

REGENERATION OF VISUAL PURPLE IN SOLUTION*

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I

INTRODUCTION

Regeneration of visual purple in solution was originally observed by Kühne (Ewald and Kühne, 1878). It was not until recently that it was again reported by Hecht, Chase, Shlaer, and Haig (1936). They found by measuring density changes at 500 $m\mu$ with Shlaer's photoelectric spectrophotometer (Shlaer, 1938) that regeneration occurred only after about 85 per cent of the visual purple originally present had been bleached and that it was maximal in solutions of pH about 7.7. Hosoya (1938) has reported that under certain conditions visual purple can be made to regenerate in solution after only a relatively small percentage of it has been bleached.

It is known that at neutral and acid pH's the photic decomposition of visual purple results in the appearance of other colored compounds (Weigert and Nakashima, 1930; Chase, 1936; Lythgoe, 1937), one or more of which become colorless at a rate dependent upon the temperature (Hosoya, 1933; Wald, 1937; Lythgoe, 1937). Since visual purple regeneration is measured optically, the presence of such additional color changes would complicate and perhaps mask the changes of visual purple itself at these pH's. Because this effect becomes greater in more acid solutions, it was apparent that pH 7.7 might not be the true maximum for regeneration.

We have extended the investigations already reported by examining in detail the effect of pH on regeneration, taking into consideration the formation and disappearance of colored decomposition products, and making the measurements in such a way as to minimize the complicating factors that are present in neutral and slightly acid solutions. We have also investigated the effect of wave length of the bleaching light, as well as that of temperature during extraction.

We have secured data which give information about the course of the regeneration reaction in solution by measuring the absorption spectrum of solutions during regeneration, and have made some investigations into the kinetics of the chemical reaction which controls the process.

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II

Materials and Methods

The visual purple was obtained from frogs (*Rana pipiens*) freshly received from Alburg, Vermont. In general, the methods of extraction were as already described (Chase and Haig, 1938). The retinas were usually hardened by immersing in 4 per cent alum solution for 2 hours before being put in the extractive, although this step was omitted in some cases. Unless otherwise specified, the extractions were made at room temperature, 20 to 24°C. All centrifuging was done at 6°C., in order to minimize decomposition of the visual purple during the periods of 1½ to 3 hours in the centrifuge, whose temperature often reaches 30° or slightly higher if no effort is made to prevent its rise.

About 12 to 16 retinas were used for each milliliter of the extractive. Either 4 per cent purified bile salts solution or 2 per cent digitalin solution (digitalin cryst., Eimer and Amend), was used, usually the latter because of its greater freedom from bacterial growth. The resulting solution, after addition of 20 per cent (by volume) of buffer solution, had an optical density at 500 m μ of about 1.8 in a depth of 20 mm. The solutions were always used as soon as possible after their preparation in order to prevent artefacts caused by changes which take place upon standing, even at low temperatures. In most cases the solutions were not more than 48 hours old; often not older than 24 hours.

A special absorption cell was used which held only 2½ to 3 ml. of solution and had an optical depth of 20 mm. The cell was rectangular and its interior cross-sectional area was only slightly greater than the cross-sectional area of the monochromatic measuring beam of the spectrophotometer.

In all our measurements we used Shlaer's photoelectric spectrophotometer (Shlaer, 1938). The data are given as photometric densities; these are equal to $\log(I_0/I_t)$ where I_0 is the intensity of the incident beam and I_t that of the beam transmitted by the solution. The photometric density is directly proportional to the concentration of absorbing substance in the solution.

As has been already shown (Chase and Haig, 1938) it is essential when studying the effect of any variable on the behavior of visual purple in solution, to use identical samples of one extraction of visual purple. This practice was consequently followed in the experiments to be reported. Each extraction was divided into several samples and these samples were then treated and measured at the same time, or as nearly the same time as possible—usually within 1 day—so that the solution itself should not undergo changes between measurements on the different samples.

III

Effect of Duration and Wavelength of Exposure

Photic decomposition of visual purple at neutral and acid pH's results in the formation of a yellow material which itself fades to a more or less colorless condition (Hosoya, 1933; Wald, 1937; Lythgoe, 1937). Therefore, when studying visual purple regeneration it is necessary to illuminate for a sufficiently long time to allow the yellow material initially formed to bleach

before the regeneration measurements are begun. Otherwise the density change measured will be the algebraic sum of a decrease in density caused by the fading of yellow color and an increase in density caused by regeneration of visual purple, with the result that the visual purple regeneration will appear less than it actually is and the shape of the regeneration curve will not be significant as far as visual purple is concerned.

Fig. 1 illustrates this effect under extreme conditions which favor its appearance. Two samples of visual purple solution, one of pH 9.9 and the other of pH 5.2, were illuminated for 5 seconds with a photoflood lamp at 3 inches distance and their density at $480\text{ m}\mu$ was then measured for 2 hours in the dark. In both samples the density decreases during the 2 hour period but the total density fall is about 10 times as great and its rate much less in the acid sample than in the alkaline sample. The fading of this decomposition product is of course less of a factor in regeneration measurements when the exposure time is longer. All of the exposures used in the experiments to be reported were therefore of long duration ($3/4$ to $1-1/2$ hours) as contrasted with the 10 minute exposure time used earlier (Hecht, Chase, Shlaer, and Haig, 1936).

