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# Inhibitory Effect of Carbohydrate on Flowering in *Lemna perpusilla*

## II. REVERSAL BY GLYCINE AND L-ASPARTATE. CORRELATION WITH REDUCED LEVELS OF β-CAROTENE AND CHLOROPHYLL[1]

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### ABSTRACT

**Flowering of *Lemna perpusilla* strain 6746 grown on 0.1 strength Hutner's medium in short days was inhibited by sucrose, glucose, fructose, and mannose, but not by various other sugars or metabolic intermediates. Only those sugars that inhibited flowering supported heterotrophic growth. Experiments with a single inductive long night indicated that an early stage in flowering was the sugar-sensitive process. Inhibition of flowering by carbohydrates was accompanied by reduced levels of chlorophyll and β-carotene. The inhibitory effects of carbohydrates on flowering were partially reversed by iminodiacetate, glycine, and L-aspartate but not by D-aspartate, ethylenediaminetetraacetate, acetate, δ-aminolevulinic acid, or mevalonic acid. The possibility is discussed that carbohydrate repression of flowering and of chloroplast pigments resulted from inadequate levels of amino acids.**

In a previous paper (14) it was shown that flowering in *Lemna perpusilla* strain 6746 grown on dilute Hutner's medium was inhibited by sucrose; increasing the concentration of the macronutrients prevented the inhibition whereas the micronutrients had no effect. Two approaches were suggested: the first, to determine which of the macronutrients were active in overcoming the inhibition; the second, to determine which organic supplements mimicked the effect of sucrose.

The first paper in this series (15) demonstrated that $Ca^{2+}$ and to a greater extent phosphate were the active ions (*i.e.*, increasing their levels in otherwise dilute medium partially prevented the inhibition of flowering by sucrose); fructose and glucose were also inhibitory, whereas the nonutilizable carbohydrate, mannitol, was not.

This paper reports on further screening of organic compounds, and on the effects of fructose given during or after a single inductive long night. It will also be shown that carbohydrate-induced inhibition of flowering is accompanied by reduced levels of chloroplast pigments.

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### MATERIALS AND METHODS

**Growth and Flowering.** Stock cultures were grown under continuous light, and experimental cultures in an 8-hr photoperiod as described previously (14, 15). Compounds were screened as follows: stocks grown with sucrose (30 mM) were the source of three-frond colonies which were planted individually onto 0.1 strength Hutner's medium (9) supplemented with the test compound. Unsupplemented medium and in some cases medium containing an inhibitory concentration of sucrose were used as controls. Since some supplements promoted frond production, dissections were done at various intervals, as noted, so that comparisons of treatment effects on flowering intensity (Fl%) could be made with cultures containing about the same number of fronds.

The ability of supplements to support heterotrophic growth was tested by incubating the cultures in darkness (24–27 C) interrupted every 24 hr with 15 min of white fluorescent light (about 40 ft-c). Light of this intensity and duration is not sufficient to maintain autotrophic growth but is required for heterotrophic growth (7, 13).

The effects of carbohydrate given during or after a single inductive long night were determined as follows. Single three-frond colonies from stock cultures grown on half-strength Hutner's medium without sucrose were planted in 0.1 strength medium and placed under continous light (warm and cool white fluorescent; about 150 ft-c) for 2 to 3 days. The cultures were then given 16 hr of darkness and returned to continuous light. For treatment with carbohydrate, fronds were transferred to 0.1 strength medium with fructose (30 mM) for the required length of time, and then transferred back to unsupplemented 0.1 strength medium.

**Chlorophyll and β-Carotene.** Flasks (125 ml) containing 50 ml of medium were inoculated with two three-frond colonies from sucrose-supplemented stocks and grown in an 8-hr photoperiod for 5 days. For each treatment, the cultures were pooled and two samples (each about 300 mg fresh wt) were homogenized and extracted with acetone at about 4 C under dim light. Extractions were repeated until the pellet was white. The supernatants were pooled and diluted with water to bring the final acetone concentration to 80%; water contributed by the tissue was taken into account, assuming a water content of 90%. The chlorophyll was estimated by $A_{652}$ measurements with the extinction coefficient of Arnon (2).

