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Differential Synthesis of an Alkaline Endonuclease in *Physarum polycephalum*

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Abstract. During the sclerotization of microplasmodia of *Physarum polycephalum* in non-nutrient salt medium or in salt medium supplemented by glucose, RNA or nucleotides a 6-fold increase in the specific activity of an alkaline endonuclease was found within 6 h after the induction. The increase was based on de novo synthesis of the enzyme and it was strongly correlated to the sharp drop in the level of cellular RNA in the first hours of the process of scerotization. The induction in exhausted growth medium or in salt medium supplemented by protein or mannitol showed a gradual 2–3-fold increase of the endonuclease in 30 h, parallel to the gradual decrease of the RNA. No changes in the specific activity of the endonuclease were found during logarithmic growth or under conditions of starvation without the induction to sclerotization.

The alkaline, polyA-specific endonuclease could possibly regulate the turnover of RNA.

Key words: *Physarum polycephalum* – PolyA-specific endonuclease – RNA turnover – Sclerotization.

Sclerotization, the formation of spherules, is a form of differentiation of the acellular slime mold *Physarum polycephalum*, which occurs under adverse growth conditions (Hüttermann, 1973; Chet and Rusch, 1969; Kikuchi, 1971). In growth medium the induction to this process starts at a not precisely known time when the trigger substance, possibly glucose, has become exhausted (Hüttermann et al., 1971; Hüttermann, 1973). The process of sclerotization can be induced at a precise time by a transfer of logarithmically growing microplasmodia from growth medium to a non-nutrient balanced salt medium (Daniel and Baldwin, 1964). The production of the extracellular slime starts in both systems 10–15 h after the time of induction (Hüttermann et al., 1974; McCormick et al., 1970a). Sclerotia are formed in salt medium after 24–35 h or in exhausted growth medium after 40–50 h (McCormick et al., 1970b; Hüttermann, 1973; Goodman and Beck, 1974).

During sclerotization the metabolism of the plasmodia changes drastically. Within a few hours the protein catabolism increases and takes over from glycogen as the carbon and energy source of the process (Goodman and Beck, 1974). The amount of protein decreases gradually to less than 50% in 24 h, although more than half of that amount is synthesized de novo during this period (Wendelberger-Schieweg and Hüttermann, 1978). Immediately after a transfer to salt medium the RNA level drops sharply and levels off at one-third (Sauer et al., 1970; Hüttermann, 1973), although at that time of the process the major part of the RNA already consists of new classes, synthesized de novo after the induction to sclerotization (Chet, 1973).

Here we give the results of a study on the quantitative changes during the process of sclerotization of one of the nucleases of *P. polycephalum* (Polman, 1974). The attention was focused on the relation between the RNA content of the microplasmodia and the level of activity of the alkaline endonuclease, which has been implicated in the degradation of RNA during the process of sclerotization. Furthermore, we tried to distinguish between effects specific for starvation and those specific for differentiation.

Materials and Methods

Microplasmodia of *Physarum polycephalum* were cultured essentially according to Daniel and Baldwin (1964) as modified by Werry (1973). Two liter Erlenmeyer flasks with 500 ml medium were rotated on a New Brunswick Shaker at 180 rpm at 26° C in the dark and 0.5 l ones with 20 ml medium at 200 rpm. All media were based on those described (Daniel and Baldwin, 1964): non-nutrient salt medium

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contained per liter 1.82 g KH_2PO_4, 0.55 g $CaCl_2 \cdot 2H_2O$, 0.55 g $MgSO_4 \cdot 7H_2O$, 0.073 g $MnCl_2 \cdot 4H_2O$, 0.055 g $FeCl_2 \cdot 4H_2O$, 0.032 g $ZnSO_4 \cdot 7H_2O$, 0.005 g hemin (Sigma) and 3.18 g citric acid. The following additions were made per liter for glucose medium: 7.16 g glucose; for protein medium: 9.1 g lactalbumin hydrolyzate (LAH, ICN Pharmaceuticals), or: 9.1 g bactotryptone (Difco) and 1.36 g yeast extract (Difco); for RNA medium: 2 g yeast RNA (Schwarz); for medium with nucleotides: 2 g acid soluble nucleotides made from yeast RNA through hydrolysis for 70 min at 70° C in 1 N $HClO_4$ and neutralization with KOH; for mannitol medium: 91.1 g D(−)Mannitol (Merck); for semi-defined growth medium: 7.16 g glucose, 9.1 g bactrotryptone and 1.36 g yeast extract. All media were made pH 4.6 with KOH. Cycloheximide (Sigma) was used at a concentration of 10 µg/ml (Chet and Rusch, 1970b; Hüttermann et al., 1971) and actinomycin D (Merck, Sharpe and Dohme) at 70 µg/ml.

