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Effects of some inhibitors of protein synthesis on the chloroplast fine structure, CO_2 fixation and the Hill reaction activity *

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Abstract

A comparative study concerning the effects of chloramphenicol (100 μg ml^{-1}), actidione (10 μg ml^{-1}), 5-bromouracil (190 μg ml^{-1}), actinomycin D (30 μg ml^{-1}) and DL-ethionine (800 μg ml^{-1}) on the chloroplast fine structure, $^{14}CO_2$ incorporation and the Hill reaction activity was the subject of the experiments presented in this paper. The experiments were conducted on bean seedlings under the conditions when chlorophyll accumulation was inhibited only partially.

The results obtained indicate that chloromphenicol is responsible for the reduction of the number of grana per section of plastid and for the formation of numerous vesicles in the stroma. In the presence of actidione, actinomycin D or DL-ethionine the lamellae are poorly differentiated into stroma and granum regions and there occur disturbances in the typical orientation of lamellae within chloroplasts. Only in the presence of 5-bromouracil the development of chloroplast structure resemble that in control plants.

A comparison of the results obtained with those published earlier (Więckowski et al., 1974; Ficek and Więckowski, 1974) shows that such processes as assimilatory pigment accumulation, the rate of CO_2 fixation, the Hill reaction activity, and the development of lamellar system are suppressed in a different extent by the inhibitors used.

INTRODUCTION

Some metabolic inhibitors are often used for studing the sites of protein synthesis within the cell. It has been well documented that in the eukaryotic plant cell both types of ribosomes (70 S in chloroplasts and 80 S in cytoplasm) are involved in the synthesis of protein constituents of the thylakoid membrane and of the chloroplast stroma (for review see Strzałka and Więckowski, 1974). Most of the commonly used

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protein or nucleic acid synthesis inhibitors also suppress directly or inhibitors applied in low concentration produce no effect or may even (Margulies, 1962; Gassman and Bogorad, 1967; and others), photosynthetic and photochemical activities of chloroplasts (Nikolayeva et al., 1969; Ireland and Bradbeer, 1971; and others), or development of the fine structure of plastids (Margulies, 1966; Młodzianowski et al., 1970; Wrischer and Vrhovec, 1972; Wrischer, 1973). The terminal effect depends also on the concentration of inhibitor, way of its introduction into the plant and time length of treatment (Nikolayeva et al., 1969; and others). Many of these inhibitors applied in low concentration produce no effect or many even stimulate some processes (Ireland and Bradbeer, 1971; Wildner, 1976, and others).

In previous papers (Więckowski et al., 1974; Ficek and Więckowski, 1974) we have shown that the sensitivity of chlorophyll accumulation to chloramphenicol, actidione, 5-bromouracil, actinomycin D, and DL-ethionine differs from that of carotenoid accumulation. In general, each of these compounds, except of 5-bromouracil, brought about preferentially the accumulation of β-carotene. Xanthophyll biosynthesis was in little suppressed by all these four compounds.

In this paper we intended to analyze the effects of chloramphenicol, actidione, 5-bromouracil, actinomycin D or DL-ethionine on the fine structure and photochemical activity of chloroplasts, and the rate of $^{14}CO_2$ fixation by leaves. These investigations were conducted under the conditions where chlorophyll accumulation was inhibited only partially by an appropriate concentration of the inhibitors. The studies were of comparative character.

MATERIAL AND METHODS

The experiments were carried out on the primary leaves of bean seedlings (*Phaseolus vulgaris* cultivar. Krakowska). The seedlings were cultivated for eight days on the liquid nutritive Hoagland solution in complete darkness, at the temperature of 28°C. Two hours before illumination of the seedlings, the shoots were cut off from the roots and the ends of excised shoots were immersed in the Hoagland medium supplemented with an inhibitor of appropriate concentration. After two hours' incubation the seedlings, still remaining in contact with the inhibitor, were submitted to continuous illumination of approx. 24 W m^{-2} light intensity * for 24 hours, and next primary leaves were harvested and examined.

* In all experiments light intensities were measured in the range of photosynthetically active radiation.

