Raffinose Synthase and Galactinol Synthase in Developing Seeds and Leaves of Legumes

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The raffinose family oligosaccharides (RFO) have been proposed to play roles in cold acclimation in plants, seed viability, and flatulence in humans after consumption of beans. Galactinol synthase (GS) and raffinose synthase (RS) are believed to be key enzymes in the biosynthesis of RFO. To begin to understand the roles proposed for RFO, we have begun a study of GS and RS in soybean and kidney beans. Both exchange and synthesis reactions of RS were detected in developing soybean seed as early as 5 days after flowering (DAF). While the RS synthesis activity leveled off at 15 DAF, the RS exchange activity continued to increase up to 60 DAF. The RS exchange and synthesis activities also differed in their reaction kinetics, pH optima, and purification properties. GS activity was highest at the late stages of kidney bean development. Consistent with the proposed role of RFO in cold acclimation, the GS activities in the developing seeds and leaves of soybean and kidney bean increased 3–4-fold upon exposure of whole plants to 4 °C. This is the first report of GS activity in kidney bean and RS in soybean.

The raffinose family oligosaccharides (RFO), which includes raffinose, stachyose, and verbascose, rank next to sucrose in their distribution in the plant kingdom. RFO have been proposed to play roles in cold acclimation, seed viability, and flatulence in humans after consumption of beans.

During winter, there is an increase in the content of sucrose and RFO with a decrease in starch in leaves and seeds. In pine needles (*Picea excelsa*), the pool size and relative turnover of sucrose, raffinose, and stachyose are 2–100-fold higher in January than in June in the northern hemisphere (Kandler and Hopf, 1980). Santarius and Milde (1977) showed that, in the frost-hardy cells of cabbage leaves, the dehardening process leads to a decrease in the concentration of sucrose and raffinose, especially in the chloroplasts. In vitro experiments demonstrated that sugars can protect thylakoids from freezing damage, raffinose being more effective on a molar basis than sucrose and glucose (Santarius, 1973). These results indicate that the commonly observed accumulation of sucrose and RFO in leaves and seeds may contribute to cold acclimation.

The role of RFO in seed viability has been proposed on the basis of observations that viable corn seeds have higher RFO content and sucrose than nonviable seeds (Ovcharov and Koshelev, 1974). During soybean embryogenesis, the loss of water at the late stage of seed maturation is accompanied by an increase in RFO and galactinol synthase (GS) activity (Saravitz et al., 1987). The RFO may provide a microenvironment of bound water and hydroxyl groups that protects membrane proteins and other biologically active molecules from denaturation during the desiccation process toward dormancy. A similar mechanism may be proposed for the cold acclimation process.

Flatulence in humans after consumption of beans or raffinose (Calloway and Murphy, 1968) is caused by the inability of humans to hydrolyze the α-1,6-galactoside link in RFO that end up in the lower gut. Bacterial fermentation of RFO leads to the formation of methane, hydrogen, and carbon dioxide. Bean scientists and growers consider flatulence to be the single most important factor that deters Americans from eating more beans.

The current knowledge on biosynthesis of RFO in plants is the following (Dey, 1985):

$$\text{UDP-galactose} + \text{myoinositol} \rightarrow \text{galactinol} + \text{UDP} \quad (1)$$

$$\text{sucrose} + \text{galactinol} \rightarrow \text{raffinose} + \text{myoinositol} \quad (2)$$

$$\text{raffinose} + \text{galactinol} \rightarrow \text{stachyose} + \text{myoinositol} \quad (3)$$

$$\text{stachyose} + \text{galactinol} \rightarrow \text{verbascose} + \text{myoinositol} \quad (4)$$

GS catalyzes reaction 1, raffinose synthase (RS) reaction 2, stachyose synthase reaction 3, and verbascose synthase reaction 4. GS and RS may be the key enzymes that channel sucrose into the RFO biosyntheric pathway. To begin to understand the roles proposed for RFO, we have begun a study of RS and GS in developing seeds and leaves of soybean and kidney bean.

