THE SULPHYDRYL–DISULPHIDE HYPOTHESIS IN RELATION TO DESICCATION INJURY OF CABBAGE LEAVES

By D. F. Gaff*

[Manuscript received September 1, 1965]

Summary

Sulphydryl (–SH) and disulphide (–SS–) levels were examined in extracts of cabbage leaves which had been desiccated to various degrees down to the death of the tissue at a water potential of −94 atm.

The soluble protein fraction showed a progressive decrease, to 50% of the control, in "reactive" –SH, i.e. the –SH in the native protein capable of reacting with Ag+. About 60% of the decrease was attributed to configurational changes, since the remainder could be accounted for by a conversion of –SH to –SS– which appeared to involve the linkage of non-protein to the soluble protein. Associated with this linkage, a decrease occurred in the titratable sulphur of the non-protein fraction, without, however, any alteration in the proportion of –SH to –SS–.

Structural protein displayed an increase in the "reactive" –SH apparently due to unfolding of the protein; at water potentials less than −40 atm (approx. 2·5 water molecules/amino acid residue). The total –SH and total –SS–, however, remained unaltered until −94 atm (approx. 2·3 water molecules/amino acid residue), at which stage a considerable conversion of –SH to –SS– occurred, involving at least some protein–protein intermolecular bonds and accompanied by the death of the tissue. This latter process agrees with the predictions of Levitt’s sulphydryl–disulphide hypothesis.

I. Introduction

The sulphydryl–disulphide hypothesis formulated by Levitt (1962) was offered primarily as an explanation of frost resistance in plants, but was considered to be equally applicable to the phenomena of heat hardiness and drought tolerance. In essence it was proposed that “...injury is due to an unfolding and therefore a denaturation of the protoplasmic proteins. This results from the formation of intermolecular –SS–bonds induced by the close approach of the protein molecules due to...dehydration. Frost resistance is a resistance towards –SH oxidation and –SH⇌–SS– interchange and, therefore, to formation of these intermolecular bonds.”

Two consequences of this hypothesis then would be that (1) protein –SS– increases at the expense of –SH during drought injury, and (2) this increase in –SS– is associated with protein aggregation. In the present paper, these two premises were examined in order to establish whether or not the sulphydryl–disulphide hypothesis could validly be applied to drought injury.

* Department of Botany, University of Missouri, Columbia, Missouri; present address: Department of Botany, Monash University, Clayton, Vic.

II. MATERIALS AND METHODS

(a) Plant Material

Cabbage plants (Brassica oleracea L.) of the genetically selected cultivar Badger Market were raised for 6 weeks in growth chambers which were illuminated for 18 hr a day by a combination of cool white fluorescent tubes and incandescent lamps (total illumination 2000 f.c., Weston meter). Temperatures were 25°C (light) and 15°C (dark) respectively for alternating periods of 12 hr. The relative humidity of the circulating air was 85% or higher. Plants were grown in vermiculite, were well watered, and supplied with a full nutrient solution once each week.

(b) Desiccation

Excised leaves were divided into two sets of comparable halves (20 g fresh weight each), which were then cut into 1-cm wide strips (to reduce cuticular restriction of water loss) and washed in distilled water to remove the contents of cut cells. One set was dried at room temperature in darkness in a desiccator over calcium chloride until the desired fresh weight was obtained, then kept in a tightly closed polythene bag until the total period of desiccation was 24 hr. The control strips were similarly enclosed for the full 24 hr without any desiccation. The water potential and the percentage survival of some desiccated strips were determined; the bulk of the tissue, however, was used in the estimation of –SH and –SS– content. Survival was measured by the ability of 50% of the tissue’s cells to accumulate neutral red, and by the ability of the tissue to regain turgor when floated on water.

(c) Extraction

Three fractions were extracted: (1) soluble non-protein fraction; (2) protein soluble in borate buffer; and (3) “structural protein” which was soluble in detergent (see Green et al. 1961).

The tissue was homogenized in a Waring Blender containing 100 ml of 0.05M Na₂B₄O₇ brought to pH 8.4 with concentrated HCl—a buffer which extracts soluble protein from chloroplast efficiently (see Zucker and Stinson 1962). The homogenate was filtered through cheesecloth and centrifuged for 5 min at 500 g to remove debris (whole cells, starch grains, and whole chloroplasts). The supernatant suspension was recentrifuged at 37,000 g for 15 min to sediment chloroplast fragments and mitochondria which were resuspended in borate buffer to give a crude extract of structural protein.