It has already been shown (Chase, 1937) that the spectral content of the bleaching source greatly influences the subsequent visual purple regeneration. If the illuminating source contains blue and violet much greater regeneration follows the bleaching than when these wavelengths are lacking. This was demonstrated by dividing a visual purple solution into two samples and bleaching one with light containing only the longer visible wavelengths while the other was bleached with light from the short-wave part of the visible spectrum. Although the two sources were adjusted in intensity to be equally effective for bleaching the visual purple in the solutions, much more regeneration occurred in the sample bleached by the source containing the blue and violet than in the other sample. Moreover, in the sample bleached by the blue and violet source there was a much greater density decrease at the shorter wavelengths during the illumination than there was during the illumination of the other sample with light containing red, orange, and yellow. This latter sample, upon being re-illuminated, but this time with blue and violet light, showed a considerable decrease in density at the shorter wavelengths and, after the light had been turned off, regenerated as much as the other sample had done. These results indicated that visual purple solutions contain a photosensitive substance whose breakdown by light is necessary before visual purple regeneration can take place. An experiment of the sort described is illustrated in Fig. 2, where density is measured at two wavelengths on two samples.

VISUAL PURPLE REGENERATION

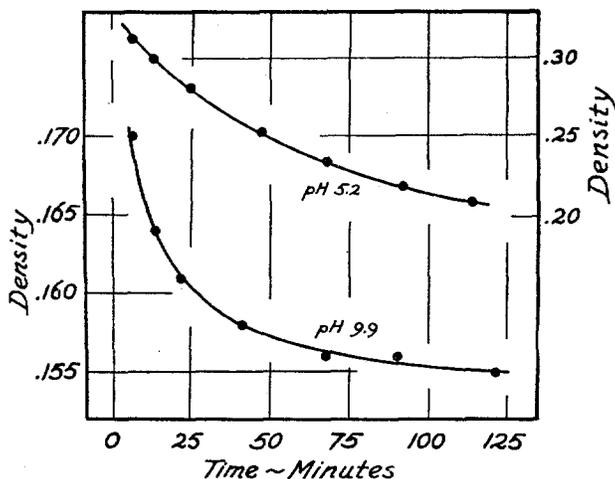


FIG. 1. The fading of two visual purple samples measured at $480\text{ m}\mu$ after a 5 second exposure to a photoflood lamp. The decrease in density is less but much faster at the more alkaline pH. In regeneration studies, illumination must be sufficiently long to allow these processes to complete themselves before density measurements are begun.

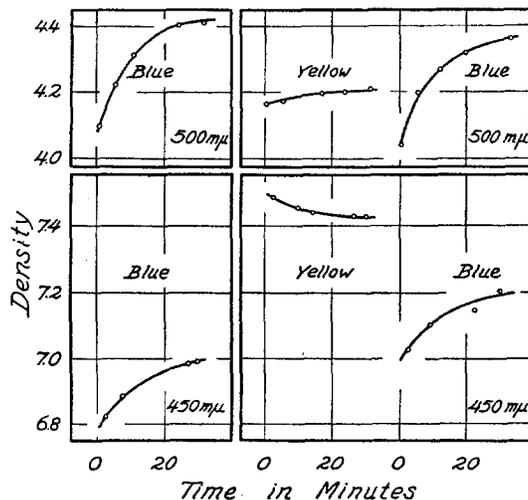


FIG. 2. Influence of the color of the bleaching source on visual purple regeneration. The sample on the left was bleached with predominantly blue and violet light; it shows distinct regeneration, both at $500\text{ m}\mu$ and $450\text{ m}\mu$. The sample on the right was bleached with light which lacked blue and violet; it shows only slight regeneration at $500\text{ m}\mu$ and fading at $450\text{ m}\mu$. A second bleaching of the latter sample with blue and violet light causes a large density decrease, particularly at $450\text{ m}\mu$, and a subsequent regeneration as great as that given by the sample shown on the left.

It was possible to obtain an approximate measurement of the absorption spectrum of this photosensitive material by considering the visual purple regeneration which followed bleaching with different wavelengths as a criterion of the absorption of these different wavelengths by the unknown photosensitive substance. Identical samples of a visual purple solution were bleached with white light filtered by four different Wratten monochromatic filters, Nos. 73, 74, 75, and 76. By means of neutral filters the intensity of these colored sources was adjusted until they were equally effective in bleaching a sample of the visual purple solution identical with those used in the experiments. When the effective intensity of the colored sources had been equalized in this way, each one was used to illuminate a single sample of the solution for 3/4 of an hour and the amount of regenera-

TABLE I

Influence of Wavelength of Bleaching Light on Subsequent Regeneration

The sources were adjusted to be equally effective in bleaching the visual purple. Exposure time was 45 minutes for the wavelengths isolated by the Wratten filters. The regeneration values given are per cent of density change at 500 $m\mu$. The pH was 7.6.

