The Action of Streptomycins on the Chloroplast System of *Euglena gracilis*

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SUMMARY

Five streptomycins bleached the chloroplast system of *Euglena gracilis*; the most effective were those which contained an aldehyde group in the streptose moiety: streptomycin and hydroxystreptomycin. Derivatives containing a secondary alcohol group in place of the aldehyde (dihydro-, dihydrodesoxy-, dihydrohydroxystreptomycin) require higher concentrations to elicit the same effect. Hydroxylamine reversed the bleaching effects of streptomycin and hydroxystreptomycin but not of dihydro-, dihydrodesoxy- or dihydrohydroxystreptomycin. These observations suggest that the aldehyde group of the streptomycins contributes to the action of these antibiotics on *Euglena* chloroplasts; the action of hydroxylamine would be to either remove the antibiotic from its site of action or to render it ineffective *in vivo* by reaction with the aldehyde group.

INTRODUCTION

An interesting and as yet inadequately explained action of streptomycin is the ability of this antibiotic to eliminate chloroplasts from *Euglena* without significantly inhibiting cell division or viability of the cell. In bacteria, streptomycin binds to ribosomes causing miscoding of messenger RNA and production of faulty protein (Davies, Gilbert & Gorini, 1964). Accordingly, Schiff & Epstein (1965) suggested that bleaching agents may act by inhibiting protein synthesis in *Euglena* chloroplasts. However, Stern, Barner & Cohen (1966) demonstrated that, in *Escherichia coli*, streptomycin lethality occurred in the absence of protein synthesis. Since the *Euglena* chloroplast represents a different system in which to study the mechanism of action of antibiotics, further light may be shed on this important subject by the use of this organism.

Aronson, Meyer & Brock (1964) proposed a mechanism of action for the streptomycins based on the ability of the antibiotics to cross-link nucleic acid. Streptomycin is a more effective cross-linking agent *in vitro* than dihydrostreptomycin, and the presence of an aldehyde group in the streptose moiety may be responsible for the increased effectiveness. According to Aronson *et al.* (1964), this aldehyde is capable of forming a second cationic attachment site by reacting with the secondary amino group at the N-methyl glucosamine moiety, resulting in a cyclic iminium salt. Since dihydrostreptomycin cannot form this cationic cyclic structure, it would be expected to be a less effective agent for precipitating nucleic acids. Streptomycin is known to be more effective as a bleaching agent in *Euglena* than dihydrostreptomycin (Zahalsky,

Hutner, Keane & Burger, 1962), which supports the model of Aronson *et al.* (1964) and suggests that the aldehyde group may play a role in chloroplast elimination. We have observed that hydroxylamine reverses the action of streptomycin in *Euglena* chloroplasts if added before about 6 to 8 divisions have taken place in the presence of the antibiotic (Ebringer, Mego & Podová, 1967). In the studies reported in the present paper we compared the actions of five streptomycins as bleaching agents in *Euglena*. These antibiotics differ only in the streptose moiety: some contain aldehyde groups and others have alcoholic groups. We also considered it of interest to compare the effect of hydroxylamine as a reversing agent on the action of these streptomycins particularly since hydroxylamine may react with the aldehyde to form an oxime.

METHODS

Euglena gracilis, strain Z, was grown in a proteose peptone-tryptone medium (Mego, 1964). Cultivation conditions were described by Ebringer (1966). Antibiotics were dissolved in distilled water, sterilized by filtration and then added to the liquid media. After a period of growth with antibiotic, the organisms were washed, harvested by centrifugation and then plated. On the ninth day after plating about 300 to 600 colonies were counted and the numbers of white and green colonies were recorded. Individual counts were made microscopically in a Burkner counting chamber.

