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In-vitro *Investigation of a New Neuromuscular Relaxant, AH8165*

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AH8165 was compared with other neuromuscular relaxants in an *in-vitro* rat phrenic nerve–diaphragm preparation. Concentrations of 6–10 μg/ml AH8165 produced progressive decreases in strength of concentration. AH8165 was 0.1 times as potent as *d*-tubocurarine, and its effects were more rapidly reversed by washing. The times to recovery from 90 per cent blockade were the same for succinylcholine and AH8165, but the time to recovery from 50 per cent blockade was shorter for succinylcholine than for AH8165. Neostigmine reversed blockade induced by *d*-tubocurarine to 80 per cent of control, while it reversed comparable blockade induced by AH8165 to only 40 per cent of control. Doses of 0.5 to 2 μg AH8165 produced contracture and increased the force of contraction of the superfused chick biventer cervicis muscle preparation. Doses of 8 to 32 μg produced decreased contracture followed by diminution of the strength of contraction. The authors conclude that AH8165 in low concentrations has a depolarizing action, which is obscured by nondepolarizing effects in higher concentrations. (Key words: Neuromuscular relaxants: AH8165.)

AH8165, a 1,1′-azobis-arylimidazo-[1,2-α]-pyridinium derivative (fig. 1), has been released in Great Britain for clinical trials as a neuromuscular relaxant. Data from investigations *in vivo* in cats, mice, and chicks indicate that the drug is a rapid-acting, nondepolarizing, neuromuscular relaxant with a short duration of action. At effective neuromuscular blocking doses AH8165 produces minimal cardiovascular effects, and blockade is readily reversible with neostigmine.[1–3]

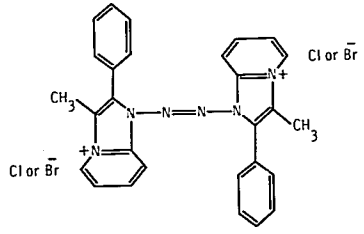

FIG. 1. Chemical structure of AH8165.

In-vitro investigations of this drug seem to be lacking. The ED_{50} of AH8165 has been compared with those of gallamine, pancuronium, and succinylcholine in various nerve–muscle preparations,[3] and Post *et al.* reported a depolarizing action of the drug at doses lower than those needed to produce neuromuscular blockade.[4] However, no attempt has been made to compare the onset of action of and rate of recovery from AH8165 with those of other neuromuscular relaxants. In addition, there has been no *in-vitro* comparison of neostigmine reversals of AH8165 and *d*-tubocurarine.

The purposes of the present study were to describe the dose–response curves for AH8165, to establish time to onset and duration of action of AH8165, to compare AH8165 with *d*-tubocurarine and succinylcholine, and to ascertain whether AHS165 in low concentrations possesses depolarizing activity.

Methods and Materials

Rat Phrenic Nerve–Diaphragm Preparation

Male Sprague-Dawley rats, weighing 100 to 200 g, were decapitated and drained of blood. The skin was incised along the mid-

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line of the chest and the thorax opened. The left phrenic nerve was isolated and removed with the diaphragm. The preparation was mounted on a muscle holder, the phrenic nerve placed across platinum electrodes, and the preparation on its mounting was placed in an organ bath containing 100 ml of Krebs solution (composition in grams per liter of distilled water: NaCl 5.5., KCl 0.35, $MgSO_4 \cdot 7\ H_2O$ 0.11, $CaCl_2$ 0.28, KH_2PO_4 0.16, $NaHCO_3$ 2.1, glucose 4.0). The solution was maintained at 37 C and bubbled with 95 per cent oxygen and 5 per cent carbon dioxide. A suture from the tendon was attached to a force-displacement transducer. An initial tension of 5 g was placed on the muscle. The nerve was stimulated at a frequency of 0.2 Hz by pulses 0.1 millisecond in duration and supramaximal in voltage.

