OBSERVATIONS ON SELECTED ISOLATED RETINAL ELEMENTS AND AN ANALYSIS OF ROD CELL RNA OF THE RABBIT

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ABSTRACT

RNA was analyzed in the whole rod cell and in the rod cell nucleus of the rabbit retina. The analysis was performed on rod cells or rod cell nuclei after they were isolated by microdissection and collected. The nuclei were denuded by selectively lysing inner and outer segments chemically. The rod cell contained an average of 0.65 $\mu\mu$g RNA. About 60% of the total RNA was nuclear. RNA concentration was of the order of 0.4% w/v. RNA base composition was determined for the whole rod cell and for the rod cell nucleus, and from it the base composition of cytoplasmic RNA was derived also. Microdissection of the retina revealed that each Müller cell had firmly attached to it a certain complement of visual cells, forming a bouquet-like arrangement. The unusual susceptibility of the inner and outer segments to lysis was regarded as an indication of an unique composition of the plasma membrane.

INTRODUCTION

An analysis of RNA in the rod cell of the rabbit retina is the subject of this report. The study utilized the sensitive ultramicroanalytical methods that Edström (1964) developed for dissected cellular material. Microdissection techniques were adapted for working with delicate retinal tissue without recourse to preliminary steps involving embedding of tissue, sectioning, and deparaffinization. Chemical analyses were performed on dissected, whole rod cells and on denuded rod cell nuclei.

PROCEDURE AND METHODS

Albino rabbits, weighing 2–4 kg, were used for experiments. The animal was anesthetized by ear vein with sodium pentobarbital (Diabutal). The eye was enucleated, and the anterior structures of the eye, including the vitreous body, were removed by the method of circumcising the eye at the ora serrata. The entire posterior hemisphere was placed for 7 min in a solution containing: 0.1 M of sucrose, 0.05 M of glycine, 0.05 M of sodium acetate, and 0.003 M of periodic acid. The retina was separated from the pigment epithelium, the emerging optic nerve was divided, and the retina was transferred to either: (*1*) a fresh solution of the same composition; or (*2*) a solution of 2% propylene glycol and 0.003 M of periodic acid. The former medium was used for the microdissection of single, whole rod cells, after a minimum fixation period of 2 hr. The latter medium was employed in order to denude visual cell nuclei by lysing selectively the inner and outer segments (see below). The nuclei were dissected free in the same medium after a further fixation period of 1 hr.

Instrumentation and Technique

The approach used for microdissection utilized a de Fonbrune oil chamber, as adapted by Edström (1964) for microanalysis of RNA from tissue units.

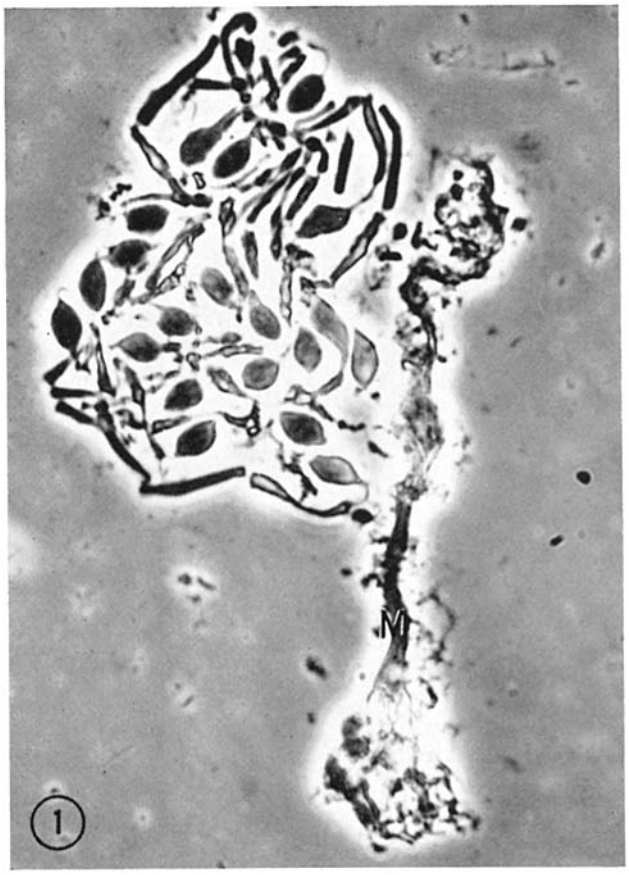

FIGURE 1 A group of intact rod cells dislodged from the Müller cell (*M*). Several hundred similarly dislodged intact rod cells were collected into a clump for RNA analysis (see Methods). Phase-contrast; × 940.

