Chloroplast Development in 4-Thiouridine Treated Radish Cotyledons^{†,††}

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Formation of protochlorophyllide and ribulose-1,5-diphosphate carboxylase, both of which were constituents proper to chloroplast, was inhibited in the radish cotyledons grown with 4-thiouridine (0.5 mM), though the growth of the seedlings, activities of a cytoplasmic enzyme glucose-6-phosphate dehydrogenase and of a mitochondrial enzyme glutamate dehydrogenase in the cotyledons were not affected. Photo-induced formation of chlorophyll pigments and of chloroplastic ribosomal RNAs in the 4-thiouridine treated cotyledons were depressed as compared with controls. Interestingly, the effect of 4-thiouridine, however, disappeared with continuous illumination. Based upon these results, specific development of the chloroplast is discussed.

We have recently focused our interest on studies of the photo-induced development of the photosynthetic organelles in higher plants, and have demonstrated the inhibitory effect of 4sU on chloroplast development of radish cotyledons in a preliminary report,¹⁾ which was the first description of the physiological action of 4sU in a higher plant. The photo-induced formation of chloroplasts in etiolated plants has been observed by many investigators to proceed independently of non-photo-synthetic organelles using antibiotics such as actinomy-

cin D,²⁾ chloramphenicol,³⁾ streptomycin,⁴⁾ *p*-chlorophenyl dimethylurea,⁵⁾ tentoxin⁶⁾ and Sandoz 6706,⁷⁾ all of which are irreversible type toxins.

The inhibitory effect of 4sU followed by the restoration on illumination is one approach to investigating the mechanism of photo-induced development of chloroplasts. We report experimental results on chloroplast development of 4sU treated radish cotyledons.

FIG. 1. 4-Thiouridine (4sU).

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^{††} Abbreviations: 4sU, 4-thiouridine; PCH, protochlorophyllide; δ -ALA, δ -Amino levulinic acid; ATA, 3-amino-1,2,4-triazole; Sandoz 6706, 4-chloro-5-(dimethylamino)-2-(α,α,α -trifluoro-*m*-tolyl)-3(2H)-pyridazinone; G6PDH, glucose-6-phosphate dehydrogenase [E.C.1.1.1.49]; GluDH, glutamate dehydrogenase [E.C.1.4.1.3]; RuDP, ribulose-1,5-diphosphate.

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3) R. W. Pollack and P. J. Davies, *Phytochemistry* **9**, 471 (1970).

4) J. Ingle, *Plant Physiol.*, **43**, 1850 (1968).

5) D. McMahon and L. Bogorad, *ibid.*, **43**, 188 (1968).

6) J. M. Halloin, G. A. deZoeten, G. Goard and J. C. Walker, *ibid.*, **45**, 310 (1970).

7) P. G. Bartels and A. Hyde, *ibid.*, **45**, 807 (1970).

MATERIALS AND METHODS

Materials and culture conditions. Radish seeds (*Raphanus sativus* Linn.) were obtained from Takii Shubyo Co. Ltd., Kyoto, and 4sU was synthesized from uridine after the methods of Fox *et al.*,⁸⁾ and purified by cellulose column chromatography using isopentyl alcohol-acetone-water (3:2:1 by volume) as the solvent.⁹⁾ The 4sU thus obtained contained no uridine, 2-thiouridine and 2,4-dithiouridine.

Radish seeds were allowed to germinate on absorbent cotton in Petri dishes containing 0.5 mM 4sU in 1 mM phosphate buffer at pH 7.0, unless otherwise noted, in the dark at 22°C for 4 days. The etiolated seedlings obtained were illuminated with Mitsubishi fluorescent lamps (4000 lux) at 22°C for the desired time. Controls were left under the same conditions except for the presence of 4sU.

