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RNA SYNTHESIS DURING CHLOROPLAST DEVELOPMENT IN *EUGLENA GRACILIS*

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Abstract—Changes in chloroplast and cytoplasmic RNA were measured during chloroplast development in *Euglena gracilis* over a 3-day period The cells were grown in the dark and then illuminated by 1500 lx of white light at a cell density which was sufficiently high to prevent any cell division in illuminated cultures Light stimulated incorporation of ^{14}C-orotic acid into both chloroplast and cytoplasmic RNA The most rapid rate of light-dependent incorporation occurred during the first 8 hr of illumination and before the main period of chlorophyll synthesis The specific activity of chloroplast RNA was higher than that of cytoplasmic RNA, particularly during the first 24 hr of illumination By 32 hr the rate of incorporation into total cell RNA had decreased to that of dark control cells While there was no significant change in the amount of RNA per cell during chloroplast development, the amount of chloroplast RNA increased to a maximum of 7 per cent of the total cellular RNA by 16 hr In growing autotrophic cultures, or in cultures in which chloroplast development was followed at low cell densities ($< 3 \times 10^6$ cells per ml), chloroplast RNA accounted for about one-fifth of the total cellular RNA

INTRODUCTION

WHEN DARK-GROWN cells of *Euglena gracilis* are exposed to light the chloroplast development that ensues over the next three days is accompanied by changes in synthesis and composition of RNA in the cell [1–3] If 5-fluorouracil, an inhibitor of ribosomal RNA synthesis, is present during the first several hours of illumination, the chloroplast development is inhibited[4] suggesting that upon illumination there is additional synthesis of ribosomal RNA which is necessary for chloroplast development. An analysis of the changes in base composition of RNA in cells during chloroplast development has indicated that the light induces the synthesis of chloroplast RNA [1] According to Zeldin and Schiff[3] the turnover of cytoplasmic RNA is also stimulated by light However, the extent of the light-dependent increase in chloroplast RNA and the relative rates of synthesis of chloroplast and cytoplasmic RNA at different stages of chloroplast development cannot be readily ascertained from published data In order to obtain information on these points we have utilized the technique of polyacrylamide gel electrophoresis, which can separate chloroplast and cytoplasmic RNA from *E gracilis*,[5] to follow the appearance of chloroplast RNA during the development of chloroplasts in a non-dividing culture. In addition, the differential incorporation of radioactive orotic acid into chloroplast and cytoplasmic RNA during early stages of chloroplast development was measured

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[1] G BRAWERMAN and E CHARGAFF, *Biochim Biophys Acta* **31,** 172 (1959)
[2] R M SMILLIE, W R EVANS and H LYMAN, *Brookhaven Symp Biol* **16,** 89 (1963)
[3] M H ZELDIN and J A SCHIFF, *Plant Physiol* **42,** 922 (1967)
[4] W R EVANS and R M SMILLIE, *J Exptl Bot* **22,** 371 (1971)
[5] N. S SCOTT, R MUNNS and R M. SMILLIE, *FEBS Letters*, **10,** 149 (1970)

