
Following radiotherapy for an extensive intraepithelial carcinoma of the conjunctiva and eyelid, a patient developed opacification of soft contact lenses used on the same side. Calcium was demonstrated within the contact lenses by cytochemical methods and by energy-dispersive x-ray microanalysis. The phenomenon was associated with unilateral tear insufficiency and an elevated tear calcium level as determined by atomic absorption spectrophotometry.

Since their introduction in 1960, soft contact lenses have been shown to be of therapeutic benefit in many ocular conditions including dry eyes and lid abnormalities. Complications from their use include corneal vascularization, infiltrates and infections, and the development of deposits on or in the lenses.1-2 This paper reports clouding of two soft contact lenses due to the deposition of calcium within them.

Case report. A 54-year-old patient was referred to Duke University Medical Center with a 4 year history of trichiasis of the left lower lid and symptoms of irritation for several months. On examination, she was found to have 20/20 vision in each eye. The right eye appeared to be normal. Examination of the left eye revealed thickening of approximately three fourths of the bulbar and palpebral conjunctiva, with scattered grayish opacities in the conjunctiva and peripheral cornea. Large portions of the upper and lower lid margins were involved by the same process. The clinical impression of intraepithelial carcinoma was confirmed by biopsies. Aside from the ocular problems the patient was well.

Because the patient was opposed to extensive surgery that would have been required for excision of the lesion, she was treated by radiation therapy. The left lens and eyebrow were shielded with lead during irradiation. After receiving 2,460 Rads, treatment was interrupted for 1 month because of conjunctivitis and erythema of the eyelids. The patient received a total dose of 4,260 Rads to the area of involvement over an 8 week period.

There was no evidence of recurrence of the carcinoma, but some keratosis of the conjunctiva and lower lid margin resulted. Because of irritation thought to be secondary to the irregular lid margin rubbing on the corneal surface, a therapeutic soft contact lens (Softcon) was placed on the eye about 1 year after completion of the radiation. While wearing the lens, the patient used gentamicin sulfate ophthalmic solution, 1 percent prednisolone sodium phosphate, and 1.40 percent polyvinyl alcohol solution. On examination 4 weeks later, the contact lens was found to be diffusely opaque. The patient was fitted with another therapeutic soft contact lens, but within 2 weeks similar deposits also developed in this contact lens. Both contact lenses were processed for microscopic examination.

There appeared to be a deficiency in the tear production in the left eye as reflected by the Schirmer tear test (SMP International, Inc., San German, Puerto Rico) (OD, 20 mm./5 minutes; OS, 3 mm./5 minutes). The tear test strips were submitted for measurement of calcium content. A serum calcium of 9.4 mg./100 ml. was found at the time of her original physical examination, and 2 years later it was 10.7 mg./100 ml. (normal, 8.5 to 10.5 mg./100 ml.).

Material and methods. Portions of each contact lens were fixed in buffered formalin, dehydrated...
in graded alcohols, embedded in paraffin, and processed for light microscopy. Cross sections of the contact lenses were stained with a variety of techniques, including the alizarin red and Von Kossa methods for calcium and phosphates, respectively.

**X-ray microanalysis.** Paraffin sections of one contact lens were cut at 8 μm and placed on a water bath at 62° C. to melt the paraffin. They were then picked up and allowed to adhere to a carbon stub. The mounted specimen was rotated in a vacuum evaporator and lightly coated with carbon. These specimens were examined in an ETEC scanning electron microscope operating at 20 Kv, accelerating voltage and subjected to energy-dispersive x-ray microanalysis with an x-ray energy spectrometer (Kevexray Model 5100; Kevex Corp., Burlingame, Calif.). By feeding the outputs of the appropriate channels of the multichannel analyzer back into the scanning system, one can “map” selected elements in a manner similar to the taking of the scanning electron micrograph. By this relatively recent technique, their spatial location within the sections was determined.

**Measurements of tear calcium.** Tears were collected from both eyes of the patient and, for control purposes, from both eyes of three normal individuals, by hooking sterile strips of absorbent paper used for the Schirmer tear test over the lower eyelid border at the junction of the middle and nasal thirds. After 5 minutes each strip was removed and placed in a sterile test tube. The terminal rounded portion of each strip which had absorbed the tears was excised transversely at the notch with clean scissors, with care taken not to touch the strip. The volume of each sample was estimated by measuring the weight of water absorbed by similar portions of 10 fresh strips, and it averaged 0.00454 ± 0.00199 ml (S.E.M.). Each sample was extracted in 5 ml of 0.015N nitric acid, and this extract was used directly for analysis. Calcium was analyzed by flameless atomic absorption techniques using an IL351A spectrophotometer equipped with an IL455 programable graphite rod atomizer (Instrumentation Laboratory, Inc., Lexington, Mass.). Standard curves were determined with a Model 31 calculator (Tektronix, Inc., Beaverton, Ore.) interfaced to the spectrophotometer. Twenty microliters of sample were dried and heated to approximately 3,000° C. over a 10 second period,
Fig. 3. A, Scanning electron micrograph of section through contact lens. Maps of calcium (B) and background (C) emitted from contact lens. (×116.)

Results.

Light microscopy. Light microscopy of both opaque contact lenses disclosed a diffuse basophilia of the deep outer portion of the contact lenses. The deposits stained readily with the alizarin red and Von Kossa methods which demonstrate calcium and phosphates, respectively (Fig. 1).

X-ray microanalysis. In the contact lens the predominantly detectable element over the diffuse background was clearly calcium with a K$_\alpha$ peak at 3.69 keV (Fig. 2). There was also an indication of phosphorus K$_\alpha$ at 2.02 keV. Comparison of the scanning electron micrograph with the calcium map (Fig. 3) unambiguously confirmed the nonuniform distribution found by alizarin red staining. The specificity of the calcium distribution is reflected by the nonlocalization in the map of the background x rays. The same procedure for phosphorus yielded a much weaker map which was nevertheless coincident with the calcium one.

Calcium in tears. A fresh test strip not exposed to tears was extracted and gave a value of 1.57 μg/ml calcium. As a check on the method, a 20 μg/ml solution of calcium was absorbed by four test strips, which were then dried, extracted, and analyzed in the same way as specimens. These gave an average concentration of 19 μg/ml. The measured calcium content in the tears from the normal individuals ranged from 16.8 to 43.2 μg/ml, whereas that on the normal side of the patient was 31.4 μg/ml and on the affected side 79.2 μg/ml.

Discussion. Although there has been speculation that deposits on or in soft contact lenses may be calcium, we are not aware of any proof. In fact, Doughman and associates, in studying opacities on nineteen lenses, found no evidence of calcium deposition, but they demonstrated lipid by histochemical methods and, in addition, found fine cracks on the anterior lens surface. These opacities occurred most frequently in patients with the dry eye syndrome. The sensitive analytical techniques used in the present study clearly establish that the opacification of the contact lenses resulted from calcification. The finding of a markedly elevated tear calcium level on the side of the affected eye in a patient with normal serum calcium levels strongly suggests that the tears were the source of the calcium deposition in the contact lenses. The elevated tear calcium presumably reflected the diminution of tearing on the same side. The present report draws attention to the fact that calcification of soft contact lenses may be clinically significant in certain instances, as when the tear calcium concentration becomes elevated.

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Actin filaments in normal and migrating corneal epithelial cells. ILENE K. GIPSON AND RONALD A. ANDERSON.

The distribution of actin filaments in the cytoplasm of normal and migrating rat corneal epithelium was studied by in situ labeling with myosin subfragment-one. In normal epithelium, actin filaments are present as an apical network under microplicae of superficial cell layers. This apical actin network is a cytoskeleton responsible for maintenance of the regular array of microplicae. A dramatic difference in distribution of actin filaments was noted between the normal cells and cells migrating to cover an abrasion. In migrating epithelial cells, actin filaments are concentrated on the basal regions of the cells. They are present as bundles of parallel filaments near the basal plasmalemma and in networks of filaments in leading edges and cell processes. These basally located actin filaments may play a role in migration of the cells during wound healing.

Although there have been many studies on corneal epithelial wound healing (for review see ref. 1), little is known about the mechanisms responsible for movement of cells to cover the wound. Recent developments in the field of cell biology may shed light on the process. Research during the past few years has shown that many types of cells other than muscle cells contain actin and myosin. Biochemical analysis indicates that 10 to 15 per cent of total protein from non-muscle cells is actin. The protein appears to be ubiquitous, present in all eukaryotic cells examined to date. A very specific histochemical procedure which permits direct localization of actin filaments has been used to demonstrate that 50 to 75 A microfilaments within a variety of cells are actin. The technique is based upon Huxley’s demonstration of binding of a fragment of myosin, heavy meromyosin (HMM), onto isolated muscle actin filaments to form arrowhead configurations or “decorated” filaments. Glycerination of cells in situ permits myosin fragments, either HMM or a smaller fragment of myosin (subfragment-one, S-1), to penetrate the cells and bind to cytoplasmic actin filaments in the characteristic arrowhead pattern. With this technique cortical networks of actin filaments have been demonstrated in a number of cell types. In addition, bundles of actin filaments have been demonstrated within actively motile cells and portions of cells undergoing changes in shape, e.g., motile epithelial cells, fibroblasts and leukocytes in culture, cells undergoing morphogenetic movements, cleavage furrows, and microvilli.

Although myosin is present in nonmuscle cells, its distribution and character is not clearly understood. The concentration of myosin within nonmuscle cells is much lower than that of actin. There are to date no direct histochemical procedures which demonstrate myosin filament distribution in cells at the electron microscope level.

Because actin filament bundles are found within motile cells (amoebae, moving cells in culture) and within portions of cells which are motile (cleavage furrows, microvilli of intestinal epithelia) and because myosin is also present in these cells, the general theory has arisen that an actomyosin system, similar in some ways to that found in muscle cells, is involved in non-muscle cell motility. Direct proof that this is the case is difficult to obtain, and exactly how the system might work to generate force is not known.

We have studied the distribution of actin filaments in normal and migrating corneal epithelium, using S-1 of myosin to localize actin filaments within the cells. We have found that actin filaments are indeed present in these cells and that the cytoplasmic location changes dramatically in the transition from normal to migrating epithelium.

Materials and methods. Normal and healing abraded corneas of adult male Simonson albino
Fig. 1. a, Superficial cells of normal rat corneal epithelium, glycerinated and treated with S-1 of myosin. A network of actin filaments (arrows) is located near the apical surface just under microplicae, of the two outer cell layers. Only a few actin filaments are present along the basal cortex of these cells. (×10,000.) b, Higher magnification of the apical network of a, demonstrating arrowheads (arrows) along actin filaments. Large arrow indicates possible attachment sites of microfilaments to the plasmalemma. (×77,000.) Inset, Section through a microplica with actin filaments extending into it. (×96,000.)
rats were used. Abrasions were produced in anesthetized animals (ketamine, 20 to 40 mg./rat) by outlining a 3 mm. central area of cornea with a trephine and removing the enclosed epithelium down to the basement membrane with a scalpel. The basement membrane was not penetrated. Normal eyes and abraded eyes, 6 hours post-abrasion, were enucleated, and corneas were excised at the limbus.

