Effect of the Protein-Synthesis-Initiation Inhibitor 2-(4-Methyl-2,6-dinitroanilino)-*N*-methylpropionamide on Ribonucleic Acid Synthesis in Radish Seedlings

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2-(4-Methyl-2,6-dinitroanilino)-*N*-methylpropionamide, an inhibitor of initiation of protein synthesis in plants, selectively alters rRNA synthesis. Ribosomal genes are transcribed in the absence of protein synthesis, but processing of the transcripts is impaired. This results in an accumulation of the 2.3×10^6 -dalton pre-rRNA. Synthesis of polyadenylated RNA is not affected by the drug.

We have reported that cycloheximide profoundly alters rRNA synthesis and has no effect on polyadenylated RNA synthesis in radish seedlings when used at a concentration of $50 \mu\text{g/ml}$ (Delseny *et al.*, 1977). We also demonstrated that much lower concentrations ($0.5 \mu\text{g/ml}$), which still completely inhibit protein synthesis, do not block the incorporation of [^3H]uridine into RNA (Delseny *et al.*, 1977). This observation casts some doubt on the concept that rRNA synthesis, in eukaryotes, is controlled by a rapidly turning-over polypeptide and is immediately dependent on concomitant protein synthesis (Warner & Soeiro, 1967; Soeiro *et al.*, 1968; Willems *et al.*, 1969). As a further probe of the requirements for rRNA synthesis we have studied the effects of another inhibitor of protein synthesis, 2-(4-methyl-2,6-dinitroanilino)-*N*-methylpropionamide, on the synthesis of rRNA and polyadenylated RNA. This drug acts at the level of initiation of protein synthesis (Weeks & Baxter, 1972; Baxter & Weeks, 1972; Baxter *et al.*, 1973). We demonstrate that it selectively alters rRNA synthesis, but that ribosomal cistrons continue to be transcribed in the absence of protein synthesis for at least 3 h.

Experimental

Conditions for radish-seed germination and labelling of seedlings have already been described in detail (Delseny *et al.*, 1975, 1977). To determine the kinetics of incorporation, homogeneous batches of 100 seedlings were incubated in the presence of 2 ml of a solution of inhibitor and labelled precursor [either a mixture of 20 ^{14}C -labelled amino acids ($40 \text{ mCi/g-atom of C}$, $10 \mu\text{Ci/ml}$) or [^3H]uridine (25 Ci/

mmol , $50 \mu\text{Ci/ml}$) from the Commissariat à l'Energie Atomique]. At the required time, a 10-seedling sample was harvested, decontaminated (by treatment with 3% calcium hypochlorite and washing with water) and ground in a mortar in the presence of 5 ml of 0.1 M-glycine/NaOH buffer (pH 9)/0.15 M-NaCl/0.01 M-EDTA. Three 0.1 ml portions were immediately taken from the homogenates, transferred on to filter-paper discs (Whatman 3MM), dried and counted to determine radioactivity uptake. Incorporation was measured in five 0.1 ml portions which were precipitated on to the discs in the presence of 20% (w/v) trichloroacetic acid. The discs were washed with 5% (w/v) trichloroacetic acid, dried and counted for radioactivity. The average count for each point was plotted against time of incubation. From the incorporation curves we calculated apparent rates of incorporation by measuring the increase in radioactivity between two successive points on the kinetic curves. (Determination of the actual rate would require knowledge of the specific radioactivity of the precursor pools.)

Perchlorate extracts were prepared by addition of conc. HClO_4 to 0.25 M in homogenates made with diluted (10 times) pH 9 buffer. These extracts were used to estimate the processing of [^3H]uridine into [^3H]UTP: after elimination of salts, they were freeze-dried and chromatographed on thin-layer cellulose plates with butanol/acetone/acetic acid/5% NH_3/water (4.5:1.5:1:1:2, by vol.) as solvent (Randerath & Struck, 1961). RNA was prepared from 50-seedling batches. They were ground in the presence of 10 ml of pH 9 buffer containing 1% sodium deoxycholate. The homogenate was deproteinized by sequential addition of 1 vol. of phenol and 1 vol. of chloroform as previously described (Delseny *et al.*, 1975). RNA preparations were analysed by 2.4% (w/v) polyacrylamide-gel electrophoresis

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(Loening, 1969) and poly(U)-Sephacryl chromatography (Harris & Dure, 1974; Delseny *et al.*, 1977). Poly(U), purchased from Sigma Chemical Co, St. Louis, MO, U.S.A., was coupled to activated Sepharose 4B from Pharmacia, Uppsala, Sweden.

