tidine reduced to half, the two peaks were of about equal width and well resolved.

Canavanine from jack bean and from alfalfa yielded guanidine and homoserine (fig. 3). All the canavanine was destroyed on reduction. On the other hand histidine was apparently unchanged.

From the table it can be seen that the canavanine obtained from alfalfa is not histidine and is similar to that obtained from the jack bean.

The amount of canavanine obtained from different samples of Weevl-Chek was variable and ranged from 14 to 20 g/kg. Other alfalfa varieties yielded as follows, Saranac, 10–13, Classic, 9–11, Arc, 9.5–12, Team, 8–11, Buffalo, 6–8.5 g/kg of canavanine.

The question arises as to how it was possible for the claim to be made that there was no canavanine in alfalfa[1]. Examining the procedure used to isolate canavanine from jack bean[11], substantial amounts of charcoal are used in the procedure. Activated charcoal will remove about 1 mg of canavanine per g of charcoal from solution[6]. When processing large quantities of jack beans or alfalfa seeds this is acceptable. For leaves and stems the canavanine would be removed. However, the author does not specify whether he extracted seeds, stems or leaves. If stems or leaves are extracted, the pentacyanoferrate reaction is not sensitive enough to detect the canavanine in alfalfa. A fluorometric procedure needs to be used[6]. In the fluorometric procedure, fluorescence intensity for canavanine is 3500 times that observed with histidine. Using 10 g/kg of alfalfa seeds as a base, to produce the fluorescence observed with alfalfa seed extracts, they would have to contain 35,000 g of histidine per kg. Using the pentacyanoferrate reaction (see fig. 1), they would have to contain at least 100 g of histidine per kg.

The histidine in alfalfa is mainly in the protein and occurs only in the expected small amounts[12,13]. In the reference referred to[11], protein is removed by neutralizing H_2SO_4 with NH_4OH. This brings the concentration of $(NH_4)_2SO_4$ to about 0.1 M in 50% ethanol. This will not precipitate all the protein. This may account for the presence of substantial amounts of histidine in the extracts.

Other statements in the paper are inexact[1]. The absorption spectrum of canavanine (fig. 1) seems to be that of a solution where the color has faded. It appears that the sensitivity with canavanine over histidine is four times. It is actually eight times (see fig. 1, this paper). The statement is also made that canavanine did not produce color with the Pauly reagent. This is true only with very low concentrations of canavanine. If 1 mg canavanine, serine, homoserine or aspartic acid is placed in a test tube and 2 ml of a 0.1% solution of diazotized sulfanilic acid, in molar NaOH is added, a deep red color will appear after about 1–2 h. This can be accelerated to minutes by warming in the flame. This color has absorption maxima at 520 and 405 nm. The color with histidine has an absorption maximum at 360 nm. In the visible (about 400 nm) this color is about 40 times as sensitive as with canavanine, and appears golden.

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Effects of methylene blue on electrical behavior of myenteric neurons

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Summary. Intracellular recording methods were used to investigate the action of methylene blue on electrical behavior of myenteric neurons in guinea pig small intestine. The neurophysiological studies were done in parallel with studies on contractile activity of the intestinal musculature. Methylene blue depolarized the membranes, increased the input resistance, augmented excitability and reduced postspike hyperpolarizing potentials in AH/Type 2 myenteric neurons. These effects, with the exception of suppression of postspike hyperpolarization, were reversed by exposure to elevated calcium. The mechanism of action of methylene blue appeared to be suppression of calcium-dependent potassium conductance in the neuronal membranes. The neuronal action of methylene blue was manifest as a release of excitatory neurontransmitter substances which evoked contraction of the small intestinal longitudinal muscle.

Key words. Guinea pig small intestine; myenteric neurons; electrical behavior; methylene blue; neuronal membranes; K^+-conductance, Ca^{++}-dependent.

Methylene blue (tetramethylthionine chloride) is an autoxidizable dye that has been used widely as a vital stain for nervous tissue in both vertebrate and invertebrate animals. Its pharmacological use as a therapeutic or diagnostic agent in humans is well known. Methylene blue was used as a stain to visualize the enteric neuronal plexuses in early extracellular studies of the electrophysiological behavior of enteric ganglion cells[1–3]. Reported results of these studies suggested that methylene blue did not alter ongoing action potential discharge of single neuronal units in the myenteric and submucosal plexuses of the intestine in a variety of species[1–3] and did not affect synaptic transmission in autonomic ganglia[4,5].

