RNA SYNTHESIS IN ISOLATED CHLOROPLASTS: CHARACTERISATION OF THE NEWLY SYNTHESISED RNA

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1. Introduction

We have recently described RNA synthesis in isolated *Euglena* chloroplasts, and have demonstrated that, in this system, some of the newly synthesised material co-electrophoresed with the 23 S and 16 S chloroplast rRNA's [1]. On this basis, it was suggested that chloroplast ribosomal genes may be transcribed in the isolated chloroplast. Subsequent work in this laboratory, using finer analytical techniques, showed that part of the newly synthesised RNA first appears as peaks with slightly lower electrophoretic mobilities than those of the 23 S and 16 S chloroplast rRNA's.

A recent paper by Munsche and Wollgiehn [2] has indicated that, after pulse labelling *in vivo* with ^{32}P, chloroplasts from the leaves of *Nicotiana* contain labelled RNA molecules with molecular weights of 1.3×10^6 daltons. Actinomycin D chase- and long-labelling conditions revealed the later appearance of label in the 23 S and 16 S chloroplast rRNA's, which have molecular weights of 1.1×10^6 and 0.56×10^6 daltons respectively. On this evidence these authors suggested that the synthesis of chloroplast rRNA in *Nicotiana* proceeds via intermediates which are larger than the final products by about 20%.

Our observations are also consistent with this interpretation. In this communication we present the results of these experiments on isolated *Euglena* chloroplasts, which indicate that the newly synthesised RNA first appears as molecules having approximate molecular weights of 1.2×10^6 and 0.7×10^6 daltons. Radioactivity in the region of the chloroplast rRNA's becomes evident only with longer labelling periods.

2. Methods

The growth of *Euglena gracilis* z and isolation of chloroplasts were performed according to techniques now standard in this laboratory [1, 3, 4]. RNA synthesis reactions and analysis of the products by sucrose gradient centrifugation and polyacrylamide gel electrophoresis have been described before [1], with the exception of the following modification. After the initial centrifugation of the chloroplast lysate through SDS-sucrose gradients, fractions containing the 23 S and 16 S rRNA's were pooled as indicated (fig. 1; cut I = '23 S fraction', cut II = '16 S fraction'), and precipitated overnight with carrier tRNA and 3 vol of ethanol. The '23 S fraction' was then electrophoresed on 2.4% polyacrylamide gels, and the '16 S fraction' on 2.8% gels.

3. Results

The use of separate gels to analyse the higher and lower molecular weight chloroplast RNA's permitted maximum resolution of closely similar RNA species in each size class. Analysis of the reaction products after incubation of isolated chloroplasts with [^{3}H]UTP for 2, 10 and 25 min is shown in fig. 2. It can be seen that label first appears in peaks with electrophoretic

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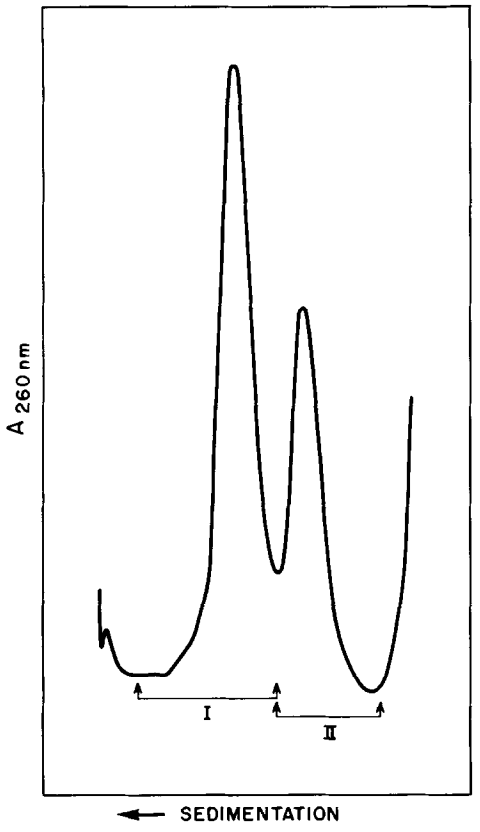