Glycerination and S-1 binding. Corneas were glycerinated for reaction with S-1 by sequential immersion in cold 50 per cent glycerol:50 per cent standard salt solution (SSS, consisting of 0.1M KCl, 5 mM MgCl, 6 mM Na-phosphate buffer, pH 7.0) with 1 mM dithiothreitol (DTT) for 4 or 18 hours; then 25 per cent glycerol:75 per cent SSS-DTT for 2 to 6 hours; and finally in 5 per cent glycerol:95 per cent SSS-DTT for 1 to 6 hours. Tissue was then washed in SSS with DTT, rinsed twice in SSS with DTT, postfixed in 1 per cent osmium tetroxide in 0.1M Na-cacodylate buffer (pH 6.0), stained in block with 2 per cent uranyl acetate in water, dehydrated in ethanol, and embedded in Epon-Araldite.

Results.

Normal epithelium. A network of “decorated” actin filaments is located just under the cell membrane and microvilli of the superficial cells (Fig. 1, a). “Decorated” filaments within the band are randomly oriented (Fig. 1, b). In some instances they extend into microvilli, and attachments of filaments to the plasma membrane are apparent. The same dense apical network of actin filaments is seen in the cell layer under the most superficial cell (Fig. 1, a). The basal cytoplasmic cortex of the superficial cells have few “decorated” filaments.

Wing and basal epithelial cells do not show any accumulation of actin filaments (Fig. 2). However, it should be noted that glycerination
Fig. 3. For legend, see opposite page.
of these "deep" epithelial cells is often incomplete, even with extended glycerination periods, so that the filaments may be obscured by remaining cytoplasm.

Migrating epithelium. Cells moving to cover the denuded basement membrane have an arrangement of microfilaments which is quite different from the normal epithelium. The greatest concentration of "decorated" actin filaments is near the basal surfaces of these cells (Fig. 3, A), although occasionally apical networks are seen. Two distinct arrangements of the basal filaments have been observed. An especially dense network of "decorated" filaments is located at the leading edges of the migrating cells and within cellular processes (Fig. 3, A and B). Other actin filaments, arranged as parallel bundles, extend back into the cell along the basal surface from the leading edge (Fig. 3, A, inset). These bundles resemble "stress fibers" in rat embryo cells6 and sheath filaments, or microfilament bundles, of spreading mouse 3T3 cells in culture.7 As is demonstrated in Fig. 3, A, several layers of cells move to cover the abrasion. The arrangement of actin filaments into leading edge networks and basal parallel bundles appears to be the same in all moving cells of the epithelial sheet.

Discussion. This study demonstrates that the distribution of actin filaments changes when cells are transformed from the normal to migrating (wound-healing) state. The actin filament networks in leading edges of migrating cells and the bundles of parallel filaments which extend back along their basal surfaces may be part of the "cytoplasmic" machinery involved in force generation for motility. Although this study does not provide direct proof that the actin filaments are responsible for motility, it does provide suggestive indirect evidence that they may play a role in the process.

Several studies of in vitro systems support this hypothesis. Contraction and movement of experimental motile model systems follows formation of large arrays of actin filaments. For example, in plasmalemma-ectoplasm "ghosts" of amoeba, assembly of actin filaments and contraction can be elicited by adding calcium ions and 1.0 mM magnesium-ATP.8 Similarly, the isolated complex of actin filaments from microvilli and the terminal web of intestinal epithelium can be induced to contract by addition of ATP and magnesium ions.9

Other in vitro data have shown a formation and distribution of actin filaments in moving cultured epithelial cells and other cell types10 which is similar to the distribution that we found in migrating corneal epithelial cells in vivo. Because of its accessibility, the cornea is an ideal system for further in vivo studies of how "muscle" cell proteins are involved in cell motility.

The function of the apical network of actin filaments in the superficial cells of normal epithelium is unknown. The network may be a cytoskeleton for maintaining microvilli in their regular array. It is also interesting to speculate that the filaments may generate a pulsation or movement of microvilli. The network resembles in some ways the terminal web of intestinal epithelium where an active pulsation of microvilli, is known to occur.9

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Key words: actin, actin filaments and cell migration, actin filaments and wound healing, cell migration and wound healing, corneal epithelium, corneal epithelial wound healing, microfilaments.

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Fig. 3. A, Low magnification of 2 to 3 cell-layer migrating epithelium glycerinated and treated with S-1. Small double arrows indicate bundles of parallel-oriented actin filaments along basal regions of cells (shown at higher magnification in inset). Large arrows indicate networks of actin filaments in leading edges of cells (shown at higher magnification in b). (×3,800.) Inset, Parallel bundle of "decorated" actin filaments from basal region of migrating cell. (×95,000.) B, Higher magnification of network of actin filaments in leading edge of migrating cell. Arrows indicate S-1 arrowheads along actin filaments. (×58,000.)
Localization of cyclic nucleotide phosphodiesterase activity within the bovine photoreceptor cell. Richard G. Pannbacker and Katherine Lovett.

Cyclic nucleotide phosphodiesterase was measured in the outer layers of the bovine retina by quantitative histochemical methods. The outer segment region contained the highest specific enzyme activity. Phosphodiesterase activity with different kinetic properties was also observed in the outer plexiform layer.

Rod outer segments can be isolated from bovine retinas by a procedure involving density-gradient centrifugation. Such a preparation has been shown to contain high levels of cyclic nucleotide phosphodiesterase activity. The bulk of this enzyme activity can be separated from the rod outer segments by dialysis followed by centrifugation, indicating that it is present in a soluble or loosely associated form. This gives rise to the question of whether the enzyme actually originated in the outer segments themselves or became associated with them during the isolation procedure. In this study, we have measured the phosphodiesterase activity of the various regions of the photoreceptor cell by a method that avoids this potential source of ambiguity: the technique of quantitative histochemistry developed by O. H. Lowry.

**Methods.** Samples were prepared for quantitative histochemical analysis essentially by the method of Lowry and associates and analyzed for phosphodiesterase activity by the method of Russel and associates. Bovine eyes were obtained from a local slaughterhouse and frozen in isopentane cooled in liquid nitrogen. Sections 10 μ thick were cut in a refrigerated microtome at −16°. Sections to be dissected were lyophilized overnight at −40°, then stored under vacuum at −20°. Alternate sections were mounted on microscope slides and stained with toluidine blue. Dissection of lyophilized sections was carried out under low-power magnification. The various layers of the retina to be isolated were identified by reference to the alternate stained sections. Samples were weighed on a quartz-fiber fishpole balance and placed in 6 by 50 mm. tubes. To each tube was added 40 μl of reagent containing pH 7.5 Tris (hydroxymethyl) aminomethane hydrochloride buffer (40 mM), magnesium chloride (5 mM), bovine serum albumin (0.2 mg. per milliliter), 2-mercaptoethanol (6.41 mM), tritiated cyclic GMP or cyclic AMP (0.1 μc) (New England Nuclear, Boston, Mass.), and nonradioactive cyclic GMP or cyclic AMP as indicated. The tubes were incubated at 37° for 1 hour, then boiled for 5 minutes. Ten microliters of 1 mg./ml. Ophiophagus hannah venom (Sigma Chemical Co., St. Louis, Mo.) were added to each and the tubes were incubated at 37° for an additional 10 minutes. Anion exchange resin (100 μl of a 1:1 suspension of AG1-X8; Bio-Rad Laboratories, Richmond, Calif.) was added, and an aliquot (50 μl) of the resulting supernatant was added to 10 ml of 3a7o scintillation cocktail (Research Products International Corp., Elk Grove Village, Ill.) and analyzed in a liquid scintillation spectrometer.

**Results.** When the outer layers of the bovine retina were analyzed for phosphodiesterase activity, with cyclic AMP or cyclic GMP employed as substrate at a concentration of 10−4M, the highest specific activity was found in the pigmented epithelium and outer segment layers (Fig. 1). A significant amount of phosphodiesterase activity was also observed in the outer plexiform layer. To determine whether this activity had the high Km for cyclic GMP characteristic of isolated...
rod outer segments, the rates at high and low cyclic GMP concentrations were compared. As shown in Table I, although samples from the outer plexiform region varied considerably in their total phosphodiesterase activity, the ratio of activities at high (10^{-4}M) and low (2.5 \times 10^{-7}M) cyclic GMP concentrations in paired adjacent samples was quite reproducible, and significantly lower than the ratio obtained with samples from the outer segment layer. The ratio of activities observed in the outer segment samples is exactly that predicted for an enzyme with a K_m of 1.8 \times 10^{-4}M cyclic GMP, the K_m obtained with isolated rod outer segments.

**Discussion.** The photoreceptor cell extends over five layers of the retina, from the pigmented epithelial layer (where outer segments interdigitate with extensions of the epithelial cells) through the outer segment, inner segment, and outer nuclear layers to the outer plexiform layer where the photoreceptor cell makes synaptic contact with horizontal and bipolar cells. In this study we found the highest phosphodiesterase activity in the layers containing outer segments. In addition to exhibiting a level of enzyme activity comparable to that observed in outer segments isolated by centrifugation, the material obtained from the outer segment layer of sections resembled isolated outer segments in the ratio of activities against cyclic AMP and cyclic GMP, and in the ratio of rates at high and low cyclic GMP concentrations.

We conclude that the phosphodiesterase activity observed in outer segments isolated from bovine retinas by centrifugation does indeed originate in the outer segment region of those retinas.

On the basis of the ratio of rates observed at high and low cyclic GMP concentrations, the outer plexiform layer contained phosphodiesterase activity with a lower apparent K_m than did the outer segment layer; however, it was not feasible to perform a detailed measurement of the K_m on samples obtained from sections. The presence of high phosphodiesterase activity in the outer plexiform layer does suggest that neurotransmitters acting in that region could act through cyclic nucleotides.

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Key words: retina, cyclic GMP, cyclic AMP, phosphodiesterase, photoreceptor.

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A guard ring pressure transensor, using the principles of surface applanation, was used to transmit intraocular pressure (IOP) from a cadaver canine eye. The transensor consisted of a passive resonant coil/capacitor combination, made pressure sensitive by the movement of a small ferrite plate which acted as its applanating surface. Oscillation induced in the transensor by a remote grid dip oscillator (GDO) was monitored by a digital frequency counter (DFC). The resonant frequency of oscillation in the transensor bore a linear relationship to the in vitro IOP in three separate experiments (coefficient of determination, $r^2 = 0.90$). These data indicate that radio telemetry is feasible, using a miniature applanating passive radio telemetric device for continual monitoring of IOP.

