PERMEABILITY PROBLEMS ENCOUNTERED WHEN TREATING CONIDIA OF NEUROSPORA CRASSA WITH RNA SYNTHESIS INHIBITORS[1]

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Summary: Conidia of Neurospora crassa were treated with three inhibitors of RNA synthesis. These cells are impermeable to actinomycin D and alpha-amanitin. To overcome this problem, the cells were treated with EDTA. It was found that EDTA prevents the uptake of radioactive RNA precursors, resulting in an apparent inhibition of RNA synthesis. Evidence is also presented that the inhibitor, proflavine, may alter permeability of conidia.

One of the most widely used inhibitors of RNA synthesis is actinomycin D. Neurospora crassa has been found (1) to be relatively impermeable to this inhibitor. This permeability problem has been overcome in germinated conidia or mycelia either by raising the temperature from 25° C to 35° C (2) or by treating the cultures with ethylenediaminetetraacetate (EDTA) (3-5). In Escherichia coli (6, 7) and in mycelia of N. crassa (8), EDTA has been shown to affect permeability and to increase sensitivity to actinomycin D. Using proflavine, Hollomon (9) found that inhibition of germination closely paralleled inhibition of RNA synthesis in N. crassa. Alpha-amanitin has been found (10) to inhibit the in vitro activity of an RNA polymerase isolated from Blastocladiella emersonii, an aquatic fungus.

During a study on active transport of amino acids, conidia of N. crassa were treated with the above inhibitors of RNA synthesis. This communication reports the permeability problems encountered. Further, it

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is shown that the use of EDTA prevents uptake of RNA precursors, resulting in an apparent inhibition of RNA synthesis.

MATERIALS AND METHODS: The wild-type strains of N. crassa, 74-OR23-1A (designated 74A), and the opposite mating type, 74-OR8-1a (designated 74a) were utilized. Dry weights were obtained on 7-day-old conidia, and a final concentration of 0.1 mg dry wt/ml was used in all experiments. After various incubation periods in the absence or presence of inhibitor and the monitoring radioactive isotope, 5 ml aliquots of cells were either delivered onto a membrane filter (uptake experiments) or into 10 ml of 10% TCA. After 30 min, the TCA solution was filtered onto a membrane filter. In both cases, the filtered samples were washed 3 times with 5 ml cold distilled water, then glued to planchets for counting in a Beckman Low Beta II proportional counter. All incubations were carried out with shaking in a constant temperature water bath. The concentrations and types of inhibitors and radioactive isotopes used are shown in the Results. EDTA was made up as a 10X stock solution (1 M) with tris buffer (1 M) and adjusted to pH 8.0. This stock was used to give final concentration of 0.1 M for all experiments.

Radioactive isotopes were obtained from either Schwarz Bio-Research, Inc., New York, or International Chemical and Nuclear Corp., Calif. Actinomycin D was obtained from Calbiochem, Calif. Alpha-amanitin was obtained from Henley and Co., Inc., New York.

RESULTS: Inhibition of RNA synthesis during the first 3 hours of conidial development was examined since this was the period used (11) in the active transport studies. A preliminary microscopic examination had shown, in agreement with Totten and Howe (2), that when the temperature is increased from 25° C to 35° C, cultures growing in the presence of actinomycin D do not conidiate. However, this treatment did not inhibit the development of conidia (germination and elongation). These results were substantiated by experiments showing no difference between counts in TCA-insoluble material from control or actinomycin D-treated conidia at either 25° C or 35° C (Table I).

Since permeability of conidia may be affected less by temperature increase than permeability of mycelia, EDTA was used to increase permeability. Because permeation is a factor in transport, the effect of EDTA on

Table I. Effect of temperature on inhibition of RNA synthesis by 10 μg/ml final concentration of actinomycin D.

RNA synthesis was monitored by ^{14}C-uridine (0.005 μCi/ml final concentration) counts/min in TCA insoluble material/mg dry wt conidia.