To change media microplasmodia were collected by centrifugation for 5 min at 200 × g and resuspended in new medium. If cycloheximide was added at the time of transfer to salt medium, it was also added to the growth medium just prior to centrifugation. The removal of cycloheximide was helped by an extra wash with salt medium during the transfer. Microplasmodia at high density were obtained by centrifugation for 5 min at 200 × g. They were incubated without the removal of medium in the dark at 26° C.

The samples, taken at the given times, contained approx. 1 – 2 ml settled plasmodia. All steps were carried out at 4° C except when noted. The plasmodia were washed once with 20 ml distilled water and twice with 10 ml buffer A (10 mM Tris · HCl, 10 mM KCl, pH 7.0) by centrifugation for 5 min at 1100 × g and gentle resuspension. After washing, two volumes of buffer A were added to the plasmodial pellet and Triton X-100 to 0.1 % (w/v). The plasmodia were sonicated in ice with a 150 Watt MSE sonifier with exponential microtip for 6 × 10 s (stage: low-4) with breaks of 20 s, dialyzed for 2 times 12 h against a 50 fold volume of buffer A and stored at −20° C.

The nuclease assay of 0.7 ml contained 0.1 ml extract in buffer A, denatured salmon sperm DNA (Sigma, 0.7 mg/ml), 0.21 M Tris · HCl pH 8.5 and 0.1 mM $MgCl_2$. The incubation at 30° C was stopped by chilling in ice and 0.05 ml bovine serum albumin (Sigma, 50 mg/ml BSA Fr. V) and 0.5 ml 2.5 N $HClO_4$ were added. The tubes were shaken vigorously, left standing in ice for 15 min and clarified by centrifugation for 10 min at 1500 × g in the cold. The absorbance of the supernatants was measured at 260 nm and corrected for blank values. One Unit of enzyme activity was defined as the amount of enzyme which produced under these conditions during an incubation at 30° C for 1 h an increase in the absorbance at 260 nm of 1.0 in the acid supernatant.

DNA, RNA and protein were determined as described by Sachsenmaier and Rusch (1964), Pieck et al. (1971) and Lowry et al. (1951).

Results

Microplasmodia of *Physarum polycephalum* in semi-defined growth medium showed the well-known logarithmic growth curve for protein, RNA and DNA for 40 – 50 h after inoculation. The amount of endonuclease activity increased in a similar pattern, which resulted in a constant specific activity for the enzyme of approx. 0.8 Units per mg protein during growth (Fig. 1 A). In some experiments the medium was inoculated with a nearly stationary culture and somewhat higher specific activities were found immediately after inoculation with a decrease to the normal level after a few hours of growth.

