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## THE INFLUENCE OF BORON ON STARCH PHOSPHORYLASE AND ITS SIGNIFICANCE IN TRANSLOCATION OF SUGARS IN PLANTS<sup>1,2</sup>

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An increase in the translocation of sugars in plants caused by boron has been demonstrated by Gauch and Dugger (6) and Sisler et al (26). Mitchell et al (22) obtained additional evidence that boron increases translocation of sugars and indirectly the translocation of growth-modifying substances. Previous work by Mitchell and Brown (21) and others showed that translocation of growth-modifying substances from leaves to other plant parts was dependent upon the translocation of sugars.

The hypothesis presented by Gauch and Dugger (6) to explain the influence of boron on translocation is based on: 1) the formation of borate-sugar complex; and 2) the movement of the complex through cellular membranes more rapidly than non-borate sugar molecules. Of the several possible explanations for the way boron could cause an increase in sugar translocation, these investigators favored the one which associated the borate ion with the cellular membrane. In this position the borate ion reacts chemically with the sugar molecule, and the resulting complex is transported across the membrane. A second reaction on the inside of the membrane releases the

sugar into the cell. Because of the rapid utilization of labeled sugars by living cells, these workers did not investigate this "carrier" concept as has been done in mineral element absorption studies (4).

Plants grown in a medium deficient in boron have been shown to accumulate carbohydrates, particularly starch, in the leaves (7). Because of this effect and the studies which show that boron increases translocation, it was decided to study the influence of boron on starch formation in vivo and in vitro.

In vitro studies by Winfield (29) showed a slight inhibition of starch phosphorylase by high concentrations of boron. He concluded that boron reacted with sugars in the same way as phosphorus; and because of the small boron:phosphorus ratio in plants, he did not believe that boron influenced the starch  $\rightleftharpoons$  glucose-1-phosphate equilibrium. Torsell (27), however, found no effect of  $10^{-3}$  M arylboric acids on starch phosphorylase.

The effect of boron on the starch phosphorylase reaction has been reinvestigated in view of the fact that boron may possibly complex glucose-1-phosphate and thus prevent its conversion to starch. In addition the influence of boron on the hexokinase reaction has also been investigated. The results of these studies are presented in this paper.

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## METHODS AND RESULTS

Ten-day-old snap bean plants, *Phaseolus vulgaris* L. var. Black Valentine, were transferred from the greenhouse to a dark room. After 48 hours in the dark, the level of starch in the primary leaves was reduced from 12 mg/gm fresh weight to 3 mg/gm fresh weight. At this time the leaves were removed, and 2- to 3-gram samples were rapidly weighed to the nearest mg and submerged in a solution of 4% glucose containing 0.001% Tween-20 (Polyoxyethylene sorbitan monolaurate) and 0.2 ml of 1 M  $K_2HPO_4$  per 100 ml. Other leaf samples were submerged in a solution containing various levels of boron, glucose, Tween-20 and phosphate. The leaf material was then subjected to vacuum infiltration according to McCready and Hassid (20). After infiltration, the leaves were removed and washed three times in tap water and twice in distilled water. They were then placed in covered dishes on moist filter paper and stored for 24 hours in the dark at 25° C. At this time the leaves were removed from the dishes and used in one of three analytical procedures: (a) starch analysis, (b) boron analysis, (c) respiration studies. The starch content was determined by digesting the alcohol insoluble residue with taka-diastase for 24 hours and subsequent acid hydrolysis to form glucose (1). Reducing sugars were determined by the method of Nelson (23). The colorimetric method of Dible et al (3) was used to analyze the plant tissue for boron content.