The following inhibitors were used: chloramphenicol (D (—) *threo*-chloramphenicol (*threo*-chloramphenicol, pharm., Polfa, Poland), actidione (Rroth), 5-bromouracil (Reagal), actinomycin D (Serva), DL-ethionine (Loba-Chimie).

Electron-microscopic studies. For the electron-microscopic studies the material was fixed in glutaraldehyde and after dehydration was embedded in Epon 812. The ultrathin sections were prepared on the Porter-Blume ultramicrotome equipped with a glass knife. The electron micrographs were take with a JEM-5Y electron microscope.

Determination of photochemical activity. Approx. 2.5 g of fresh leaves was homogenized (homogenizer type 302, Mechanika Precyzyjna) in the phosphate buffer (0.066 M, pH 7.2) containing sucrose (0.4 M) and KCl (0.01 M). The slurry was filtered through two layers of linen and the filtrate was centrifuged for 5 min at 500 × g. The supernatant was centrifuged again for 10 min at 2900 × g and the obtained pellet was resuspended in an isolating medium. The photochemical activity of chloroplasts was monitored polarographically (the Clark type electrode, and EZ-11 recorder, Czechoslovakia), using ferricyanide as an electron acceptor. Measurements were conducted at 25° C, illumination being provided by an incandescent lamp of approx. 75 W m^{-2} light intensity. The infrared radiation was absorbed by the 5 cm solution containing 70 g $Fe(NH_4)_2(SO_4)_2$ + 10 ml H_2SO_4 conc. in 500 ml H_2O.

Radioactivity measurement. The seedlings were allowed to photosynthetize in the chamber of approx. 850 ml capacity, the atmosphere of which was enriched with CO_2 to approx. 1% and contained 25 μC $^{14}CO_2$. The illumination of about 74 W m^{-2} light intensity was provided by three halogenic lamps. The light was passed through a 5 cm water filter and a glass filter of "Antisol" type to remove infrared radiation. The temperature in the chamber was maintained at 25—26° C. After illumination for 5 min discs of 5 mm in diameter were cut off from the leaf blade, and then they were west-digested by H_2SO_4 + CrO_3. Issuing CO_2 was absorbed by the base (for detailes see Shimshi, 1969). The radioactivity of the samples was counted with the Packard liquid-scintillation spectrometer, Model 2002.

RESULTS

The analysis of typical electronograms (Figs 1—6) indicates that the inhibitors applied in the concentration which retards chlorophyll accumulation by 40-70 per cent (Więckowski et al., 1974; Ficek and Więckowski, 1974) brought about a remarkable alteration in the fine structure of chloroplasts. After 24 hours' illumination the chloroplast structure of the control plants was typical of the early stages of development (Fig. 1). The lamellae were arranged along the longer axes of

plastids and formed about ten primary bundles in which granum and unappressed stroma lamellae regions were distinguishable. Each granum consisted of a few thylakoids and very rare small starch grains occurred in the plastids.

Chloramphenicol in the concentration 100 μg ml^{-1} caused a decline in the number of grana per section of chloroplast (Fig. 2), but compared with the control the grana were often better developed, i.e. they consisted of a greater number of thylakoids. In the stroma of many chloroplasts there occurred numerous vesicles of about 0.1 μm in diameter, which were sometimes connected with the inner membrane of envelope. Usually the shape of chloroplasts was irregular and revealed fission or convexities. Big starch grains were seen in the stroma.

Actidione in the concentration 10 μg ml^{-1} was responsible for the formation more ball-shaped plastids with the tendency of lamellae to accumulate in the central part of these organelles (Fig. 3). The lamellae were very often arranged concentrically. Also the stroma on the periphery of plastids was less electrondense than in the middle part. The lamellae were to a small extent differentiated into the granum and stroma regions. The unappressed lamella region showed a tendency to decrease in size.

Treatment with 5-bromouracil in the concentration of 190 μg ml^{-1} did not apparently affect the chloroplast structure in the 24 hour illuminated seedlings (Fig. 4). The lamellar system was well preserved and resembled that of control seedlings. In the presence of inhibitor only starch grains occurred more frequently than in the control material.