Fig. 1. Sucrose gradient centrifugation of chloroplast rRNA. After incubation of chloroplasts in the reaction mixture described in fig. 2, portions were brought to 2% SDS, 0.2 M NaCl and centrifuged at 2000 *g* for 10 min. The supernatant was then layered onto 15–30% sucrose gradients containing 0.5% SDS, .05 M Tris, pH 7.6, 0.2 M NaCl, and centrifuged for 16 hr at 24 000 rpm in the SW 27 rotor. Fractions were collected after passage through a continuous flow spectrophotometer at 260 nm and pooled as indicated (I = '23 S fraction'; II = '16 S fraction').

mobilities slightly lower than those of the main A_{265} peaks (figs. 2 A and D). The main absorbance peaks are the chloroplast rRNA's which co-migrate during electrophoresis with *E. coli* 23 S and 16 S rRNA's (not shown here). This similarity of electrophoretic behaviour implies identity of molecular size [5] and confirms previous molecular weight estimates of 1.1×10^6 and 0.56×10^6 daltons for the large and small chloroplast rRNA's, respectively, of *Euglena gracilis* [6]. The other A_{265} peaks on the 23 S gel are, from left to right, DNA, a possible RNA aggregate, and cytoplasmic rRNA (mol. wt. = 1.35×10^6 daltons [6]). That to the right of the 23 S peak has been discussed elsewhere [1], and is probably a cleavage product of the 23 S chloroplast rRNA. On the 16 S gels, the minor peaks are DNA, 23 S chloroplast rRNA and the cleavage product. Based on their relative electrophoretic mobilities, the peaks of newly synthesised RNA in figs. 2 A and D have molecular weights of 1.2×10^6 and 0.7×10^6 daltons respectively.

After 10 min of labelling, these molecules are still the predominant radioactive species, although definite shoulders are visible at 1.1×10^6 and 0.56×10^6 daltons (figs. 2 B and E). After 25 min, distinct peaks of radioactivity are associated with the 23 S and 16 S rRNA (fig. 2 C and F). In the case of the '23 S fraction' (fig. 2 C), the overall labelling profile reveals the accumulation of some heterogeneous, high molecular weight material. The nature of this RNA, which would have molecular weights of up to 4×10^6 daltons is not clear. However, we have occasionally noticed the appearance of these 'giant' molecules after long chases with cold UTP. They may, therefore, represent a tendency of the *in vitro* system to continue transcription beyond the normal termination sites.

The kinetics of the appearance of the labelled peaks are shown in fig. 3 b. It can be seen that the 1.2×10^6 and 0.7×10^6 daltons RNA's are synthesised rapidly for the first 10 min, but thereafter start to decay. This disappearance of label from these peaks seems to be correlated with the cessation of [^{3}H]UTP incorporation into total RNA (fig. 3 a). The 1.1×10^6 daltons peaks, on the other hand, accumulate slowly for the first 10 min, and rapidly thereafter, even though the incorporation of [^{3}H]UTP has slowed down.

4. Discussion

These results may be summarised in the following way. During the incubation of isolated chloroplasts with [^{3}H]UTP, RNA molecules with molecular weights of 1.2×10^6 and 0.7×10^6 daltons are rapidly synthesised. These molecules are unstable, and their breakdown may be observed as the incorporation reaction

