tarity exists between the toxin and the receptor protein comparable to an antigen-antibody reaction, as suggested by Changeux *et al.* (*4*). The tertiary structure of the toxin, which is held together by the disulfide bonds, has to be intact for activity.

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16 June 1971; revised 10 September 1971

Direct Test of the Positive Pressure Gradient Theory of Pseudopod Extension and Retraction in Amoebae

Abstract. *When one pseudopod of an amoeba is sucked into a capillary connected to a partial vacuum and subjected to a pressure reduction of 30 to 35 centimeters of water, extension of other pseudopods, exposed to atmospheric pressure, is not prevented. This result is interpreted to mean that cytoplasmic streaming cannot be the result of a positive pressure gradient generated along the length of the stream, for if it were, streaming would have reversed its direction under the applied pressure gradient of opposite sign and supposedly greater magnitude.*

The mechanisms of pseudopod extension and retraction in large, free-living amoebae have remained obscure and controversial despite much research.

According to the frontal contraction model of pseudopod extension (*1*), each advancing pseudopod tip is the source of a contraction drawing the viscoelastic endoplasm forward, where it becomes everted while contracting and solidifying to form the ectoplasmic tube. The advancing rim of the ectoplasmic tube is thought to consist of cytoplasm that has just contracted. The frontal contraction model received experimental confirmation when a weak photoelastic effect near pseudopod tips was predicted and observed (*2*).

Even if a motive force is applied at pseudopod tips, the bulk of endoplasmic streaming might still be attributed to a pressure gradient, as was postulated in the classical explanations of Pantin (*3*) and of Mast (*4*) and defended recently by Jahn (*5*). In fact, several investigators (*6–8*) have independently suggested that more than one force-producing mechanism could, at the same time or under different circumstances, cooperate to produce amoeboid movement.

We hope that the controversy regarding the possible role of hydrostatic pressure in pseudopod extension and retraction can be resolved in a direct and simple experiment. If a pressure gradient is responsible for flow into and out of pseudopods, then the reversal of that pressure gradient by suction on a pseudopod should cause the flow to be reversed.

Chaos carolinensis, a giant carnivorous amoeba, was cultured in Marshall's medium (*9*) and fed *Paramecium aurelia* every few days. Specimens for study were allowed to fast in fresh medium for 1 or 2 days to render them maximally active.

Amoeba proteus was raised in Prescott-James medium (*10*) and fed *Tetrahymena pyriformis* (*11*). Specimens were also allowed to fast 1 to 2 days before experiments.

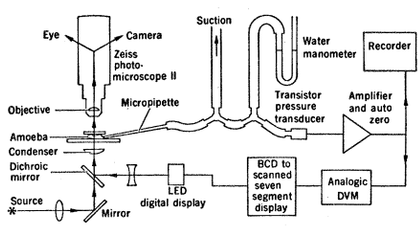


Fig. 1. Block diagram of the experimental arrangement. Abbreviations are: DVM, digital voltmeter; BCD, binary coded decimal output; LED, light-emitting diode. See text and notes for details.

