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EFFECT OF ISOPROPYL N-PHENYLCARBAMATE ON *EUGLENA*

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Introduction

Isopropyl N-phenylcarbamate (IPC) and its more stable derivate N-(3-chlorophenyl) carbamate (CIPC) belong to the group of well-known and widely used phenylurethane herbicides. Carbamate herbicides act on a variety of plant processes and cell structures (see e. g. Anderson and Thomson 1973, Dodge 1975, Wrischer and Botka 1978) but mainly as mitotic inhibitors affecting the assembly and organization of microtubules in the mitotic spindle (see. e.g. Jackson 1969, Hepler and Jackson 1969) and microtubule organizing centres (Coss et. al. 1974.) Although mostly negative results on animal cells have been obtained there is some evidence that CIPC also causes some microfilament disruption and nuclear fragmentation in mouse fibroblasts (Oliver et al. 1978).

Euglena gracilis has been proposed as a test organism for the study of herbicide action (Price and Estrada 1964). It is a system which offers several advantages: easy axenic culture, chloroplast developmet and chlorophyll synthesis show a correlation with these processes in higher plants, and a number of pigment-deficient and pigmentless mutants could be obtained so that the effect can be studied on autotrophic as well as on heterotrophic cells of the same organism. In the present work the effect of IPC on the growth and development of *Euglena gracilis* has been studied.

Material and Methods

Euglena cultures

Axenic cultures of wild type *Euglena gracilis Klebs* strain »Z« and its three bleached heterotrophic mutant strains: yellow, pale yellow and white (Marčenko 1973 1978) were grown at 30°C in a liquid medium or on agar plates in an organic medium (Marčenko 1978), supplemented with 5 — 40 mM sodium acetate.

The carotenoid content of the various strains of *Euglena* has been recorded previously (Marčenko 1973). In the yellow strain no measurable quantities of chlorophyll could be detected with certainty. Howecer, the fact that in UV light living colonies show a reddish fluorescence indicates the possibility that traces of chlorophyll — perhaps even of a form sensitive to light — are present.

Experiments on the effects of IPC on cell division

Cells were grown for 12 — 13 days at 30°C, in the dark in an organic medium (as above) with various acetate concentrations (5 — 40 mM) and plated on the same medium supplemented with 5 mM acetate to which IPC dissolved in ethanol, (10^{-4} — 1.5×10^{-3} M) was added after autoclaving. The concentration of ethanol was 82 mM, and in experiments with 1.5×10^{-3} M IPC, 123mM. Only in a few experiments IPC was added as dry powder. Cell numbers in the developing clones were scored after 24 and 48 h with a Reichert stereomicroscope.

Illumination experiments were performed at 3000 lx light intensity using white and blue fluorescent lamps.

Some experiments were done with IPC added to the liquid medium. In that case growth was measured as cell counts after 7 days, at 30°C. The cell number was determined with a haemocytometer.

Experiments on the effects of ethanol on cell division

Cells were grown for 4 days at 30°C, in the dark, in the above medium supplemented with 5 mM acetate and transferred to plates of the same medium to which ethanol (82 — 382 mM) had been added after sterilization. Cell divisions were scored as above.

Bleaching experiments

For bleaching (permanent loss of physiologically active chloroplasts), *Euglena* was cultured as above in organic medium (+ 82 mM ethanol), 5 mM acetate, 5×10^{-4} M IPC), in the dark at 30°C After 3 and 5 days of growth, cells were washed and plated on agar plates supplemented with 23 mM acetate. After 5 days of growth in the dark, at 30°C, the plates were kept at room temperature (ca 20°C) illuminated with weak light not exceeding 1000 lx (12 h light : 12 h dark) until the pigment became clearly visible. Colonies were scored as green or colourless.

