Meetings

Lens Differentiation

"What are the events, molecular and cellular, which take place and what are the regulating mechanisms that are operating during the change from relatively undetermined embryonic ectodermal cells or from differentiated, pigmented, epithelial cells into a growing mass of morphologically and chemically differentiated lens fibers?" Answers to these questions were sought at the second conference on lens differentiation, held at West Virginia University, Morgantown, West Virginia, 3–4 May 1966. Representatives of several scientific disciplines attended — experimental morphologists, cell biologists, biochemists, immunochemists, and ophthalmologists.

In a discussion of the experimental analysis of morphogenesis during embryonic lens development, Izumi Kawakami (Kyushu University, Fukuoka, Japan, and the University of Michigan) described his experiments on amphibian embryos. He was able to demonstrate the action of the cephalic mesoderm (prechordal plate and prechordal mesoderm) in the induction of nose and lens from the surface ectoderm, an event which precedes the similar action of forebrain and optic vesicle. Cephalic notochord had an ear-inducing effect acting before that of the hindbrain. There was a rostralization of these inductive capacities of head mesoderm following heat treatments or culture of the univaginated mesoderm. Since this parallels similar changes in the neural-inducing capacity of these tissues, Kawakami feels that the action of the mesoderm may be similar in both neural and sensory organ induction. Jane Oppenheimer (Bryn Mawr College) reported on her analysis of eye development following the grafting of fragments of the *Fundulus* embryonic shield in several hundred cases. These eyes either developed from the graft or were evoked by the graft from the host. Optic cups with lenses, optic cups alone, and lenses or lentoids alone were observed. These last-mentioned "free" lenses were found in both embryonic and extraembryonic regions and were often associated with entoderm, mesoderm and brain, or brain only. Preliminary experiments also indicated that prospective lens ectoderm can form a small lens when transplanted into the pericardial cavity alone but a larger lens when the optic vesicle is included. Therefore, in teleosts as well as in amphibia, it would appear that lens induction comprises both a preneural phase in which entoderm and mesoderm are the inductors and a neural phase in which the optic vesicle is the source of the stimulus for lens formation. So-called "free" lenses are formed under the influence of the former inductors in the absence of the eyecup.

Alfred Coulombre (National Institutes of Health) described his and John Everly's studies on the growth in size of the lens of the embryonic chick in different experimental situations. The volume of the lens is due mainly to the size and number of lens fibers present. New lens fibers are continually being added at the equatorial zone, mitoses being confined to the cells of the lens epithelium. The number of fibers was estimated in equatorial sections from the number of radial columns and the number of fibers per column. During development of the chick eye, there is an exponential increase in the volume of the lens. Up to 10 days of incubation, new lens fibers are laid down rapidly both in new columns and as additions to the old columns. After 10 days, no new columns are formed, the new lens fibers being added at a decreasing rate to preexisting columns. Intubated eyes (accumulation of vitreous humor and increase in intraocular pressure prevented by a glass tube inserted into the vitreous chamber) stop growing except for the neural retina, which becomes folded, and the lens, which is of normal size. However, removal of neural retina causes an arrest in lens growth. When lens epithelium and capsule are cultured in vitro in defined media, there is little mitotic activity or change in appearance of these cells. This epithelium can still form a lens if returned to the host eye. Addition of proteins from several sources to the culture soon after explantation results in the elongation of the epithelial cells and increases protein synthesis by these cells.

Of great interest were the macromolecular events associated with the differentiation of an epithelial cell into a fiber cell. Papers were devoted primarily to DNA, RNA, and protein synthesis in the lens epithelial cell and fiber cell. Through the use of sucrose density gradients, autoradiography, electrophoresis, and immunofluorescence, much information on the macromolecular events associated with fiber cell formation was obtained.

By means of autoradiographic techniques, Calvin Hanna (University of Arkansas) studied DNA synthesis and the cell cycle in the epithelial cells of the mouse lens 3 days and 12 days after birth. In the 3-day-old mouse lens, most of the cells throughout the epithelial cell layer undergo DNA synthesis, whereas at 12 days only the cells of the germinative region show DNA synthesis. Thus, the epithelial cells of the central region have entered into the stationary or G_0 phase. The time for the cell cycle in mice has been calculated to be 56 hours, the G_1 phase taking up three-quarters of this time. Hanna was also able to calculate that the length of time required for a cell to migrate from the germinative region and to differentiate into an elongated fiber cell is 6 months.