	25° C		35° C	
time (hr)	control (CPM)	actinomycin D (CPM)	control (CPM)	actinomycin D (CPM)
0	15	12	16	20
1	2268	2473	3407	3159
3	11298	11250	9898	10753

Table II. Actinomycin D and EDTA effects on RNA synthesis and transport.

RNA synthesis was monitored by ^{14}C-uridine (0.01 μCi/ml final concentration) counts/min in TCA insoluble material/mg dry wt conidia. This isotope was present during the 3 hr of incubation. Transport was assayed by adding ^{14}C-phenylalanine (0.01 μCi/ml) to the conidia after 3 hr of incubation. Actidione (10 μg/ml) was added during the 30-min transport assay. Transport values are counts/min/mg dry wt conidia. Actinomycin D was used at a final concentration of 10 μg/ml.

time (min)	control (CPM)	actinomycin D + EDTA (CPM)	EDTA (CPM)	inhibition of uridine incorpor. (%)
1. RNA synthesis:				
0	766	96		
60	11999	132		99
120	37747	197		99+
180	33483	217		99+
2. Transport:				
0	505	401	459	
10	5112	361	363	
20	6889	383	372	
30	7620	416	424	

transport was measured. Leive (6) had shown that *E. coli* treated with 2×10^{-4} M EDTA incorporated radioactive leucine and uracil at the same rate as controls. However, in mycelia of *N. crassa*, 0.5% EDTA inhibited (8) the uptake of lysine and uridine. A lower concentration of EDTA (1×10^{-1} M,

Table III. EDTA effects on uptake and incorporation of orotic acid into TCA insoluble material.

A final concentration of 0.005 μCi/ml ^{14}C-orotic acid was present during the 3 hr of incubation. Aliquots (5 ml) of conidia were delivered onto a membrane filter (uptake) or into 10 ml of 10% TCA. Values are counts/min/mg dry wt conidia.

	Uptake counts		TCA insoluble counts	
time (hr)	control (CPM)	+ EDTA (CPM)	control (CPM)	+ EDTA (CPM)
0	17	29	7	4
1	61	13	29	2
2	520	17	307	18
3	1519	13	1249	20

0.3%) has been used (3) to increase sensitivity of mycelia to actinomycin D. It was hoped that this concentration could be used to increase sensitivity to actinomycin D in conidia without affecting transport. Table II shows that, although actinomycin D plus EDTA prevents incorporation of ^{14}C-uridine into TCA-insoluble material, this concentration of EDTA inhibits the transport of ^{14}C-phenylalanine into 3-hour-old conidia.

Table III shows that this concentration of EDTA also prevents conidial uptake of RNA precursor molecules, thus making it appear as if RNA synthesis were inhibited in the actinomycin D plus EDTA-treated conidia. Other experiments using ^{14}C-uridine showed the same results. It was found that the EDTA effect on transport could be reversed if the cells were washed after a short (15 min) treatment. However, although actinomycin D was present along with EDTA for 15 min at 35° C, and added again to the incubation medium after removal of the EDTA, RNA synthesis was not significantly inhibited in conidia during the 3 hr measured.

Inasmuch as all attempts to use actinomycin D as an *in vivo* inhibitor of RNA synthesis in conidia were futile, another inhibitor, proflavine, was chosen. Hollomon (9) showed that 40 μg/ml of proflavine completely inhibited RNA synthesis in *N. crassa*. Microscopic observations, using a range of proflavine concentrations, showed that 10 μg/ml final concentration prevented germination of conidia. Figure 1 shows the effect of this concentration on transport, RNA synthesis, and protein synthesis.

