close to the cell body to near its growing tip. The region near the tip was always sensitive, but the response here differed from that of the cell body in that it was very easily desensitized by repeated pulses of ACh. Several minutes were required between pulses in order to restore maximum sensitivity (Fig. 2).

Figure 2A shows a cell with two processes, one of which was sensitive only near its tip, which had the appearance of a typical growth cone. The other process followed a tortuous path past a number of other cells (no electrical coupling was found in this instance), ending near the bottom of the photograph, and was sensitive to ACh along its length. The cell in Fig. 2B had only one process, and this was insensitive to ACh except near its tip. Insensitive regions of membrane were still able to conduct action potentials, as shown in Fig. 2B, where extracellular electrical stimulation initiated action potentials both at the axon tip and along its length.

The growth cone at the tip of a process is where new membrane is laid down. The fact that some cell processes are sensitive to ACh only at their growing tips is interesting because it demonstrates that the sensitivity somehow becomes reduced proximal to the region of membrane growth.

Parasympathetic nerve cells in adult animals (*2*) are sensitive to ACh only at spots on the cell body and not along the axons. Similarly, in adult skeletal muscles, ACh sensitivity is localized to small spots. This localization takes place during the course of development but only after the muscle fiber receives a synapse. Cutting the nerve to an adult muscle causes reversion of the pattern of distribution of ACh sensitivity to the embryonic state, an indication that the restriction of ACh sensitivity to an end plate is dependent on the nerve (*1*). Furthermore, it has been suggested that the change in the distribution of ACh sensitivity which occurs when a synapse is formed is associated with a reduction in the receptivity of the cell to further innervation (*11*).

It has yet to be demonstrated that the physiology of tumor cells propagated in culture is the same as that of nerve cells in an animal. If it is, our results suggest that the pattern of distribution of ACh sensitivity on the surface of developing nerve cells may change before synaptic connections are formed, and thereby play a part in determining which regions of a cell receive synapses. The processes of the tumor cells sensitive and insensitive to ACh could be analogous to dendrites and axons, respectively. The loss of ACh sensitivity by a growing cell process (axon) might be associated with a change in the properties of its membrane which renders it incapable of receiving synaptic connections. On the other hand, processes which retain their chemosensitivity (dendrites) would remain capable of receiving synaptic contacts.

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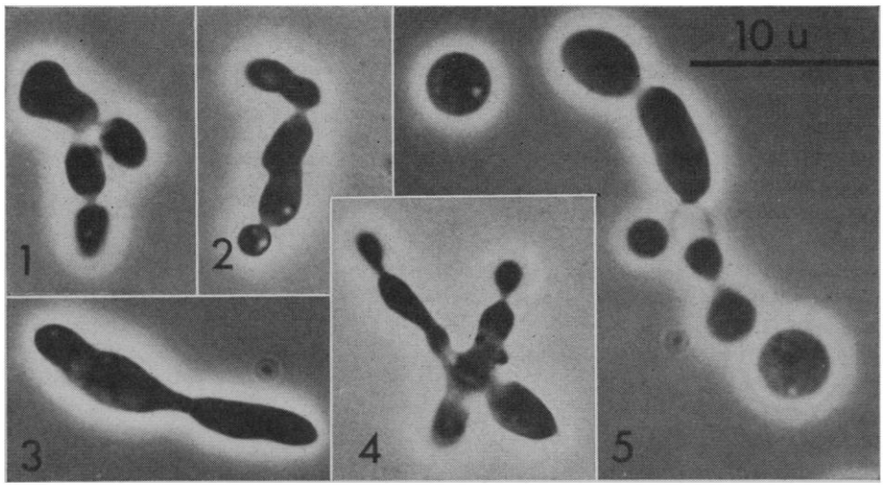
## Release of Protoplasts in the Yeast Phase of Histoplasma capsulatum without Added Enzyme

Abstract. *Cells of the yeast phase of the dimorphic systemic fungus pathogen* Histoplasma capsulatum *readily released large numbers of intact protoplasts without degradation of their cell walls by snail or microbial enzymes, previously regarded as a requirement for all yeast and mycelial fungal forms. Over 90 percent of "B" type yeast cells in the early logarithmic phase of growth released living protoplasts when incubated at 37°C with 2 molar magnesium sulfate, whereas "A" type yeast cells required prior exposure for 24 hours to 2-deoxy-*D*-glucose before incubation in the 2 molar magnesium sulfate.*