Chlorophylls were extracted with 80% acetone and were determined by measuring optical density of the extract at 665 m μ following Arnon's method.¹⁰⁾ PCH was determined by measuring the optical density of the acetone extract at 625 m μ according to Siegelman's method.¹¹⁾ Molar extinction coefficients of both pigments were $\epsilon_{665} = 73.3 \times 10^3$ and $\epsilon_{625} = 31.1 \times 10^3$, respectively. Carotenoids were extracted from the cotyledons with acetone and the extracts were evaporated *in vacuo* to give a solid substance, which was then extracted with ether. The ether solution was treated overnight with 1% potassium hydroxide in methanol. Water was added to the reaction mixture, and the upper layer (ether) was washed 4 times with saturated sodium chloride aq. solution, then dried on anhydrous sodium sulphate. Carotenoids were determined by measuring the optical density of the ether solution at 446 m μ . The amount of carotenoid was calculated as β -carotene using $E_{446}^{1\%} = 2.5$.¹²⁾

Enzyme preparation and assay method. Crude enzymes prepared after Klein's method¹³⁾ were used for the

assays of G6PDH and GluDH. A 1.5 g portion of fresh cotyledons was cut up, placed in a homogenizer (Nippon Seiki HA-2) then were homogenized in 12 ml of tris buffer (pH 7.5, 0.05 M) in the cold. The homogenate was squeezed through 4 layers of gauze and the sap obtained was centrifuged at 20,000 $\times g$ for 10 min in the cold. The supernatant was passed through a Sephadex G-25 column, and its effluent was assayed for G6PDH activity according to Kornberg's method.¹⁴⁾ Specific activity was determined by measurement of ΔE_{340} /mg of protein for the initial 3 min using NADP⁺ as cofactor. In addition, the sap obtained above was frozen and thawed 4 times, then centrifuged at 20,000 $\times g$ for 10 min in the cold and treated with a Sephadex G-25 column. The protein solution obtained was used for the assay of GluDH according to Bücher's method.¹⁵⁾ Specific activity was determined in a manner similar to the G6PDH assay but using NAD⁺ as cofactor. For the assay of RuDP carboxylase [E.C.4.1.1.39] activity, a sample was homogenized in 0.05 M tris buffer (pH 8.0) containing 0.2 mM EDTA, then it was centrifuged at 12,000 $\times g$ for 10 min. The protein solution obtained was used for the assay following a modification of the method of Klein.¹³⁾ The reaction mixture contained tris buffer (pH 8.0) 5 μ moles, RuDP 0.5 μ moles, enzyme protein 0.7 mg, and sodium bicarbonate 25 μ moles (¹⁴C-NaHCO₃ 1.0 μ Ci) in a total volume of 0.4 ml. Fifteen minutes after the addition of the labelled bicarbonate, 0.1 ml of 10% perchloric acid was added. The reaction mixture was centrifuged and 0.1 ml of the supernatant solution was dried on a planchette and its radioactivity was determined with a 2 π -gas flow counter (Aloka FC-1E).

Protein. Protein was determined colorimetrically by the method of Lowry *et al.*¹⁶⁾

δ -ALA determination. Quantitative determination of δ -ALA was performed according to Wada's method.¹⁷⁾ A trace amount of δ -ALA in the sample was deter-

8) J. J. Fox *et al.*, *J. Am. Chem. Soc.*, **81**, 178 (1959).

9) V. N. Shibaev *et al.*, "Synthetic Procedures in Nucleic Acid Chemistry," ed. by W. W. Zorbach and R. S. Tipson, Interscience Publishers, New York, N.Y., 1968 p. 503~505.

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13) B. Filner and A. O. Klein, *ibid.*, **43**, 1587 (1968).

14) A. Kornberg and B. L. Horecker, "Methods in Enzymology," Vol. 1, ed. by S. P. Colowick and N. O. Kaplan, Academic Press Inc., New York, N.Y., 1955 p. 323.

15) Th. Bücher *et al.*, *Z. Naturforsch.*, **8b**, 555 (1953).

