Molecular Aspects of Lens Cell Differentiation

John Papaconstantinou

One of the major problems confronting investigators of the mechanisms of cellular differentiation is that of learning how developing cells acquire specific biochemical characteristics and how these biochemical specificities are linked to morphological development. It is a well-documented fact that, as cells progress through specific stages of differentiation, new biochemical traits are acquired while some existing traits are lost. Thus, during differentiation there occurs a progressive cellular diversification and specialization characterized morphologically by specific cellular structure and biochemically by the synthesis of specific structural proteins and enzymes. The ultimate form of morphological and biochemical cellular specialization may be seen in the muscle cell, erythrocyte, lens cell, and so on, which have well-defined morphological characteristics and synthesize tissue-specific proteins in the form of myosin, hemoglobin, and crystallins, respectively. This ability of cells to lose and acquire specific biochemical characteristics during differentiation may be the result of differential gene action. The mechanisms by which vertebrate cells can regulate genetic expression are not known, yet it is these mechanisms which are fundamental to the regulation of morphogenesis. One of the approaches to their study is analysis on the regulation of synthesis of tissue-specific proteins. Upon entering the terminal stages of differentiation many cells initiate the synthesis of highly specific proteins. These cells remain functional for a relatively long period, and this facilitates their use for studies on the mechanisms of regulation of protein synthesis and the role of these mechanisms in cellular differentiation.

The regulation of synthesis of a specific group of proteins in lens cells is associated with a well-defined stage of cellular differentiation—the differentiation of the lens epithelial cell to the fiber cell. The biochemical basis of protein and nucleic acid changes associated with these morphological changes is the basis of the studies presented here.

The author is a member of the staff of the Oak Ridge National Laboratory, Oak Ridge, Tennessee, in the Biology Division. At the time this article was written he was affiliated with the Institute of Cellular Biology, University of Connecticut, Storrs.

Morphological Changes in Fiber Cell Differentiation

Structure of the lens. The lens is an avascular tissue composed of an outer, single layer of epithelial cells; a zone of cellular elongation, or equatorial region, composed of cells which are in the process of developing into fiber cells; and the inner fiber cells (Fig. 1).

The epithelial cells can be separated into two distinct regions (Fig. 1): the pre-equatorial or germinative region, which is composed of cells having a high rate of mitosis relative to the cells in the central region, and the epithelial cells of the central region. In lenses of embryos and newborn animals, mitotic cells may be found in the central region as well as in the germinative zone. As the animal ages, mitosis becomes negligible in the central zone and is essentially limited to the germinative region, although it is also significantly reduced in this region. Thus, the overall mitotic activity of the epithelial cells of the lens decreases as the lens ages (*1*).

On the equatorial side of the germinative epithelial cells is the region of cellular elongation. Initiation of the differentiation of epithelial cells to fiber cells occurs in this region. It is in this region that the gross morphological changes associated with fiber cell differentiation occur—that is, the transition from a cuboidal epithelial cell to the elongated fiber cell.

After the embryonic lens has been formed, fiber cells are continuously laid down throughout the prenatal and postnatal life of the animal. The bulk of the lens is composed of layer upon layer of these fiber cells, and this continuous formation of fiber cells accounts for the growth of this tissue. It may be seen, therefore, that fiber cell formation represents the final stage of lens cell differentiation and that, in the adult lens, the fiber cells formed during embryonic growth compose the central or nucleus region, while the newly formed fiber cells are found in the peripheral or cortex region (Fig. 1). This unique pattern of growth results in the formation of an adult tissue whose entire cellular history may be preserved in that tissue, within the many layers of fiber cells.

Finally, since the fiber cell loses its replicative activity, it essentially enters a permanent stationary phase. Being in the stationary phase of the cell cycle is a characteristic that the fiber cells share with the central-region epithelial cells. These two types of cells differ, however, in that the fiber cells are in an irreversible stationary phase, whereas the epithelial cells retain their ability to replicate and are in a reversible stationary phase. Thus, the advantages of using the lens for study of the regulation of tissue-specific protein synthesis in cellular differentiation are as follows: (i) it offers a pure population of cells which show distinct stages of cellular differentiation (fiber cell formation) and protein synthesis (crystallins); (ii) it offers a pure population of cells which exist in the stationary phase of the cell cycle, the fiber cells being in an irreversible stationary phase and the epithelial cells in a reversible stationary phase; and (iii) it also offers a pure population of mitotically active cells in the germinative region.

Cytological and cytochemical observations on fiber cell formation. The lens epithelial cells are characterized by their cuboidal shape, their basophilic staining properties, and their ability to replicate (*2*). In the zone of elongation (Fig. 2), where the epithelial cells begin the process of fiber cell formation, the following changes occur in the intracellular structures: (i) the cell sends out cytoplasmic processes anteriorly beneath the cuboidal epithelial cell layer and also posteriorly to form the fiber cell; (ii) the nucleus and nucleoli enlarge (*3*); (iii) the ribosomal population increases significantly, especially in the cytoplasm adjacent to the enlarged nucleus (*4*); and (iv) the nucleus is metabolically active in that it has the ability to synthesize both DNA and RNA (*5*).

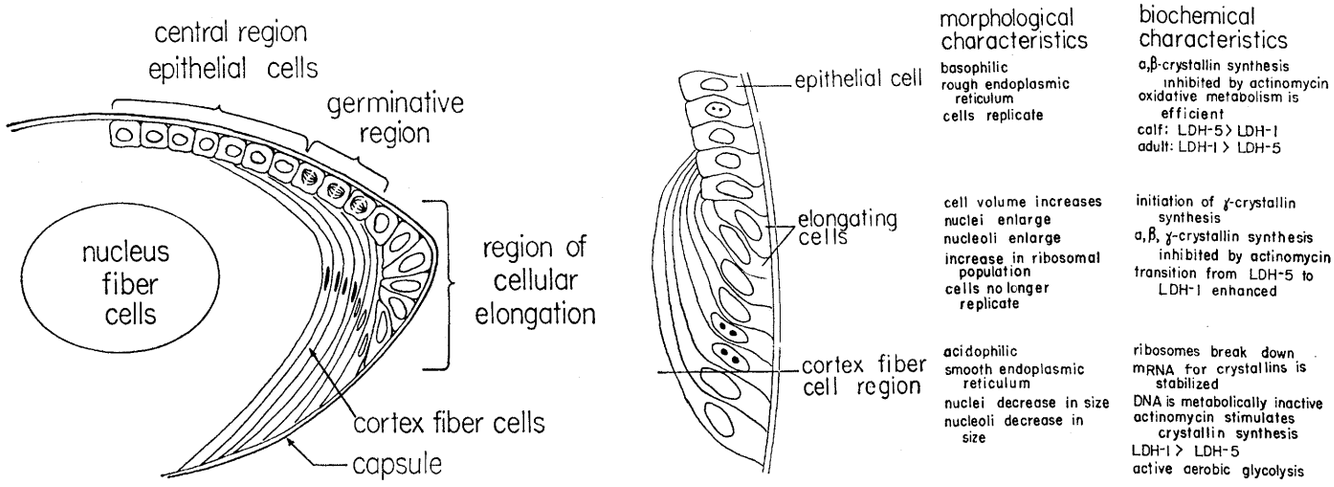


Fig. 1 (left). Diagram of the lens of the adult vertebrate. The lens is surrounded by an external noncellular capsule. Beneath the capsule are the lens epithelial cells. The region of cellular elongation is in the peripheral area. This is the region of transition where the epithelial cells begin to elongate into fiber cells. The fiber cells that are newly laid down constitute the cortex region; the fiber cells laid down during the early growth period of the lens compose the nucleus region of the adult lens. Fig. 2 (right). Diagram of the region of cellular elongation in the vertebrate lens. The major morphological and biochemical characteristics associated with lens cell differentiation are listed and are discussed in detail in the text.

