ENZYMES IN THE REGENERATION OF RHODOPSIN*

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We have shown that regenerability of a photobleached digitonin extract of rhodopsin does not depend upon the presence of the retinal isomerase of Hubbard (Hubbard, 1956) acting on all trans retinal. Regeneration kinetics were observed and no evidence for an enzyme controlled coupling reaction found.

MATERIALS AND METHODS An unpurified sample of rhodopsin was used in order to ensure the presence of additional components other than co-enzymes and cofactors that might be involved in any successive enzyme-controlled reaction. Bovine retinas (70)† were removed in the cold and under a dark red light, homogenised in 0.066 M phosphate buffer pH 6.8, washed twice for 30 minutes at 0°C followed by centrifugation at 25,000 x g for 20 minutes. No rhodopsin was found in either wash. The sedimented pellet was then extracted with 2.0% digitonin in phosphate buffer solution (35 ml) for 18 hours at 0°C and centrifuged for 20 minutes at 30,000 x g. The supernatant was separated and used as our experimental rhodopsin solution.

A "retinal isomerase" extract (Hubbard, 1956) was prepared by thawing 50 deep frozen bovine retinas, and homogenising them in 25 ml 0.066 M phosphate buffer at 0°C, pH 6.8. No care was taken to keep this system in the dark. This was centrifuged in the cold at 50,000 x g for 15-20 minutes and

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the sedimented pellet discarded. The supernatant was taken to contain the retinal isomerase (and any accompanying coenzyme or cofactor) described by Hubbard.

A highly purified sample of rhodopsin was prepared by a method previously described (Girsch and Rabinovitch, 1971), followed by chromatographic separation on a Sephadex 200 column, using a buffered pH 6.8 cetyl trimethyl ammonium bromide (CTAB) extractant.

All-trans retinal was obtained from City Chemical Corporation, New York and used as obtained. A solution of concentration 3.5×10^{-4} M in ethanol was made up. 11-cis retinal was a gift from Hoffman-LaRoche, New Jersey, and was treated similarly to the all-trans isomer. All solutions were kept at $0^{\circ}C$ in the dark until used, and exposed only to a dark red light except when photobleaching.

RESULTS Figure 1 shows a typical visible spectrum of a sample of an unpurified digitonin-extracted rhodopsin, before and after photobleaching and after the addition of approximately a 3-fold excess of 11-cis retinal. Marked regeneration is seen to occur. Difference spectra obtained from these data clearly show a peak of regenerated rhodopsin at 500 mμ, a peak of excess 11-cis retinal at 380 mμ and a peak ascribed to the Soret band of hemoglobin at 420 mμ.

The visible and uv spectrum of the "retinal isomerase" extract showed a typical protein band at 275 mμ (aromatic amino acids) and a small impurity Soret band at 420 mμ. An OD of 0.84 at 275 mμ for a 10 X diluted sample gave an estimated protein concentration of 1.2% for the undiluted extract. Figure 2a shows that the residual rate of regeneration of a photobleached rhodopsin without added retinal is small (because of the photoisomerisation of the released all-trans retinal during photobleaching) and although it is increased by addition of an excess of all-trans retinal, this is attributable to 11-cis impurity. However this same system diluted with an equal volume of undiluted "isomerase" extract shows a slight decrease in rate of regeneration instead of the increase expected from an enzy-

