Buoyant Density Studies of Chloroplast and Nuclear Deoxyribonucleic Acid from Control and 3-Amino-1,2,4-Triazole-treated Wheat Seedlings, *Triticum vulgare*[1]

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ABSTRACT

The isolation of chloroplast and nuclear DNA from dark- and light-grown, control- and 3-amino-1,2,4-triazole-treated wheat seedlings, *Triticum vulgare*, is described. Contrary to a previous report, we found that chloroplast and nuclear DNA had similar buoyant densities (1.702 grams per cubic centimeter) and that they could not be resolved by buoyant density centrifugation in CsCl. Difference in renaturation behavior of the chloroplast and nuclear DNA was used as the criterion for distinguishing one from the other. Only chloroplast DNA readily renatured whereas nuclear DNA renatured only slightly. Light-grown, 3-amino-1,2,4-triazole-treated plants were found to lack detectable quantities of chloroplast DNA whereas treated, dark-grown plants contained plastid DNA. We suggest that 3-amino-1,2,4-triazole affects the accumulation of chloroplast DNA by inhibiting the formation of chloroplast membranes, enzymes, and pigments.

The buoyant density of chloroplast DNA in CsCl has become a subject of controversy. From 1963 to 1967 several investigators (5, 6, 12, 13, 21, 24) reported that chloroplast DNA of many dicotyledonous plants had a much higher buoyant density than their corresponding nuclear DNA. The chloroplast DNA of only a few monocotyledonous plants has been studied. Mache and Waygood (16) reported that buoyant density of wheat chloroplast DNA was higher than the nuclear DNA, and their results were similar to the earlier studies. Recently, more rigorous studies of dicotyledonous plants demonstrated that buoyant density of chloroplast DNA was similar or identical to nuclear DNA and that the two types of DNA could not be separated in CsCl density gradient (1, 14, 25, 26). However, DNAs are distinguished from each other by the fact that chloroplast DNA will readily renature whereas nuclear DNA will not. In this study we report the existence in wheat seedlings of chloroplast DNA which has a buoyant density identical to the nuclear DNA.

Light-grown wheat seedlings treated with 3-amino-1,2,4-triazole have been shown to lack chloroplast ribosomes (2). Scott and Smillie (20) suggested that RNA of chloroplast ribosomes was coded for by chloroplast DNA. Evidence for this assumption comes from the following observations: (a) enucleated *Acetabularia* were able to synthesize ribosomal RNA (10), (b) ultraviolet- or heat-treated *Euglena* which lack detectable chloroplast DNA lose their ability to form chloroplast ribosomes (19), (c) chloroplast DNA hybridizes with chloroplast ribosomal RNA (24). The absence of chloroplast ribosomes in AT[2]-treated plants may be caused by inhibition of chloroplast DNA synthesis or by losing the capacity to retain chloroplast DNA. Conversely, AT may inhibit the formation of chloroplast ribosomes, thereby preventing the synthesis of enzymes necessary to replicate the chloroplast DNA. In this study, we report that chloroplast DNA is absent in the AT-treated seedlings.

MATERIALS AND METHODS

Plant Material. Wheat seedlings (*Triticum vulgare* L. var. Maricopa) were germinated and grown in Petri dishes (16 grains per dish) containing either 10 ml of 5×10^{-4} M AT or 10 ml of distilled water for 6 days in light at 1,500 ft-c, 16 hr photoperiod, 21 C, or for 6 days in darkness except for exposure to dim green light during watering at 23 C.

Isolation of Etioplasts and Chloroplasts. Several methods (6, 11, 13) of isolating chloroplasts, etioplasts, and aberrant plastids were tried but a modification of Jacobson's procedure (9) and the Whitfeld and Spencer method (26) were found to give the best plastid preparations. Wheat leaves were harvested in 60 g lots, chilled to 5 C, and chopped with razor blades in 120 ml of Jacobson media (0.5 tris-HCl, pH 8.0, 0.5 M sucrose, 1 mM MgCl₂, 4 mM mercaptoethanol, and 0.2% bovine serum albumin) or 120 ml of Honda medium (2.5% ficoll, 5% dextran, 0.25 M sucrose, 0.25 mM tris, pH 7.8, 1 mM MgCl₂, and 4 mM mercapoethanol) on ice.