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17) O. Wada *et al.*, *Brit. J. industr. Med.*, **26**, 240 (1969).

mined as follows. After the addition of a known amount of δ -ALA to an unknown sample, the δ -ALA was determined and an estimation was made for a net amount of δ -ALA in the unknown.

RNA preparations and analysis by polyacrylamide gel electrophoresis. The preparation of RNAs from the cotyledons was performed using the method described by Loening.¹⁸⁾ RNAs were determined by measuring their ultraviolet absorptions. The ratio of 260 m μ /280 m μ of the isolated RNAs was 1.82~2.20. Analysis of the RNAs by polyacrylamide gel electrophoresis was performed according to the method of Peacock which used composite gels with 3% polyacrylamide containing 0.15% bisacrylamide and 0.5% agarose.¹⁹⁾ After the prerun for 30 min, the RNAs sample (30 μ g) was applied to the vertical gel (5 $\times$ 70 mm) and electrophoresis was begun at 5 mA per tube for 150 min in a cold room. When the run was over, the gels were stained in 0.2% methylene blue as described by Peacock.²⁰⁾ Destaining was done by pouring off the excess stain and washing the gel for 24 hr with running water. The stained pattern on the gel was quantitatively estimated using a densitometer fixed with a 610 m μ filter (Asuka Ozumor 82).²¹⁾

RESULTS

Figure 2 shows the growth curves of 50 radish seedlings grown with or without 0.5 mM 4sU in the control buffer. In both total body weight and cotyledon weight, there is no or little difference between 4sU treated plants and controls, showing that 4sU (0.5 mM) did not affect germination followed by growth of the plants.

Chlorophylls as well as carotenoids of the seedlings cultured with and without 4sU were determined. The data shown in Fig. 3 indicate that 4sU inhibited normal greening, as compared with controls, especially at the early

FIG. 2. Growth Curves of Radish Seedlings.

○—○; Total body grown without 4sU
 △—△; Total body grown with 4sU
 ●—●; Cotyledon grown without 4sU
 ▲—▲; Cotyledon grown with 4sU

FIG. 3. Determination of Chlorophylls and Carotenoids in Cotyledons Cultured with and without 4sU.

●—●; Carotenoids without 4sU
 ▲—▲; Carotenoids with 4sU
 ○—○; Chlorophylls without 4sU
 △—△; Chlorophylls with 4sU

18) U. E. Loening and J. Ingle, *Nature*, **215**, 363 (1967).

19) A. C. Peacock and C. W. Dingman, *Biochemistry*, **7**, 668 (1968).

20) A. C. Peacock and C. W. Dingman, *ibid.*, **6**, 1818 (1967).

21) H. Ochiai and H. Shibata, *Bull. Faculty of Agr. Shimane Univ.*, **4**, 120 (1970) (in Japanese).

stage, but greening was restored by 3 days of illumination. Determinations of carotenoids demonstrated that both cotyledons grown with and without 4sU contained carotenoids, though to a lesser extent in the case of 4sU treated dark-grown seedlings. Carotenoids content

TABLE I. EFFECT OF SOME REAGENTS ON CHLOROPHYLL FORMATION

	Water (dark)	Water (light)	4 sU (1 mM)	Uridine (1 mM)	Glutathione (1 mM)	2-Thiouracil (1 mM)
Chlorophylls/g of fresh cotyledons (μ g)	2	452	430	492	444	302

Cotyledons cultured without 4sU in the dark for 4 days were excised and incubated with the reagents (1 mM) shown above in the dark for 12 hr; then they were illuminated (6,000 lux) for 24 hr. Chlorophyll formation was determined by methods described in "Materials and Methods" "Water (dark)" means incubation with water in the dark.

rose in a way similar to that for chlorophylls on illumination. The fact that dark-grown cotyledons treated with 4sU contained carotenoids suggests that the action of 4sU is different from that of ATA⁴¹ or Sandoz 6706,⁷¹ which resulted in albino leaves.

