IV. Regulation of din1 Expression by Sugar

<INTRODUCTION>

As described in Chapter III, I cloned a cDNA sequence complementary to transcript from a senescence-associated gene of radish, din1. The transcript from din1 showed large accumulation in fully grown cotyledons after transfer of the plants to darkness that accelerates the senescence of the tissue. The accumulation was detected within 6 h after exposure to darkness and reached a level over 100-fold higher than that in light-grown cotyledons at 24 h. din1 was activated not only by darkness but also by exposure to other senescence-accelerating stimuli such as ethylene and heat stress to about 20-fold higher level than that of light-grown plants. Among the stimuli applied to din1, dark treatment induced especially strong expression of din1 and attracted my particular attention.

In many cases, changes in gene expression caused by alteration of light condition are mediated by photoreceptors, like phytochrome, blue-light receptor (cryptochrome), and UV light receptor (Tobin and Silverthorne, 1985; Kendrick and Kronenberg, 1986). Phytochrome is the best characterized photoreceptor among them and accumulated as Pfr form under light, and in darkness phytochrome is gradually transformed to

Pr form and than degraded. Phytochrome can be maintained in Pfr form in darkness by illumination with intermittent red light, and can be transformed instantaneously to Pr form by illumination with far-red light (Butler et al., 1964). It has been known that phytochrome mediates light response of many genes, such as those of phytochrome itself (Lissemore and Quail, 1988), small subunit of Rubisco protein (Berry-Lowe and Meagher, 1985) and NADPH-protochlorophyllide reductase (Darrah et al., 1990). Blue light also controls transcription and mRNA level of many nuclear and plastid genes (Richter et al., 1987). mRNA of chalcone synthase has been shown to increase after exposure to UV light (Kreuzaler et al., 1983).

Another candidate for the sensor of light condition is plastid. Plastids change their structure and activity in response to light condition and differentiate into chloroplasts or other related organelles. Chloroplast has been suggested to release a hypothetical plastidic factor(s) to regulate nuclear photosynthetic genes at an early stage of development (Oelmuller and Mohr, 1986; Taylor, 1989). Photosynthesis also can transfer light signals to nuclear genes through photosynthate or other products by activities which are influenced by photosynthesis. It was recently reported that photosynthetic genes in maize are coordinately repressed by metabolites of photosynthetic products, such as sucrose and glucose (Sheen, 1990).

Dark treatment has long been used for induction of

senescence in leaves. However the mechanism involved in the induction by dark treatment has not been well understood. Because expression of *din1* is closely associated with the progress of senescence, elucidation of activation mechanism of *din1* by dark treatment may provide us information for the promotion mechanism of senescence by dark treatment. Therefore, I focused my study on a biochemical signal by which dark treatment activates *din1* gene, and tried to explain the way of response of the gene to a variety of stimuli.

<MATERIALS AND METHODS>

1. Plant Materials

Radish seedlings were grown on vermiculite beds at 25°C under continuous light as described in Chapter II. Experiments were done with seedlings on the 14th day of growth.

Light Treatments: For dark treatment, seedlings were transferred to the dark at 25°C. Cotyledons were harvested under dim-green light after a defined period.

For the irradiation of plants with red light and far-red light, the experimental conditions reported by Lumsden et al. (1987) were followed.

For red light irradiation, seedlings were placed in the dark and illuminated intermittently for 5 min with 55 min intervals with broad-band red light (580-710 nm) from a white light fluorescent lamp (Hitachi FL15W-F-G) covered with three layers of red plastic film.

For far-red light irradiation, seedlings were illuminated for 5 min with broad-band far-red light (710-810 nm) from a daylight fluorescent lamp (Toshiba FL 20 SD) through a red plastic sheet, Shinkolite A (Mitsubishi Rayon) and an infrared filter (Koutou Denki), and kept in the dark for the rest of period.

For blue light irradiation, seedlings were placed under blue light from a fluorescent lamp (Hitachi FL15W-F-G) covered

with three layers of blue plastic film.

Application of Inhibitors and Sugars: Tissue discs of 10 mm diameter were punched out from the central part of cotyledons of the 14-day-old seedlings. The cotyledonary discs were floated on a solution of 1 μM DCMU or 150 μM atrazine in a Petri dish. Sugars were added to water or the inhibitor solutions at indicated concentrations. The Petri dishes were placed under the same light conditions as for the growth or in the dark. The cotyledonary discs were harvested after 24 h of incubation.

Exposure to Ethylene: Cotyledonary discs were floated on water or 100 mM glucose solution, and transferred into an air-tight Plexiglas box in which ethylene was added at 10 μl/l, and kept under continuous light at $25^{\circ}C$ for 24 h.

Heat Stress: Cotyledonary discs were floated on water or 100 mM glucose solution at $35^{\circ}C$, and transferred to a growth chamber and kept at $35^{\circ}C$ for 2 h under illumination.

2. Dot-Blot Hybridization

Preparation of high-molecular-weight RNA from the treated cotyledons or cotyledonary discs, blotting of the RNA to nylon membranes and hybridization with labeled clone-1 sequence was carried out as described as Chapter II.

3. Determination of Sugar Content

Five g of cotyledons were harvested at indicated time of dark treatment and homogenized with 20 ml of 80% (v/v) ethanol in a blender and incubated at 80°C for 10 min. The homogenate was centrifuged at 10,000g for 10 min, and the pellet was reextracted with 10 ml of 80% ethanol. The supernatant fractions were combined and lyophilized for assay of sugar content. The pellet was also lyophilized and stored for assay of starch content. The lyophilized materials containing sugars were dissolved in H_2O and cleared by centrifugation at 10,000g. The supernatant was passed consecutively through columns of Dowex 50W, Dowex 1 (Dow Chemical), and a Sep-Pack Cartridge (Millipore). Contents of D-glucose, D-fructose, sucrose and starch were determined with a Biochemical Analysis System from Boehringer Mannheim.

4. Paper Chromatography of 2-Deoxyglucose

[2,6-^{3}H]2-deoxyglucose (7.4 Mbq) was lyophylized and dissolved in 40 μl of 0.1% Tween 20. Cotyledons were harvested from radish seedlings grown under continuous light or from those placed in the dark for 12 h. Four cotyledons were painted with 4 μl each of the labeling solution and incubated for 2 hours at 25 °C under the same light conditions as for the growth of the plants. The labeled cotyledons were homogenized

on ice and centrifuged at 10,000g for 5 min, and 5 μl of the supernatant was spotted on filter paper. Four cotyledons from either light-grown or dark-treated plants were homogenized in 0.5 ml of 50 mM Tris-HCl, pH 7.5 and 2 μl of labeling solution was added to a 20-μl portion of the homogenate. After 30 min of incubation at 25°C, the samples were centrifuged at 10000g for 5 min and 5 μl of the supernatants was spotted on filter paper.

Whatman 3MM filter paper was washed with 1 mM EDTA and H_2O, then dried. The labeled samples were spotted on the paper and developed in a solvent (2-propanol:pyridine:H_2O:acetic acid, 8:8:4:1, v/v). Intensity of signals was increased by ENHANCE Spray (DuPont Co.), and 2-deoxyglucose and its metabolite were detected by fluorography.

<RESULTS>

1. Involvement of Photoreceptors in the Induction of *din*1 by Darkness

As a preliminary experiment, radish seedlings were continuously illuminated with red, far-red, or blue light for 24 h, and accumulation level of *din*1 mRNA in the cotyledons was analyzed by dot-blot hybridization (Fig.4-1). Irradiation with blue light did not show any effects on dark-induced accumulation of *din*1 mRNA. However, red light and far-red light appeared to have slight influence on the level of the mRNA. As this observation indicated possible involvement of phytochrome in *din*1 regulation, further experiments with these specific lights were carried out.

If phytochrome takes part in signal transduction pathway for the induction of *din*1, the Pr form serves as an activator or the Pfr form as a repressor. In order to test this possibility, I placed radish seedlings in the dark with intermittent illumination of red light for 5 min at every 55 min intervals to keep phytochrome in Pfr form. Total cellular high-molecular-weight RNA was extracted from the cotyledons and expression level of *din*1 gene was analyzed by dot-blot hybridization. This treatment did not have any effects on the strong induction of *din*1 by darkness (Fig.4-2). Other seedlings were illuminated with far-red light for 5 min to

Fig.4-1. Effects of illumination with light of different wave length on the accumulation of transcripts from <u>din</u>1. Radish seedlings were placed under various light conditions, and total cellular high-molecular-weight RNA was prepared from the cotyledons. RNA was blotted on nylon membranes and hybridized with a probe for transcripts from <u>din</u>1 (panel 1) or with that for transcripts from the control gene (panel C). Lane L, RNA from light-grown radish cotyledons; lane D, RNA from those placed in the dark for 24 h; lane R, RNA from those illuminated with red light for 24 h; lane F, RNA from those illuminated with far-red light for 5 min then placed in the dark for 24 h; lane B, RNA from those illuminated with blue light for 24 h.

