

## PROGRESS REPORT:

# A CRITICAL APPRAISAL ON THE PHARMACOLOGY OF BETAMETHASONE

Achieved through in vitro experimentation utilising advanced

Res-Del® Tissue and Organ Perfusion Technology

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## INTRODUCTION

The ability of a physiological solution to sustain the metabolic function of living tissue *in vitro* over an experimental period is a basic requisite for any pharmacological bioassay test regime. Maintaining the viability of organ tissue explants is necessary to ensure the validity of drug responses observed during experimental investigations. Of primary importance in the design of a physiological solution is to achieve a replication of the ionic activity profile of the extracellular phase which, in mammalian species, is identifiable with the serum and interstitial fluid (1). To optimise tissue metabolism *in vitro* a perfusion solution should be isotonic and isosmotic with the extracellular fluid or blood serum and provide adequate gas transport of oxygen and carbon dioxide (2).

Res-Del® RS-I mammalian solution, a contemporary physiological solution was designed to fulfil such requirements. It is non-phosphate buffered to avoid biological complications that occur in the presence of phosphate ions (1, 3), which occur at only 12 % of the total serum value in the extracellular phase (4). This solution has been shown to maintain metabolic homeostasis by preserving the physiological and pharmacological viability of tissues and organs *in vitro* for up to 12 days (1, 5, 6, 7). It differs from other physiological solutions in that it is buffered by a BES/bicarbonate system rather than by a conventional phosphate/bicarbonate buffer system and so helps to maintain the viability of preparations over longer periods by optimising glycolysis, avoiding the precipitation of calcium and magnesium ions and, of pivotal importance, the generation of oxygen free radicals reported to occur with inorganic phosphate ions (8). A unique constituent in this solution is BES (N,N-bis[2-Hydroxyethyl]-2-aminoethane-sulfonic acid), which is a taurine-based Goods buffer (9) with zwitterionic characteristics, that sustains pH stability over a wide temperature range (10 - 37°C) while allowing optimum serum levels of bicarbonate and freely ionised levels of calcium and magnesium to be utilised (1, 10).

The present study consisted of a series of experiments to examine the viability of the rat diaphragm following exposure to different drugs under varying electrical stimulation

regimes. The effect of betamethasone upon the neuromuscular blockade induced by the neuromuscular blocking agents vecuronium, atracurium, and suxamethonium was investigated in the isolated rat hemidiaphragm preparation. Previous experiments have shown that betamethasone induced resistance to neuromuscular blockade by vecuronium and atracurium when the phrenic nerve was stimulated continuously at 0.1 pulses per second (pps) (6, 7). In the present experiment a stimulation regime was adopted using higher frequencies in order to examine the phenomena of tetanic fade and post-tetanic potentiation, which are believed to reflect changes in the rate of neurotransmitter release from motor nerve terminals.

## **METHODS**

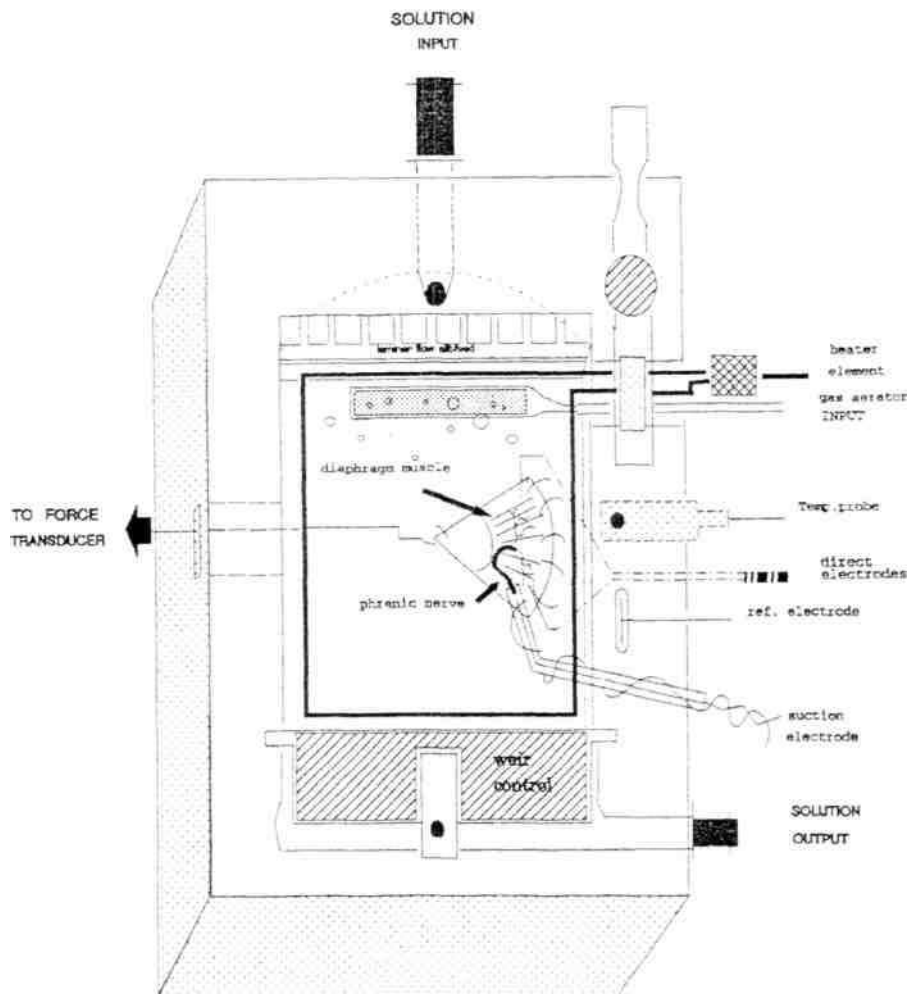
### *Animals*

This study was approved by Victoria University Animal Ethics Committee (**VUW/AEC Nos. DR91/DR94R1**). Male Wistar rats weighing 150-200g were sacrificed humanly by cervical dislocation following anaesthesia with sodium pentobarbitone (50 mg/kg). Both left (LHD) and right hemidiaphragms (RHD) from each animal were used to give a 'control' versus 'experimental' within animal comparison under different experimental conditions and effectively halved the number of animals used.

### *Assemblage of Hemidiaphragm Preparations*

Left and right hemidiaphragms were placed in two individually adjacent horizontal Res-Del® perfusion baths (Fig. 1) containing aerated RS-I. Preparations were pinned to the transparent Sylgard 184 resin base floor of the perfusion bath with fine stainless steel pins inserted through the costal muscles between the ribs. The central tendon was then attached horizontally via a fine hooked steel rod connected to an isometric Grass Ft 0.3 force-displacement transducer (Fig. 2 and ref. 5: Figs 13,14). The hook was positioned approximately 3 mm from the resin base of the bath to ensure that laminar flow of the perfusate solution perfused simultaneously under and over the preparation. The muscle was then stretched to a preload tension of 4g.

## TOP VIEW- Res-Del\* PERFUSION BATH



**Fig. 1 ISOLATED RAT HEMIDIAPHRAGM PREPARATION**

### *Perfusion Bath System*

The patented design of this organ bath allows isolated organ/tissue preparations to be placed horizontally rather than vertically as in previous conventional organ bath systems. This has several practical advantages; firstly, it allows simultaneous perfusion and perifusion of the preparation with a constant supply of fresh solution and continuous removal of waste products facilitated by a weir/spillway device. Secondly, the bath can be washed through with fresh solution at  $1 - 1000 \text{ cm}^3 \text{ min}^{-1}$  via a laminar flow slit-feed device (Fig. 1) thus removing drug solutions effectively so reducing receptor

*Perfusate and Drug Solutions:*

RS-I mammalian solution (refer Table 1) was either supplied by ICP, Auckland, NZ as a commercially prepared 10-fold concentrate or freshly made up from a 20-fold concentrate and stored at 6-8 °C. The concentrates were then diluted with deionised water and 2.1 g/L of sodium bicarbonate added immediately prior to use. The pH of aerated RS-I resided in the range 7.2 - 7.4 at 20 - 35 °C.

Component	Cone. (mmol/L)	
	110.00	
KCl	5.00	
CaCl <sub>2</sub>	1.25	<b>(Salts)</b>
MgCl <sub>2</sub>	0.45	
NaHCO <sub>3</sub>	25.00	(Buffer) <sup>a</sup>
BES	5.00	
D-glucose	10.00	
glycerol	0.11	
Na <sup>+</sup> - glutamate	0.30	
L-glutamine	0.40	(Substrates)
Na <sup>+</sup> - aspartate	0.02	
DL-carnitine	0.05	
Choline Cl <sup>-</sup>	0.01	
TPP (cocarboxylase)	40.00	<b>nmol</b>
Insulin (Porcine)	25.00	<b>mIU/L</b>

[\* aerate with carbogen, i.e. 95%O<sub>2</sub> / 5%CO<sub>2</sub>]

(pH = 7.13 - 7.41 ± 0.5 § 10-37°C)

<sup>a</sup> the concentration of the naturally occurring components in this solution, with the exception of the taurine-based Good's buffer, BES, equate to the levels found in human serum. The zwitterionic characteristics (pK<sub>a</sub> of BES ensures pH stability over the temperature range 10-38°C and allows optimal serum levels of bicarbonate to be utilised.

