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THE ABSORPTION SPECTRA OF VISUAL PURPLE AND OF INDICATOR YELLOW

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It was stated by Kühne [1878] that on exposure to light a freshly dissected retina or a solution of visual purple becomes orange, chamois, and pale yellow in turn before finally becoming colourless. From Kühne's descriptions it is clear that the whole sequence was not always observed, but he applied the term "visual yellow" or xanthopsin to the intermediate substances in general. He called the colourless phase "visual white" or leucopsin, and it seems that he was led to give a name to this invisible product on the grounds that these colourless solutions gave a green fluorescence not originally present.

Köttgen & Abelsdorff [1896], who made a careful study of the absorption of visual purple at different wave-lengths, were unable to observe the presence of visual yellow. They found that there was a progressive decrease in the density of solutions which had been exposed to light for increasing times, and that there was no increase in density at the blue end of the spectrum to indicate the appearance of a freshly formed yellow substance. This result was confirmed by Trendelenburg [1904]. Garten [1906], however, obtained clear evidence of the formation of a yellow breakdown product. Many other papers have been published on visual yellow, and on the whole the evidence has been in favour of the formation of this substance when visual purple is bleached. It might seem a simple matter to decide the issue, but there are complicating factors which make observations difficult.

Holm [1922], from visual observation of the bleaching of intact retinæ, came to the conclusion that visual yellow appeared only when high illuminations were used. He thought that visual yellow acts as a

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colour filter to protect the eye against the effects of very high illuminations. There is, however, an important factor which explains why Holm and others have observed visual yellow under some conditions and not under others. It is probable that a substance with a deep yellowish colour is nearly always formed when visual purple is bleached by light, but that it is decomposed thermally at a rather rapid rate. If, therefore, the illumination is high, the unstable intermediate substance has not had time to decompose before all the visual purple has been bleached, but if the illumination is low the first-formed intermediate substance will have decomposed before the last of the visual purple has disappeared. The yellow is masked by the purple colour.

There are most probably two distinct phases in the fading of Kühne's "visual yellow": their presence can be demonstrated by a simple experiment. Two samples of visual purple, one of which had been cooled in ice and the other warmed to about 30° C., were brought into daylight. The warmed solution after bleaching was a pale yellow; the cooled solution, however, was a deep vermilion-orange. The orange colour lasted for a considerable time at 0° C., but on warming the solution it became pale yellow. It is proposed to use the terms "transient orange" and "indicator yellow" to describe the two phases, both of which are embraced by Kühne's term "visual yellow". Later in this paper further evidence for the separate existence of transient orange will be given.

Owing to the fleeting appearance of transient orange at room temperatures its existence has apparently not been suspected by many authors, and they apply the term "visual yellow" to the comparatively stable yellow substance which is left after visual purple has been exposed to light for some minutes. The experimental details given in the papers of Garten [1906] and others make it fairly certain that these authors were dealing with the substance which is here called indicator yellow. The reactions of this latter substance provide the clue to the fact that some workers have been unable to demonstrate the formation of any yellow substance when visual purple is bleached.

It was suggested by Krause [1934] and Chase [1936] that "visual yellow" is an acid-base indicator. Nakashima [1929] had shown that "visual yellow" is a deeper yellow in acid than in alkaline solutions, and Chase published curves to show that the course of bleaching of visual purple is not the same in acid as it is in alkaline solutions. In solutions buffered between *p*H 5·8 and 7·0 there was an increase in density at the blue end of the spectrum on bleaching, showing that a yellow substance had been freshly formed. Chase did not observe an increase in density

in the alkaline solution (*p*H 9·3) but his readings were not taken at wavelengths shorter than 430 mμ, whereas results quoted in the present paper make it clear that an increase in density would not be expected at wavelengths longer than 420 mμ. Furthermore, it was shown by Chase that partially bleached alkaline solutions developed the yellow colour if they were made acid in the dark room and that the final yellow colour of an acid solution disappeared when alkali was added. Chase's observations are of great importance, and as pointed out by him and earlier by Nakashima this reaction may explain the discrepancy between the results of Garten and Köttgen & Abelsdorff.

Simple unpurified extracts of visual purple contain unstable yellow impurities, and their presence will obviously complicate the detection of a newly formed yellow substance. At 430 mμ in a simple unbleached solution (*p*H 7·0) of density 1·30, visual purple has a density of about 0·31 and the impurities a density of 0·99. The total density of the bleached solution is about 1·33, to which impurities as before contribute 0·99 and the indicator yellow a density of 0·34. The density of the impurities is about three times that of the indicator yellow, and the net increase in density on bleaching only about 0·03. It would not be surprising if the small net increase in density were missed whether the examination were made by the naked eye or spectroscopically.

Wald [1936] has also confirmed that the intact retina first bleaches to an orange colour when exposed to light, and that in time the orange is replaced by a yellow substance giving the typical indicator reactions. In a series of papers Wald [1935, etc.] describes the extraction of a carotenoid pigment from freshly bleached retinæ which he calls retinene. When retinæ have been bleached and allowed to stand they yield a substance which appears to be vitamin A. Both extractions are made with petrol ether. Wald suggests that his retinene is Kühne's "visual yellow" and that "visual white" is vitamin A. I have found that transient orange and indicator yellow in aqueous solution are not extracted by petrol ether; it would appear that these substances are neither retinene nor vitamin A and the purely descriptive terms of "transient orange" and "indicator yellow" are to be preferred.

Solutions of visual purple are difficult to prepare and their spectroscopic examination is not easy owing to their great sensitivity to light. The object of the present experiments was to obtain reliable absorption curves in the visible spectrum both for visual purple and for indicator yellow. The experiments were made with solutions buffered to different hydrogen-ion concentrations with a view to determining the degree of

dissociation of visual purple and its derivatives. This information was primarily needed in connexion with some work on the photochemical bleaching of visual purple [Dartnall *et al.* 1936], but the results obtained may also be of importance in the explanation of visual sensation, and in the solution of the problem of the chemical constitution of visual purple.

Preparation of solutions

Solutions of visual purple may be prepared by placing the dissected retina in a solution of bile salts [Kühne, 1878], of digitonin [Tansley, 1931] or in one of a variety of other substances having hæmolytic properties [Hosoya, 1933]. After half an hour or so the envelopes of the rods will have ruptured, liberating the visual purple. The mass is then centrifuged and the supernatant fluid will contain the visual purple in solution. Such a solution is usually coloured an orange red and never reddish purple as would be the case if it were pure. Spectroscopic examination shows that these solutions absorb a very large proportion of the shorter wave-lengths (Fig. 1, top curve), and the density curves published by some authors even show an increasing absorption from the red to the blue end of the spectrum with a slight unevenness in the curve at the wave-length of maximum absorption (502 mμ) of the visual purple: the density of a pure solution should decrease progressively from 502 mμ towards both ends of the spectrum. Very little improvement can be made by the choice of solvent [Hosoya & Bayerl, 1933], and there is no doubt that there are yellow substances present in the extracts which account for the absorption of the shorter wave-lengths. Simple extractions of the whole retina are usually opalescent, and although the suspended matter cannot be removed by the centrifuge, oftener than not it is precipitated in an hour or so after preparation. The presence of yellow impurities although undesirable would not prevent accurate quantitative photometric work if they were stable, but they are not, and if one measures the wave-length absorption at intervals throughout the day it is found that considerable changes are taking place in the solutions. Something can be done to stabilize the solutions by centrifuging them for as long as 2 hours [Chase, 1936] and by attention to other details [Hosoya & Saito, 1935], but even so it is probable that the changes in the yellow impurities are of the same order as those produced by the formation of "visual yellow". Kühne [1895] described a method of purification involving the precipitation of visual purple by saturation with magnesium sulphate, but I could not repeat the result. I have since found that

visual purple is precipitated by full saturation with sodium sulphate but only if the temperature is raised to 30° C.