The technique requires the use of a phase-contrast microscope with a stage fixed in the vertical sense, at least when focusing with the coarse adjustment. Two de Fonbrune micromanipulators complete the basic working unit.

Microdissection of the tissue is carried out on the under surface of a cover slip in an oil chamber (see Edström, 1964). The oil chamber is simple and can be constructed from three pieces of plate glass, 2 mm in thickness: one piece, measuring 6 × 4 cm, and two pieces, measuring 2 × 4 cm each. The latter two pieces are cemented to the former piece on its upper surface, so that a distance of 2 cm separates the two, forming a well, 2 mm in depth.

A piece of retina, 1–2 mm on a side, is excised and placed on one-half of a clean cover slip. With a pair of watchmaker's tweezers, the tissue is transferred along with a thin aqueous film. Excess fluid should be removed by capillarity. The half cover slip, which must be long enough to span the 2-cm well of the oil chamber, is inverted over the well. Dow silicone oil, *c.p.* (centipoise) 350 (Corning), is introduced under the cover slip until the space between the cover slip and the well is filled with oil. Paraffin oil is less satisfactory for microdissection purposes at the water-oil interphase.

The chamber is placed on the stage of the phase-contrast microscope. The microscope is focused at the under surface of the cover slip, where the piece of tissue lies in a thin aqueous film. The chamber is racked out of the optical axis, and the microneedles, attached to two corresponding micromanipulators, are centered in the optical axis. The tips of the needles point upward, and are inclined toward each other at an angle of about 45°. Their heights are adjusted so that the tips lie in a plane below the plane of focus. The stage is racked forward, a process which brings the tissue into the optical axis, so that microdissection can commence. Cellular elements are collected near the water-oil interphase.

RNA Analysis

For analysis of RNA content, 600 whole rod cells (Fig. 1), or 600 rod cell nuclei (Fig. 2) served as the unit sample. RNA base composition was analyzed on

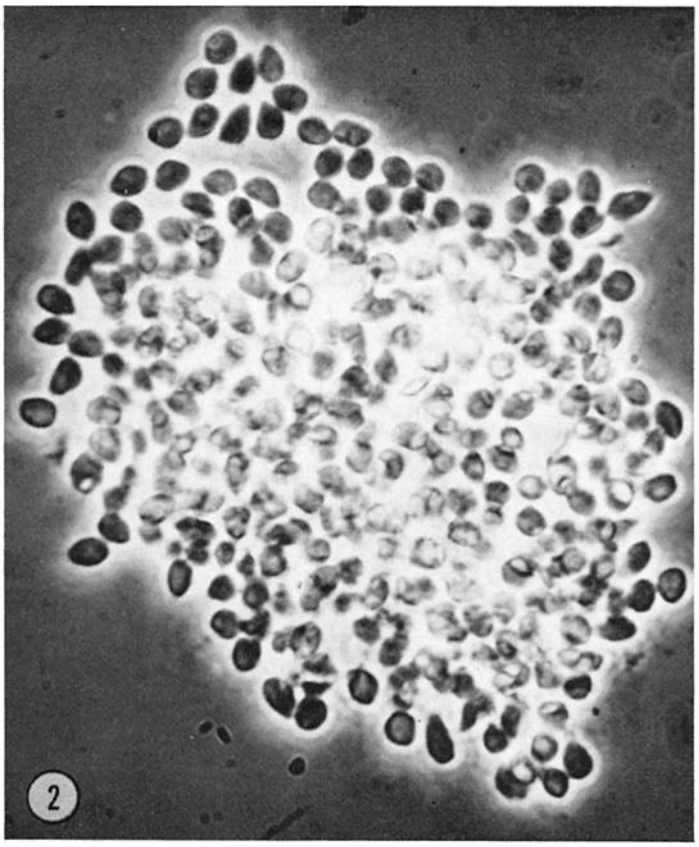

FIGURE 2 A collection of about 400 denuded rod cell nuclei similar in appearance to samples used for RNA analysis. Phase-contrast; × 1080.

