

Effect of Light and Chilling Temperatures on Chilling-sensitive and Chilling-resistant Plants

PRETREATMENT OF CUCUMBER AND SPINACH THYLAKOIDS *IN VIVO* AND *IN VITRO*¹

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MELVIN P. GARBER

Department of Horticulture, Iowa State University, Ames, Iowa 50011

ABSTRACT

The effects of chilling temperatures, in light or dark, on the isolated thylakoids and leaf discs of cucumber (*Cucumis sativa* L. "Marketer") and spinach (*Spinacia oleracea* L. "Bloomsdale") were studied. The pretreatment of isolated thylakoids and leaf discs at 4 C in the dark did not affect the phenazine methosulfate-dependent phosphorylation, proton uptake, osmotic response to sucrose, Ca²⁺-dependent ATPase activity, or chlorophyll content. Exposure of cucumber cotyledon discs and isolated thylakoids of cucumber and spinach to 4 C in light resulted in a rapid inactivation of the thylakoids. The sequence of activities or components lost during inactivation (starting with the most sensitive) are: phenazine methosulfate-dependent cyclic phosphorylation, proton uptake, osmotic response to sucrose, Ca²⁺-dependent ATPase activity, and chlorophyll. The rate of loss of proton uptake, osmotic response to sucrose, Ca²⁺-dependent ATPase activity and chlorophyll is similar for isolated cucumber and spinach thylakoids, whereas spinach thylakoids are more resistant to the loss of phenazine methosulfate-dependent phosphorylation. The thylakoids of spinach leaf discs were unaffected by exposure to 4 C in light. The results question whether the extreme resistance of spinach thylakoids treated *in vivo* is solely a function of the chloroplast thylakoid membranes and establish the validity of using *in vitro* results to make inferences about cucumber thylakoids treated *in vivo* at 4 C in light.

It is well documented that thermophilic plants are damaged by temperatures between 0 and approximately 12 C (chilling injury). Much of the earlier work concentrated on alterations in the fruit (5, 12) and vegetative parts (11, 17) of plants. Less attention has been paid to the leaves of thermophilic plants. Reported alterations in leaf metabolism include effects on the photosynthetic process (4, 9, 14, 15, 21, 29), respiration (1, 8), activation energy of enzymes (21), and altered membrane permeability (19).

The injury due to chilling temperatures is particularly acute in the presence of light. The photosynthetic rates of thermophilic plants (*Paspalum*, *Sorghum*, *Cucumis*, and *Glycine*) decrease rapidly when the temperature is lowered from 25 to 10 C in the light (9, 25). Most work on the combined effects of light and chilling temperatures has concentrated on CO₂ fixation, primarily in C₄ grasses (3, 24, 26), although available data indicate that photochemical activities are altered when leaves are chilled in light (9).

The objectives of this study were: (a) to investigate the effects of chilling cucumber cotyledons and spinach leaves in light and dark; (b) to determine whether the resistance of spinach and the sensitivity of cucumber thylakoids are functions of the thylakoid membranes; and (c) to establish the validity of using *in vitro* results to make inferences about *in vivo* phenomena in thermophilic and cool season plants exposed to light and chilling temperatures. The first two objectives will influence the direction of research on the nature of the differential resistance of cool season and thermophilic plants to light and chilling temperatures. The third objective will establish the validity of using isolated thylakoids, a simplified system, for studying the effects of light and chilling temperatures. An earlier study established the validity of using *in vitro* studies to make inferences about *in vivo* phenomena for the freeze-thaw process in plants (7).

MATERIALS AND METHODS

***In Vivo* and *In Vitro* Treatment of Thylakoids.** The exposure of cucumber cotyledon (*Cucumis sativa* L. "Marketer") and spinach leaf (*Spinacia oleracea* L. "Bloomsdale") discs to 4 C in light or dark with subsequent isolation of chloroplasts and measurement of activity constitutes the *in vivo* treatment. The isolation of chloroplast thylakoids from cucumber cotyledons and spinach leaves followed by exposure to 4 C in light or dark and subsequent assay for activity constitutes the *in vitro* treatment.

