Table 1. Effect of variation of time of hydrolysis in the Feulgen reaction on DNA content of Purkinje neurons.

Time (min)	Mean DNA (5 cells)	S.E., ±
10	24.9	0.53
12	28.8	1.17
15	27.4	0.53
18	24.9	.85
21	20.2	1.08
25	16.8	0.67
30	10.6	.86

staining is equivalent to or greater than that of diploid forms. The pallor of Purkinje cell nuclei is readily explained by their enormous volume, however, which is some fivefold to tenfold greater than the volume of most diploid nuclei, and four to five times that of tetraploid cells of liver.

3) Ages of patients from whom specimens were obtained ranged from 6½ months to 53 years. In the Purkinje neuron, tetraploidy is thus present in infancy and appears to be stable thereafter; in contrast, polyploidy in the cells of human liver develops after infancy and is an age-related phenomenon (*5*).

The significance of tetraploidy is not known. The Purkinje cell is a large neuron with an elaborate and extensive dendritic tree; it provides a complex, integrative action as the sole efferent neuron of the cerebellar cortex. In keeping with the concept that DNA is the basic determinant of cellular synthetic processes and ultimately of cellular function, the doubled content of genetic material in the mature, differentiated Purkinje cell may be expected to achieve expression in the metabolic machinery of this neuron.

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Visual Pigment Fluorescence

Abstract. *The fluorescence of cattle rod outer segments (dried) and of rhodopsin in solution lies in the range of 575 to 600 millimicrons with a quantum efficiency of 0.005 if excitation is in the visible band near 500 millimicrons. The emission is abolished by bleaching at −196°C but can be reversibly regenerated by irradiation with light of longer wavelength (600 millimicrons). This behavior reflects the known interconversion of rhodopsin to prelumirhodopsin at this temperature.*

In the initial photoprocess relating to vision, the primary function of the exciting photon is to convert the chromophore related to 11-*cis*-retinal to the all-*trans* isomer (*1*). Such a structural change appears to induce a protein rearrangement which in some unknown way could lead to membrane depolarization and complete the transduction process. A number of mechanisms to account for this transfer of information have been proposed (*2*). However, experimental evidence in favor of one mechanism over others has not been presented.

If we examine the initial photoprocess in more detail, we find that there must be some dissipative process to allow the excited state to return to the ground state (*3*). However, it is not at all clear by which mechanism this occurs. Possible mechanisms are: a reemission process (such as fluorescence or phosphorescence), radiationless transfer, exciton formation, or simply dissipation as heat. The problem is compounded in that the dissipative process itself may be the triggering mechanism, and the bleaching phenomena may be only a process controlling sensitivity (*4*).

To investigate this point more closely, we began a study of the emission characteristics of intact rod outer segments and of rhodopsin extracts obtained in 2-percent digitonin. Previous investigations of visual pigment fluorescence have dealt with the emission characteristics of the products of bleaching rather than rhodopsin. The first fluorescent species described (*5*) is a pigment (probably pigment 465) formed after the decay of metarhodopsin (probably meta II). There are apparently no reports of a fluorescence from any earlier species, including rhodopsin. If a rhodopsin fluorescence does exist, according to Hagins, it might be in the near infrared and would thus be difficult to detect. On the other hand, our experiments indicate that a rhodopsin fluorescence does exist and occurs closer in wavelength (575 mμ) to its absorption maximum (500 mμ) than was expected.

Rod outer segments were obtained from frozen retinas of cattle (*6*). The segments were isolated by a sucrose flotation method similar to that described by Wald and Brown (*7*). Digitonin solutions (2 percent) were prepared from these rods and filtered through calcium phosphate gel (*8*). As an index of purity, the ratio of the absorbancies at 400 and 500 mμ was found to fall in the range of 0.24 to 0.30, an indication of fairly pure preparations.