A new method has been devised for the preparation of visual purple solutions which are free from opalescence under most conditions, contain only a small amount of yellow impurities and which are stable for many hours at room temperature. In this new method the first step is the preparation of a suspension of retinal rods; the second step is the extraction of lipoids, etc., from the desiccated rods by petrol ether; in

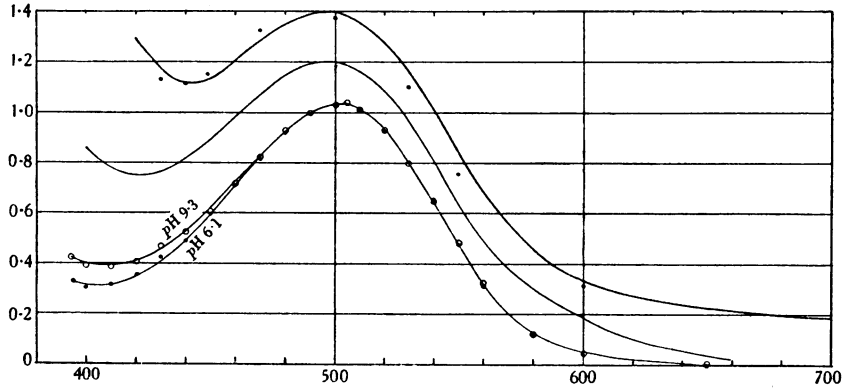

Fig. 1. Absorption curves of unbleached retinal extracts showing the elimination of impurities at different stages of purification. Values corrected so that the density of the contained visual purple at 502 mμ is 1·00 exclusive of impurities. Top curve: Extraction of whole retina with 2 p.c. digitonin solution. Extraction with 4 p.c. bile salts gives values shown by the points [Chase, 1936]. Middle curve: A suspension of "rods" was given a preliminary washing with a *p*H 4·6 buffer solution. Lower curve: Extracts used in present paper in which rods were also washed with petrol ether. Final solutions buffered to *p*H 6·1 (•—•) and *p*H 9·3 (o—o). The same preparation was used differing only in the added buffer. Abscissæ: wave-length (mμ). Ordinate: density.

the third step some of the proteins are rendered insoluble by treating the rods with an acid solution of *p*H 4·6, whilst finally the visual purple is extracted by a solution of digitonin.

The retina can rarely be removed from a frog's eye without a certain amount of pigment epithelium even after the animal has been very thoroughly adapted to darkness. An attempt was made to wash off the adhering pigment by gently rocking the retinæ in 0·6 p.c. NaCl solution. It was soon found that nearly all the rods were detached from the retina by this process. Advantage has been taken of this fact to prepare a fairly pure suspension of rods, free from the rest of the retina. The retinæ after

dissection were placed in a small test-tube about three-quarters filled with a 0·6 p.c. NaCl solution. The test-tube was then shaken vigorously and the contents poured into a filtering cone made of fine wire gauze (200 threads to the inch). The rods, pigment granules and a few isolated cells passed through freely, but the main mass of the nuclear and fibre layers of the retina were held back. The retained mass, which was usually quite colourless, was examined histologically by Dr Tansley. She found that the outer limbs of the rods were almost completely removed, and about half the inner limbs were left adhering to the nuclear and fibre layers. The frog's retina is not otherwise disrupted by shaking, but the mammalian retina appears to break up into small pieces. The retinal mass left behind in the wire gauze when treated by a solution of digitonin produced a decidedly yellow solution, which must in part account for the yellow impurities of simple extractions. The suspension of rods which passed through the gauze was centrifuged lightly; the rods rapidly passed to the bottom of the test-tube and left an opalescent supernatant fluid which could be pipetted off. The exact times for sedimentation can be determined easily by withdrawing a drop from the solution after various times of centrifuging and by microscopical examination. The visual purple is liberated from a suspension of rods by digitonin solution (2 p.c.) in 2 min. or so, compared with about ½ hour for the whole retina. About 1½ hours is necessary for complete extraction from the whole retina by 4 p.c. bile salts [Chase, 1936].

If the visual purple solutions obtained by a digitonin extract of the rods are mixed with a buffer solution of *p*H 4·6, there is a considerable precipitate. If this precipitate is allowed to form a sediment, it will be found to be coloured pink with visual purple, but some visual purple will remain in solution. It is probable, therefore, that the acid solution precipitates a protein on which some of the visual purple is adsorbed. Spectroscopic examination of the clear supernatant fluid obtained after centrifuging showed that there were less yellow impurities present and indeed the author saw a real purple tint for the first time in such a solution. To prevent the adsorption of visual purple on the precipitated protein the rods are first treated with the acid buffer and then with the digitonin solution: the residues after extraction are opaque and have scarcely any colour. The degree of improvement in the solutions can be seen in Fig. 1 (middle curve).

The solutions after the above treatment were still opalescent and absorbed an appreciable proportion of the red wave-lengths. It was thought that there were lipoids present in the solutions. The suspen-

sion of "rods" was therefore centrifuged and the debris dried in a vacuum desiccator. On the addition of petrol ether there was a moderate liberation of a yellow substance. The "rods" were again dried and then extracted with digitonin solution. The solutions showed a considerable improvement on any previously obtained, with less of the yellow impurities (Fig. 1, bottom curves): they were usually quite free from opalescence, and a solution which transmitted only 28 p.c. of the incident light at 500 mμ transmitted more than 99 p.c. at 650 mμ.

In spite of the precautions to obtain pure solutions of visual purple, it will be seen from Fig. 1 that the solutions still probably contain yellow impurities. It is likely that some of these impurities can be removed by dialysis, but the actual proof that this is so is rather difficult to obtain, and since the technique is already elaborate the additional complications were thought not to be worth while. It may be recorded, however, that probably no visual purple passed through a cellophane membrane which usually passes molecules with a molecular weight of less than 20,000. The digitonin passed through fairly quickly.

Details of preparation. All operations are carried out by the light of an ordinary ruby dark-room lamp. The frogs are kept in the dark overnight, decapitated and the heads washed and dried. With a pair of fine scissors a single cut is made right across the cornea, avoiding the iris. The head is then held in the left hand with the forefinger on the palate just behind the eye and the thumb in the corresponding position on the top of the head. On applying gentle pressure the lens is first extruded and removed, whilst still further pressure brings out the vitreous and finally the retina. The latter is then picked up with fine forceps and placed in a small test-tube containing 0·6 p.c. NaCl in water. The retina should come away whole and its shape can often be seen as it floats out in the saline solution. There is often no adhering pigment when using *Rana temporaria* but with *R. esculenta* the retina is usually not quite clean. After all the retinæ have been expressed, the test-tube containing them is shaken vigorously for 20 sec. and the contents poured into a filter cone made of fine wire gauze. The retinal residues are then shaken for 15 sec. with more 0·6 p.c. NaCl solution to remove any entangled rods. The suspension of rods which has passed through the filter is next centrifuged at about 800 r.p.m. for 1 min. and the supernatant suspension of pigment cells, etc. pipetted off. The remaining rods are treated with more saline, shaken and centrifuged again. The operation is repeated until the supernatant fluid is fairly clear and the rods are then heavily centrifuged to remove as much fluid as possible. The remaining mass, which should occupy a surprisingly small bulk, is spread over the sides of the test-tube and placed in a vacuum desiccator for about 8 hours. The dried mass is next washed with petrol ether (B.P. 40–60°) until no further yellow colour comes away. The petrol ether is then evaporated off and 0·6 p.c. NaCl solution is added to the test-tube and the latter kept on ice overnight. In the morning the saline solution is replaced by an acid buffer solution (*p*H 4·6), the mass of rods is broken up, shaken vigorously and after 2 min. the buffer is removed, the rods are washed and treated with 1 p.c. digitonin solution. The contents of the test-tube are stirred by a steel ball for 10 min. at low temperature and then centrifuged at about 4000 r.p.m. for 10 min. or so, the temperature being kept as low as possible to prevent destruction of visual purple. The supernatant fluid, which should be quite clear, is removed and stored on ice.

For ordinary estimations 1 c.c. of digitonin is used for twelve large *R. esculenta*. Dilution with an equal volume of buffer solution produces a concentration of visual purple which is quite sufficient for most purposes.

Solutions prepared in this way keep well if buffered between *p*H 6 and 9·3. Absorption curves were made on some unbleached solutions immediately after preparation and again after they had been kept at room temperature (20° C.) for 3 hours or so. During this period there was no change in density at any wave-length, showing that neither the visual purple nor the yellow impurities had undergone any change. One solution with a *p*H of about 9·3 was kept for 4 days at 0° C., and showed only a 1 p.c. loss of concentration as measured by its density. Weakly alkaline solutions kept on ice for several months retained their colour. Visual purple is destroyed slowly at *p*H 5·2 and 10·0, and very rapidly at *p*H's much less than 5 or more than 11.

Apparatus. All measurements of density were made on the apparatus described by Bayliss *et al.* [1936]. The basis of the method consists in passing a narrow spectral band from a monochromator through the unknown solution on to a photoelectric cell. The cell is illuminated for an accurately timed interval, and the current passing through it charges up one plate of a condenser to which a Lindemann electrometer is also connected. The E.M.F. which tends to develop on the electrometer is neutralized by electrostatic induction, an equal and opposite potential being applied to the other plate of the condenser. The applied E.M.F. is proportional to the light which has fallen on the photocell.

