Activation of the Ephippial Egg of *Daphnia pulex*

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ABSTRACT The ephippial eggs of *Daphnia pulex* require light for the initiation of development. The ephippial capsule prevents the completion of development but is not a barrier to an adequate light stimulus. Working with decapsulated eggs, the response to light increased to 100% within 9 days of storage in the dark and remained at 100% for up to 60 days of storage in the dark. The response was not dependent on drying the ephippia. Ephippia stored in the light did not reach 100% response to illumination when decapsulated, indicating that activation was dependent on prior dark reactions. About 4500 ft-c–min of fluorescent light energy was required for 100% activation. The effective wavelengths were between 350 and 475 mμ with 2×10^6 ergs/cm^2 sufficient to initiate nearly 100% development at 410 mμ, the most effective wavelength. Low temperature interfered with photoactivation but not with subsequent development. Chilling the ephippia resulted in an increased light requirement. Kinetic studies with chilled ephippia stored for various times in the dark indicated a diphasic process of photoactivation which has tentatively been interpreted as a light-dependent release of inhibition.

INTRODUCTION

The water flea, *Daphnia pulex*, reproduces by two means. In the first, virgin females produce eggs which develop directly in the brood chamber and are released as juveniles. Alternatively, under conditions of crowding and other factors (1, 2, 11) females produce two eggs which are deposited in a specialized portion of the female exoskeleton termed the ephippium from its resemblance to a saddle. Females release the ephippia by rising to the water surface where they remain attached to the surface film until ecdysis by which means they escape down into the medium, leaving the ephippial capsules floating on the water surface. Typically, the ephippial eggs are fertilized by males produced in an earlier generation (9). Males have been observed only rarely in the strain employed in this study. The stock is thus pseudosexual and closely resembles a strain described by Banta (1). The stock was derived from a single female obtained from General Biological Supply Company (Chicago, Ill.) in 1966.

Unlike the brood chamber eggs, ephippial eggs do not immediately complete development but remain arrested, often for many months. Recent studies have demonstrated a light requirement for the initiation of development in these eggs (8, 10, 12). The present report describes the quantitative and qualitative effects of light on the initiation of development in the ephippial eggs of this strain of *Daphnia pulex*.

MATERIALS AND METHODS

The medium used throughout was tap water (Lake Champlain) drawn from the main input into the building and accordingly not exposed to the copper, lead, and tin present in copper pipe installations. The water was stored in polyethylene containers and aerated for at least 24 hr before use. The following culture method has proven successful in producing large numbers of ephippia of high quality. Dried sheep manure was collected from a pasture near Burlington, passed once through the coarse chopper of a Universal food grinder, and added (3 g/liter) to aerated tap water. The mixture was further aerated for 24 hr and inoculated with approximately five adult *Daphnia*/liter. After inoculation, the aeration was reduced to a gentle bubbling just sufficient to maintain a slow circulation of the surface. The cultures were left undisturbed except for the periodic addition of tap water to replace losses by evaporation. The containers were hemispherical plexiglass observation blisters, originally from military aircraft. At 24°C a typical 100 liter culture produced about 6000 ephippia beginning about 20 days after inoculation, with the bulk of the ephippia being deposited over a 4 day period. Photoperiod was fixed throughout at 12L:12D with the light intensity at approximately 100 ft-c at the water surface. The ephippia were collected daily from the water surface, dried overnight on paper towels, and stored dry in shell vials either at 24°C or at 4°C. Dried ephippia are shown in Fig. 1. The ephippia were stored dry for from 1 to 20 days. Experiments were initiated by sprinkling the ephippia on the water surface in widemouthed loosely stoppered jars and storing the jars in the dark (unless otherwise noted).

