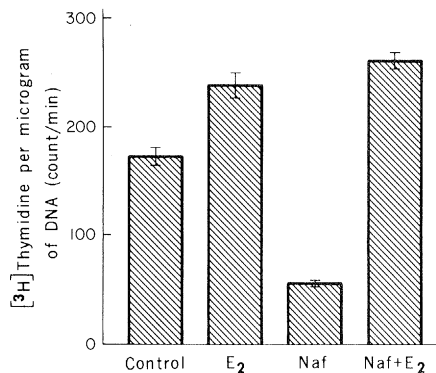


Fig. 1. Effects of estrogen ( $E_2$ ) and antiestrogen (Naf) on DNA synthesis in MCF-7 cells. These cells were grown in 5 percent  $CO_2$  in air at  $37^\circ C$  in medium consisting of Earle's basal minimal essential medium (MEM; Gibco), supplemented with nonessential amino acids (Gibco), 2 mM L-glutamine (Gibco), 0.006  $\mu g$  of insulin (Sigma) per milliliter, 5 percent calf serum (Gibco), and 50  $\mu g$  of gentamicin (Schering) per milliliter. Before confluence, the medium was changed to one containing 2 percent fetal calf serum (Gibco) which had been stripped by shaking for 30 minutes at  $45^\circ C$  with the pellet from two volumes of dextran-coated charcoal (6). Some flasks received media that also contained  $10^{-8} M$  estradiol-17 $\beta$  or  $5 \times 10^{-7} M$  Nafoxidine, or both. After 48 hours, the medium was replaced by 5 ml of MEM containing 0.5  $\mu g$  of [ $^3H$ ]thymidine and 10 mM Hepes, pH 7.4. The cells were incubated an additional 30 minutes, then rinsed with chilled MEM containing 10  $\mu g$  of unlabeled thymidine per milliliter, scraped from the plastic with the use of perforated cellophane and 1M perchloric acid, and washed three times with chilled 0.5M perchloric acid. The pellet was hydrolyzed in 0.5M perchloric acid at  $90^\circ C$  for 15 minutes. Samples were counted for radioactivity and others were assayed for DNA by the method of Burton (8). Bars represent mean  $\pm$  standard error of four culture flasks.



of their hormones. The presence of uncharged estrogen receptor in MCF-7 nuclei may explain in part the nuclear binding of estradiol seen by Brooks *et al.* (2), who suggested rather that cytoplasmic receptor exposed to estradiol at  $0^\circ C$  might have translocated into the nucleus; the interpretation of these data at the time was obscured by apparently similar observations in normal porcine uterus.

In spite of the unusual distribution of uncharged estrogen receptor which we have found in MCF-7 cells, the cytoplasmic receptor remains capable of translocating estradiol into the cell nucleus. When estradiol was added to the medium, the remaining receptor disappeared from the cytoplasm and appeared as a receptor-estradiol complex in the nucleus (Table 2). Furthermore, the previously uncharged nuclear receptor also became fully charged with estradiol.

These findings suggest the possibility that the uncharged nuclear estrogen receptor is capable of stimulating the growth of MCF-7 cells. The slight further stimulation of thymidine uptake by estradiol would then be due to the resulting small increase in nuclear receptors translocated from the cytoplasm. If this interpretation is correct, then the binding of antiestrogen molecules must inactivate the receptor, while the reversal of the antiestrogen effect by estradiol results from the blockage of antiestrogen binding and subsequent reactivation of the receptor molecule.

We have discovered at least one other cell line from another human breast cancer to have a similar distribution of estrogen receptor. Antiestrogen therapy of human breast cancer is known to cause objective tumor remission in many cases, success being mostly correlated with the

presence of estrogen receptor in the patient's tumor (7). If the high levels of free estrogen receptor in nuclei are present in some patients with breast carcinoma, hormone-ablative therapy would probably be less successful since these nuclear receptors would be fully capable of activating cell division even in the absence of hormone. In fact, this phenome-

non may explain the resistance of a number of receptor-containing breast cancers to hormone-ablative therapy. If so, antiestrogen treatment in these special cases of high estrogen receptor in nuclei may very well succeed where other endocrine therapies would fail.

