*J. theor. Biol.* (1994) **167**, 273–282

# Cell Density-sensing in *Dictyostelium* by Means of the Accumulation Rate, Diffusion Coefficient and Activity Threshold of a Protein Secreted by Starved Cells

ITA S. YUEN AND RICHARD H. GOMER†

*Howard Hughes Medical Institute and Department of Biochemistry and Cell Biology, Rice University, Houston, TX 77251-1892 U.S.A.*

(*Received on* 12 *April* 1993, *Accepted on* 26 *August* 1993)

The simple eukaryote *Dictyostelium discoideum* grows as an amoeba on leaf and soil surfaces. When starved, the amoebae aggregate and differentiate. The amoebae can also be induced to differentiate as isolated cells submerged in buffer, if the buffer contains a sufficiently high concentration of a protein (CMF) secreted by starved cells. CMF is also necessary for aggregation and differentiation on surfaces. This indicated that CMF has either an autocrine function or is part of a density-sensing system. To distinguish between these two possible functions, we first examined the rate at which CMF is accumulated and the activity threshold of cells for CMF, since both parameters will affect whether a cell can provide enough CMF to self-stimulate. We find that CMF potentiates its own accumulation, and that otherwise the accumulation rate and activity threshold are affected very little by a variety of physiological conditions. We then use diffusion calculations to show that even after many hours of continuous secretion, the CMF concentration adjacent to an isolated starved cell on a leaf or soil surface will be too low to allow differentiation, whereas an extracellular concentration of CMF sufficiently high to allow differentiation will occur when starved cells are at high densities. We find a close match between the predicted and experimentally observed density necessary for differentiation. The theoretical and observed behavior of cells at different cell densities suggests that due to its accumulation rate, diffusion coefficient, and activation threshold, CMF can function as part of a cell density-sensing system which allows *Dictyostelium* cells in the wild to co-ordinate their development.

## 1. Introduction

The simple eukaryote *Dictyostelium discoideum* normally grows as isolated cells that increase in number by fission. In the wild, the vegetative cells feed on bacteria on decaying leaves and the soil surface. When the cells eventually overgrow their food supply and starve, they stop dividing and aggregate together using relayed pulses of cAMP as the chemoattractant. The aggregated cells differentiate into a fruiting body, a mass of spore cells held up off the surface by a ~2 mm high column of stalk cells. The precursors of the spore and stalk cells (prestalk and prespore cells) begin to express cell type-specific genes in the aggregate (Loomis, 1975, 1982; Devreotes, 1989; Dottin *et al.*, 1991).

Under certain conditions, isolated cells can also differentiate in liquid culture. For instance, in submerged monolayer culture, prestalk and prespore genes can be expressed by high-density starved cells but not by low-density starved cells (Grabel & Loomis, 1976; Mehdy *et al.*, 1983). This effect is mediated by a secreted factor: low-density cells will express prestalk and prespore genes when exposed to buffer previously conditioned by a high density of starved cells (Mehdy & Firtel, 1985). The factor, called conditioned medium factor (CMF), is secreted by cells throughout development but not by vegetative cells (Gomer *et al.*, 1991); interestingly, cells need to be exposed to CMF only during the first three

† Author to whom correspondence should be addressed.

0022–5193/94/070273 + 10 $8.00/0

hours of development to allow the expression several hours later of prestalk and prespore genes (Gomer & Firtel, 1987). PSF, a protein secreted by high-density vegetative *Dictyostelium* cells (Rathi & Clarke, 1992) allows cells to sense their density relative to the food supply as they near starvation, but does not appear to be CMF (Clarke *et al.*, 1992). Similar factors have also been observed in bacteria. C-factor, a secreted 17 kDa protein, is necessary for development of starved *Myxococcus xanthus* (Kim & Kaiser, 1990); the EDF-A protein has a similar function in *Bacillus subtilis* (Grossman & Losick, 1988).

We have developed a bioassay for CMFs in which cells are starved at low density in buffer in a well of a microtiter plate or slide. Prespore and prestalk antigens, which can be detected by immunofluorescence, are expressed only if CMFs are present in the buffer (Gomer, 1987). Fractionation of conditioned medium on Sephadex G-50 shows two size classes of CMF activity. One can be purified to an 80 kDa glycoprotein while the other appears to be a set of 0·5–6 kDa proteolytic products of the 80 KDa CMF; the rescue of differentiation by the purified CMF indicates that it alone is responsible for the density sensing (Gomer *et al.*, 1991; Yuen *et al.*, 1991). The proteolytic products appear only after 20 hr, during late development, and have a much higher specific activity than the 80 kDa CMF. The derived amino acid sequence of the 80 kDa CMF shows no significant similarity to any known protein (Jain *et al.*, 1992). CMF is somewhat acidic, very hydrophilic and has no unusual motifs with the exception of an internal signal sequence, and no large regions of charge or hydrophobicity. CMF antisense transformants do not aggregate or differentiate except in the presence of exogenous CMF, suggesting that CMF is not part of a redundant pathway that parallels some other, density sensitive pathway (Jain *et al.*, 1992). From the purification of the 80 KDa CMF, we found that on average, a *Dictyostelium* cell starved at high density in the absence of cAMP pulses secretes approximately 12 molecules CMF $min^{-1}$. The threshold sensitivity of cells to CMF is roughly 0·3 ng $ml^{-1}$ for both aggregation and differentiation (Gomer *et al.*, 1991).

It is unclear what the function of CMF is. One possibility is that CMF is an autocrine factor necessary for aggregation and differentiation. In this case in the wild each cell would provide its own environment of CMF. The low cell densities in submerged culture might then represent conditions where the CMF is diluted to unnaturally low levels. Another possibility is that CMF is used for density sensing, for instance to cause a starved cell to begin development only when a high density of cells in its vicinity have also starved. Although CMF appears to mediate density sensing in the extremely unnatural condition of submerged culture with cell densities so low that aggregation does not occur, we do not know if CMF could mediate density sensing in the normal environment of a thin film of liquid on a leaf or the surface of a material such as moist dirt. In this report, we first examine whether physiological parameters other than starvation, such as cell density, light, pH and cAMP pulses affect CMF accumulation and its activity threshold, since both of these values will have a great effect on whether a cell can provide enough CMF to stimulate itself. We then use diffusion calculations to predict that at the observed secretion rate, even after many hours of continuous secretion, the CMF concentration in the vicinity of an isolated starved cell on a leaf or a dirt surface will not be sufficiently high to allow aggregation or differentiation, whereas amongst closely spaced starved cells the CMF concentration will be high enough to allow aggregation and differentiation. Observations of cells starved on a moist permeable surface verify these predictions. This then suggests that in the wild, CMF mediates density sensing.