*Histoplasma capsulatum* exists as two morphological types (*1*) in the saprophytic multinucleate heterokaryotic mycelial phase: the "A" or albino type and the "B" or brown type. The parasitic or yeast phase uninucleate forms of these two types are indistinguishable morphologically by phase microscopy or staining reactions, and they cannot be differentiated by physiological tests. We succeeded in releasing living protoplasts from both the "A" and "B" mycelial forms of *H. capsulatum* by means of wall degradation by snail gastric enzymes (*2, 3*). However, we were unable to induce significant protoplast formation from the yeast forms of the same organism by this procedure. In order to study indirectly possible differences in cell wall structure and composition, physiology, and cytology of the "A" and "B" type yeast phases, we sought to induce the release of their protoplasts in an osmotically stable and chemically defined medium.

Magnesium sulfate, at concentrations ranging from 0.8 to 1*M*, is the most consistent osmotic stabilizer of yeast protoplasts and is also an inhibitor of bacterial contamination when used in conjunction with enzyme treatment (*3*). For this reason, we tested the effect of $MgSO_4$ alone at molar concentrations of 1.0, 1.5, 2.0, 2.5, and 3.0 on the growth and morphological stability of intact *H. capsulatum* yeast cells. No enzyme or other wall-softening agents were added.

When the G-184B and G-186B yeast cultures in 2*M* $MgSO_4$ broth (*4*) were examined after 48 hours of incubation at 37°C, we noticed that large numbers of protoplasts were being released. After the cultures were incubated for 96 hours, they contained

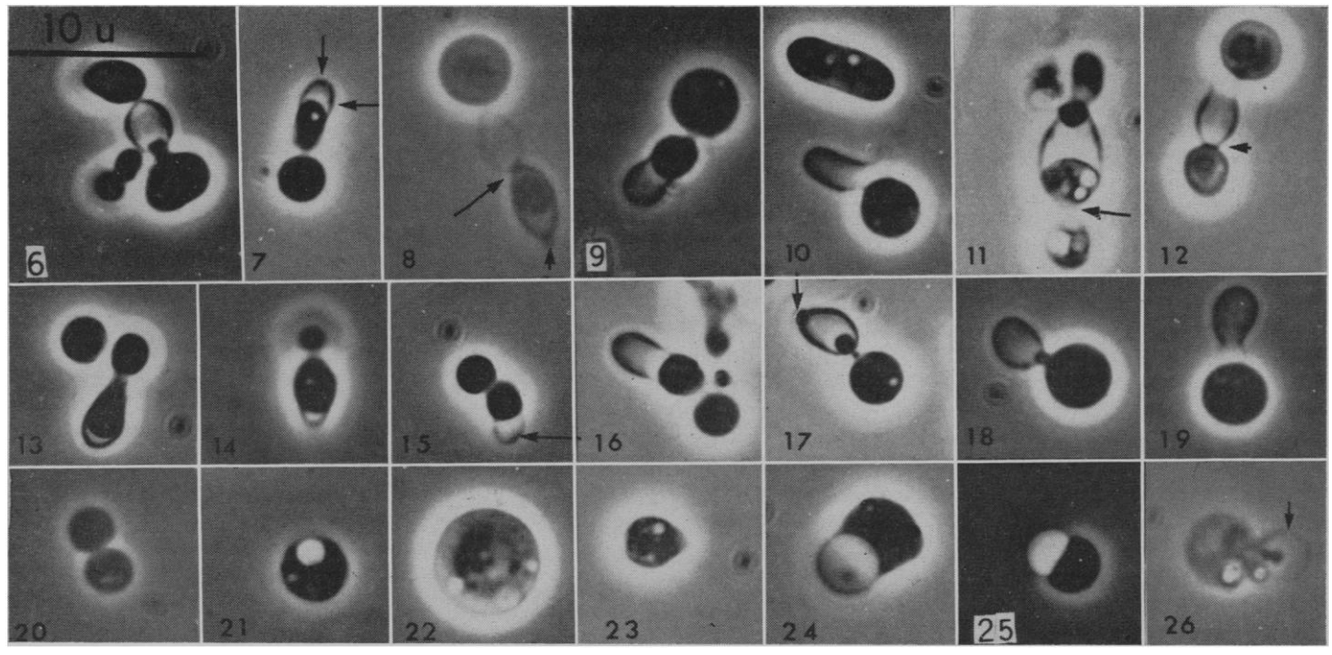



Figs. 1 to 4. Strain G-184A grown in TSB plus 2*M* $MgSO_4$ for 72 hours. Cells have lost their characteristic shape and size. No protoplasts are present. Fig. 5. Strain G-186A grown in TSB plus 2DG plus 2*M* $MgSO_4$ for 48 hours. Protoplasts are beginning to be formed in the youngest, lower terminal cell of a three-cell chain.

