Bulletin No. 385 - Biochemical and Chemical Studies on Astragalus Leaves and Roots

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Biochemical and Chemical Studies on Astragalus Leaves and Roots

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BIOCHEMICAL AND CHEMICAL STUDIES ON ASTRAGALUS LEAVES AND ROOTS

(1. Enzymatic; 2. Translocation of Se$^{75}$ with radioautographs; 3. Absorption and Exchange of Se$^{75}$ in root seedlings.)

DIVISION OF AGRICULTURAL BIOCHEMISTRY
AGRICULTURAL EXPERIMENT STATION
UNIVERSITY OF WYOMING
LARAMIE
Preface

These studies were part of a research program on the function of selenium in plant and the metabolism of selenium in animal experiments as related to toxicity and metabolism. The results of plant experiments are collected in this bulletin. The results of animal experiments will be published subsequently.

Abstracts and preliminary reports were presented during the years the work was under investigation. The data are published in this bulletin since there is lack of information on biochemical research dealing with selenium-indicator plants. In these experiments several species of *Astragalus* were used. The species selected were the most suitable for the specific experiments by their germinating and growth characteristics.

I wish to extend my appreciation to the Atomic Energy Commission for their generous support from 1950 to 1962 in making these studies possible.

Dr. Irene Rosenfeld
Research Professor
Division of Agricultural Biochemistry
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Part I
The Respiratory Enzyme Systems in Astragalus racemosus Leaves*

By Irene Rosenfeld and Felicia P. Wirtz**

The genus Astragalus, which in the taxonomy of vascular plants is placed in the order Rosales, family Fabaceae, subfamily Lotoideae, is of great interest and economic importance because some of the species are selenium converters. Basic information is needed in order to determine whether selenium functions in the enzymatic system(s) of these plants.

Numerous experiments carried out with animal and plant tissue indicate that in respiration many enzymes and intermediate steps are involved. The enzymes which carry out these reactions fall into four general groups:

1. Dehydrogenases, which remove H atoms from the substrate.
2. Decarboxylases, which remove CO$_2$ from the substrate.
3. Oxidases, which catalyze the addition of oxygen to the substrate.
4. Hydrolases, which catalyze the addition of water to a substrate.

The presence of these enzymes can be determined through the use of inhibitors both general and specific.

The presence of a high concentration of selenium in certain species of the genus of Astragalus has been demonstrated by Beath (1). Experiments with solutions and sand cultures showed that selenium-indicator plants require selenium for growth and development (2).

In recent years there has been considerable interest in the functions of enzymes or enzyme systems in higher plants. Specific enzyme systems which were demonstrated in animal tissues had been studied in higher plants by different investigators; however, comprehensive studies on the general enzyme systems in plants are limited. Bonner and Wildman (3) reported in detail on the general enzyme systems present in spinach leaf. Boswell (4) made a comprehensive study of the polyphenol oxidase and other enzyme systems present in the potato tuber.

At present there is no information on the respiration of plants which accumulate high concentrations of toxic elements such as selenium. This report deals with the general types of enzyme systems which take part in the respiration of A. racemosus leaf (selenium indicator). These studies were not intended to be a quantitative study of each or any specific enzyme system present in the A. racemosus leaves. Our aim was to get an integrated picture of the general type of enzymes present in the leaves of one species of selenium-accumulator plant.

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*This research was partially supported by grants from the AEC.
**Research Professor and Research Assistant, respectively, in the Division of Agricultural Biochemistry.
MATERIALS AND METHODS

*A. racemosus* plants were grown in a greenhouse in soil containing approximately 10 ppm of inorganic selenate (Na$_2$SeO$_4$). Forty-five to 60-day-old leaves were used for the experiments. The experiments extended over a year and a half. Seasonal variations and age may have influenced the metabolic rates during the time of the experiment, but day-to-day variations of the results were comparable with those reported by other investigators in higher plant metabolism. In a few studies younger or older leaves were used in the experiments. There were some differences in the enzymatic activity of older leaves and these exceptions will be discussed in the results. The leaves harvested on the day of the experiments were washed and blotted on filter paper and were cut with sharp scissors into two or four parts depending on the size of the leaf. The cut leaves were thoroughly mixed so that a homogeneous mixture was obtained. Preliminary experiments indicated that cutting the leaves had no influence on the rate of respiration. The respiratory rate of whole leaves was less constant than that of cut leaves. This may have been due to the slower rate of substrate penetration in the uncut leaf and a less homogeneous mixture of the tissue. Vacuum infiltration of the added substrate in the leaves described by Bonner and Wildman (3) did not increase the diffusion of the substrate into the leaves.

The homogenate technique used in the study of enzymatic reaction in animal tissue was not applicable to the *A. racemosus* leaves. It had been found that macerated leaves showed an insignificant rate of gas exchange in the first three hours with a sudden rise in respiration at the end of the fourth hour and a continued rise in subsequent hours. Freezing the leaves reduced the rate of respiration to almost zero. No further studies were made for the factors responsible for the erratic behavior of fresh macerated leaves and for the loss of activity in the frozen leaves. Storing the leaves in plastic bags for a month at 5°C reduced the moisture and increased the total solids 10-30 percent. The respiration rate of the stored leaves was inconsistent and the addition of glucose had no effect on the gas exchange. Storing the leaves at room temperature from 24 to 72 hours had no effect on the rate of respiration, but the leaves appeared withered.

Numerous experiments were conducted in order to determine the optimum amount of plant tissue to be used for these studies. These studies indicated that 200 mg of fresh leaves gave the most consistent results; therefore this amount of leaves was used in all experiments.

The influence of pH and of buffers in which the racemosus leaves were suspended was studied in preliminary experiments. The results indicated that the maximum rate of respiration with an R. Q. of one, or near one, was obtained with 0.15M sodium phosphate buffer at pH 6.0. In all experiments this buffer at pH 6.0 was used unless otherwise indicated. The addendum was added to the buffer in concentrations indicated in the results.

The direct manometric method of
Warburg described by Umbreit et al. (5) was used in these studies. All determinations were made at 30± 0.05°C. Oxygen uptake was measured in vessels having 0.2 ml 30 percent KOH in the central well. In the cyanide inhibition studies the KOH contained 2M cyanide. The substrates were added from single or double sidearms of the flasks 15 minutes after equilibration of leaves with buffer. The total volume in all experiments was 3.0 ml. Manometric readings were made at one-half hour intervals the first two hours and hourly intervals thereafter. The results are the averages of two triplicate or duplicate experiments and are expressed per 100 mg. Leaves harvested on the day of the experiment gave a reproducible rate of gas exchange; in general the results agreed within 3 percent.

RESULTS

Oxidase Systems in A. racemosus Leaves

The enzyme systems which carry out biological oxidations in plant and animal tissues have been studied in considerable detail. In plants as well as animal tissues several oxidase systems are present. The chain of respiratory enzymes which carry out the final step of oxidations is the terminal oxidase through which electrons are removed from the substrate, and finally oxygen and hydrogen ions yield water. This is the step at which oxygen is consumed. In animal tissues this enzyme is cytochrome oxidase; in plant tissues three different oxidase systems may be involved: (1) non-metal oxidase; (2) cytochrome oxidase; (3) polyphenol oxidase.

Polyphenol oxidase is responsible for darkening of plant tissues. The presence of polyphenol oxidase in higher plants was demonstrated by various investigators. Baker and Nelson (6) demonstrated that 85 percent of the O₂ uptake in potato tuber was mediated through tyrosinase-catalyzed oxidation. Bonner and Wildman (3) established the presence of polyphenol oxidase in spinach leaf. They reported that 90-100 percent or more of the oxygen consumed in the respiration of spinach leaf passes through the polyphenol oxidase system. Goddard (7) separated from wheat embryo a cytochrome oxidase which apparently was identical with that of beef heart. James and Cragg (8) have shown that ascorbic-acid oxidase may be the terminal oxidase in barley leaves, and ascorbic acid may function as a carrier between the oxidase and reduced coenzyme. Marsh and Goddard (9) reported cytochrome oxidase in carrot root. This short review indicates that there is no correlation between plants and the oxidase present in the tissues. That the heavy-metal oxidases play an important role in the respiration of A. racemosus leaf is indicated by subsequent experiments.

The respiration of racemosus leaves was inhibited by the phenols as indicated in Table 1. About 80 percent of O₂ uptake, 75 percent of CO₂ formation was inhibited by p-nitrophenol, 34
percent by 3.0 mg catechol, 12 percent by 3.0 dihydroxyphenylalanine, and no inhibition by o-nitrophenol at the end of the 4-hour experiment. Previous investigators indicated that o-phenols are not utilized as substrate by polyphenol oxidase while the p-analogues are the normal substrate of the enzyme. Our data indicate that substances which inhibited the activity of polyphenol oxidase in other plants inhibited the respiration of racemosus leaf, in varying degrees.

The compounds tested differed in several respects in their action on the respiration of racemosus leaf: (1) The transitory increase in oxygen consumption observed with the addition of catechol to polyphenol oxidase was absent or masked when catechol was added to racemosus leaves. (2) Catechol inhibited only about one-third of the respiration in 4 hours. If the experiments were continued for 8 hours, the rate of O$_2$ uptake and CO$_2$ evolution remained constant. This suggested that the limited inhibition of respiration by catechol was not due to the slow rate of penetration of substrate in the leaves. (3) p-Nitrophenol inhibited at the rate of respiration to a greater degree than catechol.

The differential effects of phenols on the polyphenol oxidase could be interpreted to indicate that the polyphenol oxidase in the A. racemosus leaves differs in some respect from the polyphenol oxidase present in other plants. It was reported by Boswell and Whiting (10) and confirmed by others that polyphenol oxidase not only oxidized the substrate but also rapidly inactivated the enzyme. This fact accounts for the transitory rise of oxygen consumption and for subsequent decrease in the gas exchange when catechol was added. The racemosus leaves had only a limited capacity for oxidation of catechol and p-nitrophenol, suggesting that other heavy-metal-containing enzymes, or an alternate pathway, may control the major part of respiration in the leaves.

