Dynamic Organization of ATP and Birefringent Fibrils during Free Locomotion and Galvanotaxis in the Plasmodium of *Physarum polycephalum*

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Abstract. Directed migration by a cell is a good phenomenon for studying intracellular coordination. Dynamic organization of both ATP and birefringent fibrils throughout the cell was studied in the multinuclear ameboid cell of the *Physarum* plasmodium during free locomotion and galvanotaxis. In a directionally migrating plasmodium, waves of ATP as well as thickness oscillations propagated from just inside the advancing front to the rear, and ATP concentration was high at the front on the average. In a DC electric field, locomotion was inhibited more strongly, ATP concentration decreased more, and birefringent fibrils were formed more abundantly at the anodal than at the cathodal side. Inside the cell there were a few undulations in the distributions of ATP and birefringent fibrils. In short, birefringent fibrils become abundant where ATP concentration decreases. The possible mechanism of the coordination in the directed migration and the implications of the scaling law are discussed.

THE cytoplasm in a cell is integrated or coordinated as a whole, and yet the principles are unknown. Directed migration by the cell is a series of metabolic processes that take place in a coherent manner throughout the cell, because this requires that one end of an ameba extends while the other retracts. The plasmodium of the true slime mold *Physarum polycephalum* is a useful organism for studying the molecular mechanisms of the intracellular coordination in an ameboid cell. Its large aggregate of protoplasm extends like a sheet at the front, and toward the rear protoplasmic veins develop in which the protoplasmic solution moves to and fro very regularly (8, 9). Cytoskeletons are observed as birefringent fibrils which appear and disappear rhythmically (15), and are found to be composed of actin/myosin together with modulating proteins (3–6, 17). The thickness of the protoplasm varies from one place to another; according to thickness phase waves propagate away or toward local stimulants when the organism is exposed to attractants and repellents, respectively (11). Accompanying the rhythmic contraction, concentrations of intracellular chemical components such as Ca^{2+} (10, 18, 26), ATP (27), NADH (13), and cyclic nucleotides (23) oscillate. Furthermore, spatial distributions of intracellular ATP concentration are correlated to various cell shapes and hence cell behavior (24).

With all these findings we may hypothesize that underlying the cellular coordination there are oscillatory chemical reactions which self-organize various spatial patterns in a time-dependent manner, and that the cell behaves according to the chemical pattern. Here we test this hypothesis. Cell polarity is induced either spontaneously or by applying a DC electric field (1, 25). The galvanotaxis has recently gained general interest because morphogenetic movements may be guided by an electric field (19). We raise the following questions: (*a*) Do chemical waves associate with the propagation of thickness oscillations?; and (*b*) How is a chemical pattern related with the organization of cytoskeleton? We show that ATP waves propagate in the same manner as those of thickness oscillation, and that birefringent fibrils become abundant and sparse corresponding to the variation of ATP concentration. The theoretical background for interpreting our results and the implications of the scaling law are discussed in relation to the cellular coordination in the directed migration.

Materials and Methods

Organism

The plasmodium of *Physarum polycephalum* (HU195 × HU200) was fed with oatflakes on a 1% agar gel. The organisms were allowed to move on 1% plain agar overnight in 22 × 33 cm^2 troughs, giving a large migrating plasmodium.

Measurement of Stationary Locomotion Velocity in a DC Electric Field

The frontal part of the large plasmodium as above was cut into several pieces, and a few pieces were placed in the middle of a narrow trough (2 × 20 cm^2). A few hours later the plasmodium extended in both directions. This bipolar plasmodium was useful for studying asymmetic effects of a DC electric field. When a unidirectionally extending plasmodium was needed, one side of the agar was blocked with a hydrophobic polymer sheet (Saran Wrap; Asahikasei Co., Tokyo, Japan) thus allowing the plasmodium to migrate in one direction. A DC current was supplied through a pair of Ag/

