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Ribonucleic Acid Synthesis During Morphogenesis in *Myxococcus xanthus*

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Ribonucleic acid synthesis was measured during the morphogenesis of *Myxococcus xanthus*. After induction of microcyst formation by the addition of glycerol to an exponential culture, net ribonucleic acid (RNA) synthesis was immediately terminated (measured either chemically or by the accumulation of acid-insoluble radioactivity). Extensive RNA turnover did take place, however, including RNA made both before and after induction. Sucrose gradient centrifugation revealed that ribosomes and ribosomal RNA were synthesized during microcyst formation even though there was no net RNA synthesis. Base analyses of the total RNA of vegetative cells and 120-min microcysts were indistinguishable.

The bacterium *Myxococcus xanthus* has a complex life cycle consisting of vegetative growth, aggregation, fruiting body formation, microcyst formation, and germination. One aspect of this life cycle, microcyst formation in liquid culture, provides a controlled system for studying biochemical events during morphogenesis. Microcyst formation can be induced by the addition of glycerol to a vegetative culture (3, 4). Transition of the vegetative cells to microcysts (resting cells) is quantitative and relatively synchronous, as measured by phase-contrast microscopy (3) and termination of deoxyribonucleic acid (DNA) synthesis (10).

In this report we present data on ribonucleic acid (RNA) synthesis during the early stages of microcyst formation. A preliminary report has appeared (Bacteriol. Proc., p. 92, 1966).

MATERIALS AND METHODS

Organism and cultivation. M. xanthus strain FB (2) was grown in 2% N-Z Case (Sheffield Chemical, Norwich, N.Y.) containing 0.1% $MgSO_4 \cdot 7H_2O$ at 30 C with gyratory shaking. The doubling time under these conditions is 270 min.

Induction of microcyst formation. Microcyst formation was induced by the following modification of the glycerol technique of Dworkin et al. (3, 4). Prewarmed 10 M glycerol was added directly to an exponentially growing culture (0.5 M final concentration), thus avoiding the centrifugation step and temperature changes previously employed. This simplified procedure was made possible by eliminating phosphate buffer from the growth medium. In all experiments, exponentially growing cultures were defined as those having an absorbance at 560 mμ of 0.5 or less; an optical density value (at 560 mμ) of 0.5 corresponds to 5×10^8 cells/ml. Incubation of the induced culture was continued as above. Microcyst formation is synchronous and morphologically complete in 120 min, as judged by phase-contrast microscopy.

Kinetics of RNA synthesis. RNA synthesis was measured by incorporation of either uridine-5-^3H or uridine-2-^{14}C (Schwarz BioResearch, Inc., Orangeburg, N.Y.) into a cold trichloroacetic acid-insoluble fraction. Since the growth medium itself contains sufficient uracil compounds to support the growth of uracil-requiring strains of *Escherichia coli* and *Bacillus subtilis*, the specific activity of the uridine cannot be specified. Samples (1 ml) from the labeled culture were pipetted into 1 ml of ice-cold 10% trichloroacetic acid and left at 0 C for at least 60 min. The samples were transferred to membrane filters (HAWP 025 00 0, 0.45 μ; Millipore Corp., Bedford, Mass.) which had been soaked in 5% trichloroacetic acid containing 0.1 mg of uridine per ml. The membranes were washed four times with 3 ml of 5% trichloroacetic acid containing 0.1 mg of uridine per ml, followed by three washes with 2 ml of anhydrous ether, and air-dried. The radioactivity was measured in a liquid scintillation spectrometer (Nuclear-Chicago Corp., Des Plaines, Ill.), using for each sample 10 ml of toluene containing 40 mg of 2,5-diphenyloxazole and 0.5 mg of *p*-bis-(2,5-phenyloxazolyl)-benzene as scintillation fluid. All reported values were corrected for background.

