, a contemporary of Herophilus, recognised the muscles as organs of contraction (2). Galen, known as the father of Anatomy because of his detailed anatomical descriptions, realised that muscle existed in an active contracted state, and an inactive relaxed state. He knew that the "power" causing contraction of the muscle travelled to the muscle via the nerve, and believed that this power originated in the soul (3). So by about 200 AD it was known that muscles provide motion to the body, that they contract
under the influence of nerves, and that some transmitter system, known as 
*speritus animalis*, conducted the signal causing contraction to the muscles via 
the nerves.

With the collapse of the Roman Empire and the resulting Dark Ages, it was 
some thirteen hundred years before any further advances in knowledge were 
made.

An explosion of interest in Science occurred during the Renaissance of the 
early fifteen hundreds. There was renewed interest in animal physiology and 
many of the ancient Greek manuscripts were rediscovered. The nature of the 
*speritus animalis*, or animal spirit, became a topic of great interest. It was 
thought to be a fluid running through channels in the nerve, and for over two 
centuries investigators sought to find this compound (4). Leeuwenhoek, the 
inventor of the microscope, used his invention to seek the cavities within 
nerves which conducted the fluid, but without success (5).

In the middle of the seventeenth century Glisson proposed that there are three 
types of impulse causing muscle contraction: an inherent excitability 
independent of other factors, the animal spirit carried by the nerves, and the 
vital spirit carried by the blood (6). About a century later Von Haller published 
his work on the sensitivity of muscle. He made a distinction between irritability 
the contraction of muscle in response to a nerve stimulus or as a result of 
direct stimulation of the muscle, and sensibility: the transmission to the soul of 
sensory information (7).
The early eighteenth century saw the first experiments on electrical phenomena and these led Stephen Hales to speculate that the force leading to muscular contraction might travel through channels within the nerve, or act "along their surfaces like electrical Powers" (8).

In the late 1780's Luigi Galvani, Professor of Anatomy at Bologna University, showed that muscle contraction could be initiated by an electric current (9). He did this by showing that the small current induced in a lightning rod by distant lightning caused muscular contraction when it passed through the body of a skinned frog. He later showed that contraction of muscle could be induced by touching a nerve with a metal rod. This led to controversy as to the origin of these bioelectric currents, with Volta maintaining that they were caused by the metal itself (10). Matteucci in 1838 detected current flow within damaged and intact muscle by means of a galvanometer (11). These findings showed that electricity was involved in nerve conduction and muscle contraction, although the mechanism was not known. The next breakthrough in knowledge occurred after the introduction of curare, the South American arrow poison, to the European scientific community.

Although he was not the first to conduct experiments on the effect of curare on animals, it is Claude Bernard who conducted the next important experiment (12). In 1850 he injected curare into the lymph sac of a frog, causing paralysis. In continuation of the experiment he showed that conductivity in the animal's nerves and irritability of the muscles to direct stimulation were unchanged. The
The conclusion of this experiment was that curare prevented the transmission of an electrical signal from the nerve to the muscle at the neuromuscular junction.

At this time the predominant belief was that there was a direct electrical connection between the nerve and muscle which was interrupted by the action of curare.

The concept of chemical transmission at the neuromuscular junction arose some twenty years later. Initial experiments by Schmiedeberg (13) in 1877 showed that the administration of muscarine caused a similar effect to vagal stimulation. Similar experiments showed a similar relationship between adrenaline and sympathetic stimulation. These findings led to an intense debate as to the nature of the signalling mechanism between muscle and nerve chemical or electrical. The proponents of electrical transmission were in the ascendancy until the early nineteen twenties.

Otto Loewi (14), in 1921, became the first person to prove the chemical transmission of nerve impulses across synapses. He stimulated the vagus nerve of a frog heart. The fluid perfusing the heart was then used to bathe the heart of another frog. The rate of the second heart decreased in concert with the first. This showed that a chemical in the perfusate, released in response to vagal stimulation, was able to effect a response in an isolated heart.

Within a few years Dale had shown that acetylcholine was released at the neuromuscular junction in response to motor nerve stimulation. Cowan showed
that acetylcholine depolarised the endplate and that this depolarisation initiated muscle contraction (15).

These findings established the theory of chemical transmission at the neuromuscular junction.

The next question was that of the nature of the interaction between acetylcholine and the postjunctional membrane. Erlich first coined the term receptor, but it was A. J. Clark who popularised the theory of receptor binding sites (16).

Clark measured the graded response to an increasing concentration of acetylcholine. When plotted on a graph the results produced a sigmoid curve. The simplest explanation for this relationship was proposed to be a monomolecular reaction between two entities, namely acetylcholine and a binding site in the cell. Further experimentation led him to conclude that these binding sites or receptors were found on the surface of the cell. These predicted entities were first seen under an electron microscope in 1971.

From the above it is clear that from the nineteen thirties we have had a basic understanding of neuromuscular physiology. Further investigation has vastly increased our knowledge of this synapse, but each new contribution to the field merely embellishes the framework set out by the early pioneers.
Choice of Model

Frog preparations were used in most of the experiments mentioned above. As the experiments performed have become more sophisticated over the years, so too has the type of nerve - muscle model.