Light and Chilling Temperature Pretreatment. Cucumber cotyledon and spinach leaf discs (No. 10 bore) were rinsed in distilled H₂O before floating on a 1% (w/v) sucrose solution at the desired temperature. The temperature of the sucrose solution was maintained by circulating chilled water through an insulated Plexiglas chamber containing two Pyrex pans with eight sample chambers. Light, provided by Sylvania flood lamps, was passed through a 2-cm thick Plexiglas chamber with running tap water to remove IR radiation. Light intensity was 21,600 ± 1,000 lux at the sample surface. The placement of leaf discs on the sucrose solution was in the presence of weak green light. Leaf discs were exposed to light after 15 min for temperature equilibration in the dark.

Isolated chloroplast thylakoids of cucumber and spinach were resuspended in 10 mM NaCl at a concentration equivalent to 200 µg Chl/ml. Chlorophyll determination was according to Arnon (2). The thylakoid suspensions were placed in test tubes contained in a Plexiglas chamber and continuously stirred while exposed to 4 C in light or dark. The sample temperature was maintained by circulating chilled water through the Plexiglas chamber. Light, provided by a Sylvania 200 w flood lamp, was passed through a 2-cm distilled H₂O filter. Light intensity at the surface of the chamber was 21,600 ± 1,000 lux.

Chloroplast Isolation. Cucumber cotyledon and spinach leaf discs were homogenized in a Waring Blendor, at low speed, for

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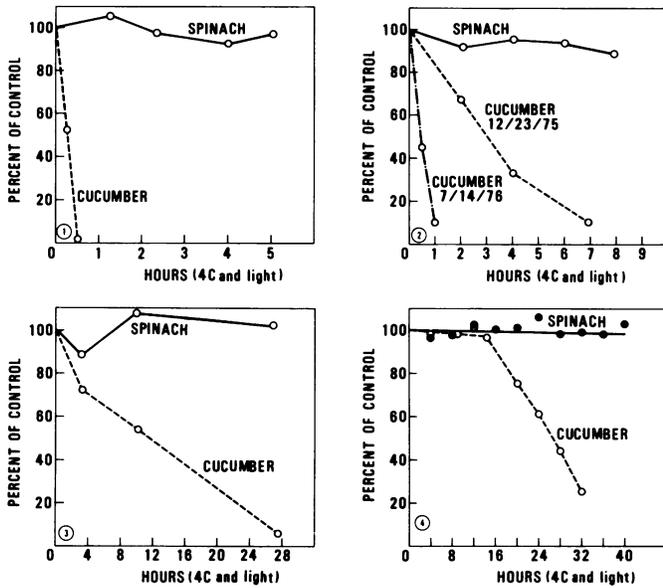


FIG. 1. PMS-dependent phosphorylation of cucumber and spinach thylakoids pretreated *in vivo* at 4 C in light. Activity of the controls was 354 and 382 $\mu\text{mol Pi}$ esterified/mg Chl \cdot hr, respectively. The control was not exposed to 4 C.

FIG. 2. Proton uptake of cucumber and spinach thylakoids pretreated *in vivo* at 4 C in light. Control activities were: spinach: 0.92 $\mu\text{g H}^+$ accumulated/mg Chl; and cucumber: (12/23/75) 0.87 and (7/14/76) 0.85 $\mu\text{g H}^+$ accumulated/mg Chl.

FIG. 3. Trypsin-activated Ca^{2+} -dependent ATPase activity of cucumber and spinach thylakoids pretreated *in vivo* at 4 C in light. Activity of the nonchilled controls was 206 and 185 $\mu\text{mol Pi}$ liberated/mg Chl \cdot hr, respectively.

FIG. 4. Chlorophyll content of spinach leaf and cucumber cotyledon discs exposed to 4 C in light.

10 to 15 sec in a volume of isolation medium, in ml, equivalent to 10 times the grams of tissue. The isolation medium consisted of 20 mM Tricine, 0.25 M NaCl, 20 mM sodium ascorbate, 0.2% BSA, and 15 mM EDTA (pH 7.8–8). The extract was filtered through eight layers of cheesecloth and centrifuged at 2,500g for 1 min. Chloroplasts were osmotically ruptured by resuspending the 2,500g pellet in 10 mM NaCl and centrifuging at 6,000g for 10 min. All operations were carried out at 0 to 4 C.

