Studies on the Flash Photolysis of Visual Pigments

2. PRODUCTION OF THERMALLY STABLE PHOTSENSITIVE PIGMENTS IN FLASH-IRRADIATED SOLUTIONS OF FROG RHODOPSIN

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Wulff, Adams, Linschitz & Abrahamson (1958) and Bridges (1961) have described the production of various pigments by flash-irradiating solutions of cattle and frog rhodopsin. In each case it was found that, after exposure to a flash, the solutions contained two types of pigment. One type, which decayed rapidly in darkness at room temperature, was called Fraction 1. The other, which was thermally stable and photosensitive, was called Fraction 2. The Fraction 2 of frog rhodopsin was shown (Bridges, 1961) to be a mixture of frog rhodopsin and a new pigment, probably based on another retinene isomer, which had λ_{max} at 486 m μ .

The present work is concerned with the composition and yield of Fraction 2 produced by flashes of various colours and intensities, and in solutions at different temperatures. The results of these experiments suggest a possible sequence of reactions occurring during and after the period of irradiation.

METHODS

Preparation of frog-rhodopsin solutions. Aqueous digitonin extracts from the dark-adapted retinæ of frogs (*Rana temporaria*) were prepared in the manner described in the preceding paper (Bridges, 1961). For work at temperatures below 0°, where such solutions would freeze, glycerol (50 or 65%, v/v) was added (cf. Broda & Goodeve, 1941), and the pH was adjusted to between 8.2 and 8.7 by the addition of aqueous 0.4 M-veronal solution (the concentration in the extract was about 0.04 M).

Hydroxylamine solutions. These were prepared as described in the preceding paper.

Absorption spectra. All spectra were measured at $25 \pm 3^\circ$ on a Unicam SP.500 spectrophotometer. Specially designed glass cells of 1 cm. optical-path length enabled measurements to be carried out on 0.6 ml. of solution.

Bleaching apparatus

Flash equipment. This was as described in the preceding paper, except that the optical-cell holder was modified so that alcohol at various temperatures could be passed through it. In this way solutions could be maintained at any desired temperature, measured by means of a copper-constantan thermocouple which was dipped into the rhodopsin solution. Condensation was prevented by circulat-

ing dry air through the interior of the apparatus. A further alteration to the optical-cell holder permitted the attachment of Ilford gelatin filters close to both optical faces; all radiation from the flash thus passed through these filters before irradiating the solution.

The spectral distribution of light reaching a solution was obtained by multiplying the relative spectral-energy curve of the flash (Robinson, 1954) by transmission spectra of the filters (measured on the Unicam SP.500). The relative energy distributions of the flash radiation after passing through the different colour filters and through one of the neutral filters used are shown in Fig. 1.

Normal white bleaching. When it was necessary to bleach solutions to completion, they were exposed for 15 min. at 25° to the light of a 15 w, 230 v lamp at a distance of 1 ft. (illumination ~ 25 ft.-candles).

Procedure. The experimental procedure was standardized as follows. (1) The absorption spectrum of the solution was

Fig. 1. Spectral-energy distribution of flash radiation after passing through some of the filters used. (A) Unfiltered (data of Robinson, 1954); (B) 'neutral' filter (nominal density of 0.31); 1, yellow, Ilford 102; 2, orange Ilford 201; 3, violet, Ilford 621; 4, green, Ilford 624; 5, red, Ilford 204.

first measured at 25° in a manner embodying a test of the thermal stability (Dartnall, 1952). (2) The solution was placed in the flash equipment and gradually cooled or warmed to the desired temperature. (3) The solution was flash-irradiated through the correct filter, brought as rapidly as possible to 25° and left for 1 hr. in darkness, after which time all thermal changes were complete (cf. the preceding paper). Subsequent operations were carried out at 25°. (4) The absorption spectrum was again measured. (5) The solution was finally exposed to 'normal' white light for 15 min. and the absorption spectrum of the fully bleached solution obtained. Difference spectra for the parent rhodopsin and for Fraction 2 could then be calculated by subtraction of the final fully bleached absorption spectrum from the initial and post-flash absorption spectra respectively (as illustrated in the preceding paper, Fig. 2).

