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EFFECT OF STRONG ILLUMINATION ON THE ION EFFLUX FROM THE ISOLATED DISCS OF FROG PHOTORECEPTORS

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SUMMARY

(1) Low levels of illumination do not modify the efflux of the radioisotopes ^{22}Na, ^{86}Rb, ^{36}Cl, and ^{45}Ca from the isolated discs of the photoreceptors of the frog (*Rana Catesbeiana*). (2) The effluxes of $^{22}Na^+$, $^{86}Rb^+$ and $^{36}Cl^-$ increase when the discs are illuminated with more than 10^4 erg/cm^2 per s for a few minutes. There is no effect on the efflux of $^{45}Ca^{2+}$ or of [^{14}C]urea. (3) The effect is greater for monochromatic lights of wavelengths in the shorter region of the spectrum. (4) The effect is also present in bleached visual membranes.

INTRODUCTION

Electrophysiological measurements suggest that the light induces a permeability change in the discs of the rod photoreceptors [1]. The methods which detect the ion movements directly have not confirmed this hypothesis unambiguously and the results are conflicting [2–11]. The differences in results may be due to the different preparations, light intensities and ions used. The problem has been systematically investigated in this study. A preparation has been used in which the plasma membrane of the cell is broken, so that the isolated discs are the only diffusion compartment, and the results have been compared to those obtained in rod outer segments where the plasma membrane is intact [12]. The light intensity has been varied from a flux of a few photons per second incident per disc to fluxes 10^7 times higher and the wavelength range varied from near ultraviolet to 665 nm. Five radioactive isotopes have been used as tracers; ^{42}K, ^{86}Rb, ^{36}Cl, ^{22}Na and ^{45}Ca.

METHODS

The outer segments of dark-adapted bullfrog (*Rana Catesbeiana*) retinas were isolated in dim red light in a solution of KCl and buffers (102 mM KCl/2.6 mM $NaHCO_3$/0.2 mM NaH_2PO_4) with the method previously described [12]. The suspension was sedimented at 150 × *g* for 1 min. The supernatant was discarded and the

pellet resuspended and forced through a microsyringe needle (inner diameter 150 μm) 15–20 times. 1 ml of the suspension was added with 20 μCi of one of the following radioactive solutions: ^{22}NaCl (0.3 Ci/mol), ^{86}RbCl ($5 \cdot 10^2$ Ci/mol), $Na^{36}Cl$ (0.15 Ci/mol), $^{45}CaCl_2$($6 \cdot 10^2$ Ci/mol), [^{14}C]urea (50 Ci/mol) (Amersham). In some early experiments, the loading solution was made hypertonic with mannitol (650 mosmol). The loading period was about 2 h and the material was at + 1 °C. The suspended discs were made to sediment with a centrifuge (7000 × g for 20 min) and the supernatant discarded. The pellet was resuspended in 0.5 ml of the KCl and buffer solution and placed in a small column of glass-wool tightly packed (3 mm ⌀) or in a glass prefilter (Millipore AP250, ⌀ 6 mm) resting over a Millipore filter (SSWP, average pore size 3 μm), Fig. 1. The material of one retina was used for one perfusion. The system retained most of the material except the smallest debris. The column was perfused with the KCl solution by a motor driven syringe at the rate of 0.4 ml/min. The pH of the perfusion ranged from 7.4 to 7.7 and the temperature was 21 °C. The perfusate was discarded for 8 min and then collected in Packard scintillation vials, usually every 45 s.

For the radioactive tracers, the efficiency of the counting was 0.7 cpm/dpm, and in the efflux we could detect changes of $0.3 \cdot 10^{14}$ Na^+, $0.3 \cdot 10^{12}$ Rb^+, $1 \cdot 10^{11}$ Ca^{2+} and $1 \cdot 10^{15}$ Cl^-. At the end of the perfusion, the glass-wool column or the glass prefilter was put in a scintillation vial. The method of counting the radioactivity and the rate constant, k, has been already described [12]. The differences in the efflux were measured between the efflux collected in the third minute after illumination and the efflux deduced by extrapolating the dark efflux at that time.