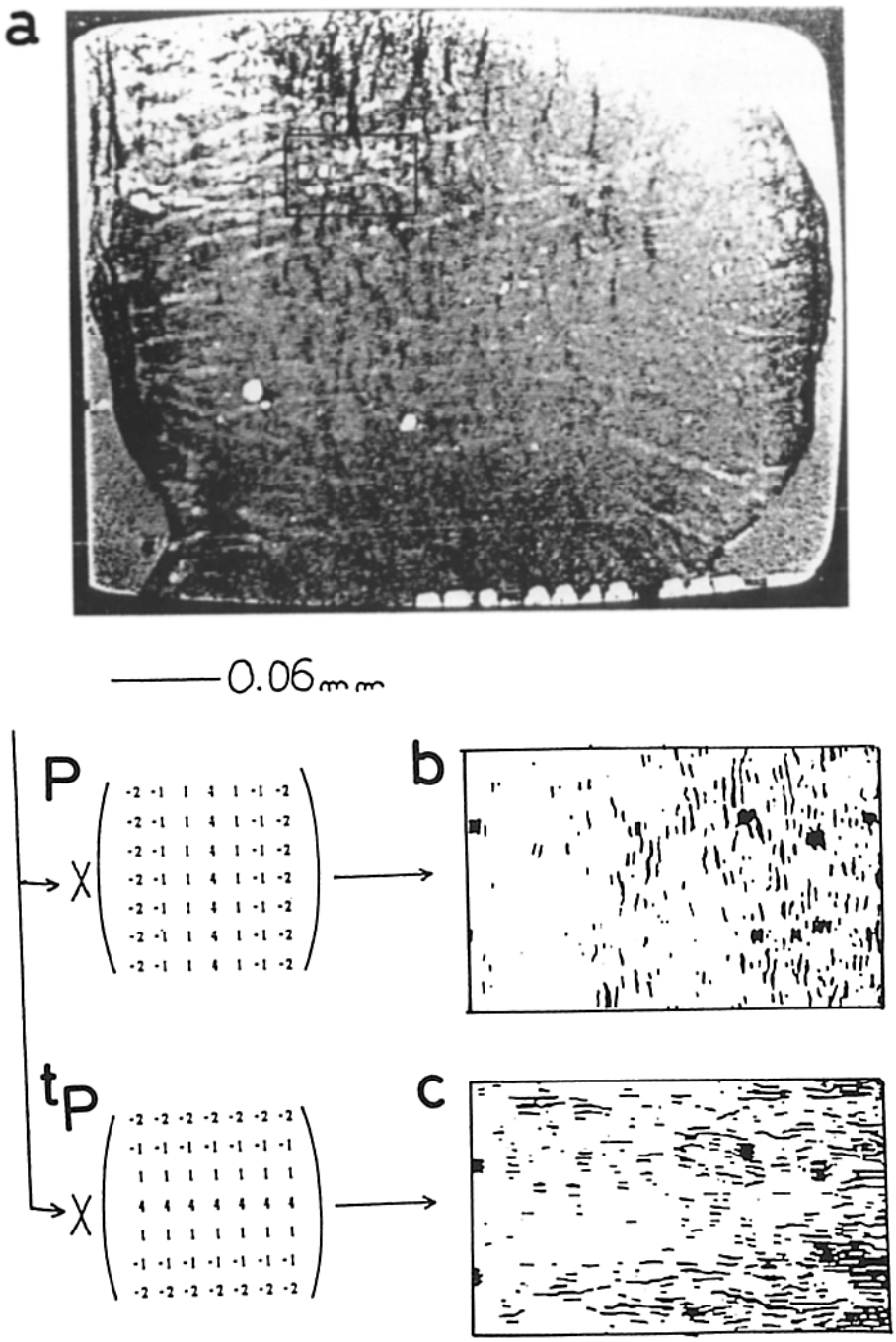

Figure 1. Image processing for extracting a fibrous structure. (*a*) A micrograph taken under a polarizing microscope. (*b* and *c*) Images processed by operating weighing matrices P and ^tP, respectively, to a small region squared in *a*. The matrix elements are shown in the figure.

AgCl electrodes placed at both ends of the trough. The position of the leading front advanced linearly with time, and the stationary locomotion velocity was determined as a function of the applied current intensity. The experiments were repeated five times at a given DC field, and results were averaged.

Measurement of Thickness Oscillations in a Directionally Migrating Plasmodium

By observing the organism through a transmitted light, the brightness level $I(x, t)$ at position x and at time t reflected the thickness of the protoplasm there, and was monitored by microcomputer image processing (11). The brightness level oscillated, and the phases of the oscillations differed from position to position. But when the organism was migrating directionally as above, the phases of the oscillations were laterally synchronous, and so the phase relationship from one end to the other in the plasmodium was studied as a one-dimensional case.

