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A biochemical role for boron

Sung Gue Lee

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A BIOCHEMICAL ROLE FOR BORON

by

Sung Gue Lee

A Dissertation Submitted to the
Graduate Faculty in Partial Fulfillment of
The Requirements for the Degree of
DOCTOR OF PHILOSOPHY

Major Subject: Biochemistry

Approved:

Signature was redacted for privacy.
In Charge of Major Work

Signature was redacted for privacy.
Head of Major Department

Signature was redacted for privacy.
Dean of Graduate College

Iowa State University
Of Science and Technology
Ames, Iowa
1968
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I. INTRODUCTION

Boron appears to be required by all seed-bearing plants and some lower plants. Many biochemical pathways appear to undergo derangement when the level of boron is inadequate in these plants. Metabolic alteration can be demonstrated in some plants as early as four hours after the discontinuance of the boron supply. Despite the attempts of numerous investigators, the biochemical role(s) of boron is still unknown. Various metabolic changes seem to occur simultaneously in boron-deficient (B-) plants, and the major effort in boron biochemistry in the past has been the distinction of the subcellular systems controlled directly by boron from those influenced indirectly.

In the early phase of the research described in this thesis, efforts were directed to the finding of biochemical reactions that are controlled directly by boron in intact plants, and in the later phase, experiments were conducted with cell-free systems to verify some of the numerous conclusions made from the experiments with intact plants.

Due to the inherent complexities of reactions in intact plants and to the apparent multiple roles of boron, the a priori nature of many assumptions was unavoidable. Regretably, only a small number of these assumptions could be tested during the given time.
II. LITERATURE REVIEW

The indispensability of boron for the sustenance of the life of higher plants has been well established since the report by Agulhon (2) in 1910. However, despite numerous investigations, the roles of boron, let alone the action mechanisms, are still largely not understood. Much of the work in this field was done before the biochemistry, particularly the metabolic pathways, of higher plants was established, and as a consequence, the assignments of the roles of boron by earlier workers were based largely upon comparative chemical analyses or anatomical observations made of boron-deficient- and normal plants. Recently, attempts have also been made to assign the roles of boron to specific enzymes from the experiments with cell-free homogenates or purified enzymes. The efforts of some of these investigators will be reviewed briefly.

From their experimental results, some investigators concluded that boron controls carbohydrate metabolism. For example, Scripture and McHargue (52) showed that the deficiency of boron causes an accumulation of reducing sugars and oligosaccharides in the tops of radish. The content of free reducing sugars in the root, on the other hand, was shown to be lower in the boron-deficient plants. Spurr (56) observed that, under boron deficiency, most of the collenchyma cell walls of celery petioles were markedly thinner and contained few lamellae but the cell walls of the phloem and ground parenchyma became thicker. From these observations, he concluded that boron affects the rate of carbohydrate condensation into cell wall materials. Ashworth (6) showed that there is more pectin in the boron-deficient sunflower tissue and Baker et al. (7)
also observed a higher concentration of pectin and possibly pentosans in the leaves of boron-deficient bean plants. Recently, Dugger, Humphreys and Calhoun (16) showed the inhibition of the activity of starch phosphorylase from potato tuber by borate. Dugger and Humphreys (15) also showed that borate enhances the biosynthesis of sucrose by sugar cane and pea seedling homogenates when glucose-1-phosphate and fructose were used as substrates. They showed also that UDPG pyrophosphorylase was enhanced by borate while UDPG transglycosylase was inhibited. Loughman (31) reported the activity of phosphoglucomutase from pea seeds to be inhibited by borate. More recently, Hinde and Finch (21) have shown the soluble fraction (supernatant fraction after centrifugation at 100,000 g) from boron-deficient *Vicia faba* root tips has greater activities of acid phosphatase and ATPase but lower activity of pyrophosphatase as compared with the enzyme activities of the soluble fraction from the normal root tips.

The results of the comparative chemical or anatomical analyses of boron-deficient or normal plants are very difficult to use as bases for the assignment of the roles of boron to specific reactions since the differences between the normal and boron-deficient plants in the distributions of various chemicals or in the cellular structures could result if any of a number of biosynthetic steps leading to the production of the end products is affected by boron. Moreover, these classical works were done with plants which already showed boron deficiency symptoms, and therefore, the observations made might have been secondary or tertiary ones. The experiments of Dugger *et al.*, although interesting, cannot be
considered to have any biological significance for the reason that the concentration of boric acid to exhibit an inhibitory effect was much too high. For example, the activity of starch phosphorylase was reduced to 78% of control by 0.01 M boric acid, a concentration of one-to-two orders of magnitude greater than the biological concentration of boric acid. Moreover, the synthesis of starch from glucose-1-phosphate has been known to be catalyzed mainly by pyrophosphorylase and ADPG-synthetase rather than by starch phosphorylase. Therefore, any effect of boron on the synthesis of starch by phosphorylase bears little significance. The work of Hinde and Finch, although very interesting in a physiological sense, does not lead to the assignment of boron to a specific enzyme or enzyme system. The greater activities of acid phosphatase and ATPase of the soluble fraction of boron deficient root tips may be either due to the boron inhibition of the activities of these enzymes or synthesis of these enzymes, or of the synthesis of natural inhibitors of these enzyme reactions.

A hypothesis related to carbohydrate metabolism which attracted much attention is that proposed by Gauch and Dugger (18). It was suggested that by complexing with sugars, borate acts as a carrier of sugars in the transport of the latter across membranes. They visualized that borate resides in the membrane, forms sugar-borate complexes at one surface of the membrane, carries the sugar across the membrane, and finally releases the sugar at the opposite side of the membrane. As supporting evidence for this hypothesis, they showed that lima bean and pea root tips showed a greater respiration rate when incubated with sucrose and borate than with sucrose alone. The augmented respiration was interpreted as increased
transport of sucrose across the plasma membrane in the presence of borate. The result and interpretation of the work by Gauch and Dugger will be discussed in greater detail later.

That phenols might be involved in boron biochemistry was first shown by Perkins and Aronoff (46) who showed the characteristic blue-white fluorescence of boron deficient necrotic tissue to be due to chlorogenic and caffeic acids. Its level was as much as 10 times that of normal tissue. More recently, Watanabe et al. (66) found that the leaves of tobacco plants, grown in boron-deficient nutrient for 38 days, produced a twenty-fold increase of scopoletin (6-methoxy-7-hydroxy-coumarin) glucoside over the leaves of normal plants. The accumulation of the phenol led them to suggest the involvement of boron in lignin biosynthesis. Lignin, a cell wall stiffening agent, is a polymer constructed from phenylpropanoid units (39). Dear and Aronoff (13) have also shown over a twofold increase in the content of chlorogenic and caffeic acids in the leaves of sunflower plants grown in boron deficient nutrient after only 24 hours following removal of B from the nutrient solution. Odnoff (43) and Neales (38) found increased lignification of cell walls of boron-deficient plants but McIlrath (36) obtained the reverse situation.

Numerous investigators have implicated boron in the metabolisms of protein and nucleic acids. Classical studies suggesting the involvement of boron in protein synthesis are those of Wadleigh and Shive (65) and Scripture and McHargue (53), who found that B- plants contained a greater amount of soluble N-compounds such as ammonia, amines, and amides compared to normal plants. Later, Whittington (67) found that boron has no effect on the net protein synthesis of the root tips of bean, while Hinde et al.
(22) studied the amino acid-dependent ATP pyrophosphate exchange activity in the same tissue and concluded that the amino acid activating activity, and thus protein synthesis is low in B- bean root tips (Vicia faba). However, their data seem to indicate that boron influences individual activating enzymes differently, increasing the activation of some amino acids and at the same time decreasing the activation of others. Currently, a number of investigators have reported evidence of the control of nucleic acid metabolism as the primary role of boron. The first report on this field is that of Shkol'nik (54) who reported that boron deficiency symptoms in sunflower plants were alleviated by adding ribonucleic acid to their nutrient solutions. Albert (4) has reported also that the content of ribonucleic acid in the root tips of tomato plants whose B had been withheld for 24 hours is lower than that in the root tips that received boron. He has shown also, however, that the cessation of the root growth occurs within 6 hours after the discontinuation of boron supply. A seemingly contradictory result has also been reported. Cory et al. (12) found that the root tips of bean plants that were grown in boron-deficient nutrient for only 4 hours incorporated $^{32}$P into ribonucleic acid much more rapidly than the normal root tips. At this time the growth of the root tips that received no further boron was not affected. Albert (25) had reported more recently that the addition of thymine, guanine and cytosine to the B- nutrient medium partially alleviates the development of boron deficiency symptoms of tomato root tips as measured by root elongation and the RNA content of the root tips, while the addition of adenine and uracil to the medium has no effect. In this experiment, the B- root tips were grown in B- nutrient for up to six days and the interpretation of these
results is very difficult.

Much of the work on the role(s) of boron, although meaningful in itself, may simply be the observations of the secondary or end results. For example, one could observe a deranged carbohydrate metabolism even when the primary role of boron is an action on nucleic acid metabolism even when the primary role of boron is an action on nucleic acid metabolism since the enzymes governing carbohydrate metabolism are coded by nucleic acids. Conversely, any derangement of the nucleic acid metabolism could be preceded by the derangement of carbohydrate metabolism, since the biosynthesis of nucleotides are directly or indirectly linked to the carbohydrate metabolism. Each additional report seemed to add more confusion to the riddle of the biochemical roles of boron.

It is therefore hoped that the work presented in this thesis will relate some of the apparently unrelated or even contradictory observations and ultimately delineate the primary role(s) of boron.
III. PRELIMINARY INVESTIGATIONS

The purpose of this investigation was to find the first subcellular, structural changes and the earliest metabolic differences caused by boron deficiency, in order to find a system or systems directly controlled by boron. Electron microscopy and radioisotope techniques were employed in the investigation.

A. Antomical Observations

Although various anatomical boron deficiency symptoms have been described in the past (54), none has been reported at subcellular levels. It was hoped, therefore, that the electron microscopic studies would reveal subcellular events that precede the development of the symptoms observable by the light microscope.

1. Methods and materials

Sunflower seedlings, germinated in moist sand, were grown in Hoagland's complete nutrient solution. Plants were made boron-deficient by transfer from complete nutrient to a minus-boron solution. This transfer was done when the first leaves were 4 to 5 cm long. Young leaves, 10 to 20 mm long, were sampled from boron deficient (B-) and normal (B+) plants. Plants were grown in B- solution up to 7 days. The first visual symptom, the browning of leaves, was apparent on the fifth day. Young (expanding) leaves were prefixed for about 16 hours with 3% glutaraldehyde, adjusted to pH 7.3 with 0.1 M phosphate buffer. One mm cubed mesophyll tissues were cut from the prefixed leaves and the tissue blocks were fixed 4 hours with 1% OsO₄, adjusted to pH 7.3 with Palade's buffer (39) at 4°. The tissue blocks were dehydrated in a graded series of aqueous ethanol
(30%, 50%, 70%, 95%, 100%) and the alcohol then replaced by 4 changes of propylene oxide. The tissue blocks were embedded in Epon resin mixture as described by Luft (33), the resin heat-polymerized, and 600 A tissue sections made with an LKB ultramicrotome equipped with a diamond knife. The tissue sections were stained with 2% uranyl acetate. The stained sections were examined with an RCA EMU F-3 electron microscope.

2. Observations

Changes in cellular structures were observed as early as three days after the removal of boron from nutrient medium. At this time no visual symptoms were recognizable. The damaged cells were localized in groups of a few to several cells near the basal margin of leaves. The damaged cells were seen as copper-brown spots under the dissecting microscope.

One of the earliest changes appears to occur in chloroplasts. B- chloroplasts appear to contain larger amount of starch, supporting the contention of Dugger et al. that boron decreases the enzymatic conversion of glucose-1-phosphate to starch (16). Also observed in B- chloroplasts is unidentified osmophilic material (product X) (fig. 2, 4). Product X appears to be confined to individual chloroplasts since the entire stromae of some chloroplasts are filled with this material while other stromae in the same cell appear to be devoid of it. As X is confined within the chloroplast membrane, it may be a polymer resulting from phenol-sensitized photooxidation incapable of diffusing through chloroplast membranes. Its deposition appears to be localized in individual cells. Nearly all of the chloroplasts in one cell may have product X while adjacent cells have none.

B- cells appear to contain a greater number than normal cells of
mitochondria with well-developed cristae. Myelin figures are commonly observed to be associated with B- mitochondria (Figure 5).

In B- cells, the ribosomal particles appear to be less in number than in B+ cells (Figure 2).

In advanced B- stages, 6 to 7 days after transfer to B- nutrient, changes also appear in the nucleus. Angular, heavily osmophilic rhombohedral structures, may be observed (Figure 6). Cell walls also undergo changes. B- leaf mesophyll cell walls appear to be thicker and have a rough serrated appearance (Figure 7).

In extremely deficient cells (6 to 7 days after), no subcellular structures are recognizable. Such cells contain a large number of heavily osmophilic bodies which have the appearance of oil droplets (Figure 7). As the deficiency becomes even more severe, these osmophilic bodies fill the entire cell (Figure 8).

3. Discussion

The development of any deficiency symptom, visual or microscopic, must be preceded by derangement of various metabolisms. Therefore, the purpose of this anatomical study was not in the finding of the molecular targets of boron action, but rather in the finding of the first subcellular location to undergo structural changes. Since the target molecules are expected to reside in such a subcellular location, further studies of the roles of boron may be restricted to such subcellular location rather than entire cell. Unfortunately, the anatomical studies did not reveal such a location unequivocally. For example, the deposition of product X in the chloroplasts and the reduction in the number of ribosomes occurred concomitantly. This may mean that the target
Figure 1. Mesophyll cells of normal (B+) sunflower leaf. 9800X.

Figure 2. Mesophyll cells of boron deficient sunflower leaf. 9800X.
Figure 3. Mesophyll cells from normal (B+) sunflower leaf. 42,100X

Figure 4. Mesophyll cells from boron deficient sunflower leaf showing deteriorating chloroplasts with dark, osmophilic product X uniformly dispersed throughout the chloroplast, starch (S), and oil droplets and cell wall (CW). Note that the ribosomal population is greater in B+ cells than in B- cells. 43,000X.
Figure 5. (top left) A three day boron deficient mesophyll cell showing mitochondria with myelin figures (arrows). 49,000X.

Figure 6. (bottom left) Six day boron deficient cell showing a nucleus with rhombohedral structures. 18,400X.

Figure 7. (top right) Seven day boron deficient cell showing deposition of osmophilic bodies (arrow) in cytoplasm. Note rough serrated cell wall structure. 28,600X.

Figure 8. (bottom right) Seven day boron deficient cell wall showing deposition of osmophilic material in cytoplasm (arrow). Note also mitochondria are undergoing disintegration. 23,700X.
molecules are not confined in a single species of subcellular organization such as the chloroplast, but present ubiquitously throughout the cell, or that the propagation of the secondary effects is too fast for anatomical studies to distinguish the location of the target molecules from the location affected indirectly.

B. Calcium and Sucrose Uptake by Isolated Chloroplasts

One of the proposed roles of boron in higher plants is its involvement in transport of sugars across membranes, boron residing in the membranes and acting as a carrier (18). The purpose of this experiment was to test the validity of this hypothesis. As the chloroplast is one of the earliest organelles to undergo changes as the boron supply to the plant is discontinued, and the uptake of ion or sugars by the chloroplasts requires the passage of these solutes through the chloroplast membrane, the capacity of isolated chloroplasts to take up radioactive sucrose or calcium should be dependent on the level of boron in chloroplasts if the above mentioned hypothesis is correct, as a necessary, but not a sufficient, condition.

In preliminary work for this experiment, the self-absorption of particles by chloroplast preparations and the efficiency of removal of adsorbed $^{45}$Ca from the chloroplast surface by washing were determined.

1. Materials
   a. Sunflower leaves Sunflower plants were grown and made boron deficient as described earlier. B- young leaves, 4 to 5 cm long, were obtained from plants grown in B- nutrient solution for 3 to 5 days, whereas B- mature leaves were harvested from plants grown in B- solution for 7 to
2. Methods

a. Preparation of the chloroplast fraction  Sunflower leaves were homogenized in polypropylene beakers with 1 ml of homogenizing medium per 1 gm tissue and with the aid of sand which had been washed previously. The tissue homogenate was filtered through 4 layers of cheese cloth and the
filtrate was centrifuged at 150 g for 2 minutes. The supernate was centrifuged at 400 g for 10 minutes, and the chloroplast pellet was suspended with the aid of a rubber policeman in 1 ml of homogenizing medium per 1 gm of the original tissue. The chloroplast suspension was centrifuged for 10 minutes at 400 g and the pellet formed was used in the following experiments.

b. **Determination of self absorption by $^{45}$Ca and $^{14}$C samples**

The chloroplast pellet obtained above was incubated for 15 minutes with solution B (but without $^{45}$Ca). The incubation mixture was centrifuged and the pellet formed was suspended in water. Aliquots of this chloroplast suspension were mixed with 0.06 μc of $^{45}$Ca in 0.1 ml of water. These mixtures were plated onto copper planchets and dried. The dried mixtures were counted with a GM counter. The radioactivities were plotted against the chlorophyll contents of these samples. Chlorophyll was determined as follows. The chlorophyll was extracted from the chloroplast pellets with boiling acetone. Two extractions for three minutes each were sufficient to extract all the chlorophyll from the chloroplast pellets of up to 0.1 ml wet volume. Acetone was then evaporated and the resulting dry chlorophyll was dissolved in ether. The absorbancy of the ethereal solution of chlorophyll was made a 662 μm with a Cary 14 spectrophotometer and converted to mg chlorophyll. The conversion was based on a specific absorptivity of 100.9 for 1 g of chlorophyll/1 liter of ether (17). The self-absorption by

$$100.9 = \frac{\text{O.D.}}{(\text{cell path})(1 \text{ g chlorophyll}/1 \text{ liter of ether})}$$
$^{14}\text{C}$ samples was obtained in the same manner as that of $^{45}\text{Ca}$.

c. The efficiency of removal of adsorbed $^{45}\text{Ca}$ from the chloroplast surface by washing with $^{40}\text{Ca}$ The chloroplast fraction prepared as described earlier was suspended in Solution A. One ml of this chloroplast suspension was mixed with 1 ml of solution B in a conical centrifuge tube and the mixture was incubated for 15 minutes at room temperature. At the end of incubation, 8 ml of ice cold solution C was added to the reaction mixture, and this mixture was allowed to stand in an ice bath for 5 minutes. The mixture was then centrifuged and an aliquot of the supernate was drawn out, plated on a copper planchet, dried and counted. The pellet was resuspended in ice cold solution C, allowed to stand in an ice bath for 5 minutes, and the suspension centrifuged. An aliquot of this supernate was drawn out and the radioactivity counted in the same manner as above. This resuspension, centrifugation and counting procedure was repeated two more times.

d. Determination of calcium uptake by isolated chloroplasts The chloroplast fraction, prepared from B+ and B- leaves by the method described earlier, were suspended in solution A and 1 ml aliquots used for chlorophyll determination. Another 1 ml aliquot of the suspensions as mixed with 1 ml of solution B and incubated in a conical centrifuge tube and shaken in a water bath at 15-50 18°C under two 150-watt lamps placed 20 cm above.

After 15 minutes of reaction, 6 ml of solution C was added to each of the reaction mixtures, after which the mixtures were centrifuged in a refrigerated centrifuge. The supernate was discarded and the chloroplast pellets were resuspended in 5 ml of ice cold solution C. The chloroplast
suspensions were allowed to stand in an ice bath for 5 minutes, after which the suspensions were centrifuged. The washing process was repeated once more. The final pellets were suspended in distilled water and plated on copper planchets, dried under an IR lamp and the radioactivities counted with GM counter.

3. Results
   a. Preparation of chloroplast fraction Figure 9 is a photomicrograph of a typical preparation of chloroplast fraction. The refractile ones are those in focus and those with darker appearances are out of focus. As may be seen from the photomicrograph, the isolated chloroplasts retained the shape of chloroplast in intact tissue.

   b. Determination of self absorptions by $^{45}\text{Ca}$ and $^{14}\text{C}$ samples

   Figure 10 shows the plots of radioactivities of $^{45}\text{Ca}$ and $^{14}\text{C}$ samples against mg chlorophyll. The self absorption coefficient $\alpha$ was obtained from

   $$\log \frac{A_0}{A} = \alpha \cdot x,$$

   where $A_0$ and $A$ are the activities of infinitely thin sample and of a sample of finite thickness express as mg chlorophyll content of the sample, and $x$ is the weight of chlorophyll in mg. The values of self absorption coefficients obtained from figure 10 are 0.301 for $^{45}\text{Ca}$ and 0.939 for $^{14}\text{C}$.

   c. The efficiency of removal of adsorbed $^{45}\text{Ca}$ from the chloroplast surface by washing with $^{40}\text{Ca}$ solution

   The radioactivities of various supernates, or washings of chloroplast fractions are shown in Table 1.
Figure 9. Photomicrograph of chloroplast preparation from sunflower leaves.
Figure 10. Apparent self absorption of emission from $^{45}$Ca and $^{14}$C by chloroplast sample. The self absorption is based on the weight of chlorophyll rather than the weight of entire chloroplast sample.
Table 1. The efficiency of removal of adsorbed $^{45}$Ca from chloroplast surface

<table>
<thead>
<tr>
<th>Sample</th>
<th>B+</th>
<th>B-</th>
</tr>
</thead>
<tbody>
<tr>
<td>1st washing</td>
<td>56,720 ± 90 cpm</td>
<td>56,670 ± 90 cpm</td>
</tr>
<tr>
<td>2nd washing</td>
<td>306 ± 5 cpm</td>
<td>655 ± 8 cpm</td>
</tr>
<tr>
<td>3rd washing</td>
<td>24 ± 3 cpm</td>
<td>42 ± 5 cpm</td>
</tr>
<tr>
<td>4th washing</td>
<td>7 ± 2 cpm</td>
<td>12 ± 2 cpm</td>
</tr>
<tr>
<td>chloroplast pellet</td>
<td>269 ± 4 c.p.m</td>
<td>244 ± 4 c.p.m</td>
</tr>
<tr>
<td>per mg chlorophyll</td>
<td>1,140 ± 17 c.p.m</td>
<td>620 ± 10 c.p.m</td>
</tr>
</tbody>
</table>

$^{45}$Ca and $^{14}$C-sucrose uptake by isolated chloroplasts

It may be seen from Table 1 that most of the adsorbed $^{45}$Ca can be washed out by three washings. Although some activity is present in the 4th washing, the magnitude of this was less than background activity. More activities were present in the 2nd, 3rd and 4th washings of B- chloroplast pellets. This is probably because the B- chloroplast material used was 1.66 times that of the B+ chloroplast material.

d. $^{45}$Ca and $^{14}$C-sucrose uptake by isolated chloroplasts

Table II shows $^{45}$Ca uptake by isolated B+ and B- chloroplast preparations from mature leaves. B- mature leaves were obtained from plants that had been grown in B- nutrient for 7 to 12 days.
Table 2. \(^{45}\text{Ca}\) uptake by isolated chloroplast fraction from mature leaves

<table>
<thead>
<tr>
<th>Exp. No.</th>
<th>Corrected counts/min./mg chlorophyll(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>B+</td>
</tr>
<tr>
<td>1</td>
<td>2,910 ± 65</td>
</tr>
<tr>
<td>2</td>
<td>4,570 ± 103</td>
</tr>
<tr>
<td>3</td>
<td>4,780 ± 112</td>
</tr>
<tr>
<td>4</td>
<td>3,970 ± 34</td>
</tr>
<tr>
<td>5</td>
<td>2,640 ± 41</td>
</tr>
<tr>
<td>6</td>
<td>2,920 ± 35</td>
</tr>
<tr>
<td>7</td>
<td>3,490 ± 32</td>
</tr>
<tr>
<td>8</td>
<td>3,450 ± 38</td>
</tr>
<tr>
<td>9</td>
<td>4,110 ± 74</td>
</tr>
<tr>
<td>Average</td>
<td>3,649</td>
</tr>
</tbody>
</table>

\(\text{\(^a\)}\)The values of cpm/mg chlorophyll shown above are the values corrected for background and self absorption. No correction was made for back scattering. The amounts of chloroplast material for these experiments range from 0.045 mg to 0.265 mg equivalent of chlorophyll.

Table 2 shows that the B+ chloroplasts have a greater capacity to take up calcium ion than B- chloroplasts. It may also be seen that the uptake capacity of B- chloroplasts varies greatly from one experiment to another. A possible explanation for this is that B- chloroplasts were obtained from leaves of plants that had been grown in B- nutrient from 7 to 12 days. Therefore, the B- chloroplasts of various preparations were likely to have been in different degrees of boron deficiency. The degree of maturity of B- leaves could have been in different degrees of boron deficiency. The degree of maturity of B- leaves could have also
contributed to the wide variations in the degrees of boron deficiency. A greater deficiency might be expected of the leaves if they expanded somewhat, while a lesser deficiency would have occurred if they did not grow at all, after the transfer of the plants to B- nutrient solution.

Young, rapidly expanding leaves show boron deficiency symptoms earlier. At the time of harvest of young leaves, some B- leaves already showed deficiency symptoms. Table 3 shows the uptake of $^{45}$Ca by chloroplasts from young leaves.

Table 3. $^{45}$Ca uptake by chloroplasts from young leaves$^a$

<table>
<thead>
<tr>
<th>Exp. No.</th>
<th>Corrected counts/min./mg chlorophyll equivalent of chloroplasts</th>
<th>B+</th>
<th>B-</th>
<th>$\frac{B-}{B+}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td>1,180 ± 33</td>
<td>990 ± 15</td>
<td>1.20</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>740 ± 23</td>
<td>690 ± 22</td>
<td>1.07</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>1,330 ± 11</td>
<td>1,430 ± 11</td>
<td>0.93</td>
</tr>
<tr>
<td>Average</td>
<td></td>
<td>1,083</td>
<td>1,037</td>
<td>1.04</td>
</tr>
</tbody>
</table>

$^a$The amount of chloroplasts used in this experiment ranged from 0.078 mg to 0.430 mg chlorophyll equivalent. In any one experiment the amount of chloroplasts used were similar for B+ and B-.

Table 3 indicates that B- chloroplasts from young B- leaves have the same capacity of calcium uptake as B+ chloroplasts. Many of the young leaves show visual deficiency symptoms at the time of harvest, while the mature leaves did not show any comparable symptoms even 7 or more days after the transfer of the plants to B- nutrient solution.

It is known that ion uptake in chloroplast is energy-dependent and various uncouplers of ATP generation abolishes the active transport of
ions (40, 41). It is also known that ion translocation into chloroplasts is activated by light (42). It is, therefore, possible that the reduced capacity of calcium uptake by the chloroplasts from boron deficient plants is due to a low level of ATP in these chloroplasts. This possibility was tested and the results are shown in Table 4.

Table 4. The effect of ATP on the uptake of $^{45}$Ca by chloroplasts from mature leaves

<table>
<thead>
<tr>
<th>Exp. No.</th>
<th>Counts/min./mg chlorophyll equivalent of chloroplasts</th>
<th>$B^+$</th>
<th>$B^-$</th>
<th>$B^-/B^+$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>No ATP</td>
<td>2,830</td>
<td>360</td>
<td>7.9</td>
</tr>
<tr>
<td></td>
<td>With ATP</td>
<td>2,210</td>
<td>510</td>
<td>4.3</td>
</tr>
<tr>
<td>2</td>
<td>No ATP</td>
<td>2,280</td>
<td>490</td>
<td>4.7</td>
</tr>
<tr>
<td></td>
<td>With ATP</td>
<td>2,210</td>
<td>950</td>
<td>2.2</td>
</tr>
</tbody>
</table>

$^a$ATP concentration was $10^{-4}$ M. Other experimental conditions were identical with previous experiments.

