reveals that this inconsistency is more apparent than real. First, the linear relationship he reported was obtained only with muscles that had been subjected to prolonged (30 to 60 hours) soaking in media low in K^+ and in which the estimated C_{Na} was relatively high (30 mM to 70 mM). Second, when C_{Na} was between 10 mM and 30 mM, this variable had little effect on the height of the overshoot. If we take into account possible discrepancies between our estimates of the extracellular space and those of Desmedt, this finding is entirely consistent with our results (Table 1). Third, in interpreting Desmedt's experiments, the possibility of a local increase in a_{Na} at the fiber membrane during depolarization should be considered. Determinations of intrafiber activity in muscles immersed for longer times in K^+-free media than those used in the present study should provide further information about this question.

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Development of Excitability in Embryonic Muscle Cell Membranes in Certain Tunicates

Abstract. *During the course of development of muscle cells in certain tunicates, a sign of regenerative membrane response appears in the gastrula stage. In the early tadpole larva, the action potential consists of a spike followed by a plateau. The latter disappears in fully differentiated cells, conceivably in association with the establishment of delayed rectification.*

The initiation of action potentials is one of the unique features of nerve and muscle cell membranes. It would, therefore, be of great interest if one could follow up the appearance and establishment of the electrical excitability of the cell membrane throughout various stages of embryonic development. For this type of study it is desired that the preparation have the following properties: (i) the destination of each blastomere should be fully determined at the very early stage of development, so that one can follow all stages of development up to fully differentiated cells; and (ii) the cells should have diameters large enough for intracellular microelectrode techniques to be applied throughout the course of cleavage. Certain tunicates (*Halocynthia roretzi* Drashe and *Halocynthia aurantium* Pallas) have large eggs (270 μm in diameter; Fig. 1A) and specific blastomeres are destined in the course of development to give rise to specific parts of the larva (*1, 2*). Moreover, the cells destined to be muscle cells remain large throughout cleavage. In fully differentiated larva there are six relatively large chains of striated muscle cells arranged longitudinally in the tail of the tadpole larva, their diameter being 15 to 20 μm (*3*) (Fig. 1A). Each chain consists of seven to eight mononuclear cells connected along the long axis of the chain (*2, 4*).

Matured eggs and sperms for fertilization were obtained from *Halocynthia roretzi* or *Halocynthia aurantium* captured in the northern part of Japan. Fertilized eggs were cultured under constant aeration at temperatures of 6° to 7°C in natural seawater. The total time of the development from the first cleavage to the hatching was about 70 hours. For the peneration of micropipettes, the follicular envelope and the chorion around the egg or the tadpole before hatching were removed by fine needles. The tunic coat of the hatched tadpole was also taken off by the needles. For the tadpole, pronase was applied for 10 minutes (at 10°C) at a concentration of 0.1 percent in the external solution to loosen the intercellular contact within the epithelium. The enzyme was washed away with a large amount of fresh artificial seawater (ASW) cooled to 0°C. Then the preparation was kept in ASW cooled to about 5.0° to −0.5°C by thermoelectric device to decrease the rate of development during the experiment. The ASW used had the composition of 452 mM NaCl, 9.8 mM KCl, 10.6 mM $CaCl_2$, and 48 mM $MgCl_2$, buffered at pH 8.0 by 10 mM tris (hydroxymethyl) aminomethane HCl. Glass microelectrodes filled with a solution of 2M potassium citrate saturated with methyl blue for marking the penetrated cell (*5*) and having a resistance of 20 to 40 megohms were used as recording electrodes. Those filled with 3M KCl and having a resistance of 5 to 10 megohms served as reference electrodes. So that we might observe the electrical responses from two cells simultaneously, we introduced a second electrode filled with potassium citrate. For checking the value of resting pitentials, we used recording electrodes filled with 3M KC1. To record potential changes while a current pulse was applied through the recording micropipette, we used the input stage of the modified bridge circuit (*6*) improved by application of FET operational amplifiers (143B, Analog Devices).