MATERIALS AND METHODS

Soybean and Kidney Bean Plants. To determine the stage of seed development where GS and RS activities are highest, soybean (*Glycine max*, variety Hudson 78) and kidney bean (*Phaseolus vulgaris*, variety California Dark Red) were grown in the greenhouse, and the flowers were individually tagged. Soybean pods were collected at 5, 15, 30, 45, and 60 days after flowering (DAF) while kidney bean pods were harvested at 27, 35, 41, and 50 DAF and stored at −80 °C until ready to be extracted.

For the cold-exposure experiments, the plants were transported to our laboratory and exposed to room temperature (23 °C) for at least 3 h with light from a 150-W bulb (Plant Light, Gro and Sho, General Electric) before sampling. The soybean plants used in these experiments were 7–8 weeks old with pods 30–40 DAF while the kidney bean plants were approximately 4–5 weeks old with 10–18-DAF pods. Leaves of the same age from different stems throughout each plant were collected. As much as possible, the seeds sampled at different times and temperatures were from the same pods of similar age. The plants were transferred to the cold room (4 °C) provided with the same lighting, and the leaves and seed were sampled at 1, 2, and 4 h.

Raffinose Synthase Assay. In addition to catalyzing the synthesis of raffinose from sucrose and galactinol, raffinose synthase has been shown to catalyze the exchange reaction between raffinose and radioactively labeled sucrose (Lehle and Tanner,

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1973). Both exchange and synthesis reactions were carried out (Lehle and Tanner, 1973) in a total volume of 70 μL. The appropriate amount of enzyme (4–200 μg of protein) was preincubated with 0.1 M Tris-HCl buffer, pH 7.3, 5 mM DTT for 15 min at 25 °C. For the exchange reaction, the rest of the incubation mixture consisted of 2 μL of [U-^{14}C]sucrose (1 μCi/μL, 350 μCi/μmol), 1.5 μL of cold 0.01 M sucrose, and 5 μL of 0.01 M raffinose. The reaction was carried out for 1–4 h at 37 °C and terminated by adding 100 μL of ethanol. The reaction mixture was centrifuged for 4 min in an Eppendorf microfuge, and the supernatant was spotted onto Whatman 3MM paper. The paper chromatogram was developed for 17 h in 1-butanol/pyridine/water/acetic acid (60/40/30/3, v/v). The paper was exposed to X-ray film for 2–4 days at −80 °C. Matching the paper with the autoradiograph, radioactive spots were cut out and counted in a scintillation counter. To confirm the location of the spots, the papers were sometimes sprayed with aniline/diphenylamine/H_3PO_4 (Block et al., 1958) to visualize the raffinose saccharides in comparison with standards. The resolution of raffinose and stachyose from sucrose was good with R_f values of 0.52, 0.32, and 0.23 for sucrose, raffinose, and stachyose, respectively.

The synthesis reaction was similarly carried out except that 5 μL of 0.01 M galactinol replaced raffinose. The controls consisted of heated enzyme extracts in place of active enzyme or omitting galactinol in the synthesis reaction or raffinose in the exchange reaction.

To determine the effects of time on the linearity of product formation, samples were incubated at 1, 2, 3, and 4 h with 60 μg of enzyme protein. The effect of pH on the velocity of the reaction was determined with use of 0.1 M phosphate for pH 5.0, 6.0, and 7.0 and 0.1 M Tris-HCl for pH 7.0, 8.0, and 8.5.

Galactinol Synthase Assay. The leaves were ground in buffer (2 mM sodium phosphate, pH 7.0, 0.02 mM DTT) at a ratio of 1/5 (g/mL) with mortar and pestle and homogenized for 25 min at 4 °C with a Polytron homogenizer (Handley et al., 1983). The homogenate was centrifuged at 27000*g* for 15 min and the supernatant recovered and stored at −20 °C until assayed. The seeds were processed in a similar manner except that the extraction buffer had the following composition: 2 mM Hepes–NaOH, pH 7.0, 0.4 mM DTT, 0.2% bovine serum albumin (BSA) (Saravitz et al., 1987).

GS was analyzed according to the procedure of Pharr et al. (1981). The complete reaction including enzyme (5–20 μL) in a total reaction volume of 100 μL contained UDP [^{14}C]galactose (2.0 μmol) with specific activity 1.04 μCi/μmol, myoinositol (2.0 μmol), $MnCl_2$ (0.7 μmol), DTT (0.05 μmol), and Hepes–NaOH buffer at pH 7.2 (2.5 μmol) for the seed or phosphate–citrate buffer, pH 5.4 (2.5 μmol) for the leaves.

