Proc. Nat. Acad. Sci. USA
Vol. 71, No. 5, pp. 2118–2122, May 1974

Cyclic-AMP-Controlled Oscillations in Suspended *Dictyostelium* Cells: Their Relation to Morphogenetic Cell Interactions

(chemotaxis/slime molds/cell aggregation/membrane receptors/dissipative structures)

GÜNTHER GERISCH* AND BENNO HESS†

* Friedrich-Miescher-Laboratorium der Max-Planck-Gesellschaft, 74 Tübingen; and †Max-Planck-Institut für Ernährungsphysiologie, 46 Dortmund, Germany

Communicated by Britton Chance, November 28, 1973

ABSTRACT **Periodic spikes of decreased optical density were recorded in cell suspensions of *Dictyostelium discoideum*. Spike formation as well as changes in the redox state of cytochrome *b* are facultatively and independently coupled to an oscillating system which is under developmental control and presumably underlies signal transmission in aggregating cells.**

Cyclic AMP triggers a double response, the slow component resembling the spikes formed during spontaneous oscillations. The fast component shows characteristics of the chemotactic response to cyclic AMP. The receptor system is suggested to sense changes of cyclic AMP concentration in time. Cyclic AMP pulses interact with the oscillating system, resulting in phase shift or suppression of spike formation, and in the induction of oscillations in an early stage of development before the onset of spontaneous oscillations. Continuous flow application of cyclic AMP does not change frequency up to flow rates which extinguish oscillations.

After the end of growth, cells of the slime mold *Dictyostelium discoideum* aggregate in response to chemotactic stimuli. This process is an example of self-organization of spatial patterns by chemical cell communication, starting with a layer of randomly distributed identical cells (1–3). Aggregation territories are controlled by centers which typically release chemotactic signals in pulses with a frequency of 0.2–0.3 min^{-1} (4, 5). The cells around a center respond by orientated cell movement, and also by producing a pulse to which the outer neighboring cells respond after a signal input/output delay of $\geqslant$ 15 sec. So waves of chemotactic pulses can be propagated over a distance much larger than the chemotactic action radius of an aggregation center (4, 5).

cAMP elicits a chemotactic response (6, 7), and when applied in pulses induces propagated waves, thus simulating transmitter action (8). Extracellular cAMP is rapidly destroyed by extracellular as well as cell-bound phosphodiesterases (9, 10). Periodic activities and cAMP effects can be recorded optically in stirred cell suspensions. This makes it possible to investigate the molecular basis of morphogenetic cell communication under conditions similar to those used in studying oscillations of the glycolytic pathway in yeast cell suspensions (11, 12). In the present paper we report that the ability to oscillate in suspensions is related to the morphogenetic capacity of the cells, and describe interactions of cAMP with the oscillating system.

Abbreviations: cAMP, cyclic adenosine 3′,5′-monophosphate; cGMP, cyclic guanosine 3′,5′-monophosphate; cIMP, cyclic inosine 3′,5′-monophosphate; t_n, developmental stage of cells timed as n hours after their removal from growth medium.

METHODS

Dictyostelium discoideum strain *Ax-2* (13) was cultivated at 22–25° axenically on growth medium containing 1.8% maltose (13) up to cell densities of 0.3 to $1.4 \cdot 10^7$/ml. The cells were washed three times in the cold with 0.017 M Soerensen phosphate buffer, pH 6.0, resuspended in the buffer, adjusted to $1 \cdot 10^7$/ml, and shaken. The time of resuspension was taken as the beginning of cell differentiation to aggregation competence. After various times, cells were centrifuged and adjusted in cold buffer to $2 \cdot 10^8$/ml. From an ice bath, 2 ml of the suspension were transferred into a cuvette with an optical pathway of 1 cm, and agitated by bubbling oxygen with a constant flow rate of 24 ± 1 ml/min through two syringes. For all measurements taken at 405 and 430 nm, the cuvette was kept at 23°. The spectrophotometer and the continuous flow equipment used are described in (14) and (12), respectively.