Method of Activation

In the course of examining the contents of rehydrated ephippia, I noticed that the eggs would develop and hatch if removed from the ephippial case. The significance of this observation will be discussed later in this paper. The operating dishes employed were standard 50 mm Syracuse dishes lined on the bottom with 2 ml of 3% agar prepared fresh each day. 10 ml of water was pipetted into the dish and a group of ephippia transferred to the water surface. Each ephippial case was then grasped at each end with a pair of sharp watchmaker's forceps, submerged, and compressed longitudinally to spring open the valves, releasing the eggs to the agar substrate. Both the decapsulation and subsequent exposure to light were performed on a flat white surface receiving 56 ft-c of white light. Samples averaged 20 eggs each. In all studies with controlled light dosages, the decapsulation was carried out under red light, the particular filter employed cutting off at 600 mμ. All white light sources were GE "Cool White" fluorescent. Monochromatic light was provided by a Bausch

and Lomb high intensity grating monochrometer equipped with a B and L 150 watt xenon light source, with the eggs illuminated from above via a front surface aluminum mirror arranged at 45° to the optical axis. White light intensities were recorded with a Model 200 Photovolt light meter. Monochromatic energies were recorded with a Model 501M Photovolt equipped with a type E sensor. The unit

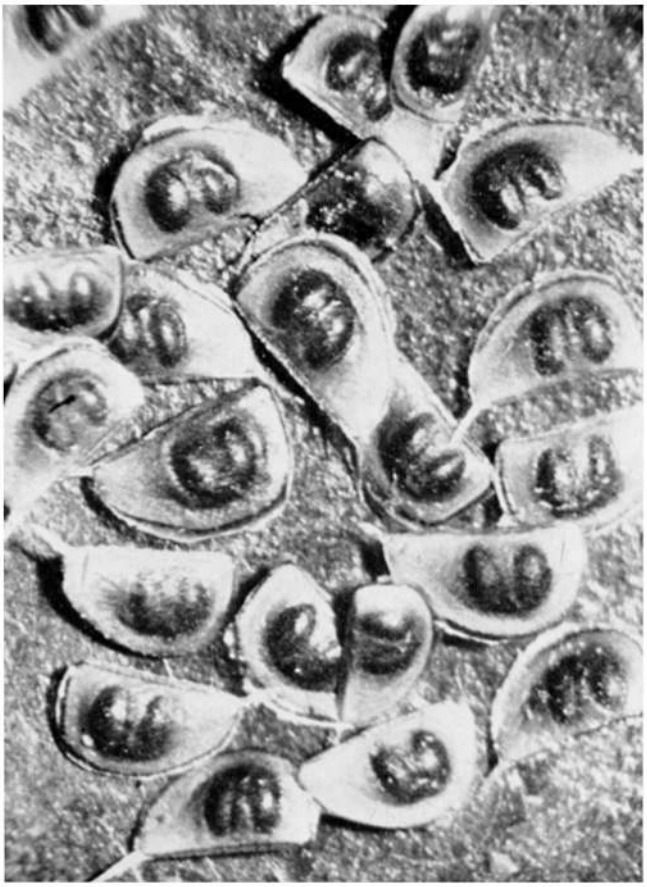

FIGURE 1. Dried ephippia. The capsules are bivalved, tightly sealed, and hinged along the straight margin. The two heavily pigmented areas on each valve mark the position and approximate size of the enclosed eggs. The larger ephippia are approximately 1 mm in length.

had previously been calibrated as a function of wavelength against a Farrand photomultiplier with a National Bureau of Standards thermocouple. Monochromator slits were fixed at 1 mm entrance and exit to give a dispersion of 6.4 mμ. The total energy applied was varied by means of time and intensity for white light, by time alone for monochromatic light. Unless noted otherwise, all material was maintained at room temperature (about 24°C).

RESULTS

Description of Development

At 24°C and 56 ft-c of light, development is complete 55 hr after decapsulation. Hatching results from the gradual swelling of a subchorionic membrane