## 2. Materials and Methods

Growth of *Dictyostelium discoideum* K Ax-3 (also known as Ax-4) cells in HL5 supplemented with antibiotics, CMF production, CMF purification and CMF assays were carried out as previously described (Gomer *et al.*, 1991; Yuen *et al.*, 1991). All operations were at 21°C unless otherwise described. PBM buffer was prepared as described; for the pH experiments, the pH was adjusted with KOH to values other than the standard value of 6·1. Conditioned medium (CM) was normally made under standard fluorescent room light; for light/dark experiments, cells were plated at $9 \times 10^4$ $cm^{-2}$ and illumination was supplemented with a Sylvania 100 W flood lamp 1 m above the plate and an adjacent plate was covered with aluminum foil. Cells were also starved for 10 hr in submerged monolayer culture in 11·2 ml of PBM at densities of 4·5, 9, 18 and $27 \times 10^4$ cells $cm^{-2}$ in VWR "100 mm" polystyrene plastic petri dishes. For the low-density experiment, cells were starved and resuspended in 56 ml of PBM to a density of $9{\cdot}1 \times 10^3$ cells $ml^{-1}$, and 1·7 units (1 unit of CMF $\approx$ 0·3 ng) of SDS-gel purified CMF (Gomer *et al.*, 1991) in 11·2 µl was then added immediately after starvation. The cells were then divided into five aliquots and plated at $1{\cdot}8 \times 10^3$ cells $cm^{-2}$ in five 100 mm plastic petri dishes. As a control, 11·2 µl of PBM was added to 56 ml of

similarly starved cells which were then divided amongst five plates. CM was collected and clarified as described above, and 450 µl of CM from cells simultaneously starved at $1{\cdot}8 \times 10^5$ cells $cm^{-2}$ was mixed with 44·5 ml of PBM. 45 ml of the two low cell-density CMs and the diluted high cell density CM were concentrated in Centriprep/10 10 kDa-cutoff concentrators (Amicon, Beverly, MA) that had previously been washed three times with 0·01% gelatin. The retained material was then collected and diluted to 450 µl.

For cAMP pulsing, cells were starved in shaking culture at a density of $5 \times 10^6$ cells $ml^{-1}$ in PBM. 10 µl of a $4 \times 10^{-5}$ M solution of cAMP in PBM was pumped into 20 ml of cells every 6 min (Mann & Firtel, 1987). 20 hr after starvation the pulses were discontinued for 10 min to allow cAMP levels to drop, and conditioned medium was then harvested. Caffeine (Sigma) was added to some cells to 3 mM upon starvation and an additional 2 mM was added every 2 hr thereafter. Other cells received cAMP (Sigma) to 0·5 mM upon starvation and as an additional 0·1 mM every hour. To remove caffeine and cAMP from the CMs, 100 µl aliquots were chromatographed through spin columns of Sephadex G-50 (Gomer *et al.*, 1991). To verify that very little cAMP was in the spin-through from the columns, one sample was spiked with 1 µCi of $^3$H cAMP (Dupont–NEN, Boston, MA).

For synchronization of cells, mid-log phase cells were pelleted by centrifugation, resuspended in PBM, repelleted, and resuspended to $1{\cdot}2 \times 10^6$ $ml^{-1}$ in 200 ml of HL-5 supplemented with antibiotics and 0·1% v/v of 10 mg $ml^{-1}$ nocodazole (Sigma) in dimethylsulfoxide. After shaking for 12 hr, the cells were pelleted by centrifugation, washed once in PBM and resuspended in 200 ml of HL-5 containing antibiotics to $1{\cdot}1 \times 10^6$ $ml^{-1}$; cells were then counted at the times indicated. Immediately after counting, 10–15 ml of cells were pelleted, washed twice in PBM, and resuspended to a density of $1 \times 10^6$ $ml^{-1}$ in PBM and shaken for 20 hr. CM was then prepared as previously described (Gomer *et al.*, 1991). Cells were also synchronized by release from stationary phase following Gomer & Firtel (1987) and by temperature shift as described by Maeda (1986).

The diffusion of CMF was calculated with programs written in Microsoft QuickBASIC for a Macintosh computer and are available upon request. To test experimentally that low-density cells on a soil-like surface cannot stimulate themselves, starved Ax-4 cells were plated at various densities on Millipore HTTP 0·4 µm pore size polycarbonate filters placed on two layers of Whatman 3 filter paper saturated with PBM. Expression of the prespore antigen requires both CMF during early development and exposure to high continuous levels of cAMP during late development (Gomer *et al.*, 1986). Accordingly, 6 hr after starvation the Millipore filters were placed on Whatman 3 filter papers soaked with 300 µM cAMP in PBM. 18 hr after starvation, the cells on the Millipore filters were fixed for 15 min by transferring them to filter papers soaked with 95% ethanol, air dried, and rehydrated in PBS/0·01% Tween-20 and stained for the prespore antigen SP70 by immunofluorescence as described (Yuen *et al.*, 1991). Nuclei were stained with 0·1 µg/ml DAPI during the final wash (Roos, 1987). 1 $cm^2$ pieces of the Millipore filters were then placed on slides and mounted under coverslips. To verify that cells had not been lost during the staining procedure, the cell density was determined by counting the DAPI-stained nuclei.

### 3. Regulation of CMF accumulation and activity threshold

When *Dictyostelium* cells starve conditions such as initial cell-density, extracellular pH or light level can vary. These parameters could conceivably influence the CMF accumulation rate or the response of cells to a given concentration of CMF. To determine if CMF accumulation is influenced by cell density, cells were starved in submerged monolayer culture at densities ranging from a high of $2{\cdot}7 \times 10^5$ cells $cm^{-2}$, a density at which the cells form aggregates in the submerged culture and secrete sufficient CMF into the buffer to allow prespore and prestalk gene expression, to a low of $1{\cdot}8 \times 10^3$ cells $cm^{-2}$, a density at which the cells are many cell diameters apart from one another, do not form aggregates, and the CMF concentration is too low to allow prespore and prestalk gene expression (Mehdy & Firtel, 1985). Conditioned medium was prepared from these cells and assayed for CMF activity. As shown in Fig. 1, the amount of CMF activity secreted per cell increases with cell density, with low density cells accumulating $\sim 2 \times 10^{-6}$ units $cell^{-1}$. To determine if the decrease in the apparent amount of CMF secreted per cell at low density is due to a loss of CMF from binding to the culture dish or during the concentration step, we made low-density CM in buffer spiked with 0·03 units CMF $ml^{-1}$ (the equivalent of $3{\cdot}3 \times 10^{-6}$ units $cell^{-1}$). The CM produced by the spiked cells contained $1{\cdot}1 \times 10^{-5}$ units $cell^{-1}$ of CMF activity. Because the CM had been concentrated by spin dialysis through a 10 kDa cutoff membrane, this CMF activity is from 80 kDa CMF and not

