THE ACTION OF ENZYMES ON RHODOPSIN

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ABSTRACT

The effects have been examined of chymotrypsin, pepsin, trypsin, and pancreatic lipase on cattle rhodopsin in digitonin solution.

The digestion of rhodopsin by chymotrypsin was measured by the hydrolysis of peptide bonds (formol titration), changes in pH, and bleaching. The digestion proceeds in two stages: an initial rapid hydrolysis which exposes about 30 amino groups per molecule, without bleaching; superimposed on a slower hydrolysis which exposes about 50 additional amino groups, with proportionate bleaching. The chymotryptic action begins at pH about 6.0 and increases logarithmically in rate to pH 9.2.

Trypsin and pepsin also bleach rhodopsin in solution. A preparation of pancreatic lipase bleached it slightly, but no more than could be explained by contamination with proteases.

In digitonin solution each rhodopsin molecule is associated in a micelle with about 200 molecules of digitonin; yet the latter do not appear to hinder enzyme action. It is suggested that the digitonin sheath is sufficiently fluid to be penetrated on collision with an enzyme molecule; and that once together the enzyme and substrate are held together by intermolecular attractive forces, and by the "cage effect" of bombardment by surrounding solvent molecules.

The two stages of chymotryptic digestion of rhodopsin may correspond to an initial rapid fragmentation, such as has been observed with many proteinases and substrates; superimposed upon a slower digestion of the fragments. Since the first phase involves no bleaching, this may mean that rhodopsin can be broken into considerably smaller fragments without loss of optical properties.

Willibald Kühne, Professor of Physiology at Heidelberg, was at once the founder of retinal biochemistry and of enzymatic histochemistry. We owe to him the term *enzyme* and the first characterization of trypsin. All these interests came together in Kühne's application of tryptic digestion to the analysis of retinal tissues (Kühne, 1878; *cf.* also Chittenden, 1882), in the course of which he observed that retinas, outer segments of rods, or fragmented rods can be digested with pancreatic trypsin without destroying rhodopsin.

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Several years later W. C. Ayres (1882), an American working in Kühne's laboratory, discovered that rhodopsin, brought into solution with the aid of bile salts, is attacked by trypsin, though immune to tryptic action until dissolved. Even after retinas had been macerated in 10 per cent saline or ammonia solution, rhodopsin was not attacked by trypsin. After extraction into bile salts solution, however, it was bleached by trypsin within an hour at 35°C.

Ayres's preparations of trypsin, made according to Kühne's directions, had no appreciable amylase or lipase activity. After such preparations had been boiled, they could be incubated for days with rhodopsin without effect. Ayres concluded that the tryptic destruction of rhodopsin is a true enzymatic action, probably proteolytic, though possibly instead a denaturation, since trypsin also coagulates proteins. In the rods, Ayres supposed that rhodopsin is bound to other molecules in some manner that protects it from tryptic action.

In the same paper, Ayres remarked that human salivary amylase (though, like trypsin, not inhibited by bile salts) does not attack rhodopsin in solution even after days of incubation.

These excellent observations have been all but forgotten. Indeed they have largely been superseded in the literature by Nakashima's report (1929) that proteolytic and glycolytic enzymes do not attack rhodopsin, whereas lipase bleaches it over yellow to colorlessness. Nakashima neither described his enzyme preparations nor did he specify whether the rhodopsin was in the retina or in solution.

The present experiments involve the action of chymotrypsin, trypsin, pepsin, and pancreatic lipase on solutions of rhodopsin.

Preparations

Rhodopsin solutions in 2 per cent aqueous digitonin were prepared as described by Wald and Brown (1951–52). Their concentrations were determined from the extinction at 500 mμ (K_{500}) on the basis that the molar extinction coefficient of cattle rhodopsin at this wavelength is 40,600 (Wald and Brown, 1953–54).

Chymotrypsin.—Crystallized bovine chymotrypsin (Armour) was dissolved in 0.0025 N HCl, and the solutions dialyzed overnight. A 0.5 per cent solution following dialysis had a Kjeldahl nitrogen content of 19.2 mg. per 100 ml.