It is apparent that added ATP increased the calcium uptake capacity of $B^-$ chloroplasts while showing no effect on that of $B^+$ chloroplasts.

A widely acknowledge hypothesis on the role of boron is its involvement in the facilitation of the sugar transport across membranes (18). The validity of this hypothesis was tested by measuring the sucrose uptake capacity of isolated chloroplasts. For comparison the calcium uptake capacity was measured with the same preparation of chloroplasts. Table 5 shows the results of this experiment.
Table 5. $^{45}\text{Ca}$ and $^{14}\text{C}$-sucrose uptake by isolated chloroplasts$^a$

<table>
<thead>
<tr>
<th>Samples</th>
<th>$^{14}\text{C}$-sucrose</th>
<th>$^{45}\text{Ca}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>B+</td>
<td>77 cpm</td>
<td>3,550 cpm</td>
</tr>
<tr>
<td>B−</td>
<td>77 cpm</td>
<td>820 cpm</td>
</tr>
</tbody>
</table>

$^a$See materials and methods for the experimental procedure.

It may be seen from Table 5 that the transport of sucrose chloroplast membrane is not influenced by boron, while that of calcium is profoundly influenced by it. Moreover, the negligible amount of sucrose transported into chloroplasts appears to suggest strongly that chloroplast membrane and possibly all other membranes are impermeable to external sucrose.

4. Discussion

Boron deficiency does not appear to have any effect on the calcium uptake capacity of chloroplasts from young leaves, even when the leaves showed visual deficiency symptoms. Although the chloroplasts from mature leaves of sunflower plants grown in B− nutrient for 7 to 12 days showed a lower capacity of calcium uptake, the addition of ATP increased the capacity, suggesting that the lower capacity of calcium uptake is not a primary effect of boron deficiency but a result of lower level of ATP in B− chloroplasts. The inability of both B+ and B− chloroplasts to take up sucrose rules out the possibility that boron resides in the membranes and facilitates sucrose transport.
C. Studies on the Early Metabolic Changes in B- Plants

The objective of this study was the finding of the earliest metabolic change resulting from boron deficiency. These earliest metabolic alterations—in an incipient stage of boron deficiency—even if the alterations are not profound, are more likely to be direct results of boron deficiency, whereas even the most pronounced metabolic changes in an advanced stage of boron deficiency may well be secondary. Since the earliest subcellular boron deficiency symptoms were observed in leaves that had been discontinued from boron supply for three days, and the anatomical deficiency symptoms are necessarily preceded by metabolic alterations, the metabolic alterations must have been initiated at some earlier time.

1. Methods and materials
   a. B+ and B- plants  
      Sunflower seeds were germinated and grown in sand for 6 days. The seedlings were then transferred to Hoagland's complete nutrient solution and further grown an additional 12 days. At this time B- plants were taken out, washed well with distilled water, transferred to B- nutrient solution and grown in this solution for 24 hours. By this date, the primary leaves had ceased to grow and were designated 'mature leaves'. The first pair of leaves were about 7 cm long. The second pair of leaves, about 3 cm long, were growing actively and were designated 'young leaves'. In the following experiments, these young and mature leaves were used unless stated otherwise.

   b. $^{14}$CO$_2$ feeding to intact plants (Light experiment)  
      The first pair of leaves were cut off from the plants prior to feeding. Two B+ and
two B- plants were fed $^{14}$CO$_2$ in a closed chamber for 10 minutes under flood light. $^{14}$CO$_2$ was generated from 0.2 to 0.4 mc of Ba$^{14}$CO$_3$ by reacting with dilute perchloric acid. Immediately after feeding, designated 0 hour, two mature leaves and two young leaves were used for analysis of $^{14}$C-compounds and the remaining leaves were used two hours later. Leaf punches were made with cork borer (i.d. = 7 mm) from these leaves and the punches were extracted three 10-minute periods with 8 ml of 80% boiling ethanol. Various aliquots of these extracts were used for the separation of $^{14}$C-compounds of each sample. $^{14}$C-compounds were separated by paper chromatography, using 80% aqueous phenol in one direction and butyric acid -n-butanol-water (2:2:1) in another. The chromatograms were radioautographed with X-ray film. The radioactive spots were cut out of chromatograms with the aid of the radioautograms and these spots were counted with a Tricarb liquid scintillation counter. The scintillant used was 0.3 gm POPOP [1,4-bis-2-(5-phenyloxazolyl)benzene] and 5 gm PPO (2,5-diphenyloxazole) dissolved in 1 liter of toluene.

c. $^{32}$P feeding to leaf slices Three disks were punched out from each of two young leaves with number 3 cork borer (i.d. = 7 mm). The leaf disks were cut to 1 mm wide slices. The slices were introduced into cellulose nitrate centrifuge tubes containing 1 ml mixture of $^{32}$P (5 µc) and one-half-strength complete nutrient solution for B+ slices or one-half-strength B- nutrient solution for B- slices. The centrifuge tubes containing the slices were inserted into heavy glass test tubes and air inside was evacuated by suctioning for two minutes. The suction was suddenly released and the $^{32}$P solution was allowed to penetrate into the slices. The leaf slices became translucent, suggesting that $^{32}$P had
penetrated. One tube each of B+ and B- slices were wrapped with aluminum foil to exclude light while one tube each of B+ and B- slices were exposed under two 150-watt tungsten lamps placed 30 cm above the tubes. The tubes were shaken with a rotary shaker for 20 minutes. At the end of 20 minutes, 50 ml of 0.01 M phosphate was added to each of the tubes and the contents of the tubes were well shaken before the liquid was drained off. The slices were washed once more with 50 ml of 0.01 M phosphate solution. The leaf slices were then extracted once with 4 ml of 95% ethanol (4 ml per three original leaf disks) for 10 minutes, twice with 50% ethanol, 3 ml and for 10 minutes for each extraction. Various aliquots of these extracts were used for the determination of total radioactivity and chlorophyll content in each sample, and for separation of various $^{32}\text{P}$-compounds. The separation of $^{32}\text{P}$-compounds were achieved by paper chromatography using 80% phenol in one direction and isobutyric acid-water-concentrated ammonia (198:99:3 v/v) in another. The chromatograms were radioautographed and the radioactive spots were cut out and counted with GM counter. Some of the unknown compounds were identified by degrading and chromatographing in various solvents.

d. $^{14}\text{C}$-glucose feeding to leaf slices Two punches each of B+ and B- leaves were made with No. 3 cork borer. The punches were cut into 1 mm slices. 2 μc of $^{14}\text{C}$-glucose was infiltrated into the slices by the same manner as $^{32}\text{P}$ feeding experiment described above. The slices were incubated with $^{14}\text{C}$-glucose for three hours in the dark. The slices were twice washed with 20 ml of 0.01 M glucose. The slices were extracted once with 5 ml of boiling 80% ethanol for 10 minutes and twice for 10 minutes and 5 ml each of boiling 50% ethanol. The extracts were separated
by means of paper chromatography using 80% phenol and BABW. The chromatograms were radioautographed, the radioactive spots were cut out of chromatograms and counted with liquid scintillation counter using POPOP and PPO in toluene.

e. $^{14}$CO$_2$ feeding to intact sunflower plants (Dark experiment)

Sunflower plants were grown in complete nutrient solution only for 6 days before transfer of B- plants to B- nutrient solution. So the plants used in this experiment were about a week younger than those used in previous experiments. At this time the primary leaves were about 3 cm. B- plants were grown in B- solution for 20 hours instead of usual 24 hours. 0.2 mc of $^{14}$CO$_2$ was fed to these young plants by the same manner that was described earlier. The distribution of various $^{14}$C-compounds in B+ and B- leaves were analyzed immediately after the feeding and also 30 hours after keeping the plants in the dark. The extraction, separation and counting of various $^{14}$C-compounds were done by the same manner that was described earlier.

2. Results

a. $^{14}$C-metabolites Table 6 shows the total radioactivities in 80% ethanol extracts per unit of chlorophyll in various samples. It may be seen from this table that both B+ and B- leaves contain the same amount of radioactive metabolites at 0 hour, but two hours after the termination of $^{14}$CO$_2$ feeding young B- leaves contain much larger amounts of radioactive metabolites than B+ leaves do.
Table 6. Total specific activities of $^{14}$C-metabolites in 80% ethanol extracts from mature (m) or young (y) leaves

<table>
<thead>
<tr>
<th>Exp. No.</th>
<th>Total specific activities (total activity c/m/mg chlorophyll) ($10^{-3}$)</th>
<th>0 hour</th>
<th>2 hour</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>m</td>
<td>y</td>
</tr>
<tr>
<td>1</td>
<td></td>
<td>1,031</td>
<td>1,226</td>
</tr>
<tr>
<td>B+</td>
<td></td>
<td>1,079</td>
<td>1,252</td>
</tr>
<tr>
<td>B−</td>
<td></td>
<td>1,410</td>
<td>1,623</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>1,273</td>
<td>1,680</td>
</tr>
<tr>
<td>B+</td>
<td></td>
<td>1,507</td>
<td>1,813</td>
</tr>
<tr>
<td>B−</td>
<td></td>
<td>1,414</td>
<td>1,900</td>
</tr>
</tbody>
</table>

*aThe standard errors were less than 5%.

*The cause of very high specific activity in the samples taken at 2 hour in Experiment 3 is not known.

In a separate experiment the residual radioactivities, or the radioactivities left after the three 80% ethanol extractions, in the leaf punches sampled two hours after $^{14}$CO$_2$ feeding were measured and the results are shown in Table 7. It may be seen from this table that young leaves have much higher residual activities than mature leaves do, and also that the residual activity is greater in B+ young leaves than in B− young leaves. No chemical analysis was made of the residues. However, the greater residual activities in young leaves suggest that the bulk of the radioactivities in the residues of young leaf punches come from $^{14}$C incorporated into proteins and nucleic acids rather than starch. The anatomical studies revealed that the number of chloroplasts is far greater
Table 7. Radioactivities in residues and in 80% ethanol extracts at 2 hours^a

<table>
<thead>
<tr>
<th>Samples</th>
<th>Residue activities (c/m)(10^{-3})</th>
<th>Activities in c/m/mg chl in extracts</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Per leaf punch</td>
<td>Per mg chlorophyll</td>
</tr>
<tr>
<td>B+ mature</td>
<td>2.9</td>
<td>40.7</td>
</tr>
<tr>
<td>B- mature</td>
<td>3.4</td>
<td>52.3</td>
</tr>
<tr>
<td>B+ young</td>
<td>43</td>
<td>525</td>
</tr>
<tr>
<td>B- young</td>
<td>23</td>
<td>307</td>
</tr>
</tbody>
</table>

^aTable 7 provides results of only a single experiment.

in mature leaves than in young leaves and the chloroplasts of mature leaves, especially those of B- plants, accumulate more starch than those of young leaves. Thus, if $^{14}$C-starch accounts for the bulk of residual activity, greater activities would be expected in the residues of mature leaf punches. The actively growing young leaves are synthesizing proteins and nucleic acids rapidly, whereas the mature leaves are not. Therefore, the high residual activities in young leaves may be attributed to rapid synthesizes of proteins and nucleic acids. It may also be concluded that the rates of synthesizes of proteins and nucleic acids are higher in B+ young leaves than B- young leaves.

Figure 11 shows the separation of 80% ethanol extracts by ascending paper chromatography using 80% phenol in one direction and BABW in another. Sucrose and glucose, and glutamic acid and serine were reseparated by descending chromatography using BABW. Table 8 shows the distribution of radioactivities in various metabolites in 80% ethanol extracts of sunflower
Figure 11. Paper chromatographic separation of 80% ethanol extract

First direction: 80% aqueous phenol
Second direction: n-butyric acid-n-butanol-water, 2:2:1, v/v.

1. Phosphorylated compounds
2. Unknown
3. Glucose
4. Serine
5. α-ketoglutaric acid
Table 8. The distribution of radioactivities in various 80% ethanol soluble metabolites in B+ and B- sunflower leaves, expressed as % of total activities of extracts

<table>
<thead>
<tr>
<th>Metabolites</th>
<th>Immediately after feeding</th>
<th>2 hours after feeding</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mature leaves B+</td>
<td>B-</td>
</tr>
<tr>
<td>sucrose</td>
<td>41.87</td>
<td>38.55</td>
</tr>
<tr>
<td>glucose</td>
<td>7.14</td>
<td>5.07</td>
</tr>
<tr>
<td>fructose</td>
<td>2.19</td>
<td>2.24</td>
</tr>
<tr>
<td>glycercic acid</td>
<td>3.50</td>
<td>2.36</td>
</tr>
<tr>
<td>glyoxylic acid</td>
<td>0.28</td>
<td>3.11</td>
</tr>
<tr>
<td>citric acid</td>
<td>1.51</td>
<td>1.32</td>
</tr>
<tr>
<td>α-ketoglutaric acid</td>
<td>3.07</td>
<td>2.82</td>
</tr>
<tr>
<td>succinic acid</td>
<td>0.33</td>
<td>0.70</td>
</tr>
<tr>
<td>fumaric acid</td>
<td>0.87</td>
<td>0.84</td>
</tr>
<tr>
<td>malic acid</td>
<td>16.43</td>
<td>17.72</td>
</tr>
<tr>
<td>chlorogenic acid</td>
<td>0.26</td>
<td>0.58</td>
</tr>
<tr>
<td>serine*</td>
<td>****</td>
<td>****</td>
</tr>
<tr>
<td>aspartic acid</td>
<td>3.23</td>
<td>5.01</td>
</tr>
<tr>
<td>alanine</td>
<td>15.13</td>
<td>12.50</td>
</tr>
</tbody>
</table>

*Serine and glutamic acid spots were almost superimposed. These were counted together for the samples at 0 hour but these spots were reserparated in the case of 2 hour samples.
leaves. The results shown in Table 8 do not contain the radioactivities of lipids. The lipids move along with solvent front in 80% phenol and tail in BABW. Although the radioactivities of these lipids were not counted, the radioautograms show that the lipid fraction of B- young leaves contains at least two to three times more radioactivity than B+ young leaves do. Unfortunately, quantitative measurements were not made.

At 0 hour, or immediately after feeding, the isotopic equilibria have not been reached, and therefore the distribution of radioactivities in various metabolites reflects to some extent the rates of syntheses and utilizations of various metabolites. Two hours after feeding, however, the isotopic equilibria have been reached and therefore the distribution of radioactivities reflects the relative equilibrium concentrations of the metabolites.

From the results shown in Table 8, the following conclusions may be made: 1) The equilibrium concentrations of glucose and fructose are higher in young B- leaves than in B+ young leaves. 2) The equilibrium concentration of α-ketoglutaric acid is higher in both young and mature B- leaves than in corresponding B+ leaves. 3) Both the rate of synthesis and the equilibrium concentration of chlorogenic acid are higher in B- leaves than in B+ leaves, and chlorogenic acid accumulates only in young leaves.

Some useful relations among various metabolites may be made from the results shown in Table 8. These relations are shown in Table 9.
Table 9. The ratios of radioactivities in some related metabolites at 2 hours after feeding $^{14}$CO$_2$

<table>
<thead>
<tr>
<th>Relations</th>
<th>Mature leaves</th>
<th>Young leaves</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>B+  B-  B~/B+</td>
<td>B+  B-  B~/B+</td>
</tr>
<tr>
<td><strong>a-Ketoglutaric acid</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Citric acid</td>
<td>0.42 1.14 2.7</td>
<td>0.14 0.35 2.5</td>
</tr>
<tr>
<td><strong>Glutamic acid</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a-Ketoglutaric acid</td>
<td>12.0 8.00 0.67</td>
<td>12.90 2.83 0.22</td>
</tr>
<tr>
<td><strong>Succinic acid</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a-Ketoglutaric acid</td>
<td>2.50 1.20 0.48</td>
<td>2.97 1.07 0.36</td>
</tr>
</tbody>
</table>

From the results shown in Table 9, the following additional conclusions may be made tentatively. 4) The conversion of citric acid to a-ketoglutaric acid via isocitric acid is faster in boron deficient leaves. 5) The synthesis of glutamic acid from a-ketoglutaric acid is slower in boron deficient leaves. 6) The conversion of a-ketoglutaric acid to succinic acid is slower in boron deficient leaves. The implications of these relations will be discussed later.

The interpretations of data as in Table 8 and 9 are often very difficult because of the simultaneous operation of vast numbers of reactions. The accumulation of a metabolite, for example, may result either from increased synthesis or from a decreased utilization of a metabolite. In order to simplify the situation, $^{14}$CO$_2$ fed plants were kept in the dark for 30 hours before analysis in the next experiment. In the dark, both photosynthesis and protein synthesis are not operating in plants (30, 38). Table 10 shows the distribution of radioactivity in various metabolites in the leaves that had been kept in the dark for 30 hours after $^{14}$CO$_2$ feeding.
Table 10. Distribution of radioactivity in various metabolites in 80% ethanol extracts from leaves stored in dark for 30 hours after $^{14}$CO$_2$ feeding, expressed as per cent of total$^a$

<table>
<thead>
<tr>
<th>Metabolites</th>
<th>B+ (%)</th>
<th>B- (%)</th>
<th>B- / B+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sucrose</td>
<td>1.30</td>
<td>1.30</td>
<td>1.0</td>
</tr>
<tr>
<td>Glucose</td>
<td>2.82</td>
<td>9.12</td>
<td>3.2</td>
</tr>
<tr>
<td>Fructose</td>
<td>5.94</td>
<td>3.92</td>
<td>0.66</td>
</tr>
<tr>
<td>Citric Acid</td>
<td>36.9</td>
<td>12.0</td>
<td>0.33</td>
</tr>
<tr>
<td>Frumaric acid</td>
<td>0.97</td>
<td>0.68</td>
<td>0.70</td>
</tr>
<tr>
<td>Malic acid</td>
<td>4.40</td>
<td>2.56</td>
<td>0.61</td>
</tr>
<tr>
<td>Glyoxylic acid</td>
<td>13.3</td>
<td>27.8</td>
<td>2.1</td>
</tr>
<tr>
<td>Glyceric acid</td>
<td>1.40</td>
<td>2.49</td>
<td>1.8</td>
</tr>
<tr>
<td>Alanine</td>
<td>1.87</td>
<td>2.77</td>
<td>1.5</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>7.48</td>
<td>18.1</td>
<td>2.4</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>9.48</td>
<td>5.63</td>
<td>0.59</td>
</tr>
<tr>
<td>Serine</td>
<td>2.91</td>
<td>4.31</td>
<td>1.5</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>9.09</td>
<td>7.89</td>
<td>0.88</td>
</tr>
</tbody>
</table>

$^a$The radioactivities in individual compounds were divided by the total radioactivity in 80% ethanol extract.

From the results of Table 10, the following conclusions may be made:

1) Glucose accumulates in boron deficient leaves but fructose is found less in the same leaves. 2) Citric acid accumulates in boron sufficient leaves but glyoxylic acid accumulates in boron deficient leaves. This may be due to the increased activity of isocitric lyase in boron deficient leaves and the small pool size of isocitric acid causes the accumulation
of citric acid in boron sufficient leaves. 3) The accumulation of
glyoxylic acid in boron deficient leaves may be partly due to reduced
malate synthetase activity since less malate was found in B- leaves.
However, the low level of malic acid in B- leaves may also be attributed
to the increased activity of NADP-malate dehydrogenase.

In an isolated system, the ratio of pyruvate/oxalacetate in B-
to pyruvate/oxalacetate in B+ leaves may be calculated from the following
reversible reaction if the concentrations of aspartic acid and alanine
are known.

\[
\text{oxalacetate} + \text{alanine} \rightleftharpoons \text{aspartate} + \text{pyruvate}
\]

From \[
\frac{(\text{aspartate})(\text{pyruvate})}{(\text{oxalacetate})(\text{alanine})} = K, \quad \frac{\text{pyruvate}}{\text{oxalacetate}} = \frac{K(\text{alanine})}{\text{aspartate}}
\]

Therefore,

\[
\frac{\frac{\text{pyruvate}}{\text{oxalacetate}}_{B-}}{\frac{\text{pyruvate}}{\text{oxalacetate}}_{B+}} = \frac{\frac{\text{alanine}}{\text{aspartate}}_{B-}}{\frac{\text{alanine}}{\text{aspartate}}_{B+}}, \text{ since } K_{B+} = K_{B-}
\]

In a complex system such as in cells, only a rough approximation may
be possible, if at all. In leaves which have been in the dark for 30
hours, protein and other macromolecule syntheses are presumably not
occurring, and therefore a rough estimation of the ratio may be permissible.
Table 11 shows this ratio and other relations between the related
metabolites.
Table 11. The relations of some related metabolites in leaves after 30 hours in the dark

<table>
<thead>
<tr>
<th>Relations</th>
<th>B+</th>
<th>B-</th>
<th>B-/B+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>0.48</td>
<td>2.33</td>
<td>4.9</td>
</tr>
<tr>
<td>Fructose</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glyoxylic acid</td>
<td>0.36</td>
<td>2.31</td>
<td>6.4</td>
</tr>
<tr>
<td>Citric acid</td>
<td>8.40</td>
<td>4.76</td>
<td>0.57</td>
</tr>
<tr>
<td>Malic acid</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pyruvic acid</td>
<td></td>
<td></td>
<td>2.50</td>
</tr>
<tr>
<td>Oxalacetic acid</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alanine</td>
<td>0.43</td>
<td>1.08</td>
<td>2.5</td>
</tr>
<tr>
<td>Malic acid</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serine</td>
<td>0.66</td>
<td>1.68</td>
<td>2.6</td>
</tr>
<tr>
<td>Malic acid</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>4.06</td>
<td>6.55</td>
<td>1.6</td>
</tr>
<tr>
<td>Alanine</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

From the relations above in Table 11, the following conclusions may also be made: 4) The ratio of pyruvate to oxalacetate is greater in B- leaves than in B+ leaves when these are stored in the dark. Not only pyruvate but alanine, serine and glycerate, which are expected to be in equilibrium with pyruvate, are found more in B- leaves. On the other hand, oxalacetate, malate, fumarate and aspartate are all low in the B- leaves. This relation appears to suggest that the oxidative decarboxylation of malate to pyruvate by NADP-malic dehydrogenase action or the decarboxylation of oxalacetate to pyruvate by the same enzyme is much greater in B- leaves than in B- leaves when these are stored in the dark. Conversely, the reductive carboxylation of pyruvate to malate may be faster in B+ leaves.
than in B- leaves.

The large amount of glyoxylate in B- seems to be due mainly to the rapid cleavage of isocitrate than to the low malate synthetase activity, since citrate would also accumulate in B- leaves rather than in B+ leaves if the reduced activity of malate synthetase were responsible primarily for the accumulation of glyoxylate in B- leaves.

It may be noted that in the dark phenylalanine accumulates in leaf tissue instead of chlorogenic acid. It may also be noted that the glyoxylic acid cycle is activated in the dark rather than the normal tricarboxylic acid cycle.

In another attempt to facilitate the interpretation of experimental results, glucose was infiltrated into leaf sliced in the dark, hoping to eliminate photosynthesis and protein and RNA syntheses, and to confine the reactions mainly to respiratory metabolism. Table 12 shows the results from this $^{14}$C-glucose feeding experiment, and Table 13 shows the relations of some of the related metabolites.

It may be seen that the results shown in Table 11 and in Table 13 qualitatively identical, suggesting that the results in these two experiments reflect catabolic reactions of carbohydrates, but that the magnitudes of differences between B+ and B- leaves in the distributions of radioactive metabolites are lesser for the results in Table 13, suggesting that the degree of boron deficiency in the leaves used for $^{14}$C-glucose feeding experiment was less severe than those used for $^{14}$CO$_2$ feeding experiment. As for the consequences of boron deficiency on the metabolic alternations, therefore, the same conclusions may be made from the results of Table 13 as was made from Table 11. Additional conclusions
Table 12. Distribution of radioactivity in various metabolites 3 hours after $^{14}$C-glucose feeding, as per cent of total\textsuperscript{a}

<table>
<thead>
<tr>
<th>Metabolites</th>
<th>B+</th>
<th>B-</th>
<th>B- / B+</th>
</tr>
</thead>
<tbody>
<tr>
<td>UDPG\textsuperscript{b}</td>
<td>0.94</td>
<td>0.87</td>
<td>0.92</td>
</tr>
<tr>
<td>Glucose-6-phosphate\textsuperscript{b}</td>
<td>1.01</td>
<td>0.84</td>
<td>0.83</td>
</tr>
<tr>
<td>Fructose-6-phosphate\textsuperscript{b}</td>
<td>1.19</td>
<td>1.01</td>
<td>0.85</td>
</tr>
<tr>
<td>Sucrose</td>
<td>1.89</td>
<td>2.72</td>
<td>1.44</td>
</tr>
<tr>
<td>Glucose</td>
<td>9.11</td>
<td>10.88</td>
<td>1.20</td>
</tr>
<tr>
<td>Fructose</td>
<td>11.60</td>
<td>8.65</td>
<td>0.75</td>
</tr>
<tr>
<td>Alanine</td>
<td>8.81</td>
<td>11.24</td>
<td>1.28</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>16.74</td>
<td>14.07</td>
<td>0.84</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>11.58</td>
<td>11.34</td>
<td>0.98</td>
</tr>
<tr>
<td>Glyoxylic acid</td>
<td>8.58</td>
<td>10.54</td>
<td>1.23</td>
</tr>
<tr>
<td>Citric acid</td>
<td>4.79</td>
<td>4.18</td>
<td>0.87</td>
</tr>
<tr>
<td>Malic acid</td>
<td>14.41</td>
<td>16.55</td>
<td>1.15</td>
</tr>
<tr>
<td>Glyceric acid</td>
<td>0.55</td>
<td>0.98</td>
<td>1.78</td>
</tr>
</tbody>
</table>

\textsuperscript{a}The activities in individual compounds were divided by the total activity of extract.

\textsuperscript{b}The identification of UDPG, glucose-6-phosphate and fructose-6-phosphate was achieved by procedures that will be discussed later.
Table 13. The ratios of radioactivity in some related metabolites, 3 hours in the dark after $^{14}C$-glucose feeding

<table>
<thead>
<tr>
<th>Relations</th>
<th>$B^+$</th>
<th>$B^-$</th>
<th>$\frac{B^-}{B^+}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose Fructose</td>
<td>0.78</td>
<td>1.26</td>
<td>1.60</td>
</tr>
<tr>
<td>Glucose Glucose-6-phosphate</td>
<td>9.0</td>
<td>13.0</td>
<td>1.44</td>
</tr>
<tr>
<td>Fructose Fructose-6-phosphate</td>
<td>9.8</td>
<td>8.6</td>
<td>0.88</td>
</tr>
<tr>
<td>Glyoxylic acid Citric acid</td>
<td>1.87</td>
<td>2.52</td>
<td>1.42</td>
</tr>
<tr>
<td>Alanine Malic acid</td>
<td>0.61</td>
<td>0.68</td>
<td>1.12</td>
</tr>
<tr>
<td>Alanine Aspartic acid</td>
<td>0.53</td>
<td>0.80</td>
<td>1.51</td>
</tr>
<tr>
<td>Glyceric acid Aspartic acid</td>
<td>0.53</td>
<td>0.80</td>
<td>1.51</td>
</tr>
<tr>
<td>Glyoxylic acid Malic acid</td>
<td>0.60</td>
<td>0.64</td>
<td>1.07</td>
</tr>
</tbody>
</table>

may be made from the results of Table 13. 1) Boron deficiency tends to shift the equilibrium between glucose and glucose-6-phosphate toward glucose formation. This may be due to an increased phosphatase activity or a decreased glucokinase activity, or both, which, in turn, may be due to direct controls of these enzyme activities by boron or indirect control of these enzyme activities by influencing the cellular concentration of ATP or inorganic phosphate. This will be discussed further later. 2) The relation between fructose and fructose-6-phosphate is opposite to that between glucose and glucose-6-phosphate, that is, the ratio of fructose
to fructose-6-phosphate is larger for B+ leaves than for B- leaves. In the results of both Tables 10 and 12, more fructose was found in B+ leaves than in B- leaves. This will also be discussed later.

b. $^{32}\text{P}$-metabolites Table 14 shows $^{32}\text{P}$ uptake by leaf slices in terms of inorganic $^{32}\text{P}$-phosphate and total $^{32}\text{P}$-organic metabolites.