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12 November 1976

## Cyclic GMP Accumulation Causes Degeneration of Photoreceptor Cells: Simulation of an Inherited Disease

**Abstract.** Guanosine 3',5'-monophosphate (cyclic GMP) metabolism in developing eye rudiments of *Xenopus laevis* embryos in culture is disrupted by the phosphodiesterase inhibitor isobutylmethylxanthine. At low concentrations of inhibitor the rudiments develop normally, but at higher concentrations of the inhibitor, cyclic GMP accumulates in the rudiments and the retinal photoreceptor cells degenerate selectively. The isobutylmethylxanthine-induced photoreceptor degeneration is associated with an accumulation of cyclic GMP and, in this respect, it simulates an early biochemical defect in the inherited degenerative disease of rd mice.

Inherited degenerative diseases of the retina cause blindness in mice, rats, dogs, and humans. About 100,000 persons are afflicted with the degenerative disease retinitis pigmentosa in the United States alone (1). Morphological and biochemical data from frogs, cattle, and human retinas show that the rod photoreceptor cells of vertebrate species are very similar. Human photoreceptor cells that degenerate from disease may have metabolic defects which are common to those of animal disorders, and animal studies may be helpful in understanding and developing treatment for human diseases causing blindness. Following this rationale, we are using animal models to describe and analyze the metabolic characteristics of normal visual cells and the causes of pho-

toreceptor degeneration that result from inherited or acquired diseases.

We advanced the hypothesis that an abnormality in guanosine 3',5'-monophosphate (cyclic GMP) metabolism leads to degeneration and death of retinal photoreceptor cells (2). Supporting data came from studies of isolated rod outer segments from normal retinas and from our investigations of inherited retinal disorders of rats and mice. Dark-adapted rod outer segments from normal retinas contain high concentrations of cyclic GMP which are reduced by exposure to light (3). These observations suggest the possible involvement of cyclic GMP in dark-light adaptation or in the visual process (4). In the Royal College of Surgeons (RCS) strain of rats, which are afflicted

with retinal dystrophy, an imbalance in the renewal of photoreceptor outer segments leads to the buildup of membranous debris, a component of which apparently disrupts metabolism of cyclic GMP in visual cells by a feedback mechanism (5). A few days after debris starts to accumulate, cyclic GMP levels become altered and the RCS photoreceptor cells begin to degenerate. In mice (C3H/HeJ or C57BL/6J *rd le*) that are homozygous for the *rd* (retinal degeneration) gene, photoreceptor cells degenerate in the second and third postnatal week because of a deficiency in cyclic GMP phosphodiesterase activity (6). As a consequence of the deficiency, the content of cyclic GMP in *rd* photoreceptor cells increases, reaching four to five times the normal level during the period of photoreceptor degeneration. We now report observations in the toad (*Xenopus laevis*) retina which support the hypothesis that a disruption in cyclic GMP metabolism can cause photoreceptor cell degeneration.

We tested the validity of this hypothesis in the normal retina by creating a drug-induced abnormality in cyclic GMP metabolism and evaluating the extent of photoreceptor cell degeneration. Eye rudiments from embryos (stage 31) of *X. laevis* were collected and grown for 1 to 3 days at 20°C, with or without isobutylmethylxanthine (IBMX), in hanging-drop culture (7). Isobutylmethylxanthine, an inhibitor of cyclic GMP phosphodiesterase, was added to the medium in concentrations ranging from  $10^{-6}$  to  $10^{-2}M$ . At the indicated intervals, rudiments were harvested; some were processed for morphological study and the rest were frozen for biochemical investigation.