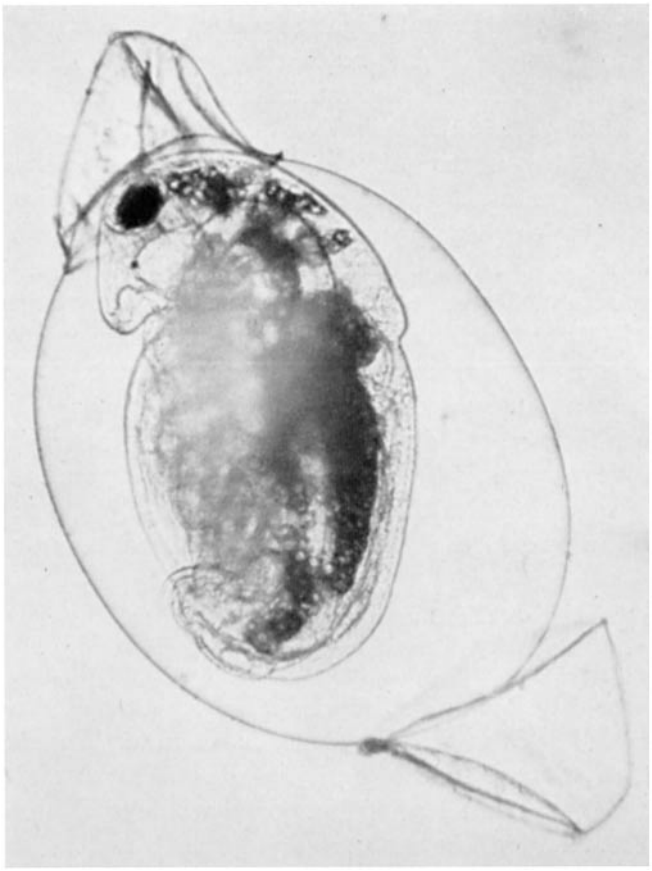

FIGURE 2. An ephippial juvenile about to hatch. The hatching membrane has swollen to its present size after first rupturing the chorion, here visible as the two hemispheres, one at each end of the membrane.

causing first the equatorial rupture of the chorion and terminating with the instantaneous rupture of the membrane after it has swollen to about twice its original linear dimensions. The swelling is clearly osmotic as it may be reversed or retarded by dilute solutions of sucrose. Fig. 2 shows an ephippial individual about to hatch. The terminal phase of swelling and rupture oc-

cupies about 3 hr at 24°C. Rupture of the membrane is apparently spontaneous and dependent on reaching a sufficient degree of stretch. The animals molt a few minutes after escape, acquiring at this molt the caudal spine characteristic of adult *Daphnia*.

Significance of Decapsulation

Since the ephippium is heavily pigmented (Fig. 1), one might suspect that it acts as a barrier to an adequate light stimulus, with decapsulation serving simply to admit sufficient light to reach the eggs. The following experiment shows that this is not the case. The material was rehydrated ephippia stored subsequently for 20 days in the dark. Two groups of ephippia were exposed to 500 ft-c for 3 hr. Then under red light, one group only was decapsulated. A third group of ephippia which had not been exposed was also decapsulated under red light. A fourth group was neither exposed nor decapsulated. All material was then placed in the dark and returned to the light 2 days later to determine the results. Only the exposed and subsequently decapsulated eggs had developed (75 %). The intact, exposed ephippia were observed for several days with no hatching resulting. When these were opened, the eggs were indistingusihable from unexposed eggs. The ephippial case is thus not a barrier to an adequate light stimulus but does constitute a powerful barrier to the completion of development. If we judge from the tightly sealed, heavy walled character of the ephippium, the inhibition is most probably a direct interference with diffusion between the egg and the external environment.

Influence of Length of Time in the Dark

In these experiments, groups of dried ephippia were rehydrated and stored in the dark at zero time. At daily intervals samples were withdrawn, decapsulated, and exposed to 56 ft-c of continuous illumination. Fig. 3 illustrates the relationship between the time in dark storage (abscissa) and the per cent hatching when decapsulated at 56 ft-c (ordinate). Developmental response began within 24 hr and increased in sigmoid fashion to 100 % within 9 days, remaining at 100 % throughout the period of continuous testing (22 days). 100 % activation was observed up to 60 days and possibly continues much longer. A similar result was obtained with undried ephippia transferred directly from the culture to the dark. Accordingly, the response is not dependent on drying. When ephippia were rehydrated and stored at 56 ft-c, the per cent development when they were decapsulated remained low and variable during the 1–10 day period, but increased to 100 % when the material was transferred to the dark. When ephippia previously stored 10 days or more in the dark were transferred to 56 ft-c the percentage activation on decapsulation declined from 100 % to about 60 % after 6 or 7 days. The decline was to about

40 % when they were transferred from the dark to 100 ft-c. These findings support the concept of dark reactions which facilitate maximum response to light exposure when this is coupled with decapsulation. No hatching was observed from intact ephippia whether they were stored in the dark, or at 56 ft-c, or following transfer from the dark to 56, 100, or 400 ft-c.