Analysis of difference spectra. Fraction 2 contains P486 and frog rhodopsin, hence its difference spectrum is displaced from that of frog rhodopsin to an extent depending on the proportion of P486. Fig. 2 illustrates the wavelength shift produced at the long-wave 50% point when varying proportions of the P486 difference spectrum are added to that of frog rhodopsin. Thus all that is necessary when determining the composition of a Fraction 2 is to compare its difference spectrum with that of frog rhodopsin, measure the displacement and then read off the percentage of P486 from Fig. 2.

Quantities of photosensitive materials are expressed in terms of their maximum change in extinction when fully bleached ($\Delta E_{\max}$). When the difference spectra of P486 and frog rhodopsin, both with $\Delta E_{\max}$ of 50, are added the $\Delta E_{\max}$ of the resulting difference spectrum is not 100 but 98.4 (see preceding paper). According to the proportions in which these difference spectra are added, the resulting $\Delta E_{\max}$ may lie between 98.4 and 100.0.

Fig. 2. Analysis of Fraction 2 difference spectra.
For explanation see text.

Consequently, after determining the percentage of P486 in Fraction 2, a corresponding small correction must be applied to the $\Delta E_{\max}$ of the Fraction 2 difference spectrum to give the total photosensitive-pigment content.

$\Delta E_{\max}$ as a measure of photosensitive-pigment concentration. For a given visual pigment under a given set of conditions (e.g. pH, temperature etc.) $\Delta E_{\max}$ is proportional to $E_{\max}$ and hence to the visual-pigment concentration (cf. Bridges, 1957). However, when the quantities of pigments in a mixture are being compared a word of qualification is necessary, since alkaline indicator yellow and retinene oxime absorb slightly differently at the respective $\lambda_{\max}$ of frog rhodopsin and P486. This, however, has only a very small effect on the estimated proportions of these pigments in a mixture. Consequently, until the respective molar-extinction coefficients are known (Dartnall, 1955), the $\Delta E_{\max}$ values provide useful means of expressing concentrations in such mixtures.

RESULTS

Effect of flash intensity on the composition and yield of Fraction 2 produced at 25°

'Fraction 2' designates any material remaining in solution after flash-irradiation and which is thermally stable and photosensitive at 25°. If the

Fig. 3. Effect of flash intensity on the yield of Fraction 2.
●, Yield of Fraction 2; +, rhodopsin component of Fraction 2.

flash intensity is decreased by means of a filter to 1/1000 of the unfiltered value, Fraction 2 is found to be entirely rhodopsin and amounts to 97% of the pre-flash rhodopsin. Clearly, in this case 'Fraction 2' is merely unbleached rhodopsin. As the flash intensity is raised the yield of Fraction 2 decreases, as shown in Fig. 3, down to a minimum of about 22%, but thereafter rises again until at the full flash intensity it is about 33% of the pre-flash rhodopsin.

Some explanation of the abscissa of Fig. 3 is necessary here. The effective fraction of light transmitted by a filter depends on the light-absorbing properties of the photo-reacting material, in this case frog rhodopsin. Therefore, to obtain the effective reduction of light intensity by any filter, neutral or coloured, relative spectral-energy curves, such as are shown in Fig. 1, must be multiplied by the extinction curve (see end of Discussion) of pure frog rhodopsin (tabulated by Dartnall, 1953). The areas beneath each of the derived filtered curves must then be divided into the area beneath the unfiltered curve. The final result, called the effective flash intensity (EFI), is therefore equal to:

$$\frac{\int_{400}^{620} A_{\lambda} \cdot E_{\lambda} \cdot d\lambda}{\int_{400}^{620} A_{\lambda} \cdot E_{\lambda} \cdot T_{\lambda} \cdot d\lambda},$$

where A_{λ} = relative spectral energy of xenon-tube emission at wavelength λ ; E_{λ} = relative extinction of frog rhodopsin at wavelength λ ; T_{λ} = percentage transmission of filter at wavelength λ .

In addition to the changes in yield of Fraction 2, the proportion of P486 (estimated as described in the Methods section) rose from 0% at low flash intensities to between 46 and 57% at full flash intensity, as shown in Fig. 4. The lower curve of Fig. 3 shows the changes in yield of the rhodopsin component only, and clearly indicates that although the proportion of rhodopsin in the mixture decreased, the actual yield rose slightly at EFI values above about 0.5. Thus although the 'Fraction 2' of low flash intensities probably consisted entirely of unbleached rhodopsin, that of higher flash intensities, in addition to containing flash-generated P486, contained also flash-generated rhodopsin.