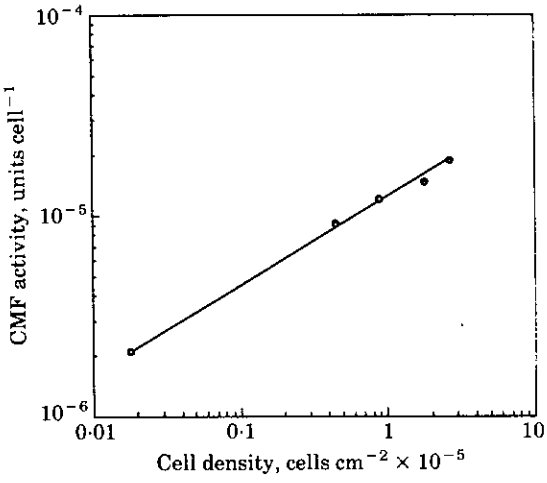


FIG. 1. CMF secretion is regulated by cell density. CMF was prepared from cells starved for 10 hr in submerged monolayer culture at the indicated densities, diluted and assayed for the ability to induce prespore antigen expression. The production of CMF per cell increases with cell density.

small molecular weight (< 6 kDa) CMF (Yuen *et al.*, 1991). This indicates that the exogenous $3{\cdot}3 \times 10^{-6}$ units cell$^{-1}$ induced the low density cells to secrete $1{\cdot}1 \times 10^{-5}$–$3{\cdot}3 \times 10^{-6}$, or $8 \times 10^{-6}$ units of CMF per cell, a roughly four-fold increase. Similar effects were seen with CM made from cells shaken in suspension culture at $5 \times 10^{6}$ and $5 \times 10^{4}$ cells ml$^{-1}$. CMF thus appears to potentiate its own accumulation. Cell density, however, does not appear to affect the activity threshold of CMF: previous results have shown that there is an activity threshold of 0·3 ng CMF ml$^{-1}$ of CMF for cells at low density in submerged culture as well as at high density on filter pads (Gomer *et al.*, 1991; Jain *et al.*, 1992).

We also examined the effect of pH on the secretion of CMF by starved cells. Cells were starved in shaking culture in PBM adjusted to pH 5·0, 5·5, 6·0, 6·5 or 7·0. The high molecular weight components of the conditioned media were isolated and assayed for CMF activity (at pH 6·1); all had approximately the same activity. CM prepared at pH 6·1 was adjusted to pH 5·0, 5·5, 6·0, 6·5, 7·0 or 7·5; all except the material adjusted at pH 7·5 had approximately the same amount of CMF activity. At this pH, cells both in the presence and absence of CMF appeared unusually splayed out by light microscopy and responded poorly to CMF. These results suggest that at pH values of 7·0 and below, the pH of the environment does not affect the accumulation of CMF or the activity threshold of CMF.

Because developmental programs such as macrocyst formation are light-sensitive (Habata *et al.*, 1991), we assayed CM from cells starved under bright light or in the dark; no difference in CMF activity was observed. This indicated that light has little effect on CMF accumulation. To determine if light affects the response of cells to CMF, we also assayed the response of cells to CM and PBM for cells starved in light, in the dark, in light for 6 hr and then placed in the dark, and finally for cells starved in the dark for 6 hr and then exposed to light. Differentiation of both prespore and prestalk cells was roughly the same for the first three conditions and slightly increased in the latter, indicating that light does not significantly affect the CMF activity threshold.

Since several *Dictyostelium* early developmental genes are regulated by pulses of cAMP, we examined whether CMF production is regulated by pulses of cAMP. At temperatures above 23°C, the conditioned medium made by cells pulsed with cAMP consistently has approximately twice the CMF activity as CM made by unpulsed cells (Table 1). This same increase in CMF production was observed for cells pulsed with cAMP starting at 2 hr after starvation, and stopping the pulses at 10 hr after starvation. Cells which were pulsed with buffer that did not contain cAMP produced a CM that had the same activity as that of unpulsed cells (data not shown). At 21°C, cAMP-pulsed and unpulsed cells consistently secrete approximately the same amount of CMF.

To determine if CMF secretion can occur in the absence of cAMP pulses, we used either caffeine to block the activation of adenylyl cyclase by the cAMP receptor or high levels of cAMP to saturate the receptor and thus block adenylyl cyclase activation (Brenner & Thoms, 1984; Mann & Firtel, 1987). Cells starved in the presence of caffeine, high continuous levels of cAMP or cAMP pulses and caffeine make CMs that are able to induce cells to differentiate about as well as the CM from untreated cells (Table 1). This suggests that CMF secretion can occur in the absence of cAMP pulses. The previous observations that the CMF activity threshold is the same for cells at very low density in submerged culture

TABLE 1

*The effect of cAMP pulses, high continuous levels of cAMP, or caffeine on the accumulation of CMF by cells at 23°C. Although pulses of cAMP enhance the accumulation of CMF by the Ax-3 cells, blocking pulses with caffeine or high continuous cAMP does not shut off CMF secretion.*

| Addition | CMF activity, units ml$^{-1}$ |
|---|---|
| Nothing | 65 |
| cAMP pulses | 123 |
| high cAMP | 72 |
| caffeine | 60 |
| cAMP pulses and caffeine | 60 |

(where cAMP pulsing would not occur) and for cells aggregating on filter pads (in the presence of cAMP pulses) suggests that cAMP pulses do not greatly affect the activity threshold of CMF.