To examine morphological degeneration by light and electron microscopy, eye rudiments were fixed for 1.5 hours with 1.8 percent glutaraldehyde and 1.0 percent  $OsO_4$  in 50 mM phosphate buffer (pH 7.3). After dehydration, the rudiments were embedded in Epon (7). To assess cyclic GMP content, frozen rudiments were lyophilized, stored at  $-70^{\circ}C$ , and extracted with 0.1N HCl; after lyophilization, the reconstituted extracts were serially diluted and assayed by the radioimmunoassay technique (8). The postincubation culture medium was lyophilized, reconstituted, and assayed directly for cyclic GMP without extraction.

Intracellular food reserves distributed among all cells of the amphibian embryo are sufficient to sustain development in vitro of retinal rudiments for several days in a simple salt solution (7). During the 3 days in culture, eyes without IBMX pass

through an orderly series of developmental changes which are similar to those observed in situ. Photoreceptor cells form a few rod outer segments in culture by day 1 (Fig. 1A); by day 3, outer segments containing more than 200 membrane disks are common (Fig. 1B). Rudiments cultured with less than  $10^{-5}M$  IBMX grow and develop normally (Fig. 1C). However, after 3 days of culture rudiments grown with  $10^{-5}M$  IBMX show slight disorganization of the rod outer segments without apparent alterations to

the inner segments or soma of the photoreceptor cell. When  $5 \times 10^{-4}M$  or greater concentrations of IBMX are added to the medium, outer segments are disorganized after day 1 (Fig. 1D). By day 2, the developing photoreceptors have elaborated more disorganized outer segment membranes and many of the cells have undergone a change in shape to assume a spherical configuration. Some of these spherical cells have been extruded from the outer retinal surface and are trapped along with their disorganized outer seg-

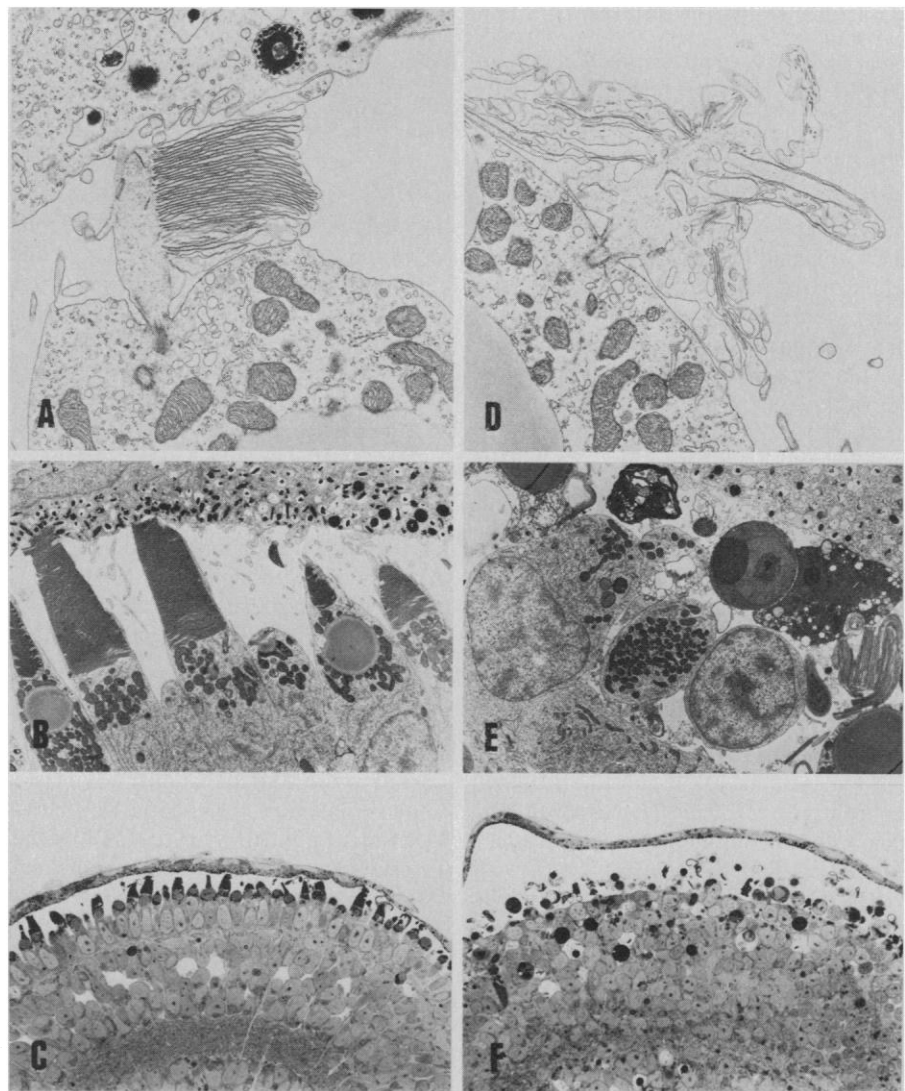
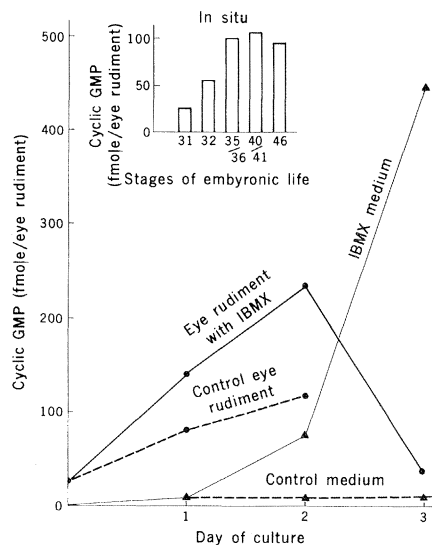