virtually pure suspensions of protoplasts (Figs. 5 and 13 to 26). In the G-184A and G-186A cultures, no protoplasts were found at any of the five concentrations of $MgSO_4$. However, swelling of the cells and loss of characteristic shape (Figs. 1 to 4) and phase refractivity indicated that changes in both the structure and composition of the walls had occurred. Eighteen-hour-old (*5*) G-184A and G-186A yeast cells grown in trypticase soy broth (TSB) (*4*) were inoculated into 50 ml of fresh TSB containing 375 mg of 2-deoxy-D-glucose (2DG), the glucan synthesis inhibitor (*6*), and incubated for 24 hours before being transferred to TSB plus 2*M* $MgSO_4$. As a result, large numbers of protoplasts were released from the "A" yeast cells within 8 to 12 hours (Figs. 6 to 12). At concentrations greater than 2.5*M* $MgSO_4$, both the "A" and "B" yeast cells were irreversibly plasmolyzed.

The protoplasts of "A" and "B" yeast cells remained viable in the TSB plus 2*M* $MgSO_4$ for at least 4 days at 25°C and at least 1 week at 4°C (Figs. 22 to 25), as determined by vital staining with 0.1 percent Janus Green B (*7*) in 2*M* $MgSO_4$. No regeneration experiments have been conducted. The protoplasts were lysed in TSB and in distilled water, and a membrane-bound vacuole with attached cytoplasmic debris was often present (Fig. 26). Empty cell walls were rarely seen at 48 to 72 hours and had completely disappeared by 96 hours without leaving any residue. After the protoplasts were left standing for several hours, most were found in the upper 2-mm layer of the medium with virtually no resolvable debris at the bottom of the flasks.

Each yeast cell released one or more protoplasts at only one point on the periphery of what appeared to be a rigid and intact cell wall (Figs. 6 to 19). The action of 2*M* $MgSO_4$, with or without 2DG, thus appears to be limited to a very small, highly susceptible area of the cell wall, namely, the most recently formed bud scar which may be delimited only by a membrane or an incomplete wall (*8*). This is supported by the observation that only young and actively dividing cells release protoplasts and at only one polar




Figs. 6 to 12. Strain G-184A grown in TSB plus 2DG plus 2*M* $MgSO_4$ showing varieties of patterns of protoplast formation. Arrows indicate old bud scars. Figs. 13 to 19. Strain G-186B grown in TSB plus 2*M* $MgSO_4$. Patterns of protoplast release are shown after 48 hours. Arrows indicate old bud scars. Fig 20. Strain G-184B budding or double protoplast. Figs. 21 to 25. Strain G-184B grown in TSB plus 2*M* $MgSO_4$ for 6 days. Large vacuoles and cell enlargement typical of old protoplasts are present. Fig. 26. Strain G-186B protoplast just before complete lysis in distilled water. Large membrane-bound vacuole is indicated by arrow.

locus (Figs. 5 to 19). Further explanation of the mode of action of 2*M* $MgSO_4$ or 2DG, or both, rests on a more definitive knowledge of the chemical composition of the cell walls of intact *H. capsulatum* yeast cells than is presently available.

Since these procedures are believed to be the first for the release of protoplasts at 37°C, they should be of particular value in further studies of yeasts pathogenic for humans. Previous investigators have never reported using osmotic stabilizing solutions above 1.3*M* (*3*), because this concentration appeared to be sufficient to maintain osmotically fragile protoplasts which had been induced by external enzymatic treatment. Whether molarities higher than 1.3 of $MgSO_4$ or other osmotic stabilizers will induce protoplast release in other actively budding yeasts remains to be determined.

