Studies in Rhodopsin

7. REGENERATION OF RHODOPSIN BY COMMUNUTED OX RETINA

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(Received 16 July 1953)

In a previous paper (Collins, Green & Morton, 1953) the regeneration of rhodopsin by eye tissue from frog and rat was studied. It was found that good regeneration *in vitro* could be obtained in a period not very different from that required for dark adaptation in man. The work using frog or rat eyes was rendered difficult and tedious because the absolute amounts of rhodopsin are quite small. It was expected that the use of ox retinas would result in more rapid progress because they are plentiful and because of their size contain at least 20 times as much rhodopsin as frog or rat retinas. Many experimental difficulties were encountered, and it was some time before a reasonable degree of regeneration was obtained with ox retina.

EXPERIMENTAL

Materials

Vitamin-A solution. Crystalline, synthetic vitamin-A alcohol or acetate (Hoffmann-La Roche), was used. The acetate was first saponified before use by *N*-KOH in ethanol at 53° for 15 min. (see Isler, Roneo, Guex, Hindley, Huber, Dialer & Koffer, 1949). The vitamin-A alcohol was extracted with light petroleum; this solution, after being washed with water, was evaporated to dryness and a solution of Tween 80 (polyoxyethylene derivative of sorbitan mono-oleate; Honeywill and Stein Ltd.) in CHCl_3 added. (In the earlier trials the detergent Dispersol A was used unsuccessfully. Tween 80 permits a greater concentration of dispersed vitamin A.) The CHCl_3 was removed *in vacuo* and the required volume of water was then added. These operations were carried out in red or dim light and the solution was stored at -78° until required, but even with these precautions it was desirable to prepare fresh solutions frequently. To determine the concentration of vitamin A in such aqueous dispersions, the method described by Collins *et al.* (1953) was used.

The adenosine triphosphate (ATP), diphosphopyridine nucleotide (DPN), cytochrome *c*, digitonin solution, buffer solution and potash alum solutions were those described by Collins *et al.* (1953).

Pyridoxal phosphate. This was supplied as the calcium salt by Roche Products Ltd.

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METHODS

Preparation of eye tissues. Ox eyes from the local abattoir were transported in light-proof containers and packed with ice. In the laboratory, all work, unless otherwise specified, was carried out in red light. The cornea, lens and vitreous humour were removed from the eye, and then the retina. In some early experiments, whole retinas were used, but the usual procedure was as follows: the retinas were comminuted (using an effective type of homogenizer described by Brendler, 1951) at 0° in whatever medium was to be employed in the subsequent experiment, but this did not include vitamin A. The volume of medium used was 1.5 ml./retina and 2 ml. samples were then taken for the experiment.

Two controls were used in every experiment. (i) Alum was added immediately to a sample (2 ml.), which was then stored in a light-proof tin until required. This sample was used to determine the total rhodopsin present. (ii) Another sample (2 ml.) was exposed to a Photoflood light (Ediswan 82, 230 v) for 1 min. at 6 in. distance, alum was added and the sample was stored until required. This sample was used to determine the trace of rhodopsin remaining after irradiation. (iii) The remainder of the solution was used (as 2 ml. samples) for regeneration experiments. The solution was exposed to a Photoflood lamp for 1 min. and then incubated at 37° for 2 or 3 hr. Vitamin-A or retinene solution was added (either just before irradiation or after the sample was returned to the red light) in sufficient quantity (0.2 ml.) to bring the final concentration to about 8×10^{-4} M.

After the period of incubation, alum was added to all the samples; after 1 hr. the mixtures were centrifuged. The sedimented material was washed with saline solution (0.9% NaCl in water) and re-centrifuged. Then 2 ml. of 1% aqueous digitonin were added and left to extract for 1 hr. The mixture was then centrifuged and the supernatant solution examined in a spectrophotometer (in a 1 cm. cell). The solution was then exposed to a Photoflood lamp for 1 min. and the absorption remeasured.

In many experiments the absorption spectra were measured in the presence of 0.5 M hydroxylamine (Wald & Brown, 1950).