Reactions were initiated with the enzyme in 1.5-mL Eppendorf microfuge tubes and incubated for 10 min at 30 °C. To determine the amount of non-myoinositol-dependent product formation, a reaction mixture lacking inositol (minus myoinositol blank) was incubated simultaneously. Reactions were terminated by the addition of 400 μL of 100% cold ethanol to each tube.

Dowex 1 anion-exchange resin was utilized to remove excess UDP [^{14}C]galactose from the terminated reaction mixtures. The resin was prepared according to the method of Splittstoesser (1969). The moist resin was then mixed with distilled water in a ratio of 1/1 (w/v). A 300-μL portion of this slurry was added to each tube. The reaction tubes were then mixed on a rotary shaker for 30 min and centrifuged in an Eppendorf microfuge for 2 min. A 100-μL aliquot of the supernatant was pipetted into 10 mL of scintillant composed of 2 parts of toluene to 1 part of Triton X-100 containing 6.0 g/L PPO and 0.3 g/L POPOP and counted in a scintillation counter.

The values reported in all the figures are averages of two to three assays done in duplicate.

Partial Purification of Raffinose Synthase from Soybean Seed. All steps were carried out at 4 °C. Frozen 45- and 60-DAF seeds were powdered with mortar and pestle and extracted with 0.1 M Tris-HCl, pH 7.3, containing 5 mM DTT with an Elvejehm homogenizer at 10 mL/g of sample. The homogenate was centrifuged for 30 min at 27000*g* to obtain a clear supernatant.

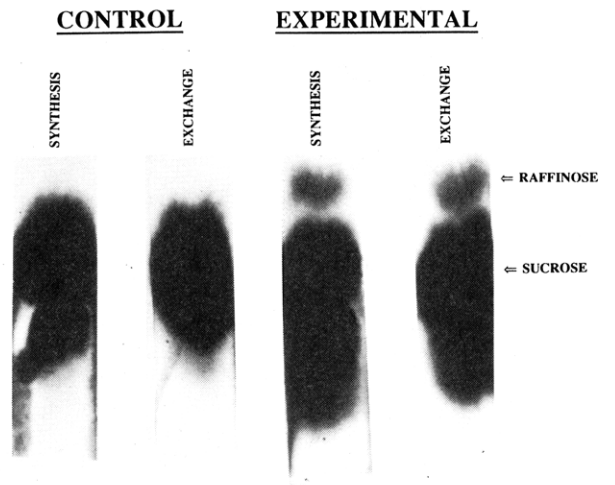

Figure 1. Exchange and synthesis reaction catalyzed by raffinose synthase in developing soybean seed. In this experiment, 156 μg of crude protein extract from 45-DAF seeds was used for each assay as described in Materials and Methods. The controls contained crude protein extract heated for 10 min at 100 °C. Reactions were carried out for 4 h at 37 °C. The direction of solvent migration is from top to bottom.

The supernatant was brought to a protein concentration of 40 mg/mL with the extraction buffer. A 2% protamine sulfate solution was added to a final ratio of 9 mg of protamine sulfate/100 mg of protein. The solution was stirred for 30 min, and the precipitate was sedimented by centrifugation as above and discarded.

To the protamine sulfate supernatant was added cold, saturated ammonium sulfate solution, pH 7.3, slowly with constant stirring to 30% saturation. After 30 min, the precipitate was collected by centrifugation as before, redissolved in 2 mL of extracting buffer, and dialyzed against 0.05 M Tris-HCl/1 mM DTT.

The 30% saturation supernatant was brought up to 60% saturation by adding cold, saturated ammonium sulfate, the mixture was allowed to stand for 30 min, and the pellet was collected by centrifugation. The pellet was redissolved in 2 mL of extraction buffer and dialyzed overnight as before.

Statistical Analysis. Significant differences among means were determined by one-way analysis of variance followed by LSD multiple comparison test using STATGRAPHICS (STSC, Inc. Rockville, MD).

