The Effects of Germine Diacetate on the Rat Phrenic Nerve–Diaphragm Preparation

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Concentrations of germine diacetate (GDA) ranging from 2.5 to 100.0 μg/ml increased the twitch tension of the indirectly-stimulated isolated phrenic nerve–diaphragm preparation of the rat to 240 to 438 per cent of control values. The intensity of action of GDA increased, and the time required for the development of its maximal effect decreased, with increasing concentrations. Repeated administrations of GDA had progressively less effect. Tetanus was poorly maintained in the presence of GDA. GDA also increased twitch tension of the directly-stimulated preparation. Muscle performance was improved by GDA during partial (40 to 60 per cent), but not during complete, *d*-Tc- or CIO-induced neuromuscular block. Improvement was due to the increase of the twitch tension of the unblocked fibers. These findings confirm earlier reports that the site of action of GDA is primarily postjunctional. (Key words: Germine diacetate; Phrenic nerve-diaphragm preparation.)

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Recently, Flacke *et al.*[1] reported encouraging results in the treatment of myasthenia gravis with a semisynthetic veratrum alkaloid, germine diacetate (GDA). Their favorable results were confirmed in a pilot study by another group of investigators (Foldes, F. F., Osserman, K., Klonymus, D., Maisel, W., Genkins, M. T., and Kronfeld, P.: Unpublished data). Before initiation of a more extensive study along these lines, it seemed worthwhile to obtain more information on the dose–effect relationship and time course of action of GDA. Since GDA has also been recommended to "antagonize" residual neuromuscular block at the end of anesthesia,[2] an investigation of the interaction of GDA and d-tubocurarine dichloride (*d*-Tc) or decamethonium dibromide (CIO) was undertaken also.

Methods

Experiments were carried out on the indirectly- and/or directly-stimulated isolated phrenic nerve–diaphragm preparation[3] of Sprague–Dawley rats weighing 250 to 300 g. The unanesthetized animals were sacrificed by rapid decapitation. Each hemidiaphragm was mounted in one chamber of a twin organ bath containing 80 ml of McEwen's solution[4] (NaCl 7.6 g, KCl 0.42 g, CaCl 0.24 g, NaH_2PO_4 0.143 g, dextrose 2.0 g, sucrose 4.5 g, distilled H_2O 1,000 ml) kept at 37 C and continuously aerated with 95 per cent oxygen–5 per cent carbon dioxide.

Indirect, supramaximal rectangular stimuli of 0.1-msec duration were applied to the phrenic nerves from a Grass Model S4 or SD5 stimulator through electrodes of the type described by Burn and Rand.[5] For direct stimu-

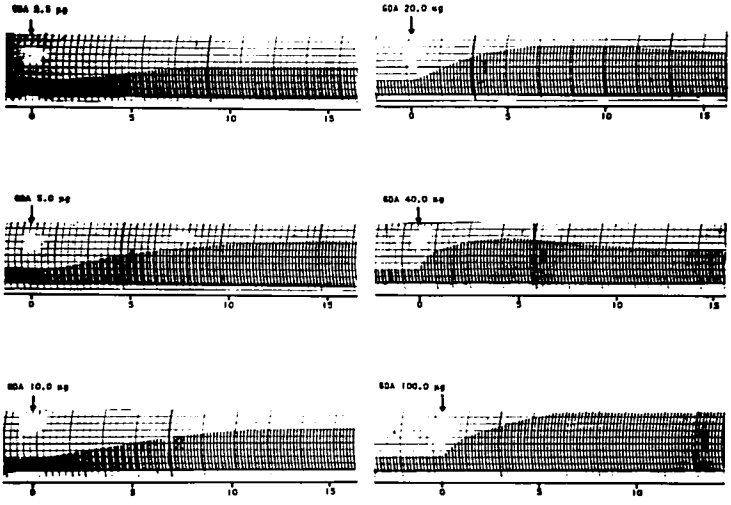

FIG. 1. Effects of increasing concentrations of GDA on twitch tension. Concentrations of GDA ranging from 2.5 to 100.0 µg/ml caused a significant increase of the twitch tension. The magnitudes of the increases were greater, and the times required for the development of the maximal effect shorter, after the three higher concentrations than after the three lower concentrations of GDA. Arabic numerals below the base lines, in this and subsequent figures, represent min from zero time.