Hanna correlated the data described previously with the effect of x-irradiation on the rate of formation of the lens fibers and the rate of replication of the epithelial cells, in an attempt to further understand the process of cataract formation. As a result of x-irradiation, the migration of the epithelial cells from the germinative region to the fiber cell region (which normally takes 6 months at this age) is slowed down, thus resulting in a slower rate of formation of fiber cells. In addition, exposure to x-irradiation induces the cells in the germinative region to undergo more rapid mitosis followed, however,

by increased cell death. Avian (chick) and reptilian lenses were found not to develop cataracts following x-irradiation. Hanna calculated that the length of time required for the cells of the chick lens annular pad to develop into a cortex fiber cell is 2 years.

Experiments designed to study the synthesis of various classes of RNA soon after embryonic induction of the chick lens were reported by Arthur Katoh and Maxwell Braverman (Argonne National Laboratory and Allegheny General Hospital, Pittsburgh). The optic vesicle and head ectoderm *anlagen* (including prospective lens) were removed from 10 to 12 somite (stage 10) chick embryos and placed in organ culture for 4 days. These parts showed no precipitin reaction with antiserum to adult lens. After 4 days in culture, lenses were formed and a positive precipitin reaction was obtained from these organ cultures, thus indicating that lens fiber differentiation had occurred.

Using this culture system, Katoh and Braverman studied the effect of actinomycin D on lens formation. It was found that 1 μg/ml of the antibiotic was extremely toxic to the lens cells and that treatment with concentrations of actinomycin D greater than 0.1 μg/ml for longer than 1 hour would kill the explants. If early *anlagen* were incubated with actinomycin D (0.1 μg/ml) for 1 hour, and then placed into culture for 4 days, no lenses were formed and no positive precipitin reactions were obtained. Using 0.05 μg/ml of the antibiotic for the same length of time resulted in some lens formation. Studies with uridine-H^3 showed a significant but not complete inhibition of RNA synthesis under treatments with actinomycin D which inhibited lens formation. These data indicate that the initiation of the formation of lens cells requires an actinomycin-sensitive reaction which may be m-RNA synthesis.

Hanna reported on the differential rate of protein and nucleic acid synthesis in lenses of the mouse and the chick. RNA synthesis in the mouse lens was found to be most active at the equatorial zone and in new fiber cells. The cortex fiber cells showed only a small degree of incorporation of uridine-H^3, whereas the nucleus fiber cells showed no turnover at all. Similar experiments on the chick lens (13- to 17-day-old embryo) showed that the heaviest RNA synthesis occurred in the outer cortex region. In addition, he calculated that the m-RNA in the lens cell lasts for 12 hours, which is considered to be a long-lived or stable template. With respect to protein synthesis in the lens cells, Hanna showed that the degree of incorporation of amino acid into protein followed the same trend as was observed for the incorporation of uridine (mouse).

In addition to the detailed studies on the differential rate of protein synthesis in the various regions of the lens, qualitative differences in specific lens proteins and their occurrence during the development of lens cells were also considered. Immunofluorescence data of Johan Zwaan and Akira Ikeda (University of Virginia) show that there is a unique structural protein of the chick lens which is the first tissue specific protein to appear during lens development. This protein, which probably corresponds to FISC of Rabaey, has been called delta-crystallin, and is the first specific antigen to appear in the lens placode of a 23-somite embryo (stage 14). Later, at stage 35, this protein can no longer be detected in the layer of epithelial cells. In addition, it has been shown that alpha-crystallin synthesis is initiated in the fiber cells of a 3- to 3½-day-old chick embryo, and spreads gradually to appear after several more days in the epithelial cells. At 8 to 10 days, the epithelial cells and cells of the equatorial zone show a strong fluorescence with antisera to alpha-crystallin whereas that of the fiber cells becomes progressively weaker. Beta-crystallins did not appear until after 7 days of incubation. Thus, the immunofluorescence data have shown the presence of a unique structural protein of the early embryonic lens, delta-crystallin. Alpha and beta crystallins appear much later in lens growth. No crystallins were detected in other ocular tissues or in the prospective lens cells before the placode stage. These data very strongly indicate, therefore, that there is a continuous regulation of synthesis of specific proteins during the growth and differentiation of cells in the lens.

Similar immunofluorescent studies were reported by José Maria Genis-Galvez (University of Salamanca, Spain, and Wayne State University, Detroit). Results of these studies were essentially the same as those reported by Ikeda and Zwaan. In addition, Genis-Galvez also presented his studies on lactate dehydrogenase and esterase isozymes in the various regions of the chick lens. Using the whole lens, all 5 LDH's had uniform activity whereas, using the cortex fiber cells alone, LDH's 1, 2, and 3 were found to have the greatest activity. Similarly the activity of esterases was found to vary with the growth of the lens.

