Bleaching of Euglenas by Antibiotics — a Specific Form of Antagonism in Actinomycetes

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ABSTRACT

The author discusses little investigated antagonistic effects of certain antibiotics, manifested by the inhibition of chlorophyll synthesis in *Euglena gracilis*. Two main types of depigmentation were found — temporary and permanent. The oldest substrains of *Euglena gracilis* var. *bacillaris*, strain Mainx, permanently bleached by erythromycin, streptomycin, carbomycin, spiramycin, kanamycin and viomycin, were kept in the light for at least two years without showing any signs of turning green. These antibiotics caused permanent apochlorosis, but reversible apoplastidy. The euglena cultures remained permanently heterotrophic, Picromycin, methymycin and neomycin. however,only temporarily inhibited chlorophyll synthesis. When transferred to medium not containing these antibiotics and incubated in the light, these cultures again produced chlorophyll.

Provasoli, Hutner and Schatz (1948) described the effect of streptomycin on green forms of *Euglena gracilis*, which became permanently bleached and hence heterotrophic. The same phenomenon was described independently a year later by Jírovec (1949).

Irreversible chlorosis of euglenas can be induced by other factors, the best known being pyribenzamine and other substances used in medicine as antihistaminics (Gross, Jahn & Bernstein, 1955; Zahalsky, Hutner, Keane & Burger, 1962). O-methylthreonine is a similar inhibitor of the synthesis of photosynthesis of photosynthetic pigments in *Euglena gracilis* (Aaronson & Bensky, 1962). Apart from the action of these chemical agents, permanent depigmentation of euglenas can also be obtained by means of physical factors, including heat (Pringsheim E. G. & Pringsheim O., 1952) and ultraviolet light (Pringsheim, 1958).

Temporary loss of chlorophyll synthesis was observed in *Euglena gracilis* cultures treated with 3-amino-1,2,4-triazole (Aaronson & Scher, 1960). This triazole is a herbicide and its bleaching effects have also been described in higher plants (Schwimmer & Weston, 1958). Its effect is similar to that of keeping euglenas on organic media in the dark. In the dark the euglenas become apochlorotic, but when they are transferred to the light, chlorophyll synthesis rapidly regenerates (Wolken, 1956).

Provasoli, Hutner and Pintner (1951) found that streptomycin had a similar effect to darkness on the chloroplasts, but that its effect was irreversible and that in the later stages the plastids broke up into fragments. Vávra (1957), however, found that streptomycin did not block multiplication of the plastids, but that, on the contrary, the number of plastids increased during contact with streptomycin and only decreased in size. His explanation is that the plastids divide, but do not grow to full size. The bleaching

Table 1. Bleaching of *Euglena gracilis* cells by antibiotics

Antibiotic	Concentration in μg./ml.	Evaluated after				
		7 days	14 days	Transferred to medium without antibiotics	Three passages without without antibiotics	Twelve passages without antibiotics
Erythromycin	50	G	G	G	G	G
	100	PG	PG	G	G	G
	200	W	W	W	W	W
	500	W	W	W	W	W
	1,000	W	W	W	W	W
	2,000	W	W	W	W	W
Streptomycin	50	G	G	G	G	G
	100	PG	PG	G	G	G
	200	W	W	W	W	W
	500	W	W	W	W	W
	1,000	W	W	W	W	W
	2,000	W	W	W	W	W
Kanamycin	50	G	G	G	G	G
	100	G	G	G	G	G
	200	G	G	PG	G	G
	500	G	G	W	W	W
	1,000	PG	PG	W	W	W
	2,000	PG	PG	W	W	W
Viomycin	50	G	G	G	G	G
	100	G	G	G	G	G
	200	G	G	PG	G	G
	500	G	G	W	W	W
	1,000	G	G	W	W	W
	2,000	G	G	W	W	W
Spiramycin	50	G	G	G	G	G
	100	G	G	G	G	G
	200	G	G	G	G	G
	500	PG	PG	G	G	G
	1,000	PG	PG	PG	G	G
	2,000	W	W	W	W	W
Carbomycin	50	PG	PG	G	G	G
	100	W	PG	G	G	G
	200	W	W	PG	G	G
	500	W	W	W	W	W
	1,000	W	W	W	W	W
	2,000	W	W	W	W	W