The hatching among decapsulated samples was remarkably synchronous, the range being typically from 53 to 57 hr following removal from the case. This synchrony suggested a definite light energy requirement for activation.

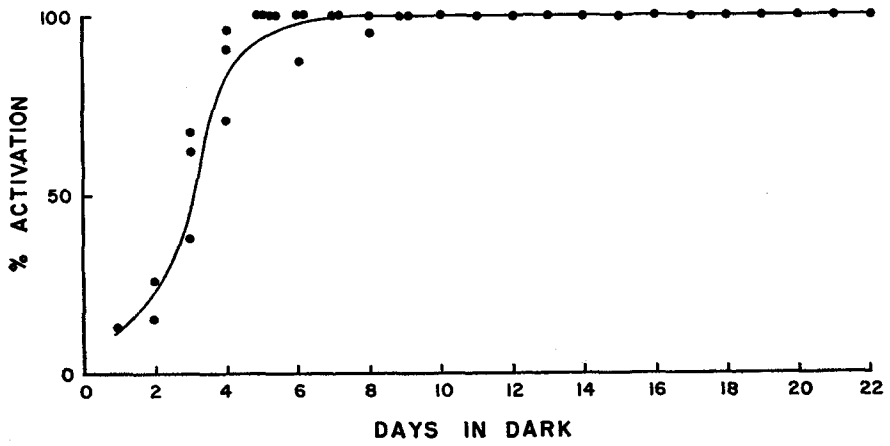

FIGURE 3. The relationship between time in the dark (abscissa) and the % activation when the ephippia were decapsulated at 56 ft-c of continuous illumination (ordinate). Zero on the abscissa marks the time of rehydration of the ephippia. Average sample size was 20 eggs.

Energy Requirement for Activation

These experiments were performed with rehydrated ephippia stored from 23 to 29 days in the dark. Each group was decapsulated under red light and then exposed for measured times to known intensities of white light, after which they were placed in the dark. The dishes were returned to the light 2 days later to determine the percentage of the eggs which had been activated and were now finishing development. Four light intensities were employed: 36, 30, 16.5, and 3 ft-c. Fig. 4 illustrates the response to light as per cent activation (ordinate) vs. energy as foot-candle–minutes (abscissa). About 4500 ft-c–min of energy results in 100 % activation, the effect being independent of the intensity in the range examined. The 36 ft-c point represents several samples, all of which yielded 100 %, while the other points represent individual determinations with an average sample size of 20 eggs. I realize there may be objections to drawing the line as an initial increase followed by a dip prior to reaching 100 %, but subsequent findings reported here indicate that photoac-

tivation is a diphasic process rather than a simple saturation by light. The line in Fig. 4 was drawn by eye to best fit the data recorded from this experimental material. An additional series was made at approximately 400 ft-c and agreed favorably with the data in Fig. 4 but was not included in the figure due to the difficulty in accurately measuring the energy incident on the eggs with the source at close range. It should be noted that 400 ft-c produced no inhibition when applied continuously.