Frog muscle has several characteristics which make it suitable for experimental purposes (17). It is very hardy, with the excised muscle able to survive for long periods in a bath of physiological salt solution. This solution is designed to correspond to amphibian extracellular fluid and provides the correct ionic environment for the muscle and prevents desiccation. Sufficient oxygen to maintain muscle function is able to diffuse through the bathing solution. The muscle is active at temperatures ranging from 0 degrees Celsius (used in muscle metabolism experiments) through to room temperature. This inherent hardness of the muscle made it suitable for the relatively simple experiments performed by the pioneers of nerve - muscle physiology. Today these preparations are used as teaching tools.

Mammalian muscle has a higher metabolic rate than the amphibian muscle described above. For this reason it has a higher oxygen requirement, which led to the need for new models able to supply more oxygen to the tissues.

The first possible model is a mammalian in vitro nerve - skeletal muscle preparation. This model is not used due to several inherent problems. Oxygen has to diffuse from the bathing solution to the muscle cells. Although oxygen can be bubbled through the solution to create a greater concentration gradient,
the innermost cells become ischaemic. Penetration of test drugs into the deepest fibres may not occur. These factors render the model useless as individual preparations may vary considerably in the rate of development of muscle ischaemia and in the rate of diffusion of test drug. By using thin strips of muscle these problems can be circumvented but the nerve supply to the muscle fibres is interrupted in the process.

The first model of practical importance is the *in vitro* phrenic nerve–diaphragm model first described by Bulbring in 1946 (18). In a freshly sacrificed rat the phrenic nerve and its attached hemidiaphragm are dissected. The costal attachments are fixed to the bottom of an organ bath and the tendinous central portion is attached to a strain gauge. The bath is then filled with a physiological salt solution containing dextrose as an energy substrate. Oxygen is bubbled through the solution continuously to oxygenate the muscle. The solution in the bath is kept at a constant temperature to ensure consistent results. The diaphragm is used because it is a thin muscle with a large surface area. This allows the best conditions for the diffusion of oxygen into the muscle. Because the muscle is used in its entirety, the nerve supply to all muscle fibres is intact.

This technique has several drawbacks. It is technically difficult to perform as the delicate muscle is easily injured during the dissection. Test drugs remain in contact with the muscle for longer than would occur *in vivo*, which may affect results. Muscle relaxants are known to affect different muscle types to a greater...
or lesser degree, so results obtained on this diaphragm muscle may not be applicable to other skeletal muscle types.

The second model used for mammalian neuromuscular studies is an \textit{in vivo} model. In an anaesthetised animal a nerve, usually the sciatic, is isolated. The tendon of a skeletal muscle supplied by the nerve is dissected and attached to a strain gauge. Venous access is obtained to allow intravenous administration of test drugs. Temperature should be kept constant. This method has several advantages. The muscle and its blood supply is minimally disturbed. Oxygen, nutrients and the test drugs are delivered to the muscle in a physiological manner. The drug is cleared from the blood by the liver by the usual physiological processes so pharmacokinetic and pharmacodynamic data may be obtained from the model. The disadvantages of this method are that there are a number of steps in the preparation of each test subject. Each test animal needs intravenous access and the airway needs to be secured by intubation or tracheostomy prior to the initiation of the experiment. Additional equipment such as a suitable ventilator is essential to maintain oxygenation and acid-base balance throughout the experiment.

The choice of preparation has led to some controversy over the years. An example of this is the conflicting evidence of the influence of magnesium on the effect of suxamethonium. Theoretically magnesium should antagonise the effect of suxamethonium. Conflicting results have however been obtained in studies investigating this interaction. A study by Ghoneim and Long (19), using the \textit{in vivo} rat phrenic nerve-diaphragm model described above, found that
magnesium potentiated the effect of suxamethonium. This was in agreement with earlier clinical (20) and in vivo (21) studies. Later clinical studies (22,23) found no potentiation of suxamethonium by magnesium. A study by Tsai, Huang and Lee in 1994 (24) clarified the matter in an in vivo model utilising a cat as subject they showed the antagonistic effect of magnesium. This confirmed both the theory of magnesium action and the impressions formed by the clinical trials. Several proposals have been made to explain the anomalous results obtained from the phrenic nerve–diaphragm model. Of these, two are probably of importance. The first is that the acetylcholinesterase required to break down the suxamethonium is deficient in this model due to consumption or being washed away in the muscle bath; and the second is that the prolonged contact of the suxamethonium with the muscle leads to a Phase II type neuromuscular block, which would be enhanced by magnesium.

Considering our requirements and the possible models available, it was easy to make a choice. As we required a model capable of assessing the potency of relaxants interacting with other drugs, the in vivo model was clearly superior to the phrenic nerve–diaphragm model, which has inherent flaws discussed above which could invalidate the data obtained from the model. Because of the differences between amphibian and mammalian muscle the frog preparation was not considered. The in vivo model is currently widely used for neuromuscular research, and we decided to develop such a model.

The question of the choice of an appropriate animal type to be utilised has both ethical and financial implications. Many animal types have been used in
research over the years including pigs (25), monkeys, cats (26) and rats (27). Of these the first two are expensive and the use of monkeys in particular would not be ethically justifiable. The use of these larger animals is usually reserved for more advanced study of new drugs where an attempt is made to predict effects such as duration of action in man. Our model will not be used for these types of studies. It was decided that smaller, cheaper animals would be utilised.

