The hydrolysis was repeated with a further 340 mg. Na oestriol 'glucuronide'. In this case 79% hydrolysis was indicated and 55 mg. dibenziminazole derivative finally obtained. This was combined with the product of the first experiment before purification.

The dibenziminazole isolated had m.p. 242–243° (decomp.). The mixed m.p. with an authentic sample of the dibenziminazole derivative of D-glucosaccharic acid (m.p. 241–243°, decomp.) was 242–243° (decomp.). The dipicrate and dihydrochloride were prepared as described by Levvy (1948). The dipicrate melted with decomposition at 210° after change of form at 142°; an authentic sample melted at 211° (decomp.), after change of form at 142°, and showed no depression in m.p. after mixture with the material isolated. The dihydrochloride melted at 265–266° and had $[\alpha]_D^{18} + 49.8 \pm 0.6$ in water (c, 2.057). On admixture with an authentic sample (m.p. 266–267°; $[\alpha]_D^{18} + 49.3 \pm 0.6$ in water (c, 2.042)) the m.p. was unchanged. Levvy (1948) found $[\alpha]_D^{18} + 52.3$ in water (c, 2.024) with his preparation of the dihydrochloride, which he showed to be the tetrahydrate. In order to avoid difficulty with varying degrees of hydration in this work, both the dihydrochloride isolated from the urinary uronic acid and the authentic dihydrochloride were recrystallized from water, and dried over CaCl_2 , under identical conditions.

SUMMARY

1. A modified method for the isolation and purification of oestriol monoglucuronidic acid and its sodium salt from human pregnancy urine is described.

2. The sodium salt has been hydrolysed by ox-spleen β -glucuronidase. The uronic acid so liberated has been oxidized to D-glucosaccharic acid which has been identified as its dibenziminazole derivative. It is therefore probable that the uronic acid moiety is D-glucuronic acid.

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Studies on Rhodopsin

1. METHODS OF EXTRACTION AND THE ABSORPTION SPECTRUM

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The history of visual pigments has been reviewed on numerous occasions: Granit (1947) has given a comprehensive and invaluable summary. As the result of the work of R. J. Lythgoe and his co-workers and of G. Wald, our knowledge of rhodopsin has been greatly enlarged. This knowledge can be conveniently summarized in the cycle shown on p. 4, based on that first proposed by Wald (1938).

It has been shown that rhodopsin is responsible for scotopic or twilight vision although not for photopic vision. Retinene has been shown in this laboratory (Morton & Goodwin, 1944) to be vitamin A aldehyde, whilst indicator yellow is now thought to be a compound between retinene and a suitably bound amino group (Ball, Collins, Dalvi & Morton, 1949) of the protein moiety.

However, although so much is now known, it would be hazardous to say that rhodopsin has ever been obtained in a state of purity. It is therefore the purpose of the present paper to consider methods of extraction, the nature of the impurities and the degree of purity actually obtained.

All successful methods for the extraction of rhodopsin involve the use of detergents such as bile salts (Kühne, 1878) or digitonin (Tansley, 1931). In the present work aqueous digitonin has been used to extract the rhodopsin from the retina. Many workers have recommended the use of alum in order to harden the retinas before extraction (Kühne, 1878; Chase & Haig, 1938, etc.). Saito (1938), Krause & Sidwell (1938) and Lythgoe (1937) developed different methods for separating the rod outer segments from the main bulk of the retinal tissue.

In the course of this study several of the above methods have been tried either singly or in combination, and for comparative purposes a working criterion of purity ($=P$) has been arbitrarily defined as the ratio of the extinction at $\lambda_{\min}$, generally near to $400\text{ m}\mu$, to that at $\lambda_{\max}$. (cf. Wald, 1938) near $500\text{ m}\mu$. A large number of preparations of rhodopsin has been obtained and the absorption spectrum measured in each.

Some values of P based on published curves or numerical data for rhodopsin solutions obtained by previous workers, are shown in Table 1.

Table 1. Values for $P_{\lambda_{\min}}$ ($E_{\min}/E_{\max}$) recorded in the absorption curves of rhodopsin and porphyropsin

Species from which retinas were obtained	$P_{\lambda_{\min}}$	Observer
Frog	0.30–0.39	Lythgoe (1937)
Ox	0.31	Krause & Sidwell (1938)
Bullfrog	0.32, 0.54–0.68	Wald (1938)
Rabbit	0.54	Wald (1938)
Various fresh-water fishes	0.50–0.77	Wald (1939)
Bullfrog	0.40–0.57	Chase & Haig (1938)
Wald (1949) gives $P_{\lambda_{\min}}$ 0.26 without stating the species.		