Suppression of the lamellar system development was also caused by actinomycin D in the concentration of 30 μg ml^{-1} (Fig. 5). The lamellae exhibited a tendancy towards formation of scarce granum stocks which were accompanied by small vesicles. The chloroplasts were usually more roundish.

Rare vesicles were encountered in the stroma of single chloroplasts of the seedlings treated with DL-ethionine in the concentration of 800 μg ml^{-1} (Figs 6, A, B). The orientation of the lamellae was often disturbed by big starch grains. There occurred some paired membranes but the plastids were devoid of grana. Unpaired stroma lamellae seemed to be less developed. The chloroplasts were usually irregular in shape.

Under the applied conditions the inhibitors did not prevent completely the $^{14}CO_2$ incorporation by leaves exposed to the light (Table 1): as compared to the control material, incorporation of the label per unit of leaf area diminished by approx. 20 per cent (actiomycin D treatment), or by approx. 50 per cent (actidione, 5-bromouracil or DL-ethionine treatment), or by 75 per cent (chloramphenicol treatment).

Under the applied conditions the photochemical activity of isolated chloroplasts calculated per unit amount of chlorophyll was even higher

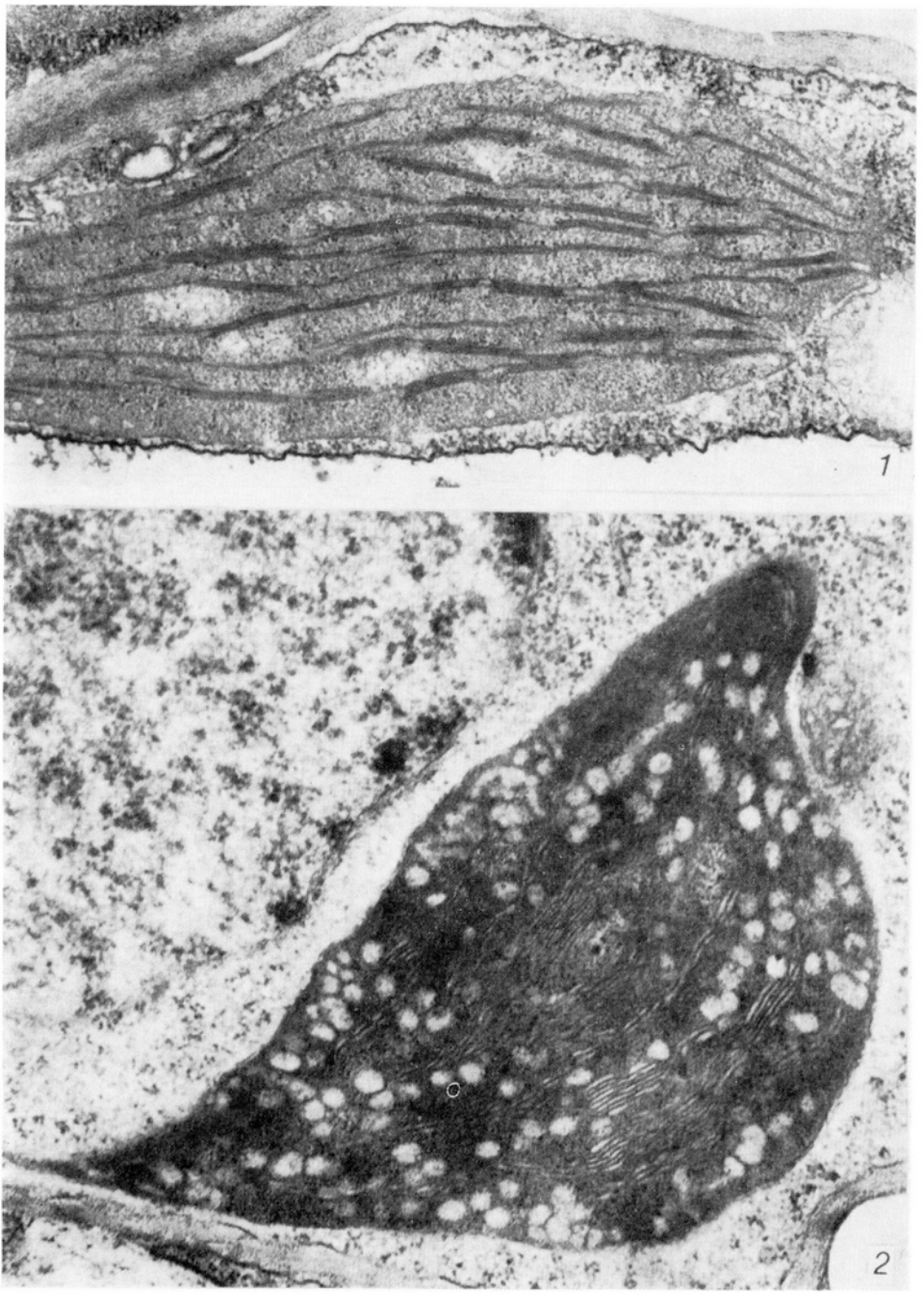

Fig. 1. Fine structure of chloroplasts of etiolated primary bean leaves exposed to light for 24 hours. No treatment with any inhibitor (control plants). Magn. 30 900 ×

Fig. 2. Effect of chloramphenicol (100 μg ml^{-1}) on the fine structure of chloroplasts of etiolated leaves exposed to light for 24 hours. Magn. 34 200 ×