Of the smaller animals the rat is commonly used in this type of experimentation. Although it has been shown that guinea pigs are physiologically more comparable to humans in their response to muscle relaxants than rats (28), there are advantages to utilising rats. The muscle relaxants are less potent in rats. This results in a quicker recovery and allows for shorter experimental times. It was for this reason, and their ready availability at our institution, that rats were chosen as the experimental animal.
CHAPTER TWO

For the reasons explained in the previous chapter an in vivo rat preparation was chosen as our experimental model. Because such a model was not available at UCT, one had to be developed. In this chapter the development of the model will be described. The first section of the chapter deals with the preparation protocol - the anaesthetising of the animal, the required dissection and the maintenance of respiration throughout the experiment. The second section of the chapter describes the workings of the apparatus and its validation. Most of the elements of the model were available to us, but some adaptations were necessary to suit our purpose.

In this in vivo nerve-muscle model the gastrocnemius muscle supplied by the sciatic nerve was used. The nerve is large and easy to isolate, while the muscle may be loosened from its insertion with minimal disruption of its nerve and blood supply.

A total of fifteen Long-Evans rats were used in the development of the model. This strain of rat is considered to tolerate anaesthesia well.

The animals were weighed prior to their being anaesthetised. The rats were anaesthetised with an intraperitoneal injection of a ketamine/xyloazine mixture which is one of the standard anaesthetic regimens used for small animal experimentation at our institution. A mixture of 9 ml ketamine 100 mg/ml and 5 ml xylazine 20 mg/ml was prepared. A dose of 0.0014 ml/g was used to
induce anaesthesia. This represents a dose of 90 mg/kg ketamine and 10 mg/kg xylazine. The intraperitoneal doses were repeated at 40 minute intervals throughout the experiment. Ketamine has been shown to prolong the effects of nondepolarising muscle relaxants in \textit{in vivo} \cite{29} and \textit{in vitro} \cite{30} studies but no noticeable differences in our muscle contraction recordings were seen in relation to the timing of injections.

The first part of the dissection was a tracheostomy, which facilitates ventilation of the animal through the course of the experiment. With the animal supine a midline incision was made in the neck. The strap muscles were parted to expose the trachea. The trachea was loosened from its surrounding fascia and incised to allow the insertion of a 14 G plastic cannula. The cannula was secured with stay sutures.

The securing of venous access was the second part of the dissection. The right femoral vein was utilised for this purpose. An incision was made in the skin of the inguinal region to expose the femoral vessels. Stay sutures were passed around the vein to prevent haemorrhage when the vessel was incised. A small incision was made in the vein and a small bore cannula (prepared by heating and stretching plastic tubing) was inserted and secured. The cannula was connected to a three way tap and flushed with saline. The dead space of the cannula was measured prior to insertion.

The third step of the dissection was the preparation of the left sciatic nerve. An incision was made in the skin of the posterior aspect of the thigh. The
hamstring muscles were parted to expose the sciatic nerve and its division into the common peroneal and tibial nerves. The peroneal nerve which runs laterally was parted to allow unopposed contraction of the gastrocnemius muscle via its supply by the tibial nerve. The sciatic nerve, freed from the surrounding tissues, was hooked with silver electrodes. Care was taken that the electrodes were not in contact with each other and they were sutured in place to maintain their positions through the course of the experiment. Various approaches to the nerve and methods of fixing the electrodes were tested during the development of the model to optimise stimulation of the nerve.

The final step was to prepare for the fixation of the gastrocnemius muscle. A suture was passed through the ankle between the gastrocnemius tendon and the malleolus to serve as an attachment to the transducer. A skin incision was made over the patella to facilitate proximal fixation of the muscle by means of an anchor pin pushed through the tendon.
All the surgical procedures described were performed on a cork dissection board. The prepared anaesthetised animal, on the dissection board, was then brought to the experimental apparatus. The tracheostomy tube was connected to a Harvard rodent ventilator (Fig.2.1) which was set to deliver a tidal volume of approximately 10 ml/kg at a rate of 40 breaths per minute.

Fig.2.1 The Harvard Rodent Ventilator
The experimental apparatus (Fig.2.2) consisted of the following:

1. A means of immobilising the limb and keeping the muscle under constant tension.

2. A means of stimulating the nerve – a Grass stimulator.

3. A means of transducing the mechanical energy produced by muscle contraction to an electrical signal – a foil type strain gauge.

4. A means of amplifying the produced signal – a purpose built amplifier.

5. A means of recording the output from the amplifier – a Philips pen recorder.

6. A means of controlling the experimental environment – a thermostatically controlled heater.

These elements of the apparatus are numbered as above on the plate below.

*Fig.2.2 The experimental apparatus*
1. Immobilisation of the limb

The knee joint was anchored by passing a 17 G needle through the patellar ligaments of the animal and positioning the needle in the groove of the proximal beam of the apparatus, pictured in Fig.2.3.

Fig.2.3 Proximal beam showing groove to facilitate fixation of knee. The strain gauge is seen in the background.
The suture passing through the ankle was attached to the strain gauge, which is itself mounted on a ratchet assembly (Fig. 2.4). The gauge was tensioned by slowly adjusting the ratchet assembly while observing the resultant trace on the pen recorder. The ratchet was clamped in place when the tension reached a predetermined reading on the paper trace.

**Fig. 2.4 The ratchet assembly with strain gauge visible at top right.**
During development of the model it was found that slight movements of the animal's body induced by the evoked muscle contractions affected the recordings obtained, so a headbox (Fig. 2.5) was added to the model. A bar was placed behind the incisors of the upper jaw and traction was applied to the head, thus stabilising the body between two anchor points. This minimized extraneous movement artefacts from the recordings obtained.

