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Stimulation by Insulin of Cell Elongation and Microtubule Assembly in Embryonic Chick-Lens Epithelia

(cell culture/colchicine/serum/crystallin synthesis/lens fiber)

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ABSTRACT **Both fetal-calf serum and insulin cause cell elongation in explanted chick-lens epithelia from 6-day-old embryos. We show that 1 μg/ml of insulin, like serum, stimulates a doubling of cell length and an assembly of longitudinally oriented microtubules; colchicine treatment inhibits this cell elongation. In contrast to serum, insulin neither promotes further lens-cell elongation nor appreciably stimulates the synthesis of bulk proteins or of delta crystallin under the present conditions. These data indicate that the early morphological events of lens fiber differentiation can be initiated by insulin in a chemically defined, serum-free medium without significant affects upon protein synthesis.**

The differentiation of fibers in vertebrate lens is characterized by a marked elongation of epithelial cells (1), the appearance of longitudinally oriented microtubules (2–6), and intensive synthesis of lens-specific proteins termed crystallins (7, 8). The early stages of lens-fiber differentiation can be studied in tissue culture. Cells of the explanted lens epithelium of a 6-day-old chick embryo elongate if serum is added to the culture medium (6, 9). During elongation of the cultured lens epithelial cells, microtubules assemble and align in the long axis of the cells, and the proportion of newly synthesized delta crystallin increases appreciably (6, 10). Delta crystallin is the predominant protein accumulated in maturing lens fibers of embryonic chicks (8, 10–12).

Recently, we reported that insulin promotes cell elongation in embryonic chick-lens epithelia cultured in a chemically defined medium lacking serum (13). In the present study, we have compared the effects of insulin with those of fetal-calf serum on cell elongation, microtubule assembly, and protein synthesis in explanted chick-lens epithelia of 6-day-old embryos. The results show that insulin—like serum—initiates a rapid doubling of cell length, accompanied by the assembly of longitudinally oriented microtubules, but—unlike serum—neither supports continued lens-cell elongation nor appreciably stimulates protein synthesis. A preliminary report of these findings has appeared (14).

MATERIALS AND METHODS

Culture of Lens Epithelia. Fertile eggs (Truslow Farms, Inc. Chestertown, Md.) of white Leghorn chickens were maintained for 6 days in a humidified, forced-draft incubator kept at 37°. The lenses were removed from the eyes and placed in plastic culture dishes (Falcon Plastics, 60 mm × 15 mm deep) containing 5 ml of sterile Ham's F-10 medium (15), either alone, supplemented with 15% fetal-calf serum (Baltimore Biological Laboratories), or supplemented with 1 μg/ml of crystalline porcine insulin (Eli Lilly and Co., potency 21.7 units/mg, glucagon content < 0.005%, zinc content 0.49%). A concentration of 1 μg/ml of insulin was chosen because it caused maximum cell elongation in the cultured epithelium (13). Fresh insulin was added daily in tests lasting longer than 24 hr. The lens epithelia were cultured as described (6). In brief, each fiber mass was separated surgically from the adhering epithelium through a tear in the posterior capsule, and a square with an average area of 0.8 mm^2 was cut with a scalpel from the center of the epithelium. This piece of epithelium remained attached to the dish, with its capsular surface toward the plastic, since the edges were pushed into the bottom of the dish. Epithelia were cultured at 37° in a water-saturated environment containing about 5% CO_2 in air. Each dish had 1 or 2 epithelia, no antibiotics were used, and the few contaminated cultures were discarded.

Histology and Measurement of Cell Length. Epithelia were fixed in acetic acid–100% ethanol 1:3 and embedded in Paraplast (16). Serial sections of 5 μm were cut normal to the surface of the epithelium and stained with Ehrlich's hematoxylin and eosin Y. Cell lengths were measured to the nearest 2.5 μm with a calibrated ocular micrometer; the mean cell length in each epithelium was determined by averaging the cell lengths in 5 sections, spaced 6–8 sections apart, in the central third of the explant.

Electron Microscopy. Epithelia were fixed in glutaraldehyde, post-fixed in osmium tetroxide, and embedded in Epon (6). Thin sections were stained with uranyl acetate and lead citrate (17) and examined in a Siemens 1A electron microscope at 75 kV.

