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AN EVALUATION OF THE CHARGE-TRANSFER MODEL FOR THE CHROMOPHORES OF THE RETINAL-CONTAINING PROTEINS, RHODOPSIN AND BACTERIORHODOPSIN

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The chromophores of rhodopsin and bacteriorhodopsin are believed to result from an electrostatic interaction between the protonated Schiff base of retinal and amino acid side chains. It has been proposed from ESR measurements on rhodopsin (Shirane, K. (1975) Nature, 254, 722–723) and model studies using retinal and tryptophan (Ishigami, M., Maeda, Y. and Mishima, K. (1966) Biochim. Biophys. Acta 112, 372–375) that the interaction is one of charge transfer and that the amino acid involved is tryptophan. Our re-examination of this work does not support the existence of a charge-transfer complex. However, additional similarities between the model system and bateriorhodopsin were observed. It is concluded that further studies in this area may yield information about the nature of the protein chromophores.

It is now generally accepted that the chromophores responsible for vision in animals and transduction of light energy in halobacteria result from an 'interaction' between the protonated Schiff base of retinal and protein amino acid side chains. The exact nature of the interaction which causes a shift in the absorption of retinal to longer wavelengths is not fully understood. Various suggestions have been made (see Refs. 1–3 and references cited therein), one being that the chromophores are charge-transfer complexes [4–11].

When the protein-bound chromophores absorb light they are rapidly converted, even at 2 K, into unstable intermediates in which part of the photons' energy is stored. It has been postulated [12,13] that the mechanism of energy storage is charge separation. A charge-transfer complex, made up of a loosely associated electron donor and acceptor, would be a plausible molecular arrangement in which charge separation might occur, since upon light absorption an electron is transferred directly from donor to acceptor. In addition, charge-transfer states, because they do not involve atomic motion, can be populated photochemically at very low temperatures [14].

The evidence available suggests that if such a complex is formed tryptophan acts as a donor. Shirane [11] and Bensasson et al. [5] claim to have detected the radical cation of tryptophan when the visual pigment rhodopsin was irradiated. Furthermore, Ishigami et al. [15] and Mendelsohn [9] claim that retinal and tryptophan form a charge-transfer complex in acidified methanol. Certainly, there is now a wealth of evidence which suggests that tryptophan is closely associated with retinal in both rhodopsin and bacteriorhodopsin [16–20] and involved in protein function [21–23]. The amino acid sequence of bacteriorhodopsin, the proton pump of halobacteria, has been determined recently and three provisional schemes of the disposition of the protein within the membrane have been published [24–26]. In two of these, a tryptophan residue is placed adjacent to the protonated retinal Schiff base.

Objections to the charge-transfer model [27,28] have been raised but in our opinion they do not rule it out. We set out to test critically the model experimentally. We have attempted to characterise the purple complex which forms when retinal and tryptophan are mixed in acid/methanol and to confirm reports that radicals are present when rhodopsin is irradiated at low temperatures.

The procedure used to obtain the putative retinal-tryptophan complex was that described by Ishigami et al. [15]. As well as all-*trans*-retinal and tryptophan, studies were made of the interaction between other retinal isomers (11- and 13-*cis*-) and several indoles (indole itself and 1-, 2- and 3-methylindoles). In each case, separate reactions were carried out with three acids (hydrochloric, sulphuric and trifluoroacetic acids) in two different solvents (methanol and dichloromethane).

Ishigami et al. [15] found that when all-*trans*-retinal and excess tryptophan were dissolved in HCl/CH_3OH an equilibrium was established between them and a purple product absorbing maximally at 540 nm. However, the formation of products absorbing at longer wavelengths is not limited to these particular reagents. All retinal isomers and the indoles examined (as well as carbazole) form such products with strong acids in methanol and dichloromethane. Absorption maxima range from 520 to 700 nm. Bandwidth (160 nm) and extinction coefficient ($6 \cdot 10^4\ l \cdot mol^{-1} \cdot cm^{-1}$) are constant and similar to those of the protein chromophores. If indole and retinal are mixed in equimolar amounts in dichloromethane acidified with trifluoroacetic acid, a quantitative yield of purple product is obtained at $-20°C$ (see Fig. 1).