*Fig. 2.5 The headbox*
2. Stimulation of the nerve.

The nerve was stimulated by means of a Grass Model S48 nerve stimulator (Fig. 2.6). This is a sophisticated stimulator capable of delivering a variety of impulses. In these experiments a square wave stimulus with a duration of 3 milliseconds was used. The magnitude of the stimulation varied in each experiment as a supramaximal stimulus was obtained for each animal. This ensured recruitment of all muscle motor units. The voltages required ranged from 10 to 25 volts.

*Fig. 2.6 The nerve stimulator.*
A stimulus isolation unit (Fig. 2.7) was connected between the stimulator and the animal to prevent extraneous currents influencing the experiment.

![Fig. 2.7 The stimulus isolation unit.](image)

The output from the stimulus isolation unit was connected to the silver electrodes mentioned earlier by means of spring clips at the ends of the leads.
3. The transducer.

Contraction of the muscle results from the nerve impulse generated by the nerve stimulator. This mechanical energy is converted to an electrical signal by the strain gauge or transducer which is housed in the locking ratchet component of the anchoring mechanism (Figs. 2.3, 2.4 & 2.8). The transducer used works on the wheatstone bridge principle. The sensing element consists of electrically conductive elastic material which changes its electrical resistance in response to deformation. In our apparatus we used a 2 element strain gauge obtained from an electric scale. It has a maximum load rating of 600g. This was the second transducer used in the apparatus—the one used during the initial stages of the development of the model was found to be insufficiently sensitive and an alternative was obtained.

Fig. 2.8 The strain gauge
4. Amplification of the signal

The electrical signal produced by the strain gauge is then transmitted to the amplifier. The amplifier was designed and built by Mr H. Hall of the Department of Human Biology. Its specifications allowed for a matched output from the transducer.

5. Recording of Data

A Philips PM 8221 pen recorder was used to record the output from the amplifier (Fig. 2.9). The output from the amplifier was calibrated to produce a 10 mV deflection of the recorder for every 300 g force exerted on the strain gauge.

Fig. 2.9 The pen recorder with the amplifier on right.
6. Controlling the experimental environment.

The experimental preparation was housed in a chamber (Fig. 2.10). It had a large vertical sliding door made of Perspex which allowed ready access to the preparation, and observation during the experiment. The chamber contained a thermostatically controlled fan heater which maintained the temperature in the chamber at 37° C. This ensured a constant ambient temperature. The circulation of the heated air caused evaporation of fluid and desiccation of exposed tissue. This was prevented by covering the tissue exposed during the dissection with gauze dampened with Ringer’s lactate solution. A constant slow flow of the Ringer’s solution kept the gauze moist.

Fig. 2.10 The model in operation. Note:
1. The headbox immobilising the head.
2. Tube from the ventilator connected to tracheostomy tube.
3. Proximal anchoring mechanism fixing the knee.
4. Suture connecting the ankle to the strain gauge.
5. Thermostatically controlled fan heater.
6. Cannula dripping Ringer’s solution onto gauze to keep tissues moist.
Throughout the development of the model attention was paid to the repeatability of the results obtained from the apparatus. It is on this property that the success of the model depends. In our model a repeated standard electrical stimulus causes a muscular contraction, which is recorded by a pen recorder. It is important that the contraction obtained causes a constant twitch height on the recorder. There are many sources of error in obtaining the recording. Although the output from the nerve stimulator is constant, variation in the connection of the electrodes to the nerve may result in only part of the nerve being stimulated, leading to a smaller than expected contraction. If the muscle tendon is not securely fastened to the strain gauge, play in the system may lead to a smaller force being exerted on the strain gauge. Movement of the experimental animal may also lead to alterations in the tension of the muscle which may also affect results. These factors were all addressed during the development of the model.
Fig. 2.11 Example of results.

Fig. 2.11 shows an example of the results obtained by the model. The graph reads from right to left. The black areas show the muscle twitches. The individual twitches cannot be distinguished because of the slow rate of paper movement (0.5 cm/min or 1 graph block every 2 minutes) and the frequency of stimulation (12 per minute). Tensioning of the strain gauge to the baseline is indicated by "a". Thereafter the nerve stimulator is switched on and a supramaximal stimulus is obtained. The system is allowed to settle for a few minutes. At "b" a dose of muscle relaxant is administered, which results in complete paralysis of the muscle indicated by a complete absence of muscle contraction. As the effect of the muscle relaxant diminishes with time, the
muscle contractions reappear ("c") and increase up to a constant level "d", after which no further increase in twitch height is seen.

With reasonably consistent results such as the above being obtained from the model, we concluded that the model was ready for the performance of scientific experiments. A total of 15 animals were required in the development of the model.
CHAPTER THREE

It is well known to anaesthetists that thiopentone reacts with various other agents. Although anecdotal reports of these interactions have been published (31), there has been very little scientific research of these reactions, which are known to be pH related. Examples of where such a pH interaction has been put to practical use is in the differentiation between local anaesthetic and cerebrospinal fluid during the performance of an epidural injection (32), and in the confirmation of tracheal intubation (33), where the carbon dioxide in exhaled air precipitates the thiopentone through which it is bubbled.