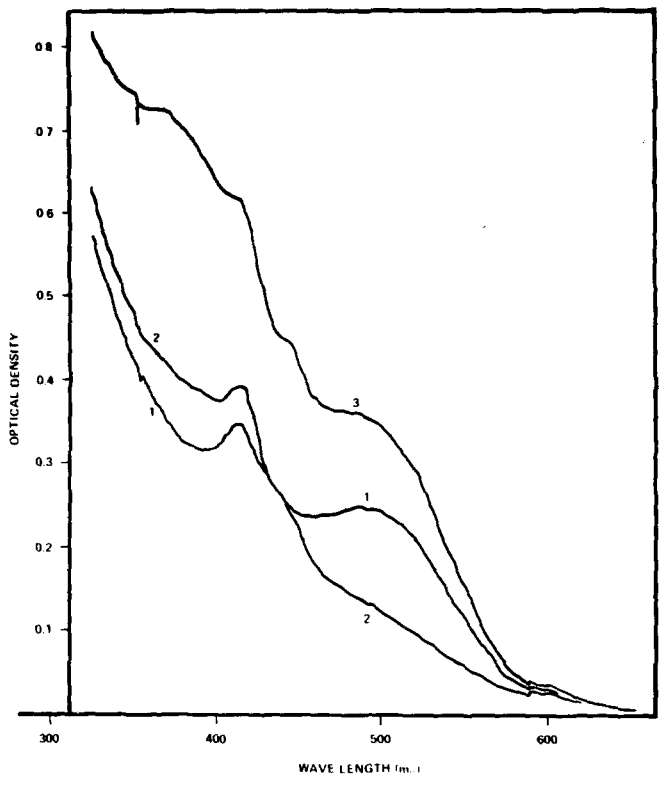

Figure 1 - Absorption spectrum of an unpurified digitonin extract of bovine visual pigment, (1) before photobleaching, (2) after photobleaching and, (3) after regeneration in the presence of an excess of 11-cis retinal.

matic isomerisation of the all-trans to 11-cis retinal. These results were repeated a number of times with a number of original preparations and with different ammonium sulfate fractions of the so-called retinal isomerase supernatant. In no case was an accelerated rate of regeneration observed that could not be accounted for by the physical process of photoisomerisation of all-trans to 11-cis retinal.

To examine further the possibility of an enzyme regulated coupling of 11-cis retinal to opsin, we have examined the kinetics of the regeneration process. Figure 2b gives a sample of the rates of regeneration of photobleached rhodopsin with increasing levels of added 11-cis retinal. These data conform well to a first order rate equation and the derived rate constants show a good linear dependence upon the 11-cis retinal con-

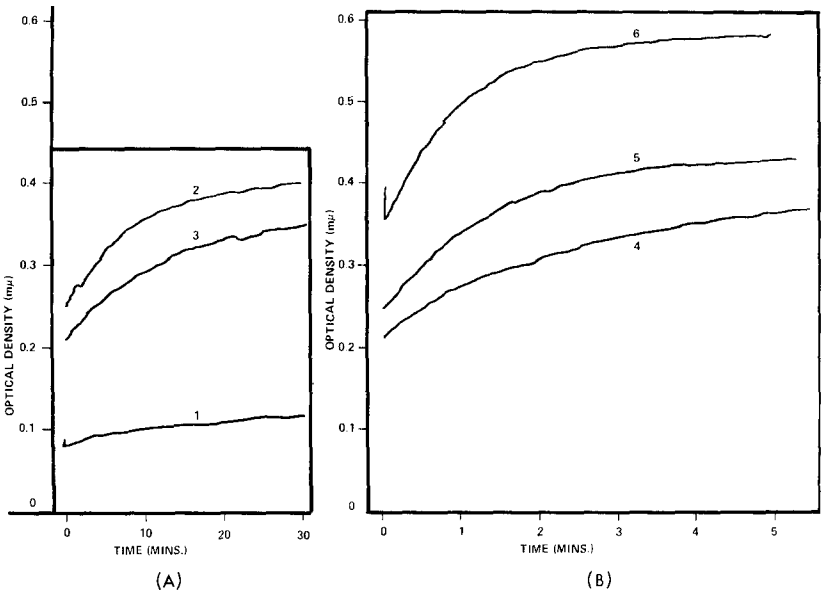

Figure 2 - Rates of regeneration of photobleached visual pigment, (1) in the absence of any added retinal, (2) with all trans retinal, (3) with all trans retinal plus "retinal isomerase", (4) with added 11-cis retinal, concentration 3.33×10^{-5}M, (5) 6.66×10^{-5}M (6) 10×10^{-5}M.