Apart from mechanical and optical improvements no important change was made in the original apparatus beyond the fitting of a KG7 photocell (G.E.C.), which permitted readings to be taken down to 395 mμ. This type of cell has its greatest sensitivity at 436 mμ for an equal-energy spectrum. When used with a prismatic monochromator and a tungsten lamp as light source, its apparent response is greatest at about 510 mμ, falling off gradually towards the ends of the spectrum. A single monochromator of the type used in these experiments passes at least 1 p.c. impure stray light. If, therefore, measurements are made at a wave-length where the photocell gives 1/100 its maximal response, it follows that the total response will be due to light of the wave-lengths set on the monochromator scale and to stray light in about equal parts. Careful attention was therefore given to the choice of auxiliary colour filters through which the light was passed before entering the monochromator. The filters finally selected were Corning R-P Ultra (395–410 mμ), Wratten 35 (420–440 mμ), Wratten 47 (450–470 mμ), Wratten

45 (480–490 mμ), Wratten 59+44 (500–510 mμ), Wratten 58 (520–560 mμ), Wratten 22 (580–650 mμ). The Corning Ultra and the Wratten 35 pass an amount of red light which is very obvious to the human eye but which has practically no effect on the KG7 photocell.

Throughout this investigation readings were taken in rotation, the first on the unknown solution, the second on the control consisting of a plain solution of digitonin. From the two measurements the density of the unknown solution could readily be calculated. Readings were always taken from the blue towards the red end of the spectrum. When two unknown solutions were being measured against a common control, 5 min. were necessary to take readings at 395 mμ; the range 400–450 mμ was covered in a further 15 min.; the range 460–500 mμ in 13 min.; and the range 510–600 mμ in 12 min. The wave-lengths at which readings were taken are given in the tables.

Spectroscopic investigations of visual purple have often been confined to a few scattered wave-lengths because the solutions were placed between the light source and the monochromator and consequently bleached rapidly. Repeated trials with the present apparatus have shown that the monochromatic patch produces no measurable bleaching during the course of an experiment. For instance, the monochromator was set at 505 mμ, near the maximal absorption of visual purple. After forty exposures the loss in concentration of the solution as measured by its density was less than 1 p.c. During the course of an experiment about twenty-four exposures are made, for the most part to wave-lengths which are much less potent than 505 mμ in bleaching visual purple.

The buffer solutions for the range *p*H 3–10 were those recommended by Clark and Lubs [Clark, 1928]. The *p*H 2 solution was that recommended by Sørensen and the *p*H 11 and 12 solutions those recommended by Kolthoff *et al.* [Clark, 1928]. The hydrogen-ion concentration of every extract was measured on a glass-electrode apparatus kindly lent by Dr P. M. T. Kerridge [1926]. Owing to the internal buffering of the solutions, the measured hydrogen-ion concentration was always nearer to neutrality than that of the added buffer. The shift was considerably less with the comparatively protein-free purified extracts than it was with simple extracts of the whole retina. If an equal volume of an *M*/20 buffer solution of *p*H 4·0 is added to an extract made from whole retinæ the colour (visual purple) is not destroyed, but with purified extracts made from rods the colour vanishes in a few seconds.

The apparatus used for the bleaching consisted of a 100 c.p. pointolite, the ball of which was focused by an *f* 3·8 lens on the cell containing the

solution. The image of the ball was 1·2 cm. in diameter, whilst the cell had a diameter of 1·0 cm. A monochromatic filter passing about 15 p.c. at its wave-length of maximum transmission (506 mμ) was placed in the beam of light. The bleaching of an alkaline solution was half completed in about 30 sec., and completed in about 6 min., but in order to ensure that all solutions were fully bleached an exposure of 8 min. was given.

With the exception of the values given in Fig. 1, all the quantitative results quoted in this paper are from five separate extractions. The same batch of *R. esculenta* was used in order to eliminate seasonal differences [Hecht & Chase, 1934] and the experiments were completed in a period of 3 weeks during July 1936.

Results

All results are given as densities. If the light incident on a solution is I_0 and the light transmitted is I_t, then the density, D, is given by the equation $D=\log_{10} I_0/I_t$. A solution which transmits 10 p.c. of the incident light has a density of 1, a solution which transmits 1 p.c. of the incident light has a density of 2.

If light passes through two solutions in contiguous cells, the total density is the sum of the densities measured separately. If one solution passes 10 p.c. and the other 1 p.c. of the incident light, the final transmission is 0·1 p.c. Expressed in densities the values are additive, density 1 + density 2 = density 3. Exactly the same applies to two substances present in one solution (Beer's Law), the final density is the sum of the densities of the components. In Fig. 2 the density of the unbleached solution at 410 mμ was 0·380 of which the visual purple contributed a density of 0·103 and yellow impurities contributed a density of 0·277. In the bleached solution the density was 0·546. Assuming that the yellow impurities remained unchanged, the density of the newly formed indicator yellow was 0·546 − 0·277 = 0·269.

The density (D) of a solution can be expressed in the form

$$I_t = I_0\, 10^{-D}.$$

In dealing with substances of known composition it is better to use the equation $I_t = I_0 e^{-\alpha c l}$ where α is the extinction coefficient per chromophoric group, c is the number of chromophoric groups per c.c., l is the length of the optical cell, and e is the natural base of logarithms. In a paper by Dartnall *et al.* [1936] on the photochemical bleaching of visual purple, the value of α at 505 mμ was found to be about $4{\cdot}0 \times 10^{-17}$ cm.^{2} for *R. temporaria*. Later determinations have given the value $5{\cdot}0 \times 10^{-17}$ cm.^{2} for *R. esculenta*. It follows that α for other wave-lengths can be calculated by multiplying the density of visual purple at that wave-length by $5{\cdot}0 \times 10^{-17}$ cm.^{2}, and dividing by 0·995 (the density at 505 mμ). This conversion is valid for visual purple densities, but since it is not known how many chromophoric groups of indicator yellow are derived from one of visual purple, the conversion of densities into extinction coefficients

would not be valid for indicator yellow. For this reason the densities of the solutions measured in the present experiments are expressed by the simplest equation using *D*. If, however, one chromophoric group of visual purple gives rise to one chromophoric group of indicator yellow, then the conversion is also valid for the latter substance.

The absorption of visual purple. If, in solution, visual purple were dissociated in the region of its chromophoric group to give free H^+ or OH^- ions, then the absorption curves would vary with the hydrogen-ion concentration of the solution. The change would be revealed either by a shifting of the point of maximum absorption or by a loss in density. When the absorption curves at the different *p*H's were compared it was found that the curves coincided at all but the shorter wave-lengths. Fig. 1 (lower curves) shows the absorption curves from the same extract of visual purple, two samples of which had been buffered at *p*H 6·1 and 9·3. It will be seen that the wave-length of greatest absorption is the same in both solutions and that both curves are of the same height at the maximum and for the longer wave-lengths. Towards the shorter wave-lengths, however, the solution at *p*H 9·3 becomes progressively denser than that at *p*H 6·1. It is possible that visual purple has absorption bands at wave-lengths outside the visible spectrum which have some absorption at the shorter visible wave-lengths. The difference between the solutions at *p*H 6·1 and 9·3 cannot, however, be due to alterations in the density of the absorption band centred at 502 mμ, because if this were so the change would be greatest at this wave-length; an alteration in the position of the maximum of absorption would also produce a change in the density at 502 mμ. The probable explanation is that the yellow impurities show some changes with hydrogen-ion concentration, but we cannot exclude the possibility that the difference in absorption is due to changes in some other part of the visual purple molecule. It will be seen in Table I that there is a progressive decrease in density in unbleached solutions for the shorter wave-lengths as the solutions are made less alkaline. At *p*H 5·2, however, the solutions are slightly denser at all wave-lengths. This is due to a slight opalescence which disappears when the solution is made more acid or more alkaline. The differences in the densities of solutions containing equal amounts of visual purple at various *p*H's, although slight, are of some importance in calculating the true absorption curves of indicator yellow. In the calculations of the absorption curves of indicator yellow advantage is taken of the fact that the relevant part of the visual purple molecule is undissociated and that the absorption curve centred at 502 mμ is unaffected by *p*H.