Results

We first checked that the D-isomer (biologically active) of 2-(4-methyl-2,6-dinitroanilino)-*N*-methylpropionamide has similar effects on protein synthesis in radish seedlings to those described in wheat germ (Baxter & Weeks, 1972; Baxter & McGowan, 1976). We found that it (0.1 mM) had no effect on ^{14}C -labelled amino acid uptake. This allows us to directly compare the apparent rates of protein synthesis in treated and non-treated seedlings. Fig. 1(a) shows that in the presence of 0.1 mM-inhibitor the rate of protein synthesis is less than 10% of that of the control. At a lower concentration it is less effective. Fig. 1(a) also shows that the inhibition is specific to the D-isomer. Fig. 1(b) demonstrates that neither uptake nor processing of ^3H uridine into ^3H UTP is significantly altered in the presence of the D-isomer of the inhibitor. A similar result is obtained with the L-isomer. Since the uptake of ^3H uridine and processing into ^3H UTP were identical in treated and non-treated samples, we can directly compare the

apparent rates of incorporation of ^3H uridine into RNA (Fig. 1c). Although the rate of incorporation sharply decreases during the first hour of treatment to about half of its level in control seedlings, RNA synthesis continues at a significant rate, since after 3 h of almost complete inhibition of protein synthesis it is still 36% of the control. In contrast with protein synthesis, the extent of inhibition does not seem to depend on the concentration of the inhibitor. Again the effect on RNA synthesis is specific to the D-isomer.

We then analysed the RNA synthesized during the treatment. Electrophoretic profiles are shown in Fig. 2. The different absorbance peaks correspond, from left to right, to DNA (sensitive to deoxyribonuclease and insensitive to ribonuclease), large cytoplasmic rRNA (1.3×10^6 daltons), large chloroplastic rRNA (1.1×10^6 daltons), small cytoplasmic rRNA (0.7×10^6 daltons) and small chloroplastic rRNA (0.56×10^6 daltons). In addition, at this stage of germination, the large rRNA contains a hidden break which is far better revealed by thermal denaturation (M. Delseny, unpublished work). In our hands, this yields a small excess of 0.7×10^6 -dalton molecules; however, newly made rRNA is in the expected ratio of 2:1 (Fig. 2e). Figs. 2(a), 2(c) and 2(e) represent control labellings of 30 min, 1 h and 3 h and show the normal processing of pre-rRNA in radish seedlings. As in many other

Fig. 1. Effects of 2-(4-methyl-2,6-dinitroanilino)-*N*-methylpropionamide on protein and RNA synthesis

Seedlings (22h old) were incubated in the presence of water, 0.1 mM- or 10 μM-D-isomer or 0.1 mM-L-isomer and the required labelled precursor for increasing periods. Uptake of ^{14}C -labelled amino acids and ^3H uridine, processing of ^3H uridine into ^3H UTP, and incorporation into macromolecules were determined as indicated in the Experimental section. From the incorporation curves the apparent rates of incorporation were calculated. All the results are presented as percentages of the control values (incubation in the presence of water). (a) Protein synthesis: Δ, 0.1 mM-D-isomer; ○, 10 μM-D-isomer; □, 0.1 mM-L-isomer. (b) Uptake of ^3H uridine (▽) and processing into ^3H UTP (○) in the presence of 0.1 mM-D-isomer. (c) RNA synthesis: ▲, 0.1 mM-D-isomer; ●, 10 μM-D-isomer; ■, 0.1 mM-L-isomer.

Fig. 2. Polyacrylamide-gel electrophoresis of high-molecular-weight RNA synthesized in the absence (a, c, e) or in the presence (b, d, f) of 0.1 mM-D-2-(4-methyl-2,6-dinitroanilino)-N-methylpropionamide. RNA was prepared from seedlings incubated for 30 min (a, b), 1 h (c, d) or 3 h (e, f) in the presence or absence of the inhibitor and with $[^3\text{H}]$ uridine. Electrophoresis was carried out for 3 h in 2.5% polyacrylamide gels. —, Radioactivity; ----, absorbance at 260 nm.