The yield of rhodopsin was given by the difference in the extinction at 500 m μ . before and after exposure to light. A further check could be obtained in the following manner: The absorption between 440 and 600 m μ . consists of that due to rhodopsin, together with irrelevant absorption, the latter largely a result of the solutions being turbid. A plot of $\log E$ against $\log \lambda$ for bleached rhodopsin solutions is very nearly a straight line, which is characteristic of turbid solutions. In this spectral region, the absorption due to

retinene follows the same relation. The procedure due to Fog (1952) was therefore applied. The three wavelengths chosen were 430, 500 and 582 m μ ., because $\sqrt{(430 \times 582)} = 500$. The general correction equation given by Fog (1952) for any solute with a distinct absorption band distorted by light scattered from suspended matter simplifies for these wavelengths to

$$E_{\text{corr.}} = 1.215E_{500 \text{ m}\mu} - \sqrt{(E_{430 \text{ m}\mu} \times E_{582 \text{ m}\mu})}.$$

The value of $E_{\text{corr.}}$ was usually in good agreement with the value obtained by difference.

The amount of regeneration was often simply expressed as a percentage of the total rhodopsin. However, as both the total rhodopsin and the residual rhodopsin varied somewhat, to facilitate comparison, the yield was more often expressed as 'net percentage regeneration' where

$$\text{percentage regeneration} = \frac{A - B}{T - B} \times 100,$$

and A is the extinction at 500 m μ . due to rhodopsin after regeneration, B is the extinction at 500 m μ . due to the residual rhodopsin after irradiation and storage in alum, and T is the extinction at 500 m μ . due to the total rhodopsin present at the beginning of the experiment.

RESULTS

Preliminary experiments

For a long time it was not here possible to obtain any regeneration with ox-eye tissues. Many different techniques were tried, but without success. At first it was thought that the interval between killing and the use of the eyes at the laboratory was too long, but failures still resulted even when the eyes were kept at 0° until arrival, and with the use of a 'complete' medium (see Collins *et al.* 1953) containing vitamin A.

At this stage it was thought worth while to ascertain whether ox retina was capable of phosphorylation and thus able to effect synthesis.

Cattle retina, kept at 0° until incubated at 37° with a 'complete' medium containing inorganic phosphate labelled with ^{32}P , incorporated the isotope into both ATP and other organic compounds.

In this experiment, the mixture which had been incubated was deproteinized with trichloroacetic acid. The inorganic phosphate was extracted from the aqueous extract by *isobutanol* (5 ml.) after the addition of ammonium molybdate (10%, w/v, 0.5 ml.) and 10N sulphuric acid (0.5 ml.). The extraction took 15 sec. and further extractions (after addition of inorganic phosphate as carrier) resulted in the complete removal of inorganic phosphate and all radioactivity associated with it. The solution was then hydrolysed for 20 min. at 100° and the inorganic phosphate formed on hydrolysis was extracted in the way described above. In two experiments, the easily hydrolysed phosphate (ATP) corresponded with 9 and 5 μg . labile P/ml. of reaction mixture having 730 and 1050 counts/min./ml., respectively. The aqueous phase, which con-

tained the less labile, organically bound phosphate, gave counts/min./ml. of 3150 and 3450, respectively, indicating that 3–4 times as much phosphorus was held in this manner as compared with the phosphorus of ATP. The method outlined here has been independently developed by other workers (see Ennor & Stocken, 1950).

These results indicated clearly that the retina could sustain synthetic processes.

From that point Tween 80 was substituted for the detergent Dispersol A which had been used initially.

Regeneration of rhodopsin using vitamin A

At first the experiments were carried out with retina and choroid and a 'complete' medium. It was found that better results were obtained if choroid was not included. This is quite different from our experience with rat-eye tissue, where the choroid and pigment epithelium is essential for optimum results. (Yield without choroid, 0.006 unit; yield with choroid, 0.019 unit.)

The next stage was to test whether the components of the 'complete' medium were all necessary. In experiments in which ATP was excluded, no fall in the amount of regeneration was observed. Further experiments showed that only vitamin A and phosphate buffer were necessary.

