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A Cellular Model for Studying Accommodation to Environmental Stressors: A Protective Response to Subtoxic Exposure to Cadmium

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A model is described for testing the effect of exposure to subtoxic challenge upon cellular integrity. The model incorporates *Physarum polycephalum* as a biological assay system, the ability of the cell to traverse the cell cycle as an indicator of cell integrity, and the use of repeated challenge by cadmium ion as a mechanism for amplifying the response to subthreshold exposure. A sensitivity profile of *Physarum*, developed by periodic exposure to 5×10^{-4} M Cd^{2+} for 30 min throughout the cell cycle, contains two peaks of sensitivity resulting in mitotic delay, one in early S and the other in late G_2. *Physarum* accommodates to a subtoxic challenge of Cd^{2+} by developing a protective response: Exposure to 10^{-4} M Cd^{2+} for 30 min in early G_2 (0.45 cycle), which does not delay mitosis, protects *Physarum* against a mitotic delay of 105 min resulting from exposure to 4×10^{-4} M Cd^{2+} for 30 min in late G_2 (0.75 cycle). Protection persists for at least two cell cycles.

INTRODUCTION

The cell, as the smallest integrating unit in biology, should be a favorable subject for studying (1) the impact of environmental stress upon a living system and (2) the capacity of that system for accommodation to stress. In addition to its relative simplicity, the cell offers promise of increased sensitivity and speed of analysis. These potential attributes suggest that studies at the cellular level may be suitable for clarifying issues which are difficult to resolve at a more complex level of organization: in tissue, organ, and whole body systems.

This project is directed to the study of such a recurring issue, the concept of a threshold (Dinman, 1972). Early studies on environmental health hazards have led to the hypothesis that the threshold in a dose–response relationship defines the point at which an organism can no longer cope with an agent without injury. This supposition implies that an organism can absorb a subthreshold challenge without consequent change in its structural or functional integrity. Although the threshold hypothesis is a time-honored and useful concept (Hatch, 1968; Stokinger, 1972), a revaluation has been prompted by the current concern raised by Dinman (1972) that subthreshold stressors do cause reactions which are not perceived and which may result over the long term in altered development or enhanced susceptibility to risk.

We have designed a simple model which uses the cell as a biological assay system. This model incorporates (1) the ability of the cell to traverse the mitotic cycle as an indicator of cellular integrity and (2) the use of multiple exposures as a mechanism for amplifying responses to subthreshold challenge.

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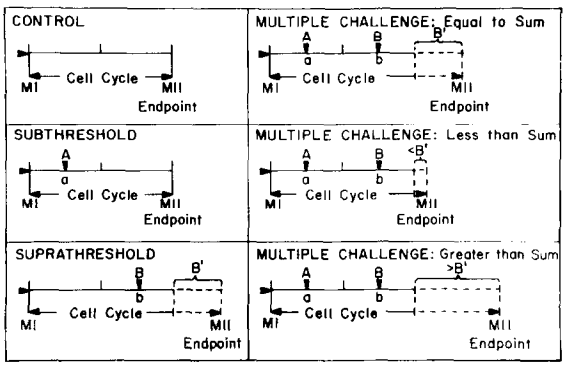

FIG. 1. A schematic representation of the model proposed for detecting cellular responses to subthreshold challenge by measuring the effect of single and multiple challenges on the duration of the cell cycle and the timing of mitosis.