Table 14. $^{32}\text{P}$ uptake by leaf slices of mg chlorophyll equivalent

<table>
<thead>
<tr>
<th></th>
<th>Light</th>
<th></th>
<th>Dark</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>B+</td>
<td>B-</td>
<td>B+</td>
<td>B-</td>
</tr>
<tr>
<td></td>
<td>9,310,000 cpm</td>
<td>6,840,000 cpm</td>
<td>6,390,000 cpm</td>
<td>5,270,000 cpm</td>
</tr>
</tbody>
</table>

Among a number of possible explanations for the results in Table 14 is one concerning ATP availability under various conditions. In the presence of light both photophosphorylation in chloroplasts and oxidative phosphorylation in mitochondria would supply ATP necessary for the active transport of $^{32}\text{P}$ into cells while in the dark only the latter would. Thus the larger amount of $^{32}\text{P}$ was uptaken by both B+ and B- leaf slices in the light than in the dark. Evidence was provided earlier that the lower capacity of B- chloroplasts to accumulate was a result of the low level of ATP in these chloroplasts. The lower capacity of B- leaf slices in $^{32}\text{P}$ dark uptake may also be attributed to the low level of ATP in B- leaves. This view will be substantiated later.

Table 15 shows the distribution of $^{32}\text{P}$ compounds in B+ and B- leaf slices.
Table 15. Distribution of $^{32}$P-metabolites, as per cent of total activity

<table>
<thead>
<tr>
<th>Metabolites</th>
<th>Light</th>
<th></th>
<th>Dark</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>B+</td>
<td>B-</td>
<td>B+</td>
<td>B-</td>
</tr>
<tr>
<td>UDPG</td>
<td>6.27</td>
<td>4.01</td>
<td>5.26</td>
<td>3.63</td>
</tr>
<tr>
<td>Glucose-6-phosphate</td>
<td>13.69</td>
<td>8.78</td>
<td>11.23</td>
<td>6.50</td>
</tr>
<tr>
<td>Fructose-6-phosphate</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATP</td>
<td>2.03</td>
<td>1.78</td>
<td>2.22</td>
<td>1.75</td>
</tr>
<tr>
<td>Unknown (spot 6)</td>
<td>0.96</td>
<td>1.00</td>
<td>1.04</td>
<td>0.65</td>
</tr>
<tr>
<td>Unknown (spot 7)</td>
<td>0.84</td>
<td>0.98</td>
<td>1.33</td>
<td>1.63</td>
</tr>
<tr>
<td>ADP</td>
<td>3.24</td>
<td>2.46</td>
<td>3.50</td>
<td>2.73</td>
</tr>
<tr>
<td>Unknown (spot 9)</td>
<td>0.89</td>
<td>1.46</td>
<td>0.84</td>
<td>1.14</td>
</tr>
<tr>
<td>Unknown (spot 10)</td>
<td>0.67</td>
<td>0.32</td>
<td>0.22</td>
<td>0.30</td>
</tr>
<tr>
<td>Unknown (spot 11)</td>
<td>5.84</td>
<td>7.18</td>
<td>3.23</td>
<td>5.35</td>
</tr>
<tr>
<td>Unknown (spot 12)</td>
<td>3.54</td>
<td>3.94</td>
<td>2.09</td>
<td>1.71</td>
</tr>
<tr>
<td>Unknown (spot 13)</td>
<td>0.44</td>
<td>0.87</td>
<td>0.69</td>
<td>0.62</td>
</tr>
<tr>
<td>Unknown (spot 14)</td>
<td>0.99</td>
<td>0.96</td>
<td>0.30</td>
<td>0.52</td>
</tr>
<tr>
<td>Total organic</td>
<td>39.41</td>
<td>35.01</td>
<td>31.95</td>
<td>26.45</td>
</tr>
<tr>
<td>Phosphate</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inorganic</td>
<td>60.59</td>
<td>64.99</td>
<td>68.05</td>
<td>73.55</td>
</tr>
<tr>
<td>Phosphate</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 16 shows the distribution of radioactivity in various $^{32}$P-metabolites in the leaves of mg chlorophyll equivalent.
Figure 12. Paper chromatographic separation of phosphorylated compounds.

First direction: 80% aqueous phenol, ascending.

Spot 2: Unknown
3: UDPG
4: Glucose-6-phosphate and Fructose-6-phosphate
5: Inorganic phosphate and ATP
6: Unknown
7: Unknown
8: ADP
9: Unknown
10: Unknown
11: Unknown
12: Unknown
13: Unknown
14: Unknown
Table 16. Distribution of radioactivity in various $^{32}$P-metabolites, per mg chlorophyll

<table>
<thead>
<tr>
<th>Metabolites</th>
<th>c/m/mg chlorophyll</th>
<th>Light</th>
<th>Dark</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>B+</td>
<td>B-</td>
<td>B+</td>
</tr>
<tr>
<td>UDPG</td>
<td>583,500</td>
<td>274,000</td>
<td>335,800</td>
</tr>
<tr>
<td>Glucose-6-phosphate</td>
<td>1,274,000</td>
<td>591,000</td>
<td>716,900</td>
</tr>
<tr>
<td>Fructose-6-phosphate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATP</td>
<td>189,000</td>
<td>125,000</td>
<td>132,800</td>
</tr>
<tr>
<td>ATP</td>
<td>301,000</td>
<td>198,000</td>
<td>233,300</td>
</tr>
<tr>
<td>Total organic phosphate</td>
<td>3,666,000</td>
<td>2,390,000</td>
<td>2,050,000</td>
</tr>
</tbody>
</table>

A classical method of determination of the rate of phosphorylation by mitochondrial preparation employs the coupling of mitochondrial phosphorylation with hexokinase reaction, glucose as phosphate acceptor and ATP as phosphate donor. Thus the relative levels of glucose and glucose-6-phosphate in a tissue reflect the level of the rate of formation of ATP in the tissue. Fructose-6-phosphate reaches a very rapid equilibrium with glucose-6-phosphate and, since the radioactivity of UDPG comes mainly from $^{32}$P-glucose-1-phosphate which is in equilibrium with glucose-6-phosphate, the radioactivities in these two compounds also reflect the level of ATP or the rate of ATP formation in the tissue. It was shown in the earlier experiments that glucose accumulates in B- leaves. Thus the following conclusion may be made:

1) The higher level of glucose and lower levels of glucose-6-phosphate, fructose-6-phosphate and UDPG in boron deficient leaves, compared to those of normal leaves, are a result of a lower rate of ATP
formation in B- leaves. The higher activity present in ADP of B+ leaves may also be the result of the higher rate of ATP synthesis in B+ leaves, since most of the activity in ADP may be originated by the transfer of $^{32}\text{P}$ from ATP to AMP.

The amount of total phosphate uptaken by B+ leaves was larger than that by B- leaves, thus more ATP must have been hydrolyzed in the active transport of inorganic phosphate into cells of B+ leaves than into cells of B- leaves. Therefore, it may also be concluded that the actual difference in the rate of ATP formation between B+ and B- leaves must be greater than the results shown in Tables 15 and 16.

The unknown compounds, spots 12, 13 and 14 moved along with the solvent fronts of both solvent systems (80% phenol and isobutyrlic acid-water-concentrated ammonium hydroxide, 198:99:3 v/v), suggesting that these compounds are higher in B- leaves than in B+ leaves. This agrees with the larger amount of $^{14}\text{C}$-lipid synthesized in B- leaves in earlier $^{14}\text{CO}_2$ feeding experiments. Since these compounds were not identified positively, a tentative conclusion will be made of lipid compounds.

2) The rate of lipid synthesis is higher in B- leaves than in $^{6}\text{B+}$ leaves. The implications of these two conclusions will be further discussed later.

c. Identification of $^{32}\text{P}$ compounds Brief mention is made of the identification of phosphorylated compounds. $^{14}\text{CO}_2$ fed sunflower leaves were infiltrated with $^{32}\text{P}$ by the method described earlier. Doubly labelled compounds were extracted by the usual way from the leaves and the compounds desired were obtained by paper chromatographic separation. All the separations for identification employed paper chromatography.
The compounds to be identified were commonly chromatographed with known compounds. After the chromatographic separation, the radioactive unknown compounds were traced by radioautographying the chromatograms, pyrimidine and purine containing known compounds were detected by shadowgraphing, and the phosphorylated compounds were detected by spraying the chromatograms with phosphomolybdate reagent. The degradation of the doubly labelled compounds yielded products containing both $^{14}$C and $^{32}$P or $^{14}$C alone. The distinction of the kinds of radionuclides in various degradation products was made by noting whether one or both of the two films placed on the chromatogram was exposed by the compounds in question. $^{32}$P containing compounds exposed both films due to high $\beta$ energy of $^{32}$P, while the compounds containing only $^{14}$C exposed the side of the film that is in direct contact with chromatogram as the weak $\beta$ emitted from $^{14}$C is unable to penetrate the film.

The shadowgram was made by placing photosensitive paper underneath the chromatogram and exposing the chromatogram under UV light, developing the imprints of UV absorbing compounds according to the usual technique of photographic development.

1) Compound 3 The "Polydase" hydrolysates of "compound 3", UTP, ATP, CTP and GTP were cochromatographed using isobutyric acid-water-concentrated ammonium hydroxide (IBWA), 198:99:3 v/v. The radioautogram made from this chromatogram showed that the hydrolysate of "compound 3" yielded four new products. Two new products, 3a and 3b, contained both $^{14}$C and $^{32}$P, one product was inorganic phosphate, and the last product, 3c, contained only $^{14}$C. The shadowgram revealed that the "Polydase" hydrolysate of each nucleotide triphosphate contained four products which
were determined to be tri, di and mononucleotides and free nucleosides. The comparison of the shadowgram and radioautogram showed one matching pair, UMP and product 3b. Since the original "compound 3" did not match with UTP and UDP, "compound 3" appeared to be UDPG.

"Compound 3" was hydrolyzed with intestinal phosphatase and the hydrolysate was cochromatographed with UDPG, glucose-1-phosphate and inorganic phosphate. The radioautogram, shadowgram and the chromatogram sprayed with molybdate reagent are shown in Figure 13. The mixture of "compound 3" and UDPG was also hydrolyzed with dilute ammonium hydroxide and the hydrolysate was chromatographed using ethyl acetate-formamide-pyridine (1:2:1 v/v), with the result being shown in Figure 14. Three radioactive spots were detected on the radioautogram and the corresponding spots were detected on the original chromatogram upon spraying with molybdate reagent. The shadowgram showed two spots corresponding with two spots on the radioautogram or the chromatogram. The spot that did not appear on the shadowgram was, therefore, concluded to be glucose-1-phosphate, a moiety of UDPG. This radioactive spot was eluted and rechromatographed with known glucose-1-phosphate. This result is shown in Figure 16. Compound 3c shown in Figure 13, therefore, appears to be glucose. Compound 3c and known radioactive glucose were spotted on a same origin of chromatogram and this mixture was chromatographed two-dimensionally with 80% phenol and BABW. The radioautogram showed a single radioactive spot, meaning that 3c was indeed glucose (Figure 17). Compound 3b of Figure 15 was also cochromatographed with known UMP and the comparison of radioautogram and shadowgram showed that "compound 3b" is indeed UMP. From all the evidence presented above, "compound 3" was
concluded to be UDPG.

2) **Compound 4**  "Compound 4", which originally was thought to be a single compound, was found to be composed of two compounds upon running chromatogram for 24 hours using IBWA. These two compounds were designated compound 4 and compound 4'.

Polydase hydrolysates of compound 4, compound 4', ATP, UTP, CTP, and GTP showed that neither of the original "compound 4" and "4'" nor their hydrolytic products corresponded to the various hydrolytic products of nucleotide triphosphates. The radioautograms showed four and three new products in the hydrolysates of "compound 4" and "4'", respectively. Upon closer examination, two new products in the hydrolysate of "compound 4" and one new product in the hydrolysate of "4'" appeared to be from initial, residual compounds 4' and 4. The true new products from "compound 4" were designated "4a" and "4b". 4b was readily shown to be inorganic phosphate and "4a" was found to contain only $^{14}$C. The new product "4'b" was shown to be inorganic phosphate and "4'a" was also found to contain only $^{14}$C. All this evidence seemed to suggest that these two compounds were sugar phosphates, and the high radioactivity in the original "compounds 4" and "4'" and also that in "4b" and "4'b" further suggested that these are hexose-monophosphate. The stability of "compounds 4" and "4'" in acid suggested that these were hexose-6-phosphates and the higher mobility of "4'" and "4'a" compared to "4" and "4a" appeared to suggest that "compound 4" was glucose-6-phosphate and "4'" was fructose-6-phosphate. These conjectures were tested by cochromatographing with known compounds. Figures 18, 19, 20, 21 and 22 show the results of such experiments.
3) ADP and ATP These were identified in a manner identical to the above.

3. Discussion
   a. Borate complexes of various compounds The ability of boric acid to control various metabolisms of plants necessarily implies the ability to interact with some constituents of plant cells. The ability of borate ion to complex with polyhydroxy compounds is well known. Borate ion, $\text{B(OH)}_4^-$, has tetrahedral symmetry and its oxygen atoms are separated by a distance of $2.4 - 2.44$ Å (11). It thus appears that the borate ion can form complexes with those polyhydroxy compounds in which the oxygen atoms of at least two hydroxyl groups are separated by $2.4 - 2.5$ Å. Thus cis-1,2-diols of five membered ring compounds ($0-0$, $2.49$ Å) react more strongly with borate ion than their trans-isomers ($0-0$, $3.4$ Å), while both cis- and trans-1,2-diols of six membered ring compounds (both $0-0$, $2.82$ Å) do not form strong borate complexes. Various $\alpha$-hydroxy acids are also capable of complexing with borate ion. Here, the $\alpha$-hydroxy oxygen and one oxygen of the carboxyl group are involved in the complex formation, and the distance between the two oxygens involved in the complex formation can easily be arranged to meet the distance requirement of complex formation. Some other requirements for a strong complex appear to be coplanarity of the two hydroxyl groups and rigidity of the plane (8, 56). Another reason for the inability of dihydroxy, six-membered ring compounds to form stable complexes appears to be the instability of any one conformation, or a rapid change from one conformation to another. 0-dihydroxy phenols are known to form particularly stable borate complexes
Figure 13. Shadowgram, radioautogram and chromatogram showing the separation of the mixture of UDPG and spot 3, and their hydrolytic products. The hydrolysis was done with porcine intestinal phosphatase for 0, ½, and 3 hours (left to right). The enzyme must have been rather inactive since most of the UDPG was unhydrolyzed. UDPG and its hydrolysates were detected in the shadowgram and the chromatogram whereas the radioactive spot 3 and its hydrolysates were detected and shown in the radioautogram.

Figure 14. Shadowgram, chromatogram and radioautogram showing the separation of alkali hydrolysates of the mixture of UDPG and spot 3. UDPG and its hydrolysates were detected in the shadowgram while spot 3 was detected by radioautography.
Figure 15. (left) Cochromatography of UMP (shadowgram) and 3b (radioautogram), a hydrolytic product of spot 3. The tailing of 3a is probably due to $^{32}$P hydrolyzed from UMP. Solvent used: methyl cellosolve-methyl isobutyl ketone-3 N NH$_4$OH (7:2:3).

Figure 16. (center) Cochromatography of glucose-1-phosphate and 3a. Solvent used: isobutyric acid-water-conc. NH$_4$OH (66:33:1).

Figure 17. (right) Cochromatography of radioactive glucose and a hydrolytic product of 3a containing $^{14}$C activity only. A single radioactive spot on the radioautogram suggests that the $^{14}$C compound from 3a is glucose. The radioactivity of known glucose and that of the $^{14}$C compound from 3a hydrolysates were similar. Solvent used: BABW.
Figure 18. (left) The hydrolytic products of spot 4 and 4’. Hydrolysis was achieved with polydase, yeast extract containing various hydrolytic enzymes. Solvent: isobutyric acid-water-conc. ammonium hydroxide, 66:33:1, v/v.

Figure 19. (right) The hydrolytic products of glucose-6-phosphate and spot 4. Solvent: isobutyric acid-water-conc. ammonium hydroxide, 66:33:1, v/v.
Figure 20. (left) Chromatogram and radioautogram showing the separation of hydrolytic product of spot 4' and fructose-1,6-bisphosphate. Solvent used: isobutyric acid-water-ammonia.

Figure 21. (center) Radioautogram showing the cochromatography of 4'a and known glucose and fructose. Solvent used: isobutyric acid-water-ammonia.

Figure 22. (right) Radioautogram showing the cochromatography of 4a and known radioactive glucose. Single spot suggests that 4a was glucose. Solvents: 2,4,6-collidine-ethyl acetate-water (2:5:5) in one direction and isopropyl alc.-pyridine-water-acetic acid (8:8:4:1) in another.
and this may be due mainly to the coplanarity and rigidity of the two hydroxyl groups.

Table 17 shows the complex forming abilities of various compounds with boric acid. The formation constants are expressed as the following equation.

\[
K = \frac{[\text{R-\(\text{O}^-\text{B}\text{OH}) (\text{H}^+)\]}}{[\text{H}_3\text{BO}_3]\text{R-\(\text{OH}^-\)}]}
\]
Table 17. Borate complex forming abilities of various compounds

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Conc. (mole/l)(^a)</th>
<th>Conductivity increment(^b)</th>
<th>K</th>
<th>Refs.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cis-1,2-cyclopentanediol</td>
<td>0.5</td>
<td>149</td>
<td>---</td>
<td>9</td>
</tr>
<tr>
<td>Trans-1,2-cyclopentanediol</td>
<td>0.5</td>
<td>0</td>
<td>---</td>
<td>9</td>
</tr>
<tr>
<td>Cis-1,2-cyclohexanediol</td>
<td>0.5</td>
<td>0</td>
<td>---</td>
<td>8</td>
</tr>
<tr>
<td>Trans-1,2-cyclohexanediol</td>
<td>0.5</td>
<td>0</td>
<td>---</td>
<td>8</td>
</tr>
<tr>
<td>Catechol</td>
<td>0.5</td>
<td>516</td>
<td>---</td>
<td>9</td>
</tr>
<tr>
<td>Catechol</td>
<td>---</td>
<td>---</td>
<td>5 x 10(^{-6})</td>
<td>49</td>
</tr>
<tr>
<td>Catechol</td>
<td>---</td>
<td>---</td>
<td>ca. 10(^{-4})</td>
<td>26</td>
</tr>
<tr>
<td>Tannin</td>
<td>0.00459</td>
<td>230</td>
<td>---</td>
<td>9</td>
</tr>
<tr>
<td>(\alpha)-D-fructofuranose</td>
<td>0.5</td>
<td>774-778</td>
<td>---</td>
<td>9</td>
</tr>
<tr>
<td>(\alpha)-D-fructofuranose</td>
<td>---</td>
<td>---</td>
<td>4.4 x 10(^{-7})</td>
<td>60</td>
</tr>
<tr>
<td>D-glucose</td>
<td>---</td>
<td>---</td>
<td>5.11 x 10(^{-8})</td>
<td>49</td>
</tr>
<tr>
<td>Sucrose</td>
<td>0.5</td>
<td>0</td>
<td>---</td>
<td>9</td>
</tr>
<tr>
<td>Gluconic acid</td>
<td>0.5</td>
<td>21,636</td>
<td>---</td>
<td>8,9</td>
</tr>
<tr>
<td>Meso-tartaric acid</td>
<td>0.0625</td>
<td>4,120</td>
<td>---</td>
<td>8</td>
</tr>
<tr>
<td>Meso-tartaric acid</td>
<td>---</td>
<td>---</td>
<td>2.98 x 10(^{-7})</td>
<td>32</td>
</tr>
</tbody>
</table>

\(^a\)Concentrations of various compounds are applicable only to conductivity measurements.

\(^b\)Conductivity increment = observed conductivity of polyol-boric acid minus the sum of the conductivities of the individual polyol and boric acid solution in Kohlrausch-Holborn units x 10\(^{6}\).

The increment of conductivity when the above compounds are mixed with boric acid comes from the fact that borate complexes of these compounds are stronger acids than boric acid alone, and thus supply more
protons to carry charge. This increment of conductivity is dependent on the concentrations of these compounds and boric acid, but not linearly, and also on the nature of the compounds. Thus, when mixed with boric acid, dl-tartaric acid exhibits a greater increment of conductivity than fructose does, while the formation constant of tartaric acid is smaller than that of fructose. Therefore comparisons of conductivity data are meaningful only among the similar compounds. The formation constants of organoborate complexes are direct measurements of the stability of these complexes, but these constants are known for a very few complexes.

The ability of glucose in solution to form complexes with boric acid comes from the conversion of glucopyranose to α-D-glucofuranose (8). α-D-glucofuranose contributed to the conductivity change but neither β-D-glucofuranose nor the pyranose show the conductivity change.

All classes of monohydroxy compounds and β-hydroxy and γ-hydroxy acids do not form stable borate complex in aqueous solution. Proteins, which do not contain cis, vicinal dihydroxy component or α-hydroxy acids, therefore, are not expected to form complexes with borate ion. This leads to the conclusion that the borate control of metabolism is not based on the interaction between an enzyme and borate per se, but rather on the interaction of the substrate with borate. The particular attention must be paid therefore to those substrates or products that are capable of forming stable complexes.

b. Interpretation of the causes of various metabolic alterations in B− leaves Numerous metabolic changes observed in B− sunflower leaves were stated in the results of isotope feeding experiments. Some of these alterations are expected to be the immediate results of boron deficiency
while others may arise from the influence of the primary metabolic changes. In the following discussion, the nature of these changes and their mutual influences will be considered. Finally, hypotheses on the modes of boron action on metabolism will be proposed.

1) Glucose and hexose-6-phosphate  The accumulation of glucose and the low level of hexose-6-phosphate in B- leaves have already been attributed to the reduced availability of ATP in B- leaves. Another cause for this alteration may be attributed to the strong inhibition of acid phosphatase activity by α-hydroxy acids, which was found by Adul-Fadl and King (1) and later confirmed by many other investigators (27, 61). It was shown earlier in Table 10 that the concentration of citric acid, an α-hydroxy acid, is much lower in B- leaves. Thus the activity of acid phosphatase is expected to be greater in B- leaves, resulting in faster conversion of glucose-6-phosphate to glucose. This may be shown as below.

\[
\text{Glucose-6-phosphate} \quad \frac{\text{faster in B- leaves because of low level of ATP available}}{\text{faster in B- leaves because α-hydroxy acid inhibition of phosphatase is reduced}} \quad \text{Glucose}
\]

According to the scheme shown above, the accumulation of glucose and the decrease in the concentration of hexose-6-phosphate are indirect results of boron deficiency. Boron has been shown to have no effect on hexokinase activity (16), but shown by Ito et al. (24) to have some inhibitory effect on acid phosphatase. This inhibition on phosphatase activity is a direct control of boron on a metabolism. However, its
biological significance is questionable because of extremely low inhibitory effect of borate. Their results show that when $2 \times 10^{-3}$ M $\alpha$-glycerophosphate or ATP was used as substrate, $2 \times 10^{-3}$ M borate inhibits the phosphatase activity only by 9% and 6% respectively. This concentration of borate is about ten times greater than the cellular concentration of borate. Moreover, the borate concentration in B-leaves is not expected to be any less than about 90% of that in B+ leaves since during the 24 hour period in which B-plants were grown in B-solution, the leaves did not grow any more than by 10%.

2) Oxidative decarboxylation It was shown that pyruvic acid (Table 11) and $\alpha$-ketoglutaric acid (Table 9) accumulate in B-leaves while malic acid and citric acid, and possibly isocitric acid, accumulate in B+ leaves, and it was proposed that the increased rate of oxidative decarboxylation is responsible for the altered equilibrium concentrations of these metabolites. The low level of citric acid or the accumulation of $\alpha$-ketoglutaric acid in B-leaves may be due either to the increased activity of aconitase or to that of isocitric dehydrogenase. The latter is considered more important because the former involves ferrous ion, which can form a much stronger citrate complex than borate, and dehydration-hydration reactions while the latter involves dehydrogenase action. This will be further discussed. The two reactions, malic acid to pyruvic acid and isocitric acid to $\alpha$-ketoglutaric acid, are similar in that both reactions use $\alpha$-hydroxy acid as their substrates, require NADP for oxidative decarboxylation, and the products are $\alpha$-keto acid. The supposition that the accumulation of $\alpha$-ketoglutaric acid in B-leaves is
the result of the increased activity of NADP-isocitric dehydrogenase rather than that of NAD-isocitric dehydrogenase is based on the following argument. While α-ketoglutaric acid accumulates in B- leaves, succinic and glutamic acids, which arise directly from α-ketoglutaric acid, are found less in B- leaves than in B+ leaves (Table 8). One logical explanation for this is that of compartmentalization of α-ketoglutaric acid pools. It is well known that NAD-isocitric dehydrogenase is a mitochondrial enzyme whereas NADP-isocitric dehydrogenase is a cytoplasmic enzyme. The activation of this cytoplasmic NADP-isocitric dehydrogenase in B- leaves will cause an overall increase in the rate of conversion of isocitric acid to α-ketoglutaric acid. However, the excessive α-ketoglutaric acid formed in the cytoplasm cannot be accessible to α-ketoglutaric dehydrogenase or glutamic dehydrogenase, and therefore the levels of both succinic acid and glutamic acid are lower in B- leaves even though the level of α-ketoglutaric acid in these leaves is much higher than that in B+ leaves.

It was mentioned earlier that borate itself is not likely to interact with proteins because proteins do not possess the necessary vicinal, cis-dihydroxy group or α-hydroxy acid. The borate control of oxidative decarboxylation, therefore, does not appear to be the direct control on the enzymes by borate ion per se. Borate, however, is capable of forming complexes with α-hydroxy acids, the substrates of the oxidative decarboxylation reactions. Therefore, it is likely that these complexes, rather than borate itself, are species that interact with the enzymes. The reaction velocities of these enzyme reactions may be influenced by the complex formation between substrates and borate even if the complexes do
not interact with the enzymes. If free acids serve as the substrates for
the enzymes and the complexes cannot, the reduction in the free acid
concentrations as a result of complex formation, will in turn result in
the reduced velocities of these enzyme reactions. Since B+ leaves contain
somewhat larger amounts of borate than B- leaves do, the concentrations of
these complexes should be somewhat greater, and that of free acids should
be somewhat lower in B+ leaves as compared to those in B- leaves. The
concentrations of these complexes are, however, very small compared to
those of free acids, since, at a given hydrogen ion concentration, the
concentrations of these complexes are proportional to the products of molar
concentrations of free acids and those of borate, which themselves are
thought to be of the order of $10^{-4}$ M to $10^{-3}$ M. With a value of $10^{-7}$ for
formation constant of a complex and at neutral pH, the concentration of
this complex would be of the order of $10^{-7}$. It may be concluded, therefore,
that the concentrations of free acids are practically unaffected by the
complex formations. Further, it may also be concluded that the control of
oxidative decarboxylation by the α-hydroxy acid-borate complexes is not a
result of reduction of substrate concentrations, but is a result of the
interaction of these complexes with the enzymes.

From the foregoing argument, the following hypothesis is proposed.
The borate complexes of free acids which serve as substrates for the NADP-
linked dehydrogenases, and can bind with the respective enzymes and behave
as inhibitors.
This may be shown schematically as below.

\[
\begin{align*}
&\text{inhibited by} \quad \text{HOCH}_2^+ - \text{CH} - \text{COO}^- \\
&\text{HOCH} + \text{NADP} + \text{HCH}_3^+ \quad \text{CH}_3 + \text{CO}_2 + \text{NADPH}
\end{align*}
\]

Some experimental evidences supporting this hypothesis will be presented in Part IV of this thesis.

