Journal of General Microbiology (1973), **75**, 51–60
Printed in Great Britain

Unbalanced Growth and Replication of Chloroplast Populations in *Euglena gracilis*

By J. R. COOK
Departments of Zoology and Botany, University of Maine, Orono, Maine, U.S.A.

(*Received* 3 *December* 1971; *revised* 29 *August* 1972)

SUMMARY

In phototrophic *Euglena gracilis*, chlorophyll content/organism is modulated both by varied amounts of chlorophyll/chloroplast and by number of chloroplasts/organism. In unlimited growth, Euglena never has less than 7 to 10 chloroplasts on the average, each containing 0·35 to 1·0 pg chlorophyll, depending on conditions and age of culture. Chloroplasts rarely contain more than 1·1 pg chlorophyll, and organismic levels greater than 10 to 12 pg are accomplished by an increased number of chloroplasts (to 60 in this study). Chloroplast replication is tightly coupled to that of the organism in log phase growth, but not in the lag and early stationary periods of growth.

INTRODUCTION

In the flagellate protozoon *Euglena gracilis*, the chloroplasts divide at about the same time as the organism (Gojdics, 1953; Leedale, 1967). In the medium used in this laboratory, only a small proportion (2 to 4 %) of the organisms are in division at any given time, and most of the organisms in exponentially expanding populations have approximately the same number of chloroplasts – 7 to 10 on the average in *E. gracilis* z strain (with optimum light). Chloroplast number is used as a taxonomic character in the green flagellates.

Cytokinesis of Euglena is typically distinguished by a vigorous back-and-forth churning of cytoplasmic contents between incipient filial cells. Leedale (1959) reported that sometimes one organism receives both nuclei, the other thus being anucleate. As it is most unlikely that chloroplasts are always distributed with great exactness, the number of chloroplasts/organism should eventually range between zero and some number much greater than 10, unless, as obviously occurs, some control mechanism either accelerates or retards the rate of chloroplast replication relative to that of the organism, thus maintaining a fairly constant chloroplast population. Thus, while chloroplast division in Euglena may be triggered by events of mitosis, it is inferred that other controls also operate to determine whether a given chloroplast shall replicate more than once, only once, or not at all, during the life of the organism.

This paper describes division rate of the organism compared with those of chloroplasts, under several different but normal physiological conditions of growth.

METHODS

Organism and media. Euglena gracilis Klebs, z strain, Pringsheim was used throughout. Nutrition was photosynthetic, with organisms grown in the salt medium of Cramer & Myers (1952). The pH of this medium, normally 6·8, was in one experiment adjusted to 3·0 with H_2SO_4. The medium contained vitamins B_1 and B_{12} at respective concentrations of 10 and 0·5 μg/l.

Culture conditions. Cultures were grown in a 9 × 50 cm Pyrex cylinder, 3·5 l capacity,

fitted with a Pyrex water-jacket through which water at constant temperature was circulated. A filtered mixture of 95 % air/5 % CO_2 was bubbled through sintered glass at the bottom of the vessel, and a Teflon-covered magnetic stirring bar mixed the culture. Sometimes a reservoir of fresh medium was connected with the vessel to permit dilution of the culture. Light was supplied by stacked incandescent lamps; light intensity incident to the culture was varied by changing the distance of the lamps and was measured with a Weston photometer.

Samples of cell suspension were removed through a siphon tube. The concentration of organisms was determined with the Coulter cell counter, and samples divided for various analyses which included chloroplast counts and chlorophyll levels, and sometimes protein and RNA levels.

Chloroplast counts. Chloroplasts were counted with the fluorescence microscope, with a Corning 7-51 exciting filter and Y-8 barrier filter. When there were more than 10 to 12 chloroplasts/organism, squashed preparations were used. These were made by using a drop small enough not to fill the area under the coverslip; the resulting compression ruptured most of the organisms. Chloroplasts thus released retained their integrity usually for some minutes, long enough to be counted. Ordinary dark-field illumination was used together with fluorescence microscopy to ensure that chloroplasts of single organisms were being counted. Initially about 100 organisms were scored in each sample; as the variance in chloroplast numbers was found to be quite constant (see Results) the number of organisms scored was later reduced to 20 to 40/sample.