Determination of pigments

Method I. Cells of wild type *Euglena* were grown on an organic medium containing 23 mM acetate for 3 months (inoculated several

times in fresh medium) in the dark, at room temperature. To prevent cell division, cells were washed and transferred into the resting medium of Stern et al. (1964) supplemented with various concentrations of IPC and kept in light (2000 lx) at room temperature for 4 days. Pigments were determined according to Röbbelen 1957 (as quoted by Urbach et al. 1976).

Method II. Cells were grown on agar plates on the same medium as above in the dark, at 30°C. After 13 days (during which time they were once replated on fresh agar), they were scraped from the agar surface and replated on fresh agar supplemented with various concentrations of IPC and kept in continuous light (2000 lx) at room temperature. 30 mg wet weight of cells was then extracted for pigment determination (as in Method I) at 24, 48 and 96 hours.

Determination of cellular paramylum content

Paramylum determination was done microscopically, the cells being scored with or without visible paramylum granules.

Results and Discussion

Effect of IPC on growth and cell division

IPC is a rather weak herbicide as compared to CIPC, which has to be applied in relatively high concentrations, preferentially as an ethanol solution added to the growth medium (Brown and Bouck 1974). Applied at concentrations of 10^{-4} M and higher it inhibits cell division and growth in *Euglena*. Cells, however, do not die immediately, but appear as senescent with lost reproductive ability. It seems to be an overall effect of herbicides that they induce changes similar to those occurring in normal senescence (Anderson and Thomson 1973). The action of IPC seems to be slow as most of the cell divisions stop only after 48 hours. The effect of the inhibitory action of IPC on the cell division of wild type Euglena is shown in Fig. 1. The lethal dose of IPC is 10^{-3} M for all investigated strains of *Euglena* as no colonies developed on plates after 5—7 day growth. However, even at this high dose and higher (1.5×10^{-3} M) some cell divisions occured (Figs. 1 and 6).

This inhibiton of cell division also depends on the nutritional history of cells. Cells previously grown on high acetate medium (40 mM) show a higher growth rate after IPC treatment during the first 48 hours, than those devoid of paramylum grown on low acetate medium (5mM). Fig 2 shows the inhibition of cell division in the yellow strain although all strains exhibited the same response. As previously acetate starved cells showed a corresponding higher sensibility towards the inhibitory action of IPC such cells were used in further experiments.

Most changes, however, are seen at an intermediate dose of about 5×10^{-4} M which affects cell division, but is mostly not lethal. This is the same dose which alters cell division and microtubule structure in the green alga *Oedogonium* (Coss and Pickett-Heaps 1974) but 10 times higher than in *Haemanthus* (Hepler and Jackson 1969). A proportion of cells in green, yellow and pale yellow *Euglena* grown in the dark in 5×10^{-4} M IPC appear monstrous due to abnormal divisions (Fig. 3—5). These cells are very unstable and are not readily formed on agar plates or in illuminated cultures as there is a rapid breakdown of cell membranes with a release of cell contents.

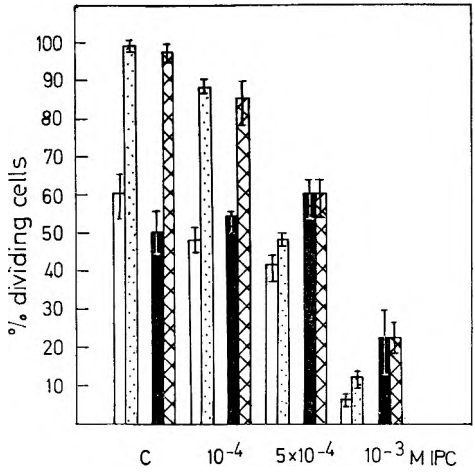

Fig. 1. Effect of IPC on cell division in wild type *Euglena*. Cells were grown for 13 days in the dark in an organic medium supplemented with 5 mM acetate and plated on the same medium to which various concentrations of IPC were added. The plates were illuminated or kept in the dark. Cell divisions were scored after 24 and 48 hours. First column: cell divisions scored after 24 h; second column: cell divisions scored after 48 h. White and dotted columns: in the light; dark and checked columns: in the dark.

