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Induction of Spherule Formation in *Physarum polycephalum* by Polyols

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A method has been developed for inducing spherule formation (spherulation) in the myxomycete *Physarum polycephalum* by transferring the culture to synthetic medium containing 0.5 M mannitol or other polyols. This morphogenetic process occurred within 12 to 35 hr after the inducer was added. The mature spherules existed as distinct morphogenetic units, in contrast to the clusters of spherules formed during starvation. Ninety per cent of the spherules germinated by 24 hr in synthetic medium. The changes in the synthesis of ribonucleic acid (RNA), deoxyribonucleic acid (DNA), and protein during plasmodial growth, spherulation, and germination of spherules are described. When spherule formation was completed, RNA, protein, and DNA decreased, compared with the values at the beginning of the conversion. The incorporation of ^{3}H-uridine into trichloroacetic acid-insoluble material was different in each of these periods, and this incorporation was sensitive to actinomycin D. The amount of glycogen increased during growth, whereas it decreased during spherulation. ^{14}C-glucose could be taken up by the cells in the presence of the inducer, and mannitol could not replace glucose as a source of energy. The mode of action of mannitol and its mechanism of induction are discussed.

When subjected to adverse environmental conditions, the slime mold *Physarum polycephalum* may form sclerotia directly from the plasmodium. The sclerotium is a resistant structure, consisting of clusters of spherules which may play an important role in the survival of the myxomycete. Jump (11) induced the formation of sclerotia by subjecting the myxomycetes to low temperature, desiccation, sublethal concentrations of heavy metals, or starvation. He also found that, under starvation conditions, 0.5 M sucrose could induce formation of sclerotia in stationary cultures after 2 to 3 days. It has been reported (15) that microcyst formation of the cellular slime mold *Polysphondylium pallidum* can be induced by high osmotic pressure caused by salts such as KCl.

Since research on spherule formation (spherulation) and on the biochemical events occurring during sclerotization has previously been carried out with a semidefined medium (3) under starvation conditions (4), we sought a method of bringing about these morphogenetic changes with a fully defined synthetic medium without subjecting the myxomycete to starvation conditions.

This paper describes a new system for spherule formation, together with some experiments designed to discover more about the mechanism of induction. In addition, experiments concerned with the macromolecular synthesis associated with this conversion and with spherule germination are described.

MATERIALS AND METHODS

Axenic cultures of *P. polycephalum* were grown as a suspension of the microplasmodia in a defined synthetic medium (AV-40; reference 5). Flasks containing 20 ml of liquid medium were shaken on a reciprocating shaker in a dark room at 22 C. After 72 hr, the plasmodia were centrifuged and then transferred to the same medium containing 0.5 M mannitol.

To measure macromolecular synthesis, 5-ml samples (containing 1.5 ml of compact cells) were serially removed and washed three times with synthetic medium. The microplasmodia or the spherules were sonically disrupted in a Branson Sonifier for 3 min at 0 C. The samples were fractionated (12).

Ribonucleic acid (RNA) was measured as ribose released after 1 hr of incubation with 30 μg of pancreatic ribonuclease (Calbiochem) per ml, by a modification (13) of the orcinol method (2).

Deoxyribonucleic acid (DNA) was analyzed by the diphenylamine method (1), and the protein was estimated by the biuret method (8). For glycogen analysis, the microplasmodia were hydrolyzed with 30% KOH at 100 C for 15 min. The polysaccharide was then precipitated with ethyl alcohol according to Van Handel (17) and measured quantitatively with anthrone reagent (15). This polysaccharide has been identified enzymatically to be glycogen (7). The isotopes ^{14}C-D-glucose (240 mc/mmole), ^{3}H-protein-hydrolysate, and uridine-5-^{3}H (4 c/mmole) were

purchased from Schwarz BioResearch Inc., Orangeburg, N.Y.; uniformly labeled-^{14}C-D-mannitol (197 mc/mmole) was purchased from International Chemical and Nuclear Corp., Irvine, Calif. To measure the incorporation of radioactive compounds, these isotopes were added to the growth medium at concentrations of 0.5 μc/ml. Samples of 5 ml were serially removed, centrifuged at 500 $\times$ g for 5 min, and then washed three times with the synthetic medium until the radioactivity of the washings decreased to background level. The microplasmodia and the spherules were sonically disrupted and fractionated as described. The radioactivity was determined in a liquid scintillation spectrometer (Packard).