Trypsin.—Crystallized bovine trypsin (Armour) was dissolved in 0.0025 N HCl, and the solution dialyzed overnight. Following dialysis a 1 per cent solution contained 71.7 mg. Kjeldahl nitrogen per 100 ml. Measured by Kunitz's casein digestion method (Northrop *et al.*, 1948, p. 308), this solution had an activity of more than 75×10^{-3} tryptic units.

Pepsin.—Crystallized swine pepsin (Armour) was prepared in 10 per cent solution in citrate buffer at pH about 4.

Pancreatic Lipase.—A partly purified preparation (Delta), dissolved in 87 per cent glycerol, was assayed by Willstätter's procedure (Sumner and Somers, 1953, p. 81). A solution containing 18 mg. of lipase powder, incubated with 2.5 gm. olive oil at 30°C., hydrolyzed 5.9 per cent in 1 hour.

Chymotrypsin

Course of Digestion.—The rhodopsin solution used in the experiment about to be described had an extinction at 500 mμ of 4.566, and hence a molar con-

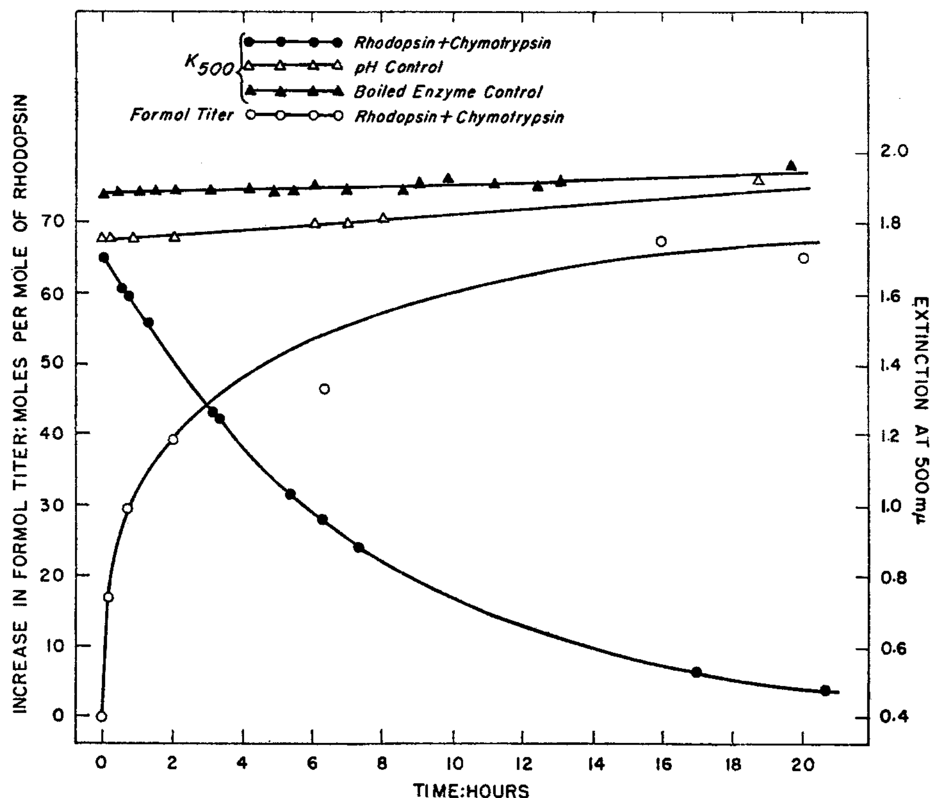

FIG. 1. Digestion of rhodopsin by chymotrypsin. Initial pH 7.82; 30°C. Abscissae, hours in the dark. Left ordinate, moles of amino groups exposed by hydrolysis, determined by formol titration, per mole rhodopsin. Right ordinate, extinction at 500 mμ, the λ_{max} of rhodopsin. The pigment bleaches as it hydrolyzes, both effects being nearly completed in 20 hours. Controls in which rhodopsin was incubated at the initial pH or with the boiled enzyme show no such effects.