Methods for β-carotene estimates, based on those of Goodwin (5), were as follows. The pigments in the acetone extract were transferred to petroleum ether (37–50 C b.p.) which was then

Table I. *Effects of Various Carbohydrates on Flowering of Lemna perpusilla 6746*

Stock cultures grown on half-strength Hutner's medium with 30 mM sucrose. Experimental cultures grown on 0.1 strength Hutner's medium in an 8-hr photoperiod. Supplements at 30 mM. Dissections on day 10. Values are means of six values ± SE.

| Supplement | No. of Fronds | Fl% |
|---|---|---|
| None | 39.7 ± 4.2 | 30.9 ± 5.4 |
| Fucose | 38.3 ± 5.8 | 30.3 ± 3.8 |
| Rhamnose | 50.0 ± 3.7 | 30.5 ± 3.9 |
| Sedoheptulose | 46.8 ± 7.2 | 47.9 ± 5.7 |
| Glucose | 85.0 ± 1.6 | 0.0 |
| Mannose | 32.2 ± 2.2 | 0.0 |

Table II. *Effects of Fructose (30 mM) Given during or after a Single Inductive Long Night*

Cultures grown on 0.1 strength Hutner's medium under continuous light for 2 days, then given 16 hr of darkness, followed by continuous light. Values are means of five cultures. See the text for details.

| Fructose Treatment | Day 4 | | Day 5 | | Day 6 | | Day 7 | |
|---|---|---|---|---|---|---|---|---|
| | No. of fronds | Fl% | No. of fronds | Fl% | No. of fronds | Fl% | No. of fronds | Fl% |
| None | 14.6 | 28.4 | 14.0 | 25.6 | 23.6 | 10.6 | 28.2 | 0.0 |
| During long night | 16.0 | 0.0 | 22.2 | 0.0 | 25.4 | 0.0 | 27.2 | 0.0 |
| During 24 hr After long night | 16.6 | 13.4 | 19.0 | 13.5 | 24.4 | 7.3 | 26.2 | 0.0 |

washed three times with distilled water and place on an alumina column. The β-carotene was eluted with petroleum ether-acetone (97:3), and was estimated by absorbancy readings at 452 nm with authentic β-carotene (Mann) as a standard.

**DNA Determinations.** The acetone-extracted pellets were extracted as follows (18): two times with 5% trichloroacetic acid and once with 95% ethanol at 4 C, followed by extractions with boiling ethanol (95%), with ethanol-ether (2:1) and ether, and then with 5% perchloric acid (90 C; 10 min). The DNA content of the perchloric acid supernatant was estimated by the diphenylamine reaction (3) with calf thymus DNA (Worthington) as the standard.

## RESULTS

**Screening Compounds for Inhibitory Effects.** Previous results (15) had shown that sucrose, glucose, and fructose but not mannitol were inhibitory. Subsequent experiments tested the effects of other sugars as well as organic acids. The results indicated that the compounds tested could be placed in one of three categories: (*a*) at concentrations as low as 7.5 mM, those which inhibited growth and/or caused abnormal vegetative growth, thus making evaluation of flowering impossible or meaningless: galactose, sorbose, arabinose, xylose, deoxyribose, erythrose, glyceraldehyde, K salts of pyruvate, malate, oxaloacetate, α-ketoglutarate, and succinate; (*b*) at 30 mM, those which had no effect on either flowering or rate of frond production: mannitol, rhamnose, fucose, sedoheptulose; (*c*) at 30 mM, those which inhibited flowering and either promoted or had no effect on rate of frond production: sucrose, glucose, fructose, mannose.

Results illustrating the last two categories are in an earlier paper (15) and in Table I. Fucose, rhamnose, and sedoheptulose had no significant effect on frond production or Fl%. Flowering was completely inhibited by mannose and glucose, but only the latter promoted frond production.

**Single Inductive Long Night.** Fructose (30 mM) was given during the long night or during the 24-hr period immediately following the long night. Replicate cultures were dissected 4, 5, 6, and 7 days after the long night (Table II). Cultures not treated with fructose had high Fl% values on days 4 and 5; a progressive decrease occurred during days 6 and 7. This reduction was not merely due to dilution caused by the appearance of fronds produced after the inductive long night. There was in fact a decline in the number of flowering fronds, apparently due to a regression in flower development. This phenomenon in 6746 has been observed before (8, 13).