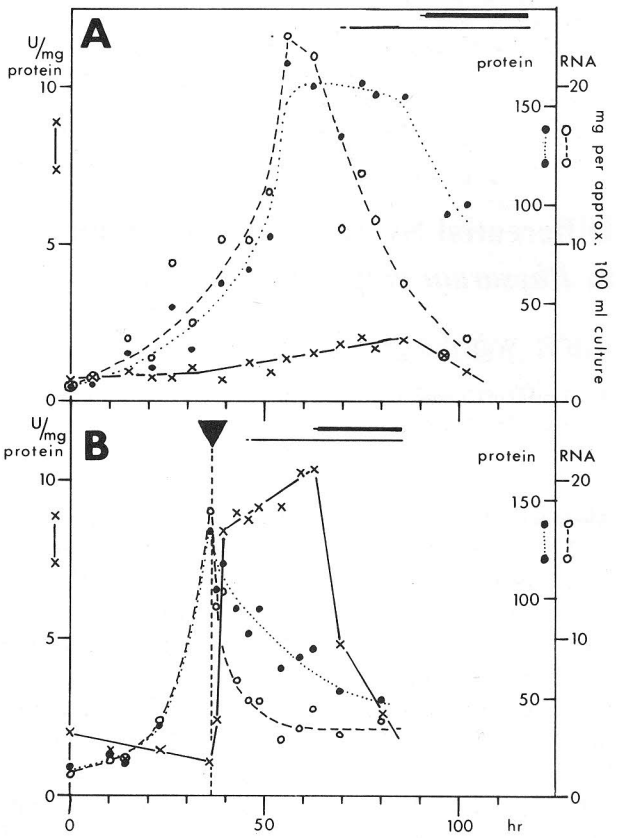

Fig. 1. Sclerotization in semi-defined growth-medium (**A**) and in non-nutrient salt medium (**B**). Endonuclease activity (×——×), protein (●·····●) and RNA (○——○) were determined as described in "Materials and Methods". The presence of slime and spherules is given at the top by the thin and thick lines, respectively. The time of the transfer in B is indicated (▶----)

A gradual rise in the specific activity of the endonuclease started 10 – 15 h before the production of the extra-cellular slime, at the presumed time of the induction to sclerotization (Fig. 1 A). This increase to 2.5 – 3 times the original level continued during the 30 h of the sclerotization process, with a decline starting at the time when the first hard-walled spherules were produced. The RNA level increased for some hours after the presumed time of the induction to sclerotization and then started to decrease gradually.

Immediately after a transfer of growing microplasmodia to non-nutrient salt medium the specific activity of the endonuclease started to increase. During the first 6 h this increase was very steep. It levelled off during the next 24 h (Fig. 1 B). A sharp drop started at the time of spherule formation. The steep increase in specific activity of the endonuclease followed by a more gradual rise showed a high correlation with the sharp drop in the level of cellular RNA immediately after the transfer, which decrease flattened out after 5 – 10 h (Fig. 1 B).

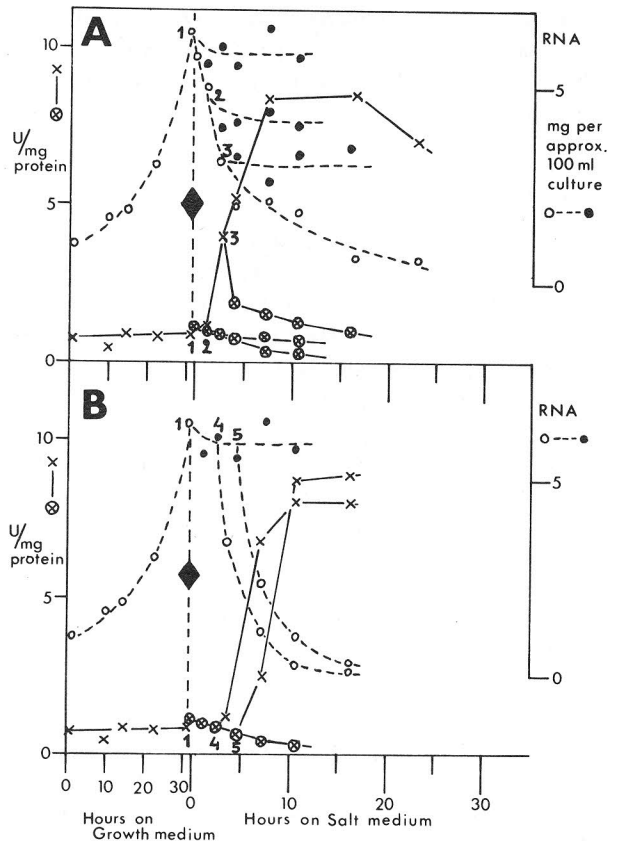

Fig. 2A and B. Sclerotization in salt medium: effect of cycloheximide. Endonuclease activity (×——×) and RNA (O---O) were determined as described in "Materials and Methods". Endonuclease activity and RNA results obtained in the presence of cycloheximide are given as (⊗——⊗) and (●---●), respectively. The transfer to salt medium is indicated (--◆--). The numbers 1, 2 and 3 give the times cycloheximide was added and 4 and 5 the times cycloheximide was removed