centration of 1.12×10^{-4}. The ratio of extinctions at 400 and 500 mμ was 0.35; in the purest preparations it is about 0.22. Its total nitrogen content was 0.894 mg. N per ml.; on the basis of a K_{500} of 1.0 this corresponds to 0.195 mg. N per ml. of solution. The lowest nitrogen contents previously found in preparations of cattle rhodopsin, for $K_{500} = 1.0$, have been 0.15 (Hubbard, 1953–54) and 0.09 (Krinsky, 1958). In the present solution therefore something less than half the nitrogen content can be ascribed to rhodopsin itself.

Mixtures of 1.0 ml. chymotrypsin solution and 0.75 ml. rhodopsin solution were incubated in the dark at 30°C. The initial pH was 7.82. At intervals, 1.0 ml. of 36 to 38 per cent neutral formaldehyde was added to a sample, and it was titrated in the light with 0.01 N NaOH to the phenolphthalein end-point.[1] An initial measurement (zero time) was made by adding formaldehyde to the rhodopsin alone, followed by the addition of enzyme and immediate titration. The increase in formol titer above that of the initial sample measured the appearance of new amino groups resulting from the hydrolysis of peptide bonds.[2]

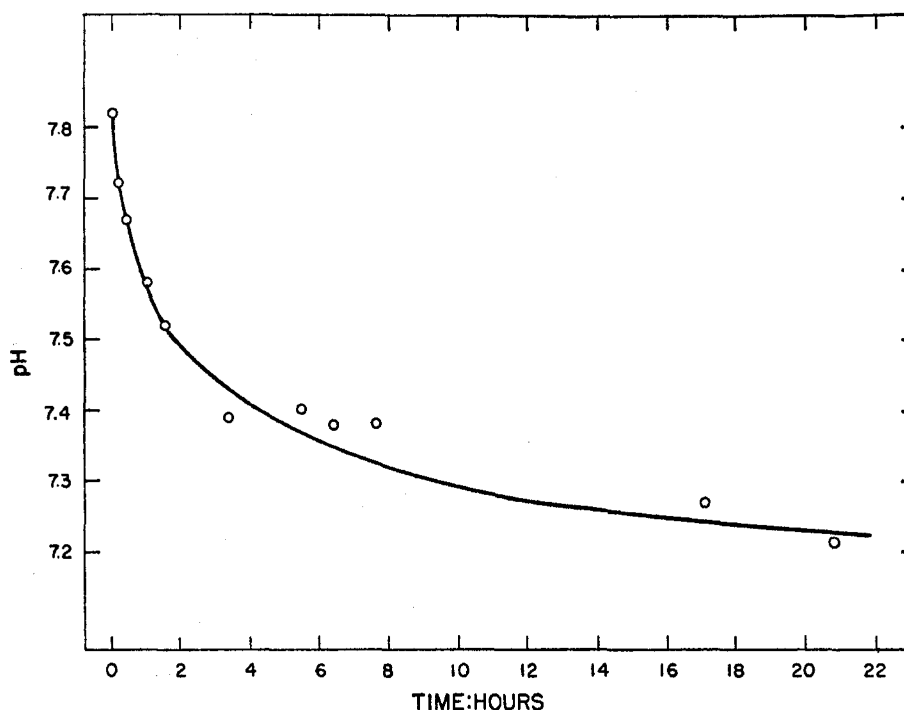

FIG. 2. Change in pH during the digestion of rhodopsin by chymotrypsin. This is the same experiment as shown in Fig. 1.

We followed also the bleaching of rhodopsin by chymotrypsin, by measuring at intervals the extinction at 500 mμ in one sample of the incubation mixture.

[1] It was necessary to add 3.0 ml. of water to the titration mixture to dilute the yellow color of retinene produced by the bleaching of rhodopsin.

[2] Several amino groups may have been merely "unmasked," without hydrolysis of peptide bonds, by the denaturing action of chymotrypsin on rhodopsin. Such effects are known to accompany under some circumstances the initial attack of proteases on other proteins (*cf.* Linderstrom-Lang, 1950). This type of activity, however, is normally expected to uncover only a few amino groups per molecule; and if it occurred here must have been of such nature as not to bleach the pigment.