lation, one platinum wire electrode was placed along the muscle fibers at the point of attachment to the rib cage and another through the central tendinous portion of the muscle. Supramaximal square-wave stimuli of 50 to 80 mv and 1- to 2-msec duration were used. With this type of stimulation the current invariably reaches the motor nerve endings of the muscle, and the resulting twitch tension is the summation of the tension output resulting from both direct and indirect stimulation. To eliminate the "indirect" component of direct stimulation, neuromuscular transmission was blocked completely with *d*-Tc before recording the control twitch tension of the directly-stimulated preparation and the effect of GDA on it.

Unless otherwise stated, the frequency of stimulation was 0.1/sec (one every 10 sec). In some experiments, tetanic (50/sec) stimuli were applied for 15 sec, and occasionally for 30 sec. Each muscle was connected to a Grass Model FT 10C force-displacement transducer and the tension developed during stimulation was recorded on a Grass Model 50 polygraph. The paper speed, unless otherwise stated, was 0.25 mm/sec.

In a few experiments, muscle action potentials also were recorded with a silver–silver chloride recording electrode inserted into the muscle in the vicinity of the central tendon. The level of the solution was lowered below the point of insertion of the recording electrode and liquid paraffin was layered on the bathing fluid to isolate the recording electrode from the plate reference electrode (Grass E5S), which was placed in the bath about 4 cm from the recording electrode. Muscle action potentials were displayed on a Tektronix Model 564 storage oscilloscope, with a type 2 A61 differential amplifier and a type

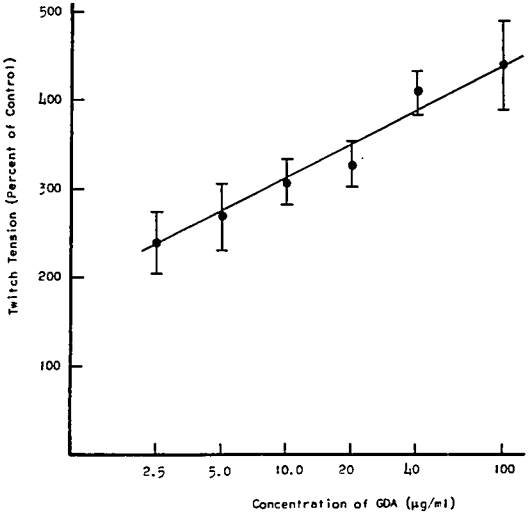

FIG. 2. Semilogarithmic dose–response curve of GDA.

2B67 time base, and photographed with a Tektronix Model C12 oscilloscope camera.

Results

The addition of 1.25 μg/ml of GDA had no apparent effect on the twitch tension of the indirectly-stimulated phrenic nerve–diaphragm preparation. Further addition of 1.25 μg/ml GDA caused a slowly-developing increase of the twitch tension. Concentrations of GDA ranging from 2.5 to 100.0 μg/ml caused progressively larger increases in twitch tension, with means ranging from 240.6 ± 33.8 (SE) to 438.0 ± 53.6 per cent of control values for the 2.5 and 100.0 μg/ml concentrations, respectively (figs. 1 and 2). The maximal effect developed more slowly (in 13.7 to 11.5 min) after addition of the three lower, than after addition of the three higher, concentrations (5.7 to 5.1 min) of GDA (table 1). The maximal effect was maintained for relatively short periods, and 30 min after the addition

TABLE 1. Effects of Various Concentrations of Germine Diacetate on the Twitch Tension of Indirectly-stimulated Muscle

GDA Concentration (μg/ml)	Number of Experiments	Onset of Action (sec)	Maximum Twitch Tension*	Time (min)† to Maximum	Twitch Tension at 30 min
2.5	5	101.0 ± 12.3	240.6 ± 13.1	13.1 ± 1.5	225.6 ± 26.5
5.0	5	50.0 ± 5.7	269.4 ± 38.3	13.7 ± 2.0	218.7 ± 25.7
10.0	8	40.0 ± 5.5	307.8 ± 25.0	11.5 ± 1.3	260.2 ± 27.7
20.0	9	14.2 ± 2.0	327.7 ± 26.6	5.7 ± 0.9	231.4 ± 13.2
40.0	6	17.8 ± 3.1	415.0 ± 23.1	5.2 ± 0.5	245.5 ± 8.5
100.0	5	16.7 ± 2.5	438.0 ± 53.6	5.1 ± 0.4	427.6 ± 53.3