In the completed fiber cell the cytoplasm loses its basophilic properties and takes on acidophilic properties; the nucleus and nucleoli decrease in size and no longer synthesize DNA and RNA; the endoplasmic reticulum, which has a granular appearance in the epithelial cell, takes on a smoother appearance in the fiber cell; and a significant decrease in the ribosomal population occurs in the differentiated fiber cell, as has been shown through electron-microscope studies (*4*). These differences in staining properties and changes in intracellular structure indicate that significant macromolecular changes are associated with fiber cell differentiation. The enlargement of the nucleus and nucleoli, for example, as well as the increase in ribosomal population is an indication of increased nucleic acid and protein synthesis during elongation. With these structural changes in mind, I would like to describe a series of biochemical events which are associated with fiber cell formation, and which may be closely linked with these cytological observations.

Biochemistry of Lens Fiber Cell Differentiation

Association of gamma-crystallin synthesis with fiber cell differentiation. Tissue-specific protein synthesis is very often observed to occur during cellular maturation—that is, during the terminal stages of cellular differentiation. The γ-crystallins are specific proteins of the lens whose synthesis is initiated during the differentiation of a lens epithelial cell to a fiber cell (*6, 7*). This is the terminal stage of lens cell differentiation.

One of the most interesting aspects of lens growth is the fact that the fiber cells are systematically laid down, layer upon layer, throughout the life of the animal. Theoretically, therefore, by peeling away the layers of fiber cells in an adult lens it should be possible to recover the cells formed at various ages. Actually, the fiber cells can be peeled off when the decapsulated lenses are placed in a buffered solution. The outer-cortex fiber cells, for example, continue to peel off until the central, nucleus region is reached. The freed cortex fiber cells can be separated from the nucleus fiber cells by decanting. Since the epithelial cells and elongating cells from the equatorial zone are removed along with the lens capsule, this provides a method for separating the lens cells into three groups—the epithelial cells, the newly formed cortex fiber cells, and the fiber cells of the nucleus region which had been laid down during the early life of the animal. Thus, using this procedure for separating the epithelial cells and fiber cells, one could proceed to determine whether the differentiation of the fiber cell is associated with the initiation of synthesis of a protein or group of proteins. Furthermore, by analyzing the fiber cells of the nucleus region of the adult lens, it may be possible to detect any differences that would suggest the existence of fetal and adult specific proteins.

In pursuit of an answer to these questions, experiments were designed to determine whether there are detectable qualitative or quantitative differences in the proteins of epithelial cells and fiber cells. There are three major groups of proteins synthesized by lens fiber cells: the α-crystallins, the β-crystallins, and the γ-crystallins (*8*). Column-chromatography procedures were established to fractionate these proteins and to determine whether homogenates of epithelial cells and fiber cells from the cortex and nucleus regions have characteristic elution patterns (*9, 10*). The elution patterns obtained are shown in Fig. 3, A–C. The most significant differences were observed with the first group of proteins eluted from the column. Upon electrophoretic analysis it was found that these proteins are the γ-crystallins (*9*). From the elution patterns of Fig. 3 it may be seen that the γ-crystallins are only slightly detectable in the epithelial cells (Fig. 3A); they are detected as a broad peak (a_1, a_2, b) in the cortex fiber cells (Fig. 3B), and as a sharp peak in the nucleus fiber cells (Fig. 3C). These observations indicate, first, that the γ-crystallins are specifically characteristic of the fiber cell. Second, the γ-crystallins of the adult cortex and adult nucleus fiber cells are qualitatively and quantitatively different with respect to their chromatographic properties on diethylaminoethyl (DEAE)-cellulose columns. Thus, the γ-crystallins formed in the fiber cells of young animals (fiber cells found in the nucleus region of the adult lens) may be chemi-

cally distinct from γ-crystallins synthesized by fiber cells of older animals (cells found in the cortex region of the adult lens).

If it is correct to conclude that the γ-crystallins are proteins specific to the fiber cells, then epithelial cells from animals of all ages should lack these proteins. The elution pattern of proteins from epithelial cells of lenses of 3-month-old calves (Fig. 4A) indicate that this is indeed the case. Although traces of γ-crystallins are detected, the amount present is much less than that detected in the fiber cells (Fig. 4, B and C). The traces of γ-crystallins found in the epithelial cell homogenates are due to the adherence of the elongating cells to the lens capsule. It is in these elongating fiber cells that the initiation of γ-crystallin synthesis occurs. (This has now been confirmed by immunofluorescence analysis by R. E. Shore.) Thus, a comparison of the elution patterns of proteins extracted from epithelial cells, cortex fiber cells, and nucleus fiber cells of adult and calf lenses shows that, at both ages, γ-crystallin synthesis is initiated during fiber cell formation. Similarly, it has been reported that γ-crystallin synthesis is associated with fiber cell formation in the regenerating salamander lens (*7*).

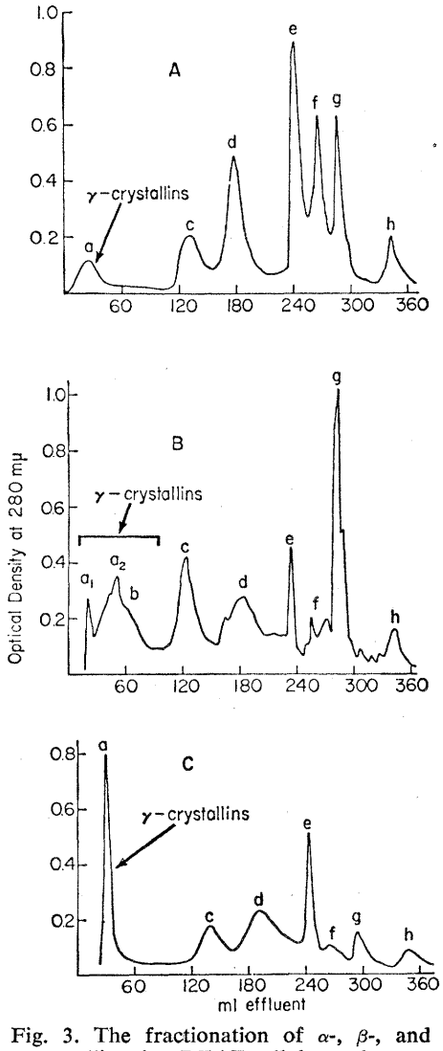


Fig. 3. The fractionation of α-, β-, and γ-crystallins by DEAE-cellulose chromatography, from adult-bovine-lens (A) epithelial cells, (B) cortex fiber cells, and (C) nucleus fiber cells. Protein concentration is expressed as optical density units at wavelength of 280 mμ. (The fractionation procedures are described in detail in *6* and *9*.)

The difference in the chromatographic properties of adult-cortex and nucleus γ-crystallins implies that the γ-crystallins of the nucleus region and those of the cortex region of the adult lens may differ distinctly in their chemical properties. These regional differences in γ-crystallins (Fig. 3, B and C) may be due to a difference in amino acids incorporated into these proteins at different ages. Thus, the γ-crystallins from lenses of younger animals (embryos and young calves) should have the same chromatographic properties as γ-crystallins from the nucleus fibers of an adult lens. Evidence in support of this supposition is presented by the elution patterns of proteins from calf-lens cortex (Fig. 4B) and calf-lens nucleus (Fig. 4C), which show that γ-crystallins in the fiber cells of these younger lenses are chromatographically similar to the γ-crystallins of the adult nucleus fibers (Fig. 3C). Similar properties have been reported for embryonic bovine lenses (*6*).