Recording of cytochrome *b* was based on oxygen-dithionite difference spectra determined at liquid air temperature in suspensions of 10^8 cells per ml using a Johnson Foundation split beam spectrophotometer. Peaks were found for cytochromes *a*, a_3 at 598, c_1 at 553, *c* at 548, and *b* at 562 and 560, as well as a Soret region with a peak at 425 and shoulders at 430 and 445 nm. The redox state of cytochrome *b* in living cell suspensions was recorded at 430 nm using 405 nm as the reference wavelength. The latter was simultaneously used for recording optical density.

RESULTS

Spikes and Sinusoidal Oscillations. After separation from the growth medium, suspended cells of *D. discoideum Ax-2* pass through a pre-aggregation phase of about 9 hr before they acquire full aggregation competence (9, 15). Within the first 5 hr of this phase, no spontaneous oscillations were observed. When, however, cells were harvested 6–14 hr after separation from the growth medium, oscillations began immediately after transfer from an ice bath into the optical cuvette. Regularly, an initial series of spikes was recorded, followed by sinusoidal oscillations (Fig. 1). Sometimes several cycles of only sinusoidal oscillations were intercalated between spike-generating cycles. The mean spike frequency was 0.14 min^{-1}, and the frequency increased upon cessation of spike formation, with a mean acceleration factor of 1.20. These results indicate that the cellular activity underlying spike formation, although being coupled to an oscillating system, is not an indispensable part of it.

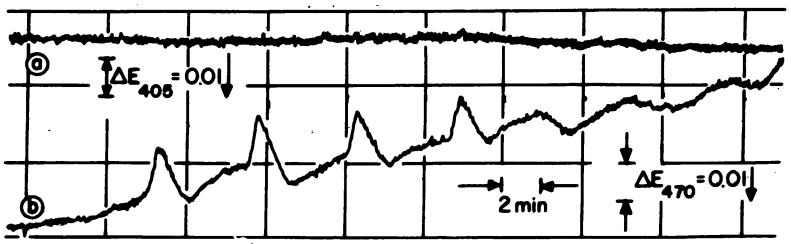

FIG. 1. Developmental control of the oscillating system. (*a*) A cell suspension recorded at t_4 (the subscript indicating hours after beginning of the preaggregation phase). (*b*) Spontaneous onset of periodic spike formation and sinusoidal oscillation at t_7. The trend of the curve, presumably indicating shift towards larger aggregates, is typical for that stage. (The optical density has been recorded at 470 nm in an exception to the usual procedure.)

The periodic signals were recorded as a decrease of optical density (E) at 405 nm, with a spike amplitude of approximately 2% of the background density. Since the amplitude decreased steadily with increasing wave length between 380 and 600 nm, the signal appearantly represents changes of light scattering, indicating changes in either cell shape, volume, or degree of agglutination. We presume that the latter contributes much to the recorded signals, since no oscillations were observed when cell agglutination was prevented by intense stirring.

Fast and Slow Responses to cAMP. Addition of cAMP to aggregation-competent cells was followed by two peaks of decreased optical density (Fig. 2). The fast peak was also found in early preaggregation cells. However, the sensitivity of these cells was about 10-fold lower (Fig. 3*a*). The maximum of the fast response was reached after half a minute, and its decay in preaggregation cells had a time constant of 7–10 sec, almost independent of initial cAMP concentrations in the range of 10^{-8} to $3 \cdot 10^{-6}$ M.

The slow response remained weak during preaggregation at any cAMP concentration tested (Fig. 2*A*, 3*a*), whereas in aggregation-competent cells it could even exceed the fast one (Figs. 2*B* and 3*a*). The length of the delay between fast and slow responses decreased from 2 min during preaggregation to 0.8 min at aggregation-competence (Fig. 3*b*). Other cyclic nucleotides tested were three orders of magnitude less active than cAMP; noncyclic nucleotides had no significant effect (Fig. 3*a*).

If a second cAMP pulse was applied at the peak of the slow response, another fast response was induced (Fig. 4), indicating that fast and slow responses were independent, although at the concentration used cAMP caused a maximal fast response and triggered an almost maximal slow response.

