Flagellum Growth and Regeneration in the True Slime Mould¹ *Didymium nigripes*

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SUMMARY

The kinetics of flagellum elongation during flagellum morphogenesis were investigated in the slime mould, *Didymium nigripes*. Growth rate varied at different temperatures. Long and short flagella elongated at independent rates and exhibited their maximum rates of elongation at different times. Limitation on final length did not seem to be a function of the precursors available for flagellum synthesis. Growth kinetics of normally growing and regenerating flagella and of amoebae previously exposed to cycloheximide suggest that control of growth rate is not merely a function of flagellum length or the diffusion of precursors to the assembly site.

The effect of cycloheximide and streptomycin on flagellum growth were compared in both morphogenetic and regenerating systems. Addition of cycloheximide to a growing or regenerating system immediately halted flagellum elongation. Amoebae exposed to cycloheximide, washed free of the drug and then incubated under standard conditions showed abnormal growth kinetics and reduced final flagellum length; these observations suggest some residual effect of this drug. Streptomycin delayed the morphogenetic process but did not affect flagellum length once morphogenesis occurred.

INTRODUCTION

Myxamoebae of the slime mould, *Didymium nigripes*, differentiate into biflagellated cells when they are suspended in liquid medium. Synchronous flagellum production in a population of amoebae is obtained if amoebae are washed free of their bacterial associate and suspended in 0.05 M phosphate buffer, pH 6.5. Under these conditions flagella begin to appear 35 to 40 min after the washing procedure has been completed. The current investigation was undertaken to clarify the details of flagellum growth and to compare the effects of cycloheximide and streptomycin on this growth.

METHODS

Growth and incubation of amoebae. Amoebae were grown and incubated as described previously (Kerr, 1972).

Flagellar amputation. Samples of flagellated amoebae to be amputated were removed from the suspension of control cells immediately before amputation. Flagella were amputated by placing a suspension of amoebae (2 to 2.5 ml) in a test-tube and agitating on a Vortex Junior mixer for 1 min, allowing to stand for 2 min and then agitating for another minute in order to achieve uniform shearing of all flagella. Under these conditions all flagella broke off 2 to 3 μ m from their point of insertion on the amoebae.

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Fig. 1. Effect of incubation temperature on flagellum elongation. Long flagellum incubated at: 23 °C, ●; 22 °C, ▲; 21 °C, ○; 18 °C, △. Short flagellum incubated at 23 °C, ■.

Table 1. Mean rate of flagellum elongation ($\mu\text{m}/\text{min}$)

Temperature	Incubation time (min)					
	45-60	60-75	75-105	105-135	135-180	180-205
Long flagellum						
18 °C	0.10	0.067	0.05	0.03	0.03	0.01
20 °C	0.12	0.16	0.05	0.06	0.06	0.00
21 °C	0.153	0.09	0.062	0.047	0.036	0.013
22 °C	0.18	0.08	0.08	0.01	0.00	0.00
23 °C	0.24	0.10	0.09	0.003	0.00	0.00
Short flagellum						
23 °C	0.04	0.053	0.073	0.01	0.00	0.00

Inhibitor studies. Inhibitors used in the experiments were cycloheximide (5 to 10 $\mu\text{g}/\text{ml}$; Sigma Chemical Company, St Louis, Missouri, U.S.A.) and streptomycin sulphate (250 to 500 $\mu\text{g}/\text{ml}$; Squibb, New York, New York, U.S.A.).

Measurement of flagellum length. Permanent slides were prepared by placing a drop containing amoebae on a slide, fixing for 5 min in Ponselle's fixative (5 parts methyl alcohol to 1 part tincture of iodine), rinsing with methyl alcohol, air drying and staining for 20 min with Giemsa's stain. Flagella were measured in the following manner: 50 flagella from each sample were drawn with the aid of the drawing-tube attachment for a Wild microscope, and the length of the lines was measured with a map mileage tracing wheel.

Fig. 2. Effect of delayed addition of cycloheximide on flagellum length in morphogenetic amoebae. Flagellum growth in untreated amoebae, ●. Flagellum growth when cycloheximide addition delayed: 15 min, ▲; 30 min, ■; 45 min, ▼; 60 min, ○; 75 min, △; 90 min, □.

The mean length of flagella from each sample was calculated. Long and short flagella were distinguished by their point of attachment and direction of protrusion from the amoebae.

RESULTS

Normal growth characteristics

Under the conditions employed, flagellum morphogenesis and elongation varied only slightly among amoebae of the same population or among separate populations. Flagella began to appear in about 10% of the amoebae after 30 min of incubation at 21 °C, were visible on 60% of the amoebae after 45 min and were present on all non-dividing amoebae after 55 to 60 min. After 2¼ to 3½ h of incubation the mean length of the long flagella was 15 μm and that of the short flagella was 7 μm. Individual amoebae exhibited maximum flagellum lengths of 20 μm and 8 μm for the long and short flagella respectively. Mean flagellar elongation was most rapid in the first hour of incubation and then decreased as the final flagellum length was approached (Table 1). After 2 h of incubation growth of both flagella was slight. Long and short flagella elongated at independent rates with fastest elongation of the short flagellum occurring when the long flagellum had almost reached its final length.