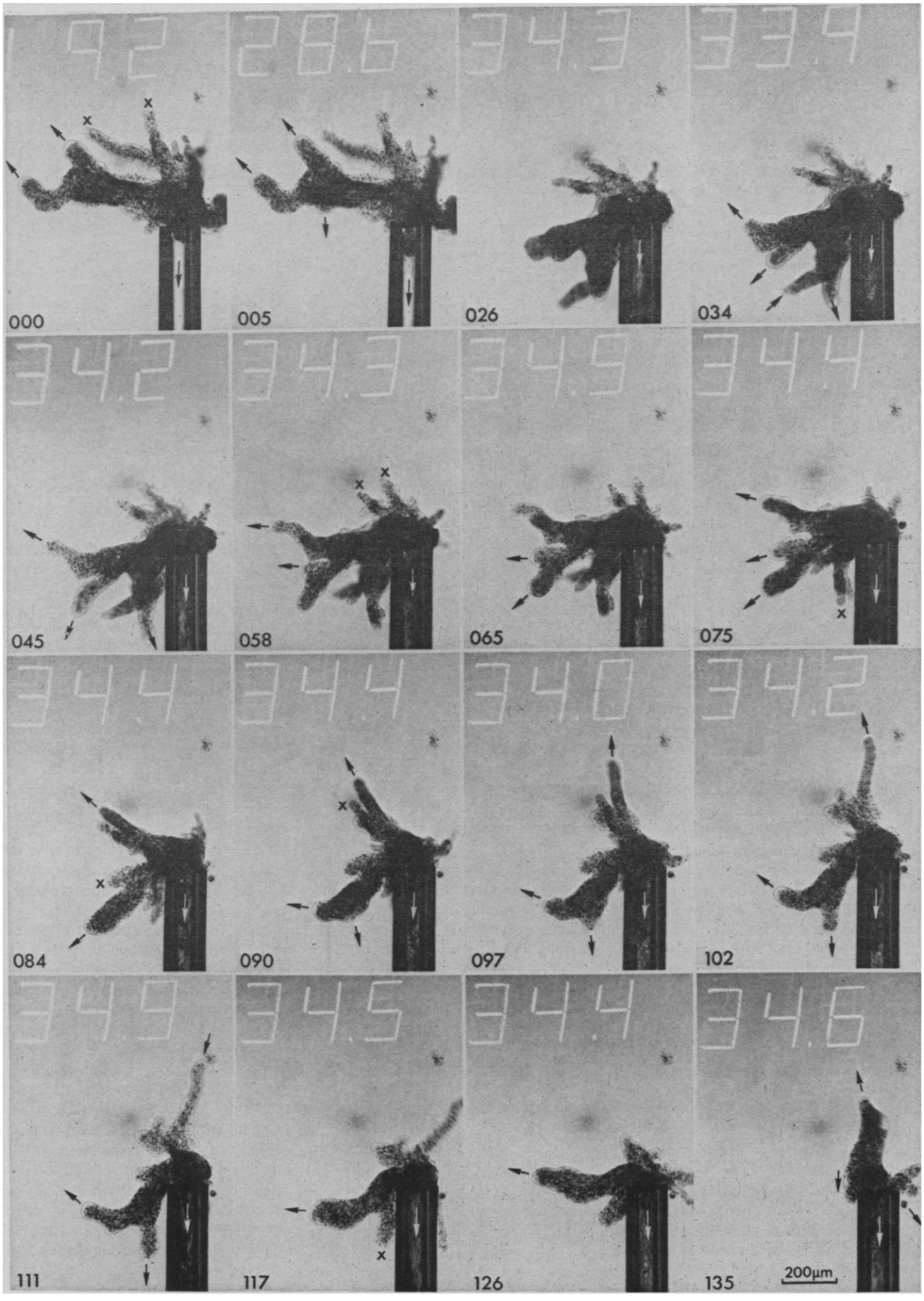
Micropipettes were pulled from Vycor glass of 6.0 mm outer diameter in a Bunsen flame. Final inner diameters of 80 to 120 μm were used for *C. carolinensis* and 30 to 70 μm for *A. proteus*. Micropipettes were connected by Y tubes to a mouth pipette, a pressure transducer, and a water manometer for calibration (Fig. 1).

Amoebae were observed with either a $\times$6, 0.16 numerical aperture (n.a.) objective (for *C. carolinensis*) or a $\times$16, 0.32 n.a. objective (for *A. proteus*) and photographed by a Zeiss photomicroscope camera on Kodak Plus-X 35-mm film.

When suction was applied, the pressure difference was recorded directly in each film exposure by projection of the illuminated digital display into the object and image planes. The digital display itself was a three-digit light-emitting diode (*12*) connected to a circuit which scanned the binary-coded decimal output of the digital voltmeter (*13*). The voltmeter registered the amplified output of the pressure transducer (*14*). When the camera was activated, the pressure difference recorded by the pressure transducer was frozen until the exposure had ended. A chart recorder kept a complete record of suction applied and the times at which the pictures were taken.

Contact of the micropipette with the uroid (tail) of either species of amoeba did not elicit a detectable response. The likelihood of response seemed to increase when the point of contact came closer to advancing pseudopod tips. Contact at a tip either caused streaming to stop or changed the direction of pseudopod extension.

Suction applied to the tail or to retracting pseudopods of either species had little effect on streaming until the pressure exceeded the yield point of the ectoplasmic gel so that cytoplasm flowed easily into the tube. The pressure required to do this varied greatly. Once endoplasm had begun to flow into the tube, the suction required to keep it flowing decreased to 0.1 to 1.0 cm of water. At this point, when small changes in externally applied suction sensitively affected the rate of flow of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200μm

endoplasm inside the capillary, one would expect that a sharp increase in applied pressure difference, for example, 30 to 35 cm of water, would establish a negative pressure gradient in the cell, opposite in polarity to the presumed positive pressure gradient that, according to the tail contraction theory, causes streaming in the amoeba.

Suction applied at the tail did not cause a reversal of cytoplasmic streaming in free pseudopods. The same was true of suction applied to advancing pseudopods. In the latter case the force required to overcome gel resistance was much less, and flow, once started, could be maintained by a negative pressure as small as 1 to 2 cm of water. Thus, when the pressure was increased to 30 to 35 cm of water, it seemed justified to assume that the pressure had been reduced in a region inside the cell.