RESULTS

Spherule induction. The presence of 0.5 M D-mannitol in the synthetic medium induced a high percentage of spherules, as determined by direct examination with phase-contrast microscopy, whereas lower or higher concentrations were less effective (Fig. 1). The spherules were formed in clusters, but several hours later most of them had separated (Fig. 3a). Figure 2 shows the increase in percentage of spherule formation during 60 hr. About 30% of the spherules had already formed after 15 hr, and most of the conversion occurred within 12 to 35 hr. When the developing spherules were transferred to fresh medium without mannitol, reversion to microplasmodia occurred rapidly; however, this reversion was inhibited when the spherules were transferred to mannitol-containing medium.

Spherule germination. In distilled water, spherules germinated after 12 hr, but they did not develop further. In the synthetic medium, spherules began germination after 5 to 6 hr. About 90% of the spherules germinated by 24 hr, and almost all of the remaining spherules subsequently germinated. Figure 3 shows phase-contrast photomicrographs of the sequence of morphological events during germination of the individual cells. It is clearly shown that the spherule germinates first as a germination tube (Fig. 3b–g), similar to spore germination by the pore method (9), and at the end of the germination the spherule is converted into a microplasmodium (Fig. 3h).

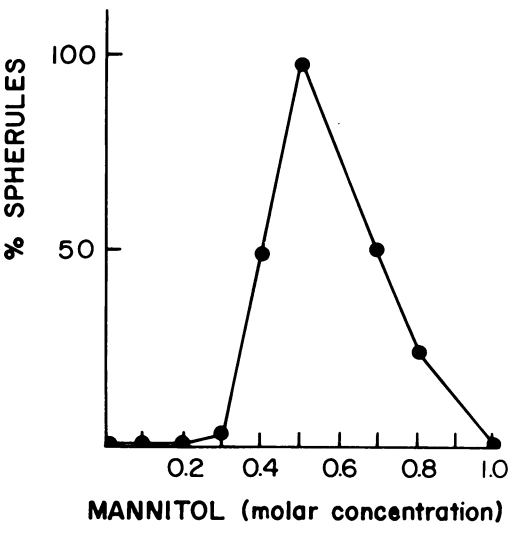

FIG. 1. *Effect of mannitol concentration on the percentage of spherule formation after 72 hr.*

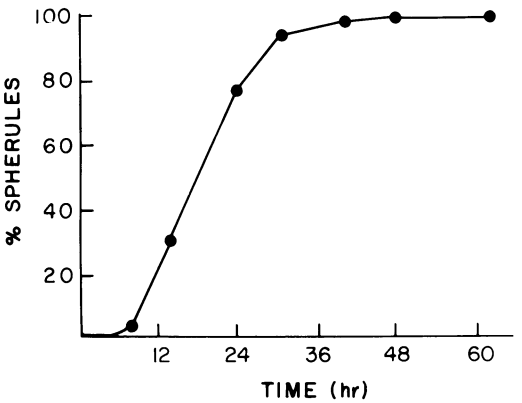

FIG. 2. *Induction of spherule formation during 60 hr in the presence of 0.5 M mannitol.*

Macromolecular synthesis. Figure 4 shows the changes in RNA, DNA, and protein content during three periods: growth, spherulation in the presence of mannitol, and germination. In the growth phase, RNA increased rapidly; similar curves were obtained for DNA and protein. The macromolecular content increased about three to four times during this period. After transfer to the mannitol-supplemented medium, a sharp decrease in total amounts of RNA and protein was observed in the initial 6 hr, whereas the decrease in DNA was only moderate. When spherule formation was completed, after 46 hr, the contents of RNA, protein, and DNA were only 13.0, 23.0, and 52.5%, respectively, compared with the values at the beginning of the process. When the spherules were transferred to fresh synthetic medium, a significant increase in RNA and protein content was observed (33.0 and 15.0%, respectively) during the next 24 hr, whereas the increase in DNA was negligible during this time.