Fig. 1 shows the effect of varying the concentration of vitamin A in the final mixture. The optimum concentration is about $7-9 \times 10^{-4}\text{M}$, and $20 \times 10^{-4}\text{M}$ gave no increased yield of rhodopsin. Collins, Love & Morton (1952) have shown that isolated rod outer segments from ox contain 22.6 mg. of vitamin A

Fig. 1. Regeneration of rhodopsin from ox retina. Regeneration is expressed as a percentage of the initial rhodopsin concentration measured on a separate portion of retinal suspension. The medium was 'complete' (see text) but the vitamin-A concentration was allowed to vary. Regeneration (60%) became independent of concentration of vitamin A above $8 \times 10^{-4}\text{M}$; (⊗) and (×) refer to separate experiments.

(derived from rhodopsin)/100 g. of wet tissue. If it is assumed that 100 g. wet tissue have a volume of 100 ml., the 22.6 mg./100 g. are equivalent to a 7.9×10^{-4} M solution of vitamin A. Vitamin-A acetate itself supported regeneration.

The effect of pH was investigated, but as the yield of rhodopsin after regeneration is variable (see below), it proved difficult to obtain a satisfactory curve showing the dependence of the extent of regeneration on pH. However, at pH's less than 7 and greater than 7.6, the yield was much less than in the region 7-7.5.

Fig. 2 shows the rate of regeneration. The average amount of regeneration for twenty-eight samples after 2 hr. incubation was 62%. The results obtained varied from 48 to 100%. The figure shows the variable nature of the results, both as to the rate of and the extent of regeneration. This suggests that there must be unidentified factors influencing the results.

The work with frog and rat eyes (Collins *et al.* 1953) showed that regeneration was poor if the eye tissues were not used as soon as possible after the death of the animal, even though a 'complete' medium had been used. Table 1 shows the effect of the lapse of time between comminution and the beginning of incubation. As can be seen, the decrease in the amount of regeneration can be counteracted by DPN. As there was usually a very short interval between comminution and the start of incubation, the addition of DPN was not usually necessary. It was only added when it was desired to take every care that conditions should be optimum.

Table 2 shows the effect of pyridoxal phosphate; in every case its use resulted in an increase in the regeneration of rhodopsin. DPN was added so that its concentration should not be limiting. In all the above experiments the vitamin A was added after the retinal homogenate had been exposed to light and had been returned to a red light, hence regeneration was obtained using *all-trans*-vitamin A.

Regeneration of rhodopsin with retinene

Wald & Brown (1950) obtained good regeneration by flooding a bleached rhodopsin solution with retinene. When this experiment was repeated in this laboratory, no regeneration could be obtained. Subsequent work by Hubbard & Wald (1952*a, b*) showed that regeneration from retinene, under the conditions used in their experiments, could only be obtained when the retinene had either been prepared from natural vitamin A or had been exposed to light. The retinene used in this laboratory had been prepared from crystalline, synthetic vitamin-A acetate, and was not unduly exposed to light, although the light was not rigidly excluded as in later experiments.

Fig. 2. Regeneration of rhodopsin in relation to time of incubation. Four experiments are shown with ox retina in a medium containing 0.034 M phosphate buffer, pH 7.4, and vitamin A. ○○, 7×10^{-4} M; × ×, 7×10^{-4} M; + +, 8×10^{-4} M; ●●, 4×10^{-4} M. Other experiments in which the final vitamin-A concentration was $8 (\pm 1) \times 10^{-4}$ M gave regenerations after 2 hr. incubation not exceeding 60%. The overall average for a series of twenty-eight experiments (2 hr. incubation, final vitamin-A concentration always 4×10^{-4} M) was 62%. The experiment '○○' (above) shows much better results, indicating that the apparent limit of about 60% is due to some unexplained inhibition.

Table 1. *The influence of the freshness of the tissue on the regeneration of rhodopsin*

(Reaction mixture 7×10^{-4} M vitamin-A alcohol, 0.02 M phosphate buffer, pH 7.4, and retinal suspension as described in the Experimental section.)

Time elapsing after comminution and the start of incubation (min.)	Expt. no.		
	1	2	3*
	Net regeneration (%)		
0	62	72	45†
30	44	44	57.5
60	34	30	37.5
90	—	17	52.5
120	—	—	57.5

* In this expt. 2.5×10^{-5} M DPN was present.

