

The action of ribonuclease on the nucleic acid system of *Spirostomum ambiguum*

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With 1 plate (fig. 1)

Summary

The experiments described in this paper have shown that the enzyme ribonuclease (RNase) is able to enter *Spirostomum ambiguum*. There is a definite concentration/time relationship in the action of RNase on the ribonucleic acid (RNA) system of the organism. The rate of action of the enzyme is proportional to its concentration in the medium. At high concentration the enzyme causes instant degeneration of the cell. At lower concentrations the enzyme acts specifically on RNA present in the cytoplasm and in the macronucleus; it causes complete loss of basiphilia. Loss of basiphilia occurs first in the cytoplasm and later in the macronucleus.

The organisms after treatment with the enzyme, when transferred back to enzyme-free Chalkley's medium, regenerate RNA. The RNA appears first in the macronucleus and later in the cytoplasm.

Introduction

RECENT work of Brachet (1955, 1957, 1960) has shown that the enzyme RNase can be successfully used in *Amoeba proteus* to study the sites of RNA and its role in protein synthesis. Similarly, Stich and Plaut (1958) have used RNase to remove cytoplasmic RNA in *Acetabularia mediterranea* in order to study protein synthesis in enucleate and nucleate fragments. They found that only the nucleate fragments could synthesize fresh RNA and show a significant rise in proteins. However, according to Brachet (1957) the penetrating capacity of RNase is limited in that it has effect on *Amoeba*, amphibian eggs, tumour cells, flagellates, and bacteria but does not have any effect on ciliates. This is apparently due to the impermeability of the outer pellicle of ciliates. It was of interest to determine whether all ciliates are impenetrable to the enzyme. The heterotrichous ciliate, *Spirostomum ambiguum* (Müller-Ehrenberg), was chosen in the present study.

Material and Methods

S. ambiguum is a freshwater ciliate belonging to the order Heterotricha. It is 1 to 3 mm long. It has a beaded macronucleus and a number of small micronuclei. The organisms were cultured in the laboratory in hay infusion with a dense population of the flagellate *Chilomonas* sp. Well-fed *Spirostomum* divides by binary fission once every 72 h. For the experimental work ciliates were isolated from cultures in their log phase. The cultures were standardized to ensure that the animals treated were under uniform physiological conditions. Before treatment, the organisms were maintained in Chalkley's medium (pH 6.8) for 18 h in order to free the cytoplasm of all food vacuoles.

Three different concentrations: 0.01 mg/ml, 0.05 mg/ml, and 0.1 mg/ml of RNase (Light, Colnbrook, Bucks., England) in Chalkley's medium were used. In each case, about 200 individuals were treated with 1.0 ml of the enzyme solution. Suitable controls were maintained and studied. After treatment, the experimental animals and controls were fixed at regular intervals in acetic acid / absolute alcohol (1:3) and stained with methyl green / pyronin according to Brachet's method with minor modifications (methyl green E. Merck 1313; pyronin BDH 418688/540420). It was found that pretreatment with buffer solution (phosphate/citrate buffer pH 4.8) before staining ensured consistent staining reactions. The intensity of staining with pyronin was taken as an index of basiphilia due to RNA.

Observations

Spirostomum is not impermeable to ribonuclease; the enzyme enters the cell readily. In all 3 concentrations used, the ciliates showed no immediate adverse reaction. The first indication of adverse effect was noticed after 4 h treatment in enzyme at 0.1 mg/ml. Vacuolation and loss of cytoplasm in small fragments from the posterior region near the contractile vacuole then occurred. This reaction was noticed only after 24 h in animals treated with enzyme at 0.01 mg/ml. However, the animals continued to live in all 3 concentrations of the enzyme for about 2 h after the first sign of adverse reaction. Restoration of the animals to Chalkley's medium without enzyme before the degenerative changes became evident, allowed survival. After adverse reactions had been shown, however, the organisms became rounded and could not be revived on transference to the culture medium.

