photometric data, and interspecies differences are not large.

Such rhodopsin preparations could however be quite variably contaminated with colourless substances such as proteins and possibly small amounts of indicator yellow and/or vitamin A, as well as purine or pyrimidine bases. The second stage must be to establish constancy in the spectrum between 300 and 400 mμ., and a third stage to establish a constant value between the absorption at 250–280 mμ. due to the carrier protein, and the absorption at 500 mμ.

Neither stage 2 nor 3 has yet been reached and nothing is known for certain about interspecies differences.

SUMMARY

1. Eyes from frogs, rats, sheep, cattle and several species of fish have been examined as sources of visual purple.

2. A combination of Saito's (1938) sugar flotation method for separating the outer segments of the rods from the retina with alum hardening of the rods proved to be superior to the other methods tried.

3. The major impurities seem to be protein (other than the rhodopsin moiety), melanin and phospholipin.

4. The ultraviolet absorption cannot be entirely accounted for as being due to tyrosine and tryptophan. A band at 260 mμ. seemed to be present and could be due to purine or pyrimidine or both, perhaps as nucleic acid.

5. The absorption maximum ($\lambda_{max.}$) for frog rhodopsin was 503 mμ., for rat 498 mμ. and sheep and ox at 500 $\pm$ 1 mμ. Char 'rhodopsin' had $\lambda_{max.}$ at 514mμ. Perch and pike porphyropsin had $\lambda_{max.}$ at 520–530 mμ., haddock rhodopsin at approx. 500 mμ.

6. A solution of frog rhodopsin has been obtained with the extinction ratio $E_{min.}/E_{max.} = 0{\cdot}24$ and hence is considerably purer than anything so far reported.

One of us (F. D. C.) participated in this work as a holder of a New Zealand National Research Scholarship. We are indebted to the Medical Research Council and the Ministry of Food for grants.

REFERENCES

Ball, S., Collins, F. D., Dalvi, P. D. & Morton, R. A. (1949). *Biochem. J.* **45**, 304.
Bliss, A. F. (1948). *J. biol. Chem.* **176**, 563.
Broda, E. E. (1941). *Biochem. J.* **35**, 960.
Broda, E. E., Goodeve, C. F. & Lythgoe, R. J. (1940). *J. Physiol.* **98**, 397.
Chase, A. M. & Haig, C. (1938). *J. gen. Physiol.* **21**, 411.
Goodeve, C. F., Lythgoe, R. J. & Schneider, F. E. (1942). *Proc. roy. Soc.* B, **130**, 380.
Goodwin, T. W. & Morton, R. A. (1946). *Biochem. J.* **40**, 628.
Granit, R. (1947). *Sensory mechanisms of the retina*, London: Oxford University Press.
Hecht, S. (1942). *Ann. Rev. Biochem.* **11**, 465.
Ishimoto, M. & Wald, G. (1946). *Fed. Proc.* **5**, 50.
Krause, A. C. & Sidwell, A. F. (1938). *Amer. J. Physiol.* **121**, 215.
Kühne, W. (1878). *On the Photochemistry of the Retina and on Visual Purple* (edited and translated by M. Foster). London: Macmillan.
Lythgoe, R. J. (1937). *J. Physiol.* **89**, 331.
Morton, R. A. & Goodwin, T. W. (1944). *Nature, Lond.*, **153**, 405.
Morton, R. A. & Stubbs, A. L. (1946). *Analyst*, **71**, 348.
Oster, G. (1948). *Chem. Rev.* **43**, 319.
Saito, Z. (1938). *Tohoku J. exp. Med.* **32**, 432.
Schneider, E. E., Goodeve, C. F. & Lythgoe, R. J. (1939). *Proc. roy. Soc.* A, **170**, 102.
Tansley, K. (1931). *J. Physiol.* **71**, 442.
Wald, G. (1938). *J. gen. Physiol.* **21**, 795.
Wald, G. (1939). *J. gen. Physiol.* **22**, 775.
Wald, G. (1949). *Docum. Ophthal.* **3**, 94.

Studies on Rhodopsin

2. INDICATOR YELLOW

BY F. D. COLLINS AND R. A. MORTON
Department of Biochemistry, University of Liverpool

(*Received* 27 *October* 1949)

The first product formed by the action of light on rhodopsin is apparently 'transient orange' (Lythgoe & Quilliam, 1938), a substance which is not stable at room temperature, but is transformed to 'indicator yellow'. As its name implies, the latter is pH sensitive; it is pale yellow in alkaline solutions and much more deeply yellow ($\lambda_{max.}$ 440 mμ.) in acid solutions (Lythgoe, 1937).

By extracting freshly bleached retinas with light petroleum, Wald (1934, 1935, 1936) obtained a new material which he called retinene. Later Morton & Goodwin (1944) showed retinene to be vitamin A aldehyde. The relationship between retinene and indicator yellow remained obscure until work in this laboratory (Ball, Collins, Morton & Stubbs, 1948 and Ball, Collins, Dalvi & Morton, 1949) showed that

retinene would combine with many amines, etc. to form compounds closely analogous to indicator yellow.

The purpose of the present paper is to examine more closely the relationship between retinene and indicator yellow on the one hand and between indicator yellow and rhodopsin on the other.

METHODS

Rhodopsin solutions were obtained by the methods discussed in the preceding paper (Collins & Morton, 1950). The pigment was destroyed photochemically ('bleached') by placing the cell containing the solution some 10–12 cm. in front of a 60 W. lamp. The absorption at 500 mμ. was measured at intervals and the bleaching continued until the extinction at 500 mμ. fell to a constant low value.

