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Modification of Dimethipin Action by Light

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Abstract. White light reduced the efficacy of dimethipin in inducing both desiccation and abscission in kidney beans (*Phaseolus vulgaris* L. cv. Black Valentine). Moreover, light reduced the previously reported inhibitory effect of dimethipin on protein synthesis (Metzger and Keng 1984) in a way that paralleled the reduction in dimethipin-induced morphological changes. Therefore the inhibition of protein synthesis by dimethipin was the parameter measured in experiments designed to characterize the light-induced reduction of dimethipin efficacy. The light effect was directly proportional to both the fluence rate and the duration of the light treatment. A similar effect of light was observed in cultured kidney bean cells devoid of chlorophyll, ruling out the participation of a photosynthetic related process. Moreover, light had no effect on either the metabolism of [2,3]-^{14}C-dimethipin in kidney bean leaves or uptake of dimethipin into cultured kidney bean cells. No evidence was obtained for photochemical decomposition of dimethipin either. Thus, the light effect is possibly the result of direct modification of the biochemical processes associated with the primary mechanism(s) of dimethipin action, or perhaps promotion of the rate of repair of dimethipin-induced cellular damage.

Dimethipin (2,3-dihydro-5,6 dimethyl-1,4-dithiin 1,1,4,4-tetraoxide) is a plant growth regulator that is used as a harvest aid on a variety of crops. The primary morphological effects of dimethipin treatment are loss of leaf turgor, desiccation, and leaf abscission (Metzger and Keng 1984). Work in this laboratory

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has focused on determining the mechanism(s) of dimethipin action. In the previous paper (Metzger and Keng 1987), we showed that the cellular basis for loss of leaf turgor was destruction of the epidermal layer leading to uncontrolled water loss. Furthermore, we have shown that the first detectable biochemical effect of dimethipin is an inhibition of protein synthesis and that this inhibition is probably important in the mechanism of action of the compound (Metzger and Keng 1984). In the course of these studies, we observed that light reduced the dimethipin-induced desiccation and abscission. Such observations could have important ramifications for efficacy of dimethipin under field conditions. Therefore, in this paper, we present the results from experiments designed to characterize the light effect in a way that provides some insights on the mechanism(s) by which light reduces dimethipin activity.

Materials and Methods

Plant Material

Kidney bean (*Phaseolus vulgaris* L. cv. Black Valentine) seedlings were grown in the greenhouse with natural lighting at 25°C in vermiculite that was continuously subirrigated with one-fourth-strength Hoagland's solution (Blankendaal et al. 1972). After 2 weeks, the trifoliate leaves were removed, and each side of both primary leaves was sprayed with a 10 ml solution containing dimethipin, 1.0% (v/v) acetone, and 0.01% (v/v) Tween 20. Control treatments consisted of a similar solution without dimethipin. When leaves were dry, the plants were then placed in a growth chamber at 25°C and ambient relative humidity. Unless otherwise stated, light intensity at plant level was 150 $\mu Em^{-2}\ s^{-1}$, PAR.

Capacity for Protein Synthesis

The ability of leaf disks to incorporate ^{14}C-leucine into protein was taken as a measure of leaf capacity for protein synthesis. In a typical experiment, 10 leaf disks (1 cm in diameter) were taken from primary bean leaves that had been previously sprayed with a test solution and floated abaxial side down on a 3 ml solution containing 0.25 μCi of ^{14}C-leucine (54 mCi $mmol^{-1}$, Amersham, Arlington Heights, IL) and 50 mg l^{-1} chloramphenicol. After 4 h, the amount of ^{14}C-leucine that was incorporated into protein was determined as described before (Metzger and Keng 1984).