Fluorescence measurements were obtained with a modified version of the Aminco-Bowman spectrophotofluorometer with the R136 photomultiplier detector. When a high-intensity monochromator (*6*) and xenon light source were substituted for the Aminco source, they gave doubly monochromatized excitation light. A marked reduction in scattering resulted with this modification, and a further reduction was obtained with the insertion of variable band-pass interference filters of the appropriate wavelength cutoffs. Wavelength standardization was obtained by a series of compounds of known fluorescence activity. Intensity corrections applied to correct the spectra were obtained with the YSI bolometer. We then estimate that, for these rather broad emission spectra, the wavelength maxima are probably not accurate to more than ± 10 mμ.

The samples were placed in 3-mm cylindrical quartz tubes maintained in position in a quartz Dewar flask. To avoid large self-absorption distortions in the emission spectra, we kept the concentrations of the rhodopsin and digitonin samples quite low (in the 10^{-5} to $10^{-6}M$ range). For the low-temperature measurements on rhodopsin, the solvent used was a mixture of glycerol and water (50 : 50). All manipulations of light-sensitive materials were carried out with only deep-red illumination. The observed emis-

sions apparently are fluorescences rather than phosphorescences because they do not pass through a rotating shutter with a resolving power of 5×10^{-4} second.

Our first results (Fig. 1) were obtained with freeze-dried rod outer segments. Illumination in the 490- to 500-mμ region produced a moderately weak fluorescence with a maximum at 600 mμ (curve 1). Although the peak height of this fluorescence decayed when partially bleached with 440-mμ light (curves 2 and 3), illumination with light of wavelength greater than 600 mμ produced a regeneration of the original fluorescence (curve 4). This emission could be abolished with extensive bleaching at room temperature.

The behavior of the emitting species under the conditions of this experiment seems to be as expected when rhodopsin undergoes the reversible conversion to prelumirhodopsin. Yoshizawa and Wald (*9*) showed that rhodopsin at −196°C can be converted to prelumirhodopsin by illumination with light in the rhodopsin absorption band. The reverse conversion from prelumirhodopsin back to rhodopsin could be accomplished by light primarily in the prelumirhodopsin absorption band and not in the rhodopsin band (that is, light of wavelengths between 600 and 630 mμ). This interconversion could be repeated a number of times; this appears to be the case with the rod fluorescence described above.

To obtain further evidence that the 600-mμ emission arises from the rhodopsin chromophore, we measured rhodopsin extracts in digitonin. A weak emission was observed at about 570 mμ, again produced by excitation in the 490-mμ region from a dilute rhodopsin sample maintained at 3°C. When more concentrated samples with A_{500} of 0.8 or greater were used, the emission was quite easily observed (Fig. 2, curve 1). At this concentration, however, self-absorption occurs and certainly distorts the resultant emission. Our experience with other fluorescing compounds indicates that, under the conditions used, there exists a red shift of perhaps 10 mμ for which correction must be made.

At liquid nitrogen temperatures, a weak emission at 575 mμ is observed from the solution of rhodopsin and digitonin in the mixture of glycerol and water. The bleaching characteristics of this outer-segment fluorescence are identical to that of the rods, including the reversibility of the effect by illumination by light of wavelengths longer than 600 mμ. One difference between these two fluorescences is the slightly lower energy of the former. This emission is centered at 600 ± 10 mμ, whereas that from rhodopsin is at 575 ± 10 mμ. The anhydrous environment of the dried rods may be responsible for this shift, but we could not check this because our apparatus was not able to measure the fluorescence of wet rods.

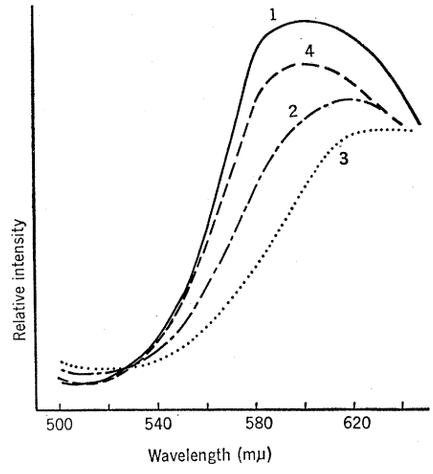

Fig. 1. Emission spectra of dried rod outer segments at −196°C. Curve 1, normal spectrum of freshly prepared rods; curve 2, after short exposure to bleaching light, $\lambda = 440$ mμ; curve 3, after prolonged exposure to bleaching light; curve 4, same as above but after short exposure (1 minute) to light of long wavelength, 600 m$\mu < \lambda < 630$ mμ.