Retinal protein solutions. (*a*) Whole retinas are bleached (whereby rhodopsin is replaced by retinene which is slowly reduced to vitamin A), the tissue is hardened in alum solution (4%, w/v) and extracted with 2% (w/v) aqueous digitonin, and centrifuged. The supernatant liquid is colourless (except occasionally for traces of haemoglobin) and free from retinene but contains protein. (*b*) Rods obtained by the sugar-flotation method are bleached by light, exhaustively extracted with light petroleum to remove retinene, hardened with alum and extracted with 2% (w/v) aqueous digitonin and centrifuged. The clear liquor contains retinal protein.

Retinene$_1$ in aqueous digitonin solutions. Retinene$_1$ previously prepared in this laboratory by oxidation of vitamin A was used either as the crystalline material or a very rich concentrate. It was dissolved in aqueous digitonin (2%, w/v), the final concentration of retinene being about 10^{-5} molar, either (1) by adding the required amount of digitonin solution to solid retinene and bringing the solution to the boil or (2) by dissolving the solid retinene in a few drops of absolute ethanol before adding the digitonin solution.

As the solutions are appreciably photosensitive the process of dissolution is best carried out in a dark room with subdued or red lighting.

Alum solution. Potash alum [$K_2SO_4.Al_2(SO_4)_3.24H_2O$] was dissolved in water (4%, w/v).

RESULTS

Irrelevant absorption. All rhodopsin solutions exhibit some absorption additional to that due to the pigment itself. The 'irrelevant' contribution is itself composite and variable. The wavelength 700 mμ. is outside the range covered by appreciable rhodopsin absorption and the *E* value at that point can be used in estimating the amount of scattered light. It often happens that as a result of bleaching rhodopsin solutions, there is an appreciable improvement in transparency in the red part of the spectrum. This complication adds to the difficulty of making strictly quantitative comparisons between unbleached and bleached solutions.

$\lambda_{max.}$ *of bleached rhodopsin solutions.* The observed absorption curve is actually a summation of con-

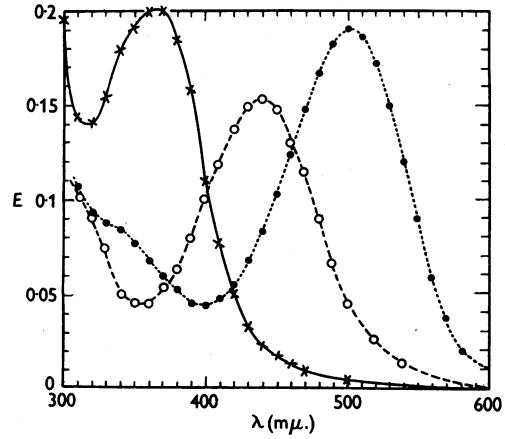


Fig. 1. Indicator yellow preparation from frog rhodopsin. ·●··●·, Rhodopsin; –○—○–, acid indicator yellow; — × — × —, alkaline indicator yellow. (All curves have had irrelevant absorption due to scattering subtracted.)

Table 1. *Observed and 'corrected' values for the wavelengths of maximum absorption shown by indicator yellow*

pH	$\lambda_{max.}$ obs. (mμ.)	$\lambda_{max.}$ corr. (mμ.)
(*a*) Bleached frog-rhodopsin preparations		
Excess alkali	360	365
9·2	~approx. 360	365
9·14	~approx. 360	365
8·05	363	367
8·0	367	367
8·0	360	365
7·6	367	373
7·5	367	367
7·4	367	367
7·4	360	370
7·0	365	367
1·76	435	440
Excess acid	435	440
(*b*) Bleached rat-rhodopsin preparations		
9·3	360	367
9·2	357	367
9·0	363	367
7·7	365	370
7·4	370	377
7·4	377	383
7·2	360	370
7·0	~370	375
7·0	365	373
6·66	~366	370
6·5	370	385
5·2	~370	367
4·0	400	417
Excess acid	435	440
	435	440
	405	435
	400	435
	430	440
	410	440

~ denotes inflexion.

tinuous absorption increasing with decreasing wavelength, and the roughly symmetrically selective absorption on either side of $\lambda_{max.}$ of indicator yellow itself. The observed $\lambda_{max.}$ as compared with the true indicator yellow maximum may therefore be displaced as a result of the irrelevant absorption. The displacement can be estimated as follows.

(*a*) The absorption spectrum of a given solution of unbleached rhodopsin is determined over the range 250–700 mμ. Using the fixation points 480, 500 and 520 mμ. the observed $E_{max.}$ (500 mμ.) is reduced to a corrected value (Collins & Morton, 1950).

(*b*) From the absorption curve for the 'reference' preparation of rhodopsin, i.e. the solution exhibiting the highest 'optical' purity so far obtained (with E_λ expressed as a fraction of $E_{max.}$) the whole curve from say 340–600 mμ. for the solution under study can be calculated by simple proportion given $E_{corr.}$ at 500 mμ.

(*c*) From the original observed curve (*a*) the corrected curve is subtracted ($E_{obs.}$–$E_{corr.}$) at wavelengths 5 or 10 mμ. apart. The difference curve represents irrelevant absorption.

(*d*) If it could be assumed that after bleaching the irrelevant absorption was substantially the same, the 'true' indicator yellow absorption would be obtained by subtracting the irrelevant absorption curve from the observed 'bleached' curve.

This procedure leads to 'corrected' values for $\lambda_{max.}$ and the best evidence for its validity is the constancy of the results.

Retinene and proteins. The general approach is illustrated by the following qualitative experiment.

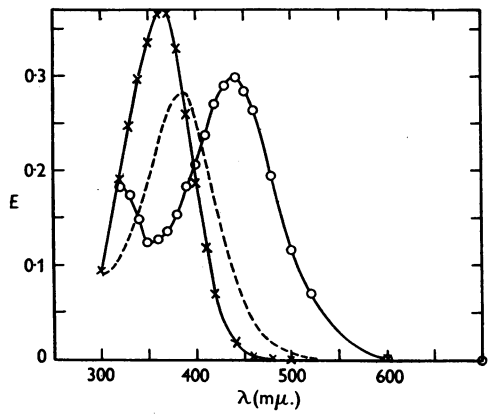


Fig. 2. Retinene and retinal protein. ----, Retinene and neutral protein solution; —×—×—, same solution plus alkali; -O—O-, previous solution plus excess acid.