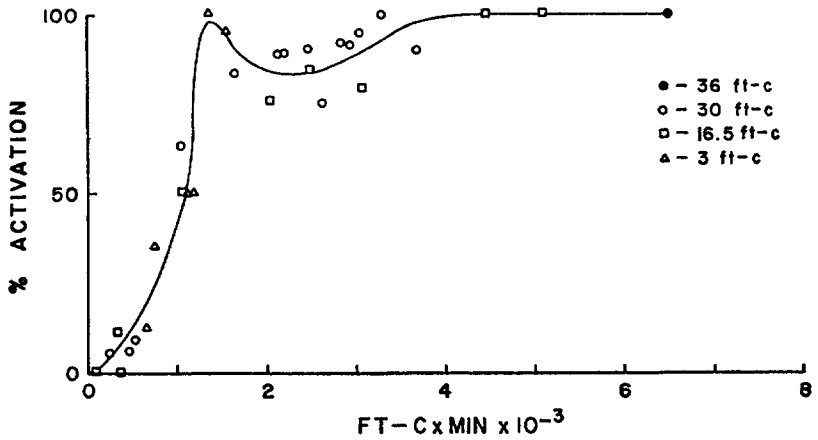

FIGURE 4. The relationship between % activation (ordinate) and the amount of white light energy applied (abscissa). The material was rehydrated ephippia stored between 23 and 29 days in the dark. The response was independent of the intensity in the range 3 to 36 ft-c.

Action Spectrum

These studies were carried out with the same material as in the preceding section except that the ephippia were older, having been rehydrated and stored in the dark for from 30 to 45 days. Preliminary experiments with filters indicated that the most effective wavelengths were near 400 mμ.

The procedure was to decapsulate a sample under red light and then expose the eggs to known amounts of monochromatic energy as calculated from the initial photometer readings. After exposure, a second reading was made of the energy. Determinations in which initial and final values failed to agree within 5 % were not considered. A search was made for the minimal amount of energy which would initiate nearly 100 % development at the most effective wavelength. This was found to be 2×10^6 ergs/cm² at 410 mμ; 4×10^6 ergs/cm² broadened the peak response but did not extend the wavelength range; 1×10^6 ergs/cm² generally lowered the response. Fig. 5 illustrates the response of the eggs to 2×10^6 ergs/cm² (ordinate) as a function of wavelength in mμ (abscissa). The line was drawn through the mean value at each

wavelength. The major peak was at 410 mμ with an apparent minor peak at 450 mμ. The great scatter makes it questionable whether the 450 mμ peak is significant. No activity was observed with 4×10^6 ergs/cm² at 550, 575, 590, and 610 mμ. No attempt was made to evaluate the effects of higher wavelengths since prolonged exposure to red light had previously proved ineffective.

Temperature Effects

The material utilized so far in this paper was maintained throughout at room temperature (about 24°C). This includes not only the temperature for ephippial production but also for dry storage and rehydration in the dark. The effects of low temperature on the reaction system are complex and must await future study for clarification. However, certain generalizations may be drawn at this time based on the following observations.

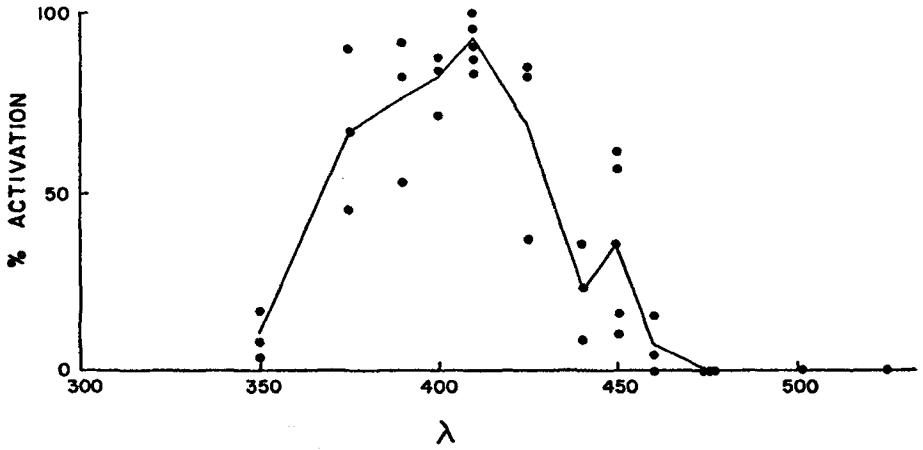

FIGURE 5. The % activation (ordinate) vs. the wavelength in millimicrons (abscissa). Each point represents the % activation of a sample of eggs which received 2×10^6 ergs/cm² of monochromatic energy at each wavelength. Note the major peak at 410 mμ. See text for details.