Fig. 2 shows the absorption curves for an unbleached extract and for the bleached solution after it had attained equilibrium. It will be apparent that there has been an increase in density at the blue end of the spectrum on bleaching, showing that a new substance has made its appearance. One cannot determine the absorption curve of visual purple itself by subtracting the bleached from the unbleached values because negative values would be obtained at the short wave-lengths. Unless one has a quite pure solution of visual purple it is necessary to make certain assumptions before its absorption curve can be determined. From what

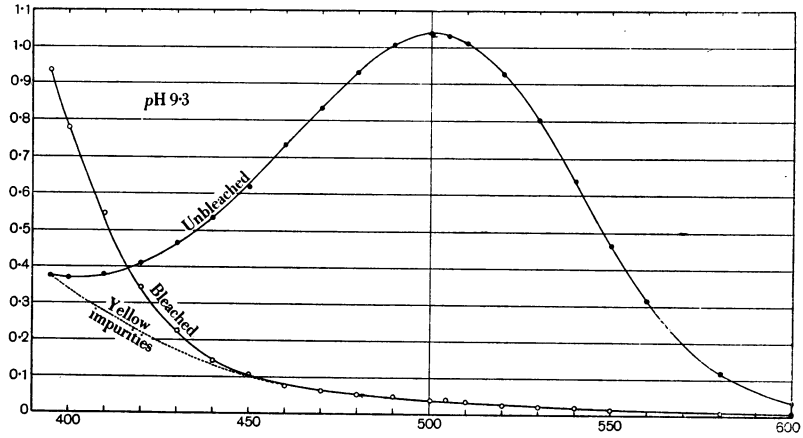

Fig. 2. Absorption curves of unbleached and bleached visual purple for one of the extracts at *p*H 9·3. The increase in density for the short wave-lengths on bleaching denotes the formation of indicator yellow. The dotted line shows the conjectural density of yellow impurities. Density of contained visual purple at 502 mμ = 1·00. Abscissæ: wave-length (mμ). Ordinate: density.

has been said under "Method of extraction" it will be clear that with successive purifications the density of the unbleached extract becomes progressively less at any one of the short wave-lengths (Fig. 1), and that with a quite pure extract it would probably be very small in the region of 400 mμ. The present solutions almost certainly contain yellow impurities, and before one can derive values for the density of visual purple at the shorter wave-lengths, it is necessary to assume values for the absorption of these impurities. If, as seems likely, the yellow impurities remain unchanged during bleaching, it would be impossible for them to have a density greater than the measured density of either the bleached or the unbleached solution at any wave-length. It is also likely that the impurities will behave similarly to the great majority of yellow biological

substances and will show a gradually increasing absorption towards the shorter wave-lengths. If the latter assumption is true then the absorption cannot be greater than that shown by the dotted line in Fig. 2. It is also probable that the absorption is not much less for the following reasons. In the first place the curve of the bleached solution rises rather abruptly from 450 mμ and becomes progressively higher as far as 395 mμ. It is probable that we are dealing with the edge of the absorption band of indicator yellow and that its absorption is very small at 460 mμ and almost negligible at 480 mμ. In the second place, it is likely that the density of visual purple decreases progressively towards the shorter wave-lengths. In the middle of the absorption band there will be a considerable difference in density between two wave-lengths 10 mμ apart and this difference will be revealed in the absorption curve of an impure extract, provided the impurities have not got any strongly marked absorption bands at these wave-lengths. At the ends of the curve the absorption band of visual purple probably flattens out as the values become small. The fact that the densities at 410 mμ were only a little greater than at 400 mμ probably means that this is the region where the absorption curve of visual purple begins to flatten out, and in order to make the density of visual purple small in this region it is necessary to assume that the density of the impurities is great. It will be assumed, therefore, that the density of the bleached solution between 395 and 460 mμ follows the dotted line in Fig. 2; that the density of the bleached solution between 460 and 480 mμ is almost wholly due to yellow impurities and that at longer wave-lengths it is wholly due to these impurities.

In the present determination of the absorption curve of visual purple, solutions buffered to a *p*H of 9·3 were used because at this value indicator yellow gives almost its lowest density values in the visible spectrum. There is actually a little less visible indicator yellow at *p*H 10·0, but visual purple is not quite stable at this alkalinity.

It was known from preliminary work that the maximum of absorption of visual purple alone was between 500 and 505 mμ. The purity of the spectral bands for most experiments was about 1·2 at 436 mμ; about 1·8 at 496 mμ; 2·0 at 546 mμ and 2·5 at 579 mμ. A special run was made on one of the denser solutions to locate the maximum more accurately. The slits of the monochromator were narrowed to give a band of wave-lengths whose purity was 0·8 mμ and many measurements of density were made in the region of the maximum. It was possible to locate the maximum absorption at 502 mμ. Five solutions were available for the calculation of the absorption curve of visual purple. For each of the

solutions a graph was drawn giving the bleached and unbleached densities; the probable values for the yellow impurities were then drawn freehand in the same way as has been done in Fig. 2. The densities read off from the freehand curve were subtracted from the densities of the unbleached solutions for the range 395–480 mμ: for the range 490–650 mμ the experimentally found bleached values were used. The densities for visual purple so obtained were for solutions of varying strength made on different days: all values were then multiplied by a factor to make the density at 502 mμ equal to 1·000. These factors were subsequently used for comparing solutions at other hydrogen-ion concentrations. The figures obtained were averaged (Table I). There was good agreement between the five experiments.

There have been very few previous determinations of the absorption spectrum of visual purple, and all have relied in one form or another on the simple procedure of subtracting the values for the bleached solution from those of the unbleached solution. Since the absorption curves of the bleached solutions vary with hydrogen-ion concentration, it follows that the apparent maximum absorption of visual purple obtained by subtracting the bleached from the unbleached values will also vary with the *p*H of the solution. The error introduced is small, however, between *p*H 6·1 and 10·0. If the true maximum of absorption of visual purple is at 502 mμ, the apparent maximum obtained by subtraction with solutions of *p*H 6·1 is at about 506 mμ. Of the previous determinations that made by Köttgen & Abelsdorff [1896] is the most important. Their maximum absorption is at about the same wave-length (500 mμ) but otherwise the agreement is not good, their values for the longer wave-lengths being as much as 20 p.c. greater than ours, whilst those for the shorter wave-lengths are much less. Their cross-over wave-length (the significance of which will be discussed later) is at about 425 mμ, from which it is concluded that the *p*H of their solutions was about 8·0. This means that their solutions did contain some indicator yellow although they believed otherwise, and the derived absorption curve for visual purple is of the shape which would be expected. Hosoya & Saito's [1935] values are in even worse agreement. Their maximum of absorption is at 510 mμ and the absorption curve for visual purple is very low indeed for the shorter wave-lengths. The present author can explain their results only by assuming that their solutions were acid and that they worked at a low temperature. From Chase's results it is possible to calculate some points on the absorption curve. From the values at 470, 480, 500 and 530 mμ in solutions at *p*H 9·3, it is clear that the maximum absorption

TABLE I. Showing the experimental values (corrected for concentration) for the densities of the unbleached and bleached solutions at various *p*H's. The values at *p*H 9·3 are the averages of five determinations. The density of the visual purple at 502 mμ in each unbleached solution is 1·00; the density in excess of 1·00 is due to yellow impurities. The densities of visual purple itself at different wave-lengths are also shown; the values are the average of five extractions and the mean variations from the average are shown. Columns "*p*H 5·2 B. 1" and "B. 2" give respectively the densities of the bleached solutions taken as soon as possible after bleaching and 3 hours later at 20° C.