unit samples containing about 600 whole rod cells, or 800–1000 rod cell nuclei. RNA content and base molar proportions were determined according to the photographic-densitometric method of Edström (1958, 1960). The lower limit of sensitivity for RNA by this method is approximately 30 $\mu\mu$g; the minimum RNA necessary for determining base composition is of the order of 300 $\mu\mu$g.

The following extraction procedure for all unit samples was employed: (*1*) 5% cold trichloroacetic acid, 5 min; (*2*) 0.01 M acetic acid, 3 min; (*3*) absolute ethanol, 5 min; and (*4*) chloroform-methanol (2:1, v/v), 5 min. The RNA was recovered after digestion with pancreatic ribonuclease (Worthington Corporation, Freehold, N.J.) (0.4 mg/ml of 0.02 M ammonium acetate, adjusted to pH 7.2 with NH_4OH). Incubation was carried out for 1.5 hr at 37°. RNA digests and electrophoretic separations of RNA hydrolyzates were photomicrographed at a wavelength of 265 mμ (Koenig, 1965).

Method for Computing Volume of Visual Cell Parts

For purposes of estimating the volume of the rod cell and its parts, the following procedure was employed. According to the theorm of Pappas, the volume of a solid generated by revolving a plane area around a coplanar axis along one side of the area is equal to the product of the area and the circumference of the circle described by its centroid; i.e., $V = 2\pi\bar{x}A$, where $\bar{x}$ is its centroid, and A is its area. The centroid can be conceived as a line, parallel to the spatially fixed axis and lying in the same plane as the area, along which the area's center of gravity is located. It can be shown that the distance between the fixed axis and the centroid is given by $3a/8$ for a parabolically shaped area, and $1a/2$ for a rectangular shaped area, where a represents the radial distance between the axis and the outer limiting boundary of the area. Since the longitudinal cross-sectional areas