Chopping was performed with razor blades attached to the ends of two blades of an electric knife (R. G. Jensen, personal communication). The homogenate was pressed through a fine wire gauze and then filtered through a double layer of Miracloth (Calbiochem, Los Angeles). The filtrate was then centrifuged at 2,500g for 10 min in a SS-34 rotor (Servall centrifuge) to give a pellet of plastids and nuclei as determined by staining with methyl green. In some of our experiments with AT-treated, light-grown plants, the filtrate was spun at 6,000g (SS-34 rotor) for 10 min. The resulting pellets were resuspended in 9 ml of the chopping medium and 3-ml aliquots were layered onto a 25-ml discon-

[2] Abbreviations: AT: 3-amino-1,2,4-triazole; NET: 0.1 M NaCl, 0.01 M EDTA, 0.01 M tris-HCl, pH 8.0; SDS: sodium dodecyl sulfate.

tinuous sucrose gradient. The gradient tubes contained either three layers of 60% (w/v) sucrose (10 ml); 45% sucrose (10 ml), and 20% sucrose (5 ml) in Honda medium or two 12-ml layers of 2 M sucrose and 1.3 M sucrose in Jacobson medium. The gradients were centrifuged for 2 hr at 25,000 rpm in SW-25.1 rotor (4 C).

Plastids isolated in Honda or in Jacobson medium formed two bands in their respective gradients. In the Honda gradient, one band was at the 20 to 45% junction and the other at the 45 to 60% junction; in the Jacobson gradient, one band was in the upper part of the 1.3 M layer and the other band at the 1.3 M to 2 M junction. Chloroplasts were removed from the gradients with a syringe and were recovered from the gradient media by diluting each fraction with an equal volume of 0.01 M tris, 0.01 M MgCl₂, pH 7.8, then centrifuging at 8,000g (SS-34 rotor) for 15 min. The chloroplast pellets were resuspended in 2% sodium dodecyl sulfate containing 0.1 M NaCl, 0.01 M EDTA, 0.01 M tris-HCl, pH 8.0, and either used directly for DNA isolation or frozen for later use. The nuclei pelleted at the bottom of the tubes in both types of gradients; these pellets were used as the source of nuclear DNA.

Both the upper and lower chloroplast bands from either type of gradient were found to contain nuclear DNA along with chloroplast DNA while the lower etioplast band from the Jacobson gradient was free of nuclear contamination. As a rule, only the upper band of plastids (Honda gradient) from AT-treated plants was collected because the lower band was contaminated with bacteria.

Chloroplasts were also isolated by the nonaqueous method of Stocking et al. (23). In some cases, chloroplast fractions were treated with 4% Triton X-100 and spun at 10,000g, and the supernatant was used for DNA isolation.

DNA Extraction and Purification. Pronase (Calbiochem, grade B) at 1 mg/ml (freed from DNAase by preincubation for 3 hr at 37 C) was added to the suspension of chloroplasts and SDS-NET buffer and then digested for 6 hr at 40 C. The solution was made 1 M with respect to NaCl before deproteinization. To remove the pronase, the digest was shaken three times with a chloroform-isoamyl alcohol mixture (25:1, v/v) over a 30 min period at 4 C. After centrifugation (5000 rpm, SS-34 rotor), the upper aqueous layer (containing the nucleic acids) was removed with a wide mouth pipet and saved. The lower layer of chloroform with protein interface was washed twice with 0.1 × SSC (SSC is 0.15 M NaCl, 0.015 M sodium citrate), and the washings were pooled with the first aqueous layer. To remove the RNA, the nucleic acid solution was treated with 50 μg/ml pancreatic ribonuclease (previously heated to 90 C for 5 min at pH 5) and 100 units/ml of T₁ ribonuclease for 2 hr at 24 C. These enzymes were removed by further chloroform-isoamyl alcohol (25:1, v/v) treatment. The DNA was precipitated from the aqueous phase with 2 volumes of ethanol (overnight at −15 C), centrifuged at 10,000g (SS-34 rotor) for 20 min, and then dissolved in 0.5 ml of 0.1 × SSC. The DNA solution was further purified by passing it through a Sepharose-4B (Pharmacia Fine Chemicals) column (1 cm × 15 cm). DNA was eluted from the column with 1 × SSC; it appeared in the effluent immediately after the void volume (4 ml) and was collected in 1-ml fractions.

Denaturation-Renaturation Studies. Ten to 20 μg/ml of DNA was denatured by adjusting the pH of the solution to 12 with NaOH for 15 min (23 C). Next, the samples were neutralized with 1 M KH₂PO₄ and were either placed on ice (denaturation) or incubated (renaturation) at 60 C in 2 × SSC for 6 hr.