† This low figure is anomalous.

Table 2. *The effect of pyridoxal phosphate on the regeneration of rhodopsin*

(Reaction mixture contained 5×10^{-4} M vitamin-A alcohol, 0.034 M phosphate buffer, pH 7.4; 0.02 M potassium L-glutamate and 2.5×10^{-5} M DPN. When added, the pyridoxal phosphate was 2×10^{-3} M.)

Conditions	Expt. no.			
	1	2	3	4
	Net regeneration (%)			
Without pyridoxal phosphate	32	23	23	23
With pyridoxal phosphate	46	43	43	50

When these results of Wald and his co-workers were published, it became desirable to investigate the effect of light on the present experiments. Table 3 shows that about the same amount of regeneration was obtained when either vitamin A or retinene was used. When either vitamin A or retinene was exposed to light before incubation, the amount of regeneration was increased. Natural vitamin A gave the same amount of regeneration as synthetic *all-trans*-vitamin A.

The experiments recorded in Table 4 were intended to test whether the regeneration occurring in whole retinal suspensions could be reproduced using only the solid portion obtained by centrifuging and washing with 0.9% saline to remove soluble substances. The figures in Table 4 refer to three experiments. In Expt. A (8×10^{-4} M retinene) it appears clear that the materials needed for regeneration are contained in the insoluble portion,

Table 3. *The effect of light on the regeneration of rhodopsin from all-trans-vitamin-A alcohol or retinene*

(Reaction mixture was $4-8 \times 10^{-4}$ M vitamin-A alcohol or $3-8 \times 10^{-4}$ M retinene and 0.034 M phosphate buffer, pH 7.4. The vitamin A or retinene was either added to the mixture just before or just after irradiation of the rhodopsin prior to regeneration. The figures represent mean percentage of net regeneration (see text), the standard error and, in brackets, the number of experiments.)

Substance added	Conditions	
	Not exposed to light	Exposed to light
Vitamin-A alcohol	55.5 ± 8.8 (8)	81.6 ± 13.0 (5)
Retinene	59.2 ± 12.3 (9)	87.5 ± 8.5 (8)

Table 4. *Regeneration of ox rhodopsin by retinene. Comparison between whole-retinal suspensions and the solid obtained from suspensions after centrifugation*

(Reaction mixture was retinene (concentration shown below) and 0.034 M phosphate buffer, pH 7.4. In samples 1 to 3 the whole suspension was used, in samples 4 to 7 the retinal suspension was centrifuged and the residue washed with 0.9% (w/v) NaCl. The washed residue was finally suspended in the phosphate buffer. The retinene was added just before or just after irradiation of the rhodopsin prior to incubation.)

Sample no.	Experiment Final concentration of retinene ($\times 10^{-4}$ M) ...	A		
		8	4 ($E_{1\text{ cm. at } 500\text{ m}\mu.}$)	4
1	Residual rhodopsin at start	0.026	0.011	0.018
2	Regenerated rhodopsin; *retinene exposed to light	0.245	0.280	0.192
3	Regenerated rhodopsin; retinene not exposed to light	0.250	0.268	0.123
4	Residual rhodopsin at start	0.015	0.003	0.022
5	Rhodopsin before bleaching	0.189	0.237	0.239
6	Regenerated rhodopsin; *retinene exposed to light	0.256	0.196	0.173
7	Regenerated rhodopsin; retinene not exposed to light	0.272	0.140	0.059

* Retinene added before bleaching, i.e. it could have been isomerized by the light.

and that soluble enzymes are not required in the formation of rhodopsin from retinene. This experiment shows the puzzling feature that the regenerations in samples 6 and 7 exceed the control reading for unbleached rhodopsin (sample 5) in the washed centrifugate. This may reflect complications due to an excess of retinene. Expts. B and C (with 4×10^{-4} M retinene) show that the power to regenerate rhodopsin lies in the insoluble part of the retinal suspension, that water-soluble enzymes are not needed with retinene, and, finally, that retinene is more effective after exposure to light.

