

References and Notes

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13. The composition of the Ringer solution was: NaCl, 100 mM; KCl, 2.5 mM; Na₂HPO₄, 2.5 mM; NaH₂PO₄, 0.45 mM; CaCl₂, 1.9 mM; NaHCO₃, 12.0 mM; glucose, 2.8 mM. This was equilibrated with 5 percent CO₂ and 95 percent O₂. The pH remained constant at 7.4 ± 0.1 unit. The temperature varied in different experiments from 13° to 15°C.
14. Differential d-c recordings were either made between the most proximal and the most distal ends of the roots with a Ag-AgCl ground in the bath or between the bath and the most distal end of the root. In the latter case the bath was left ungrounded. No qualitative differences were seen between the two methods of recording. Slow potentials were displayed on a Brush paper writer.
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16. P. D. Wall's technique [*J. Physiol. London* **142**, 1 (1958)] was employed to measure excitability of intraspinal afferent terminals. This technique is based on the concept that when primary afferent depolarization of dorsal root terminals is produced, dorsal root fibers are more easily discharged by intraspinal stimulation. This leads to an increase in the amplitude of the compound antidromic spike potential recorded from the dorsal root. A low-resistance, sodium chloride-filled glass capillary microelectrode was used for stimulation after insertion into the dorsal horn close to the terminals of the primary afferent fibers. Recording of the extracellular field potential was used to correct the location of the electrode tip in the spinal cord. Submaximum stimuli were used.
17. R. A. Davidoff, in preparation.
18. On rare occasions glycine hyperpolarized the dorsal root terminals. Bicuculline did not affect this phenomenon.
19. Amino acids have little or no effect on spinal afferent fibers proximal to the terminals (4).
20. Small, spontaneous dorsal root depolarizations have been described on exposure of the spinal cord to convulsants (2) and to excitatory agents (21).
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22. I thank V. Grayson and A. Warren for technical assistance.

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Control of Aggregation in Dictyostelium discoideum by an External Periodic Pulse of Cyclic Adenosine Monophosphate

Abstract. We have induced and controlled normal aggregation of Dictyostelium discoideum amoebas by electrophoretic release of pulses of cyclic adenosine monophosphate from a microelectrode. This has yielded information about the sequence of development of aggregation competences during interphase. We believe that modifications of the technique will have wide application in investigations of other developing systems.

The cellular slime mold *Dictyostelium discoideum* was discovered by Raper (1). His classic paper (2) is a valuable source for details of its development (3). Amoebas of *D. discoideum* live in damp environments eating bacteria which they find by chemotaxis (4, 5). When the food supply is exhausted the amoebas go through a period of differentiation, called interphase, lasting for 6 to 8 hours (6). At the end of interphase some amoebas, distributed at random, begin to emit pulses (7) of an attractant (8), almost certainly cyclic adenosine monophosphate (AMP) (9). Neighboring amoebas respond to these periodically repeated pulses by emitting their own pulse approximately 15 seconds after being signaled (10); and then moving toward the original signal source. The movement lasts for about 100 seconds (11), while the pe-

riod between pulses is approximately 300 seconds (10, 12). During the movement each amoeba is apparently refractory to further stimulation. These features of the signal and response mechanisms guarantee the outward propagation of a wave of inward movement (7, 10-13). As each amoeba acts as a local signal source, there is a tendency for amoebas to move first toward their nearest neighbors, forming streams (14), which then continue to move toward the original signal source, finally leading to aggregation. There are many further complexities in the signaling and response mechanisms which lead to aggregation of *D. discoideum*; a detailed discussion is not appropriate here, but may be found elsewhere (2, 3, 10-12, 14).

We have made a model of wave propagation during the aggregation of

D. discoideum, and from this model have been able to extract quantities for the duration and amplitude of the cyclic AMP signal (10). In order to test our model and calculations we decided to attempt to initiate and control aggregation with an external signal source.

