proteases (*8*); therefore the death of conidia germinating in the absence of inositol can be interpreted as a consequence of cell autolysis caused by the release of protease from incompletely formed protease particles. Autolysis may be complete in the case of germinating conidia because the germ tubes have not yet formed septa. At low concentrations of inositol a mycelium will grow until the exogenous inositol is exhausted. At this moment septa have already been formed, and the beginning autolysis will affect mainly the cells which have been formed last after exhaustion of the inositol. Most probably this event starts in the cells at the tip of the hyphae. The autolysis of *Neurospora* cells results in the liberation of free inositol (*5*) which may support a further limited growth of the surviving part of the hyphae. It seems very likely that this path leads to the formation of small colonies consisting of a manifold branched mycelium (*6*). According to this hypothesis there are always some cells of such a colony undergoing autolysis; in fact electron micrographs obtained from colonies showed cell damage of varying degree (*6*).

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10. Radiochemical Centre, Amersham, England; 50 μg/ml, 1 μc/100 ml of culture medium.
11. New England Nuclear Corp., Boston; 20 μg/ml; 1 μc/100 ml of culture medium.
12. Strain No. 89601, FGSC No. 546.
13. Strain No. 34486, FGSC No. 485.
14. Cultures in liquid minimal medium containing 1 percent of sucrose, grown in an oscillating incubator. Mycelia harvested 16 hours after inoculation. See (*8*).
15. D. J. L. Luck, *J. Cell Biol.* **16**, 483 (1963). The radioactivities of the phospholipid extracts were counted in a Nuclear-Chicago liquid scintillation counter.
16. Homogenization and low-speed centrifugation as described (*8*). Sucrose linear-density gradients (3.8 ml) ranging from 65 to 20 percent sucrose (weight-volume) were built on 0.5 ml of Urografin (methyl-glucamine salt of 3,5 diacetyldiamino-2,4,6-triiodobenzoic acid) with a density of 1.26 g cm^{-3}. Urografin is manufactured by Schering GmbH, Vienna, Austria; 1.0 ml of centrifuged homogenate containing 7 mg of protein were layered on top of the sucrose gradient. Centrifugation was carried out at 39,000 rev/min in a SW-39 swinging bucket rotor of a Spinco ultracentrifuge for 3 hours. After centrifugation the bottom of the tubes were punched, and the outflowing content was divided into 16 fractions.
17. For the identification of the mitochondria see (*15*).

18 November 1965

Uncoupling of a Nerve Cell Membrane Junction by Calcium-Ion Removal

Abstract. *Calcium ion participates in maintaining electrical connections between the nerve cells of Retzius* (Hirudo medicinalis). *The conductance across the junction between these cells decreases with decreasing concentration of free, extracellular* Ca^{++}. *At a certain level of* Ca^{++} *withdrawal from the cell system, junctional conductance reaches a critical low point at which the cells become functionally disconnected: the nerve impulses which are normally discharged in synchrony by the cells become asynchronous. These effects of* Ca^{++} *on junctional connection are irreversible, in contrast to those on nonjunctional surface membrane permeability.*

Close membrane complexes at cell junctions are now known to exist in a wide variety of tissues (*1*). For some tissues these junctional complexes clearly function as pathways for ionic communication between cells (*2*). We have now made an attempt at interrupting this communication by removing Ca^{++} from the tissue, a technique long used by biologists for mechanical separation of cells. The question was whether Ca^{++}, which has a well-known role in cell adhesion, also plays a role in cell-to-cell communication.

In the experimental approach to this question, we were fortunate in that cell communication is much more sensitive to Ca^{++} withdrawal than is cell adhesion. Thus, interruption of ionic communication (hereafter referred to as *uncoupling*) could be achieved with relatively mild procedures. Since Ca^{++} removal is also known to affect permeability of cell surface membranes, it was clear from the outset that uncoupling could only be demonstrated if the processes affecting junctional coupling and surface membrane permeability had different sensitivities or different rates. It turned out that the processes differed in both respects, particularly in their rates of reversal. While the processes pertaining to surface permeability were reversible within minutes, those pertaining to junctional coupling were not reversible at all. The processes could therefore be readily separated; this report deals only with Ca^{++} effects on junctional coupling obtained after reversal of the effects on surface membrane permeability.