Fig. 1 shows the results of this experiment. As the digestion proceeds, the formol titer rises and the extinction at 500 mμ reciprocally falls. At the end of

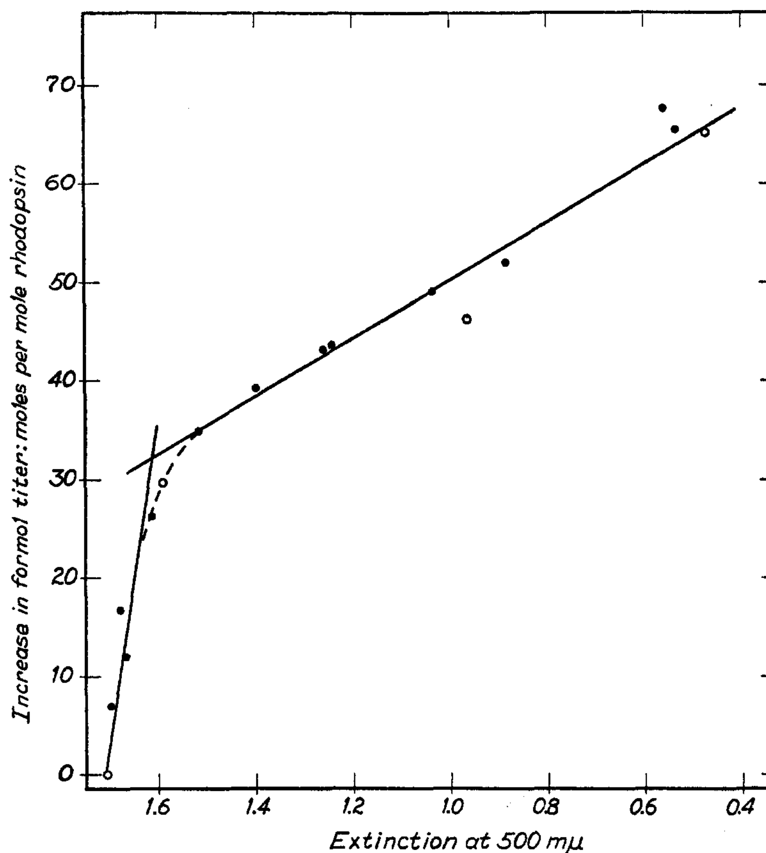

FIG. 3. Relative effects of chymotryptic digestion on the number of amino groups exposed per molecule (the formol titer) and on the extinction of rhodopsin, from the data of Fig. 1. A first rapid exposure of about 30 amino groups per molecule involves no bleaching; this is superimposed on a slow continuous hydrolysis, with proportionate bleaching.

21 hours, about 80 per cent of the rhodopsin had been bleached, and 65 new amino groups had been exposed per molecule of rhodopsin.[3]

During this experiment the pH fell from an initial value of 7.82 to 7.21. The course of this change, shown in Fig. 2, is another manifestation of the hydrolysis of rhodopsin.

[3] The complete bleaching of rhodopsin at this pH leaves a residual extinction at 500 mμ, about 10 per cent of that present originally, owing to absorption by retinene, in part bound in Schiff base linkage to amino groups (*cf.* Morton and Pitt, 1955).

A control sample of rhodopsin at pH 7.84 was incubated in the dark at 30°C., and its K_{500} measured periodically. As shown in Fig. 1 ("pH control"), the extinction rose slightly owing to a small increase in turbidity, but no bleaching was observed. Also a portion of the chymotrypsin solution was boiled for 10 minutes, then centrifuged to remove the precipitate which formed. The clear supernatant solution was incubated with an equal volume of rhodopsin solution at pH 7.88 and 26°C., and K_{500} measured at intervals. Again no bleaching occurred (Fig. 1, "boiled enzyme control").