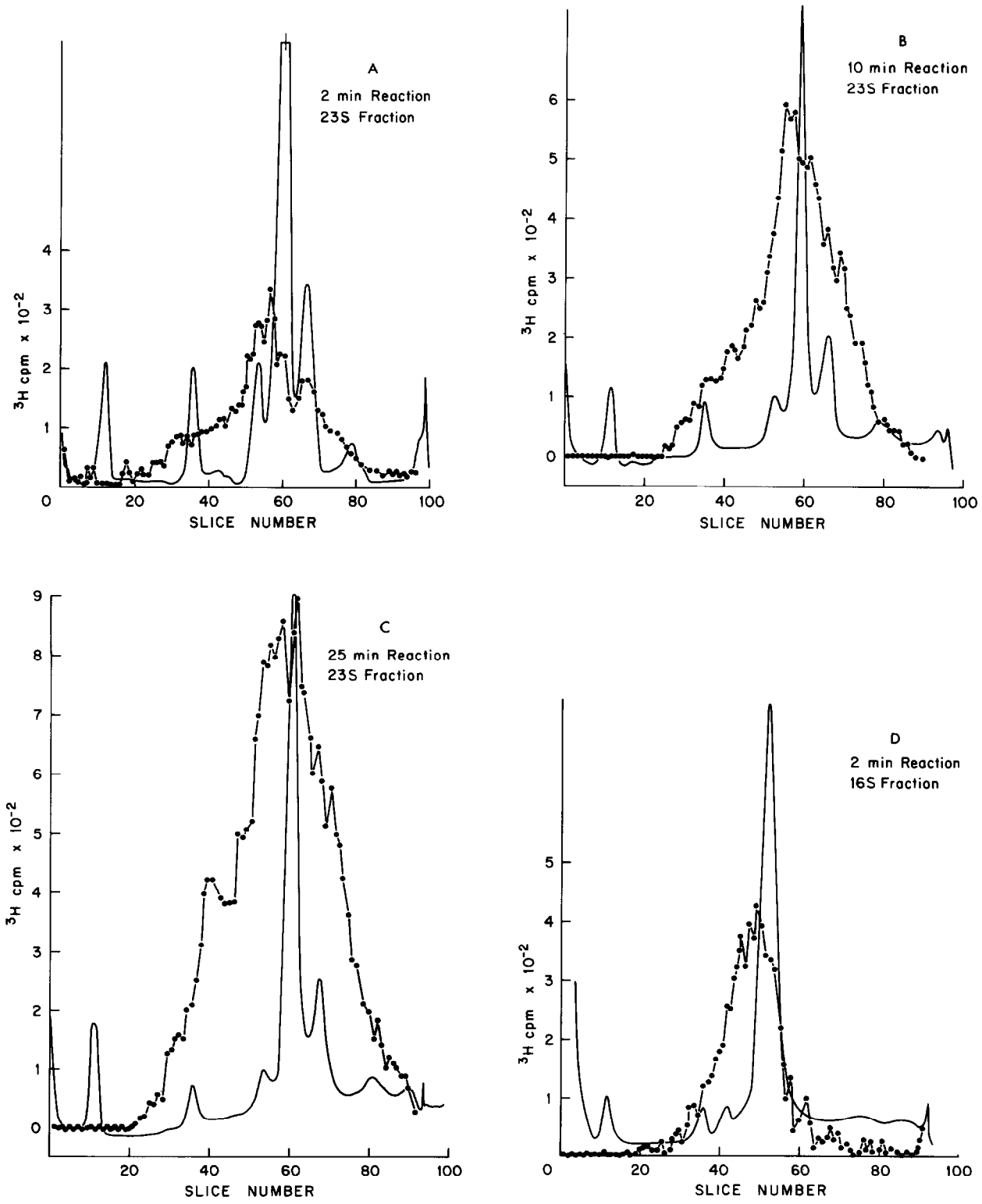

Fig. 2. Legend on opposite page under continuation of fig. 2E and F.

