Incorporation of ^3H -Uridine into RNA during Cellular Slime Mould Development

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SUMMARY

Dictyostelium discoideum wild-type amoebae incorporate ^3H -uridine into RNA at a linear rate during the developmental sequence until the terminal stage of fruiting body construction. However, 3 aggregateless strains perform in similar fashion (except for the final decrease). A sensitive criterion of bacterial contamination, making use of MAK-column chromatography of the labelled RNA, is described.

INTRODUCTION

The RNA content of *Dictyostelium discoideum* vegetative amoebae is about 15% of the total dry weight and the absolute amount of RNA decreases during fruiting body construction in proportion to the overall loss of cell material (White & Sussman, 1961). Previous experiments involving pulse labelling with ^3H -uridine and ^{32}P -phosphate have shown that appreciable RNA synthesis occurs during at least part of the development of a mutant strain (FR-17) of *D. discoideum*. Sucrose density gradient centrifugation of the labelled material revealed it to be distributed primarily in 4, 16, and 23 S components, but also to a small extent in regions other than these (Sussman & Sussman, 1965; M. Sussman, 1966). The present communication indicates that, in the wild type, RNA synthesis as reflected by ^3H -uridine incorporation proceeds at a significant rate throughout morphogenesis and decreases to a low value only at the terminal stage of fruit construction. However, three aggregateless mutants of *D. discoideum* yielded incorporation patterns essentially like that of the wild type (except for the final decrease in rate). Thus, the gross aspect of RNA synthesis does not of itself accurately reflect developmental events though differential transcription assuredly does (Sussman & Sussman, 1965).

METHODS

Organisms and experimental conditions. Wild-type *Dictyostelium discoideum* strain NC-4 (haploid) and aggregateless mutant derivatives were grown in association with *Aerobacter aerogenes* on SM agar (Sussman & Lovgren, 1965). The amoebae were harvested at room temperature in SM broth and streptomycin sulphate (0.5 mg./ml.), spun at 1000 g for 2–3 min. and resuspended in the SM + streptomycin broth at a concentration of 2×10^7 amoebae/ml. After incubation for 1 hr on a shaker at 22°, the amoebae were harvested by centrifugation, washed once with cold water and

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resuspended in water at 2×10^8 amoebae/ml. Samples (0.1 ml.) were dispensed on quarter pieces of 2 in. black Millipore filters, resting on absorbent pads saturated with 0.05 M-phosphate (pH 6.5) + streptomycin sulphate (0.5 mg./ml.) within 60 mm. Petri dishes and were incubated at 22° in a humid atmosphere. Under these conditions a very high degree of morphogenetic synchrony was achieved (Sussman & Lovgren, 1965).

Incorporation experiments. The amoebae were labelled by adding ^3H -uridine to the fluid saturating the support pads (4 mc./ μmole ; 5 $\mu\text{c.}$ /ml. pad fluid). After the desired periods of incorporation, the amoebae were harvested in cold water, spun at 7000 g for 5 min. and the pellets frozen. After thawing and brief homogenization with a Vortex mixer, 10% (w/v) trichloroacetic acid (TCA) was added along with 200 $\mu\text{g.}$ DNA as a carrier. After 30 min. in the cold the precipitates were collected on Millipore filters, washed with cold 5% (w/v) TCA and placed in vials with a toluene base scintillation fluid for counting. A Nuclear-Chicago counter was used at an efficiency of 16%.

RNA fractionation. RNA was extracted from the harvested amoebae by treatment with 3% Duponol and purified by the method of Gierer & Schramm (1956). The material was then adsorbed to a methylated albumin + kieselguhr (MAK) column and eluted in a linear NaCl gradient (Mandell & Hershey, 1960). The extinction at 260 m μ (E_{260}) was monitored in a Gilford recording spectrophotometer and 1 ml. samples were precipitated in 10% TCA for counting.

RNAse controls. In some experiments duplicate TCA precipitates were suspended in 0.075 M-phosphate (pH 7.5) with 30 $\mu\text{g.}$ of $2 \times$ recrystallized pancreatic RNAse. After 30 min. at 37° the material was reprecipitated with TCA, 10% (w/v), washed, and counted. 80–90% of the originally precipitable counts were rendered soluble by this treatment.

RESULTS

Cumulative and pulsed uridine incorporation

Wild-type amoebae, treated as described in the Methods section, were dispensed on Millipore filters and incubated at 22° for 4 hr. The Millipore filters were then shifted to new support pads containing ^3H -uridine and the organisms harvested at 2 hr intervals thereafter to determine the incorporation into TCA-insoluble material. As Fig. 1 indicates, the rate of incorporation remained constant over a 6 hr period but then declined. However, this represented equilibration, not cessation of RNA synthesis, since a second set of Millipore filters shifted to ^3H -uridine for 2 hr incubation periods between 8 and 14 hr continued to incorporate uridine at the original rate. Thus the apparent decline simply represented a resultant between the breakdown of previously synthesized RNA and the fabrication of new. To avoid this complication, the complete time course of RNA synthesis was followed by pulsed incorporation studies. Duplicate Millipore filters were exposed to ^3H -uridine between 2 and 4 hr and then harvested; two other Millipore filters between 4 and 6 hr; two more between 6 and 8 hr and so forth. In this manner a succession of 2 hr pulses was administered over a 26 hr period. Figure 2 shows the results. The counts incorporated during each pulse were added to the previous ones to provide a measure of the cumulative incorporation. The net rate is seen to have remained constant for about 20 hr and then to have declined at the end of fruit construction.