Portion (15 ml) of the supernatant from the final centrifugation was partitioned into soluble protein and non-protein fractions by means of a Sephadex column (11 × 1.9 cm), prepared from a nitrogenated suspension of Sephadex G25 in the 0.05M borate–HCl buffer, and kept under nitrogen when in use. The greenish yellow protein fraction (precipitable with trichloroacetic acid) eluted at 15–40 ml, and included an appreciable quantity of particles of microsomal size (checked by ultracentrifugation). The non-protein fraction (not precipitable with trichloroacetic acid) eluted at 50–75 ml. These three fractions were then used in the determination of –SS– and –SH.
In order to avoid oxidation of \(-\text{SH}\) to \(-\text{SS}\) during the above extraction, all the liquids employed were depleted of oxygen in vacuo, and equilibrated with purified nitrogen prior to the experiment. All further manipulations were carried out under nitrogen, and, with the exception of the resuspension of “structural protein”, were conducted at 2°C.

The crude “structural protein” suspension could be solubilized (and denatured) in 2% sodium dodecyl sulphate (SDS), forming a solution of a chloroplast pigment-protein complex (cf. cytochrome complexing with mitochondrial structural protein, Criddle et al. 1962). The pigment could not be separated by filtration on Sephadex but could be removed at acetone concentrations higher than 70%. Preliminary ultracentrifuge studies on the complex, conducted by Dr. P. Chun, indicated a sedimentation coefficient \(S\) of approximately 2.7 in 0.5% SDS (cf. \(S = 2.2\) for monomeric structural protein from beef mitochondria, Criddle et al. 1962). The protein could be purified further by precipitation and repeated washing with acetone to remove chlorophyll, carotenoids, and lipids. The precipitated protein (which was insoluble in cold water but partially soluble in 2% SDS) was used in the studies of water sorption (Table 3).

(d) Determination of \(-\text{SH} and \, -\text{SS}\-

The sulphydryl contents of the above three extracts were determined under nitrogen by amperometric titration at pH 8.5 with silver nitrate solution (Levitt et al. 1961). Titrations were made both on the undenatured protein to determine the content of “reactive” \(-\text{SH}\) accessible to \(\text{Ag}^+\) ions in the native protein, and on protein denatured with 2% SDS to find the total titratable \(-\text{SH}\). SDS produced a higher yield of titratable \(-\text{SH}\) than did 6M urea; possibly the reaction of \(-\text{SH}\) groups with cyanate, formed from urea at a pH > 6, interferes with the determination in the latter case (Stark, Stein, and Moore 1960). Alcoholic denaturation gave erratic results; moreover it has been shown (Sullivan 1963) that estimates of \(-\text{SH}\) in cysteine in alcoholic media are 120% of the known concentration.

Disulphide was determined by amperometric titration of the \(-\text{SH}\) produced by the reaction of \(-\text{SS}\) with sodium sulphite (Levitt, Sullivan, and Johansson 1962). It was necessary to denature protein samples before determining \(-\text{SS}\- content; otherwise \(-\text{SH}\) groups unmasked by configurational changes ensuing from the dissolution of the \(-\text{SS}\- bonds of the native protein were erroneously included with those produced directly from the reaction with sulphite. This error could be very considerable, e.g. in some cases the apparent \(-\text{SS}\- in the native protein was 76% of the total titratable sulphur, but 27% in the denatured protein. An empirical correction was applied for the effect of added sodium sulphite on the conductivity of the titration medium.

(e) Nitrogen Content

Replicated samples of each extract were digested in hot concentrated sulphuric acid, residual carbon was oxidized with hydrogen peroxide, and the nitrogen contents were determined by nesslerization (Umbreit, Burris, and Stauffer 1959). The standard error was approximately 2%.
(f) Water Potential

The water potential of the desiccated leaf strips was estimated by the gravimetric vapour exchange method (Slatyer 1958) using a water-bath at $27 \pm 0.1{\text{C}}$. No correction was made for respiratory loss in weight.