In view of the differences in chromatographic properties of the γ-crystallins, attempts were made to obtain additional evidence of chemical differences between the embryonic and adult γ-crystallins. Further purification of adult-cortex, adult-nucleus, and embryonic γ-crystallins was achieved by means of DEAE-cellulose fractionation (*6*). In each case the γ-crystallins were resolved into four major proteins. The purified γ-crystallins from each of these fractions were concentrated, and their relative mobilities were determined by paper electrophoresis. The electrophoretic patterns (Figs. 5 and 6) show that the γ-crystallins from embryonic and adult-nucleus fibers have the same mobility, whereas the γ-crystallins from the adult cortex have different mobility. These data are consistent with the observation that γ-crystallins of calf and adult lenses have different column-chromatography properties.

These observations have led my associates and me to conclude (i) that the γ-crystallins are tissue-specific proteins whose synthesis is associated with fiber cell differentiation; (ii) that the γ-crystallins synthesized during embryonic and early postnatal fiber cell differentiation are electrophoretically distinct from those synthesized in the fibers of the adult lens; and (iii) that the type of γ-crystallin synthesized depends on the age of the animal.

Loss of lactate-dehydrogenase-isozyme activity in lens differentiation. Another example of differential gene action associated with fiber cell differentiation, as well as with the aging

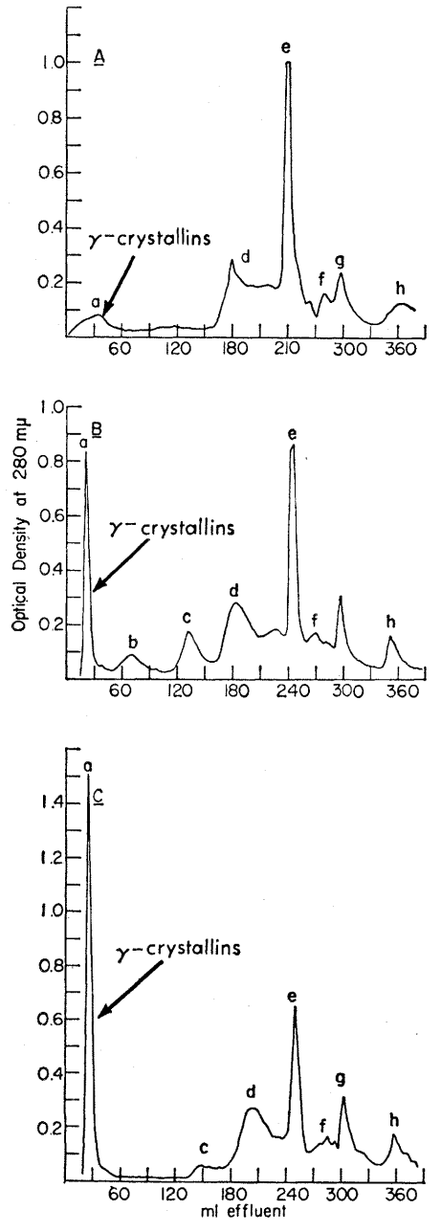


Fig. 4. The fractionation of α-, β-, and γ-crystallins by DEAE-cellulose chromatography, from calf-lens (A) epithelial cells, (B) cortex fiber cells, and (C) nucleus fiber cells.

or replicative activity of the epithelial cells, is the specific repression of one of the lactate dehydrogenase isozymes (*11*).

Lactate dehydrogenase isozymes occur in many vertebrate tissues as five electrophoretically distinct forms (*12–14*). All five isozymes are composed of four protein subunits. The extreme cathodal (LDH-5) and anodal (LDH-1) forms of these enzymes are homogeneous with respect to their subunits; however, the subunits of LDH-1 do not have the same amino acid composition as the subunits of LDH-5. By dissociation and reassociation of a mixture of LDH-1 and LDH-5, these subunits can be recombined in such a way that all five isozymes are formed (*13*). These experiments show that LDH-2, LDH-3, and LDH-4 are composed of combinations of subunits of LDH-1 and LDH-5. Since the synthesis of subunits of LDH-1 and LDH-5 is genetically regulated (*14*), any alterations in the isozymic patterns during lens cell differentiation might be attributed to the differential regulation of subunit synthesis. In the lens, alterations in isozyme patterns were thought to be associated with either a very specific stage of cellular differentiation—namely, the differentiation of an epithelial cell to a fiber cell—or with the aging of the epithelial cells (*11*).

Electrophoretic analyses of the lactate dehydrogenase isozymes show that there are five forms of the enzyme in the epithelial cells of the adult and calf lens. Typical isozymic patterns are diagrammatically presented in Fig. 7. A comparison of the patterns from calf and adult epithelial cells shows that a change in enzyme activity from predominantly anodal forms occurs. Thus, there is a transition of LDH isozyme activity in the epithelial cells during the postnatal aging of these cells. Since the epithelial cells of the lens are functionally the same during embryonic, early postnatal, and adult life, the reasons for this change in LDH activity are not obvious. There is, however, a difference between the epithelial cells of young lenses and those of adult lenses. This lies in the mitotic activity of both the germinative and the central cells as well as in the rate of fiber cell formation (*1*). As the lens reaches its maximum size in the adult, mitotic activity decreases in the germinative region and the cells of the central region enter a stationary phase. It is during this decrease in mitotic activity that the major transformation

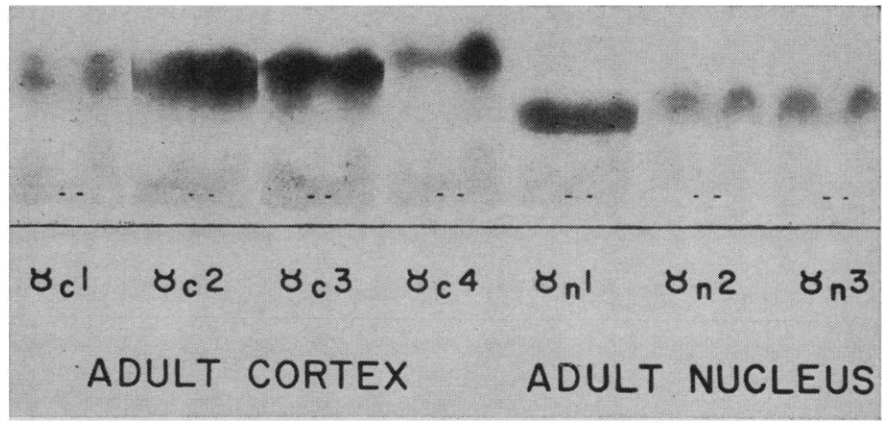


Fig. 5. Electrophoretic analysis of γ-crystallins in the cortex and nucleus of the adult lens. Electrophoresis was carried out in a solution containing 0.5 mole of tris buffer, 0.021 mole of ethylenediaminetetraacetic acid, and 0.075 mole of boric acid (*p*H 8.9) per liter, at constant voltage (5.8 volts per centimeter) for 17 hours. The γ-crystallins tested were prepared by DEAE-fractionation, precipitated in ammonium sulfate, and redissolved in 0.05*M* tris buffer.

