The steady state cellular cGMP level also depended on K^+ concentration in the equilibration medium. In the next series of experiments the cellular cGMP level was determined in four groups of specimens, each of which was incubated for 3 h in four solutions with different K^+ concentrations (0.5, 2, 8 and 20 mM) respectively. As shown in figure 2 the cGMP level increased with increasing K^+ concentration. Schultz et al. observed only a slight increase in cGMP after K^+ stimulation[10]. The relative K^+ concentration ($[K]/\sqrt{[Ca]}$) of the experimental solutions employed by them was rather high (they changed the KCl concentration from 5 to 20 mM in the presence of 0.05 mM $CaCl_2$). Therefore it is conceivable that the cGMP content before stimulation was already too high to detect any change in cellular cGMP level resulting from stimulation.

2) Effect of cGMP on ciliary activities in triton-extracted models. In order to examine the direct effect of cGMP on the ciliary motile mechanism, we observed the locomotion of triton-extracted models of *Paramecium* reactivated by ATP and Mg^{2+} in the presence of cGMP (table). As reported by Naitoh and Kaneko[5], the models swam forwards in the reactivation medium containing 3.0 mM EGTA, in which free Ca^{2+} concentration was less than 10^{-7} M (tube 1). The models began to swim backwards when free Ca^{2+} concentration in the reactivation medium was raised up to 50 μM by adding 3.0 mM $CaCl_2$ to the reactivation medium (tube 2)[11]. The models, however, swam forward even in the presence of 50 μM free Ca^{2+} if 100 μM cGMP was present in the reactivation medium (tube 3). Addition of cAMP also produced forward swimming of the models in the presence of 50 μM of free Ca^{2+} (tube 4). The production of forward swimming was observed even in the presence of 10 μM cGMP or cAMP. Forward swimming velocity was always higher when cyclic nucleotides were present in the reactivation medium. These results are consistent with those obtained by Nakaoka[12].

3) Possible role of cGMP in the control of ciliary activities. We found that both cGMP and cAMP antagonized the action of Ca^{2+} in producing ciliary reversal in triton-extracted models. In the living cells, however, only cGMP level showed a positive correlation with the recovery from ciliary reversal caused by K stimulation. Since the voltage-dependent Ca channels are located only in the ciliary membrane[13,14], the increase in cGMP level is thought to occur only in the cilia due to activation of Ca/calmodulin-regulated guanylate cyclase by Ca^{2+} taken up into the cilia through the activated Ca channels. On the basis of these facts, we propose a possible mechanism by which ciliary orientation is controlled by membrane electric events (fig. 3). Ca^{2+} taken up into the cilia through the Ca channels activated by membrane depolarization may perform a double function; 1) activation of the ciliary reversal mechanism and 2) activation of guanylate cyclase. Since the ciliary reversal takes place promptly after K stimulation, Ca^{2+} seems to interact directly with the reversal mechanism. The activation of guanylate cyclase in the ciliary membrane causes an increase in the intraciliary cGMP concentration. The increase in cGMP causes recovery of cilia from the reversed state probably through activation of a cGMP-dependent protein kinase present in the cilia[4]. Participation of the Ca-activated enzymatic systems in the recovery from ciliary reversal is consistent with the delay observed in the recovery from ciliary reversal induced by K stimulation.

It is well known that membrane hyperpolarization produces acceleration of forward swimming[7]. Schultz et al. reported that an increase in cellular cAMP level relates to the acceleration of forward swimming[10]. We found that forward swimming velocity in the reactivated triton-extracted models was always higher when cAMP was present in the reactivation medium. These facts suggest involvement of cAMP in the mechanism governing beating frequency of cilia in the normal direction.

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Superoxide dismutase activity during the plasmodial life cycle of *Physarum polycephalum*

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Summary. Superoxide dismutase activity was slow throughout the cell cycle of surface cultures of *Physarum polycephalum*. This activity increased markedly when the organism was induced to spherulate. Glutathione (GSH) and hydrogen peroxide (H_2O_2) concentrations changed very little during the cell cycle. During spherulation GSH decreased; H_2O_2 and the cyanide-resistant respiration of plasmodial homogenates increased.

Key words. Physarum polycephalum; cell cycle; superoxide dismutase; differentiation.