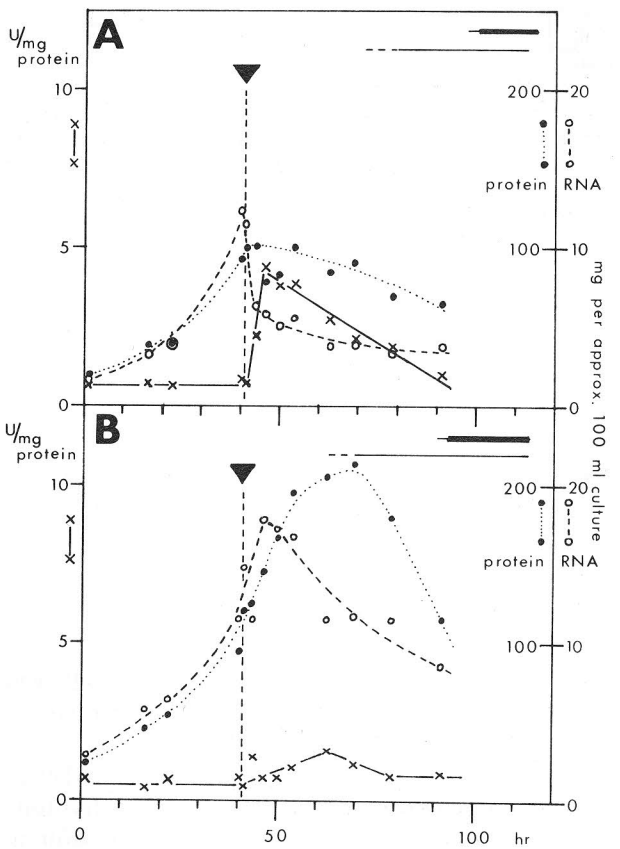

Fig. 3. Sclerotization in salt medium with added glucose (**A**) and with added protein (LAH) (**B**). Explanation of symbols: see Fig. 1

The steep increase in the specific activity of the endonuclease after a transfer to salt medium was not accompagnied by a corresponding drop in protein content. This suggested that the rate of enzyme synthesis during growth, which resulted in the constant level of specific activity, increased sharply at the time of transfer. Cycloheximide was used to test the idea of de novo synthesis of the enzyme and to study the correlation between the increase of the endonuclease and the decrease of the RNA. The addition of cycloheximide prevented any increase in the amount of endonuclease activity (Fig. 2A). The removal resulted in an immediate increase in the total and specific activity of the endonuclease, which was similar to the normal increase observed after a transfer to salt medium (Fig. 2B). These results showed that the increase of the endonuclease was based on de novo translation. Very strong effects were found for cycloheximide on the level of RNA and these effects were clearly correlated with the level of endonuclease activity. An almost immediate cessation of the decrease of RNA was found if the rise in endonuclease activity was prevented or blocked by cycloheximide (Fig. 2A) and an immediate drop in RNA was found at the moment cycloheximide was removed and endonuclease activity increased (Fig. 2B). Similar effects were found with actinomycin D but the inhibitions were not complete. When actinomycin D was added at or up to 20 min after the transfer to salt medium, the specific activity of the endonuclease still increased up to 2-fold and the level of RNA decreased slowly.

Various nutritional states of microplasmodia were chosen to distinguish between effects specific for starvation and those for differentiation. The transfer of microplasmodia to salt medium with glucose resulted in sclerotization although the process took longer than in salt medium alone and less slime was produced. The endonuclease showed a 6-fold increase in specific activity in 6 h, as was found after a transfer to salt medium, and the sharp drop in the RNA level during the initial hours after the transfer was correlated with this increase (Fig. 3A).

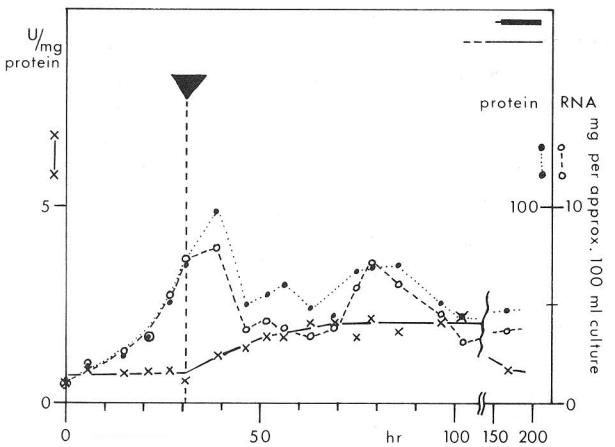

Fig. 4. Sclerotization in salt medium with mannitol. Explanation of symbols: see Fig. 1

The addition of nucleotides or RNA to the salt medium had no effect. The process of sclerotization was exactly like that with salt medium alone (Fig. 1B) and no effects were found on DNA, RNA, protein or endonuclease patterns.

