the rectum helps to account for the effectiveness of rectal probing in facilitating lordosis and inducing immobilization in rats; both effects are also elicited by probing the vaginal cervix (*6*).

Note added in proof: Kow and Pfaff (*20*) have also found that the size of the sensory field of the pudendal nerve was increased by systemic estrogen treatment.

BARRY R. KOMISARUK
Institute of Animal Behavior, Rutgers–The State University of New Jersey, Newark 07102
NORMAN T. ADLER
Department of Psychology, University of Pennsylvania, Philadelphia 19104
JOHN HUTCHISON
Sub-Department of Animal Behaviour, Cambridge University, Cambridge, England

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9. The electrodes were insulated with Teflon except at the hook; they were placed 1–2 mm apart in each nerve. Action potentials were recorded through a Grass HIP5A high impedance probe, amplified through a Grass 7P3 AC amplifier, and filtered through a Kopf spike filter. Action potentials were displayed on a Tektronix 564 storage cathode-ray oscilloscope and were monitored with a Grass AM 4 audio monitor. The nerve remained moist since body fluids continuously seeped into the recording region; we periodically blotted the region adjacent to the nerve with cotton wisps. To determine the boundaries of the responsive region, a flexible plastic ruler was used to prevent inadvertent brushing of responsive regions. Alternatively, movement of responsive regions was prevented by holding the skin well outside the responsive region and brushing toward the responsive region. The maximum sensory field dimensions were determined by brushing the fur, and when the boundary was reached, the skin was scratched. This stimulus occasionally generated action potentials in the nerve at a distance of a few millimeters beyond the boundary of the long fur. The clitoral field was found by brushing the clitoral sheath and adjacent skin. There was no significant correlation between latency (days) from ovariectomy to testing and the area of the sensory field in the uninjected rats ($r = .42$, $P > .05$, one-tailed); and there was no significant correlation between number of estrogen injections and area of sensory field in the hormone-treated rats ($r = .43$, $P > .05$, one-tailed). Anesthetic was injected intraperitoneally on the side opposite to that used for recording. The pudendal and pelvic nerves were exposed by way of a ventral approach under a dissecting microscope, by making a 6-cm incision 1 cm lateral to the midline, and locating the internal iliac vein. The electrodes were inserted into the nerves just caudal to the region where they diverge. The genitofemoral nerve lies superficially on the abdominal muscle wall and recordings were made from this nerve at about the same anterior-posterior level as the other nerves. Each rat was taped in a supine position to an electrode carrier base so that the heel of the left leg was 3.5 cm from the midline as shown in Fig. 1A and 1B. A series of dots was drawn on the fur 1 cm apart with solvent blue 38 stain (Eastman) dissolved in absolute methanol. These extended in two parallel rows, one from the tip of the clitoral sheath to the knee joint, the other 1 cm below the clitoral dot. The genital region and grid were photographed with a Polaroid MP3 camera and sensory fields were plotted directly on the photographs. All experiments were performed blind, and on any recording day two experimental and two control rats were selected for study. Each day, we did not know the treatment of any rat until all rats had been studied. Two observers plotted the extent of each receptive field. Since the genital tract could be observed in approaching the nerve for positioning the electrodes, a technician, who was not involved in any other aspect of the experiment, performed the initial part of the dissection. The technician carefully obscured the uterus and vagina with saline-soaked gauze pads. At the end of each day after recording the results, all uteri were dissected free and weighed in order to confirm the effectiveness of the estrogen. The range of uterine weights was 361 to 615 mg in the estrogen-treated rats and 69 to 125 mg in the noninjected rats. The outline of each sensory field was drawn on the calibrated photograph of each rat, and traced twice with a Keuffel and Esser model 62 0015 compensating polar planimeter. By comparing this mean area reading with the mean reading obtained by measuring the area of a 20 by 20 mm square based on the calibration of each photograph, the actual area of the sensory field was calculated.
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18 September 1972

Neurotrophic Effect on Isolated Chick Embryo Muscle in Culture

Abstract. *Acetylcholinesterase activity in cultures of dissociated skeletal muscle prepared from the thigh muscle of the 10-day-old chick embryo was increased by the presence of innervating spinal cord explants, spinal cord explants in a parabiotic environment, and by media containing brain–spinal cord extract.*