An interesting concentration/time relationship in the action of the enzyme on the RNA system of the animal was noted. Complete removal of RNA from both macronucleus and cytoplasm occurred after 2 h treatment in 0.1 mg/ml concentration, after 3 h in 0.05 mg/ml, and after 6 h in 0.01 mg/ml. The sequence of loss of basiphilia was also of interest. Basiphilia first disappeared from the cytoplasm and only later from the macronucleus. The loss was more gradual at lower concentration than at higher. Control animals in all cases showed intense basiphilia both in cytoplasm and macronucleus (fig. 1, A, B).

In another series of experiments, animals treated for 24 h with 0.1 mg/ml RNase were transferred back to Chalkley's medium. Before transfer, the animals were washed in several changes of Chalkley's medium to remove

FIG. 1 (plate). A, control *Spirostomum* after being in Chalkley's medium for 22 h. Both the cytoplasm and macronucleus show intense basiphilia. Methyl green / pyronin.

B, *Spirostomum* treated with RNase (0.1 mg/ml) for 4 h. Basiphil reaction not present in the cytoplasm or in the macronucleus. Methyl green / pyronin.

C, *Spirostomum* after treatment for 24 h in 0.01 mg/ml RNase, then transferred to Chalkley's medium and stained after 2 h. The basiphil reaction is intense in the macronucleus but very faint in the cytoplasm. Methyl green / pyronin.

D, *Spirostomum* after treatment for 24 h in 0.01 mg/ml RNase, then transferred to Chalkley's medium and stained after 5 h. The cytoplasmic and macronuclear basiphilia is almost as intense as in an untreated animal. Methyl green / pyronin.

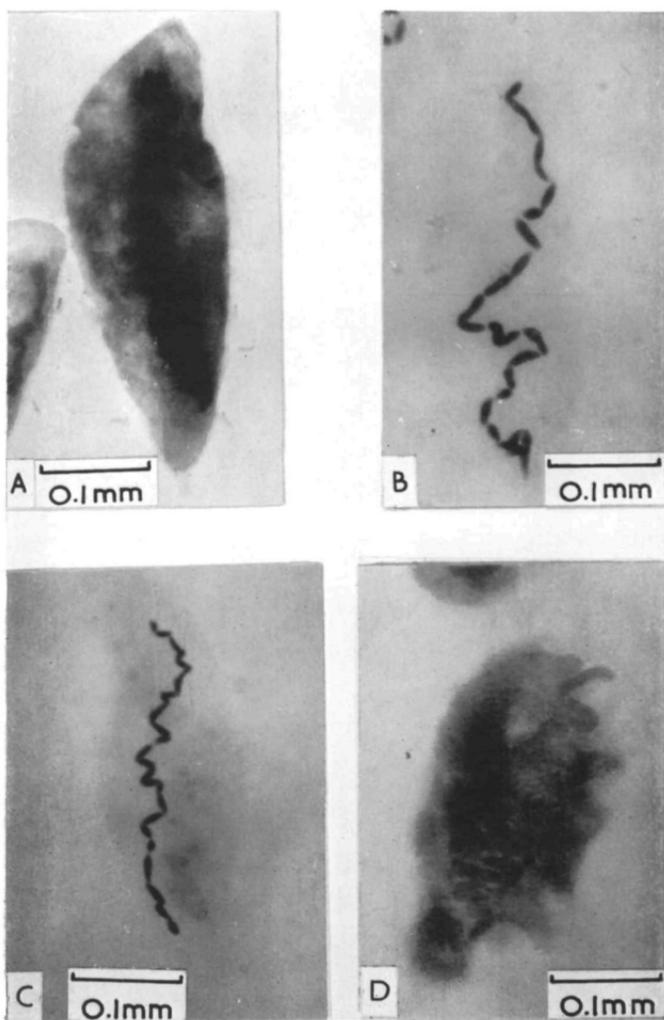


FIG. 1
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traces of the enzyme. The animals were subsequently fixed and stained at intervals of 1 h.

At the end of 1 h, both cytoplasm and macronucleus showed no basiphilia. After 2 h, strong basiphilia was noticed in the macronucleus while only faint in the surrounding cytoplasm. Thereafter, there was a gradual increase in basiphilia of the cytoplasm, and at the end of 4 h experimental animals were similar to untreated controls (fig. 1, C, D).