Biochemical determinations. Chlorophyll *a* levels were estimated spectrophotometrically in 90 % ethanol extracts of known numbers of washed organisms. Protein and RNA levels were estimated from known numbers of washed organisms by means of the biuret assay and the Schmidt & Thannhauser (1945) technique as previously described for Euglena (Cook, 1966).

Experimental design. Chlorophyll levels and chloroplast numbers were determined regularly as the cultures aged. Light intensity, temperature and culture pH were altered between experiments. An inoculum of about 500 organisms/ml was used, and harvests were started at 15 to 20×10^3 organisms/ml, except when the effect of dilution of stationary culture was to be studied specifically.

RESULTS

Average chloroplast counts ranged between 7 and 10/organism as a lower limit, and about 60/organism as an upper limit (Fig. 1). At any given age of a culture, the range of numbers/organism was relatively small (Fig. 2), and the coefficient of variation remarkably constant over wide ranges of average chloroplast numbers (Table 1).

Results of a typical experiment are shown in Fig. 3. In this example, organisms were grown in 800 ft-candle (saturating) light at 22 °C, pH 6·8. The number of chloroplasts (8·4 to 9·6) and the amount of chlorophyll (5·3 to 6·1 pg)/organism remained fairly constant throughout early log-phase growth, each chloroplast thus containing 0·6 to 0·7 pg chlorophyll. In late log phase chlorophyll concentrations increased to about 1·1 pg/chloroplast, with no significant change in the number of chloroplasts. The advent of stationary phase was accompanied by an increase in chlorophyll levels and chloroplast numbers within the organisms, both to about the same extent, so the calculated amount of chlorophyll/chloroplast remained constant at 1·1 pg. Plastid numbers increased only after each chloroplast had acquired 1·1 pg chlorophyll, which, as will be shown below, may represent the normal 'saturation' level of chlorophyll for the plastid.

The total numbers of chloroplasts/ml of culture, calculated by multiplying the average