The experiment in Fig. 2 is typical of over 20 experiments performed on each of these two species. A polypodial specimen of *C. carolinensis* was maneuvered so that the micropipette, held in a micromanipulator, would snugly fit around a slender advancing pseudopod. Suction was applied, and the pressure differences were registered automatically at the top of each frame. The time at which each frame was photographed is indicated at the lower left corner. Arrows indicate the direction of pseudopods that are advancing and retracting. A cross indicates cessation of movement. Throughout the experiment, a pressure difference sufficient to cause rates of flow several times faster than normal did not cause reversal of streaming. In fact, such pressure differences rarely showed any detectable effect on the streaming pattern except in the immediate vicinity of the capillary orifice.

The results obtained with *A. proteus* were identical except for the lower pressures required (half as great) and the shorter time required to draw the smaller volume of cytoplasm down the capillary.

In both genera of amoebae, drawing the endoplasm into a capillary sooner or later ruptured the membrane stretched ahead of it. This did not affect the results, except that some coagulation of cytoplasm mixing with the calcium-containing medium probably increased resistance to flow and thus the pressure required to maintain rapid flow.

It was inconsequential whether the endoplasm was removed by sucking on an existing pseudopod or on a capillary of small diameter inserted into the body of the amoeba.

When suction was released, the cytoplasm in the tube tended to return to the amoeba, where it had little or no effect on streaming elsewhere in the cell. If a large volume of aspirated cytoplasm was returned to the cell by application of positive pressure, cessation of streaming was typical. This was apparently not a sign of injury, however, because streaming resumed again after several seconds.

The application of suction to the endoplasm caused strain birefringence that was visible in plane-polarized light. The rheological behavior of strained endoplasm in polarized light was complicated and will be discussed fully elsewhere. The demonstration of strain birefringence shows that viscoelastic behavior can be expected of amoeba endoplasm.

Application of fluctuating positive-negative pressure gradients caused oscillations in the velocity of flow only in the immediate vicinity of the capillary orifice.

Occasionally it was possible to cause a positive pressure gradient by sucking a pseudopod into a tube slightly smaller in diameter than the pseudopod, thus squeezing the pseudopod. The result was forward flow of endoplasm into other pseudopod, making them spherical instead of cylindrical—an effect observed by Kamiya (*15*) in his double-chamber experiments.

The classical tail contraction or positive pressure gradient theory of amoeboid movement assumes that the motive force is generated by contraction of the tube of granular ectoplasm. According to this theory, the inert endoplasm is forced forward toward weak points in the ectoplasmic tube, which become the tips of advancing pseudopods.

The present results show that suction on any pseudopod (advancing or retracting) does not prevent other pseudopods from extending, even though the applied suction far exceeds published measurements of the motive force (*14*).

These findings are not altogether unexpected. Allen and Roslansky (*16*) found that when a pseudopod was advancing of its own accord into a glass capillary, a pressure difference of 1 to 2 cm of water halted local streaming but had no effect elsewhere in the cell. They noted in passing that flow induced artificially in one pseudopod had no effect on streaming in adjacent pseudopods (*17*). Allen, Colledge, and Hall (*18*) noted that amoeba cytoplasm could stream even in quartz capillaries entirely isolated from the intact cell. They interpreted this result to mean that pressure could not be responsible for streaming. The pressure theory of Pantin (3) and Mast (*4*), then popular, was thus put in doubt.

Using the double-chamber method, Kamiya (*15*) found that the pressure difference required to reverse the streaming in *C. carolinensis* could be as little as 0.05 cm of water and as high as 1.5 cm, with values to 3.0 cm when the amoeba received electrical stimulation.

The forces applied in the present study were ten times higher than this measured motive force. Kamiya also noted that flow into advancing pseudopods under an artificial pressure gradient produced spheroidal rather than cylindrical pseudopods. Using an ingenious triple-chamber method for measuring the motive force (then assumed to be pressure from contracting pseudopods) at two locations, Kamiya found independent variations. These results suggest that motive force is produced independently in different pseudopods.

At the same symposium at which Kamiya reported his double-chamber experiments, an intriguing observation by F. Kanno was reported (*19*):

> A proteus-like amoeba was sucked into a capillary with a bore of about 50–70 μ and allowed to establish vigorous unidirectional movement. Then a smaller capillary with a bore of about 12 μ was thrust about 10 μ into its tail region and cytoplasm sucked out. Movement in a forward direction continued even though over half of the cytoplasm was withdrawn from the cell. How do you think this should be interpreted?

Fig. 2. Sixteen sequential photographs of the response of *Chaos carolinensis* to the removal of cytoplasm by suction applied to one of its pseudopods by a capillary of 93-μm outer diameter. The pressure (in centimeters of water) was registered automatically at the top of each frame. The time (in seconds) at which each frame was exposed is printed in the lower left-hand corner. Arrows mark advancing and retracting pseudopods; those marked with a cross had stopped moving. Scale is on last frame (bright-field microscopy, ×6, 0.16 n.a. objective, Kodak Plus-X film).