Consider the exposure of a cell in a schematic representation of the model (Fig. 1) to a subthreshold challenge (A) at Time *a* in the cell cycle such that there is no effect on a given end point, the timing of mitosis following exposure, MII. Next, consider the exposure of a cell to a suprathreshold challenge (B) at Time *b*, later in the cell cycle than *a*, with an effect (B'), a delay in MII. Now, consider the effect of sequential exposure of a cell to the subthreshold challenge (A) at Time *a* and then to the suprathreshold challenge (B) at Time *b* on the experimental end point. The threshold concept predicts that the subthreshold challenge (A) should have no effect upon progress of the cell through the cell cycle, so that the result of additional exposure to the suprathreshold challenge (B) should be (B'), i.e., the delay in MII should be the same as in the absence of (A). In contrast, the cell may accommodate (Bernstein, 1971) to the subthreshold challenge so that multiple challenge may result in a greater or lesser delay than (B'). The delay could be greater if the subthreshold challenge were to inflict an injury which was not measurable by itself but which would be amplified by the suprathreshold challenge or if the cell were to become more sensitive and potentiate the response to supratheshold challenge. On the other hand, the reaction to multiple challenge could be less than (B') if exposure to the subthreshold challenge were to invoke a protective mechanism to diminish the effect of the suprathreshold dose.

The acellular slime mold, *Physarum polycephalum*, was selected as the biological system for testing this model. The organism was cultured in plasmodial form, which offered the advantage of a technique for the excision of identical standard-size explants (replicate disks) from a single multinucleate cell without loss of mitotic synchrony between explants (Chin *et al.*, 1972). The maintenance of synchrony between replicate disks from one plasmodium allowed us to carry and to compare nonexposed controls, subthreshold challenge controls, suprathreshold challenge controls with multiply challenged samples within one experiment.

Cadmium was selected as the stressor for this study because the effects of its widespread distribution in the environment at trace levels are not known. Although the occupational hazard of acute cadmium poisoning has been documented by Vigliani (1969), a concern for the long-term aspects of low-level exposure has

only started to surface. Cadmium has been assigned third order status in eight orders of priority in the United Nations Earthwatch Program (Jensen *et al.*, 1975). Underlying these concerns are the introduction of increasing amounts of cadmium into the biosphere as a by-product of technological progress (Page and Bingham, 1973), the low levels in tissues of infants, and the higher levels in livers and kidneys of adults, which suggest a correlation of cadmium accumulation with physical maturation (Schroeder and Balassa, 1965). The subject of cadmium toxicity has been recently reviewed by Vallee and Ulmer (1972) and by Flick *et al.* (1971); the cellular bases of cadmium toxicity are presently unknown (Kendrey and Roe, 1969).

MATERIALS AND METHODS

Cell culture and cycle. Physarum polycephalum, strain M_3CV, a generous gift of Dr. H. P. Rusch, University of Wisconsin, was cultured in an axenic medium (Chin and Bernstein, 1968) at 23°C. Synchronized plasmodia were formed by fusing a thick slurry of water-washed microplasmodia from submerged culture on dry filter paper for 4 hr. The first synchronous mitosis, MI, subsequent to fusion occurred 7.5 hr after addition of medium. Mitoses in plasmodia were naturally synchronized with a mitotic index in excess of 0.95. The timing of mitosis was determined by phase contrast microscopical examination of wet mounts prepared at regular intervals by mashing a small piece of plasmodium in a drop of salt solution (Daniel and Baldwin, 1964). Early prophase with a duration of 10 min served as the end point to identify M precisely: The marker for early prophase was the disintegrating nucleolus, which was crescent shaped and eccentric against the nuclear membrane and stood out in clear contrast to the nucleoplasm. The length of the cell cycle was determined by measuring the interval between two consecutive mitoses. The first complete cell cycle was terminated by MII 14 hr after MI.

The cell cycle in this organism consists of M, immediately followed by a 3-hr period for S and then G_2 and M. There is no G_1 (Nygaard *et al.*, 1960).

Preparation of replicate disks. Fifteen milliliters of medium was added to a petri dish containing the growing culture to float the plasmodium free of the filter paper support on which it had been synchronized and cultured. The growing edge of the plasmodium usually floated readily on the surface of the medium, while the center of the plasmodium tended to stick to the filter paper support. The plasmodium was gently freed by sliding a sterilized curved spatula between the organism and the filter paper. Care was taken not to tear or submerge any part of the plasmodium. Excess medium was removed to lower the free-floating plasmodium back onto the filter paper, and filter paper and plasmodium were transferred together to an empty petri dish. Replicate disks were cut from the growing edge of the plasmodium with a sterile 12-mm cork borer. Up to 16 disks of the same size could be excised from the growing edge of a 7-cm plasmodium.