To summarize: as the effective flash intensity is increased, the following changes in Fraction 2 are observed: (1) the total yield of photosensitive pigments decreases, passes through a minimum, then rises steadily until full flash intensity is reached; (2) the proportion of P486 increases; (3) after passing through a minimum, the yield of frog rhodopsin increases slightly.

Fig. 4. Effect of flash intensity on the percentage of pigment 486 in Fraction 2. Solutions irradiated through neutral filters: O, hydroxylamine-free solutions; +, solutions containing 0.01M-hydroxylamine. Solutions irradiated through coloured filters: ●, hydroxylamine-free solutions. The number labelling each point denotes the use of one of the following colour filters: 1, yellow, Ilford 102; 2, orange, Ilford 201; 3, violet, Ilford 621; 4, green, Ilford 624; 5, red, Ilford 204. The broken line is the theoretical relationship between flash intensity and percentage of pigment 486 (see Discussion).

Effect of flash colour on the composition and yield of Fraction 2 produced at 25°

It was noted in the preceding paper that although the difference spectrum of Fraction 1 was the same for orange and unfiltered flashes, Fraction 2 produced by an orange flash was found to contain less P486 (i.e. its $\lambda_{\max}$ was at 500 compared with 494 $m\mu$). If, however, the EFI for the orange filter is computed, and the result of this experiment is inserted into Fig. 4, it is apparent that the orange results fit the 'neutral' P486 (%) / EFI curve. Results of experiments with red, green, yellow and violet flashes similarly agree with the corresponding 'neutral' values, as illustrated. Again, in terms of the yield of photosensitive pigment in Fraction 2, no consistent wavelength effect could be detected, as shown by Fig. 5.

To summarize: the composition and yield of Fraction 2 produced by flashes of varying chromaticity is accounted for within experimental error on the basis that the coloured filters merely reduced the effective light intensity for the bleaching of rhodopsin.

Flash-irradiation in the presence of hydroxylamine

In the preceding paper the composition and yield of Fraction 2 produced at 25° by an unfiltered flash were found to be unaffected by hydroxylamine (0.01 or 0.03M). Similarly, in the present work, hydroxylamine (0.01M) was found to have no effect on the Fraction 2 produced by flashes of any intensity, as shown in Figs. 4 and 5.

Flash-irradiation of Fraction 2 at 25°. Fraction 2 produced in the presence of 0.01M-hydroxylamine by a flash of EFI 0.10 contained 12.4% of P486. Exposure of this material to a second identical flash, and leaving for 1 hr. to permit completion of thermal changes, produced a Fraction 2 which now contained 22.4% of P486. Yet a third flash raised the P486 to 44.0%. At higher flash intensities this rise was still observed but was not so marked. Thus at full flash intensity, the first-flash Fraction 2

contained 49.8% of P486, whereas second- and third-flash Fractions 2 (in the presence of hydroxylamine) respectively contained 59.0 and 62.8% of P486. Hydroxylamine (0.01M) was added to the solutions used in these experiments, so that after the decay of Fraction 1 in the first flash the solutions contained retinene oxime and Fraction 2. Thus the effect of a second flash was not complicated by regeneration of rhodopsin from irradiated indicator yellow. This is demonstrated by the fact that first-, second- and third-flash Fractions 2 formed in a hydroxylamine-free solution exposed to full-intensity flashes contained respectively 50.5, 41.6 and 39.6% of P486.

Flash-irradiation at different temperatures

The preceding results were obtained by irradiating solutions at 25°. Experiments with full-intensity flashes were also carried out at temperatures from +40° to -41°. The yield of Fraction 2, after leaving the irradiated solutions for 1 hr. at 25°, was found to increase by between 4 and 10% for every 10° fall in temperature. This is illustrated by the absorption spectra shown in Fig. 6. In this Figure, the curves labelled (b) represent absorption spectra of solutions measured after irradiation at a number of different temperatures and then leaving for 1 hr. at 25°. The yield of Fraction 2, as measured by the maximum fall of extinction when the solutions were fully bleached (curves c), is seen to rise from about 32% of the pre-flash rhodopsin at +40° to about 53% at -41°. The analysis of these and other experiments is given in Table 1, and shows that although the yield of Fraction 2 depended on the temperature, there were no significant changes in the composition of this material.

