In areas where resistance to chlorinated hydrocarbon insecticides is widespread, such a program would be especially effective if the males released also carried genes for susceptibility. Naturally, these hypotheses need to be tested in population cage experiments in the laboratory as well as in field populations (*5*).

G. B. CRAIG, JR.
W. A. HICKEY
R. C. VANDEHEY
Department of Biology, University of Notre Dame, Notre Dame, Indiana

References and Notes

1. M. Qutubuddin, *Bull. Entomol. Research* **43**, 549 (1952).
2. B. M. Gilchrist and J. B. S. Haldane, *Hereditas* **33**, 175 (1947).
3. S. R. Christophers, *Aedes aegypti* (Cambridge Univ. Press, Cambridge, 1960).
4. G. B. Craig, Jr., R. C. VandeHey, W. A. Hickey, *Bull. World Health Organization*, in press.
5. This work was supported in part by National Institutes of Health grant No. E-2753, by U.S. Army Biological Laboratories research grant No. DA-18-064-404-CML 471, and by Atomic Energy Commission research contract AT (11-1)-38.

15 August 1960

Mechanism of Fibrillation Potentials in Denervated Mammalian Skeletal Muscle

Abstract. This report concerns the origin and mechanism of fibrillation potentials. It is proposed that these potentials do not necessarily arise from degenerated end-plate organs. The precursor is the rhythmic oscillations of the membrane potential. This mechanism may also occur in the case of epileptic discharges from epileptic cells in the cerebral cortex.

An excitable element, such as a nerve cell or a muscle fiber, is said to possess the property of spontaneous activity, if it is capable of discharge at a time during which no electrical energy is supplied from outside (*1*). In denervated skeletal muscle fiber, spontaneous rhythmic fibrillation potentials can be detected. The clinical significance of fibrillation potentials has been repeatedly discussed. This report deals with the underlying mechanism by which these fibrillation potentials are produced. The conclusions presented here stem from three series of experiments previously published.

Two questions may be asked: (i) Do the spontaneous rhythmic fibrillation potentials originate from a specific focal area in the muscle fiber? (ii) Is there any difference in the membrane properties of intact and denervated muscle fibers? The following experiments were performed in order to answer these questions.

The first series of experiments (*2*) was made under hypothermia on rats in which the electrical activity was recorded from the anterior gracilis muscle with intact motor nerve supplies. The second series of experiments (*3*) was made on rats in which the sciatic nerves had been severed 3 to 21 days prior to experimentation. The third series of experiments (*4*) was made on tissue cultures in which embryonic skeletal muscle fibers of the chick were isolated. Glass micropipette electrodes, recording intracellularly from single muscle fibers, were used in all of the experiments. The recording electrode, with a tip measuring approximately 0.2 μ in external diameter, was filled with electrolyte solution and connected by a platinum wire to a cathode follower, then to a direct-coupled amplifier, and finally to an oscilloscope.

In the first series of experiments, in which the motor nerve supplies to the rat muscle fibers were intact, the resting membrane potentials were found to be extremely stable. When the intracellular electrode impaled a muscle fiber near the motor end-plates, miniature end-plate potentials (*5*) were recorded. They were thought to represent asynchronous depolarization of small and restricted areas of the muscle membrane by a release of packages of acetylcholine molecules from the motor nerve endings (*6*). The discharge of these miniature end-plate potentials, presumably arising from different areas, could be synchronously evoked by applying a stimulus to the motor nerve. The synchronous discharge of many miniature end-plate potentials gave rise to an end-plate potential; if the end-plate potential exceeded a certain depolarization level, a spike discharge resulted. The evoked spike discharge seldom repeated itself; thus the response to the stimulus was almost always one-to-one. After the subsidence of the spike discharge, the resting membrane potential resumed its previous level and remained stable. No spontaneous rhythmic spike activity was recorded from the muscle.

In the second series of experiments with denervated skeletal muscle fibers, no miniature end-plate potentials were recorded. However, oscillation of the muscle membrane was often found. The oscillating potentials differed from the miniature end-plate potentials in that they were not all-or-none and random but were graded and rhythmic. If an oscillating potential exceeded a certain magnitude, a spike discharge or a series of rhythmic spike discharges was set off. The frequency of the rhythmic spike discharges was fairly constant in recordings from any given fiber; but occasionally a spike was missing and was replaced by an oscillation of potential. Furthermore, when the membrane potential of a muscle fiber was relatively stable, and when an electrical stimulus was applied to the fiber, the stimulus frequently precipitated a series of rhythmic spike discharges or oscillations. This is different from the responses of muscle fibers with intact motor nerve supplies.

