EFFECT OF STREPTOMYCIN ON GREENING AND BIOSYNTHESIS IN *EUGLENA GRACILIS*

J. T. O. KIRK

Medical Research Council Unit for Chemical Microbiology, Department of Biochemistry, University of Cambridge, Cambridge (Great Britain)

(Received May 25th, 1961)

SUMMARY

1. An experimental system is described in which etiolated cells of *Euglena gracilis*, under conditions which do not support growth, synthesise chlorophyll in the light, rapidly and reproducibly, without a lag period. The synthesis of chlorophylls *a* and *b*, and of carotenoids, is inhibited by high concentrations of streptomycin.

2. During greening there is little or no fall in the level of the free amino compounds of the cells. Streptomycin sulphate causes an increase in the level of the free amino compounds of the cells. Inorganic sulphates have a similar, although smaller, effect.

3. At concentrations which inhibit greening, streptomycin also inhibits the incorporation of [1-^{14}C]acetate into the protein and nucleic acid of the cells, both in the light and in the dark. Incorporation of acetate carbon into polysaccharide, however, is markedly stimulated by streptomycin in the light.

4. The various effects of streptomycin on chlorophyll and carotenoid synthesis, and on acetate incorporation, are all annulled if $MnCl_2$ is added at the same molar concentration as the drug; $MgCl_2$ is ineffective.

5. It is suggested that streptomycin has a general effect on biosynthesis and that its inhibition of greening is not due to a specific inhibition of chloroplast formation, but is merely one consequence of the effect on general biosynthesis.

INTRODUCTION

PROVASOLI, HUTNER AND SCHATZ[1] found that when *Euglena gracilis* is grown in the presence of streptomycin the organism loses the ability to make chloroplasts and becomes a permanently bleached strain. High concentrations of dihydrostreptomycin also inhibit the formation of chlorophyll in the light, in non-growing etiolated *E. gracilis*[2]. VON EULER, BRACCO AND HELLER[3] found that streptomycin inhibited greening in etiolated leaves of *Brassica botrytis*, but DE DEKEN-GRENSON[4] observed no such inhibition in etiolated barley leaves, although the drug inhibited chlorophyll synthesis in germinating barley seedlings.

This paper describes an investigation of certain effects of streptomycin on the metabolism of *E. gracilis*, in the hope that this might cast some light on the mechanism by which the drug inhibits plastid formation.

Abbreviations: SM, streptomycin; Chl., chlorophyll.

METHODS

Organism and growth conditions

The organism used throughout this work was *Euglena gracilis* strain 1224/5g, from the culture Collection of Algae and Protozoa, Botany School, Cambridge. Organisms were maintained in a medium containing (% w/v): Evans Bacteriological Peptone, 3.0; Difco Yeast Extract, 0.05; $CH_3COONa \cdot 3H_2O$, 0.5; $MgSO_4 \cdot 7H_2O$, 0.02; KH_2PO_4, 0.025; $Na_2HPO_4 \cdot 2H_2O$, 0.025; $CaCl_2$ 0.002; the ingredients being dissolved in tap water to give a final pH value of about 6.2. To obtain etiolated cells, 5 ml of a light-grown culture in the early stationary phase of growth were inoculated into 100 ml of medium in a 250-ml Erlenmeyer flask. After 3 days incubation at 30° in the dark (by which time the culture was in the late logarithmic phase of growth) the cells were harvested and washed 3 times in 0.25 *M* glucose (except where otherwise stated) before use. Green cells were obtained by growing the cultures at 30° in an incubator lit by two 40-W fluorescent tubes. Light-grown cells contained about 30 μg of chlorophyll *a* and about 5 μg of chlorophyll *b*/mg dry wt. Dark-grown cells had a chlorophyll content which was about 5% of that of light-grown cells.

Cell counts and dry weight determinations

Cells were immobilized by freezing and thawing, and counted in a Neubauer counting chamber. The relationship between cell number and dry weight was determined by freeze-drying and weighing a known number of cells. It was found that both green and etiolated *E. gracilis* weighed 1.1 mg per 10^6 cells.

Pigment determination

The organisms were harvested, washed with distilled water, and approx. 5 mg dry weight of cells were extracted with 5 ml of 80% aqueous acetone for 60 min at room temperature in the dark. The absorbancy of the extract was measured at the appropriate wavelengths, by means of a Unicam SP 500 spectrophotometer. To determine the absolute amounts of chlorophylls *a* and *b*, the absorbancies at 663 mμ and 645 mμ were measured and the values of the absorption coefficients given by ARNON[5] used to calculate the concentrations. However, for a simple comparison of the amounts of chlorophyll formed under different conditions, only the absorbancy at 663 mμ was measured. The absorption spectrum of the aqueous acetone extract has a peak at 478 mμ due to carotenoids. To obtain an estimate of carotenoid synthesis, the absorbancies at 663, 645, and 480 mμ were measured. The concentrations of chlorophylls *a* and *b* were calculated and then, using the specific absorption coefficients for the chlorophylls at 480 mμ (see ref. 6), the contribution of these pigments to the absorbancy at 480 mμ was computed. Subtraction of this from the measured A_{480} gave a figure which was taken to be approximately proportional to the concentration of carotenoids.