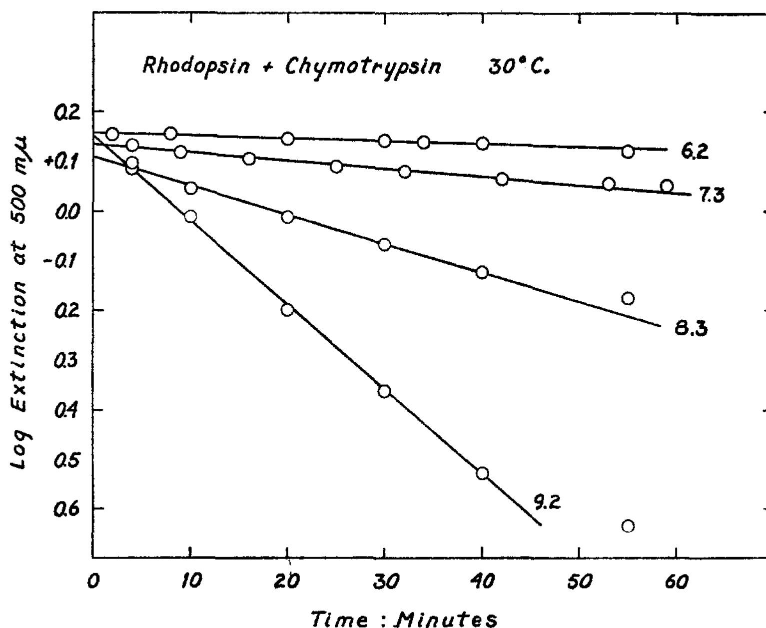

FIG. 4. Effect of pH on the rate of bleaching of rhodopsin by chymotryptic digestion. At each pH the reaction is first-order. The half-times are: 518 minutes at pH 6.2; 184 minutes at pH 7.3; 51.7 minutes at pH 8.3; and 17.7 minutes at pH 9.2.

It may be concluded that the hydrolysis and bleaching of rhodopsin observed in this experiment resulted from the enzymatic action of chymotrypsin.

Closer examination of these data reveals a significant disparity between the course of hydrolysis of rhodopsin and its bleaching. The bleaching proceeds as a simple, first-order reaction throughout its course (*cf.* Fig. 4). The hydrolysis, however, begins rapidly, and then continues more slowly. The relation between both effects is shown in Fig. 3. It is evident that the main body of the reaction is occupied with a slow release of amino groups to which bleaching is proportional; but that superimposed on this is a rapid initial release of about 30 amino groups per molecule of rhodopsin, not accompanied by bleaching. This initial effect is completed within the first hour of digestion (Fig. 1); and during this interval the pH also completes nearly half its total change (Fig. 2).

The digestion of rhodopsin by chymotrypsin therefore involves two phases: a rapid initial exposure of amino groups which probably involves no bleaching, superimposed upon a continuous, slow exposure of amino groups with proportionate bleaching. If the proportionality in the slow phase holds to the end of the reaction, complete bleaching is achieved when a total of about 80 amino groups have been exposed, *i.e.* close to 80 peptide bonds hydrolyzed, per molecule of rhodopsin: 30 in the initial rapid phase, and 50 more in the slow