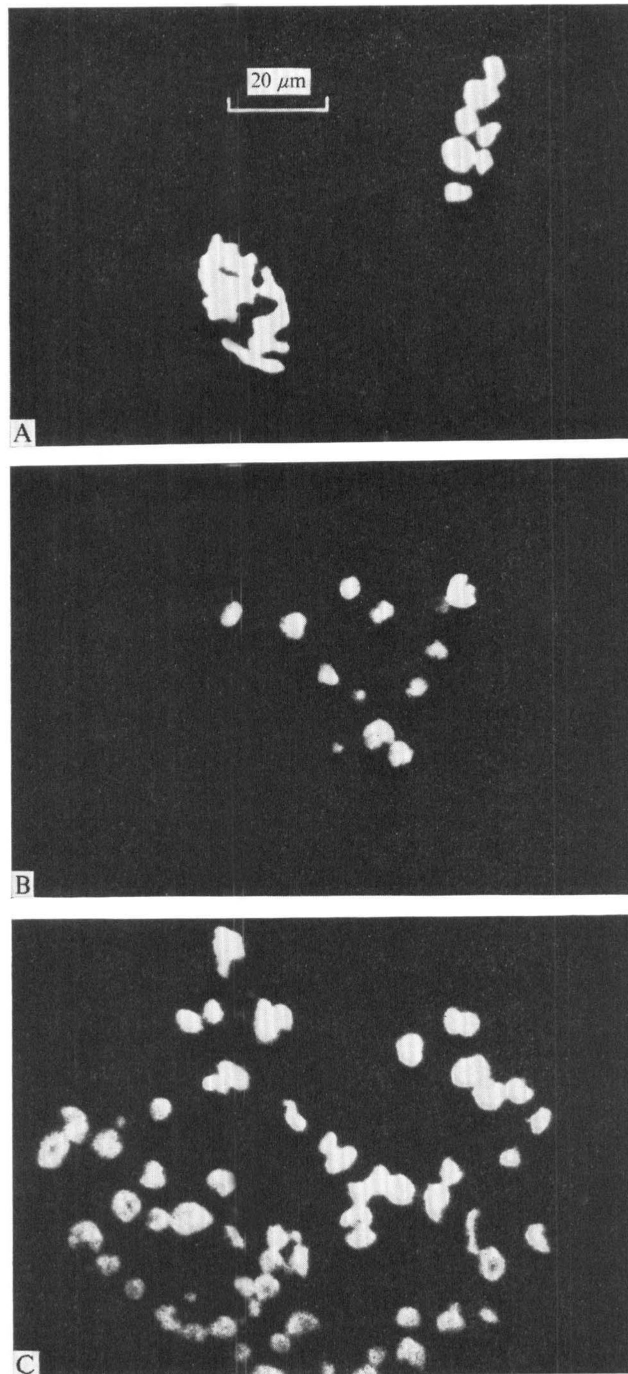

Fig. 1. (A) Two whole Euglenas containing 7 and about 15 chloroplasts. The organism with the larger number is apparently approaching division. (B) Ruptured Euglena showing 13 chloroplasts. (C) Ruptured Euglena showing 60 chloroplasts. Fluorescence micrographs.

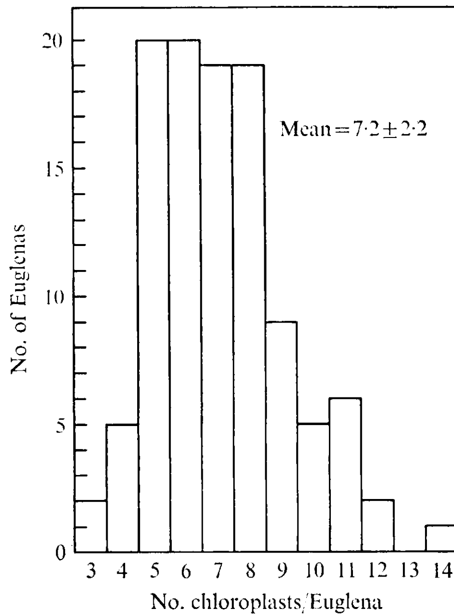

Fig. 2

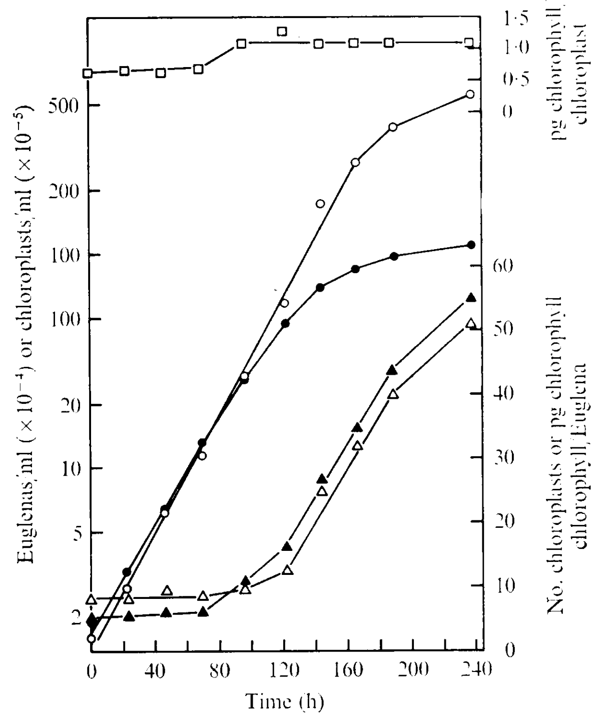

Fig. 3

Fig. 2. Frequency histogram of chloroplasts/organism in a Euglena culture (log phase at 15 °C, 800 ft-candle, pH 6·8).

Fig. 3. Plastid profile in a Euglena culture grown at pH 6·8, 800 ft-candle, 22 °C. ●, Organisms/ml; ○, chloroplasts/ml; ▲, chlorophyll/organism; △, chloroplasts/organism; □, chlorophyll/chloroplast.