The development of the excitable membrane in the embryo consisted of the following four successive stages, tentatively called A, B, C, and D. They were characterized by unique responses to depolarizing current pulses (Fig. 2) and by the amount of the resting potential.

At stage A, from the period of the first cleavage to the initiation of invagination (128 cell stage), all cells examined showed the same amount of resting potentials, -19.4 ± 7.1 mv (standard deviation, $n=9$) on the average, irrespective of whether their cell types were presumptive muscle cells or the other cells. No regenerative responses were induced when the cells were depolarized by current pulse (Fig. 2A). In the blastula or the gastrula at the next stage (B) an electrotonic

coupling was found between cells as reported for embryos of other species (*7*). Thus, similar characteristic responses were obtained in any cell with current application either to the presumptive muscle cells or to the recorded cells themselves. Some of the couplings between muscle cells remained throughout development even up to the fully differentiated stage of the tadpole larva.

At stage B, from the period of the early gastrula to that of a young tadpole larva with a tail about 50 to 70 percent of its full length, presumptive muscle cells revealed a resting potential of -70.6 ± 8.7 mv ($n=9$) (Fig. 3, curve *B*). Sudden transition of the resting potential to the hyperpolarized level occurred around the 128-cell stage, shortly after the gastrulation. The characteristic responses to a depolarizing current pulse are shown in Fig. 2B; a large increase of depolarization occurred with slight increase in intensity of the current pulse above a critical value, and the depolarization outlasted the current pulse more than a second. This may be considered as a primitive form of the action potential which is found in the next stage, C.

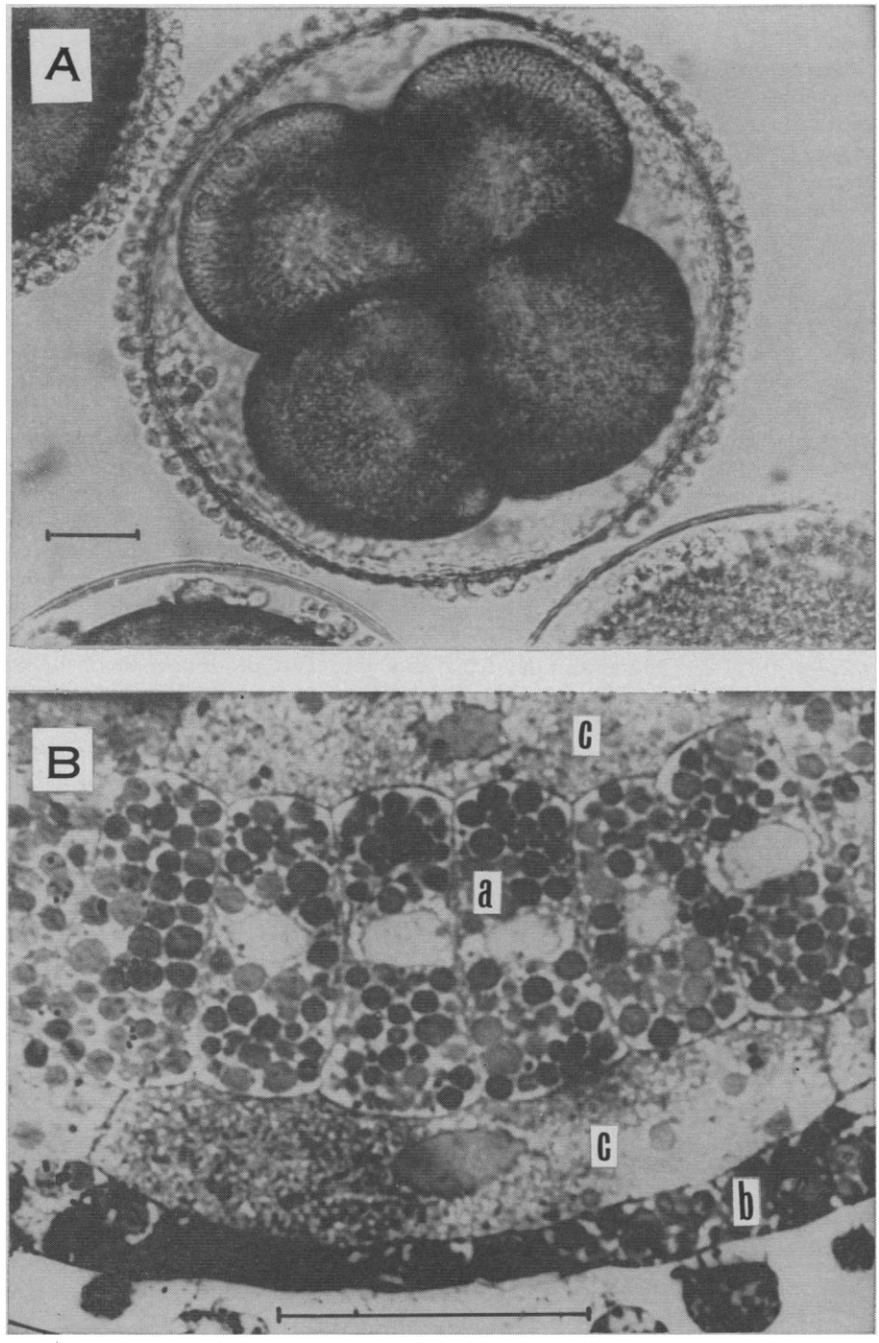
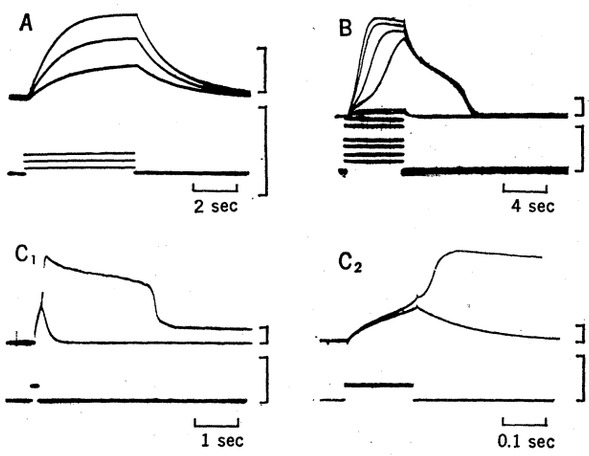