RESULTS

Exchange and Synthesis Reactions of Raffinose Synthase. Our initial assays using both exchange and synthesis reactions established the presence of raffinose synthase in developing soybean seed (Figure 1). Raffinose was absent in the control reactions containing the heat-inactivated enzymes but was present in the reaction mixtures containing the active enzymes. Specific activities of 18.2×10^{-9} and 15.4×10^{-9} μmol of raffinose/μg of protein per h for exchange and synthesis reactions, respectively, were obtained in this experiment.

The effect of incubation time on enzyme activity is shown in Figure 2. While the exchange reaction activity continued to increase up to 4 h, the synthesis activity increased very slightly up to 3 h and remained constant.

The exchange reaction exhibited a typical bell-shaped curve with an optimum between pH 7 and 8 (Figure 3). The optimum pH of the synthesis reaction was less well-defined with an apparent optimum between pH 6 and 7.

Raffinose Synthase and Galactinol Synthase Levels in Developing Soybean and Kidney Bean Seed. The exchange and synthesis reactions catalyzed by raffinose synthase were detected in soybean as early as 5 DAF

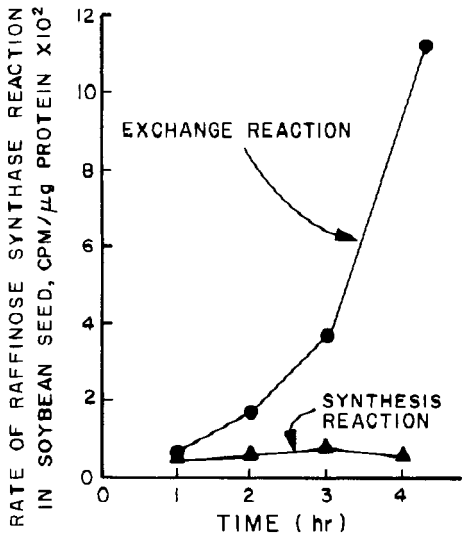

Figure 2. Rate of raffinose synthase reaction in soybean seed. Samples were incubated for 1, 2, 3, and 4 h with 60 μg of protein/reaction.

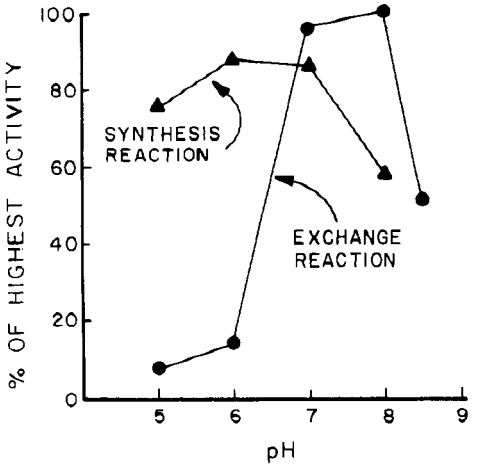

Figure 3. Effect of pH on raffinose synthase activity in soybean seed. Phosphate buffer (0.1 M) was used at pH 5.0, 6.0, and 7.0, and Tris-HCl (0.1 M) was used for a pH 7.0, 8.0, and 9.0.

(Figure 4). However, while the exchange activity continued to increase up to 60 DAF, the synthesis activity leveled off at 15 DAF and thereafter. The soybean seeds attained their heaviest weight at 30 DAF and started to dehydrate thereafter. The 60-DAF seeds were still partially green and were not completely dry yet.

The GS activity in kidney bean seed increased 1.5-fold from 27 to 35 DAF and peaked at 41 DAF when the fresh weight of the seed started to decrease as the desiccation process began (Figure 5). However, only the increase from 27 to 35 DAF was statistically significant ($\alpha = 0.006$). The seed attained its full fresh weight (average of 1200 mg) at 35 DAF.

Partial Purification of RS in Soybean Seed. Treatment of the crude extract with protamine sulfate gave approximately 2–3-fold purification (Table I). About 62% of the exchange reaction was recovered in the 30% $(NH_4)_2SO_4$ fraction with an 8-fold increase in purification, with most of the remaining activity in the 60% $(NH_4)_2SO_4$ fraction. Recoveries and fold purification were lower in the synthesis reaction. Treatment with protamine sulfate gave about 2-fold purification with 69% recovery. $(NH_4)_2SO_4$ fractionation at 30% and 60% saturations yielded only 1.5-fold purification and about 11% recovery.