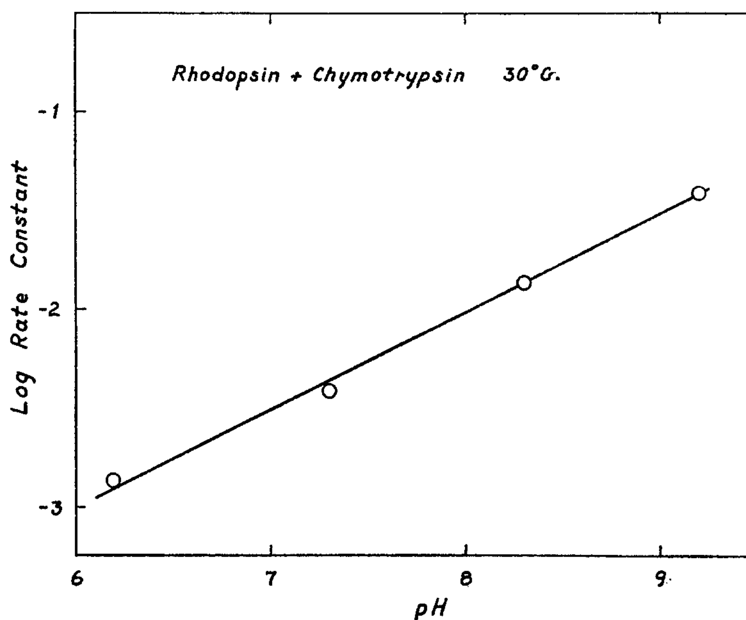

FIG. 5. pH-activity curve for the digestion of rhodopsin by chymotrypsin, in terms of the specific first-order rate constants of bleaching calculated from the data of Fig. 4.

phase associated with bleaching. As has already been said, our rhodopsin preparation was not pure, and some of the hydrolysis observed may have involved contaminants. The significance of these observations will be discussed below.

Effects of pH.—Mixtures of rhodopsin and chymotrypsin, buffered at various pH, were incubated in the dark at 30°C., and the extinction at 500 mμ was measured periodically. The results are shown in Fig. 4.

At 30°C. in the absence of enzyme, rhodopsin does not bleach appreciably within 1 hour at pH 6–8.7, and bleaches only about 5 per cent in an hour at pH 9.9 (*cf.* Radding and Wald, 1955–56). The changes shown in Fig. 4 are therefore caused wholly by the action of the enzyme.

The rate of bleaching of rhodopsin increases logarithmically as the pH rises above 6. A pH-activity curve based upon the first-order rate constants calculated from Fig. 4, is plotted in Fig. 5.

The logarithm of the rate constant when plotted against pH yields a straight line with a slope of 0.5 from pH 6.2 to 9.2.[4] That is, the rate of bleaching of rhodopsin by chymotrypsin is inversely proportional to $\sqrt{(H^+)}$. A theoretical basis for linear relationships between pH and the logarithm of the rate or the Michaelis constant has been discussed by Dixon (1953) and Laidler (1955).

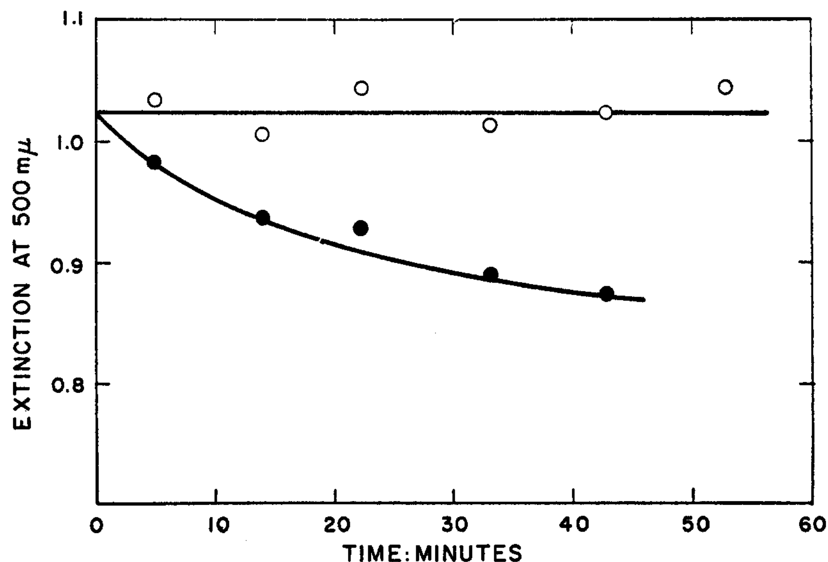

FIG. 6. Digestion of rhodopsin by trypsin (pH about 8, 30°C.). The solid circles show the bleaching of rhodopsin on incubation in the dark with trypsin, the open circles, measurements on a control mixture lacking only the trypsin.

Our measurements do not make it worthwhile to attempt to rationalize the particular value of the slope in this instance.