Recently we reported that the specific activity of superoxide dismutase (SOD; EC 1.15.1.1) in the slime mold, *Physarum polycephalum*, increases dramatically during the starvation-induced spherulation of microplasmodial cultures[1]. The increase in activity was first observed 6 h after transfering the organism from nutrient medium to a salts-only starvation medium[2]; the activity then continued to increase throughout the course of differentiation until a 21-fold enhancement of the original activity was attained. As SOD activity increased the product of its catalysis, hydrogen peroxide (H_2O_2), also increased and the intracellular level of glutathione (GSH) decreased. We have since observed that the activity of the mangano-isozyme of this en-

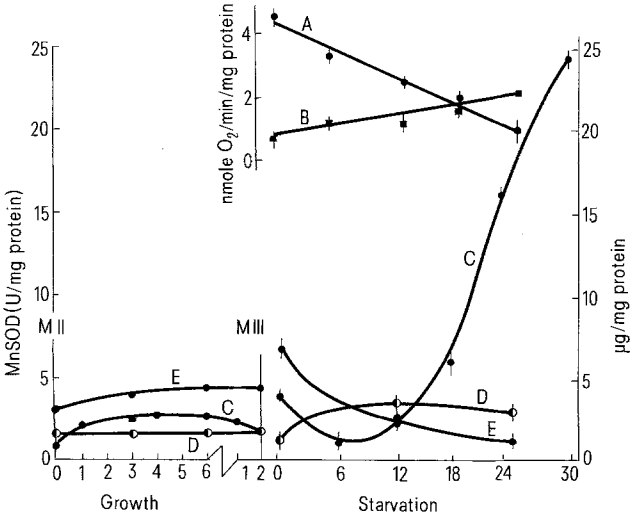
zyme (MnSOD) increases by more than 40-fold during the spherulation of the M_3cVII strain of *Physarum* microplasmodia[3]. These findings have prompted us to determine whether or not sharp increases in MnSOD activity specifically characterize the spherulating phase of the plasmodial life cycle or, instead, that fluctuations in MnSOD also occur during the diploid growth phase of the organism. We have investigated these alternatives by assaying the activity of MnSOD at selected periods throughout the cell cycle and during the subsequent differentiation of the organism into clusters of spherules (sclerotia). We have also estimated the intracellular concentrations of H_2O_2 and GSH and we have measured the cyanide-resistant respiratory activity of *Physarum* cultures incubated in the starvation medium.

Materials and methods. Microplasmodia[2] of *Physarum polycephalum* M_3cVII were fused on filter paper to form plasmodial cells[4]. The plasmodia obtained were cultured in stainless steel trays on a rocker platform as detailed by Mohberg and Rusch[4] except that two thicknesses of filter paper were used to support the organism instead of the millipore membrane-filter paper combination originally used by these workers. The synchronous cell cycle of the organism was observed by preparing smears of plasmodial samples and examining them by phase contrast microscopy[5,6] (×1000). Superoxide dismutase was assayed in individual plasmodia at the time of the second post-fusion mitosis (MII) and at 1–2 h intervals thereafter until the third mitotic division (MIII) was recorded. Glutathione and H_2O_2 were also measured throughout the cell cycle, but at 3-h intervals. Differentiation into spherules was induced by transferring cultures from growth medium to a salts-only starvation medium[2]. The differentiating cultures were assayed for MnSOD activity at the time that the plasmodia were washed and transferred to the salts medium (0-h) and at 6-h intervals thereafter throughout a 30-h period of starvation; GSH and H_2O_2 levels were measured at 12-h intervals. Superoxide dismutase (MnSOD) activity was estimated by the direct method of Misra and Fridovich[7]; this method is based on the measurement of the SOD catalyzed production of H_2O_2 in the presence of 1 mM KCN. Intracellular GSH was measured by the reduction of 5,5′dithiobis-(2-nitrobenzoic acid)[8]. H_2O_2 concentration was determined by the peroxidase catalyzed oxidation of dionisidine[9]. Respiratory rate and cyanide-resistant respiration were measured in spherulating *Physarum* samples at the time of transfer of the plasmodia to salts medium and at 6-h intervals thereafter over a 24-h period; O_2 consumption was determined with a Clark oxygen electrode (Model 53, Yellow Springs Instrument Co., Yellow Springs, Ohio) by the method of Hall[10]. Respiration was measured in homogenates of plasmodia in an equal volume of the salt medium. Cyanide-resistant respiration was measured by modification of the method of Hassan[11]; plasmodial samples were homogenized in 5 vols glucose, 5 mM, and $MgSO_4$, 0.1 mM, in 50 mM potassium phosphate buffer, pH 7.1. The rate of oxygen consumption was measured before and after the addition of KCN, 1.3 mM. Cyanide-resistant respiration was not measured directly in the salts medium (pH 3.8) because of the instability of cyanide at a pH below 7.0.