The influence of temperature on the yield of Fraction 2 does not appear to be linked in any way to accompanying changes in viscosity. Thus the yield of Fraction 2 produced at +8° in a viscous solution containing 65% (v/v) of glycerol is practically identical with that produced at the same temperature in a solution without glycerol.

DISCUSSION

Only the reactions occurring after irradiation by a single flash will be considered here. The effect of subsequent flashes is complicated by the fact that one is no longer dealing with a single pigment, but with a mixture of two pigments.

Fraction 2 present in solution after irradiation at very low flash intensities was found to consist entirely of rhodopsin, and this rhodopsin was probably merely unbleached material. If this were true of the rhodopsin component of Fraction 2 produced by a flash of any intensity, one would

Fig. 5. Comparison between the yields of Fraction 2 produced in solutions (with or without hydroxylamine) irradiated through neutral or coloured filters. Solutions irradiated through neutral filters: ○, hydroxylamine-free solutions; +, solutions containing 0.01M-hydroxylamine. Solutions irradiated through coloured filters: ●, hydroxylamine-free solutions; the points are numbered as in Fig. 4.

expect that the curve relating its yield to the flash intensity would asymptotically approach the zero line according to the equation

$$C = ae^{-kIt},$$

where a = initial rhodopsin (= 100), C = rhodopsin yield, I = effective flash intensity, t = duration

of photochemical reaction, k = constant. However, this equation does not describe the observed rhodopsin concentrations, as is apparent from the lower curve of Fig. 3. Here, the rhodopsin yield, after passing through a minimum, actually rises as the highest flash intensity is approached. This increase can only be due to the presence of rhodopsin

Fig. 6. Variation in the yield of Fraction 2 produced by irradiation (maximum flash intensity) at the various temperatures indicated. Curves (a), the pre-flash absorption, spectra; curves (b), absorption spectra measured after flash-irradiating the solution at the given temperature, then leaving it for 1 hr. at 25°; curves (c) are absorption spectra of the fully bleached solutions, i.e. after exposure for 15 min. (at 25°) to a 15w lamp. The maximum loss of extinction from curve (b) to curve (c) is a measure of the amount of Fraction 2. All spectra were measured at 25°. Pre-flash absorption spectra have been scaled so that $E_{500\text{m}\mu} = 1.00$.

Table 1. *Effect of temperature on the yield and composition of Fraction 2*

$\Delta E_{\max}$ (1 cm.)	Glycerol content* (%, v/v)	Temperature	Yield of Fraction 2† (% of original rhodopsin)	Pigment 486 in Fraction 2 (%)
0.575	50	+40°	31.83	54.0
0.578	50	+25	31.92	49.5
0.401	0	+25	34.69	49.5
0.415	65	+8	41.81	53.5
0.418	0	+8	41.84	50.0
0.534	50	-5	43.90	46.0
0.550	50	-20	48.78	50.0
0.414	65	-41	53.02	47.1

* Solutions were adjusted to pH 8.2-8.7 by addition of one-tenth of the extract volume of aqueous solutions of saturated sodium borate (for solutions with no glycerol) or 0.4M-sodium 5:5'-diethylbarbiturate (for solutions containing glycerol).

† Measured after bringing the temperature of the solution to 25° and leaving for 1 hr. at that temperature.

Fig. 7. Variation with flash intensity of the three types of substance present in a solution of frog rhodopsin after exposure to a flash. ○, Unbleached molecules (the curve represents C_A); ●, bleached molecules (the curve represents $C_B + C_D$); +, reconstituted molecules (the curve represents C_C). The broken line represents C_B , the concentration of the hypothetical intermediate B, at the end of the photochemical reaction.

reconstituted by the flash. It is therefore suggested that the curve relating flash intensity to the quantity of Fraction 2 produced (Fig. 3) is the sum of two curves. One represents unbleached rhodopsin, and diminishes with rising flash intensity according to the above equation. The other, representing the mixture of reconstituted rhodopsin and pigment 486, must increase with rising flash intensity, since the total Fraction 2 increases.

