DEPENDENCE OF CHLOROPLAST PIGMENT SYNTHESIS ON PROTEIN SYNTHESIS : EFFECT OF ACTIDIONE

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The formation of chlorophyll by etiolated plant cells in the light is sensitive to inhibitors of protein synthesis. For example, the greening of interior leaves of lettuce (von Euler, et al., 1948) or of dark-grown cells of Euglena gracilis (Kirk, 1962a, 1962b; Huzisige et al., 1957), is inhibited by streptomycin. Chloramphenicol blocks light-induced chlorophyll synthesis in etiolated bean leaves (Margulies, 1962; Gassman and Bogorad, 1965) and in dark-grown cells of Euglena gracilis (Pogo and Pogo, 1965). Greening of bean leaves is also inhibited by puromycin (Gassman and Bogorad, 1965). In the present study we have made use of actidione, which is a potent inhibitor of protein synthesis in cells of yeast (Kerridge, 1958; Siegler and Sisler, 1964a, 1964b) and higher animals (Bennett et al., 1964; Wettstein et al., 1964; Colombo et al., 1965), to study the relationship between chloroplast pigment synthesis and protein synthesis in Euglena gracilis. It appears that chlorophyll a synthesis is completely dependent upon protein synthesis, and it is suggested that this is not solely the result of a requirement for formation of the biosynthetic enzymes. Carotenoid formation appears to be much less dependent on protein synthesis than is chlorophyll formation.

METHODS

Cells of Euglena gracilis, strains 1224/5z (Z strain) and 1224/5g (G strain), from the Culture Collection of Algae and Protozoa, Botany School, Cambridge, were grown in the light or dark as previously described,

(Kirk, 1962a). Etiolated cells were washed twice with 0.15M NaCl, and suspended, at 1.0 to 2.0 mg. dry wt./ml., in 5 ml. 0.04M phosphate buffer, pH 7.0, containing 0.001M $MgSO_4$, plus additions as indicated, for greening experiments. All incubations were carried out in duplicate or triplicate in 12 ml. glass centrifuge tubes. Light and dark incubations were carried out at 30°C in a rotary illuminating apparatus with magnetic stirring (Kirk, 1962a). In experiments on leucine incorporation, 1-^{14}C-DL-leucine (50 µg and 0.01 µC/ml. was included in the medium. Cell fractionation and estimation of radioactivity were carried out as already described (Kirk, 1962b).

Pigments were extracted with 80% aqueous acetone; the absorbancy of the acetone extracts was measured at 663 mµ, 645 mµ and 480 mµ with the Hitachi Perkin-Elmer 139 spectrophotometer.

For a simple comparison of the amounts of chlorophyll a formed under different conditions, the absorbancy at 663 mµ was used: since the ratio of chlorophyll a to chlorophyll b is about 5, approximately 97% of the absorbancy at this wavelength is due to chlorophyll a. The increase in absorbancy at 480 mµ during greening is largely due to carotenoid synthesis, but there is some contribution from chlorophyll a and b. When the absorbancies at 663 mµ and 645 mµ are known, the extinction coefficients of chlorophylls a and b at 480 mµ (Mackinney, 1941) can be used to calculate the contribution of the chlorophylls to the absorbancy at 480 mµ. To permit calculation of the increase in absorbancy at 480 mµ which is due to carotenoid synthesis ($\delta A_{480}^{car.}$), the following equation has been derived:

$$\delta A_{480}^{car.} = \delta A_{480} + 0.114\ \delta A_{663} - 0.638\ \delta A_{645}$$

δA_{480} is the observed increase in absorbancy at 480 mµ, and δA_{663} and δA_{645} are the increases in absorbancy at 663 mµ and 645 mµ, respectively.

RESULTS

Effect on growth. Growth of E. gracilis, strain Z in the light was abolished by actidione at concentrations of 30 µg/ml. and above. At antibiotic concentrations of 10 and 3 µg/ml., growth (estimated turbidi-

metrically) was inhibited by 97% and 70% respectively. At 1.0 and 0.3 µg/ml growth was almost normal. Cells grown in the presence of the antibiotic contained nearly normal levels of chlorophyll. Cells from the tube containing 3 µg/ml. of actidione grew up with normal chlorophyll level on subculture into drug-free medium.

Effect on chlorophyll a and protein synthesis. Chlorophyll a synthesis by etiolated cells of the Z strain incubated for 4 hours in the light is virtually abolished by actidione at concentrations of 3 µg/ml. and above (Table 1).