Fig. 1. (A) Living four-cell stage of *Halocynthia aurantium* in its follicular envelope and its chorion. (B) Portion of the tail of 70 percent grown tadpole (*Halocynthia aurantium*). A line of cells filled with numerous yolk granules is a part of the notocord (*a*). A longitudinally extended cell between the notocord and the epithelium (*b*) is a mononuclear muscle cell (*c*) in a muscle cell chain. Fixed with glutaraldehyde and osmium tetroxide, embedded in Epon, sectioned about 1 μm, and stained with toluidine blue. Photography with Nikon type SUR-Ke microscope (scale; 50 μm).

At stage C, which corresponds to the period of the young tadpole larva before hatching, muscle cells showed resting potentials of -50.1 ± 7.6 mv ($n=7$) on the average. The resting potentials appear to be at a more hyperpolarized level at the beginning of the stage (Fig. 3, curve *C*). An action potential with an overshoot of 0 to 30 mv was generated by a depolarizing current pulse in an all-or-none fashion (Fig. 2, C_1 and C_2). The action potential consisted of an initial steep rise to a peak followed by a plateau up to 2 seconds long at 2°C. The plateau was then followed by a long-lasting depolarizing afterpotential. The duration of the action potential and apparently the amount of the resting potential also gradually decreased with the development of the tadpole larva until its hatching.

At stage D, from the time of hatching to the period just before metamorphosis of the larva, the muscle cells showed resting potentials of -42.6 ± 5.6 mv ($n=13$) and spike potentials with an overshoot of 0 to 10 mv. The spike potential had a duration of about 50 msec (measured between half-maximal points) at 1°C and was followed by relatively short-lasting after-hyperpolarization (Fig. 2D).