We used the nerve "cells of Retzius" of the ganglion of the leech *Hirudo medicinalis*. The junctional coupling between these cells provides a means for synaptic transmission (*3*) of the electrical kind now known to occur in a variety of nerve cells (*4*). The cells were suitable for our purpose because of their large size (60 to 80 μ) and good visibility, and because of the rapid exchange of ions between cells and bathing fluid (*5*).

The ganglion was isolated in physiological saline (*6*); its connective tissue capsule was opened so as to expose the nerve cells and their glia; and three or four microelectrodes were inserted into the two nerve cells arranged to measure attenuation of surface-membrane voltage or junctional resistance. For measurements of voltage attenuation, current was passed between the inside of one cell and the cell exterior, and the resulting resistive voltage drops were recorded simultaneously across the surface membranes of this cell and the adjacent one, as illustrated in Fig. 1 (inset). In an alternate arrangement, all electrodes including the ground electrode were intracellular, and the cell exterior was effectively by-passed as a current path by placing the preparation in isotonic sucrose, a medium of high resistivity. Current flowed then directly from one cell interior to the other, and junctional resistance was directly measurable. In both arrangements, the microelectrodes were left inside the cells throughout all changes of test solutions. In the saline, the cells could be kept up to 3 hours with little change in their electrical properties.

An example of partial uncoupling is illustrated in Fig. 1*A*. The preparation was bathed first in normal saline; then in Ca^{++}-free saline for 17 minutes (*7*); and finally in normal saline again. Figure 1*A* (right) shows the effects

on coupling 21 minutes after return to normal solution, when the surface membrane resistance has fully recovered to normal. What is significant here is that, while the resistive voltage in cell II is smaller than in the control, the resistive voltage in cell I is actually larger (Fig. 1). This may be expected upon uncoupling. As the resistance (r_c) across the cell junction increases, a decreasing fraction of the current reaches cell II, whereas an increasing fraction flows through the outer surface of cell I alone (Fig. 2).

The junctional resistance in this experiment increased from a normal value of approximately 24×10^6 ohms to 70×10^6 ohms. This is nearly the highest degree of uncoupling we were able to obtain by repeated washing of the prep-

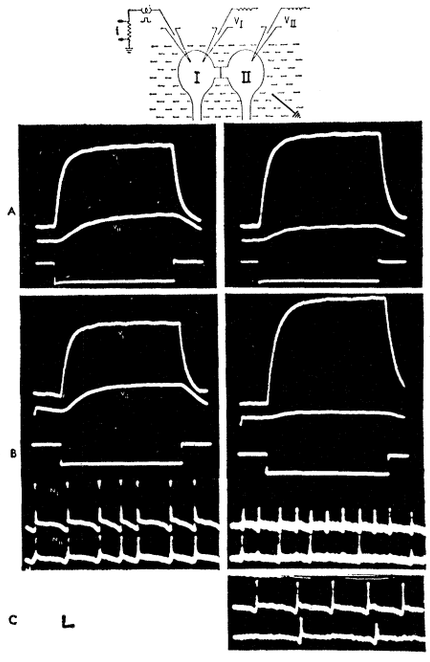

Fig. 1. Uncoupling of a nerve cell junction. Rectangular current pulses (i) of 1×10^{-8} amperes are passed between cell interior and exterior, and the resulting resistive membrane voltages V_I and V_{II} are recorded simultaneously in the two cells. (Left column) V_I, V_{II}, and samples of spontaneous nerve impulses N_I and N_{II} in the normally coupled cells; (right column) after uncoupling of the cells by bathing the preparation A in Ca^{++}-free saline; (B) in saline containing 2.5 mM EDTA; and (C) 3 mM EDTA. All records of the right column were taken after the preparation was bathed in normal saline for a time sufficient for full reversal of the Ca^{++} effects on the nonjunctional surface membranes; thus the records give purely the irreversible effects of Ca^{++}, as they relate to junctional coupling. Calibration: 10 mv for all records; 20 msec for V and i records; 200 msec for N records.