Table I shows the influence of boron on the starch content of these leaves. There is a definite decrease in the amount of starch synthesized by the leaves when infiltrated with solutions containing glucose and 20 millimoles/l or more of boron. It appears from these data that the addition of boron, at least above 10 millimoles/l, brings about some change in the metabolism of the leaf material that influences the synthesis of starch from added glucose. If this is associated with the conversion of glucose-1-phosphate

TABLE I

THE INFLUENCE OF BORON ON THE STARCH SYNTHESIS IN BLACK VALENTINE BEAN LEAVES INFILTRATED WITH 4% GLUCOSE AND VARIOUS LEVELS OF BORON (AV OF 6 EXPTS) \*

B IN INFILTRATING SOLUTION, MILLIMOLES/L	STARCH AS RED. SUGAR, MG/GM FRESH WT
Water	2.3
Glucose	3.9
1	3.9
5	3.9
10	3.6
20	2.9
50	2.6
100	2.8
L.S.D. 5%	1.1
L.S.D. 1%	1.4

\* The analysis was determined at 24 hrs after the leaves were infiltrated and placed in moist chambers in the dark at 25° C.

TABLE II

THE INFLUENCE OF INFILTRATING BLACK VALENTINE BEAN LEAF TISSUE WITH VARIOUS CONCENTRATIONS OF BORON ON THE ANALYSIS OF BORON

B IN INFILTRATING SOLUTION, MILLIMOLES/L	B IN SOLUTION, μGM/GM	B IN TISSUE, μGM/GM FRESH WT	RATIO, SOLUTION/TISSUE
1	10	1 ± 0.5	10
5	50	7 ± 3	7
10	100	15 ± 4	7
20	200	20 ± 5	10
50	500	52 ± 0.5	10
100	1000	66 ± 6	15 (Av 9.8)

to starch by starch phosphorylase then the boron is inhibiting the reaction in some manner.

Because of the high levels of boron used in the infiltrating solutions, it was thought that the results might be caused by a toxicity effect on the overall metabolism (14). The analysis of the leaf material for boron was carried out with three of the experiments. The results are given in table II. Column 2 of this table is obtained by multiplying the values in the first column by 10 (the atomic weight of boron). In this way the analytical values in column 3 can be compared to the amounts in solution. This comparison is given in column 4 of the table. Only about one-tenth the concentration of boron used in the solution for infiltration remains on or in the leaves after five washings with water. Of this one-tenth, the amount actually entering the cells and influencing physiological processes is surely much smaller. In these experiments where starch synthesis is first reduced by a significant amount, the actual boron concentration in the bean leaves is below the values given by Dible et al (3), for non-toxic levels in plants.

Additional evidence for assuming that non-toxic levels of boron were present is given in figure 1. These curves represent the oxygen uptake by leaf disks from 24 to 30 hours after infiltration with glucose and boron. There is a definite maximum response at 5 and 10 millimoles of boron per liter in the infiltrating solution; but in all concentrations used, the total oxygen uptake by the bean leaf disks, pre-treated with boron in the infiltrating solutions, was greater than that of the control disks pre-treated with glucose alone. The endogenous respiration of disks cut from water infiltrated leaves has been subtracted from all values. This experiment was repeated three times, and the same conclusions were apparent. It was also noted in all 12 experiments in which bean leaves were infiltrated with glucose and boron solutions that the leaves were turgid and appeared healthy 24 hours after infiltration. Even though high levels of boron were used in the infiltrating solutions, the effective levels are within physiological range.

An accumulation of carbohydrates in leaves of plants deficient in boron has been reported by many investigators as cited by Gauch and Dugger (7). It