While the only properly investigated periodic signaling mechanism for the control of development is that of *D. discoideum*, there are many developmental processes involving movement having a component with a period in the order of minutes (15). It has been suggested that this periodicity might represent the activity of an underlying control signal of the sort considered in Goodwin and Cohen's theoretical model for the control of development (13, 15). This gives the experimental control of *D. discoideum* aggregation added point.

Amoebas of *D. discoideum* strain NC4-H were cultured on high growth agar (3) with *Escherichia coli* B/r and centrifuged free of the food bacteria (16). Amoebas were then plated out in a plastic chamber on 2 percent agar at a density of approximately 600 per square millimeter. The thickness of the aqueous film on the agar surface was adjusted to ~1 µm. The tip of a glass micropipet, with an internal diameter of approximately 5 µm, was introduced into the surface film. The micropipet contained 1 mM cyclic AMP, and 1 mM fluorescein in a buffered salt solution, pH=6.7 (17), the same as that used to make up the agar. Its impedance was approximately 1 to 3 megohms. The fluorescein was used as a visible marker in initially setting up the system, as it dissociates to produce a negatively charged ion with approximately the same ionic weight as cyclic AMP. The electrolyte in the micropipet was biased, in order to maintain the column of cyclic AMP stationary in the pipet, through a chlorided wire via a 10-megohm resistor to approximately ½ volt above ground, to which the agar in the experimental chamber was referred, also via a silver chlorided wire. Approximately every 4 minutes and 25 seconds the bias was reversed for 2 seconds to approximately 10 to 30 volts negative with respect to ground, depending on electrode impedance, driving out the negatively charged cyclic AMP and fluorescein ions. Estimates of the partial conductance from the cyclic AMP ions indicated that a pulse of cyclic AMP

approximating that released by a group of amoebas during aggregation would thus be released into the medium. The period was chosen to be shorter than that of the modal period of approximately 5 minutes that we have observed in naturally aggregating *D. discoideum* in order that we might expect to entrain any spontaneously occurring aggregation centers.

The field of amoebas, 1.5 by 1.0 mm, centered on the microelectrode, was filmed at eight frames per minute, or other rates when appropriate, with a Nikon SKe microscope with a $\times 5$ objective and $\times 5$ eyepiece, $\times 0.5$ camera lens, and a 16-mm Bolex camera, giving a total magnification of $12\frac{1}{2}$. In later experiments the field dimensions were halved. The frame in which each pulse occurred was marked with a small light spot from a bulb in the focal plane of the camera. The developed film was projected with a stop-motion projector (Traid model 16N) and analyzed.

In 12 experiments, amoebas were attracted toward, and formed an aggregate near, the microelectrode tip. In all cases a control plate of amoebas, not pulsed with the microelectrode but otherwise treated identically, was set up and observed. In the only experiments in which we failed to induce aggregation artificially and in which the microelectrode was neither blocked nor broken, the controls failed to aggregate naturally (three cases). Furthermore, in ten pulsed controls in which the microelectrode did not contain cyclic AMP, but did contain the usual buffer and fluorescein, aggregation occurred but was unrelated to the electrode position. The results of all successful experiments were qualitatively similar. We therefore describe one experiment in detail here.

In this experiment we began pulsing

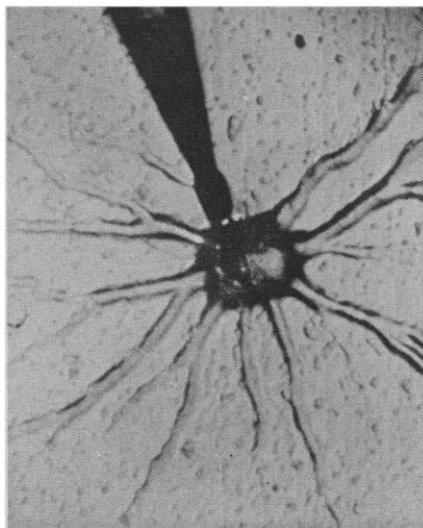


Fig. 1. Single frame from a film, showing aggregation of *D. discoideum* controlled by the microelectrode entering from top of picture. Note the well-formed streams. Frame width is 1 mm.

