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Running head: PROTOCHLOROPHYLL CONVERSION IN BARLEY

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# The Sites of Photoconversion of Protochlorophyllide to Chlorophyllide in Barley Seedlings[1]

[1]The work described in this report was carried out in the Department of Chemistry, Middle East Technical University, Ankara, Turkey.

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# ABSTRACT

The photoreduction of protochlorophyllide *a* to chlorophyllide *a* in intact 6-day old seedlings of etiolated barley (*Hordeum vulgare*) exhibits a small initial phase, followed by an induction period of about 1 hour before a rapid phase of additional chlorophyll formation begins. Cycloheximide, an inhibitor of protein synthesis has no effect on the initial phase of conversion of pre-formed protochlorophyllide, but it either abolishes or severely inhibits the subsequent phase of rapid chlorophyll synthesis within 45 minutes of its application to the seedlings. An analysis of the biphasic inhibition process suggests that the lifetime of the enzyme controlling protochlorophyllide synthesis (probably δ-aminolevulinic acid synthetase) is not longer than 10 minutes.

The rapid phase of chlorophyll formation can be effected by a series of brief (15 second) pulses of light spaced at least 5 minutes apart. When longer dark intervals are used, no increase is observed in the yield of chlorophyll per pulse. We interpret the findings to indicate that the photoconversion takes place at distinct enzymatic sites whose concentration does not increase during a period of four hours following the initial illumination. The sites can be used repeatedly with a turnover time determined by the removal of the product chlorophyllide and the synthesis and placement of a new protochlorophyllide molecule.

The penultimate stage in the synthesis of chlorophyll a in most higher plants is a photochemical step in which two protons are added stereospecifically to the porphin ring system of protochlorophyllide to form chlorophyllide a. The initial photoconversion can be monitored spectrophotometrically in intact etiolated leaves.[22] There follows a series of dark spectral shifts, during which the chlorophyllide is esterified by phytol to form chlorophyll a.[24,34] These reactions have the characteristics of an enzymatic process even apart from the stereospecific nature of the product. They are abolished by mild heating (52°C for 10 minutes)[23,29], grinding the leaf tissues with sand and buffer[5], application of a freeze-thaw cycle[9,29] and extraction of the pigments by organic solvents. Nevertheless, the initial photochemical stage can be completed within a few milliseconds by a brief flash of activating light[16,17] or, albeit slowly, by illumination at -80°C.[10] In this paper we present evidence that the number of photoconversion sites in a greening seedling does not change significantly during the first 4 hr following the initial illumination. Within this interval the photoconversion

sites can be used more than 20 times in succession.

Following an initial conversion of active protochlorophyllide present in dark-grown seedlings, there commonly follows an induction period of 1 hr or more preceding a rapid phase of further chlorophyll synthesis.[1,33] During the induction period the synthesis of protochlorophyllide is limiting, and this limitation can be overcome by feeding the plants with δ-aminolevulinic acid, a precursor of protochlorophyllide.[25] Studies using chloramphenicol[18,19] or cycloheximide[13], which are known to be inhibitors of protein synthesis, indicate that active protein synthesis is normally required in order for protochlorophyllide to be formed and sited on the enzyme where photoconversion to chlorophyllide takes place.[13,20,26,28] The action of these and other inhibitors of chlorophyll synthesis has recently been reviewed.[14,27]

The studies of the action of cycloheximide on etiolated barley seedlings described in this paper show that the inhibitor does not prevent the initial conversion of preformed protochlorophyllide, even when the seedling is exposed to the inhibitor for as long as 48 hr prior to the first illumination. On the other hand, it is highly effective in abolishing chlorophyll synthesis during the subsequent post-induction phase. During this period it acts as soon as 45 min following its application to seedling tips.

## MATERIALS AND METHODS

Barley [*Hordeum vulgaris*, var. Atlas] seeds were germin-

ated and grown in complete darkness in distilled water on cotton and filter paper. Water was added every second day. The leaves were harvested under a dim green safelight when they were 6 days old, and the upper 3 cm was used in the studies to be described. For spectrometric measurements the tightly-curled leaves were flattened between two microscope slides.