Equilibrium Centrifugation. Buoyant density centrifugation in CsCl was carried out in a four-place ANF rotor in a Spinco model E ultracentrifuge at 44,000 rpm for 20 hr at 25 C. The CsCl solution had a mean density of 1.700 g/cc. About 5 μg of wheat DNA plus 1 to 3 μg of marker DNA (1.731 g/cc; *Micro-*

coccus lysodeikticus) were used in each ultracentrifuge run. Densitometer tracing of the ultraviolet negatives was made with a Spinco Analytrol. Buoyant densities of the DNA bands were calculated according to Mandel et al. (17).

Ultrastructural Studies. For electron microscopy the pellets of isolated plastids were embedded in 1.4% agar containing 1.3 M sucrose and 0.1 M potassium phosphate buffer at pH 7.2. The agar blocks were fixed overnight in 3% glutaraldehyde (0.1 M potassium phosphate buffer pH 7.2 and 1.3 M sucrose). They were subsequently washed three times over a 3-hr period in phosphate buffer pH 7.2, fixed in 1% OsO₄ in 0.1 M potassium phosphate buffer for 6 hr, dehydrated in a graded series of acetone, and embedded in Spurr's plastic media (22). Specimens were sectioned with a Porter-Blum MT-2 ultramicrotome, post-stained with uranyl nitrate, and examined with a Phillips EM 200 electron microscope.

RESULTS

Ultrastructural Studies. The plastid fraction of AT and control, dark- and light-grown plants were examined with the electron microscope to determine if the plastids retained a morphology similar to their *in vivo* appearance. The isolated fractions consisted mainly of plastids (Fig. 1–4), although a few remnants of mitochondria and some bacteria were present in fractions from light-grown plants as observed under lower magnification with the electron microscope. Nuclei or nuclear fragments were not observed with the light or electron microscope; however, the ultracentrifugation results (Fig. 5e, 7c, 7d) showed that nuclear DNA was present in fractions from light-grown plants. Aqueously isolated chloroplasts of light-grown control plants (Fig. 1) contained grana (g) connected by anastomosing frets but lacked the outer envelope and stroma. Chloroplasts isolated by a non-aqueous method retained their stroma (s) but lacked the outer envelope (Fig. 1b). Aberrant plastids (Fig. 2) from AT-treated light-grown plants contained a few scattered internal membranes (m) and retained their outer envelope (e). Isolated etioplasts of control and treated (Fig. 3 and 4) plants were similar in appearance. They retained part of their outer membrane envelope and stroma (s); however, the prolamellar bodies were disorganized and they appeared as scattered tubules (t) dispersed throughout the stroma (s). The essential features of the plastids were observed during the isolation procedure.

Ultracentrifugation Studies. The yield of untreated wheat seedlings nuclear and plastid DNA was about 250 and 27 μg per 60 g (fresh weight) of leaf tissue, respectively. The buoyant density analytical centrifugation patterns of the etioplast, chloroplast, and nuclear DNA are shown in Figures 5 to 7. In CsCl, native DNA (Fig. 5a, 5d, 6a) from the isolated plastid and nuclear fractions formed only a single band (1.702 g/cc) regardless of the method used to isolate the fractions. These results may indicate that native nuclear and plastid DNA have similar buoyant densities. However, to determine if our chloroplast DNA was actually nuclear DNA which had contaminated the plastid fraction, we subjected our chloroplast DNA to denaturation and renaturation treatment. Several investigators (1, 14, 25, 26) have shown that the readiness of chloroplast DNA to renature after denaturation is a reliable method of distinguishing it from nuclear DNA.