* Percent of control.
† From the addition of GDA to the bathing fluid.

of GDA the twitch tension was usually less than the maximum. The decrease was relatively small and not significant with the 2.5, 5.0, 10.0 and 100.0 μg/ml concentrations, and greater with the 20.0 and 40.0 μg/ml concentrations. In most experiments where observations were continued there was a further decline at 60 min. It was difficult, especially after the addition of the higher concentrations, to remove the GDA from the preparations. Even after six washings with fresh McEwen's solution (twice in succession, three times, 5 to 10 min apart) twitch tensions remained above control values.

With the 2.5 and 5.0 μg/ml concentrations after six washings the second applications of GDA had consistently greater effects than the first applications. With the 10.0 to 100.0 μg/ml concentrations, however, twitch tensions increased less after the second than after the first administration of GDA. After the fourth administration twitch tensions were less than control values with all GDA concentrations tested.

Indirect stimulation at more rapid rates, 0.4/sec (one every 2.5 sec) or 1/sec, consistently accelerated both the development and the decay of the maximal increase in twitch tension (six experiments with 10.0 or 20.0 μg/ml GDA). The record of a typical experiment is presented in figure 3. Allowing the preparation to rest for 2 min had no effect on twitch tension.

In contrast to its significant augmentation of twitch tension, GDA caused little or no increase in tetanic tension (table 2). Because of this, whereas the ratios of the control tetanic and twitch tensions were between 4.08 and 4.4, after various concentrations of GDA these ratios ranged from 1.21 to 1.85. Simi-

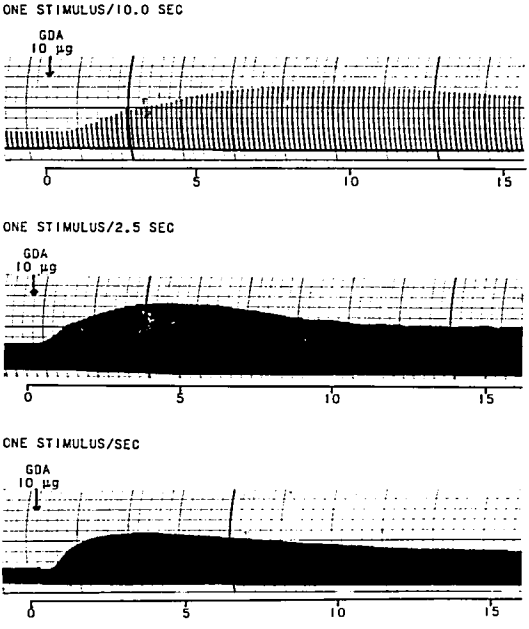

FIG. 3. Effect of rate of stimulation on the twitch tension. Increasing the rate of stimulation from 1/10 sec to 1/sec accelerated both the development and the decay of maximal effect of GDA.

TABLE 2. Comparison of the Effects of Germine Diacetate on Twitch Tension and Tetanic Tension

GDA (μg ml)	Number of Experiments	Twitch Tension (Per cent of control)		Tetanic Tension (Per cent of control)		Ratios			
		Control	After GDA	Control	After GDA	Control Tetanic Tension: Control Twitch Tension	Tetanic Tension after GDA: Twitch Tension after GDA	Twitch Tension after GDA: Control Twitch Tension	Tetanic Tension after GDA: Control Tetanic Tension
2.5	6	100.0 ± 0.0	296.0 ± 37.3	444.0 ± 53.2	519.0 ± 85.3	4.1 ± 0.5	1.9 ± 0.4	2.9 ± 0.4	1.2 ± 0.0
10.0	5	100.0 ± 0.0	390.8 ± 17.9	420.4 ± 9.8	478.0 ± 31.1	4.2 ± 0.0	1.2 ± 0.0	3.9 ± 0.2	1.1 ± 0.0
40.0	5	100.0 ± 0.0	338.4 ± 30.8	408.2 ± 29.8	422.6 ± 40.2	4.1 ± 0.3	1.2 ± 0.1	3.1 ± 0.3	1.0 ± 0.0
100.0	4	100.0 ± 0.0	374.8 ± 8.8	439.0 ± 13.6	520.5 ± 18.3	4.4 ± 0.1	1.1 ± 0.0	3.7 ± 0.0	1.2 ± 0.0

larly, the ratios of twitch tensions after and before GDA were between 3.2 and 3.4, and those of the tetanic tensions were between 1.0 and 1.2.

