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Control of Chloroplast formation in *Euglena gracilis*

ANTAGONISM BETWEEN CARBON AND NITROGEN SOURCES

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1. Cells of *Euglena gracilis* grown in the dark on high ratios of carbon source to nitrogen source ('high-carbon cells') are unable to form chlorophyll during a subsequent incubation in the light; cells grown in the dark on low ratios of carbon to nitrogen ('low-carbon cells') synthesize chlorophyll at a rapid rate during the subsequent incubation in the light. High-carbon cells will form chlorophyll rapidly if supplied with a nitrogen source during the incubation in the light: of the nitrogen sources tested, ammonium sulphate was the most effective at overcoming the block in chlorophyll synthesis. The nitrogen source does not have to be present during the actual incubation in the light: a 5hr. exposure of high-carbon cells to ammonium sulphate in the dark, followed by removal of the nitrogen source, is sufficient to bring about rapid chlorophyll synthesis during a subsequent incubation in the light. 2. The synthesis of chlorophyll by low-carbon cells exposed to the light is strongly repressed by the addition of ethanol or other utilizable carbon sources during the incubation in the light. Chlorophyll synthesis ceases altogether between 5 and 10hr. after the addition of the carbon source. Carotenoid synthesis is also inhibited, but to a smaller extent. The inhibitory effects of ethanol are prevented if ammonium sulphate is added at the same time. 3. High-carbon cells contain about four times as much carbohydrate per cell and about twice as much lipid per cell as low-carbon cells. The content per cell of total protein, soluble protein and DNA are about the same in both types of cell. The low-carbon cells sometimes, but not always, contain more RNA than the high-carbon cells. Analysis of cold-acid extracts indicates that the two kinds of cells contain about the same concentrations of pool amino acids, but that the low-carbon cells contain somewhat higher concentrations of peptides in the pool. Ion-exchange analysis of pool extracts shows a number of differences between high-carbon and low-carbon cells with respect to the concentrations of individual amino acids: in particular low-carbon cells contain higher concentrations of alanine. High-carbon cells have approximately twice as much protease activity as low-carbon cells. 4. The possible biochemical basis for the differing ability of high-carbon and low-carbon cells to form chloroplasts in the light is discussed.

When the unicellular alga *Euglena gracilis* is grown heterotrophically in the dark the cells do not form chloroplasts: they contain instead small yellow undifferentiated proplastids (Epstein & Schiff, 1961). When the dark-grown cells are exposed to light the proplastids begin to differentiate into chloroplasts: chlorophyll, carotenoids, lipids, photosynthetic enzymes and the thylakoid membranes are formed and after 1–3 days the cells contain mature, photosynthetically active, chloroplasts. This process, the development of chloroplasts on exposure to light of a cell grown in the dark, is commonly referred to, both in *Euglena* and in higher plants (in which a similar phenomenon occurs), as the greening process. In *E. gracilis* greening will take place in the absence of external nutrients: the cells can simply be suspended in buffer and illuminated. Thus they can form chloroplasts at the expense of reserves, without cell growth or multiplication. A more detailed account of the greening phenomenon, in higher plants and in *Euglena*, is given by Kirk & Tilney-Bassett (1967).

Chloroplast formation in certain algae is under

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nutritional control. It has been shown for various *Chlorella* species (Beijerinck, 1904; Kufferath, 1913; Fuller & Gibbs, 1959; Shihira-Ishikawa & Hase, 1964) and also for *E. gracilis* (Smillie & Krotkov, 1960; Buetow, 1967; Ternetz, 1912) that the addition of utilizable carbon sources to the culture medium of cells growing in the light partially or completely inhibits the synthesis of chlorophyll and the development of chloroplasts. In *E. gracilis* the chloroplast development that takes place when etiolated cells are grown in the dark and then illuminated under non-multiplying conditions is also inhibited by carbon sources, if these are added during the actual incubation in the light (App & Jagendorf, 1963). Further, the greening process in *E. gracilis* can be inhibited if high concentrations of carbon source are supplied, not during the illumination itself, but during the previous growth in the dark (Kirk & Keylock, 1967).

The present paper describes an investigation of the nutritional control of the greening process in *E. gracilis*, with particular reference to the interaction between carbon and nitrogen sources, supplied either during the period of growth in the dark or during the incubation in the light. Some of these findings have already been published as an abstract (Harris & Kirk, 1967).

METHODS AND MATERIALS

Cell growth. *E. gracilis* strain 1224/5Z (Z strain) was obtained from the Culture Collection of Algae and Protozoa, Botany School, Cambridge. Cells were grown on the basal defined medium described by Kirk & Keylock (1967) without acetate and with other modifications as indicated in the Results section. The 20ml., 200ml. or 400ml. batches of medium in 50ml., 500ml. or 1000ml. Erlenmeyer flasks were given 1·0–1·5% (v/v) inocula taken from cultures grown in the light on complex medium (Kirk, 1962). The cells were grown for 3–5 days at 28° in the dark on a New Brunswick rotary shaker. By this time they had reached the stationary phase of growth. The cultures were strained through two layers of muslin to remove large cell clumps and the cells were harvested by centrifugation at 600*g* for 3min. Cells were washed twice in 0·15M-NaCl before resuspension at a cell density of $0{\cdot}5\times10^6$–$1{\cdot}5\times10^6$cells/ml. in the basal greening medium (1·0mM-$MgSO_4$–0·04M-KH_2PO_4–Na_2HPO_4, pH7·0) with or without additions as indicated in the appropriate experimental sections.

Incubations in the light. In all experiments in which the time-course of pigment synthesis was followed over a period, 100ml. portions of the cell suspension in the basal greening medium were placed in 250ml. Erlenmeyer flasks. These were incubated at 27°–29° at a light-intensity of 200–250 ft. candles (white 'daylight' fluorescent tubes) in either the rotary illuminating apparatus described by Kirk (1964) or in the New Brunswick Psychrotherm rotary shaker. In experiments in which only one measurement of pigment synthesis, that at 24hr., was taken, 15ml. portions of the cell suspension were incubated in 50ml. Erlenmeyer flasks in the New Brunswick Psychrotherm shaker. In all incubations in the light carried out in the Psychrotherm shaker, the positions of the different flasks were altered at 4hr. intervals to ensure equal illumination.