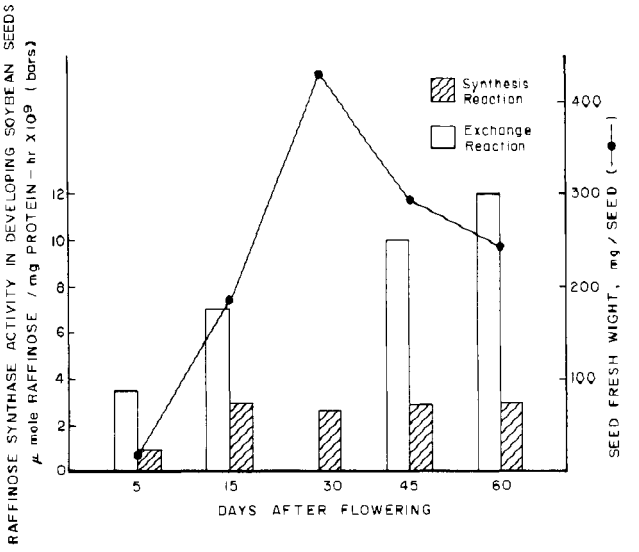

Figure 4. Raffinose synthase activity in developing soybean seed. Soybean plants of Hudson 78 variety were grown in the greenhouse, the flowers were individually tagged, and pods were collected at 5, 15, 30, 45, and 60 DAF. The assay was carried out as described in the text with 140–200 μg of protein.

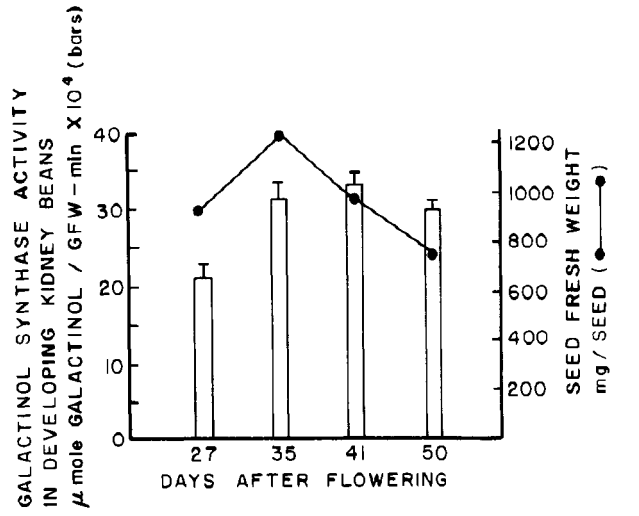

Figure 5. Galactinol synthase activity in developing kidney bean seed. Kidney bean plants variety California Dark Red were grown in the greenhouse, individual flowers were tagged, and the pods were collected at 27, 35, 41, and 50 DAF. The values of specific activity are averages of four to six replicates. To obtain the average weight of the seed, five to six seeds were weighed. GFW = grams fresh weight.

Low-Temperature Effects on GS Activity in the Seed and Leaves of Soybean and Kidney Bean. GS activity in soybean leaves increased approximately 3-fold over room temperature within 4 h after exposure to 4 °C (Table II). The increase was statistically significant ($\alpha = 0.005$ level). In soybean seed, GS declined after 1 h at 4 °C from room-temperature value, attained a maximum at 2 h (approximately 2-fold over room temperature), and decreased to 75% of the 2-h value at 4 h of 4 °C exposure (Table II). The 3.7-fold increase from 1 to 2 h at 4 °C was statistically significant ($\alpha = 0.108$ level).

In kidney bean leaves, no GS activity was measurable at 27 °C (Table III). After 1 h at 4 °C, some activity appeared and continued to increase up to 4 h, reaching a specific activity approximately 5-fold over the 1-h value. The 2- and 4-h values at 4 °C were significantly different from that at 27 °C ($\alpha = 0.004$). In kidney bean seed, the GS activity increased approximately 3-fold over 23 °C (significant at $\alpha = 0.006$) after 4 h at 4 °C (Table III).