We now report the results of a study of effects of methylene blue on intracellularly-recorded electrical behavior of small intestinal myenteric ganglion cells. These results showed that methylene blue depolarized the membrane potential and aug-

mented the excitability of the myenteric neurons. This action of methylene blue was interpreted to be a reflection of suppression of calcium-dependent potassium conductance (Ca^{++}-gK^{+}) in the somal membranes of AH/Type 2 myenteric neurons. We also report that the augmentation of enteric neuronal excitability by methylene blue was manifest as a release of excitatory neurotransmitter substances that evoked contraction of the small intestinal longitudinal muscle.

Material and methods. Tissues were obtained from the jejunum and ileum of guinea pigs (300–700 g) that had been stunned by a blow to the head and exsanguinated. Flat preparations of longitudinal muscle with the myenteric plexus attached were prepared, mounted in a superfusion chamber and visualized with an interference contrast microscope as described in an earlier report[6]. Conventional intracellular recording methods were used. Glass microelectrodes were filled with 3 M KCl and had resistances of 40–60 MΩ. The preamlifier contained negative-capacity compensation and bridge circuitry for injecting electrical current through the recording electrode and recording the resulting electrotonic potentials. All records were stored on magnetic tape for later analysis. Records were reproduced on a chart recorder (Gould) and the spike amplitudes in the illustrations are attenuated slightly by the slow frequency response of the recorder. The preparations were superfused with Krebs solution at 37–39 °C and gassed with 95% O_2–5% CO_2. Composition of the solution in mM was NaCl 120.9; KCl 5.9; $MgCl_2$ 1.2; NaH_2PO_4 1.2; $NaHCO_3$ 14.4; $CaCl_2$ 2.5; glucose 11.5. HEPES buffer was substituted for the bicarbonate-phosphate buffer system in experiments in which the calcium concentration was elevated to 7.5 mM. Methylene blue (Sigma) was applied in the superfusion for periods of 0.5 to 1 min in a concentration range of 10–250 µM.

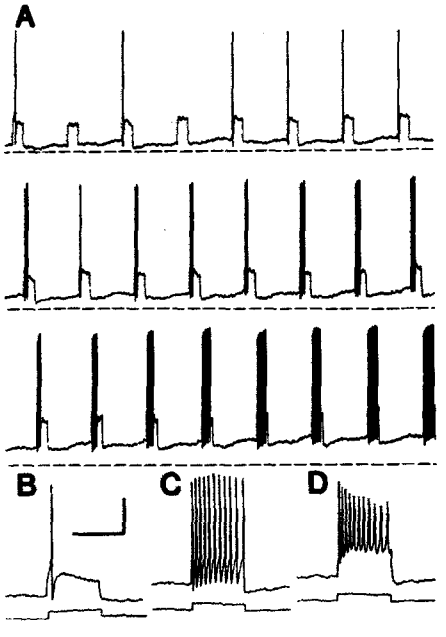

Figure 1. Effects of methylene blue on excitability of an AH/Type 2 myenteric neuron in guinea pig small intestine. A: Continuous records beginning 15 sec after exposure to methylene blue (250 µM). B: Response of cell to a single depolarizing current pulse recorded with an expanded time base before application of methylene blue. C: Multiple discharge evoked by current pulse during exposure to methylene blue. D: Multiple discharge evoked by current pulse 3.5 min after washing methylene blue from the bath. Exposure time to methylene blue was 30 sec. Vertical calibration, 20 mV; horizontal calibration, 1.5 sec for A and 300 msec in B–D. Constant-current depolarizing pulses were 0.2 pA.

Contractile activity in the longitudinal axis of the guinea pig ileum was recorded from intact segments 2–3 cm in length that were suspended between a force-displacement transducer and an anchor point in a static organ bath that contained Krebs solution. After 20–30 min of equilibration, methylene blue was added to give a final bath concentration of 10, 50, or 100 µM. Following washout of methylene blue, tetrodotoxin or atropine sulphate (Sigma) was added and methylene blue exposures were then repeated in the presence of tetrodotoxin or atropine.