Development of Kidney Bean Cell Suspension Cultures

Kidney bean seeds were surface-sterilized with a commercial chlorine bleach solution that had been diluted 10-fold with H_2O, thoroughly washed with sterilized deionized H_2O, and germinated on B5 agar medium (Gamborg and Wetter 1975). Hypocotyl sections were aseptically transferred to a B5 agar medium containing 1 mg l^{-1} 2,4-D. Callus cells that developed from the stem sections

were transferred to liquid B5 medium also containing 1 mg l^{-1} 2,4-D (50 ml in a 250-ml Erlenmeyer flask). The flasks were placed on rotary shakers (100 rpm) in the dark at 26°C. The cell suspension cultures were maintained by weekly transfer to fresh B5 medium with 1 mg l^{-1} 2,4-D. The effect of dimethipin on protein synthesis in cell suspension cultures was assessed by measuring the culture's ability to incorporate ^{14}C-leucine into protein as described by Ferrari and Widholm (1973). Ten milliliters of 1-week-old suspension cultures were added to 50 ml fresh B5 medium. Various concentrations of dimethipin and 0.25 μCi ^{14}C-leucine were dissolved in 1 ml distilled H_2O, filter-sterilized, and then added to the freshly transferred cell suspension cultures. After 24 h, the cells were harvested by filtration and then washed three times with 2 ml CH_3OH:$CHCl_3$:H_2O (12:5:3 v/v/v) mixture containing 10^{-4} M unlabeled leucine. The amount of radioactivity in the resulting residue as determined by liquid scintillation spectrometry was taken as a measure of incorporation of ^{14}C-leucine into protein.

Metabolism of ^{14}C-Dimethipin

Two-week-old kidney bean plants were decapitated, and the primary leaves were sprayed with a solution containing 0.1 mM unlabeled dimethipin, 0.01% (v/v) polyoxyethylene sorbatan monolaurate (Tween 20, Sigma Chemical Co., St. Louis, MO), and 1% (v/v) acetone. Before the leaves had dried, 10 μl of an aqueous solution containing 25,000 dpm of [2,3]-^{14}C-dimethipin (9.24 mCi $mmol^{-1}$, a gift from Uniroyal Chemical Co., Bethany, CT) was applied in 1-μl droplets throughout the upper surface of each primary leaf. After prescribed times, the primary leaves were harvested separately and frozen in liquid N_2. Individual leaves were then homogenized for 30 s in 60 ml cold 80% (v/v) aqueous acetone with a Polytron homogenizer (Brinkman Instruments, Westburg, NY) at one-half maximum speed for 30 s.

The homogenate was filtered, and the remaining residue was homogenized a second time in 60 ml 80% aqueous acetone. The mixture was stirred for 2 h at 4°C and filtered, and the two filtrates were combined. Pigments were removed from the filtrates by mixing 5 g activated charcoal (Darco G 60, Sargent-Welch Co., Skokie, IL) and 5 g Celite 545 (Fisher Scientific Co., Fair Lawn, NJ) and stirring for 30 min at 4°C. The mixture was filtered, and the filtrate was dried under reduced pressure at 35°C. The residue was redissolved in a small volume of 10% (v/v) aqueous methanol and analyzed by reverse-phase HPLC. The HPLC system consisted of two Waters 6000 A pumps (Waters Associates, Millford, MA) controlled by a Waters automated gradient controller and a Waters radial compression system containing an 8 mm i.d. Radial-Pak C_{18} reverse-phase cartridge. The column was eluted isocratically for 12 min with 10% methanol in water (both solvents contained 1% acetic acid), whereupon the proportion of methanol was increased linearly to 100% in 1 min.

The column continued to be eluted with 100% methanol for 5 min and then returned to initial condition (10% methanol). The column was equilibrated for at least 5 min before an injection was made. The flow rate was 2 ml min^{-1}. Elution of radioactive compounds from the column was monitored with a

Berthold model LB 503 radioactive flow monitor equipped with a 400-μl glass scintillator-filled flow cell. Radioactive peaks were collected and quantitated by liquid scintillation spectrometry. In this chromatographic system, ^{14}C-dimethipin eluted 9.5 min after injection.