III. RESULTS AND DISCUSSION

Two mechanisms for the dislocation of "normal" metabolism were formulated in the sulphydryl-disulphide hypothesis: (1) an inactivation of some enzymes by the oxidation of the $\text{-SH}$ groups which are often necessary for the action of the enzyme; (2) disruption of the cell organization due to denaturation of structural proteins under

<table>
<thead>
<tr>
<th>Weight of Desiccated Leaf* (as % of initial fresh wt.)</th>
<th>Water Potential† (atm)</th>
<th>Disulphide Content (as % of total titratable sulphur)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>&quot;Structural Protein&quot;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Desiccated</td>
</tr>
<tr>
<td>88</td>
<td>$-13$</td>
<td>26.7</td>
</tr>
<tr>
<td>88</td>
<td>$-20$</td>
<td>22.6</td>
</tr>
<tr>
<td>75</td>
<td>$-41$</td>
<td>27.5</td>
</tr>
<tr>
<td>50</td>
<td>$-76$</td>
<td>20.9</td>
</tr>
<tr>
<td>42</td>
<td>$-86$</td>
<td>50% of cells cease accumulation of neutral red</td>
</tr>
<tr>
<td>28</td>
<td>$-94$</td>
<td>49.3</td>
</tr>
<tr>
<td>28</td>
<td>$-194$</td>
<td>-</td>
</tr>
</tbody>
</table>

* ±2%. † ±5 atm.

Table 1

Disulphide content of desiccated and undesiccated (control) half leaves of cabbage

Each value is the mean of the data of three experiments

The proportion of $\text{-SH}$ to $\text{-SS}$ in this fraction was little affected by desiccation (Table 1). In the case of the non-protein fraction, nitrogen content was not a particularly successful common denominator on which to express sulphur contents, since desiccation affected the quantity of nitrogen per unit volume of the extract
(the ratio of desiccated to control (D/C) values for nitrogen contents of the non-protein fraction were 1·36, 1·24, 0·91, 1·02, and 1·35 respectively for the range -13 to -94 atm; the corresponding D/C values for total non-protein sulphur per unit volume of extract amounted to 1·07, 0·92, 0·68, 0·81, and 0·66). The increase in non-protein nitrogen presumably resulted from a change in the balance of the synthesis and breakdown of proteins—both desiccation and leaf detachment are known to favour a net breakdown of protein (e.g. Todd and Yo 1964). The lower value of the ratio of sulphur to nitrogen observed in the desiccated sample (D/C < 1, see above) seems to imply then a preferential incorporation or retention of cysteine residues in the soluble proteins (the increase in soluble-protein sulphur, averaging 0·55 atoms S per 1000 atoms N, accounted for 2·20 of the 2·56 atoms mean loss of non-protein sulphur per 1000 atoms N, since soluble-protein sulphur equals approximately four times non-protein sulphur). The data for soluble protein, however, indicate a modification of this explanation.

(b) Soluble Protein (including microsomes)

As predicted in the sulphhydryl-disulphide hypothesis, the level of -SS- increased with desiccation, reaching a value 50% higher than the control at the mean death point (Table 2) and was accompanied by a decrease in total -SH* which, however, was insufficient to account for the gain in -SS-. It seems likely that under dry conditions non-protein molecules may have reacted with the soluble protein -SH to become linked via -SS- bonds. In this case the changes in titratable sulphur would follow the pattern:

\[
\Delta(-SH) : \Delta(-SS-) : \Delta(\text{total } S) = -1 : +2 : +1
\]

(cf. the differences between the means of desiccated and of control treatments in Table 2: -0·55 : +1·32 : +0·78 atoms S per 1000 atoms N), whereas intramolecular and protein-protein intermolecular oxidation of -SH to -SS- would both have produced changes in the proportion -1 : +1 : 0.