TABLE 1. EFFECT OF DIFFERENT CONCENTRATIONS OF ACTIDIONE ON CHLOROPHYLL a SYNTHESIS AND ^{14}C-LEUCINE INCORPORATION. Conditions as stated in text.

Actidione, µg/ml.	-	0.32	1.0	3.2	10	100
% Inhibition of chlorophyll synth.	-	45	73	100	100	100
% Inhibition of leucine incorp.	-	50	53	70	80	86

Incorporation of 1-^{14}C-leucine into material insoluble in acetone or cold acid is also drastically inhibited by actidione : this inhibition is usually somewhat lower than the inhibition of chlorophyll formation. To confirm that incorporation of ^{14}C-leucine into protein was being inhibited, it was shown that with 10 µg/ml. actidione, the percentage inhibition of incorporation into a protein fraction isolated by trypsin treatment (Kirk, 1962a) was the same as the percentage inhibition of incorporation into the acetone/acid-insoluble fraction.

Chlorophyll a synthesis by etiolated cells of the G strain in the light was somewhat more sensitive to the antibiotic than was synthesis in the Z strain : 1 µg/ml. of actidione caused more than 90% inhibition. When etiolated cells of the Z strain were incubated with 1-^{14}C-leucine for 4 hours in the light and in the dark, in the presence or absence of actidione (10 µg/ml.), the inhibition of incorporation was essentially the same in the dark (67%) as in the light (70%).

Effect of actidione addition after chlorophyll synthesis has begun. Etiolated cells of the G strain were incubated in 12 centrifuge tubes in the light. To 3 tubes actidione at 10 µg/ml. was added at the beginning of the incubation. After 4 hours these 3 tubes, together with 3 tubes containing no antibiotic, were removed for pigment analysis. At the same time, actidione at 10 µg/ml. was added to 3 of the remaining 6 tubes and the incubation was continued for another 4 hours, at the end of which time all 6 tubes were removed for pigment analysis. The results of a typical experiment are shown in Table 2. It is clear that actidione still drastically inhibits chlorophyll a synthesis even when it is added some time after greening has commenced.

TABLE 2. EFFECT OF ACTIDIONE ADDITION, AT 0 OR 4 HOURS, ON CHLOROPHYLL SYNTHESIS. Conditions as in text.

	0-4 hours	δA_{663}	Inhibition	4-8 hours	δA_{663}	Inhibition
1	-	0.245	-			
2	+ Act.	0.019	92%			
3	-	0.245	-	-	0.388	-
4	-	0.245	-	+ Act.	0.091	77%

However, the inhibition is somewhat lower when the antibiotic is present during the 4 to 8 hour period than when it is present during the 0 to 4 hour period. It should be noted that the cells synthesize only about 40% as much chlorophyll a between 4 and 8 hours in the presence of actidione, as they do between 0 and 4 hours in the absence of antibiotic.

Effect on carotenoid synthesis. In order to determine whether carotenoid synthesis was inhibited as much as chlorophyll synthesis, etiolated cells of the G strain of E. gracilis were incubated in the light, in the presence or absence of actidione at 1 µg/ml. Tubes were withdrawn from the illumination apparatus for pigment analysis at 2, 4 and 6 hours. It was found that carotenoid synthesis was inhibited by actidione, but to a much smaller extent

than was chlorophyll synthesis: the inhibitions at 2, 4 and 6 hours were 77%, 89% and 92%, respectively, for chlorophyll formation, and 25%, 32% and 54%, for carotenoid formation. Similar results were obtained with cells of the Z strain.

This difference in sensitivity is not observed only at one concentration of actidione. The results in Table 3 show that with etiolated cells of the G strain, incubated for 4 hours in the light, a greater inhibition of chlorophyll synthesis than of carotenoid synthesis is observed at five different concentrations of antibiotic over the range 0.13 to 10 µg/ml.

TABLE 3. EFFECT OF DIFFERENT CONCENTRATIONS OF ACTIDIONE ON SYNTHESIS OF CHLOROPHYLL a AND CAROTENOIDS. Conditions as in text.

Actidione, µg/ml	0	0.13	0.32	1.0	3.2	10.0
% Inhibition of chlorophyll synth.	-	50	80	93	100	100
% Inhibition of carotenoid synth.	-	0	20	20	30	22

If the actidione is added to the medium after the cells have already been greening for some hours, carotenoid synthesis is even less inhibited than when the antibiotic is added at the time illumination begins. When etiolated cells of the G strain were exposed to actidione (10 µg/ml.) in the first 4-hour period of greening (0 to 4 hours), carotenoid synthesis was inhibited by 34%. When the drug was added after 4 hours in the light, the synthesis of carotenoid between 4 and 8 hours was inhibited by only 9% (Table 4).