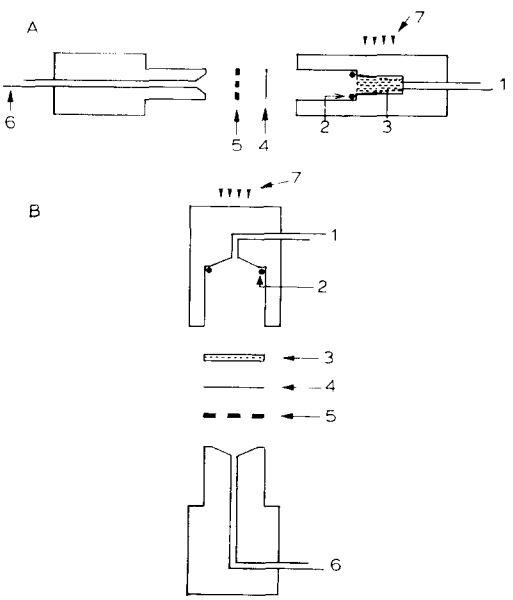

Fig. 1. Exploded view of the perfusion chambers (A) with the glass-wool column and (B) with the glass prefilter. 1, inlet tube; 2, O-ring; 3, glass bed; 4, millipore filter; 5, metal screen; 6, outlet tube; 7, light beam.

The light stimulation was made with a stimulator already described [12] and the light source was a 12 V, 100 W bulb (Zeiss) operating at a temperature of about 3200 °K, supplied by a regulated power supply (Lambda, LK 343): for ultraviolet irradiation, the radiation of a HBO 75 W (Osram) lamp was filtered with a UG1 filter 1 mm thick (Schott). A cold filter KG3 (Schott), 2 mm thick and broad-band interference filters (Filtraflex-K, Balzers) with nominal peak transmittance at 410 nm, 455 nm, 510 nm, 565 nm, 610 nm and 665 nm were used. In the first experiments, a simpler set-up was used, made of a 35 W incandescent light bulb, a condenser and a KG3 filter. The measure of the radiant flux incident on the column was made with a linear thermopile of the Moll-type (Kipp, model E4). The irradiation of the discs, however, was not uniform. On the central plane of the column of glass-wool, normal to the direction of the beam, the intensity was reduced about 100 times, but varied according to the packing of the glass-wool. The glass-prefilter reduced the light intensity by a factor of 50, but most of the rods were retained on the surface.

The amount of bleached rhodopsin was determined. The discs in the glass-wool, together with the Millipore filter, were placed in a tube with 1 ml of 2 % (v/v) solution of the detergent Emulphogene BC-720 (General Aniline and Film Corporation) for 12 h at +1 °C. The tube was then spun in a centrifuge (39 000 × g for 40 min) and a spectrum of the supernatant was taken with a DB-GT (Beckman) spectrophotometer. The absorbance at 500 nm of the difference spectrum was compared with that of a control which had not been illuminated. The percent difference was referred as to percent bleached rhodopsin.

The temperature of the perfusion was changed by immersion of the perfusion chamber in water-baths at different temperatures. A thermistor placed in the hole of the perfusion chamber measured the temperature of the fluid leaving the glass-wool column.

The loss of membranes in the perfusate from the filter was studied. Phosphate was omitted in the KCl/buffer solution and it was assumed that all the phosphorus in the perfusate was phosphorus of the membrane phospholipids. The assay for total phosphorus was made with the method of Bartlett [13].

Accumulation of ^{22}Na has been studied by a rapid filtration method on Millipore filters HAWP 304, 0.45 μm pore diameter), followed by two washings with 10 ml of cold (0 °C) KCl and buffer solution [14]. The Millipore filters were then soaked in 5 ml of scintillation medium and counted in the scintillation counter.

The equivalent exchange volume is a measure of the volume of the preparation that is at diffusional equilibrium with the radioactive loading solution. $A(t_0)$, the value at $t = 0$ of the straight line fitting the logarithm of the activity present in the material from the 8th to the 20th minute was computed. The equivalent exchange volume (V) was defined as $A(t_0)/A$, where A is the specific activity of the loading solution.

Optical microscope observation of the suspension of discs, and rod counts, were made in a Bürker chamber and electron microscope observations have been made both of a suspension and of a pellet of discs. The preparation was fixed with glutaraldehyde and stained with OsO_4, Pb citrate and UO_2 acetate.