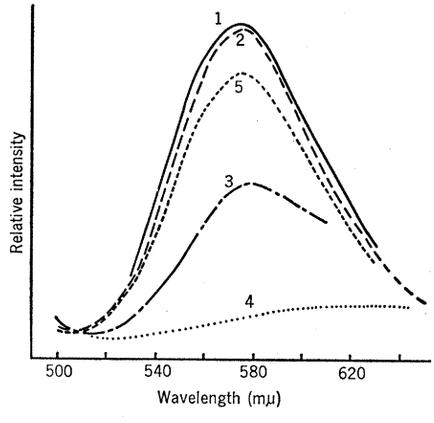

Fig. 2. Emission spectra of rhodopsin. Curve 1, rhodopsin emission at 3°C, *p*H 6.7; curve 2, emission of rhodopsin in a mixture of glycerol and water at −196°C; curve 3, same as above but exposed to a bleaching light ($\lambda = 440$ mμ) for 1 minute; curve 4, same as above but with prolonged exposure to white light at room temperature; curve 5, same as curve 3, but after exposure to light of long (600 m$\mu < \lambda < 630$ mμ) wavelength. Spectra are not corrected for self-absorption distortions.

We were able to obtain an approximate quantum efficiency for this emission by a comparative method similar to that described by Parker and Rees (*10*). Essentially the method compares the absorbancies A_s and A_u of the standard and unknown compounds and also their fluorescence intensities F_s and F_u (these are found from the areas under the fluorescence curves). If the quantum efficiency of the standard is known, then the quantum efficiency of the unknown is given by

$$\phi_u = \phi_s(F_u/F_s)\,(A_s/A_u).$$

Rhodoamine B in ethanol was used as a standard because its spectroscopic properties are similar to those of rhodopsin and its fluorescence efficiency is known (*10*). We selected 440 mμ as the excitation wavelength at which A_s and A_u were measured. With this procedure we estimate ϕ to be 0.005. This was derived for excitation at 440 mμ of digitonin and rhodopsin solutions in 50 percent glycerol at 77°K.

In that the signals are weak, it is possible that, instead of a rhodopsin fluorescence, we are observing an impurity or artifact fluorescence. This substance could have a high fluorescent efficiency and, although present in small amounts, could produce the observed spectra. If we assume that the newly generated prelumirhodopsin can quench the fluorescence of the impurity either through reabsorption or energy transfer, then the reversible change in the intensity of the 575-mμ emission would be directly related to the amount of the prelumirhodopsin species present. That is, the interconversion would affect the impurity fluorescence by changing the amount of quencher (prelumirhodopsin) present.

We may distinguish between these two explanations by the following experiment. If I_0 is the fluorescence intensity of a fresh rhodopsin preparation and I' is the intensity obtained after extensive exposure to 440-mμ radiation, then, by destroying the prelumirhodopsin present, we should expect one of two results: (i) the intensity should return to I_0 if the emission arises from an impurity; or (ii) it should remain at I' if it emanates from rhodopsin.

In principle, the experiment is simple because the prelumirhodopsin form can easily be removed by heating the sample to room temperature for about 1 minute. At this temperature, this form is converted rapidly to meta I- and meta II-rhodopsin, both of which absorb at shorter wavelengths and should not interfere with an emission at 575 mμ.

Cooling of the sample again to 77°K should allow the 575-mμ fluorescence to be measured again. This proved to be difficult because the recooling process invariably produced a glass with slightly different scattering properties from that formed initially. Therefore, the intensity of the 575-mμ emission could not be related quantitatively to the intensity observed initially. This problem was solved by the addition of a fluorescent internal standard to the rhodopsin solution. Fluorescein amine was chosen for this purpose because its emission which is at 500 mμ would not interfere with the 575-mμ fluorescence and because it was clearly resolved from the scattering background and the peak at 575 mμ. If we keep constant the intensity and resolution of the fluorescein amine emission, we may judge the quantitative behavior of the 575-mμ peak.