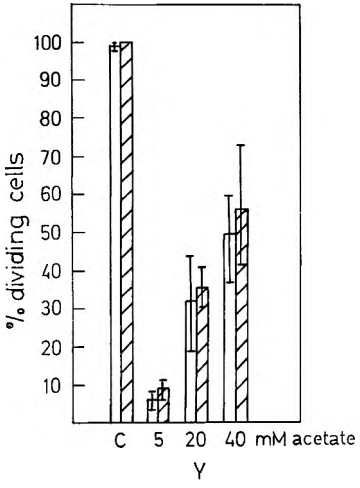

Fig. 2. Effect of IPC on cell division of carbon starved and carbon rich *Euglena* (yellow strain).
Cells were grown on an organic medium supplemented with 5, 20 and 40 mM acetate in the dark for 12 days and then plated on rich organic plates (23 mM acetate) with addition of IPC (1.5×10^{-3}M). Cell divisions were scored after 24 h (white columns) and 48 h (lined columns) growth in the dark.

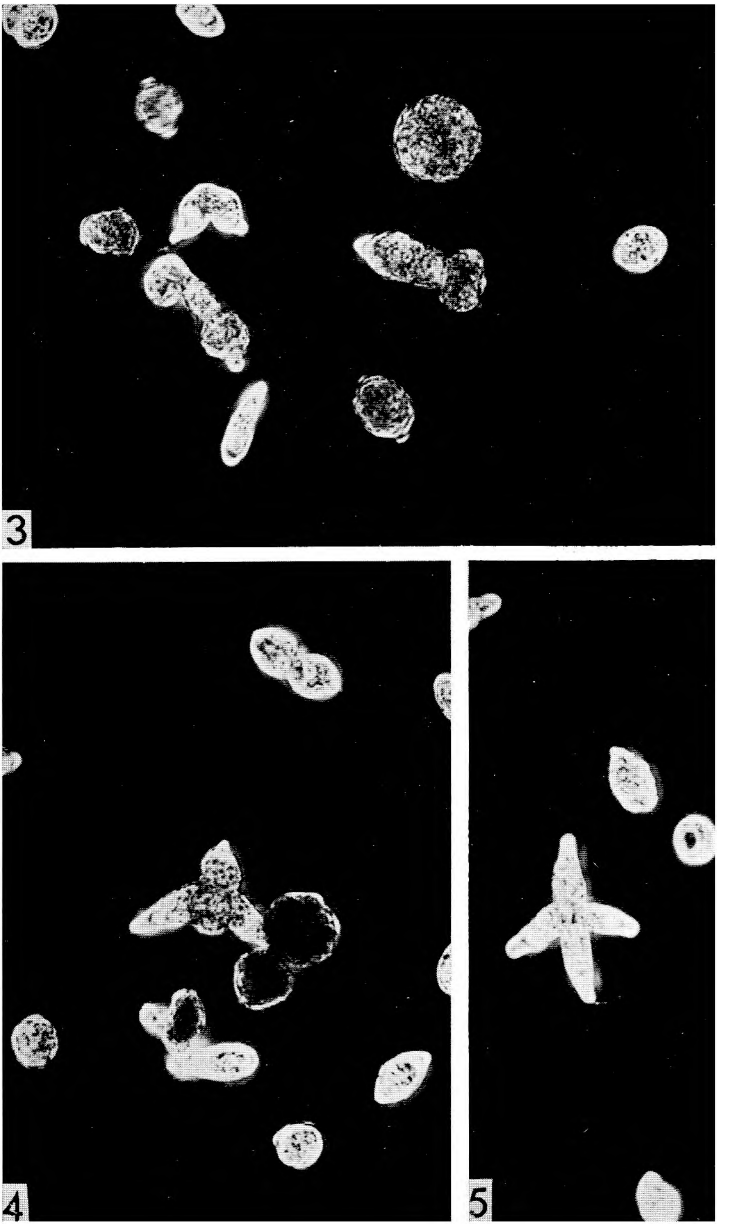

Fig. 3.—5. Monstrous cells of *Euglena* grown in IPC (wild type). Cells were grown for 7 days in an organic medium supplemented with 5×10^{-4} M IPC in the dark. Anoptral contrast, 400 : 1.

