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THE EFFECT OF ISOPROPYL N-PHENYLCARBAMATE ON THE STRUCTURE AND PHOTOSYNTHETIC ACTIVITY OF ETIOCHLOROPLASTS

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Introduction

Isopropyl N-phenylcarbamate (IPC) belongs to the group of herbicides, which inhibit the photosynthetic activity of the chloroplasts (Trebst et al. 1968, Dodge 1975). It is a rather weak herbicide, as it affects the electron transport system of isolated chloroplasts only at rather high concentrations (Moreland 1969, Schulz 1969). In addition to that, the herbicides of the carbamate group (to which IPC belongs) interfere also with many other cell functions, e. g. with the oxidative phosphorylation in mitochondria (Kirkwood 1976), with the synthesis of some enzymes, with processes of senescence in leaves (Mann et. al. 1967), and even with the formation of cytoplasmic microtubules (Hepler and Jackson 1969, Brown and Bouck 1974), causing abnormal cell divisions (Linck 1976). For this reason some authors have suggested that IPC might bind to the cell proteins and change somehow their conformation (Hansch 1969, Brown and Bouck 1974).

While the fact that IPC lowers the photosynthetic activity of the chloroplasts has been known for a long time, its influence on the structural components of the chloroplasts was much less studied. The aim of this work has been therefore to investigate the effect of IPC on the formation of structures and photosynthetic activity in etiochloroplasts of greening etiolated leaves. For comparison, the effect of IPC on the chloroplasts of green leaves has also been studied.

Material and Methods

Primary leaves of 9 days old etiolated bean plants (*Phaseolus vulgaris* cv. »starozagorski«) were detached from the seedlings and put into petri dishes on filter paper wetted either with an 1×10^{-3} M solution of IPC (»Pliva«, Zagreb, Yugoslavia) in tap water or with tap water alone. The leaves were at first incubated in darkness for 3, 6 or 12 hours and then exposed to light (2 fluorescent tubes 20 W, 4500 °K, illumination intensity 4000 lx) for 1, 6, 12, 24, 48 or 72 hours. After this treatment in light in some experiments the leaves were returned again to the dark for further 12 hours. In addition to that, green primary leaves of bean plants grown in light were maintained at the same concentration of IPC for 3, 15 or 48 hours. Pilot experiments were performed also with lower concentrations of IPC (5×10^{-4} M and 1×10^{-4} M).

The pigments were extracted in 85% acetone and the quantitative determinations were performed at wavelengths of 663, 644 and 452.5 nm (after Röbbelen 1957, cited by Urbach et al. 1976).

The photosynthetic oxygen yield of leaf pieces was calculated from manometric measurements with a Warburg apparatus (carbonate-bicarbonate buffer (Warburg et al. 1952) at 28 °C) from O_2-output in white light (illumination intensity of 5900 ± 500 lx) and from O_2-consumption (by respiration) in darkness.

For electron microscopic examination small pieces of leaves were fixed in 1% glutaraldehyde, postfixed in 1% OsO_4 and after dehydration embedded in Araldite. Thin sections were stained with uranyl acetate and lead citrate and examined in the electron microscope. Toluidine blue stained sections of the same material were used for light microscopic examinations.

For ultrastructural localization of photosystem I the leaf pieces were fixed in formaldehyde and incubated in diaminobenzidine (DAB) in light, as previously described (Wrischer 1977 a); for localization of photosystem II the prefixed leaf pieces were incubated in a ferricyanide solution in light as described by Nir and Pease (1973).

Results

The strongest inhibition of the greening of etiolated leaves was obtained with the solution of IPC at the concentration of 1×10^{-3} M. Etiolated leaves maintained at this concentration of IPC in light for 48 or 72 hours were still yellow or slightly yellow-green. Unless stated otherwise, all experiments reported here were performed with the above mentioned concentration of IPC. This solution is lethal for the leaves, when applied continuously throughout a week; however, if after several hours this solution is replaced by tap water, the retardation in greening of the leaves can be — after some days — almost annulled. Similarly, lower concentrations of IPC (e. g. 5×10^{-4} M) are much less inhibitory and an 1×10^{-4} M solution of IPC is nearly without any effect on the greening of the leaves.

If the etiolated leaves treated and the control ones (kept on tap water) are exposed to light for 2 or 3 days considerable differences arise in their anatomy. As semithin sections show, the IPC-treated leaves are much thinner than the control ones, having smaller, less vacuolated cells and smaller intercellular spaces. In fact, in their anatomy they are

very similar to normal etiolated leaves. When green leaves are treated with IPC, no anatomical changes occur.

