complete absence of contaminating mitochondria and microsomes. As seen with electron photomicrographs (RCA Model EMV-3G electron microscope using a 1% buffered osmium tetroxide fixative), the mitochondrial and microsomal fractions were found to be homogeneous enough so that experiments could be performed.

It is felt, therefore, that heparin can serve as a useful tool during ultracentrifugation experiments for the separation and isolation of subcellular particles.

Résumé. Nous avons présenté une méthode de fractionnement et d'isolement des particules subcellulaires de la muqueuse intestinale du rat. L'héparine permit l'isolement de fractions relativement homogènes.

P. GOODMAN and B. KADIS

Departments of Obstetrics and Gynecology and Biochemistry, University of Nebraska, College of Medicine, Omaha (Nebraska, USA), March 8, 1965.

STUDIA NOVA

Spontaneous Contractions and Bioelectric Activity after Differentiation in Culture of Presumptive Neuromuscular Tissues of the Early Frog Embryo

Tissue culture has recently been used to study the capacities of isolated regions of the amphibian neural plate for physiological development[1]. This experimental method has also been successfully applied to studies of the bioelectric activity of fragments of spinal cord[2,3] and cerebral cortex[4,5] isolated from later stages of mammalian (and avian) embryos and neonates. The present report describes electrophysiologic experiments on cultures of frog neurula tissue, explanted together with presumptive axial muscle, after development in vitro of spontaneous twitching. The data indicate that the characteristic, spontaneous bursts of muscle contractions in these explants may be triggered by endogenous, rhythmic neural activity.

The tissues were obtained from neurulae of *Rana pipiens* (stages 13–17, Shumway). The desired region of the neural plate, usually the prospective upper spinal cord but occasionally also the prospective hindbrain, was excised together with the underlying chorda-mesoderm. The explants (about 0.5 mm^3) were cultured on a glass, or collagen, surface in a small petri dish containing several ml of a balanced salt solution (Barth's 'X') with added globulin[6]. The cultures were examined regularly for muscular activity, which was then carefully observed and described. For bioelectric recordings, the culture coverglass was inverted so as to form the roof of a small moist chamber. The chamber was mounted on a microscope stage and micromanipulators were used to position microelectrodes at various sites in the tissue[3,7]. A given culture was studied electrically only once, and both the spontaneous activity and that evoked by electric shocks were recorded over a period of several hours (with differential-input preamplifiers and an oscilloscope).

Undisturbed cultures survived and showed muscular twitching for up to two weeks without added nutrients or change of medium. It should be emphasized that the exogenous nutritional requirements of early embryonic amphibian tissues are much simpler than those of mammalian explants since the individual cells still contain abundant endogenous yolk reserves. The explanted fragments remained as a thick mass of cells, but extensive outgrowth of muscle and connective tissue cells, and of nerve fibers, often occurred. Numerous isolated cells also grew near the main mass and were often attached to nerve fibers, which generally branched extensively from that point. In five instances an outlying single cell or small cluster, visibly connected with nerve fibers, was seen to twitch. In two of these, the twitches were synchronous with bursts of muscle contractions in the main mass, while only intermittent single twitches were seen in the other three.

Spontaneous twitching of the explant developed in more than half of the cultures prepared with neural plate and the underlying layer. This was never seen in numerous cultures of chorda-mesoderm alone despite the development of numerous muscle fibers, which could be made to twitch by electric shocks. Spontaneous activity was typically in the form of synchronous contractions of some or all of the muscle of the explant, usually with temporary desynchronization of single fibers here and there during a burst of twitches. The earliest activity seen in explants of prospective spinal cord was, characteristically, a single twitch at intervals of $^1/_2$ min or more. This occurred between the 4th and 7th days in vitro when the tissue was isolated early in neurulation (stages 13, 14), but as early as the 2nd and 3rd days if isolated somewhat later (stages 15–17). Prospective hindbrain developed relatively rapidly, for all of those cultures (isolated at stage 14) showed frequent activity on the 6th day, when half of the cord explants showed no activity or only an occasional twitch. In every case but one where single twitches had been observed to occur, *bursts* of contractions were seen on the following day. (The single exception showed only occasional single twitches for several days, and then stopped all muscular activity; a few preparations were also seen to revert to this pattern shortly before cessation of all activity.) Three of the 36 active preparations studied developed a pattern of almost continual twitching, at the same average frequency as that which occurred in the short bursts.