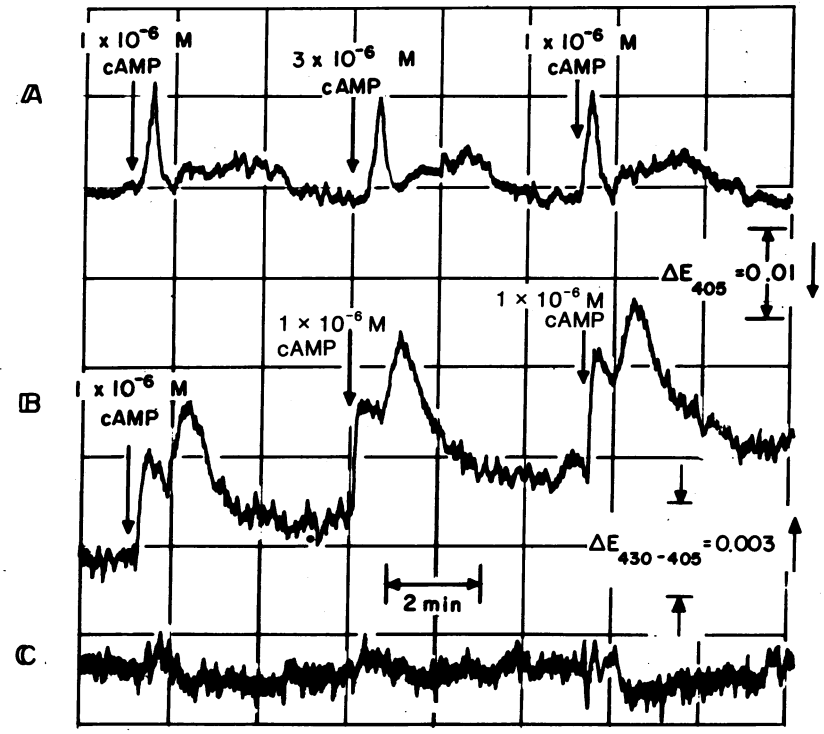

FIG. 2. (*A*) Distinct fast responses followed by weak slow responses in t_3 cells. (*B*) Slow responses exceeding the fast ones in t_{10} cells. (*C*) Cytochrome *b* absorption recorded simultaneously with (*B*). The direction of the arrow indicates increased reduction. In all figures the molarity indicated is the final concentration in the cell suspension. cAMP as well as other substances were added to 2 ml of the suspension in volumes of 0.7–2.1 μl.

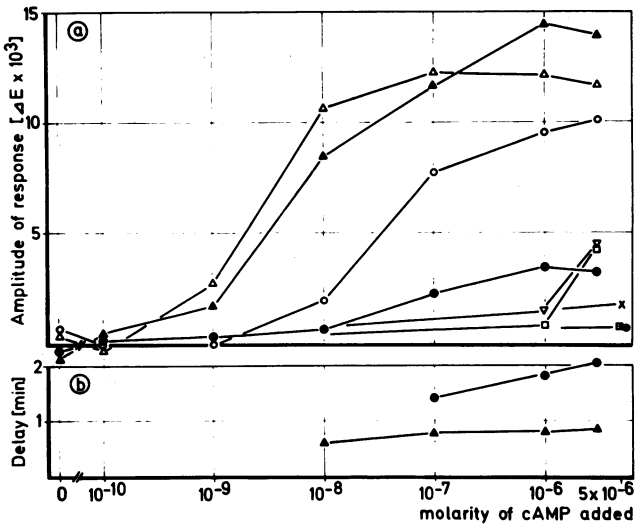

FIG. 3. Peak heights of the responses to cAMP and other nucleotides (*a*), and delays between the fast and slow responses to cAMP (*b*). (*a*) Fast responses to cAMP in early preaggregation (○) and aggregation-competent cells (△); and slow responses of the same types of cells (●, ▲). Fast responses in aggregation-competent cells to cGMP (□), cIMP, (▽), 5′-AMP (⊡), 3′-AMP (×), and ATP (⊙). The slow responses remained weak with cGMP and cIMP, or were undetectable in the case of the non-cyclic nucleotides (not shown). (*b*) Delays between the fast and slow response peaks in early preaggregation (●) and aggregation-competent cells (▲). Early preaggregation cells were centrifuged between t_0 and t_1, and measured between t_2 and t_4. Aggregation-competent cells were centrifuged from the same culture between t_8 and t_9, and measured between t_{10} and t_{15}. Data are means of at least three determinations taken at intervals of 5 min.