With stimulation rates of 50/sec, tetanic tension was relatively well maintained in the controls and with the 2.5 μg/ml concentration of GDA, but there was a progressively greater decay of the tetanic tension with increasing concentrations. With the 100 μg/ml concentration of GDA, the tetanic tension became almost zero towards the end of a 30-sec tetanus (fig. 4). The twitch tension was smaller after than before tetanus, but usually returned to the pretetanic value in several minutes.

GDA also increased the twitch tension of the directly-stimulated muscle (fig. 5). The time courses of the effects of GDA on the twitch tensions evoked by direct and by indirect stimulation were about the same.

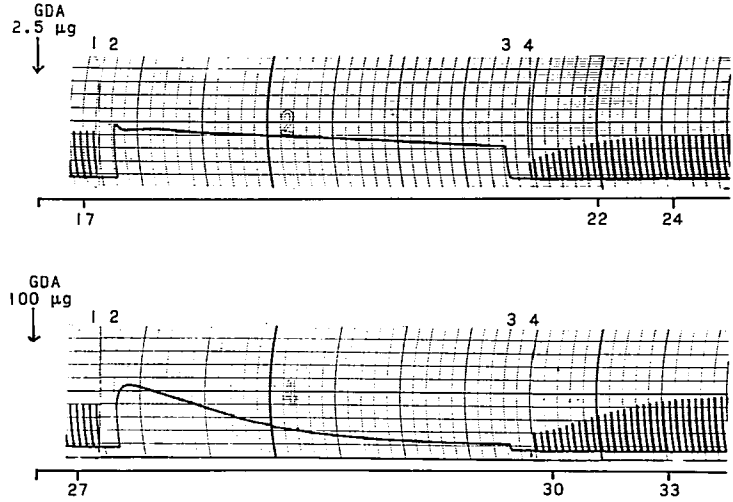

FIG. 4. Effect of GDA on the tension output during prolonged tetanus. In the presence of 100 μg/ml GDA the tension output became zero after 30 sec of tetanic stimulation. Paper speed was 5 mm/sec during tetanic stimulation.

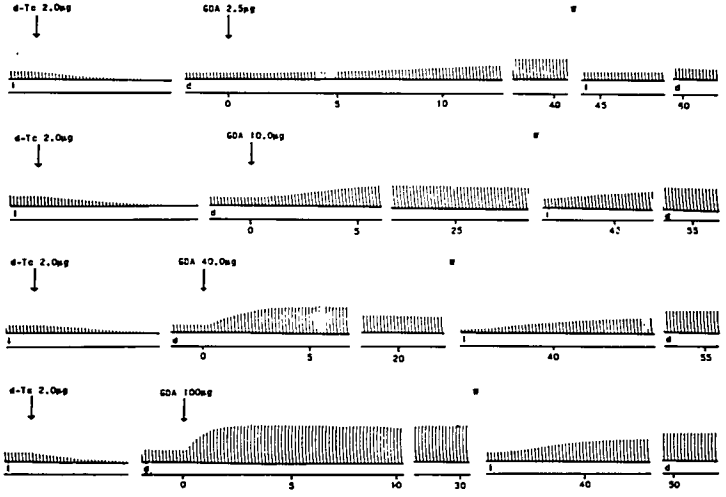

FIG. 5. Effect of GDA on twitch tension during direct stimulation. The "indirect" component of direct stimulation (for explanation see text) was eliminated by *d*-Tc-induced neuromuscular block. *i* = indirect stimulation; *d* = direct stimulation. At W the preparation was washed six times with McEwen's buffer.