RESULTS

Preliminary study of the disc preparation

One bullfrog eye gave about 10^6 rods and large rod fragments. After forcing the rods through the needle of the microsyringe, the size of the rod fragments was smaller and the plasma membrane was absent or interrupted. The preparation was of visual membranes, but swollen mitochondria and a small microbial contamination were also present.

The membranes in the radioactive solution of ^{22}Na accumulated ^{22}Na with complex two-step kinetics. The first accumulation could not be resolved by our filtration method and was completed in 1 min, and the slow increase tripled the activity in 3 h. The equivalent exchange volume (V) for $^{22}Na^+$, $^{86}Rb^+$, $^{36}Cl^-$, $^{45}Ca^{2+}$ and [^{14}C]urea in a series of experiments was estimated from the efflux curves (Table I). $^{22}Na^+$, $^{86}Rb^+$ and $^{45}Ca^{2+}$ had a greater equivalent exchange volume than $^{36}Cl^-$ and that of [^{14}C]urea was the smallest. These values for the equivalent exchange volumes were greater than 0.06 μl, the volume of the intradiscal space of our preparation, computed assuming an intradiscal thickness of 20 Å, a disc surface of 20 μm^2, and $1.5 \cdot 10^9$ discs per preparation. Thin sections of the preparation fixed in isotonic media showed the intradiscal space larger than normal, see Plate I. Another possibility is that the ions were absorbed on the surface of the discs.

The loss of membranes in the perfusate from the filter was about 2 per thousand membranes of the preparation in each collected sample. On the other hand, the radioactivity of the ionic species diffused from the preparation in the collected samples at a higher relative rate comprised between 2 and 5 %. Thus the percolation of the membrane material was not further considered.

Effect of illumination

The properties of the isolated discs and of the isolated rods were different. The illumination of the isolated rods, loaded with ^{86}Rb or ^{42}K, induced a reduction of the efflux of these ions in the solution of Ringer, Fig. 2. This effect was present for low levels of illumination down to $5 \cdot 10^{-2}$ erg/cm^2 per s and was not observed with ^{22}Na,

TABLE I

EQUIVALENT EXCHANGE VOLUMES

Tracer	V (μl)	Hours of loading
^{22}Na	0.78	2
	0.83	4
^{86}Rb	0.81	2
^{36}Cl	0.18	2
	0.23	2
	0.33	1.5
^{45}Ca	0.28	0.3
	0.52	3
	1.18	4
	1.07	4.5
[^{14}C]urea	0.03	2
	0.01	2