Fig. 1. Eye rudiments from stage 31 embryos of *Xenopus laevis* grown for 3 days in hanging-drop culture. (A) Photoreceptor with an outer segment containing approximately 35 well-oriented membrane disks in a control retina recovered after 1 day in culture ( $\times 11,200$ ). (B) Photoreceptors with outer segments composed of more than 200 membrane disks in control cultures after 3 days. The pigment epithelium, studded with dense melanosomes, is present at the upper border ( $\times 2260$ ). (C) Retina cultured for 3 days in the presence of  $9 \times 10^{-6}M$  IBMX. Well-developed photoreceptors are seen across the whole expanse of outer retina. ( $\times 300$ ). (D) Photoreceptor with highly disorganized outer segment membranes from a retina recovered after 1 day in culture with  $9 \times 10^{-4}M$  IBMX ( $\times 11,200$ ). (E) Photoreceptor debris between the outer border of the retina (lower left) and pigment epithelium (upper right) in a retina cultured for 3 days in the presence of  $9 \times 10^{-4}M$  IBMX ( $\times 2260$ ). (F) Retina cultured for 3 days in  $9 \times 10^{-4}M$  IBMX. Massive destruction of cells is seen in outer retina, with cellular debris accumulated between the retina and pigment epithelium. Only a few cells in the deeper retinal layers show degenerative changes ( $\times 300$ ). Culture of rudiments for 3 days with Squibb inhibitor SQ 65,442 at a concentration of 0.1 mM results in a similar loss of visual cells (micrographs not shown).

Fig. 2. Cyclic GMP content of eye rudiments and postincubation media displayed as a function of days in hanging-drop culture, with or without IBMX. After 1, 2, or 3 days of incubation, samples containing 20 to 40 eye rudiments cultured with or without  $9 \times 10^{-4}M$  IBMX were frozen immediately upon collection, lyophilized, and extracted with 0.1N HCl. The acid extracts were lyophilized, reconstituted with sodium acetate buffer (50 mM, pH 6.2), serially diluted, and assayed for cyclic GMP by the radioimmunoassay (8). Values for the serially diluted samples were averaged and expressed per eye rudiment. Assay variation within a single sample was approximately 10 percent. Each value represents the mean of two samples of eye rudiments cultured separately, with intersample variation not exceeding 10 percent. The postincubation medium (with or without IBMX) was collected, lyophilized, and, after reconstitution with sodium acetate buffer, assayed directly for cyclic GMP. In order to distinguish the influence of eye rudiment maturation on cyclic GMP content in situ, rudiments were collected and processed for cyclic GMP assay without culturing (inset).