The interaction of GDA and neuromuscular blocking agents was observed in 24 experiments. After incomplete neuromuscular block, produced by the addition of *d*-Tc or CIO to the bathing fluid, 10 μg/ml GDA consistently increased twitch tension (fig. 6). The administration of neostigmine methylsulfate (Prostigmin) at this time caused a further increase of the twitch tension in the presence of *d*-Tc. When partial block was induced with CIO, neostigmine added to the bath after GDA caused no further increase of the twitch tension. When complete neuromuscular block was produced by either *d*-Tc or CIO, the addition of GDA to the bath had no apparent effect. Neostigmine, however, antagonized the *d*-Tc-induced, but not the CIO-induced, complete neuromuscular block. Repeated washings with McEwen's solution, after *d*-Tc- or CIO-induced partial or complete neuro-

TABLE 3. Effects of Various Concentrations of Germine Diacetate on the Twitch Tension of Directly-stimulated Muscle

GDA Concentration (μg/ml)	Number of Experiments	Onset of Action (sec)	Maximum Twitch Tension (Per cent of Control)	Time (min) To Maximum	Twitch Tension at 30 min†
2.5	6	175.0 ± 39.0	207.7 ± 24.2	25.3 ± 0.6	196.0 ± 24.6
10.0	6	30.8 ± 3.7	356.8 ± 25.7	13.0 ± 1.0	289.5 ± 10.3
40.0	6	24.1 ± 3.5	373.5 ± 1.11	6.3 ± 0.3	226.3 ± 6.9
100.0	6	16.7 ± 2.1	326.7 ± 12.1	4.0 ± 0.0	238.2 ± 15.9

† From the addition of GDA to the bathing fluid.

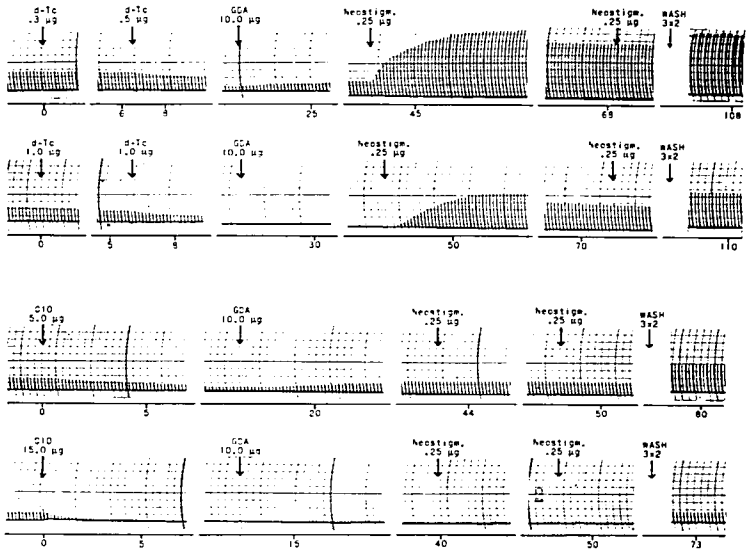

FIG. 6. The interaction of *d*-Tc and CIO with GDA during indirect stimulation. After incomplete *d*-Tc- or CIO-induced neuromuscular block (first and third tracings) the addition of GDA to the bath increased twitch tension. After complete neuromuscular block GDA alone was ineffective. In the presence of GDA, however, the addition of neostigmine consistently increased twitch tension above control values during partial or complete *d*-Tc block. After CIO and GDA, neostigmine had no visible effect. Washing the preparation after the addition of *d*-Tc or CIO and GDA and neostigmine invariably resulted in twitch tensions above control values.

muscular block followed by the addition of 10 µg/ml GDA, invariably resulted in twitch tensions above control values (fig. 6).

Recording of the muscle action potential during indirect stimulation before and after the addition of various concentrations of GDA revealed that after administration of GDA a single stimulus causes repetitive firing (fig. 7).

Choline acetylase activity and cholinesterase activity were not affected by 10^{-6} to 10^{-3} M concentrations of GDA (Foldes, F. F., and Smith, J. C.: Unpublished data).