That polyphenol oxidase is linked to other oxidation-reduction system(s) through phenolic carriers in the potato

---

### TABLE 1—Polyphenol Oxidase in A. Racemosus Leaves*

| Addenda          | mg 1st hour | O$_2$ uptake 1st hour | O$_2$ uptake 4th hour | CO$_2$ output 1st hour | CO$_2$ output 4th hour | percent inhibition O$_2$ | percent inhibition CO$_2$
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.0</td>
<td>56</td>
<td>49***</td>
<td>48</td>
<td>40***</td>
<td>18</td>
<td>12</td>
</tr>
<tr>
<td>Catechol</td>
<td>2.0</td>
<td>51</td>
<td>40</td>
<td>48</td>
<td>35</td>
<td>18</td>
<td>12</td>
</tr>
<tr>
<td>Catechol</td>
<td>3.0</td>
<td>49</td>
<td>33</td>
<td>38</td>
<td>26</td>
<td>33</td>
<td>35</td>
</tr>
<tr>
<td>o-Nitrophenol</td>
<td>0.7</td>
<td>62</td>
<td>53</td>
<td>59</td>
<td>48</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>p-Nitrophenol</td>
<td>0.7</td>
<td>56</td>
<td>9</td>
<td>61</td>
<td>10</td>
<td>82</td>
<td>75</td>
</tr>
<tr>
<td>Dopa**</td>
<td>0.7</td>
<td>60</td>
<td>46</td>
<td>50</td>
<td>39</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td>Dopa**</td>
<td>3.0</td>
<td>63</td>
<td>43</td>
<td>53</td>
<td>35</td>
<td>12</td>
<td>12</td>
</tr>
</tbody>
</table>

*85-day-old leaves.
**Dihydroxyphenylalanine.
***The final readings of the controls are considered as 100 percent.
tuber was reported by Boswell (4). He found that caffeic and gallic acids served as carriers in potato respiration. Bonner and Wildman (3) reported that dihydroxyphenylalanine (Dopa) functioned as a carrier in spinach leaf while catechol and protocatechuic acid were unable to act as respiratory carriers. The oxidation-reduction system linked with phenolic carriers was not investigated in detail; however, the addition of Dopa at 0.7 and 3 mg levels at pH 6.0 had a slight depressing effect on the gas exchange. Whether phenols serve as carriers in racemosus leaves requires more study.

The respiration of *A. racemosus* leaves was inhibited by azide and cyanide, which are known inhibitors of the oxidase system (Fig. 1). The respiration of racemosus leaves was inhibited very rapidly by 0.01M azide and at a much slower rate with the same concentration of cyanide. The sensitivity of O₂ uptake and CO₂ formation to azide and cyanide may be taken to indicate that a heavy-metal catalyst was involved. Cytochrome, polyphenol, and ascorbic-acid oxidases are known to be inhibited by the above compounds. The photo-reversibility of CO₂ inhibition has been used to differentiate between cytochrome oxidase and polyphenol oxidase. This differentiation in a photosynthetically active tissue would be of dubious value.

Various investigators have observed dissimilarities of cyanide and azide inhibition of cellular respiration. Ball (11) has suggested that Atmungsferment-azide and Atmungsferment-cyanide compounds may have different oxidation-reduction potentials which could give them different catalytic powers. Winzler (12) studying the effect of azide and cyanide on yeast respiration concluded that cyanide can inhibit respiration three different ways while azide has only one type of inhibition—its combination with the oxidized Atmungsferment. Therefore, he considered azide to be a more specific inhibitor of the Atmungsferment than cyanide.

The foregoing facts indicate that cyanide and azide do not inhibit in parallel manner under all conditions in all tissues. Our data suggest that racemosus leaves contained a respiratory enzyme which was highly azide sensitive. Since azide inhibited over 90 percent of O₂ uptake and CO₂ formation, it must be concluded that the bulk of the respiration in the racemosus leaves passed through an azide-sensitive system, probably a cytochrome oxidase.

Ascorbic acid has been suggested as a possible co-factor in one step of tyrosine oxidation (13). Experiments were carried out to determine whether in racemosus leaf ascorbic acid may act as a link to other oxidation-reduction systems. For this purpose 2,6-dichlorophenolindophenol (DCPP), a substance known to destroy ascorbic acid, was added to the substrate. The effects of DCPP on the O₂ uptake in the racemosus leaf are given in Table 2. In lower concentrations the dye had a slight stimulating effect on the O₂ uptake in the first hour. At the end of four hours the rate of O₂ uptake decreased in proportion to the amount of DCPP added. The DCPP in the substrate was completely decolorized at the end of 4 hours in all concentrations up to 1 mg. An excess of DCPP (2 mg)
FIG. 1—Inhibition of $O_2$ uptake and $CO_2$ formation by 0.001 and 0.01 M KCN and 0.01 M NaN₃ at varying intervals. $O_2$ uptake and $CO_2$ formation without the inhibitors taken as 100 percent.
TABLE 2—Effect of 2-6 Dichlorophenol-indophenol (DCPP) on Respiration of A. Racemosus Leaves*

<table>
<thead>
<tr>
<th>Addendum</th>
<th>mg</th>
<th>1st hour</th>
<th>2nd hour</th>
<th>3rd hour</th>
<th>4th hour</th>
<th>Percent inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.00</td>
<td>83</td>
<td>68</td>
<td>64</td>
<td>63**</td>
<td></td>
</tr>
<tr>
<td>DCPP</td>
<td>0.05</td>
<td>86</td>
<td>74</td>
<td>68</td>
<td>62</td>
<td></td>
</tr>
<tr>
<td>DCPP</td>
<td>0.20</td>
<td>89</td>
<td>74</td>
<td>64</td>
<td>59</td>
<td>6</td>
</tr>
<tr>
<td>DCPP</td>
<td>0.50</td>
<td>89</td>
<td>72</td>
<td>64</td>
<td>54</td>
<td>14</td>
</tr>
<tr>
<td>DCPP</td>
<td>1.00</td>
<td>80</td>
<td>67</td>
<td>55</td>
<td>52</td>
<td>17</td>
</tr>
<tr>
<td>DCPP</td>
<td>2.00</td>
<td>82</td>
<td>68</td>
<td>55</td>
<td>48**</td>
<td>24</td>
</tr>
</tbody>
</table>

*40-day-old leaves.
**The dye was not completely decolorized.
***The final readings of the controls are considered as 100 percent.

reduced the $O_2$ uptake 24 percent. Inactivation of ascorbic acid in the leaves by DCPP and the subsequent decrease in the rate of respiration suggested that the racemosus leaves may contain an ascorbic-acid-enzyme system.

The influence of various amounts of ascorbic acid on respiration of the leaves was studied and the results are presented in Table 3. The addition of 2 mg of ascorbic acid increased the $O_2$ uptake and the $CO_2$ output in the first hour 76 and 78%, respectively, over that of the control. The loss of ascorbic acid during the experiment due to high pH (6.0) may have been an important factor in the drop in respiration of the leaf at the third and fourth hour of the experiment. That the increase in $O_2$ uptake and in $CO_2$ formation was enzymatic and not due to reduction of the acid in the tissues was indicated by the sensitivity of respiration to cyanide inhibition in the presence of added ascorbic acid. The addition of 2 mg of ascorbic acid in the presence of 0.33 mg of cyanide had no effect on cyanide-inhibited respiration of racemosus

TABLE 3—Effect of Ascorbic Acid (AA) and KCN on Respiration of A. Racemosus Leaves

<table>
<thead>
<tr>
<th>Addenda</th>
<th>mg</th>
<th>$O_2$ Consumed</th>
<th>CO$_2$ Output</th>
<th>Percent increase 1st hour</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1st hour</td>
<td>4th hour</td>
<td>1st hour</td>
</tr>
<tr>
<td>Control</td>
<td>0.00</td>
<td>74**</td>
<td>60</td>
<td>76**</td>
</tr>
<tr>
<td>AA</td>
<td>1.00</td>
<td>107</td>
<td>70</td>
<td>129</td>
</tr>
<tr>
<td>AA</td>
<td>2.00</td>
<td>130</td>
<td>77</td>
<td>135</td>
</tr>
<tr>
<td>KCN</td>
<td>0.33</td>
<td>40</td>
<td>12</td>
<td>49</td>
</tr>
<tr>
<td>KCN* + AA</td>
<td>2.00</td>
<td>34</td>
<td>7</td>
<td>43</td>
</tr>
</tbody>
</table>

*0.33 mg KCN.
**The first-hour readings of control are considered as 100 percent.
leaves (Table 3). The increased rate of respiration with addition of ascorbic acid would suggest that an ascorbic-acid system may serve as a link between oxidation-reduction systems in the racemosus leaves.

Dehydrogenases of A. racemosus Leaves

Iodoacetate is known to inhibit numerous dehydrogenases, among which are malic dehydrogenase, glyceraldehyde dehydrogenase, and alcohol dehydrogenase. The inhibition by iodoacetate is assumed to be due to the oxidation of essential sulphydryl groups of the apoenzyme. Commoner and Thimann (14) have shown that iodoacetate inhibited the respiration of *Avena* coleoptiles by its action on the organic-acid metabolism. Bonner and Wildman (3) found that the cytoplasmic protein of spinach leaf was able to reduce thionine in the presence of a coenzyme. The cytoplasmic proteins contained isocitric, glutamic, malic, and alcohol dehydrogenases in addition to the enzyme aconitase. Methylene blue and thionine dyes used in plant and animal tissues as indicators for the presence of succinic dehydrogenase were not effective for determination of this enzyme in *A. racemosus* whole leaves.