Table 1. Composition of RS-I mammalian physiological solution

RS-I mammalian solution was perfused at  $4.0 \text{ cm}^3 \text{ min}^{-1}$  and continuously aerated directly in the perfusion bath with carbogen (95% oxygen / 5% carbon dioxide; NZ Industrial Gases, Wellington.) via a glass sintered aerator and maintained at  $34.0 \pm 0.5^\circ\text{C}$  by a Res-Del® 589 Temperature Control Unit. This system enabled the tissues to be perfused with a continuous flow of aerated solution while the bath temperature is held constant. Non-perfused, aerated conditions were achieved by stopping the flow of RS-I solution, during which time, drugs were added directly to the fixed bath volume and removed later by rapidly (<60 sec) flushing the bath, while keeping the tissue submerged at all times.

The drugs appropriated for this study were as follows;

Betamethasone sodium phosphate ("Betnesol"; Glaxo, New Zealand), a sterile aqueous solution was diluted to a final concentration of 1  $\mu\text{mol/L}$  with RS-I.

Atracurium besylate ("Tracurium; Wellcome New Zealand Ltd., Auckland) vials containing 50 mg/5 ml were used and added undiluted (3.0 - 7.0  $\mu\text{M}$ ) to the non-perfused bath.

Vecuronium bromide ("Norcuron<sup>R</sup>"; Pharmaco/Organon Teknika) 10 vials of freeze dried powder in a buffered base were diluted to a stock concentration of 1 mg/ml (1.0-2.6  $\mu\text{M}$ ).

Succinylcholine chloride ("Suxamethonium"; Sigma Chemical Company USA) was made up each time immediately prior to use to give a bath concentration of 2.0 - 7.0  $\mu\text{M}$ .

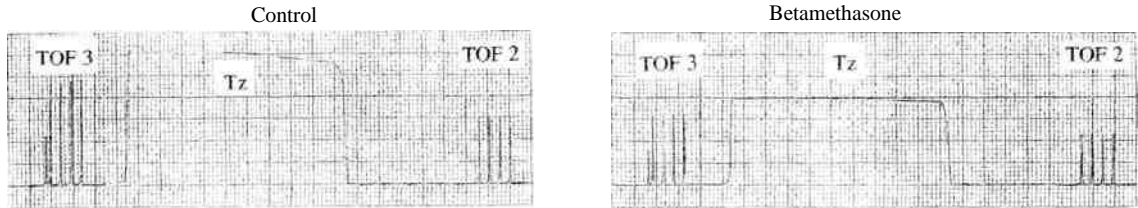
Drugs were applied to the baths with an autopipette under non-perfused conditions to give the same final drug concentration in each bath. They were added in successively increasing concentrations in ranges which gave a 30% - 70% decrease in responses for each rat hemidiaphragm preparation. Perfusion flow was halted immediately prior to drug addition and 1 minute was allowed after drug addition before the stimulation procedure was commenced. After 18 minutes the baths were rapidly washed out at  $100 \text{ cm}^3 \text{ min}^{-1}$  with RS-I and perfusion was then recommenced after 2 minutes at  $4.0 \text{ cm}^3 \text{ min}^{-1}$  until the preparation had recovered to pre-drug contractile heights as shown in Fig. 3 for suxamethonium.

Fig. 3 **The Effect of Betamethasone upon Neuromuscular Blockade Produced by Suxamethonium in the Rat Hemidiaphragm Preparation.**

Contractile performance assessed by:

- 1) TOF - Responses to train of four stimulation at 3 Hz.
- 2) Tz - Responses to tetanic stimulation at 50 Hz for 5 seconds.

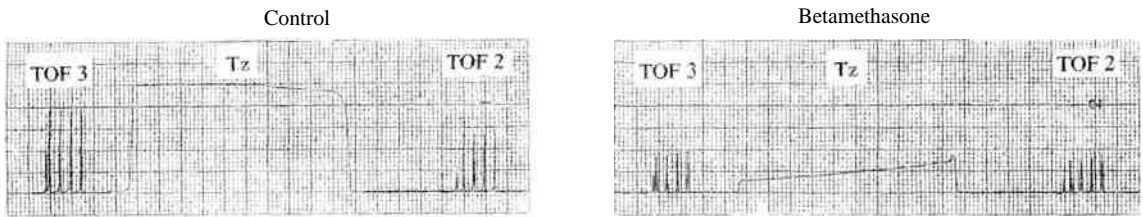
Equilibration at 4.0 ml/min for 20 minutes with RS-I mammalian solution.



Observation: No decrease in TOF ratio or Tz % responses (peak tetanic tension or tetanic fade) in control or betamethasone treated preparations.

Applied Drug : 4.0 uM suxamethonium added to control and betamethasone non-perfused Res-Del baths simultaneously.

Application Time: 18 mins

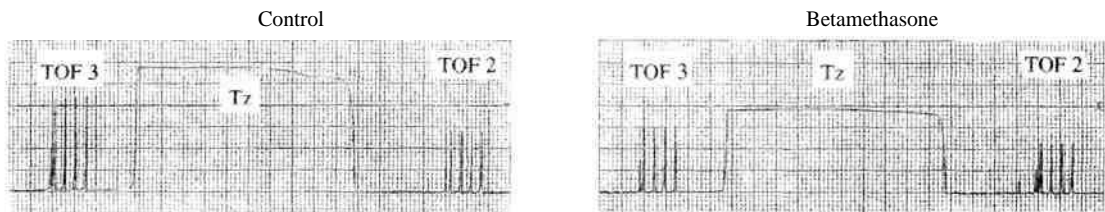


Observation:

Control - no change in TOF ratios or tetanic fade % response, but a decrease in peak tetanic tension to 82% of the pre-drug response.

Betamethasone - a decrease in TOF ratios (TOF 3 = .833 and TOF 2 = .850) accompanied by a significant decrement in tetanic response, tetanic fade showed a 34.0% response and peak tetanic tension decreased to 34.3% of the pre-drug response.

Re-equilibration at 4.0 ml/min for 20 mins with Res-Del Mammalian solution.



Observation: TOF and Tz responses returned to pre-drug levels for control and betamethasone.

### *Stimulation Procedure*

A Res-Del® suction electrode (ref 5: Fig 13) enabled localised stimulation of the submerged phrenic nerve so obviating the need to use oil layers as required when using platinum electrodes in commercially available vertical organ chambers. This suction electrode technique dramatically reduced the voltage required to achieve maximal motor unit recruitment. From an analysis of the spike discharge activity recorded *in situ* from the rat phrenic (vagal) nerve, a stimulation regime was adopted (see Table 2) in an attempt to simulate the *in situ* activity in this nerve-muscle preparation.

Spike discharge activity recorded <i>in situ</i> from the rat phrenic nerve during normal ventilation					
Spikes Action Potential Parameters	Amplitude	Duration (ms)	Interval (ms)	Discharge duration (ms)	Discharge interval (s)
Recorded <i>in situ</i> from vagal nerve	20-90 uV	1.6 - 3.0	11 - 16	170 - 320	3.2 - 4.6
Electrical parameters adopted for <b>in vitro</b> stimulation technique of the isolated rat phrenic nerve/hemidiaphragm preparation					
Stimulation Parameters	Pulse Amplitude	Pulse Duration (ms)	Inter-pulse Interval (ms)	Pulse Train Discharge Duration (ms)	Pulse Train Discharge Interval (s)
DOUBLE biphasic pulses	$3 \times R_b^1$ (5V - max <sup>m</sup> )	2.0	11	N/A	5.0 = 0.2 cps
PULSE TRAIN biphasic pulses	$3 \times R_b$ (5V - max <sup>m</sup> )	2.0	11	280	1.0 = 1.0 cps 5.0 = 0.2 cps
TETANIC(transmural) biphasic pulses	$3 \times R_b$ (5V - max <sup>m</sup> )	2.0	11	5000	0.02 = 50 cps
DIRECT (field) monophasic pulses	$3 \times R_b$ (5V - max <sup>m</sup> )	1.0	N/A	5000	as required

<sup>1</sup>  $R_b$  = Voltage of 1.0 ms biphasic pulse duration required to achieve maximal recruitment of motor units

Table 2. Electrical parameters recorded *in situ* from the vagus nerve in the rat and then simulated to stimulate isolated rat hemidiaphragm preparations

Programmable stimulus regimes were performed using a DC-neurophysiological stimulator gated by a WPI 1830 Interval Pulse Generator (Fig. 2). Indirect neural stimulation was applied using supramaximal (5 Volts) double, biphasic square-wave pulses (refer Table 2).

Direct field stimulation of the muscle fibres via stainless steel electrodes utilised single, monophasic 10 volts pulses at 50 pps for 5 seconds. The neurally and directly evoked contractile activity in the hemidiaphragm preparation was measured isometrically by a force-displacement transducer using a Gould RS3200 oscillographic recorder.