The drugs were studied to determine the following variables: rate of onset, magnitude of blockade, and rate of recovery after washing. In addition, reversibility of blockade by neostigmine was determined. Drug action was terminated by flushing the bath twice with normal Krebs solution. Ten minutes were allowed to elapse before the addition of another concentration of drug. Each drug was tested on ten diaphragms, and each diaphragm served as its own control.

Denervated Rat Diaphragm Preparation

Male Sprague-Dawley rats, weighing 90–100 g, were anesthetized with sodium pentobarbital solution, 30 mg/kg, intraperitoneally. A 1-cm incision was made through the seventh intercostal space in the left midclavicular line and the left phrenic nerve was severed at its insertion into the diaphragm.

After one week, the rats were sacrificed and the diaphragm removed in the usual manner. Stimulation of the nerve remnant failed to produce a muscle response. The muscle was mounted in a bath containing Krebs solution and stimulated by current passed between a platinum wire inserted into the tendon and a platinum electrode in the rib margin. Pulses 0.1 millisecond in duration and supramaximal in voltage were applied at a frequency of 0.2 Hz. Strength of contraction was recorded. Various concentrations of AH8165 were added to the bath as before.

Chick Biventer Cervicis Nerve–Muscle Preparation

The chick biventer cervicis nerve–muscle preparation[5] was used to examine the neuromuscular effects of AH8165.

One- or two-week-old chicks were sacrified with chloroform. The back of the head and neck was plucked and the skin incised in the midline from the skull to below the base of the neck. The biventer cervicis muscles lying on either side of the midline and immediately below the skin were exposed and removed. The lower belly of each muscle was attached to an inferiorly placed stationary rod. A suture from the tendon was passed through a hole in a small cup over the electrode to a superiorly placed force-displacement transducer. The small cup over the electrode was essential for superfusion because it eliminated the mechanical effect of the drops of Krebs solution on the motor nerve, which caused irregular twitching of the muscle.[6] An initial force of 1 g was placed on the muscle. The motor nerve within the tendon was electrically stimulated at a frequency of 0.1 Hz, with a pulse duration of 0.1 millisecond at supramaximal stimulation, usually between 20 and 100 volts. These high voltages were necessary because the motor nerve lies within the tendon. The subsequent muscle twitch obtained was recorded on a Beckman Dynograph Recorder. AH8165 was added to the cup in various concentrations to determine its effects on the following modalities: presence of contracture, magnitude of contracture, presence of blockade, and magnitude of blockade. Drug action was terminated by superfusion of normal Krebs solution. Muscle twitch was allowed to remain at pre-drug levels for at least 10 minutes before another concentration of drug was added. Each dose of AH8165 was tested on ten nerve–muscle preparations.

The chick biventer cervicis nerve–muscle preparation was used to determine whether in low concentrations AH8165 possessed any depolarizing activity. This muscle is com-

posed of two types of fibers, tonic and fast-twitch fibers.[7] The fast-twitch fibers produce a twitch response exactly like that of the rat diaphragm when electrically stimulated. The tonic fibers respond to the application of depolarizing substances with contracture. The preparation may therefore be used to test simultaneously for neuromuscular blocking activity, as reflected by reduction in the magnitude of contraction produced by neural stimulation, and for the presence of depolarization, as indicated by contracture.[5]

Statistics

Analysis of covariance was employed[8] to determine the dose–response relationships of AH8165. The ED_{50} for neuromuscular blockade was obtained from the regression line.

A four-point bioassay, as described by Finney,[9] was performed to determine the relative potencies of AH 8165 and *d*-tubocurarine. *d*-tubocurarine.

Comparisons of the effects of AH8165 with those of other neuromuscular blocking agents were made using Student's t test[8] for paired observations. In all cases $P \leq 0.05$.

