of the purified toxin in sterile water solution was 4.0 mg/kg or 160 μg per cockerel. An oral dose lethal to all the animals (LD_{100}) was observed at a dose of 6.25 mg/kg (250 μg per cockerel). Some cockerels receiving a dose of 3.12 mg/kg (125 μg per cockerel) were dead within 24 hours; those surviving for 24 hours recovered with no apparent adverse effects. Increasing the dose beyond 3.12 mg/kg resulted in death of cockerels in a correspondingly shorter time until dosages of 40 mg/kg (1.6 mg per cockerel) resulted in death within 45 minutes.

Gross and histologic lesions in cockerels, dosed at 500 μg and 250 μg, that lived more than 2 hours after dosing, were ascites with edema of the mesenteries and small hemorrhages in the proventriculus, gizzard, small and large intestine, and skin. The cockerels that died within 2 hours (given 1000 and 500 μg per cockerel) had no lesions in organs and tissues of the cardiovascular, digestive, endocrine, hematopoietic, integumentary, lymphoid, musculoskeletal, nervous, respiratory, or urogenital systems. Cockerels dosed with 125 and 62.5 μg of toxin and cockerels that were undosed also showed no lesions.

Studies with plants demonstrated growth-regulating and phytotoxic effects of the toxin. Studies were conducted with 4-mm sections cut from 5-day-old, etiolated wheat coleoptiles (*Triticum aestivum* L. 'Wakeland'). Toxin concentrations of 20 and 200 ppm were assayed in buffered solutions (at *p*H 5.6) containing 2 percent sucrose. Ten sections were placed in test tubes containing 2-ml test solutions and incubated in a roller-tube apparatus for 24 hours at 21°C (*3*). Results demonstrated that wheat coleoptiles were inhibited 24 and 57 percent ($P < .01$) at 20 and 200 ppm of toxin, respectively, relative to controls.

Further tests with aqueous solutions of the toxin containing 0.1 percent Tween 20 were conducted on intact tobacco and corn plants. Solutions were sprayed onto 6-week-old tobacco seedlings (*Nicotiana tabacum* L. 'Hick's') or introduced into the whorls of 1-week-old corn seedlings (*Zea mays* L. 'Norfolk Market White'). Single applications of 1 ml of test solutions containing 20, 200, and 2000 μg of toxin were sprayed onto tobacco seedlings; 0.1 ml of each test solution was placed into corn whorls at 2, 20, and 200 μg of toxin per plant.

Necrosis and interveinal chlorosis were observed at 200 μg per plant on both corn and tobacco plants 1 week after treatment. In addition, distortion of leaf shape and thickening of the midrib caused by hypertrophy or hyperplasia (or both) were quite prominent on tobacco leaves 1 week after treatment with toxin at 2000 μg per plant. A later effect, becoming evident 12 days after treatment, was "rosetting" (Fig. 1A) that presumably was caused by destruction of apical dominance. This effect remained prominent 21 days after treatment (Fig. 1B). Corn plants appeared to be more sensitive to the toxin, since necrosis and chlorosis were more severe than on tobacco plants treated with an equivalent amount of toxin.

The various effects of the toxin on tobacco leaves appeared to be related to the developmental stage of the leaf at the time of treatment. Effects on older leaves were limited to necrotic lesions caused by localized concentration of the toxin resulting from evaporation of spray droplets. Toxic effects on immature leaves were expressed as interveinal chlorosis and leaf-shape distortion. The rosetting or chemical pruning effect probably resulted from the action of the toxin on embryonic leaf tissue, since this effect was not apparent until about 12 days after treatment.

The rosetting or chemical pruning effect observed on tobacco was not apparent on corn, presumably because of the difference in growth patterns (lack of axillary buds in corn). Effects on corn were limited to necrosis, chlorosis, and stunting.

The long-term effect on apical growth of a single application of the toxin on tobacco and corn plants can be seen in Fig. 1, C and D. Tobacco plants treated with 2000 μg per plant showed a marked internodal shortening or chemical pruning effect 21 days after treatment. Tobacco plants treated with 200 μg per plant showed a less severe reduction in height relative to control plants (Fig. 1C). This same trend of height relative to dosage was evident on corn plants (Fig. 1D).

The effect on apical dominance and internodal shortening was evident on tobacco plants for approximately 30 days after treatment. Subsequently these plants gradually overcame the effects of the toxin, and 6 weeks after treatment there was little difference between treated and control plants.

Fusarium spp. are known to produce mycotoxins (that is, F-2 and T-2 toxins) (*4*); however, comparisons of the physical and chemical data from the *F. moniliforme* toxin with data reported for known *Fusarium* toxins suggest a structurally new toxin that produces profound effects in both plants and animals.