Filter No. and central λ of bleaching light		Regeneration	
		Solution A	Solution B
	$m\mu$	<i>per cent</i>	<i>per cent</i>
(No. 76)	440	2.9	2.9
(No. 75)	490	3.3	1.6
(No. 74)	525	1.4	1.7
(No. 73)	575	0.3	0.3

tion that occurred was measured at 500 $m\mu$ in each case. The results of two such experiments are given in Table I. The values are per cent regeneration, calculated as density regenerated at 500 $m\mu$ divided by the density at that wavelength of the visual purple originally present. The values given for the bleaching wavelengths represent the centers of the fairly narrow spectral bands transmitted by the so called monochromatic filters.

Although the per cent of regeneration that occurred in these experiments was small (probably because the bleaching intensities were limited by the low transmission of the filters), the differences in per cent regeneration following the various illuminations are quite significant, and leave no doubt that the substance whose photic decomposition is responsible for visual purple regeneration has an increasing absorption toward the shorter wavelengths and is consequently yellow in color. This yellow substance may

be a decomposition product of visual purple itself. It is also possible that a flavin—known to be present in the eye—is involved, but we have so far not been able to demonstrate this. Granit and Wrede (1937) have postulated, as a result of analysis of electroretinograms, a pigment or receptor in the frog's eye which reacts specifically to blue and violet light, so it is not impossible that this photosensitive yellow pigment may have a rôle in vision apart from aiding visual purple regeneration.

IV

Effect of pH on Regeneration

In measuring regeneration of visual purple solutions at different pH's we used three different visual purple extractions, each one furnishing three to six samples. These samples were adjusted to the desired pH values with Clark and Lubs buffers (Clark, 1928), made up to be 0.4 M. They were added to the visual purple solutions in the proportion of one part of buffer to four parts of solution, by volume. The pH of the resulting solutions was checked with a glass electrode. A 500 watt projection lamp at 10 inches served as the bleaching source. This insured adequate energy, and a 45 minute exposure allowed most of the intermediate yellow color to disappear before the regeneration measurements were begun. A heat-absorbing glass filter was interposed between the lamp and the solution.

The density of each solution was measured in the dark for three-quarters of an hour immediately following the illumination. Measurements were made at three wavelengths, 440, 500, and 560 $m\mu$. Because the transient yellow materials in the solution absorb only slightly, if at all, at 560 $m\mu$, the density changes at this wavelength were taken as representative of visual purple regeneration. After measuring regeneration for 45 minutes, the solutions were illuminated for 15 minutes more and again measured to make sure that the density change which had been observed was really caused by the building up in the dark of photosensitive substances and not simply by increases in turbidity or other artefacts.

The density values at 560 $m\mu$ following the illumination were plotted against time in the dark and smooth curves drawn. From these curves were taken the density values for 5 minutes and 30 minutes in the dark so as to avoid errors of extrapolation, and the difference between these two values was taken as a measure of the regeneration that had occurred at any given pH.

Because these samples were from three extractions of different visual purple concentration, they did not show the same amounts of regeneration.

Each set of samples was therefore used to construct a single curve showing the relation between pH of the solution and visual purple regenerated.

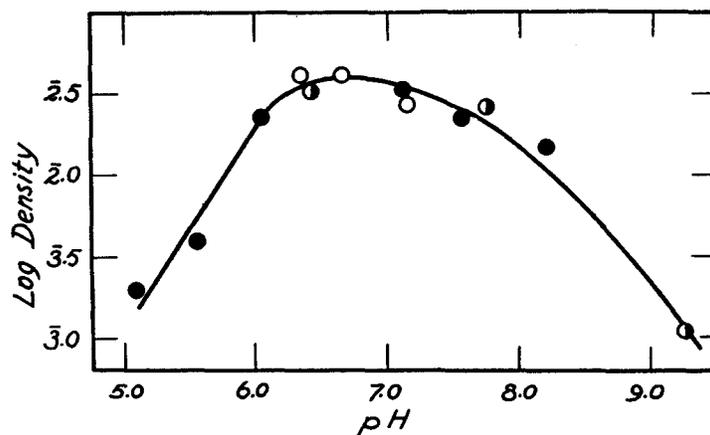


FIG. 3. Effect of pH on the regeneration of visual purple. The data, given in Table II, are from three different solutions, and represent measurements at 560 $m\mu$.

TABLE II

Effect of pH upon Amount of Regeneration

Regeneration measured by the change in density at 560 $m\mu$, after bleaching for 45 minutes with a 500 watt projection lamp at 10 inches from the solution. Data of Fig. 3.