Detailed comparative studies of the LDH isozymes in chick lens and cornea were discussed by Harry Maisel (Wayne State University). In the lens, LDH-1 was found to be most active whereas, in the cornea, LDH-5 was predominant. During embryonic development, the differentiation of corneal isozymes was exactly the opposite of that observed in the lens. Within the lens of the hatched chick, LDH-5 was most active in the epithelial cells and annular pad while LDH-1 was most active in the cortex fiber cells. Studies on lenses and corneas of cattle showed the same trend as did the chick tissues. On the other hand, lens cells of the rabbit were found to have greater LDH-5 activity, whereas the cornea had greater LDH-1 activity. This is a complete reversal of LDH activity from that seen in lenses from chicks and cattle. There is at present no explanation for the mechanism of regulation of these isozymes.

In general, the data strongly indicate that there are many stages of differentiation of lens cells which involve the regulation of DNA, all classes of RNA, and protein synthesis. Knowledge of the mechanisms of these regulations may increase our understanding of differential gene action and its role in morphogenesis.

Reports were also made on the regeneration of the lens from the iris in adult *Triturus*. Randall Reyer (West Virginia University) described the results of experiments on adult *Triturus viridescens* in which radioactive thymidine-H^3 was used as a label to trace cell movements during lens regeneration and to demonstrate the time and place of DNA synthesis. In most of the experiments, the dose used was 1.25 μc per injection, 3 or 4 injections (i.p.) being given 1 hour apart. The injections were made at intervals between 1 and 20 days after bilateral lentectomy, and the animals were sacrificed for autoradiography between 3 hours and 25 days after injection. Synthesis of DNA was first observed in the slightly swollen cells of the inner lamina of the iris epithelium,

3 and 5 days after lens removal, at regeneration stage 1. At regeneration stage 3, 10 days after lens extirpation, DNA synthesis was taking place throughout the inner iris epithelium and was most active in the depigmenting cells at the pupillary margin of the dorsal iris. The cells labeled at this time were later found in the epithelium of the lens and in both primary and secondary lens fibers. When the lens fibers began to differentiate at stage 7, DNA synthesis ceased in these cells but continued in the entire epithelium at this time and through stages 9 and 10. Cells labeled at the latter stages became secondary lens fibers after further growth of the lens. Once the visible differentiation of a fiber had begun, no DNA synthesis was observed in this cell.

Reyer pointed out that his results confirmed those reported by Eisenberg and Yamada at the December 1965 AAAS meetings, and discussed the significance of Yamada's evidence that DNA and gamma crystallins are not synthesized simultaneously.

Tuneo Yamada (Oak Ridge National Laboratory) reported on the work by Jurand, himself, and Reese on sequential changes in the pattern of ribosomes during regeneration of the lens. He began by pointing out that, during regeneration, cells become activated for protein synthesis, and that this begins before depigmentation of the dorsal iris is completed. He emphasized that immunofluorescence studies indicated that gamma-crystallin appears only during the terminal phase of the cell cycle, after the cells have stopped dividing.

During the depigmentation phase, the ribosomes in the cytoplasm, as studied by electron microscopy, are mostly single. Later, when the cells are multiplying, the ribosomes are found in clusters. A wide range of cluster size appears. When fiber differentiation is under way, the majority of the ribosomes are in middle-size clusters. In older fibers the clusters are again fewer in number. Yamada called attention to the fact that Papaconstantinou had shown by other methods that ribosomal RNA changes during the later stages of fiber differentiation. As the fibers form, a fine fibrous component, probably corresponding to lens structural proteins, appears in the cytoplasm.

Yamada also described changes in the nucleus that may be correlated with the appearance of gamma-crystallin. Fibrous (chromatic) areas could be distinguished from interchromatic space in the nucleus. During depigmentation the nuclei enlarge and the interchromatic space expands. In differentiating lens fibers a fine fibrous component appears in the interchromatic space of the nucleus together with fine granules which are larger than ribosomes. Yamada pointed out that these changes are correlated in time with the appearance of gamma crystallin as determined by immunofluorescence studies. These studies revealed the possibility that gamma crystallins may be synthesized in the nucleus as well as in the cytoplasm. As a bonus, Yamada showed some photographs demonstrating that, during the fiber-differentiation phase, mitochondria are extruded bodily into the intercellular spaces, where they change their structure.