Challenge with cadmium. The organism was challenged at specific points in the cell cycle following MI by floating replicate disks on 20 ml of medium containing the experimental concentration of $CdSO_4$. Exposure was terminated by simply lifting the disks from the exposure medium and washing them by refloating them twice successively on fresh medium. Incubation was continued on filter paper and fresh medium. Replicate disks could be challenged repetitively within one cycle.

Unexposed explants served as controls in single challenge experiments. Unexposed explants and explants exposed to each challenge singly served as controls in multiple challenge experiments. The response to Cd^{2+} was the difference in the length of the cell cycle (e.g., the timing of early prophase in MII) between replicate disks which had been exposed to cadmium and control discs which had not. At least two replicate disks were tested in each exposure, and duplicate disks usually reached MII within minutes of each other. A difference in excess of 20 min between average MII times for control and exposed disks was considered to be significant [Chin *et al.* (1972), e.g., for $n = 8$ replicate disks prepared 8 hr prior to MII, the timing of MII from the fastest time (Time 0) to the slowest time fell within a range of 14 min, with $\bar{x}_{MII} = 5.13 \pm 4.94$ min from and including Time 0; these calculations for replicate disks prepared from other plasmodia were on the same order of magnitude]. The suitability of this model for studying cellular responses to single and multiple stresses lies in the ability to mark precisely points of exposure in the cell cycle and to measure the effects of such exposure on progress through the cycle.

RESULTS

The response to single challenges was measured first to develop suitable parameters for multiple challenge experiments. A preliminary study showed that the mitotic delay resulting from a 30-min exposure to Cd^{2+} at 0.75 of the cell cycle increased linearly with increasing concentration from 2 to 10×10^{-4} M. A sensitivity profile (Fig. 2) was developed by measuring the change in response to a standard dose, 5×10^{-4} M for 30 min as a function of position in the cell cycle. The cycle begins with a period of high sensitivity which decreases sharply as the organism progresses through S to a period of low sensitivity in early G_2 (0.4 cycle). Sensitivity rises gradually through G_2 to a second maximum in late G_2 (0.8 cycle) and falls sharply again to a refractory period just prior to mitosis (0.95 cycle). The

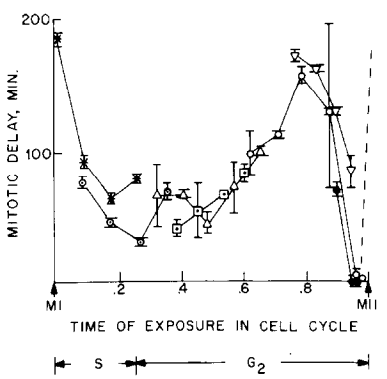

FIG. 2. A sensitivity profile: the delay in mitosis resulting from a 30-min exposure to 5×10^{-4} M cadmium ion at regular intervals throughout the cell cycle. The response was measured in the mitosis (MII) which terminated the cycle containing the challenge. Each point represents the average delay in mitosis resulting from exposure of duplicate disks. Each set of points represents a set of replicate disks excised from a common plasmodium.

profile for decreasing sensitivity to cadmium through S as well as the abrupt increase in response during transition from one cycle to the next is reproduced in the following cell cycle between MII and MIII.

Dose–response curves were obtained for early and late G_2 phases, corresponding to periods of low (0.45 cycle) and high (0.75 cycle) sensitivity. These curves, covering concentrations from 1 to 5 $\times$ 10^{-4} M, are presented in Fig. 3. The threshold concentration for Cd^{2+} at 0.45 cycle was 4 $\times$ 10^{-4} M.