Continual IOP recording would be very useful for accurate diagnosis and management of the glaucomas. Advantages of a radio telemetric system for transmitting information are lack of restraint of the subject, a steady state situation between the transducer and organ of interest, and normal activities, such as occur with intermittent measuring methods. Collins used a small passive radio telemetric pressure transducer, which he named a transensor, to transmit from within rabbits' eyes. Two opposed matched coils were placed within a small pill. The flat walls of the pill acting as diaphragms were attached one to each coil, such that compression of the diaphragms approximated the coils. Since the coils were very close to each other, slight changes in their separation affected their resonant frequency of oscillation. A remote grid dip oscillator (GDO) was used to induce oscillation in the transensor and detect the frequency of resonance, which was found to be linearly related to the IOP. This method was not developed further since it required insertion of the transensor into the eye. An external method of continual pressure monitoring has been recently reported, with use of miniature strain gauges set into a soft contact lens. It relies on changes in the angle between cornea and sclera with variations in IOP. This method of monitoring IOP is practicable only with wired telemetry at present, and therefore does not have the potential advantages of radio telemetry.

A pressure transducer was made in the form of a cylindrical guard ring applanation tonometer, the overall diameter being 6 mm., with an applanating surface of 2.5 mm. and a height of 3 mm. (Fig. 1). A similar device has been used before in radio telemetry to monitor aortic blood pressure in the dog. A small tightly wound coil of 38-gauge copper wire, wetted with 5 minute epoxy resin, was wound between two spacer washers. The ends of the coil were soldered to the wires of a miniature capacitor of 47 picofarads. The capacitor coating had been ground down to the outer surfaces of its plates before being soldered and placed in position inside one end of the coil. A small cylinder of silicone sponge rubber, cut from a standard ophthalmic plomb, was used to fill the cavity of the coil, and over this a circular flat plate of ferrite ground down to 2.5 by 0.5 mm. was placed.

The applanating surface of the coil was covered with a piece of medical-grade Dow Corning silicone rubber sheeting, 0.5 mm. thick. This was glued in place with room temperature vulcanizing rubber (RTV; adhesive A, Dow Corning Corp., Midland, Mich.), which was also molded to form the walls of the cylinder. The other end of the transducer was similarly treated, over a layer of RTV. The resonant frequency of the transensor was found to be 13.355 MHz.

The transensor was placed on the sclera of a cadaver canine eye and maintained in position with a MIRA silicone rubber implant, with a surrounding silicone rubber band (Fig. 2). The transmitting coil of a standard GDO was placed in contact with the implant so that a substantial dip was observed on its meter. The resonant frequency of the transensor was then read out on a digital frequency counter (DFC), which was coupled to the GDO. A variable-height reservoir was connected by manometer lines with a y piece to a 20-gauge needle in the anterior chamber of the eye and a Sanborn pressure transducer. For each height of the reservoir an average of 20 readings of frequency were taken and recorded on the Sanborn chart of IOP. The DFC was set to count 0.1 and 1 second samples for IOP's from
Fig. 1. Transensor designed for radiotelemetry of intraocular pressure. A, Vertical section. B, Plan view. C, Circuit diagram of the transensor.

Fig. 2. Experimental apparatus used for in vitro radio telemetric monitoring of intraocular pressure.
The mean resonant frequency in MHz with changes in IOP for three separate experiments is shown in Fig. 3. The best fit curve using linear regression by the method of least squares was determined for the data. This gave an equation of \( f = 13.359 - 0.60 T \), with \( r^2 = 0.90 \), where \( f \) = resonant frequency in MHz, \( T \) = IOP in mm. Hg, and \( r^2 \) = coefficient of determination.

The scatter with the three sets of experimental results shown here reflects the unavoidable changes in geometrical relationships and dielectric constants between transensor and GDO, especially with the presence of saline exuding through the sclera and filtration channels. Water vapor absorbed through the walls of the transensor tended to prevent its oscillation after about 3 hours of each experiment. The GDO used in these experiments is a test instrument which is not designed for high resolution of frequencies. It is not temperature stable over prolonged periods. Despite these deficiencies a satisfactory linear relationship between IOP and resonant frequency was obtained.

Further work is in progress to make a smaller version of the transensor which is more stable in conditions of moisture, and which has reproducible mechanical properties. The effects of geometrical variation between the GDO and transensor, air, tissues, and other media on total dielectric constant, and thus resonant frequency of oscillation, are also being investigated.

Once these problems are solved, it should be possible to automate frequency scanning and peak detection of the dip of a GDO system. Practical noninvasive monitoring of IOP in ambulant subjects will then be feasible.

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Key words: Intraocular pressure, continual pressure monitoring, radio-telemetry, biotelemetry, pressure transensor.

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Age-related changes in the free amino acid pool of rat lens. J. SAMUEL ZIGLER, JR., MICHAEL E. MAUNEY, AND J. B. SIDBURY, JR.*

Quite disparate values have been reported by various investigators for the concentrations of free amino acids in the normal rat lens. To test the possibility that the apparent discrepancies could result from age- or strain-related effects, assays were made on both weanling and mature specimens from two common laboratory strains of rats. Although the levels of free amino acids in the plasma were very similar in all groups, the total free amino acid concentration in the lenses of weanling rats was almost double that found in mature rats. Individual amino acids showed quite varied patterns of age-related changes in concentration. Although there is limited evidence that strain-related differences in the amino acid pool may also exist, it was concluded that age-related alterations probably account for most of the variation present in the previously reported values.

Over a period of years, a number of determinations have been reported of free amino acid levels in the ocular lens of various animals. Reddy and Kinsey (1962), among others, demonstrated that amino acids are actively transported into the lens from the aqueous humor. Other investigators (e.g., references 2 and 3) reported the effects of various cataractogenic agents on the free amino acid pool of rat lens. In the course of a study on the parachlorophenylalanine-induced cataract of the rat, it became apparent to us that wide variation existed among the values reported by different investigators for free amino acid concentrations in the normal rat lens. The present data are the results of a study undertaken to determine whether age- or strain-related differences could account for the apparent discrepancies.

Materials and methods. Female weanling rats (50 gm.) and retired breeder rats (6 to 8 months old) were obtained from the Holtzman Co., Madison, Wis. (Holtzman) and from Zivic-Miller Laboratories, Inc., Allison Park, Pa. (Sprague-Dawley). All rats were maintained on Purina Laboratory Chow (Ralston Purina Co., St. Louis, Mo.) for at least five days after arrival. They were sacrificed by decapitation, blood was collected in tubes containing heparin, and lenses were immediately removed and weighed. One lens from each animal was homogenized in 0.75 ml. of deionized water and 0.25 ml. of a solution of o-aminoisobutyric acid which was used as internal standard. Each homogenate was centrifuged at 15,000 x g for 20 minutes, and insoluble material was discarded. The supernatant was divided into two equal aliquots, and each was deproteinized by the addition of a solution of trichloroacetic acid (TCA) until the TCA concentration reached 6 percent. Precipitated protein was removed by centrifugation.

The duplicate, deproteinized aliquots of lens homogenate and duplicate aliquots of plasma (0.5 ml.) from each rat were prepared for derivatization by a clean-up procedure using Dowex 50 (Dow Chemical Co., Midland, Mich.) as previously described. Tests of this system using standard solutions of amino acids demonstrated that losses of free amino acids were negligible except for glycine where a 6 by 10 percent reduction was found. Quantitative amino acid analysis was performed by a gas chromatographic technique following conversion of the amino acids into their N-trifluoroacetyl n-butyl esters. The columns, temperature programs, and other parameters involved in the chromatography have been described.

It was found that glutathione and possibly other small peptides of the lens were not eliminated completely from the amino acids on Dowex 50 and that during the derivatization procedure some amino acid residues were split off the peptides and subsequently analyzed as part of the free amino acid pool. To correct for this artifact, the peptide components from aliquots of lens homogenates, previously passed over Dowex 50, were isolated by the method of Fazakerly and Best (1965) and subsequently derivatized and analyzed to assess the amount of artifactual amino acids produced.

The contralateral lenses from each animal were used for measurement of reduced glutathione concentrations (as acid-soluble sulhydryl) or for lens water determinations. Lens water was calculated from lens wet weight and from dry weight after drying in an oven at 95 to 100° C until constant weight was obtained. Quadruplicate determinations of both glutathione and lens water were made for each group of animals, and mean values used.

Results. Average lens wet weights for the weanling rats were 19 mg. for the Holtzman group and 20 mg. for Sprague-Dawley. The range of lens weights for all weanling rats was 18 to 21 mg. Lens weights for Holtzman retired breeders ranged from 51 to 59 mg. and averaged 55 mg. Corresponding values for Sprague-Dawley retired
Table I. Free amino acid concentration in rat lens (μmol/ml. lens water)

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<th>Weanlings</th>
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<td></td>
<td>Holtzman</td>
<td>Sprague-Dawley</td>
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<tr>
<td>Alanine</td>
<td>5.03 ± 0.29</td>
<td>5.62 ± 0.82</td>
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<td>Valine</td>
<td>0.99 ± 0.19</td>
<td>1.07 ± 0.08</td>
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<td>Glycine</td>
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</tr>
<tr>
<td>Isoleucine</td>
<td>0.39 ± 0.10</td>
<td>0.37 ± 0.06</td>
</tr>
<tr>
<td>Leucine</td>
<td>1.26 ± 0.24</td>
<td>1.35 ± 0.15</td>
</tr>
<tr>
<td>Proline</td>
<td>1.15 ± 0.23</td>
<td>0.96 ± 0.22</td>
</tr>
<tr>
<td>Threonine</td>
<td>1.61 ± 0.38</td>
<td>1.76 ± 0.12</td>
</tr>
<tr>
<td>Serine</td>
<td>1.95 ± 0.21</td>
<td>2.62 ± 0.45</td>
</tr>
<tr>
<td>Methionine</td>
<td>0.20 ± 0.15</td>
<td>0.21 ± 0.20</td>
</tr>
<tr>
<td>Hydroxyproline</td>
<td>0.18 ± 0.14</td>
<td>0.09 ± 0.09</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>0.63 ± 0.14</td>
<td>0.91 ± 0.14</td>
</tr>
<tr>
<td>Aspartic acid†</td>
<td>0.84 ± 0.27</td>
<td>3.04 ± 0.41</td>
</tr>
<tr>
<td>Glutamic acid‡</td>
<td>5.84 ± 1.36</td>
<td>3.86 ± 0.40</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>0.76 ± 0.13</td>
<td>0.86 ± 0.15</td>
</tr>
<tr>
<td>Ornithine</td>
<td>0.80 ± 0.35</td>
<td>3.00 ± 0.26</td>
</tr>
<tr>
<td>Lysine</td>
<td>2.83 ± 0.38</td>
<td>0.34 ± 0.15</td>
</tr>
<tr>
<td>Triptophan</td>
<td>0.26 ± 0.16</td>
<td>N.D.</td>
</tr>
<tr>
<td>Histidine</td>
<td>0.69 ± 0.43</td>
<td>0.89 ± 0.16</td>
</tr>
<tr>
<td>Arginine</td>
<td>1.31 ± 0.37</td>
<td>Trace</td>
</tr>
<tr>
<td>Cysteine</td>
<td>Trace</td>
<td>Trace</td>
</tr>
<tr>
<td>Glutathione</td>
<td>13.5 ± 1.86</td>
<td>13.4 ± 1.16</td>
</tr>
<tr>
<td>Totals (omitting glutathione)</td>
<td>28.98</td>
<td>25.92</td>
</tr>
</tbody>
</table>

Five lenses from five different rats were analyzed in duplicate. The results of the duplicate assays on each lens were averaged, and the mean values were used to compute the data above. Values are presented as ± standard deviation.

