Visual pigment and visual receptor cells in fetal and adult sheep

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The visual pigment, and the structure of the visual cells, were investigated by spectrophotometry and by light and electron microscopy in fetal and adult sheep. The rhodopsin system in adult sheep closely resembles that of cattle. The absorbance maxima of rhodopsin, lumirhodopsin, and metarhodopsin I are at 498, 490, and 480 nm, respectively. The estimated molar absorbance coefficient at the wavelength of maximum absorption of rhodopsin is 40,000 M⁻¹ cm⁻¹. Rhodopsin was detected from a fetal age of 85 days (term at 145 days). Partial bleaching of extracts from fetal eyes (95, 105, and 115 days) did not demonstrate cone pigments, although cones were present in fair numbers at a fetal age of about 105 days. The time course of rhodopsin formation between 85 and 140 days resembles a growth curve. The amount of rhodopsin shortly before birth (140 days) is about 0.6 times that in the adult. The number and dimensions of rod outer segments as well as packing of the discs were studied structurally and related to the rhodopsin content. A fairly good correlation was found at the earliest stages (95 and 105 days gestation age), when rhodopsin concentration was very low and rod outer segments were few and small, as well as at the latest stage (140 days). At 115 days the rhodopsin content observed by spectrophotometry was less than that indicated by the outer segment volume, probably mainly due to the outer segment discs and possibly to the rhodopsin molecules being less tightly packed than at 140 days. (INVEST OPHTHALMOL VIS SCI 23:409-418, 1982.)

Key words: retinal photoreceptors, spectrophotometry, electron microscopy, rhodopsin, fetus, ontogenesis, sheep

The structural development of the retina has been extensively studied in mammals. In several species, e.g., rat, mouse, rabbit, and cat, the litters are born with nonfunctioning retinas, since the development of the photoreceptor outer segments starts postnatally. In other mammal species, including primates, the visual cells and retinal neurons develop prenatally and motor reactions to light occurs almost immediately after birth. The
development of the outer segment discs and the time course of the development of rod and cone outer segments were studied in the frog by Nilsson.\textsuperscript{12}

Little is known about the functional maturation of the visual cells in mammals. In the rat, rhodopsin is present 7 days after birth, and an electroretinographic response appears 5 days later.\textsuperscript{1} The growth of visual cell outer segments, and the amplitude of the a- and b-waves of the electroretinogram parallel the increase in rhodopsin concentration (up to 14 days). This result is consistent with findings on rabbit\textsuperscript{13} and tadpoles.\textsuperscript{14-15} In these species, electroretinographic responses to light are not recorded until the outer segments begin to appear

In the rat, visual pigment formation and receptor function start postnatally, but experimental results show that in sheep the receptors are already sensitive to light during the gestation period (which lasts 145 days). This conclusion can be drawn from the observation that the pupillary reflex can be elicited at a gestation age of 92 days and a visually evoked cortical response at 111 days.\textsuperscript{16}

The present study concerns the time course of rhodopsin formation before birth, i.e., the approximate gestation age at which this visual pigment can first be detected, and the increase in pigment concentration with fetal age. Furthermore, the structural development of the photoreceptor outer segments was studied by light and electron microscopy in the fellow eyes to enable a correlation of morphologic and biochemical maturation. Although the sheep has a mixed retina,\textsuperscript{17} no cone pigments were detected in this study.

Materials and methods

Enucleation. Immediately after mating, the ewes were kept separate from rams. When the gestation had proceeded 85, 95, 105, 115, and 140 days (term at about 145 days), the ewes were kept in darkness the night prior to surgery, which was made under dim red light, kept in darkness for 1 to 2 hr, and frozen in liquid N\textsubscript{2} together with the adult eyes. The second fetal eye was fixed in situ for electron and light microscopy (see below).

Preparation of rhodopsin extracts

Adult sheep. The frozen eye was cut in half. After thawing, the retina was removed from the pigment epithelium, collected in Ringer’s solution, and homogenized. Photoreceptor outer segments were isolated by the standard sucrose floatation technique\textsuperscript{18} and extracted by digitonin (2%).