from LDH-5 to LDH-1 occurs. On this basis it might be proposed that the isozymic transition is associated with the entrance of the cells of the central region into the stationary phase. Although other metabolic functions may be involved in the regulation of synthesis of subunits of LDH-1 and LDH-5, there is enough evidence in the literature to warrant serious consideration of the possibility that the regulation of subunit synthesis is also associated with either the temporary or the permanent loss of mitotic activity. The erythrocyte is a prime example of a cell type which loses its mitotic activity during its maturation. Furthermore, the erythrocyte shows a trend similar to that of the lens in the regulation of LDH subunit synthesis; that is, the younger, immature erythrocytes show greater LDH-5 decreases, leaving LDH-1 (*15, 16*). Thus, as in the lens, the sequence of LDH subunit synthesis during cellular maturation is from LDH-5 to LDH-1. In addition, in the

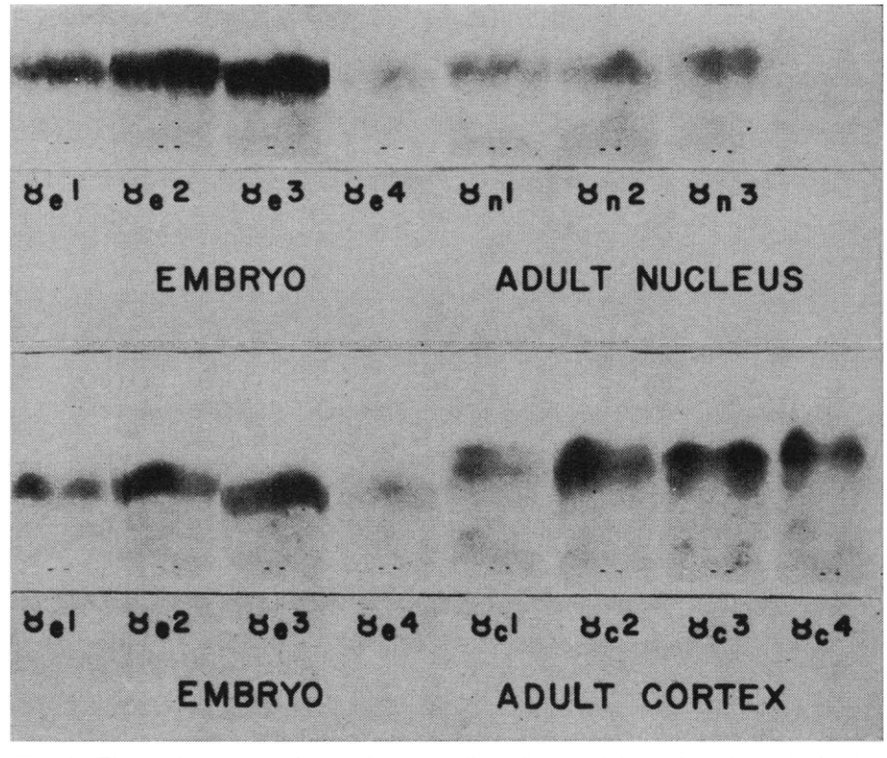


Fig. 6. Electrophoretic analysis of γ-crystallins from adult and embryonic bovine lenses. The conditions of electrophoresis are as described for Fig. 5.

erythrocyte as in the lens, LDH-5 is most active in the dividing cell.

It has recently been argued that LDH-5 is specifically localized in the cell nucleus and that the transition from LDH-5 to LDH-1 is due to nuclear loss or extrusion, such as is seen in the erythrocyte and lens fiber (*15*). This cannot be the case for the entire lens, however, since the epithelial cells of the adult show greater LDH-1 activity and still have a functional nucleus.

Let us consider now the observed differences in LDH isozyme patterns in lens epithelial cells and fiber cells. In addition to the enzyme changes in the epithelial cells alone, both calf and adult lenses show pronounced changes associated with differentiation of the fiber cell. This is the final stage of lens cell differentiation and results in the transition from a replicative cell to a nonreplicative cell. During this stage of differentiation the activity of LDH isozymes changes in such a way that LDH-1 persists. In the calf lens the fiber cells contain five detectable LDH's in which LDH-1 activity is predominant. In the adult lens the fiber cells contain essentially just LDH-1, although small amounts of LDH-2 are detectable. On the basis of the view that the synthesis of subunits of LDH-1 and LDH-5 is genetically regulated, the complete loss of LDH-3, LDH-4, and LDH-5 in the adult-lens fiber cell and the intermediate trends toward this loss in the calf cell might be attributed to the gradual suppression of synthesis of subunits of LDH-5. This suppression should decrease the availability of these subunits for recombination with subunits of LDH-1.

It appears, therefore, that during the aging of lens epithelial cells (from calf to adult) the regulation of LDH subunit synthesis results in a greater decrease in the synthesis of subunits of LDH-5 than in the synthesis of subunits of LDH-1. Furthermore, this tendency for LDH-1 to persist becomes more pronounced during differentiation of the epithelial cell to the fiber cell in both the calf and adult lens. The extreme case is seen in the adult cortex fiber cells, where LDH-1 is essentially the only one of the five isozymes remaining. Finally, I would like to correlate this finding with the replicative activity of the cell. When the epithelial cells, which retain their ability to replicate, reach a stationary phase, the synthesis of subunits of LDH-1 is greater than the synthesis of subunits of LDH-5. This is also the case when the cells enter the stage of differentiation (the fiber-cell stage) in which they ultimately lose their replicative capacity, thus entering an irreversible stationary phase.

I have presented two examples of the type of regulation of synthesis of tissue-specific proteins which is associated with a specific stage of cellular differentiation: (i) the initiation of γ-crystallin synthesis and (ii) the repression of synthesis of subunits of LDH-5. The synthesis of γ-crystallins is specifically associated with the differentiation of the epithelial cell to the fiber cell. Thus, the α- and β-crystallins are structural proteins of the epithelial cell, and the α-, β-, and γ-crystallins are structural proteins of the fiber cell. The cytological changes which occur in elongating epithelial cells, such as an enlargement of the nucleus and nucleoli and an increase in the ribosomal population, are indicative of an increase in protein synthesis and may be associated with the initiation of γ-crystallin synthesis.

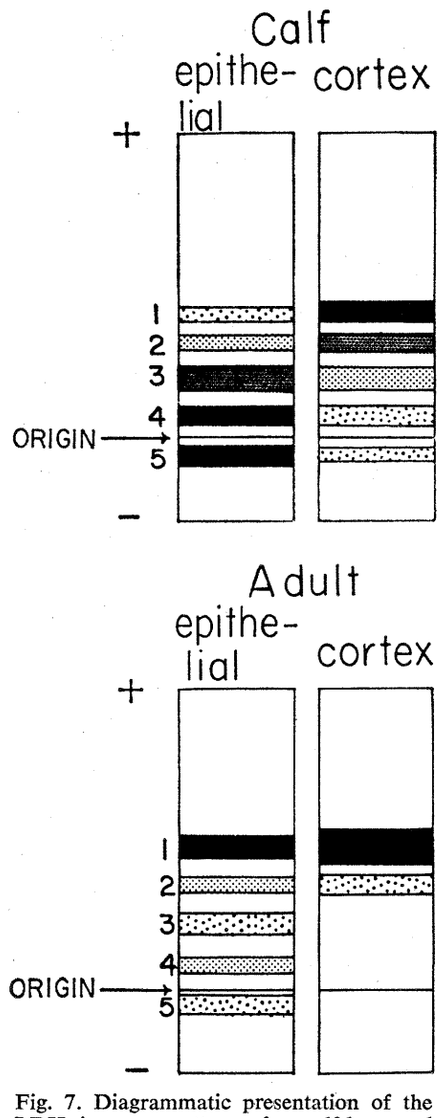


Fig. 7. Diagrammatic presentation of the LDH isozyme patterns for calf-lens and adult-lens epithelial cells and fiber cells. Each pattern was obtained by starch gel electrophoresis, and the enzymes were localized by coupling the reaction with phenazine methosulfate and nitro blue tetrazolium. The differences in intensity denote the degree of activity of the individual isozymes.