G = green cells
PG = pale green cells
W = white cells

effect of streptomycin on *Euglena gracilis* was regarded as a specific property of this antibiotic alone. This assumption was found to be wrong, however, since the author recently described the bleaching effect of erythromycin (Ebringer, 1961) and a little later of seven other antibiotics (Ebringer, 1962a).

MATERIALS AND METHODS

Strain and cultivation methods. The experiments were carried out with *Euglena gracilis* var. *bacillaris*, strain Mainx, obtained from the collection of the Protozoology Laboratory of the Czechoslovak Academy of Sciences, Prague. The following culture medium was used: Bactopeptone Difco, 5 g., sodium acetate, 2 g., glucose, 2 g., tap water, 1,000 ml. The cultures were continuously illuminated with a "Daylight" lamp (22 W/50 cm.). Biological test tubes measuring 13 × × 160 mm., containing a total of 10 ml. medium, were used for cultivation.

Preparation of antibiotic solutions. The antibiotics were added to the medium aseptically. Soluble forms of antibiotics, which were supplied in sterile packages, were prepared in the usual manner, by adding sterile distilled water to form serial dilutions. Sterile solutions of the bases of erythromycin, picromycin and methymycin, which are insoluble in water, were prepared by dissolving the antibiotic in acetone and filtering through a bacteriological filter. Excess acetone was removed from the medium by distilling in vacuo at a temperature not exceeding 50° C.

Determination of number of cells. Growth curves were obtained by counting the cells in a Bürker counting chamber.

RESULTS

As stated in previous reports (Ebringer 1961, 1962a, b), permanent loss of chlorophyll synthesis can be induced in *Euglena gracilis* by a single dose of any of the

Table 2. Temporary bleaching of *Euglena gracilis* cells by some antibiotics

Antibiotic	Concentration in μg./ml.	Evaluated after				
		7 days	14 days	Transferred to medium without antibiotics	Three passages without antibiotics	Twelve without antibiotics
Methymycin	200	PG	G	G	G	G
	500	W	PG	G	G	G
	1,000	W	W	G	G	G
Picromycin	200	G	G	G	G	G
	500	PG	PG	G	G	G
	1,000	W	W	G	G	G
Neomycin	200	G	G	G	G	G
	500	W	PG	G	G	G
	1,000	O	O	O	O	O

G = green cells.
PG = pale green cells
W = white cells
O = complete inhibition of euglenae

following antibiotics: erythromycin, carbomycin, spiramycin, streptomycin, kanamycin and viomycin. The concentrations of these antibiotics which always induce a permanent hereditary change should be differentiated (Tab. 1). With lower concentrations, it can be seen that the smaller the dose of antibiotic used for depigmentation, the more rapid the rate of reversion to green forms.

Other antibiotics — methymycin and picromycin — cause only temporary or partial depigmentation (Tab. 2). The concentration needed for bleaching is about 1,000 μg./ml. for both. When transferred to medium containing no antibiotics, the cells continue to synthesize

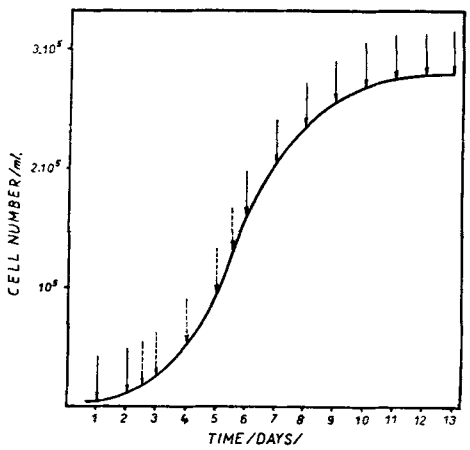

Fig. 1. Study of the bleaching effect of erythromycin (1,000 μg./ml.) on different phases of growth. The culture was always under action of antibiotic 48 hours, then transferred to medium without antibiotics.
← — — — addition of antibiotic causing permanent depigmentation of further passages of euglenas
← addition of antibiotics not inhibiting chlorophyll synthesis, so that subsequent passages of the euglenas are green.