Analysis of Proteins. After 24 hr of cultivation in Ham's F-10 medium alone or Ham's F-10 medium supplemented with fetal-calf serum or insulin, groups of 10 lens epithelia were washed with unsupplemented Ham's F-10 medium, lacking valine, and transferred with a medicine dropper to sterile polystyrene test tubes (12 mm × 75 mm). The epithelia were labeled with 100 μCi/ml of L-[2,3-^{3}H]valine (Schwarz BioResearch Corp., 17.3 Ci/mmol) at 37° in the tissue culture incubator, in a total volume of 0.25 ml of Ham's F-10 medium lacking valine. After 2 or 4 hr of incubation 0.20 ml of the medium was removed and the labeled epithelial cell proteins were separated by electrophoresis in sodium dodecyl sulfate–polyacrylamide gels (18). Epithelia were dissolved in 0.1–0.2 ml of 1.7% dodecyl sulfate–1.7% 2-mercaptoethanol–0.01 M sodium phosphate (pH 7.1), heated to 100° for 1.5 min, and electrophoresed at 200 mA for 3 hr at 20° on composite 4%

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polyacrylamide–0.5% agarose gels in a vertical slab apparatus (E-C Apparatus Corp.); the gels were stained overnight with 0.25% Coomassie Blue (Mann Research Laboratories) (6). After they were destained electrophoretically, the gels were scanned at 570 nm with a Gilford spectrophotometer equipped with a linear gel-transport attachment. The relative areas under the scans were quantitated by weighing. For determination of radioactivity, the gels were cut into 1-mm slices; each slice was dissolved in 30% hydrogen peroxide and assayed in a Beckman LS 233 liquid scintillation spectrometer using a toluene scintillation fluid containing 3.3% Liquifluor (New England Nuclear Corp.) and 8.5% Biosolv (BBS-3 from Beckman Instruments). ^3H was counted with an efficiency of about 32%. The total amount of radioactivity incorporated into the cellular proteins was determined by summing the counts in each fraction of the gel; the amount of radioactivity incorporated into delta crystallin was determined by summing the counts in fractions associated with the principal band of protein, whose migration corresponded to a molecular weight of about 45,000. This band of protein is predominantly delta crystallin, as shown by direct comparison of it with the main protein of lens fibers, which is known to be delta crystallin (8, 11, 12); both have the same mobility in dodecyl sulfate–polyacrylamide gels and have identical isoelectric points when isofocussed in sucrose density gradients (6, 10).

RESULTS

Cell elongation

The mean cell length in explanted lens epithelia almost doubled within the first 10 hr of cultivation in the presence of serum or of insulin (Table 1). Thereafter, cell elongation occurred much more slowly in both serum and insulin. After 24 hr of culture, the mean cell length in epithelia cultured in serum was not significantly different from that in epithelia cultured in insulin. The serum-treated cells continued to elongate slowly over the 3-day period of cultivation. By contrast, the epithelial cells cultured in insulin did not elongate for more than 24 hr; the cell length was actually less after 3 days of culture with insulin than after 1 day of cultivation. Epithelial cells cultured in Ham's F-10 medium alone did not elongate.

