stance in the shrimp extract, we followed the basic procedures used by Loomis (*1*) and Fulton (*7*) when they determined the compounds that induce feeding in *Hydra littoralis* (*1*) and *Cordylophora lacustris* (*7*). We found that the substance is stable to boiling, is dialyzable, and, when chromatographed on Whatman No. 1 paper in a single dimension in a mixture of butanol, acetic acid, and water (120 : 40 : 50 by volume), has an R_F of about 0.56. Testing for amino acids that might have similar R_F's, we found that neither histidine nor valine were active, nor was GSH ($10^{-4}M$) placed within the gut. The only compound present in shrimp extract that had an R_F near 0.56 and also promoted neck constrictions was the aromatic amino acid tyrosine (*p*-hydroxyphenylalanine), which was active at *p*H 6.4, 7.8, and 10.4.

To determine the degree of specificity of the endodermal receptor for tyrosine, we tested solutions ($10^{-4}M$) of various tyrosine analogs. Good neck formation was elicited by *o*-tyrosine. A solution of *m*-tyrosine, however, seemed to give the most pronounced neck constriction. Also active were dinitrotyrosine, diiodotyrosine, and 3-aminotyrosine. Weak and transient activity was elicited by monoiodotyrosine. These experiments indicate that the electrophilic group on the aromatic ring can be somewhat diverse and randomly distributed. No constriction was induced by phenylalanine, leucyl tyrosine, or tyramine. Tyrosine hydroxamide induced an occasional transient response in a few animals. Epinephrin also produced weak and transient responses.

We conclude that along the hydra's gut there are enteroreceptors specific for tyrosine. The hydroxyl, the α-amino, and the α-carboxyl groups are all necessary for the amino acid to be active. Hence, since at least three groups are required for activity, the tyrosine molecule has the three points for attachment, often associated with a specific binding of a small molecule to an active site.

The adaptive value of neck formation to hydra seems obvious. Without the neck constriction, were the distended hydra to open its mouth to receive new prey, it would lose some of the food particles being circulated in the gut. On the other hand, if more prey were captured and not swallowed, there would be an inefficient expenditure of vital nematocysts, as well as a loss of more food. By forming the neck constriction, the hydra forfeits neither part of its previous meal nor nematocysts, but is able to swallow new food as it is captured.

The initiation of neck constriction by reduced glutathione is but another of the growing list of responses in hydra controlled in part by this tripeptide. It is the specific activator of the sequence of tentacle-bending and mouth-opening in hydra's feeding behavior (*1*). This tripeptide also inhibits the animal's contraction in response to light and mechanical agitation (*8*). The so-called sweeping movements (*3*) of the "tentacle concerts" are initiated by extremely low concentrations ($10^{-9}M$) of glutathione (*9*). Electrophysiological correlates to the additions of glutathione to hydra have been shown (*10*); and now, we show glutathione has an essential function in neck formation.

The occurrence in hydra of two chemoreceptor systems that must act in concert represents, to our knowledge, the first such instance reported for the lower invertebrates. The existence of this system gives insight into how a complex chemical coordinating system might have developed, that is, by evolving receptor sites to ubiquitous small molecules emitted from the capture prey. Whether or not the glutathione-tyrosine system represents an early prototype of the more complex interacting hormonal systems of the higher invertebrates remains a question.