EXPERIMENTAL

Solutions

Potash alum. $\text{K}_2\text{SO}_4 \cdot \text{Al}_2(\text{SO}_4)_3 \cdot 24\text{H}_2\text{O}$, 4% (w/v) in water.

Digitonin solution. 2% (w/v) in water unless otherwise stated; prepared by heating the mixture of digitonin and water to boiling until solution occurs and then quickly cooling.

Sucrose. 45% (w/v) in water.

Buffer solutions. pH 9.3, $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$, 19 g./l.; pH 7, 16.4 ml. of 0.2M- Na_2HPO_4 and 3.53 ml. of 0.1M-citric acid.

Animals

In the course of this work the following animals were employed as sources of rhodopsin: frogs (*Rana temporaria*), rats (from a small animal colony), sheep and cattle (from the local abattoir), pike (*Esox lucius*), char (*Salvelinus willoughbii*), perch (*Perca fluviatilis*) and haddock (*Gadus aeglefinus*).

Apart from sheep and cattle, the animals were dark adapted for at least 3 hr. and often overnight. In the case of sheep and cattle the eyes were obtained soon after slaughter and kept in the dark until dissected.

The dissection and all subsequent operations were carried out in red light. The rats were dissected by the method of Tansley (1931) and the frogs as described by Lythgoe (1937). The eyes of the other animals had an incision made close to the ora terminalis, the junction of the retina and the conjunctiva. The incision was then continued with a pair of scissors all round the ora terminalis, thus enabling the cornea, lens and aqueous humour to be removed. The vitreous humour was next removed, although sometimes the retina adhered to it. In any case, the retina could then be obtained either from the choroid or from the vitreous humour. In some cases the retinas were hardened *in situ* with alum after the removal of the lens and vitreous humour. This was done with the haddock, pike and char.

Extraction

Four methods were used:

'No alum' method. In this case the fresh retinas were extracted with 1 or 2 ml. of 2% digitonin, generally for 1 hr. After extraction with digitonin the mixture was centrifuged strongly, the supernatant liquid containing the rhodopsin.

'Alum' method. This differed from the above in that the retinas were hardened in 4% alum solution for 1 hr. or more. The mixture was then centrifuged and the retinas washed first with 0.9% saline, and then with neutral buffer solution. Finally, the retinas were extracted with digitonin as before.

Lythgoe's method was slightly modified. Instead of an acid buffer, alum solution was used and the extraction with light petroleum omitted.

The 'sugar' method. This was based on the work of Saito (1938). The retinas were shaken up with 45% sucrose solution and centrifuged. The heavy residue consisted of a grey layer of retinal debris and a top layer of black pigment epithelium. The decanted supernatant liquid contained the 'rods' in suspension and they could be thrown down by diluting the sugar solution with three to five volumes of 0.9% saline. The precipitated 'rods' were then washed free from sugar, and centrifuged, hardened in alum

and washed with neutral buffer and finally extracted with digitonin as before. Usually, but not always, the material was left in contact with alum or digitonin for 1 hr. at each stage, the digitonin solution of rhodopsin was generally mixed with an equal volume of a buffer solution and recentrifuged before the absorption spectrum was measured.

Absorption measurements

All measurements were made with a Beckman photoelectric spectrophotometer. A 1 cm. cell was used. The compensating cell contained a solution of digitonin and buffer of the same strength as that used for the rhodopsin solution.

Although rhodopsin solutions are very sensitive to light, little photodecomposition takes place while the absorption measurements are being made. The cells were filled in the dark room (red light) and placed in the cell holder, which was carried under a black cloth to the instrument and quickly placed in position in the light-tight cell space.

RESULTS

Influence of the method of extraction on the optical properties of rhodopsin solutions

Twenty-three solutions of frog rhodopsin were obtained by one or other of the four methods of extraction described above. The mean value of $P_{\lambda \text{ min.}}$ is shown below for each of the four methods (Table 2).