approximately $1\frac{1}{2}$ hours after centrifugation. The microelectrode tip accidentally came away from the surface aqueous film three times. Each time, any responses observed immediately ceased. At first no responses were observed, but the amoebas moved weakly, at random, in the fashion characteristic of early interphase (3, 18). Approximately 4 hours after centrifugation some responses were observed. These consisted of local movement toward the microelectrode tip, within a circle of radius approximately $100\ \mu\text{m}$, beginning within at most 30 seconds of a pulse. Signal propagation throughout the field was not observed at this juncture, and no streams formed. We infer that some amoebas were capable of responding to the signal by movement, but could not relay it. Therefore the cyclic AMP detection and movement response mechanisms are developed earlier in interphase than the signal-

relaying mechanism (12, 15, 19). When the electrode became dislodged all responses immediately ceased, and the amoebas remained stationary, as is characteristic of this part of interphase.

At approximately 6 hours after centrifugation, both movement responses and a propagated signal were observed, although there was little stream formation. The signal was propagated at $42\ \mu\text{m}/\text{sec}$, close to the propagation velocity measured in natural aggregation (12). About 2 hours later well-defined streams formed over the entire field, pointing directly toward the microelectrode (Fig. 1). We infer that the ability to form the intercellular contacts found in streams develops after the ability to propagate the cyclic AMP signal. This agrees with Gerisch's finding that such contacts could form only after 9 hours from the beginning of interphase (12). Aggregation toward the microelectrode tip continued. Each periodic aggregation movement followed the cyclic AMP pulse within at most 30 seconds and consisted of an outward-propagating wave of inward movement. Aggregation continued until the aggregate formed a tip which could be seen to be secreting the mucopolysaccharide slime first produced during late aggregation (2, 3). Presumably both this secretion and the partial blocking of the microelectrode by amoebas entering its tip served to insulate the aggregate from further external signals. In any case a slug formed and crawled away to make a normal fruiting body in the fashion characteristic of *D. discoideum* (2, 3).

In this report we have briefly described the artificial control of a developmental process in a multicellular organism. The process controlled, morphogenetic movement, is of general significance in development, and there is now considerable circumstantial evidence that transmitters such as 5-hydroxytryptamine and acetylcholine, together with their associated synthetic and degradative enzymes, are involved in the control of morphogenetic movements in particular embryos at particular times (20). It is well known that cyclic AMP plays a central role in the mediation of neural and humoral signals (21); the unusual feature in *D. discoideum* aggregation is that the cyclic AMP is extracellular. There has already been speculation that adult functional control systems may have evolved from developmental control systems (13, 19). We feel that these

Table 1. Sequence of development of components of aggregation system of *D. discoideum* during interphase.

Developmental event	Time after the onset of interphase		
	Present investigation	Earlier results	
1. Weak chemotactic response to cyclic AMP	~ 2 hours	A few hours	Bonner <i>et al.</i> (24)
2. Full chemotactic response to cyclic AMP	4 hours	~ 4 hours	Bonner <i>et al.</i> (24)
3. Relaying of signal	6 hours	Qualitative description	Shaffer (7, 14)
4. Appearance of autonomously signaling cells	6 to 8 hours	6 to 8 hours	(Many) (3, 12, 14)
5. Contact formation	8 hours	~ 9 hours	Gerisch (12)

experimental results illustrate the possibility of developmental control by systems using a transmitter and rectifying signal transmission, and we suggest that such control may be widespread. Electrophoretic investigation of other developing systems, and particularly those where other periodic phenomena are known, should be illuminating. These range from periodic movements in neurulation and gastrulation (15) to periodic cell division in blastulas and periodic nuclear division in *Physarum polycephalum* (22), and in some syncytial tumors (23).