Determination of pigment and cell counts. Portions of cell suspensions were taken and the cells were harvested by centrifugation and extracted with aq. 80% (v/v) acetone. Extinctions of the extracts at 663, 645 and 480nm. were measured either in normal 1cm. cells or 2cm.-path-length micro-cells in a Unicam SP.500 spectrophotometer. The wavelength accuracy of the monochromator was checked with holmium and didymium filters. Chlorophyll concentrations were determined by using a nomogram (Kirk, 1968) based on the published extinction coefficients (MacKinney, 1941) of chlorophylls *a* and *b*. The greater part of the extinction at 480nm. is due to carotenoids: the small part due to chlorophyll is corrected for by using the equation derived by Kirk & Allen (1965). Cell counts were carried out with a Coulter counter.

Chromatography of pigments. Chloroplast pigments were extracted from the cells with methanol at 4° in dim light and transferred to diethyl ether (peroxide-free, redistilled). The ether extract was dried over anhydrous Na_2SO_4 and concentrated under a stream of N_2. Extracts were saponified, where desired, by adding 1ml. of 60% KOH to 10ml. of the methanol extract and leaving this for 20hr. at 4° in the dark and under an atmosphere of N_2. Pigments were then transferred to diethyl ether and treated as before. Preliminary experiments to separate and identify the major carotenoid pigments were carried out by t.l.c. with Kieselgel G as the adsorbent and benzene–ethyl acetate–methanol (14:5:1, by vol.) as the wash (Dr B. H. Davies, personal communication). Samples of both unsaponified and saponified extracts were chromatographed. With this system three of the four major carotenoids reported to occur in light-grown *E. gracilis* (Krinsky & Goldsmith, 1960; Krinsky, Gordon & Stern, 1964; Aitzetmüller, Svec, Katz & Strain, 1968) were detected. These were (in order of increasing R_F value) neoxanthin, diadinoxanthin and β-carotene. Zeaxanthin, reported to occur in small amounts in both light-grown and dark-grown cells of *E. gracilis* var. *bacillaris* (Krinsky *et al.* 1964), was not detected in this system nor in those used for qualitative measurements. Neoxanthin was not detected initially in dark-grown cells, but appeared after 5hr. exposure of the cells to light.

Identifications were confirmed by the relative R_F values, colour reactions with HCl vapour, absorption spectra and (for the xanthophylls) epoxide shifts on acidification. For measurements of the actual amounts of the pigments at different stages during greening, t.l.c. was found not to be suitable. Instead, the pigments were separated on paper loaded with $Al(OH)_3$ (Whatman A.H. 81). Two solvent systems (ascending) were used. The first [light petroleum (b.p. 20–40°) containing 4% (v/v) of propan-1-ol (a technique based on the work of Strain, Sherma, Benton & Katz, 1965)] gave five major spots: these were (in order of increasing R_F value) neoxanthin, diadinoxanthin and chlorophyll *b* (partially overlapping), chlorophyll *a* and β-carotene. Chromatograms were run in the dark at 4°. This system was used for estimation of chlorophyll *a*, chlorophyll *b*, neoxanthin and β-carotene. The second solvent system (run at room temperature in the dark) was that used for t.l.c.: this gave a pattern of spots on the loaded paper similar to that in the t.l.c. method, with the chlorophylls running as a single spot between diadinoxanthin and β-carotene. This

solvent system was used for estimation of diadinoxanthin and as a supplementary estimation of neoxanthin.

Chlorophyll spots were eluted with aq. 80% acetone, diadinoxanthin and neoxanthin with redistilled ethanol, and β-carotene with light petroleum (b.p. 40–60°). Chlorophylls were estimated as described in the preceding section. Carotenoid absorption spectra were determined in 1cm.- or 2cm.-path-length micro-cells in the Unicam SP.800 recording spectrophotometer. Carotenoid extinction coefficients were taken from Davies (1965), except that for diadinoxanthin, which was assumed to have $E^{1\%}_{1\,cm.}$ 2000.

Chemical analysis of cells. Cultures were washed twice in 0·15M-NaCl and resuspended (4×10^6–6×10^6 cells/ml.) in deionized water.

Dry weight. Portions (3×10^6–6×10^6 cells) of cell suspension were pipetted into weighed aluminium planchets (1 in. diam.) and dried to constant weight under an infrared lamp. Removal of water by freeze-drying gave values of dry weight not significantly different from those obtained by infrared drying.

Carbohydrate. Total cell carbohydrate was measured on portions of culture by the anthrone method of Trevelyan & Harrison (1952), with glucose as a standard.

Lipid. Portions (about 70×10^6 cells) of cell suspension were freeze-dried. The dry cells were extracted twice with 10ml. of boiling ethanol (dried, redistilled) for 15min.: the cells were removed by centrifugation and the supernatants were pooled, then evaporated to dryness under a stream of N_2 and the residue was weighed.

Nucleic acids. Cells were harvested from portions (10×10^6–12×10^6 cells) of suspension and resuspended in 0·2M-$HClO_4$. After 30min. at room temperature to remove pool materials (Kirk, 1962) the cells were removed by centrifugation and washed once more with 0·2M-$HClO_4$. The extracted cells were quickly washed with water and then extracted twice with 0·5M-$HClO_4$ at 70° for 10min. (Burton, 1956) to remove nucleic acids. The $HClO_4$ extracts were combined and absorption spectra measured on the Unicam SP.800 recording spectrophotometer. Typical hydrolysed nucleic acid absorption spectra were obtained with a peak at 260nm. Accurate values of extinction at 260nm. were measured with a Unicam SP.500 spectrophotometer. DNA was determined on samples of the extract by the diphenylamine method (Burton, 1956), with calf thymus DNA (British Drug Houses Ltd., Poole, Dorset) as the standard. The contribution of the DNA to the total extinction at 260nm. was calculated (assuming $E^{1\%}_{1\,cm.}$ 300) and subtracted from the total extinction at 260nm. The RNA concentration was calculated from the corrected extinction at 260nm. (assuming $E^{1\%}_{1\,cm.}$ 320).