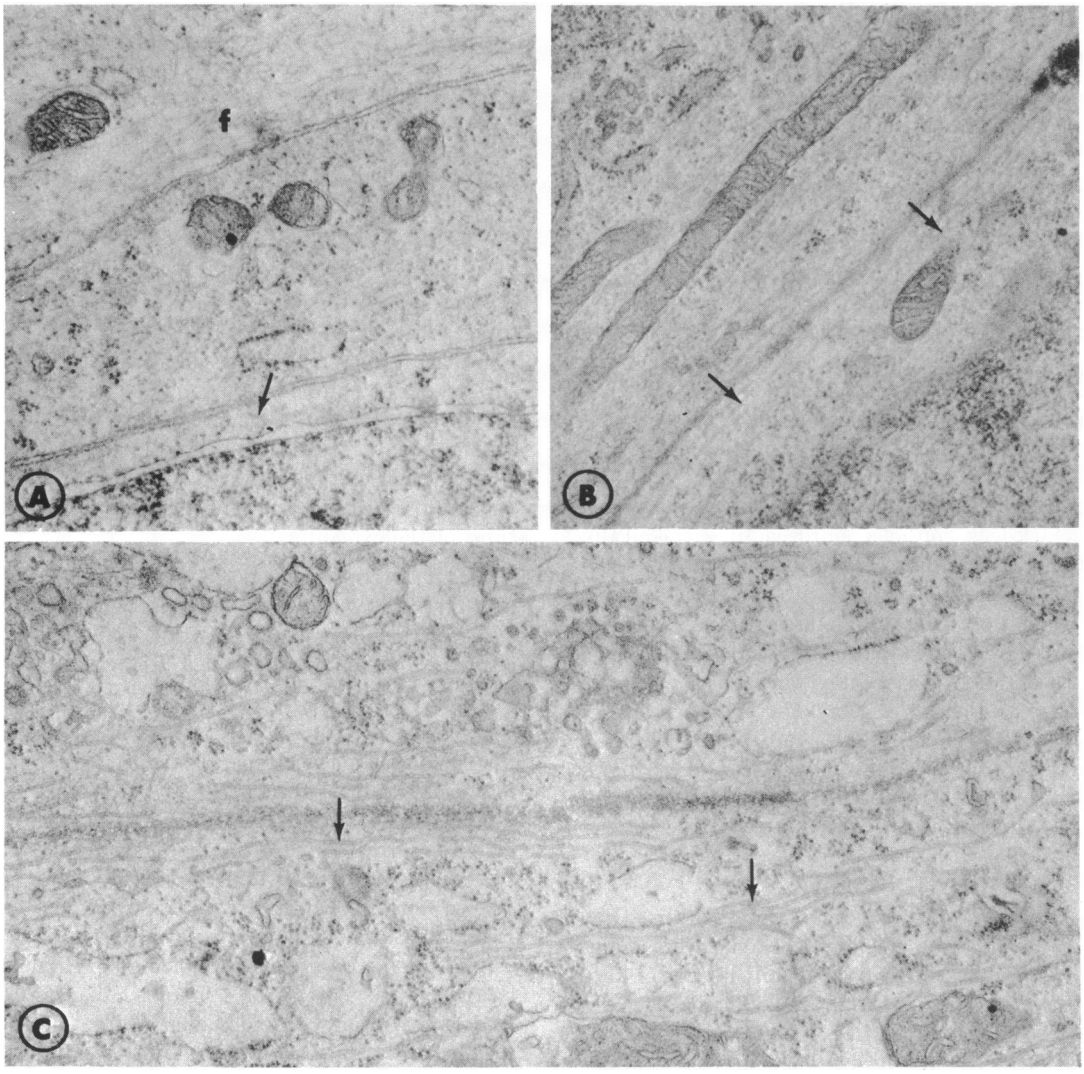

FIG. 1. Electron micrographs of lens epithelia cultured for 24 hr in Ham's F-10 medium alone (*A*), in medium supplemented with 15% fetal-calf serum (*B*), or in medium supplemented with 1 μg/ml of insulin (*C*). *Arrows* denote microtubules, which are arranged longitudinally in the cells, mostly along surface membranes. Note that cells maintained in Ham's F-10 medium alone (*A*) have few microtubules, but many filaments (*f*) that are about 100 Å in diameter. Magnification: ×30,000.

Microtubule assembly

Very few microtubules are present in the lens epithelia immediately after explantation, but numerous microtubules are visible after 24 hr of cultivation in serum (6, 19). In the present tests, after 24 hr of culture, few microtubules were seen in epithelia maintained in Ham's F-10 medium alone (Fig. 1*A*), but many were present in longitudinal arrays, mostly along surface membranes, in epithelia cultured in serum (Fig. 1*B*) or insulin (Fig. 1*C*).

Colchicine dissociates microtubules and prevents cell elongation in lens epithelia cultured with serum (19). We tested whether microtubule assembly is necessary for cell elongation in epithelia treated with insulin. Colchicine (20 μM) completely inhibited cell elongation in the presence of insulin; the mean cell lengths of epithelia cultured for 10 (2 explants) or 24 (9 explants) hr with colchicine and insulin was 10 μm, not significantly different from the cell length at the time of explantation.