The respiration of *A. racemosus* leaf was inhibited by iodoacetate as indicated in Table 4. In 2 hours 0.3 mg of iodoacetate inhibited O₂ uptake and CO₂ formation 19 and 24 percent, respectively. As the concentration of the iodoacetate was increased to 3.0 mg, there was a corresponding decrease in the rate of respiration. Increasing the inhibitor to 5.0 mg produced no further decrease in the respiration at the end of 2 hours. These results indicate that about 45 percent of O₂ uptake and CO₂ evolution in the racemosus leaves was mediated through a dehydrogenase system which was iodoacetate sensitive. In order to establish the presence or absence of the glycolytic, phosphorolytic conversion of the intermediates to pyruvic acid, which is the key figure in carbohydrate, protein, and fat metabolism, various intermediate pathways for the metabolism of organic acids were carried out.

<table>
<thead>
<tr>
<th>Addendum</th>
<th>O₂ uptake (mg)</th>
<th>CO₂ output (2nd hour)</th>
<th>Percent inhibition</th>
<th>O₂</th>
<th>CO₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.0</td>
<td>67*</td>
<td>69*</td>
<td>19</td>
<td>24</td>
</tr>
<tr>
<td>Sodium iodoacetate</td>
<td>0.3</td>
<td>54</td>
<td>52</td>
<td>46</td>
<td>42</td>
</tr>
<tr>
<td>Sodium iodoacetate</td>
<td>3.0</td>
<td>36</td>
<td>40</td>
<td>45</td>
<td>42</td>
</tr>
<tr>
<td>Sodium iodoacetate</td>
<td>5.0</td>
<td>37</td>
<td>40</td>
<td>45</td>
<td>42</td>
</tr>
</tbody>
</table>

*The final readings of control considered as 100 percent.
Role of Organic Acids in the Metabolism of A. racemosus Leaf

The use of the Thunberg technique gave indefinite results for the demonstration of succinic dehydrogenase in racemosus leaves. The inhibition by iodoacetate indicated that considerable amounts of dehydrogenases may be present in the leaves. Therefore the effect of malonate, known specific inhibitor of succinic dehydrogenase, was investigated.

The respiration of leaves was inhibited by addition of malonic acid, but it was not affected by sodium malonate, as indicated in Table 5. Sodium malonate in concentrations of 5, 10, and 20 mg at pH 6.0 had no effect on respiration of the leaves. The addition of 3 mg of malonic acid at pH 3.0 produced slight stimulation in the respiration, and this increase was maintained during the 4 hours in which the experiment was in progress. As the concentration of malonic acid was increased to 10 and 15 mg, the respiratory rate decreased 40 and 53 percent, respectively. Malonate at these concentrations not only depressed the $O_2$ uptake but at the same time the $CO_2$ output was reduced. In all cases the R. Q. was one, or near one. Beevers (15) reported that in root tissue of maize an increase in the malonic-acid concentration rapidly increased the R. Q. He concluded that in maize-root tissue there were at least two distinct effects of malonate, one aerobic and the other anaerobic. Although in these experiments high concentration of malonic acid were used, no two distinct effects on the respiration with the addition of malonic acid were observed.

Previous investigators have indicated that higher concentrations of malonate were required for inhibition of plant respiration than those reported in animal tissue. The importance of pH for malonate inhibition was reported by Bonner and Wildman (3) in their studies on respiration of spinach leaves. They found that malonate at pH 7.0 was without effect while at pH 4.5 the respiration was inhibited about 30 percent. Since the $O_2$ uptake and the $CO_2$ evolution were effected, they concluded

<table>
<thead>
<tr>
<th>Addenda</th>
<th>mg</th>
<th>$O_2$ uptake</th>
<th>CO₂ output</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1st hr.</td>
<td>2nd hr.</td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>79</td>
<td>68</td>
</tr>
<tr>
<td>Sodium malonate*</td>
<td>5</td>
<td>74</td>
<td>68</td>
</tr>
<tr>
<td>Sodium malonate*</td>
<td>10</td>
<td>77</td>
<td>67</td>
</tr>
<tr>
<td>Sodium malonate*</td>
<td>20</td>
<td>79</td>
<td>64</td>
</tr>
<tr>
<td>Malonic acid**</td>
<td>3</td>
<td>83</td>
<td>67</td>
</tr>
<tr>
<td>Malonic acid**</td>
<td>10</td>
<td>67</td>
<td>49</td>
</tr>
<tr>
<td>Malonic acid**</td>
<td>15</td>
<td>52</td>
<td>42</td>
</tr>
</tbody>
</table>

* pH 6.0.
** pH 3.0.
that succinic dehydrogenase not only was the central enzyme for the O₂ uptake but also was an essential part of the CO₂-liberating mechanism in spinach leaves. Our results agree with those of Bonner and Wildman (3) and are in disagreement with Beevers' (15) findings. These contradictions as to the effect of malonate in plant respiration reported by previous investigators may be ascribed to the fact that only undissociated malonic acid can enter into the cell, according to Turner and Hanly (16).

The role of organic acids in plant respiration has been demonstrated in Avena coleoptile (14), in potato tuber slices (17), and in spinach leaves (3). The inhibitory action of malonate on respiration of the racemosus leaf suggested that, if the malonate blocked the action of succinic dehydrogenase, the addition of succinate would reverse this inhibition. Table 6 gives the effect of succinate on malonate inhibition. Malonate inhibition was partially reversed with 15 mg succinate and completely reversed with 30 mg succinate, but 6 mg succinate had no effect on the malonate inhibition. By increasing the succinate/malonate ratio in the substrate, there was a corresponding decrease in the inhibition of the enzyme by malonate.

The addition of components of the Krebs cycle modified to some extent the malonate inhibition in the racemosus-leaf respiration as summarized in Table 7. Fumarate, malate, citrate, and α-ketoglutarate reversed the malonate inhibition. An increased O₂ uptake and CO₂ evolution observed with addition of 30 mg of fumarate would indicate that racemosus leaves contain an active fumarase. The rapid rise in the O₂ uptake and CO₂ liberation with the addition of 30 mg of citrate suggested that the leaves contained aconitase in considerable amounts. Malate and α-ketoglutarate action on malonate inhibition was not evident at the first hour. However, as the rate of respiration dropped in the control, the effect of the added substances was evident. The slower action of malate and α-ketoglutarate may be due to a slower rate of diffusion of these compounds into the leaves or the less active enzyme systems. The reversal of malonate-inhibited respiration in the leaves by addition of organic acids would suggest that a cycle similar to the

### Table 6—Effect of Succinate on Malonate-Inhibited Respiration of A. Racemosus Leaves

<table>
<thead>
<tr>
<th>Addenda</th>
<th>mg</th>
<th>O₂ uptake</th>
<th>CO₂ output</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1st hour</td>
<td>4th hour</td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>76</td>
<td>59</td>
</tr>
<tr>
<td>Malonate*</td>
<td>15</td>
<td>56</td>
<td>21</td>
</tr>
<tr>
<td>Malonate + succinate</td>
<td>6</td>
<td>46</td>
<td>24</td>
</tr>
<tr>
<td>Malonate + succinate</td>
<td>15</td>
<td>55</td>
<td>34</td>
</tr>
<tr>
<td>Malonate + succinate</td>
<td>30</td>
<td>89</td>
<td>61</td>
</tr>
</tbody>
</table>

*15 mg malonate was used with varying amounts of succinate.
TABLE 7—Effect of Organic Acids on Malonic-Acid Inhibition of A. Racemosus Leaf Respiration

<table>
<thead>
<tr>
<th>Addenda</th>
<th>mg</th>
<th>1st hour</th>
<th>4th hour</th>
<th>1st hour</th>
<th>4th hour</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>76</td>
<td>59</td>
<td>76</td>
<td>57</td>
</tr>
<tr>
<td>Malonate*</td>
<td>15</td>
<td>54</td>
<td>23</td>
<td>63</td>
<td>26</td>
</tr>
<tr>
<td>Malonate + fumarate</td>
<td>6</td>
<td>74</td>
<td>38</td>
<td>88</td>
<td>42</td>
</tr>
<tr>
<td>Malonate + fumarate</td>
<td>30</td>
<td>109</td>
<td>57</td>
<td>132</td>
<td>69</td>
</tr>
<tr>
<td>Malonate + malate</td>
<td>15</td>
<td>58</td>
<td>43</td>
<td>n.d.**</td>
<td>n.d.**</td>
</tr>
<tr>
<td>Malonate + malate</td>
<td>30</td>
<td>58</td>
<td>56</td>
<td>n.d.**</td>
<td>n.d.**</td>
</tr>
<tr>
<td>Malonate + citrate</td>
<td>30</td>
<td>92</td>
<td>64</td>
<td>105</td>
<td>68</td>
</tr>
<tr>
<td>Malonate + ketoglutarate</td>
<td>15</td>
<td>51</td>
<td>47</td>
<td>n.d.**</td>
<td>n.d.**</td>
</tr>
</tbody>
</table>

*15 mg malonic acid was used in all experiments.
**Not determined.

Krebs cycle functions in the A. racemosus leaves.