Fig. 2. contains part of the ^1H-NMR spectrum of 13-*cis*-retinal and the purple product formed from it, the visible spectrum of which is shown in Fig. 1. The absence of resonances from 13-*cis*-retinal in the product spectrum and the near doubling of the number of lines indicate that the purple con n-ical reaction product of retinal rath al-indole complex.

Retinal reacts quantitatively with indole or methyl-substituted indoles in HCl/CH_3OH. However, except for 1-methylindole, the purple products exist in equilibrium with yellow species absorbing maximally between 400 and 420 nm. The yellow species are

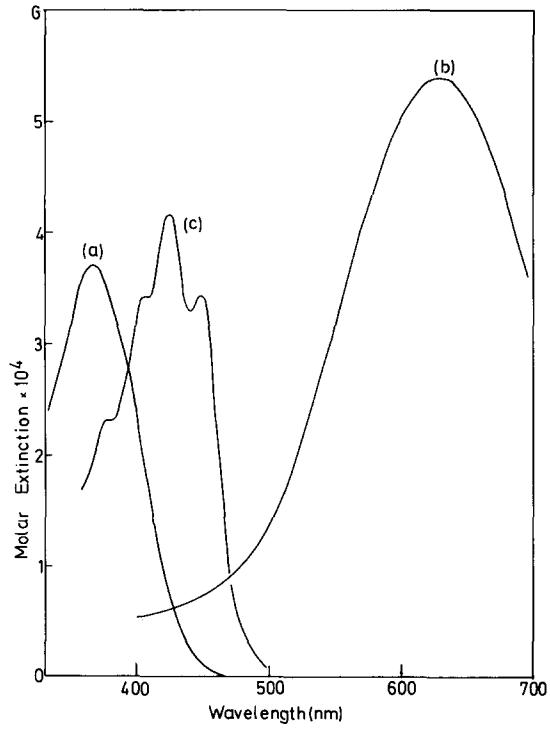

Fig. 1. (a) Visible absorption spectrum of retinal before addition of equimolar (10^{-3} M) indole and 10-fold excess trifluoroacetic acid. Sample was removed from the reaction vessel and diluted in a 1 mm cell. (b) 3 h after addition of indole and acid. Reaction temperature: $-20°C$. (c) Visible absorption spectrum of reaction product from all-*trans*-retinal and 3-methylindole in 1.5 M HCl/CH_3OH. The basified product was purified by silica gel TLC using bicarbonate-treated CCl_4 as eluant.

favoured by low acid concentration and addition of chemically inert salts (i.e., $Bu_4N^+ClO_4^-$). The electronic absorption spectrum of the all-*trans*-retinal-3-methylindole product from HCl/CH_3OH, isolated and purified under basic conditions, is shown in Fig. 1. In band position, fine structure and extinction coefficient, it resembles one of the later intermediates formed after photoexcitation of bacteriorhodopsin [29]. This intermediate, designated M_{412}, has a lifetime of a few milliseconds and its concentration under physiological conditions is very low. However, if bacteriorhodopsin is irradiated at high pH (and low temperature) [12] or in the presence of salt (and ether) [3], the M_{412} intermediate accumulates in high concentration. Thus, this retinal-indole product not