Protein. Cells (10×10^6–12×10^6) from which pool materials had been extracted were taken up in 4ml. of 1M-NaOH and brought into solution by incubation at 37° for up to 2hr. Samples of this solution were taken for protein estimation by the procedure of Lowry, Rosebrough, Farr & Randall (1951), with bovine serum albumin (British Drug Houses Ltd.) as the standard.

For estimation of soluble protein cells were suspended at a concentration of 40×10^6–50×10^6 cells/ml. in 0·05M-KH_2PO_4–Na_2HPO_4 buffer, pH7·0, and disrupted for 2½min. at 0° with the MSE 100w Ultrasonic Disintegrator. The suspension was centrifuged for 20min. at 100000*g* in the Spinco ultracentrifuge (SW 39 rotor). The supernatants were filtered through Millipore membrane filters (pore size 0·22 μm.), cooled to 4° and samples of the clear filtrate were taken for protein estimation by the procedure of Lowry *et al.* (1951) and by the biuret method (Gornall, Bardawill & David, 1949). In view of the relatively short centrifugation time used to remove large particulate material, the final extract is likely to contain ribosomal protein as well as soluble cell proteins.

Analysis of the electrophoretic pattern of the soluble proteins was carried out on Oxoid cellulose acetate strips, buffered with 0·05M-$Na_2B_4O_7$ (adjusted to pH8·5 with conc. HCl), at 200v for 5hr. in a Shandon electrophoresis tank. To 1ml. of soluble protein extract was added 4·0ml. of ethanol at −10°. After 20min. in ice the precipitate was centrifuged down, excess of supernatant removed with tissue, and the pellet taken up in 0·8ml. of 0·05M-KH_2PO_4–Na_2HPO_4 buffer, pH7. The suspension was clarified by centrifugation, the protein content determined by the biuret method and a sample containing 40μg. of protein was applied to a cellulose acetate strip 18cm. long×2·5cm. wide. At the end of the run strips were stained for 60min. in a 0·1% solution of Nigrosin in 3% (v/v) acetic acid. Excess of stain was removed by washing for 5–6hr. in 1% (v/v) acetic acid.

Samples of soluble protein, taken before ethanol precipitation and containing 80μg. of protein, were similarly applied to cellulose acetate strips. Electrophoretic patterns of such extracts, unlike those of extracts that had undergone ethanol precipitation, were diffuse and difficult to interpret.

Pool materials. For estimation of cold-acid-soluble amino compounds in dark-grown cells, cells were harvested from portions (about 70×10^6 cells) of suspension and the pool materials were removed by two successive extractions with 10ml. quantities of 0·2M-$HClO_4$ at room temperature (20°) for 30min. The extracts were combined, neutralized with $KHCO_3$ and cooled in ice to precipitate $KClO_4$, which was subsequently removed by centrifugation. After appropriate dilution the total amount of free amino compounds was estimated by the ninhydrin procedure of Cocking & Yemm (1954), with sodium glutamate as a standard.

To determine the total quantity of amino acids in the extracts, hydrolysis with conc. HCl was carried out to hydrolyse any peptides to free amino acids. To 1ml. of neutralized extract (as above) in a soda-glass tube was added 1·2ml. of conc. HCl (sp.gr. 1·18), giving a final concentration of 6·24M. The tube was sealed and placed in an oven at 105° for 20hr. The hydrolysate was evaporated to dryness in a hot-water bath under a stream of air, and the residue redissolved in water for estimation of free amino compounds by the ninhydrin procedure.

For studies on fluctuations in concentrations of pool amino compounds during greening, portions (50×10^6 cells) of culture were analysed for pool amino compounds with and without hydrolysis with conc. HCl as above.

For ion-exchange-chromatographic analysis of pool amino compounds the neutralized extracts (from 250×10^6 cells) were passed into a column (12cm.×1cm.) of Dowex-50 ion-exchange resin (H^+ form; 20–50 mesh; 8% cross-linked). Under these conditions the amino compounds are retained by the resin; salts pass through. The column was washed through with 3column vol. of water and the amino compounds were eluted with 6 column vol. of aq. 7·5M-NH_3 soln. The ammonia eluate was concentrated to a small volume in a hot-water bath under a stream of air. The solution remaining was then freeze-dried. Ion-exchange

analysis of the amino acids was carried out on a Technicon amino acid analyser by Mr K. Ireland of the Alder Hey Children's Hospital, Liverpool.

Measurement of protease activity. Cells were suspended in 0·05M-KH_2PO_4–Na_2HPO_4 buffer, pH7, at cell densities from 30×10^6 to 50×10^6 cells/ml. Suspensions were treated ultrasonically for 2½min. at 0° with an MSE ultrasonic disintegrator. The disintegrated cell preparations were immediately either assayed for protease activity or frozen and kept at −15° until required.

Proteolytic activity was measured by a modification of the method of Anson (1937). The substrate was prepared as follows: 40ml. of 2·5% (w/v) bovine haemoglobin (type II; Sigma Chemical Co., St Louis, Mo., U.S.A.) was acidified with 8ml. of 0·3M-HCl. The pH was raised to 3·5 with a small volume of 10M-NaOH, giving a final haemoglobin concentration of approx. 2% (w/v). To 2ml. of this haemoglobin solution was added 1·0ml. of citrate–phosphate buffer, pH3·5 (0·05M-citric acid adjusted to pH3·5 with 0·1M-Na_2HPO_4), and 0·3ml. of the disintegrated cell preparation. The mixture was incubated at 30° with shaking by hand at 5-min. intervals. After the appropriate period of time the reaction was stopped by the addition of 1·0ml. of 1·0M-$HClO_4$ and the incubation tubes were placed in an ice bath for 30min. to ensure complete precipitation of protein. The precipitate was removed by centrifugation and 3ml. of the clear supernatant was neutralized with 0·5ml. of 14·42% (w/v) $KHCO_3$. The $KClO_4$ was removed by chilling and centrifuging the solutions and the amount of free amino compounds in the supernatant was measured by the ninhydrin procedure (Cocking & Yemm, 1954). Hydrolysis proceeded approximately linearly for at least 7hr. For comparing proteolytic activity of different types of cells the degree of hydrolysis after 4hr. was used and the overall rate expressed as μg. of amino N liberated by protease/hr./10^6 cells.