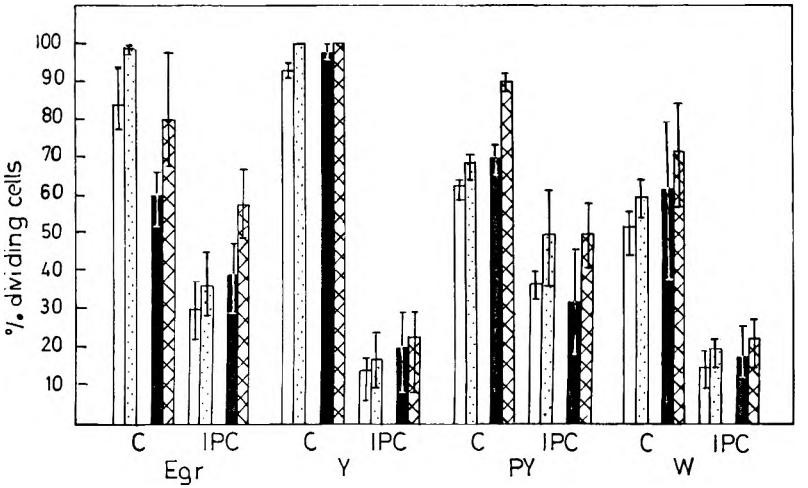

Fig. 6. Effect of IPC on cell division in the various strains of *Euglena*. Cells were grown on an organic medium supplemented with 5 mM acetate in the dark for 13 days and plated on the same medium with addition of IPC (1.5×10^{-3} M). Cell divisions were scored after 24 and 48 hours. First column: cell divisions scored after 24 h; second column: cells scored after 48 h. White and dotted columns: in the light; dark and checked columns: in the dark. E_{gr}: wild type; Y: yellow strain; PY: pale yellow strain; W: white strain.

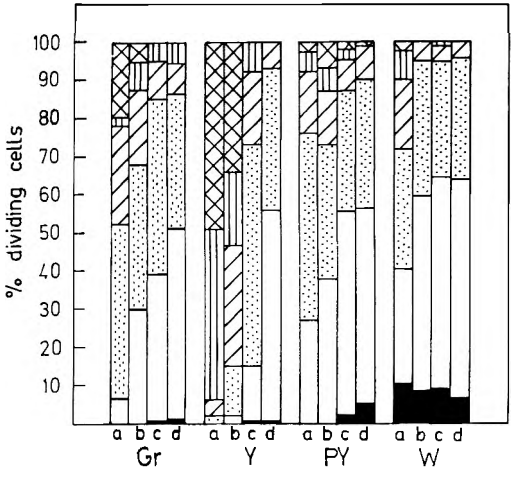

Fig. 7. Effect of ethanol on the growth of various strains of *Euglena*. Cells were grown for 4 days in the dark in an organic medium supplemented with 5 mM acetate and transferred to the same medium supplemented with 82 mM (a), 164 mM (b), 246 mM (c) and 328 mM (d) ethanol. Cells were scored after 48 hours growth in the dark. E_{gr}: wild type *Euglena;* Y: yellow strain; PY: pale yellow strain; W: white strain. Columns, black: dead cells; white: non-dividing cells; dotted: colonies with 2 — 7 cells; oblique lines: colonies with 8 cells; straight: colonies with 9 — 15 cells; checked: colonies with more than 16 cells.