Table 8 gives the results of the effect of oxaloacetate on respiration of the leaves. Malonate inhibition was reversed with the addition of oxaloacetate in the first hour, but the rate of O₂ uptake at the end of four hours decreased to about 40 percent of that of the control. Boswell (4) observed that the O₂ uptake was small, and the CO₂ output was very slightly reduced in potato tuber in the presence of malachite green and oxaloacetic acid, and he suggested that the decomposition of oxaloacetic acid was due to a decarboxylation unaccompanied by oxidation. He also stated that this reaction may not be enzymatic since oxaloacetic acid is unstable and is very rapidly decomposed in the presence of proteins. When an excess of oxaloacetate was added to racemosus leaves, the rate of O₂ uptake was constant during the last three hours of the experiment. This would suggest that the gradual decrease in O₂ uptake at the 3rd and 4th hours in the presence of malonate was due to the rapid conversion of oxaloacetate to succinate, which produced an increase in the malonate/succinate ratio with subsequent reduc-

TABLE 8—Effect of Oxaloacetate (OA) and Pyruvate on Malonic-Acid Inhibition of A. Racemosus Leaf Respiration

<table>
<thead>
<tr>
<th>Addenda</th>
<th>mg</th>
<th>1st hour</th>
<th>2nd hour</th>
<th>3rd hour</th>
<th>4th hour</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>69</td>
<td>68</td>
<td>59</td>
<td>55</td>
</tr>
<tr>
<td>Malonate</td>
<td>15</td>
<td>46</td>
<td>35</td>
<td>26</td>
<td>20</td>
</tr>
<tr>
<td>OA</td>
<td>15</td>
<td>119</td>
<td>69</td>
<td>65</td>
<td>62</td>
</tr>
<tr>
<td>Malonate* + OA</td>
<td>15</td>
<td>85</td>
<td>47</td>
<td>40</td>
<td>34</td>
</tr>
<tr>
<td>Malonate + OA** + pyruvate</td>
<td>30</td>
<td>117</td>
<td>53</td>
<td>44</td>
<td>38</td>
</tr>
</tbody>
</table>

*15 mg malonate.
**15 mg oxaloacetate (OA).
tion in the rate of respiration. The addition of oxaloacetate and pyruvate to the malonate-poisoned leaves showed an \( \text{O}_2 \) consumption similar to that of oxaloacetate. This would suggest that the limiting factor in respiration was not pyruvate, but the rapid oxidation of oxaloacetate.

That pyruvic acid is an intermediate in the normal breakdown of hexose has been demonstrated by various investigators. James and co-workers (18, 19) established that hexose diphosphate, phosphoglycerate, and pyruvic acid are intermediates in the breakdown of hexose by barley leaves. In a preliminary study the carbohydrate breakdown in \( A. \) racemosus leaves and its relation to the organic acids of the Krebs cycle was investigated. The inhibition of glycolysis by fluoride in animal and plant tissues has been long recognized. Although many enzymatic steps are inhibited by fluoride, enolase is the most sensitive to the action of fluoride. The enzyme enolase is responsible for conversion of 2-phosphoglycerate to pyruvic acid.

Investigation of the inhibitory effect of fluoride on the respiration of racemosus leaves showed considerable differences in the sensitivity of leaves to the inhibitory effects of fluoride. These differences were directly related to the age of the plants. Four-month-old leaves showed 10-15 percent decrease in respiration when compared with young leaves (5 to 8 weeks old). Less fluoride was needed to inhibit the respiration in the older leaves than in younger leaves. With older leaves 2, 4, and 8 mg of fluoride reduced the respiration 29, 50, and 64 percent, respectively. In young leaves 16 mg of fluoride inhibited only 46 percent of respiration. The differential inhibitory effect of fluoride on the respiration may have been due to the decrease of enolase in older plants. In younger and older leaves fluoride inhibited both the \( \text{O}_2 \) uptake and \( \text{CO}_2 \) output. Table 9 gives the results of the effect of sodium fluoride on the respiration of racemosus leaves. The results indicate that fluoride inhibition of the respiration was involved with the formation of pyruvic acid. The inhibition of fluoride could be eliminated by addition of 9 mg of pyruvate. However, the addition of 25 mg of glucose to the fluoride-poisoned leaves had no effect on the respiration. These results suggest that hexose breakdown in racemosus leaves, like other plant and animal tissues, passes through

<table>
<thead>
<tr>
<th>Addenda</th>
<th>mg</th>
<th>( \text{O}_2 ) uptake 1st hour</th>
<th>( \text{O}_2 ) uptake 4th hour</th>
<th>( \text{CO}_2 ) output 1st hour</th>
<th>( \text{CO}_2 ) output 4th hour</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control*</td>
<td>0</td>
<td>71</td>
<td>63</td>
<td>70</td>
<td>60</td>
</tr>
<tr>
<td>NaF</td>
<td>16</td>
<td>56</td>
<td>34</td>
<td>59</td>
<td>33</td>
</tr>
<tr>
<td>NaF** + glucose</td>
<td>25</td>
<td>60</td>
<td>39</td>
<td>67</td>
<td>39</td>
</tr>
<tr>
<td>NaF + pyruvate</td>
<td>9</td>
<td>76</td>
<td>50</td>
<td>84</td>
<td>49</td>
</tr>
</tbody>
</table>

*Leaves were 8 weeks old.
**15 mg NaF.
the phosphorylated intermediates and leads to the formation of pyruvic acid. The fact that fluoride inhibition was reversed by pyruvate, and the malonate inhibition by fumarate, suggest that these acids may be involved in respiration of racemosus leaves in a manner similar to that suggested by the Krebs cycle. Results of these interactions are shown in Table 10. The oxidation of pyruvate in the racemosus leaves is linked with that of fumarate, as indicated by the ability of pyruvate and fumarate to reverse the inhibitory effects of malonate and fluoride. Neither pyruvate nor fumarate alone increased the respiration in the fluoride-malonate-inhibited leaves. The addition of both fumarate and pyruvate brought about significant increases in the $O_2$ uptake and $CO_2$ evolution during the experiment. These results gave further evidence that organic acids are involved in the respiration of $A. racemosus$ leaves.

**DISCUSSION**

Studies on respiration in the leaves of $A. racemosus$, a selenium accumulator, indicated that its main course of respiration was carried out by enzyme systems known to be present in non-seleniferous plants. The major part of the respiration appeared to be mediated through the oxidase and dehydrogenase systems connected by a series of intermediate components of the Krebs citric-acid cycle. There are at least 14 different pathways for the metabolism of pyruvic acid in various plant and animal tissues, but the principal pathway seems to be the mechanism resembling the Krebs cycle. There is little doubt that most of the respiration was mediated by the heavy-metal-containing enzymes. The enzyme polyphenol oxidase was inhibited to a lesser degree by catechol than by phenol, which had a nitro group in the para position. o-Nitrophenol had no effect on respiration of the leaves. That poly-

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phenol oxidase acts on only specific substrates was indicated by the studies of Boswell (4), who investigated the effect of 15 phenolic compounds on the respiration of potato tuber. He found that only catechol, homocatechol, and p-cresol inactivated the enzyme, while dihydroxyphenylalanine, caffeic, and gallic acids formed cyclic redox systems with the oxidase present in potato tissue. James and Cragg (8) found no polyphenol oxidase in barley extract. They reported that the ascorbic-acid system was the active oxidizer in barley. Our results indicate that there is an ascorbic-acid system present in racemosus leaves. The increased rate of O₂ uptake and CO₂ formation suggest that an ascorbic-acid system forms a part of the oxidation-reduction system in the racemosus leaf.

Azide and cyanide, known inhibitors of oxidases, inhibited the respiration in the leaf, and the inhibition of respiration by azide was more severe than by cyanide. The bulk of O₂ uptake and CO₂ formation was inhibited by azide, which would suggest that an azide-sensitive oxidase was the terminal oxidase in respiration of the leaves. That this differential inhibition was not an artifact was indicated by some preliminary studies with radioactive selenium. When the respiration of the leaves was inhibited with azide, the Se⁷⁵ uptake was 50 percent less than in the cyanide-inhibited leaves. The significance of these findings can be evaluated only by separation of the azide-cyanide-sensitive system from the leaves.

The inhibitory effect of fluoride on respiration of the leaves suggested that hexose was phosphorylated and converted to phosphoglyceric acid and to pyruvic acid. Whether the phosphorylation of hexose in racemosus leaves is similar to that in other tissues was not investigated. However, the inhibition of respiration by fluoride was reversed by addition of pyruvate, while the addition of glucose had no effect on the rate of respiration. This would suggest that the hexose breakdown in the leaves would ultimately result in the formation of pyruvic acid.

Under aerobic conditions pyruvic acid is oxidized by the known reactions of the tricarboxylic-acid cycle. The reaction initiated by dehydrogenase in the presence of CoA and DPN requires a divalent cation and a thiamine derivative, usually phosphothiamine (20). Many of the enzymatic steps require divalent cations or anions for their activity. It is possible that selenium may be utilized in the various metabolic reactions by substituting for the cations or anions in the reaction. Selenium-indicator plants have a characteristic odor which is associated with the formation of volatile selenium compound(s), presumably methyl or ethylselenide. Rosenfeld and Beath (21) demonstrated in vitro experiments with beef liver, spleen, whole blood, and plasma that these tissues converted the selenate to selenite, volatile selenium, and elemental selenium. This conversion was enzymatic, since the tissues lost their ability to act on selenate when they were heated. Selenium is also volatilized by the plants, and it is possible that similar enzymatic reactions are responsible for its volatilization.

The difficulty of determining the
functions of selenium in indicator plants arises from the inherent characteristics of these plants, that is, selenium-accumulator plants always contain high concentrations of selenium. The seeds of selenium-indicator plants store selenium in high concentration during maturation. At the time of germination and during growth the seedlings utilize the stored selenium for the metabolic activities of the plant. If no selenium is supplied to the plant, the rate of growth is reduced and the plant withers and dies.

Since selenium is always present in the indicator plants, the study of isolated systems may give an answer or partial answer to the functions of selenium in the metabolism of selenium-indicator plants.

**SUMMARY**

The enzymatic mechanism in respiration of the leaves of *Astragalus racemosus*, a selenium-accumulator plant, was studied in vitro.

The oxidation systems studied were polyphenol, cytochrome, ascorbic-acid oxidases, and the organic acids of the tricarboxylic-acid cycle.

*A. racemosus* leaves contained only limited amounts of polyphenol oxidase, which was sensitive to catechol.

Azide and cyanide did not inhibit respiration in a parallel manner. Azide inhibited over 90 percent of O₂ uptake and CO₂ formation, suggesting that the cytochrome oxidase was the terminal oxidase in respiration of racemosus leaves.