An interaction which is of great importance to anaesthetists is that which occurs when thiopentone is mixed with aminosteroid type non-depolarising muscle relaxants. Frequently during the intravenous induction of anaesthesia the aforementioned drugs may accidentally mix in the intravenous line, resulting in the formation of a thick white precipitate which may block the intravenous cannula. It is the nature of this interaction which forms the subject of this study.

In this chapter the physical characteristics of thiopentone and rocuronium, an example of an aminosteroid non-depolarising muscle relaxant which was used in our study, will be described. Thereafter the topical literature will be reviewed and summarised.
Thiopentone has been widely used as an induction agent since its introduction to clinical practice in 1935. It is sodium ethyl (1- methyl butyl) thiobarbiturate, the sulphur analogue of pentobarbitone.

![Fig. 3.1 Structure of thiopentone](image)

A commercially supplied ampoule of thiopentone contains thiopentone and sodium carbonate as a powder in an atmosphere of nitrogen. The nitrogen atmosphere prevents the reaction of the thiopentone with carbon dioxide which leads to the formation of free thiopentone acid, which is insoluble in water. The sodium carbonate is added to raise the pH of the solution, which ensures a high proportion of ionised, and therefore soluble, thiopentone. The pH of the thiopentone solution is 10.5. The pKa is 7.6, and at physiological pH it is 61% unionised (34). Thiopentone is not stable in solution and may react with its container (35), the atmosphere (36) and a variety of drugs (35).

Pancuronium, vecuronium and rocuronium are the aminosteroid non-depolarising muscle relaxants currently in use in South Africa. As can be seen in Fig. 3.2, they have a similar structure.
As the newest of the drugs on the market, rocuronium was selected to be used in the study. Rocuronium is the 2-morpholino, 3-desacetyl, 16-allyl-pyrrolidino analogue of vecuronium (37). Rocuronium is supplied commercially in ampoules as a 1% aqueous solution. The preparation has a pH of 4.6. It is not stable in solution and needs to be stored below 10°C.
All the non-depolarising muscle relaxants exert their effects by preventing the binding of acetylcholine to the post-synaptic nicotinic receptor. All these agents have two positively charged elements at physiological pH, which are essential for their action at the neuromuscular junction. In the case of rocuronium, these groups are a quaternary ammonium and a nitrogen ring which may accept a proton. To be effective, these groups need to be separated by a chain of ten heavy atoms. This chain is formed by the steroid nucleus of the molecule (38).

The implications of the above are as follows: thiopentone must be ionised to be soluble, and requires a high pH to ensure solubility; while rocuronium is ionised at physiological pH, but requires two ionised groups to exert its effects on the acetylcholine receptor. When the two drugs are mixed a pH change occurs, resulting in the formation of a precipitate. Our study was designed to determine whether the action of rocuronium is affected by this reaction.

Although this reaction is well known, it is sketchily reported in the literature. There are letters reporting the occurrence (39,40). It is mentioned in some Anaesthetic texts (41,42), but not in others (34). In some pharmacology texts (43,44) it is not mentioned at all. The package insert for thiopentone (45) does not mention the reaction, whereas that of rocuronium (46) does. A Medline search revealed only two papers which investigated the precipitation reaction.

Morton and Lerman (47) studied the precipitate formed by the combination of thiopentone and pancuronium. Commercially prepared thiopentone was diluted to
a 2.5% solution by the addition of distilled water. The pH of the mixture was found to be 10.6. Hydrochloric acid (HCl) (0.2 N) was added to the mixture until a precipitate formed. This occurred at a pH of 9.90. The experiment was repeated using pancuronium 0.1% in the place of the HCl and a precipitate occurred at a pH of 9.25. The researchers then compared the ultraviolet absorption spectra of thiopentone, pancuronium, the precipitate resulting from their mixing and the supernatant of the solution. These are shown in Figs 3.3, 3.4, 3.5 & 3.6.

![UV absorption spectrum of thiopentone](image)

**Fig 3.3** UV absorption spectrum of thiopentone
Fig. 3.4 UV absorption spectrum of pancuronium.

Fig. 3.5 UV absorption spectrum of the precipitate from a mixture of thiopentone and pancuronium.
From these spectrophotometric data the researchers concluded that the precipitate resulting from the addition of pancuronium to thiopentone is thiopentone acid. Because there are slight differences in the pH at which the precipitate forms when HCl and pancuronium are used to acidify the thiopentone solution, and in the resultant UV absorption spectra, the authors postulated that an acid soluble thiopentone–pancuronium complex ion may also be produced. The study included no in vivo experimentation to test the biological effects of administering the resultant mixture.

Tanaguchi, Yamamoto and Kobayashi (48) performed a study similar to the aforementioned when they investigated the interaction between thiopentone and vecuronium. They prepared a precipitate by mixing equal volumes of
thiopentone 2.5% and vecuronium 0.1%. The mixture was then centrifuged and the precipitate collected, washed twice with distilled water and then redissolved in 5 ml 0.1 N sodium hydroxide. This redissolved precipitate was then analysed via ultraviolet (UV) spectrophotometry and by high performance liquid chromatography (HPLC). The results of the UV spectrophotometry phase of the experiment were in agreement with the findings of Morton and Lerman, namely that the absorption spectrum for the precipitate is nearly identical to that of thiopentone and different to that of vecuronium. These findings are demonstrated in Figs 3.7 and 3.8. The HPLC findings are that both thiopentone and the precipitate show a single peak and the same retention time, suggesting that the precipitate consists solely of thiopentone, and not a combination of thiopentone and vecuronium.