Extraction of pool materials and estimation of free amino nitrogen

Complete removal of the acid-soluble amino compounds and compounds absorbing at 260 mμ was obtained by extracting the cells with 0.2 *N* $HClO_4$ for 60 min at 20°. There was no significant increase in the A_{260} of the extracts when extraction was prolonged to 90 min suggesting that little or no hydrolysis of nucleic acid takes

place under these conditions. The substances in these extracts are referred to as the pool materials of the cells. The free amino nitrogen of the pool extracts was determined by the method of COCKING AND YEMM[7].

Preparation of pool amino acids for chromatography

The bulk of the $HClO_4$ in the extracts was removed by neutralization with KOH at 0° and the solutions were desalted by adsorption on to Amberlite IR-120 resin (H^+ form), elution with 7.5 *N* NH_4OH, and lyophilization.

Other estimations

Protein was estimated by the method of LOWRY, ROSEBROUGH, FARR AND RANDALL[8], total nucleic acid by the method of OGUR AND ROSEN[9], and carbohydrate by the method of TREVELYAN AND HARRISON[10].

Materials

Na[1-^{14}C]acetate, specific activity 5.2 μC/μmole was obtained from the Radiochemical centre, Amersham, and diluted with suitable amounts of unlabelled acetate when required. The trypsin used in the fractionation procedure was obtained from Hopkin and Williams Ltd. Streptomycin was used in the form of the sulphate (Glaxo Ltd.). Concentrations of streptomycin refer to the concentration of free base.

Fractionation procedure and estimation of radioactivity

After incubation with [^{14}C]acetate the labelled cells were fractionated as follows: (a) Incubations were terminated by chilling the tubes in an ice-bath. The cells were then harvested by centrifugation, resuspended in 0.2 *N* $HClO_4$ and after incubation for 60 min at 20° to remove pool materials, centrifuged again, and washed twice with water. The insoluble material remaining is referred to in the tables as "whole cell residue". (b) The whole cell pellet was extracted twice with absolute ethanol at 75 to 80°, for 10 min. The combined ethanol extracts are referred to as the "lipid fraction". A third extraction with hot ethanol, or with $CHCl_3$–methanol (1.75:1) removed no further labelled material. About 8% of the cell protein was found in the "lipid fraction". (c) Nucleic acids were then extracted by 2 successive treatments with 5% trichloroacetic acid[11] at 90°, for 30 min. The trichloroacetic acid was removed from the extracts with ether. On some occasions the nucleic acid extracts were further hydrolysed[12] with 1 *N* HCl to liberate free purines and pyrimidine nucleotides, and the hydrolysate was then subjected to paper chromatography in isopropanol-HCl[13]. The adenine, guanine, cytidylic acid and uridylic acid spots were eluted; the absorption at suitable wavelengths[12], and the radioactivity of the eluates were measured, and the specific activities of the 4 components calculated. (d) After removal of the nucleic acids, the pellet was washed twice with 0.01 *M* NH_4HCO_3, and then extracted twice, for 3 h at 37°, with a solution of trypsin (100 μg/ml) in 0.01 *M* NH_4HCO_3. The pellet was then washed with water, and the supernatant fluid was combined with the two extracts to give the "protein fraction". (e) The material remaining contained not less than 98% carbohydrate, and could be seen, microscopically, to consist of paramylon granules. Consequently, this is referred to as the "paramylon fraction".

Radioactive samples were pipetted on to aluminium planchettes (2 cm diameter), dried, and counted in a Panax counter with an end-window Geiger-Müller tube.

Specific activity of the paramylon glucose

The specific activity of the glucose in the paramylon fraction was obtained by hydrolysing the material in 2 *N* H_2SO_4 in a pressure cooker for 60 min at 15 pounds/in^2. The hydrolysate was neutralized with IRC-50 resin (HCO_3^- form) and subjected to paper chromatography in butanol–pyridine–water (6:4:3), isopropanol–water (9:1), and ethyl acetate–pyridine–water (8:3:1), with glucose as a marker. A single spot was obtained in every case with the same R_F as the glucose marker. The spots were developed with aniline phthalate and eluted with 80% aqueous ethanol–0.7 *N* HCl[14]. The amount of glucose present was determined by measuring the absorption at 390 mμ, and aliquots of the eluates were then plated for determination of radioactivity.

Illuminating apparatus

The etiolated organisms used in this study had a chlorophyll content which was about 5% of that of normal green cells. Organisms with no detectable chlorophyll may be obtained by growing them in the dark from an inoculum which was itself grown in the dark[2]. However, such organisms will synthesise chlorophyll only after a long lag period in the light, whereas the slightly pigmented cells used here exhibited little or no lag before chlorophyll synthesis commenced in the light. When etiolated cells were placed in an illuminated incubator, chlorophyll formation was slow—incubations had to last 2 or 3 days—and very variable from one tube to another. For rapid and reproducible rates of chlorophyll synthesis, the following conditions are found to be necessary: (a) the illumination must be intense; (b) all the tubes must receive the same illumination; (c) the cells in all the tubes must remain evenly suspended throughout the medium. Of a number of different methods tried, the one finally adopted was as follows: Incubations were carried out in 15-ml centrifuge tubes, placed (to ensure even illumination) in a circular perspex test-tube holder which rotated in front of a fixed light source. An opaque perspex reflector was fixed behind each tube. The tube-holder rotated inside a cylindrical copper casing with a removable lid. The tubes (12 in all) were illuminated through a rectangular window of a width