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References and Notes

1. W. F. Loomis, *Ann. N.Y. Acad. Sci.* **62**, 209 (1955).
2. H. M. Lenhoff, in *Chemical Zoology*, B. Scheer and M. Florkin, Eds. (Academic Press, New York, in press), vol. 2.
3. ———, *J. Gen. Physiol.* **45**, 331 (1961).
4. L. Muscatine and H. M. Lenhoff, *Biol. Bull.* **128**, 415 (1965).
5. W. F. Loomis and H. M. Lenhoff, *J. Exp. Zool.* **132**, 555 (1965).
6. This inflation of the gut with fluids seems to provide hydra a means for circulating food particles in the gut, and for regurgitating of indigestible matter, such as nauplii exoskeletons. Within 5 to 6 hours after ingestion, hydra are fully distended and take on a bloated appearance. It is at about this time that regurgitation occurs.
7. C. F. Fulton. *J. Gen. Physiol.* **46**, 823 (1963).
8. R. Roberts *et al.*, *Carnegie Inst. Wash. Publ. No. 607* (1957).
9. N. Rushforth, *Amer. Zool.* **5**, 505 (1965).
10. ——— and F. Hofman, *Biol. Bull.* **131**, 403 (1966).
11. N. Rushforth, personal communication.
12. Supported by NSF grant GB5491, NIH career development award GM5011, and a grant from the Nutrition Foundation.
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Cyclic Changes in Enzyme Activity in Synchronized Mammalian Cell Cultures

Abstract. *Activity of the enzymes glucose-6-phosphate dehydrogenase and lactate dehydrogenase increases intermittently in synchronized cultures of Chinese hamster cells. During G_1 phase (3 hours after mitosis), midway through S phase (7 hours after mitosis) and again in late S phase (10 hours after mitosis), rapid increases in activity were observed and correlated with a net increase in cell size and total protein. The evidence suggests that this is a general phenomenon which affects a whole population of proteins and may be the expression of an endogenous cellular rhythm.*

Using synchronized mammalian cells, we previously studied the relationship between DNA replication and the capacity of the cell to synthesize RNA (*1*). These studies led us to question whether enzyme levels also reflect the increased gene dosage resulting from replication of DNA and, in general, how enzyme activity changes as a function of the cell cycle. The enzymes lactate dehydrogenase (LDH) (EC 1.1.1.27) and glucose-6-phosphate dehydrogenase (G6PD) (EC 1.1.1.49) were measured during one cycle of synchronous growth and division, and the changes in activity were compared with several other parameters of cell growth, such as mean cell volume and total protein.

Synchronized cultures were obtained by treatment of an exponential population of Don C Chinese hamster cells with Colcemid for 2½ hours. Cells blocked in mitosis were selectively removed by a brief trypsinization. The collected metaphase cells were then suspended in medium at 37°C at a concentration of 1.25×10^5 cell/ml and inoculated into culture vessels at 5×10^4 cells per square centimeter of culture surface. Division of the attached cells resulted in a surface density of 1×10^5 cell/cm^2 for most of the experiment. Evidence indicates that the cells suffer no lasting effects from brief arrest with Colcemid (*2*).

Enzyme activity (Fig. 1) does not increase in a simple and regular fashion through the cycle but rather undergoes sharp periodic oscillations. Peaks occur repeatably at the end of G_1 (3 to 4 hours after mitosis), midway through S (7 hours after mitosis), and again in late S phase (10 hours after mitosis).

The periods of lowest enzyme activity occur at 4-hour intervals. In answer to our question about gene function in the cell cycle, we note that the maximum activity in an S-phase cell is twice that of a G_1 cell.

The general pattern of enzyme activity was not altered by the mode of extraction and preparation of the samples. Sonication (or freeze-thaw cycles) and extraction in the presence or absence of appropriate cofactors did not change the results. We observed, however, that the apparent amplitude of the oscillations was affected by the extent of synchrony. In experiments where the initial synchrony (percentage of mitotic cells) was 80 to 85 percent, the maximum and minimum enzyme activities were less extreme than those in Fig. 1 where initial synchrony was 96 percent. Starch-gel electrophoresis indicated that the predominant isozyme expressed by this cell line was LDH-5, although there must be some LDH-1 present because minute quantities of LDH-4 could be detected. No qualitative changes in the pattern of LDH isozymes were detected during the growth cycle.

