177. THE CATAPHORETIC MOBILITY OF VISUAL PURPLE

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THE photosensitive substance contained in the rods of the eyes of vertebrates, visual purple, is a chromoprotein, i.e. a protein to which one or more chromophores are attached. Aqueous solutions of visual purple are decomposed by light to give an unstable compound "transient orange", which in turn bleaches thermally to "indicator yellow" [Lythgoe, 1937]. Indicator yellow may also be formed without the action of light. Such treatment as is known to bring about denaturation of proteins is effective in bleaching visual purple and producing indicator yellow, e.g. heating of the solution, addition of alkali, acid or organic solvents and violent shaking. Wald [1937] found that a yellow pigment can be extracted with organic solvents from solutions of indicator yellow; this was explained by assuming that bleaching involves rupture of the bond between the protein and the chromophore.

The object of the present work was to determine the isoelectric point of visual purple and the change in its cataphoretic mobility on bleaching. A preliminary account of some of the experimental results has been given previously [Broda *et al.* 1939].

EXPERIMENTAL

To observe the mobility of visual purple a micro-electrophoretic method was chosen. The migration velocity of quartz particles (diameter $1-5\mu$) suspended in buffered solutions of visual purple was measured under the microscope. Quartz particles suspended in protein solutions are coated with a layer of the protein; the sign of the charge and the mobility are within wide limits independent of size and shape of the particles, and are identical with those of the protein [Freundlich & Abramson, 1928; Abramson, 1928].

The cell consisted of a horizontal 64 mm. glass tube of elliptical cross section ($a_1 = 4.9$ mm., a_2 (depth of the cell) = 1.6 mm.) with glass stopcocks at both ends and a ground and polished centre part for observation. The electrodes were two platinized platinum wires introduced into the ends of the cell. The tension applied was 120 V., or 18.75 V./cm. Measurements were made at one-fifth of the depth of the cell. The values for the migration velocities observed at this depth were found to be very near to the absolute values, crystalline egg albumin serving as test substance.

By reversing the current the velocities of any single particle were determined in opposite directions in the cell and an average calculated in order to eliminate errors due to accidental flow in the solution. The final values for the velocities were obtained by taking an average of the velocities of a number of particles; but the individual variations of the different particles are normally not great. Examples for such series of observations are given in Table 1.

The visual purple solutions were prepared from the retinae of Hungarian frogs as described by Lythgoe [1937] and dialysed until practically salt-free. The concentration of the original solutions was of the order of 0.3 % chromoprotein, but after dilution with buffer it was reduced to about 0.01 %. In this range the mobility of visual purple and its bleaching product did not depend on concentration indicating that the quartz particles were well coated. The buffer used was *M*/100 sodium acetate-acetic acid; thus only uni-univalent salt was introduced. The *pH* was determined with a glass electrode in conjunction with a potentiometer set manufactured by the Cambridge Instrument Co., Ltd.

When the cataphoretic mobilities are plotted against *pH*, curves are obtained which, in the case of the compounds here investigated, closely approach straight lines at *pH* values where the mobility of the (positively or negatively charged) particles is small. The isoelectric point therefore is easily found by interpolation from the mobilities determined experimentally.

The experiments on visual purple were carried out in a safe red light. In each case after a sufficient number of observations, white light was switched on in the room and the velocity of the particles was measured again during and after bleaching of the solution. To exclude the remote possibility that different particles were involuntarily selected for observation by the eye in the red and white light respectively, in some cases the white light source was switched off after bleaching was complete, and the velocity in the bleached solution was measured again in red light. There was no difference between the velocities observed in the bleached solution with red or white light.

RESULTS

At *pH* values below 5.1 a difference between the mobilities in the unbleached and bleached solutions was found. On illumination the velocities changed during a few minutes until a final value was reached; whether they increased or decreased depends on the particular *pH* value (sign of the charge). In the immediate neighbourhood of the isoelectric point the velocity change may amount to as much as 100 % and more.

As visual purple is known to be bleached by hydrogen ions the velocities obtained in unbleached solutions of comparatively high acidity cannot be taken to represent the true values. Actually no constant velocity is observed in solutions of *pH* < 4.2 even in the dark, indicating appreciable bleaching of visual purple. In such solutions the velocity increases with time until a value is reached which is identical with that of the particles in an illuminated solution. The greater the acidity of the solution, the quicker the final value is reached. Table 1 shows by way of example three series of determinations at different *pH* values. At *pH* 5.11 the velocity does not change with time either in the dark or on illumination. At *pH* 4.26 the velocity changes on illumination, but not in the dark. At *pH* 3.85 the velocity changes not only on illumination, but a gradual change occurs in the dark. At *pH* values still lower the dark change proceeds so quickly that no reliable determination of the cataphoretic mobility of unbleached visual purple appears possible. It can be calculated from Lythgoe & Quilliam's [1938] data on the velocity of bleaching of visual purple by hydrogen ions that half of the substance will be bleached at 17° and *pH* 5.11 after about 300 hr., and at *pH* 4.26 after about 12 hr. These authors do not give the bleaching velocity at *pH* values as low as 3.85, but on extrapolation from their experiments it may be estimated that the half-life time of visual purple at this hydrogen ion concentration may be of the order of 10 min.