Discussion

The findings presented are in agreement with earlier reports[6,7] that GDA significantly increases the twitch tension of indirectly- or directly-stimulated muscle. The fact that in the presence of *d*-Tc- or CIO-induced complete neuromuscular block the intensity and time course of action of GDA on directly-stimulated muscle (fig. 5) is very similar to that on unblocked, indirectly-stimulated muscle (fig. 1) confirms that the effect of GDA is primarily postsynaptic.[6,7]

In the present study, concentrations of GDA ranging from 2.5 to 100.0 µg/ml caused progressive increases in the mean twitch tension. After reaching the maximum value, the twitch tension declined gradually. Since in the presence of GDA single stimuli cause short trains of tetani (see fig. 7), it is possible that the mechanism of the decay is similar to that seen in isolated preparations subjected to tetanic

stimulation. The unexpectedly small decay of the twitch tension with the 100.0 μg/ml concentration of GDA is puzzling. It is possible that, with this concentration, the increased transmitter release observed after relatively large concentrations of GDA at the nerve terminal[7] for a limited time partially compensates for the postjunctional changes caused by prolonged exposure to GDA. Similarly, at this time we have no adequate explanation for the abrupt change in the time necessary for the development of maximal effect between the 10- and 20-μg/ml concentration of GDA.

The finding that after a single administration of GDA and repeated washings of the preparation with McEwen's solution the twitch tension remained above the control level indicates either that it is difficult to wash out GDA from the muscle or that the GDA-induced changes tend to persist. That the twitch tension becomes progressively smaller after repeated administration of GDA separated by multiple washings suggests that the first assumption is correct. It is conceivable that under these circumstances greater than optimal concentrations of GDA eventually accumulate, resulting in the decrease of the twitch tension.

As reported earlier,[6,8] it was observed that tetanic tension was much less augmented by GDA than twitch tension (see table 2). Tetanus was maintained relatively well with the 2.5 μg/ml concentration of GDA. With higher concentrations of GDA, tetanus was not maintained (fig. 4). The failure to maintain tetanus with higher concentrations of GDA may be due to exhaustion of the readily-available acetylcholine depots[9] caused by the increased acetylcholine release elicited by GDA.[7] Another possibility is that GDA, like veratrine, causes loss of K^+ from the muscle fiber.[10,11] A further possible cause of the failure to maintain tetanus in the presence of GDA may be the occurrence of presynaptic failure of propagation in the motor nerve, as described by Krnjević and Miledi.[12] On the basis of experi-

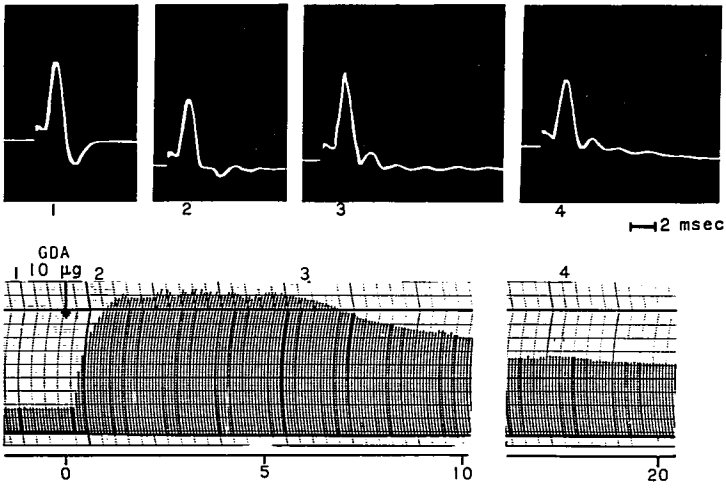

FIG. 7. Action potentials (above) and twitch tensions before and after GDA. Action potentials marked with arabic numerals were recorded at times indicated by corresponding numerals of the twitch tension tracings. Note the repetitive discharge after GDA.

mental data presently available, it is not possible to determine which of these assumptions, if any, is valid.