Results

In investigations on the mechanism of action of dimethipin, we observed that higher concentrations of dimethipin were required to induce leaf desiccation and abscission when the plants were maintained in the light than in the dark (Fig. 1). Since the first observable biochemical effect of dimethipin is an inhibition of protein synthesis (Metzger and Keng 1984), it was of interest to examine the effects of light on dimethipin-induced inhibition of protein synthesis. Primary kidney bean leaves were sprayed with solutions containing 0 (control), 0.1, or 1.0 mM dimethipin and then returned to growth chambers with continuous light or dark. After prescribed times, plants were removed from the growth chambers, and the capacity of the leaves for protein synthesis was assessed as described before.

Figure 2 shows a time course of the effects of two concentrations of dimethipin on the ability of leaves to incorporate ^{14}C-leucine into protein in both light and dark. Initially, light had little effect on dimethipin-induced reduction in a leaf's capacity for protein synthesis at either concentration of dimethipin. However, by 24 h, leaves treated with 0.1 mM dimethipin and maintained in the light had begun to recover; after 72 h, the capacity for protein synthesis recovered to almost 80% of the control value. The other treatments rapidly reached a similar low value that was maintained during the duration of the experiment (Fig. 2). These results closely paralleled the effects of light on dimethipin-induced desiccation and abscission. Neither morphological change was observed in plants treated with 0.1 mM dimethipin and maintained in the light (Fig. 1). However, leaves sprayed with 1.0 mM dimethipin and maintained in the light had similar visual symptoms and capacity for protein synthesis as those placed in the dark following treatment with 0.1 mM dimethipin. These results indicate that the light treatment reduced dimethipin activity at 0.1 mM by about 10-fold.

The effect of light was further characterized in order to gain some possible insights on the mechanism(s) by which light reduces dimethipin activity. Primary bean leaves were sprayed with a solution containing either 0 (control) or 0.1 mM dimethipin and placed in a lighted growth chamber. After various periods of time, plants were placed back in the dark for a combined total of 24 h for both light and dark periods. The leaves were then assessed for their ability to incorporate ^{14}C-leucine into protein. As shown in Fig. 3, the capacity of light to reduce dimethipin-induced inhibition of protein synthesis was positively related to the duration of the light treatment. If the converse treatment was performed, i.e., different durations of dark followed by light, an essentially inverse curve was observed (Fig. 3). Apparently there is little or no after-

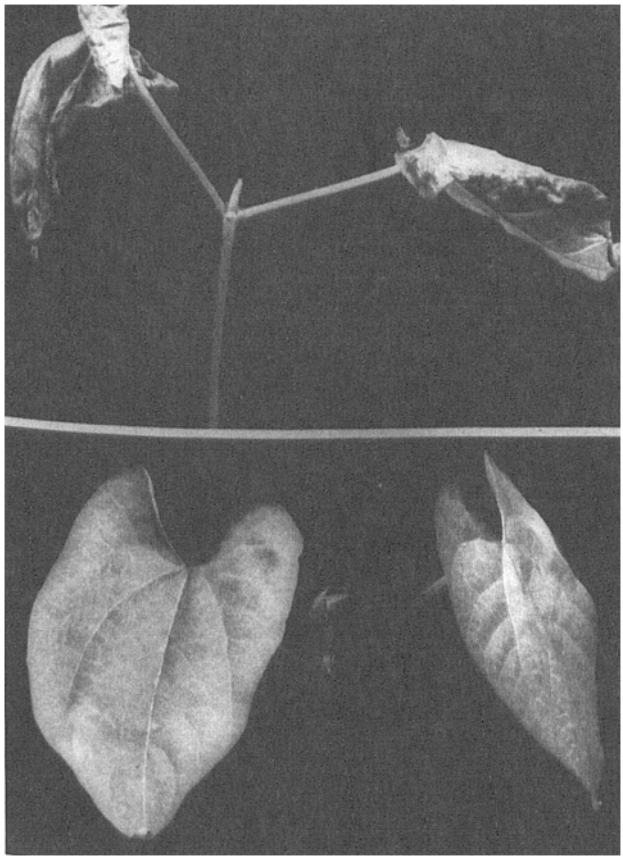

Fig. 1. Light (150 μE m^{-2} s^{-1}, PAR) prevented the appearance of desiccation symptoms on primary kidney bean leaves 72 h after a foliar application of 0.1 mM dimethipin. The plants were sprayed and placed in a growth chamber with continuous light (bottom) or darkness (top).

effect of light; that is, following removal of light, the reduction in dimethipin activity is readily reversed.