![UV absorption spectra of thiopentone and vecuronium](image)

*Fig. 3.7 UV absorption spectra of thiopentone and vecuronium*
The conclusion that can be drawn from these studies is that the precipitate formed by the mixing of thiopentone and an aminosteroid type muscle relaxant consists of thiopentone. Whether the interaction has any effect on the action of the muscle relaxant involved has not been studied to date.
CHAPTER FOUR

We have seen from our literature review that the interaction between thiopentone and aminosteroid muscle relaxants results in the precipitation of thiopentone. However the effect of this reaction on the muscle relaxant concerned has not been investigated. With this in mind an experiment was devised making use of the in vivo model we had developed. Permission to perform the study was obtained from the Animal Ethics Committee of the Faculty of Health Sciences, University of Cape Town.

The object of the experiment was to determine whether the process of precipitation which occurs when thiopentone and rocuronium are mixed, results in a loss of efficacy of the rocuronium. This was tested by comparing the effects on muscle contraction effected by rocuronium with the effects caused by rocuronium which had interacted with thiopentone. Based on clinical experience, we would not expect a difference in findings between the groups. Because only a large difference would be clinically relevant, a 50% difference between the groups was considered significant.

Methods

The experimental animal was anaesthetised, dissected and connected to the model as described in chapter 2. The nerve stimulator was adjusted to provide a supramaximal stimulus and the pen recorder was switched on. Once the system stabilised a series of three injections were made, with a 15–20 minute interval between injections. The three injections comprised rocuronium, a rocuronium–high dose thiopentone mixture and a rocuronium–low dose thiopentone mixture. Their preparation is detailed below.
Each animal was exposed to each experimental mixture, thus acting as its own control. There are six possible combinations of order of administration of drug, and two rats received each combination for a total of twelve experimental animals.

Solution G1. Rocuronium

A single commercially available ampoule of rocuronium (Esmeron® - Sanofi-Synthelabo) containing 5 ml of a 1% solution was diluted with sterile water to a volume of 25 ml. This solution contains a rocuronium concentration of 2 mg/ml.

Solution G2. Rocuronium / High Dose Thiopentone

An ampoule of a commercial preparation of thiopentone (Inframed Thiopentone sodium) was dissolved in 20 ml sterile water. To this was added 1 ampoule of rocuronium as above. The resultant precipitate was filtered. If we accept that only thiopentone precipitates in the reaction, we should be left with a solution with a rocuronium concentration of 2 mg/ml.

Solution G3. Rocuronium / Low Dose Thiopentone

An ampoule of thiopentone was prepared as described above. One ml of this solution was mixed with 1 ampoule of rocuronium and a further 19 ml of sterile water to give a final volume of 25 ml. This solution was then filtered to clear it of precipitate. Once again the rocuronium concentration should be 2 mg/ml.

The experimental subjects comprised male and female Long Evans rats with mass ranging from 340 – 540g, as tabulated below.
Table 4.1: Sex and weight of animals.

The test solutions were administered to the animals as follows. Rats were randomly selected from stock.
The dose of 0.75mg/kg rocuronium was administered. This dose had been determined to provide an adequate duration of action during the development of the model.

Results

An example of the tracings obtained is reproduced below.

Fig 4.1: Example of results.
The rocuronium/high dose thiopentone mixture (G2), caused no muscle paralysis at all and has been excluded from further analysis. The injection of G2 is represented by the circled 1 in the above figure.

Onset of action
The onset of muscle relaxation after administration of rocuronium (G1) and rocuronium/low dose thiopentone (G3) occurred within 12–36 seconds of administration. These are represented by the circled 2 and 3 on the above figure, respectively. Due to the slow paper speed setting of the pen recorder (0.5 cm/min), statistical analysis of the time of onset is not meaningful.

Muscle relaxation.
The effect of G1 and G3 on muscle relaxation is tabulated below. Complete paralysis is the time when zero twitch height was achieved, shown by “a” on Fig 4.1. The 95% recovery time (“b” on Fig 4.1) was the time from onset of paralysis to restoration of 95% of the baseline activity, and the complete recovery time (“c”) is the time from the onset of paralysis to the return to a stable contraction strength with no further recovery.
Table 4.3: Time of muscle paralysis.