Assays. Measurement of light-dependent proton uptake by chloroplast thylakoids was essentially that of Neumann and Jägendorf (18). Thylakoids equivalent to 80 $\mu\text{g Chl/ml}$ were resuspended in 5 ml of solution containing 15 mM NaCl, 0.5 mM MgCl_2 , and 40 $\mu\text{M PMS}^2$. The temperature of the reaction mixture was maintained at 16 C and the initial pH adjusted to 6.0 to 6.2. Light was provided for 2 min with a 300 w flood lamp, and the sample was titrated with 1 mM HCl. Thylakoids treated with DCCD had 5 μl of 0.1 M DCCD (in ethanol) added to the assay medium, resulting in a ratio of 100 nmol of DCCD/60 $\mu\text{g Chl}$. The procedure for DCCD treatment was similar to that of McCarty and Racker (16).

Activation of membrane-associated CF_1 by trypsin and measurement of Ca^{2+} -dependent ATPase activity were after Lien and Racker (13). Three activation periods were used to detect possible alterations in the rate of activation after light and chilling temperature treatment. Inorganic phosphate determination was a modification of Taussky and Shorr (22) as described by Wharton and McCarty (28).

Chlorophyll content of leaf discs was determined by homogenizing 10 discs in a VirTis blender for 2 min in 80% acetone. The

homogenate was filtered through Whatman No. 50 hardened filter paper with a suction flask, and Chl content was determined according to Arnon (2).

The osmotic response of thylakoids to sucrose was assayed by measuring the packed particle volume. Thylakoids, equivalent to 100 $\mu\text{g Chl}$, were suspended in sucrose solutions in thrombocytocrits and centrifuged at 6,000g for approximately 5 hr.

PMS-dependent cyclic photophosphorylation was measured as the disappearance of Pi from the reaction mixture. The reaction mixture contained in a total volume of 1 ml, 50 mM Tricine (pH 8), 50 mM NaCl, 50 $\mu\text{M PMS}$, 5 mM MgCl_2 , 2 mM K-phosphate (pH 8), 3 mM ADP (pH 7), and thylakoids equivalent to 50 $\mu\text{g Chl}$. The reaction mixture was illuminated at 20 C for 2 min with a 300 w flood lamp before stopping the reaction with 1 ml of ice-cold trichloroacetic acid. Infrared radiation was removed by passing the light through 5 cm of water. The denatured samples were centrifuged at 6,000g for 10 min and Pi content determined according to Taussky and Shorr (22).

RESULTS AND DISCUSSION

***In Vivo* and *In Vitro* Pretreatment at 4 C in Dark.** The treatments in this study consist of cucumber cotyledons and

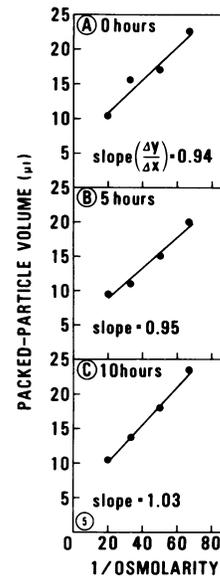


FIG. 5. Osmotic response to sucrose of spinach thylakoids pretreated *in vivo* at 4 C in light. Osmotic response to sucrose, as measured by packed particle volume, is estimated by the slope of the response line. 0, 5, and 10 hr refer to pretreatment time.

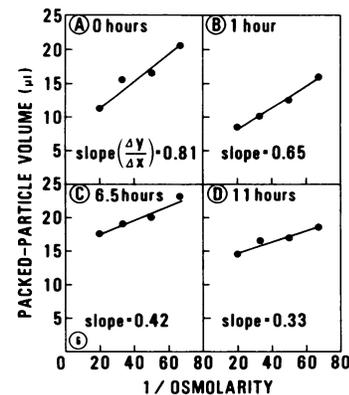


FIG. 6. Osmotic response to sucrose of cucumber thylakoids pretreated *in vivo* at 4 C in light. Slope of the osmotic response line is used as an estimate of membrane semipermeability. 0, 1, 6.5, and 11 hr refer to pretreatment times.

² PMS: phenazine methosulfate; DCCD: N,N'-dicyclohexylcarbodiimide; CF_1 : chloroplast coupling factor 1.

Table I. Effect of Light Intensity on Proton Uptake
Cucumber cotyledon discs were exposed to 4 C in light for 1 hr before the thylakoids were isolated and proton uptake measured. The control (maintained at 16 C in light) accumulated protons equivalent to 0.80 $\mu\text{g H}^+$ /mg Chl.