To a solution of egg albumin in water is added a few drops of a solution of retinene in ethanol and the mixture is divided into two portions (*a*) and (*b*); (*a*) is acidified, but no change in colour occurs, (*b*) is made alkaline and the colour becomes paler, it is then acidified and becomes orange in colour; the acidified solution (*a*) is made alkaline and then acid and the orange colour appears.

For more quantitative experiments a neutral solution containing protein from sheep retinas may be used. When retinene is added $\lambda_{max.}$ occurs at 387 mμ. On making the solution alkaline $\lambda_{max.}$ occurs at 365 mμ., and subsequent acidification brings about the appearance of an absorption band with $\lambda_{max.}$ 440 mμ. Under such circumstances the relative intensities of absorption are: retinene ($\lambda_{max.}$ 387 mμ.) $E_{max.}$ 1·0; acid indicator yellow ($\lambda_{max.}$ 440 mμ. $E_{max.}$ 1·0–1·05; alkaline indicator yellow ($\lambda_{max.}$ 370 mμ.) $E_{max.}$ 1·26–1·43.

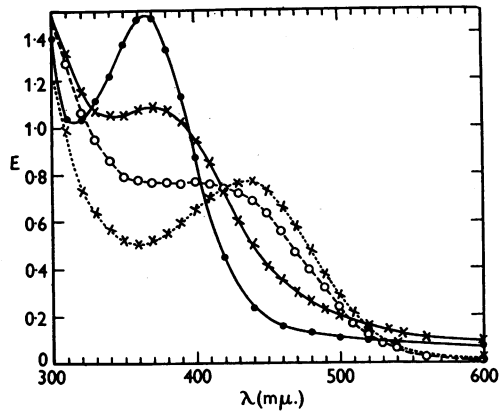


Fig. 3. Indicator yellow absorption curves uncorrected for irrelevant absorption, but with the absorption at 500 mμ. due to rhodopsin put equal to 1·00 (rhodopsin curve not shown). -●—●-, pH 8·0; -×—×-, pH 6·5; -O--O-, pH 4·0; ···×····×···, excess HCl. The isosbestic point is only apparent, because of the differences in irrelevant absorption.

The *E* values are somewhat variable as the amount of irrelevant absorption varies from one experiment to another.

If acid is added in excess to the solution showing $\lambda_{max.}$ 370 mμ. obtained by adding alkali to the retinene-protein solution, an orange precipitate is formed which can be centrifuged down and washed with water. On vigorous shaking with ethanol some of it dissolves and the solution exhibits $\lambda_{max.}$ 445 mμ.

Further characteristics of the absorption spectrum of indicator yellow. One solution (Table 1) of rat rhodopsin (pH 4) had $\lambda_{max.}$ at 417 mμ. immediately after bleaching, but on standing for 4 hr. in the dark, the maximum disappeared and only an inflexion at about 360 mμ. remained.

Other solutions of acid indicator yellow ($\lambda_{max.}$ 440 mμ.) showed on standing a drift of $\lambda_{max.}$ from 440 to 430 mμ., but if the solution was first made alkaline and then acid again $\lambda_{max.}$ reappeared at 440 mμ. This will be referred to in the discussion.

Table 2. *Quantitative relationship in the conversion of rhodopsin to indicator yellow*

(The change ($\Delta E_{500\,m\mu}$) in intensity of absorption at 500 mμ. measures the destruction of rhodopsin on complete bleaching and the rise ($\Delta E_{370\,m\mu}$) in absorption at 370 mμ. measures the formation of indicator yellow.)

Species	pH of solution	ΔE at 700 mμ.	ΔE at 370 mμ.	ΔE at 500 mμ.	$\Delta E_{370m\mu}/\Delta E_{500m\mu}$.
Frog	9·2	−0·005	0·116	−0·152	0·76
	9·14	−0·000	0·142	−0·189	0·75
Rat	9·2	−0·002	0·086	−0·117	0·74
	9·3	−0·003	0·135	−0·180	0·75
	9·0	−0·000	0·274	−0·355	0·77

For frog-rhodopsin solution at pH 1·76, $\Delta E_{500\,m\mu}$ was 0·189, and $\Delta E_{440\,m\mu}$ 0·070 and $\Delta E_{700\,m\mu}$ was not measurable, so that $\Delta E_{440\,m\mu}/\Delta E_{500\,m\mu}=0{\cdot}37$.

Extraction of retinene from retinas. A moist rod preparation (Collins & Morton, 1950, previous paper) from 12 dark-adapted frogs was first thoroughly extracted with light petroleum and then the rhodopsin was bleached. Retinene could then be extracted with light petroleum, but only very slowly.

The moist rods were dealt with in a dark room with a red light. They were placed in a centrifuge tube and shaken vigorously with light petroleum (5 ml.) and centrifuged. The liquid was poured off and replaced by a fresh portion (5 ml.) of solvent and the process repeated. In all, 10 such treatments were given in the dark room spread over a working day. Each 'washing' with light petroleum was examined for ultraviolet absorption. This was at first considerable though unselective, and the final solution was practically transparent. The residual rod preparation was then bleached by a strong light and at once re-extracted twice with light petroleum. The yield of retinene determined spectrophotometrically was 0·5 μg. After 3 hr. a second (double) extraction gave 1·0 μg. and after 5 hr. a third (double) extraction gave 0·3 μg.

Several other experiments with frog rod preparations led to the same conclusion, namely that after bleaching less retinene was obtained in the first extractions than in those made 1–2 hr. after exposure to light.