The relationship between light intensity and its ability to reduce dimethipin activity was examined. In this experiment, primary leaves of 2-week-old kidney bean plants were sprayed with either 0 or 0.1 mM dimethipin solutions and placed back in a lighted growth chamber at 25°C. Light intensity was varied by placing various numbers of layers of cheese cloth over the plants. Total darkness was achieved by covering the plants with a cardboard box. After 24 h, the leaves from three control and dimethipin-treated plants from each light treatment were assessed for the ability to incorporate ^{14}C-leucine into protein. As was found for the duration of the light treatment, there was a positive relationship between light intensity and a reduction in dimethipin activity (Fig. 4).

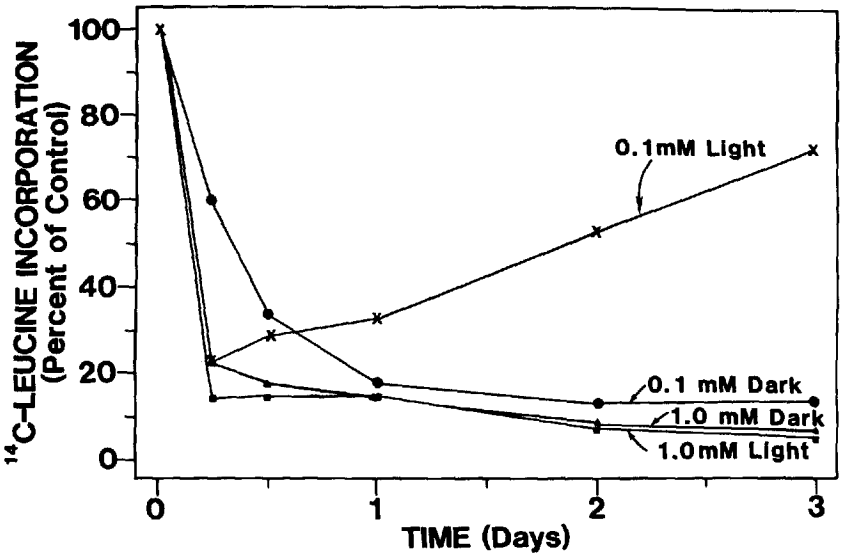

Fig. 2. Effect of light on dimethipin-induced inhibition of protein synthesis in kidney bean leaf disks. Plants were treated with either 0 (control), 0.1, or 1.0 mM dimethipin and placed in either the dark or the light. After various times, three plants from each treatment were removed, and their ability to incorporate ^{14}C-leucine into protein was evaluated as described before. Each value is expressed as percent of the control value (0 mM dimethipin) for that particular time point.

Light may deactivate dimethipin through some process requiring photosynthesis—e.g., increased metabolism, etc. If this is so, then light should have no effect on dimethipin activity in cell suspension cultures devoid of chlorophyll. On the contrary, light significantly reduced the capacity of dimethipin to inhibit protein synthesis in kidney bean cell suspension cultures (Fig. 5). Thus, the light-induced reduction in dimethipin activity is not a photosynthetically mediated process.

One possible mechanism by which light may reduce dimethipin activity is by increasing the rate of its metabolism, thereby reducing cellular levels of the bioactive compound. To test this possibility, the rate of ^{14}C-dimethipin metabolism was compared in leaves from plants maintained in light or darkness. Table 1 shows that light had no apparent effect on the rate of dimethipin metabolism in kidney bean leaves until after the appearance of visible symptoms (severe desiccation) 48 h following applicaiton of ^{14}C-dimethipin. In view of the condition of the leaves of plants maintained in the dark, it is not surprising that dimethipin metabolism virtually ceased in those plants after 48 h.