Three aggregateless strains of *Dictyostelium discoideum* yielded the same general

pattern of incorporation except that they continued to increase at a high rate over the period in which the wild-type rate declined. The data for one mutant, Agg-204, are summarized in Fig. 2.

The problem of bacterial contamination

When one examines the synthesis of developmentally regulated cell components which are unique to the slime moulds, i.e. certain enzyme activities, antigenic determinants, etc., the presence of the bacterial associate in small quantities creates no

Fig. 1

Fig. 2

Fig. 1. —○—, Cumulative incorporation of ^3H -uridine into TCA-insoluble material. The uridine was added 4 hr after the amoebae were dispensed on Millipore filters. —●—, ^3H -uridine incorporated during 2 hr exposures starting at 8, 10, 12, and 14 hr, respectively.

Fig. 2. Pulsed incorporation of ^3H -uridine by *Dictyostelium discoideum*: —○—, wild type; —●—, mutant Agg-204. Each point (the mean of duplicate samples), represents the counts/min. incorporated during exposure of the amoebae to ^3H -uridine for the preceding 2 hr. Each successive 2 hr pulse was added to the previous ones to provide a measure of cumulative incorporation. Under these conditions aggregation was completed in 10 hr, pseudoplasmodia formed by 12 hr, fruiting body construction started at 15–16 hr and was completed by 23–24 hr.

ambiguities. When, however, the component is common to both, as is true in the present instance, the possibility of bacterial contributions must be eliminated. In the case of RNA synthesis, a sensitive criterion does fortunately exist, namely the comparative elution patterns of ribosomal RNA from bacteria and that from slime mould cells during MAK-column chromatography. The former is distributed in two well-defined peaks corresponding to the 16 and 23 S fractions (Hayashi, Hayashi & Spiegelman, 1963). The latter is eluted as a single peak and is released by a salt

concentration different from those for either of the bacterial fractions. Figure 3A shows the elution of RNA synthesized by the slime mould cells prepared as described in the Methods section during 20 hr incubation on Millipore filters in the presence of ^3H -uridine. Unlabelled carrier RNA from wild-type amoebae was added for purposes of comparison. The labelled RNA is seen to have been eluted coincidentally with the carrier RNA. The degree of bacterial contamination in this experiment, as revealed

Fig. 3. A: MAK-column chromatography of RNA from amoebae prepared as in Methods and labelled with ^3H -uridine during 20 hr incubation on Millipore filters. Extraction E_{280} was measured in a continuous flow cell with the Gilford recording spectrophotometer. Radioactivity ($-\circ-$) measured in TCA-precipitable material from 1 ml. samples of the eluate. Upper curve shows the gradient of NaCl concentration in the eluate measured by a calibrated refractometer. B: Chromatography of RNA from amoebae not pretreated in SM+streptomycin medium and labelled with ^3H -uridine between 14 and 20 hr during incubation on Millipore filters.

by colony counts, is given in Table 1. Figure 3B shows the extent to which the presence of the bacterial associate even in relatively low concentration affected the elution pattern of the RNA. The amoebae were not pre-incubated in SM+streptomycin medium but instead were washed three times in cold water by 5 min. centrifugation at 1200g and were dispensed directly on to Millipore filters. The ^3H -uridine was administered over a 6 hr period (14–20 hr of incubation). The elution pattern is typical of *Aerobacter aerogenes* RNA with the exception of the barely significant peak at the refractive index of 1.3425 (similar to that of the slime mould RNA).

Table 1 shows that under these conditions the degree of contamination at the time of exposure to uridine was 3×10^5 bacteria/ 2×10^7 amoebae or about 1 part in 10^4 by weight.

DISCUSSION

The uridine incorporation data indicate that RNA synthesis was not confined to any particular stage of slime mould development but continued at an appreciable role throughout the morphogenetic sequence. However, it should be noted that no absolute rate estimates can be made until determinations of the specific activity of the UTP pool are available.

Table 1. *Extent of bacterial contamination during the incubation of amoebae Dictyostelium discoideum on the Millipore filters*

Period of incubation on Millipore filters (hr)	Viable bacteria/ 2×10^7 amoebae	
	A*	B
0	5×10^8	5×10^7
5	—	3×10^5
15	2×10^8	3×10^5

* A, amoebae treated as described in Methods; B, amoebae harvested from growth plates, washed three times by centrifugation (1200 g for 5 min.) in cold water and dispensed on Millipore filters. After incubation the amoebae were harvested in 10 ml. sterile cold water and samples (0.05 ml.) were spread on SM agar plates and incubated at 37° overnight in order to make colony counts.

In addition, the net loss of RNA content (White & Sussman, 1961) and the evidence for equilibration seen in Fig. 1 suggest an appreciable turnover, both of the RNA that was part of the vegetative amoebae and the material synthesized during development. Parallel studies with another species, *Polysphondylium pallidum* grown in axenic medium, indicate that ribosomal RNA contributed the major portion of this turnover, such that at the end of fruit construction at least 50% of the ribosomal RNA then present was synthesized during the morphogenetic sequence (R. R. Sussman, private communication). The results of MAK-column chromatography shown in Fig. 3 indicate that appreciable amounts of ribosomal RNA were also synthesized by *Dictyostelium discoideum*. The question then arises as to why amoebae which are starving and are in the process of losing a significant proportion of their dry weight during development find it necessary to synthesize new ribosomes.

At present, numerous developmental studies are being made with phagotrophs like the slime moulds and ciliated protozoa and with forms like sea urchins, sponges and *Acetabularia* which are contaminated with bacterial parasites and symbionts. The danger that the bacteria contribute to the data is always serious. For RNA synthesis, MAK-column chromatography offers a sensitive and convenient criterion of such contamination.

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