On the other hand if whole, fresh, dark-adapted frog retinas were suspended in light petroleum and exposed to light the retinene at once went into solution.

Retinene was not, however, extracted from ox or rat retinas treated similarly.

Extraction of indicator yellow from dried haddock retinas. Through the courtesy of the Director of the Torry Research Station and of Dr J. A. Lovern, one of us (F. D. C.) was able to keep live haddock in tanks so as to secure at any rate partial dark adaptation.

Alum-treated retinas were dried *in vacuo* and were found to keep fairly well. A portion of the dried material was extracted with absolute ethanol and the solution exhibited the absorption curves shown in Fig. 4. These results are discussed on p. 16.

Extraction of retinene from bleached rhodopsin solutions. It cannot be assumed without investigation that rhodopsin-containing solutions are free from vitamin A, which might indeed be present in extracts from incompletely dark-adapted retinas, especially if the enzyme-destroying alum treatment had been delayed or omitted. Under such circumstances bleached rhodopsin solutions might contain both retinene and vitamin A.

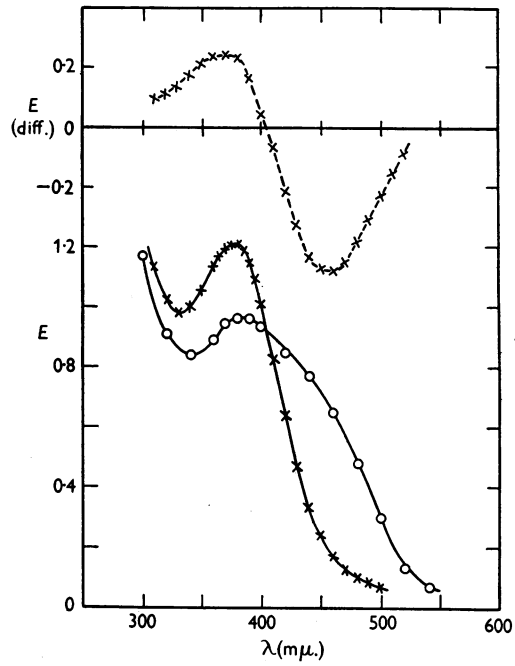


Fig. 4. −○—○−, Ethanol extract of dried haddock retinas; −×—×−, the same extract after 1 hr.; −×---×−−, a difference curve illustrating the disappearance of a maximum near 450 mμ., and its replacement by a maximum near 375 mμ.

It is possible to extract retinene from bleached rhodopsin solutions readily by means of a mixture of ethanol and light petroleum, but Bliss (1948) has shown that light petroleum alone is effective if emulsions are broken down by strong centrifuging.

This we confirm, but the extracts obtained with or without added ethanol are very impure as judged by ultraviolet absorption or by the antimony trichloride colour test. If, however, the light petroleum extract is taken to dryness and left in contact with 5% ethanolic potassium hydroxide for 0·5 hr. at room temperature and the retinene is re-extracted (chloroform) the 664 mμ. maximum in the colour test appears very clearly without turbidity (cf. Bliss, 1948). This, again, is confirmed. Three typical rhodopsin solutions gave 45, 75 and 44% recoveries of retinene at the pH values 7·4, 9·3 and 6·5 respectively, assuming that in aqueous digitonin the molecular extinction coefficients of rhodopsin at 500 mμ. and retinene at 380 mμ. were respectively 50,000 (see Discussion) and 40,000. Vitamin A was not, however, present in quantity.

In cases where vitamin A and retinene are both present the following procedure is useful. The band at 617 mμ. due to vitamin A reaches its maximum intensity after 15 sec. and that at 664 mμ. due to retinene after 100 sec. For pure vitamin A the ratio of the extinction at 664 mμ. (100 sec.) to that at 617 mμ. (at 15 sec. after mixing) is 0·094. (This figure is due to Dr J. Glover.) For retinene the ratio of the extinction at 617 mμ. (15 sec.) to that at 664 mμ. (100 sec. from mixing) is 0·560.

Hence if E_{617} is the extinction at 617 mμ. at 15 sec. and E_{664} is the extinction at 664 mμ. at 100 sec., the following equations will hold:

$$E_{664} = E_R + 0{\cdot}094E_A,$$
$$E_{617} = 0{\cdot}560E_R + E_A$$

where E_R is the extinction at 664 mμ. (100 sec.) due to retinene and E_A is the extinction at 617 mμ. (15 sec.) due to vitamin A. $E^{1\%}_{1\,cm.}$ (617 mμ.) for vitamin A is 5000 and $E^{1\%}_{1\,cm.}$ (664 mμ.) for retinene is 4000.

Bleached rhodopsin and p*-aminobenzoic acid.* *p*-Aminobenzoic acid reacts readily with retinene to give a band at 535 mμ. in acid solutions (Ball, Goodwin & Morton, 1948).

The following four experiments were carried out. (1) *p*-Aminobenzoic acid in excess was added to freshly bleached slightly acid rhodopsin and the solution was made first alkaline and then acid. (2) *p*-Aminobenzoic acid in excess was added to a slightly acid rhodopsin solution, which was then bleached, made alkaline and finally acid. (3) *p*-Aminobenzoic acid in excess was added to a freshly bleached neutral rhodopsin solution, which was then acidified. (4) *p*-Aminobenzoic acid was added in excess to a neutral rhodopsin solution, which was then bleached and made acid.

All these experiments were negative in the sense that no band at 535 mμ. was obtained, although *p*-aminobenzoic acid reacted readily with retinene in aqueous digitonin to give the 535 mμ. maximum. These experiments will be referred to in the discussion.

Retinene reductase (vitamin A dehydrogenase). A few experiments were carried out which showed that crystalline retinene was converted to vitamin A in the presence of fresh sheep retinas. However, the conversion was not quantitative and most of the retinene gave rise to products which had no specific absorption.