One way in which early developing cells differ from one another is the cell-cycle phase that they happen to be in at the time of starvation (Weijer *et al.*, 1984; McDonald, 1986; Wang *et al.*, 1988; Gomer & Firtel, 1987). To see if CMF secretion is cell-cycle dependent, we measured the CMF activity from cells starved at different cell-cycle phases. As shown in Fig. 2(a), cells synchronized by release from nocodazole divide within the first hour, increase in density only slowly until 10 hr after release, and then increase in density by roughly 35% during the next hour. Cells were taken at various times after release from nocodazole and starved for CM production; the CM was then assayed for CMF activity [Fig. 2(b)]. The synchronized cells accumulated as much CMF as did mixed

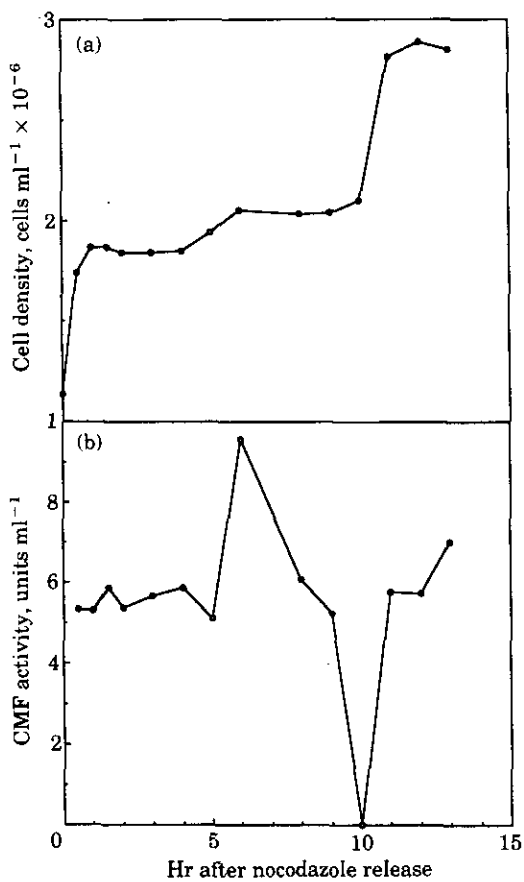


FIG. 2. Cells starved in late G2 phase secrete more CMF than cells starved in other phases; cells starved in M phase secrete very little CMF. (a) Cells were released from a block in M phase by release from nocodazole and exhibit somewhat synchronous cell division. (b) CMs from cells starved at different times after release from nocodazole block were assayed for CMF activity. The CM from a mixed phased population had approximately 6 units ml$^{-1}$ of CMF activity.

phase populations regardless of cell cycle phase, with two exceptions. Cells starved at 6 hr after release (roughly mid-G2 phase; Weijer *et al.*, 1984) produced roughly 1·7-fold more CMF than did mixed populations. Cells starved at 10 hr after release (roughly M phase; Weijer *et al.*, 1984) produced a CM which, at every concentration examined, induced differentiation in less than half of the maximum percentage of cells inducible in the assay (roughly 30%) and thus by our definition of units (Gomer *et al.*, 1991) produced 0 units of CMF activity. We observed this behavior repeatedly, with an approximately 30% variability in the CMF concentrations for each cell cycle phase, and cells synchronized by two other methods, release from stationary phase and release from temperature shift, produced similar patterns of CMF accumulation as a function of cell cycle phase at the time of starvation (data not shown). We have previously observed that the activity threshold of CMF is the same for both the prestalk and prespore antigens which we use as markers for differentiation (Gomer & Firtel, 1987; Gomer *et al.*, 1986), suggesting that the CMF activity threshold is the same for both cell types.

### 4. Diffusion of CMF allows density sensing

In the wild, *Dictyostelium discoideum* grows on the soil surface and decaying leaves. A film of free surface water is necessary for development (Gerisch, 1968; Raper, 1984). This indicates that an approximation for the physical conditions in which *Dictyostelium* cells normally aggregate ranges from a thin sheet of water (as would be formed on the surface of a leaf) to the flat surface of an effectively infinite solid (i.e. the soil surface) with a permeability and viscosity (on the microscopic scale) approximately that of moist dirt. To determine if it is physically possible for cells to secrete enough CMF to trigger differentiation at high densities, yet at low densities to not differentiate due to self-stimulation, we calculated the theoretical concentration of 80 kDa CMF in the vicinity of cells at low densities and high densities for cells in a thin film of water and for cells on a soil surface. The calculations do not consider any small molecular weight CMF since none can be detected during the first 10 hr of development (Yuen *et al.*, 1991). Several assumptions were necessary for these calculations. The first was that CMF is secreted at an even rate rather than in large bursts. Second, we assumed the secretion rate to be the same as that of the unpulsed cells shaking in buffer that were used for the large scale CMF purification (Gomer *et al.*, 1991). This is approximately 12 molecules cell$^{-1}$ min$^{-1}$ since more than 90% of CMF is secreted during the first 10 hr

of starvation and the secretion rate is roughly constant during this time (Mehdy & Firtel, 1985; data not shown). This secretion rate was calculated assuming that all of the 80 kDa protein from the final purification step had activity; if some of the purified 80 kDa protein had been inactive, the secretion rate of active factor could be as low as 4 molecules cell$^{-1}$ min$^{-1}$.

### 4.1. CALCULATIONS INDICATE THAT ISOLATED STARVED CELLS IN A THIN FILM OF WATER OR ON A PERMEABLE MATERIAL WILL NOT STIMULATE THEMSELVES WITH CMF.

We first calculated the concentration of CMF in the vicinity of an isolated cell sitting in a thin film of water, to determine whether the cell would be able to stimulate itself. The concentration $C$ at a time $\tau$ after the start of secretion and a distance $r$ from the center of the isolated cell in a thin film of water will be:

$$C = \frac{\phi}{4\pi Dh}\int_0^t \frac{e^{-r^2/4Dt}}{t}\,dt$$

(Morse & Feshbach, 1953) where $\phi = 12$ molecules min$^{-1}$, $h$ is the thickness of the film of water, and the diffusion coefficient $D$ is equal to $kT/6\pi\eta R$ with $k$ the Boltzmann constant, $T$ the absolute temperature, $\eta$ the viscosity of the medium, in this case water, and $R$ the radius of the diffusing molecule. It can be easily verified that the integral of $C$ over all space is $\phi t$. We used $k = 1{\cdot}38 \times 10^{-16}$ erg K$^{-1}$, $T = 295$ K, $\eta = 0{\cdot}955 \times 10^{-2}$ g cm$^{-1}$ sec$^{-1}$ (all from Weast, 1973) and $R = 2{\cdot}9 \times 10^{-7}$ cm (extrapolated from Moore, 1962); this value for $R$ was verified by calculating the radius of a spherical 80 kDa protein with density 1·0 g cm$^{-3}$.