Both the time of appearance of the first flagellates in the population and the rate of flagellum elongation were temperature-dependent (Fig. 1).

The effect of cycloheximide on flagellum elongation

Cycloheximide (10 μg/ml) was added to suspensions of amoebae at intervals during the period of flagellum morphogenesis and elongation. When cycloheximide was added to

Fig. 3. Effect of cycloheximide pulse on final flagellum length. Control incubated at 21 °C, ●; control washed briefly at 4 °C and then incubated at 21 °C, ○. Cycloheximide (10 μg/ml) was added to other batches of amoebae at the beginning of incubation and then removed by washing at 4 °C after intervals of time: immediately, ▲; after 15 min, △; after 30 min, ■.

differentiating amoebae before flagella protruded, a few of the amoebae continued morphogenesis and formed flagella (Kerr, 1972), but their flagella remained extremely short. The addition of cycloheximide, after flagella had appeared, immediately stopped flagellum elongation (Fig. 2).

The kinetics of flagellum elongation were studied in populations of amoebae which were exposed to cycloheximide (10 μg/ml), then centrifuged at 4 °C and resuspended in the phosphate buffer at 21 °C. In no case did the amoebae exposed to cycloheximide synthesize a flagellum as long as those of the control, and the rate of elongation was slower than in the 21 °C control (Fig. 3). Elongation of flagella in the control run through the cold washing procedure was slower than that of the control kept at 21 °C, but the two control batches of amoebae achieved the same final flagellum length.

In another series of experiments the amoebae were allowed to begin morphogenesis, then cycloheximide was added for varying periods of time, after which the amoebae were washed free of cycloheximide and resuspended in buffer at 21 °C. Again, the rate of flagellum elongation and terminal length was less than that of untreated amoebae. In general, as time of exposure to cycloheximide was increased, the terminal flagellum length after removal from cycloheximide was decreased (Fig. 4). In addition, a lag in the resumption of flagellum elongation occurred in amoebae incubated in cycloheximide for 30 min and this lag became more pronounced as the time of exposure was increased.

Fig. 4. Cycloheximide (10 $\mu\text{g/ml}$) was added after morphogenesis had begun and then removed. Amoebae not exposed to cycloheximide, ●; (a) Cycloheximide added after 15 min: not removed, ■; removed at 60 min, Δ ; removed at 90 min, ○; removed at 120 min, □. (b) Cycloheximide added after 30 min: not removed, ■; removed at 60 min, Δ ; removed at 90 min, ○; removed at 120 min, □. (c) Cycloheximide added after 60 min: not removed, ■; removed at 90 min, Δ ; removed at 120 min, ○.

The effect of streptomycin on flagellum elongation

Amoebae incubated in the presence of streptomycin (250 $\mu\text{g/ml}$) did not become flagellated during the first hour of incubation, but at 75 min flagella ordinarily began to appear and at 105 min virtually all the amoebae were flagellated (Fig. 5). Cold-washed amoebae produced flagella at a rate somewhat slower than the control. After the first 105 min amoebae exposed to streptomycin for varying periods of time up to 30 min and then washed free of the drug elongated at the same rate as the washed control. Unlike the amoebae exposed to cycloheximide, they produced flagella which grew to the normal final length. Amoebae which remained in streptomycin produced flagella which elongated rapidly (0.24 $\mu\text{m/min}$ from 60 to 90 min of incubation) after an initial delay. After 105 min of incubation these amoebae possessed flagella identical in length to those of amoebae not treated with streptomycin, and in streptomycin- and non-streptomycin-treated amoebae the flagella then elongated at identical rates until final length was achieved.

Fig. 5. Effect of streptomycin pulse on final flagellum length. Control amoebae are shown: incubated at 21 °C, ●; washed briefly at 4 °C before return to 21 °C, ○. Streptomycin-treated amoebae are shown: to which streptomycin (250 μ g/ml) was added at time 0 and not washed out, ▲; from which streptomycin was removed immediately, ▽; which remained in streptomycin for 15 min before it was removed, △; which remained in streptomycin for 30 min before removal, □.

Regenerative growth

Flagella of various lengths were broken off 2 to 3 μ m from the proximal end. The bases of flagella amputated either during elongation or after elongation had ceased resumed growth immediately until the normal maximum length was achieved (Fig. 6).

Regeneration initially proceeded at a rate more characteristic of the control population at the same length of flagella than at a rate characteristic of the control population at the same time of incubation. Under regenerative conditions the rate of flagellum synthesis did not decrease as rapidly as it did in the control and final length was achieved more rapidly. The average rate of growth measured in the regenerating systems at 21 °C was 0.14 μ m/min for the first 30 min, 0.133 μ m/min for the second 30 min, and thereafter 0.05 μ m/min until final length was achieved. Regenerating flagella reached their final lengths 30 to 60 min before amoebae in the control populations under similar conditions.