Denaturation of the native DNA from etioplast (Fig. 5b), chloroplast, and nuclear (Fig. 6b) fractions caused an increase of approximately 0.017 g/cc in their buoyant density forming a broad, single band. Following renaturation treatment, the etioplast DNA shifted back to a buoyant density close to native DNA (1.703 ± 0.001 g/cc [Fig. 5c]) whereas nuclear DNA failed to move back to the buoyant density of native nuclear DNA but rather formed a single band at a buoyant density of
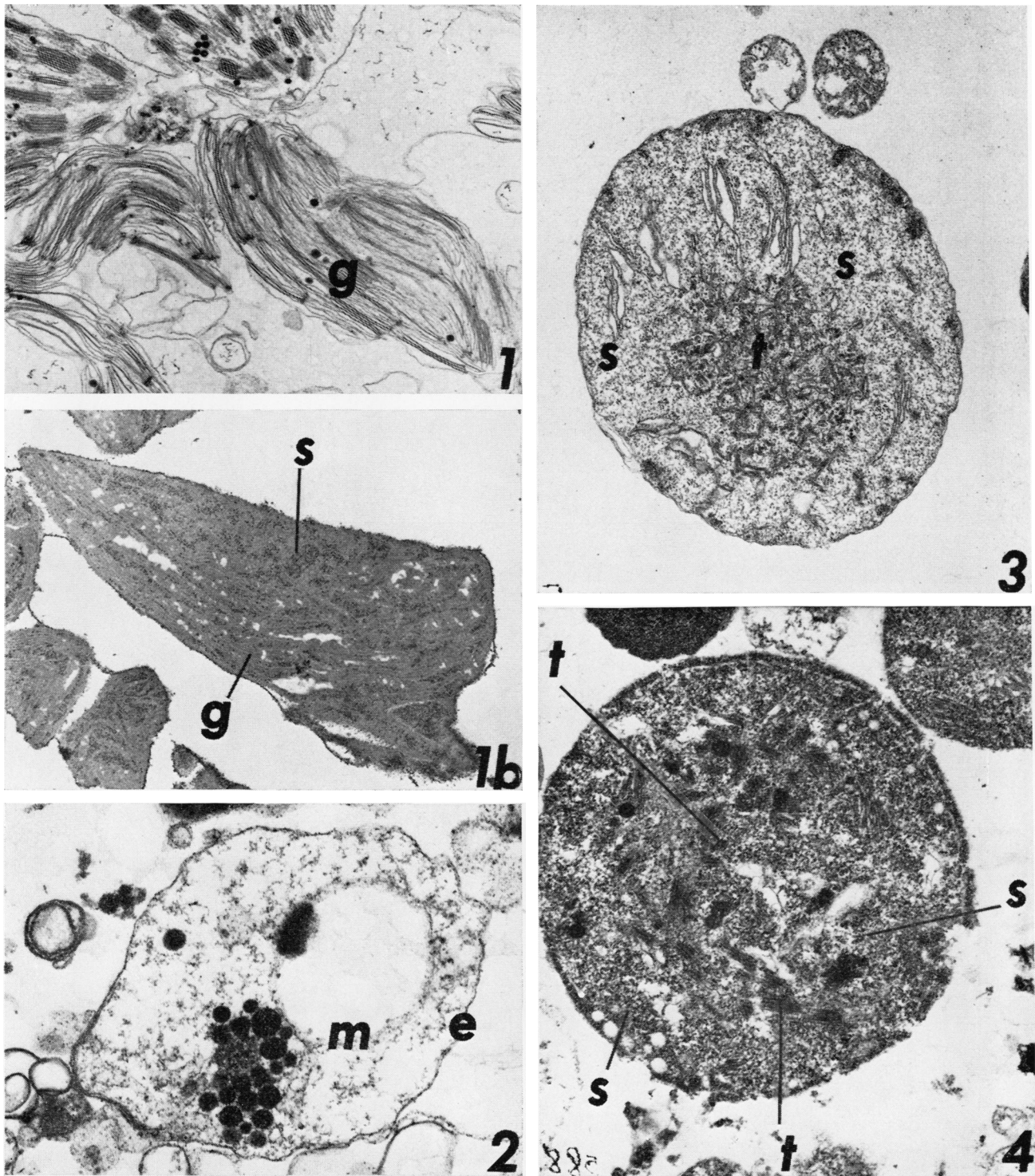
FIG. 1. Aqueous isolated chloroplasts from control, light-grown seedlings with grana (g) membranes. Note absence of stroma and outer envelope. × 9,800. b: Non-aqueous isolated chloroplast containing grana (g) and stroma (s). × 13,200.

FIG. 2. Plastid from light-grown, AT-treated plant containing only a few internal membranes (m) and outer envelope (e). × 25,700.

FIG. 3. Etioplast from control, dark-grown seedling. With prolamellar body tubules (t) scattered throughout stroma (s). × 14,600.

FIG. 4. Etioplast from AT-treated plant. Note similar appearance to Figure 3 control etioplast. × 25,700.