Finally, D. H. Reese (Oak Ridge National Laboratory) reported on his attempts, made in collaboration with Yamada, to develop an in vitro system that would permit the study of lens regeneration. When iris and neural retina were isolated, with or without cornea, no lens regeneration occurred; the iris cells did not even depigment. The investigators therefore turned to cultures of the whole eye. They studied first the ability of the whole eye to continue regeneration already begun. Waymouth's MB 252/1 was the medium of choice, and results were better in cultures maintained at 22°C than in those kept at 18°C. In eyes maintained in vivo 2 days after lentectomy and then placed in the medium, depigmentation occurred. It also occurred in eyes, placed in culture immediately after lentectomy, after about 9 to 10 days in vitro, being complete after 15 days. Beginning, intermediate, and terminal phases in the formation of primary fibers were seen after 15 to 20 days. Sato stage 7 was attained after about 21 days in vitro. Formation of secondary fibers may occur at about 25 days, the longest period of culture. The neural retina within the whole eye was in fairly good condition after 25 days in vitro, although some degeneration of rods and cones was evident in folded areas. Both the pigmented retina and the iris underwent some transformation by this time. In the case of the pigmented retina, depigmentation began in some cells at about 21 days, the nuclei of the depigmented cells enlarged, and mitoses occurred. In the cultured eyes, the space between the neural retina and the cornea was diminished. The resulting compression caused some distortion.

One of the fascinating aspects of the differentiation of the lens is the close correlation between cellular synthetic activities and morphological differentiation. William Zapisek and John Papaconstantinou (University of Connecticut and Oak Ridge National Laboratory) followed the pattern of RNA during the growth of the embryonic chick lens into the adult lens, using the methylated albumin (MAK) column and the sucrose density gradient for fractionating phenol-extracted nucleic acids. The relative amount of ribosomal RNA decreases with aging. A breakdown of ribosomal RNA associated with fiber differentiation was suggested. The product of ribosomal breakdown has MAK column properties similar to soluble RNA. After pulse-labeling RNA in the epithelial cells of the calf lens with uridine C^{14} and fractionating the phenol-extracted RNA on the methylated albumin column, Papaconstantinou, Emilia Julku, and Dorothy Schenk (University of Connecticut and Oak Ridge National Laboratory) demonstrated a single radioactive fraction. This fraction revealed a high percentage of "hybridability" with calf thymus DNA; it was suggested that it contained messenger RNA's. From the fiber cells they also obtained a similar material located at the same position in the MAK column fraction.

Ronald Reeder (Massachusetts Institute of Technology) reported on protein synthesis in the lens of the chick embryo at from 12 to 21 days of incubation. After in vitro pulse-labeling with radioactive C^{14} amino acids, the lenses were dissected into anterior epithelium, annulus, and body of lens fibers. Soluble proteins were extracted from homogenates of each of these parts and electrophoresed on urea-acrylamide gels. The radioactive proteins were located by autoradiography. Reeder found that a number of different proteins are synthesized in the anterior epithelium while synthesis in the body of the lens fibers is restricted to two major proteins. Treatment of an organ culture of the whole lens with a large dose of actinomycin D resulted in a fall in the synthesis of the protein specific for the epithelium but did not affect protein synthesis in the lens fibers for at least 6 hours. In their second paper, Zwaan

and Ikeda investigated the proteins in the lens of several species representing fish, amphibians, reptiles, birds, and mammals by the techniques of immunoelectrophoresis, immunodiffusion, and a two-dimensional polyacrylamide gel electrophoresis. They found that the crystallins are specific for the lens. Alpha, beta, and gamma crystallins are present in fish, amphibians, and mammals while alpha, beta and delta crystallins are characteristic of reptile and bird lenses. The most complex of these are the beta crystallins which, in the chick, comprise eight or nine proteins of similar size and antigenic properties but with different electrophoretic mobilities.

Alexander Kenny and G. Noel Currie (West Virginia University) reported on calcium and phosphorous metabolism in the rat lens. It had previously been established that calcium concentration increases in lenses with either spontaneously occurring or experimentally produced cataracts. This shift in calcium is counterbalanced by a drop in phosphorous, and is accompanied by a decrease in dry weight and an increase in water content in the lens. Earlier workers had suggested that the parathyroid glands were involved in the regulation of lens calcium in the dog, chronic thyroparathyroidectomy resulting in an increase in lens calcium. Kenny and Currie failed to confirm this finding in the rat. However, they were able to show that a chronic respiratory infection in rats caused fluctuations in the normal levels of calcium and phosphorous in the lens. When rats were treated with a long-acting penicillin preparation, it was possible to stabilize the levels of these elements in the lens at normal values. Under these conditions, neither parathyroidectomy nor thyroparathyroidectomy resulted in any change in calcium or phosphorous levels in the lens.