Table 2. Values of $P_{\lambda \text{ min.}}$ for rhodopsin solutions

Animal	Extraction procedure	$P_{\lambda \text{ min.}}$	No. of samples
(a) Frogs	'No alum' method	0.87	3
	Lythgoe method	0.603	3
	'Alum' method	0.517	6
	'Sugar' method	0.360	11
(b) Frogs	'Summer' Frogs	0.394	7
	'Spring' Frogs	0.30	4
	'Alum' method	0.64	8
(c) Rats	'Sugar' method	0.48	8

For a probability of 0.05 the following differences in $P_{\lambda \text{ min.}}$ would be significant; (a) 0.109, (b) 0.089, (c) 0.096.

Each method of extraction gives, on the average, a different degree of optical purity except that the 'alum' and the Lythgoe methods are much the same. The results using the 'sugar' method can be subdivided by considering the 'spring' frogs separately from the 'summer' frogs. (Inverted commas are used to indicate that the difference may not be due entirely to a seasonal change in frog retinas.)

Sixteen rat-rhodopsin solutions were obtained using the 'alum' and 'sugar' methods. The 'no alum' method gave solutions so impure that the ratio $E_{\lambda \text{ min.}}/E_{\lambda \text{ max.}}$ lacked meaning. In addition the presence of appreciable amounts of haemoglobin and impurities giving rise to turbidity emphasized the disadvantage of not using alum.

Haemoglobin was occasionally present in the 'alum' solutions, but never in the 'sugar' solutions.

For extracts made from retinas of sheep and cattle, the major difference was between the sheep rhodopsin using the 'alum' method and the bovine rhodopsin using the 'sugar' method. Bovine eyes had been tried previously, but the presence of large amounts of haemoglobin in the retina gave rise to inferior preparations. However, with the 'sugar' method, solutions of rhodopsin were obtained free from haemoglobin. With sheep retinas, solutions free from haemoglobin were only occasionally obtained, using the 'alum' method. The mean value of the ratio $P_{\lambda \text{ min.}}$ was 0.51 for bovine rhodopsin ('sugar' method, two samples) and 0.664 for sheep rhodopsin ('alum' method, 12 samples).

The extraction of rhodopsin (or porphyropsin) from fish retinas proved to be difficult. With pike, haddock, and perch, the absorption curves exhibited much irrelevant absorption, but better results were obtained later with char.

Yield of rhodopsin and method of estimation

The amount of absorption due to rhodopsin at $\lambda_{\text{max.}}$ can be calculated in two ways.

Method 1. A correction procedure for rhodopsin can be obtained analogously to that developed by Morton & Stubbs (1946) for vitamin A. They showed that if the irrelevant absorption is a linear function of the wavelength the following formula for the corrected extinction can be deduced by geometrical reasoning:

$$E_{\text{corr.}} = \frac{(\lambda_3 - \lambda_1)E_2 - (\lambda_3 - \lambda_2)E_1 - (\lambda_2 - \lambda_1)E_3}{(\lambda_3 - \lambda_1)\alpha_2 - (\lambda_3 - \lambda_2)\alpha_1 - (\lambda_2 - \lambda_1)\alpha_3},$$

where E_2 is the extinction at wavelength λ_2 (near to $\lambda_{\text{max.}}$) and E_1 and E_3 are the extinctions at wavelengths λ_1 and λ_3 respectively (on either side of $\lambda_{\text{max.}}$). α_1 , α_2 and α_3 are constants depending on the shape of the absorption spectrum of the pure substance.

For rhodopsin $\lambda_1 = 480$, $\lambda_2 = 500$ and $\lambda_3 = 520 \text{ m}\mu$. were chosen as suitable values. Substituting in the above formula, one obtains:

$$E_{\text{corr.}} = \frac{2E_2 - E_1 - E_3}{2\alpha_2 - \alpha_1 - \alpha_3} = K(2E_2 - E_1 - E_3),$$

where $K = \frac{1}{2\alpha_2 - \alpha_1 - \alpha_3}$ and is a constant. K can be

estimated by using method 2 where applicable.

Method 2. If the rhodopsin solution is alkaline (about pH 9) then the difference in the extinction at $500 \text{ m}\mu$, before and after bleaching, is equal to the extinction due to rhodopsin.