From these dose–response curves, a subthreshold challenge (10^{-4} M Cd^{2+} for 30 min at 0.45 cycle) and a suprathreshold challenge (4 $\times$ 10^{-4} M Cd^{2+} for 30 min at 0.75 cycle) were selected to test the multiple challenge model (Fig. 1). Representative results of this test are presented in Table 1. The subtoxic dose alone did not impede progress through the cell cycle, while the toxic dose per se resulted in a delay of 105 min. No delay was observed when both doses were applied at their respective positions within the cell cycle; mitosis occurred concurrently with that of unexposed controls. The subthreshold dose protected the cell from the delay which resulted from the suprathreshold dose alone.

The persistence of the protective response was explored by interposing two cell cycles between sub- and suprathreshold challenges. A plasmodium was cut in half at MI + 0.45 cycle. One half was exposed to a subtoxic dose of 10^{-4} M Cd^{2+} for 30 min. After washing, both halves were transferred to fresh medium and incubated to MIII. The half which had been exposed to the subtoxic challenge initiated MIII 11 min after the control half; this delay was not considered to be significant. At MIII + 0.72 cycle, four replicate disks were cut from each half. Two replicate disks from each half were exposed to 5 $\times$ 10^{-4} M Cd^{2+} for 30 min. The remaining disks were exposed to medium without cadmium. After two washes, all replicate disks were transferred to filter paper and fresh medium and incubated. The timing of MIV was determined for each disk (Table 2). Replicate disks which had been exposed to 5 $\times$ 10^{-4} M Cd^{2+} at MIII + 0.72 cycle entered MIV 2 hr after unexposed controls. Replicate disks which had been exposed to a subtoxic challenge of 10^{-4}

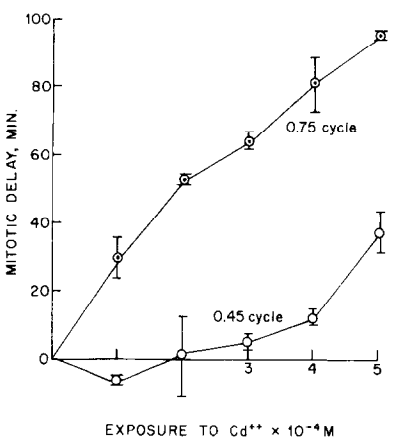

FIG. 3. Dose–response relationships for 30-min exposures to Cd^{2+} at 0.45 and 0.75 cell cycle. Each point represents the average mitotic delay in duplicate replicate disks.

TABLE 1
A TEST OF THE MODEL: CELLULAR RESPONSE TO SUBTHRESHOLD, SUPRATHRESHOLD, AND MULTIPLE CHALLENGE OF CADMIUM ION[a]

Exposure to Cd^{2+} for 30 min		Delay in MII	Average delay in MII
At 0.45 cycle	At 0.75 cycle	(min)	(min)
Control			
—	—	—	—
—	—	—	—
Subthreshold			
1×10^{-4} M	—	10	
1×10^{-4} M	—	15	13
Suprathreshold			
—	4×10^{-4} M	103	
—	4×10^{-4} M	107	105
Multiple challenge			
1×10^{-4} M	4×10^{-4} M	−11	
1×10^{-4} M	4×10^{-4} M	7	
1×10^{-4} M	4×10^{-4} M	11	
1×10^{-4} M	4×10^{-4} M	10	4

[a] Replicate disks from one plasmodium were exposed at 0.45 and 0.75 cycle to selected concentrations of cadmium for 30 min. Unexposed disks and disks exposed to only one of the two challenges were maintained as controls. The experiment was scored by measuring the effect of single and multiple exposures upon the timing of the following mitosis.

M Cd^{2+} at MI + 0.45 cycle and then to a toxic challenge of 5×10^{-4} M Cd^{2+} at MIII + 0.72 cycle entered MIV at the same time as unexposed controls. These data indicate that the protective response extended through at least two cell cycles.