Making the substitutions $z = 1/t$ and $\mu = r^2/4D$, and using equations 3·352-2, 8·214-1 and 9·73 in Gradshteyn & Ryzhik, (1965), we can show that

$$C = \frac{-\phi}{4\pi Dh}\left(0{\cdot}577215 + \ln(\mu/\tau) + \sum_{k=1}^{\infty}\frac{(-\mu/\tau)^k}{k!k}\right)$$

To correct for the presence of CMF receptors, we assumed that having CMF molecules bind to receptors would have an equivalent effect on the free CMF concentration as a lower secretion rate. Thus if $C$ is the concentration with no correction for receptors, an approximation for the corrected CMF concentration $C'$ would be $C' = (\phi t - B)C/\phi t$ where $\phi$ is the secretion rate (12 molecules min$^{-1}$), $t$ is the time, $R_T$ is the number of receptors per cell, $K_D$ is the equilibrium dissociation constant of the receptor, and $B$ is the number of CMF molecules bound per cell, subject to $B = R_T/(K_D/C' + 1)$. Solving for $C'$, we find that

$$C' = (C - K_D - R_T C/\phi t + [(K_D - C + R_T C/\phi t)^2 + 4K_D C]^{1/2})/2$$

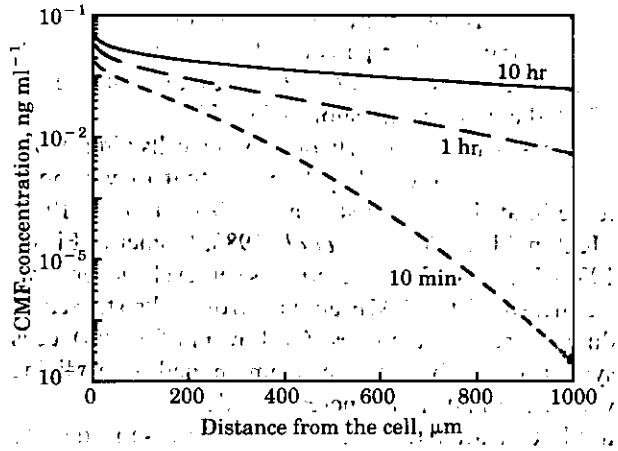


FIG. 3. Even after many hours of continuous secretion, theoretical calculations indicate that the concentration of CMF in the vicinity of an isolated cell in a 10 μm thick film of water will be well below the 0·3 ng ml$^{-1}$ threshold for CMF-dependent gene regulation. The distance is measured from the edge of the cell, assuming a 10 μm cell diameter.

This can be easily calculated and the results are shown in Fig. 3 with an arbitrary choice of a 10 μm-thick film of water (the approximate diameter of a *Dictyostelium* cell), and arbitrarily chosen $R_T = 1000$ receptors cell$^{-1}$ and $K_D = 0{\cdot}3$ ng ml$^{-1}$ (the approximate threshold activity level for CMF). The calculations were done for secretion from a point source. A more exact representation would be secretion from a spherical shell of radius 5 μm. A correction would be necessary if the concentration near the cell was high enough that a significant amount of CMF would be contained in a 5 μm radius sphere. The highest concentrations calculated (Fig. 3 and 4) for a single cell are below 0·1 ng ml$^{-1}$; this corresponds to a ratio

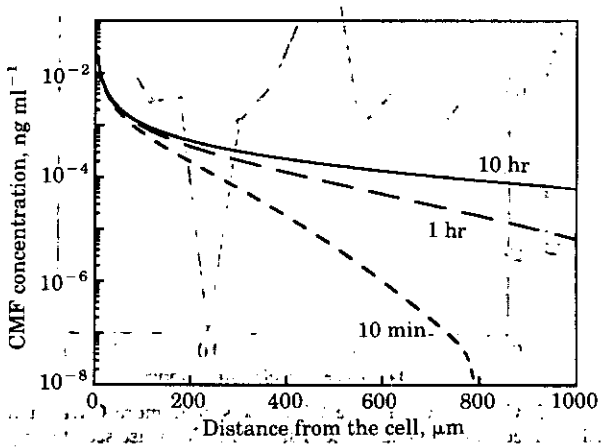


FIG. 4. Even after many hours of continuous secretion, theoretical calculations indicate that the concentration of CMF in the vicinity of an isolated cell sitting on the surface of a thick material with the properties of moist dirt will be well below the 0·3 ng ml$^{-1}$ threshold for CMF-dependent gene regulation. The distance is measured from the edge of the cell, assuming a 10 μm cell diameter.

of roughly 0·4 molecules per 5 μm sphere. Diffusion from a point source at the center of the cell thus represents a good approximation for diffusion from the surface of the cell. As expected, the CMF concentration decreases with distance from the cell; longer times of continuous secretion result in higher concentrations, especially at large distances from the cell. Most interestingly, the concentration at the very edge of the cell stays less than 0·05 ng ml$^{-1}$ up to 10 hr; this concentration is well below the 0·3 ng ml$^{-1}$ threshold for CMF-dependent gene expression (Gomer *et al.*, 1991). Similar calculations with a variety of other CMF receptor $R_T$s and $K_D$s, including no receptors, gave results that were essentially indistinguishable (data not shown). Since the effect of receptors on $C$ is to compete for ligand and thus decrease $C$, any number of receptors with any $K_D$ would lower $C$ below 0·05 ng ml$^{-1}$. We have also done calculations for thicker films of water, including an infinitely thick film which requires calculating the diffusion in three dimensions (see below). For all of these cases, after 10 hr of continuous CMF secretion, the CMF concentration at the edge of an isolated cell remains well below 0·3 ng ml$^{-1}$. Thus, even with a correction for CMF receptors and for any water film thicker than 10 μm, an isolated cell will not secrete enough CMF to stimulate itself since the concentration of free CMF adjacent to the cell will remain below 0·3 ng ml$^{-1}$. Additional calculations indicate that the theoretical CMF concentration at the edge of the cell would increase by less than 15% from the 10 hr value if the cell were to secrete CMF for 100 hr. This suggests that a cell in a thin film of water will not be able to stimulate itself even after long periods of continuous CMF secretion.