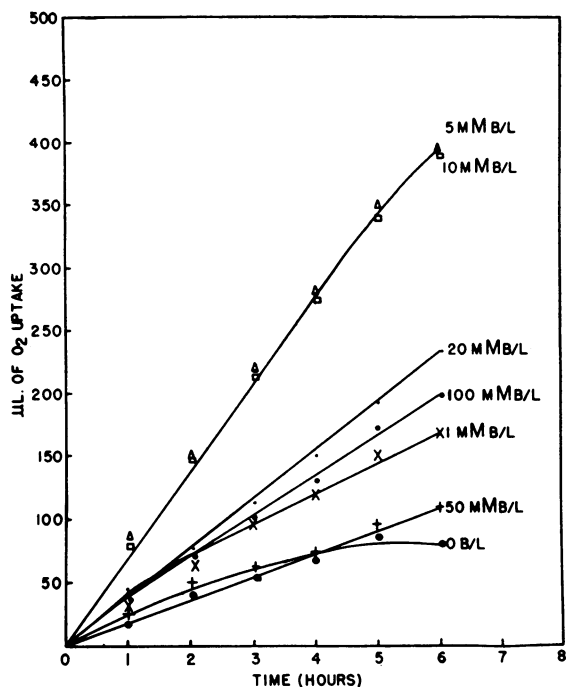


FIG. 1. The respiration of bean leaf disks 24 to 30 hrs after infiltration with 4 % glucose and various levels of boron. Fifteen disks per vessel. All treatments were duplicated.

would therefore seem logical that the addition of boron to leaves adequately supplied with boron, as described in the previous experiments, would result in a decrease in starch content if the mechanism of starch synthesis is altered by the borate ion. On the other hand, if the boron were acting as a carrier and were facilitating translocation, there should have been no difference in the starch content of the detached leaves infiltrated with glucose and boron as compared to glucose alone. However, the data presented do not negate the other hypothetical types of action previously mentioned. It was thought that an *in vitro* study might shed additional light on this problem.

Starch phosphorylase was obtained by the method of Hanes (12) from white potato. This procedure involves a fractional centrifugation of the 0.3 to 0.5 saturated ammonium sulfate precipitate. Tests of the extract for phosphatase and amylase indicated the absence of these enzymes. The preparation did contain a small amount of amylose, but not sufficient to prime the reaction ( $\text{glucose-1-phosphate} \rightleftharpoons \text{starch} + \text{phosphate}$ ) at a maximum rate. Additional starch was added to prevent this factor from being limiting. One ml of enzyme was mixed with 0.2 ml of a 5 % starch solution, 0.5 ml of sodium citrate buffer (pH 6.0) and water or boric acid to make a total volume of 2.5 ml. After equilibrating at 35°C, one ml of a 0.1 M glucose-1-phosphate solution was added. This digest mixture, as given by Green and Stumpf (10), was the

basis for variation in components during the course of the experiments.

The reaction was measured in two ways. The one more often used was to transfer a one-ml aliquot of the digest to five ml of a 6 % trichloroacetic acid solution. After several minutes the acid was neutralized with 6 % ammonium hydroxide and the inorganic phosphorus was determined by a modified Fiske and SubbaRow method (5). In some experiments the reaction was measured by determining the amount of starch formed. This method of Hanes and Cattle (13) involves the development of a starch-iodine color. The optical density is determined at 700  $m\mu$  and values are compared to a standard curve. Figure 2 indicated the kind of results obtained in the early experiments. However, the degree of response to boron was different between experiments and the influence of the ion on the reaction varied from 30 % inhibition (fig. 2) to complete inhibition by 50 millimoles/l of boron in several experiments.

To show that the influence of boron on the glucose-1-phosphate conversion to starch is on the rate of reaction and not on the final equilibrium value, the experiment reported in table III is presented. By comparing column 4 and 5 of this table, it is seen that when 10 millimoles/l of boron is present in the digest, the rate of liberation of inorganic phosphate, and evidently starch synthesis, is slower. The equilibrium value for the ratio of inorganic phosphorus to total phosphorus, however, is the same: 0.865. Hanes (12) reported an equilibrium value of 0.87 for this reaction at pH 6.0.

Because the hydrogen ion concentration influences the equilibrium value and rate of reaction, it is necessary to measure pH along with the reaction products. Columns 2 and 3 indicate that there was a shift in pH over the course of reaching an equilibrium, but in no case was the difference in pH between no boron and 10 millimoles/l of boron greater than 0.1 pH units. In view of Hanes's very extensive report on starch phosphorylase, it was apparent that the observed ef-

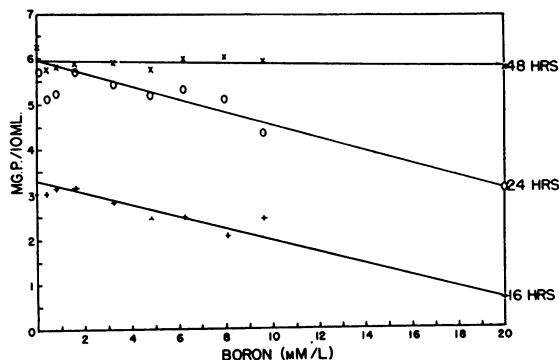


FIG. 2. The influence of boron on the starch phosphorylase enzymatic synthesis of starch from glucose-1-phosphate. Aliquots of the reaction mixtures were taken for analysis at the times shown.

