Genet. Res., Camb. (1976), **27**, *pp.* 291–296
With 1 plate
Printed in Great Britain

Effects of actinomycin D and puromycin upon excystment of *Didinium nasutum*

By H. M. BUTZEL and L. MAYER
Department of Biological Sciences, Union College, Schenectady, New York 12308, U.S.A.

(*Received* 10 *October* 1975)

SUMMARY

Activated cysts of *Didinium nasutum* continue excystment after treatment with actinomycin D or puromycin. Cysts treated at developmental stages 19–20 emerge holociliated and lacking the seizing organ. No effect of the antibiotics at any other stages was found, suggesting that it is at stage 19–20 that the initiation of normal ciliation and of mouth formation occurs. Treatment with other metabolic inhibitors either blocks excystment completely or has no effect on normal development depending upon dosage, suggesting that the action of the antibiotics is specific.

1. INTRODUCTION

Excystment of the ciliate *Didinium nasutum* has been 'staged' by Beers (1935, 1945, 1946). The resting cyst is surrounded by three walls: an outer ectocyst, a thick mesocyst and an inner thin endocyst. The first visible sign of excystment is the formation of an excystment vacuole within the cell proper (stage 14). The vacuole swells and the walls of the ectocyst and mesocyst break open as the animal, now contained within the endocyst only, emerges from the outer two walls (stages 19–20). The excystment vacuole now bursts and the cell appears shrunken and amorphous (stage 21). Within minutes the cell rounds up, develops holociliation and begins to spin rapidly within the endocyst (stage 22). By stages 23–24 the seizing organ and two ciliary tufts typical of the mature cell are seen. At stage 25 the fully developed cell escapes the endocyst and is ready to feed. The entire development from resting cyst to free-swimming form takes approximately $3\frac{1}{2}$ h from the time of initiation of excystment.

Electron-microscope studies of excystment have been carried out (Holt, 1972; Rieder, 1973) and the histochemistry of the cyst walls has been determined (Rieder, 1973) but no studies of the morphogenetic events occurring during excystment are available. This paper reports the effects of known inhibitors of transcription and translation upon the excystment process.

2. MATERIALS AND METHODS

Didinium cysts were obtained from Dr Portia Holt and stored in Knops Solution (Galtsoff *et al.* 1937) at 12 °C. Standard methods for induction of excystment using concentrated suspensions of bacteria were employed (Butzel & Horwitz, 1965; Butzel & Bolten, 1968).

Actinomycin D and puromycin were obtained from the Sigma Chemical Company. Actinomycin was made up as a stock solution of 250 μg/ml in glass distilled water and concentrations from 1·65 to 25 μg/ml were made by dilution with Knops. Puromycin was similarly made up at 600 μg/ml and diluted to either 200 or 500 μg/ml. Metabolic inhibitors were made up directly in Knops at the following concentrations: Na malonate, 10^{-6} M; NaF, 10^{-4} M; NaN_3, 10^{-3} M, 2,4-dinitrophenol, 10^{-4} M. These concentrations were established as being the strongest ones which did not result in the death of the cyst, although most were lethal for free-swimming cells.

Three methods of treating excysting cells with either actinomycin or puromycin were used. First, inhibitors were added directly to bacterial suspensions which were then used to induce excystment. The cysts were in the presence of the antibiotics from initiation to completion of excystment. It should be pointed out that no inhibition of the growth of bacterial cultures was found at the dosages employed. Secondly, cysts were allowed to initiate excystment in bacterial suspensions without the presence of antibiotics and then transferred at various stages to the inhibitor and allowed to complete excystment. Thirdly, cysts were activated in the bacteria plus antibiotic and at various stages transferred after three washings to Knops for completion of excystment.

Only the second and third methods were used for metabolic inhibitors in order to avoid the possibility of interfering with bacterial metabolism and thereby changing the rate of excystment.

Immediately after the completion of excystment the free-swimming cells were examined either by use of a 45 × stereoscopic microscope or by first immobilizing them in a microcompresser (American Biological Corporation) prior to observations with 440 × using a phase microscope. Following examination the animals were recovered and transferred to rich paramecium cultures for further determination of viability and fission rates.