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4. Mycelial *H. capsulatum* stock strains G-184A, G-184B, G-186A, and G-186B (*1*) were freshly converted to the yeast phase on brain heart infusion agar plus cystine (BHI) at 37°C in an atmosphere of 5 percent $CO_2$ and subcultured on BHI for 48 hours. The growth from two slants of each strain was removed with trypticase soy broth (TSB) (*5*) and inoculated into flasks containing 50 ml of TSB. After being incubated for 48 hours at 37°C on a reciprocating shaker, 5 ml of the yeast suspension was transferred to fresh TSB and incubated for 18 hours more on the shaker. At 18 hours these cultures are at the beginning of the logarithmic growth phase (*5*), and portions (5 ml) were used as the test samples. The five $MgSO_4$ concentrations were made by adding the appropriate amounts of anhydrous $MgSO_4$ to TSB and then the solutions were sterilized by Seitz filtration. They were each inoculated with 5 ml of 18-hour yeast suspensions, incubated at 37°C on a reciprocating shaker, and observed daily for 5 days under medium phase contrast optics on a Zeiss photomicroscope. Photomicrographs were taken with Adox KB-14 film. The 2-deoxy-D-glucose (2DG) was from Mann Biochemicals.
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## Penicillin as Epileptogenic Agent: Its Effect on an Isolated Neuron

Abstract. *Penicillin induces partial depolarization and increased excitability of the neuronal membrane of crayfish stretch receptor. Such effects suggest that the epileptic focus created by the topical application of penicillin to the mammalian cerebral cortex may result from the lowering of the threshold for impluse initiation by excitatory synaptic action within the neuron population.*

At high concentrations penicillin consistently produces seizures when topically applied to the cerebral cortex and, therefore, has become one of the most widely used epileptogenic agents. The electrocortical activity of the epileptic focus is characterized by a repetitively recurring, isolated, high-voltage negative wave—the interictal "spike" of the electroencephalograph. Simultaneously with the interictal "spike," the transmembrane potential of the neurons within the focus undergoes a typical depolarization of several millivolts which lasts as long as 100 msec. Although these events are quite stereotyped, the mode of action of penicillin as an epileptogenic agent is not clearly understood. The paroxysmal activity in the neurons of an epileptic focus might be produced by a variety of mechanisms. For example, there may be changes in the utilization of the preexisting neuronal circuitry within the population making up the focus, or some radical changes in the mode of impulse initiation in individual neurons might account for the epileptic activity (*1*).

To study the effect of penicillin on the electrical activity of an isolated neuron which is free of the complications introduced by neuronal circuitry, we chose the crayfish muscle stretch receptor. This preparation has been extensively studied physiologically (*2*). Also, the effect of another convulsant agent, strychnine, on this preparation is known (*3*) so that comparison can be made.

The slow-adapting stretch receptor was isolated and held unstretched in a plexiglass chamber containing Van Harreveld's crayfish saline (*4*). The axon, covered with Vaseline to prevent dehydration, was suspended by platinum electrodes for external recording and antidromic stimulation. Glass microelectrodes filled with 3*M* KCl were used to penetrate the soma. In some experiments, microelectrodes filled with potassium citrate were also used. No differences were noted. A bridge circuit permitted simultaneous recording and stimulation. An Ag–AgCl electrode connected to the bath through a KCl agar bridge served as ground. This reduced the large junction potential which developed when the concentrated penicillin solution was introduced into the chamber. Two procedures were used to measure the membrane potential. In those experiments where the penicillin solution was not rinsed away, the potential was calculated with the reading obtained after the withdrawal of the microelectrode from the neuron as zero potential. Where the penicillin solution was replaced with saline, the microelectrode was withdrawn, penicillin was again introduced into the chamber, and the junction potential was measured. The measurements usually coincided when both methods were used in the same preparation. The values reported are corrected for the junction potential. The sodium concentration in the crayfish saline was appropriately reduced to allow for the sodium in the penicillin salt. Both the crayfish saline and the penicillin solutions were oxygenated. To change the solution in the perfusion chamber, a volume of fluid at least 50 times that of the chamber volume (1.6 ml) was allowed to flow.

The effect of penicillin was related to the concentration. Perfusing with a solution containing 20,000 international units (I.U.) per milliliter (0.034*M*) produced (i) a decrease of the membrane potential difference, (ii) an increase of membrane excitability, and (iii) a decrease of activation of the soma by the antidromic spike.

An increase of a few millivolts in the transmembrane potential was observed in some preparations immediately after the start of perfusion (Fig. 1, B and C). This hyperpolarization lasted a few minutes and then, in all cases, disappeared. There followed a slow depolarization which, if the preparation was not rinsed with crayfish saline, brought the membrane to zero potential after 90 to 180 minutes.

The excitability of the membrane, as tested with pulses of constant intensity of outward current through the microelectrode, decreased during the early part of the hyperpolarization (Fig. 1B),