RESULTS AND DISCUSSION

Dependence of rate of greening on composition of dark-growth medium. It had previously been shown that cells of *E. gracilis* grown on particularly high concentrations of carbon source in the dark are unable to form chlorophyll when they are exposed to the light, even though the carbon source is no longer present (Kirk & Keylock, 1967). It has now been found that this inhibition can be overcome by raising the concentration of nitrogen source in the growth medium. An experiment was carried out in which the concentration of the carbon source, ethanol, was varied from 0·1 to 2·0% (v/v) and the concentration of the nitrogen source, ammonium nitrate, was varied from 0·014 to 0·09% (w/v). After growth on the different media for 4 days in the dark the cells were harvested, washed, resuspended in basal greening medium and exposed to light. The amount of chlorophyll synthesized in 24hr. was measured. The results in Table 1 show that the ability to synthesize chlorophyll in the light depends on the ratio of carbon source to nitrogen source during the previous growth in the dark, rather than on the absolute concentration of carbon or nitrogen source. For instance at a given concentration of ethanol, say 0·4% (v/v), as the concentration of ammonium nitrate was increased so the amount of chlorophyll formed during the subsequent incubation in the light rose from about zero to a high value: at a given concentration of ammonium nitrate, as the ethanol concentration during dark growth was increased the amount of chlorophyll synthesized during the incubation in the light fell to near zero. A related phenomenon in *Chlorella protothecoides* has been described by Shihira-Ishikawa & Hase (1964). They studied the effect of the ratio of the carbon source to the nitrogen source during growth in the light on chloroplast formation. In this system, too, high ratios of the carbon source to the nitrogen source tended to repress chloroplast formation, whereas low ratios promoted chloroplast formation.

Effect of nitrogen source present during incubation in the light. It is clear that the addition of extra nitrogen source to the growth medium prevents the establishment of a block in chlorophyll synthesis by high concentrations of carbon source present during growth in the dark. It has also been shown

Table 1. *Effect of ratio of carbon source to nitrogen source in growth medium on subsequent rate of greening*

Cells of *E. gracilis* were grown for 4 days in the dark on media containing different concentrations of ethanol and ammonium nitrate. The cells were then washed and resuspended in basal greening medium, and incubated in the light for 24hr.

Concn. of ethanol (%, v/v)	Concn. of NH_4NO_3 (%, w/v)	Chlorophyll synthesized (μg./24hr./10^6 cells)			
		0·015	0·03	0·06	0·09
0·1		4·68	8·80	7·50	7·65
0·2		2·65	5·85	8·40	7·71
0·4		0·33	2·55	6·43	7·69
0·7		0·00	0·59	2·76	5·66
1·0		0·00	0·04	1·83	4·04
1·5		0·03	0·19	0·41	2·14
2·0		0·23	0·36	0·51	0·31

that the block, once established, can be removed by the addition of nitrogen source during the actual greening process. Cells were grown in the dark in medium containing 2% (v/v) of ethanol and 0·03% (w/v) of ammonium nitrate and then resuspended in the basal greening medium in the light. In the absence of further additions to the greening medium, no chlorophyll synthesis occurred (Fig. 1). When 0·05% ammonium sulphate was added as a nitrogen source, chlorophyll synthesis began after about 5hr., accelerated and continued at a rapid rate throughout the rest of the incubation.

When a series of different concentrations of ammonium sulphate were supplied the rate of chlorophyll synthesis for cells at a density of 10^6cells/ml. over a 24hr. period increased linearly with ammonium sulphate concentration from zero to 125μg./ml. Further increase in the concentration of nitrogen source caused only a slight increase in chlorophyll synthesis, and at from 250μg. to 950μg. of ammonium sulphate/ml. the rate of chlorophyll synthesis remained constant.

Comparison of different nitrogen sources. Several different nitrogen sources were compared with respect to their ability to overcome the block. Cells were grown in the dark in medium containing 1·5% (v/v) of ethanol and 0·03% of ammonium nitrate. They were suspended in basal greening medium and to a series of different flasks were added a series of different nitrogen sources all at the same concentration with respect to nitrogen. The amount of chlorophyll synthesized in 24hr. in the light was measured; there was no significant synthesis when no nitrogen source was added. As shown in Table 2 ammonium sulphate was the most effective at overcoming the block and asparagine almost as

Table 2. *Ability of different nitrogen sources to relieve the block in chlorophyll synthesis induced by high concentrations of ethanol during growth in the dark*

Cells of *E. gracilis* were grown for 4 days in the dark in medium containing 1·5% (v/v) of ethanol and 0·03% of ammonium nitrate. The cells were washed, then resuspended in basal greening medium with additions as shown below, and the amount of chlorophyll synthesized in 24hr. in the light was measured.

Nitrogen source (106μg. of N/ml.)	Chlorophyll synthesized (μg./24hr./10^6 cells)	Relative rate of chlorophyll synthesis (%)
None	0·28	3·7
Ammonium sulphate	7·55	(100·0)
Asparagine	5·95	78·8
Serine	4·71	62·4
Sodium aspartate	3·66	48·5
Sodium glutamate	2·96	39·2
Glycine	2·73	36·2
Leucine	1·43	18·9
Alanine	1·08	14·3
Urea	0·95	12·6
Sodium nitrate	0·24	3·2

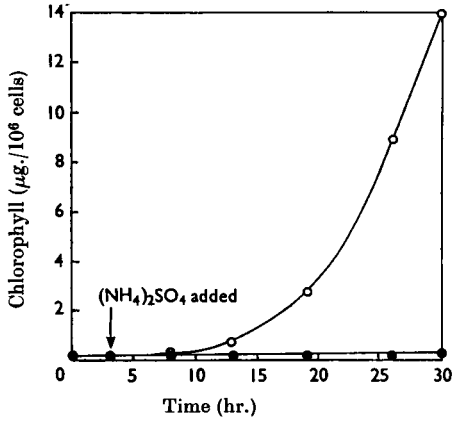

Fig. 1. Time-course of chlorophyll synthesis in the light after the addition of ammonium sulphate. Cells were grown in the dark for 4 days in medium containing 2·0% (v/v) of ethanol and 0·03% of ammonium nitrate. Cells were harvested and washed, then resuspended in basal greening medium and placed in the light. ●, Control (no additions made); ○, 0·05% of ammonium sulphate added at 3hr.