<table>
<thead>
<tr>
<th>No.</th>
<th>Solution</th>
<th>Complete Paralysis</th>
<th>95% Recovery</th>
<th>Complete Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>G3</td>
<td>108s</td>
<td>228s</td>
<td>240s</td>
</tr>
<tr>
<td></td>
<td>G1</td>
<td>180s</td>
<td>288s</td>
<td>312s</td>
</tr>
<tr>
<td>2</td>
<td>G1</td>
<td>144s</td>
<td>228s</td>
<td>252s</td>
</tr>
<tr>
<td></td>
<td>G3</td>
<td>132s</td>
<td>228s</td>
<td>252s</td>
</tr>
<tr>
<td>3</td>
<td>G3</td>
<td>144s</td>
<td>240s</td>
<td>276s</td>
</tr>
<tr>
<td></td>
<td>G1</td>
<td>168s</td>
<td>264s</td>
<td>276s</td>
</tr>
<tr>
<td>4</td>
<td>G1</td>
<td>156s</td>
<td>252s</td>
<td>276s</td>
</tr>
<tr>
<td></td>
<td>G3</td>
<td>192s</td>
<td>300s</td>
<td>312s</td>
</tr>
<tr>
<td>5</td>
<td>G1</td>
<td>108s</td>
<td>216s</td>
<td>252s</td>
</tr>
<tr>
<td></td>
<td>G3</td>
<td>180s</td>
<td>360s</td>
<td>372s</td>
</tr>
<tr>
<td>6</td>
<td>G3</td>
<td>204s</td>
<td>336s</td>
<td>384s</td>
</tr>
<tr>
<td></td>
<td>G1</td>
<td>360s</td>
<td>492s</td>
<td>504s</td>
</tr>
<tr>
<td>7</td>
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<td>G1</td>
<td>264s</td>
<td>384s</td>
<td>432s</td>
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<tr>
<td></td>
<td>G3</td>
<td>312s</td>
<td>444s</td>
<td>468s</td>
</tr>
<tr>
<td>9</td>
<td>G1</td>
<td>216s</td>
<td>432s</td>
<td>492s</td>
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<td></td>
<td>G3</td>
<td>276s</td>
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<td>568s</td>
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<tr>
<td>10</td>
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<td>204s</td>
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<tr>
<td></td>
<td>G3</td>
<td>288s</td>
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<td>600s</td>
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<td>12</td>
<td>G3</td>
<td>120s</td>
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<tr>
<td></td>
<td>G1</td>
<td>24s</td>
<td>240s</td>
<td>276s</td>
</tr>
</tbody>
</table>

The above data may be summarised as follows:
Complete paralysis - G1 vs G3; Range bracketed: 186s (24 – 396) vs 228 (108-420)

95% Recovery: G1 vs G3; Range bracketed: 310s (216–576) vs 364s (228–636)

Complete Recovery: G1 vs G3; Range bracketed: 341s (252–600) vs 389s (240–672)
Student’s t–test was chosen for statistical analysis. The calculated results are tabulated below.

<table>
<thead>
<tr>
<th>Group</th>
<th>Duration</th>
<th>95% Recovery</th>
<th>Complete Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Paralysis</td>
<td>Mean SD</td>
<td>Mean SD</td>
</tr>
<tr>
<td>G1</td>
<td>186.0</td>
<td>88.0</td>
<td>310.0</td>
</tr>
<tr>
<td>G3</td>
<td>228.0</td>
<td>104.0</td>
<td>364.0</td>
</tr>
<tr>
<td>p</td>
<td>0.3</td>
<td>0.3</td>
<td>0.3</td>
</tr>
</tbody>
</table>

As can be seen, there were no statistically significant differences between the groups. Power calculations demonstrated that, to show a 50% reduction in the effectiveness of rocuronium following admixture with thiopentone, 8 samples would be required in each group. A sample size of 12 was therefore chosen to give a large margin for error, as the likely magnitude of the effect was unknown.

Discussion

The study had two important results – firstly that the rocuronium / high dose thiopentone group showed no paralysis and secondly that the paralysis caused by the rocuronium / low dose thiopentone group, although tending to be of longer duration, was statistically no different to that caused by rocuronium alone. Bearing in mind that our experimental hypothesis considered a 50 % difference to be clinically significant, we can conclude that there was no meaningful difference between the groups.

The test drug composition was selected to imitate two clinical scenarios. The rocuronium / high dose thiopentone group would represent the admixture of rocuronium and thiopentone in a syringe prior to injection. This practice is not
common today but was used before the introduction of indwelling venous canullae (49). This study has shown that these drugs are incompatible with each other and that the rocuronium becomes ineffective.

The rocuronium/low dose thiopentone mixture represents a far more common clinical occurrence–that of the admixture of a small amount of thiopentone remaining in the intravenous line after injection with the following injection of rocuronium. This can occur in the case of a slowly flowing infusion or with the use of injection ports, where a portion of the injectate remains in the dead space of the port. This study has shown that when this occurs, there is no change in the efficacy of the rocuronium.

The question then arises as to what the mechanism of the inactivation of the rocuronium is, in the scenario of the high dose thiopentone mixture. Firstly we must determine the composition of the precipitate formed. If the rocuronium forms part of the resulting precipitate, it would explain its lack of efficacy. Our facility does not have the resources to measure the nature of the precipitate so we are led by the literature. Both the papers published which have investigated the precipitation of thiopentone and nondepolarising muscle relaxants have concluded that the precipitate consists of thiopentone alone. However, the authors made use of different methods of inducing the precipitation, and different nondepolarising agents. In Morton and Lerman's paper 0.1% pancuronium was slowly added to 10 mls of a 2.5% thiopentone solution until a precipitate formed. In the paper by Tanaguchi et al, 4 ml 2.5% thiopentone was mixed with 4 ml 0.1% vecuronium. In our study, 20 ml 2.5% thiopentone was mixed with 5 ml 1% rocuronium. Although the drugs and means of producing the precipitate differ, the similar nature of the nondepolarising drugs used
makes it likely that the precipitate formed in our experiment consists solely of thiopentone.

If we accept that the rocuronium does not form part of the precipitate, another reason for its inactivation must be proposed. Knowing that the high pH of thiopentone is responsible for many of its interactions, it was decided to further investigate in this regard. The pH of the test solutions were measured. The pH of rocuronium was 4.1, that of the rocuronium/high dose thiopentone 9.1, and that of the rocuronium/low dose thiopentone 4.5.