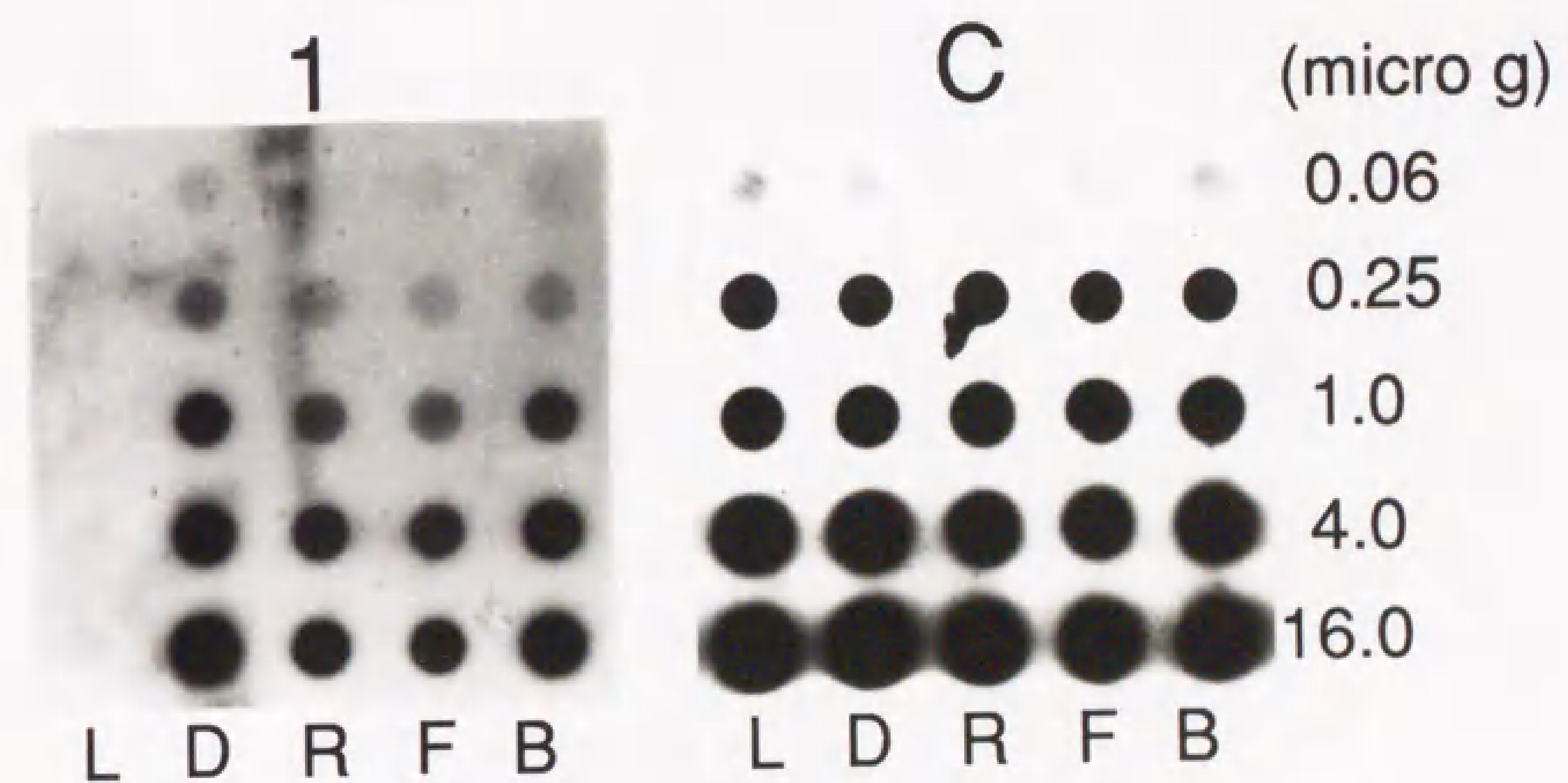
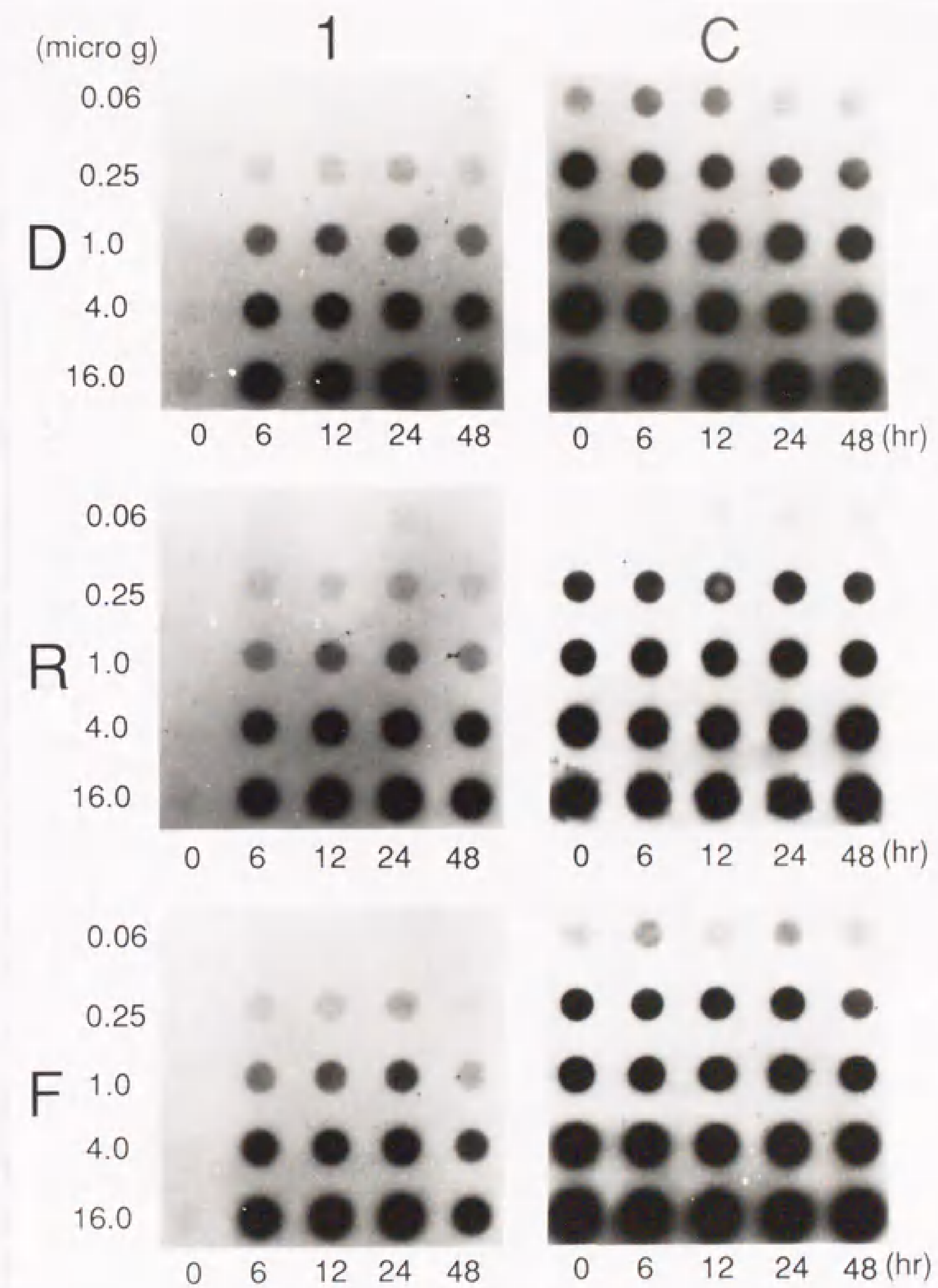


Fig.4-2. Accumulation of transcripts from <u>din</u>1 and from control gene in different light treatments. The whole seedlings on a vermiculite bed was transferred to the dark (D), intermittent irradiation of red light (R), or irradiation of far-red light for 5 min then transferred to the dark (F). After defined periods, cotyledons were harvested under dim-green light and high-molecular-weight RNA was prepared. Serially diluted RNA was applied to nylon membranes, and transcripts from <u>din</u>1 and control gene were probed with clone-1 sequence (1) and with clone-C sequence (C), respectively.



