LEOPOLD AND PRICE—SULFHYDRYLs IN PLANTS

SULFHYDRYLs IN PLANTS. II. DISTRIBUTION IN TISSUES

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The first study in this series (12) was concerned with chemical as distinct from biological reactions of plant growth regulators with -SH groups. Before information obtained could be applied to tissue systems, it was necessary that means be available for the estimation of the sulfhydryl contents of plants. While assays for specific sulfhydryl compounds in plants have been performed (24), few estimates of total sulfhydryl content have been forthcoming. Polargraphic methods have shown great promise both for nonprotein (26) and protein (3) sulfhydryls. Silver titration of sulfhydryls in homogenates with the platinum electrode has been reported for lily anthers (23), barley seeds (5), and bacterial extracts (14, 16). The results of a colorimetric assay (6) of sulfhydryl in bean leaves has also been briefly reported (9).

The present study is principally concerned with the estimation by amperometric titration of total sulfhydryl including the protein fractions of etiolated pea seedlings and mature bean leaves. A preliminary report has appeared elsewhere (20).

METHODS AND MATERIALS

The procedure used for estimating -SH contents was based on the argentaminine method of Kolthoff and Harris (10) in alcoholic ammonia and, as modified by Benesch et al (3), in aqueous TRIS (tris(hydroxymethyl)-aminomethane) buffer.

In the present study the solutions to be analyzed were stirred magnetically in 50-ml beakers (cf. 22). The bottoms were blown out slightly to form a depression in which a 2-cm teflon-covered stirring bar rotated smoothly. The procedure was otherwise similar to that of Benesch et al (3). A Rubicon (Philadelphia, Pa.) galvanometer was employed with a sensitivity of 0.001 μA per mm, and the galvanometer circuit was critically damped. Silver nitrate (0.01 M)
was added in 1-μl increments from an "Agla" micrometer syringe (Burroughs Wellcome and Co., Inc., Tuckahoe, New York) fitted with a 10-μm bent glass delivery needle.

The test material included etiolated *Pisum sativum* and *Avena sativa* seedlings as used for auxin assays, *Helianthus annuus* internodes obtained from plants grown in the greenhouse, as in auxin transport experiments (15), and the mature leaves of *Phaseolus vulgaris* grown in the greenhouse.

**Tissue Sections:** Cut sections of the tissue were washed on a coarse sintered glass funnel and ground in a conical tissue grinder with 5 ml of a mixture of 0.15 M TRIS nitrate, pH 7.4, 0.01 M KCl, with or without the addition of 8 M urea. The latter served as a mild denaturing agent, providing an estimate of the total -SH available to the tissue. The homogenate was then drained through a 30- or 60-ml Buchner-type coarse sintered glass funnel with an additional 25 ml of the TRIS mix. This step was essential to free the sample from cell wall debris which otherwise fouled the platinum electrode. A separate funnel was required for each sample, since otherwise filtration was incomplete. The filtered homogenate was finally transferred to a specially prepared 50-ml beaker (described above) and the titration begun. The whole operation from washing the sections to the beginning of the titration required three to five minutes. A standard interval between grinding and the addition of silver nitrate was chosen for each experiment.

**Subcellular Fractions:** The fractionation methods corresponded to those commonly employed for the preparation of mitochondria (8). Chilled tissue was blended for 15 seconds in a chilled Waring blender with 2 × 5 × (v/w) of a frozen slush of 0.3 M sucrose and 0.05 M potassium phosphate, pH 7. With bean leaves, 0.01 M MgCl₂ was included. The resulting suspension was strained through cheesecloth, thus separating the gross cell wall residue from the “homogenate.” The residue was then re-extracted with a small volume of the medium. The homogenate was subjected to fractional centrifugation, as indicated in the text, in a Servall SS-1 centrifuge in a 0 to 2 °C cold room. Non-protein components of the various pea and bean fractions were obtained by adding 30% sulfooxylic acid to 3% final concentration, centrifuging, and collecting the supernatants. The -SH contents of all samples were then determined by titration in a mixture of 95% ethanol, 0.25 M ammonium hydroxide, 0.05 M ammonium nitrate, and 10⁻⁵ M ethylenediaminetetraacetic acid (10). Nitrogen analyses on the various samples were obtained by micro-Nesslerization (11).

**Results**

**Application of the Method to Plant Material:** Of the methods available for estimation of sulfhydryl groups (17), amperometric titration proved the most suitable. Because of interfering substances found in unpurified plant extracts, the nitroprusside test (7) had to be excluded. The optical method of Boyer (4) employing p-chloromercuribenzoate was not practicable because of the high extinction of the plant extract. Amperometric titration with amino (3) or ammoniacal (10) silver ion, on the other hand, is not influenced by the color or turbidity of the extract, is highly specific (1, 3), sensitive, and yielded reproducible values. Recovery of cysteine or glutathione added to homogenates in alcohol-ammonia was quantitative.

