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THE RELATION OF TRANSIENT ORANGE TO VISUAL PURPLE AND INDICATOR YELLOW

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A slightly alkaline solution of visual purple which is exposed to daylight at room temperature rapidly becomes pale yellow in colour. The yellow colour is due to the formation during bleaching of a substance which has indicator properties. This substance assumes a deep chrome yellow in acid and a pale yellow colour in alkaline solution. It will be referred to as "indicator yellow" [Lythgoe, 1937]. There is, however, an intermediate stage in the bleaching process, since if the original alkaline solution of visual purple is first cooled to 0° C. and then exposed to light, the solution becomes orange in colour. The orange colour fades slowly at 0° but very rapidly if the solution is allowed to regain room temperature (20° C.), and after it has faded the presence of indicator yellow can be demonstrated in the solution. The use of the term "transient orange" to describe the substance responsible for the intermediate orange colour was suggested in a previous paper [Lythgoe, 1937]. As Wald [1936] pointed out, it is never possible to regain the original orange colour of freshly bleached visual purple once the pure yellow of the indicator substance has made its appearance.

The purpose of this paper is to advance further evidence to support the view that visual purple is decomposed by light to form transient orange and that the latter substance is thermally converted to indicator yellow at a rapid rate.

Since the thermal reaction is rapid, it can be studied accurately only by the use of temperatures in the region of 0° C. As will be shown later, it is important to realize that the "dark reaction" may be made up of two changes, viz. the decomposition of transient orange, and the subsequent decomposition of the indicator yellow formed from it. Thermal changes of

density after bleaching have been observed by many workers, notably by Hosoya [1933] and Wald [1938], but with the exception of Chase's results [1936] the solutions used have been either so warm or so acid that the destruction of transient orange must have been virtually completed before readings began. Provided only those solutions of visual purple containing a minimum of yellow impurities are used, the indicator yellow is stable on the alkaline side of neutrality [Lythgoe, 1937; Dartnall, Goodeve & Lythgoe, 1938]. The dark reaction in these solutions is composed entirely of the thermal decomposition of transient orange.

In the experiments to be described solutions of visual purple at a low temperature were bleached very rapidly by a strong light and the subsequent changes of density in the dark were followed at a number of wave-lengths.

Apparatus and material

The optical densities were measured with a slightly modified form of the photoelectric spectro-photometer described by Bayliss, Lythgoe & Tansley [1936] (Fig. 1).

The light source of the photometer was a motor-car head-lamp bulb run off accumulators. The filament of the lamp was focused by a condensing lens through preliminary light filters [Lythgoe & Quilliam, 1938] on the entrance slit of the monochromator. The spectral band emerging from the exit slit of the monochromator, after being rendered parallel, was passed through the optical cell containing the solutions under examination. The capacity of the optical cell was about 0·5 c.c. and the length of path 0·5 cm.

The light transmitted by the solution falls on a photoelectric cell (Osram KG 7). The current thus generated is led to the insulated needle of a Lindemann electrometer. The potential on the needle is reduced to zero by inducing on it an equal and opposite charge through the condensers (C_1 and C_2). One plate of the condenser is connected both to the insulated needle and to the cathode of the photocell. The other plate of the condenser is connected to a potentiometer (*Pot.*) so that, when its potential is raised, the opposite plate is charged. With the earthing switch (*E.S.*) closed the needle is at earth potential and occupies its zero position. Just before the light shines on the cell the earthing switch is opened, and when the needle begins to move it is kept at zero by adjustment of the potentiometer. By means of a shutter the light is allowed to pass into the spectroscope for intervals of 5 sec. The final reading of the potentiometer is proportional to the quantity of light which has fallen on the photocell. By taking paired readings, one on a control optical cell filled with water and the other on the unknown solution, one can measure the fraction of incident light transmitted by the latter and so calculate its optical density. The sensitivity of the instrument was controlled both by using one or two condensers (C_1, C_2) and also by means of a two-gang selector switch which applied 1·5, 3·0, 4·5, 6·0, or 9·0 V. to the ends of the potentiometer.