Burst activity in all of these explants can be characterized as follows: (a) twitches occurred at a rate of about

1 M. A. CORNER, J. comp. Neurol. *123*, 243 (1964).
2 S. M. CRAIN and E. R. PETERSON, Science *141*, 427 (1963).
3 S. M. CRAIN and E. R. PETERSON, J. cell. comp. Physiol. *64*, 1 (1964).
4 S. M. CRAIN, in Symposium on Neurological and Electroencephalographic Correlative Studies in Infancy (Ed.: P. KELLAWAY and I. PETERSÉN, Grune and Stratton, New York 1964), p. 12.
5 S. M. CRAIN and M. B. BORNSTEIN, Exp. Neurol. *10*, 425 (1964).
6 L. G. BARTH and L. J. BARTH, J. Embryol. exp. Morphol. *7*, 210 (1959).
7 S. M. CRAIN, J. comp. Neurol. *104*, 285 (1956).

5–8 per sec, with slight irregularities in the intervals between successive twitches; (b) the initial contraction was almost always relatively large and the strength of successive twitches varied greatly; (c) the durations ranged from about 0.5 to 5 sec – bursts longer than 3 sec occurred only in five cord explants, appearing during the first days of activity in four of them and decreasing thereafter to 1–3 sec; in some preparations the burst duration remained at a characteristic value for several days; (d) many cultures showed regular intervals between bursts, ranging from 1 to 20 ($\pm$ 0.5) sec, with certain values occurring more frequently (Figure 1); in the early days of activity the inter-burst intervals were usually longer and more variable. These repetitive periods of muscle burst activity were usually interrupted every few minutes by silent periods of the order of 1 min. The general character and the range of durations of endogenous bursts of muscle contractions are thus similar in *Rana* to what was found in analogous experiments with *Xenopus*, suggesting the existence of essentially the same endogenous neural rhythm in both of these anuran larvae[1].

Electrophysiologic studies of the neural regions in these explants, after 1–2 weeks in vitro, demonstrate that complex bioelectric responses can be evoked by electric stimuli. These long-lasting spike barrages, appearing at critical thresholds and showing facilitation with paired stimuli at long test intervals (Figure 2A), are remarkably similar to the response patterns characteristic of more mature cultures of mammalian spinal cord[2,3]. Although the amphibian tissue was explanted at a much earlier stage than has been attempted in the mammalian experiments, the striking resemblance in functional performance demonstrates the high degree of self-differentiation which can occur in these tiny fragments of frog neurula tissue maintained in a simple culture medium. It is likely, therefore, that the frog explants may develop complex synaptic networks within 1–2 weeks in vitro, as has been shown to occur in the mammalian cord cultures[3,8,9].

In a few favorable cultures it has been possible to evoke bursts of muscle contractions (in groups of synchronously active fibers) with single electric stimuli applied locally to a relatively distant neural region of the explant. These neurally evoked muscle twitch responses were more complex than those elicited by direct electric stimulation of the muscle tissue, and they occurred at much lower thresholds. In some cases, they could be evoked by facilitation of paired stimuli at relatively long test intervals (as in Figure $2A_{2,3}$). Their duration was often shorter than that of the characteristic, spontaneous bursts of muscle contractions in these explants. Barrages of spike potentials (of varying amplitude and duration) could be recorded from regions in which these muscle bursts were evoked, with latencies as long as 100 msec after the stimulus to a neural area (Figure $2B_{1,2}$). Simultaneous recordings near the stimulus site in the neural region indicate that the neural activity gradually spreads to the muscle zone at a slow rate suggesting circuitous, multisynaptic transmission rather than direct propagation along conductile fibers[3]. Similar sequential appearance of activity at the two sites occurs during spontaneous bursts (Figure $2B_3$).