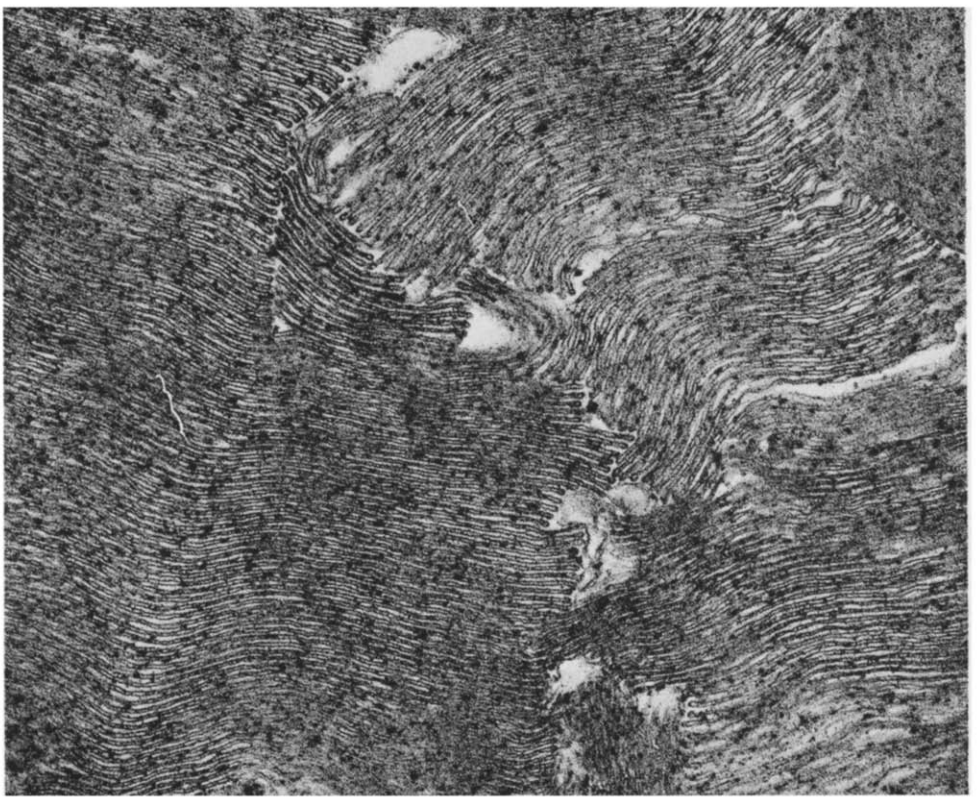

Plate 1. Thin section through a sedimented pellet of the rod fragments, seen with the electron microscope (× 60 000).

^{36}Cl or ^{45}Ca [12]. On the other hand, low levels of illumination were without effect in the prepration of isolated discs for ^{86}Rb and ^{42}K, as well as for ^{22}Na, ^{36}Cl and ^{45}Ca. Levels of illumination higher than 10^4 erg/cm^2 per s increased the efflux of ^{86}Rb, ^{22}Na and ^{36}Cl in the solution of KCl and buffers perfusing the isolated discs loaded with the radioactive ions (Fig. 3). There was no effect for ^{45}Ca (six experiments) or

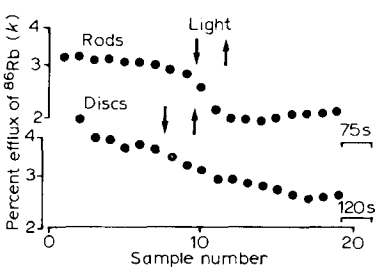

Fig. 2. Per cent loss ($k \times 100$) of ^{86}Rb per sample of perfusate from isolated rods and discs. Arrows indicate onset and offset of illumination. Ringer solution in glass-wool column. White light (500 erg/cm^2 per s). Note that the illumination reduces the efflux rate of the rods from 3 to 2 %, but does not modify the efflux of ^{86}Rb from the isolated discs.

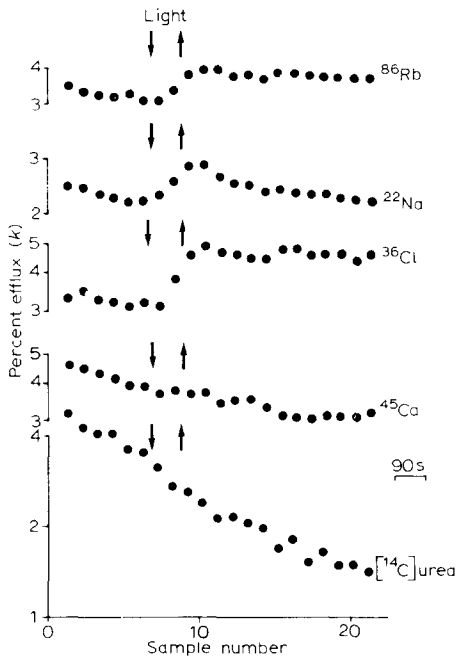

Fig. 3. Effect of light on the percent loss ($k \times 100$) of ^{86}Rb, ^{22}Na, ^{36}Cl, ^{45}Ca and [^{14}C]urea from the isolated discs. Perfusion with the solution of KCl and buffers in column. Hypertonic loading solution except for [^{14}C]urea. White light ($1.5 \cdot 10^5$ erg/cm^2 per s). Note that this strong illumination increases the efflux rate of ^{86}Rb, ^{22}Na and ^{36}Cl but not of ^{45}Ca or [^{14}C]urea.

for the non-ionic molecule [^{14}C]urea (two experiments). The amplitude of the change of the efflux was determined by taking the difference between the efflux observed in

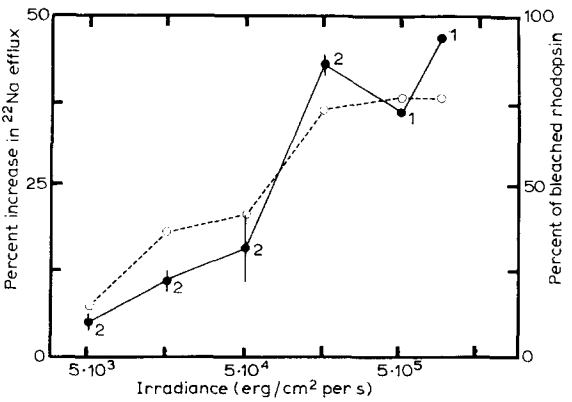

Fig. 4. Percent increase of the efflux of ^{22}Na (●) and percent of rhodopsin bleached (○) with different light intensities. Perfusion in column. The percent increase is computed 3 min after the onset of illumination. The standard deviation is indicated for each point where more than one experiment has been made. The numbers near the points indicate the numbers of the experiments.

