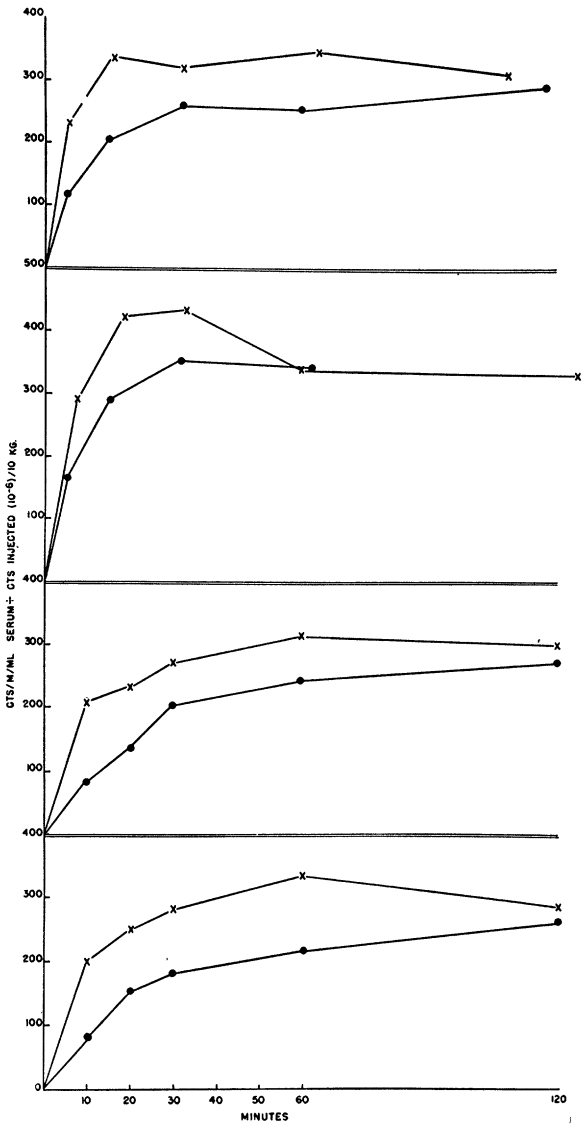

FIG. 2. Rate of serum uptake of subcutaneously injected Na^{24} in isotonic saline. Control ●——●, hyaluronidase ×——×.

on different days. Serum activity is expressed in terms of cpm/ml divided by counts injected (10^{-6}) per 10 kg body weight after correction for decay. Here, too, there was considerable variation in rate of absorption from one subject to another, despite correction of the data to constant body weight, but in each instance the serum Na^{24} value rose more rapidly when hyaluronidase was used.

From these observations, it is apparent that in the normally hydrated human subject, the rate of absorption of sodium ion from small volumes of subcutaneously injected fluid is enhanced by the addition of hyaluronidase.

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The Light Reaction in the Bleaching of Rhodopsin[1]

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The absorption of light by rhodopsin in the retina results in both the bleaching of the molecule and the excitation of rod vision. One could conclude at once that the bleaching of rhodopsin is the source of visual excitation, were it not a composite process. It consists of an initial photochemical change, followed by relatively slow thermal —i.e., "dark"—reactions. The light process converts rhodopsin to a highly unstable orange material (Lythgoe's "transient orange"), which breaks down in light or darkness to a yellow mixture of retinene$_1$ and protein (Lythgoe's "indicator yellow") (*6*, *8*). In the retina these substances are involved in further changes, but in ordinary solutions of rhodopsin this is all that occurs.

The first product formed by the action of light on rhodopsin is removed so rapidly that early attempts to measure its spectrum led only to approximations (*7*, *8*). In 1941, however, Broda and Goodeve reported an experiment that appeared to isolate the light reaction (*2*).