Bartels showed that the albino wheat leaves derived from the treatment of Sandoz 6706 could not develop chloroplasts on illumination at "high" intensity, suggesting the importance of carotenoids as "chemical buffer" or photoprotector to prevent photolysis or bleaching of chlorophylls.⁷¹ The radish cotyledons grown with 4sU in the dark for 4 days contained about 60% as much carotenoids as did the controls. The control cotyledons growing for 2.5 days in the dark contained carotenoids as much as did the 4sU treated ones grown for 4 days in the dark and these showed normal development of the chloroplast upon illumination. This suggests that the smaller content of carotenoids in the 4sU treated cotyledons did not cause the retarded greening in our experiment.

Normal cotyledons grown in the dark for 4 days were excised and incubated with various reagents for 12 hr in the dark, then were illuminated (6000 lux) for 24 hr and chlorophyll formation was determined. The data shown in Table I indicate that 4sU had no inhibitory effect on the greening of excised normal cotyledons, so that the contact of the seeds with 4sU in the germination stage should be primarily related to the inhibition of the greening on illumination. Uridine exerted only a slight promoting effect and 2-thiouracil pro-

FIG. 4. Content of PCH in Cotyledons Grown in Various Concentrations of 4sU in the Dark for 4 Days, and Chlorophylls Formation in Cotyledons after Illumination for 24 hr (6,000 lux).

○—○; PCH ●—●; Chlorophylls

duced an inhibitory effect on greening.²²¹

Figure 4 shows the content of PCH, precursor of chlorophyll biogenesis, in the cotyledons grown with various concentrations of 4sU and photo-induced chlorophyll formation in the cotyledons after 24 hr illumination (6000 lux). Both curves are almost parallel, indicating that treatment by a higher concentration of 4sU resulted in less content of PCH and less formation of chlorophylls on illumination.

On the other hand, δ -ALA, a starting material for porphyrin pigments, such as chlorophylls and cytochromes, was shown to accumulate in $40 \pm 10\%$ excess in the 4sU

22) J. Heslop-Harrison, *Planta*, **58**, 237 (1962).

FIG. 5. Accumulation of δ -ALA in Dark-grown Cotyledons Treated with and without 4sU.

Δ—Δ; treated with 4sU
O—O; treated without 4sU

(0.5 mM) treated cotyledons as compared with controls (Fig. 5). More recently Nadler and Granick²³ demonstrated that the enzymes synthesizing PCH from δ -ALA in barley were not limiting even during the lag phase in the dark, and that the limiting activity for chlorophyll synthesis appeared to be a protein (or proteins) related to the synthesis of δ -ALA. The existence of δ -ALA in excess and of PCH in lesser amounts in the 4sU treated cotyledons indicates that the formation of non-limiting enzymes synthesizing PCH from δ -ALA in the 4sU treated proplastids was arrested.

The specific activity of G6PDH, a typical enzyme in the soluble cytoplasm, was the same, in the cotyledons grown with 4sU, as the activity of controls. GluDH, a typical enzyme of mitochondria, also showed no difference in activity between the 4sU treated plants and controls as shown in Fig. 6. However, the specific activity of RuDP carboxylase, which is a proper enzyme of chloroplasts, was low in etiolated cotyledons germinated with 4sU but was significantly high in the cotyledons without 4sU. In one experiment, 4sU was

FIG. 6. Enzyme Activities in Cotyledons Cultured with and without 4sU.

Assay conditions are described in the text.

O—O; G6PDH without 4sU
Δ—Δ; G6PDH with 4sU
●—●; GluDH without 4sU
▲—▲; GluDH with 4sU
■—■; RuDP carboxylase without 4sU
□—□; RuDP carboxylase with 4sU

added to RuDP carboxylase preparations obtained from control cotyledons, but the enzyme activity was not affected. This suggests that the low specific activity of the enzyme from 4sU treated cotyledons was caused by the low formation of the enzyme in the plant.