TABLE 4. EFFECT OF ACTIDIONE ADDITION, AT 0 OR AT 4 HOURS, ON CAROTENOID SYNTHESIS. Conditions as in text.

	0-4 hours	$\delta A^{car.}_{480}$	Inhibition	4-8 hours	$\delta A^{car.}_{480}$	Inhibition
1	-	0.100	-			
2	+ Act.	0.066	34%			
3	-	0.100	-	-	0.173	-
4	-	0.110	-	+ Act.	0.157	9%

Owing to the acceleration of carotenoid synthesis with time, the amount of carotenoid formed during the second 4-hour period in the presence of actidione is greater than the amount formed during the first 4-hour period in the absence of antibiotic. This is contrary to what is observed for chlorophyll a synthesis.

DISCUSSION

It is clear that actidione is a very effective inhibitor of chlorophyll a synthesis in etiolated cells of Euglena gracilis, in the light. In view of the evidence from other systems that actidione acts directly on protein synthesis at the ribosome level (Siegler and Sisler, 1964b; Wettstein et al., 1964; Colombo et al., 1965; Bennett et al., 1965), it seems likely that in Euglena gracilis also it acts on protein synthesis. In agreement with this, actidione, as we have seen, inhibits incorporation of ^{14}C-leucine into protein in E. gracilis. The finding that the inhibition of leucine incorporation is the same in the dark as it is in the light suggests that formation of cell proteins generally, not specifically chloroplast proteins, is inhibited. This view is also supported by the fact that the antibiotic inhibits cell growth.

The fact that chlorophyll a formation can be completely prevented by quite low concentrations of actidione indicates that chlorophyll synthesis in this system is completely dependent upon the synthesis of some protein or proteins. If these proteins are enzymes involved in the biosynthesis of chlorophyll a, then the inhibition of chlorophyll a synthesis observed when actidione is added after 4 hours greening would presumably be due to the cessation of enzyme synthesis. If this is all that happens, then the amount of chlorophyll a synthesized in the next 4 hours in the presence of antibiotic should be equal to, or greater than, the amount synthesized in the first 4 hours in the absence of antibiotic, since although the cells are not making more enzyme in the second 4 hours, they should still have the enzymes they made in the first 4 hours. However, as we have seen, the amount of chlorophyll a made in the second 4 hours in the presence of actidione is

only about 40% of the amount made in the first 4 hours in the absence of actidione.

There are at least two possible explanations of this. The first is that one or more enzymes concerned with chlorophyll a synthesis undergo rapid turnover, so that when they cease to be made they rapidly disappear from the cell. This is the explanation put forward by Gassman and Bogorad (1965) to account for the fact that chloramphenicol and puromycin rapidly stop the greening of bean leaves. The second explanation, which we are more inclined to favour, is that chlorophyll a synthesis requires the formation of some protein in stoichiometric, rather than catalytic (as in the case of an enzyme), amounts. This protein could, possibly, be the protein moiety of the protochlorophyllide holochrome; that is assuming that this is used only once for the reduction of one protochlorophyllide molecule. Alternatively, if the holochrome protein is used catalytically for more than one protochlorophyllide molecule, then the protein in question might be that protein in the chloroplast lamellae to which the chlorophyll is bound. A shortage of this protein might be expected to prevent removal of the chlorophyllide (or chlorophyll) from the holochrome protein, thus preventing the holochrome protein from being used for the photoreduction of more protochlorophyllide.

The observation that carotenoid formation is much less sensitive to actidione than is chlorophyll a formation suggests that carotenoid synthesis is less closely linked to protein synthesis. The inhibition of carotenoid synthesis, such as it is, could be explained by inhibition of the formation of enzymes required for carotenoid synthesis. The relatively small size of this inhibition might be due to the fact that the etiolated cells already have enzymes for carotenoid formation, since carotenoid synthesis, unlike chlorophyll synthesis, continues (at a reduced rate) in the dark. Since addition of actidione after 4 hours greening does not actually cause a diminution in the rate of carotenoid synthesis (although it slightly reduces the acceleration in synthesis which normally takes place), it is not

necessary to postulate for carotenoid synthesis (as it is for chlorophyll synthesis) either that some protein is required in stoichiometric amounts, or that the biosynthetic enzymes are unstable. It seems likely that carotenoids differ from the chlorophylls in that the former can accumulate to some extent even though other chloroplast constituents are not being formed. This view is supported by the fact that carotenoid-containing globules can accumulate in the chromoplasts of certain fruits and flower petals.

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