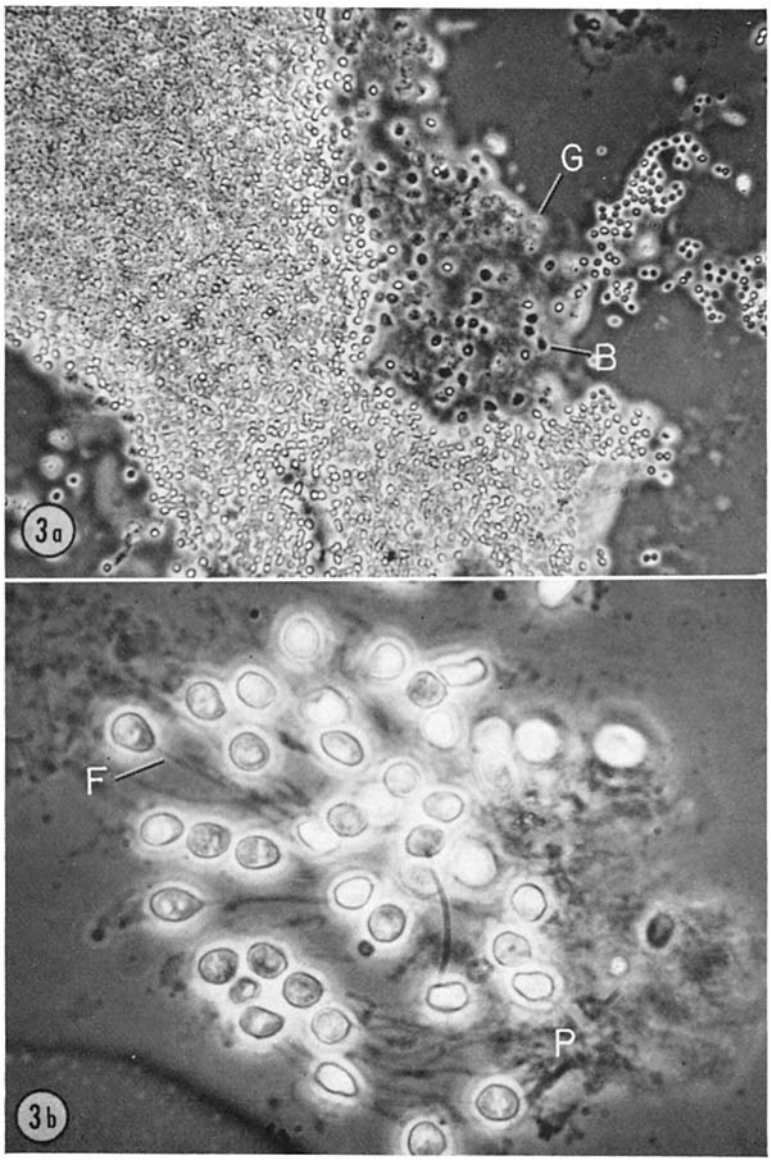

FIGURE 3 *a* A low magnification photomicrograph of a piece of retina treated with propylene glycol and periodic acid (see Methods), showing denuded visual cell nuclei. A portion of the layer of rod nuclei was removed to expose underlying bipolar (*B*) (innermost) and ganglion (*G*) cells. Phase-contrast; × 210.

FIGURE 3 *b* A high magnification photomicrograph of denuded nuclei, showing the preservation of rod fibers (*F*) and attachment to outer plexiform meshwork (*P*). Phase-contrast; × 990.

of the rod cell parts (i.e. nucleus, inner and outer segments) are symmetrical about a central axis, it is necessary to rotate the longitudinal cross-sectional area of a given part about its central axis only 1 π. Thus, the data required for computing the volume are the longitudinal cross-sectional area and the transverse radius.

The area and the radius for the rod cell nucleus, inner segment, and outer segment were determined as follows. A number of rod cells were isolated and

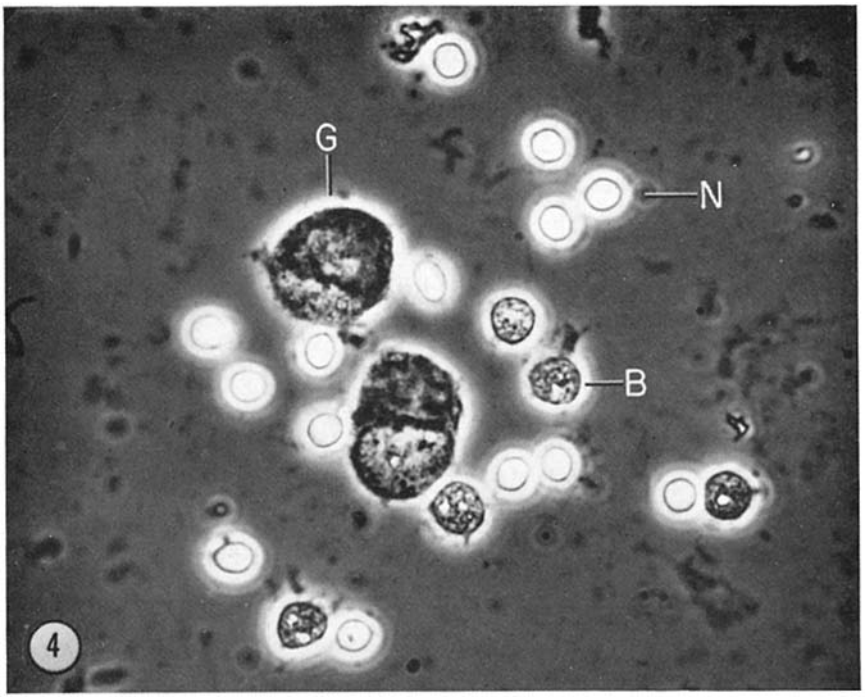

FIGURE 4 A comparison of denuded visual cell nuclei (*N*) with bipolar and ganglion cells. Note the highly refractile appearance of the visual cell nuclei and the nucleoli of the bipolar (*B*) and ganglion (*G*) cells. Phase-contrast; × 920.