Figure 7 shows the time course of RNA accumulation in the cotyledons treated with and without 4sU. The accumulation of RNA in the cotyledons of the dark-grown, 4sU treated seedlings is less than that in the untreated until 6 days growth in the dark. However, the rate of RNA synthesis in the 4sU treated plants from 2 to 4 days growth in the dark is more rapid than that of the untreated ones. The accumulation of RNAs in the 4sU treated seedlings reached about 90% that of the untreated after 4 days growth in the dark. RNA synthesis in controls was shown to be stimulated by light, reaching a maximum value in the following 24 hr, whereas the rate of accumulation of the 4sU treated RNA was lower; needing 48 hr to reach a

23) K. Nadler and S. Granick, *Plant Physiol.*, **46**, 240 (1970).

FIG. 7. Time Course of RNAs Accumulation in Cotyledons Cultured with and without 4sU.

▲—▲; cultured with 4sU
 ▲---▲; allowed to stand in the dark for 2 more days
 ●—●; cultured without 4sU
 ●---●; allowed to stand in the dark for 2 more days

maximum value. These phenomena seem to correlate with the results shown in Fig. 8, which shows the fractionation on polyacrylamide gel electrophoresis of RNAs from cotyledons cultured with (inset B, D, F and H) and without 4sU (inset A, C, E and G) at various developmental stages. According to Loening,¹⁸⁾ the peaks shown in inset C, for example, are assumed to be 25S, 23S, 18S, 16S and 13S RNA, respectively, from right to left. Both 25S and 18S RNAs, cytoplasmic ribosomal RNAs, developed similarly both in controls and in 4sU treated cotyledons, respectively, grown in the dark for 4 days. A small amount of 23S and 16S RNAs, chloroplastic ribosomal RNAs, are seen after 4 days growth

FIG. 8. Chromatograms of RNAs on Polyacrylamide Gel Electrophoresis of the Developmental Stage of Cotyledons Cultured with (Inset B, D, F and H) and without 4sU (Inset A, C, E and G).

A and B: 4 days growth in the dark.
 C and D: illuminated for 24 hr.
 E and F: illuminated for 48 hr.
 G and H: illuminated for 72 hr.

in the dark (inset A), while 16S RNA is not present in the 4sU treated ones (inset B). Inset C shows the chromatogram of RNAs obtained from greening cotyledons of controls at 24 hr illumination, demonstrating the appearance of 13S RNA. Over the same time intervals both 23S and 16S RNAs but not 13S RNA appeared in the 4sU treated cotyledons (inset D). Inset F indicates that both 23S and 16S RNAs in the 4sU treated cotyledons reached a normal ratio to cytoplasmic RNA which was comparable to that of controls shown in inset

E. From that time the chlorophyll content and carotenoid content increased as shown in Fig. 3. RNA patterns both in 4sU treated and in controls are similar after 72 hr illumination (inset H and G).

The results described above indicate that the development of chloroplastic ribosomal RNAs was selectively prevented by the presence of 4sU.

DISCUSSION

The presence of 4sU as a minor constituent has been established in transfer RNAs from *E. coli*,²⁴⁾ from *Mycoplasma*²⁵⁾ and also in ribosomal RNAs from *E. coli*,²⁶⁾ while the presence of 4sU in plant kingdom has not been reported yet.

It was demonstrated in this paper that the formation of PCH and RuDP carboxylase, both proper constituents of chloroplasts, were inhibited in radish seedlings grown with 4sU (0.5 mM), though the growth, the activity of a cytoplasmic enzyme G6PDH and the activity of a mitochondrial enzyme GluDH in these seedlings were not affected. Photo-induced formation of chlorophylls and of chloroplastic ribosomal RNAs in 4sU treated cotyledons were depressed as compared with those of controls. Interestingly, the activity of RuDP carboxylase, the formation of chlorophylls and of chloroplastic ribosomal RNAs were restored to their normal values by continuous illumination. In the 4sU treated cotyledons, the accumulation of PCH was reduced to 30%, while δ -ALA, a starting material for chlorophyll and cytochrome pigments, was accumulated $40 \pm 10\%$ in excess, as compared with controls. The results show that 4sU selectively prevented the formation of chloroplastic enzymes in the cotyledons grown in the dark