We next calculated whether an isolated starving cell sitting on the surface of a thick, moist, permeable material such as dirt or agar would be able to stimulate itself. For this geometry, the concentration of CMF at a distance $r$ from the center of a cell sitting on the surface of the material is

$$C = 2\phi\left(\frac{1}{2\sqrt{\pi D}}\right)^3 \int_0^\tau t^{-3/2} e^{-(r^2/4Dt)} dt$$

The integral of $C$ over all space is $\phi t$. Again making the substitutions $z = 1/t$ and $\mu = r^2/4D$, and using equations 3·381-3, 8·354-2 and 8·338-2 in Gradshteyn & Ryzhik, (1965), we can show that

$$C = \frac{\phi}{2\pi^{3/2} Dr}\left[\sqrt{\pi} - \sum_{n=0}^{\infty} \frac{(-1)^n (\mu/\tau)^{(n+1/2)}}{n!(n+1/2)}\right]$$

For the diffusion of CMF in soil, we made a standard correction to the diffusion coefficient, which is to assume the presence of impermeable spherical obstructions with a volume fraction of 0·5; this effectively decreases the diffusion coefficient by a factor of 2·5 (Bailey & Ollis, 1986). Observed diffusion in biofilms suggests that this is an extreme case (Siegrist & Gujer, 1985). The results of these calculations are shown in Fig. 4. The calculations were done for a point source, but as explained earlier hold for secretion from a spherical shell of the approximate cell radius, 5 μm. As for diffusion in a film of water, the CMF concentration decreases with distance from the cell; longer times of continuous secretion result in higher concentrations, especially at large distances from the cell. For large times, the concentration at the very edge of the cell remains essentially constant, at roughly 0·027 ng ml$^{-1}$, again well below the 0·3 ng ml$^{-1}$ threshold for CMF-dependent gene expression (Gomer *et al.*, 1991). This can also be seen by noting that at infinite time the sum term vanishes and $C = \phi/2\pi Dr$, which at the edge of the cell is 0·027 ng ml$^{-1}$.

### 4.2. CALCULATIONS INDICATE THAT A HIGH DENSITY OF CELLS WILL STIMULATE THEMSELVES WITH CMF

We then examined whether these same parameters would allow the extracellular concentration in the vicinity of a high density of CMF-secreting cells to go above the 0·3 ng ml$^{-1}$ stimulatory threshold. Using the above calculations for a single cell on a soil surface and ignoring effects caused by physical trapping of CMF between cells, we calculated the concentration of CMF contributed by every cell in a flat disk of cells, all embedded in a layer of water or on the surface of a thick moist permeable material. The cells in this calculation were assumed to be in a square-packed array [Fig. 5(a)]. We again did the calculations for diffusion from point sources. The correction for diffusion from 5 μm spheres would involve the CMF inside the sphere being forced outside of it. The highest CMF concentrations calculated for an array [Fig. 5(b)] are less than 2 ng ml$^{-1}$, corresponding to roughly eight molecules of CMF inside the sphere. This is less than 1 minute's worth of secretion, and thus the approximation is valid for secretion times greater than a few minutes, with the true CMF concentration being slightly higher than what we have calculated.

The concentration of CMF at the edge of the central cell in an array of 2046 total cells was calculated using the arbitrary choice of $R_T = 1000$ receptors cell$^{-1}$ and a $K_D$ of 0·3 ng ml$^{-1}$. For water film thicknesses ranging from 10 μm to infinity, we found that cells spaced 50 μm or less center-to-center would be surrounded by CMF concentrations greater than 0·3 ng ml$^{-1}$ well within 10 hr. For cell on the surface

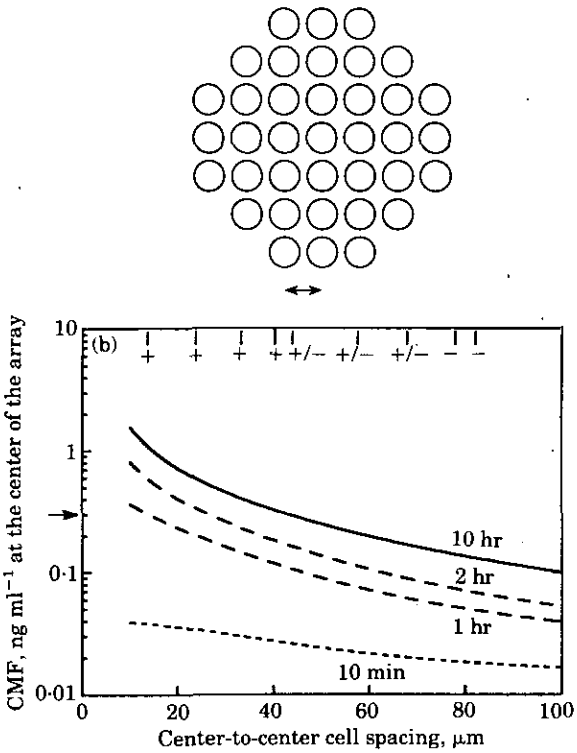


FIG. 5. A high density of starved cells secrete enough CMF to stimulate themselves. (a) For the calculations, the cells were assumed to be in a square lattice as seen from above the water film or air–dirt interface. An array of 37 cells is shown. The double headed arrow indicates the center-to-center spacing of the cells; 10 μm is the smallest possible center-to-center distance due to the size of the cells. (b) Theoretical and experimental results: the theoretical CMF concentration at the center of a circular disk of 2046 evenly spaced cells which have been secreting CMF continuously reaches 0·3 ng ml$^{-1}$ (→) within a few hours when cells are a few cell diameters apart. The calculation assumed 1000 CMF receptors on each cell and the disk of cells to be sitting on a medium which allows the diffusion of small molecules as if they were in moist dirt. To test this prediction, cells were starved on moist filter pads at a variety of average cell–cell spacings (vertical bars at top of graph) and scored for cell differentiation [(+) aggregation and roughly 50% of cells differentiating; (±) a few aggregates and 1 to 10% of cells differentiating; (−) no aggregates and no prespore-positive cells seen].

of a thick, permeable material, the concentration of CMF at the center of a confluent array (10 μm center-to-center cell spacing) reaches 0·3 ng ml$^{-1}$ in less than 1 hr [Fig. 5(b)]. When the spacing is 42 μm center-to-center (32 μm edge-to-edge distance between cells), the threshold is reached at 10 hr. This corresponds to a density of $5{\cdot}7 \times 10^4$ cells cm$^{-2}$. A larger value of $R_T$, 5000 receptors cell$^{-1}$, prevents the CMF concentration from reaching 0·3 ng ml$^{-1}$ within 2 hr, and requires center-to-center cell spacings of less than 32 μm for the CMF concentration to reach 0·3 ng ml$^{-1}$ in 10 hr. We previously found that for differentiation, cells need to be exposed to CMF at some point, but not necessarily at the beginning of development (Gomer *et al.*, 1986). With a different arbitrary choice of $R_T = 10^5$ receptors cell$^{-1}$, $K_D = 30$ ng ml$^{-1}$, and activation at 0·1% occupancy (parameters which do not allow single cells to activate themselves), an array of 2046 confluent cells will activate itself in slightly less than 2 hr, and with a center-to-center spacing of 40 μm will activate itself in 10 hr.