Presumably only the -SH accessible to Ag+ in the native protein would have been involved in protein-non-protein -SS- linkage, therefore changes in reactive -SH should equal those in the total -SH. In fact, the loss of reactive -SH (averaging 1·40 atoms S per 1000 atoms N, Table 2) was approximately twice the proportionate overall loss in -SH, consequently desiccation must have induced a considerable masking of reactive -SH, presumably as a result of conformational changes in the shrinking protein molecule. The decrease in the reactive -SH and the total -SH of the water-soluble fractions is consistent with the notion of a progressive dislocation of metabolism as desiccation induces a loss of activity of -SH-dependent enzymes, many of which have been listed by Boyer (1959). Although no study was made here to check if the activity of any enzymes did diminish, decreases have been reported in the phosphatase, peptidase, peroxidase, and dehydrogenase activity in desiccated wheat leaves (Todd and Yo 1964).

* Although neither the decrease in soluble protein -SH nor the increase in the total sulphur reached the level of statistical significance, the significant increase in protein -SS- necessitates the occurrence of at least one of these. Since both changes were of a similar order of magnitude and gave similar values of Student’s t, it seems that both changes were real, in spite of the lack of statistical significance.
Table 2

Titratable sulphur atoms per 1000 nitrogen atoms in extracts from desiccated and undesiccated half leaves of cabbage

Each value is the mean of three experiments. Asterisks denote significant differences between desiccated and undesiccated values at the following levels: *P < 0.1, **P < 0.05, ***P < 0.01

<table>
<thead>
<tr>
<th>Weight of Desiccated Leaf (as % of initial fresh wt.)</th>
<th>Water Potential of Desiccated Leaf (atm)</th>
<th>Desiccated Leaf (D)</th>
<th>Undesiccated Leaf (C)</th>
<th>Ratio D/C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Reactive</td>
<td>Reactive + Unreactive</td>
<td>Reactive</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-SH</td>
<td>-SS-</td>
<td>-SH</td>
</tr>
<tr>
<td>Soluble Non-protein Fraction</td>
<td></td>
<td>0.84</td>
<td>4.18</td>
<td>0.40</td>
</tr>
<tr>
<td>Mean total S</td>
<td></td>
<td>0.53***</td>
<td>5.26***</td>
<td>0.79</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td>8.78</td>
<td>8.34</td>
<td></td>
</tr>
<tr>
<td>Soluble and Microsomal Protein Fraction</td>
<td></td>
<td>6.09</td>
<td>4.47</td>
<td>6.45</td>
</tr>
<tr>
<td>Mean total S</td>
<td></td>
<td>5.25</td>
<td>5.35***</td>
<td>3.97</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td>10.60</td>
<td>9.82</td>
<td></td>
</tr>
<tr>
<td>Structural Protein</td>
<td></td>
<td>1.33</td>
<td>4.55</td>
<td>1.78</td>
</tr>
<tr>
<td>Mean total S</td>
<td></td>
<td>1.28***</td>
<td>4.09</td>
<td>1.87</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td>5.96</td>
<td>5.67</td>
<td></td>
</tr>
</tbody>
</table>
(c) Structural Protein

Two effects of desiccation were evident in this fraction, (1) an increase in the reactive \(-\text{SH}\), and (2) a sudden oxidation of \(-\text{SH}\) to \(-\text{SS}\) at the mean death point.

The rise in reactive \(-\text{SH}\) was not accompanied by an increase in the total \(-\text{SH}\) or by a decrease in \(-\text{SS}\), consequently the rise must have been due to an unmasking of \(-\text{SH}\) groups. This was in contrast to the desiccation-induced masking of reactive \(-\text{SH}\) in the soluble protein. The difference probably stemmed from the participation of the structural protein in the membrane structures of the cell. The rise in the reactive \(-\text{SH}\), then, may have resulted from irreversible uncoiling of the protein produced by the internal stresses set up by contraction of the molecule opposed by the forces binding the membrane together. Conceivably the conformational changes could have produced degradation of the cell membranes and an increased permeability of the cells (cf. Stocker 1961).

### Table 3

<table>
<thead>
<tr>
<th>Water Potential (atm)</th>
<th>Water Content* (as % of dry wt.)</th>
<th>No. of Water Molecules per Atom of Nitrogen in Protein†</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>135·0</td>
<td>6·3</td>
</tr>
<tr>
<td>-9</td>
<td>117·0</td>
<td>5·5</td>
</tr>
<tr>
<td>-18</td>
<td>57·8</td>
<td>2·7</td>
</tr>
<tr>
<td>-37</td>
<td>40·5</td>
<td>1·9</td>
</tr>
<tr>
<td>-46</td>
<td>41·5</td>
<td>1·9</td>
</tr>
<tr>
<td>-65</td>
<td>36·0</td>
<td>1·7</td>
</tr>
<tr>
<td>-86</td>
<td>35·8</td>
<td>1·7‡</td>
</tr>
</tbody>
</table>

* Temperature of oven 130°C.
† Calculated on the basis that the mass of the dry protein equals six times the mass of protein nitrogen.
‡ Equivalent to an average of about 2·3 water molecules per amino acid residue.