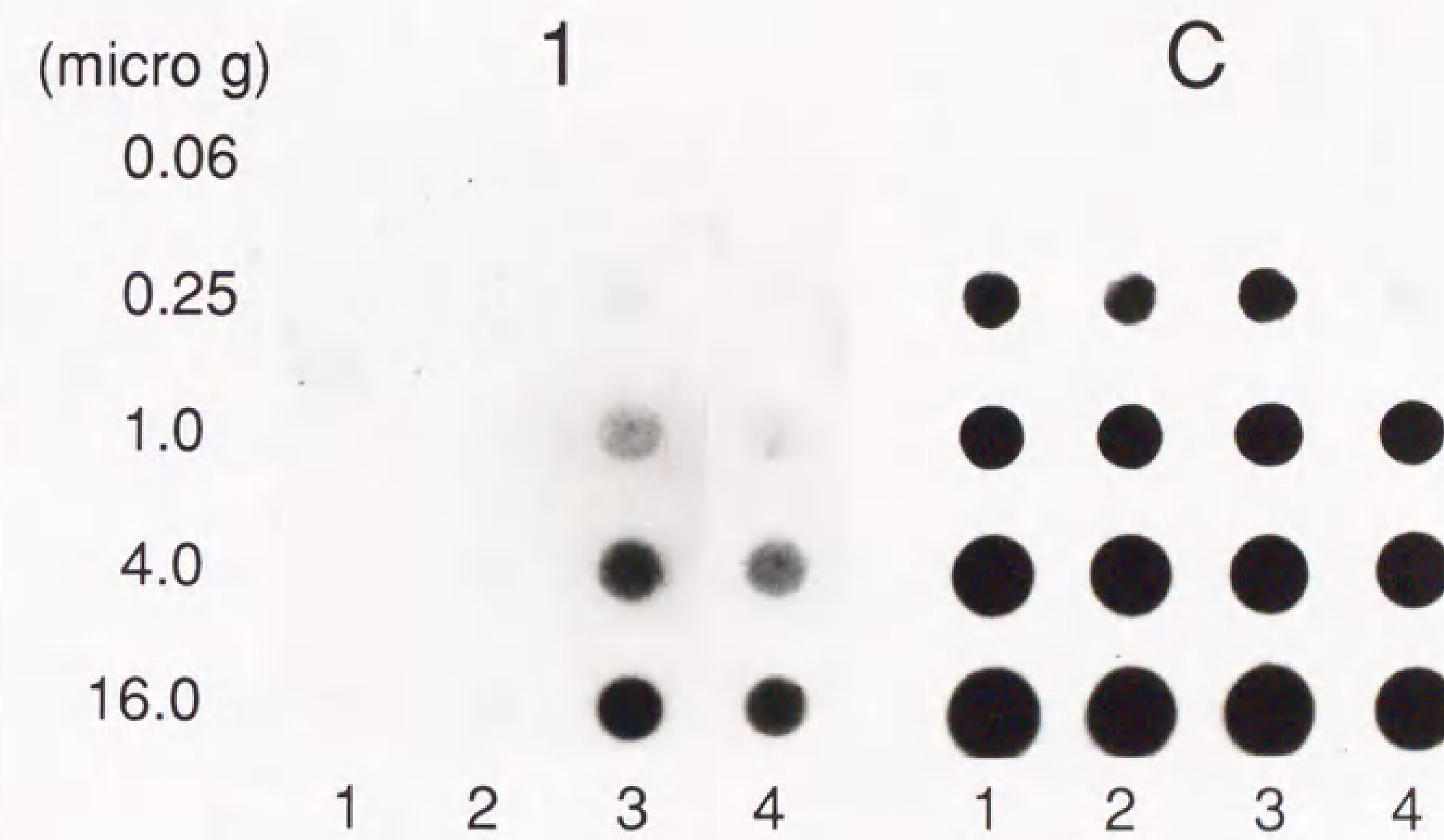
accelerate the conversion of Pfr to Pr form, and placed in the dark. Expression of _din1_ was not affected at all by this treatment either (Fig.4-2). These results indicate that phytochrome is not involved in the activation of _din1_ gene by dark treatment.

2. Photosynthesis as a Signal for the Induction of _din1_

Primary effect of dark treatment on chloroplasts is deprivation of light energy and cessation of photosynthesis. Prolonged dark treatment lowers the level of photosynthate, which may serve as a signal to activate _din1_ gene. In order to examine this possible function of photosynthesis as a signal for the induction of _din1_, I sprayed seedlings with photosynthesis inhibitors, DCMU and atrazine, placed under white light, and analyzed the level of _din1_ transcript. These inhibitors effectively induced _din1_ expression under light condition (Fig.4-3). This result suggested that _din1_ is repressed by photosynthate.

Major products of photosynthesis are CO_2 assimilates and high energy compound, ATP. The CO_2 assimilates are used to synthesize sugars, starch, amino acids, fatty acids, and other organic compounds. Among these photosynthetic products, the levels of starch and sugars in the cell are most directly affected by photosynthetic activity of the cell. However, starch is an insoluble material accumulated in the chloroplasts

Fig.4-3 Effects of photosynthesis inhibitors on the accumulation of transcripts from <u>din</u>1 and from the control gene. Cotyledonary discs were floated on water (lane 2), 1 μM DCMU (lane 3), or 150 μM atrazine (lane 4) and placed under white light for 12 h. High-molecular-weight RNA was prepared from intact cotyledons of light-grown seedlings (lane 1) and from the discs (lane 2, 3, 4). RNA was serially diluted and applied to a nylon membrane, then probed for <u>din</u>1 transcripts (1) and for transcripts from the control gene (C) with clone-1 sequence and clone-C sequence, respectively.



and can be eliminated from candidates for a signal to regulate gene expression.

I first examined whether exogenously supplied glucose, as a representative of sugars, repressed the expression of din1 (Fig.4-4). In this experiment, cotyledonary discs were prepared from 14-day-old radish seedlings with a cork borer and floated on a solution of 100 mM glucose. Glucose exogenously supplied in this way strongly suppressed the induction of din1 by dark treatment or 1 μM DCMU. An effective concentration for glucose to suppress the induction was estimated to be between 4 and 20 mM (Fig.4-5). These results suggested that glucose plays an important role in the regulation of din1 expression. However, it can not be concluded that glucose itself is a regulator of din1 gene because glucose can be readily converted to its metabolites and other sugars in the cotyledonary cells and these metabolites may also serve as a signal.

dGlc, 3mGlc, and βmGlc are analogues of glucose. dGlc and 3mGlc are generally known not to be metabolized in plant cells. If glucose itself, but not its metabolites, suppresses the induction of the gene, these analogues could also show a similar suppressive effect on the induction by dark treatment. The cotyledonary discs floated on 10 mM dGlc did not express din1 in darkness, but the discs on 10 mM 3mGlc or 10 mM βmGlc did accumulate din1 transcripts (Fig.4-6). As only dGlc showed a positive effect, the possibility of conversion of this