The lactate dehydrogenases, on the other hand, exemplify regulation which is associated not only with fiber cell formation but also with the reduction of mitotic activity of the cell. Thus, the ability of the cell to regulate LDH subunit synthesis in the absence of morphological changes brings out a significant difference between the regulation of γ-crystallin synthesis and LDH subunit synthesis. It is not known whether γ-crystallin synthesis can be induced in epithelial cells without the accompanying changes of fiber cell formation. The γ-crystallins are tissue-specific proteins whose function may be essential for the intracellular structure of the lens, whereas the LDH's are widespread and are more essential for cellular metabolic activity than for cellular structure. In both cases, regulatory mechanisms are required. It is not known whether these regulatory mechanisms are similar; the answer to this question must await further experimentation.

Nucleic Acids in Lens Fiber Cell Differentiation

Status of messenger RNA in differentiating lens cells. The synthesis of tissue-specific proteins such as hemoglobins (*17*), feather keratins (*18*), lens crystallins (*19, 20*), and zymogen granules (*21*) occurs on relatively long-lived messenger RNA (mRNA) templates. Bacterial mRNA, for example, which is considered to be short-lived, has a half-life of 2 minutes (*22*), while the half-life of mRNA for feather-keratin synthesis has been reported to be longer than 24 hours (*18*). One way to show the existence of stable mRNA is through the failure of actinomycin D to inhibit protein synthesis after RNA synthesis has been substantially inhibited. Stable mRNA appears to be a common and important feature of highly differentiated cells which synthesize very specific proteins. Although many of these proteins appear in the initial stages of cellular

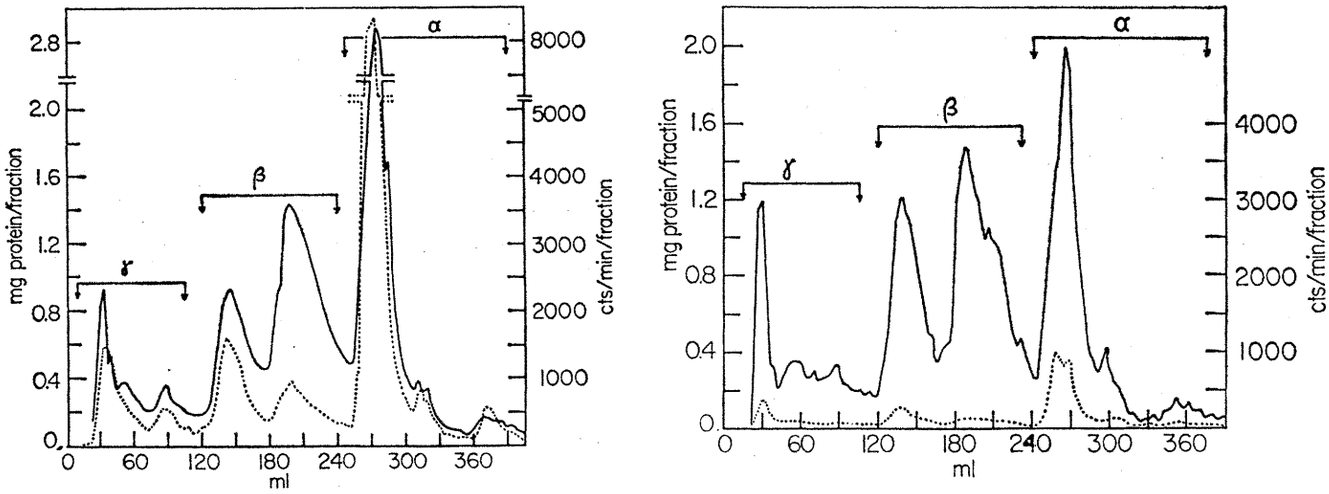


Fig. 8 (left). Fractionation of calf-lens epithelial cell crystallins on DEAE-cellulose, after incubation in ^{14}C-amino acid algal hydrolyzate for 2 hours at 37°C. (Solid lines) Total protein (in milligrams) per 3-milliliter fraction; (dotted lines) total counts per minute per 3-milliliter fraction. Fig. 9 (right). Fractionation of calf-lens epithelial cell crystallins on DEAE-cellulose, after incubation in ^{14}C-amino acid algal hydrolyzate with actinomycin D (10 micrograms per milliliter).

maturation, a basic question to be considered is whether there is, in the synthesis of these proteins, a transition from an actinomycin-sensitive to an actinomycin-insensitive period.

Experiments were carried out (*19*) to determine whether the lens crystallins of the epithelial cells and fiber cells are synthesized on long-lived or on short-lived messenger templates, and whether there is some specific stage of lens cell differentiation in which the stabilization of mRNA is initiated.

Actinomycin D, a potent inhibitor of mRNA synthesis, was used in these experiments. Epithelial and fiber cell crystallins from lenses treated with actinomycin D and from untreated lenses were fractionated on DEAE-cellulose, and the amount of radioactive amino acids incorporated into each fraction was determined. The elution patterns in Figs. 8 and 9 represent a typical protein pattern from untreated and antibiotic-treated epithelial cells. These patterns also show the degree of incorporation of radioactive amino acids into each fraction eluted from the column. It may be seen that incorporation of amino acids into the epithelial cell crystallins was extensively inhibited by actinomycin (Table 1).

The same experiments were performed with lens fiber cells. The elution patterns shown in Figs. 10 and 11 are typical for cortex fiber crystallins. The incorporation of amino acids into these proteins is also shown. The incorporation of amino acids into the crystallins is significantly greater in the actinomycin-treated cells than in untreated cells. A comparison of the specific activities of the α-, β-, and γ-crystallins from untreated and actinomycin-treated lenses shows that there is a significant stimulation of protein synthesis by the antibiotic, ranging from 66 percent for the β-crystallins to 103 percent for the α-crystallins (Table 1).

The specific activity of actinomycin-treated cells shows an inhibition of γ-crystallin synthesis in the elongating epithelial cells and a stimulation of synthesis of this same group of pro-