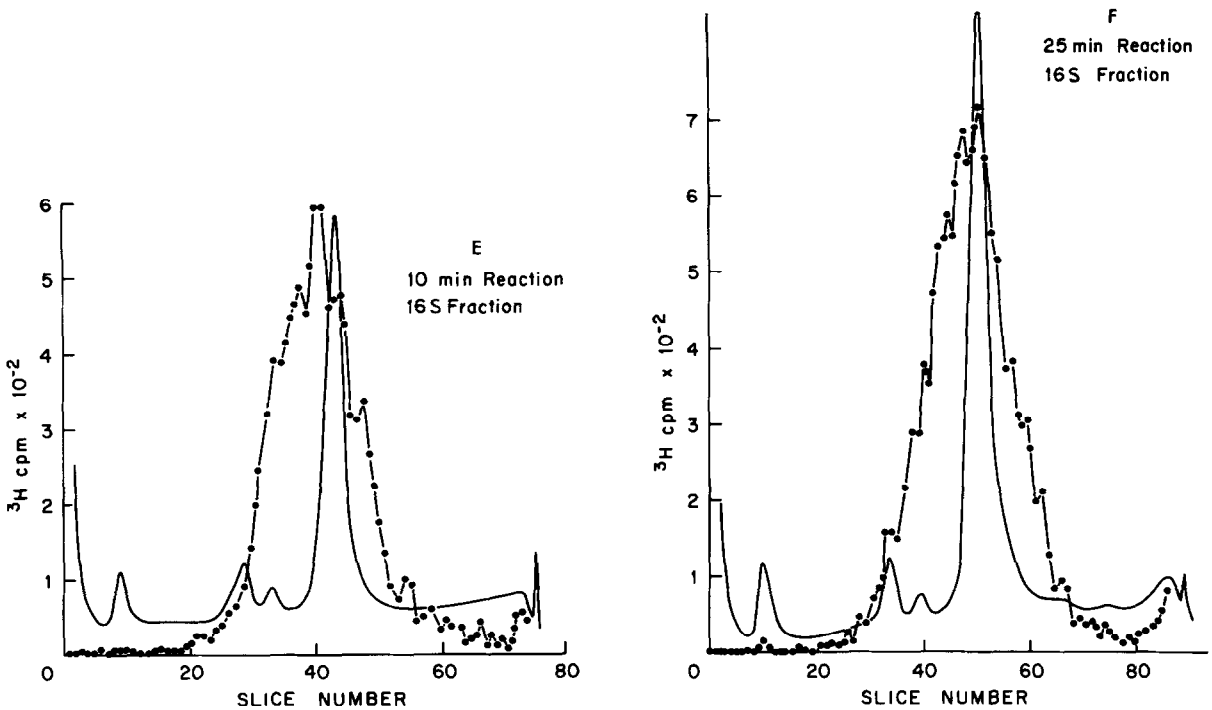

Fig. 2. Acrylamide gel electrophoresis of gradient fractions after incubation of chloroplasts with [^{3}H]UTP. Isolated chloroplasts were incubated in a reaction mix containing, per ml, 0.15 mmoles sucrose, 40 μmoles Tris–HCl, pH 7.8, 12 μmoles 2-mercaptoethanol, 20 μmoles $MgCl_2$, 0.4 μmoles each of ATP, GTP and CTP, 1 mg Macaloid, 10 μCi [^{3}H]UTP (SA. = 19.8 Ci/mmole). At the times indicated, 2 ml samples were removed, brought to 2% SDS, 0.2 M NaCl and centrifuged at 2000 *g* for 10 min. The supernatant was then layered onto SDS-sucrose gradients as described in fig. 1, and fractions I and II were pooled separately and precipitated with 3 vol of ethanol. The precipitates were collected, dissolved in electrophoresis buffer containing 5% sucrose and applied to acrylamide gels prepared as previously described [1]. Fraction I was electrophoresed for 5 hr on 2.4% gels, fraction II for 6 hr on 2.8% gels. Scanning, slicing and counting was performed as described previously [1]. (A–C) '23 S fraction': after 2 min A) 10 min B) and 25 min C) incubation *in vitro* with [^{3}H]UTP. (D–F) '16 S fraction': after 2 min D); 10 min E) and 25 min F) incubation. (——) A_{265} nm; (•—•) ^{3}H cpm.

comes to a stop. That this is not a fortuitous observation is suggested by the fact that these radioactive peaks have no counterparts in the A_{265} trace. As this metabolism proceeds, label can be seen to become associated with the A_{265} peaks of the 23 S and 16 S chloroplast rRNA's, which have molecular weights of 1.1×10^6 and 0.56×10^6 daltons respectively. These molecules appear most rapidly at a time when total RNA synthesis is slowing down and when radioactivity in the RNA's of 1.2×10^6 and 0.7×10^6 daltons is decreasing.