Ephippia were rehydrated and stored for 20 days in the dark, then transferred to a temperature of 11°C, and stored 2 more days in the dark. They were then decapsulated and exposed to continuous illumination, all at 11°C. Only 3 % of the eggs were activated, while 100 % of the room temperature controls were activated when decapsulated and exposed, all at 24°C. Further, when the 11°C ephippia were returned to room temperature, they were found to be relatively refractory to light, requiring more energy for activation when decapsulated. Thus, the low temperature regime had altered the response of the eggs to light. The following experiment reveals some information on the target of the temperature effects.

Rehydrated ephippia (stored 20 days or more in the dark at 24°C) were decapsulated and the eggs exposed for 3 hr to 36 ft-c, all at 24°C. Samples averaging 25 eggs each were then placed in the dark at 11°, 13°, 17°, 20°, 24°, and 30°C. At 24°C and lower, 100 % hatching occurred, while at 30°C 26 % of the eggs completed development. Apparently, low temperature interferes with the photoresponse of the eggs but not with their subsequent development, while 30°C interferes with development proper. Fig. 6 illustrates the relationship between rate of development (ordinate) vs. temperature (abscissa) with eggs all photoactivated at 24°C. Rate is defined as the reciprocal of the time in

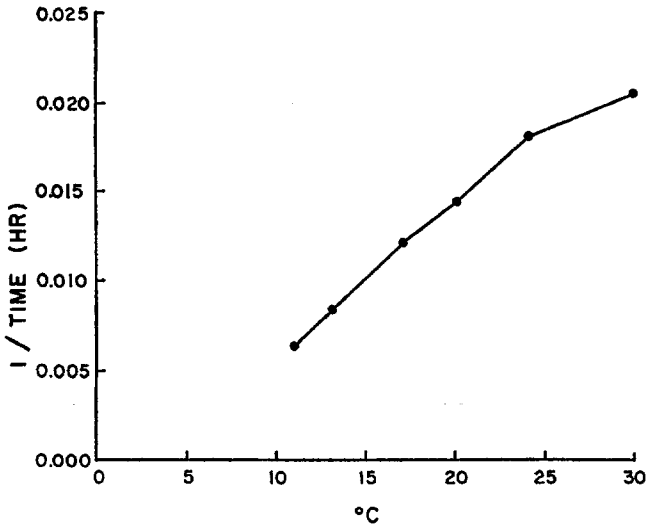

FIGURE 6. The influence of temperature on the rate of development. All material was photoactivated at 24°C and then placed at either 11°, 13°, 17°, 20°, 24°, or 30°C. The ordinate is the reciprocal of time in hours between photoactivation and hatching.

hours from photoactivation to 50 % hatching. The temperature curve is curious in exhibiting a nearly linear relationship between rate and temperature in the range 11° to 24°C.

Results with Chilled Dried Ephippia

As indicated earlier, dried ephippia were stored either at 24°C or at 4°C. When 4°C ephippia were rehydrated and stored in the dark at 24°C, the photoresponse on decapsulation was found to be markedly different from that of eggs maintained at 24°C throughout. The chilled eggs required about twice as much light energy to reach 100 % activation as did eggs stored at 24°C (Fig. 4). Figs. 7 and 8 summarize the kinetics of activation with chilled ephippial eggs. The material was rehydrated ephippia stored in the dark at 24°C for from 17 to 26 days. The light response was determined on days 17, 20, and 26

at 45 ft-c illumination (Fig. 7) and on day 22 at 400 ft-c illumination (Fig. 8). About 10,000 ft-c–min of energy were required to reach 100 % activation in contrast with about 4500 ft-c–min for unchilled eggs (Fig. 4). While the energy requirements for the final approach to 100 % were independent of the storage