1.712 ± 0.001 g/cc (Fig. 6c). After renaturation, chloroplast DNA split into two bands having buoyant densities of 1.703 ± 0.001 g/cc and 1.712 ± 0.001 g/cc (Fig. 5e) indicating a mixture of chloroplast and nuclear DNA. When this chloroplast DNA was analyzed in its native condition, only a single symmetrical band (Fig. 5d) appeared. These results show that plastid DNA of

wheat has a native buoyant density similar to nuclear DNA but can be distinguished from nuclear DNA by its denaturation-renaturation properties.

During the aqueous isolation of wheat chloroplast the stroma were lost, and the plastids appeared to be class II type chloroplasts (Fig. 1a), i.e., plastids which lack outer envelope and
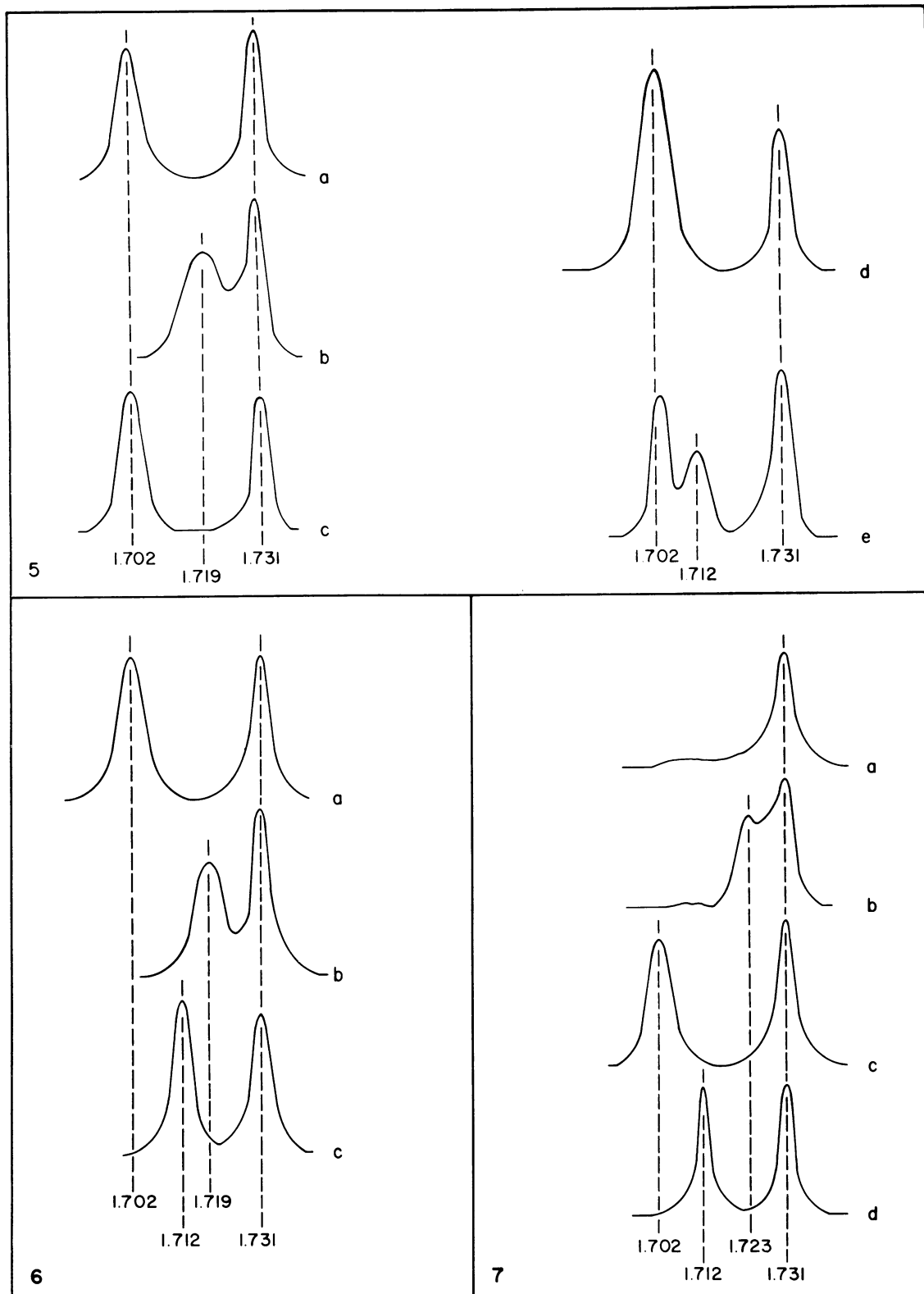
Fig. 5. Microdensitometer tracings of photographs obtained after isopycnic CsCl-density gradient centrifugation of wheat etioplast and chloroplast DNA. The concentration of DNA used in each of the figures 5–7 was not the same. a: Native etioplast DNA; b: denatured etioplast DNA; c: denatured-renatured etioplast DNA—this DNA renatured as a homogeneous fraction (1.703 ± 0.001 g/cc) returning almost to the native buoyant density (1.702 ± 0.001 g/cc); d: native chloroplast DNA; e: denatured-renatured chloroplast DNA showed two bands at 1.703 g/cc (chloroplast) and 1.712 g/cc (nuclear). Reference DNA was *Micrococcus lysodeikticus* having a buoyant density of 1.731 g/cc.