Solution	pH	Density values from smoothed curves			Log of difference	Adjusted log values
		5 min.	30 min.	Difference		
A	5.09	0.136	0.138	0.002	$\bar{3}.301$	$\bar{3}.301$
	5.55	0.153	0.157	0.004	$\bar{3}.602$	$\bar{3}.602$
	6.04	0.148	0.171	0.023	$\bar{2}.362$	$\bar{2}.362$
	7.12	0.158	0.192	0.034	$\bar{2}.532$	$\bar{2}.532$
	7.69	0.101	0.124	0.023	$\bar{2}.362$	$\bar{2}.362$
	8.19	0.122	0.137	0.015	$\bar{2}.176$	$\bar{2}.176$
B	6.42	0.093	0.108	0.015	$\bar{2}.176$	$\bar{2}.520$
	7.74	0.070	0.082	0.012	$\bar{2}.079$	$\bar{2}.420$
	9.25	0.0860	0.0865	0.0005	$\bar{4}.699$	$\bar{3}.050$
C	6.35	0.174	0.186	0.012	$\bar{2}.079$	$\bar{2}.620$
	6.67	0.156	0.168	0.012	$\bar{2}.079$	$\bar{2}.620$
	7.16	0.136	0.144	0.008	$\bar{3}.903$	$\bar{2}.440$

Since the three visual purple extractions presumably differed only in visual purple concentration, it was possible to plot the logarithms of the density changes observed and obtain three curves, which were of essentially the

same shape, but lay at different levels on the log density axis. By shifting two of these curves vertically a single curve results about which all the points fall. This curve is plotted in Fig. 3. The experimental density values used to construct the curve are given in Table II, together with their logarithmic values and the adjusted logarithmic values which appear in the figure. Over the pH range from 5.0 to 9.6 a maximum for regeneration is shown at about pH 6.7, a value more acid than that earlier reported from this laboratory by Hecht, Chase, Shlaer, and Haig (1936). The reason for this difference is that in the present measurements allowance was made for the disappearance of transient yellow intermediate products.¹

It is quite possible that the visual purple molecule or something else in the solution essential for regeneration may undergo dissociation with changes of hydrogen ion concentration and thus exert an effect upon regeneration. If this is so, we might expect that the curve relating regeneration to pH should resemble a titration curve. The curve of Fig. 3 does not, but it is possible that decomposition or the presence of colored products at pH's below 6.6 prevents the measurement of regeneration there which may theoretically occur. The abrupt drop in the curve on the acid side suggests some sort of decomposition. It is also possible that the curve of Fig. 3 may represent by its maximum an isoelectric point or some other property of a protein, since visual purple is now generally recognized to be protein in nature. The data are neither complete nor precise enough to warrant more than a mention of such possibilities. They do show that under practical conditions the maximum amount of measurable regeneration occurs at a pH of about 6.7.

v

Effect of Extraction Temperature

If some other substance besides visual purple is necessary for regeneration to occur after illumination and if this substance is extracted from the retina along with the visual purple, different temperatures during extraction might

¹ The influence of this factor is very great as can be shown if the visual purple regeneration is measured at regions in the spectrum where the unstable colored products of visual purple decomposition have a greater absorption than they have at 560 m μ . Thus, if we had taken the amount of regeneration measured at 500 m μ instead of at 560 m μ , the curve relating regeneration to pH would have been found to have its maximum at about pH 7.1 instead of pH 6.7 and there would have even been some decrease rather than increase in density at pH's below 5.6. When the regeneration as measured at 470 or 440 m μ is used, the effect of masking reactions is even more marked. Because it is as yet impossible to eliminate completely such factors, the pH maximum for regeneration may even be somewhat further toward the acid side.

be expected to change the ratio of visual purple to this other material, with a consequent difference in the amount of regeneration in the resulting solutions.

Retinas from 150 frogs were prepared for extraction in the usual way. These were then divided into three equal lots and extracted simultaneously at 7°, 20°, and 35°C., respectively. The resulting solutions were buffered at pH 6.7 and measured to determine their visual purple density. A sample of each was then illuminated for 45 minutes and measured at 500 $m\mu$ for 45 minutes immediately following the illumination. The density regenerated at 500 $m\mu$ for each sample during the 45 minute period was divided by the density at that wavelength of each sample before illumination. The sample from the 7° extraction showed 9.8 per cent regeneration, that from the 20° extraction showed 9.9 per cent regeneration, and the 35° sample regenerated 9.2 per cent. These values are sufficiently alike to show that temperature variation over the range between 7° and 35°C. during extraction is without effect upon regeneration of visual purple. Therefore, if another substance necessary for regeneration must be extracted from the retina, the solubility of this hypothetical substance in 2 per cent digitalin solution varies with temperature in the same way as that of visual purple, unless both go completely into solution at all the temperatures studied.

VI

Absorption Spectra during Regeneration

The absorption spectrum of a bleached visual purple solution was measured at different times during regeneration in order to determine whether any other reactions involving color changes occur.