Chemical analysis for RNA. RNA was estimated chemically after fractionation by a modification of the Schmidt-Thannhauser procedure (6). Washed cells were extracted with 10% perchloric acid (PCA) at 0 C for 90 min. The precipitate was collected by centrifugation at 6,000 × *g* for 15 min at 4 C, washed once with cold 5% PCA and once with 80% ethyl alcohol at room temperature, dissolved in 0.5 M KOH,

and incubated at 37 C for 14 hr. The alkali hydrolysate was chilled to 0 C, and an equal volume of cold 20% PCA was added and left in the cold for 1 hr. After centrifugation, the supernatant fluid was removed. The sediment was re-extracted with 1 ml of PCA, and the combined supernatant fluids were analyzed for RNA by the orcinol method (8).

For base analysis, the final combined supernatant fraction was neutralized with KOH, the $KClO_4$ was removed by centrifugation, and the resultant neutralized hydrolysate was concentrated by lyophilization. The nucleotides were spotted in (i) Whatman no. 40 filter paper and developed by descending chromatography with 160 ml of isopropanol plus 41 ml of HCl taken to 250 ml with water (11) or (ii) Whatman no. 3MM and separated by electrophoresis with 0.05 M ammonium acetate buffer (*p*H 3.5, 65 v/cm, 3 hr, 5 C). Ultraviolet-absorbing spots were eluted with 0.01 M HCl. For the paper chromatography, guanylic acid and adenylic acid were eluted together and determined by dichromic readings at 260 and 280 mμ (7). A blank area on the filter paper alongside each nucleotide spot was eluted and served as background.

Sucrose gradient centrifugation. Ribosomes and RNA were prepared from vegetative cells and 100-min microcysts for sucrose gradient centrifugation as follows. For ribosome preparations, washed cells were disrupted by sonic treatment. After removing any unbroken cells and large cellular debris by centrifugation at 27,000 × *g* for 10 min, the supernatant fluid was centrifuged at 100,000 × *g* for 2 hr. The sediment was resuspended in TKM buffer [2-amino-2-(hydroxymethyl)-1,3-propanediol, 0.01 M, *p*H 7.4; KCl, 0.005 M; magnesium acetate, 0.01 M].

RNA was prepared by resuspending cells in TM buffer [2-amino-2-(hydroxymethyl)-1,3-propanediol, 0.01 M *p*H 7.4; magnesium acetate, 0.001 M] containing 200 μg (per ml) of lysozyme (Worthington Biochemical Corp., Freehold, N.J.), 10 μg (per ml) of deoxyribonuclease I (Worthington Biochemical Corp.), and 0.3% Macaloid. After repeated freezing and thawing, sodium dodecyl sulfate was added to a final concentration of 1% and shaken at room temperature for 10 min. Any unbroken cells and debris were removed by centrifugation at 27,000 × *g* for 10 min.

Ribosomes or RNA (0.2 ml) were layered on a 4.8-ml 5 to 20% linear sucrose gradient and centrifuged in an SW39 rotor at 114,000 × *g* for 6 hr for RNA or 90 min for ribosomes at 10 C. Two drops were collected directly into scintillation vials. To each vial was added sequentially 0.5 ml of water and 10 ml of modified Brays solution (10 ml of *p*-dioxane, 0.6 g of naphthalene, 3 mg of 1,4-bis-[2-(4-methyl-5-phenyloxazolyl)]-benzene, 0.07 g of 2,5-diphenyloxazole). Radioactivity was measured in a Nuclear-Chicago liquid scintillation spectrometer.

RESULTS

Net RNA synthesis during microcyst formation. Exponentially growing *M. xanthus* was incubated with ^{3}H-uridine for several hours prior to induction of microcyst formation with glycerol. At zero-time (Fig. 1), the culture was divided into two portions. Glycerol was added to one portion, and an equal volume of water was added to the other. Samples were removed at timed intervals, and incorporated radioactivity was assayed. The typical data shown in Fig. 1 demonstrate that there was no net increase in the incorporation of uridine into the acid-insoluble fraction after induction. The control continued to accumulate radioactivity.