Light intensity (lux)	Proton uptake (% control)
19,000-21,600	2
5,400- 7,500	14
1,080- 1,620	4
194- 238	6

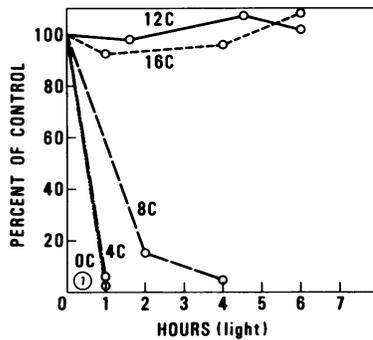


Fig. 7. Proton uptake of cucumber thylakoids pretreated *in vivo* at 16, 12, 8, 4, or 0 C in light (21,600 lux). Control activities were 0.80 to 0.85 $\mu\text{g H}^+$ accumulated/mg Chl.

spinach leaves and isolated chloroplasts of spinach and cucumber exposed to 4 C in light or dark and subsequent measurement of several membrane functions or components. The pretreatment of cucumber cotyledon and spinach leaf discs and isolated spinach and cucumber thylakoids at 4 C in dark did not affect the proton uptake, PMS-dependent phosphorylation, osmotic response to sucrose, Ca^{2+} -dependent ATPase activity, and Chl content (see Table I for data on isolated thylakoids). The treatment of plant material in this study differs from previous work in that the thylakoid functions are not measured at chilling temperatures, with the exception of osmotic response to sucrose, but at 16 to 20 C. Therefore, the "unaltered" proton uptake, PMS-dependent phosphorylation, and Ca^{2+} -dependent ATPase activity of cucumber thylakoids do not imply that these functions are normal at 4 C, but if some alteration occurs, apparently, it is readily reversible when the temperature rises to 16 to 20 C.

***In Vivo* Pretreatment at 4 C in Light.** The pretreatment of spinach leaf discs at 4 C in light did not affect the thylakoid membrane functions or components measured in this study (Figs. 1-5). In contrast, the exposure of cucumber cotyledon discs to 4 C in light resulted in the loss of PMS-dependent phosphorylation, proton uptake, osmotic response to sucrose, Ca^{2+} -dependent ATPase activity and Chl content (Figs. 1-4, and 6).

Apparently, growing conditions can cause substantial variation in the sensitivity of cucumber cotyledons to 4 C in light (Fig. 2). Plants grown in the greenhouse from April to October exhibit a more rapid loss of proton uptake than the plants grown from November to March. In fact, the thylakoids of summer-grown cucumber plants are inactivated within 1 hr when exposed to 4 C and 194 lux, the lowest light level in this study (Table I). Thus, extremely low light intensities, at 4 C, can cause rapid and complete inactivation of cucumber thylakoids. The rate and extent of loss of proton uptake were not affected by exposure of the adaxial or abaxial surfaces to equal light intensities.

To determine the upper temperature range where inactivation of cucumber thylakoids occurs, cucumber cotyledon discs were exposed to 0, 4, 8, 12, and 16 C, in light (Fig. 7). The proton

uptake of chloroplasts of leaf discs exposed to 16 and 12 C was equivalent to the control, whereas exposure to 8, 4, and 0 C resulted in a rapid decrease in proton uptake. The literature suggests that damage due to chilling temperatures is initiated at 8 to 12 C (10, 21). Seemingly, damage due to chilling in the light is initiated at the same temperatures.

Taylor and Craig (23) demonstrated that with light striking the adaxial surface, chloroplast ultrastructure is greatly altered near the adaxial surface, while chloroplast ultrastructure near the abaxial surface seemed unaffected. It is unclear in this study whether the reduction of proton uptake is due to a similar complete inactivation of some chloroplasts with others maintaining activity comparable to the control. To this end, the rate of decay of the proton gradient was measured (Table II). If some thylakoids are inactivated and others are normal, there should be a decrease in the extent of the proton gradient but not in the rate of decay. The results indicate that as the extent of the proton gradient decreases, the rate of decay also decreases. This suggests that all of the chloroplasts are inhibited to varying degrees, in contrast to the existence of "normal" and inactivated chloroplasts.