Table 1. *Plastid replication during growth of Euglena gracilis (from data used to construct Fig. 3). The coefficient of variation* (C.V.) *is the mean divided into the standard deviation* (S.D.)

Culture age (h)	No. Euglenas scored	Chloroplasts/organism Range	Mean	S.D.	C.V.
0	40	6–15	8·5	2·1	0·24
23	19	4–12	8·4	2·1	0·25
46	45	7–18	9·6	2·5	0·26
70	40	6–18	8·7	2·8	0·32
96	40	6–19	9·6	3·0	0·31
122	21	9–25	12·6	4·2	0·33
144	23	17–41	25·0	6·0	0·24
166	21	19–45	32·0	7·0	0·22
190	19	23–51	40·0	7·5	0·19
239	20	26–70	51·2	11·7	0·23

number/organism by the number of organisms/ml, are also plotted in Fig. 3. Chloroplast replication remained exponential well after the organisms had entered stationary phase.

When a stationary phase culture was diluted with fresh medium, chloroplast numbers (and chlorophyll)/organism decreased rapidly in early log phase, to a final level of about 8 (Fig. 4). The kinetics suggest that the loss was due simply to dilution through cell division, which in the early stages occurred without any chloroplast replication.

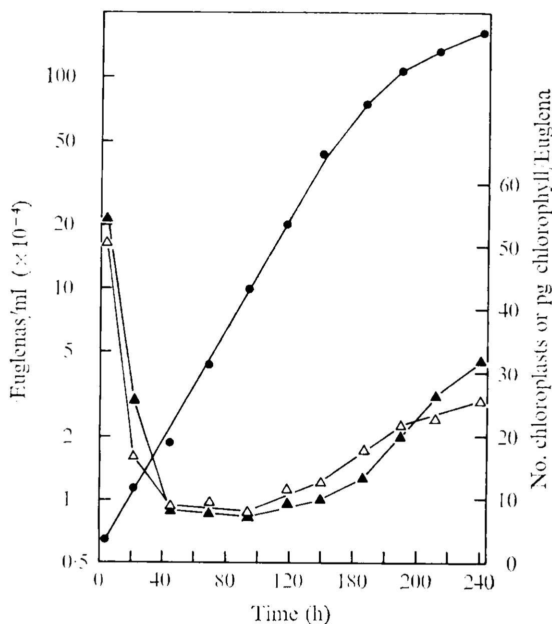

Fig. 4

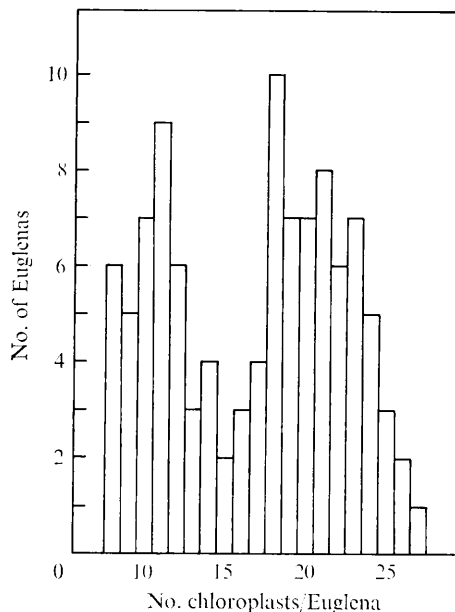

Fig. 5

Fig. 4. Plastid profile following dilution of a stationary phase culture with fresh medium. The parent culture was that shown in Fig. 3. Culture conditions and symbols used as for Fig. 3.

Fig. 5. Frequency histogram of chloroplasts/organism about 1 generation after dilution of an old culture (pH 6·8, 800 ft-candle, 22 °C).

The distribution of chloroplast numbers/organism was found to be bi-modal following dilution of old cultures, when the average number of plastids was decreasing with time (Fig. 5). This probably means only that some of the organisms had less of a lag in division than others.