The suggestion that there was no net RNA synthesis during microcyst formation was examined by direct chemical analysis. Exponentially growing cells were labeled with ^{14}C-uridine for 4.5 hr. After glycerol induction, a portion of the culture was immediately harvested and RNA was determined. The remainder of the induced culture was harvested after an additional 120 min in the isotope, and RNA was then determined. immediately after harvesting, triplicate samples were assayed for acid-insoluble radioactivity. During the 120 min in glycerol, there was a net loss of 15% of the acid-insoluble radioactivity. The recovery of the radioactivities measured prior to fractionation was used to correct for RNA losses during the extraction procedures. Chemical analyses revealed that there was a 5% decrease in the absolute amount of RNA during microcyst formation.

RNA turnover during microcyst formation. To determine whether RNA synthesis occurred dur-

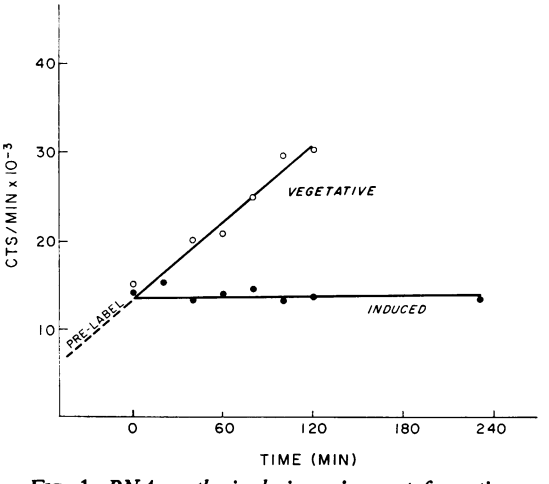

FIG. 1. *RNA synthesis during microcyst formation. ^{3}H-uridine (0.1 mc) was added to a culture of vegetative cells (25 ml, 0.2 optical density units) approximately 2 hr before induction. At zero-time, a portion of the cells was induced and the remainder retained as a vegetative control. Samples (1 ml) were removed at timed intervals and assayed.*

ing microcyst formation which did not lead to an increase in net RNA, the following type of experiment was performed. Labeled uridine and glycerol were added simultaneously to a log-growing culture. Samples were removed at intervals, and acid-insoluble radioactivity was measured. The results (Fig. 2) indicate that RNA was synthesized after induction; after 60 min, the induced culture incorporated 60% as much uridine as the control. These data can be reconciled with the absence of net RNA synthesis during microcyst formation (Fig. 1) by postulating the existence of extensive RNA turnover.

Evidence that RNA turnover also occurs beyond this 60-min period was obtained in two ways. Cells were induced in the absence of the radioactive precursor. At various times, samples were removed and incubated with the isotope for 10 min (Fig. 3). Even as late as 200 min after induction, ^{3}H-uridine was incorporated into RNA. Thus, RNA synthesis was still proceeding in the absence of RNA accumulation (Fig. 1).

Turnover was also demonstrated by "washing out" of the label from induced cells. Exponentially growing cells were labeled with radioisotope for 60 min and then induced with glycerol containing an excess of unlabeled uridine. The results (Fig. 4) indicate the loss of 50% of the radioactivity of prelabeled cells after 240 min of induction. This extensive breakdown of prelabeled RNA at the same time that there was no change in the net amount of RNA further supports the hypothesis of RNA turnover during microcyst formation. The labeled RNA of the control culture gave no evidence of breakdown in the presence of added unlabeled uridine. The reduced rate of incorporation of labeled uridine is consistent with the greatly reduced specific activity following cold uridine addition.