photographed through a phase-contrast microscope at 700 magnification with a Polaroid camera. Ten profiles of in-focus outer segments, inner segments, and nuclei were outlined on tracing paper, cut out, and weighed to the nearest microgram. Weights were converted to cross-sectional areas described by each of the profiles. Transverse diameters for each structural element were measured with an eyepiece comparator. In cases in which diameter varied along the linear extent of the element (i.e. inner segment), the smallest and greatest diameters were averaged. The nucleus was regarded as having ellipsoidal, and the inner and outer segments as having cylindrical, geometries. The equations derived for the volume computations were:

$$\text{for the nucleus: } V = A \cdot \frac{3a}{8} \cdot \pi \cdot \frac{10^{12}}{M^3}$$

$$\text{for inner and outer segments: } V = A \cdot \frac{a}{2} \cdot \pi \cdot \frac{10^{12}}{M^3}$$

where V = volume (μ^3), a = radius (cm), A = area (cm^2), and M = magnification.

RESULTS

Microscopic Observations on Dissected Visual Cells

A property of the visual cell, especially of the rod cell, in fresh tissue which immediately distinguishes it from other nervous system elements is its fragility (i.e., susceptibility to mechanical disruption). This susceptibility to disruption is reflected in yet another respect when the retina is exposed to certain chemicals which lyse the inner and outer segments, while apparently sparing the visual cell nucleus, bipolar and ganglion cell bodies. Examples of this phenomenon after treatment with 2% propylene glycol and 0.003 M periodic acid are shown in Fig. 3 *a* (see Methods).

The apparent preservation of structure of certain retinal elements does not mean that they may not be affected or modified by such treatment, however. The visual cell nuclei appear virtually denuded, although there is a "residual skeleton" of the rod fiber maintaining its synaptic connection

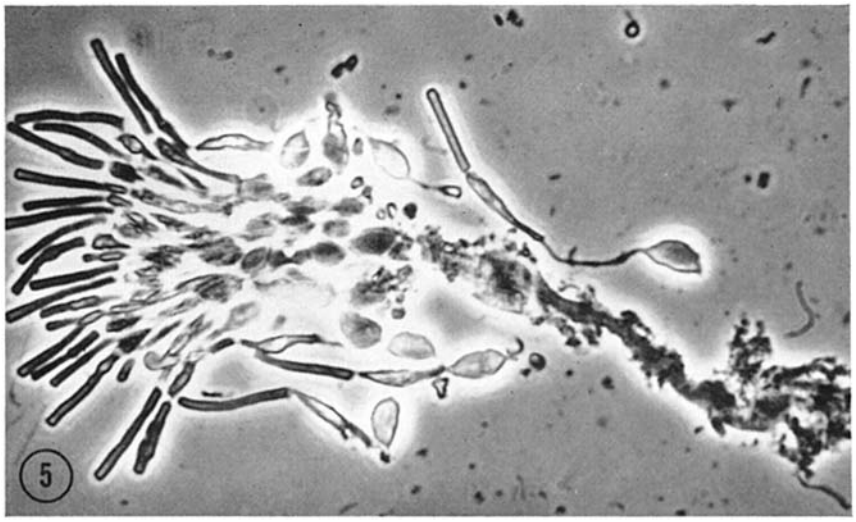

FIGURE 5 An isolated Müller cell with its attached complement of visual cells. Detached cells have been isolated from another Müller cell. Phase-contrast; × 830.

to the bipolar cell (Fig. 3 *b*); therefore, lysis does not render the visual cell nuclei free floating. The residual rod or cone fiber skeleton is extremely fragile in the rabbit after lysis of segments, but is quite resistant to disruption in the macaca monkey, the only other species examined in this respect. Other compounds (e.g. ethylenediamine dihydrochloride, iodoacetic acid, aminomethanesulfonic acid, 4-chromanol) produce similar selective lysing action (*i.e.* sparing ganglion and bipolar cells). Although the inner and outer segments seem to become solutilized when the exposure is viewed under phase-contrast microscopy, rhodopsin in rat retinae may be extracted only to a variable extent. Denuded nuclei appear refractile under phase-contrast, and in this respect they bear a resemblance to nucleoli of bipolar and ganglion cells (Fig. 4). The similarity in phase-contrast appearance would seem to suggest common regions of high protein density.