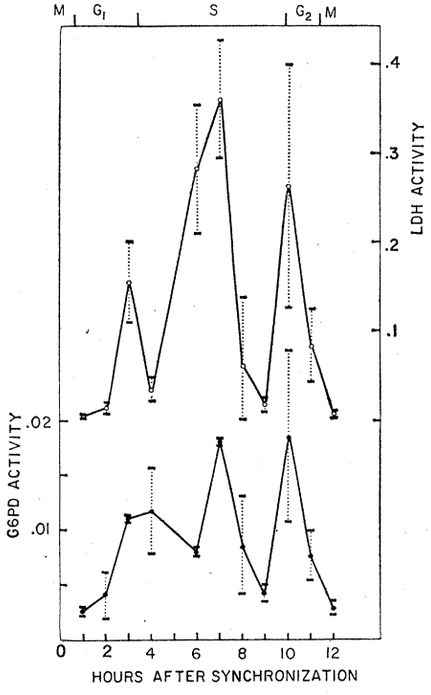

Fig. 1. Changes in enzyme activity during the cell cycle. Replicate cultures were extracted in 0.5 ml of distilled water by three cycles of freezing and thawing. The extract was then centrifuged at 27,000*g* for 1 hour, and the supernatant was immediately assayed. The LDH and G6PD activities were measured by the change in absorbance accompanying the oxidation of reduced nicotinamide-adenine dinucleotide or reduction of nicotinamide-adenine dinucleotide phosphate (NADP), respectively. The LDH reaction mixture contained $3.3 \times 10^{-4}M$ sodium pyruvate, and $0.002M$ reduced nicotinamide-adenine dinucleotide in $0.03M$ phosphate buffer *p*H 7.0. The G6PD reaction mixture contained $4 \times 10^{-3}M$ glucose-6-phosphate, $3.3 \times 10^{-2}M$ $MgCl_2$, and $1.5 \times 10^{-3}M$ NADP in $0.08M$ glycylglycine buffer *p*H 7.6. All reactions were performed at 37°C in a Gilford 2000 multiple-sample recording spectrophotometer. Results are expressed as change in absorbance per minute per 10^6 cells.

To establish that the changes in enzyme activity were a function of the cell cycle and not a result of subculturing or Colcemid treatment alone, we treated control cultures of randomly growing cells briefly (10 minutes) with Colcemid and subcultured the entire population. We did not detect the cyclic changes observed in the synchronized cells. Because this cell line is subcultured every 24 hours and is therefore constantly in the exponential phase of growth, little or no detectable lag occurs after subculture. Enzyme activity increases at the same rate as the cell number, and the slope in both cases is equivalent to a generation time of 11½ hours. When stationary phase cells are subcultured, there is often an immediate and dramatic loss in the activity of many enzymes (*3*).

To test the generality of the result obtained for enzyme activity in synchronized cells, we undertook to measure the changes in the cell volume and total cell protein (Fig. 2). The cell volume showed a rapid increase during G_1 phase; this increase began immediately after mitosis. A plateau was reached at a time corresponding to the beginning of S phase, and there was no further increase in size until mid-S phase (about 7 hours after mitosis). We observed a similar pattern for total protein, although the increase during G_1 was not as abrupt as the change in cell volume. In both instances, a pronounced increase occurred in mid-S phase. At 11 hours, we could detect the decrease in mean cell volume which accompanies the second round of division. The results were remarkably consistent from experiment to experiment (Fig. 2).

The data for mean cell volume and total protein parallel closely the changes in enzyme activity and probably reflect a rather general phenomenon affecting a whole population of proteins. One explanation for the periodic changes in activity is that enzyme synthesis is not constant through the cell cycle but occurs instead at discrete intervals. Whether the decreases in amounts of enzyme are due to a constant rate of degradation imposed on the intermittent synthesis or to some more complex system is problematical. Enzyme activity is not necessarily equivalent to enzyme synthesis, although in a rapidly dividing cell line the argument for activation of preexisting enzyme seems somewhat contrived. Certainly, the cell must double its complement of enzyme in the course of one cycle if it is to maintain balanced growth.