It is impossible with this spectrophotometer to measure completely an absorption spectrum which is changing rapidly. We therefore adopted the following method. A visual purple solution buffered at pH 7.6 was illuminated for 1 hour with a 100 watt lamp and immediately after turning off the light the density of the solution was measured at 500 $m\mu$, 470 $m\mu$, 440 $m\mu$, and 530 $m\mu$. The total time required to measure the density at these four wavelengths was about 5 minutes. The density at 500 $m\mu$ was then measured again, followed by that at 470, 440, and 530 $m\mu$, while the visual purple was regenerating, and this process was continued for 2 hours. The solution was then exposed for 15 minutes to the bleaching source and the density at the four wavelengths again determined to insure that any observed changes in the dark had been caused by regeneration of photosensitive substances. Curves were constructed showing density changes

in the dark for each of the wavelengths measured. The data used in the construction of these curves are given in Table III.

From the curves it was easy to calculate the density increase at each of

TABLE III

Course of Regeneration of Visual Purple at pH 7.6 Measured in Different Parts of the Spectrum

440 m μ		470 m μ		500 m μ		530 m μ	
Time	Density	Time	Density	Time	Density	Time	Density
<i>min.</i>		<i>min.</i>		<i>min.</i>		<i>min.</i>	
(0.0)	(0.856)	(0.0)	(0.602)	(0.0)	(0.435)	(0.0)	(0.332)
1.0	0.860	2.0	0.616	3.5	0.465	5.0	0.356
7.0	0.884	8.5	0.651	10.0	0.504	12.0	0.380
14.0	0.900	15.0	0.674	17.0	0.527	18.0	0.394
26.0	0.918	28.0	0.700	30.5	0.558	32.5	0.414
36.5	0.928	38.0	0.715	40.0	0.571	42.0	0.424
48.0	0.935	50.0	0.726	51.5	0.583	53.5	0.432
61.5	0.938	64.0	0.736	65.0	0.593	68.0	0.438
92.3	0.948	78.0	0.744	80.5	0.601	82.5	0.444
97.0	0.950	99.0	0.750	100.5	0.610	103.5	0.449
119.0	0.952	121.0	0.756	122.5	0.616	125.0	0.453
(∞)	(0.956)	(∞)	(0.760)	(∞)	(0.622)	(∞)	(0.456)

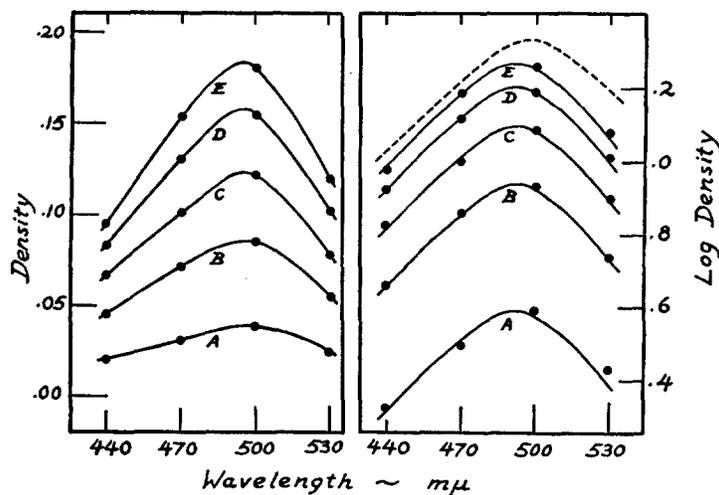


FIG. 4. The spectrum of the regenerating color at different times after bleaching; A, 5; B, 15; C, 30; D, 60; and E, 120 minutes. The log density curves on the right have the same shape and therefore show only concentration differences. The absorption spectrum of a partially purified visual purple solution before illumination is shown by the dash-line curve in terms of log density for comparison. Data are in Table III.

the five wavelengths during different times in the dark, obtaining the density for zero time in the dark by extrapolation of the curves. These values for zero time are also shown in the table, as are the final density values reached, also obtained by extrapolation.

The absorption spectrum of the regenerated material was computed for 5, 15, 30, 60, and 120 minutes in the dark and these five spectra are plotted in the left half of Fig. 4. If visual purple is the only substance that appears during the dark period the absorption spectrum constructed from the regeneration curves should be identical with the absorption spectrum of visual purple. If another colored substance (or substances) is formed at the same time but at a different rate the absorption spectra measured at different times during regeneration should not represent visual purple alone, nor should they be multiples of each other. This should also be the case if visual purple regeneration first involves the formation in the dark of some other colored substance, which then takes part in building visual purple.

It is clear that the absorption spectra of Fig. 4 are not very different from that of visual purple although there is slightly more absorption in the blue and violet than should be expected. A simple way of showing similarity or dissimilarity of these five absorption spectra among themselves is to plot them in their logarithmic form. Such curves, being independent of concentration differences, can be shifted on the log density axis. They can be superimposed only if they differ from one another in concentration alone, and not if two or more reactions involving color changes are occurring at different rates. Such logarithmic plotting is shown in the right half of Fig. 4. The curves drawn through the five sets of points are identical and it is therefore apparent that only one principal colored substance is being built up in the dark.