Incorporation of ^{3}H-uridine and the effect of actinomycin D. The incorporation of ^{3}H-uridine into material insoluble in 5% trichloroacetic acid is shown in Fig. 5. Actinomycin D, added at zero time at a concentration of 300 μg/ml, caused significant inhibition of ^{3}H-uridine incorporation

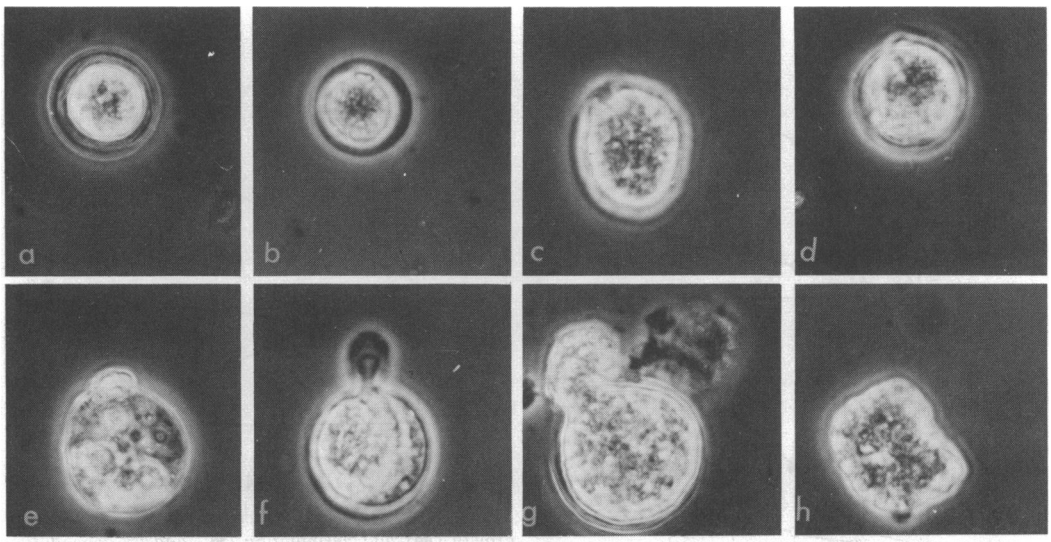

FIG. 3. *Germination of a single spherule. (a) A single spherule; (b–c) beginning of germination, 5–6 hr after transfer to a synthetic medium; (d–f) appearance of a germinating tube after 15 hr; (g) cytoplasm flows out after about 20 hr; (h) a new microplasmodium is formed after 24 hr.* (× 500).

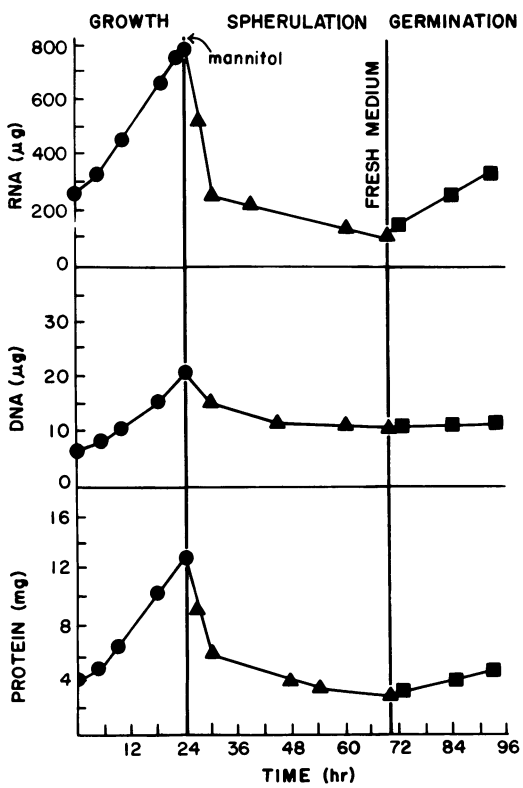

FIG. 4. *Synthesis of RNA, DNA, and protein, during growth, spherulation, and germination. (The cultures were transferred to synthetic medium containing mannitol after 24 hr of growth. The spherules were again transferred to fresh medium 46 hr after the beginning of spherulation.) The nucleic acids and protein were evaluated in 5-ml samples.*

and inhibited growth. As a result of the presence of actinomycin D, the spherules formed seemed to be abnormal; most of them were misshapen, and in some plasmodia there was an incomplete cleavage into spherules. The percentage of germination of spherules formed in the presence of actinomycin D was very low. During the growth period, as during germination, the incorporation was rapid from the very beginning, whereas during spherule formation there was a lag period of about 2 hr.