Wave-length (mμ)	Visual purple	Mean variation	*p*H 10·0		*p*H 9·3		*p*H 8·5		*p*H 7·7		*p*H 7·0		*p*H 6·1		*p*H 5·2		
			U.*	B.*	U.	B.	U.	B.	U.	B.	U.	B.	U.	B.	U.	B. 1	B. 2
395	0·001	—	0·38	0·90	0·39	0·93	0·37	0·93	0·34	0·95	0·34	0·88	0·33	0·82	0·39	0·75	0·58
400	0·016	—	0·36	0·75	0·38	0·79	0·38	0·82	0·34	0·81	0·32	0·79	0·30	0·77	0·37	0·73	0·54
410	0·103	—	0·37	0·51	0·39	0·55	0·38	0·57	0·35	0·63	0·34	0·69	0·32	0·71	0·39	0·76	0·48
420	0·196	—	0·41	0·31	0·42	0·35	0·40	0·37	0·38	0·48	0·38	0·57	0·35	0·61	0·43	0·76	0·43
430	0·306	—	0·46	0·19	0·47	0·23	0·45	0·25	0·44	0·37	0·44	0·48	0·42	0·54	0·49	0·74	0·39
440	0·416	—	0·54	0·12	0·54	0·15	0·51	0·17	0·51	0·30	0·52	0·40	0·49	0·47	0·57	0·72	0·35
450	0·530	—	0·63	0·09	0·62	0·11	0·61	0·14	0·61	0·23	0·63	0·34	0·60	0·39	0·66	0·66	0·31
460	0·661	—	0·74	0·08	0·74	0·09	0·72	0·10	0·73	0·20	0·73	0·29	0·71	0·33	0·77	0·57	0·27
470	0·780	±0·005	0·85	0·07	0·85	0·07	0·83	0·08	0·84	0·16	0·85	0·25	0·82	0·27	0·88	0·50	0·24
480	0·878	±0·005	0·94	0·07	0·94	0·06	0·93	0·07	0·93	0·14	0·94	0·21	0·92	0·23	0·98	0·41	0·21
490	0·953	±0·005	1·01	0·06	1·01	0·06	1·01	0·06	1·01	0·11	1·02	0·18	1·00	0·18	1·04	0·33	0·18
500	0·997	±0·004	1·04	0·04	1·05	0·05	1·04	0·05	1·04	0·09	1·04	0·15	1·03	0·14	1·07	0·25	0·15
505	0·993	±0·004	1·04	0·05	1·03	0·04	1·03	0·05	1·03	0·09	1·03	0·14	1·03	0·13	1·06	0·22	0·14
510	0·975	±0·002	1·02	0·04	1·02	0·04	1·01	0·04	1·01	0·08	1·01	0·13	1·01	0·12	1·04	0·19	0·13
520	0·899	±0·004	0·93	0·03	0·94	0·04	0·94	0·03	0·94	0·06	0·94	0·11	0·93	0·09	0·96	0·15	0·11
530	0·780	±0·006	0·81	0·04	0·81	0·03	0·80	0·03	0·82	0·05	0·81	0·08	0·80	0·07	0·84	0·12	0·10
540	0·622	±0·009	0·64	0·03	0·65	0·03	0·65	0·02	0·66	0·04	0·65	0·07	0·65	0·05	0·67	0·10	0·08
550	0·460	±0·008	0·48	0·03	0·48	0·02	0·49	0·02	0·49	0·03	0·48	0·05	0·47	0·04	0·50	0·08	0·07
560	0·310	±0·006	0·32	0·05	0·33	0·02	0·33	—	0·33	0·03	0·33	0·04	0·31	0·02	0·35	—	—
580	0·113	±0·006	0·13	0·02	0·13	0·02	0·12	0·01	0·13	0·01	0·13	0·01	0·12	0·01	0·15	0·05	0·05
600	0·034	±0·002	0·05	0·02	0·04	0·01	0·04	0·00	0·05	0·01	0·04	0·01	0·04	0·01	0·07	0·04	0·05
650	0·003	±0·004	0·01	0·01	0·01	0·01	0·00	0·00	0·01	0·01	0·00	0·01	0·00	0·00	0·03	0·03	0·03

* U. = Unbleached. B. = Bleached.

is not far from 500 mμ, and his values at these and other wave-lengths are scattered about the average curve obtained in the present experiment (given by the dotted line in Fig. 5).

The absorption curve of indicator yellow. The method of obtaining these curves for the *p*H range where visual purple is stable will first be described since the curves for values outside this range cannot be found with any accuracy. For one day's work not more than four samples each of 0·5 ml. of the unbuffered visual purple solution were accurately measured and to each sample 0·5 ml. of standard buffer was added. The accuracy of the dilution was checked by weighing. For each batch one

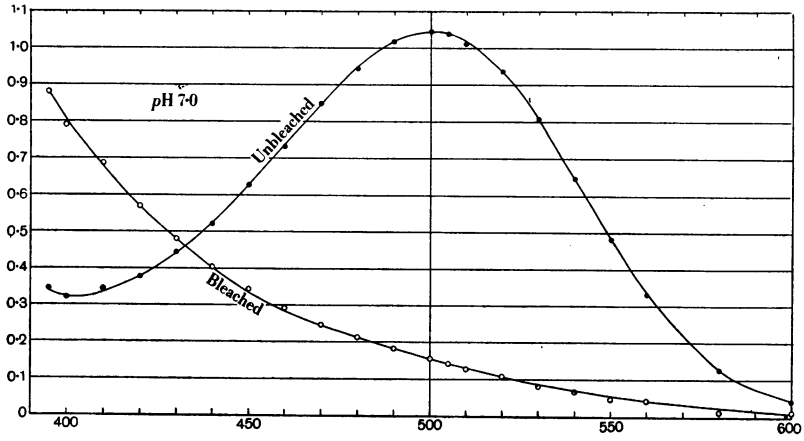

Fig. 3. Unbleached and bleached extracts at *p*H 7·0. Density of contained visual purple at 502 mμ = 1·00. Abscissæ: wave-length (mμ). Ordinate: density.

of the samples was buffered to *p*H 9·3 to act as a comparison standard between readings on different days. The two most acid samples were estimated immediately and the remaining two more alkaline samples were stored in a refrigerator and estimated later in the day. The absorption curve of the unbleached solution was first determined and the solution was then slowly bleached for a measured time in a constant illumination. The measurement of the absorption curve of the bleached solution began 10 min. after the beginning of bleaching and again after 3 hours at room temperature (19–21° C.). Some of the experimental results are given in Table I, and the values have been plotted for *p*H 9·3, 7·0 and 5·2 in Figs. 2, 3 and 4 respectively. A correction for concentration has been made by multiplying the densities by the factors mentioned on p. 344: at 502 mμ the density of the visual purple exclusive of impurities

is therefore 1·00. The formation of indicator yellow is shown by the increase in density at the short wave-lengths on bleaching. By comparing Figs. 2, 3 and 4 it will be seen that this increase is greater for the more acid solutions.

The density of the bleached solution is due partly to indicator yellow and partly to yellow impurities. Provided the absorption curve of these impurities is known, it is possible to find the density of the indicator yellow itself at any wave-length. It will be remembered that the densities of the yellow impurities themselves vary with hydrogen-ion concentration. It is therefore necessary to determine these densities for each sample. This was done by assuming that the visual purple content

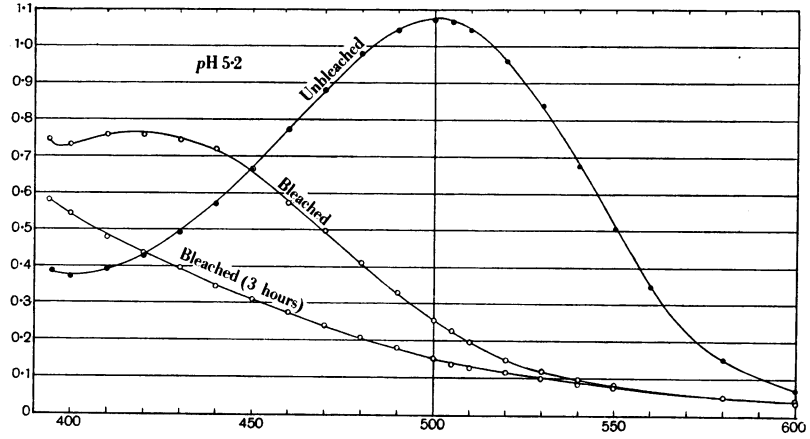

Fig. 4. Unbleached and bleached extracts at *p*H 5·2. The bleached solutions were estimated immediately after bleaching and again 3 hours later. Correction for concentration as in Figs. 2 and 3. Abscissæ: wave-length (mμ). Ordinate: density.

of the four samples was the same for each; the values for the yellow impurities were obtained by subtracting the density of the standard visual purple curve from the total density of the unbleached solution. An example will make this clear. At 520 mμ the density of visual purple itself was 0·90. For the extract at *p*H 7·0 the unbleached density at 520 mμ was 0·94; subtracting the visual purple density we obtain 0·04 for the density of the yellow impurities. The density of the bleached *p*H 7·0 solution at 520 mμ was 0·11; subtracting 0·04, the density of impurities, we obtain a value of 0·07 for the density of indicator yellow.

Fig. 5 gives the absorption curves of indicator yellow for the range *p*H 5·2–10·0. Most of the bleached solutions showed practically no loss

in density after standing at room temperature for 3 hours. The solution at *p*H 6·1 did, however, show a slight change and the solution at *p*H 5·2 showed a considerable loss of density with time (Figs. 4 and 6). It

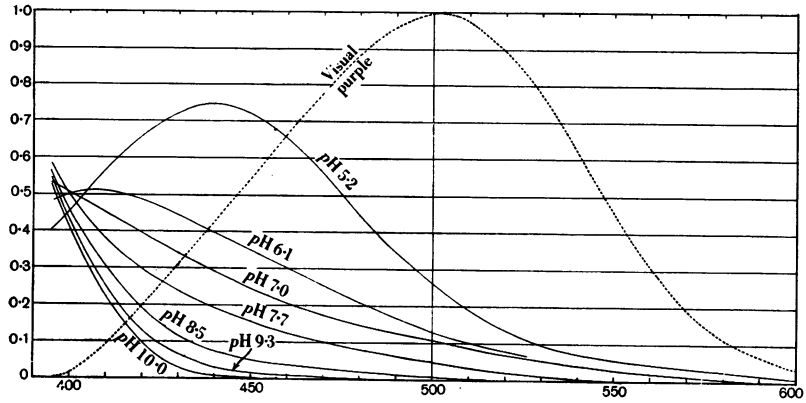

Fig. 5. The absorption curves of indicator yellow alone for hydrogen-ion concentrations which do not destroy visual purple. The density of the parent visual purple would be 1·00 at 502 mμ and its absorption curve is shown by the dotted line. The curves for *p*H 5·2 and 6·1 have been corrected for fading. Abscissæ: wave-length (mμ). Ordinate: density.

