5. R. H. Green and D. W. Woolley, *J. Exptl. Med.* 86, 55 (1947); D. W. Woolley and R. H. Green, *J. Bacteriol.* 54, 63 (1947); D. W. Woolley, *J. Exptl. Med.* 89, 11 (1949).
6. T. M. Rivers *et al.*, *Viral and Rickettsial Infections of Man* (Lippincott, Philadelphia, ed. 2, 1953).
7. This work was supported in part by a grant from the Office of Naval Research (contract No. N6 ONR-24309) and in part by a grant from the Division of Research Grants and Fellowships, U.S. Public Health Service.
8. J. E. Salk, *J. Immunol.* 49, 87 (1944).
9. M. G. Sevag, *Biochem. Z.* 273, 419 (1934).
10. The combustion analyses were performed by the Clark Microanalytical Laboratory, Urbana, Illinois.
11. R. Dreywood, *Ind. Eng. Chem. Anal. Ed.* 18, 499 (1946).
12. L. A. Elson and J. J. Morgan, *Biochem. J. (London)* 27, 1834 (1933).
13. H. M. Randall *et al.*, *Infrared Determination of Organic Structures* (Van Nostrand, New York, 1949).
14. We wish to thank the Wilson Laboratories, Chicago, Ill., for supplying us several times with concentrates from 600-lb lots of pork lung.
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Concentration of Visual Purple in a Retinal Rod of Rana pipiens

The density of the photosensitive pigment in the distal segments of the retinal rods and cones determines the effectiveness of radiation incident upon the retina in evoking a visual response. Only the light that is absorbed by the pigment in the visual cells can be effective in initiating a visual stimulus. Since there are significant losses of light in the transparent media of the refractive apparatus and in the neural layers of the retina, it is of importance to know what portion of the light that finally reaches the distal segments will be absorbed. This can be evaluated by a knowledge of the density of the pigment within the distal segments of the retinal rod.

Several methods have been applied to the determination of the density of visual purple in the distal segment of the retinal rod. One procedure has been to determine the maximum amount of pigment that is extractable from a known number of retinas and to estimate how much light this quantity of pigment would absorb if it were dispersed in an area corresponding to the retinal distribution of rods (*1*). A second method is to estimate the total visual pigment in the outer limb by measuring the minimum molecular weight per chromophore and computing the total content by assay of the carotenoid component (*2, 3*). An additional and completely unrelated method is to compare the scotopic luminosity curve and the spectral absorption curve of visual purple as used by Hecht *et al.* (*4*) in order to estimate the absorption by rod segments in the retina.

A more direct method is to assay the extracted visual purple and to reduce the data to the dimensions of a single distal rod segment, as was attempted by Broda *et al.* (*5*) and Denton (*6*). The problem of light absorption in the distal segments has become increasingly important in the consideration of the mechanism of the initiation of visual response.

In the series of estimations reported here (*7*), *Rana pipiens* were used. The animals were adapated to the dark for 24 hours, the eyes were removed and sectioned, retina and pigment layer were removed, and the pigment layer was separated from the retina. The retinas were washed in Ringer's solution and then very gently shaken in it. This served to detach the distal segments of the rods. The rod segments were separated from the remainder of the retinal debris by straining through fine wire mesh and recovered from the Ringer's solution by low-speed centrifugation. The accumulated rods were resuspended in a known volume of Ringer's solution, and the total number of rod segments in the solution was estimated by means of a hemocytometer (Table 1, column 3). The visual purple was extracted in 5 ml of digitonin solution after the suspended rod segments had been collected by centrifugation at high speed. The absorption spectrum of the resulting solution was measured with a Beckman DU spectrophotometer. The density at 500 mμ is given in Table 1, column 4. The residue remaining after the centrifugation of the first digitonin extract was extracted a second time with fresh digitonin, but it did not contain a measurable quantity of visual purple.

The dimensions of the distal segments of the rods were measured by means of an ocular micrometer. Fifty separate measurements gave an average length of 52.1 μ and a diameter of 7.6 μ. From these figures, the cross-sectioned area of a single rod was computed to be 45.36 μ^2.

Since the number of rod segments in each preparation is known, the contribution of a single rod segment to the density of the total visual purple solution can be computed. This is shown in Table 1, column 5.