Cell division and growth is most sensitive to IPC in green *Euglena*, particularly when grown in the light. The effect of IPC on the growth and cell division of *Euglena* at different light regimes is shown in Table I and Fig. 1. There is no pronounced difference of IPC-induced inhibition of cell division in light or in the dark in heterotrophic strains of *Euglena* (Fig. 6). In green *Euglena*, this difference is more pronounced at higher doses of IPC. This difference is statistically significant. A photoinhibition of cell division in the white strain was observed, which is in accordance with the findings of Cook (1968), namely that an inhibition is most pronounced in colourless *Euglena*.

Table 1. Effect of IPC on growth of wild type *Euglena*. Cells were grown in an organic medium supplemented with 82 mM ethanol for 5 days and kept under different illumination regimens.

IPC Concentration	Illumination regimen	Cell number/ml
0	Light : Dark 12:12 h	1×10^6
0	Dark	4.5×10^6
2.5×10^{-4} M	Light : Dark 12:12 h	8.5×10^5
2.5×10^{-4} M	Dark	1.5×10^6
3.5×10^{-4} M	Light : Dark 12:12 h	7.5×10^4
3.5×10^{-4} M	Dark	2.3×10^5
5.0×10^{-4} M	Light : Dark 12:12 h	5.0×10^3
5.0×10^{-4} M	Dark	2.1×10^4

IPC used as dry powder, instead of being dissolved in ethanol, had a much smaller effect on cell division, so most experiments were performed with IPC dissolved in ethanol.

Ethanol itself influences the growth of *Euglena*. It stimulates the growth of *Euglena* in the dark and inhibits chlorophyll synthesis (Kirk and Keylock 1967). As it is rapidly oxidized to acetate, it similarly represents a source of carbon and energy needed for cell division and growth (Buetow 1976, Garlaschi et al. 1974).

Ethanol stimulated the growth of all investigated strains in the dark. However, cells of *Euglena*, grown on acetate, appeared more normal than those grown on ethanol where giant cells could often be observed (Marčenko, unpublished). It has also been shown that the effect of ethanol on cell division depends on the previous nutritional history of cells. Cells transferred from a high acetate medium (20—40 mM, filled with paramylum granules) exhibit higher division rates when transferred to ethanol medium (82 mM) than those transferred from low acetate medium (5mM), almost without microscopically visible paramylum granules (Marčenko, unpublished).

To test the difference in the sensitivity of various strains of *Euglena* to ethanol, a test was devised with cells growing on agar plates and cell divisions scored as described in Materials and Methods. It has been shown that the yellow strain has the highest adaptability to ethanol as it shows the highest growth rate when grown on ethanol (Fig. 7). The difference in the proportion of dividing cells in the yellow strain on ethanol (except at the highest concentration) and other strains is statistically significant. The cells of this strain also appear to be most normal morphologically. Higher concentrations of ethanol, howewer, act inhibitorily (Fig. 7).

White, pigmentless *Euglena* shows the poorest growth on ethanol. It is the strain with the lowest growth rate (even in media without ethanol) and a low plating efficiency. A portion of cells is not viable and dies during cell division. The percentage of dead cells is independent of the ethanol concentrations used (Fig. 7) and depends, most likely, on some lethal factor in the gonome.