Observations and Results

Rat Phrenic Nerve–Diaphragm Preparation

Concentrations of AH8165 of less than 6.0 μg/ml produced 5 to 10 per cent increases in strength of contraction in some muscle preparations. However, this was not consistent, and occurred in only half the preparations tested. Increasing concentrations of AH8165 to 6 to 10 ng/ml produced progressive diminution of the force of contraction. When maximal blockade was reached, it remained stable at that level for as long as two hours. The best-fitting regression line was calculated (fig. 2) and an ED_{50} of 7.4 μg/ml determined from it.

The rates of onset to 90 per cent blockade by AH8165, *d*-tubocurarine, and succinylcholine were compared. AH8165 was found to have a more rapid onset of blockade than *d*-tubocurarine, but a slower onset than succinylcholine (fig. 3).

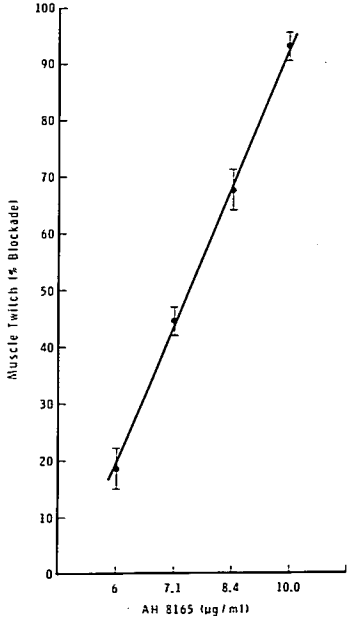

FIG. 2. The effect of AH8165 on muscle twitch of the rat phrenic nerve–diaphragm preparation. The points represent the actual data points; the line is the best-fitting regression line. The scale of the abscissa is logarithmic. Values are means ± SE; n = 10. The ED_{50} calculated from the line is 7.4 μg/ml.

The time to 100 per cent recovery from blockade after washout of AH8165 was examined. This was considered to be the time from addition of normal Krebs solution until muscle contraction had returned to pre-drug control levels. Immediately upon addition of normal Krebs solution, recovery began; it was quite rapid (fig. 4). Time to recovery was directly related to concentration of AH8165. Time to 100 per cent recovery from an 18.7 per cent blockade, produced by 6.0 μg/ml, was 0.9 ± 0.2 minutes (SE). Recovery to 100 per cent from a 93 per cent blockade, produced by 10.0 μg/ml, was 3.1 ± 0.4 minutes.

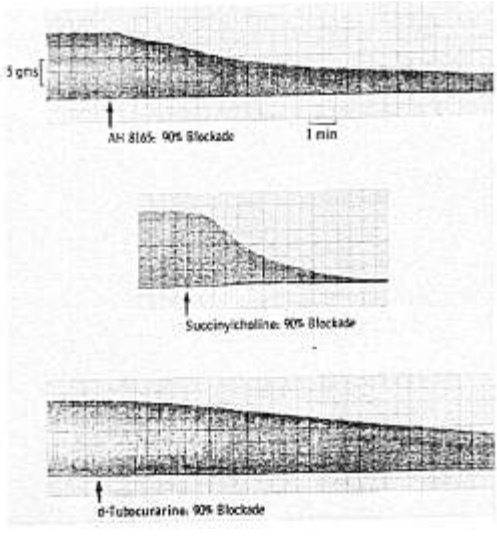

FIG. 3. Typical records of the onsets of 90 per cent blockades induced by AH8165, succinylcholine, and *d*-tubocurarine in the rat phrenic nerve–diaphragm preparation. Arrows indicate administration of the drugs.

A comparison of AH8165 with *d*-tubocurarine was undertaken. The dose–response curves of AH8165 and *d*-tubocurarine were parallel, and AH8165 was 0.1 times as potent as *d*-tubocurarine (fig. 5).