In etiolated leaves, maintained on IPC in light, the concentration of total chlorophyll is much lower than in those kept on tap water. After e. g. 48 hours in light the total chlorophyll concentration reaches only 6.5% of that of untreated leaves, while at the same time the concentration of total carotenoids remains almost unchanged (Table 1).

When green leaves are maintained on IPC, the content of the total chlorophyll drops somewhat, but only in the case when young, i. e. not fully grown leaves are used for the experiment (Table 2).

Table 1. Total chlorophyll, total carotenoids and photosynthetic activity of *etiolated leaves* maintained 48 h in light on tap water and IPC.

	Total chlorophyll (mg/g fr. wt.)	Total carotenoids (mg/g fr. wt.)	Photosynthetic activity (μg O_2/mg fr. wt./h)	Photosynthetic efficiency (μg O_2/mg chlorophyll/h)
Tap water	0.522	0.10	0.041	78.54
IPC 1×10^{-3} M	0.034	0.09	0.011	323.53

Table 2. Total chlorophyll, total carotenoids and photosynthetic activity of young *green leaves* maintained 48 h in light on tap water and IPC.

	Total chlorophyll (mg/g fr. wt.)	Total carotenoids (mg/g fr. wt.)	Photosynthetic activity (μg O_2/mg fr. wt./h)	Photosynthetic efficiency (μg O_2/mg chlorophyll/h)
Tap water	1.765	0.20	0.073	41.36
IPC 1×10^{-3} M	1.453	0.20	0.031	21.34

The total photosynthetic activity of etiolated leaves held in light is significantly affected by IPC. After a 48-hours treatment it reaches only 27.5% of that of the leaves maintained on water; but, if calculated to the content of the chlorophyll present (expressed as photosynthetic efficiency), it is more than 4 times higher (Table 1). The O_2-consumption in darkness (respiration) of the treated leaves is also much higher than in the control: after a 48-hours light-treatment it is 0.023 μl O_2/mg fresh weight/h in leaves maintained on water, and 0.055 μl/mg fresh weight/h in IPC-treated leaves.

IPC also considerably lowers the photosynthetic activity of green leaves — to about 43% of the control. The same inhibition is obtained both with a shorter treatment (3 hours), and a longer one (48 hours; Table 2). In these green leaves the O_2-consumption in darkness is not considerably influenced by IPC.

The electron microscopic analysis has shown that there are no structural changes in etioplasts of leaves maintained on IPC in darkness, even when this treatment is prolonged to 12 hours. During the first three hours in light, which follow the dark pretreatment, there are no differences between treated and control leaves — neither in the time of the transformation of the prolamellar bodies, nor in the formation of the primary thylakoids. The differences begin to appear, however, after about three hours in light, when the greening of the control leaves begins, i. e. when the first grana are formed. This process is strongly inhibited by IPC. While after 48 hours in light the plastids of control leaves are already transformed into chloroplasts, as they contain at this time already numerous grana (Fig. 1), in the plastids treated with IPC, the grana are, if present at all, small and scarce (Fig. 2). In addition to that, these plastids are still roundish and not elongated as in the untreated leaves. If maintained constantly on IPC they do not differentiate further, and remain in the stage of etiochloroplasts till the death of the leaf.

The fine structure of other cell constituents is not markedly affected by IPC. After a 48-hours treatment mitochondria contain numerous tubules, and the dictyosomes are often surrounded by a large number of vesicles (Fig. 2).

In order to find out whether in the etiochloroplasts the IPC does block the formation of all membrane structures or only the development of grana, and to determine the role of light in this inhibition, the following experiments were performed: The etiolated leaves were first incubated in darkness on IPC and then exposed to light for 1 or 2 hours, in order to allow the prolamellar bodies in these etiochloroplasts to lose their crystalline lattice. Then the leaves were returned to the dark. After 12 hours in darkness the etiochloroplasts contained prolamellar bodies with a regular lattice of tubules, similar to that usually formed in darkness in normal etiochloroplasts. On the contrary, if the treatment in light was prolonged (e. g. to 12 hours) so that no tubular remnants of the prolamellar bodies were left in the plastids, and when

Fig. 1. Etiolated bean leaf maintained 48 h in light on tap water. Part of a young chloroplast with numerous small grana. 31,000 : 1.