As yet, there is little information concerning enzyme activity and the kinetics of enzyme synthesis through the mammalian cell cycle. In synchronous bacterial cultures, the potential for induced enzyme synthesis doubles once in each cell generation, and the doubling

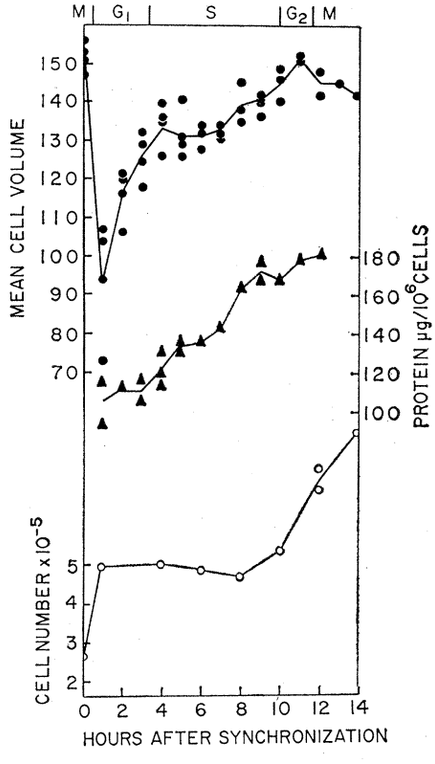

Fig. 2. Cell volume and protein measurements. Cells were removed from the culture vessel in a small amount of 0.1-percent trypsin and then suspended in 0.9-percent NaCl counting fluid. Mean cell volume was determined in the Coulter counter with a volume computer. Values plotted were arbitrary machine units. Protein measurements were made by washing the samples in Earle's balanced salt solution, extracting the cells twice in cold 10-percent trichloroacetic acid, and rinsing the cell sheet in 95-percent ethanol. Dried cell sheets were dissolved in the alkaline solution of the Lowry procedure, and the usual protein analysis was completed. Determination of the duration of G_1, S, and G_2 has been described (*1*).

times for several enzymes have been correlated with the time of replication of the corresponding gene locus (*4*). However, while the uninduced rate of enzyme synthesis shows cyclic changes, the change does not necessarily occur at the time of replication. Several mathematical models which suggest that the synthesis of enzymes is entrained to DNA replication by concomitant RNA synthesis and which contend that the quantities of RNA and enzyme oscillate under the control of a repressor have been proposed. These periodic events are considered to be part of the timekeeping mechanism of the bacterial cell (*5*). While no such precise system of control for mammalian cells has yet been demonstrated, we have observed that the quantities of several enzymes change periodically through the cell cycle and that these changes are paralleled by net increases in mean cell volume and total protein. Bursts of ribosomal RNA synthesis also occur in G_1 and mid-S phases. Possibly, the bursts in enzyme activity are causally related to the synthesis of ribosomal RNA.

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References and Notes

1. R. R. Klevecz and E. Stubblefield, *J. Exp. Zool.* **165**, 259 (1967).
2. R. R. Klevecz, *J. Cell Biol.* **26**, 53A (1965); E. Stubblefield and R. R. Klevecz, *Exp. Cell Res.* **40**, 660 (1965); E. Stubblefield, R. R. Klevecz, L. Deaven, *J. Cell. Physiol.* **69**, 345 (1967).
3. R. DeMars, *Metabolic Control Mechanisms in Animal Cells* (National Cancer Institute, Washington, D.C., 1964), pp. 181–196; C. DeLuca, *Exp. Cell Res.* **43**, 39 (1966); F. H. Ruddle and J. Rapola, *J. Cell Biol.* **31**, 97A (1966).
4. M. Masters and A. B. Pardee, *Proc. Nat. Acad. Sci. U.S.* **54**, 64 (1965); P. Kuempel, M. Masters, A. B. Pardee, *Biochem. Biophys. Res. Commun.* **18**, 858 (1965).
5. B. C. Goodwin, *Nature* **209**, 479 (1966).
6. Supported by research grant GM 9966 and training grant F2-CA-33,311 from NIH.
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Composite Membranes: The Permeation of Gases through Deposited Monolayers