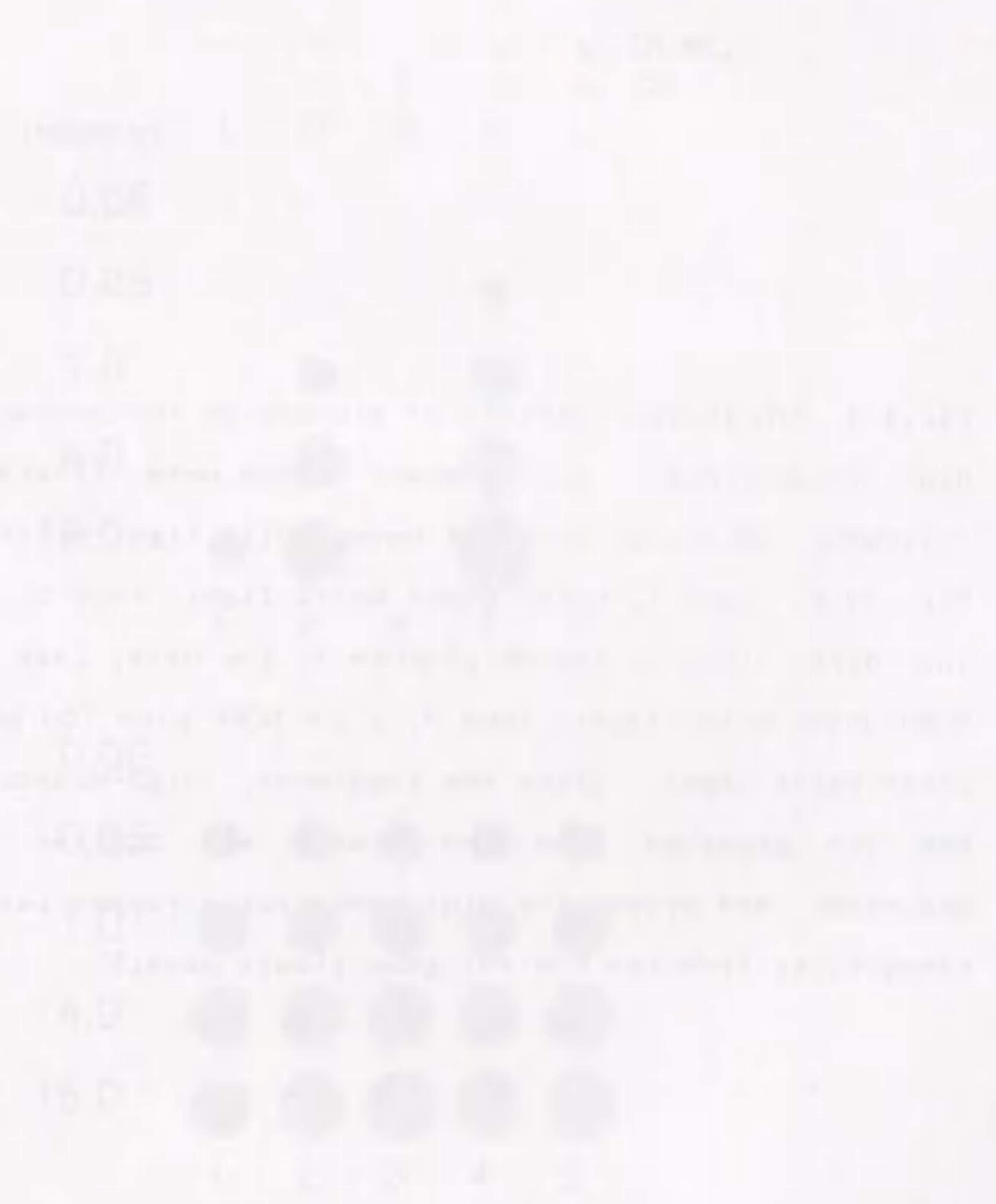


Fig.4-4 Inhibitory effects of glucose on the accumulation of din1 transcripts. Cotyledonary discs were floated on the following solutions and kept under white light or in the dark for 12 h. Lane 1, water under white light; lane 2, water in the dark; lane 3, 100 mM glucose in the dark; lane 4, 1 μM DCMU under white light; lane 5, 1 μM DCMU plus 100 mM glucose under white light. After the treatment, high-molecular-weight RNA was prepared from the tissue and blotted on nylon membranes, and probed for din1 transcripts (upper panel) or for transcripts from the control gene (lower panel).

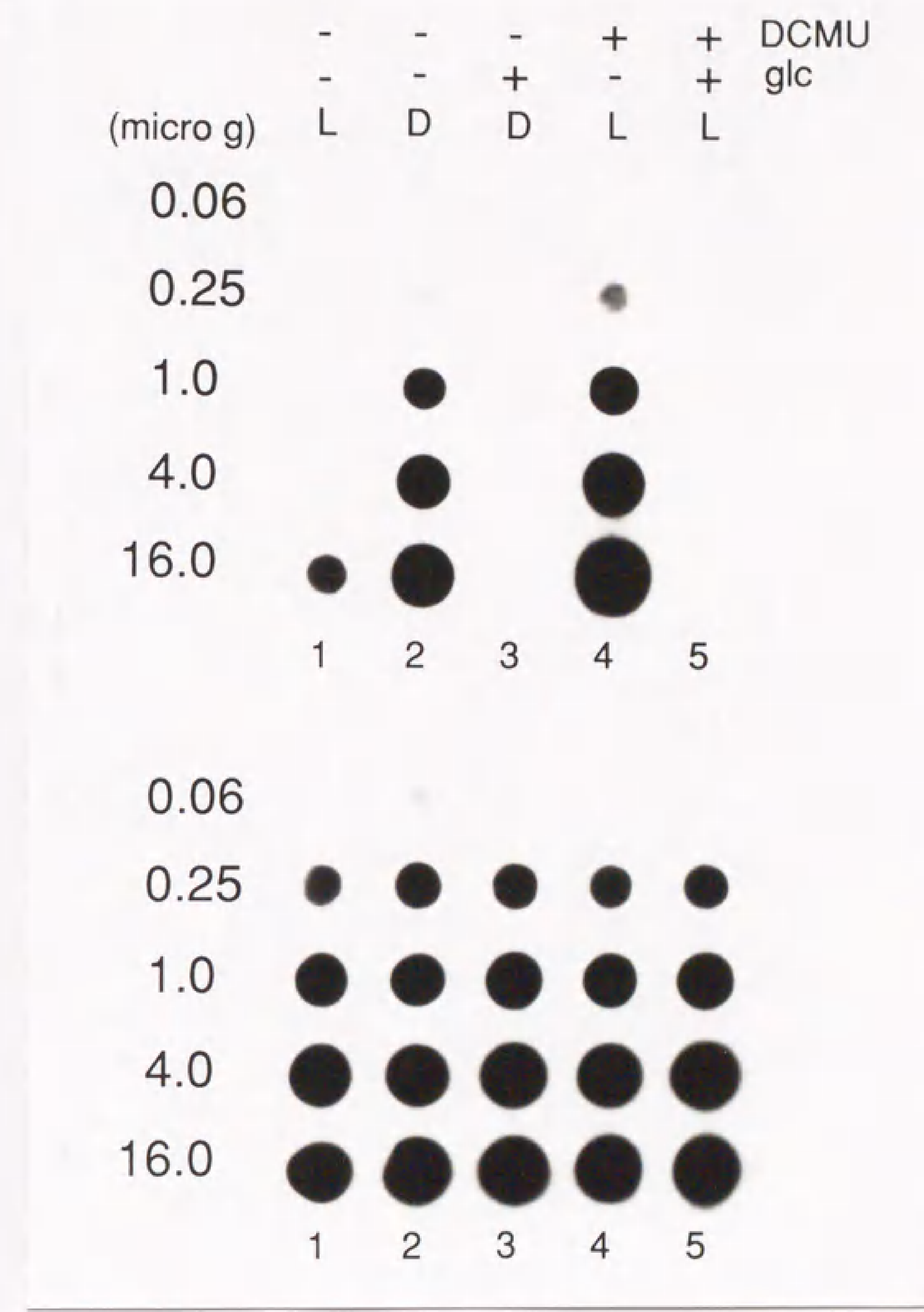
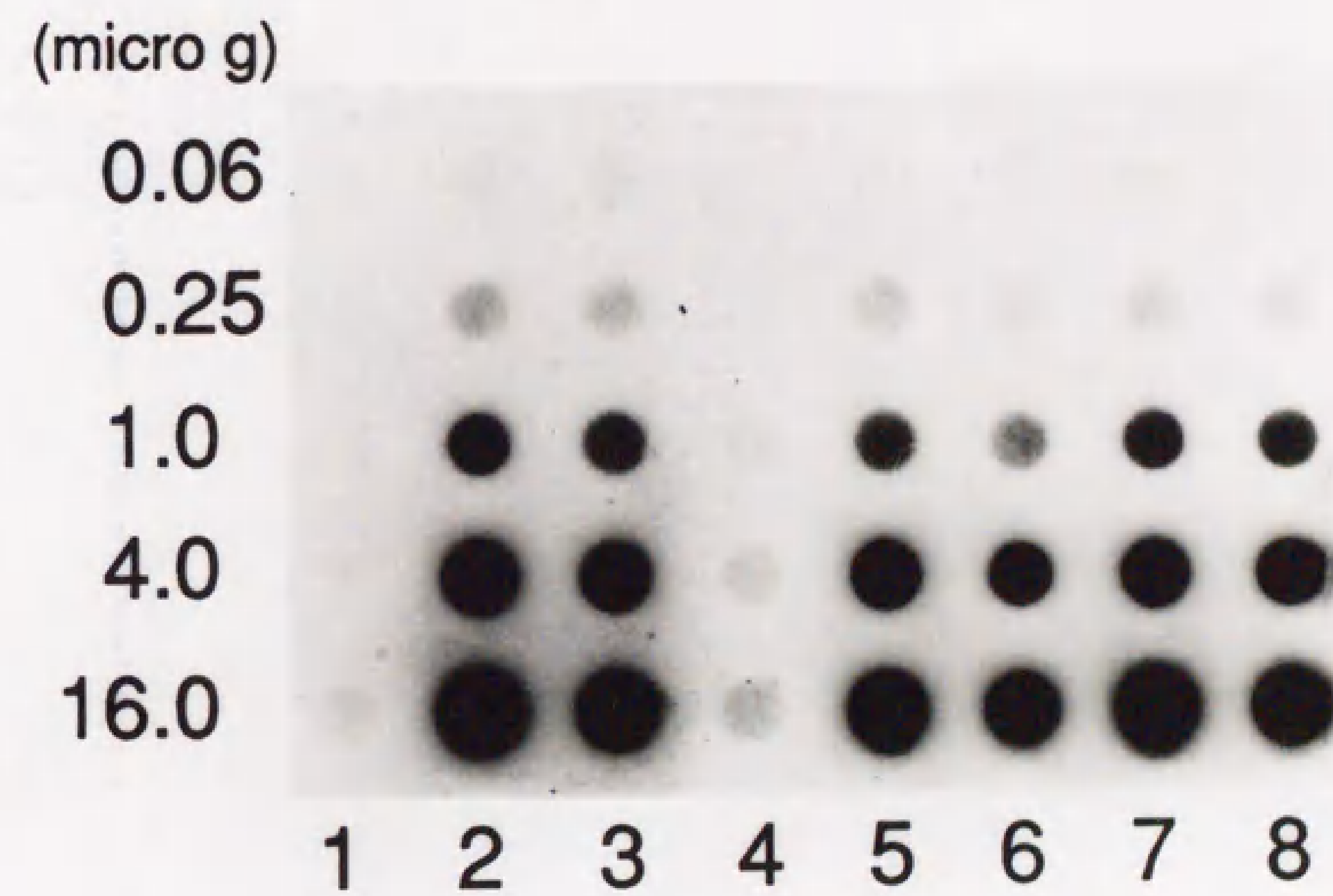