According to the foregoing argument, the alteration of the rates of oxidative decarboxylation of α-hydroxy acids is a direct and immediate result of boron deficiency.

The apparent increase of NADP-isocitric dehydrogenase and NADP-malic dehydrogenase activity may be explained equally well with an entirely distinct but mechanistically similar model. In this model, borate is involved in the carrier and active transport of α-hydroxy acids across the mitochondrial membrane. Since the α-hydroxy acids are produced in mitochondria, they must be transported out into the cytoplasm in order to be acted upon by cytoplasmic dehydrogenases. It may be visualized that borate complexes of these acids are firmly bound to the carrier molecules and are unable to dissociate. Since a greater number of carrier molecules are expected to be tied down by borate complexes of
α-hydroxy acids in B+ leaves as a result of higher level of borate, the rate of α-hydroxy acid transport into cytoplasm is expected to be slower in B+ leaves. Consequently, a lesser amount of α-hydroxy acids will be available to the cytoplasmic, NADP-linked dehydrogenase.

A similar result may be expected if borate of α-hydroxy acids are incapable of binding with carrier molecules. These two possibilities are shown schematically in Figures 23a and 23b.

In the foregoing, attention was paid to the formation of borate complexes of α-hydroxy acids and their possible influence on the metabolism of free α-hydroxy acids. The possibility of the formation of borate-coenzyme complex must also be considered. Both NAD and NADP contain two ribose molecules, and ribose possesses vicinal, cis-hydroxy groups capable of forming complex with boric acid. Therefore, there is the possibility that it is borate complex of NADP that inhibits the dehydrogenase activities.
Figure 23a. Borate-substrate complex cannot dissociate from carrier

Figure 23b. Borate-substrate complex cannot bind with carrier
3) **Accelerated relief of inhibition**  

One intriguing question yet to be answered is how could such profound changes in the equilibrium concentrations of metabolites or in the reaction velocities of the aforementioned dehydrogenase be possible when the actual difference between B+ and B- leaves in the concentration of borate, and hence that of the corresponding complexes is slight. Normally the reaction velocity of an enzyme increases when substrate-concentration increases and therefore the accumulation of intermediary metabolites is prevented in a chain of reactions. Consequently, the accumulation of large amounts of citric or malic acid in B+ leaves requires a special mechanism.

The large accumulation of these acids in B+ leaves may be explained with the hypothesis proposed earlier. At early hours, after the transfer of sunflower plants to B- solution, the borate concentration in these incipient B- leaves is expected to be only slightly less than that in B+ leaves. Since the concentration of borate and those of the α-hydroxy acids, B- leaves are expected to contain slightly less amounts of these borate complexes. Consequently, the dehydrogenases in B- leaves are subjected to slightly less inhibition, resulting in a slightly faster conversion of these α-hydroxy acids to α-keto acid products or slightly faster diminution of α-hydroxy acids. The slight diminution in the α-hydroxy acid concentrations in the above process will diminish the borate-α-hydroxy acid concentrations, or inhibitor concentrations, further. Therefore, the reaction velocities also will be increased further. The continuation of this process for a number of times will ultimately result in a profound decrease in the α-hydroxy acid concentration and increase in α-keto acid products in B- leaves.
The novelty of the hypothesis stated earlier, namely, that borate $\alpha$-hydroxy acids, rather than borate per se, are actual inhibitors of the NADP-linked dehydrogenase reactions is that the profound alterations in the equilibrium concentrations of these reversible reactions cannot be explained by any other plausible mechanisms.

The concept of accelerated relief of inhibition may also be applicable to the carrier hypothesis.

4) **Cleavage and condensation of C-C bond**

a) **Isocitric lyase** The storage of sunflower leaves in the dark for 3 hours causes an accumulation of glyoxylic acid with a concomittant decrease in the level of malic acid (cf. Table 8 and 12). Upon longer storage of these leaves in the dark, both glyoxylic acid and citric acid accumulate with further decrease in malic acid (Table 10). These results are illustrated in Figure 24. If an assumption is made that aconitase activity does not show circadian oscillation, the accumulation of glyoxylic acid in the dark could result if either isocitric lyase is activated or malate synthetase is inactivated. If the former is responsible, one may expect that the overall activity of the "glyoxylic acid cycle" is high in the dark. On the other hand, if the inactivation of malate synthetase is responsible, the overall activity of the "glyoxylic acid cycle" would be expected to be slow. One of the results of the activation of the "glyoxylic acid cycle" is a rapid conversion of isocitric acid to malic acid. This is illustrated in Figure 25. In the complete absence of this cycle, the conversion of isocitric acid to malic acid is achieved through path (3), whereas both paths (1) and (2) will also contribute to the conversion of isocitric acid to malic acid if
glyoxylic acid cycle operates. Therefore, the activity of glyoxylic acid cycle will be reflected in the equilibrium concentrations of malic acid and isocitric acid or citric acid. The accumulation of citric acid and the low amount of malic acid in the dark suggest, therefore, that glyoxylic acid cycle is not appreciably active or may be completely inactive in the dark. The accumulation of glyoxylic acid will, however, decrease isocitric lyase activity eventually as equilibrium is reached in the concentrations of glyoxylate and isocitrate.

The oxidation of two molecules of acetyl coenzyme A via the "Tricarboxylic Acid Cycle" (TCA) will produce 4 CO₂ and 24 ATPs. On the other hand, in a complete turn of glyoxylic acid cycle, two molecules of acetyl coenzyme A will produce either one molecule of oxalacetic acid and 8 ATPs or regenerate back one molecule of acetyl coenzyme A and produce 2 CO₂, 5 ATPs and one molecule of NADPH. These are shown below:

\[
\begin{align*}
    \text{acetyl coA} + \text{oxalacetate} & \rightarrow \text{citrate} + \text{coA} \\
    \text{citrate} & \rightarrow \text{isocitrate} \\
    \text{isocitrate} & \rightarrow \text{glyoxylate} + \text{succinate} \\
    \text{succinate} + 2 \text{ADP} + 2 \text{Pi} & \rightarrow \text{fumarate} + 2 \text{ATP} \\
    \text{fumarate} & \rightarrow \text{malate} \\
    \text{glyoxylate} + \text{acetyl coA} & \rightarrow \text{malate} + \text{coA} \\
    2 \text{malate} + 6 \text{ADP} + 6 \text{Pi} & \rightarrow 2 \text{oxalacetate} + 6 \text{ATP} \\
    \text{2 acetyl coA} + 8 \text{ADP} + 8 \text{Pi} & \rightarrow \text{oxalacetate} + 8 \text{ATP} + 2 \text{coA}
\end{align*}
\]
Figure 24. The accumulation of glyoxylic acid and citric acid, and concomitant decrease in the level of malic acid in the leaves stored in the dark, as per cent radioactivities of the total activity in soluble fraction.

\[= \text{citric acid}\]
\[= \text{glyoxylic acid}\]
\[= \text{malic acid}\]

Figure 25. A possible cause of the accumulation of glyoxylic acid in the dark
ACTIVATION OF ISOCITRIC LYASE?

INACTIVATION OF MALATE SYNTHETASE?

ACTIVATION OF ACONITASE?

ACETYL COA

GLYOXYLATE

CO A

MALATE

CITRATE \rightarrow ISOCITRATE

\alpha-KETOGLUTARATE \rightarrow SUCCINATE \rightarrow FUMARATE

SUCCINATE \rightarrow FUMARATE

SUCCINATE \rightarrow FUMARATE

CO A

MALATE

\text{\textbf{% OF TOTAL RADIOACTIVITY}}

\text{\textbf{LIGHT}}

\text{\textbf{DARK 3 HRS.}}

\text{\textbf{DARK 30 HRS.}}
Thus it can be seen that the operation of glyoxylic acid is conducive to the increase of pool sizes or of the accumulation of the intermediary organic acids of the TCA cycle, but not to the production of ATP, while the operation of TCA cycle will produce ATP more efficiently but will not increase the pool sizes of the intermediary carboxylic acids.

It is known that both photosynthesis and protein synthesis (30, 38) occur in high plants in the presence of light. In the presence of light, the supply of carbohydrate is presumably abundant. At the same time, the utilization of amino acids, including keto acids such as α-ketoglutaric acid and oxalacetic acid, will also increase because of active protein synthesis. The draining of these α-keto acid intermediary metabolites of the TCA cycle, will eventually deplete oxalacetic acid, which is necessary for the continuation of the cycle, unless the production of organic acids is supplemented in some other way. The operation of the glyoxylic acid cycle is one effective way to supply oxalacetic acid and compensate the excessive drainage of organic acids during the active synthesis of proteins.

In the dark, however, the (light-activated) protein synthesis ceases and as a result the draining of α-keto acids will also be discontinued. The operation of glyoxylic acid cycle is, therefore, not only unnecessary but may be harmful, since the pool size of carboxylic acid intermediary metabolites will tend to increase autocatalytically. Moreover, the cessation of photosynthesis in dark will result in the dwindling of the supply of carbohydrate. The synthesis of macromolecules may also diminish, as the available carbohydrate may be used mainly for energy production. This is achieved most effectively by the operation of TCA cycle and inoperation of glyoxylic acid cycle during the night.
Although teleological in nature, the above arguments also support the view that the glyoxylic acid cycle is inactive in the dark and the accumulation of glyoxylic acid in leaves stored in the dark is a result of the inactivation of malate synthetase and not a result of the activation of isocitric lyase.

It was shown in Tables 11 and 8 and also in Figure 24 that the glyoxylate/citrate ratio is very much higher in B- leaves than in B+ leaves. Assuming that malate synthetase activity was greatly reduced in the leaves stored in the dark for 3 hours and completely inactivated in the leaves stored in the dark for 30 hours, the ratio of glyoxylate to citrate reveals the quasi-equilibrium concentrations of these metabolites, or the ratio of the rate of forward reaction to the rate of backward reaction. If an assumption is made that aconitase activity is not influenced by borate, it may be concluded that isocitric lyase activity or the rate of cleavage of isocitrate to glyoxylate and succinate, is higher in B- leaves than in B+ leaves. Alternatively, the backward reaction, i.e. the condensation reaction, is faster in B+ leaves than in B- leaves. The equilibrium for this reaction, is faster in B+ leaves than in B- leaves. The equilibrium for this reaction is far toward the cleavage of isocitrate and therefore, the former situation is expected to be more meaningful. At $10^{-4}$ M each of glyoxylate and succinate, the concentration of isocitrate is about $3 \times 10^{-6}$ according to the literature (53).

The boron control mechanism proposed previously for NADP-linked dehydrogenases may be applicable to isocitric lyase. Thus, while free isocitric acid serves as a substrate for isocitric lyase, the borate
complex of isocitrate behaves as an inhibitor of this enzyme, illustrated below:

\[
\text{Enz + Isocitrate} \xrightleftharpoons{\text{Succinate}} \text{Enz-Isocitrate} \xrightleftharpoons{\text{Enz + Glyoxylate}} \text{Glyoxylate} \xrightarrow{\text{No Rx}} \text{Succinate}.
\]

The assumption that aconitase activity is not influenced by borate is based on the \textit{a priori} proposition made earlier that isocitric acid is partitioned favorably to the cytoplasmic NADP-isocitric dehydrogenase rather than the mitochondrial NAD-isocitric dehydrogenase. If aconitase activity, and not isocitric dehydrogenase activity, is influenced by borate, then both NAD-isocitric dehydrogenase and NADP-isocitric dehydrogenase activity will be influenced by borate to roughly similar extents. Evidence was shown earlier that the activities of these two enzymes were influenced by borate differently. However, if the \textit{a priori} proposition was incorrect, the accumulation of citric acid in B+ leaves may be the result of the inhibition of aconitase by citrate-borate complex. Nevertheless, the overall conclusion that glyoxylic acid cycle is more active in B- leaves would still be valid.

It is pertinent to recall the earlier conclusion that NADP-linked dehydrogenase activities are higher in B- leaves. The examination of Table 18 suggests that the conclusion is valid only for leaves stored in the dark, but not for those exposed to light.
Table 18. Comparison of the ratios of various related metabolites

<table>
<thead>
<tr>
<th>Relation</th>
<th>Exposed to light</th>
<th>Stored in dark</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>B⁺</td>
<td>B⁻</td>
<td>B⁻/B⁺</td>
</tr>
<tr>
<td>Citric acid</td>
<td>0.39</td>
<td>0.30</td>
<td>0.78</td>
</tr>
<tr>
<td>Malic acid</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pyruvic acid</td>
<td></td>
<td></td>
<td>0.72</td>
</tr>
<tr>
<td>Oxalacetic acid</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alanine</td>
<td>0.21</td>
<td>0.17</td>
<td>0.81</td>
</tr>
<tr>
<td>Malic acid</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

^ Obtained for young leaves, exposed to 400 watts for 2 hours, placed 40 cm above the leaves.

For the leaves stored in the dark, the pyruvate/oxalacetate ratio and the alanine/malate ratios for B⁻ leaves are 2.5 times greater than those of B⁺ leaves, suggesting that these enzyme activities are greater in B⁻ leaves than in B⁺ leaves. But for the leaves exposed to light, these ratios suggest that these enzyme activities are lower in B⁻ leaves than in B⁺ leaves. This apparent reversal may be due merely to the over-activation of glyoxylic acid cycle in B⁻ leaves in the light. The higher rate of malic acid supply in B⁻ leaves provided by glyoxylic acid in the presence of light, would more than compensate the higher rate of the loss of malic acid by NADP-malic dehydrogenase activity in these leaves. In the absence of light, no such compensation can be made since the glyoxylic acid cycle becomes dormant. The faster diminution of malic acid in B⁻ leaves as a result of higher activity of the dehydrogenase is revealed in the high alanine/malic acid ratio, once the masking effect by glyoxylic acid pathway is removed.
b) Other TCA cycle enzymes  The boron-controlled inhibition mechanism of this type may be further extended to other similar enzyme reactions. The reverse reactions of malic synthetase and the condensing enzyme are similar to isocitric lyase in that all three reactions involve the cleavage of α-hydroxy acids (where the length of the arrow indicates the approximate rate of the reaction in each direction):

Isocitric lyase

\[
\begin{align*}
\text{HOCH-COOH} \\
\text{HC-COOH} \\
\text{HCH-COOH}
\end{align*}
\xrightarrow{\text{Isocitric lyase}}
\begin{align*}
\text{COOH} \\
0=\text{CH} \\
\text{CH}_2\text{COOH}
\end{align*}
\]

Malic synthetase

\[
\begin{align*}
\text{HOCH-COOH} \\
\text{CH}_2 \\
\text{COOH}
\end{align*}
\xrightarrow{\text{Malic synthetase}}
\begin{align*}
\text{COOH} \\
0=\text{CH} \\
\text{CH}_3 \\
\text{COO-CoA}
\end{align*}
\]

Condensing enzyme

\[
\begin{align*}
\text{CH}_2\text{-COOH} \\
\text{HOC-COOH} \\
\text{CH}_2\text{-COOH}
\end{align*}
\xrightarrow{\text{Condensing enzyme}}
\begin{align*}
\text{CH}_2\text{COOH} \\
0=C-\text{COOH} \\
\text{CH}_3 \\
\text{COO-CoA}
\end{align*}
\]

If the argument employed for isocitric lyase is applicable to both malic synthetase and condensing enzyme, the rates of cleavage of these α-hydroxy acids would be faster and conversely the rate of condensation would be slower in B- leaves. Unlike isocitric lyase, the equilibria of these two reactions lie toward condensation rather than cleavage. The influence of boron deficiency on these two reactions may be, therefore, less profound than that on isocitric lyase. The full significance of
boron control on these two enzymes can be appreciated only after further experiments. Argument was made earlier that the overall rate of the glyoxylic acid pathway is faster in B- leaves than in B+ leaves. Therefore the acceleration of the pathway by increased isocitric lyase activity must be greater than the retardation of the pathway by the increased cleavage reaction of the malate synthetase in B- leaves.

c) Aldolase and fructose-6-phosphate 1-kinase It was shown earlier that both glucose-6-phosphate and fructose-6-phosphate accumulate in B+ leaves (Table 12 and 16) while various three-carbon compounds accumulate in B- leaves. A possible reason for this is the higher activity of aldolase in B- leaves. A possible reason for this is the higher activity of aldolase in B- leaves. Fructofuranose, unlike glucopyranose, is capable of forming a complex with borate. Formation of the complex appears to be possible even when carbons 1 and 6 of fructofuranose are phosphorylated:

![Chemical structure](image)

As in the previous arguments, the borate complex of fructose-1,6 phosphate may behave as an inhibitor of aldolase action. The borate control of aldolase activity is somewhat analogous mechanistically to that of isocitric dehydrogenase since both of these reactions involve the cleavage of C-C bond.
If the slower rate of conversion of fructose-6-phosphate to glyceraldehyde-3-phosphate in B+ leaves is indeed due to the slow rate of the aldolase reaction, the possibility exists that all similar classes of reaction will be slow in B+ leaves. For example, transaldolase and transketolase reactions involve the cleavage of C-C bonds of various sugar phosphates which are capable of forming complexes with borate.

5) Consequences of altered metabolism of organic acids

a) Reduction in the ATP generation

It was mentioned earlier that a complete cycling of two molecules of acetyl coenzyme A through the TCA Cycle produces 4 CO₂ and 24 ATPs, whereas a complete cycling of two molecules of acetyl coenzyme A through the "glyoxylic acid cycle" produces one molecule of oxalacetic acid and 8 ATPs. If malic acid is oxidatively decarboxylated by NADP-malic dehydrogenase to pyruvic acid, and this, in turn, is converted back to acetyl coenzyme A, the "glyoxylic acid cycle" will produce 2 CO₂s, 5 ATPs, and one NADPH for each molecule of acetyl coenzyme A consumed. It is clear therefore that B- leaves will produce less ATP. Moreover, the preferential utilization of NADP over NAD in the oxidation of α-hydroxy acids in B- leaves will lower the level of ATP still further since NADPH produced in cytoplasm is not likely to be coupled with ATP generation as efficiently as NADH produced in mitochondria.

The above conclusion, derived from theoretical considerations, agrees well with the actual results of ³²P feeding experiments and ⁴⁵Ca uptake by chloroplasts.
b) **Protein synthesis and the level of soluble metabolites**

In the presence of light, the accelerated "glyoxylic acid cycle" is the most prominent feature in the altered metabolism of organic acids in B- leaves. As this cycle is conducive to the enlargement of the pool size of organic acids and related amino acids, the level of the "soluble fraction" in B- leaves is expected to be higher. This prediction agrees well with the experimental results (Table 6 and 7). In the dark, however, the "glyoxylic acid cycle" is not operational, and thus NADP-linked oxidative decarboxylation is most prominent among the altered metabolisms of organic acids in B- leaves.

The consequence of the activation of these cytoplasmic, NADP-linked dehydrogenases may be a diminution of the pool size of organic acids since the channelling off of α-hydroxy acids into cytoplasm by the action of these dehydrogenases will result in a diminution of the supply of oxalacetic acid necessary for the continuation of the TCA Cycle. Therefore, the level of soluble metabolites will be lower in the dark in B-, than in B+ leaves.

The reversal of the diurnal-nocturnal variation of the level of soluble metabolites resulting from boron deficiency is expected to influence the physiology as well as biochemistry of boron deficient leaves. Young, growing leaves normally receive organic compounds, notably sugars, from mature leaves. The more-than two-fold increase of soluble metabolites (and hence the chemical potentials of these metabolites) will probably alter the transport of organic metabolites into these young leaves since the transport, especially the passive transport of organic compounds, is related to the chemical potentials on
both sides of the cellular membrane. It is not unlikely that the B-
young leaves export, rather than import, various organic compounds to
mature leaves during the day time.

At night, the shortage of ATP may retard the rate of active
transport of minerals into the B- young leaves.

The first step of protein synthesis involves the activation of amino
acids by ATP. The low level of ATP in B- leaves is expected to limit the
rate of amino acid activation, and hence, protein synthesis. The
evidence for a lower rate of protein synthesis was shown in Table 7.

c) High level of NADPH in B- leaves One result of the
activation of NADP-linked dehydrogenases is over-production of NADPH. The
high availability of NADPH may increase the rate of various syntheses
requiring NADPH. The higher rate of \(14\text{C}\) incorporation into lipid
material observed indirectly in B- leaves may be the result of the high
NADPH level in these leaves. The generation of tetrahydrofolic acid, the
immediate precursor of \(N^{10}\)-formyl tetrahydrofolic acid—which is the
formyl transfer agent in purine biosynthesis—requires NADPH also.

d) The reduction in the level of citric acid in B- leaves

The reduction in the level of citric acid may facilitate the
hydrolysis of various phosphorylated compounds, including ATP, by acid
phosphatase. The inhibition of acid phosphatase activity by \(\alpha\)-hydroxy
acids was mentioned earlier.

6) The interpretation of the results of other investigators

a) Sucrose permeability As mentioned earlier, Gauch
and Dugger showed that lima bean and pea root tips exhibit a higher rate
of respiration when incubated with sucrose plus borate than when incubated
with sucrose alone. They attributed the augmented respiration to an increased transport of sucrose across the plasma membrane in the presence of borate. Their interpretation seems to be wrong for the following reasons. i) Sucrose cannot penetrate the chloroplast membrane, as shown in the experiment of $^{14}$C-sucrose uptake by chloroplasts. Animal mitochondria are known to be impermeable to sucrose (34), and it is likely that plant mitochondria are also impermeable to sucrose. ii) Sucrose does not have the ability to form complexes with borate, as shown earlier. Therefore, if the membranes of those organelles are "typical", borate is not likely to influence the transport of sucrose across membranes in any way.

The augmented respiration in the presence of borate can be explained by the \textit{a priori} conclusions made earlier in this experiment. It was mentioned that isocitric acid metabolism is directed toward the glyoxylic acid cycle in B-leaves, and the consumption of two molecules of acetyl coenzyme A results in the production of one molecule of oxalacetic acid if they are channeled through this glyoxylic acid pathway, whereas two molecules of acetyl coenzyme A will produce 4 CO$_2$ if channeled through TCA Cycle. Therefore, the augmented respiration observed by these workers in the presence of borate is not because of a larger amount of sucrose transported into the tissue but because of the increased concentration of borate in these tissue and the resultant metabolic vectoring of organic acids to the TCA Cycle.

b) \textbf{Increased acid phosphatase activity} The higher activities of acid phosphatase and ATPase observed by Hinde and Finch (21) in the soluble fraction of B- \textit{Vicia faba} root tips may be due to the low
level of α-hydroxy acids, the inhibitors of acid phosphatase, in B-leaves.

7) **Autocatalytic acceleration of phenol synthesis in B-leaves and its consequences** Particular attention must be paid to phenol metabolism in relation to the roles of boron in higher plants. A survey of past literatures reveals that those plants in which phenol metabolism is naturally active are extremely sensitive to boron deficiency whereas those plants in which phenol metabolism is not so active are usually less sensitive to boron deficiency. The examples of the former are sunflower, tobacco, tomato and potato plants. An example of the latter is the soybean plant. Thus, sunflower plants take 4 days of growing in B-nutrient solution to show visual boron deficiency symptoms, soybean plants require a month or more of growing in B-nutrient solution to develop boron deficiency symptoms.

It was shown earlier that chlorogenic acid accumulates in the presence of light (Table 8) and phenylalanine accumulates in the dark (Table 10) in B- sunflower leaves. It was also shown that α-hydroxy phenols are by far the best ligands for the formation of borate complexes. It is therefore likely that the complexing ability of phenols is related to the accumulation of chlorogenic acid and phenylalanine in B-leaves. This assumption is particularly interesting in view of the fact that the previously postulated hypothesis, namely the inhibition of enzyme activities by borate-substrate complexes, can be applied to explain the accumulation of phenols. Figure 25 shows the metabolic pathway leading to the formation of chlorogenic acid and phenylalanine, and the possible sites of enzyme inhibition by borate-substrate complexes. According to
this scheme, the production of erythrose-4-phosphate will be faster in B-leaves as a result of reduction in 6-phosphogluconate-borate complex and ribose-5-phosphate-borate complex in B-leaves. 6-phosphogluconic dehydrogenase activity is particularly expected to be higher in B-leaves since this enzyme is similar to NADP-isocitric dehydrogenase and NADP-malic dehydrogenase, whose increased activities in B-leaves were mentioned earlier. Thus the substrate for this enzyme, 6-phosphogluconic acid is an α-hydroxy acid and the reaction is an oxidative decarboxylation involving NADP. Transaldolase and transketolase actions, on the other hand, are analogous to isocitric lyase since the cleavage of C-C bond is involved in these reactions.

The conversion of 5-dehydroquinic acid to 5-dehydroshikimic acid may also be faster since 5-dehydroquinic acid is an α-hydroxy acid. The increased rates of various reactions in B-leaves that lead to the synthesis of 5-dehydroshikimic acid will, in turn, cause a faster syntheses of phenylalanine and chlorogenic acid. If the rate of utilization of these products does not increase at a same pace, these products will accumulate in B-leaves.

Unlike α-hydroxy acids or sugars, the amount of borate bound to α-hydroxy phenol-borate complexes is not negligible compared to the amount of free borate. The ratio of free borate to chlorogenic acid-borate complex, for example, approaches to 1:1 at neutral pH and $10^{-3}$ M free chlorogenic acid as based on the formation constant of this complex, which will be shown in Part IV of this thesis. Thus the reduction in the concentration of free borate in B-leaves as a result of over-production of chlorogenic acid in B-leaves, although by a small amount initially,
will result in the increase of the concentration of the complex and in the
decrease of free borate. Consequently, a lesser amount of borate is
available for the formation of α-hydroxy acid-borate complexes, which, in
turn, will result in even faster synthesis of chlorogenic acid. The
continuation of this autocatalytic acceleration of chlorogenic acid
synthesis will result in a significant reduction of the concentration of
free borate. Thus when the amount of total borate in B- leaves is as high
as 95% of that in B+ leaves, the amount of free borate in B- leaves may
be 70% or less of that in B+ leaves.

The accumulation of phenolic compounds may activate various phenol
oxidases. Phenols combine with proteins reversible by hydrogen bonding,
while the oxidized phenols, or quinones, condense with proteins
irreversibly by covalent bonding (30). The extreme sensitivity of various
phenol producing plants to boron deficiency may probably due to this
inactivation of enzymes by phenols. Thus the control of phenol synthesis
is probably the most important role of boron in the phenol-producing plants.

As a possible cause of rapid phenol synthesis in B- leaves, still
another factor may be considered. It was shown earlier that B- leaves
contain a lesser amount of fructose-6-phosphate and a larger amount of
glyceric acid and possibly glyceraldehyde-3-phosphate compared to B+
leaves. Then the equilibrium concentration of erythrose-4-phosphate, an
immediate precursor of shikimic acid pathway, is expected to be higher in
B- leaves. This may be seen from the following transketolase reaction.

Glyceraldehyde-3-P + Sedoheptulose-7-P $\rightleftharpoons$ Fructose-6-P + Erythrose-4-P

In the above reaction, the larger amount of glyceraldehyde-3-phosphate
and lesser amount of fructose-6-phosphate in B- leaves will force the
reaction to the left, causing rapid formation of erythrose-4-phosphate.