With pea stem sections in the TRIS mix, a partial destruction of sulfhydryl groups occurred between grinding and the addition of silver ion. An experiment in which pea stem section homogenates were allowed to incubate from 4 to 30 minutes at room temperature indicated a logarithmic decline in the sulfhydryl content. Extrapolation to zero time indicated at four minutes a loss of 12% of the initial -SH; as it was technically not possible to decrease the interval, no information was available on the actual losses in the moments following disruption of the tissue. When pea sections were ground in TRIS with added glutathione the -SH recovered at four minutes was also 12% less than theoretical. Since the use of a standard interval between grinding and titration yielded reproducible values, this destruction of sulfhydryls proved a relatively minor problem. The use of TRIS retains a distinct advantage over alcoholic ammonia; for the degree of denaturation of protein can be more closely controlled (3).

Since it was planned to use the sulfhydryl assay

![Fig. 1. Sensitivity of titrations with pea stem homogenates. -SH content of pea stem section homogenates determined for various sizes of sample. Sections homogenized and titrated in 0.15 M TRIS nitrate, pH 7.4, 0.01 M KCl, and 8 M urea.](image-url)
in conjunction with growth studies, it was essential that the method be sufficiently sensitive for small quantities of tissue. Sample titrations were, therefore, performed with varying quantities of etiolated pea stem sections. The results are illustrated in figure 1. It can be seen that the assay is linear to at least sixty 10-mm sections. Thirty sections or about 600 mg fresh weight provided convenient sulphydryl values.

**Sulphydryl Content of Tissues**

Oat coleoptile sections and pea stem sections were ground and titrated in TRIS-nitrate mixture containing 8 M urea. The results are summarized in table I. Included is a measurement of 6-cm sunflower internodes, kindly prepared by Dr. Ethel Niedergang-Kamien (15).

### Table I

**Sulphydryl Content of Avena, Helianthus and Pisum Tissues**

<table>
<thead>
<tr>
<th>Plant</th>
<th>Age</th>
<th>Tissue</th>
<th>Micro-moles/gm fresh wt</th>
<th>Micro-moles/100 sections (10 mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Avena sativa</td>
<td>72 hrs</td>
<td>Etiolated coleoptiles</td>
<td>0.32</td>
<td>0.46</td>
</tr>
<tr>
<td>(oat)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Helianthus annuus</td>
<td>3 wks</td>
<td>Internodes</td>
<td>0.08</td>
<td>0.16</td>
</tr>
<tr>
<td>(sunflower)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pisum sativum</td>
<td>7 days</td>
<td>Etiolated subapical</td>
<td>0.86</td>
<td>1.41 ± 0.08*</td>
</tr>
<tr>
<td>(pea)</td>
<td></td>
<td>internodes</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

-SH of whole tissue extract estimated by amperometric titration in TRIS-8 M urea medium. See Methods. * Twenty-seven separate experiments.

### Table II

**Intracellular Fractionation of -SH Content of Pisum Seedlings**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Approx. force-time integral during centrifugation (g-min)</th>
<th>Nitrogen content (mg N/gm fresh wt)</th>
<th>% of homogenate</th>
<th>Sulphydryl content (micromoles/gm fresh wt)</th>
<th>% of homogenate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogenate</td>
<td>. . . .</td>
<td>. . . .</td>
<td>. . . .</td>
<td>. . . .</td>
<td>. . . .</td>
</tr>
<tr>
<td>Starch</td>
<td>500*</td>
<td>0.29</td>
<td>0.07</td>
<td>2.4</td>
<td>2</td>
</tr>
<tr>
<td>&quot;Plastid&quot;</td>
<td>4,500**</td>
<td>1.88</td>
<td>0.60</td>
<td>15.3</td>
<td>23</td>
</tr>
<tr>
<td>Mitochondria A</td>
<td>50,000†</td>
<td>0.93</td>
<td>0.18</td>
<td>7.6</td>
<td>7</td>
</tr>
<tr>
<td>Mitochondria B</td>
<td>500,000††</td>
<td>0.71</td>
<td>0.08</td>
<td>5.8</td>
<td>3</td>
</tr>
<tr>
<td>&quot;Soluble&quot;</td>
<td>500,000††</td>
<td>7.44</td>
<td>0.17</td>
<td>60.5</td>
<td>68</td>
</tr>
<tr>
<td>Recovery</td>
<td>. . . .</td>
<td>. . . .</td>
<td>. . . .</td>
<td>. . . .</td>
<td>. . . .</td>
</tr>
</tbody>
</table>

Fractions prepared from sucrose-phosphate homogenates of 3-day-old etiolated seedlings. -SH determined by titration in alcohol ammonia. * 1000 x g for 30 sec. ** 1500 x g for 5 min. † 10,000 x g for 5 min. †† 16,700 x g for 30 min.
Table III