Fetal sheep. A circular sample (50 mm\textsuperscript{2}) was removed from the posterior pole (temporally to the optic nerve) of the frozen eye by means of a hollow metal tube with sharpened edges. The frozen sample that contained all tissue layers of the eye was transferred to a Petri dish and warmed to room temperature. The thawing was observed in a dissecting microscope by infrared light. The photoreceptor layer was removed by a fine forceps and transferred to a tube containing 1.5 ml of phosphate buffer (0.066M, pH 6.5). The sample was then centrifuged for 4 min (Eppendorf 3200 centrifuge, 12,000 rpm), and the supernatant was discarded. The residue was washed twice in phosphate buffer (0.07M, pH 6.5; volumes were 10 to 50 µl). To extract the visual pigment quantitatively from the retinal tissue, the extraction procedure was repeated until no further pigment was detected.

Spectrophotometry. Spectrophotometric measurements of visual pigment extracts were carried out in microcells (light path 10 mm, volume 8 µl). The microcells were filled, using a micropipet (Pipetman) under inspection by infrared light. The cuvettes were covered at both ends with a small quartz coverslip.

All measurements were made at a temperature of 18° C, with the exception of low-temperature measurements, which were made as follows. Rhodopsin extracts were mixed with glycerol (1:2), transferred to cuvettes (light path 3 mm), and mounted on a cold-finger, which was inserted into a quartz Dewar bottle containing methanol. The temperature of the bath was adjusted by a cryostat (Colora KTS 90). To avoid waste water outside the cells, the experiments were carried out in N\textsubscript{2} atmosphere.

The absorption measurements were made by a double-beam spectrophotometer (Hitachi 356). The spectrophotometer was equipped with a “baseline compensator” (Hitachi), which allowed accurate measurements of small absorbance changes.
Visual pigment extracts were illuminated by monochromatic light (interference and heat filters, Schott & Gen.) from a xenon arc lamp (150 W, Zeiss).

Electron microscopy. The fellow eyes of fetuses at a gestation age of 95, 105, 115, or 140 days were fixed in situ by perfusion via the left cardiac ventricle with 2% glutaraldehyde in 0.1M sodium cacodylate buffer at pH 7.2. All perfusions were made between 10:00 A.M. and 3:00 P.M. under dim red light. Each eye was enucleated and the anterior segment was removed. Fixation was continued for a minimum of 3 days by immersion in the same fixative at 4° C. The eyecup was then cut in smaller pieces and the sclera was dissected away. After postfixation in osmium tetroxide (1%) in Veronal buffer, and dehydration in acetone, the specimens were embedded in Westopal V. Pieces of retina and pigment epithelium from the posterior pole of the eye, either overlying the tapetum or close to this area, were cut for electron microscopic examination. The sections were subsequently stained with uranyl acetate and lead citrate and examined in an electron microscope (Philips EM 300).

Light microscopy. Sections (1 μm thick) were cut from the same embedded material. They were oriented for longitudinal as well as for cross sections of the photoreceptor cells in the posterior pole of the eye. After staining with toluidine blue, the sections were photographed in a Zeiss photomicroscope. Paper copies (enlarged photographically 7 times) were used for measurements of outer segment dimensions (at least 100 rods and 30 cones were measured) as well as for determination of rod and cone distribution (at least 200 receptors were counted).

Results

Spectral absorption of rhodopsin in adult sheep. The only data available on the absorbance of sheep rhodopsin are values for the wavelength for maximal absorption (λmax) 500 ± 1 nm.19 In the present study, the absorbance spectra of visual pigment from adult sheep were therefore determined for comparison with those of fetal sheep.

Fig. 1A shows the spectral absorbance of the extracted visual pigment (curve 1). Bleaching (λ ≈ 528 nm) of the visual pigment in the presence of NH₂OH (final concentration 0.1M) resulted in the formation of retinal oxime with an absorbance maximum at about 370 nm (curve 2).

Fig. 1B. Change in spectral absorbance (○) after illumination of sheep rhodopsin extract, and nomogram (○) for rhodopsin (λmax = 500 nm).

The change in spectral absorbance due to the illumination is illustrated by the difference spectrum seen in Fig. 1B. The spectrum shows that the maximally absorbed wavelength was 498 nm. A nomogram20 for a rhodopsin absorbing maximally at this wavelength well agrees with the observed absorbance changes. The ratio of the absorbance changes is similar to that of other vertebrate rhodopsins, indicating that the molar absorbance coefficient (at λmax) is approximately 40,000 M⁻¹·cm⁻¹.