TABLE 2
PROTECTION OVER TWO CELL CYCLES[a]

Exposure to Cd^{2+} for 30 min		Delay in MIV	Average delay in MIV
At MI + 0.45 cycle	At MIII + 0.72 cycle	(min)	(min)
Control			
—	—	—	—
—	—	—	—
Subthreshold			
1×10^{-4} M	—	−18	
1×10^{-4} M	—	−12	−15
Suprathreshold			
—	5×10^{-4} M	110	
—	5×10^{-4} M	120	115
Multiple challenge			
1×10^{-4} M	5×10^{-4} M	−5	
1×10^{-4} M	5×10^{-4} M	4	−1

[a] The experimental protocol was similar to that described in Table 1, except that the timing of the exposures was altered.

DISCUSSION

These experiments demonstrate that the model proposed in this paper is conceptually appropriate and technically useful for measuring the ability of a cell to respond to what would have ordinarily been considered a subthreshold exposure. Whether these results, developed in a eukaryotic syncitium, can be reproduced in a more advanced eukaryotic (i.e., mammalian) cell is of immediate interest to us and remains to be demonstrated. The mode (Fig. 1) provides that either the enhancement of a toxic dose or protection against it by prior administration of the subthreshold dose would indicate the ability of the cell to accommodate to low level environmental stress. In these experiments, protection was observed: Prior exposure to 10^{-4} M cadmium ion for 30 min at 0.45 cell cycle, which itself does not delay mitosis, elicits a protective response against a mitotic delay of 105 min resulting from exposure to 4×10^{-4} M cadmium for 30 min at 0.75 cycle (Table 1). The importance of the protective response to the cell is implied by its duration through at least two more cell cycles (Table 2).

These results substantiate our concern for identifying the essential qualities of the threshold concept (Dinman, 1972). The protective response shows that the threshold in a traditional dose–response relationship (as measured in Fig. 2) may fail to define the limit at which an organism is most sensitive to an agent. The threshold may reflect the lowest technical sensitivity for measuring a response or the failure, as in this case, to examine a more appropriate response with greater sensitivity. These considerations suggest that reactions to subthreshold challenges, which may be initially overlooked, may ultimately be expressed over the long term in modified responses to subsequent challenges or in altered development of the organism. An organism may be more responsive to its environment than we have previously conceived. Of particular interest to the long-term aspects of cadmium toxicity are reports that this metal may be involved in teratogenesis (Ferm, 1971) and carcinogenesis (Lucis *et al.*, 1972). The responses provided by this model (protection in this paper and potentiation in the following paper), if they are found in higher mammalian (human) cells, will suggest that greater awareness and consideration be taken in setting permissible limits for exposure to environmental agents.

The protective response observed above is reminiscent of protection against cadmium toxocity in rats, as reported by Gunn *et al.* (1966): A single parental injection of cadmium chloride, 0.03 to 0.04 mmole/kg, selectively damages the testis, causing massive necrosis and vascular occlusion. Repair begins a few weeks following cadmium administration. A second injection of cadmium 8 months later does not produce any vascular injury or damage to interstitial tissue. If a similar protective mechanism is involved in *Physarum* and the rat, the study of *Physarum* at the cellular level offers the advantages of greater sensitivity, simplicity, and speed.

The model presented here capitalizes on transit through the cell cycle as a sensitive parameter of cellular integrity. The sensitivity profile (Fig. 1) reflects the changing patterns of metabolic events through the cell cycle (Mitchison, 1972). The peak of sensitivity in S may reflect interference with DNA replication (Sachsenmaier and Rusch, 1964) and, more specifically, may reflect cadmium competi-

tion for a zinc ion requirement in DNA polymerase (Springgate *et al.*, 1973). At present, we have no indication of the cellular target(s) which is sensitive to cadmium in late G_2, responsible for mitotic delay, and spared by the protective response. The refractoriness of the cell cycle to Cd^{2+} at 0.95 cycle (45 min before M) (Fig. 2) suggests that all cadmium-sensitive processes for mitosis are no longer liable to cadmium exposure at this time.