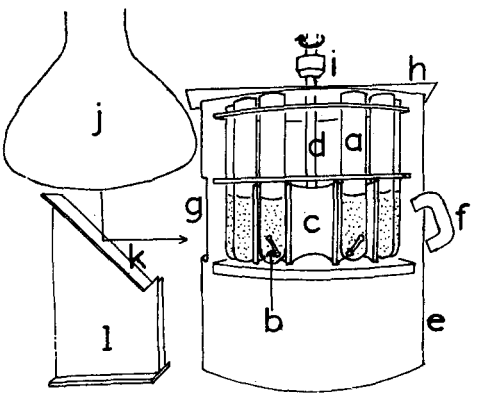

Fig. 1. Details of illuminating apparatus. a, 15-ml centrifuge tube; b, "flea" (0.5 in iron nail, polythene-covered); c, reflector; d, brass spindle; e, cylindrical copper casing; f, permanent magnet; g, window (4.5 cm wide); h, removable lid; i, chuck of electric motor; j, reflector spotlight; k, mirror; l, perspex stand for mirror. The apparatus is placed in a water bath at a depth such that the water in the bath is just above the level of the medium in the tubes.

such that 2 tubes were illuminated at once. Intense illumination was provided by a 150-w Reflector Spotlight (Ecko Ltd.), the light being reflected through the window on to the tubes by a mirror at 45°. The apparatus was placed in a water bath maintained at 30°.

In order to keep the cells suspended, magnetic stirring was used. One pole of a permanent magnet was fitted through a slot in the casing, and a polythene-covered "flea" placed in each tube. The tube-holder was normally made to rotate at 60 rev. per min which was sufficient to keep the cells suspended throughout the medium. If any tendency for the cells to form floating clumps appeared, the tubes were taken out at about hourly intervals during the incubation and briefly shaken.

Each tube contained 5 ml of a cell suspension with the density between 0.7 and 1.4 mg dry wt. cells/ml. When it was wished to incubate tubes in the dark and light at the same time the "dark" tubes were painted on the outside with black enamel.

The apparatus is illustrated in Fig. 1.

RESULTS

The chlorophyll-forming system

Time course and optimal conditions: In the apparatus described above, etiolated cells suspended in 0.04 *M* phosphate buffer, pH 7.0, containing 0.001 *M* $MgCl_2$ (basal medium) synthesised from 4.0–6.3 μg of chlorophyll *a* and from 1.0–1.5 μg of chlorophyll *b*/mg dry wt. in 6 h. These rates are about 7 times as fast as those reported for partially-decolourised *E. gracilis* by HUZISIGE, TERADA, NISHIMURA AND UEMURA[2]. There was not more than 6% increase in the total protein and nucleic acid, or in the number of organisms during a 6-h incubation under these conditions. Although rates of chlorophyll synthesis can vary by up to 50% from one culture to another, the rates obtained with cells of the same culture were extremely reproducible. When 12 tubes, each containing cells at a density of 0.95 mg dry wt./ml in basal medium, were incubated for 6 h, the standard deviation of the rates of chlorophyll synthesis was found to be 1.87% of the mean value, and the difference between the highest and the lowest rate was 5.6% of the mean.

Chlorophyll synthesis decreased considerably after about 9 or 10 h (Fig. 2) by which time the chlorophyll content of the organisms was about 30% of that of light-grown organisms. Carotenoids were also synthesised, but the percentage increase was much less than in the case of the chlorophylls. The rate of chlorophyll synthesis over 6-h periods varied by not more than 20% with pH over the range 5.5–8.0, and not more than 10–15% increase in rate was obtained by adding any of the following nutrients, alone or in various combinations, over the concentration ranges indicated: sodium acetate (16–800 μg/ml), ammonium sulphate (10–500 μg/ml), pyridoxal (5 μg/ml), a mixture of 18 amino acids (4–200 μg of each amino acid/ml), a mixture of 5 purines and pyrimidines (1–5 μg of each base/ml), peptone (2000 μg/ml), or yeast extract (20–2000 μg/ml).

Effect of streptomycin: 200 μg streptomycin/ml is the minimum concentration which will convert *E. gracilis* strain 1224/5g to the bleached form under conditions supporting growth. The synthesis of chlorophyll in etiolated cells is inhibited by streptomycin, and Fig. 3 shows the percentage inhibition produced by increasing concentrations of drug in basal medium. The inhibition produced by 2.0 mg strepto-

mycin/ml is the same for cells suspended at 0.5, 1.0, and 2.0 mg dry wt/ml. For a given concentration of streptomycin the percentage inhibition increases markedly with pH, being 3 times as great at pH 7.0 as it is at pH 5.5. In the case of bacteria the inhibitory action of streptomycin against growth is greater at higher pH values[15].