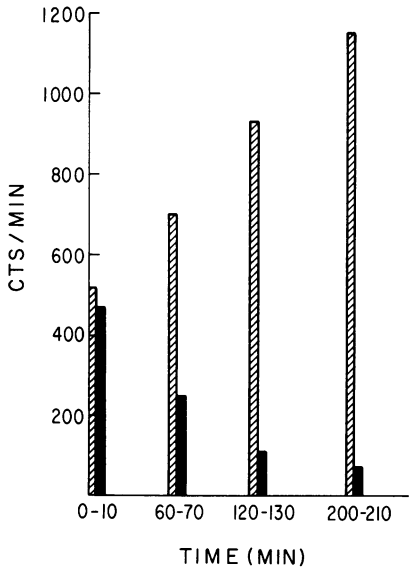

FIG. 3. *Short-term labeling of RNA during microcyst formation. Duplicate 1-ml samples of vegetative (cross-hatched) and induced cells (solid) were exposed to ^{3}H-uridine (2.5 μc/ml) for 10 min and then assayed. The abscissa represents time after induction.*

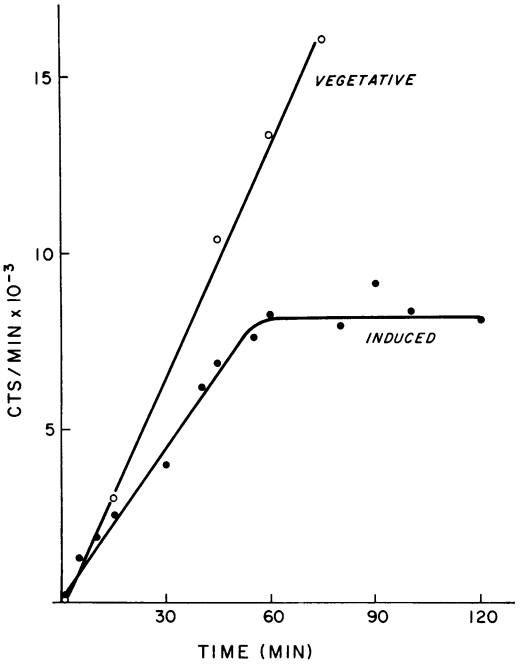

FIG. 2. *Kinetics of ^{3}H-uridine incorporation after induction. Vegetative cells were divided into two portions. Glycerol and ^{3}H-uridine (2.5 μc/ml) were added simultaneously to one, and only ^{3}H-uridine (2.5 μc/ml) to the other. Samples (1 ml) were removed at timed intervals and assayed.*

It is clear from the above experiments that some RNA made previous to induction must become labile upon induction. An experiment was performed to examine the stability of RNA made after glycerol induction. Vegetative cells were incubated with radioactive uridine for 60 min prior to induction. At zero-time, glycerol was added together with excess unlabeled uridine. A parallel culture was labeled for 60 min after induction and then chased by unlabeled uridine. The rate of disappearance of radioactivity from the two cultures is a reflection of the relative stability of the RNA made before and after induction. The data in Fig. 5 demonstrate that RNA made before and after induction was degraded during subsequent microcyst formation. The relative stabilities of the RNA preparations

cannot be ascertained, however, since the radioactive uridine monophosphate and uridine diphosphate formed by breakdown may be utilized to different extents in the two cases.

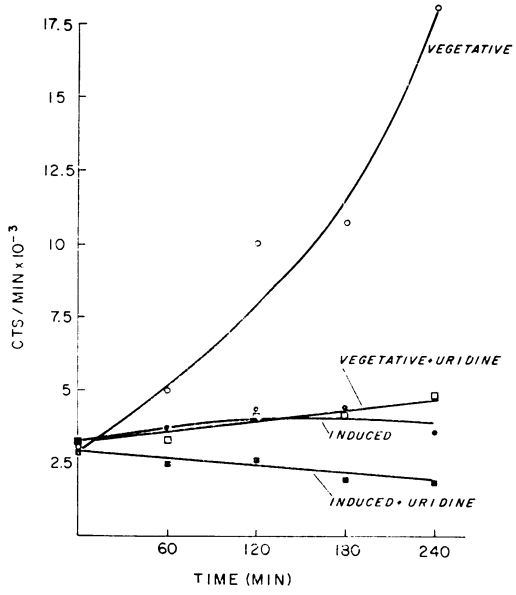

FIG. 4. *Effects of an unlabeled uridine chase on prelabeled vegetative and induced cells. Vegetative cells were labeled with ^{3}H-uridine (1.25 μc/ml) for 60 min and then divided into four portions. Cold uridine (50 μg/ml) was added as a chase to one vegetative and one induced portion. The abscissa represents time after induction and uridine addition.*