Discussion

Contrary to Brachet's statement (1957) that the enzyme RNase does not penetrate into all cells and that '... ciliates are insensitive to its action', the present study has shown that RNase enters *S. ambiguum*. The exact mode of entry is not clear, however. It might enter by active penetration through the pellicle, or alternatively through the gullet. That the enzyme primarily affects the pellicle is indicated by the fact that at higher concentrations (more than 0.1 mg/ml) the pellicle is instantly discarded, whilst even at lower concentrations (less than 0.1 mg/ml) prolonged exposure to the enzyme first causes degeneration of the pellicle and later of the animals. More recently, Bhide and Brachet (1960) working on the action of RNase on onion root-tip cells state 'it is not unreasonable to assume that in plant root-tip cells and animal cells, the uptake of RNase is due to the binding of the enzyme to specific (perhaps RNA containing) sites and to subsequent pinocytosis'.

Brachet (1955, 1957, 1960) in his experiments on the action of RNase on *A. proteus* has not recorded sequential loss of basiphilia first from the cytoplasm and later from the nucleus, as we have observed in *Spirostomum*. According to Brachet, RNase penetrates *Amoeba* rapidly by pinocytosis and within a period of 5 to 15 min there is a lowering of basiphilia, especially in the nucleoli. He suggests (1957) that in *Amoeba* an enzyme-substrate complex is formed and is followed by the breakdown of RNA in the cell system. Although the mode of action of the enzyme is not clear in the case of *Spirostomum*, from the present series of experiments we can state that RNase causes the breakdown of both cytoplasmic and macronuclear RNA as indicated by the loss of basiphilia.

Brachet (1957) has also recorded that in *Amoeba* regeneration of RNA and the revival of the animal to normal condition is only possible if extraneous RNA (yeast) is added to the medium. The situation is different in *Spirostomum*, as treated individuals on being transferred to Chalkley's medium can regenerate fresh RNA even without the presence of extraneous RNA. In starfish oocytes where incorporation of labelled amino-acids takes place if the RNase-treated cells are washed and left in normal sea-water, Ficq and Errera (1955) suggest that this is due to breakdown of an RNA-RNase complex. In *Spirostomum* a similar situation may exist, but the possibility of new synthesis of RNA from nucleotides already present in the cell cannot be ruled out.

The regeneration of RNA in the macronucleus and in the surrounding cytoplasm, followed by its later appearance throughout the cytoplasm, is in

agreement with observations on the nuclear synthesis of RNA in other cell systems. Nuclear origin of RNA has been suggested by several workers. Goldstein and Plaut (1955) demonstrated the synthesis of RNA first in the nucleus and later its movement into the cytoplasm. Prescott (1957) found no incorporation of labelled uracil in enucleate fragments of *Amoeba*, indicating that the entire cytoplasmic RNA comes from the nucleus. Scholtissek, Schneider, and Potter (1958) have reported that in liver cells nuclear RNA is synthesized and then rapidly transferred to the cytoplasm. Goldstein and Micou (1959) have demonstrated the transfer of RNA from nucleus to cytoplasm in cultured amnion cells. More recently, Taylor (1960), using tritium-labelled nucleosides, has demonstrated synthesis of RNA in the nucleus and its transference to the cytoplasm in cultured cells of embryonic connective tissue of the Chinese hamster. Also Woods (1960), using tritiated cytidine, a precursor of both RNA and DNA, has suggested that RNA is synthesized in the nucleus, especially in the nucleolus, and later moves as large molecules to the cytoplasm in root meristem cells of *Vicia faba*. Whether labelled nucleolar material moves into the cytoplasm as fully synthesized RNA or as intermediate precursors is not clear. Our own observations based on the intensity of basiphilia show that RNA appears first in the macronucleus and then in the cytoplasm. It may be plausible to suggest that RNA is synthesized in the macronucleus of *Spirostomum* and later transferred to the cytoplasm. Confirmation of this suggestion is only possible if labelled precursors are used to study the course of RNA synthesis in this ciliate.

We wish to thank Prof. B. R. Seshachar, of the Department of Zoology, University of Delhi, for guidance and encouragement. One of us (R. V. D.) wishes to thank the Ministry of Education, Government of India, for the award of a scholarship.

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