It is also very likely that the concentration of phosphoenolpyruvate is high in B- leaves since, as mentioned earlier, alanine, serine, glyceral acid and pyruvic acid accumulate in B- leaves. The accumulation of phosphoenolpyruvate and a rapid formation of erythrose-4-phosphate may therefore be a cause of the activation of shikimic acid pathway and the accumulation of phenols in B- leaves. This argument is valid only if the concentrations of these two precursors of phenols are limiting factors of phenol synthesis.
Figure 26. The metabolic pathway leading to the formation of chlorogenic acid and phenylalanine, and possible sites of enzyme inhibition by substrate-borate complexes.
IV. SUBSTANTIATIVE INVESTIGATIONS

THE INFLUENCE OF BORON ON THE ACTIVITIES OF 6-PHOSPHOGLUCONIC DEHYDROGENASE AND VARIOUS C-C BOND CLEAVAGE ENZYMES

It was shown earlier that phenolic compounds such as chlorogenic acid and phenylalanine accumulate in B-leaves and their accumulation was attributed to the release of the borate inhibition on the activities of various enzymes of the pentose phosphate shunt and the shikimic acid pathway. As possible candidates of such enzymes, 6-phosphogluconic dehydrogenase, transketolase, transaldolase and aldolase were mentioned. The influence of borate on the activities of these enzymes, studied with cell-free systems, are presented in this chapter.

A. Materials and Methods

1. Syntheses of radioactive substrates

   a. Glucose-6-phosphate-UL-^{14}C

      Uniformly $^{14}$C-labelled glucose-6-phosphate was made by reacting glucose-6-phosphate-UL-^{14}C and ATP with yeast hexokinase purchased from Calbiochem Company. The reaction mixture contained the following (in micromoles): glucose-$^{14}$C, 0.25 (50 μc; ATP, 0.4; MgCl$_2$, 2.0; glycylglycin, 5; and about 5 μg of hexokinase. The reaction mixture volume was 1.0 ml and the pH about 7.5, as estimated by pH paper. The reaction was carried out for four hours and the reaction products separated by means of a paper electrophoresis procedure to be described in detail later.

   b. Glucose-6-phosphate-C-1-^{3}H

      Glucose-6-phosphate labelled with tritium at carbon 1 position was made from corresponding H-labelled glucose by the manner identical to the above.
c. 6-phosphogluconic acid-UL-C-14  6-phosphogluconic acid-UL-C-14 was prepared by reacting glucose-6-phosphate-UL-C-14 and NADP with glucose-6-phosphate dehydrogenase purchased from Calbiochem. The reaction mixture contained approximately 0.1 micromole of glucose-6-phosphate-UL-C-14, 0.3 micromoles NADP, 2.0 micromoles MgCl₂, 5.0 micromoles glycyl-glycine and about 10 µg of glucose-6-phosphate dehydrogenase. The reaction volume was 1.0 ml and the pH of original glycylglycine buffer was 8.0. After reacting for 6 hours the reaction mixture was separated by means of paper electrophoresis.

2. Preparation of enzymes
   a. 6-phosphogluconic dehydrogenase  20 to 50 gm of expanding sunflower leaves were homogenized with a porcelain mortar and pestle with the aid of acid-washed sand in 60 to 150 ml, pH 6.2 buffer of 0.05 M citrate, 0.2 M ascorbate and acid-washed charcoal, and 0.05 gm/ml of buffer. The pH of the homogenate usually increased to 6.3 to 6.5 due to exceptionally high pH of sunflower leaf tissue. The homogenate was filtered through fine glass wool and the filtrate centrifuged at 12,000 g for 20 minutes. Most of the charcoal was precipitated together with unbroken cells, chloroplasts and mitochondria. The supernatant was mixed with one-fifth its volume of protamine sulfate solution (10 mg/ml) and the mixture centrifuged at 12,000 g for 20 minutes. The precipitate was discarded and 0.27 g of ammonium sulfate was added per ml of supernatant. The mixture was centrifuged as above and the precipitate formed was discarded. To the supernatant an additional 0.05 gm of ammonium sulfate was added and the mixture centrifuged. The supernatant was discarded and the precipitate dissolved in 2 to 5 ml of 0.1 M glycylglycine buffer of
pH 8.0. The protein solution was diluted to 100 to 250 ml with distilled water and to this 0.1 ml of calcium phosphate gel (7 mg/ml) to every ml of the diluted protein solution. The mixture was centrifuged and the precipitate formed was discarded. The supernatant was made 50%-saturated with ammonium sulfate and the mixture centrifuged. The supernatant was discarded and the precipitate was dissolved in 1 to 2 ml of 0.1 M glycylglycine buffer of pH 7.8. This second ammonium sulfate fraction was diluted to desired volume with distilled water and used as the source of 6-phosphogluconate dehydrogenase. The protein content of the partially purified 6-phosphogluconate in the reaction mixture was measured by Folin's method.

b. Various enzymes of the pentose shunt phosphate pathway

20 gm of expanding sunflower leaves were homogenized, filtered, centrifuged and treated with protamine sulfate in a manner identical to the procedure described for the preparation of 6-phosphogluconate dehydrogenase, except that the buffer for homogenation contained $10^{-4}$ M thiamine pyrophosphate. The protamine sulfate treated supernate was 55% saturated with ammonium sulfate and the precipitate was collected by centrifugation. The precipitate was dissolved in 1 ml of 0.1 M glycylglycine buffer and then diluted to 20 ml with distilled water. This dilute enzyme preparation was used in various experiments.

3. Borate control of the activities of 6-phosphogluconate dehydrogenase and other enzymes of the pentose phosphate pathway

a. Borate control of 6-phosphogluconic dehydrogenase activity

All reaction mixtures contained the following: 0.25 micromoles 6-phosphogluconate, 0.006 micromoles $^{14}$C-6-phosphogluconate-UL (ca. 1.1 mc),
0.5 micromoles NADP, 25 micromoles Hepes buffer (purchased from Calbiochem Co.), 15 micromoles Ca(NO₃)₂ and 0.087 mg enzyme. The reaction mixture volume was 1.625 and the final pH was 7.8. The B⁺ reaction mixture contained 1 or 5 micromoles of borate preadjusted to pH 7.8 with ammonium hydroxide. The molar concentrations of the above are 1.61 x 10⁻⁴ M 6-phosphogluconate, 3.08 x 10⁻⁴ M NADP, 1.54 x 10⁻² M Hepes buffer (pK 7.55), and 9.3 x 10⁻³ M Ca(NO₃)₂, and no borate, 6.1 x 10⁻⁴ or 3.05 x 10⁻³ M borate. The reaction was initiated by adding 1 ml of dilute enzyme solution to 0.625 ml of the mixture of substrate and cofactors in buffer. 0.3 ml aliquots of the reaction mixtures were taken out at 10, 20, 33, 46 and 60 minutes after the initiation of the reaction and the aliquots were introduced into 2 ml of boiling 80% ethanol to terminate the reaction. After reducing the mixture volume by flash evaporator, the reaction mixtures were banded onto Whatman No. 4 filter paper and were separated electrophoreostically using a CO₂-saturated diethylamine buffer. The radioactive bands were cut out and radioactivity counted with a scintillation counter by the same method described earlier.

b. Properties of crude enzyme preparation It was essential that the crude enzyme preparation contain phosphoribose isomerase, phosphoketopento epimerase and 6-phosphogluconic dehydrogenase in addition to transaldolase and transketolase, because none of the direct substrates for the last two enzymes were available. These enzymes had to be coupled with 6-phosphogluconic dehydrogenase, phosphoribose isomerase and phosphoketopento epimerase, as 6-phosphogluconate was available at the time of this experiment. It was also necessary that the crude enzyme preparation
not contain any enzyme that might interfere with the assays of transketolase and transaldolase activities. Therefore, prior to the experiment on the borate control of these two enzyme, the crude enzyme preparation was tested for the presence of various enzyme activities. All these enzyme assays were done with a Cary 14 spectrophotometer by measuring oxidation or reduction of pyridine nucleotides at 340 nm. The activities of glucose-6-phosphate dehydrogenase and 6-phosphogluconic dehydrogenase were assayed directly by measuring NADP reduction in the presence of the crude enzyme preparation and glucose-6-phosphate and 6-phosphogluconate respectively. The activities of various other enzymes in the crude preparation were measured by adding triosephosphate isomerase and \( \alpha \)-glycerophosphate dehydrogenase, both of which were obtained from Calbiochem Co., to the crude enzyme preparation and then measuring the oxidation of NADH in the presence of various substrates. In the enzyme assays, coupled with triosephosphate isomerase and \( \alpha \)-glycerophosphate dehydrogenase activities, advantage was taken of the high value of the equilibrium ratio of \( \alpha \)-glycerophosphate to dihydroxyacetonephosphate. The reported equilibrium constants for the reversible reactions of triosephosphate isomerase (37) and \( \alpha \)-glycerophosphate dehydrogenase (10) are as follows:

\[
glyceraldehyde-3-phosphate/k_{\text{hydroxyacetonephosphate}} = 0.045,
\]

\[
(dihydroxyacetonephosphate)(\text{NADH})/(\alpha\text{-glycerophosphate})(\text{NAD}) = 5.5 \times 10^{-12}.
\]

Various experimental conditions are shown in Table 19 and the results are shown in Figures 28a to 28h.
Table 19. The contents of reaction mixtures of various assays

<table>
<thead>
<tr>
<th>Substrates (all at 1 micromole)</th>
<th>ATP</th>
<th>NAD + P_i</th>
<th>NADH</th>
<th>NADP</th>
<th>TPI</th>
<th>GDP</th>
<th>Enzymes assayed</th>
<th>Figures</th>
</tr>
</thead>
<tbody>
<tr>
<td>ribose-5-P</td>
<td>0</td>
<td>0</td>
<td>0.5</td>
<td>0</td>
<td>+</td>
<td></td>
<td>phosphoribose isomerase</td>
<td>28a</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>phosphoketopento epimerase</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>transketolase</td>
<td></td>
</tr>
<tr>
<td>fructose-1,6-diP</td>
<td>0</td>
<td>0</td>
<td>0.5</td>
<td>0</td>
<td>+</td>
<td></td>
<td>aldolase</td>
<td>28b</td>
</tr>
<tr>
<td>glucose-6-P</td>
<td>1</td>
<td>0</td>
<td>0.5</td>
<td>0</td>
<td>+</td>
<td></td>
<td>phosphohexose isomerase</td>
<td>28c</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>fructose-6-P-1-kinase</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>aldolase</td>
<td></td>
</tr>
<tr>
<td>ribose-5-P glucose-6-P</td>
<td>1</td>
<td>0</td>
<td>0.5</td>
<td>0</td>
<td>+</td>
<td></td>
<td>transaldolase</td>
<td>28d</td>
</tr>
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<td>enzymes of Figure 29a</td>
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<td></td>
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<td></td>
<td></td>
<td></td>
<td>enzymes of Figure 29c</td>
<td></td>
</tr>
<tr>
<td>ribose-5-P</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>-</td>
<td></td>
<td>phosphohexose isomerase</td>
<td>28e</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>transaldolase</td>
<td></td>
</tr>
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<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>enzymes of Figure 29a</td>
<td></td>
</tr>
<tr>
<td>glucose-6-P</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>-</td>
<td></td>
<td>glucose-6-P dehydrogenase</td>
<td>28f</td>
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<tr>
<td>6-phosphogluconate</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>-</td>
<td></td>
<td>6-phosphogluconic</td>
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<td>dehydrogenase</td>
<td></td>
</tr>
<tr>
<td>ribose-5-P</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td></td>
<td>-</td>
<td>glyceraldehyde-P</td>
<td>28h</td>
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<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>dehydrogenase</td>
<td></td>
</tr>
</tbody>
</table>

*a All reaction mixtures contained 20 micromoles of MgCl₂, 40 micromoles of Hepes buffer and 0.2 ml of crude enzyme preparation described in the text. The reaction volume and pH were 2.2 ml and 7.7. The oxidation of reduction of pyridine nucleotides were measured at 340 μM with a Cary 14 spectrophotometer.

*b TPI = triosephosphate isomerase and GDP = α-glycerophosphate dehydrogenase
c. Borate control of the activities of transaldolase, transketolase and aldolase  
The borate control of these enzymes was determined by examining the rates of conversion of isotopically labelled 6-phosphogluconate and glucose-6-phosphate into various products of transketolase and transaldolase reactions. The reaction mixtures contained the following:

- substrate: 0.8 micromoles 6-phosphogluconate-UL-\(^{14}\)C (3 \(\mu\)c) and 0.8 micromoles glucose-6-phosphate-C-1-\(^{3}\)H (about 8 \(\mu\)c) in 0.8 ml of water. The specific activities of radioactive substrates were diluted with non-active substrates.
- cofactors, coenzymes and enzymes: 1 micromole ATP, 2 micromoles NADP, 1 micromole NADH, 10 micromoles MgCl\(_2\), 30 micromoles Hepes buffer, about 10 \(\mu\)g of triosephosphate isomerase and about 20 \(\mu\)g of \(\alpha\)-glycerophosphate dehydrogenase, both of which were purchased from Calbiochem Co., and 0.5 ml of crude sunflower enzyme mixture preparation. These were in 3.0 ml of aqueous solution and the pH of the mixture was 7.7. To this mixture 0.2 ml of water, 0.2 ml of 0.1 M borate or 0.2 ml of 0.01 M borate was added to make the mixture volume 3.2 ml. The pH of the borate solutions were preadjusted to 7.7.

The reaction was initiated by introducing the substrates into test tubes containing the cofactors, coenzymes and enzymes. At various times after the initiation of the reaction 1 ml aliquots were drawn out and mixed with 4 ml of boiling absolute ethanol. The reaction mixtures were vacuum dried, and the dried mixtures were dissolved in 0.2 ml each of distilled
water. To each of these mixtures a small amount (about 0.05 ml wet volume) of weak cation exchange resin, Amberlite IRC 50, was added to remove excess cations. The pH of the mixture was about 5.5 after mixing with the resin as judged with pH paper. The mixtures and three successive washings of the resin were banded onto Whatman No. 4 filter paper and the reaction products were separated electrophoretically. Radioactive products were detected with radioautogram and their activities counted in the usual manner with a liquid scintillation counter. Tritium was counted with a window setting between 50 and 450, and $^{14}$C was counted with window setting between 450 and 1000. 50% and 15% gain were used for $^3$H and $^{14}$C respectively.

B. Results

1. **Borate control on 6-phosphogluconic dehydrogenase**

   The radioautograms showed three radioactive bands. One was readily identified as unreacted 6-phosphogluconate. The mobility of the second band was identical with that of ribose-5-phosphate. As will be shown later, the mobilities of ketose phosphate and aldose phosphate isomers are identical in the electrophoresis procedure using CO$_2$-saturated diethylamine. Therefore this second band could be either ribulose-5-phosphate alone or mixture of ribulose-5-phosphate, ribose-5-phosphate and xyulose-5-phosphate. This band was assumed to be ribulose-5-phosphate. The third band which had very weak activity was proved to be a glucose-6-phosphate contaminant. The radioactivity in this band remained the same throughout the reaction, suggesting that glucose-6-phosphate was not involved in any reaction. Therefore it may be concluded that the enzyme preparation did not contain hexosephosphate isomerase, transaldolase or transketolase. It is also
likely, therefore, that the enzyme preparation did not contain phosphoribose isomerase or phosphoribose epimerase. Table 20 shows the distribution of radioactivity in the substrate and corresponding products at various times after the initiation of the reaction.

Table 20. Distribution of radioactivity in substrate and product

<table>
<thead>
<tr>
<th>time</th>
<th>borate conc.</th>
<th>counts/min</th>
<th>6-phosphogluconate</th>
<th>ribulose-5-phosphate</th>
<th>total</th>
<th>R-5-P total</th>
<th>% of B-</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td></td>
<td>140,700</td>
<td>15,700</td>
<td>156,400</td>
<td>0.100</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>10 min.</td>
<td>6.1×10⁻⁴ M</td>
<td>147,700</td>
<td>8,600</td>
<td>156,300</td>
<td>0.055</td>
<td>55</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3.05×10⁻³ M</td>
<td>156,300</td>
<td>6,200</td>
<td>162,500</td>
<td>0.040</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td></td>
<td>113,800</td>
<td>29,900</td>
<td>143,700</td>
<td>0.208</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>20 min.</td>
<td>6.1×10⁻⁴ M</td>
<td>123,900</td>
<td>17,100</td>
<td>141,000</td>
<td>0.121</td>
<td>58</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3.05×10⁻³ M</td>
<td>136,100</td>
<td>12,100</td>
<td>148,200</td>
<td>0.082</td>
<td>39</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td></td>
<td>99,500</td>
<td>44,800</td>
<td>144,300</td>
<td>0.310</td>
<td>100</td>
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</tr>
<tr>
<td>33 min.</td>
<td>6.1×10⁻⁴ M</td>
<td>112,300</td>
<td>26,500</td>
<td>138,800</td>
<td>0.191</td>
<td>62</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3.05×10⁻³ M</td>
<td>139,000</td>
<td>19,900</td>
<td>158,900</td>
<td>0.125</td>
<td>40</td>
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<tr>
<td>0</td>
<td></td>
<td>85,700</td>
<td>52,700</td>
<td>138,400</td>
<td>0.381</td>
<td>100</td>
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</tr>
<tr>
<td>46 min.</td>
<td>6×10⁻⁴ M</td>
<td>120,900</td>
<td>35,400</td>
<td>156,300</td>
<td>0.226</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3.05×10⁻³ M</td>
<td>129,400</td>
<td>25,800</td>
<td>155,200</td>
<td>0.166</td>
<td>43</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td></td>
<td>77,900</td>
<td>63,500</td>
<td>141,400</td>
<td>0.449</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>60 min.</td>
<td>6×10⁻⁴ M</td>
<td>104,600</td>
<td>43,200</td>
<td>147,800</td>
<td>0.292</td>
<td>65</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3.05×10⁻³ M</td>
<td>131,500</td>
<td>29,200</td>
<td>160,700</td>
<td>0.182</td>
<td>41</td>
<td></td>
</tr>
</tbody>
</table>

The experimental conditions were shown in Materials and Methods.
Figure 27. Borate inhibition of 6-phosphogluconate dehydrogenase activity. All reaction mixtures contained 0.25 micromoles 6-phosphogluconate, 0.006 micromoles $^{14}$C-6-phosphogluconate-UL (ca. 1.1 μc), 0.5 micromoles NADP, 25 micromoles Hepes buffer, 15 micromoles Ca(NO$_3$)$_2$, and 0.087 mg enzyme. The reaction mixture volume and pH were 1.625 ml and 7.8. B+ reaction mixture contained 1 or 5 micromoles of borate.
The fraction of radioactivity in ribulose-5-phosphate, or the ratio of radioactivity in ribulose-5-phosphate to the total radioactivity, for various samples is shown in Figure 27. It can be seen from Table 20 and Figure 27 that 6-phosphogluconic dehydrogenase activity is indeed inhibited in the presence of borate, and therefore, the prediction made in Part III that the activity of NADP-linked dehydrogenases catalyzing oxidative decarboxylation is higher in B-leaves is substantiated. However, the result shown in Table 20 does not tell whether the enzyme activity was inhibited by the borate-substrate complex or by borate alone.

2. Properties of the crude enzyme preparation

The contents of the reaction mixtures of various assays were summarized in Table 20, and the activities of various enzymes are shown in Figure 28. Figure 28a shows the presence of transketolase, phosphoribose isomerase and phosphodetopento epimerase. Ribose-5-phosphate was evidently converted to sedoheptulose-7-phosphate and glyceraldehyde-3-phosphate by transketolase action. Since glyceraldehyde-3-phosphate, an excellent acceptor substrate for both transketolase and transaldolase, is expected to be converted rapidly to α-glycerophosphate, sedoheptulose-7-phosphate is expected to accumulate in this system as glyceraldehyde-3-phosphate disappears.

Figure 28b shows the presence of aldolase in the crude enzyme preparation.

Figure 28c shows that the activity of either phosphohexose isomerase or fructose-6-phosphate-1-kinase or both is low in the crude enzyme preparation. It is likely that the low rate of NADH oxidation in this system is due to the low activity of fructose-6-phosphate-1-kinase rather
Figure 28. Assay of various enzyme activities of the pentose phosphate shunt in the crude enzyme preparation. The experimental conditions are given in Table 19.
than phosphohexose isomerase since phosphohexose isomerase activity in intact tissue is high and phosphoribose isomerase activity is also high in the crude enzyme preparation. It is known that in most biological systems the low activity of fructose-6-phosphate-1-kinase activity is the rate limiting reaction of glycolysis (3). A direct test for this enzyme was not possible because fructose-6-phosphate was not available.

Figure 28d shows that the rate of oxidation of NADH is slower when both ribose-5-phosphate and glucose-6-phosphate were present than when ribose-5-phosphate alone is present in the reaction mixture. This is strange because the rate of NADH oxidation in the presence of the two substrates was expected to be the sum of the rates in the presence of a single substrate. One possible explanation for this observation is that the crude enzyme preparation contains high phosphohexose isomerase and glucose-6-phosphate reaches rapid equilibrium with fructose-6-phosphate. Thus, in effect, the concentration of the donor substrate for transketolase is greatly augmented as a result of the rapid conversion of glucose-6-phosphate to fructose-6-phosphate, and fructose-6-phosphate formed removes glyceraldehyde-3-phosphate by transaldolase action, and this in turn reduces the amount of dihydroxyacetone phosphate necessary to oxidize NADH.

Figure 28e shows the presence of transaldolase in addition to the enzymes of Figure 28a in the crude enzyme preparation. The low rate of NADP reduction probably is due to the limited amount of glucose-6-phosphate, the substrate for glucose-6-phosphate dehydrogenase since ribose-5-phosphate would be expected to have been converted and distributed among the pool of ribose-5-phosphate, ribulose-5-phosphate, xylulose-5-phosphate, sedoheptulose-7-phosphate, glyceraldehyde-3-phosphate, fructose-6-phosphate
and glucose-6-phosphate before being acted upon by glucose-6-phosphate dehydrogenase.

Figure 28f and 28g show the presence of both glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase activity in the crude enzyme preparation.

Figure 28h shows the absence of glyceraldehyde-3-phosphate dehydrogenase activity in the crude enzyme preparation. The absence of this enzyme activity is probably due to the charcoal treatment.

3. Borate control of transketolase, transaldolase and aldolase

The results of a multienzyme system such as the combination of the pentose phosphate shunt and the glycolytic pathway are not easy to interpret. To make the matter worse, it was found belatedly that the crude enzyme preparation for sunflower leaves used in this experiment was too active. For this reason, only the result of the sample taken at 10 minutes after the initiation of the reaction will be considered. The distribution of radioactivity in various reaction products at this time is shown in Table 21 and the electrophoretic separation of various reaction products is shown in shadowgram made from radioautogram in Figure 29. The uppermost band corresponds to 6-phosphogluconate and fructose-1,6-diphosphate. The next band corresponds to α-glycerophosphate and the band in the middle corresponds to pentose phosphate. This band has identical mobility as ribose-5-phosphate but is expected to be a mixture of ribulose-5-phosphate, xylulose-5-phosphate and ribose-5-phosphate. The second lowest band is a mixture of glucose-6-phosphate and fructose-6-phosphate, and the lowest band corresponds to sedoheptulose-7-phosphate. The 6-phosphogluconate band and the band containing glucose-6-phosphate and fructose-6-phosphate
Figure 29. Shadowgram made from the radioautogram of paper electrophoretogram showing the separation of various reaction products of crude sunflower enzymes of the pentose phosphate shunt from $^{14}$C-6-phosphogluconate-UL and $^{3}$H-glucose-6-phosphate-C-1.

Legends: 6-P-G; 6-phosphogluconate
$\alpha$-GP; $\alpha$-glycerophosphate
P-5-P; pentose phosphates
H-6-P; hexose-6-phosphates
S-7-P; sedoheptulose-7-phosphate
were eluted and reseparated by means of paper chromatography using isobutyric acid-water-concentrated ammonia (66:33:1 v/v).

Table 21. Distribution of radioactivity in various reaction products from $^{14}$C-6-phosphogluconate-UL and $^3$H-glucose-6-phosphate by crude enzyme prepared from sunflower leaves

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Concentration of boric acid (in M)</th>
<th>Radioactivity $^{14}$C (in 1,000 cpm)</th>
<th>Radioactivity $^3$H (in 1,000 cpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6-phosphogluconate</td>
<td>0</td>
<td>430.2</td>
<td>125.3</td>
</tr>
<tr>
<td></td>
<td>$5 \times 10^{-4}$</td>
<td>552.0</td>
<td>115.7</td>
</tr>
<tr>
<td></td>
<td>$5 \times 10^{-3}$</td>
<td>662.3</td>
<td>104.8</td>
</tr>
<tr>
<td>a-glycerophosphate</td>
<td>0</td>
<td>145.1</td>
<td>37.9</td>
</tr>
<tr>
<td></td>
<td>$5 \times 10^{-4}$</td>
<td>107.3</td>
<td>30.1</td>
</tr>
<tr>
<td></td>
<td>$5 \times 10^{-3}$</td>
<td>64.1</td>
<td>26.9</td>
</tr>
<tr>
<td>pentose-5-phosphates</td>
<td>0</td>
<td>69.2</td>
<td>40.9</td>
</tr>
<tr>
<td></td>
<td>$5 \times 10^{-4}$</td>
<td>98.6</td>
<td>44.1</td>
</tr>
<tr>
<td></td>
<td>$5 \times 10^{-3}$</td>
<td>95.9</td>
<td>60.8</td>
</tr>
<tr>
<td>glucose-6-phosphate</td>
<td>0</td>
<td>11.5</td>
<td>39.0</td>
</tr>
<tr>
<td></td>
<td>$5 \times 10^{-4}$</td>
<td>10.9</td>
<td>62.2</td>
</tr>
<tr>
<td></td>
<td>$5 \times 10^{-3}$</td>
<td>9.0</td>
<td>66.7</td>
</tr>
<tr>
<td>fructose-6-phosphate</td>
<td>0</td>
<td>55.4</td>
<td>215.1</td>
</tr>
<tr>
<td></td>
<td>$5 \times 10^{-4}$</td>
<td>60.8</td>
<td>356.8</td>
</tr>
<tr>
<td></td>
<td>$5 \times 10^{-3}$</td>
<td>58.9</td>
<td>426.6</td>
</tr>
<tr>
<td>sedoheptulose-7-phosphate</td>
<td>0</td>
<td>137.0</td>
<td>375.7</td>
</tr>
<tr>
<td></td>
<td>$5 \times 10^{-4}$</td>
<td>133.8</td>
<td>257.1</td>
</tr>
<tr>
<td></td>
<td>$5 \times 10^{-3}$</td>
<td>132.7</td>
<td>140.2</td>
</tr>
</tbody>
</table>
From the results shown in Table 21, some relations among various compounds may be made, and these are shown in Table 22.