*Corrected values (see text).
†Includes asparagine.
‡Includes glutamine.

breeders were a range of 51 to 65 mg, with a mean of 56 mg. Water content as a percentage of wet weight was found to be 58.4 percent in the Holtzman weanlings, 58.5 percent for Sprague-Dawley weanlings, and 54.6 percent for both groups of retired breeders. The range for any particular group was within ± 2 percent of the mean.

Free amino acid concentrations in the lenses of each of the four groups of rats are reported in Table I. It is immediately apparent that there is a markedly decrease in free amino acid concentration in the lens of mature rats as compared to weanlings. The decrease in total concentration is about 50 percent in the Sprague-Dawley rats and 41 percent in the Holtzman. Although individual amino acid-concentrations generally follow the pattern indicated by the total concentrations, some deviate substantially (e.g., alanine, glutamic acid, and arginine).

In Table II the plasma concentrations of free amino acids are reported. In contrast to the lens data, free amino acid levels in the plasma are generally quite similar in all groups; only hydroxyproline is consistently and markedly affected by age. To investigate the interrelationship of plasma and lens amino acid concentrations, a linear model was postulated and tested by regression analysis. Correlation coefficients for the various groups of rats ranged from 0.69 to 0.91, indicating a rather good fit for the linear model. The values for certain amino acids were found to be consistently displaced from the plotted regression line. To demonstrate this fact, the ratio of lens concentration to plasma concentration was calculated for each amino acid of every group. Table III contains these data. It is clear that concentrations within the lens exceed plasma levels and that this difference is most pronounced in weanling rats. The aromatic amino acids phenylalanine and tyrosine, as well as glutamic acid, are concentrated to the greatest extent in the lens relative to circulating levels.

Analysis of interference due to amino acids derived from peptide breakdown during the amino acid derivatization procedure indicated that only glycine and glutamic acid were factitiously generated in measurable quantities. Glycine and glutamic acid are the C-terminal and N-terminal amino acids, respectively, of the major peptides in the lens, i.e., glutathione, ophthalmic acids, and several related tripeptides. The amount of artifactual glutamic acid was negligible compared to the free amino acid level. Corrections were necessary for glycine because in the weanling rat lens about 35 percent of the total glycine analyzed was found to be factitiously generated from lens peptides; in mature rat lens 10 percent was arti-
factual. This finding suggests that under the deriva-
tization conditions the glycine-cysteine bond of
 glutathione may be more labile than the glutam-
ic acid-cysteine bond. Perhaps a more likely
explanation stems from the fact that the initial
step in the derivatization is esterification of the
free carboxyl group. Thus, amino acids cleaved
off during the second stage of derivatization would
not be fully derivatized unless they were original-
ly C-terminal. Partially derivatized amino acids
would not be volatile and thus would not affect
the analysis.

Discussion. Comparison of our lens amino acid
concentrations for weanling rats with the corre-
sponding values of Kinoshita and associates (1962)\(^{2}\)
indicates substantial agreement except for our
markedly higher values for glycine and serine. It
should be noted that Harris and Gruber (1973)\(^{3}\)
found concentrations similar to ours for these
amino acids. Patterson and associates (1965)\(^{4}\) re-
ported lens amino acid concentrations for mature
rats which agree well with ours. Concerning the
relationship of age to lens free amino acid
concentration it has been reported that in the
rabbit there is no change with age\(^{5}\) whereas in the
bovine lens there is a modest decrease in the
mature lens relative to calf lens.\(^{6}\)

It is apparent that the concentrations of free
amino acids in the rat lens are much higher than
the circulating plasma levels. It has been shown
that amino acid synthesis in rat lens accounts
for only a minor portion of the amino acid pool,
thus active transport mechanisms are responsible
for the high lens levels.\(^{7}\) A previous study has
demonstrated that in vitro the transport of \(\alpha-
aminoisobutyric acid\) is greater in young rat lens
than in the mature lens.\(^{8}\) Our data show that
in weanling rats most lens amino acid concentra-
tions are three to six times greater than those of
the plasma whereas in mature rats levels in the
lens are generally two to four times the circulat-
ing levels. The data for the majority of amino
acids fit these averages quite well, as the linear
correlation coefficients cited above demonstrate.
Examination of the residuals from the linear re-
gression plots reveals, however, that certain amino
acids consistently and substantially differ from
these average values (e.g., tyrosine, phenylalanine,
and glutamic acid). These findings probably re-
semble to the fact that amino acid transport in the
lens involves a number of distinct systems with
different affinities.\(^{9}\) The fact that two of the
most highly concentrated amino acids, tyrosine
and phenylalanine, are structurally very similar
suggests that they are transported by the same
system and that it is an especially active or
efficient one.

It has been established by this study that
there are substantial age-related changes in the
free amino acid concentration of rat lens. Pre-
vious unpublished work from this laboratory in-
volving rats from a variety of strains and ages,
Table III. Ratios of lens-to-plasma free amino acid concentrations

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Weanlings</th>
<th></th>
<th>Retired breeders</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Holtzman</td>
<td>Sprague-Dawley</td>
<td>Holtzman</td>
<td>Sprague-Dawley</td>
</tr>
<tr>
<td>Alanine</td>
<td>7.1</td>
<td>6.8</td>
<td>2.0</td>
<td>1.7</td>
</tr>
<tr>
<td>Valine</td>
<td>4.5</td>
<td>5.9</td>
<td>2.3</td>
<td>2.6</td>
</tr>
<tr>
<td>Glycine</td>
<td>3.6</td>
<td>2.8</td>
<td>2.3</td>
<td>2.1</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>4.3</td>
<td>6.2</td>
<td>3.2</td>
<td>3.2</td>
</tr>
<tr>
<td>Leucine</td>
<td>6.0</td>
<td>6.8</td>
<td>4.4</td>
<td>2.6</td>
</tr>
<tr>
<td>Proline</td>
<td>3.8</td>
<td>3.2</td>
<td>2.7</td>
<td>2.0</td>
</tr>
<tr>
<td>Threonine</td>
<td>5.0</td>
<td>7.3</td>
<td>2.1</td>
<td>1.8</td>
</tr>
<tr>
<td>Serine</td>
<td>4.4</td>
<td>9.4</td>
<td>3.9</td>
<td>2.7</td>
</tr>
<tr>
<td>Methionine</td>
<td>3.3</td>
<td>3.5</td>
<td>2.4</td>
<td>3.2</td>
</tr>
<tr>
<td>Hydroxyproline</td>
<td>3.1</td>
<td>1.3</td>
<td>4.5</td>
<td></td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>10.0</td>
<td>12.3</td>
<td>12.4</td>
<td>7.3</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>7.0</td>
<td>9.1</td>
<td>4.9</td>
<td>6.4</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>11.5</td>
<td>7.1</td>
<td>7.0</td>
<td>8.2</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>14.1</td>
<td>13.6</td>
<td>12.6</td>
<td>9.0</td>
</tr>
<tr>
<td>Ornithine</td>
<td>5.3</td>
<td>8.6</td>
<td>3.3</td>
<td>2.8</td>
</tr>
<tr>
<td>Lysine</td>
<td>4.8</td>
<td>5.4</td>
<td>5.1</td>
<td>1.9</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>3.2</td>
<td>6.8</td>
<td>0.5</td>
<td>1.0</td>
</tr>
<tr>
<td>Histidine</td>
<td>7.7</td>
<td>N.D.</td>
<td>7.4</td>
<td>N.D.</td>
</tr>
<tr>
<td>Arginine</td>
<td>3.3</td>
<td>2.5</td>
<td>6.8</td>
<td>2.5</td>
</tr>
</tbody>
</table>

in which lenses were pooled before amino acid analysis, suggests strongly that the decrease in the lens free amino acid pool occurs before the age of 6 months and that little further change occurs at least up to the age of 15 months. The existence of strain-related effects on amino acid levels is uncertain. Most amino acids are of similar concentration in lenses from specimens of similar age but different strain. There are one or two instances (e.g., glutamic acid) which are suggestive of possible strain-related effects on the amino acid pool, but in the absence of further data it is impossible to be certain. At any rate, it seems likely that many of the discrepancies found in the literature are the result of age-related changes in concentration. Many of the previous studies which have measured rat lens free amino acids have involved animals between 1 and 6 months of age, the period during which a major decrease in the amino acid pool occurs. Teleologically, the higher amino acid concentrations present in the young rat lens are reasonable since that is the period of greatest growth and protein synthesis for the lens. Lowered concentrations in mature lenses may reflect reduced activity or efficiency of active transport systems or increased permeability of lens membranes.

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Key words: rat lens, free amino acid pool, age-related effects, amino acid transport, strain-related effects.

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HLA antigens and primary open-angle glaucoma in black Americans. BERNARD BECKER AND DONG H. SHIN.

Black patients with primary open-angle glaucoma, when compared to nonglaucomatous individuals, demonstrate significantly increased prevalences of the HLA antigens B7 and B12 and significantly decreased frequencies of A1 and A11. White patients with primary open-angle glaucoma have in common with blacks the increases in B7 and B12 and the decrease in A11, but present no deficit of A1. In addition, white patients with primary open-angle glaucoma demonstrate a significant increase of A3 and a decrease of Bw35, both of which are not found in blacks.

A close association of HLA-B7 and HLA-B12 antigens with primary open-angle glaucoma (POAG) has been observed in both black and white patients. HLA-B7 and HLA-B12 in nonglaucomatous patients with elevated intraocular pressure also proved to have predictive value for the development of glaucomatous visual field loss. Interestingly, HLA-B7 and HLA-B12 seemed to relate to cupping of the optic nervehead and positive family history of glaucoma, but not to the level of intraocular pressure. Recently A-locus as well as B-locus antigens have been reported in association with diseases. An increased prevalence of HLA-A3 and marked decrease of HLA-Bw35 were found in white patients with POAG. The present investigation has extended the study to A-locus antigens in black Americans with POAG.

Materials and methods. Thirty-five black patients with POAG and 28 black volunteers with NN-NG response0 (less than 31 mm. Hg after 6 weeks of topical 0.1% dexamethasone 4 times daily) were typed for histocompatibility antigen according to the lymphocyte microcytotoxicity technique used previously. All had negative glucose tolerance tests and no evidence of pigmented dispersion, trauma, or congenital or inflammatory ocular diseases. They had been followed in the Glaucoma Center of Washington University for 1 to 15 years and had been subjected to complete eye examinations, including appplanation tonometry, tonography, gonioscopy, ophthalmoscopy, and Goldmann perimetry at regular intervals. Statistical analysis employed Yates’ corrected chi-square test or Fisher’s exact test.