Table 1. *Three typical series of observations*

pH		Velocity (reciprocal values in arbitrary units)						
5.11	Dark:	-	3.45	3.25	3.20	3.40	3.25	
			3.20	3.45	3.75	3.50	3.15	
			3.30	3.30	3.45	3.45	3.20	
		Interval	3.10	3.15	3.45	3.40	3.30	3.30
			3.00	3.20	3.25	3.25	3.50	3.10
	Illuminated:		3.05	3.20	3.35	3.35	3.40	3.20
			3.00	3.35	3.20	3.45	3.30	
			3.70	3.30	3.40	3.20	3.60	
			3.35	3.30	3.30	3.30	3.45	
4.26	Dark:	+	10.4	11.8	17.1	10.7		
			12.6	14.0	14.6	14.8		
			11.5	12.9	15.8	12.7		
	Illuminated:		7.5	7.4	7.6	6.5	5.8	7.2
			7.9	7.6	7.2	8.3	8.0	7.8
			7.7	7.5	7.4	7.4	6.9	7.5
3.85	Dark:	+	4.60	4.55	4.35	4.70	4.20	
			4.70	4.55	4.15	3.90	4.40	
			4.65	4.55	4.25	4.30	4.30	
		Interval	3.50	3.50	4.30	4.00	3.90	
			3.90	3.60	4.20	4.20	4.50	
			3.70	3.55	4.25	4.10	4.20	
		Interval	3.50	3.70	3.85	3.75	3.70	
			3.70	3.70	3.90	4.05	4.05	
			3.60	3.70	3.85	3.90	3.85	
	Illuminated:		3.60	2.90	2.90	3.10	2.85	
			3.15	2.70	3.00	3.10	3.10	
			3.40	2.80	2.95	3.10	3.00	
		Interval	2.90	2.50	2.90	2.90	2.90	
			3.20	2.65	2.95	2.45	2.90	
			3.05	2.60	2.90	2.70	2.90	

Notes. The upper and lower figures in the same column refer to the velocities in opposite directions obtained by reversing the current.

The time intervals are about 5 min.

The values at pH 4.26 (dark) scatter considerably, as the velocity is so small that water currents influence the result.

The results obtained with a particularly pure preparation of visual purple are shown in Fig. 1. According to these the most probable values of the isoelectric points of visual purple and its bleaching product are pH 4.47 and 4.57. In agreement with this a solubility minimum of the bleaching product was found between pH 4 and 5. The isoelectric point being far on the acid side of the neutral point the compounds are to be ranged among the so-called acid proteins. This explains the earlier observation [Lythgoe & Quilliam, 1938] that buffered solutions of visual purple tend to become acid on prolonged dialysis.

While the shift on illumination is observed in every experiment, the absolute values of the cataphoretic velocities of the individual samples show slight variations. These could be due (1) to partial denaturation during preparation or to the presence of varying amounts of (2) non-protein or (3) protein impurities. (1) Denaturation cannot occur to any marked extent. While visual purple invariably gives indicator yellow on denaturation, pure visual purple solutions do not show indicator properties. (2) Phospholipins are present in the solutions

[Krause, 1937; Broda, Goodeve & Lythgoe, unpublished]. It was found, however, that addition of large amounts of lecithin to a solution of a test substance (gelatin) does not influence its cataphoretic behaviour. Furthermore, the solutions, from their preparation, contained varying amounts of digitonin. This could be removed quantitatively from the solution by a short shaking with a solution of cholesterol in light petroleum; but in practice this treatment was

Fig. 1. The cataphoretic mobility of visual purple and its bleaching product. Open circles: visual purple; full circles: bleaching product.

unnecessary, as addition of much digitonin to a gelatin solution did not change the mobility of the particles. (3) Washing of the rod tissue with buffer of pH 4.5, which is a normal step in the preparation of the solutions, removes small amounts of a colourless protein. This is detected by its ultraviolet absorption band which disappears on addition of alcohol to the buffer solution. Part of this protein may conceivably remain in the tissue and be dissolved together with the visual purple when digitonin acts on the rods.

DISCUSSION

The chemical significance of the charge difference. It is well known that intense irradiation of proteins, especially with ultraviolet light, may cause denaturation or far-reaching decomposition obviously leading to changes in their electrical properties. But apparently no protein has been known to undergo a shift in cataphoretic mobility on moderate illumination. This unique electrochemical behaviour of visual purple is connected with its high photochemical sensitivity as expressed in terms of light absorption.