Interaction of Cyclic AMP with the Oscillating System. When cells were stimulated in the preaggregation phase by cAMP pulses applied at a frequency of 0.2 min^{-1}, precocious onset of oscillations was observed at 2.5–4 hr after end of growth (Figs. 5–7). The mean spike frequency was 0.14 min^{-1}, similar to that in aggregation-competent cells. After stimulation by cAMP, the cell suspensions sometimes passed an initial period of irregular activity until rhythms became dominant (Fig. 6). From that time on the cAMP-induced oscillations were rather stationary, and, therefore, were preferentially used for exploring the interference of cAMP with established oscillations.

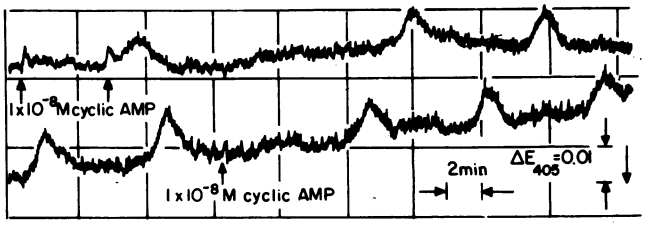

FIG. 5. Onset of oscillation in t_4 cells after several cAMP pulses, and phase shift induced by cAMP. It is typical that oscillation begins after a series of cAMP pulses applied at intervals of 5 min as soon as the slow response becomes prominent. In the case shown, spike formation was in phase with this induced response, with one spike missing. A phase shift was induced in the oscillating system by injecting cAMP shortly before the midst of the period between two spikes.

When applied to already oscillating cell suspensions, single cAMP pulses induced one of three reactions: phase shifts, irregular out-of-phase-activity, or suppression of oscillations (Fig. 6). In Fig. 5 cAMP applied within the first half-period after the spike caused a phase delay; in Fig. 7 cAMP applied after the midst of the period elicited a precocious spike. In this case, one spike was skipped, but from the next spike on the system oscillated in the new phase. When cAMP was applied just before the regular appearance of a spike, the next following spike was inhibited but no phase shift was induced (not shown).

We have repeatedly observed that cAMP concentrations close to threshold levels affected oscillations when applied after the midst of the period, but were ineffective when applied at an earlier phase (Fig. 7), which indicates reduced sensitivity to cAMP in the first part of a period. Attempts to restore oscillations after their suppression by cAMP were unsuccessful. When such cells were synchronized by cAMP pulses applied at frequencies of 0.14–0.22 min^{-1}, fast and slow responses could be elicited by such pulse (Fig. 2*b*) but no rhythmic activity continued after cessation of cAMP stimulation.

When cAMP was added continuously at flow rates of 3 and 50 nmoles min^{-1} $liter^{-1}$ to cells of the early preaggregation phase, no activities resembling the fast responses to cAMP pulses or premature commencement of oscillation during the period of cAMP application were observed. Established oscillations were suppressed by constant flow application of cAMP. Lower flow rates did not influence the frequency of