To test the hypothesis that at an air–water interface, cells below a certain density will not stimulate themselves to differentiate, cells were starved on moist filter pads at densities ranging from $1{\cdot}7 \times 10^3$–$5{\cdot}0 \times 10^5$ cells cm$^{-2}$. The approximate cell spacing was calculated as (density)$^{-1/2}$. Immunofluorescence staining showed that no prespore cell differentiation occurred for spacings of more than 70 μm, and that spacings of 40 μm or less caused strong differentiation, closely matching the calculated spacing at which the CMF concentration exceeds 0·3 ng ml$^{-1}$ [Fig. 5(b) at top]. We previously observed that differentiation of cells at low cell densities in buffer requires only the addition of purified CMF and then cAMP at six hours (Jain *et al.*, 1992). Since the cells in this experiment were exposed to cAMP at 6 hr, this suggests that the absence of differentiation at low cell densities in this experiment is due to a lack of CMF.

The calculations indicate that in 10 hr a single cell in isolation should be unable to differentiate whereas 2046 confluent cells should differentiate. This then forces the prediction that for cells on a soil surface there should be a critical number of confluent cells necessary for differentiation, with any smaller number of cells unable to differentiate (at least until the 80 kDa CMF breaks down to the much more potent small CMF; Yuen *et al.*, 1991). Repeating the calculations for successively smaller arrays of cells, we found that for aggregates of fewer than roughly 45 cells, the CMF concentration fails to rise to 0·3 ng ml$^{-1}$ for any number of receptors.

## 5. Discussion

### 5.1. MEASURED ACTIVITY THRESHOLD AND ACCUMULATION OF CMF

In order to calculate the concentration of CMF in the vicinity of cells, we have examined some of the parameters that might affect the CMF activity threshold and accumulation rate. None of the parameters that we have examined affect the CMF activity threshold of 0·3 ng ml$^{-1}$, with the exception of high pH, which also affects the gross morphology of the cells and thus is probably simply affecting viability. Although we have been actually measuring accumulation, the observation that very little CMF

activity is lost when conditioned medium from high density cells is allowed to sit at room temperature for 2 days (Yuen *et al.*, 1991) suggests that the CMF degradation rate is low and that the CMF secretion rate will be closely approximated by accumulation/time. The measured CMF secretion per cell increases by approximately nine-fold over a 150-fold range of cell densities (a determination of the exact secretion rate would require a correction for different surface to volume ratios at different cell densities). This increase is probably not due to cell contact, since there is an increase in CMF secretion per cell at the higher cell densities, even though all of the cells are in aggregates at these densities. The multiplicative effect of adding CMF to low density cells suggests that the increase is due to a positive feedback effect of CMF itself. The amount of CMF secreted per cell by high-density cells in submerged monolayer conditions (Fig. 1) is similar to that of cells starved in shaking culture, which is 160 units per $9 \times 10^6$ cells or $1{\cdot}7 \times 10^{-5}$ units cell$^{-1}$ (Gomer *et al.*, 1991).

A variety of conditions have been examined for effects on CMF production at normal growth temperatures (21°C), and none cause the effective CMF secretion to exceed roughly 12 molecules cell$^{-1}$ min$^{-1}$. We previously found that CMF accumulation in a different buffer is pH sensitive (Gomer & Firtel, 1987); in this case pHs above 6·3 caused a decrease in accumulation. Since the change in accumulation is buffer-specific, pH *per se* is probably not regulating CMF secretion.

High CMF levels are required for the cAMP pulse-mediated aggregation (Jain *et al.*, 1992) and these pulses in turn increase CMF accumulation two-fold at higher temperatures (Table 1), suggesting the possibility of a positive feedback loop. There are two major classes of signal transduction pathways downstream of the cAMP receptor. One pathway involves activation of adenylyl cyclase, while the other does not (reviewed in Devreotes, 1989). The pathway activating adenylyl cyclase can be blocked with caffeine (Brenner & Thoms, 1984; Mann & Firtel, 1987). Since caffeine blocks the cAMP pulse-mediated increase in CMF production at 23°C, this may be the pathway responsible for the cAMP-pulse-increased CMF accumulation (Table 1).

The accumulation of CMF by cells at most phases of the cell cycle indicates that both prespore and prestalk cells (Gomer & Firtel, 1987) secrete CMF. It is unclear whether the slightly increased accumulation of CMF by cells starved in mid- to-late G2 (prespore phase; Gomer & Firtel, 1987) has a functional significance. The activities of other molecules (measured at the aggregation stage of development) that regulate aggregation, such as cAMP receptors and phosphodiesterase, also vary depending on cell-cycle phase at the time of starvation (Wang *et al.*, 1988). The decrease in CMF activity accumulated by cells starved in M phase may be due to a block in the secretion pathway similar to that observed for *Xenopus* cells in M phase (Kanki & Newport, 1991), and suggests that these cells may not progress through the cell cycle after starvation.

### 5.2. CALCULATED CONCENTRATION OF CMF

The diffusion calculations indicate that even after ten hours of secreting CMF, the level of 80 kDa CMF in the vicinity of an isolated starved cell, either in a film of water with a thickness of 10 μm or more, or on the surface of a permeable material, will be too low to induce the expression of CMF-requiring genes (Figs 3 and 4). The fact that cells at low densities cannot stimulate themselves suggests that isolated cells cannot provide enough CMF for themselves and that thus CMF does not simply have an autocrine function for cells on solid or permeable surfaces. For the same secretion rate, the level of 80 kDa CMF in the vicinity of a high density of starved cells will induce the expression of CMF-requiring genes [Fig. 5(b)]. This would allow CMF to function as part of a density-sensing mechanism in *Dictyostelium* by triggering aggregation when a majority of the cells in a given area had starved, as signalled by CMF secretion. The calculations assumed a density-independent CMF secretion rate; the lower amount of CMF secreted by low-density cells and the increased amount of CMF activity per cell from high-density cells would tend to magnify the density-sensing effect. The differentiation of starved cells on the surface of moistened filter pads at average cell–cell spacings below 40 μm and their lack of differentiation at spacings above 70 μm suggest that the diffusion calculations are a good model for the density-sensing process and verify the prediction that cells at low densities cannot stimulate themselves. The small amount of differentiation at average spacings between 40 and 70 μm may be due to local density fluctuations. Both the lack of differentiation at low cell densities and the differentiation at high cell densities are predicted to occur for at least two widely different values of the number of CMF receptors per cell and their affinities for CMF. The calculations also allow us to estimate how much the parameters can be varied and still allow the density-sensing mechanism to function. For instance, for cells on a soil surface, the CMF secretion rate could be increased ten-fold and would still be too low for an isolated cell to trigger itself. Thus the increased CMF activity secreted by

mid-G2 phase cells will still be below the threshold for activation of low-density cells. Lowering the CMF secretion rate however requires a reduction in the number of receptors per cell or the diffusion coefficient for high-density cells to be able to differentiate, suggesting that for one set of parameters the secretion rate can vary only within a factor of 10. We have thus shown that cells can sense their local density by simultaneously secreting and recognizing a molecule. In addition, the diffusion calculations led to the prediction that a planar aggregate would need to contain more than 45 cells for the CMF concentration to reach its activation threshold. This suggests that for other geometries a similar density-sensing mechanism with different secretion rate, diffusion coefficient, and activation threshold parameters could function in determining the total number of cells in a tissue.