To compare the rates of recovery from blockades produced by AH8165 and *d*-tubocurarine, the data of figure 5 were used to select concentrations of the drugs that would produce blockades of equal mag-

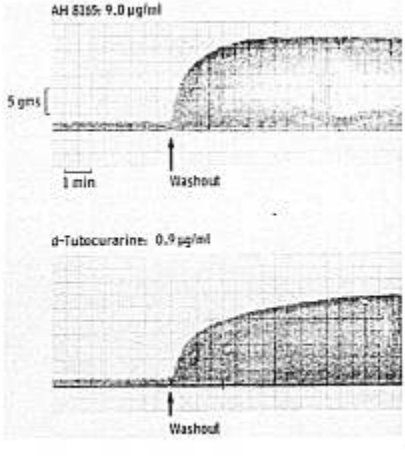

FIG. 4. Recoveries from blockades induced by AH8165 and *d*-tubocurarine in the rat phrenic nerve–diaphragm preparation. *Upper trace:* blockade produced by AH8165, 9.0 µg/ml. *Lower trace:* blockade produced by *d*-tubocurarine, 0.9 µg/ml. Arrows represent washout with normal Krebs solution.

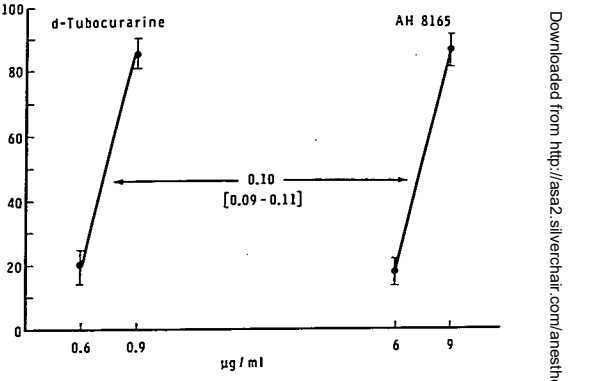

FIG. 5. Comparative effects of AH8165 and *d*-tubocurarine on muscle twitch of the rat phrenic nerve–diaphragm preparation. Points represent actual data points. Values are means ± SE, n = 10. The four-point bioassay was performed and the resultant potency ratio of AH8165 to *d*-tubocurarine was calculated to be 0.1. The 95 per cent confidence interval was 0.09 to 0.11.

nitude. The muscle preparation was then washed with normal Krebs solution and the time to 100 per cent recovery and rate of recovery were evaluated. Recoveries from the effects of the two agents were very different (table 1). AH8165 (9.0 µg/ml) and *d*-tubocurarine (0.9 µg/ml) produced blockades of 86.2 per cent ± 3.9 and 86.4 per cent ± 4.4, respectively. However, the time to 100 per cent recovery from *d*-tubocurarine blockade was longer. A typical record can be seen in figure 4.

A comparison of AH8165 with succinylcholine was also undertaken. The dose–response curves of the two agents were not parallel. In order to compare the rates of recovery from blockades produced by AH8165 and succinylcholine, the drugs were titrated to produce blockades of equal magnitude. It was found that there was a significantly greater time to 100 per cent recovery from the 50 per cent blockade produced by AH8165. However, there was no significant difference between the rates of recovery to 100 per cent from 90 per cent blockades produced by succinylcholine and AH8165 (fig. 6).

Neostigmine reversals of blockades of equal magnitude produced by AH8165 and *d*-tubocurarine were compared. Neostigmine, 1 µg/ml, produced 80.1 ± 5.2 per cent reversal of a 90 per cent blockade by *d*-tubocurarine, but produced only 38.7 ± 4.3 per cent reversal of the 90 per cent blockade AH8165 (fig. 7). The slope of reversal from the former was also significantly greater than that from the latter. In contrast to its effect on *d*-tubocurarine, neostigmine could not completely reverse blockade produced by AH8165. Ten µg/ml and 50 µg/ml of neostigmine were administered to a preparation in which 90 per cent blockade had been

TABLE 1. Recovery of Blockades of Contraction of the Rat Phrenic Nerve–Diaphragm Preparation Induced by AH8165 and *d*-Tubocurarine*