When Euglena was grown at a reduced light intensity, the results were qualitatively similar to those shown in Fig. 3 and 4. With other conditions the same as described for Fig. 3 and 4 (i.e. a temperature of 22 °C at pH 6·8), culture at 300 ft-candle (Fig. 6) resulted in a somewhat larger number of chloroplasts in log-phase growth as the major difference when compared to culture at 800 ft-candle. The lower light intensity yielded the same log-phase growth rate but a much reduced final Euglena density; 300 ft-candle is about the lowest intensity that supports optimal growth rates of Euglena (Cook, 1963).

The smallest number of chloroplasts observed at 300 ft-candle was about 15/organism, increasing to about 45 by stationary phase (Fig. 6). After dilution of the stationary-phase culture, a reduction to about 20 chloroplasts/organism occurred.

In the experiment shown in Fig. 6, the amount of chlorophyll/plastid varied considerably more than it was found to do in other experiments, at least before dilution; after dilution, chlorophyll concentrations equilibrated at about 1·1 pg/chloroplast. It would appear that, at this low light intensity, a long period of adaptation is required before the plastid system assumes a balanced growth rate.

Euglenas grown at pH 3 (22 °C, 800 ft-candle) were similar to those grown at pH 6·8 (Fig. 3), except that chlorophyll/chloroplast was somewhat reduced when the organisms contained more than about 15 pg chlorophyll. These results are summarized in Fig. 8.

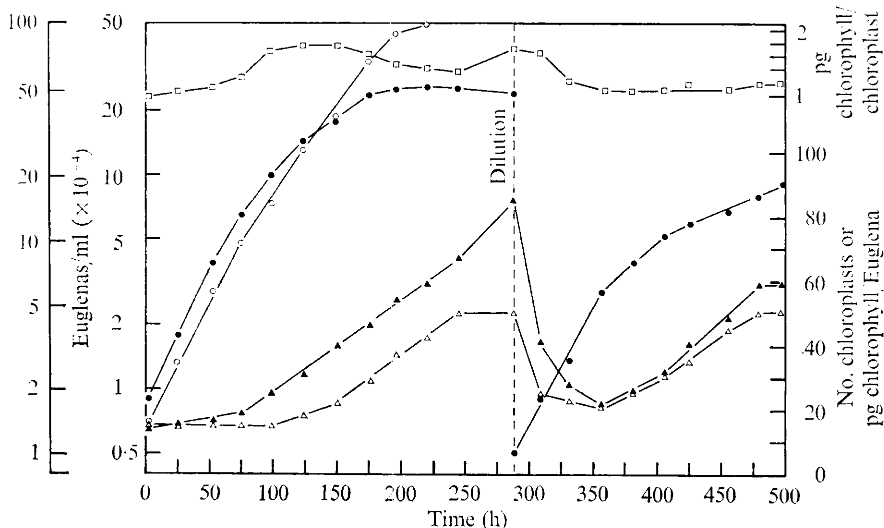

Fig. 6. Plastid profile in a Euglena culture grown at pH 6·8, 300 ft-candle, 22 °C. Symbols as for Fig. 3. The culture was diluted with fresh medium at 287 h.

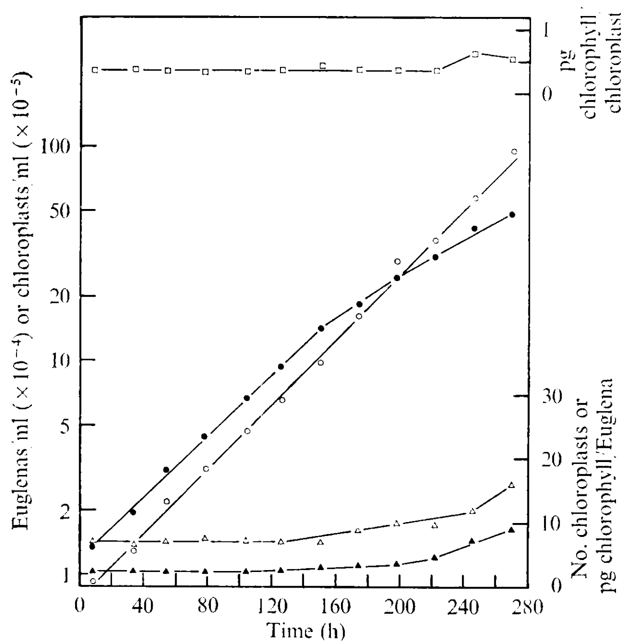

Fig. 7. Plastid profile in a Euglena culture grown at pH 6·8, 800 ft-candle, 16 °C. Symbols as for Fig. 3.