Experimentally, it is not possible to arrive at an estimate of the proportion of unbleached rhodopsin in any given Fraction 2. If, however, it is assumed

that pigment 486 represents a constant proportion (47%) of the reconstituted photopigments at all flash intensities, then it is a straightforward matter to calculate the yield of reconstituted material at any particular intensity. Values for the unbleached rhodopsin may then be obtained which, as shown in Fig. 7, agree with corresponding values calculated from the above equation, when $kt = 9$. Therefore the assumption appears to be justified.

The increase with rising flash intensity of the reconstituted photopigment (Fig. 7) suggests that it is formed by the irradiation of some substance which is an intermediate in the sequence of reactions after absorption of a single light quantum by the rhodopsin molecule, as shown in Scheme I. In this scheme, A represents the original rhodopsin and B represents the intermediate state convertible by light into another state C. Both B and C are slowly converted into bleached molecules (indicator yellow ultimately) and reconstituted photopigment molecules respectively. The independence of the composition of Fraction 2 on the wavelength of the irradiating light would make it likely that conversion of C into photosensitive material is slow compared with the duration of the flash. Were this not so, long-wavelength flashes would produce Fractions 2 rich in pigment 486 and short-wavelength flashes would produce Fractions 2 poor in pigment 486 (relative to the corresponding 'neutral' flash composition).

If the yields of A, B and C are calculated from the kinetics of this Scheme (assuming all reactions to be first-order) the results describe the observed yields of bleached and reconstituted molecules in Fig. 7. A better fit of these results is, however, obtained by assuming that the intermediate 'B' decomposes at a rate comparable with the duration of the flash, as shown in Scheme II. If the kinetics of these reactions are first-order we have

$$-\frac{dC_A}{dt} = k_1 IC_A \quad \text{or} \quad C_A = ae^{-k_1 It},$$

Scheme I

Scheme II

where $a = C_A + C_B + C_C + C_D = 100$, C_A , C_B , C_C and C_D represent the percentage yields of A, B, C and D respectively. Similarly,

$$\frac{dC_C}{dt} = k_2 IC_B$$

and

$$\frac{dC_D}{dt} = k_3 C_B.$$

Therefore

$$\frac{dC_B}{dt} = -\frac{dC_A}{dt} - \left[\frac{dC_C}{dt} + \frac{dC_D}{dt} \right].$$

From the above equations

$$\frac{dC_B}{dt} = k_1 I e^{-k_1 I t} - [k_2 I + k_3] C_B.$$

This may be integrated to

$$C_B = \frac{ak_1}{\frac{k_3}{I} + k_2 - k_1} [e^{-k_1 I t} - e^{-(\frac{k_3}{I} + k_2) t}]$$

(cf. Rutherford, Chadwick & Ellis, 1930). Since:

$$\frac{C_C}{C_D} = \frac{k_2 I}{k_3},$$

therefore
$$C_D = \frac{a - (C_A + C_B)}{1 + (k_3 I / k_2)}.$$

Knowing C_A , C_B and C_D it is then possible to calculate C_C .

When all thermal changes have ceased after flash-irradiation, the yield of bleached molecules is given by $(C_B + C_D)$, and of reconstituted molecules by C_C . As shown in Fig. 7, the curves relating these two quantities to the light intensity are in satisfactory agreement with the experimental points when $k_1 t = 9$, $k_2 t = 9$ and $k_3 t = 18$. The broken curve of Fig. 4, representing the calculated percentage of pigment 486 in Fraction 2, is

similarly not incompatible with the experimental observations. If it is assumed that the rates of the photochemical reactions are independent of temperature, the rate of conversion of B into D has a temperature coefficient of between 1.14 and 1.08.

It should be noted that if the extinction curve of frog rhodopsin changes with temperature in the same way as that of cattle rhodopsin, then the effective flash intensity would be 1.09 at -45° . (This has been calculated from the data published by Wald, Durell & St George, 1950.) However, if this value is inserted into the equations shown above, the yield of Fraction 2 increases only by about 7% from $+23^\circ$ to -45° . As shown in Table 1, the observed increase from $+25^\circ$ to -41° was about 60%.