followed by growth in the light. Furthermore, based upon the results obtained from the behaviors of RNAs on polyacrylamide gel electrophoresis, we concluded that 4sU depressed photo-induced chloroplastic RNA synthesis, which should be a prior process to the formation of either enzymes²⁷⁾ or structural proteins²⁸⁾ in chloroplasts. This supports the hypotheses that the enzymatic or structural proteins involved in chloroplast development are formed on the chloroplastic ribosomal RNAs and also that chloroplasts are semi-autonomous organelles.

More recently Pollack *et al.*³⁾ investigated the developmental kinetics of cytoplasmic and chloroplastic ribosomal RNA and chlorophyll in normal pea buds upon transfer from dark to light, and demonstrated that chloroplastic ribosomal RNA showed no lag period in its rate of formation, while chlorophyll showed a lag of 24 hr after initiation of the light, which suggests that there is a direct dependence of the production of chlorophyll on the amount and appearance of chloroplastic ribosomal RNA. This is also true of our 4sU treated radish cotyledons.

These phenomena might be related to the biochemical property of 4sU as a thiol nucleoside and/or to the photo-chemical property of 4sU as a photosensitizer, which has a maximum absorption at 330 m μ . It was demonstrated that ribosomes of *E. coli* which were shown to contain 4sU-phosphate as a constituent of the ribosomal RNA by Parish *et al.*²⁶⁾ could be dissociated reversibly into 50S and 30S subunit by treatment with *p*-chloromercuribenzoic acid.²⁹⁾ On the other hand, ribosomes of *Pseudomonas aeruginosa* and *Bacillus subtilis* which contained no detectable amount of 4sU-phosphate were shown to be insensitive to the thiol reagent. Parish, therefore, concluded

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25) H. Hayashi, H. Fisher and D. Söll, *Biochemist-ry*, **8**, 3680 (1969).

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27) M. Gassman and L. Bogorad, *Plant Physiol.*, **42**, 774 (1967).

28) J. T. O. Kirk, *Planta*, **78**, 200 (1968).

29) F. Miyazawa and T. Tamaoki, *J. Mol. Biol.*, **24**, 485 (1967).

that 4sU moiety in the *E. coli* ribosomes must have contributed to the stability of 70S ribosomes. The presence of 4sU in plant kingdom has not been reported yet, but it is assumed that the stability of chloroplastic ribosomes (70S) may be influenced by the presence of 4sU, thiol nucleoside. In addition, Sawada demonstrated that bovine pancreatic ribonuclease [E.C.2.7.7.16] was inactivated by irradiation with ultraviolet light of 330~365 m μ in the presence of a substrate analogue, 4sU-2(3)-phosphate.³⁰⁾ The proplastid or chloroplast, in our experiments, might preferentially suffer from the inhibitory effect by 4sU due to its higher sensitivity to 4sU or to its poorer metabolic activity of 4sU. What step(s) of chloroplastic RNA synthesis undergo the inhibitory effect of 4sU is not clear. The

question remains as to whether the 4sU in the plant acts either as such or as any metabolite of 4sU. Investigations of these problems are in progress in our laboratory.

Moreover, the mechanism by which the selective effect of 4sU disappeared during continuous illumination needs to be determined. One of us demonstrated that 4sU showed specific photochemical behaviour distinguished from other constituents of RNAs.³¹⁾ One possible explanation of the detoxication is that 4sU, free or bound in the plant, might receive photo-induced transition into uridine via uridine-4-sulfonic acid,³²⁾ based on the photochemical properties of 4sU.

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32) E. B. Ziff and J. R. Fresco, *J. Am. Chem. Soc.*, **90**, 7338 (1968).