It should be possible to control all these periodic cellular processes by using similar, but appropriately modified, external signals delivered from a source of cellular dimension such as a micro-electrode tip. Moreover, it should be possible to elucidate the several functional elements of a developmental control system and to establish the order in which functional competence emerges for each element during development. For example, our present experiments with *D. discoideum* show unequivocally that the several components of the aggregation control system develop sequentially during interphase. These results are summarized in Table 1 together with related results by earlier workers.

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Mammary Carcinoma: A Specific Biochemical Defect in Autonomous Tumors

Abstract. *Rat mammary carcinoma (R3230AC) which does not regress after ovariectomy has a markedly reduced amount of cytoplasmic estradiol binding protein. Cytoplasm from the tumor fails to interact with estradiol sufficiently to permit estradiol binding to tumor chromatin. This defect can be corrected in vitro by substituting cytoplasm, containing the binding protein, from rat uterus, thus permitting estradiol binding to tumor chromatin. The results indicate that the hormonal autonomy of this carcinoma is due to a lack of estradiol binding protein and not to the inability of estradiol to interact with the cell genome.*

We recently reported that dimethylbenzanthracene-induced mammary carcinomas which regress after ovariectomy contain a specific cytoplasmic 17 β -estradiol binding protein (EBP), whereas mammary carcinomas which continue to grow after ovariectomy lack this protein (1). In further consideration of this defect in autonomous mammary tumors we found a 90 percent reduction in cytoplasmic EBP in a transplantable rat mammary carcinoma (R3230AC) which does not regress after ovariectomy but has retained the capacity to respond partially to estrogen by stimulating certain enzyme activities (2). In classical estrogen target tissues or hormone-dependent mammary carcinoma, estrogen enters the cell, interacts with the specific EBP, migrates to the nucleus, and then binds to chromatin. Since the R3230AC tumor fails to accumulate intranuclear estradiol significantly, it became of interest to study the in vitro binding of estradiol to chromatin prepared from R3230AC tumor nuclei in the presence and absence of EBP. These experiments were undertaken to determine whether the estradiol autonomy of these cells is due to a lack of EBP or the inability of the genome to interact properly with the hormone-binding protein complex. We now report that, in the presence of R3230AC cytoplasm, estradiol fails to bind R3230AC chromatin. In the presence of uterine cytoplasm which contains abundant EBP,

estradiol binds appreciably to R3230AC chromatin.

Chromatin from R3230AC tumor nuclei was prepared by techniques described (3, 4). The ratio of histone protein to DNA and that of nonhistone protein to DNA were 0.90 to 1.12 and 1.11 to 1.42, respectively, as determined

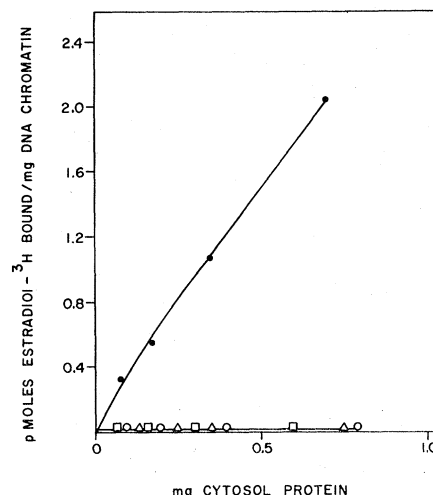


Fig. 1. Binding to chromatin of [^3H]estradiol in the presence of increasing concentrations of cytoplasm from rat uterus (solid circles), R3230AC tumor (open circles), rat brain (squares), and rat muscle (triangles). The incubation mixture included R3230AC chromatin (DNA content 44 μg), cytoplasm which had been previously incubated as described in the text. The concentration of chromatin in this experiment was too high to demonstrate saturation by uterine EBP.