Propagation of the thickness oscillations before and after an application of a DC electric field was determined as follows. The time courses of the brightness level $I(x, t)$ at various positions along the plasmodium are shown in Fig. 4. With the approximation that a propagating wave does not change its form during propagation, the oscillation at point x and time t with a frequency w is given by $I(x, t) = I(\mathbf{k} \times x - wt)$, where $\mathbf{k}$ is the phase gradient vector indicating the wave propagation. The phase gradient vector $\mathbf{k}$ is then calculated as $\mathbf{k}/w = -\text{grad}(I)/(\text{d}I/\text{d}t)$. Based on this notion, we evaluated the $\mathbf{k}$ vector graphically. Curves were drawn that connected the equal phases of the oscillations in the neighboring points (Fig. 4, *dotted lines*), and spatial gradients of these curves were determined at various positions. The experiments were repeated at least 10 times.

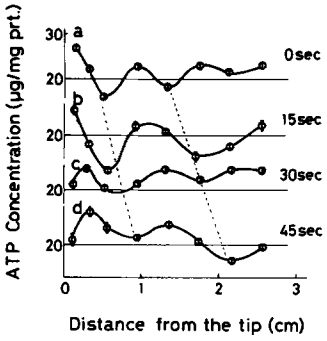

Figure 2. Propagation of ATP waves in a unidirectionally migrating plasmodium. (*a–d*) A series of one-dimensional distributions of ATP concentration at 15-s intervals. Dotted lines connect minima in the distributions. Bars indicate SD for ATP (n = 3) and protein (n = 2) assays.

Measurement of Intracellular ATP Distribution

The organism was allowed to extend uni- or bidirectionally either on wet filter paper or on a plain agar in a narrow space as above. For measuring the two-dimensional distribution of ATP concentration, the whole plasmodium was frozen in liquid N_2, and cut into ~40 pieces. Each piece was put into a hot buffer (95°C, 10 mM Tris-HCl, pH 7.1) for 10 min to extract ATP. Amounts of ATP and the proteins were determined by the luciferin-luciferase and the Lowry methods, respectively (24). The ATP concentration is defined as the amount of ATP divided by the amount of protein.

Distributions of ATP concentration in the plasmodium were laterally uniform (see Fig. 5). This lateral synchrony was used to demonstrate chemical waves in the plasmodium by measuring time courses of the one-dimensional distribution of ATP. The organism was cut at appropriate time intervals into strips together with filter paper (~5 mm wide and 5 cm long) and each strip was frozen immediately in liquid N_2. The strip was then cut into ~10 pieces, and each piece was subjected to ATP and protein assays as above. The ATP and protein assays were repeated three times and duplicately, respectively, for each sample, and the experiments were repeated four times independently.

Analysis of Spatio-Temporal Distribution of Birefringent Fibrils with Use of Microcomputer Image Processing

Tiny plasmodia ~1 mm wide were allowed to migrate overnight in a narrow gap between two sheets of agar gel (3). The birefringent fibrils in this thin-spread plasmodium were observed with a polarizing microscope (Optiphoto; Nihon Kogahu Co., Tokyo, Japan), appearing dark where longitudinal and bright where lateral as seen in Fig. 1 *a*. The video images were averaged to decrease the noise level (Image Sigma II; Nippon Avionics Co., Tokyo, Japan) and taken to a microcomputer as 256 × 256 pixels with 256 brightness levels through a video digitizer (ALT 256-8-40MA; Altec System Co., Osaka, Japan). The fibrous structures $A(j, j)$ were extracted by operating a weighing matrix P or its transposed matrix ^tP to a pixel of the original frame $G(i, j)$ at a point (i, j):

$$A(i, j) = \sum_k \sum_l G(i + k, j + l) * P(k, l)$$

where P was chosen as a 7 × 7 matrix whose elements are shown in Fig. 1. The above operation gives lateral or horizontal fibers (Fig. 1, *b* and *c*). The amount of birefringent fibers in a given area was obtained by adding the brightness of each pixel in these processed images. The image was divided into 16 × 16 parts, and the density of birefringent fibers in each part was measured at 5-s intervals (see Fig. 7). The spatial density distribution of birefringent fibers was obtained by adding the birefringence in each part for 2 min (a period of the oscillations), and depicted as a smooth line

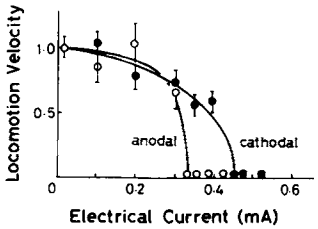

Figure 3. Inhibition of the locomotion by the plasmodium with electric current. Open and closed circles indicate cases where the advancing fronts are at anodal and cathodal sides, respectively. Bars indicate SD (n = 5).