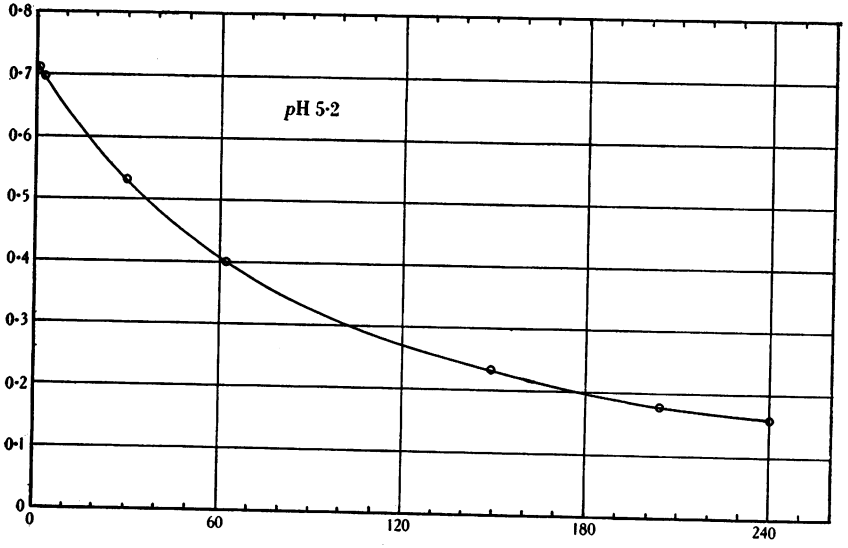

Fig. 6. The fading of indicator yellow with time in a solution buffered at *p*H 5·2. Bleaching was for 8 min. and the time intervals were measured from the end of bleaching. The density of the parent visual purple would be 1·00 at 502 mμ. Abscissæ: time (min.). Ordinate: density at 450 mμ.

follows that the readings taken late in a run will be relatively too low compared with those taken early. Readings on the bleached solutions at *p*H 5·2 and 6·1 were taken at 450 mμ immediately after bleaching and at regular intervals afterwards. On the assumption that a single substance was disappearing, the densities at the other wave-lengths were corrected to give the density at the end of bleaching.

The curves shown in Fig. 5 are very similar to the absorption curves of some ordinary laboratory indicators and there is a suggestion of an "iso-bestic" point at about 400 mμ. The absorption curve of visual purple is drawn on the same graph and for an equivalent concentration; that is to say, if a sample of visual purple had an absorption curve as drawn, then on bleaching, the absorption curve of the indicator yellow would be of the same size as that given in the figure. The wave-length at which the curve for visual purple crosses the curves for the indicator yellow is also of interest. We will call this the "cross-over" wave-length. At this wave-length the loss of density due to the bleaching of visual purple is exactly equalled by the gain in density due to the formation of indicator yellow. The cross-over wave-length is very constant between experiments on different days at the same *p*H value, and since it is independent of the amount of yellow impurities or the degree of bleaching it is a useful guide to the *p*H of the solutions whose absorption curves are published in the literature. For the more deeply coloured acid solutions the cross-over wave-length is longer than for the alkaline solutions. Some published curves show a cross-over wave-length between 490 and 500 mμ, and sometimes a second cross-over point in the red end of the spectrum. In our experience this result is always associated with intercurrent precipitation in the solutions measured.

Visual purple is destroyed by moderately strong acid or alkaline solutions, and it is not possible to produce accurate curves for indicator yellow in such solutions owing to the variations in the density of the yellow impurities with *p*H. The curves given in Fig. 7 for *p*H 10·8, 4·0 and 3·3 are probably a fair approximation. As before the comparable curve for visual purple is drawn. It will be seen that the curves taken at *p*H 4·0 and 3·3 are very similar to those at *p*H 5·2 (Fig. 5) and any differences which exist are possibly due to variations in the yellow impurities. The curves are, however, quite different for those taken in the range *p*H 10·0–6·1 and suggest that some tautomeric change has occurred. The height of the indicator yellow curve at *p*H 4·0 is not very different from the density of the visual purple from which it was derived. The colour of a solution at *p*H 3·3 is a deep chrome yellow.

The solutions were prepared by rapidly bleaching 0·5 ml. of visual purple solution and then adding 0·5 ml. of the appropriate buffer. The densities of the final solutions were estimated in the usual way with a comparison standard, buffered at *p*H 9·3. At *p*H 4·0 the indicator yellow was slowly decomposed but not quite so rapidly as it was at *p*H 5·2. A series of readings at 450 mμ was taken on the solutions at different times after bleaching. As with the solutions at *p*H 5·2 and 6·1, the readings at other wave-lengths were corrected on the assumption that a single substance was disappearing, the densities being calculated to give the values

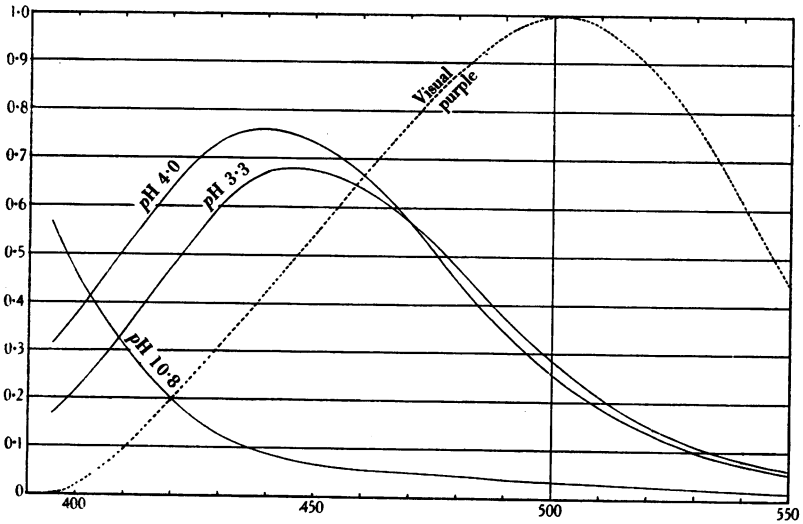

Fig. 7. The absorption curves for indicator yellow alone for hydrogen-ion concentrations which destroy visual purple. The density of parent visual purple would be 1·00 at 502 mμ and its absorption curve is shown by the dotted line. The curve for *p*H 4·0 has been corrected for fading. Abscissæ: wave-length (mμ). Ordinate: density.

immediately after bleaching. At *p*H 3·3 the indicator yellow is comparatively stable. The true values for the densities of indicator yellow were calculated as before by subtracting the densities of the yellow impurities from the values actually obtained. Since it is not possible to obtain an experimental value for the impurities at *p*H's where visual purple is decomposed, the values for the yellow impurities at the nearest *p*H on the same day's runs were used.

Nakashima [1929] found that when the colour of visual purple is destroyed by the addition of an acid, the solution becomes a deeper yellow than when an alkali is added. This observation was confirmed, and

it was shown in addition that the absorption curve of the indicator yellow is almost the same when the visual purple is "bleached" by acid as when an unbuffered solution is bleached by light and then buffered. Two 0·5 ml. samples of visual purple were taken: one was bleached by light and 0·5 ml. of buffer was added to bring the *p*H to 4·0; the other sample was bleached by the addition of 0·5 ml. of the same buffer solution in the dark room. The absorption curves (Table II) of the two solutions showed

TABLE II. Showing the experimental values (corrected for concentration) for the densities of bleached solutions at various *p*H's. As in Table I a solution in which the visual purple alone had a density of 1·00 at 502 mμ would bleach to give a solution with the densities given.