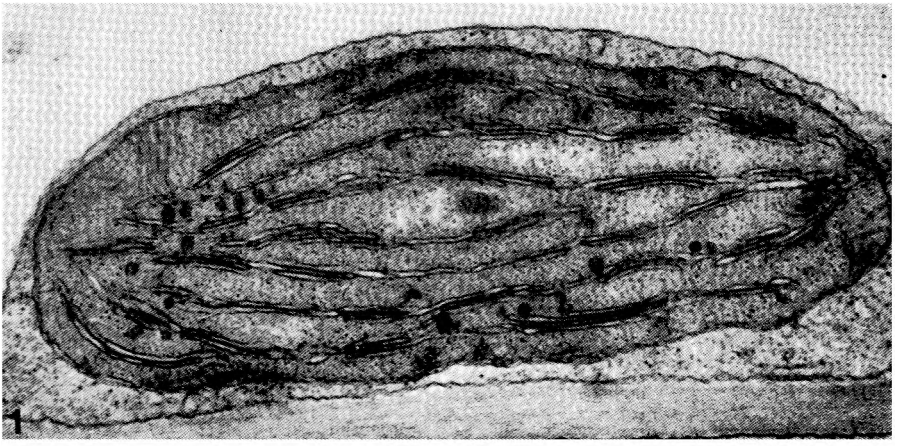
Fig. 2. Etiolated bean leaf treated 48 h in light with IPC. There are only single thylakoids or pairs of thylakoids in the etiochloroplasts. 29,000 : 1.

Fig. 3. Etiolated bean leaf kept on IPC: after incubation in darkness, 2 h in light and then 12 h in darkness. Part of an etiochloroplast with a prolamellar body containing irregularities (holes) in the lattice. 28,000 : 1.

Fig. 4. Green bean leaf held 48 h in light on IPC. Part of a chloroplast with some dilated thylakoids. 30,000 : 1.

Fig. 5. DAB-reaction in etiolated bean leaf kept 24 h in light on IPC. A positive, although weak, reaction is present in the thylakoids of the etiochloroplast. The tubules of the mitochondria also react with DAB. 24,000 : 1.

Fig. 6. DAB-reaction in etiolated bean leaf held 24 h in light on water. The reaction is positive in all thylakoids of the young chloroplasts. The tubules of the mitochondrion are DAB-positive. 26,000 : 1.



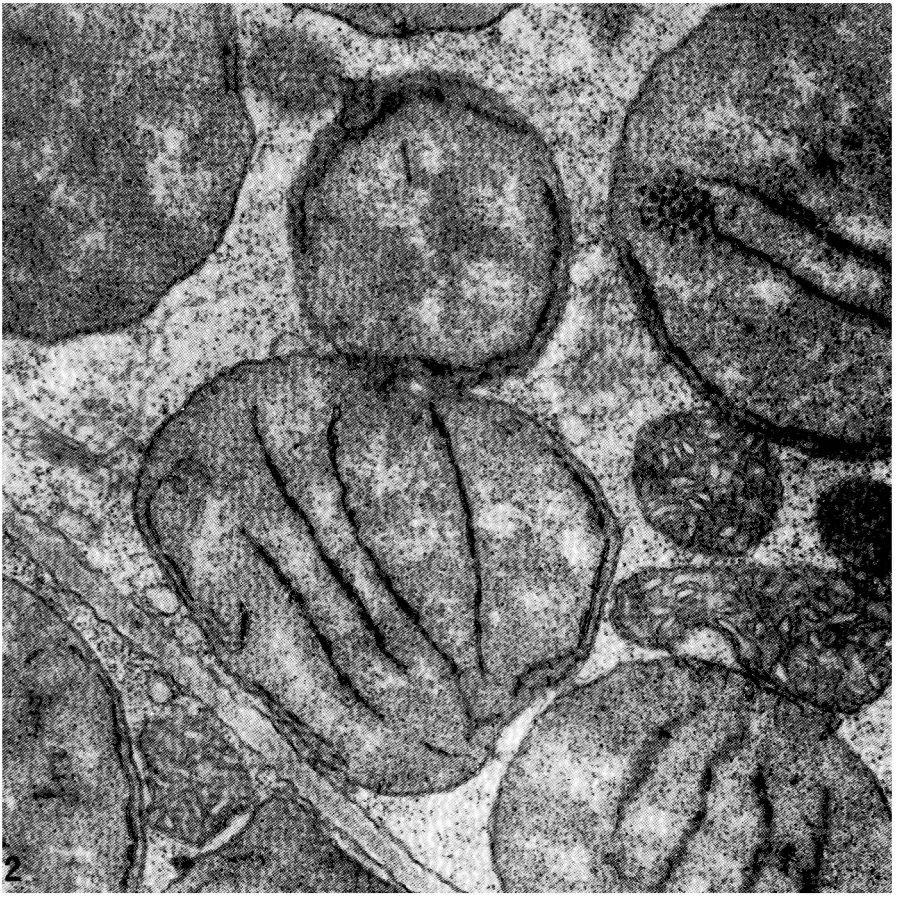
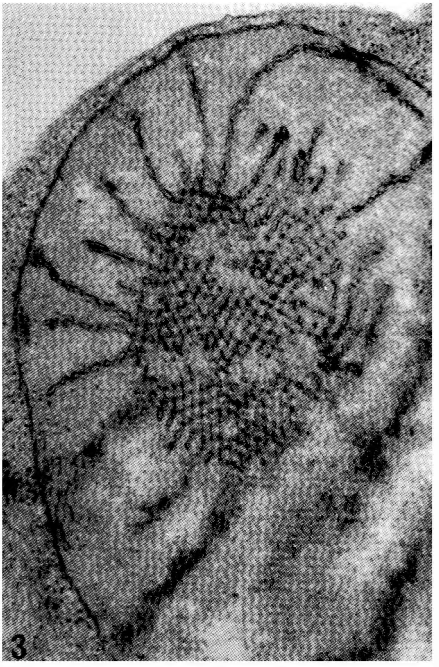