The addition of 2,6-dichlorophenol-indophenol destroyed ascorbic acid and reduced the rate of respiration. The addition of ascorbic acid increased the O₂ uptake and CO₂ formation. That this reaction was enzymatic and not artifact was indicated by the fact that KCN-depressed respiration was not reversed by the addition of ascorbic acid. Ascorbic-acid oxidase may be the co-enzyme of the oxidation-reduction system directly involving oxygen.

Malonate inhibition was reversed by addition of succinate. Fumarate, malate, oxaloacetate, citrate, and α-ketoglutarate reversed malonate inhibition in varying degrees.

Fluoride inhibited the respiration by preventing the formation of pyruvate. Fluoride inhibition was decreased by addition of pyruvate but was not affected by addition of glucose.

Fluoride-malonate inhibition could be reversed by addition of both fumarate and pyruvate.

These results suggest that the respiration in the leaves of *A. racemosus* was mediated by a cycle similar to, if not identical with, the Krebs citric-acid cycle.

**REFERENCES**

Part II

A. Translocation of Radioactive Selenium in Astragalus bisulcatus

By Irene Rosenfeld and Harold F. Eppson*

THE TRANLOCATION of Se\textsuperscript{75} was studied in a selenium-accumulator plant. There is considerable evidence of a characteristic translocation of organic substances and minerals in plants.

The first step in translocation of the elements which are available to the root surfaces is by the xylem vessels. The root will retain some of the available substance for its growth; the rest is transmitted to the aerial parts of the plant. The deposition of radioiron in leaves has been shown by Rediske (1). He observed that under changing pH he was able to increase the iron uptake in plants. Biddulph (2) studied the \textsuperscript{32}P, \textsuperscript{35}S, and \textsuperscript{45}Ca uptake in the leaves. He reported that, when \textsuperscript{32}P and \textsuperscript{35}S were added to a solution, these elements were rapidly taken up by the plants and, if the plants were placed in phosphorus- and sulfur-free solutions, redistribution of the available \textsuperscript{32}P to new growth regions was made during the period of growth. The distribution of \textsuperscript{45}Ca in plants differed from sulfur and phosphorus in that, once \textsuperscript{45}Ca was deposited, it remained at the site of deposition.

MATERIALS AND METHODS

Se\textsuperscript{75}Cl\textsubscript{4} and K\textsubscript{2}SeO\textsubscript{4} were adsorbed on 100 gms of a 50-50 mixture of Amberlite IR4B and IR120. The resin was divided into three parts. Each part contained 5 x 10\textsuperscript{5} c/m Se\textsuperscript{75} and 90 mg. K\textsubscript{2}SeO\textsubscript{4}. The resin with the adsorbed selenium was mixed with 4 kgs. of soil and \textit{A. bisulcatus} seeds were planted. Samples were collected at 3 and 6-month intervals during growth and analyzed for selenium. The total selenium and the radioactive selenium present in the samples were determined.

The radioactive selenium in the leaves was localized by radioautographs on X-ray film 210 days after planting of the seeds. The time of exposure of the film was 4 and 8 days.

*Professor and Associate Professor, respectively, of Agricultural Biochemistry. The technical assistance of Sadako Hayase is acknowledged.
RESULTS AND DISCUSSION

The results of the selenium uptake from the soil are given in Table 1. In three months the leaves, stems, and roots had absorbed a considerable amount of selenium, and the selenium was distributed in all parts of the plant. The distribution of total selenium and Se\textsuperscript{75} indicated that, as selenium is taken up by the root of the plant, it is translocated to the leaves and stems, since these organs contain a larger amount of radioactive selenium than the roots. At the end of six months there was considerable reduction in the amount of selenium in the leaves and roots. The stems contained approximately the same amount of total selenium, but the concentration of radioactive selenium was higher than it was at the end of three months, suggesting that selenium does not remain immobile in the plant after it is absorbed from the soil but is translocated within the same plant during its growth. The differences in distribution of radioactive and non-radioactive selenium in the plants may be due to the non-uniform distribution of the two forms of selenium on the resin mixture which supplied the selenium to the plants.

The distribution of the radioactive selenium in the plants grown in the greenhouse was studied by radioautographs. Fig. 1 shows the distribution of Se\textsuperscript{75} in the leaves harvested 210 days after the seeds were planted. The newly formed leaves and the growing leaves and stems contained more radioactivity than the older leaves. These results suggest that during growth selenium-accumulator plants either concentrate or need a greater amount of selenium for their metabolic activities. The younger leaves on the left contain higher concentrations of Se\textsuperscript{75} when compared with the older leaves on the right.

Some leaves which were yellow and had lost most of the chlorophyll were selected for radioautograph studies. Fig. 2 shows the radioautograph of the yellow plant. This radioautograph indicates that with the loss of chlorophyll there was also a loss of radioactive selenium. This poses the question whether there is any relation between the presence of selenium in the plant and the pigmentation of the plant. This question can be answered only by chemical studies and isolation of the chlorophyll.

<table>
<thead>
<tr>
<th>Harvested after planting</th>
<th>Crock no.</th>
<th>Leaves</th>
<th>Stems</th>
<th>Roots</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Total selenium*</td>
<td>Se\textsuperscript{75}**</td>
<td>Total selenium*</td>
</tr>
<tr>
<td>3 months</td>
<td>I</td>
<td>12.1</td>
<td>3.6 x 10\textsuperscript{4}</td>
<td>6.2</td>
</tr>
<tr>
<td>6 months</td>
<td>II</td>
<td>3.6</td>
<td>1.5 x 10\textsuperscript{4}</td>
<td>6.9</td>
</tr>
<tr>
<td>6 months</td>
<td>III</td>
<td>2.2</td>
<td>5.0 x 10\textsuperscript{4}</td>
<td>4.0</td>
</tr>
</tbody>
</table>

*mg Se/gm tissue.
**c/m/gm (corrected for decay).
FIG. I—Radioautograph of *A. bisulcatus* showing the Se$^{75}$ distribution 210 days after the seeds were planted. From left to right the leaves show the differential uptake of Se$^{75}$ in the leaves.

FIG. II—Radioautograph of chlorotic leaves of *A. bisulcatus* showing less Se$^{75}$ uptake than normal leaves. Midribs of leaflets and rachis of leaves contain the most radioactivity.
Fig. 3 shows two branches of *A. bisulcatus*, one actively growing and the other in a state of senescence. The leaves which are ready to drop contain very little Se$^{75}$ while the stem appeared to contain a great deal of radioactivity, indicating that either the stems withdrew the selenium from the dying leaves, or there was some damage in the transport of Se$^{75}$ to the leaves.

These radioautographs indicate that tissues undergoing the most active growth appear to have the greatest capacity for accumulating selenium in contrast with cells that are physiologically less active. Selenium is highly mobile in plants. The internal redistribution of this element occurs readily and more or less continuously during the life of the plant. Older leaves and other structures lose selenium, which is then translocated to growing regions. It is possible that the mobility of selenium in the plants is related to the physiological activity of the plant.

**FIG. III—Two branches of *A. bisulcatus*: one actively growing (8-day exposure time) with high concentration of Se$^{75}$; the other branch, having lost several leaves, dying with only a small amount of radioactivity (exposure time 2 weeks).**

**SUMMARY**

The translocation of Se$^{75}$ in *A. bisulcatus*, a selenium accumulator, was investigated *in vivo*. Analysis of leaves, stems, and roots of *A. bisulcatus* grown in soil containing Se$^{75}$ and K$_2$SeO$_4$ indicates that not all parts of the plant contained the same amount of selenium. The roots as they absorb the selenium...
from the soil do not store it but translocate it to the aerial parts of the plant. The stems served as a translocating organ and always contained a large amount of selenium.

Radioautographs of the leaves indicated that there is definite relation between metabolic activity and presence of selenium in the plant. As the leaves lose their pigments there is a decrease in the selenium content.

REFERENCES


B. Localization of Se\(^{75}\) in Astragalus bisulcatus Leaf Fractions

By Irene Rosenfeld

The preceding results indicated that the high concentration of selenium in indicator plants was present in the leaves and leaflets of these plants. In order to get a better understanding of the occurrence of selenium in "selenium-accumulator" plants, some of the morphological components of the leaves of Astragalus bisulcatus were separated to determine the distribution of Se\(^{75}\) in relation to the proteins in these fractions.

Chloroplasts contain physiologically active substances such as chlorophylls, protein, carotenes, and lipids (1, 2, 3). They are also a site of enzymatic activity. It was, therefore, of interest to see whether selenium was localized in this fraction. The leaves of A. bisulcatus were fractionated into various components, and the relation between the protein and selenium content of the various fractions was determined.

METHODS

A. bisulcatus was grown in the greenhouse in soil containing radioactive selenium (indicated in Section A, page 21). Mature leaves from the plants were harvested three hours before use. The harvested leaves were washed, the midribs removed, and the remainder cut into small pieces. The cut pieces were placed in the dark for 3 hours to reduce the amount of starch since starch interfered with the fractionation. All subsequent steps in preparation of the
material were carried out at about 4°C. About 10 grams of leaves were put into a Waring blender with 150 ml. of a cold solution of M/2 sucrose in 0.01 M phosphate buffer at pH 6.8. High dilution was used to facilitate blending and to dilute the possibly toxic vacuolar contents. The tissue was blended at full speed for 2 minutes.

After blending, the slurry was fractionated according to the scheme shown in Fig. 4.

Most of the whole cells and cell walls (residue) were strained out with shark-skin filter paper lining the basket centrifuge (5). The chloroplasts and nuclei pass through this filter. In order to remove the whole cells as much as possible, the filter paper was sealed with waterproof tape, but a few epidermal hair and mesophyll cells frequently appeared in the strained homogenate. This method was found to be the most effective for separation of the cellular components. The method was a modification of that of McClendon (6) and Wildman and Bonner (5).