In the tissue culture experiments, series after series of rhythmic activity synchronous with the fibrillary movements of the muscle fiber observed through the microscope were recorded by the intracellular electrode. This spontaneous activity was similar to that recorded from denervated skeletal muscle fibers and consisted of oscillating and spike potentials. The rhythmic oscillations occurred independently but gave rise to the rhythmic spike discharges. Therefore, they were the precursor of these spike discharges. Upon stimulation the cultured muscle fiber responded with a series of rhythmic spike discharges similar to the response of denervated muscle fibers. The response was sometimes a series of rhythmic oscillating potentials superimposed on a sustained depolarization following an initial spike discharge.

Earlier experiments with axons of the squid or lobster showed that oscillating potentials could be produced by sustained membrane depolarization (*7*, *8*). An increase in the oscillation of potential in axons of the squid and cerebral cortical neurons of the cat could be produced readily by veratrine and strychnine (*9*, *10*). These observations were obtained even when no electrical energy was supplied from outside the system. The spontaneous rhythmic spike discharges followed a decrease in the membrane potential and an increase in the oscillating potential.

In denervated mammalian skeletal muscle fibers, Ware, Bennett, and McIntyre also found a decrease in the resting membrane potential (*11*). They reported that the resting membrane potential dropped from a mean value of about −100 to −77 mv 22 days after denervation. Although their findings need to be confirmed (*3*), they are in keeping with the observations that sustained depolarization was accompanied by membrane oscillations or rhythmic spike discharges in nerves of the squid (*7*), lobster and crayfish (*8*, *12*), visual cells of the limulus crab (*13*), and cortical neurons of the cat (*10*).

It is known that a tropic change occurs in muscles deprived of motor nerve supplies. After denervation, an increase in hexokinase and a decrease in cytochrome oxidase activity were observed (*14*). Other metabolic changes remain to be found.

This study of denervated skeletal muscles in vivo and embryonic muscles in tissue culture explants indicates that no presynaptic activity is required for the production of rhythmic fibrillation

potentials. Furthermore, no neuronal elements or end-plate organs were found in the cultured muscle. The spontaneous rhythmic discharges must arise at some point along the muscle fiber itself.

The above observations would suggest that the presence of a degenerated end-plate organ in denervated muscle is not important for the generation of fibrillation potentials. It is likely, as found by McIntyre and his associates, that a decrease in resting membrane potential occurs as a result of a change in muscle metabolism following denervation. The decrease in resting membrane potential leads to instability and oscillation of the membrane potential and, finally, to rhythmic spike discharges. It is probable that this sequence of events also occurs in the case of epileptic discharges from pathological nerve cells in the cerebral cortex (*15*).

CHOH-LUH LI
National Institute of Neurological Diseases and Blindness, National Institutes of Health, Bethesda, Maryland

References and Notes

1. B. D. Burns, *Mammalian Cerebal Cortex* (E. Arnold, London, 1958).
2. C. Li, *Am. J. Physiol.* **194**, 200 (1958).
3. C. Li, G. Shy, J. Wells, *J. Physiol. (London)* **135**, 522 (1957).
4. C. Li, K. Engel, I. Klatzo, *J. Cellular Comp. Physiol.* **53**, 421 (1959).
5. P. Fatt and B. Katz, *J. Physiol. (London)* **115**, 320 (1951).
6. ———, *ibid.* **117**, 109 (1952).
7. A. Arvanitaki, *J. Physiol. (Paris)* **37**, 895 (1940); K. S. Cole, *Arch. sci. physiol.* **3**, 253 (1949).
8. F. Brink, D. W. Bronk, M. G. Larabee, *Ann. N.Y. Acad. Sci.* **47**, 457 (1946).
9. A. M. Shanes, *J. Gen. Physiol.* **33**, 57 (1949).
10. C. Li, *J. Neurophysiol.* **22**, 436 (1959).
11. F. Ware, Jr., A. L. Bennett, A. R. McIntyre, *Am. J. Physiol.* **177**, 115 (1954).
12. C. Eyzaguirre and S. W. Kuffler, *J. Gen. Physiol.* **39**, 87 (1955).
13. M. G. F. Fuortes, *J. Physiol. (London)* **148**, 14 (1959).
14. F. Humoller, D. Hatch, A. R. McIntyre, *Am. J. Physiol.* **167**, 656 (1951); **170**, 371 (1952).
15. C. Li, S. Chou, S. Howard, *Epilepsia*, 1960, in press; presented in part at a seminar in the department of physiology, University of Chicago, Chicago, Ill., on 27 May 1960.

29 August 1960

Preparation of Monolayer Cell Cultures from Tissues of Some Lower Vertebrates

Abstract. Cold trypsin dispersion at *p*H 7.2 was used to obtain cultivable cells and cell groups from tissues of six species of fresh-water bony fishes, a frog, and a turtle. The cells readily attached to glass and were capable of at least limited, and in some cases extended, division in media consisting of commercially available components.