Numerous experimental observations have indicated that motor neurons have important trophic effects on muscle (*1*). Recent reports indicate that many in vivo observations of trophic influences of neurons can be reproduced in cells grown in tissue or organ culture. Peterson and Crain (*2*) observed that neuritic contacts between explants of spinal cord and muscle enhanced muscle development in explants of fetal rodent skeletal muscle. Kano and Shimada (*3*) found that, when the dissociated thigh muscle of the embryonic chick was grown in culture together with explants of spinal cord, the formation of functional neuromuscular junctions restricted the acetylcholine-sensitive area of muscle cells to the junctional region. In noninnervated fibers the whole surface was sensitive to acetylcholine. Lentz (*4*) found that sensory ganglia in direct contact with organ cultures of forelimb muscle from adult newts produced a delay in the decrease in cholinesterase activity that normally occurred as a result of denervation. A similar effect was produced when the sensory ganglia were separated from the muscle by a Millipore filter. The addition of homogenates of nerve tissue to the culture media also produced greater activity of muscle cholinesterase than occurred in untreated muscle cultured

for the same period of time. We have investigated the influence of innervation, the presence of noninnervating spinal cord explants and of a brain–spinal cord extract on the level of acetylcholinesterase (AChE) activity in cultures of the dissociated skeletal muscle of the chick.

Spinal cord explants were taken from 10-day-old chick embryos, placed in pairs on collagen-coated cover slips, and cultured in roller tubes. After 4 days in vitro, spinal cord cultures were presented with a drop of cell suspension of dissociated muscle taken from 10-day-old chick embryos. Thigh muscle was dissociated by exposure to 1 percent trypsin (Difco, 1 : 250) in calcium- and magnesium-free Puck's solution for 30 minutes. After being washed three times in Puck's solution, loosened tissues were transferred to the nutrient media and dissociated into single cells by gentle pipetting. Control muscle cultures without spinal cord explants were also maintained in vitro in an environment identical to the combined cord-muscle cultures. After 2 days in Maximow's slides, the cultures were transferred to roller tubes and maintained for another 8 days. The nutrient media consisted of 35 parts of horse serum, 35 parts of medium 199, 5 parts of 10-day-old chick embryo extract (whole brain and spinal cord excluded), 22 parts of Hanks balanced salt solution, and 3 parts of 20 percent glucose. At the end of the culture period, the cultures were rinsed with saline and homogenized with a glass homogenizer. Acetylcholinesterase activity of muscle culture homogenates was measured by determining the rate of hydrolysis of acetyl-β-methylcholine with the use of radiometer titrator and titrigraph (*5*). The protein content of the homogenates was measured by the method of Bonting and Jones (*6*).

The morphological development of muscle in tissue culture was similar to that described (*7, 8*), although the occurrence of mature myotubes was somewhat earlier in our study as compared to previous studies (Fig. 1). Within 4 to 5 days in vitro, fully differentiated myotubes with characteristic cross-striations were present. At the same time both synchronous and asynchronous contractions were observed. Microscopic examination of living cultures after 3 to 4 days in vitro revealed possible nerve-muscle contacts in the form of axons that appeared either to terminate on, overlay, or run in parallel with the myotubes. Impregnation with silver by a modification of Bodian's Protargol method (*9*) made motor neurons and axons readily apparent. Terminal axons formed bulbous swellings or small rings, which were in contact with the striated myotubes (Fig. 2) (*7, 8*). Electron microscopic examination of the nerve-muscle contact region revealed terminal axons closely associated with muscle fibers (Fig. 3). The nerve terminals contained small mitochondria and numerous spheroid vesicles 300 to 500 Å in diameter. Myofilaments were observed within the muscle. It was apparent that the structures described above are in fact neuromuscular junctions as described in previous tissue culture studies (*8, 10*). Neuromuscular junctions developed in vitro have been demonstrated to be functional synaptic contacts by electrophysiological studies (*3, 11*). From the findings described above it is evident that neuromuscular junctions are formed in our cultures after 7 days in vitro.

Table 1. Changes in acetylcholinesterase activity in cultures of embryonic chick skeletal muscle resulting from the influence of innervating nerve fibers, parabiotic spinal cord explants, and a brain–spinal cord extract (*12*). Each value is the mean ± standard error for four assays (ten cultures per assay).