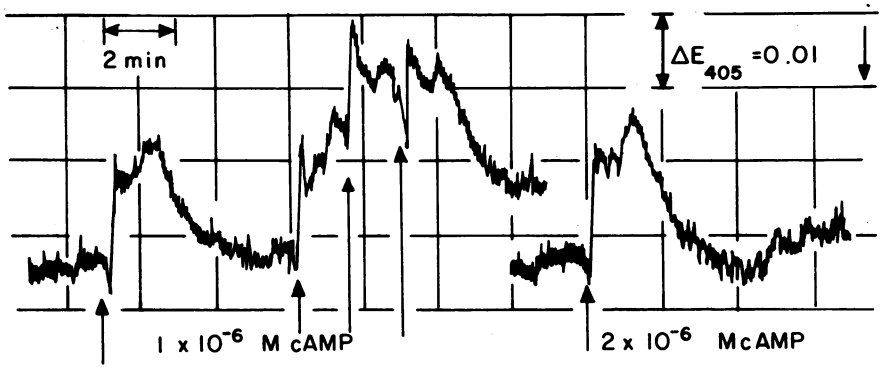

FIG. 4. Superposition of fast and slow responses. A total of $2 \cdot 10^{-6}$ M cAMP was divided into two injections and the second one applied simultaneously with the slow response to the first; the response was higher than after a single injection of either $1 \cdot 10^{-6}$ or $2 \cdot 10^{-6}$ M cAMP. A third injection did not further increase the response. Negative spikes appearing at the moment of cAMP injection were artifacts produced by inserting a capillary. The cells were in the t_{12} stage.

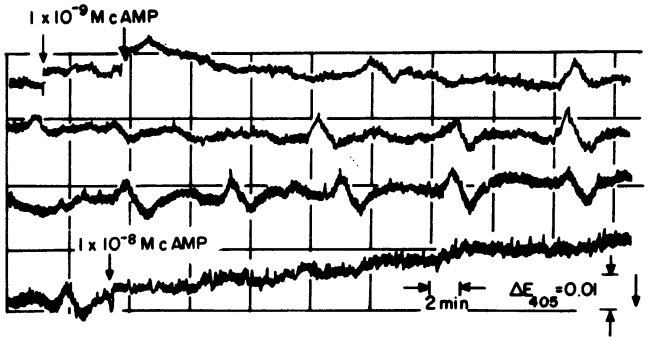

FIG. 6. Induction and suppression of oscillation in t_4 cells by cAMP. The induced activity was not clearly periodic from the very beginning; but after more than one hour frequency and pattern became constant. cAMP suppressed the oscillation almost completely. (The interrupted lines at the time of the initial cAMP additions indicate changes in optical density caused by diluting the cell suspension, in this case, with 10 μl of solution.)

oscillations (Fig. 8), suggesting that the frequency does not depend on the extracellular steady-state levels of cAMP below those levels suppressing oscillations.

Oscillations of the Mitochondrial Electron Transport System. Regularly, spike formation was accompanied by a shift of cytochrome *b* into a more reduced state (Fig. 7). Two observations indicate that the electron transport is coupled to the oscillator underlying spike formation, rather than to spike formation *per se:* In Fig. 7, a cytochrome *b* shift occurred also in that period after cAMP addition in which no spike was formed. Conversely, strong cAMP-induced spikes were not accompanied by a significant cytochrome *b* shift (Fig. 2*C*).

DISCUSSION

The phenomena observed in cell suspensions showed various aspects of pulse generation, wave propagation, and chemotactic response in cell layers, suggesting that basic mechanisms of the morphogenetic signal system can be investigated under homogeneous conditions in stirred cell suspensions. The oscillating system acting under suspension conditions was under developmental control. The sensitivity to cAMP measured in suspension increased during the pre-aggregation phase (Fig. 3); previously an increase was also found using chemotaxis as an assay (7). The fast peak of the response to cAMP showed that the cAMP receptor functioned already when the slow peak was still rudimentary. The chemotactic response has been found to develop earlier than the ability to propagate waves (8). We suppose that the fast peak is equivalent to the chemotactic response, and that the slow response to cAMP corresponds to the periodic spikes formed by oscillating cells in response to a self-produced factor. This factor should be identical with the transmitter mediating wave propagation in cell layers, which has been suggested to be cAMP too (8).

A half-maximal fast peak was obtained by $1 \cdot 10^4$ cAMP molecules per cell, and the response was still detectable at a molarity of 10^{-9}, when the molecule to cell ratio was $3 \cdot 10^3$. The low concentration necessary and the high nucleotide specificity of the receptor system are similar to those measured for chemotaxis (18).