Table 22. The distribution of radioactivity among the related compounds

<table>
<thead>
<tr>
<th>Relations</th>
<th>Ratios of radioactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>no borate</td>
</tr>
<tr>
<td>$^{14}$C-a-glycerophosphate</td>
<td>0.338</td>
</tr>
<tr>
<td>$^{14}$C-6-phosphogluconate</td>
<td></td>
</tr>
<tr>
<td>$^{3}$H-sedoheptulose-7-phosphate</td>
<td>1.75</td>
</tr>
<tr>
<td>$^{3}$H-fructose-6-phosphate</td>
<td></td>
</tr>
<tr>
<td>$^{3}$H-glucose-6-phosphate</td>
<td>0.18</td>
</tr>
<tr>
<td>$^{3}$H-fructose-6-phosphate</td>
<td></td>
</tr>
<tr>
<td>$^{14}$C-glucose-6-phosphate</td>
<td>0.30</td>
</tr>
<tr>
<td>$^{3}$H-glucose-6-phosphate</td>
<td></td>
</tr>
<tr>
<td>$^{3}$H-a-glycerophosphate</td>
<td>0.18</td>
</tr>
<tr>
<td>$^{3}$H-fructose-6-phosphate</td>
<td></td>
</tr>
</tbody>
</table>

The results of Tables 21 and 22 may be interpreted as follows:

a. The ratio of $^{14}$C-a-glycerophosphate to $^{14}$C-6-phosphogluconate

The lower ratio of $^{14}$C-a-glycerophosphate to $^{14}$C-6-phosphogluconate in B+ reaction mixture may be the result of the borate inhibition of one or all of the enzymes that lead to the formation of a-glycerophosphate from 6-phosphogluconate. It was shown earlier that 6-phosphogluconate dehydrogenase activity is inhibited in the presence of borate. If the inhibition of 6-phosphogluconate dehydrogenase alone is responsible for the slow rate
of α-glycerophosphate formation, it would be expected that various intermediary compounds accumulate in B- reaction mixture. Since, however, the 14C activities of pentose phosphate and sedoheptulose-7-phosphate in B- reaction mixture are not greater than those of B+ reaction mixtures, it may be concluded that these intermediary compounds are used at faster rates as they are formed at faster rates in B- reaction mixtures. Since excessive amounts of both triose isomerase and α-glycerophosphate dehydrogenase were added to the reaction mixture, the slower rate of α-glycerophosphate cannot be caused by the borate inhibition on the activities of these enzymes. From the foregoing argument, it may be concluded that other enzymes of the pentose shunt as well as 6-phospho-gluconate dehydrogenase are inhibited in the presence of borate. This may be shown schematically as below.

\[ \text{6-phosphogluconate} \rightarrow \text{Ribulose-5-phosphate} \]

- inhibition of 6-phosphogluconate dehydrogenase in the presence of borate

\[ \text{Ribulose-5-phosphate} \rightarrow \text{Ribose-5-phosphate} \]

- inhibition of isomerase in the presence of borate?

\[ \text{Ribulose-5-phosphate} \rightarrow \text{Xylulose-5-phosphate} \]

- inhibition of epimerase in the presence of borate?

\[ \text{Ribose-5-phosphate} + \text{Xylulose-5-phosphate} \rightarrow \text{Sedoheptulose-7-phosphate} + \text{Glyceraldehyde-3-phosphate} \]

- inhibition of transketolase in the presence of borate?
The inhibition of any one of the above three enzymes, namely, phosphoribose isomerase, phosphoketopento epimerase, and transketolase, by borate will result in the slower rate of glyceraldehyde-3-phosphate formation, and hence α-glycerophosphate formation. Since pentose phosphates were not separated from one another, a direct conclusion as regards to which of the above three enzymes are inhibited in the presence of borate is not possible.

b. The distribution of $^{14}$C and $^3$H in sedoheptulose-7-phosphate

It may be seen from the results shown in Tables 21 and 22 that borate influences the appearance of tritium but not the appearance of $^{14}$C in sedoheptulose-7-phosphate. Since most of the $^3$H in sedoheptulose-7-phosphate is expected to have come from H-fructose-6-phosphate and $^{14}$C in the same compound to have come from $^{14}$C-6-phosphogluconate, the distribution of $^3$H and $^{14}$C in sedoheptulose-7-phosphate appears to suggest that its formation from fructose-6-phosphate is inhibited by borate but its formation from pentose phosphates is not influenced by borate. In order to interpret this observation, the following considerations may be mandatory: 1) Since most of the $^3$H activity is present in fructose-6-phosphate rather than glucose-6-phosphate, the phosphohexose isomerase reaction is a very fast reaction and the equilibrium between glucose-6-phosphate and fructose-6-phosphate is reached rapidly. 2) Since the $^3$H activity appears in 6-phosphogluconate, some carbon other than C-1 must be tritiated in the course of reaction. The most likely way to introduce $^3$H to carbon other than C-1 is by the hydride transfer reaction during the rapid interconversion of glucose-6-phosphate and fructose-6-phosphate (23, 59). Therefore, the $^3$H present in
The $^3$H activity in 6-phosphogluconate shown in Table 21 is the activity present at the time of the termination of experiment, and therefore $^3$H activity present in 6-phosphogluconate during the early stages of reaction must have been much lower. Hence, most of $^3$H activity present in pentose-5-phosphates and sedoheptulose-7-phosphate may be considered to have come from $^3$H-fructose-6-phosphate rather than from $^3$H-6-phosphogluconate. For example, the activity of $^3$H in 6-phosphogluconate at the time of termination of the reaction is approximately 20% of the activity of $^{14}$C in the same compound, while $^3$H activity in pentose phosphates is about 60% of $^{14}$C activity in these compounds. The contribution of $^3$H-6-phosphogluconate to the activity of $^3$H in pentose phosphates will become significant only after the conversion of an appreciable amount of $^3$H-glucose-6-phosphate-C-2 to $^3$H-6-phosphogluconate-C-2, which in turn is dependent on the conversion of an appreciable amount of $^3$H-glucose-6-phosphate-C-1 to $^3$H-glucose-6-phosphate-C-2. Thus during the earlier stages of the reaction the concentration of $^3$H-6-phosphogluconate may be considered to have been very low, and therefore, the contribution of $^3$H-6-phosphogluconate to the $^3$H activities of various reaction products may be considered to be very small compared with the contribution of $^3$H-fructose-6-phosphate.

3) The formation and the appearance of $^3$H activity in sedoheptulose-7-phosphate is dependent on the actions of transketolase and transaldolase. The reaction mechanism of transaldolase involves the transfer of the first three carbon units of ketoses to aldoses, the former being the donor substrate and the latter being the acceptor substrate. According to the review on transaldolase by Racker (48), a general rule for the substrate specificity of this enzyme is that
the size of both reactants and products of the reaction should be no larger than a seven-carbon skeleton and no smaller than a three-carbon skeleton. Thus, only fructose-6-phosphate and sedoheptulose-7-phosphate can serve as good donor substrates and glyceraldehyde-3-phosphate and erythrose-4-phosphate can serve as good acceptor substrates. Then, the transaldolase reaction may be considered to be restricted to the reactions shown below.

\[
\text{Sedoheptulose-7-phosphate} + \text{Glyceraldehyde-3-phosphate} \rightarrow \text{Fructose-6-phosphate} + \text{Erythrose-4-phosphate}
\]

\[
\text{Sedoheptulose-7-phosphate} \rightarrow \text{Erythrose-4-phosphate} + \text{Sedoheptulose-7-phosphate}
\]

The reaction mechanism of transketolase involves the transfer of the first two carbon units from a ketose to an aldose. The requirement of the sizes of substrates for this enzyme is the same as that for transaldolase mentioned earlier. An additional requirement for the donor substrates is that the hydroxyl groups at C-3 and C-4 to be in the trans-position (55). For example, ribulose-5-phosphate cannot serve as a donor substrate while xylulose-5-phosphate can. Thus, the donor substrates are xylulose-5-phosphate, fructose-6-phosphate and sedoheptulose-7-phosphate; while the acceptor substrates are glyceraldehyde-3-phosphate, erythrose-4-phosphate and ribose-5-phosphate.

With the specificities of substrates for the two enzymes, the transfers of $^3$H and $^{14}$C during the reaction in this experiment may be studied. In the reaction schemes shown below $^{14}$C is represented by C$^*$ and
the presence and position of tritium is represented by subscript \( t \). In the very early stage of reaction, \( ^3\text{H} \) is not likely to be present in ribose-5-phosphate or xylulose-5-phosphate that originate from 6-phosphogluconate, as \( ^3\text{H} \) is present only in the C-1 position of fructose-6-phosphate. The reactions of the above two enzymes in this stage of reaction may be shown as below (the carbons are numbered from right to left):

**Reaction 1**

\[
\begin{align*}
\text{C}^*\text{C}^*\text{C}^*\text{C}^* + \text{C}^*\text{C}^*\text{C}^*\text{C}^* & \xrightarrow{\text{TK}} \text{C}^*\text{C}^*\text{C}^*\text{C}^*\text{C}^* + \text{C}^*\text{C}^*\text{C}^* \\
(\text{ribose-5-P}) & \xrightarrow{\text{TK}} (\text{xylulose-5-P}) & \xrightarrow{(\text{sedoheptulose-7-P}) (\text{G-3-P})}
\end{align*}
\]

**Reaction 2**

\[
\begin{align*}
\text{C}^*\text{C}^*\text{C}^*\text{C}^* + \text{C}^*\text{C}^*\text{C}^*\text{C}^*\text{C}^* & \xrightarrow{\text{TK}} \text{C}^*\text{C}^*\text{C}^*\text{C}^*\text{C}^*\text{C}^* + \text{C}^*\text{C}^*\text{C}^* \\
(\text{ribose-5-P}) & \xrightarrow{(\text{fructose-6-P})} (\text{fructose-6-P}) & \xrightarrow{(\text{sedoheptulose-7-P}) (\text{erythrose-4-P})}
\end{align*}
\]

The \(^{14}\text{C}\)-glyceraldehyde-3-phosphate formed in reaction 1 will be converted quantitatively \( \alpha\)-glycerophosphate immediately because of the reason mentioned earlier in section 3b of materials and methods. In the later stages of reaction, \(^3\text{H}\) will appear in C-1 of ribose-5-phosphate and xylulose-5-phosphate, and C-2 of fructose-6-phosphate. With these substrates and the substrates formed during the very early stage of the reaction, the reactions of transaldolase and transketolase may now be shown as follows.

**Reaction 3**

\[
\begin{align*}
\text{C}^*\text{C}^*\text{C}^*\text{C}^*\text{t} + \text{C}^*\text{C}^*\text{C}^*\text{C}^*\text{C}^*\text{t} & \xrightarrow{\text{TK}} \text{C}^*\text{C}^*\text{C}^*\text{C}^*\text{C}^*\text{t} + \text{C}^*\text{C}^*\text{C}^* \\
(\text{ribose-5-P}) & \xrightarrow{(\text{xylulose-5-P})} (\text{sedoheptulose-7-P}) & \xrightarrow{(\text{G-3-P})}
\end{align*}
\]

**Reaction 4**

\[
\begin{align*}
\text{C}^*\text{C}^*\text{C}^*\text{C}^*\text{C}^*\text{t} + \text{C}^*\text{C}^*\text{C}^*\text{C}^*\text{C}^*\text{C}^*\text{t} & \xrightarrow{\text{TK}} \text{C}^*\text{C}^*\text{C}^*\text{C}^*\text{C}^*\text{C}^*\text{t} + \text{C}^*\text{C}^*\text{C}^* \\
(\text{ribose-5-P}) & \xrightarrow{(\text{fructose-6-P})} (\text{sedoheptulose-7-P}) & \xrightarrow{(\text{G-3-P})}
\end{align*}
\]
In the reactions shown above, transketolase was denoted by TK and transaldolase by TA.

As mentioned earlier, the $^{14}$C activity in sedoheptulose-7-phosphate does not appear to be influenced by borate as much as $^3$H activity in this compound is. According to the reaction schemes shown earlier, the inequality of the influence of borate on the appearance of $^{14}$C and $^3$H can best be explained if borate inhibits Reactions 2, 4 and 7 more strongly.
than Reactions 1, 3, 5 and 10. In Reactions 2, 4 and 7, $^3\text{H}$ in sedoheptulose-7-phosphate formed comes from $^3\text{H}$-fructose-6-phosphate with a high specific activity of $^3\text{H}$, while in Reaction 1 no $^3\text{H}$ is introduced into sedoheptulose-7-phosphate. In Reaction 3, $^3\text{H}$ comes from $^3\text{H}$-pentose phosphates with a low specific activity of $^3\text{H}$. Finally, in Reaction 5 and 10 the increase in the specific activity of $^3\text{H}$ is small because the additional $^3\text{H}$ activity introduced into sedoheptulose-7-phosphate originates from ribose-5-phosphate which has a low specific activity of $^3\text{H}$. A lower ratio of $^3\text{H}$ activity to $^{14}\text{C}$ activity would also result if borate inhibits transaldolase activity but does not inhibit transketolase activity. However, this possibility is ruled out because if borate has no influence on transketolase, $^{14}\text{C}$ activity in pentose phosphates would be expected to be higher because of higher activity of 6-phosphogluconate dehydrogenase in the absence of borate. The results presented in Table 21 show that the $^{14}\text{C}$ activity of pentose phosphates is higher in the B- reaction mixture.

An alternative explanation for the low ratio of $^3\text{H}$ to $^{14}\text{C}$ in the presence of borate is that both transketolase reaction and transaldolase reaction are inhibited by borate when the donor substrate is fructose-6-phosphate. We are reminded here that furanose sugars with vicinal hydroxy groups in the cis-position form stable complexes with borate. Among the various sugar phosphates shown in Reactions 1 through 10, only ribose-5-phosphate and fructose-6-phosphate ($\beta$-D-fructofuranose), in both of which the hydroxyl groups at C-2 and C-3 are in cis-position, can form stable complexes with borate. It appears, then, that all reactions involving ribose-5-phosphate and fructose-6-phosphate should be inhibited by borate, and both $^{14}\text{C}$ and $^3\text{H}$ activities should be low in sedoheptulose-7-phosphate.
in the presence of borate. This, however, is not true, and the possible reason that reactions involving fructose-6-phosphate, but not ribose-5-phosphate, may be explained as follows.

In early studies on the reaction of transketolase and transaldolase by Racker and his colleagues (49), the two- or three-carbon unit that is transferred from the donor to acceptor substrate has never been found in the reaction products, and this led them to conclude that the two- or three-carbon units transferred are not released from the enzyme. This may be shown schematically as (the numbers below carbons are the carbon numbers of donor substrate):

\[
\text{ENZYME} + \text{Fructose-6-phosphate} \rightarrow \text{ENZYME-(C-C-C)} + \text{C-C-C-P}_\text{i} \\
1\ 2\ 3 \quad 4\ 5\ 5 \\
\text{(glyceraldehyde-3-P)}
\]

\[
\text{ENZYME-(C-C-C)} + \text{Acceptor} \rightarrow \text{ENZYME} + \text{C-C-C-Acceptor} \\
1\ 2\ 3
\]

It may be seen from the above scheme that carbons numbered 1, 2 and 3 of fructose-6-phosphate are complexed with the enzyme. Since carbons numbered 2 and 3 of fructose-6-phosphate are involved in complex formation with borate, the borate complex in the reaction medium can reduce the reaction velocity of the enzyme reaction simply by decreasing the concentration of free fructose-6-phosphate, the true substrate for the enzyme, or by acting as a competitive inhibitor of the enzyme reaction. The latter is preferred since the decrease in the concentration of free fructose-6-phosphate based on the formation constant of the complex shown in Table 21 is not large enough to account for the low ratio of $^3$H to $^{14}$C in sedoheptulose-7-phosphate in B+ reaction mixture. If the borate complex
of fructose-6-phosphate is tightly bound to the enzyme then this complex can act efficiently as an inhibitor. Ribose-5-phosphate, on the other hand, is an acceptor substrate and it is unlikely that number 2 and 3 carbons of ribose-5-phosphate are bound to the enzyme. Therefore, the complex-forming ability of ribose-5-phosphate would be expected to reduce the concentration of free ribose-5-phosphate slightly but the complex itself is not likely to act as a competitive inhibitor.

The low ratio of $^3\text{H}$ to $^{14}\text{C}$ in sedoheptulose-7-phosphate can best be explained by the proposed mechanism mentioned above, but final conclusions cannot be made for the inhibition mechanism without additional experimentation.

c. The appearance of $^3\text{H}$ in $\alpha$-glycerophosphate

The examination of Reactions 1 through 10 indicates that $^3\text{H}$ will not be introduced into $\alpha$-glycerophosphate by transketolase or transaldolase action. The most probable way to introduce $^3\text{H}$ into $\alpha$-glycerophosphate is the cleavage of either fructose-1,6-diphosphate or sedoheptulose-7-phosphate or both, by the aldolase reaction. As shown earlier in Figure 28 the crude enzyme preparation contained very weak activity of fructose-6-phosphate-1-kinase, and this probably is the reason for the low $^3\text{H}$ activity in $\alpha$-glycerophosphate. Since the $^3\text{H}$ activity in $\alpha$-glycerophosphate is lower in the reaction mixture containing borate, it may be concluded that borate inhibits the aldolase reaction also.

d. The activities of $^3\text{H}$ and $^{14}\text{C}$ in glucose-6-phosphate and fructose-6-phosphate

It may be seen from the result shown in Table 21 that the activity of $^3\text{H}$ in both glucose-6-phosphate and fructose-6-phosphate is lowered by borate, while the activity of $^{14}\text{C}$ in these compounds
does not appear to be influence by borate. This observation may also be explained by the same mechanism that was elaborated for the inequal influence of borate on the distribution of $^3$H and $^{14}$C in sedoheptulose-7-phosphate. Thus the borate inhibition of Reactions, 2, 4 and 7 will result in a slower rate of the disappearance of fructose-6-phosphate. The $^{14}$C activity is introduced into glucose-6-phosphate and fructose-6-phosphate by Reactions 8 and 9, however. Neither of the donor substrates, xylulose-5-phosphate and sedoheptulose-7-phosphate are capable of forming borate complexes. Thus Reactions 8 and 9 are not likely to be influenced by borate. In Reactions 8 and 9, the concentrations of erythrose-4-phosphate and sedoheptulose-7-phosphate are expected to be higher in B- reaction medium. These differences in the concentrations of substrates may account for the slight differences in the $^{14}$C activities in B- and B+ glucose-6-phosphate.

e. The ratio of $^3$H activities in glucose-6-phosphate and fructose-6-phosphate. The results presented in Tables 21 and 22 shows that the equilibrium between the concentrations of glucose-6-phosphate and fructose-6-phosphate is not affected by borate. This suggests that phosphohexose isomerase is not influenced by borate.

C. Discussion

From the results of the earlier experiments with intact tissue, evidence suggesting the borate inhibition of cleavage reactions were presented. The lower levels of citrate and fructose-6-phosphate in B-leaves were attributed to the removal of borate inhibition of isocitrate lyase and aldolase activity. The results presented in Part IV substantiate
the earlier contentions on the borate inhibition of the activities of these enzymes. The biological significance of the borate inhibition of aldolase activity is not clear since the rate-limiting reaction in the glycolysis is normally considered to be the fructose-6-phosphate-1-kinase reaction (3).

The inhibition by borate of 6-phosphogluconate dehydrogenase activity, transketolase, and transaldolase activities may be biologically significant since the pentose shunt is probably a main source of phosphoribosyl pyrophosphate necessary for nucleic acid synthesis. In B- leaves the nucleic acid synthesis is more active during the early stage of boron deficiency before the secondary effects of boron deficiency become dominant. Thus Cory et al. (12) found a higher rate of $^{32}$P incorporation into ribonucleic acid when the bean root tips used in their experiments were grown in B- nutrient solution only for 4 hours, while Timashov (60) and Albert (4) found a larger amount of ribonucleic acid in B+ compared to plants grown in B- nutrient over a week or longer.

The difference in the findings of Cory et al. may be explained by the feedback inhibition and gene repression concepts. These are known to occur for L-histidine. Since both histidine and ribonucleic acids arise from a same precursor, namely, phosphoribosyl pyrophosphate, it is not probable that the excessive production of histidine resulting from the activation of the pentose phosphate shunt and the increased supply of phosphoribosyl pyrophosphate may ultimately be the cause of the feedback repression of the synthesis of various enzymes common to the two pathways.
V. AUXILIARY EXPERIMENTS

A. Paper Electrophoretic Separation of Various Phosphoric Esters

Both in the enzymatic preparation of 6-phosphogluconic acid from glucose-6-phosphate and in the experiments on borate control of various enzyme activities, a convenient method of separating various phosphorylated compounds was needed. Although numerous have appeared in the past on the methods of separation of certain combinations of sugar phosphates or nucleotides, no information was found on the separation of various phosphorylated compounds resulting from the above experiments. An example is the separation of 6-phosphogluconic acid from glucose-6-phosphate. The paper chromatographic separation was attempted with a number of known solvent systems for phosphorylated compounds but no satisfactory result was obtained. The electrophoretic separation of 6-phosphogluconic acid from various sugar phosphates appeared to be promising since 6-phosphogluconic acid, due to its additional charge from the carboxyl group in a basic buffer, was expected to have a higher mobility than sugars of comparable sizes. The buffers reported for paper electrophoresis was considered unsatisfactory, however, for the following reasons. In 0.25 M NaHCO$_3$-Na$_2$CO$_3$ buffer system (48) the phosphate compounds eluted from paper will be badly contaminated with sodium salts. In Na-citrate buffer system (57) phosphates cannot be detected by chemical means. Although this problem can be solved if radioisotopically labelled compounds were used, only a very few such compounds were available. Borate buffer and phosphate buffer were also not satisfactory for obvious reasons. Therefore, a new buffer system that may supplement various disadvantages
of existing buffers was sought.

The requirements of such a buffer was thought to be as follows:

1) The buffer should be readily removeable from the paper, 2) It should not interfere with chemical detection of phosphate compounds, 3) It should have a pH range 9 to 10. This pH was based on the fact that the pK's of phosphoric acid are 2.12, 7.21 and 12.32, and the first and the second pH's of various phosphorylated compounds are from 0.96 for glucose-6-phosphate to 2.10 for glyceraldehyde-3-phosphate and 6.11 for glucose-6-phosphate to 6.75 for glyceraldehyde-3-phosphate. When the phosphate moiety alone is considered, either pH 4 (half-way between the first and the second pK's) or 9 (half-way between the second pK's and the third pK of inorganic phosphate) would be ideal. However, pH 4 is close to the pK of carboxyl moiety of 6-phosphogluconic acid or PGA.

CO₂-saturated diethylamine was found to meet the aforementioned requirements. This buffer can be decomposed at 80°C, does not interfere with molybdate reagent, and has pH range 9 to 9.7.

1. Materials and method

0.5 M CO₂ saturated diethylamine was prepared by dissolving dry ice in diethylamine and diluting with distilled water. To 103 ml (molar volume) of diethylamine in a 2 liter Erlenmeyer flask bathed in a dry ice-acetone mixture, small pieces of dry ice, about the size of a walnut, were dropped. The great amount of heat evolved was dissipated by the dry ice made contact with diethylamine but due to their high density the fumes did not escape the flask unless an excessive amount of dry ice was added to diethylamine. Solid diethylamine carbonate was formed in the flask.
This was dissolved in sufficient amount of distilled water to make 0.5 M diethylamine carbonate. A little more dry ice was added to the buffer until the pH of the buffer reached 9 to 9.5. For sugar phosphates a pH of 9.5 to 9.7 was best and for nucleotides a pH of 9 to 9.2 appeared to be better.

Electrophoresis was done using 45 cm-long, 15 cm-wide Whatman No. 3 or 3MM paper at 600V and 42 milliampere for 2½ hours. After electrophoresis the paper was dried in the hood and heated in an 80°C oven for 30 minutes. The paper was sprayed with Haness-Isherwood molybdate reagent (19), heated in 80°C oven for 5 minutes, then exposed to U.V. light until blue spots appear on a white background.

2. Results and discussion

The electrophoretic separation of various sugar phosphates and nucleotides is shown in Figure 30. Fructose-1,6-diphosphate and 6-phosphogluconate have similar mobilities, and glucose-6-phosphate, glucose-1-phosphate, ADP and ATP are too close to each other to be separated satisfactorily.

The paper chromatography employing isobutyric acid-water-concentrated ammonia is excellent for many sugar phosphates and nucleotides, but inorganic phosphate makes long streaks, as was shown earlier in Figures 14 and 18, and it is often impossible to separate ADP and ATP from the inorganic phosphate. It is especially true for the experiments in which the yields of various organic phosphate products are very small compared to the amount of inorganic phosphate substrate or reactant such as perfusion of inorganic phosphate to intact tissue. For the mixture of
inorganic phosphate and various organic phosphates, an excellent separation of these compounds may be obtained by the combination of the electrophoresis introduced here and the paper chromatography employing isobutyric acid-water-concentrated ammonia. The electrophoresis may also be used to separate oligonucleotides of various sizes and phosphates of oligosaccharides.
Figure 30. Paper electrophoretic separation of various phosphorylated compounds. Electrophoresis was done with 45 cm-long, 15 cm-wide Whatman No. 3 paper at 600V and 42 milliampere using CO$_2$ saturated 0.5M diethylamine, pH 9.3, for 2½ hours.
B. Determination of Formation Constants of the Borate Complexes of Chlorogenic Acid and 6-Phosphogluconic Acid

The formation of complexes between borate and a large variety of diols, polyols and α-hydroxyl carboxylic acids is well known. The enhanced acidity, conductivity and ionophoretic mobility of various diols, polyols and α-hydroxy acids in aqueous solution of borate has been attributed to the formation of ionic species of (I) and (II) (20).

\[ \text{H}_3\text{CO}_3 + \text{HO-R-OH} \rightleftharpoons \text{R} - \text{OB}^- + \text{H}^+ \text{ Type I.} \]

\[ \text{R} - \text{OB}^- + \text{HO-R-OH} \rightleftharpoons \text{R} - \text{B}^- + \text{H}_2\text{O} \text{ Type II.} \]

Much of the information on the formation of borate complexes of these types is qualitative. Although quantitative formation constants of borate complexes have been reported for some compounds, the values appear to be highly unreliable as different investigators report values for the same complex which vary as much as an order of magnitude. For example, the values of the formation constant for borate-catechol complex (Type I, above) range from \(5 \times 10^{-6}\) (49) to \(1 \times 10^{-4}\) (26) and those for borate-mannitol \(1.92 \times 10^{-7}\) (14) to \(8.04 \times 10^{-6}\) (5). Possible reasons for these wide variations will be discussed later. A relatively new method has been introduced for determination of borate complex dissociation constants with chlorogenic acid and 6-phosphogluconic acid by absorption spectrophotometry and optical rotatory dispersion.
1. **Borate complexes of chlorogenic acid**

The absorption maxima of orthohydroxy phenols have been shown to increase and shift to longer wave length in the presence of complexing agents or alkali (29). This bathochromic shift was used to calculate the dissociation constants of the borate complexes of chlorogenic acid. The following assumptions were made in the determination of the constants:

1. The functional groups involved in the complex formation are diols of caffeic acid moiety and the α-hydroxy acid of the quinic acid moiety of chlorogenic acid. The cis-diols of quinic acid at number 4 and 5 carbon positions are not believed to be involved in the complex formation since cis-1,2-cyclohexanediol does not appear to form a measurable borate complex (Table 17). (2) The change of absorption spectrum of chlorogenic acid upon addition of borate is due to complex formation between borate and diol groups of the caffeic acid moiety of chlorogenic acid. This is based on the fact that similar changes of absorption spectrum are observed for free caffeic acid solution but not for free quinic acid solution when borate is added. Moreover, at 355 nm, the wavelength at which all spectroscopic measurements were made, α-hydroxy acids have no absorption. (3) The molar absorptivity of complex Type II, in which two caffeic acid moieties are complexed with a molecule of borate, is twice that of Type I, in which only one caffeic acid is complexed with boric acid. In other words, the molar absorptivity of a subspecies of Type II, QCa-B-CaQ is twice that of B-CaQ, where CaQ or QCa is chlorogenic acid and Q is then the quinic acid, and Ca the caffeic acid moieties. A necessary (though not sufficient) condition for the validity of this assumption is that both QCa-B-CaQ and B-CaQ have identical isosbestic points. As shown in Figure 31, five
Figure 31. Absorption spectra of aqueous solution of chlorogenic acid and the mixture of chlorogenic acid and various concentrations of boric acid. The concentration of chlorogenic acid was $8 \times 10^{-5}$ M in all solutions and the concentrations of boric acid are: none (curve 1), $4 \times 10^{-5}$ M (curve 2), $4 \times 10^{-4}$ M (curve 3), $4 \times 10^{-3}$ M (curve 4), and $4 \times 10^{-2}$ M (curve 5). The pH's of all solutions were 7.6.
Figure 32. Bathochromic shift of the absorption spectrum of chlorogenic acid resulting from the addition of borate (upper two curves) or alkali (bottom curve). All measurements were done at 355 nm.
isobestic points were observed when boric acid concentrations varied from $4 \times 10^{-5}$ M to $4 \times 10^{-2}$ M with the concentration of chlorogenic acid of $8 \times 10^{-5}$ M throughout. At $4 \times 10^{-2}$ M boric acid, nearly all complexes are likely to be of Type II, whereas at $4 \times 10^{-2}$ M boric acid nearly all complexes are likely to be in the form of Type I. Identical spectral changes were obtained upon addition of alkali to the solution of chlorogenic acid resulting either from the addition of base or boric acid is due to the removal of hydrogens from diols of caffeic acid moiety of chlorogenic acid and the mechanism of removal does not have any influence on the spectral change. This is shown in Figure 32.

a. Theoretical consideration  In the case of catechol, which has a single binding site for boric acid, the absorbance of the solution containing boric acid and catechol may be expressed by equation 1.

$$A = e_c(C) + e_{bc}(BC) + 2e_{bc}(BC_2)$$

Eq. 1,

where $A$ is absorbancy of the solution; $e_c$ and $e_{bc}$ are the extinction coefficients or molar absorptivities of free catechol and catechol boric acid complexes; respectively $C$, $BC$ and $BC_2$ are the molar concentrations of free catechol, complex Type I and Type II respectively. For the extinction coefficient of complex Type II, $2e_{bc}$ is used, since this complex contains two catechol molecules complexed to boric acid. The values of $e_c$ and $e_{bc}$ may be obtained readily by measuring the absorptions of catechol alone and catechol plus a saturating amount of boric acid. Denoting the sum of complex Types I and II by $P$ and the total concentration of catechol by $C_t$, the following expressions follow from Eq. 1.
\[ A = e^c (C_t - BC - 2BC^2) + e^c (BC) + e^c (2BC^2) \]

\[ = e^c (C_t) + (e^c - e^c)(BG + 2BC^2) \]

\[ \frac{A - e^c (C_t)}{e^c - e^c} = BC + 2BC^2 = P \quad \text{Eq. 2} \]

Since the left hand side of Equation 2 is a measurable quantity, both \( P \), the amount of catechol in the complexes, and \( C \), the amount of free catechol, may also be calculated.

\[ C = C_t - P \quad \text{Eq. 3} \]

The quantities \( BC \) and \( BC^2 \) may be expressed by two dissociation constants, \( K_1 \) and \( K_2 \) as below, where \( B_f \) represents the amount of free boric acid.

\[ K_1 = \frac{(B_f)(C)}{BC} \quad \text{Eq. 4} \]

\[ K_2 = \frac{(C)(BC)}{BC^2} \quad \text{Eq. 5} \]

From Equations 2, 4 and 5, the following expression may be made.

\[ P = (BC)(1 + \frac{2C}{K_2}) = \frac{(B_f)(C)}{K_1} \left( 1 + \frac{2C}{K_2} \right) \quad \text{Eq. 6} \]

and

\[ \frac{1}{(B_f)(C)} = \left( \frac{1}{K_1} + \frac{2C}{K_1 K_2} \right) \frac{1}{P} = \left( \frac{1}{K_1} + \frac{2C}{K_1 K_2} \right) \frac{1}{P} - \frac{2}{K_1 K_2} \quad \text{Eq. 7} \]

In Equation 7 a plot of \( \frac{1}{(B_f)(C)} \) against \( \frac{1}{P} \) results in a straight line.