In five experiments, we consistently found the intensity of the 575-mμ emission after heating to be that observed immediately after the 440-mμ irradiation and not that found initially. Apparently, the 575-mμ fluorescence is not dependent on the amount of the prelumirhodopsin form present, and thus it probably does not come from an impurity. It is reasonable to assume then that this emission arises from rhodopsin.

If the photoconversion of rhodopsin to prelumirhodopsin is carried out by light of 440-mμ wavelength, Yoshizawa and Wald (*9*) found only a 57-percent conversion. Apparently, there is a photoequilibrium in which the prelumirhodopsin form, which also absorbs at 440 mμ, is driven back to rhodopsin. In measurements of the 575-mμ fluorescence, we find a similar situation; that is, irradiation at 440 mμ apparently reduces the intensity of the 575-mμ emission, but only to a value I', which is of the order of 50 percent of the initial intensity I_0. If we destroy the prelumirhodopsin species formed after a given irradiation, the resultant intensity now at the value I' can be further reduced with irradiation at 440 mμ to a value about half I' or $I_0/4$. We have not been able to record absorption spectra for these irradiated samples; however, the behavior described above qualitatively parallels that found by Yoshizawa and Wald for their rhodopsin samples.

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Histone Acetylation in Insect Chromosomes

Abstract. *Acetylation of histones takes place along the salivary gland chromosomes of* Chironomus thummi *when RNA synthesis is active. It can be observed but not measured quantitatively by autoradiography of chromosome squashes. The "fixatives" commonly used in preparing squashes of insect chromosomes preferentially extract the highly acetylated "arginine-rich" histone fractions; the use of such fixatives may explain the reported absence of histone acetylation in* Drosophila melanogaster.

Acetylation of histones does occur on insect chromosomes, and a recent report to the contrary by Ellgaard (*1*) is based on inadequate methods of histone fixation. Ellgaard stated that there is "a definite absence of chromosomal acetylation" and that there is no preferential acetylation of histones in regions of gene activation for RNA synthesis. His conclusions were based entirely upon negative results obtained in the autoradiography of chromosome squashes after excised salivary glands were incubated in the presence of sodium acetate-^{3}H (*1*). If correct, these results would constitute a serious objection to the view that the acetylation of histones is correlated with gene activation for RNA synthesis (*2–4*). However, it is not likely that they are correct.

The conditions employed in preparing the *Drosophila melanogaster* chromosome squashes [treatment with a mixture of ethanol and acetic acid (3 : 1), followed by 45 percent acetic acid (*1*)] are not suitable for studies of histone metabolism; such "fixatives" not only dissolve free histones, but also extract the greater part of the radioactive histone from the chromatin of other nuclear types, such as calf-thymus nuclei labeled in vitro with ^{14}C-acetyl coenzyme A, or rat-liver nuclei labeled in vivo with sodium acetate-(methyl-^{3}H). We have evidence that there is a definite incorporation of radioactive acetate into the chromosomes of another insect species *Chironomus thummi*; the incorporated radioactivity is detectable by autoradiography (even after the poor histone fixation procedures usually employed in the preparation of salivary chromosome squashes).

In studies of histone acetylation in mammalian tissues, it has been observed that the radioactive acetate is largely incorporated into "arginine-rich" histone fractions (*2–4*). In rat liver, for example, more than 60 percent of the newly incorporated acetyl groups appear in the arginine-rich f2al fraction prepared by the method of Johns (*5*). In this protein, the acetyl group is believed to be attached to the nitrogen of the amino-terminal serine residue (*4, 6*). Many acetyl groups also appear in the arginine-rich f3 histone fraction, where they are joined to the protein by both *N*-acetyl and *O*-acetyl linkages (*4*). Both of these arginine-rich histone fractions are soluble in ethanol after treatment with acids, and this solubility is the basis of one of the most important methods for their fractionation (*5*).

When arginine-rich histones are prepared from calf-thymus nuclei and suspended in the mixture of ethanol and acetic acid used in preparing insect chromosome squashes, much of