In one experiment three preparations were used as follows. (1) Three light-adapted retinas were covered with a solution of retinene in 2% (w/v) aqueous digitonin. (2) Three light-adapted retinas were kept at 100° for 5 min. and then covered with a solution of retinene in aqueous digitonin. (3) Three light-adapted retinas were left untreated.

The three preparations were left for 2 hr. and were then dried with anhydrous Na_2SO_4 and extracted with light petroleum. This solvent was removed *in vacuo* and replaced by 10 ml. *cyclo*hexane. The amounts of vitamin A were estimated by the three-point correction procedure of Morton & Stubbs (1946).

No trace of retinene could be found (absence of $\lambda_{max.}$ or inflexion at 370 mμ. and of a maximum at 664 mμ. with the antimony trichloride reagent).

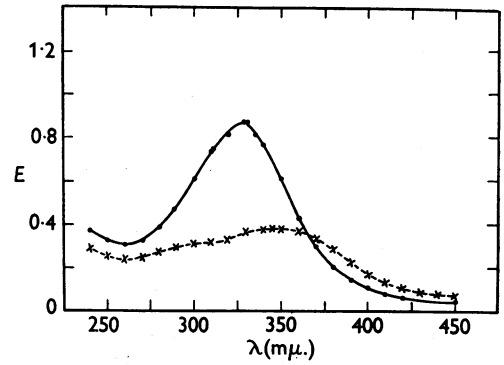


Fig. 5. --×--×--, Retinene after incubation with boiled retinas; ———, after incubation with fresh retinas. Much retinene has been destroyed in the absence of retinene reductase, but with the fresh retinas the absorption curve is mainly due to vitamin A.

The amounts of vitamin A were as follows: (1) 4·0 μg.; (2) 0·5 μg. and (3) 0·8 μg. Approximately 18 μg. of retinene had been added to (1) and (2). Therefore in (1) 18% of the retinene had been converted to vitamin A while 82% was unaccounted for. Fig. 5 shows the absorption curves obtained in another experiment.

DISCUSSION

In favourable circumstances the irrelevant absorption is not changed as a result of irradiation, but in some cases careful comparison of intensities of absorption, particularly on the long-wave side of 650 mμ., indicates a change in the light loss due to scattering.

A decrease in the size or number of colloidal particles would be needed to account for the decreased scattering (cf. Oster, 1948) which in one instance was followed by measuring the change in absorption from 600 to 1000 mμ. The immediate importance of this variable phenomenon is that quantitative interpretation must rest on selected absorption curves where the change in scattering is very small (Table 2).

The position of the absorption maximum of acid indicator yellow at 440 mμ. is well verified within narrow limits, but that of alkaline indicator yellow has not so often been recorded.

The values of the two maxima observed in the present work (440 and 365 mμ.) were in good agreement, whether the indicator yellow came from rat or frog retinas, provided that the pH was definitely on the acid or alkaline side. For more nearly neutral solutions the results were more variable (cf. Table 1), especially for rat preparations.

Now in a simple two-component system with maxima at 440 and 365 mμ., the bands being of the same order of intensity and half-width, there should clearly be an isosbestic point. When samples of the same indicator yellow solution were examined at different pH values no definite isosbestic point could be recorded (cf. Lythgoe, 1937). Further, trial summations of the two curves show that in a two-component system there would be two distinct maxima over a considerable range of mixtures (cf. Fig. 6). In fact, in no case has bleached rhodopsin solution been obtained which had more than one maximum.

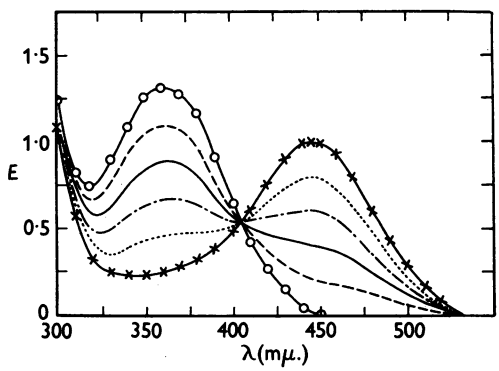


Fig. 6. Theoretical mixture curves for acid and alkaline indicator yellow in various proportion. Note the absence of an intermediate $\lambda_{max.}$ in the summations.

It must therefore be concluded that freshly bleached solutions of rhodopsin contain a third absorbing component, retinene itself being by far the most likely.

Mixtures of retinene ($\lambda_{max.}$ 385 mμ., ethanol) and alkaline indicator yellow ($\lambda_{max.}$ 365 mμ.) will have a single shifting maximum intermediate between 385 and 365 mμ. because the curves overlap more than those of the two forms of indicator yellow. Therefore the position of $\lambda_{max.}$ in many neutral solutions could be accounted for if a mixture of retinene and alkaline indicator yellow were present. Now the extinction of alkaline indicator yellow at 365 mμ. is 1·26–1·43 times that of retinene at 385 mμ. (p. 12). If a neutral bleached rhodopsin solution with $\lambda_{max.}$ between 375 and 385 mμ. is made alkaline, $\lambda_{max.}$ shifts to about 365 mμ. and the intensity of absorption increases, a fact which suggests that alkali forms indicator yellow from retinene.

The solution in which $\lambda_{max.}$ was 417 mμ (Table 1) could have contained a mixture of acid indicator yellow and retinene, but the evidence is inconclusive.

The reactions of retinene throw light on the whole problem. Retinene will only combine with proteins or aliphatic amines, e.g. methylamine (cf. Ball *et al.* 1949) in an alkaline medium and, if the retinene is about 10^{-5} molar, the amine or protein has to be present in large excess ($> 10^{-3}$ molar). In alkaline solutions $\lambda_{max.}$ is at 365 mμ., as for alkaline indicator yellow. If the solution is made acid, $\lambda_{max.}$ becomes 440 mμ., as for acid indicator yellow. If the solution is neutralized, $\lambda_{max.}$ tends towards 385 mμ.; $\lambda_{max.}$ at 440 mμ., would not be obtained unless the neutralized solutions are first made alkaline and then acid.