The holder (*C.H.*) of the optical cell was hollow, and water was pumped through it to maintain the solution under examination at any desired temperature. In practice, three large reservoirs were used. One contained water at room temperature. The second was packed with ice and the

water from the melting ice was pumped through the holder, maintaining the solution in the optical cell at about 2·9° C. The water in the third reservoir was kept at about 10° C. by a thermoregulator [Lythgoe & Quilliam, 1938].

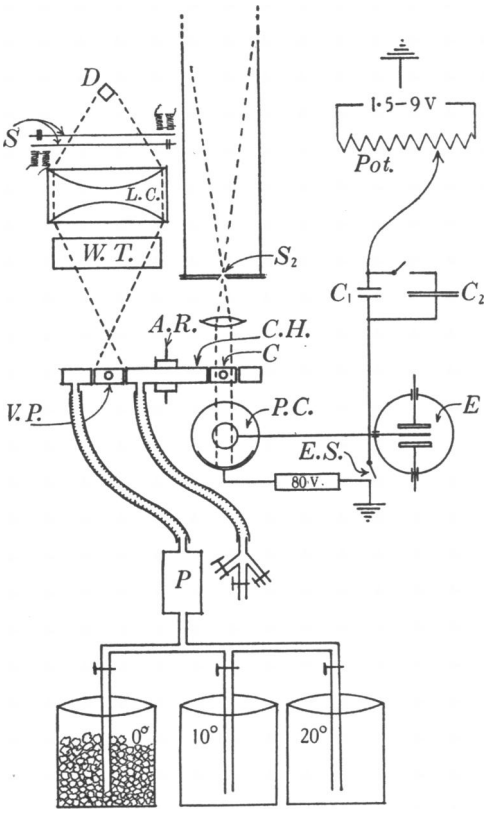

Fig. 1. Diagram of apparatus. *V.P.* solution under investigation; *C*, control solution; *C.H.* hollow cell holder; *A.R.* axis of rotation of cell holder; S_2, exit slit of spectroscope; *P.C.* photocell; *E*, Lindemann electrometer; C_1 and C_2, condensers; *E.S.* earthing switch; *D*, diamond of Pointolite; *L.C.* lantern condenser; *W.T.* water trough; *S*, blades of shutter and controlling electromagnets; *P*, water pump; 0°, 10°, 20°, the water reservoirs. The taps controlling the water circulation are also shown.

By means of two three-way taps, one in the outgoing and one in the returning water systems, it was possible to change quickly from one reservoir to another, so selecting the required temperature. A small glass ball was put into the optical cell and its movement on rotation of the cell holder served to stir the solution.

The formation of dew on the optical cell at low temperatures was prevented by blowing dry air on to the cell face and adjacent parts. The air was dried by passing it first through a metal spiral which was immersed in a freezing mixture, and secondly, through a Silica gel breather. This apparatus was found to be very efficient in preventing the formation of dew. One charge of freezing mixture (3 parts crushed ice: 1 part NaCl) was found to last for the whole of 1 day's experiments.

The visual purple was bleached by a 1000 C.P. Pointolite, the diamond (*D*) of which was focused on the optical cell by means of a large lantern condenser (*L.C.*). A trough of water, 4 cm. thick, was interposed between the condenser and the cell. A thermocouple placed in the cell during bleaching showed that the layer of water removed all the heat rays from the bleaching light. The visual purple was bleached for an accurately timed exposure controlled by two shutters (*S*), one made to open, the other to close the aperture in front of the Pointolite. The shutters were released electromagnetically by two trip switches operated by a disc rotating at a constant speed. It was found that an exposure of 1 sec. produced almost complete bleaching of the visual purple, but an exposure of 3 sec. was used in practice.