Absorption spectra of the mounted leaf segments were measured by placing them directly in front of the photomultiplier of a Unicam SP800 UV-Visible Spectrophotometer. The large photosensitive surface of the end-window photomultiplier enabled reasonably sharp spectra to be obtained for the leaves, despite their pronounced light scattering. The amounts of protochlorophyllide and chlorophyll(ide) were estimated from the magnitudes of the absorbance peaks at 650 and 683 (678) nm, respectively.

Solutions of cycloheximide (0.5 mg-ml$^{-1}$; Upjohn, Kalamazoo, Michigan) and chloramphenicol (0.5 mg-ml$^{-1}$; as succinate, Na salt, Lepetit) in water were prepared before the start of each experiment and stored at 0°C until used. The inhibitors were applied by floating the leaf segments on 10 ml of the solution in covered petri dishes.

Continuous illumination was provided by a bank of five fluorescent lamps (General Electric, 20 watt, 820 lumens, daylight) at a distance of 30 cm from the samples. Brief pulses of illumination were provided by a hand torch held 15 cm from the samples and providing an incident intensity of about

25 foot-candles. A duration of 15 sec was sufficient to saturate short-term protochlorophyllide conversion. To provide reproducibility for the spectral measurements, the leaves were illuminated in the sample compartment of the spectrophotometer and were left *in situ* during the dark intervals between illumination pulses.

## RESULTS

*Inhibition of Chlorophyll Formation by Cycloheximide*. In preliminary experiments using equal concentrations (0.5 mg-$ml^{-1}$) of cycloheximide or of chloramphenicol, the cycloheximide was found to be the more potent inhibitor of chlorophyll formation during extended illumination of etiolated barley seedlings. For this reason cycloheximide was chosen for the experiments which follow.

The normal course of protochlorophyllide to chlorophyll conversion, in the absence of any inhibitor, produced a small initial absorption peak at about 678 nm upon illumination with white light. Associated with a 15 sec illumination with either weak (25 ft-c) or strong (2000 ft-c) light were the following absorbance changes: $\Delta A_{678} = +0.02 \pm 0.005$ and $\Delta A_{650} = -0.01 \pm 0.005$ at the chlorophyll and protochlorophyllide maxima, respectively. Following a lag period of about 1 hr, a rapid phase of chlorophyll synthesis began and after 6 hr of strong illumination $\Delta A_{678} = 0.72$ was obtained.

The effect of the time of cycloheximide application was studied using a regimen in which leaves were exposed to

cycloheximide at different times (0 to 48 hr) before the first illumination. Following a 6 hr period of strong illumination, the absorption spectrum of each leaf was recorded.

[Table I] The results are summarized in Table I. Although some effect of the time of application of inhibitor relative to the time of first illumination was observed, two important findings can be seen: (1) Even when the inhibitor was applied at the same time as the first illumination, there was over 90% inhibition of chlorophyll formation during 6 hr illumination. (2) Continuous application of the inhibitor during 48 hr prior to (and during) the illumination did not abolish completely the formation of chlorophyll in the light.

In order to estimate the time required for cycloheximide to act, we illuminated leaves continuously for five hours and then transferred them in the light to a solution containing 0.5 $mg\text{-}ml^{-1}$ of cycloheximide. Leaves for a control were left under the original conditions (distilled water). Samples were removed at intervals (3 leaves per sample) and their absorption spectra recorded. The results of this experiment are shown

[Fig. 1] in Figure 1. Within the reproducibility of the data, the cycloheximide appears to act very quickly (within 1 hour) to block completely the further formation of chlorophyll. It is significant that neither an increase nor a decrease in chlorophyll absorption was observed during the next 19 hr, whereas the chlorophyll in the control leaves increased steadily to a point beyond which the absorbance at 678 nm could not be measured accurately. The time of action of

cycloheximide is measured somewhat more accurately in an experiment described below.

Pulsed Illumination Studies. Because of our concern over possible adverse effects resulting from the high illumination intensity of the experiments described above, the studies to be described now were carried out using relatively low intensity (25 ft-c) white light. A pulse duration of 15 sec was found to saturate the photoconversions and was adopted as a standard period of illumination.