Changes in the pool amino compounds

Effect of light: In spinach chloroplasts, the ratio by weight of protein to chlorophyll is usually about 7 to 1 (see ref. 16). Thus, when chlorophyll formation takes place in etiolated *E. gracilis*, chloroplast protein is probably formed at the same time. In order

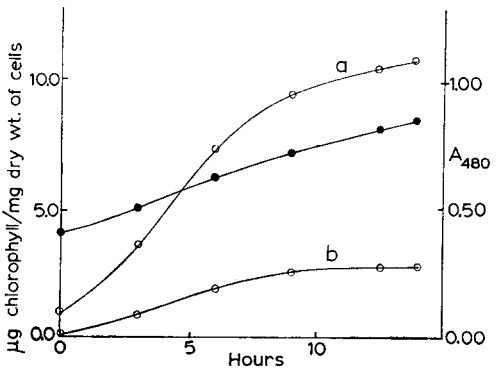

Fig. 2. Time course of pigment synthesis. Etiolated cells (1.12 mg dry wt/ml) suspended in basal medium. O—O, chlorophylls (curve a, chlorophyll *a*; curve b, chlorophyll *b*); ●—●, absorbancy at 480 mμ due to carotenoids.

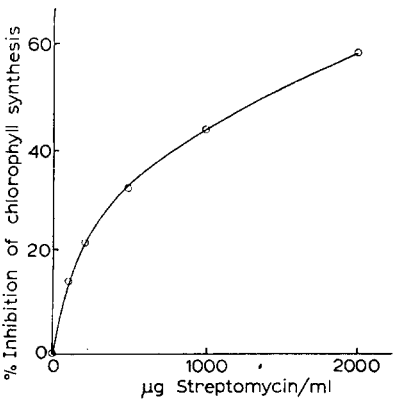

Fig. 3. Effect of streptomycin on chlorophyll synthesis. Etiolated cells (0.95 mg dry wt/ml) incubated for 6 h in basal medium, in the presence of increasing concentrations of streptomycin.

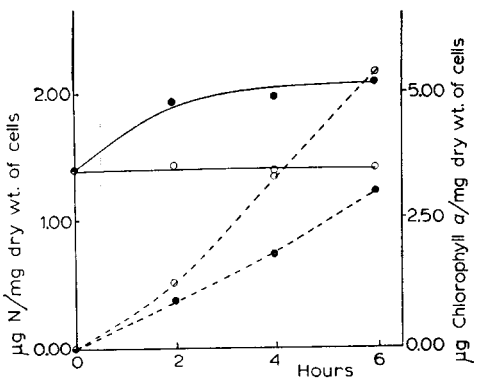

Fig. 4. Effect of streptomycin on pool amino compounds during greening. Etiolated cells (1.06 mg dry wt/ml incubated in basal medium in the light. O, in the absence of streptomycin; ●, in the presence of streptomycin (1.0 mg/ml). ———, free amino nitrogen content of extracts; – – – –, increase in chlorophyll *a* content of cells.

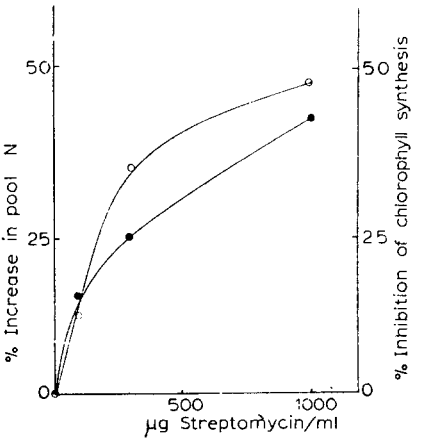

Fig. 5. Correlation of inhibition of chlorophyll synthesis with the effect on pool amino compounds. Etiolated cells (1.1 mg dry wt/ml) incubated in basal medium for 4 h in the light, O—O, % increase in free amino nitrogen of pool extracts produced by different concentrations of streptomycin; ●—●, % inhibition of chlorophyll synthesis produced by different concentrations of streptomycin.

to determine whether pool amino acids contribute to this protein synthesis, etiolated organisms were incubated in basal medium in the light or the dark, and the level of the amino compounds in the pool was measured before and after incubation. In one experiment the free amino nitrogen content fell in the light from 0.89 to 0.78 μg/mg dry wt. of cells over a period of 12 h, during which time 11.4 μg of chlorophyll were formed/mg dry weight of cells. In the dark the free amino nitrogen content increased by 0.15 μg/mg dry wt. of cells. These results suggest that if the pool amino acids are used for the synthesis of chloroplast protein, then their removal is balanced by the breakdown of other proteins or peptides. During the incubation there was no fall in the absorption at 260 mμ of the extracts either in the light or in the dark, suggesting that if there is any synthesis of chloroplast nucleic acids during this time, then the materials for it are also not derived solely from the pool.