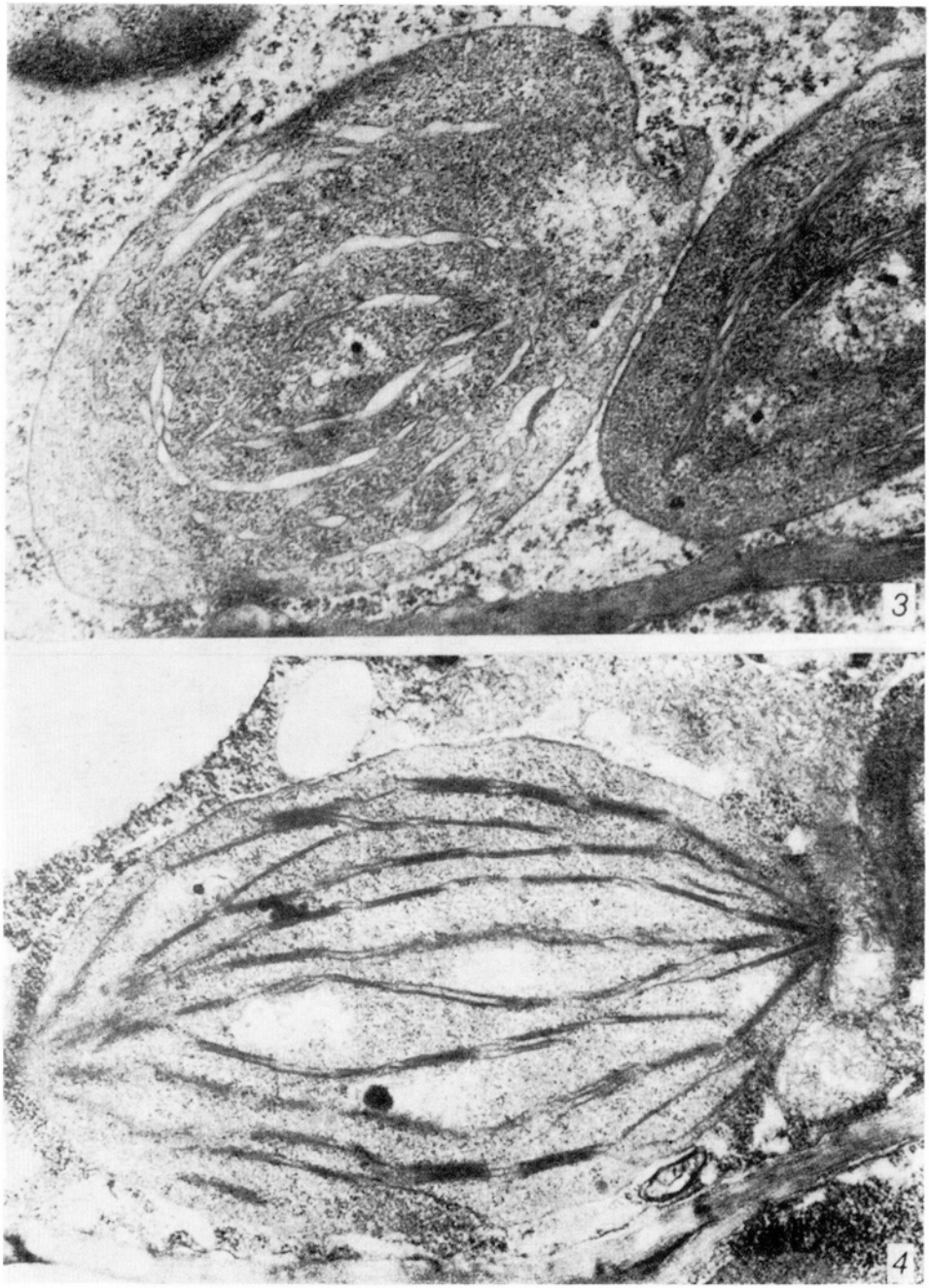

Fig. 3. Effect of actidione (10 μg ml^{-1}) on the fine structure of chloroplasts of etiolated leaves exposed to light for 24 hours. Magn. 30 900 ×

Fig. 4. Effect of 5-bromouracil (190 μg ml^{-1}) on the fine structure of chloroplasts of etiolated leaves exposed to light for 24 hours. Magn. 24 600 ×