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15 January 1973

Electrogenic Sodium Pump in Squid Giant Axon

Abstract. *Squid giant axon possesses a hyperpolarizing electrogenic sodium pump which is stimulated by internal sodium and by external potassium. This conclusion is based on the following observations: strophanthidin depolarizes the membrane and enhances the depolarizing effect of 5 or 10 millimolar external potassium; the magnitude of these effects is directly related to the internal sodium concentration; both effects are abolished by cyanide.*

The giant axon of the squid is one of a few archetypal preparations from which our knowledge of active ion transport has been derived. Ironically, it is one of the last ones to join the growing list of demonstrably electrogenic sodium pumps in animal cells (*1*).

Table 1. Effect of strophanthidin on squid giant axon membrane potential under various conditions. The values are means ± standard errors; the number of experiments is given in parentheses. The Na_i concentration was estimated by flame photometry. The strophanthidin-induced depolarization excludes seven axons for which the depolarization was unstable. The ratio of the slopes obtained by plotting the potential against log K_o after and before exposure to strophanthidin is m_a/m_b.

Condition of axons	Resting potential (mv)	Na_i (mmole/kg)	Strophanthidin-induced depolarization (mv)	m_a/m_b for K_o between	
				5 and 10 m*M*	1 and 10 m*M*
Fresh	−59.2 ± 0.9 (13)	56 ± 4 (4)	1.36 ± 0.03 (8)	1.33 ± 0.08 (6)	2.15 ± 0.31 (4)
Cold stored*	−63.7 ± 1.8 (8)	67 ± 6 (2)	2.31 ± .55 (6)	1.74 ± .57 (3)	3.18 ± .18 (4)
Stimulated 9 × 10^4 times in Na-ASW	−71.0 ± 4.0 (4)	101 ± 7 (2)	4.68 ± .47 (4)	1.84 ± .11 (4)	2.86 ± .95 (2)
Stimulated 9× 10^4 times in Li-ASW	−62.7 (1)	27 (1)	0.7 (1)	1.06 (1)	
Exposed to 2 m*M* CN for 60 minutes	−66.0 (1)	85 (1)	0.0 (1)	0.94 (1)	

* Stored in ASW at 4°C for 8 to 28 hours before use.

Hodgkin and Keynes (*2*) reported a small (1.6 ± 0.9 mv, $N = 5$) increase in the resting membrane potential of squid giant axons after the injection of enough sodium chloride to raise the internal sodium concentration, Na_i, by about 40 m*M*, and suggested that this effect might be due to active sodium extrusion making a direct contribution to the resting potential. It had already been found (*3*) that active sodium extrusion seemed to exceed active potassium uptake by a factor of 3/2, and it was calculated that the contribution of such a putative electrogenic pump to the resting potential would be of the order of 1.8 mv. Other authors have since confirmed that, in squid giant axon, active sodium extrusion usually exceeds active potassium uptake (*4*, *5*), although Mullins and Brinley (*5*) have shown that the Na/K ratio may vary from about 1/1 to 3/1 or higher, depending on conditions.

Electrogenic sodium pumping in squid axon would have at least two experimentally verifiable consequences: (i) membrane depolarization on stopping the pump and (ii) (if the pump under study is subject to stimulation by external potassium) a partial masking of the well-known depolarizing effect of external potassium, K_o, on the membrane potential. Demonstration of an electrogenic effect of the expected magnitude would strengthen the belief that the pump transports mainly Na^+ and K^+ and no appreciable amounts of, say, protons or anions as well. We now report more direct experimental evidence for a contribution of the sodium pump of squid giant axon to the resting membrane potential.

Carefully cleaned giant axons of *Loligo pealei* (diameters, 420 to 560 μm) were mounted vertically in a microinjection chamber (*6*). Artificial seawater (ASW) solutions (at 21° to 23°C) flowed past the axons, some of which had been injected with ^{22}Na. Resting membrane potentials were continuously recorded, by using an Orion model 601 *p*H meter, from an axial glass capillary (130 μm in diameter with a 10-μm tip) filled with 3*M* KCl (0.3 megohm). Commercial calomel half-cells were used as reference electrodes. With both electrodes immersed in ASW, drift amounted to 50 μv per 45 minutes. The resting potentials of axons in ASW were stable, typically to within 200 μv during a 20-minute period, and were demonstrably more stable (within about 50 μv) for the time required to complete a change of solution. We attribute this low drift to the use of stable half-cells and a *p*H meter amplifier.