Intracellular Fractionation of -SH Content of Phaseolus vulgaris Leaves

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Approx. Force-time Integral in Centrifugation (g-min)</th>
<th>Nitrogen Content</th>
<th>-SH Content</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Micromoles/gm Fresh wt</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mg N/gm Fresh wt</td>
<td>% of Total Homogenate</td>
</tr>
<tr>
<td>Homogenate</td>
<td>. . . . . .</td>
<td>6.95</td>
<td>100</td>
</tr>
<tr>
<td>Starch</td>
<td>1,000 *</td>
<td>0.28</td>
<td>4</td>
</tr>
<tr>
<td>Chloroplast</td>
<td>4,000 **</td>
<td>0.16</td>
<td>2</td>
</tr>
<tr>
<td>Mitochondria-chloroplast</td>
<td>500,000 †</td>
<td>1.18</td>
<td>17</td>
</tr>
<tr>
<td>Soluble</td>
<td>500,000 †</td>
<td>5.55</td>
<td>80</td>
</tr>
<tr>
<td>Recovery</td>
<td>. . . . . .</td>
<td>. . .</td>
<td>. . . .</td>
</tr>
</tbody>
</table>

Fractions prepared from sucrose-phosphate-magnesium chloride homogenates of mature leaves of Phaseolus vulgaris. -SH determined by titration in alcohol ammonia.

* 1000 x g for 1 min.
** 1000 x g for 4 min.
† 16,700 x g for 30 min.

lected at about 1000 g-minutes; a small fraction of relatively pure chloroplasts were sedimented at 4000 g-minutes; and a mixture of mitochondria and the remaining chloroplasts obtained at 500,000 g-minutes. The sulfhydryl and nitrogen data are summarized in table III. In bean leaves the "soluble" fraction again had the bulk of the protein sulfhydryl, but more than 10% of the recovered non-protein sulfhydryl appeared in the mitochondria-chloroplast fraction.

From tables II and III it may be calculated that the sum of the total protein -SH contents of the several fractions is considerably less than that of the homogenate, whereas the non-protein -SH nearly coincides. Why the protein -SH should be specifically affected is not known. It is possible that the particulate component became susceptible to autoxidation during washing.

In both tissue systems described the cell debris was neglected because of the technical problem of keeping the platinum electrode free of fiber. A few titrations of preparations of washed fiber were successful, and in the case of pea seedlings a figure of about 1% of the homogenate -SH was found.

Discussion

The inherent errors as well as the advantages of the amperometric methods for sulfhydryl analysis have been discussed in detail by Benesch et al (3). Application of the method to certain plant material has presented only one difficulty, the relatively rapid decrease in -SH content after grinding. Losses in the pea stem between grinding and titration were estimated at 12%. It would be advantageous to have present in the homogenizing medium a reagent to mask the -SH groups, as is employed in the chromatographic separation of non-protein sulfhydryls (18). Recent experiments indicate that the amperometric method can be modified to include this precaution by homogenizing the sample in an estimated 10% excess of silver nitrate in TRIS buffer and back-titrating with standardized glutathione.

The application of amperometric titration to plant tissues permitted certain preliminary observations. First, the -SH contents of the four plant tissues examined varied over a 50-fold range (table I and III). The physiological consequences of these differences may prove of importance. Second, comparisons can be made with measurements in the animal literature. Flesch and Kun (6) assembled data for various mammalian tissues for which the -SH contents were typically 1 to 2 micromoles per gram; liver, for example, was reported as containing 1.5 micromoles per gram. Plant tissues appear to be in the same order of magnitude: nearly 2 micromoles per gram for pea seedlings (table II) and nearly 5 micromoles per gram for bean leaves (table III). Ehrenberg (5), also employing silver titration, found about 2 micromoles -SH per gram in barley embryos. About 90% of the plant tissue sulfhydryl can be attributed to proteins, a proportion very similar to that in red blood cells (2). The values for micromoles -SH per mg protein X for plant tissues are as follows: 0.16 for pea seedlings, 0.18 for oat coleoptiles, 0.45 for pea stems, 0.57 for bean leaves, and up to 0.8 for lily anthers (23). The corresponding values for crystallized enzymes were found by Benesch et al (23) to range from 5 to 12.

The employment of the amperometric method of sulfhydryl estimation in growth studies is reported in the following contribution (19).

Summary

1. Amperometric silver titration was employed for the estimation of the sulfhydryl contents of etiolated

5 There is a discrepancy between these figures and those of Benesch and Benesch (1). The latter's measurements of glutathione exceed the total -SH levels reported by Flesch and Kun.
pea seedlings, oat coleoptiles, bean leaves, and sunflower internodes.

2. Preliminary estimates of the protein and non-protein sulphydryl contents of sub-cellular fractions are also reported. The -SH : protein N ratios were fairly uniform among the fractions.

3. Plant sulphydryl contents per unit of fresh weight were found to compare closely with values reported for mammalian tissue.

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LITERATURE CITED