Although electrophysiologic analysis of these frog explants is still at a preliminary stage, the bioelectric data suggest that the regular endogenous bursts of muscle contractions may, indeed, be triggered by periodic bursts of neural activity. This interpretation agrees with microscopic observations during ontogenetic development of

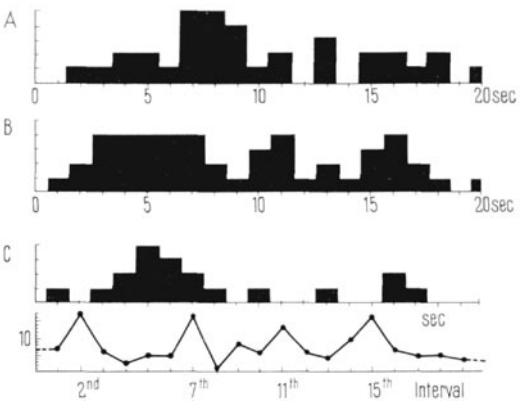

Fig. 1. A, Duration of intervals between bursts of twitching in cultured frog embryo spinal cord with somites (8–9 days in vitro). Composite of 35 measurements on 5 cultures. B, The same in 5–6 day old cultures. 50 measurements on 6 cultures, two of them also used later for the measurements in A. C, Distribution of the intervals between 20 successive bursts in a single 6-day-old culture. D, The intervals shown in C plotted successively in time. The intervals immediately preceding and following this sequence were longer than 1 min.

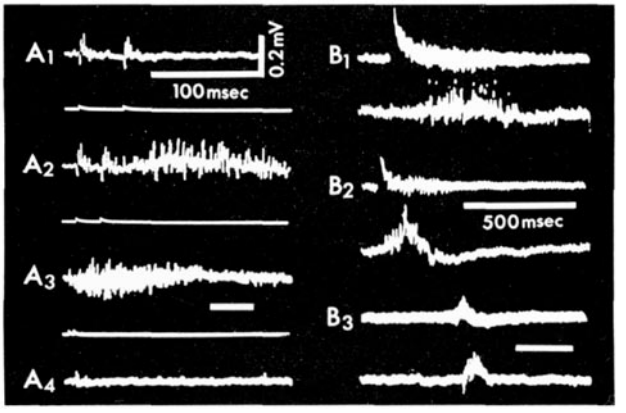

Fig. 2. Bioelectric potentials recorded with extracellular microelectrodes (tip diameters: 5–25 μ) in cultured frog embryo spinal cord with somites (1–2 weeks in vitro). A_1, Brief responses in a neural region following each of two electric stimuli, applied 400 μ from recording site, about 40 msec apart (through saline-filled pipettes with 10 μ tips). Lower sweep shows stimulus signals. A_2, Long-lasting, complex barrage evoked after stimulus test-interval was reduced to 20 msec. At slower sweep rate (A_3), entire barrage response evoked by paired stimulus is recorded. Only a brief potential occurs after single stimulus (A_4). B_1, Simultaneous records from a neural region (upper sweep) and a neuromuscular region (lower sweep), about 400 μ apart, following a stimulus applied near former site. Note much longer latency before appearance of bioelectric response at neuromuscular region (concomitant with burst of muscle contractions in this vicinity of explant). B_2, Similar evoked responses, at slower sweep rate; note larger amplitude of response in neuromuscular region. B_3, Spontaneous potentials recorded at same sites as in $B_{1,2}$. Note that here, too, the activity appears earlier in the neural region (upper sweep). Time and amplitude calibrations apply to all succeeding records, unless otherwise noted; upward deflection indicates negativity at active recording electrode.