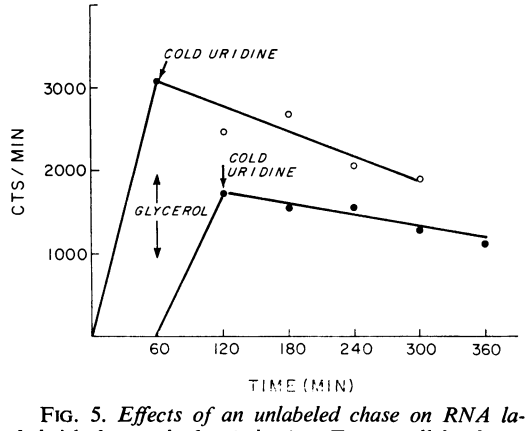

FIG. 5. *Effects of an unlabeled chase on RNA labeled before and after induction. Two parallel cultures were labeled with ^{3}H-uridine (1.25 μc/ml) for 60 min prior to addition of unlabeled uridine. One culture was induced 60 min prior to addition of the radioisotope, and the other was induced simultaneously with addition of the isotope. The loss of acid-insoluble radioactivity was measured.*

Fractionation and characterization of RNA made after induction. Base analyses of RNA from vegetative cells and from 120-min microcysts showed no significant differences (Table 1). The low uracil fraction of the total RNA might be partially due to uridine derivatives in the soluble RNA, which were not included in the analyses.

The type distribution of RNA made after induction was investigated. Cells were labeled with ^{3}H-uridine for 30-min intervals at various times after induction. Each sample was combined with ^{14}C-uridine-labeled vegetative cells, and the mixture was fractionated into a high-speed supernatant liquid and sediment. RNA made during microcyst formation was distributed similarly to the vegetative cell pattern (Table 2). The high-speed sediment containing induced ^{3}H-RNA and vegetative ^{14}C-RNA was then centrifuged in a 5 to 20% sucrose gradient. A peak of tritiated 70*S* ribosomes was found coincident with the ^{14}C ribosomes (Fig. 6). Extracted RNA was also banded in a sucrose gradient (Fig. 7), to demonstrate that the radioactivity in the 70*S* peak came at least partially from ribosomal RNA and not just from attached messenger RNA. The data clearly indicate the existence of 23*S* and 16*S* peaks. There is a slight displacement of the ^{3}H-16*S* peak, which could be due to the contribution of nonribosomal RNA of high molecular weight. A large amount of RNA of lower molecular weight was also found. Since no low-molecular-weight marker was used in this experiment, it is

TABLE 1. *Base analyses of vegetative and microcyst RNA*[a]

Base	Total RNA (mole %)		Ribosomal RNA (mole %), vegetative
	Vegetative	Microcyst	
Adenine	27.0	26.0	25.0
Guanine	34.5	34.5	32.5
Cytosine	21.5	22.5	23.0
Uracil	17.0	17.0	19.5

[a] Data represent the average of at least three determinations.

TABLE 2. *Distribution of uridine incorporated during microcyst formation*

Fraction[a]	Uridine incorporated (counts/min)		
	0–30 min	60–90 min	90–120 min
Sediment	39,500	79,500	65,000
Supernatant fluid	4,300	9,500	7,500

[a] Obtained by centrifugation at 100,000 × *g*.