The final concentration of hydroxylamine sulphate was always 10^{-1} M. Hydroxylamine was present for only 20 min. and was then removed by washing on the centrifuge. The chlorophyll content was determined according to the method of Arnon (1949). Sulphates of the streptomycins were obtained by the courtesy of the following: dihydrodesoxystreptomycin sulphate from Dr Kazuo Kagiwada, Kaken Chemical Co. Ltd., Tokyo, Japan; hydroxy- and dihydrohydroxystreptomycin sulphate from Dr J. C. Sylvester, Abbott Laboratories, North Chicago, Illinois, U.S.A; dihydrostreptomycin sulphate, from Dr Alvaro Zugaza, Antibiotics, S.A. Madrid, Spain.

The following abbreviations will be used: SM, streptomycin; OHSM, hydroxystreptomycin; DHSM, dihydrostreptomycin; DHOHSM, dihydrohydroxystreptomycin; DHDSM, dihydrodesoxystreptomycin; HA, hydroxylamine.

RESULTS

Figure 1 shows that bleaching action of the various streptomycins depended on the concentration and duration of action. The most effective were SM and OHSM, which produced 100% bleached colonies in 24 hr at 500 $\mu\text{g./ml.}$, in 3 days at 100 $\mu\text{g./ml.}$, only after 5 days at 10 $\mu\text{g./ml.}$ DHSM, DHOHSM and DHDSM, however, required 4 days to produce 100% bleached colonies at 500 $\mu\text{g./ml.}$ Even after 6 or more days these antibiotics did not produce total bleaching at 10 $\mu\text{g./ml.}$

Table 1 shows that OHSM inhibited growth slightly more than the other antibiotics. In order to diminish this toxicity effect, we used 100 $\mu\text{g./ml.}$ in further experiments since this concentration produced total bleached colonies. SM was used in this concentration, while DHSM, DHOHSM and DHDSM were used at 500 $\mu\text{g./ml.}$

Hydroxylamine reversed only the effects of SM and OHSM (Table 2). The reversal was nearly complete when this agent was added before 4.5 divisions. After about 5 divisions only a small percentage of the organisms were reversed. Reversal effectiveness

Fig. 1. Proportions of bleached colonies obtained after growth of *Euglena gracilis* in various concentrations of streptomycins for varying lengths of time. (a) 500 µg./ml. (b) 100 µg./ml. (c) 10 µg./ml. After the indicated period of cultivation with the streptomycins, the organisms were washed, plated on antibiotic-free media and bleached and green colonies counted after 9 days. A, SM; B, OHSM; C, DHSM; D, DHOHSM; E, DHDSM (for abbreviations, see text).

was approximately the same for SM and OHSM but completely ineffective for the other antibiotics.

Table 3 shows the chlorophyll content of the cultures during growth in the presence of the various antibiotics. Although 100% of bleached colonies were obtained after plating in many cases, the cultures contained considerable quantities of chlorophyll. These results suggest that chlorophyll content of the cultures does not represent a true measure of the bleaching effectiveness of these agents.

Table 1. *Effects of various streptomycins on growth of Euglena gracilis*

Antibiotic ($\mu\text{g./ml.}$)...	500	200	100	10
SM†	438*	440	530	572
OHSM	355	398	446	485
DHSM	418	470	520	536
DHOHSM	460	497	510	560
DHDSM	400	468	550	575

* The numbers represent thousands of organisms/ml. medium after 6 days at 24°. † For abbreviations see text.

Table 2. *Prevention of streptomycin-bleaching of Euglena gracilis by hydroxylamine*

Organisms in media containing streptomycins were allowed to grow for approximately the numbers of divisions in column 1. Samples were removed, treated with HA for 20 min., washed and plated on agar (column '+ HA'). The column '- HA' indicates the results without hydroxylamine treatment. Colonies were counted after 9 days growth on agar at 24°.

Numbers of divisions	% permanently bleached colonies									
	SM		OHSM		DHSM		DHOHSM		DHDSM	
	-HA	+HA	-HA	+HA	-HA	+HA	-HA	+HA	-HA	+HA
1.0	87	8	93	4	25	26	40	48	52	49
2.5	100	1	100	7	40	39	64	63	57	62
3.9	100	1	100	2	63	59	80	80	85	89
4.5	100	2	100	2	84	86	91	92	91	95
4.9	100	40	100	50	100	100	100	100	100	100
5.4	100	93	100	98	100	100	100	100	100	100
5.9	100	100	100	100	100	100	100	100	100	100

For abbreviations and concentrations see text.