Effect of streptomycin: In order to determine whether streptomycin causes an accumulation of amino compounds in *E. gracilis*, as it does in barley seedlings[4], etiolated cells were incubated in the light, in the presence and absence of streptomycin, and the free amino nitrogen content of the pool was followed over a period of 6 h. Fig. 4 shows that in the absence of streptomycin the level of the amino compounds remains the same within experimental error, but in the presence of streptomycin there is an immediate rise in the level of acid-soluble amino compounds. Streptomycin had no effect on the level of 260-mμ absorbing material in the pool. Any increases in the rate of excretion into the supernatants of amino compounds or of substances absorbing at 260 mμ in the presence of streptomycin were within experimental variation. Fig. 5 shows that at different concentrations of streptomycin, the percentage increase in the amino compounds of the pool can be correlated with the percentage inhibition of chlorophyll synthesis.

In an attempt to decide whether the increase in the free amino content related to all the pool components, samples of the pools were extracted from cells treated with streptomycin and from untreated control cells. These were then chromatographed on paper in phenol–water (100:20). All the ninhydrin-positive spots were more intense in the sample from drug-treated cells than in the controls. In order to obtain more

TABLE I

EFFECT OF STREPTOMYCIN ON POOL AMINO ACIDS

Etiolated cells incubated for 4 h in the light, in basal medium, in the presence or absence of streptomycin (1.0 mg/ml). The pool extracts from 495 mg dry weight of drug-treated, and of control, cells (from 15 different experiments) were analysed. SM, streptomycin.

	μmoles/g cells		*+SM/−SM*
	−SM	*+SM*	
Cysteic	0.97	1.18	1.22
Aspartic	1.05	0.87	0.83
Threonine	0.13	0.84	6.46
Serine	1.63	3.18	1.95
Glutamic	3.08	3.46	1.12
Cystine	0.66	0.38	0.58
Valine	2.47	4.41	1.78
Isoleucine	0.72	1.82	2.53
Leucine	0.25	0.92	3.68
Tyrosine	0.32	0.69	2.16
Phenylalanine	1.80	2.20	1.22

precise information on the nature of the compounds involved in the increase, the pool extracts from 15 experiments were combined and the neutral and acidic amino acids analysed by ion-exchange chromatography[17]. Table I shows that, with the possible exception of aspartic and cystine, there is an increase in the amount of all the amino acids in the presence of streptomycin, although there are considerable variations in the amount of increase of individual amino acids. However, it is not possible to deduce from these data that the total increase in amino compounds is due to amino acids.

To determine whether the accumulation of amino compounds occurred only in the light, etiolated cells were pre-incubated with streptomycin (1.0 mg/ml) in the dark for 2 h to enable them to take up the antibiotic. The organisms were then washed to remove the streptomycin, resuspended in basal medium, and the suspension divided into two parts, one of which was incubated for 4 h in the light, the other in the dark. It was found that in the light the level of the pool amino compounds increased by 33%; in the dark it increased by 37%. This result suggests that the effect of streptomycin on the pool amino compounds is not specifically connected with any light-requiring process, such as chloroplast formation.

Effect of inorganic sulphates: The streptomycin used throughout this work was in the form of the sulphate. Hence it was possible that some of the effects observed might have been due to the sulphate ion, or to non-specific electrolyte effects. To test this possibility cells were incubated in basal medium, in the light, with streptomycin sulphate or with Na_2SO_4 (at the same concentration with respect to sulphate). It is apparent from the results presented in Table II that Na_2SO_4 also causes an increase in the amino compounds of the pool. This increase may reach 75% of that caused by streptomycin sulphate. $MgSO_4$ had the same effect as Na_2SO_4 on the amino compound pool, but neither of these salts inhibited chlorophyll synthesis. The fact that streptomycin sulphate and inorganic sulphates both cause an increase in the pool amino compounds casts doubt on the view that this particular effect of streptomycin sulphate is related to its inhibition of chloroplast formation.

Incorporation of [1-^{14}C]acetate by E. gracilis

Characteristics of the incorporation: *E. gracilis* can use acetate as its sole carbon source for growth. A study of the effects of streptomycin on the incorporation of labelled acetate by this organism might cast some light on the mechanism by which

TABLE II

EFFECT OF Na_2SO_4 ON AMINO COMPOUNDS OF POOL

Etiolated cells incubated in the light in basal medium, supplemented with streptomycin sulphate (1.0 mg base/ml, *i.e.* $2.6 \cdot 10^{-3}$ *M* SO_4^{2-}), or with Na_2SO_4 ($2.6 \cdot 10^{-3}$ *M*), or without addition, SM, streptomycin.

Expt.	*Time (h)*	*Increase in amino nitrogen (%)*		*+ Na_2SO_4 / + SM·SO_4*
		+ SM·SO_4	*+ Na_2SO_4*	
1	4	50	13	0.26
2	4	62	29	0.47
3	4	28	15	0.54
4	3	29	9	0.31
5	2	34	26	0.76

the drug inhibits chlorophyll formation. It was found that when etiolated cells were suspended at a density of 1.0 mg dry wt./ml in basal medium supplemented with Na-[1-^{14}C]acetate (0.02 μC and 200 μg/ml) and $(NH_4)_2SO_4$ (300 μg/ml), and exposed to light, they incorporated ^{14}C in a linear manner for more than 4 h. No matter how small an amount of [^{14}C]acetate was added, the cells never incorporated more than 50% of the ^{14}C, suggesting, perhaps, that the acetate was incorporated by a route which involved decarboxylation of half the molecules. The incorporation in a given time was nearly twice as great in the light as it was in the dark.