The influence of culture temperature (pH 6·8, 800 ft-candle) on the plastid system was more pronounced. At 30 °C (the optimal temperature for growth of this strain of Euglena) organisms in log phase had the same number of choroplasts as found at 22 °C (see Fig. 3) but slightly reduced amounts of chlorophyll; chlorophyll/chloroplast averaged about 0·6 pg. At 16 °C, chlorophyll levels were very much reduced (approaching 2 pg/organism as a lower limit, compared with 6 pg at 22 °C). The average number of chloroplasts was slightly

Table 2. *Biochemical characteristics of Euglena gracilis over the growth curve (from Fig. 2)*

	Thousands of Euglenas/ml									
	18	32	64	130	270	460	690	850	970	1090
Chlorophyll/organism (pg)	5·3	5·5	5·8	6·1	11	16	27	35	44	55
Protein/organism (pg)	380	380	390	400	450	500	550	550	550	530
RNA/organism (pg)	42	41	44	46	50	55	60	60	55	55

reduced to about 7/organism during log phase growth, increasing to about 16 in early stationary phase (Fig. 7). These chloroplasts thus contained only about 0·35 pg chlorophyll over most of the growth cycle. Similar to the results found at 22 °C, the chloroplast population in organisms grown at 16 °C replicated exponentially well after the organisms had entered early stationary phase (Fig. 7).

In several experiments, cellular RNA and protein levels were measured. Results of a typical experiment are summarized in Table 2. During log and early stationary phases of growth, RNA and protein content of the organisms were proportional to chlorophyll content. After the log-phase growth rate slowed, however, protein and RNA content/organism became constant (or even decreased) in most of the experiments, while chlorophyll levels were still increasing. Smillie & Krotkov (1960) likewise found a decrease in RNA during stationary phase.

DISCUSSION

It is established that chloroplasts divide at about the same time as the organism in many species of *Euglena*, including *Euglena gracilis*, z strain (Gojdics, 1953; Leedale, 1967). During log-phase growth, the rate of chloroplast replication was very tightly coupled to the rate of organism replication (Fig. 3, 6, 7). The average number of chloroplasts/organism varied not at all, and the range of numbers was so small that some control of replication rates is suggested.

In early stationary phase, however, chloroplasts multiplied more rapidly than did the organisms (Fig. 3, 6, 7); and after dilution of an old culture, organisms multiplied more rapidly than did the chloroplasts (Fig. 4, 6). Coupling of the two was thus not rigid. Further, the chloroplasts did not behave as a unit. When the chloroplasts replicated more rapidly than the organisms, some chloroplasts clearly divided more than once in a generation; but not all of them did so. If this had been so, one would have seen a step-wise increase in plastid numbers, e.g. from 10 to 20 over a doubling of organism number. That this did not occur is clear from results presented in Fig. 3, 4, 6, 7.

In this study, only light intensity caused a significant change in the number of plastids/organism. During log-phase growth – the only portion of the growth curve where chloroplast numbers remained constant – Euglena had about 7 to 10 chloroplasts at 800 ft-candle and about 15 at 300 ft-candle. The increase in plastid numbers as cultures entered stationary phase was almost certainly a direct consequence of mutual shading, with an effective reduction of light intensity. It may be emphasized that increased plastid numbers were not the result of increased rates of plastid replication; these rates remained constant while the rate of division of organisms decreased (see Fig. 3, 6, 7).