	Concentration (µg/ml)	Per Cent Blockade	Time to 100 Per Cent Recovery (Min)	Slope of Initial Recovery (g/Min)
AH8165	6.0	18.9 ± 4.9	1.7 ± 0.3	0.28 ± 0.09
d-Tubocurarine	0.6	18.2 ± 3.7	2.1 ± 0.5	0.04 ± 0.01†
AH8165	9.0	86.2 ± 3.9	4.1 ± 0.4	0.52 ± 0.06
d-Tubocurarine	0.9	86.6 ± 4.4	5.3 ± 0.5†	0.42 ± 0.05†

* Values = means ± SE, n = 10.
† Significant difference, $P \leq 0.05$, t-test for paired data.

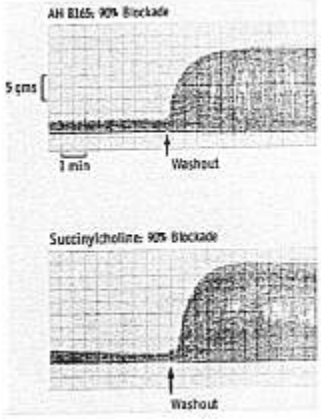

FIG. 6. Recoveries from blockades induced by AH8165 and succinylcholine in the rat phrenic nerve–diaphragm preparation. *Upper trace:* 90 per cent blockade by AH8165. *Lower trace:* 90 per cent blockade by succinylcholine. Arrows represent washout with normal Krebs solution.

produced by AH8165 in an attempt to increase reversal, but no increase was found (fig. 8). At 50 μg/ml neostigmine, a progressive decrease in the strength of contraction followed the initial incomplete reversal of AH8165. This probably was the beginning of a depolarizing blockade caused by the anticholinesterase agent itself (fig. 8).

Denervated Rat Diaphragm Preparation

The effect of AH8165 directly on muscle tissue was evaluated. Low concentrations of AH8165 that occasionally increased the strength of contraction in innervated muscle had no effect in denervated muscle. Similarly, concentrations of AH8165 that previously had diminished the strength of contraction in the rat phrenic nerve–diaphragm preparation also had no effect on the strength of contraction of denervated muscle. AH8165 concentrations 10, 50, and 100 times that which produced 100 per cent blockade in the innervated diaphragm preparation had no effect on the strength of contraction of denervated muscle. It was concluded that AH8165 had no direct effect on muscle tissue.

Chick Biventer Cervicis Nerve–Muscle Preparation

Doses of AH8165, 0.5 to 4 μg, added to the superfusate produced contracture of the tonic

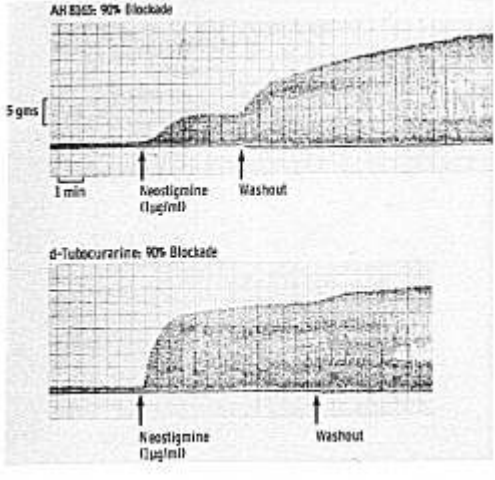

FIG. 7. Neostigmine reversal of blockades induced by AH8165 and *d*-tubocurarine in the rat phrenic nerve–diaphragm preparation. *Upper trace:* 90 per cent blockade produced by *d*-tubocurarine. The first arrow represents administration of neostigmine, 1 μg/ml; the second arrow represents washout with normal Krebs solution.