Fig.4-5. Effective concentration of glucose for repression of din1. Cotyledonary discs prepared from 14-day-old radish seedlings were floated on the following solutions and kept under light (lane 1) or in darkness (lane 2-6) for 12 h. Total cellular high-molecular-weight RNA was prepared from the discs, blotted on nylon membrane and hybridized with a probe of clone-1 sequence. Lane 1 and lane 2, water; lane 3, 100 mM glucose; lane 4, 20 mM glucose; lane 5, 4 mM glucose; lane 6, 1mM glucose. (Lane 7, RNA of cotyledon discs incubated at 35°C for 2 h on water; lane 8, RNA of cotyledons incubated at 35°C for 2 h on 100 mM glucose.)

(micro g)
0.06
0.25
1.0
4.0
16.0
1 2 3 4 5 6 7 8

Fig.4-6. Effects of glucose analogs. Effects of dGlc, 3mGlc and βGlc on the accumulation of din1 transcripts. Cotyledonary discs were prepared from 14-day-old radish seedlings and floated on the following solutions and kept under light (lane 1) or in the dark (lane 2, 3, 4, 5, 6, 7 and 8) for 12 h. Lane 1, water; lane 2, water; lane 3, 1 mM dGlc; lane 4, 10 mM dGlc; lane 5, 1 mM 3mGlc; lane 6, 10 mM 3mGlc; lane 7, 1 mM βGlc; lane 8, 10 mM βGlc. Total cellular high-molecular-weight RNA was prepared from the treated discs, blotted on nylon membrane and hybridized with a probe of clone-1 sequence.



glucose analogue into other forms was examined by tracing the fate of ^{3}H-dGlc added to the tissue. Unexpectedly, dGlc was converted to an unidentified compound in radish cells (Fig.4-7). I found in the literature that dGlc is converted to 2-deoxysucrose in maize plants (Saglio, 1985). These findings may suggest that a metabolite(s) of glucose rather than glucose itself serve as a signal to suppress the induction of the gene.

Sucrose and fructose at 10 mM suppressed the induction of din1 as efficiently as glucose (Fig.4-8). All of these sugars were ineffective at 1 mM. Mannitol did not show any effects up to 100 mM (Fig.4-9). These observation indicate that the suppressive effect is not caused by the osmotic pressure of the added sugars.

3. Sugar Content in Cotyledons after Exposure to Darkness

As described above, the induction of din1 gene was totally suppressed by exogenous addition of sugars to the cotyledons. This observation suggested that intracellular level of sugar serves as a biochemical signal for the expression of din1 and that the dark treatment activates the gene by lowering the sugar level. To confirm this mechanism, I measured the actual level of sugars in the cotyledonary cells after exposure to darkness (Fig.4-10).

Starch was completely exhausted within 6 h (Fig.4-10). The levels of sucrose and fructose decreased immediately after

Fig.4-7. dGlc is metabolized in radish cotyledons. [2,6-^{3}H]2-deoxyglucose was incubated with an extract of light-grown cotyledons (lane 2) or that of cotyledons placed in the dark for 12 h (lane 3) for 30 min at 25°C. Other sets of cotyledons were harvested from light-grown plants and those placed in the dark for 12 h, painted with [2,6-^{3}H]2-deoxyglucose and incubated at 25°C for 2 h (lane 4 and 5, respectively). The cotyledons were homogenized and centrifuged. [2,6-^{3}H]2-deoxyglucose (lane 1) and the extracts were spotted on Whattmann filter paper and chromatograghed. An arrow indicates the origin of development.

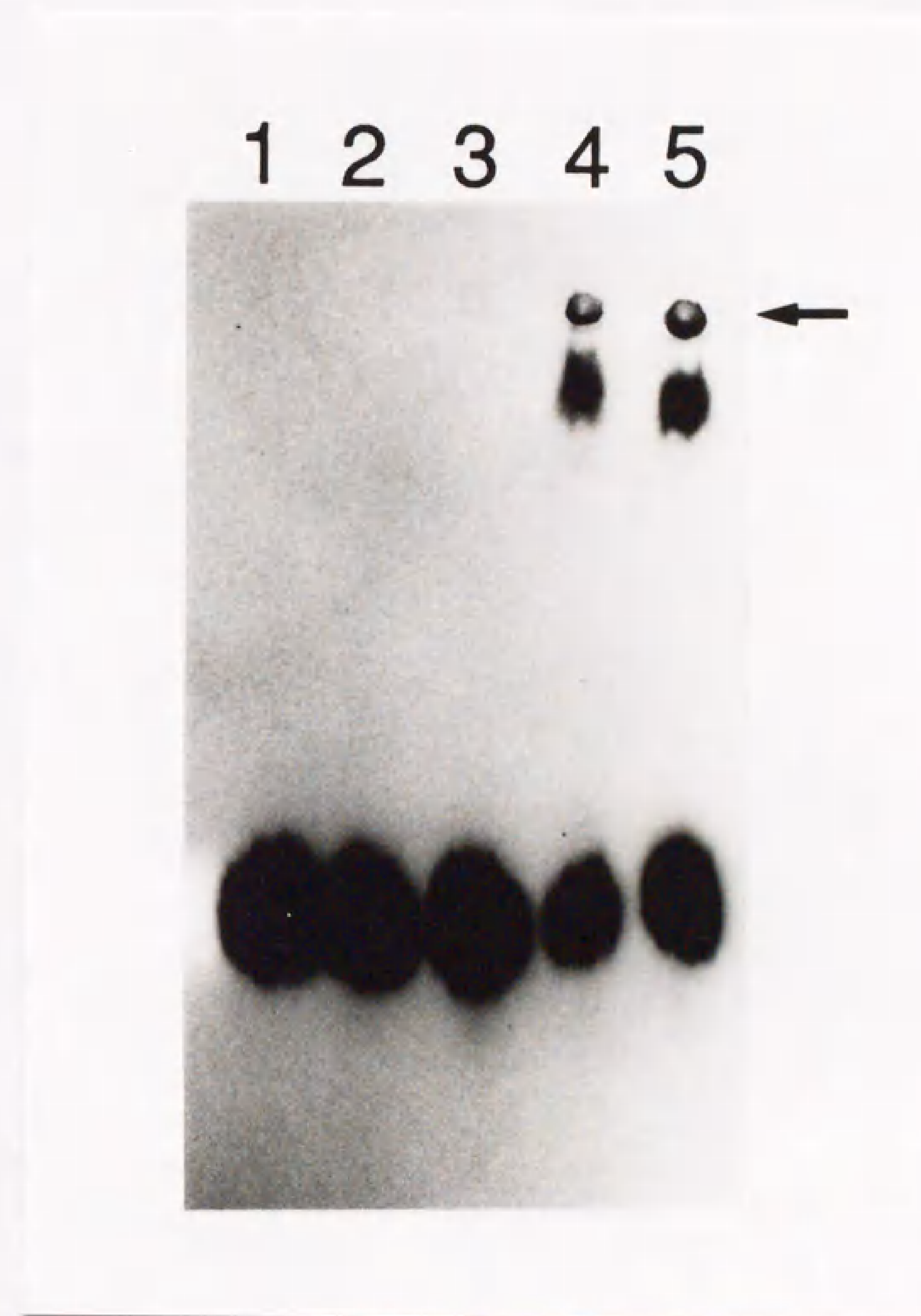


Fig.4-8. Effects of different sugars on the accumulation of din1 transcripts. Cotyledonary discs were floated on the following solutions in the dark for 12 h, except those which were placed under white light (lane 1); water, lane 1 and 2; sucrose, 1 mM (lane 3) and 10 mM (lane 4); fructose, 1 mM (lane 5) and 10 mM (lane 6); glucose, 1 mM (lane 7) and 10 mM (lane 8). High-molecular-weight RNA was prepared from the tissue and blotted on a nylon membrane, then probed for din1 transcripts.