TABLE III

THE INFLUENCE OF BORON ON THE RATE GLUCOSE-1-PHOSPHATE IS CONVERTED TO STARCH BY POTATO STARCH PHOSPHORYLASE ENZYME (AV OF 2 EXPTS)

HRS FROM START	pH		MG P/10 ML DIGEST	
	- B	+ B, 10 MILLI- MOLES/L	- B	+ B, 10 MILLI- MOLES/L
0	6.35	6.35	0.2	0.2
6	6.35	6.35	1.2	0.6
17	6.20	6.30	4.5	2.4
24	5.75	5.72	9.0	6.5
42	5.75	5.72	9.0	9.0

fects of boron on the reaction were not due to a difference in pH.

With more active preparations of the starch phosphorylase enzyme, it was possible to observe a measurable reaction in much shorter time than indicated in figure 2 and table III. Table IV is a summary table of 18 experiments in which 0, 1, 10, and 50 millimoles/l of boron were used in the digests. In these experiments various preparations of enzyme and concentrations of substrate were used. Consequently, the results are given as a percentage of the phosphate liberated in the control digest. This table shows that with different preparations of the enzyme and over a range of substrate concentrations, the conversion of glucose-1-phosphate to starch is inhibited by the presence of borate ions.

To characterize the nature of the inhibition of boron on this reaction, a series of experiments were carried out in which the concentrations of glucose-1-phosphate and boron were varied. A reciprocal plot of the velocity of reaction and substrate concentration is given in figure 3. From the plot of the reactions at different boron concentrations, it appears that the inhibition is one in which the inhibitor (boron) complexes with the substrate (glucose-1-phosphate) and in so doing reduces the reactive substrate concentration, at the more dilute levels, to a value that is limiting the reaction. It can be seen that if

TABLE IV

SUMMARY OF 18 EXPERIMENTS SHOWING THE INFLUENCE OF BORON ON THE IN VITRO CONVERSION OF GLUCOSE-1-PHOSPHATE TO STARCH BY STARCH PHOSPHORYLASE ENZYME \*

CONC, MILLIMOLES/L	MG P/DIGEST AS % OF CONTROL
0	100
1	94 ± 5.5
10	78 ± 5.5
50	50 ± 18

\* The temperature and pH were constant in all experiments. Because of the difference in the activity of various enzyme preparations extracted in the course of this work the time of measuring the reaction varied between experiments from 30 min to 4 hrs.

higher concentrations of substrate were not used in this experiment, the results would indicate a competitive type inhibition. The Michaelis constant for the association of substrate and boron cannot be determined by enzymological measurement. If this constant were known for the glucose-1-phosphate and boron, it would be possible to substitute the value into the proper equation and the problem would be reduced to the Michaelis-Menten equation in its simplest form. The curves in figure 3 are similar to the example given by Friedenwald and Maengwyn-Davies (11) for coupling of inhibitor and substrate. Considering the ability of boron to complex poly-hydroxy compounds, this type of inhibition seems possible.