Effect of streptomycin: Etiolated cells were incubated for 4 h in basal medium supplemented with Na-[1-^{14}C]acetate and $(NH_4)_2SO_4$ in the presence and absence of streptomycin (4 mg/ml), in the light or in the dark. In a typical experiment the streptomycin inhibition of the net synthesis of chlorophyll *a* was 53%, of chlorophyll *b* 59%, and of carotenoids 59%. The results relating to incorporation of [^{14}C]acetate are shown in Table III where it can be seen that a concentration of streptomycin which inhibits chlorophyll synthesis also inhibits, to about the same extent, the incorporation

TABLE III

EFFECT OF STREPTOMYCIN ON INCORPORATION OF [1-^{14}C]ACETATE

Etiolated cells (1.07 mg dry wt/ml) incubated for 4 h in the light or in the dark, in basal medium supplemented with $(NH_4)_2SO_4$ (300 μg/ml), and Na-[1-^{14}C]acetate (0.03 μC and 200 μg/ml) in the presence or absence of streptomycin at 4.0 mg/ml. SM, streptomycin.

Counts/min	*Light*			*Dark*		
	Counts/min/mg cells		*Percent change*	*counts/min/mg cells*		*Percent change*
	— SM	*+ SM*		*— SM*	*+ SM*	
Whole cell residue	1138	926	— 19	602	466	— 23
Lipid	461	375	— 19	227	218	— 4
Protein	427	142	— 67	167	67	— 60
Paramylon	139	384	+ 176	164	152	— 7
Nucleic acid:						
Guanine	—	—	— 66	—	—	— 50
Adenine	—	—	— 54	—	—	— 51
Cytidylic	—	—	— 69	—	—	— 58
Uridylic	—	—	— 47	—	—	— 35

TABLE IV

EFFECT OF STREPTOMYCIN ON INCORPORATION OF [1-^{14}C]ACETATE INTO PARAMYLON GLUCOSE

Experimental conditions as in Table III. Specific activities measured after hydrolysis and chromatography. The specific activity of the glucose obtained from cells incubated in the light in the absence of streptomycin has been made equal to 1.00. SM, streptomycin.

	Ratios of total counts/min in paramylon fraction	*Ratios of specific activities (range of values obtained with 3 different solvent systems)*
Light	1.00	1.00
Light + SM	2.93	2.98–3.32
Dark	1.05	1.20–1.29
Dark + SM	1.34	1.45–1.57

of acetate carbon into protein and nucleic acid whether incubation takes place in the light or dark.

The stimulation by streptomycin of incorporation into the paramylon fraction in the light was shown to represent increased incorporation into polysaccharide by comparing the specific activities of the glucose in the different samples after chromatography in three different solvents. Table IV shows that the specific activity of the glucose increases to the same extent as does the total radioactivity of the paramylon fraction.

To investigate the possibility that streptomycin inhibited the utilization of polysaccharide, cells (washed in 0.85 % NaCl instead of glucose solution) were incubated in basal medium lacking acetate but with $(NH_4)_2SO_4$, in the light or in the dark, and in the presence or absence of streptomycin. The total carbohydrate content was measured. As shown in Table V, streptomycin appears to reduce the rate of utilization of carbohydrate both in the light and in the dark.

TABLE V

EFFECT OF STREPTOMYCIN ON CHANGES IN TOTAL CARBOHYDRATE CONTENT

Etiolated cells (1.22 mg dry wt/ml) incubated for 6 h in the light or in the dark, in basal medium supplemented with $(NH_4)_2SO_4$ (300 μg/ml), in the presence or absence of streptomycin (4.0 mg/ml). Cells washed before determination of carbohydrate. SM, streptomycin.

	μg glucose/mg dry wt. of cells		*Percent decrease*
	0 h	*6 h*	
Light	476	341	28
Light + SM	476	398	16
Dark	476	392	18
Dark + SM	476	417	12

Effect of metals on the inhibitory action of streptomycin: A number of cations have been shown to antagonise the inhibitory effect of streptomycin on the growth of bacteria[18]. ROSEN AND GAWLIK[19] found that salts of Ca^{2+}, Mn^{2+}, Sr^{2+}, and Mg^{2+}, overcame the bleaching action of streptomycin on growing *E. gracilis*.

The effects of various metal salts on the inhibition of chlorophyll synthesis by streptomycin in non-growing, etiolated *E. gracilis* have been studied. Cells were incubated in the presence or absence of streptomycin, plus or minus the metal salt. In all cases the metal was used at the same molar concentration as the streptomycin, *i.e.* $6.9 \cdot 10^{-3}$ *M*. The results are presented in Table VI which shows that $CaCl_2$ and $MnCl_2$ overcame the inhibitory action of streptomycin almost completely, whereas NaCl, KCl, $MgCl_2$ and $MgSO_4$ had little or no significant effect. In the presence of $CoCl_2$ or $ZnSO_4$, streptomycin caused no further inhibition of chlorophyll synthesis, but the interpretation of this is rather difficult as both salts were themselves markedly inhibitory.