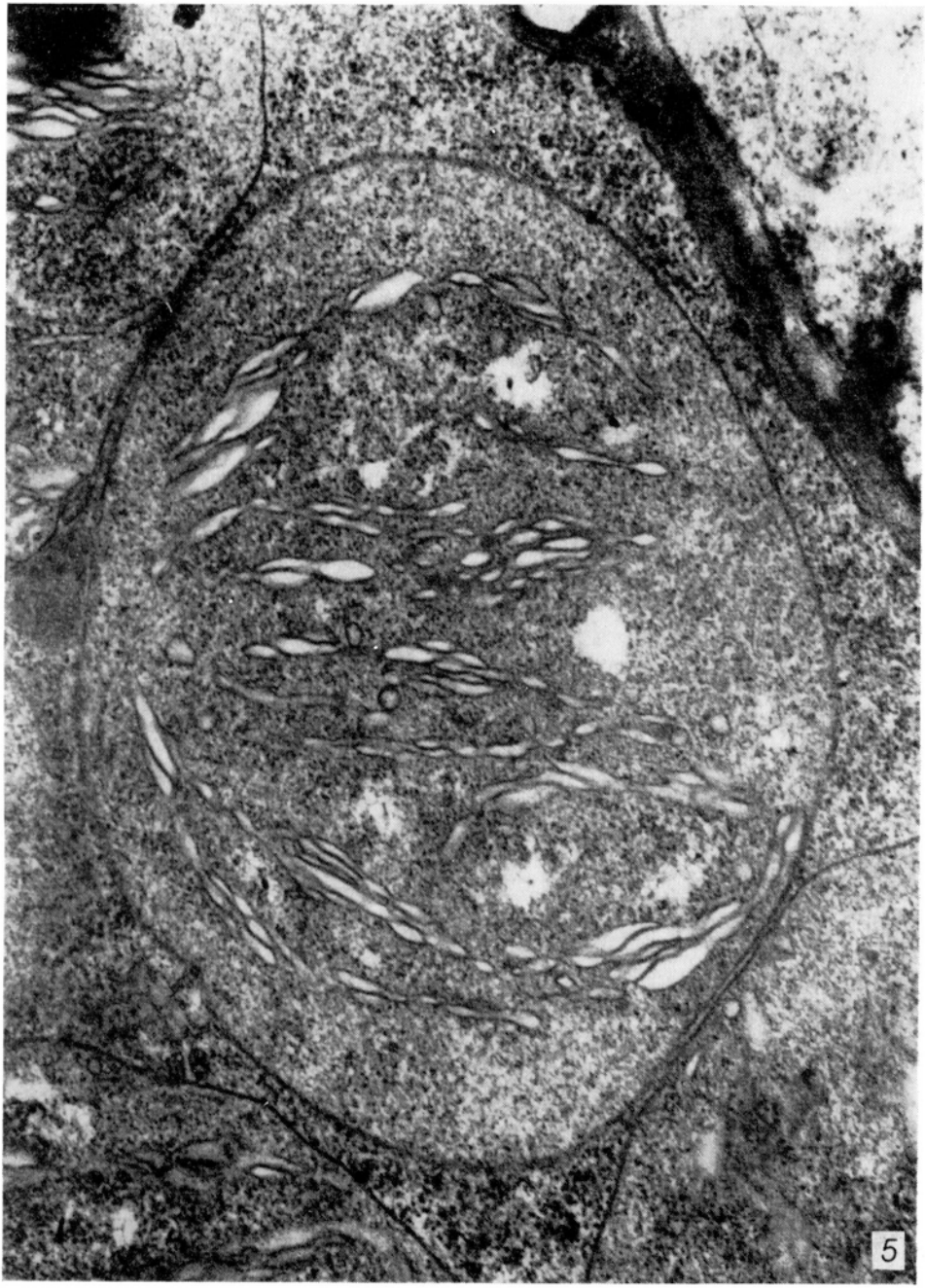

Fig. 5. Effect of actinomycin D (30 μg ml^{-1}) on the fine structure of chloroplasts of etiolated leaves exposed to light for 24 hours. Magn. 33 900 ×