Since glucose-1-phosphate forms a weakly ionized compound with borate and glucose forms a strongly ionized compound (17, 18), it was thought that boron might also alter the phosphorylation of glucose by influencing the hexokinase reaction. The experiments

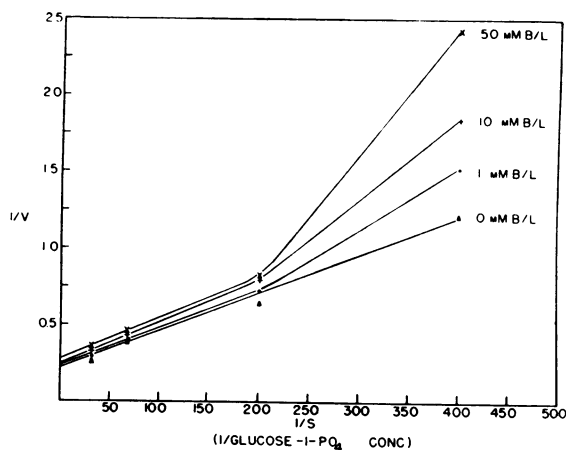


FIG. 3. The reciprocal plot of glucose-1-phosphate concentration vs rate of reaction.

conducted to test this hypothesis were carried out in vitro with hexokinase from yeast (Crude type II, Sigma Chemical Co.), and from pea seedlings and cotyledons. The method of separation of the insoluble or mitochondrial fraction from pea seedlings and the soluble fraction from pea cotyledons as well as the method of assay for hexokinase activity used in these experiments are described by Saltman (25). The reaction mixture of 1.5 ml contained 0.01 M adenosine triphosphate, 0.0033 M glucose, 0.01 M  $MgCl_2$ , 0.06 M TRIS (tris hydroxymethyl-aminomethane) buffer, pH 8.0, enzyme and  $H_3BO_3$  at 10, 50, and 100 millimoles/l. The control reaction did not contain boric acid. There was no effect of borate ion on the action of hexokinase in phosphorylating glucose. With yeast hexokinase 1.4 millimoles of glucose were phosphorylated per control reaction in 10 minutes at 30° C, whereas the reactions containing the boron, 1.5, 1.6, 1.4 millimoles of glucose were phosphorylated in the same time and at the same temperature. The reactions were duplicated and the experiments were repli-

cated twice. The standard errors of all the values were within  $\pm 0.2$  millimoles glucose. Glucose was estimated by a modification of the glucose oxidase methods<sup>3</sup> of Whistler et al (28), and Comer (2).

With the mitochondrial fraction from pea seedlings the glucose phosphorylated in 30 minutes at 35°C was 1.7 millimole per reaction for the control and 1.7, 1.7, 1.8 millimole per reaction for 10, 50 and 100 millimole/l of boron.

The soluble fraction of hexokinase from pea cotyledons was only one-third as active as the insoluble fraction from the seedlings; however, there was no difference in the amount of glucose phosphorylated between no boron and 10, 50 and 100 millimoles/l of boron.

### DISCUSSION

In the recent review of the physiological action of boron in higher plants (7), it was pointed out that 15 distinct postulated roles of boron have appeared in the literature. Many of these roles can be understood more fully when evaluated in terms of the concept that boron increases the translocation of sugars. The validity of this concept has been borne out by several kinds of experimental results (6, 26). The inability, however, to elucidate the mechanism of action has resulted in several working hypotheses (7). That these have remained hypotheses denotes the complexity of the problems associated with organic transport in plants. As is often the case, a different approach to the same problem is fruitful in understanding physiological problems such as this one.

A study of the effect of boron on the starch phosphorylase system seemed to be the best approach at this time. Winfield (29) had previously investigated the influence of boron on starch phosphorylase as well as potato aldehyde oxidase and tyrosinase. His report showed no effect from boron on the latter two enzymes in an *in vivo* study. In other experiments conducted with starch phosphorylase enzyme preparation, Winfield reported a 21% inhibition of the synthesis of starch from glucose-1-phosphate by a 0.1 M concentration of boron. This is approximately 6,000 ppm, six times the highest concentration used in the experiments reported in this paper. With the same concentration of boron, Winfield showed a 17% increase in the rate starch was degraded to glucose-1-phosphate under the optimum condition for the reaction. He proposed that boron entered into the "condensation reaction of sugar molecules in the same fashion as does phosphate ion," and that "it seems unlikely that boron influences the starch  $\rightleftharpoons$  glucose-1-phosphate equilibrium in normal plants where phosphate concentrations would be much greater than borate." The data presented in this paper indicate that the equilibrium is not changed but rather the rate of reaching equilibrium is changed. The compe-

tion between borate and phosphate as postulated by Winfield does not seem likely; rather, his results, as well as the results presented in this paper, are thought to be caused by the formation of a glucose-1-phosphate-borate complex.