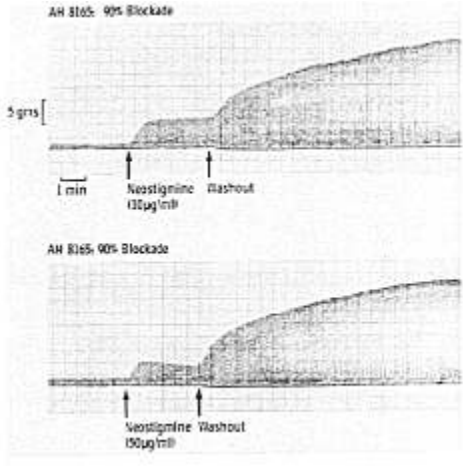

FIG. 8. The effects of neostigmine, 10 μg/ml, and neostigmine, 50 μg/ml, on the blockade induced by AH8165 in the rat phrenic nerve–diaphragm preparation. *Upper and lower traces:* 90 per cent blockade by AH8165. In the upper trace the first arrow represents administration of 10 μg/ml neostigmine and the second arrow represents washout with normal Krebs solution. In the lower trace the first arrow represents administration of 50 μg/ml neostigmine and the second arrow represents washout with normal Krebs solution.

component of the muscle. The increase in the magnitude of contracture was dose-related. Doses of 0.5 to 2 μg also potentiated the strength of contraction of the fast-twitch component. A dose of 4 μg produced 5 per cent blockade of the force of contraction. A dose of 8 μg appeared to mark a turning point in the muscle response to AH8165. This dose

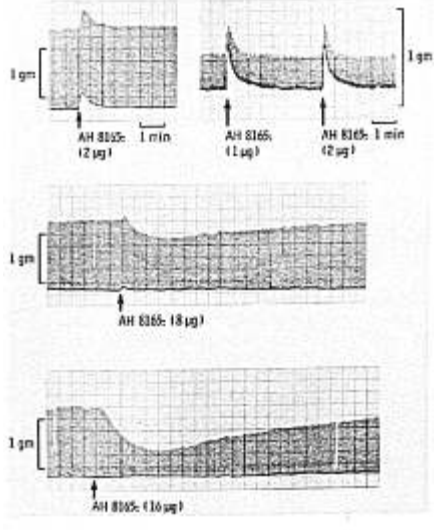

FIG. 9. Typical records of the effect of AH8165 on the chick biventer cervicis nerve–muscle preparation. *Upper left trace:* effect of 1 μg AH8165. The arrow represents the point of drug administration. *Middle trace:* effect of 8 μg AH8165 on the same preparation. *Lower trace:* effect of 16 μg AH8165 on the same preparation. *Upper right trace:* effects of 1 μg and 2 μg AH8165 on another preparation in which the tonic component appeared to predominate.

produced 40 per cent depression of the strength of contraction along with the first observed decrease in the magnitude of contracture. A dose of 16 μg decreased tonic muscle contracture to negligible values and produced 60 per cent blockade of the fast-twitch component. Contracture was absent with 32 μg AH8165 and blockade of contraction was nearly 90 per cent. Typical records of the drug effects on both tonic and fast-twitch components of muscle can be seen in figure 9.

Generally, blockade of contraction occurred after contracture had subsided. In one preparation, onset of blockade was observed to occur concomitantly with contracture (fig. 9). The latter is typical of the effects of depolarizing drugs. However, most muscles manifested blockade of twitch after contracture of the tonic muscle fibers had subsided.

Discussion

It is interesting to compare the results of this study with results of other studies in regard to onset of blockade, duration of and recovery from blockade, potency, and mechanism of action of AH8165.

AH8165 was shown to have an onset of action which was more rapid than that of *d*-tubocurarine, but less rapid than that of succinylcholine. This is in contrast to studies in man which show the onset of action of AH8165 to be more rapid than[10,11] or about the same as[12,13] that of succinylcholine. We found the duration of action of AH8165 to be longer than that of succinylcholine and comparable to that of *d*-tubocurarine. No reversal of blockade was observed within two hours unless neostigmine was added, in which case rapid but only partial reversal occurred. Others who have studied AH8165 in the rat phrenic nerve–diaphragm preparation gave no mention of duration of action.

