appear after 20 cell doublings in the absence of isoantiserum. Fluctuation analysis, generally used to distinguish between inductive and selective mechanisms of variation (*9*), cannot be applied to these experiments because of the inability of antiserum to eliminate all sensitive cells in a single exposure. An alternative means of making this distinction is to determine the antiserum sensitivity of clones isolated from strains showing partial resistance after several selective cycles (for example, Fig. 1, curve B). If induction is the mechanism, the clones isolated from partially resistant strains should belong to one population with intermediate resistance; whereas if the mechanism is selection for preexisting variants, the clones should form two populations, either fully sensitive or fully resistant. These studies are in progress.

Although the locus specifying the isoantigen reactive with AS in the cell strains used in these experiments is not known, it is of interest that monospecific antiserums of HL-A specificity are cytotoxic for human fibroblasts (*10*). By using these antiserums in an isoantigenic selective system in conjunction with the approximately 30 other markers known to be detectable in human fibroblasts, it may be possible to detect mitotic recombination in these cells and to determine human linkage.

R. ADMAN*
D. A. PIOUS†

Department of Pediatrics, University of Washington School of Medicine, Seattle 98105

References and Notes

1. S. M. Gartler and D. A. Pious, *Humangenetik* **2**, 83 (1966); R. S. Krooth, G. A. Darlington, A. A. Velazquez, *Annu. Rev. Genetics* **2**, 141 (1968).
2. D. A. Pious, *Biochem. Genet.* **2**, 185 (1968).
3. R. Payne and M. R. Rolfs, *J. Clin. Invest.* **37**, 1756 (1958).
4. B. W. Papermaster and L. A. Herzenberg, *J. Cell Physiol.* **67**, 407 (1966); D. A. Pious, *Genetics* **56**, 601 (1967).
5. C′ obtained from individual rabbits was tested for potency and toxicity prior to use; only potent and relatively nontoxic C′ was used, at the highest concentration found to be nontoxic. This concentration varied from 20 to 25 percent.
6. J. F. Morgan, H. J. Morton, R. C. Parker, *Proc. Soc. Exp. Biol. Med.* **73**, 1 (1950).
7. P. Gorer and P. O'Gorman, *Transplant. Bull.* **3**, 142 (1956).
8. E. A. Boyse, L. J. Old, E. Stockert, N. Shigeno, *Cancer Res.* **28**, 1280 (1968).
9. S. E. Luria and M. Delbrück, *Genetics* **28**, 491 (1943).
10. R. A. Adman and D. A. Pious, unpublished result.
11. Supported in part by NIH grant GM 15883, a PHS postdoctoral fellowship (R.A.), and a research career development award from NICHD (D.A.P.)
* Present address: Department of Genetics, University of Washington.
† Requests for reprints should be sent to D. A. Pious.

23 December 1969

Acetylcholine Sensitivity of Muscle Fiber Membranes: Mechanism of Regulation by Motoneurons

Abstract. *Inhibitors of RNA and protein synthesis prevent the development of acetylcholine supersensitivity in organ-cultured rat diaphragm muscle but do not affect established acetylcholine sensitivity. The restriction of this sensitivity in innervated muscle apparently involves neuronal restriction of gene activity in muscle fibers.*

The distribution of active acetylcholine (ACh) receptors in skeletal muscle fiber membranes (ACh sensitivity) is controlled by motoneurons (*1*). This neuronal influence is a prime example of "neurotrophic effects" (*2*). Innervated muscle fibers are highly sensitive to ACh only in the area of neuromuscular junction but, following denervation, become quite sensitive to ACh over their entire length. The mechanism by which motoneurons regulate the distribution of ACh sensitivity and the mechanism by which ACh sensitivity is increased in denervated muscle fibers have remained unknown. I now report that the increase in ACh sensitivity in denervated muscle fibers is prevented when actinomycin D or puromycin or cycloheximide is used to inhibit RNA or protein synthesis. I conclude that the neuronal restriction of ACh sensitivity in muscle fibers involves regulation of gene activity in muscle fibers.

The left phrenic nerves of female Sprague Dawley rats weighing 100 to 180 g were cut approximately 5 to 10 mm from the diaphragm surface. At appropriate times after denervation diaphragms were transferred to organ culture (*3*). For culture each hemidiaphragm was cut to form two radial straps 6 to 8 mm wide. Each strap was pinned, slightly stretched, through a single layer of cheesecloth to a stainless steel grid. The ends of the grid were bent so that the diaphragm strap rested upon the surface of the culture medium (7 ml of medium in a 15- by 60-mm disposable plastic culture dish). Trowell T-8 medium (without chloramphenicol) was supplemented with 1 percent rat or horse serum and equilibrated at 37°C with 5 percent CO_2 in oxygen. When needed, actinomycin D