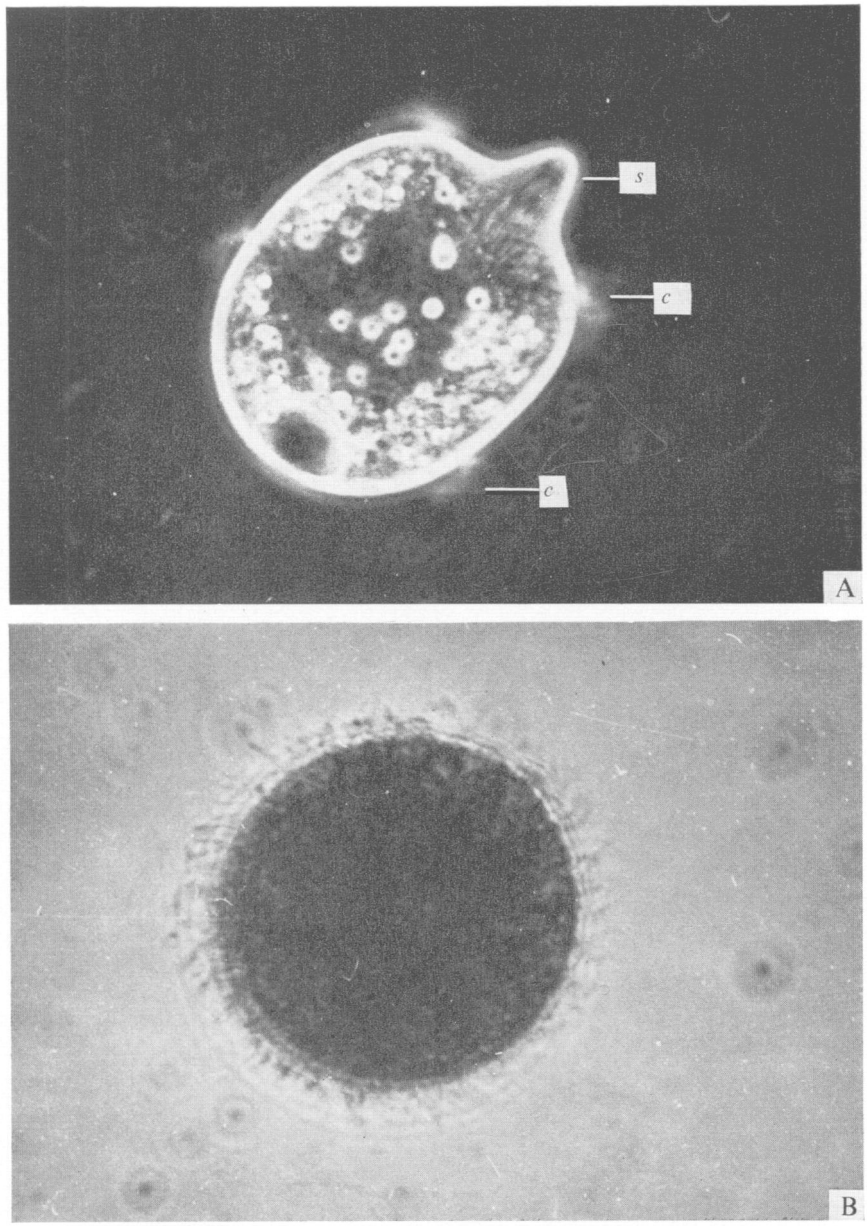
3. RESULTS

(i) *Effects of actinomycin D*

Cysts placed in actinomycin at any stage and at all dosage levels continued excystment at the same rate and percentage of excystment as untreated cysts (Table 1). At dosages from 3·25 to 25 μg/ml two striking differences were found. First, all treated cysts emerged from the endocyst holociliated instead of possessing the normal two ciliary tufts. Secondly, all cysts at these dosages emerged lacking the seizing organ so that the final product of excystment was a spherical, holociliated cell lacking all visible signs of the ingestatory apparatus (Plate 1).

Treatment of cells at specific stages of excystment showed that only cysts in the presence of the inhibitor at stages 19–20 were affected; cells treated before or after this time emerged from the cyst completely normal in both ciliation and in possession of a seizing organ (Table 2).

At the lowest dosage used, washed cells reisolated into well-fed paramecium cultures formed ciliary tufts and seizing organs and fed and divided normally.



(A) Normal excysted cell showing two ciliary tufts (*c*) and the seizing organ (*s*). (B) Excysted cell treated with actinomycin D at stage 20. Ciliary tufts and seizing organ are absent and cell is holociliated.

https://doi.org/10.1017/S0016672300016475 Published online by Cambridge University Press

At 6·25–12·5 μg/ml of actinomycin most animals became normal and began to divide after a delay of several hours. Cells treated with 25 μg/ml developed the ciliary tufts but failed to form the seizing organ and died without feeding.