Qualitatively similar results were obtained with cultures treated with fructose after the long night; there was, however, about a 50% reduction in the flowering intensity on days 4 and 5 compared to the untreated controls. In cultures given fructose during the long night, Fl% values were zero, regardless of dissection time.

**Heterotrophic Growth.** It was of interest to determine which of the compounds tested were utilizable. One way to do this was to test their ability to support growth under essentially nonphotosynthetic conditions (see "Materials and Methods"). Of the substances listed in categories *b* and *c*, only glucose, fructose, sucrose, and mannose supported heterotrophic growth. As examples, mean frond numbers on day 8 for the aforementioned sugars (15 mM) were 25, 21, 23, and 12, respectively, whereas all other compounds tested did not increase frond numbers over the initial inoculum.

**Reversal by Amino Acids.** Previous work (15) showed that wash water from the cation exchange resin, Chelex, prevented sucrose-induced inhibition of flowering. According to technical literature supplied by the manufacturer (BioRad), Chelex, upon standing, releases iminodiacetate. Authentic iminodiacetate was therefore tested and found to prevent the effect of sucrose. The next question to be answered was whether the effect of iminodiacetate was a result of chelation or due to its entry into the metabolism of the fronds. For example, it was possible that the iminodiacetate was broken down to glycine and acetate, and that one or both of these were the active material.

To distinguish between these possibilities, a few approaches were used. The chelator EDTA, at concentrations as high as 0.085 mM, had no effect on the sucrose inhibition. Further, acetate (3 mM) did not overcome the inhibition by sucrose and, in fact, seemed to be inhibitory itself. However, most of the reduction in Fl% seemed to have been simply due to a reduction in frond production.

Table III. *Effects of Glycine and Iminodiacetate on Flowering of Lemna perpusilla 6746*

Cultures grown as in Table I. Sucrose at 30 mM. Those with sucrose dissected on day 6, those without sucrose on day 10. Values are means of five cultures ± SE.

| Supplement | Concn | Without Sucrose | | With Sucrose | |
|---|---|---|---|---|---|
| | | No. of fronds | Fl% | No. of fronds | Fl% |
| | mM | | | | |
| None | ... | 29.6 ± 1.2 | 68.1 ± 1.5 | 26.2 ± 0.4 | 6.8 ± 4.5 |
| Glycine | 0.15 | 16.6 ± 1.4 | 52.3 ± 2.8 | 26.2 ± 0.4 | 29.8 ± 1.4 |
| | 0.60 | 13.2 ± 1.0 | 28.0 ± 3.3 | 19.4 ± 1.0 | 28.8 ± 4.7 |
| Iminodiacetate | 0.15 | 30.4 ± 0.8 | 69.7 ± 1.0 | 27.4 ± 0.4 | 13.3 ± 7.6 |
| | 0.60 | 29.4 ± 0.4 | 65.3 ± 1.0 | 27.0 ± 0.3 | 23.0 ± 7.5 |

In contrast, partial reversal of the sucrose inhibition occurred in the presence of glycine. This is indicated in the results of an experiment comparing the effect of glycine with that of iminodiacetate (Table III). At 0.6 mM glycine and iminodiacetate were equally effective in preventing inhibition by sucrose; at 0.15 mM glycine was more effective than iminodiacetate.

Another amino acid, L-aspartic acid, was also found to be effective in reversing the sucrose inhibition. This allowed for another test of the chelation idea, namely, a comparison of the effects of L-aspartic acid with that of the D form. Both have equal chelation properties, but the D form is metabolically inactive. The results (Table IV) show that neither form affected flowering in the absence of sucrose. The inhibitory effect of sucrose was partially reversed by L-aspartic acid but not by the D form. Neither form had a significant effect on frond production in the presence or absence of sucrose.

In short, the ability of glycine and L-aspartic acid to overcome the flower-inhibitory effect of sucrose appears to have been due to their effects as metabolites, and not as chelators. In addition, glycine partially reversed the inhibitory effects of glucose, fructose, and mannose (Table V).