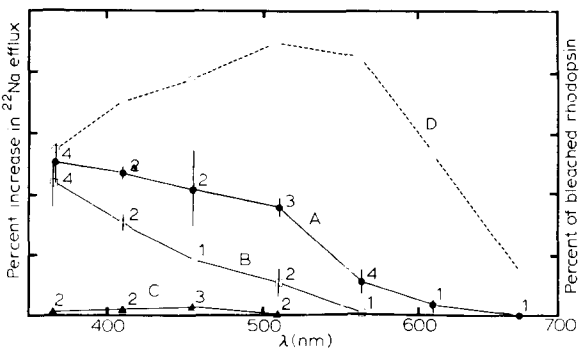

Fig. 5. Percent increase of the efflux of ^{22}Na in non-bleached discs (A), in discs bleached with green light (λ = 565 nm) (B) and blue light (λ = 410 nm) (C). Test irradiance $2 \cdot 10^4$ erg/cm^2 per s for A and $5 \cdot 10^4$ erg/cm^2 per s for B and C. Bleaching with $2 \cdot 10^6$ erg/cm^2, 15 min before the test light. Points are mean values. The standard deviations and the number of experiments are also reported. The percent of rhodopsin bleached in the experimental conditions of A is also reported (D). Perfusion in glass prefilter.

the third minute after the onset of illumination and the efflux expected at that time in darkness. Bleaching of 50 % or more of the rhodopsin was needed for a sizeable effect, Fig. 4. The effect has been studied with ^{22}Na in the following experiments. A spectral study has been made. The discs were exposed to monochromatic lights in the wavelength range 365–665 nm with a constant energy flux ($2 \cdot 10^4$ erg/cm^2 per s). The effect was greater in the shorter wavelength region of the spectrum and was absent in the yellow region (Fig. 5, Curve A).

This study was repeated in discs previously bleached with $5 \cdot 10^6$ erg/cm^2 either of green (λ = 565 nm) or blue (λ = 410 nm) light. In the discs bleached with green light, a subsequent green illumination was without effect, but blue light still gave a sizeable increase of the efflux (Fig. 5, Curve B). The discs bleached with blue light, on the other hand, were characterized by an efflux rate higher than normal, that could not be further increased by additional light in the whole wavelength region (Fig.5, Curve C).

In a group of six experiments, the isolated discs were bleached with green light (λ = 565 nm) and then illuminated with blue light (λ = 410 nm) at different intervals of time ranging from immediately after up to 1 h after bleaching. The effect was always present and there was no significant change of size.

In a preparation, the influence of temperature on the efflux of ^{22}Na in darkness has been studied. In the range from 0 to 30 °C, the Q_{10} of the efflux rate was about 2. This value is higher than for diffusion in water but not sufficiently high to suggest a metabolic drive. This mechanism is also made unlikely by the observation that the light effect was present at 0 °C (two experiments) and even when the uncoupling agent 2,4-dinitrophenol (10^{-4} M) was present in the perfusion (one experiment).

DISCUSSION

The high photosensitivity of the isolated rods is lost when the structure of the rods is broken and the discs isolated. The integrity of the rod structure seems therefore