Also of interest was the observation that no acetone-soluble metabolites were detected despite the disappearance of half of the parent compound in 24 h. However, nearly all of the missing radioactivity was recovered in the 80% acetone-insoluble residue (data not shown). These ^{14}C-labeled metabolites were not soluble in any organic solvents tested including methanol, acetonitrile, and ethylacetate (data not shown). Thus the nature of the metabolite(s) remains unknown.

Light could also alter uptake of dimethipin in a way that reduces the cellular

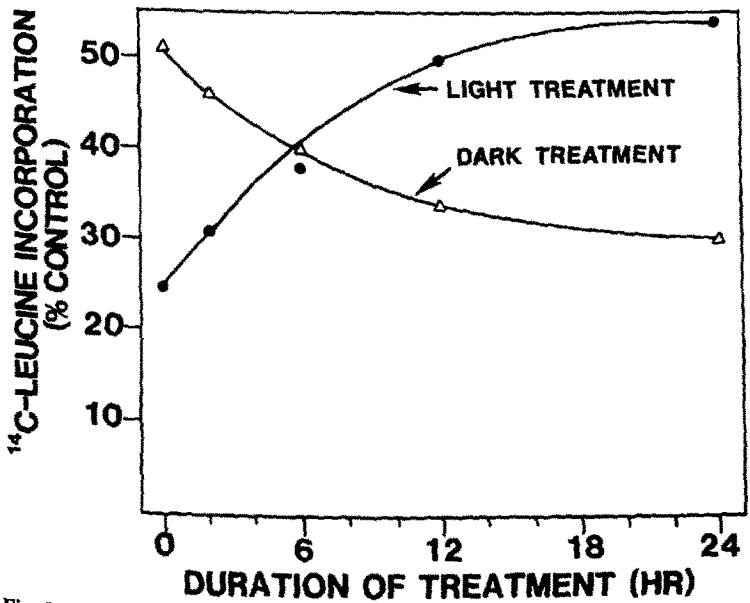

Fig. 3. Effects of different durations of light or dark pretreatment on dimethipin-induced inhibition of protein synthesis. Primary bean leaves were sprayed with 0 (control) or 0.1 mM dimethipin and returned to a growth chamber with light or dark. For the light pretreatments, three plants were removed from the lighted growth chamber and placed in a dark chamber. Conversely, the dark pretreatments consisted of maintaining plants in the dark growth chamber for various periods of time whereupon three plants were then placed in the lighted growth chamber. In both experiments, the capacity of the leaves to incorporate ^{14}C-leucine into protein was assessed as described before after the sum of the lengths of the light and dark treatments equaled 24 h following the spray treatment. Each value represents the percent control (0 mM dimethipin) value for each treatment.

concentration. This possibility was examined by comparing the uptake of ^{14}C-dimethipin in cell suspension cultures maintained in light or darkness. Ten milliliters of 1-week-old suspension cultures was added to 50 ml B5 medium containing either 0.01 or 0.1 mM unlabeled dimethipin and 5.0×10^4 dpm ^{14}C-dimethipin. After 24 h in either light or darkness, the cells were harvested by filtration and washed, and the remaining radioactivity was measured. Table 2 shows that light had no significant effect on dimethipin uptake at either concentration.

The reduction of dimethipin activity by light was not due to photodecomposition either. To demonstrate this, two experiments were performed. In the first, a 0.1 mM solution of dimethipin containing 0.01% Tween 20 and 1% acetone was stored in continuous light (150 μE m^{-2} s^{-1}, PAR) for 1 week at 25°C. The ability of this solution to induce typical morphological symptoms and reduce leaf capacity for protein synthesis was the same as a solution stored in the dark (data not shown). In the second experiment, a 0.1 mM solution of dimethipin with 100,000 dpm of ^{14}C-dimethipin, 0.01% Tween 20, and 1% acetone was stored in continuous light for 1 week at 25°C. No loss of parent compound was observed when analyzed by reverse-phase HPLC (data not shown).