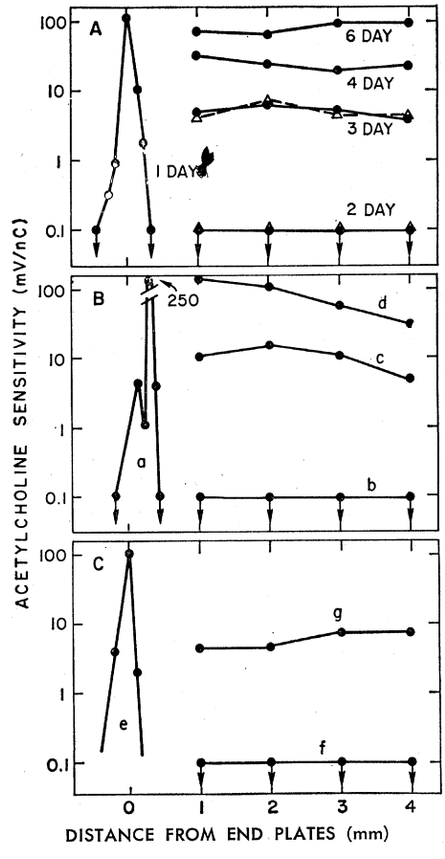

Fig. 1. (A) Acetylcholine sensitivity of rat diaphragm muscle fibers 1, 2, 3, 4, and 6 days after section of the phrenic nerve (•—•) or 2 and 3 days after transfer of normal muscle to organ culture (△—△). The arrows indicate that the sensitivity was less than 0.1 mv/nC and usually below the limit of detection. (B) Acetylcholine sensitivity of muscle fibers: (*a*) single end plate after 3 days' culture in 1 μg of actinomycin D per milliliter (ACh sensitivity was demonstrated in 25 such end plates); (*b*) after 2 days' denervation in vivo and subsequently 1, 2, or 3 days' culture in 1 μg of actinomycin D per milliliter (data from 105 fibers in 12 cultures); (*c*) after 3 days' denervation in vivo and subsequently 2 days' culture in 1 μg of actinomycin D per milliliter; (*d*) after 4 days' denervation in vivo and subsequently 1 day in culture in 1 μg of actinomycin D per milliliter. Data in (*c*) and (*d*) are representative of 152 fibers in 19 control cultures. (C) Acetylcholine sensitivity of muscle fibers: (*e*) single end plate after 3 days' culture in 10 μg of cycloheximide per milliliter (ACh sensitivity was demonstrated in 22 such end plates); (*f*) after 2 days' denervation in vivo and subsequently 1, 2, or 3 days' culture in 10 μg of cycloheximide per milliliter (data from 121 fibers in seven cultures); (*g*) after 3 days' denervation in vivo and subsequently 2 days' culture in 10 μg of cycloheximide per milliliter (data from 27 fibers in two cultures).

or puromycin or cycloheximide was added.

For about 3 days cultures containing up to 1 μg of actinomycin D per milliliter or 100 μg of cycloheximide per milliliter maintained large muscle fiber resting potentials (50 to 70 mv) and the ability to contract upon electrical stimulation. Cultures containing 10 μg of puromycin per milliliter usually lasted less than 2 days. Since nearly complete development of ACh sensitivity requires about 6 days, most experiments involved cutting the phrenic nerve and, 1 to 4 days later, transferring the denervated diaphragm to culture.

Acetylcholine sensitivity was measured by recording transmembrane potentials with an intracellular micropipette while ACh was applied iontophoretically to the outer surface of the muscle fiber membrane from a micropipette containing 3*M* AChCl solution (*4*). Sensitivity is expressed as millivolts of depolarization per nanocoulomb (nC) of iontophoretic current. Iontophoretic current pulses of 5×10^{-9} to 2×10^{-7} amp and durations of 1 to 100 msec were employed. Iontophoretic pulses were adjusted to yield maximum values of ACh sensitivity. Latency between onset of iontophoretic current and beginning of depolarization was always less than 10 msec. An ACh sensitivity of 0.01 mv/nC (0.2 mv depolarization produced by an iontophoretic current pulse of 2×10^{-7} amp for 100 msec) was considered the minimum detectable ACh sensitivity in any experiment. When no ACh sensitivity could be detected, the accurate placement of micropipettes was confirmed by advancing the ACh micropipette into the fiber, passing current, and recording electrotonic pulses (*4*). Measurements were made on four to eight fibers at positions 1, 2, 3, and 4 mm from the area of neuromuscular junctions. Separation of ACh and recording micropipettes was usually less than 200 μm. Acetylcholine sensitivity around individual end plates was also mapped. After electrophysiological analysis the location of end plates was verified by staining acid-fixed preparations with thiolacetic acid–lead reagent (for cholinesterases) (*5*) and Schiff reagent (for the phrenic nerve) (*6*).