Type of culture	Methylcholine hydrolyzed per milligram of protein		
	μmole/hr	Increase (%)	*P*
Control	2.55 ± 0.16		
Innervated (cultures)	5.48 ± 0.33	115	< .001
Control	3.28 ± 0.11		
Parabiotic spinal cord tissue	6.25 ± 0.51	90	< .001
Control	2.20 ± 0.15		
Brain–spinal cord extract	3.82 ± 0.21	72	< .001

The effect of innervation on cholinesterase activity of muscle was studied by comparing AChE activity of innervated muscle cultures and that of noninnervated control muscle cultures. In the cord-muscle cultures prior to homogenization, the spinal cord explants were removed by dissection under an inverted microscope. The AChE activity in the innervated muscle cultures was 115 percent higher than in control muscle cultures (Table 1). These results are consistent with the view that innervation influences the development of AChE in muscle.

The effect of the presence of spinal cord explants in a parabiotic condition on AChE activity of muscle cultures was also studied. Explants of 10-day-old chick embryo spinal cord were cultured on collagen-coated cover slips in roller tubes for 4 days, and then these cord cultures and dissociated muscle cell cultures (see above) that had been grown for 2 days on cover slips in Maximow's assembly were positioned

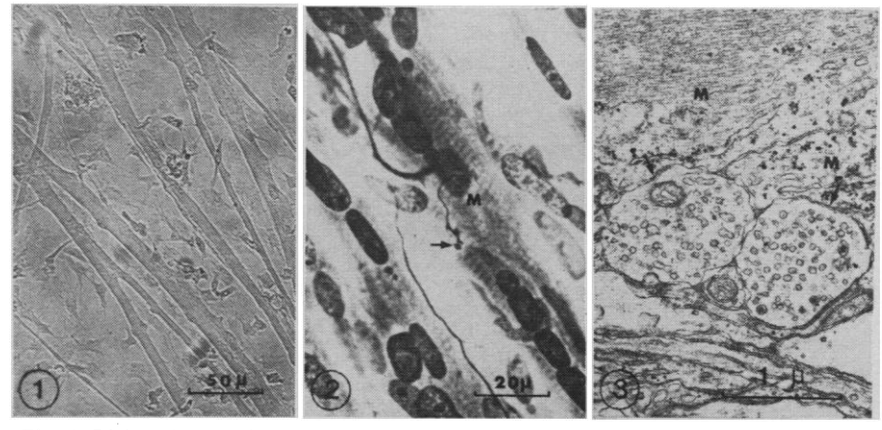

Fig. 1. Light microscopic appearance of living cultures (muscle culture, maintained for 7 days in vitro) showing well-developed myotubes with cross striations. Fig. 2. A terminal bulb (arrow) of an incoming nerve fiber (spinal cord–muscle culture, maintained for 7 days in vitro) is shown making contact with striated myotube. Bodian method. Fig. 3. Two nerve terminals (spinal cord–muscle culture maintained 7 days in vitro) containing numerous synaptic vesicles establishing synaptic contacts (arrows) with myotubes (*M*).

back-to-back in roller tubes, and maintained another 8 days in vitro. With this procedure there was no direct physical contact between the two tissues other than the common culture medium. Muscle cultures exposed to spinal cord explants in this parabiotic condition and control muscle cultures were assayed for AChE activity. In muscle cultures, which were exposed to the media bathing the physically separated spinal cord explants for 8 days, AChE activity was almost twice that of the control cultures (Table 1), an indication that direct contact between nerve and muscle is not essential for regulation of AChE activity.

The results also indicate that the trophic effect can be mediated by a diffusible substance produced by spinal cord tissue. We therefore sought to determine whether a brain–spinal cord extract would promote AChE synthesis. For that purpose, dissociated muscle cultures (see above) were maintained in nutrient fluids containing 5 percent brain–spinal cord extract of 10-day chick embryos in place of ordinary embryo extract (brain and spinal cord excluded). After 10 days in vitro, experimental groups of muscle cultures grown in medium with brain–spinal cord extract and control muscle cultures were assayed for AChE activity. The AChE activity was higher in the muscle cultures grown in the presence of brain extract as compared to the control muscle cultures grown in the absence of brain extract (Table 1). These results show that brain–spinal cord extract is effective in inducing AChE activity in muscle cultures.

Our results agree with both in vitro and in vivo experimental findings of trophic effects of neurons as they related to the control of cholinesterase levels in skeletal muscle (*1*), and in addition confirm the finding of Lentz (*4*) that this effect can be produced in the absence of functional synapses.