Etiolated leaves were given a pre-illumination pulse and then allowed to stand in the dark for periods of 1 to 5 hr before application of a second identical pulse. Absorption spectra were recorded before and after each pulse. In each case an absorbance increase $\Delta A = +0.02 \pm 0.005$ at 678 and an absorbance decrease $\Delta A = -0.01 \pm 0.005$ at 650 nm were observed for each of the two pulses. Within the precision of the measurements, the yields were found to be the same for the first and the second pulse, regardless of whether the dark interval between pulses was 1, 2, 3, 4, or 5 hr. There was no evidence of any additional convertible protochlorophyllide formed after the first hour of darkness.

In order to determine the turnover time of the photoconversion process during the rapid phase of chlorophyll synthesis following the induction period, a series of spaced pulses was applied to a single leaf beginning 1 hr (in darkness) after a pre-illumination pulse. Spectra of the leaf were measured between each pair of pulses, and the specimens

remained in position in the spectrophotometer compartment during the entire course of each experiment. The results
Table II] of these experiments are summarized in Table II. The yields per pulse are presented as average values over several pulses (in order to increase the precision). Apart from the first two pulses, each of which occurred following a long dark interval, no significant trends were noted within a single sequence. The results show that for dark intervals between 5 and 20 min the yield of chlorophyll per pulse is independent of the duration of the interval. Shorter dark intervals produce a regular decrease in the yield per pulse, reaching half the maximum value at an interval of 2.0 to 2.5 min of darkness. For the shortest intervals studied the 15 sec period of illumination is not brief enough to be ignored. Nevertheless, the results point to the facts that the half-time for turnover of the photoconversion is about 2.5 min and that dark intervals longer than 5 min do not produce any increase in the amount of photoconvertible protochlorophyllide. Furthermore, the rate of chlorophyll formation using 15 sec pulses spaced at 5 min intervals, $\Delta A_{678} = 0.015$ per pulse, is comparable with the steady state rate observed under continuous, higher intensity illumination (Fig. 1) of $\Delta A_{678} = 0.016$ per 5 min.

The first two pulses in each experiment described in Table II appear to produce somewhat larger yields per pulse than do those of the subsequent sequence. The effect appears to be real, but is barely outside the experimental uncertainity.

Thus, there may be a small increase in the photoconvertible protochlorophyllide produced during prolonged dark periods, but further experiments are required to document this difference.

Time of Action of Cycloheximide Using Pulsed Illumination. The effect of single pulses of light on one leaf could be more easily observed than could the increase in chlorophyll content in different leaves under continuous illumination. Therefore, we used the pulse scheme to study the time of action of cycloheximide during the phase of rapid chlorophyll synthesis. For this experiment a leaf was illuminated continuously (strong light) for 3 hr, its spectrum was measured, and then it was transferred to cycloheximide (0.5 mg-ml$^{-1}$) in the dark. Three 15 sec pulses of weak light were given at 5 min intervals. The leaf was then removed and mounted in the spectrophotometer. The total exposure to cycloheximide was 15 min, but no attempt was made to wash off the adhering solution prior to the transfer to the spectrophotometer. The pulses were then continued at 5 min intervals, and the spectrum was recorded in each interval

Fig. 2] between pulses. The results are presented in Figure 2. During the first 45 min (9 pulses) following the application of the cycloheximide, the yield per pulse was not significantly different from that in the absence of cycloheximide. After the ninth pulse the production of chlorophyll ceased abruptly. The sharp transition observed suggests that the inhibition process occurs in two stages.

# DISCUSSION

Inhibitors of protein synthesis, such as chloramphenicol or cycloheximide, have been shown to block the formation of chlorophyll in algal systems and in higher plants.[13,14,19,20] Two mechanisms have been proposed to account for the action of these inhibitors. In one of these the inhibitor is thought to block the synthesis of enzymes needed to form the precursors of protochlorophyllide.[6] Feeding experiments indicate that the blockage occurs early in the biosynthetic pathway, prior to the formation of δ-aminolevulinic acid.[7,8,20] The second mechanism, favored by Kirk[12,13], proposes that the synthesis of new protein is required for the incorporation of chlorophyll into the growing lamellar structures of developing plastids. Because of conflicting reports of the ability of δ-aminolevulinic acid to overcome the inhibition in different organisms, it is difficult at the present to formulate a single hypothesis encompassing all of the findings.