The electrical stimulation protocol consisted of Train of Four stimulation (TOF), where 3 pps was applied for the duration of 4 twitches and tetanisation (Tz), whereby 50 pps was applied for 5 seconds to achieve maximal recruitment of motor units. Similar techniques have been used to assess the degree of neuromuscular blockade in clinical anaesthesia whereby, the ulnar nerve in the forearm is stimulated and responses are monitored by the muscle tension developed in the adductor pollicis muscle of the thumb (11).

Tetanic fade is thought to be reflective of changes in presynaptic events because there is overwhelming evidence that depression of twitch tension and fade are consequences of separate actions (12). The aim of tetanic stimulation (Tz) is to apply a sufficiently high frequency of stimulation so that a maximal number of motor units are recruited to give an indication of the extent of the neuromuscular blockade (see Table 2). The latter technique also stresses the nerve terminal such that in the presence of a neuromuscular blocker, the large safety factor which exists at the neuromuscular junction is surpassed. The resulting wane in transmitter output is unmasked and is expressed as a fading tension response known as tetanic fade (12).

**TABLE 3: Synopsis of Twitch Tension Activity in Relation to Neuromuscular**

Mechanisms.

Contractile Parameter	Physiological Significance	Reference
peak tetanic tension	- mainly attributed to block of postjunctional acetylcholine receptors.	(12,13)
tetanic fade and train of four fade	- where drugs act on nerve endings to impair a component of evoked acetylcholine release in a use-dependent manner.	(12,13) (14,15)
post-tetanic potentiation	- where prior repetitive activity produces an increase in transmitter release from the presynaptic nerve terminal.	(16)

*Statistical Treatment of Data.*

Data for percent response peak tetanic tension and percent response tetanic fade were analysed using an analysis of covariance to test for a betamethasone effect upon the blocking action of each drug. Each drug was considered separately using the aforementioned test regime with dose as the covariate.

**RESULTS**

*RS-I Solution*

An initial experiment was carried out at  $25 \pm 0.5$  °C to determine the longevity of the preparations under perfused and non-perfused conditions using this perfusion technology. After 9 hours, peak tetanic tension had decreased by 9.4 % (LHD) and 25.7 % (RHD) **but** tetanic fade responses showed no decrease in response over this time period. In addition, it was also observed that there was no significant difference between perfused and non-perfused preparations over time for tetanic fade when using RS-I solution (Fig. 4).

A confident comparison was therefore achieved between the contractile responses recorded during pre-drug perfusion with drug-induced responses under non-perfused conditions using this technology. These preliminary findings enabled drug tests to be performed on tissues for many hours (i.e. 6 - 8 hours) with reliability and repeatability so dramatically reducing (ca. 60 %) the number of animals required to be sacrificed.

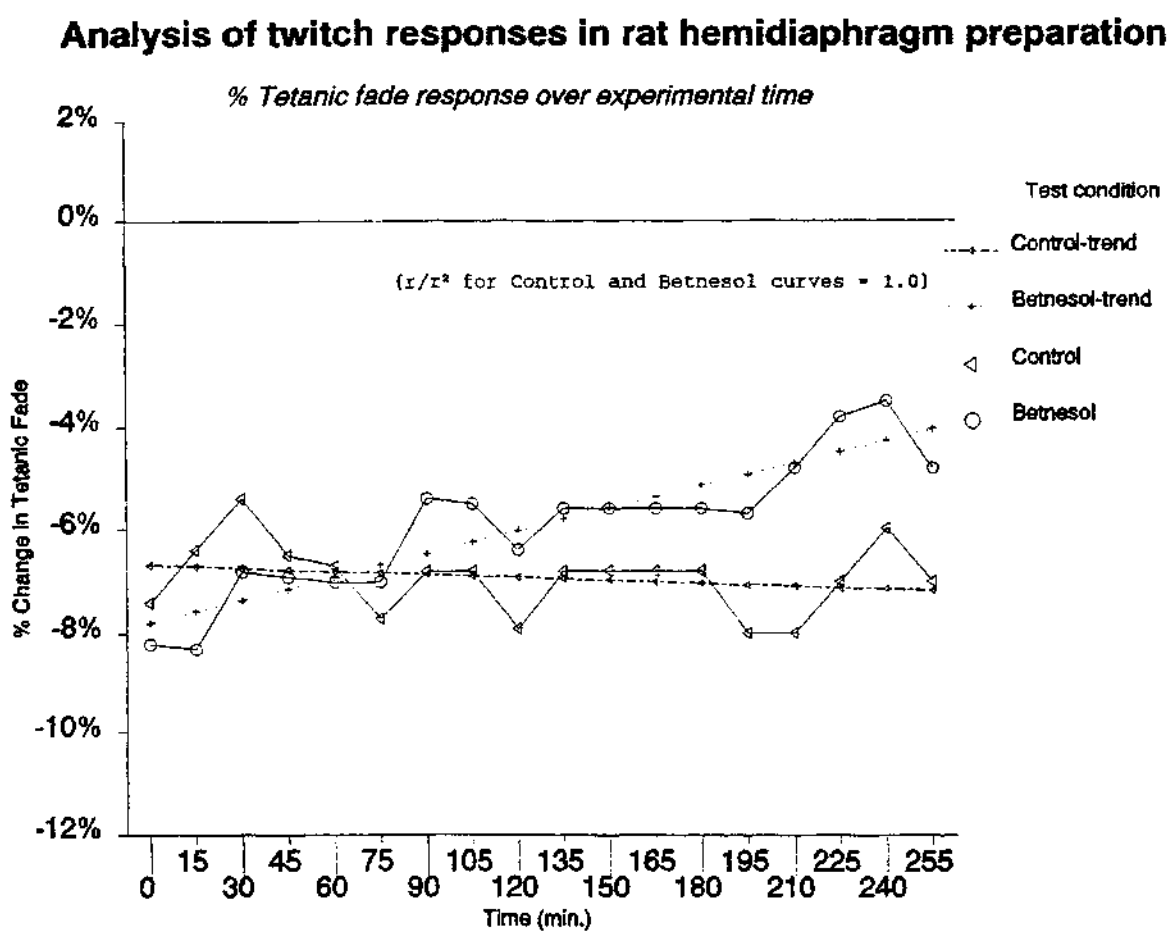
Interestingly, experiments conducted at  $34.0 \pm 0.5$  °C showed that after 6 hours peak tetanic tension decreased by only 33 % using RS-I compared to a 78 % decrease in preparations perfused with conventional Krebs and Henseleit solution (2, 4, 6).

#### *Betamethasone*

An experiment was carried out to observe the effect of betamethasone alone upon muscle contractility (Fig. 4). The control preparation which received only RS-I for the duration of the experiment showed no statistical decrease in percent tetanic fade response over time. The experimental preparation was perfused with RS-I for 90 minutes prior to being perfused with 1  $\mu\text{mol/L}$  betamethasone for 6½ hours. Regression analysis showed that the slope for the experimental responses was twice that of the control indicating that betamethasone had decreased tetanic fade over time to a greater extent than under control conditions. Confidence intervals (95 %) for the two curves separated at 100 minutes indicating a significant difference between control and experimental regression curves after the addition of betamethasone. Peak tetanic tension showed no decrease in the control over time and a slight decrease (ca. 5 %) with betamethasone over time.

The observed two-fold decrease in the rate of tetanic fade with betamethasone and separation of confidence intervals from the control suggests that betamethasone was having a potentiating effect upon neurotransmission. A decrease in fade generally indicates an increase in transmitter output, thus it appears that betamethasone was having an effect presynaptically. The degree of this effect however was small, namely, a 3 % increase in comparison to a 1.5 % increase between the controls after the same period of time. This

facilitatory effect is consistent with that previously reported (17, 18), but more trials would be needed to show whether such a small change is consistent across animals or whether it is due to random variation. Betamethasone showed no significant change in peak tetanic tension when compared with the control.