Manganese (in the form of the chloride or the sulphate) also appeared to reverse the inhibition by streptomycin of carotenoid synthesis; $MgSO_4$ was ineffective. As shown in Table VII $MnCl_2$ overcomes the various effects that streptomycin has on the incorporation of [1-^{14}C]acetate. $MgCl_2$ appears to be without effect.

TABLE VI

EFFECT OF METALS ON THE INHIBITORY ACTION OF STREPTOMYCIN

Etiolated cells incubated for 4 h in the light, in 0.04 *M* Tris buffer, pH 7.6, in the presence or absence of $6.9 \cdot 10^{-3}$ *M* streptomycin (4.0 mg/ml), plus or minus the metal salt ($6.9 \cdot 10^{-3}$ *M*). SM, streptomycin.

Expt	*Salt*	*SM*	*μg chlorophyll a/mg dry wt./h*	*Percent inhibition by SM*	*Percent antagonism by salt*
1	—	—	0.92		
	—	+	0.29	68	
	NaCl	—	0.96		
	NaCl	+	0.35	64	6
	KCl	—	0.97		
	KCl	+	0.49	49	28
2	—	—	0.71		
	—	+	0.16	77	
	$MgCl_2$	—	0.75		
	$MgCl_2$	+	0.23	69	10
3	—	—	1.05		
	—	+	0.30	71	
	$CaCl_2$	—	1.05		
	$CaCl_2$	+	0.99	6	92
4	—	—	1.08		
	—	+	0.44	59	
	$MnCl_2$	—	1.00		
	$MnCl_2$	+	0.95	5	92
	$CoCl_2$	—	0.35		
	$CoCl_2$	+	0.37	— 6	110
5	—	—	1.25		
	—	+	0.52	58	
	$MgSO_4$	—	1.31		
	$MgSO_4$	+	0.60	54	7
	$MnSO_4$	—	1.19		
	$MnSO_4$	+	1.19	0	100
6	—	—	0.86		
	—	+	0.34	60	
	$ZnSO_4$	—	0.56		
	$ZnSO_4$	+	0.66	— 18	130

$$\text{Percent antagonism} = 100\left(1 - \frac{\text{Percent inhibition in presence of salt}}{\text{Percent inhibition in absence of salt}}\right)$$

It would appear, then, that $MnCl_2$ but not $MgCl_2$, when added at the same molar concentration as the streptomycin antagonises the effects of the drug on chlorophyll synthesis, on carotenoid synthesis, and on incorporation of [1-^{14}C]acetate.

DISCUSSION

DE DEKEN-GRENSON[20] found that when etiolated chicory leaves formed chlorophyll in the light, there was an increase in the protein of the chloroplast and other fractions which was balanced by a fall in the level of the free amino acids of the pool. However, BRAWERMAN AND CHARGAFF[21] reported that when etiolated *E. gracilis*

TABLE VII

EFFECT OF $MnCl_2$ AND $MgCl_2$ ON THE ACTION OF STREPTOMYCIN ON INCORPORATION OF [1-^{14}C]ACETATE

Etiolated cells (0.92 mg. dry wt/ml) incubated for 4 h in the light in 0.04 *M* Tris buffer, pH 7.6, supplemented with $(NH_4)_2SO_4$ (300 μg/ml) and Na-[1-^{14}C]acetate (0.03 μC and 164 μg/ml) in the presence or absence of streptomycin (4.0 mg/ml). Three parallel incubations were done — in the presence of $6.9 \cdot 10^{-3}$ *M* $MnCl_2$, in the presence of $6.9 \cdot 10^{-3}$ *M* $MgCl_2$, and in the absence of any metal salt. SM, streptomycin.

Salt added	*None*			*$MnCl_2$*			*$MgCl_2$*		
	counts/min/mg cells		*Percent change*	*counts/min/mg cells*		*Percent change*	*counts/min/mg cells*		*Percent change*
	—SM	*+ SM*		*—SM*	*+ SM*		*—SM*	*+ SM*	
Whole cell residue	1214	1161	— 5	1225	1248	+ 2	1241	1256	+ 1
Lipid	461	397	— 14	496	441	— 11	409	379	— 7
Protein	452	70	— 84	406	391	— 4	384	90	— 76
Nucleic acid	95	27	— 72	69	73	+ 5	84	28	— 67
Paramylon	153	588	+ 285	216	264	+ 22	207	691	+ 233

formed chlorophyll in the light, the increase in the protein of the chloroplast fraction was balanced by a fall in the level of the soluble proteins of the cell. These workers suggested that the chloroplast proteins are already present in the etiolated cells and, in the light, merely have to be built into the chloroplast structure. The fact, reported here, that there is little or no fall in the level of the free amino compounds of the cell during greening is consistent either with the hypothesis of BRAWERMAN AND CHARGAFF[21] or with the view that other proteins or peptides break down, and that the amino acids liberated are then utilised for the synthesis of chloroplast protein. On this latter hypothesis, the level of the free amino compounds at any moment would be a function of the relative rates of breakdown and resynthesis of protein.