chlorophyll. Higher antibiotic concentrations were not used, since orientation tests showed that higher picromycin and methymycin concentrations caused complete inhibition of the cells, which died soon after the antibiotics had been added. When soluble forms of these antibiotics have been obtained, their bleaching properties will probably be greater, as in the case of the less readily soluble base of erythromycin, whose bleaching effect was weaker than that of soluble erythromycin salts. Neomycin also had only a weak inhibitory effect on chlorophyll synthesis in *Euglena gracilis*. It could not be used in concentrations of more than 500 μg./ml., however, since higher concentrations were toxic for euglenas.

The given erythromycin concentrations had practically no effect on proliferation of the cells: the same applies to carbomycin and spiramycin. With antibiotics

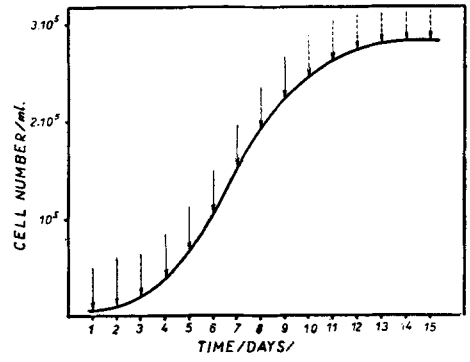

Fig. 2. Study of bleaching effect of viomycin (1,000 μg./ml.) on different phases of growth. The culture was always under action of antibiotic 48 hours, then transferred to medium without antibiotics.
← — — — addition of antibiotic causing permanent depigmentation of further passages of euglenas
← addition of antibiotics not inhibiting chlorophyll synthesis, so that subsequent passages of the euglenas are green.

of the streptomycin group, however, the situation was different. Although kanamycin, in the given concentrations, likewise had practically no inhibitory effect on proliferation, streptomycin and viomycin both inhibited it. The most toxic of all, however, was neomycin. In this connection it should be noted that inhibition of proliferation of euglenas basically correlates with the toxicity of these antibiotics for higher animals.

In addition to inhibiting chlorophyll synthesis, antibiotics of the erythromycin and streptomycin group also interfere with the development of plastids. Erythromycin inhibition of plastid development has already been described (Ebringer, 1962c). It was later found that antibiotics could be divided into three groups, according to their effect on the plastids and inhibition of chlorophyll synthesis:

Group 1: erythromycin, carbomycin, spiramycin and streptomycin. These antibiotics interfere with the normal development of plastids. After the antibiotic has been added to the medium the plastids gradually decreased in size until they almost disappeared. After a few days they again regenerated and gradually increased in size; now, however, they were only leucoplasts. Inhibition of the plastids was also accompanied by inhibition of chlorophyll synthesis and the cells were rapidly bleached (Fig. 1). Apochlorosis was permanent, but apoplastidy was only temporary.

Group 2: viomycin and kanamycin. On adding these antibiotics to euglena cultures, the cells remained green and the plastids also appeared intact. The bleaching effect of the tested concentrations of these antibiotics was manifested only in subsequent passages. When transferred to media containing no antibiotics, the euglena cells did not produce chlorophyll, but remained permanently bleached and were heterotrophic. These antibiotics affected the cells in the later stages of growth. This was particularly the case with viomycin, which only bleached euglenas in the resting phase (Fig. 2). Apochlorosis was again permanent.