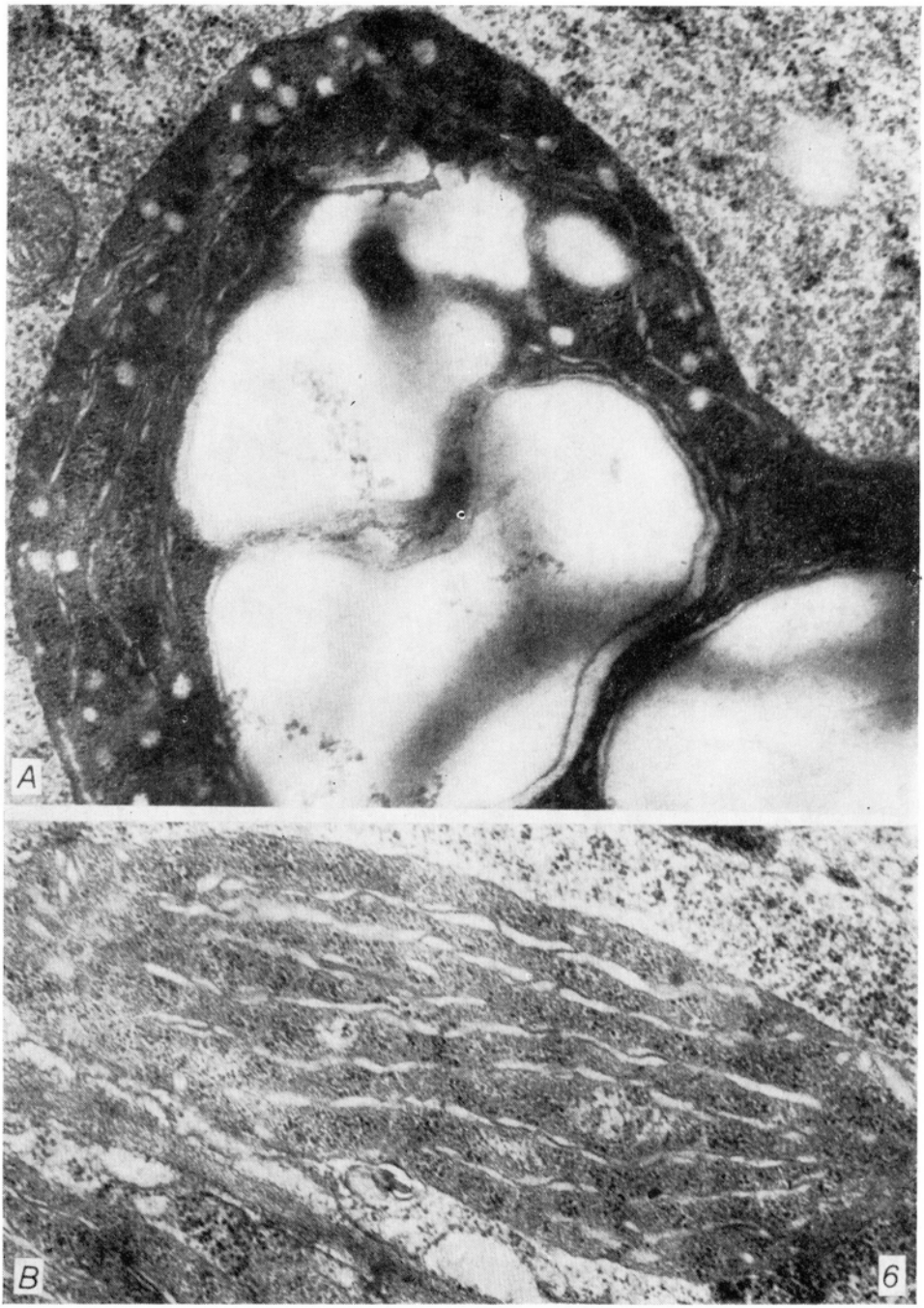

Fig. 6A, B. Effect of DL-ethionine (800 μg ml^{-1}) on the fine structure of chloroplasts of etiolated levaes exposed to light for 24 hours, Magn. 33 300 ×

in the chloroplasts isolated from the inhibitor treated seedlings than in chloroplasts isolated from the control seedlings (Table 1).

Table 1

Effect of some inhibitors of protein synthesis on the incorporation of $^{14}CO_2$ by illuminated leaves and on the photochemical activity of isolated chloroplasts

Mean values and standard deviations for three to four experiments. For other details see text

	$^{14}CO_2$ incorporation cpm	oxygen evolution* µmol O_2 mg^{-1}chl h^{-1}
	x 10^3	
Control	4.2±0.2	16±1
Chloramphenicol (100 µg ml^{-1})	1.1±0.5	20±2
Actidione (10 µg ml^{-1})	2.3±0.8	43±13
5-bromouracil (190 µg ml^{-1})	2.4±0.5	54±13
Actinomycin D (30 µg ml^{-1})	3.2±0.3	48±10
DL-ethionine (800 µg ml^{-1})	2.5±0.9	98±26

* The reaction mixture of 3.1 ml contained chloroplast suspension in isolating medium and 6 mM $K_3Fe(CN)_6$.

DISCUSSION

While interpreting the experimental results concerning the effect of any metabolic inhibitor one should take into consideration not only its specificity of action and concentration but also the sensitivity of the organism and the length of the time of treatment (Margulies, 1962; Döbel, 1963; Ireland and Bradbeer, 1971; Wara-Aswapati and Bradbeer, 1974; McMahan, 1975). Difference in procedures of plant treatment with an inhibitor may yield different results. In this study the fine structure of plastids, $^{14}CO_2$ incorporation, and