All these facts are consistent with neutral bleached rhodopsin solutions consisting of a mixture of indicator yellow and retinene.

The fact that $\lambda_{max.}$ of acid indicator yellow gradually shifts from 440 mμ. towards 400 mμ. on standing suggests that acid indicator yellow slowly changes to retinene. Most neutral bleached rhodopsin solutions had $\lambda_{max.}$ at approx. 365 mμ., which suggests that alkaline indicator yellow is formed before and not after free retinene.

The results of extracting bleached rhodopsin with light petroleum become clearer if summarized as follows:

(1) Fresh retinas immediately after bleaching—retinene is extracted readily.

(2) Bleached rhodopsin solutions—retinene is extracted partially (30–50 %).

(3) Bleached retinas after light petroleum extraction—retinene is extracted slowly.

(4) Bleached retinas dried after alum treatment—acid indicator yellow is extracted.

(1) was with frog retinas, (2) with rat, frog and sheep rhodopsin solutions, (3) was with frog retinas and (4) was with haddock retinas. (1) was not possible with rat or bovine retinas unless ethanol was added.

The interspecies differences are not sufficiently well characterized to warrant any conclusions at present, but they seem to be genuine.

Of more immediate importance is that these facts confirm the results of Bliss (1948). In every case so

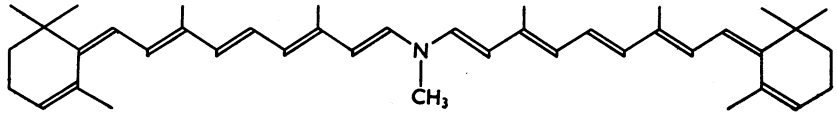
far examined the conversion of indicator yellow to retinene can be observed (or inferred), but never the reverse process. It must again be concluded that indicator yellow is formed first and may then be either wholly or partially converted to retinene.

Aminobenzoic acid reacts readily with free retinene to form a substance with $\lambda_{max.}$ at 535 mμ. The fact that *p*-aminobenzoic acid does not react with bleached rhodopsin solutions is a further indication of the absence of free retinene immediately after bleaching (p. 14).

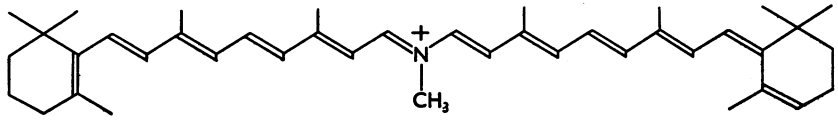
In their work on indicator yellow analogues, Ball *et al.* (1949), have shown that retinene will combine with various amines to form substances which are pH sensitive. With aromatic amines $\lambda_{max.}$ in acid is >490 mμ. and hence natural indicator yellow cannot contain an aromatic nucleus conjugated with the amino group. Ball *et al.* (1949) conclude that, as retinene combines as readily with benzylaniline as with aniline, the resulting compounds must each contain one molecule of retinene and one of amine.

With aliphatic amines or with aromatic amines in which the aromatic ring is not joined directly to the amino group, $\lambda_{max.}$ is at 440 mμ. in acid and at 365 mμ. in alkali. Unlike the aromatic amines aliphatic amines will only react with retinene in an alkaline medium. Dimethylamine does not react but methylamine does, indicating that the most probable reaction is one between two molecules of retinene and one molecule of amine.

The simplest compound containing two molecules of retinene and one of methylamine will be:



[Note break in conjugation (cf. vitamin A_2 with $\lambda_{max.}$ 350 mμ.)] and addition of acid would give:



[Note 'full' conjugation restored (cf. carotene) with $\lambda_{max.}$ 450 mμ.]

The first structure would correspond to alkaline indicator yellow and would be expected to show $\lambda_{max.}$ 365 mμ. The second structure would correspond to acid indicator yellow and $\lambda_{max.}$ at 440 mμ. is consistent with the available spectroscopic data.

If these are truly analogues to indicator yellow its structure must be similar. Instead of having a methyl group attached to the central nitrogen atom the protein will no doubt be attached at that point.

On two occasions it has been reported that acid indicator yellow is soluble in organic solvents. In the present work (p. 13) haddock indicator yellow has been obtained in ethanol, whilst Bliss (1946) obtained a substance with $\lambda_{max} = 460$ mμ. in chloroform. Acid indicator yellow analogue prepared from β-alanine or methylamine is soluble in chloroform and $\lambda_{max.}$ is at 460 mμ. There can be very little doubt that the proposed general structure of indicator yellow is substantially correct. However, if the nitrogen is attached to a protein, it is at present difficult to see how indicator yellow can become detached.

The relationship between the values of $E_{max.}$ for rhodopsin, retinene and indicator yellow may now be discussed. Let ϵ_v, ϵ_a, ϵ_b and ϵ_r stand for molecular extinction coefficients at 500, 440, 370 and 385 mμ. respectively, for rhodopsin ($\lambda_{max.}$ 500 mμ.) acid indicator yellow ($\lambda_{max.}$ 440 mμ.) alkaline indicator yellow ($\lambda_{max.}$ 365–370 mμ.) and retinene ($\lambda_{max.}$ 385 mμ.) where $E = \epsilon cl$, E being extinction ($\log I_0/I$); c, molar concentration and l cell thickness, 1 cm. By 1 g.mol. is meant here $6{\cdot}06 \times 10^{-23}$ chromophoric or prosthetic groups. This definition does not prejudge the question of the number of molecules of coloured component/molecule of carrier protein.