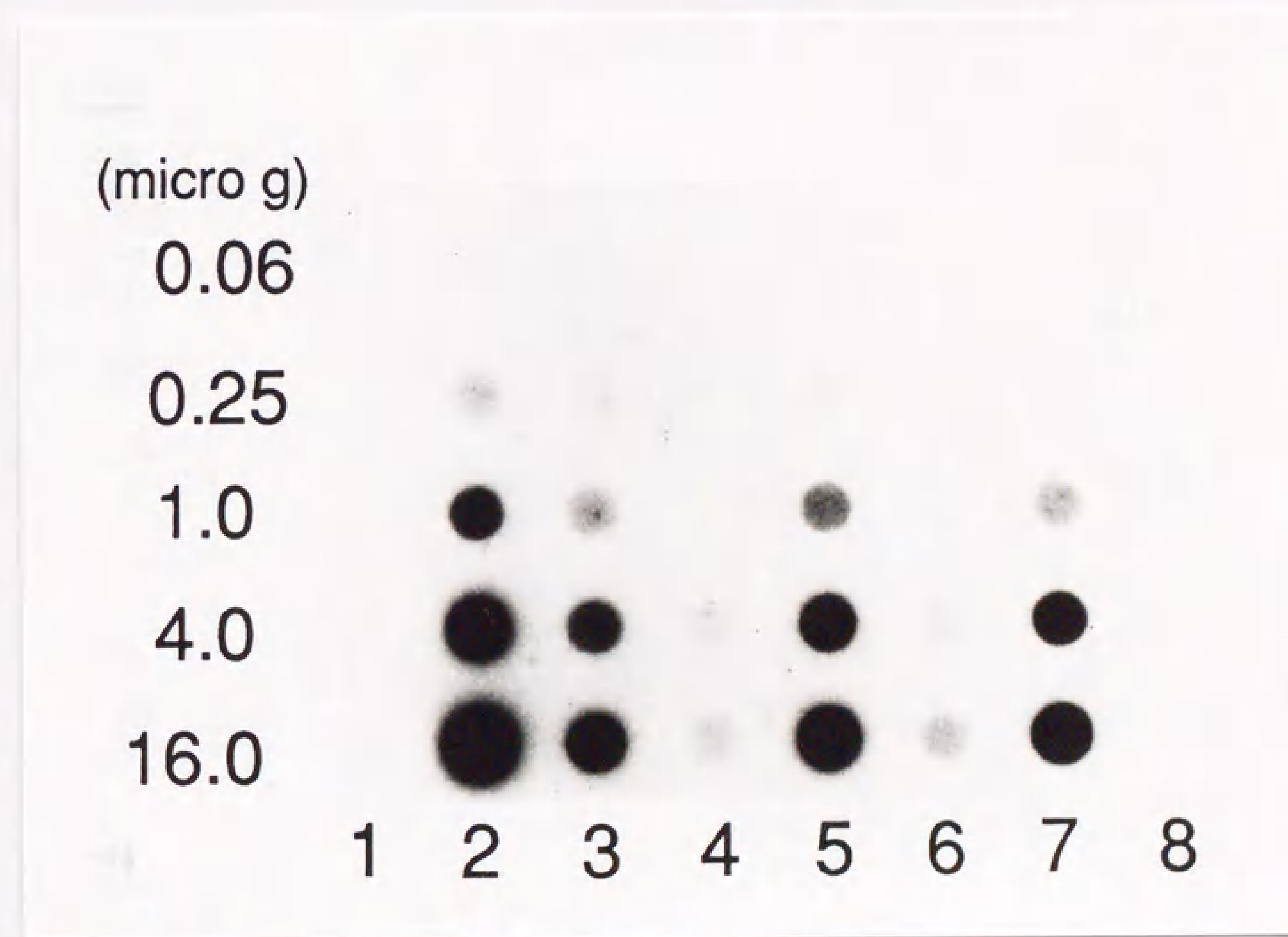


Fig.4-9. Effect of osmotic pressure on the accumulation of din1 transcripts. Cotyledonary discs were floated on the following solutions in the dark for 12 h, except those which were placed under white light (lane L). Lane L, water; lane D, water; lane M1, 100 mM mannitol; lane M2, 10 mM mannitol; lane M3, 1 mM mannitol. (Lane G1, 100 mM dGlc; lane G2, 10 mM dGlc; lane G3, 1 mM dGlc.)