Results and discussion. AH/Type 2 myenteric neurons were identified by specific electrophysiological criteria that were described in an earlier review[7]. The effects of methylene blue

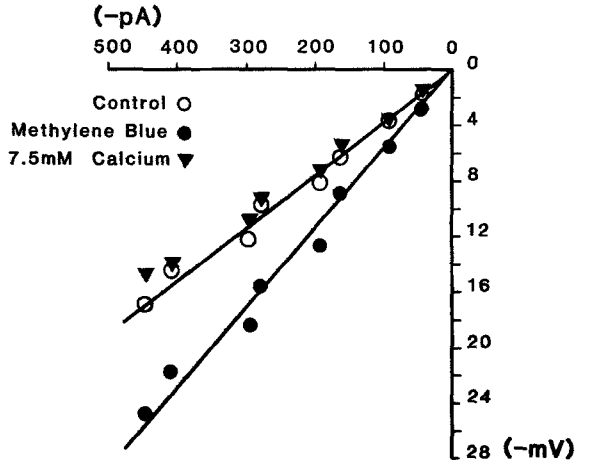

Figure 2. Current-voltage relationships for an AH/Type 2 myenteric neuron from guinea pig small intestine. Current-voltage plots are given for the cell prior to exposure to methylene blue (O), the peak response about 3.5 min after a 30-sec exposure to methylene blue (●), and about 2 min after Krebs solution containing 7.5 mM calcium entered the bath at the peak of the methylene blue response (A). Lines were fitted visually to the data points; no line is drawn to fit data points for elevated calcium. Increased slope after methylene blue exposure reflects increase in input resistance was reversed to control by treatment with elevated calcium.

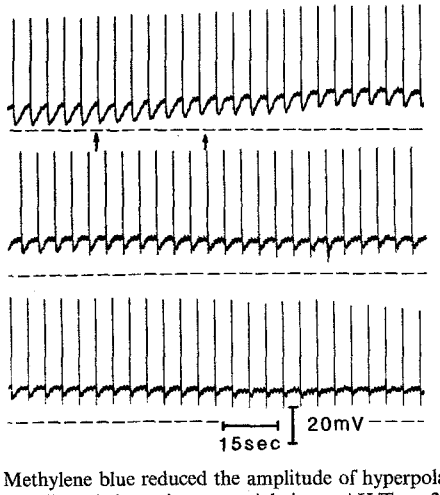

Figure 3. Methylene blue reduced the amplitude of hyperpolarizing potentials that followed the action potentials in an AH/Type 2 myenteric neuron of guinea pig small intestine. The records are continuous. Action potentials were evoked by intrasomatic injection of constant-current depolarizing pulses of sufficiently short duration to evoke only a single action potential. Arrows indicate time at which 100 µM methylene blue entered and left the tissue bath.

were studied on 29 AH/Type 2 neurons from 25 guinea pigs. In 24 of the cells, application of methylene blue was followed by depolarization of the membrane potential, increase in the input resistance of the cell, reduction of the hyperpolarizing after-potentials associated with the action potentials and augmentation of excitability (fig. 1–3). The membrane depolarization ranged from 8 to 22 mV. Increases in input resistance were reflected by an increase in the slope of ohmic current-voltage plots (fig. 2) after treatment with methylene blue. Augmentation of excitability was reflected always by a significant increase in the mean number of action potentials that was evoked by intrasomatic injection of depolarizing current pulses after exposure to the dye (fig. 1). The hyperpolarizing after-potentials were reduced both in duration and amplitude and these changes in the postspike hyperpolarizing potentials could not be attributed to the changes in resting membrane potential evoked by methylene blue (fig. 3).

The effects of methylene blue were dose-dependent and developed over a time course of 3 to 6 min after a 0.5–1 min exposure to the substance (fig. 1, 3). The effects were not quickly reversed when methylene blue was removed from the superfusion solution and were aparent for periods of 15–30 min after withdrawal of the dye.