The solutions were prepared from *Rana esculenta* by the method already described [Lythgoe, 1937]. Their hydrogen-ion concentrations were measured with a glass electrode in conjunction with a valve electrometer [Lythgoe & Quilliam, 1938].

Method

Fresh preparations were always used and the absorption curve of the unbleached solution of visual purple was first measured. The holder for the optical cell was then rotated into the bleaching position and the solution was exposed to the light from the Pointolite for 3 sec. The cell holder was immediately rotated to the estimating position and a density measurement made. Each reading on the solution was paired with a control reading on an optical cell filled with distilled water. Usually the control followed immediately after the reading on the solution, but when it was desired to follow quick changes at several wave-lengths a set of control readings was taken at these wave-lengths both before and after the readings on the solution. The response of the apparatus was found to change very little over a period of 5 min. or so. After bleaching, density readings on the solution were then made in rotation at the wave-lengths under investigation until the end of the experiment.

Results

In one experiment (Fig. 2) the solution (*p*H 7·13) was cooled to 3° C. and the absorption curve of the unbleached visual purple was determined. The solution was then bleached, and density readings were taken in the range 460–490 mμ. The order in which readings were taken is indicated by the arrows in Fig. 2. The readings at 470, 480 and 490 mμ. were taken

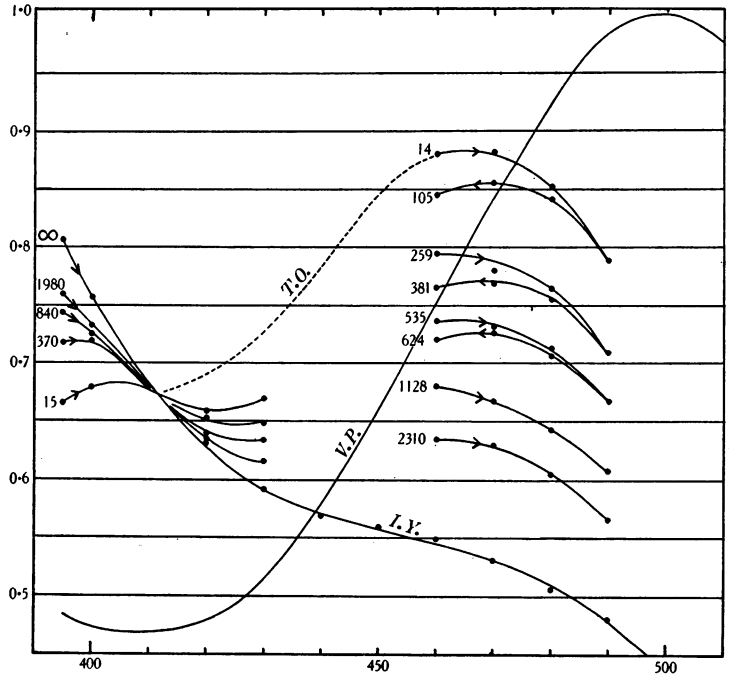

Fig. 2. The conversion of transient orange into indicator yellow. *p*H 7·13. 3° C. Ordinate: density. Abscissae: wave-lengths. *V.P.* the unbleached visual purple. The numbers against the points give the times (sec.) at which the readings were taken; the arrows show the direction in which they were taken. The readings on the curve marked *T.O.* were taken within 1¼ min. after bleaching. After the solution had been at 3° for 2500 sec. it was warmed to 20° for 20 min. and cooled again. The curve marked *I.Y.* was then taken. The graph embodies readings on two identical solutions.

at 29, 44 and 59 sec. respectively after the end of bleaching, but the dark reaction is so fast, especially in its early stages, that there had already been a considerable loss of density before readings were begun. After readings in the range 460–490 mμ. had been repeated at intervals for about ¾ hr. the temperature of the solution was raised to about 20° C. for 20 min. and again cooled. The curve so obtained is marked *I.Y.*,

and it will be seen that there has been a further loss of density. This is the absorption curve of indicator yellow and we know from other experiments that there would be only small further changes in density on keeping the solution.