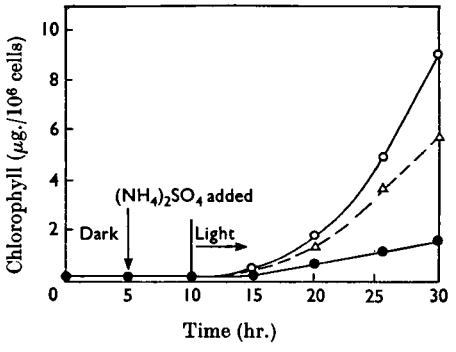

Fig. 2. Effect of short exposure to ammonium sulphate on subsequent chlorophyll synthesis in the light. Cells were grown in the dark for 4 days in medium containing 1·5% (v/v) of ethanol and 0·03% of ammonium nitrate. Cells were harvested and washed, then resuspended in basal greening medium. ●, Control (no additions made); ○, 0·05% ammonium sulphate was added at 5hr., and the cell suspension was kept in the dark until 10hr., and then placed in the light; △, 0·05% ammonium sulphate was added at 5hr., and the cell suspension was kept in the dark until 10hr., when ammonium sulphate was removed and the cell suspension then placed in the light.

effective. All the other compounds tested had activity except sodium nitrate: it may be that *E. gracilis* is unable to utilize NO_3^- ions.

Effect of short incubation with nitrogen source. To overcome the block in chlorophyll synthesis the nitrogen source does not have to be present actually during the incubation in the light. After growth in the dark at high ethanol concentrations a relatively short exposure to nitrogen source in the dark will bring about chlorophyll synthesis during a subsequent incubation in the light. Cells were grown in the dark with 1·5% (v/v) ethanol and 0·03% ammonium nitrate, and then suspended in the basal greening medium. After a preliminary 5hr. incubation in the dark, 0·05% ammonium sulphate was supplied to two batches of cells; to a third batch no addition was made. After a further 5hr. in the dark one of the batches of cells that had been supplied with ammonium sulphate was washed twice and resuspended in basal greening medium. All three batches of cells were then exposed to light. The cells that had not been supplied with a nitrogen source formed chlorophyll at a very low rate (Fig. 2). The cells that had been exposed to ammonium sulphate for 5hr. in the dark synthesized chlorophyll at a very much higher rate. The cells that were supplied with ammonium sulphate both during the 5hr. dark period and the subsequent incubation in the light formed chlorophyll at a higher rate still.

Effect of carbon source present during incubation in the light. After the study of the effect of addition of a nitrogen source on chlorophyll synthesis in the light by cells previously grown in the dark at high concentrations of carbon source, the converse experiment was now carried out, i.e. a study of the effect of addition of a carbon source on chlorophyll synthesis in the light by cells previously grown in the dark on high concentrations of nitrogen source. Cells were grown in the dark in a medium containing 0·4% (v/v) of ethanol and 0·06% of ammonium nitrate and, as expected, carried out rapid chlorophyll synthesis when resuspended in basal greening medium and exposed to light (Fig. 3). When 0·4% (v/v) of ethanol was added early in the greening process chlorophyll formation was unaffected for the first few hours, but ceased completely 8–12hr. later. Ethanol had much the same effect when added about half-way through the greening process (Fig. 4). Essentially similar results were obtained with other carbon sources, namely acetate, pyruvate, succinate, malate, glucose and fructose. In all these experiments the carbon source in question was also used as the sole carbon source in the dark-growth medium to ensure that the greening cells had acquired the ability to utilize the compound. App & Jagendorf (1963) had reported that ethanol, acetate and malate cause a partial inhibition of greening. In essence, the present results confirm this with the difference that here chlorophyll synthesis ceased altogether a short time after addition of the carbon source, whereas App & Jagendorf (1963) found that greening

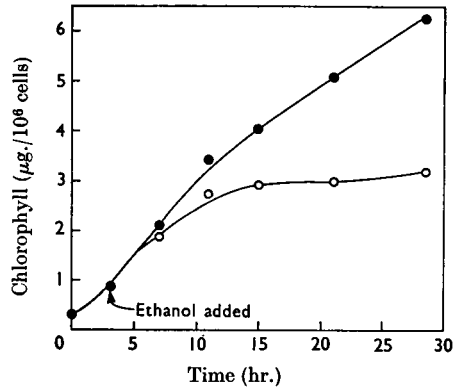

Fig. 3. Effect of adding ethanol on subsequent chlorophyll synthesis in the light. Cells were previously grown in the dark for 4 days in medium containing 0·4% (v/v) of ethanol and 0·06% of ammonium nitrate. Cells were harvested and washed, then resuspended in basal greening medium. ●, Control (no additions made); ○, 0·4% (v/v) of ethanol added at 3hr.

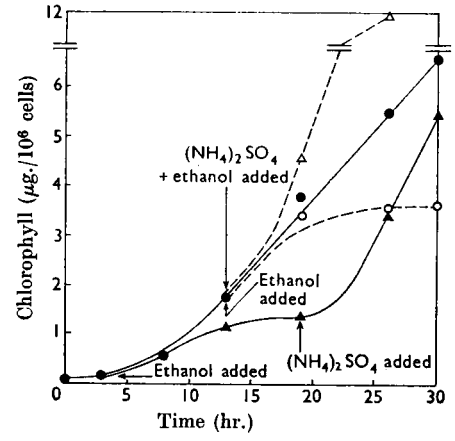

Fig. 4. Effect of adding ethanol and ammonium sulphate on subsequent chlorophyll synthesis in the light. Cells were previously grown for 4 days in the dark in medium containing 0·4% (v/v) of ethanol and 0·06% of ammonium nitrate. Cells were harvested and washed, then resuspended in basal greening medium. ●, Control (no additions made); ▲, 0·6% (v/v) of ethanol added at 3hr. followed by the addition of 0·05% of ammonium sulphate at 19hr.; ○, 0·6% (v/v) of ethanol added at 13hr.; △, 0·6% (v/v) of ethanol + 0·05% of ammonium sulphate added at 13hr.

continued at a diminished rate in the presence of the carbon source.