Protein synthesis

Lens epithelia cultured for 24 hr in Ham's F-10 medium alone, or supplemented with fetal-calf serum or insulin, were labeled with L-[2,3-^{3}H]valine for 2 hr; their proteins were examined on dodecyl sulfate–polyacrylamide gels in order to compare the effect of fetal-calf serum with that of insulin on the synthesis of total cellular proteins and of delta crystallin (Fig. 2). Delta crystallin was the principal band of protein, which migrated with a molecular weight of about 45,000 (6, 10). The scanning patterns show that the serum-treated epithelia contained 3.2-times more protein than those cultured in Ham's F-10 medium alone, while the insulin-treated epithelia had only 1.4-times more protein than those maintained in unsupplemented medium. Similar relative differences existed for the amounts of delta crystallin present. The profile of radioactivity indicates that serum stimulated incorporation of [2,3-^{3}H]valine into total cellular proteins by 3.1-fold and into delta crystallin by 4.4-fold; by contrast, insulin stimulated incorporation into total cellular proteins by only 1.2-fold and into delta crystallin by 1.3-fold. As expected (6), serum treatment increased the proportion of newly synthesized delta crystallin from 28% to 40% of the total protein synthesized. Insulin treatment, however, only increased the proportion of newly synthesized delta crystallin to 30% of the total protein synthesized. Four other experiments of this type gave similar results.

Additional experiments were conducted to determine whether these differences were due to synthesis or to degradation of protein. Two types of experiments were performed. In the first test, lens epithelia were labeled for 2 hr with radioactive valine and subsequently incubated for 22 hr in Ham's F-10 medium supplemented with fetal-calf serum or insulin. In the second test, epithelia were cultured for 20 hr in Ham's F-10 medium alone or supplemented with fetal-calf serum or insulin, labeled for 2 hr with radioactive valine, and finally incubated for another 2 hr in the presence of 20 μg/ml of cycloheximide. Cycloheximide, an inhibitor of protein synthesis in eukaryotic cells (20), suppressed valine incorporation into protein by more than 99%. In both experiments, there was no significant difference in the amount of degradation of total proteins or of delta crystallin in epithelia cultured in unsupplemented or supplemented medium. A maximum of 25% of the total proteins labeled was degraded during the chase; there were less than 10% differences in the amounts of label present in delta crystallin before and after the chase. Thus, the differences in the accumulation of proteins in serum- or insulin-treated epithelia were due to differences in synthesis, rather than to differences in degradation, of these proteins.

TABLE 1. *Mean cell lengths in cultured lens epithelia from 6-day-old chick embryos*

Hours cultured	Cell length (μm ± 2 SEM)	
	Serum*	Insulin†
0	13.5 ± 3.3	—
5	19.8 ± 2.7	19.0 ± 1.3
10	25.4 ± 3.4	22.5 ± 2.2
24	30.4 ± 1.8	25.8 ± 2.2
72	35.0 ± 5.2	19.3 ± 2.1

* Number of epithelia measured was as follows: Not cultured, 5; cultured 5 hr, 6; cultured 10 hr, 8; cultured 24 hr, 16; cultured 72 hr, 24.

† Number of epithelia measured was as follows: Cultured 5 hr, 5; cultured 10 hr, 10; cultured 24 hr, 27; cultured 72 hr, 16.