The results summarized in Table I show that the protochlorophyllide formed in the dark in etiolated barley seedlings is capable of being photoconverted into chlorophyll in the presence of 0.5 $mg\text{-}ml^{-1}$ of cycloheximide, even when the inhibitor was applied as much as 48 hr prior to the first illumination. As the time of prior application is shortened, the initial yield of chlorophyll increases somewhat owing to the increased content of protochlorophyllide accumulating in the seedlings between 4 and 6 days old. We conclude from these observations

that the active protochlorophyllide formed in the dark-grown plastids is in a very stable structure and does not require ongoing synthesis of protochlorophyllide in order to retain its activity over a period of two days.

When cycloheximide is added 3 hr after the start of illumination there is no significant decrease in chlorophyll formed during the next 45 min. Then, during the next 10 min the activity falls to zero and no further chlorophyll is formed (Fig. 2). Whether the inhibitor stops protein synthesis quickly and leaves a substantial excess of the essential enzyme(s) or, alternatively, a period of 45 min is required for the inhibitor to act, it is evident that we are dealing with a short-lived (~10 min) enzyme that requires constant and active re-synthesis in order to maintain chlorophyll formation in the light.

The postulate, referred to in the Introduction, that protophotochloride is converted at specialized sites of an enzymatic nature was supported by the isolation of soluble protochlorophyllide-protein complexes which retain the ability to carry out the photoconversion.[2,15, 21, 30] Because of the low concentration of these complexes relative to the amount of chlorophyll synthesized during the first two days of illumination of etiolated seedlings, it is reasonable to suppose that the same photoconversion sites are used repeatedly in building up the chlorophyll content. Both Boardman[3] and Bogorad, et al.[4] have presented evidence that the chlorophyll, once formed,

is translocated to a separate macromolecular structure which sediments in the ultracentrifuge differently from the protochlorophyllide protein. Bogorad, et al.[4] discuss several possible models for the photoconversion and translocation process. Further support for a limiting number of photoconversion sites was provided by Sundqvist[31] who found that the presence of an excess of protochlorophyllide, resulting from feeding the leaves with the precursor δ-aminolevulinic acid, does not increase the yield of chlorophyllide over that obtained during a brief illumination of untreated leaves.

In the studies that we have carried out, we find that the yield of chlorophyll formed using brief saturating pulses of illumination given directly to a dark-grown barley seedling is nearly the same as the yield per pulse during the phase of rapid chlorophyll synthesis following the induction period. During the rapid synthesis phase a dark interval of 5 min between light pulses is sufficient to saturate the amount of protochlorophyllide produced and longer dark intervals do not increase the yield of chlorophyll per pulse. The half-time for steps required to replace a converted protochlorophyllide molecule on the active site is about 2.5 min. A similar conclusion can be drawn from the rate of active protochlorophyllide synthesis in the dark following brief illumination of wheat seedlings.[32] We find that this process can be repeated at least 20 times without any increase or decrease in the yield per pulse. The turnover time of 2.5 min is significantly longer than the 20 sec required for the regeneration of active

protochlorophyllide in bean leaves which had been fed δ-aminolevulinic acid and had built up a pool of inactive protochlorophyllide in the dark.[11] Thus, it appears that about 2 min is required for the biosynthesis of protochlorophyllide from its precursors and an additional 20 sec for the placement of the molecule on the photoconversion site.

Our findings are entirely consistent with the postulate that the number of protochlorophyllide photoconversion sites remains constant during the first few hours of illumination of etiolated barley seedlings, and that the same sites can be used repeatedly for the photoreduction.