The effects of arylboric acids on the growth of roots from wheat plants has recently been investigated by Torssell (27). He attributes the root growth-promoting property of these compounds to a characteristic of the dihydroxyboron group which in some way regulates the growth of the cells. He further states that this characteristic is not associated with auxin effects, but rather with the ability of arylboric acid to complex the carbohydrates in the polysaccharide chains of the cell wall and slow down the stabilization of the wall. "When the normal stabilization of the cell wall is prevented by arylboric acids, it will result in an increased cell elongation."

Further results by Torssell (27), cited as "in press," indicate that phenylboric acid in concentrations  $\leq 10^{-3}$  M did not influence starch phosphorylase,  $\alpha$  amylase, nor invertase. Boric acid at  $10^{-3}$  M was also found by the present investigators to be ineffective on starch phosphorylase.

Reed (24) showed that the catechol oxidase activity in the phloem cells of boron-deficient celery plants was higher than in normal celery plants. The cortical cells of boron-deficient plants also had a higher oxidase and phosphatase activity than did similar cells in plants supplied with boron. Abnormally high rates of oxygen consumption were noted by MacVicar and Burris (19) in whole tissue homogenates and chloroplast suspensions from boron-deficient plants. They associated this with an increase in polyphenoloxidase activity as a result of boron deficiency. Of the reactions studied by these investigators, the oxidation of dihydroxyphenyl-L-alanine showed significant inhibition with 0.001 M boron concentration. Higher concentrations of boric acid (0.01 M to 1 M) inhibited ascorbic acid oxidase from cabbage leaves. They justify the high levels of boron by saying that "within the cells a particular compound may be highly concentrated in local areas. With cell-free preparations it may be necessary to reproduce this high local concentration in the entire medium to achieve the activity apparent in the intact cell." This point is well taken and was also the reasoning behind using high levels of boron in the experiments reported here.

In a recent review (7), other investigations were cited with respect to experimental evidence showing inhibition or stimulation from added boron on tyrosinase, catalase, invertase, and diastase. Gilbert and Swallow (8) found that Q-enzyme was inhibited by sodium borate even in the presence of activating ions.

It is recognized that the inhibition of the starch phosphorylase reaction by boron may be only one of the many reactions so influenced. Any enzymatic reaction involving a substrate capable of complexing boron may be influenced by the element. On the other hand, boron could be a specific inhibitor irrespective

<sup>3</sup>The components of these methods are sold by Worthington Biochemical Corp. under the trade name of Glucostat.

of the degree to which it forms a complex with a substrate.

Compared to the glucose-borate complex the glucose-1-phosphate-borate complex is weakly ionized. According to Kyme and Cohn (17) and Goodman et al (9) glucose-1-phosphate is elutriated from an ion exchange column more rapidly with a weak borate solution than are the hexoses. Sucrose also forms a very weakly ionized complex with borate and may be rapidly elutriated from an ion exchange column. The authors point out that there is in general a direct relationship between the retention of sugar or sugar-phosphate on an ion exchange column and the increase in conductivity of the complex when sugar and borate are mixed (18), as well as a change in the optical rotation and pH (16) proportional to the degree of complexing. McPherson and Percival (18) found that the addition of borate to sucrose depressed the conductivity which was indicative of no complex formation. Isbell et al (16), on the other hand, found that the addition of borate to sucrose caused a change in the specific rotation as well as the pH, indicating the formation of a complex. The sucrose-borate complex formed is presumed to be of the 1,3 diol type. Glucose-1-phosphate, irrespective of the limitations set by MacPherson and Percival (18), also forms a complex with borate. Kyme and Cohn (17) found a slight increase in the volume necessary to elutriate glucose-1-phosphate from an ion exchange column as the borate concentration was increased from  $10^{-5}$  to  $10^{-2}$  M.