At present the identity of the hypothetical state B is unknown. Clearly it cannot be indicator yellow (see preceding paper), and furthermore the absence of any consistent wavelength effect in the coloured-flash experiments illustrated in Figs. 4 and 5 shows that the photoreacting intermediate B had an absorption spectrum identical with or very similar to that of frog rhodopsin. Substance B may be the result of the primary photochemical event, i.e. it is possibly an electronically excited state of the rhodopsin molecule. Again, it may be a new substance of the type found in the immediate post-flash period by Wulff *et al.* (1958). Were the latter the case, there need be no conflict with the conclusions of the coloured-flash experiments, for the light-absorbing properties of these substances differed little from those of the parent rhodopsin.

Effective flash intensity

It might be expected that the light-absorbed curve rather than the extinction curve of rhodopsin should be used to estimate the effective fraction of

light transmitted by the filters. The light absorbed at any wavelength λ may be calculated from the expression

$$I_{\text{abs.}} = I_{\text{inc.}} (1 - 10^{-E_{\lambda}}),$$

where $I_{\text{abs.}}$ = light absorbed, $I_{\text{inc.}}$ = light incident on the solution, E_{λ} = extinction at wavelength λ . If $E_{\lambda} \leq 0.05$ this expression approximates to $I_{\text{abs.}} = 2.303 I_{\text{inc.}} E_{\lambda}$ (cf. Dartnall, 1957). Since $E_{\lambda} = \alpha_{\lambda} cl$ (where α_{λ} = extinction coefficient at wavelength λ ; c = concentration; l = optical path length), it follows that E_{λ} is proportional to the number of chromophores in solution, and hence only when $E_{\lambda} \leq 0.05$ is the light absorbed per molecule constant for a given value of $I_{\text{inc.}}$. The amount of light absorbed per molecule progressively diminishes as E_{λ} is raised above about 0.05.

Under the present experimental conditions, however, the yield of Fraction 2 produced by flash-irradiation of rhodopsin solutions was independent of the parent rhodopsin concentration and hence of the extinction of those solutions (Fig. 8). The $\Delta E_{\text{max.}}$ of these solutions ranged from about 0.3 to 1.3 yet the yield of Fraction 2 was always constant. It must therefore be accepted that, under the present experimental conditions, the light absorbed per rhodopsin molecule was constant over the range of extinctions tested. Therefore, since the light absorbed was proportional to the extinction, it is the extinction curve of rhodopsin which is used in assessing the 'effective flash intensity'.

Fig. 8. Yield of Fraction 2 produced by irradiating rhodopsin solutions of varying concentrations. (a) Effective flash intensity = 1.00; (b) effective flash intensity = 0.10.

SUMMARY

1. Alkaline (pH 8.2–8.7) aqueous digitonin solutions of frog rhodopsin were exposed at different temperatures to intense 4 msec. flashes from a 400J xenon-filled discharge tube. At all temperatures below 0° the solutions contained 50 or 65 % (v/v) of glycerol to prevent freezing. Some experiments were also carried out at higher temperatures on solutions which contained glycerol.

2. Exposing a solution at any temperature to a full-intensity flash and leaving for 1 hr. in darkness at 25° gives rise to a thermally stable photosensitive residue (Fraction 2) composed of the parent rhodopsin and a new pigment with $\lambda_{\text{max.}}$ at 486 m μ (pigment 486 or P 486).

3. The effects of altering flash intensity and colour on the composition and yield of Fraction 2 produced in solutions irradiated at 25° were investigated. If the flash intensity was reduced to about $\frac{1}{1000}$ of its unfiltered value, 'Fraction 2' amounted to about 97 % of the pre-flash rhodopsin and contained no P 486. This material was probably unbleached rhodopsin. As the flash intensity was raised, the yield of Fraction 2 steadily diminished to a minimum of about 22 % of the pre-flash rhodopsin, but thereafter rose to the full flash-intensity level of about 33 %.

4. At the same time, the proportion of P 486 in Fraction 2 rose from 0 % at low flash intensities to between 46 and 57 % at the maximum intensity. However, although the proportion of rhodopsin in Fraction 2 exhibited a corresponding decrease, the actual yield, after passing through a minimum, rose slightly towards the higher flash intensities.