These workers prepared solutions of rhodopsin in mixtures of glycerol and water (3:1) such as Kühne had examined more than 60 years before (*3*). On cooling to −73° C such solutions vitrify. The absorption band of rhodopsin was reported to shift about 10 mμ toward the red and to become much narrower in shape. On exposure to light at −73° C, the absorption maximum moved about 5 mμ toward the blue, and fell some 12 % in height. This was the light reaction. The photoproduct remained stable at the low temperature. It did not appear to vary in spectrum between pH 6 and 9, confirming an earlier conclusion of Lythgoe and Quilliam concerning transient orange. On warming to room temperature in the dark it decomposed spontaneously to indicator yellow.

We have reexamined this behavior of rhodopsin at low temperatures. Spectra were measured with the Beckman spectrophotometer in a specially designed Dewar flask, silvered except for a window. The rhodopsin solution, well buffered in a glycerol-water mixture (2:1) was suspended inside the vessel in a quartz cell over liquid air or solid carbon dioxide. The temperature of the solu-

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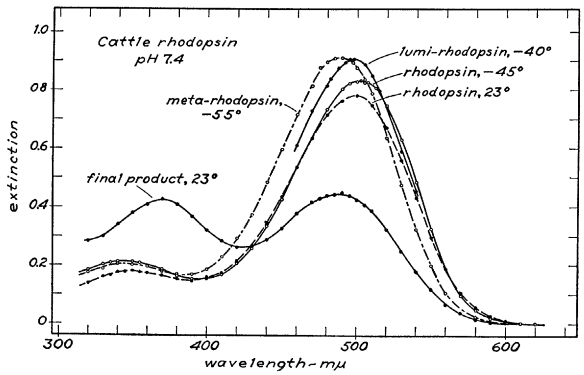

FIG. 1. Bleaching of rhodopsin at low temperatures in a glycerol-water mixture (2:1). The spectrum was measured at 23° C and again at −45° C. The solution was then exposed to intense white light at the low temperature until all changes were completed (lumi-rhodopsin). It was then warmed to −15° C in darkness, left at this temperature until all changes were completed, and recooled to −55° C prior to remeasurement (meta-rhodopsin). Finally the solution was warmed to room temperature in darkness, and the spectrum of the final product was measured. This was a mixture of regenerated rhodopsin and of retinene$_1$ (max 375 mμ) + protein in roughly equal amounts. All these spectra have been corrected for changes in volume of the solvent with the changing temperatures.

tion was measured with a thermocouple immersed in it. Parallel experiments with cattle and bullfrog preparations yielded similar results, with one small difference as noted below.

A series of measurements made with cattle rhodopsin in neutral solution is shown in Fig. 1. The maximum absorption in the glycerol-water mixture at room temperature lies at about 500 mμ. On cooling to −30°–−100° C, the maximum shifts progressively 5–9 mμ toward the red, and rises 6%–14% higher than would be caused by contraction of the solvent; but the shape of the band changes very little. On exposing the solution to light at these low temperatures, the maximum shifts about 5 mμ toward the blue, rising about 5% in height in cattle rhodopsin, falling about this amount in frog rhodopsin, still with little change in shape. This is the light reaction. We shall call its product *lumi-rhodopsin.*

If the solution of lumi-rhodopsin is warmed to about −20° C, a further change occurs in darkness. The absorption band shifts another 7–9 mμ toward the blue, with little further change in height or shape. (The solution shown in Fig. 1 had been warmed to −15° C until all changes were completed, then recooled to −55° C for measurement.) We shall call this second product *meta-rhodopsin.* Neither its spectrum nor that of lumi-rhodopsin changes consistently with pH, between pH 3.9 and 10.1, nor is either substance affected appreciably by further exposure to light. Note that the change from lumi- to meta-rhodopsin at −20° C is unmistakably a distinct process. Not only is no retinene$_1$ formed during this conversion, but absorption in the region of the retinene$_1$ maximum (about 375 mμ) tends to fall a little.

