and 40 minutes,  $20.4 \pm 1.4$  (S.E.; n = 5) and  $38.6 \pm 2.8$  (S.E.; n = 5) percent of the <sup>14</sup>C had been released from the choroid plexuses. Moreover, when 1.0  $\mu M$  FA was placed in the second medium, <sup>14</sup>C release at 20 and 40 minutes increased to  $28.0 \pm 3.4$ (S.E.; n = 4) and  $45.5 \pm 1.0$  (S.E.; n = 4) (P < .05). Moreover, when the second medium was kept at 1°C (without FA), only  $15.7 \pm 3.9$  (S.E.; n = 3) was released after 40 minutes (P <.05). Thus, a significant portion of the <sup>14</sup>C was not irreversibly bound inside the choroid plexuses.

In conclusion, we have demonstrated in the choroid plexus a specific, saturable uptake system for folates that is distinct from the weak carboxylic acid, weak basic, and ascorbate transport systems of the choroid plexus (9, 14). That is, substances that completely saturated these systems had no effect on folate uptake. The affinity for the folate transport system is:  $FA > (\pm)$ -MTHF = (+)MTHF > methotrexate.Evidence in favor of the choroid plexus being a locus of folate transport from blood to CSF would include: (i) The observation that the  $K_t$  (18.0nM) for MTHF in the isolated choroid plexus (that is, the concentration of MTHF at which the uptake system is half-saturated) was approximately that of the blood (1). Moreover, this value was not significantly different from the  $K_t$  (12) nM) observed for folate transport from blood into human CSF (6). Thus, a slight increase in the concentration in the plasma would readily saturate folate entry into CSF as observed in vivo (6). The ascorbate transport system from blood to CSF is similar (10). (ii) The finding that, although there was significant intracellular binding of MTHF inside the choroid plexus, there was ready release of intracellular MTHF, a release that was decreased by incubation in the cold. However, questions as to whether reduced folates enter brain primarily from CSF via the choroid plexus rather than directly from blood need to be postponed until this folate transport system in the choroid plexus can be conclusively shown to transport reduced folates in significant amounts from blood to CSF in vivo.

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# **Toxicology and Pharmacological Action of Anabaena** flos-aquae Toxin

Abstract. Calves, rats, ducks, and goldfish given lethal oral doses of bacteriafree lyophilized cell suspensions of toxic Anabaena flos-aquae died as a result of respiratory arrest. Experiments with selected animals and pharmacological preparations showed that the main effect of the toxin was production of a sustained postsynaptic depolarizing neuromuscular blockade.

Algal poisonings, caused by toxic blooms of the blue-green alga Anabaena flos-aquae (Lyngb.) de Bréb., have been an infrequent but repeated occurrence in several countries of the world (1). Most economic loss has been confined to livestock and waterfowl that have drunk wind-concentrated



Fig. 1. Male mallard duck given an oral MLD (milligrams per kilogram) of A. flos-aquae lyophilized cells, showing opisthotonus. Death occurred in 15 minutes.

surface blooms, dominated by toxic strains of this alga, which develop in eutrophic freshwater lakes and sloughs during the summer months.

We have studied the toxic effects of lyophilized cells in calves, rats, mice, ducks, and goldfish and have also investigated the mechanism of action of toxin extracts of the algae. Bacteriafree cultures of A. flos-aquae NRC-44-1 were used (2). Mass cultures were concentrated by flash evaporation and lyophilized. Aqueous suspensions of lyophilized cells from different batches of the alga had a consistent minimum lethal dose (MLD) of 60 mg kg-1 administered intraperitoneally when bioassayed on male mice (15 to 23 g) or male rats (300 to 400 g).

A toxin extract was prepared from the lyophilized cells by a modification of the procedure of Stavric and Gorham (3). The algal powder was extracted four times with absolute ethanol at room temperature. The extracts were pooled and evaporated to dryness at  $35^{\circ}$  to  $40^{\circ}$ C in a rotary evaporator. The residue was taken up in chloroform. The chloroform-soluble fraction was washed four times with acidic water (*p*H 4) in which the toxin is soluble and stable. These water fractions containing the toxin were then dried in air. The presence of the toxin was verified by its absorbance at 229 nm (3). This extract had an MLD of about 0.3 mg kg<sup>-1</sup> (intraperitoneal, mouse).