The logarithmic form of the absorption spectrum of a partially purified unbleached visual purple solution is also shown in Fig. 4 by the dash-line curve to emphasize the small departure of the absorption spectrum of the regenerated substance from that of visual purple. It is possible that this small extra absorption represents the simultaneous regeneration in the dark of the yellow photosensitive material described in section III. However, several yellow pigments are now known to appear in visual purple solutions so it is not at present profitable to try to identify this extra absorption at the shorter wavelengths, especially as its occurrence is rather variable.

In another experiment conducted at pH 6.7 and involving density measurements at 440, 470, 500, 530, and 560 $m\mu$, measuring at 500 $m\mu$ and two other wavelengths on each of two identical samples, the same results were obtained except that the extra absorption at the shorter wavelengths

was greater. Still other experiments agreed in this respect with the one illustrated. It is possible that the greater absorption at shorter wavelengths in the more acid solutions may be caused by a natural acid-base indicator in the regenerating solution, as Chase has shown occurs during bleaching of visual purple (Chase, 1936), but the data are not conclusive on this point. It is also possible that visual purple regenerated in solution is not the same as the extracted visual purple before illumination.

Visual purple regeneration in solution then does not occur through consecutive reactions involving color changes, unless such changes take place during the initial bleaching of the visual purple, or during the first few moments in the dark, too rapidly to be measured.

The occurrence of this extra absorption at the shorter wavelengths in the case of the regenerating visual purple, offers an obstacle to the calculation of regenerated visual purple in terms of per cent of that present in the unilluminated solution. If the density of the regenerated material at 450 $m\mu$, for example, is compared with the density at that wavelength of the visual purple before illumination (using either the classical absorption spectrum or the absorption spectrum of the unbleached solution), the apparent percentage regeneration may be as much as twice as great as it is if these calculations are made using the density measured at 500 $m\mu$.

Having recognized this fact, it is nevertheless possible to use percentages of regeneration as a means of comparing the regeneration capacity of various extractions, provided that calculations are always made in terms of a single wavelength, preferably 500 $m\mu$. Measured at this wavelength and in solutions of the optimum pH, regeneration during the current work was found to vary in different extractions from 6 per cent to 18 per cent, with 12 per cent the value most often encountered. The greatest amount of regeneration which we obtained involved a density increase at 500 $m\mu$ of about 0.18 in a solution which had a visual purple density of 1.0 before illumination. The optical depth of the absorption cell in this case was 10 mm.

VII

Successive Regenerations

Kühne reported (Ewald and Kühne, 1878) that both in the isolated retina and in solution visual purple could be made to undergo several successive regenerations, each time to a less extent, until finally none occurred. In repeating his observations we were interested to see whether the gradual failure to regenerate after successive bleachings was caused by the disappearance of a reactant or by the destruction of a catalyst of some sort.

If a catalyst is failing, the second and third regenerations should be at a slower rate than the first, but should ultimately produce the same concentration of visual purple. On the other hand, if a reactant is being used up, the amount of visual purple regenerated should be less in the second regeneration than in the first, and still less in the third, but the curves for all three should be of the same form. We illuminated a visual purple solution (buffered at pH 7.7) for 3/4 hour and then measured the density change at

TABLE IV
Successive Regenerations of Visual Purple

Density measurements at 500 μ . The first bleaching was for 45 minutes; the second and third were for 15 minutes each. pH 7.7. Density values for zero and infinite regeneration time were obtained by extrapolation of the smoothed curves. These data are plotted in Fig. 5.

First regeneration		Second regeneration		Third regeneration	
Time	Density	Time	Density	Time	Density
<i>min.</i>		<i>min.</i>		<i>min.</i>	
(0.0)	(0.0775)	(0.0)	(0.0775)	(0.0)	(0.0775)
0.7	0.0812	1	0.0804	5	0.0860
4	0.0872	5	0.0872	10	0.0900
7	0.0968	11	0.0948	17	0.0956
12	0.1056	18	0.1024	28	0.1008
19	0.1152	25	0.1076	42	0.1052
26	0.1244	31	0.1120	51	0.1076
33	0.1304	39	0.1160	64	0.1104
40	0.1352	51	0.1204	79	0.1124
49	0.1404	64	0.1252	100	0.1152
62	0.1456	72	0.1272	113	0.1164
70	0.1496	82	0.1284	119	0.1172
80	0.1536	87	0.1300		
89	0.1556	96	0.1320		
100	0.1580	108	0.1332		
110	0.1600	115	0.1344		
117	0.1612	120	0.1352		
(∞)	(0.1675)				

500 μ for 2 hours in the dark. The solution was then re-illuminated for 15 minutes and measured in the dark for another 2 hours. After this it was illuminated a third time for 15 minutes and again measured for 2 hours in the dark. Fig. 5 shows the results of this experiment, the data being given in Table IV. The two curves through the lower sets of points are calculated multiples of the smooth curve drawn through the upper set of points. The fact that the experimental points fall upon these calculated curves so well

indicates that in successive regenerations a reactant is used up and an enzyme or catalyst apparently is not the controlling factor. It is possible that the reactant in question is normally supplied from the pigment layer as Kühne believed (Ewald and Kühne, 1878). In such a case there would of course be only a limited quantity of it in the solution, which should cause exactly the behavior observed in this experiment.