If it is assumed that the absorption spectrum of rhodopsin is unaffected by the pH, the value of K obtained in alkaline solutions will apply to all solutions.

$$K \text{ (for frog rhodopsin)} = 4.86 \pm 0.09,$$

$$K \text{ (for rat rhodopsin)} = 4.59 \pm 0.06.$$

Table 3. Relationship between the yield of rhodopsin/sheep retina and the volume of digitonin solution used

Average number ($\bar{n}$) of retinas ex- tracted/ml. of digitonin solution (v = volume used, $n/v = c$)	Yield of rhodopsin $E_{1\text{ cm.}/c}$ (E at 500 m μ ., 1 cm. cell)	No. of separate experiments
6.7	0.011	2
5.3	0.012	3
4.7	0.016	3
4.0	0.021	4
3.2	0.047	3
2.4	0.134	6
1.6	0.101	19
1.3	0.130	10
0.6	0.164	1

Table 4. Mean yields of rhodopsin using retinas from the different species under comparable conditions of extraction

Species	Mean yield $E_{1\text{ cm.}/c}$ (E at 500 m μ ., 1 cm. cell)	Standard deviation	No. of separate experiments
Frog	0.034	0.015	19
Toad	0.015	—	1
Guinea pig	0.003	—	1
Sheep	0.116	0.036	36
Rat	0.021	0.008	16
Goat	0.26	—	1
Ox	0.175	—	1

An absorption spectrum of pure frog rhodopsin

The 'purest' solution of rhodopsin (i.e. the one showing the least irrelevant absorption) so far obtained had $P_{\lambda \text{ min.}} = 0.24$. The absorption at

Fig. 1. — Absorption spectrum of the 'purest' frog rhodopsin so far obtained. $\odot$, points from Granit's (1947) cat scotopic dominator curve. $\times$, relative photochemical efficiencies [$(\epsilon_{\lambda} \times \gamma_{\lambda})/(\epsilon_{500\text{ m}\mu} \times \gamma_{500\text{ m}\mu})$] after Schneider, Goodeve & Lythgoe (1939)].

700 m μ . was 0.003 and this quantity has been subtracted from the values at all the other wavelengths. This, of course, is an underestimation of the absorption due to the scattering of light (p. 8), but the true value will not be less than 0.003. The re-

sulting absorption spectrum is shown in Fig. 1 with the extinctions at all wavelengths expressed as a fraction of that at $\lambda_{\text{max.}}$ (500 m μ .).

Another solution which was unusually pure was char (*Salvelinus willoughbii*) 'rhodopsin', $\lambda_{\text{max.}}$ 514 m μ . and $P_{\lambda \text{ min.}} = 0.23$. (This species yields a mixture of porphyropsin and rhodopsin.)

Wavelength of maximum absorption in rhodopsin solutions

Scattering of light is appreciable in rhodopsin solutions; they are in fact often a little turbid. By assuming the applicability of Rayleigh's law (light scattering $E_{\lambda} = c/\lambda^4$) and giving to c a series of hypothetical values, a range of scattering curves can be obtained. Each of these can be superimposed on the experimental curve in which $P_{\lambda \text{ min.}}$ is least.

Fig. 2. Comparison of $\lambda_{\text{max.}}$ for frog and rat rhodopsin. $\times$, rat. $\circ$, frog.

The results will be curves corresponding with 'impure' rhodopsin solutions in which the 'impurities' are responsible for light scattering conforming to the Rayleigh Law. Inspection of such curves, shows that if in the summation curves $P_{\lambda \text{ min.}}$ is < 0.32 , the position of $\lambda_{\text{max.}}$ is not appreciably different from that observed for the best preparations. When $P_{\lambda \text{ min.}}$ is > 0.33 but < 0.45 the displacement is about 1 m μ .; if $P_{\lambda \text{ min.}}$ is > 0.45 but < 0.55 , it is 2 m μ . and when the ratio is > 0.56 but < 0.63 it is 3 m μ .