Results. As previously noted in both blacks and whites, HLA-B7 and HLA-B12 antigens were significantly increased in black patients with POAG over that in NN-NG responders (Table I). Either B7 or B12 antigen was present in 94 per cent of black glaucoma patients as compared to 32 per cent of the NN-NG group (p < 0.0005). HLA-A1 was present in only 2 (6 per cent) of 35 black patients with POAG, but in 8 (29 per cent) of 28 NN-NG responders (p < 0.02). The HLA-A11 antigen failed to appear in the black glaucoma patients tested but occurred in 5 (18 per cent) of the black NN-NG individuals (p < 0.02). Either A1 or A11 was found in only 2 (6 per cent) of the black glaucoma group as compared to 12 (43 per cent) of the black NN-NG category (p < 0.0005). In the black population no significant difference was found between POAG patients and NN-NG responders with respect to prevalences of HLA-A3 and HLA-Bw35 (Table I).

Discussion. The present study confirms the previous observation that the prevalences of HLA-B7 and HLA-B12 in POAG patients, both black and white, are increased over those of NN-NG responders. In addition, HLA-A1 and HLA-A11 are found to be in significant deficit in black patients with POAG as compared to black individuals of the NN-NG category. Either antigen, HLA-A1 or HLA-A11, is present in 6 per cent of black glaucoma patients but in 43 per cent of the black NN-NG group. In white glaucoma patients HLA-A11 is also in deficit, but there is no significant decrease of HLA-A1 (Table II). Furthermore the significant increase in prevalence of HLA-A3 and the decrease of HLA-Bw35 observed in the white patients with POAG (Table II) are not found in blacks with this disease (Table I). The frequencies of HLA-A3 and HLA-Bw35 in blacks with POAG are not significantly different from those of nonglaucomatous blacks (Table I). Thus the white and black pa-

### Table I. HLA antigens in black patients

<table>
<thead>
<tr>
<th>Antigens</th>
<th>POAG patients</th>
<th>NN-NG responders</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>2 (6%)</td>
<td>8 (29%)</td>
<td>&lt;0.02*</td>
</tr>
<tr>
<td>A3</td>
<td>5 (14%)</td>
<td>6 (21%)</td>
<td>NSf</td>
</tr>
<tr>
<td>A11</td>
<td>0 (0%)</td>
<td>5 (18%)</td>
<td>&lt;0.02*</td>
</tr>
<tr>
<td>B7</td>
<td>18 (51%)</td>
<td>5 (18%)</td>
<td>&lt;0.025*</td>
</tr>
<tr>
<td>B12</td>
<td>19 (54%)</td>
<td>5 (18%)</td>
<td>&lt;0.01f</td>
</tr>
<tr>
<td>Bw35</td>
<td>9 (26%)</td>
<td>8 (29%)</td>
<td>NSf</td>
</tr>
<tr>
<td>B7 or B12</td>
<td>33 (94%)</td>
<td>9 (32%)</td>
<td>&lt;0.0005f</td>
</tr>
<tr>
<td>A1 or A11</td>
<td>2 (6%)</td>
<td>12 (43%)</td>
<td>&lt;0.0005*</td>
</tr>
<tr>
<td>A11 or</td>
<td>9 (26%)</td>
<td>12 (43%)</td>
<td>NSf</td>
</tr>
</tbody>
</table>

NN-NG = Intraocular pressure less than 31 mm. Hg after 6 weeks of topical 0.1% dexamethasone 4 times daily.

*Fisher’s exact test.

(Yates’ corrected chi-square test.)
Table II. HLA antigens in white patients

<table>
<thead>
<tr>
<th>POAG patients</th>
<th>NN-NG responders</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of patients</td>
<td>35</td>
<td>50</td>
</tr>
</tbody>
</table>

Antigens:
- A1: 7 (20%) | 13 (26%) | NS†
- A3: 15 (43%) | 11 (22%) | NS†
- A11: 1 (3%) | 9 (18%) | <0.035*
- B7: 19 (54%) | 7 (14%) | <0.0005†
- B12: 17 (48%) | 9 (18%) | <0.01†
- Bw35: 3 (9%) | 18 (36%) | <0.005*
- A1 or A11: 7 (20%) | 19 (38%) | NS†
- A11 or Bw35: 4 (11%) | 22 (44%) | <0.005†

NN-NG = Intraocular pressure less than 31 mm Hg after 6 weeks of topical 0.1% dexamethasone 4 times daily.
NS = Not significant.
†Yates' corrected chi-square test.

Racial differences in HLA antigens include prevalences, linkage disequilibria between A and B loci antigens, and correlations between HLA antigens and diseases. For example, HLA-B8 is increased in white patients with Graves’ disease, but not in Japanese. Instead, Japanese patients with Graves’ disease present increases in HLA-Bw35.

The resistance to glaucomatous damage seen in white patients with elevated intraocular pressure and HLA-A11 or HLA-Bw35 suggests parallel studies of HLA-A1 and HLA-A11 in blacks.

From the Glaucoma Center, Washington University School of Medicine, St. Louis, Mo. This study was supported in part by grant EY 00336 from the National Eye Institute and by a fellowship grant from Fight For Sight, Inc., New York, N. Y. (Dr. Shin). Submitted for publication Oct. 5, 1976. Reprint requests: Glaucoma Center, Washington University School of Medicine, 680 S. Euclid, St. Louis, Mo. 63110.

Key words: primary open-angle glaucoma, HLA antigens.

REFERENCES

Inhibition of leukocyte migration with melanoma-associated antigens in choroidal tumors. DEVRON H. CHAR.

Patients with choroidal melanomas and simulating choroidal tumors were tested in a masked manner with a 3M KCl antigen derived from a metastatic choroidal melanoma. Good discrimination between patients having ocular melanomas and con-
trol subjects, using a soluble melanoma antigen to test in vivo cell-mediated immunity to melanoma antigens. In this study we have used the LMI assay with an antigen processed from a metastatic choroidal melanoma, to determine whether the results of this in vitro test would differentiate patients with choroidal melanoma from those patients with simulating choroidal lesions.

Methods and materials. All patients were examined in the Ocular Oncology Clinic of the University of California, San Francisco, and informed consent was obtained from each subject prior to inclusion in this study. All subjects were examined with slit-lamp biomicroscopy, direct and indirect ophthalmoscopy, Goldmann perimetry, fluorescein angiography, and ultrasound. Many patients also had skin tests performed with a soluble melanoma antigen, and some had radioactive phosphorous uptake tests. The diagnosis of each patient was made independently of immunologic test results, and the LMI test was performed with the technician “masked” as to the clinical diagnosis of each patient. Ten patients had pathologically confirmed choroidal melanomas, and two patients have small melanomas which are being closely followed. The following types of control subjects were tested: four patients with choroidal nevi, two with choroidal hemangiomas, one with retinal pigment epithelial hyperplasia, three with metastatic breast carcinoma to the choroid, and one with thyroid carcinoma metastatic to the choroid and one with adenocarcinoma metastatic to the choroid.

The methodology used in the preparation of 3M KCl extracts, and the performance of the LMI assay has been previously described. All metastatic melanoma from the liver was obtained in a sterile manner after the death of a patient with a primary choroidal melanoma. The tissue was finely minced, suspended in a solution of 3M KCl at 4° C. for 24 hours, centrifuged, dialyzed against normal saline, and concentrated to approximately 9 mg./ml. This antigenic preparation was cultured to ensure sterility and stored at -70° C. until used.

Heparinized venous blood (50 ml.) was collected from each subject. The leukocytes were separated by Plasmagel sedimentation, repeatedly washed in McCoy's 5A medium, and then resuspended in McCoy's medium with 10 per cent heat-inactivated fetal calf serum at a concentration of 2 × 10^6/ml. One milliliter of each subject's leukocytes was then incubated with and without 700 µg of antigen in a humidified 5 per cent CO2 incubator at 37° C. for 45 minutes. After the incubation, the cell suspensions were drawn up into 25 µl pipettes in quadruplicate and centrifuged. The pipettes were then cut slightly below the fluid-cell interface. These pipettes were separately placed in McCoy's medium in Sterilin chambers and incubated on a level shelf at 37° C. for 16 hours in a humidified 5 per cent CO2 incubator. At the end of this incubation the areas of migration of the leukocytes with and without antigen were measured by projection planimetry. The migration index was calculated by:

\[
\text{Migration Index} = \frac{\text{Mean area of migration of 4 replicates with antigen}}{\text{Mean area of migration of 4 replicates without antigen}}
\]

In almost all of the LMI tests the replicates values were within 10 per cent of each other, and often they were within 3 per cent of each other. Statistical analysis was done by the Mann-Whitney test.

Results. The results of the LMI assays are presented in Fig. 1. There is a statistically significant difference between the LMI results in patients having ocular melanomas versus those having benign and metastatic simulating choroidal lesions (p < 0.01). The specificity of the LMI assay with this extract derived from a metastatic choroidal melanoma was excellent. With a value of less than 82 considered as a positive LMI result, no patients with simulating lesions had a positive LMI test. Four of the 12 patients with ocular melanomas had LMI scores above 82, so that the sensitivity of this assay was only 66 per cent. Two additional subjects had LMI scores
of less than 82 (data not shown). The affected eyes in these two patients have not yet been enucleated. In both, the diagnosis is probably an early choroidal melanoma; however, a choroidal nevus in one case, and a melanocytoma in the second cannot be excluded. In normal subjects’ tests no LMI scores less than 94 were observed (data not shown.) In the patients with ocular melanomas there was no correlation between LMI results and histologic cell type.

Discussion. Cellular reactivity, measured in a LMI assay to an antigen derived from a metastatic choroidal melanoma, was observed to discriminate between patients with choroidal melanomas and patients with simulating lesions. No patients with simulating choroidal lesions had positive LMI results versus eight out of 12 patients with ocular melanomas. In two additional patients who had positive tests, we are unsure of the diagnosis, but both probably have early melanomas. Investigators have used modifications of the LMI assay to demonstrate cell-mediated immunity in human tumors.5-7 The sensitivity of this assay has been variable. In our present investigation less than 70 per cent of patients tested have had positive LMI results with antigens derived from their histologic tumor type. Different antigenic preparations processed in the same manner often yield marked differences in reactivity,7 and we have observed a much lower incidence of positive reactions with some melanoma-associated antigens (unpublished observations). More extensive antigen purification may increase the incidence of specific reactivity. In skin-testing cancer patients with different tumor antigen preparations, we have observed an increased sensitivity with the more purified antigens. However, even with more highly purified antigens we have noted a significant incidence of false negative (10 per cent) results (unpublished data). It is probable that a different density of tumor antigens is present on different allogeneic melanoma cells, and this would partially account for the differences in reactivity noted between different antigen preparations.8 Furthermore, the difference in antigen density on allogeneic melanoma cells may explain the marked variation in antigen concentrations used in the LMI to achieve specific migration inhibition. Some antigen preparations can be tested at 50 ug/ml, whereas others require up to 800 ug/ml to elicit specific cellular reactivity.