The behavior of visual purple which has been extracted from the retinas by 4 per cent sodium desoxycholate solution may be significant in this connection. Visual purple solutions obtained by use of this extractant do not regenerate, even at pH's that are optimal for bile salts and digitalin extractions. The simplest explanation is that the sodium desoxycholate combines with the visual purple molecule or with its decomposition products in such a way that rebuilding of the visual purple after illumination does not take place. Alternatively, the desoxycholate may combine with or fail to extract from the retina some other substance which normally enters into the reactions through which visual purple regeneration is accomplished.

VIII

Reaction Kinetics

The reaction kinetics of the bleaching of visual purple by light have been shown to be first order (Hecht, 1921; Chase, 1936). Regeneration of visual purple, on the other hand, might be expected to be the result of a bimolecular reaction, especially in view of the dependence of dark adaptation on vitamin A (Fridericia and Holm, 1925; Tansley, 1931; Hecht and Mandelbaum, 1938; Wald, Jeghers, and Arminio, 1938). However, if one of the reactants were present in any great excess, a first order equation should describe the data.

The precautions necessary in order to favor the maximum amount of visual purple regeneration, uncomplicated by the simultaneous appearance or disappearance of other colored substances, have been mentioned. The absorption of these substances is greater at shorter than at longer wavelengths so that density measurements made at 560 $m\mu$ should be expected to be more indicative of changes in visual purple concentration, uncomplicated by concentration changes of yellow substances, than would measurements made at 500 $m\mu$. On the other hand, measurements made at 560 $m\mu$ would be subject to larger experimental errors than measurements made at 500 $m\mu$ because the absorption of visual purple is much less at 560 $m\mu$ than at 500 $m\mu$. We therefore measured the density change at several wavelengths simultaneously following an exposure of 1 hour, which allowed

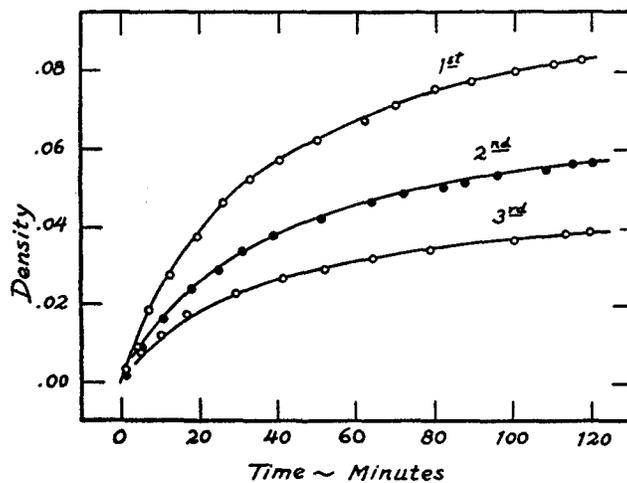


FIG. 5. Three successive regenerations in the same solution, measured at 500 $m\mu$. The colored material bleaches to the same density each time and the three curves are multiples of one another. The data are given in Table IV.

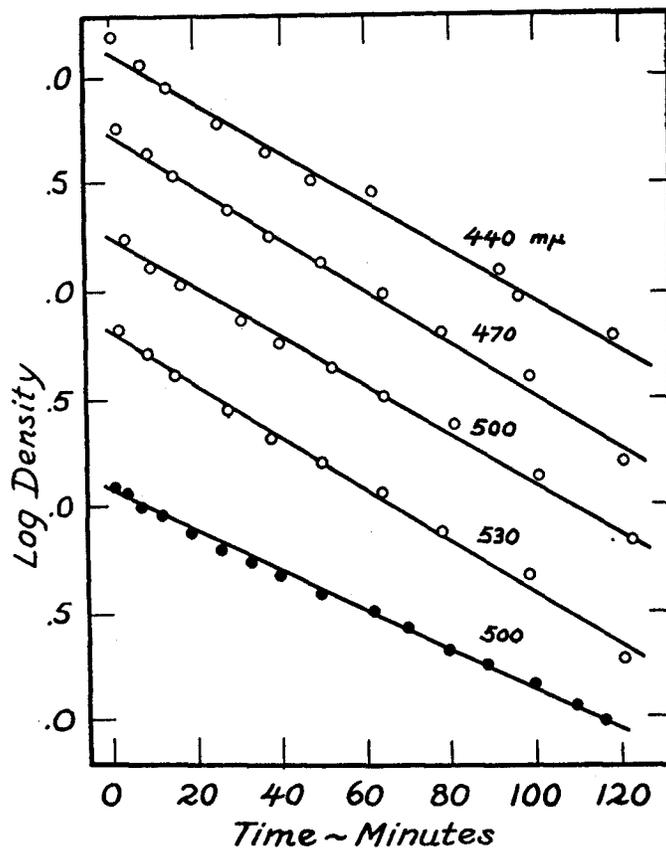


FIG. 6. Description of visual purple regeneration by the first order equation. The four upper sets of data are from Table III; the lowest set (solid black circles) are from the first regeneration given in Table IV.

most of the fading reaction to complete itself before the light was turned off and the measurement of regeneration was begun.