Possible reasons for the loss of PMS-dependent phosphorylation and proton uptake include the release of CF_1 , inactivation of CF_1 , and loss of membrane semipermeability. The Ca^{2+} -dependent ATPase activity of cucumber thylakoids, a quantitative estimate of CF_1 , decreases when cucumber cotyledons are exposed to 4 C in light (Fig. 3). However, the addition of DCCD to partially inactivated cucumber thylakoids did not restore or stimulate proton uptake, suggesting that the CF_1 enzyme is inactivated and not merely released from the membrane. The osmotic response of cucumber thylakoids to sucrose, an indicator of membrane semipermeability, decreased when cotyledon discs were exposed to 4 C in light. However, substantial loss of Ca^{2+} -dependent ATPase activity and osmotic response to sucrose occurs after complete loss of PMS-dependent phosphorylation and proton uptake (Fig. 8).

The decrease in Chl content of cucumber cotyledons, exposed to 4 C in light, begins after a substantial decrease in PMS-dependent phosphorylation, proton uptake, osmotic response to sucrose, and Ca^{2+} -dependent ATPase activity. The lag phase in the loss of Chl is unlike the kinetics of inactivation of membrane functions, where a lag phase was not detected.

Figure 8 summarizes the temporal relationship of all parameters measured. The sequence of activities or components lost during inactivation of cucumber cotyledons (starting with the most sensitive) is: PMS-dependent phosphorylation, proton uptake, osmotic response to sucrose, Ca^{2+} -dependent ATPase activity, and Chl.

***In Vitro* Pretreatment at 4 C in Light.** The *in vitro* pretreatment at 4 C in light was an attempt to establish the validity of using isolated thylakoids to study the effects of chilling in light and determine if the resistance of *in vivo* treated spinach thylakoids is a function of the thylakoid membranes. Isolated cucumber thylakoids exhibited a rapid loss of PMS-dependent phosphorylation, proton uptake, osmotic response to sucrose, Ca^{2+} -

Table II. Rate of Decay of the Proton Gradient
Cucumber cotyledon discs and isolated cucumber thylakoids were exposed to 4 C in light (21,600 lux) before the proton uptake and rate of decay were measured. The control was maintained at 16 C.

Sample	Time in hr at 4 C in light	Δ pH	Rate of decay ¹
Control	0	0.315	0.50
Cotyledon discs	0.5	0.150	0.375
	1.0	0.035	0.250
Isolated thylakoids	2	0.277	0.42
	4	0.126	0.25
	6	0.063	0.20
	9	0.028	0.10

¹Expressed as pH units Min^{-1} $400 \mu\text{g Chl}^{-1}$.

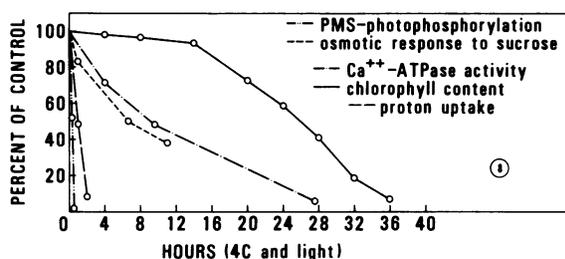


FIG. 8. Sequence of activities and components lost during the pretreatment of cucumber thylakoids *in vivo* at 4 C in light. Sequence of loss (starting with the most sensitive) is: PMS-dependent phosphorylation, proton uptake, osmotic response to sucrose, Ca^{2+} -dependent ATPase activity, and Chl.

dependent ATPase activity and Chl (see Table III and Fig. 9). The sequence of activities and components lost during inactivation was the same as with *in vivo* pretreated thylakoids (compare Figs. 8 and 10). The most noticeable difference is the slower inactivation of membrane components for *in vitro* treated thylakoids. This probably can be accounted for by the method of treatment. The mechanism of inactivation appears similar to the *in vivo* treatment. There is a decrease in Ca^{2+} -dependent ATPase activity but the addition of DCCD to partly inhibited thylakoids did not stimulate proton uptake. Also, the reduction in the extent of the proton gradient was accompanied by a reduction in the rate of decay (Table II). The inactivation of *in vitro* treated cucumber thylakoids resembles *in vivo* treated thylakoids and the results seem to establish the validity of using *in vitro* results to make inferences about *in vivo* phenomena for cucumber thylakoids exposed to 4 C in light.