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Table I. The Effect of the Application of Cycloheximide Before and During Illumination of Etiolated Barley Leaves. [Cycloheximide concentration 0.5 mg-ml$^{-1}$]

| Time of Application of Cycloheximide (hours) | Absorbance of Maximum at 678 nm after 6 Hours of Illumination | % Inhibition of Chlorophyll Formation (relative to control minus cycloheximide) |
|---|---|---|
| 0 | 0.05 | 93 |
| -2 | 0.04 | 94.5 |
| -4 | 0.03 | 96 |
| -6 | 0.02 | 97 |
| -8 | 0.02 | 97 |
| -12 | 0.01 | 98.5 |
| -24 | 0.01 | 98.5 |
| -48 | 0.01 | 98.5 |
| Control | 0.72 | ---- |

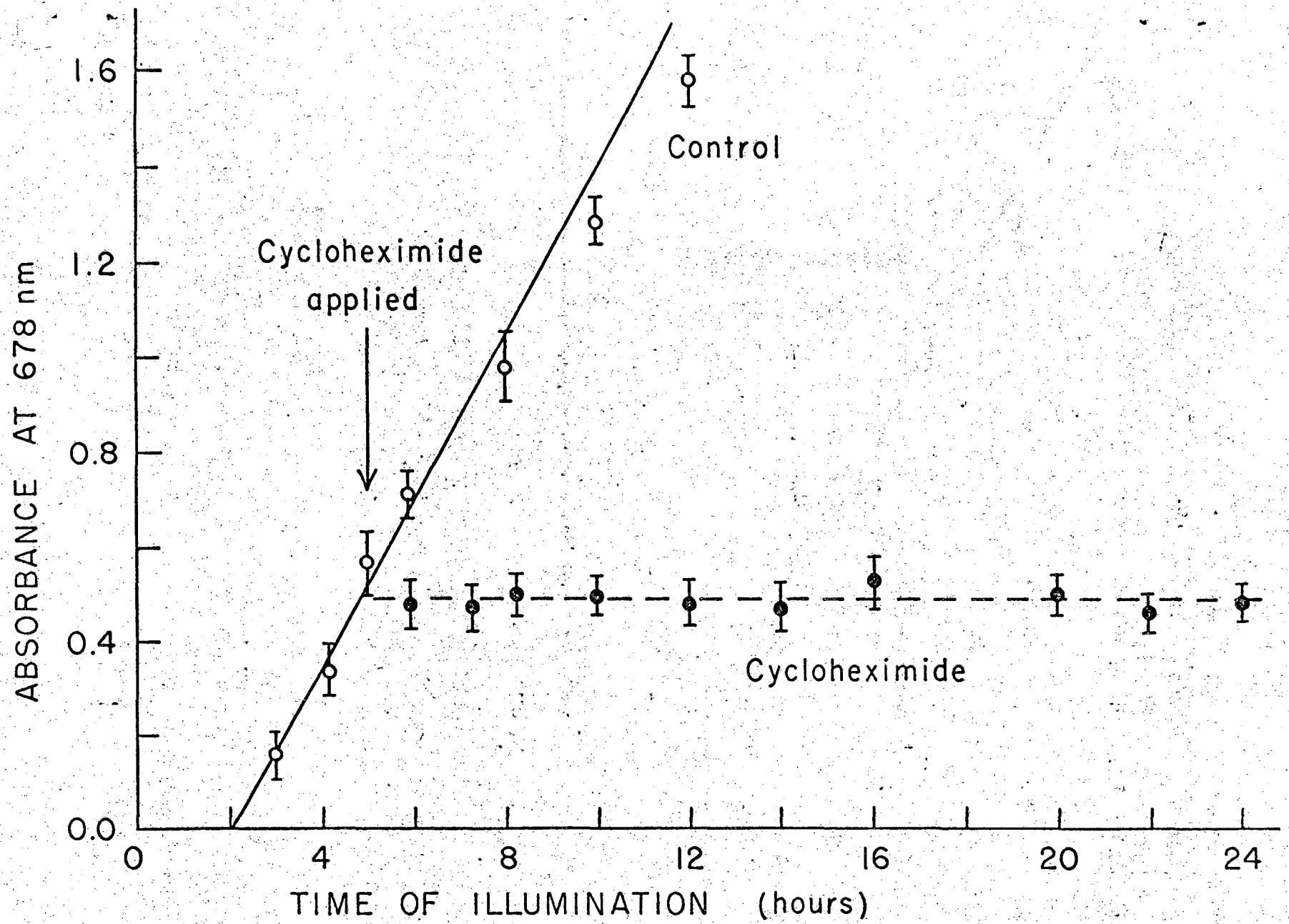


Fig. 1

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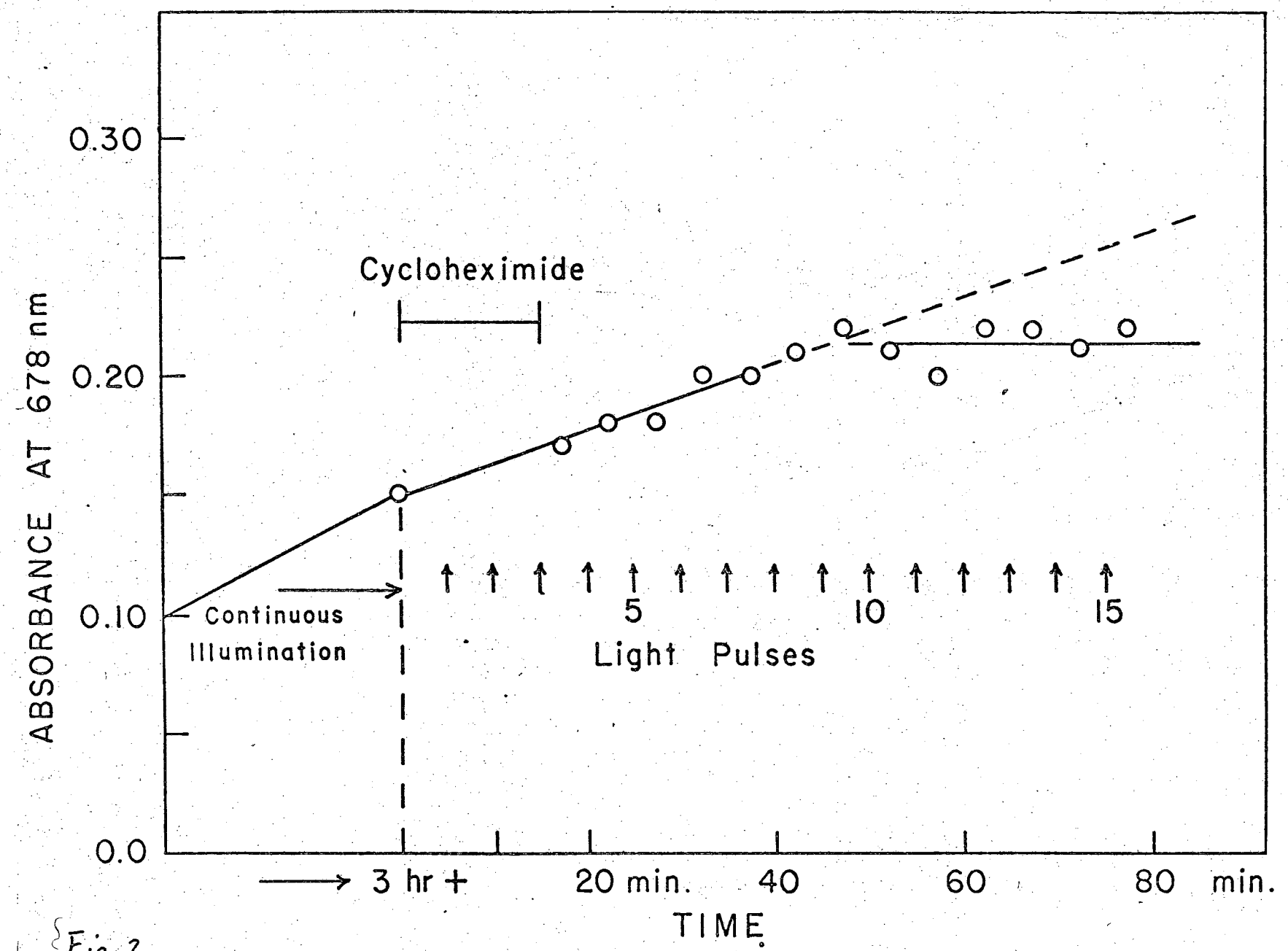


Fig. 2

XRI 7012-4950

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