Fig. 1—2.



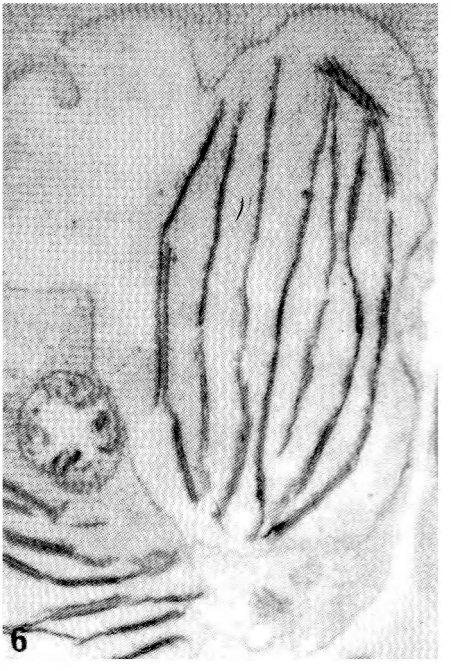


Fig. 3—6.

after that time the leaves were returned to the dark for additional 12 hours, the prolamellar bodies did not appear anew. In the intermediate stages the prolamellar bodies were only partly rebuilt in darkness; in these cases the crystalline lattice contained some irregularities — i. e. holes (Fig. 3).

The chloroplasts of green primary leaves, which were treated with IPC, did not show considerable morphological differences if compared with the control leaves. When the treatment lasted 48 hours or longer, there was only a slight dilatation of some thylakoids (Fig. 4).

Photooxidation of diaminobenzidine (DAB) was used to localize the activity of photosystem I in the thylakoids of IPC-treated etiochloroplasts. This cytochemical method showed that in the thylakoids, which succeeded to form after 24 or 48 hours in light on IPC, the DAB-reaction was definitively positive, although very weak (Fig. 5). In these etiochloroplasts only the membranes of the plastid envelope were without any reaction. In the control leaves in the course of their greening in light a positive DAB-reaction appeared much earlier. It was detectable in the thylakoids already during the third hour after the onset of illumination (Wrischer 1977 b), and after 24 hours in light all the stroma and grana thylakoids were clearly dark, according to the osmiophilic DAB--deposits present in the thylakoids (Fig. 6). In addition to the thylakoids, a positive DAB-reaction appeared also in the mitochondrial tubules (Figs. 5, 6), as the cytochrome oxidase system reacts also with DAB (Seligman et al. 1968). In the thylakoids of chloroplasts of green leaves treated with IPC, the DAB-reaction remains positive.