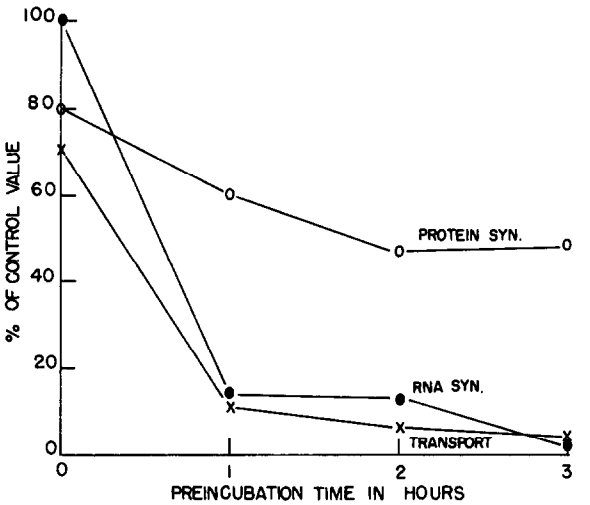

Fig. 1. Inhibition of protein synthesis, RNA synthesis, and transport by 10.0 µg/ml final concentration of proflavine.

Protein synthesis was monitored by ^{14}C-phenylalanine (0.01 µCi/ml final concentration) and RNA synthesis by ^{14}C-uridine (0.005 µCi/ml final concentration) counts/min in TCA-insoluble material/mg dry wt conidia. These isotopes were present during preincubation. The zero point represents a 30-45 sec pulse.

Transport was assayed at the beginning of preincubation (0 hr) and at hourly intervals during preincubation by 30-min pulse-labeling with ^{14}C-phenylalanine (0.01 µCi/ml final concentration) in the presence of 10 µg/ml (final concentration) actidione. Values were calculated as percentage of counts/min in untreated (no proflavine present) controls.

A protein synthesis-dependent increase in transport activity occurs (11) during early development of conidia. It might be assumed that this developmental event, not transport (permeation), is being affected by proflavine. However, for the zero preincubation time, prior to any developmental increase in transport activity, there is a 30% decrease in transport due to the presence of proflavine. Actidione (10 µg/ml) was added during the 30-min transport assays (after the preincubation periods) to prevent further protein synthesis. Subsequent transport assays, after 1 hr periods of preincubation in the presence of proflavine, show a continuing decrease in counts from the zero level, as would be seen if permeability is being altered by the proflavine.

Alpha-amanitin has been shown (10) to inhibit the in vitro activity of an RNA polymerase which functions in an aquatic fungus in the synthesis of

Table IV. Effect of alpha-amanitin on RNA synthesis.
RNA synthesis was monitored by ^{14}C-uridine (0.005 μCi/ml final concentration) counts/min in TCA insoluble material/mg dry wt conidia. A final concentration of 2.5 μg/ml alpha-amanitin was used.

time (hr)	control (CPM)	+ alpha-amanitin (CPM)
0	58	69
1	1434	1291
2	5593	5516
3	8113	8574

DNA-like RNA. If N. crassa is permeable to this inhibitor, it may be used to study in vivo inhibition of mRNA synthesis. Table IV shows no effect by ten times the concentration of alpha-amanitin used by Horgen and Griffin (10) on the incorporation of uridine into TCA-insoluble material during the first three hours of conidial development. These results could indicate that conidia are impermeable to this inhibitor. Further, microscopic observations showed no effect by this concentration on conidia through 12 hours. After 24 hr, the pads in control and treated flasks were pulled, dried, and weighed. The control pad weighed 24.2 mg; the treated pad weighed 22.0 mg. Apparently, N. crassa is impermeable to this inhibitor, since it would be difficult for a mycelial pad to form without RNA synthesis.

DISCUSSION: It has been shown (2) that 5.0 μg/ml of actinomycin D inhibits the formation of conidia at 35° C. At this temperature and twice this concentration of actinomycin D, the development of conidia through elongation does not appear to be altered. Thus actinomycin D cannot be used in conidia of N. crassa unless some other means of rendering the cells permeable to this drug is found. Further, impermeability would seem to be a reasonable cause for the lack of an effect by alpha-amanitin.

In addition, the results obtained both when proflavine is used as an inhibitor and when the cells have been treated with EDTA indicate an alteration in the permeability of conidia to amino acids and RNA precursors. Thus any results which utilize isotopes in the medium as monitors to show in vivo inhibition of synthesis due to proflavine, or any inhibitor used in conjunction with high concentrations of EDTA, in conidia of N. crassa must be suspect

until proof of synthesis inhibition is established.

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