Fig. 6. Microdensitometer tracings of ultraviolet absorption photographs of wheat nuclear DNA banded in CsCl density gradients. a: native nuclear DNA; b: denatured nuclear DNA; c: denatured-renatured nuclear DNA which partly renatures with a return to buoyant density of 1.712 g/cc.

Fig. 7. Densitometer tracings of ultraviolet photographs from the plastid fraction of 3-amino-1,2,4-triazole-treated wheat seedlings. a: In most experiments plastid DNA was undetected; b: bacteria DNA (1.723 g/cc) contaminating the plastid fraction; c: Native DNA from plastid fraction (1.702 g/cc); d: DNA shown in Figure 5c (1.702 g/cc) renatured to density of 1.712 g/cc, identical to renatured nuclear DNA.

stroma matrix. To determine if any chloroplast DNA was lost from the stroma during the aqueous isolation procedure, we isolated the chloroplasts by the nonaqueous methods (Fig. 1b). The nonaqueously isolated chloroplasts contained a single DNA band with buoyant density of 1.702 ± 0.001 g/cc. Upon denaturation and renaturation, this band of DNA divided into two bands having buoyant densities of 1.703 ± 0.001 g/cc (chloroplasts) and 1.712 ± 0.001 g/cc (nuclear), and the patterns were identical to Figure 5d and 5e obtained with aqueously isolated chloroplasts.

The DNA of plastid fraction from AT-treated, light-grown seedlings (1,500 ft-c) gave three different patterns in CsCl. The first pattern (10 out of 15 experiments) showed no DNA bands (Fig. 7a). The second pattern showed a single DNA band with a buoyant density of 1.723 g/cc (Fig. 7b). An ultrastructural examination of this fraction (lower band on the Honda gradient) showed that it was contaminated with bacteria. This fraction was not collected thereafter. The third pattern showed a single DNA band with 1.702 ± 0.001 g/cc density (3 out of 15 experiments, Fig. 7c). When this DNA was subjected to denaturation and renaturation (Fig. 7d), a single band developed having a density of 1.712 ± 0.001 g/cc, identical to the buoyant density of nuclear DNA (Fig. 6a, 6c). These results indicated that light-grown, AT-treated plants lacked chloroplast DNA or quantities of it that could be detected by our methods. In our denaturation-renaturation experiments, we were not able to detect quantities of DNA below 2 to 3 μg/ml concentrations because during the experimental procedure the volume of the DNA solution was increased, thereby decreasing the concentration of DNA below detectable amounts. Chloroplast DNA of AT-treated seedlings was isolated from leaf samples identical in weight to the controls. This was done so that if any DNA was present it would be in quantities that could be detected. The etioplast fractions of AT-treated, dark-grown plants were found to contain DNA. This DNA was found to be identical to etioplasts of control plants (Fig. 5a–c) with respect to its buoyant density and denaturation properties. AT appeared to have no effect on plastid DNA of dark-grown plants.

DISCUSSION

Mache and Waygood (16) reported that the buoyant density of native wheat chloroplast DNA was 1.714 g/cc, being distinguishably denser than wheat nuclear DNA. We have never detected this DNA band in any of our preparations, but agree with them that native nuclear DNA has a buoyant density of 1.702 g/cc (Fig. 6a). In this study, chloroplast and nuclear DNA had identical buoyant densities (1.702 g/cc) in CsCl, or at least were not resolvable from each other. This conclusion is based on the observation that chloroplast DNA renatures readily after denaturation whereas nuclear DNA does not. Mache and Waygood (16) did not perform denaturation-renaturation experiments with their chloroplast DNA, and we were unable to compare our results with theirs directly.