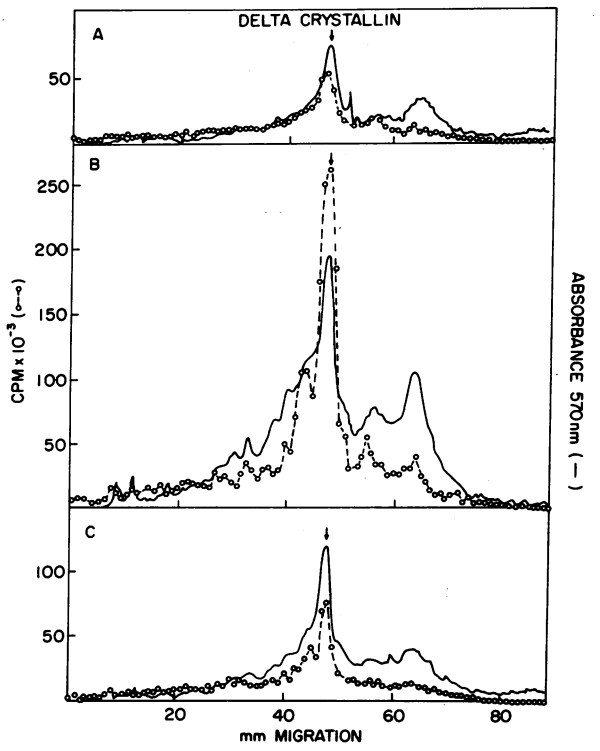

FIG. 2. Electrophoretic patterns of absorption and of radioactivity of stained Na dodecyl sulfate–polyacrylamide gels containing the proteins of 10 lens epithelia cultured for 24 hr in Ham's F-10 medium alone (*A*), supplemented with 15% fetal-calf serum (*B*), or supplemented with 1 μg/ml of insulin (*C*). The epithelia were labeled with [^{3}H]valine for 2 hr before electrophoresis. The molecular weight of delta crystallin is about 45,000, as judged by its position relative to markers of bovine-serum albumin, ovalbumin, and cytochrome *c*, which were electrophoresed on parallel slots of the gel slab.

DISCUSSION

Serum (6, 9) or insulin (13) stimulates cell elongation in explanted 6-day-old lens epithelia of chick embryos. Longi-

tudinally oriented microtubules assemble in elongating lens cells treated with serum (6). Microtubules also become oriented along surface membranes when embryonic-lens epithelial cells are stimulated to elongate by the addition of insulin to a chemically defined, serum-free medium. Our previous investigations have shown that the lens-cell elongation initiated by insulin is not due to the small quantities of glucagon or zinc that contaminate the insulin preparation, and is not a nonspecific response promoted by the addition of protein to the medium, since neither fetal-calf serum nor cytochrome *c* cause cell elongation when added to a final concentration of 1 μg/ml, which is the optimum insulin concentration to induce cell elongation (13). The effect of insulin on microtubule assembly in cultured embryonic-lens epithelia resembles the marked effect of insulin on microtubule assembly in explanted rat adipocytes (21).

Although it is not known whether or not the mechanism of cell elongation is the same in serum-treated and insulin-treated lens epithelia, there are several similarities in the two cases. First, the initial doubling of cell length stimulated by serum or insulin occurs rapidly and synchronously for the first 10 hr. This finding indicates that the cells can elongate during most of the cell cycle, whether stimulated by serum or insulin. Lens epithelial cells stimulated by serum or by insulin assemble longitudinally arranged microtubules. Serum-induced cell elongation is inhibited by dissociation of the microtubules with vinblastine sulfate or with colchicine (19, 22); similarly, lens-cell elongation stimulated by insulin is inhibited by colchicine treatment. The colchicine inhibition of cell elongation promoted by fetal-calf serum is not caused by cell death because protein synthesis is unaffected and the proportion of cells in mitosis increased with the duration of the experiment, and is not due to mitotic arrest of each cell because colchicine prevents elongation of all the cells in the explant, including those not in mitosis (19). The appearance of longitudinally oriented microtubules in developing fibers of intact lenses (2–6) and experiments with colcemid (5) also implicate microtubules in lens-cell elongation during embryonic development. Indeed, longitudinally arranged microtubules have been correlated with elongated cells or cell processes in many different systems (see ref. 19 for references).

The possibility remains that mitosis also contributes to the lens epithelial-cell elongation, by cell elongation due to an increase in cell density, an effect that could squeeze the cells and result in their elongation.

Insulin can substitute for serum to promote growth (23–29) and, thus, like serum, elicit a "pleiotypic response" (30) in cultured cells. However, the effect of insulin on explanted embryonic lens epithelia of chicks differs from that of serum in at least two respects. Insulin does not promote lens epithelial-cell elongation for more than 24 hr, while cells maintained in fetal-calf serum continue to elongate slowly for at least a month (Piatigorsky and Rothschild, in preparation), and insulin does not stimulate the synthesis of lens proteins as effectively as serum. It is likely that the failure of the cultured lens cells to elongate for more than one day in media containing insulin is related to the comparatively low rate of protein synthesis in the insulin-treated epithelial cells. It is also possible that insulin receptors are released from the epithelial cell surface, as has been demonstrated to occur in lymphocytes cultured in serum-free medium (31). Perhaps higher concentrations of insulin would provide, like serum, a more general stimulation of the cultured-lens epithelial cells. In one test, we found that [^{3}H]valine incorporation was 1.6-times higher and delta crystallin content was 1.2-times greater in lens epithelia cultured with 10 μg/ml of insulin than in those cultured with 1 μg/ml of insulin. These values represented less than half the stimulation of protein synthesis afforded by serum. At 1 μg/ml, however, insulin promotes lens-cell elongation and microtubule assembly without an appreciable stimulation of protein synthesis, a result consistent with earlier experiments with cycloheximide (19) indicating that the early events of cell elongation can occur in the absence of concomitant protein synthesis; thus, insulin treatment provides a chemically defined culture system in which to study lens epithelial-cell elongation and microtubule assembly.