**Chlorophyll and β-Carotene.** Fronds in which flowering was inhibited by carbohydrates appeared lighter green than untreated controls. Experiments were therefore done to determine the effects of sucrose on chlorophyll and β-carotene levels. Since previous results (14, 15) had shown that flowering was inhibited by sucrose in 0.1 strength medium, but not in half-strength, both dilutions were tested. The results of one such experiment (Table VI) show that chlorophyll and, to a greater extent, β-carotene were reduced by sucrose in cultures grown on 0.1 strength medium; in half-strength medium, sucrose appeared to increase pigment levels. The other flower-inhibitory sugars, glucose, fructose, and mannose, also markedly reduced the pigment levels in cultures grown on 0.1 strength medium (Table VII).

These results suggested the possibility that the metabolic change induced by the carbohydrates involved a biosynthetic pathway leading to a chloroplast pigment as well as to a substance required for flowering. A test of this hypothesis involved determining whether a precursor of chlorophyll, δ-aminolevulinic acid (0.015 mM), or a precursor of β-carotene, mevalonic acid (0.015 and 0.03 mM), could overcome the flower-inhibitory effect of sucrose. Neither precursor reversed the inhibition. Since the main interest of this study was the flowering effect, no tests were done to determine whether the precursors reversed the inhibition of chloroplast pigments. However, fronds treated with δ-aminolevulinic acid or mevalonic acid were not darker green than untreated controls.

Table IV. *Effects of L- and D-Aspartic Acid on Flowering of Lemna perpusilla 6746*

Cultures grown as in Table I. Sucrose, 30 mM; amino acids at 0.015 mM. Those with sucrose dissected on day 8, those without sucrose on day 10. Values are means of six cultures ± SE.

| Supplement | Without Sucrose | | With Sucrose | |
|---|---|---|---|---|
| | No. of fronds | Fl% | No. of fronds | Fl% |
| None | 32.8 ± 3.5 | 68.3 ± 4.5 | 64.2 ± 8.6 | 4.2 ± 1.7 |
| D-Aspartic acid | 41.4 ± 5.7 | 73.9 ± 3.3 | 72.3 ± 3.0 | 8.4 ± 1.5 |
| L-Aspartic | 34.4 ± 4.0 | 71.7 ± 3.4 | 68.8 ± 1.9 | 37.5 ± 2.9 |

Table V. *Glycine reversal of sugar-induced inhibition of flowering of Lemna perpusilla 6746*

Cultures grown as in Table I. Dissections on day 8. Glycine at 0.3 mM.

| Supplement | Concn | Without Glycine | | With Glycine | |
|---|---|---|---|---|---|
| | | No. of fronds | Fl% | No. of fronds | Fl% |
| | mM | | | | |
| Sucrose | 30 | 71.8 ± 3.7 | 0.0 | 48.0 ± 5.2 | 60.5 ± 5.7 |
| Glucose | 30 | 55.2 ± 1.3 | 0.0 | 42.6 ± 3.1 | 23.8 ± 3.3 |
| Fructose | 10 | 30.0 ± 1.5 | 0.0 | 28.0 ± 0.4 | 14.4 ± 7.0 |
| Mannose | 30 | 19.0 ± 1.2 | 0.0 | 23.4 ± 1.2 | 26.3 ± 5.5 |

Table VI. *Effects of Concentration of Medium and of Sucrose on Chlorophyll and β-Carotene Levels*

Sucrose at 30 mM. Each value mean of two samples. Cultures grown under 8-hr photoperiod for 5 days. See the text for details.

| Medium | β-Carotene | | Total Chlorophyll | |
|---|---|---|---|---|
| | *ng/mg fresh wt* | *ng/μg DNA* | *ng/mg fresh wt* | *ng/μg DNA* |
| Half-strength | 12 | 77 | 464 | 3155 |
| Half-strength + sucrose | 15 | 124 | 551 | 4682 |
| 0.1 strength | 15 | 100 | 500 | 3127 |
| 0.1 strength + sucrose | 4 | 27 | 299 | 2140 |

Table VII. *Effects of Various Sugars on β-Carotene and Chlorophyll Levels*

Cultures grown on 0.1 strength medium with fructose and mannose at 20 mM; glucose at 30 mM. Eight-hour photoperiod for 5 days. Each value mean of two samples. See the text for details.