The difference of the electric charges before and after bleaching must be due to a difference in the ionized groups in the adsorbed protein layer. The production of a similar electric charge difference in haemoglobin through chemical reaction has been observed by Hastings *et al.* [1924] and by German & Wyman [1937]; the titration curve of haemoglobin differs from that of oxyhaemoglobin, the oxidized compound being a stronger acid between pH 6.1 and 9.0 and a stronger base between pH 4.5 and 6.1. In view of Wald's assumption that bleaching of visual purple involves rupture of the bond between the protein and the chromophore, it is interesting that the isoelectric point of globin, freed from the chromophore haem by acids, is 0.7 pH unit higher than that of the chromoprotein haemoglobin [Roche, 1929].

The greater positive charge of visual purple after bleaching may be due to a decrease in the number of ionized acid groups as well as to an increase in the number of ionized alkaline groups. The first might be caused by a reduction of the strength of acid groups present (but not by the total suppression of one of them, as then the charge difference would remain unaltered at higher pH). The second may be brought about by a strengthening of alkaline groups present, or even by a liberation of new alkaline groups. Unfortunately, the instability of visual purple in acid solutions prevents a decision whether the charge difference is maintained at low pH (indicating that new alkaline groups are liberated on bleaching), or whether, as in the system haemoglobin-oxyhaemoglobin, the two curves unite at low as well as at high pH (indicating that only a modification in the strength of existing groups has occurred, and that the total number of dissociable groups has not changed). In the latter case the dissociation region is merely shifted on transformation, and the mobility or titration curves, even if they are extended over the widest pH range, cannot provide a criterion whether the groups transformed are acid or alkaline.

The dissociation constant of the group affected can be roughly estimated from the relationship between the charge difference and pH from 5.1 to 3.85, if one univalent group is assumed to be responsible. From the curvature of this function it follows that the midpoint of the dissociation region is somewhat below pH 4, and that the dissociation constant is therefore somewhat greater than 10^{-4} . This refers to the group in the bleached compound if the group is alkaline, and to the group in visual purple if the group is acid.

Dissociation constants as high as 10^{-4} are so far unknown among the basic groups of protein constituents, but are common among their acid groups. In the living retina ($pH \sim 6.5$) the dissociation of the group will be practically complete.

The group apparently has nothing to do with the indicator properties of the bleaching product. The transformation region of indicator yellow is above pH 5 [Lythgoe, 1937].

The numerical value of the charge difference. The electric charge Q of a protein molecule at any pH value can be estimated by means of Abramson's [1932] equation

$$Q = 6\pi\eta vr \left([r \sqrt{m} \cdot 0.33 \times 10^8] + 1 \right)$$

(η = viscosity of the solution in poises, r = radius of the molecule, m = ionic strength of the solution, v = cataphoretic mobility in cm./sec./electrostatic units/cm.) As pointed out above, the mobility of the protein is identical with that of the coated quartz particle. Accordingly the charge difference ΔQ produced by bleaching visual purple at pH 3.85, i.e. the maximum charge difference which can be measured fairly safely, can be calculated from the mobility difference of $0.71 \mu/\text{sec.}/V./\text{cm.}$, if the protein is assumed to be spherical, and its

molecular weight and specific gravity are 270,000 [Hecht & Pickels, 1938] and 1.3, respectively:

$$\Delta Q = 6\pi \cdot 0.01 \cdot 37.3 \times 10^{-8} \cdot 2.13 \times 10^{-4} ([37.3 \times 10^{-8} \sqrt{(0.01) \cdot 0.33 \times 10^8} + 1) \\ = 33.5 \times 10^{-10} \sim 7 \text{ elementary charges.}$$

The dissociation constant of the group concerned being a little greater than 10^{-4} , the charge difference at pH 3.85 is due to not more than about half ionization of the group; should a modification in the strength of one of the existing groups be the reason of the charge difference, this may correspond to less than half ionization at the midpoint of the dissociation region, as then the ionization of the original, non-modified group has to be taken into account. Thus at full ionization not less than 14 elementary charges per visual purple molecule, or not less than one charge per weight of 19,300, would be obtained on bleaching. Similarly, on oxidation of haemoglobin (mol. wt. 66,800) at least three additional charges per molecule, or one charge per weight of 22,300, are produced [Hastings *et al.* 1924; German & Wyman, 1937]. The charge difference of not less than 14 compares with a number of 16 Svedberg units in the molecule of the visual purple protein, probably each unit carrying one chromophore [Broda *et al.* 1940]; it may be that light or hydrogen ions transform one group in each Svedberg unit, while bleaching the chromophore attached to it.

SUMMARY

The cataphoretic mobilities of visual purple and its bleaching product have been determined between pH 3.5 and 5.1 and found to differ. The most probable value of the isoelectric point of visual purple is pH 4.47 and that of the bleaching product 4.57.

The shift in the mobility is due to a change in the ionized (acid or alkaline) groups in the protein molecule on bleaching. The nature of this change is discussed. The dissociation constant of the group concerned is of the order 10^{-4} .

The charge difference at the midpoint of the dissociation region of the group amounts to about 7 elementary charges per molecule of the chromoprotein. The molecules contain at least as many groups capable of transformation as chromophores.

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