Centrifugation was carried out in an International refrigerated centrifuge size 1, using the regular angle head or the multi-speed attachment. For convenience the suspensions were indicated as follows: S-1, that fraction which remained in suspension in the supernatant liquid at a speed up to 5000 x g; S-2, that which remained in suspension at 19,000 x g for 30 minutes; S-3, the amber solution free of chloroplast and pigment (19,000 x g for 1 hour); and S-4, the S-3 solution freed of protein by precipitation with trichloroacetic acid. The residues (up to 60 x g) contained the cell walls and the whole cells. The residues were pooled and diluted to volume and an aliquot was analyzed. The green sediments, which contained the chloroplasts and other particulate matter of the cells from 2200 x g to 19,000 x g, were pooled and analyzed in toto. All fractions were analyzed for nitrogen and Se$^{75}$.

The radioactivity of the leaf homogenate and of the separated fractions was measured by use of a gas-flow counter. The total activity present in the whole homogenate was taken as 100 percent.

The nitrogen determinations were carried out on duplicate aliquots of the various fractions by the Kjeldahl method or by direct Nesslerization. This method directly determined the total nitrogen; the protein was calculated by the usual factor (N x 6.25).

RESULTS AND DISCUSSION

Separation of the pigments from the protoplasmic components of the leaves presented considerable difficulties. Various investigators have studied the factors which influence the fractionation of chloroplasts from other components (4, 5, and 6).

The chloroplasts of A. bisulcatus appeared to be extremely fragile. Most of the chloroplasts broke and the grana was liberated which remained in colloidal suspension in the supernatant liquid. By fractional centrifugation the pigmented fractions were separated from the other components of the leaves.
FIG. IV—A. bisulcatus leaves
and
M/2 sucrose and M/100 phophate buffer pH 6.8
Waring blender 2 minutes
Leaf homogenate
Strained through sharkskin filter paper
500 rpm 20 minutes

Residues:

1. Cell walls and whole cells
   Green suspension
   60 x g 10 min.

2. Whole cells
   Green suspension

Green sediments:

1. Whole chloroplasts
   Green suspension
   2200 x g 30 min.

2. Broken chloroplasts and nuclei
   Green suspension (S-1)
   5000 x g 15 min.

3. Green pigment and mitochondria
   Green suspension (S-2)
   19,000 x g 30 min.

4. Green pigment
   Amber solution cytoplasmic fluid (S-3)
   19,000 x g 1 hr.
   TCA supernatant (S-4)
TABLE 2—Distribution of SE\textsuperscript{75} and Its Relation to Plant Proteins in the Leaf of A. bisulcatus

<table>
<thead>
<tr>
<th>Fractions</th>
<th>Se\textsuperscript{75} (%)</th>
<th>Protein (N x 6.25) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total macerate</td>
<td>100.0</td>
<td>100.0</td>
</tr>
<tr>
<td>Residues (cell walls &amp; unbroken cells)</td>
<td>13.0</td>
<td>15.0</td>
</tr>
<tr>
<td>Green sediments (chloroplast, grana, and other particulate matter)</td>
<td>0.2</td>
<td>22.0</td>
</tr>
<tr>
<td>S-1 Supernatant 5000 x g</td>
<td>63.0</td>
<td>45.5</td>
</tr>
<tr>
<td>S-2 Supernatant 19,000 x g</td>
<td>19.0</td>
<td>13.2</td>
</tr>
<tr>
<td>S-3 Supernatant (cytoplasmic fluid)</td>
<td>12.0</td>
<td>7.5</td>
</tr>
<tr>
<td>S-4 TCA supernatant</td>
<td>2.0</td>
<td>3.1</td>
</tr>
</tbody>
</table>

The suspending media and the pH of the media had considerable effect on separation of the fractions. The chloroplasts swelled and disintegrated after a few minutes in distilled water. The swollen chloroplast became granular and vacuolated. The grana appeared discoid and green, with smooth surfaces; it remained intact for several days in distilled water. The use of M/2 sucrose somewhat reduced the disintegration but did not completely eliminate this factor.

The second major problem in separation of the leaf components was the acid content of the vacuole, which caused coagulation of the protein. The normally soluble proteins were denatured and flocculated by the acid vacuolar juices, and the apparent cytoplasmic protein yield was at first very low. The use of phosphate buffer at pH 6.8 appeared to give the most consistent results. Further studies on the effect of pH and media on the separation of the cytoplasmic components of the leaf are needed before morphological components of the cells can be chemically characterized.

Table 2 presents the results on the Se\textsuperscript{75} and protein distribution in the various fractions of the leaves. These results indicate that the selenium existed in two forms: (1) in combination with proteins of the cells and (2) in combination with the cytoplasmic fluid of the cells. Considerable amounts of the isotope and nitrogen were present in the fractions which contained the cell walls and some of the whole cells. This may be due to adsorption or adherence of the denatured proteins on cells and cell walls. Denaturation of protein was due to the acidity of the vacuolar sap. The protein composition of the chloroplast must differ con-
siderably from that of the protoplasm and cytoplasmic sap, since they did not bind any appreciable amount of Se$_{75}$.

The quantity of chloroplastic material in leaf cells has been investigated by Galston (7), by determination of the chlorophyll/nitrogen ratio. He found that between 30 and 40 percent of the total leaf nitrogen was contained in the chloroplast of the tobacco leaf. Using the same ratio for computation, about 20 percent of total leaf nitrogen was present in the chloroplast of A. bisulcatus. The low uptake of Se$_{75}$ by the chloroplasts of A. bisulcatus and the low nitrogen content may be due to the loss of soluble proteins when fragmentation of the chloroplasts occurred during fractionation of cytoplasmic components of the leaf. The proteins in the chloroplastic material may be predominantly one type of protein or a mixture of several types of proteins; or they may be proteins combined with chlorophyll. However, these data suggest that either the chloroplast has proteins which differ from those of the cytoplasmic proteins, or proteins which have the capacity to bind elements such as selenium are present in the soluble form and are easily dissolved in sucrose and phosphate-buffer solution.

The three fractions of the homogenized plant supernatant contained most of the leaf proteins as well as Se$_{75}$. Supernatant (S-1) contained 63 percent of the Se$_{75}$ and had the highest concentration of protein. After precipitation of the protein the supernatant (S-4) contained only 3.1 percent of protein and 2.0 percent of Se$_{75}$.

These results indicate that more than 60 percent of Se$_{75}$ was bound with the proteins, and the remainder was present in the low-molecular-weight vacuolar substance and tissue fluids. It appears that in plant as well as in animal tissues, selenium has a characteristic affinity for the tissue proteins. It would be of considerable interest to determine whether the binding of selenium in plant as well as in animal tissue is due to the same group of proteins.

**SUMMARY**

A method for the fractionation of leaf protoplasm and chloroplasts was developed for a selenium-indicator plant, A. bisulcatus. Factors influencing the separation were discussed.

The distribution of Se$_{75}$ indicated that selenium does not occur in firm combinations with the proteins of the chloroplasts.

Most of the Se$_{75}$ was present in the cytoplasmic fractions. The fractions which contained the highest concentration of protein had proportionally high concentration of selenium.
REFERENCES

Part III
Absorption and Exchange of Se$^{75}$ by Astragalus preussii Root Seedlings*

By Irene Rosenfeld**

Although considerable attention has been given to the problem of salt absorption and transport in plants, no other group of plants studied affords a more provocative problem than the group of selenium-accumulating plants, Astragalus. Members of this genus have been known to accumulate 3,000 or more ppm selenium in seeds, and 300 to 800 ppm selenium in stems and roots, respectively. In a great number of species, selenium is obligatory, and reproductive flowering does not take place if selenium is not present. However, this requirement of selenium in even the most obligatory species may be supplied by a minute amount of selenium (1). The accumulation of selenium in these plants provides an excellent experimental subject for study of the problem of salt absorption and transport.

Most workers in the field of salt absorption acknowledged that 1) salt accumulation is the concentration of ions against a concentration gradient and implies metabolic activity on the part of the cells; 2) that ion accumulation occurs selectively, i.e., anions and cations enter independently and at different rates; 3) that the ion-absorption process is an exchange process in which, for every cation or anion absorbed, an $H^+$ or an $OH^-$ or an $HCO_3^-$ ion is released; and 4) that isotopic exchanges between accumulated isotopes and those of the culture medium occur: this type of exchange occurs in the absence of metabolic activity (2).

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*Preliminary report given at Oak Ridge Institute of Nuclear Studies, 1954.
**Professor of Agricultural Biochemistry. The technical assistance of Sadako Hayase is acknowledged.
In order to clarify subsequent discussion, it is necessary to define certain terms which will be used in this report. If ion enters the cells without chemical, electrovalent, or energy changes in the cells, then the cell is permeable to that ion. Uptake and absorption are terms used to cover the entry of ions into the roots by metabolic processes. Ionic exchange refers to the net change or exchange of Se for Se$^{75}$.

The relation between salt uptake and respiration has been postulated by earlier investigators. Subsequent workers [(3), (4), (5)] contributed to the evidence that salt accumulation in storage tissue depended on aerobic respiration and on other associated activities such as utilization of sugars and synthesis of organic acids. Later workers showed that the same dependence on metabolic function was also a necessary part of ion absorption by the root portion of the plant. Steward (6) and other workers further indicated that the most actively absorbing area was the meristematic root tip and that there was a decreasing longitudinal gradation in capacity for salt accumulation. The exact cause of the higher salt accumulation by actively metabolizing young cells is not known, but it is postulated that it may be due in part to the greater metabolic rate of this segment of the root.

Overstreet and Jacobson (7) and Jacobson and Overstreet (8) in their reports on ion absorption found similarly corroborating evidence by using radioactive tracers. They found marked differences between exchange rates of live and ether-killed roots by the use of radioactive ions Rb$^+$, Br$^-$, I$^-$, and Sr$^{++}$. These differences in the uptake of ions by live and dead roots not only were governed by electrovalent or chemical effects, but other unknown factors were also involved.

In these studies the mechanism of selenium uptake by root seedlings of a selenium indicator, A. preussii, was investigated; the results of these experiments follow.