To test whether the eyes were dark-adapted, the extract was divided. One part was incubated by 11-cis-retinaldehyde (30 min, 18° C), after which NH₂OH (final concentration 0.1M) was added to both samples. They were then illuminated to bleach the visual pigment. A comparison of the maximal absorbance change of the two samples
Fig. 2A. Changes in spectral absorbance after illumination of rhodopsin extract at —60° C (curve 1) and warming to 18° C (curve 2). Nomogram ($\lambda_{\text{max}} = 490 \text{ nm}$) is indicated by the dashed line.

Fig. 2B. Changes in spectral absorbance after illumination of rhodopsin extract at —25° C (curve 1) and warming to 18° C (curve 2). Nomogram ($\lambda_{\text{max}} = 480 \text{ nm}$) is indicated by the dashed line.

Fig. 3. Absorbance spectra of rhodopsin, lumirhodopsin, and metarhodopsin I.

Visual pigment in fetal sheep. To minimize the loss of outer segments during the preparation of visual pigment extracts from fetal eyes, the sucrose flotation was omitted. The spectral absorbance changes upon illumination of visual pigment from fetuses at gestation ages of 105 and 115 days are seen in Fig. 4. Maximal absorbance decrease is seen at about 500 nm. The absorbance changes agree well with those for adult sheep rhodopsin,
indicating that the extract contained almost exclusively rhodopsin. This assumption was further supported by partial bleaching (635, 570, 530, and 498 nm) of the extract with and without NH₂OH, which did not reveal measurable amounts of visual pigments other than rhodopsin at 95, 105, and 115 days.

When bleached extracts from retinas at 95 days were incubated (pH 6.8, 20° C, 30 min) with 11-cis-retinaldehyde, the total amount of bleached rhodopsin was regenerated. There was no increase in amount of regenerated pigment. The result shows that the eyes were completely dark-adapted at the outset of the experiments. It also indicates that no free opsins is present in the fetal retina.

The time course of rhodopsin formation during fetal development was determined by quantitative extraction of samples (50 mm²) from retinas at gestation ages of 85, 95, 105, 115, and 140 days. Since photoreceptor development centrally in the eye precedes that in the periphery, all samples were taken from the central part of the retina. The extracts were bleached in the presence of NH₂OH. Fig. 5 shows the increase in amount of rhodopsin with fetal age. A just-measurable amount (about 0.003 of adult content) was found at 85 days gestation age.

The rate of increase from 85 to 140 days approximated a normal growth curve. A similar

Fig. 4. Absorbance changes of visual pigment extracts from fetal retinas at a gestation age of 105 (○) and 115 (△) days, and those of rhodopsin from adult sheep (●).

Fig. 5. Increase in rhodopsin concentration with fetal age. Left abscissa, Relative concentration. Right abscissa, Moles per 50 mm² retina sample. Data points are observed concentration. Solid line, Calculated increase with time (logistic curve); dashed line, standard deviation of observed adult concentration.
Figs. 6 to 10. For legends see facing page.
lar increase occurs postnatally in the rat. A normal growth curve is a logistic curve with three phases: initial exponential increase, approximately linear increase, and exponential approach to a final value. The mathematical expression of the logistic curve is

\[ y(t) = y_1 + \frac{e^{\alpha t} - 1}{e^{\alpha t} - 1} \]

where \( y(t) \) is the rhodopsin concentration at time \( t \), \( y \) is the adult concentration, \( \alpha \) is the growth rate constant, and \( t_M \) is the time when rhodopsin concentration is 50% of adult content. Observed data were as follows: \( \alpha = 0.092 \) (calculated from the concentrations at 85, 95, 105, and 115 days, Fig. 5) and \( t_M = 137 \) days. Inserting these values into the equation results in the growth curve seen in Fig. 5. From the curve can be estimated that during the first phase the concentration doubled in about 7.5 days and that the concentration 5 days before birth was about 0.6 times that in the adult. The postnatal age at which adult concentration is first reached was not determined.