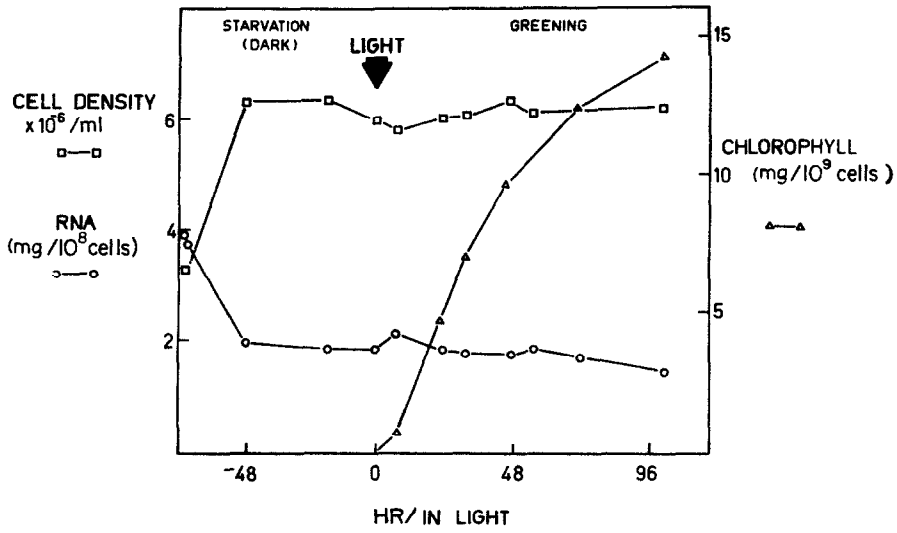

FIG 1 CHANGE IN CELL COMPONENTS DURING CHLOROPLAST DEVELOPMENT
Dark-grown cells were maintained on a carbon-free medium for 3 days in the dark and then illuminated for 4 days The culture was sampled at intervals for RNA (○——○), chlorophyll (△——△) and number of cells (□——□)

RNA Content of Cells during Chloroplast Development

Figure 1 shows changes in RNA and cell concentrations when dividing cells of dark-grown *E. gracilis* were shaken in the dark in a carbon-free medium and subsequently illuminated Cells were grown in the dark until a cell density of 4×10^6 cells per ml had been reached. The cells were transferred to a medium lacking carbon substrates for growth and maintained in darkness. Within 24 hr the number of cells had doubled and the amount of RNA per cell had decreased by half There was no change in RNA per cell during the next 2 days in the dark or during the following 4 days of illumination Thus the RNA content of the culture remained constant from the beginning of the period of carbon starvation

In other experiments, if the cell density was higher than 4×10^6 cells per ml at the time of transfer to a carbon-free medium, the cell number increased to a maximum of $7–8 \times 10^6$ cells per ml with a proportionate reduction of the RNA content per cell

In contrast to RNA, the DNA content per cell remained constant at 6 mg DNA per 10^9 cells throughout both the starvation and greening periods and so there was a doubling of the DNA content of the culture during the first 24 hr of starvation in the dark.

Amount of Chloroplast RNA in Developing Chloroplasts

The RNA extracted from chloroplasts from cells illuminated for 12–72 hr was characterized by polyacrylamide gel electrophoresis Chloroplast ribosomal RNA, which consists of 23S and 16S species,[5] was the principal high molecular weight RNA present in the chloroplasts prepared after 16 hr (Fig 2a) or longer periods of illumination However, in the chloroplast fraction isolated after only 12 hr of illumination some of the RNA appeared to be

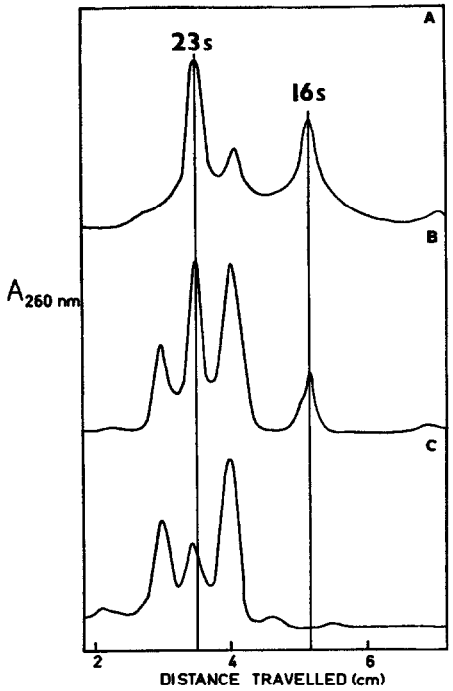

FIG 2 POLYACRYLAMIDE GEL ELECTROPHORESIS OF RNA FROM DEVELOPING CHLOROPLASTS
Chloroplast and cytoplasmic fractions were prepared from cells after 12 or 16 hr of illumination and the RNA was extracted with phenol and separated on polyacrylamide gel The absorbance profiles of the gels are shown. (a) chloroplast RNA (16 hr of light), (b) 'chloroplast' RNA (12 hr of light), (c) cytoplasmic RNA (16 hr of light)

cytoplasmic in origin (Fig. 2b), since peaks attributable to cytoplasmic RNA (Fig. 2c) were also present

The RNA content of the isolated chloroplasts was estimated chemically and Table 1 (Experiment 1) shows the amount of chloroplast RNA (mostly ribosomal RNA, as transfer RNA is lost during the isolation procedure) as a percentage of the total cellular RNA at different stages of chloroplast development. After 12 hr of illumination the chloroplast fraction contained 7 per cent of the total RNA, but after correcting for cytoplasmic RNA present in this fraction, chloroplast RNA accounted for only 2 per cent of the total RNA After 24 hr of illumination the chloroplast RNA content of the cell had reached 7 per cent and remained at around this level for the next 2 days (Table 1)

This value for the percentage of total RNA contained in the chloroplast of *E. gracilis* is only one-third of that estimated previously for autotrophic cells using another method [2] At the light intensity and cell densities used in the standard greening conditions (Fig. 1) the cells do not divide even after the chlorophyll content has reached or surpassed that found in dividing autotrophic cells When the experiment was carred out at cell densities which were sufficiently low to allow cell division to take place once chloroplast development was completed, the percentage of total RNA found in the chloroplasts was more than doubled (Table 1, Experiments 2 and 3) and was comparable to values found for growing autotrophic cells (Table 1, Experiments 4 and 5). The rate of chlorophyll synthesis was similar at all cell densities

TABLE 1 AMOUNT OF RNA IN CHLOROPLASTS

Exp	Hr of illumination	Chloroplast RNA (% of total cellular RNA)
1	12	2*
	16	6
	24	7
	48	6
	72	7
2	48	14
	72	16
3	72	18
4	—	17
5	—	17

Expt 1–3, greening cells Expt 1, standard conditions (see Fig 1), Expt 2 standard conditions, except cell density at 72 hr was 2 8 × 10^6 cells per ml, Expt 3, as Expt 2 except cell density was 1 3 × 10^6 cells per ml Expt 4, autotrophic cells at 1 5 × 10^6 cells per ml Expt 5, autotrophic cells at 2 0 × 10^6 cells per ml A correction for the loss of chloroplasts during the isolation procedure was made by measuring the yield of chlorophyll

* Corrected for cytoplasmic contamination (see text)

Incorporation of Orotic Acid by Growing Autotrophic Cells

Autotrophic cells from a culture in the exponential phase of growth were incubated for various periods of time with ^{3}H-orotic acid While the specific activities of the chloroplast and cytoplasmic RNA extracted with phenol increased more than 10-fold between 3 and 6 hr after addition of the isotope, the increase between 6 and 14 hr was comparatively small (Table 2) and the ratio of the specific activity of cytoplasmic RNA to chloroplast RNA remained constant at 1·2 1

TABLE 2 SPECIFIC ACTIVITY OF RNA FROM GROWING AUTOTROPHIC CULTURES

Time of incubation	Cytoplasmic RNA (cpm per mg × 10^{-3})	Chloroplast RNA (cpm per mg × 10^{-3})	Cytoplasmic RNA / Chloroplast RNA
3	3 4	2 2	1 5
6	40	32	1 2
14	54	44	1 2

Cells were grown autotrophically and ^{3}H-orotic acid (62 mCi/m-mole) was added (700 μCi/l) when the cell density had reached 10^6 cells per ml RNA was extracted from the chloroplast and cytoplasmic fractions with phenol and the specific activity determined as described

Incorporation of Orotic Acid during Chloroplast Development in a Non-Dividing Culture

Cells at different stages of chloroplast development were incubated with ^{14}C-orotic acid for 16 hr and the rate of incorporation into total RNA measured Other aliquots of cells from the original culture were treated similarly but were kept in the dark Figure 3 shows that the rate of incorporation was approximately linear over 16 hr of labelling of all cultures

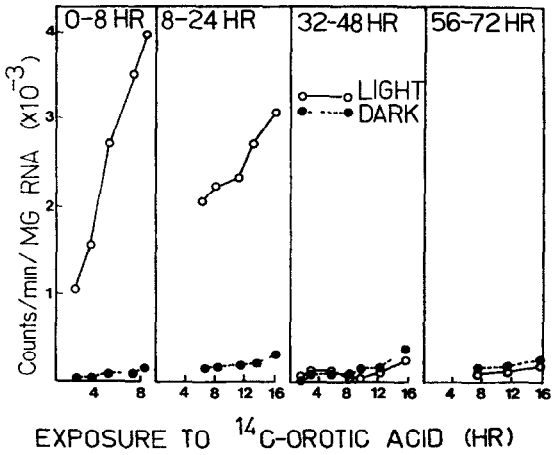

FIG 3 INCORPORATION OF OROTIC ACID INTO CELLULAR RNA BY CELLS ILLUMINATED FOR INCREASING PERIODS OF TIME

Dark-grown cells were shaken in darkness in a carbon-depleted medium for 30 hr Eight 50 ml samples of cells were placed either in light (1500 lx) and 5% CO_2 in air or in darkness ^{14}C-orotic acid (40 μCi/l, 0 8 mCi/m-mole) was added after 0, 8, 32 and 56 hr to individual flasks in the light and in the dark The values shown at the top of the graphs indicate the time periods (as measured from the beginning of the illumination) during which ^{14}C-orotic acid was fed Total RNA was extracted from the cells with KOH

examined. The rate of incorporation was greatest in cells labelled during the first 8 hr of illumination and resulted in 2 per cent of the isotope being incorporated into RNA Cells which had been illuminated for more than 32 hr and also cells kept in darkness took up much less isotope (about 0·1 per cent)

To determine the distribution of the labelled RNA, the cells were fractioned into chloroplast and cytoplasmic fractions at different stages of chloroplast development immediately following a 16-hr labelling period Table 3 shows that during the early stages of chloroplast development, when the light-stimulated incorporation of ^{14}C-orotic acid into RNA was at its highest (Fig 3), the specific activity of total chloroplast RNA (extracted with KOH)

TABLE 3 THE INCORPORATION OF ^{14}C-OROTIC ACID INTO CHLOROPLAST AND CYTOPLASMIC RNA DURING CHLOROPLAST DEVELOPMENT

Period of labelling (hr of illumination)	Specific activity ratio of chloroplast ribosomal RNA to cytoplasmic ribosomal RNA	Specific activity ratio of chloroplast total RNA to cytoplasmic total RNA
0–16	5 2 1	4 1 1
8–24	7 0 1	2 9·1
32–48	—	2 3 1
56–72	1 2 1	1 7 1

Experimental conditions were similar to those given in Fig 3 ^{14}C-orotic acid (20 μCi, 0 8 mCi/m-mole) was added to one litre of cells Total RNA was obtained by KOH digestion and ribosomal RNA was obtained from sucrose gradient centrifugation of RNA extracted with phenol The amount of orotic acid taken up and hence the specific activity of the RNA varied slightly from experiment to experiment and so the results are expressed as a ratio

was about four times that of the total cytoplasmic RNA. This ratio subsequently decreased so that when chloroplast development was completed, the specific activity of the RNA in the chloroplast was only 1 7 times higher than cytoplasmic RNA

Samples of the chloroplast and cytoplasmic fractions were extracted with phenol and the RNA was fractionated on a sucrose gradient as described under Experimental In cells illuminated for 16 hr, over 90 per cent of the RNA from the chloroplast was ribosomal (sedimenting at 16S and 23S) and there was good agreement between the distribution of fractions absorbing at 260 nm and the radioactivity throughout the gradient On the other hand, centrifugation of the RNA from the cytoplasmic fraction in a sucrose gradient revealed that about 25 per cent of the absorbancy and almost 50 per cent of the radioactivity was in low molecular weight RNA (3S–5S) This is reflected in comparisons shown in Table 3 between the specific activities of ribosomal RNA from the chloroplast and cytoplasmic fractions During the early stages of chloroplast development, the ratio of specific activity of chloroplast ribosomal RNA to that of cytoplasmic ribosomal RNA was even higher than the corresponding ratio for total RNA (Table 3)

Although the chloroplast RNA had a consistently higher specific activity than the cytoplasmic RNA, it made up only a minor portion of the cellular RNA and the total radioactivity found in each chloroplast fraction isolated was about one-third of that incorporated by the whole cell

DISCUSSION

Under the standard experimental conditions employed in these studies, illumination of dark-grown cells of *Euglena gracilis* resulted in a rapid development of chloroplasts, but there was no appreciable change in the total RNA of the cells Zeldin and Schiff[3] and Brawerman and Chargaff[6] also observed no change in total RNA during greening of *E gracilis* although net increases in RNA of 30–50 per cent have been reported in other papers [2,7] The differences may be related to the extent to which the experimental conditions used were conducive to cell division A medium rich in nitrogen and yeast extract supported an increase in cellular RNA,[7] but a medium lacking these components did not [6] In our experiments the medium contained a nitrogen source and no carbon substrate but the low light intensity (optimal though for chloroplast development) and high cell density used excluded cell division In these cells chloroplast RNA reached a maximum by 16 hr of illumination, but accounted for only 7 per cent to the total cellular RNA This percentage was more than doubled in growing autotrophic cells or in greening cells at a low cell density so that the cells divided after chloroplasts were formed This additional chloroplast RNA then is not essential for the development of existing chloroplasts but may be synthesized prior to chloroplast division

Even under experimental conditions where no cell division took place, light nevertheless stimulated the incorporation of orotic acid into both chloroplast and cytoplasmic RNA (Table 3) Zeldin and Schiff[3] also found that light stimulated the uptake of ^{32}P into cytoplasmic RNA during chloroplast development in *E gracilis* In our studies, the effect of light on the incorporation into RNA was confined to the early stages of chloroplast development (Fig 3, Table 3) Between 32 and 72 hr of illumination there was little difference in the rate of incorporation in illuminated and non-illuminated cultures, although during this

[6] G BRAWERMAN and E. CHARGAFF, *Biochim Biophys Acta* **31**, 164 (1959)
[7] G. BRAWERMAN, A O. POGO and E CHARGAFF, *Biochim Biophys Acta* **55**, 326 (1962).

period in the light chlorophyll synthesis continued (Fig 1) After 16 hr of illumination the total RNA in the chloroplast had four times the specific activity of that in the cytoplasm and moreover the activity of chloroplast ribosomal RNA was five times higher than the cytoplasmic ribosomal RNA. After 24 hr of illumination, the chloroplast ribosomal RNA was seven times more highly labelled than the cytoplasmic ribosomal RNA Developing chloroplasts show a rapid increase in size between 12 and 24 hr of illumination[8] and this would seem to be accompanied by a rapid increase in amount of chloroplast RNA (Table 1) and by a high incorporation of precursor on a specific activity basis (Table 3) It is not known to what extent this incorporation is affected by a change in precursor pool size or by ribonuclease activity during this period.

The importance of RNA synthesis in the early stages of illumination is consistent with the effects of 5-fluorouracil This compound inhibits chloroplast development only when added during the first 14 hr of illumination [4]

The maintenance of a high level of incorporation of ^{14}C-orotic acid into cytoplasmic RNA during the early stages of chloroplast development (Table 3, Fig 3) coincides with the increase in the synthesis of some enzymes localized in the cytoplasm that may be important in providing energy for chloroplast development [9,10] Thus the early stages of chloroplast development may also involve mobilization of the cytoplasmic protein synthesizing system

The cytoplasmic protein synthesizing system may be active in chloroplast development in other ways Some chloroplast proteins may be synthesized in the cytoplasm and then transferred to the chloroplasts[9] and this may be true of certain lipids and other non-protein components of chloroplasts There is some evidence from the effects of chloramphenicol and cycloheximide upon the increase in phospholipids which accompanies chloroplast development in *E gracilis* that this increase occurs as the result of synthesis in the cytoplasm.[11]

EXPERIMENTAL

Cell cultures *Euglena gracilis* strain Z was grown in the dark in the basal medium described by Hutner *et al* [12] and supplemented with vitamin B_{12} (5 μg/l) In order to study chloroplast development, the dark-grown cells were resuspended in a carbon-depleted medium (growth medium from which all carbon compounds except EDTA and vitamins had been omitted) to a density of 3–8 $\times$ 10^6 cells per ml, shaken in the dark for 2 or 3 days and then exposed to white fluorescent light (1500 lx) The atmosphere was 5% CO_2 in air and the temperature was 25°

Autotrophic cells were grown in the carbon-depleted medium at a light intensity of 7000 lx The cultures were shaken in 5% CO_2 in air at 25°

Preparation of chloroplast and cytoplasmic fractions Chloroplasts were prepared as previously described[13] with a yield of 20–50% (determined by recovery of chlorophyll) The chloroplasts formed in cells that had been exposed to light for only 12 hr were small[8] and the isolation method was modified as follows The cells were broken at 8800 p s i in a French Pressure Cell and centrifuged at 200 *g* for 2 min to remove paramylon granules The chloroplasts were collected at 10,000 *g* for 15 min and separated from the cell debris by flotation on 58% (w/v) sucrose

The cytoplasmic fraction was obtained from the cell homogenate after removal of the chloroplasts and other particulate material by centrifugation at 20,000 *g* for 30 min

Extraction, estimation and determination of radioactivity in RNA Total RNA was extracted with KOH

[8] Y Ben-Shaul, J A. Schiff and H T Epstein, *Plant Physiol* **39**, 231 (1964)
[9] R M Smillie, D Graham, M R Dwyer, A Grieve and N F Tobin, *Biochem Biophys Res. Commun.* **28**, 604 (1967)
[10] M R Dwyer and R M. Smillie, *Biochim Biophys Acta* **216**, 392 (1970)
[11] D G Bishop and R. M Smillie, *Arch Biochem Biophys*, **139**, 179 (1970)
[12] S H Hutner, M K Bach and G I M Ross, *J Protozool* **3**, 101 (1956)
[13] N S Scott, V C Shah and R M Smillie, *J Cell Biol* **38**, 151 (1968)

and estimated in triplicate as described by Smillie and Krotkov [14] The concentration of RNA was measured using $E^{1cm\ 1\mu g/ml}_{260nm} = 0.031$ (calculated from the base composition of *Euglena gracilis* RNA reported by Eisenstadt and Brawerman[15])

Undegraded RNA was extracted with phenol as previously described [5] The yield varied from 50 to 80 per cent

Before determining the radioactivity incorporated into this RNA, small molecular weight contaminants were first removed by precipitating the RNA with cetyltrimethylammonium bromide[16] at a concentration of 2–5 mg per mg RNA After this treatment over 95 per cent of the remaining radioactivity was solubilized by ribonuclease (10 μg/ml, 30° for 30 min) or KOH (0 3 M, 90° for 15 min) In different experiments, from 85 to 100 per cent of the radioactivity was able to be precipitated by cold 5% trichloracetic acid

Samples of RNA were either plated on glass fibre filters and counted in toluene scintillant (3 g PPO and 0 2 g POPOP per litre of toluene) or made to 1 0 ml with water and counted in 10 ml of Triton–toluene scintillant (2 vol toluene scintillant and 1 vol Triton X-100) All samples were counted in a Packard Liquid Scintillation Spectrometer, Model 3375, at 75 to 80 per cent efficiency for ^{14}C and 30% for ^{3}H

Analyses Sucrose density gradients[17] of 12–24% bentonite-treated sucrose in 0 1 M sodium acetate buffer, pH 5, 0 05 M NaCl and 0 0001 M $MgCl_2$ were overlaid with RNA which had been precipitated with cetyltrimethylammonium bromide and were centrifuged in an SW39 rotor on a Spinco Model L centrifuge for 16 hr at 30,000 *g* Each gradient was analysed with a continuous flow cell in a Shimadzu MPS-50L recording spectrophotometer and 30 fractions were collected for measurement of radioactivity

RNA (15–30 μg) was separated on 2 6% polyacrylamide gels[18] by electrophoresis at 5 ma/gel for 12 hr After soaking in water, the gels were scanned at 260 nm The positions of 23S and 16S ribosomal RNA were established by comparison with ribosomal RNA from *E coli*, and the amount of RNA in the individual peaks was determined by planimetry The 16S ribosomal RNA (Fig 2b) is characteristic of chloroplasts, constituting one-third of the chloroplast RNA,[5] and does not appear in cytoplasmic RNA preparations (Fig 2c) Thus, where necessary a correction for contamination of chloroplast fractions with cytoplasmic RNA was calculated from the proportion of 16S RNA in a fraction

Chlorophyll was estimated by the method of Arnon [19]

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[14] R M SMILLIE and G KROTKOV, *Can J Bot* **38,** 31 (1960)
[15] J M EISENSTADT and G BRAWERMAN, *J Mol Biol* **10,** 392 (1964)
[16] R K RALPH and A R BELLAMY, *Biochim Biophys Acta* **87,** 9 (1964)
[17] H NOLL, *Nature, Lond* **215,** 360 (1967)
[18] U E LOENING, *Biochem J* **102,** 251 (1967)
[19] D I ARNON, *Plant Physiol* **24,** 1 (1949)

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