Such information with regards to the differences in the degree of borate complexing is of interest when the results with hexokinase and phosphorylase are compared. Glucose, which complexes strongly with boron, was phosphorylated by hexokinase at the same rate with and without boron. On the other hand, the conversion of glucose-1-phosphate to starch by starch phosphorylase was partially inhibited by 10 to 100 millimoles of borate per liter.

For purposes of developing a working hypothesis that included the findings reported here and the now well established effects of boron on translocation of sugars, the relationship between sucrose phosphorylase and substrate explained by Gottschalk and referred to by Hassid (15) is pertinent. For bacterial sucrose phosphorylase to synthesize or hydrolyze sucrose, a particular spatial arrangement of the substrate molecule is necessary. In order to satisfy the specificity of this enzyme, the glucose acceptor (fructose in the case of the disaccharide sucrose) must possess adjacent to the glucosidic oxygen an -OH group *cis* disposed and co-directional to the -OH group of the glucose part of the molecule. As pointed out by Zittle (30) borate ions also form compounds with cyclic diols that have *cis* hydroxy groups. In these experiments the added borate could combine with the substrate, glucose-1-phosphate, and effectively reduce the concentration of the substrate available for enzyme action to a lower, limiting value. This appears to be the case as shown by the double reciprocal plot

in figure 3. The equilibrium value for the reaction between boron and a diol is dependent on the molar ratio of boron to diol as well as the hydrogen ion and water concentration (16). If non-borated substrate is removed from solution by enzymatic condensation onto existing starch molecules, the borated substrate  $\rightleftharpoons$  non-borated substrate equilibrium will shift to the right, and in time all the available substrate will enter into the enzymatic reaction. This could explain why the rate of reaching equilibrium and not the final equilibrium value *in vitro* is influenced by borate ions.

At any one time the quantity of soluble carbohydrates moving in the translocating tissue is a function, in part, of the quantity of soluble carbohydrate in the cells of the leaves. This would be true for carbohydrates synthesized in the leaf cells or added in some manner such as injection or infiltration. If there exists in the cells an inhibitor which prevents or slows down the condensation of soluble carbohydrates to starch, then it seems reasonable to assume that within a period of time, more of the carbohydrate will move out of the leaf cells to other plant parts than if the inhibitor were not present. This hypothesis could account for the translocation effect observed when boron is added to the sugar in "feeding" experiments (6, 22), or when boron is included in the mineral nutrient solution used for growing plants as compared with boron deficiency. Sisler et al (26) observed that the characteristic symptoms of brittleness in tomato appeared eight days after removing boron from the nutrient solution. After only four days, however, these plants showed a decrease in the amounts of  $C^{14}$  labelled carbohydrates that were translocated as compared to normal plants.

As a result of the present experiments and their interpretation, it is proposed that one of the ways in which boron may bring about an increase in translocation of sugars in plants is by decreasing the enzymatic conversion of glucose-1-phosphate to starch. With an increase in the steady state concentration of glucose-1-phosphate, the amount that may be available for other reactions, such as the synthesis of sucrose or other hexose phosphates, is increased. An increase in these soluble carbohydrates *in situ* may therefore result in an increase in translocation from the site of synthesis to some other plant part.

#### SUMMARY

Bean leaves infiltrated with 4% glucose after a dark period synthesized more starch than leaves infiltrated with 4% glucose and various levels of boron. Analysis for boron concentration in the leaves indicated that the level of the element was within physiological range for plants.

*In vitro* studies in the glucose-1-phosphate  $\rightleftharpoons$  starch reaction show that boron influences the rate of the reaction but not the final equilibrium.

A plot of 1/rate of reaction vs 1/glucose-1-phosphate concentration indicates that, at low levels of substrate, the boron combines with the substrate and influences the rate of starch synthesis.

Because boron influences the synthesis of starch, a hypothesis is presented which could explain why translocation of sugars in plants is increased when the element is present in sugar "feeding" and in C<sup>14</sup> carbohydrate synthesis experiments.

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