The data of Table III are typical of a number of measurements of visual purple regeneration. If visual purple is formed from its precursors in the illuminated solution, then if the reaction is monomolecular, or if it is bimolecular with one reactant present in excess, the first order equation should describe the regeneration process.

In the equation, $Kt = \log \frac{a}{(a-x)}$, x represents the concentration of visual purple formed during the time in the dark, t , and a represents the concentration of reactant present at zero time. This quantity, a , can then be assumed to be equal numerically to the difference between the density of the solution at the end of the reaction and that at the beginning. $(a-x)$ can be calculated for any time, t , by subtracting the density regenerated at that time from the constant value of a .

If the logarithm of $(a-x)$ is plotted against t a straight line should describe the data if the reaction is first order. Fig. 6 shows the data from Table III and from the first double column of Table IV plotted in this way. It is apparent that the first order equation describes the regeneration of visual purple in solution fairly well. The small deviations which appear must represent complicating factors that are not understood at present since they look regular rather than random. For an approximate description of visual purple regeneration, however, the first order equation is quite adequate.²

IX

Retinal Metabolism and Regeneration

Warburg and Negelein (1929), Kubowitz (1929), and Nakashima (1929) studied the metabolism of the retinas of white rats, frogs, and fish, and failed to report any effect of light. More recently Jongbloed and Noyons (1936) have presented data which indicate that the oxygen consumption and CO₂ production of frog half-eyes are increased about 25 per cent in the dark fol-

² In a few measurements of visual purple regeneration the data could not be described by the first order equation nor by that of a second order reaction. Inspection of such curves always showed an abnormally high rate of density increase during the first 15 minutes of the dark period with the change practically over by the end of one-half hour, whereas it usually continues for 2 hours or longer. It seems unprofitable to attempt an explanation of these infrequent cases until more is known of the other color changes that may occur during visual purple regeneration.

lowing an exposure to light. They concluded that the excess metabolism is probably concerned with the resynthesis of visual purple.

In 1934, one of us (E. L. S.) began a series of respiration experiments with frog retinas (*Rana pipiens*). Measurements were made with the Warburg apparatus, using from 6 to 8 retinas or half-eyes in vessels of about 8 ml. capacity at temperatures of 25° and 30°C. No detectable differences in oxygen consumption were found between light and dark retinas under a variety of conditions. Measurements were made with isolated retinas in Ringer's solution and also with half-eyes in vitreous humor. Changes in pH from 5.0 to 9.0 had no effect on the oxygen consumption in Ringer. These observations were all made with summer frogs. Since Jongbloed and Noyons worked with winter frogs some of the measurements were repeated during the winter of 1937-38 with the same results as before.

The illumination system involved a 500 watt lamp with a condensing system, so that a high light intensity was available. Measurements were made with intensities of from 1000 to 100,000 meter candles. In some experiments, after a bleaching period of 15 minutes the eyes were removed from the manometer vessel and the visual purple was found to be completely bleached out. Other half-eyes were subsequently measured over a period of 2 hours in the dark. When these were removed they showed a definite pinkish color in the retina, indicating that regeneration of visual purple had taken place, although not completely as compared with freshly extirpated retinas from dark-adapted frogs.

If any increase in oxygen consumption occurs in the retinas of *Rana pipiens* concurrent with the regeneration of visual purple, it cannot be larger than 5 per cent.

SUMMARY

1. Measurements of visual purple regeneration in solution have been made by a procedure which minimized distortion of the results by other color changes so that density changes caused by the regenerating substance alone are obtained.

2. Bleaching a visual purple solution with blue and violet light causes a greater subsequent regeneration than does an equivalent bleaching with light which lacks blue and violet. This is due to a photosensitive substance which has a gradually increasing effective absorption toward the shorter wavelengths. It is uncertain whether this substance is a product of visual purple bleaching or is present in the solution before illumination.

3. The regeneration of visual purple measured at 560 $m\mu$ is maximal at about pH 6.7 and decreases markedly at more acid and more alkaline pH's.

4. The absorption spectrum of the regenerating material shows only a concentration change during the course of regeneration, but has a higher absorption at the shorter wavelengths than has visual purple before illumination.

5. Visual purple extractions made at various temperatures show no significant difference in per cent of regeneration.

6. The kinetics of regeneration is usually that of a first order process. Successive regenerations in the same solution have the same velocity constant but form smaller total amounts of regenerated substance.

7. *In vivo*, the frog retina shows no additional oxygen consumption while visual purple is regenerating.

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