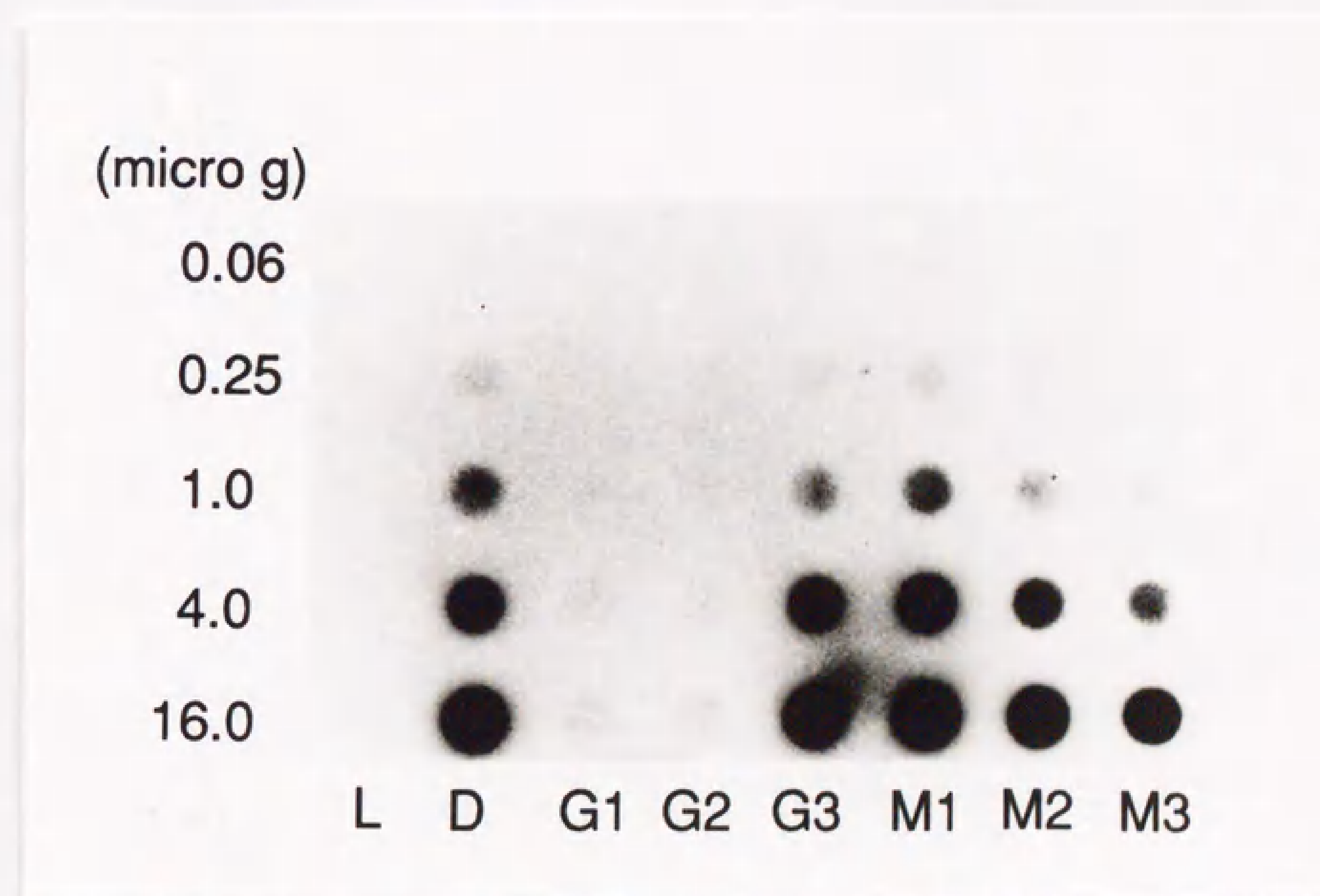
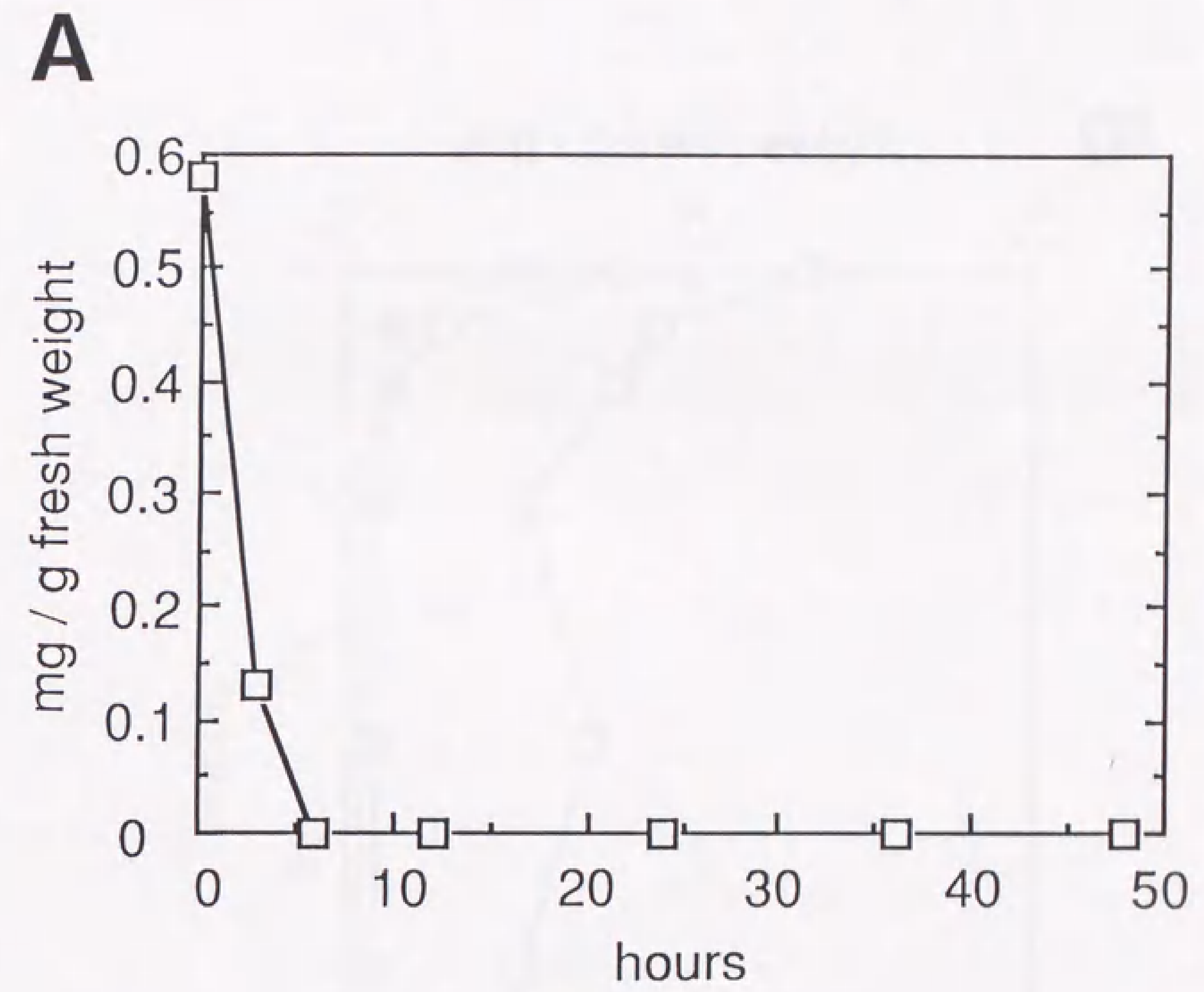
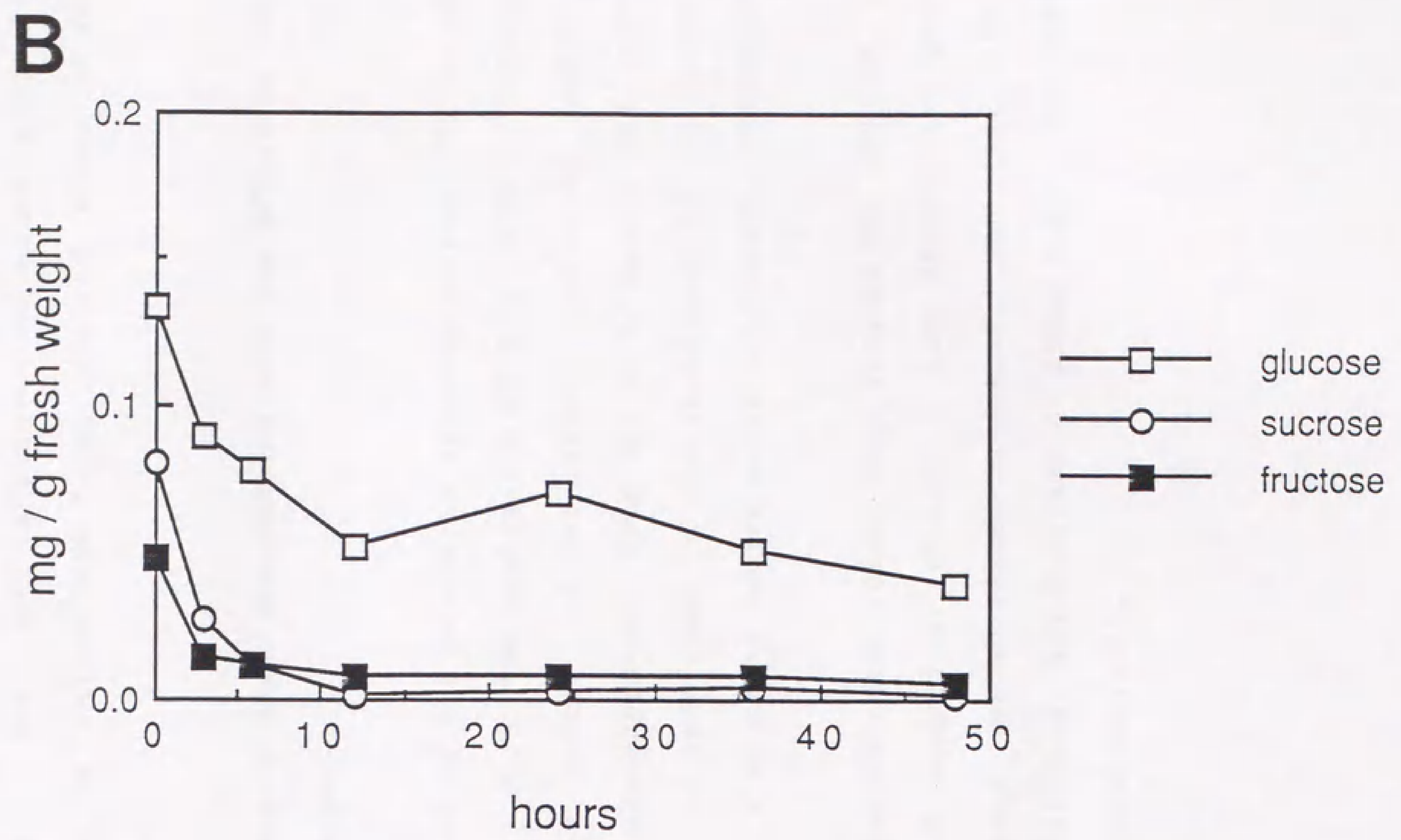


Fig.4-10. Changes in starch and sugar contents during dark treatment. Radish seedlings on vermiculite beds were transferred to darkness and cotyledons were harvested at 0, 3, 6, 12, 24, 36, 48h. Water-soluble sugars were extracted with 80% ethanol, and contents of glucose, sucrose, and fructose were determined. Starch was determined with the ethanol-insoluble materials. Content was plotted against the time of dark treatment. A: Content of starch. B: Content of sugars.




B
mg / g fresh weight
0.2
0.1
0.0
0
10
20
30
40
50
hours
glucose
sucrose
fructose