DISCUSSION

If the results of the present paper are compared with the previous ones (Collins *et al.* 1953) the conditions required for the regeneration of rhodopsin are strikingly different. With frog tissue, the retina and choroid must be incubated in a 'complete' medium including ATP and vitamin-A alcohol. With rat, both the retina and choroid are required, but the medium need only contain vitamin A and a buffer (phosphate, pH 7.4). With ox, only the retina is required along with vitamin A and a buffer (pH 7.4). These differences are presumably correlated with the dependence of the visual cells on a retinal or a choroidal circulation, and on whether the tissue is poorly or richly supplied with metabolites. Ox retina, isolated by the same techniques as were used in the regeneration experiments, is capable of phosphorylation, and is thus capable of supporting many synthetic processes. Therefore these interspecies differences do not necessarily imply any fundamental differences in the mechanism of

regeneration. The retina and choroid of frog may be poorly supplied with metabolites and coenzymes, while the choroid of the rat may be well supplied. The fact that ox retina appears to be well supplied with metabolites and coenzymes increases the experimental difficulties, as it is difficult to determine which metabolites and coenzymes are directly needed for the regeneration of rhodopsin.

In the case of pyridoxal phosphate, only a small effect would be expected if it were added to a comminuted retinal suspension already containing a nearly adequate amount. The fact that pyridoxal phosphate has such a significant effect suggests that it is directly concerned in the regeneration of rhodopsin, and not with an indirect process.

The results were quite variable and, even with pyridoxal phosphate, 100% regeneration was not obtained, although on other occasions 100% regeneration was obtained without pyridoxal phosphate. Nor did the use of a 'complete' medium result in consistently better results. Therefore all the factors necessary for the regeneration of rhodopsin are not yet known.

Hubbard & Wald (1952*a, b*) have shown that light has an important effect on regeneration from retinene. They found that *all-trans*-retinene added to a bleached rhodopsin solution produced no regeneration, but if the retinene had previously been exposed to a bright light, as much as 85% regeneration resulted. The same result was obtained if the retinene had been prepared from natural vitamin-A esters. They suggested that only a *cis* isomer of retinene was effective in regeneration. Hubbard & Wald (1951) obtained good regeneration using the system, vitamin A (as the alcohol, prepared from a fish-liver oil), DPN, crystalline alcohol dehydrogenase, and opsin. Opsin was obtained from rod outer segments which had been hardened in alum, washed with a neutral buffer and finally extracted with aqueous digitonin. This digitonin solution would contain, among other things, the protein moiety of rhodopsin. Using this system, they obtained up to 65% regeneration in 9 hr. or more. Later, Hubbard & Wald (1952*a, b*) tested *all-trans*-vitamin A and an isomer, neovitamin A; both were inactive. According to current theories (Pauling, 1949), only four stereoisomers of vitamin A or retinene are possible. Hubbard, Gregerman & Wald (1953) have now prepared five 'isomers' of retinene. Only 'neoretinene *b*' gave rhodopsin on incubation with the system described above. 'isoRetinene *a*' yielded a 'rhodopsin' with $\lambda_{\max}$ displaced to 487 m μ . The other forms of retinene were inactive.

In the simple system used by Wald and his co-workers, only specific forms of vitamin A and retinene were capable of supporting regeneration. In the present work a more complex system was used and regeneration was obtained from *all-trans*-

vitamin A or retinene. The simplest explanation is that there is an enzyme capable of catalysing the change of *all-trans*-vitamin A or retinene to the active form, which then combines with opsin. The fact that irradiation of the vitamin A or retinene prior to incubation increased the amount of regeneration would suggest that the amount of this hypothetical enzyme might be a limiting factor in the retinal suspension used in the present work. It cannot be stated whether this enzyme would act on *all-trans*-vitamin A or its aldehyde, as they are equally effective in regeneration, perhaps because the equilibrium catalysed by alcohol dehydrogenase is reached quickly, in which case it would be immaterial whether the alcohol or aldehyde were used.