Trypsin

Mixtures of equal volumes of rhodopsin solution, 4 per cent trypsin, and borate buffer at pH 8.62, were incubated in the dark at 30°C., and the K_{500} followed for 43 minutes. A control mixture was incubated with the trypsin omitted.

Fig. 6 shows that in the presence of the enzyme, the extinction of rhodopsin fell 14.5 per cent, whereas that of the control mixture remained unchanged.

[4] Because of instability of rhodopsin at alkaline pH it is not possible to extend the pH-activity curve beyond pH 9.2.

Pepsin

A solution of swine pepsin in citrate buffer at pH 4.08 was mixed with half its volume of rhodopsin solution. A control mixture was prepared lacking the pepsin. Both solutions were incubated at room temperature in the cell compartment of the Beckman spectrophotometer, and K_{500} was followed for 20 hours. At the end of this period the pH of both solutions was 4.15.

The results of this experiment are summarized in Table I. During the first 5 hours, the rhodopsin exposed to pepsin bleached to 61.7 per cent of its initial extinction, whereas the control remained unchanged. After about 20 hours of incubation, the digestion mixture had bleached to 27.4 per cent of its initial extinction, the control to 79.4 per cent. The complete bleaching of rhodopsin at

TABLE I

Bleaching of Rhodopsin by Pepsin

pH 4.1; *ca.* 25°C.

Time	Per cent of initial extinction at 500 mμ	
	Control	Rhodopsin + pepsin
hrs.		
1.2	99.3	89.3
2.0	97.6	70.8
4.75	97.6	61.7
19.67	79.4	27.4

this pH leaves a residual extinction at 500 mμ of about 10 per cent, owing to retinene. Therefore rhodopsin had bleached about 81 per cent in the digestion mixture, as compared with 23 per cent in the control.

Pancreatic Lipase

On incubation at 30°C. with the lipase preparation described above, at pH 7.3–7.9, rhodopsin did not bleach significantly in 2 hours. After 20 hours, the extinction at 500 mμ was still 84 per cent of the initial value, representing a bleaching of about 18 per cent. A control mixture of rhodopsin and glycerol at the same pH and temperature did not bleach at all. Incubation with the enzyme preparation appeared therefore to have had a small effect.

Assay of this enzyme preparation, however, showed that it possessed some proteolytic as well as lipolytic activity. By Kunitz's procedure (Northrop *et al.*, 1948, p. 308), 1 ml. of the enzyme solution assayed at approximately 20×10^{-3} tryptic units. This should have been sufficient to account for the slow bleaching of rhodopsin observed. This experiment therefore yields no evidence that pancreatic lipase bleaches rhodopsin in solution.

DISCUSSION

The general result of these experiments is that rhodopsin in digitonin solution is digested and bleached by a variety of proteases. This is in good accord with the view that the rhodopsin chromophore, neo-*b* retinene, is bound to the protein opsin at a site composed primarily of amino acids.

The rhodopsin preparations used in these experiments are known to contain phospholipide. Broda (1941) has reported that partly purified solutions of frog rhodopsin contain large amounts of lipide-soluble phosphorus. Recently Krinsky (1958) has found that treatment of the outer segments of cattle rods with phospholipase C, which splits phosphoryl choline from lecithin, leaves rhodopsin intact and regenerable after bleaching. Rhodopsin prepared from such material contains about 20 atoms of lipide-soluble P per molecule. It is not yet clear that this forms part of the structure of rhodopsin; but it certainly forms part of such preparations as are used in the present experiments. It is possible that pancreatic lipase attacks this portion of our preparations; we did not test for this. Since, however, lipase does not appreciably bleach rhodopsin, one can conclude that any digestion it may accomplish leaves the site of attachment of retinene to opsin intact.