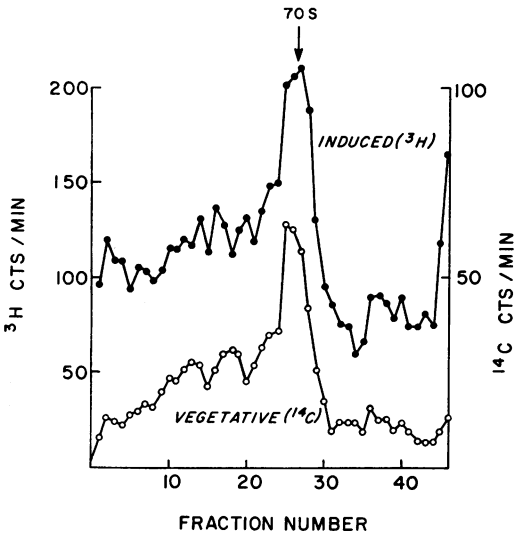

FIG. 6. *Sedimentation analysis of ribosomes from vegetative cells and microcysts. Induced cells were exposed to* ^{3}*H-uridine (3 μc/ml) from 70 to 100 min after glycerol induction and then combined with vegetative cells labeled with* ^{14}*C-uridine (0.01 μc/ml) for one generation. Ribosomes were isolated from the combined cells, layered on a sucrose gradient, and centrifuged for 2 hr at 114,000 × g.*

difficult to estimate the sedimentation constant of this material. However, similar runs suggest that this RNA has a broad peak at approximately 9S and could be microcyst messenger RNA or ribosomal RNA breakdown products.

DISCUSSION

Net RNA synthesis during microcyst formation. The data presented demonstrate the lack of accumulation of RNA during the formation of microcysts in liquid culture. The use of radioactive uridine incorporation as a measure of total RNA is justified, since the base composition of vegetative cells and microcysts are indistinguishable and the incorporated radioactivity is alkali-sensitive. Since the base composition of total and ribosomal RNA differ significantly, whereas the base composition of the total RNA of the two cell types remains constant, it is suggested that the type distribution of RNA is not drastically altered during microcyst formation. For example, the mole fraction of uracil in total RNA is 0.17 and in ribosomal RNA it is 0.195 (standard deviation, 0.009).

It was reported by Sadler and Dworkin (10) that RNA synthesis increased in a two-step manner after induction. The discrepancy between