In salt media supplemented with protein, either as an amino acid hydrolyzate (LAH) or as bactotryptone with yeast extract like in semi-defined growth medium, the process of sclerotization yielded identical results. The increase in the protein level after the transfer (Fig. 3B) was very similar to that found during sclerotization in growth medium (Fig. 1A). This similarity was also observed in the pattern of RNA, a transient increase followed by a gradual decrease, and in the pattern of the specific activity of the endonuclease, which showed a gradual 2 – 3-fold increase in 20 – 30 h. The correlation between the increase of the endonuclease and the start of the decrease of the RNA was identical to the one in exhausted growth medium.

This correlation was also present after a transfer of microplasmodia to salt medium with 0.5 M mannitol: a 2.5 – 3-fold increase in 30 h in the specific activity of the endonuclease and a gradual decrease in the level of RNA (Fig. 4).

The condition of starvation without the process of sclerotization is found in microplasmodia when they are centrifuged down from the growth medium to a high density. During at least 10 h at high density the specific activity of the endonuclease and the protein were constant, the DNA increased gradually to 145% and the RNA decreased very slowly to 70%. This result showed that under conditions of starvation without the induction to sclerotization no significant variations occurred in the levels of endonuclease activity and RNA, and that the increase in the specific activity of the endonuclease was correlated with sclerotization and not with starvation. This result showed further that the centrifugation procedure during all the transfers of the other experiments had no effect on protein, RNA or endonuclease.

Discussion

The alkaline endonuclease studied in this paper is identical with the DNA-degrading activity at pH 8.5 described by Polman et al. (1974). The assay conditions were adjusted to minimize the interference by other nucleolytic activities, especially the phosphodiesterases described by Hüttermann (1972). A 10-fold variation in activity of these enzymes had no effect on the level of nuclease activity measured. The alkaline nuclease has been purified from microplasmodia and was shown to be a single-strand-specific zinc-metallo endonuclease with a high specificity for polyA (results to be published elsewhere).

During growth of microplasmodia the increase in endonuclease activity parallelled the increase in protein. This constant rate of nuclease synthesis resulted in the stable level of specific activity of approx. 0.8 Units per mg protein. A similar constant value of specific activity was found throughout the cell cycle of surface plasmodial cultures (results not shown). Changes in the rate of nuclease synthesis and subsequently in the specific activity of the enzyme were found during the process of sclerotization.

The results obtained with cycloheximide and actinomycin D treatments indicated that the increases in specific activity were based on de novo synthesis and not on activation. The addition of cycloheximide to surface plasmodial cultures suppressed the increase in nuclease activity (Polman, 1974), the addition to sclerotizing microplasmodia blocked all increases and its removal gave the normal pattern of increasing endonuclease activity. The incomplete inhibition by actinomycin D, which even at concentrations of 0.3 mg/ml for several hours did not give complete inhibition of transcription (Chet, 1973; Chet et al., 1973; Hüttermann et al., 1970), was probably caused by the slow uptake in vivo. A definitive proof for de novo synthesis by density labelling with deuterated amino acids (Hüttermann and Gebauer, 1972) could not be obtained because the addition of a deuterated algal hydrolyzate would have changed the pattern of salt medium (Fig. 1B) into the pattern of salt medium with added protein (Fig. 3B).

In general, two systems for the sclerotization of microplasmodia were found. Sclerotization in salt medium was typical for one group, which included salt medium with the additions of glucose, RNA or nucleotides. The rate of nuclease synthesis increased sharply at the time of transfer, which resulted in the typical

six fold increase in specific activity. After 6 h the rate of synthesis decreased and probably stopped. The subsequent slow increase (Fig. 1 B) or decrease (Fig. 3 A) in specific activity resulted from a decreasing amount of total endonuclease activity and a relatively faster (Fig. 1 B) or slower (Fig. 3 A) declining level of protein. This protein was catabolized as the carbon and energy source of the process of sclerotization (Goodman and Beck, 1974). The need for protein catabolism was probably partially prevented by the addition of glucose to the salt medium. The final drop in endonuclease activity was caused by the inclusion of the enzyme in the sonication-resistant spherules (Chet and Rusch, 1970b).