After the completion of this run the cell was refilled with unbleached visual purple from the same preparation and bleached as before, but density readings were now taken in the range 395–430 mμ. It will be seen from Fig. 2 that during the dark after bleaching there has been a progressive decrease in density at 420 and 430 mμ. At 395, 400, and, to some

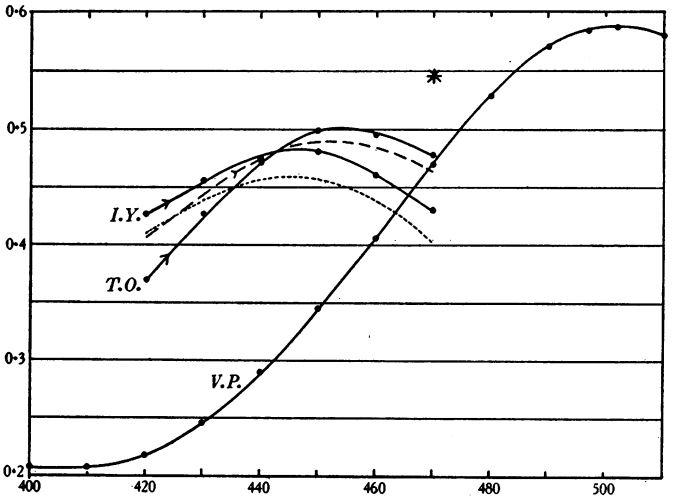

Fig. 3. The conversion of transient orange into indicator yellow at *p*H 4·90. 2·9° C. Ordinate: density. Abscissae: wave-lengths. *V.P.* unbleached visual purple. The taking of readings was begun immediately after bleaching and the arrows show the direction in which they were taken. The curve marked *T.O.* was taken within 3 min. of bleaching but it is not a true curve of transient orange. The asterisk records the density at 470 mμ. of an identical solution 10 sec. after bleaching. The readings for the upper dotted line were begun 270 sec. after bleaching. The curve marked *I.Y.* taken 1 hr. after bleaching is not very different from the true curve for indicator yellow. The lower dotted line represents readings taken 2½ hr. after bleaching; it records the decomposition of indicator yellow.

extent, at 410 mμ., however, there has been an increase in density in the dark. At about 412 mμ. the density must have remained unaltered during the dark reaction. The increase in density at 395 and 400 mμ. is of importance in the interpretation of the changes which occur during the dark reaction.

Fig. 3 shows the results of an experiment on a solution of visual purple at *p*H 4·90. The procedure was similar to that in the first experiment

except that the range of wave-lengths was different. After bleaching, the densities in the dark decreased between 450 and 470 mμ. but increased between 420 and 440 mμ. The density at about 443 mμ. must have remained constant during the dark reaction. As with the experiment at *p*H 7·13, there had been a considerable loss of density before the first of the readings at 470 mμ. was taken. The asterisk in Fig. 3 marks the density at this wave-length 10 sec. after bleaching in another sample of the solution at *p*H 4·90. The curve marked *I.Y.* was taken 1 hr. after bleaching, and it is not very different from the curve for indicator yellow at this acidity [Lythgoe, 1937]. The subsequent behaviour of this solution in the dark was different from that of more alkaline solutions, since after a further period of 1⅓ hr. at 2·9° C. there was a loss of density at all wave-lengths (lower dotted line in Fig. 3). The explanation of this phenomenon is that the indicator yellow was beginning to decompose since it is unstable at this acidity [Lythgoe, 1937].

Similar results were obtained in a number of other experiments at other acidities, but in markedly acid or alkaline solutions the early changes of density in the dark after bleaching are too rapid to admit of detailed study.

The results of several experiments at a variety of hydrogen-ion concentrations (*p*H 4·90–9·25) showed that, on bleaching visual purple, the density at 470 mμ. shows a marked increase whilst that at 480 mμ. shows a small decrease. The maximum density of a freshly bleached solution is probably at about 470 mμ. The curves marked *T.O.* (Figs. 2 and 3) are only a first approximation to the true absorption curve of transient orange.