Table 5. Positions of $\lambda_{\max}$ for rhodopsin preparations after correcting for the effect of scattering

	$P_{\lambda \min.}$	$\lambda_{\max.}$ observed (m μ .)	$\lambda_{\max.}$ corr. (m μ .)
Frog	0.40	502	503
	0.38	502	503
	0.32	502	502
	0.24	503	503
	0.35	501	502
	0.32	502	502
	0.36	502	503
	Mean	502	502.6 $\pm$ 0.5
Rat	0.54	496	498
	0.49	496	498
	0.46	496	498
	0.49	497	499
	0.39	497	498
	0.41	497	498
	0.63	495	498
	0.54	496	498
	0.46	497	499
	Mean	496.5	498.2 $\pm$ 0.4
Ox	0.49	498	500
	0.50	487	499
Sheep	0.38	498	499
	0.42	498	499
Haddock	0.93	approx. 500	—
Char	0.23	514	514
	0.49	512	514
Pike	0.86	approx. 525	—
Perch	0.82	524	—
	0.74	528	—
	0.83	520	—

— Means that correction for irrelevant absorption is of doubtful value.

Ultraviolet absorption shown by rhodopsin solutions

As rhodopsin is a conjugated protein, the observed ultraviolet absorption will be a summation of three contributions: (i) that due to the prosthetic grouping, (ii) that due to selectively absorbing amino-acid residues in the carrier protein, and (iii) that due to impurities including proteins other than rhodopsin.

All preparations show selective absorption in the region 260–280 m μ ., but those which exhibit low values for $P_{\lambda \min.}$ tend to have the lowest intensity of absorption in the ultraviolet region.

If the absorption spectrum of a pure protein, excluding nucleoproteins, is considered, the curve is found to agree very well with a summation of contributions due to the amounts of tyrosine, tryptophan and phenylalanine, found to be present by ordinary methods of analysis of protein hydrolysates.

The ultraviolet absorption maximum shown by the best rhodopsin preparations occurs near 270 m μ . (pH 9) and a preliminary consideration of its origin is worth while.

An approximate estimate of the absorption due to tyrosine and tryptophan was made in the following rather circuitous manner. The absorption due to rhodopsin at 500 m μ . was determined by the three-point fixation formula. By using the absorption spectrum of the purest rhodopsin solution so far obtained, the absorption at 360 m μ . due to rhodopsin could be calculated. This wavelength was chosen as probably the shortest wavelength in the standard curve at which the irrelevant absorption was negligible. By subtracting the absorption shown by the best rhodopsin at 360 m μ . from the total absorption, the amount of the irrelevant absorption at that wavelength could be obtained. By assuming that the irrelevant absorption was due to scattered light and obeyed Rayleigh's Law, the irrelevant absorption at 290 and 280 m μ . could then be calculated. If it be assumed that the only absorption at 280 m μ . and 290 m μ . is that due to tyrosine, tryptophan and irrelevant contaminants, then the amounts of tyrosine and tryptophan can be calculated according to the procedure of Goodwin & Morton (1946). Their data have been modified so as to apply to the wavelengths 290 and 280 m μ . and at a pH of 9.3.

E_{280} is the extinction at 280 m μ . after subtracting the irrelevant absorption, and similarly for E_{290} . If x is the extinction at 280 m μ . due to tyrosine and y is

Table 6. A trial analysis of the ultraviolet absorption band of rhodopsin solutions

$P_{\lambda \min.}$ (approx. 400 m μ .)	$\lambda_{\max.}$ ultraviolet region (m μ .)	* $P_{\lambda \max.}$ (approx. 270 m μ .)	$P_{280 \text{ m}\mu.}$ (corrected for irrelevant absorption)	$P_{280 \text{ m}\mu.}$ (due to tyrosine)	$P_{280 \text{ m}\mu.}$ (due to tryptophan)
0.32	277	3.7	3.4	1.6	1.8
0.37	272	5.7	4.5	2.3	2.2
0.46	269	5.8	4.8	2.2	2.6
0.52	267	21.4	13.3	8.5	4.8
0.56	267, ~280	14.3	10.2	7.2	2.9
0.66	265	8.6	6.9	5.1	1.8
0.70	270	9.2	7.2	4.3	2.9
0.73	266	24.6	19.4	10.9	8.5
0.77	270	6.3	4.4	1.5	2.9
0.79	275	22.8	20.7	11.9	8.8
0.92	~272	11.7	11.3	3.9	7.4

* i.e. $E_{270 \text{ m}\mu.}/E_{500 \text{ m}\mu.}$

~ denotes inflexion.

the extinction at 280 m μ . due to tryptophan then the following formulae hold at pH 9.3.

$$E_{280} = x + y,$$

$$E_{290} = 0.442x + 0.791y.$$

Further, knowing x and y and the full absorption curves of tyrosine and tryptophan at pH 9.3 it is possible to calculate the absorption due to those two substances at any other wavelength. If the absorption so calculated as due to the tyrosine and tryptophan content of the solution, is subtracted from the total absorption a difference curve is obtained. This curve always had an inflexion or maximum at about 260 m μ . (Fig. 3).