Two male, 6-week-old calves were given lyophilized algae in aqueous suspension by stomach tube directly into the reticulorumen. One, given 120 g (equal to 27 intraperitoneal mouse MLD's kg-1) developed muscle fasciculations and loss of muscle coordination. The animal collapsed from respiratory arrest within 7 minutes. Intubation and artificial respiration restored normal heart rate, electrocardiogram, blood pressure, blood gases, and pH. Normal respiration failed to return within 6 hours. A second calf received a total of 8.75 MLD's in successive doses of 2.5, 2.5, 1.25, and 2.5 MLD's at 10-minute intervals. The effects were similar to those with the first calf, and respiratory collapse occurred 3 hours after administration of the final bolus. Artificial respiration was maintained for 30 hours with some intermittent but no permanent resumption of spontaneous respiration. Rats given lethal doses of toxin either orally or intraperitoneally show a latent period followed by twitches, gasping, and convulsions, with death occurring in 14 to 16 minutes. Rats were also maintained by artificial respiration for periods up to 8 hours, but again spontaneous respiration failed to return. Mice show signs similar to those of rats with death times of 4 to 5 minutes. The oral MLD of calves was estimated at 6 to 8 times that of the mouse intraperitoneal MLD kg<sup>-1</sup>; rats 25 times; ducks 6 times; and goldfish 2 times. Goldfish given oral or intraperitoneal doses of the toxin show a latent period of 2 to 4 minutes followed by muscle rigidity. Death appears to be due to inability to operate the respiratory pump and occurs in 12 to 14 minutes with the mouth contracted open. Goldfish were not affected when placed in a mature algal culture, lyophilized culture (2 mg ml<sup>-1</sup>), or in an aqueous

Fig. 2. (Top) Respiration of an anesthetized rat treated with lyophilized cells. (Bottom) Contractions of anterior tibialis muscle induced by multiple square wave stimulation (1 pulse per 2 seconds) of the sciatic nerve. (A) Before intraperitoneal injection of 1 MLD of aqueous lyophilized cells. The respiratory rate was approximately 75 min<sup>-1</sup> (inspiration below and expiration peaks above midline). (B) Tracing 5 to 7 minutes after injection. The respiratory rate has decreased to about 40 min<sup>-1</sup>. (C) At 10 to 11 minutes, the respiratory rate was 35 min<sup>-1</sup>; the muscle contractions show a decrease, indicating neuromuscular blockade. (D) At 14 to 15 minutes respiratory arrest occurred after 16 minutes. The anesthetic was urethane (25 percent) and was given at a rate of 0.5 ml per 100 g of body weight. A trachea cannula was connected to a Grass volumetric pressure transducer PT5-A. The tendon was connected by a string to a Grass force-displacement transducer FT10G. In all cases transducer outputs were connected to a Hewlett-Packard carrier amplifier 8805B, and recordings were made on an HP model 7754A four-channel physiograph.

medium containing the toxin extract at 6  $\mu$ g ml<sup>-1</sup> for up to 8 hours. This indicates that the toxin is not readily absorbed across the gill membranes.

Male mallard ducks given lyophilized algae in suspension either orally or intraperitoneally developed opisthotonus and muscular rigidity similar to that reported by Buttle and Zaimis (4) after the administration of decamethonium to chicks (Fig. 1). Opisthotonus has earlier been reported as a sign for pigeons given doses of algae from a mixed blue-green bloom that contained A. flos-aquae (5). Signs similar to opisthotonus have also been described in pigeons given a mixture of algae in which Anabaena was only a small component (6). The opisthotonus observed in ducks, because of the length of the neck in this animal, has some of the appearance of fowl botulism "limberneck" (7). It is, however, physiologically distinct and is not related.

Some tachyphylaxis (8) or resistance to successive sublethal doses of the toxic algal suspensions was observed in the responses of most species studied.



Fig. 3 (left). Response of a gastrocnemius muscle sciatic nerve preparation from an anesthetized male mallard duck to intravenous (jugular) injection (at arrow) of 25  $\mu$ g of toxin extract (MLD; intraperitoneal; mouse = 0.3 mg kg<sup>-1</sup>) in Krebs



saline. The anesthetic was intravenous sodium pentobarbital (Nembutal, 25 mg/kg). The rising baseline, which indicates depolarization, was sustained for 22 minutes before it returned to the control level. The nerve was stimulated at 1 pulse per 2 seconds. Fig. 4 (right). Typical depolarizing syndrome in day-old chicks to an intravenous dose of (A) decamethonium (0.2 mg); (B) toxin extract (0.2 mg); and (C) typical flaccid paralysis due to lethal intravenous injection of *d*-tubocurarine. This was especially the case with the second calf which received sequential sublethal doses.