Results and discussion. The activity of MnSOD was quite low in *Physarum* plasmodia during their growth throughout the cell cycle (fig., growth C). The rate of this activity increased following MII, becoming stationary after about 3 h; activity then remained constant for the remainder of the cycle until MIII when it declined to approximately the level observed at MII. Although MnSOD activity increased during interphase the activity observed throughout the cell cycle was very low when compared to the activity exhibited by spherulating plasmodia (fig., starvation C). Immediately after washing the plasmodia and transferring them to the salts medium an inrease in MnSOD was observed (Oh). Activity then declined over the next 6 h to a level approximating that of the growing plasmodium at mitosis. This period of decline corresponds, temporally, to the biochemical reorientation period described by Goodman and Beck[12]. After 6 h in the starvation medium an abrupt increase in MnSOD was observed, culminating in an approximate 25-fold increase in activity after 30 h. The plasmodium is in the process of spherulation at this time; no additional samples were assayed. The pattern of an initial decline in MnSOD activity followed by a dramatic increase is highly repeatable in spherulating microplasmodia[1,3]. The demonstrated absence of such a pattern during plasmodial growth confirms that this profile of activity is characteristic of the differentiating plasmodium (fig.).

The intracellular concentrations of H_2O_2 and GSH in growing plasmodia were comparable to those recorded for differentiation (fig., D,E); however, significant changes in these concentrations occurred during differentiation (fig., starvation). A slight increase was observed for GSH during growth but the levels of both GSH and H_2O_2 were virtually constant prior to transfer of the plasmodia to the differentiation medium (fig., growth). The product of superoxide dismutation is H_2O_2; therefore it was not surprising that the increase in MnSOD activity during differentiation was accompanied by an increase in H_2O_2 (fig, starvation D). However, the increase was not strikingly large and it did not parallel the increase in MnSOD; instead, H_2O_2 concentration reached a steady state within 12 h after transfer of the plasmodia to salts. We cannot account for the control of H_2O_2 levels in *Physarum* via the most familiar mechanisms; *Physarum* apparently has no catalase and its glutathione peroxidase activity is quite low during spherulation[1]. One of several possible explanations is that some H_2O_2 is eliminated by spontaneous reaction with endogenous metal ions[13]; the salts medium is rich in transition metals[2].

If the increase in MnSOD during spherulation is an inductive response to an increase in its substrate, the superoxide free radical (O_2^-), the decline in intracellular GSH may also be accounted for (fig., starvation E). Glutathione is believed to quench O_2^- by reacting directly with it[14,15]. The content of GSH in *Physarum* begins to decline soon after transfer of the organism to salts. This decline may be a consequence of its consumption during the spontaneous reduction of O_2^-; GSH is the major source of reducing equivalents in cells[16]. The GSH decline fol-



Parameters of oxygen metabolism in *Physarum polycephalum* during the cell cycle (growth) and during spherulation (starvation). Oxygen consumption was measured in plasmodial homogenates in the presence of 1.3 mM KCN in pH 7.1 buffered glucose (B) and in pH 3.8 salts medium (A). Superoxide dismutase activity (C), hydrogen peroxide (D) and glutathione concentrations (E) were measured during the growth of plasmodia from the second (MII) through the third (MIII) post-fusion mitosis and during starvation-induced differentiation. Oxygen consumption is expressed as nmoles O_2/min/mg protein. Hydrogen peroxide and glutathione concentrations are recorded as µg/mg protein.

lows an initial increase repeatedly observed when the cycling organism was transferred to salts. The need for free radical defenses during spherulation is also suggested by changes in the nature of oxygen consumption by the differentiating plasmodium. The rate of aerobic respiration was higher in homogenates of plasmodia prepared at the time of transfer to the salts medium (0-h starvation) than after 24 h of starvation, when the rate of oxygen consumption approached zero (fig., starvation B).