When c and l are kept constant E_v, E_a, E_b and E_r will represent extinctions for rhodopsin, acid and alkaline indicator yellow and retinene respectively, and E_λ, e.g. $E_{500\ m\mu.}$ will represent observed values.

On bleaching an alkaline solution of rhodopsin the maximum at 500 mμ. is replaced by a maximum at 365 mμ. due to indicator yellow. The absorption at 500 mμ. initially may be written $E_{500\ m\mu.} = E_v + d$, where d represents irrelevant absorption at 500 mμ., d is assumed not to change with bleaching by light, so that (at pH 9·3) $E'_{530\ m\mu.} = d$, and $\Delta E_{500\ m\mu.} = E_v$.

Similarly the absorption at 370 mμ. before and after bleaching is written $E_{370\ m\mu.}$ and $E'_{370\ m\mu.}$. The absorption at 370 mμ. due to rhodopsin will be a constant fraction α of E_v, so that $E_{370\ m\mu.} = \alpha E_v + d'$, where d' is the irrelevant absorption. After bleaching (at pH 9·3)

$$E'_{370\ m\mu.} = d' + E_b$$

and

$$\Delta E_{370\ m\mu.} = E_b - \alpha E_v$$

and

$$\Delta E_{370\ m\mu.}/\Delta E_{500\ m\mu.} = (E_b - \alpha E_v)/E_v = E_b/E_v - \alpha.$$

From Table 2 $E_b/E_v = 0{\cdot}75 + \alpha$.

According to our best curve for rhodopsin (Collins & Morton, 1950) $\alpha = 0{\cdot}31$ which agrees well with the ratio 0·29 for the relative photosensitivities of rhodopsin at 365 and 500 mμ. (Schneider, Goodeve & Lythgoe, 1939). Accepting the latter figure

$$E_b/E_v = 1{\cdot}04.$$

Similarly it can be shown that

$$E_a/E_v = 0{\cdot}81.$$

Now let the chromophoric group (i.e. the prosthetic group responsible for the 500 mμ. maximum) of rhodopsin contain p retinene (or vitamin A, i.e. C_{20}) residues, and acid indicator yellow n such residues and alkaline indicator yellow m residues. On bleaching a rhodopsin solution of concentration c_v, the concentration of acid indicator yellow will be pc_v/n or that of alkaline indicator yellow pc_v/m. Assuming complete interconvertibility

$$E_v = \epsilon_v c_v \quad \text{and} \quad E_a = \epsilon_a c_a = \epsilon_a . pc_v/n.$$

Hence $$E_a/E_v = \epsilon_a . p\ c_v/n\epsilon_v c_v = \epsilon_a p/\epsilon_v n. \qquad (1)$$

Also $$E_r = \epsilon_r c_r'$$

(where c' and c are not equal) and

$$E_a = \epsilon_a c_a' = \epsilon_a . c_r'/n,$$

hence $$E_a/E_r = \epsilon_a c_r'/n . \epsilon_r c_r' = \epsilon_a/\epsilon_r . n. \qquad (2)$$

Similarly $$E_b/E_v = \epsilon_b . p/\epsilon_v m \qquad (3)$$

and $$E_b/E_r = \epsilon_b/\epsilon_r . m. \qquad (4)$$

It has been shown that when $E_v = 1{\cdot}00$

$$E_a = 0{\cdot}81 \quad \text{and} \quad E_b = 1{\cdot}04.$$

For the compound formed between β-alanine and retinene, a good analogue of indicator yellow (Ball *et al.* 1949), it has been found that when $E_r = 1{\cdot}00$, $E_a = 1{\cdot}02$ and $E_b = 1{\cdot}25$, and for the product of the interaction of retinene and protein from retinas (p. 12) $E_a = 1{\cdot}0$–$1{\cdot}05$ and $E_b = 1{\cdot}25$–$1{\cdot}43$ when $E_r = 1{\cdot}00$. Finally, ϵ_r in aqueous digitonin solution $= 39{,}200$. Substituting these numerical values in the relevant equations (1) to (4): $\epsilon_a = 40{,}000n$; $\epsilon_v = 49{,}300p$; $\epsilon_b = 49{,}000m$ and $\epsilon_v = 47{,}200p$. The mean value for ϵ_v will be about $48{,}000p$.

As indicated previously there is good reason to believe that $m = n = 2$. Hence $\epsilon_a = 80{,}000$ and $\epsilon_b = 98{,}000$. At present it is not possible to determine p.

From the result of Schneider *et al.* (1939), $\epsilon_v . \gamma$ (γ = quantum efficiency) = 24,000. Therefore

$$48{,}000p . \gamma = 24{,}000 \quad \text{or} \quad p . \gamma = 0{\cdot}5.$$

If $p = 1$, $\gamma = 0{\cdot}5$; if $p = 2$, $\gamma = 0{\cdot}25$, etc. Therefore γ is at most 0·5 and may be 0·25 or even less.

Retinene reductase (*vitamin A dehydrogenase*). There is no doubt that crystalline retinene (prepared from vitamin A, Ball, Goodwin & Morton, 1948) is converted by an enzyme present in the retina to vitamin A. However, before the present work was completed, Wald (1949) showed that the process can be realized *in vitro* by means of coenzyme I, retinene reductase and a suitable substrate such as fructose 1:6-diphosphate. Bliss (1949) has shown that this process is reversible.

Conclusions

The structure of indicator yellow is of vital importance to the structure of rhodopsin. It is clear that although transient orange (Lythgoe & Quilliam, 1938) is the primary product of irradiation, indicator yellow is the first reasonably stable product. The original attachment between carrier protein and chromophoric groups is clearly unbroken and the C-N link of the indicator yellow must also occur in rhodopsin.

The nature of this link is probably the same in the indicator yellow analogue as in indicator yellow itself and by implication, the structure of the chromophore is that suggested on p. 16.