In anesthetized rats, intravenous injection of extract blocked responses of the anterior tibialis muscle to stimulation of the sciatic nerve (Fig. 2). The effect was not reversed by neostigmine. In vitro, on the rat phrenic nerve hemidiaphragm preparation, the extract blocked responses of the muscle to nerve stimulation. In anesthetized ducks, responses of the gastrocnemius muscle to stimulation of the sciatic nerve were blocked, and the muscle contracted (Fig. 3) in a manner similar to that reported for other avian species given depolarizing neuromuscular blocking agents. Blockade was not reversed by neostigmine but could be prevented by prior injection of d-tubocurarine. In chicks, injection of extract produced a typical depolarizing muscle relaxant syndrome (Fig. 4). On the frog rectus muscle, an extract of the algae gave a contractile response which was qualitatively similar to acetylcholine. d-Tubocurarine shifted dose-response lines of acetylcholine or the extract to the right in a parallel manner which indicated competitive inhibition. On the guinea pig ileum, large doses of extract caused a contraction that could be abolished by hexamethonium without affecting responses to acetylcholine.

It is concluded that lyophilized cells and extracts of the toxic strain A. flosaquae NRC-44-1 contain a material which has many of the characteristics of a depolarizing neuromuscular blocking agent (9) that is very rapidly absorbed by the oral route. This toxin is considered to be the very fast death factor (VFDF) reported by Gorham et al. (10) as being produced by the parent strain NRC-44. The structure of the toxin, first described by Stavric and Gorham (3) as a tertiary amine, has since been deduced on chemical and spectroscopic grounds (11) and confirmed (12). An active fraction in crude synthetic material (13) shows similar toxicology and pharmacology to that of the lyophilized cells and extract. WAYNE W. CARMICHAEL

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## **Glucagon: Role in the Hyperglycemia**

### of Diabetes Mellitus

Abstract. Glucagon suppression by somatostatin reduces or abolishes hyperglycemia in dogs made insulin-deficient by somatostatin, alloxan, or total pancreatectomy. This suggests that the development of severe diabetic hyperglycemia requires the presence of glucagon, whether secreted by pancreatic or newly identified gastrointestinal A cells, as well as a lack of insulin. Glucagon suppression could improve therapeutic glucoregulation in diabetes.

The metabolic derangements of diabetes mellitus have traditionally been ascribed entirely to insulin deficiency. We propose that, in addition to lack of insulin, the presence of the insulinopposing hormone glucagon is involved in the development of severe diabetic hyperglycemia. Two lines of evidence favor this. (i) Relative hyperglucagonemia has been found in every form of spontaneous (1) and experimental (2) diabetes examined thus far, including that produced by total pancreatectomy (3), a procedure which had been erroneously assumed to cause a deficiency of glucagon as well as of insulin. (ii) A simultaneous deficiency of both insulin and glucagon produced by the administration of somatostatin (4) does not give rise to hyperglycemia (5). The obvious physiologic and clinical importance of this question prompted us to study the role of glucagon in the pathogenesis of diabetic hyperglycemia.

Our results suggest that the development of diabetic hyperglycemia does, indeed, require the presence of glucagon. When both insulin and glucagon are suppressed to unmeasurable concentrations by somatostatin, hyperglycemia occurs only when glucagon concentrations are restored exogenously. As for the presence of hyperglycemia after pancreatectomy, we have con-

firmed the remarkable finding of Vranic et al., of Matsuyama and Foa, and of Mashiter et al. (3) that glucagon is present in the plasma of totally depancreatized dogs. Moreover, when release of glucagon after pancreatectomy is completely blocked by somatostatin, hyperglycemia is also blocked despite the absence of insulin. Glucagon of depancreatized dogs, which mimics the secretory behavior of pancreatic glucagon with respect to its responses to arginine, insulin, and somatostatin infusion, probably originates in the gastrointestinal tract, where we find cells with granules indistinguishable from those in pancreatic A cells and a glucagonlike polypeptide immunometrically, biologically, and physicochemically similar to pancreatoglucagon. Finally, suppression of glucagon in alloxan-diabetic dogs results in rapid reduction of hyperglycemia to hypoglycemic concentrations, which suggests a potentially valuable new approach to the treatment of diabetes.

We studied insulin deficiency produced in dogs by somatostatin, by alloxan, and by total pancreatectomy. Pancreatoglucagon was measured with the highly specific antiserum 30K (6). Gut glucagon-like immunoreactivity (GLI), a polypeptide which differs immunochemically from pancreatic glu-