Discussion

When visual purple is bleached by light at room temperature the indicator yellow formed is relatively stable provided the solution is on the alkaline side of neutrality. The intermediate substance, transient orange, might be related to these substances in two ways. It might be a true intermediate substance, thus:

$$V.P. \xrightarrow{\text{light}} T.O. \xrightarrow{\text{thermal}} I.Y. \qquad \text{.....(i)}$$

On the other hand, both indicator yellow and transient orange might be formed simultaneously, the latter then decomposing, thus:

$$V.P. + \text{light} \begin{cases} \nearrow I.Y. \\ \searrow T.O. \xrightarrow{\text{thermal}} \text{colourless breakdown products.} \end{cases} \qquad \text{.....(ii)}$$

If the latter were true then with the disappearance of transient orange there would be a progressive loss of density at all wave-lengths in the dark, the loss being greatest at the wave-length of its greatest density. The increasing density in the dark at the shorter wave-lengths is very strong evidence that another substance is being formed during the dark reaction. It will be shown later that the indicator reaction develops in the solution only as the orange colour disappears, and scheme (i) is therefore the more likely to be true. It would appear therefore that at *p*H 7·13 transient orange has a maximum absorption at about 470 mμ., whilst the indicator yellow formed from it has a maximum absorption in the near ultra-violet with a decreasing absorption towards the longer wave-lengths [Krause & Sidwell, 1938]. At about 412 mμ. the densities of transient orange and indicator yellow have the same value, and no matter what their relative proportions at any time and in any one solution the density at that wave-length will be unaltered. During the dark reaction therefore we find an increase in density at wave-lengths shorter than 412 mμ., a decrease in density at wave-lengths longer than 412 mμ. with a maximum loss at about 470 mμ., whilst at the cross-over wave-length of 412 mμ. there is no change in density.

It has been shown that, in weakly acid solutions, indicator yellow is unstable, and that a cross-over wave-length is observed only during the early stages of the dark reaction. We also find that only those preparations of visual purple which are relatively transparent to the blue wave-lengths give clear-cut results, the impure solutions giving a gradual loss of density at all wave-lengths similar in appearance to the destruction of indicator yellow in acid solution. In such solutions the dark reaction is made up of (1) the conversion of transient orange into indicator yellow which we regard as the true dark reaction, (2) the decomposition of the indicator yellow formed. Both reactions probably occurred simultaneously in the experiments recorded by Hosoya [1933] and Wald [1937].

Wald followed the dark reaction at 29·3° C. (*p*H 6·9). He appears to have obtained a slight increase in absorption below 435 mμ. with a maximum of absorption at 480 mμ. His absorption curve taken 1·2 min. after bleaching is very much lower than ours taken at the same time interval, and it seems probable that most of the transient orange had already been decomposed at the high temperature used. For the most part the bleached solutions of Wald and of Hosoya showed a slow fall in density at all wave-lengths and in our view this reaction was predominantly due to the decomposition of indicator yellow.

Recently Wald [1938] has published results to show that in acid

solution (*p*H 3·9 and 5, and to some extent at *p*H 6·9) there is an increase in density during the dark reaction which is greatest at 440 mμ. We have never found any trace of this rise in the few experiments we have made in this range of acidities and we feel that our failure to find it must be due to some fundamental difference between our solutions and those used by Wald. It should be emphasized however that we invariably worked at temperatures below 3° C., and that at the temperature used by Wald (25° C.) the reactions recorded in the present paper would have been practically completed before he had taken his first reading.