It is apparent from these results that a concentration of streptomycin (4 mg/ml) which inhibits chlorophyll synthesis, also inhibits, to about the same extent, the incorporation of acetate carbon into protein and nucleic acid in organisms incubated in the light or in the dark. This suggests that the effect of streptomycin on greening is not due to a specific inhibition of chloroplast formation but is one result of a general inhibition of biosynthesis. The results are consistent either with an effect on carbon metabolism, prior to the formation of amino acids, nucleotides, and pigments, or with an inhibition of protein and nucleic acid synthesis. In the latter case, chlorophyll synthesis might be inhibited simply because there would not be any protein for it to be attached to.

If some of the acetate has to be converted to polysaccharide before being utilised for biosynthesis, the increase in the labelling of the paramylon fraction observed in organisms incubated with streptomycin in the light, could be due to an inhibition of polysaccharide utilization rather than to a stimulation of its synthesis. Alternatively, the inhibitory effects of streptomycin might cause an accumulation of labelled intermediates which, since they cannot be used for the biosynthesis of proteins etc., are converted into polysaccharide.

The antagonistic effect of certain metals on the action of streptomycin could be due to combination with the drug (which is a chelating agent[22,23]) to give complexes which do not penetrate the cells or are otherwise inactive. Alternatively, the metals

could act by displacing the drug from its site of action within the cell. The fact that $MgCl_2$ is relatively ineffective suggests that in this system, at least, streptomycin does not act by specifically competing with, or binding, Mg^{2+}.

It is not self-evident that the mechanism by which streptomycin inhibits chlorophyll formation in washed suspensions of non-growing, etiolated cells is the same as the mechanism by which it induces loss of the ability to make chloroplasts in growing cells. It should be pointed out that although a streptomycin concentration of 200 μg per ml has relatively little effect on greening in non-growing, etiolated cells, nevertheless it is sufficient to convert growing *E. gracilis* to the bleached form. However, this could be due to the fact that in the growth experiments organisms were exposed to the drug for 3–4 days, whereas in the washed cell experiments exposure was only for 4–6 h.

It is hoped that further work along the lines reported in this paper may give some indication of the primary site of action of this antibiotic and throw some light on the relationship between the "bleaching effect" and the inhibition of biosynthetic processes.

ACKNOWLEDGEMENTS

This work was carried out during the tenure of a Medical Research Council Scholarship for Training in Research Methods. The author is indebted to Dr. B. A. NEWTON for his interest and advice: and to Professor E. F. GALE, F.R.S., for valuable criticism. He is also grateful to Mrs. B. D. BROWN for carrying out the ion exchange analysis of the pool amino acids.

REFERENCES

1 L. PROVASOLI, S. H. HUTNER AND A. SCHATZ, *Proc. Soc. Exptl. Biol. Med.*, 69 (1948) 279.
2 H. HUZISIGE, T. TERADA, M. NISHIMURA AND T. UEMURA, *Biol. J. Okayama Univ.*, 3 (1957) 209.
3 H. VON EULER, M. BRACCO AND L. HELLER, *Compt. rend.*, 227 (1948) 16.
4 M. DE DEKEN-GRENSON, *Biochim. Biophys. Acta*, 17 (1955) 35.
5 D. I. ARNON, *Plant Physiol.*, 24 (1949) 1.
6 G. MACKINNEY, *J. Biol. Chem.*, 140 (1941) 315.
7 E. C. COCKING AND E. W. YEMM, *Biochem. J.*, 58 (1954) xii.
8 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.
9 M. OGUR AND G. ROSEN, *Arch. Biochem. Biophys.*, 25 (1950) 262.
10 W. E. TREVELYAN AND J. S. HARRISON, *Biochem. J.*, 50 (1952) 298.
11 W. C. SCHNEIDER, *J. Biol. Chem.*, 161 (1945) 293.
12 R. MARKHAM, *Modern Methods of Plant Analysis*, Vol. IV, Springer-Verlag, Berlin, 1955, p. 246.
13 G. R. WYATT, *Biochem. J.*, 48 (1951) 584.
14 C. M. WILSON, *Anal. Chem.*, 31 (1959) 1199.
15 E. P. ABRAHAM AND E. S. DUTHIE, *The Lancet*, 250 (1946) 455.
16 J. BONNER, *Plant Biochemistry*, Academic Press, Inc., New York, 1950, p. 259.
17 S. MOORE, D. H. SPACKMAN AND W. H. STEIN, *Anal. Chem.*, 30 (1958) 1185.
18 R. DONOVICK, A. P. BAYAN, P. CANALES AND F. PANSY, *J. Bacteriol.*, 56 (1948) 125.
19 W. G. ROSEN AND S. R. GAWLIK, *J. Protozool.*, 8 (1961) 90.
20 M. DE DEKEN-GRENSON, *Biochim. Biophys. Acta*, 14 (1954) 203.
21 G. BRAWERMAN AND E. CHARGAFF, *Biochim. Biophys. Acta*, 31 (1959) 164.
22 W. O. FOYE, W. E. LANGE, J. V. SWINTOFSKY, R. E. CHAMBERLAIN AND J. R. GUARINI, *J. Am. Pharm. Assoc.*, 44 (1955) 261.
23 W. G. ROSEN, *Plant Physiol.*, 32 (1957) *Suppl.* viii.