One of the constant features of the retina is the Müller cell (Fig. 1) and its complement of visual cells. In the rabbit, approximately 20 visual cells are firmly held attached by Müller cell processes to each Müller cell, resembling a bouquet (Fig. 5). The number of visual cells associated with each Müller cell was found to be considerably fewer in the monkey and many more in the rat, suggesting that this characteristic may be species-dependent.

Neither the bipolar nor the ganglion cells seem constrained by the Müller cell in the manner it exhibits with visual cells. The visual cell, in effect, must be plucked from its niche with the needle tip in order to dislodge it. With care, the synaptic spherule can be isolated with the rod cell (Fig. 6). Just proximal to the synaptic spherule, the rod fiber thins out appreciably for a short distance. In the isolation and collection of whole rod cells for RNA analysis, however, no attempt was made to preserve the rod fiber and synaptic spherule.

The over-all dimensions of the rod cell seem to remain fairly uniform. The striking difference from one cell to another in each Müller cell bouquet is the length of the rod fiber and the relative distance between nucleus and inner segment (Fig. 6). The nucleus lying innermost in the nuclear layer (i.e., closest to the bipolar cell) is an example of the extreme because this nucleus has no rod fiber, and the synaptic spherule appears as a protuberance at the base of the nucleus (Fig. 6). In this case, the nucleus is well removed from the inner segment, the latter constituting the cell's cytoplasmic site of protein synthesis (Droz, 1963; Young, 1966).

RNA Content of the Rod Cell

Quantitative RNA analysis was performed on samples of whole rod cells and on samples of denuded rod cell nuclei. In order to determine

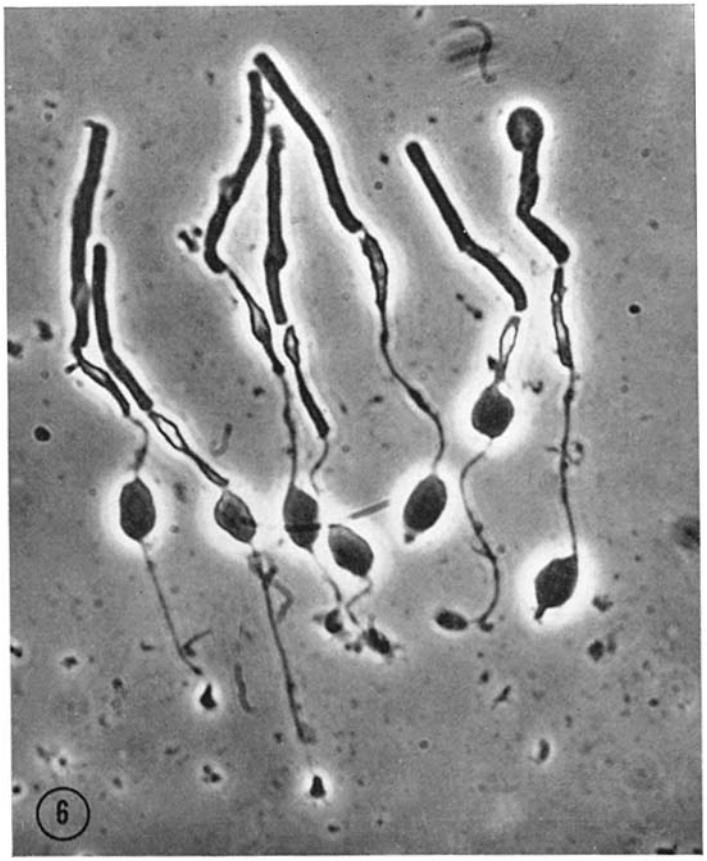

FIGURE 6 A comparison of various intact rod cells taken from different regions of the visual cell nuclear layer of the retina. Phase-contrast; × 990.