Table 1. *Effect of actinomycin D and puromycin upon excystment*

Treatment	No. of cysts tested	Excystment (%)
B	90	95
B + actinomycin	259	93
B + puromycin	20	100

Cysts in various media from initiation to excystment. B, Bacterial suspensions. Actinomycin concentrations from 1·65 to 25 μg/ml; puromycin 250 or 500 μg/ml.

Table 2. *Effect of actinomycin upon various stages of excystment*

Stage	Treatment		No. treated	Excystment		% yielding normal fission rates
	From	To		Normal	Abnormal	
13	K	K	30	29	1	100
13	K	A	24	3	21	84
14	K	A	23	5	18	100
19	K	A	13	0	13	85
20	K	A	8	8	0	100
22	K	A	6	6	0	100
14	A	K	7	7	0	100
19	A	K	10	0	10	100
20	A	K	6	0	6	84
22	A	K	7	0	7	100

'From' indicates medium in which excystment was begun and cells developed until designated stage. 'To' indicates medium into which cells were transferred at designated stage. K, Knops solution; A, actinomycin. 'Normal' and 'abnormal' refer to condition of ciliation and seizing organ at time of excystment. Actinomycin dosages from 3·25 to 12·5 μg/ml.

(ii) *Puromycin effects*

Although there was slightly more variability in the effects of puromycin, the same general results found for actinomycin were found. The rate and percentage of excystment did not differ from untreated cysts (Table 1) and those treated at stages 19–20 emerged holociliated and without seizing organs (Table 3). Most of the puromycin-treated animals were also able to develop the seizing organ and normal ciliation as well as to feed and give rise to normal cultures.

(iii) *Other inhibitors*

No effect upon development of ciliature or seizing organs was found in any of the other metabolic inhibitors at dosages which were not lethal to the cysts (Table 4). At dosages of Na malonate, NaF, NaN_3 or 2,4-dinitrophenol 10 × those used all cysts failed to initiate or complete excystment.

Table 3. *Effect of puromycin upon various stages of excystment*

Stage	Treatment: From	Treatment: To	No. treated	Excystment: Normal	Excystment: Abnormal	% yielding normal fission rates
13	K	K	39	39	0	97
13	K	P	38	0	38	47
14	K	P	7	0	7	100
19	K	P	12	5	7	100
20	K	P	16	11	5	69
22	K	P	14	14	0	100
14	P	K	5	2	3	100
19	P	K	13	3	10	92
20	P	K	20	6	14	95
22	P	K	17	0	17	76

'From, to, normal, abnormal' as in Table 2. K, Knops solution; P, puromycin. Puromycin used at either 200 or 500 μg/ml.

Table 4. *Total number of cysts developing normally in various inhibitors*

(No abnormally excysted cells were found in these experiments.)

Compound ...	NaF	NaN_3	Na malonate	2,4 DNP
Molarity ...	10^{-4}	10^{-5}	10^{-6}	5×10^{-4}
Stages 14–15	15	4	14	24
Stages 19–20	31	18	22	30
Stages 21–24	22	19	20	15

4. DISCUSSION

None of the inhibitors employed changed the time required for excystment nor the ability to excyst. Only actinomycin D and puromycin, and not other inhibitors of metabolism, altered the pattern of events during morphogenesis. The specificity, both in timing and in effect, of these two antibiotics known to interfere with transcription and translation was unique. The initiation, as well as successful completion, of excystment in the presence of actinomycin may suggest that these processes do not require the addition of new messenger RNA for the process of excystment itself but that such information is required later for the formation of normal ciliation and for normal stomatogenesis. The limitation of the actinomycin and puromycin effects to stages 19–20 suggests that it is at this time, during the emergence of the cyst from the ectocyst and mesocyst walls, that the initiation of events leading to the formation of the seizing organ and ciliary tufts begins.

Although it is possible that the outer two cyst walls might be a barrier to the penetration of the reagents used here prior to stages 19–20, this seems unlikely. Cysts placed in actinomycin at any stage of development rapidly take on the bright orange colour of actinomycin, indicating that the antibiotic has been taken up by the cells. The vital stain, Nile Blue Sulphate, also readily penetrates into the cells. In addition, reagents believed to interfere with membrane functions, such as

charged detergents, effectively block excystment prior to stage 19 and have no effect afterwards (Butzel & Davich, 1974), further indicating the ability of reagents to enter the cysts and the specificity of the actinomycin and puromycin effect.