ment membranes in the space between the retina and pigment epithelium. Most of these disorganized photoreceptors remain viable at this time and only occasional pycnotic cells are encountered. Within some of the extruded photoreceptors after 2 days of culture, mitochondria are swollen and the outer mitochondria membranes have tortuous, crenulated profiles. Other extruded photoreceptors contain mitochondria that are identical to those in control cultures. Altered mitochondria are more frequently encountered in rod photoreceptors than in cones. By day 3, most of the extruded photoreceptors are necrotic along with the few photoreceptors remaining in the outer retina (Fig. 1E). Cell death is selective for photoreceptors at concentrations of IBMX less than  $10^{-3}M$  (Fig. 1F). Higher concentrations of IBMX result in cell death throughout the inner retinal layers as well.

The effectiveness of IBMX as an inhibitor of cyclic GMP phosphodiesterase was evaluated by measuring cyclic GMP in control and treated rudiments. Without IBMX there is an increase in cyclic GMP content of the control rudiments during the culture period (Fig. 2) which is similar to that observed in situ (Fig. 2 inset). The increase in cyclic GMP content between embryonic stages 31 and 42 probably reflects the growth and development of photoreceptor outer segments, which are rich in cyclic GMP (3). In the presence of  $9 \times 10^{-4}M$  IBMX, cyclic GMP content in the eye rudiments increases about 80 percent above that of the control in day 1 of culture, and it becomes progressively greater than the control throughout day 2. During day 3, when photoreceptor cells are degenerating, the cyclic GMP content

in the eye rudiments decreases. The concentration of cyclic GMP in the incubation medium increases during day 2 and becomes very high by day 3 (Fig. 2). This indicates that the photoreceptor cells release cyclic GMP into the medium when they degenerate.

These data show that the addition of a cyclic GMP phosphodiesterase inhibitor (IBMX) to the culture medium results in elevation of cyclic GMP in the rudiments and disorganization and death of the retinal photoreceptor cells.

It is proposed that cyclic GMP plays a role in dark-light adaptation or in the visual process (4). If cyclic GMP is associated with such a basic function of rod photoreceptor cells, then it is possible that inherited or acquired diseases result from errors in cyclic GMP metabolism. We have shown that in photoreceptor degeneration induced by IBMX in toads or by the *rd* gene in mice, cyclic GMP metabolism is abnormal before the cells show morphological abnormalities. In both disorders, the disruption in cyclic GMP me-

tabolism results from a deficiency in cyclic GMP phosphodiesterase activity, and cyclic GMP content increases above normal before the photoreceptor cells degenerate. To our knowledge, this is the first simulation in normal retinas of an inherited retinal disease. Since both the IBMX-induced photoreceptor degeneration in toads and the gene-induced visual cell degeneration in *rd* mice are associated with a disruption in cyclic GMP metabolism, the etiology of these disorders may stem from the as yet undescribed role of cyclic GMP in the metabolism or function of the visual cells.

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## Chlamydiae (with Phages), Mycoplasmas, and Rickettsiae in Chesapeake Bay Bivalves

**Abstract.** *Intracytoplasmic chlamydia-like organisms, some with phages, rickettsia-like organisms, and mycoplasma-like organisms have been found in clams and oysters from the Chesapeake Bay area by electron microscopy. None of these organisms have been previously detected in mollusks, nor have phages been previously observed in Chlamydia sp.*

Amorphous, basophilic, finely granular intracytoplasmic inclusions in digestive tubule cells of both hard clams, *Mercentaria mercenaria* (Fig. 1A), and soft

clams, *Mya arenaria*, and in gut goblet cells of American oysters, *Crassostrea virginica*, from Chesapeake Bay or Chincoteague Bay, or both, were exam-