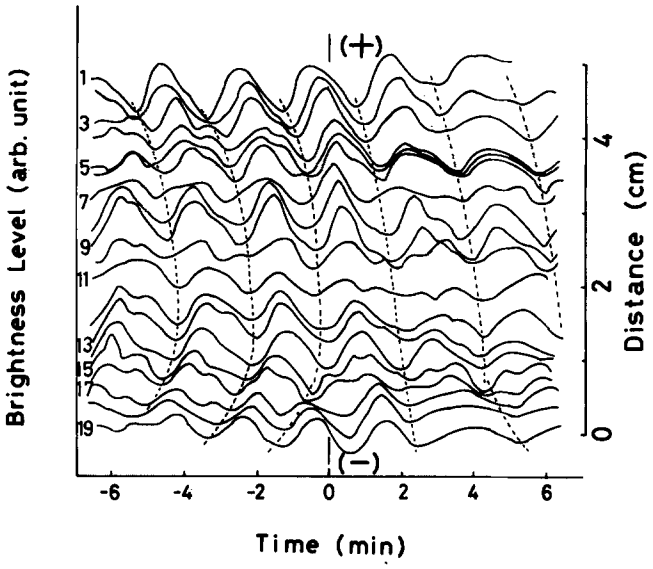

Figure 4. Propagation of the thickness oscillations in a bipolar plasmodium before and after the stimulation with electric current. Brightness levels are traced with time at positions (numbered from 1 to 19 in the figure) which are located equidistantly from one end to the other end of a bipolar plasmodium ~5 cm long. At time zero a DC field (0.4 mA) is applied. Signs, + and −, indicate polarity of the field. Dotted lines connect minima of the oscillations at neighboring positions, which are then regarded as time–distance relationship (see the ordinate on the right-hand side). The slopes in these curves are proportional to the k vectors. Before the stimulation, near the middle of the plasmodium, the phases of the oscillations are almost the same; i. e., waves do not propagate (k is almost zero). On the other hand, near both ends waves propagate toward inside. Soon after the stimulation, waves propagate from anodal to cathodal side.

by using a Spline function (see Fig. 8). Weak DC currents (Power Supply KS7510; Marysol Sangyo Co., Tokyo, Japan) were passed through Ag/AgCl electrodes placed at both ends of a thin agar gel sufficiently apart (3 cm) from the cell to avoid effects of electrode deposits. The experiments were repeated more than five times at a 0.4-mA current, where the strongest asymmetric effects were observed.

All experiments were performed at 23 ± 1°C. All results reported are typical excepting locomotion experiments.

Results

Propagation of ATP Waves in a Directionally Migrating Plasmodium

A series of one-dimensional distributions of ATP at 15-s intervals is shown in Fig. 2, where the organism is migrating unidirectionally leftward in the figure. ATP concentration is high on the average at the frontal region, and undulates over the whole plasmodium. These undulations in the ATP distribution propagate from just inside of the front toward the middle, as shown by dotted lines in the figure. The propagation velocity reaches ~1 cm/min.

Asymmetric Inhibition of the Locomotion in the Plasmodium by the DC Electric Field

Electric current inhibits the locomotion of the *Physarum* plasmodium at both anodal and cathodal sides (Fig. 3). As the electric current increases, the locomotion velocity (v)