Further amputation studies were done to determine what length flagella the amoebae were capable of synthesizing from the available precursors. The same populations of amoebae were subjected to amputation procedures five times at hourly intervals and the subsequent serial regenerations were measured (Fig. 7). Amoebae in these experiments synthesized a sum length of long flagellum equal to 42 to 45 μ m before the experiment was

Fig. 6. Regeneration of flagella amputated at various stages of the elongation process. Control amoebae, ●. Amputation at 60 min, ○; at 90 min, ▲; at 120 min, △; at 210 min, ■; at 270 min, □.

terminated. Lengths of short flagellum being synthesized in these experiments were not recorded. Although initial regeneration kinetics were normal for amoebae regenerating for the fourth or fifth time, normal final length was not achieved after amputation repeated this many times.

Flagellated amoebae were amputated at several times during flagellum growth and then incubated in the presence of cycloheximide ($7.5 \mu\text{g/ml}$); no growth occurred after cycloheximide addition. The rate of elongation in regenerating amoebae treated with streptomycin (250 to $500 \mu\text{g/ml}$) did not differ from that observed in untreated amputated amoebae.

DISCUSSION

Control of elongation

Flagellum assembly is ultimately dependent upon protein synthesis, but experiments described here showed that control over the rate of flagellum assembly must be a process distinct from control over the rate of flagellum-protein synthesis. The two types of flagella reach different final lengths and elongate at different rates; thus the amoebae can distinguish between the two types of flagella and it is suggestive that control over assembly might reside in the flagellar apparatus itself. Decreasing kinetics of elongation have been reported in regenerating flagella of *Ochromonas danica*, *Astasia longa*, *Euglena gracilis* (Rosenbaum & Child, 1967) in *Peranema trichophorum* (Tamm, 1967) and in *Chlamydomonas reinhardtii* (Rosenbaum, Moulder & Ringo, 1969). In *O. danica*, *P. trichophorum* and *C. reinhardtii* the flagella are known to elongate from the distal end. The hypothesis has been advanced

Fig. 7. Serial amputation and regeneration of flagella.

(Tamm, 1967; Rosenbaum *et al.* 1969) that the rate of assembly may be governed by the rate of diffusion of flagellar precursors to the assembly site at the tip of the flagellum. That additional controls must be operating in the *Didymium* system is suggested by several results. First, during normal growth of the short flagellum, the maximum growth rate is not achieved when the flagellum is at its shorter lengths (Fig. 1). Secondly, in regenerating flagella, elongation proceeds at a rate more rapid than in the control of the same length after the first hour of the process. Lastly, the process of flagellum growth in *Didymium nigripes* has been shown to be extremely temperature-sensitive; one would not expect a process governed by diffusion rate to show such great variation over a temperature range so narrow as the one investigated.

Tamm (1967) previously attempted to compare regenerative and replicative (normal growth after division) flagellum growth in the protozoan *Peranema trichophorum*. Tamm noted variability in the regeneration kinetics of single cells at different stages of the cell cycle; this made any comparison of replicative and regenerative growth more difficult. Techniques for synchronizing the cell cycle of *Didymium nigripes* amoebae have not yet been developed. However, amoebae used in the present studies may be somewhat synchronous because they were in stationary phase of growth and had not fed for several hours before the experiments. In the *Didymium* system regenerative and morphogenetic growth are clearly separable after the first 30 min. The faster growth rate of the regenerating flagella is quite pronounced and results in a 30 to 60 min advantage in achieving final length.

Control over final length

One interesting question to be resolved is why the flagella typically cease their elongation when a final length of about 15 μm has been achieved. The possibility that these stationary-phase non-feeding amoebae contain raw material reserves sufficient for only 15 μm of flagellum has been excluded by the serial amputation experiments. The concept that cellular components involved in protein synthesis are operational for only a fixed period of time has been negated by temperature-dependent variations in growth rate which result in identical final length and by growth-rate variations observed in regenerative and streptomycin-delayed growth.

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REFERENCES

- KERR, S. J. (1972). Inhibition of flagellum morphogenesis in the true slime mould, *Didymium nigripes*. *Journal of General Microbiology* **72**, 419–427.
- ROSENBAUM, J. L. & CHILD, F. M. (1967). Flagellar regeneration in protozoan flagellates. *Journal of Cell Biology* **34**, 345–364.
- ROSENBAUM, J. L., MOULDER, J. E. & RINGO, D. (1969). Flagellar elongation and shortening in *Chlamydomonas*. *Journal of Cell Biology* **41**, 600–619.
- TAMM, S. L. (1967). Flagellar development in the protozoan *Peranema trichophorum*. *Journal of Experimental Zoology* **164**, 163–186.