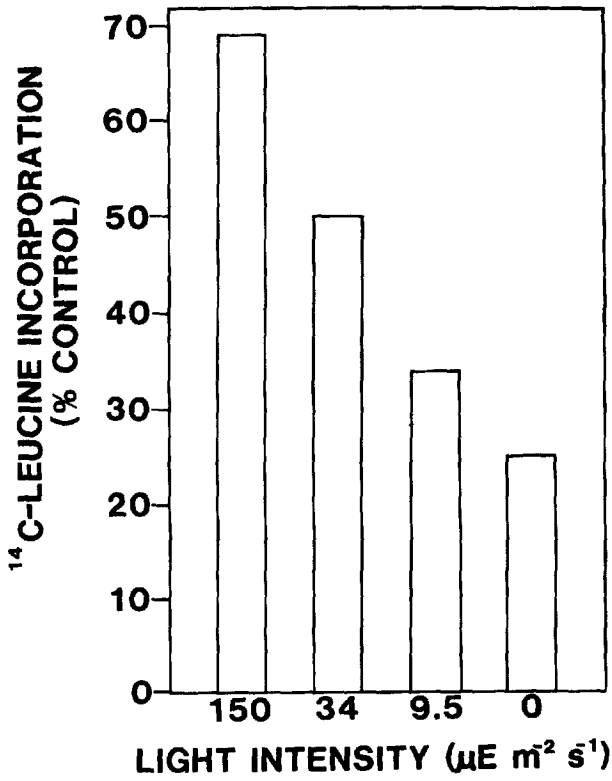

Fig. 4. Relationship between intensity and the capacity of light to reduce dimethipin-induced inhibition of protein synthesis in primary bean leaves. Leaves were sprayed with either 0 (control) or 0.1 mM dimethipin and then subjected to one of four different light regimes for 24 h. The leaves were then assessed for the ability to incorporate ^{14}C-leucine into protein as described before. Each value represents the percent control value for each treatment.

Discussion

The mechanism(s) by which light modifies dimethipin action remain unclear. It does not appear to be mediated through some photosynthetic process, since light also reduced dimethipin-induced inhibition of protein synthesis in kidney bean cell suspension cultures (Fig. 5). Moreover, the light effect cannot be attributed to differences in the rates of dimethipin degradation in plants maintained in light or darkness following dimethipin treatment (Table 1). Nor did light affect cellular uptake of ^{14}C-dimethipin in kidney bean cell suspension cultures (Table 2). Finally, no evidence was obtained indicating that light effect was due to photochemical decomposition. These results indicate that either light may directly modify the biochemical processes associated with the primary mechanism(s) of dimethipin action or it may possibly enhance the rate of repair of dimethipin-induced cellular damage.

At present, it is not known which of these two possibilities is correct. However, some interesting parallels exist between dimethipin and malformin action. Malformin is a cyclic peptide isolated from the culture filtrate of the fungus *Aspergillus niger* van Tiegh (Takahashi and Curtis 1961) that induces abscission in a number of species (Curtis 1968). Similar to dimethipin, the biological activity of malformin is reduced by light. Malformin reacts nonenzy-