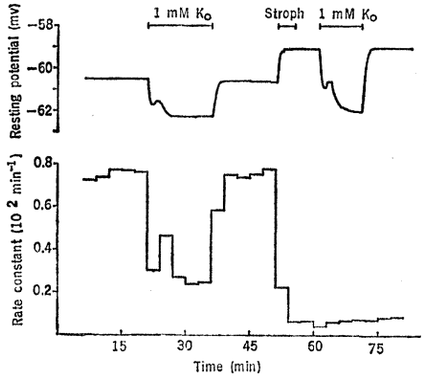

Fig. 1. Effect of external potassium (K_o) and strophanthidin (*Stroph*) on the resting membrane potential and sodium efflux from squid giant axon. The axon was exposed to seawater containing 10 m*M* K^+ except where indicated, when the concentration of K_o was 1 m*M*. During the period indicated, the seawater also contained strophanthidin ($10^{-5}M$); recovery of the sodium pump from inhibition by strophanthidin is known to be extremely slow in squid axon. (Upper trace) Continuous recording of resting membrane potential. (Lower trace) Rate constant for ^{22}Na efflux. The undulation in the potential trace when the change was made to 1 m*M* K_o was not always observed, and could have been due to mixing artifacts. The diameter of this axon was 450 μm; the temperature was 22°C.

On exposure of the axons to ASW containing $10^{-5}M$ strophanthidin, the sodium efflux was reduced to low levels, and the resting membrane potential underwent a sudden depolarization (Fig. 1), averaging 1.4 mv in fresh axons. In addition, as shown in Table 1, the strophanthidin-induced depolarization was larger in axons whose sodium content had been raised by storage in the cold or by repeated stimulation, was smaller in an axon previously depleted of Na by tetanizing in ASW in which all the sodium was replaced by lithium (Li-ASW), and was abolished by previous exposure to cyanide (*7*). In all axons except the one treated with cyanide, the depolarization produced by raising the concentration of K_o from 1 to 10 m*M* was enhanced after exposure to strophanthidin. A strophanthidin-induced increase in permeability to potassium can safely be excluded as the cause for this phenomenon, since this would also require a hyperpolarization of the membrane by strophanthidin at either value of K_o, rather than a depolarization, as observed (Fig. 1). Furthermore, the enhancement of depolarization by K_o after exposure to strophanthidin was greater at increased concentrations of Na_i (see the last two columns of Table 1). The described behavior is to be expected if the normal depolarizing effect of K^+ on the membrane is antagonized by a hyperpolarizing electrogenic sodium pump which is stimulated by external potassium as well as internal sodium.

Our conclusion that the sodium pump of squid giant axon normally operates in electrogenic fashion seems warranted on the basis of the aggregate evidence: (i) Strophanthidin produces a sudden membrane depolarization. (ii) This depolarization is larger in sodium-loaded nerves and smaller in sodium-depleted ones, and is (iii) abolished by previous exposure to cyanide. (iv)

Treatment with strophanthidin increases the sensitivity of the resting membrane potential to external K^+. (v) This increase is related to the internal sodium concentration, and is (vi) prevented by previous exposure to cyanide.

This demonstration that the sodium pump of squid axon, too, is electrogenic reinforces the hypothesis (*1*) that a truly neutral Na-K pump may not, in fact, exist.

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7. In 20 axons the drug-induced depolarization was stable, as in Fig. 1; in another seven axons the membrane potential decreased and then returned within 1 to 3 minutes to a value somewhere between the original resting potential and the peak depolarization. We could not correlate this unusual behavior with obvious parameters such as age, resting potential, or sodium content.
8. These experiments were performed at the Marine Biological Laboratory, Woods Hole, Massachusetts. Supported by PHS grants NS09380 and NS08977. P.D.W. was holder of a research career development award (GM 46373) of the National Institute of Neurological Diseases and Stroke.
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Polyadenylic Acid in Visna Virus RNA

Abstract. *Visna virus 70S RNA contains long stretches of polyadenylic acid [poly(A)]. The homogeneity in length of poly(A) regions is observed in 70S RNA from visna virus and all RNA tumor viruses tested, and not with other types of RNA. By this criterion visna virus resembles RNA tumor viruses.*

Data have been presented showing that the genomic RNA of all RNA tumor viruses (oncornaviruses) that have been studied contain polyadenylic acid [poly(A)] residues of large and rather homogeneous size (*1*). In that study, poly(A) was detected by molecular hybridization to [^{3}H]polyuridylic acid [poly(U)]; although more conventional assay techniques have obtained the same result (*2*). The genome of the RNA-containing tumor virus appears to be unique in having 70*S* RNA with long, relatively homogeneous stretches of poly(A). Poly(A) regions from most mature RNA preparations are heterogeneous in size. This appears to be the case for pure RNA preparations such as poliovirus RNA (*3*) and hemoglobin messenger RNA (mRNA) (*4*), as well as for the bulk poly(A)-containing RNA in the cytoplasm of cells (*5*). Similar results are presented in this report. The one case where poly(A) in RNA appears to be of uniform size is that of newly synthesized cellular RNA (*6*); however, unlike the RNA tumor virus genome, *this RNA* is of heterogeneous size.