The identity of cellular targets for cadmium will be resolved as a study of the nature of the protective response develops. We are interested in determining the lower limit of subthreshold exposure for inducing protection, the upper limit of toxic exposure for which protection is complete, the timeliness of both subthreshold and toxic challenges, the specificity for cadmium ion, the availability of the protective response in other biological systems, and its exploitability.

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REFERENCES

Bernstein, I. A. (1971). "Biochemical Responses to Environmental Stress." Plenum, New York.

Chin, B., and Bernstein, I. A. (1968). Adenosine triphosphate and synchronous mitosis in *Physarum polycephalum*. *J. Bacteriol.* **96**, 330–337.

Chin, B., Friedrich, P. D., and Bernstein, I. A. (1972). Stimulation of mitosis following fusion of plasmodia in the myoxmycete *Physarum polycephalum*. *J. Gen. Microbiol.* **71**, 93–101.

Daniel, J. W., and Baldwin, H. H. (1964). methods of culture for plasmodial myxomycetes. *In* "Methods of Cell Physiology" (D. M. Prescott, Ed.), Vol. 1, pp. 9–41. Academic Press, New York.

Dinman, B. D. (1972). "Non-concept" of "no threshold" chemicals in the environment. *Science* **175**, 495–497.

Ferm, V. H. (1971). Developmental malformations induced by cadmium. A study of timed injections during embryogenesis. *Biol. Neonate* **19**, 101–107.

Flick, D. F., Kraybill, H. F., and Dimitroff, J. M. (1971). Toxic effects of cadmium: A review. *Environ. Res.* **4**, 71–85.

Gunn, S. A., Gould, T. C., and Anderson, W. A. D. (1966). Loss of selective injurious vascular response to cadmium in regenerated blood vessels of testis. *Amer. J. Pathol.* **48**, 959–969.

Hatch, T. F. (1968). Significant dimensions of the dose–response relationship. *Arch. Environ. Health* **16**, 571–578.

Jensen, C. E., Brown, D. W., and Mirabito, J. A. (1975). Earthwatch. Guidelines for implementing this global environmental assessment program are presented. *Science* **190**, 432–438.

Kendrey, G., and Roe, F. J. C. (1969). Cadmium toxicology. *Lancet* **1**, 1206–1207.

Lucis, O. J., Lucis, R., and Aterman, K. (1972). Tumorigenesis by cadmium. *Oncology* **26**, 53–67.

Mitchison, J. M. (1972). "The Biology of the Cell Cycle." Cambridge Univ. Press, London.

Nygaard, O. F., Guttes, S., and Rusch, H. P. (1960). Nucleic acid metabolism in a slime mold with synchronous mitosis. *Biochim. Biophys. Acta* **38**, 298–306.

Page, A. L., and Bingham, F. T. (1973). Cadmium residues in the environment. *In* "Residue Reviews" (F. A. Gunther, Ed.), Vol. 48, pp. 1–44. Springer–Verlag, New York.

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

Schroeder, H. A., and Balassa, J. J. (1961). Abnormal trace metals in man: Cadmium. *J. Chron. Dis.* **14**, 236–258.

Springgate, C. F., Mildvan, A. S., Abramson, R., Engle, J. L., and Loeb, L. A. (1973). *Escherichia coli* deoxyribonucleic acid polymerase I, a zinc metalloenzyme. *J. Biol. Chem.* **248**, 5987–5993.

Stokinger, H. E. (1972). Concepts of thresholds in standards setting. *Arch. Environ. Health* **25**, 153–157.

Vallee, B. L., and Ulmer, D. D. (1972). Biochemical effects of mercury, cadmium and lead. *Annu. Rev. Biochem.* **41**, 91–127.

Vigliani, E. C. (1969). The biopathology of cadmium. *Amer. Indust. Hyg. Assoc. J.* **30**, 329–340.