From the intercept of this plot with ordinate, \( \frac{2}{K_1 K_2} \) can be obtained, and with this value known, \( \frac{1}{K_1} \) can be obtained from the intercept of the plot.
with abscissa. The experimental verification of this depends upon a knowledge of $B_f$. $B_f$ may be estimated by the following relation:

$$B_t - P = B_t - (BC + (BC + 2BC^2) < B_f = B_t - (BC + BC^2) < B_t - \frac{1}{2}P = B_t - (\frac{1}{2}BC + BC^2)$$  \text{ Eq. 8}

where $B_t$ is the total concentration of boric acid.

In the above relation, the concentration of $B_f$ is larger than $B_t - P$ and smaller than $B_t - \frac{1}{2}P$. When the boric acid concentration is much larger than the catechol concentration, the amount of $2BC^2$ is expected to be negligible compared to $BC$, and therefore, $B_t - P$ may be used for $B_f$.

In the above treatment, both $K_1$ and $K_2$ can be calculated from a single plot, and the absorbancies of various species are related to the molar concentrations rather than activities of these species, the dissociation constants expressed in equations 4 and 5 are directly correct values and need not be corrected for activities, whereas when pH or conductivity enhancement are used for the calculations of the dissociation constants, the values obtained are in terms of chemical activities.

In the case of chlorogenic acid, the foregoing principles cannot be used directly, since a molecule of chlorogenic acid has at least two binding sites, namely, the diols of the caffeoyl moiety and the α-hydroxy acid of quinoyl moiety. Therefore, the average number of boric acids bound to chlorogenic acid would be somewhat greater than 1. If both sites of chlorogenic acid are complexed fully with boric acid at a saturating concentration of boric acid, the number of boric acids per molecule of chlorogenic acid will be 2, and at lesser concentrations, the average will be between 1 and 2.
The concentrations of various species in the solution mixture of boric acid and chlorogenic acid are represented by the following notations:

- $B^n C$ \textsuperscript{n} \text{ sum of various subspecies of complex Type I}
- $B^n C_2$ \textsuperscript{n} \text{ sum of various subspecies of complex Type II}
- CaQ \text{ free chlorogenic acid}
- $(\text{CaQ})_t$ \text{ total chlorogenic acid}
- $B^f$ \text{ free boric acid}
- $B^t$ \text{ total boric acid}

Among the complexes of Type I, the following subspecies may be visualized:

- B-CaQ \text{ boric acid bound to the caffeoyl moiety}
- CaQ-B \text{ boric acid bound to the quinoyl moiety}
- B-CaQ-B \text{ boric acid bound to both moieties}

Among complexes of Type II the following subspecies may be visualized:

- CaQ-B-CaQ, QCa-B-CaQ, CaQ-B-QCa, CaQ-B-CaQ-B, QCa-B-CaQ-B, B-CaQ-B-CaQ and B-CaQ-B-QCa.

There is no reason not to believe that various copolymers of chlorogenic acid and boric acid exist, e.g. CaQ-B-CaQ-B-QCa-B-QCa-B-QCa etc. However, at the concentrations of boric and chlorogenic acids used, the concentrations of these polymers will be vanishingly small, and may be ignored for all practical purposes. Thus various subspecies of complex Types I and II only will be considered in the following arguments.

The ratio of the amount of boric acid complexed to complexes Type I and II to the total amounts (concentrations) of these complexes are
denoted by \( n \) and \( n' \) respectively.

Some relations between the total concentration of complex Type I and the concentrations of various subspecies are shown in the following equations.

\[
\frac{B \cdot C}{n} = B \cdot CaQ + CaQ \cdot B + B \cdot CaQ \cdot B \quad \text{Eq. 9}
\]

\[
n = \frac{B \cdot CaQ + CaQ \cdot B + 2B \cdot CaQ \cdot B}{B \cdot CaQ + CaQ \cdot B + B \cdot CaQ \cdot B} = 1 + \frac{B \cdot CaQ \cdot B}{B \cdot C} \quad \text{Eq. 10}
\]

The concentrations of the three subspecies of complex Type I may be shown by the following relations.

\[
k_1 = \frac{(B_f)(CaQ)}{B \cdot CaQ} \quad \text{Eq. 11}
\]

\[
k_1' = \frac{(B_f)(CaQ)}{CaQ \cdot B} \quad \text{Eq. 12}
\]

where \( k_1 \) and \( k_1' \) are the dissociation constants for caffeoyl and quinoyl moieties. The concentration of \( B \cdot CaQ \cdot B \) may be considered to be related to \( k_1 \) and \( k_1' \) by the relation shown in Equation 13.

\[
B \cdot CaQ \cdot B = \frac{(\alpha)(B_f^2)(CaQ)}{k_1 k_1'} \quad \text{Eq. 13}
\]

where \( \alpha \) is an allosteric constant. If the binding of boric acid to \( B \cdot CaQ \) or \( CaQ \cdot B \) is not influenced by the boric acid already bound to chlorogenic acid, the value of term \( \alpha \) is unity.

From Equation 10 and 13, \( \frac{B \cdot C}{n} \) may be expressed as below.

\[
\frac{B \cdot C}{n} = \frac{(B \cdot CaQ \cdot B)}{(n - 1)} = \frac{(\alpha)(B_f^2)(CaQ)}{(n - 1)(k_1 k_1')} \quad \text{Eq. 14}
\]
Various relations for complex Type II may be obtained similarly. Among the seven subspecies of complex Type II shown earlier, four species contain two molecules of boric acid per molecule of chlorogenic acid. The ratio of the sum of the concentration of the subspecies with two molecules of boric acid to \( B_n C_2 \) may be obtained from the relations shown below.

\[
k_1 = \frac{(CaQ-B)(CaQ)}{CaQ-B-CaQ} = \frac{(\phi)(B-CaQ)(CaQ)}{QCa-B-CaQ} = \frac{(\beta)(B-CaQ-B)(CaQ)}{QCa-B-CaQ-B} = \frac{(\gamma)(B-CaQ-B)(CaQ)}{B-CaQ-B-CaQ}
\]

Eq. 15

\[
k_1' = \frac{(CaQ-B)(CaQ)}{CaQ-B-QCa} = \frac{(\phi)(B-CaQ)(CaQ)}{CaQ-B-CaQ} = \frac{(\beta)(B-CaQ-B)(CaQ)}{CaQ-B-CaQ-B} = \frac{(\gamma)(B-CaQ-B)(CaQ)}{B-CaQ-B-QCa}
\]

Eq. 16

\( \phi, \beta \) and \( \gamma \) in Equations 15 and 16 are allosteric constants. The relation between \( n' \) and the concentrations of various subspecies of complex Type II may be shown as in Equation 17.

\[
n' = \frac{CaQ-B-CaQ + QCaQ-B-CaQ + CaQ-B-QCa + 2(QCa-B-CaQ-B + B-CaQ-B-CaQ + 2B-CaQ-B-QCa)}{B-CaQ-B-QCa} = 1 + \frac{(QCa-B-CaQ-B + B-CaQ-B-CaQ + CaQ-B-CaQ-B + B-CaQ-B-QCa) B_n C_2}{B_CaQ-B-QCa}
\]

Eq. 17

Equation 17 may be rearranged by combining with Equations 15 and 16.

\[
n' = 1 + \frac{(B-CaQ-B)(CaQ)(\frac{1}{k_2} + \frac{1}{k_2'})(\beta + \gamma)}{B_n C_2}
\]

Eq. 18
In the special case arising when \( \beta + \gamma \) is 1, \( n' \) becomes \( n \). Since \( \beta + \gamma \) is constant, \( n' \) may be considered to be proportional to \( n \), and the following expression may be made.

\[
n' - 1 = (n - 1)b = \frac{(B-CaQ-B)(CaQ)\left(\frac{1}{k_2} + \frac{1}{k_2'}\right)(\beta + \gamma)}{B_nC_2}
\]

and by substituting Equation 13 to the above,

\[
n' - 1 = (n - 1)b = \frac{(a)(B_f^2)(CaQ^2)\left(\frac{1}{k_2} + \frac{1}{k_2'}\right)(\beta + \gamma)}{(k_1k_1')(B_nC_2)}
\]

The ratio of the amount of caffeoyl moiety complexed with borate to the total amount of complex Types I and II may be denoted by \( m \) and \( m' \).

\[
m = \frac{(B-CaQ) + (B-CaQ-B)}{(B-CaQ) + (B-CaQ-B) + (CaQ-B)} = \frac{k_1' + aB_f}{k_1 + k_1' + aB_f} = \frac{k_1}{k_1 + k_1' + aB_f}
\]

The value of \( m' \) may also be expressed in terms of \( k_2 \) and \( k_2' \). However, since the value of \( m' \) is dependent on \( m \) and complex Type II contains two molecules of chlorogenic acid, the value of \( m' \) is approximately 2\( m \).

\[
m' \approx 2m
\]

The value of \( n \) shown in Equation 10 may be expressed in terms of \( k_1 \) and \( k_1' \) as shown in Equation 22.
From Equations 20 and 22, $k$ and $k'$ can be expressed in terms of $m$ and $n$.

$$k_1 = \frac{(1 - m)}{(n - 1)}(\alpha B_f)$$  \hspace{1cm} \text{Eq. 23}

$$k_1' = \frac{(m - 1 - n)}{(n - 1)}(\alpha B_f)$$  \hspace{1cm} \text{Eq. 24}

The quantities $B_C$ and $B_C^2$ may also be expressed by two pseudoconstants $K_1$ and $K_1'$.

$$K_1 = \frac{(B_f^n)(CaQ)}{(B_C)}$$  \hspace{1cm} \text{Eq. 25}

$$K_1' = \frac{(B_C)(CaQ)}{(B_C^2)}$$  \hspace{1cm} \text{Eq. 26}

Seven subspecies were included in complex Type II arbitrarily earlier. An exact expression of $m'$ based on these seven subspecies may be as follows:

$$m' = \frac{(CaQ-B-CaQ) + (2OCa-B-CaQ) + (CaQ-B-Ca0-B) + (20Ca-B-CaQ-B) + (CaQ-B-CaQ) + (QCa-B-CaQ) + (CaQ-B-CaQ-B) + (QCa-B-CaQ-B) + (B-Ca?Q-Ca2) + (CaQ-B-CaQ) + (CaQ-B-CaQ-B) + (QCa-B-CaQ-B) + (B-CaQ-B-QCa) + (B-CaQ-B-CaQ) + (CaQ-B-QCa)}{(B-CaQ-B-QCa) + (2B-CaQ-B-CaQ) + (QCa-B-CaQ-B) + (QCa-B-CaQ-B) + (B-CaQ-B-QCa) + (B-CaQ-B-CaQ) + (CaQ-B-QCa)}$$. It may be seen that the value of $m'$ is greater than 1 but less than 2.

Equations 25 and 26 are pseudoconstants because their values are
dependent on the value of $n$, which, in turn, is dependent on $B$.

In Equations 14 and 19, $B_n$ and $B_n^C_2$ are expressed in terms of $B_f$ and $CaQ$. The absorbancy of the mixture of chlorogenic acid and various species of borate complexes of chlorogenic acid may be expressed as in Equation 27.

$$A = e_c (CaQ + CaQ-B + 2CaQ-B-QCa) + e_{bc} (mB_n C) + e_{bc} (2mB_n C_2)$$  \text{Eq. 27}

Equation 27 may be rearranged as follows:

$$A = e_c (CaQ_t - mB_n^C - 2mB_n^2C_2) + e_{bc} (mB_n C) + e_{bc} (2mB_n C_2)$$

\[= e_c (CaQ_t + m(e_{bc} - e_c)(B_n C + 2B_n C_2)).\]

$$\frac{A - e_c (CaQ_t)}{m(e_{bc} - e_c)} = B_n C + 2B_n^2C_2 = P = CaQ_t - CaQ$$  \text{Eq. 28}

Equation 28 is approximate, as it is based on the supposition that $m' = 2m$. Any error resulting from this supposition becomes negligible if the total amount of $B_n C_2$ is very much smaller than $B_n C$ in the solution mixture or if $k_1$ is very much smaller than $k_1'$.

By combining Equation 28 with Equations 14 and 19, the following expression may be made:

$$P = \frac{(a)(B_f^2)(CaQ)}{(n - 1)(k_1k_1')} + \frac{2(a)(B_f^2)(CaQ)(\frac{1}{k_1} + \frac{1}{k_1})(\alpha + \gamma)}{b(n - 1)(k_1k_1')}.$$
\[
\frac{(n - 1)}{(B_f^2)(CaQ)} = \left[ \frac{\alpha}{k_1k'_1} + \frac{2CaQ(\beta + \gamma)}{bk_1k'_1} \left( \frac{1}{k_2} + \frac{1}{k'_2} \right) \right] \frac{1}{P} - \frac{2(\alpha)(\beta + \gamma)}{bk_1k'_1} \left( \frac{1}{k_2} + \frac{1}{k'_2} \right) \]

Eq. 29

Equation 29 is in a similar form as Equation 7 except that the left hand side of the Equation contains another variable, \((n - 1)\), which is dependent on \(B_f\) according to Equation 22. The value of \(P\) in the right hand side of Equation 29 is a function of \(m\), which in turn, is a function of \(B_f\) according to Equation 20.

In Equation 29, \(B_f\) is an independent variable and \(CaQ\), \((n - 1)\) and \(P\) are dependent variable whose values are dependent on \(B_f\). Both \(P\) and \(CaQ\) are also dependent variable of \(m\) as shown in Equation 2, the value of \(m\) itself being a dependent variable of \(B_f\). Thus, if a set of true values of \((n - 1)\) and \(P\) and \(CaQ\) are inserted into Equation 29 at various \(B_f\), the plot of \(\frac{(n - 1)}{(B_f^2)(CaQ)}\) against \(\frac{1}{P}\) will yield a straight line, or since both \(CaQ\) and \(P\) are dependent on \(m\), a straight line will be obtained if true values of \((n - 1)\) and \(CaQ\) and \(P\) obtained from the true values of \(m\) at various \(B_f\) are inserted into Equation 29. It may be seen however, that numerous sets of \(m\) and \(n\) values other than the true values of \(m\) and \(n\) can also satisfy the straight line condition. Since \(m\) and \(n\) are both functions of \(B_f\), the values of \(m\) and \(n\) at various concentrations of boric acid may be calculated if either \(m\) or \(n\) is known at any one concentration of boric acid. But neither of these can be obtained directly from spectroscopic measurements. The permissible range of \(m\) values may be obtained, however,
from the spectroscopic measurements. For example, suppose that the quinic acid moiety cannot form a boric acid complex. Then both B-CaQ-B and CaQ-B of Equation 20 disappear, and the value of m becomes 1.

Alternatively, assume that the complex-forming ability of the quinic acid is very much greater than that of caffeic acid. Then, the quinoyl moiety is fully complexed (CaQ-B) prior to the formation of B-CaQ-B. The latter form will be the first observable complex contributing to an increment of absorptivity. Under these conditions, the concentrations of CaQ, B-CaQ and the complexes of Type II will become vanishingly small and the absorptivity increment observed may be considered to be due entirely to B-CaQ-B. With this condition, the values of m in Equation 20 and \((n - 1)\) in Equation 10 will become as shown below.

\[
m = n - 1 = \frac{B-CaQ-B}{CaQ-B + B-CaQ-B} = \frac{B-CaQ-B}{CaQ-B} \quad \text{Eq. 30}
\]

Since the subspecies of complexes that are involved in the increase of absorbancy at 355 nm are B-CaQ, B-CaQ-B and their homologs of complex Type II, the sum of the concentrations of these subspecies at a given concentration of boric acid is defined by \( A - e_{\text{CaQ}}(CaQ_i) \) regardless of the magnitude of the complex-forming ability of the quinoyl moiety. Thus, if the complex-forming ability of the quinoyl moiety is much larger than that of caffeoyl moiety, B-CaQ will vanish and B-CaQ-B will increase, and if the complex-forming ability of quinoyl moiety is small compared to that of caffeoyl moiety, the concentration of B-CaQ will increase at the expense of B-CaQ-B, but the sum of the concentrations of these two subspecies will remain constant. In other words, the magnitude of the dissociation constant
k_i in Equation 23 will remain constant even if the complex-forming ability of the quinoyl moiety is varied at will from zero to infinity, or as the value of m is varied from the lowest to the highest permissible limits mentioned earlier. At a given concentration of boric acid, the term B_f as well as k_i is constant. Therefore, if the value of m is varied within the boundary condition, n should also vary such that the ratio of (1 - m) to (n - 1) remains constant. Thus, a straight line will be obtained when (1 - m) is plotted against (n - 1), the slope being \( \frac{k_i}{aB_f} \). This plot may be defined if two points of the straight line can be obtained. These two points can be obtained by the following manner.

At the lower limit of m values, that is, when the complex-forming ability of the quinoyl moiety is very much larger than that of the caffeoyl moiety, any detectable amount of caffeoyl complex is formed only at some very high concentration of boric acid. The solution of chlorogenic acid and boric acid is then expected to be comprised only of B-CaQ-B and CaQ-B and the sum of these two species is equal to CaQ_t, as was mentioned earlier. The absorbancy of such a solution mixture would then be expressed as below.

\[
A = \epsilon_c (CaQ + CaQ-B) + \epsilon_{bc} (B-CaQ-B) = \epsilon_c (CaQ)_t - B-CaQ-B) + \epsilon_{bc} (B-CaQ-B)
\]

\[
= \epsilon_c (CaQ)_t + (\epsilon_{bc} - \epsilon_c)(B-CaQ-B).
\]

From the above relation, when \( k_i > k_i' \),

*This statement is absolutely true only when the concentration of boric acid is infinitely larger than that of chlorogenic acid so that the decrease in the concentration of boric acid due to the complex formation with the quinic acid moiety is negligible compared with the total concentration of boric acid. Therefore at a relatively low concentration of boric acid this statement is only approximately true.*
From Equations 30 and 31,

\[
\frac{A - e_c (\text{CaQ})}{e_{bc} - e_c} = B - \text{CaQ} - B
\]

From Equations 30 and 31,

\[
m = n - 1 = \frac{A - e_c (\text{CaQ})}{(\text{CaQ})_t (e_{bc} - e_c)}
\]

The value of \(m\) and \(n - 1\) shown in Equation 32 is the value at the lower limit.

Since the values of \(m\) and \(n - 1\) are both 1 at the high limit, and their values at the lowest limit of \(m\) may be calculated from the absorption data, both \((1 - m)\) and \((n - 1)\) at these two limits may be obtained. The values of \((1 - m)\) and \((n - 1)\) are proportional to each other at a given concentration of boric acid, according to Equation 23. Hence, the ratio of \((1 - m)\) to \((n - 1)\) at a given concentration of boric acid may be obtained, as can the value of \(k_1/\alpha\) if the value of \(B_f\) can be estimated at a given \(B_t\) value. When the complex-forming ability of the quinic acid is far greater than that of the caffeic acid, and the quinoyl moiety is fully complexed at a given concentration of \(B_t\), \(B_f(q)\), the concentration of free boric acid under this assumption may be expressed by Equation 33.

\[
B_f(q) = B_t - (\text{borate complexed to quinoyl moiety}) - (\text{borate complexed to caffeoyl moiety}) = B_t - (\text{CaQ})_t - m(\text{CaQ})_t = B_t - (\text{CaQ})_t (1 + m)
\]

In the foregoing discussion, two necessary conditions for the true values of \(m\) and \(n\) were mentioned: 1) The values of \(m\) and \(n\) should be such that the plot of Equation 29 at various concentrations of boric acid must yield a straight line, 2) The values of \(m\) and \(n\) must be such that these
values satisfy the ratio of \((1 - m)\) to \((n - 1)\) determined by the manner described earlier.

A large number of combinations of \(m\) and \(n\) will satisfy the condition imposed by the individual conditions, but only one combination of sets of \(m\) and \(n\) will satisfy both conditions and these values of \(m\) and \(n\) may be considered to be true values.

b. **Experiments and results** Chlorogenic acid and boric acid were obtained from Nutritional Biochemical Corporation and Mallinckrodt Company respectively. All spectroscopic measurements were done with a Cary 14 spectrophotometer at 355 nm using quartz cuvettes with a 1 cm light path length. Five measurements of the absorbancy of \(10^{-4}\) M chlorogenic acid adjusted to pH 7.6 with Hepes (0.05 M final concentration) buffer were 0.650, 0.660, 0.655, 0.655 and 0.652. The average of these were taken as 0.655 and the molar extinction coefficient of chlorogenic acid at pH 7.6 was taken as 6.550. Various concentrations of boric acid, preadjusted to the proximity of pH 7.6, were mixed with chlorogenic acid and the mixtures were buffered with 0.05 M Hepes buffer to bring the pH exactly to 7.6. The final concentration of chlorogenic acid in various solution mixtures were always \(10^{-4}\) M. Table 22 shows the absorbancies of the mixture of chlorogenic acid and the various concentrations of boric acid.

It may be seen from the result shown in Table 22 that the limiting absorbancy is attained at boric acid concentration of \(0.1\) M or higher. If the complex-forming ability of the quinic acid moiety is much greater than that of caffeic acid, all increase of absorbancy observed must be due to the subspecies B-CaQ-B and its homologs in complex Type II. If, however,
the complex-forming ability of the caffeic acid moiety is far greater than that of the quinic acid moiety, the increase observed at relatively low concentrations of boric acid is expected to be mainly due to B-CaQ and its homologs in complex Type II. At a very high concentration of boric acid, both moieties will be saturated and the increase will be mainly due to the subspecies B-CaQ-B. Thus, the ratio of B-CaQ to B-CaQ-B is expected to be much greater at boric acid concentrations of 0.1 M than at 1.0 M. The absorbancies of the mixture of chlorogenic acid and these two different concentrations of boric acid are identical suggesting that the molar extinction coefficient of B-CaQ and B-CaQ-B are identical. The molar extinction coefficient of these two species are therefore taken as 18,580.

Table 22. The absorbancies of the mixture of $10^{-4}$ M chlorogenic acid and various concentrations of boric acid at 355 nm$^a$

<table>
<thead>
<tr>
<th>Boric acid concentrations (M)</th>
<th>Absorbancy</th>
<th>Boric acid concentrations (M)</th>
<th>Absorbancy</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.655</td>
<td>1 $\times$ 10$^{-1}$</td>
<td>1.858</td>
</tr>
<tr>
<td>1 $\times$ 10$^{-3}$</td>
<td>1.045</td>
<td>2 $\times$ 10$^{-1}$</td>
<td>1.859</td>
</tr>
<tr>
<td>2 $\times$ 10$^{-3}$</td>
<td>1.242</td>
<td>4 $\times$ 10$^{-1}$</td>
<td>1.857</td>
</tr>
<tr>
<td>3 $\times$ 10$^{-3}$</td>
<td>1.364</td>
<td>6 $\times$ 10$^{-1}$</td>
<td>1.858</td>
</tr>
<tr>
<td>4 $\times$ 10$^{-3}$</td>
<td>1.445</td>
<td>7 $\times$ 10$^{-1}$</td>
<td>1.858</td>
</tr>
<tr>
<td>1 $\times$ 10$^{-2}$</td>
<td>1.651</td>
<td>8 $\times$ 10$^{-1}$</td>
<td>1.859</td>
</tr>
<tr>
<td>2 $\times$ 10$^{-2}$</td>
<td>1.784</td>
<td>9 $\times$ 10$^{-1}$</td>
<td>1.857</td>
</tr>
<tr>
<td>3 $\times$ 10$^{-2}$</td>
<td>1.816</td>
<td>10 $\times$ 10$^{-1}$</td>
<td>1.858</td>
</tr>
<tr>
<td>5 $\times$ 10$^{-2}$</td>
<td>1.851</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^a$All solution mixtures contained $10^{-4}$ M chlorogenic acid and the pH of the solutions was 7.6 throughout.
In calculations of m, n, a and the various dissociation constants, measurements at 1 x 10^-3 M, 2 x 10^-3 M, 4 x 10^-3 M and 1 x 10^-2 M boric acid were used. The following procedure was used to obtain these quantities.

1) **Determination of the ratio of m to n at various concentrations of boric acid**

   a) At all concentrations of boric acid both m and n will become 1 at the limit of m, or when the quinic acid does not complex with boric acid at all. Thus, both \((1 - m)\) and \((n - 1)\) become 0. The origin in the plot of \((1 - m) = \frac{k_1}{a_B} (n - 1)\) will constitute one of the two points necessary to define the slope of the plot.

   b) The second point of this plot is obtained from the hypothetical situation where the quinic acid moiety has an exceedingly large formation constant for boric acid complex compared to that of caffeic acid moiety. The values of m, \((n - 1)\), \((1 - m)\) and \(\frac{(1 - m)}{(n - 1)}\) were calculated from the results shown in Table 22 by using Equations 30 and 32, and are shown in Table 23.