TABLE I

RNA Content, RNA Concentration, and Volume of Selected Parts of the Rabbit Rod Cell

	Whole cell (±SD)	N	Nucleus (±SD)	N	Nuclear RNA	Inner segment (±SD)	Outer segment (±SD)
					%		
RNA content ($\mu\mu$g)	0.65 ± .23	(10)	0.39 ± .11	(9)	60	0.26*	— (?)
Volume (μ^3)	176‡		91.6 ± 12.2			52.4 ± 4.8	31.8 ± 2.4
RNA concentration (w/v)	0.36%		0.42%			0.49%	—

* Difference between whole cell and nucleus.

‡ Sum of nucleus, inner and outer segments only.

the proportion of total RNA that was nuclear, a sample of whole cells and a sample of nuclei were taken from the same animal, but each pair of samples was obtained from different rabbits. The results (Table I) indicated that the average RNA content of the rabbit rod cell was 6.5 × 10^{-13} g. Approximately 60% of this amount (viz., 0.39 $\mu\mu$g) was nuclear RNA.

As a basis for comparison with other cell types, it was necessary to convert absolute quantities into concentrations. For this purpose, average volumes were computed (see Methods). The volume values (Table I) were intended only as rough estimates for establishing order of RNA concentration owing to possible inherent errors in the determinations of volume (e.g., shrinkage, irregular geometrical form, etc.).

RNA Base Composition of the Rod Cell

The base composition of the intact rod cell and that the rod cell nucleus is shown in Table II. With these data and those bearing on the fractional distribution of RNA between the nucleus and the inner segment, it was possible to derive the base composition of extranuclear, or cytoplasmic, RNA (Table II).

TABLE II

Base Molar Proportions of the Rabbit Rod Cell and Its Parts*

Base	Whole cell (±SEM)	N	Nucleus (±SEM)	N	Cytoplasm (derived)‡
Uracil	21.43 ± 0.64	10	24.4 ± 0.82	22	16.92
Cytosine	27.38 ± 0.85	10	25.83 ± 0.74	22	29.70
Guanine	28.55 ± 0.78	10	26.45 ± 0.65	22	31.70
Adenine	22.63 ± 0.32	10	23.25 ± 0.59	22	21.70

* Expressed as per cent of sum.

‡ Cytoplasm base ratio $= \frac{1.0\ (\text{Whole cell base ratio}) - 0.6\ (\text{nuclear base ratio})}{0.4}$

Since ribosomal RNA would be likely to predominate in the cytoplasm, the high molar proportions of cytosine and guanine of cytoplasmic RNA are consistent with this expectancy. With 60% of the cell's RNA in the nucleus, it is understandable also that nuclear RNA should determine to a large extent the over-all base composition of the whole rod cell.

DISCUSSION

Some years ago, Ehrlich and Dische (1950) analyzed the nucleic acids of the whole retinae of several species, and included for comparison nucleic acid analyses of a few selected tissues, such as liver, brain cortex, and optic nerve. They found that the DNA:RNA ratio was higher in the retina than in the other tissues examined. For example, in the rabbit retina ratios of 3.6 and 5.3 were obtained; that of the rabbit cortex was 0.57, a difference of about 8-fold. On the basis of present findings, the high DNA:RNA ratio of the retina can be attributed to the low RNA content of the visual cell, on the one hand, and to their large numbers, on the other. Thus, if the rod cell is assumed to be diploid, its DNA content should be of the order of 7 $\mu\mu$g (see Edström and Kawiak, 1961). Therefore, with an average RNA content of 0.65 $\mu\mu$g, the rod cell DNA:RNA ratio would be about 11. Ganglion cells, which average about 50 $\mu\mu$g RNA (Edström and Eichner, 1957), yielding a DNA:RNA ratio of 0.14, probably contribute substantially toward reducing the ratio for the whole retina to that observed by Ehrlich and Dische (1950).