When old cultures were diluted with fresh medium, the effective light intensity was increased, and presumably served temporarily to inhibit chloroplast replication. Subsequent

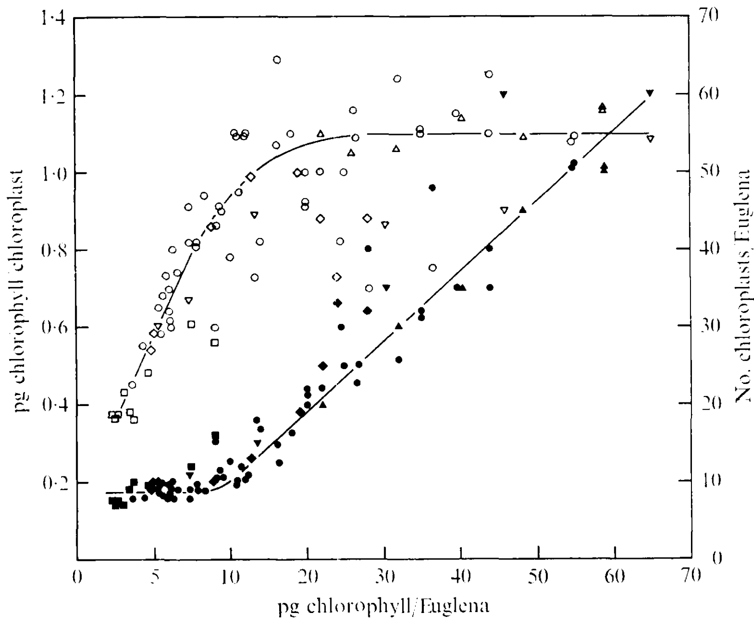

Fig. 8. Chloroplasts/organism (closed figures; right ordinate) and chlorophyll/chloroplast (open figures; left ordinate) as a function of total chlorophyll/organism. The abscissa is expanded between the limits of 0 to 10 pg. All at pH 6·8, 22 °C, 800 ft-candle, except as follows: diamonds, pH 3; triangles, 300 ft-candle; squares, 15 °C; and inverted triangles, 30 °C.

division of the organisms led to a reduction in plastid numbers. Such populations generally displayed a bi-modal distribution of chloroplast numbers (Fig. 5). This lack of homogeneity prevented meaningful calculations of chlorophyll content/chloroplast, and is an important exception to a generalization made below.

Other workers have described variations in chloroplast numbers of Euglena. Carell (1969) reported increased plastid numbers when Euglenas were grown in limiting concentrations of vitamin B_{12}; the kinetics were very similar to those described here, with the increase in numbers occurring in stationary phase. Vitamin B_{12} levels in the Cramer–Myers medium (used in this study) were 5 times greater than minimal requirements (Robbins, Hervey & Stebbins, 1953). Gross & Villaire (1960) also reported complex variations in chloroplast numbers of Euglena with culture age, but they used an organic medium which would have caused some repression of chlorophyll synthesis. Davis & Epstein (1971) found that plastid 'ploidy' of Euglena is more labile during heterotrophic than in phototrophic growth.

Chlorophyll levels have often been used as a measure of chloroplast numbers in Euglena. This error derives from the implied, or sometimes explicity stated, conviction that there is a perfect correlation between the amount of chlorophyll and the number of chloroplasts. Extreme amounts of chlorophyll found in this study ranged between 0·36 and 1·75 pg/plastid. Levels below 1·1 pg (to 0·36 pg) were common; levels above 1·1 to 1·2 pg/plastid were so rare that it may be concluded that 1·2 pg is the normal upper limit for chlorophyll content of the average plastid in Euglena, under conditions of phototrophic nutrition. These results are shown reduced in Fig. 8, which is a plot of chlorophyll/plastid as a function of chlorophyll/organism, derived from 10 different experiments of the sorts shown in Fig. 3, 4, 6, 7, where Euglenas were grown in different light intensities, at different pH, or at different temperatures. Excluded from the plot are those values found immediately after dilution of

old cultures, and those from the first part of Fig. 6, which is interpreted to be an extended period of adaptation to low light intensity. Elevated temperatures (30 °C) and low pH (3·0) also led to some relatively extreme values of chlorophyll/plastid, particularly when the average Euglena contained more than 10 pg chlorophyll, but these values are included in Fig. 8.