Salt medium with added protein or growth medium exhausted for essential components (Hüttermann et al., 1971; Hüttermann, 1973), were examples for the second system of sclerotization. The transient increase of cellular RNA indicated that the protein taken up by the microplasmodia made the synthesis of nucleotides possible. A gradual decrease of RNA followed and this increased turnover of RNA was parallelled by the slightly increased rate of nuclease synthesis.

The observed correlations of the specific activity of the endonuclease with the cellular RNA level, the specificity of the endonuclease for polyA and the described regulation of mRNA turnover by the endonucleolytic degradation of polyA tails (Adams and Jeffery, 1978), suggest that the enzyme functions in the turnover of RNA, particularly mRNA. The normal RNA turnover during growth increased sharply after a transfer to salt medium. The nuclease, made in increasing amounts immediately after induction, could cause this increased RNA turnover and could supply, possibly in a concerted action with other nucleolytic activities (Hüttermann and Chet, 1971; Brand and Hüttermann, 1972; Chet et al., 1973), the nucleotides required for the synthesis of new classes of RNA, needed for differentiation (Chet and Rusch, 1970a; Chet, 1973). The attempt to supply these nucleotides from exogeneous sources as nucleotides or RNA failed; the normal, sharp drop in cellular RNA makes it reasonable to assume that they were not taken up by the microplasmodia. After a transfer to salt medium with protein the turnover of RNA remained constant for several hours. This resulted in some increase in cellular RNA after the transfer. The increasing rate of RNA turnover was parallelled by the increased rate of nuclease synthesis.

In all systems with sclerotization some form of starvation was always present. The system of salt medium with mannitol was reported to be a condition of starvation without the process of sclerotization (Hüttermann and Chet, 1971). However, we found that sclerotization did occur, although delayed by approximately 50 h, and that the patterns of RNA and nuclease were similar to those in salt medium with protein. Whether the induction to this differentiation occurred at the time of transfer with a delay of 50 h of the process of sclerotization, or after the mannitol had been metabolized (Knowles and Carlile, 1978) is not known. Starvation of microplasmodia by high plasmodial density (Nations et al., 1974; McAlister et al., 1977) showed that starvation alone, at least when no change in the turnover of RNA is found, was not correlated with changing synthesis rates of the endonuclease.

References

Adams, D. S., Jeffery, W. R.: Poly(A) degradation by two distinct processes in the cytoplasmic RNA of *Physarum polycephalum*. Biochemistry **17**, 4519–4524 (1978)

Brand, G., Hüttermann, A.: Activity and isoenzymes of RNases during starvation induced sperulation of *Physarum polycephalum*. Abstr. Physarum Meeting, Göttingen, pp. 36–37 (1972)

Chet, I., Rusch, H. P.: Induction of spherule formation in *Physarum polycephalum*. J. Bacteriol. **100**, 673–678 (1969)

Chet, I., Rusch, H. P.: RNA differences between spherulating and growing microplasmodia of *Physarum polycephalum* as revealed by sedimentation pattern and DNA-RNA hybridization. Biochim. Biophys. Acta **209**, 559–568 (1970a)

Chet, I., Rusch, H. P.: RNA and protein synthesis during germination of spherules of *Physarum polycephalum*. Biochim. Biophys. Acta **224**, 620–622 (1970b)

Chet, I., Retig, N., Henis, Y.: Changes in RNases during differentiation (spherulation) of *Physarum polycephalum*. Biochim. Biophys. Acta **294**, 343–347 (1973)

Chet, I.: Changes in RNA during differentiation of *Physarum polycephalum*. Ber. Deutsch. Bot. Ges. **86**, 77–92 (1973)

Daniel, J. W., Baldwin, H. H.: Methods of culture for plasmodial myxomycetes. In: Methods in Cell Physiol., Vol. 1 (D. M. Prescott, ed.), pp. 9–42. New York: Academic Press 1964

Goodman, E. M., Beck, T.: Metabolism during differentiation in the slime mold *Physarum polycephalum*. Can. J. Microbiol. **20**, 107–111 (1974)

Hüttermann, A., Porter, M. T., Rusch, H. P.: Activity of some enzymes in *Physarum polycephalum*. II. Arch. Mikrobiol. **74**, 283–291 (1970)