Fig. 3. Attempted analysis of rhodopsin absorption in the region 240–300 m μ . O—O—O—O, observed curve;, calculated irrelevant absorption assumed due to scattering; x—x—x—x, difference curve after subtracting absorption attributable to tyrosine and tryptophan (note 260 m μ . maximum).

Absorption due to 'impurities' generally

This absorption, down to about 260 m μ ., could be calculated as follows: the corrected absorption at 500 m μ . due to rhodopsin could be calculated by the three-point correction procedure and then by using the data for the purest rhodopsin the absorption due to rhodopsin at any particular wavelength could be calculated. When this was subtracted from the original absorption the spectrum of the impurities could be obtained (Fig. 4).

DISCUSSION

In 1942 Hecht had stated that the rhodopsin solutions obtained by Wald (1938), Lythgoe (1937), Krause & Sidwell (1938), Saito (1938) and Chase & Haig (1938), were substantially pure and that the absorption spectrum had been adequately characterized. One of the purposes of the present paper is to show that pure rhodopsin has not yet been

Fig. 4. Example of irrelevant absorption in different frog rhodopsin preparations obtained by subtracting the 'best' curve from the observed curves. (Scale displaced to avoid overlapping.)

obtained. The ratio $P_{\lambda \text{ min.}}$ for the purest solution previously obtained was 0.30 (Lythgoe, 1937), but in the present work a value of 0.24 was obtained.

It is evident from the results obtained with different methods of extraction, that the sugar method for separating the outer segments of the rods, followed by hardening with alum, is the best method. Even then, however, the value obtained for $P_{\lambda \text{ min.}}$ is very variable. The poor results obtained with Lythgoe's (1937) method may be due to insufficient experience of it, but the fact that superior results were obtained with the sugar method under comparable conditions confirms the efficacy of the latter. Bliss (1948) has independently used a similar method of extraction for cephalopsin.

The unexplained difference between the 'spring' and 'summer' frogs and the great variability in the purity of successive preparations, obtained by the same method, demonstrates that there is some factor which has so far eluded control.

The porphyropsin and rhodopsin solutions obtained from fish were, with the exception of char, very impure. This seemed to be due to the large amounts of melanin associated with the pigment epithelia which were very often difficult if not impossible to separate from the retina.

The influence of the ratio of the volume of digitonin solution to the volume of retinal tissue being ex-

tracted is interesting. It can be shown that for a given volume of retinal tissue there is a minimum volume of digitonin below which the extraction is not quantitative. One of the chief advantages of the sugar method is that the volume of tissue is reduced to a very small amount and better extraction occurs.

An examination of the results presented above enables some conclusions to be drawn concerning the nature of the impurities. The absorption curves shown in Fig. 4 represent an approximation to that of the 'impurities'. The effect of colloidal particles in scattering the light is shown by the gradual rise in absorption from 700 m μ . to the ultraviolet. This portion of the absorption curve does not obey Rayleigh's Law exactly, but the divergence is not great. From this it can be concluded that the particles are not greatly different in size from the wavelength of visible light (0.4–0.7 μ .) (cf. Oster, 1948).

However, below about 400 m μ . the smooth curve due to the impurities is broken by an abrupt increase. This must be due to specific absorption and will correspond to the 'yellow impurities' of Chase & Haig (1938) and others.

Although the figures in Table 6 are only approximate, three facts emerge clearly: (1) the ratio of the intensity of the protein absorption to that due to rhodopsin is very variable and strongly suggests that some of the 'impurity' is protein in nature. (2) An approximate estimate of tyrosine and tryptophan content, calculated as a percentage of the rhodopsin molecule, can be obtained as follows.

$E_{1\text{ cm.}}^{1\%}$ (500 m μ .) for rhodopsin is about 6.6 (calculated from the data of Broda, Goodeve & Lythgoe (1940)).

$E_{1\text{ cm.}}^{1\%}$ (280 m μ .) for tyrosine (pH 9.3) is 78 and for tryptophan 262.