Assuming that Beer's law is valid over the ranges of concentration encountered, we can estimate the density of the visual purple in a single rod segment by reducing the cross-sectional area of the solution to that of a single rod. Since the density of the solution for a depth of 1 cm is known from direct measurement, this manipulation is in effect equivalent to computing the density of the solution when its cross-sectional area is equal to that of a single rod and when its depth is permitted to increase until the volume is identical with that of the original 5-ml extraction. This procedure yields a concentration factor by which the contribution of a single rod segment to the density is multiplied to yield the density of pigment in a distal segment of the rod. The concentration factor and the computed density of the visual purple in the rod segment are shown in Table 1. The density of pigment in the rod segment is 0.642 at a depth equivalent to the axial length of the rod, 52 μ, and the density per micron along the axis of the rod is 0.012.

This value is higher than that reported by Broda *et al.* (*5*) or that computed by Hubbard (*3*), and it is of the same order of magnitude as that reported by Denton for another species of frog (*6*). Since this method of assay requires the solubilization of the pigment, the unavoidable losses during processing mean that these are minimal values. In addition, if the distal segments of the rod are not removed intact, there must be a portion of the visual pigment which remains in the retinal layers. The density per micron depth of a distal segment of a rod is a more general and useful value and is obtained by using the value of 52.1 μ for the length of the segments analyzed. This value of 0.01233 per micron is also shown in Table 1.

An additional consideration in estimating the maximal absorption by visual purple in a single rod is the degreee of orientation of the anisotropic molecules (*8*). The absorption of unpolarized light by a solution of randomly oriented anisotropic molecules would be lower than that of a rod that consisted of a series of parallel planes of optically oriented

Table 1. Density of visual purple in retinal rods. The concentration factor is 0.1102×10^8; the computed density of the pigment in the rod segment along the axis (density per rod) (factor) is 0.642; and the density per micron along rod axis is 0.012.

Preparation No.	Number of retinas	Total rods	Optical density at 500 mμ for 1-cm depth of solution	Density per single rod in 5-ml solution at 1-cm depth
1	25	4.06×10^6	0.256	0.063×10^{-6}
2	100	18.175×10^6	1.049	0.058×10^{-6}
3	100	26.18×10^6	1.410	0.054×10^{-6}
			Av.	0.0583×10^{-6}

molecules in which the axes of the successive planes were randomly oriented, but would be greater than that of a case in which the optical axes of the planes were all parallel. The latter situation places an upper limit on the absorption by the completely oriented element of 50 percent of the incident light. Until the detailed orientation of the visual purple molecules is known, this question cannot be resolved.

A density of 0.642 in the distal segment of the rod means that approximately 75 percent of the light incident on the rod segment is absorbed if its path is parallel to the axis of the rod segment. However, the retinal rods do not occur on the optical axis but are distributed peripherally. As a result, a beam of light which is less than the diameter of a rod in cross section may follow a path that will intersect several rods despite the curvature of the retina in the optic cup. If the beam falls at such an angle that it traverses the longest diagonal axis of a single rod, the probability of absorption would increase, for the path length through the rod is greater, and a somewhat thicker layer of photosensitive pigment would be traversed. In this case, the absorption would be 80 percent. If the beam intersected a closely packed, parallel bundle of rod segments at an angle of 45°, the absorption would be nearly 90 percent. Therefore, in species possessing a fovea, peripheral vision not only has the advantage of the higher absorption because of higher pigment concentration but also has the increased possibility of light gathering by the obliquity of the incident light.

As shown by Hecht *et al.* (*4*), a single quantum hit in each of only a small number of rods is required to produce a visual sensation in man at threshold. With absorptions by the individual elements of this order of magnitude or even considerably lower in the case of the mammalian eye, as suggested by Hubbard (*3*), the probability is high that the effective photochemical event will occur in the surface region of the receptor cell and that the resulting activated products will be in the optimal position for the initiation of the process that leads to membrane asymmetry and the initiation of an impulse, if indeed it can be assumed at this time that a mechanism as direct as this is involved in the initiation of the impulse.

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References and Notes

1. G. Wald, *J. Gen. Physiol.* 21, 795 (1938); A. König, *Sitzber. kgl. preuss. Akad. Wiss.* No. XXX, part 2, 577 (1894).
2. F. D. Collins, R. M. Love, R. A. Morton, *Biochem. J. (London)* 51, 669 (1952); G. Wald and P. K. Brown, *J. Gen. Physiol.* 37, 189 (1953).
3. R. Hubbard, *J. Gen. Physiol.* 38, 381 (1954).
4. S. Hecht, S. Shlaer, M. H. Pirenne, *ibid.* 25, 819 (1942).
5. E. E. Broda, C. F. Goodeve, R. J. Lythgoe, *J. Physiol. (London)* 98, 397 (1940).
6. E. J. Denton, *ibid.* 127, 81 (1955).
7. This work was supported by grant B-238 from the National Institute of Neurological Diseases and Blindness, National Institutes of Health, U.S. Public Health Service.
8. B. Commoner and D. Lipkin, *Science* 110, 41 (1949).