Glycogen content. The glycogen content changed during growth, spherule formation, and germination (Fig. 6). The amount of glycogen more than doubled during growth, whereas it decreased greatly 20 hr after transfer to a mannitol-supplemented medium. The glycogen increased relatively quickly during the first 6 hr of germination and then increased more slowly.

The capacity of the plasmodium to incorporate ^{14}C-glucose and ^{14}C-mannitol into glycogen in the presence of 0.5 M D-mannitol was examined (Fig. 7). The amount of mannitol incorporated into glycogen and also into the 5% trichloroacetic acid-insoluble material was very small.

Effect of other compounds on spherule formation. In an attempt to learn more about the mechanism of action of mannitol, various compounds were tested. It was found that all the polyols

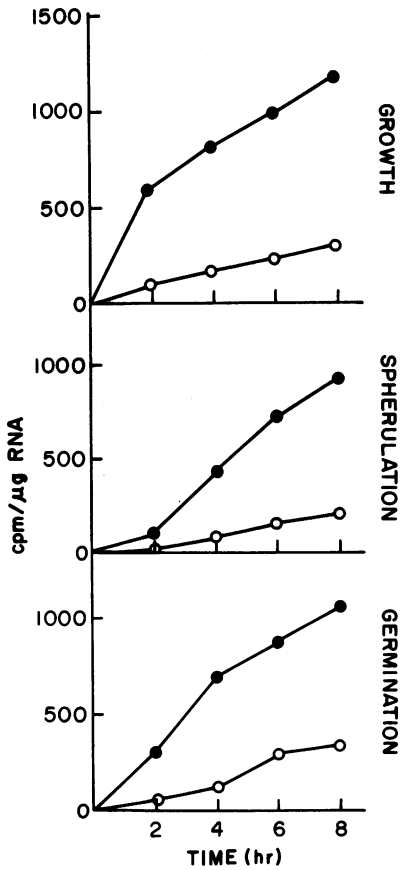

FIG. 5. *Incorporation of ^{3}H-uridine into RNA during growth, spherulation, and germination* (●) *and the effect of actinomycin D on this incorporation* (○).

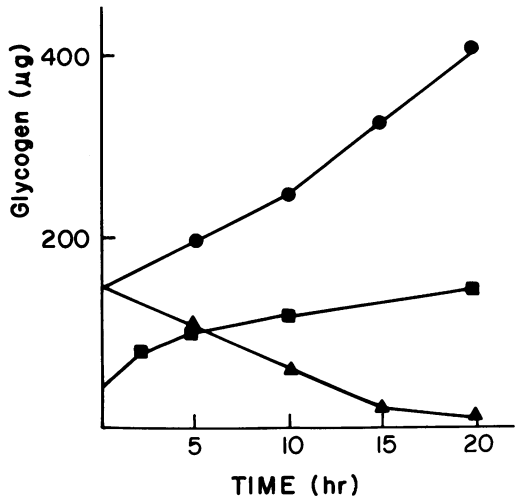

FIG. 6. *Synthesis of glycogen in the cells during growth* (●) *and germination* (■), *and its degradation during spherulation* (▲). *The glycogen was evaluated in 5-ml samples.*

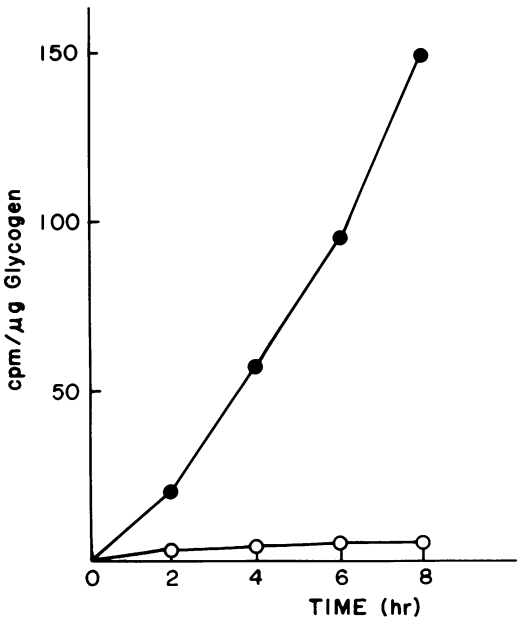

FIG. 7. *Incorporation of ^{14}C-glucose* (●) *and ^{14}C-mannitol* (○) *into glycogen in the presence of 0.5 M mannitol.*