These observations might suggest that the appearance of label in the RNA's of 1.1×10^6 and 0.56×10^6 daltons is not strongly dependent upon RNA synthesis in the isolated chloroplast. This would imply that these molecules arise by cleavage of other molecules already labelled in these experiments. Such molecules would be expected to be larger than, and labelled prior to, the RNA's of 1.1×10^6 and 0.56×10^6 daltons, and to be in a state of active turnover. The RNA's with molecular weights of 1.2×10^6 and 0.7×10^6 daltons described in this paper exhibit such properties. Whether they can be regarded as being equivalent to the chloroplast rRNA precursors formed *in vivo* in *Nicotiana* [2] is at present a matter for conjecture.

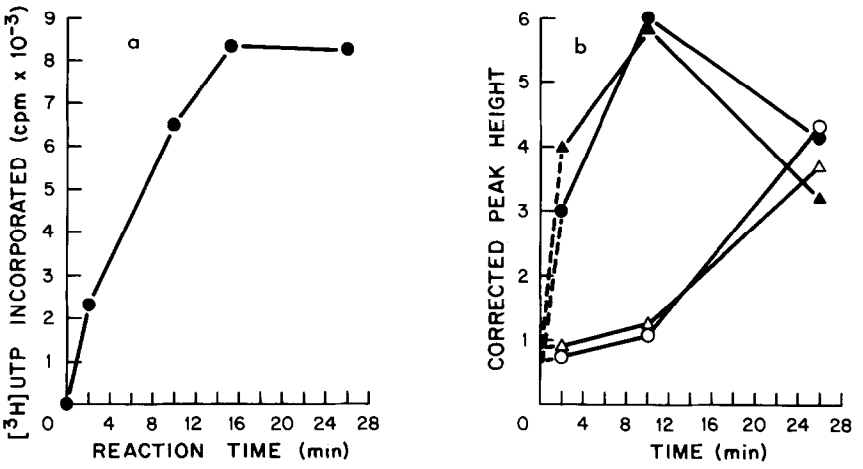

Fig. 3: a) Kinetics of [^{3}H]UTP incorporation into isolated chloroplasts. 0.03 ml samples from the reaction mix described in fig. 2 were applied to paper discs, precipitated with 10% trichloroacetic acid, and washed and counted as described previously [1]; b) Kinetics of labelling of chloroplast RNA's. The amount of radioactivity appearing in the various peaks in fig 2 was calculated in the following way. The recovery of RNA from the sucrose gradients was normalised with respect to the 23 S and 16 S absorbance peaks in fig. 2, and idealised curves, based on the radioactivity profiles, were generated for the 1.2×10^6, 1.1×10^6, 0.7×10^6 and 0.56×10^6 daltons fractions. The height of these peaks was measured and, after correction for overlap of neighbouring peaks, was expressed in arbitrary units. (▲---▲) 1.2×10^6 daltons peak; (△---△) 1.1×10^6 daltons peak; (● --- ●) 0.7×10^6 daltons peak; (○ --- ○) 0.56×10^6 daltons peak).

References

[1] Carritt, B. and Eisenstadt, J.M. (1973) European J. Biochem., in press.
[2] Munsche, D. and Wollgiehn, R. (1973) Biochim. Biophys. Acta 294, 106
[3] Preston, J.F., Parenti, F. and Eisenstadt, J.M. (1973) Planta 107, 351.
[4] Harris, E.H., Preston, J.F. and Eisenstadt, J.M. (1973) Biochemistry 12, 1227.
[5] Loening, U.E. (1969) Biochem. J. 113, 131.
[6] Rawson, J.R. and Stutz, E. (1969) Biochim. Biophys. Acta 190, 386.