**Morphology.** Part of the photoreceptor layer and the pigment epithelium from the extratapetal area of a fetus at 140 days gestation age are seen in Fig. 6. Rods and cones were present in the same proportions as in the adult retina, i.e., one cone per 14 ± 0.7 rods (mean and standard error of mean, here as in the following). The same was true for the earlier stages: 115 days, one cone per 16 ± 0.8 rods; 105 days, one cone per 15 ± 0.6 rods; 95 days, one cone per 14 ± 0.6 rods. At 95 days the receptor types were determined on the basis of inner segment structure and location of the nuclei. The narrow rod inner segments at 140 days contained lightly stained mitochondria. The diameter of the cone inner segments was approximately twice that of the rod inner segments, and the mitochondria in the cones were substantially more electron dense than those in the rods. The rod outer segment length was 8.2 ± 0.2 \( \mu \)m (corresponding to 0.63 times that in the adult) and the diameter was 1.2 ± 0.02 \( \mu \)m (0.92 adult diameter). The cone outer segment length was 3.2 ± 0.2 \( \mu \)m (0.8 adult length). No reliable measurements of cone diameter could be made because of the shape of the cone outer segment. The discs of both rods and cones were slightly distorted, particularly at the outer segment tips, but not much more than can be seen in the adult retina. They were also less tightly packed than in adult receptors. Otherwise no major difference was observed between the visual cells in this retina and those in the adult. Both diameter and length of the rod outer segments were observed to increase with gestation age. This fact suggests that the volume of the rod outer segments is a better morphologic correlate to the rhodopsin content than their length, in spite of differences between stages in packing of the discs. The volume at 140 days was calculated to be about 0.54 times that of adult volume. No structures that could be identified with certainty as phagosomes were observed in the electron microscope. In light microscopic sections, three structures appearing to be phagosomes were found in the pigment epithelium overlaying 450 rods (cut longitudinally). In the pigment epithelium melanosomes and granules presumably con-

![Fig. 6. Part of pigment epithelium (P) and photoreceptor layer from tapetal area of eye of sheep fetus at a gestation age of 140 days. RI, Rod inner segment; RO, rod outer segment; CI, cone inner segment; CO, cone outer segment; MI, mitochondria; M, melanosome; G, granule. Same abbreviations apply to Figs. 7 to 10.](image)

![Fig. 7. Cone and rod from fetus at 115 days gestation age.](image)

![Fig. 8. Rod with short outer segment and its connecting structure (arrow) from extratapetal area of fetus at a gestation age of 105 days.](image)

![Fig. 9. Cone with short outer segment from extratapetal area. Gestation age 105 days.](image)

![Fig. 10. Rod outer segment at very early stage of development showing only a few discs. Arrow, Connecting structure. Extratapetal area. Gestation age 95 days.](image)
taining lysosomes were seen. The pigment epithelium overlaying the tapetum lacked melanosomes. Otherwise, the pigment epithelium and the rest of the retina were similar in the tapetal and extratapetal areas at this and earlier stages.

Also at 115 days gestation age, well-developed rods and cones were seen (Fig. 7). The rod outer segment length and diameter were $5.7 \pm 0.2$ and $0.9 \pm 0.02 \mu m$ (corresponding to $0.44$ and $0.69$ adult size). The volume was calculated to be about $0.21$ times that in the adult. The cone outer segment length was $2.6 \pm 0.1 \mu m$ (0.65 adult length). The sparse (in comparison with adults) packing of the discs was more apparent at this and earlier stages than at 140 days. Four structures appearing to be phagosomes were found in light microscopic sections representing the same length of the pigment epithelium as that studied at 140 days.

Both rod (Fig. 8) and cone (Fig. 9) outer segments were also present at a gestation age of 105 days. However, even at the posterior pole not all receptors showed outer segments, and more cones than rods seemed to lack outer segments. In sections from a zone slightly outside the central area the decrease in number of outer segments was more apparent at this and earlier stages than at 140 days. Four structures appearing to be phagosomes were found in light microscopic sections representing the same length of the pigment epithelium as that studied at 140 days.

At the youngest gestation age investigated (95 days) the outer segments were very few and small. Those present seemed to be rod outer segments. Only in one eye could an outer segment be attributed with certainty to a cone. Fig. 10 shows a small developing rod outer segment, with only a few discs, and its connecting structure. Outside the posterior pole outer segments were lacking entirely.

**Volume of rod outer segments compared to rhodopsin concentration.** The very few and small outer segments seen at 95 days gestation age correspond with the low (about 1%) rhodopsin concentration. At 105, 115, and 140 days the average volume of the rod outer segments was less than 0.1, about 0.21 and 0.54 times that of adult volume. The corresponding figures for the rhodopsin concentration (Fig. 5) were 0.03, 0.09, and 0.58 times those of adult concentrations.