**Fig. 4 Tetanic fade responses in the isolated rat hemidiaphragm over time**

### *Vecuronium*

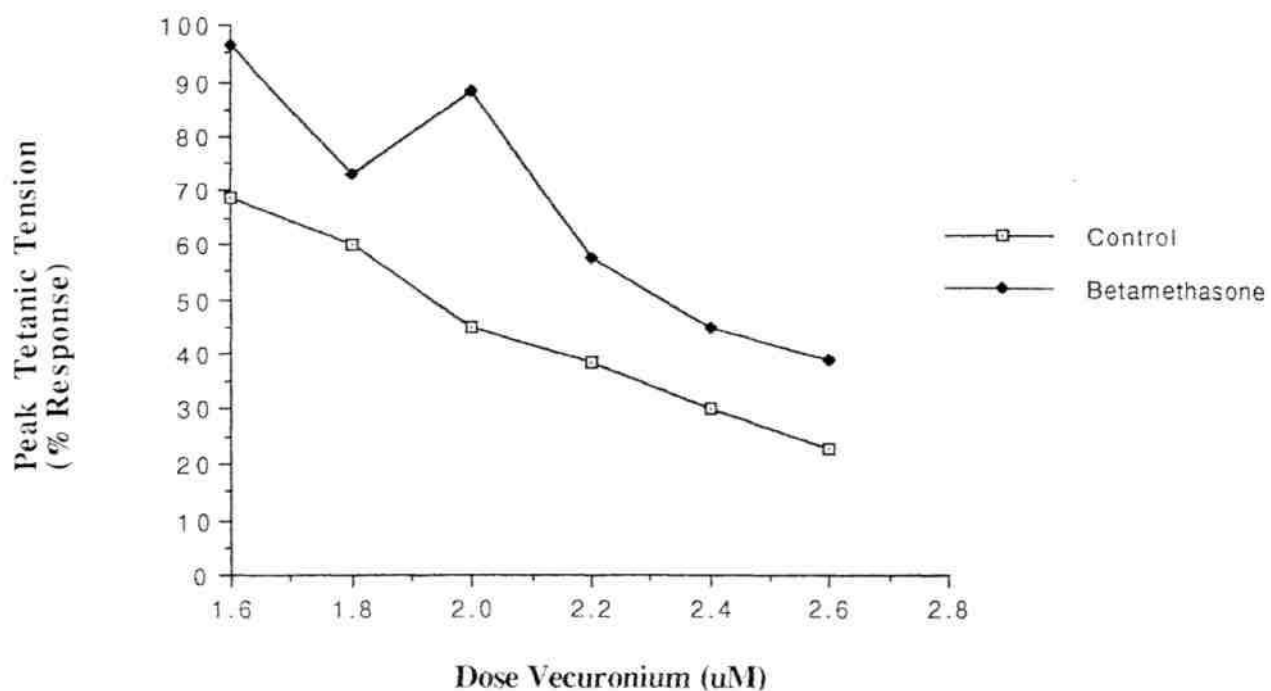
The results presented of peak tetanic tension responses (Fig. 5a) suggests that betamethasone treatment can decrease the degree of neuromuscular blockade produced by all doses of vecuronium in comparison to that observed in control preparations.

At higher doses of vecuronium (Fig. 5b) betamethasone produced less than 50 % tetanic fade while the control preparation fade increased with dose until there was no response to tetanic stimulation after 5 seconds (recorded as 0 % response tetanic fade).

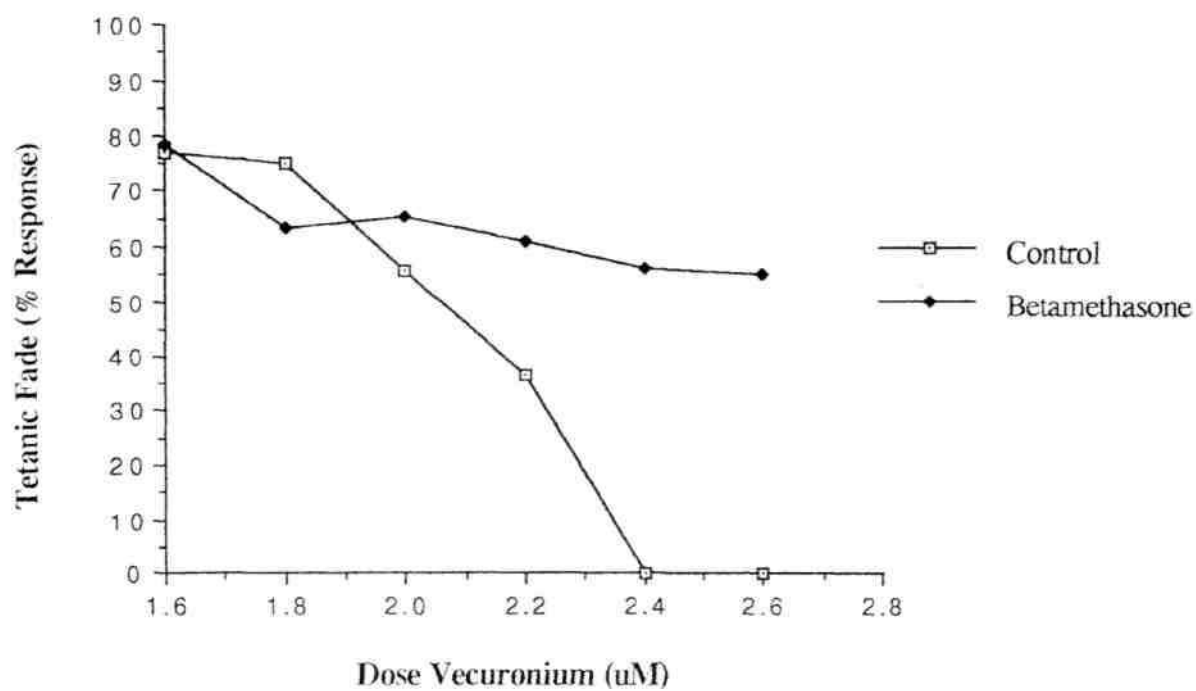
This finding agrees with that of other researchers (6, 7), confirming clinical observations that vecuronium blockade is decreased by pretreatment with betamethasone. This is also consistent with the observation that when another steroid drug, dexamethasone, was administered simultaneously with d-tubocurarine, the LD<sub>50</sub> of d-tubocurarine was increased (19).

The observed control responses suggests that vecuronium may have acted upon the presynaptic nerve terminal such that transmitter output was decreased over the duration of tetanic stimulation resulting in a decrease in twitch response over this period. This would agree with findings in the cat soleus nerve-muscle preparation that higher doses of vecuronium suppress the repetitive firing of motor nerve terminals (20). However, in combination with betamethasone (Fig. 5b), vecuronium produced much less tetanic fade at the same concentrations which suggests that betamethasone may be acting presynaptically to prevent the decline in transmitter output caused by vecuronium.

(a)



(b)

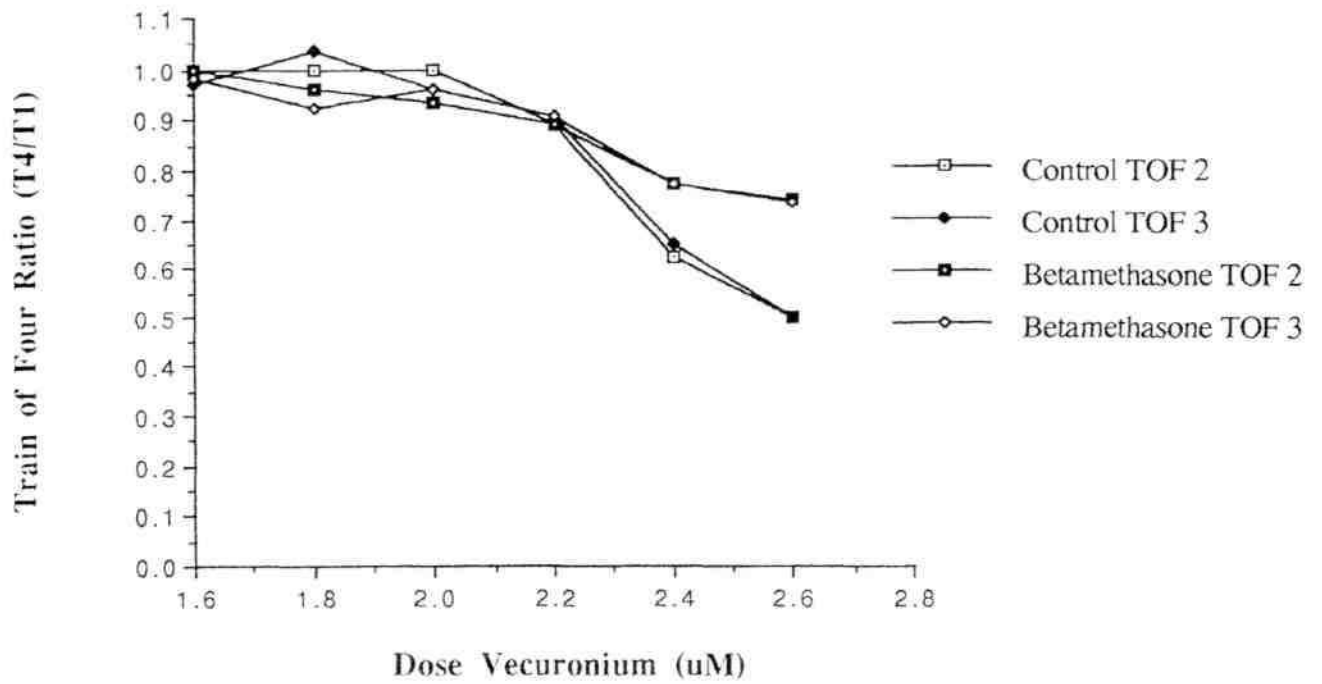


tetanic tension and (b) percent response tetanic fade in control and betamethasone treated preparations.µ