When oxygen consumption by the homogenates was measured in a medium containing KCN in buffered glucose (pH 7.1) an increase in rate was observed after 6 h of starvation; logarithmically growing plasmodia exhibited only a slight capacity for cyanide-resistant respiration at the time of transfer to the differentiation medium. After 24 h in salts the cyanide-resistant respiration of the homogenates approximately doubled under the assay conditions employed (fig., starvation A). An increase in the generation of O_2^- would be expected to require an increase in the rate of cyanide-resistant oxygen consumption; more oxygen would be univalently reduced to O_2^- and less reduced to water under conditions favoring this form of oxygen utilization. The increase observed is therefore consistent with the changes observed in MnSOD, H_2O_2 and GSH. The relationship between respiration, cyanide-insensitive respiration and O_2^- production has been described by Hassan[11]. We have previously reported that coupled mitochondria from *P. polyephalum* exhibit qualitatively typical respiratory patterns when provided with succinate, malate, isocitrate and α-glycerophosphate as substrates[17]. We presume that the MnSOD measured in this study is also mitochondrial. This presumption will be tested in future experiments directed specifically toward an evaluation of the role of *Physarum* mitochondria in free radical production.

Our observations suggest that the process of spherulation in *P. polycephalum* involves a cell state that is characterized by oxidative stress. It has previously been speculated that this form of differentiation is triggered by anoxic conditions[18]. This suggestion has never been seriously tested. We have observed that when *Physarum* microplasmodia are cultured in salts in an atmosphere of nitrogen, the microplasmodia do not spherulate; instead, they form a plasmodial collar on the flask within 24 h. They ultimately die without exhibiting any signs of differentiation. We have also observed that the rate at which microplasmodia spherulate tends to parallel the increase in MnSOD activity. A white mutant strain of *Physarum* (LU887 × LU897) that does not spherulate in salts also does not exhibit an increased MnSOD activity in salts; a closely related yellow strain (LU897 × LU863) spherulates more slowly than does the M_3cVII strain of this study and it also exhibits a more gradual increase in MnSOD activity. When the closely related yellow and white strains are fused the resulting heterokaryon spherulates at a rate intermediate between the rates of its parent strains; the increase in MnSOD activity of the heterokaryon is also intermediate between the increases observed in the parent strains[3]. Based on these findings and the results of the present study we conclude that sharp increases in MnSOD activity are characteristic of starvation-induced spherulation and that these increases are not observed at other times during the diploid phase of the life cycle of *P. polycephalum*. We surmise that either oxy-free radicals or the antioxidant defenses against these free radicals play a causative role in spherulation.

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Synthetic activities of mass cultures and clones of human gingival fibroblasts[1]

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Summary. The percentage of synthesis dedicated to collagen is elevated in low-density cultures of human gingival fibroblasts, as is per-cell total protein synthetic activity and glycosaminoglycan accumulation. These observations can be explained, in part, by a decrease in membrane transport of precursor substance in high-density cultures. Synthetic activity by human fibroblasts can be reliably assayed in vitro using as few as 500 cells sparsely seeded. Such low-cell number assay is essential for study of single-cell clones, where replicative life span is limited.
Key words. Gingiva; collagen; fibroblast; single-cell clones.

Study of the synthetic activities of cultured fibroblast-like cells from various donor sites may aid in the elucidation of normal cell biology, as well as the pathogenesis of connective tissue disorders. It has been demonstrated, however, that varying the conditions of culture can lead to widely disparate experimental results[2-4]. For example, differences in in vitro cell density may greatly alter the data obtained in experiments designed to evaluate cellular biochemical functions. Furthermore, since normal diploid fibroblasts routinely exhibit finite replicative life spans and synthetic capacities in vitro, the question of culture 'age' becomes critical. This is particularly important in experiments utilizing clones derived from single cells. For example, it is generally accepted that fibroblast senescence ensues after circa 40 population doublings[5]; thus, by the time only 1000 cells have