More detailed studies were carried out to determine whether the different chloroplast pigments show the same sensitivity to repression. It was found that total carotenoid synthesis is also inhibited by the addition of ethanol to a greening cell suspension. However, inhibition is less than with chlorophyll: at a time when total chlorophyll synthesis has ceased altogether (as in Fig. 3) carotenoid synthesis continues at a rate that is about 60% lower than that in the control (without ethanol). Synthesis of the individual pigments was studied by paper-chromatographic techniques. On addition of 0·4% (v/v) of ethanol to a greening cell suspension, the syntheses of chlorophyll *a* and of chlorophyll *b* are inhibited to the same extent. Inhibition of synthesis of carotenoids sets in at about the same time as inhibition of chlorophyll synthesis. Formation of β-carotene and neoxanthin is inhibited more than the synthesis of diadinoxanthin.

The inhibitory effects of ethanol on chlorophyll synthesis are prevented if ammonium sulphate is added to the suspension at the same time (Fig. 4). Indeed the rate of synthesis in the presence of ethanol plus ammonium sulphate is higher than in the absence of any addition. If ammonium sulphate is added about 10hr. after the addition of ethanol, i.e. at a time when chlorophyll synthesis has ceased, then after a further 5hr. or so chlorophyll synthesis recommences again at a rate faster than in the control without ethanol or ammonium sulphate.

Finally, in experiments with cells grown in the dark on high concentrations of ethanol, the block in chlorophyll synthesis is removed by the addition of 0·05% of ammonium sulphate (as in Fig. 1), but a further addition of 1·5% (v/v) of ethanol 10hr. later (when chlorophyll is being synthesized at a rapid rate) will once more diminish the rate of chlorophyll synthesis.

Chemical composition of cells grown on different media. There is a clear-cut difference in the physiological properties of cells grown in the dark on high ratios of carbon source to nitrogen source ('high-carbon cells') and those grown on low ratios of carbon source to nitrogen source ('low-carbon cells'): the former have little or no ability to synthesize chlorophyll in the light, the latter can form chlorophyll rapidly. Studies were now carried out on the chemical composition of these two kinds of cells to determine whether there is any gross difference that might help to provide a biochemical basis for their different physiological properties. Results of a typical analysis are shown in Table 3. High-carbon cells have a much higher dry weight than low-carbon cells, being 2½–3 times as heavy per cell. Most of this increase is accounted for by carbohydrate (probably in the form of the storage polysaccharide, paramylon): the high-carbon cells contain about 4 times as much carbohydrate per cell as the low-carbon cells. High-carbon cells also have an increased lipid content, this being about twice that in low-carbon cells. Both kinds of cell have about the same protein and DNA content per cell. In other experiments it was shown that the two kinds of cells also have about the same contents of soluble protein. Soluble protein extracts from high-carbon and low-carbon cells show essentially the same pattern on cellulose acetate electrophoresis: in both cases the same three major bands are seen with similar intensities. In some experiments (e.g. the one described in Table 3) the low-carbon cells had 50–100% more RNA per cell than the high-carbon cells: however, little significance can be attached to this finding since in certain other experiments the two kinds of cell had the same RNA content.

Comparison of concentrations of pool amino compounds. It was thought possible that the differing abilities of high-carbon and low-carbon cells to form chlorophyll might be due to their having different concentrations of low-molecular-weight nitrogenous constituents in the pool available for biosynthesis of chloroplast components. Accordingly the concentration of pool amino compounds in these cells was determined by measuring the amount of ninhydrin-positive

Table 3. *Chemical composition of cells grown on low or high ratios of carbon source to nitrogen source*

Cells of *E. gracilis* were grown for 4 days in the dark on media containing either 2·0% (v/v) of ethanol and 0·03% of ammonium nitrate (high-carbon cells), or 0·4% (v/v) of ethanol and 0·06% of ammonium nitrate (low-carbon cells). In a subsequent experiment it was shown that in a 24hr. incubation in the light the high-carbon cells synthesized no chlorophyll; the low-carbon cells formed chlorophyll rapidly.

	Composition (μg./10^6 cells)					
	Dry wt.	Carbohydrate	Lipid	Protein	DNA	RNA
High-carbon cells	2720	2320	143	225	5·2	10·0
High-carbon cells	2520	2180	136	220	4·9	9·3
Low-carbon cells	980	510	71	238	4·5	15·5
Low-carbon cells	980	514	75	235	4·4	16·0

material in cold-acid extracts of cells grown as described in Table 3. No difference was found, there being 0·5–0·7 μg. of amino nitrogen/10^6 cells both in high-carbon and low-carbon cells.

Since the pool extracts may contain peptides as well as amino acids, the value for total free amino nitrogen may not be a true measure of the amount of readily available nitrogen in the pool. Therefore to determine the total amount of amino acids in the pool, whether free or in peptides, the extracts were subjected to hydrolysis by concentrated acid to convert peptides into amino acids: the concentration of free amino nitrogen was measured before and after hydrolysis. Hydrolysis invariably increased, by 2½- to 5-fold, the concentration of free amino nitrogen in the pool extracts. The increase was greater in extracts from low-carbon cells than in extracts from high-carbon cells. Although before hydrolysis the concentration of free amino nitrogen in the pool was the same in both types of cell, after hydrolysis the average concentration of free amino nitrogen was 1·6 μg. of nitrogen/10^6 cells in high-carbon cells and 2·9 μg. of nitrogen/10^6 cells in low-carbon cells, a difference of 75%.