The specificity observed in this assay appears quite promising for use as an ancillary diagnostic aid, although we do not have pathologic confirmation of the diagnosis in four patients’ clinically diagnosed ocular melanomas. In other reports using an LMI assay with standard and tumor-associated antigens, the specificity has been approximately 80 to 90 per cent.9 10 Tumor masses are composed of tumor cells and supporting structures. It is possible that these supporting stromal elements possess a number of normal cross-reacting tissue-associated antigens which are responsible for some of the nonspecific reactivity observed. From previous work, we think that histocompatibility antigens and bacterial contamination are unlikely sources of this cross-reactivity.9 Even with more extensive antigen purification we have observed some nonspecific reactivity in an in vivo assay using a soluble melanoma antigen (unpublished data). We are currently investigating other techniques of antigen preparation in an attempt to minimize nonspecific reactivity. However, it is doubtful that this can be entirely eliminated.

Metastatic lesions to the choroid can be extremely difficult to diagnose. Often the choroidal lesion may be the first presentation of a systemic tumor, and tests such as the radioactive phosphorous test are often falsely positive in patients with metastatic disease.10 It is possible that the LMI assay might be clinically useful in such cases even if its sensitivity is only 70 per cent, especially since we have noted that none of the patients with metastatic simulating lesions had positive LMI results.

The number of patients tested with this antigen was low, and there appeared to be no correlation between histologic cell type and LMI result in patients with choroidal melanomas. In most previous human cancer studies, the LMI results did not correlate well with the disease status; however, we have previously noted a slight preponderance of positive LMI reaction in early versus advanced systemic melanomas.3 It is possible that the LMI assay may provide information of prognostic importance in patients with primary choroidal melanomas, but our follow-up in patients is too short to ascertain this.

From the Department of Ophthalmology, University of California School of Medicine, San Francisco. Supported in part by the Cancer Research Coordinating Committee, University of California, Berkeley, Grant #75 SF 11, Fight for Sight, Inc., New York, N. Y., and National Institutes of Health Training Grant EY 01759. Submitted for publication Sept. 29, 1976. Reprint requests: Devron H. Char, M.D., Department of Ophthalmology, U-490, University of California School of Medicine, San Francisco, Calif. 94143.

Key words: melanoma, leukocyte migration, inhibition, tumor immunity.

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Thymus-derived lymphocytes in the Vogt-Koyanagi-Harada syndrome. DEVRON H. CHAR, JENNIFER BRUNN, and WILLIAM WEST.

Previous investigations have suggested that immunologic factors may play a role in the Vogt-Koyanagi-Harada syndrome, a disease involving the eye, central nervous system, and dermis. Among patients with this disease we have observed a decrease in their peripheral blood lymphocytes, as measured by two types of rosette assays. These results strengthen the concept that an altered immunologic status may be important in the pathophysiology of this disease.

The Vogt-Koyanagi-Harada syndrome (VKH) is a diffuse bilateral chronic uveitis associated with a number of systemic findings including alopecia, poliosis, vitiligo, dysacusis, and meningeval irritation. Previous investigations have demonstrated that some patients with this disease have cellular reactivity to uveal antigens; however, one cannot determine from these studies whether an altered immune status is of importance in the pathophysiology of the disease.

Thymus-derived peripheral blood lymphocytes form nonimmune rosettes with sheep red blood cells. In some human diseases in which altered immunologic status occurs, the number of rosette-forming T cells is decreased. We have studied patients with VKH to determine whether an alteration in their immunologic status, as measured by changes in the number of thymus-derived peripheral blood lymphocytes, exists.

Method. Eight patients with VKH and 16 age-matched controls were tested in two rosette assays. The patients were all evaluated in the Uveitis Survey Clinic of the University of California, San Francisco. Two patients had active disease, one of which was on alternate-day oral prednisone; his last dose having been taken approximately 36 hours prior to testing; six patients were in the inactive phase of the disease.

Peripheral venous blood (10 ml.) was drawn into a 12 ml. syringe containing 100 units of preservative-free heparin. The blood was diluted to 40 ml. with phosphate-buffered saline (PBS) and centrifuged on 10 ml. layer of Ficoll-Hypaque at 400 × g for 20 minutes. A single sheep was used as a source of red cells, and a single batch of heat-inactivated fetal calf serum (FCS) was used in all tests. Sheep red blood cells (SRBC’s) were obtained weekly and diluted 1:2 with Alsever’s solution.

Two rosette assays were used in this study. One was done at an incubation temperature of 4° C, and the second assay was done with fewer SRBC’s at an incubation temperature of 29° C. All tests were performed in triplicate, the technician being unaware of the clinical data.

In the 4° rosette assay, which measures total T cell rosettes, 0.2 ml. of FCS and 0.1 ml. of lymphocytes (5 × 10⁶/ml.) were mixed and added to 0.2 ml. of SRBC’s (0.3 × 10⁶/ml.). This suspension was incubated at 37° C for 5 minutes, centrifuged at 100 × g for 5 minutes, and incubated for 4 hours at 4° C. The cells were then gently resuspended in RPMI 1640, and 200 cells were counted, a positive cell being defined as one with three or more SRBC’s around it forming a rosette. The 29° assay, which measures approximately the same subpopulation of T cells as the “active” rosette test, was done in a similar manner except that the concentration of SRBC’s was 1 × 10⁶/ml. and the incubation was carried out at 29° C. The data were analyzed with Student’s t test.
**Results.** Fig. 1 summarizes the results obtained in this study. In both the 4° and 29° rosette assays, patients with VKH showed significantly lower values of rosetting cells than did the normal controls (p < 0.005). However, some overlap between normal controls and patients did occur in both of these assays. There appeared to be no correlation between those results and the clinical status of the patients; however, the small number of patients tested makes valid conclusions on this point impossible.

**Discussion.** Previous workers have suggested that immunologic factors are important in the pathophysiology of VKH; however, supportive data have been extremely limited. Although a number of investigators have observed that patients with VKH had a heightened cellular reactivity to xenographic uveal antigens versus normal controls, the significance of these studies is unclear. It is possible that an altered immunologic reactivity to uveal antigens or to melanosomes, in general (e.g., the systemic findings of vitiligo, alopecia, and poliosis) are important in the development of this disease. However, cellular reactivity to uveal antigens might also occur secondarily to uveal inflammation.

Decreased numbers of thymus-derived lymphocytes, as measured by nonimmune rosette assays, are observed in patients with alterations in their immunologic status, including congenital immunodeficiency disease, sarcoidosis, leprosy, collagen vascular disease, and cancer. We do not think that extraneous factors capable of lowering the rosette values were present in the patients tested. Therefore, our observation that VKH patients manifest an alteration in the number of peripheral blood thymus-derived lymphocytes would support the concept that an immune mechanism may be important in the development of the disease. This possibility is further supported by the fact that some patients who have been treated with immunosuppressive agents have experienced a remission of their disease (unpublished observation).

It is presently unclear what subpopulation of T cells is diminished in patients with VKH. Current studies in our laboratory seem to implicate suppressor cells, although it is possible that a serum substance may also be important; additional work is needed to delineate this.

We wish to thank Drs. Samuel J. Kimura and C. Richard O'Connor for the referral of patients.

From the Department of Ophthalmology, University of California School of Medicine, San Francisco, and National Cancer Institute, Bethesda, Md. This research was supported by NIH Grants EY 01759, EY 01597, Fight for Sight Grant-In-Aid G 574, and UC Berkeley Cancer Coordinating Committee Grant 75 SF 11. Submitted for publication Sept. 1, 1976. Reprint request: Devron H. Char, M.D., Department of Ophthalmology, University of California School of Medicine, San Francisco, Calif. 94143.

Key words: Vogt-Koyanagi-Harada syndrome, rosettes, cell-mediated immunity.

**REFERENCES**


Pineal reactivity of anti-retina sera.

CAROLYN M. KALSW AND W. B. WACKER.

Specific immunofluorescence has been demonstrated in the guinea pig pineal gland by homologous sera from guinea pigs injected with an extract of retina homogenate. This fluorescence appears to be in the cytoplasm and is evenly distributed among the cells of the pineal gland. Specific immunofluorescence in the retina has been previously demonstrated by these sera.

Experimental allergic uveitis (EAU) has been elicited in guinea pigs by injection of an extract of homologous retina homogenate (S preparation). 1-3 Complement fixation and immunodiffusion methods have been used to establish the tissue specificity of the activity of the antisera from such animals (anti-S sera). 1-4 Because these techniques require the use of tissue homogenates, any small discrete areas of tissue capable of cross-reactivity might not be detected as a result of dilution by homogenization procedures. Therefore, we used an indirect fluorescent antibody technique on tissue sections to re-examine the tissue specificity of the activity of anti-S sera. 5-7 Because these techniques require the use of tissue homogenates, any small discrete areas of tissue capable of cross-reactivity might not be detected as a result of dilution by homogenization procedures. Therefore, we used an indirect fluorescent antibody technique on tissue sections to re-examine the tissue specificity of the activity of anti-S sera. 5-7

The functional and structural relationship of pineal gland and retina in lower vertebrates 5-7 would suggest that even in mammals there might be an antigenic cross-reaction between the two tissues. The small size of the pineal gland would make any cross-reactivity difficult to detect by complement fixation or immunodiffusion, especially if brain homogenate were used as a source of antigen. The present paper describes the occurrence of pineal reactivity of anti-S sera as detected by immunofluorescence in horizontal brain sections.

Materials and methods. Female Hartley guinea pigs weighing 200 to 400 gm. were used in this study.

Anti-S sera 5 antisera to a partially purified S preparation, i.e., ammonium sulfate precipitated and Sephadex G-100 chromatographed (anti-FII sera), 6 and antisera to a suspension of the sedimentable portion of retina homogenate (anti-P sera) 7 were prepared as previously described. Antisera to uvea and lens were prepared by injection into the foot pad of a 0.1 ml. emulsion made up of equal volumes of homogenate of 10 mg. of tissue and Bacto adjuvant complete H37-Ra (Difco Laboratories, Inc., Detroit, Mich.). These sera were collected 22 days postimmunization. The number of each type of sera tested were anti-S, 97 sera; anti-FII, 20 sera; anti-P, 7 sera; anti-uvea, 10 sera; anti-lens, 5 sera.

The sera were examined for immunofluorescent activity in tissues that had been fixed at 4° C. in 95 percent ethanol and embedded in paraffin according to the technique of Sainte-Marie. 8 Brains were sectioned in the horizontal plane through the area of the pineal gland. Tissue sections were cleared and stained according to an indirect fluorescent antibody protocol 5 with various sera used in the first layer and fluorescein-labeled IgG fraction of rabbit anti-guinea pig gamma globulin (Cappel Laboratories, Downingtown, Pa.) in the second layer. Incubations were for 15 min. at room temperature.