The intravenous administration of relatively large (1.25 to 3.15 mg/kg) doses of GDA has been recommended [2] to antagonize the effects of residual neuromuscular block at the end of anesthesia. In the present study, GDA antagonized partial *d*-Tc- or ClO-induced neuromuscular block. In agreement with the conclusions of other investigators,[13] our data indicate that the increased twitch tension brought about by GDA in the presence of partial neuromuscular block was not caused by the antagonism of the residual neuromuscular block, but by the stimulation of the tension output of the unblocked muscle fibers. As pointed out by Hofmann,[7] the postsynaptic effects of GDA require muscle fibers capable of generating action potentials. Since even the slow (1 to 2 mg/min) intravenous infusion of GDA causes nausea and other unpleasant sensations in conscious subjects (personal observations), the rapid (60 to 180 mg/min) intravenous administration of GDA recommended for the reversal of residual neuromuscular block is feasible in anesthetized patients only. Increased salivation, contraction of abdominal muscles, hiccoughing and transient elevation of blood pressure, however, occur even in anesthetized subjects.[2] In view of all this, fractional doses of atropine and anti-ChE remain preferable to GDA for the reversal of residual non-depolarization block. GDA, however, may prove to be the agent of choice for the treatment of antibiotic-induced neuromuscular block that can be only partially (*e.g.*, neomycin, streptomycin) or not at all (*e.g.*, polymyxin A) antagonized by anti-ChE and/or calcium.[14]

Although its etiology is still unclarified, it is generally accepted that the muscle weakness and fatigability of myasthenia gravis result from impaired neuromuscular transmission. Since its primary effect is postjunctional, GDA will improve muscular performance in myasthenia gravis only if there are a sufficient number of functioning endplates.[7] The fact that GDA is also capable of increasing the amount of acetylcholine released at motor nerve terminals has no practical significance. The high concentration of GDA required for a significant increase of acetylcholine release inhibits conduction in the terminal nerve fibers,[7] adversely affects its postjunctional effect and produces unpleasant side-effects. Consequently, it is probable that in the treatment of myasthenia gravis GDA will be an adjuvant. It is reasonable to assume that combining moderate doses of anti-ChE and GDA will produce better therapeutic effects than anti-ChE alone. We recommend that future clinical trials with GDA and other related veratrum alkaloids be conducted along these lines.

AUTHOR'S NOTE: Since the submission of this manuscript, it has been reported [15] that GDA is unstable in aqueous solution and hydrolyzes rapidly to germine-3-monoacetate (GMA-3), two unidentified acetate esters, and germine. It is probable, therefore, that some of the effects attributed to GDA were caused by GMA-3 and its breakdown products.

The germine diacetate used in this study was supplied by Merck Sharp and Dohme, Inc. The authors are indebted to Dr. Werner Flacke of the Department of Pharmacology of the Harvard Medical School for his willingness to discuss the experimental findings and their interpretation.

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Drugs

BARBITURATE SLEEP Two subjects received 200 mg of sodium amylobarbitone for 26 nights. All-night sleep records showed that the drug shortened the time delay to the onset of sleep, increased the total sleep period, lengthened orthodox sleep time and depressed rapid-eye-movement sleep. After five nights, rapid-eye-movement sleep returned to baseline values, *i.e.*, tolerance to the drug developed. On stopping the drug, withdrawal phenomena were seen, even to this small dose. (*Evans, J. I., and others: Sleep and Barbiturates: Some Experiments and Observations, Brit. Med J. 4: 291 (Nov.) 1968.*)

MORPHINE TOLERANCE Development of tolerance to and physical dependence on morphine in mice can be prevented by concomitant administration of cycloheximide. The fact that the rate of synthesis of brain 5-hydroxytryptamine (5-HT) increases with tolerance to morphine suggests that the protein involved may be associated with 5-HT synthesis. Inhibition of this synthesis with p-chlorophenylalanine markedly decreases development of tolerance to and physical dependence on morphine. (*Way, E. L., Loh, H. H., and Shen, F.: Morphine Tolerance, Physical Dependence and Synthesis of Brain 5-hydroxytryptamine, Science 162: 1290 (Dec.) 1968.*)

SEDATIVES AND COAGULATION Plasma warfarin levels and prothrombin times were determined in volunteers after sedative or placebo therapy for 21 days. Phenobarbital, 120 mg, and glutethimide, 1 g, reduced the half-life of warfarin nearly 50 per cent. Chloral betaine, 1.74 g, reduced the half-life of warfarin about 20 per cent. The proposed mechanism for this modification of warfarin inactivation is induction of microsomal enzymes of the liver. (*MacDonald, M. G., and others: The Effects of Phenobarbital, Chloral Betaine, and Glutethimide Administration on Warfarin Plasma Levels and Hypoprothrombinemic Responses in Man, Clin. Pharmacol. Ther. 10: 80 (Jan.) 1969.*)