Hence % tyrosine = $\frac{P_{280} \text{ (due to tyrosine)}}{78} \times \frac{6.6}{1.0} \times \frac{100}{1}$
and

% tryptophan = $\frac{P_{280} \text{ (due to tryptophan)}}{262} \times \frac{6.6}{1.0} \times \frac{100}{1}$.

The rhodopsin in the solution with $P_{\lambda \text{ min.}} = 0.32$ appeared to have 14 % tyrosine and 5 % tryptophan. In contrast the rhodopsin in the solution with $P_{\lambda \text{ min.}} = 0.79$ appeared to have 102 % tyrosine and 22 % tryptophan, which simply means that the solution is grossly impure. As most proteins have less than 5 % tryptophan and not much more tyrosine this is very strong evidence for the presence of protein impurities or large amounts of tyrosine-containing material. (3) The absorption at 270 m μ . cannot satisfactorily be accounted for in terms of tyrosine and tryptophan. The band at 260 m μ . which is deduced could be due to purines and/or pyrimidines, perhaps as nucleic acids.

From these results and from previous work the following conclusion can be drawn concerning the

nature of the impurities. Colloidal particles cause general absorption, approximately obeying Rayleigh's Law and 'yellow impurities' cause absorption below 400 m μ . This absorption may be due to phospholipin breakdown products (Broda, 1941, and Ishimoto & Wald, 1946), protein (including nucleoprotein) and melanin. This last is rendered likely by the apparently large amounts of tyrosine present and by the fact that melanin is found in neighbouring tissues such as the pigment epithelium. There is good evidence that all three of these impurities are present but it is doubtful whether phospholipins, unless oxidized, would contribute any specific absorption.

A criterion for pure rhodopsin must be that the shape of the absorption spectrum is unaltered in any wavelength region by further 'purification'. As some of the impurities may be transparent it will be necessary to employ other criteria in addition to the spectroscopic one.

Although pure rhodopsin has not yet been obtained some deductions may be made concerning it. The ratio $P_{\lambda \text{ min.}}$ is not greater than 0.24 and a lower limit can be set in two ways. If the prosthetic group of rhodopsin contains vitamin A or retinene, then a resemblance in absorption spectrum should be apparent. Both pure vitamin A and pure retinene have a minimum and the ratio $P_{\lambda \text{ min.}}$ is 0.20 for vitamin A and 0.24 for retinene dissolved in digitonin solution. Goodeve, Lythgoe & Schneider (1942) showed that the ratio of the photosensitivity at 400 m μ . to that at 500 m μ . for rhodopsin was 0.22. The photosensitivity is the product of the molecular extinction coefficient and of the quantum efficiency and hence this ratio should correspond to $P_{\lambda \text{ min.}}$. Therefore 0.20 can be taken as the lower limit. This suggests that our purest rhodopsin solution is not far from being 'optically' pure from 400–500 m μ . In Fig. 1 it is compared to Granit's (1947) scotopic luminosity curve and with the photosensitivity curve of Goodeve *et al.* (1942).

It is proposed in a further communication to consider the absorption in the region 310–390 m μ . which decreases considerably as the protein absorption at 270 m μ . is reduced. The purer the solution the more apparent is the inflexion at 340 m μ . in the rhodopsin spectrum.

The fact that $\lambda_{\text{max.}}$ varies from species to species is not entirely unexpected (cf. haemoglobin). The difference is small but quite definite; 503 m μ . for frogs to 498 m μ . for rats.

Conclusions

The approach to pure rhodopsin will have to be made in stages. The first stage is clearly to obtain a reproducible absorption curve over the region 400–700 m μ . This has been very satisfactorily attained since there is good agreement between the electrophysiological, photochemical and spectro-

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 m μ ., and a third stage to establish a constant value between the absorption at 250–280 m μ . due to the carrier protein, and the absorption at 500 m μ .

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 m μ . 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 m μ ., for rat 498 m μ . and sheep and ox at 500 ± 1 m μ . Char 'rhodopsin' had $\lambda_{\max}$ at 514 m μ . Perch and pike porphyropsin had $\lambda_{\max}$ at 520–530 m μ ., haddock rhodopsin at approx. 500 m μ .

6. A solution of frog rhodopsin has been obtained with the extinction ratio $E_{\min}/E_{\max} = 0.24$ and hence is considerably purer than anything so far reported.

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Studies on Rhodopsin

2. INDICATOR YELLOW

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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 m μ .) 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