5. The composition and yield of Fraction 2 produced in solutions irradiated at 25° by flashes of varying chromaticity is accounted for on the basis that the coloured filters merely decreased the effective light intensity for the bleaching of rhodopsin.

6. Hydroxylamine (0.01M) had no effect on the composition and yield of Fraction 2 produced in solutions irradiated at 25° by flashes of any intensity.

7. If a solution were exposed, at 25°, to a number of flashes at intervals of about 1½ hr. the Fraction 2 produced by each successive flash was found to contain a progressively increasing proportion of P 486.

8. In solutions irradiated at various temperatures with full-intensity flashes and left for 1 hr. in darkness at 25°, the yield of Fraction 2 increased by between 4 and 10 % for every 10° drop in temperature between +40° and –41°. The changing viscosity of these solutions did not appear to be responsible for this effect. No significant alteration in the percentage of P 486 was observed.

9. It is suggested that Fraction 2 is made up of unbleached parent rhodopsin and a reconstituted constant-composition mixture of rhodopsin and P486. This reconstituted material is produced by the irradiation of an intermediate substance which is unstable even at -40° .

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Adenylic Deaminase of Pea Seeds

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Adenylic deaminase catalyses the deamination of adenosine 5'-monophosphate with the formation of inosine 5'-monophosphate and ammonia. The enzyme was first reported in extracts from rabbit muscle by Schmidt (1928), who showed that it was specific for adenosine 5'-monophosphate as substrate and was distinct from adenosine deaminase. Kalckar (1947) obtained active deaminase preparations from rabbit muscle after precipitating myosin and fractionating the enzyme with ammonium sulphate. Nikiforuk & Colowick (1956) purified adenylic deaminase some 40-fold from crude bicarbonate extracts of rabbit muscle and studied a number of the properties of the enzyme. Separation from myosin and further purification enabled Lee (1957) to crystallize the rabbit-muscle enzyme.

Adenylic deaminase has been found in several other animal tissues such as heart muscle, liver and kidney but the activities are lower than in skeletal muscle (Lee, 1957). The enzyme occurs as a complex with myosin (Kalckar, 1947; Humphrey & Webster, 1951).

The occurrence of the enzyme in a higher-plant tissue was investigated. In the present investigation adenylic deaminase was found in a particulate fraction from pea seeds and a preparation free from interfering enzymes was obtained. A number of properties of the pea-seed adenylic deaminase were studied.

EXPERIMENTAL

Special chemicals. Adenine, adenosine, adenosine 5'-monophosphate (AMP), adenosine diphosphate, adenosine triphosphate, inosine 5'-monophosphate (IMP) and diphosphopyridine nucleotide were obtained from Pabst Laboratories, Milwaukee, Wis., U.S.A., triphosphopyridine nucleotide and *p*-hydroxymercuribenzoate from Sigma Chemical Co., St Louis, Mo., U.S.A., and cysteine hydrochloride from British Drug Houses Ltd.

Preparation of 5'-adenylic acid deaminase from pea seeds. Preliminary experiments showed that the adenylic-deaminase activity was associated with a particulate fraction in pea seeds and the following preparative procedure was adopted. Pea seeds (*Pisum sativum* L., var. Laxton's Progress) were soaked overnight in water and 50 g. of the soaked seeds was blended for 3 min. at 4° with 50 ml. of 40 mM- NaHCO_3 . The homogenate was squeezed through muslin, centrifuged at 500 g for 5 min. and the supernatant centrifuged at 20 000 g for 30 min. The precipitate from the high-speed centrifuging was suspended in 10 ml. of water, centrifuged at 20 000 g for 30 min. and the resultant precipitate was dispersed in water to give a final volume of 5 ml.

The light-green enzyme preparation could be stored at -15° for 5 weeks with 10% loss of deaminase activity. Heating at 60° for 5 min. resulted in 95% loss of activity. No phosphatase activity was detected when samples of reaction mixtures containing the enzyme preparation and AMP or IMP were examined for the formation of inorganic phosphate or, on paper chromatograms, for the production of adenosine or inosine.

Reaction mixtures. The reaction mixtures were maintained at 30° . The determination of enzyme activity was a