Fig. 3. Kinetics of accumulation of polyadenylated RNA in the presence or absence of 0.1 mM-D-2-(4-methyl-2,6-dinitroanilino)-*N*-methylpropionamide

The total amount of [³H]uridine incorporated into polyadenylated RNA is determined by chromatography on poly(U)-Sephadex columns. Each point represents the average of four determinations (two extractions and duplicate assays for each extraction). ○, Control seedlings; ●, seedlings treated with 0.1 mM-inhibitor.

plants (Grierson & Loening, 1974), a 2.3×10^6 -dalton molecule is cleaved into molecules of 1.4×10^6 and 0.9×10^6 daltons. These molecules are in turn precursors of the mature rRNA species. Figs. 2(b), 2(d) and 2(f) show the profiles of molecules synthesized in the presence of 0.1 mM-2-(4-methyl-2,6-dinitroanilino)-*N*-methylpropionamide during the same period. Clearly the presence of the 2.3×10^6 -dalton pre-rRNA molecule in all three profiles indicates that the ribosomal cistrons continue to be transcribed in the absence of protein synthesis. The main target of the drug seems to be the processing of the 2.3×10^6 -dalton species, since virtually no 1.4×10^6 -dalton RNA is detected after 30 min of treatment. After longer labelling this species very slowly accumulates. A notable point is that there is an increase in the amount of radioactivity in the 2.3×10^6 -dalton molecules relative to the control. Electrophoretic patterns obtained after treatment with the L-isomer are identical with the control patterns (not shown).

Finally we examined the effect of the D-isomer on the synthesis of presumptive mRNA, the polyadenylated RNA. RNA preparations labelled with [³H]uridine were fractionated by poly(U)-Sephadex chromatography. The fractions corresponding to each class of RNA were pooled and their trichloroacetic acid-insoluble radioactivity was measured. Fig. 3 shows that overall radioactivity incorporated into polyadenylated RNA molecules is fairly similar in treated and non-treated seedlings. However, as a consequence of the partial inhibition of rRNA synthesis the ratio of polyadenylated RNA to non-

polyadenylated RNA changes. For example, after 3 h of treatment with inhibitor about 25% of the radioactivity corresponds to polyadenylated RNA instead of the 10% in the control. We have checked that the inhibitor has no significant effect on the size distribution of polyadenylated molecules. The electrophoretic profiles (not shown) are similar to those previously published (Harris & Dure, 1974; Grierson & Covey, 1975; Payne, 1977; Delseny *et al.*, 1977).

Discussion

The results presented here provide new data on the effects of a pre-emergence herbicide 2-(4-methyl-2,6-dinitroanilino)-*N*-methylpropionamide. This drug, which is known to be a specific inhibitor of protein-synthesis initiation in eukaryotes, also affects rRNA synthesis. Our data indicate that it has little or no effect on polyadenylated-RNA synthesis, confirming observations on sea-urchin embryos (Aronson, 1977). The use of this initiation inhibitor helps us to confirm that transcription of ribosomal cistrons continues when protein synthesis is almost completely inhibited and therefore transcription is not immediately dependent on concomitant protein synthesis. On the other hand, the processing of the 2.3×10^6 -dalton precursor seems to be very sensitive to inhibition of protein-synthesis initiation. This situation somewhat differs from that reported in HeLa cells: 1,3-bis-(2-chloroethyl)nitrosourea, an inhibitor of initiation in these cells, selectively inhibits synthesis of 45S pre-rRNA, but allows previously made molecules to mature normally (Penman *et al.*, 1976). Our results, however, do not rule out the possibility that the ribosomal genes are transcribed at a lower rate. Indeed, the amount of radioactivity in the 2.3×10^6 -dalton molecules is higher in treated than in non-treated seedlings, but the accumulation is only moderate. We cannot discriminate between two possibilities: (1) ribosomal genes are transcribed at a normal rate, but, processing being altered, most of the large precursor is degraded, yielding only a moderate increase relative to the control; (2) ribosomal genes are transcribed at a lower rate and processing is slower, equally resulting in a moderate accumulation of 2.3×10^6 -dalton RNA. Finally it is worth emphasizing that 2-(4-methyl-2,6-dinitroanilino)-*N*-methylpropionamide could be a useful tool for investigating the properties of pre-ribosomal particles containing 2.3×10^6 -dalton RNA and of messenger ribonucleoprotein (or their nuclear precursors), which are as yet completely unknown in higher plants.

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