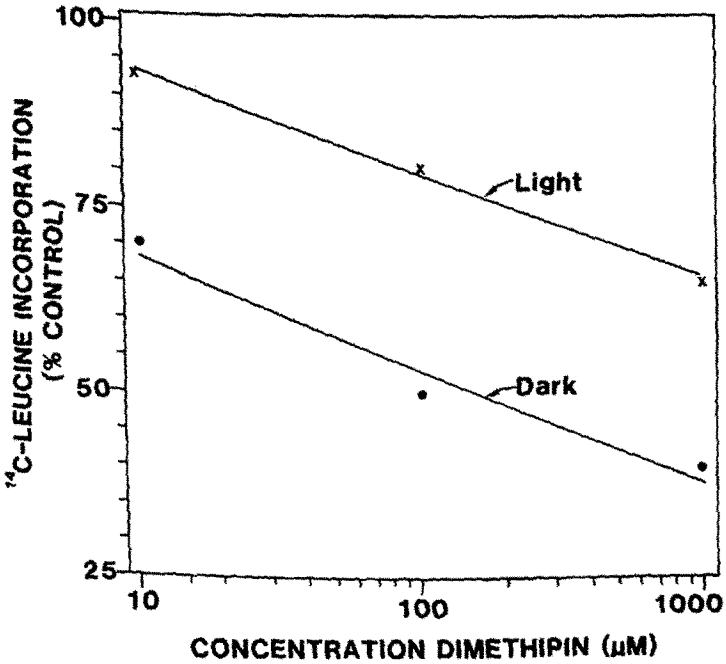

Fig. 5. Effect of light on the ability of dimethipin to inhibit the incorporation of ^{14}C-leucine into protein by kidney bean cell suspension cultures. An aliquot (10 ml) of 1-week-old cultures was added to fresh culture media containing various concentrations of dimethipin and 0.25 µCi ^{14}C-leucine. After 24 h in light or darkness, the cells were harvested, and the amount of ^{14}C-leucine incorporated into protein was determined as described before. Each value represents the percent control value for each treatment.

Table 1. Effect of light on the metabolism of ^{14}C-dimethipin in primary kidney bean leaves.

Time after application of ^{14}C-dimethipin	Amount of ^{14}C-dimethipin remaining (%)	
	Light	Dark
24 h	47	46
48 h	31	34
72 h	18	33

Primary leaves were first sprayed with a solution containing 0.1 mM unlabeled dimethipin and, while still wet, treated with 10 µl of an aqueous solution containing 25,000 dpm of [2,3]-^{14}C-dimethipin. The plants were placed in growth chambers with light or darkness. After prescribed times, the leaves were harvested, and the amount of ^{14}C-dimethipin remaining was determined as described.

matically with compounds containing free thiol groups to form insoluble products (Iriuchijima and Curtis 1970). Dimethipin reacts with free thiols in a similar fashion (J. P. McManus, Uniroyal Chemical Company, Naugatuck, CT; personal communication). In the case of malformin, this reaction is considered

Table 2. Effect of light on the uptake of ^{14}C-dimethipin by cultured kidney bean cells.

Dimethipin concentration	^{14}C-dimethipin content (DPM/200 mg fresh wt.)	
	Dark	Light
0.01 mM	114.7 ± 13.2	125.6 ± 7.4
0.1 mM	152.8 ± 24.0	136.8 ± 20.1

Ten milliliters of 1-week-old bean cell suspension culture was added to 50 ml fresh culture media containing either 0.01 or 0.1 mM dimethipin (unlabeled) and 5.0×10^4 dpm of ^{14}C-dimethipin. After 24 h, the cells were harvested, and the amount of uptake of ^{14}C-dimethipin was determined.

essential in the mechanism of action of the compound (Suda and Curtis 1964). It remains to be seen if dimethipin has an analogous mechanism of action.

Nevertheless, it is interesting to speculate that light inhibits the activity of both malformin and dimethipin by reducing the access to free thiol groups. For example, light may cause a conformation change in an essential protein(s) in a way that free thiol groups cannot react with the compounds, thus preventing or minimizing damage. Such changes could be subtle enough that differences in metabolism under light and dark conditions would be undetectable. Alternatively, light may increase the level of some essential peptide such as glutathione to a point where reaction with dimethipin never reduces the cellular content of the free compound below some critical level. This possibility is consistent with the observation (Fig. 2) that light could not reverse the effects of dimethipin at higher concentrations.

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