**Figure 6: Drug dose response curve for vecuronium of (a) train of four ratios (TOF 15**

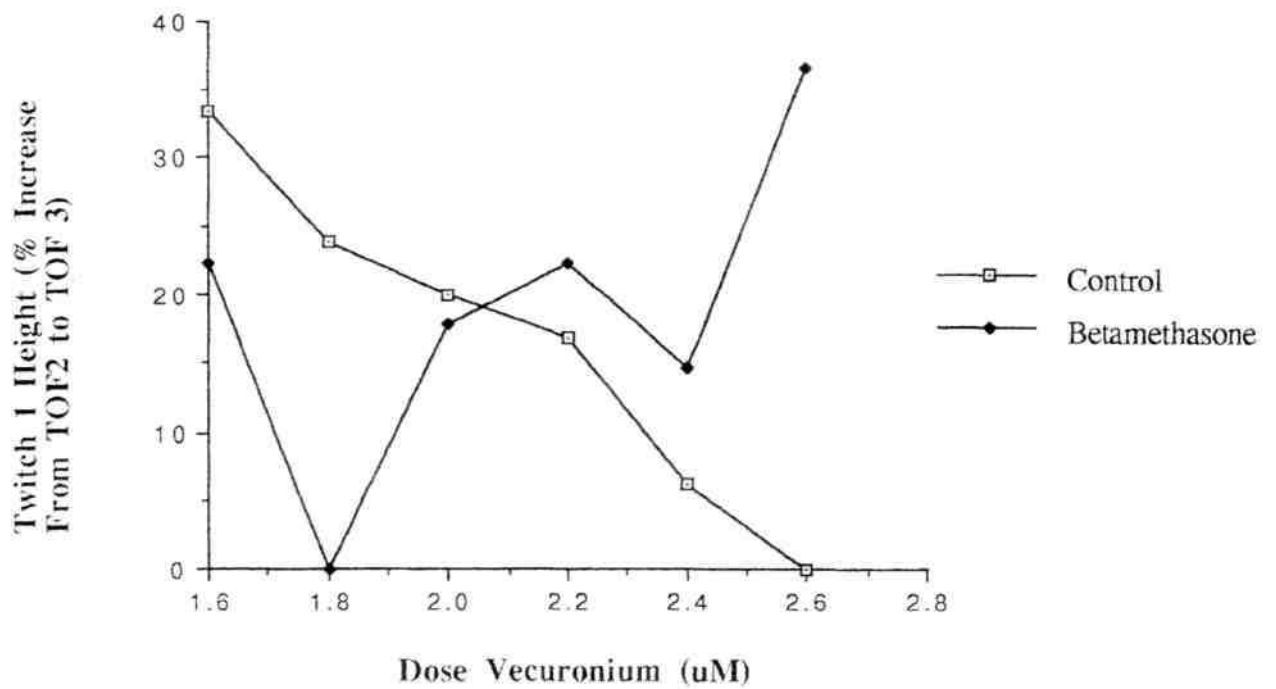
**(a)**

**2 and TOF 3) and (b) post-tetanic potentiation in control and betamethasone treated**



**(b)**

**preparations.**



The fade observed in train of four ratios (Fig. 6a) also suggests a presynaptic mode of action for betamethasone whereby less fade was evident compared to the control indicating that betamethasone may be antagonising the effect of vecuronium presynaptically. While post-tetanic potentiation (Fig. 6b) responses were inconsistent for betamethasone, post-tetanic potentiation steadily decreased and was inhibited at 2.6  $\mu$ M in the control preparation but was still observed in the betamethasone treated preparation.

The phenomena of post-tetanic potentiation has been described as a presynaptic mechanism where upon, following high frequency stimulation, neurotransmitter output at low frequency stimulation is increased with a consequent increase in twitch contraction (12) caused by a resultant increase in release of acetylcholine quanta from the presynaptic terminal (16).

Vecuronium was found to decrease post-tetanic potentiation (Fig. 6b) with increasing doses, and to eventually inhibit it altogether. This is in accordance with other findings that post-tetanic potentiation is suppressed at higher doses of vecuronium (20). In the presence of betamethasone post-tetanic potentiation was decreased to a lesser extent than in the control with increasing concentrations of vecuronium and was not inhibited at any dose (Fig. 6b). This result could again reflect a presynaptic effect of betamethasone in which the expected inhibition of post-tetanic potentiation by vecuronium is offset.

Essentially, the data for vecuronium suggest that betamethasone reduced the neuromuscular blocking action of vecuronium and that this is likely to have occurred due to effects that are presynaptic in origin. It may be speculated that vecuronium produces fade and inhibition of post-tetanic potentiation by blocking a presynaptic acetylcholine receptor linked to a positive feedback mechanism for acetylcholine synthesis and/or release (21, 22).

Interestingly, the corticosteroid prednisolone has been shown to facilitate the spontaneous release of acetylcholine in the rat diaphragm (17), as has dexamethasone in the mouse

diaphragm (18). If betamethasone similarly acts to enhance acetylcholine release, this could explain some of the results observed in the present study. Increased acetylcholine levels in the synaptic cleft would provide increased competition with vecuronium for postsynaptic nicotinic receptors, so decreasing the vecuronium block. The reversal of neuromuscular blockade induced by non-depolarizing blockers using anticholinesterase drugs follows a similar mechanism, except, that the increased acetylcholine level in the synaptic cleft is due to the inhibition of acetylcholinesterase.

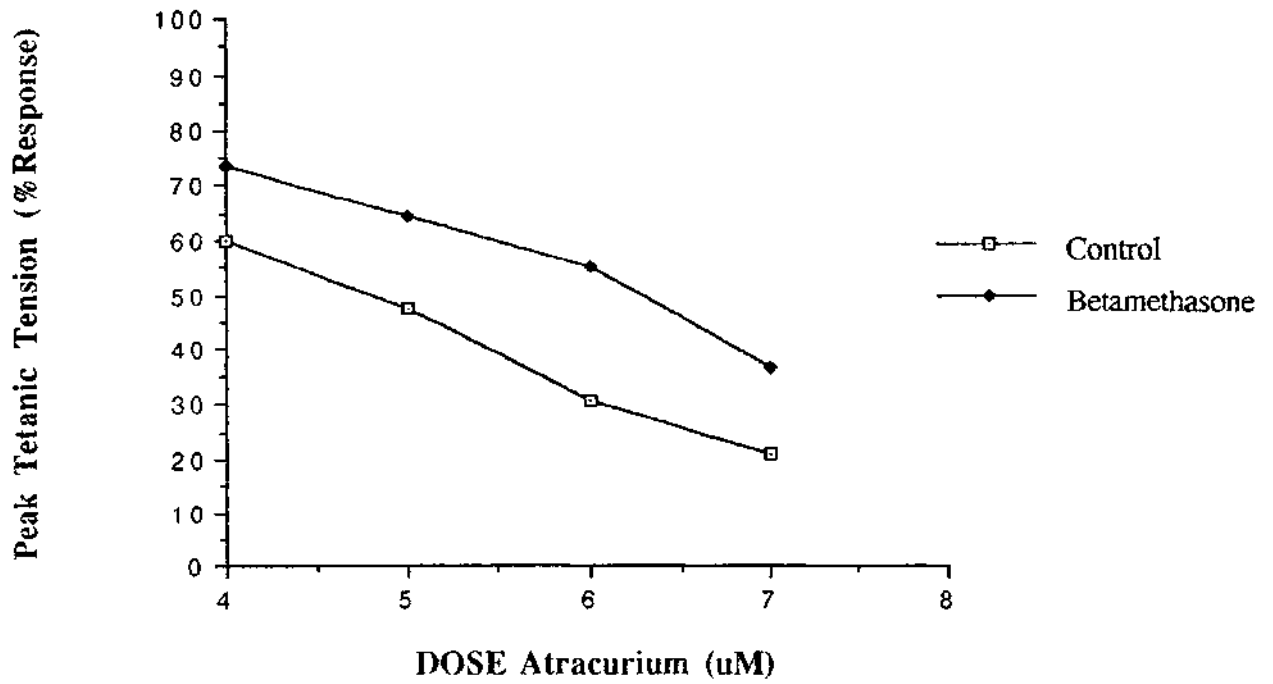
If vecuronium is acting presynaptically to reduce acetylcholine output and betamethasone is increasing acetylcholine output then competition is also likely to similarly occur at presynaptic receptor sites so preventing vecuronium from binding with these receptors. Neostigmine has been shown to decrease fade induced by non-depolarising drugs presumably by an increase in acetylcholine concentration in the synaptic cleft (12). While vecuronium is suggested to inhibit a positive feedback mechanism by blocking either a nicotinic or muscarinic presynaptic receptor, the increased release of acetylcholine may effectively compete with vecuronium to act upon receptors linked to a positive feedback mechanism, leading to further acetylcholine release. This may provide a plausible explanation for the present findings whereby betamethasone could offset the blocking action of vecuronium both pre- and postsynaptically.

#### *Atracurium*

Similar results (Figs. 7 and 8) to those achieved with vecuronium were observed with atracurium, whereby betamethasone treatment decreased the neuromuscular blockade induced by atracurium. Atracurium alone was found to produce tetanic fade which increased with dose (Fig. 7b), as did the train of four fade response (Fig. 8a) suggesting a presynaptic inhibitory action for the drug in addition to postsynaptic blockade. This is in line with observations using the rat cut fibre diaphragm preparation that atracurium is capable of producing stimulus, frequency-dependent inhibition of acetylcholine release from the nerve terminal (23).

Betamethasone gave an increase in post-tetanic potentiation at all doses of atracurium (Fig. 8b), which was greater than that observed in the control. Atracurium decreased post-tetanic potentiation over increasing doses, suggesting that a presynaptic, positive feedback mechanism was being inhibited. This effect was probably achieved through a similar mechanism to that proposed for the effect of betamethasone upon the vecuronium blocking actions pre- and postsynaptically.