The recovery of cells after treatment with effective dosages of actinomycin or puromycin and the subsequent removal of the cells to normal growth conditions demonstrated that the effects of low dosages of the inhibitors are not permanent, but in some cases a lag occurs prior to complete recovery of normal feeding and fission rates. Such a temporal block in morphogenesis and fission at a particular time in the cell cycle has been reported for other ciliates treated with actinomycin D. For example, actinomycin at specific stages blocks fission and stomatogenesis in *Paramecium aurelia* (Hanson & Kaneda, 1968) and in *Tetrahymena pyriformis* (Whitson & Padilla, 1965). This reagent also interferes with regeneration of the oral apparatus at a specific stage in *Stentor coeruleus* (James, 1967) and in *Stylonichia mytilus* (Sapra & Ammerman, 1974). Stomatogenesis is a major restructuring of the ciliate during its life-cycle either during excystment, fission or regeneration and it is not perhaps surprising to find that interference with the formation of messenger RNA at a time when many new structures are being laid down is widespread among the ciliates, and that there are specific times during the life-cycle when this RNA apparently is synthesized.

A possible analogy in the Didinium work to metazoan development may exist in that the information for the excystment process is apparently contained within the resting cyst and new information for completion of the morphogenesis of the cell is needed only later in development. If this analogy is valid, study of the didinial system may illuminate more general processes of activation and of development.

Work supported by an anonymous grant to Union College for Genetic Research. We wish to thank Dr Portia Holt for supplying the cysts used in this work and to acknowledge the technical assistance of Mrs Elizabeth Howard. A brief extract of this work has appeared since the preparation of this paper (Butzel & Mayer, 1974).

REFERENCES

Beers, C. D. (1935). Structural changes during encystment and excystment in the ciliate *Didinium nasutum*. *Archives für Protistenkunde* **84**, 133–155.

Beers, C. D. (1945). The excystment process in the ciliate *Didinium nasutum*. *Journal of the Elisha Mitchell Society* **61**, 264–275.

Beers, C. D. (1946). Excystment in *Didinium nasutum* with special references to the role of bacteria. *Journal of Experimental Zoology* **103**, 201–231.

Butzel, H. M. & Bolten, A. B. (1968). The relationship of the nutritive state of the prey organism *Paramecium aurelia* to the growth and development of *Didinium nasutum*. *Journal of Protozoology* **15**, 256–258.

Butzel, H. M. & Davich, M. (1974). The relationship of the cyst walls to excystment of *Didinium nasutum* as demonstrated by various agents acting upon these walls. *Journal of Protozoology* **21** (suppl), 441.

Butzel, H. M. & Horwitz, H. (1965). Excystment of *Didinium nasutum*. *Journal of Protozoology* **12**, 413–416.

BUTZEL, H. M. & MAYER, L. (1974). Effects of actinomycin D and puromycin upon excystment of *Didinium nasutum*. *Journal of Protozoology* **21** (suppl), 419.

GALTSOFF, P. S., LUTZ, F. E., WELCH, P. S. & NEEDHAM, J. G. (1937). *Culture Methods for Invertebrate Animals*, p. 101. Ithaca, New York: Comstock Publishing Company.

HANSON, E. D. & KANEDA, M. (1968). Evidence for sequential gene action within the cell cycle of *Paramecium aurelia*. *Genetics* **60**, 793–805.

HOLT, P. A. (1972). An electron microscope study of the Rhabdophorine ciliate, *Didinium nasutum*, during excystment. *Transactions of the American Microscopical Society*. **191**, 144–168.

JAMES, E. (1967). Regeneration and division in *Stentor coeruleus*: the effects of microinjected and externally applied actinomycin D and puromycin. *Developmental Biology* **16**, 577–593.

RIEDER, N. (1973). Elektronenoptische und histochemische Untersuchungen an der Cystenhülle von *Didinium nasutum O. F. Müller*. *Archives für Protistenkunde* **115**, 125–131.

SAPRA, G. R. & AMMERMAN, D. (1974). An analysis of the developmental program in relation to RNA metabolism in the ciliate *Stylonichia mytilus*. *Developmental Biology* **36**, 105–112.

WHITSON, G. L. & PADILLA, C. M. (1965). The effects of actinomycin D on stomatogenesis and cell division in temperature-synchronized *Tetrahymena pyriformis*. *Experimental Cell Research* **36**, 667–671.