Hüttermann, A., Elsevier, S. M., Eschrich, W.: Evidence for the de novo synthesis of glutamate dehydrogenase during the spherulation of *Physarum polycephalum*. Arch. Mikrobiol. **77**, 74–85 (1971)

Hüttermann, A., Chet, I.: Activity of some enzymes in *Physarum polycephalum*. III. Arch. Mikrobiol. **78**, 189–192 (1971)

Hüttermann, A.: Isoenzyme pattern and de novo synthesis of phosphodiesterase during differentiation (spherulation) in *Physarum polycephalum*. Arch. Mikrobiol. **83**, 155–164 (1972)

Hüttermann, A., Gebauer, M.: Differential protein synthesis during differentiation (spherulation) of *Physarum polycephalum*: lack of synthesis of glucose-6-phosphate dehydrogenase. Arch. Mikrobiol. **85**, 91–94 (1972)

Hüttermann, A.: Biochemical events during spherule formation of *Physarum polycephalum*. Ber. Deutsch. Bot. Ges. **86**, 55–76 (1973)

Hüttermann, A., Gebauer, M., Brand, G., Wessel, I.: Studies on the regulation of carbohydrate synthesis during growth and differen-

tiation of *Physarum polycephalum*. I. Arch. Microbiol. **98**, 215–223 (1974)

Kikuchi, M.: Studies on sclerotia of a myxomycete *Physarum polycephalum* I. Sc. Rep. T. K. D. Sect. B **14**, 215–226 (1971)

Knowles, D. J. C., Carlile, M. J.: Growth and migration of plasmodia of the myxomycete *Physarum polycephalum:* the effect of carbohydrates, including agar. J. gen. Microbiol. **108**, 9–15 (1978)

Lowry, O. H., Rosebrough, N. J., Farr, A. L., Randall, R. J.: Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**, 265–275 (1951)

McAlister, L. E., Allison, V. F., Jeter, J. R., Nations, C.: High-density induction of a quiescent cell state in *Physarum polycephalum*. J. Cell Sci. **25**, 179–190 (1977)

McCormick, J. J., Blomquist, J. C., Rusch, H. P.: Isolation and characterization of an extracellular polysaccharide from *Physarum polycephalum*. J. Bacteriol. **104**, 1110–1118 (1970a)

McCormick, J. J., Blomquist, J. C., Rusch, H. P.: Isolation and characterization of a galactosamine wall from spores and spherules of *Physarum polycephalum*. J. Bacteriol. **104**, 1119–1125 (1970b)

Nations, C., LeStourgeon, W. M., Magun, B. E., Rusch, H. P.: The rapid intranuclear accumulation of preexisting proteins in response to high plasmodial density in *Physarum polycephalum*. Exp. Cell Res. **88**, 207–215 (1974)

Pieck, A. C. M., Kuyper, Ch. M. A., Rijken, A. A. M.: Comparison of the pool of nucleotides from liver cells under different physiological conditions. Proc. Koninkl. Acad. Wetensch. (Amsterdam) **74**, 311–317 (1971)

Polman, B. J. J.: DNA degrading enzymes of the slime mold *Physarum polycephalum* Schwein. Ph. D. Thesis, Nijmegen (1974)

Polman, B. J. J., Janssen, H. M. J., Kuyper, Ch. M. A.: Activities of DNA degrading enzymes in the slime mold *Physarum polycephalum*. Arch. Microbiol. **96**, 119–124 (1974)

Sachsenmaier, W., Rusch, H. P.: The effect of 5-fluoro-2′-deoxyuridine on synchronous mitosis in *Physarum polycephalum*. Exp. Cell Res. **36**, 124–133 (1964)

Sauer, H. W., Babcock, K. L., Rusch, H. P.: Changes in nucleic acid and protein synthesis during starvation and spherule formation in *Physarum polycephalum*. Arch. Entwicklungsmech. Organ. **165**, 110–124 (1970)

Wendelberger-Schieweg, G., Hüttermann, A.: Amino acid pool and protein turnover during differentiation (spherulation) of *Physarum polycephalum*. Arch. Microbiol. **117**, 27–34 (1978)

Werry, P. A. T. J.: DNA synthesis in *Physarum polycephalum*. Ph. D. Thesis, Nijmegen (1973)

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