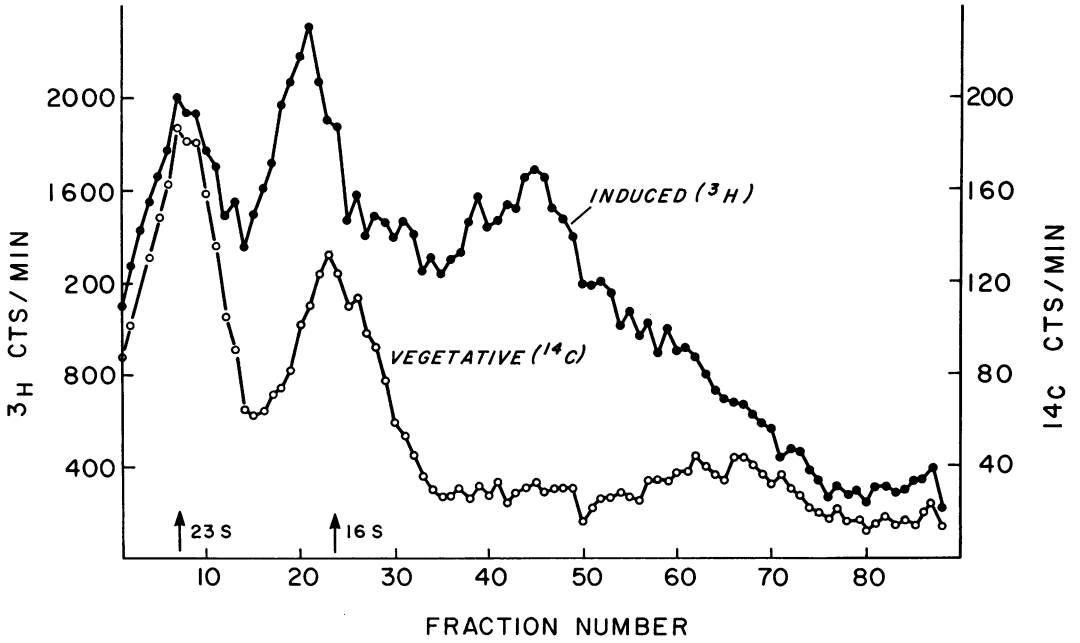

FIG. 7. *Sedimentation analysis of RNA made during microcyst formation. Induced cells were exposed to* ^{3}*H-uridine (3 μc/ml) from 70 to 100 min after induction and then combined with vegetative cells labeled with* ^{14}*C-uridine (0.009 μc/ml) for one generation. RNA was extracted, layered on a sucrose gradient, and centrifuged for 6 hr at 114,000 × g.*

our results and theirs might be due to solubilization of polysaccharides and glycoproteins during the hot-acid extraction used by those workers. With the orcinol procedure, such material would have been included in total ribose. This work has been repeated by Dworkin and the absence of net RNA synthesis affirmed. They have not, however, been able to demonstrate the abrupt termination of uridine incorporation at 60 min after induction (*personal communication*).

The termination of net RNA synthesis is, within experimental error, an immediate consequence of glycerol addition. The mechanism for this phenomenon is unknown. The following data demonstrate that the cells remain metabolically active for several hours after induction: (i) electron microscopy has revealed extensive changes in fine structure for at least 5 hr (Bacon and Eiserling, *in preparation*); (ii) DNA continues to accumulate for 150 min (9); and (iii) protein increases 33% during the first 110 min (11). These results also suggest that RNA turnover takes place in the absence of net RNA synthesis.

Problems in the quantitation of RNA turnover. Theoretically, the rate of RNA turnover can be measured quantitatively by blocking synthesis with actinomycin D. In *M. xanthus*, however, the concentration (10 μg/ml) of the drug sufficient to completely inhibit RNA synthesis results in the degradation of over 40% of the vegetative RNA.

The data obtained from the pulse and chase experiments (Fig. 3–5), demonstrating RNA turnover during microcyst formation, cannot be used to make quantitative statements as to the extent of this turnover, since complex precursor pool effects may exist. In one extreme case, it is possible that nucleosides and their derivatives formed by the breakdown of pre-existing RNA are used exclusively for resynthesis. In the other extreme case, the exogenously added uridine would equilibrate completely and rapidly with the internal pools. This can occur either by pool expansion or exchange reactions. Since we have been able to demonstrate the utilization (at least, to some extent) of exogenously added uridine in the absence of net synthesis, the first extreme case is clearly excluded. Similar equilibration problems have been investigated with reference to *B. subtilis* and *E. coli* (D. Nierlich, Bacteriol. Proc., p. 130, 1966). In these bacteria, added nucleosides do not equilibrate with the internal pools. Until the specific activities of the exogenous uridine and of the pools are compared in *M. xanthus*, we cannot state whether the added uridine equilibrates partially or completely. Since microcyst formation is not a steady-state phenomenon, the participation of added uridine in RNA turnover may also vary with time. Therefore, we can make only qualitative statements and minimum estimates of RNA turnover.

In the pulse experiments (Fig. 3), the incorporation of uridine during microcyst formation decreased from 90% during the first 10 min to 10% at 200 min, relative to the vegetative control. Since there is no net RNA synthesis, these percentages define minimal turnover rates.