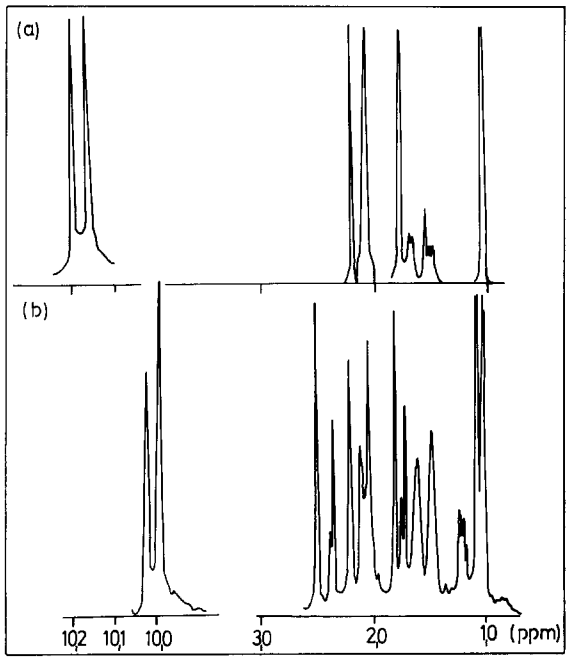

Fig. 2. ^{1}H- NMR spectra of (a) 13-*cis*-retinal (b) reaction product of equimolar 13-*cis*-retinal and indole in $C^2H_2Cl_2$ containing CF_3COO^2H. Shows ranges 10.3–9.9 and 3.0–0 ppm. The ordinate of the 10.3–9.9 ppm range is expanded 10-fold. N.B., indole protons do not give signals in either range. The resonances between 10.2 and 10.0 ppm originate from the retinal aldehyde proton and between 1.0 and 3.0 ppm from retinal aliphatic protons.

only resembles the M_{412} intermediate spectrally, but also in reactivity. Attempts to characterise it more fully were frustrated by its propensity to decompose in concentrated solution.

We followed the example of Shirane [11] in using ESR spectroscopy to scan for radicals in the irradiated proteins. Suspensions of membranes containing rhodopsin and bacteriorhodopsin were diluted 1 : 1 with glycerol and introduced into 3 mm diameter quartz ESR tubes. On freezing in liquid nitrogen, a clear glass was formed. Experiments were carried out on samples in darkness or illuminated by light with wavelengths 550 nm or longer. The ESR spectrometer was a Varian E9 model, operating at 9 GHz and fitted with an Oxford Instruments liquid helium cryostat (type CA 5245) and temperature controller. ESR signals were sought over a range of 200 G on either side of the field setting required for g 2. Experiments were carried out at both liquid nitrogen (77 K) and liquid helium (4 K) boiling points because a recent study [30] has shown that in the case of at least one of the visual pigments, the first intermediate formed upon light absorption is only stable below 32 K. Trial experiments with the tetracyanoethylene-indole charge-transfer complex indicated that even if tryptophan radical cations had been generated from a few per cent of the protein chromophores, under the conditions of the experiment, they would have been well within the limits of detection.

The bacteriorhodopsin-containing membrane, the so-called purple membrane, was isolated from *Halobacterium halobium* cells by standard procedures [31]. Membrane suspensions had an absorbance of 1 at 570 nm in a 1 cm path-length cell.

Rhodopsin-bearing rod outer segment membranes were prepared in dim red light from freshly isolated frog retinas (*Rana temporaria*) using discontinuous sucrose density centrifugation: homogenised retinas were suspended in amphibian Ringer's solution [32], pH 7.4, containing 38% (w/v) sucrose and covered with a layer of sucrose-free Ringer's solution. After centrifugation for 2 min at 12 000 × g (Eppendorf Microcentrifuge 5412), rod outer segment membranes were harvested from the interface, washed with Ringer's solution and resuspended in sufficient glycerol/Ringer's solution (1 : 1) to give an absorbance of 1 at 500 nm in a 1 cm path-length cell.

We are unable to confirm the observation of Shirane [11] that free radicals are present when rhodopsin is irradiated at 77 K. In fact, radicals could not be detected when either rhodopsin or bacteriorhodopsin were irradiated at 4 and 77 K. Under the conditions of the experiments, significant quantities of the initial chromophore photoproducts should have been present. Therefore, it would seem that if photon energy is stored in charge separation it is not by way of an intermolecular electron transfer as occurs in organic charge-transfer complexes.

The purple retinal-tryptophan reaction product also had no ESR spectrum when irradiated at low temperature. Under identical conditions much lower concentrations of the tetracyanoethylene-indole complex gave a detectable signal.

Thus, we conclude that the protein chromophores are not charge-transfer complexes. However, the resemblance between the retinal-indole reaction pro-

ducts and the protein chromophores is striking and warrants further investigation.

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