The term visual yellow is used to describe all the coloured breakdown products of visual purple. As we have seen, the term really embraces two substances which we call transient orange and indicator yellow. Are there other links in the chain? Unless there is an extremely rapid change before the appearance of transient orange, it would seem that so far as the visible spectrum is concerned there are no further links. From Fig. 2 it will be clear that since there is a single cross-over wave-length between the indicator yellow and the substance which is formed initially on bleaching, any further intermediate substance would have to possess the same density at that wave-length. Furthermore, the greatest loss of density occurs at about 470 mμ. during the whole of the true dark reaction. If two substances were concerned it would be unlikely that their absorption curves would be identical. The problem can be investigated quantitatively. If the fading of the orange colour in the dark involves nothing more than the quantitative conversion of transient orange to indicator yellow, then the progressive losses or gains occurring at one wave-length should be proportional to the losses or gains in density at any other wave-length. In other words, if we plot densities at one wave-length against the densities recorded at the same times at another wave-length, the points should fall on a straight line if the fading in the dark involves only a single chemical change. In Fig. 4 the densities at all the wave-lengths investigated (ordinates) have been plotted against the densities at 460 mμ. at the same times (abscissae). Actual density readings at 460 mμ. for all the times involved were not available but they were obtained by interpolation from a graph relating time with density at 460 mμ. This wave-length was chosen because we had many well-spaced readings for it. It will be seen that the points fall on straight lines within experimental error. This seems to be conclusive evidence that transient orange is a single absorbing molecular species and that its conversion to indicator yellow is the only reaction during the true dark reaction which can be studied in the visible spectrum.

The absorption curve of transient orange is not very markedly affected by hydrogen-ion concentration. This can be shown by the following test-tube experiment. Two samples of visual purple at about *p*H 5·0 were cooled to 0° C. and bleached side by side in daylight. Both

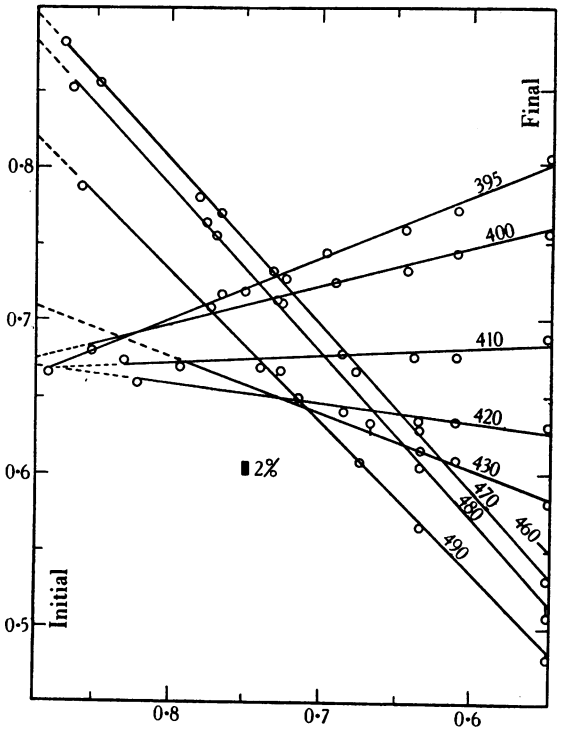

Fig. 4. The readings are the same as those shown in Fig. 2. Ordinate: density readings at a number of wave-lengths (395, 400, 410, 420, 430, 470, 480, 490 mμ.). Abscissae: the density readings at 460 mμ. at corresponding times (interpolated). The points lie on a series of straight lines within experimental error. (The short thick vertical line represents the extent of the displacement which would have occurred with a 2% error in the measurement of light transmissions.) The figure shows that the loss or gain of density at any wave-length is strictly proportional to the loss of density at 460 mμ.: the conversion of transient orange to indicator yellow is a simple chemical change.