The photoreduction of ferricyanide has been used to localize the photosystem II in the plastids (Nir and Pease 1973, Kirchanski 1976). This cytochemical method is still unprecise, as the coarsely granular reaction products lie irregularly aggregated in the grana regions, preferentially in the thylakoid gaps (i. e. between thylakoids). In addition to that, some unspecific precipitates appear also in the plastid stroma. In spite of these disadvantages it could be shown that in IPC--treated etiochloroplasts the reaction was positive, as some granular precipitates lie between two thylakoids in the places where they were in contact. The reaction seemed to be weaker than in the control material. The chloroplasts of the IPC-treated green leaves showed also a weak positive photoreduction of ferricyanide.

Discussion

The experiments reported in this paper show that IPC strongly affects the normal differentiation of etiochloroplasts only at the concentration of 1×10^{-3} M. Lower concentrations are much less effective, or even without any effect. IPC inhibits the formation of grana, the accumulation of chlorophyll and the photosynthetic activity of the etiochloroplasts. Otherwise, these etiochloroplasts have a very high photosynthetic activity, if calculated to the content of chlorophyll present in them. This fact could be explained by the assumption that IPC inhibits the differentiation of the plastids leaving them in the early etiochloroplast stage, where the photosynthetic rate per unit chlorophyll is always high (Leech 1977). This assumption is in accordance with the fact that IPC affects also other processes which are connected with the light induced cell differentiation and leaf development. So it has

been noticed that in IPC-treated leaves the O_2-consumption in darkness (respiration) is as high as in normal etiolated leaves during the first stages of greening. Further, the differentiation of the anatomical structure of the leaves is also strongly inhibited by IPC.

It is known that IPC inhibits the Hill reaction in isolated chloroplasts (Dodge 1975). According to the results on bean etiochloroplasts obtained with the cytochemical methods diaminobenzidine and ferricyanide — which are supposed to react with photosystem I and II respectively (Nir and Pease 1973, Kirchanski 1976) — it seems probable indeed, that both photosystems are underdeveloped owing to the IPC-inhibition of the overall plastid development.

The results reported in this paper show, that etiochloroplasts, treated with IPC, are able to rebuild prolamellar bodies in darkness only when some tubular elements are still present, but not when the components of the prolamellar bodies are already completely built into the thylakoid system. This fact supports the idea, that the formation of all membrane structures, either thylakoids or tubules, in the etiochloroplasts is strongly or even completely inhibited by IPC.

On the other hand, the results obtained with chloroplasts of green leaves treated with IPC show that, although their fine structure is little disarranged, an obvious inhibition of the photosynthetic activity is nevertheless present. These results would favour the opinion that IPC binds to some membrane components, probably to proteins (Hansch 1969), in such a way that this prevents a normal photosynthetic activity of the thylakoids.

Summary

The effect of isopropyl N-phenylcarbamate (IPC) on the development of the structure and function of etiochloroplasts in greening bean leaves has been studied by pigment analysis, measurements of photosynthetic activity and ultrastructural analysis.

The investigations have shown that IPC strongly inhibits the development of the thylakoid system, the synthesis of the chlorophyll and the photosynthetic activity of the etiochloroplasts; cytochemical analyses indicate that the development of both photosystem I and photosystem II are partly inhibited by IPC. The experiments have shown further that the production of all new membrane structures in the etiochloroplasts is strongly affected by IPC, so that these organelles constantly remain in the early etiochloroplast stage of development.

When green leaves are treated with IPC, there are no large ultrastructural changes in the chloroplasts, although the photosynthetic activity is lowered to less than a half of that of the control leaves.

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SADRŽAJ

Istražen je utjecaj N-fenil-izopropil-karbamata (IPC) na strukturu i funkciju etiokloroplasta u listovima graha tijekom njihova ozelenjavanja. Istraženi su sastav pigmenata, fotosintetska aktivnost i ultrastruktura tretiranih i kontrolnih listova.

Istraživanja su pokazala da IPC snažno inhibira razvoj tilakoidnog aparata, sintezu klorofila i fotosintetsku aktivnost etiokloroplasta, a citokemijski nalazi upućuju na to da on djelomično inhibira i fotosistem I i fotosistem II. Pokusi pokazuju nadalje da IPC snažno inhibira sintezu *de novo* svih membranskih struktura u etiokloroplastima, tako da oni zaostanu u svom ranom razvojnom stadiju.

Ako se zeleni listovi graha tretiraju IPC-om, ultrastruktura njihovih kloroplasta samo je neznatno izmijenjena. Istovremeno pak njihova je fotosintetska aktivnost smanjena na manje od 50% aktivnosti kontrolnih listova.

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