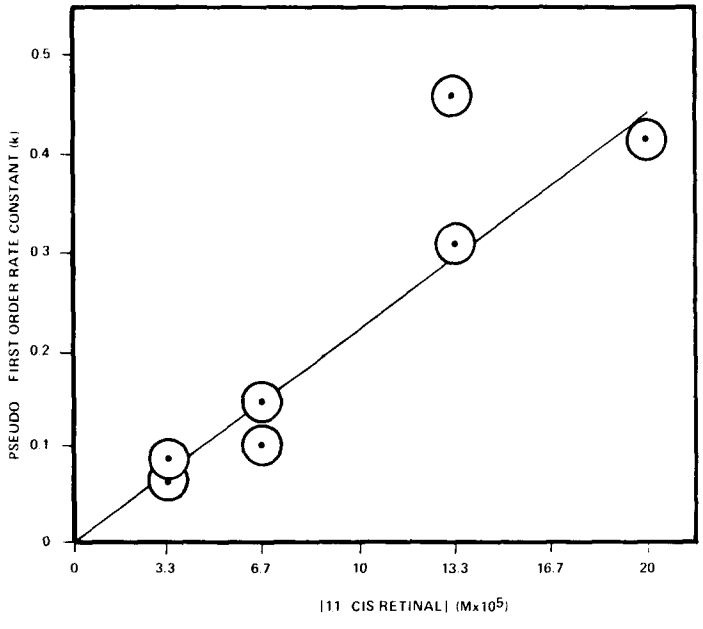

Figure 3 - Dependence of the first order rate constant for the regeneration of rhodopsin from photobleached rhodopsin in essentially constant concentrations of 11-cis retinal.

centration in the regenerating system (Figure 3).

Figure 4 shows the visible spectrum of the purified rhodopsin and its completely photobleached product. Although behaving normally with respect to bleaching, this material was totally incapable of regeneration under any of the conditions described above. Addition of equal volumes of this CTAB rhodopsin and a regenerable digitonin visual pigment reduced the regeneration rate to zero.

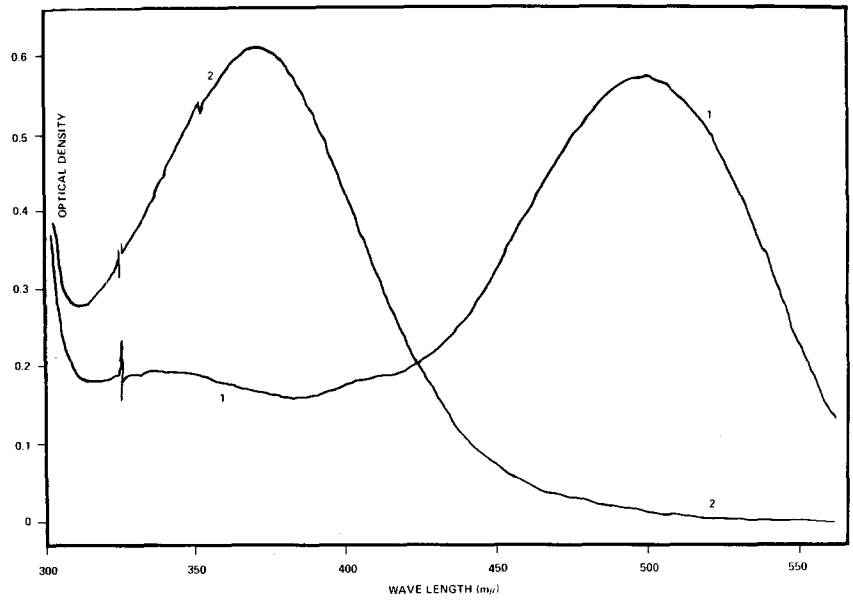

Figure 4 - Visible spectrum of a highly purified rhodopsin in CTAB (1) before photobleaching, (2) after photobleaching.

DISCUSSION The data in Figures 1 and 2 show that, unlike a CTAB-extracted rhodopsin which is incapable of regeneration (Snodderly, 1967), these extracts of visual pigment will regenerate readily with 11-cis retinal in a manner very dependent upon retinal concentration. The "retinal isomerase" extract had no effect upon the regeneration, and this was confirmed many times with different preparations and subsequent ammonium sulfate fractions. This has forced us to the conclusion that the existence of an isomerase remains unproven. Clearly some mechanism is required for converting all-trans to 11-cis retinal in the regeneration process,

but some other way must be sought to demonstrate it.

In the examination of the kinetics of regeneration, the fact that the process seems to be strictly first order throughout the reaction, with a direct dependence upon 11-cis retinal concentration, precludes the possibility that an enzyme controlled coupling reaction is involved. Enzyme kinetics demands that the rate of product formation be first order only initially, reducing to zero order as the reaction goes to completion.

The effect of a CTAB rhodopsin on a proven regenerable system rules out the possibility that an essential non-enzymatic system is present in the latter but not in the former. This suggests an inhibitory effect of CTAB on regeneration, and this was demonstrated by addition of 0.07% buffered CTAB solution. The rate of regeneration was markedly reduced and totally halted at 0.28%.

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