(a)



(b)

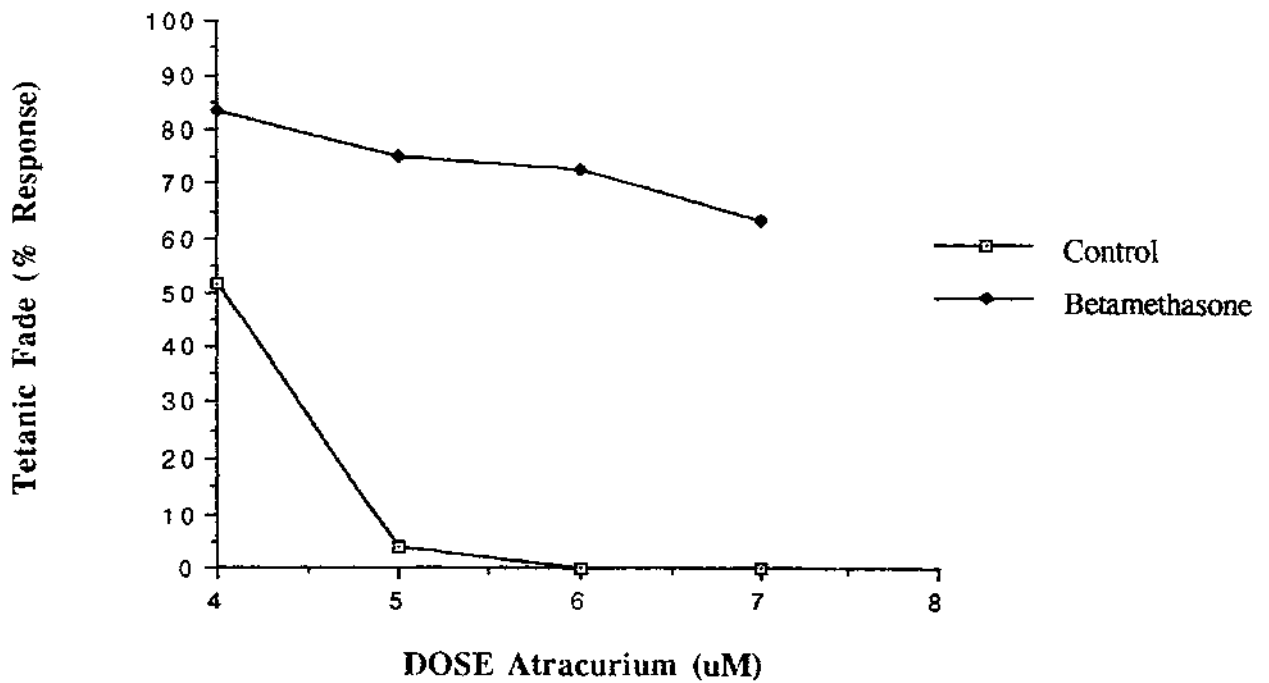
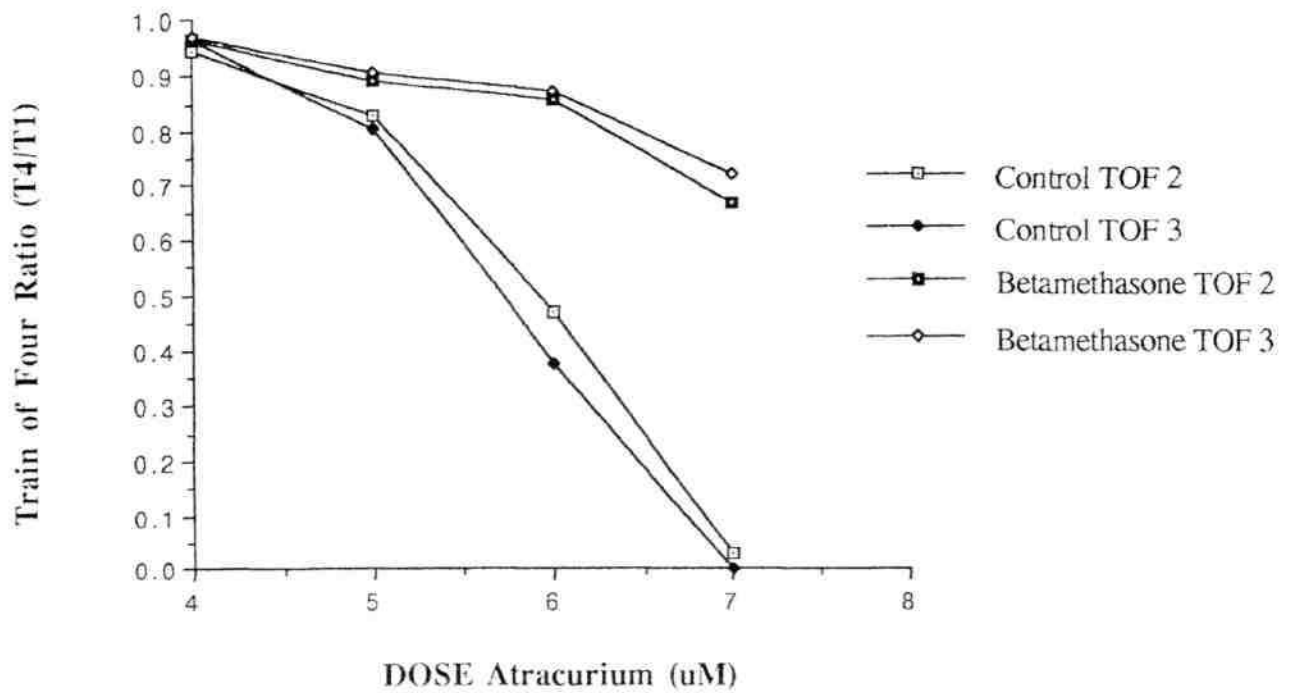
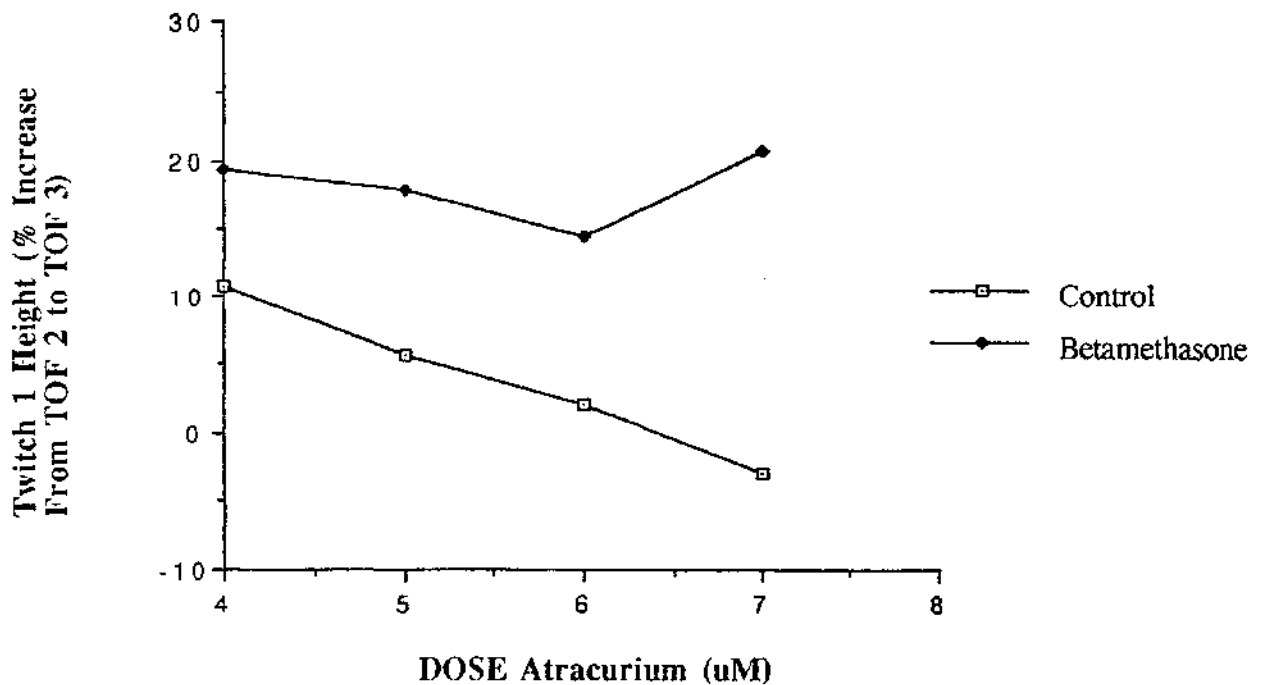


Figure 7: Drug dose response curve for atracurium of (a) percent response peak tetanic tension and (b) percent response tetanic fade in control and betamethasone treated preparations.

(a)



(b)



**Figure 8:** Drug dose response curve for atracurium of (a) train of four ratios (TOF 2 and TOF 3) and (b) post-tetanic potentiation in control and betamethasone treated preparations.