Wave-length	*p*H 10·8 bleached	*p*H 4·0 bleached by light	*p*H 4·0 bleached by buffer	*p*H 3·3 bleached
395	0·93	0·65	0·66	0·54
400	0·82	0·67	0·68	0·55
410	0·58	0·74	0·77	0·63
420	0·39	0·77	0·85	0·71
430	0·28	0·81	0·90	0·76
440	0·21	0·79	0·90	0·79
450	0·15	0·74	0·85	0·76
460	0·13	0·64	0·74	0·72
470	0·12	0·54	0·63	0·63
480	0·10	0·44	0·52	0·53
490	0·08	0·34	0·41	0·43
500	0·07	0·26	0·31	0·34
505	0·06	0·23	0·27	0·29
510	0·06	0·19	0·22	0·25
520	0·05	0·14	0·16	0·18
530	0·04	0·10	0·12	0·13
540	0·03	0·08	0·09	0·10
550	0·02	0·06	0·06	0·06
580	0·01	0·04	0·04	0·03
600	0·01	0·03	0·03	0·01
650	0·00	0·03	0·03	—

the same maximum and general shape of curve; in addition, the rate of loss of density was the same in both. Indicator yellow is probably the same both when visual purple is decomposed by light and when it is decomposed by the acid buffer. The density of the solution bleached by light was, however, slightly less; the difference is too great to be accounted for by experimental error.

The regeneration of visual purple. There is an increase in density of bleached solutions at *p*H 7·0 and 7·7 when they are kept in the dark for 3 hours. This increase is due to the regeneration of visual purple; it is greatest in the region of 500 mμ and has been a constant feature in experiments made during the last year. The increase would be accounted for if about 3 p.c. of the original visual purple were reformed. Hecht *et al.* [1936] have also noted the same phenomenon. It is stated that more than

half the increase in density is accomplished in the first 10 min. In the present work the reading at 500 mμ was not taken until about 30 min. after the end of bleaching—it is not surprising that Hecht's value (15 p.c.) is greater than ours. From earlier results got by the present author it is clear that regeneration is not confined to the *p*H range of 6·6–8·0; 10 p.c. regeneration has been repeatedly found at *p*H 9·3 (at 20° C.), but it appears to be a much slower process occupying about 24 hours. It is obvious that for this work one must use solutions which do not precipitate. Of rather greater interest than the mere phenomenon of regeneration is the fact that the density of the indicator yellow always shows a decrease when regeneration occurs. It is therefore probable that indicator yellow is taking part in the reformation of visual purple. The changes involved are, however, too small to admit of quantitative treatment. The cross-over wave-length, where the density of visual purple equals that of indicator yellow, remains much the same during regeneration.

Indicator yellow. By the use of the term "indicator yellow" it has been assumed that the substance formed from visual purple some little time after it has been bleached by light is an acid-base indicator. There are two reasons for this belief, first, that the densities of the bleached solutions vary with the hydrogen-ion concentration, the differences being often maintained for long periods. Secondly, that the changes are reversible. These reasons will be considered in more detail.

The yellow colour at *p*H 4·0, 5·2 and 6·1 is gradually lost and it is possible that more alkaline solutions are paler for the simple reason that more of the yellow substance has been broken down. If, however, the yellow substance were decomposed at an increasing rate when the solutions are more alkaline, it should be easy to detect the progressive loss of density with time at intermediate hydrogen-ion concentrations. Such a loss in density did not occur for the range *p*H 7·0–10·0 over a period of 3 hours. Furthermore, the acid solutions should remain the yellowest, whereas it was found that after 3 hours the measured density of the solution at *p*H 5·2 was less than that at *p*H 7·0. We are safe in assuming, therefore, that the end product of bleaching is stable between 7 and 11, but is unstable between *p*H 4·0 and 6·1. Hosoya [1933–4] who measured the loss of density with time in bleached solutions was probably investigating changes in yellow impurities as well as in what is here called indicator yellow. The changes can be followed in test-tubes by visual observation. In a series of bleached solutions at different *p*H's, the solutions at *p*H 10·0 and 9·3 are very pale lemon yellow. The depth of yellow increases gradually to *p*H 4·0 where it is very marked. The

solution at *p*H 3·3 has a slightly different reddish hue and is chrome yellow. If these solutions are kept at room temperature in sealed tubes for 24 hours or so, there is no appreciable change in most of the solutions, the solutions at *p*H 4·0 and 5·2 have, however, suffered a considerable loss in density and are the palest of all, being almost colourless.

The reversibility of the indicator change can be demonstrated quite readily in the test-tube. 0·5 ml. of a solution of visual purple was made

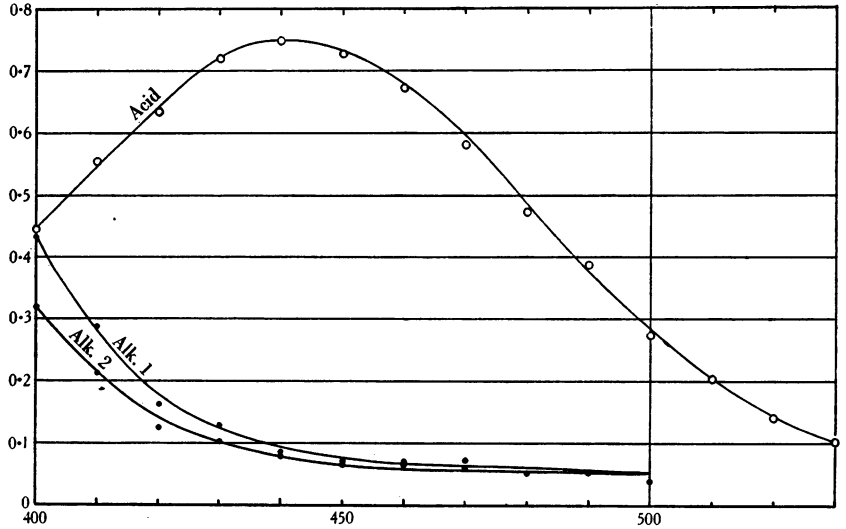

Fig. 8. The reversibility of the indicator yellow reaction. The absorption curve for an alkaline solution was first determined (Alk. 1). A curve was then taken on the acidulated solution (*p*H 5·0) and finally the solution was made alkaline again (*p*H 9·5) and another curve was taken (Alk. 2). Density of parent visual purple would be 1·00 at 502 mμ. Abscissæ: wave-length (mμ). Ordinate: density.

alkaline by the addition of an equal volume of 0·02 *N* NaOH. After bleaching the colour was pale lemon yellow. 0·1 ml. 0·2 *N* HCl was then added to make the solution acid; the colour became a deep lemon yellow, whilst on making the solution alkaline again the original pale yellow was restored. The change can be repeated any number of times. The absorption curves of these solutions were measured and the result is given in Fig. 8. The figures have been corrected for losses of density due to dilution and have been multiplied by a factor to make the density of the parent visual purple equal to 1·0. It will be seen that the density of the final alkaline solution (Alk. 2) is rather less than the density of the alkaline solution before the addition of acid (Alk. 1). The difference is

probably to be accounted for by the destruction of indicator yellow in the acid solution.

It has been shown that indicator yellow is unstable in solutions of certain acidity. In alkaline solutions, on the other hand, indicator yellow although scarcely visible to the eye, is quite stable and after several hours it is still capable of regaining its full yellow colour when it is acidulated. A solution of visual purple was made alkaline with 0·02 *N* NaOH bleached and divided into four samples which were stored at room temperature (20° C.). The first sample was then estimated as it stood and another sample was made acid before estimation. The estimation was repeated in the same way on the other samples later in the day. It was found that the density of a solution which had been acidulated late in the day was almost exactly the same as the density of a solution which had been acidulated 6 hours earlier.

Indicator yellow apart from its instability at some *p*H's behaves in many respects like some of the more complicated laboratory indicators. There are many ways in which the very complicated reactions of indicator yellow (Figs. 5 and 7) could occur, but fortunately they can be explained in terms of indicators of known behaviour. It would seem that for the *p*H range of about 6–10 there is an alkaline form with a narrow absorption band which is probably maximal in the near ultra-violet and an acid form with a broad absorption curve. It would appear that at about 400 mμ the densities of the acid and alkaline forms are the same, and the density at this point (iso-bestic point) should be unaltered whatever the relative proportions of the two forms. It is only the simplest indicators which have a well-defined iso-bestic point and the amount of scatter for indicator yellow is no greater than it is for an indicator such as phenol red. It can be shown theoretically that an indicator must pass from the almost purely acid form to the almost purely alkaline form in a range of about four *p*H units. If any further change in the absorption curve takes place outside this range it is due to a tautomeric change within the molecule. The densities of the indicator yellow for the range *p*H 10·0 to 6·1 do not conform to the standard equation relating *p*H and the degree of dissociation. The probable reason is that at *p*H 6·1 the molecule of indicator yellow has begun to undergo a tautomeric change. Changes of this nature are common in indicators, for example in thymol blue, and it appears that indicator yellow has a separate tautomeric form between *p*H 5·2 and 3·3. This range would, however, be very difficult to investigate quantitatively because of the thermal instability. It is a pity that the human eye is not sensitive to change of hue in the wave-

lengths being considered: if it were, the reactions of indicator yellow would form a striking colour display.