photochemical activity were investigated under the conditions of only partial inhibition of the development of photosynthetic apparatus. No attempt was made here to carry out study in the conditions where nucleic acid or protein synthesis had been suppressed completely. Therefore there exist some discrepancies between the results described in this paper and those published e.g. by Nikolayeva et al. (1969), Bishop et al. (1973) and others. For example, we never observed the chloroplasts to contain prolamellar bodies after a 24 hour-illumination of etiolated seedlings in the presence of any inhibitor used. Wrischer (1967, 1973) has found

those bodies in bean plastids after 6 hours' illumination of the seedlings treated with 10^{-2} M ethionine or after 12 hours with 0.1 per cent of 2-thiouracil. Wrischer has pointed out that the prolamellar bodies did not disappear quickly in young plants only.

Chloramphenicol did not cause the formation of giant grana like those having been revealed by Młodzianowski et al. (1970) in the chloroplasts of protonema of *Funaria hygrometrica*. But, consistently with the results of Margulies (1966), Wrischer (1967) and Młodzianowski et al. (1970), we found numerous vesicles in the stroma of the chloroplasts treated with antibiotic. It seems possible that the membranes of these vesicles are built of proteins which are synthesized on 80 S ribosomes in the cytoplasm. These proteins could not be involved in the formation of normal lamellae due to the lack of constituents which are formed on 70 S ribosomes in chloroplasts.