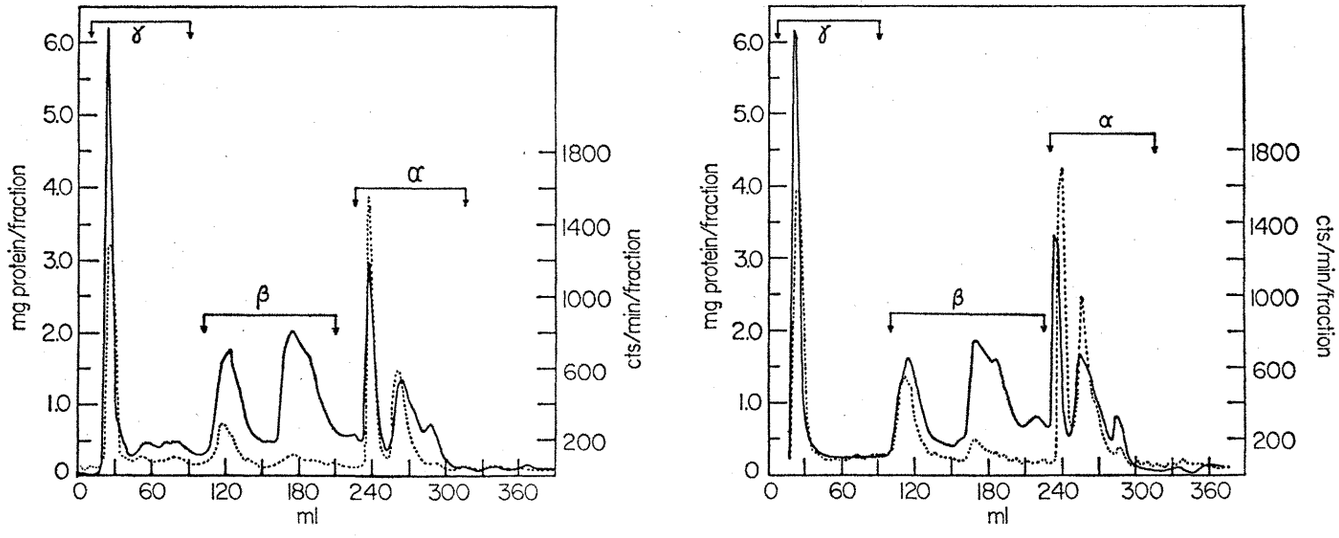


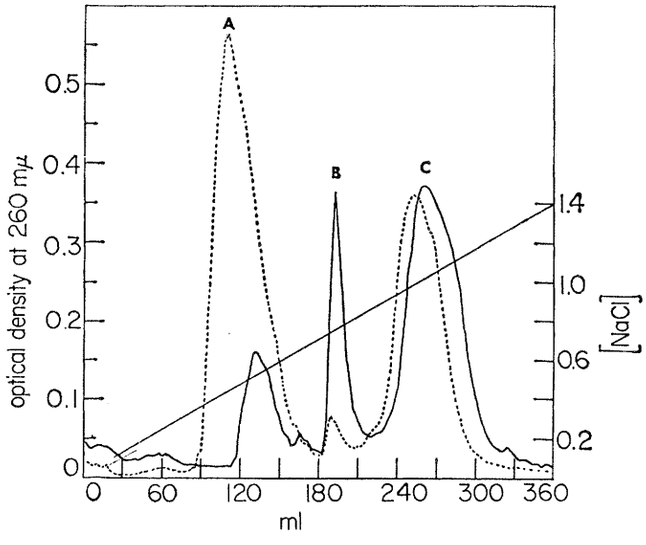
Fig. 10 (left). Fractionation of cortex fiber cell crystallins of calf lens after incubation in ^{14}C-amino acid algal hydrolyzate at 37°C. The experimental conditions are as described in Fig. 8. Fig. 11 (right). Fractionation of cortex fiber cell crystallins of calf lens after incubation with ^{14}C-amino acid algal hydrolyzate and actinomycin D (10 micrograms per milliliter). The experimental conditions are as described for Fig. 9.

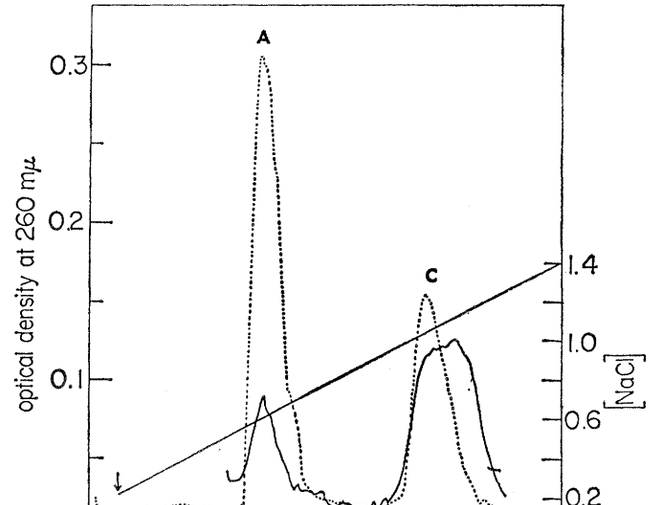
Table 1. The effect of actinomycin D on lens protein synthesis.

	Epithelial cells [count min^{-1} (mg of protein)$^{-1}$]			Fiber cells [count min^{-1} (mg of protein)$^{-1}$]		
	Control	Actinomycin D (10 μg/ml)	Inhibition (%)	Control	Actinomycin D (10 μg/ml)	Stimulation (%)
γ-Crystallins	1590	314	80	235	396	68
β-Crystallins	963	166	83	99	165	66
α-Crystallins	1980	572	71	340	690	103

teins in the fiber cells. Thus, at the time γ-crystallin appears, the synthesis of this protein as well as of the α- and β-crystallins is sensitive to inhibition by actinomycin D, and, upon differentiation to a fiber cell, the synthesis of these same proteins occurs on stabilized templates. Because of the stimulation of protein synthesis in the fiber cells by actinomycin D, experiments were carried out to determine the metabolic activity of RNA under the same conditions which produce this stimulation. Epithelial cell and fiber cell RNA was extracted from lenses incubated with radioactive uridine in the presence and absence of actinomycin D. The transfer RNA (tRNA) and ribosomal RNA (rRNA) were resolved by sucrose gradient centrifugation. When the fractions from the gradient had been counted, it was found that actinomycin D inhibited the synthesis of all species of RNA by 95 percent or more (*23*). These data indicate that the inhibition of protein synthesis in the epithelial cells is due to the inhibition of mRNA synthesis. In the fiber cell, where there is a natural slowing down of RNA synthesis, whatever synthesis does occur is also potently inhibited by actinomycin D. It is under these conditions that protein synthesis in the fiber cells is stimulated. From these observations it may be concluded that the synthesis of crystallins in calf-lens epithelial cells occurs on rapidly-turning-over RNA templates, whereas these same proteins are synthesized on stable templates in the fiber cells. Thus, it may be concluded that the differentiation of epithelial cells to fiber cells involves the stabilization of these templates.

Ribosomal breakdown in lens fiber cells. Ribosomes are a necessary basic unit of the protein-synthesizing machinery of the cell and are of special interest with respect to morphogenesis and the synthesis of proteins related to morphogenesis. From both electron-microscope and biochemical studies, a great deal of information has been obtained with respect to synthesis of ribosomes and lens cell differentiation. Electron-microscope examination of epithelial cells in the region of cellular elongation has shown that, at this stage of cellular differentiation, there





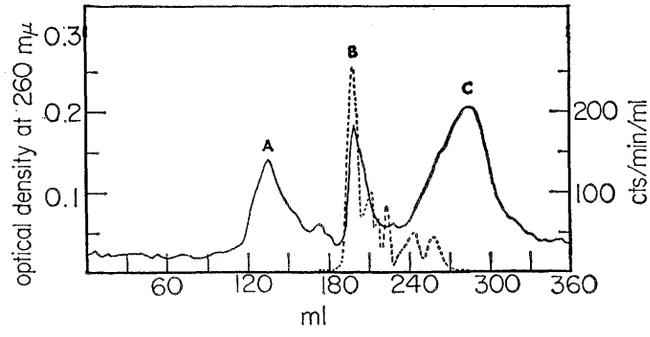


Fig. 12 (above). Fractionation of phenol-extracted nucleic acids from (solid line) epithelial cells and (dashed line) cortex fiber cells of calf lens, on methylated albumin columns. Nucleic acid concentration is expressed as optical density units at wavelength of 260 mμ (O.D.$_{260}$). A total of 35.4 O.D.$_{260}$ units from epithelial cells were placed on the column; 45.0 O.D.$_{260}$ units from the fiber cells were placed on the column. The nuclei acids were eluted with salt solution in a linear gradient ranging from 0.2 to 1.4 mole of NaCl per liter in 0.05*M* sodium phosphate, p*H* 6.8. Fig. 13 (top right). Fractionation of deoxyribonuclease-treated phenol-extracted nucleic acids from (solid line) epithelial cells and (dashed line) cortex fiber cells of calf lens. The conditions of fractionation are as described for Fig. 12. Fig. 14 (bottom right). Fractionation of phenol-extracted nucleic acids from calf-lens epithelial cells on methylated albumin columns. The intact lenses were first incubated in ^{3}H-thymidine. (Solid line) Nucleic acid concentration in units of O.D.$_{260}$; (dashed line) nucleic acid concentration in counts per minute per milliliter.