The effects of methylene blue were reminiscent of the actions of the neurotransmitter substances for slow synaptic excitation of myenteric neurons[8] and of the actions of 5-hydroxytryptamine and substance P on these neurons[9,10]. Several lines of evidence suggest that the mechanism of action of the neurotransmitters for the slow synaptic excitatory event and for 5-hydroxytryptamine and substance P is suppression of Ca^{++}-gK^{+} in the somal membranes of the AH/Type 2 ganglion cells[11]. The close similarity between the actions of methylene blue, slow synaptic neurotransmitters, 5-hydroxytryptamine and substance P suggests that methylene blue may also act to suppress Ca^{++}-gK^{+}. We tested this suggestion by investigating the effects on the methylene blue response of elevating the concentration of calcium in the bathing solution from 2.5 mM to 7.5 mM.

Elevation of the concentration of calcium in the superfusion solution after the onset of action of methylene blue aborted most of the changes that were produced by the dye (fig. 2). When the elevated calcium solution entered the organ bath at the peak of the methylene blue response, the membrane potential repolarized to normal over a time span of 2-3 min and the input resistance declined to or slightly less than normal (fig. 2). Unlike the membrane potential and input resistance, the hyperpolarizing after-potentials never recovered to the level prior to methylene blue treatment in the elevated calcium solutions. These results are consistant with the suggestion that the mode of action of methylene blue is suppression of the inward fluxes of calicum which maintain the elevated levels of intracellular calcium that are necessary for continued activation of potassium channels in the resting state of the somal membranes. It appears that methylene blue interferes with the calcium channels that are opened during the action potential as well as the channels that are open during rest. It also seems that elevated levels of calcium are more effective in reversing the action of the dye on the resting channels than the spike-associated channels, because elevated calcium did not readily reverse the action of methylene blue on the postspike hyperpolarizing potentials. This may be the case also for sympathetic neurons where it has been suggested on the basis of studies of neuronal release of norepinephrine that methylene blue acts to suppress Ca^{++}-gK^{+}[12].

The observations reported here raise a question of the extent to which the results of earlier extracellular electrical studies were influenced by the use of methylene blue to visualize the myenteric plexus[1–3]. An adequate assessment of this is impossible; nevertheless, it is significant that in the intracellular studies methylene blue never evoked any spike discharge that resembled the burst-type or single-spike patterns that were characteristically found with extracellular recording of single units in the myenteric plexus[1–3]. It may be that the action of methylene blue is restricted to the somal membranes of AH/Type 2 myenteric neurons which have a Ca^{++}-gK^{+} system.

The augmentation action of methylene blue on excitability of myenteric ganglion cells appeared to be translated into enhanced release of excitatory neurotransmitter substances at the longitudinal muscle of the small intestine, because application of methylene blue evoked contractions in the longitudinal axis in 21 of 22 preparations (fig. 4). The amplitude of these contractile responses were reduced by 65–100% of the control responses when methylene blue was added in the presence of either atropine (1 μM) or tetrodotoxin (2 μM). This suggests that a significant component of the contractile action of methylene blue on the intestinal musculature is mediated by release of excitatory neurotransmitters that is dependent on action potential discharge in the enteric neurons. The suppressive action of atropine indicates that the principal excitatory neurotransmitter is acetylcholine.

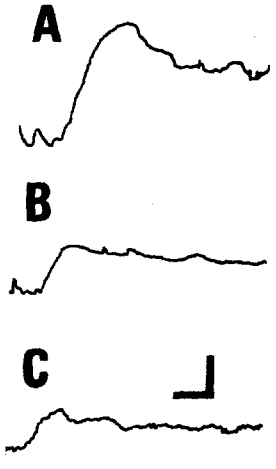

Figure 4. Effects of methylene blue on contractile responses in longitudinal axis of a segment of guinea pig small intestine. A: Methylene blue (10 μM) added to organ bath evoked a contractile response. B: Contractile response to methylene blue was suppressed in the presence of 1 μM atropine in the same preparation. C: Contractile response to methylene blue was suppressed also in the presence of 2 μM tetrodotoxin. Horizontal calibration 3 sec; vertical calibration 1 g.

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