In Fig. 3, relations of applied current intensity and response potential at all stages are plotted on the same set of coordinates. The value of the potential on the *y*-axis of each curve corresponds to the resting membrane potential at the respective stage. In each experiment, the membrane potential at a steady level after application of a current step or at least 5 seconds after onset of the current was plotted as a function of the current intensity. At stage A, the curve was almost linear (Fig. 3, curves *Aa* and *Ab*), although a slightly steeper slope was found for the hyperpolarizing current compared with the one found for the depolarizing current. The effective resistance was in



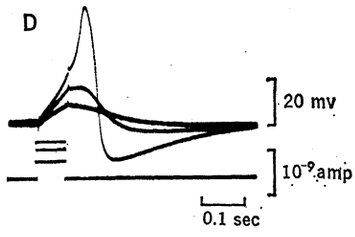


Fig. 2. Membrane responses of blastomere and muscle cells of a developing tunicate to depolarizing currents at each stage of development. (A) At 64-cell stage; resting potential (RP), — 25 mv. (B) At the middle of the gastrula stage, recorded from a cell in the presumptive muscle region (right side of blastopore); RP, — 70 mv. (C_1 and C_2) From a muscle cell of the tadpole with a 90 percent grown tail. C_1 and C_2 illustrate an all-or-none responding action potential with a slow and a rapid sweep, respectively; RP, —59 mv. (D) The spike potential from a muscle cell of the fully grown tadpole after its hatching; RP, — 45 mv. All records were taken with single electrodes filled with potassium citrate and connected to the modified bridge circuit.

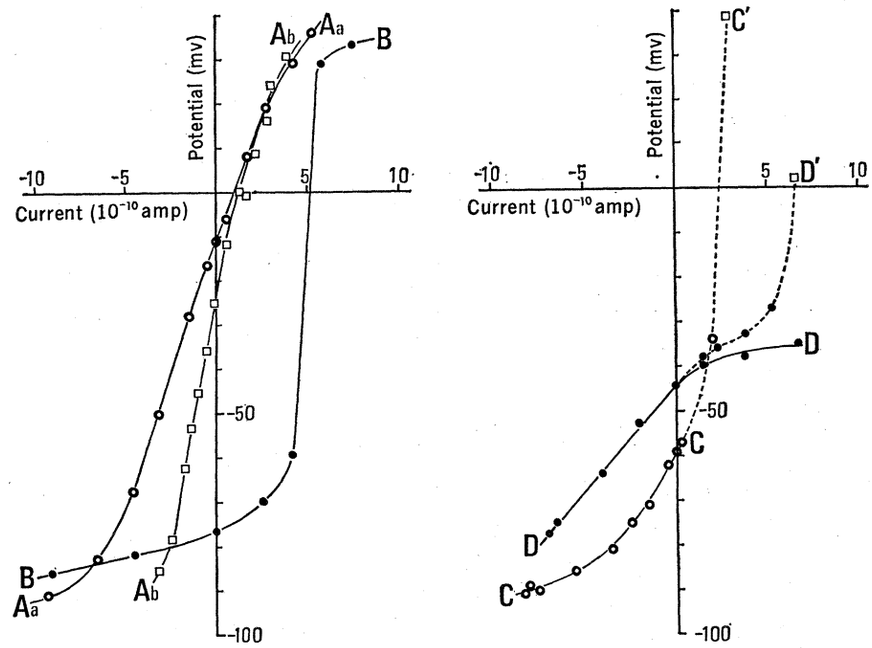


Fig. 3. Relations of stimulus current and response potential for the blastomere membrane in the egg and the muscle cell membrane in the tadpole. (Left side) Curve *Aa* from an egg just before the first cleavage. Curve *Ab* from a blastomere at the 64-cell stage. Curve *B* from a cell in presumptive muscle region (left side of blastopore) at the middle stage of the gastrula. All measurements were made 5 seconds after the onset of the current. (Right side) Curve *C* and *C'* from a muscle cell of the young tadpole with a 90 percent grown tail. Curve *D* and *D'* from a muscle cell of a fully grown tadpole after its hatching. The potential measurement in *C* and *D* (solid) were made at a steady potential after the current step was applied (duration of the current, 250 msec in *C* and 600 msec in *D*). Curve *D* of the depolarized region especially illustrates relations of current and voltage when the regenerative responses were thoroughly inactivated with the current pulse of 600 msec in duration. Curve *C'* and *D'* (dotted) show the relations at the peak of the regenerative responses. The open square symbol indicates the measurement at the peak of spike potential. In all records, currents were injected through the recording electrodes with the aid of the modified bridge circuit.