With regard to recovery, the strength of contraction of the indirectly stimulated diaphragm preparation was found to return rapidly to pre-drug levels following washout of AH8165. This corroborates other findings in the same preparation.[3] The rate of recovery and time to 100 per cent recovery were greater after all levels of blockade produced by AH8165 than after comparable blockades produced by *d*-tubocurarine. By contrast, the rate of recovery from blockade produced by AH8165 was less than or equal to that from blockade produced by succinylcholine. In studies *in vivo* it was found that recovery from blockade produced by AH8165 was faster than recoveries from blockades produced by *d*-tubocurarine, gallamine, and pancuronium.[2] This could indicate that the binding of AH8165 to the receptor is less than that of *d*-tubocurarine but greater than or equal to that of succinylcholine. This is in contrast to *in-vivo* findings in anesthetized cats that suggested that AH8165 stayed at the receptor site for a shorter period than succinylcholine.[3]

Previous studies have indicated that AH8165 is a nondepolarizing neuromuscular relaxant,[1–3] but there has been no comparison of the dose–response curve of AH8165 with those of other nondepolarizing muscle relaxants. We found AH8165 to have a dose–response curve parallel to that of *d*-tubocurarine, and from these curves we calculated AH8165 to be 0.1 times as potent as *d*-tubocurarine. This confirms an earlier study in which a tenfold difference in potency was noted.[14] The ED_{50} determined from our regression line was 7.4 μg/ml. This differs from an ED_{50} of 0.68 μg/ml reported by Brittain and Tyers.[3] They applied interrupted tetanic stimulation as bursts of 45 Hz for 0.2 seconds every 15 seconds, while we applied single pulses every 5 seconds. Since the magnitude of neuromuscular blockade is a function of the frequency of nerve stimulation,§ it is not surprising that lower doses of AH8165 were needed to produce neuromuscular blockade in the experiments of Brittain and Tyers.

Previous studies have indicated AH8165 to be readily reversed by neostigmine.[1–3,10,11] Blodd *et al.*[12] compared the reversibility by neostigmine of AH8165 with that of *d*-tubocurarine and found the recovery of strength of contraction after the former to be more rapid. From this they inferred that AH8165 would also be more readily reversed by neostigmine than would pancuronium and gallamine. In the present investigation neostigmine produced only 38 per cent reversal of

§ Standaert FG: Personal communication.

blockade by AH8165, while *d*-tubocurarine blockade was completely reversed. One wonders what roles certain factors *in vivo*, such as circulation and redistribution, play in the apparent ready reversibility reported by the previous investigators.

There has been little effort to understand the mechanism of action of AH8165, but most investigators have accepted it as nondepolarizing. That AH8165 and *d*-tubocurarine have parallel dose–response curves is compatible with this. On the other hand, the fact that neuromuscular blockade cannot be reversed completely by neostigmine suggests that AH8165 does not have the same mode of action as *d*-tubocurarine. Our observation of facilitation of strength of contraction by low concentrations of the drug suggests that AH8165 has some depolarizing activity. This was confirmed in the chick biventer cervicis muscle preparation. In low doses the agent produced contracture of the tonic muscle component and potentiated the force of contraction of the fast-twitch component. These are both indicative of depolarizing activity. With higher doses contracture was diminished and the force of contraction of the fast-twitch fibers was reduced. This is indicative of nondepolarizing activity. From these findings it is concluded that AH8165 in low concentrations has a depolarizing effect, which is masked by the nondepolarizing blockade in high concentrations. These findings confirm the work of Post *et al.*,[4] who found that low doses of AH8165 increased miniature endplate potential (MEPP) frequency in the *in-vitro* frog sartorius muscle and potentiated contraction of the cal soleus muscle *in vivo*, and that high doses reduced MEPP amplitude. This dual action demonstrates a basic difference between AH8165 and both *d*-tubocurarine and succinylcholine.

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