Results. All anti-S sera tested showed specific fluorescence in the pineal gland (Fig. 1). The fluorescence appeared to be in the cytoplasm and was evenly distributed among the cells (Fig. 2). There was no specific fluorescence detectable in the stroma of the pineal gland. For orientation we have included a section of normal pineal gland stained with hematoxylin and eosin (Fig. 3). These same sera also showed specific fluorescence in the photoreceptor cells of the retina, as previously described. 6 Similar fluorescent patterns in both pineal gland and retina were also demonstrated by anti-FII sera. Anti-P sera, which exhibited a specific immunofluorescence pattern distinct from that of anti-S sera, i.e., restricted to the outer segments of the photoreceptor cells, showed no specific fluorescence in the pineal gland. Furthermore, neither anti-uvea nor anti-lens sera showed any such activity on pineal gland or retina. (One anti-uvea serum showed a very weak reaction which might have resulted from contamination of uvea with some photoreceptor outer segments during dissection.) This anti-S activity has not been demonstrated in any other tissues tested, including heart, liver, kidney, uterus, striated muscle, esophagus, trachea, skin, lung, and brain.

Discussion. This study reveals that there is an antigenic cross-reaction between guinea pig retina and pineal gland as demonstrated by a fluorescent antibody technique. Specificity of the cross-reaction is supported by the reactivity of anti-FII.
sera and the nonreactivity of anti-P sera and of antisera to other ocular tissues. Although studies with anti-pineal sera and other serologic tests, especially immunodiffusion, will further establish the specificity of the reaction, neither antigenic identity nor monospecificity can be firmly set until the antigen(s) is obtained in pure form. Studies are progressing in these areas.

Although recent work has emphasized the secretory nature of the pineal gland in higher
Fig. 3. Hematoxylin-eosin-stained section of the pineal gland of a normal guinea pig. (Original magnification x93.75.)

vertebrates, demonstration of an antigenic cross-reaction that is specific for retina and pineal gland is not unexpected in view of the phylogenetic relationship of retina photoreceptors and pineal gland. Indeed, this finding emphasizes an evolutionary tie between sensory pineal photoreceptors of lower vertebrates and secretory pinealocytes of mammals. The results are also consistent with a recent demonstration of a similarity of morphology between neonatal pinealocytes and developing photoreceptor cells in the rat. This unique relationship between retina and pineal should assist in the biochemical identification of the cross-reacting antigen(s).

Further studies will investigate the role of this cross-reacting antigen(s) in EAU. Obviously it must first be established that the pineal gland is affected during the course of EAU. This is possible since there seems to be at least a modest breach in the blood-brain barrier in the area around the pineal. It is interesting to speculate as to whether the pineal gland would be involved before or after the retina during the development of EAU. This question of initial insult is especially important if we are to relate the animal model to the human disease process.

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From the Department of Ophthalmology, University of Louisville School of Medicine, Louisville, Ky. This investigation was supported by United States Public Health Service Research Grants R23 EY 01516 and EY 00254 from the National Eye Institute. Submitted for publication Sept. 1, 1976. Reprint requests: Dr. Carolyn M. Kalsow, Kentucky Lions Eye Research Institute, 301 E. Walnut St., Louisville, Ky. 40202.

Key words: retina, pineal gland, immunofluorescence, guinea pig, cross-reaction.

REFERENCES

Retina viability in the Necturus. JAMES P. CULBERT AND WILLIAM W. DAWSON.

Electroretinograms (ERG's) were recorded from the eyes of paralyzed normal and decapitated mudpuppies (Necturus maculosus). Fully dark-adapted responses to a range of stimulus intensities were compiled hourly under both conditions. Statistical analyses indicate no significant change in responsiveness in paralyzed normal animals during periods up to 6 hours. After decapitation, there was a significant decline of sensitivity to light during the first 3 hours. An oxygen-sensitive component similar to the c-wave was observed with DC recording but only in normal eyes. Anoxic mudpuppy eyes show an ERG decline with an average time constant of 1.5 hours.

The Necturus maculosus (mudpuppy) possesses a very small eye, but the retina is composed of unusually large cells. The size of the retinal cells in amphibia has been used to advantage by many who have recorded extracellular and intracellular potentials from most of the layers and cell types. Important correlations have been made between intracellular recordings and intracellular staining of many retinal cells.

Kuffler, Nicholls, and Orkand have noted that Necturus glial cell resting potentials are more easily obtained in well-circulated anesthetized animals than in vitro eyes. But there have been no systematic examinations of the longevity of the decapitated preparation upon which most of the current literature is based.

Single-cell electrophysiological measures are particularly susceptible to variations with time which are not necessarily related to the physiological state of the tissue. We elected to use the electroretinogram (ERG) as a noninvasive measure of retinal condition over extended periods in the whole unanesthetized animal and in decapitated animals.

Methods. Twelve mudpuppies between 11 and 14 inches in length were maintained at 15°C. in filtered, dechlorinated water treated with malachite green to inhibit fungal growth. The storage temperature was chosen to approximate the animals' normal environment, 6 to 10°C. A 12 hour light-dark cycle was maintained. Live minnows were supplied for food. The experimental preparations were immobilized by the intramuscular injection of pancuronium bromide (0.15 mg. per kilogram). In our hands, pancuronium bromide caused almost no undesirable side effects in test animals in contradistinction to gallamine or curare. Pancuronium has been reported to have superior characteristics in other animals. Most of our Necturus were allowed to recover normally from the neuromuscular block. When returned to their home tanks, they appeared to function naturally and to compete successfully for minnows during feeding. Following immobilization, the animals were maintained in a chamber supplied with circulating water (20°C.) saturated with 100 per cent oxygen which was bubbled past the external gills. Each animal's head was tilted so that the eyes were above the water level. Electrocardiographic (ECG) electrodes were attached to the animal, and the ECG was monitored throughout the experiment. If ECG rate or amplitude changes were noted before decapitation, the experiment was terminated. Before recording, the brille (second cornea) was dissected away under red light and a soft, coiled, stainless steel (20 mil) wire was placed against the cornea with a reference electrode behind the eye on the posterior portion of the orbit. Attempts to dilate the pupil with 1 per cent Mydriacyl (Alcon) were not successful. Diffuse stimulation of the eye was provided by a General Radio Strobolume, a xenon flash lamp with accessory lenses. Flash duration was 30 microseconds. Peak irradiance at the cornea was 30 watt-sec. per square centimeter, measured by a UDT 40A PIN silicon photodiode. The stimulus was regulated over a 6 log unit range with neutral density filters.

Signals were recorded under two conditions; the control group animals were in vivo. The experimental group was recorded in vivo for 3 hours. Then they were recorded for 4 hours following decapitation. There were six animals in each group. After 2 hours' dark adaptation, the eye was stimulated by a range of light intensity from full intensity to 6 log units below 30 watt-sec.
Fig. 1. ERG obtained with DC recording in a normal dark-adapted Necturus. Probable wave types are indicated. Stimulus duration was 500 msec. (lower trace).

Fig. 2. ERG b-wave, amplitudes of one paralyzed whole animal, elicited 1 to 6 hours after dark adaptation by a range of stimulus intensities. Ordinate values are relative to maximum (100 per cent) response. Results of stimulation at the same intensities are presented for each subsequent hour up to six. X-axis units are referred to a maximum irradiance of 30 watt-sec per square centimeter.

per square centimeter. Responses to four stimuli, one/10 sec., were computer averaged. The stimuli were delivered in ascending series of intensity. The sequence of stimulation was repeated once each hour for 6 to 8 hours in both conditions. In the experimental group the procedure was the same as in the control group. However, after 3 hours of recording of stable ERG amplitudes, the preparation was surgically decapitated just anterior to the gill structure. Water was then drained from the chamber but oxygen flow was continued. Recording was continued after decapitation until the ERG b-wave was no longer discernible from the averaged noise level. Amplification bandpass 3 dB points were 0.1 Hz and 3 kHz. The measurements of the ERG c-wave were made with DC differential amplifiers and chlorided silver wire electrodes in saline-filled, cotton-wick glass electrodes. These stimuli were presented with an infrared-filtered, 150 W, tungsten source with a mechanical shutter which provided for 0.5 sec. exposure.

Results. DC recording of the ERG in normal eyes produced records as in Fig. 1. Components are a-, b-, and c-waves according to Granit's criteria. AC recording produced data in Figs. 2, 3, and 4. The control preparations produced amplitude-intensity functions as shown in Fig. 2. Experimental animals produced results as shown by Fig. 3. Decapitation occurred immediately before the records at hour 4. The signals recorded from the in vivo and the in vivo-decapitated animals were not significantly different prior to decapitation (Fig. 4). There was large individual variance in amplitude, and all data have been referred to the individual's maximum response. Since decapitation movement required repositioning of the electrode, a second normalization to the maximum
Fig. 3. ERG b-wave amplitude of one paralyzed whole animal subsequently decapitated just before measure at hour 4. Otherwise, as Fig. 2.

Discussion. In the older literature, studies of tissue respiration in the mammalian retina indicate that retinal oxygen consumption is higher than that found in either liver or brain. Consequently, the retina is probably the most metabolically active tissue in the vertebrate body. Research on the anoxic retina provides electrophysiological confirmation. These researchers found that activity of the optic nerve declines most rapidly in the anoxic rabbit retina followed soon by reduced ERG b-waves. In the hypoxic cat retina, the reverse sequence was reported by Adams, Perez, and Dawson for b-wave amplitude, which decreased more rapidly than optic nerve responses when the partial pressure of arterial oxygen was reduced to 40. The link between b-wave amplitude and hypoxia seems firmly established in mammals. In lower vertebrates, research by Miller and Dowling gave strong support to the Müller cells as the origin of the b-wave. Müller cell function has been related to redistribution of K+ ions. Both receptors and interneurons are dependent upon extracellular K+ concentration for control of membrane potentials. If redistribution of K+ was altered by Müller cell deactivation due to hypoxia, it would be sur-
Fig. 4. Summary of ERG b-wave amplitudes produced by a fixed 0.3 watt-sec per square centimeter stimulus at several times after paralysis (filled circles) or after paralysis with subsequent decapitation (open circles). Decapitation was done just before the hour 4 measure. Values are group means; limit marks are ± S.D. No S.D. could be calculated at 4 hours since decapitation required reassignment of the maximum amplitude value (100 on ordinate).

prising that normal photoreceptor and interneuron processes could continue.