It had previously been reported (Kirk, 1962) that there is only a slight decrease in the concentration of pool amino compounds during greening. This was confirmed in the present work, and it was further shown that the same is true for pool extracts that have undergone acid hydrolysis. Cells were grown for 4 days in the dark on a low-carbon medium [0·2% (v/v) ethanol, 0·06% ammonium nitrate] and then suspended in basal greening medium in the light. At the beginning of the incubation, and again after 9hr. greening, samples were taken for extraction of pool materials with cold perchloric acid. The concentration of free amino nitrogen was measured before and after hydrolysis with concentrated hydrochloric acid. The free amino nitrogen content of untreated extracts fell from 0·60 μg. of nitrogen/10^6 cells at zero time to 0·45 μg. of nitrogen/10^6 cells after 9hr. in the light. The free amino nitrogen content of acid-hydrolysed extracts fell from 2·43 μg. of nitrogen/10^6 cells at zero time to 1·96 μg. of nitrogen/10^6 cells after 9hr. in the light. Thus there is relatively little fall in the concentration of free amino acids or peptides in the cell pool during greening. In this experiment 1·36 μg. of chlorophyll was synthesized/10^6 cells over the 9hr. period.

Chromatography of pool amino acids. It had already been shown that there was no significant difference in the concentrations of free amino compounds in pool extracts from high-carbon and low-carbon cells. However, it seemed possible that there might be differences in the relative amounts of the different amino acids. To test this possibility cells were grown in the dark on media containing either 2·0% (v/v) of ethanol and 0·03% of ammonium nitrate (high-carbon cells) or 0·2% (v/v) of ethanol and 0·06% of ammonium nitrate (low-carbon cells). Pool extracts were prepared from 250 × 10^6 cells of each type and analysed by ion-exchange chromatography. The results are shown in Table 4. The major differences are that the high-carbon cells have higher concentrations of cysteic acid, aspartic acid, glutamic acid, glycine, methionine, tyrosine, phenylalanine, histidine and an amino acid eluted in the position of 3-methylhistidine, and lower concentrations of alanine, ornithine, lysine and arginine.

Comparison of protease activities. Previous studies (Kirk, 1962), confirmed in the present work, showed there is little change in the concentration of pool amino compounds during greening. This had led

Table 4. *Amino acid analyses of pool material extracted from cells grown on low or high ratios of carbon source to nitrogen source*

Cells of *E. gracilis* were grown for 4 days in the dark on media containing either 2·0% (v/v) of ethanol and 0·03% of ammonium nitrate (high-carbon cells) or 0·2% (v/v) of ethanol and 0·06% of ammonium nitrate (low-carbon cells). Cells were washed, then extracted twice with 0·2 M-$HClO_4$. Amino acid analysis was carried out on a Technicon amino acid analyser. In a subsequent experiment it was shown that in a 24hr. incubation in the light the high-carbon cells synthesized no chlorophyll; the low-carbon cells formed chlorophyll rapidly.

Amino acid (in order of elution from column)	Amino acid composition (μmoles/10^9 cells)	
	Low-carbon cells	High-carbon cells
(Cysteic acid)	0·621	1·200 running with solvent front
Aspartic acid	0·124	0·181
Threonine	0·207	0·200
Serine	0·207	0·194
Glutamic acid	0·124	0·206
Proline	—	—
Glycine	0·234	0·413
Alanine	3·117	0·736
Cystine	—	—
Valine	0·159	0·187
Methionine	0·400	2·729
Isoleucine	0·000	0·000
Leucine	0·069	0·000
Tyrosine	0·069	0·129
Phenylalanine	0·069	0·323
Post-NH_3 peak	(0·517)	(0·516)
Ornithine	1·104	0·710
Lysine	3·090	1·516
Histidine	0·145	0·581
3-Methylhistidine	0·759	2·729
Tryptophan	0·221	0·187
Post-tryptophan	(0·690)	(1·940)
Arginine	2·048	0·832

to the conclusion (Kirk, 1962; Kirk & Tilney-Bassett, 1967) that during greening of *Euglena* in the absence of a nitrogen source there must be breakdown of other cell proteins to provide the amino acids for the synthesis of chloroplast proteins, and also the nitrogen source for the formation of chlorophyll itself. For such breakdown to take place the cell must contain one or more proteolytic enzymes. The existence of such an enzyme in *E. gracilis* has in fact been reported by Bertini, Brandes & Buetow (1965). It seemed possible that the inability of high-carbon cells to form chloroplasts could be due to the absence of proteolytic enzymes. Accordingly experiments were carried out to determine whether there was any difference in the protease activity of high-carbon and low-carbon cells. Cells were grown in the dark on media containing 2·0% (v/v) of ethanol, 0·03% of ammonium nitrate (high-carbon cells) or 0·2% (v/v) of ethanol and 0·06% of ammonium nitrate (low-carbon cells). The protease activities of ultrasonically treated extracts were measured. In a typical experiment the values (μg. of amino nitrogen liberated from haemoglobin/hr. by protease from 10^6 cells) were 1·00 for high-carbon cells and 0·50 for low-carbon cells. Thus the high-carbon cells, far from being deficient in protease activity, consistently had about twice the activity observed in low-carbon cells.

It is noteworthy that these proteolytic activities appear to be sufficient to break down cell proteins at a rate rapid enough to supply amino acids for chloroplast protein synthesis. For instance, the particular batch of low-carbon cells used in the experiment described here synthesized chlorophyll during a subsequent incubation in the light at the rate of 7·84 μg./24hr./10^6 cells (i.e. 0·326 μg./hr./10^6 cells). If we assume that the ratio of chloroplast protein synthesized to chlorophyll synthesized is about the same as the ratio of protein to chlorophyll in an intact chloroplast, i.e. about 14:1 (a value based on reported data for spinach chloroplasts; see Kirk & Tilney-Bassett, 1967), then associated with the synthesis of this amount of chlorophyll we might expect, at most, about 4·56 μg. of chloroplast protein to be formed/hr./10^6 cells. This corresponds to 0·57 μg. of amino nitrogen/hr./10^6 cells. As shown above, the proteolytic activity in the low-carbon cells is about sufficient to supply amino acids at this rate. Preliminary experiments have indicated that the proteolytic activity of low-carbon cells increases still further during incubation in the light in basal greening medium, the activity rising by about 60% in 9hr. The occurrence of such an increase is to be expected in the light of the report of Bertini *et al.* (1965) that incubation of cells of *E. gracilis* under starvation conditions causes an increase in protease activity.