[8] R. P. Bunge, M. B. Bunge, and E. R. Peterson, J. Cell. Biol. *24*, 163 (1965).

[9] E. R. Peterson, S. M. Crain, and M. R. Murray, Z. Zellforsch. *66*, 130 (1965).

amphibian neuromuscular systems in situ and in vitro (as noted above)[1,10]. It also supports SZEPSENWOL's conclusions regarding the neural basis of spontaneous muscle twitching in cultured chick embryo myotomes[11,12]. Spontaneous, but less regular, muscle contractions have also been seen in recent studies of cord-innervated skeletal muscle tissue explanted from 12-day mouse embryo myotomes[13]. Electrophysiologic experiments with these mouse myotome explants have permitted, moreover, extensive analysis of neuromuscular transmission during spontaneous as well as evoked activity, including selective block of cord-innervated muscle responses with *d*-tubocurarine[14]. It should also be noted that a sequence of repetitive potentials occurs sporadically (and may often be evoked by single stimuli) in cultures of various mammalian CNS tissues, involving a pattern which is remarkably similar to that characteristic of the endogenous muscle bursts in the frog explants. This consists of a series of diphasic, oscillatory potentials occurring at a rate of 5–15 per sec, and lasting about 0.5–3 sec[2–5]. It is of interest that a similar pattern of repetitive discharge, in response to a single stimulus, develops in neuronally isolated slabs of neonatal cat cerebral cortex, in situ[15]. In some mouse cerebral cultures, this oscillatory discharge occurs spontaneously, especially after various neuropharmacologic agents, with an interval between bursts of 1–5 sec[16]. Development of these stereotyped, repetitive discharges in such diverse CNS tissues, under such widely different environmental conditions, suggests that a basic type of neural network underlies this common pattern of activity[1,4,16].

Zusammenfassung. Gewebekulturen von *Froschneurula* mit zugehörigen Muskelprimordien differenzieren in vitro und zeigen charakteristische Serien von Muskelkontraktionen. Elektrophysiologisch wurde nachgewiesen, dass die Muskelzuckungen durch endogene, komplexe, rhythmische Nervenaktivität hervorgerufen werden können.

M. A. CORNER[17] and ST. M. CRAIN[18]

Central Institute for Brain Research, Amsterdam (The Netherlands) and Departments of Anatomy and Neurology, College of Physicians and Surgeons, Columbia University, New York (USA), September 18, 1964.

[10] M. A. CORNER, J. Embryol. exp. Morphol. *12*, 665 (1964).
[11] J. SZEPSENWOL, Anat. Rec. *95*, 125 (1946).
[12] J. SZEPSENWOL, Anat. Rec. *98*, 67 (1947).
[13] M. B. BORNSTEIN and L. M. BREITBART, Anat. Rec. *148*, 362 (1964).
[14] S. M. CRAIN, Anat. Rec. *98*, 273 (1964).
[15] D. P. PURPURA and E. M. HOUSEPIAN, Exp. Neurol. *4*, 377 (1961).
[16] S. M. CRAIN, Biophysical Society, 8th Annual Meeting, Chicago, Abstr. WG1 (1964); in preparation.
[17] Supported in part by award from the National Multiple Sclerosis Society (USA). Thanks are due to Dr. MARGARET R. MURRAY and to the staff of her tissue culture laboratory for their hospitality and assistance during this period.
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CORRIGENDUM

W. L. BENCZE, R. W. J. CARNEY, L. I. BARSKY, A. A. RENZI, L. DORFMAN, and G. DE STEVENS: *Synthetic Estrogens and Implantation Inhibitors.* Experientia *21*, 261 (1965). The fourth line above the formulae should read as follows: coupling constant of 5 c/s for the C_1 hydrogen which was . . .

The chemical formula of compounds IV, VI, and VIII (*cis* configuration) should be pictured as follows: