THE EARLY SYNTHESIS AND POSSIBLE FUNCTION OF A 0.5 x 10^6 M_r RNA AFTER TRANSFER OF DARK-GROWN SPIRODELA PLANTS TO LIGHT

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SUMMARY. A 0.5 x 10^6 M_r RNA found in plastids of the aquatic angiosperm Spirodela, is synthesized at a much higher rate than any other rapidly labeling RNA species about 3-3½ h after dark-grown plants are transferred to light. The pulse labeling kinetics of the 0.5 x 10^6 M_r RNA after transfer to light, argue against its involvement in the biogenesis of plant rRNAs. Although poly(A) RNA is found in Spirodela, poly(A) sequences are not detected in the 0.5 x 10^6 M_r RNA; yet a sucrose gradient fraction which includes RNA of this M_r stimulates amino acid incorporation by an E. coli cell free extract more than other RNA fractions. The possible involvement of the 0.5 x 10^6 M_r RNA as a chloroplast messenger is discussed.

A 0.45 - 0.50 x 10^6 M_r RNA obtained from whole cell RNA of plants was for a number of years suggested to be an undegraded cleavage product of the nucleocytoplasmic (n.c.) r preRNA (1,2,3). The 0.45 - 0.50 x 10^6 M_r (0.5 x 10^6 M_r) RNA was found to be undermethylated in Spirodela (3). More recently, 0.45 - 0.50 x 10^6 M_r RNA was localized in isolated chloroplasts (4,5,6) and such isolated organelles were able to synthesize this RNA species in vitro (4). Another type of 0.45 - 0.50 x 10^6 M_r RNA has also been reported to be a part of the break-down products of heavy (1.05 x 10^6 M_r) chloroplast rRNA (7,8,9).

We now report differences in the rates of [5-^{3}H]uridine incorporation into rapidly labeling RNAs of axenically cultured dark-grown Spirodela plants under the influence of light. Our kinetic evidence shows that the 0.45 - 0.50 x 10^6 M_r RNA of Spirodela clearly does not belong to the family of rRNAs; however it may have properties expected from a chloroplast messenger.

MATERIALS AND METHODS

The axenic culture and labeling of Spirodela oligorrhiza (Kurtz) Heglem. (Duckweed), the deproteinization and DNAase treatments of nucleic acids, polyacrylamide gel electrophoresis of purified RNA, gel scanning, slicing, radioactivity and apparent molecular weight (M_r) determinations were as previously described (2,10). Dark-grown plants had the same culture conditions but were grown in light-tight boxes and transferred under dim green light of about 1 foot candle. Chloroplasts were isolated as described earlier (4,6).

RNA with polyadenylic acid sequences (Poly(A) RNA) was separated from bulk RNA by oligo(dT)-cellulose chromatography (11) by applying about 50 A_{260} units of whole cell RNA dissolved in application buffer (0.01 M Tris·HCl (pH 7.5)-0.5 M KCl) to the oligo(dT) column, and eluting the poly(A) RNA which was retained

by the column with 0.01 M Tris·HCl (pH 7.5). The poly(A) RNA fraction which was brought to a final concentration of 0.5 M NaCl, as well as the rest of the RNA which had not been adsorbed to the column, were precipitated with ethanol for polyacrylamide gel electrophoresis. The base composition of poly(A) segments (12) of RNAse treated, oligo(dT) bound RNA was determined by high voltage paper electrophoresis of the alkaline hydrolysate of this RNA which had been obtained from plants grown in 1/20 normal concentration of phosphate and labeled for 1 hr in $^{32}P_i$ (100 μCi/ml).

RESULTS AND DISCUSSION

The rapidly labeling RNA species from light and dark grown plants. Chloroplasts which were isolated from light grown plants of Spirodela labeled for 2 h with [5-^{3}H] uridine, contained radioactivity in RNAs of 2.7, 2.3(?),1.2,0.75 and 0.5 x 10^6 M_r above the polydisperse RNA (6). The rapidly labeling RNA species which could be separated from whole cell RNA of light-grown plants which were labeled as above but for only 45 min, are shown in Fig. 1A. These included the 2.0-2.4, 1.4 and 1.0 x 10^6 M_r n.c. r preRNAs (1,2,9,13) and the 0.7 x $10^6 M_r$ n.c. light rRNA, in addition to those chloroplast RNAs (1.2 and 0.5 x $10^6 M_r$) which could be resolved in gels of whole cell RNA after a very short label.

The radioactivity patterns of the rapidly labeling RNAs of whole cell RNA from light and dark grown plants were very similar (Figs. 1A and B), except that the amount of [5-^{3}H] uridine incorporation was reduced by more than 90% in dark-grown plants. This is consistent with earlier data of reduced chloroplast rRNA synthesis in dark-grown plants (8,14). The equivalent reductions of incorporation into 0.5 x 10^6 M_r and all other rapidly labeling RNAs in the dark (Fig. 1B), does not resolve the question whether the 0.5 x 10^6 M_r RNA belongs to the family of n.c. (1, 13) or chloroplast rRNAs (15) or whether it is completely unrelated to the ribosomal RNA species.

The incorporation of [5-^{3}H] uridine into RNA after transfer of dark-grown plants to light. When we exposed dark-grown plants for 3½ h to light and then pulse labeled for 45 min only, we observed a marked increase in the [5-^{3}H] uridine incorporation into 0.5 x 10^6 M_r RNA (Fig. 1C). During such a short pulse there is very little processing to n.c. or chloroplast rRNA which would ordinarily conceal many rapidly labeling RNA species (2). It can be seen from Table 1 that the ratio cpm after 3½ h light/cpm in continuous dark is 10x greater for the 0.5 x 10^6 M_r RNA, than the ratios of any of the other RNA species which can be resolved in whole cell RNA under these conditions. It is also clear that the increase in incorporation into all other RNA species is minimal.

Pulse labeling of 0.5 x 10^6 M_r RNA at various times after transfer of plants from dark to light. Two time course experiments on the incorporation into various RNA species during 45 min of label in light, ½-23 h after transfer of dark-grown

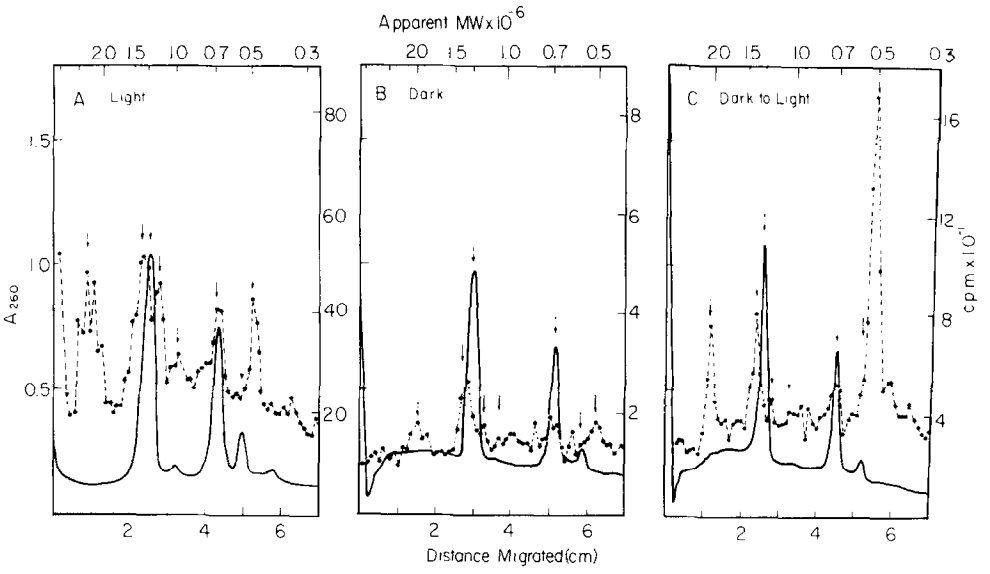

Fig. 1. The effect of light on the rate of synthesis of 0.5 x 10^6 $\underline{M}_r$ RNA. Plants were labeled with [5-^{3}H] uridine, (100 µC/ml) for 45 min: A. light-grown; B. dark-grown; C. 3½ h after transfer of dark-grown plants to light. Whole cell RNA was fractionated on polyacrylamide gels as described in Methods. ---●---●--- ^{3}H; ———— A_{260}.

Table 1. Relative increase in incorporation of [5-^{3}H] uridine into the various RNA fractions 3½ h after transfer of dark-grown plants to light*

	RNA fractions (apparent $\underline{M}_r$ x 10^{-6})						
	2.3	1.4	1.2	0.7	0.5	Polydisperse	Total
	cpm after 3½ h light / cpm in dark						
$\bar{X}$:	1.10	0.85	1.59	1.06	10.49	1.38	1.58
$S_{\bar{x}}$** :	0.26	0.14	0.41	0.20	1.47	1.14	0.06

* Based on data of four separate experiments with identical conditions to those of Fig. 2B and C.

** $S_{\bar{x}} = \sqrt{\frac{\Sigma(X-\bar{X})^2}{(n-1)n}}$; n = 4 except in 1.2 x 10^6 $\underline{M}_r$ where n = 3.

plants to light, are summarized in Fig. 2. The relative rate of incorporation per unit of molecular weight of 0.5 x 10^6 $\underline{M}_r$ RNA increased 1.5 h after transfer to light, reached a maximum at 3.0-3.5 h and decreased again between 3.5 and 9 h. The total incorporation into 4 other RNA species (Fig. 2A) which could be resolved within 45 min of label during continuous light, represented only 15%

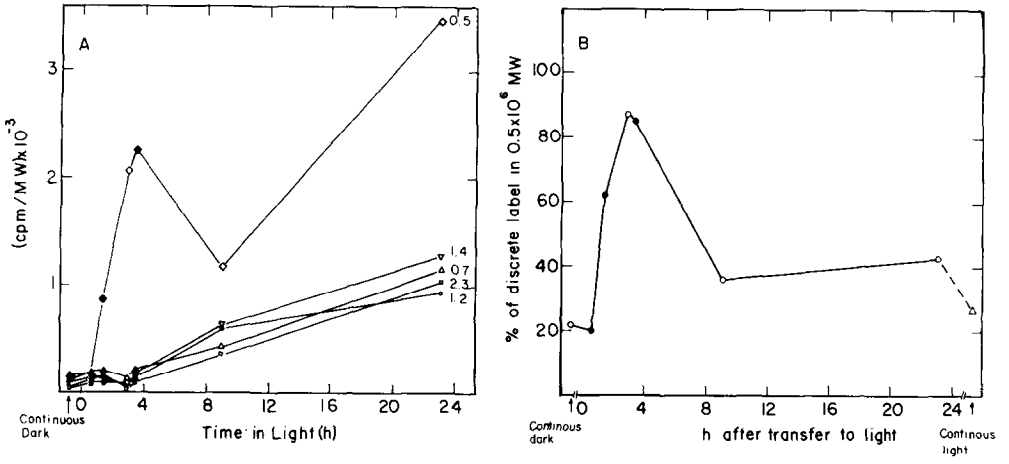

Fig. 2. The time course of relative pulse labeling of the 0.5 x 10^6 M_r RNA after transfer of dark-grown plants to light. Samples of 100 dark-grown plants were labeled with [5-^{3}H] uridine (100 µC/ml) for 45 min in the dark as well as at various times from 30 min to 23 h after transfer to light. Whole cell RNA was extracted and the total incorporated radioactivity of each of the discrete RNA species from polyacrylamide gels was integrated and then divided by the M_r to get A: the relative number of labeled molecules. B: the percent of the total number of these molecular weight units in 0.5 x 10^6 M_r. The continuous light point was obtained from a different experiment (Fig. 1A). Open and closed symbols represent separate experiments.

of the incorporation per unit M_r 3 h after transfer to light (Fig. 2B); the remaining 85% of radioactivity was in 0.5 x 10^6 M_r RNA (Fig. 2B). Incorporation into the 2.3, 1.4, 1.2 and 0.7 x 10^6 M_r RNAs increased gradually only after 3½ h of light at a much lower rate per unit molecular weight than the 0.5 x 10^6 M_r RNA; this difference in the rate of incorporation was maintained at least until 23 h (Fig. 2A). The 0.5 x 10^6 M_r RNA may not have quite reached the steady state light level within the time of the experiments (Fig. 2B). Results identical to those of Fig. 2 have been reported.

These results show clearly that the 0.5 x 10^6 M_r RNA must be synthesized independently of all the nucleo-cytoplasmic and chloroplast rRNAs and their precursors. The rRNA species appear much later than the 0.5 x 10^6 M_r RNA after transfer of plants from dark to light, with separate kinetics.

The timing of the wave of increased incorporation into the 0.5 x 10^6 M_r RNA during greening is interesting as it occurs so early (Fig. 2). The dark-grown Spirodela plants are colorless and are selected from young fronds which develop and continue to reproduce indefinitely without illumination but with sucrose and minerals. When such dark-grown plants are transferred to light, they begin to green about 24 h later (8). The wave of a relatively high rate of 0.5 x 10^6 M_r RNA synthesis (Fig. 2) shortly after the transfer of dark-grown plants to light, may reflect a certain degree of synchrony of the development of etioplasts (16) to chloroplasts. Such synchrony has been reported in dark-grown seedlings of

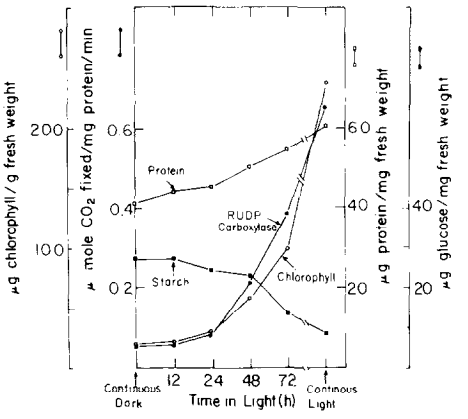

Fig. 3. Changes in the activity of RUDP carboxylase and in the amounts of chlorophyll, soluble proteins and starch after illumination of dark-grown plants. Samples of 100 mg fresh weight were homogenized in 1 ml buffer (50 mM HEPES, 1mM EDTA, 1 mM dithiothreitol (DTT), pH 8) at various times after transfer of dark-grown plants to light. After centrifugation at 12,000 x g for 10 min, the pellet was used to measure starch (22). The supernatant was used to determine the level of soluble proteins (23) and the activity of RUDP carboxylase (24). Chlorophyll was extracted with 80% acetone from 10 mg fresh weight and its amount was determined (25).

Phaseolus (17,18) and in *Euglena* (19) after transfer to light.

Could the 0.5 x 10^6 M_r RNA be a chloroplast mRNA? - Poly(A) RNA and size distribution of RNAs with template activity? Spinach chloroplast RNA contains mRNA activity which has been used in the *in vitro* translation of what seems to be the large subunit of RUDP carboxylase (fraction I protein) in an *E. coli* cell free protein synthesizing system (20). In *Spirodela* there is a lag of about 24 h after transfer of dark-grown plants to light, until both chlorophyll is formed and the activity of the plastid located enzyme RUDP carboxylase increases (Fig. 3). The whole cell soluble protein level increases somewhat earlier. Siddell and Ellis (21) detect protein synthesis in plastids 3 h after onset of illumination of etiolated pea seedlings. It is therefore conceivable that the early labeling 0.5 x 10^6 M_r RNA signifies the increased synthesis of mRNA of one of the proteins essential for normal development and functioning of the chloroplasts after illumination of dark-grown plants.

It had been shown earlier that 0.5 x 10^6 M_r RNA of *Spirodela* was undermethylated (3), raising the question whether this RNA species contains poly(A) sequences. Pulse-labeled whole cell RNA from light-grown plants which had an electrophoretic pattern similar to that shown in Fig. 1A, was applied to an oligo(dT)-cellulose column (11). The polyacrylamide gels of the unbound RNA which passed through the column, contained approximately the same 0.5 x 10^6 M_r

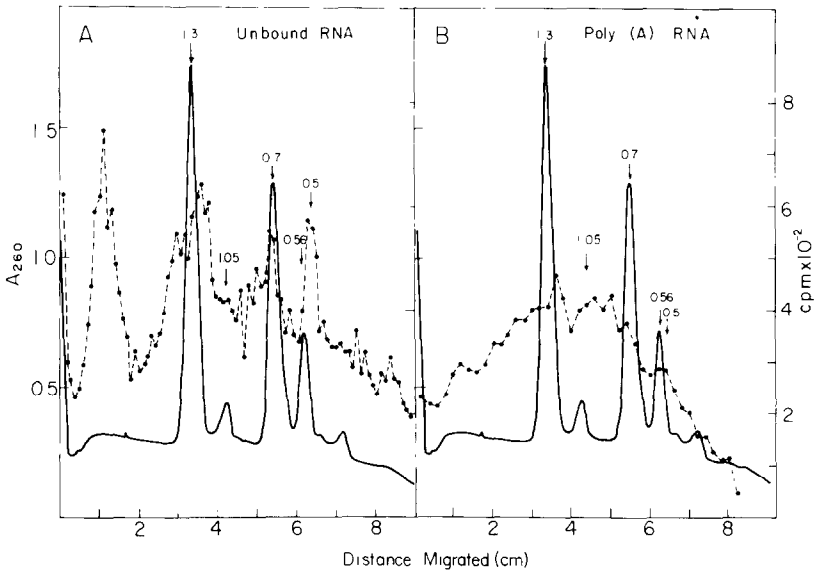

Fig. 4. Oligo(dT) binding of Spirodela RNA. The RNA of 500 light-grown plants, labeled for 1 h with [5-^{3}H] uridine (100 µC/ml), was applied to an oligo(dT) cellulose column (11); the unbound RNA (A) and the bound poly(A) RNA (B) portions were fractionated simultaneously on polyacrylamide gels and the radioactivity of B was superimposed on the A_{260} scan of A. ---●---●--- ^{3}H; ——— A_{260}.

radioactivity (724 cpm, Fig. 4A) as that which had been added to the column (665 cpm; gel not shown). The same was true for the rapidly labeling RNAs of the nucleo-cytoplasmic and chloroplast rRNA systems. Although the binding of Spirodela RNA to both oligo(dT) and millipore filters is rather low (about 1% of A_{260}) sufficient radioactive RNA counts bound to the column. This characteristic heterogeneous poly(A) RNA had a polydisperse distribution with no distinct peak in the 0.5 x 10^6 M_r region of the gel (Fig. 4B); the same was true in a gel of poly(A) RNA (not shown) obtained after rebinding the unbound fraction (Fig. 4A) to the oligo(dT) cellulose column at 4°C (26). The eluate of $^{32}P_i$ pulse labeled whole cell RNA which had been bound to the oligo(dT)-cellulose column after DNAase and RNAase digestion contained 76 mole % adenylic acid using the methods outlined in (12).

The fact that poly(A) sequences were not detected in the 0.5 x 10^6 M_r RNA does not necessarily preclude it from being a messenger (26,27,28). Though chloroplasts contain mRNA activity (20), no poly(A) sequences have so far been reported in RNA of this organelle. It seemed worthwhile therefore to pursue the possibility of finding template activity in a 0.5 x 10^6 M_r RNA-containing fraction of a sucrose gradient of Spirodela RNA. Whole cell RNA from light-

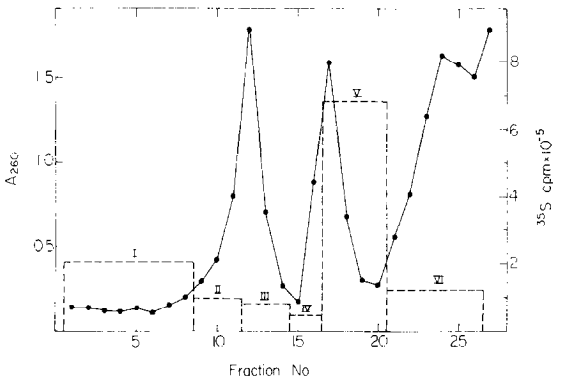

Fig. 5. The template activity of Spirodela RNAs. About 1 mg of whole cell RNA from light-grown plants was first layered on a 5-40% sucrose gradient containing 0.1 M NaCl, 10 mM Tris·HCl pH 7, and 1 mM EDTA-Na_2, centrifuged in an SW 25 Spinco rotor at 23,000 rpm for 20 h at 4°C and the various RNA fractions which were pooled as indicated in the figure, were then precipitated in ethanol, redissolved and incubated (15-25 µg RNA per fraction) in a modification of the amino acid incorporating system described by Capecchi (29) and Hartely et al. (20). This contained in a 50 µl reaction mixture: 10 µl preincubated S 30 extract from E. coli (30),2.5 µmoles Tris HCl·pH 7.8, 0.5 µmoles Mg acetate, 2.0 µmoles NH_4Cl, 0.25 µmoles phosphoenolpyruvate (PEP), 0.15 µmoles ATP, 0.1 µmoles GTP, 0.002 µmoles each of a mix of 19 amino acids (minus methionine), 0.25 µmoles DTT, 5 µg leucovorin (folinic acid) - purchased from Lederle, 0.25 µg PEP kinase and 2 µCi [^{35}S] methionine (250 Ci/mmole). The reaction mixture was incubated for 20 min at 35°C. Aliquots were dried on filter paper discs, washed in cold TCA, then heated to 90°C, washed as described by Roberts and Paterson (31) and counted. ——●——●——, A_{260}; -----[^{35}S] methionine cpm incorporated per pooled RNA fraction(A_{260}) of the sucrose gradient.

grown plants was fractionated on a sucrose gradient. The bulked fractions delineated by the histogram of Fig. 5 were used as probes for template activity. Incorporation of [^{35}S] methionine into TCA precipitable products was determined after incubation of each of the bulked RNA fractions with an E. coli S30 extract (29,30,31). Maximum incorporation was obtained with RNA fraction V (Fig. 6) containing the 0.5 x 10^6 M_r species. This suggests two possible interpretations: 1. The different mRNAs which can be translated by the E. coli S30 extract may have a polydisperse size distribution which fortuitously happens to have a peak of template activity in this molecular weight region. 2. The major mRNA translated in the E. coli system could be the 0.5 x 10^6 M_r RNA of the chloroplast. We prefer the second interpretation: the product of the cell free translation could conceivably be the large subunit of RUDP carboxylase which appears to be quantitatively the main protein which is translated in chloroplasts in vivo and in vitro (20,32). The molecular weight of the large subunit of RUDP carboxylase has been reported as 52,000 in spinach chloroplasts (20) and 59,000 in chloroplasts of Euglena (24). The translation of these proteins would require RNA

templates of 0.38 and 0.43 x 10^6 M_r respectively. These calculated template molecular weights are somewhat smaller than the RNA discussed in this paper, which has a range of apparent molecular weights from 0.45 - 0.50 x 10^6.

An approximately 60,000 M_r product which corresponds to the M_r of the large subunit of Spirodela RUDP carboxylase has been translated with an S30 E. coli extract (Sagher, Edelman, Rosner, Gressel and Jakob, in preparation). RNAs from whole cells as well as from isolated chloroplasts of Spirodela were used as templates confirming the work of Hartley et al (20). However direct evidence that the 0.5 x 10^6 M_r RNA is the messenger of the 60,000 M_r product and that this product is indeed the large subunit of the RUDP carboxylase is not yet available.

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REFERENCES

1. Grierson, D. and Loening, U.E. (1972). Nat. New Biol. 235, 80-82.
2. Rosner, A., Posner, H.B. and Gressel, J. (1973). Plant Cell Physiol. 14, 555-564.
3. Posner, H.B., Gressel, J. and Rosner, A. (1974). Plant Cell Physiol. 15, 807-811.
4. Hartley, M.R. and Ellis, R.J. (1973). Biochem. J. 134, 249-262.
5. Grierson, D. and Loening, U.E. (1974). Eur. J. Biochem. 44, 501-507.
6. Posner, H.B. and Rosner, A. (1975). Plant Cell Physiol. 16, 361-365.
7. Leaver, C.J. and Ingle, J. (1971). Biochem. J. 123, 235-243.
8. Rosner, A., Porath, D. and Gressel, J. (1974). Plant Cell Physiol. 15, 891-902.
9. Grierson, D. (1974). Eur. J. Biochem. 44, 509-515.
10. Gressel, J., Rosner, A. and Cohen, N. (1975). Anal. Biochem. In press.
11. Aviv, H. and Leder, P. (1972). Proc. Nat. Acad. Sci. U.S. 69, 1408-1412.
12. Sagher, D., Edelman, M. and Jakob, K.M. (1974). Biochim. Biophys. Acta 349, 32-38.
13. Loening, U.E., Grierson, D. and Gressel, J. (1972). Symp. Biol. Hung. 13, 193-199.
14. Detchon, P. and Possingham, J.V. (1973). Biochem. J. 136, 829-836.
15. Bohnert, H.J., Schmitt, J.M. and Herrmann, R.G. (1975). 10th Meet. Fed. Europ. Bioch. Societies Abst. 152.
16. Kirk, J.T.O. and Tilney-Basset, R.A.E. (1970). The Plastid, pp. 64-70, W.H. Freeman, London.
17. Gyldenholm, A.O. (1968). Hereditas 59, 142-168.
18. Bradbeer, J.W. (1969). New Phytol. 68, 233-245.
19. Heizmann, P. (1974). Proc. 3rd Intern. Cong. Photosynth. pp. 1745-1754, Avron, M., ed., Elsevier, Amsterdam.
20. Hartley, M.R., Wheeler, A. and Ellis, R.J. (1975). J. Mol. Biol. 91, 67-77.
21. Siddell, S.G. and Ellis, R.J. (1975). Biochem. J. 146, 675-685.
22. McCready, R.D., Guggolz, J., Silviera, V. and Owens, H.S. (1950). Anal. Chem. 22, 1156-1158.
23. Lowry, O.H., Rosenbrough, N.J., Farr, A.L. and Randall, R.J. (1951). J. Biol. Chem. 193, 265-275.
24. Rabinowitz, H., Reisfeld, A., Sagher, D. and Edelman, M. (1975). Plant Physiol. In press.
25. Arnon, D.I. (1949). Plant Physiol. 24, 1-15.

26. Nakazato, H., Venkatesau, S. and Edmonds, M. (1975). Nature 256, 144-146.
27. Perry, R.P., Kelley, D.E. and LaTorre, J. (1972). Biochem. Biophys. Res. Commun. 48, 1593-1600.
28. Adesnik, M. and Darnell, J.E. (1972). J. Mol. Biol. 67, 397-406.
29. Capecchi, M.R. (1966). J. Mol. Biol. 21, 173-193.
30. Nirenberg, M.W. (1963). In: Methods in Enzymology 6, 17-23. Colowick and Kaplan, eds., Academic Press, New York.
31. Roberts, B.E. and Paterson, B.M. (1973). Proc. Nat. Acad. Sci. U.S. 70, 2330-2334.
32. Blair, G.E. and Ellis, R.J. (1973). Biochim. Biophys. Acta 319, 223-234.