Table I. Partial Purification of Raffinose Synthase from Soybean[a]

fraction	total protein, mg	exchange reaction: total act., μmol h^{-1}	exchange reaction: sp act., μmol mg^{-1} h^{-1}	exchange reaction: fold purifien	exchange reaction: rec, %	synthesis reaction: total act., μmol h^{-1}	synthesis reaction: sp act., μmol mg^{-1} h^{-1}	synthesis reaction: fold purifien	synthesis reaction: rec, %
crude extract	672	6317×10^{-5}	9.4×10^{-8}	1.0	100	961.0×10^{-5}	1.43×10^{8}	1.0	100
protamine sulfate	248	6051×10^{-5}	24.4×10^{-8}	2.6	95.8	664.6×10^{-3}	2.68×10^{-8}	1.9	69.2
30% $(NH_4)_2SO_4$ fraction	50.4	3901×10^{-5}	77.4×10^{-8}	8.2	61.8	100.8×10^{-5}	2.00×10^{-8}	1.4	10.5
60% $(NH_4)_2SO_4$ fraction	53.6	2087×10^{-5}	38.9×10^{-3}	4.1	33.0	114.2×10^{-5}	2.13×10^{-8}	1.5	11.9

[a] Average of duplicate trials.

Table II. Effects of Low Temperature on Galactinol Synthase Activity in Leaves and Seed of Soybean

sample treatment	GS act.,[a] μmol galactinol/(GFW min) $\times 10^4$
Leaves	
23 °C	146.8[a]
4 °C, 4 h	419.0[b]
Seeds	
23 °C	863.8[cd]
4 °C, 1 h	400.0[e]
4 °C, 2 h	1473.8[d]
4 °C, 4 h	1298.4[cd]

[a] Values are averages of four to six replicates; those with no common superscripts are significantly different from each other at $\alpha = 0.005$ for leaves and $\alpha = 0.108$ for seed.

Table III. Effects of Low Temperature on Galactinol Synthase Activity in Leaves and Seed of Kidney Bean

sample treatment	GS act.,[a] μmol galactinol/(GFW min) $\times 10^4$
Leaves	
27 °C	nil
4 °C, 1 h	10.75[ab]
4 °C, 2 h	26.75[b]
4 °C, 4 h	51.45[c]
Seeds	
23 °C	9.63[e]
4 °C, 4 h	31.55[d]

[a] Values are averages of four to six replicates; those with no common superscripts are significantly different from each other at $\alpha = 0.004$ for the leaves and $\alpha = 0.006$ for the seed.

DISCUSSION

Raffinose Synthase. Although expected, this is the first report of raffinose synthase activity in the developing soybean seed. We found in our initial experiments that the rate of the exchange reaction was higher than that of the synthesis reaction. This was confirmed in later experiments. For instance, while the rate of the exchange reaction continued to increase up to 4 h of incubation, that of the synthesis reaction increased only very slightly up to 3 h and remained constant. Similar observations were reported in *Vicia faba* by Lehle and Tanner (1973), who found that the exchange reaction activity of raffinose synthase was considerably more stable than the synthesis activity. This observation also explains why Moreno and Cardini (1966) were able to detect only the exchange reactions in wheat germ extracts.

Both exchange and synthesis reactions were observed in the developing soybean seed as early as 5 DAF. However, the exchange activity continued to increase up to 60 DAF while the synthesis activity leveled off at 15 DAF and thereafter. Consistent with our findings is the observation that raffinose and stachyose levels increased at the later stages of soybean seed development (Yazdi-Samadi et al., 1977; Saravitz et al., 1987).

Differences were observed in the recoveries and fold purification of the exchange and synthesis reactions. The highest purification (8-fold) of the exchange activity was obtained in the 30% $(NH_4)_2SO_4$ fraction, which accounted for 62% of the original total activity. On the other hand, the protamine sulfate treatment yielded the highest fold purification (1.9-fold) for the synthesis reaction and accounted for approximately 70% of the original total activity. Further treatment with $(NH_4)_2SO_4$ did not improve the purity of the enzyme and resulted in much lower recoveries (Table I).

The differences observed between the exchange and synthesis reactions may be explined in two ways:

One, the two reactions are catalyzed by the same enzyme with two different active sites, one for the exchange reaction and one for the synthesis reaction. The synthesis reaction active site, which binds both sucrose and galactinol, would be less stable than the exchange reaction site. Lehle and Tanner (1973) reported parallel activities of the exchange and synthesis reactions of raffinose synthase in *V. faba* during purification from the crude extract except in the last step of hydroxyapatite chromatography. These workers concluded that one enzyme catalyzed both synthesis and exchange reactions.