**MATERIALS AND METHODS**

_A. preussii_ seeds were soaked in concentrated sulphuric acid for 20 minutes and then rinsed in water for 3 hours. The seeds which floated were discarded, and uniform seeds were selected for germination. The seeds were germinated on distilled-water-moistened blotters in an incubator at 20°C. for 7 or 8 days. There was no apparent difference between the metabolic rates of 7 or 8-day-old root seedlings, hence, both ages of root seedlings were used (Rosenfeld, unpublished results). The cotyledons were removed and the primary root plus the hypocotyl were used in the experiments.

Radioactive selenium (Se$^{75}$) was obtained from Oak Ridge. The selenium was in the form of the tetrachloride (SeCl$_4$), which had a specific activity of 72 millicuries per gram of selenium. In water SeCl$_4$ disassociates in the following manner to form selenious acid:

$$\text{SeCl}_4 + 3\text{H}_2\text{O} = \text{H}_2\text{SeO}_3 + 4\text{HCl}$$

Sodium selenite (Na$_2$SeO$_3$) formed in the buffer solution and consequently the tracer was the anion, -Se$^{75}$O$_3^-$.
Absorption Experiments

The solution used for absorption studies (absorption solution) contained $3.3 \times 10^7$ c./m Se$^{75}$ in 23 ml. of Ringer-Phosphate buffer at pH 7.2, with selenium molarity of 0.003M. Ten roots of equal length and diameter were selected. The roots were weighed and placed in a glass-stoppered flask containing the absorption solution. The flasks were rotated on an automatic shaker during absorption. The duration of each absorption period is indicated in the results. The roots were removed after the absorption period with a forceps from the absorption solution and washed rapidly 8 times in a solution containing 0.007 mg. of Se/ml. The last wash water contained 10 to 50 c./m (as Se$^{75}$), indicating that the nonabsorbed Se$^{75}$ was removed from the surfaces.

The absorption of Se$^{75}$ by the roots was followed by acid extraction or maceration for determination of the total uptake of Se$^{75}$ by the roots or their cellular components, or else the roots were transferred directly to the exchange solution for exchange studies.

Exchange Experiments

After absorption of Se$^{75}$ and washing, the roots were transferred to an exchange solution and for varying time intervals, as indicated in the results. Ringer-Phosphate buffer, pH 7.2, containing 1 mg./selenium/ml., was used as the exchange solution. The exchange experiments were of two types. The first type of exchange experiments consisted of groups of 10 roots which were exchanged for 2, 5, 8, 10, and 15 minutes—in some cases the time was extended to $1/2$ and 1 hour. The roots were washed 8 times in the same manner as in the absorption experiments, and the Se$^{75}$ uptake was determined. In the second type of experiment the roots were transferred at 1-minute intervals to the Na$_2$SeO$_3$ solution. This procedure was initiated in order to minimize reabsorption of the exchanged Se$^{75}$. In this procedure the washing of the roots was omitted between transfers. The roots were washed once after the final transfer.

Acid Extraction

After absorption or exchange experiments, the distribution of the Se$^{75}$ was studied. These studies included the total Se$^{75}$ in the roots, acid-extractable Se$^{75}$, and the “bound” Se$^{75}$, which was not removed with the acid (1N H$_2$SO$_4$) used. The acid-soluble fraction will be designated in the results as sap, and the “bound” fraction will be designated as Se$^{75}$ in the root skeleton.

The above procedures were followed in all experiments with live and dead roots. The roots were killed by immersion in 95 percent alcohol overnight. These roots will be designated in the text as dead roots.
The experiments were carried out at room temperature (25°C.) and at 0°C., as indicated in the results. The temperature of absorption and of exchange was always the same, i. e., if the absorption was carried out at 0°C., then the exchange reaction was carried out at the same temperature.

**Determination of Se\(^{75}\) and Se**

Radioactivity of the absorption solution, exchange solution, total tissue, and sap was determined by plating aliquots from a known volume in nickel planchets. The samples were dried in a constant-temperature oven at 50°C. overnight. The root skeletons were dried in toto at 50°C. overnight. The radioactivity of all samples was measured in a Geiger-Mueller counter. The results are expressed as the Se\(^{75}\) uptake, or percent exchanged from the total, by taking the total absorbed as 100 percent. This presentation seemed most appropriate, since there was some variation among the roots, and considerable variation of stored selenium among the root seedlings.

Total selenium was determined by the Gooch and Peirce method as described by Trelease and Beath (1).

**RESULTS**

**Absorption Studies**

It appears from the data that the absorption of Se\(^{75}\) in live roots was greatly influenced by temperature, as indicated in Fig. 1. At 0°C., when the metabolic processes of the roots had essentially ceased, there was a decrease in the absorption of the radioactive isotope. The decrease in the isotope uptake at low temperature (0°C.) may be due to the effect of temperature on the physical properties of the roots, such as viscosity of the protoplasm as well as altered membrane structure, which would have profound effects on the mobility of the ions within the cells and on the movement of the ions outside the cells. Ulrich (9) observed that, in abscissed barley roots, KCl accumulation and respiration were influenced by temperature changes of the substrate.

The large amount of Se\(^{75}\) present in dead roots (0°C. and 25°C.) would suggest that either the differential permeability of the cells was destroyed by killing the roots with alcohol, or there was an increase in the non-specific adsorption of Se\(^{75}\) on the colloidal surfaces of the roots. Present evidence indicates that healthy plant-cell membranes have very low permeability to ions, while the permeability is greatly increased with injury to the cell membranes (10).

In order to determine whether an increase in time would increase the isotope uptake, as well as the accumulation of Se\(^{75}\) in the roots, the absorption time was increased to 1/2, 1, 2, 3, 4, 6, and 8 hours. The absorbed Se\(^{75}\) in the roots was extracted with 1N H\(_2\)SO\(_4\) and the radioactive sele-
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FIG. I—Absorption of Se\textsuperscript{75} by \textit{A. preussii} (10 live roots, L. R., 10 dead roots, D. R.) at \(0^\circ\text{C.}\) and \(25^\circ\text{C.}\). Results are the averages of 5 absorption experiments. Duration of absorption 15 minutes. Absorption solution contained \(3.3 \times 10^7\) c/m Se\textsuperscript{75} 0.003 M Se in Ringer-phosphate buffer pH 7.2.

ium present in the sap and the root skeleton determined. In summarizing these experiments, the results were the following: (1) The radioactive selenium was present in high concentration in the sap in the dissolved state, since the selenium ion was easily extracted with 1N H\textsubscript{2}SO\textsubscript{4} from both living and dead roots. (2) In live roots the concentration of Se\textsuperscript{75} in the sap varied from 86 to 88 percent of the total uptake. In dead roots the sap contained from 98 to 99 percent of the total Se\textsuperscript{75} (Fig. 2). These results suggest that in dead roots the major part of the Se\textsuperscript{75} present was due to the decrease in selective permeability of the cells. In live roots no accumulation of Se\textsuperscript{75} took place with increase in absorption time.

Overstreet and Broyer (2) reported that the absorption or uptake of radioactive potassium in a “high salt plant” was not an accumulation but was due to the establishment of ionic equilibrium with the potassium already present in the plant. In \textit{A. preussii}, a selenium accumulator, the initial selenium is far greater than the ionic accumulation in normal plants. Quantitative analyses for selenium were carried out on groups of 200 and 300 seeds, 40 to 50 live-root seedlings, and 50 dead roots after alco-
TABLE 1—Concentration of Selenium in A. preussii in Random Samples of Seeds and Roots

<table>
<thead>
<tr>
<th>Parts analyzed</th>
<th>No. of seeds or roots in each analysis</th>
<th>Weights gr.</th>
<th>Se p.p.m.</th>
<th>Percent variation from Lowest value</th>
<th>Highest value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Seeds</td>
<td>200</td>
<td>1.0031</td>
<td>1067</td>
<td>40</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>0.9711</td>
<td>844</td>
<td>11</td>
<td>29</td>
</tr>
<tr>
<td></td>
<td>300</td>
<td>1.4578</td>
<td>761</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>0.9325</td>
<td>900</td>
<td>18</td>
<td>16</td>
</tr>
<tr>
<td>Live Roots</td>
<td>50</td>
<td>2.6767</td>
<td>369</td>
<td></td>
<td>52</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>2.6217</td>
<td>460</td>
<td>25</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>2.7839</td>
<td>581</td>
<td>57</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>42</td>
<td>2.2909</td>
<td>764</td>
<td>107</td>
<td></td>
</tr>
<tr>
<td>Dead Roots</td>
<td>50</td>
<td>2.7834</td>
<td>169</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alcohol used to kill the roots</td>
<td>...</td>
<td>...</td>
<td>162</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>Average percentage variation in seeds</td>
<td>23</td>
<td>22</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Average percentage variation in live roots</td>
<td>63</td>
<td>39</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

FIG. II—"Bound" Se\textsuperscript{75} in 10 live and 10 dead-root skeletons at room temperature (R. T.) and 0°C. residue Se\textsuperscript{75} acid extraction at various time intervals. Experimental procedure same as in Fig. I.

The results of these determinations are presented in Table 1. In seeds, the concentration of selenium varied from 1067 to 761 ppm, the average percent variation from the lowest to the highest value being 23 and 22 percent respectively. In live roots the selenium varied from 369 to 764 ppm, the average variation from the lowest to highest value being 63 and 39 percent, respectively. It appears that, during growth, the root seedlings utilized about 50 percent of the stored selenium. The root seedlings had the characteristic odor of volatile selenium, indicating that during metabolic activities of the cells the selenium was utilized and some of the selenium was eliminated from the cells as volatile selenium. That selenium uptake is connected with the metabolic activities of cellular respira-
tion was indicated in our studies on *A. racemosus* leaves. The addition of 0.001M azide and 0.01M cyanide inhibited the respiration in the leaves at a differential rate. The inhibition of respiration with azide and cyanide reduced the absorption of Se\textsuperscript{75} at a rate comparable to the reduction of O\textsubscript{2} uptake and CO\textsubscript{2} formation (11).