One possible explanation for the existence of CMF is that *Dictyostelium* cells need to coordinate aggregation. The cells eat soil bacteria, and thus may starve asynchronously since some cells in an aggregation field will still be digesting bacteria while others will not have found a bacterium for several hours. For maximum spore dispersal, fruiting bodies probably need to raise as many spores as possible as high off the ground as possible, and thus large aggregates would be advantageous compared to small aggregates. Without a mechanism to sense the density of starved cells, cohorts of cells which starved at the same time would each form a small, ineffective fruiting body. The behavior of the cells lacking CMF, the results of the diffusion calculations, and the experimental verification of the predicted cell density necessary for differentiation suggest that the function of CMF may be to coordinate development of large fruiting bodies, by permitting aggregation when most of the cells in a given area have starved, as signalled by CMF secretion.

We thank Subha Arimilli and Robin Ammann for assistance with the experiments, James Bryers, Robert Gomer, John Olson, Maureen Price, Tim Spann and Michael Stern for helpful discussions, and an anonymous referee for improving the paper and noticing the asymptotic limit for the three-dimensional diffusion. This work was supported by GM 42604 from the National Institutes of Health. R. H. G. is an assistant investigator of the Howard Hughes Medical Institute.

Note added in proof: We have begun to characterize the CMF receptor and have found $R_T = 3{\cdot}9 \times 10^4$ receptors cell$^{-1}$ and $K_D = 170$ ng ml$^{-1}$ (Jain, R. & Gomer, R. H. (1994). *J. Biol. Chem.* **269,** 9128–9136). When these value used in the calculations described above, results essentially identical to those shown here are obtained.

## REFERENCES


BAILEY, J. E. & OLLIS, D. F. (1986). In: *Biochemical Engineering Fundamentals* pp. 522–525. New York: McGraw-Hill.

BRENNER, M. & THOMS, S. D. (1984). *Dev. Biol.* **101,** 136–146.

CLARKE, M., DOMINGUEZ, N., YUEN, I. S. & GOMER, R. H. (1992). *Dev. Biol.* **152,** 403–406.

DEVREOTES, P. (1989). *Science*, **245,** 1054–1058.

DOTTIN, R. P., BODDULURI, S. R., DOODY, J. F. & HARIBABU, B. (1991). *Dev. Genet.* **12,** 2–5.

GERISCH, G. (1968). In: *Current Topics in Developmental Biology*, Vol. 3, pp. 157–197. New York: Academic Press.

GOMER, R. H., ARMSTRONG, D., LEICHTLING, B. H. & FIRTEL, R. A. (1986). *Proc. natn. Acad. Sci.* U.S.A. **83,** 8624–8628.

GOMER, R. H. (1987). In: *Methods in Cell Biology*, Vol. 28 (Spudich J. A., ed.) pp. 471–486. Orlando, FL: Academic Press.

GOMER, R. H. & FIRTEL, R. A. (1987). *Science* **237,** 758–762.

GOMER, R. H., YUEN, I. S. & FIRTEL, R. A. (1991). *Development* **112,** 269–278.

GRABEL, L. & LOOMIS, W. F. (1976). *Dev. Biol.* **64,** 203–209.

GRADSHTEYN, I. S. & RYZHIK, I. M. (1965). In: *Table of Integrals Series and Products*. New York: Academic Press.

GROSSMAN, A. D. & LOSICK, R. (1988). *Proc. natn. Acad. Sci. U.S.A.* **85,** 4369–4373.

HABATA, Y., URUSHIHARA, H., FANG, H. & YANAGISAWA, K. (1991). *Cell Struct. Funct.* **16,** 185–187.

JAIN, R., YUEN, I. S., TAPHOUSE, C. R. & GOMER, R. H. (1992). *Genes Dev.* **6,** 390–400.

KANKI, J. P. & NEWPORT, J. W. (1991). *Dev. Biol.* **146,** 214–227.

KIM, S. K. & KAISER, D. (1990). *Cell* **61,** 19–26.

LOOMIS, W. F. (1975). In: *Dictyostelium discoideum. A Developmental System*. New York: Academic Press.

LOOMIS, W. F. (1982). In: *The Development of Dictyostelium discoideum*. New York: Academic Press.

MAEDA, Y. (1986). *J. gen. Microbiol.* **132,** 1189–1196.

MANN, S. K. O. & FIRTEL, R. A. (1987). *Molec. Cell. Biol.* **7,** 458–469.

MCDONALD, S. A. (1986). *Dev. Biol.* **117,** 546–549.

MEHDY, M. C. & FIRTEL, R. A. (1985). *Molec. Cell. Biol.* **5,** 705–713.

MEHDY, M. C., RATNER, D. & FIRTEL, R. A. (1983). *Cell* **32,** 763–771.

MOORE, W. J. (1962). In: *Physical Chemistry*. Englewood Cliffs, NJ: Prentice-Hall.

MORSE, P. M. & FESHBACH, H. (1953). In: *Methods of Theoretical Physics*. New York: McGraw-Hill.

RAPER, K. B. (1984). In: *The Dictyostelids*. Princeton, NJ: Princeton University Press.

RATHI, A. & CLARKE, M. (1992). *Mech. Dev.* **36,** 173–182.

ROOS, U.-P. (1987). In: *Methods in Cell Biology*, Vol. 28 (Spudich, J. A., ed.) pp. 261–279. Orlando, FL: Academic Press.

SIEGRIST, H. & GUJER, W. (1985). *Water Res.* **19,** 1369–1378.

WANG, M., AERTS, R. J., SPEK, W. & SCHAAP, P. (1988). *Dev. Biol.* **125,** 410–416.

WEAST, R. C. (1973). In: *Handbook of Chemistry and Physics*. Cleveland: CRC Press.

WEIJER, C. J., DUSCHL, G. & DAVID, C. N. (1984). *J. Cell Sci.* **70,** 111–131.

YUEN, I. S., TAPHOUSE, C. R., HALFANT, K. A. & GOMER, R. H. (1991). *Development* **113,** 1375–1385.