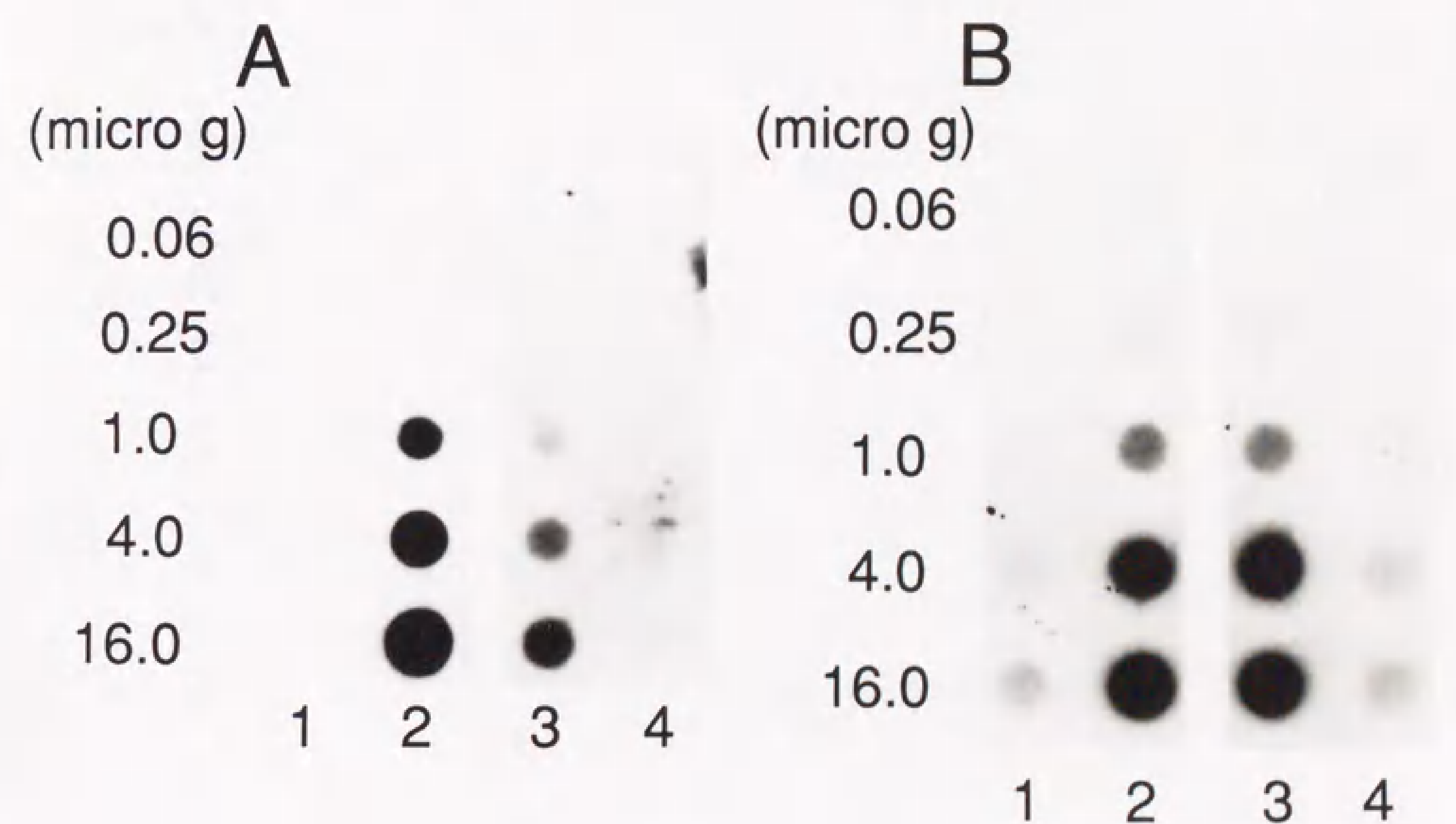
transfer to darkness and became stable at a very low level, that is, about 1/10 and 1/5 of that in light-grown cotyledons, respectively. The level of glucose decreases gradually and remained at a rather high level, that is, about a half of initial level.

4. Dominance of Sugar Suppression over the Effect of Ethylene and Heat Stress

Exposure of the plants to ethylene or heat stress has been shown to induce the expression of din1 gene (Chapter II). Plant cells accept various stimuli such as light, heat stresses, phytohormones from the environment and the plant itself at the same time. I was interested in the response of din1 when a plant is subjected to different contemporaneous stimuli.

When cotyledonary discs were floated on 100 mM glucose solution and exposed to ethylene or heat stress, the expression of din1 gene was abolished in either case (Fig.4-11). It appears that suppressive effect of added sugar overcomes the action of these stimuli.

Fig.4-11. Glucose effects on the induction of din1 by heat stress and ethylene. A: Cotyledonary discs were floated on prewarmed water (lane 3) or 100 mM glucose (lane 4) and placed in a growth chamber at 35°C for 2 h. High-molecular-weight RNA was prepared from the tissue and blotted on a nylon membrane, then probed for din1 transcripts. In order to compare strength of hybridization signals, RNA prepared from cotyledons of light-grown seedlings (lane 1) and from 12 h dark-treated seedlings (lane 2) were blotted on the same membrane. B: Cotyledonary discs were floated on water (lane 3) or 100 mM glucose solution (lane 4) and transferred into a Plexiglas box. Ethylene was injected into the box at 10 μl/l and kept under white light for 12 h. Lane 1, RNA prepared from cotyledons of light-grown seedlings; lane 2, RNA from those of 12 h dark-treated seedlings.



<DISCUSSION>

Dark treatment has long been used to promote senescence of leaves. *In vitro* translation experiments indicated that dark treatment induces expression of a number of genes (Malik, 1987; Kawakami and Watanabe, 1988). In spite of these studies, mechanism by which dark treatment promotes the progress of senescence has been left to be uncovered. The only hypothesis explaining the relation between dark treatment and senescence is that light conditions determine the extent of stomatal opening, which in turn controls progress of senescence (Thimann, 1979). Since *din1* gene is associated to the progress of senescence as described in Chapter II, understanding of activation mechanism of *din1* by dark treatment will contribute to elucidate the mechanism of dark acceleration of leaf senescence.

As illumination with red, far-red, or blue light did not affect the expression of *din1*, I abandoned the idea that phytochrome or blue-light receptor is involved in the regulation of the gene. Instead, I found that photosynthetic activity of the cell is the key to understand the biochemical signal released by dark treatment to activate the gene.

Cotyledons floated on DCMU or atrazine solution expressed *din1* under white light. These reagents inhibit photoelectron flow and subsequent synthesis of ATP. This inhibition results

in the cessation of CO_2 fixation and other activities of chloroplasts. The induction by the inhibitors was suppressed by addition of glucose. These results support the idea that products of CO_2 fixation contribute to the repression of din1 under light conditions. These inhibitors stop photoelectron flow and at the same time severely damage structure of chloroplast under illumination. The fact that addition of glucose suppressed the induction by the inhibitors ruled out the possibility that stress caused by the destruction of chloroplast structure might have induced din1 expression.

Exogenous supply of sucrose, fructose, and glucose suppressed the induction of din1 gene. Sucrose was slightly more effective, probably because sucrose is a disaccharide and it produces metabolites in double quantities of monosaccharides. The observation that unmetabolizable 3mGlc was not effective suggest that a metabolite(s) of glucose rather than glucose itself serves as a signal for the repression of din1 gene. The possibility for glucose to be a direct signal is, however, not excluded because 3mGlc is known to be imported into the cell slower than glucose (Lin et al., 1984; Xia and Saglio, 1988), and because methylation may interfere the function of glucose as a regulatory factor. Genes for storage proteins of tuberous organs are known to be regulated by sugars, but in this case a high level of sucrose induces expression of the genes (Wenzler et al., 1989).

As described in Chapter II, din1 expression is induced

within 6 h after transfer to the dark. Actual contents of glucose, fructose, and sucrose in cotyledonary cells decreased rapidly after exposure to the dark. This time course of reduction of the sugar level mirrors the timing of the accumulation of din1 transcripts. Exogenously supplied sugars suppressed the induction of the gene. These observations altogether suggest that the level of the unidentified metabolite(s), which represses din1 expression, therefore repression of din1 also depend on the level of sugars in the leaf cell.

Expression of some photosynthetic genes in maize are coordinately repressed by such metabolites of photosynthetic products as sucrose and glucose (Sheen, 1990). The photosynthetic genes are repressed by addition of sugars at rather high concentrations (approximately by half at 30 mM). din1 is also repressed by sugars but at relatively low concentrations (almost completely at 10 mM or less) when sugars are exogenously supplied, and the gene is activated in the cotyledonary cells when photosynthesis is hindered. The repression of the photosynthetic genes by sugars was suggested to be related to the repression of the genes in nonphotosynthetic tissues in which sugar level is kept high. The repression of din1 gene by sugars appears to be quite distinct not only in the effective sugar concentrations but also in the physiological meanings. I have not calculated actual concentrations of sugars in the cells of cotyledons

which were floated on sugar solutions, but the critical concentration for the suppression seems to be within a physiological range in photosynthetic cells (Gerhardt et al., 1987). Thus, din1 is repressed when the cells are actively fixing CO_2 by photosynthesis and is activated when they are starved of photosynthate by a cause whatever it is.

Function of din1 product is not known. However, as described in Chapter II, the expression of din1 is highly associated with the progress of senescence. Experimental data presented in this chapter revealed that din1 is regulated by sugar level which is normally determined by photosynthetic activity. From these observations, I would like to propose a hypothesis to explain how dark treatment promotes leaf senescence; leaf cells sense their own photosynthetic activity through the accumulation level of photosynthate which is also controlled by the respiratory activity and the rate of export of the products to sink tissues. When the sugar level is lowered by any reason, the leaf cell expresses such senescence-associated genes as din1 and starts senescing. Sugar or its metabolite(s) has been suggested to be one of the factors which strongly modulate the progress of senescence by observations that carnation flower fed with sucrose delays progress of senescence and sufficient supply of respiratory substrates retarded the senescence (Nichols, 1973). The results of my research strongly suggested that the supplied sugar represses expression of senescence-associated genes and as a result

retards senescence. This hypothesis needs to be examined by further experimentation.