is a significant increase in the ribosomal population (*4*). This increase in ribosomes during the formation of the fiber cells may be related to the initiation of γ-crystallin synthesis, and possibly to an overall increase in protein synthesis which would be required for the increase in cellular volume that accompanies fiber cell formation. Observations with the electron microscope have also shown that, in the completed fiber cell, the ribosomal population decreases to a significantly low level. These observations are in good agreement with results of our biochemical analyses of the ribosomal RNA in epithelial and fiber cells (*4*). In these studies we have used methylated albumin columns (*24*) to fractionate nucleic acids from lens epithelial cells and fiber cells as a means of detecting any qualitative or quantitative differences that may occur in the nucleic acids of these cells. An elution pattern of the epithelial cell nucleic acids is shown in Fig. 12. The tRNA (peak *A*), which is involved in the activation of amino acids for protein synthesis, is eluted at NaCl concentrations between 0.4*M* and 0.6*M*; DNA (peak *B*) is eluted at NaCl concentrations between 0.6*M* and 0.8*M*, and rRNA (peak *C*), at NaCl concentrations between 0.8*M* and 1.0*M*. [This sequence of elutions agrees well with a similar elution system used to fractionate tRNA, DNA, and rRNA from *Escherichia coli* (*24*)]. In Fig. 12 a pattern for the RNA extracted from fiber cells is superimposed on the corresponding pattern for RNA from epithelial cells to facilitate comparison. There are striking differences between the two patterns: the pattern for fiber cell RNA shows a significantly larger amount of material eluted in peak *A* (tRNA) than in peak *C* (rRNA); in addition, there is a sharp decrease in the DNA (peak *B*) of the fiber cell pattern relative to the epithelial cell pattern. The quantitative difference between the tRNA and rRNA of epithelial and fiber cells is better seen in the patterns of Fig. 13. Phenol-extracted nucleic acids from epithelial and fiber cells were treated with deoxyribonuclease to remove DNA prior to fractionation on methylated albumin columns. The DNA (peak *B* of Fig. 12) is completely removed by this treatment, and now the epithelial and fiber cell patterns are almost alike except for the quantitative differences between peaks *A* and *C*. This apparent decrease in rRNA and increase in tRNA might be explained in one of two ways: (i) the increase in tRNA may actually be due to the accumulation of ribosomal breakdown products whose chromatographic properties are similar to those of tRNA; or (ii) the majority of the ribosomes break down completely, so that the ratio of tRNA to rRNA increases, thus giving the appearance of an increase in the tRNA. A similar ribosomal breakdown has been reported in *E. coli* under conditions of magnesium starvation (*25*).

Inactivation of DNA. One intracellular alteration that is characteristic of the terminal stages of lens cell differentiation is the inactivation of the nucleus. This results in the loss of cellular replication and, in the lens, is associated with the stabilization of mRNA and ribosomal breakdown. The methylated-albumin-column patterns of Fig. 12 indicate that there is a significant decrease in the DNA of the cortex fiber cells. These observations are in agreement with cytological reports that the nucleus of the cortex fiber cell decreases in size and is ultimately lost in the older fiber cells.

Mitotic activity has never been observed in the lens fiber cells, and the view that the fiber cells have lost their ability to replicate is now generally accepted. Furthermore, through cytological studies it is also well known that the nucleus of the fiber cell decreases in size and is gradually lost. Although small amounts of DNA could be detected in the nucleic acids extracted from the fiber cells, it is not known whether this DNA is turning over.

In view of this, we performed experiments to determine whether ^{3}H-thymidine is incorporated into the DNA of the fiber cells. From epithelial cells and fiber cells of calf lens incubated in ^{3}H-thymidine, the nucleic acids were extracted with phenol and fractionated on methylated albumin columns (Fig. 14). The DNA (peak *B*) fractions were counted, and it was found that ^{3}H-thymidine had been incorporated into the DNA of the epithelial cells. In a corresponding experiment with the fiber cell DNA fraction there was no incorporation of ^{3}H-thymidine into the fiber cell DNA. It was concluded from these experiments that DNA synthesis in the epithelial cells is gradually turned off as fibrogenesis comes to completion. Thus, with the loss of nuclear activity in the fiber cell, there occurs a stabilization of mRNA and a gradual breakdown of the ribosomes.

Summary and Conclusion

I have presented a series of observations on macromolecular interactions which occur during the terminal stages of lens cell differentiation. These are summarized in Fig. 2. Other cell types that undergo similar changes are the erythrocyte and skin cells (epidermis) during the process of keratinization. These other cells are also involved in the synthesis of highly specific proteins, and there are indications that molecular alterations similar to those described for the lens may also occur in these cells (*26*). Thus, elucidation of a specific series of macromolecular interactions such as those described may provide a basis for the biochemical definition of the terminal stages of cellular differentiation. Differentiation of the reticulocyte, for example, involves inactivation of the nucleus, stabilization of mRNA, and possibly a ribosomal breakdown such as I have described here (*26*). Furthermore, elucidation of the mechanisms of reactions involving the initiation of tissue-specific protein synthesis and subsequent nuclear inactivation, stabilization of mRNA, and breakdown of the ribosomes may provide a basis for defining the mechanisms of terminal cellular differentiation.

The lens cell has reached its highest form of cellular differentiation when it has formed the fiber cell. With respect to the mechanism of lens fiber cell formation, we would like to know whether specific biochemical changes such as γ-crystallin synthesis are intimately linked to fiber cell formation—that is, whether γ-crystallins are required to bring about the formation of a fiber cell. The potential for synthesizing γ-crystallins is inherent in the genome of the cell. This part of the genome is nonfunctional in the epithelial cell. Can these genes be activated without bringing about a simultaneous cellular elongation, nuclear inactivation and loss of cellular replication, stabilization of mRNA, and breakdown of the ribosomes? The degree of coupling or uncoupling of tissue-specific-protein synthesis to morphogenesis is an important part of the mechanism of cellular differentiation. We feel that we have now reached the stage where we can begin to answer these questions.