Conclusions. The findings reported in this paper indicate that a major nutritional factor controlling chloroplast formation during greening is the ratio of carbon source to nitrogen source during the previous growth in the dark and also during the actual period of greening in the light. Raising the ratio of carbon source to nitrogen source tends to prevent chloroplast pigment synthesis, lowering it promotes pigment synthesis.

There are many similarities between this *Euglena* system and the nutritional control of chloroplast formation in *Chlorella protothecoides*, which has been investigated by Hase and his co-workers (this work is reviewed by Kirk & Tilney-Bassett, 1967). In the *Chlorella* system, too, the essential determining factor appears to be the ratio of carbon source to nitrogen source. The *Euglena* and *Chlorella* systems cannot, however, be regarded as simply equivalent because there is a fundamental difference between them. *Chlorella* forms chloroplasts both in the light and in the dark, and the studies carried out are therefore of direct nutritional effects on chloroplast formation under conditions in which chloroplast formation can normally take place. *E. gracilis* does not form chloroplasts in the dark, and what we are primarily concerned with is the relationship between the ability to form chloroplasts in the light and the nutritional treatment during the previous growth in the dark, in which no chloroplast formation was possible. However, despite the difference between the two systems, it does seem probable that the fundamental metabolic mechanism by which the ratio of carbon source to nitrogen source controls chloroplast formation is the same in both algae.

Considering first the effect of the ratio of carbon source to nitrogen source during growth of *E. gracilis* in the dark, there is clearly some fundamental difference in the constitution of the high-carbon cells and low-carbon cells: the latter will develop chloroplasts in the light, the former will not. What is the nature of the block in the high-carbon cells? There are two kinds of explanation. Either these cells lack something that is required for chloroplast formation or they possess something that prevents chloroplast formation. Considering the first type of explanation in more detail it could mean for instance that the cells lack some nitrogenous constituent that is required in substrate quantities for chloroplast formation. It seems rather unlikely that this nitrogenous substance is an amino acid, since the ion-exchange analysis of the pool extracts indicates that the high-carbon cells do not totally lack any of the major pool amino acids present in low-carbon cells. An alternative possibility is that the missing nitrogenous constituent is a reserve protein or peptide that would normally be broken down to yield amino acids for synthetic purposes during greening. The measurements of proteolytic activity

indicate that the high-carbon cells do at least have the enzymic machinery for breaking down proteins, but we do not know whether they have the necessary substrate. It is perhaps significant that cellulose acetate electrophoresis showed no difference in the patterns of the soluble proteins of high-carbon and low-carbon cells. The measurements of amino nitrogen after acid hydrolysis of pool extracts suggest that the low-carbon cells have a somewhat higher concentration of peptides in the pool, but the concentrations in the high-carbon cells are not so much lower as to account for the total inability of these cells to form chloroplasts. Whatever the hypothetical nitrogenous substance is, we would expect the low-carbon cells to have enough of it to support greening when exposed to the light. The ability of nitrogen sources added during greening to overcome the block in the high-carbon cells implies that the cells are then able to synthesize whatever nitrogenous components they need.

An example of the second type of explanation (that the cells possess something that prevents chloroplast formation) is the hypothesis that cells grown on high-carbon media accumulate some substance that represses genes required for chloroplast formation. This substance must persist for as much as 30hr. in the absence of any carbon source in the light, thus keeping the essential genes repressed throughout this time. The ability of nitrogen sources added during greening to lift the repression could be due to the metabolic conversion of this repressing substance, in the presence of a nitrogen source, into something else. For instance, the repressor could be some low-molecular-weight carbon compound that, in the presence of a nitrogen source, is converted into a nitrogenous substance (such as an amino acid) that is not a repressor. An alternative example of this type of theory is that the inhibitory substance is not a gene repressor but is an inhibitor of an enzyme in some biosynthetic pathway, such as the chlorophyll biosynthetic pathway.

If we now consider the ability of ethanol and other carbon sources to block chloroplast formation when added during the actual greening process then again we can put forward the same two types of explanation. First of all the ethanol might sequester some essential nitrogenous metabolite by causing it to be converted into a compound that cannot be used for chloroplast formation. Considering the second type of explanation it is possible that, in the presence of a carbon source, the cell makes a substance that represses genes required for chloroplast formation: or, the cells may make an inhibitor that inhibits an essential biosynthetic enzyme.

We believe that both these types of explanation, i.e. that the high-carbon cells lack something that is required for chloroplast formation, or that they possess something that prevents chloroplast formation, are consistent with our results. However, while accepting that either type of explanation (and indeed possibly other types of explanation altogether) may be true, we are inclined to favour, as a working hypothesis, the second type of explanation. We consider that an explanation on the basis of repression is intrinsically more probable, partly by analogy with the phenomenon of repression, particularly catabolite repression, in bacteria, and partly because certain of our results can more plausibly be accommodated to a mechanism of this type. For instance, the dark-grown high-carbon cells have much higher concentrations of carbohydrate and lipid. It seems possible that, although the carbohydrate (which is probably mainly polysaccharide) and lipid are not themselves directly repressing chloroplast formation, they may give rise by the usual metabolic pathways to a high concentration of some low-molecular-weight carbon compound that is the actual repressor. The higher concentrations of alanine in the pool contents of the low-carbon cells are also consistent with such a hypothesis. If the repressor were an intermediate of carbohydrate metabolism at, say, the C_3 level, e.g. pyruvate or phosphoenolpyruvate, the effect of addition of a nitrogen source, added either during growth in the dark or incubation in the light, could be to lower the concentration of this repressing metabolite, e.g. converting it into alanine. A parallel effect of high concentrations of nitrogen source during growth would be the prevention of the accumulation of lipid and carbohydrate.

In conclusion, then, we believe that the inability of cells grown on high concentrations of carbon source to form chloroplasts could be due either to the lack of some essential nitrogenous metabolite or reserve material, or to the presence of a substance that either represses essential genes or inhibits biosynthetic enzymes. Of the different possible explanations we consider the hypothesis that the high-carbon cells contain a repressor for genes concerned with chloroplast formation to be marginally the most plausible.

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