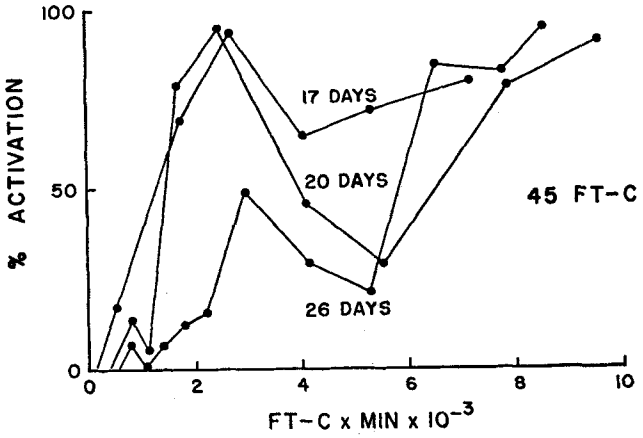

FIGURE 7. The relationship between % activation (ordinate) and the amount of white light energy applied (abscissa). The material is comparable with that represented in Fig. 4 except that these ephippia were stored at 4°C while dry. The days represent the time in storage in the dark at 24°C. Light intensity was 45 ft-c. Note that the kinetics change with time of storage. See text for details.

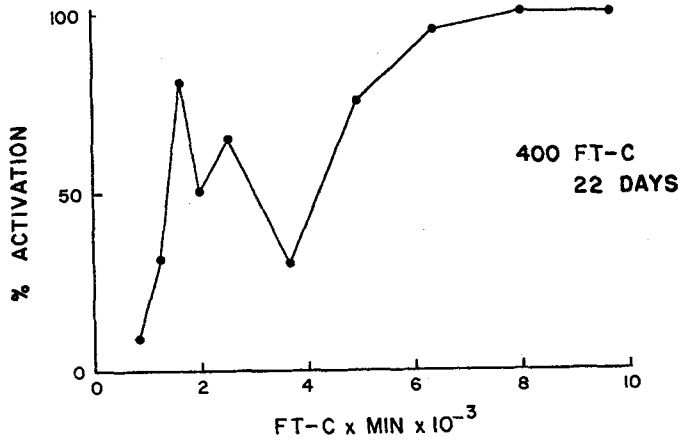

FIGURE 8. The same material as in Fig. 7, with the response recorded after 22 days of storage and at 400 ft-c. See text for details.

time, the response to lower energy varied with the storage time, in each case consisting of an initial increase followed by a decline before the final increase to 100 % activation. As time in storage increased, the height of the preliminary peak declined and the subsequent decline became more pronounced. Note

that the effect was basically the same both at 45 ft-c and 400 ft-c. These data suggested a two-step process of photoactivation, a possible mechanism for which is developed in the discussion.

DISCUSSION

Hypothetical Scheme for Activation

The following discussion is based largely on the kinetic evidence for egg activation derived from chilled ephippia (Figs. 7 and 8). Two alternative hypotheses are considered.

1. Light causes the release of some stored factor essential for development but inhibitory at high concentration, with a low energy requirement for decomplexing the factor. Higher energies either photoinactivate or photoconvert the substance to a noninhibitory form, thus reversing the inhibition. There is some evidence against this interpretation. Notice in Fig. 7 that the first or low energy peak in activation becomes suppressed as the length of time for storage in the dark increases. If the only requirement were the release of an adequate amount of the necessary factor, all the low energy peaks would have the same height, in principle 100 %. Such is not the case.

The second hypothesis overcomes this objection by assuming only a release of inhibition as follows.

2. An active site is covered with the factor which accumulates in complexed form as the length of time in the dark is increased. Low levels of light energy decomplex the factor, exposing the active site but, as in the first hypothesis, with inhibition at a high concentration of the free factor. Higher energy levels serve either to photoinactivate the factor or otherwise convert it into a noninhibitory form, thus relieving the inhibition. This hypothesis has the advantage of accounting for the progressive decline in the low energy peak as well as its shift to higher energy levels, the idea being that the active site becomes more and more effectively blocked with the factor as time in the dark increases.