References and Notes

1. C. Hanna and J. E. O'Brien, *Arch. Ophthalmol.* **66**, 103 (1961); E. Cotlier, *ibid.* **68**, 801 (1962); A. G. Mikulicich and R. W. Young,

Invest. Ophthalmol. **2**, 344 (1963); B. D. Srinivasan and C. V. Harding, *ibid.* **4**, 452 (1965).
2. D. G. Cogan, *Exp. Eye Res.* **1**, 291 (1962).
3. M. Hertl, *Z. Zellforsch. Mikroskop. Anat. Abt. Histochem.* **43**, 228 (1955).
4. G. Eguchi, *Embryologia* **8**, 247 (1964); S. Karasaki, *J. Ultrastructure Res.* **11**, 246 (1964).
5. C. Hanna, *Invest. Ophthalmol.* **4**, 480 (1965); R. Reeder and E. Bell, *Science* **150**, 71 (1965).
6. J. Papaconstantinou, *Amer. Zool.* **4**, 279 (1964); *Biochim, Biophys. Acta* **107**, 81 (1965).
7. C. Takata, J. F. Albright, T. Yamada, *Science* **147**, 1299 (1965).
8. C. T. Mörner, *Z. Physiol. Chem.* **18**, 16 (1894); A. C. Woods and E. L. Burkey, *J. Amer. Med. Ass.* **89**, 102 (1927); E. L. Burky and A. C. Woods, *A.M.A. Arch. Ophthalmol.* **57**, 41 (1928); G. Viollier, H. Lobhart, H. Sullmann, *Helv. Physiol. Pharmacol. Acta* **5**, C10 (1947); H. Sullmann and G. Viollier, *ibid.* **6**, C66 (1948).
9. J. Papaconstantinou, R. A. Resnik, E. Saito, *Biochim. Biophys. Acta* **60**, 205 (1962).
10. A. Spector, *ibid.* **39**, 191 (1960); *Invest. Ophthalmol.* **4**, 579 (1965).
11. J. A. Stewart and J. Papaconstantinou, *Federation Proc.* **24**, 667 (1965); *Biochim. Biophys. Acta* **121**, 69 (1966).
12. N. O. Kaplan and M. M. Ciotti, *Ann. N.Y. Acad. Sci.* **94**, 701 (1961); R. D. Cahn, N. O. Kaplan, L. Levine, E. Zwilling, *Science* **136**, 962 (1962); C. L. Markert and H. Ursprung, *Develop. Biol.* **5**, 363 (1962); D. T. Lindsay, *J. Exp. Zool.* **152**, 75 (1963); D. M. Dawson, T. L. Goodfriend, N. O. Kaplan, *Science* **143**, 929 (1964); W. E. Nance, A. Clafin, O. Smithies, *ibid.* **142**, 1075 (1963).
13. C. L. Markert, *Science* **140**, 1329 (1963).
14. C. R. Shaw and E. Barto, *Proc. Nat. Acad. Sci. U.S.* **50**, 211 (1963).
15. E. S. Vesell, *Science* **150**, 1735 (1965).
16. ——— and A. G. Bearn, *J. Gen. Physiol.* **45**, 553 (1962); ———, *Proc. Soc. Exp. Biol. Med.* **111**, 100 (1962); W. H. Starkweather, L. Cousineau, H. K. Schock, C. J. Zarafonetis, *Blood* **26**, 63 (1965).
17. J. Bishop and R. Schweet, *Biochim. Biophys. Acta* **49**, 235 (1961). F. Wilt, *J. Mol. Biol.* **12**, 331 (1965).
18. T. Humphreys, S. Pennman, E. Bell, *Biochem. Biophys. Res. Commun.* **17**, 618 (1964).
19. J. Papaconstantinou, P. V. Koehn, J. A. Stewart, *Amer. Zool.* **4**, 321 (1964); paper presented at the 148th meeting of the American Chemical Society, 1964; J. Papaconstantinou, J. A. Stewart, P. V. Koehn, *Biochim. Biophys. Acta* **114**, 428 (1966).
20. R. B. Scott and E. Bell, *Science* **145**, 711 (196→ *ibid.* **147**, 405 (1965).
21. N. K. Wessels, *Develop. Biol.* **9**, 92 (1964); → *J. Cell Biol.* **20**, 415 (1964); *J. Exp. Zool.* **157**, 139 (1964).
→ C. Levinthal, A. Kenyon, A. Higa, *Proc. Nat. Acad. Sci. U.S.* **48**, 1631 (1962).
23. J. A. Stewart and J. Papaconstantinou, unpublished observations.
24. J. D. Mandell and A. D. Hershey, *Anal. Biochem.* **1**, 66 (1960); N. Sueoka and T. Cheng, *J. Mol. Biol.* **4**, 161 (1962).
25. S. Natori, R. Nozawa, D. Mizuno, *Biochim. Biophys. Acta* **114**, 245 (1966).
26. J. F. Bertles and W. S. Beck, *J. Biol. Chem.* **237**, 3770 (196→ J. A. Grasso, H. Swift, G. A. Ackerman, *J. Cell Biol.* **14**, 235 (1962); I. L. Cameron and D. M. Prescott, *Exp. Cell Res.* **30**, 609 (196→ J. A. Grasso, J. W. Wodward, H. Swift, *Proc. Nat. Acad. Sci. U.S.* **50**, 134 (1963); P. A. Marks, R. A. Rifkind, D. Danon, *ibid.*, p. 3→ *J. Cell Biol.* **22**, 599 (1964).
27. This research project was supported by National Science Foundation grant No. CRMS-185 and by U.S. Public Health Service grant No. NB-04455-02.

Mass Drug Catastrophes and the Roles of Science and Technology

Walter Modell

Drug disaster—qua drugs—attracted little attention until very recently despite the fact that no drug catastrophes of modern times compare even remotely with those of the past, probably because we have come to expect only good from drugs.

The roles of science and technology in the causation, control, and prevention of poisoning from the new drugs can best be developed against the background of the history of mass poisoning and drug catastrophe. A logical, meaningful definition of "drug" is essential to such an examination if one is to establish the relative importance of different kinds of mass poisonings and chemical catastrophes. In a purely biologic sense and as the pharmacologist views it, any substance that by its chemical nature alters structure or function in the living organism is a drug. Drug action is therefore a general biologic phenomenon; usefulness in disease and adverse effect are merely results of pharmacologic action. Pharmacologic effects are exerted by foods, vitamins, hormones, microbial metabolites, plants, snake venoms, stings, products of decay, air pollutants, pesticides, minerals, synthetic chemicals, virtually all foreign materials (very few are completely inert), and many materials normally in the body.

Early man knew much more about poisons than about drugs with therapeutic value (*1*). Even later, although Hippocrates saw little use for drugs in therapy, when they wanted to dispose of Socrates, the Greeks had an herb for it. Toxicology paved the way to pharmacology. If the surgeon can be said to have been fathered by the barber, then the modern pharmacotherapist is the direct descendant of the Borgias. It seems only yesterday (perhaps it was less than 30 years ago) that strychnine was an important drug in every physician's pharmacopoeia; today it is archaic, not because it is poisonous but because it has no demonstrable medical use.

The ancients knew nothing of modern approaches, nor did they have the understanding that can turn poisons, like curare, into agents of therapeutic value. They learned by accident alone, and, their methods of observation being limited, the effects that were first attributed to drugs were more likely to be adverse than therapeutic. Man learned early that the wild parsnip caused quick death, and bites of certain snakes, a more lingering one; that the sting of certain insects caused local or even serious systemic reactions; that certain fish were not "seafood"; and that toadstools were not for eating. He knew of fishberries, strychnine, hemlock, and curare. Cleopatra's testing of the poison of her asp on her slaves before she applied it to herself is typical of the pharmacological experiments of the time (*1*).

Poisoning, accidental and deliberate, was well known in peace and war. The environment had a full complement of potent poisons with which man had to learn to deal, along with wind, water, heat, cold, and famine, in order to survive. Poisoning from strange foods and foods from strangers' kitchens was a common danger for the wealthy and those in power; the food taster or tester was the equivalent of the "informed" subject of a modern acute experiment in clinical pharmacology. Even as late as the 17th century there was more exact knowledge of and concern with poisons than with medicinal effects of drugs.

Today, poisoning is uncommon; the physician no longer tends to think of it in making a differential diagnosis involving even the most bizarre symptoms. Poisoning, innocent or homici-

The author is in the department of pharmacology of Cornell University Medical College, 1300 York Avenue, New York 10021. The article is based on his report to the Committee on Science in the Promotion of Human Welfare, AAAS.