the range of 50 to 200 megohms with the hyperpolarizing current. It should be noted here that, since all cells at this stage as well as in the gastrula period of stage B were electrotonically coupled, the effective resistance at these stages could not be the same as that of the penetrated cell alone.

At stage B, the curve became S-shaped (Fig. 3, curve *B*). The slope resistance became low for the range of the potential—more negative than the resting and more positive than the zero potential. Between these two regions the slope was very steep. This region of the curve corresponds to the early sign of regenerative depolarization caused by a current pulse as shown in Fig. 2B. In other words, a mechanism producing the voltage-dependent inward movement of certain cations may be induced at this stage. Studies of the ionic mechanisms at this stage are now in progress.

At stage C, the curve for muscle cells was similar to that found at stage B for the range of membrane potentials below the threshold for the action potential (Fig. 3, curve *C*, solid). The curve shows a flection around the value for the resting potential. Curve *C'* (dotted) includes the point representing the peak of the characteristic long-lasting action potential.

Curve *D'* (dotted) in Fig. 3 was, unlike the other curves, obtained by plotting the membrane potential at the initial peak of the local response or the spike potential against the applied current intensity at stage D. The curve *D'-D* (solid plus dotted) looks similar to curves *B* and *C* with respect to the steep rise at a certain region of depolarization. However, curve *D-D* (solid), representing the relation obtained with a current pulse (600 msec long) long enough to inactivate spike generation in muscle cells, that is, the curve at steady state, shows a marked decrease in the resistance with the depolarizing current. Thus, the decrease in spike duration during the transition from stage C to D may indicate the appearance of a mechanism of delayed rectification.

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8. We thank the members of the Laboratory of Physiology, Ocean Research Institute, University of Tokyo, for teaching us embryological techniques; Dr. A. Kanemitsu of the University of Tokyo for preparing Fig. 1; Prof. S. Hagiwara and Dr. A. D. Grinnell for their comments on this manuscript; and the marine biological laboratory at Asamushi of Tohoku University, the Marine Laboratory of Fishery at Onagawa of Tohoku University, and Otaru City Aquarium and Wakkanai City Aquarium in Hokkaido for collecting materials. Supported by a grant from the Japan Educational Ministry to K.T.

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Genetic Restriction of Energy Conservation in Schizophyllum

Abstract. *A genetically determined malfunction of energy conservation reduces assimilation of substrate to half or less its normal level while leaving oxidative processes apparently unchanged. This metabolic defect, which differs in many ways from any previously described, is related to the function of the* B *incompatibility factor and occurs both in the common* A *heterokaryon* (A = B ≠)*—in which two compatible, wild* B *factors interact—and in mutant-*B *homokaryons.*

A malfunction of energy metabolism that differs from any previously known occurs as a normal feature in the tetrapolar basidiomycete *Schizophyllum commune*. Previously reported cases of genetically altered respiratory processes are of two general types. In *petite* mutants of yeast and *mi* or *poky* mutants of *Neurospora*, critical oxidative processes are absent or severely reduced (*1*). Several mutations in yeasts that affect later stages in the respiratory system have been found in a search for mutants that have intact oxidative systems uncoupled from adenosine triphosphate (ATP) formation (*2*). The phenomenon reported here more closely resembles the latter type of defect but differs in several significant ways from any of the cases now known.

Perhaps a closer parallel is found in the high rate of respiration leading to thermogenesis and little generation of ATP in brown adipose tissue of certain mammals (*3*).