Previous results obtained in cell layers suggested a refractory phase following excitation (16, 17). The differential

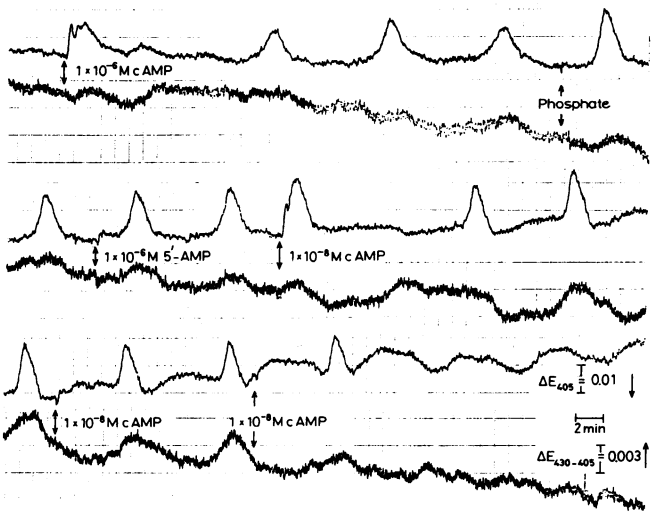

FIG. 7. Spike formation (*upper curves*) correlated with changes in the redox state of cytochrome *b* (*lower curves*). This continuous record of a cAMP-induced oscillation in $t_{2.5}$ cells shows (*1*) precocious spike formation associated with a phase shift, caused by injecting cAMP shortly after the midst of a period; (*2*) inefficacy of the same small quantity of cAMP when injected within the first third of a period; (*3*) inactivity of phosphate and 5′-AMP; (*4*) gradual appearance of sinusoidal oscillation between the spikes, and final replacement of the latter (see also Fig. 1*b*); (*5*) a 14% increase of frequency when spike formation ceased; (*6*) oscillation of the redox state of cytochrome *b* in phase with spike formation, recorded as in Fig. 2*B*.

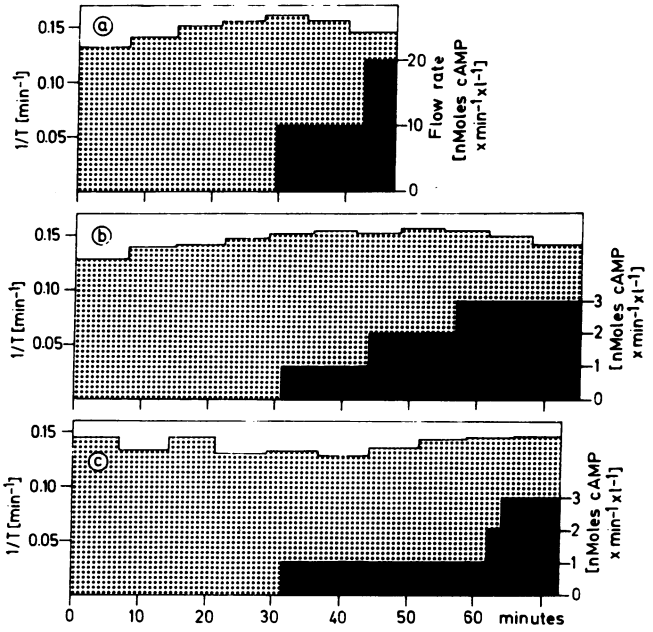

FIG. 8. Frequency of oscillation under the influence of a continuous flow of cAMP. *Black* (*right ordinate*): Rate of cAMP application. *Dotted* (*left ordinate*): 1/T, the reciprocal value of a period length. (*a*) Spike frequency of cAMP-induced oscillation in t_5 cells. (*b*) The same between t_4 and t_5. (*c*) Frequency of spontaneous sinusoidal oscillation between t_{10} and t_{12}. In (*a*) and (*c*) the end of the graph was the time when oscillation became suppressed, in (*b*), when flow was stopped.