This preliminary study has utilized the most extreme mudpuppy preparation, decapitation with unopened eye. Others have ranged from isolated eye cup5 to only cornea and lens removed from the decapitated animal.6 Results from our preparation suggest that all ERG signal components are affected by the loss of the blood supply and the resulting anoxia. A separate analysis for a-wave amplitude was not done since, in mudpuppy, the a-wave can be very small at low stimulus values. With DC recording, it was possible to demonstrate a late positive signal with all the characteristics of the c-wave only when there was evidence for good physiological condition. In this context, it is significant that the c-wave could not be demonstrated in the decapitated preparation described by Bortoff7 or by Miller and Dowling.3

Doubt has been cast upon the physiological generality of much of the research on Limulus completed in the last 30 years since Barlow and Kaplan8 reported that the standard, isolated eye preparation resulted in large differences in function when compared to data from the intact Limulus. This research indicates that cells in the retina of Necturus, like Limulus, are susceptible to oxygen deficit. The time constant for ERG signal decline in the anoxic bovine eye is about 2 minutes in contrast to 1.5 hours in mudpuppy.10 Whole Necturus may be maintained with normal eye signals and recover from over 7 hours of paralysis by pancuronium bromide.

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This research was assisted by the NSF Grant GB-31649X to William Dawson. James Culbert is a Fellow of the Center for Neurobiological Sciences which is supported by Grant No. 5T01 MH 10320-10. Submitted for publication Aug. 18, 1976. Reprint requests: Dr. William W. Dawson, Box J-284, J. Hillis Miller Health Center, University of Florida, Gainesville, Fla. 32610.

Key words: mudpuppies, anoxia, ERG, retina.

REFERENCES

The rod outer segment phospholipid/opsin ratio of rats maintained in darkness or cyclic light. Daniel T. Organisciak and Werner K. Noell.

Rhodopsin (opsin), lipid, and fatty acids were measured in rod outer segments (ROS's) of rats maintained for at least 2 weeks in continuous darkness or in 12 hours per day cyclic light. Average rhodopsin per eye was 1.5 nmol, for the 5 ft.-c. cyclic light groups compared to 2.4 nmol, for the dark groups of the same age. The phospholipid/opsin ratio was significantly higher after cyclic light maintenance, suggesting that slow adaptive processes control the opsin density of the ROS membranes. Estimates indicate that ROS length also depends on the long-term light environment. ROS lipid and fatty acid composition were not consistently different in dark and light groups.

During a study of the role of retinol in the genesis of the irreversible retinal damage by visible light, it was found that the intensity of the damaging effect depends upon the light environment in which the rat has been reared or maintained, prior to the damaging exposure. Long-term maintenance in weak cyclic light provided protection compared to maintenance in continuous darkness. This led to the suggestion that the long-term light environment has a controlling influence upon the molecular organization of the membranes of the outer segments of rod photoreceptors where the damage appears to be initiated. In the following discussion we will report on lipid and opsin measurements of isolated rod outer segments (ROS's) from rats maintained under different light conditions.

Materials and methods. The animals used were Sprague-Dawley albino rats obtained from Charles River Breeding Laboratories either at the age of 21 days or when weighing between 150 and 175 g. They were maintained in continuous darkness or on a 12-hour light-12-hour dark cycle. The latter rats were in plastic cages with stainless steel wire tops. Illumination of the desired level, measured at the cage tops, was achieved by white plexiglass sheets, 1/4 inch thick, in front of the incandescent light source. All rats were in the same dark environment for 16 to 18 hours before the retina was removed in dim red light.

ROS's were isolated and purified by sucrose gradient density centrifugation, and aliquots of the isolated ROS's, generally from 5 to 7 animals, were analyzed for rhodopsin content. Rhodopsin was also measured for the whole eye of animals of every group. Lipids were quantitatively extracted, organic phosphorus determined, and samples analyzed from lipid and fatty acid composition. The opsin content of the Emulphogene BC-720 extracts was calculated from the A570/A560 absorbance ratio of extracts from alun-treated ROS's by normalizing to a ratio of 1.7. Extracts from nonalum-treated ROS's were used to indicate the purity of the outer segment preparation. The absorbance ratios of these samples were 10 to 20 percent higher than in the alun-treated ones, indicating that rhodopsin was the major protein present and that contamination of the sample by tissue other than ROS's was probably small.

The measurements were performed over a period of 1 year on a total of 12 groups of cyclic light-treated rats and their respective, dark-maintained mates. Illumination was 2 ft.-c. in two groups, 5 ft.-c. in six groups, 10 ft.-c. in two groups, and 50 ft.-c. in one group. The duration of cyclic light maintenance varied between 10 days and 3 months.

Results. As shown by the data of Table I, maintenance of the albino rats in weak cyclic light produced two major differences in comparison to rats maintained in the dark environment: the opsin content of the outer segments was decreased and the phospholipid/opsin ratio increased.

Row 1 of Table I lists average results for Group I. The 90- to 160-day-old rats had been either in a dark environment for at least 4 weeks (D) or in 5 ft.-c. cyclic light for 18 to 95 days (L). The average rhodopsin content per eye of the L rats (1.8 nmol.) was 25 percent lower than that of D rats (p < 0.01). The average ratio of
Table I. ROS rhodopsin and phospholipids related to cyclic light (L) and dark (D)

<table>
<thead>
<tr>
<th>Group</th>
<th>ROS yield</th>
<th>TPL/opsin</th>
<th>TPL/eye</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(nmol.)</td>
<td>(nmol.)</td>
<td>(nmol.)</td>
</tr>
<tr>
<td>Group I:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D, 26-140 days</td>
<td>2.45 ± 0.13</td>
<td>65.6 ± 4.4</td>
<td>159</td>
</tr>
<tr>
<td>L (5 ft.-c.), 18-95 days</td>
<td>1.78 ± 0.16</td>
<td>77.0 ± 6.7</td>
<td>140</td>
</tr>
<tr>
<td>Group II:</td>
<td>2.48</td>
<td>1.74</td>
<td>61.8</td>
</tr>
<tr>
<td>D, 140 days</td>
<td>17</td>
<td>1.74</td>
<td>61.8</td>
</tr>
<tr>
<td>L (5 ft.-c.), 18 days</td>
<td>1.94</td>
<td>1.72</td>
<td>76.4</td>
</tr>
<tr>
<td>Group III:</td>
<td>2.44</td>
<td>1.73</td>
<td>64.9</td>
</tr>
<tr>
<td>D, 91 days</td>
<td>26</td>
<td>1.73</td>
<td>64.9</td>
</tr>
<tr>
<td>L (5 ft.-c.), 91 days</td>
<td>1.72</td>
<td>1.73</td>
<td>73.0</td>
</tr>
<tr>
<td>Group IV:</td>
<td>2.35</td>
<td>1.70</td>
<td>62.6</td>
</tr>
<tr>
<td>D (58 days), D (32 days)</td>
<td>20</td>
<td>1.70</td>
<td>62.6</td>
</tr>
<tr>
<td>L (10 ft.-c.), 14 days</td>
<td>1.56</td>
<td>1.67</td>
<td>82.6</td>
</tr>
</tbody>
</table>

Values are expressed as the means ± S.D.; n = 6 except as indicated.

Rhodopsin of isolated outer segments per eye in % of total rhodopsin per eye measured in animals of same group.

A_{278}/A_{500} represents the difference in absorption before and after bleaching of the sample.

A_{278}/A_{500} of the ROS extracts was the same (1.74 in both groups, indicating that opsin also was probably 25 percent lower in L than in D. At least 10 days of continuous darkness was required for rhodopsin per eye of the cyclic light group to reach a level as high as in the dark group, the half-time of this rise was about 6 days. The ROS total phospholipid content (TPL) is given per opsin content on a molar basis. In the cyclic light subgroup of Group I (Table I), TPL/opsin was 77.0 compared to an average of 65.6 for the dark-maintained subgroup (p = 0.01). Hence, 17 percent more phospholipids per opsin appear to be contained in the isolated ROS fragments of L than of D. Assuming that homologous samples of the ROS membranes are measured (see ROS yield, Table I), it follows that the rhodopsin density of the ROS membranes is greater in D than L. Multiplying TPL/opsin by the rhodopsin per eye, one obtains an estimate of the phospholipids of the outer segment population of the whole eye. As shown in Table I (TPL/eye), the difference between L and D is 12 percent. Under the assumption of a constancy of phospholipid per disc and of the number of rods per eye, this would indicate that ROS length is less in L than D.

Group II of Table I gives the value from a single experiment. The animals had been in continuous darkness for 122 days (since age 21 days) and then were divided into D and L (5 ft.-c.) subgroups for the next 18 days. As with the average results, the light environment reduced rhodopsin content and increased TPL per opsin, but TPL/eye was about the same as in D.

The data of Group III are from animals which, from the age of 21 days were either for 91 days continuously in dark or for 91 days in 5 ft.-c. cyclic light. The same differences are measured as in Group II except that TPL/eye is 20 percent lower in L than D, suggesting that in comparison with experiment II, a change in the phospholipid content per one outer segment or in ROS "length" develops slower in a new light environment than a change in rhodopsin. The difference in TPL/eye was significant at a 0.02 level for 4 weeks or longer maintenance in 5 ft.-c.

Group IV is from animals of the same shipment as Group III. The L rats were kept in the cyclic light only for 58 days and then switched to the dark environment for 32 days before measurements. Results with this group are indistinguishable from those obtained with D in Group III.

Group V indicates that the effects of 10 ft.-c. cyclic light for 14 days are about the same as those with 5 ft.-c. for 91 days. Maintenance in 50 ft.-c. from the age of 3 weeks for 3 months resulted in very low rhodopsin values (1.2 nmol.), indicating visual cell loss by light damage. The TPL/opsin ratio was not higher than after maintenance in 5 ft.-c. TPL/eye was 32 percent lower than in D.

The major glycerophospholipids and fatty acid composition of ROS's was not significantly different in L compared to D. However, in one group (5 ft.-c., 36 days), 22:6 in phosphatidylethanolamine was 68 percent of the D measurement, whereas 22:6 in phosphatidylethanolamine and phosphatidylcholine was 94 and 88 percent of D, respectively.

Discussion. The present study was undertaken to reveal, if possible, differences in the membrane structure of the outer segment after maintenance...
in weak cyclic light in comparison to maintenance in a light-deprived environment. Our results are interpreted to show that slow adaptive mechanisms control the opsin density of the ROS membranes and, to a lesser degree, also ROS phospholipid weight, suggesting a control of ROS length. Prolonged maintenance in the dark environment, associated typically with the enhancement of the susceptibility to damage by light, is best viewed as a nonphysiological condition of light deprivation. Weak cyclic light may match the wild-life condition to which the rat is evolutionarily adapted. Hence, if the ROS composition relates at all to the susceptibility to damage by light, it follows that the closer packing of rhodopsin in the lipid matrix of the membranes in the light-deprived condition makes these membranes more vulnerable to light, possibly by virtue of a change in certain protein-lipid interactions. We had hoped that the dark condition might be characterized by an increase in the highly unsaturated fatty acids of the ROS's, but unequivocal evidence on this point was not obtained.

\*A 12.5 percent increase in outer segment length by 6 days in total darkness has been recently reported for the frog by Basinger and associates (Science 194: 1074, 1976).

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