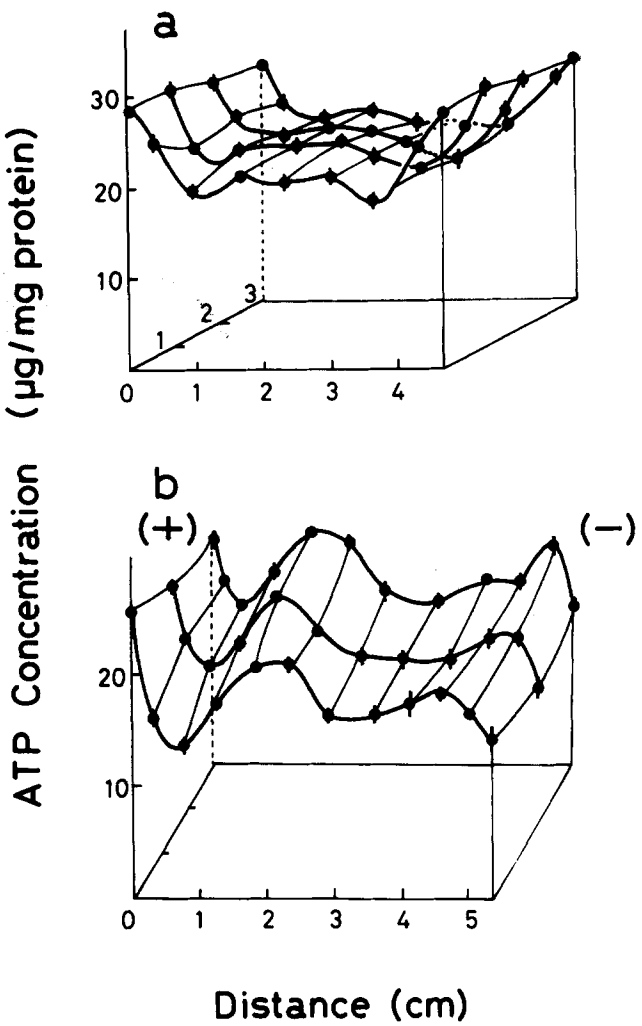

Figure 5. Typical two-dimensional distribution of ATP concentration in a bipolar plasmodium. (*a*) A plasmodium extending evenly to two directions. (*b*) A plasmodium 1 h after the application of a DC electric current (0.4 mA), showing galvanotaxis toward a cathode (−). Bars indicate SD as in Fig. 2.

decreases at first gradually and then steeply near the critical current I_{crt}, above which the locomotion is suppressed completely. An empirical equation for the inhibition looks like critical phenomena:

$$v/v_o = (1 - I/I_{crt})^a$$

where v_o is the locomotion velocity at $I = 0$, and the value of the exponent a is found to be 0.21. The value of I_{crt} at the cathodal side is ~1.4 times larger than that at the anodal. Thus, a current can be selected such that the anodal inhibition is much stronger than the cathodal (ref. 1). In the following, the electric current that yielded the strongest asymmetric effect was applied.

Propagation of Thickness Oscillation in the Plasmodium before and after Applying a DC Electric Field

The thickness (brightness level) of the plasmodium changes periodically at a given point, and variations at 19 positions from end to end in a bipolar plasmodium are shown in Fig. 4. Before the stimulation (time zero), the oscillation propagates from two ends toward the inside, the propagation velocity being higher near the ends (~1 cm/min). This feature coincides with that of the ATP wave (Fig. 2). After the stimulation, the thickness decreases at the anodal side, and the oscillation weakens and prolongs at the cathodal side. Overall, the waves propagate unidirectionally from anodal to cathodal side.

Changing Patterns of ATP Distribution with a DC Electric Current

Changes in two-dimensional distribution of ATP before and after an application of a DC electric field to bipolar plasmodia are shown in Fig. 5. In the control (*a*) ATP distribution is symmetric longitudinally, being high at both ends and undulating inside. After a long exposure to the DC field (*b*), ATP distribution turns into asymmetric; ATP becomes high near the anodal end, while ATP lowers near the cathodal.

Transients of ATP distribution are shown in Fig. 6. Shortly after the application of a DC field, ATP decreases more in the anodal side than in the cathodal, resulting in an overall gradient of ATP concentration with a few humps inside (*b*). As the time passes, ATP concentrations at the cathodal end and just inside the anodal decrease, and two large and broad peaks appear inside (*c* and *d*), leading to the asymmetric ATP distributions (Fig. 5).

Changes in the Distribution of Birefringent Fibrils with a DC Electric Current

When the cell does not show polarity, the birefringent fibrils appear and disappear periodically and synchronously throughout the cell (Fig. 7) (3). The DC field causes the amount of the fibrils at the anodal side to increase, while the oscillation of the fibrils becomes weak at the cathodal.

Changing patterns in the distribution of the fibrils after applying a DC field are shown in Fig. 8. At first, birefringent fibrils increase both just inside the anodal front and at the extreme end of the cathodal (*b*). At a later stage, birefringent fibrils increase at two particular positions inside, and yet there is on the average a gradient in the amount of birefringent fibrils, being more abundant toward the anodal (*c* and *d*).

Discussion

Our present and previous results indicate that ATP concen-

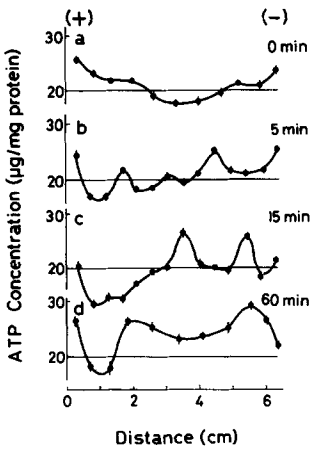

Figure 6. Typical changing patterns of ATP distribution with a DC electric field. Symmetric ATP distribution (*a*) in a bipolar plasmodium turns into asymmetric with a DC electric field (*b–d*). Bars indicate SD as in Fig. 2.