### *Suxamethonium*

Betamethasone decreased peak tetanic tension to a greater extent than the control at all but the lowest dose of suxamethonium applied. The results (Fig. 9a) suggest that betamethasone potentiated the extent of the suxamethonium-induced block over the linear dose range of the curve which is opposite to its effect with vecuronium and atracurium. This is probably because at low doses, where suxamethonium itself shows little blocking action, a betamethasone effect may not be noticeable, whereas at high doses of suxamethonium many postsynaptic receptors are blocked and little difference would be observed in the resulting twitch contraction due to the non-competitive actions of suxamethonium.

Assuming that betamethasone increases acetylcholine release (17, 24), potentiation of the suxamethonium block by betamethasone is likely to result from an increase of acetylcholine in the synaptic cleft. Accumulation of acetylcholine would result in increased channel opening and is therefore likely to enhance, rather than reverse, ion channel block in the presence of suxamethonium, a non-competitive blocker (25). It has been reported that anticholinesterase agents, which block acetylcholinesterase and therefore increase acetylcholine levels in the synaptic cleft, are synergistic with depolarising neuromuscular blocking agents, particularly in their initial phase of action (26).

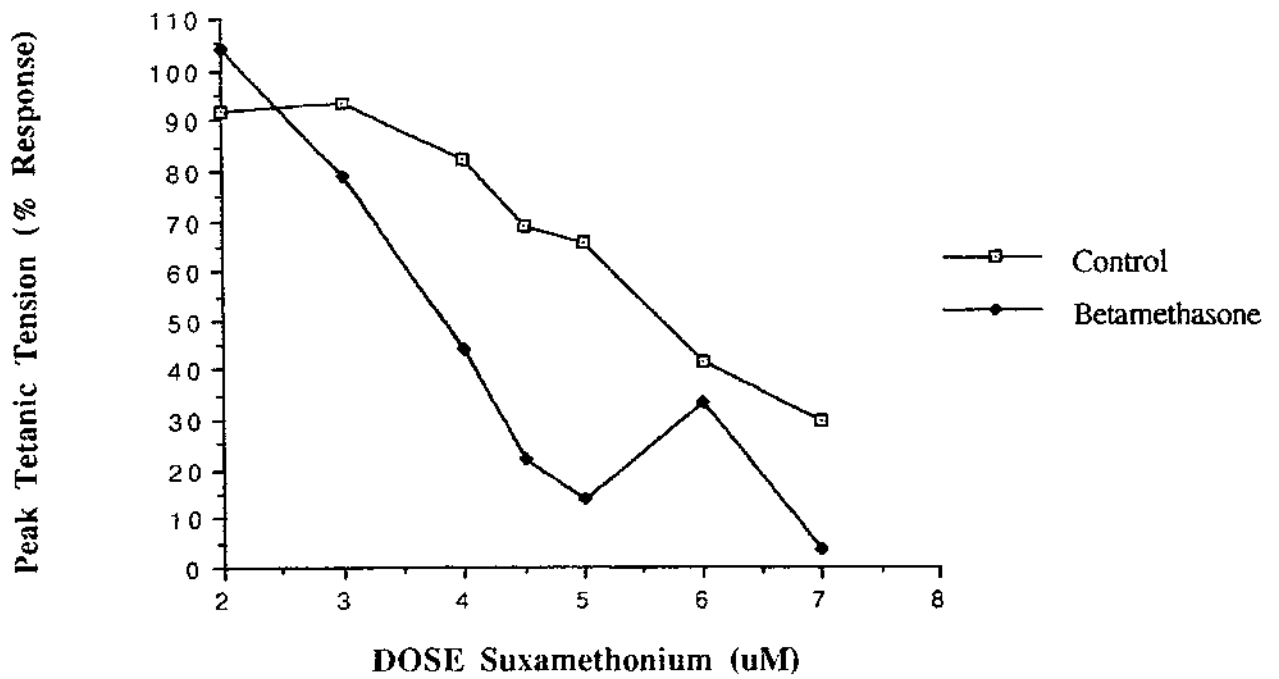
The non-competitive nature of suxamethonium is reflected clinically, where neostigmine does not improve recovery from suxamethonium-induced neuromuscular blockade (27). Attenuation of the neuromuscular blocking action of suxamethonium by betamethasone may not have been noticed in clinical use because suxamethonium is used only as a short-term blocking drug whereby its effect wears off very quickly.

Betamethasone with suxamethonium showed much greater fade when compared with the suxamethonium control (Fig. 9b), which displayed little fade even at a dose of 7  $\mu\text{M}$ , at which point, betamethasone fade was maximal. The occurrence of fade with

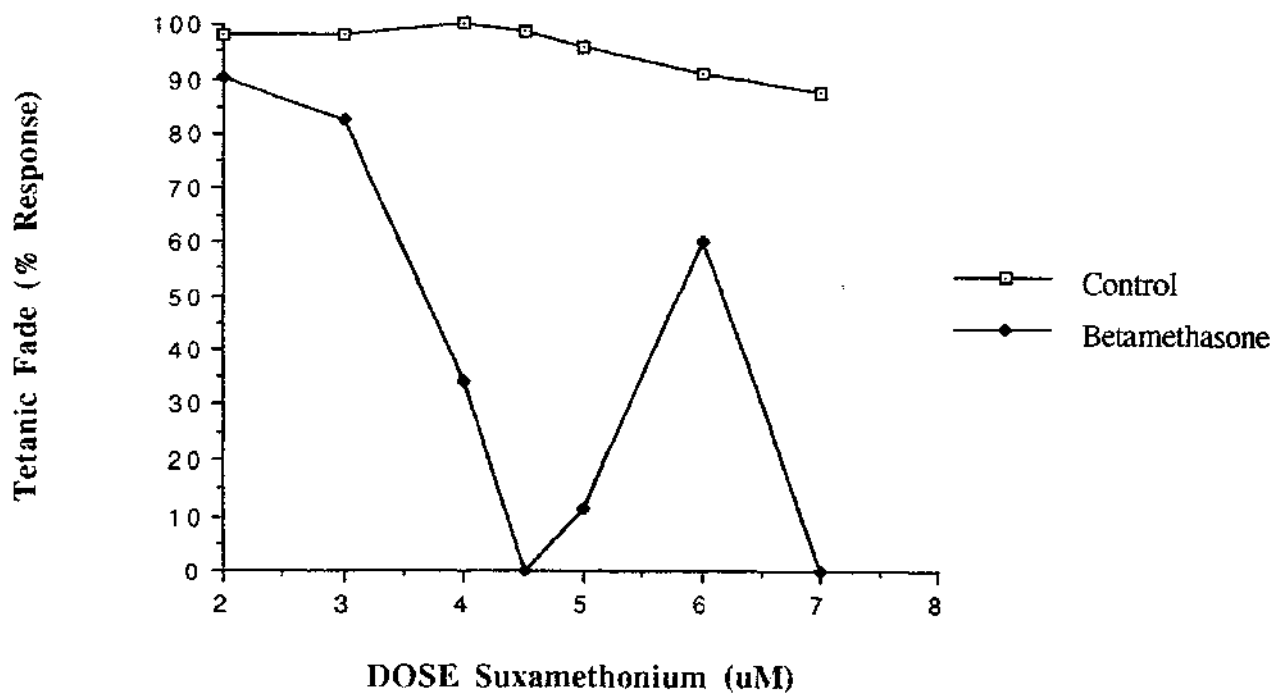
suxamethonium is characteristic of phase-II block (28), and suggests that suxamethonium was having some presynaptic effect, possibly blocking a positive (nicotinic) feedback mechanism for acetylcholine upon its own release. In contrast, betamethasone greatly increased tetanic fade at increasing concentrations of suxamethonium. It may be postulated that in the presence of betamethasone, acetylcholine output was significantly increased which could, in addition to the positive feedback, nicotinic receptors being blocked by suxamethonium, also activate the presynaptic negative feedback, muscarinic receptors so decreasing transmitter release resulting in an accentuation of tetanic fade.