sensitivity of the oscillating system to cAMP after and before a spike might be an expression of refractoriness, although in no phase were the cells absolutely insensitive. However, the cAMP effects in cell suspensions still leave us with several unanswered questions: (*1*) How is the independence of fast and slow responses, shown in Fig. 4, explained? (*2*) Cells that had stopped oscillating after cAMP application were able to respond to cAMP and had produced it before, supposing that the factor responsible for the spikes is cell-produced cAMP. What then is the nature of the block that prevents spontaneous oscillation of these cells? (*3*) Does the suppression of oscillations by cAMP have any biological function? Bacteria as well as cAMP released by them disturb aggregation patterns, shifting the cell population back from aggregation to food seeking (18). The paralysis of periodic signal generation in aggregation centers and of wave propagation may be a mechanism involved.

The insensitivity of the oscillation frequency to continuous flow of cAMP also needs to be clarified. In line with the receptor systems sensing chemotactic factors in bacteria (19, 20) and nematodes (21), the cAMP receptor system in *D. discoideum* appears to respond to rapid changes of concentration in time, rather than to concentration *per se*. The contribution of cAMP destruction by phosphodiesterases to the time-pattern of the response remains to be investigated.

We thank Mr. H. Schlüter, Mrs. I. Lauterborn, and Mrs. M. Bürk for valuable cooperation. The work was supported by the Deutsche Forschungsgemeinschaft and Stiftung Volkswagenwerk.

1. Gerisch, G. (1971) *Naturwissenschaften* **58**, 430–438.
2. Robertson, A. & Cohen, M. H. (1972) *Annu. Rev. Biophys. Bioeng.* **1**, 409–464.
3. Segel, L. A. & Stoeckly, B. (1972) *J. Theor. Biol.* **37**, 561–585.
4. Gerisch, G. (1968) *Curr. Top. Develop. Biol.* **3**, 157–197.
5. Shaffer, B. M. (1962) *Advan. Morphog.* **2**, 109–182.
6. Konijn, T. M., van de Meene, J. G. C., Bonner, J. T. & Barkley, D. S. (1967) *Proc. Nat. Acad. Sci. USA* **58**, 1152–1154.
7. Bonner, J. T., Barkley, D. S., Hall, E. M., Konijn, T. M., Mason, J. W., O'Keefe, G. & Wolfe, P. B. (1969) *Develop. Biol.* **20**, 72–87.
8. Robertson, A., Drage, D. J. & Cohen, M. H. (1972) *Science* **175**, 333–335.
9. Malchow, D., Nägele, B., Schwarz, H. & Gerisch, G. (1972) *Eur. J. Biochem.* **28**, 136–142.
10. Pannbacker, R. G. & Bravard, L. J. (1972) *Science* **175**, 1014–1015.
11. Hess, B. & Boiteux, A. (1968) *Biochim. Biophys. Acta (Library)* **11**, 148–162.
12. Hess, B. & Boiteux, A. (1968) *Hoppe-Seyler's Z. Physiol. Chem.* **349**, 1567–1574.
13. Watts, D. J. & Ashworth, J. M. (1970) *Biochem. J.* **119**, 171–174.
14. Hess, B., Kleinhans, H. & Schlüter, H. (1970) *Hoppe-Seyler's Z. Physiol. Chem.* **351**, 515–531.
15. Beug, H., Katz, F. E. & Gerisch, G. (1973) *J. Cell Biol.* **56**, 647–658.
16. Cohen, M. H. & Robertson, A. (1971) *J. Theor. Biol.* **31**, 101–118.
17. Gerisch, G. (1965) *Roux' Arch. Entwicklungsmech.* **156**, 127–144.
18. Konijn, T. M. (1972) *Advan. Cyclic Nucleo. Res.* **1**, 17–31.
19. Macnab, R. M. & Koshland, D. E., Jr. (1972) *Proc. Nat. Acad. Sci. USA* **69**, 2509–2512.
20. Berg, H. C. & Brown, D. A. (1972) *Nature* **239**, 500–504.
21. Ward, S. (1973) *Proc. Nat. Acad. Sci. USA* **70**, 817–821.