tested, but only several sugars, could induce the morphogenetic process (Table 1). The hexosamines were toxic to the mold. The addition of 0.08 to 0.6 M of the alcohols (ethyl alcohol, propanol, isopropanol, ethylene glycol) or the salts (ammonium sulfate, potassium chloride, sodium glycerophosphate), both of which had morphogenetic activity in other systems (14, 16), had no effect on spherule formation with *Physarum*.

The polyols were the best inducers of spherule formation in a synthetic medium but had a very slight effect in a non-nutrient salt medium.

DISCUSSION

Our experiments describe a new method of inducing spherule formation in *P. polycephalum* by the addition of 0.5 M mannitol and other polyols to cultures grown on a synthetic medium. This system is different from others described (4, 11), since the whole morphogenetic process occurs in a defined growth medium without requiring a period of starvation in a non-nutrient medium. The discrete spherules induced by polyols should provide useful material for studying the process of germination of individual spherules and the biochemical reactions leading to spherule formation.

The total amounts of RNA, protein, and DNA

were shown to increase during growth, but all showed a decrease during spherulation. The incorporation of ^{3}H-uridine as a measure of RNA synthesis was studied, and the rate was found to be different in each of the three stages: growth, spherulation, and germination. The incorporation was very rapid during growth, but after addition of mannitol a lag phase of 2 hr was observed, and the rate of incorporation was lower. Although there was a threefold drop in total RNA from 2 to 6 hr postinduction, the specific activity of the RNA increased about sevenfold during the same period, indicating a high turnover during the morphogenetic process. There was a very short lag phase at the beginning of germination, but after 1 hr the rate increased and the incorporation was similar to that during the growth stage. The lag in incorporation at the start of germination may be owing to the lower permeability of the spherule walls or to the high osmotic pressure conditions resulting from the presence of mannitol. It was found that the incorporation of ^{3}H-uridine in all three stages was partially inhibited by actinomycin D.

When actinomycin D was added with the mannitol, it prevented the development of completely normal spherules. The percentage of germination of these spherules was very low. These results suggest that new RNA synthesis is essential to the process of spherule formation.

When the incorporation of ^{14}C-glucose into glycogen was compared with that of ^{14}C-mannitol, it was found that the incorporation of mannitol was very low, and it does not seem likely that mannitol can replace glucose as a carbon source.

The glycogen content increased during growth and germination, but decreased to a very low level during spherule formation. This decrease was not as sharp as that found during starvation (J. J. McCormick, *personal communication*), but indicates that the slime mold may need this energy source during conversion. In contrast with spherule formation under starvation conditions, in our system the glucose entered the cell and became incorporated into glycogen (Fig. 7).

The most effective inducers were found to be polyols; the only other compounds tested which induced spherule formation were several sugars. The fact that high concentrations of salts did not induce spherule formation suggests that this morphogenetic process is not the result simply of high osmotic pressure, as was found in microcyst formation of *P. pallidum* (16). The inducers stimulating spherule formation in *Physarum* seem to have a similar effect to that observed with another group of inducers affecting microcyst formation in *Myxococcus xanthus* (6). It has been suggested that inducers of microcyst formation may act by altering the membrane-DNA relationship (14), and it was found that mannitol inhibits specific enzyme production in higher plants (10). It may be that one of these situations exists also with spherule induction in *P. polycephalum*. Further elucidation of the problem will involve a study of the initial reaction brought about by the inducer and the characterization of the macromolecular components synthesized during both spherulation and germination.

TABLE 1. *Effect of carbohydrates on spherule formation*

Compound	Percentage of spherules after 48 hr
Mannitol	98–100
Galactitol	95–98
Erythritol	95
Arabitol	95
Ribitol	90
Glycerol	85
Inositol	85
Sorbitol	80
Lactose	80
Fructose	70–80
Mannose	65
Sorbose	50
Sedoheptulose	30
Cellobiose	10
Ribose	8
Glucose	5
Galactose	5
Arabinose	5
Glucuronic acid	0
Phosphoglyceric acid	0

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