The development of extrajunctional ACh sensitivity in organ-cultured rat diaphragm follows a time course similar to that in vivo. Fibers rarely have a sensitivity greater than 0.1 mv/nC 48 hours after denervation, and virtually all fibers have a sensitivity of 2 to 20 mv/nC by 72 hours (Fig. 1A). Actinomycin D (1 μg/ml) added to organ cultures of 0-, 1-, or 2-day denervated rat diaphragms prevents the rise of extrajunctional ACh sensitivity (Fig. 1B). However, actinomycin D has no effect upon the ACh sensitivity at the neuromuscular junction or upon established extrajunctional ACh sensitivity. One microgram of actinomycin D per milliliter retards the rate of RNA synthesis by 80 percent (measured as incorporation of 5-^{3}H-uridine into trichloroacetic acid–precipitable, alkali-labile material), while the rate of protein synthesis (incorporation of ^{14}C-leucine into trichloroacetic acid–precipitable, hot trichloroacetic acid–stable material) declines only 15 percent in 48 hours.

Cycloheximide (1 to 100 μg/ml) or puromycin (10 μg/ml) added to diaphragm cultures likewise halts the development of extrajunctional ACh sensitivity (Fig. 1C). Neither inhibitor has any effect upon end-plate ACh sensitivity or upon established extrajunctional ACh sensitivity. Puromycin is quite toxic, and most cultures become electrically inexcitable after 48 hours' exposure. Cycloheximide is much less toxic and at 10 μg/ml immediately retards the rate of protein synthesis by 95 percent while the rate of RNA synthesis declines slowly to about 40 percent of control levels in 48 hours. Concentrations of actinomycin D and of cycloheximide which do not block RNA or protein synthesis (0.01 μg/ml) have no effect upon the increase in ACh sensitivity.

While it remains possible that unknown side effects of the inhibitors caused the observed results, some tentative conclusions can be drawn regarding the molecular basis of ACh sensitivity and its regulation. First, since ACh sensitivity remains undiminished for 3 days in the virtual absence of protein synthesis, I conclude that the rate of turnover of ACh receptors is probably very slow. Second, since inhibition of RNA or protein synthesis will prevent the rise of extrajunctional ACh sensitivity in denervated muscle fibers, I conclude that new species of RNA and protein are required for the expression of new ACh sensitivity. Perhaps these requirements reflect the synthesis of ACh "receptors" from newly activated genetic information. A corollary of the second conclusion is that the neuronal regulation of ACh sensitivity probably involves regulation of gene activity in muscle fibers.

DOUGLAS M. FAMBROUGH
Department of Embryology,
Carnegie Institution of Washington,
Baltimore, Maryland 21210

References and Notes

1. W. B. Cannon and A. Rosenblueth, ***The Supersensitivity of Denervated Structures*** (Macmillan, New York, 1949); J. V. Luco and C. Eyzaguirre, *J. Neurophysiol.* **18**, 65 (1955); J. Axelsson and S. Thesleff, *J. Physiol. (London)* **147**, 178 (1959); R. Miledi, *ibid.* **151**, 1, 24 (1960); J. Diamond and R. Miledi, *ibid.* **162**, 393 (1962); R. Miledi, E. Stefani, J. Zelená, *Nature* **220**, 497 (1968).
2. L. Guth, *Physiol. Rev.* **48**, 645 (1968); *Neurosci. Res. Program Bull.* **7**, 1 (1969).
3. R. Miledi and O. A. Trowell, *Nature* **194**, 981 (1962).
4. W. L. Nastuk, *Fed. Proc.* **12**, 102 (1953); J. del Castillo and B. Katz, *J. Physiol. (London)* **128**, 157 (1955).
5. M. Crevier and L. F. Bélanger, *Science* **122**, 556 (1955); G. B. Koelle and R. S. Horn, *J. Histochem. Cytochem.* **16**, 743 (1968).
6. H.-M. Liang, *Anat. Rec.* **99**, 511 (1947).
7. I thank Mrs. Arlyne Musselman for technical assistance.

11 December 1969

1,3-Bis(p-chlorobenzylideneamino)guanidine Hydrochloride (Robenzidene): New Poultry Anticoccidial Agent

Abstract. *At 66 parts per million in the feed, robenzidene is highly effective in preventing chicken coccidiosis caused by any one of eight* Eimeria *species. Three times this dose is safe for broiler chickens. In dogs and rats, the toxicity was relatively low over a period of 90 days. In laboratory trials, the drug completely prevents oocyst production by seven species and greatly reduces oocyst production by* Eimeria maxima.

Over the past 20 years anticoccidial drugs have been added to feed for chickens to help prevent coccidial diseases. Nevertheless, coccidiosis continues to be one of the three most prevalent poultry diseases (*1*). Reports of drug resistance in field strains of the protozoa responsible, *Eimeria* species, have appeared with increasing frequency, sometimes soon after introduction of new agents (*2*). The recommended concentrations of anticoccidial agents for continuous preventive use in feed are effective in reducing economic losses, but do not completely prevent production of oocysts and thereby permit