Treatment with actidione did not cause such a visible damage of the chloroplast fine structure as was reported e.g. by Wrischer and Vrhovec (1972). We never observed crystalloids to occur in the stroma, and the bundles of lamellae were less bent. But in agreement with the results of these authors we observed enlargement of the grana region.

The obtained data indicate that various inhibitors modified differently the developmental pattern of chloroplast fine structure, even under the conditions when the chlorophyll accumulation was prevented to the same extent, e.g. the accumulation of chlorophyll in leaves treated with actidione, actinomycin D or DL-ethionine was prevented in approx. 60-70 per cent, but under the same conditions one could see certain differences in the chloroplast fine structure (Figs 3, 5, 6). Moreover, no correlation occurred in plants treated with each of used inhibitors between the extent of inhibition of chlorophyll accumulation (Więckowski et al., 1974, Ficek and Więckowski, 1974) and that of $^{14}CO_2$ incorporation or of oxygen evolution by chloroplasts. For example, the accumulation of chlorophyll in chloramphenicol-treated leaves was reduced to about 60 per cent and under the same conditions the incorporation of $^{14}CO_2$ in the light was reduced to about 25 per cent of the control. Similar tendancy occurred in 5-bromouracil-treated leaves. On the contrary, green pigment accumulation in leaves treated with actidione, actinomycin D, or ethionine was diminished to about 30-50 per cent while the $^{14}CO_2$ incorporation to about 60-70 per cent of the control. Thus the accumulation of chlorophyll is more resistent to the action of chloramphenicol or 5-bromouracil than that of carbon dioxide fixation. The reverse situation occurred in the leaves treated with actidione, actinomycin D, or ethionine: the accumulation of chlorophyll was inhibited in a higher extent than that of CO_2 fixation.

An enhanced activity of Hill reaction in the presence of inhibitors used is presumably also the result of a delay in development of photosynthetic

apparatus and the rate of oxygen evolution by chloroplasts isolated from 24 hour-illuminated leaves in the presence of inhibitor corresponds to that noted in the earlier stages of leaf development (Więckowski, 1972).

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Wpływ niektórych inhibitorów biosyntezy białek na ultrastrukturę chloroplastów, asymilację CO_2 i aktywność reakcji Hilla

Streszczenie

Przeprowadzono porównawcze badania nad wpływem chloramfenikolu (100 μg ml^{-1} aktidionu (10 μg ml^{-1}), 5-bromouracilu (190 μg ml^{-1}), aktynomycyny D (30 μg ml^{-1})i DL-etioniny (800 μg ml^{-1}) na ultrastrukturę chloroplastów, asymilację $^{14}CO_2$ i aktywność reakcji Hilla. Doświadczenia wykonano na fasoli. Warunki doświadczeń zostały tak dobrane, aby akumulacja chlorofilu w młodocianych liściach była zahamowana tylko częściowo.

Otrzymane wyniki wskazują, że chloramfenikol jest odpowiedzialny za redukcję liczby gran w chloroplaście i za powstawanie licznych pęcherzykowatych struktur na terenie stromy. W obecności aktidionu, aktynomycyny D i etioniny lamelle ulegają w mniejszym stopniu zróżnicowaniu na odcinki granowe i stromy. Ponadto obserwuje się zaburzenia w typowej orientacji lamell wewnątrz chloroplastu. Tylko w obecności 5-bromouracylu wykształcają się chloroplasty, które swoją strukturą przypominają chloroplasty roślin kontrolnych.

Porównanie otrzymanych danych z wynikami opublikowanymi wcześniej (Więckowski et al., 1974; Ficek i Więckowski, 1974) wskazuje, że u roślin traktowanych różnymi inhibitorami biosyntezy kwasów nukelinowych lub białek nie istnieje ścisła zależność pomiędzy stopniem zahamowania biosyntezy chlorofilu a stopniem zmian w aktywności asymilacji CO_2, aktywności reakcji Hilla i przebiegiem rozwoju systemu lamellarnego chloroplastów.