Comparison of RNA synthesis during endospore and microcyst formation. Microcyst and spore formation are both intracellular differentiations which can be stimulated by appropriate environmental conditions and which result in profound morphological and biochemical changes. In microcyst formation, the stimulus can be the addition of glycerol to a vegetative culture. Within the first few minutes after glycerol addition, net RNA synthesis and chromosome initiation are terminated (9). Thus, by use of this simple triggering device, a more rapid and synchronous transformation than exists during sporulation can be achieved. It is interesting to note that 0.1 M dimethylsulfoxide can also be used to induce microcyst formation (D. Zusman, *unpublished data*). This supports the hypothesis of Sadler and Dworkin that the inducers act in a physical rather than metabolic manner (10). Another advantage in studying biochemical events during microcyst formation is that the entire vegetative cell is converted into the microcyst. In endospore formation, compartmentalization makes the interpretation of experiments more difficult.

The most striking similarity between sporulation and microcyst formation with regard to RNA synthesis is the extensive RNA turnover in the absence of net synthesis. From the onset of sporulation ($t_0 - t_1$), net RNA synthesis ends, yet there is a turnover of all classes of RNA (1). During sporulation, this turnover leads to an enhanced 4*S* RNA fraction (1). Although a direct examination of this sort has not been carried out in *M. xanthus*, data from base analyses lend no support for a similar accumulation.

It has been suggested that the spore genome, repressed during vegetative growth, may contain 100 or more genes (5). Although no such estimate is presently available for the "cyst genome" of *M. xanthus*, the resistance properties and structural features of the microcyst suggest at least several cyst-specific genes. It should be interesting to examine the size of the "cyst genome" and the properties of the cyst-specific RNA. As more information becomes available on the kinetics and type distribution of this RNA, a comparison with the better-studied endospore

system should lead to an improved understanding of the general problems of morphogenesis and differentiation.

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LITERATURE CITED

1. BALASSA, G. 1966. Renouvellement des arn et des protéines au cours de la sporulation de *Bacillus subtilis*. Ann. Inst. Pasteur **110**:316–347.
2. DWORKIN, M. 1962. Nutritional requirements for vegetative growth of *Myxococcus xanthus*. J. Bacteriol. **84**:250–257.
3. DWORKIN, M., AND S. GIBSON. 1964. A system for studying microbial morphogenesis: rapid formation of microcysts in *Myxococcus xanthus*. Science **146**:243–244.
4. DWORKIN, M., AND W. SADLER. 1966. Induction of cellular morphogenesis in *Myxococcus xanthus*. I. General description. J. Bacteriol. **91**:1516–1519.
5. HALVORSON, H. O. 1965. Sequential expression of biochemical events during intracellular differentiation. Symp. Soc. Gen. Microbiol. **15**:343–368.
6. LESLIE, I. 1955. The nucleic acid content of tissues and cells, p. 2–5. *In* E. Chargaff and J. M. Davidson [ed.], The nucleic acids, vol. 2, Academic Press, Inc., New York.
7. LORING, H. S. 1955. Hydrolysis of nucleic acids and procedure for the direct estimation of purine and pyrimidine fractions by absorption spectrophotometry, p. 199–202. *In* E. Chargaff and J. M. Davidson [ed.], The nucleic acids, vol. 1, Academic Press, Inc., New York.
8. MEJBAUM, W. 1939. Uber die Bestimmung kleiner Pentosemengen, insbesondere in Derivaten der Adenylsäure. Z. Physiol. Chem. **258**:117–120.
9. ROSENBERG, E., M. KATARSKI, AND P. GOTTLIEB. 1967. Deoxyribonucleic acid synthesis during exponential growth and microcyst formation in *Myxococcus xanthus*. J. Bacteriol. **93**:1402–1408.
10. SADLER, W., AND M. DWORKIN. 1966. Induction of cellular morphogenesis in *Myxococcus xanthus*. II. Macromolecular synthesis and mechanism of inducer action. J. Bacteriol. **91**:1520–1525.
11. WYATT, G. R. 1955. Separation of nucleic acid components by chromatography on filter paper, p. 255–257. *In* E. Chargaff and J. M. Davidson [ed.], The nucleic acids, vol. 1, Academic Press, Inc., New York.