| Supplement | β-Carotene | | Total Chlorophyll | |
|---|---|---|---|---|
| | *ng/mg fresh wt* | *ng/μg DNA* | *ng/mg fresh wt* | *ng/μg DNA* |
| None | 20 | 120 | 631 | 3785 |
| Glucose | 6 | 69 | 198 | 2395 |
| Fructose | 6 | 46 | 259 | 1902 |
| Mannose | 5 | 61 | 300 | 3542 |

## DISCUSSION

**Carbohydrate-Amino Acid Interaction.** The flower-inhibitory effect of sucrose, fructose, and glucose reported in previous papers (14, 15) was mimicked by mannose, but not by any of the other compounds tested, *e.g.*, mannitol. Only those sugars that inhibited flowering were able to support heterotrophic growth. These results are consistent with the idea that the inhibition was not merely due to osmotic effects of the sugars. The sugar-sensitive process was an early stage in the flowering process, possibly induction or initiation. Relatively low concentrations of either glycine or L-aspartic acid partially reversed the inhibitory effects of the sugars on flowering but did not promote growth. These amino acids were not simply satisfying a requirement for additional nitrogen, since supplementing 0.1 strength medium with either $NO_3^-$ or $NH_4^+$ did not reverse the inhibition of flowering by sucrose (15).

If it is assumed that the ability of glycine or L-aspartic acid to reverse the inhibitory effect of carbohydrate reflected inadequate levels of these amino acids *in vivo*, two questions may be considered. The first is how these inadequate levels prevented flowering. One possibility is that glycine and L-aspartic acid or some compound derived from them are precursors of a substance specifically required for flowering. Results reported for short day and long day plants on the effects of amino acid analogues (4,

11, 19) and of exogenously supplied amino acids (12) have indicated that the amino acid status is important during early stages of the flowering process, especially during the dark inductive period. It has been suggested that not all amino acids are required, and that synthesis of a "flowering hormone" is involved (4). The present results add evidence for a regulatory role of amino acids and further indicate an interaction with carbohydrates.

Thus the second question to be considered is how sugars might lead to inadequate levels of amino acids. Previous results (15) showed that phosphate caused a pronounced reversal of the sucrose effect. Phosphate occupies a key position in the regulation of respiration. It is therefore possible that low levels of phosphate slowed down glycolysis, causing a relatively high diversion of glucose through the pentose phosphate pathway. Such a diversion would be enhanced by sucrose induction of glucose-6-P dehydrogenase (see Ref. 17) and might lead to lower levels of amino acid precursors. The ability of L-aspartic acid to reverse the inhibitory effect on flowering without promoting growth could therefore be interpreted to mean that levels of amino acids *in vivo* were adequate for vegetative growth, but not for flower induction.

Kandeler (10) has shown that flowering of the long day plant *Lemna gibba* is inhibited by sugar and by high levels of carbon dioxide, and on the basis of these and other results he has suggested that excessive consumption of ATP prevented flowering. The reversal of the sucrose effect in 6746 by phosphate supplements (15) is consistent with this idea. Thus glycine and L-aspartic acid might have affected ATP levels by acting as precursors for respiratory intermediates.

**Chloroplast Pigments.** Although there have been reports of carbohydrate repression of chlorophyll formation in algae (see, for examples, Refs. 1 and 16), the results with *Lemna* seem to be the first report of such an effect in a higher plant. In *Chlorella protothecoides*, reductions in the formation of carotenoids and especially chlorophyll occurred when glucose-urea ratios were high (16). In contrast, the synthesis of $\beta$-carotene in *Lemna* was more sensitive to carbohydrate repression than was chlorophyll.

The mechanism of the effect of sugar on chloroplast pigments in *Lemna* may be explained on the basis of the previous assumption, namely, inadequate levels of amino acids. Glycine is a precursor of $\delta$-aminolevulinic acid, which is required for chlorophyll synthesis. The resulting inhibition of chloroplast formation might have caused reductions in carotene synthesis. However, the apparent inability of $\delta$-aminolevulinic acid and mevalonic acid to reverse the sucrose effect on chlorophyll is inconsistent with this hypothesis, although compartmentalization of biosynthetic pathways (see Ref. 6) complicates interpretation of these negative results.

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