Table 3. *Chlorophyll content (as % of a control culture without added antibiotic) of Euglena gracilis and the percent streptomycin-bleached colonies obtained 9 days after plating on agar*

Antibiotic ($\mu\text{g./ml.}$)	500	Colonies	200	Colonies	100	Colonies	10	Colonies
	Chloro- phyll		Chloro- phyll		Chloro- phyll		Chloro- phyll	
SM	0	100	0	100	0	100	29	100
OHSM	0	100	0	100	0	100	69	100
DHSM	21	100	30	100	36	100	86	87
DHOHSM	26	100	35	100	43	100	55	85
DHDSM	31	100	32	100	33	100	61	79

For details and abbreviations see text.

DISCUSSION

The results presented in this paper establish that those streptomycins containing an aldehyde group on the streptose moiety were the most effective bleaching agents (see Fig. 2) and support the model proposed by Aronson *et al.* (1964) discussed in the introduction. There is no direct evidence yet to suggest that these antibiotics bind directly to chloroplast DNA; however, some mechanism must be proposed to account for the ability of streptomycin permanently to eliminate chloroplasts from *Euglena*. Zimmer, Triebel & Thrum (1967) have shown that certain antibiotics, including streptomycin and dihydrostreptomycin, interact with DNA *in vitro* to increase the melting

Fig. 2. The structures of streptomycins.

point of this nucleic acid. Their evidence suggests that the antibiotics cross-link DNA by electrostatic interactions. It does not seem unreasonable, therefore, to postulate that the antibiotics may prevent the separation of DNA strands after replication in the chloroplast. Hydroxylamine may act by releasing the antibiotics from electrostatic interaction with chloroplast DNA perhaps by reacting with the aldehyde group. This hypothesis would require either a different mechanism of action for the non-aldehyde-containing antibiotics or a less efficient binding to DNA.

Approximately six divisions were required before treatment with hydroxylamine became ineffective as a reversing agent. This correlates with the observation of Mego & Buetow (1967), who found that approximately this number of divisions was required for irreversible chloroplast loss induced by growth at elevated temperature. Again, it does not seem unreasonable to postulate that inhibition of DNA replication may require this number of divisions to 'dilute out' the relevant nucleic acid.

Other inhibitors of DNA synthesis are also effective bleaching agents in *Euglena*. These include nalidixic acid, porfiromycin, streptonigrin, novobiocin, rubiflavin, sarkomycin (Ebringer & Mego, unpublished observations), some mutagens (McCalla, 1967) and ultraviolet light (Lyman, Epstein & Schiff, 1961; Gibor & Granick, 1962).

An argument against the theory that bleaching agents act by inhibition of protein

synthesis in chloroplasts is the effect of chloramphenicol. This antibiotic is effective only in organisms containing 70S ribosomes (Vazquez & Monro, 1967) including *Euglena* chloroplasts. Chloramphenicol has no effect on protein synthesis in the cytoplasm of *Euglena* which contains 80S ribosomes. This substance, therefore, should be an ideal bleaching agent, since rapid cell division can proceed while chloroplast protein synthesis is inhibited. Chloramphenicol is not a bleaching agent, however, and only produces a temporary inhibition of chloroplast protein formation (Anderson & Smillie, 1966; Pogo & Pogo, 1965). Other inhibitors of protein synthesis such as the tetracyclines and puromycin are also not effective bleaching agents (Selsky, 1967). Although aureomycin has been reported to cause bleaching of *Euglena* (Robbins, Hervey & Stebbins, 1953), we have not been able to demonstrate more than a temporary inhibition of chloroplast formation under a variety of conditions.

We have also presented evidence that measurement of chlorophyll may not always be a valid index of permanent bleaching effectiveness of antibiotics. This may account for the observations of Linnane & Stewart (1967) in which these authors reported that macrolidic antibiotics were not bleaching agents in *Euglena*.

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