Another aspect of these experiments deserves notice. In our preparations rhodopsin forms with digitonin a large micelle. The micellar weight is about 300,000, of which rhodopsin with its associated phospholipide accounts for about 40,000 (Hubbard, 1953–54). Since the molecular weight of digitonin is 1229, this means that in each micelle one molecule of rhodopsin is associated with—probably surrounded by—about 200 molecules of digitonin. One would expect this involvement to offer serious hindrance to reactions which depend upon collisions with other molecules. For this reason we were surprised some years ago to find that opsin in digitonin solution reacts rapidly and smoothly with retinene, also held in solution as a micelle with digitonin (Wald and Brown, 1950). The present experiments display still more extraordinary behavior, for here rhodopsin reacts with enzyme proteins despite its envelopment in what we suppose to be a sheath of digitonin.

It seems necessary to assume that the digitonin coating is reasonably fluid, that collisions with other molecules penetrate it, and that relatively weak and labile forces of attraction between rhodopsin or opsin and other molecules succeed in holding it aside. The intimate picture of the reaction of rhodopsin with an enzyme probably begins with a collision which penetrates the screen of digitonin, bringing the proteins into contact. Thereafter they are held together by intermolecular electrostatic and van der Waals forces, and hydrogen bonds; and by what we may call—to borrow a phrase from photochemistry—a "cage effect," in that two such heavy particles once in contact must tend to be held together by the bombardment of solvent molecules all around them, and

ordinarily must collide repeatedly before separating. For these reasons both particles can be expected to "crawl over" each other for a time, so affording opportunity for the enzyme action to develop. This consideration is important for the discussion which follows.

We have found that the action of chymotrypsin on rhodopsin includes two phases: an initial rapid hydrolysis that exposes about 30 amino groups per molecule without bleaching, superimposed on a much slower hydrolysis that exposes about 50 additional amino groups per molecule with proportionate bleaching. How can this observation be explained?

It is possible that the first phase involves a rapid hydrolysis of contaminants, with, of course, no bleaching. We have noted that our rhodopsin preparations contained impurities which accounted for about half their nitrogen content. Much of this extraneous nitrogen has since been shown to be present in phospholipides (Krinsky, 1958) and small molecular peptides (Albrecht, 1957). The latter should not have been attacked vigorously by chymotrypsin, since it hydrolyzes primarily peptide linkages of the aromatic amino acids and methionine (Neurath, 1952), none of which occurs in more than traces in the peptides isolated by Albrecht from rhodopsin preparations. It is unlikely that contaminants of this nature could expose amino groups with the speed or in the quantity involved in the rapid initial phase of chymotryptic digestion.

It seems more probable that the rapid phase of hydrolysis without bleaching can be ascribed to rhodopsin itself. If so, what may it mean?

Some years ago Tiselius and Eriksen-Quensel (1939) observed that when pepsin acts upon ovalbumin, the first effect is a rapid fragmentation, without observable intermediates, from the initial substrate weight of 34,400 to a product of fairly uniform weight about 1080, apparently an octa- to decapeptide. The authors concluded that pepsin attacks ovalbumin in an all-or-nothing way, fragmenting each molecule that is attacked, completely to this level. Since these first observations, this mode of action, though by no means universal, has been observed with a wide variety of proteinases and substrates (Haugaard and Roberts, 1942; Winnick, 1944; Beloff and Anfinsen, 1948), and has been carefully discussed by Linderstrom-Lang (1950). Winnick, for example, has shown that a number of proteinases, including chymotrypsin, act on casein to produce initially fragments of the average sizes of penta- to heptapeptides.

It is possible that the action of chymotrypsin on rhodopsin involves such an initial fragmentation into smaller units, which, since it is not accompanied by bleaching, must leave the site of attachment of the retinene chromophore intact. Bleaching might then be caused by the slow hydrolysis of the chromophoric site itself; the proportionality between hydrolysis and bleaching in the slow phase of the reaction suggests that this is so. Alternatively it may be that the initial fragmentation leaves the chromophoric site unstable, so that the

fragments bleach spontaneously, without further hydrolysis. In that case, the proportionality between bleaching and hydrolysis in the slow phase of the reaction is fortuitous. These alternatives can readily be tested. Whichever of them proves to be true, the present experiments suggest that the protein moiety of rhodopsin might be cleaved to a fraction of its original size without loss of optical properties. This possibility is being investigated further in our laboratory.

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