Transient orange. If two samples of alkaline visual purple, one of which has been cooled in ice and the other warmed, are placed in the light, the cooled solution bleaches to a vermilion-orange colour. On subsequent warming of the latter solution it loses its orange colour and becomes identical in colour with the solution which has been bleached in the warm. It seems probable that there is an intermediate phase in the bleaching of visual purple by light.

It has been noticed by Dartnall [1936] that during the bleaching of visual purple by monochromatic light, a "dark reaction" occurred. This dark reaction showed that when a partially bleached solution of visual purple was kept in the dark, there was a further loss of density roughly proportional to the amount of freshly bleached visual purple. The present author has made some preliminary experiments in which a sample of visual purple was bleached in a high illumination for about 15 sec., and the subsequent change of density was followed. It was found that at *p*H 7·0 and 9·0 there was an abrupt loss of density at 460 mμ due to the bleaching of visual purple followed by a further loss of density over a period of about 10 min. at *p*H 9·0 and 15 min. at *p*H 7·0. There appeared to be practically no change at 435 mμ (*p*H 7·0) and a slight gain in density at 410 mμ (*p*H 7·0). The changes in density are probably due to the same process as Dartnall's dark reaction and mark the breakdown of transient orange to the stable indicator yellow. Transient orange is apparently not formed in solutions at *p*H 5·2.

Discussion and conclusions

A tentative scheme for the bleaching of visual purple both by light and by moderately strong acids and alkalis is given in Fig. 9. The scheme has been drawn up merely to describe the facts recorded in this paper in a condensed form. According to the scheme when visual purple is bleached, transient orange is formed. The latter undergoes a thermal change leaving indicator yellow in the solution. Fairly strong acids and alkalis when added to visual purple form indicator yellow immediately. The reversible changes in the indicator yellow itself on altering the hydrogen-ion concentration appear to be extremely rapid. It is possible to reach the final indicator yellow stage at *p*H 4·0 by two routes, viz. either by adding acid buffer direct to a visual purple solution or by the action of light on a solution of about *p*H 6·5 and subsequent acidulation. There are also alternative routes to the indicator yellow of *p*H 11.

In view of the fact that indicator yellow behaves in all ways like an acid-base indicator such as thymol blue, it is possible that transient orange is a manifestation of indicator reactions. Most laboratory acid-base indicators take up their final colour very quickly, but there are exceptions of which China blue is an example. China blue may take some minutes before it comes to equilibrium in an acid or alkali solution and it is possible that the transient orange is behaving similarly.

It is well known that the staining reactions of the retina are changed after it has been exposed to light. The changes are difficult to interpret [Tansley, 1935], but most authors believe that the retina becomes acid

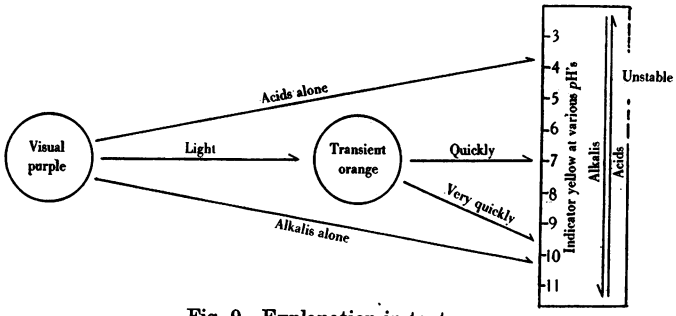

Fig. 9. Explanation in text.

when bleached. If this is true and if the reactions which occur in solutions of visual purple also occur in the intact retina, it means that the bleached whole retina must contain a yellow substance whose density is comparable with the visual purple which it has replaced. Considerable changes in the luminosity curve may be produced by these internal yellow filters.

Summary

1. A method is described for the preparation of reasonably pure solutions of visual purple which if buffered between *p*H 6·0 and 9·3 are quite stable at room temperature for several hours.

2. The absorption curves of visual purple extracts are unaffected by hydrogen-ion concentration except for small variations which can be explained by changes in the impurities present. If, therefore, the visual purple molecule is dissociated in solution, it is not in the region of its chromophoric group.

3. By making certain assumptions about the densities of impurities present, a fairly accurate absorption curve for visual purple has been obtained. The maximum absorption is at 502 mμ. The curve is different

from those obtained by many previous authors because their solutions were probably more acid than *p*H 9·0.

4. During the course of bleaching a well-defined intermediate substance is formed to which the name "transient orange" has been given. Transient orange is thermally decomposed to a yellow substance which has been named "indicator yellow".

5. Indicator yellow is pale yellow in alkaline solution, a deeper yellow of the same apparent hue in neutral solution and a deep chrome yellow in acid solution. Absorption curves for indicator yellow have been determined between 395 and 650 mμ over a range of hydrogen-ion concentration from *p*H 3·3 to 10·8. The accepted explanation of the results is that indicator yellow is an acid-base indicator, and for this belief fresh evidence has been produced. For the range *p*H 6·1 to 10·0 there is some indication of an "iso-bestic" point at about 400 mμ. The alkaline form at *p*H 10·0 has a narrow absorption band with a maximum which is probably in the near ultra-violet: the more acid form has a broad absorption band. Between *p*H 6·1 and 3·3 it appears that a tautomeric change takes place in the molecule and the absorption curves are quite different from those taken between *p*H 6·1 and 10·0. Indicator yellow is thermally unstable between *p*H 4·0 and 5·2 and slight changes can be found at *p*H 3·3 and 6·1. Between *p*H 7·0 and 10·8 it remains unchanged for several hours at 20° C.

As revealed by their absorption curves the reactions of indicator yellow are completely reversible.

6. Visual purple is stable between *p*H 5·2 and 10·0: outside this range it loses its colour and the indicator yellow formed is indistinguishable from that formed by first bleaching the solution in light and then adding the appropriate buffer.

7. A little regeneration of visual purple in bleached solutions occurs between *p*H 7·0 and 9·3 but only slowly in the more alkaline solutions.

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REFERENCES

Bayliss, L. E., Lythgoe, R. J. & Tansley, K. (1936). *Proc. Roy. Soc.* B, **120**, 95.
Chase, A. M. (1936). *J. gen. Physiol.* **19**, 577.
Clark, W. M. (1928). *The Determination of Hydrogen Ions.* London.
Dartnall, H. J. A. (1936). Ph.D. thesis. Univ. of London.
Dartnall, H. J. A., Goodeve, C. F. & Lythgoe, R. J. (1936). *Proc. Roy. Soc.* A, **156**, 158.
Garten, S. (1906). *v. Graefes Arch. Ophthal.* **63**, 112.
Hecht, S. & Chase, A. M. (1934). *Proc. Nat. Acad. Sci.*, Wash., **20**, 238.
Hecht, S., Chase, A. M., Shlaer, S. & Haig, C. (1936). *Science*, **84**, 331.
Holm, E. (1922). *C. R. Soc. Biol.*, Paris, **87**, 465.
Hosoya, Y. (1933–4). *Pflügers Arch.* **233**, 57.
Hosoya, Y. (1934). *Arch. Sci. biol.* **18**, 183.
Hosoya, Y. & Bayerl, V. (1933). *Pflügers Arch.* **231**, 563.
Hosoya, Y. & Saito, Z. (1935). *Tohoku J. exp. Med.* **27**, 172.
Kerridge, P. M. T. (1926). *J. sci. Instr.* **3**, 404.
Köttgen, E. & Abelsdorff, G. (1896). *Z. Psychol.* **12**, 161.
Krause, A. C. (1934). *The Biochemistry of the Eye.* Baltimore.
Kühne, W. (1878). *The Photochemistry of the Retina etc.* London.
Kühne, W. (1895). *Z. Biol.* **32**, 21.
Nakashima, M. (1929). Trans. XIII Ophthal. Cong. **1**, 317.
Tansley, K. (1931). *J. Physiol.* **71**, 442.
Tansley, K. (1935). *Ibid.* **84**, 34 *P.*
Trendelenburg, W. (1904). *Z. Psychol. Physiol. Sinnesorg.* **37**, 1.
Wald, G. (1935). *J. gen. Physiol.* **19**, 351.
Wald, G. (1936). *Ibid.* **20**, 45.