A solution of rocuronium in the same concentration as the experimental solutions was prepared (2 mg/ml). The pH of the solution was manipulated by the slow titration of sodium hydroxide to achieve the same pHs as the experimental solutions. No precipitation resulted. This would support the experimental evidence of the two papers quoted above that the precipitate consists of thiopentone alone.

The experiment was then repeated using these rocuronium preparations in one animal. The high pH solution caused no muscle paralysis at all while the paralysis resulting from the other two solutions are: Plain rocuronium: Complete paralysis 168 s, 95% recovery 228 s, Complete recovery 252 s. Rocuronium pH 4.5: Complete paralysis 192 s, 95% recovery 360 s, Complete recovery 384 s. These findings fall within the ranges of results obtained for the respective rocuronium/thiopentone solutions. This would suggest that the pH change caused by the thiopentone solution is the mechanism by which the rocuronium is inactivated rather than an interaction with the thiopentone itself. This view is supported by the in vitro experiment by Tannaguchi et al quoted
earlier, where no spectrophotometric evidence of a thiopentone–vecuronium molecule was found. More study is required before this hypothesis can be accepted.

The action of rocuronium, as of all the clinically used muscle relaxants, is effected by its interaction with the acetylcholine receptor. This interaction is dependant on the presence of a quaternary nitrogen atom within the molecule. It is possible that an increase in the pH of the rocuronium solution in some way interferes with the positively charged quaternary ammonium to prevent binding with the acetylcholine receptor. This possible interaction has not been studied.

As stated above, no previous studies have investigated the effect of this reaction with thiopentone on the potency of the nondepolarising muscle relaxants. The findings of our study represent new scientific information. A study using suxamethonium, a depolarising muscle relaxant has been performed. This study by Fraser, quoted in Dundee's book (50), found that thiopentone precipitated immediately after mixing with suxamethonium, but which redissolved in an excess of thiopentone. If the mixture was allowed to stand for 90 minutes, a 50% reduction in efficacy of the suxamethonium resulted. Further detail of the experiment could not be obtained.

Rocuronium, pancuronium and vecuronium are commonly used aminosteroid nondepolarising muscle relaxants. They are all acidic in nature with pH's of 4 to 5. They are all relatively unstable molecules requiring refrigeration or, in the case of vecuronium, reconstitution from powder form prior to use. Thiopentone precipitates when mixed with all these drugs. Therefore it is likely that the result of this
experiment may be extrapolated to include all the aminosteroid nondepolarising muscle relaxants.

The clinical implication of this study is the following: The precipitation reaction which occurs when a small amount of thiopentone mixes with an aminosteroid nondepolarising muscle relaxant in the intravenous line causes no diminution of the effect of the muscle relaxant.
CHAPTER FIVE

In this dissertation we have firstly shown the importance of animal experimentation in the development of our current theories of neuromuscular physiology. There is still ample scope for research in this field of physiology. There are more sophisticated models available, such as an isolated perfused peroneal nerve anterior tibialis model (51), but models of the type we have developed are more commonly used in current research, and probably have a wider application.

The object of development of the model, as stated at the beginning of this dissertation, was as both a research and educational tool. The experiments described in this dissertation show that the model is suitable for use in a research setting. As far as teaching is concerned, it has been introduced and now forms an integral part of the senior physiology curriculum and is used as a demonstration practical for undergraduate science students.

During performance of the experiment some shortcomings in the design of the model became evident, which will be addressed in future. The most important problem is that of data capture. The current model utilises an elderly pen recorder which is difficult to maintain in good working order. There are plans to replace the recorder with a computerised system of data recording.

The second major drawback of the current design is the lack of monitoring of the experimental animal. In our experiments the animals were not fully monitored. Factors such as hypothermia and hyper- or hypocapnia could well
influence the results obtained. Although the temperature in the chamber was kept constant we did not regulate the animals' temperatures. Likewise with the ventilation of the animals—they were ventilated according to their mass but with the small tidal volumes involved over- or underventilation could easily occur.

These improvements in the experimental model will be addressed in future. Temperature and end tidal carbon dioxide monitoring can very easily be applied. Intra-arterial blood pressure monitoring is possible to be used, but will increase the preparation time prior to experimentation.

In conclusion then, an in vivo nerve–muscle model has been developed by the University Departments of Anaesthesia and Human Biology. An experiment conducted with the aim of validating the model has been described. This has shown that the model is a valid experimental tool and has opened the way for further research in this field of physiology.
References


2. Ibid. pp 6-7

3. Ibid p 9

4. Ibid p 26

5. Ibid p 310

6. Ibid p 308

7. Ibid p 308

8. Ibid p 310


10. Ibid. pp 26-28

11. *Machina Carnis* p 311


14. Ibid p 21

15. *Muscle Relaxants in Anesthesiology* p 6


18. Ibid pp 48-51


33. Dean, V. S; Dingley, J et al. The use of bromothymol blue and sodium thiopentone to confirm tracheal intubation. *Anaesthesia* 1996 January 51 pp 29-32


40. Schmidt, U.; Basta, S. R.; Shank, E. S. Precipitation of Thiopental by Rapacuronium. Anesthesiology 2001; 95 (1): 273


45. Thiopentone Sodium Package Insert. Intramed November 1998

46. Esmeron® Package Insert. Sanofi-Synthelabo September 2002