On allowing the solution of meta-rhodopsin to rise to room temperature in darkness, it goes over to a mixture of regenerated rhodopsin and retinene$_1$ + protein in roughly equal amounts. On a second exposure to light at room temperature, the regenerated rhodopsin bleaches to a final mixture of retinene$_1$ and protein alone.

The changes in color associated with the light reaction and with the further change to meta-rhodopsin are very small—from red to orange-red, with little change in depth. By merely looking at these solutions before and after exposure to light, one could not have been certain that any change had occurred at all.

Many years ago, Kühne observed that when retinas have been thoroughly dried over sulfuric acid, their rhodopsin scarcely seems affected by even an hour's exposure to direct sunlight (*5*). It occurred to us that here, as in the cold, the light reaction might be completed, but with so little change in color as to escape notice.

To examine this possibility we prepared dry gelatine films of rhodopsin of a quality suitable for accurate spectrophotometric examination. Weigert and his coworkers had prepared similar films in their studies of photodichroism (*10*). Solutions of frog or cattle rhodopsin in 4% digitonin were mixed with one-third their volume of warm 10% gelatine, and were poured on heavy celluloid film. After setting, the film was dried in a desiccator over calcium sulfate ("drierite") for at least 3–4 days. By this time the rhodopsin film had curled off or could be peeled off the celluloid. All these operations were carried out in darkness or under dim red light. As blanks, similar films were prepared in which digitonin solution alone was substituted for the rhodopsin.

Measurements made with a dry gelatine film of cattle rhodopsin are shown in Fig. 2. Bullfrog preparations exhibit almost identical behavior. The absorption spectrum of rhodopsin in the film is remarkably similar to its spectrum in aqueous solution. The maximum lies at 495–498 mμ. On exposure to a short, intense burst of light—in Fig. 2 the approximately 0.02-sec flash of a photoflash lamp—the maximum shifts about 6 mμ toward the blue, and rises about 10% in height. This is lumi-rhodopsin. Within the next 15 min at room temperature in light or darkness the spectrum shifts a further 5–10 mμ toward the blue, simultaneously falling slightly in height. This is the conversion of lumi- to meta-rhodopsin. The meta-rhodopsin remains stable for days in the dry state, and is not affected by further exposure to light. On wetting the film with water, however, meta-rhodopsin goes over in the dark to a mixture of regenerated rhodopsin and retinene$_1$ + protein in roughly equal amounts. Prior to measuring the spectrum of this product, the film is redried. On reexposing the dried film to light, the regenerated rhodopsin goes through the same changes as did the original pigment.

Thus in the dry state, as in the cold, rhodopsin goes through the light reaction unhindered, and the dry photoproduct also is converted slowly to meta-rhodopsin; but neither regeneration nor formation of retinene$_1$ can occur.

The bleaching of rhodopsin therefore involves the following stages: Light converts rhodopsin to lumi-rhodopsin. At temperatures in the neighborhood of −20° C, or at room temperature even in the dry state, lumi-rhodopsin goes to meta-rhodopsin. The nature of these changes

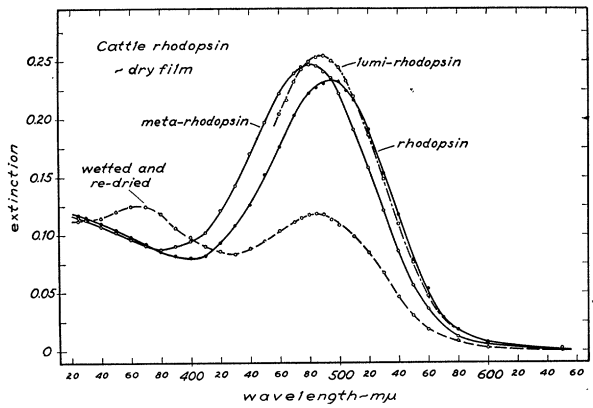

FIG. 2. Bleaching of rhodopsin in gelatine film. The spectrum of rhodopsin was measured in a film dried over calcium sulfate. It was then exposed to the instantaneous illumination of a photoflash lamp, and the spectrum recorded within the first minute thereafter (lumi-rhodopsin). After about 1 hr in darkness at room temperature the spectrum was remeasured (meta-rhodopsin). These changes were complete; a second exposure to a photoflash lamp produced no further effect. The film was then soaked in *m*/15 phosphate buffer, pH 7.2, for 10 min and redried, all in darkness. The spectrum of the final product shows a mixture of regenerated rhodopsin and of retinene$_1$ + protein in roughly equal amounts.