There are few fields of biological interest that have not found some use for the rapidly advancing techniques of cell and tissue culture. Paul's recent text on tissue culture (*1*) describes cultivation of tissues from cold-blooded animals, but the paucity of information reflects the scant attention that has been devoted to in vitro cultivation of tissues from the lower vertebrates. Madin *et al.* (*2*) reviewed the cultivation of tissues of domestic and laboratory animals but actually discussed only mammals. The recently compiled bibliography of tissue culture (*3*) contains more than 15,000 entries, but less than 0.002 percent deal with fish or fish tissue. Wolf and Dunbar (*4*) cited the principal works on fish tissue culture; all reports dealt solely with the technique of explantation.

The purpose of this report is to describe a method employed routinely in this laboratory during the past 9 months to obtain monolayer cultures of cells from several tissues of propagated trouts. Exploratory work with tissues from other easily obtained fishes, an amphibian, and an aquatic reptile has shown that the method is equally efficient for obtaining cultivable cells from these other animals.

Tissues were dispersed with a modification of the cold trypsinization described by Bodian (*5*). Since trout tissue explants prospered in vitro only when maintained at a *p*H between 7.2 and 7.4, the final *p*H of the digestion mixture was buffered at 7.2. Dulbecco and Vogt's phosphate-buffered saline (*6*) was modified to contain 1.065 g of Na_2HPO_4 and 0.249 g of KH_2PO_4 and constituted 87.5 percent of the digestion mixture. The balance consisted of a 2.5-percent trypsin solution (10 percent) (*7*) and, because it seemed to benefit the cells, human serum (2.5 percent). Potassium penicillin G and streptomycin sulfate were added at the rate of 200 unit/ml.

One to several volumes of saline-washed, scissor-minced tissue were used for each 10 volumes of the digestion mixture. All tissues were obtained as cleanly and aseptically as possible and, throughout preparation, were kept in vessels on or near ice. Most tissue was used soon after removal, but on occasion trout tissue was held at 4°C for 24 hours before digestion.

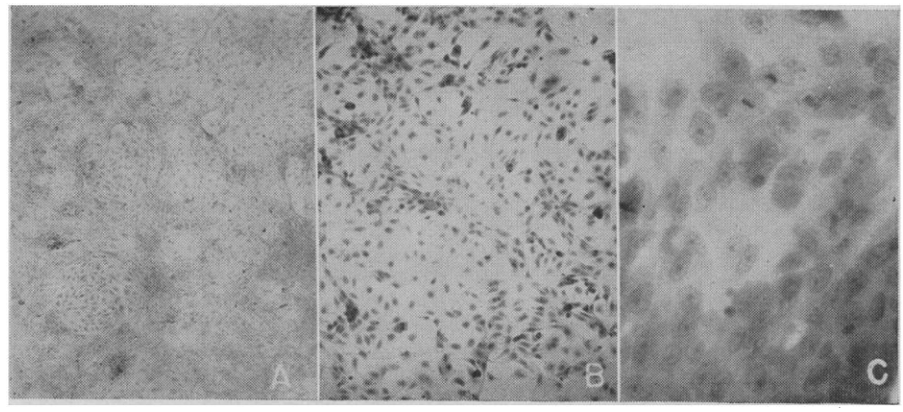

Fig. 1. *A*, Primary cultures of pooled gonad from immature rainbow trout (*Salmo gairdneri*) (× 24). *B*, Original cultivation of kidney from the bullfrog (*Rana catesbeiana*) (× 48). *C*, Original cultivation of ovary from the painted turtle (*Chrysemys picta*), showing cells in various stages of division (× 290). Hematoxylin and eosin stain.

Dispersion was effected at 4° to 6°C in fluted erlenmeyer flasks on magnetic stirrers which had separate rheostats and, therefore, did not heat the mixture. Cells obtained from the first hour's harvest generally gave poor results. In practice, the first hour's harvest was discarded and replaced with an equal volume of fresh digestion mixture. Depending upon the relative speed of dispersion, the process was continued for three or more hours. Best results were obtained when the time did not exceed 20 hours. Overnight treatment, which usually released from 10^5 to 10^6 or more cells per milliliter, became the routine procedure.

Cells were sedimented from the harvest fluid by cold centrifugation at 200*g* for 20 minutes. The resulting packs averaged 0.1 or more of the starting tissue volume. The cells were washed once in cold salt solution that contained 2 to 5 percent serum and were then suspended in the growth medium. Dilutions of 1:400 to 1:600 (1 to 3 × 10^5 cells per milliliter) were normally made, although considerably higher dilutions were possible. The initial *p*H was between 7.2 and 7.4, and static vessels were incubated routinely at either 12.5°C or, preferably, 19°C.

Mammalian-type media (*7*), used in all work, readily stimulated attachment and division of cells from fishes, a frog, and a turtle (Fig. 1). These results are in accord with the determinations of Phillips *et al.* (*8*), which first showed that, except for phosphorus, the inorganic constituents of trout blood are remarkably like those of human blood.