Either hypothesis must assume that the decomplexed as well as the complexed factor has similar if not identical absorption properties since final activations of 100 % have been obtained with monochromatic light at both 410 and 450 mμ. Although not presented in detail at this time, monochromatic kinetics at 410 and 450 mμ resemble the white light data presented in Figs. 7 and 8. Unfortunately, neither of the hypotheses is entirely satisfactory, the first for reasons already discussed and the second for the following reasons. If activation were simply a release of inhibition, then 100 % activation would be obtained from ephippia stored in the light and then decapsulated. However, as pointed out earlier, when dark-adapted ephippia were transferred to con-

tinuous light, on decapsulation the percentage development declined with time. Similarly, ephippia rehydrated and stored in the light do not reach 100 % activation at 56 or 100 ft-c illumination. It is necessary to postulate either dark preparatory reactions independent of the primary photoreaction process or alternatively to postulate that photoreaction involves a simultaneous release of inhibition and activation. The complex nature of the activation offers a possible explanation for the scatter characteristic of the action spectrum (Fig. 5). It is possible that further studies with interrupted light or inhibitors may reveal more about what is clearly a complex process.

Nature of the Receptor

At present very little can be concluded from the observation of a peak response at 410 mμ. Both the range and the peak of the present system resemble positive and negative phototaxic movements in *Euglena* strains both possessing and lacking eyespots (5). This and related papers have been reviewed by Clayton (3). Unfortunately the nature of the photoreceptors remains unknown.

The older literature dealing with crustacean pigments has been reviewed by Goodwin (4). More recently Herring (6, 7) has published an extensive analysis of carotenoid metabolism in *Daphnia magna*. One argument against carotenoids or their derivatives as receptors is offered by Herring's conclusion that aside from vision there is apparently no carotenoid requirement in *Daphnia magna*. However, if light activation of the ephippial egg proves to be as suggested here a release of inhibition, the possibility remains that carotenoids or their derivatives, while not necessary for growth and differentiation, may be required for light-initiated development. In short, and this point has not been settled, carotenoid-free ephippial eggs may not require light for the initiation of development. Experiments are in progress to resolve this question.

Significance of Ephippial Activation

It is impossible to directly compare these findings with those from the literature as earlier studies were made with eggs contained in intact ephippia. In general, these earlier studies have shown that a long period of storage is required before development may be initiated in ephippial eggs (8, 12). As a result, the production of ephippial eggs and their subsequent activation have been regarded as examples of diapause induction and release, respectively (11, 12). However, it is clear from the present study that a primary barrier to development resides in the ephippial case proper rather than in any intrinsic mechanism of the egg. The capacity to complete development is present from the very beginning, the only requirements being the release from the capsule and illumination following an adequate period in the dark—9 days for 100 % activation (Fig. 3). It should be noted that some activation (about 15 %, Fig. 3) may be obtained from eggs only 48 hr in total age, based on the fact that

ephippia collected at 24 hr intervals can be no older than 24 hr at the time of collection and exhibit 15 % activation after 24 hr of rehydration. A similar result has been obtained with undried ephippia transferred directly to the dark at the time of their collection. With respect to photoperiodic control of the mechanism, it should be noted that 100 % activation was obtained from eggs which had received only two light stimuli, the first at the time of their production and the second at the time of their decapsulation and subsequent exposure, the entire intervening period having been spent in darkness. It is thus unnecessary to postulate in a strict sense a phasic or photoperiodic control of the mechanism. However, it is certainly possible that under natural conditions seasonal variations in photoperiod could be instrumental in controlling activation. It is of some interest to note that chilling the ephippia results in an increased light requirement, which might be effective in preventing activation during subsequent short day periods. The evaluation of this aspect of the problem is difficult due to an inability to evaluate the energies reaching the eggs contained in intact ephippia, especially since the capsules vary substantially in their pigmentation.

Since the ephippial eggs may be dried, it is clear that a primary significance of this stage in the life history resides in the capacity to repopulate temporary bodies of water. To be effective in such an adaptation it is important that development not proceed immediately, or even synchronously after a long period of storage. Synchronous activation could be disastrous to a temporary pond population if a second dry period happened to precede ephippial egg production by the population. By constituting a very effective and probably variable obstacle to development, the ephippial capsule acts to prevent highly synchronized activation; hence a reserve source of developmental potential is preserved. The production of ephippial eggs by a crowded population permits the individuals to continue their reproductive activity but at the same time to seal off the products so that they do not contribute further to the competition for limiting factors in the environment. Viewed in this way, ephippial production becomes an important factor in the regulation of population density as well as the sole mechanism for reproductive continuity in a periodically unfavorable environment.

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