Group 3: picromycin, methymycin and neomycin. These antibiotics only temporarily bleached *Euglena gracilis* cells (temporary apochlorosis).

DISCUSSION

Antibiotics of the erythromycin group, i.e. macrolidic antibiotics, are characterized by the presence of macrocyclic lactone rings, to which one or several sugar components are joined. The author assumes that the mechanism of the antibacterial effect of these antibiotics is the same. This is borne out by reciprocal cross resistance and by their bleachng effects (described above).

Antibiotics of the streptomycin group have a qualitatively similar activity spectrum and (with the exception of viomycin) a similar chemical structure. The molecules of streptomycin antibiotics (except viomycin, which is a polypeptide) usually consist of carbohydrate elements and aminocyclical components. The interrelationship of these antibiotics is also confirmed by frequent cross resistance, the similarity of their side-effects on the macroorganism and their above bleaching effects. All antibiotics of the erythromycin and streptomycin group are produced by the genus *Streptomyces*.

Since all bleaching antibiotics (except viomycin) contain specific amino sugars, it was first assumed that the bleaching mechanism could be explained on the basis of their interference with the chloroplast nucleic acids. Viomycin, however, is a polypeptide without a sugar component, so that this theory was incomplete. The course of viomycin bleaching also differs from that of the other antibiotics (Fig. 1 and 2).

It is interesting to note that all the bleaching antibiotics inhibit *Mycobacterium tuberculosis* extremely strongly. If some correlation exists between the depigmentation of euglenas and inhibition of *Mycobacterium tuberculosis*, this phenomenon could probably be utilized for screening antibiotics with a tuberculostatic effect. In that case *Euglena gracilis* could also be used for studying combinations of antibiotics for the treatment of tuberculosis. It was found that mixtures of these antibiotics had a pronounced synergic effect on depigmentation of *Euglena gracilis*.

The phenomenon of the change of

autotrophic to heterotrophic organisms by antibiotics might also help to explain the formation of some heterotrophic flagellates from green partners. A number of authors assume (Provasoli, 1957) that some natural leucoflagellates are formed by the action of streptomycin or by the combined action of this antibiotic and physico-chemical factors on coloured species. The discovery of further "bleaching" antibiotics makes this concept even more feasible, since antibiotic mixtures seem to have a synergic effect on depigmentation (Ebringer, 1962c). This means that, on using a mixture, the euglenas are bleached by a much lower antibiotic concentration than on using one antibiotic only. It can therefore be assumed that "loss" mutations also occur in nature and that the mutagens are bleaching antibiotics. It can be assumed from the above results that inhibition of chlorophyll synthesis is another widespread manifestation of antagonism, which is at present known only in the genus *Streptomyces*.

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ОБЕСЦВЕЧИВАНИЕ ЭВГЛЕНЫ АНТИБИОТИКАМИ — ОСОБАЯ ФОРМА АНТАГОНИЗМА У АКТИНОМИЦЕТОВ

Л. Эбрингер

Исследовалось малоизученное до сих пор антагонистическое действие некоторых антибиотиков. Этот антагонизм проявляется в угнетении синтеза хлорофилла у Euglena gracilis. Мы заметили, что, по существу, имеют место два вида депигментации: временная и устойчивая. Наиболее старые субкультуры Euglena gracilis var. bacillaris, штамм Mainx, устойчиво обесцвеченные эритромицином, стрептомицином, карбомицином, спирамицином, канамицином и виомицином, мы поддерживаем на свету уже не менее 2 лет, причем не наблюдали их позеленения. Перечисленные антибиотики вызывают

устойчивый апохлороз, но обратимую апопластичность. Такие культуры эвглены становятся устойчиво гетеротрофными. Наоборот, пикромицин, метимицин и неомицин вызывают лишь временную утрату способности к синтезу хлорофилла. После пересева в среду без этих антибиотиков обесцвеченные культуры снова начинают продуцировать хлорофилл.