The metabolic malfunction in *Schizophyllum* is associated with a specific phenotype that results from either of two distinct genotypes. Sexual development follows from the mating of two homokaryons that are heteroallelic for both of two incompatibility factors, the *A* factor and the *B* factor. A mating between two homokaryons that differ at only one factor results in the operation of only part of the morphogenetic sequence. Matings where the *A* factor is homoallelic and the *B* factors heteroallelic (*A* = *B* ≠) yield heterokaryons with greatly reduced growth and altered hyphal morphology with gnarled, irregularly branched hyphae and frequent protoplasmic extrusions (*4, 5*). This heterokaryon is very closely mimicked for every aspect studied by a homokaryon carrying a primary mutation in the *B* factor. The phenotypic similarities of the two types of mycelia include gross and microscopic morphology, nuclear distribution, sexual interactions, ultrastructure, and enhanced glucanase activity (*6, 7*). Identical developmental controls seem to be released by two compatible interacting *B* factors in the heterokaryon and by the single mutant-*B* factor in the homokaryon (*7–9*).

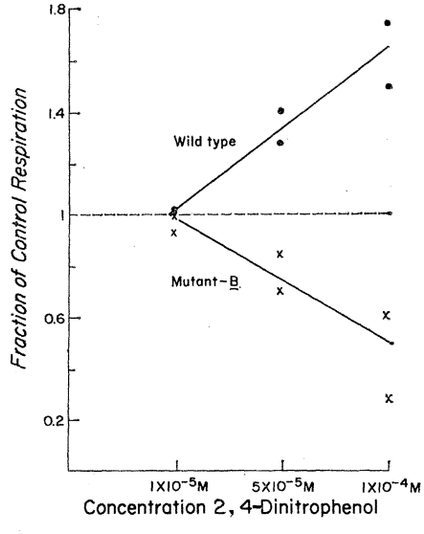

Fig. 1. Effect of DNP on respiration of mutant-*B* and wild-type homokaryons. Strains were grown in liquid for 2 to 3 days and washed twice; respiration was measured manometrically. Each respirometer flask contained 3 ml of mycelial suspension, 0.1*M* phosphate buffer at *p*H 6, an appropriate concentration of DNP in the main chamber, and 10 percent KOH in the central well.

Our study has been made primarily with the mutant-*B* homokaryon—a homogeneous system in contrast to the *A* = *B* ≠ heterokaryon, which contains homokaryotic as well as heterokaryotic elements (*5, 8*).

Studies on the *B* mutant at 30°C in shaking-liquid cultures in minimal medium containing 2 percent glucose have shown values for growth rate, total growth capability, and ratio of dry weight formed to glucose used reduced to half or less compared to the corresponding values for a wild-type strain that is isogenic to the mutant except for the *B* factor. Residual glucose in the medium was measured by an enzymatic assay (Glucostat Special, Worthington). Growth was measured by increase in dry weight, determined by filtering the mycelium on tared filter paper and drying at 105°C for 24 hours. These results parallel the preliminary findings for the *A* = *B* ≠ heterokaryon (*5*). Enriched media such as yeast extract, peptone, and malt extract do not restore the mutant to normal growth.

Respiration of these obligate aerobes (there was no detectable growth after 9 days in a tank flushed with nitrogen and maintained under positive pressure) was measured manometrically (*10*) at 30°C on a dry weight basis. Mycelia were grown in a liquid, minimal, glucose medium for 2 to 3 days; the cultures were incubated on a rotary shaker, and the harvested cells were washed twice by centrifugation. The mycelial pellets were then suspended in the above growth medium, and a portion (3 ml) was pipetted into each respirometer flask. Respiration was measured for 80 minutes. The endogenous rates of the wild-type strain correspond to those found previously (*11*). The mutant *B* showed an accelerated respiration rate (Table 1).

To determine whether, in the mutant, the inefficient use of glucose and the accelerated respiration rate were linked to a more extensive oxidation of glucose, we grew and prepared mycelia in two ways: (i) as above for the determination of respiratory rates and (ii) colonies were washed after 3 to 4 days' growth from cellophane membrane on minimal medium. Measurements were made manometrically (at 30°C) of CO_2 production and O_2 consumption