is still obscure; one thinks of ionization or the formation of a free radical, followed by molecular rearrangement, but this must await further study. Given access to water and a high enough temperature to permit thermal activation, meta-rhodopsin is transformed on the one hand to rhodopsin, on the other to retinene$_1$ and protein.

This is not a wholly exhaustive description of the bleaching process under all conditions, but it probably goes as far with the early stages of bleaching as will be found useful. Our observations in extreme cold indicate complexities in the light reaction itself, not unexpected in so complex a molecule as rhodopsin. The primary reason for stopping with the stages we have named is their stability under specific conditions of bleaching. The relatively stable end product of bleaching rhodopsin at temperatures below $-40°$ C is lumi-rhodopsin. The relatively stable product of bleaching rhodopsin at about $-20°$ C or in the dry state is meta-rhodopsin. The stable products of bleaching ordinary solutions of rhodopsin at room temperature are retinene$_1$ and protein, or a mixture of these substances with regenerated rhodopsin if the solutions are replaced in darkness following irradiation.

Further complexities can also be found in the later stages of bleaching. In acid solutions (pH about 4) meta-rhodopsin yields an orange intermediate with maximal absorption at about 440 mμ, which is transformed only in the course of several hours at room temperature to retinene$_1$ (*8*). Also retinene$_1$ has the capacity to couple spontaneously with a wide variety of amino compounds (*1*). It is formed in association with rhodopsin-protein, and undoubtedly remains coupled in part with this molecule; but in part it also leaves this protein to go into the free state and to couple with other retinal molecules. One such reaction which has already been demonstrated in more complete systems than those just described is the migration of retinene$_1$ from rhodopsin-protein to the apoenzyme, retinene reductase, prior to its reduction to vitamin A_1 (*9*).

The light reaction, however, seems to possess much the same characteristics under all the circumstances we have explored. It proceeds at roughly the same rate at very low temperatures and in the dry state as in aqueous solutions in the warm. Accurate measurements made with dry films show it to be a first-order process, proportional in rate to the light intensity. These are the conventional properties of a simple photochemical process, the rate of which depends only upon the rate of absorption of quanta of light. When Hecht, years ago, found these properties in the over-all bleaching of rhodopsin in solution (*4*), it was because his experiments were performed in such a way that the light reaction was the limiting process.

The observations we have described reveal a striking parallel between the bleaching of rhodopsin and the photographic process. In both cases light produces a scarcely visible change—a "latent image"—followed by gross thermal changes. The parallel is particularly close in the dry rhodopsin film, in which light produces a stable latent image composed of meta-rhodopsin, which can be "developed" at any later time simply by wetting. Here, development induces bleaching, and results in a positive.

These observations also reflect upon the relation between the bleaching of rhodopsin and the excitation of rod vision. We are still as far from knowing the mechanism of excitation in the retinal receptors as in muscle or nerve. Nevertheless, something of the relation between visual stimulation and bleaching can be inferred from the times involved in both processes. When an eye is exposed to light, an electrical discharge can be recorded from the optic nerve within periods of the order of tenths of a second, even in cold-blooded animals. The excitation of the rods themselves must occur much earlier than this. It seems probable that the formation of retinene$_1$ is too slow a process to account for this response. More likely it depends upon the light reaction itself, or upon some further change—such as the conversion of lumi- to meta-rhodopsin—so intimately associated with the light reaction as to follow it to completion within a small fraction of a second.

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