Rigid proof is lacking because the analogues have not yet been obtained crystalline and in any case are very readily hydrolysed.

The broad picture of retinal pigments will remain incomplete, however, until a photosensitive substance showing $\lambda_{max.}$ 500 mμ. can be prepared.

SUMMARY

1. The absorption maximum ($\lambda_{max.}$) of alkaline indicator yellow is 365 mμ. and that of acid indicator yellow is 440 mμ.

2. Retinene is present in varying amounts in bleached rhodopsin solutions and appears to be formed by the hydrolysis of indicator yellow.

3. Structures are proposed for indicator yellow.

4. Under certain conditions acid indicator yellow appears to be partially soluble in ethanol.

5. Relationships between the molecular extinction coefficients of rhodopsin, indicator yellow and retinene are discussed.

6. The relationship of indicator yellow to rhodopsin is discussed.

One of us (F. D. C.) participated in this work as a holder of a New Zealand National Research Scholarship. We are indebted to the Medical Research Council and the Ministry of Food for a grant.

REFERENCES

Ball, S., Collins, F. D., Dalvi, P. D. & Morton, R. A. (1949). *Biochem. J.* **45**, 304.
Ball, S., Collins, F. D., Morton, R. A. & Stubbs, A. L. (1948). *Nature, Lond.*, **161**, 424.
Ball, S., Goodwin, T. W. & Morton, R. A. (1948). *Biochem. J.* **42**, 516.
Bliss, A. F. (1946). *J. gen. Physiol.* **29**, 249.
Bliss, A. F. (1948). *J. biol. Chem.* **176**, 563.
Bliss, A. F. (1949). *Biol. Bull.* **97**, 50.
Collins, F. D. & Morton, R. A. (1950). *Biochem. J.* **47**, 3.
Lythgoe, R. J. (1937). *J. Physiol.* **89**, 331.
Lythgoe, R. J. & Quilliam, J. P. (1938). *J. Physiol.* **94**, 399.
Morton, R. A. & Goodwin, T. W. (1944). *Nature, Lond.*, **153**, 405.
Morton, R. A. & Stubbs, A. L. (1946). *Analyst*, **71**, 348.
Oster, G. (1948). *Chem. Rev.* **43**, 319.
Schneider, E. E., Goodeve, C. F. & Lythgoe, R. J. (1939). *Proc. roy. Soc.* A, **170**, 102.
Wald, G. (1934). *J. gen. Physiol.* **18**, 905.
Wald, G. (1935). *J. gen. Physiol.* **19**, 351.
Wald, G. (1936). *J. gen. Physiol.* **19**, 781.
Wald, G. (1949). *J. gen. Physiol.* **32**, 367.

Studies in Rhodopsin

3. RHODOPSIN AND TRANSIENT ORANGE

BY F. D. COLLINS AND R. A. MORTON
Department of Biochemistry, University of Liverpool

(*Received* 27 *October* 1949)

The existence of transient orange was first established by Lythgoe & Quilliam (1938), who showed that if a rhodopsin solution was irradiated at a temperature near 0°, a comparatively stable orange colour resulted, but if the solution was warmed to room temperature it became much paler (colourless at alkaline pH). The unstable orange substance showed $\lambda_{max.}$ at about 470 mμ. and, on warming, this 'transient orange' changed to 'indicator yellow'. Further work (Broda & Goodeve, 1941) showed that at $-70°$ transient orange was stable and insensitive to light. Lythgoe accordingly formulated the photodecomposition of rhodopsin as follows:

$$\text{rhodopsin} + h\nu \rightarrow \text{transient orange},$$

followed by a thermal process

$$\text{transient orange} \rightarrow \text{indicator yellow}.$$

Lythgoe (1940), in a review of dark adaptation, referred to a possible regeneration of rhodopsin from transient orange, but he did not live to develop his ideas. He also suggested that the regenerated rhodopsin had a maximum differing a little from that of untreated rhodopsin, but did not indicate in which direction the shift occurred.

Chase & Smith (1939) exposed rhodopsin solutions to light of various wavelengths, and with blue light obtained 15% regeneration. The regenerated rhodopsin showed $\lambda_{max.}$ slightly less than 500 mμ., but they were not quite certain that the difference was outside experimental error.

The whole problem seems ripe for reinvestigation.

EXPERIMENTAL

Solutions

Sucrose. 45 g./100 ml. water.

Alum. ($K_2SO_4.Al_2(SO_4)_3.24H_2O$): 4 g./100 ml. water.

Digitonin. A 1% (w/v) solution in water was prepared just before use by boiling the requisite amounts of digitonin and water until all the digitonin had dissolved, and then cooling.

Buffer solutions. McIlvaine's standard buffer solutions were used (Clark, 1928) for the pH range 6–8 and for pH 9·2 a solution of borax ($Na_2B_4O_7.10H_2O$), 19 g./l., was used.

Light petroleum. B.p. 40–60°.

Solid carbon dioxide was used to freeze the solutions, generally in an ethanol bath, which was cooled by the refrigerant to $-70°$.

The absorption spectra were determined by means of the Beckman quartz photoelectric spectrophotometer using a 1 cm. cell.

Preparation of rhodopsin solutions

The procedure was that described by Collins & Morton (1950*a*) and will only be briefly referred to here. All work was done in red light unless otherwise stated.

The retinas from frogs (*Rana temporaria*), rats or bullocks (eyes obtained from an abattoir soon after killing) were shaken with sucrose solution and centrifuged. The supernatant liquid, containing a suspension of rods, was decanted. The suspension was then diluted with water and the rods thrown down by recentrifuging. The rods were then hardened in alum for 2 hr. The alum solution was removed by centrifuging and the rod preparation was then extracted with digitonin solution for 1 hr. The digitonin extract, after centrifuging to remove retinal debris, was then mixed with