The course of regeneration can be formulated as follows: The initial step is almost certainly

The next reaction would be the conversion of retinene into active retinene. The nature of the active form is at present unknown, but there seem to be at least three possibilities. (i) It could be a *cis* isomer, as first suggested by Hubbard & Wald (1952*a*). There appear to be five 'isomers' of retinene, in apparent conflict with Pauling's (1949) theory; one or more of these could be non-stereoisomeric (e.g. a dimer), but the active form could still be a *cis* isomer. (ii) The active form could contain an α -ionone ring, as suggested by Hubbard *et al.* (1953). (iii) The active form could be a dimer of retinene. Hence, the second step can be written as follows:

The work reported in this paper suggests that the isomerase or dimerase is insoluble in water and is inactivated by alum.

Wald and his co-workers have shown that 'active retinene' reacts spontaneously with opsin to form rhodopsin. At present the details of this reaction are unknown, but two important facts are relevant. Wald & Brown (1952) have shown that sulphydryl groups are liberated on irradiation of rhodopsin, and that retinene will combine with sulphydryl compounds in slightly acid or neutral media. Collins (1953) has shown that the first stable product after irradiating rhodopsin is indicator yellow (first described by Lythgoe, 1937), and that this material is not an artifact as suggested by Ball, Collins, Morton & Stubbs (1948) and by Wald (1951). Retinene has been shown to combine with primary amines in an alkaline medium to form Schiff's bases, which have absorption spectra closely resembling that of indicator yellow (Ball, Collins, Dalvi & Morton, 1949; Collins & Morton, 1950). The above evidence suggests that retinene can combine with sulphydryl groups at pH 7 and not with amino

groups, but that after irradiating rhodopsin, the retinene is combined with an amino group and not a sulphhydryl group. A possible mechanism is as follows:

Rhodopsin could be the compound with either a retinene-to-sulphur bond or a retinene-to-nitrogen bond. However, the fact that compounds of retinene and sulphhydryl compounds absorb at short wavelengths (about 330 m μ .), while the compounds of retinene and a primary amine can absorb at long wavelengths (440 m μ .), and even at 500 m μ . under certain conditions (Collins, 1953), strongly suggests that rhodopsin contains a retinene-to-nitrogen bond.

The role of pyridoxal phosphate is not implicit in the foregoing. If it be assumed that it is acting as a coenzyme of transamination, its function may be to maintain an essential amino group, present in opsin. It is even conceivable that pyridoxal phosphate may be transformed into pyridoxamine phosphate, which may be part of the opsin molecule.

SUMMARY

1. The regeneration of rhodopsin has been studied with comminuted ox retina. The reaction mixture need only contain *all-trans*-vitamin-A alcohol and phosphate buffer (pH 7.4). The average amount after 2 hr. at 37° was 62 %, but the results were variable and ranged from 48 to 100 %.

2. Pyridoxal phosphate was found to increase the extent of regeneration.

3. The effect of light on the vitamin A or retinene used showed that vitamin A and retinene were

equally effective and that prior exposure to light resulted in increased regeneration in both cases.

4. The implications of these results are discussed in connexion with current theories of the mechanism of the regeneration of rhodopsin.

We wish to thank the Medical Research Council for financial support. We are indebted to Dr T. B. Parks of Milwaukee for a gift of DPN and to Roche Products Ltd. for a gift of pyridoxal phosphate.

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Comparative Detoxication

2. GLUCOSIDE FORMATION FROM PHENOLS IN LOCUSTS

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(Received 22 September 1953)

It has recently been shown that locusts, like mammals, can acetylate aromatic amino compounds (Myers & Smith, 1953*a*) and form substituted hippuric acids from aromatic acids (Friedler & Smith, 1953).

When phenols are absorbed by mammals, a considerable proportion of the dose is eliminated in the urine as ethereal sulphates and glucosiduronic acids (glucuronides) (Williams, 1947). With large doses of

phenols glucosiduronic acid formation is usually predominant (Bray, Humphris, Thorpe, White & Wood, 1952). Glucosiduronic acids are also formed in birds (Sperber, 1947) and frogs can convert menthol into menthylglucosiduronic acid (Schmid, 1936).

Apart from a preliminary report on part of the present work (Myers & Smith, 1953*b*), the only reference to the conjugation of an ingested phenol