Abstract. *We have measured permeabilities of deposited monolayers of stearic acid varying in thickness from 8 to 48 deposited layers. These results are compared with permeabilities of polymeric films and insoluble monolayers. The technique may have applicability in the fabrication of synthetic membranes.*

We have measured the rate of permeation of various gases through built-up monolayers of stearic acid. These multilayers were deposited onto permeable support films by using the conventional Langmuir-Blodgett method (*1*). By means of tracer techniques we were able to observe both the regularity and the concentration of the deposited films. In all, permeabilities have been measured for CO_2, N_2, and He diffusing through multilayers varying in thickness from 8 to 48 deposited layers, a range of 200 to 1200 Å. Our results indicate that this technique may be useful in fabricating composite films for a variety of applications, including model systems for the study of natural membranes (*2*, *3*).

The support film used in these tests was an experimental silicone polymer (XD-1) (*4*). Properties which the support film must have are: it must be mechanically manipulatable; it must have suitable deposition characteristics for the monolayer which is to be deposited on its surface; and it must have a high permeability. Of the materials tested XD-1 provided the best combination of these properties (*5*).

The film was prepared immediately before use by casting a 0.02-cm-thick layer of XD-1 (10 percent by weight) in chloroform onto a clean glass surface. After allowing for solvent evaporation the film was removed from the glass and stretched over a solid support consisting of a disc of porous stainless steel (4.4 cm outside diameter) mounted in a stainless steel ring. Two layers of film were necessary to guard against possible pinholes in the film. The properties of the silicone polymer are such that it readily adheres to itself or a clean metal surface; simply stretching the film over the edge of the support gave a uniform, tightly held surface. The thickness of the resulting double layer of film varied from 0.8 to 2×10^{-3} cm. Stearic acid-1-C^{14} (9.19 mc/mmole) (*6*) was used for the depositions. All data reported here are for stearic acid deposited at a constant surface pressure of 29 dyne/cm with oleic acid used as a piston oil (*7*). The substrate solution used for the deposition was $10^{-4}M$ $BaCl_2$, $2 \times 10^{-4}M$ $KHCO_3$, and $10^{-7}M$ $CuCl_2$ with a *p*H of 7.5 and temperature less than 22°C. Under these conditions the deposited monolayer is not simply stearic acid but a mixture of stearic acid and stearate soaps formed with the various substrate cations (*8*).

For the depositions two supported films were mounted back to back in a dipping device which enabled us to lower and raise the films through the water surface at a uniform and reproducible rate. The change in concentration of the monolayer during the deposition was measured by comparing the corrected count rate of the stearic acid on the support film to that above the monolayer on the substrate solution. For this comparison the necessary background, dead time, and backscatter corrections were made (*5*). The ratio of the corrected count above the film to that over the water surface is defined as the deposition ratio. Allowing for geometric and statistical errors in the counting, the precision of the deposition ratio is approximately ±5 percent. In addition, the uniformity of the deposited layers was checked by autoradiography.

The mass transfer equipment consisted of two identical mass transfer cells. The film was placed in one cell, an impermeable plate was placed in the second cell. The film divided the mass transfer cell into two parts. To initiate an experiment a piston device was used to increase stepwise the pressure in both cells. The resulting gas pressure difference between the high pressure side of the two constant-volume cells was measured with a sensitive, variable-reluctance pressure transducer (*9*). From the transient pressure response the mass transfer coefficient, or a related quantity, the permeability, could be calculated. For simplicity, the mass transfer coefficient has been defined as $k_p = N/\Delta p$, where N is the mass flux through the film in milliliters (STP) per square centimeter per second under Δp, the pressure difference of the diffusing gas in centimeters of mercury. Consistent with this definition, k_p here has the units of: ml(STP)/cm^2-sec-cm-Hg.

To determine the mass transfer characteristics of the deposited multilayers it was necessary to perform two experiments, one with the silicone support film alone, and a second with the composite film. From these results, the resistance of the multilayer could be calculated. The resistance to transfer, that is, the reciprocal of the mass trans-