Figure 9: Drug dose response curve for suxamethonium of (a) percent response peak **23**

(a)

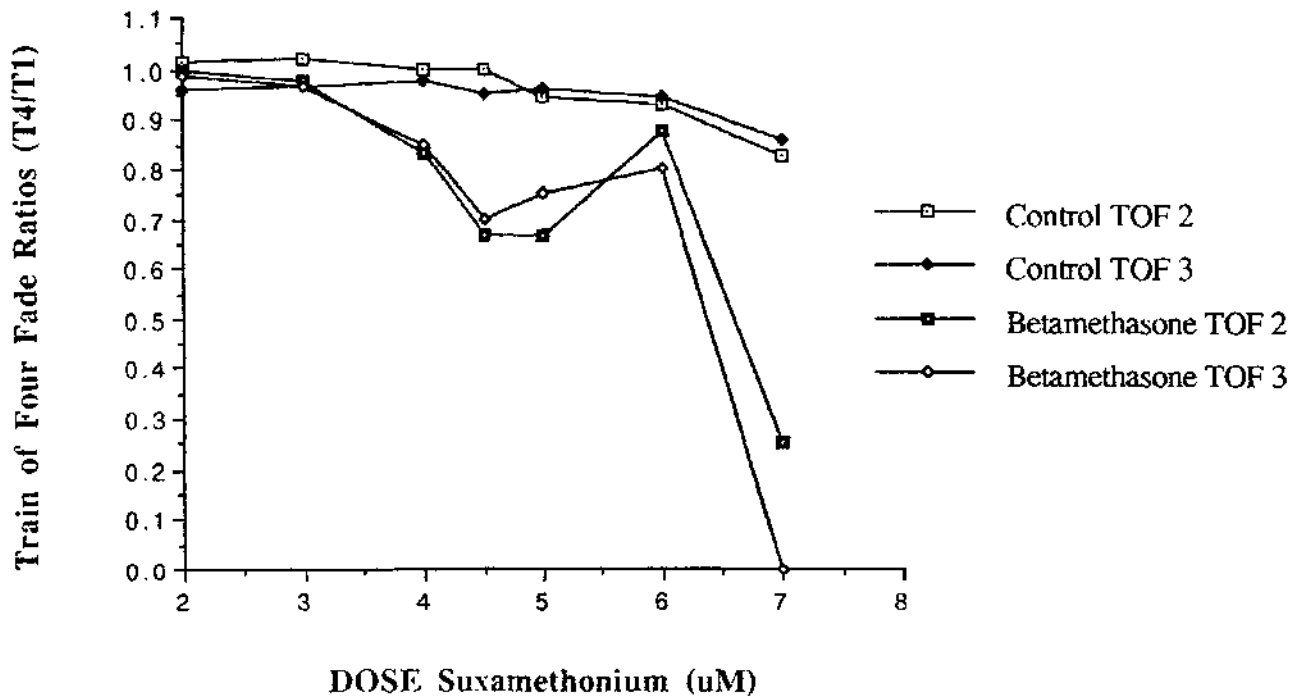


(b)



tetanic tension and (b) percent response tetanic fade in control and betamethasone treated preparations.

(a)



(b)

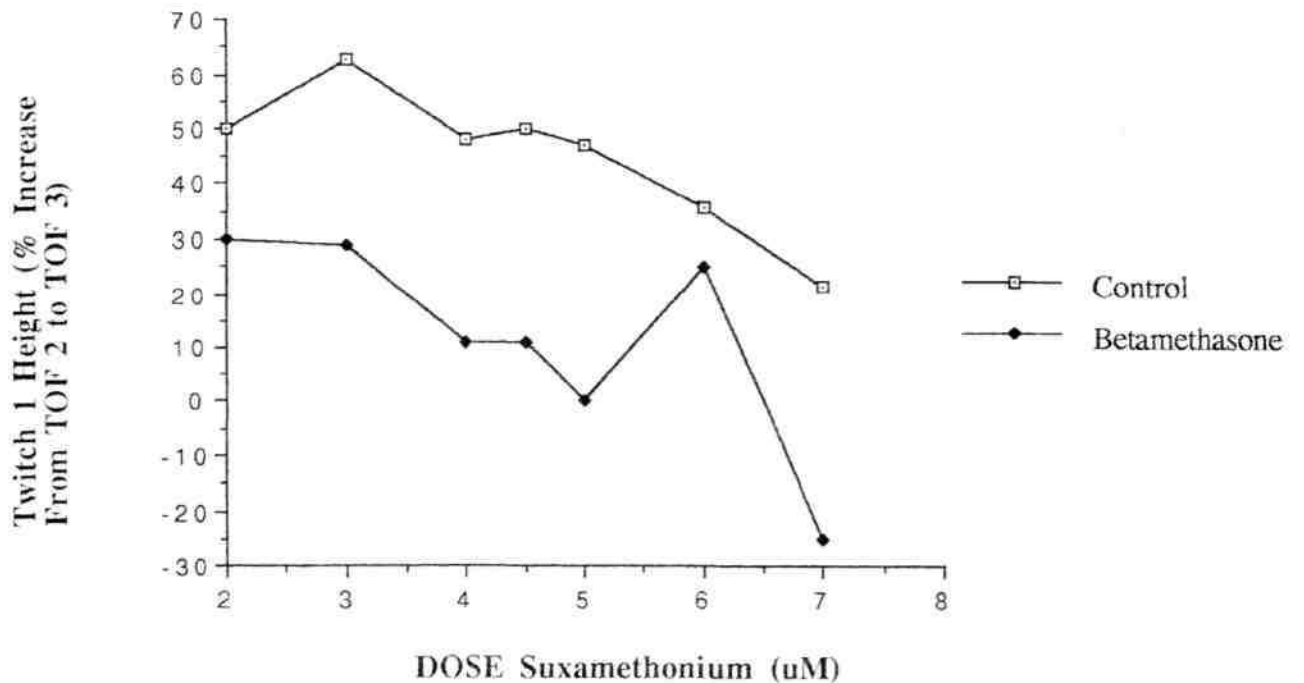


Figure 10: Drug dose response curve for suxamethonium of (a) train of four ratios (TOF 2 and TOF 3) and (b) post-tetanic potentiation in control and betamethasone treated preparations.

Evidence for the occurrence of a presynaptic action by suxamethonium is also suggested in the train of four responses which showed some fade for the suxamethonium control but greater fade in the presence of betamethasone and was further accentuated at higher concentrations of suxamethonium (Fig. 10a).

The hypothesis for a presynaptic action for suxamethonium is supported by an observed increase in post-tetanic potentiation at lower doses of suxamethonium followed by a decrease with increasing dose. Post-tetanic potentiation at a dose of 7  $\mu$ M suxamethonium (Fig. 10b) even showed a % percent decrease in twitch height compared with that prior to tetanic stimulation. This may suggest an initial presynaptic facilitatory effect of suxamethonium upon presynaptic nicotinic receptors at lower doses followed by a delayed presynaptic blocking effect

The observation that betamethasone attenuated suxamethonium-induced neuromuscular blockade but decreased that induced by atracurium or vecuronium may be explained in terms of the differences between depolarising and non-depolarising drug receptor interactions as previously discussed. However, the differences in fade responses between suxamethonium and the non-depolarising drugs are more complex. There is ample evidence that depression of peak tetanic tension and fade are consequences of separate independent mechanisms because, for the same degree of twitch depression, different neuromuscular blocking drugs produce different degrees of tetanic fade (25). Thus differences between the drugs in fade responses cannot be discounted as being the same postsynaptic mechanism as for peak tetanic tension.

## INFERENCES

For all three blocking drugs the effect of betamethasone was statistically insignificant. A strong interaction was found between the dose of each blocking drug and betamethasone (shown by non-parallel curves between the control and experimental) confounding any interaction between the drug and steroid. The blocking drugs also showed an interaction

between each animal and betamethasone, although to a lesser extent for suxamethonium. This was due largely to differences between rats and low sample numbers.

As well as decreasing the postsynaptic blockade by vecuronium and atracurium it is likely that betamethasone also affects their presynaptic receptor blocking actions, probably via an increase in acetylcholine concentration in the synaptic cleft which thereafter affects the activities of presynaptic receptors with these neuromuscular blocking agents. Thus the presence of betamethasone with atracurium or vecuronium may lead to a decrease in a presynaptic, negative-feedback mechanism upon acetylcholine output, resulting in the observed decrease in fade.

In the case of suxamethonium, the presynaptic effect of betamethasone is probably via a similar increase in neurotransmitter concentration, but the effect of this is to attenuate the presynaptic suxamethonium block. This could occur in a similar way to attenuation of the postsynaptic block by suxamethonium, in that, the increase in acetylcholine results in activation of more presynaptic receptors so facilitating the binding of suxamethonium to these receptors (25) and the observed synergy of betamethasone with suxamethonium.

From this study it may be inferred that the neuromuscular blocking agents examined bind to different types of presynaptic nicotinic and muscarinic cholinergic receptors, and so interfere with mechanisms that mediate different presynaptic events, as has been shown for many cholinergic agonists and antagonists. Such differences cannot be implied unequivocally from these experiments as the dose ranges of the drugs used would have to be standardised and tested to observe any true differences between the drug interactions. Likewise although muscle contractility returned to pre-drug responses following drug wash out and perfusion, the possibility of an earlier dose affecting the response of the tissue to latter doses cannot be discounted. This is known as correlated errors and could be taken into account by administering doses randomly.

Equally the possibility that betamethasone had a cumulative effect over time cannot be ruled out. This could in part account for the strong interaction that was found between betamethasone and the doses of neuromuscular blocking drugs tested as these were applied in successively increasing concentrations over time. An increase in the number of experiments performed may be advantageous as the significance level for the observed responses may have increased.

Despite these reservations certain trends were observed for particular drug responses over extended time periods which raise interesting questions regarding the long-term, accumulative action of the drugs examined. In terms of the proposed mechanisms which exist at the neuromuscular junction to regulate neurotransmitter output from the nerve terminal, the role of strategically placed nicotinic and muscarinic auto-presynaptic receptors (21, 22) needs further investigation. Studies using microelectrode technology to examine changes in quantal content and release of neurotransmitter would be beneficial to further elucidate the specific sites of action of these drugs at the mammalian neuromuscular junction.

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