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1. Coulombre, A. J. (1965) in *Organogenesis*, eds. DeHaan, R. & Ursprung, H. (Holt, Rinehart and Winston, Inc., New York), pp. 219–251.
2. Byers, B. & Porter, K. R. (1964) *Proc. Nat. Acad. Sci. USA* **52**, 1091–1099.
3. Porte, A., Stoeckel, M. E. & Brini, A. (1968) *Arch. Ophthalmol.* **28**, 681–706.
4. Kuwabara, T. (1968) *Arch. Ophthalmol.* **79**, 189–195.
5. Pearce, T. L. & Zwaan, J. (1970) *J. Embryol. Exp. Morphol.* **23**, 491–507.
6. Piatigorsky, J., Webster, H. deF. & Craig, S. P. (1972) *Develop. Biol.* **27**, 176–189.
7. Papaconstantinou, J. (1967) *Science* **156**, 338–346.
8. Clayton, R. M. (1970) in *Current Topics of Developmental Biology*, eds. Moscona, A. A. & Monroy, A. (Academic Press, New York), Vol. 5, pp. 115–180.
9. Philpott, G. W. & Coulombre, A. J. (1965) *Exp. Cell Res.* **38**, 635–644.
10. Craig, S. P. & Piatigorsky, J. (1973) *Biochim. Biophys. Acta*, in press.
11. Zwaan, J. & Ikeda, A. (1968) *Exp. Eye Res.* **7**, 301–311.
12. Yoshida, K. & Katoh, A. (1971) *Exp. Eye Res.* **11**, 184–194.
13. Piatigorsky, J. (1973) *Develop. Biol.* **30**, 214–216.
14. Piatigorsky, J., Rothschild, S. & Wollberg, M. (1972) *J. Cell Biol.* **55**, 204 abstr.
15. Ham, R. G. (1963) *Exp. Cell Res.* **29**, 515–526.
16. Piatigorsky, J. & Rothschild, S. S. (1972) *Develop. Biol.* **28**, 382–389.
17. Venable, J. H. & Coggeshall, R. (1965) *J. Cell Biol.* **25**, 407–408.
18. Shapiro, A. L., Viñuela, E. & Maizel, J. V., Jr. (1967) *Biochem. Biophys. Res. Commun.* **28**, 815–820.
19. Piatigorsky, J., Webster, H. deF. & Wollberg, M. (1972) *J. Cell Biol.* **55**, 82–92.
20. Ennis, H. L. & Lubin, M. (1964) *Science* **146**, 1474–1476.
21. Soifer, D., Braun, T. & Hechter, O. (1971) *Science* **172**, 269–271.
22. Piatigorsky, J., Webster, H. deF. & Wollberg, M. (1971) *J. Cell Biol.* **47**, 158*A*–159*A*.
23. Lieberman, I. & Ove, P. (1959) *J. Biol. Chem.* **234**, 2754–2758.
24. Waymouth, C. & Reed, D. E. (1965) *Tex. Rep. Biol. Med.* **23**, Suppl. 1, 413–419.
25. Temin, H. M. (1967) *J. Cell Physiol.* **69**, 377–384.
26. Schwartz, A. G. & Amos, H. (1968) *Nature* **219**, 1366–1367.
27. Blaker, G. J., Birch, J. R. & Pirts, S. J. (1971) *J. Cell Sci.* **9**, 529–537.
28. Harding, C. V., Reddan, J. R., Unaker, N. J. & Bagchi, M. (1971) *Int. Rev. Cytol.* **31**, 215–300.
29. Gerschenson, L. E., Okigaki, T., Andersson, M., Molson, J. & Davidson, M. B. (1972) *Exp. Cell Res.* **71**, 49–58.
30. Hershko, A., Mamont, P., Shields, R. & Tomkins, G. M. (1971) *Nature* **232**, 206–211.
31. Gavin, J. R. III, Buell, D. N. & Roth, J. (1972) *Science* **178**, 168–169.