Several explanations may be given for the conflicting results. First, it is possible that wheat chloroplasts contain more than one type of chloroplast DNA as recently reported by Bard and Gordon for spinach (1). They indicated that chloroplast DNA having a buoyant density similar to nuclear DNA made up 65% of the total chloroplast DNA and this DNA would be easiest to detect. Second, it may be that 1.714 g/cc DNA is not chloroplast in origin but rather comes from mitochondria. Hotta et al. (8) reported that a cellular fraction from wheat roots which was composed of proplastids and mitochondria contained two DNA bands having buoyant densities of 1.716 and 1.707. We suggest that the heavier band (1.716 g/cc) may be mitochondrial DNA and the lighter band (1.707 g/cc) proplastid DNA. They found that nuclear DNA of the root had a buoyant density of 1.707

g/cc, which was identical to the lighter band (1.707 g/cc) of the proplastid fraction. These results suggest that nuclear and proplastid, but not mitochondrial DNA, may have similar buoyant densities. Third, we may have lost the 1.714 g/cc DNA during aqueous isolation procedure. This probably did not happen since several ultrastructural studies (3, 27) have shown that chloroplast DNA fibers were in intimate contact with the grana and stroma lamellae membranes and not free in the stroma. Nieman and Paulsen (18) showed that chloroplast DNA resisted removal by repeated washings of the chloroplasts. Our DNA patterns from nonaqueously isolated chloroplasts which retained their stroma were identical to the DNA patterns of aqueously isolated chloroplasts. These results indicated that we were not losing the chloroplast DNA.

We tried to detect the 1.714 g/cc DNA by extracting DNA from a crude 2,500g chloroplast and nuclear pellet or from the supernatant of the Triton X-100-treated pellet and running DNA on a preparative CsCl equilibrium gradient. Triton X-100 was used in some experiments because it lyses chloroplasts but not nuclei or bacteria, thereby enabling us to separate nuclei and bacteria from chloroplasts by centrifugation (1). Fractions from the high density side of preparative run were collected, reduced in volume, and reanalyzed in an analytical centrifuge. We failed to detect the 1.714-g/cc DNA band.

There seems to be no a priori reason why the buoyant density of chloroplasts from monocotyledons should differ from that of nuclear DNA. Presently, we are unable to compare our results with other published reports since very little work has been done with monocotyledons.

Since AT has no observable effect on etioplast DNA of AT-treated, dark-grown plants, AT must work along with light to inhibit the formation or accumulation of chloroplast DNA in the leaf. Burns et al. (4) suggested that the mode of action of AT was to reduce the concentration of carotenoid pigments of the chloroplasts. This reduction of carotenoids may render the chlorophyll pigments susceptible to photooxidation in bright light. The chlorophyll molecules may then be converted to a highly reactive species, which then destroys the chloroplast DNA. Some support of this inference is shown by the work of Leff and Krinsky (15). They reported that photooxidized chlorophyll could function as a photosensitizer which reacts with chloroplast DNA resulting in a genetic alternation. Ultrastructural studies have shown that much of the chloroplast DNA exists in pockets in the stroma. In this case, some secondary photoproduct of chlorophyll destruction may diffuse out from the thylakoids and act on the plastid DNA. This mechanism may not be too likely.

AT may also affect the chloroplast DNA indirectly by inhibiting formation of grana membranes in the plastids as well as by pigment destruction. Several studies (3, 27) have shown that chloroplast DNA is bound to the plastid membranes. Since plastids of AT-treated plants lack internal membranes, the DNA, if synthesized, could not be stabilized on membranes and consequently could be lost. Many explanations can be offered to explain the effect of AT on the chloroplast DNA. However, at this time, we feel that the best interpretation of our results is that AT brings about a metabolic change in the developing plastids of light-grown plants which either destroys the chloroplast DNA or blocks the synthesis of chloroplast enzymes or structural components necessary for DNA synthesis, thereby resulting in loss of chloroplast DNA.

The mode of action of AT in higher plants is still unknown. Hilton (7) reported that the primary site of action of AT in heterotropic microorganisms is the inhibition of dehydratase enzyme in the histidine pathway or inhibition of purine and one-carbon metabolism. However, these mechanisms do not explain AT action on higher plants.

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