Also plotted in Fig. 8 are plastid numbers as a function of chlorophyll content in Euglena. The upper extreme was found to be about 60 chloroplasts/organism, but this number might well be exceeded under other culture conditions. Of greater interest is the lower extreme, 7 to 10 plastids/organism regardless of the amount of chlorophyll contained. On the basis of these data, it is concluded that the normal plastid complement of *Euglena gracilis* z strain is never less than 7 to 10 chloroplasts/organism. Epstein & Allaway (1967) found that *Euglena gracilis* var. *bacillaris* contained only 5 to 7 chloroplasts when growth was limited by phosphate or sulphate – half the normal level of about 12 when grown in complete medium.

This study permits a few generalizations concerning growth and replication of plastid populations in Euglena. It is evident that during phototrophic growth of Euglena, unlimited except perhaps by light, chlorophyll levels in the organism are modulated in two ways. From minimal levels approaching 2 pg (possibly less) up to about 10 pg chlorophyll/organism, the modulation occurs by alteration of chlorophyll levels in a constant number of 7 to 10 chloroplasts. Chlorophyll levels greater than 10 to 15 pg/organism are achieved by an increase in the number of chloroplasts, each of which remains fairly constant in chlorophyll content at about 1·1 pg/plastid. Finally, while the chloroplasts may at times replicate more rapidly or more slowly than the organism, chloroplasts and organisms replicate at identical rates throughout most of log phase growth. The latter point remains the most intriguing problem in the control of plastid replication in Euglena, and its solution almost certainly will prove to be the most elusive.

This work was supported by United States Public Health Service Grant GM-12179. The author is United States Public Health Service Research Career Development Awardee K4-GM9395.

REFERENCES

CARELL, E. F. (1969). Studies on chloroplast development and replication in *Euglena*. I. Vitamin B_{12} and chloroplast replication. *Journal of Cell Biology* **41**, 431–440.

COOK, J. R. (1963). Adaptations in growth and division in *Euglena* effected by energy supply. *Journal of Protozoology* **10**, 436–444.

COOK, J. R. (1966). Photosynthetic activity during the division cycle in synchronized *Euglena gracilis*. *Plant Physiology* **41**, 821–825.

CRAMER, M. & MYERS, J. (1952). Growth and photosynthetic characteristics of *Euglena gracilis*. *Archiv für Mikrobiologie* **17**, 384–402.

DAVIS, E. A. & EPSTEIN, H. T. (1971). Some factors controlling step-wise variation of organelle number in *Euglena gracilis*. *Experimental Cell Research* **65**, 273–280.

EPSTEIN, H. T. & ALLAWAY, E. (1967). Properties of selectively starved *Euglena*. *Biochimica et biophysica acta* **142**, 195–207.

GOJDICS, M. (1953). *The Genus Euglena*, pp. 1–268. Madison, Wisconsin: University of Wisconsin Press.

GROSS, J. A. & VILLAIRE, M. (1960). Chloroplast development and numbers in relation to culture age in *Euglena*. *Transactions of the American Microscopical Society* **79**, 144–153.

LEEDALE, G. F. (1959). Formation of anucleate cells of *Euglena gracilis* by miscleavage. *Journal of Protozoology* (Suppl.) **6**, 26.

LEEDALE, G. F. (1967). *Euglenoid Flagellates*, pp. 1–242. Englewood Cliffs, New Jersey: Prentice-Hall.

ROBBINS, W. J., HERVEY, A. H. & STEBBINS, M. E. (1953). *Euglena* and vitamin B_{12}. *Annals of the New York Academy of Sciences* **56**, 818–830.

SCHMIDT, G. & THANNHAUSER, S. J. (1945). A method for the determination of desoxyribonucleic acid ribonucleic acid, and phosphoproteins in animal tissues. *Journal of Biological Chemistry* **161**, 83–89.

SMILLIE, R. M. & KROTKOV, G. (1960). Phosphorus-containing compounds in *Euglena gracilis* grown under different conditions. *Archives of Biochemistry and Biophysics* **89**, 83–90.