samples turned orange. On making one sample alkaline (about *p*H 8·5), there was only a slight change of colour to a slightly more orange hue than that of the acid sample. This experiment is difficult to perform satisfactorily, since if a solution is ever either too acid or too alkaline the transient orange is very rapidly decomposed. The indicator reactions of transient orange must be much less marked than those of indicator

yellow. This can be shown by another test-tube experiment. One sample of a preparation of visual purple at *p*H 5·0 was cooled to 0° C.; another sample remained at 20° C. On bleaching side by side in daylight the cooled sample turned orange, whilst the warmer one turned deep pure yellow. Equal volumes of an alkaline solution were added to each test-tube, but whereas the warmer sample gave the typical indicator reaction and turned a pale yellow, the cooled sample was very little affected, showing that if any change with altered acidity had occurred it was comparatively slight. Furthermore, there could not have been any considerable quantities of indicator yellow in the solution otherwise its colour change would have been seen. When the solutions were again made acid the warmer sample regained its original deep yellow colour but the cooled solution again remained unchanged. When the latter solution was warmed it became yellow in colour, and exhibited typical indicator properties.

Several experiments were performed in which the density of a visual purple solution was measured immediately after bleaching either at 470 or at 480 mμ. Within the *p*H range 4·9–9·25 there was always an increase in density at 470 mμ. and a small decrease in density at 480 mμ. This means that at some wave-length between 470 and 480 mμ. the density of the freshly bleached solution is identical with the density of the unbleached solution at the same wave-length. Since the absorption curve of visual purple at these wave-lengths is unaffected by hydrogen-ion concentration, it follows that the absorption curve of transient orange is not very markedly affected by this condition. Any effect which may exist is small compared with the acid-alkali reaction of indicator yellow. Strict experimental verification will be difficult, since we find that the conversion of transient orange is markedly accelerated in acid or alkaline solutions. A few seconds must elapse before the first reading can be taken on a freshly bleached solution, and during this time the amount of transient orange converted to indicator yellow will vary considerably in solutions of different acidity. The fact that transient orange has an absorption curve which is largely independent of hydrogen-ion concentration shows that it is structurally different from indicator yellow.

The results from an investigation now in progress on the influence of temperature and acidity on the decomposition of transient orange also show that this substance is quite different both from visual purple and from indicator yellow.

SUMMARY

1. Visual purple solutions were rapidly bleached in an intense light and the changes in density—the "dark reaction"—were followed at several wave-lengths.

2. The substance first formed by the action of light (transient orange) has an absorption equal to that of the parent visual purple at a wave-length between 470 and 480 mμ. The initial product of bleaching is very little affected by hydrogen-ion concentration.

3. In a solution at *p*H 7·13 the density at wave-lengths longer than 412 mμ. decreased during the dark reaction with a maximum loss in the region of 470 mμ., but at wave-lengths shorter than 412 mμ. the solution increased in density showing that another substance was being formed (indicator yellow).

4. These changes correspond to the conversion of unstable transient orange to indicator yellow which is a comparatively stable substance in alkaline solution.

5. The early changes in a more acid solution (*p*H 4·90) are similar, but subsequently the absorption curves show that the indicator yellow begins to decompose. The dark reaction is therefore composed of two separate reactions: (i) the thermal conversion of transient orange into indicator yellow—the true dark reaction, (ii) the thermal decomposition of indicator yellow to colourless products.

6. Evidence is advanced to support the view that transient orange is a distinct molecular species.

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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.
Dartnall, H. J. A., Goodeve, C. F. & Lythgoe, R. J. [1938]. *Proc. Roy. Soc.* A, **164**, 216.
Hosoya, Y. [1933]. *Pflüg. Arch. ges. Physiol.* **233**, 57.
Krause, A. C. & Sidwell, A. E. [1938]. *Amer. J. Physiol.* **121**, 215.
Lythgoe, R. J. [1937]. *J. Physiol.* **89**, 331.
Lythgoe, R. J. & Quilliam, J. P. [1938]. *J. Physiol.* **93**, 24.
Wald, G. [1936]. *J. gen. Physiol.* **20**, 45.
Wald, G. [1937]. *Nature, Lond.*, **139**, 587.
Wald, G. [1938]. *J. gen. Physiol.* **21**, 795.