T. H. Oh, D. D. Johnson
S. U. Kim*

Departments of Pharmacology and *Anatomy, University of Saskatchewan, Saskatoon, Canada*

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12. The reason for the differences in AChE activity in the control groups shown in Table 1 is not known. These values were obtained from three separate experiments. In each experiment the control and experimental muscle cultures were obtained from embryonic muscle subjected to the same dissociation procedure and subsequently subjected to the same culture medium and environment. It is possible that slight variations in the trypsinization procedure, in the culture medium, or in the environment are responsible for the variations in AChE activity. Similar variations in AChE activity of cells grown in culture have been reported [I. Werner, G. R. Peterson, L. Shuster, *J. Neurochem.* **18**, 141 (1971)].

* Present address: Division of Neuropathology, University of Pennsylvania, Philadelphia 19104.

5 September 1972

Australia Antigen (Hepatitis B Antigen): A Conformational Antigen Dependent on Disulfide Bonds

Abstract. *Reduction and alkylation of purified hepatitis-associated Australia antigen (hepatitis B antigen) resulted in a total loss of serologic activity. The reduced and alkylated protein formed a single band with a sedimentation coefficient of 31S on analytical ultracentrifugation, and no subunits were detected by Sephadex gel filtration. Although this preparation induced a delayed hypersensitivity response when injected into guinea pigs, it failed to stimulate humoral antibody formation. The data suggest that hepatitis B antigen is a conformational antigen critically dependent upon the disulfide bonds of the protein moiety.*

The discovery of Australia antigen and its association with post-transfusion "serum" hepatitis caused by hepatitis type B virus (*1*) stimulated studies of the natural history, epidemiology, and prevention of hepatitis type B (*1*, *2*). Purified hepatitis B antigen, prepared devoid of plasma protein by zonal ultracentrifugation, has been used for studies of structure and composition (*3*). Determining the immunochemical basis of the serologic specificity of the hepatitis B antigen is essential for understanding its molecular biology. The fact that the antigen is unusually resistant to enzymatic proteolysis (*4*) necessitated an alternate approach to its structural analysis. The antigen was found to contain 6.5 moles of cysteine per 100 moles of protein (6.5 mole percent) (*5*). Reduction and alkylation of the disulfide bonds of the cystine residues resulted in complete loss of antigenic activity.

Hepatitis B antigen was purified from the plasma of a healthy carrier by a combination of isopycnic banding and rate sedimentation on cesium chloride gradients (*5*). The purified antigen, composed exclusively of particles 20 nm in diameter, was devoid of detectable human plasma proteins as determined by gel diffusion analysis with antiserum to normal human serum. Inert indicator red cells, coated by exposure to antigen solution (0.5 mg/ml) and then to 1.25 m*M* chromic chloride, were not agglutinated by serial dilutions of antiserum to human serum, but were agglutinated by antiserum to hepatitis B antigen at a dilution of 1 : 6000 (*6*).

A solution of purified protein (0.5 mg/ml) in 0.55*M* tris(hydroxymethylaminomethane (tris) buffer, *p*H 8.0, was treated with 0.2*M* β-mercaptoethanol for 3 hours at 37°C, chilled to 0°C, and alkylated with 0.2*M* iced

Table 1. Effect of various treatments on serologic specificity of hepatitis B antigen. The antigen solution (0.5 mg/ml, in tris-HCl buffer, *p*H 8.0) was treated with 0.2*M* β-mercaptoethanol or 0.05*M* dithiothreitol with and without alkylation with 0.2*M* iodoacetamide. All materials were dialyzed against saline, then against 1.0*N* acetic acid. The reduced alkylated and reduced nonalkylated proteins were tested with antiserum to hepatitis B antigen by hemagglutination inhibition (HI) and countercurrent electrophoresis (CE). A + for hemagglutination inhibition indicates a titer comparable to control, untreated antigen.

Treatment	HI	CE
β-Mercaptoethanol	+	+
β-Mercaptoethanol, iodoacetamide	−	−
β-Mercaptoethanol, urea	+	+
β-Mercaptoethanol, urea, iodoacetamide	−	−
Iodoacetamide	+	+
Dithiothreitol	+	+
Dithiothreitol, iodoacetamide	−	−
Acetic acid	+	+
Urea	+	+
Control (untreated)	+	+