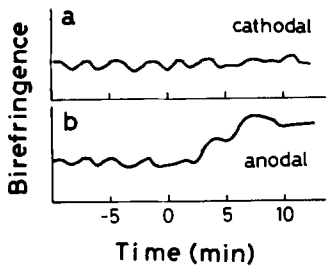

Figure 7. Typical time courses of birefringence before and after application of a DC electric field. Oscillations are synchronous before stimulation (time zero) with a 0.4-mA current.

tration in the plasmodium oscillates, propagates as waves, and distributes nonuniformly in such a manner that the different distribution patterns correspond to different cell behaviors. General features of these phenomena are explained theoretically in terms of the self-organization in chemical systems far away from equilibrium (16). Here the nonlinear chemical kinetics coupled with diffusion, for example, self-organize chemical patterns that depend on kinetic parameters and boundary conditions, and hence the size of the system. But qualitative features may not depend on the particular system, because the kinetic equations with different parameters can often be reduced to the same equations by scaling. This scaling law may hold well in the plasmodium, because ATP distributions are similar in small (~2-cm) and large (~40-cm) plasmodia (24).

On the assumption that scaling is valid in the plasmodium, we can compare changes in ATP distribution (Fig. 6) with those in birefringent fibrils (Fig. 8). The ATP concentration decreases most just inside the anodal end where the birefringent fibrils increase most. Both ATP and birefringent fibrils distribute with gradients throughout the plasmodium with a few undulations, although detailed distributions of undulations are somewhat different between the two. So we may conclude that on the first approximation the more ATP decreases, the more abundantly birefringent fibrils are formed. Does ATP regulate the cytoskeletal organization directly? Some evidence supports this: ATP causes contraction in a reconstituted actomyosin thread (12), and high ATP dissolves actin.

Our results also provide a new look at the mechanism of galvanotaxis in ameboid cells. Previous studies seem chiefly to try to identify the location of active sites in the cell: either the preferential migration toward the cathode in the *Physarum* plasmodium is explained by the selective suppression of the pseudopodial extension at the anodal side (1); or the protoplasm moves toward the cathodal because the protoplasm at the anodal side contracts and pushes the protoplasmic sol toward the cathodal side (7, 22). Our results are consistent with these theories based on local mechanism but, more importantly, showed the protoplasm react as a whole to the electric field and self-organize new distributions of intracellular ATP and cytoskeletons (Figs. 5, 6, and 8).

Furthermore, our results and theory described above give a mechanism of why the cell is able to behave coordinately as an integrated unity. If the cytoskeletons are controlled by chemical patterns such as ATP, cellular coordination is automatically satisfied because the chemical patterns themselves are formed by taking into consideration the kinetic parameters and boundary conditions. An alternative approach for cellular coordination in directed migration is trying to correlate the particular arrangements of organelles such as a microtubule-organizing center with cell polarity (21, 28). This approach seems awkward in multinucleated cells such as the *Physarum* plasmodium, and also fails to answer what brings about a polar arrangement of organelles in terms of physical chemistry. Our theory answers why the cell polarity originates in terms of physical chemistry if the scaling law is applicable.

The next step of the research will be to make clear what enzymatic processes are responsible for the organization of chemical patterns in the *Physarum* plasmodium. Until now mitochondria have been shown to be involved in keeping the oscillatory contraction (20). Glycolytic system (14) and mitochondria (2) should be two potent candidates, because oscillations occur autonomously, chemical patterns are formed, and waves propagate in these systems. It remains unclear how these phenomena in cell-free systems are related to cellular functions.

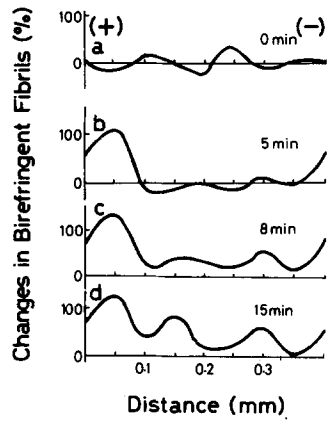

Figure 8. Typical changing patterns of birefringent fibrils with a DC electric field. The fibrils become abundant toward the anodal side, accompanying undulations. Data points are connected smoothly with a Spline function.

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