Two, the two reactions can be catalyzed by two different enzymes. The marked differences in the stabilities and pH profiles of the synthesis and exchange reactions support this model, although more studies on purified enzymes are needed to verify it.

Galactinol Synthase. We established that GS activity in kidney bean was highest at the late stage of seed development when the seed's fresh weight started to decrease as the desiccation process began. Our results are consistent with data obtained from soybean (Saravitz et al., 1987) where GS activity was highest at the stage when the seed starts to lose water. Also at this stage, the RFO were found to increase in soybean (Saravitz et al., 1987; Yazdi-Samadi et al., 1977). In four soybean genotypes of different maturity groups, Saravitz et al. (1987) showed a positive correlation between GS activity and concentration of stachyose that accumulated. Furthermore, they demonstrated a linear relationship between galactinol formed, estimated from the GS activity and the level of galactose in the RFO formed during seed development. These data support the proposed critical role of GS in regulating the channeling of sucrose into the RFO biosynthetic pathway.

We also showed that low temperature induced and/or increased GS activity in the leaves and seeds of soybean and kidney bean. This is consistent with the observed increase in the content of sucrose and RFO accompanied by a decrease in starch in leaves and seeds of plants during winter, which has been proposed to contribute to cold acclimation (Santarius and Milde, 1977; Santarius, 1973). Since soybean and kidney bean are not frost-hardy plants, we also assessed the effects of low temperature on a member of the pine tree family, *P. pinea*. GS activity in pine tree needles increased 2-fold over the room-temperature value at 1 h, 4 °C and 7-fold at 2 h, 4 °C and declined to about 52% of the 2-hvalue at 4 h, 4 °C.

Interestingly, after 3 h of reexposure to room temperature, GS activity decreased to a value almost equal to the initial room-temperature activity.

That GS activity was increased or induced by low temperature suggests that this enzyme may play a key role in the biosynthesis of RFO in response to low temperature. The increase or induction may be due to gene activation or a posttranslational process. If the former is true, the GS gene would be an interesting model for studying gene activation by low temperature in plants. Knowledge of molecular mechanisms of gene activation by low temperature in plants would be of potential importance in designing genetic engineering strategies for frost resistance or cold acclimation in plants. We are purifying GS from kidney bean in preparation to cloning its gene.

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Registry No. GS, 81725-96-4; RS, 62213-45-0.

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A Stable Form of Vitamin C: L-Ascorbate 2-Triphosphate. Synthesis, Isolation, and Properties

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L-Ascorbate 2-triphosphate (AsTP) was synthesized by phosphorylating L-ascorbate (AsA) with sodium trimetaphosphate (STMP). The regioselectivity of the reaction was optimum (95% of 2-phosphorylation) when 1.62 M AsA was reacted with 1.3 equiv of STMP at pH 10.4–10.7 and 35 °C for 24 h or 55 °C for 5 h. The reaction yielded 4% unreacted AsA, 86% AsTP, 3% L-ascorbate 2-diphosphate (AsDP), 3% of a 4,5-elimination compound (ENE), and 1% L-ascorbate 2-monophosphate (AsMP). Those derivatives of AsA were isolated by anion-exchange chromatography and identified by UV and ^{1}H, ^{13}C, and ^{31}P NMR spectroscopy. UV absorbance indicated that the L-ascorbyl moiety in AsTP was 1, 2, and 3 orders of magnitude more stable than AsA toward O_2 oxidation at 28–45 °C and pH 3, 6, and 8, respectively. AsMP, AsDP, and AsTP were readily hydrolyzed by acid phosphatase to give stoichiometric release of AsA. AsTP appeared to be equivalent to AsA as a source of vitamin C in guinea pigs.

The principal mechanism causing loss of vitamin C in foods and feeds is initiated by O_2 oxidation of L-ascorbic acid (AsA) catalyzed by Fe(III) and Cu(II) ions. The product, dehydroascorbic acid, retains vitamin potency but is rapidly decomposed to many compounds void of activity (Liao and Seib, 1988). In foods and feeds with high

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