Isolated spinach thylakoids exposed to 4 C in light were inactivated in a manner similar to isolated cucumber thylakoids (Table III). In fact, the sequence and rate of inactivation were similar to *in vivo* and *in vitro* treated cucumber thylakoids. The only difference was the slower loss of PMS-dependent phosphorylation for spinach thylakoids. The inactivation of *in vitro* treated spinach thylakoids is in contrast to the *in vivo* treatment where spinach thylakoids were unaffected. The results cast some doubt on whether the resistance of *in vivo* treated spinach thylakoids is solely a function of the thylakoid membrane. The resolving of spinach thylakoid resistance by *in vitro* treatment may prove comparable to the biochemical procedure of first resolution and then reconstitution, which has been useful in elucidating membrane structure and function (6, 20, 27).

The inactivation at 4 C in light and the lack of effect at 4 C in dark for cucumber thylakoids indicate that the alterations at

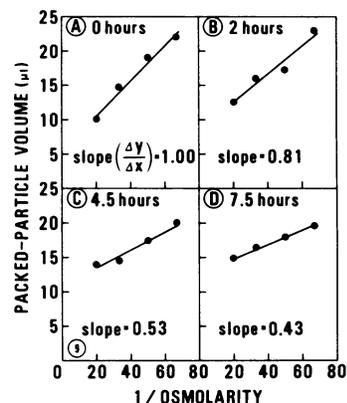


FIG. 9. Osmotic response to sucrose of cucumber and spinach thylakoids pretreated *in vivo* at 4 C in light. Slope of the osmotic response line is an indication of membrane semipermeability. 0, 2, 4.5, and 7.5 hr represent the duration of pretreatment.

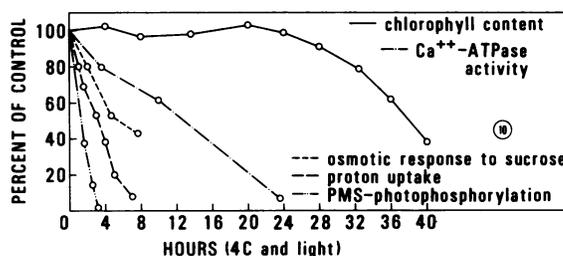


FIG. 10. Sequence of activities and components lost during pretreatment of cucumber thylakoids *in vitro* at 4 C in light. Sequence of loss (starting with the most sensitive) is: PMS-dependent phosphorylation, proton uptake, osmotic response to sucrose, Ca^{2+} -dependent ATPase activity, and Chl.

chilling temperatures in dark are readily reversible, while chilling under light results in inactivation that is irreversible or more slowly reversible than the dark pretreatment. This has practical implications for thermophilic crops planted in early spring when chilling temperatures persist until sunlight strikes the plants.

In summary, the chilling of cucumber cotyledons under light is more damaging to the chloroplast thylakoids than chilling in dark, with similar results for pretreatment *in vivo* and *in vitro*. The chloroplasts of the chilling resistant spinach are unaffected when exposed to 4 C and light *in vivo*, but behave as cucumber thylakoids after pretreatment *in vitro*.

Table III. Pretreatment of Cucumber and Spinach Thylakoids In Vitro

Cucumber and spinach thylakoids were exposed to 4°C in light (21,000 lux) or dark before measurement of membrane functions. The results are expressed as percent of the control (not exposed to chilling temperatures). The control activities for cucumber and spinach thylakoids are, respectively: 421 and 492 $\mu\text{moles Pi esterified/mg Chl/hr}$; 0.86 and 0.92 $\mu\text{eg H}^+$ accum/mg Chl; 185 and 210 $\mu\text{moles Pi liberated/mg Chl/hr}$ and 200 $\mu\text{g Chl/ml}$.

Sample	Pretreatment conditions		Time of pretreatment (hr)																			
			ATP				Proton uptake				Ca ⁺⁺ -ATPase				Chl							
	Light	Dark	1	2	3	4	5	6	2	4	6	8	4	8	12	16	24	8	16	24	32	40
Cucumber	+		90		97				104	101	98	101	92	86	96		106	100	98	99	96	100
	+		26	10	2				73	40	24	12	80	62		34	4	98	102	101	80	51
Spinach	+		108	104		92	99		96	96	97	98	106	104		106	100	103	101	99	102	100
	+		86	44	26	2			70	50	20	10	74	52		30	6	97	99	101	83	48

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