The list of RNA tumor viruses having a genomic 70*S* RNA with long, relatively homogeneous stretches of poly(A) now includes Rous sarcoma virus, avian myeloblastosis virus, feline leukemia virus (Rickard), feline sarcoma virus (Gardner), Rauscher leukemia virus, murine sarcoma virus (Kirsten-Gross), murine mammary tumor virus (C_3H), and a primate virus, the Mason Pfizer agent. Since this configuration has not been observed in RNA from any other source, it is reasonable to assume the situation is diagnostic and to search for it in agents where oncogenic potential is controversial.

Visna virus constitutes such an agent. This RNA-containing particle causes a chronic neurological disorder in sheep (*7*). Although similar in several characteristics to the tumorigenic RNA-containing viruses (*8, 9*) it was not classified with this group. The reports that visna virus preparations contain RNA-dependent DNA polymerase activity (*10–12*) and 70*S* RNA (*9, 13*) led to the suspicion that this virus may also be tumorigenic. These biochemical observations encouraged further biological evaluation and led to the finding that visna virus can induce transformation in vitro (*14*).

We report here that the 70*S* RNA of visna, like that of the RNA tumor viruses, also contains large, homogeneous residues of poly(A).

On rate zonal centrifugation, RNA isolated from visna virus shows (Fig. 1) a peak of poly(A)-containing material in fractions 7 and 8. This is the same position where the poly(A)-containing RNA of avian myeloblastosis virus was found in a separate gradient (not shown). Hybridization of the 70*S* material to [^{3}H]poly(U) was completely abolished by preliminary treatment with 0.4*M* NaOH for 18 hours at 30°C, a finding consistent with its identification as an RNA molecule. Of two preparations of visna virus RNA examined, both showed this 70*S* poly(A)-containing material; however, the gradient which is not presented also showed a poly(A)-containing peak in fractions 3 and 4. We have observed similar rapidly sedimenting RNA, larger than the 70*S* RNA species, in preparations of RNA from tumor

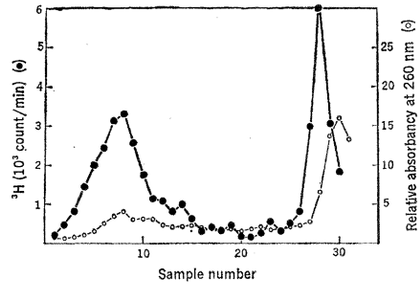

Fig. 1. 70*S* poly(A)-containing RNA from visna virus. The procedure for the growth and purification of visna virus was essentially the same as described previously (*10, 11*). Virus was precipitated in 6 percent polyethylene glycol (6000 daltons). The resuspended virus was clarified by centrifugation at 5000*g* for 10 minutes, and then sedimented at 100,000*g* for 60 minutes. The virus was resuspended in TNE buffer (0.01*M* tris, *p*H 7.5, 0.1*M* NaCl, 0.001*M* EDTA) and centrifuged on preformed gradients of 20 to 60 percent sucrose in TNE. The virus band was removed, diluted with TNE buffer, centrifuged for 1 hour at 100,000*g*, and resuspended in TNE buffer. The virus suspension was extracted twice with TNE buffer saturated with a mixture of phenol and cresol (1 : 0.15) and once with chloroform. The resulting aqueous phase was treated with potassium acetate to a concentration of 2 percent, and nucleic acids were then precipitated with 2.2 volumes of ethanol. The precipitate was centrifuged, dissolved in 0.5 ml of TNE buffer, and applied to a linear glycerol gradient (10 to 30 percent) in TNE buffer. The RNA was sedimented for 4 hours at 40,000 rev/min (4°C in a SW41 Beckman rotor). Fractions of 0.4 ml were collected. The poly(A) content of 0.025 ml of each fraction obtained from glycerol gradient centrifugation was determined by hybridization to [^{3}H]poly(U) (2×10^7 count/min per microgram) as previously described (*1*).