Effects of IPC on chlorophyll synthesis

IPC inhibits chloropyll synthesis in green Euglena as shown in Table II. Ethanol itself inhibits chlorophyll synthesis so the chlorophyll content is low. The chlorophyll content in the control without ethanol is approximately 1.6 times higher than in the control with ethanol. Both methods used showed a similar inhibitory effect of IPC. The difference

Table 2. Effect of IPC on chlorophyll synthesis

	Total chlorophyll in µg/100 mg wet weight			
	Dark	Light 24 h	Light 48 h	Light 96 h
Control (+ 82 mM ethanol)	3.3*	7.8	73.5	127.0
IPC 5×10^{-4} M (+ 82 mM ethanol)	—	4.9	39.1	75.7
IPC 10^{-3} M (+ 82 mM ethanol)	—	3.8	19.8	74.1

* The cells were not completely bleached during the dark period

between the various IPC doses used was highest after 48 hours. Later, some recovery of chlorophyll synthesis occurred. Method I has the advantage of measuring the chlorophyll content of cells under resting conditions while, with Method II, some interfering cell growth occurred. However, in the resting medium with IPC cells soon fall to the botom which prevents their even illumination.

The effects of IPC on chlorophyll are in accordance with the findings of Wrischer and Botka (1978) on greening of etiolated leaves.

Effect of IPC on paramylum synthesis

IPC inhibits de nuovo paramylum synthesis in the dark in dividing cells of *Euglena* (5×10^{-4} M). In green and yellow *Euglena* this concentration of IPC probably affects the utilization of ethanol (and/or acetate) as cells completely devoid of paramylum granules appear even in carbon-rich media. In the white strain, which shows a much smaller growth rate, an inhibition of paramylum synthesis by IPC was not observed, as paramylum breakdown in this strain seems to be also much slower.

A similar inhibition of carbon reserve substance (starch) formation by IPC was observed by Herichová (1972, 1973).

Bleaching experiments

Although IPC inhibits chlorophyll synthesis, it does not cause any permanent bleaching of green *Euglena*. For bleaching experiments cells were grown in 5×10^{-4} M IPC for 5 days washed and transferred to agar plates. The percentage of colourless colonies developed after the illumination of the plates did not exceed that of the control cultured without IPC.

Summary

The effects of the herbicide isopropyl N—N phenylcarbamate (IPC) on wild type *Euglena* and three bleached mutants (yellow, pale yellow and white) have been studied.

IPC is a slowly acting, rather weak, herbicide which inhibits cell division in all *Euglena* strains studied. This inhibition depends on the nutritional history of cells and the adaptability of the strain to the ethanol used to dissolve IPC.

Wild type *Euglena* is most sensitive to IPC, especially if cultured in the light, although the lethal dose has been found to be the same for all strains studied (10^{-3} M).

Most metabolic changes, such as appearance of monstrous forms, and inhibition of chlorophyll and paramylum synthesis, are seen at an intermediate dose of about 5×10^{-4} M.

IPC does not cause bleaching i. e. irreversible permanent loss of chloroplasts.

This study confirms that IPC does not only act on autotrophic plant cells, but on cells of heterotrophic organisms as well.

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SAŽETAK

DJELOVANJE N-FENIL-IZOPROPIL-KARBAMATA NA EUGLENU

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Proučavano je djelovanje herbicida N-fenil-izopropil-karbamata (IPC) na divlji tip euglene i njene izbliježene mutante (žutu, blijedo žutu i bijelu).

IPC je sporo-djelujući, dosta slab herbicid, koji inhibira staničnu diobu u svim proučavanim sojevima euglene. Ova inhibicija ovisi o prethodnim hranidbenim uvjetima i prilagodljivosti dotičnog soja na etanol u kojem je IPC otopljen.

Divlji tip euglene je pokazao najveću osjetljivost na IPC, naročito pri uzgoju na svjetlosti, iako je letalna doza (10^{-3} M) ista za sve proučavane sojeve.

Većina metaboličkih promjena, kao što su pojava monstruoznih oblika, inhibicija sinteze klorofila i paramiluma opaža se kod srednje doze od gko 5×10^{-4} M.

IPC ne izaziva izbljeđivanje tj. ireverzibilni trajni gubitak kloroplasta.

Ovo istraživanje potvrđuje da IPC ne djeluje samo na autotrofnu biljnu stanicu već također i na stanice heterotrofnih organizama.