The dead roots contained 50 percent less selenium than the live roots. This loss probably was due to the alcohol extraction, since the selenium was present in the alcohol, which was used for killing the roots. According to Beath and Eppson (12), the selenium in *A. preussii* leaves is present in organic form. It appears that in root seedlings the selenium is also in organic combination. The organic selenium in the seedlings was partially alcohol soluble. The rate of ionic combination and absorption was influenced by the ionic content and the metabolic activity of the absorbing cells; therefore variations in the absorption of Se\textsuperscript{75} were due to the initial variation of selenium in the root seedling. Hoagland and Broyer (4) found that barley roots, which have a high concentration of a particular ion, will not accumulate or apparently even absorb any more of that ion. The ability of the roots to absorb the selenium ions in high concentration may be a unique characteristic of selenium-accumulator or selenium-indicator plants.

**Exchange Experiments**

Experiments were carried out to determine the extent to which metabolic processes such as ion exchange reaction take part in the absorption of Se\textsuperscript{75}. The rate of exchange in a buffer solution containing 1 mg. Se/ml. (as selenite 0.12 M) was more rapid at 25°C., as indicated in Fig. 3. At 25°C. at the end of five minutes, dynamic equilibrium was established between the root and the exchange solution, which was indicated by the constant amount of Se\textsuperscript{75} present in the roots after 5 minutes. No equilibrium was established between the cellular Se\textsuperscript{75} and the exchange-solution selenium at 0°C. in 15 minutes. In order to determine whether equilibrium between the Se\textsuperscript{75} in the roots and exchange fluid would occur at 0°C. with increase in time, the experiment was continued for 60 minutes, as indicated in Fig. 4. During the one-hour experiment the Se\textsuperscript{75} in the roots gradually decreased, but no equilibrium between the root exchange solution was established. The roots behaved as roots killed with alcohol.

The non-metabolic character of exchange in live roots at 0°C. is also evident when comparisons are made on the rate of exchange with dead roots. The percentage of Se\textsuperscript{75} which remained in the dead-root sap and skeleton at various time intervals at 0°C. is shown in Fig. 5. The rate of exchange of Se\textsuperscript{75} for Se in the exchange solution in dead roots appeared to be similar to that in live roots at 0°C. Both reactions appear to be non-metabolic, since during the experiments no equilibrium was established with the exchange solution and roots. The above data suggest that dynamic equilibrium is reached only in metabolic reaction, while non-metabolic
FIG. III—Absorption exchange of \( \text{Se}^{75} \) in \textit{A. preussii} roots (10 live roots, L. R., 10 dead roots, D. R.) at room temperature (R. T.) and \( 0^\circ \text{C} \). Percentage of \( \text{Se}^{75} \) which remained in the roots after various time intervals of exchange. Absorption time 15 minutes (100% uptake); absorption solution \( 3.3 \times 10^{-7} \text{ c/m Se}^{75} \) 0.003 M Se in Ringer-phosphate buffer pH 7.2. Exchange solution, 1.0 mg Se/ml. in Ringer-phosphate buffer pH 7.2. The brackets and arrows indicate experimental variations in repeated experiments. \( x \) and \( o \) indicate the means of the experimental results.
reactions are characterized by the slow exchange rates.

Overstreet and Jacobson (7) and Jacobson and Overstreet (8) in their exchange studies at 0°C. with radioactive Rb⁺, I⁻, Sr⁰⁰, and PO₄ observed that the rate of exchange of the radioactive elements in "low salt" barley roots with inert isotope in the exchange solution showed considerable variation. The exchange curves for the various ions were characteristically different. With ether-killed tissue all elements were released very rapidly by isotopic exchange. The release of Se⁷⁵ by dead roots in these experiments was not as rapid as those observed by Jacobson and Overstreet (8). However, these differences may have been due to the basic differences between A. preussii and barley roots used in the experiments.

The similarity of the exchange curves in live and dead roots at 0°C. indicates that, if selenium represents chemical attachment with the root pectins and proteins, the binding complexes are affected by temperature changes or by killing the tissues with alcohol.

In live roots at 25°C., the binding complexes appeared to be very labile, since the selenium ion in the exchange solution rapidly equilibrated with Se⁷⁵ present in the roots. If the selenium is
in the chemical combination of $R'\cdot SeO_4$ or $R'\cdot SeO_3$, it dissociates rapidly, which would account for the failure in isolating organic-selenium compounds from the various selenium-indicator plants, and for the rapid breakdown of organic selenium to elemental selenium during the various steps of isolation (13).

Since ion exchange is influenced by the relative concentration of the absorbed ions, and the competing ions and the exchange are not unidirectional, a study on the rate at which $Se^{75}$ leaves the roots, (if reabsorption of $Se^{75}$ from the exchange solution was reduced to a minimum), was carried out. The results on the exchange of $Se^{75}$ for selenium in 1-minute intervals up to 6 minutes, and 2-minute intervals from 6 to 8 minutes at 25°C. in live roots, are presented in Fig. 6. The highest percentage of exchange occurred at the end of the first minute, at which time the roots contained the most radioactive ion. At the end of one minute, 42 percent of the $Se^{75}$ from the root exchanged with non-radioactive selenium, from 1 to 2 minutes 17 percent, from 2 to 3 minutes 12 percent, and from 6 to 8 minutes 2.5 percent $Se^{75}$ had exchanged. At the end of 6 minutes equilibrium was established between the outgoing and incoming $Se^{75}$.

These results suggest that in live roots at 25°C, a considerable amount of the exchanged $Se^{75}$ was reabsorbed during exchange. The reabsorption did not shift the time during which equilibrium was established between the root and the exchange solution.

**DISCUSSION**

These experiments have encompassed several principal aspects on the problem of membrane permeability as evidenced by $Se^{75}$ uptake and exchange: (1) the temperature effect, (2) differentiation between metabolic exchange and non-metabolic processes, and (3) the relation of the ionic concentration of the cells to the rate of absorption and exchange. It is apparent from the results that selenium was absorbed by the roots very rapidly, and the absorption was related to the metabolic activities of the cells. When the metabolic activities of the cells were reduced by lowering the temperature to 0°C., there was a decrease in the ionic absorption.

Hoagland and Broyer (4) found that under decreased aerobic respiration the cell membranes behave as if they were relatively impermeable to salts. Since live and dead roots at 0°C. failed to reach ionic equilibrium, it appears that live roots at 25°C. (R. T.) through active work increased the rate of uptake and exchange to the point where it exceeds that afforded by simple absorption. The absorption exchange at 25°C. was not directly comparable to simple diffusion of ions but intimately related to the metabolic activities of the cells. The effect of temperature may have been due to the changes in the physical characteristics of the roots and to changes in the mobility of the ions in the absorption, or exchange, solutions. These factors should be taken into account when the temperature effects on accumulation, ionic exchange, absorption, adsorption, or permeability are considered. In dead cells the physical
and chemical characteristics of the cells were altered, there was complete loss of selective permeability of cells, and large amounts of Se\textsuperscript{75} entered into the roots. The lowering of temperature to 0\textdegree C. reduced the ionic uptake in the dead cells to a lesser degree than in live cells. That absorption and exchange of the ions occurs simultaneously in living cells was evident from the results when the roots remained in the same exchange solution for 2, 5, 8, 10, and 15 minutes, or when the roots were transferred to fresh exchange solution at 1-minute intervals, thereby minimizing reabsorption. In the first case at the time of equilibrium the concentration of ions in the roots was higher than in the second experiments, indicating that reabsorption and exchange of Se\textsuperscript{75} took place rapidly in the roots. It is evident that in live cells the trend is toward isotopic equilibrium between the sub-

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FIG. VI—Total Se\textsuperscript{75} absorbed by the roots taken as 100 percent. Percentage of Se\textsuperscript{75} exchanged for nonradioactive isotope by live roots at 25\textdegree C, when the roots were transferred at 1-minute intervals in order to minimize the reabsorption of Se\textsuperscript{75}. Absorption time, absorption, and exchange solutions same as in Fig. III.
strate and root, which must involve certain metabolic activities of the cells.

The root seedling contained variable amounts of selenium. These variations were due to the differences in amounts of initially stored selenium in the seeds. Since the roots contained a high concentration of stored selenium, the only possible exchange-absorption reaction which would take place would be ionic exchange between Se$^{75}$ in the substrate and selenium in the roots. It remains to be determined whether the outward movement of the ions likewise depends upon the same metabolic factors.

There appeared to be a different level of equilibrium for the sap and the root skeleton, as was indicated in the studies with acid extraction of the roots after absorption or exchange. It is possible that eventually all parts of the root would come to isotopic equilibrium, but the roots initially had a high concentration of selenium. Such isotopic equilibrium between sap, root skeleton, and surrounding media may never be reached under experimental conditions.

**SUMMARY**

The mechanism of ion uptake with Se$^{75}$ in live metabolically active, live metabolically inactive (0°C.), and dead roots of selenium accumulator, *A. preussii*, was investigated.

The root seedlings contained a high concentration of selenium, and absorption of radioactive selenium was mainly through active absorption and ionic exchange for the selenium present in the roots.

Live roots under favorable conditions (25°C.) attained ionic equilibrium at a rapid rate. Evidence was presented to show that the inward and outward movement of ions at 25°C. was maintained by cellular metabolism.

The non-metabolic absorption exchange of Se$^{75}$ depended on the cellular permeability to the selenium ion. At 0°C. both live and dead roots failed to establish equilibrium between the roots and the exchange solution.

Acid extraction of the roots indicated that the sap of both live and dead roots contained the highest concentration of the absorbed Se$^{75}$.

Live root skeleton "bound" more than 10 percent of the Se$^{75}$ which was not acid-extractable. About 1 percent of the Se$^{75}$ was "bound" in the dead-root skeleton, and this was not removed during extraction.
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11. Rosenfeld, I., and F. P. Wirtz. Part I in this bulletin.