A comparison of RNA concentration in the rod cell with that reported in the literature (Edström, 1956; Brattgård, Edström, and Hydén, 1957; Hydén, 1959; Edström and Eichner, 1957) for various nerve cells indicates that the red cell contains about ¼th the concentration of a nerve cell body, at best. Of course, it is important to note that concentration values depend, in some instances, on whether the volume determination was carried out on fixed or unfixed material; high values are obtained generally with fixed cells, owing to shrinkage. In the case of the rod cells, which is considered fixed material, the actual concentration, therefore, may be even somewhat lower than indicated.

In the estimation of extranuclear RNA concentration, RNA distribution in the rod cell was assumed to be limited to the region of the inner segment. This assumption was based upon: (*1*) the absence of ribosomes in the outer segment according to electron-microscopy (Sjöstrand, 1953; De Robertis, 1956); and (*2*) the radioautographic observations of Droz (1963) and Young (1966), which indicated that outer segment protein was

first synthesized in the inner segment and translocated subsequently to the outer segment. The aforementioned findings were consistent with the lack of local protein synthesizing machinery in the outer segment.

Most of the nerve cell's RNA is cytoplasmic (see Hydén, 1960), which, in turn, is ribosomal. Therefore, ribosomal RNA will determine in large measure the over-all RNA base composition of the cell. This is not the case, however, with the rod cell, owing to the fact that only 40% of total RNA is extranuclear. The over-all low concentration of RNA in the rod cell, coupled with the low cellular proportion of ribosomal RNA, may not necessarily reflect a correspondingly low rate of protein synthesis, especially if the RNA's distribution is circumscribed (i.e., highly localized). In any case, the protein-synthesizing capacity, of the red cell, when compared with that of the nerve cell, would seem to be low.

Standard histological techniques fail to show a discrete nucleolar entity in the rod cell nucleus In the nerve cell, the nucleolus is quite prominent and comprises about 25% of the nuclear RNA (Hydén, 1960). Present evidence (see Bonner, 1965; Prescott, 1964) supports the concept that precursor ribosomes are formed in the nucleolus. Close similarity has been demonstrated between the base composition of nucleolar RNA and that of cytoplasmic RNA (*i.e.* ribosomal) (Edström, Grampp, and Schor, 1961). In the present study, nuclear RNA was quite different from cytoplasmic RNA. However, this is not necessarily indicative of a lack of nucleolar RNA, or of an inability to form ribosomes after cellular differentiation. The rate of ribosomal RNA renewal or turnover may be very low and may not require an extensive nucleolar machinery. This important question would seem to merit further investigation.

Finally, some comments seem warranted regarding the ease with which inner and outer segments can be lysed in vitro (see Results) without similarly lysing ganglion and bipolar cells. Selective lysing of visual cells may be attributed to a plasma membrane that has unusual properties differing from those of ganglion and bipolar cells. Its susceptibility to dissolution or disruption may stem from a chemical composition and molecular organization that are peculiar only to the visual cell. Under certain experimental and hereditary conditions, the inner and outer segments of the visual cell show a striking tendency to undergo dissolution, culminating generally in cell death. The cell death is fairly selective, affecting rod cells more readily than cone cells, and affecting bipolar and ganglion cells least, or not at all. Conditions causing visual cell death include: (*1*) chemical agents, such as iodoacetate (Noell, 1952), iodate (Grignolo, Orzalesi, and Calabria, 1966); (*2*) X-irradiation (Noell, 1962 *b*); (*3*) vitamin A deficiency (Dowling and Gibbons, 1961); (*4*) high O_2 pressure (Noell, 1962 *a*); (*5*) hereditary factors (Noell, 1963; Dowling and Sidman, 1962); and (*6*) excessive light exposure (Noell, 1965). In the instances in which the ultrastructure was examined during the early stages leading to cell death (e.g., Grignolo, Orzalesi, and Calabria, 1966; Dowling and Sidman, 1960; Dowling and Gibbons, 1961; Noell, 1965), the disruption of the ordered membrane structures of the outer segment was an incipient sign.

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