At \(-94\) atm, slightly beyond the mean death point, the percentage of \(-\text{SS}\)-increased to twice the value of the control. The increase in \(-\text{SS}\) was real and was accompanied by a corresponding decrease in total \(-\text{SH}\) content with very little change in total S, i.e. the \(-\text{SS}\) was formed by oxidation of the \(-\text{SH}\). The fraction of the total sulphur oxidized to \(-\text{SS}\) (50%) represented most of the oxidation that is stereochemically possible (see Table 4) in the immobilized structural protein. The fall in reactive \(-\text{SH}\) at \(-94\) atm relative to the level at \(-41\) and \(-76\) atm suggested that the oxidation may have involved the formation of some protein–protein intermolecular \(-\text{SS}\) bonds.

The increase in \(-\text{SS}\) should, according to the sulphydryl–disulphide hypothesis, be a cause of death and certainly the data show the structural protein \(-\text{SS}\) increase
was associated with the death of the tissue. The rise in −SS− must produce a considerable disorganization of the membrane structures of the cell, possibly similar to those reported in the electron microscope studies of Schnepf (1961). However, there was no clear evidence to indicate the direction of causation. Although the water imbibed by the structural protein has fallen to a level where its influence in maintaining the configuration of the protein must have been critically reduced (Table 3), the removal of steric hindrance was not responsible for the −SS− rise, since virtually all the −SH groups involved could be oxidized in vitro at water potentials of only −5 atm (Table 4), consequently the rise in −SS− might have been due to the failure of some other mechanism which prevents the oxidation of −SH in vivo.

**Table 4**

**Oxidation of Structural Protein Extracts by Aeration**

Air bubbled through protein extracts at rate of 7 litres per hour

<table>
<thead>
<tr>
<th>Condition of Protein during Aeration</th>
<th>−SS− Content (as % of total titratable S) after</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 Min</td>
</tr>
<tr>
<td>Denatured in SDS, 0·05M borate buffer, pH 8·4</td>
<td>26</td>
</tr>
<tr>
<td>Undenatured, 0·05M borate buffer, pH 8·4</td>
<td>13</td>
</tr>
<tr>
<td>Undenatured, 0·05M phosphate buffer, pH 5·8</td>
<td>10</td>
</tr>
</tbody>
</table>

**IV. Conclusions**

The injury and death of desiccated cabbage leaves stemmed from degradation of the lipoprotein membranes produced initially by irreversible conformational changes in the structural protein molecule, and at the death point by the oxidation of −SH to −SS− that was predicted in Levitt’s hypothesis. However, this latter conversion was not due to steric alterations allowed by water loss, but to a failure of some other mechanism preventing oxidation.

The −SS− bonding of non-protein molecules to the soluble protein by oxidation or interchange with the surface −SH may be primarily a hardening response rather than an injury effect, as it coincides with Levitt’s sixth mechanism of hardening (Levitt 1962). However, when the progressive masking of surface −SH by conformational changes was taken into account, the overall loss in surface −SH was considerable, and a consequent dislocation of −SH-dependent enzyme activity may have been a major factor in the disorganization and death of the tissue.

The contrasting patterns of sulphydryl–disulphide changes for the soluble protein and for the structural proteins probably arise from the close packing of the structural protein molecules, and from the intermolecular forces binding them into the structure of the cell membranes.
V. Acknowledgments

The work presented here was supported by a U.S. National Science Foundation Grant (NSFGB230) received by Professor Levitt, under whose guidance the work was performed while the author was on leave of absence from the Botany Department of the University of Melbourne.

VI. References


Chibnall, A. C. (1939).—“Protein Metabolism in Plants.” (Yale Univ. Press: New Haven, Connecticut.)