   **Table 23.** The calculated values of m, \((n - 1)\), \((1 - m)\) and \(\frac{(1 - m)}{(n - 1)}\) for the hypothetical situation where the quinic acid moiety becomes saturated first

<table>
<thead>
<tr>
<th>Bt (in M)</th>
<th>m = (n - 1)</th>
<th>(1 - m)</th>
<th>(\frac{(1 - m)}{(n - 1)})</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.001</td>
<td>0.324</td>
<td>0.678</td>
<td>2.09</td>
</tr>
<tr>
<td>0.002</td>
<td>0.488</td>
<td>0.512</td>
<td>1.05</td>
</tr>
<tr>
<td>0.004</td>
<td>0.657</td>
<td>0.343</td>
<td>0.522</td>
</tr>
<tr>
<td>0.010</td>
<td>0.828</td>
<td>0.172</td>
<td>0.208</td>
</tr>
</tbody>
</table>
Since the first point of the plot of \( (1 - m) = \frac{k_1}{\alpha B_f} (n - 1) \) passes through the origin at all concentrations of boric acid, the ratio \( \frac{(1 - m)}{(n - 1)} \) shown in Table 23 is the ratio attainable from the true values of \( m \) and \( n \) at any given concentration of boric acid.

c) If the concentration of free boric acid is known, the quantity \( \frac{k_1}{\alpha B_f} \) may be calculated from the ratio of \( (1 - m) \) to \( (n - 1) \) in Table 23. When the concentration of boric acid is much greater than that of chlorogenic acid, both \( B_t \) and \( B_q \) of Equation 33 approach the concentration of free boric acid \( B_f \), and therefore, an approximate value of \( \frac{k_1}{\alpha B_f} \) may be estimated. Table 24 shows \( \frac{k_1}{\alpha B_f} \) calculated with \( B_t \) and \( B_q \) in place of \( B_f \).

Table 24. Approximate value of \( \frac{k_1}{\alpha B_f} \)

<table>
<thead>
<tr>
<th>( B_t ) (in M)</th>
<th>( B_q (1 - m) )</th>
<th>( B_t (1 - m) )</th>
<th>( \frac{k_1}{\alpha} = \frac{B_q (1 - m)}{\alpha (n - 1)} )</th>
<th>( \frac{k_1}{\alpha} = \frac{B_t (1 - m)}{\alpha (n - 1)} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.001</td>
<td>5.89 \times 10^{-4}</td>
<td>6.78 \times 10^{-4}</td>
<td>1.82 \times 10^{-3}</td>
<td>2.09 \times 10^{-3}</td>
</tr>
<tr>
<td>0.002</td>
<td>1.00 \times 10^{-4}</td>
<td>1.02 \times 10^{-4}</td>
<td>2.05 \times 10^{-3}</td>
<td>2.09 \times 10^{-3}</td>
</tr>
<tr>
<td>0.004</td>
<td>1.35 \times 10^{-4}</td>
<td>1.37 \times 10^{-4}</td>
<td>2.06 \times 10^{-3}</td>
<td>2.09 \times 10^{-3}</td>
</tr>
<tr>
<td>0.010</td>
<td>1.69 \times 10^{-4}</td>
<td>1.72 \times 10^{-4}</td>
<td>2.06 \times 10^{-3}</td>
<td>2.08 \times 10^{-3}</td>
</tr>
</tbody>
</table>

It may be seen in Table 24 that the values of \( \frac{k_1}{\alpha} \) obtained with \( B_q \) and \( B_t \) become close at higher concentrations of boric acid, as was expected since both \( B_q \) and \( B_t \) approach the value of \( B_f \) as \( B_t \) becomes much greater than \((CaQ)_t\). The result shown in Table 24 suggests that the value of \( \frac{k_1}{\alpha} \) is in the neighborhood of \( 2.06 \times 10^{-3} \text{ M} \) or \( 2.07 \times 10^{-3} \text{ M} \).
2) Selection of $m$ and $n$ that satisfy Equation 29

The true values of $m$ and $n$ must also meet the requirement that a straight line be obtained when \( \frac{(n - 1)}{B^2(CaQ)} \) is plotted against \( \frac{1}{P} \), where both of these variables are also dependent variables of $m$ and $n$.

a) Boundary conditions for $m$ values

The higher limit of $m$ is 1 for all concentrations of boric acid as mentioned earlier and the lower limits of $m$ at various concentrations of boric acid are given in Table 23. Any $m$ within this boundary may be chosen arbitrarily for one reference concentration of boric acid. As an example, let us choose $10^{-2}$ M boric acid as the reference.

b) Calculation of $n$ at the reference concentration of boric acid

The $n$ value at $10^{-2}$ M of boric acid was the ratio of $(1 - m)$ to $(n - 1)$ given in Table 23.

Example: For an arbitrarily chosen $m$ of 0.90 at $10^{-2}$ M $B_t$,

\[
(n - 1) = \frac{(1 - 0.90)}{0.208} = 0.481
\]

c) Calculation of $n$ values at various concentrations of boric acid

Equation 22 shown earlier may be rewritten as follows:

\[
(n - 1) = \frac{\alpha B}{k_1 + k_1' + \alpha B} = \frac{B}{F + B}, \quad \text{where} \quad F = \frac{k_1 + k_1'}{\alpha}.
\]

At higher concentrations of boric acid $B_t$ may replace $B_f$ in the above equation. Then, since $(n - 1)$ is fixed at the reference concentration of boric acid, $F$ also becomes fixed. $F$ is considered a constant, and the approximate values of $(n - 1)$ at various concentrations of boric acid are
calculated.

Example: For \((n - 1)\) value of 0.481 at \(B_t = 10^{-2}\) M,

\[
(n - 1)_{B_t} = 10^{-2} = \frac{1 \times 10^{-2}}{F + 1 \times 10^{-2}} = 0.481, \quad F = 1.08 \times 10^{-2}.
\]

\[
(n - 1)_{B_t} = 2 \times 10^{-3} = \frac{2 \times 10^{-3}}{1.08 \times 10^{-2} + 2 \times 10^{-3}} = 0.156
\]

The \((n - 1)\) values calculated are approximate values since \(B_t\) instead of \(B_f\) was used. The error resulting from this may not be severe because \(B_f\) appears both in the denominator and numerator, and the magnitude of \(B_t\) used was much greater than that of \((CaQ)_t\). This error can be corrected, however.

d) **Calculation of \(m\) values at various concentrations of boric acid**

The values of \((1 - m)\) are calculated by using the approximate values of \((n - 1)\) calculated in the example c) and the ratios of \((1 - m)\) to \((n - 1)\) given in Tables 23. The values of \(m\) is calculated from \((1 - m)\).

Example: From \((n - 1) = 0.156\) and \(\frac{(1 - m)}{(n - 1)} = 1.05\) at \(B_t = 2 \times 10^{-3}\) M,

\((1 - m) = 0.156 \times 1.05 = 0.164\), and \(m = 0.836\) at \(B_t = 2 \times 10^{-3}\) M.

e) **Calculation of \(P\) values**

With \(m\) values known, \(P\) may be calculated by using Equation 27.

Example: From \(m = 0.836\), \(A = 1.242\), at \(B_t = 2 \times 10^{-3}\) M and with \(e_c = 6,550\) and \(e_{bc} = 18,580\),

\[
P = \frac{A - \frac{e_c(CaQ)_t}{m e_{bc} - e_c}}{e_c} = \frac{1.242 - (6,550)(10^{-4})}{(0.836)(18,580) - (6,550)} = 0.587
\]

\[= 6.530 \times 10^{-5}\text{ at } B_t = 2 \times 10^{-3}\text{ M.}\]
f) **Calculation of \( B_f \) and \( \text{CaQ} \)** Since \( P \) is the molar quantity of chlorogenic acid in various complexes as shown in Equation 28, \( \text{CaQ} \), the molar concentration of free chlorogenic acid, is obtained by subtracting \( P \) from \( \text{(CaQ)}_t \). Since \( n \) is the ratio of boric acid to chlorogenic acid in the total of various complexes, the amount of boric acid in these complexes may be considered to be \( nP \). Thus, \( B_f \) may be calculated by subtracting \( nP \) from \( B_t \).

**Example:** From \( P = 6.530 \times 10^{-5} \) at \( B_t = 2 \times 10^{-3} \) M and \( \text{(CaQ)}_t = 1 \times 10^{-4} \) M, \( \text{CaQ} = \text{(CaQ)}_t - P = 1 \times 10^{-4} - 0.584 \times 10^{-4} = 0.416 \times 10^{-4} \), and \( B_f = B_t - nP = 2 \times 10^{-3} - (1.156 \times 10^{-4} \times 0.416) = 1.952 \times 10^{-3} \).

**g) Reiteration** If desired, the steps from 2c) and 2f) may be reiterated with the values of \( B \) obtained in 2f).

**Example:** With \( B_f = 9.84 \times 10^{-3} \) M and \( (n - 1) = 0.481 \) at \( B_t = 1 \times 10^{-2} \) M, \( F = \left( \frac{1}{n - 1} \right) (B_f) = (1.08)(9.84 \times 10^{-3}) = 1.063 \times 10^{-2} \).

\( (n - 1) \) at \( 2 \times 10^{-3} \) M \( B_t \) will be

\[
(n - 1)_B = \frac{1.52 \times 10^{-3}}{1.062 \times 10^{-2} + 1.952 \times 10^{-3}} = 0.154.
\]

Note that the first approximation of \( (n - 1) \) at \( B_t = 2 \times 10^{-3} \) M that was calculated in 2c) was 0.156.

**h) Plotting of Equation 29** By the manner described above, \( \text{CaQ}, B_f, P \) and \( (n - 1) \) were calculated at various \( B_t \). Sets of matching values of \( \text{CaQ}, B_f, P \) and \( (n - 1) \) were also obtained on the bases of numerous values of \( m \) chosen arbitrarily at the reference concentration of boric acid. With these sets of values, Equation 29 was plotted.
The best fitting straight line was obtained when the values of \( m \) at the reference concentration of boric acid was 0.996 (Figure 33). The closeness of this \( m \) value to the high limit of the permissible range of \( m \) values suggests that the complex forming ability of the caffeoyl moiety is very much greater than that of the quinoyl moiety.

When \( \frac{(n - 1)}{(B_0^2)(CaQ)} \) is plotted against \( \frac{1}{P} \) with the set of the values of \( B_0^2 \), CaQ, P and \( (n - 1) \) calculated on the basis of \( m \) value of 0.996 at \( B_t = 10^{-2} \) M, the intercept of the plot with abscissa and the ordinate were 765/M and \( 7.90 \times 10^5/M^3 \).

When \( \frac{(n - 1)}{(B_0^2)(CaQ)} \) is zero (intercept of the straight line with abscissa), Equation 29 becomes

\[
\frac{a}{k_1 k_1'} + \frac{(2a)(CaQ)(\beta + \gamma)}{bk_1 k_1'} \left( \frac{k_2 + 1}{k_2} \right) \frac{1}{P} = \frac{(2a)(\beta + \gamma)}{bk_1 k_1'} \left( \frac{1}{k_2} + \frac{1}{k_2} \right).
\]

Substituting the term on the right hand side of the Equation with \( 7.90 \times 10^{-5} \) (intercept of ordinate) and \( \frac{1}{P} \) with 765, the above Equation becomes as below.

\[
\frac{a}{k_1 k_1'} + 7.90 \times 10^5 \times (CaQ)_t = 7.90 \times 10^5 .
\]

\[
\frac{a}{k_1 k_1'} = \frac{7.90 \times 10^5}{765} - \left[ 7.90 \times 10^5 \times (CaQ)_t \right] = 1,033 - 79 = 954
\]

\[
\frac{k_1 k_1'}{a} = 1.048 \times 10^{-3} .
\]

Since the values of \( m \), \( n \) and \( B \) are now known, the values of \( \frac{k_1}{a} \) and \( \frac{k_1'}{a} \) may also be calculated. Table 25 shows the concentrations of free boric acid calculated at various \( B_t \) when \( m \) value at \( 10^{-2} \) M \( B_t \) is 0.996, and the values of \( \frac{k_1}{a} \) and \( \frac{k_1'}{a} \) calculated with the values of \( \frac{(1 - m)}{(n - 1)} \) shown.
Figure 33. The plot of \( \frac{(n - 1)}{(B^2)(CaQ)} \) against \( \frac{1}{p} \) of equation 29 with 0.996 for the value of \( m \) at \( B = 1 \times 10^{-2} \) M.
\[ \frac{n^{-1}}{(B^2)(C)} \times 10^{-7} \]
in Table 23 by using the relation given in Equation 23.

Table 25. The concentration of free boric acid at various concentrations of total boric acid and 10^{-4} M chlorogenic acid, and the values of k_1/\alpha and k_1'/\alpha based on these values of B_f.

<table>
<thead>
<tr>
<th>B_t (M)</th>
<th>B_f (x 10^3 M)</th>
<th>( \frac{k_1}{\alpha} (x 10^3 \text{ M}) )</th>
<th>( \frac{k_1'}{\alpha} (\text{M}) )</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.001</td>
<td>0.968</td>
<td>2.022</td>
<td>0.495</td>
</tr>
<tr>
<td>0.002</td>
<td>1.950</td>
<td>2.047</td>
<td>0.487</td>
</tr>
<tr>
<td>0.004</td>
<td>3.933</td>
<td>2.053</td>
<td>0.473</td>
</tr>
<tr>
<td>0.010</td>
<td>9.916</td>
<td>2.062</td>
<td>0.486</td>
</tr>
</tbody>
</table>

It was stated earlier that Equation 23 is only approximately true at finite concentrations of boric acid but precise at an infinitely high concentration of boric acid. Therefore the values of k_1/\alpha and k_1'/\alpha shown in Table 25 are approximate and the correct values of k_1 and k_1' may be obtained by extrapolating these values to an infinitely high concentration of boric acid. The k_1/\alpha and k_1'/\alpha obtained graphically at infinitely high concentrations of boric acid are 2.066 \times 10^{-3} and 0.470 respectively.

Now k_1, k_1' and \alpha may be calculated as follows:

\[
k_1 = \frac{k_1 k_1'/\alpha}{k_1'/\alpha} = \frac{1.048 \times 10^{-3}}{0.470} = 2.23 \times 10^{-3}
\]

\[
k_1' = \frac{k_1 k_1'/\alpha}{k_1/\alpha} = \frac{1.048 \times 10^{-3}}{2.066 \times 10^{-3}} = 0.507
\]

\[
\alpha = \frac{k_1}{k_1/\alpha} = \frac{k_1'}{k_1'/\alpha} = 1.13.
\]
It may be seen that the complex-forming ability of the caffeoyl moiety is over two hundred times greater than that of the quinoyl moiety. Due to the very large value of the dissociation constant of the quinoyl acid moiety the plot of Equation 29 crosses both the ordinate and abscissa near the origin. It may also be seen that the value of allosteric constant is close to 1, suggesting that the allosteric hinderance of the binding of the second molecule of boric acid to the chlorogenic acid by the boric acid already bound to it is negligible.

It is likely then that the allosteric constants β and γ are also 1. Under this condition b and α in Equation 29 become unity and the value of \( \frac{1}{k_2 + 1/k_2'} \) may also be calculated. Thus, from the intercept of ordinate axis and the value of \( \frac{\alpha}{k_1 k_1'} \), \( \beta + \gamma = 2\alpha = 2.26 \) and \( b = 1 \),

\[
\frac{1}{k_1} + \frac{1}{k_1'} = \frac{\text{Intercept of ordinate}}{\alpha/k_1 k_1'} = \frac{7.90 \times 10^5}{2.26 \times 9.54 \times 10^{-2}} = 378.
\]

In the absence of allosteryism, it is likely that the ratio of \( k_1 \) to \( k_1' \) is similar to that of \( k_1 \) to \( k_1' \). If this relation is true, then both \( k_2 \) and \( k_2' \) may also be calculated. Thus, the values of \( k_2 \) and \( k_2' \) calculated under the assumption \( k_1/k_1' = k_2/k_2' \), are \( 2.66 \times 10^{-3} \) and 0.603 respectively. It is interesting to note that the value of \( k_1 \) (see Eq. 11) is smaller than \( k_2 \) (see Eq. 15) by a proportion (1.19) that is similar to the value of \( \alpha \).

The apparent dissociation constants of chlorogenic acid shown in Equation 25 and 26 may be obtained from the values of \( k_1, k_1', k_2 \) and \( k_2' \). Thus from Equations 25 and 14, \( K_1 \) may be expressed as follows:
The quantities \((n - 1)\) and \((n - 2)\) in the above Equation may be obtained from \(k_1\) and \(k_1'\) by the relation shown in Equation 22.

The formation constants, which are the reciprocals of the dissociation constants, calculated for complex Type I are 449 and 1.97 for the caffeic acid moiety and the quinic acid moiety respectively. These formation constants are the reciprocals of Equations 11 and 15, or \(\frac{(B-CaO)}{(B_\ell)(CaQ)}\) and \(\frac{(CaO-B)}{(B_\ell)(CaQ)}\) respectively. When these values are multiplied by hydrogen ion concentration at pH 7.6, \(2.51 \times 10^{-8}\), in order to compare with the values of formation constants cited from literature shown in Table 17, these constants become \(1.13 \times 10^{-5}\) for the caffeic acid moiety and \(4.95 \times 10^{-8}\) for the quinic acid moiety. The formation constant of complex Type I of catechol in literature (see Table 17) varies from \(1 \times 10^{-4}\) to \(5 \times 10^{-6}\), while the formation constant of tartaric acid in literature (see Table 17) is \(2.98 \times 10^{-8}\).

2. 6-phosphogluconic acid

6-phosphogluconic acid exhibits a negative Cotton effect with a maximum at 210 nm. Upon addition of boric acid, 6-phosphogluconic acid exhibits a positive Cotton effect of greater magnitude with a maximum at 222 nm. The Cotton effect at this wavelength is believed to be due to the \(n \rightarrow \pi^*\) transition of the acyl chromophore according to Urry and Eyring (64), and the Cotton effect observed from various carboxylic acids and their derivatives has been reviewed more recently by Klyne (28). This change in
the optical rotation was used to calculate the dissociation constants of the borate-6-phosphogluconate complex. Figure 31 shows the optical rotatory dispersion curves of 6-phosphogluconate at various concentrations of boric acid. As may be seen, the limiting rotation does not appear to have reached even at 1.8 M boric acid. Because of the low solubility of boric acid the preparation of boric acid of higher concentration was not possible. Therefore, the molecular rotation of the complex was calculated indirectly.

At a high ratio of boric acid to 6-phosphogluconic acid, complex Type II (as mentioned earlier) is expected to be negligibly small and the contribution of this type of complex to the total rotation observed may be ignored. The number of borate-binding sites in 6-phosphogluconate is not known, but it is likely that 6-phosphogluconate can bind with 2 boric acids at the most, as shown below:

\[
\begin{align*}
\text{O}=\text{C}-\text{O} & \quad \text{OH} \\
\text{H} & \quad \text{B} \\
\text{H} & \quad \text{C} \\
\text{H} & \quad \text{O} \\
\text{H} & \quad \text{C} \\
\text{H} & \quad \text{O} \\
\text{H}_2 & \quad \text{C}-\text{O} & \quad \text{OH}_1
\end{align*}
\]

The increased optical rotation observed may be due entirely to complex formation at the site involving the carboxy group and α-hydroxyl group. At the present, we will be concerned only with the borate complex formed at this site. With this restriction, the total rotation of the mixture of 6-phosphogluconic acid and boric acid may be expressed as in Equation 35.
where \( \alpha \) is the rotation, in degrees/dm, of the mixture of 6-phosphogluconic acid and boric acid; \( \phi_g \) and \( \phi_{bg} \) are the molecular rotations, in degree/dm/M, of 6-phosphogluconic acid and its borate complex; \( G_f \) is the sum of molar concentrations of free 6-phosphogluconic acid and any possible species of complexes which do not involve the acyl group and the \( \alpha \)-hydroxyl group; and \( BG \) is the molar concentration of the complex in which boric acid is bound to the acyl group and \( \alpha \)-hydroxyl group. Equation 35 may be rewritten as in Equation 36.

\[
\alpha = \phi_g (G_f - BG) + \phi_{bg} (BG),
\]

\[
\alpha = \phi_g G_f + (\phi_{bg} - \phi_g) (BG), \text{ and}
\]

\[
\frac{\alpha - \phi_g G_f}{\phi_{bg} - \phi_g} = BG
\]

BG may be expressed in terms of the dissociation constant \( k_1 \), of the complex:

\[
BG = \frac{(B_t - BG) (G_f - BG)}{k_1}
\]

When \( B_t \) is much greater than \( G_f \), \( B_t \) may replace \( (B_t - BG) \), viz.,

\[
BG = \frac{B_t (G_f - BG)}{k_1} = \frac{B_t G_f}{k_1 + B_t}
\]
From Equations 36 and 37,

\[ \frac{1}{\alpha - \phi G_t} = \frac{k_1}{(\phi_{bg} - \phi_e) B_t G_t} + \frac{1}{(\phi_{bg} - \phi_e) G_t} \]  

Eq. 38

The left hand side of Equation 38 is measurable quantity, while on the right side, \( B_t \) and \( G_t \) are known quantities. Since \((\phi_{bg} - \phi_e)\) and \( k \) are both constants, Equation 38 can be plotted.

a. **Experiments and results**

2M borate was made by dissolving reagent grade boric acid with enough sodium hydroxide to solublize the boric acid. The final pH of the stock solution of boric acid was made 6.3. Upon dilution of the concentrated borate solution, the pH always increased. The final pH of all solution mixtures of 6-phosphogluconic acid and boric acid was adjusted to 7.6 by adding a small volume of additional sodium hydroxide solution. Figure 33 shows the optical rotatory dispersion (ORD) measurements of 6-phosphogluconic acid and mixtures of 6-phosphogluconic acid and various concentrations of borate, while Table 26 shows the magnitude of optical rotations of various solutions at 222 nm.

<table>
<thead>
<tr>
<th>( B_t ) (M)</th>
<th>( \alpha ) (deg/dm)</th>
<th>( \alpha - \phi G_t )</th>
<th>( \frac{1}{\alpha - \phi G_t} )</th>
<th>( \frac{1}{B_t} ) (M(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.80</td>
<td>8.69</td>
<td>9.60</td>
<td>0.104</td>
<td>0.556</td>
</tr>
<tr>
<td>1.20</td>
<td>8.06</td>
<td>8.97</td>
<td>0.112</td>
<td>0.833</td>
</tr>
<tr>
<td>0.80</td>
<td>7.10</td>
<td>8.01</td>
<td>0.125</td>
<td>1.25</td>
</tr>
<tr>
<td>0.50</td>
<td>5.84</td>
<td>6.75</td>
<td>0.148</td>
<td>2.00</td>
</tr>
<tr>
<td>0.00</td>
<td>-0.91</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
</tbody>
</table>
From the measured value of \(-0.91\) for the optical rotation of \(2 \times 10^{-3}\) 6-phosphogluconic acid, the value of \(\phi_g\) was calculated and value of 405 was obtained. Using the values of \(\frac{1}{a - \phi_b G_t}\) and \(\frac{1}{\beta_t}\) shown in Table 23, Equation 38 plotted and this is shown in Figure 34. The intercept of this plot with the abscissa and the ordinate were \(-2.99/M\) and \(8.62 \times 10^{-2}/\text{deg/dm}\). According to the relation in Equation 38 and \(2 \times 10^{-3} M\) for \(G_t\),

\[
0 = \frac{1}{\phi_{bg} - \phi_g} (-2.99) \frac{k_1}{2 \times 10^{-3}} + \frac{1}{\phi_{bg} - \phi_g} 2 \times 10^{-3}
\]

\(-2.99k_1 = 1\). Therefore, \(k_1 = 0.334\).

And also from Equation 38,

\[
8.62 \times 10^{-2} = \frac{1}{\phi_{bg} - \phi_g} 2 \times 10^{-3}
\]

\(\phi_{bg} - \phi_g = 5,800\).

Since \(\phi_g\) is 405, \(\phi_{bg}\) is 6,205.

With the value of \(\phi_{bg}\) known, \(k_1\)', the dissociation constant of boric acid complex of 6-phosphogluconic acid at an other site than the acyl group and \(\alpha\)-hydroxy group may be calculated in the manner described for chlorogenic acid.
Figure 34. Optical rotatory dispersion of $2 \times 10^{-3}$ M 6-phosphogluconate (bottom curve) and the mixture of $2 \times 10^{-3}$ M 6-phosphogluconate and varying concentrations of boric acid. The pH of all solutions were made 7.6 without using buffer. The curves at center are the baselines.
Figure 35. Graphical determination of dissociation constant of 6-phosphogluconic acid by using the plot of
\[ \frac{1}{\alpha - \phi G_t} \] against \[ \frac{1}{B_t} \] of Equation 38 on page 166.
VI. SUMMARY AND CONCLUSIONS

The following changes were observed to occur in sunflower leaves resulting from boron deficiency.

1) Glucose accumulates.

2) The levels of hexose-6-phosphate, UDPG, ADT and ATP decrease.

3) Various three carbon compounds related to pyruvate accumulate.

4) The levels of malate and citrate decrease.

5) The level of α-ketoglutarate increases while that of succinate and glutamate decreases.

6) The level of glyoxylate, which accumulates only in the dark, becomes much greater.

7) Phenols (chlorogenic and caffeic acids) accumulate in the presence of light and phenylalanine accumulates in the dark.

8) The level of 80% ethanol soluble fraction increases but the level of 80% ethanol insoluble fraction decreases in young leaves.

9) The activities of 6-phosphogluconate dehydrogenase, aldolase, transaldolase and transketolase increase.

10) The number of mitochondria appears to increase but the population of ribosomes appears to decrease.

11) Some unknown compound, "Product X" becomes deposited in chloroplasts, and these chloroplasts have a lower capacity to take up calcium ion.

12) Rhombohedral structures appear in the nucleus.

In the above, the observations 1) through 8) were obtained from the leaves of sunflower plants grown in B- nutrient for 24 hours or less, (observation 9) was made with partially purified enzymes, and the
observations 10) through 12) were made from the leaves of sunflower plants grown in B- nutrient for 4 days up to 12 days.

From the aforementioned observations, the following hypotheses are proposed tentatively as the mechanism of boron in higher plants.

1) Borate inhibits the activities of various NADP-linked dehydrogenase enzymes involved in the cleavage of C-C bonds. Examples of the former are 6-phosphogluconate dehydrogenase, NADP-malic dehydrogenase and NADP-isocitric dehydrogenase. Examples of the latter are aldolase, transaldolase, transketolase and isocitrate lyase.

2) The low levels of α-ketoglutarate and glutamate are the result of the channeling of isocitrate to the cytoplasmic NADP-isocitrate dehydrogenase as against the mitochondrial NAD-isocitrate dehydrogenase.

3) The activation of the NADP-linked dehydrogenases and the enzymes involved in C-C cleavage in B- leaves result in the activation of the glyoxylic acid pathway, the pentose phosphate pathway and the shikimic acid pathway.

4) The activation of the glyoxylic acid pathway results in the accumulation of soluble compounds and decreased synthesis of ATP.

5) The activation of the pentosephosphate shunt and the shikimic acid pathway results in the overproduction of phenols, which is the immediate cause of the death of the plants.

6) As a possible mechanism of the inhibition of various enzyme activities in the presence of borate, the inhibition mechanism involving the substrate-borate complex as an inhibitor is proposed.
As auxiliary experiments, optical methods of determination of the
dissociation constants of borate complexes and an electrophoretic
separation of phosphorylated compounds were presented.
VII. ACKNOWLEDGMENT

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VIII. BIBLIOGRAPHY