Our present results and those of the earlier authors are consistent with the view that streaming does not depend upon an internal hydrostatic pressure gradient. There remains the possibility that local pressure gradients (for example, those caused by frontal contraction) might be present. The fact that pseudopod extension is initiated at the pseudopod tip (*1, 20–22*), as well as the photoelastic cycle at pseudopod tips (*2*), argue that the motive force is applied as tension to the viscoelastic endoplasm at pseudopod tips. Available evidence suggests that the frontal contraction model applies at least to the "*Chaos-Amoeba* group" (*23*). Griffin (*24*) has suggested that anteriorly flattened species of amoebae (such as *Amoeba verrucosa* and *A. striata*) are driven by a similar contraction. The views of Abé (*25*) concerning *A. striata* would be similar if it should turn out that "gel formation" and cytoplasmic contraction are one and the same process at the front ends of amoebae.

As Griffin (*26*) has pointed out, a totally different mechanism of pseudopod extension is evident in the giant herbivorous amoeba, *Pelomyxa palustris*. Here the extremely simple monopodial movement is consistent with a positive pressure gradient model. Many other forms of pseudopods show interesting functional and ultrastructural diversity, which may reflect different mechanisms of pseudopod extension and retraction. The results presented here should not be generalized to all other types of cell movement. They do indicate, however, a reasonable experimental approach to answering the question of whether hydrostatic pressure is, or is not, the motive force for a given biological movement phenomenon involving cytoplasmic streaming.

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14. Model PT-M3, Stow Laboratories, Stow, Mass.
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25. T. H. Abé, *Cytologia* **26**, 378 (1961); *ibid.* **27**, 111 (1962).
26. *Pelomyxa palustris* and a related form, *Pelomyxa villosa*, are giant polynucleate herbivorous amoebae which move monopodially and respond hardly at all to stimuli. They differ dramatically in morphology, physiology, ecology, and manner of movement from the "*Chaos-Amoeba* group." See J. L. Griffin, in *Prmiitive Motile Systems in Cell Biology*, R. D. Allen and N. Kamiya, Eds. (Academic Press, New York, 1964), p. 303.
27. Supported by NIH grants GM 08691-05 and GM 14891-05. This work was initiated at Princeton University. We thank R. Loos for drawing Fig. 1, R. Speck for photographic assistance, and D. Rice for culturing amoebae.
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29 March 1971; revised 5 August 1971

Suppression of Urethan-Induced Lung Adenomas in Mice Treated with Trehalose-6,6-Dimycolate (Cord Factor) and Living Bacillus Calmette Guérin

Abstract. *Trehalose-6,6-dimycolate (cord factor), a glycolipid from mycobacteria, suppressed the development of urethan-induced tumors in the lungs of mice to a similar degree as living* Mycobacterium bovis *(strain BCG) bacilli. The inhibition was apparently due to the host cellular reaction caused locally by cord factor.*

Experimental intravenous infection with bacillus Calmette Guérin (BCG) induces granuloma formation in the lungs, spleen, and liver of mice, increases antibody formation against unrelated antigens, and enhances the activity of the reticuloendothelial system (*1*). Nonspecific resistance to heterologous bacterial infections as well as to grafted tumors of animals sensitized with BCG is apparently related to the above changes (*2*). Relevant to these activities are also the observations that experimental leukemia can be cured in some cases (*3*), that remissions in human leukemia can be prolonged with BCG (*4*), and that, according to the results of a recent statistical study, leukemia mortality is about two times less among BCG-vaccinated than among nonvaccinated subjects (*5*). It has become evident that a chemically defined glycolipid from tubercle bacilli, trehalose-6,6-dimycolate (cord factor), is able to elicit a typical granulomatous response in the lungs of mice after an intravenous injection of amounts as small as 1 to 5 μg. The cellular composition was indistinguishable from that in granulomas formed after an infection with living BCG. Repeated injections of cord factor induced a more extensive cellular response than did one injection of the same total amount of cord factor, a reaction very similar to that which appears after repeated injections of intact tubercle bacilli and which is known as the accelerated tubercle formation (*6, 7*). Macrophages from mice first treated intraperitoneally with cord factor and another mycobacterial fraction (wax D) showed increased acid phosphatase activity. Such animals were resistant to infection with heterologous bacteria and Ehrlich ascites cells (unpublished data).

Cord factor (5 to 10 μg) when injected into the foot pads of mice, induced histological changes similar to changes that followed injections of living BCG. Both materials induced, in the draining lymph nodes, hyperplasia of the lymphoid tissue in the paracortical zone, accumulation of macrophages, and formation of granulomas composed of macrophages, epitheloid cells, and lymphocytes (*8*). Because no