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Radiocarbon Age Estimates Obtained by an Improved Liquid Scintillation Technique

The physical and chemical techniques used in our laboratories for radiocarbon dating have already been described (*1*, *2*). The purpose of this work is to indicate an important modification in method and to report on the samples measured to this time (*3*).

In the liquid scintillation method of radiocarbon dating, the sample carbon is introduced into the sensitive volume as the solvent component of the scintillator or as a suitable diluent. Because of the low energy of the C^{14} beta spectrum and the resultant feeble light pulses, it is necessary to use a scintillator with as high a fluorescence efficiency as possible. This is particularly important for a single-channel counter used at ambient temperatures. We therefore first attempted the synthesis of the solvent component of the liquid scintillator from sample carbon (*1*), for this insures maximum luminescence output in the final scintillator solution.

On the other hand, for routine operation, the complexity of the chemical syntheses of such solvents as toluene and xylene suggests the use of an appropriate diluent whose chemical synthesis is less involved, and whose presence in the liquid scintillator does not appreciably reduce the luminescent output. In an earlier report (*2*), we showed that methanol could be used as a diluent in a liquid scintillator for the detection of natural radiocarbon with a 30-percent efficiency. An examination of Fig. 1 in that report shows, however, that labeled carbon concentrations of greater than 0.1 g/ml of scintillator are not feasible, for then the C^{14} spectrum and the photomultiplier noise spectrum overlap too markedly. A specially selected K1190 photomultiplier was used in this work. Subsequently, three of six C467 Dumont tubes tested have been found to give approximately the same low noise level as this selected experimental tube.

Recently (*4*) it has been shown that the use of naphthalene as the second solvent reduces the quenching action of many materials in liquid scintillators. Using this technique, we were able to introduce appreciable quantities of trimethyl borate (*5*) into a liquid scintillator without seriously affecting the efficiency of fluorescence. Since the methyl borate can be prepared quite readily from methanol, the possibility of its use as the labeled component for routine radiocarbon dating has been investigated.

The preparation of methanol from sample carbon was performed by procedures already described (*2*). The conversion of the methanol to methyl borate was accomplished by a modification of a procedure of Schlesinger *et al.* (*6*). In principle, the method involved the esterification of boric oxide with methanol and the separation of the resultant azeotrope. In a typical synthesis, 18.80 g (0.59 mole) of methanol was placed in a 100-ml flask fitted with a semimicro fractionating column, and 13.72 g of finely powdered boric oxide was added in four portions through a side arm. Following the addition of each portion, the contents of the reaction vessel were refluxed gently. Upon completion of the addition, the reaction mixture was refluxed a further 2 to 3 hours. The contents were then distilled through the column, and the methanol-methyl borate azeotrope was collected in a receiver immersed in dry ice. The azeotrope was then separated by rapid addition of 4.2

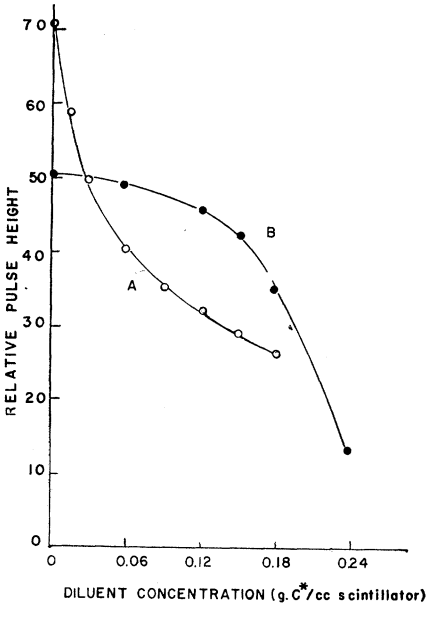

Fig. 1. Dilution curves showing pulse height, relative to anthracene at 100, as a function of diluent concentration expressed as grams of labelled carbon per milliliter of scintillator. *A*, Diluent methanol in solution of 4 g/lit of terphenyl and 0.1 g/lit of 1,4 di-2-(5-phenyloxazolyl)-benzene in xylene; *B*, diluent methyl borate in solution of 3 g/lit of phenylbiphenylyloxadiazole, and 70 g/lit of naphthalene in xylene.