needed in order to maintain the process of amplification that allows a few bleached photopigment molecules to excite the photoreceptor cell. The second observation is that a high intensity illumination of the isolated discs increases the efflux of $^{86}Rb^+$, $^{22}Na^+$ and also $^{36}Cl^-$ ions. The effect observed with these small ions is thus independent of the electric charge they carry. This result is in contrast with some of the previous data on the influence of light on ion movement in rod visual membranes [2, 3, 5, 15], but agrees with the report of Etingof [7] for both Na^+ and K^+ (assuming K^+, to behave as Rb^+), with the osmotic study of Heller et al. [4] and with the results of Buckser and Buckser [6] for ^{22}Na. The high levels of illumination used by some researchers, in preparations where both isolated rods and discs are present, are likely to yield an effect in both of these structures. This may lead to equivocal results, as is well illustrated by the photic decrease of the efflux of ^{86}Rb in the rods and the increase in the discs. The fact that no photic effect was detected in discs in the low luminosity range with our method does not exclude the possibility that, in the intact rod, there is a physiologically significant release of ions by the discs. The high equivalent exchange volumes determined for the different ions may be due to ions bound to the surface rather than in the disc. These bound ions could contribute to the background of radioactivity in the washout masking small responses. For Ca^{2+}, our results are negative in the entire luminosity range tested, and the possibility that light released more than a few hundred Ca^{2+} per disc can be excluded, assuming that complete isotopic exchange had been obtained. This negative result is in agreement with the report of Yoshikami and Hagins [8] but not with other preliminary reports [9–11]. Additional evidence is therefore needed. The relevant question as to whether or not the changes in efflux are due to a permeability change has not been answered in this study. Alternatively, the changes might reflect a variation in activity of the ions bound to the membrane. Experiments on the loading kinetics, which might have been very illuminating on this point, did not show a difference.

The spectral sensitivity of the effect is greater in the blue region of the spectrum and is different from the absorption of the native photopigments [16–19]. This observation rules out the physiologic significance of the effect, since vision is a much more efficient process in the green than in the blue region of the spectrum. Nevertheless, the wavelength range in which an effect was obtained covers, in part, the region of absorption of the native photopigments and of the photoproducts. The effect in the discs bleached with green light indicates that molecules different from the native photopigments can give rise to the effect. The absence of a photic effect after bleaching with blue light may be due to saturation. This interpretation is in agreement with the high efflux rate in this last condition. The molecules that are responsible for the effect have not been identified, but there is the possibility that the photopigments and the products of bleaching absorb the energy that causes the effect. The process may be similar to the permeability change induced by illumination of the red cells in the presence of a dye [20], with the difference that the light-absorbing molecules are naturally present in the visual membranes.

REFERENCES

1 Falk, G. and Fatt, P. (1973) J. Physiol. 229, 185–220
2 Bonting, S. L. and Bangham, A. D. (1967) Exp. Eye Res. 6, 400–413

3 Duncan, G., Daemen, F. M. and Bonting, S. L. (1969) Fed. Proc. Fedn. Am. Soc. Exp. Biol. 28, 331
4 Heller, J., Ostwald, T. J. and Bok, D. (1970) Biochemistry 9, 4884–4889
5 Anton'ov, V. F., Fanasyev, A. L. A., Ostrovsky, M. A. and Federovich, I. B. (1971) Biophysics 16, 78–86
6 Buckser, S. and Buckser, C. (1971) Exp. Eye Res. 12, 138–146
7 Etingof, R. N. (1972) Vision Res. 12, 929–941
8 Yoshikami, S. and Hagins, W. A. (1973) in Biochemistry and physiology of visual pigments (Langer, H., ed.), pp. 245–256, Springer-Verlag, Berlin
9 Poo, M. M. and Cone, R. (1973) Exp. Eye Res. 17, 503–510
10 Mason, W. T., Fager, R. S. and Abrahamson, E. W. (1974) Nature 247, 562–563
11 Hendriks, Th., Daemen, F. J. M. and Bonting, S. L. (1974) Biochim. Biophys. Acta 345, 468–473
12 Cavaggioni, A., Sorbi, R. T. and Turini, S. (1973) J. Physiol. 232, 609–620
13 Bartlett, G. R. (1959) J. Biol. Chem. 234, 466–468
14 Kepes, A. (1969) in Biophysics (Passow, H. and Stampfli, R., eds), pp. 49–62, Springer-Verlag, Berlin
15 Bowmaker, J. K. (1970) Vision Res. 10, 601–604
16 Reuter, T. E., White, R. H. and Wald, G. (1971) J. Gen. Physiol. 58, 351–371
17 Reuter, T. E. (1966) Vision Res. 6, 15–38
18 Liebman, P. A. and Entine, G. (1968) Vision Res. 8, 761–775
19 Dartnall, H. J. A. (1967) Vision Res. 7, 1–16
20 Davson, H. (1964) General Physiology, pp. 1041–1046, J. A. Churchill