Edward Cotlier and Jean Fox (Washington University) described the isolation of rubella virus from the lens of human congenital rubella syndromes. Congenital cataracts were among the many anomalies noted in infants born to mothers who had been infected with rubella virus during the first trimester of pregnancy. Tissue culture and Echo-11 interference techniques were used to demonstrate that the rubella virus was recoverable from the lens and aqueous humor of children as old as 9 to 10 months of age. From the times at which the mothers of affected children were known to have contracted the virus, it is possible to conclude that the virus particle must have traversed the lens capsule in order to infect the lens. Experimental infection of pregnant rats and hamsters with human rubella virus resulted in a wide range of congenital anomalies, including cataracts, among the offspring.

One of the most important considerations in the analysis of the cell population dynamics of the vertebrate lens is the factor, or complex of factors, which controls mitotic activity in the epithelium of this structure. Two of the papers in the last session of the conference directed attention to this aspect of lens development. Nancy Rafferty (Johns Hopkins University) reported on injury-associated changes in the epithelium of the frog lens. This investigator confirmed her earlier findings that injury to the epithelium results in a marked increase in its proliferative rate. Studies of cell density were coupled with autoradiography of three regions at different distances from the center of the epithelium. By studying changes in these three regions as a function of time following injury to the center of the lens epithelium, it was possible to show that injury to the epithelium shortens the G_2 from 6 hours to 2½ or 3 hours. DNA synthesis and the ensuing cell divisions begin in the cells of the proliferative zone near the equator following injury to the center of the epithelium. DNA synthetic activity and mitosis sweep centrally toward the wound as time elapses. This observation indicates either that the stimulated cells of the proliferative zone actually migrate toward the wound as they continue to proliferate, or else that the injury stimulus proceeds from the proliferative zone toward the wound, stimulating new cells along the way to divide.

Clifford Harding, W. L. Wilson, and Jean Wilson (Oakland University) also considered the control of mitosis in the lens epithelium. They have demonstrated a large and rapid increase in mitotic activity in explanted lenses of the rabbit which are exposed to serum. This increase in mitotic activity is preceded by a latent period. Dialysis of the serum yielded two fractions, neither of which was capable of stimulating mitotic activity in the epithelium of the rabbit lens. Recombining these

fractions reconstituted the mitogenic activity of the serum.

The last item in the conference was in marked contrast to the preceding biochemical discussions. This was a demonstration by T. W. Williams (West Virginia University) of stereophotomicrographs of the major structures of the primate eye and its adnexa. Many of these were of specially injected preparations which showed the major vessels of the eye. Others demonstrated the iris, the ciliary body, and the relations of the fibers of the ciliary zonule to ciliary body and lens.

The several themes represented by the different papers presented in this conference are examples of the diverse approaches used in the study of the developing vertebrate lens. It is an indication of the speed with which this field is moving that, even after the passage of only 2 years between the first and the second conference, very little in the second conference was a repetition of anything presented in the first. This conference was supported by grant (No. GB-4845) from the National Science Foundation. The participants would like to acknowledge this support with thanks.

RANDALL W. REYER
Department of Anatomy,
West Virginia University
ALFRED J. COULOMBRE
National Institutes of Health,
Bethesda, Maryland
TUNEO YAMADA
JOHN PAPACONSTANTINOU
Biology Division,
Oak Ridge National Laboratory,
Oak Ridge, Tennessee

Forthcoming Events

January

20–21. **Blood**, 15th symp., Wayne State Univ., Detroit, Mich. (W. H. Seegers, Dept. of Physiology, Wayne State Univ. School of Medicine, Detroit 48207)

20–2. International College of **Surgeons**, 3rd Caribbean surgical congr. and cruise. (S. E. Henwood, 1516 Lake Shore Dr., Chicago, Ill. 60610)

22–3. **Electron Microscopy**, workshop, Northeastern Univ., Boston, Mass. (M. D. Maser, Millard Fillmore Hospital, 3 Gates Circle, Buffalo, N.Y. 14209)

23–24. **Avionics**, symp., Montreal, Canada. (Secretary, Canadian Aeronautics and Space Inst., 77 Metcalfe St., Ottawa, Ont.)

23–24. **Coupled Reactor Kinetics**, natl. mtg., Texas A&M Univ., College Station. (C. G. Chezem, Dept. of Nuclear Engineering, Texas A&M Univ., College Station 77843)