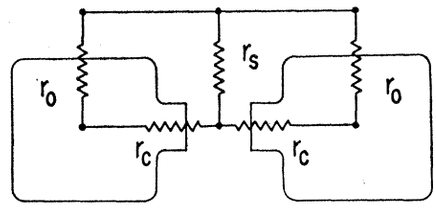

Fig. 2. Electrical analog of cell junction. r_o, Resistance across the nonjunctional cell surface membrane; r_c, across the cell junction; r_s, along the intercellular surface exterior. In the normally coupled junction r_s is high in relation to r_c.

aration in Ca^{++}-free solutions. For more uncoupling, chelating agents had to be used.

Figure 1B illustrates a case in which disodium ethylenediamine tetraacetate (EDTA) was used. Junctional resistance increased from approximately 30×10^6 ohms to 190×10^6 ohms. This is approximately the level of junctional resistance at which interaction of impulse activity is cut off between the cells. Normally the cells produce impulses in synchrony. This is one of the functional adaptations of this type of junction (*4*). When the junctional resistance reaches the level above, synchronization first fails partially (Fig. 1B); and then completely (Fig. 1C).

Magnesium ion seems to substitute for Ca^{++} in maintaining intercellular communication. In a series of experiments in which all the $CaCl_2$ of the bathing solution was replaced by equimolar $MgCl_2$, no uncoupling was found. Subsequent removal of $MgCl_2$ resulted in uncoupling.

It appears from the present results that Ca^{++} is normally required for maintaining electrical connection between these cells. At present we have nothing definite to offer as to the mode of Ca^{++} action. But some light is thrown on this question by the fact that, when uncoupled, the cells seal themselves off and actually increase their total surface resistance.

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6. The solution contained in mmole per liter: NaCl, 113.5; KCl, 4.3; $CaCl_2$, 1.8; maleic acid, 10; glucose, 10; tris buffer, 10; NaOH ~ 10 adjusted to pH 7.4 (from Nicholls and Kuffler, *5*). The Ca^{++} concentration of the test solution referred to in the text as Ca^{++}-free was below 10^{-5} mM, the level of Ca^{++} contamination in the present experiments. In the test solutions containing EDTA, the chelator was added to the Ca^{++}-free solution in the concentrations stated for each case. Molarities and osmolarities were constant within 2 percent, and pH within 3 percent in all solutions.
7. Equilibrium between the actual exterior of the nerve cells and the bathing solution is reached here within 30 seconds as shown for a variety of ions by Nicholls and Kuffler (*5*).

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Uncoupling of an Epithelial Cell Membrane Junction by Calcium-Ion Removal

Abstract. *Calcium takes part in maintaining ion communication between salivary gland cells (*Chironomus thummi*). Its withdrawal from the cell systems results in virtual disconnection of ion communication, at Ca^{++} concentrations which do not noticeably affect cell adhesion. The junctional membrane surfaces, which are normally quite freely permeable to ions, become as impermeable as the nonjunctional membrane surfaces; each cell seals itself off irreversibly as a unit. In maintaining ion communication Mg^{++} substitutes for Ca^{++}.*

This report deals with the action of Ca^{++} on an epithelial cell junction. Among the various epithelial cells which are now known to present intercellular ion communication (*1*), we chose the salivary gland cells of the midge *Chironomus thummi* for the present experiments. These have junctional membrane complexes of the septate type (*2*). The choice was guided by the size of the cells (100 to 200 μ), by their simple arrangement in single chains, and, particularly, by the stability of their cell surface membrane resistance (*3*).