**Discussion**

The present measurements show that the absorbance characteristics of sheep rhodopsin and its intermediates in the bleaching sequence are similar to the bovine rhodopsin system. Even though the sheep has a mixed retina with a rather large number of cones, partial bleaching did not demonstrate any cone pigment in adult eyes. This negative result was obtained in spite of the fact that measurements were made in the absence of NH$_2$OH, since cone pigments may be less stable in the presence of this compound. The failure to detect cone pigments might be due to loss of cone outer segments during the dissection of the retina, but it is more likely that the amount of cone pigment (relative to rod pigment) was too small to be detected. As in the adults, no cone pigment could be demonstrated in fetal eyes. Even at the earliest stage when visual pigment was detected, the wavelength for maximal absorption corresponded to that of rhodopsin. The electron microscopy showed that cone and rod outer segments develop simultaneously in approximately the same proportion as in the adult retina, with the possible exception that at the earliest stage (95 days) there may be more rod outer segments. A parallel development of cones and rods occurs in the frog, whereas in the chick the temporal sequence is less clear. Since cones are much fewer than rods and cone outer segments much shorter than those of rods, the amount of

cone pigment is considerably smaller than that of rods and therefore was not detected.

The pigment epithelium in adult sheep contains phagosomes related to the turnover of the photoreceptor outer segments. Very few phagosomes were seen during gestation. This observation might indicate that during intrauterine development (term at 145 days) there is less turnover of photoreceptor outer segments than in the adult sheep. At least in some species, photoreceptor turnover is closely related to time of day and cyclic lighting. It is not excluded that a diurnal rhythm in photoreceptor turnover occurs also in fetal sheep. If so, phagosomes might have been found in larger number and at all gestation ages if fixation had been made at a different time of day or under different light conditions. In the guinea pig phagosomes occur from the early stages of outer segment formation, indicating that in this species phagocytotic activity occurs throughout the intrauterine period of outer segment growth.

The low (relative to adult) concentrations of rhodopsin at 95 and 105 days corresponded well with the few and small outer segments seen at these gestation ages. At 140 days, i.e., shortly before birth, relative rhodopsin concentration and relative rod outer segment volume also agreed, whereas at 115 days the figure for the relative outer segment volume (0.21) exceeded that for relative rhodopsin concentration (0.09). There may be several reasons for this discrepancy. One contributing factor probably is that the discs are less tightly packed at the early stages (95 to 115 days) compared with the latest stage (140 days). In the rat the tightest packing of the discs probably starts after the eyes have opened and the rod outer segments have almost ceased to grow (day 14 to 22 after birth). Absence of light exposure seems to cause a further increase in rhodopsin concentration. In dark reared albino rats the rhodopsin content increases 50% compared with controls, while the rod outer segment length increases only 25% and while no apparent increase was found in rod outer segment diameter, rod outer segment disc packing density, or eye size. As shown in the present study, in sheep the packing increases in utero while the outer segments are still growing (and have not yet been exposed to light). The increase in packing is most apparent shortly before birth, probably causing a larger rise in rhodopsin concentration at this stage compared with earlier stages. In addition, the packing of rhodopsin molecules within the disc membrane may vary with the gestation age. The rhodopsin-to-lipid ratio may be low (compared with that of the adult) when the outer segments begin to form and increase during the later part of the gestation. Finally, at early stages of development the outer segments are very labile, suggesting that outer segments may have been lost during the preparation procedure before the extraction. Such a loss may have led to figures too low for the rhodopsin concentration at the early stages.

The present results show that during fetal development the increase in rhodopsin concentration follows a growth curve, the concentration 5 days before birth being about 0.6 times that in the adult. In the rat the formation of rhodopsin starts postnatally. When the eyes open (on day 14) the rhodopsin concentration is about one half that in the adult. In both species the photoreceptors are thus not completely developed when the eyes are first exposed to visual stimuli. The further increase in rhodopsin concentration up to adult level in the rat continues to follow a growth curve. Assuming a corresponding postnatal increase in rhodopsin concentration in the sheep, the adult level would not be reached until at least 40 days after birth.

In sheep the pupillary reflex can be elicited at a gestation age of 92 days. At this stage (day 95) rod and cone outer segments are very few and small, and the rhodopsin concentration is about 0.01 times that in the adult. The reflex cannot be elicited at 81 days, when the rhodopsin content is less than 0.003 times that in the adult. Rhodopsin formation thus only slightly precedes the first signs of functional maturation of the visual system. This result agrees with the observation that in the guinea pig the pupillary reflex
reflex is elicited on day 41 to 45 of gestation (term on day 62), approximately corresponding to the time (day 45) when the photoreceptor outer segments begin to form.

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